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IN VITRO ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACTS DERIVED FROM LEAVES OF JUVENILE AND MATURE SHOOTS OF *FICUS PUMILA* L. (MORACEAE)

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The aim of this study was to investigate the *in vitro* antioxidant activity of aqueous extracts from the leaves developed on the shoots of various developmental stages (juvenile and mature/generative) of *Ficus pumila* L. using the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] on the equine erythrocytes' suspension model. Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w). The equine erythrocyte aliquots were used in the study. A volume of 0.1 ml of the *F. pumila* extract was added to 1.9 ml of clean equine erythrocytes. For positive control (blank), phosphate buffer was used. The treatment with the extract obtained from leaves of mature shoots reduced the erythrocytes TBARS level by 22% (p = 0.029), while TBARS level was increased by 15.5% (p >0.05) when incubated with an extract derived from leaves of juvenile shoots as compared to untreated erythrocytes. When equine erythrocytes were incubated with extract obtained from leaves of mature shoots, the ketonic derivatives level was significantly decreased by 6.9% (p = 0.040), while non-significantly decrease both aldehydic and ketonic derivatives of OMP was observed after incubation with extract derived from juvenile shoots (by 8.18 and 12.5%, p >0.05). The treatment by *F. pumila* leaf extract (from juvenile and mature shoots) caused the increase of TAC in erythrocyte suspension as compared to untreated erythrocytes. Thus, extracts derived from both juvenile and mature shoots resulted in an increase of total antioxidant capacity of equine erythrocytes' suspension.

Keywords: *Ficus pumila* L., leaf extract, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

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Introduction

Antioxidant compounds present in many plants, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity, can protect cells against damage caused by reactive oxygen species (ROS) (Cai et al., 2004; Das et al., 2014). A large number of medicinal plants have been investigated for their antioxidant properties. Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress (Akinmoladun et al., 2010).

Mopuri et al. (2018) have investigated the *in vitro* effects of the various parts of *Ficus carica* L. (fig) on antioxidant, antidiabetic, and antiobesogenic activity. The fruit ethanolic extract contained a high quantity of polyphenols and flavonoids compared with all other extracts. The activity of the ethanolic extract of *F. carica* fruit was significantly higher than all other extracts and parts of the plant in terms of antioxidative, antidiabetic, and antiobesogenic effects. The ethanolic extract of the fruit of *F. carica* may have potential antidiabetic and antiobesogenic agents. The research of Eshwarappa et al. (2015) highlights the chemical composition and antioxidant potential of leaf gall extracts (aqueous and methanol) of *Ficus racemosa* L. (syn. *Ficus glomerata* Roxb.), which is extensively used in the preparation of traditional medications to treat various metabolic diseases. Higher content of both total phenolics and flavonoids were found in the methanolic extract and the significantly high antioxidant activity can be positively correlated to the high content of total polyphenols/flavonoids of the methanol extract (Eshwarappa et al., 2015). In the study of Abrahim et al. (2018), the *Ficus deltoidea* Jack varieties have excellent antioxidant activity with no cytotoxic effects on normal liver cells. The leaves caused no signs of toxicity in a normal liver cell line. Misbah et al. (2013) have investigated the antidiabetic and antioxidant activities of the fruits from different varieties of *F. deltoidea*, employing *in vitro* methods. The antioxidant activities of the fruits of *F. deltoidea* might be asserted by the phenolic content but other polar plant components were possibly involved in the antidiabetic properties. The study of these compounds having both antihyperglycemic and antioxidant activities may provide a new approach in the treatment of diabetes mellitus (Misbah et al., 2013).

In our previous studies, *in vitro* protective effects of extracts obtained from various tropical and subtropical plants against hemolysis, lipid peroxidation, and oxidative damage of proteins in equine erythrocytes were evaluated (Tkachenko et al., 2017, 2018; Buyun et al., 2018). In case of *Ficus* genus, we have focused on the evaluation of antioxidant effect of leaf extract obtained from *F. religiosa* L. based on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification (OMP), total antioxidant capacity (TAC)] using the equine erythrocytes model. Treatment by plant extract reduced the erythrocytes TBARS level by 25.3% (p = 0.009), while plasma TBARS level was increased by 75.6% (p = 0.000), as compared to untreated erythrocytes. When plasma was incubated with extract, the ketonic derivatives level was significantly increased by 22.8% (p = 0.000), while non significantly decrease both aldehydic and ketonic derivatives of OMP was observed. Treatment by *F. religiosa* extract caused the increase of TAC in plasma and

erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant. Consequently, all these data suggest that *F. religiosa* could be explored for its antioxidant potential (Tkachenko et al., 2018).

Hence, it becomes necessary to search new sources for noble antioxidants, especially those that would be safe and cheap and thus easily affordable by all populations. The present study was designed to investigate the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] using the equine erythrocytes' model to evaluate the antioxidant activities of the aqueous extracts of leaves derives from vegetative and mature shoots of *Ficus pumila* L.

Ficus pumila is a climbing fig commonly used as an ornamental plant. It is reported to be native to East Asia, specifically South China through to Malaysia, but now it is cultivated in numerous countries around the world (Starr et al., 2003). It was reported that *F. pumila* is a very aggressive vine that rapidly grows on almost any surface, climbing by means of clusters of adventitious roots that emerge from internodes and sometimes form into an aggregate structure called an adhesive disk or pad (Groot et al., 2003).

Ficus pumila is a functionally dioecious species, i.e. it consists of female individuals with syconia which include only long-style female flowers and "male" individuals whose syconia include both male flowers and short-style flowers (Chen et al., 2008). The juvenile foliage is much smaller and thinner than mature leaves produced as the plant ages.

According to Yong et al. (2013), the leaves of this plant have antimicrobial, antileishmanial, and anti-inflammatory effects among many other medicinal benefits. These benefits could be attributed to the wide array of chemicals contained in it (Larbie et al., 2015). The Okinawan folks in Japan use *F. pumila* as a beverage or herbal medicine to treat diabetes and high blood pressure (Leong et al., 2008). The preparation of Ooitabi leaves in water provides a sufficient amount of flavonoid glycosides to the Okinawan although 50% of aqueous ethanol extracted these flavonoid glycosides more effectively. The potential of Ooitabi leaves as a natural source of antioxidants for health management was proved by Leong et al. (2008). Also, it was shown that the latex of *F. pumila* can cause phytophotodermatitis (Rademaker and Derraik, 2012), a toxic reaction caused by direct skin exposure to certain plant compounds, followed by exposure to ultraviolet (UV) light. Therefore, this plant, like all other ornamental *Ficus* spp. plants are very likely to contain certain important phytochemicals with pharmacological properties that could be useful (Larbie et al., 2015).

In this study, we report the data on the antioxidant properties of *F. pumila* in different plant parts (vegetative and mature shoots) to identify the plant part that gives the highest antioxidant activities and this may justify important ethno-medical uses of this medicinal plant as antioxidants possessing diverse biological actions.

Material and methodology

Collection of Plant Materials

The leaves of *F. pumila* (juvenile and mature shoots) were collected in M.M. Gryshko National Botanic Garden (Kyiv, Ukraine) (Figure 1, 2). The whole collection of tropical and subtropical plants at M.M. Gryshko National Botanic Garden (Kyiv, Ukraine) (including *Ficus* spp. plants) has the status of a National Heritage Collection of Ukraine. Plant samples were thoroughly washed to remove all the attached material and used to prepare extracts.



Figure 1 General view of *Ficus pumila* specimen with mature shoots climbing on the ventilation facility in the exhibition glasshouse at National Botanic Garden (Photo: Lyudmyla Buyun)

It is generally accepted that flowers and fruits rarely appear on indoor plants. Nevertheless, we have observed the pear-shaped fruits on the *F. pumila* plants cultivated under glasshouse conditions at NBG (Figure 2, B). However, we have not verified that this syconium included true long-style female flowers. Therefore in our study, we judged it appropriate to consider two different developmental stages of *F. pumila* shoots (juvenile and mature), taking into consideration rather morphological and morphometric characteristics of the leaves and shoots, but not plant individual sex differentiation (Figure 2A, 2B).



Figure 2 Leaf morphology of *F. pumila* (Photo: Lyudmyla Buyun)
A – shoot with juvenile leaves; B – fruit-bearing shoot with mature leaves

Preparation of Plant Extracts

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Horses

Eighteen clinically healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30' 48.0" E 16º 57' 44.9"), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. Before sampling, all horses were thoroughly examined clinically by a veterinarian and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma Blood was stored into The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control, phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method

for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of per 1 L was calculated using $1.56 \cdot 10^5$ mM^1/cm^1 as the extinction coefficient.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2.4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP430).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ±S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney *U*-test (Zar, 1999). In addition, the relationships between oxidative stress biomarkers were evaluated using Spearman's correlation analysis. All statistical calculations were performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

The TBARS content as a biomarker of lipid peroxidation in the equine erythrocytes suspension after *in vitro* incubation with *F. pumila* leaf extract (juvenile and mature shoots) was assessed and shown in Figure 3A. As can be seen in Figure 1A, treatment by extract obtained from leaves of mature shoots reduced the erythrocytes TBARS level by 22% (p = 0.029), while TBARS level was increased by 15.5% (p >0.05) when incubated with an extract derived from leaves of juvenile shoots as compared to untreated erythrocytes.

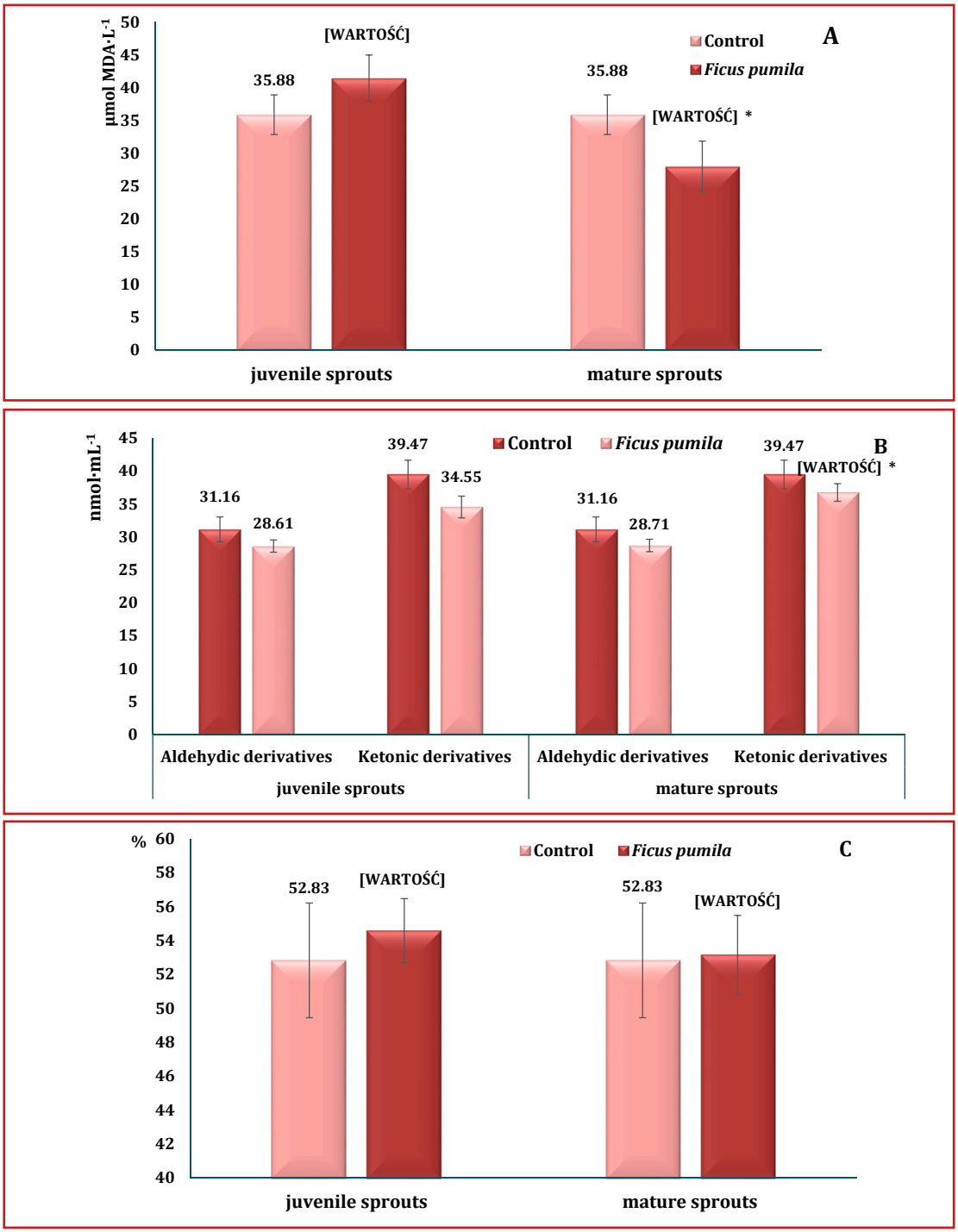


Figure 3 The TBARS content as a biomarker of lipid peroxidation (A), aldehydic and ketonic derivatives of oxidatively modified proteins (B), and total antioxidant capacity (C) in the equine erythrocytes' suspension after *in vitro* incubation with *Ficus pumila* leaf extract obtained from juvenile and mature shoots (M ±m, *n* = 18)

When equine erythrocytes were incubated with extract obtained from leaves of mature shoots, the ketonic derivatives level was significantly decreased by 6.9% ($p = 0.040$) (Figure 3B), while non-significantly decrease both aldehydic and ketonic derivatives of OMP was observed after incubation with extract derived from juvenile shoots (by 8.18 and 12.5%, $p > 0.05$).

As shown in Figure 3C, treatment by *F. pumila* leaf extract (juvenile and mature shoots) caused the increase of TAC in erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant.

Several correlations between checked parameters were found (Figure 4). Erythrocyte TBARS level correlated positively with TAC level in the equine erythrocytes' suspension after *in vitro* incubation with *F. pumila* leaf extract obtained from mature shoots ($y = 42.931 + 0.3655 \times x$; $r = 0.609$; $p = 0.0073$; $r^2 = 0.3713$) (Figure 4A). Decreased TBARS level induced the decrease of aldehydic derivatives of OMP in the equine erythrocytes' suspension after *in vitro* incubation with *F. pumila* leaf extract obtained from juvenile shoots ($y = -15.774 + 1.9995 \times x$; $r = 0.5254$; $p = 0.0252$; $r^2 = 0.2760$). The ketonic derivatives of OMP correlated positively with aldehydic derivatives of OMP ($y = 2.463 + 1.1215 \times x$; $r = 0.6342$; $p = 0.0047$; $r^2 = 0.4021$). The increased level of TAC in erythrocytes' suspension after incubation with leaf extract obtained from *F. pumila* (mature shoots) positively correlated with TAC level (Figure 4).

In the present study, we used an *in vitro* equine erythrocytes model to investigate the antioxidant effect of aqueous extract of *F. pumila* leaves. Many results also clearly suggest that treatment by herbal extracts *in vivo* and *in vitro* study prevents organ damage by a decrease of lipid peroxidation and protection of the antioxidant defense system. On this basis, the current investigation was conducted to evaluate the antioxidant effects of *F. pumila* leaf extract obtained from juvenile/vegetative and mature shoots. The main finding of this study was that the aqueous extract obtained from the mature shoots of *F. pumila* was able to decrease the lipid peroxidation and protein damage biomarkers in the equine erythrocytes' suspension after *in vitro* incubation.

Several methods have been developed to measure the free radical scavenging capacity, regardless of the individual compounds, which contribute towards the total capacity of a plant product in scavenging free radicals (Lo et al., 2004). For instance, Larbie et al. (2015) have determined the antiproliferative activity as well as phytochemicals content, total phenolic content (TPC), and antioxidant activity of the stem and leaf extracts (FPS and FPL) of *F. pumila* using standard methods. The hydroethanolic extracts of the leaves and stems were rich in tannins, general glycosides, saponins, terpenoids, alkaloids, flavonoids (leaves only), and sterols (stem only). Strong total antioxidant activities were observed with FPL and FPS with EC50 values of 0.07 and 0.089 mg/ml, respectively. All the crude extracts showed an anti-proliferative effect on the three human leukemic cell lines used (Jurkat, CEM, and HL-60). However, FPL gave the strongest inhibition concentration at 50% values of 130.97 (Jurkat) and 56.31 µg/ml (HL-60). These findings suggest that crude extracts of FPS and FPL have an anti-proliferative effect on the leukemia cells. The antioxidant properties of the plant including phenolics may be partly responsible for the anti-proliferative activity (Larbie et al., 2015).

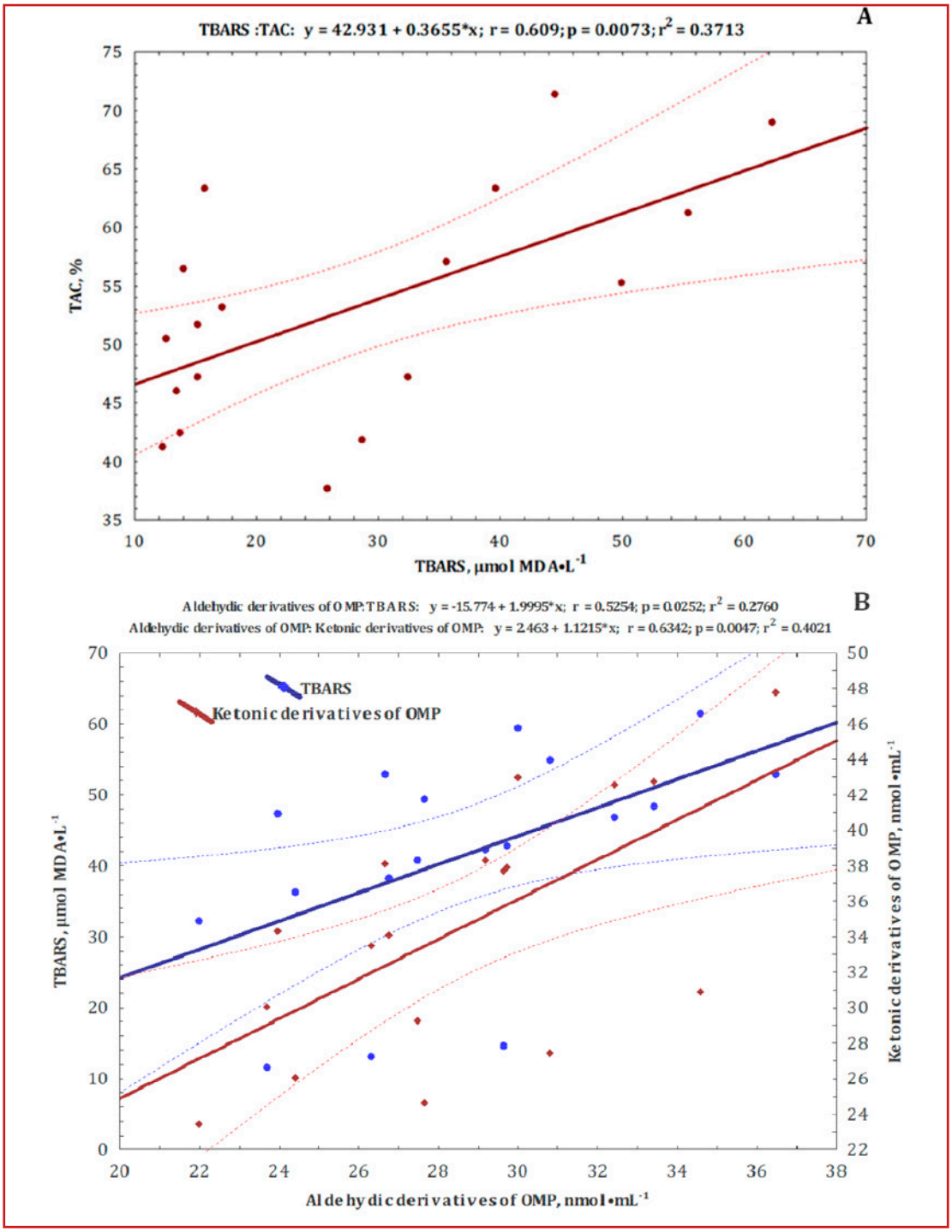


Figure 4 Correlations between oxidative stress biomarkers – TBARS and TAC level (A), aldehydic and ketonic derivatives of OMP and TAC level (B) in the equine erythrocytes' suspension after *in vitro* incubation with *Ficus pumila* leaf extract obtained from mature shoots (A) and juvenile shoots (B)

Collectively, the current available data suggest that the phenolics present in plants belonging to the *Ficus* genus are important antioxidants because they have ideal structural chemistry for free radical scavenging activities, and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis (Ribeiro et al., 2008). Moreover, polyphenols exhibit a wide range of biological effects such as protection of lipid oxidation *in vivo* with significant consequences in atherosclerosis and also protect DNA from oxidative damage with important consequences in the age-related development of some cancers (Reddy et al., 2012; Das et al., 2014). Our findings suggested that leaves of *F. pumila* rich in antioxidant contents which are the major contributor to scavenge the free radicals in oxidation pathways.

We believe, that there is another important aspect in preliminary pharmacological studies of plants that should not be neglected. More than 15,000 angiosperm plant species are dioecious, i.e., having distinct male and female individuals (Seethapathy et al., 2018). The male and female plants also have remarkable differences in ecological adaptability, physiological and biochemical characteristics (Hultine et al., 2016; Li et al., 2016). The allocation of resources between male and female plants is different, and also variation in secondary metabolites is reported among dioecious plants (Li et al., 2016; Seethapathy et al., 2018). However, little is known about the ethnobotany of dioecious species and whether preferences exist in pharmacological investigations for a specific gender of plant samples screened for specific kinds of biological activity.

Consequently, in the current study, we have undertaken an attempt to investigate the *in vitro* antioxidant activity of extracts from the leaves developed on the shoots of various developmental stages (juvenile and mature/generative) of *F. pumila* plants.

The results obtained from the correlation between total antioxidant capacity and oxidative stress biomarkers suggested that antioxidant compounds are dominant contributors to the antioxidant activity of the extracts obtained from *F. pumila* leaves. Our future phytochemical screening of leaves also will reveal the presence of various classes of secondary metabolites which have great importance in medicinal chemistry and natural product research for their high antioxidant properties.

Conclusions

In the present study, analysis of oxidative stress biomarkers showed that the treatment by extract obtained from leaves of mature shoots of *Ficus pumila* reduced the lipid peroxidation and oxidatively modified protein contents in the equine erythrocytes. Extracts derived from both juvenile and mature shoots resulted in an increase of total antioxidant capacity of equine erythrocytes' suspension. However, further detailed investigation, especially *in vivo* and *in vitro* antioxidant and toxicity studies are needed to justify its use as a natural source of antioxidants.

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ANTIBACTERIAL PROPERTIES OF ETHANOLIC EXTRACTS OBTAINED FROM LEAVES OF SOME *THYMUS* L. (LAMIACEAE) REPRESENTATIVES AGAINST *ACINETOBACTER BAUMANNII*

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The *Thymus* L. is one of the most widely used genera in folk medicine, where it is popular for its stimulatory action on all organism functions. The purpose of this study was to evaluate the antimicrobial effects of five ethanolic extracts obtained from leaves of some *Thymus* L. representatives (*Thymus serpyllum* L., *Th. pannonicus* All., *Th.* × *porcii* Borbás, *Th. pulegioides* L., *Th. alpestris* Tausch ex A. Kern.) against *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin (specimen 3680, UK NEQAS). Freshly leaves were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity. Antimicrobial activity was determined using the agar disk diffusion assay. The present study has shown that ethanolic extracts obtained from leaves of *Thymus* species inhibited mild activity against *A. baumannii*. The mean diameter of inhibition zone for *Th. serpyllum* was (10.45 ±0.81) mm, for *Th. pannonicus* (10.82 ±0.63) mm, for *Thymus* × *porcii* (9.57 ±0.75) mm, for *Th. pulegioides* (10.54 ±0.52) mm, and for *Th. alpestris* (10.62 ±0.54) mm. It should be noted that the most antimicrobially effective plant against *Acinetobacter baumannii* was *Thymus pannonicus*. The present study lays the basis for future research, to validate the possible use of *Thymus* species as a candidate in the treatment of bacterial infections. The knowledge about the chemical profile of the extract will help in explaining the observed activity and designing experiments for activity fractionation for isolation of the active principle. The identification of precise molecular mechanisms addressing how these extracts inhibit bacterial growth needs to be explored.

Keywords: *Thymus* species, leaf extracts, agar disk diffusion assay, antibacterial activity, inhibition zone diameter

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Introduction

Acinetobacter baumannii is an opportunistic pathogen and one of the six most important multidrug-resistant microorganisms in hospitals worldwide responsible for hospital-acquired nosocomial infections (Antunes et al., 2014; Lee et al., 2017). This human pathogen is responsible for a vast array of infections, i.e. ventilator-associated as well as bloodstream infections in critically ill patients, and mortality rates can reach 35% (Antunes et al., 2014). The spread of multidrug-resistant *Acinetobacter* strains is cause for concern (Harding et al., 2018). Due to the prevalence of infections and outbreaks caused by multi-drug resistant *A. baumannii*, few antibiotics are effective for treating infections caused by this pathogen (Lee et al., 2017).

Because of the limited number of available antibiotics, we used medicinal plants with an antibacterial effect. In our previous study, we have revealed that the ethanolic extracts obtained from leaves of *Thymus* L. representatives exhibited different activity against clinical isolates of methicillin-resistant *Staphylococcus aureus*. Results obtained for antibacterial activity exhibited various degrees of the zone of inhibition and significant activity was observed for *Thymus pulegioide*s L. (21–26 mm) followed by *Th. pannonicus* All. (14–18 mm) and *Th. serpyllum* L. (13–18 mm), respectively while some extracts (*Th.* × *porcii* Borbás and *Th. alpestris* Tausch ex A. Kern.) showed mild activity at the same concentration. The above results revealed that the selected plants can be further utilized for isolation of the active ingredients as the crude extracts were found good for inhibition of MRSA. It is therefore important to investigate the potential of these plants as novel antimicrobial agents, targeting the multidrug-resistant bacteria and clinical importance (Honcharenko et al., 2018a). Additionally, the ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *Salmonella enteritidis* studied and presented in the work by Honcharenko et al. (2018b). The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* (13.1 ±0.85 mm) followed by *Th. alpestris* (12.6 ±0.25 mm), *Th.* × *porcii* (12.2 ±0.55 mm), and then by *Th. serpyllum* (10.5 ±0.23 mm) (Honcharenko et al., 2018b). Moreover, the ethanolic extracts obtained from leaves of *Thymus* representatives exhibited intermediate activity against β-lactamase producing *Pseudomonas aeruginosa* (Honcharenko et al., 2018c). The mean diameter of inhibition zone for *Th. serpyllum* was (11.3 ±0.3) mm, for *Th. pannonicus* (11.5 ±0.5) mm, for *Th.* × *porcii* (9.8 ±0.5) mm, for *Th. pulegioides* (11.2 ±0.7) mm, and for *Th. alpestris* (12.8 ±0.8) mm. The effects varied significantly according to the *Thymus* taxa. It should be noted that the most antimicrobial effective plant against β-lactamase producing *P. aeruginosa* was *Th. alpestris*, being highly active with the ethanolic extract (mean diameter of inhibition zone was 12.8 ±0.8 mm). The antibacterial activity of extracts was greatest for *Th. alpestris* followed by *Th. pannonicus* followed by *Th. serpyllum* and then by *Th. pulegioides* (Honcharenko et al., 2018c).

Our previous studies lay the basis for future research, to validate the possible use of *Thymus* species as a candidate in the treatment of bacterial infections and a potential target for the activity-guided isolation of active constituents in order to explore the mechanism of action and relevant uses in the medicine and veterinary. Recent studies have shown that *Thymus* species have strong antimicrobial and antioxidant activities (Jia et al., 2010). The thyme

essential oil has also remarkable antibacterial effects, which are associated with the presence of their phenolic components, carvacrol, and thymol. In a study by Yamazaki et al. (2004), carvacrol had the most powerful effect against *Listeria monocytogenes*, followed by thymol, eugenol, cinnamaldeyde, and isoeugenol. In comparative studies including the oregano and thyme essential oils, the two oils demonstrated remarkable antibacterial activity.

Thymol is a naturally occurring phenol monoterpene derivative of cymene and isomer of carvacrol and is one of the major constituents (10–64%) of the essential oils of thyme (*Thymus vulgaris* L.). Nowadays, thymol and thyme present a wide range of functional possibilities in the pharmacy, food, and cosmetic industry due to several studies that have evaluated the potential therapeutic uses of this compound for the treatment of disorders affecting the respiratory, nervous, and cardiovascular systems. Moreover, this compound also exhibits antimicrobial, antioxidant, anti-carcinogenesis, anti-inflammatory, and antispasmodic activities, as well as a potential as a growth enhancer and immunomodulator (Salehi et al., 2018).

Schelz et al. (2006) observed that the essential oil of *Thymus vulgaris* inhibited the growth of *Staphylococcus epidermidis* and *Escherichia coli* more efficiently than the essential oils of orange, eucalyptus, fennel, geranium, cedar, ginger, turpentine, rosemary, and tea tree, whereas of 15 essential oils used against foodborne (*Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* 0157:H7) and food spoilage bacteria (*Brochothrix thermosphacta* and *Pseudomonas fluorescens*), the oregano, thyme, and cinnamon oils demonstrated the most powerful antibacterial effect (Mith et al., 2014). Additionally, the oregano, thyme, and basil oils exhibited sufficient to moderate effects against multi-resistant clinical isolates of *Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Sakkas et al., 2016).

These data have prompted us to verify the antibacterial effects of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine against *Acinetobacter baumanii*. Therefore, the aim of this study was to evaluate the antimicrobial effects of five ethanolic extracts obtained from leaves of some *Thymus* representatives (*Thymus serpyllum*, *Th. pannonicus*., *Th.* × *porcii*, *Th. pulegioides*, *Th. alpestris*) against *Acinetobacter baumanii* strain.

Material and methodology

Collection of Plant Materials

Samples were harvested in June–August, 2016. Leaves of *Thymus serpyllum* were collected among the grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 03′ 58.9′′, E 26° 13′ 37,5′′, 257 m a.s.l.). Leaves of *Th. pannonicus* were harvested among grass in the roadside between the two cultivated fields (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 09.6′′, E 26° 13′ 19.2′′, 283 m a.s.l.). Leaves of *Th. pulegioides* were collected among grass nearby land parcels (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 02.8′′, E 26° 14′ 13.9′′, 306 m a.s.l.). Leaves of *Th.* × *porcii* (a hybrid between *Th. pannonicus* and *Th. pulegioides*) were sampled in the grass stand, on the side of the footpath of the race track

(Medovoi Pechery Str., Lviv, Ukraine; N 49° 49‘ 15.1“, E 24° 05‘ 12.5“, 348 m a.s.l.). Leaves of *Th. alpestris* were harvested on the side of the road below the stream, in mountain valley Shumneska (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 32.3“, E 24° 21‘ 26.4“, 1,259 m a.s.l.). Identification of these five taxa was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all the attached material and used to prepare ethanolic extracts.

Preparation of Plant Extracts

Freshly leaves were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial strain

For the study, specimen 3680 (UK NEQAS, The United Kingdom National External Quality Assessment Service) was used. It contained an *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin. The organism was borderline susceptible to imipenem and meropenem and only 14.3% of participants reported intermediate or resistant (Seaton et al., 2017).

Agar diffusion susceptibility testing

Antimicrobial activity was determined using the agar disk diffusion technique (Bauer et al., 1966). The *Acinetobacter baumannii* strain was obtained from the Department of Bacteriology, Regional Hospital in Koszalin (West-Pomeranian Voivodeship, Poland). The strain was grown in a test tube containing 45 mL of sterile nutrient broth (Oxoid™ Ltd.) at 37 °C for 24 hours. The purity of the inoculum was confirmed by plating on appropriate selective media and microscopic examination of the Gram-stained smear.

The culture was inoculated onto Mueller-Hinton (MH) agar plates. Sterile filter paper discs impregnated with extracts were applied over each of the culture plates. Isolates of bacteria were then incubated at 37 °C for 24 h. The plates were then observed for the zone of inhibition produced by the antibacterial activity of various ethanolic extracts obtained from leaves of *Thymus* representatives. The presence of inhibition zones around each of paper discs after the period of incubation was regarded as the presence of antimicrobial action while the absence of any measurable zone of inhibition was interpreted as the absence of antimicrobial action. Negative control discs impregnated with sterile ethanol were used in each experiment. The antimicrobial activities of the extracts tested were evaluated at the end of the inoculated period by measuring the inhibition zone diameter around each paper disc in millimeters. The plates were observed and photographs were taken. For each extract, eight replicate trials were conducted. Zone diameters were determined and averaged.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Poland). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Antimicrobial activity of various ethanolic extracts obtained from leaves of *Thymus* species against *Acinetobacter baumannii* measured as inhibition zone diameter is shown in Figure 1 and 2. The present study has shown that ethanolic extracts obtained from leaves of *Thymus* species inhibited mild activity against *A. baumannii*. The mean diameter of inhibition zone for *Th. serpyllum* was (10.45 ±0.81) mm, for *Th. pannonicus* (10.82 ±0.63) mm, for *Thymus* × *porcii* (9.57 ±0.75) mm, for *Th. pulegioides* (10.54 ±0.52) mm, and for *Th. alpestris* (10.62 ±0.54) mm (Figure 1 and 2).

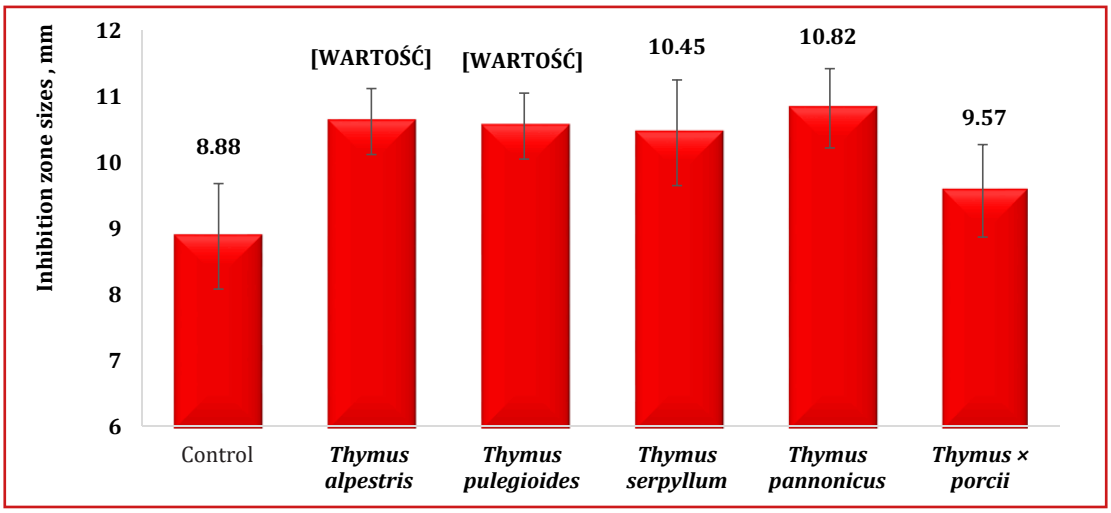


Figure 1 The mean of inhibition zone diameters of various ethanolic extracts obtained from leaves of *Thymus* species against *Acinetobacter baumannii* (M ±m, *n* = 8)

Detailed data regarding the zones of inhibition by the various plant extracts were recorded and presented in Figure 2.

The main purpose of our study was to assess the antimicrobial effects of five ethanolic extracts obtained from leaves of *Thymus* species against *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin (specimen 3680, UK NEQAS). Ethanolic extracts of the plant materials decrease the growth of the strain studied. The effects varied significantly

according to the *Thymus* species. It was observed in the present study that ethanolic extracts inhibited the growth of bacteria tested moderately. The highest antimicrobial effect was recorded for *Th. alpestris*, *Th. pannonicus*, and *Th. pulegioides* (Figure 1 and 2). It should be noted that the most antimicrobially effective plant against *Acinetobacter baumannii* was *Th. pannonicus* (mean diameter of inhibition zone was 10.82 ±0.63 mm) (Figure 1).



Figure 2 Antimicrobial activity of various ethanolic extracts obtained from leaves of *Thymus*. pannonicus (37), *Thymus* × *porcii* (38), *Th. pulegioides* (39), *Th. serpyllum* (40), *Th. alpestris* (41, 42) against *Acinetobacter baumannii* measured as inhibition zone diameter

Many works support the traditional use of *Thymus* species as valuable plant-based compounds for the treatment of wounds. Ustuner et al. (2019) have observed that *Thymus sipyleus* Boiss. subsp. *rosulans* (Borbas) Jalas (TS) extracts can promote the healing process by enhancing fibroblast migration, proliferation, and collagen synthesis as well as suppressing pro-inflammatory cytokines.

Fresh and dried *Thymus* species, as well as their processed products, have been widely used as flavorings since ancient times; however, during the last few decades, they also have become a subject for a search of natural antioxidants and antimicrobial agents (Jia et al., 2010). For example, Demirci et al. (2018) have evaluated *in vitro* antimicrobial activities of the essential oil derived from *Thymus sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus* by agar diffusion, microdilution, and vapor diffusion methods against selected rhinosinusitis associated strains such as *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, *Streptococcus pyogenes*, *S. pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Additionally, the *in vitro* anti-inflammatory activity was evaluated by 5-lipoxygenase (5-LOX) inhibitory effect of the essential oil spectrophotometrically. The antibacterial activity against rhinosinusitis pathogens varied between 160 and 1,250 µg/

mL minimum inhibitory concentrations, with the best inhibitory effects observed against the *S. aureus*, *S. pyogenes* and *M. catarrhalis*. The anti-inflammatory activity of the oil was determined as 12.1 ±1.8% in 100 μg/mL. The results Demirci et al. (2018) showed the *in vitro* antimicrobial and anti-inflammatory potential of the oil also in the vapor phase against sinusitis supporting the traditional use.

Antimicrobial activities of the essential oils from *Thymus marschallianus* Will. and *Thymus proximus* Serg. growing in the wild in Xinjiang against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, yeast, *Rhizopus*, and *Penicillium* were investigated by Jia et al. (2010). The inhibition zones (IZ) and minimum inhibitory concentration (MIC) values were 5.0 to 35.7 mm in diameter and 1.81 to 4.52 μL/mL, respectively. Benameur et al. (2019) have evaluated the susceptibility of blaESBL producing Enterobacteriaceae to Slovakian *Thymus vulgaris* essential oil alone and in combination with cefotaxime. Thymol (34.5%), *p*-cymene (22.27%) and linalool (5.35%) were the major components present in the essential oil. The identified strains were multi-drug resistant (MDR). *Thymus vulgaris* essential oil showed high activity against all MDR strains, including blaESBL producing isolates, with inhibition zones and MIC values in the range of 24–40 and 2.87–11.5 μg/mL, respectively. *Thymus vulgaris* essential oil in combination with cefotaxime showed a synergistic action against blaSHV-12 producing *Escherichia coli* (FICI 0.28) and an additive effect vs ESBL producing *Enterobacter cloacae* (FICI 0.987).

Thymus vulgaris essential oil could be an alternative to classical antibiotics against bacterial biofilms, which show increased tolerance to antibiotics and host defense systems and contribute to the persistence of chronic bacterial infections (Perez et al., 2019). Lagha et al. (2019) have studied the antibacterial activity of five medicinal plant essential oils against urinary tract infections caused by *E. coli* using disc diffusion and minimal inhibition concentration (MIC) methods. In addition, the biofilm inhibitory action of oils was realized by crystal violet. Gas chromatography-mass spectrometry analysis showed variability between oils in terms of compound numbers as well as their percentages. Antibacterial activity was observed only in cases of *Origanum majorana*, *Thymus zygis*, and *Rosmarinus officinalis*, while *Juniperus communis* and *Zingiber officinale* did not show any effect towards *E. coli* isolates. *T. zygis* essential oil demonstrated the highest antibacterial activity against *E. coli* isolates, followed by *O. majorana* and *R. officinalis*. Further, oils showed high biofilm inhibitory action with a percentage of inhibition that ranged from 14.94 to 94.75%. *R. officinalis* oil had the highest antibiofilm activity followed by *T. zygis* and *O. majorana* (Lagha et al., 2019).

The synthesized silver nanoparticles (AgNPs) using *Thymus kotschyanus* Boiss. & Hohen. revealed significant antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, while the growth inhibition of AgNPs at 1,000–500 μg/ml occurred against *Klebsiella pneumonia* and at 1000–250 μg/ml of AgNPs was observed against *E. coli* (Gholami et al., 2018).

Manconi et al. (2018) have extracted, characterized, and formulated *Thymus capitatus* (*Tymbra capitata*) essential oil in phospholipid vesicles: liposomes, glycerosomes and Penetration Enhancer-containing Vesicles (PEVs). The antibacterial activity of the oil was demonstrated

against cariogenic *Streptococcus mutans*, *Lactobacillus acidophilus*, and commensal *Streptococcus sanguinis*. The combination of antioxidant and antibacterial activities of *Thymus* essential oil formulations may be useful for the treatment of oral cavity diseases.

In vitro assessment of antioxidant and antimicrobial activities of methanol extracts and essential oil of *Thymus hirtus* sp. *algeriensis* was carried out by Fatma et al. (2014). The essential oils of *Thymus hirtus* sp. *algeriensis* were screened for their antibacterial activity against six common pathogenic microorganisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteridis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes*) by well diffusion method and agar dilution method (MIC). All the essences were found to inhibit the growth of both Gram-positive and Gram-negative bacteria organisms tested. These activities were correlated with the presence of phenolic compounds in the active fractions. HPLC confirmed the presence of phenolic compounds in methanol extracts (Fatma et al., 2014).

The biological activities of *Thymus* plants and essential oils depend on their chemical composition, which is determined by the genotype and influenced by environmental conditions (Jia et al., 2010). The main constituents of *Thymus vulgaris* leave essential oil are 2 phenolic compounds, thymol (2-isopropyl-5-methylphenol) and its conformational isomer, carvacrol (5-isopropyl-2-methylphenol). Further components in the essential oil are thymol methyl ether, cineol, cymene, α-pinene, and borneol (Pina-Vaz et al., 2004). The antimicrobial activities of *Thymus vulgaris* oil are mostly believed to be related to the thymol and carvacrol contents of the oil (Fani and Kohanteb, 2017). The main compounds identified in the infusion of *Th. pulegioides* essential oil were: carvacrol (38.96%), thymol (25.17%), linalool (9.75%) and thymoquinone (7.27%), while in hydrodistillate: carvacrol (63.20%), linalool (16.95%) and thymol (15.55%) (Rădulescu et al., 2009). It is well documented that the presence of these chemicals is responsible for various medicinal properties of plants. There are many reports available to support the role of phytochemicals and their activity against specific diseases.

CONCLUSIONS

The ethanolic extracts obtained from leaves of *Thymus* species showed different antibacterial activities against *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin (specimen 3680, UK NEQAS). It should be noted that the most antimicrobially effective plant against *Acinetobacter baumannii* was *Thymus pannonicus*. The present study lays the basis for future research, to validate the possible use of *Thymus* species as a candidate in the treatment of bacterial infections.

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MOLECULAR VARIABILITY OF OLD MAIZE (*ZEA MAYS* L.) BASED ON GENE SPECIFIC MARKERS

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Maize (*Zea mays* L.) or corn is a plant belonging to the family of grasses and is one of the most important cereal crops worldwide as a human nutrient, a basic element of animal feed and raw material for manufacture of many industrial products. Maize is the oldest plant to have a fully established gene map with the basic genome consisting of 10 chromosomes and is an excellent plant for the detection of genotoxins, mutagenic and clastogenic substances in the environment. The SSR molecular markers were used to assess genetic diversity in 20 old European maize genotypes. Five SSR primers revealed a total of 33 alleles ranging from 5 (UMC1962) to 8 (UMC1370) alleles per locus with a mean value of 6.60 alleles per locus. Variations in DNA sequences lead to polymorphism. Greater polymorphism is indicative of greater genetic diversity. The PIC values ranged from 0.780 (UMC1962) to 0.842 (UMC1370) with an average value of 0.814 and the DI value ranged from 0.794 (UMC1962) to 0.848 (UMC1370) with an average value of 0.823. 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis. Probability of identity (PI) was low ranged from 0.005 (UMC1241 and UMC1370) to 0.011 (UMC1859) with an average of 0.007. A dendrogram based on UPGMA analysis separated 20 maize genotypes into two clusters. The first cluster contained two maize genotypes Bučiansky Konský Zub (SK) and Moldavskaja (SUN). Cluster two was divided into two main clusters 2a and 2b. SSR markers are useful in the assessment of maize diversity, the detection of duplicate samples in genotypes collection, and the selection of a core collection to enhance the efficiency of genotypes management for use in maize breeding and conservation.

Keywords: Old Maize, Genetic diversity, SSR markers, Dendrogram, PIC

Introduction

With the advent of the first maize hybrids, in 1933 in the US and around 1950 in Europe, maize cultivation has undergone a complete change. Numerous open-pollinated landraces adapted to specific regions were substituted by a limited number of hybrids bred from a large genetic basis (Gay, 1984). Today, the main maize hybrids cultivated in the world involve a restricted

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number of key inbred lines. Therefore, the genetic diversity of those cultivars is almost certainly limited, in comparison to the large genetic diversity available in genebanks. A few years ago, the threat of genetic erosion led to a significant interest in the assessment of genetic diversity in germplasm collections and a huge number of studies on various crops (Dubreuil and Charcosset, 1998). Molecular markers based on polymerase chain reaction (PCR) methods, such as simple sequence repeats (SSRs) or microsatellites, have become important genetic markers in a wide range of crop species, including maize. SSRs markers have many advantages over other types of molecular markers, such as co-dominance, abundant in genomes, highly polymorphisms, locus specificity, good reproducibility, and random distribution throughout the genome (Sun et al., 2011). These features, coupled with their ease of detection, make them ideal for identifying and distinguishing between accessions that are genetically very similar (Saker et al., 2005). For the analysis of genetic diversity of maize genotypes were used several dominant molecular markers: amplified fragment length polymorphism (AFLP) (Roy and Kim, 2016), random amplified polymorphic DNA (RAPD) (Balážová et al., 2016; Vivodík et al., 2017a), start codon targeted (SCoT) (Vivodík et al., 2017b), inter-simple sequence repeat (ISSR) (Žiarovská et al., 2013) and sequence-related amplified polymorphism (SRAP) (Abd El-Azeem et al., 2015). And codominant molecular markers were also used for the analysis of maize genotypes: simple sequence repeat (SSR) (Shiri et al., 2014), expressed sequence tag (EST)-SSR (Galvão et al., 2015) and using protein markers (SDS-PAGE) (Vivodík et al., 2017c).

The present study aimed to examine the genetic variability within and among old maize genotypes cultivated in Europe, using 5 SSR markers. The data collected will contribute to identification, rational exploitation and conservation of germplasms of maize genotypes.

Material and methods

Plant material and DNA extraction

Maize genotypes (20) were obtained from the Gene Bank VURV Praha-Ruzine (Czech Republic) and from the Gene Bank in Piešťany, the Slovak Republic. DNA of 20 genotypes of maize was extracted from leaves of 10-day old seedlings using the Gene JET Plant Genomic DNA Purification Mini Kit. Maize genotypes were grown in a growth chamber on humus soil.

SSR amplification and statistical analysis

SSR analysis: Amplification of SSR fragments was performed according to (Elçi and Hançer, 2015) (Table 1). Polymerase chain reaction (PCR) was performed in 20 µl of a mixture containing 7.5 µl H2O, 10.0 µl Master Mix (Genei, Bangalore, India), 0.75 µl of each primer (10 pmol) and 1 µl DNA (100 ng). Amplification was performed in a programmed thermocycler (Biometra, Germany) and amplification program consisted of an initial denaturing step at 94 °C for 2 min, followed by 35 cycles of amplification [95 °C (30 s), 1 min at the 55 °C, 72 °C (30 s)] and a final elongation step at 72 °C for 10 min. Amplification products were confirmed by electrophoresis in 7% denaturing polyacrylamide gels and silver stained and documented using gel documentation system Grab-It 1D for Windows.

Table 1 List of 5 SSR primers (Elçi and Hançer, 2015)

SSR marker	F primer	R primer
UMC1859	ATATACATGTGAGCTGGTTGCCCT	GCATGCTATTACCAATCTCCAGGT
UMC1241	TGAAGCAAGTCACTGGTAAGAGCA	TGACACACCCATACTTCCAACAAG
UMC1370	GGGAGCACACACAGTAGTACTCGAT	AGAGGCTCTCCTCCTTCAAGCTC
UMC1962	ATAAGTGGGGGAGGCGAGCTA	GAGAACCAACCACCAAAGAAGTCC
UMC1380	CTGCTGATGTCTGGAAGAACCCT	AGCATCATGCCAGCAGGTTTT

Results and discussion

Five maize SSR primers were used for the identification and estimation of the genetic relations among 20 old European maize genotypes. All 5 SSR primers generated clear banding patterns with high polymorphism (Figure 1). Five SSR primers revealed a total of 33 alleles ranging from 5 (UMC1962) to 8 (UMC1370) alleles per locus with a mean value of 6.60 alleles per locus (Table 2). Variations in DNA sequences lead to polymorphism. Greater polymorphism is indicative of greater genetic diversity. The PIC values ranged from 0.780 (UMC1962) to 0.842 (UMC1370) with an average value of 0.814 and the DI value ranged from 0.794 (UMC1962) to 0.848 (UMC1370) with an average value of 0.823 (Table 2). 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis. Probability of identity (PI) was low ranged from 0.005 (UMC1241 and UMC1370) to 0.011 (UMC1859) with an average of 0.007 (Table 2). A dendrogram based on UPGMA analysis separated 20 maize genotypes into two clusters. The first cluster contained two maize genotypes Bučiansky Konský Zub (SK) and Moldavskaja (SUN). Cluster two was divided into two main clusters 2a and 2b. The main cluster 2a contained genotype Dnepropetrovskaja (SUN) and main cluster 2b was divided into two subclusters 2ba and 2bb. Subcluster 2ba contained three genotypes – Iregszemeseil 2 hetes (HUN), Aranyozon sarga lofogu (HUN) and Mikulická (CZE) and subcluster 2bb contained other 14 genotypes of maize. We could not distinguish two genotypes, M Silokukurica (HUN) and Bezuncukskaja (SUN) (subcluster 2bb), which can be caused due to close genetic background (Figure 2).

Table 2 List of SSR primers, the total number of bands and the statistical characteristics of the SSR markers used in maize

Marker name	Number of alleles	DI	PIC	PI
UMC1859	7	0.808	0.799	0.011
UMC1241	6	0.830	0.823	0.005
UMC1370	8	0.848	0.842	0.005
UMC1962	5	0.794	0.780	0.010
UMC1380	7	0.835	0.827	0.006
Average	6.60	0.823	0. 814	0.007

Note: DI – diversity index; PIC – polymorphic information content; PI – probability of identity.

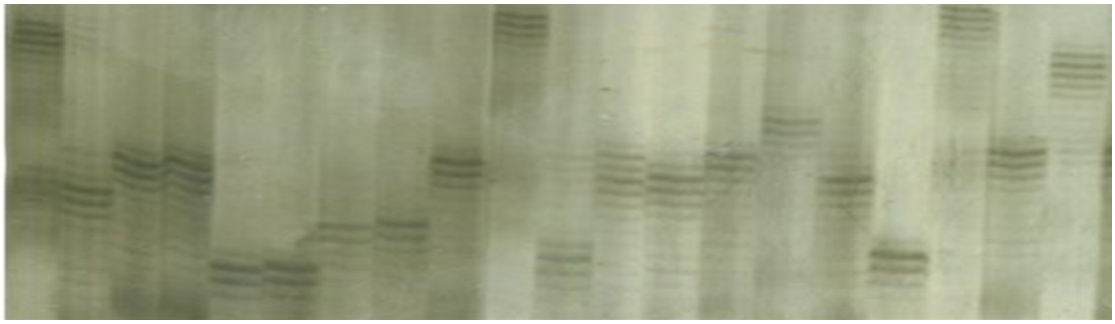


Figure 1 PCR amplification products of 20 genotypes of maize produced with SSR primer UMC1859. Lanes 1–20 are maize genotypes

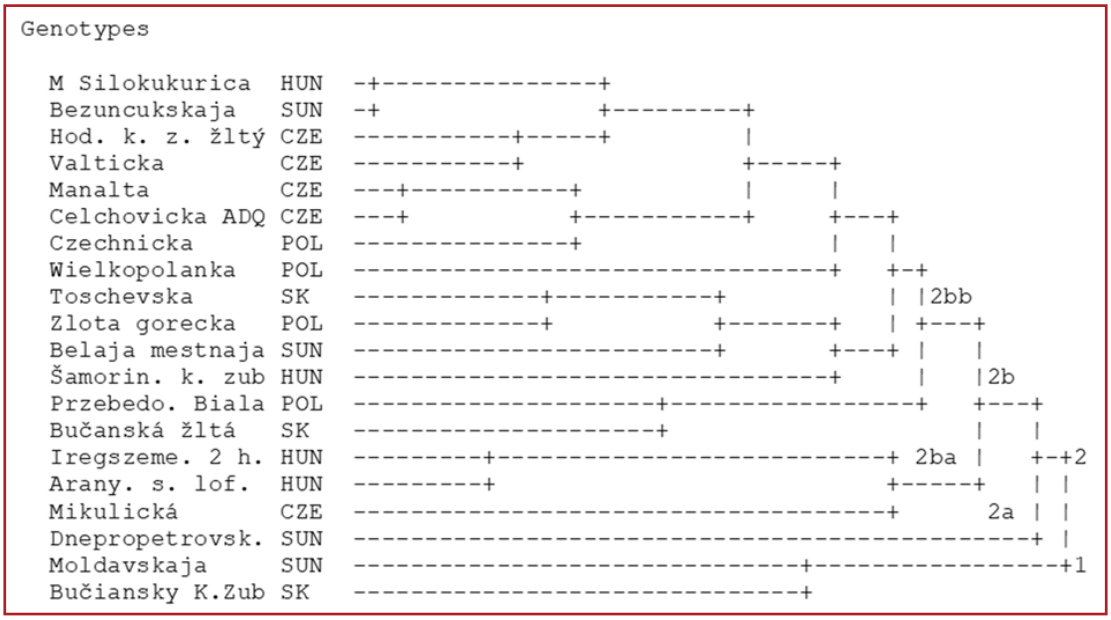


Figure 2 Dendrogram of 20 old maize genotypes prepared based on 5 SSR markers
CZE – Czechoslovakia; HUN – Hungary; POL – Poland; SUN – Union of Soviet Socialist Republics; SK – Slovakia

Efendi et al. (2015) study the level of homozygosity and genetic diversity by codominant SSRs markers. The research aimed to select homozygosity and analyze genetic diversity of 51 maize inbreds using 36 SSRs markers. The research was aimed to select among 51 maize inbreds with high homozygosity and to investigate the genetic diversity using 36 SSRs markers. The result shows that there were 30 inbreds indicating homozygosity level of more than 80%. The diversity of those inbreds was moderately high, with genetic similarity of between 0.22 and 0.87 distributed within six heterotic groups. Genetic diversity of 38 maize hybrids was studied by Shiri et al. (2014) using 12 SSR primers and morphological traits under two different irrigation conditions. The 38 hybrids were evaluated in two trials, one under well-watered (WW) conditions and one under drought-stress (DS) conditions, using an RBCD

design with three replications for two years (2008–2009) in Moghan, Iran. The total number of PCR-amplified products was 40 bands, all of them polymorphic. Primer Phi031 generated the highest number of bands (6). Among the studied primers, UMC2359, PHI031 and UMC1862 showed the maximum polymorphism information content (PIC) and the greatest diversity. The aim of the study Salami et al. (2016) was to evaluate the genetic diversity of Benin's maize accessions by SSR marker. Thus, one hundred eighty seven maize accessions from three areas (South, Center and North) were analyzed using three SSR markers. A total of 227 polymorphic bands were produced and showed high genetic diversity (Shannon index = 0.51). The polymorphic information content (PIC) values for the SSR loci ranged from 0.58 to 0.81, with an average of 0.71. Genetic distance-based UPGMA dendrogram showed a genetic differentiation between accessions and they were grouped into four clusters in each area. Nine flint and nine dent accessions from six agro-ecological groups (races), chosen on the basis of diverse pedigrees, were analyzed by Ignjatovic-Micic et al. (2015) for genetic relatedness using phenotypic and simple sequence repeat (SSR) markers. One of the aims was to establish a reliable set of SSR markers for a rapid diversity analysis using polyacrilamide gels and ethidium bromide staining. In the principal component analysis (PCA) the first three principal components accounted for 80.86% of total variation and separated most of the flint from dent landraces. Ten SSR primers revealed a total of 56 and 63 alleles in flint and dent landraces, respectively, with low stuttering and good allele resolution on the gels. High average PIC value (0.822) also supports informativeness and utility of the markers used in this study.

Conclusion

In conclusion, a high level of genetic diversity exists among the old maize accessions analyzed. According to analysis, the collection of 20 diverse accessions of maize was clustered into two clusters. The first cluster contained two maize genotypes Bučiansky Konský Zub (SK) and Moldavskaja (SUN). Cluster two was divided into two main clusters 2a and 2b. Main cluster 2a contained genotype Dnepropetrovskaja (SUN) and main cluster 2b was divided into two subclusters 2ba and 2bb. Subcluster 2ba contained three genotypes – Iregszemeseil 2 hetes (HUN), Aranyozon sarga lofogu (HUN) and Mikulická (CZE) and subcluster 2bb contained other 14 genotypes of maize. We could not distinguish two genotypes, M Silokukurica (HUN) and Bezuncukskaja (SUN) (subcluster 2bb), which can be caused due to close genetic background. An SSR marker system is a rapid and reliable method for cultivar identification that might also be used in quality control in certified seed production programs, to identify sources of seed contamination, and to maintain pure germplasm collections.

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ALLELOCHEMICALS FROM *CASTANEA SATIVA* MILL.: PLANT-ROOT ENVIRONMENT INTERACTIONS

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The paper is devoted to the study of the allelopathic properties of the root environment of *Castanea sativa* Mill. plants of different ages under the conditions of introduction. The objects of the research were 10 and 40-year-old plants of *C. sativa*, which are growing in Forest-Steppe of Ukraine in M.M. Gryshko National Botanical Garden of National Academy of Sciences of Ukraine. Rhizosphere soil samples were collected at 0–30 cm layer. The fallow soil was used as a control. Allelopathic and biochemical analyzes of root environment of *C. sativa* were conducted in dynamics during the growing season. Allelopathic activity of the root environment was studied by direct bioassay method. The soil redox potential (Eh) was measured by potentiometric technique. Phenolic compounds were extracted from the soil by desorption method using an ion exchanger KU-2-8 (H^+). Allelopathic analysis of the root environment of chestnut plants showed phytotoxicity within the range of 19.9–61.9% compared with control. Phytotoxicity increased under the influence of 40-year-old plants. The redox status of the root environment of *C. sativa* was characterized by the predominance of reducing conditions, the intensity of which increased under the influence of the older plants. The values of redox potential of the rhizosphere soil were 1.2–3.5 times lower than control. This indicates the accumulation of mobile organic compounds in the root environment of *C. sativa*. The content of phenolic compounds in the root environment of *C. sativa* was 1.4–2.5 times higher than control. The accumulation of organic compounds of phenolic nature caused obviously an increase in the phytotoxicity of the root environment of *C. sativa* along with the age of the plants. Thus, the seasonal dynamics of allelopathic activity, redox conditions, and the content of phenolic allelochemicals in the root environment of *C. sativa* depended on the age of plants.

Keywords: *Castanea sativa*, root environment, allelopathic activity, phenolic allelochemicals, redox potential

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Introduction

Sweet chestnut (*Castanea sativa* Mill., Fagaceae family) is a valuable food, ornamental, honey and medicinal plant. *C. sativa* is a rich source of broad-spectrum bioactive compounds and nutrients, that give it medicinal properties (Leonardi et al., 1996; Chiarini et al., 2013; Fuente-Maqueda, 2018; Grygorieva et al., 2018; Cerulli et al., 2018; Esposito et al., 2019). *C. sativa* exhibits antibacterial, antifungal, antioxidant and cardioprotective effects (Basile et al., 2000; Chiarini et al., 2013; Esposito et al., 2019). *C. sativa* pollen is highly prized for its nutritional, healing and dietary qualities (Žiarovská et al., 2015). *C. sativa* is an important source of commercially extracted tannins (Melicharová and Vizoso-Arribe, 2012). *C. sativa* is widely used for the commercial production of nuts, used both in fresh and in the form of paste, puree, crumb, flour and confectionery (Demiate et al., 2001; Melicharová and Vizoso-Arribe, 2012; Grygorieva et al., 2017). Chestnut is a moderate heliophile species and has rather high photosynthetic rates, contributing to its fast growth (Covone and Gratani, 2006). In connection with this, *C. sativa* is cultivated for high-quality hardwood timber, used in the furniture industry, as structural material for buildings, as well as for fences, parquets, shelves for wine barrels and baskets (Melicharová and Vizoso-Arribe, 2012).

C. sativa grows in nature mainly in the Mediterranean and Atlantic regions of Europe, Asia Minor and North Africa (Villani et al., 1994; Rubio et al., 2002; Conedera et al., 2004; Krebs et al., 2004; Dengiz et al., 2011; Melicharová and Vizoso-Arribe, 2012; Miguez-Soto et al., 2019). *C. sativa* is spread in Ukraine predominantly in the Precarpathian and Transcarpathian regions. Recently, the economic efficiency of using of *C. sativa* in natural habitats significantly reduced as a result of damage from pests and diseases, and the effects of global climate change (Melicharová and Vizoso-Arribe, 2012; Conedera et al., 2016; Miguez-Soto et al., 2019). Therefore, it is extremely important to cultivate *C. sativa* outside the natural range to preserve its gene pool.

The success of the introduction of new plant species is largely determined by the allelopathic factor, that regulates the processes of growth and development both in plantings of different plant species and in monoculture (Zaimenko et al., 2017; Pavliuchenko et al., 2018). The study of species interactions between *C. sativa* and *Pseudotsuga menziesii* (Mirb.) Franco showed, that mixed plantations had a positive effect on the productivity of the chestnut (Nunes et al., 2011). The ethyl acetate soluble fraction of the aqueous extract from *C. sativa* leaves showed potent allelopathic effect on *Raphanus sativus* seed germination, root and epicotyl growth (Basile et al., 2000).

The purpose of the work was to investigate the allelopathic properties of the root environment of *C. sativa* plants of different ages under the conditions of introduction.

Material and methodology

Plant material and soil source

The objects of the research were 10 and 40-year-old plants of *C. sativa*, which are growing in Forest-Steppe of Ukraine in M.M. Gryshko National Botanical Garden of National Academy of

Sciences of Ukraine. Rhizosphere soil samples were collected at 0–30 cm layer. The fallow soil was used as a control. The soil is a grey forest. Allelopathic and biochemical analyses were conducted in dynamics on phases of plant development during flowering (I), fruit formation (II) and the end of the growing season (III).

Allelopathic activity

Allelopathic activity of the root environment was studied by direct bioassay method on cress (*Lepidium sativum* L.) root growth (Grodzinskij et al., 1990).

Biochemical analyses

The redox potential (Eh) was measured in soil suspension modelling soil solution at the soil to distilled water ratio as 1 : 1 by potentiometric technique (Labuda and Vetchinnikov, 2011; Fiedler et al., 2007). Phenolic compounds were extracted from the soil by desorption method using an ion exchanger KU-2-8 (H^+) (Grodzinskij et al., 1988).

Data analysis

Experimental data were statistically analyzed using the software package Microsoft Excel.

Results and discussion

Allelopathic analysis of the root environment of chestnut plants showed phytotoxicity within the range of 19.9–61.9% compared with control (Figure 1). Phytotoxicity increased under the influence of 40-year-old plants. The allelopathic activity of the root environment was maximal at the end of the growing season, which is obviously due to the release of organic compounds from plant residues.

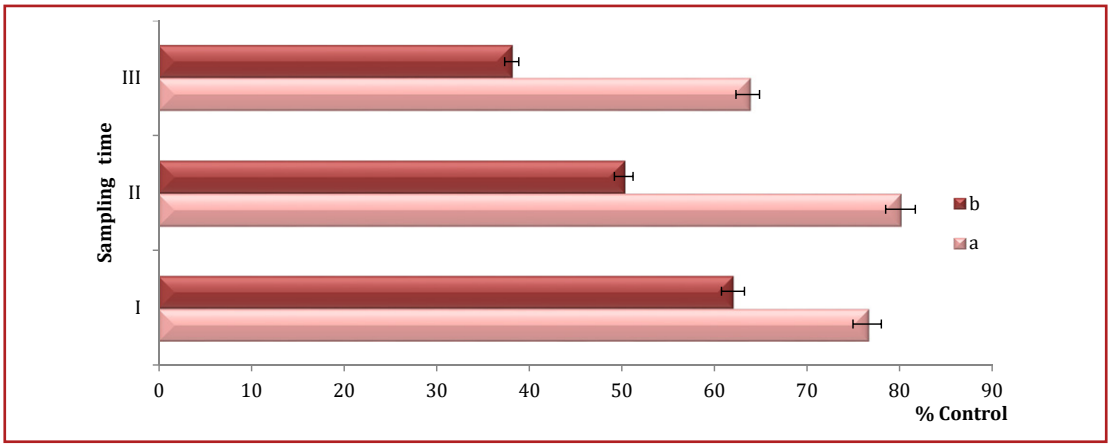


Figure 1 Allelopathic activity of the root environment of *Castanea sativa* (bioassay – root growth of *Lepidium sativum* L.) (%) control
a – 10-year-old plants; b – 40-year-old plants

The degradation of toxic organic compounds depends on their innate chemical properties and environmental conditions (Fiedler et al., 2007). Thus, anaerobic conditions can enhance the

solubility of toxic forms of organic compounds (Fiedler et al., 2007). Therefore, it is extremely important to know the nature of oxidation-reducing processes in the root environment.

The biochemical status of the root environment of *C. sativa* was assessed by the values of the redox potential. The redox status of the root environment of *C. sativa* was characterized by the predominance of reducing conditions, the intensity of which increased under the influence of the older plants (Figure 2).

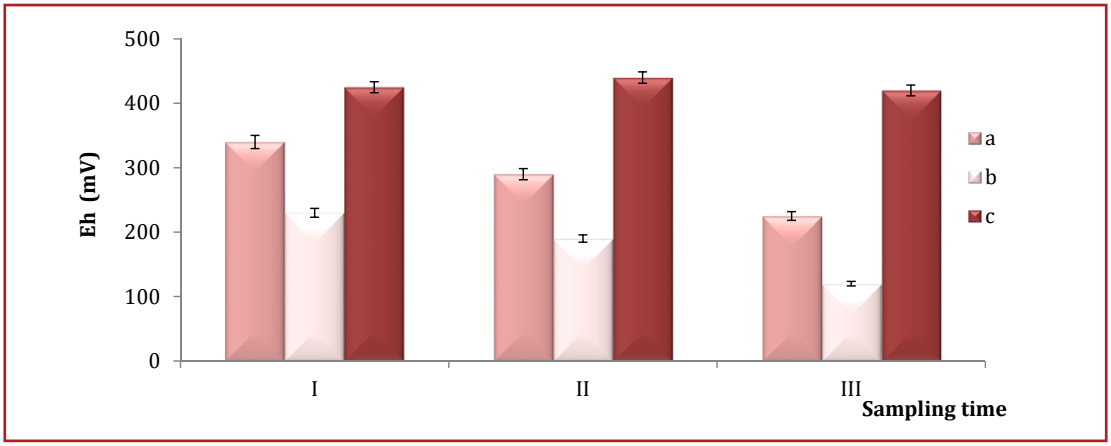


Figure 2 The redox potential (Eh) of *Castanea sativa* rhizosphere soil (mV)
a – 10-year-old plants; b – 40-year-old plants; c – control

At the same time, weakly oxidizing conditions in the control were detected. The values of the redox potential of the rhizosphere soil were 1.2–3.5 times lower than control. This indicates the accumulation of mobile organic compounds, which may have allelopathic properties.

It is known that phenolic compounds are one of the main classes of allelochemicals, since they influence on various physiological and biochemical processes of plants, regulate species interactions in both mixed and pure plantations (Li et al., 2010). Therefore, it was necessary to analyze the content of phenolic allelochemicals in the root environment of *C. sativa*.

The content of phenolic compounds in the root environment of *C. sativa* was 1.4–2.5 times higher than control (Figure 3). The concentration of phenolic compounds was higher in the root environment of the older *C. sativa* plants. The dynamics of accumulation of phenolic compounds in the rhizosphere soil has shown a tendency to gradually increase their content during the growing season.

Litterfall, wood, and leaves of *C. sativa* are an important source of organic matter and nutrients for the soil (Leonardi et al., 1996; Nunes et al., 2011). However, secondary metabolites, including phenolic nature, from different tree organs can also be released into the rhizosphere soil of *C. sativa* through root exudates, leaching, and decay. *C. sativa* contains a significant pool of phenolic compounds, in particular tannins and flavonoids (Chiarini et al., 2013; Fuente-Maqueda, 2018; Esposito et al., 2019). Tannins and phenolic compounds characterized in *C. sativa* bark, including ellagic acid, gallic acid, and ellagitannins (vescalin, castalin,

vescalagin, and castalagin), were reported (Chiarini et al., 2013). Other minor compounds from *C. sativa* bark, detected in trace levels, 5-*o*-galloylhamamelose (3.5-dimethoxy-4-hydroxyphenol)-1-*o*-β-D-(6-*o*-galloyl)-glucoside isomer, *m*-digallic acid, kurigalin isomer, and chestanin were reported (Chiarini et al., 2013). Glucose esters with gallic acid (-mono, -di, and -trigalloylglucose), ellagitannins (vescalagin/castalagin), phenolic acids (gallic, ellagic, protocatechuic, chlorogenic acid), flavonoids (apigenin, quercetin, and quercetin 3-*O*-glucoside) has been identified in chestnut burs (Esposito et al., 2019). A methanol extract of the leaves of *C. sativa* contained as main compounds crenatin, chestanin, gallic acid, cretanin, 5-*O*-*p*-coumaroylquinic acid, *p*-methyl gallic acid and quercetin-3-*O*-glucoside (Cerulli et al., 2018). HPLC analysis of ethyl acetate fraction of *C. sativa* leaf aqueous extract identified the following flavonoids: rutin, hesperidin, quercetin, apigenin, kaempferol, morin, galangin, and naringin (Basile et al., 2000). The ethyl acetate extract from *C. sativa* leaves and therein identified apigenin, rutin, quercetin inhibited seed germination, root and epicotyl growth of *Raphanus sativus* (Basile et al., 2000).

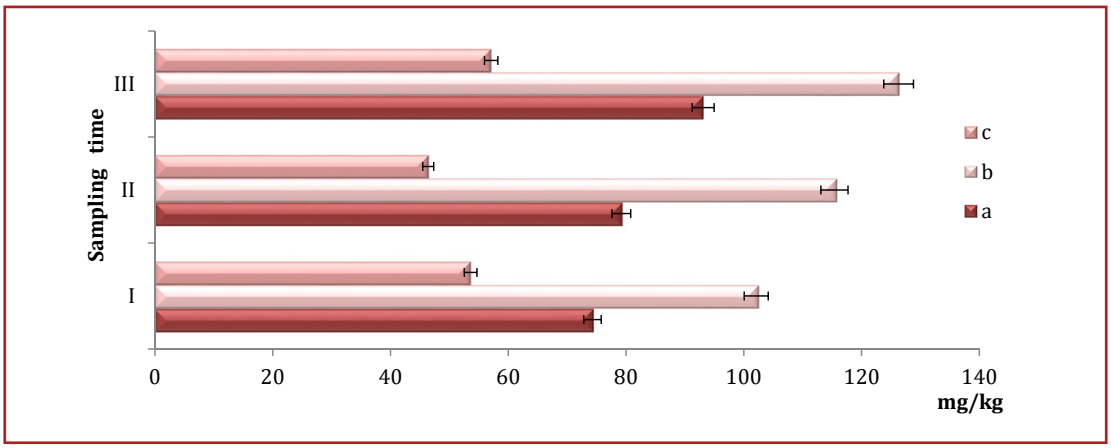


Figure 3 Phenolic compounds content in the root environment of *Castanea sativa (*mg/kg)
a – 10-year-old plants; b – 40-year-old plants; c – control

In connection with the foregoing, it can be stated that the accumulation of phenolic allelochemicals obviously leads to increased phytotoxicity of the root environment of the older *C. sativa* plants.

Conclusions

The accumulation of organic compounds of phenolic nature caused obviously an increase in the phytotoxicity of the root environment of *C. sativa* along with the age of the plants. Thus, the seasonal dynamics of allelopathic activity, redox conditions, and the content of phenolic allelochemicals in the root environment of *C. sativa* depended on the age of plants.

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OPEN ACCESS

MORPHOLOGICAL AND ANATOMICAL STRUCTURAL FEATURES OF SHOOTS OF SPECIES OF THE GENUS *THYMUS* L., INTRODUCED IN BOTANICAL GARDEN OF LVIV NATIONAL UNIVERSITY NAMED AFTER IVAN FRANKO

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The aim of work is the exploration of morphological and anatomical features of species of genus *Thymus* L., which are exhibited at a 'medical plants' collection in Botanical Garden of Ivan Franko National University of Lviv, namely *Th. vulgaris* L., *Th. zygis* L., *Th. camphoratus* Hoffmanns. Link, *Th. callieri* Borb. Anatomical structure studies of a lamina of leaves were conducted with the help of 'Leica DM 500' microscope. Raw of species of genus *Thymus* is aboveground mass on a stage of flowering. In explored species leaves are contrary crisscrossed, in the lower part of sprout they are small petioled and upwards they become sedentary. The pattern of the lamina is changed in a similar way. In all species, these laminas are wrapped up. Pubescence of leaves is made of simple, non-glandular hairs and glandular essential oils (capitate and peltate) trichomes. Leaves *Th. vulgaris* are lanceolate, dark green above, wrapped below, where they are gray from dense pubescence with simple, unicellular, curved hairs. Leaves of *Th. zygis* are grey because of thick pubescence. It is created of a single cell, upwards sharpened hairs, and two-cell curved hairs, where three cellular hairs can be met. In *Th. callieri* leaves are green, over the edge of lamina they have pubescence of long, 5–7 cellular impacted hairs and between them short, 1–3 cellular hairs are situated. Simple hairs also are situated along veins as well. In all of the species capitate trichomes are situated on the surface of a lamina. Except this, on the surface of lamina essential peltate hairs with multicellular glands are situated. Cells of the upper epidermis of *Th. vulgaris* and *Th. zygis* they are oval, *Th. callieri* has epidermis cells with winding walls. The stomata apparatus is of a diacetate type. The amount of stomata is bigger from the abaxial side than from adaxial surface leaves. Essential oil fittings are situated above big veins in mesophile of a leaf. Their numerous cells contain thick cytoplasmic content. The mesophyll is formed from spongy tissue, which has 1 large and, rarely, 2 small essential oil containers (*Th. vulgaris*). Other species have 3–5 essential oil fittings. All species formed inflorescence of the complex tassel. The apex of inflorescences grows monopodially. It is racemous (open). The partial inflorescences are dichasium and

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located in the axils of opposite leaves. The calyx is campanullate with unevenly lobed and pubescent teeth of the lower lip. The corolla is double-lobed, formed from 5 grown petals.

Keywords: *Thymus* spp., morphology and anatomy, leaves, trichomes

Вступ

Колекція лікарських рослин Ботанічного саду Львівського національного університету імені Івана Франка є однією з найчисельніших в Україні та нараховує понад 300 видів, які походять з різних регіонів світу. Рід чебрець (*Thymus* L.) представлений у ній середземноморськими видами *Thymus vulgaris* L., *Th. zygis* L. та *Th. camphoratus* Hoffmanns Link. Останній є ендеміком південного й південно-західного узбережжя Португалії та входить до списку пріоритетних видів Бернської конвенції (*Flora Europaea*, 1972). З видів української флори у Ботанічному саду інтродуковано *Th. serpyllum* L. (борові піски у соснових і мішаних лісах лісостепової зони), *Th. marschallianus* Willd. (узлісся, схили, відслонення у лісостепових і північних степових районах) та *Th. callieri* Borb. (кам'янисті степи і схили степової зони) (Kotov, 1960).

Види роду *Thymus* багаті ефірними оліями і проявляють антимікробні, антиоксидантні властивості, мають широке застосування у медицині, аромотерапії, косметології та в кулінарії (Marino et al., 1999; Lee et al., 2005; Hazzit et al., 2006; Kryvtsova et al., 2017, 2018; Salamon et al., 2018; Santoro et al., 2018). У Державну Фармакопею України (2014) входять два європейські види: *Th. vulgaris* і *Th. zygis* та один – *Th. serpyllum*, що росте в Україні. Інші види широко застосовуються у народній медицині.

Лікарською рослинною сировиною (ЛРС) цих видів є трава. Thymi herba – це *Th. vulgaris* або *Th. zygis*, або суміш цих видів, повинна містити ефірної олії не менше 12 мл/кг, у перерахунку на безводну сировину, в якій сумарний вміст тимолу та карвакролу має бути не менше 40 %. У траві *Serpylli herba* (*Th. serpyllum*) вміст ефірної олії повинен бути не менше 3,0 мл/кг, у національній статті *Serpylli herba* – не менше 1,5 мл/кг. Окрім того, науковцями з різних країн (Senatore, 1996; Mockute et al., 2001; Vaičiulytė et al., 2016; Svydenko et al., 2016) встановлено, що компонентний склад ефірних олій видів роду *Thymus* залежить від кліматичних умов. Так, для популяцій, що ростуть у країнах з теплим кліматом, зокрема, Середземномор'я, характерний хемотип монотерпенового фенолу – тимолу і карвакролу; другий хемотип – монотерпенових вуглеводнів – *p*-цимену і γ-терпінену, характерний для країн з континентальним кліматом.

Метою нашої роботи було визначення морфолого-анатомічних ознак будови надземних органів видів роду *Thymus*, інтродукованих у Ботанічному саду, які можуть бути діагностичними для лікарської рослинної сировини.

Матеріали і методи

Об'єкти дослідження

Об'єктом дослідження були види роду *Thymus*: *Th. vulgaris*, *Th. zygis* і *Th. callieri* – перспективні ефіроолійні і лікарські рослини, які експонуються в колекції лікарських рослин Ботанічного саду Львівського національного університету імені Івана Франка.

Методи дослідження

Дослідження проводили у вегетаційному періоді 2018–2019 років. Для цього у фенофазі повного цвітіння відбирали нездерев'янілі стебла, розташовані на них листки серединної формації, суцвіття та квітки з приквітками. Анатомічні дослідження проводили за методикою Pausheva (1988), з використанням мікроскопу «Leica DM 500». Для вивчення особливостей анатомічної будови виготовляли поперечні зрізи листків і стебел товщиною 10–20 мкм з допомогою мікротому та леза. Вивчали структуру епідермісу верхнього і нижнього боків листкової пластинки. Для освітлення тимчасових препаратів використовували гліцерин, а фарбування проводили розчином йоду в йодистому калії та кислим фуксином. Морфологічні дослідження проводили з використанням бінокулярного мікроскопу МБС 1. Мікрофотографії робили цифровою фотокамерою DCM500. У роботі використовували термінологію з «Державної Фармакопеї України» (2014).

Результати та їх обговорення

Для ідентифікації лікарської рослинної сировини важливе значення має вивчення морфолого-анатомічних ознак будови органів рослини, які також лежать в основі систематики рослинного світу. У «Флорі Європи» (*Flora Europaea*, 1972) рід *Thymus* поділяється на два підроди: моновидовий – *Coridothymus* (Reichenb. Fil.) Borbas і *Thymus*, який включає 65 видів. Досліджувані види *Th. vulgaris* і *Th. zygis* належать до секції *Thymus* (Vulgares Velen., Zygis Willk.), а *Th. callieri* Borb. (*Th. glabrescens* subsp. urumovii (Velen.) Jaias) – до секції *serpyllum* (Miller) Bentham. У виданні «Флора України» (Kotov, 1960) є інформація про *Th. callieri*, який належить до секції *Verticillati* Klok.

Діагностичними в межах роду *Thymus* Nachychko (2015) пропонує використовувати такі ознаки: тип життєвої форми, опушення стебла і листків, форма і спосіб жилкування листкової пластинки, ступінь вираження черешка, а також тип суцвіття та форма і опушення чашечки. Для ідентифікації ЛРС видів роду *Thymus* використовують будову листка, його опушення, наявність ефіроолійних структур та будову квітки (Tykhonenko et al., 2010).

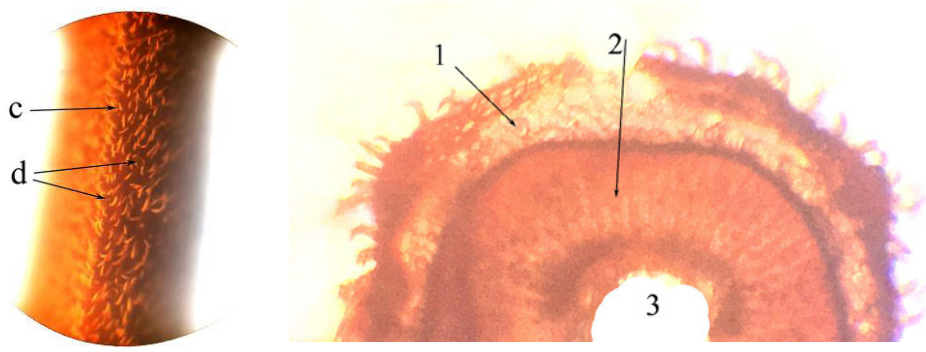
На рівні секцій види класифікуються на основі ряду ознак. Так, види секції *Thymus* – невеликі чагарники з повстистими листками, що мають чітко виражену середню жилку і загорнуті донизу краї. Приквітки схожі на листки, переважно зеленуваті, чашечка дзвониковидна, верхні зубці ланцетно-трикутні, віночок довший за чашечку.

Види секції *Serpyllum* – невеликі чагарники або багаторічні трави. Стебла звичайно трав'яні або здерев'янілі в основі, округлі, опушені по колу, або з двох протилежних

боків. Листки не повстисті, краї не загнуті, приквітки схожі на листки, чашечка дзвониковидна.

В умовах Ботанічного саду досліджувані види роду *Thymus* напівчагарнички, в яких основи пагонів (скелетні осі) здерев'янілі, округлі, а термінальні пагони – трав'янисті. На поперечному перерізі трав'яні стебла округлі, опушені по колу переважно одноклітинними (у *Th. vulgaris* вони загнуті), рідше двоклітинними, відлеглими волосками. У *Th. callieri*, біля суцвіття, опушення інтенсивніше і утворене довгими дво- і триклітинними волосками (рис. 1. III. A1, A2). На стеблі у *Th. vulgaris* і *Th. zygis* розташовані великі головчасті, сидячі волоски (Рисунок 1. I. II. A: 1d).

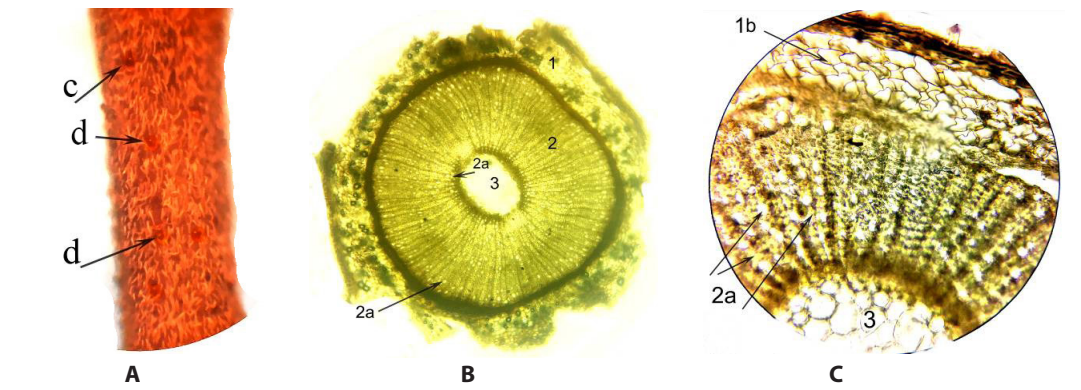
Стебло, фрагменти якого можуть траплятися в сировині, на поперечному перерізі має кору, центральний циліндр та серцевину (Рисунок 1. B, C). Кора у всіх видів тонка, утворена з великих паренхімних клітин, під якими знаходиться флоема (Рисунок 1. B, C: 1b). У видів зі слабо чотиригранними стеблами (*Th. callieri*) спостерігається потовщення по ребрах стебла, утворене з коленхіми (Рисунок 1.III.C,D:1a). Центральний циліндр найширший у *Th. zygis* (Рисунок 1.II.B). У *Th. vulgaris*, *Th. zygis* і *Th. callieri* центральний циліндр непучкового типу, утворений з суцільного шару вторинної ксилеми, спостерігається сильна склерифікація міжсудинної паренхіми (Рисунок 1. I, II, III. B, C: 2a). У середині стебла є серцевина, утворена з великоклітинної паренхіми, яка після відмирання утворює порожнину у дворічних і старіших частинах стебел.



I. *Th. vulgaris*

Рисунок 1 I. Поперечний переріз через стебло
A – зовнішній вигляд стебла (A1 – посередині, A2 – вгорі, під суцвіттям); B – поперечний переріз стебла; C – фрагмент поперечного перерізу стебла: 1 – кора: a – ребра з коленхімою; b– паренхіма кори; c – прості волоски; d – головчасті волоски; 2 – центральний циліндр: a – судини; b – провідний пучок; c – міжпучкова паренхіма; 3 – серцевина

Figure 1 I. Cross-section through the stem.
A – appearance of the stem (A1 – in the middle, A2 – above, under the inflorescence); B – cross-section of the stem; C – fragment of the cross-section of the stem: 1 – bark: a – ribs with collenchyme; b – parenchyma bark; c – simple hairs; d – head hairs; 2 – the central cylinder: a – vessels; b – a conducting bundle; c – an interpunctional parenchyma; 3 – core



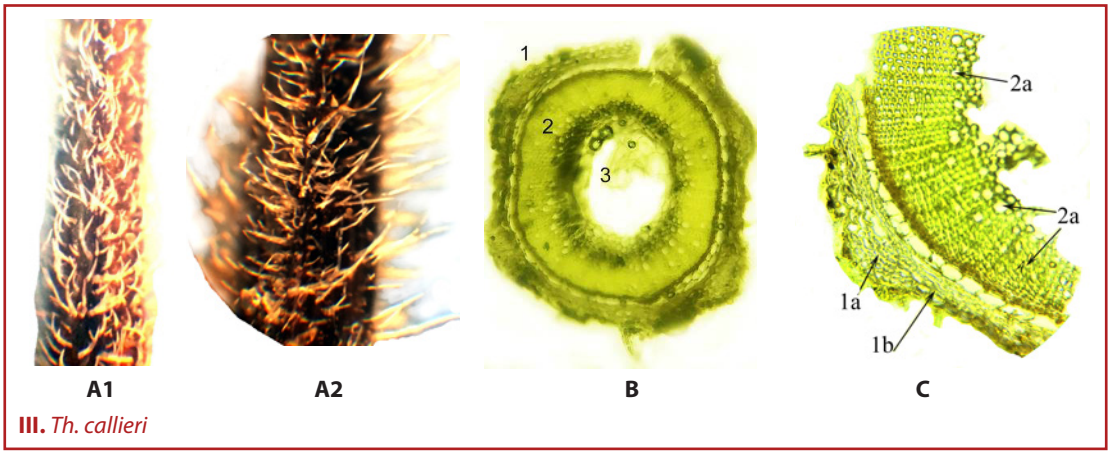


Рисунок 1 II. III. Поперечний переріз через стебло

А – зовнішній вигляд стебла (А1 – посередині, А2 – вгорі, під суцвіттям); В – поперечний переріз стебла; С – фрагмент поперечного перерізу стебла: 1 – кора: а – ребра з коленхімою; b– паренхіма кори; с – прості волоски; d – головчасті волоски; 2 – центральний циліндр: а – судини; b – провідний пучок; с – міжпучкова паренхіма; 3 – серцевина

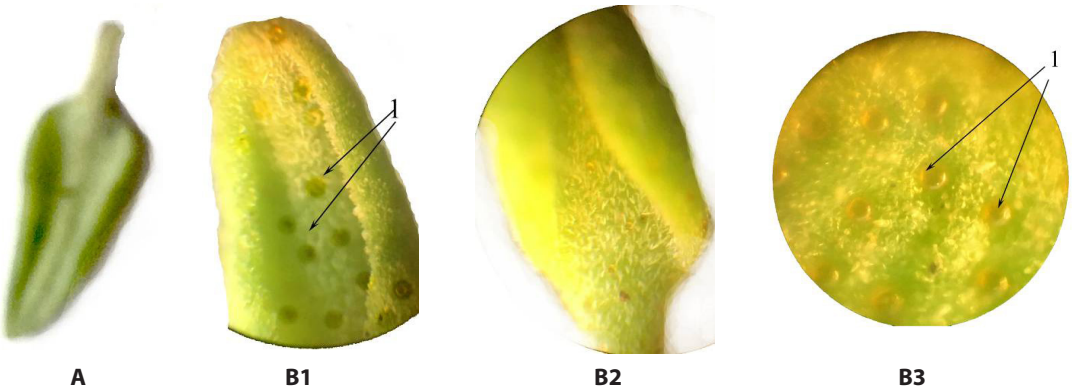
Figure 1 II. III. Cross-section through the stem.

A – appearance of the stem (A1 – in the middle, A2 – above, under the inflorescence); B – cross-section of the stem; C – fragment of the cross-section of the stem: 1 – bark: a – ribs with collenchyme; b – parenchyma bark; c – simple hairs; d – head hairs; 2 – the central cylinder: a – vessels; b – a conducting bundle; c – an interpunctional parenchyma; 3 – core

У досліджуваних видів листкорозміщення навхрест супротивне, у пазухах розвиваються бічні пагони. В нижній частині пагона листки короткочерешкові, у верхній – сидячі. З такою ж закономірністю міняється і форма листкової пластинки (Рисунок 2. I, II, III. А). У *Th. vulgaris* листки видовжено ланцетні, лопатковидні біля основи, темнозелені з адаксіальної і сірі від опушення – з протилежної сторони, дещо видовжуються вгору по пагону. У *Th. zygis* листки дрібні, від довгастоланцетоподібних до лінійних, сірі від густого опушення з обох боків. У *Th. callieri* листки видовженоланцетні, вгору по пагону стають лінійноланцетні, тупуваті на верхівці. У всіх видів край пластинки листка суцільний з загорнутими донизу краями.

Опушення листків утворене з незалозистих одно- і багатоклітинних волосків та залозистих головчастих, які поділяють на капітатні і пельтатні. Перші мають одноклітинну головку, одноклітинну ніжку, або є сидячі, і базальну клітину. У пельтатних волосків («ефіроолійні залозки» (Denisova, 1975) головка сидяча, утворена з 4 клітин і оточена навколо 8–16 розетковими клітинами епідермісу.

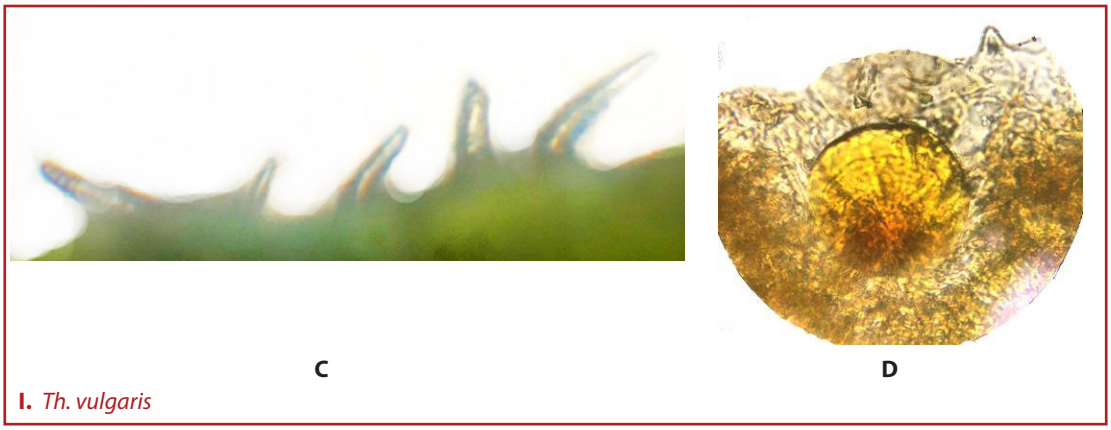
Листкові пластинки *Th. vulgaris* з адаксіальної сторони зелені, опушені простими переважно одноклітинними, зігнутими волосками, між якими є головчасті з одноклітинною ніжкою (Рисунок 2. I. B3). З абаксіальної сторони пластинки сірі від опушення простими одно, рідко двоклітинними, загнутими волосками (Рисунок 2. I. B1, B2; C). Подібне опушення листків *Th. vulgaris* L. описано в літературі (Blažeković et al., 2006; Boz et al., 2009). У *Th. zygis* опушення листків густе, повстисте, з обох боків листка, утворене одноклітинними, овальними при основі та загостреними до верхівки волосками (як зубчики) та двоклітинними волосками. Деякі з них колінчасто зігнуті. Рідше між ними є довгі, триклітинні волоски (Рисунок 2. II. B, C). Загалом, ці два види є дуже близькі між собою, лише *Th. vulgaris* характерний для флори Італії, Болгарії, а *Th. zygis* – поширений в Іспанії та Португалії (Flora Europea, 1972). Особини *Th. callieri* по краю листкової пластинки опушені довгими, 5–7-клітинними, перетиснутими в одному чи двох місцях волосками. Між ними є короткі, 1–3-клітинні волоски. Прості, короткі волоски також розташовані по жилках листка (Рисунок 2. IIIA, B).



I. *Th. vulgaris*

Рисунок 2 I. Зовнішня будова листка
A – зовнішній вигляд листка: (A1 – абаксіальна; A2 – адаксіальна поверхня листка); B – збільшений його фрагмент (B1, B2 – абаксіальна; B3 – адаксіальна поверхня листка; C – опушення листків; D – пельтатний волосок (ефіроолійна залозка): 1 – головка; 2 – навколоепідермальні розеткові клітини

Figure 2 I. Exterior structure of leaf
A – appearance of leaf (A1 – abaxial, A2 – adaxial leaf surface); B – enlarged fragment (B1, B2 – abaxial, B3 – adaxial leaf surface); C – pubescence of leaves; D – peltate hair („essential oil gland"): 1 – head; 2 – around epidermal socket cells





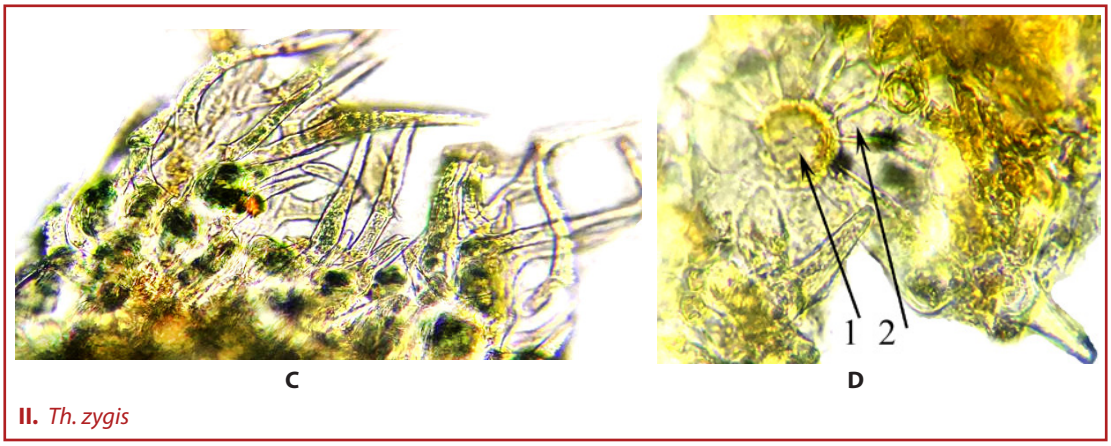
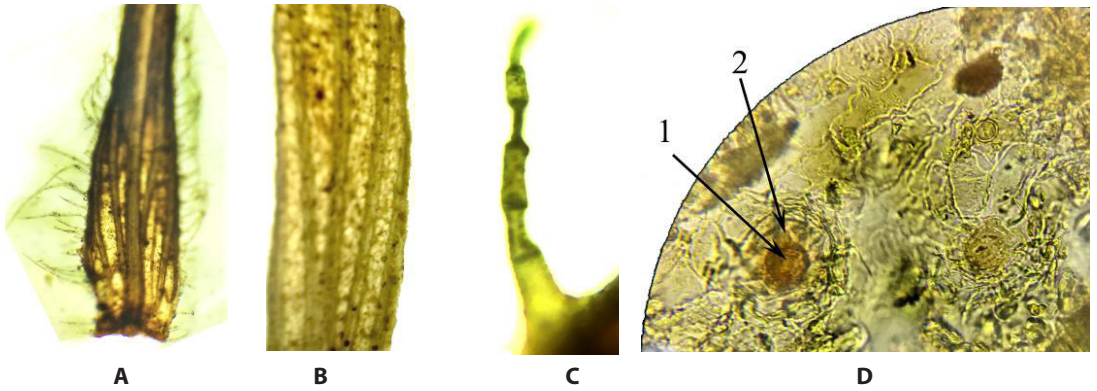


Рисунок 2 I. II. Зовнішня будова листка

А – зовнішній вигляд листка: (А1 – абаксіальна; А2 – адаксіальна поверхня листка); В – збільшений його фрагмент (В1, В2 – абаксіальна; В3 – адаксіальна поверхня листка; С – опушення листків; D – пельтатний волосок (ефіроолійна залозка): 1 – головка; 2 – навколоепідермальні розеткові клітини

Figure 2 I. II. Exterior structure of leaf

A – appearance of leaf (A1 – abaxial, A2 – adaxial leaf surface); B – enlarged fragment (B1, B2 – abaxial, B3 – adaxial leaf surface); C – pubescence of leaves; D – peltate hair („essential oil gland"): 1 – head; 2 – around epidermal socket cells



III. *Th. callieri*

Рисунок 2 III. Зовнішня будова листка

А – зовнішній вигляд листка: (А1 – абаксіальна; А2 – адаксіальна поверхня листка); В – збільшений його фрагмент (В1, В2 – абаксіальна; В3 – адаксіальна поверхня листка; С – опушення листків; D – пельтатний волосок (ефіроолійна залозка): 1 – головка; 2 – навколоепідермальні розеткові клітини

Figure 2 III. Exterior structure of leaf

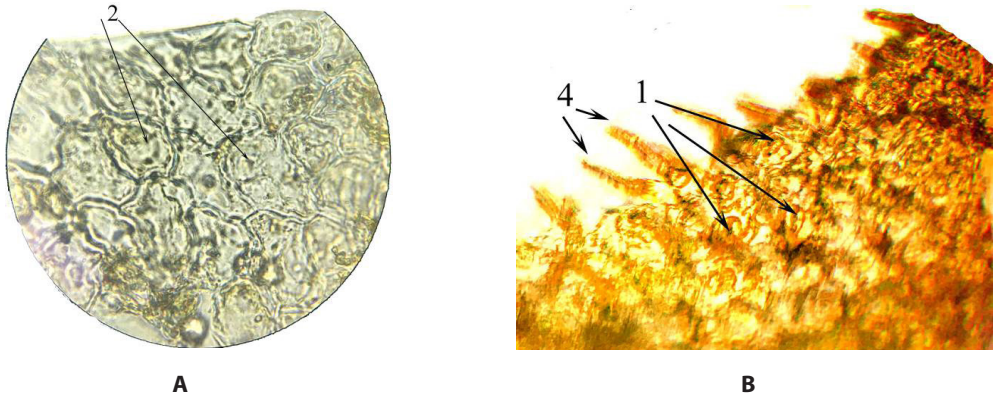
A – appearance of leaf (A1 – abaxial, A2 – adaxial leaf surface); B – enlarged fragment (B1, B2 – abaxial, B3 – adaxial leaf surface); C – pubescence of leaves; D – peltate hair („essential oil gland"): 1 – head; 2 – around epidermal socket cells

Заслуговує уваги жилкування листків. Так, жилкування листків *Th. callieri* камптодромне, з добре вираженими центральною і бічними жилками, які до краю пластинки зникають (Рисунок 2. IIIA). У *Th. vulgaris* і *Th. zygis* (Рисунок 2. I. IIA) жилкування гіфодромне. Жилки виступають лише на абаксіальній поверхні пластинки.

Окрім того, у всіх видів на поверхні листків є головчасті залозисті волоски з одноклітинною головкою, сидячі або з одноклітинною ніжкою. Листкові пластинки також вкриті пельтатними волосками (ефіроолійними залозками), кількість яких на адаксіальній поверхні є значно більшою, ніж на абаксіальній. У Середземноморського виду *Th. zygis* (Рисунок 2. II. B, D) їх діаметр становить 0,2–0,3 мм, а розташовані вони по 2–3 на 1 мм^2, утворені з 4-клітинної головки, оточеної 10–12 розетковими клітинами. На абаксіальній поверхні листка *Th. zygis* (Рисунок 2. IIA) вони сконцентровані біля черешка та по краю пластинки, а вся поверхня опушена довгими і короткими волосками, між якими трапляються головчасті. Подібну будову і розташування мають залозки у *Th. vulgaris* (Рисунок 2. IB1, B2, B3; D).

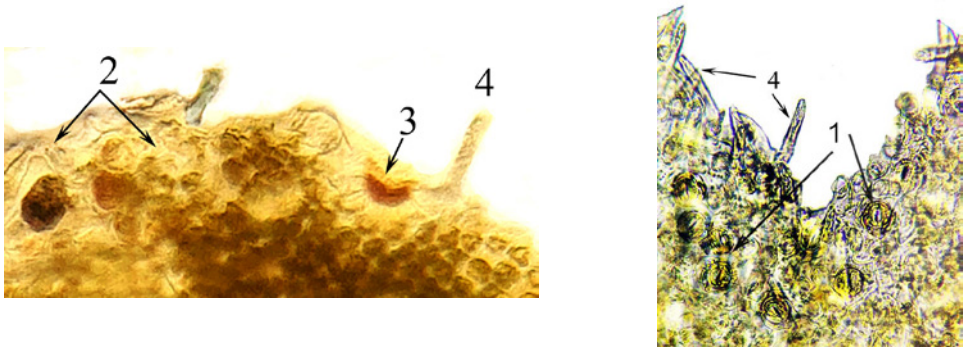
У *Th. callieri* кількість ефіроолійних залозок на листку менше, в середньому 0,8–1,0 на 1мм^2. Вони трохи менші за розмірами – 0,15 мм у діаметрі, головка утворена з 4 клітин і оточена 8 клітинами епідермісу (Рисунок 2. IID).

Відрізняються види між собою за будовою епідермісу. Клітини епідермісу з обох боків листкової пластинки у *Th. vulgaris* неправильно овальні, у *Th. zygis* – округлобагатокутні, у *Th. callieri* – овальні, зі звивистими стінками з адаксіального боку, а з абаксіального – клітини епідермісу видовжені (Рисунок 3). На поверхні верхнього епідермісу у *Th. vulgaris* видно краплі воскового нальоту (Рисунок 3. IA).



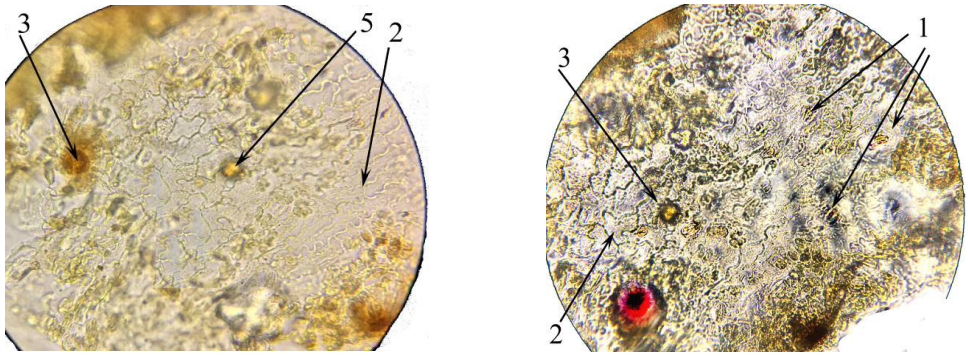
A B

I. *Th. vulgaris*



A B

II. *Th. zygis*



A B

III. *Th. callieri*

Рисунок 3 I. II. III. Будова епідермісу листкової пластинки

А – епідерміс адаксіального (верхнього) боку листкової пластинки; В – епідерміс абаксіального (нижнього) боку; 1 – продихи, 2 – клітини епідермісу; 3 – пельтатні волоски; 4 – прості волоски; 5 – капітатні волоски

Figure 3 I. II. III. The structure of the epidermis of the leaf blade

A – epidermis of the adaxial (upper) side of the leaf blade; B – epidermis of the abaxial (lower) side; 1 – stoma; 2 – epidermal cells; 3 – peltate glandular hairs; 4 – simple hairs; 5 – capitate glandular hairs

У всіх видів продиховий апарат діацитного типу, продихи овальні у *Th. callieri*, а у двох інших видів – округлі та погано помітні між густим опушенням. З абаксільного боку листка продихи розташовані значно щільніше, ніж з адаксіального, де вони поодинокі.

На поперечному перерізі листка видно, що у мезофілі *Th. vulgaris*, *Th. zygis* і *Th. callieri* (Рисунок 4B) добре виражена лише губчаста тканина, в якій знаходяться великі ефіроолійні вмістища. Вони розташовані над крупними жилками, а в їхній основі видно судини і склеренхіму судинноволокнистого провідного пучка. Чисельні клітини вмістищ мають густий цитоплазматичний вміст. При гіфодромному жилкуванні у *Th. vulgaris* видно лише одне ефіроолійне вмістище, над центральною жилкою. На деяких листках цього виду, з обох боків від центрального вмістища, може бути по одному, дуже малому вмістищі (Рисунок 4 I, II, IIIA). Тоді як у пластинці *Th. zygis*, крім великої середньої, є ще по 1–2 пари бічних жилок, які не помітні на поверхні, але над ними розташовані вмістища. У *Th. callieri* на поперечному перерізі листка в мезофілі видно 3–5 внутрішніх ефіроолійних вмістищ.

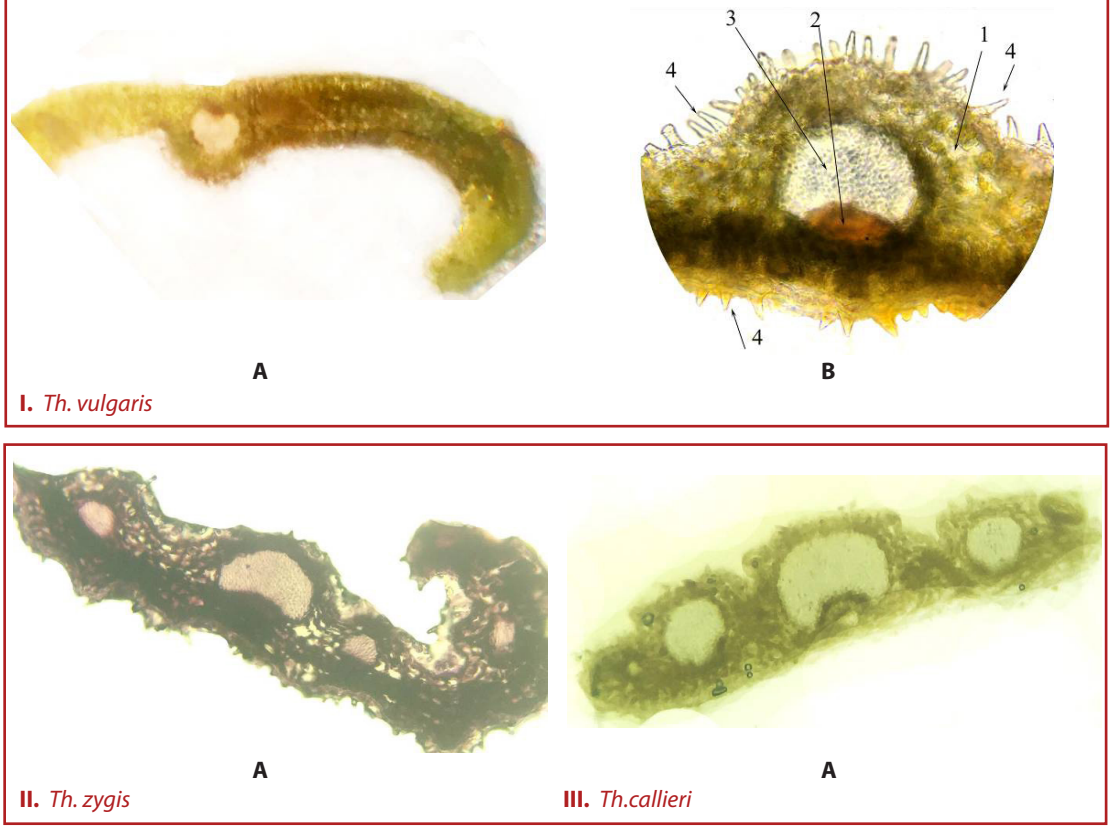


Рисунок 4 I. II. III. Поперечний переріз через листкову пластинку
А – поперечний переріз через листок; В – фрагмент перерізу (збільшено у 140 раз): 1 – мезофіл листка; 2 – судинноволокнистий провідний пучок; 3 – ефіроолійні вмістища; 4 – прості волоски

Figure 4 I. II. III. Cross-section through a sheet
A – cross-section through a leaf; B – section fragment (increased by 140 times): 1 – mesophyll leaf; 2 – vascularfibrous conductive bundle; 3 – essential oils conceptacle; 4 – simple hairs

Ще однією важливою діагностичною ознакою лікарської рослинної сировини є будова генеративних органів. У *Th. callieri* суцвіття компактне, головкоподібне, наприкінці цвітіння стає переривчастим, з кількома несправжніми «кільцями» квіток в основі. У *Th. vulgaris* і *Th. zygis* в умовах культури, видовжене, китицеподібне, квітки зібрані кільцями в пазухах приквіток, подібних на листки. При детальнішому аналізі виявилося, що у всіх видів формується китицеподібне суцвіття тирсоїдного типу, у якого верхівка наростає моноподіально, формуючи пазушні симподіальні парціальні суцвіття, які є дихазіями (*Th. vulgaris* і *Th. callieri*). Розташовані парціальні суцвіття навхрест супротивно в пазухах приквіток, подібних до листків (Рисунок 5 (1, 2).

До діагностичних систематичних ознак видів роду належить також будова чашечки квітки. Чашечка зросла основами чашолистків, п'ятизубчаста, двогуба, верхня губа має 3 короткі зубці, нижня – 2 довгі. У *Th. zygis*, *Th. vulgaris* і у *Th. callieri* чашечка дзвоникоподібна, форма зубців її верхньої губи трикутна, нижньої – з видовжено трикутними, відігнутими зубцями, сильно опушеними по краю дво і триклітинними волосками. Після цвітіння у *Th. zygis* зав'язь маточки закривається волосками (Рисунок 5A). Зросла частина чашечки у всіх видів опушена короткими і довгими волосками, особливо у *Th. callieri*, – дво- і триклітинними (Рисунок 5C), між якими трапляються головчасті, ефіроолійні волоски.

Віночок також двогубий, 5 пелюсток зрослись основами в трубочку, верхня губа суцільна, у *Th. callieri* слабо виїмчаста, нижня – трилопатева. Віночок у *Th. vulgaris* і *Th. zygis* світлорожевий, зовні густо вкритий головчастими волосками (Рисунок 5A). Андроцей утворений з 4 тичинок – дві довші, дві коротші, пиляки пурпурові. Маточка одна, приймочка довга, дволопатева.

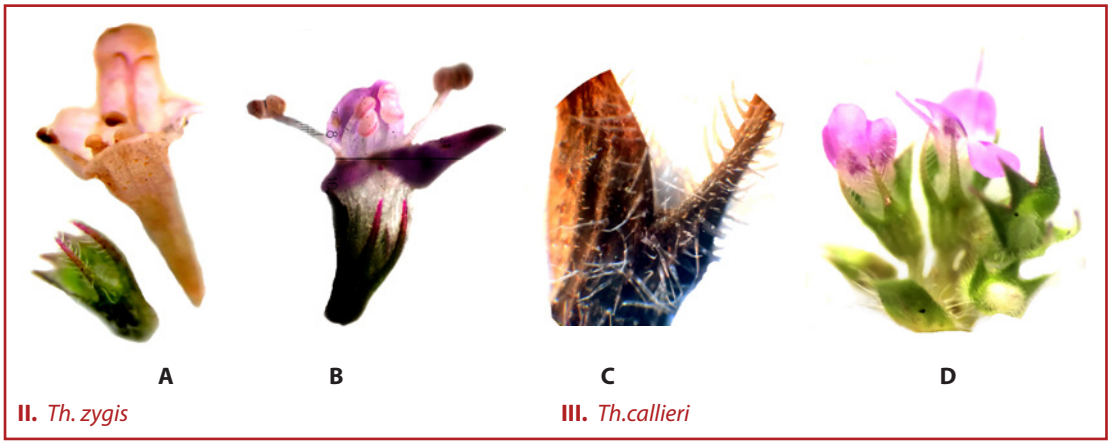


Рисунок 5 II. III. Будова генеративних органів рослин
A – квітка і чашечка; B – квітка; C – чашечка; D – парціальні суцвіття

Figure 5 II. III. Structure of plant generative organs
A – flower and calyx; B – flower; C – calyx; D – partial inflorescences

Висновки

За результатами проведених досліджень та аналізу отриманих даних встановлено ознаки, які можуть бути діагностичними при визначенні лікарської рослинної сировини досліджених видів роду *Thymus*.

У всіх видів листки навхрест супротивні, з загорнутими донизу краями пластинки, в нижній частині пагона короткочерешкові, у верхній – сидячі. Листки *Th. vulgaris*, темнозелені зверху, знизу – сірі від густого опушення простими, одноклітинними, загнутими волосками, між якими трапляються двоклітинні. У *Th. zygis* листки сіруваті з обох боків пластинки від опушення, в якому переважають двоклітинні і триклітинні колінчасто зігнуті волоски. У *Th. callieri* листки по краю опушені довгими, 3–7 клітинними перетиснутими волосками і простими одноклітинними – по жилках. У *Th. vulgaris* і *Th. zygis* пельтатні трихоми великі, розташовані густіше з верхнього боку листка, ніж з нижнього. У *Th. callieri* на листку, з обох боків розташовані дрібні пельтатні та булавовидні залозисті волоски. Клітини епідермісу овально–багатокутні, у *Th. callieri* – овальні, зі звивистими стінками. Продиховий апарат діацитного типу. Мезофіл утворений з губчастої тканини в якій є ефіроолійні вмістища.

Досліджені види мають суцвіття тирсоїдного типу, з парціальними суцвіттями, що є дихазіями. Чашечка дзвоникоподібна, зубці нижньої губи сильно опушені по краю дво-, триклітинними волосками. Чашечка зовні опушена простими одноклітинними, а у *Th. callieri* – дво- і триклітинними волосками. Віночок двогубий, світлорожевий, зовні густо вкритий головчастими волосками.

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REPRODUCTIVE CAPABILITY OF ENGLISH ROSES IN CONDITIONS OF FOREST-STEPPE OF UKRAINE

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Reproductive capability of English roses has not been well studied experimentally. But these researches are important to rose hybridization because cultivars often have low fertility. In the article offered morphological indicators of reproductive success and evaluate assess the reproductive potential of 7 cultivars of English roses from collection of 55 varieties. The quantitative indicators of the generative organs of the studied varieties are estimated according to the methodological instructions given in the Atlas of the morphological features of the varieties of roses. Biometric indicators were investigated: number of petals, stamens and pistils. Part of the stamens turned into petals, and therefore the amount of pollen decreased. As a result of studying the flower structure of English roses, it was determined that their inherent feature is the phenomenon of trimorphous geterostyly, namely there are three forms of the flower of cultivars: short-, medium- and macro-styles. Potential and real seed productivity is identified. The highest number of seeds in the hip is recorded in cultivar origin. Seed production shows that fertilization has occurred. The data should be used when planning rose breeding, namely for the selection of parent plants: the cultivars with well-knotted seeds should be used as seed plants.

Keywords: structure of the flower, reproduction ability, English roses.

Вступ

У ботанічних садах та дендропарках створюють колекції рослин, які є джерелом для теоретичних обґрунтувань і практичних рекомендацій у галузі інтродукції, акліматизації, збереження генофонду рідкісних рослин, генетики та селекції (Rubtsova, 2006).

Інтродукція видів роду *Rosa* L. в дендропарку «Олександрія» розпочалась наприкінці XVIII ст. Найчисленніша колекція сортових троянд (понад 100 сортів) була створена в парку на початку 70-х років та з різних причин на початок XXI ст. вона була загублена (Rubis, 2014; Rubtsova, 2016).

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В дендропарку «Олександрія», який є пам'яткою садово-паркового мистецтва України кінця XVIII – початку XIX ст., інтродукція троянд почалась 200 років тому. У праці І. Фундуклея зазначено, що в оранжереї парку культивували "*Rosa chinensis* Hack., яка заплітала своїми гілками всю стіну оранжереї, на якій були тисячі квіток" (Funduklej, 1852). За часів графів Браницьких троянди активно використовувались в паркових композиціях (Cherevchenko, 2003), де їм навіть було присвячено окрему композицію – "Острів Троянд". Відомо, що графиня Олександра Браницька подарувала ботанічному саду Харківського університету 37 видів рослин, серед яких була *Rosa centifolia* L. (Alekhin, 2004). За даними Epanchina (1891)(Dovidkovyi posibnyk, 1996) наприкінці XIX ст. в ландшафтних парках використовували наступні види роду *Rosa*: *R. foetida persiana* (Lemaire) Rehder, *R. rubrifolia* var. *livida*, *R. pimpinellifolia* fl. pl., *R. rugosa* fl. pl., *R. cinnamomea* fl. pl. (Syn.: *R. pendulina* Linnaeus), *R. hudsoniana* Redoute, *R. nitida*, *R. villosa*.

На початку 70-х років XX ст. в парку вирощували близько 2000 кущів троянд понад 100 сортів (Grisyuk, 1961).

За даними каталогу „Дерева і кущі дендрологічного парку "Олександрія" НАН України" (Dovidkovyi posibnyk, 1996) в 1996 р. в парку зростало 7 видів роду *Rosa*: *R. canina*, *R. corymbifera*, *R. bifera* Poir., *R. eglantera* L., *R. pomifera* Herrm., *R. rugosa* Thunb., *R. tomentosa* Smith.

Інтродукція англійських троянд в дендрологічному парку розпочалася в 2007 році, коли було закладено колекційну ділянку розарій. Вони вирізняються високими декоративними якостями (Rubis, 2014), тому їх використання в селекційних програмах є актуальним. Зараз на території розарію зростає 243 сорти.

Мета досліджень – вивчити репродуктивні стратегії англійських троянд, які проявляються в морфологічних особливостях для майбутнього використання результатів досліджень у селекції

Матеріали та методика

Об´єкти

7 сортів англійських троянд з колекції дендропарку „Олександрія": Abraham Darby, The Pilgrim, Othello, Graham Thomas, Pat Austin, Mary Rose, Heritage. Досліди проведено у 2015–2018 рр.

Методика

За методичними вказівками, викладеними в Атласі морфологічних ознак сортів троянди (Gacenko, 2009), досліджували біометричні показники: кількість пелюсток (махровість), тичинок, маточок у квітках та горішків у плодах. У будові генеративних органів вивчали співвідношення довжини тичинок та маточок. Оцінювали потенційну та фактичну продуктивність при вільному запиленні досліджуваних сортів (Korchagin, 1960). Статистичну обробку даних виконали за рекомендаціями Zajcev (1990).

Результати та обговорення

Відомо більш ніж 250 сортів англійських троянд, але дослідження репродуктивних стратегій зосереджено на сортах інших садових груп (De Vries DP, 1983; Gudin, 2000; Macphail, 2009; Vaskivska, 2017), тому дослідження морфологічних показників квіток англійських троянд є актуальним.

Аналіз біометричних показників показав, що всі досліджені сорти є махровими. Зазвичай англійські троянди мають махрові квітки, але є невелика кількість немахрових та напівмахрових сортів. В досліджуваних колекціях сортів з немахровими та напівмахровими квітками немає (Rubtsova, 2017). Середня кількість пелюсток становить від 46 (сорт Graham Thomas) до 144 (сорт The Pilgrim) (Таблиця 1).

Махровість у троянд є наслідком перетворення тичинок на пелюстки (Rubtsova, 1982), через що в квітках є перехідні структури від тичинок до пелюсток. Зазвичай у видів роду *Rosa* L. квітка має п'ять пелюсток, тому можна припустити, що досліджувані сорти втратили таку кількість тичинок: Abraham Darby – 101, The Pilgrim – 139, Othello – 49, Graham Thomas – 41, Pat Austin – 88, Mary Rose – 66, Heritage – 73). Через втрату тичинок зменшується кількість пилку. Кількість тичинок у досліджуваних махрових сортів є значною: від 20 (сорт Pilgrim) до 156 (сорт Abraham Darby) (Таблиця 1).

Таблиця 1 Кількісні показники генеративних органів англійських троянд
Table 1 Quantitative indicators of generative organs of English rose

Сорт	Середня кількість, шт.				
	пелюсток (махровість)	тичинок	маточок	горішків (насінин) у плоді	% зав'язування горішків (насіння)
Abraham Darby	106 ±5,2	156 ±10,1	30 ±5,6	10 ±2,1	33,3
The Pilgrim	144 ±12,8	20 ±2,2	24 ±3,7	–	–
Othello	54 ±3,4	88 ±5.6	62 ±6,4	10 ±3,7	16,1
Graham Thomas	46 ±3,5	84 ±6,1	32 ±5,2	6 ±1,3	18,7
Pat Austin	93 ±5,2	60 ±8,3	48 ±4,1	6 ±2,2	12,5
Mary Rose	71 ±5,7	38 ±5,4	28 ±5,3	8 ±2,4	28,6
Heritage	78 ±6,3	98 ±8,7	48 ±4,8	25 ±3,5	52,1

Плід троянд – цинародій (багатогорішок). Кожний горішок є однонасінним. Циннародій формується з квітки з апокарпним гінецеєм, тобто гінецеєм, який складається з декількох вільних маточок, кожна з яких утворена одним плодолистиком, тому кількість маточок у квітки троянди є показником потенційної кількості горішків (насіння) в плоді.

Кількість маточок (потенційна кількість горішків у циннародії) у досліджуваних сортів становила від 24 (сорт The Pilgrim) до 48 (сорт Heritage). Кількість горішків у плодах є невеликою – від 6 до 25. У сорту Heritage зафіксовано найбільшу середню кількість горішків у плоді – 25 шт. Найвищий відсоток зав'язування горішків (насіння)

зареєстровано у сортів Heritage (52,1 %) та Abraham Darby (33,3 %). Сорт Pilgrim за період спостереження горішків не зав'язував.

За результатами дослідження будови квітки англійських троянд було встановлено, що їм притаманне явище триморфної гетеростилії, тобто у сортів є три форми квіток: коротко-, середнє- та довгостовпчасті (Рисунок 1). Отримані дані підтверджують результати досліджень будови квіток інших садових груп (Rubtsova, 1982; Vaskivska, 2017; Macphail, 2009).



Короткостовпчаста квітка (сорт Graham Thomas) | Середньостовпчаста квітка (сорт Othello) | Довгостовпчаста квітка (сорт Heritage)

Рисунок 1 Будова квіток у чайно-гібридних троянд
Figure 1 The structure of English rose flowers

Домінують короткостовпчасті квітки (сорти Graham Thomas, Mary Rose, Abraham Darby, Pat Austin). Довгостовпчасті квітки виявлено у двох сортів – Heritage, The Pilgrim, середньостовпчасті квітки – ще у одного сорта – Othello. Наші дослідження не виявили зв'язку між гетеростилією квіток чайно-гібридних троянд та зав'язуванням горішків (насіння) (Таблиця 2).

Таблиця 2 Зав'язування горішків у сортів англійських троянд з різною будовою квітки
Table 2 Formation of hips in English Rose cultivars with different structure of flower

Будова квітки					
Короткоствпчасті		**Середньостовпчасті**		**Довгостовпчасті**	
Назва сорту	**% зав'язування горішків (насіння)**	**Назва сорту**	**% зав'язування горішків (насіння)**	**Назва сорту**	**% зав'язування горішків (насіння)**
Graham Thomas	18,7	Othello	16,1	Heritage	52,1
Mary Rose	28,6			The Pilgrim	0
Abraham Darby	33,3				
Pat Austin	12,5				

У сортів з довгостовпчастими квітками (Heritage, The Pilgrim) самозапилення квіток виключено, у сортів з короткостовпчастими квітками (Graham Thomas, Mary Rose, Abraham Darby, Pat Austin) ймовірність самозапилення є високою.

Висновки

Колекція англійських троянд у дендропарку „Олександрія“ нараховує 55 сортів (23 % загальної кількості сортів колекції). Проаналізовано біометричні показники та морфологічні особливості будови квіток 7 сортів англійських троянд колекції. Встановлено показники репродуктивної стратегії: потенційна та фактична кількість насінин у плоді. Одержані дані необхідно враховувати при плануванні селекційної роботи з трояндами, а саме – при підборі батьківських рослин: сорти, які добре зав'язують горішки (насіння) слід використовувати як материнські.

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SPECIFIC OF THE ASSIMILATION SURFACE OF COLUMNAR APPLE-TREE

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The aim of this study was to determine the anatomical structure of leaves and clean productivity of photosynthesis (CPP) of assimilation surface plants of different originally columned cultivars of an apple-tree in the conditions of Forest-Steppe of Ukraine. The objects of research served seven columnar cultivars in plantings of primary varietal apple. According to the results of the study, the leaves of all varieties grown in the Kyiv region are found to contain three layers of palisade fabric, the thickness of which varied by cultivar. Much less control (cv. Bolero) it was the Favorite, the Tantsivnytsia, the President, the largest – in the leaves of the cultivar Bilosnizhka (124.1 microns). The ratio of palisade tissue to spongy was 1.13–1.24. The varietal difference in parameters was detected the assimilation surface and its main parameters are revealed photosynthetic activity. The highest level of clean productivity of photosynthesis leaves is observed in plants of the currency Valiuta, Bilosnizhka and Favorite – 9.1–13.9 g/m^2 per day. In the Bolero, Sparta and Tantsivnytsia cultivars, the rate of dry matter accumulation is slightly lower (8.3–8.7 g/m^2). The specificity of complex fruit formations of trees of the studied cultivars varied depending on age sections of the trunk. A comprehensive assessment of the morphophysiological components was found to be investigated varieties differ in potential productivity and efficiency of its realization in economic harvest.

Keywords: columnar apple-tree, cultivars, anatomical structure of leaves, leaf area, clean productivity of photosynthesis

Вступ

Листок яблуні є основним асимілюючим органом рослини, в якому утворюються органічні речовини, що слугують структурно-енергетичним матеріалом для всього організму (Blanco and Folegatti, 2003; Fedoryako, 2004). Пластинчаста форма листка забезпечує найбільшу поверхню на одиницю об'єму тканин, що створює найліпші умови для повітряного живлення. Площа окремого листа і загальна листкова поверхня рослини дозволяють оцінити її фотосинтетичний потенціал і функціональну активність. Крім цього, листок володіє найбільшими пристосовними властивостями

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до умов навколишнього середовища, що виражається в зміні площі асиміляційної поверхні рослини в залежності від факторів довкілля (Schulze et al., 2005; Gibson and Gibson, 2006; Valk, 2009). Продуктивність окремої рослини і всього агроценозу визначається інтенсивністю і продуктивністю фотосинтезу, а також загальною площею листя. Останнє має великий вплив на загальний рівень фотосинтезу у рослин та їхню врожайність.

Найвищі врожаї, відповідно до теорії фотосинтетичної продуктивності рослин, можуть бути отримані при інтенсивному збільшенні площі листя. На прикладі різних сільськогосподарських культур переконливо доведено, що формування листкової поверхні як нижче, так і вище певного фізіологічного оптимуму, негативно позначається на продуктивності вирощуваних рослин. В цілому для рослини кількість поглиненої листям сонячної радіації зростає пропорційно збільшенню листкової поверхні лише до певної межі. На думку Nichiporovich (1956; 1961), площа листя агроценозів може досягати 40–50 тис.м^2/га; подальше збільшення її не призводить до підвищення продуктивності. Для насаджень яблуні з великими кронами оптимум визначено в розмірі 37–45 тис.м^2 листя на 1 га (Ursulenko, 1967). Ряд дослідників пропонує підтримувати листковий покрив у насадженнях яблуні на рівні 35–40 тис.м^2/га і навіть 40–50 тис.м^2/га (Lukyanov, 1969). За проектованої врожайності в 50 т плодів теоретично обгрунтована площа листя складе 50 тис м^2/га, тому що для садів з округлою кроною багатьма дослідниками вважається оптимальним навантаження 1кг плодів на 1м^2 листя (Kudryavets and Drugova, 1976). За надмірної кількості листя в кронах погіршується світловий режим, що знижує продуктивність фотосинтезу. Тому окремі вчені вважають, що збільшення листкової поверхні в яблуневих садах понад 20–25 тис.м^2/га є недоцільним (Friend and Helson, 1976; Devyatov, 1981). Від загальної площі листя та їх активної діяльності залежить диференціація генеративних бруньок, ріст плодів. Тому співвідношення між площею листків на дереві і масою утворених плодів повинне бути оптимальним; останнє залежить від типу насаджень і підщепи.

Листя забезпечує синтез органічних речовин, які витрачаються на ріст і формування врожаю, а також накопичення пластичних речовин під урожай наступного року (Kudryavets, 1984). Це стає можливим, якщо в кроні дерева на один плід припадає 15–25 листків (Khromenko, 2000). Збалансоване співвідношення між процесами росту і плодоношення у яблуні відзначається за наявності в кроні не більше 20 % (від числа, що розпустилися) квіткових бруньок (Karpov, 1983). За деякими даними, величина цього показника повинна становити 30–35 %. У дослідах Khromenko (1986) для отримання максимальної маси одного плоду було досить 300 см^2 листкової поверхні, але ці листки не забезпечували закладання квіткових бруньок, а площа листя в 540–600 см^2 на плід сприяла його нормальному розвитку і закладанню 17 % квіткових бруньок. Рекомендована площа листя в 20–25 тис.м^2/га при навантаженні 600 см^2 листкової поверхні на плід може забезпечити врожайність до 40–60 т/га.

За даними Ovsyannikov (1983), при використанні 50 % асимілятів на врожай для формування плоду масою 100г потрібно 340–504 см^2 листкової поверхні. Аналогічні

результати наводяться і в інших працях (Fisenko and Sabadan, 1999; Khromenko, 2000). Таким чином, розвиток оптимальної для кожного агроценозу площі листя з високою асиміляційною здатністю є одним з основних чинників його високої продуктивності, і управління цим процесом за допомогою агроприйомів може слугувати основою підвищення врожайності.

Встановлено, що у звичайних сортів яблуні на кількість плодів, що зав'язалися (початок X-го етапу органогенезу), впливає і є визначальним фактором площа листя на плодовому утворенні під суцвіттям. За повідомленнями Buntsevych (2008), зі збільшенням листкової площі під суцвіттям в умовах, що виключають адитивність, кількість зав'язуваних плодів збільшується: кільцівки із листковою поверхнею 21 см^2 залишаються без зав'язі, із площею листя 28 см^2 зав'язують один-два плодики, пагони з 34 см^2 листя зав'язують три і більше плодики. В роки високого врожаю у звичайних сортів яблуні, за інформацією Fisenko (2004), площа листкової поверхні значно зменшується, а виробництво плодів на 1м^2 листя досягає 3,7–4,4 кг. Подібної інформації по колоноподібних сортах на даний час немає.

Метою наших досліджень є визначення анатомічної структури листків, площі та чистої продуктивності фотосинтезу (ЧПФ) асиміляційної поверхні рослин різних за походженням колоноподібних сортів яблуні в умовах Лісостепу України.

Матеріали і методи дослідження

Об'єкти досліджень

Дослідження проводили у відділі селекції плодових і ягідних культур Інституту садівництва НААН України (ІС НААН). Об'єктами дослідження слугували сім сортів яблуні колоноподібного типу в незрошуваних насадженнях яблуні, які закладено на Київщині в 2002 (Болеро, Спарта, Танцівниця) та 2010 рр. (Фаворит, Білосніжка, Валюта, Президент) згідно методики первинного сортовипробування. Дерева на середньорослій підщепі 54–118 висаджено за схемою 4 × 1 м. Ґрунт дослідної ділянки темно-сірий опідзолений середньосуглинковий на карбонатному лесі, типовий для правобережної частини західного Лісостепу. Система утримання грунту у міжряддях саду – дерново-перегнійна, у пристовбурних смугах – гербіцидний пар. Агротехнічний догляд за насадженнями проводили відповідно до зональних рекомендацій (Kondratenko et al., 1997).

Клімат регіону розташування досліджуваних рослин

Клімат регіону розташування насаджень досліджуваних сортів помірно-континентальний. Середньорічна температура повітря становить 7,3 °С, щорічна кількість опадів – 597 мм, сума активних температур 2 580 °С. В період дослідження погодні умови характеризувались сталістю. Термічний режим в цілому сприяв доброму росту й розвитку рослин. Сума активних температур 10 °С і вище істотно перевищувала середні багаторічні значення і становила 3 200 °С (2017 р.) і 3 681 °С (2018 р.). Умови зим 2016–2017 та 2017–2018 років були сприятливими для успішної перезимівлі

рослин. Річна кількість опадів становила 385,5 мм (2017) і 360,0 мм (2018). У 2017 році під час цвітіння спостерігались заморозки (мінус 2–4 °С), а наступного року на початку росту зав'язей – град.

Методики проведення досліджень

Фізіологічні дослідження включали в себе визначення площі листкової пластинки – ваговим методом з відбором висічок і подальшими розрахунками даного показника за методикою Fulga (1975); ЧПФ визначали методом Koshelev (1987); анатомічну структуру листкової пластинки досліджували за Khrzhanovsky (1989). Показники фотосинтетичної діяльності обраховували за формулами, запропонованими Ovsyannikov (1976) та Kudryavets (1987).

Аналіз даних

Експериментальні дані були статистично проаналізовані за допомогою програмного пакету Microsoft Excel.

Результати та їх обговорення

Анатомічна будова листків – це сортова ознака, яка також залежать від умов освітлення крони; останнє істотно впливає на асиміляційну спроможність сортів. Так, за Jackson (1970), потовщення палісадної паренхіми та збільшення кількості хлоропластів відбувається завдяки підвищенню освітленості листків.

Листки колоноподібних сортів яблуні темно-зелені, товсті, з довгими черешками, що є також характерним і для розповсюджених сортів із спуровим типом плодоношення. У листків рослин усіх досліджуваних сортів, вирощуваних в умовах оптимального освітлення крони, відмічено наявність трьох шарів основної асиміляційної тканини, в якій активно відбуваються процеси фотосинтезу. За оптимальних умов у сортів з добре розвиненою палісадною паренхімою, як доводить Dunstone (1973), накопичується більше сухої речовини в перерахунку на одиницю площі листка. Листки досліджуваних сортів різнились за товщиною палісадної тканини (Таблиця 1; $НІР_{05}$ = 8,811). Значно меншою щодо контролю вона була у сортів Фаворит, Танцівниця та Президент. Найбільшу товщину основної асиміляційної тканини відзначено в листків сорту Білосніжка, рослини якого формують листки з великими клітинами епідермісу. Особливістю палісадної паренхіми листків майже у всіх сортів є дуже щільне розміщення клітин у тканині.

Щодо губчастої хлоренхіми, в якій активно проходять процеси газообміну і транспірації та менш активно – фотосинтезу, дослідженнями було визначено, що її товщина у листках усіх сортів була в межах 90,13–109,06 мкм. Значно нижче контролю її товщина відмічена у сортів Фаворит, Танцівниця, Президент та Валюта ($НІР_{05}$ = 6,948).

Співвідношення товщини палісадної і губчастої паренхіми, що утворюють м'якоть листка, у сортів становило 1,13–1,24; найвищим воно було у листків сортів Фаворит, Валюта та Президент, але істотної відмінності між досліджуваними сортами не виявлено ($НІР_{05}$ = 0,092).

Таблиця 1 Біометричні показники анатомічної структури листкової пластинки колоноподібних сортів яблуні (ІС НААН, 2017–2018 рр.)

Table 1 Biometric indices of the anatomical structure of the leaf blade of columnar apple cultivars (IH NAAS, 2017–20018)

Сорт	Загальна товщина листка (мкм)	Товщина тканин листка, мкм				Відношення (палісадна/ губчаста)
		губчаста	палісадна	епідерміс		
				верхній	нижнній	
Президент*	229,82	91,90	110,58	14,01	11,87	1,21
Валюта*	241,08	96,73	118,26	13,21	10,33	1,23
Фаворит*	225,76	89,61	110,77	13,33	10,63	1,24
Білосніжка*	255,78	109,06	124,09	13,23	12,07	1,15
Танцівниця**	215,57	90,13	102,70	12,11	9,43	1,14
Спарта**	248,81	104,39	117,64	14,12	11,53	1,13
Болеро (К) **	251,86	104,60	121,55	13,65	11,83	1,16
$НІР_{05}$	13,309	6,948	8,811	1,191	1,207	0,092

Примітка: * 7–8-річні дерева; ** 15–16-річні дерева

У цілому за період дослідження найбільшим розміром листкової пластинки на плодових утвореннях характеризувалися дерева сорту Білосніжка та Фаворит, нижче контролю були листкові пластинки Танцівниці (Таблиця 2).

Таблиця 2 Характеристика листків дерев колоноподібних сортів яблуні на підщепі 54–118 (ІС НААН, 2017–2018 рр.)

Table 2 Characteristics of leaves of trees of columnar apple cultivars on rootstock 54–118 (IH NAAS, 2017–2018)

Сорт	Середня площа листкової пластинки ($см^2$)	Загальна листкова поверхня ($м^2$/дерево)	Загальна листкова поверхня (тис. $м^2$/га)	Площа листя ($см^2$/100 грам плоду)
Президент*	17,98	1,39	3,48	199,31
Валюта*	17,99	1,78	4,45	199,59
Фаворит*	20,34	1,98	4,94	743,20
Білосніжка*	25,55	2,08	5,21	795,12
Танцівниця**	13,40	0,45	1,12	75,63
Спарта**	18,83	2,30	5,76	580,67
Болеро (К)**	18,18	3,30	8,24	311,90
$НІР_{05}$	1,462	0,295		

Примітка: * 7–8-річні дерева; ** 15–16-річні дерева

Площа загальної листкової поверхні на одному дереві була найбільшою у контрольного сорту Болеро (3,30 $м^2$), найнижчою – у Танцівниці – 0,45 $м^2$. У перерахунку на гектар, за фактичною схемою садіння вона становила 1,12–8,24 тис.$м^2$ і була значно нижчою,

ніж у садах звичайних (традиційних) сортів яблуні. За оптимальної схеми садіння (2,5 + 0,9 × 0,5 м), запропонованою Всеросійським селекційно-технологічним інститутом садівництва і розсадництва (ВСТИСП) (Khromenko and Vorobyov, 2015), площа загальної листкової поверхні для досліджуваних сортів становитиме 6,62–48,53 тис.м^2/га. У середньому за роки дослідження формування плоду масою 100 грам у сорту Білосніжка забезпечувала найбільша площа листкової поверхні – 795,12 см^2, що за Ovsyannikov (1976; 1983), на 37 % вище за потрібне для звичайних сортів. Нижчим за контроль даний показник є в сортів Танцівниця, Валюта та Президент. У дерев сорту Танцівниця, який є природним карликом, формування 100 грам плоду забезпечує в середньому 75,63 см^2 листкової поверхні, що відповідає 5–6 листкам. У сортів Валюта і Президент ріст і розвиток одного плоду в середньому відбувається за наявності 17 листків.

Облиственість складних плодових утворень дерев досліджуваних сортів різнилась залежно від віку ділянки стовбура. Так, у яблунь сорту Танцівниця найбільшу площу листя мали 15-річні плодухи, в сорту Спарта – 19-річні. На деревах сорту Президент відмічено рівномірну облиственість плодух на 4–9-річних ділянках стовбура.

Найвищий рівень ЧПФ листя спостерігався в рослин сорту Валюта (13,9 г/м^2.доба), у сортів Білосніжка, Фаворит, Болеро, Спарта і Танцівниця накопичення сухої речовини на 34,5–40,3 % нижче (Рисунок 1).

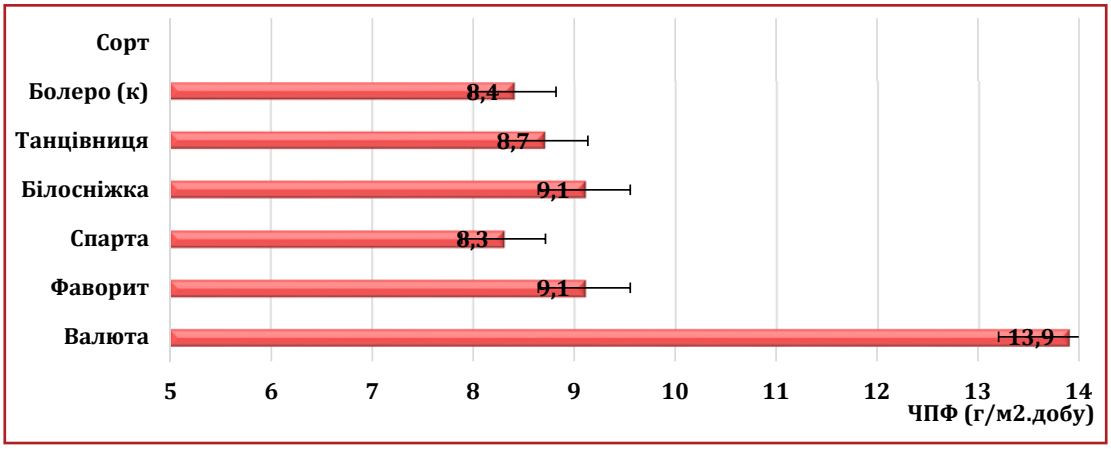


Рисунок 1 Рівень ЧПФ листків колоноподібних сортів яблуні
Figure 1 Level of clean productivity of photosynthesis of leaves of columnar apple cultivars

Порівнюючи ці дані з наведеними у науковій літературі про те, що чиста продуктивність фотосинтезу деяких сортів яблуні досягає 10–12 г сухої речовини на 1 м^2 листової поверхні за добу, а в середньому за вегетацію реальна ЧПФ не перевищує 40–50 % потенційної (4–6 г/м^2 за добу), відзначаємо, що рівень ЧПФ листя у колоноподібних сортів є високим. Сорти Білосніжка та Фаворит хоч і мають велику за площею листкову поверхню і листки з товстим шаром палісадної паренхіми, проте неефективно використовують біологічний потенціал у процесі формування плодів.

Результати дослідження показали, що велика маса плоду не завжди обумовлює високу врожайність. Так, у сорту Танцівниця за маси плоду 127 грам сформована порівняно висока врожайність – 7,2 кг/дер. У той же час, у сортів Спарта, Фаворит та Білосніжка маса плодів становила 176–228 грам, врожайність була нижчою на 57–82 %, ніж у сорту Болеро. Сорти Президент і Валюта формували плоди середнього розміру (146 і 152 г, відповідно), що дозволило одержати врожай на рівні 4,5–16,0 кг/дер. Урожайність досліджуваних колоноподібних сортів яблуні не залежала від походження: серед вітчизняних, а також інтродукованих було виділено сорти як з високою, так і низькою врожайністю.

Висновки

Серед досліджуваних об'єктів рослини сорту Білосніжка формують найтовщу листкову пластинку і найбільший за висотою шар палісадної тканини; в сорту Танцівниця ці показники найменші. Товщина палісадної тканини листків різнилася за сортами. Співвідношення товщини палісадної і губчастої паренхіми становило 1,13–1,24, найвищим воно було в листків сортів Фаворит, Валюта та Президент. Загальна листкова поверхня на одному дереві залежала від сорту. Формування плоду масою 100 грам в сорту Білосніжка забезпечує найбільша площа листкової поверхні – 795,12 см^2; нижча, відносно контролю – у сортів Танцівниця, Валюта та Президент. У дерев сорту Танцівниця формування 100 грам плоду забезпечує в середньому 75,63 см^2, що відповідає 5–6 листкам. У сортів Спарта, Фаворит та Білосніжна на формування одного плоду "працювали" 56–71, Болеро – 27, Валюта і Президент – 17 листків. Найбільший рівень ЧПФ спостерігався в листків сорту Валюта (13,9 г/м^2 добу). У сортів Білосніжка, Фаворит, Болеро, Спарта і Танцівниця накопичення сухої речовини суттєво не відрізнялось і дорівнювало 8,3–9,1 г/м^2 добу. Урожайність досліджуваних колоноподібних сортів яблуні не залежала від походження: серед вітчизняних, а також інтродукованих було виділено сорти як з високою, так і низькою врожайністю.

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BIOLOGICAL ACTIVITY OF EXTRACTS FROM SOME SPECIES OF CONIFEROUS PLANTS

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The development and use of biological preparations based on plant secondary metabolites is an important trend in modern organic farming. Among secondary metabolites, a significant place is belong to substances of the class of terpenoids (isoprenoids), which possess a wide spectrum of biological activity and practically no toxicity to warm-blooded animals and humans. One of the main sources of terpenoids is conifers, such as *Juniperus sabina* L. (Cupressaceae) and *Pinus sylvestris* L. (Pinaceae), which in quantitative content and qualitative composition are vastly superior to other widely distributed species in the plant world. Thus, the secondary metabolites of savin juniper and scots pines advantageous as a raw material for the production of various biological preparations for organic agriculture. The aim of this study was to determine the biological activity of extracts and essential oils from species of *Juniperus sabina* and *Pinus sylvestris*, growing in the Republic of Moldova (RM) and Slovak Republic, Nitra (SN) and Pianiny mountains (PM). The antioxidant activity of ethanolic extracts of Coniferous was determined by two procedures with appreciation of their radical scavenging capacities against DPPH and peroxyl free radicals and was evaluated in equivalent of standard substances (Trolox and gallic acid) and by indexes IC_{50}. According to the determined indexes the antioxidant activity of tested extracts from Coniferous plants was qualified in followed order: *J. sabina* SN > *J. sabina* PM > *J. sabina* RM> *P. sylvestris* RM ≥ *P. sylvestris* SN. Moreover, it was established that both ethanolic extracts and essential oil *J. sabina* possessed high efficiency against *Leptinotarsa decemlineata* Say and *Galleria mellonella* L. Mortality of imago and larvae consisted in average 6.7–53.3 and 66.7–100% respectively, ovicidal activity was 100%, and antifeedant effect persisted at the level of 1–3 points. The treatment of feed and insects with the essential oils of *P. sylvestris* needles resulted in the death of only 6.7–13.3% of the wax moth larvae.

Keywords: Coniferous plant, extract, essential oil, antioxidant activity, biopesticidal property

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Introduction

Plants play a significant role in human life. They are a source of food, serve as raw materials for the construction and manufacture of various products, applied in almost all areas of human activity, including medicine, perfume, light and coatings industries, etc. Generally, the phytochemical constituents of plants break up into two categories based on their role in basic metabolic processes, namely primary and secondary metabolites. Primary plant metabolites are involved in basic life functions; therefore, they are more or less similar in all living cells. These include amino and nucleic acids, proteins, lipids, carbohydrates, enzymes, vitamins, and some organic acids. The role of secondary plant metabolites (SPMEs) in growth, photosynthesis, reproduction, and development of plants has not been found yet. At the same time these substances play an important role in plant survival in the environment and serve to establish ecological relationships between plants and other organisms. In the course of their vital activity, higher plants accumulate a big number of SPMEs (estimated 200,000), belong to the wide variety of chemical classes of compounds: alkaloids, terpenoids, saponins, phenolic compounds (flavonoids, tannins) and their glycosides (Crozier et al., 2006). Due to chemical structure, the SPMEs can exhibit a large spectrum of biological activity (growth regulator, immunomodulator, antioxidant, antimicrobial, pesticide, etc). The main advantages of preparation based on secondary plant metabolites are their high activity at low concentrations, specificity, harmlessness for the environment, low toxicity for warm-blooded animals, humans, and many species of beneficial insects, including pollinators. In this context, the development and use of biological preparations based on plant secondary metabolites is an important trend in modern organic farming.

Savin juniper or savin (*Juniperus sabina* L., Cupressaceae) is one of the most widespread conifer species in the world, native to the mountains of central and southern Europe, western and central Asia, as well as Northern Africa (Algeria). It grows in a wide variety of ecological conditions: on the sand, chalk deposits, rocks, foothills and high in the mountains, rising to 3,300 m, occurring on southern stony and fine-earthed northern slopes. Needles of *J. sabina* contain bitter glycoside pinopicrin, gallic acid, tannins, flavonoids, resin, wax, vitamin C, organic acids and more. In addition, savin juniper contains essential oil (up to 2.5–4.8%), which has a specific odor and consists of alcohol sabinol and various terpenic compounds (Ayoshina and Velichko, 2004; Myrzagaliyeva and Medeubaeva, 2014; Novikov et al., 2014). Such a variety of chemical compounds, as well as the availability of extraction of biologically active substances from various plant organs, has led to the fact that the savin is widely used in medicine and homeopathy, perfumery, in microscopic technique (immersion oil), for the production of various alcohol drinks, as well as dyes, lacquers, joinery, bath besom, etc. (Lagoni, 2002; Aleksandrova and Aleksandrov, 2005; Farjon, 2005, 2013; Nosov, 2007; Semenyutina et al., 2014).

Scots pine (*Pinus sylvestris* L., Pinaceae) is a long-living, coniferous tree that is the most widely distributed pine, native to Eurasia. It is readily identified by its combination of fairly short, blue-green needles and orange-red bark. The needles are 3–5 cm long and occur in fascicles of two. The cones are pointed ovoid in shape and 3–7 cm long. The threes normally grow up to 25 m in height and in exceptional cases up till 40 m. Scots pine is, especially in the

north of Europe, an economically important species. The wood is strong and easy to work with, making it excellent for general constructions, furniture, and the pulp and paper industry. Pine, as well as juniper, is also used for stabilizing sandy soils (Gardner, 2013; Daugavietis and Spalvis, 2014). Scots pine is an important timber tree. Earlier it uses as mining props and for interior construction, for street paving blocks. At the moment most of the production goes to the paper industry. Other uses of scots pine wood are railway sleepers, fencing, crates, pallets, boxes, laminated wood, particleboard, fibreboard, and various wood-based materials (Gardner, 2013; Daugavietis and Spalvis, 2014).

The secondary metabolites of savin juniper and pines have long attracted the attention of researchers as a raw material for the production of various biological preparations for organic agriculture. Earlier, it was shown that the *J. sabina* and *P. sylvestris* possessed potential biological activity (Elisovetcaia et al., 2017, 2018a, b; Gladei et al., 2018; Elisovetcaia and Brindza, 2018). The purpose of this research was to establish the antioxidant and biopesticidal activity of extracts and essential oils from species of *Juniperus sabina* and *Pinus sylvestris*, growing in the Republic of Moldova and Slovak Republic.

Material and methodology

Scientific researches were carried out in the laboratory conditions during 2017–2019 years, in the Laboratories of Bioreglators Natural and Integrated Protection, Institute of Genetics, Physiology and Plant Protection (IGPPP), Chisinau, Republic of Moldova and in the Institute of Biodiversity Conservation and Biosafety, Department of Genetics and Plant Breeding (FAaFR), Slovak University of Agriculture in Nitra.

Plant materials

The needles (twigs) of *Juniperus sabina* were collected in spring and autumn 2017–2018, and *Pinus sylvestris* in autumn 2018 and in winters 2018–2019 in the different zones from Slovak Republic (Nitra and Tatras Mountains, Pianin region) and Republic of Moldova, dried and crushed used the laboratory mill Type MRP-1 (asynchronous motor, 'Agropribor', Russia) and rotary chopper PULVERISETTE, powdered to uniform size of particles passing through a sieve with apertures 2.0 mm in diameter. The plant materials were collected by trimming 30–40 cm of regrown basal branches. The identification of the *J. sabina* and *P. sylvestris* plants was carried out by consultation with the specialists from the Botanical Garden (Institute) and the Forest Research and Management Institute – ICAS, Chisinau, as well as by morphometric analysis of juniper and pine needles. The following abbreviation was accepted depending on the region where the plant materials were collected: Slovakia, Nitra – SN; Slovakia, Tatras Mountains, Pianin region – SM and Republic of Moldova – RM.

Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

The content of moisture and dry substances

The content of moisture and dry substances were determined both for fresh and dried herbal raw materials, as well as for extracts according to the standard methods using Moisture analyzer MAX series, RADWAG 26 – 600 Radom (Markova et al., 2003).

Preparation of ethanolic extracts

The dry, crushed and powdered plants raw materials from *Juniperus sabina* and *Pinus sylvestris* were extracted with 96% ethyl alcohol in the ratio plant materials: solvent – 1 : 5. The mixture was shacked for 4 hours on a laboratory shaker, then infused during 72 hours, followed by evaporation of the solvent. The precipitation was diluted with 96% ethanol and the extract, containing in 100 ml 20 g of dry substances, was obtained.

Essential oil

Essential oil extraction was carried out by the hydrodistillation Ginsberg method (Rassem et al., 2016; Timasheva et al., 2016). A samples (200 g) fresh or dry plant raw materials were placed in a 2 liter flat bottomed flask, 1,600 ml of water was added and boiled. The duration of this procedure was 4.0 hours. Essential oil was evaporated from plant raw materials by heating a mixture of water and plant materials at atmospheric pressure, followed by the liquefaction of the vapors in a condenser (reflux) and collection in receiving vessel Ginsberg. The yield of essential oil was expressed as a mass-volume percent of absolute dry weight of the plant raw materials.

Determination of peroxyl radical scavenging activity (PRSA)

Determination of PRSA of extracts is based on the degree of inhibition of potassium iodide oxidation by antioxidants that scavenge peroxyl radicals, generated from thermal degradation of 2.2'-azobis(2-amidinopropane)-dihydrochloride (Ivanova, 2016). Iodine amount released during oxidation was determined by potentiometric titration using the automatic titrator "Titroline easy" (Germany) with steps of 0.01 ml. PRSA of the tested sample was expressed as the inhibition ratio for iodine release in comparison with control sample (without antioxidant). The PRSA of standard antioxidants and plant extracts progressed in a dose-dependent manner. The linear relationship between PRSA and concentration of samples containing antioxidants had high correlation coefficients (r^2 = 0.848–0.997). The 50% inhibitory concentration IC (50) of each extract was calculated from curves of dose-dependent PRSA. The doses of standard antioxidants that scavenged 50% of free radicals were 35.37 ±3.36 μM for gallic acid and 138.71 ±22.33 μM for Trolox. PRSA of extracts was expressed in gallic acid (GAE) equivalent.

DPPH radical scavenging activity

DPPH radical scavenging activity of extracts was measured using 2.2-diphenyl-1-picrylhydrazyl (DPPH) by procedure widely reported (Clarke et al., 2013). The tested extracts of different concentrations (from 0.1 to 1%) were prepared. The sample (0.1 mL) was mixed with 3.9 mL of DPPH solution (0.025 g DPPH in 100 mL methanol). The absorbance of the reaction mixture was determined after 10 min. in darkness using the Visible Spectrophotometer GENESYS™ 20 (4001-000 UV/Vis, ThermoFisher Scientific) at 515 nm. The inhibition

concentration of 50% free radicals (IC_{50}) was calculated from the curve of activity-dose dependence in limits of extract concentration from 25 to 500 μg/ml. The Trolox (6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid, 0–210 μg/L, R^2 = 0.998) serve as standard antioxidant substances and radical scavenging activity of tested extracts were expressed in mg Trolox equivalents per g DW.

Determination of ovicidal, insecticidal, antifeedant, repellent and deterrent activities in the laboratory conditions

Determination of biopesticidal activity of alcohol extracts and essential oil were carry out on the egg-laying, imago and 2–3 instars larvae of Colorado Potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) and on the 2–3 instars larvae wax moth *Galleria mellonella* L. (Linnaeus, 1758) (Lepidoptera: Pyralidae) according to classic method which was modified us (Elisovetcaia, 2010). The biological material of *L. decemlineata* was obtained from a natural population of insects, but *G. mellonella* was reared in the laboratory conditions using an artificial nutrient medium.

Laboratory testing of the extracts against *L. decemlineata* L. was carried out in a climate cell with adjustable conditions at set temperatures of +22–24 °C, air humidity of 70–80% and 16-hours photoperiod. Young leaves of potatoes with standard sizes have served as a substrate for feeding *L. decemlineata*. Leaves were treated using a method of immersing into the extracts' and oils' solutions, kept in laboratory exhaust hood for 1 hour until full evaporation of the solvent and after which they were placed into Petri dishes with insects. The control was a variant of leaves treatment with a water-ethanolic solution of corresponding concentration.

Laboratory testing of the extracts and oils from *J. sabina* and *P. sylvestris* against *G. mellonella* was carried out in a climate cell Versatile Environmental Test Chamber SANYO with adjustable conditions at set temperatures of +28.0–0.2 °C, air humidity of 70% and 16-hours photoperiod. For laboratory experiments, Petri dishes were used. An artificial nutrient medium (ANM) has served as a feed for *G. mellonella* with the addition of the extracts and oil. Were 11 variants: first to third three controls – treatment of the feed (200 μkl/2.0 g feed) and dorsal field of the insects (5 μkl) with 0.5% ethanolic solution and without treatment as well as fourth to eleventh – treatment of the feed (200 μkl/2.0 g feed) and treatment of the dorsal field of the insects (5 μkl) with ethanol extracts (0.1%) and essential oil from *J. sabina* and *P. sylvestris*. The feed was treated by essential oils and the plant extracts using a micropipette and thoroughly mixed kept outdoors for an hour and then placed into Petri dishes with insects. We observed the movement of larvae during the first hour after the accommodation and then daily monitored the behavior of the larvae. Also, the feed was periodically weighed (on 3, 7, 14 and 18 days), up to the beginning of pupation of the larvae.

Insecticidal activity

The insecticidal activity (efficiency) was determined using the number of dead insects for three days (for *L. decemlineata*) and two weeks (for *G. mellonella*) in comparison with the control according to a classic method which was modified by us (Elisovetcaia, 2010).

Antifeedant activity

The antifeedant activity of the extracts and oils under laboratory conditions against Colorado potato beetle was established on the third day after the beginning of the experiment according to a standard scale in points (Elisovetcaia, 2010); against larvae *G. mellonella* was noted by the amount of feed eaten by one larva in three days compared to the control.

Repellent effect

Repellent effect was recorded on the basis of the behavior of the larvae and imago *L. decemlineata* and of the larvae *G. mellonella* after placing them in Petri dishes with the treated leaves or nutrient medium.

Effect on pupal and imago phases (deterrent activity)

The effect of alcohol extracts and essential oils from plants of *J. sabina* and *P. sylvestris* was evaluated when larvae fed with treatment meal, as well as with topical dorsal application. Observations on insects were carried out periodically for a month. It was noted the beginning of pupation and the emergence of the imago, as well as the number of eggs laid.

Ovicidal activity

The extracts (0.1–20.0%) and oils (0.1%) from *J. sabina* were tested on ovicidal activity using egg clutches of *L. decemlineata*, collected from untreated potato plants. Each variant consisted of three replications, three-egg clutches each. Leaves with egg clutches were treated using a method of immersing into the extract for several seconds and then kept in an exhaust hood for 1 hour until full evaporation of the solvent. Then they were placed into double dishes. Leaves with egg clutches treated with water-ethanolic solution of corresponding concentration served as a control. The ovicidal activity was determined on the 10^{th} day according to classic method which was modified us (Elisovetcaia, 2010).

Statistic analysis

Statistical processing of obtained data was carried out according to the one-way ANOVA test and Microsoft Excel software.

Results and discussion

It was found that the moisture of the needles of juniper savin and scots pine varies depending on the place of growth and on the collection period (Table 1). On average, the annual content of moisture of needles *J. sabina* fluctuated from 45.15 ±1.30 (February) to 59.08 ±2.45% (May). The needles of *J. sabina* growing in Nitra accumulated the maximum amount of moisture, the smallest – in Moldova. At the same time, the annual average of needles *P. sylvestris* moisture fluctuated from 47.10 ±2.15 (March) to 58.60 ±2.30% (September). The plants of *P. sylvestris* growing in Moldova accumulated the maximum amount of moisture, the smallest – in Nitra. A comparative analysis of the annual average of moisture content of raw materials showed that there is an insignificant difference between the plant samples from different collection regions, for *J. sabina* and *P. sylvestris*. However, it should be noted that during the season

(spring, autumn, winter) the moisture content of the raw material varies significantly in all regions for both Coniferous plants (Table 1).

Table 1 Annual average of moisture content in needles of *Juniperus sabina* L. and *Pinus sylvestris* L. from different places of growth

Variant	Average of moisture content depending on the time of collection (%)			Annual average of plant raw materials moisture (%)	Difference between variants	Least significant difference
	spring	autumn	winter			
Juniperus ***sabina*****, Moldova, Chisinau (RM)**	47.80 ±1.75	46.20 ±1.50	45.15 ±1.30	46.38 ±0.92	–	*$LSD_{0.05}$ =8.27, p >0.05
Juniperus ***sabina*****, Slovakia, Pianiny, Tatras Mountains (SM)**	51.03 ±2.12	50.17 ±2.45	49.30 ±1.90	50.17 ±2.45	-3.79	
Juniperus ***sabina*****, Slovakia, Nitra (SN)**	59.08 ±2.45	52.50 ±2.15	47.10 ±2.45	52.89 ±5.99	-6.51	
	**$LSD_{0.05}$ = 0.79, p ≤0.05					
Pinus sylvestris**, Moldova, Chisinau (RM)**	50.80 ±3.25	58.60 ±2.30	50.36 ±1.85	53.25 ±4.84	-	*$LSD_{0.05}$ = 12.35, p >0.05
Pinus sylvestris**, Slovakia, Nitra (SN)**	48.10 ±1.88	56.80 ±2.78	49.07 ±2.01	51.32 ±4.76	1.93	
	**$LSD_{0.05}$ = 0.91, p ≤0.05					

Notes: RM – Republic of Moldova; SN – Slovakia, Nitra; SM – Slovakia, Pianiny; Tatras mountains region

The analysis of the antioxidant capacities of extracts *J. sabina* and *P. sylvestris*, growing in different zones of Slovakia and in R. of Moldova is shown in Table 2. The extracts of *P. sylvestris* from Slovakia and Moldova possessed the highest IC_{50} in comparison with the extracts of *J. sabina*. The extract of *J. sabina* from Nitra had significantly lower IC_{50} than the extracts from Pianiny Mountains and Moldova. The lowest IC_{50} represents the highest potency of extracts to scavenge of DPPH free radicals. It was noted, that the DPPH radical scavenging activity of ethanolic extracts of J sabina depended on places of growth. At the same time, it was revealed that the antioxidant activity of *P. sylvestris* extracts growing in different regions does not significantly differ from each other ($LSD_{0.05}$ = 5.3; p ≤0.05). The ethanolic extracts of *J. sabina* from Nitra showed the highest antioxidant activity against both DPPH and peroxyl free radicals. The antioxidant activity of extracts of *J. sabina* from Moldova was significantly lower than other samples of *J. sabina* (Table 2). Despite the fact that extracts from scots pine showed an activity of 2.6–4.4 times lower than that of savin juniper, their antioxidant activity can also be considered high. Thus, all tested samples conform

to diminution of antioxidant activity that was presented by followed order: *J. sabina* SN > *J. sabina* PM > *J. sabina* RM> *P. sylvestris* RM ≥ *P. sylvestris* SN. It should be noted that to scavenge the same amount of DPPH free radicals the extracts were used in concentrations of 14–16 times more than for peroxyl radical scavenging. The results (Table 2) obtained by both procedures for determinations of antioxidant activities have close correlation, Pearson coefficient of correlation equal to 0.98–0.99. These results coincided well with our previous studies (Gladei et al., 2018; Elisovetcaia et al., 2018a).

Table 2 Radical scavenging activity of extracts from needles of *Juniperus sabina* L. and *Pinus sylvestris* L., various places of growing

Sample	DPPH antioxidant activity		Peroxyl radical scavenging activity		Gallic acid equivalent (GAE) (μMol/g DW)
	IC_{50} (mg/ml)	Trolox equivalent (μg/g DW)	IC_{50} (mg/ml)	Trolox equivalent (μg/g DW)	
***J. sabina* RM**	2.60 ±0.17	53.77 ±3.48	0.16 ±0.02	217.06 ±0.28	221.45 ±1.35
***J. sabina* SN**	1.64 ±0.01	85.57 ±0.21	0.13 ±0.01	267.15 ±0.35	303.99 ±1.16
***J. sabina* SPM**	1.93 ±0.01	72.58 ±0.32	0.15 ±0.04	231.53 ±0.41	261.27 ±5.31
***P. sylvestris* RM**	6.68 ±0.64	20.96 ±0.53	0.41 ±0.03	84.71 ±0.18	92.14 ±7.19
***P. sylvestris* SN**	7.28 ±0.55	19.23 ±0.55	0.51 ±0.04	68.10 ±0.14	75.29 ±0.39

Determination of the biopesticidal activity of obtained extracts and oils from *J. sabina* against Colorado Potato beetle (*Leptinotarsa decemlineata* Say) in the laboratory conditions

Ovicidal activity

It was established, that both 5.0% ethanolic extracts and 0.1% essential oils (from Moldova and Slovakia) in the laboratory condition possessed higher ovicidal activity against *L. decemlineata* (Table 3). In none of the variants of the *J. sabina* hatching of the larvae of the Colorado, beetle was observed. Whereas in the control hatching of the larvae reached 100% on the second day of the experiment. With a decrease in the concentration of alcohol extracts (from 0.1 to 20%), ovicidal activity significantly decreases – $LSD_{0.05} = 61.5$; $p \leq 0.05$ (Figure 1).

Early, we found that at a concentration of 1.0–2.5% (dry solids), the ethanol extract of *J. sabina* exhibits low insecticidal activity in regard to the egg-laying of the Colorado potato beetle (Elisovetskaya, 2015). The new obtained data showed that needles of the *J. sabina* contain biologically active substances with ovicidal properties, which, however, are insufficient quite extracted with 96% ethanol. However, it was found that *J. sabina* essential oil is most promising as an ovicidal means against Coleopteran insects if it is used indoors, such as greenhouses, storages, etc.

Table 3 Insecticidal, antifeedant and ovicidal activity of plant extract and essential oil from *Juniperus sabina* L. against *Leptinotarsa decemlineata* Say in the laboratory condition, 2018

Variants		Concentration (dry residue for ethanolic extract) (%)	Insecticidal activity (%)		Antifeedant activity (point)		Ovicidal activity (%)
			imago	larvae of II-III instars	imago	larvae of II-III instars	0
Control		–	0	0	5	5	
			Ethanolic extract				
J. sabina	Slovakia	20.0	13.3	73.7	2–3	1	100
	Moldova	20.0	6.7	66.7	2–3	1	100
Essential oil							
J. sabina	Slovakia	0.1	53.3	100	1	1	100
	Moldova	0.1	26.7	100	1	1	100
			$LSD_{0.05}$ =15.4 $p \leq 0.05$		$LSD_{0.05}$ =10.9 $p \leq 0.05$		

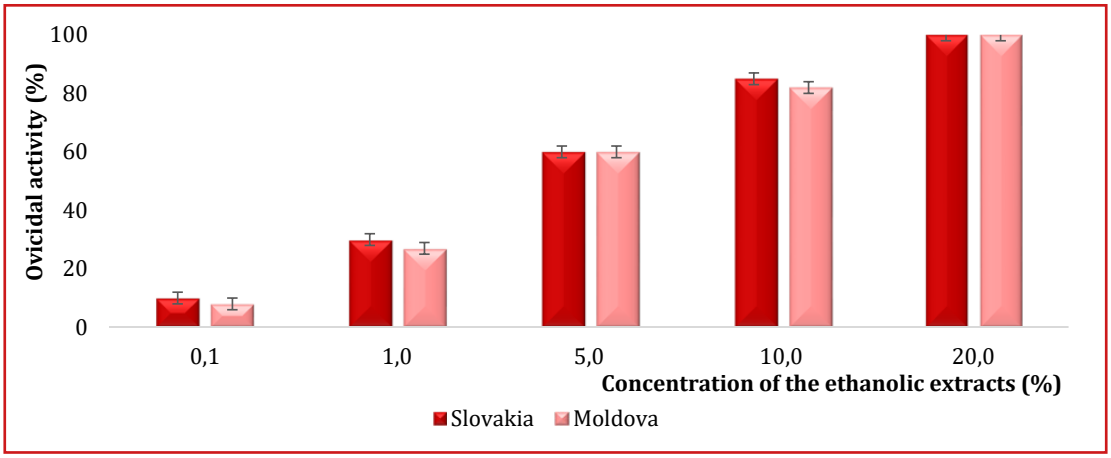


Figure 1 Ovicidal activity ethanolic extracts (from Moldova and Slovakia) depending on the concentration, against *Leptinotarsa decemlineata* Say in the laboratory condition ($LSD_{0.05}$ = 61.5; $p \leq 0.05$)

Insecticidal and antifeedant activity

As a result of laboratory testing, it was revealed that essential oils from *J. sabina* were more effective than ethanolic extracts against imagoes and larvae of potato beetle (Table 3). It was established that the character of extracts effects on insects is intestinal and insignificant contact. Essential oils have a pronounced contact action, as well as intestinal and „fumigant". The lowest insecticidal activity was observed by application of alcohol extracts against adult insects. In variants with essential oil, the death of larvae reached 100% in both variants

(Moldova and Slovakia). It was determined, that ethanolic extract and essential oil of *J. sabina* from Slovakia showed higher insecticidal properties against the Colorado beetle than the same from Moldova. Statistical analysis demonstrated, that the difference was significant only for variant with imago – between essential oils from Moldova and Slovakia ($LSD_{0.05}$ =15.4, $p \leq 0.05$). There was no significant difference between the other variants (except control). All plant preparations showed high antifeedant properties to the larvae and imago Colorado potato beetle, which were slightly lower for imago in ethanol extracts compared to essential oils (Table 3). Thus these extracts and essential oils showed high level insecticidal activity, combining contact, intestinal and fumigant action, against larvae Coleopterans, which coincided very well with our previous studies (Elisovetcaia et al., 2017; 2018a).

Repellent and deterrent activities

Our observation for *L. decemlineata* during the first hour after the accommodation in the Petri dishes showed that larvae in the experiment moved in the opposite direction from the feed and kept along the walls of dishes, trying not to approach the treatment leaves of the potato. They circled on the perimeter of the Petri dishes, sometimes approached to the treated feed, but, in this case, larvae immediately, very quickly, moving in the opposite direction. This fact indicates the presence of the repellent effect of extracts and essential oil from *J. sabina*. Comparative analysis showed that the control larvae moved to the feed in the first 5–7 seconds.

Summing up what has been said, it was determined, that mortality of imago and larvae *L. decemlineata* when using plant preparations from *J. sabina* consisted in average 6.7–53.3% and 66.7–100% respectively, ovicidal activity was 100%, antifeedant effect persisted at the level of 1–3 points for imago and 1 point for larvae. As well as were established repellent and deterrent activities for the extracts and essential oil from *J. sabina* relative to *L. decemlineata*. The highest insecticidal, antifeedant, repellent and deterrent properties against Colorado potato beetle were shown by essential oil *J. sabina* from Slovakia. These results are consistent with those obtained previously (Elisovetcaia et al., 2017) and prove the presence of identical properties in the plant *J. sabina*, regardless of the region of growth.

Determination of biopesticidal activity of obtained extracts and oils from *J. sabina* and *P. sylvestris* against wax month *Galleria mellonella* in the laboratory conditions

As a result of laboratory testing, it was revealed that extracts and essential oils from *J. sabina* were more effective against larvae *G. mellonella* than extracts and essential oils from *P. sylvestris* (Table 4).

Insecticidal activity

It was found that the greatest insecticidal effect was achieved in the variant with the dorsal application of essential oil from *J. sabina* – 65.0% mortality of larvae. Statistical analysis of the data showed that there is no significant difference between all the variants with extracts and essential oils from *P. sylvestris*. At the same time, in case of *J. sabina*, insecticidal activity significantly depends both on the method of extracting biologically active substances, and on the means of treatment – introduction extract or oil into the feed or topically on the dorsal

field of the insects. It was established that biologically active substances contained in pine needles have a more intestinal effect than contact, while for *J. sabina* the exact opposite is true (Table 4).

Table 4 Effect of essential oil and ethanolic extract from *Juniperus sabina* L. and *Pinus sylvestris* L. on the survival of larvae, the formation of pupae molting of imago and on the feed consumption of the larvae *Galleria mellonella* Linnaeus

Variants	Mortality larvae (%)	Pupation (%)	Imago molted (%)	Mortality imago (%)	Average amount of feed consumption (larva/3 day) (g)
Control (0.5% Ethanol*)	0	100	100	0	0.0693 ±0.0006
Control (0.5% Ethanol)**	0	100	100	0	0.0690 ±0.0005
Control***	0	100	100	0	0.0696 ±0.0007
***Js* 0.1% Ethanolic extract ***	10.0	30.0	24.0	16.7	0,0126 ±0.0004
Js* 0.1% Ethanolic extract*	20.0	48.0	36.0	55.0	0,0252 ±0.0005
Js* Essential oil	20.0	15.0	24.0	66.7	0,0105 ±0.0003
Js* Essential oil*	65.0	15.0	8.0	50.0	0,0087 ±0.0002
Ps* 0.1% Ethanolic extract	13.3	46.7	46.7	0	0.0219 ±0.0004
Ps* 0.1% Ethanolic extract*	6.7	46.7	53.3	0	0.0183 ±0.0003
Ps* Essential oil	13.3	40.0	40.0	0	0.0213 ±0.0004
Ps* Essential oil*	6.7	46.7	53.3	0	0.0177 ±0.0005
	$LSD_{0.05}$ = 5.1, $p \leq 0.05$			$LSD_{0.05}$ = 0.0042, $p \leq 0.05$	

Notes: * treatment of the feed, 200 µkl; ** treatment of the dorsal field, 5 µkl; *** without total treatment

Antifeedant activity

As a result of the experiment it was established, that the extracts and essential oils from *J. sabina* and *P. sylvestris* have high antifeedant activity against larvae *G. mellonella*. Amount of feed consumption of the larvae wax moth in the laboratory conditions (during the first three days of the experiment) both when using extracts from *J. sabina* and *P. sylvestris*, and their essential oils, was significantly less than in the control variants (Table 4). There was no significant difference in the feed consumption between the different control variants. At the same time, it was noted that was a significant difference between the variants with extracts and oils from *J. sabina* and *P. sylvestris*. Thus, it was found that extract and essential oil from *J. sabina* more effectively reduced feed consumption than from *P. sylvestris*. It is also noted that between the means of treatment (insects or feed) the difference is not significant, except for the ethanol extract from *J. sabina* – the dorsal application was more effective. In other words,

in the variant with topical application of essential oil from *J. sabina* to the dorsal field of insects the lowest feed consumption was observed (Table 4). Earlier, Chinese researchers (Gao et al., 2004) have proved the high insecticidal and antifeedant activities of deoxypodophyllotoxin (DPP), isolated from a petroleum ether extract of *Juniperus sabina*, against lepidopteran larvae *Pieris rapae* L. (Lepidoptera: Pieridae) At 0.015–0.100 g/litre, DPP showed insecticidal activity, with an LC_{50} of 0.020 g/litre. Antifeedant activity against fifth-instar larvae of *P. rapae* was at 0.05–1.00 g/litre and its AFC_{50} (concentration for 50% antifeedant activity) values at 12 and 48 h were 0.170 and 0.060 g/litre, respectively. Our studies showed that ethanol extracts and essential oils from *J. sabina* also exhibit pronounced insecticidal and antifeedant properties against another representative of Lepidoptera – *G. mellonella* (Pyralidae). However, it was found that the level of biopesticidal activity significantly depends on the extraction method and ontogenetic phase of the pest development. Differences in the activity level of extracts from *J. sabina* confirm the theory that secondary plant metabolites have a selective effect and affect multiply on the various species of insects.

Repellent and deterrent activities

The repellent activity of extracts and essential oils from *J. sabina* and *P. sylvestris* was confirmed by observing the behavior of larvae *G. mellonella* during the first hours after the accommodation in the Petri dishes. Insects moved in the opposite direction from the feed and circled around the feed for a long time, not approaching it. The time between replanting and the start of nutrition in the experimental variants was 9–40 (and more) times higher than the control and ranged from 90–120 seconds to 3–5 minutes. New data are consistent with our previous data (Elisovetcaia and Brindza, 2018).

The deterrent properties of the extracts and oils from *J. sabina* and *P. sylvestris* were manifested in suppressing the nutrition of the wax moth larvae, reducing the number of the pupated larva, molted imago, as well as in suppressing oviposition of the adults *G. mellonella* in comparison with the control (Table 4). Feed consumption in the experimental variants with extracts and essential oils from *J. sabina* and *P. sylvestris* was 2.75–6.6 times lower than in control; pupation decreased by 2.1–6.7 times, and the molting of imago – by 1.9–8.0 times. It was observed that essential oils had also deterrent effect on egg-laying of the wax moth – the number of eggs laid in the variants treated with extracts and essential oils was 25–100% lower than in the control.

Thus, all tested plant preparations from Coniferous (*J. sabina* and *P. sylvestris*) showed high insecticidal, antifeedant, repellent and deterrent properties against Lepidopterans (*G. mellonella*), as well as ethanolic extracts and essential oil from *J. sabina* and against Coleopterans (*L. decemlineata*). These data significantly supplement the previously obtained results (Elisovetcaia and Brindza, 2018).

Conclusions

For the first time was carried out a comparative analysis of the antioxidant and biopesticidal activity of two species of conifers – *Juniperus sabina* and *Pinus sylvestris*, growing in the Republic of Moldova (RM) and Slovak Republic, Nitra (SN) and Pianiny mountains (PM). The ethanolic extracts of *J. sabina* from Nitra showed the highest antioxidant activity against both DPPH

and peroxyl free radicals. All tested samples conform to diminution of antioxidant activity were presented by followed order: *J. sabina* SN > *J. sabina* PM > *J. sabina* RM> *P. sylvestris* RM ≥ *P. sylvestris* SN (DPPH – 85.57 ±0.21 > 72.58 ±0.32 > 53.77 ±3.48 > 20.96 ±0.53 >19.23 ±0.55 and peroxyl free radicals – 267.15 ±0.35 > 231.53 ±0.41 > 217.06 ±0.28 > 84.71 ±0.18 > 68.10 ±0.14). Moreover, it was established that both ethanolic extracts and essential oils from *J. sabina* possessed high biopesticidal efficiency against *Leptinotarsa decemlineata* and *Galleria mellonella*. Ovicidal activity of the extracts and essential oils from *J. sabina* against *L. decemlineata* reached 100%, mortality of imago and larvae consisted on average 6.7–53.3 and 66.7–100% respectively. The antifeedant effect of all plant preparations persisted at the level of 1–3 points (foliage ingestion did not exceed 5–25%). It was established that the essential oils were more effective across the board than ethanolic extracts. It was revealed that against larvae *G. mellonella* extracts and essential oils from *J. sabina* were more effective than from *P. sylvestris*. The greatest insecticidal effect was achieved in the variant with the dorsal application of essential oil from *J. sabina* – 65.0% mortality of larvae. The extracts and essential oils from *J. sabina* and *P. sylvestris* have a high antifeedant, repellent and deterrent activities, that expressed in the suppressing the nutrition of the wax moth larvae, reducing the number of pupated larva, molted imago, as well as in suppressing oviposition of the adults *G. mellonella* in comparison with the control. Feed consumption in the experimental variants with extracts and essential oils from *J. sabina* and *P. sylvestris* was 2.75–6.6 times lower than in control; pupation decreased by 2.1–6.7 times, and the molting of imago – by 1.9–8.0 times. Also the number of eggs laid in the variants treated with extracts and essential oils was 25–100% lower than in the control.

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OPEN ACCESS

REACTIONS OF POLLEN OF PLANTS FROM GENUS *LYSIMACHIA* L. ON DIFERRENT ENVIROMENTAL CONDITIONS

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A cytological study of pollen of different species of the genus *Lysimachia* L. has been carried out. This is part of the comprehensive study of biological, anatomical, cytological, biochemical and molecular genetic features of the common species in Ukraine of this genus. The genetic collection of the genus *Lysimachia* has been begun to create on the territory of Feofania Park in Kyiv which is the government designated park. The general appearance and sculpture of pollen grains and the sensitivity of pollen of some species of the genus *Lysimachia* to different conditions of plant cultivation were studied. The pollen morphology and structure of his shell in of *L. nummularia* L., *L. punctata* L. and *L. vulgaris* L. studied using the scanning electron microscope. The obtained results made it possible to confirm that the shape of pollen grains, the structure of its shell are genetically determined and bear the specific features of the taxon. Growth and development of plants of *Lysimachia* in different conditions of the environment affect the biometric indices of pollen and its fertility. The sensitivity of pollen to exogenous conditions is elevated during the flowering of plants, compared to the phase of budding. There was a tendency towards a decrease in the diameter of pollen grains with the flower opening of the *L. punctata* and *L. vulgaris* in the cultivation of plants in conditions with increased exposure to anthropogenic factors. A high level of pollen fertility for plants of *L. nummularia* has been established.

Keywords: *Lysimachia* L., pollen, sensitivity

Вступ

Процеси формування і розвитку пилку більшості рослин є дуже чутливими до впливу зовнішніх факторів різної етіології, які істотно впливають на поведінку пилкових зерен при проростанні (Bessonova, 1992; Dzyuba, 1999; Glazunova, 2001). Ці фактори значно, а іноді й невпізнаванно, змінюють характер росту пилкових трубок і процес проростання (Golubinskiy, 1974; Petrovich, 1976; Frenkel and Gadun 1982). Ще в минулому столітті паліноіндикація якості оточуючого середовища визнана надійною ланкою в ланцюгу екологічного моніторингу, оскільки реакція чоловічої генеративної сфери рослин (зокрема – пилку) і тварин загалом адекватні (Bessonova, 1992; Dzyuba, 1999;

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Dzyuba et al., 2001). Основою паліноіндикаційного методу є аналіз і облік морфологічних та функціональних характеристик пилку (Dzyuba, 2006, 2014). Паліноіндикація із використанням лісових деревостанів і трав'янистих рослин належить до сучасних методів фітоіндикації стану не лише природних і квазіприродних, але й селітебних ландшафтних комплексів. Пилок використовують як складову інтегрального контролю при визначенні екостанів промислово-господарського, урбанізованого та рекреаційного природокористування (Pogosyan et al., 1991; Bessonova et al., 1994; Gorovaya et al., 2003; Kavelenova, 2003; Bessonova et al., 2013; Grytzay and Tryphonov, 2015). На основі якісного і кількісного аналізу мінливості життєвих показників пилку вищих рослин в Україні здійснюється індикація якості навколишнього природного середовища (Наказ МОЗ України № 116 від 13. 03. 2007 р.).

Рід *Lysimachia* містить приблизно 140–200 видів з поширенням по всьому світу і за результатами сучасних досліджень досить активно доповнюється новими видами (Loc and Hu, 2011; Zhang et al., 2012; Zhou et al., 2015; Wang et al., 2016). В Україні наведені 5 видів цього роду (*Lysimachia nummularia* L. (вербозілля лучне або монетне), *L. vulgaris* L. (в. звичайне), *L. nemorum* L. (в. гайове), *L. punctata* L. (в. крапчасте), *L. verticillaris* L. (в. кільчасте) (Didukh et al., 2010). З них чотири (за винятком *L. verticillaris*) зростають на території парку-пам'ятки садово-паркового мистецтва загальнодержавного значення «Феофанія» (далі – ППСПМ «Феофанія»). Досить часто *L. nummularia*, *L. punctata* і *L. vulgaris* трапляються в природних і напівприродних локалітетах та культурфітоценозах Києва. Цитологічне вивчення пилку різних видів роду *Lysimachia* є частиною комплексного вивчення біологічних, анатомічних, цитологічних і біохімічних особливостей поширених в Україні представників цього роду.

Це дослідження передбачає визначення чутливості пилку вербозілля до різних умов вирощування, враховуючи їх цінність для збагачення різноманіття, зокрема, декоративних рослин та раціонального використання природних ресурсів.

Матеріали та методи

Об'єкти дослідження

Досліджувався пилок видів *Lysimachia nummularia*, *L. punctata*, *L. nemorum* і *L. vulgaris*, відібраний у фазу бутонізації та цвітіння рослин з різних за антропогенним навантаженням місцезростань в м. Києві, населених пунктів Київської і Полтавської областей. У зазначених міських насадженнях рослини *L. nummularia* були відібрані з таких територій: ділянка 1 – ППСПМ 'Феофанія' (синантропна ділянка, паркова частина); ділянка 2 – ППСПМ 'Феофанія' (напівприродна (порушена) лучна ділянка); ділянка 3 – ППСПМ 'Феофанія' (лісове угруповання); ділянка 4 – с. Новосілки Київської області (синантропна ділянка); ділянка 5 – парк 'Перемога' м. Київ (синантропна ділянка). Рослини *L. punctata* були відібрані з об'єктів декоративного озеленення (ділянка 1 – ППСПМ 'Феофанія'; ділянка 2 – вул. академіка Заболотного, м. Київ; ділянка 3 – смт. Гребінка Полтавської області). Пилок *L. vulgaris* відбирали як в напівприродних локалітетах та малопорушених умовах (ділянка 1 – ППСПМ 'Феофанія'; ділянка 2 – острів

Муромець, м. Київ), так і з об'єктів озеленення територій загального користування (ділянка 3 – Оболонська набережна, м. Київ; ділянка 4 – клумба в центральній частині м. Буча Київської обл.).

Морфометричні характеристики

Вивчалась загальна будова пилкових зерен досліджуваних видів. Сухі пилкові зерна покривали дрібнозернистим золотом за допомогою JEOL Smart Coater та вивчали за допомогою скануючого електронного мікроскопа JEOL JCM-6000 (JEOL Ltd., Токіо, Японія) з використанням програмного забезпечення Semafore (JEOL, Sollentuna, Швеція). Для дослідження брали свіжозібраний матеріал у фазу цвітіння рослин цього роду зі створеної колекції на території ППСПМ 'Феофанія'. Опис пилкових зерен – за прийнятою термінологією (Kupriyanova and Aleshina, 1972, 1978; Tokarev, 2002; Tsymbalyuk and Mosyakin, 2013; Punt et al., 1994). Вимірювання морфометричних параметрів проводили на 30 пилкових зернах кожного зразка з використанням програми AxioVision Rel. 4.8.2.0. Довжину пилкового зерна визначали за довжиною полярної осі (пряма лінія, що з'єднує проксимальний і дистальний полюс), а ширину за розміром екваторіальної осі (перпендикулярна до полярної осі та розташована в екваторіальній площині). Розміри пилкових зерен подані в мікрометрах (мкм).

Визначення фертильності пилку

Фертильність пилку досліджуваних видів визначали на препаратах, фарбованих за методом йодної реакції (Pausheva, 1988). Для кожного виду досліджували по 100 пилкових зерен на 4 препаратах у фазу бутонізації та цвітіння. В роботі використане наступне наукове обладнання Інституту: стереомікроскоп Leica M205C з комплектуючими; мікроскоп прямий бінокулярний Nikon Eclipse E100; фотоапарат Nikon Coolpix L830. Обробка даних здійснювалась за допомогою ліцензійних програм (Helicon Focus PRO, QuickPHOTO MICRO, Davis WeatherLink) з використанням комп'ютерів на базі Intel Core-i7/DDR 3 2Gb/HDD 500 Gb/DVD-RW.

Статистичний аналіз

Були визначені основні характеристики згідно методів описової статистики: середнє арифметичне (M), похибка середнього значення (m), G – дисперсія; V – коефіцієнт варіації, %.

Результати та їх обговорення

З використанням скануючої електронної мікрографії визначені типові структури, форми та розміри пилкових зерен для кожного генотипу досліджених видів роду *Lysimachia*. За зробленим раніше описом (Kupriyanova and Aleshina, 1978; Kurmanov and Ishbirdin, 2012) пилкові зерна *L. nummularia* – 3-борозно-орові, кулясті, злегка сплюснуті по екватору; в обрисах з полюса слабо 3-лопатеві, з екватора – широкоеліптичні, майже округлі; полярна вісь – 27,6–28,8 мкм, екваторіальний діаметр 22,8–25,2 мкм. Борозни широкі, довгі, з рівними краями і притупленими кінцями. Ори екваторіально витягнуті, еліптичні, 2,4–3,0 мкм завдовжки і 1,2–1,8 мкм в ширину.

Скульптура сітчаста, рівноямчаста, комірки сітки кутасті або округлі, товстостінні, діаметр комірок зменшується в напрямку до борозенок; покрив тонкий, переривчастий, потовщується над групами стовпчиків. Сучасні дослідження морфології пилку і структури оболонки 20 видів *Lysimachia* з 12 районів Китаю доповнили окремі описи: пилкові зерна сфероїдальні, субсфероїдальні, продовгувато сфероїдальні, продовгуваті. Відношення полярного діаметра до екваторіального 0,97–1,65. Розмір дрібний до середнього 10–28 × 8,5–26,6 мкм. Товщина екзини 0,8–1,5 мкм. Орнаментація горбкувато-дрібноямчаста. Визначені 4 типи пилку на основі розміру, форми, структури, орнаментації секзини: тип Capillipes (підрід Idiophyton), тип Heterogenea (підрід Palladia), тип Hutsunae і тип Christinae (підрід *Lysimachia*). Створено ключ і еволюційний розвиток чотирьох типів. Найпримітивніший тип Capillipes вирізняється дрібними пилковими зернами з горбкувато-дрібноямчастою без колумел структурою, тип Christinae – найбільш спеціалізований з добре розвинутою колумелятною і сітчастою орнаментацією (Shao and Zhang, 2005).

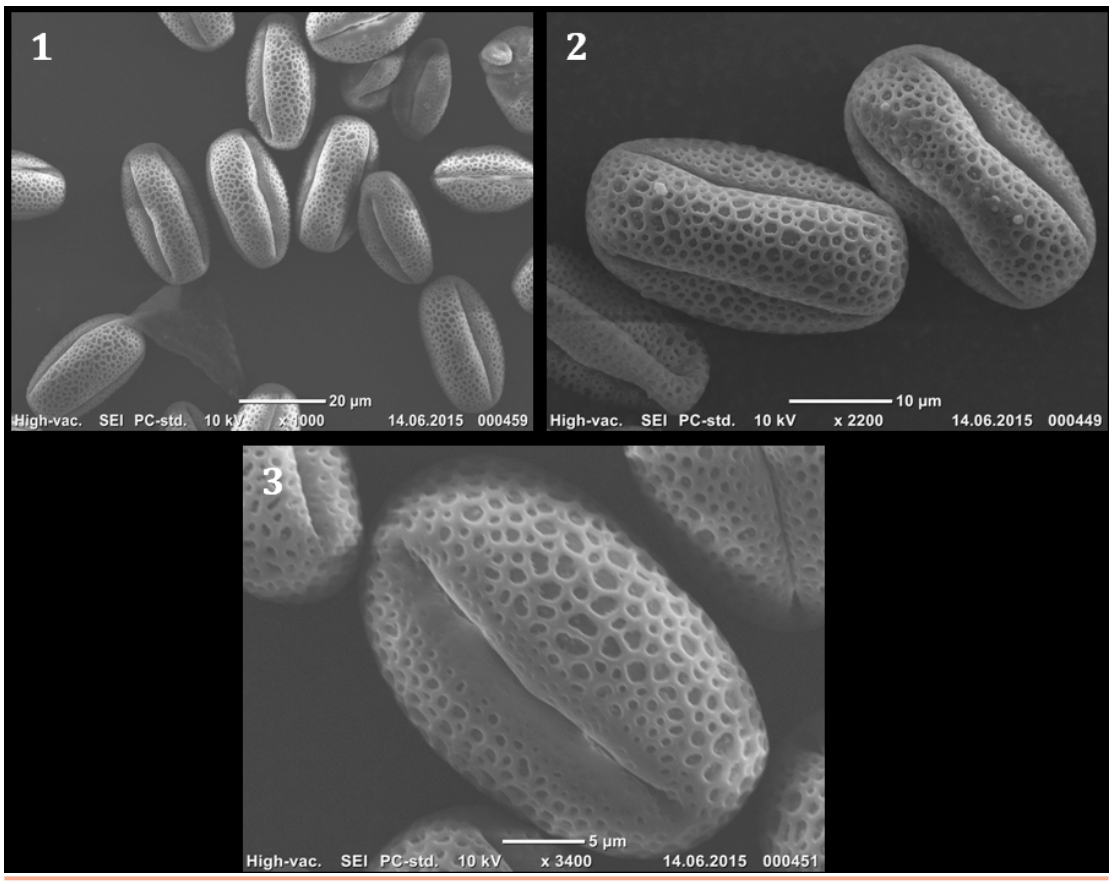


Рисунок 1 Пилкові зерна *Lysimachia nummularia* L.
1 – загальний вигляд (збільшення × 1 000); 2 – загальний вигляд (збільшення × 2 000); 3 – скульптура поверхні (збільшення × 3 400)

Figure 1 Pollen grains of *Lysimachia nummularia* L.
1 – general view (× 1,000); 2 – general view (× 2,000); 3 – reticulate exine (× 3,400)

Отримані результати дослідження пилкових зерен *L. nummularia* (Рисунок 1), *L. punctata* (Рисунок 2) та *L. nemorum* (Рисунок 3), зібраного на території ППСПМ 'Феофанія', дали можливість підтвердити, що форма пилкового зерна, структура його оболонки детерміновано генетично та несуть специфічні ознаки таксону, до якого належить рослина. Лише за розміром пилкові зерна *L. nummularia* дещо менші (довжина 30–32 мкм, діаметр 12–13 мкм), ніж у *L. punctata* (довжина 32–34 мкм, діаметр 17–18 мкм). Загалом, сформований пилок рослин *L. nummularia* з колекції парку 'Феофанія' на 8,7–11,1 % має більшу полярну вісь, але менший екваторіальний діаметр, ніж у вище наведеному описі (Kupriyanova and Aleshina, 1978; Kurmanov and Ishbirdin, 2012). Відношення полярного діаметра до екваторіального також більше і становить 1,21–1,14. Тобто, досліджені нами пилкові зерна цього виду мають виражену продовгувато-сфероїдальну форму, порівняно з більш округлим пилком у вказаному описі.

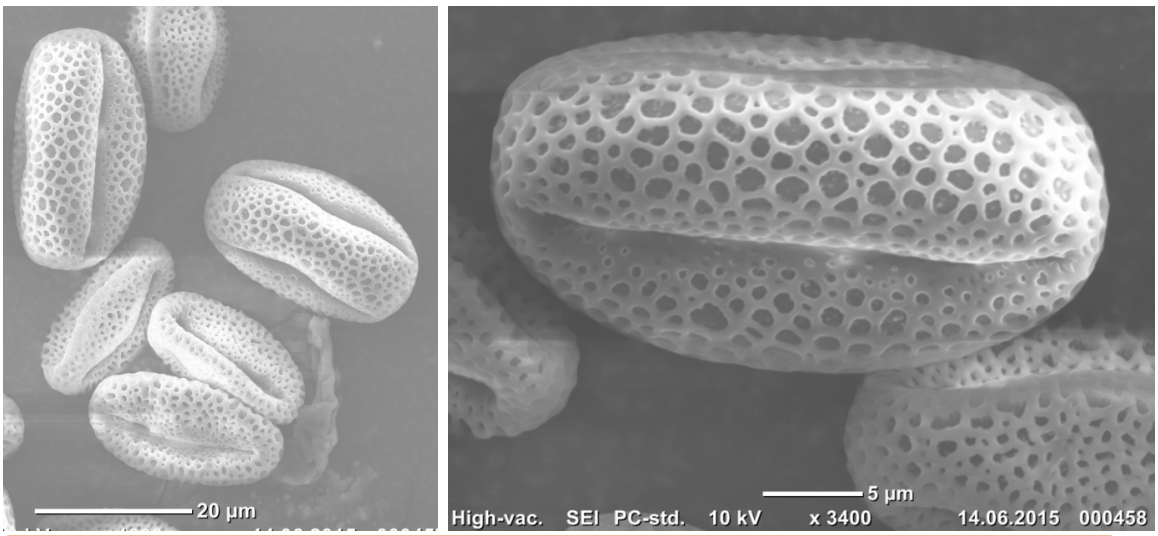


Рисунок 2 Пилкові зерна *Lysimachia punctata* L.
1 – загальний вигляд (х 1300); 2 – скульптура поверхні (збільшення × 3 400)

Figure 2 Pollen grains of *Lysimachia punctata* L.
1 – general view (× 1,300), 2 – reticulate exine (× 3,400)

Дослідження стану пилкових зерен *L. nummularia*, *L. punctata* та *L. vulgaris* в процесі розвитку рослин в колекції ППСПМ 'Феофанія', на території озеленення м. Києва і околиць виявило його чутливість до впливу умов вирощування. Для рослин *L. nummularia* відмічено тенденцію продовження росту пилкових зерен з наступним розвитком квітки. Це спостерігалось у рослин з синантропних ділянок територій з незначним антропогенним навантаженням – паркова частина ППСПМ 'Феофанія' і с. Новосілки в околицях Києва (Таблиця 1). Водночас, в популяції цього виду з синантропної ділянки на території парку 'Перемога' м. Києва в період масового квітування рослин не відмічено істотного збільшення діаметру пилкових зерен.

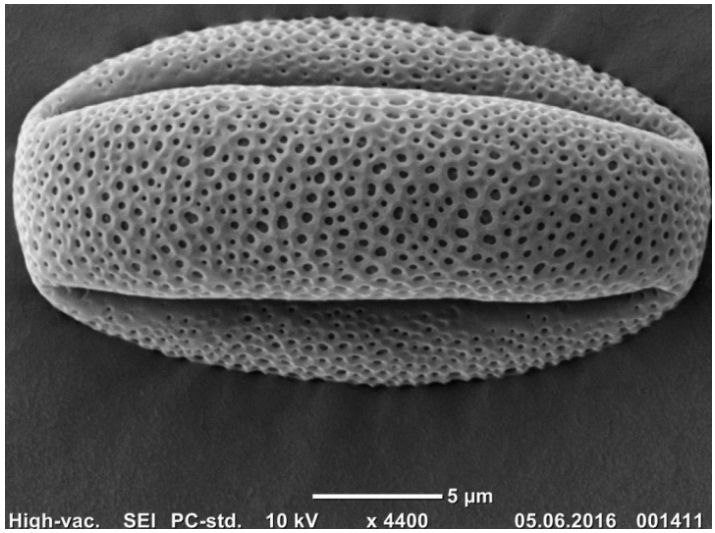


Рисунок 3 Пилкове зерно *Lysimachia nemorum* L.
1 – загальний вигляд і скульптура поверхні (× 4 400)

Figure 3 Pollen grain of *Lysimachia nemorum* L.
1 – general view and reticulate exine (× 4,400)

У рослин *L. punctata* та *L. vulgaris* з розкриттям квітки збільшення діаметру пилкових зерен припиняється. При цьому слід врахувати відмінність у розмірах квіток досліджених видів. Якщо середній діаметр квітки *L. nummularia* – 13–16 мм на різних ділянках ППСПМ 'Феофанія', то діаметр віночка у *L. punctata* та *L. vulgaris* досягає 18–20 мм, відповідно (Shpulyar, 2014; Radchenko et al., 2016). Зменшення біометричних показників пилку у фазу цвітіння рослин (на 26,82 %) відмічено у *L. punctata* при їх використанні у вуличному озелененні (вул. Заболотного, м. Київ). Слід зазначити, що навіть у фазу бутонізації в рослин з цієї ділянки пилок був дрібніший, ніж у рослин з декоративних насаджень ППСПМ 'Феофанія' (в середньому 21,36 і 22,94 мкм, відповідно).

Тенденцію до зменшення діаметру пилкових зерен після завершення фази бутонізації відмічено і у *L. vulgaris*, але це зменшення було менш вираженим. При цьому в рослин з лучного угруповання (острів Муромець) та синантропного угруповання (Оболонська набережна, м. Київ) вже в закритому бутоні формувався крупніший пилок, ніж в більш порушених умовах (озеленення центру м. Буча). Найбільш виражене зменшення розмірів пилку з розкриттям квітки було в рослин в умовах з підвищеним впливом антропогенних факторів (Оболонська набережна, м. Київ).

Подібні процеси інгібуючого впливу комплексу екзогенних факторів антропогенного середовища на ріст та життєві показники чоловічого гаметофіту нами встановлювались раніше для інших рослин (Matyashuk and Mazura, 2013; Matyashuk et al., 2014; Matyashuk et al., 2014; Matyashuket al., 2014).

Таблиця 1 Середній діаметр пилку *Lysimachia* spp. в різні фази розвитку з різних місцезростань
Table 1 The average diameter of pollen of *Lysimachia* spp. in different phases of development from different locations

Моніторингова ділянка	Бутон			Квітка		
	M ±m (мкм)	G	V (%)	M ±m (мкм)	G	V (%)
***Lysimachia nummularia* L.**						
м. Київ, ППСПМ 'Феофанія'	22,37 ±0,2224	2,47	0,0702	25,03 ±0,3059	4,67	0,0864
м. Київ, Парк 'Перемоги'	26,12 ±0,2170	2,35	0,0587	26,79 ±0,1546	1,19	0,0408
с. Новосілки, вул. Васильківська	22,3 ±0,2899	2,01	0,0636	24,85 ±0,1783	1,58	0,0507
***Lysimachia punctata* L.**						
м. Київ, ППСПМ 'Феофанія'	22,94 ±0,2350	2,76	0,0745	22,47 ±0,2324	2,70	0,0731
м. Київ, вул. Заболотного	21,36 ±0,1670	1,32	0,0552	15,63 ±0,2989	4,68	0,1352
***Lysimachia vulgaris* L.**						
м. Київ, Острів Муромець	25,4 ±0,1504	1,13	0,0417	24,96 ±0,2509	3,14	0,0710
м. Київ, Оболонська Набережна	26,07 ±0,2191	2,35	0,0588	24,6 ±0,1855	1,72	0,0533
м. Буча Київська обл.	22,94 ±0,1837	1,68	0,0566	22,86 ±0,2050	2,10	0,0634

Примітка: M – середнє арифметичне: m – похибка середнього значення; G – дисперсія; V – коефіцієнт варіабельності (%)

Пилок досліджених видів роду *Lysimachia* потенційно має високий рівень фертильності. Так, при вирощуванні *L. nummularia* в сприятливих умовах (паркова частина і лучна ділянка ППСПСМ 'Феофанія') формується понад 90 % життєздатного пилку. Хоча при цьому відзначено дуже низьку продуктивність плодоутворення та формування повноцінного насіння у цих рослин. Майже на усіх досліджених ділянках у рослин цього виду відмічалась вища фертильність пилку в закритому дозрілому бутоні, ніж у фазу масового квітування. Так, на ділянках паркової та лучної частини ППСПМ 'Феофанія', а також на синантропній ділянці с. Новосілки (відповідно, ділянки 1, 2, 4) у бутоні формувалось понад 90–92 % фертильного пилку (Рисунок 4, 5). З початком квітування у рослин на ділянках 1 та 2 зменшення середньої фертильності пилку було незначним. У рослин цього виду з лісової ділянки парку 'Феофанія' та з парку 'Перемога' (3 та 5 ділянки, відповідно) формувалось від 73 до 75 % фертильного пилку в бутоні. Але при цьому під час масового цвітіння у рослин з цих місцезростань було відмічено найменшу втрату фертильності пилку. Максимальна втрата (з 92,4 до 57,4 %) життєвого потенціалу сформованих пилкових зерен з розкриттям квітки спостерігалась у рослин на ділянці 4 (с. Новосілки Київської області).

Так само високий потенціал фертильності пилку відмічений для *L. punctata* (Рисунок 6). Рослини цього виду з ППСПМ 'Феофанія' вирізнялись значним зростанням фертильності пилку з розвитком квітки. Зростала чутливість сформованого пилку цього виду до умов вирощування при використанні рослин в озелененні населених пунктів – вул. академіка Заболотного, м. Київ та смт. Гребінка Полтавської області

(ділянки 2, 3, відповідно). Було відмічене зниження фертильності пилку з розкриттям квітки (в середньому до 50–55 %) на цих ділянках.

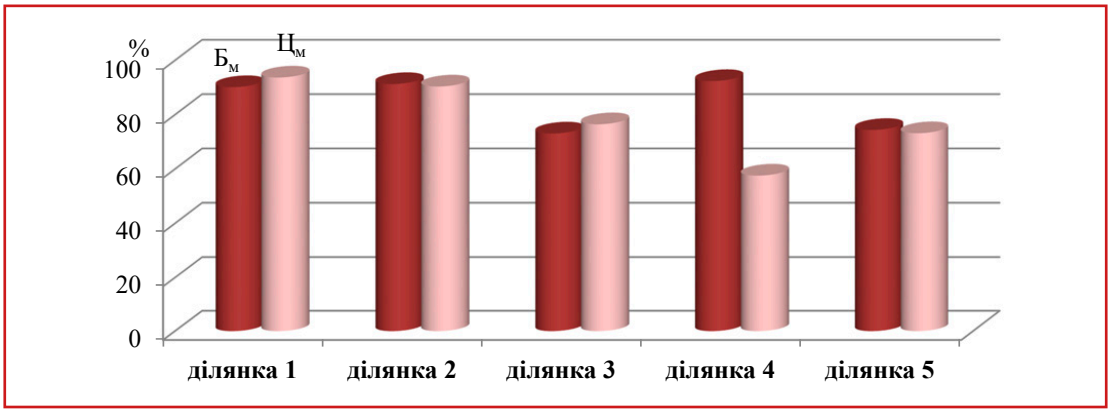


Рисунок 4 Фертильність пилку *Lysimachia nummularia* L. в фазу бутонізації ($Б_м$) та цвітіння ($Ц_м$) в різних умовах вирощування
ділянка 1 – ППСПМ 'Феофанія' (синантропна ділянка, паркова частина); ділянка 2 – ППСПМ 'Феофанія' (напівприродна (порушена) лучна ділянка); ділянка 3 ППСПМ 'Феофанія' (лісове угруповання); ділянка 4 – с. Новосілки Київської області (синантропна ділянка); ділянка 5 – парк 'Перемога' м. Київ (синантропна ділянка)

Figure 4 The fertility of pollen of *Lysimachia nummularia* L. in budding stage ($Б_м$) and flowering period ($Ц_м$) in different growing conditions
1 – the territory of Feofania Park in Kyiv (sinanthropic area, park part); 2 – the territory of Feofania Park in Kyiv (semi-natural (disturbed) meadow area); 3 – the territory of Feofania Park in Kyiv (forest grouping of plants); 4 – the village of Novosilky, Kyiv region (sinanthropic area); 5 – Peremoga Park in Kyiv (sinanthropic area)

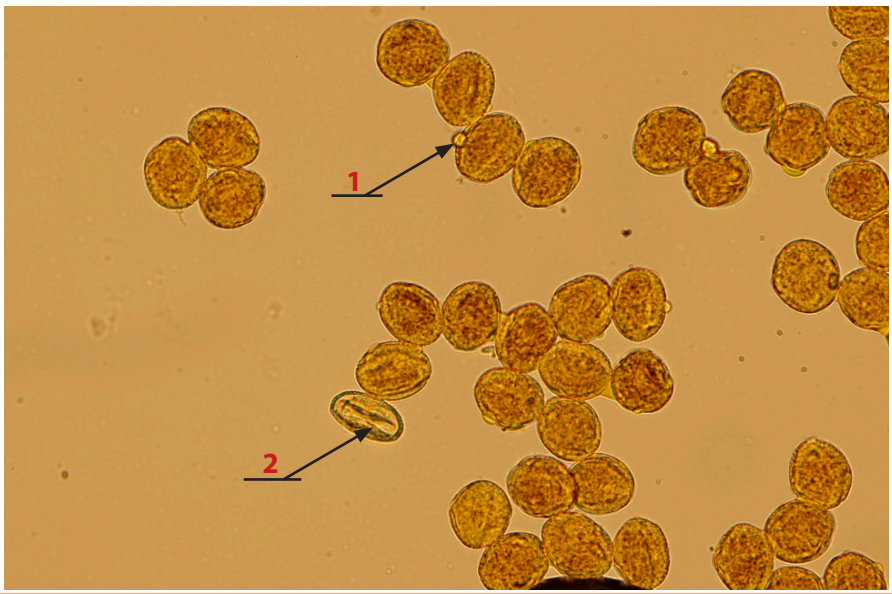


Рисунок 5 Пилкові зерна *Lysimachia nummularia* L. (ділянка ППСПМ 'Феофанія')
1 – фертильні; 2 – стерильні (при збільшенні × 400)

Figure 5 Pollen grains of *Lysimachia nummularia* L. (from the territory of Feofania Park in Kyiv)
1 – general view of fertile; 2 – sterile pollen (× 400)

Досить високу фертильність пилку відмічено у *L. vulgaris*, особливо при вирощуванні в напівприродних локалітетах та малопорушених умовах (ділянки 1, 2). У фазу бутонізації в рослин на цих ділянках фертильність пилку досягала в середньому 90% і зберігалась досить високою з подальшим розкриттям квітки (Рисунок 7). Простежувалась чутливість пилку цього виду до умов вирощування у фазу масового цвітіння, коли фертильність на ділянках з підвищеним антропогенним навантаженням (міське озеленення м. Києва і м. Буча Київської області) істотно знижувалась (до 49 % на ділянці 4).

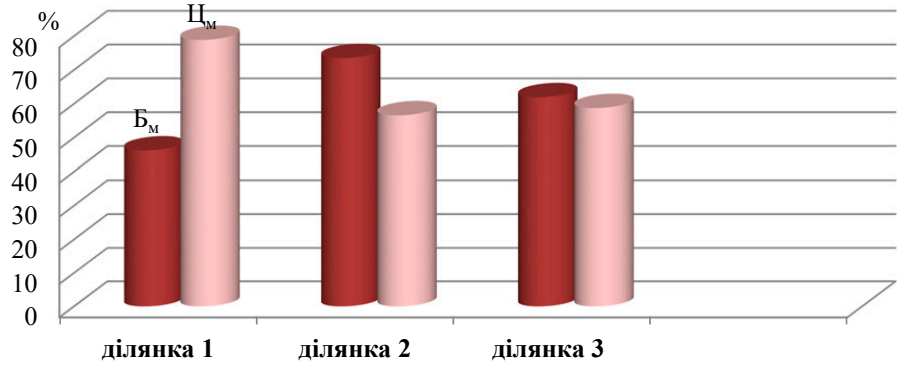


Рисунок 6 Фертильність пилку *Lysimachia punctata* L. в фазу бутонізації (Б$_м$) та цвітіння (Ц$_м$) в різних умовах вирощування
ділянка 1 – ППСПМ 'Феофанія'; ділянка 2 – вул. академіка Заболотного, м. Київ; ділянка 3 – смт. Гребінка Полтавської області

Figure 6 The fertility of pollen of *Lysimachia punctata* L. in budding stage (Б$_м$) and flowering period (Ц$_м$) in different growing conditions
1 – the territory of Feofania Park in Kyiv; 2 – Zabolotny str., Kyiv; 3 – village Hrebinka, Poltava region

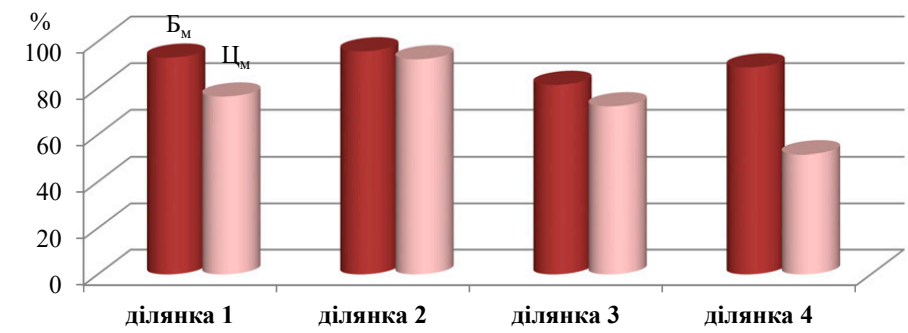


Рисунок 7 Фертильність пилку *Lysimachia vulgaris* L. в фазу бутонізації (Б$_м$) та цвітіння (Ц$_м$) в різних умовах вирощування
ділянка 1 – ППСПМ 'Феофанія'; ділянка 2 – острів Муромець, м. Київ; ділянка 3 – Оболонська набережна, м. Київ; ділянка 4 – м. Буча Київської області

Figure 7 The fertility of pollen of *Lysimachia vulgaris* L. in budding stage (Б$_м$) and flowering period (Ц$_м$) in different growing conditions
1 – the territory of Feofania Park in Kyiv; 2 – Muromets island, Kyiv; 3 – Obolon naberezhna, Kyiv; 4 – Bucha (city), Kyiv region

Висновки

Дослідження стану пилку видів роду *Lysimachia* з різних місцезростань м. Києва та його околиць виявило високий життєвий потенціал пилку *L. nummularia* в різних умовах вирощування та високу стійкість його до впливу зовнішніх факторів. Висока фертильність пилку *L. nummularia* і *L. punctata* відмічається при зростанні рослин в більш сприятливих умовах. Встановлено тенденцію до зменшення кількості фертильного пилку з початком цвітіння цих рослин, порівняно з більш життєздатним станом пилку в закритому бутоні. За чутливістю до дії комплексу екзогенних чинників можливо використовувати пилок *L. nummularia* і *L. punctata* для індикації якості навколишнього урбанізованого середовища.

Подяка

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PHOTOSYNTHETIC PIGMENTS IN SHOOTS OF *VACCINIUM CORYMBOSUM* L. (CV. ELLIOTT)

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Shoots of *Vaccinium corymbosum* L. (Ericaceae), which introduced in Ukraine are thought to be responsible for several therapeutic properties. Acetone (80 and 100%), ethanol (96%), and dyethyl ether extracts were prepared from aerial parts of *V. corymbosum* cv. Elliott in order to examine chlorophyll *a* and *b*, and carotenoids content. The quantitative content of chlorophyll *a* and *b*, and carotenoids in the shoots of *V. corymbosum* of the Elliott variety has been investigated and the dynamics of their seasonal accumulation, depending on the vegetation phase, has been analyzed. The content of pigments was determined spectrophotometrically at wavelengths that correspond to the absorption maxima of chlorophylls *a* and *b* and carotenoids for each solvent. For comparison, *Vaccinium uliginosum* L. (bog bilberry) shoots were used, as an aboriginal European species of Ericaceae. It was established that the best solvent for extraction of chlorophylls in *V. corymbosum* shoots is 100% acetone, as compared to 96% ethanol and diethyl ether. The highest content of both chlorophylls was in the flowering and fruiting stages. The maximum values for chlorophylls *a* and *b* content were recorded in autumn period – end of September-beginning of October (phase III of growing season): 78.57 ±2.02 mg/100 g DW for chlorophyll *a*, and 81.13 0.72 mg/100 g DW for chlorophyll *b*. In the 80% acetone as extragent the maximum values for both chlorophylls were observed in Phase II of fruiting 73.24 ±0.27 mg/100 g DW for chlorophyll *a* and 48.51 ±0.55 mg/100 g DW for chlorophyll *b*. In all the variants studied, the minimum value of chlorophylls *a* and *b* was observed in the IV winter growing season. The content of chlorophylls in the shoots of V. corymbosum is higher compared to the *V. uliginosum* during fruiting period, and the total content of carotenoids in the *V. corymbosum* and *V. uliginosum* shoots was not significantly different at the fruiting stage.

Keywords: *Vaccinium corymbosum* L. cv. Elliott, shoot, chlorophyll, carotenoids

Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is a long-blooming deciduous shrub belonging to the section Cyanococcus, genus *Vaccinium*, heather family (Ericaceae). The record of the name of *Vaccinium corymbosum* derives from WCSP (data supplied on 2012-03-23) which

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reports it as an accepted name with original publication details: Sp. Pl. 350 1753., derived exclusively from North America, which is found in wild form in marshy regions north USA and Canada (Vander Kloet, 1980; Song and Hancock, 2011). In the ecological aspect, highbush blueberry belongs to the xeromorphic oligotrophs and obligates mycorrhiza species, as well as to plants with incompletely specialized flowering shoots (Pliszka, 2002). *V. corymbosum* is a plant that has a high commercial value and is cultivated worldwide. Species of highbush blueberries introduced and grown in Ukraine are outside their ecological optimum and are vulnerable to changes in environmental factors (Shlapak et al., 2013; Vasyuk, and Haritonova, 2016). A sensible indicator of the physiological state of plants is the quantitative and qualitative changes in the pigment complex, which is interesting and relevant for *V. corymbosum*. Herbal pigments are not only responsible for the absorption, transmission, and transformation of light energy into photosynthesis, but also biologically active sources for therapeutic applications, including antioxidant, antitumor, anti-inflammatory action, photothermal, photodynamic therapy (Wang and Wink, 2016; Park et al., 2018). It is known that chlorophylls and carotenoids correlate with phenolic compounds of plants, and also play an important role in the prevention of thalassemia and hemolytic anemia; and reduce the risk of cardiovascular disease (Niroula et al., 2019), exhibit antioxidant and immunomodulatory activity that prevents age disease, skin disease due to exposure to ultraviolet radiation, diabetes and some types of digestive tract cancers in human (Clotault et al., 2008; Bernal et al., 2010).

The aim of the study was to investigate the features of photosynthetic pigment accumulation (chlorophylls *a* and *b* and carotenoids) in shoots of *V. corymbosum* L. cv. Elliott and to trace their changes in different vegetation periods in order to evaluate the possibility of use as a medicinal plant raw material; to compare the pigment content in *V. corymbosum* and *V. uliginosum* shoots in the fruiting phase.

Material and methodology

Plant material

Shoots of *Vaccinium corymbosum* cv. Elliot grown on the experimental plot of 'Berry Partner' Ltd. in the village Sokilnyky of Lviv region of Ukraine were collected in the seasons 2017–2018. *V. corymbosum* plants were obtained by *in vitro* propagation on Woody Plant Medium (Yavorska et al., 2016). Collection of *V. corymbosum* c.v. Elliott plant materials, was carried out during flowering (I), fruiting period (II), in autumn after fruiting (III), the period preparation for winter rest (IV). Shoots of *Vaccinium uliginosum* (an native European species of the Ericaceae family) harvested during fruiting were used for comparison. The plant material was air-dried in the dark at a temperature of 22–24 ºC and ground to a powder before use.

Extraction of photosynthetic pigments

Pigments of airborne shoots of highbush blueberry were extracted with acetone (80 and 100%), ethanol (96%), diethyl ether. The content of pigments was determined spectrophotometrically (as described in Musienko et al., 2001). The optical density of the extract was determined at wavelengths that correspond to the absorption maxima of

chlorophylls *a* and *b* and carotenoids for each extractant. Shredding of dry material carried mechanically (using a grinder), and then passed through sieves with pore diameter 2.5 mm and used for the extraction process. 100.00 or 200.00 mg plant material was ground, placed in a porcelain mortar and triturated refilling 2–5 mL portions of chilled solvent in the presence of calcium carbonate (0.2 g). The extract obtained was centrifuged at 5.000 rpm for 10 minutes. The supernatant was collected, and the precipitate resuspendable to discoloration. The extracts were combined, the volume was adjusted to 25 or 50 ml, and the optical density at the appropriate wavelength was determined using a solvent as a solution for comparison. Depending on the nature of the solvent used to extract the pigments, their concentration was calculated according to the corresponding equation.

Determination of the total content of photosynthetic pigments

The concentration of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), chlorophyll *a* + *b* (Chl *a* + *b*), and carotenoids were calculated according to the formulae. If 100% acetone was employed in pigment extracting, the optic density was defined in optimal λ waves (in λ=662; 644; 440.5 nm). The pigment concentration was estimated by Holm-Wettstein formula (Holm, 1954; Wettstein, 1957):

$$C_{Chl\ a,}\ mg/L = 9.784 \cdot D_{662} - 0.990 \cdot D_{644}$$

$$C_{Chl\ b},\ mg/L = 21.426 \cdot D_{644} - 4.650 \cdot D_{662}$$

$$C_{Chl\ (a+b)},\ mg/L = 5.134 \cdot D_{662} + 20.436 \cdot D_{644}$$

Calculations were performed according to the formula proposed by Shlyk (Shlyk, 1968) too:

$$C_{Chl\ a},\ mg/L = 11.7 \cdot D_{662} - 2.02 \cdot D_{644}$$

$$C_{Chl\ b},\ mg/L = 21.19 \cdot D_{644} - 4.54 \cdot D_{662}$$

$$C_{Chl\ (a+b)},\ mg/L = 7.14 \cdot D_{662} + 19.10 \cdot D_{644}$$

If 80% acetone was used for the extraction of pigments, the absorption was measured at λ 665, 663, 649, 646, 470 nm. The pigment concentration was estimated by the formula proposed by Vernon (Vernon, 1960):

$$C_{Chl\ a},\ mg/L = 11.63 \cdot D_{665} - 2.39 \cdot D_{649}$$

$$C_{Chl\ b},\ mg/L = 20.11 \cdot D_{649} - 5.18 \cdot D_{665}$$

$$C_{Chl\ (a+b)},\ mg/L = 6.45 \cdot D_{665} + 17.72 \cdot D_{649}$$

The calculations were performed according to the formula proposed by Lichtentaller and Welburn (1983) too:

$$C_{Chl\ a},\ mg/L = 12.21 \cdot D_{663} - 2.81 \cdot D_{646}$$

$$C_{Chl\ b},\ mg/L = 20.13 \cdot D_{646} - 5.03 \cdot D_{663}$$

Calculations of the content of chlorophylls and carotenoids in the extract, where 96% ethanol was used as a solvent, were carried out according to the formula proposed by Wintermans and de Mots (1965) aftermeasurement of absorbance at 649 and 665 nm:

$$C_{Chl\ a}, \text{ mg/L} = 13.70 \cdot D_{665} - 5.76 \cdot D_{649}$$

$$C_{Chl\ b}, \text{ mg/L} = 25.80 \cdot D_{649} - 7.60 \cdot D_{665}$$

Calculations of the content of chlorophylls and carotenoids in the extract, where 100% diethyl ether was used as an solvent, were carried out according to the formula proposed by Wintermans and de Mots (1965) after measurement of absorbance at 480, 642.5 and 660 nm:

$$C_{Chl\ a}, \text{ mg/L} = 9.93 \cdot D_{660} - 0.777 \cdot D_{642.5}$$

$$C_{Chl\ b}, \text{ mg/L} = 17.6 \cdot D_{642.5} - 2.81 \cdot D_{660}$$

$$C_{Chl(a+b)}, \text{ mg/L} = 7.12 \cdot D_{660} + 16.8 \cdot D_{642.2}$$

$$C_{Car}, \text{ mg/L} = (1{,}000 \cdot D_{480} - 0.52 \cdot C_{Chl\ a} - 7.25\ C_{Chl\ b})/226$$

The results were recalculated and expressed in mg per g of dry matter. To calculate the content of pigments in the plant material in mg/g of dry or fresh weight, according to following formula:

$$A = C \cdot V / H \cdot 1{,}000$$

where A – content of pigments, mg/g dry or fresh weight; C – pigment concentration, mg/l; V – volume of extract, ml (25 or 50 ml); H – weight of plant material (0.100–0.200 g).

All extraction procedures were performed under subdued light to avoid pigment degradation. Values represent mean ± SE (n = 5).

Results and discussion

Investigation of the content of pigments, namely chlorophylls, is an important indicator in plant physiology, biochemistry, ecology and has a taxonomic value. When applying plant raw materials for the manufacture of drugs or in the treatment of the use of teas or tinctures that make *ex tempore* it is important to know their composition, and hence the content of chlorophylls and carotenoids. For the initial screening of plant material, as well as for checking its quality in routine analyzes, rapid methods, in particular spectrophotometric, are most suitable. Determining the concentration of chlorophylls *a* and *b* in solution without separating them is difficult, however, existing differences in the absorption spectra, namely certain wavelengths where the absorption of one pigment significantly exceeds the absorption of another, allow quantitative determination of chlorophylls *a* and *b* without their separation. In this study we used acetone (100%, 80%), ethanol (96%) and diethyl ether as solvents, which are effective extractants and provide close estimates of the content of chlorophylls (Ritchie, 2006). Table 1 presents the quantitative content of Chl *a*, Chl *b*, Chl *a* + *b* in acetone and ethanol extracts of *V. corymbosum* shoots in different phenological periods and *V. uliginosum* L. (during fruiting).

Table 1 Chlorophylls contents (mean ±SD) in shoots of *Vaccinium uliginosum* L. and *V. corymbosum* L. cv. Elliott

Plant material	Phenological phases*	Chl *a* (mg/100 g DW)	Chl *b* (mg/100 g DW)	Chl *a* + *b* (mg/100 g DW)
In 100% acetone				
according to Holm-Wettstein formulas				
V. uliginosum	II	29.60 ±0.83	20.29 ±0.43	49.88 ±0.43
***V. corymbosum* cv. Elliott**	I	56.20 ±0.38	12.94 ±0.15	69.14 ±0.24
	II	63.98 ±0.46	61.22 ±1.52	125.13 ±1.98
	III	69.84 ±1.69	81.13 ±0.72	150.97 ±0.98
	IV	28.95 ±0.05	11.73 ±0.04	40.66 ±0.07
according to Shlyk formulas				
V. uliginosum	II	33.91 ±0.99	20.19 ±0.43	54.1 ±0.59
***V. corymbosum* cv. Elliott**	I	65.49 ±0.46	13.03 ±0.14	78.52 ±0.32
	II	72.47 ±0.59	60.82 ±1.50	133.35±2.08
	III	78.57 ±2.02	80.54 ±0.71	157.85 ±1.15
	IV	33.52 ±0.06	11.71 ±0.02	45.22 ±0.07
In 80% acetone				
according to Vernon formulas				
V. uliginosum	II	28.91 ±0.03	17.22 ±0.03	46.14 ±0.01
***V. corymbosum* cv. Elliott**	I	29.02 ±1.31	14.23 ±0.72	43.26 ±0.59
	II	65.54 ±0.71	48.51 ±0.55	111.84 ±3.65
	III	38.89 ±0.15	27.99 ±0.15	66.92 ±0.06
	IV	29.83 ±0.45	2.15 ±0.18	31.99 ±0.27
according to Lichtenthaler formulas				
V. uliginosum	II	29.81 ±0.73	12.26 ±0.31	42.07 ±0.61
***V. corymbosum* cv. Elliott**	I	36.90 ±0.60	5.04 ±0.26	41.90 ±0.37
	II	73.24 ±0.27	29.56 ±0.44	102.60 ±0.36
	III	37.8 ±0.14	24.05 ±0.09	61.85 ±0.07
	IV	26.7 ±0.12	2.39 ±0.05	29.09 ±0.07
In 96% ethanol				
according to Wintermans and de Mots formulas				
V. uliginosum	II	18.94 ±0.02	25.73 ±0.02	44.67 ±0.02
***V. corymbosum* cv. Elliott**	I	19.41 ±0.24	20.87 ±0.23	40.28 ±0.06
	II	22.43 ±0.12	6.32 ±0.05	28.75 ±0.08
	III	17.74 ±0.33	26.64 ±1.22	44.38 ±0.92
	IV	16.79 ±0.15	4.21 ±0.09	21.01 ±0.06

Notes: *phases of vegetation: I – the period of flowering; II – the period of fruiting ; III – in autumn after fruiting period; IV – the period preparation for winter rest.

Depending on the solvent, as shown in Table 1, the absolute values of chlorophyll content vary considerably. Thus, the content of Chl *a* in the 100% acetone extracts of *V. corymbosum* shoots in the phase I and phase III is 1.5–2.0 times higher compared to the corresponding 80% acetone extracts. Quantitative values of chlorophyll content in 96% ethanol extracts are significantly lower (2–3 times) compared to 80 and 100% acetone extracts. The quantity of chlorophylls in the extracts of *V. uliginosum* and V. corymbosum in the use of diethyl ether as a solvent (Table 2) is significantly lower compared to acetone and ethanol extracts, respectively.

The results obtained indicate that the content of chlorophylls and carotenoids in shoots of blueberries c.v. Elliott increases from flowering phase to fruiting phase (Table 1, 2), regardless of the extractant used to determine them. Further increases in the content of chlorophylls and carotenoids in the autumn (late September – early October – phase III) were detected only when 100% acetone was used as the extractant: 78.57 ±2.02 mg/100 g DW for chlorophyll *a* and 81.13 ±0.72 mg/100 g DW for chlorophyll *b*. Apparently, due to the accumulation of other groups of substances, 80% acetone and diethyl ether do not remove the entire amount of pigments from shoots in other investigated phases of highbush blueberry. The minimum content of chlorophylls *a* and *b* was observed at the beginning of winter (phase IV).

The content of chlorophylls *a* and *b* in shoots of bog bilberry was 2–3 times lower than that of highbush blueberry in the fruiting phase in all investigated variants, except for the use of diethyl ether as an extragent, where the absolute values of chlorophylls *a* and *b* were slightly different both investigated species.

Table 2 Chlorophylls and Carotenoid contents (mean ±SD) in shoots of *Vaccinium uliginosum* L and *V. corymbosum* L. cv. Elliott

Plant material	Chl *a* (mg/100 g DW)	Chl *b* (mg/100 g DW)	Chl *a* + *b* (mg/100 g DW)	Car (mg/100 g DW)
		In Diethyl ether		
***V. uliginosum* II**	16.90 ±0.26	18.20 ±0.09	35.07 ±0.20	4.39 ±0.12
***V. corymbosum* cv. Elliott**				
I	16.68 ±0.25	8.98 ±0.14	25.65 ±0.14	3.81 ±0.04
II	18.85 ±1.94	12.4 ±0.63	31.23 ±0.06	3.72 ±0.05
III	19.37 ±1.80	27.04 ±2.89	44.95 ±1.11	4.01 ±0.17
IV	11.66 ±0.31	6.5 ±0.19	18.26 ±0.24	2.73 ±0.04

Notes: *phases of vegetation: I – the period of flowering; II – the period of fruiting ; III – in autumn after fruiting period; IV – the period preparation for winter rest.

The content of carotenoids in the extracts of *V. corymbosum* shoots with ethyl ether was growing from the beginning of the vegetation till autumn and almost halved in the winter period of vegetation (IV period of winter rest). The increase in carotenoids content in *V. corymbosum* occurred during the growing season and decreased twice with the onset

of winter rest (Table 2). The quantity of carotenoids in the extracts of *V. uliginosum* shoots is higher compared to *V. corymbosum* during fruiting (Phase II).

Since chlorophylls and carotenoids in food and herbal medicines are predominantly composed of lipophilic derivatives, and exhibits antioxidant, anti-mutagenic activity, and anti-inflammatory action, and could induction of apoptosis (Ferruzzi et al., 2007; Niyogi et al., 2015), it is important to know their content and to study the mechanisms of action.

Conclusions

According to the results of our studies, the following conclusions can be made: for extraction of chlorophylls from *V. corymbosum* shoots, the best solvent is 100% acetone, as compared to 96% ethanol and diethyl ether; the highest content of both chlorophylls was in the flowering and fruiting stages; the minimum values for chlorophylls *a* and *b* content were recorded in winter period; the content of chlorophylls in the shoots of *V. corymbosum* is higher compared to the *V. uliginosum* in fruiting period; the total content of carotenoids in the *V. corymbosum* and *V. uliginosum* shoots was not significantly different at the fruiting stage. This results will have great significance for comprehensive study and further use of *V. corymbosum* shoots as medicinal importance plant material.

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STOMATAL STRUCTURE IN *SOLIDAGO* L. SPECIES AS THE INDEX OF THEIR ADAPTATION OPPORTUNITIES

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The study of the adaptability of alien plant species is very important. According to this characteristic there is possible to forecast their further spreading in the secondary distribution range. This is especially relevant for the species having already "escaped" from cultivation and started actively invading the natural communities. Well known that the structure of the leaf blade reflects the adaptability of the species. The purpose of this study is to compare the stomatal structure of cultivated *Solidago* L. species to assess their adaptive capacity. Alien specimens from the collection of Padova botanical garden (Italy): *S. altissima* L., *S. caesia* L., *S. canadensis* L., *S. graminifolia*(L.) Salisb., *S. juncea* Aiton, *S. latifolia* L., *S. lepida* DC., *S. rugosa* Mill., *S. sempervirens* L., *S. serotinoides* Á. Löve & D. Löve (= *S. gigantea* var. leiophylla Fernald), *S. uliginos*a Nutt. were the objects of the study. The stomata were characterized by 21 quantitative characteristics. Various strategies were being implemented to increase the total transpiration area of plants: increasing the size of stomata; increasing the number of stomata; increasing the area of the leaf blade; increasing the number of leaves on the shoot; increasing the number of the shoots. In terms of the relative transpiration area index (I_{ot}, %), the species are divided into three groups with a high (25–50), medium (15–25) and low (<15) I_{ot}. Our article is considered as a basis for monitoring the dissemination of alien species. The high value of I_{ot} indicates the greater adaptiveness of alien species and can be used to predict the further expansion of their secondary distribution range and increase the chances of transforming into an invasive taxon.

Keywords: *Solidago*, leaves, stoma, invasion plants

Introduction

North American *Solidago* L. species have been cultivated in European botanical gardens since the end of the 17th century (Kowarik, 2003). The most complete collection of this genus is represented in the oldest botanical garden of Europe – in Padua (Italy). The garden was established in 1545 by the decision of the Venetian Senate with the aim of growing "medicinal herbs" for the Faculty of Medicine of the University of Padua. In 1997, like the "prototype of all botanic gardens", it was listed by UNESCO as a World Heritage Site (Buffa et al., 1999).

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Solidago altissima L., *S. caesia* L., *S. canadensis* L., *S. graminifolia* (L.) Salisb., *S. juncea* Aiton, *S. latifolia* L., *S. lepida* DC., *S. rugosa* Mill., *S. sempervirens* L., *S. serotinoides* Á. Löve & D. Löve (= *S. gigantea* var. leiophylla Fernald) and *S. uliginos*a Nutt. grow in the historic part of the garden. All these species naturally grow on the territory of North America and have established into Europe through an intentional introduction. Three of them (*S. altissima*, *S. canadensis*, and *S. gigantea*) are naturalized in Europe (Tutin et al., 1976) and invaded the natural phytocenoses, i.e. belong to the group of invasive species (Lambdon et al., 2008; Vinogradova et al., 2010). In recent years *S. graminifolia* has been an active expansion of the secondary distribution range. It is marked as invasive in Poland and Belarus. The aboriginal *S. virgaurea* L. is included in the collection, too.

The quantitative characteristics of stomata are highly dependent on environmental conditions; therefore, it is not always possible to carry out a comparative analysis of different taxa on these parameters. Here, in the Botanical Garden of Padua, the plants grow side by side, on the same agricultural background, in partial shade. This allows comparing the characteristics of the stomatal apparatus of various species. It is known that the adaptive capacity of plants can be assessed by the morpho-anatomical feathers of the leaves (Marin et al., 1988; Kiseleva, 2009; Egorova et al., 2016; Vinogradova et al., 2018). For example, a small number of stomata and their small size provide the permanent opening of stomata and excessive transpiration, which may indicate low adaptability of plants to light and humidity conditions. The correlation between some micromorphological structures and the degree of plant's adaptation to various ecological conditions is proved for many species: *Crataegus* spp. (Foroughbakhch et al., 2000; Ganeva et al., 2009), *Calystegia soldanella* (L.) Roem. & Schult., *Euphorbia paralias* L. and *Otanthus maritimus* L. (Ciccarelli et al., 2009), *Amelanchier ovalis* Medic (Ganeva and Uzunova, 2010b), *Prunus armeniaca* L. (Laajimi et al., 2011), *Pinus roxburghii* Sarg. (Tiwari et al., 2013), *Mespilus germanica* L. (Koçyiğit et al., 2015), *Cornus* spp. (Klymenko and Klymenko, 2017), *Typha domingensis* Pers. (Akhta et al., 2017), *Cydonia oblonga* Mill., *Pseudocydonia sinensis* (Thouin) C.K. Schneid. and *Chaenomeles japonica* (Thunb.) Lindl. ex Spach (Vinogradova et al., 2018).

The evaluation of adaptive capacity is very relevant for alien species. It gives a possibility to predict the further expansion of their secondary distribution range. For genus *Solidago*, this is doubly relevant since already several species of this genus "escape" from cultivation and began to be actively invaded the natural cenoses. There are data on the leave's structure only for alien *S. canadensis*, *S. gigantea* (= *S. serotinoides*) and *S. graminifolia* as well as aborigen *S. virgaurea* (Szymura and Wolsky, 2011; Vinogradova, 2012). Data for other *Solidago* spp. concerned only macromorphology of these species (Weber, 2000). Our work is the first study of leaf anatomy for the most part of *Solidago* spp., which is cultivated in Europe.

The purpose of this work is a comparative analysis of the stomatal characteristics of cultivated *Solidago* species to assess the adaptive capabilities of these alien species.

Material and methods

Plant material

Samples of the following alien *Solidago* species from the collection of the botanical garden in Padua are the objects of the study: *S. altissima*, *S. caesia*, *S. canadensis*, *S. graminifolia*, *S. juncea*, *S. latifolia*, *S. lepida*, *S. rugosa*, *S. sempervirens*, *S. serotinoides*, and *S. uliginos*a. During the visit to the garden at the beginning of June 2017, we collected leaves per each species. For the analysis, 3 shoots of each species were selected, and from each shoot 3 leaves were selected from the basal part of the stem (total 9 leaves per each species).

Ultrastructure of leaves

To study the features of the stomatal apparatus, the method of obtaining lacquer replicas was used. Unfortunately, it was not possible to obtain clear prints of the epidermis in *S. latifolia* due to a thin wax coating covering the leaves, and this species is not included in our study. The following micro-morphological features were analyzed: the number of guard cell of the stoma, the length of the polar axis of the stoma (l), the length of the equatorial diameter (d), the shape of the stomata (by the ratio l/d), the area of the one stoma (s ellipse= $\pi(l/2)\ (d/2)$), the number of stomata in the field of the microscope view (n), the total transpiratory area (= $s \times n$). The relative transpiration area (relative transpiration index, I_{ot}) was calculated as the ratio of the total average transpiration area to the total area of the underside of the leaf blade, according to the formula:

$$I_{ot} = \frac{\sum n\pi LD}{\sum S} 100\%$$

These seven signs were studied for both the upper and lower sides of the leaf blade, and then their ratio was determined. Thus, the stomatal apparatus of 10 species are characterized by 21 quantitative traits.

Morphology of the stomatal apparatus was examined with the Keyence VHX-1000E digital microscope. The number of stomata in the field of the microscope view with an increase of × 1,500 was determined in 10 replicates. The total number of stomata for each species to determine their size is 50–100.

Analysis of data

Basic statistical analyses were performed using PAST 3.15. Data were submitted ANOVA and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$). All data are presented as M ±m, where M – arithmetic average, m – arithmetic average deviation.

Results and discussions

The size of the leaf blades in the studied species decreases in the following order: *S. sempervirens* → *S. juncea* → *S. altissima* → *S. serotinoides* → *S. canadensis* → *S. rugosa* → *S. lepida* → *S. caesia* → *S. uliginos*a → *S. graminifolia*. In three species (*S. altissima*, *S. canadensis*, and *S. rugosa*) the leaves are amphystomatic, i.e. the stomata are located only on the lower

(abaxial) side of the leaf; in other species, the leaves are hypostomatic, and the stomata are located on both the lower and the upper (adaxial) side of the leaf blade (Figure 1A, B).



Figure 1A Plant habitus and microphotographs of stomata on the abaxial and adaxial side of the leaf blade
1 – *Solidago altissima* L.; 2 – *Solidago caesia* L.; 3 – *Solidago canadensis* L.; 4 – *Solidago graminifolia* (L.) Salisb.; 5 – *Solidago juncea* Aiton; 6 – *Solidago lepida* DC.



Figure 1B Plant habitus and microphotographs of stomata on the abaxial and adaxial side of the leaf blade
7 – *Solidago rugosa* Mill.; 8 – *Solidago sempervirens* L.; 9 – *Solidago serotinoides* Á. Löve & D. Löve; 10 – *Solidago uliginosa* Nutt.

The samples were significantly divided into 2 groups (Figure 2, Table 1), according to the size of stomata from the abaxial side of the leaf blade. The first group, characterized by large stomata (36–40 × 28–30 μm), includes *S. sempervirens*, *S. altissima*, and *S. juncea*.

All other species are included in the second group (19–28 × 16–22 μm), with the exception of *S. serotinoides*. *S. serotinoides* are distinguished by the highest variability of this feature (27–41 × 20–34 μm) and in Figure 2 is not even displayed since it occupies an intermediate position between the two groups and overlaps all other samples.

In most samples, the shape of the stomata is rounded: the ratio of the average length of the polar axis to the average length of the equatorial diameter is 1.2–1.4. Only in *S. serotinoides* on the adaxial side of the leaf blade, stomata are elongated, and this ratio is 1.7.

Table 1 Quantitative characteristic of stomata in *Solidago* L. species

Species	Polar axis (*l*) (μm)			Equatorial diameter (*d*) (μm)			*l*/*d*		
	min	max	$\bar{x}$ ±S*x*	min	max	$\bar{x}$ ±S*x*	min	max	$\bar{x}$ ±S*x*
Abaxial side of the leaf blade									
S. sempervirens	36	44	39.7 ±0.8	25	36	30.3 ±0.8	1.0	1.5	1.3 ±0.0
S. altissima	26	51	36.4 ±0.8	23	31	27.7 ±0.4	1.1	1.9	1.3 ±0.0
S. juncea	29	37	32.6 ±0.4	24	32	28.2 ±0.4	1.0	1.3	1.2 ±0.0
S. uliginosa	23	36	27.7 ±0.7	17	25	21.6 ±0.4	1.1	1.6	1.3 ±0.0
S. caesia	18	30	26.3 ±0.7	14	23	20.0 ±0.7	1.1	1.6	1.3 ±0.0
S. canadensis	21	32	26.3 ±0.6	15	28	20.2 ±0.4	0.9	2.1	1.3 ±0.0
S. lepida	17	28	23.9 ±0.5	11	21	18.0 ±0.4	1.0	1.9	1.3 ±0.0
S. graminifolia	17	25	22.6 ±0.4	13	23	19.0 ±0.4	1.0	1.8	1.2 ±0.0
S. rugosa	14	28	19.4 ±1.2	11	21	15.9 ±0.8	0.9	1.5	1.2 ±0.0
S. serotinoides	27	41	32.8 ±0.9	20	34	24.5 ±0.8	1.0	2.0	1.4 ±0.1
Adaxial side of the leaf blade									
S. sempervirens	33	42	37.1 ±0.6	27	35	30.3 ±0.4	1.1	1.4	1.2 ±0.0
S. altissima	stomata are absent								
S. juncea	28	43	33.5 ±0.9	18	30	25.5 ±0.8	1.1	1.8	1.3 ±0.1
S. uliginosa	21	32	24.9 ±1.0	19	23	21.3 ±0.4	1.0	1.5	1.2 ±0.1
S. caesia	23	25	24.3 ±0.7	14	20	17.3 ±1.8	1.3	1.6	1.4 ±0.1
S. canadensis	only one stoma								
S. lepida	18	27	23.2 ±0.5	15	23	17.4 ±0.5	1.1	1.7	1.3 ±0.0
S. graminifolia	18	27	23.1 ±0.3	13	23	19.0 ±0.4	1.0	1.8	1.2 ±0.0
S. rugosa	stomata are absent								
S. serotinoides	26	31	29.0 ±1.5	15	18	17.0 ±1.0	1.4	2.0	1.7 ±0.2

Note: min, max – minimal and maximal measured values; $\bar{x}$ – arithmetic mean; S*x* – standard error of the mean

The most species (5 of 7) have hypostomatic leaves, i.e., the number of stomata is greater on the abaxial side of the leaf than on the adaxial side, and stomata are larger (Figure 2). In *S. graminifolia*, the size of the stomata on the adaxial and the abaxial side of the leaf blade is not significantly different, and the number of stomata on the underside of the leaf (average 5.6) is lower than on the adaxial side (average 6.8 in the field of the microscope view). In *S. sempervirens*, the stomata on the abaxial side of the leaf are larger than on the adaxial one (as for the most species), but their number on the abaxial side (as in *S. graminifolia*) is lower than on the adaxial side (2.4 contrary 3.0). These parameters are shown in Figure 3, 4.

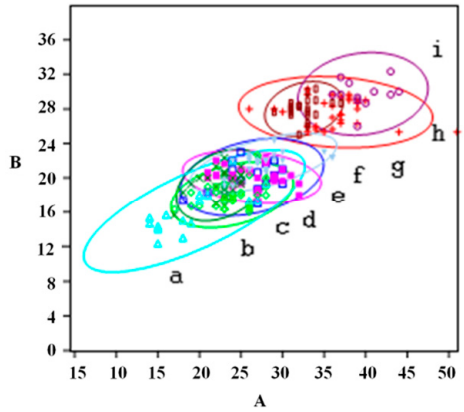


Figure 2 The stomatal size of *Solidago* species on the underside of the leaf blade
a – *Solidago rugosa* Mill.; b – *Solidago graminifolia* (L.) Salisb.; c – *Solidago lepida* DC.; d – *Solidago canadensis* L.; e – *Solidago caesia* L.; f – *Solidago uliginosa* Nutt.; g – *Solidago juncea* Aiton; h – *Solidago altissima* L.; i – *Solidago sempervirens* L.

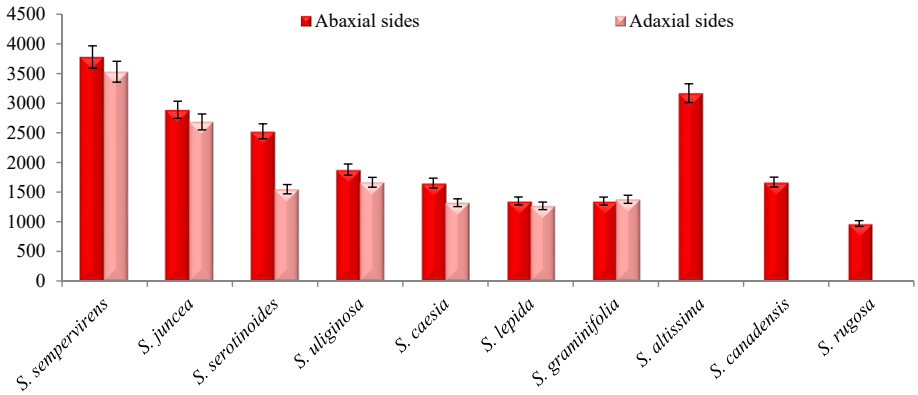


Figure 3 The average area of the one stoma on the leaf blades of *Solidago* spp. (μm^2) (each value represents the mean of three independent experiments (±SD)

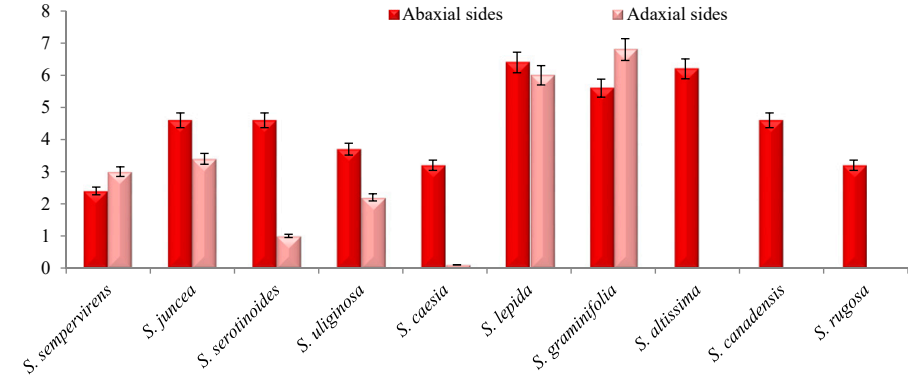


Figure 4 The average number of stomata in the field of the microscope on the leaf blades of *Solidago* spp. (each value represents the mean of three independent experiments (±SD)

When comparing these two figures, it is clearly seen that the average area of one stoma negatively correlates with the average number of stomata, therefore the variability of the average transpiration area is relatively low (Figure 5). In order to make a correct comparison of species over the area of transpiration, the relative transpiration index (I_{ot}) have been calculated: the ratio of the total average transpiration area to the total area of the abaxial side of the leaf blade.

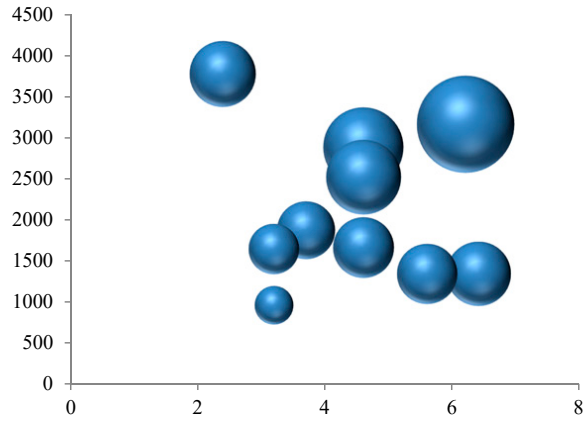


Figure 5 The average total transpiration area in the field of view of the microscope on the underside of the leaf blade of *Solidago* spp. (μm^2)

According to this index I_{ot}, the studied species line up in the following order (%): *S. altissima* (50), *S. juncea* (34), *S. serotinoides* (29), *S. sempervirens* (23), *S. lepida* (22), *S. canadensis* (19), *S. graminifolia* (19), *S. uliginos*a (18), *S. caesia* (13) and *S. rugosa* (8).

Our data on the stomatal characteristics in various *Solidago* species are correlated quite well with the invasive activity of these taxa. In this respect, the relative transpiration area index is especially indicative. *S. altissima* and *S. serotinoides*, which are widespread in the secondary distribution range, have at the same time the largest relative transpiration area (from 25 to 50%). *S. juncea*, which also has a high value of this indicator, is already beginning to be invaded the natural cenoses of Russia. This species was collected by A.A. Notov in the Tver region (Herbarium collections MW, MHA). *S. sempervirens*, *S. lepida* and *S. uliginos*a have an index from 15 to 25%. They are similar on this parameter with the invasive *S. canadensis* and *S. graminifolia*, they are able to adapt well to the conditions of the Old World. They are less decorative and their presentation in the culture is not enough. This prevents (for now!) to the wide distribution of these species. *S. caesia* and *S. rugosa* are poorly adapted to the conditions of the new homeland (I_{ot} <15 %), the latter also had short shoots with few leaves in the beds in the Padua Botanical Garden.

Thus, we can take into consideration the stomatal structure (in addition to other features) for the prediction of the possible expansion of some alien species in their secondary distribution range. If an alien species has I_{ot} >25%, it is more likely to transform into an invasive taxon. Such species require stronger control measures of their dissemination.

Conclusions

In *Solidago* species cultivated in the Botanical Garden of Padua, the size of stomata is generally negatively correlated with the average number of stomata. Various strategies are being implemented to increase the total transpiration area of the plants. At the cellular level, this is an increase in stoma size (*S. sempervirens*, *S. altissima*, *S. juncea*) and an increase in the number of stomata (*S. altissima*) up to the formation of hypostomatic leaves, i.e. the location of numerous stomata on both sides of the leaf blade (*S. lepida*, *S. graminifolia*). At the organism level, the strategy of alien species is either to increase the area of the leaf blade, which is especially characteristic of rosette leaves (*S. sempervirens*, *S. juncea*, *S. uliginos*a), or to increase the number of leaves on the shoot (*S. lepida*) or to increase the number of shoots (*S. serotinoides*, *S. graminifolia*). According to the index of the relative area of transpiration, species are divided into a group with a high (25–50%) relative area of transpiration (*S. altissima*, *S. serotinoides*, *S. juncea*), an average (15–25%) area (*S. sempervirens*, *S. lepida*, *S. uliginos*, *S. canadensis*, and *S. graminifolia*) and a low (less than 15%) relative area of transpiration (*S. caesia* and *S. rugosa*).

The high value of the index of the relative area of transpiration indicates greater adaptability of alien species and can (among other features) be used to predict the further expansion of their secondary distribution range and increase the chances to transform into an invasive taxon.

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ANTIMICROBIAL ACTIVITIES OF THREE COMMERCIAL ESSENTIAL OILS DERIVED FROM PLANTS BELONGING TO FAMILY PINACEAE

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The aim of this study was to compare the antibacterial effects of several essential oils derived from plants belonging to family Pinaceae and subfamily Abietoideae (cedar oil, fir oils derived from Silver fir *Abies alba* Mill. and Siberian fir *Abies sibirica* L., respectively) against some Gram-positive and Gram-negative bacteria. The Kirby-Bauer disk diffusion test for measuring zone diameters of bacterial growth inhibition was used. The fir oil derived from Silver fir *Abies alba* showed considerably more profound activity than the cedar oil and fir oil derived from Siberian fir *Abies sibirica*. Maximum antibacterial activity was shown by essential oil of silver fir oil against *Escherichia coli*, methicillin-sensitive *Staphylococcus aureus* (MSSA), and *Pseudomonas aeruginosa*. Silver fir essential oil was found to be active against *K. pneumoniae* while Siberian fir essential oil showed the mild activity of clear inhibition zone against *P. aeruginosa* and methicillin-sensitive *Staphylococcus aureus*. Silver fir essential oil has the highest antibacterial potential from all tested essential oils and could be a promising candidate concerning possible applicability in the prevention of bacterial growth. Moreover, essential oils may play an important role in the discovery of new drugs for the treatment of a wide range of pathogenic microorganisms in the near future. This study provides insight into the *in vitro* antibacterial activity of a wide variety of essential oils derived from many different plant genera against pathogenic bacteria. The data contributes to the ongoing scientific investigation regarding the application of essential oils as natural antibacterial agents.

Keywords: *Abies alba* Mill., *Abies sibirica* L., essential oils, antibacterial activity, Gram-positive and Gram-negative bacteria, Kirby–Bauer disk diffusion test

Introduction

Pinaceae, the largest family of conifers, comprises more than 230 species in 11 genera – *Abies* Mill., *Cathaya* Chun & Kuang, *Cedrus* Trew, *Keteleeria* Carrière, *Larix* Mill., *Nothotsuga* Hu ex C.N.Page, *Picea* A. Dietr., *Pinus* L., *Pseudotsuga* Carrière, *Pseudolarix* Gordon, and *Tsuga*

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(Endl.) Carrière (Sudianto et al., 2016; Ran et al., 2018). The pine family is one of the most ecologically and economically important groups of living plants. Many of the species that are highly valuable for their timber include firs (*Abies*), cedars (*Cedrus*), larches (*Larix*), spruces (*Picea*), pines (*Pinus*), Douglas firs (*Pseudotsuga*), and hemlocks (*Tsuga*) (Lin et al., 2010). The Pinaceae are exclusively distributed in the northern hemisphere, except for one species, *Pinus merkusii* Jungh. & de Vriese, whose habitat crosses the equator in Sumatra (Thieret, 1993). Twelve genera (i.e., *Abies*, *Cathaya*, *Cedrus*, *Hesperopeuce*, *Keteleeria*, *Larix*, *Nothotsuga*, *Picea*, *Pinus*, *Pseudolarix*, *Pseudotsuga*, and *Tsuga*) have been recognized in the family since the pioneering work of Van Tieghem published in 1891 (Lin et al., 2010).

The morphology and anatomy of Pinaceae are well studied relative to other plant groups (Gernandt et al., 2018). Hart (1987) divided the family into two groups: the presence of resin canals in the seeds and cleavage polyembryony supported the monophyly of *Abies*, *Cedrus*, *Keteleeria*, *Pseudolarix* and *Tsuga* and the presence of resin canals in the secondary xylem and leaves having an endodermis with thickened Casparian strips supported the monophyly of *Cathaya*, *Larix*, *Picea*, *Pinus*, and *Pseudotsuga*.

Pinaceae species often form the dominant component of boreal, coastal, and montane forests in the Northern Hemisphere (Farjon, 1990; Liston et al., 2003). For instance, *Pinus*, the largest genus of the family, with more than 110 species, occupies an extended geographic range – North America, the northern part of Asia, and Europe (Farjon, 1990).

The cedar genus *Cedrus*, consisting of 4–5 species (Farjón, 1990), is native to the mountains of the western Himalayan and Mediterranean regions (Lin et al., 2010). *Cedrus* is traditionally placed in the Abietoideae along with other four genera, *Abies*, *Keteleeria*, *Pseudolarix*, and *Tsuga*. *Cedrus* (true cedar) is one of 11 commonly accepted genera in Pinaceae, first described by Trew in 1757 (Farjón, 2001). It comprises four species with a highly disjunctive distribution in circum-Mediterranean and western Himalayas (Farjón, 1990, 2001), i.e. *Cedrus deodara* (Roxb.) G. Don in the Hindu Kush, Karakoram and Indian Himalayas, *Cedrus libani* A. Rich. in Turkey, Lebanon and Syria, *Cedrus brevifolia* (Hook. f.) Henry in Cyprus, and *Cedrus atlantica* (Endl.) Manetti ex Carriére in North Africa (Algeria, Morocco) (Qiao et al., 2007).

The *Cedrus* species have many medicinal properties. The extract of essential oil from tree wood has a significant anti-inflammatory effect, severe analgesic, and anticonvulsant action. The wood ethanol extract shows antihyperglycaemic activity, anxiolytic and anticonvulsant effect, while the branch's bark air-dried aqueous extract has an antiarthritic activity. Petroleum ether extract of the heartwood presents severe diuretic and anti-urolithiasis effect (Tewari, 1994; Bhatnagar and Moitra, 1996; Dirr, 2016). In the traditional Ayurveda therapeutic system, all plant parts of the Deodar Cedar are used against various diseases. Among others, they are used to treat inflammation, dyspepsia, insomnia, cough, and cold. They are also given against fever, urinary discharges, bronchitis, leucoderma, elephantiasis, tuberculosis glands, mental disorder, and skin and blood diseases. Wood is used as a diuretic, expectorant, for relieving rheumatism, as well as for treating epilepsy and urinary tract diseases. The bark is used in formulations that are administered as astringents, antipyretics, and antidiarrheals. Finally, Cedar oleoresin is applied for the healing of wounds and the treatment of skin rashes

(Tewari, 1994; Bhatnagar and Moitra, 1996; Dirr, 2016). Cedar of Lebanon has ethnobotany and pharmaceutical value while its aromatic wood is used in the construction, woodworking, and instrumental industries. Also, the tree is widely grown for ornamental purposes and is used both in gardening and in landscape architecture (Chaney, 1993).

Silver fir (*Abies alba* Mill.) is a large conifer that can be found in central Europe and some parts of Southern and Eastern Europe. It is one of the tallest tree species of the genus *Abies* in Europe. This tree is considered an important ecological and functional balancer of European forests and a fundamental species for maintaining high biodiversity in forested ecosystems (Mauri et al., 2016). Siberian fir (*Abies sibirica* L.), a large evergreen conifer with a high part of greenery, is one of the dominant species in European Russia, west and east Siberian taiga, using for therapy and prophylaxis in official and folk medicine for ages (Koctesha et al., 1997). The essential oils obtained from the leaves were also used in the past to heal bruises as well as for treating coughs and colds (Farjon, 2010) and to help respiratory system and have easing and soothing effect for muscle (Yang et al., 2009).

Up to 2008, 277 compounds were isolated from 19 plants of *Abies* species. The chemical constituents are mostly terpenoids, flavonoids, and lignans, together with minor constituents of phenols, steroids, and others. The crude extracts and metabolites have been found to possess various bioactivities including insect juvenile hormone, antitumor, antimicrobial, anti-ulcerogenic, anti-inflammatory, antihypertensive, antitussive, and CNS (central nervous system) activities (Yang et al., 2008).

Essential oils are complex mixtures of compounds, mainly monoterpene and sesquiterpene hydrocarbons (10 and 15 carbon atoms, respectively) and their oxygenated derivatives (alcohols, aldehydes, esters, ketones) as well as phenylpropanoids (Lee et al., 2009; Saad et al., 2013), which have antitumor, antioxygen, anti-aging, anti-mutation, and sedative effects (Kwak et al., 2006; Lee et al., 2007).

Essential oils (EO) that are lipophilic liquids, extracted from diverse plants containing different natural, biologically active components have antimicrobial and antioxidant properties (Khorshidian et al., 2018). The essential oils are obtained from plant materials (leaves, buds, fruits, flowers, herbs, twigs, bark, wood, roots and seeds). Having a density generally lower than that of water, essential oils are volatile, liquid, limpid, lipid-soluble, rarely colored, and soluble in organic solvents (Chouhan et al., 2017). Essential oils from different plant species contain more than 200 constituents which are comprised of volatile and non-volatile components. The application of essential oils as antimicrobial, anticancer, anti-inflammatory and anti-viral agents is due to their effective and efficient properties, inter alia (Aziz et al., 2018).

Due to the hydrophobicity of essential oils' components, they easily pass through the bacterial cell membrane interfering with molecular transport mechanisms leading to cell inactivation (Burt, 2004; Chouhan et al., 2017; Khorshidian et al., 2018). This eventually results in the death of a bacterial cell due to leakage of critical molecules and ions from the bacterial cell to a great extent. Some compounds modulate drug resistance by targeting efflux mechanisms in several species of Gram-negative bacteria (Chouhan et al., 2017).

The effect of the antibacterial activity of essential oils may inhibit the growth of bacteria (bacteriostatic) or destroy bacterial cells (bactericidal). Nevertheless, it is difficult to distinguish these actions. In relation to this, antibacterial activity is more frequently measured as the minimum bactericidal concentration (MBC) or the minimum inhibitory concentration (MIC) (Burt, 2004; Swamy et al., 2016).

The long-known antimicrobial actions of essential oils are now being extensively scientifically reviewed and applied in health and industry fields (Sienkiewicz et al. 2012). Numerous experimental studies have confirmed the inhibitory action of essential oils against bacteria, fungi, yeasts, viruses, and protozoa. They have also been reported to exhibit anti-inflammatory and immunostimulatory activities (Dorman and Deans, 2000; Król et al., 2013).

In this regard, the antibacterial properties of three commercial essential oils derived from plants belonging to family Pinaceae and subfamily Abietoideae (cedar oil, fir oils derived from Silver fir *Abies alba* and Siberian fir *Abies sibirica*, respectively) against some Gram-positive and Gram-negative bacteria were studied in the present research. To this intent, the antimicrobial susceptibility test was used (the Kirby–Bauer disk diffusion test for measuring zone diameters of bacterial growth inhibition).

Material and methods

Essential oils

Essential oils were provided by Polish essential oil manufacturers (Etja, Elbląg, Poland). Overall, essential oils from 3 plant species (cedar oil, fir oils derived from Silver fir *Abies alba* and Siberian fir *Abies sibirica*, respectively). The investigated samples did not contain additives or solvents and were confirmed to be natural by the manufacturers. The samples were stored in resalable vials at 5 °C in the dark but were allowed to adjust to room temperature prior to investigation. Geographical origins were excluded as information was mostly not available.

Clinical Isolates

Clinical specimens submitted for routine culture and antibiotic susceptibility testing of hospitalized patients during the period of April to May 2019, at the microbiology laboratory of the Koszalin Regional Hospital were processed. The sources of the clinical isolates were pus, wound, and urine of seven different patients. The purity, as well as the identity of each isolate, was confirmed in the laboratory conditions by standard microbiological methods. The isolates were further identified on the basis of 16S ribosomal RNA (rRNA) gene sequence homology.

Antimicrobial susceptibility testing

First, the samples were examined by a microscopic Gram stain examination. Samples with Gram-negative results were inoculated on plates of nutrient agar, Clede agar, MacConkey's, and blood agar (Merck, Germany) and then incubated at 37 °C for 24 hours. The colony that showed fermenting of lactose on MacConkey agar and Cled agar media were purified and identified according to their morphology as circular, rose-pink to red colonies on MacConkey

agar medium and yellow colonies on Cled agar. The isolates were identified by some biochemical reactions (e.g. catalase enzyme, potassium hydroxide test, Indole and methyl red test, Voges Proskauer reaction, urease and citrate, H_2S and oxidase test).

P. aeruginosa isolates were tested for MBL production by the imipenem-EDTA disk diffusion test (Yong et al., 2002). Susceptibility testing of the isolates was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI). The following antimicrobial agents were used: amikacin (30 μg), ampicillin (10 μg), aztreonam (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), cephalexin (30 μg), gentamicin (10 μg), erythromycin (15 μg), imipenem (10 μg), meropenem (10 μg), clindamycin (2 μg), aztreonam (30 μg), norfloxacin (10 μg), oxytetracycline (30 μg), oxacillin (1 μg), enrofloxacin (5 μg), tetracycline (30 μg), amoxicillin (25 μg), piperacillin (100 μg), piperacillin-tazobactam (100/10 μg), tobramycin (10 μg), ceftazidime (30 μg), chloramphenicol (30 μg), doxycycline (30 μg), cefadroxil (30 μg), pefloxacin (5 μg), and cefepime (30 μg). MIC was determined by E-test strips (according to manufacturer's instruction) and agar dilution method on all MBLs-producing isolates (the Guidelines of Clinical and Laboratory Standard Institute). The resistance breakpoints were the same as the ones defined by the *National Committee for Clinical Laboratory Standards* (NCCLS, 2000).

Phenotypic Disc Confirmatory Test was performed as recommended by the CLSI (Clinical and Laboratory Standards Institute). Disks of ceftazidime (CA, 30 μg) and ceftazidime-clavulanic acid (CAC, 20 + 10 μg) or ceftriaxone (CE, 30 μg) and ceftriaxone clavulanic acid (CEC, 20 + 10 μg) were placed on Muller Hinton Agar (MHA). An increase in the zone diameter (= 5 mm) for CAC versus CA or CEC versus CE is confirmed as β-lactamase (ESBL)-producing strain *E. coli* strain. The *Staphylococcus aureus* isolates were characterized by Gram-staining and their ability to produce coagulase and clumping factor using Slidex Staph Plus (BioMerieux). Additionally, the species were identified using the biochemical identification system ID 32 Staph (BioMerieux).

The antibacterial susceptibility profile of the isolates revealed that many isolated strains were classified as multi-drug resistant (MDR) bacteria.

Isolate 1 – *Pseudomonas aeruginosa* was resistant to gentamicin (10 μg), cefotaxime (10 μg), and amikacin (30 μg);

Isolate 2 – *Enterococcus faecalis* was resistant to gentamicin (10 μg);

Isolate 3 – *Pseudomonas aeruginosa* was resistant to piperacillin-tazobactam (100/10 μg), ceftazidime (30 μg), piperacillin (100 μg), and cefepime (30 μg);

Isolate 4 – *Enterococcus faecium* was resistant to gentamicin (10 μg) and ampicillin (10 μg);

Isolate 5 – *Klebsiella pneumoniae* was resistant to piperacillin-tazobactam (100/10 μg), gentamicin (10 μg), tobramycin (10 μg), and ciprofloxacin (5 μg);

Isolate 6 – *Escherichia coli*, not β-lactamase (ESBL)-producing strain, was a sensitive strain to antibiotics tested;

Isolate 7 – methicillin-sensitive *Staphylococcus aureus* (MSSA) was resistant to tobramycin (10 μg), piperacillin (100 μg), clindamycin (2 μg), and erythromycin (15 μg).

Bacterial Growth Inhibition Test of Essential Oils by the Disk Diffusion Method

Strains tested were plated on TSA medium (Tryptone Soy Agar) and incubated for 24 hr at 25 °C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. Muller-Hinton agar plates were inoculated with 200 µl of standardized inoculum (10^8 CFU/mL) of the bacterium and spread with sterile swabs. Sterile filter paper discs impregnated by sample were applied over each of the culture plates, 15 min after bacteria suspension was placed. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) (Bauer et al., 1966). A negative control disc impregnated by sterile ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hr at 37 °C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks.

The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated six times. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Three commercial essential oils derived from plants belonging to subfamily Abietoideae (cedar oil, fir oils derived from Silver fir *Abies alba* and Siberian fir *Abies sibirica*, respectively) were tested against Gram-positive and Gram-negative pathogenic bacteria locally isolated from the patients. The fir oil derived from Silver fir *Abies alba* showed considerably more activity than the cedar oil and fir oil derived from Siberian fir *Abies sibirica* (Figure 1). Maximum antibacterial activity was shown by essential oil of silver fir oil against *E. coli* with the inhibition zone size (25.7 ±1.13) mm, methicillin-sensitive *Staphylococcus aureus* (MSSA) – (23.8 ±1.25) mm, and *Pseudomonas aeruginosa* – (22.4 ±1.1) mm (Figure 1, 2). Silver fir essential oil was found to be active with the inhibition zone diameter of (19.4 ±0.98) mm against *K. pneumoniae* while Siberian fir essential oil showed mild activity with (9.4 ±0.1) mm and (9.4 ±0.25) mm of clear inhibition zone against *P. aeruginosa* and *S. aureus* MSSA (Figure 1).

These findings are in line with the results from previous works and enhance the often requested need for chemical characterizations of antimicrobial essential oils to identify the active compounds and their interdependencies. Antimicrobial properties of essential oils against a wide range of microorganisms have been reported in various studies. Due to the hydrophobicity of essential oils' components, they easily pass through the bacterial cell membrane interfering with molecular transport mechanisms leading to cell inactivation (Burt, 2004; Khorshidian et al., 2018).

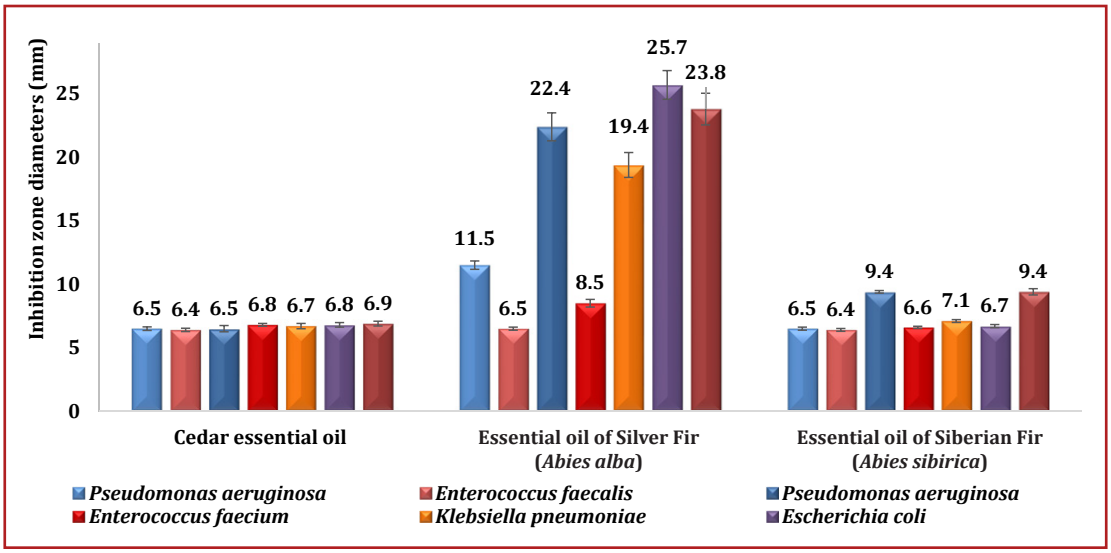


Figure 1 Antibacterial activity of examined essential oils (cedar oil, fir oils derived from Silver fir *Abies alba* and Siberian fir *Abies sibirica*) against Gram-positive and Gram-negative pathogenic bacteria locally isolated from the patients. The data were presented as the mean ± the standard error of the mean (S.E.M.)

The chemical composition, including the enantiomeric excess of the main terpenes, the antimicrobial and antiradical activities, as well as the cytotoxicity of *Abies alba* and *A. koreana* seed and cone essential oils, were investigated in the study by Wajs-Bonikowska et al. (2015). In the examined oils and hydrolats, a total of 174 compounds were identified, which comprised 95.6–99.9% of the volatiles. The essential oils were mainly composed of monoterpene hydrocarbons, whereas the composition of the hydrolats, differing from the seed oils of the corresponding fir species, consisted mainly of oxygenated derivatives of sesquiterpenes. The seed and cone essential oils of both firs exhibited DPPH-radical-scavenging properties and low antibacterial activity. Moreover, they evoked only low cytotoxicity towards normal fibroblasts and the two cancer cell lines MCF-7 and MDA-MBA-231. At concentrations up to 50 μg/ml, all essential oils were safe in relation to normal fibroblasts. Although they induced cytotoxicity towards the cancer cells at concentrations slightly lower than those required for the inhibition of fibroblast proliferation, their influence on cancer cells was weak, with IC_{50} values similar to those observed towards normal fibroblasts (Wajs-Bonikowska et al., 2015).

Studies by Yang et al. (2009) dedicated to investigation of the chemical composition, cytotoxicity and its biological activities of Silver fir (*Abies alba*) essential oil have revealed that the composition of the oil was follow: bornyl acetate (30.31%), camphene (19.81%), 3-carene (13.85%), tricyclene (12.90%), dl-limonene (7.50%), α-pinene (2.87%), caryophyllene (2.18%), β-phellandrene (2.13%), borneol (1.74%), bicyclo[2.2.1]hept-2-ene,2.3-dimethyl (1.64%) and α-terpinene (1.24%). The results also indicated that the oil showed no cytotoxic effect, at concentrations of 1 and 5%, for as long as 24 and 3 h, respectively. The antiradical capacity was evaluated by measuring the scavenging activity of the essential oil on the

2.2-diphenylpicrylhydrazyl (DPPH) and 2.2'-azino-bis 3-ethyl benzothiazoline-6-sulfonic acid (ABTS) radicals. The oil was able to reduce both radicals dose-dependently, and the concentration required for 50% reduction against DPPH radicals (2.7 ±0.63%) was lower than ABTS radicals (8.5 ±0.27%). The antibacterial activity of the oil was also evaluated using a disc diffusion method against *Staphylococcus aureus*, *Streptococcus mutans*, *Listeria monocytogenes*, *Acinetobacter baumannii*, *Escherichia coli*, and *Vibrio parahaemolyticcus*. The oil exhibited no antibacterial activity against all the bacterial strains tested except *S. aureus* of mild activity (Yang et al., 2009).

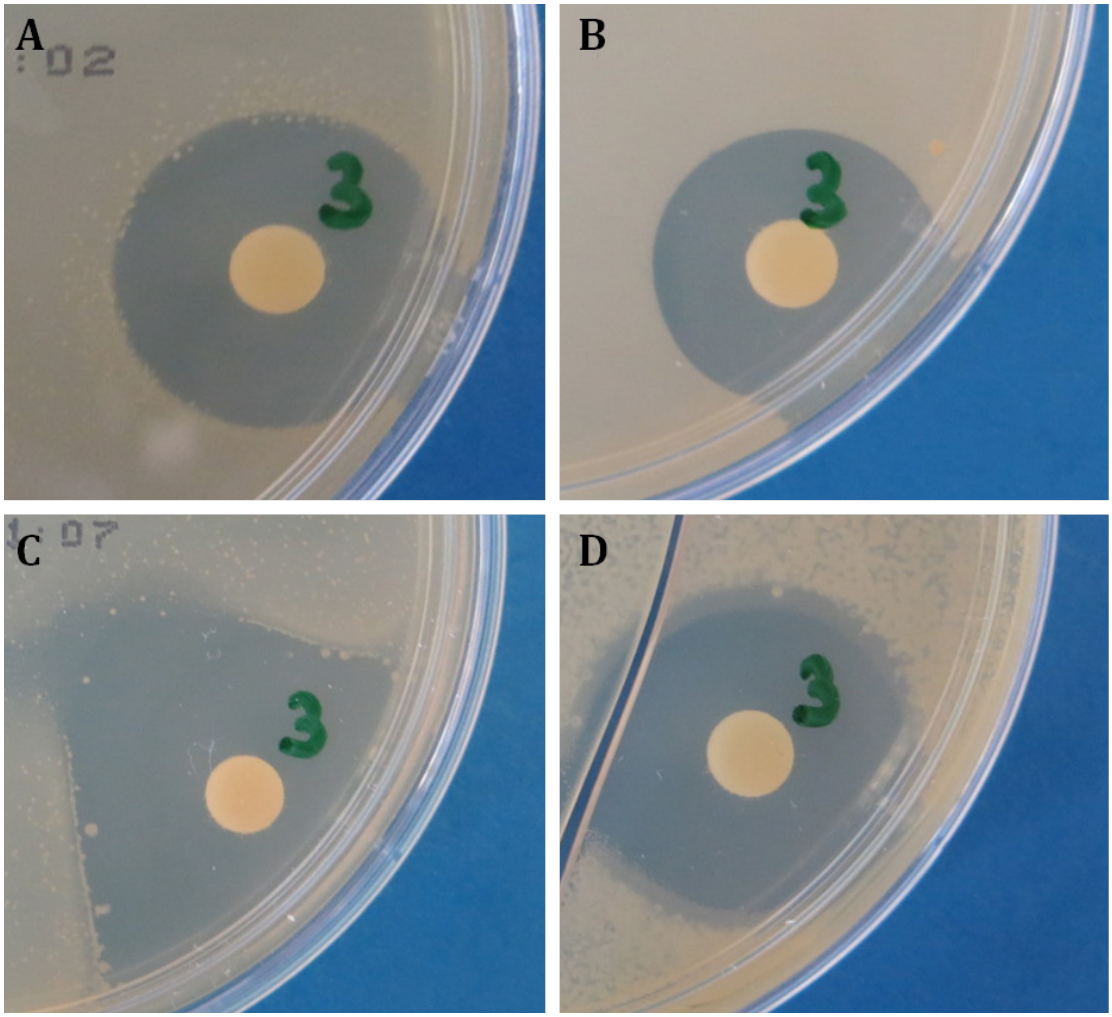


Figure 2 Antibacterial activity of essential fir oils derived from Silver fir *Abies alba* in disc diffusion assays. Example of a disc diffusion assay plate showing the halos in the bacterial lawn resulting from the antibacterial activity of Silver fir oil against *Pseudomonas aeruginosa* (isolate 3, A), *Klebsiella pneumoniae* (isolate 5, B), *Escherichia coli* (isolate 6, C), and methicillin-sensitive *Staphylococcus aureus* (isolate 7, D)

Lanzerstorfer et al. (2019) have evaluated the efficacy of the dispersion of selected essential oils in reducing microbial contamination in two hospital wards. The study was carried out at two wards of a 1,227-bed acute-care hospital in Austria. The concentration of airborne bacteria and fungi was measured in the patient rooms before and after the dispersion of a mixture of *Citrus limon* and *Abies alba* essential oils. Before dispersion of the essential oils in both wards, the mean concentration of bacteria was in a typical range (123 colony forming units (CFU)/m^3 and 10^4 CFU/m^3) while the mean concentration of fungi differed substantially (155 CFU/m^3 and 28 CFU/m^3). After the dispersion of the essential oils, a-reduction in both bacterial and fungal contamination was observed. In the first two hours, the mean concentration of airborne bacteria and fungi was reduced by approximately 40 and 30–60% respectively (Lanzerstorfer et al., 2019).

The antiproliferative, antimicrobial and antioxidative effects of fir (*Abies alba* Mill.) honeydew honey from the mountain region of Croatia (Gorski Kotar) as a potential replacement for standard antibiotics and chemotherapeutic agents were studied by Broznić et al. (2018). Cell viability, annexin V assay, and flow cytometry analysis served to analyze the antiproliferative effect on, apoptosis induction in and cell death of cancer cell lines: HeLa, MCF-7, SW620, CFPAC-1, MIA PaCa-2 and normal diploid human fibroblasts (BJ). Antimicrobial activity was tested against *Staphylococcus* and *Acinetobacter* strains by agar well diffusion and microdilution assays. The $DPPH^{\cdot}$ assay determined the radical scavenging activity, while mathematical models helped to evaluate the kinetic data of $DPPH^{\cdot}$ inhibition. Antiproliferative effect on all tested cell lines and the prominent effect on normal diploid human fibroblasts (BJ), colorectal adenocarcinoma (SW620, metastatic) and breast epithelial adenocarcinoma (MCF-7, metastatic) was observed. The mechanisms of the antiproliferative effect included the accumulation of cells in the sub-G1 phase in all tested cells and induction of apoptosis in SW620 and MCF-7 cells predominantly. The antibacterial assays showed that antibiotic-resistant strains of both bacteria, including multi-resistant strain *A. baumannii* ATCC® BAA-1605™, were sensitive to all tested honey samples (Broznić et al., 2018). Results of well diffusion tests showed that all tested *Staphylococcus* strains were more sensitive than *Acinetobacter* strains to all honey samples with inhibition zones between 13 and 21 mm, while the inhibition zone of *Acinetobacter* strains varied between 7 and 14 mm. There were no significant sensitivity differences between the antibiotic-resistant and susceptible bacterial *Staphylococcus* and *Acinetobacter* strains to the tested honey samples (Broznić et al., 2018).

Multiple studies have been dedicated to investigating the chemical compositions and antibacterial and antifungal activities of essential oils extracted from *Abies* plants (Lee and Hong, 2009; Satou et al., 2011). Various bioactive compounds such as lignans and triterpenoids have been isolated from several *Abies* species (Roshchin et al., 1998; Kim et al., 1999). Recently, the chemical composition of the essential oil prepared from the needles of A. koreana and its antibacterial activity against nine bacterial strains were reported (Jeong et al., 2007).

The antimicrobial activity of essential oils isolated from nine *Abies* species (*Abies koreana* Wills, *A. alba* Mill., *A. pinsapo* Boiss., *A. concolor* (Gord. et Glend.) Lindl. ex Hildebr., *A. firma* Sieb. et Zucc. (all of these exotic for Turkey), *A. nordmanniana* (Stev.) Spach. subsp *nordmanniana* which are plantation forms and *A. cilicica* (Ant. et Kotschy.) Carr. subsp. *cilicica*, *A. cilicica*

(Ant. et Kotschy.) Carr. subsp. *isaurica* Coode et Cullen, *A. nordmanniana* (Stev.) Spach. subsp. *bornmüelleriana* Mattf. which are natural forms against nine bacteria (*Escherichia coli*, *Bacillus megaterium*, *B. cereus*, *B. subtilis*, *B. brevis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus aureus*) and two yeasts (*Saccharomyces cerevisiae* and *Candida albicans*) have been investigated by Bagci and Diğrak (1996) using a disc diffusion method. The results suggested that the essential oils tested for antimicrobial activity can be classified into three groups according to the strength of their antimicrobial activities. The essential oils of *A. pinsapo* and *A. concolor* does not have any antimicrobial activity, while the essential oils, isolated from *A. alba* and *A. firma* had a modest activity. The essential oils of *A. koreana*, *A. cilicica* subsp. *cilicica*, *A. cilicica* subsp. *isaurica*, *A. nordmanniana* subsp. *nordmanniana*, and *A. nordmanniana* subsp. *bornmüelleriana* had possessed the highest antimicrobial activity against the tested bacteria and yeast species. The essential oils of the nine *Abies* species tested for antimicrobial activity were more active against yeast species than against bacteria, and the antimicrobial activity of essential oils was variable, depending on the bacterial strains and the source of the essential oil (Bagci and Diğrak, 1996).

The study of Coté et al. (2016) supports the use of oleoresin of *Abies balsamea* (L.) Mill. by the used by Native Americans of the boreal forest of Canada and French Canadians to treat various infections, suggesting that oleoresin has antibacterial properties. The antibacterial activity of the oleoresin was investigated against *E. coli*, *S. aureus* and two methicillin-resistant *S. aureus* (MRSA) strains using a new sensitive assay developed to evaluate hydrophobic matrix and compounds. The results showed that whole oleoresin was inactive against Gram-negative *E. coli* (MIC_{90} >90 µg/ml) but active against Gram-positive *S. aureus* and MRSA with MIC_{90} ranging from 18.2 to 30 µg/ml. The oleoresin is mainly composed of monoterpene (28%), sesquiterpenes (2%), and diterpenes (45%). Resin acids were found, in part, responsible for the antibacterial activity of the whole oleoresin. Isopimaric acid and levopimaric acid are the most active with an MIC_{90} of respectively 9.7 µg/ml and 10µg/ml.

This study also revealed results that differ greatly from those reported by others. The lacking growth inhibition by cedar oil and Siberian fir oil may be due to the comparably low concentrations used. Authors, who found these oils to be inhibitory, used way higher concentrations. In this case, clarification can only be achieved by chemical analysis of the respective oils which has not been performed as part of our investigations, due to the more broadened approach.

The essential oils extracted from the leaves and the shoots of five *Abies* species (Pinaceae) growing in Japan, i.e., *A. firma*, *A. homolepis*, *A. veitchii*, *A. mariesii*, and *A. sachalinensis*, were characterized by GC-FID and GC/MS analyses (Satou et al., 2011).

The antimicrobial activities of several parts of various trees grown in the Kahramanmaraş region of Turkey were investigated by Diğrak et al. (1999). Chloroform, acetone and methanol extracts of leaves, resins, barks, cones and fruits of *Pinus brutia* Ten., *Juniperus oxycedrus* L., *Abies cilicia* Ant. & Kotschy Carr., *Cedrus libani* A. Rich. and *Pinus nigra* Arn. were prepared and tested against *Bacillus megaterium* DSM 32, *Bacillus subtilis* IMG 22, *Bacillus cereus* FMC

19, *Escherichia coli* DM, *Klebsiella pneumoniae* FMC 3, *Enterobacter aerogenes* CCM 2531, *Staphylococcus aureus* Cowan 1, *Mycobacterium smegmatis* RUT, *Proteus vulgaris* FMC 1, *Listeria monocytogenes* Scoot A, *Pseudomonas aeruginosa* DSM 5007, *Candida albicans* CCM 314, *Candida tropicalis* MDC 86 and *Penicillium italicum* K. The antifungal effects were not observed for the whole extracts, *E. coli* was not inhibited by any of the plant extracts except by the chloroform and acetone extracts of the leaves of A. cilicia, which showed inhibition zones of 16-18 mm, respectively. All the plant extracts used in the study of Diğrak et al. (1999) inhibited the growth of the other bacteria studied.

Increasingly, it has been recognized that the water-soluble extract from pine needles of *Cedrus deodara* (WEC) might be a new potential source of natural antibacterial agents applicable to food. Zeng et al. (2012) have studied the antibacterial activity of WEC against five food-borne bacteria, and its related mechanism was investigated by a transmission electron microscope. *In vitro* antibacterial assay showed that WEC possesses a remarkable antibacterial activity against tested food-borne bacteria including *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus cereus*, with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in the ranges of 0.78–12.5 and 1.56–25 mg/ml, respectively. In a food system of fresh-squeezed tomato juice, WEC was observed to possess an effective capacity to control the total counts of viable bacteria. Shikimic acid was isolated from WEC and identified as the main antibacterial compound.

Extracts of woods commonly used for animal bedding were tested for antimicrobial activity. Essential oils from Alaska cedar (*Chamaecyparis nootkatensis*), western juniper (*Juniperus occidentalis*) and old-growth Douglas fir (*Pseudotsuga menziesii*), as well as methanol extracts of wood from these trees plus western red cedar (*Thuja plicata*) and ponderosa pine (*Pinus ponderosa*), were tested for antimicrobial activity against anaerobic bacteria and yeast in study by Johnston and co-workers (2001). The test microbes included *Fusobacterium necrophorum*, *Clostridium perfringens*, *Actinomyces bovis* and *Candida albicans* which are common to foot diseases and other infections in animals. The essential oils and methanol extracts were tested using a standardized broth assay. Only extracts of Alaska cedar and western juniper showed significant antimicrobial activity against each of the microbes tested. The essential oil of Douglas fir did show antimicrobial activity against *A. bovis* at the concentrations tested. The methanol extracts of the heartwood of Douglas fir and the sapwood of ponderosa pine showed no antimicrobial activity. The major chemical components of western juniper (cedrol and alpha- and beta-cedrene) and Alaska cedar (nootkatin) were also tested. In western juniper, alpha- and beta-cedrene were found to be active components. Nootkatin showed activity only against *C. albicans*. The inhibitory activity in Alaska cedar oil was high enough to justify further efforts to define the other chemical components responsible for the antimicrobial activity.

Chaudhary et al. (2009) reported 36 constituents in the oil of woodchips of *C. deodara*. In the pentane fraction, 27 compounds were identified representing 90.89% of the constituents detected. A total of 11 sesquiterpene hydrocarbons and 16 oxygenated sesquiterpenes were identified constituting 59.68 and 31.21%, respectively. In the acetonitrile fraction, 31 compounds were identified representing 89.24% of the constituents detected assigning to three different classes: oxygenated monoterpene (3.73%), sesquiterpene hydrocarbons

(14.44%), and oxygenated sesquiterpenes (71.07%). The major constituents in the pentane fraction were himachalenes (52.35%) and atlantones (15.45%). The other constituents were himachalene oxide (9.12%), himachalol (2.04%), α-dehydro-ar-himachalene (1.94%), cis-α-bisabolene (1.58%), and γ-dehydro-ar-himachalene (1.44%). The major constituents in the acetonitrile fraction were atlantones (41.40%) followed by himachalenes (11.70%). Further chromatography led to an increase in the percentage of himachalenes and atlantones from pentane and acetonitrile fraction to 90.89 and 95.74%, respectively (Chaudhary et al., 2009, 2011). Generally, the *Cedrus* oils contain high percentages of the himachalenes (Chaudhary et al., 2011).

Takao et al. (2012) have prepared essential oil (EO) from waste wood chips made from used sake barrels (USBs) of Japanese cedar (i.e., EO-USB) by steam distillation. They have found that EO-USB and three commercially purchased EOs derived from xylem tissue of Japanese woods, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtusa*) and false arborvitae (*Thujopsis dolabrata*), suppressed fungal growth activity against Trichophyton rubrum, which is the cause of tinea disease. The magnitude of the suppressive effects of the EOs ranked as follows: *T. dolabrata* > USB = *C. japonica* > *C. obtusa*. These EOs also inhibited the activity of DNA polymerase in an extract from *T. rubrum* mycelia with the following ranking: *T. dolabrata* > USB = *C. japonica* > *C. obtusa*. In addition, 50 μg/ml of EO-USB showed antifungal properties, killing *T. rubrum* mycelia at 27–42 °C in 20 min. Three prepared sesquiterpenes, δ-cadinene, epi-cubenol, and β-eudesmol, inhibited the fungal growth and DNA polymerase activities of *T. rubrum*, and epi-cubenol showed the strongest inhibition among the compounds tested. These sesquiterpenes had no inhibitory effects on the activities of other DNA metabolic enzymes, such as DNA topoisomerase II, IMP dehydrogenase, polynucleotide kinase, and deoxyribonuclease from *T. rubrum*. These results suggest that EO-USB containing epi-cubenol may be useful for its anti-tinea disease properties, which are based on DNA polymerase inhibition (Takao et al., 2012).

A comprehensive overview of new discoveries in essential oil research with new insights on antimicrobial activity, as well as immunomodulatory, anti-apoptotic, anti-angiogenic and antitumoral properties of these compounds was provided by Saad et al. (2013). The interest in using essential oils and their active components as modulators of cellular physiology, homeostasis, and fate is due to the high diversity in the composition of essential oils in addition to the presence of a high molecular assortment in each composition. It was assumed that this natural and sustainable resource of diverse molecular composition and structure will open an opportunity for targeting a wide spectrum of cellular processes (Saad et al., 2013).

Additionally, the numerous modes of essential oils action have been proposed involving, for example, degradation of the bacterial cell wall, modification of proteins of the cytoplasmic membrane, alteration of membrane permeability, inactivation of extracellular enzymes, reduction of intracellular ATP, leakage of cellular contents, coagulation of cytoplasm, and interruption of electron flow and active transport (Burt, 2004; Llana-Ruiz-Cabello et al., 2015; Radaelli et al., 2016).

Conclusions

In summary, this study provides insight into the *in vitro* antibacterial activity of a wide variety of essential oils derived from many different plant genera against pathogenic bacteria. The data contributes to the ongoing scientific investigation regarding the application of essential oils as natural antibacterial agents. After benchmarking essential oils from plants belonging to subfamily Abietoideae, silver fir essential oil is identified as a promising candidate concerning possible applicability in the prevention of bacterial growth.

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FICUS MACROPHYLLA DESF. EX PERS. LEAVES (MORACEAE) POSSESS ANTIMICROBIAL POTENTIAL FOR THE PREVENTION OF BACTERIAL INFECTIONS: PRELIMINARY *IN VITRO* STUDY

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The aim of this study was to test the efficacy of ethanolic extract prepared from *Ficus macrophylla* Desf. Ex Pers. leaves against Gram-positive and Gram-negative bacteria in order to evaluate the possible use of this plant in the prevention of bacterial infections. The testing of the antibacterial activity of the plant extract was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. Gram-negative bacteria *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922), as well as Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (NEQAS 3679) and *Streptococcus pneumoniae* (ATCC 49619), as well as fungus *Candida albicans* locally isolated were used as test organisms. Our results revealed that the ethanolic extract of *F. macrophylla* leaves possessed mild activity against the Gram-positive and Gram-negative bacteria. However, *S. pneumoniae* and *E. coli* appeared to be less sensitive to the *F. macrophylla* extract. Thus, *F. macrophylla* possesses the medicinal potential for the therapy of bacterial infections induced by *S. aureus* and may be used as a natural antiseptic and antimicrobial agent in medicine. Moreover, the antibacterial activity of this plant would help for the development of a new alternative medicine system which has no adverse effects. Further investigation is necessary to identify those bioactive compounds, which will be a platform for clinical applications. Finally, we believe that these findings are important in order to evaluate the significance of collections of tropical plants maintained under glasshouse conditions at Botanical Gardens worldwide and to plan the conservation strategy by the establishment of national collections of plants with valuable characteristics with the prospects of their use as sources of antimicrobial agents.

Keywords: *Ficus macrophylla*, ethanolic extract, antibacterial activity, inhibition zone diameters, Kirby-Bauer disc diffusion method

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Introduction

The family Moraceae traditionally treated as a member of the order *Urticales* comprises 37 genera and 1,050–1,100 species worldwide, most of which are distributed in the tropics and subtropics. The Moraceae are largely woody and highly diverse in the morphology of both vegetative and reproductive structures. They include terrestrial trees, shrubs, climbers, hemi-epiphytes, and rarely subshrubs and herbs. Their leaves are usually alternate and then arranged in spirals or distichous, although some species have a characteristic phyllotaxis, which may differ between the main axis and lateral branches of a plant. The lamina is simple and entire in most of the species, sometimes more or less deeply lobed, and the latter can feature either only juvenile leaves or both juvenile and adult ones. The flowers are small, pedicellate or sessile, unisexual, and are arranged into inflorescences which typically occur in pairs in the leaf axils or (more rarely) are solitary and cauliflorous. The inflorescence of Moraceae is considerably diverse with regard to structure, shape, and degree of condensation and fusion. Two of its main types can be recognized, the one being largely bisexual and circular in outline and the other unisexual and elongate, racemose or spicate, although within these types variations occur. The fruits are drupaceous or achene-like. Among the members of the order, Moraceae are distinguished by the combination of milky latex, anatropous ovules, and apical placentation (Berg, 2001; Datwyler and Weiblen, 2004; Clement and Weiblen, 2009).

The pantropical genus *Ficus* L. is by far the largest in the family and includes approximately 750 species with the hotspot of species richness in SE Asia. Some *Ficus* species are reported to be among the oldest human food sources (Kislev et al., 2006).

The genus is represented by evergreen and (semi)deciduous trees, shrubs, and lianas, often with aerial adventitious roots. More than half of the species are hemi-epiphytes that start life as an epiphyte in the crown of another tree and then send roots down to the ground enveloping the trunk of the host tree. In the most extreme case, the adventitious roots growing down from the branches form additional woody trunks, enabling the tree to spread out laterally and cover a wide area (the banyan-type tree). The leaves are spirally arranged, distichous, (sub)opposite or subverticillate. The most striking feature of *Ficus* is the pollination syndrome based on the species-specific mutualism with insects from the family Agaonidae (Hymenoptera) commonly referred to as “fig wasps”.

Flowering phenology is central to the ecology and evolution of most flowering plants. It was assumed that in highly-specific pollination systems, such as that involving fig trees (*Ficus* species) and fig wasps (Agaonidae), any mismatch in timing has serious consequences because the plants must balance seed production with the maintenance of their pollinator populations (Zhao et al., 2014).

The inflorescence represents an urceolate receptacle (called syconium or fig) with a narrow orifice (ostiole) at the top lined with numerous unisexual flowers. The ostiole functions as a passage for the fig wasps entering the syconium to pollinate flowers and lay eggs. Several types of flowers occur inside the figs, namely the long-styled pistillate “seed flowers” producing seeds, short-styled pistillate “gall flowers” targeted by the fig wasps to lay eggs in, and staminate flowers from which the pollen attaches to newly born wasp individuals

aiming to leave the syconium. Figs of monoecious species contain all the three flower types, the long style of seed flowers preventing wasps from laying eggs in. In (gyno-)dioecious species, syconia developed on separate plants contain either only the seed flowers or both the gall and staminate ones, and pollination of the "right" seed-producing flowers appears rather accidental due to the wasps being incapable of distinguishing between these syconia types. The figs are also featured in a pronounced protogyny when the pistils precede anthers in maturation; its functional role is to enable pollination by one generation of fig wasps and collecting pollen by the next one that came out from the gall flowers. The fruitlets of *Ficus* function mostly as seeds of a fleshy fruit (mature syconium), which appears the functional entity for dispersal by fruit-eating animals (Cook and Rasplus, 2003; Berg and Corner, 2005).

Along with being an object of extreme interest for researchers during the last two centuries, *Ficus* has a long history of use by humans as a food source, in medicine, planting, and other industries and fields of human activity, partly owing to its great diversity and wide distribution range. Among popular ethnomedicinal uses of *Ficus* are treatments of skin damages, disorders of the digestive system and related organs, and parasitic infections. Besides these, the range of healing targets for particular *Ficus* species compiled from local medicines can be competitive with that of broad-spectrum traditional remedies (Lansky and Paavilainen, 2011).

Over the past several decades, the incidence of nosocomial fungal infections (i.e., invasive fungal infections acquired in a healthcare-associated setting) has dramatically increased. Factors responsible for the rise of these infections include aging populations in countries with advanced medical technologies, the resultant increase in the incidence of many cancers, increasingly intensive myeloablative therapies for these cancers, increasingly intensive care for critically ill patients (Perlroth et al., 2007).

Among the species of genus *Ficus*, *Ficus macrophylla* Desf. ex Pers. (Moreton Bay fig) is a monoecious evergreen tree reaching up to 30 m in height, hemi-epiphytic or terrestrial, with glabrous or puberulous leafy twigs, native to eastern Australia (Figure 1).

This species normally starts life in the forest as an epiphyte growing on the branch of another tree. As it grows larger, it sends down aerial roots which root into the ground below, providing the plant with extra nutriment and allowing it to out-compete the host tree, eventually smothering it. The trees gradually reach large proportions, with immense buttresses, trunks up to 8 meters or more in circumference, and branches both high and spreading. Aerial roots (if produced) grow mainly from large, framework branches near the ground, and these may produce a few extra trunks or props. Its leaves are 7–30 cm long and 4–13 cm wide, alternate, ovate to elliptic, with acute to the obtuse apex and rounded to obtuse base. The upper leaf surface is glabrous, glossy green, sometimes puberulous on the midvein, while the lower surface is mostly silvery to rusty or brownish, tomentose with weak ferruginous hairs, rarely glabrous. The syconia are pedunculate, spheroid to cubical or oblong, 18–25 mm long and 15–24 mm in diameter, puberulous to pilose, glabrescent, red to red-brown with pale spots when mature (Dixon, 2001).



Figure 1 A specimen of *Ficus macrophylla* Desf. Ex Pers. at Botanic Garden of Ivan Franko Lviv National University (Lviv, Ukraine) (A, B), branch with leaves (C), adaxial and abaxial leaf surface (D, E, F)

Moreover, Australian Aborigines found a myriad of uses for the *F. macrophylla* long before European settlement. The most obvious use is the year-round fruit, the figs. Next, the inner bark or roots were used to make a sturdy cloth and cord for bags as well as woven fishing nets. Also, the branches, as well as the bark, were used to make waterproof dug-out canoes. Lastly, the milky sap, which exudes when the tree is cut was prepared as a medicine to treat infections and to dress small wounds (http://hpathy.com/).

The literature survey indicates that the genus of *Ficus* has multiple pharmacological actions that include antidiabetic, antioxidant, antidiarrhoeal, anti-inflammatory, antipyretic, antifungal, antibacterial, hypolipidemic, anti-filarial, and hepatoprotection (Yadav et al., 2015). Moreover, their leaves are used for alleviation of infectious and inflammatory conditions in many countries (Yadav et al., 2015). Widespread medicinal use and significant biological activities of the extracts from the plants of genus *Ficus* justified a continued investigation of *F. macrophylla*. Based on the above considerations, the aim of this study was to test the efficacy of ethanolic extract prepared from *F. macrophylla* leaves against Gram-positive and Gram-negative bacteria to evaluate the possible use of this plant in the prevention of bacterial infections.

Material and methods

Plant materials

The leaves of *F. macrophylla* were sampled in M.M. Gryshko National Botanic Garden (Kyiv, Ukraine) and Botanic Garden of Ivan Franko National University in Lviv (Lviv, Ukraine). The whole collections of tropical and subtropical plants both at M.M. Gryshko National Botanic Garden and Botanic Garden of Ivan Franko National University in Lviv (including *Ficus* spp. plants) have the status of a National Heritage Collection of Ukraine and are supported through State funding (Buyun et al., 2018). At the M.M. Gryshko National Botanic Garden, the plant of *F. macrophylla* has been in cultivation since 1950. The species author abbreviations were followed by Brummitt and Powell (1992).

Preparing Plant Extracts

The sampled leaves of *F. macrophylla* were brought into the laboratory for antimicrobial studies. Freshly collected leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 10) at room temperature. The extract was then filtered and investigated for their antimicrobial activity. The extract was stored at 4 °C until use.

Bacterial strains

The testing of the antibacterial activity of the plant extract was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). Gram-negative bacteria *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922), as well as Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (NEQAS 3679) and *Streptococcus pneumoniae* (ATCC 49619), as well as fungus *Candida albicans* locally isolated were used as test organisms. The clinical strain of *C. albicans* was also used in this study. *C. albicans* were differentiated from other *Candida* and *Cryptococcus* species by its ability to grow on the Levine formula of EMB agar and to produce germ tubes within 3 h, and pseudohyphae and budding cells at 18–24 h when incubated at 35 °C in 5–10% CO_2. The addition of tetracycline to the Levine formulation aids in the selection of *C. albicans* from clinical sources that are contaminated with bacteria. Susceptibility testing of the isolate was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI).

Evaluation of Antibacterial Activity of Plant Extracts by the Disk Diffusion Method

Strains tested were plated on TSA medium (Tryptone Soy Agar) and incubated for 24 hr at 37 °C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). Muller-Hinton agar plates were inoculated with 200 µl of standardized inoculum (10^8 CFU/mL) of the bacterium and spread with sterile swabs.

Growth from freshly subcultured *C. albicans* isolates was suspended in 10 mL of sterile saline to obtain turbidity of 0.5 McFarland standard. Using a sterile swab, the Sabouraud dextrose

agar plates were evenly inoculated with the *C. albicans* suspension. The plates were then incubated at 27 °C for 48 h. The antifungal activity was evaluated by measuring the diameter of inhibition zones (mm). Each test was repeated eight times.

Sterile filter paper discs impregnated by extract were applied over each of the culture plates, 15 min after bacteria suspension was placed. A negative control disc impregnated by sterile ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hr at 37 °C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks. The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of extract tested. All statistical calculation was performed on separate data from each bacterial and fungal strains. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

The results of the antimicrobial activity of ethanolic extract obtained from *F. macrophylla* leaves are presented in Figure 2 and 3. The antibacterial activity against each bacterium was observed to be varied.

In particular, our results revealed that the ethanolic extract of *F. macrophylla* leaves possessed mild activity against the Gram-positive bacteria (14.06 ±0.67 mm inhibition zone diameter for *S. aureus* and 12.5 ±1.61 mm methicillin-resistant *S. aureus*), the Gram-negative bacteria (10.88 ±0.54 mm for *K. pneumoniae*, 10.19 ±0.25 mm for *P. aeruginosa*) and fungus strain (10.13 ±0.74 mm for *C. albicans*) (Figure 2). However, *S. pneumoniae* and *E. coli* appeared to be less sensitive to the *F. macrophylla* extract; the inhibition zones were 9.75 ±0.58 mm for *E. coli* 9.0 mm and 9.5 ±0.49 mm for *S. pneumoniae*, respectively (Figure 2).

In agreement with the results obtained from the present study, previous studies undertaken by numerous researchers have found that various *Ficus* species possess noticeable antibacterial activity against bacterial and fungus strains. According to Atindehou et al. (2002) tested crude ethanol extracts from 115 plant species against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), Gram-positive bacteria (*Enterococcus faecalis* and *S. aureus*), and fungi (*Candida albicans* and *Cladosporium cucumerinum*). Among the examined plants, there were three *Ficus* species, namely *F. exasperata*, *F. mucuso*, and *F. sur*. The Gram-negative bacteria appeared unaffected by any plant extract tested, whereas the Gram-positive bacteria and fungi were inhibited by at least several plant species. Among *Ficus* species tested, *F. exasperata*

and *F. mucuso* had no significant effect on any microorganism, while *F. sur* appeared among the most active plant species against Gram-positive bacteria. No effect was shown for *F. sur* against *C. albicans*.

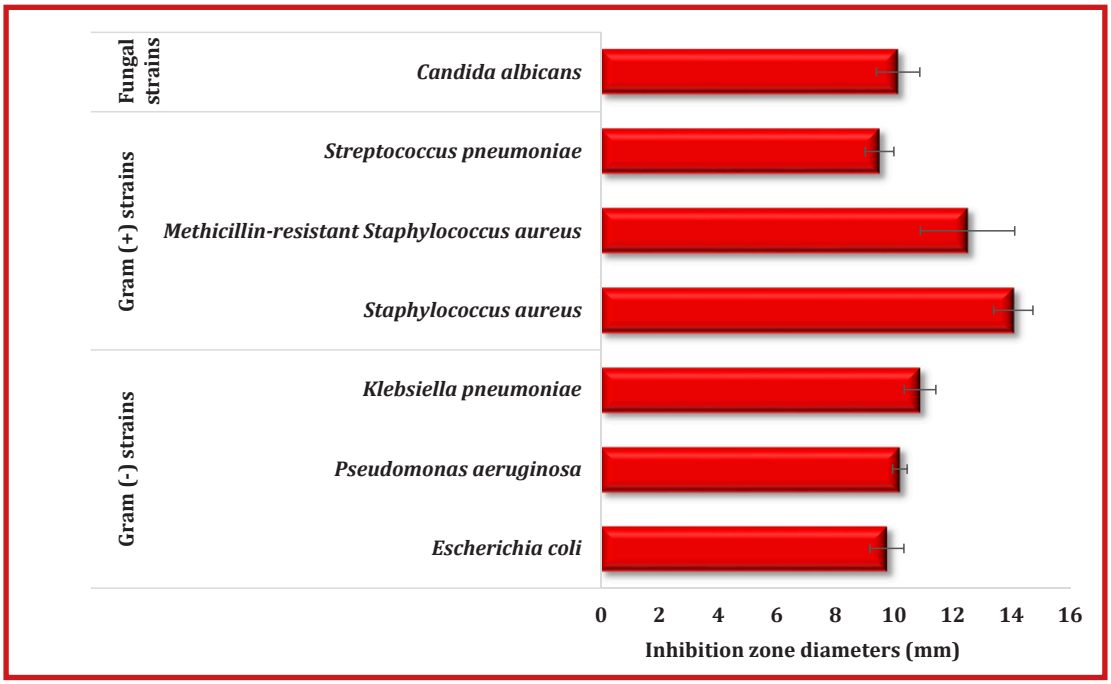


Figure 2 Antimicrobial activity of the ethanolic extract obtained from *Ficus macrophylla* Desf. Ex Pers. leaves against bacterial and fungal strains measured as inhibition zone diameter (M ±m, n = 8). The data were presented as the mean ± the standard error of the mean (S.E.M.)

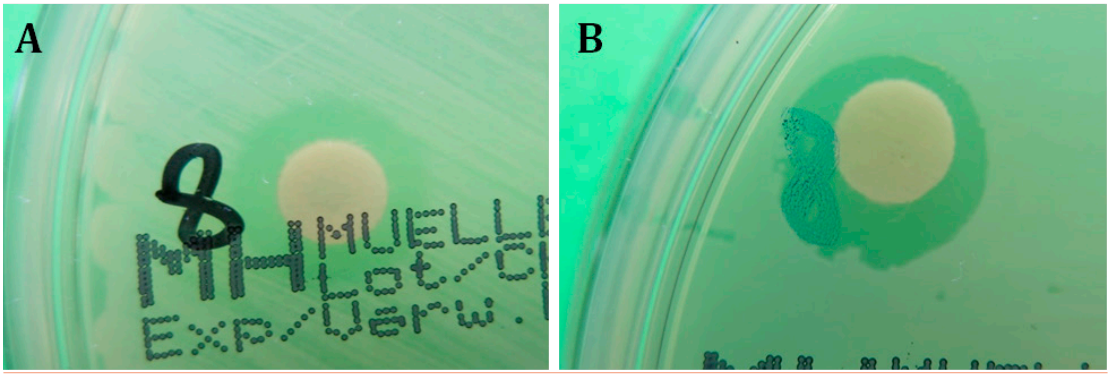


Figure 3 A disc diffusion assay plate showing the halos in the bacterial lawn resulting from the antibacterial activity of the ethanolic extract of *Ficus macrophylla* leaves against *Escherichia coli* (A) and *Pseudomonas aeruginosa* (B)

Antifungal activity of African plant methanol extracts against *Cryptococcus* neoformans and five *Candida* species (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*) analyzed by Hamza et al. (2006) showed *C. albicans* among the least susceptible pathogens. The stem bark extract from *F. sur* was ineffective against all tested organisms.

Many studies have demonstrated that plants belonging to the *Ficus* genus possess antibacterial activity. For example, Steenkamp et al. (2007) tested a wide spectrum of South African medicinal plant species for *in vitro* activity of their crude methanol and water extracts (of bark, roots, and fruits) against *Candida albicans* standard strain (ATCC 10231) and five clinical isolates (U1, U7, M42, M43, and M44). Among *Ficus* species examined, whose fruits were analyzed, *F. capensis* Thunb. (currently *F. sur* Forssk.) extracts showed no effect and *F. sycomorus* L. water extract was only active against two *Candida* isolates (M42 and M43; MIC 3,72 mg/ml). Samie and co-workers (2010) reported no effect of *F. sycomorus* bark acetone and hexane extracts against *C. albicans* as well as other fungi studied (i.e., *C. krusei* and *Cryptococcus* neoformans). The authors suggested such a discrepancy with the study of Steenkamp et al. (2007) to have resulted from the use of solvents of opposite polarity (hence this feature may potentially influence the effectiveness of antimicrobial agents), phytochemical differences in plant parts used, and geographical location of specimens sampled.

On the other hand, Kubmarawa and co-workers (2007) carried out an antimicrobial and phytochemical screening of 50 Nigerian plant species ethanolic extracts, among which were five species of *Ficus* (i.e., *F. abutifolia* (Miq.) Miq., *F. platyphylla* Del., *F. polita* Vahl, *F. sycomorus* L., and *F. thonningii* Blume). Microbial strains used in the study were *Bacillus subtilis* NCTC 8236, *E. coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 13709, and *Candida albicans* ATCC 10231. *Ficus* stem bark extracts demonstrated comparatively low antimicrobial activity, with the broadest activity spectrum being of *F. thonningii* extract (active against all microorganisms except *P. aeruginosa* and *S. aureus*). Extracts from *F. polita* and *F. sycomorus* showed no activity at all. *C. albicans* showed generally low susceptibility compared to other organisms tested. Among *Ficus* species, only *F. thonningii* extract affected *C. albicans* (with MIC value of 1.0 mg/ml). Phytochemical analysis revealed the presence of only saponins and volatile oil in *F. thonningii* extract and saponins and flavonoids in *F. polita* extract, while richer chemical content was found in *F. abutifolia* (tannins, alkaloids, and volatile oil), *F. platyphylla* (saponins, flavonoids, alkaloids, and volatile oil), and *F. sycomorus* (glycosides, tannins, flavonoids, and volatile oil) extracts. However, the authors do not make any speculations regarding the contribution of particular chemical classes to the antimicrobial activity of plant extracts tested. Authors also suggest the presence of some compound classes (such as alkaloids) in plants to be affected by climatic and environmental factors.

A number of experimental insights have elucidated the antimicrobial and antifungal efficacy of extracts derived from various organs of *Ficus* plants. Assessment of antimicrobial activity of the methanolic extract, its fractions and isolated compounds from *F. polita* Vahl. roots against a number of microbe strains (*Candida albicans* ATCC9002, *Escherichia coli* ATCC8739 and AG100, *Klebsiella pneumoniae* ATCC11296, *Providencia smartii* ATCC29916, *Pseudomonas aeruginosa* PA01, *Salmonella typhi* ATCC6539, and *Staphylococcus aureus* ATCC25922)

revealed that *C. albicans* susceptible to the crude extract and two its fractions, namely hexane 100% and Hex-EtOAc 75%, with MICs 64, 32, and 64 μg/ml respectively (Kuete et al., 2011). Ethyl acetate and hexane-ethyl acetate fractions, as well as isolated compounds (the latter showing relatively high inhibition of tested bacteria), appeared the least effective against *C. albicans* with MICs 128 μg/ml and higher.

The microbial activity of ethyl acetate, methanol and aqueous extracts of leaves of *Ficus arnottiana* Miq. was evaluated for potential antimicrobial activity against medically important bacterial and fungal strains: Gram-positive – *Staphylococcus aureus*, *Bacillus subtilis*; two Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* human pathogenic bacteria; and two fungal strains *Fusarium solanii*, *Candida albicans* (Kaur and Singh, 2016).

The development of antifungal agents to clinical use is particularly challenging due to significant commonality in the molecular targets expressed by eukaryotic fungal and mammalian cells. Many compounds with potent antifungal activity are therefore unacceptably toxic to humans. The lack of emerging therapeutic options is particularly concerning in an era of increasing antifungal resistance. Thus, there is a pressing need for innovative strategies to prevent and treat fungal OIs, particularly among the most vulnerable, immunosuppressed patients (Eades and Armstrong-James, 2019).

Supposedly, the broad antibacterial activities of this extract could be a result of the plant secondary metabolites. The phytochemical screening on genus *Ficus*, reveals the presence of sterols and/or terpenes (Kuo and Li, 1997; Kuo and Chaiang, 1999), coumarins (Chunyan et al., 2009), furanocoumarin glycosides (Chang et al., 2005), isoflavones (Li and Kuo, 1997), lignans (Li and Kuo, 2000), phenolic acids (Oliveira et al., 2009), and chromone (Basudan et al., 2005).

In general, *Ficus* species are rich sources of polyphenolic compounds. In particular, flavonoids and isoflavonoids are responsible for the extract's strong antioxidant activity that may be useful in preventing diseases involving oxidative stress (Sirisha et al., 2010). All the detected phenolic acids are known to have antimicrobial and antioxidant properties (Jaafar et al., 2012). The antimicrobial property of *F. macrophylla* extract may be due to its constituents. As it was suggested by Kumar and Pandey (2013), antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form a complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. In addition, lipophilic flavonoids may also disrupt microbial membranes (Kumar and Pandey, 2013).

It was evidenced that phytochemicals are able to inhibit peptidoglycan synthesis, damage microbial membrane structures, modify bacterial membrane surface hydrophobicity and also modulate quorum-sensing (QS) (Rasooli et al., 2008).

Conclusions

To conclude, the ethanolic extract obtained from *F. macrophylla* leaves showed varying inhibitory activities against all the test organisms. *F. macrophylla* possesses the medicinal potential for the therapy of bacterial infections induced by *S. aureus* and may be used as a natural antiseptic and antimicrobial agent in medicine. Moreover, the antibacterial activity of this plant would help for the development of a new alternative medicine system which has no adverse effects. Further investigation is necessary to identify those bioactive compounds, which will be a platform for clinical applications. Additionally, we have to keep in mind that, even though we have revealed potent *in vitro* antimicrobial activity of *F. macrophylla* extract for certain bacteria, it may not be expressed *in vivo*.

Finally, these findings are important in order to evaluate the significance of collections of tropical plants maintained under glasshouse conditions at Botanic Gardens worldwide and to plan the conservation strategy by the establishment national collections of plants with valuable characteristics with the prospects of their use as sources of antimicrobial agents.

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ANTIBACTERIAL EFFICACY OF LEAF EXTRACT OBTAINED FROM *FICUS LINGUA* WARB. EX DE WILD. & T. DURAND (MORACEAE) AGAINST *AEROMONAS* SPP. STRAINS

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Although many species within the genus *Ficus* L. have been encompassed by pharmacological investigations in previous years, there are many species that have not been studied and whose ethnobotanical relevance is yet to be investigated. With this background, an attempt was made to study the *in vitro* antimicrobial activity of the ethanolic extracts of various plants belonging to the genus *Ficus* against fish pathogens. This is especially important in view of the increasing resistance of bacteria observed during the last years and is an alternative to antibiotic therapy. Therefore, the aim of this study was to screen the antimicrobial activity of the ethanolic extract of *Ficus lingua* Warb. ex De Wild. & T. Durand leaf against fish pathogen – three *Aeromonas* strains (*Aeromonas sobria*, *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida*). The focus of this paper is on the analytical methodologies, which include the extraction of active compounds and antimicrobial activity assay by the disk diffusion technique. It was revealed that the leaf extract of *F. lingua* used in this study has bactericidal properties which make it very attractive for use in fish aquaculture. Its uses will reduce the side effects of applying synthetic compounds. The ethanolic extract obtained from leaves of *F. lingua* exhibited the maximum antimicrobial activity against *Aeromonas sobria* strain (inhibition zone diameter was 19.38 ±1.27 mm), *Aeromonas hydrophila* (16.06 ±1.05 mm), and *Aeromonas salmonicida* subsp. *salmonicida* (11.25 ±1.16 mm). The most susceptible strain to the antimicrobial activity of *F. lingua* was *Aeromonas sobria*. However, further study is needed to determine the effects of the active compounds presented in the leaf extract of *F. lingua* on fish metabolism in *in vitro* and *in vivo* study. Present results suggest the possibility of using such extracts in *in vivo* studies in order to corroborate if it could be possible using those extracts in aquaculture in order to achieve protection against pathogenic infections.

Keywords: *Ficus lingua* Warb. ex De Wild. & T. Durand, *Aeromonas sobria*, *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida*, antimicrobial activity, disc diffusion technique, ethanolic extract

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Introduction

The aquaculture industry is one of the fastest-growing sectors in recent decades. The global aquaculture production of food fish reached 62.7 million tonnes in 2011 and is continuously increasing with an estimated production of food fish of 66.5 million tonnes in 2012 (a 9.4% increase in 1 year (FAO, www.fao.org/fishery/topic/16140; Clarke et al., 2013). High intensity of aquacultural practices led to negative situations, i.e. environmental harm (e.g. poor quality of water), increased number of various opportunistic microorganisms and stress conditions for fish growth and health (Beltrán et al., 2018). Disease prevention is the key issue to maintain the sustainable development of the aquaculture sector, both environmentally and economically. Widespread use of antibiotics in aquaculture has led to the development of antibiotic-resistant bacteria and the accumulation of antibiotics in the environment, resulting in water and soil pollution (Clarke et al., 2013). It has been reported that antimicrobials used in aquaculture are administered to fish mostly in food and only rarely by injection or bath (Armstrong et al., 2005; Sørum, 2006). Consequently, this method of administration leads to their affecting both diseased and healthy fish (metaphylaxis) in the population (Sørum, 2006; Okocha et al., 2018). Moreover, it was highlighted that the application of antibiotics or other chemical treatments might have an effect on non-target organisms (e.g. other non-target culture fish and shellfish), whereas, the residues from plant-derived compounds tend to be biodegradable in the water (Kumar and Bossier, 2018).

In recent years the use of antibiotics to treat and control fish diseases has been banned in the EU (Beltrán et al., 2018). Prophylactic methods based on stimulation of the fish immune system (i.e., vaccination, probiotics, and immunostimulation) have been successfully used for this purpose and have become an integrated part of the management of modern aquaculture processes (Magnadottir, 2010).

Due to the increasing demand for chemical diversity in screening programs, an extensive search for therapeutic drugs from natural products to be used in aquaculture has grown throughout the world (Defoirdt et al., 2011; Cobello et al., 2013; Kumar and Bossier, 2018).

Plant-based products, such as phenolics, polyphenols, alkaloids, quinones, terpenoids, lectins, and polypeptides have been shown to be very effective alternatives to antibiotics and other synthetic compounds for an efficient treatment against microbial infection in aquaculture (Citarasu, 2010; Wunderlich et al., 2017; Kumar and Bossier, 2018).

The pantropical genus *Ficus* L., with its approximately 750 species, is the largest within the family and one of the most speciose genera of flowering plants and is a well-known genus used worldwide since ancient times in traditional and folk medicine (Berg and Wiebes, 1992; Cook and Rasplus, 2003; Berg and Corner, 2005). *Ficus* trees have a number of uses in various industries and fields of human activity. Virtually all parts of their body are utilized in ethnomedicine to cure disorders of digestive and respiratory systems, skin diseases, parasitic infections, etc. Some species have been cited to have analgesic, tonic, and ecbolic effects (Lansky and Paavilainen, 2011). Numerous studies have demonstrated that the plants belonging to the genus *Ficus* present a wide variety of secondary metabolites, most of the phenolic compounds such as flavonoids, terpenoids, phenolic acids, alkaloids, tannins and

active proteins and fatty acids among others, which are the principal components responsible for its activities and allows its use in traditional medicine (Ahmed and Urooj, 2010; Singh et al., 2011; Dangarembizi et al., 2012; Badgujar et al., 2014; Bunawan et al., 2014). Among the pharmacological properties demonstrated for the compounds present in genus *Ficus* are anticonvulsant, anti-inflammatory, analgesic, antimicrobial, antiviral, hypolipidemic, antioxidant, immunomodulatory, antiasthmatic, parasympathetic modulatory, estrogenic, antitumor, antiulcer, antianxiety, antihelmintic, analgesic, tonic, anti-diabetic, antipyretic, anti-inflammatory, antitussive, hepatoprotective activities etc. (Ahmed and Urooj, 2010; Lansky and Paavilainen, 2011; Singh et al., 2011; Dangarembizi et al., 2012; Badgujar et al., 2014; Bunawan et al., 2014; Yadav et al., 2015). For all these reasons, plants belonging to the genus *Ficus* could be considered a priori as a good source of new natural compounds to treat, prevent and control fish diseases in aquaculture.

At the current time, there are intense and active investigations into natural products with biocidal activities for fish (Galina et al., 2009). It is well known that many active compounds of plants are responsible for potential bio-activities. For that reason, there has been considerable interest in the use of medicinal plants in aquaculture with a view to providing safe and eco-friendly compounds for replacing antibiotics and chemical compounds as well as to enhance immune status and control fish diseases (Awad and Awaad, 2017). In addition to the immunostimulant properties, it has also been demonstrated that many medicinal plants are also able to have other positive effects on fish, such as the stimulation of fish growth, weight gain and early maturation of cultured species (Galina et al., 2009; Biller-Takahashi and Urbinati, 2014; Newaj-Fyzul and Austin, 2015; Vallejos-Vidal et al., 2016).

Ficus lingua Warb. ex De Wild. & T. Durand is an evergreen shrub, sometimes with a climbing habit, or can become a tree with a spreading, much-branched crown; it can grow up to 30 meters tall (African Flowering Plants Database, Conservatoire et Jardin Botaniques; Tropical Plants Database). It often starts life as an epiphyte in the branch of a tree and can eventually send down aerial roots that, once they reach the ground, provide extra nutrients that help the plant grow more vigorously. These aerial roots can completely encircle the trunk of the host tree, constricting its growth - this, coupled with the more vigorous top growth, can lead to the fig outcompeting and killing the tree in which it is growing (Protabase – Plant Resources of Tropical Africa). The tree is traditionally used as a source of fiber from which cloth can be made. The cloth is traditionally made by removing pieces of bark from the bole and large branches, then soaking it in water for several days, drying it in the shade and then beating it with a mallet to make it supple enough for use It is sometimes grown to provide shade and as an ornamental and bonsai tree (Protabase – Plant Resources of Tropical Africa; Tropical Plants Database).

The range of *F. lingua* is tropical Africa – Liberia, through the moister parts of Africa to Uganda and Kenya, south to DR Congo, Malawi, Mozambique, and Swaziland. The habitat – evergreen humid forest; coastal bushland; coral outcrops; Hemi-epiphytic, strangler or secondarily terrestrial; sometimes growing on rocks. At elevations up to 1,200 meters (African Flowering Plants Database, Conservatoire et Jardin Botaniques; Protabase – Plant Resources of Tropical Africa).In this study, we evaluated the antimicrobial activity of the ethanolic extract of *Ficus*

lingua against fish pathogen – three *Aeromonas* strains (*Aeromonas sobria*, *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida*) in order to evaluate the possible use of this plant in preventing infections caused by these bacteria in aquaculture.



Figure 1 Twig of *Ficus lingua* Warb. ex De Wild. & T. Durand

The role of *Aeromonas* spp. as a causative agent of fish diseases has been known for decades, longer than their comparable role in causing systemic illnesses in humans (Janda and Abbott, 2010).

The current investigation was conducted as a part of an ongoing project between the Institute of Biology and Environmental Protection (Pomeranian University in Slupsk, Poland), National Veterinary Research Institute (Puławy, Poland), M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine (Kyiv, Ukraine), and Ivan Franko National University in Lviv (Lviv, Ukraine) undertaken in the frame of cooperation program aimed at assessment of medicinal properties of tropical plants, cultivated *in vitro*.

Materials and methodology

Collection of plant material and preparing plant extract

The leaves of *Ficus lingua* were sampled at National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine) and Botanic Garden of Ivan Franko Lviv National University (Lviv, Ukraine). The sampled leaves were brought into the laboratory for antimicrobial studies. Freshly collected leaves were washed, weighed, and homogenized in 96% ethanol (in proportion

1 : 10) at room temperature, and centrifuged at 3,000 g for 5 minutes. Supernatants were stored at -20 °C in bottles protected with the laminated paper until required.

Bacterial strains for antimicrobial activity assay

Three *Aeromonas* strains: *Aeromonas sobria* (K825) and *Aeromonas hydrophila* (K886), as well as *Aeromonas salmonicida* subsp. *salmonicida* (St30), originated from freshwater fish species such as common carp (*Cyprinys carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), respectively, were isolated in Department of Fish Diseases, The National Veterinary Research Institute in Pulawy (Poland). Bacteria were collected from fish exhibiting clinical disorders. Each isolate was inoculated onto trypticase soy agar (TSA) (BioMérieux) and incubated at 27 ±2 °C for 24 h. Pure colonies were used for biochemical identifications, according to the manufacturer´s instructions, except the temperature of incubation, which was at 27 ±1 °C. The following identification systems were used in the study: API 20E, API 20NE, API 50CH (BioMérieux). Presumptive *Aeromonas* isolates were further identified to the species level by restriction analysis of 16S rDNA genes amplified by polymerase chain reactions (PCR) (Kozińska, 2007).

Bacterial growth inhibition test of plant extracts by the disk diffusion method

Antimicrobial susceptibility of the tested *Aeromonas* isolates was performed by the Kirby-Bauer disc diffusion method (1966), according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (2014). Each inoculum of bacteria in the density of 0.5 Mc McFarland was cultured on Mueller–Hinton agar for 24 h at 28 ±2 °C prior to the determination of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria provided by the Clinical and Laboratory Standards Institute (2014). The results of the disk diffusion test are "qualitative," in that a category of susceptibility (i.e., susceptible, intermediate, or resistant) is derived from the test rather than a MIC (Jorgensen and Ferraro, 2009). Seven drugs representing different antimicrobial classes as quinolones, tetracyclines, sulphonamides, and phenicols were used (Table 1).

Table 1 Antimicrobial agents used in the study

Group of antimicrobial agents	Symbol (Oxoid®)	Active substance(s)	Concentration (µg)
Sulphonamides	S3	sulphonamides	300
	SXT	sulphonamides and trimethoprim	25
Quinolones	OA	oxolinic acid	2
	UB	flumequine	30
	ENR	enrofloxacin	5
Tetracycline	OT	oxytetracycline	30
Phenicols	FFC	florfenicol	30

After incubation, the inhibition zones were measured. Interpretation criteria have been adopted from that available for *Aeromonas salmonicida* (CLSI, 2006)

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Poland). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Results on *in vitro* antimicrobial activity assessment of ethanolic extract obtained from *F. lingua* leaves against three *Aeromonas* strains (*Aeromonas sobria*, *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida*) expressed as a mean of diameters of inhibition zone is presented in Figure 2.

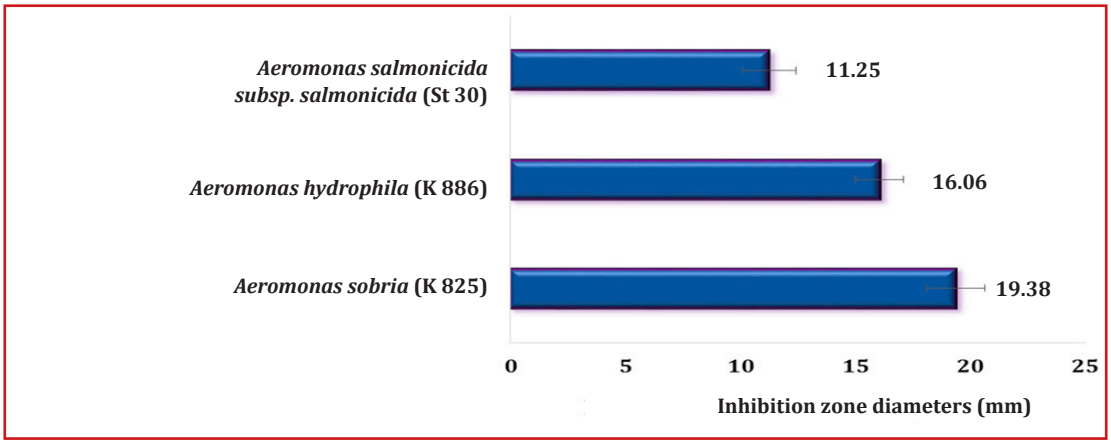


Figure 2 The mean inhibition zone diameters induced by ethanolic extract obtained from leaves of *Ficus lingua* against *Aeromonas sobria*, *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida* strains (1,000 µL inoculum) (M ±m, *n* = 8)

Our results of the disc diffusion screening revealed, that *F. lingua* possessed antibacterial properties against *Aeromonas* strains. The ethanolic extract obtained from leaves of *F. lingua* exhibited the maximum antimicrobial activity against *Aeromonas sobria* strain (inhibition zone diameter was 19.38 ±1.27 mm), *Aeromonas hydrophila* (16.06±1.05 mm), and *Aeromonas salmonicida* subsp. *salmonicida* (11.25 ±1.16 mm) (Figure 2). The most susceptible strain to the antimicrobial activity of *F. lingua* was *Aeromonas sobria*.

In response to the development of drug-resistant pathogens in aquaculture remarkable progress in the field of antibacterial herbal therapy has been made in recent years based on *ex situ* cultivated plants, *Ficus* genus, in particular (Tkachenko et al., 2016a-d; 2017a-c; 2018; 2019).

In our previous studies, the therapeutic potential for the use of various plants of the *Ficus* genus in the control of bacterial diseases was evaluated against fish pathogens in *in vitro* study with promising results (Tkachenko et al., 2016, 2017, 2018, 2019). Most ethanolic extracts obtained from *Ficus* spp. according to previous studies proved effective against the bacterial strain of Gram-negative *A. hydrophila* tested, with 10–12 mm zones of inhibition were observed. *A. hydrophila* demonstrated the highest susceptibility to *F. pumila*. The highest antibacterial activity against *A. hydrophila* (200 µL of standardized inoculum) was displayed by *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* leaf extracts (Tkachenko et al., 2016). Among various species of *Ficus* genus exhibiting moderate activity against *A. hydrophila* (400 µL of standardized inoculum), the highest antibacterial activity was displayed by *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* leaf extracts (Tkachenko et al., 2016). Antibacterial properties of plant extracts have been by far the most studied bioactivity with potential applications in aquaculture systems (Reverter et al., 2014). Castro et al. (2008) have revealed by agar diffusion assay that 31 methanolic extracts of Brazilian plants presented antibacterial activity against the fish pathogenic bacteria, i.e. *Streptococcus agalactiae*, *Flavobacterium columnare*, and *A. hydrophila*. *F. columnare* being the most susceptible microorganism to most of the tested extracts. Similarly, Wei and Musa (2008) also studied the susceptibility by assay of minimum inhibitory concentration (MIC) of two Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*), four Gram-negative bacteria (*C. freundii*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*) and 18 isolates of *Edwardsiella tarda* to aqueous extract of garlic (500, 250, 125, 62.5 mg/mL), and found that all garlic extracts were effective against the tested pathogenic bacteria.

The antimicrobial activity of *F. religiosa* L. bark, leaf, stem, and fruit aqueous extracts against a number of major pathogens (*Aeromonas hydrophila*, *Enterobacter aerogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Aspergillus niger*, and *Candida albicans*) and conducted their phytochemical analysis was screened by Rajiv and Rajeshwari (2012). All tested extracts appeared active against the pathogens at concentrations 25–100 mg/ml, the widest inhibition zone (15–16 mm) resulting from the highest concentration. Fruit extract showed generally the weakest activity and only the leaf extract affected the whole set of tested organisms at maximal concentration. Antibacterial properties of the extracts were generally better pronounced than antifungal ones. *E. coli* was most strongly inhibited by the maximal concentration extracts from the stem (inhibition zone diameter 16 mm) and leaves (15 mm) and it was unaffected by bark extract. The reference antibiotic tetracycline showed a very similar activity (16 mm) for *E. coli* at concentration 5 µg/ml. Qualitative phytochemical analysis showed the bark extract to have the richest chemical composition (sugar, alkaloids, phenols, and tannins present), being poorer in fruits (phenols and flavonoids), stem (sugar and tannins), and leaves (only tannins). Glycosides and terpenoids featured all extracts tested. Hence the most specific chemicals appeared to be alkaloids (found only in bark) and flavonoids (only in fruits), while tannins were common for the plant parts with the highest antimicrobial activity in general (i.e., bark, leaves, and stem). Although the authors present the results of the phytochemical analysis, they do not make any inferences concerning the possible contribution of particular chemical classes to the antimicrobial activity of plant extracts.

Several works have studied the immune parameters of various fish species after the dietary administration of plant extracts as well as *in vitro* and *in vivo* antibacterial and immunostimulant activity against the fish pathogens. In general, treated fish showed increased humoral (e.g. plasma protein, lysozyme, and complement activity) and cellular (phagocytic and respiratory burst activities) immune activities. For example, Sheikhlar et al. (2017) have tested *in vitro* antimicrobial activities of aqueous and methanol extracts of lemon *Citrus limon* peel, *Euphorbia hirta* (aerial parts), and fenugreek *Trigonella foenum-graecum* seeds against the bacterium *Aeromonas hydrophila*. A swab paper disk method showed that the methanol extract of *E. hirta* (EHE) had the largest inhibition zone and the lowest minimum inhibitory concentration compared to all other herbal extracts. Based on these results, EHE was included in the diets of Sharptooth Catfish *Clarias gariepinus* at 0 (control), 2, 5, or 7 g/kg of diet (experiment 1). After 30 d, the growth, feed intake, hepatosomatic index (HSI), and plasma biochemical parameters were measured. With a separate batch of Sharptooth Catfish, the efficacy of the EHE diets in conferring fish resistance to *A. hydrophila* over 30 d was compared to that of a diet containing oxytetracycline (OTC; experiment 2). The un-treated fish (Control group) were fed the control diet and were not injected with *A. hydrophila*, while the Control-AH and OTC-AH groups were infected with *A. hydrophila* and were fed either the control diet or the diet containing OTC at 1 g/199 g. The other three treatments included fish that were injected with *A. hydrophila* but fed diets with increasing EHE at 2, 5, or 7 g/kg. Experiment 1 showed no change to growth, feeding efficiency, HSI, or plasma biochemical parameters. In experiment 2, however, fish that were fed dietary EHE at 5 g/kg had significantly lower mortality than the Control-AH group, with further resistance observed for fish fed EHE at 7 g/kg. Dietary OTC was more effective than EHE as a prophylactic to *A. hydrophila* infection in Sharptooth Catfish. Nevertheless, EHE can potentially be a valuable dietary supplement to improve the resistance of Sharptooth Catfish to *A. hydrophila* infection.

Herbals such as *Ixora coccinea*, *Daemia extensa*, and *Tridax procumbens* were selected by Anusha et al. (2014) to screen *in vitro* antibacterial and immunostimulant activity against the freshwater fish pathogen *Aeromonas hydrophila* using different organic polar and non-polar solvents. Initial screening results revealed that ethyl acetate extracts and its purified fraction of *I. coccinea* were able to suppress the *A. hydrophila* strains at more than 15 mm of the zone of inhibition and positive immunostimulant activity. In order to study the *in vivo* immunostimulant influence of the compounds, the crude extracts (ICE) and purified fractions (ICF) were incorporated into the artificial diets at the concentration of 400 mg/kg and fed to the ornamental goldfish *Carassius auratus* for 30 days. After termination of the feeding experiment, they were challenged with highly virulent *A. hydrophila* AHV-1 which was isolated from infected goldfish and studied the survival, specific bacterial load reduction, serum biochemistry, hematology, immunology, and histological parameters. The control diet-fed fishes succumbed to death within five days at 100% mortality whereas ICE and ICF fed groups survived 60 and 80% respectively after 10 days. The diets also helped to decrease the *Aeromonas* load after challenge and significantly improved the serum albumin, globulin, and protein. The diets also helped to increase the RBC and hemoglobin levels significantly from the control group. The immunological parameters like phagocytic activity, serum bactericidal activity, and lysozyme activity were significantly increased in the experimental diets.

Macrophages and erythrocytes were abundantly expressed in the treated groups. Anusha et al. (2014) revealed that the Phthalate derivatives from *I. coccinea* help to stimulate the immune system against *A. hydrophila* challenge in *C. auratus*.

The effects of dietary inclusion of peels and cloves of garlic (*Allium sativum*) on the growth performance and disease resistance of African catfish (*Clarias gariepinus*) fingerlings against *Aeromonas hydrophila* infection were studied by Eirna-liza et al. (2016). Seven isonitrogenous (36% protein) experimental diets were formulated to contain graded levels of garlic (peels and cloves) at 0, 10, 20 and 30 g/kg. Fish were fed twice a day for 12 weeks. The results demonstrated that no significant differences were observed with respect to growth performance or feed utilization efficiency, specific growth rate (SGR), and feed conversion ratio (FCR) of fish fed with different inclusion levels of garlic peels and cloves as compared to control group. The plasma biochemical results showed higher total protein, albumin and globulin content in the control group as compared to the experimental groups, but these results were not significant. After the fish were challenged with *A. hydrophila*, low survival (13%) was found in the control group which was significantly lower as compared to all the treatment groups (>35%). Meanwhile, the highest survival (64%) was observed for fish fed with garlic cloves at 20 g/kg (T3). The results showed that the inclusion of garlic cloves at 20 g/kg could enhance the resistance of African catfish fingerlings to *A. hydrophila* infection (Eirna-liza et al., 2016).

Harikrishnan and Balasundaram (2008) have evaluated the antimicrobial potency of aqueous and ethanolic decoction (individual extract) and concoction (mixed extract) of three common medicinal herbs, turmeric *Curcuma longa*, Tulsi plant *Ocimum sanctum*, and neem *Azadirachta indica*, against the *in vitro* growth of *A. hydrophila*. Among the decoctions, *A. indica* exhibited the most potent antibacterial property against *A. hydrophila*. Among the concoctions, both the aqueous and ethanolic triherbal extracts mixed in the ratio of 1 : 1 : 1 had higher antibacterial activity than the other concoctions and decoctions. Goldfish *Carassius auratus* were challenged with *A. hydrophila* intramuscularly in the caudal region with two separate doses (days 1 and 3) of 50 µL/fish (1.8×10^3 colony-forming units per milliliter). On days 9 (early) and 15 (late) of infection, fish were held in a net and dip treated for 5 min daily in a 1-L solution of 1% aqueous triherbal concoction. Red blood cell (RBC) count, hemoglobin, and hematocrit levels of the infected group were significantly higher than those of the control group. In the early treated group, all of the affected profile values returned to near normal, while the late-treated group registered a partial recovery, such as improved RBC count. The derived hematological values, such as mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, of the early and late-treated groups also significantly declined but were restored to near normal only in the early treated group. The dip treatment of *A. hydrophila*-infected goldfish in an aqueous triherbal concoction had a synergistic restorative effect on the hematological variables (Harikrishnan and Balasundaram, 2008).

Ngugi et al. (2015) results demonstrate that using stinging nettle (*Urtica dioica*) can stimulate fish immunity and make adult Victoria Labeo (*Labeo victorianus*) more resistant to bacterial infection (*A. hydrophila*). They investigated the effects of dietary administration of *U. dioica* on growth performance, biochemical, hematological and immunological parameters in juvenile

and adult Victoria Labeo against *A. hydrophila*. Fish were divided into 4 groups and fed for 4 and 16 weeks with 0%, 1%, 2% and 5% of *U. dioica* incorporated into the diet. The use of *U. dioica* in the diet resulted in improved biochemical, hematological and immunological parameters. Among the biochemical parameters; plasma cortisol, glucose, triglyceride, and cholesterol decreased while total protein and albumin in fish increased with increasing dietary inclusion of *U. dioica*. Among the hematology parameters: red blood cell (RBC), white blood cell (WBC) counts, hematocrit (Htc), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and neutrophils increased with increasing dietary inclusion levels of *U. dioica*, some depending on the fish age. Serum immunoglobulins, lysozyme activity, and respiratory burst were the main immunological parameters in the adult and juvenile *L. victorianus* measured and they all increased with increasing herbal inclusion of *U. dioica* in the diet. Dietary incorporation of *U. dioica* at 5% showed significantly higher relative percentage survival (up to 95%) against *A. hydrophila* (Ngugi et al., 2015).

Mansouri Taee et al. (2017) examined the effects of dietary Myrtle (*Myrtus communis* L.) on non-specific immune parameters and bactericidal activity of skin mucus in rainbow trout (*Oncorhynchus mykiss*) fingerlings. The treatments were feeding trout with experimental diets containing different levels (0, 0.5, 1 and 1.5%) of Myrtle powder. The fingerlings were fed on the experimental diet for sixty days and then skin mucus non-specific immune parameters, as well as bactericidal activity, were measured. At the end of the trial, the highest skin mucus soluble protein level was observed in the group fed with 1.5% Myrtle. The alkaline phosphatase (ALP) activity was significantly increased in fish groups fed 1 and 1.5% Myrtle compared with the control group. However, the evaluation of skin mucus lysozyme activity showed no significant difference between the treatments and the control group. Also, no antibacterial activity was detected against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* in all treatments and control groups. Whereas skin mucus of rainbow trout showed antimicrobial activity against fish pathogens (*Aeromonas hydrophila* and *Yersinia ruckeri*) in 1 and 1.5% Myrtle treatments. The results of Mansouri Taee et al. (2017) indicated beneficial effects of dietary Myrtle on mucosal immune parameters of fingerling rainbow trout.

The ability of some herbs and seaweeds to inhibit the activity of bacterial fish pathogens is of great interest. The antibacterial screening of extracts of the leaves of *Finlaysonia obovata* with hexane, chloroform, and alcohol was carried out by Mishra and Sree (2008) against freshwater fish pathogenic bacteria viz., *Micrococcus* sp. (multidrug-resistant strain), *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, *Staphylococcus aureus*, *Escherichia coli*, *Edwardsiella tarda* by the disc-assay method. The hexane and chloroform extracts were found active against four and five pathogens, respectively.

The different bactericidal activity observed in this study may be due to the different components extracted. Among the most abundant components present in leaves of various *Ficus* plants, flavonoids, phenolic acids and especially terpenoids present bactericidal activity. Guimarães et al. (2019) have investigated the antibacterial activity of 33 free terpenes commonly found in essential oils and evaluate the cellular ultrastructure to verify possible damage to the cellular membrane. The higher antimicrobial activity was related to the presence of hydroxyl groups (phenolic and alcohol compounds), whereas hydrocarbons resulted in less activity.

The first group, such as carvacrol, l-carveol, eugenol, trans-geraniol, and thymol, showed higher activity when compared to sulfanilamide. The mechanism causing the cell death of the evaluated bacteria is based on the loss of cellular membrane integrity of function (Guimarães et al., 2019). Moreover, the natural phenols and their semisynthetic derivatives were tested by Pinheiro et al. (2018) for their antimicrobial activity against the bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Listeria innocua*, *Pseudomonas aeruginosa*, *Salmonella enterica typhimurium*, *Salmonella enterica* ssp. *enterica*, and *Bacillus cereus*. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were determined using concentrations from 220 to 3.44 μg/mL. Most of the tested compounds presented MIC values ≤220 μg/mL for all the bacteria used in the assays (Pinheiro et al., 2018).

The relationship between the structure and antibacterial activity of 22 polyphenols was analyzed by Taguri et al. (2006) using minimum inhibitory concentration (MIC) as a criterion against 26 species of bacteria that can grow in Mueller-Hinton medium. There was no clear correlation between Gram-staining and bacterial susceptibility to polyphenols, and the extent of the susceptibility was approximately dependent on the species of bacteria. In the same Gram-negative bacteria, the antibacterial activity of the polyphenols against *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* was comparatively strong. On the other hand, the activity against 11 species of the Enterobacteriaceae was comparatively weak, and the activity against six species of aerobic bacteria causing plant disease was moderate. Polyphenols having pyrogallol groups showed strong antibacterial activity, and those with catechol and resorcinol rings showed lower activity. The structure-activity relationship was extended to 26 polyphenol-rich plant extracts which could have potent antibacterial activity (Taguri et al., 2006).

It was assumed that the important function of plant-based compounds as antimicrobial includes binding to substrate or metal ions and making unavailable for microbial pathogens, microbial cell membrane disruption, binding to bacterial cell adhesins or other proteins and inhibiting the binding of bacteria to cell membranes, inactivating the microbial enzymes, blocking the viral cell fusion or adsorption in host cell (Kumar and Bossier, 2018). In addition, it was evidenced that phytochemicals are able to inhibit peptidoglycan synthesis, damage microbial membrane structures, modify bacterial membrane surface hydrophobicity and also modulate quorum-sensing (*QS*) (Rasooli et al., 2008).

Conclusions

Based on the above investigation it can be concluded that the leaf extract of *F. lingua* has bactericidal properties which make it very attractive for use in fish aquaculture. Based on the above investigation it can be concluded that Its uses will reduce the side effects of applying synthetic compounds. The ethanolic extract obtained from leaves of *F. lingua* exhibited the maximum antimicrobial activity against *Aeromonas sobria* strain (inhibition zone diameter was 19.38 ±1.27 mm), *Aeromonas hydrophila* (16.06 ±1.05 mm), and *Aeromonas salmonicida* subsp. *salmonicida* (11.25 ±1.16 mm). The most susceptible strain to the antimicrobial activity of *F. lingua* was *Aeromonas sobria*. However, further study is needed to determine the effects of the active compounds presented in the leaf extract of *F. lingua* on fish metabolism in *in vitro*

and *in vivo* study. Based on the above investigation it can be concluded that Present results suggest the possibility of using such extracts in *in vivo* studies in order to corroborate if it could be possible using those extracts in aquaculture in order to achieve protection against pathogenic infections.

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EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACTS OBTAINED FROM *AGLAONEMA COMMUTATUM* SCHOTT AND ITS CULTIVARS AGAINST *CITROBACTER FREUNDII*

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Due to the indiscriminate use of antimicrobial drugs, the emergence of human pathogenic microorganisms resistant to antibiotics has been increased. This has caused many clinical problems in the treatment of infectious diseases. *Aglaonema* plants have been widely used in recent years because of its anti-aging and longevity properties, natural anti-allergic and anti-inflammatory properties. There is a need to evaluate extracts of this plant in order to provide scientific proof for its wide application in the traditional medicine system. In the present study, we focused on investigating the *in vitro* antibacterial activity of ethanolic extracts obtained from *Aglaonema commutatum* Schott and its cultivars (Malay Beauty, Silver Queen, and Silver King), cultivated under glasshouse conditions at M.M. Gryshko National Botanical Garden, National Academy of Science of Ukraine against *Citrobacter freundii* strain locally isolated from human materials. The testing of the antibacterial activity of the plant extracts was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. The extracts from *A. commutatum* and cultivars Silver Queen exhibited higher inhibitory activity (P <0.05) than the extracts from cv. Melay Beauty and cv. Silver King. The highest *in vitro* inhibition was scored by *A. commutatum*, followed by cultivats Silver Queen, Malay Beauty, and Silver King. The ethanolic extracts obtained from the leaves of *Aglaonema commutatum* and its cultivars (Malay Beauty, Silver Queen, and Silver King) has the potential for use as natural antimicrobial agents. Further *in vivo* and *in vitro* antimicrobial, phytochemical and toxicological studies are required to evaluate the chemotherapeutic effect of the plant.

Keywords: *Aglaonema commutatum* Schott, leaf ethanolic extracts, antibacterial activity, inhibition zones, disc diffusion assay

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Introduction

Citrobacter species belong to a group of facultative, anaerobic, Gram-negative bacilli within the family Enterobacteriaceae found infrequently as normal inhabitants of the intestinal tract of humans (Plakkal et al., 2013; Liu et al., 2018). They are frequently found in water, soil, food, and the intestines of animals and humans. Previously recognized as environmental contaminants or colonizers with low virulence, they are now known to cause a wide spectrum of infections involving the urinary tract, liver, biliary tract, peritoneum, intestines, bone, respiratory tract, endocardium, wounds, soft tissue, meninges, and the bloodstream (Liu et al., 2018).

In the last decade, the demand for antimicrobial agents is increasing due to the continuous emergence of clinical bacterial strains resistant to one or several conventional antibiotics (Fassi Fehri et al., 2007). The limited effective life span of current antibiotics, the lack of compliance of patients, the unmonitored use in agriculture, and the slow rate in releasing new antimicrobial agents have led to an alarming increase in antimicrobial resistance (Othman et al., 2019). Plants promise a source of natural antimicrobial agents (Kusuma et al., 2014) because they have a great ability to produce secondary metabolites such as phenolics and polyphenols, alkaloids, terpenoids, and essential oils, lectins, and others (Othman et al., 2019). It was assumed that these metabolites can act as chemical mediators, intermediating between the plant and the environment and playing a crucial role in the protection of the plant (Taiz and Zeiger, 2010). Because of this chemical potential (a reaction against some organisms), these substances have been researched to find new molecules with antimicrobial, antimalarial, insecticidal properties. It has been reported that the antimicrobial activity of plants is related to the defense mechanism against microorganisms. Therefore, it is possible to control infectious agents using natural products responsible for the inhibitory effect on pathogenic microorganisms using various plant extracts (Fukuyama et al., 2012). Other applications for natural antioxidants may include bioactive nutraceuticals, bio-pharmaceuticals, and food additives (Kusuma et al., 2014).

Aglaonema (Araceae) is an important ornamental foliage plant genus, one of the most beautiful foliage plants, as are many members of this monocotyledonous family in which flowers are borne on a type of inflorescence called a spadix. The genus *Aglaonema* is comprised of 21 species that inhabit humid and heavily shaded forests of many territories of Asia (Chen et al., 2003; Govaerts and Frodin, 2002). *Aglaonema* contains many cultivars that are important tropical foliage plants due to their tolerance of drought and low light and low relative humidity levels encountered under interior conditions (Chen et al., 2002). It has a good combination of leaf color, such as green and red, green and white, pink and green, red, among others (Mariani et al., 2011). *Aglaonema* plants have been widely used in recent years because of its anti-aging and longevity properties, natural anti-allergic and anti-inflammatory properties (Kiatsongchai, 2015; Islam et al., 2019). Moreover, a decoction of the roots is drunk to treat dropsy and fever (Perry, 1980). Anti-hyperglycemic effects of N-containing sugars from *Aglaonema treubii* Engl. in diabetic mice were noted (Nojima et al., 1998). It was shown that the genus contains polyhydroxy alkaloids that exhibit the glycosidase inhibitor activity (Ismail and Ahmad, 2017).

The inhibitory activity of *Aglaonema modestum* Schott ex Engl. on nitric oxide synthase (iNOS) in lipopolysaccharide-activated macrophages was also investigated (Ryu et al., 2001).

The glycosidase and glycosyltransferase inhibitors have attracted considerable interest in their potential roles in curing various diseases such as cancer, diabetes and virus infection including AIDS (Winchester et al., 1992; Jacob, 1995; Asano et al., 2000). In recent years, there has been a study of genetic relationships in 9 species of *Aglaonema* by amplified fragment length polymorphism (AFLP) marker (Chen et al., 2004).

In addition, the genus *Aglaonema* has the capability to remove pollutants from the indoor air such as benzene, toluene, TCE, m-xylene, hexane, etc. (Kaur and Kumar, 2015). Song and co-workers (2007) have examined the reduction of indoor air contaminants by plants placed in an indoor space using *Aglaonema brevispathum*, *Pachira aquatica*, and *Ficus benjamiana*, which were verified as air-purifying plants by NASA. The concentration of Volatile Organic Compounds (Benzene, Toluene, Ethylbenzene, and Xylene) was monitored three hours after the plants were placed and three days after the plants were placed. The amount of reduction in the concentration of Toluene and Formaldehyde was monitored 3 hours and 3 days after the plants were placed in the space. The reduction in the concentration of Benzene, Toluene, Ethylbenzene, Xylene, and Formaldehyde was significantly greater when plants were present. When plants were placed near a window, the reduction of concentration was greater. The more plants were used, the more a reduction of indoor air contaminants occurred. The effect of reducing the concentration of air contaminants increased when the number of plants increased, and when the plants were placed in a sunny area. The concentration of Toluene was reduced by 45.6 μg/m3 when 10% of the model space was occupied by *Aglaonema brevispathum* (Song et al., 2007).

Studies have been conducted on the constituents of methanol crude extracts, derived from the leaves, stems, and roots of *Aglaonema simplex*, an aquatic plant that has been widely used as ornamental plants (Ismail and Ahmad, 2017). The results showed that the extracts contained secondary metabolites belonging to the terpenoids, steroids, phenolics, alkaloids, and glycosides. Thus, *A. simplex* is suggested as one of the potential sources of the phytochemicals for the treatment of atherosclerosis (Ismail and Ahmad, 2017).

Roy et al. (2011) have screened phytochemical substances and to assay cytotoxicity and antibacterial activities of ethanolic extracts of leaves of two medicinal plants, *Aglaonema hookerianum* Schott (Araceae) and *Lannea grandis* Engl. (Anacardiaceae) available in Bangladesh. The brine shrimp lethality bioassay showed that the ethanolic extracts of *Aglaonema hookerianum* and *Lannea grandis* possessed cytotoxic activities with LC_{50} 5.25, 5.75 μg/mL and LC_{90} 10.47, 9.55 μg/mL, respectively. Two extracts obtained from leaves were examined for their antibacterial activities against some gram-positive bacteria such as *Bacillus subtilis*, *Bacillus megaterium*, and *Staphylococcus aureus*, also gram-negative strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Salmonella paratyphi*, and *Vibrio cholerae*. The agar disc diffusion method was applied to observe the antibacterial efficacy of the extracts. Results indicated that both plant extracts (μg/mL) displayed antibacterial activity against all of the tested microorganisms. The ethanolic extracts of leaves of *Aglaonema hookerianum* showed significant antimicrobial activity (zone of inhibition: 15.08 ±0.45 to 20.37 ±0.45 mm) against all tested bacterial strains and the highest zone of inhibition was observed against *S. paratyphi* (20.37 ±0.45 mm). The ethanolic

extracts of *Lannea grandis* leaves also showed significant activity against all tested bacteria with a zone of inhibition ranging from 13.93 ±0.09 to 18.25 ±0.54 mm. These results were also compared with the zones of inhibition produced by the commercially available standard antibiotic, Amoxicillin at a concentration of 10 µg per disc. Observed antibacterial properties of the ethanolic extract of *Aglaonema hookerianum* and *Lannea grandis* showed that both plants might be useful sources for the development of new potent antibacterial agents (Roy et al., 2011).

Expanding the same research, the aim of this study was to evaluate the antibacterial activity of ethanolic extracts obtained from *Aglaonema commutatum* Schott and its cultivars, cultivated under glasshouse conditions at M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine against *Citrobacter freundii* strain locally isolated from human materials.

Materials and methodology

Collection of Plant Materials

The leaves of *Aglaonema commutatum* Schott and its cultivars (Malay Beauty, Silver Queen, Silver King), cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine.

Preparation of Plant Extracts

The leaves were brought into the laboratory for antimicrobial studies. Freshly sampled leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial test strain and growth conditions

The non-repetitive clinical strain of *Citrobacter freundii* isolated from patients with uretic infection was collected from Koszalin Hospital during March–April, 2019. The purity, as well as the identity of isolate, was confirmed in the laboratory conditions by standard microbiological methods and were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2014).

Susceptibility testing of the isolate was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI, 2014). The antibiotics tested were piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, cefuroxime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamicin, trimethoprim-sulphamethoxazole, ciprofloxacin, levofloxacin, tetracycline, tigecycline, and polymyxin B. Results were interpreted according to CLSI criteria. MIC was determined by *E*-test strips (according to manufacturer's instruction) and agar dilution method (according to the Guidelines of Clinical and Laboratory Standard Institute). The resistance breakpoints were the same as the ones defined by the *National Committee for Clinical Laboratory Standards* (NCCLS, 2014).

The cultivation medium was trypticase soy agar (Oxoid®, UK), supplemented with 10% defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37 °C. The cultures were later diluted with a sterile solution of 0.9% normal saline to approximate the density

of 0.5 McFarland standard. The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration. *Citrobacter freundii* strain studied was susceptible to all antibiotics used.

Determination of the antibacterial activity of plant extracts by the disk diffusion method

The testing of the antibacterial activity of the plant extracts was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). The strain was inoculated onto Mueller-Hinton (MH) agar plates. Sterile filter paper discs impregnated with extracts were applied over each of the culture plates. Isolates of bacteria were then incubated at 37 °C for 24 h. The plates were then observed for the zone of inhibition produced by the antibacterial activity of ethanolic extracts obtained from the leaves of *A. commutatum* and its cultivars (Malay Beauty, Silver Queen, Silver King). A negative control disc impregnated with sterile ethanol was used in each experiment. At the end of the period, the inhibition zones formed were measured in millimeters using the vernier. For each extract, eight replicates were assayed. The plates were observed and photographs were taken. The susceptibility of the test organisms to the plant extracts was indicated by a clear zone of inhibition around the holes containing the plant extracts and the diameter of the clear zone was taken as an indicator of susceptibility. Zone diameters were determined and averaged.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 8.0 software (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

The ability of the selected ethanolic plant extracts obtained from leaves of *A. commutatum* and its cultivars to inhibit *C. freundii* growth was determined in this study. The results revealed that four extracts exert antibacterial activity against this microorganism. However, the extracts from *A. commutatum* and cv. Silver Queen exhibited higher inhibitory activity (P <0.05) than the extracts from cultivars Melay Beauty and Silver King. Maximum *in vitro* inhibition was scored by *A. commutatum*, followed by cultivars Silver Queen, Malay Beauty, and Silver King, which presented inhibition zones of (14.5 ±0.9) mm, (13.5 ±0.6) mm, (11.2 ±0.7) mm, and (8.4 ±0.6) mm, respectively. In the case of the positive controls, 96% ethanol possesses a mild anti-*C. freundii* effect, which presented inhibition zones of (8.8 ±0.59) mm (Figure 1).

Detailed data regarding the zones of inhibition by the various plant extracts screened were recorded and presented in Figure 2.

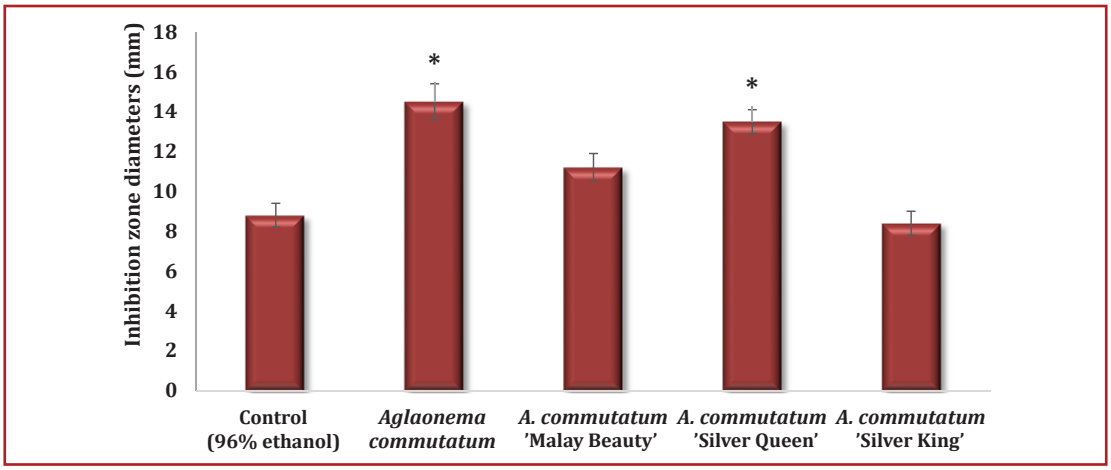


Figure 1 Antimicrobial activity of various extracts obtained from leaves of various plants belonged to the *Aglaonema* genus against *Citrobacter freundii* measured as inhibition zone diameter. * denote significant differences between the control and plant treatment groups (p <0.05)

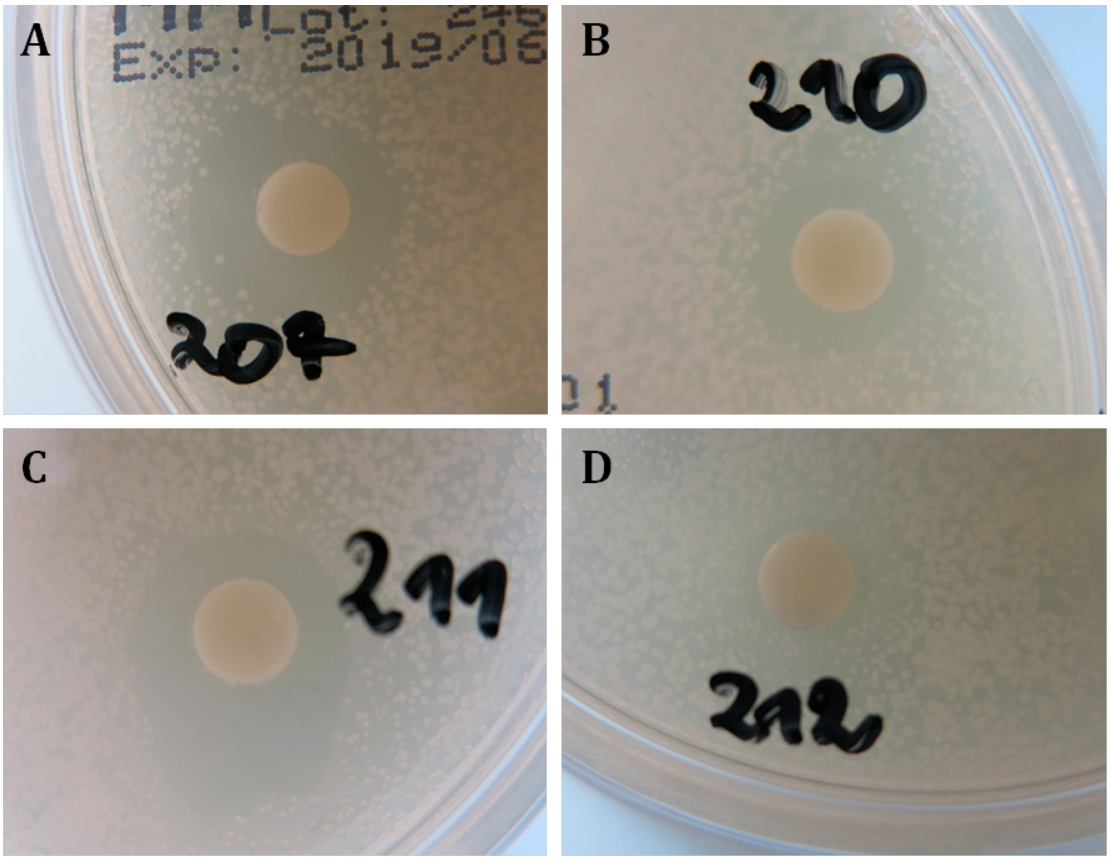


Figure 2 Inhibition zones induced by various ethanolic extracts obtained from leaves of *Aglaonema commutatum* (A) and cultivars Malay Beauty (B), Silver Queen (C), Silver King (D) against *Citrobacter freundii* growth

In line with the growing interest in the antibacterial potential of different tropical and subtropical plants, we examined the antibacterial properties of ethanolic extracts obtained from *Aglaonema commutatum* and its cultivars against *Citrobacter freundii*. The results from the screening study were obtained with the disc diffusion method. The only extract that did not exhibit any activity against the pathogen was the ethanolic extract of *A. commutatum* 'Silver King'. The largest zone of inhibition diameter was that of the ethanolic extracts of *A. commutatum* and cv. Silver Queen against *Citrobacter freundii*.

Very little information is available concerning the antimicrobial activity of the studied plant species. A literature survey of Roy et al. (2013) reveals that research works on antibacterial activity have been conducted on different plants of Araceae. Most of the plants under investigation have shown significant activity against different pathogenic bacteria. From the available data, regarding the zone of inhibitions indicate that the bacterial strains whose activities have been inhibited most by the secondary metabolites present in the crude extracts of the plants are *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. A maximum zone of inhibition has been observed in the case of ethanol extract obtained from the tuber of *Typhonium trilobatum* having a 32 mm zone of inhibition against *Staphylococcus aureus* (Roy et al., 2013).

Yunahara et al. (2014) have determined the antibacterial and antioxidant activity of different extracts of leaves Keladi tikus [*Typhonium flagelliforme* (Lodd) Blume]. Their results showed the ethyl acetate, *n*-butanol and water fraction had antibacterial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*, while the *n*-hexane fraction had no activity against bacteria tested (Yunahara et al., 2014).

These findings were supported by other data reported in the studies conducted by Baba and Malik (2015), in which it was evaluated the antioxidant and antimicrobial activity of a methanolic extract of the roots of *Arisaema jacquemontii*. The plant belongs to the family Araceae and is commonly known as cobra lily. It is used as a food, an antihelminthic and in the treatment of respiratory infections, dermatitis and as an antidote for snakebites. The extracts had antibacterial activity against both Gram-positive and Gram-negative bacteria, with MICs of 0.24–0.41 mg/mL. The extract had the greatest activity against *Salmonella enteritidis* and *Micrococcus luteus* and the least against *Streptococcus faecalis* and *Staphylococcus aureus*. The root extract also had significant antifungal activity, with values of 28.32–36.50%, the greatest activity being seen against *Fusarium oxysporum* and the least against *Aspergillus flavus* (Baba and Malik, 2015). Out of various human cancer cell lines employed in sulphorhodamine B (SRB) assay, the tuber lectin from *Arisaema jacquemontii* was found to have appreciable inhibitory effect on the *in vitro* proliferation of HCT-15 (Dukes' type C, colorectal adenocarcinoma), HOP-62 (Lung adenocarcinoma), SW-620 (Dukes' type C, colorectal adenocarcinoma), HT-29 (colorectal adenocarcinoma), IMR-32 (neuroblastoma), SKOV-3 (adenocarcinoma), Colo-205 (Dukes' type D, colorectal adenocarcinoma), PC-3 (grade IV, adenocarcinoma), HEP-2 (Carcinoma), and A-549 (Carcinoma) cancer cell lines by 82, 77, 73, 70, 41, 41, 37, 29, 21 and 21%, respectively (Kaur et al., 2006).

Luteolin isolated from *Eminium spiculatum* (Blume) Kuntze (Araceae) exhibited moderate antibacterial activity against *Escherichia coli* and resistant strains of *Staphylococcus aureus* in 1 µg per mL concentration (Afifi and Abu-Dahab, 2012). Luteolin, luteolin-7-O-glucoside, and vitexin inhibited ADP and collagen-induced platelet aggregation in a concentration-dependent manner. For the determination of the antiproliferative activity, breast cancer cell lines MCF-7 and T47D were used in the study of Afifi and Abu-Dahab (2012). Luteolin demonstrated the highest inhibitory activity with IC_{50} values of 14.92 and 18.49 µmol per L for MCF-7 and T47D, respectively (Afifi and Abu-Dahab, 2012).

The antimicrobial, phytotoxic, haemagglutination and antioxidant potential of crude methanolic extract (Crd. MeOH Ext.) and four organic fractions of *Arisaema tortuosum* were investigated by Azam et al. (2016). All fractions have been screened for antimicrobial properties against eight bacterial pathogens and six fungal pathogens using agar well diffusion and tube dilution method, respectively. Furthermore, the organic fractions were also screened for its phytotoxicity against *Lemna minor*. Haemagglutination was performed against all human blood groups while free radical scavenging activity was performed to investigate the antioxidant potential of *A. tortuosum*. Results obtained for antibacterial activity exhibited a various degree of the zone of inhibition and significant activity was observed for *Pseudomonas aeruginosa* (27.16 ±0.60) followed by *Bacillus cereus* (18.55 ±0.69) for Crd. MeOH Ext. and chloroform ($CHCl_3$) fraction, respectively while some strains showed resistant at the same concentration. Similarly, non-significant antifungal activity was observed for the plant extracts. However, the highest activity among the strains was observed for *Alternaria alternata* (22 ±1.24%) and *Aspergillus niger* (20 ±1.00%) for ethyl acetate (EtOAc) fraction and Crd. MeOH Ext., respectively. Based on the results of Azam et al. (2016) study, it can be concluded that *A. tortuosum* has significant antimicrobial and moderate phytotoxic potential and therefore can lead to antibiotics and herbicide production.

Dzotam et al. (2016) have investigated *in vitro* antibacterial activity of the methanol extracts of three Cameroonian food plants, *Moringa oleifera* Lam. (Moringaceae), *Xanthosoma mafaffa* (L.) Schott (Araceae), *Passiflora edulis* Sims (Passifloraceae) against multidrug-resistant (MDR) bacteria. The study was extended to the ability of the studied extracts to potentiate the activity of some commonly used antibiotics against some of the tested MDR bacteria. The studied microorganisms included sensitive and resistant strains of *Escherichia coli* (ATTC8739, AG100, AG100A, AG102, AG100ATet, W3110), *Enterobacter aerogenes* (ATCC13048, EA289, EA27, EA298, CM64), *Klebsiella pneumoniae* (ATCC11296, KP55, KP63, K24), *Pseudomonas aeruginosa* (PA01, PA124), *Providencia stuartii* (ATCC29914, NEA16) obtained clinically or from the American Type Culture Collection (ATCC). The broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of the extracts, as well as those of antibiotics in association with the extracts. It appears that the extracts from *P. edulis* inhibited the growth of 17/19 (89.5%) bacteria with a concentration ranged from 128 to 1,024 µg/mL. The two other samples showed selective activities, their inhibitory activity being recorded on 13/19 (68.4%) and 11/19 (57.9%) tested bacteria for *M. oleifera* and *X. mafaffa* extracts respectively. The lowest MIC value (128 µg/mL) was obtained with *P. edulis* and *M. oleifera* extracts on *Escherichia*

coli AG100. Extracts showed antibacterial activities with minimum inhibitory concentrations ranging from 128–1,024 µg/mL on the majority of the 19 tested Gram-negative bacterial strains. Extract from the pericarp of *P. edulis* inhibited the growth of 89.5% of the 19 tested bacterial strains, the lowest minimum inhibitory concentration (MIC) value of 128 µg/mL being recorded against *Escherichia coli* AG100 strain (Dzotam et al., 2016).

Conclusions

The ethanolic extracts obtained from the leaves of *Aglaonema commutatum* Schott and its cultivars (*A. commutatum* 'Malay Beauty', *A. commutatum* 'Silver Queen', and *A. commutatum* 'Silver King') have the potential for use as natural antimicrobial agents. Considering our outcome of this study, further *in vivo* and *in vitro* antimicrobial, phytochemical and toxicological studies are required to evaluate the chemotherapeutic effect of the plant.

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IN VITRO ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT DERIVED FROM LEAVES OF *SANSEVIERIA AETHIOPICA* THUNB. (ASPARAGACEAE)

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This study was performed to assess the antibacterial properties of ethanolic extract prepared from *Sansevieria aethiopica* leaves against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains, clinically important bacteria, which are indicator organisms commonly used in various projects in order to monitor antibiotic resistance. For this study, a panel of organisms including *Staphylococcus aureus* ATCC 25923 (mecA negative), *S. aureus* ATCC 29213 (mecA negative, Oxacillin sensitive, weak β-lactamase-producing strain), *S. aureus* NCTC 12493 (mecA positive, Methicillin-resistant, EUCAST QC strain for cefoxitin), *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27583 were used. The results of antibacterial assays showed that plant extract has exhibited the highest antibacterial activity against *S. aureus* as compared to the *E. coli* and *P. aeruginosa* strains. The diameters of inhibition zones were (26.35 ±1.26) mm, (16.15 ±1.47) mm, and (21.6 ±1.23) mm for *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, and *S. aureus* NCTC 12493, respectively. Conversely, the extract has shown less antimicrobial activities against *P. aeruginosa*. The mean of the inhibition zone was (12.49 ±1.09) mm. Finally, the ethanolic extract of *S. aethiopica* leaves exhibited mild antibacterial activity against *E. coli* [mean of inhibition zone ranged (18.62 ±1.32) mm for *E. coli* ATCC 25922 and (16.38 ±1.02) mm for *E. coli* ATCC 35218]. Thus, the extract obtained by leaves of *Sansevieria aethiopica* showed potent antimicrobial activity. It can be assumed that the presence of the plant extracts could be used for the treatment of various infections because of its effective zone of inhibition of bacteria growth. However, isolation and characterization of the active ingredients in this plant together with their mechanisms of actions on pathogens are still open for further investigations.

Keywords: *Sansevieria aethiopica* Thunb., leaf extract, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains, antibacterial activity, disc diffusion technique, ethanolic extracts

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Introduction

Genus *Sansevieria* Thunb., belonging to Asparagaceae family (Lu and Morden, 2014), comprises ca. 70 species worldwide, distributed mainly in dry or arid areas of the Old World tropics and subtropics (Staples and Herbst, 2005), with a distribution range from Africa to South-East Asia and the islands of the Indian Ocean (Alfani et al., 1989; Carlquist and Schneider, 2007). Representatives of this genus are usually xerophytic perennial rhizomatous plants that occur in dry tropical and subtropical parts of the world (Staples and Herbst, 2005). The habitats of *Sansevieria* in the Old World are often described as open, sunny places, but frequently with subsurface moisture availability (Carlquist and Schneider, 2007). It is assumed that species in this genus represent an important example of the evolution of Asparagaceae for their rapid adaptation to a diversity of habitats (Lu and Morden, 2014). The recent classification has treated *Sansevieria* as a synonym of Dracaena based on their overlapping morphological characteristics (Bos, 1998; Lu and Morden, 2014).

Sansevieria, a genus with diverse ethnobotanical uses in its geographical distribution range, has occupied an important place among plant genera applied for the treatment of a broad spectrum of diseases and disorders (Khalumba and Mbugua, 2005; Staples and Herbst, 2005; Takawira-Nyenya et al., 2014). Members of *Sansevieria* are of great economic importance as ornamental plants mainly due to the multicolored and mottled leaves and the interesting wide variation in leaf shapes (Nazeer and Khoshoo, 1984). *Sansevieria* species are also used as a source of fiber and in traditional African medicine. For example, in Africa (i.e., Tanzania, South Africa, and Zimbabwe), leaves and rhizomes of *S. hyacinthoides* (L.) Druce is squeezed and the juice is used in the treatment of ear infections, earaches, toothache, hemorrhoids, ulcers and intestinal worms, stomach disorders and diarrhea (Wyk and Gericke, 2000). Plant preparations of the *Sansevieria liberica* Gerome and Labroy are commonly used across Nigeria for the treatment of inflammatory conditions (Akindele et al., 2015), while the aqueous root extract of *S. liberica* is used in traditional African medicine (TAM) for the treatment of diarrhea (Adeyemi et al., 2009). Moreover, the anticancer activity of root extracts of *S. liberica* using a combination of *in vitro* and *in vivo* models has been reported (Akindele et al., 2015). It was reported that the whole plant of *S. roxburghiana* Schult. & Schult. f. is traditionally used as cardiotonic, expectorant, febrifuge, purgative, tonic, in glandular enlargement and rheumatism (cited by Haldar et al., 2010). Antitumor activity of *S. roxburghiana* rhizome against Ehrlich ascites carcinoma in mice was evaluated by Haldar et al. (2010).

There are some studies that revealed the antimicrobial activity of *Sansevieria* species (Onah et al., 1994; Aliero et al., 2008; Philip Deepa et al., 2011; Sheela et al., 2012). Similarly, in our previous study, we have evaluated the antibacterial capacity of ten species of *Sansevieria* genus against *Staphylococcus aureus* in order to validate scientifically the inhibitory activity for microbial growth attributed by their popular use and to propose new sources of antimicrobial agents (Buyun et al., 2016). The selected bacterial strain *S. aureus* is widespread and causes serious problems due to their pathogenicities and high levels of drug resistance. This has caused many clinical problems in the treatment of infectious diseases because the commercially available antibiotics commonly used are sometimes associated with adverse effects such as hypersensitivity, allergic reaction, and immunosuppression in the host. Thus,

the search for the discovery of new antimicrobial agents is an urgent need. The results proved that the inhibition zones ranged between 16 and 34 mm. *S. fischeri* and *S. francisii* extracts were particularly active against tested strain (diameters of inhibition zones were 34 mm). This was followed by the activities of *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* leaf extracts (diameters of inhibition zones ranged from 25 to 31 mm). The ethanolic extracts of *S. canaliculata* and *S. trifasciata* showed less antimicrobial activities (16.0 to 16.5 mm). The results proved that the ethanolic extracts of *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* exhibited a favorable antibacterial activity against *S. aureus*. By the agar diffusion method, the ethanolic extracts of *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, and *S. metallica* leaves showed anti-*S. aureus* activity, evidencing that ethanol is an efficient organic solvent to be used for the extraction of bioactive plant materials (Buyun et al., 2016). As previously mentioned, our results also revealed that the ethanolic extracts obtained from leaves of *S. kirkii*, *S. arborescens*, *S. roxburghiana*, *S. francisii*, *S. forskaliana*, *S. cylindrica*, *S. trifasciata*, *S. canaliculata*, *S. caulescens*, *S. metallica*, *S. aethiopica* possess antibacterial potency against *Escherichia coli* isolates and may be used as natural antiseptics and antimicrobial agents in medicine (Tkachenko et al., 2017).

Sansevieria aethiopica Thunb. is an evergreen, succulent, perennial plant producing long, narrow, erect or slightly spreading sword-shaped leaves up to 75 cm long from a rhizomatous rootstock (Figure 1). The plant can spread to form colonies. Good quality fiber is obtained from the leaves of wild plants, which are also used for local medicinal purposes for the treatment of oral, ear and other fungal infections (Tropical Plants Database, Ken Fern). The leaf fibers of *S. aethiopica* are used to make strong twines and ropes. Like several other species of the genus it is especially valued to make bowstrings, hence the vernacular name. The Himba of Namibia uses the fiber to make clothing. In Botswana, it is used to make fishing lines and nets and the rope is used to make sleeping mats by tying thick papyrus stems together (Kirby, 1963; Obermeyer, 1992; Newton, 2001; Praptosuwiryo, 2003; Takawira-Nyenya, 2006).

The rhizomes are a source of drinking water obtained by chewing and spitting out the fibers. *S. aethiopica* is planted as an ornamental in pots and gardens. The medicinal use of rhizomes and leaves is widespread in southern Africa. This plant is used for the treatment of oral, ear and other fungal infections (Hutchings et al., 1996). In Zimbabwe, the leaves are heated and the sap is squeezed into the ear to treat ear infections, while the rhizome is warmed and used for treating toothache (Newton, 2001; Praptosuwiryo, 2003; Takawira-Nyenya, 2006). The leaves are bruised, then heated for a short time. They are then twisted by hand and the fluid thus obtained is dripped into the ear as a cure for ear problems (Leffers, 2003). Fresh or boiled rhizomes are eaten to treat hemorrhoids, stomach-ache, ulcers, diarrhea, and internal parasites. In Namibia, Bushmen apply the heated, pounded leaves to a stiff neck to give relief. Leaf sap is applied to wounds to accelerate healing and to maternal breasts to stimulate milk production (Newton, 2001; Praptosuwiryo, 2003; Takawira-Nyenya, 2006).

These data have prompted us to focus on the assessment of antibacterial activities of the leaves of *Sansevieria aethiopica* plants growing in the M.M. Gryshko National Botanic Garden (Kyiv, Ukraine). Thus, the current study was designed to test the antibacterial efficacy of ethanolic extract prepared from *Sansevieria aethiopica* leaves against *Escherichia coli*, *Staphylococcus*

aureus, and *Pseudomonas aeruginosa* strains, clinically important bacteria, which are indicator organisms commonly used in various projects in order to monitor antibiotic resistance.



Figure 1 *Sansevieria aethiopica* Thunb.
A – specimen of *S. aethiopica* cultivated under glasshouse conditions at NBG (Kyiv, Ukraine) (photo by Denis Krupoderov); B – Growth habit of *S. aethiopica* (cultivated plants with inflorescences developing under glasshouse conditions at M.M. Gryshko National Botanical Garden, Kyiv, Ukraine) (Photo: Lyudmyla Buyun)

Materials and methodology

Collection of Plant Material

The leaves of *S. aethiopica* were sampled in M.M. Gryshko National Botanic Garden (Kyiv, Ukraine) (Figure 1). The whole collection of tropical and subtropical plants at M.M. Gryshko National Botanic Garden (Kyiv, Ukraine) has the status of a National Heritage Collection of Ukraine. The sampled leaves were brought into the laboratory for antimicrobial studies.

It should be noted that in the updated version of the World Checklist of Selected Plant Families (WCSP) *Sansevieria aethiopica* Thunb. (Asparagaceae) is considered as a synonym of currently accepted *Dracaena aethiopica* (Thunb.) Byng & Christenh [http://wcsp.science.kew.org/].

Preparation of Plant Extracts

The leaves were brought into the laboratory for antimicrobial studies. Freshly crushed leaves and pseudobulbs were washed, weighed, and homogenized in 96% ethanol (in proportion

1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial test strain and growth conditions

For this study, a panel of organisms including *Staphylococcus aureus* ATCC 25923 (mecA negative), *S. aureus* ATCC 29213 (mecA negative, Oxacillin sensitive, weak β-lactamase-producing strain), *S. aureus* NCTC 12493 (mecA positive, Methicillin-resistant, EUCAST QC strain for cefoxitin), *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27583 were used. The cultivation medium was trypticase soy agar (Oxoid, UK), supplemented with 10% defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37 °C. The cultures were later diluted with a sterile solution of 0.9% normal saline to approximate the density of 0.5 McFarland standard. The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration.

Determination of the antibacterial activity of plant extracts by the disk diffusion method

Antimicrobial activity was determined using the agar disk diffusion assay (Bauer et al., 1966). Strains were inoculated onto Mueller-Hinton (MH) agar plates. Sterile filter paper discs impregnated with extract were applied over each of the culture plates. Isolates of bacteria were then incubated at 37 °C for 24 h. The plates were then observed for the zone of inhibition produced by the antibacterial activity of ethanolic extract obtained from the leaves of *S. aethiopica*. A negative control disc impregnated with sterile ethanol was used in each experiment. At the end of the period, the inhibition zones formed were measured in millimeters using the vernier. For each extract, eight replicates were assayed. The plates were observed and photographs were taken. The susceptibility of the test organisms to the plant extracts was indicated by a clear zone of inhibition around the holes containing the plant extracts and the diameter of the clear zone was taken as an indicator of susceptibility. Zone diameters were determined and averaged.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica software, version 8.0 (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

The aim of our study was to examine the antibacterial properties of *Sansevieria aethiopica* leaves against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains. The results of antibacterial activity screening are given in Figure 2 and 3, which clearly indicate that the extract has shown antibacterial activity against the entire tested organisms. The extract has shown better activity against *S. aureus* compared to the *E. coli* and *P. aeruginosa* strains. The diameters of inhibition zones were (26.35 ±1.26) mm, (16.15 ±1.47) mm, and (21.6 ±1.23) mm for *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, and *S. aureus* NCTC 12493, respectively. The extract has shown less antimicrobial activities against *P. aeruginosa*. The mean of the inhibition zone was (12.49 ±1.09) mm. Finally, the ethanolic extract of *S. aethiopica* leaves exhibited mild antibacterial activity against *E. coli* [mean of inhibition zone ranged (18.62 ±1.32) mm for *E. coli* ATCC 25922 and (16.38 ±1.02) mm for *E. coli* ATCC 35218] (Figure 2 and 3).

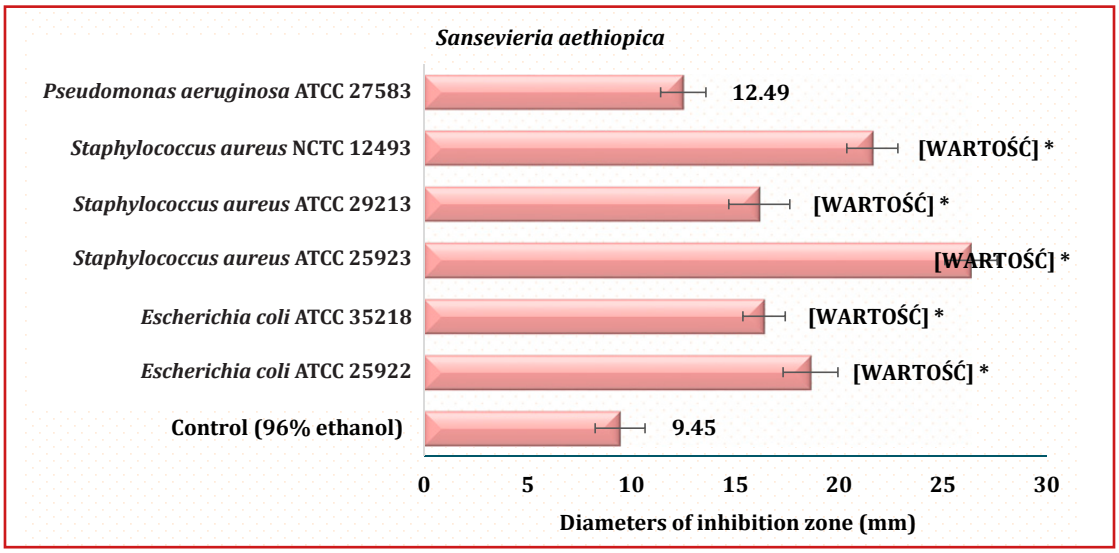


Figure 2 The mean inhibition zone diameters of ethanolic extracts obtained from leaves of *Sansevieria aethiopica* against *S. aureus* (ATCC 25923, ATCC 29213, NCTC 12493), *Escherichia coli* (ATCC 25922, ATCC 35218), and *Pseudomonas aeruginosa* (ATCC 27583) (M ±m, *n* = 8)

Consequently, in this study, the antibacterial activity of *S. aethiopica* leaf extract (Figure 2 and 3) was investigated against the standard Gram-positive strains: *Staphylococcus aureus* (ATCC 25923, ATCC 29213, NCTC 12493) and Gram-negative strains: *Pseudomonas aeruginosa* (ATCC 27583) and *Escherichia coli* (ATCC 25922, ATCC 35218) by the disc diffusion technique. The mean of inhibition zone diameters of ethanolic extracts obtained from leaves of *S. aethiopica* was statistically increased against *S. aureus* by 179% (ATCC 25923), by 71% (ATCC 29213), and by 129% (NCTC 12493), respectively as well as against *E. coli* strains by 97% (ATCC 25922) and by 73% (ATCC 35218) compared to control sample (96% ethanol) (Figure 2).

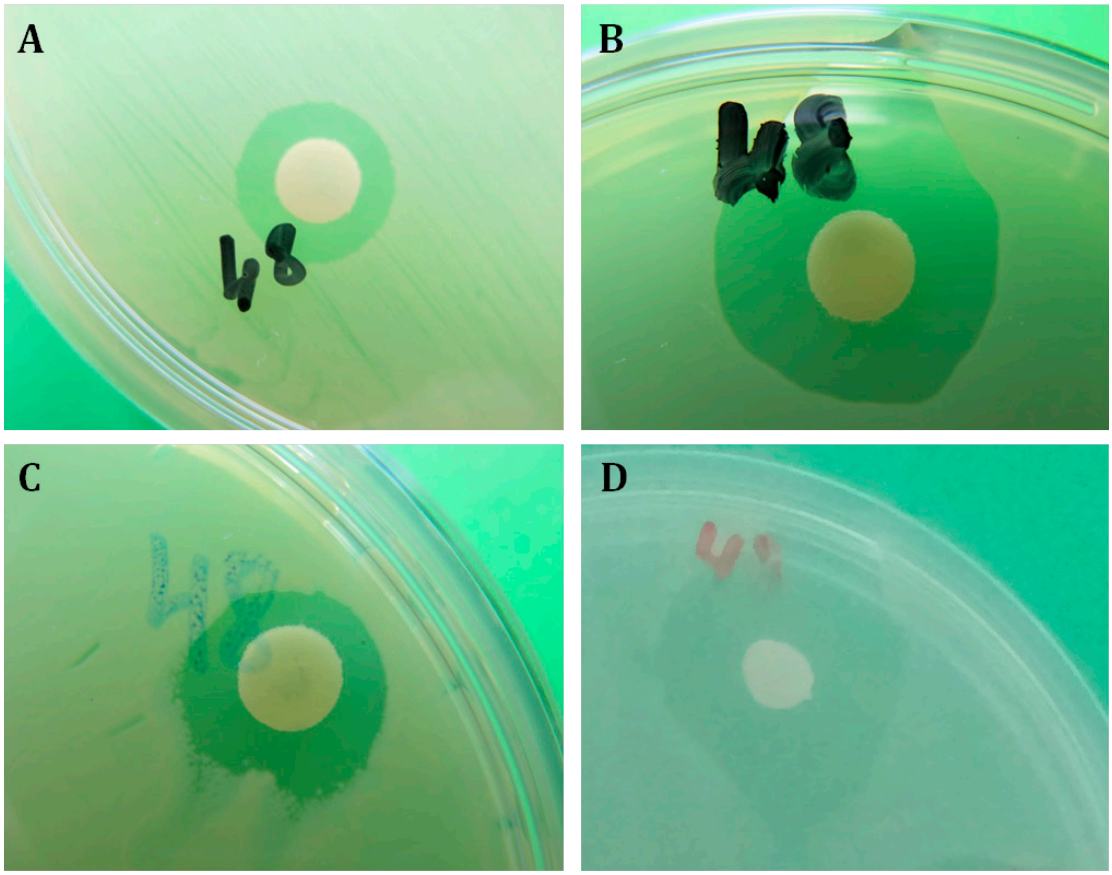


Figure 3 Antimicrobial activity of ethanolic extract obtained from leaves of *S. aethiopica* against *Escherichia coli* ATCC 25922 (A), *Escherichia coli* ATCC 35218 (B), *Pseudomonas aeruginosa* ATCC 27583 (C), and *Staphylococcus aureus* NTCC 12493 (D) measured as inhibition zone diameter

The comparison of our data, with those published by other authors, reveals that these results support the reports of other researchers that plant extracts are very effective in the treatment of bacterial infection. For example, David and Jide Afolayan (2017) have studied the effect of extracts of *S. aethiopica* on *C. albicans* ATCC 10231 and their possible mechanisms of actions were proposed. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the extracts were determined using the macro broth dilution method while the structural changes in the fungus after treatment with the extract were determined by an electron microscope. Standard methods were used to determine the effects of the extracts on the proton pumping, internal pH and ergosterol synthesis. The MIC of the extracts ranged between 1.5625 and 3.125 mg/ml. Acetone extract had the highest MIC of 3.125 mg/ml and both Acetone and ethanolic extracts had MFC of 6.250 mg/ml. Acetone and methanolic extracts had fungicidal effects on the test yeast. The extracts-treated cells showed alterations in the morphology; wrinkled surfaces, shrinkages, tears, and holes. Proton pumping activity was lower in the treatment group compared to the control while the internal pH of test fungus ranged

between 5.40 and 6.03. These researchers have observed a decrease in ergosterol content in the candidal cells treated with the plant extracts. At ½MIC of acetone, methanol and ethanol extracts of the plants the amount of 24(28)-dihydroergosterol to ergosterol was 0.0972/0.5128, 0.0939/0.3571 and 0.1032/0.3702 g/dry weight respectively. The extracts were able to inhibit the growth, affect the intracellular pH (by extension the membrane integrity) and interfere with the sterol metabolism in *C. albicans* ATCC 10231 (David and Jide Afolayan, 2017).

David and Afolayan (2013a) have also investigated the *in vitro* interactions between extracts of *S. aethiopica* and gentamicin and the activities of the resultant iso-effective combinations against biofilm of *Enterobacter faecalis* ATCC 29212 and *E. faecalis* KZN. *In vitro* interactions between the plant extracts and gentamicin were studied using the checkerboard microdilution method and anti-biofilm activity of the iso-effective combinations was determined by semi-quantitative adherence assay. Acetone extract of *S. aethiopica* has the highest inhibitory activity. The minimum concentration of gentamicin that inhibited the two isolates was the same (0.016 mg/ml). Different isoeffective points were observed with fractional inhibitory concentration indexes ranged between 0.375 and 1.9313. The maximum biofilm reduction was observed when the two antibacterial agents were combined. The combinations of the agents were able to break the barrier created by the biofilm. This is necessary before dental pathogen embedded in the biofilm could be eradicated or reduced. The efficacy of the extracts of *S. aethiopica* (singly and in combination with gentamicin) justifies its usage in oral hygiene and also suggests it as an important candidate for the formulation of paste or tincture for oral hygiene and treatment of enterococcal infections (David and Afolayan, 2013a).

In another study, David and Afolayan (2013b) have screened *S. aethiopica* leaf extracts for antifungal activity against *Candida albicans* ATCC 10231. A micro broth dilution method was used to determine the minimum inhibitory concentrations (MICs) and a biofilm enumeration assay was employed to determine the minimum biofilm inhibition concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) of the extracts. Electron microscopy was used to determine the effects of the extracts on the ultrastructure of the biofilm of the test fungus. Acetone extract had the least effect on *C. albicans* ATCC 10231 with MIC of 3.125 mg/ml. For the extracts, the MBICs and MBECs were higher than the corresponding MICs. The MBEC : MIC and MBIC : MIC were 8 : 1 and 2 : 1, 4 : 1 and 2 : 1, and 8 : 1 and 8 : 1 for acetone, ethanolic and methanolic extracts, respectively. Extract treated cells showed a change in the morphology of the cells. Extracts of *S. aethiopica* were able to affect the proliferation of both planktonic and sessile cells of *C. albicans* (David and Afolayan, 2013b).

Moreover, in our previous study, we also have assessed the *in vitro* antibacterial activity of ethanolic extract prepared from *S. cylindrica* leaves against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains. *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* NCTC 12493, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27583 were used to screen for antibacterial activity of leaf extract by the disc diffusion assay (Kirby–Bauer method). The results of antibacterial activity clearly showed that the extract has shown antibacterial activity against the entire tested organisms. The extract has shown better activity against *S. aureus* and *P. aeruginosa* strains compared to the *E. coli* strains. The diameters of inhibition zones were (22.5 ±1.24) mm, (20.5 ±1.3) mm, and (16.4 ±0.95) mm for *S. aureus*

ATCC 25923, *S. aureus* ATCC 29213, and *S. aureus* NCTC 12493, respectively. The extract has shown less antimicrobial activities against *P. aeruginosa*. Finally, the ethanolic extract exhibited mild antibacterial activity against *E. coli* (Buyun et al., 2018). The preliminary screening assay indicated that the leaves of *S. parva* with antibacterial properties may offer alternative therapeutic agents against bacterial infections. The results proved that the leaf extract from *S. parva* exhibits a favorable antibacterial activity against Gram-positive strains: *Staphylococcus aureus* (ATCC 25923, ATCC 29213, NCTC 12493) and Gram-negative strains: *Pseudomonas aeruginosa* (ATCC 27583) and *Escherichia coli* (ATCC 25922, ATCC 35218) (Tkachenko et al., 2019).

By the agar diffusion method, the ethanolic extracts from *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, and *S. metallica* showed anti-*S. aureus* activity, evidencing that ethanol is an efficient organic solvent to be used for the extraction of bioactive plant materials. It would be reasonable to suggest that the bioactivity of *Sansevieria* plant extracts is attributed to phytochemical constituents. The microbial growth inhibition capacity relies on the rich variety of phytochemicals including carbohydrates, saponin, flavonoids, phenols, alkaloid, anthocyanin and cyanine, glycosides, proteins, and phytosterols (Deepa Philip et al., 2011).

Prakoso et al. (2018) have explored the role of *Aloe vera* (L.) Burm.f. (AV), *Ananas comosus* (L.) Merr. (AC), and *Sansevieria masoniana* Chahin. (SM) on the skin wound infected with methicillin-resistant *Staphylococcus aureus* (MRSA). Forty-five adult female Sprague Dawley rats weighing 250–300 grams were divided into 5 groups. All the groups were exposed to two round full-thickness punch biopsy and infected with MRSA. The group C was the control group/untreated; group BC was treated with base cream/without extract; group AV was treated with 75% AV cream; group AC was treated with 75% AC cream, and group SM was treated with 75% SM cream. The wounds were observed on days 5, 10, and 15. The base cream formulation in this study has no potential effect on wound healing. The treatment groups (AV, AC, and SM) showed a better result of healing on the wound that was infected with MRSA in all parameters compared with groups C and BC (P <0.05). The topical application of AV, AC, and SM increased the wound contraction, skin tensile strength, angiogenesis, fibroblast, and collagen deposition in wound tissue, and it starts on day 5 (P <0.05). The healing of skin wounds was measured by a percentage of closure, skin tensile strength, and histopathology. The result showed that AV, AC, and SM have a similar potential effect on healing in the wound that was infected with MRSA compared to the groups C and BC (P <0.05). The study of Prakoso et al. (2018) proved that AV, AC, and SM are widely used as traditional medicine and have a potential role in activating cluster of differentiation 8 [$CD8^+$, a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR)] to infiltrate the wound tissue as the mechanism to eliminate MRSA infection (Wu and Xu, 2014). As T-cells are cytotoxic, $CD8^+$ plays an important role not only in controlling the infection but also in eliminating the infected cells (Prakoso et al., 2014).

The resin extracts of *Dracaena cinnabari* Balf. f. (Asparagaceae) and its solution in methanol were investigated for their *in vitro* antifungal activities against six human pathogenic fungal strains by the agar diffusion method, for antioxidant activities using the DPPH assay and for cytotoxic activity using the neutral red uptake assay by Al-Fatimi (2018). The test organisms: *Candida krusei* (ATCC 90878), *Absidia corymbifera* (100798), *Aspergillus fumigatus* (13550/99),

Trichophyton mentagrophytes (05/2004) *Microsporum gypseum* and *Mucor* sp. were used. In comparison with different resin extracts, the methanolic solution of the whole resin showed the strongest biological activities. The best antifungal activity was demonstrated by the methanolic solution of the crude resin (20–30 mm) against *Aspergillus fumigatus*, *Microsporum gypseum* and *Trichophyton mentagrophytes* followed by less polar dichloromethane and ethyl acetate extracts (18 to 20 mm), compared with the antifungal reference nystatin. In contrast, the residue of the resin dissolved in methanol showed weak activity. The methanolic solution of the whole resin was evaluated for the MIC. The resin solution showed a MIC at 500 µg/mL with inhibition zones 8 mm and 10 mm towards *M. gypseum* and *T. mentagrophytes*, respectively. It showed strong antifungal activity, especially against *Microsporum gypseum* and *Trichophyton mentagrophytes* besides antioxidant activities and toxicity against FL-cells, a human amniotic epithelial cell line (Al-Fatimi, 2018).

The antibacterial activity of the *S. aethiopica* extract may be due to the presence of various active metabolites. The phytochemicals, antioxidants and antibacterial potential of acetone and methanolic extracts of *S. aethiopica* leaf against bacterial pathogens responsible for otitis were evaluated by David and Afolayan (2016). The phenolic contents of the extracts were 57.13 and 19.06 mg tannic acid/g in acetone and methanolic extracts respectively. Flavonols and proanthocyanidin recorded the least values in methanolic and acetone extracts respectively. The extracts have good antioxidant properties although lower than the standard chemicals used as controls. The extracts expressed antibacterial effects on both Gram-negative and Gram-positive bacteria however, their activity was more pronounced on Gram-negative organisms. Although slightly toxic, the extracts have both bacteriostatic and bactericidal effects on the selected bacteria associated with otitis, especially Gram-negative (David and Afolayan, 2016). For example, the phytochemical screening revealed the high presence of alkaloids in the methanolic extract of *S. roxburghiana* compared to acetone, chloroform, and ether. Flavonoids were present in ethanolic and ether extracts in moderate proportions; saponins were present in ethanolic and methanolic extracts in moderate proportions. Steroids were shown in higher proportions in methanol, chloroform and ether and moderate in acetone; terpenoids presence were was shown in chloroform and absent in all rest of the extracts. Tannins were high in acetone and methanol and moderate in ethanol and chloroform. Phenols were only in methanol fractions, while quinones were presented in methanol, chloroform, and ether at moderate levels (Kumar and Kumari, 2015).

Phytochemical analysis revealed the presence of oils, reducing sugars, alkaloids, saponins, anthraquinones, and tannins in the root extract of *S. liberica* (Adeyemi et al., 2009).

S. roxburghiana Schult. & Schult.f. and *S. trifasciata* Prain plants were used to screen pharmacologically important phytochemical constituents against Gram-positive and Gram-negative bacterial strains in the study of Kingsley et al. (2013). The methanol extract from the leaves of *S. roxburghiana* and *S. trifasciata* plants showed good inhibition against all the pathogens. *S. roxburghiana* exhibited good inhibition effect against *S. aureus* and *P. aeruginosa* whereas *S. trifasciata* manifested good antimicrobial effect against *E. coli*, *S. aureus*, and *P. aeruginosa*. The combined effect of antibiotics and plant extract has enhanced the antimicrobial effect of the extracts obtained against pathogenic microorganisms. The

percentage inhibition of combined effect was calculated and it was observed that the leaves of *S. roxburghiana* possess antimicrobial effect (50%) against *S. aureus* combined with norfloxacin whereas the leaf extract of *S. trifasciata* when combined with tetracycline it showed 36% of inhibition against *S. aureus*. Both of the plant extracts were effective against Gram-positive and Gram-negative pathogenic microorganisms. The components present in the plant extracts are responsible for the inhibition effects as all the antimicrobial and phytochemical agents are being carried by the nature of the solvent used. The compounds separated through thin layer chromatography of *S. roxburghiana* and *S. trifasciata* with 50 mg/mL concentration exhibited good antimicrobial effect against pathogenic microorganisms (Kingsley et al., 2013).

Therefore, based on data derived from previous and recent studies, the therapeutic actions of extracts derived from *Sansevieria* spp. plants are more likely related to the antibacterial properties of their constituents.

Conclusions

The extract obtained by leaves of *Sansevieria aethiotica* showed potent antimicrobial activity. The extract has shown better activity against *S. aureus* compared to the *E. coli* and *P. aeruginosa* strains. It can be assumed that the presence of the plant extracts could be used for the treatment of various infections, caused by the tested organisms, because of its effective zone of inhibition of bacteria growth. The result lends credence to the ethnobotanical use of this plant in treating microbial infection and shows that *Sansevieria aethiotica* could be exploited for new potent antimicrobial agents. However, isolation and characterization of the active ingredients in this plant together with their mechanisms of actions on pathogens are still open for further investigations.

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PLANT GENETIC RESOURCES INFORMATION SYSTEM OF SLOVAKIA AS THE PRIMARY SOURCE OF THE INFORMATION

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Thanks to the support of two projects from European Regional Development Fund of European Union the National Agricultural and Food Centre, Research Institute of Plant Production in Piešťany (Slovak Republic) provides the development and deployment of an information system GRISS designed for complex information management research of plant genetic resources for food and agriculture and to support of management processes accessions of plant genetic resources stored in Genebank according to international principles and in accordance with the National Programme of Conservation of Plant Genetic Resources for Food and Agriculture in Slovak Republic. Genebank is responsible for the providing of the Genetic Resources Information System of Slovakia (GRISS). An information system is used through the Web interface used by the curators of collections of plant genetic resources and users from breeder's institutions, universities, public institutions, and others. Currently, the Information System provides passport information on more than 27.000 accessions and more than 23.000 accessions stored in active and basic collection in the Genebank. At present, the Slovakian collection in the European Search Catalogue for Plant Genetic Resources (EURISCO) represents 17.142 accessions from which 640 accessions are included in the European Genebank Integrated System (AEGIS). On request of plant breeders, researchers, but also private persons, the genebank is distributing research material amounting to over 2.000 samples in response to more than 50 individual requests per year. The information system GRISS is freely accessible at http://griss.vurv.sk.

Keywords: plant genetic resources, accessions, information system, GRISS, web application, passport data, evaluation data

Introduction

The *ex situ* conservation of plant genetic resources started by the mid-twentieth century as a reaction to the rapid loss of agricultural biodiversity, mainly due to the replacement of landraces by improved varieties (Gepts, 2006; Van de Wouw et al., 2009; Khoury et al., 2014). Genebanks were created in the mid-twentieth century to preserve cultivated biodiversity. The Genebank in the Research Institute of Plant Production in Piešťany (RIPP) is a coordination of

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activities under the National Programme of Conservation of Plant Genetic Resources for Food and Agriculture in the Slovak Republic. The National program is a long-term program of the Ministry of Agriculture and Rural Development of Slovak Republic. Genebank is responsible not only for the methodological leadership and coordination of the international cooperation but also for documentation of plant genetic resources (PGR) in the form of national information system GRISS (Genetic Resources Information System of Slovakia). An integral part of activities with plant genetic resources in the Genebank of Slovak republic consists of records keeping and documentation of all *ex situ* seed accessions of generatively propagated plant species stored in the active and basic collection as well as fruit species, vegetatively propagated species and *in vitro* maintained cultures. Other activities belonging to informatization include the following activities such as reception of accessions, germination control, seed monitoring, regeneration of accessions as well as distribution of accessions to users. There is no need to emphasize that such a volume of informally demanding activities could not be effectively managed without a sufficient information system. GRISS acts as a data source for global information systems, such as the Global Biodiversity Information Facility (GBIF) (Endersen, Knupffer, 2012) or the European Search Catalogue for Plant Genetic Resources (EURISCO) (Faberová, 2010; Weise et al., 2017).

The aim of this paper is to present the highly complex work of the Slovak genebank maintenance is supported by the management component of the new Information System GRISS.

Material and methodology

Conceptual and technological framework

GRISS is a new plant germplasm information system (IS) that will replace the earlier offline version of the genetic resources documentation system – EVIGEN used since the opening of Gene Bank in 1996 (Žáková, 2004; Žáková, 2005). IS GRISS is fully compatible with the passport descriptors standards of FAO/Bioversity Multicrop Passport Descriptors – MCPD v.2.1 (Alercia et al., 2015). IS GRISS is conceptually, methodically and methodologically compatible with the largest online search systems in Europe and USA, represented by GBIS and GRIN (Oppermann et al., 2015; Faberová, Papoušková, 2013; Papoušková, 2016).

Base system unit

The basic unit kept separately in a genebank is called an accession, which is represented by physical objects such as seed samples, plants from an accession grown together in a field plot or material preserved *in vitro*. All these iterations of an accession share their origin and common information.

Taxonomy

Information about scientific names of genebank accessions is fundamental. The taxonomic hierarchy, e.g. genus, species, and infraspecific taxa, is reflected in the system. The taxonomic system of IS GRISS is based on the concept (Marhold and Hindák, 1998).

Information management

IS GRISS is determined to comprehensive management of accessions of plant genetic resources stored in the National Agricultural and Food Centre, Research Institute of Plant Production in Piešťany in the Genebank in accordance with international principles and in accordance with the National Programme (Benediková et. al., 2014). GRISS presents the platform for information about *ex situ* plant collections maintained in Slovak republic. IS GRISS allows users to search and obtain information about a number of crop species such as cereals, legumes, fodder crops, medicinal and aromatic plants, wild species, landraces, and breeding lines. IS GRISS allows search by crop, taxonomy, country of origin, acquisition, accession status, and other passport descriptors. The collected germplasm is freely available for use in scientific research programs. IS GRISS provides access to information's not only the managers of genebanks but also provides information to other curators of collections, scientists, plant breeders, farmers, students and the general public responses.

Search catalog

All information collected by the information system is provided by the user via the online search system. The information system GRISS is freely accessible at the web portal http://griss.vurv.sk (Mendel et al., 2013; Mendel et al., 2016).

Ordering Germplasm

To support the key task of genebanks, to provide seed samples to breeders, researchers and the public domain, comprehensive information needs to be managed. User need to be logged in to order germplasm

Results and discussion

IS GRISS is designed as a web application that provides a sophisticated web interface for simple data input via a web browser. Information system GRISS allows curators of collections of plant genetic resources automated support for all activities related to the creation and management of passport data and characterization/evaluation data. It enables effective management of the collections. Information system mainly used to curators of collections to prepare, management and archiving of protocols, preparation and editing passport and evaluation data for accessions. IS GRISS at every moment provides an overview of stored or processed accessions in all collections, allow accessions lists to effectively filter according to many criteria, manage the process of accessions regeneration, control and evaluate users requests to provide individual items. It provides mechanisms for access to stored data and their individual analysis and export data to <.xls> file.

IS GRISS in terms of user target groups and related functions is divided into two functional and interdependent parts:

a) Front Office – public part of IS, a web application designed primarily to public users from the professional community, facilitates communication and dissemination of knowledge accumulated from the research of plant genetic resources in Slovakia. In particular, it is the communication of basic information on the accessions of

plant genetic resources stored in the Genebank. Registered users (applicants) also have the opportunity online to order accessions of plant genetic resources through the ''shopping cart''.

b) Back Office – non-public part of IS accessible only for authorized users – curators of collections of plant genetic resources through a user name and password. This section is primarily dedicated to curators of genetic resources, laboratory technicians, system admins and genebank management to comprehensive management of all *ex situ* accessions of plant genetic resources stored in an active and base collection in the Genebank of Slovak republic.

Information system GRISS is used by all institutes cooperating within the National Programme of Conservation of Plant Genetic Resources for Food and Agriculture in the Slovak Republic. Internally is composed of 3 parts: passport data, characterization/evaluation data and storage data.

Passport data

Passport data present basic information on crops – a unique identifier = national accession number, genus, species, name of genetic resources, as well as information about holding institute code, country of origin, status of accessions, source of the material, date of collection, type of storage, whether the genotype has safety duplication in another genebank, MLS, etc. Passport descriptors also contain information about wild material received from collection missions such as the collector's institute name, collection number, collection name, geographic data and description of the collection site. All passport data are compliant with the Multicrop Passport Descriptors standard – MCPD v.2.1 (Alercia et al., 2015). The passport part of the National inventory of Slovakia is replicated to the global system for plant genetic resources in Europe – EURISCO (Weise et al., 2017). At present, the Slovakian collection in EURISCO catalog represents 17.142 accessions from which 640 accessions are included in the AEGIS system (Engels et al., 2011). The national accession number is assigned to the genetic resource as the unique identifier. The accession number, in a key field is the first step to include an accession into the documentation system. Curators of collection assign the national accession number to the accessions according to set rules. The national accession number consists of holding institution code, crop code and the serial number of the accession with the crop collection (Mendel, 2019).

Characterization and evaluation of data

C & E data constitute the basis for a standardized characterization system that provides an internationally agreed format and universally understood 'language' for plant genetic resources data (Gotor et al., 2008). Recently, more attention has been paid to increasing the effective utilization of genetic resources through their characterization and evaluation. The evaluation is based on 2–3 years of field experiments where accessions are evaluated by IPGRI/ Bioversity International through crop standard descriptors list these allowing for simple and rapid discrimination between all phenotypes (Oppermann et al., 2015). In contrast to the passport descriptor set with universal usability, characterization and evaluation descriptors

are specific for Genera, characterization and evaluation data are coded on a scale 1–9 according to descriptor lists. Field trials are usually complemented by laboratory tests according to specific crop needs. Descriptive data that is of major importance to users is available to vary degrees from 12.000 genetic resources, i.e. 67% of all accessions due to field and laboratory tests. In general, descriptors contain morphological, biological, biochemical, cytological and yield data. Each year approximately 2000 PGR's are involved. The data obtained from the experiments are processed and included in the information system.

Storage data

Information on storage documentation is contained in the third part of the GRISS system. Besides the accession number, there is an acquisition number, the code for the location of the accession in storage, germination ability, moisture content, amount of seeds in the container, storage date of accession is entered. Also documented is how much, when and to whom parts of the accession have been distributed. All other data are available in the passport part via accession number. Each accession stored in the genebank should have an accession number, which is assigned by the curator of the collection. End-users are mainly research institutes, other genebanks, breeders and universities. A smaller proportion of the accessions is distributed to museums for exposure purposes. Accessions are distributed free of charge on the basis of signed SMTA (Standard Material Transfer Agreement). On request of plant breeders, researchers, but also private persons, the genebank is distributing research material amounting to over 2.000 samples in response to more than 50 individual requests per year compared to the German genebank which distributes 30.000 samples per year (Oppermann et al., 2015).

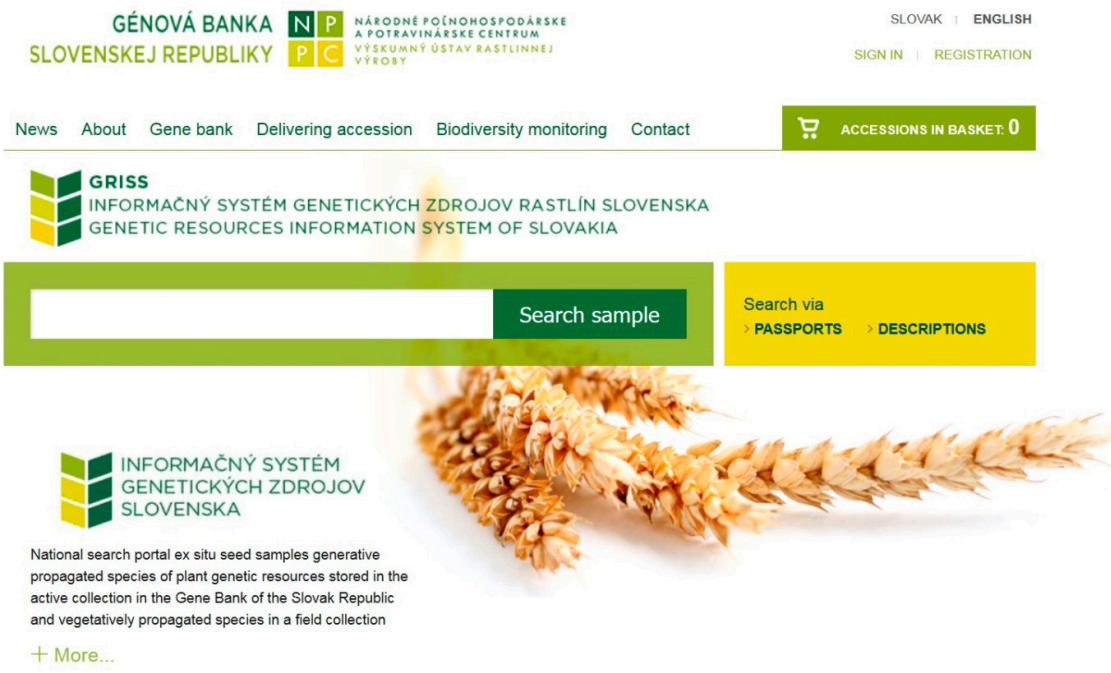


Figure 1 Users interface at http://griss.vurv.sk

The Slovakian *ex situ* plant germplasm collection is illustrated (Table 1). The largest proportion of the accessions belongs to cereals and legumes. More information about accessions can be obtained from the web portal of IS GRISS.

Table 1 Structure of National Collection of Plant Genetic Resources of Slovak Republic in the Genebank

Crop Group	Number of accessions	
	Active Collection	Basic Collection
Aromatic & Medicinal plants	467	43
Beet	152	56
Cereals	11,299	1,817
Flowers	28	62
Grasses	203	89
Vegetables	325	143
Legumes	3,430	1,044
Oilseeds	608	288
Fodder crops	960	83
Industrial crops	476	240
Corn	841	416
Pseudocereals	251	18
Total	19,040	4,298

Conclusions

Thanks to the new IS GRISS for PGR data documentation mainly good interconnection of all three main working areas: passport, characterization/evaluation, and storage parts and profiting from the construction of descriptor lists published by Bioversity. The main effect of the new IS GRISS is overall increase in the quality of documentation of plant genetic resources in the Slovak Republic, where the curators of the new system enable more effective management of PGR collections as well as significantly improve the efficiency of selecting the most suitable resources for the specified use (breeding material or experimental material etc.). The system also facilitates getting information about PGR and also enabled online orders of PGR provided from the gene bank for home and foreign users. At international level has improved compatibility with other documentation systems and international data exchange, mainly for EURISCO. IS GRISS was built as an open system and modular scalable system. The modular system architecture allows its future expansion with additional subsystems such as barcode, image analysis and geographic information systems (GIS). The concept of a comprehensive information system solutions, including interface based on the use of open standards and platforms ensuring low-cost ratio for future growth.

Acknowledgements

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OPEN ACCESS

IN VITRO CULTURE AND SOME BIOCHEMICAL CHARACTERISTICS OF *FITTONIA ALBIVENIS* (LINDL. EX VEITCH) BRUMMITT

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In this work we described a procedure of *Fittonia albivenis* (Lindl. ex Veitch) Brummitt *in vitro* conservation and microclonal propagation by enhanced bud proliferation from shoot tips as well as studying some biochemical characteristics of plants cultured both *in vivo* and *in vitro*. Multiple shoot formation from apical and axillary buds started in 4 months on MS/2 medium without growth regulators. Multiplication coefficients ranged from 6.0 ±1.9 to 14.7 ±2.6 for different plant clones. So, as a result of our work, the effective system of microclonal propagation of *F. albiviens* on hormone-free media was elaborated. It have been shown that application of plant growth regulators seems just to shorten the time period of *Fittonia in vitro* microclonal propagation but does not have any significant effect on multiplication coefficients. The total content of bioactive substances (flavonoids and phenolics) was about 0.2–0.45% of dry weight, that is significantly less than its amount in known sources of polyphenols (e.g., ground coffee). However, the data indicate the significant differences in the content of these bioactive substances between *in vivo* growing plants with dark-green (clone #1) and light-green leaves (clone #2) as well as different reactions to the transfer to the *in vitro* conditions. For some clones the significant increase in both phenolics and flavonoids amount was observed after such transfer. The clones with highest productivity have been identified and will be used in further researches.

Keywords: *Fittonia albivenis*, *in vitro* propagation, phenolics, flavonoids

Introduction

Acanthaceae family consists of 4,300 species in 346 genera and is, therefore, one of the most diverse families of flowering plants. Besides the fact that Acanthaceae plants possess an important ecological role in their native habitats they contain many important secondary metabolites and are used for treatment of many diseases. For example, some of them have the ability to kill or retard the growth of many infectious microbes including Pseudomonas

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species (Shinwari et al., 2017); show antiplasmodial, antitumor and antioxidant activities (Charoenchai et al., 2010), etc.

Fittonia albivenis (Lindl. ex Veitch) Brummitt (synonyms *F. argyroneura* Coem., *F. verschaffeltii* (Lem.) Van Houtte) is a species of Acanthaceae family native to the Peruvian rainforests and known as 'nerve plant' or 'mosaic plant'. According to leaf coloration, *Fittonia* plants are separated into two groups: white-veined plants belong to the *Argyroneura* group while red or pink-veined plants belong to the Verschaffeltii group. Available literature reports several directions of *Fittonia's* practical application. Due to its attractive foliage it is often used as ornamental plant. Leaf venation characteristics make *Fittonia* an object for studying leaf anatomy and physiology (Wook et al., 2012; Bercu and Popoviciu, 2015). Several publications point to *Fittonia's* ethnopharmacological properties. It is reported that aerial parts of *F. albivenis* in combination with *Tabernaemontana sananho* Ruiz and Pav. are efficient for treatment of snakebite wounds (Sanz-Biset et al., 2009; Félix-Silva et al., 2017). *Fittonia* aerial parts are also used in Peruvian Amazon as antidiarrheal for children (Sanz-Biset et al., 2009). Whole plant infusions or decoctions were used as a rinse of the mouth and for toothache therapy (Quattrocchi, 2012). It has been reported that indoor air is polluted with volatile organic compounds (VOCs) that can cause some acute and chronic diseases. An alternative way to reduce the level of VOCs in indoor air is the use of plants. Among indoor plants screened for their ability to remove important VOCs with differing chemistries *F. albivenis* was identified as a species with superior benzene, toluene, and trichloroethylene removal efficiency (Yang et al., 2009; Dela Cruz et al., 2014).

All these data make *Fittonia albivenis* an interesting object for further studies. However, its growing *ex situ* apart from the natural habitat in rainforests requires special conditions and makes it necessary to elaborate efficient methods of cultivation and biomass accumulation for the research activities and practical application. *In vitro* culture can be a promising approach in this respect. The role of biotechnological methods in plant biodiversity conservation and practical application is steadily growing but available *ex situ* plant depositories focus mainly on agricultural crops while there appear to be no major depositories of the medicinal and other wild-growing plants (Cordell and Corvald, 2005). In our research project concerning formation and maintaining *ex situ*/*in vitro* bank of plants representing different taxonomic groups of the world flora the *ex situ* bank contains seed samples of 31 monocotyledonous and 134 dicotyledonous plant families. Aseptic plants, cell lines and embryogenic cultures of 18 monocotyledonous and 92 dicotyledonous families form *in vitro* bank (Belokurova and Kuchuk, 2014). Acanthaceae family is represented in seed bank by some species of *Acanthus*, *Indoneesiella*, *Ruellia* and *Crossandra* genera while in *in vitro* bank *Crossandra nilotica* Oliv., *Indoneesiella echioides* (L.) Sreem., *Ruellia humilis* Nutt., *Ruellia strepens* L. are cultured.

Publications concerning *Fittonia in vitro* cultivation are not numerous. They focus on searching basic principles of tissue culture for this species and studying chimeric stability with different proliferation methods (Chen and Li, 1987; Yang, 2000; Pan et al., 2005; Wang et al., 2015a, b). In addition, information about bioactive compounds synthesized by *Fittonia* plants is very scarce.

The aim of this research was to enrich *in vitro* bank with one more (*Fittonia albivenis*), to elaborate efficient protocols of its *in vitro* multiplication, and to determine the content of some bioactive substances in soil-growing initial plants and aseptically cultured lines. The work is being carried out within the research project 'Germplasm bank of the world flora' (Belokurova and Kuchuk, 2014).

Material and methodology

Plant material

Eleven clones of *F. albivenis* soil-growing plants were used as initial material for studies. These plants were purchased on the local market and grown in the green-house of the Institute of Cell Biology and Genetic Engineering. Aseptic plants obtained during this study were cultivated in the thermal rooms of Germplasm bank of world flora of Institute of Cell Biology and Genetic Engineering. The clones differed with leaf coloration (dark or light green with white, or red, or rose veins of the various value of color) and growth rates in the soil.

In vitro culture and microclonal propagation

Stem fragments with apical and axillary buds of soil-growing plants (five explants of each plant clone) were carefully washed in stream water and used to start *in vitro* culture. Surface sterilization has been carried out using 70% ethanol (30 seconds), then with commercial bleach (resulted concentration of sodium hypochlorite – 0.5%) for 10 minutes. The treated buds were then thrice washed with sterile distilled water, dried on sterile filter paper and placed for cultivation on MS culture medium (Murashige and Skoog, 1962) with twice reduced macro-components and sucrose (medium MS/2). Plant material was cultured at 25 °C and 16-hour-photoperiod with subcultivations to the fresh media every 4 weeks. Growing shoots were cut into pieces containing an axillary bud and subcultured separately. Propagation rates were estimated in 2 months after the beginning of multiple shoot formation (6 months after starting *in vitro* culture) as a number of shoots formed in each cluster. Twenty clusters of each plant clone were used for calculations of statistical confidence.

Determination of the total phenolic content

The Folin-Ciocalteu method was used (Singleton and Rossi, 1965). The method is based on the reaction of compounds having a phenolic group with a Folin-Ciocalteu reagent to form blue-colored complexes whose optical density is measured at a wavelength of 760–765 nm. 200 µl of 10% aqueous Folin-Ciocalteu reagent was added to 100 µl of *Fittonia* ethanol extracts and the obtained solutions were stirred for 20–30 seconds. Then 800 µl of 7.5% aqueous Na_2CO_3 was added and the resulting solutions were left for 2 hours at room temperature; after that the optical density was measured. Ferulic acid was used as a standard substance so phenolic content was expressed as milligram of ferulic acid per gram of dry mass.

Determination of the total flavonoids content

The total flavonoids content was determined spectrophotometrically by measuring the optical density of red-colored complexes formed due to the reaction of flavonoids with aluminum

chloride (Pękal and Pyrzynska, 2014). $NaNO_2$ (360 µl of 5% solution) was added to 1 ml of ethanol extracts and left to incubate for 5 minutes following by adding 600 µl of 2% $AlCl_3$. The solutions were carefully stirred and left for 6 minutes. Then 600 µl of 1 M NaOH was added and incubated for 10 minutes. The optical density of formed red solutions was measured at a wavelength of 510 nm. Rutin was used as a standard substance so flavonoids content was expressed as milligram of rutin per gram of dry mass.

Statistics

Statistical processing of the results was performed using descriptive statistics. In the description of the content of phenolics and flavonoids, the values are presented in the form M ± m, where M is the mean, m is the standart error oth the mean; in the description of the *in vitro* multiplication coefficient m is the standard deviation.

Results and discussion

In our work on the maintenance and study of *in vitro* bank of the world flora we deal in most cases with a restricted quantity of initial plant material for starting *in vitro* culture. As a rule seed samples are used as starting explants but apical and axillary buds can be used as well if the living plants are available. Using shoot tips alternatively to seeds is preferential to maintain clone stability. In this work despite the little quantity of available *Fittonia* buds used as initial material the procedure of surface sterilization was quite successful. Surface sterilization efficiency reached 60–100% for nine of eleven plant clones that allowed obtaining 3–5 aseptic buds of each clone for further induction and growing of shoots. It is sufficient quantity to induce aseptic plants to be multiplied in *in vitro* bank for species conservation.

Hormone-free culture medium MS/2 was used to induce bud development and formation of *Fittonia* shoots as a common procedure for many other species maintained in our *in vitro* collection. Each aseptic bud formed slowly growing single shoot with 4–6 leaves within a period of approximately 3 months. The shoots were divided into the internodes with axillary buds and cultured on the same medium. Their growth was very slow and further development delayed while the basal parts of the stem were gradually enlarging along with some local necrosis of the tissues. Formation of multiple shoot clusters started in 4–5 months in all studied plant clones. Single shoots were cut from the clusters and transferred for rooting to the fresh culture medium of the same composition. Rooting took place in 1.0–1.5 months with the efficiency 90–95%.

Table 1 demonstrates the propagation efficiency of the studied *Fittonia* clones. In general they did not significantly differ according to their multiplication rates although the mean values of the coefficients in some cases were quite far from each other. At the same time significant differences in mean values were detected for clone #2 and #3; #2 and #4; #2 and #10; #2 and #11; clone #3 and #9. Plants #3 showed the lowest multiplication coefficients and growth rate. It should be mentioned that the rates of *in vitro* shoot multiplication of each plant clone in general corresponded to their growth rates in the soil (clones #2 and #9 were the most rapidly growing while clones #3 and #10 were very slowly growing in the soil).

Regular transfer of shoot clusters to the fresh MS/2 medium resulted in increasing multiplication efficiency. As an example, the total quantity of regenerated plants of the most rapidly growing clone #2 per 8-months cultivation reached more than 1,500 plants ready to be transferred to the soil. After 14-months cultivation the propagation rates decreased, and plants were maintained *in vitro* by routine subculturing of shoot tips.

Table 1 Some characteristics of the studied *Fittonia albivenis* (Lindl. ex Veitch) Brummitt clones *in vitro*

Clone	Leaf coloration	*In vitro* multiplication coefficient	Total phenolic content1	Total flavonoids content2
#1	Dark-green leaves and dark-red veins	9.6 ± 4.4	3.14 ±0.21	2.26 ±0.21
#2	Light-green leaves and white veins	14.7 ±2.6	3.88 ±0.30	2.07 ±0.11
#3	Dark-green leaves and rose veins	6.0 ±1.9	not studied	not studied
#4	Green leaves and crimson veins	9.6 ±2.4	2.60 ±0.62	2.13 ±0.20
#6	Dark-green leaves and light-rose veins	10.2 ±3.8	4.32 ±0.16	3.14 ±0.8
#8	Almost entirely pink	9.5 ±3.8	2.78 ±0.53	4.00 ±1.08
#9	Almost entirely dark-pink	11.5 ±3.4	3.70 ±0.80	3.33 ±0.35
#10	Bright-green leaves and crimson veins	7.2 ±2.6	3.97 ±0.43	2.96 ±0.30
#11	Dark-green leaves and dark-rose veins	8.3 ±3.4	3.41 ±0.56	3.02 ±0.37

Notes: 1 – expressed as mg ferulic acid/g of dry weight; 2 – expressed as mg rutin/g of dry weight

There are few publications concerning *in vitro* culture of *F. albivenis* focusing on elaboration of efficient methods of surface sterilization and mass propagation of this species (Chen et al., 1987; Yang, 2000; Pan et al., 2005; Wang et al., 2015) as well as studying chimeric stability with using different proliferation methods (Wang et al., 2015). In the earlier studies (Chen et al., 1987) different combinations of plant growth regulators (6-benzylaminopurine (BA), 1-Naphthaleneacetic acid (NAA), 2.4-Dichlorophenoxyacetic acid (2.4-D), and Indole-3-butyric acid (IBA) were used to study *F. albivenis in vitro* morphogenesis. In other work shoot clusters were induced on MS culture medium supplied with BA and NAA. BA concentrations ranged from 0.1 to 2.0 mg/l while studied NAA concentrations were from 0 to 1.0 mg/l. The highest multiplication coefficient (5.6 during 1-month cultivation) was observed on 1 mg/l BA, and it's increasing up to 2.0 mg/l did not induce higher multiplication rates and even lowered it (Yang, 2000). Pan et al. (2005) have reported that the effect of clump-germ induction from shoot tips was the best on the medium MS + 0.1 mg/l BA + 0.1 mg/l IBA.

In our research, a hormone-free medium was used for growing *F. albivenis* plants to ensure their clonal stability *in vitro* and to minimize any possible morphological and/or other types of variations. As our main aim was *in vitro* conservation the multiple shoot formation can be estimated as some type of "side effect" in the course of cultivation without exogenous plant growth regulators. The rates of multiplication were quite high as those on culture media supplied with growth regulators reported in the literature (Yang, 2000). Comparison of our

results and the other published data shows that application of plant growth regulators seems just to shorten the time period of *Fittonia in vitro* microclonal propagation but does not have any significant effect on multiplication coefficients.

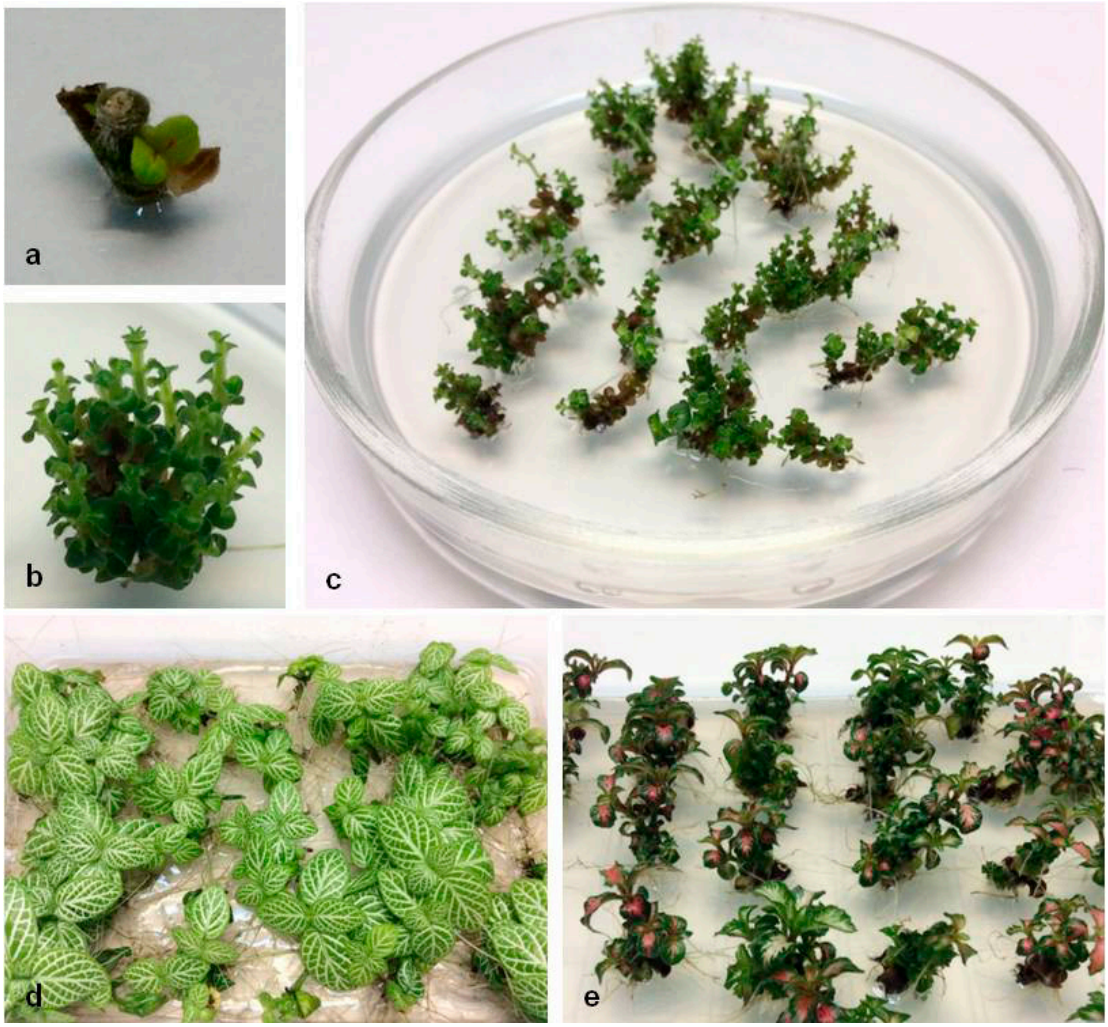


Figure 1 *Fittonia albivenis* multiple shoot formation on hormone-free MS/2 medium
a – a stem fragment with axillary bud used as initial explant; b and c – formation of shoot clusters; d and e – *in vitro* rooted plants of the clones #2 (d) and #9 (e) ready to be transferred to the soil

The results of the determination of total phenolics and flavonoids content are shown in Table 1 and Figure 2 and 3.

The total content of flavonoids and phenolics was about 2.0–4.5 mg (of rutin or ferulic acid, accordingly) on 1 g of the dry weight of plant (0.2–0.45% of dry weight). It is about 10–50 times less than these bioactive substances amount in known sources of polyphenols – e.g., total polyphenol content in ground coffee is about 52–57 mg gallic acid/g dry weight (Ignat et al., 2011). However, the data indicate the significant differences in the content of these bioactive

substances between plants with dark-green and light-green leaves which were cultivated *in vivo*. The dark-green plants (clone #1 *in vivo*) accumulate more phenolic substances and, especially, flavonoids than that with light-green leaves (clone #2 *in vivo*). These clones have also different reactions to the cultivation *in vitro*. In such conditions the content of the investigated substances did not change in dark-green leaves clone (clone #1 *in vitro*) whereas the significant increase in both phenolics and flavonoids amount was observed for light-green leaves one (clone #2 *in vitro*).

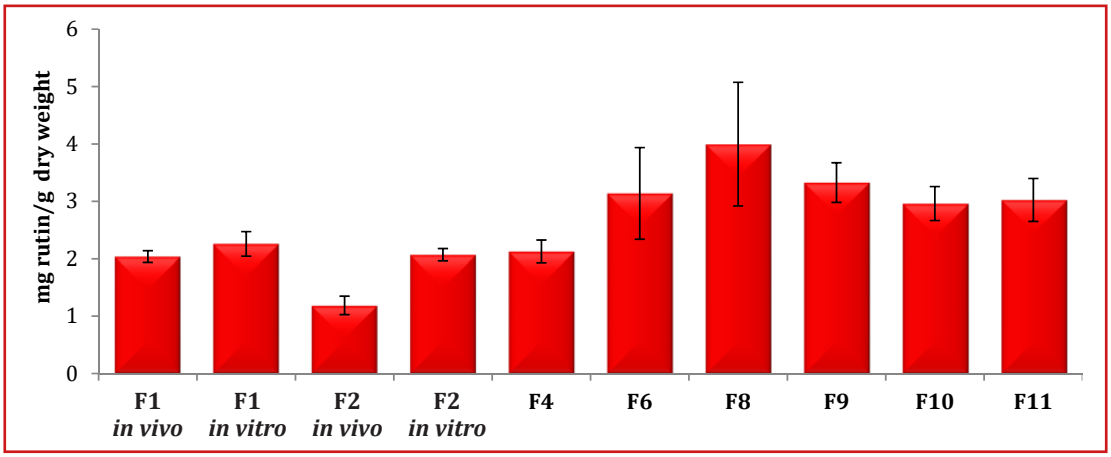


Figure 2 Total flavonoids content of *Fittonia* extracts of *in vitro* and *in vivo* cultivated plants (mean ± standart error of the mean)

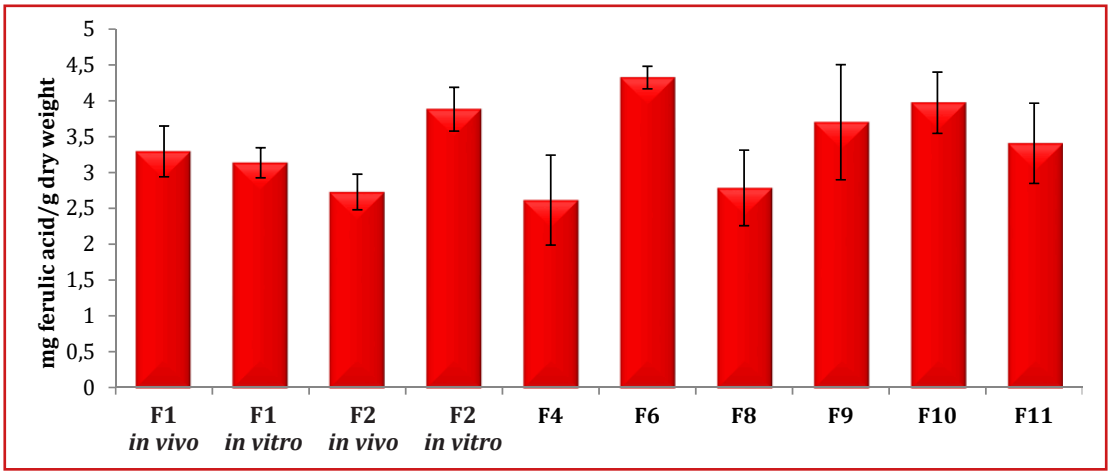


Figure 3 Total phenolics content of *Fittonia* extracts of *in vitro* and *in vivo* cultivated plants (mean ± standart error of the mean)

There is also a significant difference in the amount of flavonoids between different *Fittonia* clones cultivated under *in vitro* conditions. E.g., the flavonoids content was the largest for the clone #8 and it was twice as large as its amount in most of the studied clones (clones #1–4,

11). The amount of phenolic compounds in investigated clones differed to a lesser extent. It should also be noted that there was no significant correlation between the amount of studied bioactive compounds and the coloration of the leaves and/or their veins of the investigated plants.

Conclusions

As a result of our work, the effective system of microclonal propagation of *F. albiviens* on hormone-free media was elaborated. The rates of multiplication were quite high as those on culture media supplied with growth regulators reported in the literature (Yang and Zheng, 2000). It have been shown that application of plant growth regulators seems just to shorten the time period of *Fittonia in vitro* microclonal propagation but does not have any significant effect on multiplication coefficients. The total content of bioactive substances (flavonoids and phenolics) was about 0.2–0.45% of dry weight, that is significantly less than its amount in known sources of polyphenols (e.g., ground coffee). However, the data indicate the significant differences in the content of these bioactive substances between investigated clones as well as different reactions to the transfer to the *in vitro* conditions. For some clones the significant increase in both phenolics and flavonoids amount was observed after such transfer. The clones with highest productivity have been identified and will be used in further researches.

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ANTICANDIDAL PROPERTIES OF LAVANDIN ESSENTIAL OILS

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The hydrodistillated essential oils of lavandin cultivars (*Lavandula hybrid* Medic.) Inii and Rabat, which characterized by relatively high amount of monoterpenes were tested for anticandida effects against five pathogenic fungi using *in vitro* microbial growth inhibition assays; and were compared with that of a commercial antifungal reagent, decasanum and essential oil of *Lavandula angustifolia* Mill. (lavender oil). The oils of lavandins Inii and Rabat exhibited considerable antifungal activity against all tested *Candida* spp. Both the tested oils of lavandins Inii and Rabat demonstrated inhibitory action against all the *Candida* species at low concentrations (100 or 200 µL). The most sensitive were *C. pseudotropicalis*, *C. parapsilosis*, and *C. kefir*, some less sensitive were *C. curvata* and *C. tenuis*. However, they were moderately active against Decasanum. The anticandidal effect of essential oil of both lavandins can be due to the interaction of many of their compounds especially linalool, linalyl acetate, camphor, and 1.8-cineole which amount is prevalence. It can be concluded that the oils of lavandins Inii and Rabat have potential against pathogenic *Candida pseudotropicalis*, *C. parapsilosis*, *C. curvata*, *C. kefir*, and *C. tenuis*. Essential oils of lavandin cultivars Inii and Rabat are offered for further research as possible alternatives or additional therapeutic agents for candidiasis in diabetics, patients with periodontitis, immune and urogenital system disorders. Particularly promising may be Inii and Rabat essential oils in the treatment of patients with triazole and echinocandin resistant forms of candidiasis.

Keywords: lavandin, essential oil, anti-*Candida*

Introduction

Antimicrobial agents are some of the most widely used therapeutic drugs worldwide. 'A post-antibiotic era – in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century' – Dr. Keiji Fukuda, World Health Organisation (WHO) Assistant Director – General (WHO, 2014.). This provision remains relevant today. Although a considerable amount of research has gone into the study of

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the role of microbiocidal versus microbiostatic agents in the treatment of different infectious diseases, there is no accepted standard of practice (Finberg et al., 2004). This is especially true when searching for antifungal agents, since in the last few decades opportunistic fungal infections have been increasingly recognized as major causes of various human diseases, especially among immunocompromised patients (Li et al., 2015). Recognition of the side effects of chemically synthesized antimicrobial drugs on the host is generally accepted (Kumarasamy et al., 2010). In recent years, there has been a growing interest in researching and developing new anticandidal agents from various sources to combat their resistance. It remains possible to find anticandidal agents among substances of natural origin, in particular among substances synthesized by plant varieties created by breeding methods. The ability of plant oils to inhibit the growth of fungi is known (Kalemba and Kunicka, 2003; Elshafie et al., 2016; Vorobets and Rivis, 2017). For example, lavender essential oil inhibited both the growth and the activity of *Candida albicans* more efficiently than the main used drug clotrimazole (Bona et al., 2016). Lavandins are interspecies hybrids obtained as the results of natural and artificial selection of *Lavandula angustifolia* Mill. and *Lavandula latifolia* Medic. Some of them were created by Ukrainian breeders, and among them are lavandins 'Inii' and 'Rabat' (Svydenko, 2001). Phytochemical screening of both Inii and Rabat cultivars for active components revealed a high content of essential oils (Svydenko and Glushchenko, 2018). The aim of this study is to show the anticandidal activity of Inii and Rabat lavandins essential oil against the clinically important strains of yeasts.

Material and methodology

The plant material of lavandins Inii and Rabat was obtained from the experimental lots of the Institute of Rice of National Academy of Agrarian Sciences of Ukraine, Kherson region, Ukraine in July 2017–2018. Flowering tops of the plants were harvested manually, at the maximum flowering stage.

Extraction of essential oils and identification of their compounds

Aerial parts of lavandins Inii and Rabat were subjected to hydrodistillation in a Clevenger apparatus to obtain essential oil, and final yield of extraction is 1.8 and 1.7%, respectively. Volatile compounds were identified by gas chromatography (gas chromatograph Agilent Technology 6890N and GC/mass spectrometry (Svydenko, 2001). The essential oils thus obtained served as material for subsequent microbiological studies. These oils were stored at 4 °C in the dark until analyzed. Essential oils of lavandins Inii and Rabat were evaluated for antifungal activity against *Candida* spp.

Fungal Strains

In this study *Candida pseudotropicalis* (D-14-C), *C. parapsilosis* (D-35-C), *C. curvata* (D-15-C), *C. kefir* (D-30-C), and *C. tenuis* (D-45-C) were used as tested microorganisms. The strains used from the Microbial Culture Collection of the Department of Microbiology of Ivan Franko National University of Lviv.

Media, chemicals, and culture conditions

The standard agar diffusion method was used to determine the sensitivity of microorganisms to essential oils (EOs) in modification by the wells (Collins et al., 2004; Magaldi et al., 2004). The method is not considered to be most appropriate for the study of essential oils, but acceptable as a preliminary study (Kalemba and Kunicka, 2003; Balouiri et al., 2016), simple, and easy to reproduce. Nevertheless, an approximate minimum inhibitory concentration can be calculated for some microorganisms and antibiotics by comparing the inhibition zones (Nijs et al., 2003). Into 9 cm sterile Petri dishes poured up to 20 ml of the sterile medium of Sabouraud Dextrose Agar. After hardening dense nutrient medium on the surface was spread 0.3 ml suspension of microorganisms. The suspensions were prepared in separate samples in distilled sterilized water from two-day cultures of fungi of the genus *Candida*. The concentration of microorganisms in the suspension was determined according to the state standard of turbidity №5. After 20–30 minutes, the wells were made on the surface of the seeded medium with a 5 mm stamp (1–2 pc). After that, in the wells, the sample of essential oil in the amount of 100 or 200 μL was applied. Stuffed Petri dishes were incubated in a thermostat at +30 °C for 48 hours for all tested *Candida* spp. depending on the growth in the control medium. The diameter of zones of inhibition around the of test cultures was measured in mm after two days, including the diameter of the well.

Antifungal standard, and Substances

Decasanum and Essential Oil of Lavender (*Lavandula angustifolia* Mill., Lamiaceae family) were used as controls so as they were found to inhibit *C. albicans* and other *Candida* strains (D'Auria et al., 2005; Vorobets et al., 2018). When the strain showed no activity, the value considered was equal to zero or total growth inhibition. Values ranging from 6 to 8 mm and less were considered as non-active.

Statistical analysis

A bioassay was carried out in triplicate and repeated twice. Values mentioned are the mean of triplicate observations and standard deviation from the mean.

Results and discussion

Our results indicate that all *Candida* strains used in this study were completely sensitive to Inii and Rabat lavender essential oils (Table 1). Both the tested oils demonstrated inhibitory action against all the *Candida* species at low concentration (100 or 200 μL). The most sensitive were *C. pseudotropicalis*, *C. parapsilosis*, and *C. kefir*, some less sensitive were *C. curvata* and *C. tenuis*. The presence in the well of 200 μL of essential oil of Lavandin Inii and Lavandin Rabat caused total growth inhibition of strains *C. kefir*, *C. tenuis*, and *C. parapsilosis*. Significant inhibition of growth of strains *C. pseudotropicalis* and *C. curvata* was also observed.

Table 1 Antifungal activity of lavandins' inflorescences oil, zone of inhibition (mm)

Strains	*Candida pseudotropicalis*	*Candida curvata*	*Candida kefir*	*Candida parapsilosis*	*Candida tenuis*
Method of diffusion in agar in modifying the wells, 100 µL					
Lavandin Inii Essential Oil	40.2 ±0.84	30.6 ±0.89	40.4 ±0.55	40.0 ±0.71	30.8 ±0.84
Lavandin Rabat Essential Oil	40.2 ±0.45	40.4 ±0.55	40.4 ±0.55	40.0 ±0.71	39.8 ±0.84
Control1: *Lavandula angustifolia* Essential Oil	40.4 ±0.55	40.8 ±0.45	40.2 ±0.45	40.4 ±0.55	40.0 ±0.71
Control 2: Decasanum	9.4 ±0.89	8.2 ±0.84	11.8 ±1.30	11.0 ±0.71	7.2 ±0.45
Method of diffusion in agar in modifying the wells, 200 µL					
Lavandin Inii Essential Oil	40.4 ±0.55	40.1 ±0.71	total growth inhibition	total growth inhibition	total growth inhibition
Lavandin Rabat Essential Oil	40.2 ±0.45	40.2 ±0.71	total growth inhibition	total growth inhibition	total growth inhibition
Control1: *Lavandula angustifolia* Essential Oil	41.3 ±0.58	42.3 ±0.58	42.3 ±1.53	41.3 ±0.58	41.3 ±0.58
Control 2: Decasanum	12.3 ±0.23	11.3 ±0.32	12.±0.32	12.4 ±0.42	9.4 ±0.45

All *Candida* spp. which we used for the investigation of their resistances against lavandins oils are among very virulent. *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. kefir* are among the most common *Candida* species in human infections (Diekema et al., 2012; Lockhart et al., 2012). Several articles suggest that a significant proportion of patients with hematologic malignancies, especially patients with acute myelogenous leukemia undergoing induction chemotherapy, are colonized with *C. kefir*, with a substantial risk for subsequent bloodstream infection and colonization and infection follow a seasonal distribution, with higher rates during the warm months of the year. *C. glabrata*, generally considered to be a species with low virulence but with a higher mortality rate than *C. albicans* (Colombo et al., 2003; Pappas et al., 2004). *C. glabrata* isolates and all *C. krusei* isolates are resistant to fluconazole (Shorr et al., 2011; Andes et al., 2012; Kullberg and Arendrup, 2015). Patients with diabetes are at increased risk of developing vulvovaginal candidiasis due to non-albicans *Candida* spp. such as *C. glabrata* and *C. tropicalis* (Goswami et al., 2006). *C. glabrata* rapidly develop resistance to multiple drug classes, including triazoles and echinocandins (Vale-Silva and Sanglard, 2015; Healey et al., 2016); so, as *Candida parapsilosis* (Souza et al., 2015). The detection of new anticandidal agents is relevant. The oils of both studied lavandins inhibited growth of *C. kefir*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, so they possess biological activity. The biological activity of a compound *in vitro* and *in vivo* depends on its chemical structure. Our previous studies have shown that essential oils of both studied lavandins contain many groups of compounds. The anticandidal effect of essential oil of both lavandins can be due to the interaction of many of them.

Essential oils of lavandins could be of great interest in the biomedical field, opening new directions for the design of film-coated surfaces with anti-biofilm properties as it was proposed earlier for other EOs. The mass fraction of essential oil in inflorescences of lavandins during growing in the Kherson region varies in quite significant limits: from 0.96 to 2.2% of freshly harvested raw materials or from 2.2 to 5.5% from absolutely dry (Svydenko and Glushchenko, 2018). Thirty-three compounds were identified in the studied Lavandin Inii and forty-one in Lavandin Rabat essential oils by GC-MS analysis (Svydenko, 2001). The main components of essential oils of lavandins are given in Table 2.

Table 2 Main components of lavandins' inflorescences essential oil (% per 100 g of plant materials)

Lavandin Inii		**Lavandin Rabat**	
Compound	**%**	**Compound**	**%**
Linalool	57.79	**Linalool**	49.45
Linalylacetate	11.11	**Camphor**	15.40
1.8-cineole	7.38	**Linalylacetate**	8.68
Camphor	4.64	**1.8-cineole**	7.18
Lavandulyle acetate	2.67	**Borneol**	6.18
α-terpineol	2.63	**Lavandulyle acetate**	1.60
trans-Linalool oxide	2.61	**α-terpineol**	1.18
tri-enol acetate	1.94	**Lavandulole**	1.08
Borneol	1.26	**trans-Linalool oxide**	0.95
Lavandulole	0.99	**α-bisabolole**	0.64
2.6-dimetyl-3.7-oktadien-2.6-diol	0.97	**Hexyl acetate**	0.66
α-terpineol	0.74	**Hexyl butyrate**	0.66
caryophyllenoxide	0.71	**cis-Linalool oxide**	0.65
α-bisabolol	0.67	**Neryl acetate**	0.56
1-okten-3-ol	0.61	**β-farnesene**	0.50
Hexanol	0.56	**Limonene**	0.41
Geranilacetate	0.43	**Geranilacetate**	0.39
Terpinolene	0.39		
Neryl acetate	0.28		

Source: Svydenko and Glushchenko, 2018

An essential oil obtained from inflorescences of lavandins mainly contains terpenoids, which obviously can cause anti-candidiasis effect. The major volatile compounds were linalool, linalyl acetate, 1.8-cineole, and camphor. A total of four constituents, representing 80.9 and 80.7% from the total oil, were identified by GC/MS in Lavandin Inii and Lavandin Rabat, respectively. Fungicidal activity of lavender essential oil and linalool has been shown by other authors (Serra et al., 2018). Linalool is among the components of essential oils with the

revealed properties to prevent the formation of at least 80% regrowth of *Candida albicans*, after the biofilm was treated with antimicrobials (Serra et al., 2018). Anticandidal activity of the essential oils of thyme, pennyroyal, and lemon on different species of *Candida* including *C. albicans*, *C. krusei* and *C. glabrata*, which are isolated from patients who suffered from vulvovaginal candidiasis have been shown (Mahdavi and Esmailzadeh, 2009). *In vitro* study found that oil of *L. angustifolia* inhibited growth of *C. albicans*, as did it is component linalool (D'Auria et al., 2005). The efficacy of clinical use of linalool has been confirmed in several *Candida* strains (*C. albicans*, *C. krusei*, and *C. tropicalis*) that contaminated the oral cavity in patients with oral candidiasis (Dias et al., 2018). Its' constituents linalool and linalyl acetate are detectable in the blood five minutes after topical application and largely disappear from the blood within 90 minutes.

With these results, it is possible to establish that the anticandidal effects attributed to lavandins Inii and Rabat are due in part to the activity of essential oil components, which has also been linked to other species of Lamiaceae family.

Conclusions

The results of the present study demonstrated the important antifungal activity of essential oils of lavandin Inii and Rabat. Essential oils of both investigated lavandins are a promising use in healthcare decontamination against *Candida* spp. Both lavandins grow well in the South of Ukraine and possible to obtain good quality and quantity of raw material for use in pharmacy and medicine. This may help us to design highly specific antifungal drugs that avoid or minimize host side effects.

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BIOECOLOGICAL CHARACTERISTICS PLANTS OF *SALVIA VERTICILLATA* L. UNDER CONDITION FOREST-STEPPE ZONE OF UKRAINE

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The genus *Salvia* L., is one of the largest in the Lamiaceae family. *Salvia verticillata* L. is used for various diseases in folk medicine and as honey plants. The purpose of the work is to investigate the variability of bioecological and morphological characteristics of plants and seeds of the species *S. verticillata*, to select high-yield forms for use in Ukraine at the level of cultivars. Conducted comparative studies of biometric and morphological features, quantitative and qualitative characteristics seeds of the plants species *S. verticillata* were determined. It is established that the mass of 1,000 seeds of *S. verticillata* species is rather unstable during the years of storage. The mass of 1,000 seeds *S. verticillata* decreases significantly after three years of storage. It is established that in the first two years storage of seeds *S. verticillata* its germination is maintained at a fairly high level, in the third year it decreases three times, in 4–5 years the single seeds germinate, and for the sixth year the germination is equal to zero. Depending on the row spacing and the number of cuts, yield and productivity of the plants *S. verticillata* were determined both for fresh green mass and seeds. Biometric studies have shown the relationship between the row spacing and the number of generative shoots and inflorescence on plants, and the productivity of plantings. At a feeding area of 35 × 15 cm, the plants had thin stems (112.5 ±3.6 pc/m^2) and the lowest seed productivity (0.8 g/plant). At a feeding area of 55 × 15 cm, the plants had more thick stems (152.0 ±4.0 pc/m^2) and the highest seed productivity (up to 5 g/plant). Plants grown with rows of 55 cm had the highest productivity of green mass. It is proved that under conditions of the Forest-Steppe zone during the first flowering raw material of plants species *S. verticillata* for technical processing should be harvested, and during the second flowering – seeds should be collected. Seeds of *S. verticillata* during storage should be updated every 2–3 years. On the basis of gene pool of spicy aromatic plants and introductory populations in the NBS of NASU for the first time in Ukraine the cultivar *S. verticillata* – 'Musketeer' was created and was entered in the Register of Varieties of Ukraine in 2016.

Keywords: *Salvia verticillata*, bioecological characteristics, seeds, green mass, productivity

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Вступ

Рід *Salvia* L., що належить до підродини Nepetoideae, є одним з найбільших у родині Lamiaceae, і налічує близько 900 видів (Kovalova, 2009; Dolya et al., 2012). Велика кількість рослин серед видів роду є ароматичними, що робить їх важливими з комерційної точки зору. Представники роду містять значну кількість ефірної олії та використовуються як кулінарні трави і ароматизатори при виготовленні страв і напоїв (Lawrence, 1992), парфумерних виробів (Ahmed, 2011). Фітосировина видів роду *Salvia* та отримані з неї біологічно активні сполуки, займають важливе місце у виготовленні сучасних лікарських засобів та харчових продуктів. Деякі з них використовують як стимулятори і регулятори системи травлення з додатковими антисептичними властивостями. Інші види рослин *Salvia* відомі своїми жарознижуючими, знеболюючими та відхаркувальними властивостями і можуть бути застосовані в терапії грипу і простуди. Деякі види використовують у терапії психозів, депресій, неврозів у зв'язку з седативними властивостями відповідних відварів (Okuno, 2004; Naghibi, 2005). На вітчизняному фармацевтичному ринку, у фітотерапії та народній медицині поширені препарати, в яких використовується надземна частина деяких видів шавлії, переважно *S. officinalis* L. та *S. sclarea* L. Вони також використовуються у харчовій промисловості у складі пряних сумішей для ароматизації продуктів харчування (Korablova and Rakhmetov, 2012). Ряд видів *Salvia* використовують як декоративні рослини (Cervelli, 2011) та як фітосировину для лікарських засобів (Okuno, 2004; Naghibi, 2005; Kyseleva and Smyrnova, 2009).

Шавлію кільчасту *S. verticillata* L. використовують за різних захворювань у народній медицині та як медоносні рослини (Liu, 2011). Дослідження останніх років свідчать також про значний антидіабетичний ефект при використанні засобів із *S. verticillata* (Attitalla, 2011; Eidi, 2011; Semenchenko, 2014; Mahdizadeh, 2018). Однак, детальних досліджень культури в Україні не проводили. Саме тому, на нашу думку, *S. verticillata* потребує більш ретельного вивчення в сенсі її вирощування та комплексного використання у різних галузях промисловості.

Метою роботи було дослідити варіабельність біоекологічних та морфологічних особливостей рослин і насіння виду *S. verticillata*, відібрати високопродуктивні форми для використання в Україні на рівні сортів.

Матеріали та методи

Експериментальна робота виконана в період з 2012 по 2018 роки в умовах Лісостепу в Національному ботанічному саду ім. М. М. Гришка НАН України із залученням колекційних зразків та рослин *Salvia verticillata* L., інтродукованих з Тернополя та Скадовська (Rakhmetov et al., 2015).

Для вивчення морфобіологічних особливостей і господарсько-цінних ознак *Salvia verticillata* були застосовані польові досліди в комплексі з лабораторними дослідженнями. Вивчали особливості росту і розвитку, морфогенез вегетативних і репродуктивних органів, насінну продуктивність. В період вегетації щодекади

проводили фенологічні спостереження і біометричні вимірювання (Dospekhov, 1986). Насінну продуктивність визначали за методом Работнова (Rabotnov, 1950) і методичними вказівками з насіннєзнавства інтродуцентів (Emelianov, 1969). Облік врожаю проводили в період масового цвітіння рослин за методикою польових дослідів (Dospekhov, 1986). Сировину зрізували вручну і відразу зважували. Статистичний аналіз проводили за допомогою пакета Агрос (Martynov et al., 2000).

Результати та їх обговорення

S. verticillata – багаторічні трав'янисті рослини, у яких на зиму вся надземна частина відмирає, а весною з бруньок відновлення відростають нові пагони наступного року. У рослин *S. verticillata* головний пагін не виражений. Залежно від площі живлення, рослини мають від 1–2 до 7–9 пагонів різної довжини.

Фенологічні цикли росту і розвитку рослин проходять упродовж 8–9 місяців (Рисунок 1). Встановлено, що *S. verticillata* починає квітувати і плодоносити вже на першому році життя (Cervelli et al., 2011), незалежно від способу розмноження – насінням чи саджанцями. Більшість рослин *S. verticillata* у перший рік життя зацвітають досить пізно – у серпні.

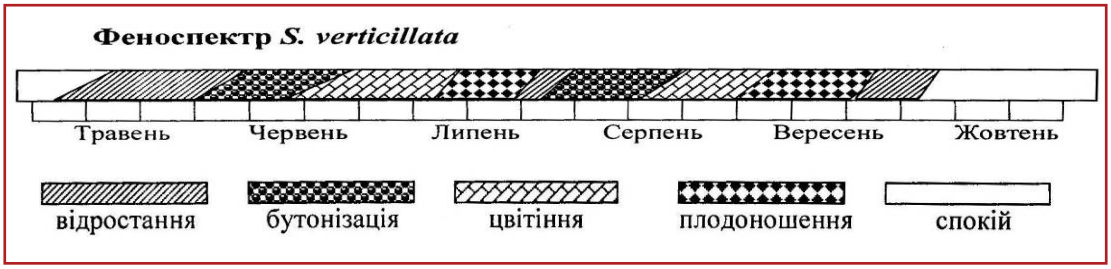


Рисунок 1 Феноспектр рослин виду *Salvia verticillata* L. на 2-й та подальші роки вегетації

Figure 1 Phase spectrum of plants species *Salvia verticillata* L. on the 2nd and subsequent years of growth

Фенологічні спостереження показують, що у наступні роки життя рослини *S. verticillata* схильні до тривалого цвітіння протягом вегетаційного сезону, і мають два максимуми (Рисунок 2a). Перший максимум – у середині червня. Рослини *S. verticillata* характеризуються високою регенераційною здатністю. Через деякий час після зрізування рослин і збору насіння (Рисунок 2c, d) відростають нові квітконоси і у кінці серпня рослини знову починають квітувати (Cervelli et al., 2011).

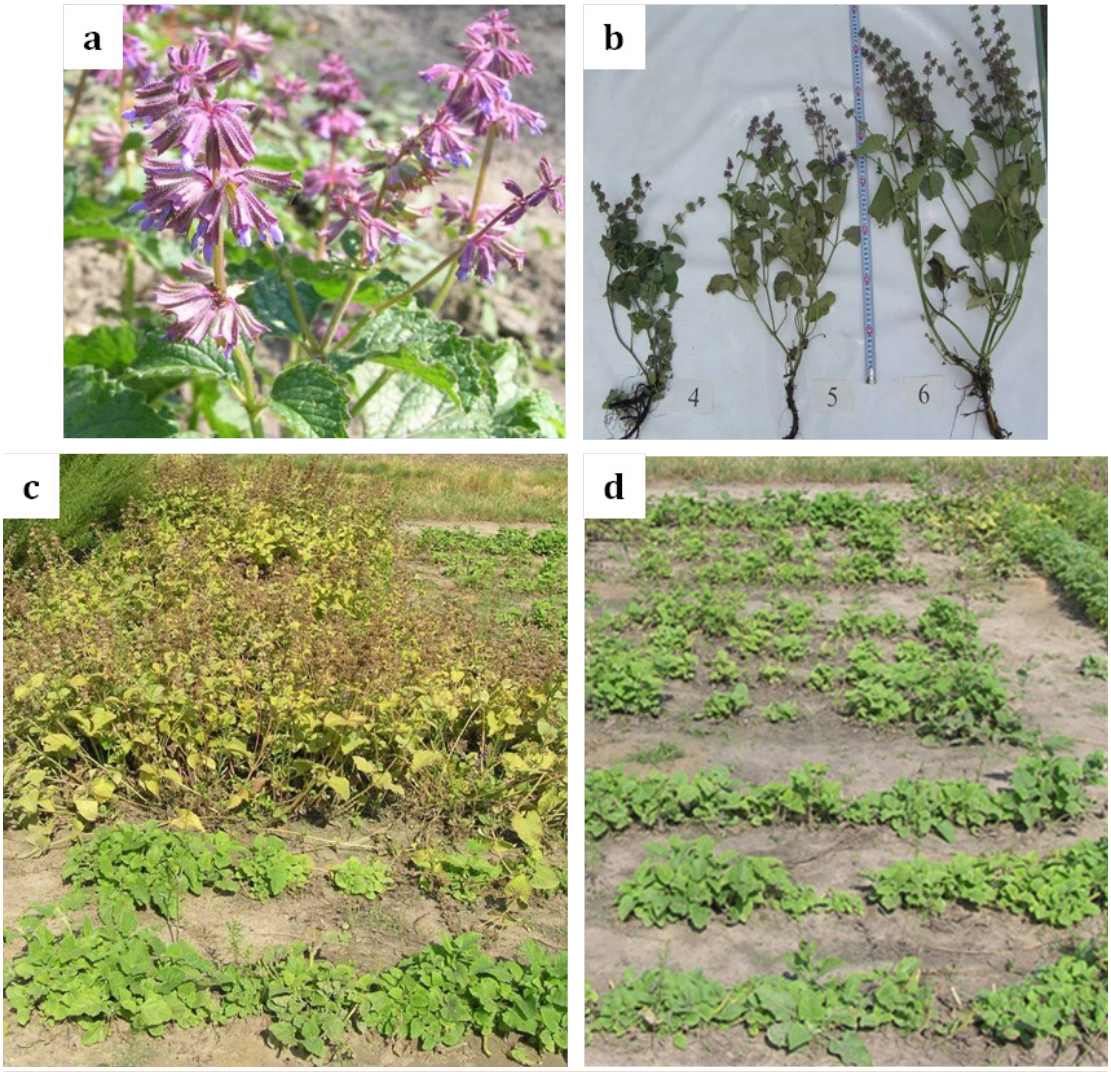


Рисунок 2 Рослини
a – *Salvia verticillata* L. у фазі цвітіння; b – висота рослин залежно від площі живлення (35, 45, 55 см); c – фаза достигання насіння; d – відростання рослин після скошування

Figure 2 Plants
a – *Salvia verticillata* L. in the flowering phase; b – plant height depending on the width of the row spacing (35 cm, 45 cm and 55 cm); c – the phase of the seeds maturation; d – plant regrowth after cutting

Біометричні та фенологічні спостереження дозволили встановити адаптаційні можливості рослин, основні закономірності сезонної ритміки росту та розвитку, характерні особливості онтогенезу. Проведено порівняльні дослідження біометричних показників і морфологічних особливостей, визначено кількісні та якісні характеристики насіння рослин видів *S. verticillata* власної репродукції (Рисунок 3).

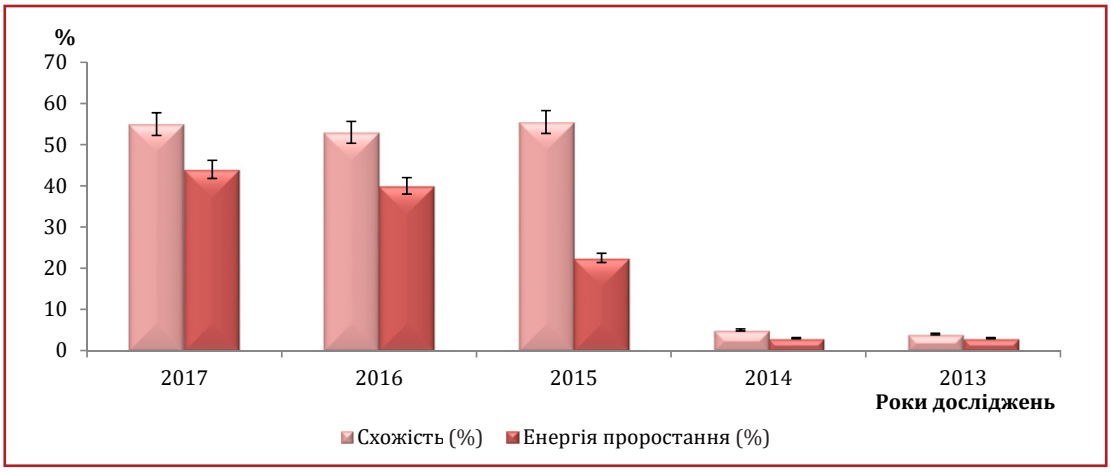


Рисунок 3 Вплив тривалості зберігання насіння *Salvia verticillata* L. на його схожість та енергію проростання

Figure 3 Influence of the storage period seed *Salvia verticillata* L. on its germination and germination energy

Встановлено, що маса 1000 шт. насінин виду *S. verticillata* є досить нестабільною за роками зберігання. У *S. verticillata* маса 1000 насінин після трьох років зберігання значно зменшується (Dolya et al., 2010). Встановлено, що у перші два роки зберігання насіння *S. verticillata* його схожість тримається на досить високому рівні, на третій рік знижується втричі, на 4–5-й роки проростають поодинокі насінини, а вже на шостий рік схожість дорівнює нулю. Одержані результати свідчать про те, що насіння *S. verticillata* при зберіганні потрібно оновлювати кожні 2–3 роки, що узгоджується з даними Kovalova et al. (2009).

У лабораторних умовах насіння *S. verticillata* починає проростати на 3–4 добу за температури +30 °С. У ґрунті насіння проростає упродовж 18–22 діб. Так при сівбі у середині квітня сходи з'являлися на початку травня, а при сівбі у першій декаді травня – в останній декаді травня.

На дослідних ділянках шавлії кільчастої, закладених насінним та посадковим матеріалом, для встановлення особливостей морфогенетичного потенціалу виду визначали вплив площі живлення на біометричні показники рослин в процесі онтогенезу (Рисунок 2b). Під час біометричних вимірювань рослин *S. verticillata* враховували пагони довжиною до та понад 50 см. Біометричні дослідження продемонстрували залежність між площею живлення та кількістю генеративних пагонів і кільчаток на рослинах. При площі живлення 35 × 15 см рослини мали більш тонкі стебла, проте кількість і коротких, і довгих стебел була вищою ніж за площі 45 × 15 см (Таблиця 1). Незначну перевагу за біометричними показниками мають рослини на варіанті з міжряддям 55 × 15 см. Вищу продуктивність одержано за рахунок збільшення кількості довгих пагонів на одиниці площі. Встановлено, що загальна кількість пагонів була найменшою за площі живлення 45 × 15 см, що цілком узгоджується з показниками урожайності та продуктивності рослин *S. verticillata*

(Cervelli et al., 2011, Semenchenko et al., 2014). Біометричні показники генеративних пагонів у різних варіантах мало різняться між собою.

Таблиця 1 Кількість та довжина пагонів *Salvia verticillata* L. залежно від площі живлення
Table 1 Number and length of *Salvia verticillata* L. shoots depending on row spacing

Ширина міжряддя	Повторність	Кількість пагонів (шт./м^2)		
		загальна	до 50 см	>50 см
55 см	1	163,0 ±4,1	54,0 ±2,9	109,0 ±3,5
	2	141,0 ±3,9	41,0 ±2,4	100,0 ±3,5
	Середнє	152,0 ±4,0	47,5 ±2,6	104,5 ±3,5
45 см	1	85,0 ±3,1	29,0 ±1,5	56,0 ±2,9
	2	97,0 ±3,4	40,0 ±2,4	57,0 ±2,9
	Середнє	91,0 ±3,3	34,5 ±1,8	56,5 ±2,9
35 см	1	122,0 ±3,7	56,0 ±2,9	66,0 ±3,1
	2	103,0 ±3,5	38,0 ±2,1	65,0 ±3,1
	Середнє	112,5 ±3,6	47,0 ±2,5	65,5 ±3,1

Урожайність і продуктивність надземної маси рослин *S. verticillata* визначали залежно від площі живлення та кількості скошувань як на фітосировину з гарним біохімічним складом (Korablova et al., 2018), так і для насіння. Після першого літнього скошування рослини добре регенерують і знову проходять фази відростання, бутонізації, цвітіння та дають повноцінне дозріле насіння (Рисунок 4).

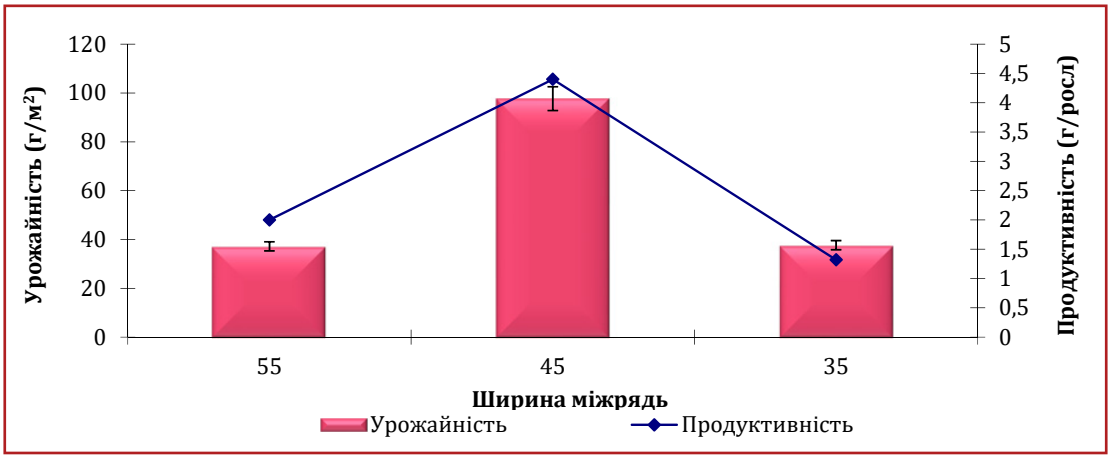


Рисунок 4 Урожайність насіння рослин *Salvia verticillata* L. та продуктивність залежно від площі живлення

Figure 4 Yield of seeds plants *Salvia verticillata* L. and their productivity depending on row spacing

Найвищі показники продуктивності зеленої маси мали рослини, вирощені з міжряддями 55 см. Така ж залежність зберігалася під час другого скошування, але урожайність була на 30–40 % нижче. Вищу продуктивність одержано за рахунок збільшення кількості і маси великих пагонів на одиниці площі. Продуктивність насіння була вищою за міжрядь у 45 см.

Створено селекційний розсадник перспективних форм *S. verticillata*, де досліджували формову різноманітність та проводили оцінку продуктивності та декоративних якостей рослин. Аналізували стійкість рослин до ушкодження хворобами та шкідниками. Встановлено, що рослини *S. verticillata* стійкі до шкідників, проте можуть уражатися борошнистою росою. Біометричні спостереження дозволили встановити адаптаційні можливості, основні закономірності сезонної ритміки росту та розвитку, характерні особливості онтогенезу видів роду *Salvia*, відібрати перспективні форми рослин. У 2013 році на базі генофонду пряноароматичних рослин та інтродукційних популяцій у НБС НАНУ вперше в Україні створено сорт *S. verticillata* – Мушкетер, який занесено до Реєстру сортів України у 2016 р. (Derzhavnyi reiestr..., 2018).

За даними досліджень опрацьовано та затверджено в установленому порядку Методику проведення експертизи сортів виду *S. verticillata* на відмінність, однорідність і стабільність. У 2016 році *S. verticillata* було включено до ТУ України «Зелень пряноароматичних рослин (свіжа і сушена) та їх насіння», які регламентують використання рослинної сировини у харчовій промисловості.

Висновки

Встановлено, що в умовах Правобережного Лісостепу рослини *S. verticillata* проходять повний цикл розвитку, дають життєздатне насіння і стабільно здатні до насінного розмноження. Результати досліджень схожості, енергії проростання та маси 1000 шт. насінин залежно від тривалості зберігання свідчать про те, що насіння *S. verticillata* при зберіганні потрібно оновлювати кожні 2–3 роки.

Таким чином, отримані дані з вивчення біоморфологічних особливостей інтродуцентів роду *Salvia* у нових умовах мають як наукове, так і практичне значення для добору господарсько-цінних видів, створення нових сортів та збагачення флори України.

Доведено, що сировину рослин виду *S. verticillata* в умовах Правобережного Лісостепу доцільно заготовляти на технічну переробку під час першого цвітіння, а під час другого – збирати насіння. При роздільному вирощуванні рослин необхідно сівбу на зелену масу проводити з міжряддями 55 см, а на насінниках – доцільно скоротити міжряддя до 45 см.

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PROSPECTS OF USING SPICES IN TECHNOLOGY SOFT-RIPENED GOAT CHEESE

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The problem of balanced nutrition is one of the most important social problems in our time. In the context of changes in population nutrition and protein deficits, increasing attention is being paid to increasing the use of protein resources for nutritional purposes, including the creation of new products. The value of such products is determined not only by the amount of protein, but also by its quality. Goat's milk is rich in casein and albumin, which contain important amino acids for the human body. Soft-ripened cheese based on goat milk is characterized with high nutrition and biological value, as well as, nutrient availability. Peculiarities of fractional composition of casein of goat milk determine the necessity of searching for methods of adjusting the technological properties of milk during production of cheese. The studies have substantiated feasibility of using spices – turmeric, ginger and fenugreek in the technology of soft-ripened cheese based on goat milk. It is proved, that adding these spices in the dry state with the particles sized up to 0.4 mm to a normalized mixture before thermal conditioning in amount of 1.0 ±0.1% reduces the indicator of active acidity of normalized mixtures and amount of whey, excreted in the course of processing, by an average of 0.2 pH, provides formation of a more dense clot, speeds up the excretion of whey and increases the product yield by an average of (5.0 ±0.2%). The formation of a dense clot in the control sample occurred within 60 min, while in the model specimens containing 1% of spices in the dry state – in 45 minutes and hydrated – in 50 minutes. Such a technological effect is apparently associated with adsorption of the components of spices on casein micelle surface and, as a consequence, with reducing their surface potential and subsequent aggregation. The quality indices of goat's soft cheese made from goat's milk with spices for at least 5 days at temperature (4 ±2 °C) and relative humidity (85 ±5%) were established.

Keywords: goat milk, soft cheese, spices, turmeric, ginger, fenugreek

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Вступ

Проблема збалансованого харчування є однією з найважливіших соціальних проблем в наш час. Відповідно до змін у структурі харчування населення й дефіцитом білка все більша увага приділяється питанню збільшення використання білкових ресурсів на харчові цілі, у тому числі створенню нових продуктів. Повноцінність таких продуктів визначається не тільки кількістю білка, але і його якістю. У концепції правильного харчування першорядна роль відводиться молочним продуктам. Це повною мірою стосується і сирів м'яких, що є джерелом повноцінного та легкозасвоюваного білка.

Завдяки високій харчовій, біологічній цінності і фізіологічній активності продукти переробки козиного молока можуть бути використані у дієтичному та функціональному харчуванні для усіх категорій населення (Haenlein, 2004, Vargas et al., 2008).

Особливий інтерес представляють гіпоалергенні та біологічні властивості козиного молока. У порівнянні з коров'ячим козине молоко багатше на казеїн і альбумін, які містять важливі для організму людини амінокислоти. У фракційному складі білків козиного молока є знижений вміст αS1 – казеїну (що сприяє гіпоалергенності), підвищений вміст β-казеїну (що впливає на швидкість утворення в шлунку дрібнодисперсного легкозасвоюваного згустку) та висока ступінь дисперсності жирової фази за рахунок меншого розміру жирових міцел. Середній розмір жирових кульок козячого молока, за дослідженнями вчених, складає у середньому 2 мкм (Protasova, 2011) та 5,33–6,14 мкм (Felik et al., 2012), для коров'ячого молока цей показник становить 21,1–31,2 мкм (Ryzhkova, 2017). Жирові міцели меншого розміру рівномірно розташовані по всій масі козиного молока, що характеризує його гомогенність. Ліпіди козиного молока містять значно більше довголанцюгових кислот (С 4 : 0 – С 12 : 0), аніж ліпіди коров'ячого молока. Завдяки цьому відбувається краще перетравлювання ліпідів козиного молока, оскільки ліпаза діє на етерні зв'язки таких жирних кислот з більшою активністю. В козиному молоці у порівнянні з коров'ячим менший вміст ненасичених моно- і більший поліненасичених жирних кислот, у тому числі незамінних – лінолевої та ліноленової (Andrusenko and Omelianchuk, 2008). Високий вміст ненасичених жирних кислот забезпечує нижчу точку плавлення жирів козиного молока. Окрім того, ненасичені жирні кислоти сприяють прояву фізіологічної дії деяких вітамінів (тіамін, аскорбінова кислота) (Symonenko and Dymytrieva, 2015).

Засвоюваність козиного молока та молочних продуктів з нього досить висока – 94–98 % (Kunizhev and Andrusenko, 2000; Ostroumova, 2005). Особливість виробництва сирів з козиного молока пов'язана з його меншою здатністю до згортання ферментами, що пояснює фракційний склад білка (мало α-фракцій (10–15 %), та більш низька титрована кислотність (Ballabio et al., 2001; Bevilacqua et al., 2011; Clark and Sherbon, 2014). Особливості складу козиного молока визначають його технологічні властивості – більш повільну порівняно з коров'ячим молоком здатність до зсідання під дією сичужного ферменту, утворення ніжного згустку та, як наслідок, підвищені втрати сухих речовин під час оброблення згустку. Тому актуальним напрямом

наукових досліджень є корегування технологічних властивостей козиного молока з метою підвищення економічної ефективності виробництва сирів на його основі.

Зважаючи на це, актуальним є удосконалення технології м'яких сирів на основі козиного молока із застосуванням функціонально-технологічних інгредієнтів рослинного походження, що дозволять не тільки цілеспрямовано змінювати його властивості, а й можуть слугувати додатковим джерелом біологічно активних речовин (Teterina et al., 2015; Kochubei-Lytvynenko et al., 2017).

Перспективним в цьому напрямі є використання прянощів. Прянощі у своєму складі містять комплекс біологічно активних речовин, здатних впливати на технологічні властивості. На нашу думку, завдяки антиоксидантним та антимікробним властивостям прянощі можна застосовувати для збереження та поліпшення технологічних, смакових та якісних характеристик м'яких сирів.

Біологічна активність прянощів пояснюється наявністю в них фенольних сполук. Значна кількість природних антиоксидантів фенольного класу утворюється внаслідок метаболізму рослин (Dillard and German, 2000). Такі сполуки є ефективними інгібіторами вільно-радикальних процесів, мають широке використання внаслідок антимікробних властивостей по відношенню до збудників захворювань харчового походження.

Метою досліджень є розробка ресурсозаощаджуючої технології сирів м'яких продовженого терміну зберігання на основі козиного молока з прянощами.

Для досягнення поставленої мети були визначені наступні завдання: обґрунтувати вибір функціонально-технологічних інгредієнтів для подальшого застосовування у складі м'яких сирів; визначити спосіб підготовки та раціональну кількість введення прянощів до молочної основи; дослідити вплив прянощів на показник активної кислотності молока, процес сичужного зсідання та оброблення згустку; визначити якість сирів м'яких з козиного молока з прянощами за органолептичними властивостями та показником активної кислотності; дослідити динаміку активної кислотності продуктів у процесі зберігання.

Матеріали і методи

Для дослідження були обрані наступні види прянощів: гуньба сінна (*Trigonella foenum-graecum* L.), імбир лікарський ((*Zingiber officinale* Poscoe), куркума ароматна (*Curcuma aromatica* Salisb.).

У якості об'єктів дослідження було обрано модельні зразки сичужного м'якого сиру з козиного молока з імбирем, гуньбою та куркумою (вміст прянощів від 0,2 до 1,0 % з інтервалом у 0,2 %).

З метою визначення раціонального способу підготовки обрані прянощі до молочної основи вносили у сухому меленому вигляді або після попередньої гідратації (змішування з молоком, співвідношення прянощі : гідратуюче середовище як 1 : 2, підігрів до температури 45–50 °С з подальшим витримуванням протягом 15 хвилин),

суміш козиного молока та прянощів піддавалась пастеризації при 76 °С упродовж 20–25 с, охолодженню до (35 ±2) °С та фільтруванню.

З метою дослідження впливу прянощів на функціонально-технологічні властивості м'яких сирів з козиного молока проводилися дослідження їх органолептичних та фізико-хімічних показників. У дослідних зразках визначали показники активної кислотності на етапах виробництва та під час зберігання, показники вологоутримуючої здатності, вмісту вологи та сухих речовин (метод висушування), вихід готового продукту (г із 1000 г молока).

Вологоутримуюча здатність (ВУЗ) м'якого сиру визначалась гравіметричним методом по Грау-Хамму у модифікації А. А. Алексєєва, що ґрунтується на визначенні кількості вологи, яка виділяється з продукту при пресуванні під вагою 5000 г на скляній пластині розміром 110 × 110 × 5 мм і яка вбирається фільтрувальним папером. Наважку сиру 0,3000 ±0,0005 г вміщують на кружальце з поліетиленової плівки діаметром 40 мм, на неї накладається фільтр такого ж діаметру. Плівку з наважкою та фільтром розміщують на скляній пластині та покривають скляною пластиною такого ж розміру і на неї встановлюють гирю масою 0,5 кг та піддають пресуванню упродовж 7 хвилин. Після цього фільтр з наважкою звільняють від навантаження і пластини. Залишки сиру разом з поліетиленом знімають з фільтрувального паперу та зважують. Вологоутримуючу здатність визначають за формулою:

$$ВУЗ = 100\ (а - б)/а \quad (1)$$

де: *ВУЗ* – вологоутримуюча здатність сиру, %; *а* – кількість вологи в наважці сиру, г; *б* – кількість вологи, що виділилася з наважки сиру, г

$$а = 0{,}3\ В_{сиру}/100 \quad (2)$$

де: 0,3 – наважка сиру, г; $В_{сиру}$ – масова частка вологи у сирі, %

Результати та їх обговорення

Прянощі є джерелом ряду біологічно активних речовин, що виявляють фізіологічну активність, сприяють покращенню травлення, виявляють антиоксидантні та антимікробні властивості.

Зокрема, насіння гуньби містить 20–30 % білків і до 4 % пептидів. Пептиди, що містяться в насінні, мають катіонну природу і проявляють виражену антимікробну та фунгіцидну активність. Насіння містить до 45–50 % вуглеводів, які характеризуються вираженим накопиченням галактуронової кислоти (більше 60 % від вмісту). Вміст ліпідів становить 7–10 %, містить 65 % нейтральних ліпідів (із них на частку похідних олеїнової кислоти припадає понад 17 %), 28 % гліколіпідів і 7 % фосфоліпідів, що узгоджується з даними Khole et al., 2014. Завдяки багатому хімічному складу гуньба володіє гіпоглікемічними, протимікробними, протизапальними, анаболічними, антикоагулянтними властивостями.

Коренеплоди куркуми містять 3–5 % ефірної олії. До складу прянощів входять: білки, крохмаль, смола, гуміарабік та ліпіди. Із мінеральних речовин міститься кальцій, залізо, фосфор та йод. У куркумі присутні вітаміни групи В – B_1, B_2, B_3, а також аскорбінова кислота. Яскравий жовто-помаранчевий колір обумовлений добре розчинним в жирі помаранчевим барвником куркуміном – поліфенолом $C_{21}H_{20}O_6$, який міститься в прянощі у вигляді альфа-йод-куркуміну. Куркума – сильний антиоксидант, який можна прирівняти за дією до вітамінів С і Е, виявляє протизапальні властивості.

Коренеплоди імбиру містять до 4 % ефірної олії, головною складовою частиною якої є цінгіберен (до 70 %), він надає продукту характерного аромату, а також багато білків, вуглеводів (переважно у формі крохмалю), клітковини. Згідно з даними Borysova and Makarova (2013) мінеральний склад представлений солями магнію, фосфору, кальцію, заліза, натрію, калію та цинку. Багатий імбир вітамінами С, B_1, B_2 і А.

Кількісний вміст окремих біологічно активних речовин вищезазначених прянощів наведено у Таблиці 1.

Таблиця 1 Порівняльна характеристика вмісту біологічно активних речовин окремих прянощів (Орловська, 2011)
Table 1 Comparative characteristics of the content of biologically active substances of individual spices (Orlovskaia, 2011)

Група біологічно активних речовин	Найменування речовини	Гуньба сінна	Імбир	Куркума
Органічні кислоти	Кавова кислота	24,7	2,72	17,86
	Хлорогенова кислота	3,54	–	–
	Неохлорогенова кислота	1,46	–	8,17
	Цикорієва кислота	–	7,45	–
	Ферулова кислота	–	0,65	3,28
Флавоноїди	Лютеолін-7-глікозид	1,34	1,58	–
	Дигідрокверцетин	10,81	–	–
	Рутин	2,34	–	5,46
	Вітексин	9,22	–	6,89
	Гіперозид	1,35	–	3,57
	Гесперидин	1,05	2,09	–
	Лютеолін	–	0,44	–
	Кверцетин	–	2,33	3,7
	Апігенін	–	0,62	–
	Кемпферол	–	0,08	–
Фенольні кислоти	Саліцилова кислота	2,52		–
	Галова кислота	11,73	24,12	11,49
Кумарини	Скополетін	18,49	–	–
	Умбелліферон	2,35	–	–
Дубильні речовини		–	5,61	–

З огляду на те, що обрані прянощі – гуньба сінна, імбир та куркума містять комплекс біологічно активних речовин різної хімічної природи, їх використання у технології сирів м'яких з козиного молока напевне, матиме і технологічний ефект. Дослідниками Haenlein (2004), Khole et al. (2014) вивчались антиоксидантні властивості прянощів. Orlovskaia (2011) відмічає не тільки антиоксидантні, а й антимікробні властивості, тоді як вплив активних складових прянощів на фізико-хімічні та технологічні властивості козячого молока не вивчались.

Тому у подальших дослідженнях нами визначався вплив способу підготовки прянощів – імбиру, куркуми та гуньби сінної на показник активної кислотності козиного молока, процес сичужного згортання, вихід та стабільність під час зберігання сиру м'якого з козиного молока.

Визначено активну кислотність козиного молока з прянощами у сухому та гідратованому вигляді до пастеризації. Спостерігалась тенденція зниження значення активної кислотності суміші зі збільшенням вмісту прянощів (Рисунок 1).

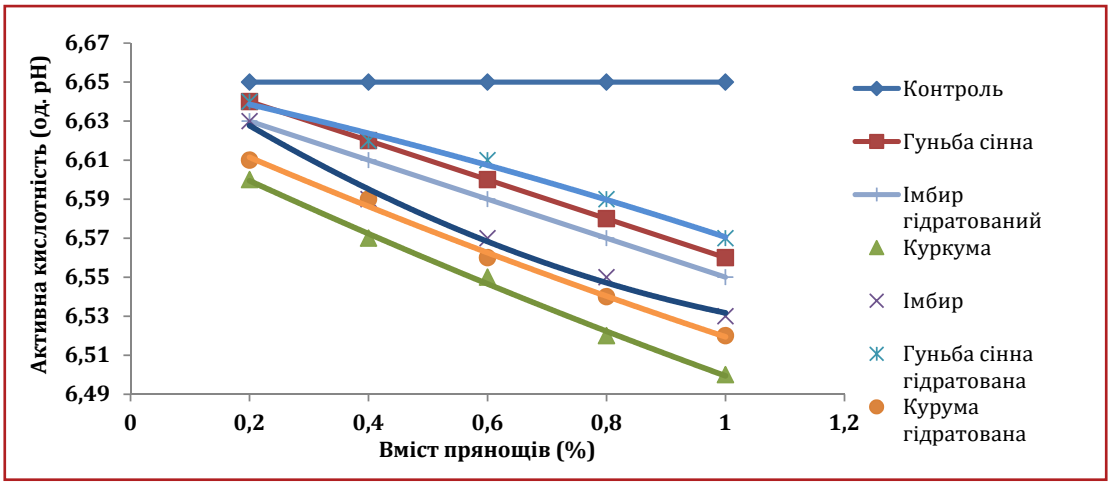


Рисунок 1 Активна кислотність козиного молока залежно від виду та кількості внесених прянощів
Figure 1 The active acidity of goat milk, depending on the type and amount of spices used

Найнижче значення активної кислотності спостерігалось у зразку з додаванням куркуми, що пояснюється вмістом органічних та фенольних кислот, а саме кавової, неохлорогенової, ферулової та галової. Найменший вплив на показник активної кислотності козиного молока має вміст гуньби. Дещо вище значення активної кислотності зразків з додаванням прянощів після гідратації пояснюється можливою деструкцією термонестійких сполук (зокрема, карбонової кислоти) під час теплового оброблення.

На етапі розрізання та постановки сирного зерна контролювалась активна кислотність сироватки, помітна була тенденція значення активної кислотності при збільшенні кількості введення прянощів (Рисунок 2) зі збереженням закономірностей

динаміки показника активної кислотності в модельних зразках козиного молока з прянощами.

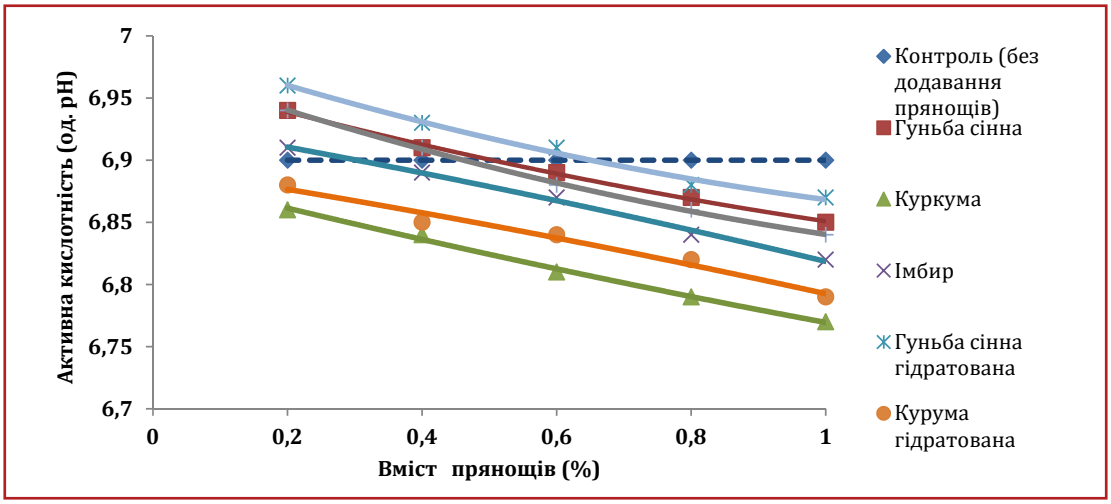


Рисунок 2 Вплив виду та кількості прянощів на активну кислотність сироватки з козиного молока під час виробництва сиру м'якого

Figure 2 Influence of species and number of spices on the active acidity of whey of wheat milk during the production of soft cheese

Утворення щільного згустку в контрольному зразку відбулося упродовж 60 хв, в той час як у модельних зразках з вмістом 1 % прянощів у сухому вигляді – за 45 хвилин та у гідратованому – за 50 хвилин.

Під час розрізання сирний згусток з вмістом прянощів у сухому вигляді в кількості 1 % мав правильний злам, більш інтенсивне відділення сироватки, що мала прозорий колір у порівнянні з контрольним зразком.

На етапі відділення сироватки достатньо щільне зерно утворювалось в модельних зразках з вмістом 1 % прянощів у сухому вигляді за 25 хвилин, гідратованому за 30 та у контрольному зразку за 35 хвилин. Таким чином встановлено, що внесення прянощів у кількості 1 % в сухому вигляді дозволить дещо скоротити час на технологічні операції по сквашуванню та постановці сирного зерна, забезпечить отримання більш щільного згустку з меншими втратами жиру та білку в сироватку.

В готовому продукті було визначено вологоутримуючу здатність та вихід продукту. Показники свідчать про те, що в модельних зразках з вмістом прянощів в сухому вигляді вміст сухих речовин, вихід продукту і відповідно вологоутримуюча здатність вищі у порівнянні з контрольним зразком (Рисунок 3).

Такий ефект можна пояснити адсорбцією складових прянощів на поверхні міцел казеїну козиного молока, що на думку авторів, сприяє зниженню поверхневого заряду та частковій агрегації казеїнових міцел, що забезпечуватиме покращення здатності до сичужного зсідання та сприятиме утворенню більш міцного згустку.

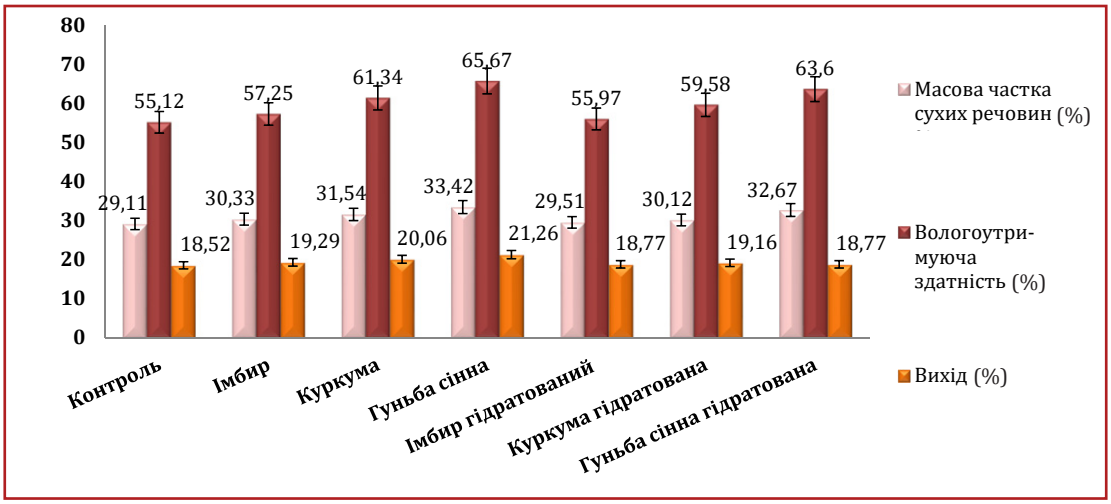


Рисунок 3 Масова частка сухої речовини, вологоутримуюча здатність та вихід сиру м'якого з козиного молока з прянощами (кількість прянощів – 1,0 %)

Figure 3 Mass fraction of dry matter, moisture retaining ability and yield of soft cheese from goat's milk with spices (1.0% spice content)

Була проведена органолептична оцінка модельних зразків сирів м'яких з козячого молока з прянощами. Визначено раціональну кількість введення прянощів – 1,0 %, зразки з таким вмістом прянощів отримали найвищу оцінку за органолептичними показниками. Сири м'які з прянощами мали смак та аромат, властивий для цієї групи продуктів з приємним вираженим пряним присмаком. Колір – рівномірний за усією масою, для сиру з куркумою – із злегка жовтуватим відтінком, для сиру з гуньбою та імбирем – із сіро-кремовим відтінком, що гармоніював із смаковими характеристиками. Консистенція – однорідна, в міру щільна, без ознак відділення сироватки.

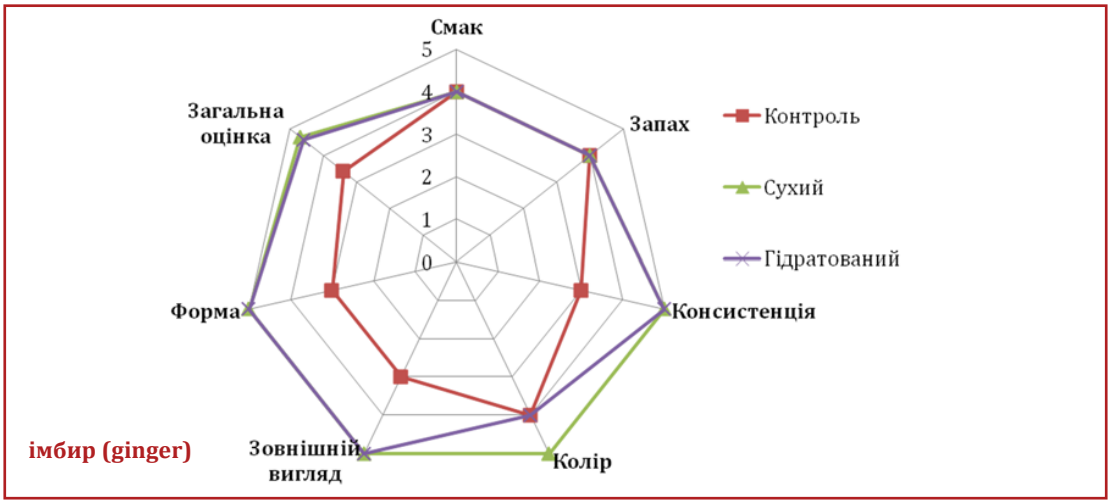


Рисунок 4А Профілограми органолептичних властивостей сиру м'якого з козиного молока з прянощами

Figure 4A Profilograms of organoleptic properties of soft cheese from goat's milk with spices

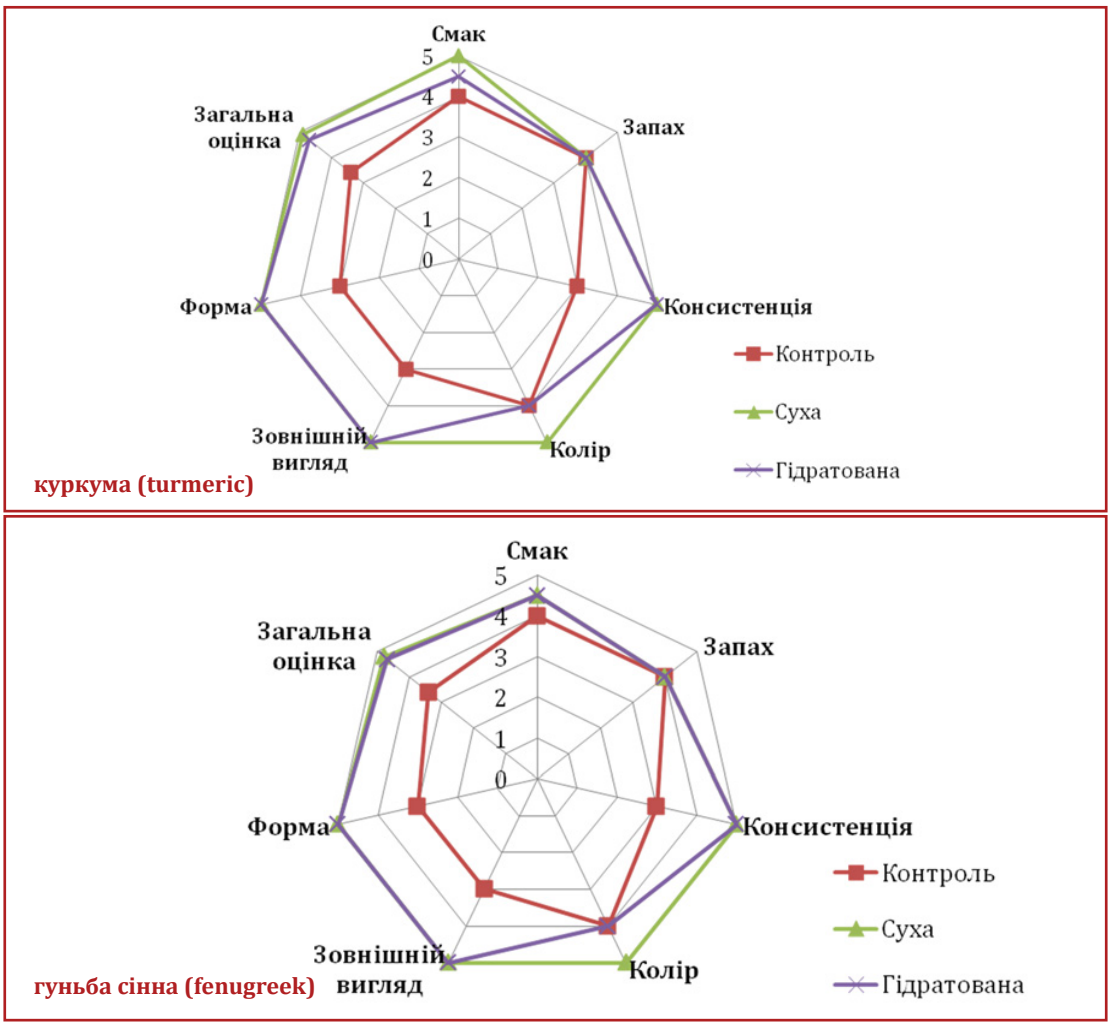


Рисунок 4В Профілограми органолептичних властивостей сиру м'якого з козиного молока з прянощами

Figure 4B Profilograms of organoleptic properties of soft cheese from goat's milk with spices

Профілограми органолептичних властивостей сирів м'яких з козиного молока з прянощами наведено на Рисунку 4А–В.

Упродовж зберігання досліджувалась динаміка показника активної кислотності (Рисунок 5), яка показала, що модельні зразки з прянощами є більш стійкими до зниження активної кислотності в порівнянні з контролем. Такий ефект можна пояснити вмістом у прянощах фенольних сполук, що виявляють антиоксидантні властивості та слугують захисним бар'єром під час окиснення молочних жирів, що узгоджується з даними Korablova and Rakhmetov (2012) та Korablova (2012). Причому, стабільність зразків із внесенням прянощів у сухому вигляді та гідратованому є практично на одному рівні. Тому із урахуванням органолептичної оцінки модельних зразків сирів

м'яких з козиного молока рекомендовано пряноші вносити у сухому вигляді без попередньої гідратації.

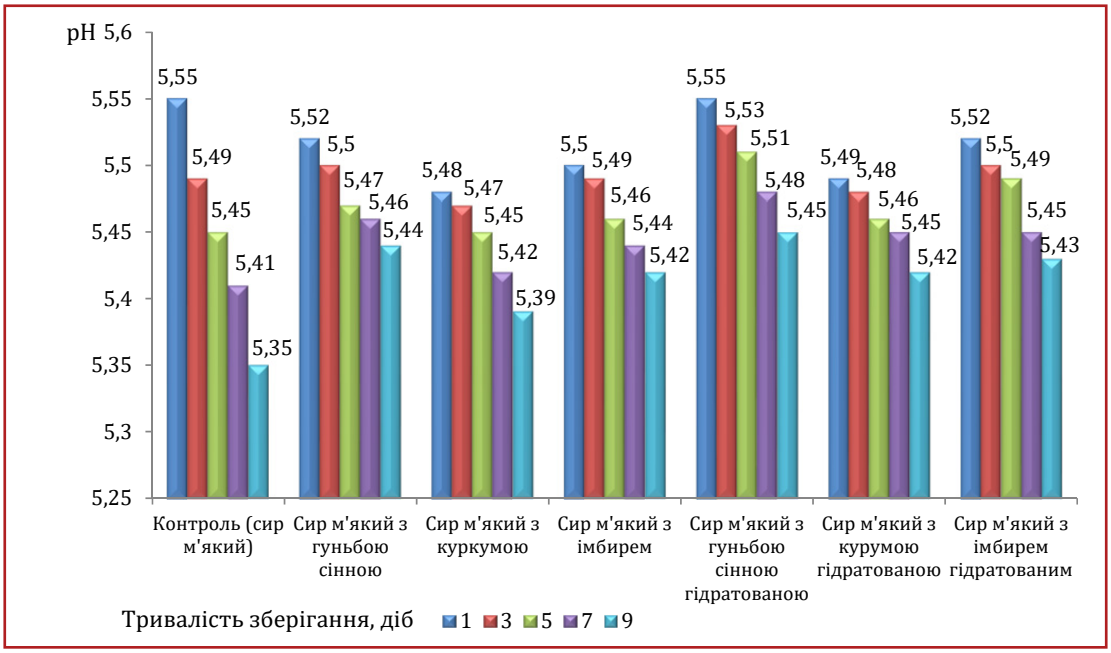


Рисунок 5 Динаміка показника активної кислотності сирів м'яких з козиного молока з прянощами протягом зберігання (температура (6 ±2 °С), відносна вологість повітря не більше 85 %)

Figure 5 Dynamics of the index of active acidity of soft cheeses of goat's milk with spices during storage (temperature (6 ±2 °С) °С, relative humidity not more than 85 %)

Висновки

Обґрунтовано доцільність удосконалення технології свіжих м'яких сирів з козиного молока шляхом використання натуральних функціонально-технологічних добавок.

Здійснено аналіз компонентного складу біологічно активних сполук гуньби, куркуми, імбиру та експериментально доведено доцільність їх введення до складу рецептур м'яких сирів з козиного молока. Визначено технологічні параметри підготовки прянощів для введення до молочної основи – в сухому меленому вигляді у нормалізовану суміш до теплового оброблення, рекомендована кількість прянощів –1,0 ±0,2 %. Встановлено, що введення прянощів знижує показник активної кислотності козиного молока та сироватки, виділеної у процесі оброблення сичужного згустку, у середньому на 0,2 од. pH, забезпечує утворення більш щільного згустку, пришвидшує відділення сироватки, що, вочевидь, пов'язано із адсорбцією на поверхні міцел казеїну компонентів прянощів і, як наслідок, зниженням їх поверхневого заряду та агрегацією;

Встановлено збереження показників якості розроблених сирів м'яких з козиного молока з прянощами протягом не менше 5 діб за температури (4 ±2 °С) та відносної вологості повітря (85 ±5 %).

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MINERAL COMPOSITION OF VEGETABLE BIOMASS OF *AMARANTHUS* L. SPECIES

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The mineral composition is explored of the four species of *Amaranthus* L.: *A. lividus* L., *A. tricolor* L., *A. acutilobus* Uline et W. L. Bray, and *A. blitoides* S. Wats. The leaves were analyzed when the plants were in the vegetative phase of development. The element concentrations determined by techniques of atomic absorption spectrometry with inductively coupled plasma spectrometry. The order of the elements is as follows: *A. acutilobus*, *A. lividus*: K > Ca > Mg > S > P > Fe > Al > Na > Zn > Mn > B > Ba > Ni > Pb > V > Cd > Cr; *A. blitoides*: K > Ca > Mg > S > P > Fe > Al > Na > Zn > Mn > Ba > B > Ni > Pb > V > Cd > Cr; *A. tricolor*: K > Ca > S > Mg > P > Fe > Al > Na > Zn > Mn > B > Ba > Ni > Pb > V > Cd > Cr. Potassium is the main ash element in the leaves of all studied species of *Amaranthus*. Its content varied from 39,010.00 to 44,926.67 mg/kg. *A. tricolor* is significantly different among other species for the ratio of sulfur and magnesium. In its leaves, a large amount of sulfur is found. The species with ascending stems (*A. acutilobus* and *A. blitoides*) contained sulfur up to 4020.00 and species with erect stems (*A. lividus* and *A. tricolor*) within 5,724.00–6,314.40 mg/kg. Maximum content of Mg was 6,734.33, P 1,706.33, Fe 1,133.28 mg/kg. The content of heavy metals was in a safe quantity, except nickel. The highest coefficients of variation (%) are characteristic of the content of barium (45.07), sodium (31.18), vanadium (28.22) and potassium (26.62). The most stable is the content of phosphorus (3.25%). The mineral composition of leaves of amaranth showed its high value as a vegetable product.

Keywords: *Amaranthus acutilobus*, *Amaranthus blitoides*, *Amaranthus lividus*, *Amaranthus tricolor*, mineral composition

Introduction

The use of plants of the genus *Amaranthus* L. today is a promising trend in vegetable growing. In the world, they are widely used in countries of Asia and Africa (Olasantan, 1992; Timothy et al., 1996; Kachigumaetal., 2015). For example, in South Korea, amaranth leaves are used in dried form and exported to the United States (Pemberton end Lee, 1996). Amaranth is one of the four most used plants of natural flora in South Africa. Shackleton et al. (1998) have shown that more than half of the respondents (59%) have consumed it as a nutritional supplement to oatmeal and corn.

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Among the Ukrainian developments, there are recipes of jelly marmalade with the addition of amaranth leaves powder (Karnaushenko and Ustimenko, 1995; Molodozhen, 1996). It is proved that the introduction of such an additive increases the kinematic viscosity and density of jelly masses, enriches with vitamins, protein, micro and macro elements. The addition of leafy flour reduces the use of citric acid to 50% and eliminates the use of colorants due to the reaction of melayidine formation, and also extends the shelf life of finished products. Plants of the genus *Amaranthus* are common in the culture of Ukraine, mainly as fodder and grits. The least attention was paid to the use of amaranth in vegetable growing. The most commonly cultivated are *Amaranthus cruentus* L. (syn. *A. paniculatus* L.) and *A. caudatus* L., rarely *A. lividus* L., *A. tricolor* L., *A. hypochondriacus* L. and *A. mantegazzianus* Passer. The *A. acutilobus* Uline et W. L. Bray and *A. blitoides* S. Wats. known as plants of natural flora, but have a great potential for use.

The rich chemical composition of the amaranth causes the effectiveness of its use as a medicinal plant. It detects radio and cytoprotective, anti-inflammatory, detoxifying, antitumor action, enhances protective functions, and also positively affects the potential of the human organism (Fisun et al., 2002; Yerashova et al., 2002).

The above-ground biomass of amaranth, simultaneously with the widespread use as a source of amino acids, pectin, and trace elements, has an antibacterial effect and is used in the form of decoctions for the treatment of colds. Extractive drugs can be used in the treatment of tonsillitis, cystitis, and pyelonephritis. The therapeutic ability of the leaves of amaranth is found both when they are used for animal feed and food for humans. Amaranth has show antioxidant, tread, and immunomodulatory properties due to the high content of biologically active substances (rutin, amaranthine, vitamins A, C, E, unsaturated fatty acids, squalene) and can be used for the treatment of radiation sickness and other diseases (Zayachkivska and Kozak, 1997; Makeev et al., 2002).

Material and methodology

Taking into account world experience, as well as our own observations, we have been chosen following species for the research: *A. acutilobus*, *A. blitoides*, *A. lividus*, *A. tricolor*. *A. tricolor* was presented by two samples, obtained from Germany and China (cultivar H Yue Ye Te Tsai) (Figure 1).

Experiment organization

Plants grown on the experimental site of the Department of Cultural Flora of M.M. Gryshko National Botanical Garden (NBG) of NAS of Ukraine were studied. The data of the quantitative elemental composition were obtained in the Center of collective use by devices Spectrometric Center of Elemental Analysis «SCEA» of NBG. Plants were in the vegetative phase of development. The leaves were taken for analysis since in this period of development they form the largest part of the plant.



Figure 1 Plants in the vegetative phase of development
a – *Amaranthus acutilobus* Uline et W. L. Bray; b – *A. Lividus* L.; c – *A. blitoides* S. Wats.; d – *A. tricolor* L.; e – *A. tricolor* 'H YueYeTeTsai'

Methodology of element concentration

For the determination of element concentrations, crushed material was dried at 35 °C and dissolved in concentrated nitric acid. Samples were placed in a chemical microwave oven. In it, under the action of the given parameters of pressure and temperature occurred decomposition of biomaterial. The extract was obtained were injected directly to the mass spectrometer and for using a concentric sprayer was translated into an aerosol with subsequent submission to argon plasma, where ionization took place. Then the separation of ions by mass-charging ratio took place and measuring the intensity of the signals the segmented ion analytes. The element concentrations determined by techniques of atomic absorption spectrometry with inductively coupled plasma spectrometry (ICP mass spectrometry). The concentration of chemical elements in the extract was determined on the device ICAP 6000 Series ICP Spectrometer (Thermo Scientific). Element concentrations expressed as mg/kg dry weight. The repetition of experiment – triple (Kovtun-Vodyanytska, 2016).

Statistical analysis

For statistical evaluation were used standard methods using statistical software Microsoft Excel 2010.

Results and discussion

Determination of the elemental composition of amaranths leaves revealed the high content of valuable macro and microelements. The most accumulated potassium and calcium – extremely important elements in the human diet (Vitamin and mineral requirements in human nutrition, 2004). In general, the elements contained in the leaves of different species of amaranth can be ranked by number (from greater to smaller) into the following rows:

A. acutilobus, *A. lividus*: K > Ca > Mg > S > P > Fe > Al > Na > Zn > Mn > B > Ba > Ni > Pb > V > Cd > Cr;

A. blitoides: K > Ca > Mg > S > P > Fe > Al > Na > Zn > Mn > Ba > B > Ni > Pb > V > Cd > Cr;

A. tricolor: K > Ca > S > Mg > P > Fe > Al > Na > Zn > Mn > B > Ba > Ni > Pb > V > Cd > Cr.

As the analysis showed, potassium is the main ash element in the leaves of all studied species of *Amaranthus*. Its content varied from 39,010.00 to 44,926.67 mg/kg (Table 1). Amaranth plants accumulated calcium from 28,523.33 to 36,126.67 mg/kg, which makes the raw material a valuable nutritional supplement. The magnesium content in all samples was observed at a rather high level (4,586.00–6,734.33 mg/kg), the lowest was in *A. acutilobus*. *A. tricolor* is significantly different among other species for the ratio of sulfur and magnesium, since both samples of this species revealed more sulfur than magnesium. A large amount of sulfur was found in the leaves of the species under study, with a significant difference between species with ascending stems (*A. acutilobus* and *A. blitoides*) containing sulfur up to 4,020.00 and species with erect stems (*A. lividus* and *A. tricolor*) – within 5,724.00–6,314.40 mg/kg. The same tendency is typical for phosphorus, sodium manganese and boron – they were detected the least in *A. acutilobus* and *A. blitoides*. Instead, iron and aluminum in the leaves of these species are larger than in samples with erect stems.

Table 1 Elemental composition of leaves of *Amaranthus* species (mg/kg)

Elements	*A. acutilobus*	*A. blitoides*	*A. lividus*	*A. tricolor*	*A. tricolor* 'H YueYeTeTsai'	V (%
K	42,111.67* ±2,245.98**	42,083.33 ±1,738.18	44,926.67 ±1,837.36	39,010.00 ±2,404.69	39,276.67 ±605.77	26.62
Ca	28,523.33 ±1,254.80	35,516.67 ±3,72.11	31,396.67 ±6,40.72	34,590.00 ±716.49	36,126.67 ±1,931.07	9.63
Mg	4,586.00 ±187.21	6,241.00 ±91.54	6,734.33 ±215.34	4,835.33 ±174.26	5,996.00 ±185.62	16.32
S	4,003.60 ±118.14	4,020.00 ±89.03	6,314.40 ±200.12	5,724.00 ±81.94	6,170.40 ±61.77	21.88
P	1,590.33 ±2.27	1,578.33 ±2.94	1,616.67 ±7.12	1,661.00 ±16.72	1,706.33 ±9.91	3.25
Fe	1,056.52 ±136.40	1,133.28 ±69.04	726.05 ±18.12	746.37 ±14.87	863.53 ±21.15	20.22
Al	840.00 ±127.99	881.67 ±160.72	635.67 ±80.11	667.00 ±78.64	587.67 ±85.06	18.04
Na	201.67 ±13.23	199.83 ±10.34	401.70 ±46.50	362.17 ±9.11	327.77 ±16.71	31.18
Zn	65.25 ±11.64	48.05 ±0.91	67.19 ±4.37	52.98 ±5.04	60.42 ±12.89	13.82
Mn	44.53 ±1.02	43.71 ±2.88	49.16 ±5.28	46.12 ±3.02	52.79 ±0.81	7.89
B	30.86 ±2.11	33.65 ±2.97	34.35 ±0.59	36.61 ±2.24	37.49 ±1.76	7.56
Ba	29.25 ±4.75	40.40 ±2.49	14.99 ±0.80	17.21 ±0.60	17.65 ±1.31	45.07
Ni	1.80 ±0.12	2.10 ±0.03	1.64 ±0.08	1.81 ±0.12	1.77 ±0.13	9.26
Pb	1.48 ±0.08	1.79 ±0.29	1.24 ±0.37	1.60 ±0.38	1.39 ±0.04	13.91
V	1.23 ±0.21	1.30 ±0.06	0.71 ±0.08	0.83 ±0.07	0.78 ±0.09	28.22
Cd	0.23 ±0.03	0.18 ±0.02	0.17 ±0.01	0.16 ±0.01	0.26 ±0.01	21.51
Cr	–***	–	–	–	–	–

Notes: V, % – variation coefficient; * – arithmetic mean; ** – Standard error of the mean; *** – <0.0000.

The zinc content ranges from 48.05 to 67.19 mg/kg. The highest variation is observed between samples for the content of barium (45.07%), the quantity of which is significantly (approximately twice) predominant in *A. acutilobus* and *A. blitoides*.

A. lividus is most distinguished among the studied samples because of predominance of potassium (44,926.67 mg/kg) and magnesium (6,734.33 mg/kg) content. The main source of valuable macronutrients was *A. lividus* and *A. tricolor* 'H YueYeTeTsai',the last one had the highest calcium and phosphorus content (36,126.67 and 1,706.33 mg/kg, respectively).

Ni, Pb, V, Cd, and Cr form a group of trace elements, the content of which does not exceed 10 mg/kg. Their significance is extremely important for metabolic processes in the organism, but the amount of consumption is regulated by maximum permissible standards (Vitamin and mineral requirements in human nutrition, 2004). According to the WHO requirements for medicinal plant material (Kovtun-Vodyanytska, 2016), their maximum content is as follows: Ni – 1.5, Pb – 10, Cd – 0.3, Cr – 1.5 mg/kg. The vanadium toxicity threshold is 10–20 mg/kg daily ration (Smolyar and Petrashenko, 2010). Our data indicates that the content of these elements is in a safe quantity, except nickel. Its accumulation in amaranth leaves is probably caused by high background content in the soil. It is significant that no sample contained chromium (Table 1).

The largest variation between samples is observed in the content of barium (45.07%). The great variability is the content of sodium (31.18%), vanadium (28.22%) and potassium (26.62%). The most stable is the content of phosphorus (3.25%), boron (7.56%), manganese (7.89%), nickel (9.26%) and calcium (9.63%), because their variation coefficients do not exceed 10%.

Our research analyzed the mineral composition of the four species of *Amaranthus*. For plants grown in Ukraine, this is the first study to compare plants of these species: *A. acutilobus*, *A. blitoides*, *A. lividus*, *A. tricolor*. The tradition of using of amaranths as a vegetable is especially widespread in Africa. Kachiguma et al. (2015) provide data on the content of some mineral elements for *A. lividus* compared to *A. hybridus* L., *A. cruentus* and *A. hypochondriacus*. The most important thing is that our samples were dominated by potassium (up to 44,926.67 mg/kg), while in samples grown in the Central Malawi calcium more than potassium. The content of potassium in *A. lividus* (44,926.67 mg/kg) in our experiment is three times greater than the African samples (1,473–1,563 mg/100 g) (Kachiguma et al., 2015). Amaranths from Central Malawi accumulated much less calcium than in Ukraine, their maximum values were 2,381 mg/100 g and 36,126.67 mg/kg, respectively. Amaranths collected by researchers in Africa accumulation magnesium 383.4–513.9, iron – from 14.21 to 27.06 and zinc from 1.03 to 3.46 mg/100 g. The content of iron and zinc was much lower than in our samples, but the quantity of magnesium is comparable to our data.

Conclusions

The results of the analysis of the mineral composition of leaves of different species of *Amaranthus* showed their high value as a vegetable product. *A. acutilobus*, *A. blitoides*, *A. lividus*, *A. tricolor* have high levels of potassium, calcium, and magnesium, which makes it

possible to use them in dietary nutrition and fodder production. The growth of amaranths in Ukraine provides a high concentration of a wide range of macro and microelements in the raw material.

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INFLUENCE OF FERMENTATION CONDITIONS ON THE CONTENT OF PHENOLIC COMPOUNDS, ANTHOCYANINS AND THE SPECTRAL CHARACTERISTICS OF CABERNET SAUVIGNON WINES

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The wine characteristics have been determined by the variety potential and specificity, but also by the yeast strain carrying out the alcoholic fermentation. Phenolic components and anthocyanins play a significant role in the formation of the organoleptic wine qualities, especially of red wines. The yeast affects the colour of the wine mainly with the extracellular β-glucosidase they produce. The objective of the study was to investigate the influence of the technological factors yeast strain, temperature and inoculum amount of yeast culture on the variation of the total phenolic compounds and anthocyanins during the alcoholic fermentation and on the spectral characteristics of Cabernet Sauvignon wines. The experiment was carried out with grapes of Cabernet Sauvignon variety with sugars content of 23.10% and titratable acids of 6.15 g/dm^3. The alcoholic fermentation was conducted with inoculum amount of yeast culture 2, 3 and 4% of the strains *Bordeaux* and *8-11* of *Saccharomyces cerevisiae* species. The fermentation temperature was 20, 24 and 28 °C. The course of the process was followed by the variation in the dry matter, measured daily with the Abbe refractometer. The dynamics of the total phenolic compounds and the anthocyanins during the fermentation was determined by measuring on 1st, 5th, 10th and 20th day. After the alcoholic and the malolactic fermentations in the experimental wines were also analyzed the content of flavonoid phenolic compounds, non-flavonoid phenolic compounds and their spectral characteristics. The results demonstrated that under the experimental conditions both strains *Bordeaux* and *8-11* exhibited the best fermentation activity at 28 °C. The temperature factor had a stronger impact on the variations of total phenolic compounds and anthocyanins in the course of the alcoholic fermentation. The quantitative maximum of both indicators was recorded on the 5th day of the process. In the experimental wines, the variants fermented under the conditions of 28 °C/4% contained more total phenolic compounds, anthocyanins, flavonoid phenolic compounds and non-flavonoid phenolic compounds. The red colour ratio also was the highest. The wines of *8-11* strain were distinguished by a higher content of total phenolic compounds, anthocyanins and, respectively a more intense colour.

Keywords: wine, yeast, alcoholic fermentation, phenolic compounds, spectral characteristics.

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Introduction

The wine characteristics have been determined by the variety potential and specificity, but also by the yeast strain carrying out the alcoholic fermentation. It has a significant influence on its composition with its metabolism and physiological features.

Phenolic components play a significant role in the formation of the organoleptic wine qualities, especially of red wines. The amount of phenolic compounds in grapes depended on number of factors, as the main of them being the variety, the soil and weather conditions in the growing area, the applied agricultural and technical practices, the degree of maturity, etc. Most of the phenolic compounds are contained in the seeds and the skins, from where they pass into the must and the wine (Abrasheva et al., 2003).

The wine anthocyanin profile has also been determined by the variety specifics (Burns et al., 2003; Otteneder and Marx, 2004). The anthocyanins are localized mainly in the grapes skin, in free or bound state in the form of glycosides (Singleton and Trousdate, 1992; Fisher and Strasser, 1999). The fermentation conditions and certain technological factors such as the time of contact with the solids, the temperature, the yeast strain, also influence the amount of phenols and anthocyanins in wine (Stoyanov et al., 2004).

In the production of red wines, with the increase of the alcoholic content during the fermentation, the extraction of phenolic and colouring substances is enhanced as the optimal temperature for this being 28–30 °C (Getov, 2002). The yeast affects the colour of the wine mainly with the extracellular β-glucosidase they produce. Strains with low β-glucosidase activity produce wines with higher anthocyanin content (Tsvetanov et al., 1994; Tsvetanov et al., 1995). The higher activity does not result in a direct decrease in their amount but it is directly related to their colouring ability, converting them into copolymerization aglycons (Spasov et al., 1998; Rodrigues et al., 2004). Part of the anthocyanins during the fermentation are adsorbed by the yeast cells, as well as in the oxidation, variations in the medium pH, interacting with other substances and pass into insoluble form and precipitate (Chobanova, 2012). As a result, both quantitative and qualitative variations in the phenolic and anthocyanin composition of the wine are observed at the end of the process.

The objective of the study was to investigate the influence of the technological factors yeast strain, temperature and inoculum amount of yeast culture on the variation of the total phenolic compounds and anthocyanins during the alcoholic fermentation and on the spectral characteristics of Cabernet Sauvignon wines.

Material and methodology

Plant material

The experiment was carried out with grapes of Cabernet Sauvignon variety. Each technological variant was crumbled and crushed separately and by equal distribution of the grapes, it was ensured the uniformity of the raw material.

Alcoholic fermentation

Conditions

The alcoholic fermentation was carried out under the following conditions:

- fermentation substrate – 4.0 kg of grape pulp, sulphuring with 50 mg/kg SO_2, with sugars content of 23.10% and titratable acids of 6.15 g/dm^3;
- 48-hour active yeast culture of the *Bordeaux* and *8-11* collection strains of *Saccharomyces cerevisiae* species in quantity of 2%, 3%, 4% (the strains were provided by the yeast collection of the University of Food Technologies, Plovdiv, Bulgaria);
- fermentation temperature – 20 °C, 24 °C, 28 °C.

The course of the alcoholic fermentation was followed by the variation in the dry matter, measured daily by means of an Abbe refractometer at a constant value.

Determining the variations in total phenolic compounds and anthocyanins

The content of the total phenolic compounds and anthocyanins during the fermentation was determined, taking the following steps: start (day 1), vigorous fermentation (day 5), silent fermentation (day 10) and after the malolactic fermentation (day 20). Their amount was analyzed in the clear part, after centrifugation of the samples, using a UV-Vis spectrophotometer Cary 50 Varian, respectively by the methods:

- total phenolic compounds (TPC), g/dm^3 gallic acid – method of Singleton and Rossi with a Folin-Ciocalteu reagent and measurement of sample absorption spectrophotometrically at λ 750 nm (Ivanov et al., 1979);
- anthocyanins, mg/dm^3 – method of Gayon and Stonestreet by pH changing and using of buffer solutions with pH 0.6 and pH 3.5 and the absorption of both samples was measured spectrophotometrically at λ 520 nm (Ivanov et al., 1979).

Determination of the content of phenolic compounds, anthocyanins and spectral characteristics of the experimental wines

After the end of the alcoholic fermentation (found by chemical analysis of the sugars) and the spontaneous malolactic fermentation (established by paper chromatography), the experimental wines were decanted and by UV-Vis spectrophotometer Cary 50 Varian, they were further analyzed for the content of:

- flavonoid phenolic compounds (FPC), mg/dm^3 catechin equivalent and non-flavonoid phenolic compounds (NPC), mg/dm^3 caffeic equivalent – Somers method with analytical and calculation part – 0.2 cm^3 of the test sample was added to 10 cm^3 1n HCl and between 3rd and 4th hour the absorbance of the solution was measured at 280 and 320 nm (Chobanova, 2007)
- spectral characteristics – determined by measuring the sample absorbance in a 0.1 cm cuvette at λ 420, 520 and 620 nm and equated to a 1 cm cuvette (Chobanova, 2007):
  - colour intensity *IC'* [abs. unit] – the Glories method, was the sum of the measurements at the three wavelengths:

$$IC' = A_{420} + A_{520} + A_{620}$$

– colour tint *T* [abs. unit] – the Sudraud method represented the absorbance at 420 nm and 520 nm ratio:

$$T = A_{420}/A_{520}$$

– *dA*% – the Glories method, the indicator expressed the participation of free and bound flavylium forms of anthocyanins in the total wine colour:

$$dA\% = \left\{1\text{-}\left[(A_{420} + A620)/2 \times A_{520}\right]\right\} \times 100$$

– yellow colour ratio in the total wine colour – the Glories method, absorbance measurement at 420 nm:

$$\%\ yellow\ colour = (A_{420}/IC') \times 100$$

– red colour ratio in the total wine colour – the Glories method, absorbance measurement at 520 nm:

$$\%\ red\ colour = (A_{520}/IC') \times 100$$

– blue colour ratio in the total wine colour – the Glories method, absorbance measurement at 620 nm:

$$\%\ blue\ colour = (A_{620}/IC') \times 100$$

Results and discussion

The investigation of the studied technological factors impact on the time of the alcoholic fermentation found that *Bordeaux* and *8-11* strains exhibited good fermentation activity. With the temperature and the inoculum amount of the yeast culture increase, within one temperature range, the process began and ended earlier. The dynamics of the dry matter in the fermentation of the grape pulp with both strains were similar. At 20 °C, the process started and ran more slowly, due to the slower cell multiplication. The yeasts exhibited the best activity at 28 °C and the duration of the process was shorter by 3–4 days compared to that at 20 °C. With the increase of the inoculum quantity of yeast culture (2%, 3%, 4%) the fermentation occurred faster because of the higher number of active yeast cells in the medium.

The results of the dry matter variation during the fermentation of Cabernet Sauvignon grape pulp with the studied strains are presented in Figure 1.

In the course of the alcoholic fermentation, the variation of TPC and anthocyanins in the grape pulp was monitored. The results of their dynamics have confirmed the findings of other authors (Sims and Bates, 1994; Spranger et al., 1998), namely reaching the maximum during the exponential phase of yeast development followed by a decrease in their concentration due to the absorption by the cells or the involvement in the condensation processes. The effect of the inoculum amount of the yeast culture of the studied strains was similar and relatively weaker. The influence of the factor temperature was stronger as an indicator of the fermentation process. The higher temperature benefited the colouring matter extraction from

the solids during the fermentation. The studied indicators from the grape pulp composition showed a tendency of going up with the increase of the yeast culture amount and temperature, reaching the maximum rates at 28 °C/4%.

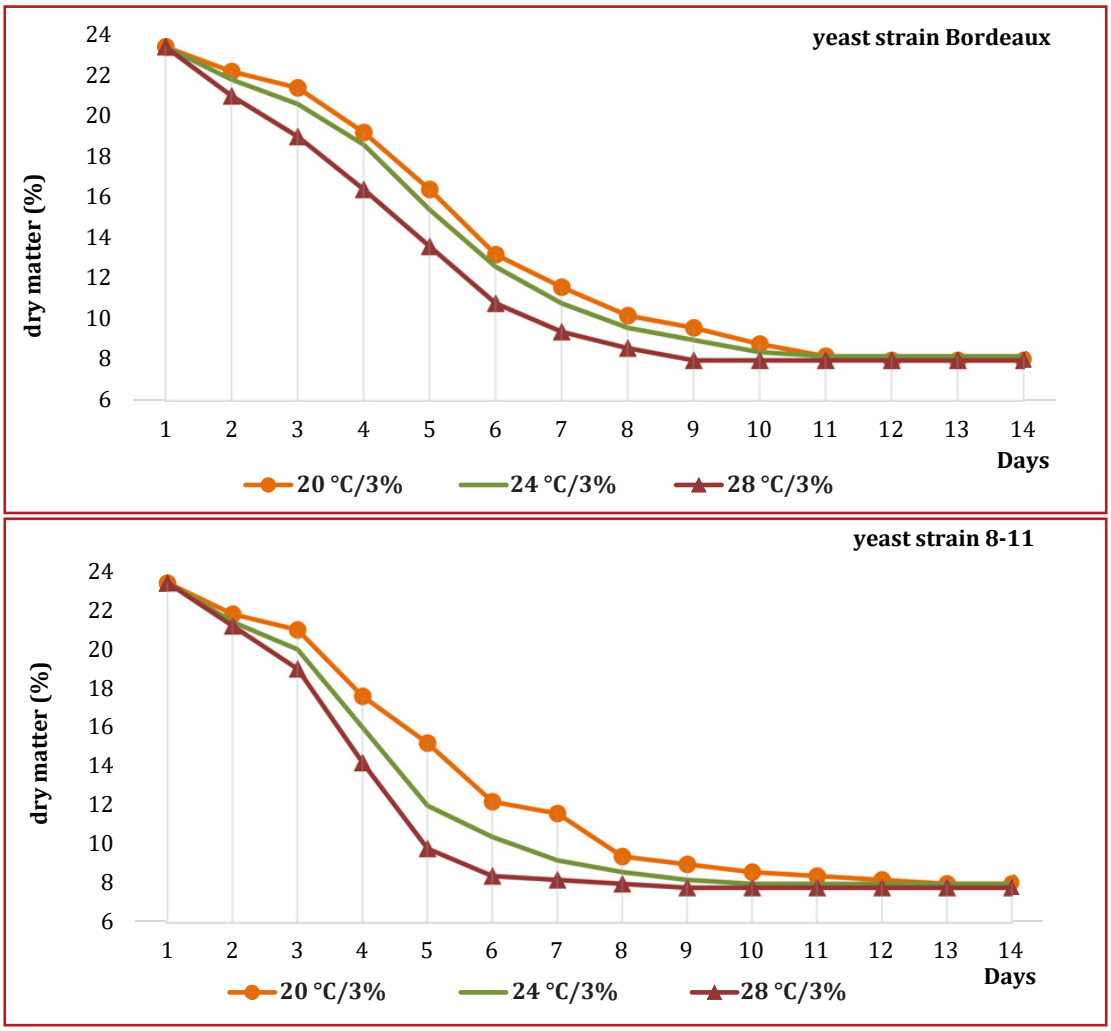


Figure 1 Dynamics of dry matter during the alcoholic fermentation of Cabernet Sauvignon grape pulp with the studied strains

Figures 2 and Figure 3 represent the variations in the TPC concentration and anthocyanins during the fermentation with *Bordeaux* and *8-11* strains at 3% inoculum and temperature 20, 24, 28 °C. The results of the process with 2 and 4% inoculum at the same temperature intervals were similar.

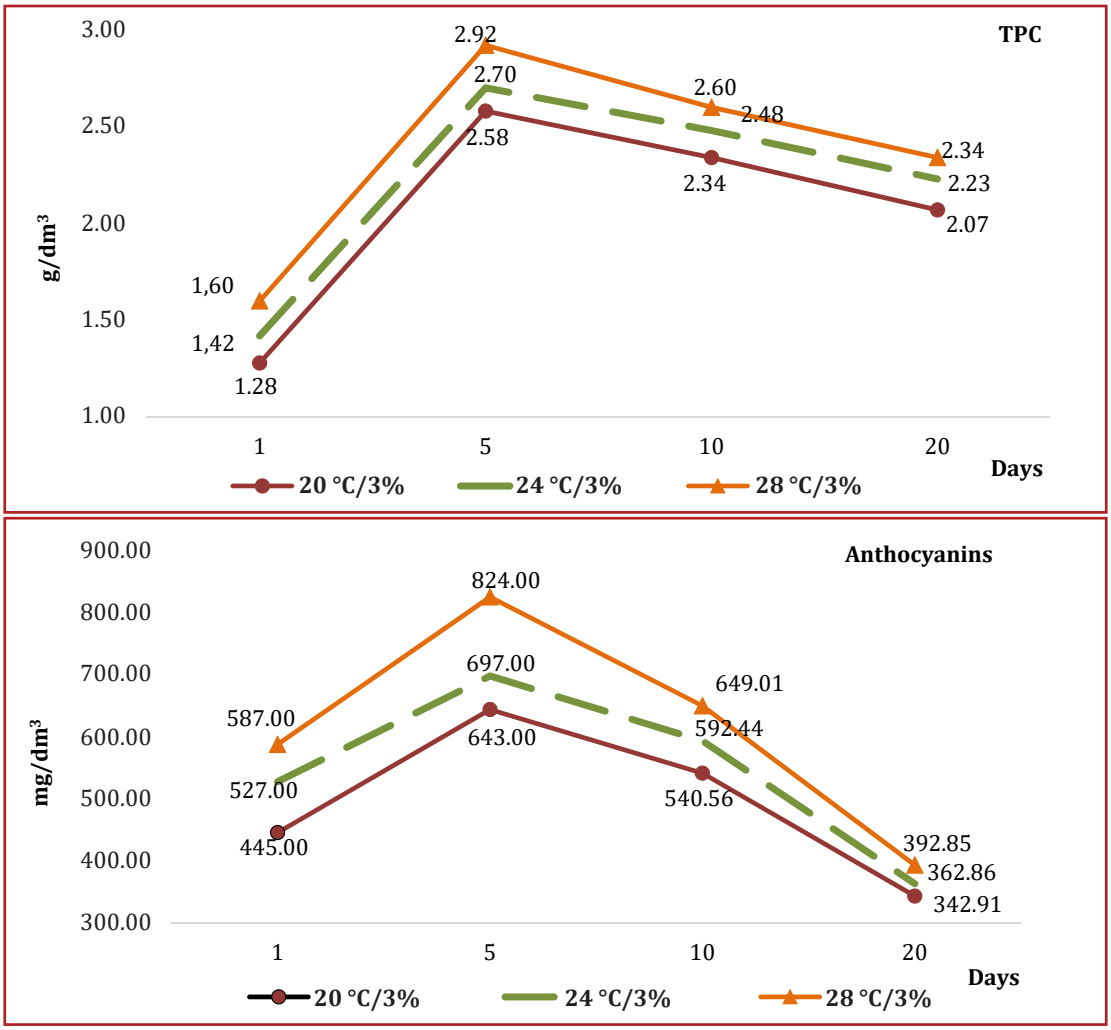


Figure 2 Variations in TPC and anthocyanins during the alcoholic fermentation with *Bordeaux* strain

The yeast effect appeared to be mainly on the amount of anthocyanins through the activity of the produced β-glucosidase. The *Bordeaux* strain had a higher β-glucosidase activity compared to the *8-11* strain (Yoncheva et al., 2007). Anthocyanins reduction during the fermentation was associated with the active cell development during the logarithmic phase, when cells released a significant amount of enzymes, including glucosidases, attacking their molecules (Kanev and Patokova, 2004).

The TPC and anthocyanins ratio was the highest for both strains, in wines fermented at 28 °C. These results have confirmed that it was the optimal temperature for obtaining intensely coloured red wines with a high content of phenolic substances. The quantitative variation of anthocyanins during the fermentation indicated that the maximum was on the fifth day. In the conditions of the experiment at the same fermentation temperature, the amount of anthocyanins in the wines obtained with an inoculum of yeast culture 3 and 4% was higher

for both strains *Bordeaux* and *8-11*. Due to the lower β-glucosidase activity of *8-11* strain, the wines produced with it contained more anthocyanins.

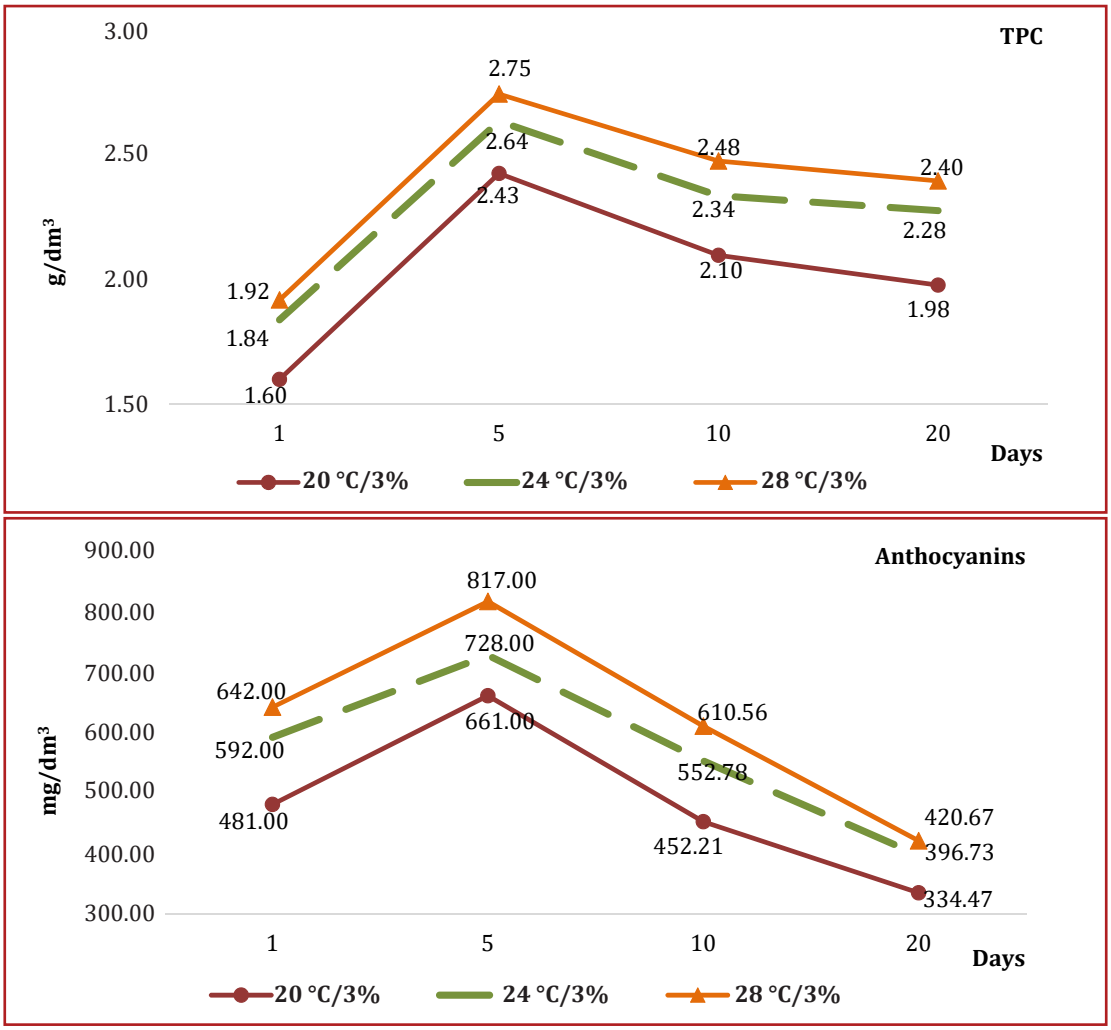


Figure 3 Variation in TPC and anthocyanins during the alcoholic fermentation with *8-11* strain

The rate of TPC in the experimental samples is proportional to that of the anthocyanins, so their variation in the course of the alcoholic fermentation was similar. In wines made with *Bordeaux* strain they ranged from 1.88 (20 °C/2%) to 2.38 g/dm^3 (28 °C/4%), while in the samples obtained with *8-11* strain – from 1.88 (20 °C/2%) to 2.44 g/dm^3 (28 °C/4%).

This trend was similar for the obtained young wines – the variants fermented under the conditions of 28 °C/4% contained more TPC, anthocyanins, as well as FPC and NPC (Table 1). That was due to the more intense extraction processes. The increase of FPC during the fermentation could be explained by the extraction from the solids and the rise in the amount of anthocyanins that were related to this group of compounds, while the higher rates of NPC

were probably due to the formation of new phenolic acids during the sugars decomposition. Their concentration, however, did not reach such rates to affect negatively the wine quality.

Table 1 Phenolic compounds and spectral characteristics of the experimental Cabernet Sauvignon wines

Variants	20 °C			24 °C			28 °C		
	2%	3%	4%	2%	3%	4%	2%	3%	4%
Indicators	***Saccharomyces cerevisiae Bordeaux***								
TPC (g/dm^3)	1.88	2.07	1.94	2.14	2.23	2.22	2.27	2.34	2.38
NPC (mg/dm^3 caffeic equivalent)	157.18	164.61	173.83	173.27	175.21	174.27	177.49	184.93	205.57
FPC (mg/dm^3 catechin equivalent)	2,935.77	3,148.63	3,270.06	3,299.35	3,559.36	3,522.93	3,672.24	3,777.20	3,790.79
Anthocyanins (mg/dm^3)	313.60	342.91	326.92	348.23	362.86	350.28	380.43	392.85	422.80
Colour intensity IC' [abs. unit]	9.12	9.25	9.37	9.43	9.47	9.52	9.65	9.78	10.22
Colour tint T [abs. unit]	0.476	0.465	0.473	0.470	0.389	0.477	0.516	0.545	0.512
dA %	70.10	70.40	69.60	69.80	68.20	69.70	67.00	66.10	66.70
% Yellow colour	29.80	29.20	29.40	29.30	29.90	29.70	31.10	30.80	30.70
% Red colour	62.60	62.80	62.20	62.30	61.10	62.20	60.30	60.00	60.00
% Blue colour	7.60	8.00	8.40	8.40	9.00	8.10	8.60	9.20	9.30
	Saccharomyces cerevisiae 8-11								
TPC (g/dm^3)	1.88	1.98	1.91	2.10	2.28	2.25	2.22	2.40	2.44
NPC (mg/dm^3 caffeic equivalent)	164.50	173.16	172.50	181.26	179.15	196.69	195.58	194.93	208.90
FPC (mg/dm^3 catechin equivalent)	3,100.78	3,047.92	3,297.21	3,295.06	3,548.64	3,609.35	3,691.50	3,711.50	3,852.93
Anthocyanins (mg/dm^3)	310.36	334.47	328.28	372.48	396.73	388.15	385.68	420.67	424.94
Colour intensity IC' [abs. unit]	9.48	9.50	9.54	9.78	10.20	10.85	11.12	11.65	11.86
Colour tint T [abs. unit]	0.496	0.500	0.551	0.562	0.562	0.494	0.492	0.504	0.463
dA %	68.80	68.50	65.20	63.10	64.40	67.90	68.60	67.30	71.30
% Yellow colour	30.60	30.70	30.50	30.30	30.90	30.10	30.20	30.50	29.50
% Red colour	61.60	61.30	60.90	60.50	60.40	60.90	61.50	60.40	61.20
% Blue colour	7.80	8.00	8.60	9.20	8.70	9.00	8.30	9.10	9.30

Note: TPC total phenolic compounds, FPC flavonoid phenolic compounds, NPC flavonoid phenolic compounds

The wine composition rates and its spectral characteristics were within the typical ranges of Cabernet Sauvignon wines. With increasing the temperature and ratio of yeast culture, their amount went up due to the better extraction conditions. The highest rates were recorded in the variants fermented at 28 °C with 4% inoculum (Table 1). The wines of *8-11* strain were characterized by a slightly higher content of TPC, anthocyanins and, respectively a more intense colour. The values of dA% indicator, determining the brightness of the red colour were directly correlated with them. That affected positively the colour characteristics of the samples. The ratio of the three main colours was within the typical ranges for young red wines. The red colour share was the greatest in the variants obtained at 28 °C. The higher rate of the yellow colour in the samples of both strains resulted in a slight brick-coloured tinge, which was also confirmed by the tint values. That could be assumed as a prerequisite for the faster positive colour development during the wine aging.

Conclusions

Based on the study results it might be summarized:

- *Bordeaux* and *8-11* strains showed the best fermentation activity at 28 °C. When increasing the inoculum amount of the yeast culture the fermentation also took place faster.
- The impact of the temperature on the variation of TPC and anthocyanins in the course of the alcoholic fermentation was more pronounced than the inoculum quantity of the yeast culture.
- The maximum concentration rate of TPC and the anthocyanins during the fermentation was recorded on the fifth day.
- In the experimental wines, the variants fermented at 28 °C/4% contained more TPC, anthocyanins, FPC, NPC and the share of the red colour was the highest.
- The wines of *8-11* strain had higher TPC content and because of the lower β-glucosidase activity of the strain they contained more anthocyanins and therefore had a more intense colour.

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IMPACT OF PLANTS SECONDARY METABOLITES ON WHITE CABBAGE PRODUCTIVITY

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Improving the sustainability of vegetable crops to adverse growing conditions is a significant reserve for increasing yields with the already achieved level of intensification of their production. The implementation of resource-saving and especially environmentally safe technologies for the production of vegetables brings to the fore the use of biologically active substances (BASs) of natural origin as growth regulators. It is BASs with their exogenous use that can in low concentrations influence the intensity of the physiological processes in the plants, especially under unfavorable conditions for their growth. The purpose of this study was to use a complex of biologically active substances isolated from some representatives of the wild flora of Republic of Moldova (family of Plantaginaceae Juss) as growth regulators and to establish their effect on seed viability, yield and biochemical composition of white cabbage (*Brassica oleracea* L.). The use of the preparation Ecostim and the complex of bio-regulators from *Veronica officinalis* for pre-sowing seed soaking created the most favorable conditions for the growth and development of white cabbage, significantly increasing the seed germination, stimulating the processes of root formation of seedlings and its survival. The ability of these bio-regulators to improve the production process of white cabbage was established. Seed treatment with the solutions of Ecostim and complex of bio-regulators from *Veronica officinalis* contributed to an additional 11.1 tons and 6.7 tons of standard cabbage heads per hectare, respectively. It was shown that the pre-sowing treatment of seeds also had a positive effect on the biochemical indexes of cabbage, increasing the content of vitamin C and sugars. The performed research, its scientific and applied results allowed us to recommend the pre-sowing seed treatment with solutions of the preparation Ecostim and the complex of bio-regulators from *Veronica officinalis* as an element in the technology of cabbage growing.

Keywords: bioregulator, Ecostim, *Veronica officinalis*, white cabbage, yield

Введение

Повышение устойчивости овощных культур к неблагоприятным условиям произрастания является существенным резервом увеличения урожайности при уже достигнутом уровне интенсификации их производства. Реализация

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ресурсосберегающих и особенно экологически безопасных технологий производства овощей на первый план выводит применение в качестве регуляторов роста биологически активные вещества (БАВ) природного происхождения (Alexeeva, 2008; Botnari et al., 2017). Целесообразность использования данных соединений объясняется тем, что продукты вторичного метаболизма растений – флавоноиды, иридоиды, стероиды в свободном и гликозилированном состоянии в последние десятилетия привлекают пристальное внимание исследователей в связи с широким спектром их действия. Именно БАВы при экзогенном их применении способны в малых концентрациях влиять на интенсивность протекания физиологических процессов в растении, особенно при неблагоприятных условиях выращивания последних (Botnari et al., 2017; Mashcenko et al., 2018).

Климат Республики Молдовы – умеренно континентальный. Явления засухи и переменчивый характер погоды составляют негативный аспект климата в регионе. В связи с этим в республике проводятся исследования по созданию научной базы системы устойчивого земледелия, обеспечивающего получение экологически чистой сельскохозяйственной продукции, и исключающего загрязнение земельных и водных ресурсов (Natsional'naya programma deystviy po bor'be s opustynivaniem, 2000).

Пренебрежение современными экологическими принципами земледелия привело к снижению плодородия почв, что способствовало уменьшению урожайности овощных культур (Pechatnova, 2014). Так, средняя урожайность капусты белокочанной по годам, согласно данным Национального бюро статистики РМ, колеблется в пределах 14–20 т/га.

Причинами низкой урожайности капусты являются не только агрометеорологические условия вегетационного периода, но и несовершенство овощных севооборотов, не всегда правильное использование минеральных и органических удобрений, отсутствие сортов местной селекции (Borisov, 2015). Поэтому особое значение имеет использование новых приемов, способных увеличить продуктивность культуры. Одним из них является применение экологически чистых, биологически активных ростовых веществ, которые способствуют не только повышению урожая, но и устойчивости растений к комплексу болезней, сглаживанию колебаний в метеоусловиях, а также повышают всхожесть семян, засухоустойчивость растений, улучшают укоренение рассады, регулируют плодообразование и ускоряют созревание плодов (Potapova, 2009; Mashcenko et al., 2018; Musaev, 2018;)

В связи с этим, целью данной работы было использование комплекса биологически активных веществ, выделенных из некоторых представителей дикорастущей флоры Республики Молдовы (сем. Plantaginaceae Juss.), в качестве регуляторов роста и изучение их влияния на жизнеспособность семян, урожайность и биохимические показатели капусты белокочанной (*Brassica oleracea* L., сем. Brassicaceae Burnett).

Материалы и методы

Объекты исследования

Из надземной части льнянки обыкновенной (Linaria vulgaris Mill.) (I), коровяка густоцветкового (*Verbascum densiflorum* Bertol.) (II), льнянки дроколистной (*Linaria genistifolia* L.) (III), вероники лекарственной (*Veronica officinalis* L.) (IV), собранных в период максимального накопления биологически активных соединений, а именно в период цветения, исчерпывающей экстракцией водным этанолом с последующей очисткой полученных экстрактов адсорбционно-распределительной хроматографией на колонках с сорбентом получили сумму БАВов (Mashcenko and Borovskaya, 2018).

Схема лабораторного тестирования

Выделенные суммарные фракции охарактеризовали с помощью спектральных и хроматографических методов анализа и использовали в лабораторном тестировании их биологической активности для предпосевного замачивания семян капусты белокочанной с низкой всхожестью (62,4 %). Для сравнения использовали препарат Экостим, созданный на основе стероидного гликозида фуростанолового ряда –томатозида, разрешенный к применению на территории Республики Молдовы в качестве регулятора роста овощных культур.

В эксперименте использовали водные растворы БАВов с предполагаемой биорегуляторной функцией в диапазоне концентраций 0,0001–0,1 %, экспозиция замачивания – 24 часа. Контролем служили семена, замоченные в дистиллированной воде. Каждый эксперимент проводили в 4-х кратной повторности по 100 семян каждая. Проращивали семена в термостате по общепринятой методике (International rules for seed testing, 2017). В лабораторных условиях определяли влияние биорегуляторов на следующие параметры: энергия прорастания, общая всхожесть семян, длина зачаточных корешков и проростков капусты.

Производственные испытания

Для производственных испытаний в качестве объекта исследования были взяты семена капусты белокочанной позднеспелого сорта Барыня. Семена замачивали в 0,01%-ных водных растворах биорегуляторов, проявивших при лабораторном тестировании наибольший положительный эффект на всхожесть, а именно препарата Экостим и комплекса биорегуляторов, полученных из вероники лекарственной. По истечении 45-ти дней рассаду высаживали в открытый грунт. Почва опытного участка – чернозем обыкновенный среднемощный. Орошение капельное. Площадь опытного участка составляла 930 м^2.

Результаты и их обсуждение

Раннее нами было испытано действие вторичных метаболитов высших растений – стероидных гликозидов, выделенных из семян культурных растений, на семенную продуктивность капусты белокочанной, в результате чего указанные препараты

были рекомендованы в качестве физиологических стимуляторов данного параметра капусты (Buharov et al., 2016).

Поскольку капусту белокочанную в Республике Молдова выращивают в основном рассадным и лишь частично безрассадным способом, а при посеве семян с недостаточной энергией прорастания всходы часто не могут преодолеть почвенную корку, образовавшуюся в результате весенних осадков и быстрого подсыхания почвы, мы решили использовать природные биорегуляторы, которые хорошо зарекомендовали себя в качестве индукторов энергии прорастания семян на других культурах, для стимулирования жизнеспособности семян капусты белокочанной при экзогенном их применении (Botnari et al., 2017).

Лабораторное тестирование исследуемых соединений выявило стимулирующее влияние всех изучаемых веществ на первичные процессы метаболизма семян капусты, выразившееся в повышении энергии прорастания, общей всхожести, увеличении длины зачаточных корешков и проростков. По показателям энергии прорастания выделился вариант, где для замачивания семян применяли раствор суммы верофозидов, полученных из *V. officinalis*, в концентрации 0,001 %. В этом случае энергия прорастания превышала контроль на 48,2 %.

Общая всхожесть во всех вариантах использования растворов биорегуляторов для предпосевной обработки семян превосходила контроль на 15,2–56,6 % (Рисунок 1). Заслуживает внимания вариант с использованием 0,001 %-ного раствора соединений из *V. officinalis*, в котором общая всхожесть превышала контроль в 1,7 раза.

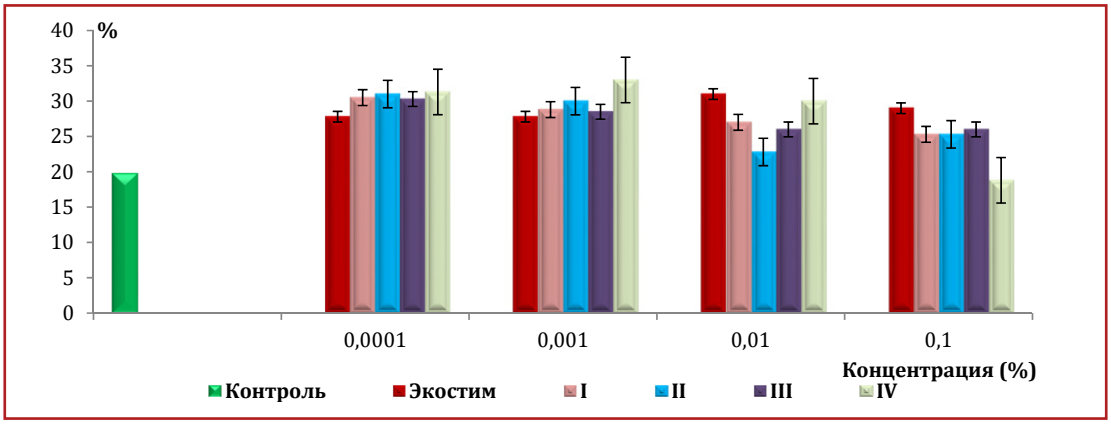


Рисунок 1 Влияние биорегуляторов растительного происхождения на всхожесть семян капусты белокочанной

Figure 1 Impact of plant bioregulators on the germination of white cabbage seeds

Семена капусты сохраняют всхожесть 3–4 года, но одной из причин ее невысокой урожайности может быть плохое укоренение рассады, не способной противостоять неблагоприятным условиям, особенно дефициту влаги. Для получения гарантированного урожая капусты при возделывании ее в рассадной культуре

особое значение имеет приживаемость рассады (Lunev, 2006). Решению этой проблемы способствует стимулирование роста корешков и проростков, что может быть обеспечено применением природных биорегуляторов для предпосевного замачивания семян. Даже незначительное увеличение этих показателей благоприятно сказывается на росте и развитии рассады после высадки в поле, что в дальнейшем гарантирует получение качественной продукции и максимального урожая капусты. Стимулирующий эффект исследуемых биорегуляторов на длину зачаточных корешков и проростков наблюдали во всех вариантах опыта, причем данные показатели заметно превосходили контроль (до 20,0 %).

Применение регуляторов роста в производственных условиях ускорило интенсивность развития растений. Полученная из обработанных биорегуляторами семян рассада хорошо укоренилась после пересадки в грунт. Растения отличались интенсивным развитием и сочной окраской листовой розетки. В то же время на контрольном участке, где была высажена рассада, полученная из необработанных семян, встречались ослабленные растения с преждевременным пожелтением и признаками высыхания листьев, а в дальнейшем с преобладанием антоциановой окраски розетки (Рисунок 2).



Рисунок 2 Производственное испытание биорегуляторов на капусте
а – контрольный участок; б – экспериментальный участок

Figure 2 Production testing of bioregulators on cabbage
a – control plot; b – experimental plot

Ранее было установлено, что препараты группы стероидных гликозидов стимулируют и повышают устойчивость растений к стрессовым условиям произрастания, в конечном итоге увеличивая биологическую продуктивность и выход товарной продукции овощных культур (Maksimovskih, 2011; Borisov, 2015; Balashova et al., 2017). В нашем случае относительная прибавка урожайности на участке, где для предпосевной обработки семян использовали раствор препарата на базе стероидного гликозида Экостим, составила 12,1 т/га кочанов. Тогда как замачивание семян в растворе суммы верофозидов способствовало увеличению выхода конечной продукции по сравнению с контрольным опытом только на 2,8 т/га (Таблица 1).

Таблица 1 Влияние биорегуляторов на урожайность капусты белокочанной
Table 1 Impact of bioregulators on white cabbage yield

Вариант	Средняя масса стандартного кочана (кг)	Урожайность ± к контролю		
		(т/га)	(%)	(т/га)
Контроль	2,9	81,5	–	–
Экостим	3,4	93,6	14,8	12,1
Верофозид	3,2	84,3	3,4	2,8
$НСР_{0,05}$		4,23		

Следует отметить, что предпосевная обработка семян капусты привела к значительному увеличению средней массы стандартных кочанов в сравнении с контролем и, как результат, к улучшению товарного качества конечной продукции. На производственных участках с применением растворов Экостима и верофозидов дополнительно получено 11,1 и 6,7 т/га стандартных кочанов, соответственно.

Важно отметить существенное влияние испытанных биорегуляторов на улучшение биохимических показателей капусты. Содержание такого ценного витамина как аскорбиновая кислота в кочанах, полученных на участке с применением для предпосевной обработки 0,01 %-ного водного раствора суммы верофозидов, превосходит данный показатель контрольного варианта на 7,7 %. Применение биорегуляторов растительного происхождения в нашем опыте оказало положительное влияние и на общее содержание сахаров в капусте. Так предпосевная обработка с применением водного раствора препарата Экостим в концентрации 0,01 % способствовала повышению данного показателя относительно контроля на 14,0 % (Таблица 2).

Таблица 2 Влияние биорегуляторов на биохимический состав кочанов капусты белокочанной
Table 2 Impact of bioregulators on the biochemical composition of the white cabbage heads

Вариант	Сухое вещество		Общий сахар		Витамин С	
	(%)	(%) к контролю	(%)	(%) к контролю	(мг/100 г)	(%) к контролю
Контроль	10,4	--	5,7	--	50,5	--
Экостим	10,1	-2,8	4,9	14,0	49,8	-1,4
Верофозид	10,5	1,0	5,9	3,5	54,4	7,7

Выводы

Изучено влияние биологически активных веществ растительного происхождения на всхожесть, рост, развитие, урожайность и биохимические показатели капусты белокочанной. Показано, что применение препарата Экостим и комплекса биорегуляторов из *Veronica officinalis* для предпосевного замачивания семян создает наиболее благоприятные условия для роста и развития капусты белокочанной, существенно повышая энергию прорастания, всхожесть семян, стимулируя процессы корнеобразования рассады и ее приживаемость.

Установлена способность данных биорегуляторов совершенствовать продукционный процесс капусты белокочанной. Обработка семян растворами препарата Экостим и верофозидов способствовала получению дополнительно стандартных кочанов капусты 11,1 и 6,7 т/га, соответственно.

Показано, что предпосевная обработка семян капусты положительно влияла на ее биохимические показатели, повышая содержание витамина С и сахаров.

Выполненные исследования и научно-прикладные результаты позволяют рекомендовать предпосевную обработку семян 0,01%-ными растворами препарата Экостим и биорегуляторов из *Veronica officinalis* в качестве элемента в технологии выращивания капусты.

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IDENTIFICATION OF TUNISIAN CASTOR GENOTYPES (*RICINUS COMMUNIS* L.) USING SCOT MARKERS

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In the present investigation, 20 genotypes of Tunisian castor were analyzed using 5 start codon targeted (SCoT) markers. These primers produced total 33 fragments across 20 Tunisian castor genotypes, of which 26 (78.70%) were polymorphic with an average of 5.20 polymorphic fragments per primer and number of amplified fragments ranged from 4 (SCoT 66) to 9 (SCoT 65). The polymorphic information content (PIC) value ranged from 0.652 (ScoT 65) to 0.816 (SCoT 61) with an average of 0.738. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. The hierarchical cluster analysis showed that the Tunisian castor genotypes were divided into 3 main clusters. Cluster 1 contained 4 castor genotypes from region Gabes (G-1, G-2, G-4 and G-5). Cluster 2 contained 4 castor genotypes from region Mornag (M-1, M-2, M-4, M-5). Cluster 3 contained 12 Tunisian castor genotypes and was divided into subcluster 3a, 3b, and 3c. Subcluster 3a contained one genotype from region Mornag (M-3), one genotype from region Mednine (MD-5) and 2 genotypes from the region Kebili (K-2 and K-3). Subcluster 3b contained 2 castor genotypes from region Kebili (K-1 and K-5). Subclaster 3c contained five genotypes from region Ksar jedid (KJ-1, KJ-2, KJ-3, KJ-4, KJ-5) and one genotype from region Kebili (K-4). Two Tunisian castor genotypes of 3c subcluster (K-1 nad K-4) were genetically the closest. We can assume that they have a close genetic background. The present study shows the effectiveness of employing SCoT markers in analysis of castor and would be useful for further studies in population genetics, conservation genetics, and genotypes improvement.

Keywords: Tunisian Ricin, molecular markers, SCoT analysis, polymorphism, dendrogram, PIC

Introduction

Castor bean (*Ricinus communis* L.) has recently been highly rated as a source of raw material (oil) for biodiesel production, because beyond its high oil content (25–55%), it is a culture of great social appeal in Brazil by intensive use of workmanship in the field and allows for intercropping with other crops like beans, groundnuts or maize (Madail et al., 2007). In addition, castor bean cultivation is encouraged in areas of low water availability and is genetically improved to produce biofuel (Evangelista et al., 2004).

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These types of molecular techniques included random amplified polymorphic DNA (RAPD) (Štefúnová et al., 2015), amplified fragment length polymorphism (AFLP) (Molin et al., 2013), inter-simple sequence repeat (ISSR) (Žiarovská et al., 2013) and simple sequence repeats (SSRs) (Shehata et al., 2009). These marker systems are useful for biodiversity analyses, phylogenetic studies, germplasm management, cultivar identification, and other applications (Luo et al., 2010). Recently, a simple novel DNA marker technique namely start codon targeted (SCoT) polymorphism, was developed by Collard and Mackill (2009). Primers for SCoT marker analysis were designed from the conserved region surrounding the translation initiation codon, ATG (Sawant et al., 1999). Suitability of SCoT markers for the construction of genetic maps, fingerprinting and phylogenetic studies has been proved by many authors. In many crops, such as citrus (Mahjbi et al., 2015) and castor (Kallamadi et al., 2015).

The goals of this study were to examine the effectiveness of SCoT markers (SCoT 66, SCoT 65, SCoT 63, SCoT 62, SCoT 61) for analysis of genetic diversity of Tunisian castor and to study genetic relationships among 20 Tunisian castor accessions.

Material and methods

Plant material and DNA extraction

Ricin lines (20) were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia. Regions of origin of analyzed genotypes of Tunisian ricin: KJ – Ksar jedid, K – Kebili, G – Gabes, M – Mornag, MD – Mednine. Genomic DNA was isolated from the 14 days leaves with GeneJET Plant Genomic DNA Purification Mini Kit according to the manufacturer's instructions. Castor genotypes were grown in a growth chamber on humus soil.

SCoT amplification and statistical analysis

A total of 5 SCoT primers (SCoT 66, SCoT 65, SCoT 63, SCoT 62, SCoT 61) developed by Collard and Mackill (2009) were selected for the present study (Table 1). Each 15 µL amplification reaction consisted of 1.5 µL (100 ng) template DNA, 7.5 µL Master Mix (Genei, Bangalore, India), 1.5 µL 10 pmol primers, and 4.5 µL distilled water. Amplification was performed in a programmed thermocycler (Biometra, Germany) using the following program: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; a final extension at 72 °C for 5 min. Amplified products were separated on 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system Grab-It 1D pre-Windows.

The SCoT bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The binary data generated were used to estimate the level of polymorphism by dividing the polymorphic bands by the total number of scored bands and to prepare a dendrogram. A dendrogram was constructed based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the help of SPSS professional statistics version 17 software package. For the

assessment of the polymorphism between genotypes maize and usability SCoT markers in their differentiation, we used polymorphic information content (PIC) (Weber, 1990).

Table 1 List of used SCoT primers (Collard and Mackill, 2009)

SCoT Primers	Primer sequence (5´-3´)
SCoT 66	ACCATGGCTACCAGCGAG
SCoT 65	ACCATGGCTACCACGGCA
SCoT 63	ACCATGGCTACCACGGGC
SCoT 62	ACCATGGCTACCACGGAG
SCoT 61	CAACAATGGCTACCACCG

Results and discussion

In this work, 5 primers were screened for PCR amplification of DNA and SCoT analysis in 20 Tunisian castor genotypes. Table 1 and Table 2 shows sequences of these primers, the total number of amplified fragments from 20 Tunisian castor genotypes, the number of polymorphic bands and the polymorphic information content for each primer. Five primers produced 33 DNA fragments (Table 2) with an average of 6.60 bands per primer. From these five primers, primers SCoT 65 was the most polymorphic, where 9 polymorphic amplification products were detected. The lowest number of amplified polymorphic fragments (4) was detected by primer SCoT 66. Of the 33 amplified bands, 26 (78.70%) were polymorphic, with an average of 5.20 polymorphic bands per primer. To determine the level of polymorphism in the analyzed group of castor genotypes, polymorphic information content (PIC) was calculated (Table 2).

Table 2 The statistical characteristics of the SCoT markers used in castor

SCoT Primers	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	PIC
SCoT 66	4	3	75.00	0.729
SCoT 65	9	7	77.78	0.652
SCoT 63	8	6	75.00	0.780
SCoT 62	5	4	80.00	0.715
SCoT 61	7	6	85.71	0.816
Average	6.60	5.20	78.70	0.738
Total	33	26	–	–

The polymorphic information content (PIC) value ranged from 0.652 (ScoT 65) to 0.816 (SCoT 61) with an average of 0.738. The dendrogram of genetic relationships among 20 Tunisian castor genotypes based on 5 SCoT markers was constructed (Figure 1).

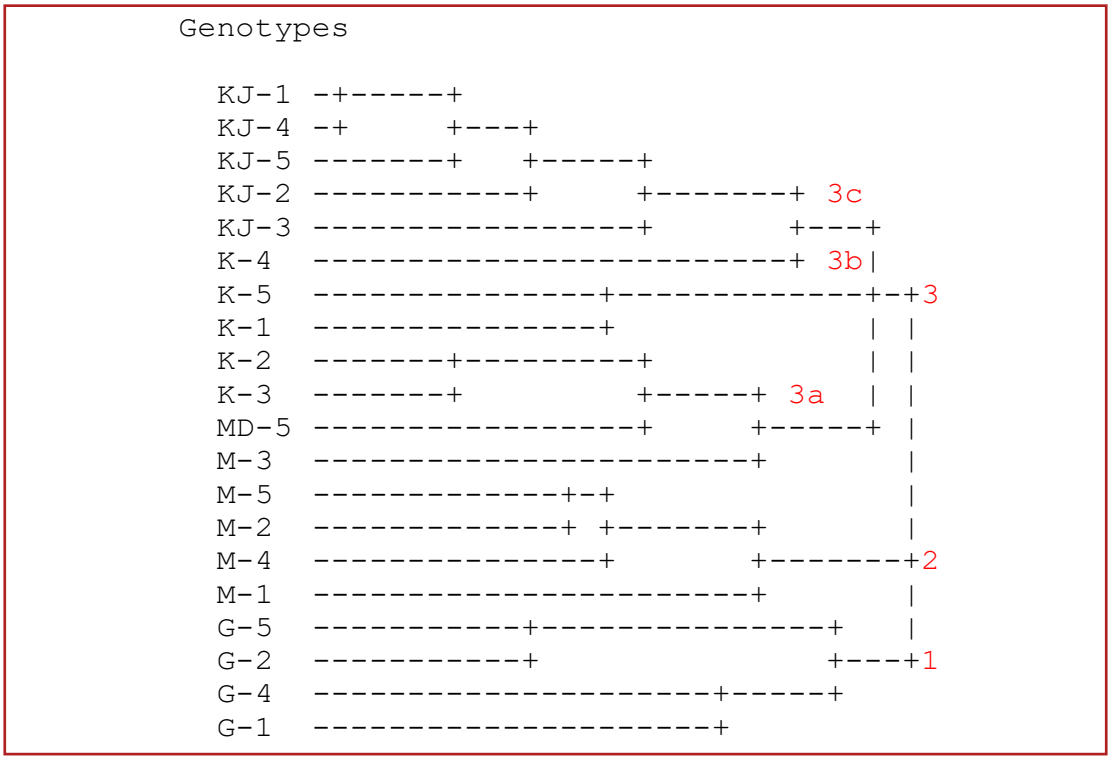


Figure 1 Dendrogram of 20 Tunisian castor genotypes prepared based on 5 SCoT markers. Regions of origin of analyzed genotypes of Tunisian ricin
KJ – Ksar jedid; K – Kebili; G – Gabes; M – Mornag; MD – Mednine

The hierarchical cluster analysis showed that the Tunisian castor genotypes were divided into 3 main clusters. Cluster 1 contained 4 castor genotypes from region Gabes (G-1, G-2, G-4, and G-5). Cluster 2 contained 4 castor genotypes from region Mornag (M-1, M-2, M-4, M-5). Cluster 3 contained 12 Tunisian castor genotypes and was divided into subcluster 3a, 3b, and 3c. Subcluster 3a contained one genotype from region Mornag (M-3), one genotype from region Mednine (MD-5) and 2 genotypes from the region Kebili (K-2 and K-3). Subcluster 3b contained 2 castor genotypes from region Kebili (K-1 and K-5). Subcluster 3c contained five genotypes from region Ksar jedid (KJ-1, KJ-2, KJ-3, KJ-4, KJ-5) and one genotype from region Kebili (K-4). Two Tunisian castor genotypes of 3c subcluster (K-1 nad K-4) were genetically the closest. We can assume that they have a close genetic background (Figure 1). Lower average polymorphism (21%) obtained by SCoT technique was detected by Kallamadi et al. (2015) who analysed molecular diversity of castor (*Ricinus communis* L.). Out of a total of 108 bands, 23 (21%) were polymorphic with an average of 2.1 polymorphic bands per primer. The total number of bands per primer varied from 5 and 20 in the molecular size range of 100–3,000 bp. The PIC varied from 0.06 for SCoT28 to 0.45 for SCoT12 with an average of 0.24. In the present study of Vivodík et al. (2018), the representatives of the genus castor collected

from 12 different parts of Tunisia were differentiated by the DNA fingerprinting patterns using 37 SCoT primers. PCR amplification of DNA using 37 primers for SCoT analysis produced 268 DNA fragments that could be scored in all 56 genotypes of Tunisian castor. The number of amplified fragments varied from 4 (SCoT 45, SCoT 31 and ScoT 17) to 10 (SCoT 3, SCoT 11, SCoT 14, SCoT 18 and SCoT 12). Of the 268 amplified bands 230 were polymorphic, with an average of 6.22 polymorphic bands per primer. To determine the level of polymorphism in the analysed group of Tunisian castor genotypes polymorphic information content (PIC) was calculated. The lowest values of polymorphic information content were recorded for SCoT 17 (0.411) and the the highest PIC values were detected for SCoT 14 (0.868) with an average of 0.751. Petrovičová et al. (2017) study genetic variability among the set of 45 rye genotypes using 8 SCoT markers. Amplification of genomic DNA of 45 genotypes, using SCoT analysis, yielded 114 fragments, with an average of 14.25 polymorphic fragments per primer. The hierarchical cluster analysis showed that the rye genotypes were divided into 2 main clusters. Chai et al. (2017) investigate the optimal number of individuals that may represent the genetic diversity of a single population, using Start Codon Targeted (SCoT) markers. Two cultivated varieties and two wild accessions were evaluated using five SCoT primers, also testing different sampling sizes: 1, 2, 3, 5, 8, 10, 20, 30, 40, 50, and 60 individuals. Cluster analysis by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and structure placed the 240 individuals into four distinct clusters. Balážová et al. (2017) used eighteen primers to study polymorfism of 8 oat genotypes. Altogether 153 different fragments were amplified of which 67 were polymorphic with an average number of 3.72 polymorphic fragments per genotype. The number of polymorphic fragments ranged from one (SCoT9, SCoT62) to nine (SCoT40). The polymorphic information content ranged from 0 (SCoT9, SCoT62) to 0.876 (SCoT40) with an average of 0.524.

Conclusion

In summary, SCoT marker analysis was successfully developed to evaluate the genetic relationships among the genus castor accessions originated from the various area. The hierarchical cluster analysis showed that the Tunisian castor genotypes were divided into 3 main clusters. Cluster 1 contained 4 castor genotypes from region Gabes (G-1, G-2, G-4, and G-5). Cluster 2 contained 4 castor genotypes from region Mornag (M-1, M-2, M-4, M-5). Cluster 3 contained 12 Tunisian castor genotypes and was divided into subcluster 3a, 3b, and 3c. Subcluster 3a contained one genotype from region Mornag (M-3), one genotype from region Mednine (MD-5) and 2 genotypes from the region Kebili (K-2 and K-3). Subcluster 3b contained 2 castor genotypes from region Kebili (K-1 and K-5). Subcluster 3c contained five genotypes from region Ksar jedid (KJ-1, KJ-2, KJ-3, KJ-4, KJ-5) and one genotype from region Kebili (K-4). Two Tunisian castor genotypes of 3c subcluster (K-1 and K-4) were genetically the closest. We can assume that they have a close genetic background. Polymorphism revealed by SCoT technique was so abundant and could be used for molecular genetics study of the castor accessions, providing high-valued information for the management of germplasm, improvement of the current breeding strategies, and conservation of the genetic resources of castor species.

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CHANGE OF CELLS AND LAYERS SIZES OF HEMP (*CANNABIS SATIVA* L.) BAST FIBERS IN THE SYNTHETIC BREEDING PROCESS

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The sizes (length and width) of cells and layers of primary and secondary bast fibers of hemp (*Cannabis sativa* L.) were reduced due to self-pollination (the phenomenon of inbred depression). The average length of the primary bast fibers cells decreased from 66.15 to 60.45 μm and cell width decreased from 40.05 to 36.30 μm. The average length of the secondary bast fibers cells decreased from 26.55 to 21.75 μm and cell width decreased from 15.75 to 14.25 μm. The values of these anatomical characteristics have increased as a result of free pollination and hybridization of inbred lines and the creation of a synthetic population (the phenomenon of heterosis). The sizes of the primary bast fibers cells in synthetic population plants were 73.95 × 47.40 μm and the sizes of the secondary bast fibers cells was 32.70 × 22.05 μm. The thickness of the primary fiber layer in the Hlukhivsky 58 variety was 230.25, inbred lines were 155.25, and the synthetic population was 264.00 μm. The thickness of the second fiber layer was 99.75, 77.25 and 116.75 μm, the total thickness of the fiber layer was 330.00, 232.50 and 380.75 μm, respectively. The proposed method for the creation of synthetic populations of monoecious hemp by the scheme "variety – inbred lines – synthetic population" is effective at increasing the fiber content of plants.

Keywords: hemp, inbreeding, synthetic breeding, fiber, cell

Вступ

Коноплі посівні (*Cannabis sativa* L.) після певного періоду забуття набувають все більшої популярності і поширення як сільськогосподарська культура комплексного використання, основними напрямами якого є волокнистий, насіннєво-олійний, біоенергетичний і медичний, однак традиційно вони є луб'яною рослиною, у зв'язку з чим розроблені дієві методи і способи селекції, які дали змогу суттєво збільшити вміст волокна в стеблах (Laiko, 2012; Vyrovets, 2015). Ось чому вивченню анатомічної будови поперечного зрізу стебла і волокнистого шару зокрема, завжди приділялась значна увага, що дозволяло встановити чинники формування високої кількості і якості волокна в онтогенезі та виявити цінні генотипи.

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Особливістю анатомічної будови є те, що коноплям властиве формування первинного і вторинного лубу. Первинний луб (флоема) розміщений за первинною твірною тканиною перицикломом (перикамбієм), внаслідок діяльності якого він і виникає. Флоема складається із ситоподібних (решітчастих) трубок, товстостінних прозенхімних клітин первинних луб'яних волокон і тонкостінних клітин луб'яної (флоемної) паренхіми. Первинні луб'яні волокна мають довжину до 50–75 мм і зливаються у суцільне кільце пучків. Тип пучків – колатеральні, складні, відкриті, розміщені в одне коло. За первинним лубом залягає вторинний луб (вторинна кора), що також складається з ситоподібних трубок, вторинних луб'яних волокон і луб'яної паренхіми. Вторинне волокно має довжину не більше 4 мм. За луб'яною паренхімою залягає вузька смужка генеративної тканини – камбію, внаслідок діяльності якого відбувається перехід до вторинної будови (Myhal, 2011).

Волокнисті пучки конопель (часто вони зливаються у суцільне кільце) складаються з окремих клітин, які називаються елементарними волокнами. У більшості випадків луб'яні волокна мають веретеноподібну форму з загостреними чи злегка притупленими кінцями. Важливими ознаками елементарних волокон є форма, розмір клітини (діаметр, або довжина і ширина) та каналу, товщина оболонок та їх шаруватість. Вони значною мірою визначають якісні показники волокна як сировини для переробної промисловості (Myhal, 2011).

Розміри клітин первинних луб'яних волокон на поперечному зрізі у середньому становлять від 5 до 55 мкм, а вторинних – від 10 до 20 мкм. Характерною особливістю конопель є шаруватість оболонки елементарних волокон. Первинна оболонка впритул прилягає до серединної пластинки й органічно поєднується з нею. Первинна оболонка тонка й не буває шаруватою. Вторинна оболонка складає основну масу клітини луб'яних волокон. Вона виникає за первинною оболонкою і має шарувату структуру, що значно потовщує її (Myhal, 2011). Вторинна клітинна оболонка є нерівномірною за складом і структурою. У ній виділяють три відмінні шари, умовно позначені як S1, S2 і S3. Після відкладання шару S1, товщина якого залишається незмінною протягом усього періоду формування вторинної оболонки, відкладається шар S3, який потім модифікується в S2, що певний час збільшується. Отже, формування вторинної оболонки супроводжується інтенсивною модифікацією новоутворених шарів. Інтрузивний ріст волокон у довжину передує утворенню вторинної клітинної оболонки, а не поєднується з ним, як вважали раніше. Саме формування потовщеної клітинної оболонки дозволяє рослинним волокнам набувати міцності, необхідної для реалізації їх механічних функцій (Chernova, 2007; Snegireva et al., 2015; Chernova et al., 2018).

До фази бутонізації у стеблах конопель спочатку формується більш якісне первинне волокно, а з початком закладання генеративних органів спостерігається утворення гіршого за якістю вторинного волокна, хоча у жіночих і однодомних рослин ініціація росту вторинного волокна може розпочинатися раніше (Mediavilla et al., 2001). На ранніх стадіях онтогенезу клітини вторинних луб'яних волокон зустрічаються лише внизу стебла, суттєве утворення вторинного волокна спостерігається на початку цвітіння або при досягненні рослиною висоти більше 2,25 м і/або діаметра більшого за

1 см (Hernandez, 2007). Кількість клітин первинних луб'яних волокон на поперечному зрізі стебла зростає приблизно аж до початку суцвіття, а потім зменшується, а кількість клітин вторинних луб'яних волокон поступово зменшується від другого міжвузля, і приблизно в зоні початку суцвіття вторинне волокно стає відсутнім (Chernova, 2007; Snegireva et al., 2015).

У селекції на підвищення волокнистості (маси і вмісту волокна) використовуються різні методи і прийоми. Одним із шляхів створення нового селекційного матеріалу в цілому і збільшення вмісту волокна в стеблах конопель зокрема є створення самозапилених ліній (Layko et al., 2000; Mishchenko and Laiko, 2013) та подальша їх міжлінійна, сортолінійна і лінійносортова гібридизація з добором в гібридних популяціях. Доведено, що таким гібридам характерні ефект гетерозису за багатьма цінними господарськими ознаками (Mishchenko, 2016a), порівняно висока стабільність ознаки однодомності (Mishchenko, 2015) і відсутності канабіноїдних сполук (Mishchenko, 2016b) як на ранніх етапах селекції, так і в процесі подальшого розмноження. Важливе значення при цьому відіграє оптимальний підбір компонентів схрещувань на основі встановлення комбінаційної здатності різних самозапилених ліній (Mishchenko, 2017), що суттєво може підвищити продуктивність.

Разом з тим, інбридинг конопель може бути використаний з метою очищення популяції від одного або декількох небажаних рецесивних алелів і стабілізації таким чином певних селекційних ознак чи їх комплексу в існуючому селекційному матеріалі (сорті) шляхом створення однорідних самозапилених ліній з наступним об'єднанням їх в єдину синтетичну популяцію. Комбінація (суміш) самозапилених ліній рідко використовується як метод для покращення перехреснозапильних культур, тому що потомство, яке утворюється у результаті самозапилення, зазвичай має низьку життєздатність насіння, також при комбінації самозапилених ліній спостерігається тенденція до збільшення частки рослин, схильних до самозапилення (Briggs and Noulz, 1972). Не зважаючи на це, даний метод існує і може застосовуватися на практиці, зокрема не тільки для поліпшення селекційного матеріалу, а й для створення нового, оскільки в процесі близькоспорідненого розмноження популяція диференціюється за багатьма ознаками на низку самозапилених ліній, які мають досить суттєві відмінності порівняно з вихідною формою.

Мета і завдання дослідження – встановити особливості зміни розмірів (довжини і ширини) клітин та шарів первинних і вторинних луб'яних волокон в процесі синтетичної селекції, яка здійснюється за схемою «сорт – самозапилені лінії – синтетична популяція».

Матеріали та методи

Вихідний матеріал та методика створення синтетичних популяцій

Для самозапилення добирали селекційні сім'ї сорту однодомних конопель Глухівські 58 з бажаними ознаками чи їх комплексом (у даному випадку з високими показниками загальної довжини і маси стебла, маси і вмісту волокна). Насіння

висівали в умовах вегетаційного будинку, рослини ізолювали до цвітіння індивідуальними ізоляторами з агроволокна. Самозапилення проводили протягом 4–6 поколінь до настання інбредного мінімуму, отримання однорідних та стабільних ліній. Паралельно із самозапиленням здійснювали вивчення інбредних ліній у розсаднику оцінки за основними селекційними ознаками за площі живлення рослин 30 × 5 см. Для розмноження добирали лінії з наступними характеристиками: найменший ступінь депресії, відсутність шкідливих мутацій і морфозів, бажаний рівень прояву селекційних ознак, відсутність у потомстві рослин з канабіноїдними сполуками, переважання у статевому складі однодомної фемінізованої матірки та відсутність плосконі однодомних конопель. Решту самозапилених ліній вибраковували. На завершальних етапах близькоспорідненого розмноження залишали лише ті лінії, які в результаті самозапилення утворювали найменшу кількість насіння, а за даними розсадника оцінки за умови відсутності просторової ізоляції і вільного запилення пилком різного походження характеризувалися високою насіннєвою продуктивністю. Такий підхід дозволяв добирати генотипи, які меншою мірою здатні до самозапилення, а більшою мірою – до перехресного запилення (чи самонесумісності), що у майбутньому підвищувало життєздатність і продуктивність потомства. Для отримання синтетичної популяції проводили вільне схрещування кращих 5–7 самозапилених ліній, досить подібних одна до одної. Для цього їх висівали в умовах вегетаційного будинку у двократній повторності з випадковою послідовністю на ділянці розміром 2,5 × 2,5 м, яку перед цвітінням ізолювали груповим ізолятором. Отримували насіння syn-1. У розсаднику оцінки вивчали потомство кожної лінії за комбінаційною здатністю, на основі чого для отримання наступного покоління синтетичної популяції з кожної лінії (як правило, використовували 3–5 ліній), що добре себе зарекомендувала за досліджуваними ознаками і властивостями, добирали однакову кількість (масу) насіння, змішували і висівали для отримання syn-2 і потім syn-3.

Анатомічний аналіз

Для анатомічного аналізу відбирали типові стебла конопель з однаковим діаметром (1 см) у фазу початку дозрівання насіння – код за шкалою BBCH 81 (Mishchenko et al., 2017), коли волокно повністю сформоване. Площа живлення рослин 30 × 5 см. Зі стебла живих рослин конопель на рівні четвертого міжвузля брали відрізок кори з частиною деревини завдовжки 2–3 см. За допомогою гострого леза готували поперечні зрізи та тимчасові анатомічні препарати. Анатомічний аналіз волокнистих структур стебел конопель, зокрема довжину і ширину клітин та товщину шарів первинних і вторинних луб'яних волокон, проводили з використанням мікроскопа при збільшенні в 600, 300 та 150 разів. Виміри здійснювали за допомогою окуляр-мікрометра з умовними поділками (умовними одиницями), які потім виражали в мікрометрах (мкм). Проводили по 10 вимірювань 10 стебел сорту, самозапилених ліній і синтетичної популяції.

Статистичний аналіз

Статистичну обробку експериментальних даних проведено за параметрами варіаційної статистики (середнє арифметичне, похибка вибіркового середнього, коефіцієнт варіації) та *t*-критерієм Стьюдента (Dospekhov, 1973).

Результати та їх обговорення

У поколіннях від самозапилення рослин однодомних конопель, як правило, спостерігається явище інбредної депресії, яке супроводжується зменшенням показників селекційних ознак, появою мутацій та морфозів (Mishchenko, 2011), зміною статевої структури (Mishchenko, 2013). Воно продовжується до настання інбредного мінімуму, який у конопель, залежно від ознаки, наступає у 3–6 поколінні. У окремих сімей різко виражена інбредна депресія не спостерігається або ж продуктивність знижується не на достовірному рівні значимості. Ці ж закономірності характерні і для ознак волокнистості: довжини і діаметру стебла, маси стебла і волокна, вмісту волокна (Mishchenko and Laiko, 2013).

Ефективність створення синтетичних популяцій однодомних конопель з метою поліпшення існуючого селекційного матеріалу (сорту) чи створення нового не викликає сумнівів. Так, у синтетичної популяції, створеної на основі вільного запилення інбредних ліній за описаною експериментальною методикою, відбувається підвищення ознак продуктивності і навіть гетерозисний ефект, який полягає у перевищенні вихідних форм (сортів) за ознаками довжини стебла (264,0 порівняно з 223,9 см), маси стебла (18,23 і 17,84 г), маси волокна (6,05 і 5,62 г) і вмісту волокна (33,53 і 32,70 %) (Таблиця 1). Разом з тим логічно виникає питання, за рахунок чого змінюються ознаки волокнистості, зокрема кількість чи вміст волокна у рослинах. Відповідь на це проблемне питання дає анатомічний аналіз поперечного зрізу стебла, а саме – встановлення розмірів клітин первинних і вторинних луб'яних волокон, товщини шарів первинного і вторинного волокна, які в сукупності з висотою рослин, технічною довжиною (частиною стебла до зони суцвіття) і діаметром стебла формують продуктивність рослин конопель за волокном, що було передбачено наступним етапом наших досліджень.

Порівняно з вихідним сортом Глухівські 58, у самозапилених ліній спостерігається тенденція до зменшення розмірів клітин елементарних луб'яних волокон. Довжина клітин первинних луб'яних волокон у середньому зменшується з 66,15 до 60,45 мкм, ширина – з 40,05 до 36,30 мкм. Довжина клітин вторинних луб'яних волокон у середньому зменшується з 26,55 до 21,75 мкм, ширина – з 15,75 до 14,25 мкм. У синтетичної популяції встановлено збільшення розмірів досліджуваних клітин. Вони з високою достовірністю перевищують не тільки показники самозапилених ліній, а й сорту. Так, довжина клітин первинних луб'яних волокон у рослин синтетичної популяції становила 73,95, ширина – 47,40 мкм, вторинних луб'яних волокон – 32,70 і 22,05 мкм відповідно (Таблиця 2).

Таблиця 1 Зміна ознак волокнистості конопель сорту Глухівські 58 в процесі синтетичної селекції

Table 1 Changes in the characteristics of the fiber content of the Hlukhivsky 58 hemp variety in the synthetic breeding process

Варіант	Ознаки							
	довжина стебла (см)		маса стебла (г)		маса волокна (г)		вміст волокна (%)	
	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)
Сорт	223,9 ±4,57	9,1	17,84 ±1,284	34,6	5,62 ±0,412	40,7	32,70 ±0,616	7,7
Самозапилена лінія	212,9 ±6,02	11,0	16,74 ±1,824	42,2	5,49 ±0,608	42,9	32,72 ±0,413	4,9
Синтетична популяція	264,0 ±4,02	5,9	18,23 ±1,214	25,8	6,05 ±0,371	23,7	33,53 ±0,712	8,9

Примітка: $\bar{x}$ – середнє арифметичне; $s_{\bar{x}}$ – похибка вибіркового середнього; V (%) – коефіцієнт варіації

Таблиця 2 Зміна розмірів клітин елементарних луб'яних волокон на поперечному зрізі стебла конопель сорту Глухівські 58 в процесі синтетичної селекції

Table 2 Change of the sizes of elemental bast fibers cells on the stem cross-section of the Hlukhivsky 58 hemp variety in the synthetic breeding process

Варіант	Розміри клітин первинних луб'яних волокон, мкм							
	первинних				вторинних			
	довжина		ширина		довжина		ширина	
	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)
Сорт	66,15 ±2,980	20,1	40,05 ±1,901	21,2	26,55 ±1,255	21,1	15,75 ±0,718	20,4
Самозапилена лінія	60,45 ±2,222	16,4	36,30 ±1,150	14,2	21,75 ±0,894	18,4	14,25 ±0,481	15,1
Синтетична популяція	73,95 ±2,898	17,5	47,40 ±2,626	24,8	32,70 ±1,845	25,2	22,05 ±1,603	32,5
P_{1-2}					P <0,01			
P_{2-3}	P <0,001		P <0,001		P <0,001		P <0,001	
P_{1-3}			P <0,05		P <0,01		P <0,001	

Примітка: $\bar{x}$ – середнє арифметичне; $s_{\bar{x}}$ – похибка вибіркового середнього; V (%) – коефіцієнт варіації; (P) – рівень значимості

Аналогічно зменшується товщина і кількість вторинних оболонок первинних і вторинних клітин луб'яних волокон, які значною мірою детермінують якість волокна для промислового виробництва.

Відомо, що якісне волокно конопель визначають правильно сформовані елементарні волокна, які мають округлу чи ізодіаметричну форму, невеликий діаметр клітин, опуклий контур оболонки (без зиґзаґів), малий канал (точковий або щілиноподібний), товсту оболонку з помірно рідкою шаруватістю і компактне з'єднання волокон в пучках (Myhal, 2011).

Також установлено, що у процесі створення вихідного матеріалу за схемою «сорт – самозапилені лінії – синтетична популяція» змінюється товщина волокна на поперечному зрізі стебла. У результаті інбридингу вона зменшується, а у результаті вільного самозапилення при створенні синтетичної популяції – збільшується. Товщина шару первинного волокна у сорту Глухівські 58 становила 230,25, самозапилених ліній – 155,25, синтетичної популяції – 264,00 мкм, вторинного волокна – 99,75, 77,25 і 116,75 мкм, загальна товщина шару волокна – 330,00, 232,50 і 380,75 мкм відповідно. За досліджуваними ознаками різниця між сортом і самозапиленими лініями, самозапиленими лініями і синтетичною популяцією, вихідним сортом і синтетичною популяцією за невеликим винятком є достовірною на високому рівні значимості (Таблиця 3).

Таблиця 3 Зміна товщини шару волокна на поперечному зрізі стебла конопель сорту Глухівські 58 в процесі синтетичної селекції

Table 3 Change of the thickness of the fiber layer on the stem cross-section of the Hlukhivsky 58 hemp variety in the synthetic breeding process

Варіант	Товщина шару волокна, мкм				
	первинного		вторинного		загальна
	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x} \pm s_{\bar{x}}$	V (%)	$\bar{x}$
Сорт	230,25 ±8,992	17,5	99,75 ±4,900	22,0	330,00
Самозапилена лінія	155,25 ±8,236	23,7	77,25 ±4,390	25,4	232,50
Синтетична популяція	264,00 ±10,347	17,5	116,75 ±8,186	31,4	380,75
P_{1-2}	P <0,001		P <0,01		–
P_{2-3}	P <0,001		P <0,001		–
P_{1-3}	P <0,05				–

Примітка: $\bar{x}$ – середнє арифметичне; $s_{\bar{x}}$ – похибка вибіркового середнього; V (%) – коефіцієнт варіації; P – рівень значимості.

Порівняно з іншими генотипами, для сорту Глухівські 58 та селекційного матеріалу, створеного з його використанням, характерні досить великі розміри клітин луб'яних волокон. Зауважимо, що ознаки як волокнистого шару в цілому, так і окремих клітин луб'яних волокон зокрема, у сортів і колекційних зразків конопель різного генетичного походження характеризуються високим ступенем мінливості, тому у селекційних дослідженнях проблема їх подальшого вивчення залишається актуальною (Kryvosheieva, 2000; Onupriienko, 2008b; Myhal, 2011; Mishchenko and Kmets, 2017). Особливо виявлено значні відмінності в анатомічній будові, розмірах та розміщенні клітин елементарних волокон та шарів первинного і вторинного волокна у рослин високоволокнистих сортів, порівняно з низьковолокнистими (Kryvosheieva, 1999; Onupriienko, 2008a). Виходячи з цього, аналіз анатомічної будови стебла конопель до цвітіння може бути використано у практичній селекції, наприклад, при доборі рослин у селекційних розсадниках (Laiko et al., 2013) або у фазу біологічної стиглості для визначення якості волокна за непрямими ознаками – формою, розмірами, будовою

вторинних оболонок і каналу клітин елементарних волокон та співвідношенням товщини шарів більш якісного первинного волокна, порівняно з більш грубим вторинним тощо.

У самозапилених ліній відбувається зменшення розмірів клітин елементарних волокон (особливо первинних), товщини шарів залягання волокна і маси волокна з рослин. Ступінь інбредної депресії у кожному конкретному випадку залежить від генотипу селекційного матеріалу. Зменшення маси волокна з рослини під впливом близькоспорідненого розмноження може відбуватися як шляхом зменшення розмірів клітин елементарних волокон, так і кількості таких клітин (і відповідно товщини шару волокна), або залежати від обох вказаних явищ одночасно (Mishchenko et al., 2015). Нашими дослідженнями установлено, що у конопель в процесі самозапилення відбувається депресія, а в результаті вільного запилення інбредних ліній і створення синтетичної популяції внаслідок отримання високогетерозиготних організмів спостерігається явище гетерозису не лише за морфологічними, а й анатомічними ознаками, які у своїй сукупності підвищують волокнистість селекційного матеріалу.

Висновки

Запропоновано і апробовано методику створення синтетичних популяцій однодомних конопель. У результаті самозапилення зменшуються розміри (довжина і ширина) клітин та шарів первинних і вторинних луб'яних волокон (явище інбредної депресії), а в результаті вільного запилення інбредних ліній і створення синтетичної популяції спостерігається збільшення величини даних ознак (явище гетерозису). Волокнистість рослин змінюється внаслідок сукупного впливу морфологічних і анатомічних ознак.

Подяка

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OPEN ACCESS

CONTENTS OF CATECHINS AND ANTHOCYANINS IN THE ABOVE-GROUND ORGANS OF PLANTS OF *PRUNUS SPINOSA* L.

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Carrying out phenological and biochemical studies, the prospect of using the studied varieties of a thorn in breeding programs for obtaining new adaptive, large-fruited varieties and cultivars have been established. The data on the content of catechins and anthocyanins in the bark, leaves, flowers, and fruits of cultivated plants and wild varieties of *Prunus spinosa* L. are presented. As a result of the study, it was found that the wild variety of *Prunus spinosa* has a significantly higher content of anthocyanins and catechins in the above-ground organs than cultural varieties. This indicates a higher adaptive capacity of wild varieties to unfavorable environmental factors in comparison with cultural varieties, as catechins and anthocyanins, as well as other representatives of flavonoids, are used as biochemical markers of species adaptability. According to the content of anthocyanins and catechins can predict the success of the introduction. The highest amount of catechins and anthocyanins found in the leaves. The flowers have the lowest content of anthocyanins and catechins. In the study of the contents of catechins and anthocyanins in fruits of plants cultivated and wild varieties of *Prunus spinosa*. It was found that the wild variety of *Prunus spinosa* (genotype Sp1) has the lowest content of catechins in the presence of the highest content of anthocyanins.

Keywords: *Prunus spinosa*, aboveground organs, variety, anthocyanins, catechins

Introduction

Modern botanical research of cultivated fruit plants is impossible without genetic analysis, selection, and use of the gene pool of their wild relatives (as they are a valuable reserve of genetic material), which provides resistance to biological factors of the environment.

Among the common plants of the family *Rosa*ceae Juss. (*Malus* Mill., *Pyrus* L., *Cydonia* Mill., etc.) subfamily Amygdaloideae Arn. is one of the most cultivated in temperate climatic latitudes, including the territory of Ukraine (Komarnickij et al., 1975). One of the reasons for this spread was probably the location of ancient human settlements in the area of the historical center of the origin of plants (Lee et al., 2001).

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Today, among wide varieties of species *Prunus* L. significant interest attracts thorns (*Prunus spinosa* L.), which is characterized by high productivity, frost and winter hardiness, resistance to pests and diseases, and is a valuable breeding material for further study. By successive selections and folk breeding among wild thorns were identified large-fruited varieties (Woldring, 2000). Therefore, the comparative ecological and biological, biochemical studies of cultivated and wild representatives of the species *Prunus* spinosa is relevant.

Occupying a fairly large area of distribution of *Prunus spinosa* is marked by a large polymorphism. In the middle of the species are not rare varieties with different ecological fitness, winter hardiness, and drought resistance. That is the last two characteristics are the most important when it is grown in culture and breeding. There are also large-fruited thorns whose average mass reaches 15 g, they grow in the South of the Volga region and Crimea (Eremin, 2003).

Phenological observations have shown that the biological characteristics of the studied representatives are closely related to the climatic conditions of the growth area. Thus, the phase of Bud blooming in the varieties of thorns of large-fruited and wild was observed in the first decade of April at an average daily temperature of +6–8 °C. Flowering varieties of thorns small-fruited and thorns "large-fruited" was observed earlier, for 4–6 days from their wild representative. The beginning of mass flowering occurred at a temperature of +10–15 °C.

Fruiting varieties of thorns cultural variety 1 and cultural variety 2 was noted earlier-in the third decade of August, the first decade of September, and lasted 25–30 days. It is established that the wild-growing thorn begins to bear fruit later, from the second decade of September to the third decade of October, and has a longer period of fruiting, in comparison with the previous ones (Golubkova et al., 2017).

Prunus spinosa is a honey plant. Juice from fruits and bark are used to color the tissues, the fruits themselves and the aboveground part of the plant are widely used in folk medicine. Planting of thorns is often used in landscaping, as a hedge. The fruits contain 8.9% sugar, 2.0% organic acids, up to 1.5% pectin. In the nuclei – up to 37% fat, amygdalin, and other substances. Thorn plants serve as a stock for undersized varieties of peach, apricot, and plum (Makarov, 1972; Koch et al., 2016).

Prunus spinosa is a valuable genetic resource and is a raw material for the pharmaceutical and agricultural industries, therefore, the main task of the acclimatization Department of fruit plants M.M. Gryshko National Botanical Gardens was the enrichment of biodiversity of stone fruit crops rare plants for wide use in the culture and learning biology.

Prunus spinosa has unique medicinal properties. If in many medicinal plants healing power has some one part, the thorn in this regard is unique – for medicinal purposes use not only its berries but also leaves, roots, flowers, branches and bark (Marchelak et al., 2017).

Thorn helps with diseases of the stomach, intestines, kidneys, and liver, as well as metabolic disorders, with a lack of vitamins in the body. It is a good sweatshop and antipyretic. The juice of the thorn berries has antiviral properties, so its purpose makes sense in disorders of the gastrointestinal tract as a result of helminthosis (Yuksel, 2015). Medicinal properties of

the fruit known in dysentery, food poisoning, colitis, non-specific varieties. Pharmacological preparations of blackthorn have anti-inflammatory, astringent, diuretic, expectorant, laxative and antibacterial properties (Chopik et al., 1983).

In homeopathic practice use leaves, young branches, bark, rhizomes, flowers, and fruits of thorns. Water infusion from the flowers of the plant is an effective laxative, soothing the nervous system, a diaphoretic and diuretic (Ozcan et al., 2008). Bark, young branches, roots of thorns have a diaphoretic and anti-sporadic effect (Ahmad et al., 1998). The leaves and flowers of the blackthorn are in the variety of decoction with the aim of increasing the elasticity of blood vessels, improve cell metabolism, increase the protective forces of the body (Marchelak et al., 2017). A major role in the formation of medicinal properties of thorn due to the action of the secondary metabolites of the class of flavonoids – catechins and anthocyanins (Lila, 2004, Horbowicz et al., 2008), which are also considered as environmental markers of plants and participate in adaptive reactions of the plant organism (Minaeva, 1978; Lukner, 1979; Levon et al., 2017).

The aim of this study was to determine the content of anthocyanins and catechins in the above-ground organs of *Prunus spinosa* plants, which will allow evaluating the level of adaptation of its different varieties.

Material and methodology

Biological material

For the study, we used a wild variety of *Prunus spinosa* (genotype Sp1), small-fruited variety (genotype Sp2) and large-fruited variety (genotype Sp3). Thorn large-fruited from the collection of M.M. Gryshko National Botanical Garden of NAS of Ukraine. All of them are grafted on the seedlings of cherry plum, which makes it possible to effectively use the genetic material, without the variation of root seedlings, together with other cultural varieties of the subfamily Prunoideae.

Biochemical analysis

Quantitative determination of the content of anthocyanins and catechins was carried out in the dried leaves and fruits of the middle tier of sprouts dried under shading in natural conditions in a well-ventilated area at a temperature of 20–25 and crushed in an electric mill.

The quantitative determination of catechins is based on the reaction of their condensation with vanillin (4-hydroxy-3-methoxybenzaldehyde). The reaction catalyst was concentrated hydrochloric acid. The optical density of the solution of condensation products was proportional to the initial concentration of catechins. The determination of the optical density was carried out with a green filter (maximum absorption 520 ±10 nm) (Kriventsov, 1982).

The quantity of anthocyanins was determined by the method of photoelectric colorimetry at a wavelength of 530 nm using an alcoholic extract of plant material homogenate was acidified to 3.5% hydrochloric acid (Kriventsov, 1982).

Measurements were carried out on a photoelectrocolorimeter Zalimp KF 77 (Poland).

Obtained data calculated using formulas:

$$C_{ant} = \frac{D \cdot V \cdot R \cdot H \cdot K}{l \cdot m}$$

$$C_{cat} = \frac{D \cdot V \cdot R \cdot H}{l \cdot m}$$

where: D – the optical density of the solution; V – the total amount of the extract and the average sample (ml); R – dilution ratio of the solution; l – working length of the cuvette (cm); H – aliquot part; m – linkage, the average of the sample (g); K – conversion factor

The number of parallel measurements was 3. The accuracy of the method was in the range of 2.5–4.8%. The obtained data were presented in mg/100 g of dry matter (DM) in terms of anthocyanidin.

Statistical analysis

Statistically processed data is shown on histograms as arithmetic means and their standard errors. The significance level was set at $\alpha = 0.05$. The statistical analysis was performed with IBM SPSS Statistics, release 23.0.

Results and discussion

According to our previous data on the biochemical properties of *Prunus spinosa*, the aboveground organs of this plant are rich in biologically active compounds. The content of cyanogenic glycoside prunasin in sprouts is in the range of 131–691 mg/100 g DM (Levon et al., 2007), catechin content in fresh fruits – 78.5 mg/100 g DM (Mustafaeva, 2013), vitamin C – 25.5 mg/100 g DM (Celik et al., 2017). Eight flavonoids were isolated from the flowers of *Prunus spinosa*: kaempferol, quercetin, kaempferol 3-O-alpha-L-arabinofuranoside, quercetin 3-O-alpha-L-arabinofuranoside, kaempferol 3-O-alpha-L-ramnopyranoside, kaempferol 7-O-alpha-L-ramnopyranoside, kaempferol 3-O-beta-D-xylopyranoside, kaempferol 3-O-(2''-E-p-coumaroyl)-alpha-L-arabinofuranoside. The last three have been found for the first time in this plant (Olszewska et al., 2001). Steroids and terpenoids are abundant in *Prunus spinosa*: oleanolic acid, alpha-amyrin, sitosterol, beta-Sitosterol-beta-D-glucoside (Wolbis et al., 2001).

Prunus spinosa has been found to be a good source of phenylpropanoid esters: 5-hydroxy-6-methoxy-7-O-beta-D-glucosyl coumarin (Crespo et al., 1992). *Prunus spinosa* is also a rich source of epicatechin (Kolodziej), cyanidin-3-glucoside (Werner et al., 1989) and caffeic acid (Olszewska et al., 2000). In the fruits of *Prunus spinosa* was discovered aliphatic components nonacosane ($C_{29}H_{60}$) and 10-nonacosanol (Kaminska et al., 1970).

As a result of our research, it was found that among all the studied varieties the leaves had the highest content of anthocyanins (255–281 mg/100 g DM) (Figure 1). The content of anthocyanins in the bark ranged from 50 to 163 mg/100 g DM. In the flowers, the low content of anthocyanins (12–81 mg/100 g of DM) was found. Among the small-fruited (Sp2) and

large-fruited cultural varieties (Sp3) of *Prunus spinosa*, the flowers of the large-fruited variety had a much higher content of anthocyanins than the small-fruited variety.

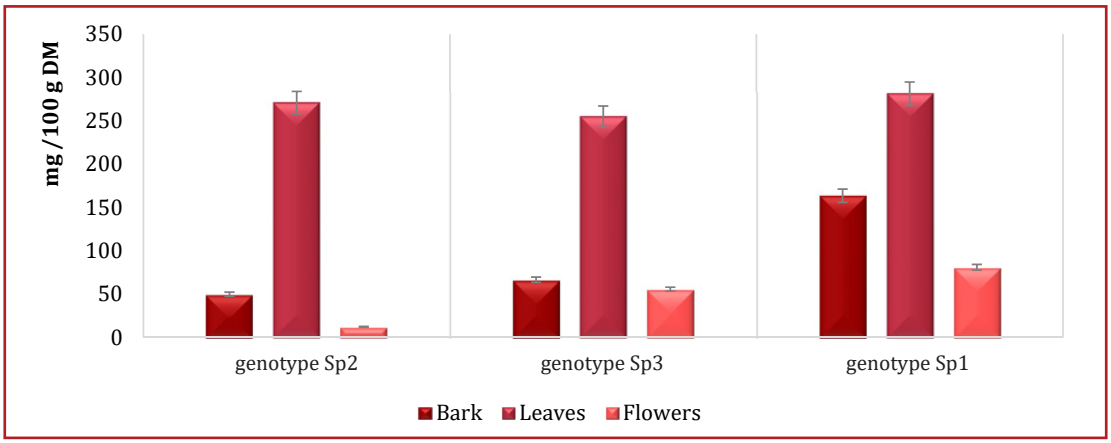


Figure 1 The content of anthocyanins in the above-ground organs of plants of *Prunus spinosa* L. (mg/100 g DM)

The greatest content of catechins determined in the leaves (617–1031 mg/100 g DM) (Figure 2). The content of catechins in the bark of the studied objects was much lower than in the leaves (440–684 mg/100 g DM). Flowers had a low content of catechins (162–216 mg/100 g DM). Among the small-fruited (Sp2) and large-fruited (Sp3) cultural varieties of *Prunus spinosa*, the flowers of the large-fruited variety had a slightly higher content of catechins (171 mg/100 g DM) than the small-fruited variety (162 mg/100 g DM).

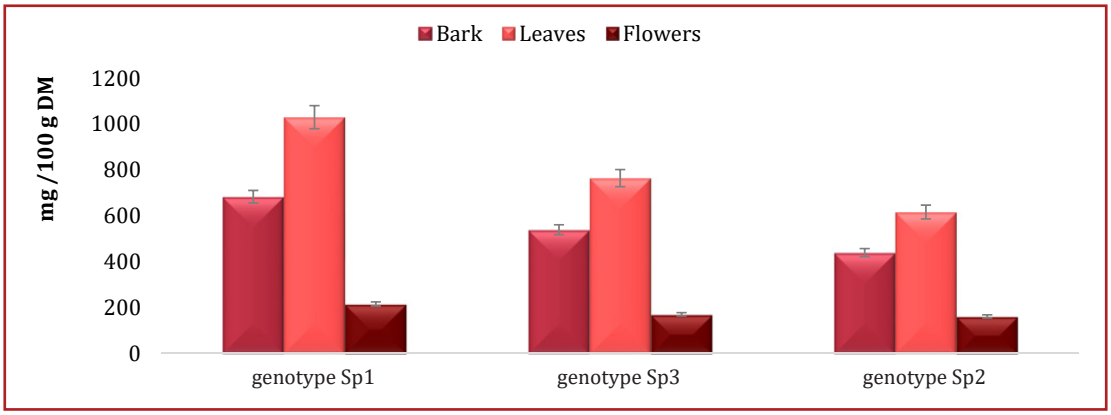


Figure 2 The content of catechins in the above-ground organs of plants of *Prunus spinosa* L. (mg/100 g DM)

It is interesting to note the fact that the wild variety of *Prunus spinosa* (Sp1) had a much higher content of anthocyanins and catechins in the aboveground organs than cultural varieties. This indicates a higher adaptive capacity of wild varieties to adverse environmental factors in

comparison with cultural varieties, as the accumulation of anthocyanins and catechins is one of the biochemical markers of the level of adaptation of species (Levon et al., 2016, 2017).

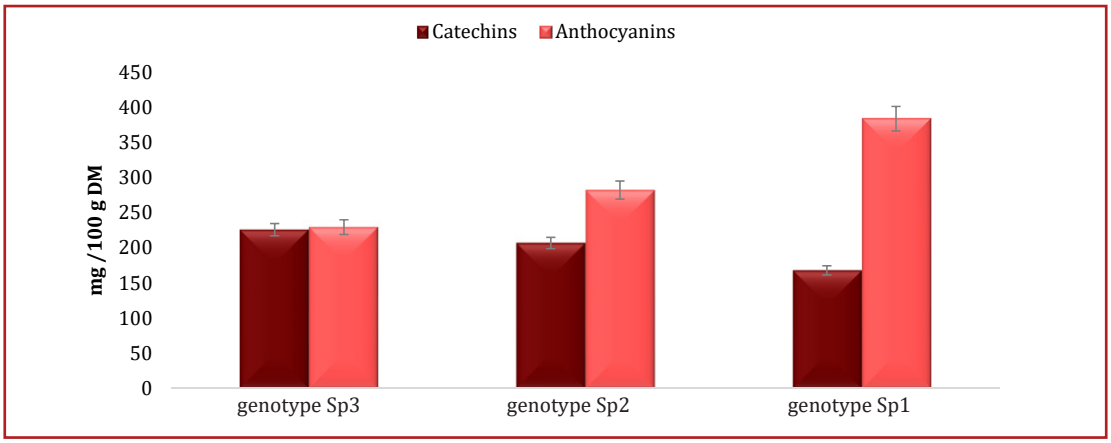


Figure 3 The content of catechins and anthocyanins in the fruits of plants of *Prunus spinosa* L. (mg/100 g DM)

In the study of the content of catechins and anthocyanins in the fruits of cultural and wild varieties of *Prunus spinosa* (Sp2), it was found that among all the studied varieties the greatest content of catechins were the fruits of the large-fruited cultural variety (Sp3) of *Prunus spinosa* (226 mg/100 g DM) (Figure 3). The wild variety (Sp1) of *Prunus spinosa* had a significantly lower content of catechins than the cultural variety and it was 168 mg/100 g DM. According to the content of anthocyanins in the fruits of cultural and wild varieties of *Prunus spinosa*, a radically opposite result observed. The greatest content of anthocyanins had fruits of wild variety (Sp1) *Prunus spinosa* – 384 mg/100 g DM. Low content of anthocyanins had fruits of large-fruited (Sp3) cultural variety *Prunus spinosa* (230 mg/100 g DM). This pattern can be explained by the oxidation-reduction reaction. Catechins are oligomers/polymers which give anthocyanins by cleavage of the C–C bond under strongly acidic conditions in the presence of molecular oxygen (Bilia et al., 1996). Consequently, the oxidation of catechins leads to an increase in the content of anthocyanins.

Conclusions

The study showed that the above-ground organs of different varieties of *Prunus spinosa* have a high content of anthocyanins and catechins in the conditions of M.M. Gryshko National Botanical Garden of the NAS of Ukraine. The wild variety of *Prunus spinosa* (genotype Sp1) has a higher content of these compounds. The study of the quantitative content of anthocyanins and catechins in the above-ground organs of *Prunus spinosa* varieties showed that they are of interest to the food and pharmaceutical industry and can serve as a raw material for P-vitamin preparations, because the content of vitamin P in the fruit of the wild blackthorn is not inferior rosehip and chokeberry and superior currants.

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PROMISING PLUM CULTIVARS AND SELECTION FROM THE UKRAINIAN BREEDING PROGRAM

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The authors present the research results of new plum cultivars and promising selections (*Prunus domestica* L.), bred at the L.P. Symyrenko research pomology station of the Institute of Horticulture NAAS of Ukraine, under the conditions of the Ukrainian Western Forest-Steppe in order to select the most promising in the relation of economic-valuable traits. Field, laboratory, comparative research methods have been applied as well as statistical ones. The cultivars with compact crown habit and high unit yield capacity, in particular Oda, Nenka, selection № 8087 (Aria), which crown volume have never exceed 20 m^3 and the unit productivity was 2.0–2.5 kg/m^3, were distinguished. In addition to these, high yield capacity per area unit had such varieties as Originalna, Prestige and selection № 8121 (Yantarna Mlyivska). It was established that their fruits are characterized by a high average mass (41.4–58.3 g), high flavor properties (8.4–8.5 points) and can be used in commercial orchards of Ukraine. Thus, among the studied varieties there were determined the best according to the complex of valuable economic features: in early ripening group – Nenka and Oda, the middle ripening – elite selections No. 8087 (Aria), No. 12456 (Dobra), No. 8121 (Yantarna Mlyievska), and the late-ripening ones Originalna and Prestige, which can be widely used in commercial orchards of Ukraine for significantly increase of yields and profitability.

Keywords: *Prunus domestica*, cultivars, fruits, growth, yield

Вступ

Слива є цінною плодовою породою завдяки унікальним дієтичним та технологічним властивостям її плодів.

В Україні на кінець 2017 року загальна площа під садами цієї культури становила 20,4 тис. га. Частка сливи в структурі кісточкових є досить високою – 29,6 %, займаючи друге місце після вишні (31,1 %). Серед провідних світових виробників плодів сливи Україна знаходиться на дванадцятому місці з обсягом у 2017 р. –

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200,5 тис. т і врожайністю 10,9 т/га, що становить 1,5 % від загального обсягу світового виробництва (Derzhavna Sluzhba Statystyky Ukrayiny, 2019).

Визначальним фактором у ефективному виробництві плодової продукції належить сорту. Його частка в підвищенні врожайності плодових культур оцінюється в 40–70 % (Savieliev, 2003). При цьому залучення у виробництво кращих нових сортів не потребує додаткових капітальних вкладень (Shestopal', 2006).

Кліматичні умови України є оптимальними для успішного промислового вирощування сливи, водночас її прибуткове виробництво можливе лише за умов використання відповідних сортів. Основні площі промислових садів в нашій державі представлені такими сортами як Ганна Шпет та Стенлей, питома вага яких у виробництві садивного матеріалу за 2015 рік становила відповідно 14,9 та 75,8 % (Bazilevs'ka, 2015).

Тенденції на світовому ринку свідчать про те, що зростає попит на крупноплідні сорти сливи ранніх та пізніх строків достигання масою не менше 45–50 г високих смакових якостей, які можуть реалізовуватися у свіжому вигляді за найвищою ціною. Найбільш популярними в Європі насьогодні є Президент, Стенлей, Чачакська найболіа, також набувають поширення стійкі до іржі та вірусу шарки сорти Топхіт, Топенд плюс та ін. (Rozpara, 2004; Botu, 2012; Bolat, 2017).

Водночас, актуальним залишається добір сортів і для переробної промисловості, яким би були властиві стабільна врожайність і легке відокремлювання невеликої кісточки від м'якоті плоду.

Крім того, у зв'язку із зміною клімату основною вимогою до сорту є висока стійкість до несприятливих абіотичних та біотичних факторів довкілля.

Отже, одним із пріоритетних завдань в селекції сливи є створення високопродуктивних адаптивних до умов вирощування крупноплідних сортів з високою товарною якістю плодів.

Матеріали та методика

Місце проведення досліджень

Досліди проведено протягом 2016–2018 рр. на Дослідній станції помології ім. Л.П. Симиренка (м. Мліїв, Черкаська обл.), яка входить до структури Інституту садівництва Національної академії аграрних наук України (далі – ІС НААН), і на сьогодні є провідною установою зі створення сортів сливи в Україні.

Мета досліджень

полягала у виділенні сортів, які б відповідали вимогам сучасного садівництва і могли значно покращити районований сортимент.

Об'єкти досліджень

Вивчалися 14 сортів та 9 елітних форм сливи різних строків достигання, зокрема: ранньостиглі сорти і форми (III декада липня – I декада серпня) – Ненька (контроль), Ода і Герман; середньостиглі (II–III декада серпня) – Ренклод Карбишева (контроль), № 12516, № 7756 (Ізюминка), № 8087 (Арія), № 9605, № 9996 (Юна), № 12456 (Добра), Заманчива, Чачакська краща і Янтарна Мліївська; пізньостиглі (I–II декада вересня) – Стенлей (контроль), № 7794 (Розважлива), №8110 (Фантазія), № 8164 (Престиж), №8143, Рекорд, Топхіт, Блюфрі, Президент і Штутгарт.

Методика проведення досліджень

Зазначені сорти і форми щеплено на сіянцях аличі та висаджено у 2000 р. за схемою 6 × 4 м. Кількість облікових дерев на ділянці – 10, повторень – 3, розміщення варіантів рендомізоване. Комплексну оцінку сортів та гібридів проводили за загальноприйнятими методиками (Siedov and Ogolcova, 1999; Metodyka.., 2004).

Результати та обговорення

При створенні інтенсивних насаджень велике значення має сила росту сортів сливи. Для зручності догляду за такими садами більшу перспективу мають сорти з компактним габітусом крони в поєднанні з слаборослими підщепами, що дозволить більш щільно розміщувати дерева на одиниці площі (Eriomin, 2003; Sobol' and Kolesnyk, 2005) та механізувати процес збирання врожаю (Rozpara, 2012). Попередні дослідження в Інституті садівництва НААН показали, що для створення щільних садів придатні сорти Богатирська, Ода, Стенлей та Вереснева (Zavgorodnyaya and Pavlyuk, 1988).

Дослідженнями встановлено істотні відмінності за силою росту та габітусом крони дерев сливи. Найбільшою висотою (більше 4 м) та об'ємом крони (понад 25 м^3) відзначалися сорти Рекорд, Блюфрі, Президент та Ренклод Карбишева. Виділено слаборослі сорти Ода і Ненька, у яких висота дерев не перевищує 3,0 м, а об'єм 15 м^3, що дає можливість використовувати їх в садах із щільним розміщенням дерев.

Компактним габітусом крони також відрізнялися дерева елітних форм № 7756 (Ізюминка), № 8087 (Арія) та № 9605, у яких об'єм крони не перевищував 20 м^3. Дерева всіх сортів за роки досліджень мали добрий загальний стан та високу зимостійкість.

Найбільш раннє достигання плодів відмічається у сортів Ненька (III декада липня) та Ода (I декада серпня), яким у районованому сортименті за строками достигання і слаборослістю не має аналогів.

До групи середньостиглих віднесено Заманчиву, Трудівницю Млієва, Кантату та елітні форми № 8121 (Янтарна Мліївська), № 12456 (Добра), № 7756 (Ізюминка), № 9996 (Юна), № 8087 (Арія). Їх плоди достигають у другій декаді серпня. Пізнім достиганням плодів (вересень) на рівні контрольного сорту Стенлей відзначається Волошка, Оригінальна, Окраса саду та форми № 8164 (Престиж), № 8110 (Фантазія), № 7794 (Розважлива).

Найголовнішим критерієм господарської оцінки сортів сливи є їх врожайність. За цим показником виділилися з ранньостиглих сортів Ненька і Ода (14,6–15,7 т/га), середньостиглих – елітна форма № 8121 (Янтарна Мліївська) – 16,5 т/га та з групи пізніх – Оригінальна і № 12456 (Добра) – (16,7 т/га) та № 8124 (Престиж) – 16,0 т/га. Оцінку основних сортів та елітних форм наведено в Таблиці 1.

Таблиця 1 Основні господарсько-біологічні показники сортів та елітних форм сливи (середнє за 2016–2018 рр.)
Table 1 Basic economic and biological indices of varieties and elites forms of plum (average for 2016–2018)

Сорт, форма	Урожайність		Маса плоду (г)		Дегустаційна оцінка (бал)
	т/га	кг/м^3	середня	максимальна	
Ранньостиглі					
Ненька (к)	14,6	2,4	49,6	63,6	8,5
Ода	15,7	2,5	41,4	43,9	8,4
Середньостиглі					
Ренклод Карбишева (к)	12,3	1,2	41,8	54,3	8,4
№ 7756 (Ізюминка)	10,4	1,4	37,2	40,5	8,4
№ 8087 (Арія)	13,8	2,0	43,2	51,8	8,4
№ 12456 (Добра)	16,7	1,5	47,2	57,1	8,5
№ 8121 (Янтарна Мліївська)	16,5	1,9	58,3	71,5	8,0
Пізньостиглі					
Стенлей (к)	15,4	1,7	43,9	56,1	8,3
Волошка	15,1	1,4	57,2	66,5	8,3
Оригінальна	16,7	1,6	40,4	52,7	8,5
№ 8115 (Окраса саду)	14,6	1,4	62,0	75,1	8,5
№ 8124 (Престиж)	16,0	1,8	49,8	58,4	8,0
№ 7794 (Розважлива)	13,1	1,5	33,4	37,8	8,5
№ 8110 (Фантазія)	14,8	1,6	43,2	48,9	8,5
НІР$_{05}$	1,8	0,2	4,6	6,7	0,7

Сорти з високою питомою продуктивністю дають можливість більш ефективно використовувати відведену земельну площу, що підвищує ефективність виробництва плодів сливи. За цим показником з розрахунку на 1 м^3 крони виділяються слаборослі сорти Ненька і Ода (2,4–2,5 кг/м^3), а також елітні форми № 8087 (Арія) – 2,0, № 8121 (Янтарна Мліївська) – 1,9, № 8124 (Престиж) – 1,8, № 8110 (Фантазія) і Оригінальна (1,6) та контрольний сорт Стенлей – 1,7 кг/м^3.

Величина та смакові властивості плодів є основними показниками при визначенні їх якості. Велику середню масу від 36 до 45 г формують практично всі досліджувані

сорти за виключенням елітної форми № 7794 (Розважлива), дуже велику (понад 45 г) зафіксовано у Неньки (49,6 г), Волошки (57,2 г) та елітних форм № 12456 (47,2), № 8121 (58,3), №8115 (62,0) і № 8124 (49,8 г). У цих сортів та елітних форм середня та максимальна маса плодів була в 1,2–1,4 рази більшою, ніж в контролі.

У плодів усіх сортів, які вивчалися, кісточка добре відділяється від м'якоті, що є важливою властивістю при виготовленні соків, желе, виноматеріалів та високоякісного чорносливу. Зовнішній вигляд та дегустаційну оцінку проводили за дев'ятибальною шкалою. Найбільш привабливими виявилися плоди сорту Ненька, Оригінальна та елітних форм № 12456, № 8121 та № 8124. Ці ж сорти виділяються за смаковими якостями, вмістом цукрів (9,36–12,1 %), сухих розчинних речовин (13,95–16,49 %) й органічних кислот (0,78–0,86 %). Тому не випадково, що у Державному реєстрі сортів рослин, придатних для поширення в Україні на 2019 рік налічується 14 сортів сливи, з яких 9 – мліївської селекції, які повинні вирішити нагальні питання промислового вирощування сливи (Derzhavnyi Reiestr, 2019).

Наводимо коротку характеристику найбільш перспективних сортів сливи різних сортів достигання Дослідної станції помології ім. Л.П. Симиренка ІС НААН.

Ода (Рисунок 1) – ранньостиглий сорт (I декада серпня), отриманий від штучного запилення сорту Волошка сумішшю пилку сортів Кірке, Артон, Угорка ажанська і Катерина синя. Дерево середньорослe, утворює округлу середньої густоти крону.



Рисунок 1 Плоди сорту Ода
Figure 1 Fruits of cv. Oda

Вступає в плодоношення на 3–4 рік після садіння, забезпечуючи врожай 40–45 кг з дерева. Плоди великі, одномірні, овальної форми, кісточка вільна. Середня маса 40–50 г. Основне забарвлення плоду зелене, покривне – фіолетово-буре. Шкірочка середньої щільності з сильним восковим покриттям. М'якуш жовтий, ніжний, соковитий, кисло-солодкого смаку. Плоди містять 17,68 % сухих речовин, 7,9 – цукру, 0,75 % – органічних кислот.

Добра (Рисунок 2) – середньостиглий сорт (II–III декада серпня), отриманий від запилення гібридної форми № 8143 пилком сорту Персикова. Дерево середньорослe з обернено пірамідальною середньої густоти кроною. Вступає в плодоношення на 3–4 рік після садіння, урожайний (35–40 кг/дер.). Плоди крупні, одномірні, овальної форми. Середня маса 47–57 г. Основне забарвлення плоду жовто-зелене, покривне – від червоно-бурого до темно-синього. Шкірочка середньої щільності з легким восковим нальотом. М'якуш жовто-зелений, щільний, соковитий, хрящуватий, кисло-солодкого смаку. Плоди містять 18,94 % сухих речовин, 12,60 – цукру, 0,87 % – органічних кислот.



Рисунок 2 Плоди сорту Добра
Figure 2 Fruits of cv. Dobra

Оригінальна (Рисунок 3) – пізньостиглий сорт (III декада серпня – I декада вересня), отриманий шляхом відбору серед сіянців від вільного запилення сорту Волошка. Дерево середньорослe з округлою припіднятою середньої густоти кроною. Вступає в плодоношення на 3–4 рік після садіння, урожайний (40–45 кг/дер.). Плоди великі, одномірні, округлої форми. Середня маса 40–50 г. Шкірочка еластична, середньої щільності з легким восковим покриттям. М'якуш жовтий, щільний, соковитий,

хрящуватий, кисло-солодкого смаку. Плоди містять 16,90 % сухих речовин, 9,2 – цукру, 0,77 % – органічних кислот.

Зазначені сорти сливи включено до Державного реєстру сортів рослин, придатних для поширення в Україні та рекомендовано для промислових насаджень у зоні Лісостепу.



Рисунок 3 Плоди сорту Оригінальна
Figure 3 Fruits of cv. Originalna

Висновки

Таким чином, за комплексом господарсько-цінних ознак серед сортів, які вивчалися з групи ранньостиглих виділяються Ненька та Ода, середньостиглих – елітні форми № 8087 (Арія), №12456 (Добра), № 8121 (Янтарна Мліївська) та пізньостиглих – Оригінальна і № 8124 (Престиж). Використання цих сортів і форм в промислових садах дозволить значно підвищити їх врожайність та прибутковість господарств.

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SLOPES OF THE RAILWAYS OF THE MOSCOW AS AN ECOLOGICAL NICHE FOR RARE AND ENDANGERED PLANT SPECIES

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The role of the railways slopes as an ecological niche for rare and endangered plant species was reflected on the example of the Moscow metropolis. A comparative analysis of the information from the database on the flora of the railways in Moscow (Barinov, 2017) and data from the Red Book of Moscow (Red Book..., 2011) was carried out. The database was created on the basis of herbarium materials stored in the Herbarium of the Main Botanical Garden (MHA) and Herbarium of the M.V. Lomonosov Moscow State University (MW). It includes information on 480 natural and 606 alien species. The railways were considered in a broad sense (including railway tracks, platforms, stations, adjacent slopes and cuvettes, exclusion zones). The length of the Moscow railways in the old borders of the city (until 2012) is more than 300 km. 480 aboriginal species was registered on the railway, 34 (7%) of them are considered as rare species and were included in the 'Red Book of Moscow'. Two species *Botrychium lunaria* and *Allium angulosum* have been receive category 0, four *Antennaria dioica*, *Nonea pulla*, *Dactylorhiza fuchsii*, and *Thymus marschallianus* – category 1. Labels of all herbarium specimens are given. Some railway's populations of rare species are quite stable. Thus, *Allium angulosum* on the meadow near the railway station Bojnja, and *Thymus marschallianus* on the railway embankments with meadow-steppe plant communities in front of Butovo station exist here more than 20 years. The record belonged to *Thymus* × *loevyanus*: this hybridogenic taxon grows in the same locality – on the railway embankments with meadow steppe cenoses near the railway station Butovo since 1946 (more than 65 years). Since in Moscow even mosaic meadow communities are extremely rare, it is necessary to use all measures to maintain the remnants these natural phytocenoses, the disappearance of which is the main threat to the reduction of natural biodiversity. The task is to develop measures to preserve indigenous species on the roadside, in spite of the prevailing practice of covering slopes with rolling lawns. The proportion of natural meadow species can be increased with a suitable mode of mowing and abandoning of herbicides and possibly also by artificial seeding of native plants. In a metropolis, such an approach will be the only possible way to increase the ecological significance of roadside habitats that will be able to assume the function of ecological corridors not only for native plants but also insects and even, possibly, animals. It is concluded that in large cities, railway slopes can make up for the lack of suitable habitats for both natural vegetation in general, and for rare and endangered plant species.

Keywords: biodiversity, endangered plant species, Moscow, railway, rare plants, Red Book

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Введение

Луга, даже антропогенно нарушенные, являются биотопами, находящимся под угрозой уничтожения. В Москве и Московской области многовидовых лугов большой площади не осталось совсем, лишь в редких случаях можно найти мозаичные сообщества. Из-за постоянно усиливающегося землепользования доля придорожных местообитаний в общей площади полуестественной растительности быстро растет. Типичные луговые виды растений, приспособленные к непрерывному выпасу или скашиванию, которые сохраняют «открытой» окружающую среду, начинают произрастать в альтернативных местообитаниях по обочинам дорог. Хотя растительность обочин считается ценотически ненасыщенными сообществами (Kopecky and Hejny, 1974; Schaffers and Sýkora, 2002), они, тем не менее, могут быть богаты видами.

В 1980-х годах в Европе признана важность обочин в поддержании биоразнообразия и разработаны меры для сохранения на них богатых видами сообществ (Parr and Way, 1988; Cale and Hobbs, 1991; Jefferson et al., 1991; Harrington, 1994; Allem, 1997; Tikka et al., 2001). Доказано, что в сильно фрагментированном городском ландшафте железнодорожные откосы могут выполнять функцию экологических коридоров для аборигенных видов (Penone et al., 2012). Признавая экологическое значение придорожных местообитаний, европейские ботаники проводят инвентаризацию синтаксонов, встречающихся вдоль обочин дорог. В Нидерландах, например, на обочинах железных дорог выявлено 15 полуестественных богатых видами растительных сообществ, относящихся к 11 союзам, входящим в 10 ассоциаций, причем все эти сообщества произрастали и за пределами придорожных местообитаний. Аналогичные исследования проведены в Бельгии (Schaffers and Sýkora, 2002) и Финляндии (Tikka et al., 2000).

Снижение доли естественных фитоценозов является главной угрозой для многих редких и исчезающих видов сосудистых растений. И откосы железных дорог могут быть экологической нишей для этих видов. Эти местообитания сходны с сухими и мезофитными лугами, и их растительность поддерживается косьбой и удалением дающих тень древесных растений. Разумеется, откосы железных дорог не являются полноценной заменой полуестественным лугам. Но присутствие луговых видов на обочинах может быть повышено за счет подходящего режима скашивания и отказа от использования гербицидов, что будет способствовать формированию на обочинах дорог естественной растительности.

В Великобритании некоторые дорожные откосы рассматриваются в качестве объектов особого научного интереса (Parr and Way, 1988), а в Голландии 8 % придорожных растений классифицируются как редкие или довольно редкие виды (Tikka et al., 2001). В Финляндии 4 % исчезающих на национальном уровне видов регулярно встречаются на обочинах, например, *Campanula cervicaria* L., *Dianthus arenarius* L. и *Hypericum montanum* L. (Tikka et al., 2001). В Богемии (Чехия) в этот перечень входят *Dactylorhiza majalis* (Rchb.) Hunt et Summerh. и *Gentianopsis ciliata* (L.) Ma (Jandová et al., 2009).

Сходные тенденции отмечены и в России. В Самаре и Самарской области (Ivanova, 2008) на железной дороге произрастают луговые (*Centaurea jacea* L., *Dianthus pratensis* Bieb.,), степные (*Astragalus danicus* Retz., *Salvia tesquicola* Klok. and Podeb., *Stipa capillata* L.) и лесные (*Corylus avellana* L., *Pulmonaria obscura* Dumort., *Euonymus verrucosa* Scop.) растения. Отмечены и редкие виды (*Centaurea ruthenica* Lam., *Astragalus zingeri* Korsh., *Stipa capillata* L. и др.). На железнодорожных насыпях южной части Приволжской возвышенности (в границах Саратовской области) также найдены (Skvortsova and Berezutskyi, 2008; Ryibakova, 2008) популяции охраняемых растений (*Chartolepis intermedia*, *Astragalus cornutus*, *Glycyrrhiza glabra*, *Iris halophila*, *Stipa pennata*, *Adonis wolgensis*, *Dodartia orientalis* и др.). На железнодорожных насыпях Куйбышевской и Южно-Уральской железных дорог в Башкирии выявлено 7 видов, занесенных в Красную книгу Республики Башкортостан: *Glycyrrhiza korshinskyi*, *Linaria altaica*, *Rosa pimpinellifolia*, *Stipa korshinskyi*, *Stipa lessingiana*, *Stipa sareptana*, *Thermopsis schischkinii* (Husainova, 2016).

В связи с этим целью настоящей работы является выявление роли откосов железных дорог Москвы как экологической ниши для редких и исчезающих видов природной флоры.

Материал и методика

Объекты исследования

Изучена флора железных дорог Москвы, протяженность которых в старых границах мегаполиса (до 2012 г.) составляет более 300 км. Железные дороги рассматривались в широком смысле (включая железнодорожные пути, платформы, станции, прилегающие склоны и кюветы, зоны отчуждения).

Методы исследования

Проведен сравнительный анализ сведений из базы данных по флоре железных дорог г. Москвы (Barinov, 2017), и данных Красной книги г. Москвы (Krasnaya Kniga…, 2011). База данных создана на основе гербарных материалов, хранящихся в Гербарии Главного ботанического сада (MHA) и Гербарии МГУ им. М.В. Ломоносова (MW), в нее включены сведения по 480 природным и 606 чужеродным видам (Vinogradova et al., 2017). В отдельных случаях для уточнения сведений использовались и некоторые литературные источники (Ignatov et al., 1990; Adventivnaya flora.., 2012). Все гербарные этикетки цитируются в работе без редакторской правки.

Результаты и дискуссия

Аналогично данным, полученным в других регионах, на железной дороге Москвы в сохраненных полуестественных луговых сообществах произрастают редкие и уязвимые виды растений. Отмечено 34 вида, включенные в Red Book of Moscow (2011). Два вида: *Botrychium lunaria* и *Allium angulosum* имеют категорию 0, четыре вида: *Dactylorhiza fuchsii*, *Nonea pulla*, *Thymus marschallianus* и *Antennaria dioica* имеют

категорию 1, тринадцать видов имеют категорию 2, двенадцать видов имеют категорию 3 и три вида имеют категорию 5 (Таблица 1).

Таблица 1 Виды из Красной Книги г. Москвы, обнаруженные на железных дорогах
Table 1 Plant species are included in the 'Red Book of Moscow' which grow at the railway

Категории редкости видов	Наименование видов
Категория 0 (исчезнувшие виды):	*Botrychium lunaria* (L.) Sw., *Allium angulosum* L.
Категория 1 (виды, находящиеся под угрозой исчезновения):	*Antennaria dioica* (L.) Gaertn., *Nonea pulla* (L.) DC., *Dactylorhiza fuchsii* (Druce) Soo, *Thymus marschallianus* Willd.
Категория 2 (редкие виды или малочисленные виды с сокращающейся или сократившейся численностью)	*Astragalus glycyphyllos* L., *Veronica teucrium* L., *Lithospermum officinale* L., *Geranium robertianum* L., *Campanula bononiensis* L., *Allium oleraceum* L., *Festuca valesiaca* Gaud., *Filipendula vulgaris* Moench (= *F. hexapetala* Gilib.), *Listera ovata* (L.) R. Br., *Phleum phleoides* (L.) Karst., *Viola tricolor* L., *Thymus* × *loevyanus* Opiz. (=*Th. marschallianus* Willd. ×*Th. pulegioides* L., *Thymus* × *glabrescens* Willd.), *Anthyllis vulneraria* L. (= *A. macrocephala* Willd.= *A. schiwereckii* (DC.) Blocki)
Категория 3 Уязвимые виды	*Astragalus danicus* Retz., *Polygonum bistorta* L. (= *Bistorta major* S.F. Gray), *Fragaria viridis* (Duch.) Weston, *Iris pseudacorus* L., *Campanula rotundifolia* L., *Campanula persicifolia* L., *Trollius europaeus* L., *Dactylorhiza incarnata* (L.) Soo, *Anthemis tinctoria* L., *Eryngium planum* L., *Steris viscosa* (L.) Rafin.
Категория 4 Виды неопределённого статуса	на железных дорогах Москвы не выявлены
Категория 5 Восстановившиеся виды	*Campanula patula* L., *Convallaria majalis* L., *Leucanthemum vulgare* Lam.

Категория 0 (исчезнувшие виды)

Botrychium lunaria (L.) Sw. (гроздовник полулунный). В Московской области изредка встречается во всех её частях, кроме Заокской. На территории, ныне занимаемой Москвой, в XIX- начале XX вв. вид отмечали в Раменках, по р. Сетуни, в Хорошеве, между сёлами Братцево и Спасское, в Петровско-Разумовском и Останкине (Krasnaya Kniga..., 2011).

В 1990 г. на железной дороге в трансформированной долине р. Нищенки этот вид был найден 'Москва. Малая Окружная ж.д. Юго-Восточный сектор, 800 м от сорт.ст. Андронова к сорт. ст. Лефортово по склону неглубокой ж.д. выемки. Колония. 30.07.1990. В.Д. Бочкин' (МНА). После 2000 г. ни в данном пункте, ни где-либо ещё не наблюдался.

Allium angulosum L. (лук углова́тый, или мыши́ный чесно́к). В Московской области довольно редок, известен из южных и восточных районов. На территории, ныне занимаемой Москвой, в XIX веке отмечен на Ключиках в Перове, под Симоновым монастырём, в Верхних Котлах и на р. Москве близ Серебряного Бора. В прошлом

чаще произрастал в естественных местообитаниях – на пойменных заливных лугах, а позднее приобрёл черты редкого заносного вида. В последние десятилетия встречались единичные экземпляры на открытых нарушенных склонах, луговинах вблизи железных дорог, приречных лугах и песках. В 1980 г. найден у пересечения канала им. Москвы с Ленинградским шоссе, в 1989–1991 гг. – у ж.д. ст. 'Бойня' (Krasnaya Kniga.., 2011). Вид в этом местообитании довольно устойчив, поскольку более чем через 20 лет обнаружен там же 'Москва. Юго-Восток. Малая окружная ж.д. Середина груз. ст. Бойня. Луговина по полотну ж.д. Несколько куртин. N 55º43,702' – E 37º 42,032'. 11.07.2013. В.Д. Бочкин, С.Р. Майоров (МНА)'.



Рисунок 1 Гербарные сборы *Allium angulosum* на грузовой станции Бойня
Figure 1 Name and description of the image Herbarium specimens of *Allium angulosum* at the cargo station Bojnia

Категория 1 (виды, находящиеся под угрозой исчезновения)

Antennaria dioica (L.) Gaertn. (кошáчья лáпка двудóмная). В Московской обл. до недавнего времени вид считался обычным во всех районах, но в ближнем Подмосковье резко сократил численность и распространение. На территории, ныне занимаемой Москвой, в XIX – первой половине XX вв. кошачья лапка была распространена достаточно широко, указывалась, например, для Лосиного Острова, Сокольников, Кунцева, Петровско-Разумовского и Останкина. В 1980–2000 гг. в пределах МКАД известны лишь единичные находки, из них только 3 на природных территориях – в окрестностях Знаменского-Садков, Черепкове и на Крылатских холмах (Krasnaya Kniga.., 2011). Произрастал этот вид и по железной дороге: 'г. Москва, в кустарнике возле насыпи Белорусской ж.д. (совская ветка) между ст. Рабочий поселок и кольцевой автодорогой. 25.05.1980. М.С. Игнатов; г. Москва. Малая окружная ж.д. Юго-восточный сектор. 800 м от сорт. ст. Андроновка к сорт.ст. Лефортово. Луговина по склону неглубокой ж.д. выемки. 30.07.1990. В.Д. Бочкин' (МНА). Кошачья лапка росла также в единичных экземплярах в Коломенском, но после 2003 г. там не найдена. В 2009 г. популяция кошачьей лапки в долине р. Сходни в Куркине насчитывала 8 цветущих и не менее 10 вегетирующих экземпляров (Krasnaya Kniga.., 2011).

Nonea pulla (L.) DC. (нонея тёмно-бурая, дурнушка тёмно-бурая, кривоцвет тёмный, монашка тёмно-бурая, нонея бурая, нонея тёмная). В Московской обл. известен во всех ботанико-географических р-нах, кроме Верхне-Волжской низменности. Обычен в южн. части Подмосковья, в северной встречается редко и преимущественно во вторичных местообитаниях. На территории, ныне занимаемой Москвой, в конце XIX в. нонею регистрировали близ Серебряного Бора и в Выхине, но лишь как заносный вид на ж.д., в 1980–1990 гг. – также на железной дороге: 'Москва по полотну Окружной ж.д. возле пересения ее с Октябрьской ж.д. 10.06.1981. М.С. Игнатов; Москва, около полотна Окружной ж.д. возле стадиона им. Ленина. Единично. 25.05.1981. М.С. Игнатов; Москва, Курская ж.д., около ст. Текстильщики, на насыпи ж.д. 20.05.1987. В.Д. Бочкин; Москва, Северо-Запад. Малая окружная ж.д. Сорт. ст. Серебряный бор. По полотну ж.д. Одно растение. Венчик темно-красный. 08.06.1990. В.Д. Бочкин' (МНА). В последнее время найдена в Коломенском на Дьяковском участке в виде малочисленных локальных популяций и единичных экземпляров (Krasnaya Kniga.., 2011).

Dactylorhiza fuchsii (Druce) Soo (пальчатокоренник Фукса). В Московской обл. обычен и встречается повсеместно. На территории, ныне занимаемой Москвой, в XIX – первой половине XX вв., возможно, этот вид отмечали в Лосином Острове, Битцевском лесу, на Воробьёвых горах, в Кунцеве, Всехсвятской роще, Петровско-Разумовском, Останкине, хотя ряд указаний может относиться к пальчатокоренникам пятнистому, балтийскому или гебридскому. В 1950–1980-е гг. этот (или похожий) вид зарегистрирован только на территории МСХА им. К.А.Тимирязева в 1958 и 1962 гг., а также в ГБС (Krasnaya Kniga.., 2011). Встречен он и по железной дороге 'Москва, юг. Павелецкая ж.д. Луговина по полотну ж.д. около пл. Бирюлево-Товарная. Луговина. Одно растение. 20.06.1991. В.Д. Бочкин' (МНА). В последнее время обнаружен в пойме р. Котловки в 2009 г., на юге Битцевского леса в 2008 г., в Зап. Знаменском карьере в 2008–2009 гг., пойме р. Сетуни в Жуковке в 2009 г., на Щукинском п-ве в 2007, 2009 и 2010 гг., долине Машкинского ручья в Куркине в 2009 г. (Krasnaya Kniga.., 2011).

Thymus marschallianus Willd. (тимьян Маршалла). Встречается в южной части Московского региона. На территории, ныне занимаемой Москвой, вид отмечали в 1865 г. в Н.Котлах, в 1985 г. в Коломенском и в 1996 г. близ Троекуровского пруда, за МКАД – в окрестностях Знаменского-Садков. В 1987 г. найден у ж.д. ст. Бутово (Красная книга.., 2011), через год собран там же: 'Насыпь с лугостепной растительностью справа у ж.д. на Симферополь перед ст. Бутово. Юг Москвы. Р/н Бутово-Знаменского. 06.06.1988. В.Куваев' (МНА). В этом же месте вид обнаружен и через 10 лет: «Юг Москвы. Окрестности Знаменского района. Насыпь Курского направления Моск.ж.д. справа 0,5–0,8 км перед ст. Бутово. Лугостепная растительность. 11.06.1997. В. Куваев» и еще через 10 лет «Юг Москвы вне МКАД. Справа от Курской ж.д. <1 км перед ст. Бутово. Высокая насыпь со степненно-луговой растительностью. 11.06.2007. В. Куваев, Б. Когут; Лугостепь на высокой насыпи справа от М-Курской ж.д. ~ 1 км перед ст. Бутово. 29.05.2008. В. Куваев, Ю. Насимович» (МНА). Таким образом, на ж.д. насыпи с луговостепной растительностью крупная популяция *Thymus marschallianus* стабильно

растет уже около 20 лет. В 2001–2010 гг. подтверждено произрастание этого вида и на Дьяковском городище в Коломенском (Krasnaya Kniga.., 2011).

Категория 2. Редкие виды или малочисленные виды с сокращающейся или сократившейся численностью

Виды этой категории встречаются на железных дорогах гораздо чаще, чем предыдущие группы, и популяции их более многочисленны.

Allium oleraceum L.'Москва, север, Савеловская ж.д., 1км от пл. Окружная к пл. Дегунино. Болото у основания ж.д. насыпи. Луговина по краю болота, среди ив. Большая колония. 22.06.1992. В.Д. Бочкин' (МНА).

Anthyllis vulneraria L. 'Москва. Близ пл.Петровско-Разумовская. По ж.д. откосу. 29.08.1966. М. Лутонина»; 'Москва, Октябрьская ж.д. Левая колея. В 400м от проспекта Мира (пл. Рижская) к пл. Останкино. По полотну ж.д. Луговина. 16.07.1990. В.Д. Бочкин'; 'Москва, юг, Павелецкая ж.д.. По полотну ж.д. Бирюлево-Пассажирская. Луговина. Большая колония. 20.06.1991. В.Д. Бочкин'; 'Москва. Юго-запад. Киевская ж.д. 900 м от пл. Москва-сортировочная к пл. Матвеевская (возле пересечения с р. Сетунь). По полотну высокой ж.д. насыпи. Большая колония. 21.06.1991. В.Д. Бочкин' (МНА).

Astragalus glycyphyllos L. 'Москва, вдоль полотна Курской ж.д. между ст.Люблино и Текстильщики. 09.06.1980. Собрал М.С. Игнатов, определил В.Д. Бочкин'; 'Москва, Бирюлевский лес. Южная часть Царицынского лесного массива. На железнодородном полотне. Один экз. 55º35'с.ш., 37º42' в.д., 170 м над у.м. 07.07.1992. Ю.А. Насимович' (МНА).

Campanula bononiensis L. 'Москва, Рижская ж.д. 400 м от пл. Рижского вокзала к пл. Дмитровская. Придорожная луговина. Колония. 04.07.1991. В.Д. Бочкин'; 'Москва, Северо-Запад, Рижская ж.д. 700 м от пл. Трикотажная к пл. Павшино. Луговина по склону неглубокой ж.д. выемки. В массе. 10.08.1991. В.Д. Бочкин' (МНА).

Festuca valesiaca Gaud. 'Москва, в зарослях кустарников на ст. Покровская Курской ж.д. 28.06.1980. Алексеев'; 'Москва, Киевская ж.д. По полотну грузовой колеи ж.д. станции Москва-сортировочная. Отдельные куртины, рассеяно.06.06.1989. В.Д. Бочкин'; 'Москва. Малая Окружная ж.д. Ветка к мясокомбинату в 700 м от ст. Угрешская к ст. Андроновка. На песке вдоль полотна. Около сорт.ст. Бойня. Большая колония. 11.06.1989. В.Д. Бочкин'; 'Москва, Малая Окружная ж.д. Сорт.ст. Бойня (возле ст.метро Волгоградский проспект). По полотну ж.д. Колония. 04.07.1990. В.Д. Бочкин'; 'Москва. Юго-Запад. Малая Окружная ж.д. 500 м от Новодевичьей набережной к сорт. ст. Воробьевы горы (р-н Лужников). На высокой ж.д. насыпи. По полотну тупиковой ветки. Колония. 12.06.1990. В.Д. Бочкин'; 'Лугостепь по высокой насыпи справа от М-Курской ж.д., примерно 800 м перед ст. Бутово. 29.05.2008. В. Куваев, Ю. Насимович' (МНА).

Filipendula vulgaris Moench 'Москва, юг. Павелецкая ж.д. 100м от пл. Чертаново к пл. Бирюлево-товарная. Луговина по склону ж.д. выемки. Колония.19.06.1991. В.Д. Бочкин' (МНА).

Geranium robertianum L. 'Москва. Сорт. ст. Моссельмаш Октябрьской ж.д. (в 180 м от пл. Моссельмаш в сторону ст. НАТИ), вдоль полотна ж.д. 55.8603º с.ш., 37.5330º в.д. Немногочисленная популяция. 05.06.2013. К.Ю. Теплов'; 'Москва, Ю-В Курская ж.д. сорт.ст. Люблино, 100 м от диспетчерской вышки от Москвы. По полотну ж.д. Много. N 55º40,267' – E 37º43,845' 27.08.2013. В.Д. Бочкин'; 'Москва, откос ОЖД близ Лосиного острова и Бермудского треугольника, не менее десятка розеток в условиях легкого затенения подростом деревьев. 23.04.2014. Ю.А. Насимович' (MHA).

Listera ovata (L.) R. Br. 'Москва, север. Савеловская ж.д. 1,8 км от пл. Окружная к пл. Дегунино. Болото у основания ж.д. насыпи. Несколько десятков растений. 22.06.1992. В.Д. Бочкин' (MHA).

Lithospermum officinale L. 'Москва, северо-запад. Рижская ж.д. 100 м от пл. Трикотажная к пл. Павшино. Придорожная луговина. В массе. 10.08.1991. В.Д. Бочкин' (MHA).

Phleum phleoides (L.) Karst. 'Москва. Северо-Восток. Малая окружная ж.д. По полотну ж.д. около сорт. ст. Ростокино. (возле пересечения с Ярославской ж.д.). 05.06.1990. В.Д. Бочкин'; 'Москва, Север. Савеловская ж.д. По полотну сорт. ст. Бескудниково. Одна куртина. 30.06.1992. В.Д. Бочкин' (MHA).

Thymus × *loevyanus* Opiz. 'Близ ст. Бутово. Травянистый склон к ж.д. 22.06.1946. Ворошилов'; 'Юг Москвы. Окрестности Знаменского. Насыпь Моск.-Курской ж.д. справа примерно в 0,5 км перед ст. Бутово. Остатки лугостепной растительности. 08.07.1996. В. Куваев'; 'Юг Москвы. Окрестности Знаменского. Насыпь Курского направления Моск. ж.д. справа 0,5–0,8 км перед ст. Бутово. Лугостепная растительность. 11.06.1997. В. Куваев'; 'Юг Москвы. Лугостепь на возвышенности насыпи справа у М-Курской ж.д. перед станцией Бутово. 05.09.2005. В. Куваев'; 'Юг Москвы вне МКАД. Справа от ж.д. на Симферополь, примерно в 1 км перед ст. Бутово. Высокая насыпь с лугостепной растительностью. 03.08.2006. Собрал В. Куваев, определил С. Майоров'; 'Юг Москвы, вне МКАД. Справа от Курской ж.д., больше чем в км перед станцией Бутово. Высокая насыпь с остепненно-луговой растительностью. 11.06.2007. В. Куваев, Б. Когут'; 'Москва. Восточная опушка Северного Бутовского лесопарка. Бутовская лугостепь (склон железнодорожной выемки по направлению к Москве от ст. Бутово). Начало сентября 2012. Собрал – М.В. Островская, определил Ю.А. Насимович' (MHA).

В этом местообитании, как видно из гербарных этикеток (Рисунок 2), данный гибридогенный таксон произрастает уже более 65 лет!

Veronica teucrium L. 'Москва. Павелецкая ж.д. 800 м от пл. Бирюлево-Пассажирская к пл. Булатниково. Придорожная луговина. Колония. 20.06.1991. В.Д. Бочкин'; 'Москва, юг. Курская ж.д. 400 м от ст. Царицыно к пл. Покровская. Придорожная луговина. Колония. 20.06.1991. В.Д. Бочкин'; 'Юг Москвы вне МКАД. Справа от М-Курской ж.д., примерно в 1 км перед ст. Бутово. Высокая насыпь с остепненно-луговой растительностью. 11.06.2007. Собрал – В. Куваев, Б. Когут, определил – В. Куваев, Н. Решетникова' (MHA).

Viola tricolor L. 'Москва. Юго-Восток. Малая Окр. ж.д 400 м от пересечения с Курской ж.д по ветке к платф. Чухлинка Горьковской ж.д. Свалка строительного мусора. Колония. N 55°43,372' E37°43,187'. 25.9.2012. В.Д. Бочкин' (МНА).



Рисунок 2 Гербарные этикетки, иллюстрирующие стабильное произрастание *Thymus* × *loevyanus* в одном и том же местообитании в течение 1946–2012 годов

Figure 2 Herbarium labels illustrating stable of *Thymus* × *loevyanus*' population in the same habitats during 1946–2012

Категория 3. Уязвимые виды

Anthemis tinctoria L. 'Москва. Октябрьская ж.д. Левая колея. Около пл. Рижская. По полотну грузовой колеи. Колония. 16.07.1990. В.Д. Бочкин'; 'Москва, юго-запад. Киевская ж.д. 700 м от пл. Москва-сортировочная к пл. Матвеевская. Луговина по полотну ж.д. Большая колония. 21.06.1991. В.Д. Бочкин'; 'Москва, Дубининская ул., д 63. Заводские ж.д. пути ЗВЧ (Завод Владимира Ильича). Павелецкая ж.д. На сорном месте. Колония. 15.07.2000. В.Д. Бочкин'.

Astragalus danicus Retz. 'Москва, близ ст. Петровско-Разумовская. По ж.д. откосу. 20.06.1966, собрал – Дубницкий, определил – В. Макаров'; 'Москва, по откосу насыпи Окружной ж.д. возле стадиона им. Ленина. 04.06.1981. М.С. Игнатов'; 'Москва. Малая Окружная ж.д. По полотну сорт.ст. Андроновка. На луговине. Большая колония. 18.05.1989. В.Д. Бочкин'; 'Москва. Север. Малая Окружная ж.д. По полотну тупиковой ветки около сорт.ст.Владыкино (со стороны сорт.ст. Ростокино). Луговина. Колония. 05.06.1990. В.Д. Бочкин'; 'Москва, юго-восток. Малая окружная ж.д. По полотну груз.ст.Бойня. Придорожная луговина. Колония. 06.06.1991. В.Д. Бочкин'; 'Москва, север. Савеловская ж.д. 600 м от пл. Окружная к пл. Дегунино. (возле пересечения с р Лихоборкой) Луговина на склоне высокой ж.д. насыпи. Большая колония. 22.06.1992. В.Д. Бочкин'; 'ЮВАО. Нижегородская улица, возле пересечения с 3-м кольцом вдоль полотна Горьковской ж.д. у пересечения с 3-м кольцом. Придорожная луговина. Одна куртина. 11.10.2008. В.Д. Бочкин'; 'Москва. Юго-Восток. Малая окр.ж.д. 400 м от ТТК по ветке от Бойни к Чухлинке. На вершине высокой ж.д. насыпи. На песке. Куртина. N 55° 43,16 – E° 42,46. 25.09.2012. В.Д. Бочкин'; 'Москва. Юго-восток. Горьковская ж.д. 700 м от пересечения с Малой окр.ж.д. (сорт. ст. Андроновка) к ст. Серп и Молот. Придорожная луговина. Куртина. N 55°44,083 – E 37°42,993. 18.05.2013. В.Д. Бочкин'.

Campanula persicifolia L. «Москва. Курская ж.д., 1.4 км от пл. Битца к пл.Бутово (возле ВИЛРа). Придорожная луговина, по склону ж.д. выемки. Колония. 09.09.1990. В.Д. Бочкин»; «Москва, северо-запад. Рижская ж.д. 700 м от пл.Трикотажная к пл. Павшино. Луговина по склону неглубокой ж.д. выемки. Колония. 10.03.1991. В.Д. Бочкин»; «Москва, север. Савеловская ж.д. 700 м от пл.Бескудниково к пл. Лианозово. По склону ж.д. насыпи. Луговина. Колония. 30.06.1992. В.Д. Бочкин».

Campanula rotundifolia L. 'Москва, Владыкино, между Окружной ж.д. и оградой ГБС. В старых посадках березы по насыпи вдоль ж.д. Колония. 21.07.1986. Собрал – В.Д. Бочкин, определил – В. Макаров'; 'Москва, Малая окружная ж.д., Юго-восточный сектор, 800 м от сорт. ст. Андроновка к сорт. ст. Лефортово. Луговина по склону неглубокой ж.д. выемки. Большая колония. 30.07.1990. В.Д. Бочкин'; 'Юг Москвы. Разреженная растительность суховитой бровки пр.берега руч.Козловка у впадения в р. Битца близ ст.Битца Курской ж.д. 22.06.2005. В. Куваев'.

Dactylorhiza incarnata (L.) Soo 'Москва. Рижская ж.д. 100м от пл. Рижского вокзала к пл. Дмитровская. Придорожная сырая луговина. 3 растения. 04.07.1991. В.Д. Бочкин'; 'Москва, север. Савеловская ж.д. 800 м от пл. Окружная к пл. Дегунино. Болото у основания высокой ж.д. насыпи. Сырой луг вдоль болота. Колония. 22.06.1992. В.Д.

Бочкин'; 'Москва, север. Савеловская ж.д. 1,9 км от пл. Окружная к пл. Дегунино. Болото у основания ж.д. насыпи. Несколько десятков растений. 22.06.1992. В.Д. Бочкин' (MHA).

Eryngium planum L. 'Москва, по насыпи ж.д. между станциями Текстильщики и Люблино. 07.05.1980. М.С. Игнатов'; 'Москва, Шмитовский пр., склон насыпи Окружной ж.д. Одно растение. 06.08.1981. А.Н. Швецов'; 'Москва, Курская ж.д., около ст. Люблино в районе Люблинских прудов, в лесополосе, по опушке. Колония. 09.06.1987. В.Д. Бочкин'; 'Москва, северо-запад. Рижская ж.д., 1,5 км от пл. Тушино к пл. Покровско-Стрешнево. (возле пересечения с Волоколамским шоссе). Луговина по склону высокой ж.д. насыпи. Много. 08.08.1991. В.Д. Бочкин'.

Fragaria viridis (Duch.) Weston 'Москва, Казанская ж.д. 100 м от пл. Вешняки в сторону пл. Плющево (к Москве). Возле автомобильной эстакады. Вдоль ж.д. полотна. Придорожная луговина. Колония. 16.05.1989. В.Д. Бочкин'; 'Москва, Октябрьская ж.д., 300 м от пересечения с ул. Дыбенко к пл.Левобережная. Вдоль ж.д. По склону ж.д. Колония. 25.07.1990. В.Д. Бочкин'; 'Москва, юг. Павелецкая ж.д. 1км от пл. Нижние котлы к пл.Коломенское (возле пересечения с Варшавским шоссе). Придорожная луговина. По склону глубокой ж.д. выемки. Колония. 17.06.1991. В.Д. Бочкин'; 'Москва, Рижская ж.д., 4000 м от пл. Дмитровская к пл. Гражданская. Луговина по склону ж.д. насыпи. Большая колония. 04.07.1991. В.Д. Бочкин'.

Iris pseudacorus L. 'Москва, ст. Кусково Горьковская ж.д., между рельсов старой ветки по которой давно уже не ходят поезда. Весьма крупный куст. 13.06.1980. М.С. Игнатов'; 'Москва, Октябрьская ж.д., 1,5 км от пл.Ховрино к пл.Левобережная. Заболоченный придорожный кювет. (около пересечения с Малой окружной ж.д.). 25.07.1990. В.Д. Бочкин'; 'Москва, Рижская ж.д. 200 м от пл. Рижского вокзала к пл. Дмитровская. Заболоченная придорожная луговина. Колония. 04.07.1991. В.Д. Бочкин'.

Polygonum bistorta L. 'Москва, север, Савеловская ж.д., 2,2 км от пл. Окружная к пл. Дегунино. Болото у основания ж.д. насыпи. Большая колония. 22.06.1992. В.Д. Бочкин'; 'Москва, север, Савеловская ж.д. 900 м от пл. Окружная к пл. Дегунино. Болото у основания ж.д. насыпи. Колония. 22.06.1992. В.Д. Бочкин'.

Steris viscosa (L.) Rafin. 'Москва, Ярославская ж.д., 2 км от пл.вокзала к пл. Москва. Луговина по склону ж.д. выемки. Несколько растений. 20.06.1990. В.Д. Бочкин'; 'Москва, Юг. Курская ж.д., 100 м от пересечения с Павелецкой ж.д. к пл. Покровская. Луговина по склону ж.д. выемки грузовой колеи. Одно растение. 19.06.1991. В.Д. Бочкин'; 'Москва, юг. Курская ж.д. 700 м от ст. Царицыно к пл. Покровская. Грузовая колея. Луговина по склону ж.д. выемки. В массе. 20.06.1991. В.Д. Бочкин'.

Trollius europaeus L. 'Москва, север, Савеловская ж.д., 1,2 км от пл.Окружная к пл. Дегунино. У основания ж.д. насыпи. Сырой луг. Колония. 22.06.1992. В.Д. Бочкин'.

Таким образом, в Москве 7 % придорожных растений (34 из 480) классифицируются как редкие или довольно редкие виды, и некоторые дорожные откосы должны рассматриваться как объекты особого внимания, например, насыпь с лугово-степной растительностью перед железнодорожной станцией Бутово, на которой редкие

виды удерживаются довольно долгое время. Поскольку в Москве даже мозаичные луговые сообщества встречаются крайне редко, необходимо задействовать все меры поддержания остатков этих природных фитоценозов, исчезновение которых является главной угрозой снижения естественного биоразнообразия. Остро стоит задача разработки мер для сохранения на обочинах дорог аборигенных видов, вопреки сложившейся практике застилания откосов рулонными газонами. Доля природных луговых видов может быть увеличена при подходящем режиме скашивания и отказа от использования гербицидов, а также, возможно, за счет искусственного подсева семян аборигенных растений. В условиях мегаполиса такой подход явится единственно возможным способом повышения экологического значения придорожных местообитаний, которые смогут принять на себя функцию экологических коридоров для аборигенных видов не только растений, но и насекомых и даже, возможно, животных.

Выводы

На откосах железных дорог г. Москвы отмечено 34 вида, включенные в «Красную книгу города Москвы», причем двум из них *Botrychium lunaria* и *Allium angulosum* присвоена категория 0, а четырем *Antennaria dioica*, *Nonea pulla*, *Dactylorhiza fuchsii* и *Thymus marschallianus* – категория 1. Некоторые «железнодорожные популяции» редких видов довольно устойчивы: и *Allium angulosum* на луговине возле ж.д станции Бойня, и *Thymus marschallianus* на ж.д. насыпи с луговостепной растительностью перед ж.д станцией Бутово существуют уже более 20 лет. Рекорд установил *Thymus* × *loevyanus*: этот гибридогенный таксон растет в одном и том же местообитании – на ж.д. насыпи с луговостепной растительностью перед ж.д станцией Бутово с 1946 года (более 65 лет).

Таким образом, в условиях крупных городов железнодорожные откосы могут восполнять отсутствие подходящих местообитаний как для естественной растительности в целом, так и для редких и исчезающих видов растений.

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OPEN ACCESS

PROMISING CONSTRUCTIONS OF SWEET CHERRY (*PRUNUS AVIUM* L.) ORCHARDS UNDER CONDITIONS OF UKRAINIAN FOREST-STEPPE

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The purpose of the research was the comparative estimation of the modern sweet cherry (*Prunus avium* L.) orchards, created according to the foreign and domestic technologies with using the best large-fruited cultivars, high-productive rootstocks, modern methods of the pruning under the optimum dense trees distribution. Field, laboratory and comparative methods of the investigations were applied as well as statistical. The growth processes intensity in the orchards of cv. Regina on Gisela 5 appeared lower than on the middle rootstocks Gisela 6 and VSL-2. Among the pruning methods, the fan-trained crown proved averagely in the research years by 1.2–2.6 times less productive for all the explored cultivar-rootstock combinations as compared to the variants with the rounded small-size and multi-ax flattened crowns. High-productive constructions of the orchards with Talisman and Annushka varieties on the middle rootstocks VSL-2 were selected with a planting plan of 889 trees/ha and rounded small-size and multi-ax flattened crowns. At the age of six years those orchards provide the competitive yield level (8.1–12.5 t/ha) and fruits marketability (94.1–95.7%). Comparison of the best domestic varieties such as Talisman and Annushka with Regina variety showed that they are competitive and not inferior of foreign ones not only in yield but also in marketable fruit quality. The largest fruits were found out in Talisman variety – 18.4 g with a diameter of 35 mm.The Regina orchards on Gisela 5 and Gisela 6 were low-productive and did not ensure the necessary production marketability level comparing with the Ukrainian orchards.

Keywords: sweet cherry, orchard constructions, yield, fruit mass, marketability

Вступ

На сучасному етапі розвитку інтенсивного садівництва ключовим аспектом новітніх технологій промислового вирощування плодів черешні є, передусім, безумовне дотримання вимог глобальних торговельних мереж до товарної якості продукції, що потребує застосування крупноплідних сортів, високопродуктивних клонових підщеп, специфічних агротехнічних заходів тощо.

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Зважаючи на це, останнім часом у світі триває пошук таких сорто-підщепних комбінувань та способів формування крони, які б забезпечували найбільшу величину плодів для їх реалізації за найвищою ціною (Kappel,1996; Balmer, 2001; Bujdosó, 2004; Whiting, 2005).

На сьогодні найбільш розрекламованими серед них іноземними розсадницькими компаніями є німецькі підщепи серії Gisela, які в поєднанні з скороплідним сортом Регіна стали своєрідним символом інтенсивної культури черешні (Sitarek, 2014; Melnyk, 2017).

Закордонні спеціалісти широко пропонують створювати насадження зі щільністю садіння від 1 250 дер./га, використовуючи зазначені сорто-підщепні комбінування переважно з лідерними формуваннями крони, оскільки на їх переконання саме таке поєднання сорту, підщепи і форми крони сприяє отриманню високої товарної якості продукції (Hrotko, 2011; Lugli, 2011; Sitarek, 2014). Витрати на закладення 1 га такого саду становлять від 9,5 (Melnyk, 2017), а якщо устаткувати його системою краплинного зрошення, захистом від заморозків, граду, дощу та птахів, до 62,5 тис. євро на 1 га (Shevchuk, 2014). При цьому середня урожайність таких насаджень становить 10–15 т/га (Makosh,1999).

Водночас вітчизняними вченими внаслідок довготривалих досліджень розроблено і запропоновано виробництву нові альтернативні типи інтенсивних насаджень черешні з округлою малогабаритною кроною, які потребують в 4,7 рази менше капітальних вкладень на їх створення порівняно з іноземними і забезпечують середню урожайність залежно від типу насаджень – 10,7–19,2 т/га (Kishchak, 2017).

Зважаючи на ці обставини, нами було закладено дослід з порівняльної оцінки сучасних інтенсивних садів з використанням кращих вітчизняних та зарубіжних крупноплідних сортів, високопродуктивних підщеп, сучасних способів формування крон за оптимально щільних схем розміщення дерев. Тому визначення і добір в них ефективних елементів технології і визначало актуальність нашої роботи.

Матеріали та методика

Місце та схема проведення досліду

Навесні 2013 року в Інституті садівництва НААН України було закладено дослід з порівняльної оцінки сучасних інтенсивних насаджень черешні з використанням перспективних вітчизняних та зарубіжних сорто-підщепних комбінувань. Досліджувалися іноземний сорт Регіна на підщепах Гізела 5 і Гізела 6 та вітчизняні сорти Мелітопольська мирна, Талісман і Аннушка, щеплені на ВСЛ-2. Насадження на Гізелі 5 та Гізелі 6 закладалось дворічним кронованим садивним матеріалом іноземного походження, а на ВСЛ-2 – стандартним некронованим однорічним. У дерев зазначених сорто-підщепних комбінувань формували три форми крони – округлу малогабаритну (схема садіння 4 × 2,5 м), веретеноподібну та багатовісну сплощену (4,0–4,5 × 2,0 м).

Особливості формування і обрізування дерев

Округлу малогабаритну крону (далі – округла) формували згідно з рекомендаціями ІС НААН, веретеноподібну шляхом відгинання гілок, які рівномірно спірально розміщували навколо центрального провідника за Kishchak (2013). У багатовісної сплощеної (далі – сплощеної) крони на стовбурі на висоті 70–100 см від рівня ґрунту виділяли 5–6 гілок – вісей, на яких формували обростаючі напівскелетні гілки не старші 4-річного віку. Гілки з широкими кутами відходження від стовбура, що сильно росли в бік міжрядь, утримували обрізуванням в межах проекції плодової стіни, а під меншими 45° залишали і спрямовували вертикально.

Ширина такої крони в поперек ряду не повиненна перевищувати 2,5 м. На однорічних довгих гілках-вісях навесні в період набухання бруньок проводили кербування для кращого галуження пагонів і більшого утворення плодоносних гілочок.

Методика проведення досліду

У кожному варіанті по 9 облікових дерев, повторність триразова. Ґрунт дослідної ділянки темно-сірий опідзолений легкосуглинковий на карбонатному лесі утримували під чорним паром, без зрошування.

Обліки та спостереження за основними показниками росту і плодоношення проводили за загальноприйнятими методиками Karpenchuk and Melnyk (1987) та Sedov фтв Oholtsova (1999).

Результати та обговорення

Дослідженнями українських та зарубіжних вчених встановлено вплив сорто-підщепних комбінувань черешні (Sitarek, 2010; Long, 2015), різних способів формування і обрізування дерев (Tretiak, 1990; Long, 2015) на їх біометричні показники.

Результати досліджень ростових процесів в різних конструкціях насаджень черешні показало, що менша їх інтенсивність відмічається в насадженнях сорту Регіна на Гізелі 5, що має прояв у зменшенні на 21,1–34,7 % сумарного приросту та 14,1–25,4 % об'єму крони порівняно з насадженнями на середньорослих підщепах Гізела 6 та ВСЛ-2.

Протиградове накриття у цьому насадженні спричиняло стримування лінійного росту дерев у висоту, яку зафіксовано у 5-річному віці незалежно від підщепи і форми крони на рівні встановлення сітки, тобто 3,5 м. Натомість показник об'єму крон дерев різнився залежно від сили росту підщепи. Так, на напівкарликовій підщепі Гізела 5 він становив 6,7–7,0 м^3, а на середньорослих (ВСЛ-2 та Гізела 6), особливо у варіанті з округлою малогабаритною кроною, де дерева інтенсивніше розросталися в бік ряду та міжрядь, – 7,3–8,9 м^3 (Таблиця 1).

У 2018 році параметри дерев доводилося сильно обмежувати для залишення світлового проходу 2,0, а висоти – 3,4–3,5 м, тому об'єм крони залишався на рівні значень минулого року.

Таблиця 1 Біометричні показники росту дерев черешні в різних конструкціях саду, 2018 р. Садіння 2013 р.
Table 1 Biometrical indices of the sweet cherry trees growth in different orchard constructions, 2018, planted in 2013

Форма крони (Фактор В)	Схема садіння (м)	Діаметр штамба (см)	Висота дерев (м)	Об'єм крони ($м^3$)
Регіна на підщепі Гізела 5 (Фактор А)				
Округла (к)	4,5 × 2,5	11,8	3,3	7,1
Веретеноподібна	4,0 × 2,0	12,0	3,5	6,7
Сплощена	4,0 × 2,0	10,9	3,4	6,7
Регіна на підщепі Гізела 6				
Округла (к)	4,5 × 2,5	12,4	3,4	8,1
Веретеноподібна	4,0 × 2,0	12,0	3,5	6,7
Сплощена	4,0 × 2,0	12,3	3,5	7,3
Мелітопольська мирна на підщепі ВСЛ-2				
Округла (к)	4,5 × 2,5	12,1	3,4	8,9
Веретеноподібна	4,5 × 2,0	11,1	3,5	7,3
Сплощена	4,5 × 2,0	11,1	3,4	8,3
Талісман на підщепі ВСЛ-2				
Округла (к)	4,5 × 2,5	12,0	3,4	8,1
Веретеноподібна	4,5 × 2,0	11,2	3,4	7,5
Сплощена	4,5 × 2,0	11,2	3,4	8,1
Аннушка на підщепі ВСЛ-2				
Округла (к)	4,5 × 2,5	12,1	3,4	8,5
Веретеноподібна	4,5 × 2,0	11,4	3,4	8,3
Сплощена	4,5 × 2,0	11,4	3,4	8,1
$НІР_{05}$ (А)		0,45	0,04	0,06
$НІР_{05}$ (В)		0,58	0,05	0,08

Водночас, за показником діаметра штамба суттєвої різниці між варіантами не відмічено і на шостому році після садіння він становив як на напівкарликовій підщепі Гізела 5, так і середньорослих ВСЛ-2 та Гізела 6 – 11,4–12,0 см.

Різні способи формування крони також вплинули на величину показників росту дерев черешні. Так, при формуванні веретеноподібної крони застосовували вищий ступінь обрізування дерев, що не завжди сприяло кращому пагоноутворенню. Нові ж пагони, які відростали після сильного вкорочення гілок на заміщення, як правило, займали вертикальне положення і були дуже довгими (до 1,0–1,5 м), що знову ж потребувало вкорочення. У сорту Регіна на Гізелі 5 у варіанті з веретеноподібною кроною загальна кількість пагонів у сумі за 6 років була нижчою і становила 142 шт.,

що в 1,2 рази менше, ніж у контролі (округла малогабаритна крона). Така ж тенденція спостерігається і при формуванні дерев інших сорто-підщепних комбінувань.

Водночас, серед сортів, які вивчалися, саме сорт Регіна відзначався високою пагоноутворювальною здатністю при формуванні веретеноподібної крони. Тому у дерев з використанням цього сорту з підщепами серії Гізела найкраще застосовувати зазначену систему формування. Ось чому саме таке поєднання сорту, підщепи і системи формування має на сьогодні високий рекламний успіх.

Поряд з цим дерева сортів класичної селекції, зокрема Талісман, Мелітопольська мирна, складніше формувати з веретеноподібною кроною через низьку пагоноутворювальну здатність і чітко виражене ярусне закладання скелетних гілок порівняно з сортами нового покоління (Регіна, Аннушка). Тому вирощувати сорти такого типу у високощільних насадженнях з формуванням веретеноподібних крон є недоцільним, оскільки вони будуть низькопродуктивними.

Крім цього, вивчення нових способів формування крони дерев показало, що найбільший ефект досягається за використання кербування однорічних гілок, внаслідок чого кількість бічних розгалужень з плодовими бруньками на деревах збільшується в 1,3–1,4 рази, і як наслідок, зменшується довжина пагонів. Особливо помітним це було у дерев сорту Регіна на Гізелі 5 в варіанті з багатовісною сплощеною кроною, де безпосередньо при формуванні осей застосовували кербування на однорічних довгих гілках, а показник середньої довжини пагонів в 2016–2017 рр. зменшився в 1,2–1,6 рази.

В середньому за роки досліджень показник довжини пагонів не залежав від способу формування і знаходився в межах 56,2–67,9 см.

У 2015 році відмічено перше цвітіння дерев, ступінь якого був вищим в насадженнях сорту Регіна на Гізелі 5 та Гізелі 6 і в насадженні сорту Аннушка на ВСЛ-2 з формуванням веретеноподібної крони (8,0–8,5 б). Проте через ранньовесняні заморозки 21–22 квітня (мінус 0,8–1,0 °С) зав'язування було низьким, а кількість плодів, що утворилася, становила 2–5 штук на дерево. Лише у дерев сорту Аннушка на ВСЛ-2, цвітіння яких також відбувалося в більш пізні строки (25–27.04), зав'язування було кращим (8,8–10,0 %) і вони утворили від 7,2 (багатовісна сплощена) до 31,7 (веретеноподібна) штук плодів, що свідчить про скороплідність даного сорту і ефективність зазначеного формування крони.

У дерев сорту Регіна на підщепах Гізела 5 і Гізела 6, які також цвіли в пізні строки, зав'язування було низьким (2,8–6,7 %), тому кількість плодів не перевищувала 2–4 шт. з розрахунку на дерево, що ставить під сумнів його скороплідність зважаючи на те, що насадження закладалося дворічними саджанцями.

У 2016 році через зниження температури повітря в період цвітіння пізніх сортів до +1,5–5,9 °С відсоток зав'язування плодів черешні був низьким і становив по сорту Регіна на підщепах Гізела 5 та Гізела 6 від 4,2 до 12,9 %, Талісман на ВСЛ-2 – 10,8–15,7 %, Аннушка на ВСЛ-2 – 7,5–12,3 % (Таблиця 2).

Таблиця 2 Зав'язування плодів черешні в різних конструкціях саду. Садіння 2013 р.
Table 2 Sweet cherry fruits infructescence in different orchard constructions, planted in 2013

Форма крони (Фактор В)	Схема садіння (м)	Зав'язування плодів за три роки (%)			
		2016	2017	2018	середнє
Регіна на підщепі Гізела 5 (Фактор А)					
Округла (к)	4,5 × 2,5	9,6	32,6	5,0	15,7
Веретеноподібна	4,0 × 2,0	8,4	36,7	7,3	17,5
Сплощена	4,0 × 2,0	12,2	42,6	4,1	19,6
Регіна на підщепі Гізела 6					
Округла (к)	4,5 × 2,5	12,9	35,1	3,8	17,3
Веретеноподібна	4,0 × 2,0	7,2	39,1	6,9	17,7
Сплощена	4,0 × 2,0	4,2	40,0	2,5	3,6
Мелітопольська мирна на підщепі ВСЛ-2					
Округла (к)	4,5 × 2,5	9,8	38,1	16,6	21,5
Веретеноподібна	4,5 × 2,0	10,9	42,1	17,9	23,6
Сплощена	4,5 × 2,0	20,0	40,2	21,9	27,4
Талісман на підщепі ВСЛ-2					
Округла (к)	4,5 × 2,5	13,0	31,2	15,0	19,7
Веретеноподібна	4,5 × 2,0	15,7	41,0	14,7	23,8
Сплощена	4,5 × 2,0	10,8	47,6	28,8	29,1
Аннушка на підщепі ВСЛ-2					
Округла (к)	4,5 × 2,5	7,5	51,3	16,8	25,2
Веретеноподібна	4,5 × 2,0	12,3	40,1	15,8	22,7
Сплощена	4,5 × 2,0	9,6	47,6	14,7	24,0
$НІР_{05}$		1,1	4,4	1,4	

Тому врожай плодів був невисоким і становив від 1,5 кг/дер. (Мелітопольська мирна на підщепі ВСЛ-2) до 5,2 кг/дер. (Регіна на підщепі Гізела 6).

У 2017 році за сприятливих умов перезимівлі генеративні бруньки в усіх типах саду добре збереглися, тому цвітіння було дружним, а відсоток зав'язування плодів – високим (31,2–51,3 %). Очікуваний урожай п'ятирічних насаджень черешні залежно від сорту знаходився в межах 6,4–16,2 т/га.

Водночас 11 травня 2017 року через значне зниження температури повітря до мінус 2 °С відмічено сильне підмерзання зав'язі, що призвело до втрати врожаю і внаслідок чого продуктивність насаджень становила в середньому 0,3–1,6 т/га.

У 2018 році незважаючи на те, що грудень та січень характеризувалися підвищеними температурами повітря, в другій декаді лютого відмічали їх зниження до -21,3 °С. Це спричинило навесні у дерев черешні значне побуріння тканин під бруньками.

Внаслідок негативної дії низьких температур та повітряної посухи в період цвітіння спостерігали масове опадання зав'язі, що вплинуло на зниження рівнів їх урожайності.

За таких умов кращу адаптивність виявили насадження черешні на середньорослій підщепі ВСЛ-2, яка навіть за меншої в 1,4 раза щільності розміщення дерев порівняно з напівкарликовою Гізела 5 забезпечили вищу продуктивність. Зокрема, у вітчизняних сортів Талісман та Аннушка на підщепі ВСЛ-2 (889 дер./га) з формуванням округлої малогабаритної крони врожайність становила 11,5–12,5 т/га, що в 6,0–7,4 раза вище, ніж у насадженнях німецького сорту Регіна на підщепах Гізела 5 та Гізела 6 (1 250 дер./га) з формуванням у дерев веретеноподібної крони (Таблиця 3).

Таблиця 3 Урожайність черешні залежно від конструкцій саду. Садіння 2013 р.
Table 3 Sweet cherry productivity depending on the orchard constructions, planted in 2013

Форма крони (Фактор В)	Схема садіння (м)	Урожайність (кг/дер.)				Урожайність (т/га)			
		2016	2017	2018	середнє	2016	2017	2018	середнє
Регіна на підщепі Гізела 5(Фактор А)									
Округла (к)	4,5 × 2,5	3,6	0,3	2,5	4,3	3,2	0,3	2,2	1,9
Веретено подібна	4,0 × 2,0	3,0	0,5	1,5	5,4	3,8	0,6	1,7	2,0
Сплощена	4,0 × 2,0	3,8	0,9	2,0	7,3	4,7	1,0	2,2	2,6
Регіна на підщепі Гізела 6									
Округла (к)	4,5 × 2,5	5,2	0,3	2,4	5,6	4,6	0,3	2,2	2,4
Веретено подібна	4,0 × 2,0	2,0	0,3	1,7	5,0	2,7	0,3	1,9	1,6
Сплощена	4,0 × 2,0	3,1	0,5	1,5	6,2	3,9	0,5	1,7	2,0
Мелітопольська мирна на підщепі ВСЛ-2									
Округла (к)	4,5 × 2,5	1,5	0,2	9,2	5,9	1,4	0,2	8,2	3,3
Веретено подібна	4,5 × 2,0	2,0	0,3	4,5	5,3	2,2	0,3	4,0	2,2
Сплощена	4,5 × 2,0	1,9	0,4	7,3	7,1	2,1	0,4	8,1	3,5
Талісман на підщепі ВСЛ-2									
Округла (к)	4,5 × 2,5	2,2	0,3	14,0	7,5	2,0	0,3	12,5	4,9
Веретено подібна	4,5 × 2,0	2,7	0,3	3,7	5,1	3,0	0,3	4,1	2,5
Сплощена	4,5 × 2,0	2,5	0,3	7,3	6,4	2,8	0,3	8,1	3,7
Аннушка на підщепі ВСЛ-2									
Округла (к)	4,5 × 2,5	2,4	1,0	13,0	8,0	2,1	0,9	11,5	4,8
Веретено подібна	4,5 × 2,0	2,7	1,1	5,9	7,0	3,0	1,2	6,6	3,6
Сплощена	4,5 × 2,0	2,2	1,4	11,0	8,2	2,4	1,6	12,2	5,4
$НІР_{05}$ (А)		0,61	0,08	1,39		0,69	0,08	1,5	
$НІР_{05}$ (В)		0,47	0,06	1,07		0,53	0,06	1,2	

Слід зазначити, що за роки досліджень насадження з веретеноподібною кроною мали найнижчі показники урожайності не тільки по сорту Регіна, а й по інших досліджуваних сортах, що пояснюється особливостями формування крон з регулярною заміною напівскелетних гілок з плодовою деревиною. Середня урожайність у варіантах із такими кронами за 2016–2018 рр. не перевищувала 1,6–3,6 т/га і була на 25–49 % меншою порівняно з округлою малогабаритною кроною, при цьому показник середньої маси плодів у таких дерев практично не збільшувався.

Аналогічні результати отримали ще у 2007 р. чеські вчені Blažková та Hlušičková (2007), які показали, що у сорту Регіна на 10 досліджуваних клонових підщепах, в т.ч. серії Gisela, сумарний урожай за роки досліджень був на 40–60 % нижчим, ніж у Лапінса. При цьому середня маса плодів у обох сортів була на одному рівні.

Крім того, на Конференції Міжнародної асоціації розвитку інтенсивного садівництва (IFTA) по культурі черешні, яка відбулася у 2014 р. в м. Келовні (Канада) професор Lynn Long (США) за наслідками багаторічних досліджень засвідчив, що сорт Регіна є низькопродуктивним порівняно з традиційними американськими Бінгом та Світхартом, тому вважає недоцільним його застосування в промислових насадженнях (Long, 2014).

У 2018 році склалися сприятливі умови для формування плодів черешні високої товарної якості. Достатні запаси продуктивної вологи в ґрунті, які сформувалися внаслідок випадання великої кількості опадів в березні та відсутність їх під час достигання плодів забезпечили високу товарність вітчизняних сортів на рівні 87,3–96,2 %.

Випадання двох дощів 13 та 14 червня (по 4,76 мм) у період збирання врожаю не викликали розтріскування плодів навіть у сортів, схильних до нього.

Найбільшу кількість опадів в червні (22,92 мм) відмічали 19.06, коли практично весь урожай основних сортів було зібрано і лише плоди найпізнішого за строком достигання сорту Регіна залишилися на дереві. Після випадання такої кількості опадів у сорту Талісман кількість плодів, що розтріскалися, становила 10,3 %, Аннушка – 18,1, а у сорту Регіна вона була найбільшою, зокрема на підщепі Гізела 6 – 16,9–18,7, а на Гізелі 5 – 19,3–21,1 %. В період повного достигання плодів цього сорту після опадів першого (8,96 мм) та другого липня (3,64 мм) вона збільшилася до 22,4 %. Внаслідок цього товарність плодів сорту Регіна становила лише 76,5–81,1 %, а Талісману і Аннушки 94,1–95,7 %.

На зниження товарності плодів сорту середнього строку достигання Мелітопольська мирна вплинуло більше пошкодження їх птахами (2,1–4,4 %), та як наслідок, загнивання (3,2–5,5 %). У зв'язку з цим, кількість непошкоджених плодів становила 87,3–90,8 %, а їх середня маса – 8,5–8,7 г (Рисунок 1).

В цілому за роки досліджень середня маса плоду не залежала від способів формування і становила у сорту Регіна на Гізелі 5 – 9,3–9,6 г з дещо вищим показником на Гізелі 6 – 9,9–10,1 г. На цьому ж рівні знаходилися плоди сортів Талісман і Аннушка – 9,1–10,0 г, а меншими вони були у сорту Мелітопольська мирна – 8,1–8,3 г. Найкрупніші окремі плоди формувались у сорту Талісман, де їх маса сягала 18,4 г з діаметром

35 мм та Аннушки – 12,1 г і 31 мм, тоді як у сорту Регіна маса найкрупніших плодів становила відповідно 12 г з діаметром 30 мм.

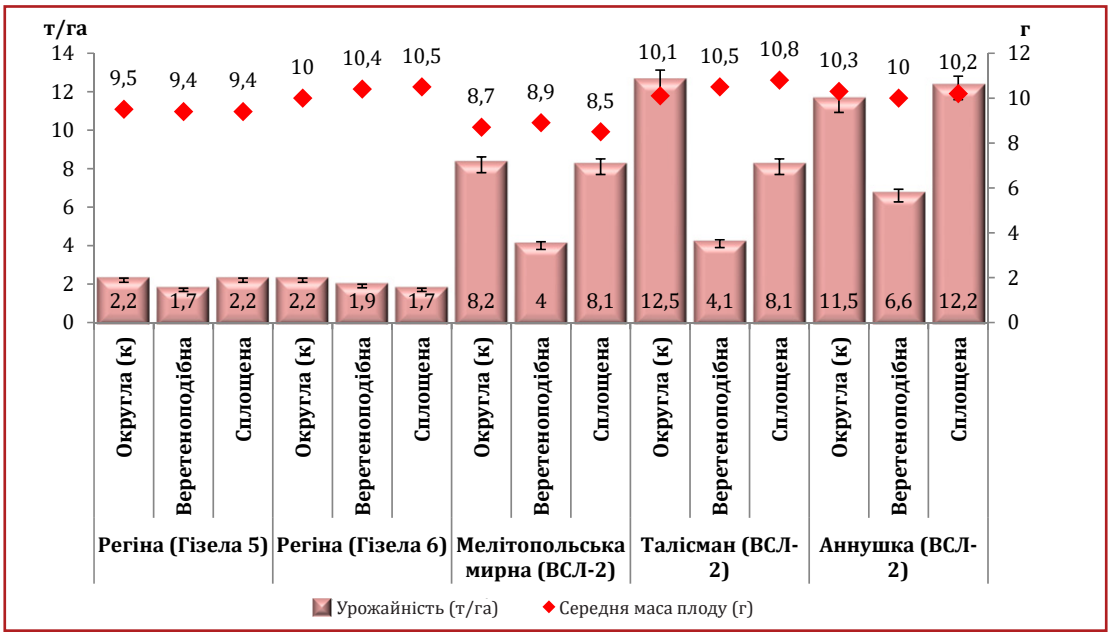


Рисунок 1 Урожайність та середня маса плоду черешні в різних конструкціях саду, 2018 р.
Figure 1 Sweet cherry yield productivity and fruit mass in different orchard constructions, 2018

Отже, порівняння кращих вітчизняних сортів з популярними сортами іноземної селекції, показало, що вони є конкурентоспроможними і не поступаються зарубіжним не тільки за урожайністю, а і товарною якістю плодів.

Висновки

В різних конструкціях саду серед досліджуваних способів формування веретеноподібна крона виявилася менш продуктивною для всіх досліджуваних сорто-підщепних комбінувань і в середньому за 2016–2018 роки поступалася в 1,2–2,0 рази за цим показником варіантам з округлою малогабаритною та багатовісною сплощеною кроною.

За роки досліджень сорт Регіна в усіх конструкціях саду забезпечував в 1,3–2,6 рази меншу урожайність, ніж вітчизняні сорти на середньорослій підщепі ВСЛ-2, що свідчить про його непридатність для створення високопродуктивних насаджень. Кращими за продуктивністю в період росту і плодоношення виділилися насадження з використанням сортів черешні Талісман і Аннушка на середньорослій підщепі ВСЛ-2 з щільністю садіння 889 дер./га і формуванням округлої малогабаритної та багатовісної сплощеної крони, де в 6-річному віці досягнуто конкурентоспроможний рівень урожайності (8,1–12,5 т/га) та товарності плодів (94,1–95,7 %). Зазначений тип саду забезпечує отримання стабільних врожаїв та продукції високої товарної якості, що відповідає вимогам глобальних торговельних мереж.

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POSSIBILITY OF REPRODUCTION OF *LINUM USITATISSIMUM* L. FROM SEEDS WITH LOW GERMINATION AND VIABILITY *IN VITRO* CONDITIONS

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The development of a method for the reproduction of flax (*Linum usitatissimum* L.) from seeds of low germination and viability was the goal of our research. To prevent damage to the seed embryo and weak seedlings for sterilization of the seeds aqueous NaOCl solution at a reduced concentration (1.5%) and a reduced exposure (10 minutes) was applied. The effect of a combination of various combinations of phytohormones and other biologically active substances on the germination of flax seed was established. Cultivation of seeds in the Murashige and Skoog medium with macro elements and microelements in the full dose which includes 2.5 mg/L glycine, 0.2 mg/L thiamine, 1.0 mg/L pyridoxine, 5.0 mg/L ascorbic acid, 0.2 mg/L kinetin (KIN), 0.2 mg/L gibberellic acid (GA_3), 4.0 m/L succinic acid, 12.5 g/L sucrose and does not contain nicotinic acid increases the seeds germination to 10.0–25.0%. In this case, the explants were cultivated for 2–3 days at a temperature of 19–21 °C and further at a temperature of 22–24 °C. The White medium which contains 0.1 mg/L of 1-naphthylacetic acid (NAA) and 12.5 g/L of sucrose was used for microclonal propagation of the generated shoots. The proposed method provides the obtaining of valuable flax seed breeding material from the seed of low germination and viability, a high coefficient of its reproduction, which accelerates the breeding process.

Keywords: flax, seeds, germination, reproduction, *in vitro*

Вступ

Льон звичайний (*Linum usitatissimum* L.) є важливою сільськогосподарською культурою комплексного використання. Здебільшого його вирощують для отримання натурального волокна, що може використовуватися у текстильній промисловості, насіння, харчової або технічної олії. Не зважаючи на те, що льон культивують декілька тисячоліть, він і сьогодні залишається предметом численних наукових досліджень, присвячених філогенезу і таксономії (Optasiuk and Shevera, 2011; Zelentsov et al., 2016), селекції (Lohinov, 2007; Kryvosheieva, 2017), технології вирощування (Shuvar, 2015; Vyshnivska et al., 2017), біотехнології (Polyakov, 2000; Evtimova et al., 2005; Millam et al., 2005; Burbulis and Blinstrubiene, 2011) тощо.

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За умови зберігання у звичайних умовах складських чи лабораторних приміщень насіння льону здатне до проростання протягом 8–10 років. Розмноження зразків колекції льону здійснюється також через декілька років і в незначних обсягах, тому воно часто характеризується низькою життєздатністю і, за потреби, не може у достатній мірі забезпечити відтворення селекційного матеріалу. Крім того, льон має низький коефіцієнт розмноження насіння. Таким чином, актуальним є розробка способу розмноження рослин льону з насіння з низькою схожістю та життєздатністю в умовах *in vitro* для збереження цінних генотипів в сільськогосподарській біотехнології і селекції.

Відомим є спосіб розмноження рослин міскантусу з низькою схожістю та життєздатністю (Hontarenko and Lashuk, 2015), який включає використання як експлантів насіння міскантусу, застосування гіпохлориту натрію (NaOCl) для стерилізації насіння, висаджування його на агаризоване живильне середовище з макро- і мікроелементами та додаванням регуляторів росту у певних концентраціях, отримання калусів у культурі *in vitro*, культивування калусів та регенерацію з них мікропросток. Також відомим є спосіб відновлення схожості насіння перцю (Ivchenko, 2016), в основу якого покладено пророщування насіння в умовах *in vitro* на середовищі Мурасіге і Скуга, модифікованому гібереловою кислотою ($ГК_3$) (0,1 мг/л) і кінетином (КІН) (3 мг/л), та на середовищі Мурасіге і Скуга, модифікованому бурштиновою кислотою (БК) (3 мг/л). Установлено, що культивування на живильних середовищах дозволяє вивести зі стану органічного спокою від 5 до 100 % насінин дикорослих видів томата, які зберігалися у неконтрольованих умовах протягом тривалого часу (до 15-ти років) (Shabetia et al., 2014; Miroshnychenko, 2016). Серед луб'яних культур подібний спосіб відновлення схожості насіння запропонований для конопель, у яких воно здатне до проростання не більше 3–4 років, та має значні межі варіювання енергії проростання і схожості: розмах варіації становить від 1 до 68 (Mishchenko, 2013). Виявлено, що додавання до середовища Мурасіге і Скуга 0,4 мг/л $ГК_3$ і 4,0 мг/л БК підвищує схожість насіння на 7,5–24,0 % (Mishchenko and Laiko, 2017; Mishchenko, 2018).

Мета і завдання дослідження – розробити спосіб розмноження льону звичайного з насіння з низькою схожістю та життєздатністю *in vitro* та підвищити коефіцієнт розмноження шляхом висаджування насіння на живильне агаризоване середовище певного складу (який би не викликав активного калусоутворення чи пригнічення проростків) з подальшим мікроклональним розмноженням отриманих пагонів.

Матеріали та методи

Рослинний матеріал

У дослідженнях, проведених у 2019 р., використані колекційні зразки льону звичайного UF0402071 Есмань (країна походження – Україна) і UF0402132 Elise (країна походження – Нідерланди) урожаю 2012, 2014 і 2017 рр.

Особливості культивування *in vitro*

Насіння стерилізували водним розчином гіпохлориту натрію (NaOCl) у концентрації 1,5, 3,0 і 6,0% з експозицією 10, 15 і 20 хв. Тричі промивали стерильною дистильованою водою і висаджували на агаризоване живильне середовище. За основу було взяте середовище Мурасіге і Скуга (MS) (Murashige and Skoog, 1962), яке доповнювали фітогормонами, регуляторами росту та вітамінами у різних поєднаннях і концентраціях, зокрема досліджено наступні варіанти: 1) $ГК_3$ 0,1 мг/л, КІН 0,1 мг/л; 2) $ГК_3$ 0,1 мг/л, БК 2,0 мг/л; 3) БК 2,0 мг/л; 4) $ГК_3$ 0,2 мг/л, КІН 0,2 мг/л; 5) $ГК_3$ 0,2 мг/л, БК 4,0 мг/л; 6) БК 4,0 мг/л; 7) $ГК_3$ 0,4 мг/л, КІН 0,4 мг/л; 8) $ГК_3$ 0,4 мг/л, БК 4,0 мг/л; 9) $ГК_3$ 0,2 мг/л, КІН 0,2 мг/л, БК 4,0 мг/л; 10) 6бензиламінопурин (БАП) 0,2 мг/л, БК 2,0 мг/л; 11) індол-3-оцтова кислота (ІОК) 0,6 мг/л, БК 2,0 мг/л; 12) контроль – безгормональне середовище. У базовому середовищі було збільшено на чверть концентрацію гліцину (2,5 мг/л) та вдвічі збільшено вміст тіаміну (0,2 мг/л) і піридоксину (1,0 мг/л), додано 5,0 мг/л аскорбінової кислоти, 12,5 г/л сахарози. Модифіковане середовище не містило нікотинової кислоти. Експланти культивували 2–3 доби при температурі 19–21 °С і надалі при температурі 22–24 °С, фотоперіоді 16 год і відносній вологості повітря 60–80 %. Мікроклональне розмноження проводили при досягненні пагонами висоти 10–15 см. Для мікроклонального розмноження використовували середовище White (1943), Murashige and Skoog (1962), Gamborg and Eveleigh (1968).

Морфометричний та статистичний аналіз

Схожість насіння визначали на 10-ту добу, вимірювання мікроклонів проводили на 35 ту добу від початку культивування. Статистичну обробку експериментальних даних проведено за параметрами варіаційної статистики та *t*-критерієм Стьюдента (Dospekhov, 1973).

Результати та їх обговорення

Розроблений спосіб розмноження рослин льону звичайного з насіння з низькою схожістю та життєздатністю включає використання як експлантів насіння, застосування гіпохлориту натрію для його стерилізації, висаджування насіння на агаризоване живильне середовище, мікроклональне розмноження утворених пагонів *in vitro*.

Насіння льону стерилізують водним розчином гіпохлориту натрію (NaOCl). Використання даного стерилізуючого агента у зниженій концентрації до 1,5 % і за зменшеної експозиції до 10 хв, триразове промивання стерильною дистильованою водою запобігають ушкодженню зародка насінини, утворенню слабких проростків льону та появі видимих мутацій. За таких умов вихід стерильних експлантів становить 97,5–100,0 %.

Потім простерилізоване насіння висаджують на живильне середовище Мурасіге і Скуга з макро- і мікроелементами у повній дозі, до складу якого входить 2,5 мг/л гліцину, 0,2 мг/л тіаміну, 1,0 мг/л піридоксину, 5,0 мг/л аскорбінової кислоти,

0,2 мг/л КІН, 0,2 мг/л $ГК_3$, 4,0 мг/л БК, 12,5 г/л сахарози і яке не містить нікотинової кислоти, культивують 2–3 доби при температурі 19–21 °С і надалі при температурі 22–24 °С, фотоперіоді 16 год і відносній вологості повітря 60–80 %. Було використано різні поєднання і концентрації фізіологічно активних речовин для підвищення (відновлення) схожості, однак вищезазначене виявилось найбільш ефективним (Таблиця 1).

Таблиця 1 Схожість насіння льону в залежності від умов культивування
Table 1 Seed germination of flax depending on conditions of cultivation

Варіант	Схожість насіння залежно від року урожаю (%)					
	UF0402071 Есмань			UF0402132 Elise		
	2012 р.	2014 р.	2017 р.	2012 р.	2014 р.	2017 р.
Лабораторна схожість	11,25	63,75	80,00	10,00	60,00	78,75
MS безгормональне	15,00	68,75	80,00	11,25	65,00	78,75
MS + $ГК_3$ 0,1 мг/л, КІН 0,1 мг/л	20,00	70,00	85,00	15,00	70,00	80,00
MS + $ГК_3$ 0,1 мг/л, БК 2,0 мг/л	16,25	70,00	80,00	15,00	66,25	80,00
MS + БК 2,0 мг/л	15,00	70,00	81,25	15,00	65,00	80,00
MS + $ГК_3$ 0,2 мг/л, КІН 0,2 мг/л	25,00	76,25	90,00	20,00	70,00	85,00
MS + $ГК_3$ 0,2 мг/л, БК 4,0 мг/л	25,00	70,00	86,25	16,25	70,00	85,00
MS + БК 4,0 мг/л	18,25	70,00	85,00	15,00	65,00	77,50
MS + $ГК_3$ 0,4 мг/л, КІН 0,4 мг/л	30,00	76,25	91,25	25,00	75,00	90,00
MS + $ГК_3$ 0,4 мг/л, БК 4,0 мг/л	26,25	75,00	95,00	25,00	70,00	85,00
MS + $ГК_3$ 0,2 мг/л, КІН 0,2 мг/л, БК 4,0 мг/л	36,25	80,00	100,0	30,00	80,00	95,00
MS + БАП 0,2 мг/л, БК 2,0 мг/л	10,00	65,00	75,00	5,00	61,25	76,25
MS + ІОК 0,6 мг/л, БК 2,0 мг/л	10,00	68,75	87,50	10,00	65,00	80,00

Слід зазначити, що амінокислотний та вітамінний склад забезпечують оптимізацію біохімічних процесів у пагоні. Підвищена концентрація аскорбінової кислоти до 5,0 мг/л, яка є антиоксидантом, попереджує утворення фенольних сполук, що спричиняють пригнічення росту і розвитку або ж ведуть до загибелі експлантів. Цитокінін КІН, $ГК_3$ та БК саме у концентраціях 0,2, 0,2 і 4,0 мг/л відповідно ініціюють та стимулюють поділ клітин у зародку насінини, ріст сім'ядоль, зародкових стебельця і корінця за одночасної збереженості генетичної автентичності зразка,

що встановлено експериментально, дозволяють призупиняти дію накопиченого в тканинах зиготичних зародків насінини в процесі зберігання природного інгібітора росту – абсцизової кислоти (АК). Таким чином, зазначені регулятори росту впливають на регуляторні механізми, пов'язані з порушенням органічного спокою зрілих зародків льону.

Змінна температура культивування, дещо знижена на початку пророщування насіння і підвищена через 2–3 доби, підвищує інтенсивність ростових процесів. Вищезазначені умови дозволяють отримати на 10,0–25,0 % більше проростків порівняно з лабораторною схожістю, тобто розроблений спосіб підтверджує свою ефективність.

Після того, як пагони досягнуть довжини 10–15 см, проводять мікроклональне розмноження (Рисунок 1).

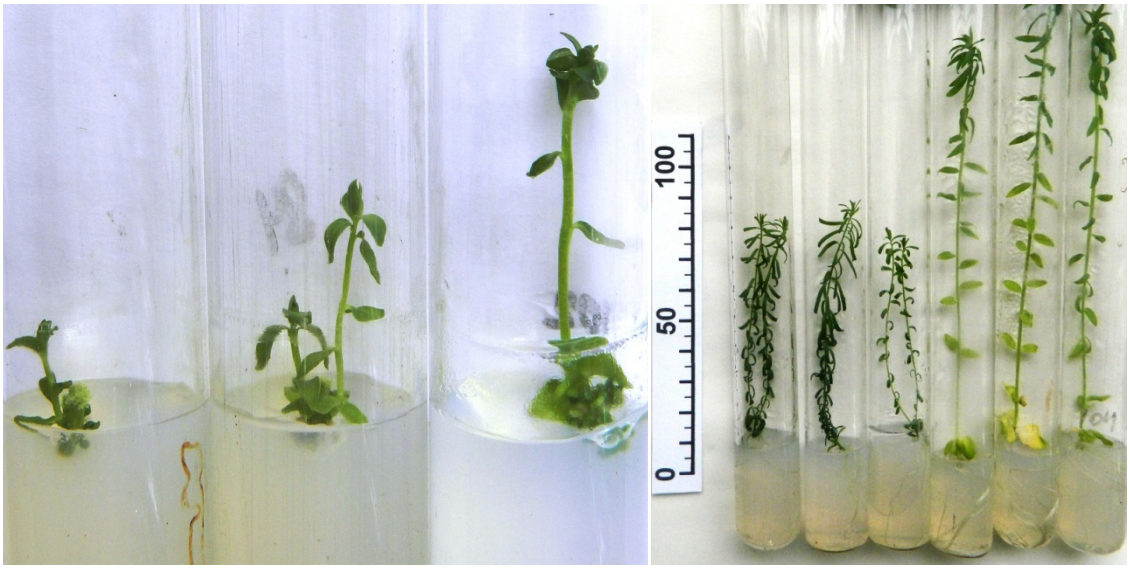


Рисунок 1 Ріст мікроклонів льону *in vitro*
Figure 1 Growth of microclone flax *in vitro*

Верхівкові (апікальні) і бічні (латеральні) бруньки або живці з меристемами пересаджують на живильне середовище Уайта, до складу якого входить 0,1 мг/л 1нафтилоцтової кислоти (НОК) і 12,5 г/л сахарози, що дозволяє отримувати з 1-го проростка в середньому 15–25 мікроклонів з високою частотою ризогенезу (близько 85 %). Якщо генотип є чутливим до індукції ризогенезу, то додавання НОК є необов'язковим, також можливим є використання живильних середовищ Мурасіге і Скуга, Гамборга і Евелега. Пагони, що досягли 7–10 см, адаптують *in vivo*.

Мікроклональне розмноження отриманих в результаті дії екзогенних регуляторів росту пагонів на штучному живильному середовищі за наявності вітамінного комплексу дозволяє отримувати з 1-го пагона в середньому 16–21 мікроклонів. Включення у середовище КІН, $ГК_3$ і БК у певних концентраціях сприяє на достовірному

рівні збільшенню висоти рослин як у зразка UF0402071 Есмань (13,37 см для без гормонального середовища і 14,60 см для середовища з регуляторами росту), так і UF0402132 Elise (11,98 см, порівняно з 14,03 см), а також кількості мікроклонів, отриманих з одного пагона (16,2 і 21,1, 15,6 і 20,3 відповідно), без зміни генетичної автентичності (Таблиця 2).

Таблиця 2 Залежність ознак висоти пагонів та кількості мікроклонів з одного пагона від умов культивування (зразок UF0402071 Есмань – чисельник, зразок UF0402132 Elise – знаменник)

Table 2 Dependence of the signs of the shoots height and the number of microclones from one shoot from the cultivation conditions (sample UF0402071 Есмань – numerator, sample UF0402132 Elise – denominator)

Статистичний показник	Варіант		*t*ф
	MS безгормональне	MS + $ГК_3$ 0,2 мг/л, КІН 0,2 мг/л, БК 4,0 мг/л	
	Висота пагонів (см)		
Середнє арифметичне та похибка вибіркової середньої	13,37 ±0,318 11,98 ±0,685	14,60 ±0,168 14,03 ±0,168	3,42 2,91
Коефіцієнт варіації (%)	10,6 25,6	5,1 5,4	
Розмах варіації ($x_{max} - x_{min}$)	5,0 10,4	1,3 2,5	
Середньоквадратичне стандартне відхилення	1,142 3,062	0,751 0,753	
	Кількість мікроклонів з одного пагона (шт.)		
Середнє арифметичне та похибка вибіркової середньої	16,2 ±0,559 15,6 ±0,456	21,1 ±0,761 20,3 ±0,665	5,19 5,83
Коефіцієнт варіації (%)	15,5 13,1	16,1 14,6	
Розмах варіації ($x_{max} - x_{min}$)	10 9	14 12	
Середньоквадратичне стандартне відхилення	2,498 2,038	3,401 2,975	

Примітка: $t_{0,05} = 2,01$; $t_{0,01} = 2,68$; $t_{0,001} = 3,50$

Як і коноплі посівні (Mishchenko and Laiko, 2017; Mishchenko, 2018), льон звичайний є досить чутливим до наявності в середовищі фітогормонів екзогенного походження, використання порівняно високих концентрацій КІН (3 мг/л), що запропоновано для відновлення схожості перцю (Ivchenko, 2016), веде до інтенсивного утворення калусних тканин, нездатних до органогенезу (формування пагонів). Виявилось, що концентрації 0,2 мг/л у комплексі з іншими біологічно активними речовинами ($ГК_3$ і БК) вже достатньо для підвищення схожості насіння. Спосіб розмноження рослин міскантусу з насіння з низькою схожістю та життєздатністю, який включає використання як експлантів насіння міскантусу, отримання калусів у культурі *in vitro*,

культивування калусів та регенерацію з них мікропроростлин (Hontarenko and Lashuk, 2015), не доцільно застосовувати щодо досліджуваного нами виду, оскільки льон звичайний досить чутливий до культури *in vitro*, що може викликати появу різного роду змінених ознак і нових форм (Kubrak and Shapturenko, 2013).

Культивування соматичних тканинних структур і регенерація з них рослин дозволяє отримувати генетично змінені рослини-регенеранти, які можуть суттєво відрізнятись генотипічно і фенотипічно від своїх вихідних форм. Саме проходження клітинами стадії калусогенезу веде до появи різного роду змін в генотипі, а рівень сомаклональної мінливості залежить від різних факторів, зокрема вихідного матеріалу, умов культивування *in vitro* складу живильних середовищ, наявності екзогенних регуляторів росту тощо. Таким чином, можна втратити генетичну автентичність зразків льону. Корисним даний метод буде для створення нового селекційного матеріалу.

Висновки

Запропонований спосіб забезпечує отримання цінного селекційного матеріалу льону звичайного з насіння з низькою схожістю та життєздатністю, високий коефіцієнт його розмноження, що прискорює селекційний процес.

Подяка

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SCREENING FOR ANTIMICROBIAL ACTIVITY OF NINE ETHANOLIC EXTRACTS OBTAINED FROM LEAVES OF *BEGONIA* PLANT: A POSSIBLE ALTERNATIVE IN THE TREATMENT OF INFECTIONS CAUSED BY *CITROBACTER FREUNDII*

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Many plants of the family *Begoniac*eae are used in the treatment of different diseases. Traditionally leaves of *Begonia* L. are used to decrease of fleeting pain in the limbs and joints, for blood purification, to reduce the body temperature, for treating anemia, for the treatment of respiratory infections, diarrhea, blood cancer, and skin diseases, peptic ulcer, conjunctivitis, colic and dyspepsia, dysentery and mouth ulcer. Moreover, the leaves of *Begonia* species are used for the treatment of cancer; besides, they possess anti-HIV activity. Some of the plants of the genus *Begonia* were previously reported for their antimicrobial activities. The antimicrobial activity of ethanolic extracts obtained from the leaves of *Begonia solimutata* L.B. Sm. & Wassh., *Begonia goegoensis* N.E.Br., *Begonia foliosa* Kunth, *Begonia* × *erythrophylla* Hérincq, *Begonia thiemei* C.DC., *Begonia peltata* Otto & Dietr., *Begonia heracleifolia* Cham. & Schltdl., *Begonia dregei* Otto & Dietr., and *Begonia mexicana* G. Karst. ex Fotsch was evaluated against the clinical strain of *Citrobacter freundii* strain. The testing of the antibacterial activity of the plant extracts was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. All ethanolic extracts obtained from leaves of *Begonia* species exhibited high activity against *C. freundii*. The most effective plants among species screened against *Citrobacter freundii* locally isolated were *B. thiemei*, *B. foliosa*, and *Begonia* × *erythrophylla* being highly active with the ethanolic extract (diameters of inhibition zone were ranged from 16.5 to 26 mm). The highly active antimicrobial effects of extracts obtained from *B. thiemei*, *B. foliosa* noted against *Citrobacter freundii* are worthy of highlighting. The identification of active compounds and their mode of action requires further investigation for antibacterial drug development.

Keywords: *Begonia*, leaf ethanolic extracts, antibacterial activity, inhibition zones, disc diffusion technique

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Introduction

Begonia L. is one of the most species-rich angiosperm genera with approximately 1,500 species currently recognized (Frodin, 2004). Representatives of *Begonia* genus are widely known as popular ornamental plants but the medicinal importance of its members is sparsely known. A few papers have been published, providing information regarding the medical properties of various *Begonia* species (Indrakumar et al., 2014; Amutha and Sreedevikumari, 2016; Shrestha et al., 2016). For instance, its leaves, flowers, and roots are used in diverse ailments in traditional and folklore remedies. Moreover, the leaves of *Begonia* species are used for the treatment of cancer and possess anti-HIV activity (Wu et al., 2004).

Plants belonging to the *Begonia* genus can be good candidates for as an alternative therapy in restricting the resistant infectious organisms. Some of the plants of the genus *Begonia* were previously reported for their antimicrobial activities (Holetz et al., 2002; Indrakumar et al., 2014; Amutha and Sreedevikumari, 2016; Shrestha et al., 2016). The antimicrobial activities of volatile compounds of intact plants of 24 *Begonia* species have been assessed against several pathogenic microorganisms (i.e. *Staphylococcus epidermidis*, *Escherichia coli*, and *Candida albicans*). As a result, 14 *Begonia* species, possessing well expressed phytoncide activity have been recommended to use as indoor plants, based on their ability to reduce microbial air pollution indoor by a factor of 1,5–3,0 in particular, by decreasing the *Staphylococcus aureus* load (Karpova et al., 2009, Tsybulia et al., 2011).

The genus *Citrobacter* belongs to the family of Enterobacteriaceae and comprises 11 different species of facultatively anaerobic, motile, Gram-negative bacilli, which are oxidase-negative and typically utilize citrate as the sole carbon source (Hodges et al., 1978; Janda et al., 1994; Samonis et al., 2009). Among *Citrobacter* species, the most commonly isolated from human clinical specimens are *C. koseri* (formerly named *C. diversus*), *C. freundii*, *C. youngae*, *C. braakii*, and *C. amalonaticus*, while the majority of cases of infection are associated with *C. koseri* and *C. freundii* (Janda et al., 1994; Samonis et al., 2009). *Citrobacter freundii* is recognized as an emerging opportunistic pathogen and is known to cause a variety of ailments (e.g., urinary tract infections, wound infections, gastrointestinal infections, septicemia, meningitis), especially in immunocompromised patients and in-hospital settings (Brenner et al., 1993; Gupta et al., 2003; Samonis et al., 2009; Ranjan and Ranjan, 2013; Leski et al., 2016). This emergence has coincided with the finding that *C. freundii* is often resistant to multiple antibiotics, suggesting that both clinical and environmental strains may be important reservoirs of antimicrobial resistance determinants (ARDs) (Pepperell et al., 2002; Leski et al., 2016). *Citrobacter* infections were found to represent 0.8% of Gram-negative infections in a large surveillance study (Jones et al., 2000; Samonis et al., 2009). Moreover, in hospital settings, *Citrobacter* spp. have accounted for 3–6% of all isolates of Enterobacteriaceae (Lipsky et al., 1980; Samonis et al., 2009; Lavigne et al., 20117). The mortality rate of hospitalized patients with *Citrobacter* infections has been observed to be 6.8% (Mohanty et al., 2007).

Considering the points highlighted above and based on previous results obtained in our laboratory, the aim of the present study was to find out *in vitro* possible antimicrobial action of the ethanolic extracts from leaves of nine *Begonia* species against *Citrobacter freundii* strain locally isolated.

Materials and methodology

Collection of Plant Materials. Collection of Plant Material

The leaves of *Begonia* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. The leaves of *Begonia solimutata* L.B. Sm. & Wassh., *Begonia goegoensis* N.E.Br., *Begonia foliosa* Kunth, *Begonia* × *erythrophylla* Hérincq, *Begonia thiemei* C.DC. (syn. *Begonia macdougallii* Ziesenh.), *Begonia peltata* Otto & Dietr. (syn. *Begonia kellermanii* C.DC.), *Begonia heracleifolia* Cham. & Schltdl., *Begonia dregei* Otto & Dietr., *Begonia mexicana* G. Karst. ex Fotsch was sampled for our study. The antimicrobial screening of *Begonia* leaf extracts has been carried out.

Preparation of Plant Extracts

The leaves were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial test strain and growth conditions

The non-repetitive clinical strain of *Citrobacter freundii* isolated from patients with uretic infection was collected from Koszalin Hospital during March-April, 2019. The purity, as well as the identity of isolate, was confirmed in the laboratory conditions by standard microbiological methods and were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (2014).

Susceptibility testing of the isolate was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI, 2014). The antibiotics tested were piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, cefuroxime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamicin, trimethoprim-sulphamethoxazole, ciprofloxacin, levofloxacin, tetracycline, tigecycline, and polymyxin B. Results were interpreted according to CLSI criteria. MIC was determined by *E*-test strips (according to manufacturer's instruction) and agar dilution method (according to the Guidelines of Clinical and Laboratory Standard Institute). The resistance breakpoints were the same as the ones defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2014).

The cultivation medium was trypticase soy agar (Oxoid, UK), supplemented with 10% defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37 °C. The cultures were later diluted with a sterile solution of 0.9% normal saline to approximate the density of 0.5 McFarland standard. The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration. *Citrobacter freundii* strain studied was susceptible to all antibiotics used.

Determination of the antibacterial activity of plant extracts by the disk diffusion method

The testing of the antibacterial activity of the plant extracts was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). The strain was inoculated onto Mueller-Hinton (MH) agar plates. Sterile filter paper discs impregnated with extracts were

applied over each of the culture plates. Isolates of bacteria were then incubated at 37 °C for 24 h. The plates were then observed for the zone of inhibition produced by the antibacterial activity of ethanolic extract obtained from the leaves of various extracts of plants belonging to the *Begonia* genus. A negative control disc impregnated with sterile ethanol was used in each experiment. At the end of the period, the inhibition zones formed were measured in millimeters using the vernier. For each extract, eight replicates were assayed. The plates were observed and photographs were taken. The susceptibility of the test organisms to the plant extracts was indicated by a clear zone of inhibition around the holes containing the plant extracts and the diameter of the clear zone was taken as an indicator of susceptibility. Zone diameters were determined and averaged.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 8.0 software (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Antimicrobial activities of various ethanolic extracts obtained from leaves of various plants belonged to the *Begonia* genus against *C. freundii* measured as an inhibition zone diameter are presented in Figure 1 and 2. The present study has shown that all ethanolic extracts obtained from leaves of *Begonia* species exhibited high activity against *C. freundii*. The diameter of the inhibition zone for *B. solimutata* was (15.5 ±1.1) mm, for *B. goegoensis* – (18.1 ±1.2) mm, for *B. foliosa* – (21.5 ±1.5) mm, for *Begonia* × *erythrophylla* – (18.8 ±1.2) mm, for *B. thiemei* – (22.5 ±1.5) mm, for *B. peltata* – (16.8 ±1.3) mm, for *B. heracleifolia* – (13.2 ±1.1) mm, for *B. dregei* – (11.8 ±0.8) mm, and for *B. mexicana* – (12.5 ±0.6) mm (Figure 1 and 2).

Detailed data regarding the zones of inhibition by the various plant extracts were recorded and presented in Figure 2.

It should be noted that the most effective plants among species screened against *Citrobacter freundii* locally isolated were *B. thiemei*, *B. foliosa*, and *Begonia* × *erythrophylla*, being highly active with the ethanolic extract (diameters of inhibition zone were ranged from 16.5 to 26 mm). The highly active antimicrobial effects of extracts obtained from *B. thiemei*, *B. foliosa* noted against *Citrobacter freundii* are worthy of highlighting.

In our previous study (Tkachenko et al., 2016), we have also demonstrated that the ethanolic extracts obtained from leaves of *Begonia* species had moderate activity against *Escherichia coli*. The diameters of inhibition zone for *B. solimutata* were 14 mm, 11.5 mm for *B. goegoensis*, 13 mm for *B. foliosa*, 13.5 mm for *Begonia* × *bunchii*, 15 mm for *B. thiemei*,

19 mm for *B. peltata*, 12 mm for *B. heracleifolia*, 11.5 mm for *B. dregei*, and 16 mm for *B. mexicana*. The highest antimicrobial effect was recorded for *B. peltata*, *B. mexicana*, and *B. thiemei*. The most antimicrobial effective plant against *E. coli* was *B. peltata*, being highly active with the ethanolic extract (diameter of inhibition zone was 19 mm). The highly active antimicrobial effects noted against *E. coli* are worthy of highlighting (Tkachenko et al., 2016). Moreover, the ethanolic extract from the leaves of *B. goegoensis* exhibited strong activity against *Pseudomonas aeruginosa* (inhibition zone diameter ranged from 12 mm to 13 mm), while methanolic leaf extract screened revealed less profound activity (within 11–12.5 mm) in our previous study (Tkachenko et al., 2017). Moreover, it has been observed that ethyl acetate, hexane and dichloromethane extracts obtained from leaves of *B. goegoensis* revealed no antibacterial activity against *P. aeruginosa* and β-lactamases producing *P. aeruginosa* (MBL-positive *P. aeruginosa*) strains. MBL-positive *P. aeruginosa* was also susceptible to ethanolic and methanolic extracts (inhibition zone diameter ranged from 12.5 mm to 15.5 mm) (Tkachenko et al., 2017).

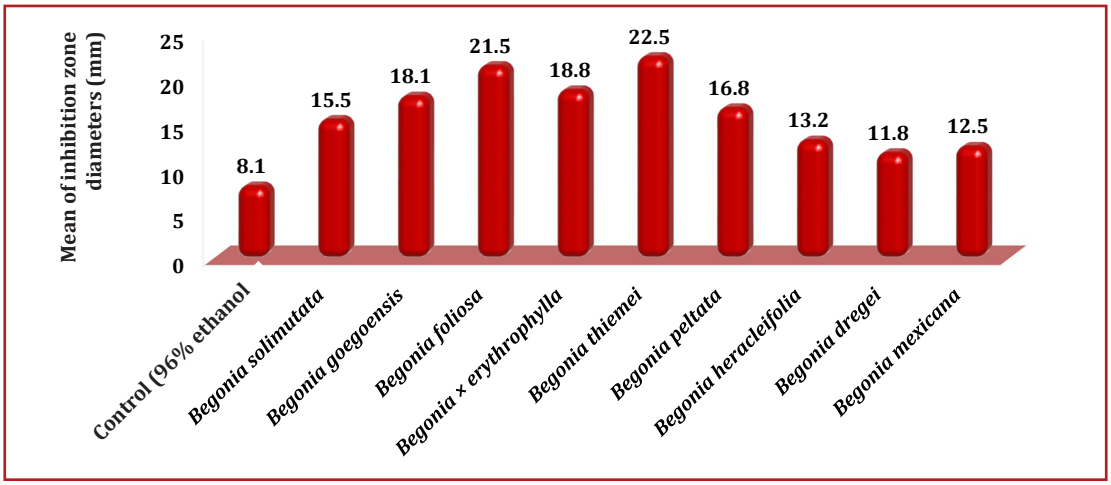


Figure 1 Antimicrobial activity of various extracts obtained from leaves of various plants belonged to the *Begonia* genus against *Citrobacter freundii* measured as inhibition zone diameter

Similar antimicrobial effect of various plants belonged to *Begonia* genus was also demonstrated by other researchers. For example, Siregar et al. (2018) have demonstrated the antibacterial potency of simple fractions of ethyl acetate extract of *Begonia baliensis* Girm. from Bukit Sangyang, Penebel, Tabanan-Bali. The chemical compounds of ethyl acetate extracts were isolated and separated by column chromatography. The obtained fractions were analyzed for antibacterial activity by disc diffusion assay against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus epidermidis* and *Staphylococcus aureus*. The chromatographic column yielded 14 simple fractions, whereas antibacterial test results showed 5 active fractions. Fraction 3 was active against *S. epidermidis*, fraction 5 against *E. coli* and *S. epidermidis*, while fractions 10, 11 and 12 were active only against *Bacillus subtilis* (Siregar et al., 2018).

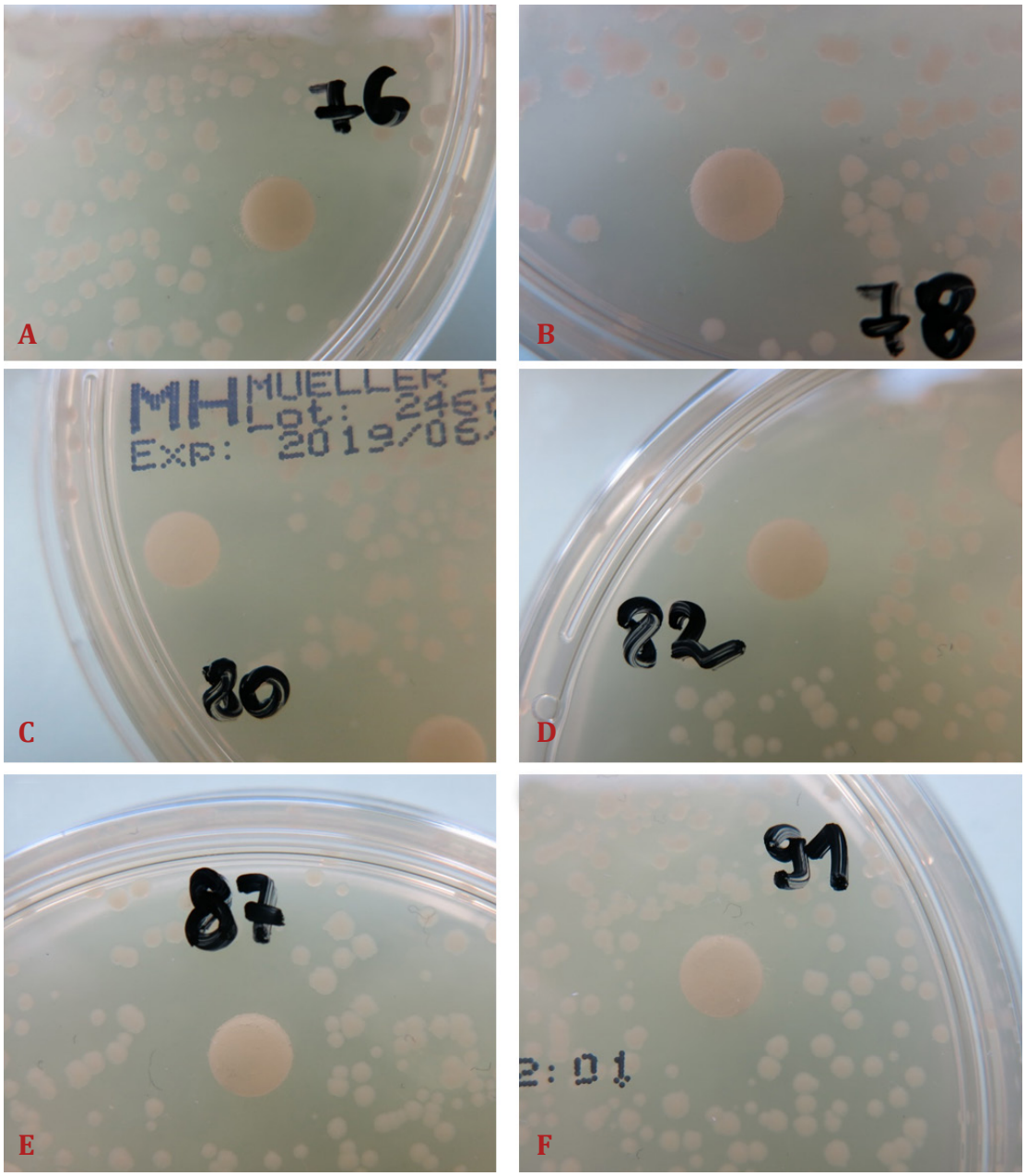


Figure 2 Inhibition zones induced by various ethanolic extracts obtained from leaves of *Begonia goegoensis* (A), *B. foliosa* (B), *Begonia* × *erythrophylla* (C), *B. thiemei* (D), *B. peltata* (E), and *B. heracleifolia* (F) against *Citrobacter freundii* growth

Antimicrobial properties of *Begonia fischeri* var. *palustris* plantlets were assessed by Karpova et al. (2019). Flavonoid composition of the leaves of *in vitro* plantlets and greenhouse stock plants had no substantial differences. Significant differences between flavonoid contents of the leaves [13.6 and 15.5 mg/g of dry weight (DW), respectively], were not found.

Aqueous ethanolic extracts of plants showed antimicrobial effects against reference strains of *Bacillus subtilis*, *Streptococcus pyogenes* and *Staphylococcus aureus*. The concentration of flavonoids in acetone and ethanol extracts of exudative compounds of the leaves of *in vitro* plantlets was 0.02 and 2.0 mg/g DW, respectively (Karpova et al., 2019).

Jeeva and Antonisamy (2012) have investigated the antibacterial activity and phytochemical properties of *Begonia floccifera* Bedd. methanolic flower extracts against the selected pathogens (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, *Serratia marcescens*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*) by the disc diffusion method. The results of the phytochemical screening revealed that phenol, tannins, xanthoproteins, steroids, phytosterols, triterpenoids, sapogenins, coumarins, and carbohydrates are comprised in the methanolic extracts of *B. floccifera*. The antibacterial activity has been observed in the methanolic extracts of *B. floccifera* against the tested bacteria with varied activity. The maximum diameter of inhibition zone was 28 mm for *B. cereus*, 25 mm for *S. aureus*, 15 mm for *E. coli*, 13 mm for *P. mirabilis*, 7 mm for *K. pneumonia*. The other pathogens viz., *P. aeruginosa*, *S. typhi*, *S. marcescens*, *Enterobacter* spp., *E. faecalis*, and *S. pyogenes* showed the minimal susceptibility to inhibition by extracts tested. Thus, methanolic flower extracts of *B. floccifera* can be used to treat nausea, vomiting, diarrhea, urinary tract infections, nosocomial infections, pneumonia, septicemias, etc. (Jeeva and Antonisamy, 2012).

Phytochemical investigation of the various extracts of the leaves of *Begonia malabarica* Lam. resulted in the isolation and identification of six known compounds, viz. friedelin, epi-friedelinol, β-sitosterol, luteolin, quercetin, and β-sitosterol-3-β-d-glucopyranoside in the study of Ramesh et al. (2002). The aqueous and organic solvent extracts were also tested against ten human pathogenic bacteria and four fungal strains by the agar well diffusion method. All the extracts were devoid of antifungal activity against the tested fungi. The hexane extract did not show any activity. The aqueous extracts showed activity against the Gram-negative bacteria except for *Vibrio parahaemolyticus*. The chloroform and methanol extracts showed activity against all the tested bacteria (Ramesh et al., 2002). Shobi et al. (2018) have studied the antibacterial activity of di-butyl phthalate isolated from *B. malabarica*. It was extracted with various solvents and bioactive compounds were isolated using chromatographic techniques. One of the bioactive compounds isolated from it was a colorless or pale yellow oily compound that is soluble in chloroform. The structure of the compound was elucidated as di-butyl phthalate with the help of spectral data. The compound is reported to have antibacterial and anticancer properties. Dibutyl phthalate showed a 9 mm zone of inhibition against *Staphylococcus epidermidis*, *Strephtococcus pneumoniae*, *Escherichia coli*, *Micrococcus luteus*, *Klebsiella pneumonia*e, *Shigella flexneri*, *Vibrio cholerae* and *P. aeruginosa* at the concentration of 100 mg/ml. Similar zone of inhibition recorded at all the concentrations of *E. coli* and *Pseudomonas aeruginosa*. Eight mm zone of inhibition was recorded against *Strephtococcus pneumoniae* at 50, 25 and 12.5 mg/ml. *Staphylococcus epidermidis*, *Klebsiella pneumonia*e, and *Shigella flexneri* also showed an 8 mm zone of inhibition at 25, 12.5 and 6.25 mg/ml concentrations (Shobi et al., 2018).

Other researchers also demonstrated the necessitates of using medicinal plants as an alternative therapy in restricting the resistant infectious organisms. In the study of Narayanan et al. (2011), the antibiotic resistances of organisms isolated from urinary tract infected patients were evaluated using the National Committee for Clinical Laboratory Standards (NCCLS) method and Multiple Antibiotic Resistance (MAR) index values, and the MAR values were also calculated for plant extracts. The 10 common medicinal plants collected from Kolli Hills, Namakkal, South India were extracted using the chloroform, methanol, acetone, ethanol and saponification procedure. The efficacy of the extracts on the uropathogens was tested by agar disc diffusion method in order to analyze the inhibitory activity of plant extract on the organisms. *Azadiracta indica* A. Juss., *Tinospora cordifolia* (Wild.) and *Euphorbia hirta* Linn. exhibited high inhibitory activity against most of the 11 tested organisms followed by *Cassia javanica* Linn. and *Phyllanthus niruri* Linn. The maximum zone size of 46.3 mm was exhibited by methanol extract of *P. niruri* Linn. against *Pseudomonas aeruginosa*. *Asparagus racemosus* Willd. and *Eupatorium triplinerve* Vahl had the least activity against resistant pathogens. Saponified lipids of most of the plants exhibited maximum antibacterial activity. Among the tested organisms, *P. aeruginosa* and *Staphylococcus epidermidis* were the most susceptible and *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter koseri*, and *Citrobacter freundii* were the least inhibited by most of the extracts of medicinal plants (Narayanan et al., 2011).

Variation in the chemical profile of extracts could influence their biological activities. Therefore, it was important to evaluate the chemical composition of extracts to correlate with their antimicrobial activities. A study conducted by Kalpanadevi and Mohan (2012) has shown that the extracts of *B. malabarica* and *B. floccifera* leaves contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. *In vitro* assay systems confirmed *B. malabarica* and *B. floccifera* whole plants as natural antioxidants. The phenolics and flavonoids could be the reason for its antioxidant activity. The preliminary phytochemical studies revealed the presence of flavone, sterol, triterpene in hexane, chloroform, and methanol extracts; phenol in chloroform and methanol extracts of *B. malabarica* and quinone, saponin, tannin, and starch in methanol extract. All the extracts did not answer for alkaloids (Ramesh et al. 2002). Preliminary phytochemical screening of *B. floccifera* and *B. malabarica* conducted by Ariharan et al. (2012) showed the presence of vitamin C in the leaves of both plant species assayed. Apparently, the antimicrobial activity of the leaf extracts of these *Begonia* species screened against pathogenic strains of Gram-positive (*Staphylococcus aureus*, *S. epidermidis*) and Gram-negative (*Pseudomonas aeurogenosa*, *Salmonella typhimurium*) bacteria could be due to the presence of this phytoconstituent. Additionally, the flavonoids content (including glycosides of quercetin and kaempferol), anthocyanins and ascorbic acid in overground part of plants of 7 species and cultivars of genus *Begonia* L. (*B. bahiensis*, *B. bowerae*, *B. carolineifolia*, *B. fischeri*, *B. heracleifolia*, *B. 'Erythrophylla'*, *B. 'Helen Teupel'*) were determined by Karpova et al. (2009). The flavonoids content was 24–650 mg% of dry weight, including glycosides of quercetin – 3–76 mg%. Kaempferol glycosides were detected only in species of section Gireoudia (1.2–5.7 mg%). The contents of anthocyanins were between 60 and 157 mg%, ascorbic acid – 5–43 mg% of fresh weight. These results suggest that studied plants of *Begonia*

species can be considered as the sources of biologically active compounds with antioxidant and antimicrobial activities (Karpova et al., 2009).

Conclusions

This *in vitro* study corroborated the antimicrobial activity of the selected plants belonged to *Begonia* genus. All these plants were effective against *Citrobacter freundii* strain locally isolated. This study also showed that *B. thiemei*, *B. foliosa*, and *Begonia* × *erythrophylla* could be potential sources of new antimicrobial agents. The identification of active compounds and their mode of action requires further investigation for antibacterial drug development.

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ACCUMULATION OF NUTRIENTS IN THE RAW OF *CRAMBE* L. SPECIES

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Investigation of accumulation of different compounds in above-ground part of these plants an important aspect for evaluation of perspective of use. The aim of this study was to compare the peculiarities of the biochemical composition of *Crambe* species dynamically. Plant material collected from the experimental collection of M.M. Gryshko National Botanical Garden of the NAS of Ukraine. It was studied above-ground parts of *C. cordifolia* Steven, *C. koktebelica* (Junge) N. Busch, *C. maritima* L., *C. steveniana* Rupr. At the spring vegetation, budding stage, flowering, and fruitage. Following biochemical parameters was studied: dry matter by drying to consist weight at the 105 °C; content of sugars by Bertrand's method using of glucose scale; ascorbic acids with 2.6-dichlorophenolindophenol, tannins with indigo carmine discoloration, organic acids by sodium hydroxide titration with phenolphthalein; carotene with gasoline galosh spectrophotometrically; ash in muffle over. The dry matter during vegetation was from 9.76 (*C. cordifolia*, budding) to 22.54 (*C. maritima* at the fruitage) %, total content of sugars from 6.54 (*C. maritime* at the fruitage) to 33.18 (*C. cordifolia* at the budding) %, ascorbic acid from 139.85 (*C. maritima* at the spring vegetation) to 987.02 (*C. maritime* at the budding) mg%, β-carotene from 0.39 (*C. maritima* at the budding) to 1.82 (*C. cordifolia* at the fruitage) mg%, tannins from 1.28 (*C. cordifolia* at the fruitage) to 7.47 (*C. steveniana* at the budding) %, content of organic acids from 3.12 (*C. steveniana* at the fruitage) to 6.28 (*C. koktebelica* at the spring vegetation) %, ash from 6.11 (*C. maritima* at the spring vegetation) to 14.61 (*C. cordifolia* at the flowering) %. Correlation analysis showed that between the content of tannins and organic acids existed positive moderate correlation (r = 0.52). Between other parameters found the weak, very weak positive or negative correlation. Thus, comparing biochemical analyze identified that raw of four species of *Crambe* is a valuable source of nutrients during vegetation. The high content of sugars, ascorbic acid, and carotene can recommend these plants as forage crops. Also, raw of these plants can be recommended for farther pharmacological investigations.

Keywords: *Crambe*, biochemical composition, raw

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Introduction

Plants from Brassicaceae Burnett have a long story of an important role in human life as food, forage, medicinal plants, etc. They are source of biologically active compounds that determine their pharmacological properties (Raiola et al., 2018; Shankar et al., 2019). Chemical investigation of Brassicaceae characterized by presence of phytonutrients such as tocopherols, vitamin E, carotenoids, polyphenol compounds, amino acids, glucosinolates, etc. (Avato and Argentieri, 2015). Oil plants from this family revealed antioxidant activity by DPPH method in methanol extracts as well as in water extracts (Rakhmetov et al., 2018).

Genus *Crambe* L. has a wide area of distribution in the world (Prina, 2009). Species of *Crambe* genus are very promising species due to their biological and biochemical properties (Kalista, 2017). They belong to the Brassicaceae and in the flora of Ukraine there are 8 species (Iljinska et al., 2007). The most known and useful plants from Brassicaceae are the source of oil, content of which can be varied from 17 to 40% and upper (Vergun et al., 2017a). *Crambe abissinica* the most known species last time due to content of oil and other nutrients such as protein, amino acids, phenolic compounds, etc. (Lovatto et al., 2017). Also, the utilization of oil produced by *C. abissinica* is favored over high erucic acid rapeseed oil due to its higher natural content of C22:1 (Zorn et al., 2019). According to Stankevych (2017), plants of this species are high-yield cultures, which may compete with other oil cultures of Brassicaceae by a complex of parameters.

Under-ground parts of *Crambe cordifolia* are the rich source of polyphenol compounds with antioxidant activity as showed the study of different extracts. Also, they exhibited antimicrobial activity (Rashid et al., 2018).

The aim of the research is the determination of selected biochemical compounds in the raw of the above-ground part of plants of *Crambe* species, taking into account that there is limited information about accumulation some nutrients.

Materials and methodology

Biological material

It was investigated species of the genus of *Crambe*: *C. cordifolia* Steven, *C. koktebelica* (Junge) N. Busch, *C. maritima* L., *C. steveniana* Rupr. An experiment carried out during 2018–2019 at the laboratory of Cultural Flora Department of M.M. Gryshko National Botanical Garden of the NAS of Ukraine. Plants samples took at the start of spring growth, budding stage, flowering stage, and fruitage. All investigated plants are perennial.

Biochemical analyses

Dry matter determination

Plant samples were dried in drying oven at the 105 °C till constant weight in aluminum boxes. Results are given in percentages (Hrytsajenko et al., 2003).

Total content of sugars determination

The total content of sugars was investigated by Bertrand's method in water extracts. 4 g of fresh mass mixed and homogenized with distilled water (approximately 50 ml) in the 100 ml test-tubes and heated in the water bath at 70 °C during 15–20 min. After cooling in the obtained mixtures added 1 ml of phosphate-oxalate mixture. After this was added 1.5 ml of lead acetate. The obtained mixture brings to the mark (100 ml) with water. After filtration from obtained solution took 50 ml and mixed with 8 ml of 20% HCl (at the 70 °C in water bath for 5 min) and after cooling was neutralized by 12% NaOH and brought to the mark by distilled water (100 ml). 3 ml of obtained solution mixed with 6 ml of Fehling's solution reagent (6 min boiling in the water bath). Obtained mixture analyzed for total content of sugars. Results are given by percentages (Hrytsajenko et al., 2003).

Total content of ascorbic acid

Determination of ascorbic acid content conducted by method offered by K. Murri. 2 g of fresh mass mixed with 50 ml of 2% oxalic acid. Obtained mixture put into the dark for 20 min. Content of ascorbic acid of obtained extracts determined by a 2.6-dichlorophenol-indophenol method that based on the reduction properties of ascorbic acid. Obtained results expressed in the mg% DW (Hrytsajenko et al., 2003).

Total content of carotene

The concentration of total carotene determined according to Pleshkov (1985) using extraction with rubber solvent (petrol). 1 g of absolutely dried raw mixed with 20 ml of Kalosha petrol for 2 hours. After this obtained filtrate measured spectrophotometrically at the wavelength 440 nm at the Unico spectrophotometer. Obtained results expressed in mg% DW.

Total content of tannins

The content of tannins was determined with indigo carmine as indicator (Ermakov et al., 1972). 5 g of fresh mass mixed with distilled water (approximately 50 ml) in 100 ml taste-tubes. Obtained mixture heated in the water bath at 70 °C for 2 hours. After cooling, adding water to the 100 ml and following filtration 10 ml of filtrate used for determination of total content of tannins. This procedure used 700 ml distilled water and 25 ml of 1% solvent of indigo carmine. Obtained results expressed in %.

Total content of organic acids

The total content of organic acids determined with phenolphthalein and results calculated with malic acid coefficient (Krishchenko, 1983). 10 ml of filtrate (the same procedure described for determination of total content of tannins) titrated with 1 N solvent of NaOH in presence of phenolphthalein. Obtained results expressed in percentages.

Total content of ash

The total content of ash determined by combustion in oven at the 300–700 °C for 3 days with step increasing of temperature (Hrytsajenko, 2003).

Statistical analysis

Experimental data were analyzed using Excel 2016. Correlation analysis was performed using Pearson's criterion. Mean values of three replicates and standard deviations given in Figures 1–7.

Results and discussion

Plants of the *Crambe* genus in M.M. Gryshko Botanical Garden have grown and have investigated in collection of forage plants last some decades. Previous studies concerning their antioxidant activity and accumulation of nutrients in the underground parts of plants (Vergun et al., 2018).

We determined that the content of dry matter during vegetation was from 9.76 to 22.54% depending on species and stage of growth (Figure 1). The dry weight of different microgreens Brassicaceae according to Xiao et al. (2016) was 4.9–10.3% depending on species. Other species from this family *Camellina sativa* L., their cultivars and varieties had dry matter content in range 18.13–23.38% in stage of flowering (Vergun et al., 2017b).

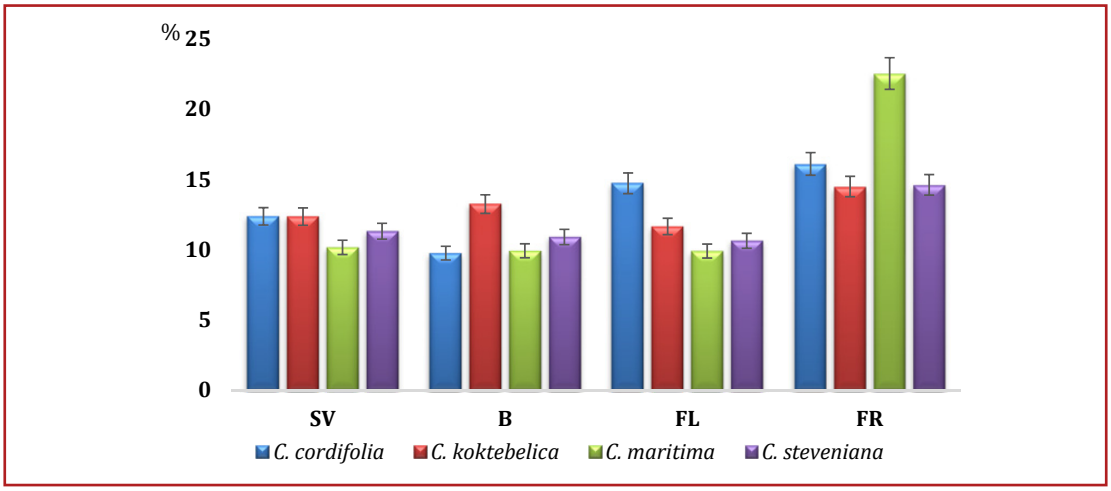


Figure 1 Total content of dry matter in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

The total content of sugars in the *Crambe* species determined from 6.54 to 33.18% (Figure 2). It should be noticed that *C. cordifolia* demonstrated the most content of sugars in the stage of budding. Also, high content of sugars found in *C. steveniana* in the period of flowering. Minimal value of this parameter fixed in the fruitage for *C. maritima*. Plants of *Camellina sativa* had content of sugars in range of 4.76–8.12% in stage of flowering (Vergun et al., 2017b). As reported Rosa et al. (2001), total content of sugars in broccoli cultivars was 114.5–229.2 mg/g.

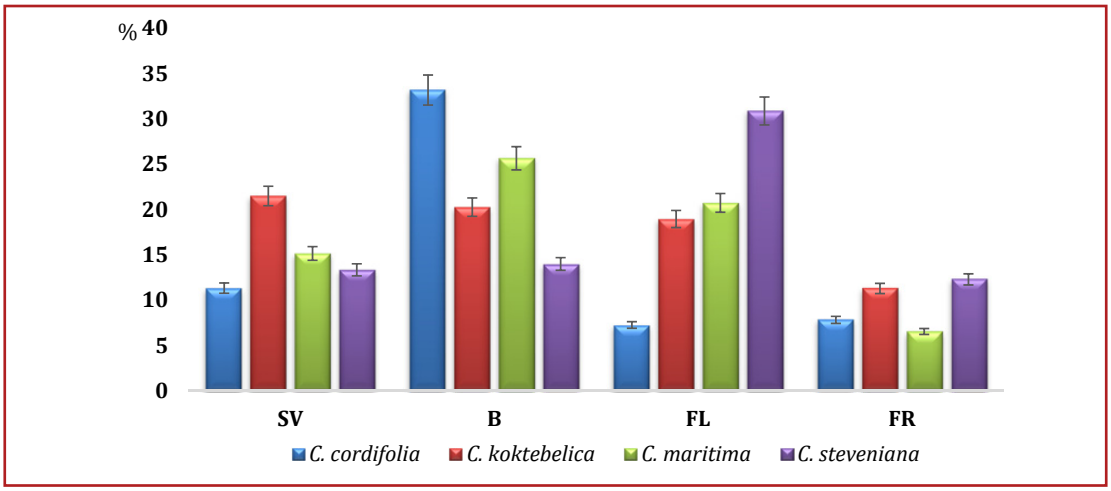


Figure 2 Total content of sugars in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

Ascorbic acid plays an important role as co-factor for some enzymes and acts in numerous processes. It demonstrated strong antioxidant activity that makes this compound wide distributed in the cosmetic industry (Golonka et al., 2017). As reported Lokato et al. (2013), ascorbic acid participates in some physiological processes such as immune stimulation, synthesis of collagen, hormones, iron absorption, etc. The content of ascorbic acid varied from 139.85 to 987.02 mg% DW depending on species (Figure 3). Plants of *Camellina sativa* had value of this parameter in range of 207.23–410.23 mg% DW (Vergun al., 2017b). Other cruciferous species as *Armoracia rusticana* had ascorbic acid content in leaves up to 350 mg% FW (Rivelli et al., 2017).

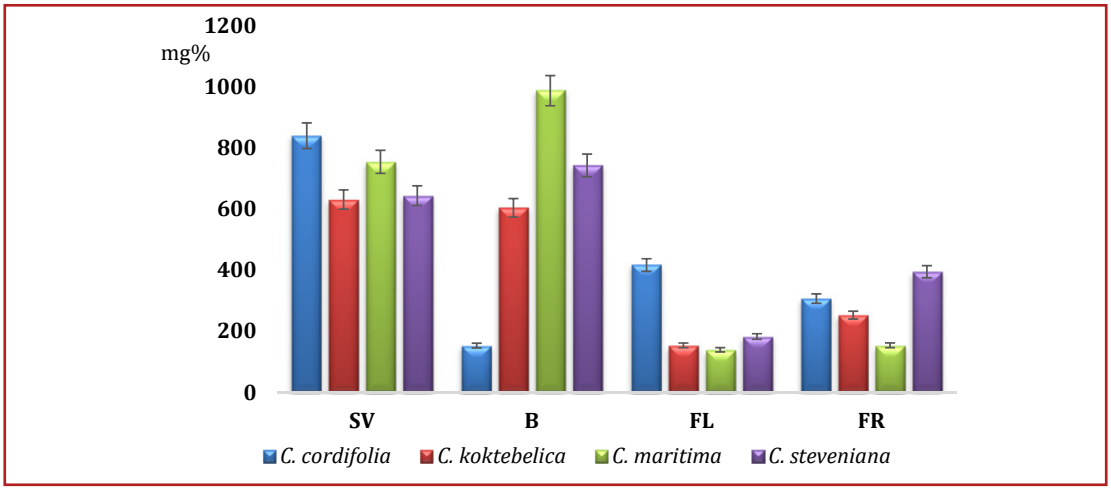


Figure 3 Total content of ascorbic acid in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

The study of the accumulation of β-carotene focused on its pharmacological properties such as anti-carcinogenic and anti-mutagenic. β-carotene an important provitamin A, which demonstrates the protective action in human organisms, for example, as powerful antioxidants (Bogacz-Radomska and Harasym, 2018). Content of β-carotene in raw of *Crambe* species determined from 0.387 to 2.408 mg% (Figure 4).

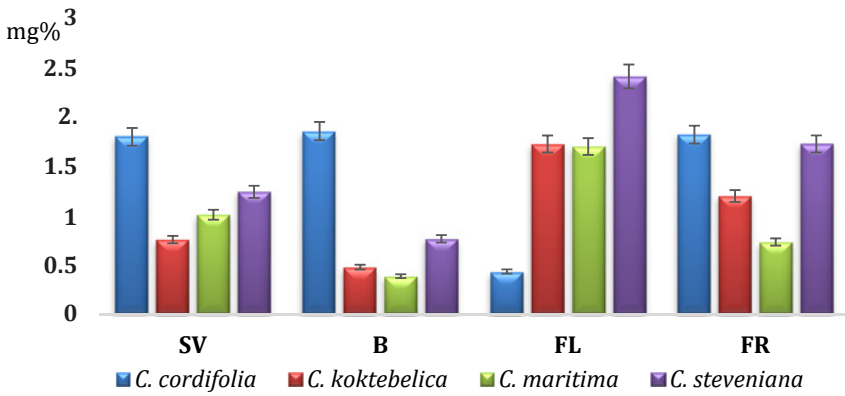


Figure 4 The content of β-carotene in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

The study of the content of tannins in raw of investigated plants showed accumulation of them from 1.28 to 6.47% (Figure 5). Tannins are water-soluble polyphenols that are present in many plant food productions. From the one side food rich in tannins has low nutritional value, however, they also characterized by antimicrobial activity (Chung et al., 1998). Previous study concerning to Brassicaceae species showed that rapeseed meal contains 3% of tannins (Mitaru et al., 1982).

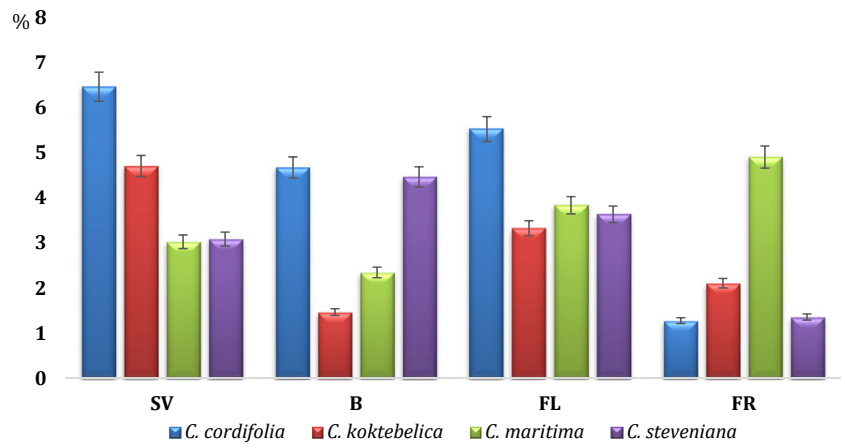


Figure 5 Total content of tannins in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

Accumulation of organic acids in raw of *Crambe* species during vegetation represented in Figure 6. We found that sum of organic acids of investigated species was from 3.12 to 6.28% depending on period of growth and species. Maximal values of this parameter were found at the period of spring vegetation for all species and at the budding stage for *C. cordifolia*. A high content of organic acids evidently due to their role in photosynthetic intermediates (López-Bucio et al., 2000).

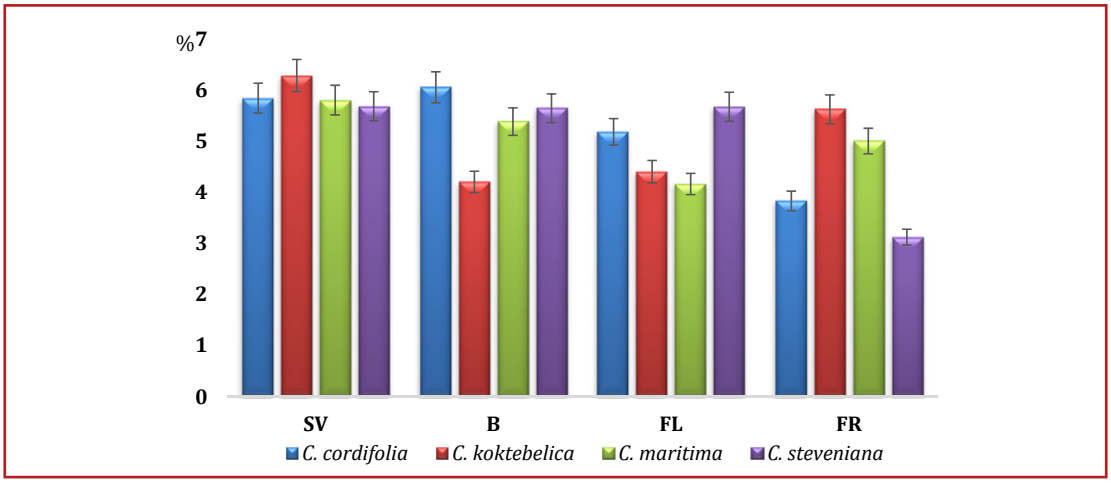


Figure 6 Total content of organic acids in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

One of the important biochemical parameters to determine nutritional value of raw, especially food, is ash that concludes mainly mineral components (Godočiková et al., 2019). Also, according to Prochnow et al. (2009), content of ash important parameter for evaluation of quality of phytofuel. So, with increase of ash concentration heating value is decreases.

The concentration of ash in Brassicaceae plants can vary. For example, the total content of ash in stems of *Lepidium sativum* was 17.1% as reported Sandekar et al. (2018).

In our study content of ash for different species of *Crambe* was from 6.11 to 14.61% (Figure 7). It should be noted that raw *C. cordifolia* demonstrated maximal ash content at every period among other species. As reported Tutus et al. (2010), ash content of *C. orientalis* and *C. tataria* was 7.83 and 9.31%, respectively.

Correlation analysis showed that between tannins and organic acids, ascorbic acid and organic acids, sugars and organic acids found a moderate correlation that was 0.52, 0.46 and 0.40, respectively. Weak relation found between carotenoids and sugars and tannins and ash (0.28 for both). All other parameters showed very weak or absent correlation.

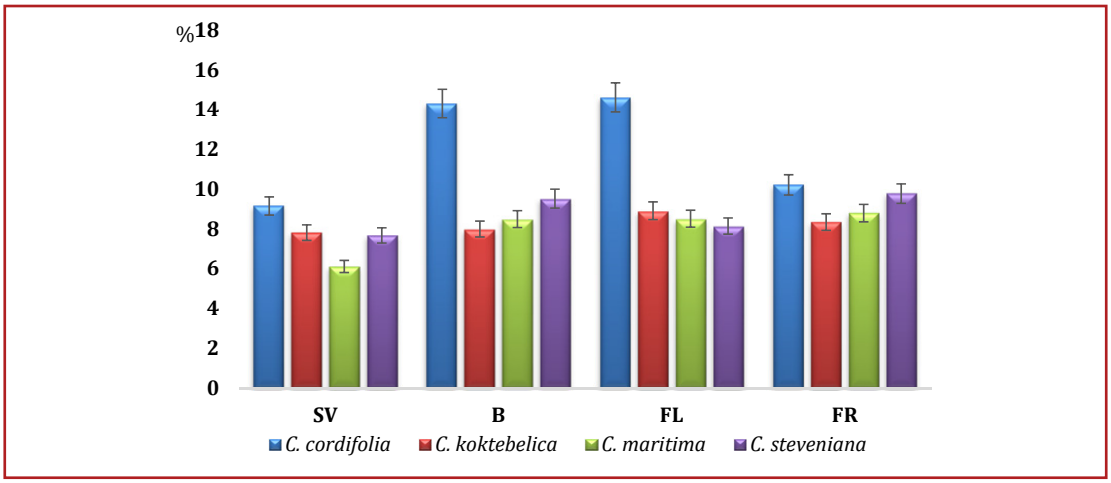


Figure 7 Total content of ash in the above-ground part of plants of *Crambe* spp.
SV – spring vegetation; B – budding stage; FL – flowering stage; FR – fruitage

Conclusions

Investigation of the biochemical composition of four species of genus *Crambe* showed that investigated plants are valuable sources of nutrients. Accumulation of selected biochemical compounds in above-ground parts of these plants depends on stage of growth. Maximal values of dry matter and ascorbic acid found for *C. maritima*, total content of sugars and organic acids for *C. koktebelica*, carotene for *C. steveniana*, ash for *C. cordifolia*. Due to high content of ascorbic acid, carotene, sugars and other biochemical parameters of raw, *Crambe* species can be recommended as forage plants. Also, it required father pharmacological investigations of these plants as food and medicinal plants.

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EVALUATION OF THE *IN VITRO* ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS DERIVED FROM LEAVES OF *CAMELLIA JAPONICA* CULTIVARS (THEACEAE) AGAINST ENTEROBACTER CLOACAE STRAIN

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The increasing incidence of antibiotic resistance among bacterial and fungal pathogens necessitates plants as an alternative therapy in restricting the resistant infectious organisms. The present study was undertaken to determine the antibacterial activity of ethanolic extracts derived from the leaves of *Camellia japonica* cultivars. The aim of the presented study was to assess the *in vitro* antimicrobial activity of ethanolic extracts derived from the leaves of *C. japonica* 'Kramer's Supreme', 'C.M. Wilson', 'La Pace', 'Mrs. Lyman Clarke', 'Benikarako', 'Fanny Bolis' against clinical cefuroxime-resistant *Enterobacter cloacae* strain. Ethanolic extracts were prepared by freshly crushed leaves and evaluated for their antimicrobial activity against *Enterobacter cloacae* strain locally isolated using disc diffusion assay. Among the six plant extracts screened, *C. japonica* 'Mrs. Lyman Clarke' exhibited the highest inhibitory zones against the tested strain (the mean value of the zone of inhibition was 12.5 ±0.7 mm). The least activity is attributed to *C. japonica* 'La Pace' extract. Based on our current investigations it can be concluded that *C. japonica* and its cultivars possess a mild antibacterial efficacy. This mild activity on the *E. cloacae*, Gram-negative organisms, is most likely due to the protective nature of the outer membrane of their cell walls. It may also be assumed that the antimicrobial potential of various samples of these plants might be due to a wide variety of compounds present in these plants. The findings reported herein give scientific credence to the traditional uses of these plants and suggest that extracts derived from the leaves of *C. japonica* and its cultivars merit further phytochemical study as natural antibiotics to identify the secondary metabolites.

Keywords: *Camellia japonica* L., leaves, *Enterobacter cloacae*, antibacterial activity, disc diffusion technique, ethanolic extracts

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Introduction

Despite the alarming rise in antimicrobial resistance over the last decades, resistance to antimicrobial drugs is nothing new (Carranza et al., 2015). Subsequent antibiotics use resulting in the emergence of resistant bacterial strains one to four years after their introduction and pose a greater threat to public health (Reddick and Alto, 2014). Therefore, alternative strategies must be considered if antimicrobial resistance is to be successfully combated. One alternative is the use of medicinal plants, which for many cultures continue to play a crucial role in the primary care of patients (Farnsworth, 1990; Fabricant and Farnsworth, 2001). Ethnobotany, ethnomedicine, and traditional medicine can provide information that is useful as a 'pre-screen' to select plants for experimental pharmacological studies (Farnsworth, 1990). It is widely accepted that the antimicrobial activities and physiological effects from plant therapies are due to the biosynthesis of secondary metabolites produced by plants as a defense mechanism. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions (Ramakrishna and Ravishankar, 2011).

Camellia L., containing about 280 species, is a genus with high economic, ecological and phylogenetic values in the family Theaceae (Vijayan et al., 2009; Yang et al., 2013). It is native to Southern, Eastern Asia and China, which possess more than 80% of the species and are the center of species diversity (Gao et al., 2005).

Among the *Camellia* species, the economic value of the *C. japonica* L. ranks the highest due to its beautiful ornamental flowers and medicinal properties. It is native to South Korea, Japan, and China. *Camellia japonica*, which comprises thousands of cultivars, is the most widely cultivated species. Besides, it has been used in traditional medicine in Japan, China, and Korea (Yoon et al., 2017). Extensive studies have been conducted in order to assess the phytochemical composition of various parts of *C. japonica* plants, including saponins in its fruits and seeds and triterpenes in its flowers and seed oil (Akihisa et al., 1997; Yoon et al., 2017). It has been reported that leaves of *C. japonica* possess antioxidant, antifungal, and cytotoxic properties (Park et al., 2002; Thao et al., 2010; Onodera et al., 2016).

Green tea (*Camellia sinensis* L.) has received much attention as a beverage worldwide during the last few decades due to its various beneficial effects on human health, including different types of cancer, heart disease, and liver disease, etc. (Chacko et al., 2010; Bashir et al., 2014). Studies reveal that green tea possesses diverse pharmacological properties to lower the incidence of metabolic syndromes, such as obesity, type II diabetes, and cardiovascular risk factors (Chacko et al., 2010). Tea leaves have been reported to contain over 700 bioactive compounds of which one third is contributed by polyphenols (Khan and Mukhtar, 2007; Vijayan et al., 2009; Mahmood et al., 2010).

Long-term consumption of tea catechins could be beneficial against high-fat diet-induced obesity and type II diabetes and could reduce the risk of coronary disease (Chacko et al., 2010). Green tea was also reported as useful against HIV strains (Fassina et al., 2002). An aqueous extract of *Camellia sinensis* found to be effective against Gram-positive, Gram-negative, and fungi (Khan et al., 2019). The most important finding of this study is its aqueous extract inhibitory effect against drug-resistant microorganisms, e.g. methicillin-resistant

Staphylococcus aureus (MRSA), *Pseudomonas aeruginosa*, and *Candida albicans* (Khan et al., 2019).

Species of the *Enterobacter cloacae* complex are widely encountered in nature. They can act as pathogens (Mezzatesta et al., 2012). Today, 22 species belong to the *Enterobacter* genus (Davin-Regli et al., 2019). During the last three decades, *Enterobacter aerogenes* and *E. cloacae* have been reported as important opportunistic and multi-resistant bacterial pathogens for humans in hospital wards (Davin-Regli and Pagès, 2015). The biochemical and molecular studies on *E. cloacae* have shown genomic heterogeneity, comprising six species: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter nimipressuralis*, *E. cloacae* and *E. hormaechei* are the most frequently isolated in human clinical specimens (Mezzatesta et al., 2012). These species are also described in the environment and have been reported as opportunistic pathogens in plants, animals, and humans (Davin-Regli et al., 2019). The dissemination of *Enterobacter* sp. is associated with the presence of redundant regulatory cascades that efficiently control the membrane permeability ensuring the bacterial protection and the expression of detoxifying enzymes involved in antibiotic degradation/inactivation (Davin-Regli and Pagès, 2015).

Enterobacter is a versatile bacterium able to promptly respond to the antibiotic treatment in the colonized patient (Davin-Regli and Pagès, 2015). It is able to manage different mechanisms of resistance *via* various local and global regulator genes and the modulation of the expression of different proteins, including enzymes (β-lactamases, etc.) or membrane transporters, such as porins and efflux pumps (Davin-Regli et al., 2019). In fact, they are capable of overproducing AmpC β-lactamases by derepression of a chromosomal gene or by the acquisition of a transferable ampC gene on plasmids conferring the antibiotic resistance. Many other resistance determinants that are able to render ineffective almost all antibiotic families have been recently acquired (Mezzatesta et al., 2012). In addition, these bacterial species are able to acquire numerous genetic mobile elements that strongly contribute to antibiotic resistance (Davin-Regli and Pagès, 2015).

Increasing resistance among pathogens to conventional antibiotics and undesirable side effects of existing therapies have made traditional medicinal plants an attractive source to screen for antimicrobial agents (Choi et al., 2017).

In line with this effort, the present study was aimed to determine the antibacterial activity of six cultivars *Camellia japonica* against clinical cefuroxime-resistant *Enterobacter cloacae* strain.

Materials and methodology

Collection of Plant Material

The leaves of cultivars *Camellia japonica* 'Kramer's Supreme', 'C.M.Wilson', 'La Pace', 'Mrs. Lyman Clarke', 'Benikarako', 'Fanny Bolis' cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanical Garden (Kyiv, Ukraine).



Figure 1 *Camellia japonica* L. plants cultivated at the glasshouses of M.M. Gryshko National Botanic Garden, NAS of Ukraine

Preparation of Plant Extracts

The collected leaves were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial strain

The testing of the antibacterial activity of the plant extract was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). The non-repetitive clinical strain of cefuroxime-resistant *E. cloacae* isolated from bloodstream infection was collected from Koszalin Hospital during March-April, 2019. The purity, as well as the identity of isolate, was confirmed in the laboratory conditions by standard microbiological methods and were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (2014).

Susceptibility testing of the isolates was performed by the disk diffusion technique according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI). The antibiotics tested were piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, cefuroxime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamicin, trimethoprim-sulphamethoxazole, ciprofloxacin, levofloxacin, tetracycline, tigecycline, and polymyxin B. Results were interpreted according to CLSI criteria. MIC was determined by *E*-test strips (according to manufacturer's instruction) and agar dilution method (according to the Guidelines of Clinical and Laboratory Standard Institute). The resistance breakpoints were the same as the ones defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2014).

Enterobacter cloacae strain studied was resistant to amoxicillin, cefuroxime, trimethoprim-sulphamethoxazole, and cefotaxime.

The Disk Diffusion Method for Evaluation of Antibacterial Activity of Plant Extracts

Strain tested was plated on TSA medium (Tryptone Soy Agar) and incubated for 24 hr at 37 °C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity

adjusted equivalent to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). Muller-Hinton agar plates were inoculated with 200 µl of standardized inoculum (108 CFU/mL) of the bacterium and spread with sterile swabs.

Sterile filter paper discs impregnated by extract were applied over each of the culture plates, 15 min after bacteria suspension was placed. A negative control disc impregnated by sterile 96% ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hr at 37 °C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks. The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean. All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each extract. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica software, version 8.0 (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (R) ≤10 mm (Okoth et al., 2013).

Results and discussion

Figure 2 and 3 summarize the results of bacterial growth inhibition of *E. cloacae* by ethanol extract derived from the leaves of various *C. japonica* cultivars.

The crude extracts were analyzed for their antibacterial effect by the determination of their inhibitory zones against *E. cloacae* strain. Of the six plant extracts screened, *C. japonica* 'Mrs. Lyman Clarke' exhibited the highest inhibitory zones against the tested strain (the mean of the zone of inhibitions was 12.5 ±0.7 mm). The *C. japonica* 'Benikarako', 'Fanny Bolis', and 'Kramer's Supreme' exhibited zone of inhibitions 10.3 ±0.7, 10.6 ±0.6, 10.2 ±0.6 mm against *E. cloacae* strain, respectively, as shown in Figure 2. The antibacterial effect of *C. japonica* 'La Pace' was almost comparable to the effects of positive control (9.1 ±0.5 mm). The least activity is attributed to *C. japonica* 'La Pace' extract.

In this study, we investigated the antimicrobial activity of plant extracts by agar well diffusion. In our study, *Camellia japonica* 'Kramer's Supreme', 'C.M. Wilson', 'La Pace', 'Mrs. Lyman Clarke', 'Benikarako', 'Fanny Bolis' were less potent against the test bacterium as can be assumed from the observed zone of growth inhibitions of *Enterobacter cloacae*. It is noteworthy to mention that this mild activity on the *E. cloacae*, Gram-negative organisms, is most likely due to the protective nature of the outer membrane of their cell walls. Hence, these extracts may not have good candidates as drugs lead for antibacterial activity because of these high values of

diameters of inhibition zone due to the importance of the potency of the antibacterial agent in drug development, amongst other factors.

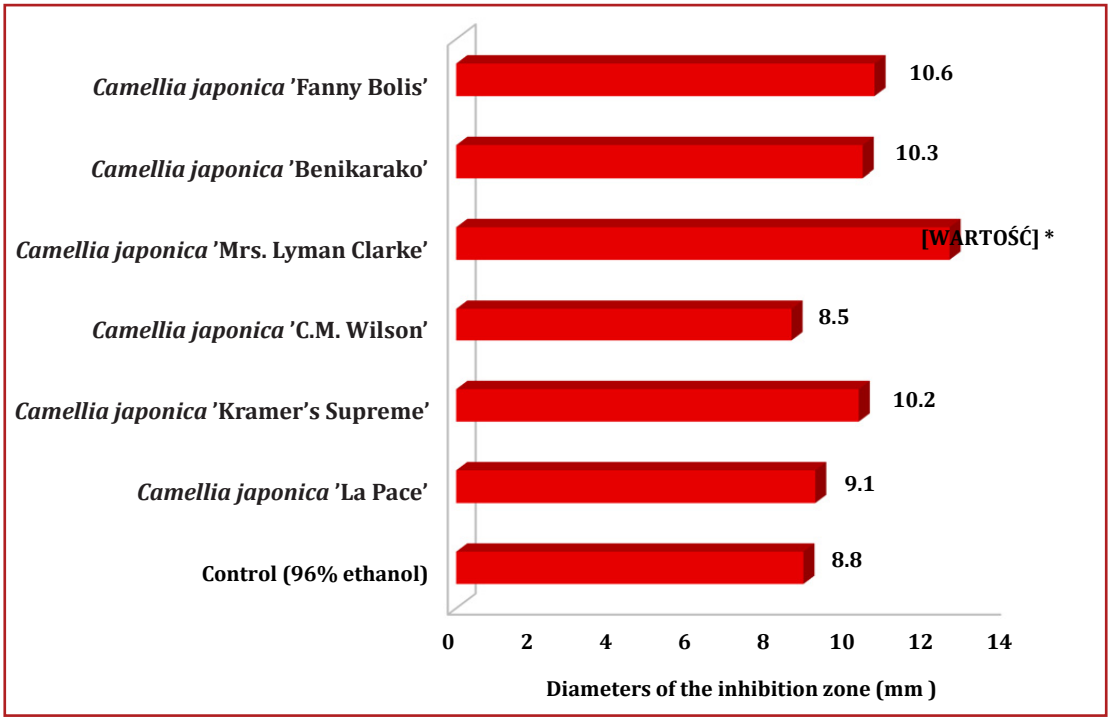


Figure 2 Zone of growth inhibitions of *Enterobacter cloacae* induced by the extracts obtained from leaves of various *Camellia* cultivars in millimeter (n = 8)
* changes are statistically significant (p <0.05) compared to 96% ethanol (control)

In a separate study, we tested these extracts for toxic effects on human erythrocytes. *C. japonica* and its cultivars were found to be non-toxic on the concentrations tested for antimicrobial activity (data not shown). Results obtained in our previous study showed that there is a possibility of using extracts derived from leaves of various *C. japonica* cultivars in intensive aquaculture farms. Leaf extracts of *Camellia japonica* may be used as an antioxidant agent in aquaculture. The lipid peroxidation (2-thiobarbituric acid reactive substances (TBARS) as biomarker) level in the muscle tissue of rainbow trout (*Oncorhynchus mykiss* Walbaum) after incubation with extracts obtained from leaves of various *Camellia japonica* cultivars was evaluated in our previous study (Kharchenko et al., 2017a). Moreover, all extracts (except *C. japonica* 'Benikarako' and 'Fanny Bolis') reduced the TBARS level in the extracts-treated muscle tissue, but these results were non-significant. Furthermore, the use of such plant products as antioxidants and immunostimulants in aquaculture systems may also have environmental value because of their biodegradability (Kharchenko et al., 2017a). The superoxide dismutase (SOD) activity, an antioxidant enzyme, was increased in the muscle tissue after incubation with *C. japonica* 'Kramer's Supreme' extract (by 52%, p = 0.004), 'C.M. Wilson' (by 88%, p = 0.001), 'Mrs. Lyman Clarke' (by 87.2%, p = 0.000), and 'Fanny Bolis' (by 40.7%, p = 0.044) compared to the control group. The SOD activity in the muscle tissue

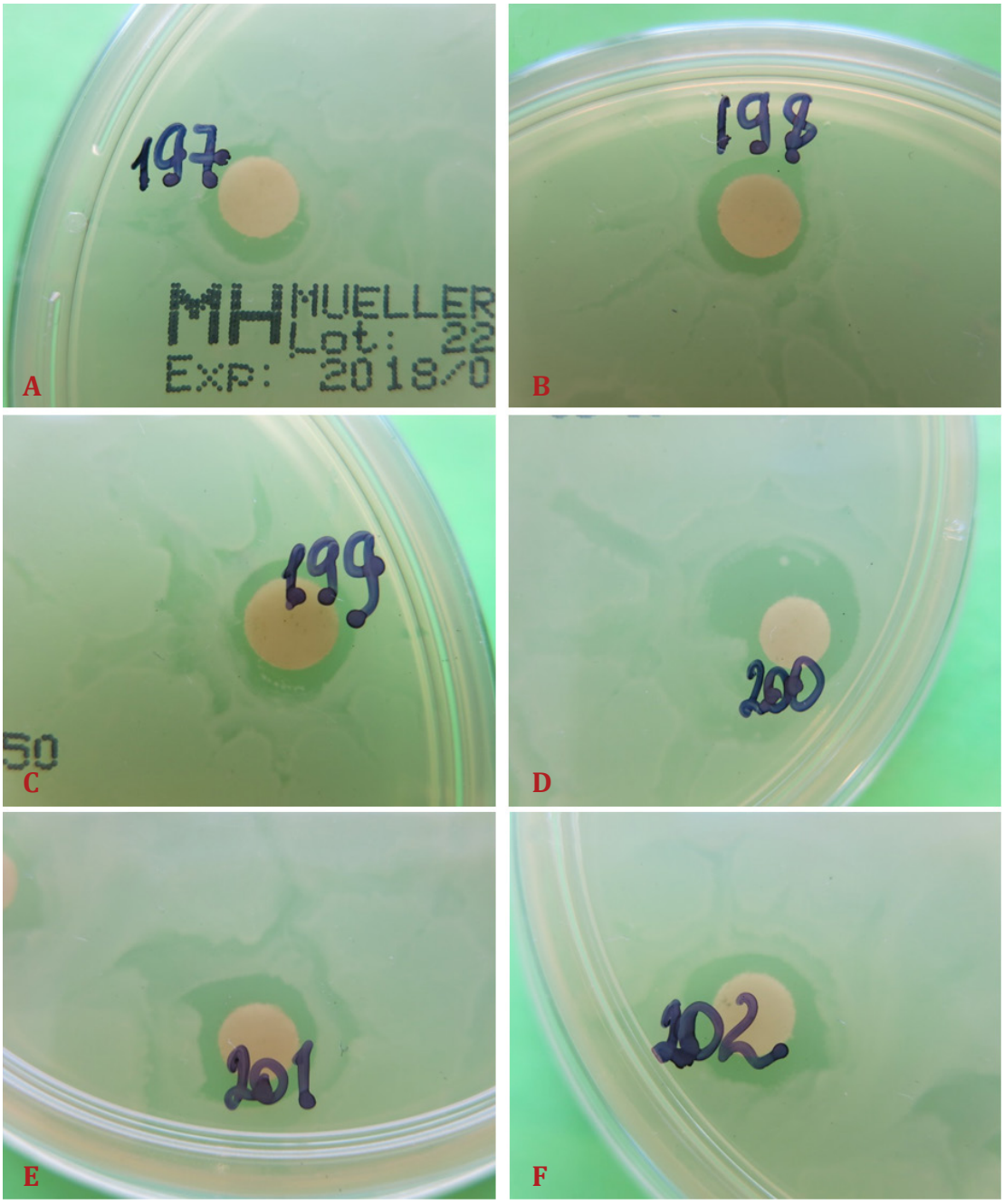


Figure 3 Antimicrobial activity of ethanolic extracts of various *Camellia* cultivars analyzed by Kirby-Bauer disc-diffusion assay: *Camellia japonica* 'La Pace' (A), 'Kramer's Supreme' (B), 'C.M. Wilson' (C), 'Mrs. Lyman Clarke' (D), 'Benikarako' (E), and 'Fanny Bolis' (F)

after incubation with *Camellia japonica* 'La Pace' and 'Benikarako' was also non-significantly increased by 31.5 and 27.3% (p <0.05), respectively (Kharchenko et al., 2018). The results of the investigation revealed quite a high level of total antioxidant capacity (TAC) in samples of muscle tissue incubated with leaf extracts of *C. japonica* 'C.M. Wilson' and 'Benikarako' cultivars. The levels of TAC were increased by 41.7 and 44.8% (p <0.05) as compared with a control group of muscle tissue homogenates. Leaf extracts of *C. japonica* 'La Pace' and 'Kramer's Supreme' cultivars being incubated with muscle tissue have not changed the level of TAC, while the effect of the leaves extracts of *C. japonica* 'Mrs. Lyman Clarke' and 'Fanny Bolis' on the decreasing of TAC level was insignificant (p >0.05). The results of the study suggested the high antioxidant capacity of *Camellia* cultivars screened give reason to believe that application of these plant extracts signifies a rational curative strategy to prevent and cure various fish diseases involving oxidative stress by increasing the ability of a fish organism to adapt (Kharchenko et al., 2017b).

The results obtained in the current study can be correlated with the previous publications. The potential presence of naturally occurring antimicrobials in petals of *Camellia japonica* against foodborne pathogens in microbiological media and food was studied by Kim and co-workers (2001). Petals of the camellia flower were extracted with methanol and fractionated into basic, acidic, and neutral fractions. The acidic fraction produced an inhibitory zone of 14 to 19 mm (diameter) in a disk assay against the pathogens *Salmonella typhimurium* DT104, *Escherichia coli* O157: H7, *Listeria monocytogenes*, and *Staphylococcus aureus* on agar plates. An aqueous extract from the petals of *C. japonica* had an inhibitory effect on the growth of all pathogens at 37 °C in microbiological media by increasing the lag phase. None of the microorganisms was inhibited completely. Milk was used as a model food system. Aqueous extract at a concentration of 100 mg/ml was bacteriostatic against all the foodborne pathogens in the milk stored at 25 °C for up to 4 days (Kim et al., 2001). Antimicrobial and anti-biofilm activities of the methanol extracts of *Camellia japonica* against dental pathogens *Streptococcus mutans* and *Candida albicans* were evaluated by Choi et al. (2017). As a result, it was found out that *C. japonica* significantly inhibited the growth of both bacteria by over 76% (Choi et al., 2017).

Antimicrobial properties of fermented *C. japonica* leaf extracts against Gram-positive *Staphylococcus epidermidis* and *Bacillus subtilis*, and Gram-negative *Klebsiella pneumonia* and *Escherichia coli* bacteria were evaluated by disc diffusion method (Moon and Kim, 2018). Ethanol extracts showed higher antimicrobial activities against all bacterial strains tested with higher inhibitory effects on pancreatic lipase than methanol extracts. The results of this study highlight the possible use of fermented *C. japonica* leaf extracts as a source of antioxidant, antibacterial and antiobesity agents. Moreover, ethanol was recommended as a solvent for extracting antioxidants, antibacterial and antiobesity agents from this plant (Moon and Kim, 2018).

Synergistic combinations of antimicrobial agents with different mechanisms of action can be more successful strategies to combat infections involving multidrug-resistant (MDR) bacteria. In the study by Farooqui et al. (2015), the synergistic antimicrobial activity of *Camellia sinensis* and *Juglans regia* which are commonly used plants with different antimicrobial agents was studied. Antimicrobial susceptibility of 350 Gram-positive and Gram-negative strains belonging to 10 different bacterial species, was tested against *Camellia sinensis* and *Juglans regia* extracts.

Camellia sinensis showed higher antibacterial activity against MDR *Salmonella typhi* than to other Gram-negative isolates. By testing a variety of clinical strains in a time-kill kinetic assay, Farooqui et al. (2015) further showed that green tea extract is bacteriostatic in nature and more efficient against MDR strains of *S. typhi* compared to pan-susceptible strains. *Camellia sinensis* also showed synergistic activity with nalidixic acid (FICI 0.37) against MDR *S. typhi* strains (Farooqui et al., 2015). The leave extracts of *Camellia* varieties indicated the presence of active biological compounds such as flavonoids, proteins, phenols, alkaloids and glycosides which exhibited strong activity against selected bacterial strains (Farooqui et al., 2015).

The crude extracts of six different plants of green tea *Camellia assamica* and *Camellia sinensis* were tested by Bashir et al. (2014) against three Gram-positive and four Gram-negative bacteria using the agar disk diffusion method at 50 mg/ml concentration. The maximum inhibition of *Staphylococcus aureus* was recorded by dimethyl sulphoxide extracts of green tea varieties. The measured zone of inhibition of dimethyl sulphoxide extracts by Qimen was (10.00 ±0.0 mm), Japanese (10.00 ±0.0 mm), Turkish (10.00 ±0.0 mm), Indonesian (8.33 ±1.0 mm), P3 clone (10.00 ±0.0 mm) and Sri Lankan (10.00 ±0.0 mm). Maximum scavenging potential activity was found with ethanol, methanol and dimethyl sulphoxide extracts. Spot screening indicated that the presence of active biological compounds such as flavonoids, proteins, phenols, alkaloids, and glycosides also exhibited strong activity against tested bacterial strains (Bashir et al., 2014).

Effect of tea extracts and standard flavanols against five pathogenic bacteria viz., *Listeria monocytogenes* (MTCC-839), *Pseudomonas aeruginosa* (MTCC-741), *Bacillus cereus* (MTCC-1272), *Staphylococcus aureus* (MTCC-96) and *Escherichia coli* (MTCC-443), and eleven indigenous potential bacterial probiotics belonging to genera *Enterococcus*, *Bacillus* and *Lactobacillus* spp. obtained from fermented foods of Western Himalayas, was investigated by Sourabh et al. (2014). As is generally well known, the antimicrobial effect of plant extracts, including extract derived from *Camellia* spp. plants are closely associated with the content of secondary metabolites, such as phenolic and/or flavonoid compounds (Kang et al., 2003; Choi et al., 2017). In turn, the phenolic compound content is also closely related to the antioxidant activity of plant extracts.

Phenolic compounds of nutraceutical importance viz., catechins (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG) were also estimated in fresh green tea shoots of *Camellia sinensis* (L) O Kuntze cultivar. EGCG, ECG, and EGC exhibited antibacterial activity but, C and EC did not show this activity. It has been reported that tea extracts having high concentrations of EGCG and ECG were more potent in antibacterial action against bacterial pathogens. Tea extracts and standard flavan-3-ols augmented viability of potential probiotics in an order of EGCG > EGC > ECG > EC > C. Tea extracts and standard flavanols had no antibacterial activity against *Escherichia coli* (MTCC-443) but, in combination with probiotic culture supernatants, this activity was seen (Sourabh et al., 2014).

Sharma et al. (2012) have explored the anti-microbial activity and the basic mechanism of aqueous green tea leaf extract on selected bacterial strains. *Staphylococcus epidermidis*, *Micrococcus luteus*, *Brevibacterium linens*, *Pseudomonas fluorescens*, and *Bacillus subtilis* were

found to be sensitive to green tea extract *via* disc diffusion assay (zone of inhibition ≥7 mm). The effect of aqueous extract on the adhesion of different bacteria to Vero cells indicated that it inhibits the adhesion at its minimum inhibitory concentration (MIC) value (0.156–0.313 mg/ml) (Sharma et al., 2012).

The use of natural plant materials such as green tea and rosemary could improve the microbial quality of foods in addition to their functional properties. The antimicrobial effects of green tea and rosemary added to foods as antagonists to foodborne pathogens were determined in laboratory media by Lee et al. (2009). The growth of each pathogen (*Bacillus cereus*, *Salmonella typhimurium*, *Enterobacter sakazakii*, *Escherichia coli* O157: H7, *Staphylococcus aureus*, and *Listeria monocytogenes*) in tryptic soy broth or a rice cake with or without the addition of green tea or rosemary leaf powders before autoclaving or cooking, respectively, was investigated after inoculation. The addition of 1% green tea or rosemary produced similar results for inhibiting the growth of pathogens in tryptic soy broth. However, green tea was more effective than rosemary for inhibiting the growth of *L. monocytogenes*. Both botanicals had inhibitory effects against all pathogens tested in this study. Green tea was particularly effective against *B. cereus*, *S. aureus*, and *L. monocytogenes*, and rosemary was strongly inhibitory against *B. cereus* and *S. aureus*. The addition of 1 or 3% green tea or rosemary to rice cakes did not significantly reduce total aerobic counts; however, levels of *B. cereus* and *S. aureus* were significantly reduced in rice cakes stored for 3 days at room temperature (Lee et al., 2009).

Many of the direct effects of tea catechins are a result of the catechins binding to the bacterial lipid bilayer cell membrane which then causes damage to the membrane (Sirk et al., 2008, 2009; Reygaert, 2014). Molecular dynamics simulations were performed by Sirk et al. (2008) to study the interactions of bioactive catechins (flavonoids) commonly found in green tea with lipid bilayers, as a model for cell membranes. Simulations in the study of these researchers showed that the seven tea catechins evaluated had a strong affinity for the lipid bilayer *via* hydrogen bonding to the bilayer surface, with some of the smaller catechins able to penetrate underneath the surface. Epigallocatechin-gallate (EGCG) showed the strongest interaction with the lipid bilayer based on the number of hydrogen bonds formed with lipid headgroups (Sirk et al., 2008). The molecular structure and aggregated condition of the catechins significantly influence their absorption, as well as their ability to form hydrogen bonds with the lipid headgroups (Sirk et al., 2009). This damage can then lead to a variety of related antimicrobial effects.

Cho et al. (2007) have found that when exposed to Korean green tea (*Camellia sinensis*) polyphenols, the bacterial response of *Escherichia coli* was changed regulation of 17 individual genes. Nine upregulated proteins were identified (including GroEL and proteins involved in cellular defense, such as GyrA, RpoS, SodC, and EmrK), whereas the expression of eight proteins was downregulated by exposure to TPP (including proteins involved in carbon and energy metabolism, such as Eno, SdhA, and UgpQ, as well as those involved in amino-acid biosyntheses, such as GltK and TyrB). One of the major outcomes of this change in regulation was damage to the bacterial cell membrane (Cho et al., 2007). The catechins primarily act on and damage bacterial membranes. The observation that Gram-negative bacteria are more resistant to bactericidal catechins than Gram-positive bacteria can be explained to some

extent by the presence of negatively charged lipopolysaccharide (Ikigai et al., 1993). Bacterial cell membrane damage inhibits the ability of the bacteria to bind to host cells (Sharma et al., 2012), and inhibits the ability of the bacteria to bind to each other to form biofilms, which are significant in pathogenesis (Blanco et al., 2005).

Recently, a 'green method' for the synthesis of gold nanoparticles (AuNPs) has been reported by Sharma et al. (2019), where *C. japonica* leaves extract was used as a reducing agent. Furthermore, the resulting antimicrobial activity of the synthesized AuNPs was tested against seven different microbial strains such as *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Klebsiella pneumonia*e, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*. The AuNPs showed a moderate activity at the dosage rate of 200 μg/mL (Sharma et al., 2019). The antimicrobial action of gold nanoparticles against Gram-positive and Gram-negative bacterial strains was explained by Shamaila et al. (2016). For the Gram-positive bacteria the structure of the membrane, i.e., the peptidoglycan layer's thickness, which is an important part of pathogenic bacteria, is 50% higher than in Gram-negative bacteria. Consequently, larger doses of nanoparticles are required for Gram-positive bacteria (Shamaila et al., 2016).

Bacterial membrane damage also results in an inability of the bacteria to be able to secrete toxins (Sugita-Konishi et al., 1999; Shah et al., 2008). Production and extracellular release of maltose-binding protein, a periplasmic protein, was also inhibited by epigallocatechin gallate (EGCg) and gallocatechin gallate (GCg), indicating that their inhibitory effect on release from the periplasm into the outer milieu is not specific to Vero toxins (VTs) from enterohemorrhagic *Escherichia coli* (EHEC), but general to the proteins accumulated in EHEC periplasm (Sugita-Konishi et al., 1999). The observation that secretion of key components of Staphylococcal virulence in study of Shah et al. (2008) can be compromised by a naturally occurring polyphenol supports the notion that (-)-epicatechin gallate (ECg) and related compounds may have therapeutic utility for the control of infections that are currently difficult to treat due to the propensity of methicillin-resistant *S. aureus* to accumulate antibiotic resistance genes.

Zhang and Rock (2004) have found that green tea components (especially EGCG) inhibit specific reductases (FabG, FabI) in bacterial type II fatty acid synthesis. The presence of the galloyl moiety was essential for activity, and EGCG was a competitive inhibitor of FabI and a mixed type inhibitor of FabG demonstrating that EGCG interfered with cofactor binding in both enzymes. EGCG inhibited acetate incorporation into fatty acids *in vivo*, although it was much less potent than thiolactomycin, a validated fatty acid synthesis inhibitor. Inhibition of fatty acid synthesis by green tea has also been found to inhibit bacterial production of toxic metabolites. The inhibitory effect on the production of toxic end metabolites of bacteria can be attributed to the presence of the galloyl moiety, which is ester-linked with the 3-OH of the catechin moiety in the polyphenolic compounds (Sakanaka and Okada, 2004).

Okamoto et al. (2003, 2004) also found that green tea catechins have an inhibitory effect on protein tyrosine phosphatase and cysteine proteinases in certain anaerobic oral bacteria (*Prevotella intermedia*, *Porphyromonas gingivalis*). The inhibitory effect observed is due to the presence of galloyl moiety in the structure (Okamoto et al., 2003, 2004).

The catechins inhibit bacterial DNA gyrase by binding to the ATP binding site of the gyrase B subunit. In the group of four tested catechins, epigallocatechin gallate (EGCG) had the highest activity, followed by epicatechin gallate (ECG) and epigallocatechin (EGC) (Grandišar et al., 2007). The green tea polyphenols can inhibit the enzyme dihydrofolate reductase in bacteria and yeast, effectively blocking the ability of the microorganisms to synthesize folate. In elucidating its mechanism of action, Navarro-Martínez et al. (2005) have shown that epigallocatechin gallate is an efficient inhibitor of *Stenotrophomonas maltophilia* dihydrofolate reductase (Navarro-Martinez et al., 2005). EGCG also acts as an antifolate compound on *Candida albicans*, disturbing its folic acid metabolism (Navarro-Martinez et al., 2006). The bioflavonoids obtained from green tea could also inhibit the activity of bacterial ATP synthase, reducing the ability of the microorganisms to produce enough energy (Chinnam et al., 2010).

Conclusions

Based on our current investigations it can be concluded that *Camellia japonica* and its cultivars possess a mild antibacterial efficacy. It may also be concluded that the antimicrobial potential of various samples of these plants might be due to a wide variety of compounds present in these plants. The findings reported herein give scientific credence to the traditional uses of these plants and suggest that extracts derived from the leaves of *Camellia japonica* and its cultivars merit further chemical study as natural antibiotics to identify the secondary metabolites.

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ANTIBACTERIAL ACTIVITY OF THE ETHANOLIC EXTRACTS DERIVED FROM LEAVES AND PSEUDOBULBS OF SOME ORCHIDS BELONGING TO *COELOGYNE* GENUS AGAINST *ENTEROBACTER CLOACAE* STRAIN

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Orchids belong to the largest family of angiosperms possessing the antimicrobial, antioxidant, hepatoprotective, anti-inflammatory, anti-arthritic and wound healing properties revealed in pre-clinical studies. The study of orchids also can provide health professionals with alternative, feasible, and low-cost therapies for treating bacterial and fungal diseases. For this reason, orchids may be used as new antimicrobial agents. Our previous study reported that the selected orchids belonging to the genus *Coelogyne* Lindl. exhibited fairly strong antioxidant and antimicrobial activities. This paper focused on the assessment of the antibacterial activity of eight plants, i.e. *Coelogyne cristata* Lindl., *C. fimbriata* Lindl., *C. flaccida* Lindl., *C. huettneriana* Rchb.f., *C. ovalis* Lindl., *C. speciosa* (Blume) Lindl., *C. tomentosa* Lindl. and *C. viscosa* Lindl. towards clinical cefuroxime-resistant *Enterobacter cloacae* strain. Antibacterial activity was evaluated qualitatively and quantitatively using disc diffusion assay. The ethanolic extracts obtained from leaves and pseudobulbs of five orchids belonging to the *Coelogyne* genus were found to exhibit fairly strong antibacterial activity towards *Enterobacter cloacae* strain used, the diameter of inhibition zones varied from 8.0–25.5 mm. It has been observed that ethanolic extract obtained from pseudobulbs of eight species from the *Coelogyne* genus revealed the highest antibacterial activity (11.0–25.5 mm as the diameter of inhibition zone) as compared to ethanolic extracts obtained from leaves of the same *Coelogyne* species. These findings provide a clear demonstration of the generally overlooked importance of the collection of tropical plants accumulated at the Botanic Gardens, orchids collection, in particular, as an important source of new chemical substances with potential therapeutic effects, including antifungal activity. Further studies aimed at the isolation and identification of active substances from the extracts obtained from leaves and pseudobulbs of *Coelogyne* species could also disclose compounds with better therapeutic value. It is believed that screening of all the investigated plants for other biological activities is essential.

Keywords: orchids, leaf extracts, *Enterobacter cloacae*, antibacterial activity, disc diffusion technique, ethanolic extracts

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Introduction

Enterobacter cloacae are part of the normal flora of the gastrointestinal tract of 40 to 80% of people and are widely distributed in the environment. Species of the genus *Enterobacter* are opportunistic pathogens and are capable of causing opportunistic infections in hospitalized patients (intensive care units, emergency units, urology department) (Michalska and Gospodarek, 2007). Species of the *Enterobacter cloacae* complex are widely encountered in nature (Mezzatesta et al., 2012). *Enterobacter aerogenes* and *E. cloacae* have been reported as important opportunistic and multidrug-resistant bacterial pathogens for humans during the last three decades in hospital wards. These Gram-negative bacteria have been largely described during several outbreaks of hospital-acquired infections (Davin-Regli et al., 2015). In recent years, *Enterobacter cloacae* have emerged as an important nosocomial pathogen (Dalben et al., 2008). The biochemical and molecular studies on *E. cloacae* have shown genomic heterogeneity, comprising six species: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter nimipressuralis*. *E. cloacae* and *E. hormaechei* are the most frequently isolated in human clinical strains (Mezzatesta et al., 2012). The dissemination of *Enterobacter* spp. is associated with the presence of redundant regulatory cascades that efficiently control the membrane permeability ensuring the bacterial protection and the expression of detoxifying enzymes involved in antibiotic degradation and inactivation. In addition, these bacterial species are able to acquire numerous genetic mobile elements that strongly contribute to antibiotic resistance. Moreover, this particular fitness helps them to colonize several environments and hosts and rapidly and efficiently adapt their metabolism and physiology to external conditions and environmental stresses (Davin-Regli et al., 2015). They are capable of overproducing AmpC β-lactamases by de-repression of a chromosomal gene or by the acquisition of a transferable ampC gene on plasmids conferring the antibiotic resistance (Mezzatesta et al., 2012).

It has been highlighted that the pathogenic mechanisms and factors contributing to the disease associated with the *E. cloacae* complex are not understood yet (Davin-Regli et al., 2015). Nevertheless, it was assumed that an ability of *E. cloacae* to form biofilms and to secrete various cytotoxins (enterotoxins, hemolysins, pore-forming toxins) is important for its pathogenicity (Mezzatesta et al., 2012). Moreover, certain *Enterobacter* spp. have been reported as plant-growth enhancers since they possess multiple growth-promoting activities (Ramesh et al., 2014).

Plant-derived antimicrobial agents have received much attention due to their effectiveness against drug-resistant strains, and diverse antimicrobial activities including antibiofilm activity (Khan et al., 2017; Lu et al., 2019).

A wide range of phytochemicals has been reported as antibacterial compounds, belonging to different classes, such as alkaloids, terpenoids, polyphenols, tannins, and phenanthrenes. It was emphasized that among them, phenanthrenes are a relatively uncommon class of aromatic metabolites in the plant kingdom, which are mainly found in the Orchidaceae and Juncaceae (Kovács et al., 2008; Chen et al., 2018; Tóth et al., 2018).

Although the antimicrobial activity of many orchid species, including *Coelogyne* species, has been effectively established against a wide spectrum of microorganisms (Kovács et al., 2008; Haque and Ghosh, 2015; Chen et al., 2018), bacterial drug resistance continues to be a worldwide public health issue in the treatment of infectious diseases, thereby stimulating search for new alternatives with fewer side effects (Mambe et al., 2019).

The orchid genus *Coelogyne* Lindl. comprises over 200 species, distributed from Southeast Asia to the south-western Pacific Islands (Clayton, 2002; Chen and Clayton 2009). It is currently divided into 4 subgenera and 19 sections (Gravendeel, 2005). *Coelogyne* species grow in the primary forest from sea level up to 4,000 m altitude. They are predominantly epiphytes, but in humid environments, some species can grow as lithophytes over rocky cliffs or even as terrestrials (Clayton, 2002). The *Coelogyne* genus is characterized by sympodial growth habit, pseudobulbs of one internode, a winged column, and massive caudicles (Dressler, 1981). Within Coelogyninae subtribe this genus is distinguished by a free, never-saccate lip, with high lateral lobes over the entire length of the hypochile and smooth, papillose, toothed or warty keels on the epichile (Seidenfaden and Wood 1992).

Correspondingly, taking into consideration the development of resistance by Gram-negative bacteria the present study was aimed to compare the antibacterial activity of the leaf and pseudobulb extracts of eight orchid species, i.e. *Coelogyne cristata* Lindl., *C. fimbriata* Lindl., *C. flaccida* Lindl., *C. huettneriana* Rchb.f., *C. ovalis* Lindl., *C. speciosa* (Blume) Lindl., *C. tomentosa* Lindl. and *C. viscosa* Lindl. against clinical cefuroxime-resistant *Enterobacter cloacae* strain.

Materials And Methodology

Collection of Plant Material

The leaves and pseudobulbs of orchids, i.e. *C. flaccida* Lindl., *C. huettneriana* Rchb.f., *C. speciosa* (Blume) Lindl., *C. fimbriata* Lindl., *C. tomentosa* Lindl., *C. ovalis* Lindl., *C. cristata* Lindl., *C. viscosa* Rchb.f. (Figure 1) cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (Kyiv, Ukraine). Since 1999 the whole collection of tropical and subtropical plants (including orchids) has had the status of a National Heritage Collection of Ukraine and is supported through State funding. Besides, M.M. Gryshko National Botanic Garden collection of tropical orchids was registered at the Administrative Organ of CITES in Ukraine (Ministry of Environment Protection, registration No. 6939/19/1-10 of 23 June 2004).

Preparation of Plant Extracts

The collected leaves and pseudobulbs were brought into the laboratory for antimicrobial studies. Freshly crushed leaves and pseudobulbs were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

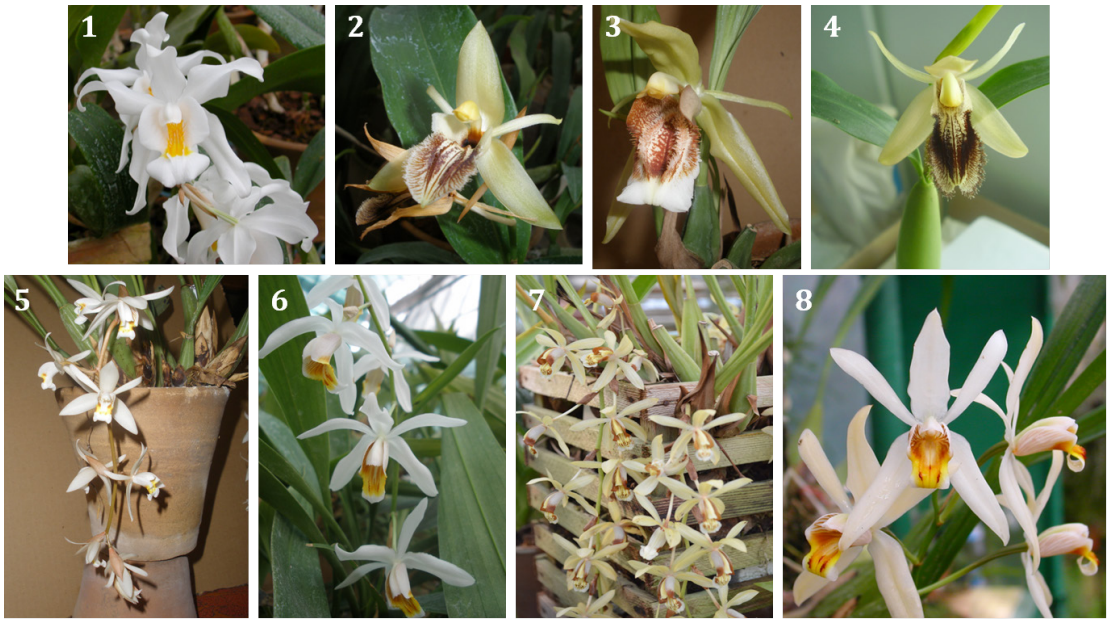


Figure 1 The general views of flowers of *Coelogyne* species from the collection of M.M. Gryshko National Botanical Garden (Kyiv, Ukraine) (Photo: Lyudmyla Buyun, Oleksandr Gyrenko)
1 – *Coelogyne cristata* Lindl.; 2 – *Coelogyne fimbriata* Lindl.; 3 – *Coelogyne speciosa* (Blume) Lindl.; 4 – *Coelogyne ovalis* Lindl.; 5 – *Coelogyne flaccida* Lindl.; 6 – *Coelogyne huettneriana* Rchb.f.; 7 – *Coelogyne tomentosa* Lindl.; 8 – *Coelogyne viscosa* Rchb.f.

Bacterial strain

The testing of the antibacterial activity of the plant extract was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). The non-repetitive clinical strain of cefuroxime-resistant *E. cloacae* isolated from bloodstream infection was collected from Koszalin Hospital during March-April, 2019. The purity, as well as the identity of isolate, was confirmed in the laboratory conditions by standard microbiological methods and were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (2014).

Susceptibility testing of the isolates was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI). The antibiotics tested were piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, cefuroxime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamicin, trimethoprim-sulphamethoxazole, ciprofloxacin, levofloxacin, tetracycline, tigecycline, and polymyxin B. Results were interpreted according to CLSI criteria. MIC was determined by *E*-test strips (according to manufacturer's instruction) and agar dilution method (according to the Guidelines of Clinical and Laboratory Standard Institute). The resistance breakpoints were the same as the ones defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2014).

Enterobacter cloacae strain studied was resistant to amoxicillin, cefuroxime, trimethoprim-sulphamethoxazole, and cefotaxime.

The Disk Diffusion Method for Evaluation of Antibacterial Activity of Plant Extracts

Strain tested was plated on TSA medium (Tryptone Soy Agar) and incubated for 24 hr at 37 °C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). Muller-Hinton agar plates were inoculated with 200 µl of standardized inoculum (108 CFU/mL) of the bacterium and spread with sterile swabs.

Sterile filter paper discs impregnated by extract were applied over each of the culture plates, 15 min after bacteria suspension was placed. A negative control disc impregnated by sterile 96% ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hr at 37 °C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks. The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each extract. The data were analyzed using an one-way analysis of variance (ANOVA) with Statistica software, version 8.0 (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Ethanolic extracts obtained from leaves and pseudobulbs of eight orchids belonging to the *Coelogyne* genus resulted in considerable suppression of clinical cefuroxime-resistant *Enterobacter cloacae* strain growth. Moreover, differential efficacy on the test organism was noted between *Coelogyne* species as well as between extracts obtained from leaves and pseudobulbs tested. Consequently, the extracts from various species displayed varied antibacterial potency against cefuroxime-resistant *Enterobacter cloacae* (Figure 2, 3).

As presented in Figure 2, tested extracts showed variable antibacterial activities with inhibition zone diameter values ranging from 8.1 to 17.8 mm (crude extracts derived from the leaves) and from 11.5 to 24.3 mm (for pseudobulb extracts). In our study, the marked antibacterial efficacy against cefuroxime-resistant *Enterobacter cloacae* strain was observed in the case of ethanolic extracts obtained from leaves of *C. tomentosa* (mean diameter of inhibition zones was 15.5 ±0.9 mm), *C. speciosa* (15.9 ±0.7 mm), and *C. huettneriana* (17.8 ±1.2 mm). A statistically significant increase (p <0.05) in inhibition zone diameters of strain growth was

100% (for *C. huettneriana*), 79% (for *C. speciosa*), and 74% (for *C. tomentosa*). Leaf extracts of *C. cristata*, *C. ovalis*, *C. fimbriata*, *C. viscosa*, and *C. flaccida* displayed less inhibitory activity against strain tested (mean diameter of inhibition zones was ranging from 8.0 to 13.5 mm) (Figure 2, 3).

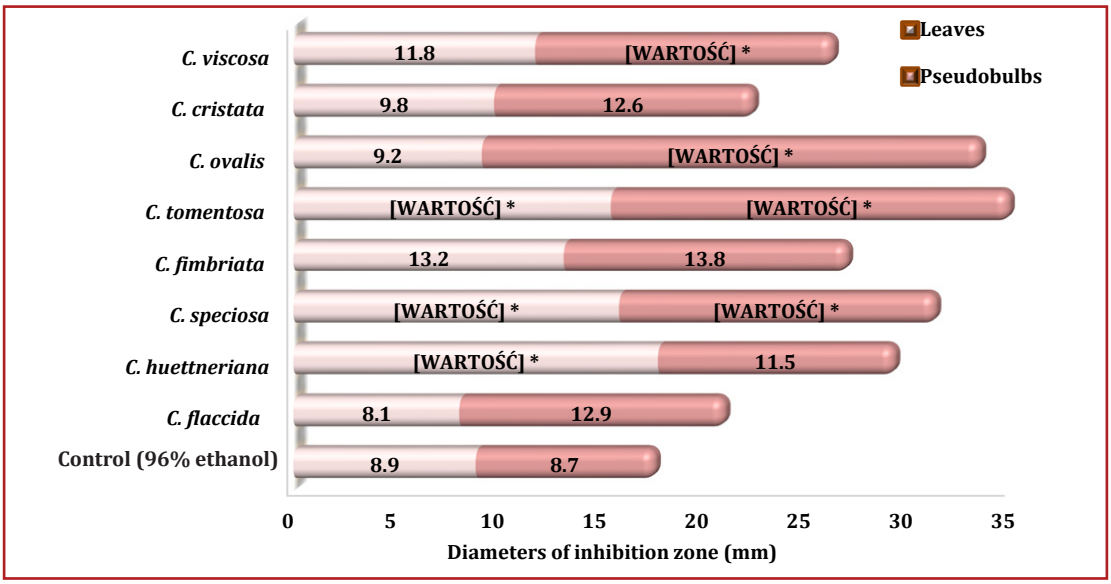


Figure 2 Antimicrobial activity of ethanolic extracts obtained from leaves and pseudobulbs of eight *Coelogyne* species against cefuroxime-resistant *Enterobacter cloacae*, measured as diameters of inhibition zone (n = 8)

On the other hand, the ethanolic extracts obtained from pseudobulbs of *C. ovalis* (mean diameter of inhibition zones was 24.3 ±1.3 mm), *C. tomentosa* (19.4 ±1.1 mm), and *C. speciosa* (15.4 ±0.8 mm) revealed statistically significant antibacterial activity against cefuroxime-resistant *Enterobacter cloacae* strain compared to control (96% ethanol). A statistically significant increase (p <0.05) in inhibition zone diameters of strain growth was 179% (for *C. ovalis*), 123% (for *C. tomentosa*), and 77% (for *C. speciosa*) (Figure 2, 3).

The present study has shown that ethanolic extracts derived from the leaves and pseudobulbs of eight species from the *Coelogyne* genus exhibited marked antibacterial activity against *Enterobacter cloacae* (inhibition zone diameter were ranged from 8.0 to 25.5 mm) (Figure 2, 3). Moreover, it has been observed that ethanolic extract obtained from pseudobulbs of eight species from *Coelogyne* genus revealed the highest antibacterial activity (11.0–25.5 mm as the diameter of inhibition zone) compared to ethanolic extracts obtained from leaves of eight species from *Coelogyne* genus (Figure 2, 3).

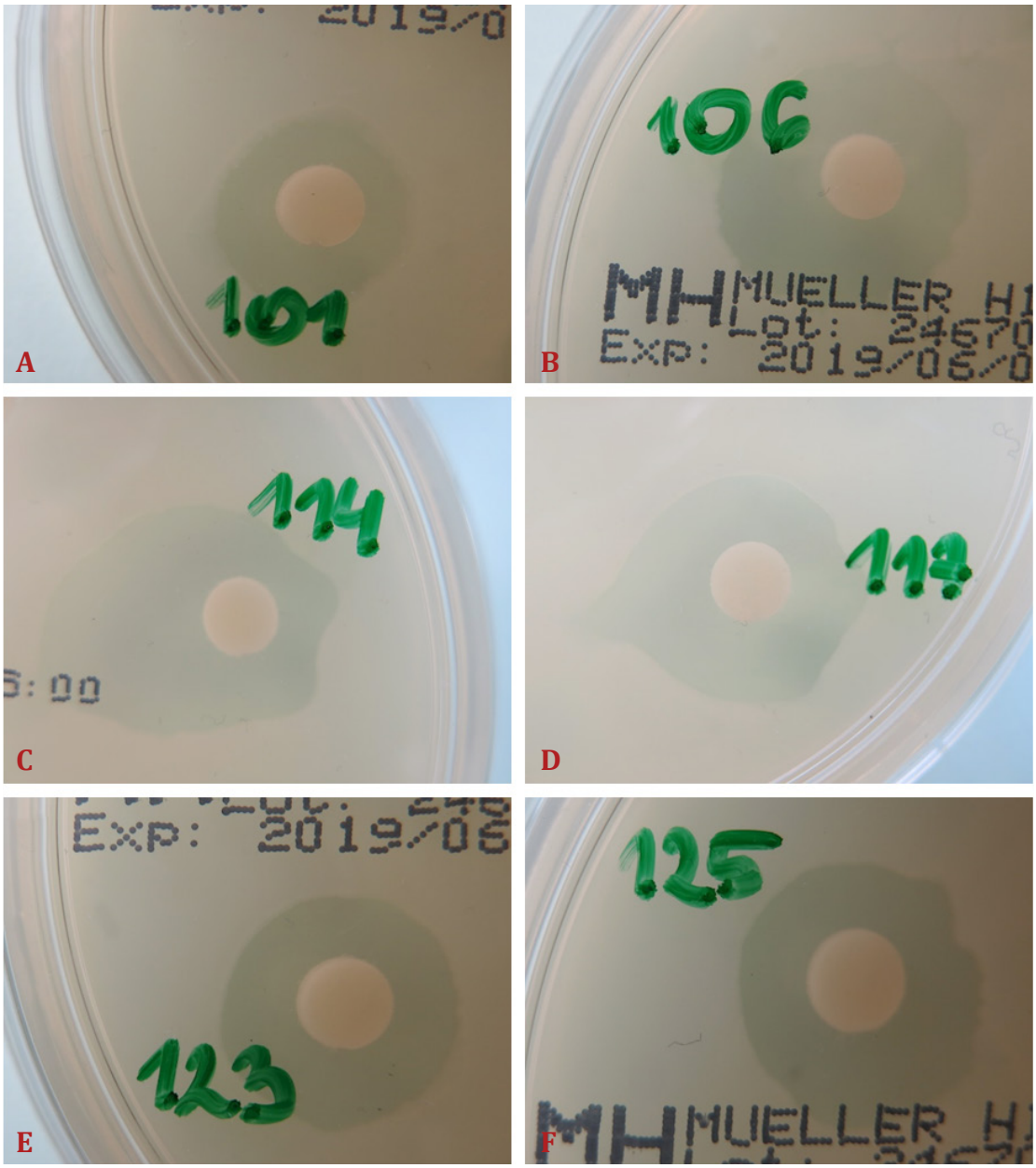


Figure 3 Examples of a disc diffusion assay plate showing the halos in the bacterial lawn resulting from the antibacterial activity of extracts obtained from *Coelogyne tomentosa* Lindl. leaves (A) and pseudobulbs (B), *Coelogyne ovalis* Lindl. pseudobulbs (C), *Coelogyne huettneriana* Rchb.f. leaves (D), *Coelogyne speciosa* (Blume) Lindl. leaves (E) and pseudobulbs (F)

In recent years, the assessment of antibacterial properties of orchids has received considerable attention (Majumder et al., 1995, 2001, 2011; Singh et al., 2012; Tkachenko et al., 2015; Soumiya and Christudhas, 2017; Buyun et al., 2016, 2017, 2018). A number of orchid species are used as a potent inhibitor against Gram-positive and Gram-negative

bacteria and also proved to be a potent antimicrobial agent (Singh et al., 2012). Bhatnagar and Ghosal (2018) have conducted phytochemical profiling and evaluation of the antibacterial and antimycobacterial activity of orchid of Arunachal Pradesh, *Pleione maculata*. The whole plant material was divided into diethyl ether (Et2O), n-butanol (n-BuOH) and aqueous (Aq) fractions. The five different MDR bacterial clinical isolates including *Escherichia coli* (2461), *Staphylococcus aureus* (2413), *Enterococcus* sp. (2449), *Acinetobacter* sp. (2457), *Serratia* sp. (2442), *Mycobacterium tuberculosis* H37Rv were used in the study. The screening result identified Et2O fraction as the most active fraction showing significant zone of inhibition against *E. coli* and *Staphylococcus aureus* sp. and moderate antimycobacterial activity (MIC value 104.16 µg/mL) against H37Rv strain of *Mycobacterium tuberculosis*. The largest zone of inhibition (24.1 ±0.3 and 20.7 ±0.5 mm) was demonstrated by the Et2O fraction against *E. coli* and *S. aureus* sp. while in case of n-BuOH fraction the zone of inhibition was 18.7 ±0.7 mm and 17.1 ±0.6 mm against *S. aureus* and *Serratia* sp. respectively. However, the aqueous extract was not as effective against any bacterial pathogens as compared to organic solvent fractions even though slight antibacterial activity was observed. The Et2O fraction also inhibited the growth of *Serratia* sp. and *Enterococcus* with an inhibition zone 15.2 ±0.5 mm and 13.0 ±0.5 mm. The *Acinetobacter* sp. was least inhibited by the plant fractions. Analysis of the results showed that Et2O fraction of *P. maculata* exhibited moderate antimycobacterial activity against H37Rv strains with MIC 104.16 µg/mL followed by n-BuOH fraction with MIC value 166.66 µg/mL respectively. However, the aqueous residue of the plant did not show any activity against *Mycobacterium tuberculosis* strain. Phytochemical screening of the fractions revealed the presence of triterpenes, phlobatannins, alkaloids and cardiac glycosides in the most active Et2O fraction (Bhatnagar and Ghosal, 2018).

It was shown that blestriacin, a dihydro-biphenanthrene, extracted from the fibrous roots of *Bletilla striata* exhibited significantly anti-Gram-positive bacterial activity against *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, and *Bacillus subtilis* (Qian et al., 2015)

Phytochemical analysis revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, sterols in the hydro-alcoholic extract of *Coelogyne cristata* (Mitra et al., 2018). This orchid species is traditionally used in Indian subcontinent as a stimulant and tonic in aged patients suffering from persistent diseases, like asthma, degenerative changes and blood-borne diseases (Kumari et al., 1986; Singh et al., 2012; Mitra et al., 2018).

Soumiya and Christudhas (2017) have determined the phytochemical screening and antimicrobial activity of *Cymbidium aloifolium* (L.) Sw. orchids in Kanyakumari district of South India. The phytochemical screening and antimicrobial activity were performed using different extracts (methanol, ethanol, chloroform, acetone and aqueous) of *C. aloifolium*. Four different bacteria (Gram-positive: *Staphylococcus aureus* and *Bacillus subtilis*; Gram-negative: *Escherichia coli* and *Klebsiella pneumonia*e) and two fungi (*Penicillium* sp. and *Aspergillus niger*) were used in the Soumiya and Christudhas (2017) study. All the extracts showed different degrees of inhibitory action that potential against tested bacteria under this study. The antibacterial activity of *C. aloifolium* leaf extract revealed that the maximum inhibition zone of 34 mm recorded in aqueous extract against *S. aureus*. Minimum inhibitory activity was recorded in acetone and chloroform extract. The aqueous extract had the highest

activity against *Aspergillus niger* and *Rhizopus* with an inhibition zone of 45 and 42 mm, respectively. Minimum inhibitory activity was recorded in acetone and chloroform extract. The antimicrobial activity of *C. aloifolium* leaf extract revealed that the maximum inhibition zone of (43.5 ±0.84) recorded in aqueous extract against *Rhizopus* sp. followed by *Aspergillus niger* (42 ±0.62). The aqueous extract showed the inhibitory activity of (34.5 ±0.40) against *Staphylococcus aureus*. Methanol extract also exhibited some activity against *Staphylococcus aureus* (30±0.23) and *Klebsiella pneumonia* (25 ±0.47). The next activity was noticed in ethanol extract against *Bacillus subtilis*, *Aspergillus niger*, *Klebsiella pneumonia*, and *Staphylococcus aureus*. No activity was noticed in ethanol extract against *Escherichia coli* and *Rhizopus* sp. The acetone extract showed significant activity against *Bacillus subtilis*, *Aspergillus niger*, and *Rhizopus* sp. Among the different extracts, aqueous extracts showed better results than the positive control. Minimum inhibitory activity was recorded in methanol extract and acetone extracts (Soumiya and Christudhas, 2017).

The antimycobacterial, leishmanicidal and antibacterial activity of three orchids *Rhynchostylis retusa* (L.) Blume, *Tropidia curculigoides* Lindl. and *Satyrium nepalense* D. Don, traditionally used in tuberculosis, asthma and cold stage of malaria in folk Indian medicine, was demonstrated in the Bhatnagar et al. (2017) study. The dried material of each plant was divided into three parts. Solvent extraction and fractionation afforded altogether 30 extracts and fractions, which were evaluated against *Mycobacterium tuberculosis* (H37Rv and MDR strain) for antimycobacterial activity; promastigotes and amastigotes of *Leishmania donovani* for leishmanicidal activity and two Gram-positive and three Gram-negative clinical isolates for antibacterial activity. The most significant antimycobacterial activity was observed with n-hexane fraction of the flower of *Satyrium nepalense* with MIC of 15.7 µg/mL. The most promising leishmanicidal activity was observed with diethyl ether fraction of the roots of *Rhynchostylis retusa* with IC_{50} values of 56.04 and 18.4 µg/mL against promastigotes and intracellular amastigotes respectively. Evaluation of antibacterial activity identified *S. nepalense* flower n-hexane and *R. retusa* roots diethyl ether as potential fractions with MIC values of ≤100 µg/mL against selected clinical isolates. *S. nepalense* as the most promising plant followed by *R. retusa* and *T. curculioides* was demonstrated. Also, the fractions exhibited cell cytotoxicity well within the permissible limit (Bhatnagar et al., 2017).

Nagananda and Satishchandra (2013) have evaluated the antibacterial and antifungal activity of medicinally important orchid *Dendrobium nodosum* Dalzell (syn. *Flickingeria nodosa* (Dalz.) Seidenf) against human pathogens with cold and hot successive extracts. The antimicrobial activities of the plant extracts were evaluated against 7 bacterial and 6 fungal strains using well diffusion method on Mueller-Hinton agar medium. The cold water extract has antibacterial activity against *S. aureus* and *S. citreus* with a maximum zone of inhibition. The cold chloroform extract has good antifungal activity against *T. mentagrophytes* (Nagananda and Satishchandra, 2013).

Previously, we have given considerable attention to evaluation of the antibacterial effects of ethanolic extracts obtained from leaves and pseudobulbs of plants belonging to various *Coelogyne* species, maintained under glasshouse conditions. For example, the assessment of antifungal potential of the leaves of eight orchid species, i.e. *Coelogyne cristata*, *C. fimbriata*,

C. flaccida, *C. huettneriana*, *C. ovalis*, *C. speciosa*, *C. tomentosa* and *C. viscosa* against fungus strain, *Candida albicans* was conducted by Buyun et al. (2018). Marked antifungal efficacy was observed in case of ethanolic extracts derived from leaves of *C. flaccida* (mean diameter of inhibition zones was 19.5 mm), *C. viscosa* (18.6 mm), *C. huettneriana* (18.2 mm), and *C. fimbriata* (17.5 mm). Extracts of *C. cristata*, *C. ovalis*, and *C. tomentosa* displayed less pronounced inhibitory activity against test fungus (mean diameter of inhibition zones ranging from 16.0 to 17.5 mm). Similarly, the ethanolic extracts from the pseudobulbs of eight *Coelogyne* species exhibited strong activity against *C. albicans* (inhibition zone diameter ranged from 16.0 to 23.5 mm). Moreover, it has been observed that ethanolic extract obtained from pseudobulbs of *C. speciosa* revealed the highest antibacterial activity (21 mm as diameter of inhibition zone) among various *Coelogyne* species screened. The results also indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results (Buyun et al., 2018).

In vitro antimicrobial activity of various extracts obtained from vegetative parts of *Coelogyne speciosa* against Gram-positive (*Staphylococcus aureus* ATCC 25923) and Gram-negative bacteria (*Escherichia coli* ATCC 25922) was also demonstrated in our study (Buyun et al., 2016, 2017). The ethanolic extracts from leaves and pseudobulbs of *C. speciosa* exhibited strong activity against *S. aureus* (inhibition zone diameter was 19.0 and 21.5 mm, respectively), while the methanolic extract from leaves and pseudobulbs revealed mild activity (8.0 and 8.0 mm). Moreover, it has been observed that ethyl acetate, hexane and dichloromethane extracts obtained from leaves and pseudobulbs of *C. speciosa* displayed no antibacterial activity against *S. aureus*. Our results also showed that ethanolic extract from leaves of *C. speciosa* exhibited strong activity against *E. coli* (inhibition zone diameter was 21 mm), while others extract from pseudobulbs revealed minimum activity (inhibition zone diameter did not exceed 12 mm). Those extracts in ethyl acetate, hexane, and dichloromethane both from the leaves and pseudobulbs revealed no antibacterial activity against *S. aureus* (Buyun et al., 2016, 2017).

Finally, the Global Strategy for Plant Conservation (GSPC) outlines 16 targets encompassing knowledge, conservation, sustainable use, awareness, and capacity-building activities at Botanic Gardens (Mounce et al., 2017). Therefore, our recent works have exemplified the conviction that living plant collections accumulated at Botanic Gardens as germplasm repositories, are able to provide great opportunities for multi-focused approach, aimed at both *ex situ* biodiversity conservation and pharmacological screening as well (Tkachenko et al., 2015, 2018; Buyun et al., 2018). Consequently, we believe that our activity could be considered as an implementation of the Global Strategy for Plant Conservation.

Conclusions

In conclusion, this study has provided informative data about the antimicrobial potential of the tested plant extracts by suggesting that ethanolic extracts derived from the leaves and pseudobulbs of eight species from *Coelogyne* genus exhibited marked antibacterial activity against *Enterobacter cloacae* (inhibition zone diameter ranged from 8 to 25.5 mm). Moreover, it has been observed that ethanolic extract obtained from pseudobulbs of eight species from *Coelogyne* genus revealed the highest antibacterial activity (11.0–25.5 mm as the diameter of

inhibition zone) as compared to ethanolic extracts obtained from leaves of eight *Coelogyne* species. Consequently, the present study, which revealed the antibacterial properties of the plant extracts, is a justification of the use of orchids belonging to *Coelogyne* genus to treat some of the infectious diseases caused by antibiotic-resistant *Enterobacter cloacae* strains. This fact is withstood by the bactericidal effect of the extracts derived from the leaves and pseudobulbs. Nevertheless, there is still room for an in-depth investigation, in order to make these plants best use in medicine and veterinary and to select them as an alternative to combat bacterial resistance

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ANTIBACTERIAL ACTIVITIES OF ETHANOLIC EXTRACT OBTAINED FROM *RHODODENDRON MYRTIFOLIUM* SCHOTT & KOTSCHY LEAVES AGAINST CLINICALLY ISOLATED BACTERIAL STRAINS

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The plants belonging to the genus *Rhododendron* L. and isolated compounds demonstrate diverse biological activities including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, insecticidal and cytotoxic activity, as well as antibacterial activity. Our study was designed the effects of *Rhododendron myrtifolium* Schott & Kotschy leaf extract against bacterial strains were monitored *in vitro* by the disk diffusion method. The aim of this study was to assess possible antibacterial effects of an ethanolic extract derived from *Rhododendron myrtifolium* leaves against *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* strain locally isolated from human biological fluids. Thus, the study contributes to on-going investigations on the bioactivity potential of plant species such as the *Rhododendron*. Leaves of *Rhododendron myrtifolium* were harvested on the side of the road between the Menchul valley and Rogneska valley (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine). The results revealed that extract exerts antibacterial activity against *Citrobacter freundii*. However, the *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* were resistant to *R. myrtifolium* leaf extract. Maximum *in vitro* inhibition was scored against *Citrobacter freundii*, followed by *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, which presented inhibition zones of (12.1 ±0.9) mm, (9.1 ±0.5) mm, (7.5 ±0.6) mm, and (7.2 ±0.5) mm, respectively. In the case of the positive controls, 96% ethanol possesses a mild antibacterial effect, which presented inhibition zones of (6.5 ±0.7) mm. The results from the screening study performed by the disc diffusion method revealed that *R. myrtifolium* possesses a mild antibacterial activity against *C. freundii*. However, further investigation is needed to determine the bioavailability of the active compounds and to determine the dose and toxicity before it can be used as therapeutic agents.

Keywords: *Rhododendron*, leaf extracts, agar disk diffusion assay, antibacterial activity, inhibition zone diameter

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Introduction

Antimicrobial resistance has become a pre-eminent concern in medicine and public health. This problem is widespread, and the causative factors are uncontrolled (Mah and Memish, 2000). Control of infections caused by multi-drug resistant Gram-positive and Gram-negative bacteria has become a major problem in various countries in the prevention of infectious diseases. Currently, the spread of multi-drug resistant bacteria is not only through nosocomial infections, but also occurs in the community (Radji et al., 2013). Several multi-drug resistant bacteria that are most commonly found, especially through nosocomial infections, are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Rice, 2008). In the past few decades, these strains become increasingly important pathogens in hospitals and play an important role in the colonization and infection of hospitalized patients by a variety of nosocomial infections including bacteremia, urinary tract infections, and nosocomial pneumonia (Radji et al., 2013).

Treatment of these infections is often very difficult due to the cross-resistance of these bacteria with a large group of antibiotics. Therefore, it seems reasonable to explore alternative antimicrobial agents for control multi-drug resistant bacteria. Recently, there has been growing interests to find antimicrobial compounds from medicinal plant extracts as an alternative approach to discover new antimicrobial compounds (Ríos and Recio, 2005; Radji et al., 2013). *Rhododendron* species have been proved to possess medicinal and health promotion properties, including the ability to inhibit the growth of some types of pathogenic bacteria (Innocenti et al., 2010; Silici et al., 2010; Popescu and Kopp, 2013; Rezk et al., 2015; Wang et al., 2015; Li et al., 2016; Hakeem Said et al., 2017; Shrestha et al., 2017).

The genus *Rhododendron* L. (Ericaceae) is one of the most species-rich among angiosperms, comprising over 1000 species spreading across the northern hemisphere and with the center of diversity in southeastern Asia (Irving and Hebda, 1993). These plants are morphologically diverse, including evergreen and deciduous shrubs, subshrubs, and trees, mostly terrestrial but sometimes chasmophytic or epiphytic, with greyish-brown striate bark, spirally arranged branches, and leaves, often in pseudo-whorls, and umbellate to pyramidal racemes of 1–30 conspicuous fragrant flowers. Because of its foliage and flowers, the genus is well-known in ornamental cultivation and gardening, with numerous varieties artificially bred thanks to rhododendrons' natural ability to interspecific hybridization (Cullen, 2005). A noteworthy European member of the genus is *R. myrtifolium* Schott & Kotschy, an evergreen clump-forming dwarf shrub up to 50 cm in height, occurring in high-mountain habitats of the eastern and southern Carpathian Mountains and northern Balkans, largely within altitudes of 1,400–2,500 m. The species is featured in small narrowly elliptic to obovate coriaceous leaves abaxially covered with glandular scales containing essential oils, terminal inflorescences of tubular-campanulate pinkish flowers, and long-pedunculate dry multilocular capsules containing numerous diminutive seeds (Cullen, 1980; Mircea, 2005; Boratyński et al., 2006; Voloschuk and Prokopiv, 2011). Although endangered in countries of its distribution, *R. myrtifolium* has been used in folk medicine for the preparation of herbal teas (Dihoru and Boruz, 2014; Nedelcheva and Draganov, 2014) and presents a major touristic attraction during

its mass flowering period in mountains (Rivers, 2017). Its evolutionary closest relatives, *R. ferrugineum* L. and *R. hirsutum* L. (e.g., Sosnovsky et al., 2017) have been shown to possess cytotoxic, antibacterial, and antiviral effects of their extracts (Louis et al., 2010; Gescher et al., 2011; Seephonkai et al., 2011; Rezk et al., 2015b), while the biochemical features and bioactive potentials of *R. myrtifolium* remain unexplored.

Rhododendron species have been traditionally used in China, Nepal, Russia, and North America against inflammation, pain, skin ailments, common cold, and gastrointestinal disorders and for treating human diseases like asthma and skin diseases. These species are known to be a good source of polyphenolic plant secondary plant metabolites (Shrestha et al., 2017). The plant extracts belonging to the genus *Rhododendron* and isolated compounds demonstrated diverse biological activities including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, insecticidal and cytotoxic activity (Popescu and Kopp, 2013), as well as antibacterial activity (Innocenti et al., 2010; Silici et al., 2010; Popescu and Kopp, 2013; Rezk et al., 2015; Wang et al., 2015; Li et al., 2016; Hakeem Said et al., 2017; Shrestha et al., 2017).

The aim of this study was to assess possible antibacterial effects of an ethanolic extract derived from *Rhododendron myrtifolium* leaves against *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* strain locally isolated from human biological fluids. Thus, the study contributes to on-going investigations on the bioactivity potential of plant species such as the *Rhododendron*. Hence, the effects of *Rhododendron myrtifolium* leaf extract against bacterial strains were monitored *in vitro* by the disk diffusion method.

Materials and methodology

Collection of Plant Materials

Leaves of *Rhododendron myrtifolium* were harvested on the side of the road between the Menchul valley and Rogneska valley (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 28.4“, E 24° 20‘ 05.6“, 1,485 m a.s.l.) (Figure 1). Plant samples were thoroughly washed to remove all the attached materials and used to prepare the ethanolic extract.

Preparation of Plant Extracts

Freshly leaves were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1 : 19, w/w) at room temperature. The extract was then filtered and investigated for antimicrobial activity.

Antimicrobial susceptibility testing

Non-repetitive clinical strains of isolated from biological materials infected patients were collected from Koszalin Hospital during March-April, 2019. The purity, as well as the identity of isolate, was confirmed in the laboratory conditions by standard microbiological methods and were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (2014).



Figure 1 *Rhododendron myrtifolium* plant (Photo: Viktor Nachychko, Yevhen Sosnovsky)

First, the samples were examined by a microscopic Gram stain examination. Samples with Gram-negative results were inoculated on plates of nutrient agar, Clede agar, MacConkey's, and blood agar (Merck, Germany) and then incubated at 37 °C for 24 hours. The colony that showed fermenting of lactose on MacConkey agar and Cled agar media were purified and identified according to their morphology as circular, rose-pink to red colonies on MacConkey agar medium and yellow colonies on Cled agar. The isolates were identified by some biochemical reactions (e.g. catalase enzyme, potassium hydroxide test, Indole and methyl red test, Voges Proskauer reaction, urease and citrate, H2S and oxidase test).

Susceptibility testing of the isolates was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI, 2014). The following antimicrobial agents were used (μg): amikacin (30), ampicillin (10), aztreonam (30), ceftazidime (30), ciprofloxacin (5), cephalexin (30), gentamicin (10), erythromycin (15), imipenem (10), meropenem (10), clindamycin (2), aztreonam (30), norfloxacin (10), oxytetracycline (30), oxacillin (1), enrofloxacin (5), tetracycline (30), amoxicillin (25), piperacillin (100), piperacillin-tazobactam (100/10), tobramycin (10), ceftazidime (30), chloramphenicol (30), doxycycline (30), cefadroxil (30), pefloxacin (5), and cefepime (30). MIC was determined by E-test strips (according to manufacturer's instruction). The resistance breakpoints were the same as the ones defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2000).

Phenotypic Disc Confirmatory Test was performed as recommended by the CLSI (Clinical and Laboratory Standards Institute, 2014). The antibacterial susceptibility profile of the isolates revealed that many isolated strains were classified as multi-drug resistant (MDR) bacteria.

For the current study, four bacterial strains were used for the study of susceptibility or resistance of bacteria to the phytochemicals:

- Isolate 1 – *Citrobacter freundii* strain was susceptible to all antibiotics used;
- Isolate 2 – *Enterobacter cloacae* strain was resistant to the amoxicillin, cefuroxime, trimethoprim-sulphamethoxazole, and cefotaxime;
- Isolate 3 – *Klebsiella pneumoniae* was resistant to piperacillin-tazobactam (100/10 μg), gentamicin (10 μg), tobramycin (10 μg), and ciprofloxacin (5 μg);
- Isolate 4 – *Escherichia coli*, not β-lactamase (ESBL)-producing strain, was a sensitive strain to antibiotics tested.

Bacterial Growth Inhibition Test by the Disk Diffusion Method

Strains tested were plated on TSA medium (Tryptone Soy Agar) and incubated for 24 hr at 25 °C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. Muller-Hinton agar plates were inoculated with 200 μl of standardized inoculum (10^8 CFU/mL) of the bacterium and spread with sterile swabs. Sterile filter paper discs impregnated by sample were applied over each of the culture plates, 15 min after bacteria suspension was placed. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) (Bauer et al., 1966). A negative control disc impregnated by sterile ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hr at 37 °C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks.

The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated six times.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica8.0 software (StatSoft, Poland). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

The ability of the ethanolic extract obtained from leaves of *R. myrtifolium* to inhibit *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* growth was determined in this study. The results revealed that extract exerts antibacterial activity against *Citrobacter freundii*. However, the *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* were resistant to *R. myrtifolium* leaf extract. Maximum *in vitro* inhibition was scored against *Citrobacter freundii*, followed by *Escherichia coli*, *Klebsiella*

pneumoniae, and *Enterobacter cloacae*, which presented inhibition zones of (12.1 ±0.9) mm, (9.1 ±0.5) mm, (7.5 ±0.6) mm, and (7.2 ±0.5) mm, respectively. In the case of the positive controls, 96% ethanol possesses a mild antibacterial effect, which presented inhibition zones of (6.5 ±0.7) mm (Figure 2).

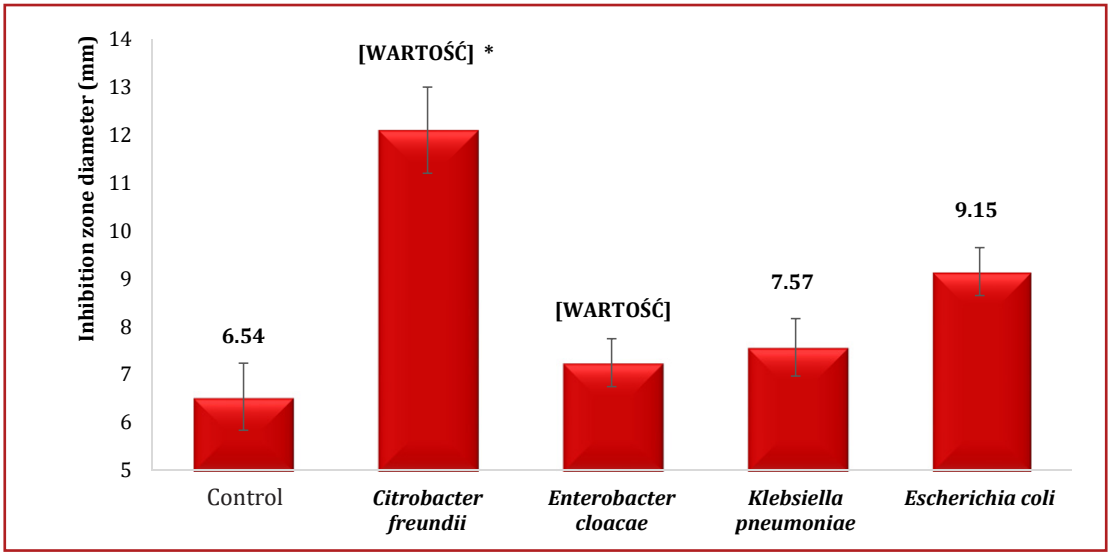


Figure 2 The mean of inhibition zone diameters of the ethanolic extract obtained from leaves of *Rhododendron myrtifolium* against *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* strains locally isolated (M ±m, $n = 6$)
* – the changes are statistically significant ($p < 0.05$) compared to the control group (96% ethanol)

Detailed data regarding the zones of inhibition by the ethanolic extract obtained from leaves of *R. myrtifolium* against *Citrobacter freundii* and *Enterobacter cloacae* were recorded and presented in Figure 2.

In line with the growing interest in the antibacterial potential of different plants, we examined the antibacterial properties of the ethanolic extract obtained from *R. myrtifolium* against *Rhododendron myrtifolium* leaves against *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* strains. The results from the screening study performed by the disc diffusion method revealed that *R. myrtifolium* possesses a mild antibacterial activity against *C. freundii* (Figure 2, 3A).

The results of this study are consistent with other studies that have previously been reported that extracts of *Rhododendron* leaves have antibacterial activity against resistant bacteria strains. Crude extracts of *Rhododendron* leaves were tested for their antibacterial activity using agar diffusion and minimum inhibitory concentration assays and results were presented in the study of Hakeem Said et al. (2017). The antibacterial activity of *R. collettianum* Aitch. & Hemsl. was compared to a series of inactive extracts. Three metabolites were found to distinguish *R. collettianum* from other species indicating the ability to suggest potential bioactive substances (Hakeem Said et al., 2017).



Figure 3 Antimicrobial activity of ethanolic extract obtained from leaves of *R. myrtifolium* against *Citrobacter freundii* (A) and *Enterobacter cloacae* (B) measured as inhibition zone diameter

The polyphenolic profile of fruits, flowers, and leaves of different ages of *Rhododendron ambiguum* Hemsl. and *Rhododendron cinnabarinum* Hook. f. was studied by Shrestha et al. (2017). Fifty-nine different polyphenols including isomers were identified in these species. The leaves and fruits contained more polyphenols than the flowers. Also, the antibacterial activity of these parts (leaves, fruits, and flowers) against gram-positive bacteria was studied. There was no bioactivity observed for crude extracts of all samples against *Escherichia coli*. Against Gram-positive bacteria, the bioactivity of *R. ambiguum* ranged between 0.5 and 0.7 cm, while for *R. cinnabarinum* between 0.5 and 0.8 cm. Antibacterial effects of fruit and leaf extracts were in the same order of magnitude. However, there was a reduced antibacterial activity observed for the flowers of *R. cinnabarinum* and *R. ambiguum*. This could be due to the evolutionary aspect as flowers have a short blooming period in a year compared to the leaves and fruits. In general, *B. thioparus* was the most sensitive bacteria species towards the plant parts for both *Rhododendron* species (Shrestha et al., 2017).

A higher antibacterial effect of *Rhododendron* species against Gram-positive and higher effect for *R. cinnabarinum* was demonstrated in the antibacterial screening by Rezk et al. (2015a). *Erwinia amylovora* 1189 (wild type), *Escherichia coli* TG1 (wild type), *Pseudomonas syringae* DC3000 (wild type) as well as the respective mutants with deletions in acrAB, tolC, or mexAB. Additionally, knockout mutants of *E. amylovora* and *E. coli* with deletions in both, acrAB and tolC were subjected to the antimicrobial analysis. The leaf extracts of 17 *Rhododendron* species exhibited significant growth-inhibiting activities against Gram-positive bacteria. In contrast, only very few of the leaf extracts affected the growth of Gram-negative bacteria. Five out of the 120 *Rhododendron* leaf extracts showed no growth inhibitory activity against any of the Gram-positive tester organisms: *R. elliottii* Watt ex Brandis, *R. hylaeum* Balfour & Farrer, *R. ponticum* L., *R. keiskei* Miquel, and *R. eriocarpum* (Hayata) Nakai. However, crude extracts

obtained from 38 other *Rhododendron* species exhibited moderate antimicrobial activity against 12 out of the 26 tested bacterial species. The crude extracts derived from the remaining 77 *Rhododendron* species showed significant bioactivities against at least one of the 26 tester organisms. The spectrum of microbial susceptibilities towards *Rhododendron* extracts varied widely and showed dramatic differences, i.e. some of the tester organisms were susceptible to leaf extracts from most of the *Rhododendron* species while other bacterial tester organisms were susceptible towards very few *Rhododendron* leaf extracts. *Bacillus thioparus* was the most sensitive tester species susceptible to all 77 potentially bioactive *Rhododendron* leaf extracts. The other tested Gram-positive bacteria showed similar susceptibility only towards leaf extracts derived from the 17 most bioactive *Rhododendron* species. All leaf extracts with antimicrobial bioactivity were extracted from representatives of the subgenus *Rhododendron*, with 15 from the sub-section *Rhododendron* and two belonging to the section *Pogonanthum*. The use of bacterial multidrug efflux pump mutants revealed remarkable differences in the susceptibility towards *Rhododendron* leaf extract treatment. In contrast, susceptibilities of the tested Gram-negative bacterial strains were classified into either low or moderate extent. Only one Gram-negative species, *Sinorhizobium meliloti*, belonging to the order of α-proteobacteria exhibited susceptibility to most of the bioactive *Rhododendron* extracts and was therefore similar in its susceptibility to the majority of Gram-positive bacteria. The results of Rezk et al. (2015a) suggested that common genetic traits are responsible for the production of bioactive secondary metabolite(s) which act primarily on Gram-positive organisms, and which may affect Gram-negative bacteria independence of the activity of multidrug efflux pumps in their cell envelope. In other studies, Rezk et al. (2015b) have assessed the cytotoxicity exerted by leaf extracts from plants of the genus *Rhododendron* towards epidermal keratinocytes and intestine epithelial cells. Different concentrations of DMSO-dissolved remnants of crude methanol *Rhododendron* leaf extracts were incubated for 24 h with cultured epidermal keratinocytes (human HaCaT cell line) and epithelial cells of the intestinal mucosa (rat IEC6 cell line) and tested for their cytotoxic potential. In particular, the cytotoxic potencies of the compounds contained in antimicrobial *Rhododendron* leaf extracts were assessed by quantifying their effects on plasma membrane integrity, cell viability and proliferation rates, cellular metabolism, cytoskeletal architecture, and determining initiation of cell death pathways by morphological and biochemical means. Extracts of almost all *Rhododendron* species, when applied at 500 µg/mL, were potent in negatively affecting both keratinocytes and intestine epithelial cells, except material from *R. hippophaeoides* var. *hippophaeoides*. Extracts of *R. minus* and *R. racemosum* were non-toxic towards both mammalian cell types when used at 50 µg/mL, which was equivalent to their minimal inhibitory concentration against bacteria. At this concentration, leaf extracts from three other highly potent antimicrobial *Rhododendron* species proved non-cytotoxic against one or the other mammalian cell type: Extracts of *R. ferrugineum* were non-toxic towards IEC6 cells, and extracts of *R. rubiginosum*, as well as *R. concinnum*, did not affect HaCaT cells. In general, keratinocytes proved more resistant than intestine epithelial cells against the treatment with compounds contained in *Rhododendron* leaf extracts (Rezk et al., 2015b).

The essential oil of *Rhododendron anthopogon* was investigated by Innocenti et al. (2010). The anti-microbial activity of *R. anthopogon* essential oil was evaluated by reference microdilution assays against a series of Gram-positive and Gram-negative reference strains. Gram-positive

and Gram-negative bacteria: *Staphylococcus aureus* (ATCC 20202), *Enterococcus fecalis* (ATCC 29216), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27753) were evaluated to measure the anti-bacterial activity; a total of six strains of *Candida albicans* and nine strains of *Candida* spp., all clinical isolates, were selected to test the antifungal activity. Furthermore, *Mycobacterium tuberculosis* reference strain H37Rv was employed to determine the anti-tubercular activity. Although topical anti-inflammatory activity was obtained only at very high concentrations, a remarkable anti-microbial activity was detected against *B. subtilis* and *M. tuberculosis*. Moreover, the *R. anthopogon* essential oil inhibited most clinical strains of *Candida* spp. at doses comparable with reference antifungal drugs and the strongest activity was found against a clinical isolate of *C. pseudotropicalis*. Moreover, the oil was able to reduce cancer cell growth independently of the cell line and the treatment protocols used (Innocenti et al., 2010).

The properties of *Rhododendron* species which inhibit bacterial growth are mainly related to their chemical components including phenolic compounds, especially flavonoids, essential oils, procyanidins, chromones, terpenoids, and steroids against various Gram-positive and Gram-negative bacteria. It has many biological properties such as antioxidant, anti-inflammatory, antiviral, antibacterial, anticancer, antidiabetic, immunomodulatory, cardioprotective and hepatoprotective among others due to their polyphenolic constituents (Demir et al., 2016).

The biological activities of major procyanidins isolated from the leaf extract of *R. formosanum* Hemsl. were investigated by Wang et al. (2015). Four compounds, including two procyanidin dimers, procyanidin A1 (1) and B3 (2), and two procyanidin trimmers, procyanidin C4 (4) and cinnamtannin D1 (5), were isolated and identified on the basis of spectroscopic data. The structure of a new procyanidin dimer, rhodonidin A (3), was elucidated by 2D-NMR, CD spectrum, and MS. The procyanidin trimmers and rhodonidin A are reported for the first time in Ericaceae. The biological activities of these procyanidins were evaluated using anti-bacterial and anti-oxidative assays. Only the new compound 3 demonstrated strong antibacterial activity against *Staphylococcus aureus* at a MIC value of 4 μg/mL. All compounds showed pronounced antioxidant activities and the activities are enhanced as the amount of OH groups in procyanidins increased (Wang et al., 2015).

The proanthocyanidins of the leaves of 16 taxa of the *Rhododendron* genus [*Rhododendron catawbiense* Michx. cv. *Grandiflorum*, *Rhododendron* ‚Cunningham‘s White‘ (syn. *Rhododendron caucasicum* Pall. × *Rhododendron ponticum* L.), *Rhododendron smirnowii* Trautv., *Rhododendron calophytum* Franch., *Rhododendron dichroanthum* ssp. *scyphocalyx* (Balf. f. & Forrest) Cowan, *Rhododendron micranthum* Turcz., *Rhododendron praevernum* Hutch., *Rhododendron ungernii* Trautv., *Rhododendron kaempferi* Planch., *Rhododendron degronianum* ssp. *heptamerum* var. *hondoense* (Nakai) H. Hara, *Rhododendron fortunei* Lindl., *Rhododendron ponticum* L., *Rhododendron galactinum* Balf. f. ex Tagg., *Rhododendron oreotrephes* W. W. Sm., *Rhododendron brachycarpum* ssp. *brachycarpum* D. Don ex G. Don, and *Rhododendron insigne* Hemsl. & E. H. Wilson] were investigated qualitatively by liquid chromatography-mass spectrometry by Jaiswal et al. (2012). Four caffeoylquinic acids, six p-coumaroylquinic acids, epigallocatechin, gallocatechin, catechin, epicatechin, epigallocatechin gallate, catechin gallate, epicatechin gallate, gallocatechin gallate, two quercetin-O-hexosides, quercetin-O-galloyl-hexoside, quercetin-O-pentoside,

quercetin-O-rhamnoside, quercetin-O-pentoside-O-hexoside, quercetin-O-rhamnoside-O-hexoside, quercetin-O-feruloyl-hexoside, quercetin-O-(p-hydroxy)benzoyl-hexoside, taxifolin-O-pentoside, myricetin-O-rhamnoside, two myricetin-O-pentosides, three myricetin-O-hexosides, and two myricetin-O-galloyl-hexosides were detected (Jaiswal et al., 2012).

The seven flavanones were isolated from the aerial parts of *Rhododendron hainanense* Merr. and were tested for their antimicrobial activities against six bacteria and six plant pathogenic fungi by Li et al. (2016). Within the series of flavanones tested, farrerol (1) displayed moderate antibacterial activities against *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Erwinia carotovora*, with MICs ranging from 15.6 to 125 μg/mL. Furthermore, farrerol (1) exhibited excellent inhibitory activities against six plant pathogenic fungi: *Fusarium oxysporum* sp. *niveum*, *Colletotrichum gloeosporioides*, *Penicillium italicum*, *Rhizoctonia solani*, *Fusarium oxysporum* sp. *cubenserace* and *Phytophthora melonis*, with EC50 values of 9, 18, 35, 39, 46 and 66 μg/mL, respectively (Li et al., 2016).

The antioxidant properties and cytotoxic activity of species belonging to the *Rhododendron* genus were also studied. For example, Demir et al. (2016) have evaluated the antioxidant properties and cytotoxic activity of the extract of flowers of *Rhododendron luteum* Sweet on three cancers (human breast, colon, and liver carcinoma) and human foreskin fibroblast cells for the first time. *R. luteum* extract exhibited selective cytotoxicity against colon and liver cancer cells compared to normal fibroblast cells, while this selective cytotoxicity was not observed in breast cancer cells (Demir et al., 2016). The essential oil of *Rhododendron anthopogon* D. Don. showed marginal antibacterial and cytotoxic activities against MCF-7, MDA-MB-231, and 5637, but no antifungal effects (Dosoky et al., 2016).

Fifty *Rhododendron* honey samples obtained from the Black Sea Region of Turkey were also screened for total phenolic content and for potential antioxidant activity by Silici et al. (2010). The antimicrobial activity was studied by using eleven bacteria and two yeasts. The 13 microorganisms containing eleven bacteria and two yeasts were used as test organisms: *Aeromonas hydrophila* ATCC 7965, *Bacillus cereus* FMC 19, *Bacillus subtilis* ATCC 6630, *Escherichia coli* O157:H7 RS_932, *Listeria monocytogenes* 1/2B, *Mycobacterium smegmatis* RUT, *P. mirabilis* BC_3624, *P. aeruginosa* ATCC 27853, *Salmonella typhimurium* NRRLE_4463, *Staphylococcus aureus* ATCC 28213, *Candida albicans* ATCC 1223, *Saccharomyces cerevisiae* BC 5461, and *Yersinia enterocolitica* ATCC 1501. The honey samples showed the highest antimicrobial activity against *P. aeruginosa* and *P. mirabilis*. In addition, *S. aureus*, *A. hydrophila*, *L. monocytogenes*, *B. subtilis*, *M. smegmatis*, and *S. typhimurium* were moderately sensitive to the antimicrobial activity of *Rhododendron* honey. However *B. cereus*, *E. coli* O157:H7, and *Y. enterocolitica* were the most resistant microorganisms. In addition, honey samples also had no inhibitory effects on two yeasts; *C. albicans* and *S. cerevisiae* (Silici et al., 2010).

Conclusions

The results revealed that extract exerts antibacterial activity against *Citrobacter freundii*. However, the *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* were resistant to *R. myrtifolium* leaf extract. Maximum *in vitro* inhibition was scored against *Citrobacter freundii*, followed by *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. It can be

concluded that *Rhododendron myrtifolium* leaves extract can be as complementary medicine in treating diseases caused by multidrug-resistant strains of *Citrobacter freundii*. However, further investigation is needed to determine the bioavailability of the active compounds and to determine the dose and toxicity before it can be used as therapeutic agents.

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ESTIMATION OF PHOTOSYNTHETIC PIGMENTS IN THE LEAVES OF *CRAMBE* SPP. DURING VEGETATION

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This study investigated the content of chlorophyll *a* (Chl *a*), b (Chl *b*), carotenoids (car) and their ratio in four species of *Crambe* L. during vegetation: *C. cordifolia* Steven, *C. koktebelica* (Junge) N.Busch, *C. maritima* L., *C. steveniana* Rupr. collected in the M.M. Gryshko National Botanical Garden of the NAS of Ukraine (Kyjiv). The middle height increment of investigated plants between stages of growth is measured. The concentration of chlorophylls *a*, *b* and *car* measured spectrophotometrically on UNICO 2800 at the wavelength 662, 644 and 440, respectively. Extraction procedure conducted in acetone. Obtained results are given in mg/g of fresh mass. The height increment in a period of spring vegetation-budding was 26.3–80.2 cm, budding-flowering 12.3–136.0 cm, flowering-fruitage 1.3–16.6 cm depending on species. *C. cordifolia* had the most value of this parameter at the budding-flowering period and *C. maritima* had the least value at the flowering-fruitage period. Content of Chl *a* at the spring vegetation period was 0.321–0.490 mg/g, at the budding period 0.237–0.680 mg/g, at the flowering period 0.349–0.680 mg/g and at the fruitage 0.478–0.962 mg/g depending on species. Content of Chl *b* was 0.133–0.203 mg/g at the spring vegetation stage, 0.158–0.275 mg/g at the budding stage, 0.158–0.275 mg/g at the flowering stage and 0.227–0.439 mg/g at the fruitage depending on species. The concentration of *car* was 0.038–0.078 mg/g at the spring vegetation period, 0.043–0.104 mg/g at the budding period, 0.070–0.201 mg/g at the flowering period and 0.117–0.435 mg/g at the fruitage depending on species. Correlation between the content of Chl *a* and increment of plants was strong at the spring vegetation (r = 0.78), moderate at the budding period (r = 0.52) and strong at the flowering (r = 0.76). At the flowering stage found a very strong correlation (r = 0.90) between Chl *b* content and increment of plants. Thus, obtained data showed that between the accumulation of photosynthetic pigments of investigated plants and increment of plants exists a positive correlation, which depends on the period of growth. Further, it's can be used for the study of total productivity and some grow parameters of these plants.

Keywords: *Crambe*, chlorophylls, carotenoids

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Introduction

Plants of the *Crambe* L. genus have studied in the Department of Cultural Flora of M.M. Gryshko National Botanical Garden. Investigation of the biochemical composition of underground parts of these plants showed high content of ascorbic acid, dry matter, sugars, etc. (Vergun et al., 2018). These plants also a good source of nutrients in the above-ground parts (Vergun et al., 2019). Some of these crops have industrial and pharmaceutical importance due to the content of oil in seeds and biologically active compounds, among which different groups of phenolic compounds (Bukhari et al., 2013). Also, these plants tested as forage and medicinal crops, on the other hand, selected species of this genus listed in the Red Book of Ukraine (Kalista, 2017).

The study of raw of these plants demonstrated different biological activities such as antioxidant, antimicrobial, cytotoxic, allelopathic, etc. (Razavi et al., 2009; Bukhari et al., 2013; Rashid et al., 2018; Kalista et al., 2019). One is the most important species of this genus is *C. abyssinica* that can produce oil with rich content of fatty acids (Bondioli et al., 1998). Oil from this species used for industrial products, such as industrial lubricants, corrosion inhibitors, etc. (Jasper et al., 2010). They also prospective candidates for use in biodiesel production (Silva et al., 2012).

Ecological aspects of *C. abyssinica* study showed results of the effect of different levels of soil salinity on growth parameters. As a result, this plant can be recommended as an alternative source of green biomass even under some levels of salinity (Ionov et al., 2013; Vasconcelos et al., 2015).

The aim of this study was to determine the accumulation of photosynthetic pigments in *Crambe* spp. leaves during vegetation. As reported by Dey et al. (2016), an important parameter of crop health is the leaf chlorophylls measurements. Plant pigments are necessary for photosynthesis and content of them can provide information about the physiological status of plants (Zhou et al., 2019).

Materials and methodology

Biological material

In this study leaves of four species of *Crambe* were evaluated: *C. cordifolia* Steven, *C. koktebelica* (Junge) N.Busch, *C. maritima* L., *C. steveniana* Rupr. Leaves collected from the experimental collection of the M.M. Gryshko National Botanical Garden of the NAS of Ukraine (Kyiv) during vegetation. Every two weeks was measured height of plants and during vegetation conducted phenological properties. Increment of plants measured at the stage of spring vegetation, budding, flowering and fruitage.

Determination of photosynthetic pigments

0.1 g of fresh leaves mass homogenized with 10–20 ml of 100% acetone and 0.5 mg $MgCO_3$ powder was added. The extract was then centrifuged at 2,500 rpm for 5 min. The measurement of pigments was conducted spectrophotometrically: chlorophyll *a* (Chl *a*) at the wavelength 662 nm, chlorophyll *b* (Chl *b*) at the wavelength 640 nm and carotenoids (*Car*) at

the wavelength 440 nm. For the measuring used 2,800 Uv/VIS Spectrophotometer (Unico). The results expressed in mg/g of fresh weight. The concentration of chlorophyll *a*, chlorophyll *b* and carotenoids were calculated using the equation proposed by Holm-Wettstein (Musienko et al., 2001).

Statistical analysis

Basic statistical analyses were performed using Excel 2016. Average and standard deviation given in Figure 1–5. Correlation coefficients were calculated by CORR analysis.

Results and discussion

Ecological aspects of plant study basically related to the conditions of growth. One of the most important ecological factors in plant life is light (Lichtenthaler, 1987).

Plant height in crops is generally considered to be a quantitative trait controlled by multiple loci and significantly affected by environmental factors (Chai et al., 2019). In our study, we used to measure the height of plants, increment and content of chlorophylls in leaves dynamically. The increment of plants during vegetation was measured and results represented in Figure 1. This parameter of investigated plants in the period of spring vegetation was from 16.3 to 26.6 cm, in the budding stage from 26.3 to 80.2 cm, in the flowering stage from 12.3 to 136.0 cm, the fruiting stage from 1.3 to 16.6 cm depending on species.

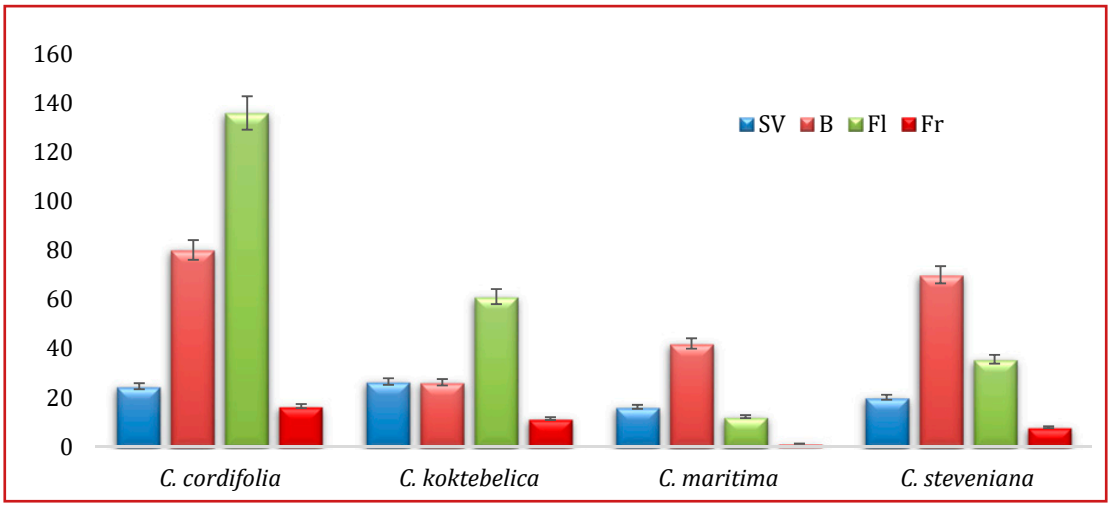


Figure 1 Increment of plants of *Crambe* L. species during vegetation(cm)
SV – spring vegetation; B – budding; Fl – flowering; Fr – fruitage

Investigation of leaf chlorophyll one of the most important vegetation parameter and is an indicator of photosynthetic activity (Lichtenthaler, 1987; Carmona et al., 2015). Chlorophyll responsible for green color and conversation of light into chemical energy and mainly determines the photosynthetic rate and primary productivity (Li et al., 2019). The most known method of investigation of chlorophylls and carotenoids content is conducted

spectrophotometrically (Lichenthaler, 1987). However, nowadays exist special equipment (portable chlorophyll meter) for measuring chlorophylls in field conditions is very simple and allows them to obtain results immediately (Dey et al., 2016; Kaspary et al., 2019).

Photosynthetic pigments are a group of nutrients that can demonstrate an antioxidant property (Zawiślak and Nurzyńska-Wierdak, 2014). Chlorophyll *a* and *b* associated with the light-harvesting process and are an indicator of plant health (Gogoi and Basumatary, 2018). Usually content of chlorophylls studies with the influence of different ecological factors (Li et al., 2019). The study of chlorophyll content can be used for vegetation health monitoring, forage quality assessment, biomass estimation, productivity measures, etc. (Hallik et al., 2017).

Content of Chl *a* in the stage of spring vegetation found from 0.32 to 0.49 mg/g FW, in the stage of budding from 0.24 to 0.68 mg/g FW, in the stage of flowering from 0.35 to 0.68 mg/g FW, and in stage of fruiting from 0.48 to 0.96 mg/g FW depending on species (Figure 2). It should be noted that in this period accumulation of Chl *a* in the leaves during vegetation for investigated species was uneven.

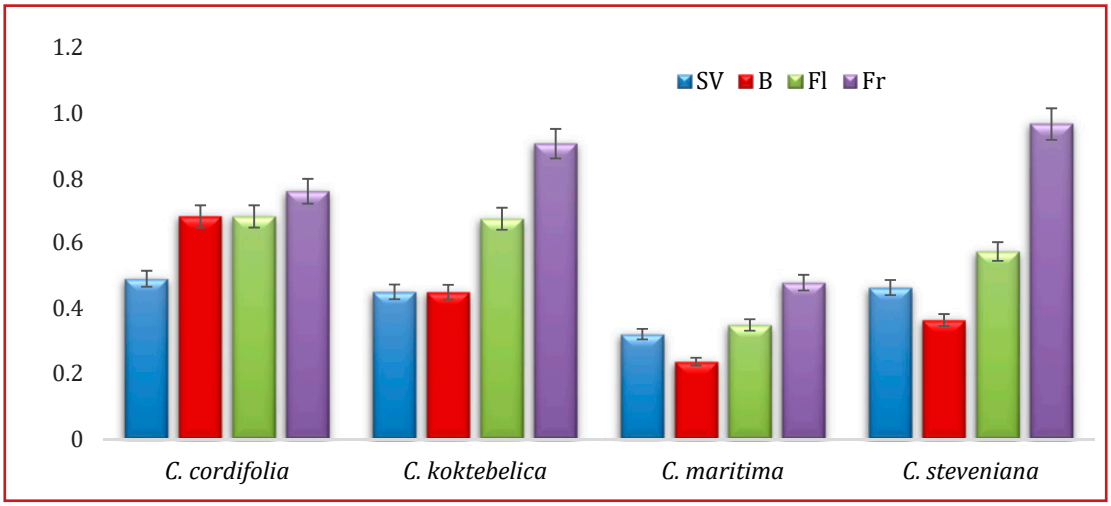


Figure 2 Content of chlorophyll *a* in the leaves of *Crambe* L. species during vegetation (mg/g) FW. SV – spring vegetation; B – budding; Fl – flowering; Fr – fruitage

Chlorophyll content changing with the change of the external environment and definite as an important diagnostic indicator for plant growth study (Li et al., 2019). Content of chlorophyll *b* in the leaves of *Crambe* in a period of spring vegetation was from 0.13 to 0.20 mg/g FW, in the period of budding from 0.16 to 0.28 mg/g FW, in the period of flowering from 0.15 to 0.27 mg/g FW, and fruitage from 0.28 to 0.44 mg/g FW (Figure 3). Content of chlorophylls decrease more rapidly than carotenoids if plants in the stress conditions or during leaf senescence (Gitelson and Merzlyak, 1994; Peñuelas et al., 1995; Filella et al., 2009).

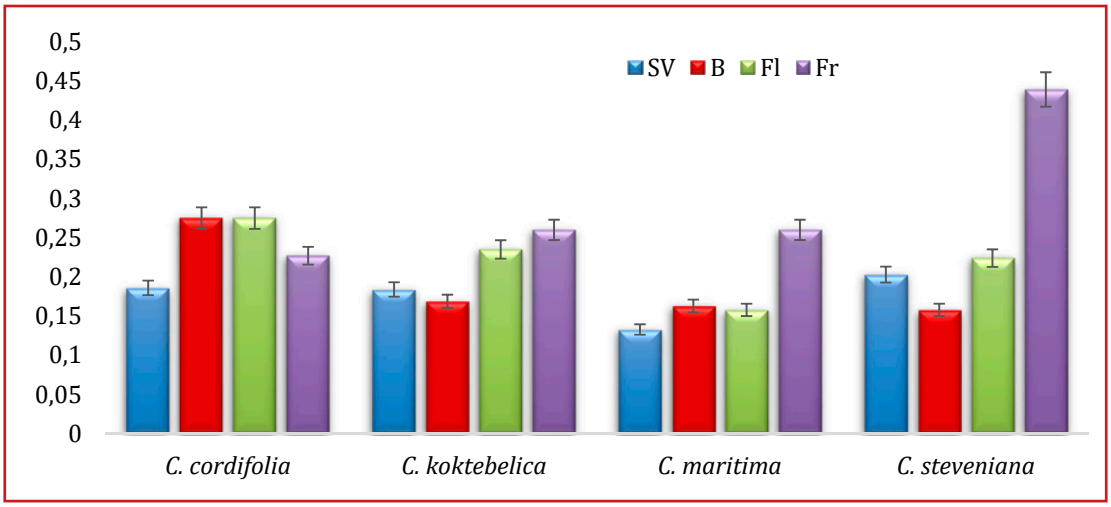


Figure 3 Content of chlorophyll *b* in the leaves of *Crambe* L. species during vegetation (mg/g)
FW. SV – spring vegetation; B – budding; Fl – flowering; Fr – fruitage

Carotenoids are involved in various defense mechanisms. They can inhibit the formation of free radicals preventing their negative effect on the organism. Carotenoids characterized as photoprotective agents with the ability to reduce sunburns, photo-allergy and some kinds of cancer (Veeru, 2009). This is mainly related to the yellow color during chlorophyll degradation (Li et al., 2019). In the study of Ivanišova et al. (2017), total carotenoid content and antioxidant activity of plant extracts by the phosphomolybdenum method had a strong correlation. Content of carotenoids in the stage of spring vegetation was from 0.04 to 0.10 mg/g FW, budding stage from 0.04 to 0.10 mg/g FW, flowering stage from 0.07 to 0.20 mg/g FW, fruitage stage 0.12 to 0.44 mg/g FW (Figure 4).

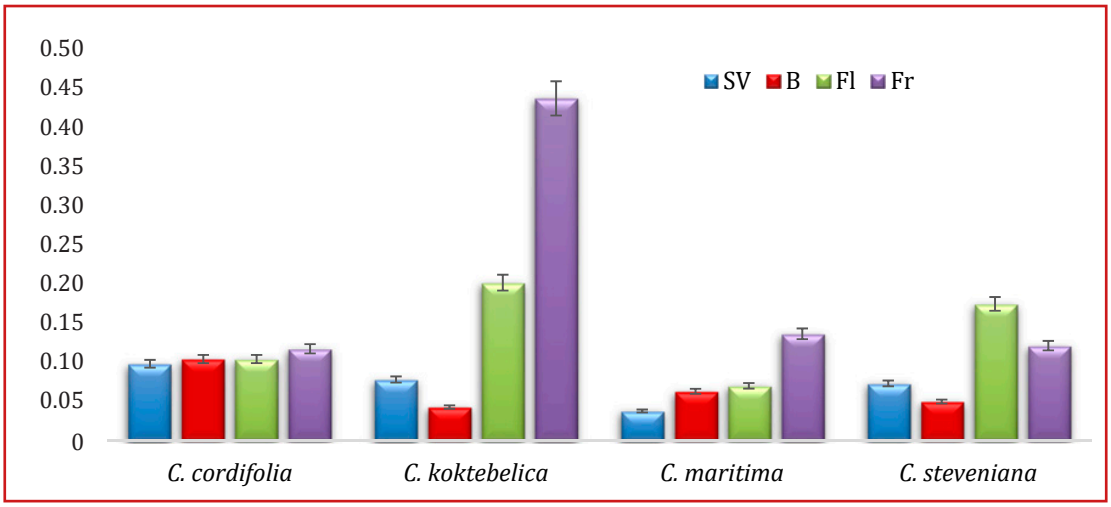


Figure 4 Content of carotenoids in the leaves of *Crambe* L. species during vegetation (mg/g)
FW. SV – spring vegetation; B – budding; Fl – flowering; Fr – fruitage

Any stress effects on regulation processes in plant organisms and photosynthetic properties. This can be noticed in the relation between carotenoids and chlorophylls. Carotenoids/chlorophylls ratio, in this case, increased (Filella, 2002). As reported before, the knowledge of the proportion between chlorophylls and carotenoids in leaves can provide more information for indicating plant physiological status (Zhou et al., 2019). According to Gogoi and Basumatary (2018), chlorophyll a/*b* (Chl *a*/*b*) ratio indicates the developmental state of the photosynthetic apparatus in plant organisms and connected with plant growth. The Chl a/b ratio in this study was from 2.28 to 2.64 in the period of spring vegetation, from 1.45 to 2.65 in the period of budding, from 2.28 to 2.87 in the period of flowering, from 1.84 to 3.50 (Figure 5). As reported by Anderson (1986), the typical Chl a/b ratio for shade plants is 1.6–2.2 and light plants 2.6–3.4.

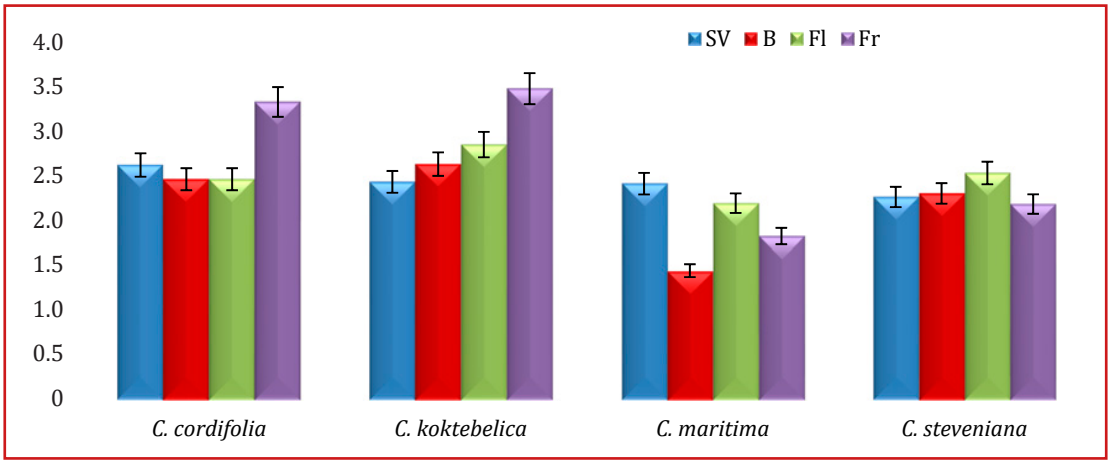


Figure 5 Relation between the content of chlorophyll *a* and chlorophyll *b* in the leaves of *Crambe* L. species
SV – spring vegetation; B – budding; Fl – flowering; Fr – fruitage

Correlation analysis of obtained results showed very strong, strong, moderate, etc., relations between investigated parameters (Table 1).

It should be noted that very strong correlation at the period of spring vegetation found between the content of *car* and increment of plants and between all investigated pigments. At the budding stage determined very strong correlation between Chl *a* and Chl *b* and between Chl *b* and car. At the flowering stage, very strong correlation found between chlorophylls content, increment of plants and Chl *b*, and between ratio of chlorophylls and car. At the fruitage period found strong correlation between increment of plants and chlorophylls ratio that had the highest value during vegetation.

Table 1 Coefficient of correlation between increment of plants and accumulation of pigments during vegetation

Characters	Increment	Chl *a*	Chl *b*	*Car*
Spring vegetation				
Chl *a*	0.782	1		
Chl *b*	0.620**	0.929**	1	
Car	0.836**	0.962	0.794**	1
Chl *a*/*b*	0.455	0.239**	-0.136*	0.492*
Budding				
Chl *a*	0.521*	1		
Chl *b*	0.625	0.898	1	
Car	0.694**	0.701**	0.940**	1
Chl *a*/*b*	0.130	0.719**	0.341**	0.014
Flowering				
Chl *a*	0.762*	1		
Chl *b*	0.901	0947**	1	
Car	-0.013*	0.636	0.389*	1
Chl *a*/*b*	0.260	0.797**	0.564	0.923
Fruitage				
Chl *a*	0.548	1		
Chl *b*	-0.259**	0.547	1	
Car	0.175**	0.353**	-0.261	1
Chl *a*/*b*	0.880	0.470	-0.478**	0.592

Note: Significant correlations are indicated by $^{**}p < 0.01$ and $^{*}p < 0.05$

Conclusions

Obtained data showed that maximal values of chlorophyll *a* found at the stage of fruitage for all investigated species. Minimal values of it found at the stage of spring vegetation for *C. cordifolia* and *C. koktebelica*, and at the stage of budding for *C. maritima*, *C. steveniana*. The least value of chlorophyll *a*/*b* ratio found for *C. maritima* at the stage of budding indicates some changes in the photosynthetic apparatus.

It is found a correlation between the content of chlorophyll *a* and the growth of the plant in the start of vegetation between the process of growth and accumulation of photosynthetic pigments exists the correlation. Very strong and strong correlation found between increment and *car* content at the spring vegetation and budding period, respectively. Very strong correlation identified between the increment of plants and the ratio of chlorophylls at the end of vegetation.

Thus, the obtained results showed that a positive correlation between the accumulation of photosynthetic pigments of investigated plants and the increment of plants depends on the

period of growth. Taking into account fewer reviews about pigment status of *Crambe* spp., obtained data can be used for the study of total productivity and some grow parameters of these plants.

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OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTES AFTER *IN VITRO* TREATED WITH LEAF EXTRACT OBTAINED FROM *THYMUS* × *PORCII* BORBÁS (LAMIACEAE)

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Antioxidants from natural products are important to protect against free radicals. The literature has documented the antioxidant activity of the *Thymus* genus (Lamiaceae) in inhibiting the formation of free radicals generation. In line with our previous study, we continue to assess the antioxidant potential of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine on equine erythrocytes' model. Therefore, in the present study, the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity (TAC)], as well as HCl-induced hemolysis in the equine erythrocytes, was used for assessing the antioxidant activity of extract obtained from the leaves of *Th.* × *porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*). Leaves of *Th.* × *porcii* were sampled in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv, Ukraine; N 49° 49' 15.1", E 24° 05' 12.5", 348 m a.s.l.). Equine erythrocytes aliquots were used in the study. For positive control (blank), phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, samples were used for the biochemical assays. The aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered after *in vitro* incubation with an extract obtained from leaves of *Th.* × *porcii*. The *Th.* × *porcii* extract caused to increase of TBARS content as a biomarker of lipid peroxidation in the extract-treated erythrocytes, and these results were statistically significant. Total antioxidant capacity was non-significantly increased. When *Th.* × *porcii* extract (5 mg/mL) was added to the erythrocyte suspension, the maximum level of hemolysis occurred after 8.5 min of incubation with 0,1M HCl (17.91 ±1.87%). The total duration of hemolysis after *Th.* × *porcii* extract (5 mg/mL) incubation was 11.5 min. Our results showed that HCl-induced hemolysis in a typical time-dependent manner. the extract obtained from leaves of *Th.* × *porcii* (5 mg/mL) has a mild cytotoxic activity on the equine erythrocytes increasing the level of lipid peroxidation biomarker and hemolysis rate. Investigation of the mechanism of action revealed that this extract has hemolytic activity. Therefore, *Th.* × *porcii* extract at a concentration of 5 mg/mL induced the increase of hemolyzed erythrocytes and caused to decrease in hemolysis duration. Screening of *Thymus*

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species for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

Keywords: *Thymus*, leaf extract, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity, hemolysis

Introduction

In recent years, the use of plants and food supplements containing botanical ingredients, as an alternative therapy for various diseases, has been intensified due to their high content of chemical agents such as polyphenols, i.e. flavonoids, tannins, and alkaloids (Nabavi et al., 2015). The genus *Thymus* consists of approximately 215 species currently recognized (Morales, 2002). These herbaceous perennials and sub-shrubs distributed in Europe, Northwest Africa, Ethiopia, Asia and Greenland (Morales, 2002; Bartolucci et al., 2013). It is one of the most widely used genera in folk medicine, where it is popular for its stimulatory action on all organism functions (Viuda-Martos et al., 2011).

These plants possess a variety of activities including antimicrobial, antioxidant, anti-inflammatory, cytotoxic, analgesic, and anti-diabetic due to the presence of second metabolites, i.e. flavonoids, phenylpropanoids, tannins, organic acids, terpenoids, and phytosterols (Li et al., 2019). Thymol (10–64%) is one of the major constituents of the essential oils of thyme (*Thymus vulgaris* L.), a medicinal plant with several therapeutic properties (Salehi et al., 2018). These multiple therapeutic effects of thymol are largely attributed to its anti-inflammatory (*via* inhibiting recruitment of cytokines and chemokines), antioxidant (*via* scavenging of free radicals, enhancing the endogenous enzymatic and non-enzymatic antioxidants and chelation of metal ions), antihyperlipidemic (*via* increasing the levels of high density lipoprotein cholesterol and decreasing the levels of low density lipoprotein cholesterol and low density lipoprotein cholesterol in the circulation and membrane stabilization) (*via* maintaining ionic homeostasis) effects (Nagoor Meeran et al., 2017).

The antimicrobial activity of *Thymus* species, as well as thymol, has been well studied. In our previous study (Honcharenko et al., 2018b, c), the ethanolic extracts obtained from leaves of various *Thymus* representatives exhibited intermediate activity against β-lactamase-producing *Pseudomonas aeruginosa* and *Salmonella enteritidis* strains locally isolated. The effects varied significantly according to the *Thymus* taxa. It should be noted that the most antimicrobial effective plant against β-lactamase-producing *P. aeruginosa* was *Th. alpestris* Tausch ex A. Kern., being highly active with the ethanolic extract (mean diameter of inhibition zone was 12.8 ±0.8 mm). The antibacterial activity of extracts was greatest for *Th. alpestris* followed by *Th. pannonicus* All. followed by *Th. serpyllum* L. and then by *Th. pulegioides* L. (Honcharenko et al., 2018c). The ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *S. enteritidis*. The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* followed by *Th. alpestris*, *Th.* × *porcii*, and then by *Th. serpyllum*. These plant extracts could be a potential source of new antibacterial agents (Honcharenko et al., 2018b).

Thymol also increases the activity of endogenous antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and the level of other non-enzymatic antioxidants such as vitamin C, vitamin E, and reduced glutathione (Nagoor Meeran and Prince, 2012), and thereby the total antioxidant status *in vivo* (Youdim and Deans, 2000). In our previous study (Honcharenko et al., 2018a), we have assessed the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] in the equine erythrocytes after treatment with *Thymus serpyllum* L. extract. Lipid peroxidation biomarker, aldehydic and ketonic derivatives of oxidatively modified proteins, total antioxidant capacity was non-significantly altered after *in vitro* incubation with an extract obtained from *Th. serpyllum*.

In line with our previous study, we continue to assess the antioxidant potential of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine on equine erythrocytes' model. Therefore, in the present study, the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity (TAC)], as well as HCl-induced hemolysis in the equine erythrocytes, was used for assessing the antioxidant activity of extract obtained from the leaves of *Th.* × *porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*).

Materials and methodology

Collection of Plant Materials

Leaves of *Th.* × *porcii* (a hybrid between *Th. pannonicus* and *Th. pulegioides*) were sampled in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv, Ukraine; N 49° 49' 15.1", E 24° 05' 12.5", 348 m a.s.l.) (Figure 1). Identification of this species was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all the attached material and used to prepare the extract.

Preparation of Plant Extract

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. The extract was stored at -20 °C until use.

Horses

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30' 48.0" E 16º 57' 44.9"), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for



Figure 1 *Thymus* × *porcii* plant (Photo: Viktor Nachychko, Vitaliy Honcharenko)

hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control (phosphate buffer) was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes aliquots were used in the study.

2-Thiobarbituric Acid Reactive Substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for

determining the malondialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The µmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of extracts obtained from leaves of *Th.* × *porcii* against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of Total Antioxidant Capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Assay of Acid Resistance of Erythrocytes

The acid resistance of erythrocytes was measured spectrophotometrically with 0.1M HCl (Terskov and Gitelson, 1957). The assay is based on the measuring of the dynamics of erythrocytes disintegration into hemolytic reagent action. The time of hemolytic reagent action serves as the measure of erythrocyte resistance. The assay mixture contained 5 mL of 1% erythrocyte suspension and 0.05 mL of 0.1M HCl. The absorbance was read at 540 nm every 30 seconds after HCl addition till the end of hemolysis. The difference of absorbance at the beginning and at the end of hemolysis was determined as 100% (total hemolysis). The disintegration of erythrocytes (%) at every 30seconds was expressed as a curve.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the values (significance level, p <0.05) was examined using the Mann-Whitney U test (Zar, 1999). All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

After incubation of equine erythrocytes with a leaf extract obtained from *Th.* × *porcii*, the aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered. The *Th.* × *porcii* extract caused to increase of TBARS content as a biomarker of lipid peroxidation in the extract-treated erythrocytes, and these results were statistically significant (by 57.4%, p <0.05). The aldehydic and ketonic derivatives level was non-significantly decreased by 1.8 and 1.7% (p >0.05), respectively (Figure 2).

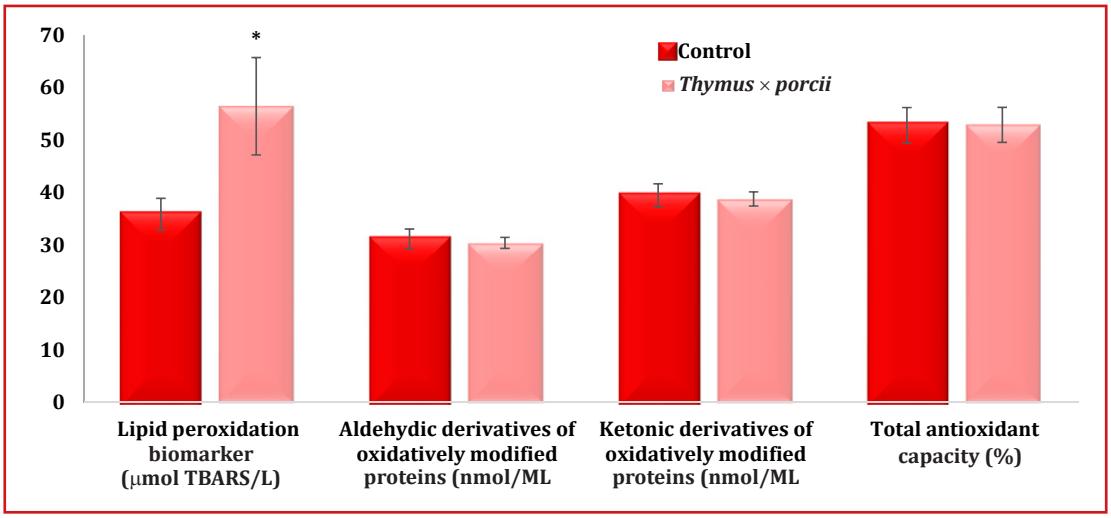


Figure 2 The TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes suspension after *in vitro* incubation with leaf extract obtained from *Thymus* × *porcii* (M ±m, n = 18)

Many *in vitro* studies confirmed the antioxidant properties of thyme extracts. Many results also clearly suggest that treatment by *Thymus* extracts *in vivo* and *in vitro* prevents organ damage *via* protection of the antioxidant defense system and scavenge of hydroxyl free radicals by producing of phenoxyl radicals, major transient species (Nagoor Meeran et al., 2017). For example, the phenolic constituent profiles and the antioxidant, anti-proliferative, neuroprotective, anti-aging and anti-diabetic activities of both *Th. pulegioides* aqueous decoctions (AD) and hydro-ethanolic extracts (HE) were studied by Taghouti et al. (2018). Rosmarinic acid was the main phenolic compound, accounting for 35.2 or 47.8% of total identified phenols in AD or HE, respectively. Furthermore, large amounts of luteolin-O-hexuronide, eriodictyol-O-hexuronide, and chrysoeriol hexoside were found. Both extracts showed significant *in vitro* antioxidant activity and anti-proliferative activity against Caco-2 cells and reduced hepatotoxicity (HepG2 cells). In general, both *Th. pulegioides* extracts showed poor anti-diabetic activity, moderate anti-aging effects and high neuroprotective activity with both AD and HE extracts, at 0.5 mg/mL, showing 80% inhibition of the acetylcholinesterase activity and 94% inhibition of the tyrosinase activity (Taghouti et al., 2018).

Six different assays were employed in study of Kindl et al. (2015) in order to evaluate the antioxidant properties of the ethanolic extracts of selected *Thymus* species growing in Croatia (*Th. longicaulis*, *Th. praecox* subsp. *polytrichus*, *Th. pulegioides*, *Th. serpyllum* subsp. *serpyllum*, *Th. striatus*, and *Th. vulgaris*) as well as elucidate its mode of action. The tested *Thymus* extracts and pure compounds at different concentrations (0.4–25 μg/mL) significantly inhibited DPPH• in a concentration-dependent manner. The activities of plant extracts were 11–28%, 23–52%, and 52–85% at 1.56 μg/mL, 3.13 μg/mL, and 6.25 μg/mL, respectively. At the mentioned concentrations, *Th. serpyllum* subsp. *serpyllum* as well as a commercial sample of *Th. vulgaris* were the least effective (Kindl et al., 2015).

The erythrocytes of mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure (Pagano and Faggio, 2015). Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity (Webster and Toothill, 1987) and red cell damages by free radicals would probably be associated with hemolysis (Farag and Alagawany, 2018). Therefore, the next purpose of our study was to evaluate the effect of *Th.* × *porcii* extract (5 mg/mL) on the *in vitro* HCl-induced hemolysis of the equine erythrocytes (Figure 3).

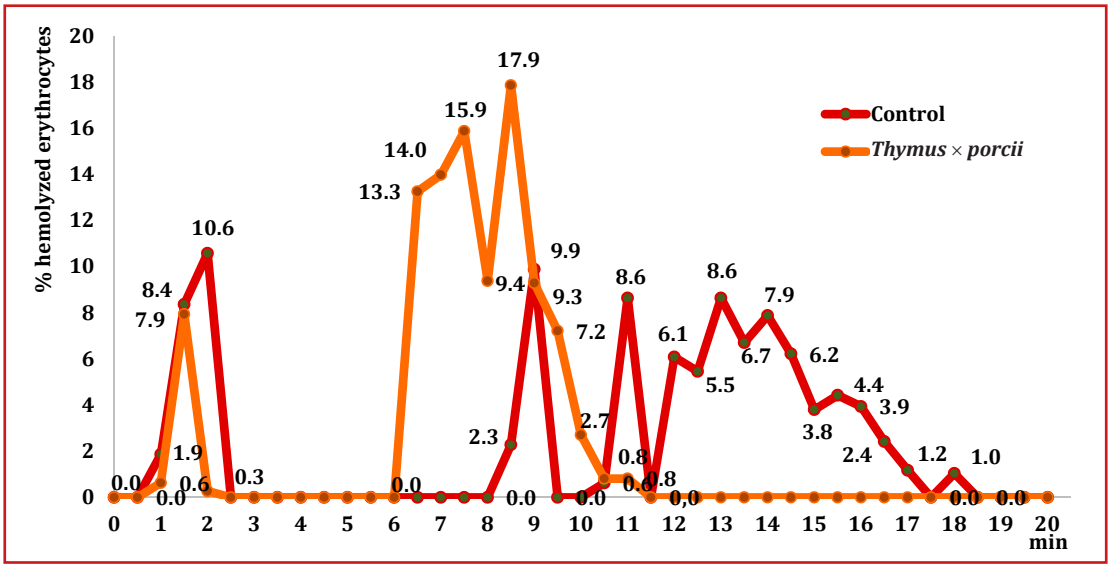


Figure 3 Effect of *Thymus* × *porcii* extract (5 mg/mL) on the HCl-induced hemolysis *in vitro* of the equine erythrocytes (M ±m, *n* = 18)

In the control group (erythrocyte suspension), erythrocytes incubated with 0,1M HCl remained stable and demonstrated slight hemolysis. The maximum level of hemolysis was (10.6 ±0.91%); the total duration of hemolysis was 19 min. When *Th.* × *porcii* extract (5 mg/mL) was added to the erythrocyte suspension, the maximum level of hemolysis occurred after 8.5 min of incubation with 0,1M HCl (17.91 ±1.87%). The total duration of hemolysis after *Th.* × *porcii* extract (5 mg/mL) incubation was 11.5 min. Our results showed that HCl-induced hemolysis in a typical time-dependent manner. Therefore, *Th.* × *porcii* extract at

a concentration of 5 mg/mL induced the increase of hemolyzed erythrocytes and caused to decrease in hemolysis duration (Figure 3).

Many both *in vitro* and *in vivo* studies have shown that plants belonging to the *Thymus* genus possess antioxidant properties. In the study of Afonso et al. (2018), *Th. zygis*, *Th. pulegioides*, and *Th. fragrantissimus* has grown under organic cultivation regime were characterized regarding nutrients and phenolic compounds, as well as the antioxidant and antibacterial properties of these species, were screened. The plants were particularly notable for their high K/Na ratio, polyunsaturated fatty acids content and low omega-6/omega-3 fatty acids ratios, which are valuable features of a healthy diet. Caffeic acid and/or its derivatives, mainly rosmarinic acid and caffeoyl rosmarinic acid, represented the majority of the phenolic constituents of these plants, although they were less representative in *Th. pulegioides*, which in turn was the richest in flavones. These species also exhibited the highest antioxidant capacity while *Th. zygis* was the most active towards Gram-positive and Gram-negative bacteria (Afonso et al., 2018).

Other plants belonging to the *Thymus* genus also possess antioxidant activity against high-fat-diet-induced hyperlipidemia and atherosclerosis (Yu et al., 2016), cisplatin-induced nephrotoxicity (El-Sayed et al., 2015), doxorubicin-induced cardiotoxicity (El-Sayed et al., 2016), UV radiation-induced oxidative stress-mediated skin damages (Sun et al., 2017). For example, *Th. vulgaris* is a potential botanical agent for use against UV radiation-induced oxidative stress-mediated skin damages. In the study of Sun et al. (2017), UVB-induced skin damages have been shown to be ameliorated by treatment with *Th. vulgaris* in hairless mice (HR-1) skin, demonstrated by decreased matrix metalloproteinases (MMPs) and increased collagen production. These researchers also have examined the photoprotective effects of *Th. vulgaris* against UVB and elucidated the molecular mechanism in normal human dermal fibroblasts. *Th. vulgaris* remarkably prevented the UVB-induced reactive oxygen species and lactate dehydrogenase. A dose-dependent increase in glutathione, NAD(P)H: quinone oxidoreductase-1 and heme oxygenase-1, by *Th. vulgaris* was confirmed by increased nuclear accumulation of Nrf2. Furthermore, 5-Methoxyindole-2-carboxylic acid was introduced as a specific inhibitor of dihydrolipoyl dehydrogenase (DLD). Moreover, Nrf2 expression was regulated by DLD, which was a tricarboxylic acid cycle-associated protein that decreased after UVB exposure. Besides, *Th. vulgaris* significantly diminished UVB induced phosphorylation of mitogen-activated protein kinases pathway, containing extracellular signal-regulated kinase, Jun N-terminal kinase, and p38, which consequently reduced phosphorylated c-fos and c-jun (Sun et al., 2017).

The *in vitro* antioxidant activity of thymol and investigated the effect of thymol on high-fat-diet-induced hyperlipidemia and atherosclerosis was studied by Yu et al. (2016). New Zealand white rabbits were fed with regular chow, high-fat and high-cholesterol diet (HC), T3, or T6 (HC with thymol supplementation at 3 mg/kg/d or 6 mg/kg/d, respectively) for 8 weeks. Aortic intimal thickening, serum lipid parameters, multiple inflammatory markers, proinflammatory cytokines, and atherosclerosis-associated indicators were significantly increased in the HC group but decreased upon thymol supplementation. In the study of Yu et al. (2016), thymol exhibits antioxidant activity and may suppress the progression of high-

fat-diet-induced hyperlipidemia and atherosclerosis by reducing aortic intimal lipid lesion, lowering serum lipids and oxidative stress, and alleviating inflammation-related responses.

The possible protective effects of thymol and carvacrol against cisplatin (CP)-induced nephrotoxicity was assessed by El-Sayed et al. (2015). Administration of thymol [20 mg/kg, orally (p.o.)] and/or carvacrol (15 mg/kg, p.o.) for 14 days before CP injection and for 7 days after CP administration restored the kidney function and examined oxidative stress parameters. Thymol was more effective nephroprotective than carvacrol. Moreover, a combination of thymol and carvacrol had a synergistic nephroprotective effect that might be attributed to antioxidant, anti-inflammatory, and anti-apoptotic activities (El-Sayed et al., 2015). Moreover, El-Sayed et al. (2016) also demonstrated that thymol and carvacrol prevent doxorubicin-induced cardiotoxicity by abrogation of oxidative stress, inflammation, and apoptosis in rats. The administration of thymol (20 mg/kg p.o.) and/or carvacrol (25 mg/kg p.o.) for 14 days before doxorubicin administration and for 2 days after doxorubicin injection ameliorated the heart function and oxidative stress parameters. Summarily, thymol was more cardioprotective than carvacrol. Moreover, a combination of thymol and carvacrol had a synergistic cardioprotective effect that might be attributed to antioxidant, anti-inflammatory, and anti-apoptotic activities (El-Sayed et al., 2016).

Thymol and carvacrol administration also decreased the oxidative damage and increased the antioxidant levels and improved the sperm quality parameters Güvenç et al. (2019). However, the combined use of these two active ingredients had a limited therapeutic effect on the mentioned parameters. These researchers have investigated the effects of different doses of thymol and carvacrol on sperm quality oxidative stress and the antioxidant system. During the study, sperm quality parameters (motility, concentration, abnormal spermatozoa, and live-dead sperm ratio), biochemical parameters [malondialdehyde (MDA), reduced glutathione(GSH), glutathione peroxidase (GSH-Px), catalase (CAT), AST, ALT, GGT, urea, and creatinine] were analyzed, and histopathological examination was performed. The study results showed that monotherapies of thymol and carvacrol significantly decreased MDA levels in testicles, liver and kidney tissues compared to the control group. GSH levels increased only with the thymol administration and GSH-Px and catalase activity increased only with the carvacrol administration compared to the control group. The combined administration of these two agents did not cause any significant change in any parameter. Regarding the sperm quality parameters, only the spermatozoa concentration and motility increased significantly in the thymol and carvacrol groups compared to the control group. In respect of spermatozoon anomaly, there was a significant decrease in thymol and carvacrol monotherapy groups. The histopathological analysis of the testicle, liver and kidney tissues of the animals showed no difference between the groups (Güvenç et al., 2019).

In conclusion, the extract obtained from leaves of *Th.* × *porcii* (5 mg/mL) has a mild cytotoxic activity on the equine erythrocytes increasing the level of lipid peroxidation biomarker and hemolysis rate. Investigation of the mechanism of action revealed that this extract has hemolytic activity. These findings suggest the use of *Th.* × *porcii* extract as a source of prooxidant compounds and warrant further studies to evaluate their therapeutic potential.

Conclusions

The aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered after *in vitro* incubation with an extract obtained from leaves of *Th.* × *porcii*. The *Th.* × *porcii* extract caused to increase of TBARS content as a biomarker of lipid peroxidation in the extract-treated erythrocytes, and these results were statistically significant. Total antioxidant capacity was non-significantly increased. When *Th.* × *porcii* extract (5 mg/mL) was added to the erythrocyte suspension, the maximum level of hemolysis occurred after 8.5 min of incubation with 0,1M HCl (17.91 ±1.87%). The total duration of hemolysis after *Th.* × *porcii* extract (5 mg/mL) incubation was 11.5 min. Our results showed that HCl-induced hemolysis in a typical time-dependent manner. Therefore, *Th.* × *porcii* extract at a concentration of 5 mg/mL induced the increase of hemolyzed erythrocytes and caused to decrease in hemolysis duration. Screening of *Thymus* species for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

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EFFECT OF *RHODODENDRON MYRTIFOLIUM* SCHOTT & KOTSCHY LEAF EXTRACTS ON HCL-INDUCED HEMOLYSIS IN HUMAN ERYTHROCYTES

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Many species of the genus *Rhododendron* L. contain a large number of chemical compounds that possessed the antioxidant properties. *Rhododendron myrtifolium* Schott & Kotschy possesses cytotoxic, antibacterial, and antiviral effects, while its biochemical features and bioactive potentials remain unexplored. In the current study, the *in vitro* hemolytic activity of *R. myrtifolium* Schott & Kotschy extract in three different doses on human erythrocytes was evaluated. The total hemolysis (%) was obtained using 0.1 M HCl compared to the phosphate saline buffer as a control sample. Therefore, the aim of the current study was to assess possible antioxidant and anti-hemolytic effects of extract derived from *R. myrtifolium* leaves using oxidative stress biomarker [2-thiobarbituric acid reactive substances (TBARS) as biomarker of lipid peroxidation] and HCl-induced hemolysis assay on human erythrocytes' model after incubation with plant extracts in three doses (5.0, 2.5, and 1.25 mg/mL). Our results revealed that the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in different doses (1.25, 2.5, 5.0 mg/mL) caused the statistically significant increase of TBARS level. The highest value of TBARS level was observed after the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in 5.0 mg/mL. At this dose, protuberances and ruffled edges on erythrocytes were also noted. On the other hand, *R. myrtifolium* extract at 1.25 mg/mL caused the highest percent of hemolyzed erythrocytes and decrease hemolysis duration. This study demonstrated, that among the leaf extract of *R. myrtifolium*, the dose of 5 mg/mL exhibited the highest level of lipid peroxidation biomarker and morphological changes in the human erythrocytes. In addition, the study also demonstrated that *R. myrtifolium* leaf extract at dose 1.25 mg/mL possessed hemolytic effect *in vitro* compared to other doses studied. Therefore, further investigation of it's *in vitro* and *in vivo* activity is warranted.

Keywords: *Rhododendron*, leaf extracts, 2-thiobarbituric acid reactive substances (TBARS), hemolysis, erythrocyte suspension

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Introduction

As one of the largest genera of vascular plants, *Rhododendron* L. (Ericaceae) is one of the most species-rich among angiosperms, comprising over 1,000 species spreading across the northern hemisphere and with the center of diversity in southeastern Asia (Irving and Hebda, 1993). The phytochemistry and bioactivity of this genus have been investigated, and many plants have been demonstrated to have significant biological activities, including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, antioxidant, insecticidal and cytotoxic activity (Demir et al., 2016). *In vivo* and *in vitro* testing of plant extracts and isolated compounds determined diverse biological activities including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, insecticidal and cytotoxic activity (Popescu and Kopp, 2013). Some members of the genus *Rhododendron* were used in traditional medicine for arthritis, acute and chronic bronchitis, asthma, pain, inflammation, rheumatism, hypertension, and metabolic diseases (Popescu and Kopp, 2013).

Recent studies have shown that many species of the genus *Rhododendron* contain a large number of phenolic compounds possessed the antioxidant properties that could be developed into pharmaceutical products (Qiang et al., 2011; Lin et al., 2014, 2016). Phenolic compounds decrease the risk of serious health concerns due to their antioxidants properties to the oxidative damage by reactive oxygen species (ROS). Plant phenolic compounds can act as antioxidants, structural polymers (lignin), attractants (flavonoids and carotenoids), UV screens (flavonoids), signal compounds (salicylic acid, flavonoids) and defense response chemicals (tannins, phytoalexins) (Lin et al., 2016). Therefore, the using of antioxidant components from natural resources such as plants is getting significant attention since the last few decades (Yasin et al., 2017).

Determination of cellular toxicity of a drug towards human erythrocytes is the basic step of drug development against any particular disease. Hemolytic assays for the plant extracts were carried out due to the fact that compounds possessing potent biological activity may not be appropriate in pharmacological preparations if they have hemolytic effects. This assay is usually used to check the biocompatibility of potential drug against erythrocytes in which the interaction of molecules with the cells (RBCs) results in the formation of pores in the membrane that ultimately leads to the release of hemoglobin (Mishra et al., 1981; Lieber et al., 1984; Katsu et al., 1986; Baumann et al., 2000). In the current study, the *in vitro* hemolytic activity of *Rhododendron myrtifolium* Schott & Kotschy extract in three different doses on human erythrocytes was evaluated. The total hemolysis (%) was obtained using 0.1 M HCl compared to the phosphate saline buffer as a control sample.

R. myrtifolium is an evergreen clump-forming dwarf shrub up to 50 cm in height, occurring in high-mountain habitats of the eastern and southern Carpathian Mountains and northern Balkans, largely within altitudes of 1400–2500 m. The species is featured in small narrowly elliptic to obovate coriaceous leaves abaxially covered with glandular scales containing essential oils, terminal inflorescences of tubular-campanulate pinkish flowers, and long-pedunculate dry multilocular capsules containing numerous diminutive seeds (Cullen, 1980, 2005; Mircea, 2005; Boratyński et al., 2006; Voloschuk and Prokopiv, 2011). Although

endangered in countries of its distribution, *R. myrtifolium* has been used in folk medicine for the preparation of herbal teas (Dihoru and Boruz, 2014; Nedelcheva and Draganov, 2014) and presents a major touristic attraction during its mass flowering period in mountains (Rivers, 2017). Its evolutionary closest relatives, *R. ferrugineum* L. and *R. hirsutum* L. (e.g., Sosnovsky et al., 2017) have been shown to possess cytotoxic, antibacterial, and antiviral effects of their extracts (Louis et al., 2010; Gescher et al., 2011; Seephonkai et al., 2011; Rezk et al., 2015a, b), while the biochemical features and bioactive potentials of *R. myrtifolium* remain unexplored.

We continue to assess the dose-dependent antioxidant potential of crude aqueous extracts from the leaves of *R. myrtifolium* sampled in the western part of Ukraine using a human erythrocytes' model. The aim of the current study was to assess possible antioxidant and anti-hemolytic effects of extract derived from *R. myrtifolium* leaves using oxidative stress biomarker and HCl-induced hemolysis assay on human erythrocytes' model after incubation with plant extracts.

Material and methodology

Collection of Plant Materials

Leaves of *Rhododendron myrtifolium* were harvested on the side of the road between the Menchul valley and Rogneska valley (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09' 28.4", E 24° 20' 05.6", 1,485 m a.s.l.). Plant samples were thoroughly washed to remove all the attached materials and used to prepare the ethanolic extract.

Preparation of Plant Extract

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Human blood samples

Blood (10–20 ml) was obtained from normal volunteers via venipuncture (4 males and 5 females aged 28–53-years old). The Research Ethics Committee of Regional Medical Chamber in Gdańsk (Poland) approved the study (KB-31/18). All patients provided written informed consent before the start of the study procedures. Human erythrocytes from citrated blood were isolated by centrifugation at 3,000 rpm for 10 min and washed two times with 4 mM phosphate buffer (pH 7.4) and then re-suspended using the same buffer to the desired hematocrit level. Cells stored at 4 °C were used within 6 h of sample preparation. An erythrocytes' suspension at 1% hematocrit was incubated with 4 mM phosphate buffer (pH 7.4) (control) and pre-incubated with the extracts (5.0, 2.5, and 1.25 mg/mL, respectively) at 37 °C for 60 min. This reaction mixture was shaken gently while being incubated for a fixed interval at 37 °C. For positive control (phosphate buffer) was used. Erythrocytes' aliquots were used in the study.

2-Thiobarbituric Acid Reactive Substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of MDA per L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

Assay of Acid Resistance of Erythrocytes

The acid resistance of erythrocytes was measured spectrophotometrically with 0.1 M HCl (Terskov and Gitelson, 1957). The assay is based on the measuring of the dynamics of erythrocytes disintegration into hemolytic reagent action. The time of hemolytic reagent action serves as the measure of erythrocyte resistance. The assay mixture contained 5 mL of 1% erythrocyte suspension and 0.05 mL of 0.1 M HCl. The absorbance was read at 540 nm every 30 seconds after HCl addition till the end of hemolysis. The difference of absorbance at the beginning and at the end of hemolysis was determined as 100% (total hemolysis). The disintegration of erythrocytes (%) at every 30seconds was expressed as a curve.

Morphological alterations of erythrocytes

The smears were fixed by dipping the slides in absolute methanol, allowing them to air-dry, and then staining with May-Grunwald solution for 5 min, followed by 6% Giemsa stain for 15 min. Slides were selected on the basis of staining quality. In each group, 10,000 cells (minimum of 1,000 per slide) were examined under a 40× objective with a 10× eyepiece (Microscope Leica DM300) to identify morphologically-altered erythrocytes in separate studies.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney *U*-test (Zar, 1999). In addition, the relationships between oxidative stress biomarkers were evaluated using Spearman's correlation analysis. All statistical calculations were performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

The TBARS content as a biomarker of lipid peroxidation in the human erythrocytes' suspension after *in vitro* incubation with *R. myrtifolium* leaf extract was shown in Figure 1.

Our results revealed that treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in different doses (1.25, 2.5, 5.0 mg/mL) caused the statistically significant increase of TBARS level by 47.1% (p <0.05), 31.2% (p <0.05), and 105.6% (p <0.05), respectively (Figure 1). The highest value of TBARS level was observed after the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in 5.0 mg/mL.

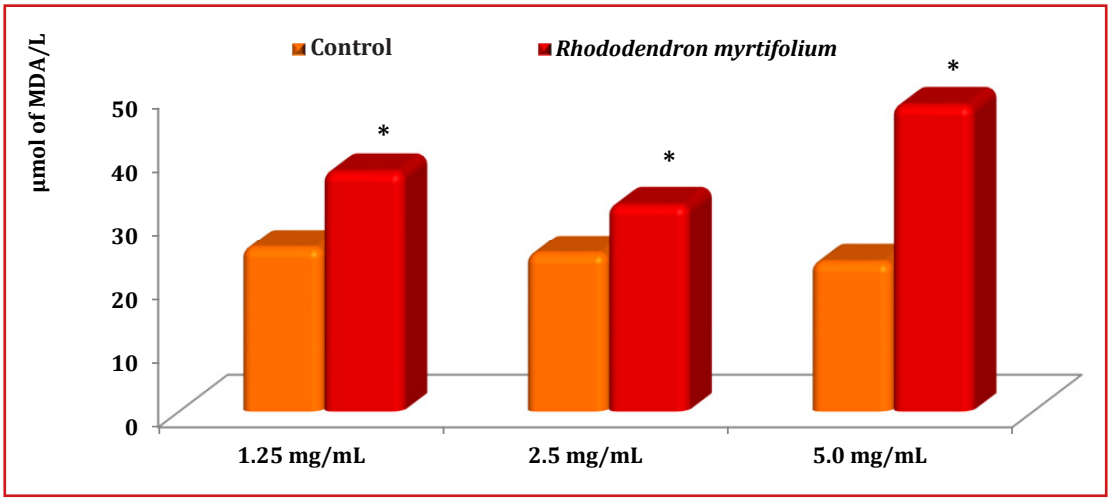


Figure 1 The TBARS content as a biomarker of lipid peroxidation in the human erythrocytes' suspension after *in vitro* incubation with *Rhododendron myrtifolium* leaf extract in different doses (1.25, 2.5, 5.0 mg/mL) (n = 9)

The representative Figure 2 shows the observed values of % hemolysis with time at 1.25, 2.5, 5.0 mg/mL after the treatment by extract obtained from *R. myrtifolium* leaves.

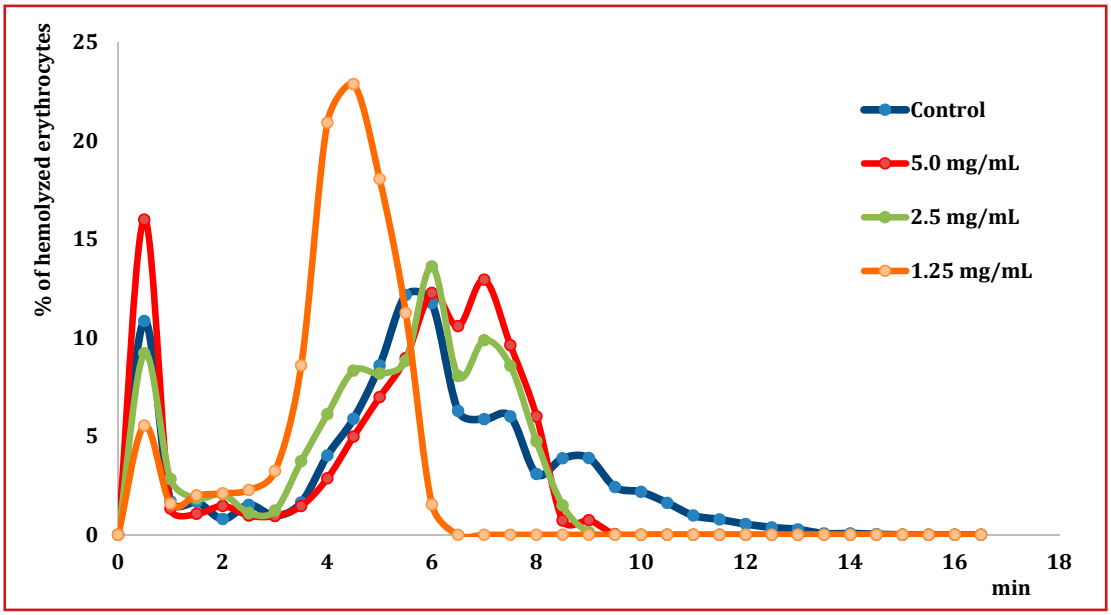


Figure 2 Effects of *R. myrtifolium* extract in different doses (1.25, 2.5, 5.0 mg/mL) on HCl-induced hemolysis *in vitro* in human erythrocytes (n = 9)

In the control group (untreated erythrocytes' suspension), erythrocytes incubated with 0.1 M HCl remained stable and demonstrated slight hemolysis. The maximum level of hemolysis was (12.17 ±1.02%); the total duration of hemolysis was 15.0 min. When *R. myrtifolium* extract

(5 mg/mL) was added to the erythrocytes' suspension, the maximum level of hemolysis has occurred after 0.5 min of incubation with 0.1 M HCl (16.0 ±1.11%). The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.0 min. At the concentration of *R. myrtifolium* extract of 2.5 mg/mL, it decreased the hemolysis (the maximum % of hemolysis was 13.6 ±0.98% at 6.0 min, the total duration of hemolysis was 9.5 min). Interestingly, *R. myrtifolium* extract at 1.25 mg/mL caused the highest % of hemolyzed erythrocytes (the maximum % of hemolysis was 22.9 ±2.32% at 4.5 min, the total duration of hemolysis was 6.5 min). Therefore, we selected *R. myrtifolium* extract at a concentration of 2.5 mg/mL for the next study. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract at different doses (Figure 2).

Effect of *R. myrtifolium* extract at 1.25, 2.5, 5.0 mg/mL on morphological changes of human erythrocytes for 1 h of incubation is shown in Figure 3.

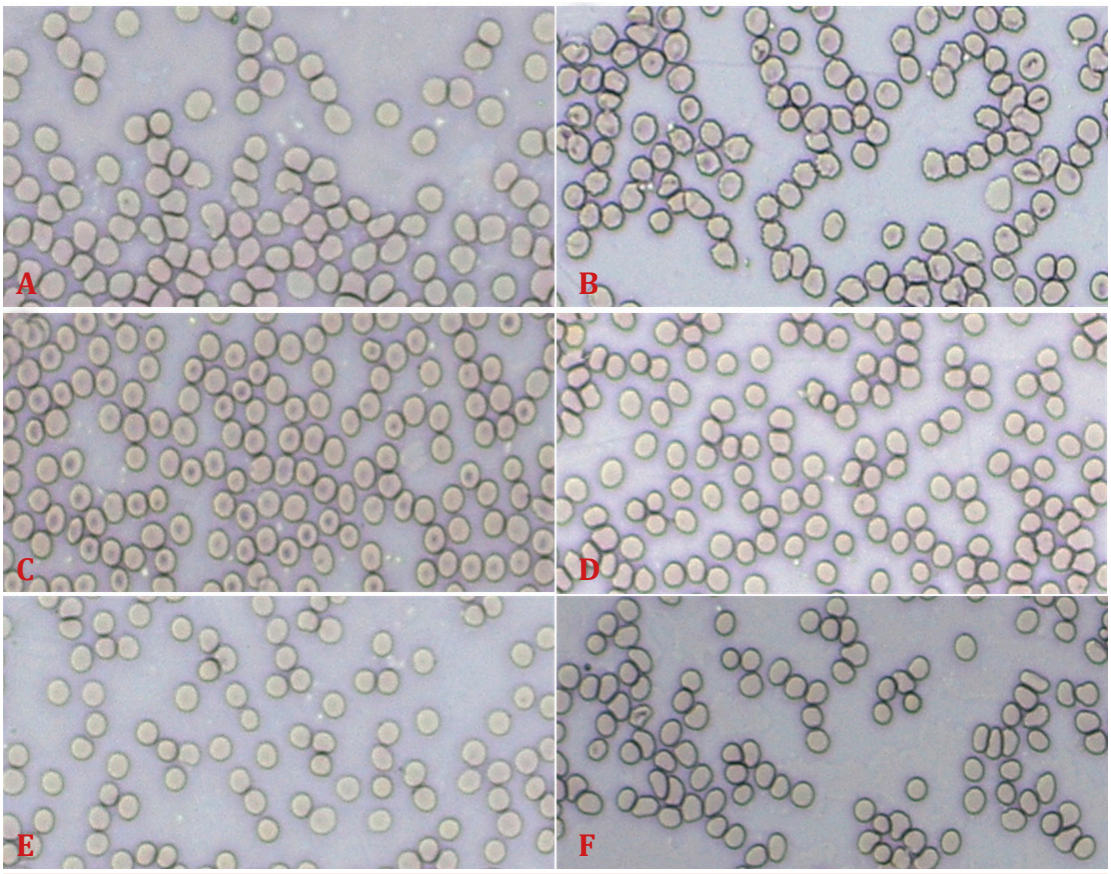


Figure 3 Effect of *R. myrtifolium* extract in different doses on morphological changes of human erythrocytes for 1 h of incubation. (A), (C), and (E) Control samples, (B) *R. myrtifolium* extract (5.0 mg/mL), (D) *R. myrtifolium* extract (2.5 mg/mL), (F) *R. myrtifolium* extract (1.25 mg/mL)

As shown in Figure 3 (A, C, E), the untreated erythrocytes are a normal biconcave shape. After exposure to *R. myrtifolium* extract in dose 5 mg/mL, protuberances and ruffled edges

on erythrocytes were noted (Figure 3B). No changes in the size and shape of cells, as well as protuberances on their surfaces, were demonstrated after exposure to *R. myrtifolium* extract in both doses 2.5 mg/mL and 1.25 mg/mL (Figure 3D, F).

Our results demonstrated that the extract deriving from the leaves of *R. myrtifolium* showed large variations in TBARS levels (Figure 1). The highest TBARS level was determined for a dose of 5 mg/mL. At this dose, protuberances and ruffled edges on erythrocytes were also noted (Figure 3D). On the other hand, *R. myrtifolium* extract at 1.25 mg/mL caused the highest % of hemolyzed erythrocytes and decrease hemolysis duration (Figure 2).

In vivo and *in vitro* testing of *Rhododendron* plants and isolated compounds determined diverse biological activities including cytotoxic activity (Popescu and Kopp, 2013). In several studies demonstrated that *Rhododendron* spp. can cause intoxications in humans (Popescu and Kopp, 2013; Denim et al., 2016). The toxicity is due to grayanotoxins, diterpenes which activate voltage-gated sodium channels and lead to gastrointestinal, cardiac and central nervous system symptoms (Popescu and Kopp, 2013). For example, diterpenes, such as rhodojaponin III, were considered as the toxic agents associated with the toxicities of *R. molle* G. Don. In China, the *R. molle* is traditionally and commonly recognized as a toxic plant as other plants in rhododendrons (Cai et al., 2018). *Rhododendron honey*, locally known as „mad honey", contains grayanotoxin (GTX) and thus induces toxic effects when consumed in large amounts. But, it is still popularly used for treating medical conditions such as high blood pressure or gastrointestinal disorders (Sibel et al., 2014). The effect of grayanotoxin from *R. honey* on antioxidant parameters measured from rats fed with *R. honey* was evaluated by Sibel et al. (2014). It has been observed that both grayanotoxin and high dose *R. honey* treatments showed an oxidant effect on blood plasma and organ tissues of Sprague-Dawley female rats. The biochemical analysis of the various parameters (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, creatine kinase, and creatine kinase muscle and brain) showed a significant elevation with increasing concentration of honey (Sahin et al., 2016). On the other hand, a low dose (1 g/kg/bw) of *R. honey* produces no acute adverse effects on the renal functions of rats (Silici et al., 2016).

Traditionally, *Rhododendron* species is a very important medicinal plant having oxytocic, estrogenic, anti-inflammatory, analgesic, and hepatoprotective activities; it also inhibits the prostaglandin synthetase. For example, *R. luteum* Sweet may be a great source of antioxidant and antitumor natural agents due to their capability of decreasing cancer cell proliferation (Denim et al., 2016). The essential oil of *R. tomentosum* Harmaja with the rich polyphenolic fraction possesses analgesic, anti-inflammatory, antimicrobial, antiviral, antifungal and insecticidal potential, demonstrated by *in vivo* and *in vitro* studies. In addition, recent scientific research reported promising antidiabetic, antioxidant and anticancer properties (Dampc and Luczkiewicz, 2013). The biological activities of major procyanidins isolated from the leaf extract of *R. formosanum* Hemsl. were investigated by Wang et al. (2015). All compounds showed pronounced antioxidant activities and the activities are enhanced as the amount of OH groups in procyanidins increased. Therefore, the pleiotropic effects of procyanidins isolated from the leaves of *R. formosanum* can be a source of promising compounds for the development of future pharmacological applications (Wang et al., 2015).

The ethyl acetate fraction of *Rhododendron fauriei* Franch. (syn. *R. brachycarpum*) leaves extract has strong antioxidant, α-glucosidase, and prevention of DNA damage activities, and furthermore, ethyl acetate fraction significantly protected the cells from high glucose-induced cell death. In the study of Yang et al. (2017), the effects of *R. fauriei* and its different fractions (*n*-hexane, ethyl-acetate, and *n*-butanol) on antioxidant activity, DNA damage prevention and the activity of α-glucosidase were studied. Pretreatment of pancreatic β-cells from the Syrian golden hamster (HIT-TI5) with the ethyl acetate fraction at concentrations of 300 μg/ml significantly protected the cells from high glucose-induced cell death (Yang et al., 2017).

The total phenols and total flavonoid contents, as well as antioxidative properties of the crude extract and solvent fractions of *R. anthopogonoides* Maxim., were determined using seven antioxidant assays in the study of Jing et al. (2015). Additionally, the protective effect of the extracts on hypoxia-induced injury in PC12 cells was also investigated. The gathered data demonstrated that ethyl acetate and *n*-butanol fractions were able to protect PC12 cells against hypoxia-induced injury through direct free radical scavenging and modulation of endogenous antioxidant enzymes (Jing et al., 2015).

Our results suggested that extracts from these plants can be further used for the isolation and structural characterization of valuable bioactive compounds and investigated in *in vivo* experimental model of the disease.

Conclusions

Our results revealed that the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in different doses (1.25, 2.5, 5.0 mg/mL) caused the statistically significant increase of TBARS level. The highest value of TBARS level was observed after the treatment of human erythrocytes by extract derived from *R. myrtifolium* leaves in 5.0 mg/mL. At this dose, protuberances and ruffled edges on erythrocytes were also noted. On the other hand, *R. myrtifolium* extract at 1.25 mg/mL caused the highest percent of hemolyzed erythrocytes and decrease hemolysis duration. This study demonstrated, that among the leaf extract of *R. myrtifolium*, the dose of 5 mg/mL exhibited the highest level of lipid peroxidation biomarker and morphological changes in the human erythrocytes. In addition, the study also demonstrated that *R. myrtifolium* leaf extract at dose 1.25 mg/mL possessed hemolytic effect *in vitro* compared to other doses studied. Therefore, further investigation of it's *in vitro* and *in vivo* activity is warranted.

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OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTE SUSPENSION AFTER *IN VITRO* INCUBATION WITH LEAF EXTRACT OBTAINED FROM *RHODODENDRON MYRTIFOLIUM* SCHOTT & KOTSCHY (ERICACEAE)

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A noteworthy European member of the genus is *Rhododendron myrtifolium* Schott & Kotschy, an evergreen clump-forming dwarf shrub up to 50 cm in height, occurring in high-mountain habitats of the eastern and southern Carpathian Mountains and northern Balkans, largely within altitudes of 1,400–2,500 m. It has been shown that *R. myrtifolium* possesses antibacterial and antiviral effects of their extracts, while the biochemical features and bioactive potentials of *R. myrtifolium* remain unexplored. In the current study crude aqueous extract from the leaves of *R. myrtifolium* was assessed for antioxidant activities, cytotoxicity, and anti-hemolytic potential. The aim of this study was to assess possible antioxidant and anti-hemolytic effects of an extract derived from *R. myrtifolium* leaves using oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS) as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, the total antioxidant capacity (TAC)] and HCl-induced hemolysis assay on equine erythrocytes' model. Our results demonstrated that the treatment by extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL decreased the TBARS level in the equine erythrocytes' suspension, while aldehydic and ketonic derivatives of oxidatively modified proteins and total antioxidant capacity increased. These changes were statistically non-significant. When *R. myrtifolium* extract was added to the erythrocyte suspension, the maximum level of hemolysis has occurred after 6.0 min of incubation with 0.1 M HCl. The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.5 min compared to 12 min in the control sample. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract. On the other hand, no changes in the size and shape of cells, as well as protuberances on their surfaces and/or cells, after exposure to *R. myrtifolium* extract was observed. Importantly, no ruffled edges (echinocyte or crenated cells) were noted. The erythrocytes maintained the normal biconcave shape, except a very few cells, underwent a slight change in conformation. Therefore, extract obtained from the leaves of *R. myrtifolium* in dose 5 mg/mL Interestingly, when assayed for its cytotoxic evaluation against equine erythrocytes,

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the extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL showed mild hemolytic activity, while the oxidative stress biomarkers were non-significantly changed. Our results further suggest that *Rhododendron myrtifolium* could be further investigated for the isolation and purification of the active constituents.

Keywords: *Rhododendron myrtifolium* Schott & Kotschy, leaf extracts, 2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins, the total antioxidant capacity, hemolysis

Introduction

The genus *Rhododendron* L. (Ericaceae) is one of the most species-rich among angiosperms, comprising over 1,000 species spreading across the northern hemisphere and with the center of diversity in southeastern Asia (Irving and Hebda, 1993). This genus is a rich source of phenolic compounds, especially flavonoids, essential oils, chromones, terpenoids, and steroids. It has many biological properties such as antioxidant, anti-inflammatory, antiviral, antibacterial, anticancer, antidiabetic, immunomodulatory, cardioprotective and hepatoprotective among others due to their polyphenolic constituents (Demir et al., 2016). *In vivo* and *in vitro* testing of plant extracts and isolated compounds determined diverse biological activities including anti-inflammatory, analgesic, anti-microbial, anti-diabetic, insecticidal and cytotoxic activity (Popescu and Kopp, 2013).

Plants belonging to the genus *Rhododendron* generally used for traditional medicine. For example, *R. anthopogonoides* Maxim, a kind of traditional Tibetan medicine, has been used to remove body heat, body detoxification, cough, asthma, stomachic and swelling, eliminate abundant phlegm and inflammatory for a long time (Jing et al., 2015). The ethyl acetate and *n*-butanol fractions of *R. anthopogonoides* had significant antioxidant activity and could prevent PC12 cells against hypoxia-induced injury. So it might be regarded as an excellent source of antioxidants and had great potential to explore as a therapeutic agent for preventing hypoxia-related sickness in the future (Jing et al., 2015). *R. fauriei* Franch. (syn. *R. brachycarpum*) is a flowering plant generally used for traditional medicine in Korea to treat hypertension, neuralgia, and sterility. Previous studies have also shown that *R. fauriei* extract alleviates inflammation and antimicrobial activity (Yang et al., 2017). The roots of *R. mucronulatum* Turz. have been used in Oriental traditional medicine for the treatment of dysuria, fever, an increase of digestive activity and tonics in China and Korea (Choi et al., 2011).

The various studies also reveal the strong antioxidant activity of plants belonging to the *Rhododendron* genus. The biological activities of major procyanidins isolated from the leaf extract of *R. formosanum* Hemsl. were investigated by Wang et al. (2015). All compounds showed pronounced antioxidant activities and the activities are enhanced as the amount of OH groups in procyanidins increased. The pleiotropic effects of procyanidins isolated from the leaves of *R. formosanum* can be a source of promising compounds for the development of future pharmacological applications (Wang et al., 2015). On the other hand, Guo et al. (2017) have revealed that the ultrasound-assisted extraction of *R. aganniphum* Balf. f. & Kingdon-Ward presented good radical scavenging activity, superoxide and hydroxyl radicals *in vitro*

and could be used as a natural antioxidant in the food and medical industries. The methanolic extracts of *R. pseudochrysanthum* Hayata leaves have excellent antioxidant activities and great potential as a source for natural health products (Lin et al., 2014).

A noteworthy European member of the genus is *R. myrtifolium* Schott & Kotschy, an evergreen clump-forming dwarf shrub up to 50 cm in height, occurring in high-mountain habitats of the eastern and southern Carpathian Mountains and northern Balkans, largely within altitudes of 1,400–2,500 m. The species is featured in small narrowly elliptic to obovate coriaceous leaves abaxially covered with glandular scales containing essential oils, terminal inflorescences of tubular-campanulate pinkish flowers, and long-pedunculate dry multilocular capsules containing numerous diminutive seeds (Cullen, 1980, 2005; Mircea, 2005; Boratyński et al., 2006; Voloschuk and Prokopiv, 2011). Although endangered in countries of its distribution, *R. myrtifolium* has been used in folk medicine for the preparation of herbal teas (Dihoru and Boruz, 2014; Nedelcheva and Draganov, 2014) and presents a major touristic attraction during its mass flowering period in mountains (Rivers, 2017). Its evolutionary closest relatives, *R. ferrugineum* L. and *R. hirsutum* L. (e.g., Sosnovsky et al., 2017) have been shown to possess cytotoxic, antibacterial, and antiviral effects of their extracts (Louis et al., 2010; Gescher et al., 2011; Seephonkai et al., 2011; Rezk et al., 2015b), while the biochemical features and bioactive potentials of *R. myrtifolium* remains unexplored.

In the current study crude aqueous extracts from the leaves of *R. myrtifolium* was assessed for antioxidant activities, cytotoxicity, and anti-hemolytic potential. The aim of this study was to assess possible antioxidant and anti-hemolytic effects of an extract derived from *R. myrtifolium* leaves using oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS) as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, the total antioxidant capacity (TAC)] and HCl-induced hemolysis assay on equine erythrocytes' model.

The erythrocytes of mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure (Pagano and Faggio, 2015). Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity (Webster and Toothill, 1987) and red cell damages by free radicals would probably be associated with hemolysis (Pagano and Faggio, 2015; Farag and Alagawany, 2018). Red blood cells along with its membrane have always been an important medium for the study due to the important role it plays in varied physiological and metabolic processes (Jha et al., 2009; Karabulut et al., 2009; Pandey et al., 2009). The erythrocyte could be isolated and handled easily so that they could provide a good model for many assays (Alagawany et al., 2016; Farag and Alagawany, 2018). Additionally, the high concentration of polyunsaturated fatty acids in RBCs membrane, the high oxygen tension, and redox-active hemoglobin molecules [the source of reactive oxygen species (ROS) in erythrocyte] make them a good biological lipid membrane model especially for screening the oxidative stress conditions induced by various substances (Farag and Alagawany, 2018).

Material and methodology

Collection of Plant Materials

Leaves of *Rhododendron myrtifolium* were harvested on the side of the road between the Menchul valley and Rogneska valley (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 28.4“, E 24° 20‘ 05.6“, 1,485 m a.s.l.). Plant samples were thoroughly washed to remove all the attached materials and used to prepare the ethanolic extract.

Preparation of Plant Extract

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Horses

Eighteen clinically healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30’ 48.0” E 16º 57’ 44.9”), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. Before sampling, all horses were thoroughly examined clinically by a veterinarian and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma Blood was stored into The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control, phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of per 1 L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2.4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Assay of Acid Resistance of Erythrocytes

The acid resistance of erythrocytes was measured spectrophotometrically with 0.1 M HCl (Terskov and Gitelson, 1957). The assay is based on the measuring of the dynamics of erythrocytes disintegration into hemolytic reagent action. The time of hemolytic reagent action serves as the measure of erythrocyte resistance. The assay mixture contained 5 mL of 1% erythrocyte suspension and 0.05 mL of 0.1 M HCl. The absorbance was read at 540 nm every 30 seconds after HCl addition till the end of hemolysis. The difference of absorbance at the beginning and at the end of hemolysis was determined as 100% (total hemolysis). The disintegration of erythrocytes (%) at every 30seconds was expressed as a curve.

Morphological alterations of erythrocytes

The smears were fixed by dipping the slides in absolute methanol, allowing them to air-dry, and then staining with May-Grunwald solution for 5 min, followed by 6% Giemsa stain for 15 min. Slides were selected on the basis of staining quality. In each group, 10,000 cells (minimum of 1.000 per slide) were examined under a 40× objective with a 10× eyepiece (Microscope Leica DM300) to identify morphologically-altered erythrocytes in separate studies.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney U-test (Zar, 1999). In addition, the relationships between oxidative stress biomarkers were

evaluated using Spearman's correlation analysis. All statistical calculations were performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes' suspension after *in vitro* incubation with *R. myrtifolium* leaf extract was shown in Figure 1.

As can be seen in Figure 1, treatment by extract caused the reduced erythrocytes' TBARS level by 7.5% (p >0.05), while the ketonic aldehydic and ketonic derivatives level was increased (by 5.4 and 5.3 5, p >0.05, respectively) as compared to untreated erythrocytes. However, these changes were statistically non-significant. The TAC level was statistically non-significant changes.

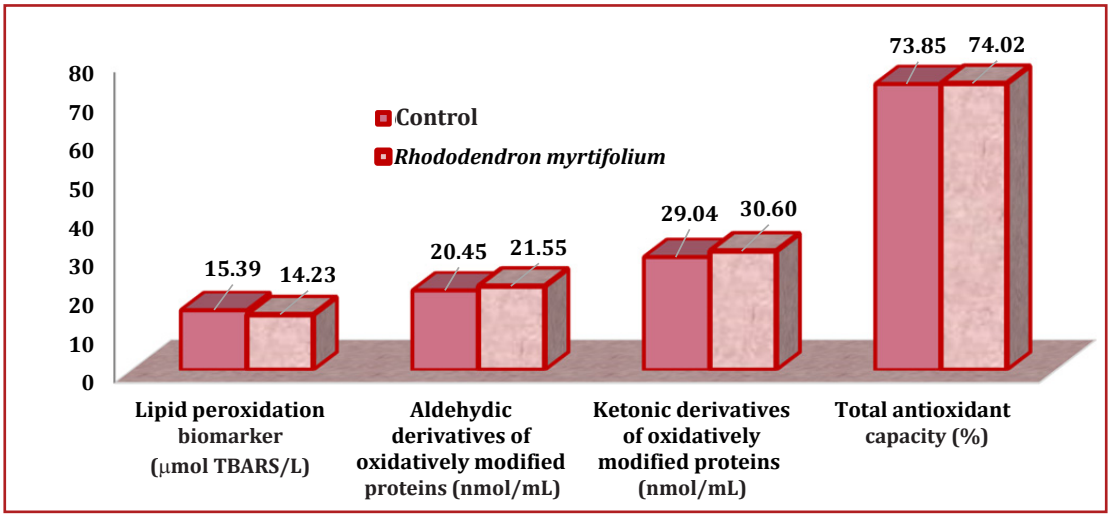


Figure 1 The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes' suspension after *in vitro* incubation with *Rhododendron myrtifolium* leaf extract (M ±m, n = 18)

The representative Figure 2 shows the observed values of % hemolysis with time at 5 mg/mL after the treatment by extract obtained from *R. myrtifolium* leaves.

In the control group (untreated erythrocytes' suspension), erythrocytes incubated with 0.1 M HCl remained stable and demonstrated slight hemolysis. The maximum level of hemolysis was (14.6 ±0.85%); the total duration of hemolysis was 12.0 min. When *R. myrtifolium* extract was added to the erythrocyte suspension, the maximum level of hemolysis has occurred after 6.0 min of incubation with 0.1 M HCl (18.1 ±1.21%). The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.5 min. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract (Figure 2).

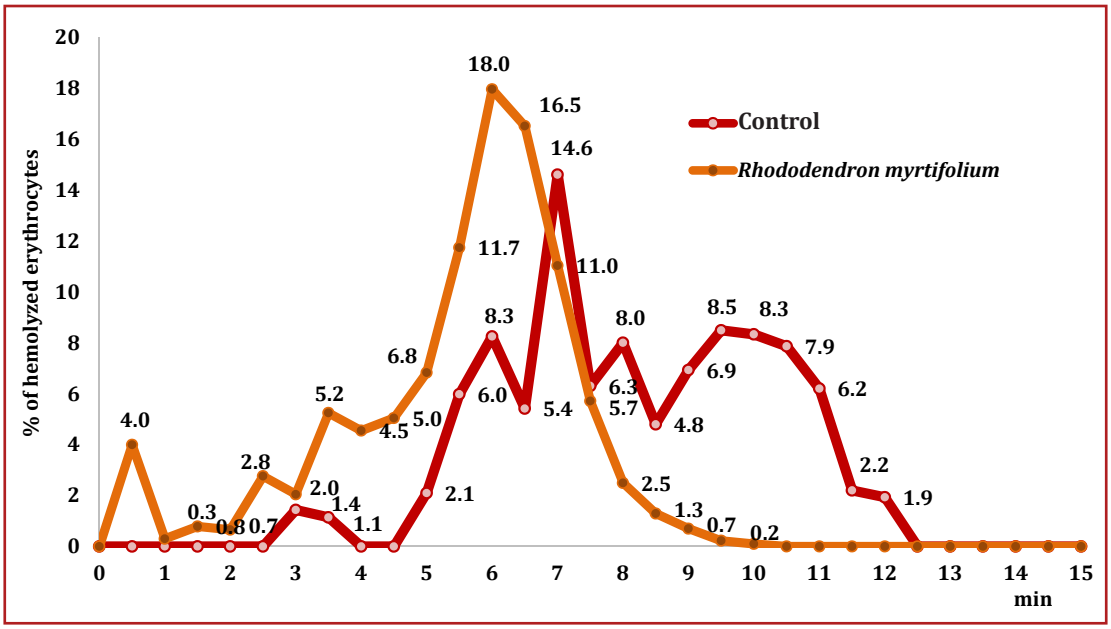


Figure 2 Effects of *Rhododendron myrtifolium* leaf extract (5 mg/mL) on HCl-induced hemolysis in equine erythrocytes (n = 18)

The morphological changes of equine erythrocytes for 1 h of incubation with *R. myrtifolium* extract is shown in Figure 3.

The normal red blood cell shape is a biconcave discocyte. Intercalation of a drug in the outer half of the membrane lipid bilayer leads to echinocytosis, intercalation in the inner half to stomatocytosis (Reinhart et al., 2014). The observation of the photomicrographs revealed that the untreated erythrocytes are a normal biconcave shape (Figure 3A). No changes in the size and shape of cells, as well as protuberances on their surfaces and/or cells, after exposure to *R. myrtifolium* extract was observed. Importantly, no ruffled edges (echinocyte or crenated cells) were also demonstrated. The erythrocytes in the presence of *R. myrtifolium* extract maintained the normal biconcave shape, except a very few cells, underwent a slight change in conformation (Figure 3).

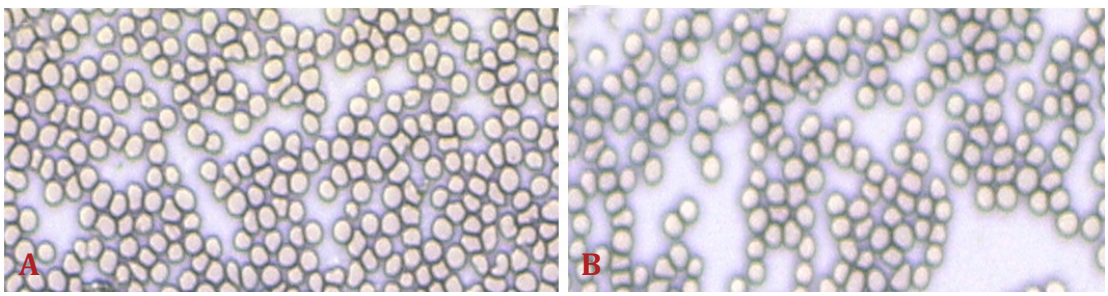


Figure 3 Effect of *Rhododendron myrtifolium* extract on morphological changes of equine erythrocytes for 1 h of incubation. Control sample (A), *R. myrtifolium* extract (5 mg/mL) (B)

The results of this study are consistent with other studies that have previously been reported that chemicals derived from various *Rhododendron* plants are promising candidates as antioxidant and anti-inflammatory agents. For example, Choi et al. (2011) have investigated the antioxidative and anti-inflammatory effects of compounds derived from *R. mucronulatum* Turcz., their 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities and the protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-stimulated HaCaT cells and their end products, nitric oxide (NO) and prostaglandin E2 (PGE2), respectively. All compounds showed potent DPPH radical scavenging compared with positive controls (L-ascorbic acid). Also, taxifolin and taxifolin 3-O-β-D-glucopyranoside dose-dependently inhibited the expressions of inflammatory mediators, NO and PGE2, suggesting they are promising candidates as anti-inflammatory agents (Choi et al., 2011).

On the other hand, *R. album* Blume may prove to be an effective therapeutic agent for the treatment of inflammatory diseases. The biological effects of *R. album* methanol extract (RAME) on inflammation was investigated in lipopolysaccharide (LPS)-stimulated mouse RAW264.7 cells by Park et al. (2015). These researchers have investigated the effects of RAME on the production of nitric oxide (NO) and prostaglandin E2 (PGE2) in LPS-stimulated RAW264.7 cells. To explore the anti-inflammatory mechanisms of RAME, they measured the mRNA and protein expression of pro-inflammatory mediators induced by RAME in the LPS-stimulated RAW264.7 cells by RT-PCR and western blot analysis, respectively. RAME significantly inhibited the production of NO, PGE2, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α in the LPS-stimulated RAW264.7 cells. It also suppressed the mRNA and protein expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and mitogen-activated protein kinases (MAPKs) with a concomitant decrease in the nuclear translocation of nuclear factor-κB (NF-κB) in the LPS-stimulated RAW264.7 cells. Therefore, RAME inhibits LPS-induced inflammatory responses. These effects were considered to be strongly associated with the suppression of NF-κB activation (Park et al., 2015).

Medicinal plants and herbs are possessed compound that acts as antioxidants mainly due to the presence of polyphenolics and flavonoids. Antioxidant flavonoids have been isolated from the flower of *R. yedoense* var. *poukhanense* by Jung et al. (2007). One new flavonoid and three known flavonoids, quercetin-5-O-beta-D-glucopyranoside (1), quercetin (3), and quercitrin (4), were isolated from the butanol and ethyl acetate extracts of the plant. The new flavonoid was identified as myricitrin-5-methyl ether (2). The isolation of these flavonoids from this plant, for the first time, is a valuable finding. The flavonoids were evaluated for their antioxidant activities using 1.1-diphenyl-2-picrylhydrazyl free radical (DPPH), TBARS (2-thiobarbituric acid reactive substance) and superoxide anion radical ($O_{2}^{\cdot-}$) in the xanthine/xanthine oxidase assay system. The antioxidant activities of myricitrin-5-methyl ether (2) and quercetin (4) were higher than that of L-ascorbic acid when evaluated using a TBARS assay (Jung et al., 2007).

The timing of the harvest of plants impacts the plant's phenolic content and its antioxidant and anti-inflammatory activities. For instance, Black et al. (2011) have assessed seasonal variation of phenolic constituents and medicinal activities of Northern Labrador tea, *R. tomentosum* ssp. *subarcticum*. The antioxidant potency was measured in a DPPH radical scavenging

assay, and the anti-inflammatory activity was determined with a TNF-α production assay. The most abundant constituent was (+)-catechin, which made up 19% of the total weight of characterized phenolics. There was significant seasonal variation in the quantity of all constituents assessed, whereas there was no seasonal variation of their total sum. The antioxidant activity was positively correlated with phenolic content and negatively correlated with daylight hours. The anti-inflammatory activity was negatively correlated with caffeic acid derivative 1 and daylight hours (Black et al., 2011).

The antioxidant and antimutagenic effects of the hexane, chloroform, and ethyl acetate fractions of *R. arboreum* Sweet leaves and their chemical composition was carried out by Gautam et al. (2018). The different fractions inhibited lipid peroxidation, repressed the production of nitric oxide radicals, and prevented deoxyribose degradation. The antimutagenic activity of the leaf fractions was analyzed against 4-nitro-O-phenylenediamine, sodium aside and 2-aminofluorene mutagens in two test strains (TA-98 and TA-100) of Salmonella typhimurium (Gautam et al., 2018). The evaluation of the antioxidant properties and cytotoxic activity of dimethyl sulfoxide extract of flowers of *R. luteum* was done by Denim et al. (2016). *R. luteum* extract exhibited selective cytotoxicity against colon and liver cancer cells compared to normal fibroblast cells, while this selective cytotoxicity was not observed in breast cancer cells (Denim et al., 2016).

Our results suggested that extracts from these plants can be further used for the isolation and structural characterization of valuable bioactive compounds and investigated in *in vivo* experimental model of the disease.

Conclusions

In our study, treatment by extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL decreased the TBARS level in the equine erythrocytes' suspension, while aldehydic and ketonic derivatives of oxidatively modified proteins and total antioxidant capacity increased. These changes were statistically non-significant. When *R. myrtifolium* extract was added to the erythrocyte suspension, the maximum level of hemolysis has occurred after 6.0 min of incubation with 0.1 M HCl. The total duration of hemolysis after *R. myrtifolium* extract incubation was 10.5 min compared to 12 min in the control sample. The results showed that HCl-induced hemolysis was increased by the treatment of *R. myrtifolium* extract. On the other hand, no changes in the size and shape of cells, as well as protuberances on their surfaces and/or cells, after exposure to *R. myrtifolium* extract was observed. Importantly, no ruffled edges (echinocyte or crenated cells) were noted. The erythrocytes maintained the normal biconcave shape, except a very few cells, underwent a slight change in conformation. Therefore, extract obtained from the leaves of *R. myrtifolium* in dose 5 mg/mL Interestingly, when assayed for its cytotoxic evaluation against equine erythrocytes, the extract obtained from leaves of *R. myrtifolium* in dose 5 mg/mL showed mild hemolytic activity, while the oxidative stress biomarkers were non-significantly changed. Our results further suggest that *Rhododendron myrtifolium* could be further investigated for the isolation and purification of the active constituents.

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ASSESSMENT OF OXIDATIVE STRESS BIOMARKERS IN THE EQUINE BLOOD AFTER *IN VITRO* INCUBATION WITH LEAF EXTRACT OBTAINED FROM *DENDROBIUM PARISHII* RCHB.F.

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The main aim of the study was an assessment of the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] in the plasma and equine erythrocytes after treatment with *Dendrobium parishii* Rchb. f. leaf extract. The leaves of *D. parishii* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w). The equine plasma and erythrocyte aliquots were used in the study. The pellet of blood was resuspended in phosphate buffer (pH 7.4). A volume of 0.1 ml of the *D. parishii* extract was added to 1.9 ml of clean equine erythrocytes or 1.9 mL of plasma. For positive control (blank), phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, samples were used for the biochemical assays. The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered in the erythrocytes' suspension after *in vitro* incubation with an extract obtained from *D. parishii*. More significant changes were observed in the plasma. The *D. parishii* extract caused to increase in the formation of intracellular aldehydic and ketonic derivatives of oxidatively modified proteins in the extract-treated plasma, but these results were non-significant. Total antioxidant capacity was non-significant decreased both in plasma and erythrocytes. Screening of *Dendrobium* plants for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

Keywords: *Dendrobium parishii* Rchb. F., leaf extract, equine erythrocytes, plasma, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

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Introduction

The genus *Dendrobium* Sw. is one of the largest genera in the family Orchidaceae, comprising with approximately 1,200–2,000 species, distributed in the tropical and subtropical regions of Asia and North Australia (Wood, 2006; Hou et al., 2017).

Dendrobium species are important commercial plants widely used as herbal medicines for hundreds of years in China due to their pharmacological properties (Lee et al., 2018; Cheng et al., 2019). Recently, within the *Dendrobium* genus, most studies have focused on the bioactivity and structural types of some small molecules, such as bibenzyl, phenanthrene, anthracene, fluorene, coumarin, flavone, cinnamate, sesquiterpene, sterol, fatty acid, and alkaloids (Honda and Yamaki, 2000; Zhao et al., 2017; Paudel et al., 2018; Ivannikov et al., 2019a,b). Among them, phenolic derivatives have been extensively studied for their biological activities, especially in the field of cancer, inflammation, and neurodegeneration (Lee et al., 2018; Sut et al., 2017). Numerous researchers have investigated polysaccharide, the major active constituents in *Dendrobium*. The polysaccharides in many plants are attracting worldwide attention because of their biological activities and medical properties, such as anti-viral, antioxidant, immune-stimulating and antitumor activities, anti-chronic inflammation, anti-hypertensive, neuron-protective effects (Lee et al., 2018). Polysaccharides extracted from some *Dendrobium* species, such as *Dendrobium officinale* Kimura & Migo (syn. *D. huoshanense* Z.Z. Tang & S.J. Cheng) and *Dendrobium chrysotoxum* Lindl., have been studied (Luo et al., 2009). Alkaloids exhibit antioxidant, anticancer, and neuroprotective activities. Other compounds manifest antioxidant, anticancer, and immunomodulatory (Ng et al., 2012).

Dendrobium parishii was studied for chemical constituents by solvent extraction, followed by column chromatography by Kongkatitham et al. (2018). Seven compounds including two new (1–2) and five known compounds (3–7) were isolated and screened for antioxidant activity using 2.2-Diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and deoxyribose assays. The new compound (compound 2) showed the highest antioxidant activities and was further evaluated for its antioxidant and anti-inflammatory effects, as well as mechanisms of action in RAW264.7 murine macrophage cells under oxidative stress and inflammation induced by hydrogen peroxide (H_2O_2) and lipopolysaccharide (LPS), respectively. Compound 2 at 12.5, 25 and 50 μg/mL significantly decreased ROS in H_2O_2 treated RAW264.7 cells in a dose-dependent manner and enhanced antioxidant enzyme [superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)] activities. For the anti-inflammatory activity, compound 2 reduced the expression of iNOS and COX-2 in LPS treated RAW264.7 cells. The results indicate that compound 2 has the potential to be developed as an antioxidant and anti-inflammatory agent (Kongkatitham et al., 2018).

Equine erythrocytes are more sensitive to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage, i.e. methemoglobin formation, alteration of aggregation, and reduction of cellular deformability (Baskurt and Meiselman, 1999). Erythrocytes from horses are slower than erythrocytes from other species studied in their ability to regenerate glutathione (GSH) after it has been oxidized *in vitro* (Harvey et al., 2003). These reduced abilities may be related to the fact that horse erythrocytes have lower

glutathione reductase (GR) activities than erythrocytes from humans and most domestic animal species, and the Michaelis-Menton constant (Km) of GSSG for GR is higher in horses than in three other species measured. Moreover, sulfhydryl groups in proteins and unsaturated lipids in membranes are especially susceptible to oxidation. Oxidative denaturation and the precipitation of the globin portion of hemoglobin into large aggregates result in the formation of Heinz bodies that can bind to and alter membranes. Membrane structure also is altered by the oxidation of sulfhydryl groups and by lipid peroxidation (Harvey, 1997).

The list of *Dendrobium* species used in Traditional Chinese Medicine comprises 41 species, including *D. parishii* Rchb. f., distributed in Yunnan, Guizhou, India, Myanmar, Thailand, Laos, Vietnam (Cheng et al., 2019).

Therefore, in the current study, the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] in the equine plasma and erythrocytes was used for assessing the antioxidant activity of *Dendrobium parishii* Rchb. F. leaf extract, considering the tremendous medicinal importance of this endangered orchid species.

Material and methodology

Collection of Plant Materials

The leaves of *D. parishii* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine (Figure 1). Since 1999, the whole collection of tropical and subtropical plants (including orchids) has the status of a National Heritage Collection of Ukraine. Besides that, the NBG collection of tropical orchids was registered at the Administrative Organ of CITES in Ukraine (Ministry of Environmental Protection, registration No. 6939/19/1-10 of 23 June 2004). Plant samples were thoroughly washed to remove all the attached material and used to prepare extracts.

Preparation of Plant Extracts

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extract was then filtered and used for analysis. The extract was stored at -20 °C until use.

Horses

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30’ 48.0” E 16º 57’ 44.9”), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.



Figure 1 *Dendrobium parishii* Lindl. plant, cultivated at M.M. Gryshko National Botanic Garden glasshouses (Kyiv, Ukraine). Photo: Lyudmyla Buyun

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control (phosphate buffer) was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malondialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of the extract obtained from leaves of *D. parishii* against free radical-induced protein damage in equine erythrocytes and plasma, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocyte suspension and plasma was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2.4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney *U*-test (Zar, 1999). All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

The data on the TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine plasma and erythrocytes suspension after *in vitro* incubation with leaf extract obtained from *Dendrobium parishii* was presented in Figure 2.

As shown in Figure 2, treatment by extract caused to increase of the plasma TBARS level by 46.4% (p = 0.000), while erythrocyte TBARS level was non-significantly increased by 6.3% (p >0.05) when compared to untreated erythrocytes.

When equine erythrocytes were incubated with an extract obtained from *D. parishii*, the aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered. The *D. parishii* extract caused to increase in the formation of intracellular aldehydic and ketonic derivatives of OMP in the extract-treated plasma (by 2.3 and 17.4%, p >0.05). In the erythrocyte suspension, ketonic derivatives of OMP was decreased by 3.2% (p >0.05), but these results were non-significant. Total antioxidant capacity was non-

significantly decreased both in plasma (by 15.2%, p >0.05) and erythrocytes (by 14.1%, p >0.05) (Figure 2).

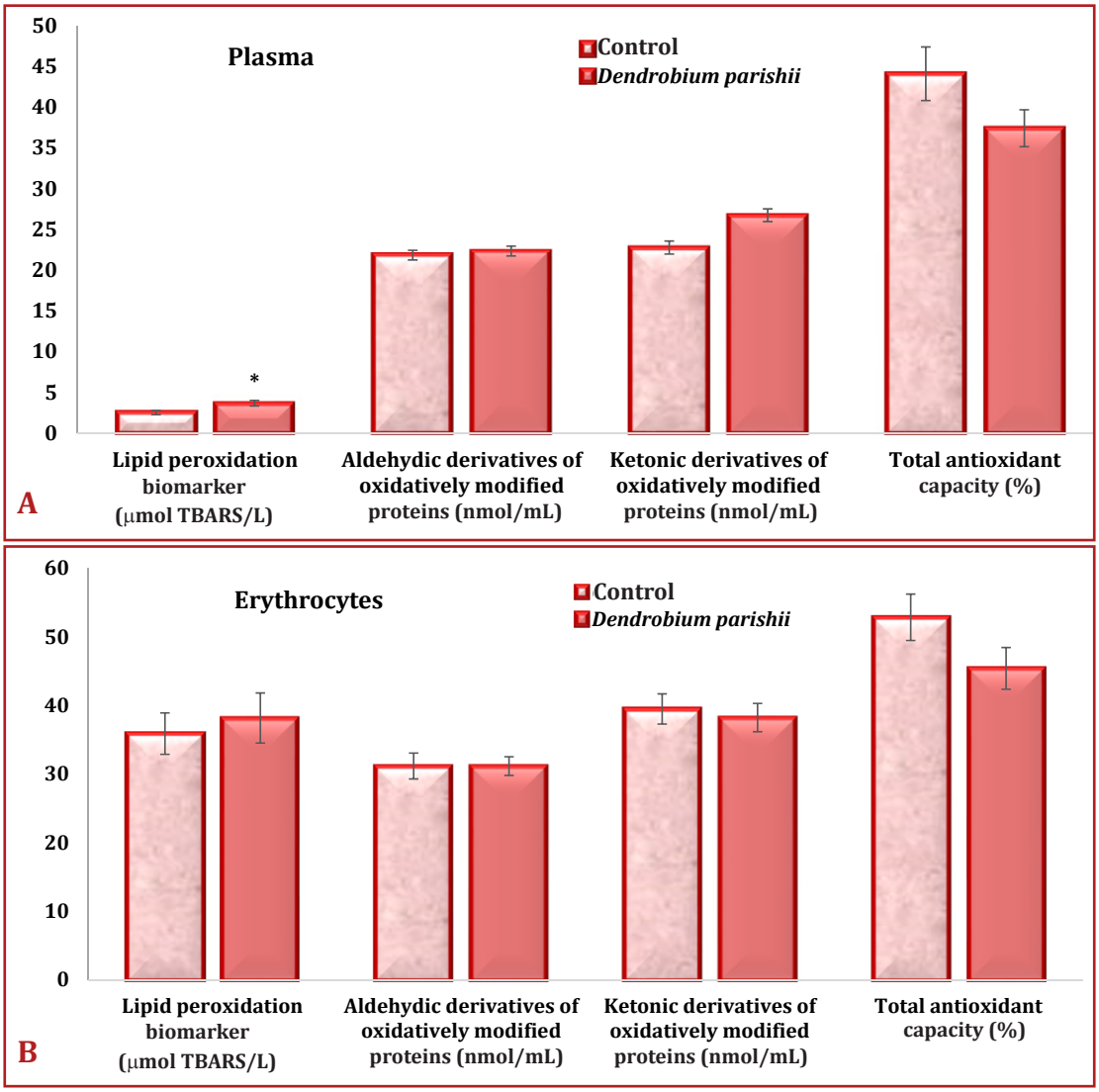


Figure 2 The TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine plasma and erythrocytes suspension after *in vitro* incubation with leaf extract obtained from *Dendrobium parishii* (M ±m, n = 18)

Many studies have confirmed the antioxidant properties of *Dendrobium* plants. Antioxidant properties of crude extract, partition extract, and fermented medium from *Dendrobium* plant flower were investigated by Abu et al. (2017). The 100% methanolic crude extract showed the highest total phenolic content and the best antioxidant properties. A correlation relationship between antioxidant activity and total phenolic content showed

that phenolic compounds were the dominant antioxidant components in this flower extract. The microbial fermentation on the *D. sabin* flower medium showed potential in increasing the phenolic content and DPPH scavenging activity. The TPC of the final fermented medium showed approximately an 18% increment, while the DPPH of fermented medium increased significantly to approximately 80% at the end of the fermentation. The flower showed very good potential properties of antioxidants in crude extract and partition extract as well as better antioxidant activity in the flower fermented medium (Abu et al., 2017).

Phytochemical screening of the leaf extracts of *D. parishii* has been conducted by Ivannikov et al. (2019a). It was established that the phenolic acids and flavonoids in the glycoside form were the main groups of bioactive compounds revealed in the extracts. Moreover, these researches have employed nanotechnology to produce composites for delayed release of bioactive compounds using *D. parishii* leaf extracts and fumed silica (Ivannikov et al., 2019a).

The fact that plant extracts of *Dendrobium moniliforme* (L.) Sw. have a number of bioactive compounds (water-soluble polysaccharides, phenanthrenes, bibenzyl derivatives, and polyphenol compounds) that showed antioxidant and cytotoxic activities suggests the potential pharmacological importance of this plant. Paudel et al. (2018) have explored the antioxidant and cytotoxic activities of *D. moniliforme* extracts and detected their bioactive compounds. The total flavonoid content (TFC) content was highest (116.65 µg GAE/mg of extract) in *D. moniliforme* chloroform extract (DMC) and the total flavonoid content (TFC) content was highest (116.67 µg QE/mg of extract) in *D. moniliforme* acetone extract (DMA). *D. moniliforme* hexane extract (DMH) extract showed the highest percentage of DPPH (2.2-diphenyl-1-picrylhydrazyl) radical scavenging activity (94.48%), followed closely by *D. moniliforme* ethanol extract (DME) (94.45%), DMA (93.71%) and DMC (94.35%) at 800 µg/ml concentration. The antioxidant capacities of DMC, DMA, DMH and DME, which were measured in IC_{50} values, were much lower 42.39 µg/ml, 49.56 µg/ml, 52.68 µg/ml, and 58.77 µg/ml respectively than the IC_{50} of *D. moniliforme* methanol extract (DMM) (223.15 µg/ml). DMM at the concentration of 800 µg/ml most inhibited the growth of HeLa cells (78.68%) and DME at the same concentration most inhibited the growth of U251 cells (51.95%). The cytotoxic capacity (IC_{50}) of DMM against HeLa cells was 155.80 µg/ml of extract and that of DME against the U251 cells was 772.50 µg/ml of extract. A number of bioactive compounds were detected in both DME and DMM (Paudel et al., 2018). Chan and co-workers (2018) have investigated the polyphenol content, antioxidant activity, and inhibition ability of mushroom tyrosinase and melanogenesis of *Dendrobium officinale* Kimura & Migo (syn. D. tosaense Makino) (DT) extract.

Sukumaran and Yadav (2016) have determined *in vitro* free radical scavenging and anti-inflammatory activity of *D. macrostachyum* Lindl. Sequential stem and leaf extracts were assessed for its antioxidant and anti-inflammatory activity by *in vitro* methods. The stem ethanolic extracts exhibited significant IC_{50} value of 10.21, 31.54 and 142.97 µg/ml respectively for DPPH, ABTS radical scavenging and reducing power activity. The ethanol and water extract was highly effective as albumin denaturation inhibitors (IC_{50} = 114.13 and 135.818 µg/ml respectively) and proteinase inhibitors (IC_{50} = 72.49 and 129.681 µg/ml respectively). Membrane stabilization was also noticeably inhibited by the stem ethanolic

extract among other extracts (IC_{50} = 89.33 µg/ml) but comparatively lower to aspirin standard (IC_{50} = 83.926 µg/ml). The highest total phenol content was exhibited by the ethanolic stem and leaf extracts respectively at 20 and 16 mg of gallic acid equivalents of dry extract. On LCMS analysis 20 constituents were identified and it included a chemotaxonomic marker for *Dendrobium* species. The results of Sukumaran and Yadav (2016) showed a relatively high concentration of phenolics, high scavenger activity and high anti-inflammatory activity of the stem extract compared to the leaf extract.

In the study, conducted by Luo and Fan (2011), the polysaccharide DFHP (Mw 209.3 kDa) was first isolated from the stem of *Dendrobium fimbriatum* Hook. var. *oculatum* Hook. with the dominance of mannose, glucose, galactose. Free radicals scavenging activities *in vitro* indicated that DFHP has significant radicals scavenging abilities on ABTS and Hydroxyl radicals. The scavenging effects were powerful and close to the positive control (Vc). Therefore, the polysaccharide DFHP should be explored as a novel potential antioxidant. On the other hand, DFHP exhibited a weak scavenging effect on DPPH radical compared to the reference (Luo and Fan, 2011).

The protective effects of *Dendrobium officinale* polysaccharides against H_2O_2-induced injury in H9c2 cells were demonstrated. The results also indicated the anti-oxidative capability of *D. officinale* polysaccharides. Zhao et al. (2017) have studied the protective effect of *D. officinale* polysaccharides against H_2O_2-induced oxidative stress in H9c2 cells. MTT assay was carried out to determine the cell viability of H9c2 cells when pretreated with *D. officinale* polysaccharides. Fluorescent microscopy measurements were performed for evaluating the apoptosis in H9c2 cells. Furthermore, the effects of *D. officinale* polysaccharides on the activities of antioxidative indicators (malondialdehyde, superoxide dismutase), reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) levels were analyzed. *D. officinale* polysaccharides attenuated H_2O_2-induced cell death, as determined by the MTT assay. *D. officinale* polysaccharides decreased malondialdehyde levels, increased superoxide dismutase activities, and inhibited the generation of intracellular ROS. Moreover, pretreatment with *D. officinale* polysaccharides also inhibited apoptosis and increased the MMP levels in H9c2 cells (Zhao et al., 2017).

HPLC-profiling of metabolites extracted from *Dendrobium parishii* seeds was undertaken by Buyun and Grakhov (2014). It was revealed that seeds of this species comprise, together with phenolics, the low polar inhibitors, apparently, also terpenoid or steroid derivatives.

A polysaccharide is the main active ingredient in *D. officinale*; its antioxidant activity is a hot research topic nowadays. The antioxidant activities of the polysaccharide *in vitro* assay indicate that the *D. officinale* polysaccharide has a good scavenging activity of 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical, higher scavenging activity of hydroxyl radical, and metal chelating activities (Luo et al., 2016). Zhang et al. (2017) have adopted a model of H_2O_2-induced H9c2 cardiomyocytes apoptosis, aiming to study the effect of *D. officinale* Polysaccharide (DOP-GY) for cardiomyocyte apoptosis caused by oxidative stress and its possible mechanism. The pretreatment of DOP-GY (low dose: 6.25 µg/mL, medium-dose: 12.5 µg/mL, high dose: 25 µg/mL) followed by a 2h incubation with 200 µM H_2O_2 elevated the survival rate, cut the

lactate dehydrogenase (LDH) leakage, reduced lipid peroxidation damage, improved the activity of the endogenous antioxidant enzymes. In addition, the pretreatment of DOP-GY significantly inhibited the production of ROS, declined the mitochondrial membrane potential, down-regulated pro-apoptosis protein and up-regulated anti-apoptosis protein. The protective effect was correlated with the PI3K/Akt and MAPK signal pathway. The study of Zhang and co-workers (2017) suggests that DOY-GY has the potential to exert cardioprotective effects against H_2O_2-induced H9c2 cardiomyocyte apoptosis (Zhang et al., 2017).

The crude *D. officinale* polysaccharide (DOP) was obtained by hot water extraction- ethanol precipitation method, and four new polysaccharide fractions (DOP-40, DOP-50, DOP-60, and DOP-70) were further obtained from the crude DOP by fractional precipitation with ethanol method by Xing et al. (2018). The antioxidant activities of them were evaluated by the reducing power assay, and the superoxide anion, 2.2-diphenyl-1-picrylhydrazyl (DPPH), and hydroxyl free radicals scavenging assays, respectively. Finally, the anticancer activities of them were investigated via the MTT assay and the western blot analysis using HepG2 cells. Among these four purified fractions were mainly composed of D-mannose and D-glucose with different molar ratios, and their average molecular weights were 999, 657, 243 and 50.3 kDa, respectively. What's more, DOP-70 always exhibited the strongest antioxidant and anticancer activities, while DOP-40 and DOP-60 showed very close antioxidant and anticancer activities which were better than that of DOP-50. The western blotting analysis also showed that DOP-40, DOP-60, and DOP-70 induced apoptosis in HepG2 human liver cancer cells through the Bcl-2 and Bax-dependent pathway (Xing et al., 2018).

Liao et al. (2015) have compared the radical scavenging activity of five different acidic polysaccharides, and to find the correlation with the functional groups. Five kinds of *Dendrobium* polysaccharides had different abilities to scavenge ABTS+ free radicals and hydroxyl free radicals. Moreover, the study had shown that five kinds of antioxidant activity of acidic polysaccharides had an obvious correlation between uronic acid and sulfuric acid. The antioxidant activity of each sample was positively correlated with the content of uronic acid and negatively correlated with the content of sulfuric acid. Sulfuric acid can inhibit the antioxidant activity of acidic polysaccharide but uronic acid can enhance the free radical scavenging activity. By analyzing the structural characteristics of five acidic polysaccharides, all samples have similar structures, however, *D. denneanum* Kerr, *D. devonianum* Paxton and *D. officinale* which had β configuration have higher antioxidant activity than *D. nobile* and *D. fimbriatum* which had a configuration (Liao et al., 2015).

The anti-diabetic activity of *Dendrobium loddigesii* polyphenols (DJP) was evaluated by Li et al. (2018). In particular, the serum biochemical index and tissue appearance were evaluated. In order to gain an insight into the anti-diabetic mechanism, the oxidative stress index, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and gut microbiota modulation was determined by ELISA, immunohistochemistry or high throughput sequencing 16S rRNA gene. The results revealed that DJP had the effects to decrease the blood glucose, body weight, low-density lipoprotein cholesterol (LDL-C) levels and increase insulin (INS) levels in the mice. DJP improved the mice's fatty liver and diabetic nephropathy. DJP showed the anti-oxidative abilities to reduce the malondialdehyde (MDA) level and increase the contents of

superoxide dismutase (SOD), catalase (CAT) as well as glutathione (GSH). DJP exerted the anti-inflammatory effects of decreasing expression of IL-6 and TNF-α. After treatment of DJP, the intestinal flora balance of the mice was ameliorated, increasing *Bacteroidetes* to *Firmicutes* ratios as well as the relative abundance of *Prevotella*/*Akkermansia* and reducing the relative abundance of S24-7/*Rikenella*/*Escherichia coli*. The study of Li et al. (2018) revealed for the first time that DJP improves the diabetic db/db mice symptoms of diabetes and complications, which might be due to the effects that DJP induced the decrease of inflammation as well as oxidative stress and improvement of intestinal flora balance.

Pan et al. (2012) have studied the preventive effects of galactoglucomannan from *D. officinale* (syn. *D. huoshanense*) on sodium selenite-induced hepatic damage and fibrosis in rats. Oral administration of the galactoglucomannan brought about a decline in the activities of serum lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, and the levels of H_2O_2 and malonic dialdehyde. The level of glutathione was increased, hepatic plasma membrane fluidity was reinstated, and maintained the activities of antioxidative enzymes including catalase, glutathione *S*-transferase, and superoxide dismutase were maintained. The prevention of selenite-induced hepatic damage and fibrosis was in keeping with the attenuated expression of type I collagen and transforming growth factor-β1. Thus, the galactoglucomannan stands the chance of being developed into an antifibrotic agent for forestalling hepatic damage and fibrosis (Pan et al., 2012).

Conclusions

The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives level, as well as total antioxidant capacity, were non-significantly altered in the erythrocytes' suspension after *in vitro* incubation with an extract obtained from *D. parishii*. More significant changes were observed in the plasma. *D. parishii* leaf extract caused to increase in the formation of intracellular aldehydic and ketonic derivatives of OMP in the extract-treated plasma, but these results were non-significant. Total antioxidant capacity was non-significant decreased both in plasma and erythrocytes. The lack of clinical safety and toxicity data for *D. parishii* and many other herbs that are increasingly being used suggests the necessity of further investigations regarding their influence on organs and tissues function, including the evaluation of molecular mechanisms involved in order to exploit them for potential therapeutic benefits. Screening of *Dendrobium* plants for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

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ASSESSMENT OF BIOMARKERS FOR ANTIOXIDANT DEFENSE IN THE EQUINE ERYTHROCYTES AFTER INCUBATION WITH *BEGONIA PSILOPHYLLA* IRMSCH. LEAF EXTRACT

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In this study, the investigation of the effect of *Begonia psilophylla* Irmsch. leaf extract on the antioxidant defenses [catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity (TAC)] in the equine erythrocyte suspension and plasma was undertaken. In relation to blood cells, circulating erythrocytes are regularly exposed to stress conditions and are especially vulnerable as they have no membrane repair mechanism or regenerative capacity. Freshly sampled *B. psilophylla* leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in ratio 1 : 19, w/w) at room temperature. The extract was then filtered and used for analysis. A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control, the 0.1 M phosphate buffer was used. After incubation, the mixture at 37 °C for 60 min it was centrifuged at 3,000 rpm for 5 min with continuous stirring. Erythrocytes and plasma aliquots were used in the study. The presence of the extract during incubation of erythrocyte suspension and plasma caused a non-considerable increase of catalase and glutathione peroxidase activity, while the activity of glutathione reductase was not changed compared to control samples. At the same time, *B. psilophylla* extract caused a statistically significant decrease in ceruloplasmin level by 47.6% (p <0.05). The total antioxidant capacity in the equine erythrocytes' suspension and plasma after *in vitro* incubation with *B. psilophylla* leaf extract was non-significantly changed. Based on the collected data, positive trends were observed in the regressions of GPx activity against catalase activity (r = 0.809, p = 0.0005), and ceruloplasmin level (r = 0.553, p = 0.017) for in the equine erythrocytes' suspension after *in vitro* incubation with *B. psilophylla* leaf extract. Therefore, our study suggests that crude extract obtained from *B. psilophylla* leaves has an effective antioxidant and anti-inflammatory effect after the treatment of equine erythrocytes. It could be interpreted that *B. psilophylla* extract showed the anti-inflammation effect expressed as decreasing the ceruloplasmin level in the plasma. The pronounced effect of *B. psilophylla* leaf extract, probably, could be attributed to its secondary metabolites content. However, the components responsible for the antioxidative activity of *B. psilophylla* extract is currently unclear. Therefore, further investigations need

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to be carried out to isolate and identify the phytochemical constituents and antioxidant compounds present in the plant extract.

Keywords: *Begonia psilophylla* extract, equine erythrocytes, total antioxidant capacity, superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin

Introduction

The health benefits of plants and plant food-based diets could be related to both integrated antioxidant and anti-inflammatory mechanisms exerted by a wide array of phytochemicals present in fruit, vegetables, herbs, and spices. Therefore, there is increasing interest in identifying foods, food extracts and phytochemical formulations from plant sources that are able to efficiently modulate oxidative and inflammatory stress to prevent diet-related diseases (Serafini and Peluso, 2016). Antioxidants from plants are a large group of bioactive compounds (i.e., flavonoids, phenolic compounds, sulfur-containing compounds, tannins, alkaloids, phenolic diterpenes, and vitamins) demonstrating different antioxidant activities. For example, flavonoids have the ability to scavenge free radicals and can form complexes with catalytic metal ions rendering them inactive. Studies have shown that spices and herbs such as rosemary, sage, and oregano are excellent sources of antioxidants with their high content of phenolic compounds (Yashin et al., 2017).

Antioxidants help prevent cellular damage caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide anion radical ($O_{2.}–$) (Halliwell and Gutteridge, 1989). Antioxidants can be enzymes or molecules such as vitamins E and C, urea, glutathione, etc. Antioxidant enzymes include superoxide dismutase (SOD), which catalyzes the dismutation of $O_{2.}–$ to water and oxygen, catalase (CAT), which reduces H_2O_2 to water and oxygen, and glutathione reductase (GR), which regenerates reduced glutathione (GSSG) used as a direct scavenger of ROS or as a substrate for the antioxidant enzyme glutathione peroxidase (GPx) (Halliwell and Gutteridge, 1989).

A diverse group of antioxidants such as polyphenols, ascorbic acid, vitamin A, α-lipoic acid, thioredoxin, glutathione, melatonin, coenzyme Q, beta carotenoids, alpha-tocopherols as well as antioxidant enzymes has been widely investigated for the prevention and treatment of diseases resulting from oxidative damage (Ighodaro and Akinloye, 2017).

Begonia is a mega-diverse genus containing more than 1800 species, with a very high proportion of microendemics and hotspots of diversity in the Andes and Southeast Asia (Hughes et al., 2018). The first living plant in *Begonia* was introduced to Europe during the eighteenth century, and thereafter over 400 natural species have been introduced for horticulture and many cultivars have been developed (Tebbitt, 2005). Begonias are among the most popular ornamental plants in the world thanks to their large, showy, and long-lasting multicolor flowers (Sakhanokho et al., 2013; Twyford et al., 2014). They are used as garden plants and potted plants, in hanging baskets, and as greenhouse flowers, as well as potherbs or leafy vegetables in many parts of the world. The roots and tubers of some species have been reported to possess antimicrobial activities and are used to treat various ailments (Sakhanokho et al., 2013).

In our previous study, we have assessed the percentage of equine erythrocyte hemolysis induced by treatment with extracts of various species of *Begonia* genus to exemplify their further potential development and use as a drug against metabolic diseases in medicine and veterinary (Tkachenko et al., 2017). Our study demonstrated that among 30 species of *Begonia* genus, the most species screened possessed anti-hemolytic activity. The results of these biological assays demonstrated that compounds present in *B. glabra*, *B. aconitifolia*, *B. sanguinea*, *B. thiemei*, *B. masoniana*, B. × *credneri*, *B. oxyphylla*, *B. subvillosa*, *B. ulmifolia*, *B. conconvulaceae* can prevent the formation of methemoglobin and reduce hemolysis, while *B. erythrophylla*, *B. psilophylla*, and *B. arborescens* var. oxyphylla extracts can facilitate the formation of methemoglobin and hemolysis in healthy equine blood. Extracts from leaves of *B. foliosa*, *B. rex*, *B. solimutata*, *B. mexicana*, *B. goegoensis*, *B. imperialis* var. *smaragdina*, *B. pustulata*, *B. peltata*, *B. cucullata*, *B. angularis*, *B. boisiana*, *B. venosa* exhibited the decrease of percentage hemolysis of equine erythrocytes, but these alterations were non-significant (Tkachenko et al., 2017).

Moreover, we also assessed the antioxidant effect of leaf extract obtained from *Begonia rex* Putz. on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification] and antioxidant defenses [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity (TAC)] using the equine erythrocytes model. The extract during incubation of erythrocyte suspension caused a non-considerable TBARS formation (by 18%, $p > 0.05$), while the content of aldehydic and ketonic derivatives of oxidatively modified proteins was decreased (by 7 and 8%, $p > 0.05$, respectively) compared to control. The aqueous leaf extract of *B. rex* has proven to be the most effective to increase the catalase and GPx activity. The increase of the catalase and GPx activity was induced by TAC enhancement by 34% ($p > 0.05$). The SOD activity was non-significantly decreased by 17% ($p > 0.05$). *B. rex* extract caused a statistically significant decrease in ceruloplasmin level by 64% ($p < 0.05$). These *in vitro* assays indicate that *B. rex* leaf extract screened is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity of *B. rex* extract is currently unclear (Buyun et al., 2018). *In vitro* microbiological investigation of ethanolic extracts obtained from leaves of various *Begonia* species against bacteria and fungi strain was revealed the antibacterial properties of these plants (Tkachenko et al., 2016, 2017a, b).

In this study, we have focused on the antioxidant effect of leaf extract obtained from *Begonia psilophylla* on antioxidant defenses biomarkers [catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity] using the equine plasma and erythrocytes model. Thus, equine erythrocytes were proved to be a good tool for analyzing the oxidative stress biomarkers as a mechanism of antioxidant action of *B. psilophylla* leaf extract.

Material and methodology

Collection of Plant Materials

The leaves of *Begonia psilophylla*, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. The biochemical screening of *Begonia* leaf extracts has been carried out in the laboratory of the Institute of Biology and Earth Sciences, Pomeranian University in Slupsk (Poland). Our current scientific project has been undertaken in the frame of the cooperation program between the Institute of Biology and Earth Sciences (Pomeranian University in Slupsk, Poland) and M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine, aimed at assessment of medicinal properties of tropical plants. It has encompassed some tropical mega-diverse genera, including genus *Begonia* with a near pantropical distribution.

Preparation of Plant Extracts

Freshly collected leaves *B. psilophylla* were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in ratio 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Horses

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30’ 48.0” E 16º 57’ 44.9”), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters that were in the reference ranges. The females were non-pregnant.

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (phosphate buffer) was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes and plasma aliquots were used in the study.

Assay of Superoxide dismutase activity

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0) by Kostiuk and co-workers (1990) method. Activity is expressed in units of SOD per mL of blood.

Assay of Catalase activity

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al. (1988). One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 mmol H_2O_2 per min per L of blood.

Assay of Glutathione peroxidase activity

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by detecting the non-enzymatic utilization of GSH (the reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to by the method of Moin (1986). The assay mixture contained 0.8 mL of 0.1 M Tris-HCl buffer with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 mL of 4.8 mM GSH, 0.2 mL of hemolyzed erythrocytes (1 : 20), 1 mL of 20 mM t-butyl hydroperoxide, and 0.1 mL of 0.01 M 5.5-dithiobis-2-nitrobenzoic acid. The rate of GSH reduction was followed spectrophotometrically at 412 nm. GPx activity is expressed as μmol GSH per min per mL of blood.

Assay of the Ceruloplasmin level

The ceruloplasmin (CP, EC 1.16.3.1) level in the plasma was measured spectrophotometrically at 540 nm, as described by Ravin (1961). The assay mixture contained 0.1 mL of plasma, 0.4 M sodium acetate buffer (pH 5.5), and 0.5% *p*-phenylenediamine. The mixture was incubated at 37 °C for 60 min. Before cooling at 4 °C for 30 min, the mixture was added to 3% sodium fluoride for inhibition. Ceruloplasmin was expressed in mg per L of plasma.

Measurement of Total antioxidant capacity (TAC)

The TAC level in the plasma and erythrocytes' suspension was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the parameters (significance level, p <0.05) was examined using the Mann-Whitney *U*-test (Zar, 1999). In addition, the relationships between antioxidant defense biomarkers were evaluated using Spearman's correlation analysis. All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

Figure 1A summarizes the results obtained by incubating equine erythrocyte suspension in the presence of the aqueous extract of *B. psilophylla*. As seen, the presence of the extract during incubation of erythrocyte suspension and plasma caused a non-considerable increase of catalase and glutathione peroxidase activity, while the activity of glutathione reductase was non-changed compared to control samples.

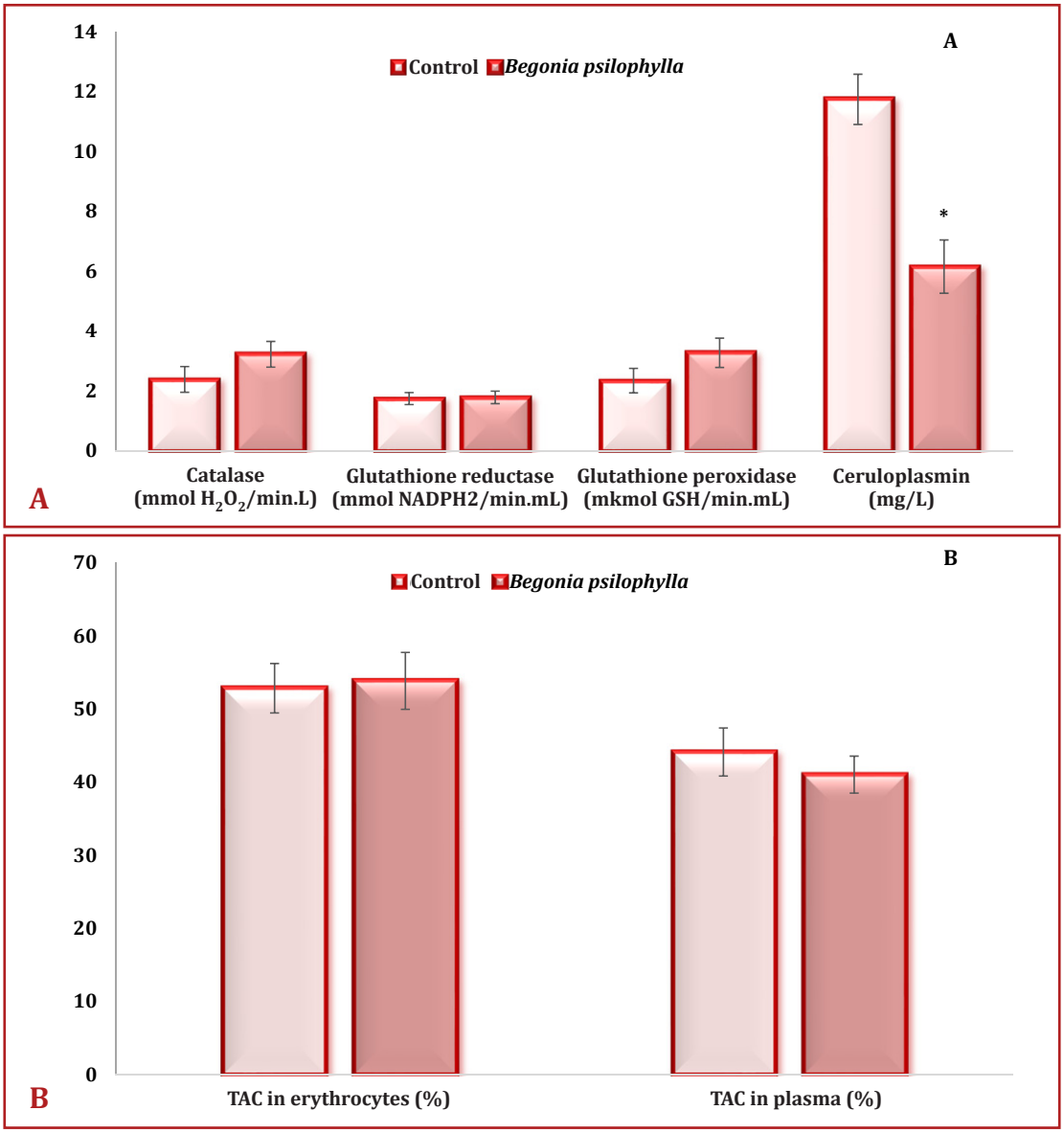


Figure 1 The catalase, glutathione reductase, glutathione peroxidase, and ceruloplasmin activity (A), as well as total antioxidant capacity (TAC) in the equine erythrocytes' suspension and plasma (B) after *in vitro* incubation with *Begonia psilophylla* leaf extract (M ±m, *n* = 18)

In our study, the aqueous leaf extract of *B. psilophylla* has proven to be the most effective to increase the catalase and GPx activity (by 35.3%, p >0.05 and 39.7%, p >0.05) (Figure 1A). Else, *B. psilophylla* extract caused a statistically significant decrease in ceruloplasmin level by 47.6% (p <0.05) (Figure 1A).

Ceruloplasmin is a serum ferroxidase that contains greater than 95% of the copper found in plasma. This protein is a member of the multicopper oxidase family, an evolutionarily conserved group of proteins that utilize copper to couple substrate oxidation with the four-electron reduction of oxygen to water (Hellman and Gitlin, 2002). It has been proposed to function in copper transport, oxidation of organic amines, Fe^{2+}–oxidation and the regulation of cellular iron levels, and regulation of catechols metabolism, radical scavenging and other antioxidant processes (Healy and Tipton, 2007). Therefore, we suggested that *B. psilophylla* extract could exhibit the anti-inflammatory effect decreasing ceruloplasmin level after *in vitro* incubation with leaf extract.

The total antioxidant capacity (TAC) in the equine erythrocytes' suspension and plasma after *in vitro* incubation with *B. psilophylla* leaf extract was non-significantly changed (increased by 1.9% in the erythrocytes and decreased by 7% in the plasma) (Figure 1B).

Based on the collected data, positive trends were observed in the regressions of GPx activity against catalase activity (r = 0.809, p = 0.0005), and ceruloplasmin level (r = 0.553, p = 0.017) for in the equine erythrocyte suspension after *in vitro* incubation with *B. psilophylla* leaf extract (Figure 2).

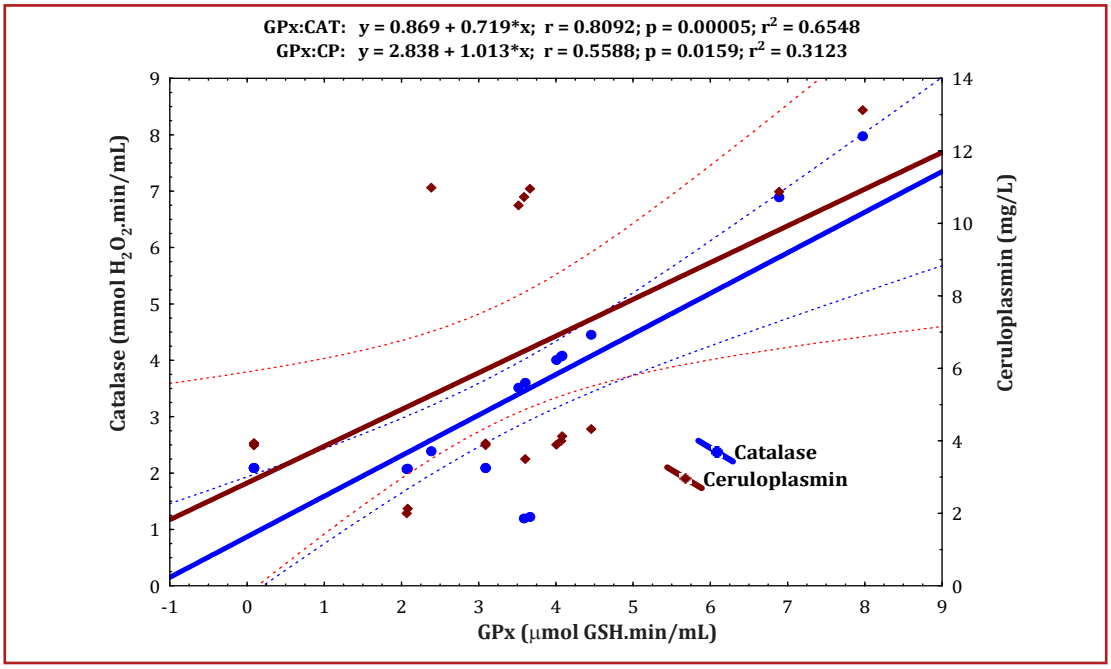


Figure 2 Correlations between oxidative stress biomarkers GPx activity, catalase activity and ceruloplasmin level in the equine erythrocyte suspension after *in vitro* incubation with *Begonia psilophylla* leaf extract

Our study demonstrates changes in the antioxidant defenses biomarkers of equine erythrocytes incubated with *B. psilophylla* leaf extract (Figures 1 and 2). Accordingly, our study suggests that crude extract obtained from *B. psilophylla* leaves has an effective antioxidant and anti-inflammatory effect after the treatment of equine erythrocytes. *B. psilophylla* extract showed the anti-inflammation effect expressed as decreasing on the ceruloplasmin level in the plasma. The pronounced effect of leaf *B. psilophylla* extract could be attributed to its secondary metabolites, e.g. polyphenols, flavonoids, anthocyanin contents.

It has become increasingly clear that all secondary metabolite components in *Begonia* species displayed antioxidant and antimicrobial properties through different biological mechanisms (Hossain and Nagooru, 2011). It is reasonable to suggest, that variation in the chemical profile of plants could influence their biological activities. Therefore, it was important to know the chemical composition of extracts to correlate with their antioxidant activities.

Aswathy et al. (2016) have demonstrated that *Begonia rex-cultorum* (Baby rainbow) and *Begonia malabarica* exhibited the highest antioxidant activities. The anthocyanin concentration positively correlates with the antioxidant activities among the cultivars. The diversity in radical scavenging in these assays may be due to factors like stereoselectivity of the radicals or due to the differential solubility of anthocyanin molecules in the crude extract (Aswathy et al., 2016).

A study conducted by Kalpanadevi and Mohan (2012) has shown that the extracts of *B. malabarica* and *B. floccifera* contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. The methanol extracts of whole plants of *B. malabarica* and *B. floccifera* showed potent *in vitro* antioxidant activities using various models, i.e. DPPH, hydroxyl, superoxide and ABTS radical scavenging activity. *B. malabarica* and *B. floccifera* whole plant extracts (methanol) exhibited potent *in vitro* antioxidant activity in DPPH radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging, ABTS radical cation scavenging and reducing power in comparison to the known antioxidants, such as ascorbic acid and Trolox. It was observed that methanol extracts of the whole plant of *B. malabarica* had higher activity than that of the whole plant extract of *B. floccifera*. At a concentration of 1 mg/mL, the scavenging activity of methanol extract of the whole plant of *B. malabarica* reached 96.14% while at the same concentration, that of the *B. floccifera* was 63.51%. At a concentration of 1 mg/mL, the scavenging activity of methanol extract of the whole plant of *B. malabarica* exhibited higher activity than ascorbic acid. Superoxide radical scavenging activity of *B. malabarica* and *B. floccifera* whole plant extracts was studied and compared with ascorbic acid. It was observed, that the superoxide radical scavenging activity of *B. malabarica* and *B. floccifera* extracts increased with increasing concentration. At a concentration of 1 mg/mL, the superoxide radical scavenging activity of methanol extracts of *B. malabarica* and *B. floccifera* were found to be 81.55% and 62.56%, respectively. Among plant extracts screened, *B. malabarica* exhibited higher activity (79.11%) at a concentration of 1 mg/mL than Trolox. The reducing power of extracts increased with an increase in concentration. Nevertheless, the reducing power values of the methanol extracts of *B. malabarica* whole plant was slightly higher than that of ascorbic acid (Kalpanadevi and Mohan, 2012).

Many studies have suggested that plant secondary metabolites obtained from Begoniaceae representatives are responsible for their antioxidant activity. Literature data confirmed that extracts from various parts of the *Begonia* plants exhibited strong antioxidant properties, effectively deactivating the stable, synthetic DPPH radical. For example, Indrakumar and co-workers (2014) have evaluated the antimicrobial and *in vitro* antioxidant potential of extracts of *B. dipetala*. Antimicrobial activity, DPPH free radical scavenging activity, Superoxide anion scavenging activity, Nitric oxide scavenging activity, and Ferric reducing antioxidant power assay were carried out on different concentration of the extracts. The reducing power assay of the ethanolic extract showed a reduction at various concentrations similar to that of standard ascorbic acid. The *in vitro* antioxidant studies clearly indicate that the ethanolic extract of *B. dipetala* has significant antioxidant activity (Indrakumar et al., 2014).

Methanol and ethyl acetate extract of *B. trichocarpa* has shown a marked dose-dependent antioxidant activity in both 2.2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method and Nitric acid scavenging method in the study of Sindhu et al. (2016). Additionally, total phenol content of different extracts of *B. trichocarpa* was estimated. It was established that methanol leaf extracts contain 49.96% of phenol content and 23.71% anthocyanin content.

Consequently, it was assumed that the antioxidant activity of *B. trichocarpa* may be due to the high phenol content and the presence of anthocyanin (Sindhu et al., 2016).

Previous studies have reported both protective and destabilizing effects of various plant extracts on erythrocyte membrane stability (Awe et al., 2009; Chikezie et al., 2011). It was suggested that the presence of phytochemicals such as tannins, saponin was responsible for destabilizing effects of certain plant extracts on erythrocyte membrane. *Carica papaya* L. leaf extracts, on the contrary, exhibited a significant inhibition of hemolysis *in vitro* and could have a potential therapeutic effect on disease processes causing destabilization of biological membranes (Ranasinghe et al., 2011).

The results of this research indicated that crude extract obtained from *B. psilophylla* leaves was highly effective for the control of oxidative stress. Protective effect of *B. psilophylla* extracts was evident by amelioration in plasma antioxidant enzymes‘ activities as compared to control. The antioxidant defense system was improved through suppression on the ceruloplasmin level by treatment of equine plasma with *B. psilophylla* leaf extract. Finally, we can report that *B. psilophylla* crude extract is a good antioxidant agent for oxidative stress prevention.

Conclusions

The results of current study revealed that crude extract obtained from *B. psilophylla* leaves exhibited a marked antioxidant effect after the treatment of a suspension of equine erythrocytes and plasma. The findings suggest that protective effect of *B. psilophylla* extract is evident by amelioration in antioxidant enzymes‘ activities. The antioxidant defense system was determined by the increase of glutathione peroxidase and catalase activity, as first line defense antioxidant enzymes, after the treatment with *B. psilophylla* extract. Moreover, *B. psilophylla*

extract showed the anti-inflammation effect exhibited as a decreasing ceruloplasmin level in the plasma. The pronounced effect of *B. psilophylla* leaf extract, probably, could be attributed to its secondary metabolites content. However, the phytochemicals responsible for the antioxidative activity of *B. psilophylla* extract is currently unclear. Therefore, further investigations need to be carried out to isolate and identify the phytochemical content and antioxidant compounds present in the *B. psilophylla* leaf extract, which can effect the erythrocyte function.

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PRELIMINARY *IN VITRO* ASSESSMENT OF EFFECTS OF LEAF EXTRACTS OF VARIOUS *SANSEVIERIA* THUNB. SPECIES ON THE LIPID PEROXIDATION LEVEL IN THE EQUINE PLASMA

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The aim of this study was to evaluate the *in vitro* effect of buffer extracts obtained from the leaves of various *Sansevieria* species against lipid peroxidation in the equine plasma. The succulent leaves of living plants of *Sansevieria francisii* Chahin, *S. caulescens* N.E.Br., *S. suffruticosa* N.E.Br., *S. roxburghiana* Schult. & Schult.f., *S. metallica* Gérôme & Labroy, *S. gracilis* N.E.Br., *S. hyacinthoides* (L.) Druce, *S. cylindrica* Bojer ex Hook., *S. canaliculata* Carrière, *S. aethiopica* Thunb., *S. kirkii* Baker, *S. trifasciata* Prain, *S. forskaliana* (Schult. & Schult.f.) Hepper & J.R.I. Wood, *S. fischeri* (Baker) Marais, *S. dooneri* N.E.Br., *S. intermedia* N.E.Br., *S. parva* N.E.Br. were sampled for the study. Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and investigated for their pro-oxidant and antioxidant activity. A volume of 0.1 mL of the various extracts was incubated with 1.9 mL of equine plasma at 37 °C for 60 min with continuous stirring. The results of the study showed that the TBARS level as a biomarker of lipid peroxidation was significantly increased after incubation with extracts of selected species belonging to the *Sansevieria* genus and these results were statistically significant. The most potent effect was demonstrated by the *S. intermedia*, *S. forskaliana*, *S. trifasciata*, *S. parva*, and *S. caulescens* leaf extracts compared to phosphate buffer as control sample (increased by 65.6, 53.2, 51.6, 50, and 48%, respectively). *S. kirkii*, *S. aethiopica*, and *S. suffruticosa* caused the less increase of the TBARS level after 1-h incubation with equine plasma (by 18.8, 27.6, and 30.8%, respectively, $p < 0.05$). To conclude, *Sansevieria* species possess a promising antioxidant and pro-oxidant potential. Further studies involving phytochemical identification of the main compounds in plants are necessary to affirm and maximize the possible use of the plants as a therapeutic remedy for the prevention of oxidative stress. The dose-depended antioxidative and pro-oxidative effects of various *Sansevieria* species in both plasma and erythrocyte suspension will be further studied in detail.

Keywords: *Sansevieria* Thunb., extracts, 2-thiobarbituric acid reactive substances (TBARS), oxidative stress, equine plasma

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Introduction

Many studies clearly demonstrate that various plants of the *Sansevieria* genus are effective agents in the treatment and prevention of many diseases and disorders. This justifies the use of the plant extract for the treatment of diarrhea. The use of these plants in folk medicinal remedies for treating various health problems has been reported. Most cases confirm the therapeutic value of the plants. These plants have been tested in the treatment of hemorrhoids, pain, smallpox, chicken-pox, and measles, venereal diseases, malnutrition, paralysis, epilepsy, convulsions, and spasm, pulmonary troubles, and a vermifuge, as well as a remedy for parasitic infections. In studies carried out in Nakuru and Maragua districts of Kenya by Khalumba et al. (2005), they identified five use categories of *Sansevieria* plants, namely medicine (33% of the reports), fibers (24%), soil conservation (22%), fodder (18%), and other uses (14%) for four species, *Sansevieria ehrenbergii* Schweinf. ex Baker, *S. parva* N.E.Br., *S. raffillii* N.E. Br., and *S. suffruticosa* N.E. Br. Chhabra et al. (1987) mentioned the use of *S. bagamoyensis* N.E.Br. for the treatment of convulsive fever in Tanzania. Watt and Breyer-Brandwijk (1962) listed the use of *S. hyacinthoides* (L.) Druce in the treatment of a toothache and earache and the use of the rhizome decoction of *S. kirkii* as a purgative both reported from East Africa. Yet, Kiringe (2006) reported on the use of *S. volkensii* Gürke for the treatment of sexually transmitted diseases such as gonorrhea. In Kenya, Owuor and Kisangau (2006) included the use of *S. parva* leaf sap for the treatment of snakebite wounds and *S. kirkii* Baker extracts for treatment of snakebite wounds. The ethanol and water extracts of *S. trifasciata* Prain leaves showed a dose-dependent and significant increase in pain threshold and possess mild analgesic properties (Anbu et al., 2009). It was suggested that *S. trifasciata* can be good medicinal plant especially as antibacterial agent due to the high toxicity level (Berame et al., 2017).

For instance, the results obtained in a study of Adeyemi et al. (2009) suggest that the aqueous root extract of *S. liberica* Gérôme & Labroy possesses antidiarrhoeal property due to inhibition of gastrointestinal propulsion and fluid secretion, possibly mediated through inhibition of the nitric oxide pathway. Moreover, Akindele et al. (2015) have evaluated the anticancer activity of root extracts of *S. liberica* using a combination of *in vitro* and *in vivo* models. The aqueous root extract of *S. liberica* is used in African folklore medicine for ailments including chronic pain, inflammatory conditions, and convulsive disorders (Amida et al., 2007). The root part of *S. liberica* is used in ethnomedicine in the treatment of fever, headache and cold, as well as analgesic, antibiotic and anti-inflammatory (Watt and Breyer-Brandwijk, 1962). Preparations of the *S. liberica* are commonly used across Nigeria for the treatment of inflammatory conditions (Akindele et al., 2015). Nevertheless, in spite of these data, Takawira-Nyenya et al. (2014) reported that the documentation of ethnobotanical uses of genus *Sansevieria* is incomplete. Results of Amida et al. (2007) indicated that the aqueous root extract of *S. liberica* shows that it is relatively safe when given orally, and there is a pointer toward possible usefulness to boost red blood cells and increase sperm quality, but findings indicate potential to affect hepatic cells at high doses, when administered chronically (Amida et al., 2007). The treatment with the plant extracts of the rhizomes of *S. liberica* protects the liver against carbon tetrachloride-induced hepatotoxicity in Wistar albino rats (Ikewuchi et al., 2011). This hepatoprotective activity may have been produced via inhibiting lipid peroxidation by

exerting a membrane-stabilizing action or inhibiting cytochrome P_{450} aromatase (Ikewuchi et al., 2011).

Recently, tropical and subtropical plants containing antioxidants have become an area of scientific research because they have greater health benefits with various pharmacological activities. The antioxidant and anti-proliferative activities of the methanol extract *S. roxburghiana* Schult. & Schult. f. and its fractions have been explored by Maheshwari et al. (2017). The results of these researchers observed that the ethyl acetate fraction of *S. roxburghiana* exhibited effective antioxidant and anti-proliferative activities. It was suggested that the phenolic compounds identified in the ethyl acetate fraction could be responsible for the activities (Maheshwari et al., 2017).

In our previous study (Buyun et al., 2016; Tkachenko et al., 2017), we have evaluated the antibacterial capacity of ten species of *Sansevieria* genus in order to validate scientifically the inhibitory activity for microbial growth attributed by their popular use and to propose new sources of antimicrobial agents. Also antimicrobial activities of extracts obtained from leaves of various species of *Sansevieria* genus were investigated (Buyun et al., 2016–2018; Tkachenko et al., 2016, 2017). Moreover, our previous study (Tkachenko et al., 2018) have demonstrated their protective effects against lipid peroxidation. Specifically, the results indicated that extracts of *S. francisii*, and *S. forskaliana* led to a decrease of TBARS concentration, a biomarker of lipid peroxidation, in erythrocytes (by 16.9% and 8.4%, p >0.05, respectively). When erythrocytes were incubated with *S. aethiopica*, *S. caulescens*, *S. roxburghiana*, *S. gracilis*, the TBARS level was similar to that of the untreated erythrocytes. In the meantime, the treatment of *S. canaliculata*, *S. suffruticosa*, *S. metallica*, *S. fischeri*, *S. dooneri*, *S. trifasciata*, *S. parva*, *S. intermedia*, and *S. kirkii* non-significantly increase the formation of intracellular TBARS in the extract-treated erythrocytes by approximately 7–20%, respectively. However, *S. hyacinthoides* and *S. cylindrica* had a significant increase of TBARS level in the extract-treated erythrocytes (by 29.7% and 21%, p <0.05, respectively). Despite the medicinal relevance of plants, our studies have suggested that these plants are potentially pro-oxidant in dose studied (5 mg per mL) (Tkachenko et al., 2018).

Therefore, the aim of this study was to evaluate the *in vitro* effect of buffer extracts obtained from the leaves of various species belonging to the *Sansevieria* genus against lipid peroxidation in equine plasma.

Material and methodology

Collection of Plant Material

The leaves of *Sansevieria* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Specifically, the leaves of *Sansevieria francisii* Chahin, *S. caulescens* N.E.Br., *S. suffruticosa* N.E.Br., *S. roxburghiana* Schult. & Schult.f., *S. metallica* Gérôme & Labroy, *S. gracilis* N.E.Br., *S. hyacinthoides* (L.) Druce, *S. cylindrica* Bojer ex Hook., *S. canaliculata* Carrière, *S. aethiopica* Thunb., *S. kirkii* Baker, *S. trifasciata* Prain, *S. forskaliana* (Schult. & Schult.f.) Hepper & J.R.I. Wood, *S. fischeri* (Baker) Marais, *S. dooneri* N.E.Br., *S. intermedia* N.E.Br., *S. parva* N.E.Br. were

sampled for the study. Various databases available for searching collections of living plants, e.g. World Checklist of Selected Plant Families (WCSP, 2018), International Plant Names Index, The Plant List, have been used for the taxonomic identity of plants screened.

Preparation of Plant Extracts

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and investigated for their antioxidant activity. The extract was stored at -20 °C until use.

Horses

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30' 48.0" E 16º 57' 44.9"), aged 8.9±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were taken simultaneously in all horses from the jugular vein in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Whole blood was stored in sterile tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min at 4 °C using a refrigerated centrifuge to remove plasma. The separated erythrocytes were washed three times in 4 mM phosphate buffer saline (PBS), pH 7.4. After centrifugation, the supernatant and the buffy coat were carefully removed with each wash. Washed erythrocytes were finally re-suspended to the desired hematocrit level in 4 mM PBS. The erythrocytes and plasma were stored at 4 °C. A volume of 0.1 mL of the various extracts was added to 1.9 mL of equine plasma. For positive control, PBS was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Plasma aliquots were used in the study.

Quantitative estimation of lipid peroxidation by determination of 2-thiobarbituric acid reactive substances (TBARS)

The most important product of lipid peroxidation reacting with 2-thiobarbituric acid (TBA) is malonic dialdehyde (MDA) (Lykkesfeldt, 2007). Therefore, the lipid peroxidation was determined by quantifying the concentration of TBARS by Kamyshnikov (2004) for determining the malonic dialdehyde (MDA) concentration. Briefly, 0.1 mL of plasma was added to 1 mL of 20% of trichloroacetic acid (TCA) and 1 mL of 0.8% of 2-thiobarbituric acid (TBA). The mixture was heated in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at

540 nm. The concentration of MDA (μmol per L) was calculated using 1.56 10^5 mM/cm as the extinction coefficient.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). In order to find significant differences (significance level, p <0.05) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

Figure 1 illustrates the level of 2-thiobarbituric acid reactive substances (TBARS) in equine plasma induced by the treatment of leaf extracts of various species belonging to the *Sansevieria* genus as compared with treatment by phosphate buffer as a control samples.

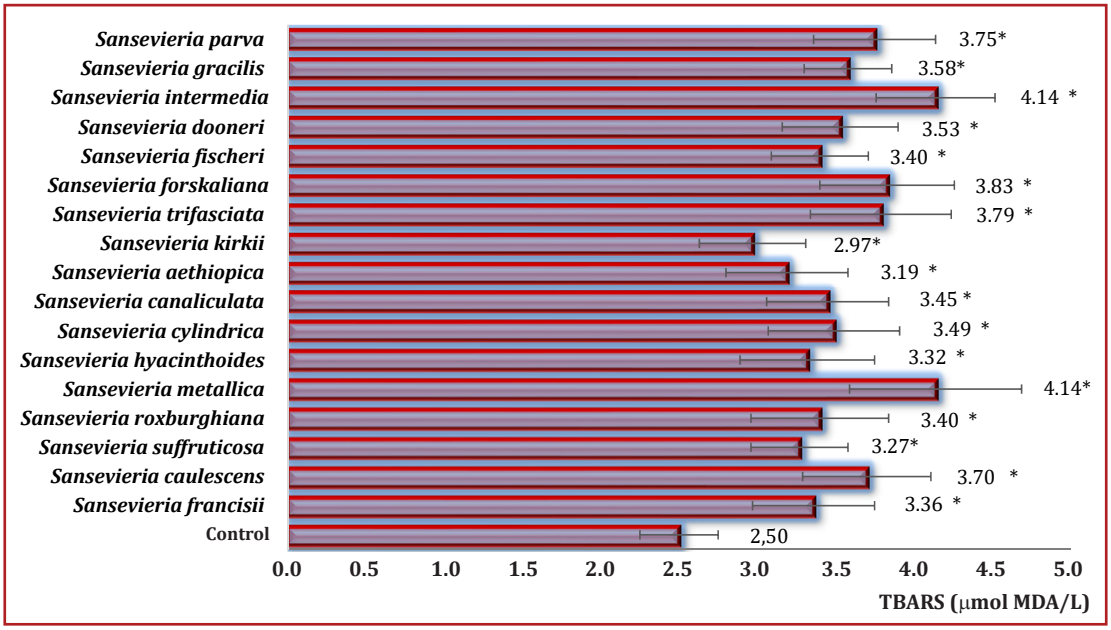


Figure 1 The level of 2-thiobarbituric acid reactive substances (TBARS) in equine plasma induced by the treatment of leaf extracts derived from various species of the *Sansevieria* genus as compared with treatment by phosphate buffer (control). The data were analyzed using one-way analysis on ranks (ANOVA) using the Kruskal-Wallis test by ranks
* *P*-value <0.05 was considered as significant (n = 18)

As presented in Figure 1, when equine plasma was incubated with extracts of various *Sansevieria* species, the TBARS level was significantly increased and these results were statistically significant. At 1 h of incubation, the TBARS concentration of untreated plasma was 2.50 ±0.25 μmol/L. Extracts of various *Sansevieria* species led to an increase of TBARS

concentration in plasma: *S. francisii* (by 34.4%, $p = 0.007$), *S. caulescens* (by 48%, $p = 0.000$), *S. suffruticosa* (by 30.8%, $p = 0.009$), *S. roxburghiana* (by 36%, $p = 0.007$), *S. metallica* (by 65.6%, $p = 0.000$), *S. hyacinthoides* (by 32.8%, $p = 0.014$), *S. cylindrica* (by 39.6%, $p = 0.003$), *S. canaliculata* (by 38.0%, $p = 0.003$), *S. aethiopica* (by 27.6%, $p = 0.032$), *S. kirkii* (by 18.8%, $p = 0.050$), *S. trifasciata* (by 51.6%, $p = 0.000$), *S. forskaliana* (by 53.2%, $p = 0.000$), *S. fischeri* (by 36.0%, $p = 0.003$), *S. dooneri* (by 41.2%, $p = 0.001$), *S. intermedia* (by 65.6%, $p = 0.000$), *S. gracilis* (by 43.2%, $p = 0.000$), and *S. parva* (by 50.0%, $p = 0.000$). These changes were statistically significant ($p > 0.05$) (Figure 1). The most potent effect was demonstrated by the *S. intermedia*, *S. forskaliana*, *S. trifasciata*, *S. parva*, and *S. caulescens* compared to phosphate buffer as a control sample (increased by 65.6, 53.2, 51.6, 50.0, and 48.0%, respectively). *S. kirkii*, *S. aethiopica*, and *S. suffruticosa* caused the less increase of the TBARS level after 1-h incubation with equine plasma (by 18.8, 27.6, and 30.8%, respectively, $p < 0.05$) (Figure 1).

Therefore, the results indicated that the treatment of equine plasma with extracts of various *Sansevieria* species significantly enhance the formation of intracellular TBARS in the extract-treated plasma samples by approximately 18–66%, respectively (Figure 1).

The chemical compounds responsible for the toxic effects of plants are probably produced as part of the plant's defense mechanism against pest and herbivores or to gain an advantage over competing for plants (Ighodaro et al., 2017). According to the results obtained, we addressed the hypothesis that by-products in the extracts of various *Sansevieria* species can be responsible for their pro-oxidant activity. In previous phytochemical screening of the *Sansevieria* plants different groups of chemical were observed to be isolated: carbohydrates, saponins, glycosides, flavonoids, steroids in the leaves (Mimaki et al., 1996, 1997). The screening of the plant material revealed the presence of the alkaloids, tannins, and antrhaquinones in the leaves and roots of *S. trifasciata* (Berame et al., 2017). Additionally, phytochemical analysis of extracts of *S. cylindrica* leaves showed the presence of steroids, flavonoids, saponins, tannins, and phenolic acids (Ahamad et al., 2017). The interest in the possible health benefits of flavonoids has increased owing to their potent antioxidant and free radical scavenging activities observed *in vitro*. Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented and their pro-oxidant properties have been actually described *in vivo* (Procházková et al., 2011). It was reported that compounds with antioxidant activity can act as pro-oxidants under certain conditions or in high concentrations. Studies evidently indicate that natural antioxidants, including polyphenols, flavonoids, anthocyanins, and carotenoids, can act as pro-oxidants, which produce reactive oxygen species and cause oxidative stress (Eghbaliferiz and Iranshahi, 2016). Due to their pro-oxidant properties, they are able to cause oxidative damage by reacting with various biomolecules, such as lipids, proteins, and DNA (Procházková et al., 2011). The pro-oxidant activity is typically catalyzed by metals, particularly transition metals such as Fe and Cu, present in biological systems (Eghbaliferiz and Iranshahi, 2016).

A series of 40 flavonoids were investigated by Baldin et al. (2017) with the purpose of correlating these properties via structure and activity analyses based on integrated networks and QSAR models. The classical groups for the antioxidant activity of flavonoids were combined in order to explain the influence of antioxidant and pro-oxidant activities on the

anti-parasitic properties. Flavonoids have demonstrated *in vivo* and *in vitro* leishmanicidal, trypanocidal, antioxidant, and pro-oxidant properties. The chemotherapy of trypanosomiasis and leishmaniasis lacks efficacy, presents high toxicity, and is related to the development of drug resistance. The dual activity of flavonoids presenting both anti- and pro-oxidant activities revealed that the existence of a balance between these two features could be important to the development of adequate therapeutic strategies (Baldin et al., 2017).

These results are also comparable to those found by Cherrak et al. (2016) who have revealed that flavonoids possess both pro-oxidant and antioxidant activity depending on the nature and concentration of the flavonoids and metal ions. Natural flavonoids such as quercetin, (+) catechin and rutin as well as four methoxylated derivatives of quercetin used as models were investigated to elucidate their impact on the oxidant and antioxidant status of human red blood cells (RBCs). The impact of these compounds against metal toxicity was studied as well as their antiradical activities with DPPH assay. Antihemolytic experiments were conducted on quercetin, (+) catechin and rutin with an excess of Fe, Cu, and Zn (400 μM), and the oxidant (malonic dialdehyde, carbonyl proteins) and antioxidant (reduced glutathione, catalase activity) markers were evaluated. The results showed that Fe and Zn have the highest pro-oxidant effect (37 and 33% of hemolysis, respectively). Quercetin, rutin and (+) catechin exhibited strong antioxidant properties toward Fe, but this effect was decreased with respect to Zn ions. However, the Cu showed a weak antioxidant effect at the highest flavonoid concentration (200 μM), while a pro-oxidant effect was observed at the lowest flavonoid concentration (100 μM) (Cherrak et al., 2016).

A wide range of polyphenols possesses anticancer and apoptosis-inducing properties (Khan et al., 2012). Notably, an important aspect of the chemopreventive action of polyphenols is their differential activity in selectively targeting cancer cells while sparing normal cells. Although polyphenols are generally recognized as antioxidants, they also act as pro-oxidants inducing DNA degradation in the presence of metal ions such as copper. Since it is known that tissue and cellular copper levels are significantly elevated in a number of malignancies, cancer cells would be more subject to redox cycling between copper ions and polyphenols to generate reactive oxygen species (ROS) responsible for DNA breakage (Khan et al., 2012).

Our present findings are in agreement with results obtained in our previous study including the assessment of antioxidant activity of extracts obtained from leaves of selected *Sansevieria* species (Tkachenko et al., 2017, 2018; Pażontka-Lipiński et al., 2018). When erythrocytes were incubated with leaf extracts of various species from the *Sansevieria* genus, the aldehydic derivatives level was significantly reduced by 13.6% (p <0.05) for *S. forskaliana* extract. Moreover, all extracts (except *S. francisii* extract) reduced the formation of intracellular aldehydic derivatives of oxidatively modified proteins (OMP) in the extracts-treated erythrocytes, but these results were non-significant. Treatment by extracts of various *Sansevieria* species reduced the concentration of ketonic derivatives of OMP when compared to untreated erythrocytes. The most potent effect was demonstrated by the *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* compared to control samples (phosphate buffer) (16.1, 14.7, 13.4, 12.9, 12.9, 12.7, and 12.1%, respectively). However, there were no significant changes in other extracts. The experimental evidence

obtained in our previous study indicated that various species of *Sansevieria* genus are a rich source of compounds that manifest antioxidant activity and can effectively protect erythrocytes against oxidative-induced damage. Thus, *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* may be considered as a valuable source of natural antioxidants that may potentially be recommended for applications in medicine and veterinary practice. According to the above-mentioned antioxidant mechanisms, extracts of various *Sansevieria* species may inhibit the formation of protein carbonyl by scavenging free radicals formed *in vitro*. According to many supporting documents, it can be assumed that secondary plant metabolites, i.e. polyphenolic compounds in extracts of various species from *Sansevieria* genus extract may contribute to the antioxidant activity. Therefore, these findings may be potentially contributive to the validation of the medicinal use of various *Sansevieria* species for the prevention of oxidative stress (Tkachenko et al., 2017). Moreover, the results of our previous study (Tkachenko et al., 2018) also showed that the leaves of *S. francisii* and *S. forskaliana* led to a non-significantly decrease of 2-thiobarbituric acid reactive substances (TBARS) concentration in erythrocytes. However, *S. hyacinthoides* and *S. cylindrica* had a significant increase of TBARS level in the extract-treated erythrocytes (by 29.7 and 21%, p <0.05, respectively). The outcome of this study suggests that *Sansevieria* species has a promising antioxidant and prooxidant potential (Tkachenko et al., 2018).

Similar findings were discovered in our other several works, which have been conducted to evaluated the lipid peroxidation biomarkers and total antioxidant capacity in the muscle tissue of rainbow trout (*Oncorhynchus mykiss* Walbaum) under incubation with extracts derived from the leaves of various *Sansevieria* species, aimed at the further improving methods for preventing and treating fish diseases by increasing the natural resistance of fish organism using antibacterial and antioxidant agents in aquaculture (Maryniuk et al., 2017, 2018). It has been found that the most potent antioxidant effect was demonstrated for the extracts of *S. caulescens*, *S. suffruticosa*, *S. hyacinthoides*, *S. canaliculata*, *S. aethiopica*, *S. gracilis*, and *S. parva* as compared to phosphate buffer control (46.6, 66.8, 77.3, 49.8, 71.1, 63.4, and 39.4%, respectively). Likewise, the results showed that extracts of *S. hyacinthoides* and *S. aethiopica* efficiently increased the total antioxidant capacity in rainbow trout muscle tissue (Maryniuk et al., 2017). Among plant extracts screened for *in vitro* antioxidant properties in rainbow trout muscle tissue, the strongest toxicity responses were exhibited by *S. cylindrica*, *S. canaliculata*, *S. trifasciata*, *S. metallica* leaf extracts (Maryniuk et al., 2017).

Moreover, we also assessed the *in vitro* antibacterial activity of ethanolic extract prepared from various *Sansevieria* plants (Buyun et al., 2018). The results of antibacterial activity clearly showed that the *S. cylindrica* extract has shown antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains, clinically important bacteria, which are indicator organisms commonly used in programs to monitor antibiotic resistance. The extract has shown better activity against *S. aureus* and *P. aeruginosa* strains compared to the *E. coli* strains. The extract has shown less antimicrobial activities against *P. aeruginosa*. Finally, the ethanolic extract exhibited mild antibacterial activity against *E. coli* (Buyun et al., 2018).

Thus, it can be inferred from this study, that *Sansevieria* species have a promising antioxidant and pro-oxidant potential. However, the precise mechanisms underlying these potential need to be evaluated in future studies. Further studies involving bioassay-guided identification of the main compounds in plants are necessary to affirm and maximize the possible use of the plant as a therapeutic remedy for the prevention of lipid peroxidation in the blood. The obtained information may be useful in the clinical usage of plants in medicine and veterinary. Finally, these findings justify the traditional uses of *Sansevieria* plants for therapeutic purposes.

Conclusions

The results of the study showed that the TBARS level was significantly increased after incubation with extracts of selected species belonging to the *Sansevieria* genus and these results were statistically significant. Moreover, it was revealed that *Sansevieria* leaf extracts were found to have different levels of antioxidant properties in the test model. The most potent effect was demonstrated by the *S. intermedia*, *S. forskaliana*, *S. trifasciata*, *S. parva*, and *S. caulescens* compared to phosphate buffer as a control sample (increased by 65.6, 53.2, 51.6, 50, and 48%, respectively). *S. kirkii*, *S. aethiopica*, and *S. suffruticosa* caused the less increase of the TBARS level after 1-h incubation with equine plasma (by 18.8, 27.6, and 30.8%, respectively, p <0.05). *Sansevieria* species possess a promising antioxidant and pro-oxidant potential. Further studies involving phytochemical identification of the main compounds in plants are necessary to affirm and maximize the possible use of the plants as a therapeutic remedy for the prevention of oxidative stress. It was concluded, that the dose-depended antioxidative and pro-oxidative effects of various *Sansevieria* species in both plasma and erythrocyte suspension will be further studied in detail.

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CYTOTOXIC EFFECTS OF LEAF EXTRACTS OF SOME *THYMUS* L. (LAMIACEAE) REPRESENTATIVES USING *IN VITRO* HUMAN BLOOD MODEL

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In the course of *in vitro* systems search for the toxicity screening of plant extracts, different cellular models have been applied to examine their adverse effects in our previous studies. In this study, the main aim was to assess the dose-dependent pro- and antioxidant potential of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine on human erythrocytes' model. For this purpose, we used 2-thiobarbituric acid reactive substances (TBARS) as a biomarker of lipid peroxidation used as an assessment of oxidative stress in erythrocytes' suspension after incubation with plant extracts in two doses (5 mg/mL and 0.5 mg/mL). An erythrocytes' suspension at 1% hematocrit was incubated with 4 mM phosphate buffer (pH 7.4) (control) and pre-incubated with the extracts (5 mg/mL and 0.5 mg/mL, respectively) at 37 °C for 60 min. The treatment by extracts obtained from various plants belonging to the *Thymus* genus in dose 5 mg/mL increased the TBARS level as a biomarker of lipid peroxidation in the human erythrocyte suspension when compared to untreated erythrocytes. The most potent prooxidative effect was demonstrated by the *Th. alpestris* Tausch ex A. Kern., *Th. serpyllum* L., *Th.* × *porcii* Borbás, and *Th. pannonicus* All. compared to phosphate buffer as a control samples. The minimum increase of TBARS content in human erythrocyte suspension was induced by *Th. pulegioides* L. extract. In the case of dose 0.5 mg/mL, *Th. alpestris*, *Th. pannonicus*, *Th.* × *porcii* caused also increased the TBARS level (by 58%, 51%, 43.1%, p <0.05, respectively) compared to untreated erythrocytes. On the other hand, *Th. serpyllum* and *Th. pulegioides* extracts decreased the TBARS level (by 16.5 and 2.7%, respectively), but these changes were no significant. Moreover, *Th. serpyllum* extract in dose 0.5 mg/mL caused the increase of TBARS level (by 48.8%, p <0.05) compared to those in dose 5 mg/mL. The extracts obtained from leaves of *Th. alpestris*, *Th. pannonicus*, and *Th.* × *porcii* in both doses (5 and 0.5 mg/mL) has a mild cytotoxic activity on the human erythrocytes increasing the level of lipid peroxidation biomarker. These findings suggest that *Th. alpestris*, *Th. pannonicus*, *Th.* × *porcii* extracts possessed prooxidant effects in both doses, while effects of *Th. serpyllum* and *Th. pulegioides* extracts to erythrocyte suspension were

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dose-dependent. Further research is needed to determine the effects of the active compounds of various plants belonging to the *Thymus* genus on erythrocytes' metabolism.

Keywords: *Thymus*, leaf extracts, human erythrocytes, lipid peroxidation, *Th. alpestris*, *Th. serpyllum*, *Th.* × *porcii*, *Th. pannonicus*, *Th. pulegioides*

Introduction

Plants from the genus *Thymus* are important medicinal herbs, which are known are rich in different active substances such as thymol, carvacrol, p-cymene, and terpinene (Morales, 2002; Nabavi et al., 2015). It is one of the most widely used genera in folk medicine, consisting of approximately 215 species currently recognized. It is popular for its stimulatory action on all organism functions (Viuda-Martos et al., 2011).

Nowadays, thymol and thyme present a wide range of functional possibilities in the medicine, pharmacy, food, and cosmetic industry (Salehi et al., 2018). Thymol, which is a monocyclic monoterpene, chemically known as 2-isopropyl-5-methylphenol found in oil of thyme various other kinds of plants (Aydın et al., 2017). For centuries, it has been used in traditional medicine and has been shown to possess various pharmacological properties, i.e. antioxidant, free radical scavenging, anti-inflammatory, analgesic, antispasmodic, antibacterial, antifungal, antiseptic and antitumor activities with multiple therapeutic actions against various cardiovascular, neurological, rheumatological, gastrointestinal, metabolic and malignant diseases (Nagoor Meeran and Prince, 2012; Nagoor Meeran et al., 2017). The interest in the formulation of pharmaceuticals, nutraceuticals, and cosmeceuticals based on thymol is due to several studies that have evaluated the potential therapeutic uses of this compound for the treatment of disorders affecting the respiratory, nervous, and cardiovascular systems (Salehi et al., 2018). Thymol has also been reported as an anti-cancer agent, but its anti-cancer mechanism has not yet been fully elucidated (Deb et al., 2011).

In the course of *in vitro* systems search for the toxicity screening of plant extracts, different cellular models have been applied to examine their adverse effects in our previous studies. For example, we have assessed the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] in the equine erythrocytes after treatment with *Thymus serpyllum* L. extract. Lipid peroxidation biomarker, aldehydic and ketonic derivatives of oxidatively modified proteins, total antioxidant capacity was non-significantly altered after *in vitro* incubation with an extract obtained from *Th. serpyllum* (Honcharenko et al., 2018c). According to the results obtained, we addressed the hypothesis that by-products in the leaf extracts obtained from various *Thymus* representatives may be a major contributor to an increase of the antioxidant capacity of muscle tissue of rainbow trout after incubation *in vitro*. We have investigated the influence of leaf extracts obtained from various *Thymus* representatives on the total antioxidant capacity (TAC) in the muscle tissue of rainbow trout after incubation with extracts under *in vitro* conditions. The most potent antioxidant effect was demonstrated for the extracts of *Th. alpestris* Tausch ex A. Kern., *Th.* × *porcii* Borbás, *Th. pannonicus* All.,

Th. serpyllum L., and *Th. pulegioides* L. compared to phosphate buffer as a control samples (59.5, 48, 45.9, 43.9, and 38.8%, p <0.05, respectively). The results showed that the leaf extract of *Th. alpestris* efficiently increased the TAC level in muscle tissue. Our results provide a new perspective on the use of various *Thymus* species as a medicinal plant to improve the antioxidant response of rainbow trout (Honcharenko et al., 2018b).

Moreover, we also demonstrated the antibacterial activity of some *Thymus* representatives against β-lactamase-producing *Pseudomonas aeruginosa* and *Salmonella enteriditis* strains locally isolated (Honcharenko et al., 2018a, d). The effects varied significantly according to the *Thymus* taxa. It should be noted that the most antimicrobial effective plant against β-lactamase-producing *P. aeruginosa* was *Th. alpestris*, being highly active with the ethanolic extract (mean diameter of inhibition zone was 12.8 ±0.8 mm). The antibacterial activity of extracts was greatest for *Th. alpestris* followed by *Th. pannonicus* followed by *Th. serpyllum* and then by *Th. pulegioides* (Honcharenko et al., 2018d). The ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *S. enteritidis*. The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* followed by *Th. alpestris*, *Th.* × *porcii*, and then by *Th. serpyllum*. These plant extracts could be a potential source of new antibacterial agents (Honcharenko et al., 2018a).

This study describes the interaction of aqueous extracts of leaf extracts obtained from various *Thymus* representatives with human erythrocytes. The cell membrane is a diffusion barrier which protects the cell interior. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with foreign species. Erythrocytes were chosen because although less specialized than many other cell membranes they carry on enough functions in common with them, i.e. active and passive transport, the production of ionic and electric gradients, etc. Therefore, their structure can be considered representative of the plasma membrane in general (Suwalsky et al., 2006, 2008). The erythrocytes could be isolated and handled easily so that they could provide a good model for many assays (Alagawany et al., 2016; Farag and Alagawany, 2018). Additionally, the high concentration of polyunsaturated fatty acids in the membrane, the high oxygen tension, and redox-active hemoglobin molecules [the source of reactive oxygen species in erythrocyte] make them a good biological lipid membrane model especially for screening the oxidative stress conditions induced by various substances (Farag and Alagawany, 2018).

We continue to assess the dose-dependent pro- and antioxidant potential of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine on human erythrocytes‘ model. Thus, the main aim of our study was to assess the dose-dependent pro- and antioxidant potential of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine on human erythrocytes‘ model. For this purpose, we used 2-thiobarbituric acid reactive substances (TBARS) as a biomarker of lipid peroxidation used as an assessment of oxidative stress in erythrocytes‘ suspension after incubation with plant extracts in two doses (5 mg/mL and 0.5 mg/mL).

Material and methodology

Collection of Plant Materials

Leaves of *Thymus serpyllum* L. were collected among the grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 03′ 58,9′′, E 26° 13′ 37,5′′, 257 m a.s.l.). Leaves of *Th. pannonicus* All. were harvested among grass in the roadside between the two cultivated fields (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 09,6′′, E 26° 13′ 19,2′′, 283 m a.s.l.). Leaves of *Th. pulegioides* L. were collected among grass nearby land parcels (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 02,8′′, E 26° 14′ 13,9′′, 306 m a.s.l.). Leaves of *Th.* × *porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*) were sampled in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv, Ukraine; N 49° 49‘ 15.1“, E 24˚ 05‘ 12.5“, 348 m a.s.l.). Leaves of *Th. alpestris* Tausch ex A. Kern. were harvested on the side of the road below the stream, in mountain valley Shumneska (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 32.3“, E 24° 21‘ 26.4“, 1259 m a.s.l.). Identification of these five taxa was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all the attached material and used to prepare the extract.

Preparation of Plants Extracts

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. The extracts were stored at -20 °C until use.

Human blood samples

Blood (10–20 ml) was obtained from normal volunteers via venipuncture (4 males and 5 females aged 28–53-years old). The Research Ethics Committee of Regional Medical Chamber in Gdańsk (Poland) approved the study (KB-31/18). All patients provided written informed consent before the start of the study procedures. Human erythrocytes from citrated blood were isolated by centrifugation at 3,000 rpm for 10 min and washed two times with 4 mM phosphate buffer (pH 7.4) and then re-suspended using the same buffer to the desired hematocrit level. Cells stored at 4 °C were used within 6 h of sample preparation. An erythrocytes' suspension at 1% hematocrit was incubated with 4 mM phosphate buffer (pH 7.4) (control) and pre-incubated with the extracts (5 mg/mL and 0.5 mg/mL, respectively) at 37 °C for 60 min. This reaction mixture was shaken gently while being incubated for a fixed interval at 37 °C. For positive control (phosphate buffer) was used. Erythrocytes' aliquots were used in the study.

2-Thiobarbituric Acid Reactive Substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method

for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The μmol of MDA per L was calculated using $1.56 \cdot 10^5$ mM/cm as the extinction coefficient.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the values (significance level, p <0.05) was examined using the Kruskal-Wallis H-test (Zar, 1999). All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

As shown in Figure 1, treatment by extracts obtained from various plants belonging to the *Thymus* genus in dose 5 mg/mL increased the TBARS level, when compared to untreated erythrocytes. The most potent prooxidative effect was demonstrated by the *Th. alpestris*, *Th. serpyllum*, *Th.* × *porcii*, and *Th. pannonicus* compared to phosphate buffer as control samples (an increase of TBARS content by 82.0, 78.7, 53.7, and 34.1%, p <0.05, respectively). The minimum increase of TBARS content in human erythrocytes' suspension was induced by *Th. pulegioides* extract (by 11.2%, p <0.05) (Figure 1).

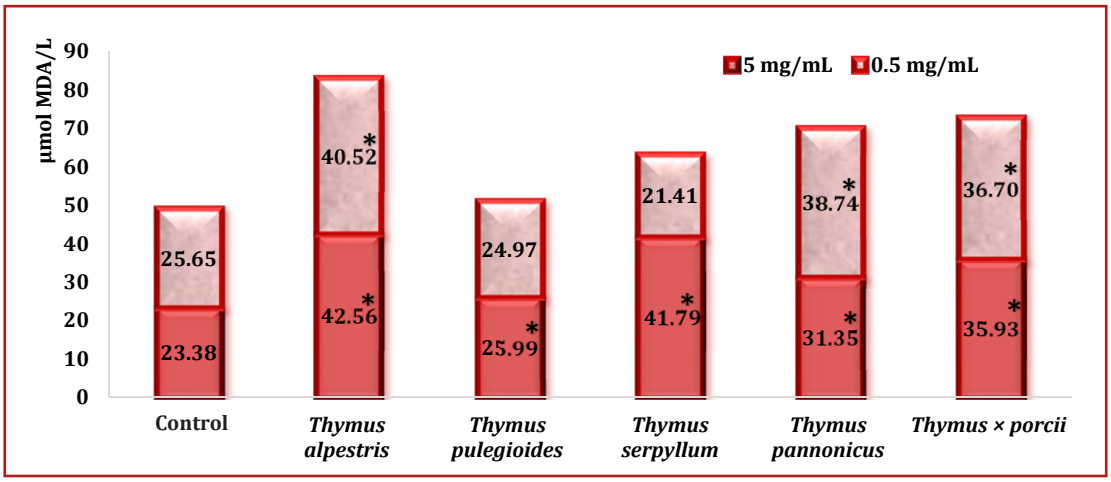


Figure 1 The TBARS content as a biomarker of lipid peroxidation in the human erythrocytes suspension after *in vitro* incubation with leaf extract obtained from *Thymus alpestris*, *Th. pulegioides*, *Th. serpyllum*, *Th. pannonicus*, and *Th.* × *porcii* (n = 9). * – changes were statistically significant (p <0.05) compared to the untreated control (phosphate buffer as a control sample)

Extracts obtained from various plants belonging to the *Thymus* genus in dose 0.5 mg/mL caused increased the TBARS level, i.e. in the case of *Th. alpestris* (by 58%, p <0.05), *Th. pannonicus* (by 51%, p <0.05), *Th.* × *porcii* (by 43.1%, p <0.05) compared to untreated

erythrocytes. *Th. serpyllum* and *Th. pulegioides* extracts decreased the TBARS level (by 16.5 and 2.7%, respectively), but these changes were no significant (Figure 1). Moreover, *Th. serpyllum* extract in dose 0.5 mg/mL caused the increase of TBARS level (by 48.8%, p <0.05) compared to those in dose 5 mg/mL (Figure 1).

Many results also clearly suggest that treatment by *Thymus* extracts *in vivo* and *in vitro* prevents organ damage via protection of the antioxidant defense system and scavenge of hydroxyl free radicals by producing of phenoxyl radicals, major transient species (Nagoor Meeran et al., 2017). Thymol is also a potent antioxidant and anti-inflammatory agent in human cells. For example, Braga et al. (2006) have investigated whether thymol can interfere with the production of reactive oxygen species (ROS), nitric oxide and nitric oxide-derived peroxynitrite released by human neutrophils after activation by formyl methionyl-leucyl-phenylalanine (fMLP) and paramethoxyamphetamine (PMA) with and without the addition of the L-arginine (L-Arg) nitric oxide donor to the medium. In cell-free systems using H_2O_2/ $HOCl^-$ and 3-morpholinosydnonimine (SIN-1) as radical producers, significant scavenging activity of thymol was present already at 0.08 and 0.68 μg/ml respectively, and these are very low concentrations. These findings can be related to the phenolic structure of thymol, because phenolic compounds have redox properties and play an important role in absorbing and neutralizing free radicals and peroxynitrite, and in decomposing peroxides (Braga et al., 2006).

Many studies using human cells model were conducted for assessment of the antioxidative activity, chemopreventive efficacy and cytotoxicity of *Thymus* extracts. For instance, Kozics et al. (2013) have investigated the composition and the quantitative estimation of *Salvia officinalis* L. and *Thymus vulgaris* L. extracts, the protective effects of plant extracts against hydrogen peroxide- and 2,3-dimethoxy-1,4-naphthoquinone-induced DNA damage, and levels of enzymatic and non-enzymatic antioxidants [superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione] in human HepG2 cells. To measure the antioxidative activity of plant extracts three assays were used, i.e. 1.1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2.2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The results of Kozics et al. (2013) showed that the oxidant-induced DNA lesions were significantly reduced in cells pre-treated with the plant extracts studied. The observed DNA-protective activity could be explained by both elevations of GPx activity in cells pre-treated with *S. officinalis* and *Th. vulgaris* and antioxidant activity of those extracts (Kozics et al., 2013). Moreover, the results of Horváthová et al. (2016) have indicated that the consumption of *S. officinalis* and *Th. vulgaris* extracts positively affect the resistance of rat liver cells against oxidative stress and may have hepatoprotective potential. Intake of sage and thyme had no effect either on the basal level of DNA damage or on the activity of SOD in rat hepatocytes and did not change the biochemical parameters of blood plasma. Simultaneously, the activity of GPx was significantly increased and the level of DNA damage induced by oxidants was decreased. Moreover, the sage extract was able to start up the antioxidant protection expressed by an increased content of glutathione (Horváthová et al., 2016).

Galasso et al. (2014) have assessed the chemopreventive efficacy of the *Th. longicaulis* C. Presl. extracts collected at different phases during its life cycle, by means of their anti-

inflammatory, cytotoxic and antioxidant activities. To this purpose, each extract underwent an extensive screening towards five human cell lines: CCRF-CEM (leukemia); U251 (glioblastoma); MDA-MB-231 (breast cancer); HCT-116 (colon cancer) and MRC-5 (lung fibroblasts) through [2.3bis(2-metoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H tetrazolium hydroxide] test. The ability of the extracts to counteract cyclooxygenase-2 (COX-2) expression was also evaluated by COX-2 expression assay in human THP-1 monocyte-derived macrophages. COX-2 inhibition could represent a valuable anticancer strategy as it is associated with carcinogenesis and over-expressed in a variety of human malignancies. Extract, which was particularly rich in rosmarinic acid and methylapigenin, exhibited a strong antioxidant and anti-inflammatory effectiveness (Galasso et al., 2014).

The study of Caprioli et al. (2018) confirms the very low cytotoxicity of the *Th. lanceolatus* Desf. ethanolic extract and highlights its nutraceutical properties as an antioxidative and preservative agent and its possible use as an ingredient in functional foods. The biological activity of *Th. lanceolatus* ethanolic extract on proliferation, viability and ROS protection was investigated towards K562, CaCo-2, and SH-SY5Y human cancer cell lines. Cell proliferation was inhibited in K562 and SH-SY5Y cells in the first 48 h at 500 μg/mL but slowly resumed after 72 h. A weak cytotoxic effect can be observed at 24, 48 and 72 hours: 12.8, 14.9 and 24.2% reduction in K562 viability, and 11, 15 and 12.7% in SH-SY5Y. No cytotoxicity was observed in CaCo-2 cells under the same experimental conditions. Even at the lowest concentrations (50 μg/mL), the extract was efficiently able to protect human cells against t-BHP-induced oxidative damage. For instance, the highest concentration of the extract (100 μg/mL) decreases ROS generation by about 30% in SH-SY5Y and 70% in CaCo-2 and K562 cells (Caprioli et al., 2018).

Thymol may have antiproliferative potential against brain tumor cells involving oxidative alteration. *In vitro* antiproliferative [by 3-(4.5 dimetylthiazol-2-yl)-2.5 diphenlytetrazolium bromide (MTT) test], genotoxic [by single-cell gel electrophoresis (SCGE)] and oxidative effects [by total antioxidant capacity (TAC) and total oxidative status (TOS) analysis] of thymol (0–400 mg/L) were assessed on cultured primary rat neurons (CPRNs) and N2a neuroblastoma cells by Aydın et al. (2017). The obtained data from MTT analysis revealed that thymol (only at 400 mg/L) led to significant decreases in the cell viability in cultured primary rat neurons. Moreover, thymol inhibited cell growth in N2a cells at concentrations of 200 and 400 mg/L. DNA damage rates were statistically no-significant in both treated cell types as compared to the control group. The results of Aydın et al. (2017) also showed that 10, 25 and 50 mg/L of thymol application into the cell cultures supported antioxidant capacity in primary rat neurons but not in N2a cells.

Thymol also induces mitochondrial dysfunction and apoptosis and may be efficacious against multiple cancers. De La Chapa et al. (2018) have evaluated the effects of thymol against oral squamous cell carcinoma (OSCC) and its anticancer mechanism-of-action. Thymol had significant, long-lasting antiproliferative effects *in vitro*. *In vivo*, thymol displayed significant antitumor effects in Cal27-derived tumors. Thymol‘s anticancer effects were confirmed in HeLa-derived xenografts demonstrating that thymol effects are not tumor-type specific. Thymol induces significant ΔΨm depolarization and apoptosis (De La Chapa et al., 2018).

Deb et al. (2011) have investigated the anticancer activity of thymol on HL-60 (acute promyelocytic leukemia) cells. Thymol demonstrated dose-dependent cytotoxic effects on HL-60 cells after 24h of exposure. However, thymol did not show any cytotoxic effect in normal human peripheral blood mononuclear cells. The cytotoxic effect of thymol on HL-60 cells appears to be associated with the induction of cell cycle arrest at sub G0/G1 phase, and apoptotic cell death based on genomic DNA fragmentation pattern. Thymol also showed a significant increase in the production of ROS activity, an increase in mitochondrial H_2O_2 production and depolarization of mitochondrial membrane potential. On performing Western Blot analysis, thymol showed an increase in Bax protein level with a concomitant decrease in Bcl2 protein expression in a dose-dependent manner. Activation of caspase-9, -8 and -3 and concomitant PARP cleavage, which is the hallmark of caspase-dependent apoptosis was also shown by Deb et al. (2011). Moreover, to rule out the involvement of other mechanisms in apoptosis induction by thymol, these researchers also studied its effect on the apoptosis-inducing factor (AIF). Thymol induced AIF translocation from mitochondria to cytosol and to the nucleus, thus indicating its ability to induce caspase-independent apoptosis. Thymol-induced apoptosis in HL-60 cells involves both caspase-dependent and caspase-independent pathways (Deb et al., 2011).

The *Th. serpyllum* may protect against hypertension in an experimental model of essential hypertension. Mihailovic-Stanojevic et al. (2013) have evaluated the total phenol and flavonoid contents, antioxidant capacity, free radical scavenging activity and potential antihypertensive effect of the aqueous extract obtained from *Th. serpyllum* (wild thyme, TE) in spontaneously hypertensive rats (SHR) and in normotensive Wistar rats. The ferric reducing/antioxidant power and antioxidant capacity analysis revealed strong antioxidative properties of wild thyme. Bolus injection of wild thyme (100 mg/kg body weight i.v.) induced a significant decrease of systolic and diastolic blood pressure and total peripheral resistance in SHR, without effects on these parameters in normotensive Wistar rats. The cardiac index remained unchanged after *Th. serpyllum* treatment in all experimental rats. A given dose of *Th. serpyllum* did not show significant nitric oxide-scavenging activity *in vivo* (Mihailovic-Stanojevic et al., 2013).

In our study, treatment by extracts obtained from various plants belonging to the *Thymus* genus in dose 5 mg/mL increased the TBARS level, when compared to untreated erythrocytes. On the other hand, extracts obtained in dose 0.5 mg/mL caused the less increased the TBARS level compared to untreated erythrocytes. Moreover, *Th. serpyllum* and *Th. pulegioides* extracts decreased the TBARS level (by 16.5 and 2.7%, respectively), but these changes were no significant.

In conclusion, the extracts obtained from leaves of *Th. alpestris*, *Th. pannonicus*, and *Th.* × *porcii* in both doses (5 and 0.5 mg/mL) has a mild cytotoxic activity on the human erythrocytes increasing the level of lipid peroxidation biomarker (TBARS level). These findings suggest that *Th. alpestris*, *Th. pannonicus*, *Th.* × *porcii* extracts possessed prooxidant effects in both doses, while effects of *Th. serpyllum* and *Th. pulegioides* extracts to erythrocyte suspension were dose-dependent.

Conclusions

In conclusion, the treatment by extracts obtained from various plants belonging to the *Thymus* genus in dose 5 mg/mL increased the TBARS level as a biomarker of lipid peroxidation in the human erythrocyte suspension, when compared to untreated erythrocytes. The most potent prooxidative effect was demonstrated by the *Th. alpestris*, *Th. serpyllum*, *Th.* × *porcii*, and *Th. pannonicus* compared to phosphate buffer as a control sample. The minimum increase of TBARS content in human erythrocyte suspension was induced by *Th. pulegioides* extract. In the case of dose 0.5 mg/mL, *Th. alpestris*, *Th. pannonicus*, *Th.* × *porcii* caused also increased the TBARS level (by 58, 51, 43.1%, p <0.05, respectively) compared to untreated erythrocytes. On the other hand, *Th. serpyllum* and *Th. pulegioides* extracts decreased the TBARS level (by 16.5 and 2.7%, respectively), but these changes were no significant. Moreover, *Th. serpyllum* extract in dose 0.5 mg/mL caused the increase of TBARS level (by 48.8%, p <0.05) compared to those in dose 5 mg/mL. Further research is needed to determine the effects of the active compounds of various plants belonging to the *Thymus* genus on erythrocytes' metabolism.

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ANTIOXIDANT POTENTIAL OF LAMIACEAE HERBS FROM BOTANICAL GARDEN OF SLOVAK UNIVERSITY OF AGRICULTURE IN NITRA

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Polyphenol compounds are the secondary metabolites of plant origin that are widely distributed. They exhibit a wide spectrum of biological activities, among which is antioxidant activity. Investigation of polyphenol content and their antioxidant activity of aromatic herbs was the main goal of this study. Plant material of this study was *Hyssopus officinalis* L., *Lavandula angustifolia* Mill., *Nepeta kokanica* (Regel) Kuntze, *Rosmarinus officinalis* L., *Salvia officinalis* L., *Thymus serpyllum* L., and *Th. vulgaris* L. (Lamiaceae Lindl.) harvested from the collection of the Botanical Garden of Slovak University in Nitra at the period of flowering. It was determined the total content of polyphenols (gallic acid equivalent (GAE)), flavonoids (quercetin equivalent (QE)), phenolic acids (caffeic acid equivalent (CAE)), antioxidant activity (Trolox equivalent (TE)) and reducing power (TE) of extracts. All measurements carried out on the spectrophotometer Jenway (6405 UV/Vis, England). In our study total content of polyphenols among investigated plant extracts was from 39.43 to 62.87 mg GAE/g of DW (dry weight); the content of flavonoids was from 20.80 to 36.80 mg QE/g of DW; content of phenolic acids was from 4.91 to 24.30 mg CAE/g of DW; antioxidant activity by DPPH-method was from 7.78 to 8.50 mg TE/g of DW; antioxidant activity by phosphomolybdenum method was from 122.92 to 225.36 mg TE/g of DW. Thus, we found that among investigated plants *S. officinalis* extracts had the highest amount of polyphenol compounds, phenolic acids, and flavonoids. Antioxidant activity and reducing power was maximal for plants of *H. officinalis*. All investigated plants are a potential source of natural antioxidants and have potential as therapeutic agents.

Keywords: Lamiaceae, polyphenols, flavonoids, phenolic acids, antioxidant activity.

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Introduction

Lamiaceae Lindl. is a well-known family, which includes approximately 250 genera that widely distributed in the world (Cigremis et al., 2010) and has been the subject of numerous studies for identifying the antioxidant potential (Tzima et al., 2018). Herbs from Lamiaceae such as rosemary, oregano, hyssop, lavender, thyme are known as raw with high antioxidant activity (Soleimani et al., 2011; Dauqan et al., 2017; Hawrył et al., 2019). Herbs from Lamiaceae have a long history of use as medicinal plants. It is a group of aromatics, medicinal, spicy plants also (Carović-Stanko et al., 2016; Kovtun-Vodyanytska, 2017).

Plants containing biologically active compounds such as vitamins, flavonoids, polyphenols possess high antioxidant activity (Gupta and Sharma, 2006). Plants from Lamiaceae such as *Rosmarinus officinalis* (Nieto et al., 2018), *Salvia officinalis* (Abdelkader et al., 2014; Gantner et al., 2018), *Hyssopus officinalis* (Vlase et al., 2014), *Lavandula angustifolia* (Turgut et al., 2017; Alexa et al., 2018) possess the high antimicrobial activity (Assis et al., 2018). Also, it is found that *Hyssopus officinalis* have been used as a carminative, tonic, antiseptic, expectorant, cough reliever, etc. (Khazaie et al., 2008). This species known as a culinary herb and medicinal plant may use as natural food ingredients comparing with synthetic antioxidants (López-Fernandez et al., 2003; Armatu et al., 2010). The essential oil from *Hyssopus officinalis* also a rich source of biologically active compounds (Fathiazad et al., 2011).

Investigations with *Rosmarinus officinalis* showed the antiproliferative effect of plant extracts on human prostate cancer cell lines (Bourhia et al., 2019), antifungal (Hendel et al., 2016), antimutagenic potential, reducing the DNA damage and lipid peroxidation resulting from treatment with arsenic exposure (Farias et al., 2018). The study of *Salvia officinalis* showed that raw of it demonstrated numerous activities: the anticancer, anti-inflammatory, antinociceptive, antioxidant, antimicrobial, hypoglycemic, hypolipidemic, etc. (Ghorbani and Esmaeilizadeh, 2017). Aerial parts of *Thymus serpyllum* has used as anthelmintic, antiseptic, antispasmodic, carminative, deodorant, diaphoretic, disinfectant, expectorant, sedative and tonic raw (Jarić et al., 2015).

Ahmad et al. (2015) described ethnobotanical use of some Lamiaceae species: flowers, leaves and essential oil of *Hyssopus officinalis* exhibited expectorant and antiviral activities; *Nepeta kokanica* leaves and flowers characterized by anthelmintic, emetic, digestive activity. Also, some studies demonstrated the allelopathic activity that is characterized by a low level of toxicity (Yunosheva and Ellanska, 2018).

Taking into account the useful properties of these plants the aim of the research is the determination of the total antioxidant activity and the content of phenolic compounds of the leaves of selected Lamiaceae herbs in the period of flowering.

Materials and methodology

Biological material

Plant material of this study was above-ground parts plants of *Hyssopus officinalis* L., *Lavandula angustifolia* Mill., *Nepeta kokanica* (Regel) Kuntze, *Rosmarinus officinalis* L., *Salvia officinalis* L.,

Thymus serpyllum L., and *Th. vulgaris* L. harvested from the collection of the Botanical Garden of Slovak University in Nitra at the period of flowering. Plant raw dried in the oven (Binder 115, Germany) at 38 °C temperature, 48 hours. Biochemical analyses conducted at the Slovak Agricultural University in Nitra.

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and CentralChem (Slovakia).

Preparation of sample extracts

The dry above-ground parts of plants were used for the detection of total phenolic content and total flavonoid content. An amount of 0.2 g of each sample was extracted with 20 mL of 80% ethanol for 24 h. Then, the sample in 80% ethanol was centrifuged at 4605 RCF (Rotofix 32 A, Hettich, Germany) for 10 min and the supernatant was used for measurement with the DPPH and molybdenum reducing antioxidant power methods.

Determination of antioxidant activity

Total polyphenol content

The total polyphenol content (TPC) was measured by the method of Singleton and Rossi (1965) using the Folin-Ciocalteu reagent. A quantity of 0.1 mL of each sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured with the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25–300 mg/L–1; R^2 = 0.998) was used as the standard. The results were expressed in mg/g DM gallic acid equivalent (GAE).

Total flavonoid content

The total flavonoid content (TFC) was determined by the modified method described by Shafii et al. (2017). An aliquot of 0.5 mL of the sample was mixed with 0.1mL of 10% (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M potassium acetate and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (1–400 mg/L; R^2 = 0.9977) was used as the standard. The results were expressed in mg/g DM quercetin equivalent (QE).

Total phenolic acid content

Total phenolic acid (TPA) content was determined using the method of Farmakopea Polska (1999). A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% $NaNO_2$ + 10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1–200 mg/L, R^2 = 0.999) was used as a standard and the results were expressed in mg/g DM caffeic acid equivalents (CAE).

Free radical scavenging activity

Free radical scavenging activity of samples was measured using the 2.2-diphenyl-1-picrylhydrazyl (DPPH) (Sanches-Moreno et al., 1998). An amount of 0.4mL of the sample was mixed with 3.6mL of DPPH solution (0.025 g DPPH in 100 mL methanol). The absorbance of the reaction mixture was determined with the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10–100 mg/L; R^2 = 0.989) was used as the standard and the results were expressed in mg/g DM Trolox equivalents (TE).

Molybdenum reducing antioxidant power

Molybdenum reducing (MRP) antioxidant power of samples was determined by the method of Prieto et al. (1999) with slight modifications. The mixture of the sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then rapidly cooled. The absorbance at 700 nm was detected with the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10–1,000 mg/L; R^2 = 0.998) was used as the standard and the results were expressed in mg/g DM Trolox equivalent (TE).

Statistical analysis

Data were analyzed with ANOVA test and differences between means compared through the Tukey-Kramer test (α = 0.05).

Results and discussion

Polyphenols are the group of biologically active compounds, the main classes of which are phenolic acids and flavonoids. Epidemiological studies showed that diet dense in polyphenols has a higher chance of cardiac safety. Polyphenols exhibit anticancer and anti-diabetic activity (Abbas, 2017). Plant phenolic compounds play a protective role against degenerative diseases due to their antioxidative action (Beer et al., 2002).

The number of different groups of polyphenol compounds varied with the investigated plant species (Figure 1). Total phenolics were ranged from 39.43 (*Lavandula angustifolia*) to 62.87 (*Salvia officinalis*) mg CAE/g DW.

According to Fathiazad et al. (2011), the content of polyphenol compounds of n-butanol and ethyl acetate extracts of *Hyssopus officinalis* was 246 mg GAE/g and 51 mg GAE/g, respectively. In our study ethanol extracts had value 45.47 mg GAE/g DW.

As reported by Al'udatt et al. (2015), the total phenolic content of different extracts of *Thymus vulgaris* was from 0.92 to 2.49 mg GAE/g. In other studies, polyphenol content for *Th. vulgaris* was 0.58 mg GAE per 100 g DW (Wojdyło et al., 2007), 158 mg GAE/mg DW (Köksal et al., 2017), 32 mg GAE/mg DW (Armatu et al., 2010). In our study, this parameter had value 52.1 mg GAE/mg DW.

The total phenolic content of herb extracts of *Salvia officinalis* in some reports was 8.25 mg GAE per 100 g DW (Wojdyło et al., 2007), 31.5 mg GAE/g (Abdelkader et al., 2014), 32 mg GAE/g (Armatu et al., 2010). In our study extracts of this species had the most content of polyphenols and was determined as 62.87 mg GAE/g DW.

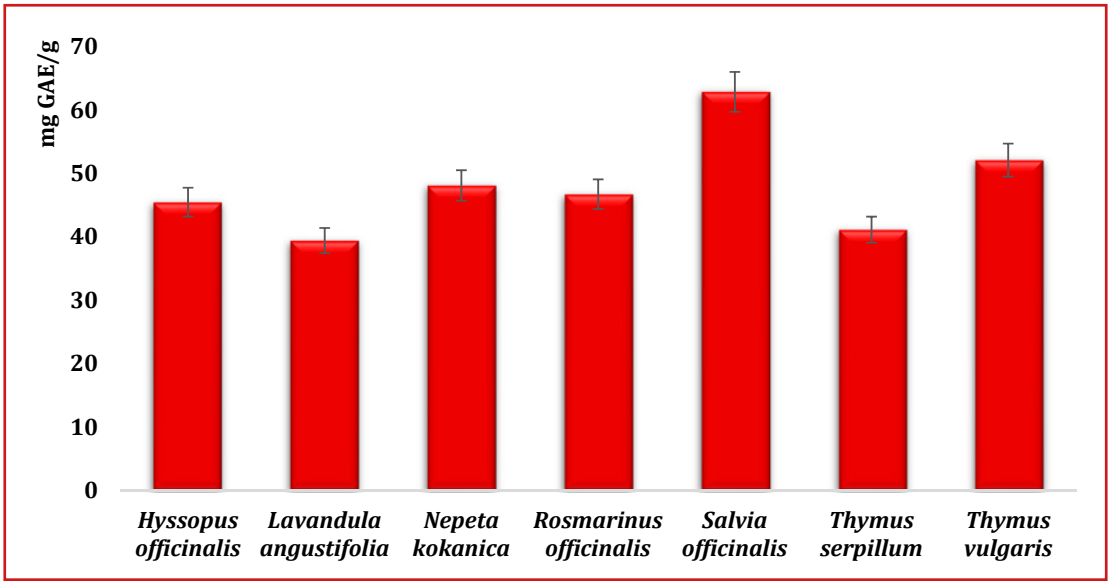


Figure 1 Total content of polyphenol compounds of ethanol extracts of Lamiaceae herbs in the period of flowering

According to Wojdyło et al. (2007), the total phenolic content of herb extracts of *Rosmarinus officinalis* 1.71 mg GAE per 100 g DW. Bourhia et al. (2019) resulted, that ethanol extracts of *R. officinalis* demonstrated polyphenol content from 74.15 to 146.63 mg GAE/mg depending on the origin. According to Hendel et al. (2016), total polyphenol content in this species was 128.98 mg GAE/g. Armatu et al. (2010) found 22 mg GAE/g polyphenol content of this species. In our research value of this parameter for *R. officinalis* was 46.73 mg GAE/g DW. Andrys et al. (2017) reported that polyphenol content in *Lavandula angustifolia* extracts was 12.3–21.7 mg GAE/g. Duda et al. (2015) found polyphenol content in *L. angustifolia* 12.44 –18.16 mg GAE/g DW. It should be noted that in our study ethanol extracts of *L. angustifolia* had the least value of polyphenol compounds content among other investigated species.

But should be taken into account that fact that the physicochemical nature of the individual phenolics in the extracts may be more important in contributing to the antioxidant activity than the total phenolic content (Santos et al., 2012).

The simplest phenolic compounds found in plants are derivates of benzoic and cinnamic acids, which related to the group of phenolic acids among them gallic, coumaric, caffeic, ferulic, etc (Beer et al., 2002). Some phenolic acids found in free (many fruits and vegetables) and bound form (Abbas,2017). The phenolic acid content of investigated extracts was from 4.91 (*Nepeta kokanica*) to 24.30 (*Salvia officinalis*) mg CAE/g DW (Figure 2).

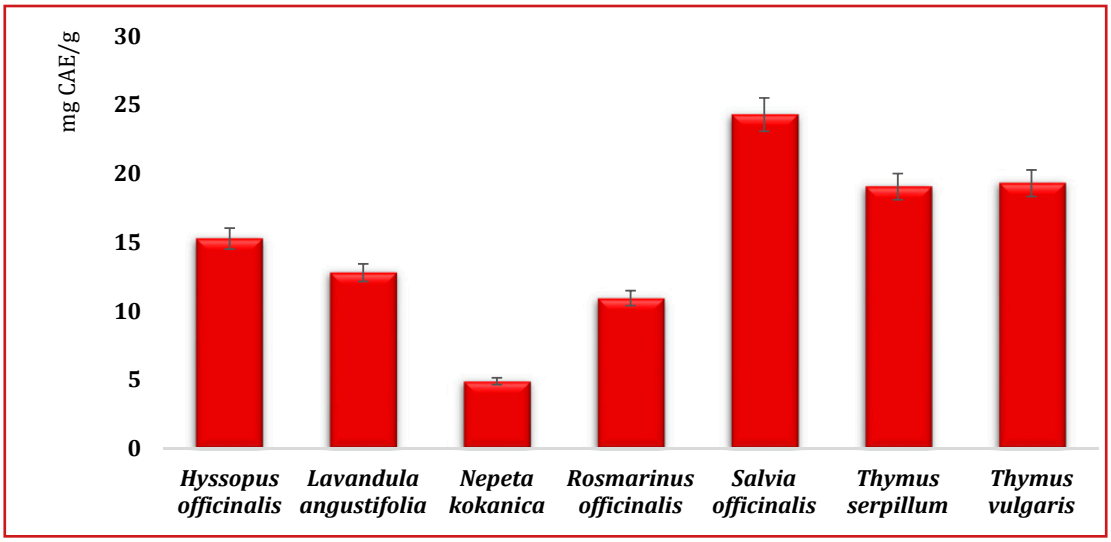


Figure 2 Total content of phenolic acids of ethanol extracts of Lamiaceae herbs in the period of flowering

Flavonoids are polyphenol compounds that are widely distributed in plants that perform many functions such as chemical messengers, physiological regulators, cell cycle inhibitors (Köksal et al., 2017). The content of flavonoids was from 20.80 to (*Thymus* serpillum) to 36.80 (*Salvia officinalis*) mg QE/g DW (Figure 3).

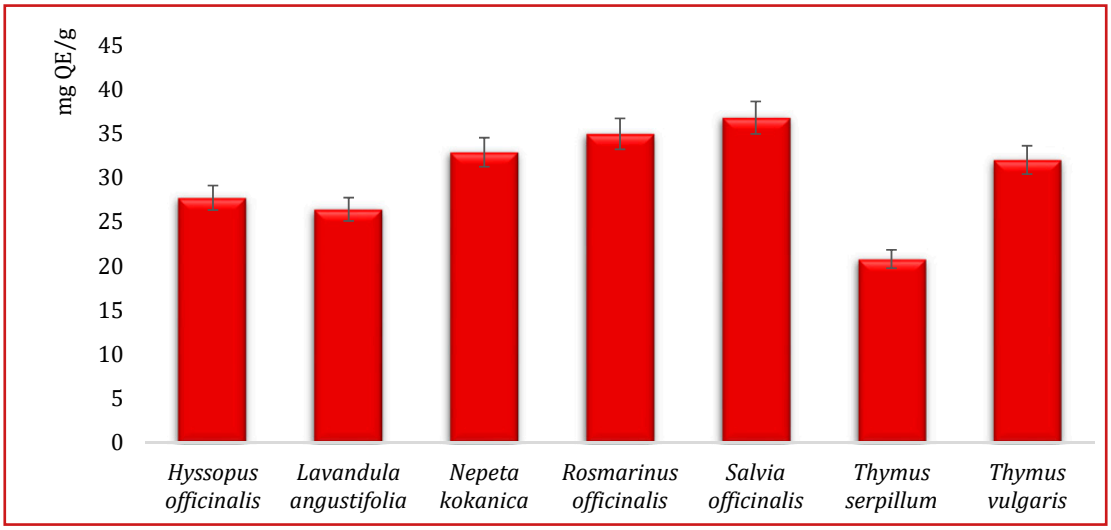


Figure 3 Total content of flavonoids of ethanol extracts of Lamiaceae herbs in the period of flowering

Purified flavonoids from *Hyssopus officinalis* demonstrated a high antioxidant activity. The most known flavonoid from the aerial part of this species is apigenin (Fathiazad et al., 2011). Duda et al. (2015) found the content of flavonoids in *Lavandula angustifolia* 3.37–4.85 mg QE/g DW. As reported Hendel et al. (2016), flavonoids content for *Rosmarinus officinalis* was

38.02 mg QE/g. Abdelkader et al. (2014) determined in extracts of *Salvia officinalis* flavonoids 18.46 mg QE/g. The study of Köksal et al. (2017) reported that the content of flavonoids in ethanol extracts of *Thymus vulgaris* was 36.6 mg QE/mg.

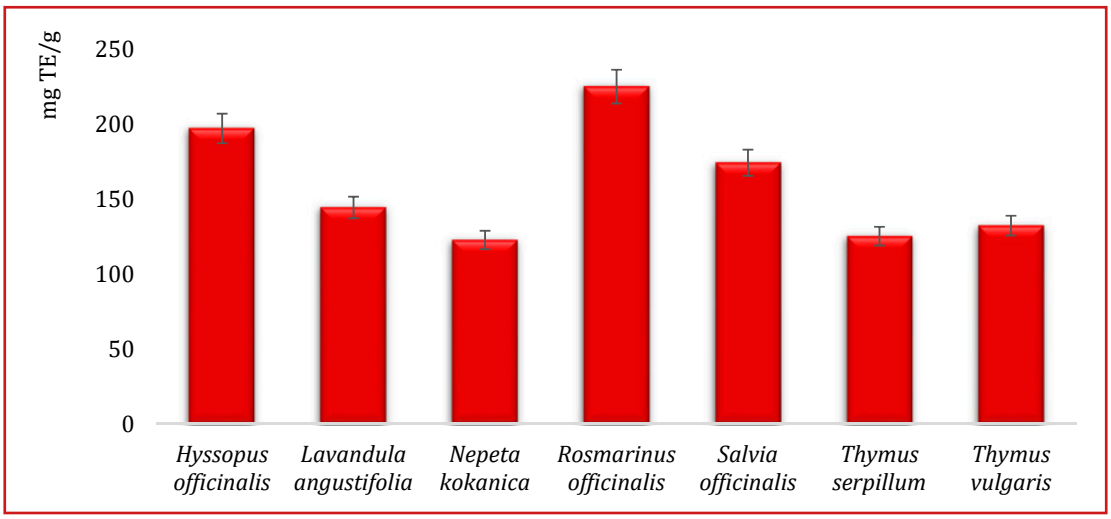


Figure 4 Antioxidant activity by phospomolybdenum method of ethanol extracts of Lamiaceae herbs in the period of flowering

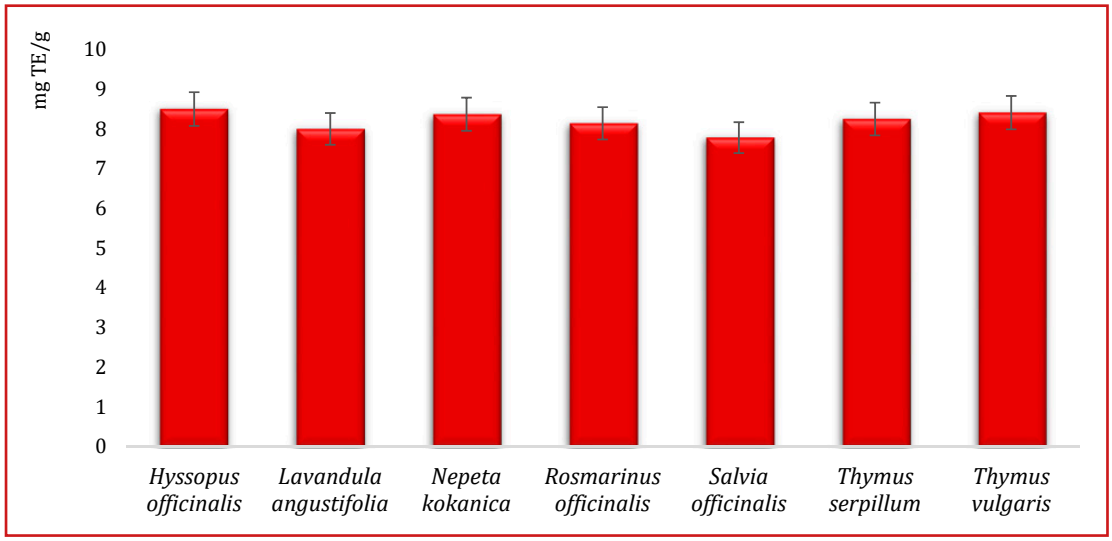


Figure 5 Antioxidant activity by DPPH-method of ethanol extracts of Lamiaceae herbs in the period of flowering

Exist numerous methods of measuring antioxidant activity for the screening of new plant antioxidants such as DPPH, DMPD, ABTS, ORAC, nitric oxide method, etc. (Gupta and Sharma, 2006). Antioxidant activity of investigated plants by phosphomolybdenum method was from 122.92 (*Nepeta kokanica*) to 225.36 (*Rosmarinus officinalis*) mg TE/g DW (Figure 4).

Antioxidant activity by phosphomolybdenum method, which also called reducing power of extract, in our previous study with *Scutellaria baicalensis* showed a strong correlation with all investigated groups of phenolic compounds (Vergun et al., 2019).

The antioxidant activity of ethanol extracts of investigated plants was from 7.78 (*Salvia officinalis*) to 8.50 (*Hyssopus officinalis*) mg TE/g (Figure 5). Amamra et al. (2018) found a correlation between antioxidant activity by DPPH and other methods and content of polyphenols and flavonoids (from 0.73 to 0.98) in different extracts of *Thymus vulgaris*.

Conclusions

Taking into account the results of this study and results obtained from other researchers should be concluded that a group of investigated Lamiaceae species is a valuable source of antioxidant compounds that can be recommended for use in the food and pharmacological industry. It should be noted that *Salvia officinalis* ethanol extracts were the leader of containing the polyphenols, phenolic acids and flavonoids among other investigated species. The study of plant raw for the screening of antioxidant compounds gives the possibility to identify new species for human use considering also the origin of plants.

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DETERMINATION OF ANTIOXIDANT CAPACITY AND POLYPHENOLS CONTENTS IN FRUITS OF GENOTYPES OF *CHAENOMELES JAPONICA* (THUNB.) LINDL.

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In traditional Chinese medicine, the fruits of various species of *Chaenomeles* Lindl. have been used for thousands of years in the treatment of rheumatoid arthritis, hepatitis, asthma, and the common cold and also have reported anti-inflammatory, antinociceptive, antimicrobial, antioxidant, immunoregulatory, antitumor, hepatoprotective, antiparkinsonian, and antioxidant activities. Fruits contain many bioactive components, including polyphenols, triterpenes, and organic acids. Cold-pressed *Chaenomeles japonica* (Thunb.) Lindl. seed oil is one of the richest sources of natural micro constituents such as fatty acids, carotenoids, squalene, polyphenols, phytosterols, tocopherols and other compounds with high biological activity. The aim of this work was to evaluate the biological activity of fruits of *Chaenomeles japonica* genotypes, as potential species for cultivation and use in Ukraine. The objects of the research were 13 genotypes of *Chaenomeles japonica* selection in M.M. Gryshko National Botanical Garden (Kyiv, Ukraine). The raw materials were collected in the season of full ripeness (October). Antioxidant activity (AOA) was measured using three different photometric methods (DPPH – 2.2-diphenyl-1-picrylhydrazyl, ABTS – 2.2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, FRAP – ferric-reducing antioxidant power). Total polyphenol content (TPC) was evaluated using the Folin-Ciocalteu reagent assay. The results for AOA (μmol Trolox/g) determined by the DPPH method varied from 10.08 to 21.42, those obtained by the ABTS method varied from 31.13 to 70.87, and those obtained by the FRAP method varied from 14.97 to 42.51. The results for TPC varied from 162.46 to 408.09 mg/100 g. The results showed that all fruit extracts exhibited strong antioxidant activities, which generally correlated positively with the total phenol contents. Due to the content of biologically active substances, the fruits are valuable raw materials for various types of processing in the confectionery, distillery, pharmaceutical industries, production of natural low-calorie products for children, diet and treatment and prophylactic nutrition. This means that genotypes of *Chaenomeles japonica* may be used as a source of new health resources when improving the nutritional properties of the world's less traditional fruit species.

Keywords: *Chaenomeles japonica*, genotypes, fruits, antioxidant activity, phenols contents

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Introduction

The genus *Chaenomeles* Lindl. belongs to the Rosaceae Juss. family and comprises four species: *C. cathayensis* (Hemsl.) C.K.Schneid., *C. japonica* (Thunb.) Lindl., *C. speciosa* (Sweet) Nak. and *C. thibetica* T.T.Yu (Phipps et al., 1990). *Japanese quince* (*Chaenomeles japonica*), a shrub naturally occurs in central and southern Japan (Phipps et al., 1990). Compared to the other three species *Japanese quince* best suited to the climate of northern Europe, where it was introduced in 1869 (Weber, 1964).

In traditional Chinese medicine, the fruits of various species of *Chaenomeles* have been used for thousands of years in the treatment of rheumatoid arthritis, hepatitis, asthma, and the common cold (Zhang et al., 2010, 2014) and also have reported anti-inflammatory, antinociceptive, antimicrobial, antioxidant, immunoregulatory, antitumor, hepatoprotective, antiparkinsonian, and antioxidant activities (Xie et al., 2007; Zhao et al., 2008; Zhang et al., 2010; Zhang et al., 2014).

Fruits by contains many bioactive components, including polyphenols, triterpenes, and organic acids (Lesińska, 1987; Lesińska et al, 1988; Hellín et al., 2003; Ros et al., 2004; Strek et al., 2007; Hallmann et al., 2011; Du et al., 2013; Baranowska-Bosiacka et al., 2017; Klymenko et al., 2017; Watychowicz et al., 2017). Cold-pressed *Chaenomeles japonica* seed oil is one of the richest sources of natural micro constituents such as fatty acids, carotenoids, squalene, polyphenols, phytosterols, tocopherols and other compounds with high biological activity (Górnaś et al., 2014).

The aim of this work was to evaluate the biological activity of fruits of *Chaenomeles japonica* genotypes, as potential species for cultivation and use in Ukraine.

Materials and methodology

Biological material

The objects of the research were 13 genotypes (Ch-01 – Ch-13) of *Chaenomeles japonica* selection in M.M. Gryshko National Botanical Garden of NAS of Ukraine (Kyiv). The raw materials were collected in the season of full ripeness (October).

Chemicals and spectral measurements

1.1-diphenyl-2-picrylhydrazyl (DPPH) ferrous chloride, tripyridyltriazine (TPTZ), kaliumperoxodisulfat, 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid were obtained from Sigma Chemical Co. (Sigma-Aldrich, Poland, Poznań). Methanol was obtained from POCh Poland. All chemicals and solvents were of analytical grade. All UV–V measurements were recorded on a Shimadzu UV–2401PC (Kyoto, Japan).

Preparation of extracts for analysis of total polyphenols and antioxidant activity

The amount of about 2 g of fruits was homogenized and extracted with 80% aqueous methanol to a final volume of 20 mL at room temperature. The extraction was performed in the ultrasonic

bath (Polsonic, Poland) for 15 minutes. All extracts were filtered through pickling mixture paper filters (Whatman filter No.1), and then they were subjected to analyses.

Determination of total polyphenol content (TPC)

Total phenolic contents of fruits were determined by using the Folin–Ciocalteu reagent method according to Gao et al. (2000). Plant extracts (0.1 mL) were mixed with 0.2 mL of Folin-Ciocalteu reagent and 2 mL of H_2O, and after 3 min 1 mL 20% sodium carbonate. Total polyphenols were determined after 1 h of incubation at room temperature in the dark. The absorbance of the resulting blue colour was measured at 765 nm with a Shimadzu UV-VIS spectrophotometer. The standard curve was prepared using different concentrations of gallic acid. The results were calculated as mg of gallic acid equivalent (GAE)/1 g. All the determinations were performed in triplicates.

Determination of Reducing power, (FRAP) radical scavenging activity

Ferric reducing antioxidant power (FRAP) was measured suing Benzie and Strain (1996). An aliquot (1.0 ml) of the diluted extract was added to 3 ml of FRAP solution (acetate uffer (300 µM, pH 3.6), a solution of 10 µM TPTZ in 40 µM HCl, and 20 µM $FeCl_3$ at 10 : 1 : 1 (*v*/*v*/*v*) ratio). The mixture was shaken and left at room temperature for 10 min. The absorbance was read at 593 nm after 10 minutes using a Shimadzu UV2401PC spectrophotometer. The standard curve was prepared using different concentrations of Trolox. The results of the assay were expressed in µM Trilox per 1 g. All the determinations were performed in triplicates.

Determination of (DPPH) radical scavenging activity

The DPPH free radical scavenging activity of fruits extracts was measured from bleaching of the purple colour of (2.2-diphenyl-1-picryl hydrazyl) out as described by Yen and Chen (1995). Exactly 0.5 ml solution of different concentrations of extracts was added to 2 ml of DPPH. The mixture was shaken and left at room temperature for 10 min. The absorbance was measured at 517 nm, using a Shimadzu UV2401PC spectrophotometer. The standard curve was prepared using different concentrations of Trolox. The results of the assay were expressed in µM Trolox per 1 g. All the determinations were performed in triplicates.

Determination of ABTS radical scavenging activity

ABTS (2.2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) assay was based on the method of Re et al. (1999). Briefly, ABTS radical cation is generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate via incubation at room temperature (23 °C) in the dark for 12–16 h. The ABTS solution was diluted with an absorbance of 0.700 ±0.040 at 734 nm. The reagent blank reading was taken (A_0). After the addition of 3.0 ml of diluted $ABTS^+$ solution to 30 µl of plant extracts, the absorbance reading was taken exactly 6 min after the initial mixing (A_t). The standard curve was prepared using different concentrations of Trolox. Results of antioxidant activity were expressed in µMol Trolox equivalents (TE)/g. All the determinations were performed in triplicates.

Statistical analysis

Basic statistical analyses were performed using PAST 2.17. The variability of all these parameters was evaluated using descriptive statistics. Correlation coefficients were calculated by CORR analysis.

Results and discussion

Last years in the gardening of our country, as well as other countries, new fruit and berry plants till recently meeting only in nature, are entered. The non-traditional fruits plants differ by high maintenance of valuable biologically active substances and have an important economic value. Works on the introduction and selection of new for a culture in Ukraine species are conducted the M.M. Gryshko National Botanical Garden of Ukraine (NBG) in Kyiv. By basis for the creation of cultivars sewed rich genetic fund of fruits plants, collected in collections of NBG, which presented more than by 120 species and varieties of growing wild and 2000 cultivars of different species (Klymenko and Grygorieva, 2013; Klymenko et al., 2014, 2017). The Department of Fruit Plants Acclimatisation of NBG explores biochemical composition and biological activity different from species of non-traditional plants namely *Cornus mas* L. (Klimenko, 2004; Kucharska et al., 2015), *Chaenomeles* spp. (Klymenko and Mezhenskyj, 2013), *Pseudocydonia sinensis* Schneid. (Monka et al., 2014; Grygorieva et al., 2019), *Asimina triloba* (L.) Dunal (Klymenko and Grygorieva, 2016; Brindza et al., 2019), *Morus nigra* L. (Kucelova et al., 2016), *Ziziphus jujuba* Mill. (Ivanišová et al., 2017), *Castanea sativa* Mill. (Grygorieva et al., 2018e), *Diospyros virginiana* L. (Grygorieva et al., 2018c, d), *Sambucus nigra* L. (Horčinová Sedláčková et al., 2018), *Mespilus germanica* L. (Grygorieva et al., 2018b), *Elaeagnus multiflora* Thunb. (Grygorieva et al., 2018a), *Aronia michurinii* A. Skvorts. et Yu. Maitullina. (Vinogradova et al., 2018).

Chaenomeles spp. collection fund of NBG a great diversity (Figure 1). The collection of *Chaenomeles* in the department is represented by several species, in particular, *Ch. japonica* (Thunb.) Lindl., *Ch. speciosa* (Sweet) Nakai, *Ch.* × *superba* (Frahm) Rehder, *Ch. cathayensis* (Hemsl.) Rehd. and numerous hybrid varieties – fruit and decorative. It is a representative genetic bank of species for the reproduction and introduction of *Chaenomeles* as valuable fruits, medicinal and decorative plants in farmer and amateur plants. Selection work with *Chaenomeles* in the NBG has been conducted for more than 50 years, and four varieties of *Chaenomeles japonica* are incorporated in the Register of Plant Varieties of Ukraine. There are 17 more promising genotypes (Klymenko and Mezhenskyj, 2013).

Phenolic compounds can act as low molecular weight antioxidants, protect cells from the effect of oxidative stress developing in conditions of hypothermia. Having high reaction activity due to the presence in the structure of aromatic rings and free hydroxyl groups, they can easily engage in free-radical reactions and connect the active forms of oxygen and peroxy radicals produced in the cells under stress conditions. Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiative or aggression by pathogens. They are formed in all organs of plants from sugars and participate in the process of cellular respiration, transferring hydrogen from oxidative molecules. Phenolic compounds exhibit a strong effect on the growth of plants, inhibiting germination of seeds, growth of

stems and roots. They have strong anti-bacterial properties and provide plant immunity to fungal and especially to bacterial infection.



Figure 1 Fruits of different cultivars of *Chaenomeles japonica* (Thunb.) Lindl. selection of M.M. Gryshko National Botanical Garden (Kyiv, Ukraine)

The amount of total polyphenols contents varied with the genotypes (Figure 2). Total polyphenols contents were ranged from 162.46 ±2.41 to 408.09 ±8.67 mg/100 g.

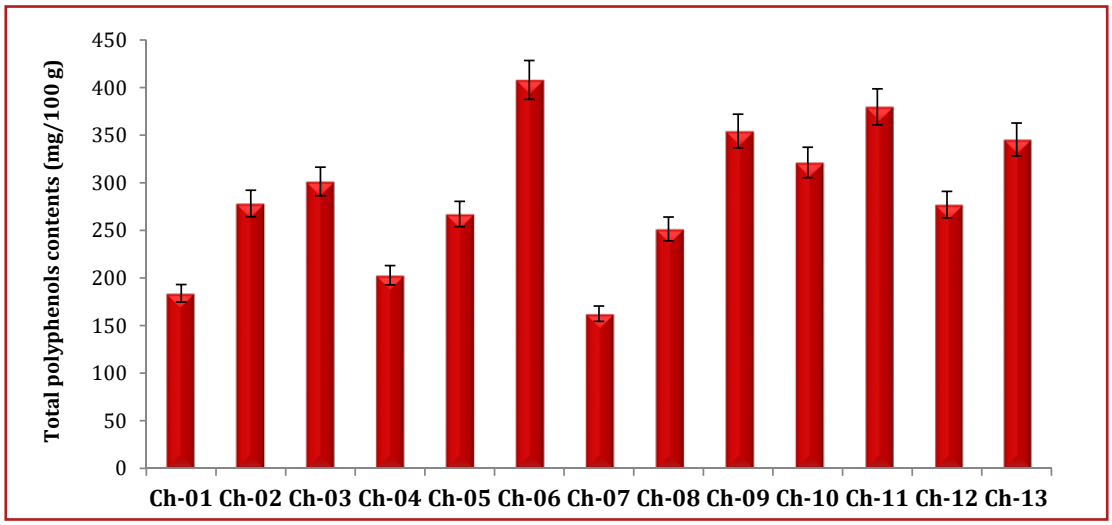


Figure 2 Total polyphenols contents of genotypes of *Chaenomeles japonica* (Thunb.) Lindl. fruits extracts based on gallic acid equivalents (GAE, Line) (mg/100 g)

Our results are consistent with data of researches investigated the polyphenols of *C. sinensis* and *C. speciosa* (Miao et al., 2016). Miao et al. (2016) report that great in *C. sinensis* total polyphenol content fruits from 345 to 590 mg GAE/g DW and in *C. speciosa* from 356 to 404 mg GAE/g DW. Du et al. (2013) in fresh weight identified total polyphenol content 19.35 ±0.59 mg GAE/g FW.

Antioxidants are substances that can prevent or slow damage to cells caused by free radicals, unstable molecules that radicals, that the body produces as a reaction to environmental and other pressures. 'Antioxidant' is not really the name of a substance, but rather it describes what a rang of substances can do. Flavonoids, flavones, catechins, polyphenols, and phytoestrogens are all types of antioxidants and phytonutrients, and they are all found in plants – based foods. The best sources of antioxidants are plant – based foods, especially fruits and vegetables. Food that is particularly high in antioxidants are often referred to as a 'super food' or 'functional food'.

The DPPH radical scavenging activity of each *Chaenomeles japonica* genotypes extracts is shown in Figure 3. Antioxidant activity of fruits genotypes ranged from 10.08 (Ch-07) to 21.42 (Ch-06) µMol Trolox/g.

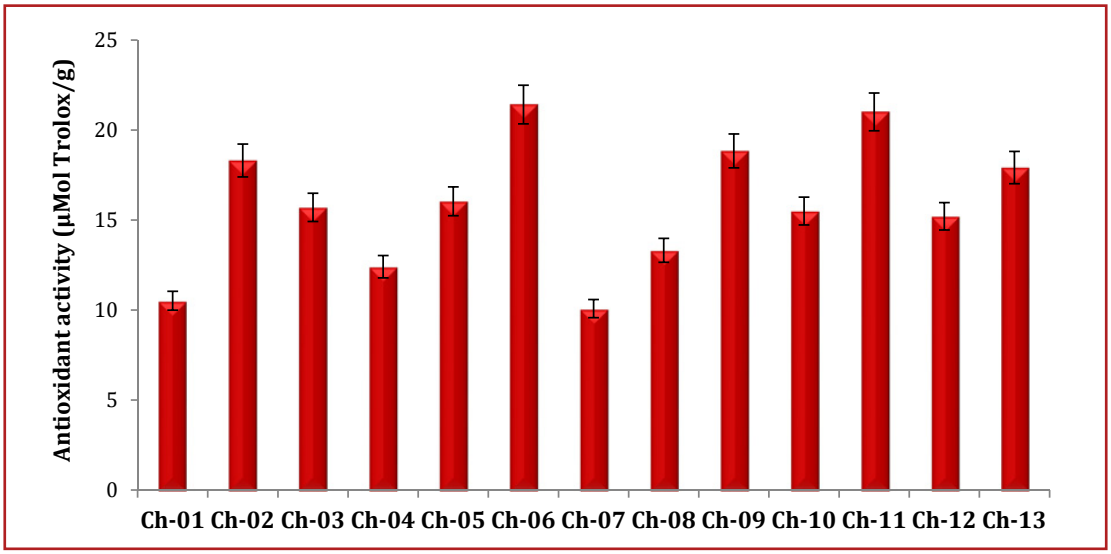


Figure 3 Antioxidant activity of *Chaenomeles japonica* (Thunb.) Lindl. extracts determined by DPPH method (µMol Trolox/g)

Other stable free radical cation, $ABTS^{+}$, was used to evaluate the antioxidant activity of the *Chaenomeles japonica* extracts.

As shown in Figure 4 antioxidant activity ranged from 31.13 (Ch-01) and 31.54 (Ch-07) to 70.29 (Ch-09) and 70.87 (Ch-06) µMol Trolox/g and had the most results comparing with other methods.

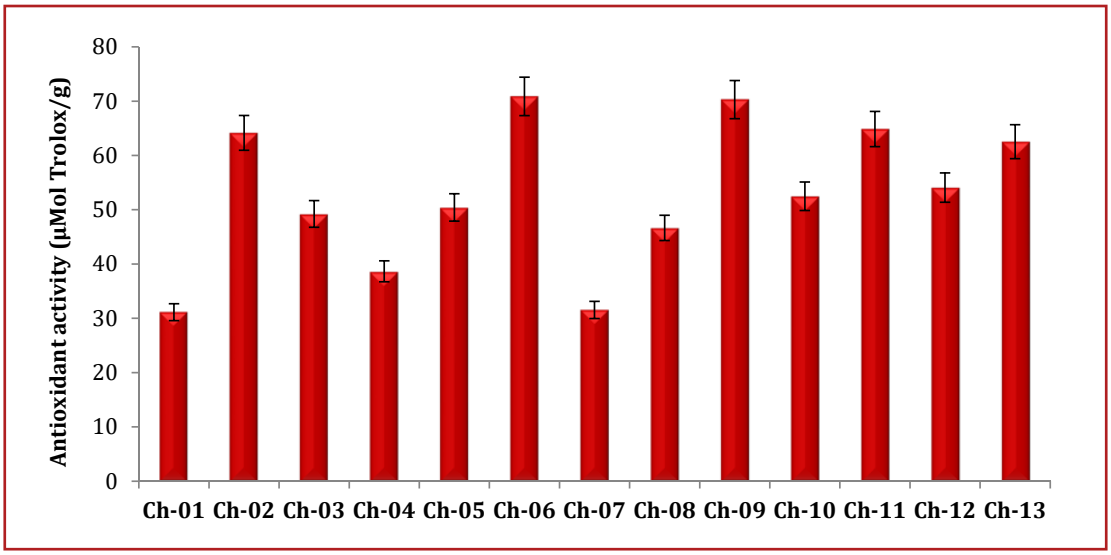


Figure 4 Antioxidant activity of *Chaenomeles japonica* (Thunb.) Lindl. extracts determined by ABTS method (µMol Trolox/g)

Antioxidant activity of *Chaenomeles japonica* genotypes evaluated by FRAP method (Figure 5) ranged from 14.97 (Ch-07) and 15.64 (Ch-01) to 40.22 (Ch-11) and 42.51 (Ch-06) µMol Trolox/g.

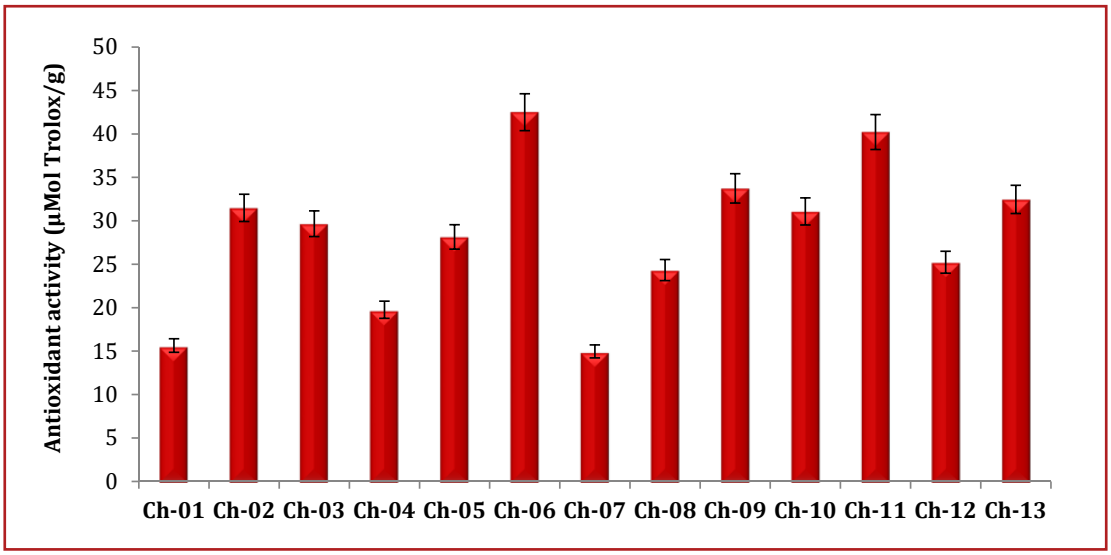


Figure 5 Antioxidant activity of *Chaenomeles japonica* (Thunb.) Lindl. extracts determined by FRAP method (µMol Trolox/g)

Our results compared favourably with previous studies on *C. japonica* (Nawirska et al., 2007; Tarko et al., 2010; Du et al., 2013), and showed equivalent antioxidant activity. Nawirska et al. (2007) investigated the antioxidant activity of *C. japonica* by ABTS, DPPH and FRAP methods.

Results were ranged 13.97 ±0.06, 18.21 ±0.34 and 6.12 ±0.01 μMol Trolox/g DW, respectively. Du et al. (2013) investigated the antioxidant activity of *C. japonica* by ABTS (118.15 ±4.10 μMol Trolox/g FW) and FRAP (19.10 ±0.76 μMol Trolox/g FW) methods.

The Pearson correlation coefficients between antioxidant activities and TPC were depicted in Figure 6.

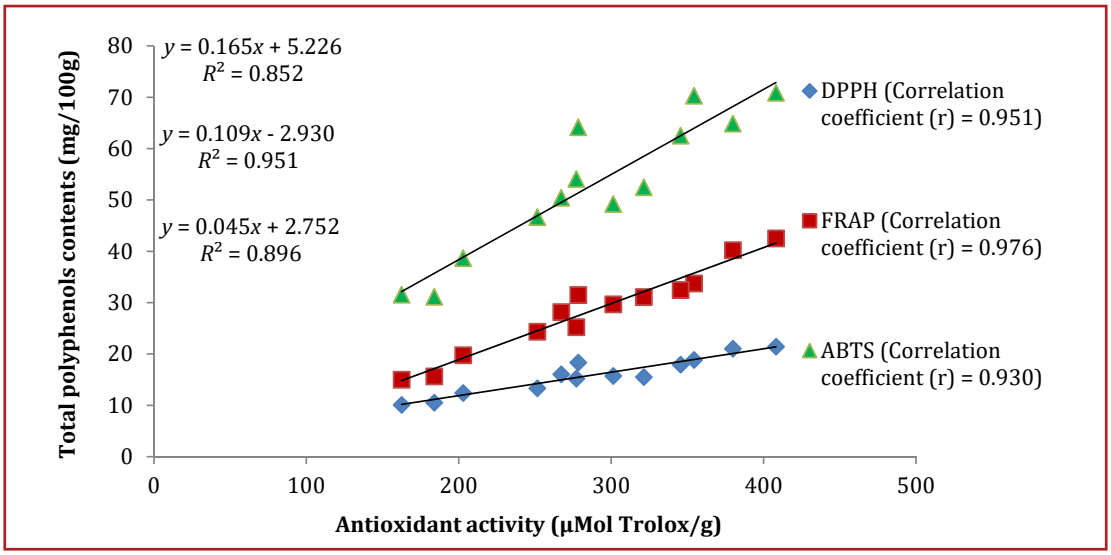


Figure 6 Correlation between antioxidant activity and TPC of *Chaenomeles japonica* (Thunb.) Lindl. fruits extract

The findings of this study indicate that total polyphenols present dramatically high and positive correlations with ABTS scavenging capacity, DPPH and FRAP (r = 0.930, p <0.05; r = 0.951, p <0.05 and r = 0.976, p <0.05, respectively), which confirmed that fruits had strong antioxidant activities as previously reported (Rasmussen et al., 2005; Du et al., 2013).

Conclusions

This study demonstrates the potential of fruits *Chaenomeles japonica* in Ukraine, as a prospective source of valuable polyphenol contents with high antioxidant activities and health beneficial properties. The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicated that these compounds contribute to the strong antioxidant activity. Due to the content of biologically active substances, the fruits are valuable raw materials for various types of processing in the confectionery, distillery, pharmaceutical industries, production of natural low-calorie products for children, diet and treatment and prophylactic nutrition. Our results point to the fact that observed plants of *Chaenomeles* are suitable natural sourced have high biological efficiency – antioxidant activity. This means that some *Chaenomeles* cultivars be used as a source of new health sources when improving the nutritional properties of the world's less traditional fruit species.

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ANTIOXIDANT ACTIVITIES AND PHENOLIC COMPOUNDS IN FRUITS OF CULTIVARS OF CORNELIAN CHERRY (*CORNUS MAS* L.)

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Cornelian cherry (*Cornus mas* L.) is a plant grown in the Eastern and Southern regions of Europe and the Middle East, where it has been used in domestic cuisines for centuries and is one of the most valuable fruit plants in the family Cornaceae Bercht. et J. Presl. It is a very ancient cultural plant in Ukraine known from times of Kyiv on Rus. It wildly used as a valuable food and medicinal plant. All parts of the plant cornelian cherry widely used in folk medicine for the prevention and treatment of many diseases. The aim of this study was to evaluate the antioxidant activity and phenolics content of *Cornus mas* 20 cultivars collected in the M.M. Gryshko National Botanical Garden of NAS of Ukraine. The content of the total antioxidant activity and phenolic compounds from the fruits of the *Cornus mas* twenty cultivars were compared. Antioxidant activity (AOA) was measured using three different photometric methods – DPPH (2.2-diphenyl-1-picrylhydrazyl), ABTS (2.2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (Ferric reducing antioxidant power). Total phenolic content (TPC) was evaluated using the Folin-Ciocalteu reagent assay. The results for AOA (μMol Trolox/g) determined by the DPPH method varied from 5.94 (Kozerog) to 16.56 (Kostia), ABTS method varied from 13.560 (Koralovyj Marka) to 33.96 (Semen), FRAP method varied from 8.45 (Koralovyj) to 22.49 (Kostia). The results for TPC varied from 91.34 (Kozerog) to 289.79 (Bolgarskyj) mg/100g. There was recorded a positive linear correlation between antioxidant activity and total phenolic content in the examined plant material. Obtained data showed that investigated cultivars have a potent antioxidant activity that can be used for further investigation and utilization of cornelian cherry. The results of our study also showed that cornelian cherry is an important traditional plant that deserves special attention for widespread growth in Ukraine due to its high biochemical characteristics, as well as useful nutritional and medicinal properties. The fruits of cornelian cherry are promising sources of biologically active substances. Cornelian cherry fruits are a rich source of nutrients for humans. Currently, this plant is less known and this new source can be potential as a functional food or value-added ingredient in the future in our dietary system.

Keywords: cornelian cherry, *Cornus mas*, cultivars, fruits, antioxidant activity, phenolics compounds

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Introduction

The genus *Cornus* L. of family Cornaceae Bercht. et J. Presl. consists of about 65 species and is widely distributed in the northern hemisphere with centers of diversity in southern and central Europe, southwest Asia, eastern and western North America and mountains of Central America, South America and East Africa (Eyde, 1988). Among the *Cornus* species, four species namely *Cornus mas* L., *C. officinali*s Siebold & Zucc., *C. chinensis* Wangerin and *C. kousa* F. Buerger ex Hance bear edible fruits that are consumed in different parts of Europe and Asia (Seeram et al., 2002).

The cornelian cherry (*Cornus mas*) is one of the most valuable fruit plants and known in garden cultivation for 4000 years. This plant comes from the foothills of the Caucasus and from there it spreads over Turkey, Romania, Bulgaria, and Italy and to the inland European continent (Dolejsi et al., 1991; Klimenko, 2004a, b; Brindza et al., 2009).

Cornelian cherry was and still is, known for its flavor, nutritional and medicinal benefits. Chemical composition of fruits of cornelian cherry rich enough. Biologically active compounds, such as vitamin C, organic acids (mainly malic acid), pectins (Seeram et al., 2002; Kucharska et al., 2011; Kucharska, 2012), phenolic acids (gallic and ellagic, and derivatives of hydroxycinnamic acids) (Pantelidis et al., 2007; Kucharska, 2012), flavonoids (anthocyanins, flavonols) (Tural and Koca, 2008; Pawlowska et al., 2010; Kucharska et al., 2015), triterpenoid (ursolic acid) (Yayaprakasam et al., 2006), iridoids (Kucharska et al., 2015; Klymenko et al., 2016), and recently identified iridoids (loganic acid, cornuside, loganin, sweroside) (West et al., 2012; Deng et al., 2013) have been reported in fruits of cornelian cherry. These compounds are purported to be beneficial for the prevention of heart disease and diabetes. Fruits and drugs of them (decoctions, tinctures, teas, concentrates of fresh and dried raw materials) exhibit antiscorbutic, antibacterial, anti-inflammatory, antioxidant, general health-improving, tonic, astringent, temporary hypotensive, and diuretic properties (Seeram et al., 2002; West et al., 2012). Fresh fruits recommend 10–12 g/day for neurasthenia, common weakening, joint pains, infectious hepatitis, and others. Cornelian cherry fruits also were used in folk medicine for the treatment of various fever-related diseases (flu, sore throat, and malaria) and gastrointestinal disorders.

The fruits, flowers, leaves, seeds, and bark in *Cornus mas* have been known and appreciated in folk medicine for years, especially in Asia and is a rich source of anthocyanins, phenolic acids, tannins, carotenoids, flavonoids, flavanols, iridoids, organic acids, vitamins (Du and Francis, 1973a, 1973b; Jensen et al., 1973; Zargari, 1997; Klimenko, 2004; Brindza et al., 2007; Horvath et al., 2007; Leskovac et al., 2007; Pantelidis et al., 2007; Kucharska et al., 2009; Savikin et al., 2009; Yilmaz et al., 2009; Pawlowska et al., 2010; Hassanpour, 2011; Deng et al., 2013; Cetkovska et al., 2014; Drkenda et al., 2014; Krivoruchko, 2014; Sochor et al., 2014; Behrangi et al., 2015; Milenkovic-Andjelkovic et al., 2015; Rudrapaul et al., 2015).

The pharmacological studies showed antibacterial (Jayaprakasam et al., 2006; Mirbadalzadeh and Shirdel, 2010; Radovanović et al., 2013; Asgary et al., 2014; Soltani et al., 2015; Shishehbor et al., 2016), hypolipidemic (Jayaprakasam et al., 2006; Sozański et al., 2016), antioxidant (Tural and Koca, 2008; Popović et al., 2012), anticoagulant (Rafieian-Kopaei et al., 2011),

antiparasitic (Mehrabi et al., 2012), cardioprotective (Alavian et al., 2014; Badalica-Petrescu et al., 2014; Darbandi et al., 2016), anticancer (Rezaei et al., 2014), hepatoprotective (Somi et al., 2014), and anti-inflammatory (Moldovan et al., 2016; Sozański et al., 2016) effects of the *Cornus mas* fruits.

There are many recipes of different foods prepared from cornelian cherry and supplied in private communications, such as cornelian cherry in sugar, lavash, syrup, juice, jam, candied fruits, jelly, marmalade, compote, wine, fruit drink, marinated cornelian cherry, cornelian cherry liqueur, pickles and so on (Brindza et al., 2007; Pantelidis et al., 2007; Klymenko et al., 2017). Refreshments from cornelian cherry are very tasty. Methods of using cornelian cherry as food raw materials are very diverse. Local people where cornelian cherries are known from immemorial time to prepare national dishes from cornelian cherry. Especially appreciated are Caucasus products called 'Turshu' and 'Lavash' – these are concentrates (they are stored for a long time) used as a product with a high content of vitamins, and fine seasoning for various dishes, especially meat. Fresh fruits can be stored for a long time, frayed or sprinkled with sugar. Very tasty are cornelian cherries frozen and canned in sugar syrup.

The aim of this study was to evaluate the antioxidant activity and phenolics content of twenty *Cornus mas* cultivars. Obtained data showed that investigated cultivars have a potent antioxidant activity that can be used for further investigation and utilization of *Cornus mas*.

Material and methodology

Biological material

The fruits of 20 cultivars of *Cornus mas* collected and selection in the M.M. Gryshko National Botanical Garden of NAS of Ukraine (NBG) were the objects of these investigations. The raw material was collected in the period (September) of full ripeness.

Chemicals and spectral measurements

1.1-diphenyl-2-picrylhydrazyl (DPPH) ferrous chloride, tripyridyltriazine (TPTZ), kaliumperoxodisulfat, 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid were obtained from Sigma Chemical Co. (Sigma-Aldrich, Poland, Poznań). Methanol was obtained from POCh Poland. All chemicals and solvents were of analytical grade. All UV–V measurements were recorded on a Shimadzu UV–2401PC (Kyoto, Japan).

Preparation of extracts for analysis of total polyphenols and antioxidant activity

The amount of about 2 g of fruits was homogenized and extracted with 80% aqueous methanol to a final volume of 20 mL at room temperature. The extraction was performed in the ultrasonic bath (Polsonic, Poland) for 15 minutes. All extracts were filtered through pickling mixture paper filters (Whatman filter No. 1), and then they were subjected to analyses.

Determination of total polyphenols content (TPC)

Total phenolic contents of fruits were determined by using the Folin-Ciocalteu reagent method according to Gao et al. (2000). Plant extracts (0.1 mL) were mixed with 0.2 mL of Folin-Ciocalteu reagent and 2 mL of H_2O and after 3 min 1 mL 20% sodium carbonate. Total polyphenols were determined after 1 h of incubation at room temperature in the dark. The absorbance of the resulting blue colour was measured at 765 nm with a Shimadzu UV-VIS spectrophotometer. The standard curve was prepared using different concentrations of gallic acid. The results were calculated as mg of gallic acid equivalent (GAE)/1 g. All the determinations were performed in triplicates.

Determination of Reducing power, (FRAP) radical scavenging activity

Ferric reducing antioxidant power (FRAP) was measured suing Benzie and Strain (1996). An aliquot (1.0 ml) of the diluted extract was added to 3 ml of FRAP solution (acetate buffer (300 µM, pH 3.6), a solution of 10 µM TPTZ in 40 µM HCl, and 20 µM $FeCl_3$ at 10 : 1 :1 (*v*/*v*/*v*) ratio). The mixture was shaken and left at room temperature for 10 min. The absorbance was read at 593 nm after 10 minutes using a Shimadzu UV2401PC spectrophotometer. The standard curve was prepared using different concentrations of Trolox. The results of the assay were expressed in µM Trilox per 1 g. All the determinations were performed in triplicates.

Determination of (DPPH) radical scavenging activity

The DPPH free radical scavenging activity of fruits extracts was measured from bleaching of the purple colour of (2.2-diphenyl-1-picrylhydrazyl) out as described by Yen and Chen (1995). Exactly 0.5 ml solution of different concentrations of the extract was added to 2 ml of DPPH. The mixture was shaken and left at room temperature for 10 min. The absorbance was measured at 517 nm, using a Shimadzu UV2401PC spectrophotometer. The standard curve was prepared using different concentrations of Trolox. The results of the assay were expressed in µM Trolox per 1 g. All the determinations were performed in triplicates.

Determination of ABTS radical scavenging activity

ABTS (2.2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) assay was based on the method of Re et al. (1999). Briefly, ABTS radical cation is generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate via incubation at room temperature (23 °C) in the dark for 12–16 h. The ABTS solution was diluted with to an absorbance of 0.700 ±0.040 at 734 nm. The reagent blank reading was taken (A_0). After the addition of 3.0 ml of diluted $ABTS^+$ solution to 30 µl of plant extracts, the absorbance reading was taken exactly 6 min after the initial mixing (A_t). The standard curve was prepared using different concentrations of Trolox. Results of antioxidant activity were expressed in µMol Trolox equivalents (TE)/g. All the determinations were performed in triplicates.

Statistical analysis

Basic statistical analyses were performed using PAST 2.17; hierarchical cluster analyses of similarity between phenotypes were computed on the basis of the Bray-Curtis similarity

index. Data were analysed with ANOVA test and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$). Correlation coefficients were calculated by CORR analysis.

Results and discussion

Cornelian cherry is well known across Europe. In countries such as Austria, Bulgaria, England, Hungary, Moldova, Poland, Slovakia and Ukraine, this species has been known in the culture for hundreds of years and is widely used as a valuable edible, medicinal and ornamental plant. But only in recent decades, breeding programs aimed at the development of large fruit and high-yielding trees have been launched in several countries. Today, the selection of large-fruited cultivars are characterized by the following: different fruit shapes, smaller stones, desirable chemical composition, and different maturity dates on the same plant. Both producers and consumers desire such fruits.

In the NBG for 50 years selection work with cornelian cherry has been carried out. 14 cultivars were created which are included in the Register of Varieties of Ukraine. The gene pool of more than fifty cultivars and varieties created. Best cultivars of our selection are Evgenia, Semen, Coralovyi Marka, Svetlyachok, Elena, Vydubetskyi, Elegantnyi, Luk'yanovskyi, Exoticheskyi, Radost, Nikolka, Vavilovets, Vladimirskyi, Grenader (Klymenko et al., 2017a, b).

The first stage was the analytical selection when we used the results of the spontaneous selection. As a result of second stage – synthetic selection (cultivated forms of cornelian cherry of diversified origin were used) cultivars were developed that are characterized by steady annual fructification, with diverse biochemical properties and practical and valuable characteristics (mass, shape, color fruit, ripening and other), high productivity, and frost resistance under the conditions of the Forest-Steppe (Klymenko et al., 2017a, b). The shiny fruit is red, dark red cherry red, pink, or yellow, while the fruit can be oval, pear or bottle-shaped (Figure 1). Yellow fruited cultivars are either rare or no longer occur in the wild (Klimenko, 2004). The average fruit mass is 5.0–8.0 g, the maximal average is 9.0–10.0 g. The mass of the stone 7.5–11.0% from the mass of the fruit. They contain 8.0 to 15.0% of sugars; 1.3–1.9% of organic acids; 101.0–193.0 mg% of vitamin C; 670.0–850.0 mg% of anthocyanin the skin, and 36.0–121.3 mg% in the pulp (Klymenko et al., 2017a, b).

Plants have long been a source of dietary antioxidants. It is believed that two-thirds of the world's plant species have medicinal importance, and almost all of these have excellent antioxidant potential (Krishnaiah et al., 2011). Antioxidant activity of plants as *Chaenomeles japonica* (Thunb.) Lindl. Ex Spach (Zhang et al., 2010), *Cornus mas* L. (Kucharska et al., 2011; Milenkovic-Andjelkovic et al., 2015), *Pseudocydonia sinensis* Schneid. (Monka et al., 2014; Grygorieva et al., 2019), *Morus nigra* L. (Kucelova et al., 2016), *Ziziphus jujuba* Mill. (Ivanišová et al., 2017), *Diospyros virginiana* L. (Grygorieva et al., 2018), *Sambucus nigra* L. (Horčinová Sedláčková et al., 2018), *Asimina triloba* (L.) Dunal (Brindza et al., 2019) were discussed recently.



Figure 1 Fruits of different cultivars of *Cornus mas* L. selection of M.M. Gryshko National Botanical Garden (Kyiv, Ukraine)

Antioxidant activity of *Cornus mas*

The DPPH radical scavenging activity of each *Cornus mas* cultivars extracts is shown in Figure 2.

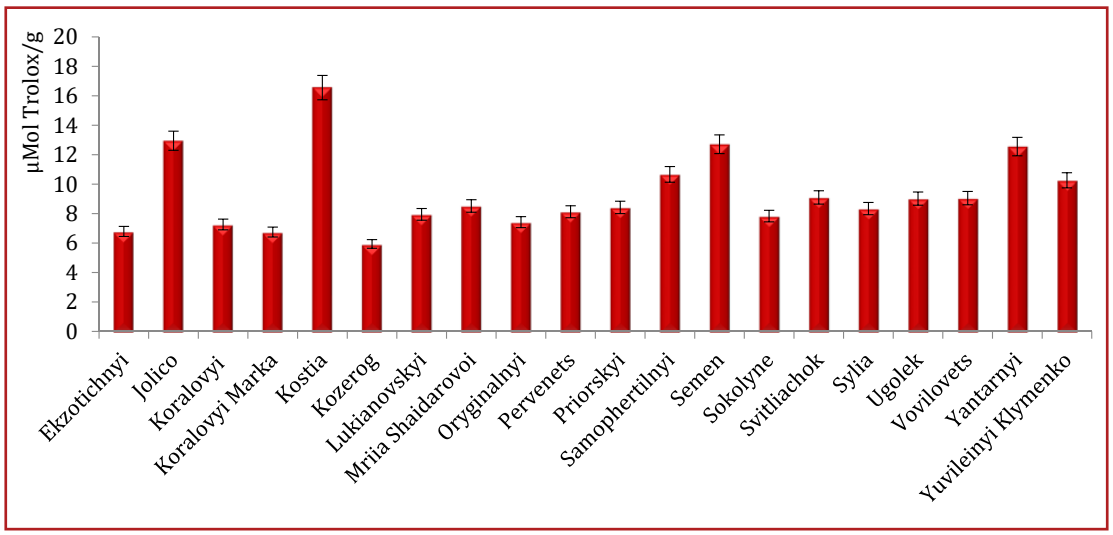


Figure 2 Antioxidant activity of *Cornus mas* L. cultivars evaluated by DPPH method (µMol Trolox/g)

Antioxidant activity of cornelian cherry cultivars ranged from 5.94 (Kozerog) to 16.56 (Kostia) µMol Trolox/g.

Milenkovic-Andjelkovic et al. (2015) study, 100 ml of cornelian cherry fruit extract can scavenge 94–109 mg of dissolved DPPH radicals. Tural and Koca (2008) reported that the methanolic extracts of the cornelian cherry fruits showed EC50 (mg/ml) (DPPH reduction) values as 0.52. Dragovic-Uzelac et al. (2007) determined that DPPH values in two different

cornelian cherry types were between 33.41 and 39.89 mmol Trolex equivalent/kg f.w. Paulovicsova et al. (2009), found that the antiradical activities were 77.59–84.56 and 88.85% in three cornelian cherry types. Hassanpour et al. (2011) stated that the efficiency of DPPH radicals' scavenging depends on the total polyphenolic concentration, total flavonoids, and ascorbic acid content. In the cited study, fruit extracts scavenged DPPH radicals at levels varying from 38.98 to 77.6%. According to Kucharska et al. (2011), antioxidant activity in Polish breeding cultivars was the highest in Dublany cultivar (20.72 µMol Trolox/g) and the lowest in Juliusz cultivar (10.85 µMol Trolox/g).

Other stable free radical cation, $ABTS^{+}$, was used to evaluate the antioxidant activity of the cornelian cherry extracts (Figure 3).

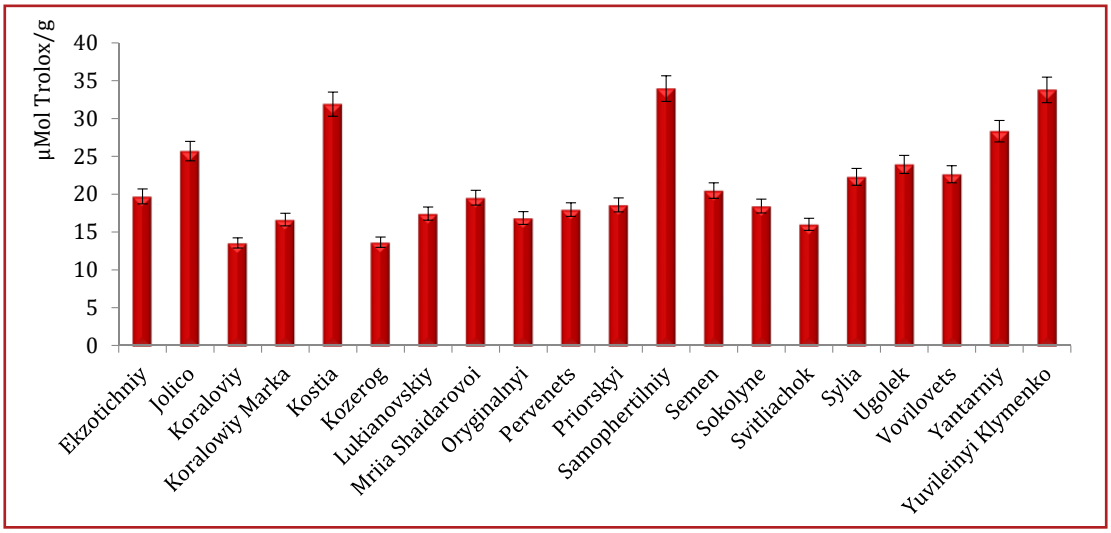


Figure 3 Antioxidant activity of *Cornus mas* L. cultivars evaluated by $ABTS^{+}$ method (µMol Trolox/g)

As shown in Figure 2 antioxidant activity of extracts of cornelian cherry cultivars measured by ABTS·+ ranged from 13.56 (Koralovyi Marka) to 33.96 (Semen) µMol Trolox/g and had the most results comparing with other methods. These results indicated that the cornelian cherry extracts have different radical scavenging activity depending on cultivars.

Moldovan et al., (2016) reported that the antioxidant activity of fresh cornelian cherries was 677.88 ±19.25 mol Trolox equivalents/100 g FW (ABTS assay). According to Rop et al. (2010), the content ranges of fruits from 3.65 (cv. Devin, Polish breeding) to 10.28 (Vydubeckyi, Ukrainian breeding) g GAE kg-1 FM. According to Kucharska et al. (2011) antioxidant activity in Polish breeding cultivars the content ranges of fruits from 18.87 (cv. Juliusz) to 38.96 (cv. Szafer) µMol Trolox/g.

Antioxidant activity of cornelian cherry cultivars evaluated by the FRAP method (Figure 4) ranged from 8.45 (Koralovyi) to 22.49 (Kostia) µMol Trolox/g. FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced oxidant system.

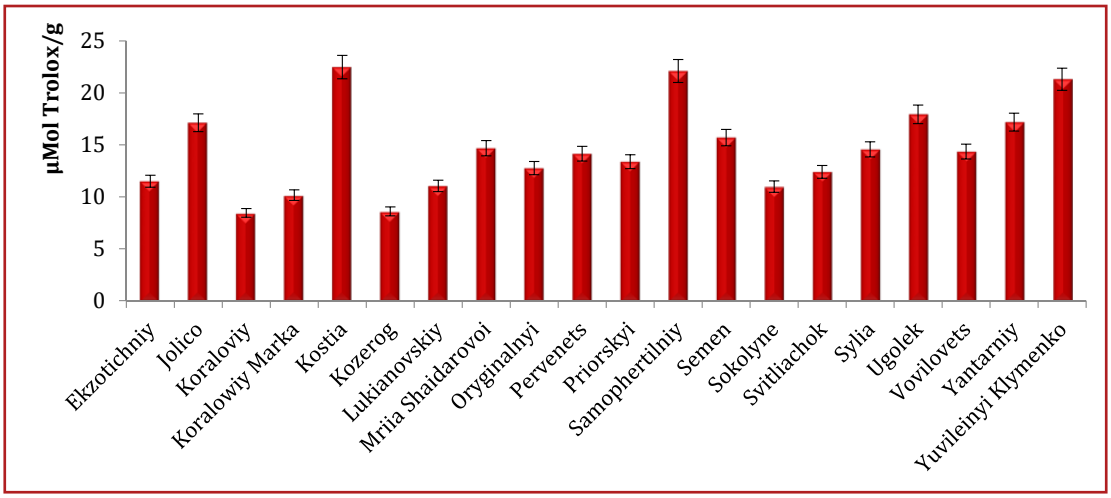


Figure 4 Antioxidant activity of *Cornus mas* L. cultivars evaluated by FRAP method (µMol Trolox/g)

According to Kucharska et al. (2011) antioxidant activity in Polish breeding cultivars the content ranges of fruits from 21.17 (cv. Juliusz) to 41.08 (cv. Szafer) µMol Trolox/g. According to Biaggi et al. (2018), the Cornelian cherry extract can reduce 20.41 µmol Fe^{2+} per gram of solution. A study conducted by Yilmaz et al. (2009) presents a significant range of FRAP values, in which the minimum is 73 and the maximum reaches 114 µmol AA (ascorbic acid) equivalent per gram of DW. Pantelidis et al. (2007) confirm that the FRAP value is approximately 84 µmol AA/g DW. However, Popovic et al. (2012) present a different range for this parameter, which is probably caused by a data presence question. In their study, the ferric-reducing antioxidant power ranges from 2 to 65 µmol/ml Fe^{2+}.

The cluster analysis (CA) was performed according to the hierarchical cluster analysis (HCA) method using the mean value to distinguish similar groups among the various antioxidant capacities. In this study, twenty genotypes were grouped into five main clusters based on the highest similarities (Figure 5). Yuvileinyi Klymenko, Yantarnyi, Semen and Kozerog (the last cultivar showed a high value only with the ABTS method) represented the highest values by the FRAP, ABTS, and DPPH assays; the third group had the lowest values of antioxidant activity; the fourth group includes the largest number of varieties with similar data as determined by the DPPH, FRAP and ABTS methods: Group 1: Yuvileinyi Klymenko, Yantarnyi and Semen; Group 2: Kostia, Samophertilnyi, Jolico and Ugolek, Group 3: Koralovyi Marka, Lukianivskyi and Sokolyne; Group 4: Svitliachok, Oryginalnyi, Sylia, Priorskyi, Pervenets and Mriia Shaidarovoi; Group 5: Ekzotychnyi, Vovilovets and Koralovyi.

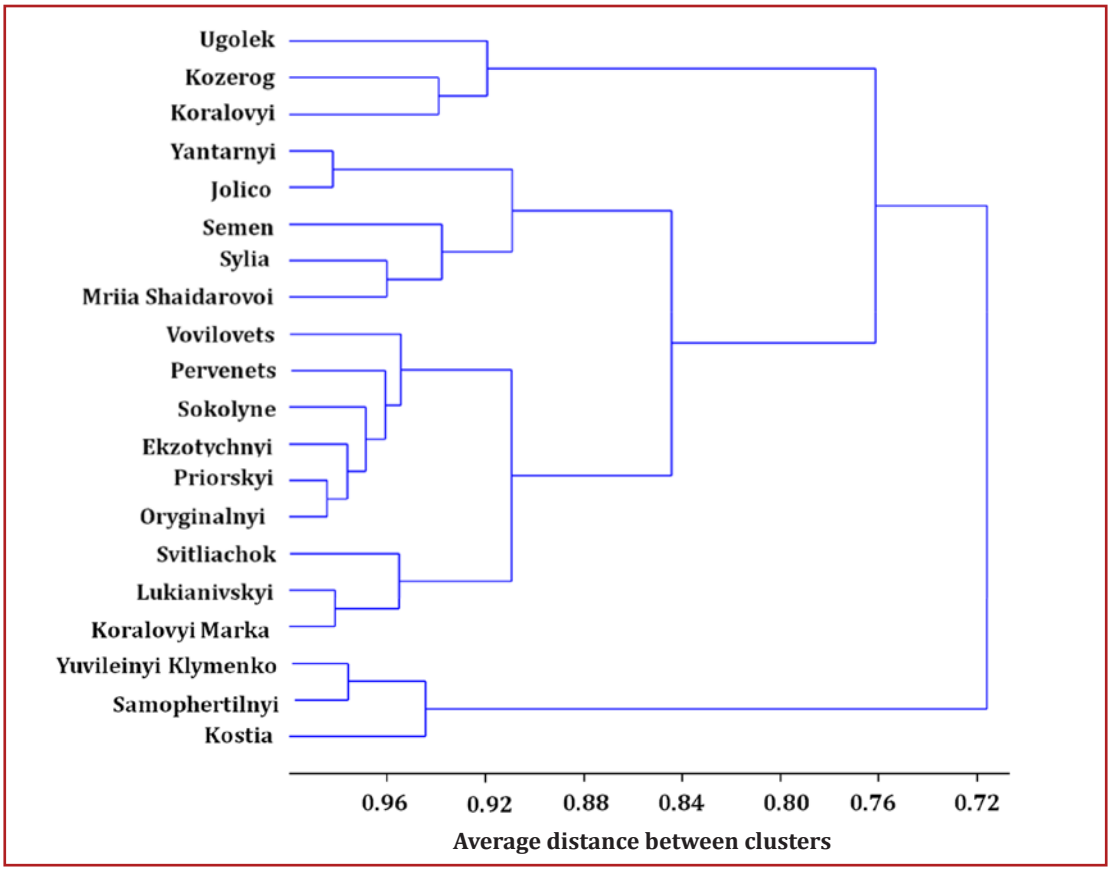


Figure 5 Cluster diagram of the antioxidant activity (FRAP, ABTS, and DPPH methods) of 20 cultivars *Cornus mas* L.

Assessment of total polyphenol content

The results for TPC determined by the Folin-Ciocalteu method varied from 91.34 (Kozerog) to 289.79 mg/100 g (Yuvileinyi Klymenko) (Figure 6).

The obtained results were within the limits of the values published by several authors for cornelian cherries from Greece, Czech Republic, Poland, Romania, Azerbaijan, Turkey (Tural and Koca, 2008; Yilmaz et al., 2009; Hashempour et al., 2010; Rop et al., 2010; Kucharska et al., 2011; Cetkovska et al., 2014). Rop et al. (2010) stated that the total amount of polyphenolic content was cultivar dependent. In their study, the total polyphenolic content was the lowest for the cv. Devin Czech breeding (261 mg GAE/100 g FW), and the cv. Vydubieckii Ukrainian breeding had the highest concentration of polyphenol (811 mg GAE/100 g FW). The total phenolic content of *Cornus mas* of Greece cultivars was 1,592 mg GAE; of Azerbaijan cultivars, 1,097–2,695 mg GAE; of Turkey cultivars, 2,659–7,483 mg GAE (Yilmaz et al., 2009; Cetkovska et al., 2014). According to Cetkovska et al. (2014), total polyphenol content in Ukrainian breeding cultivars was the highest in Ekzotichnyi cultivar (614.3 mg GAE per 100 g DW) and the lowest in Lukianivskyi cultivar (182.3 mg GAE per 100 g DW). According to Romanian

researchers, the determined total phenolic content of the fresh ripe fruits of Cornelian cherry acquired from a local market from Cluj-Napoca was 489.94 ±17.88 mg/100 g FW expressed as GA equivalents (Moldovan et al., 2016). Kucharska et al. (2011) presented research cultivars Polish breeding, that the richest in polyphenols was cv. Szafer (464 mg/100 g) and the poorest was cv. Juliusz (262 mg/100 g).

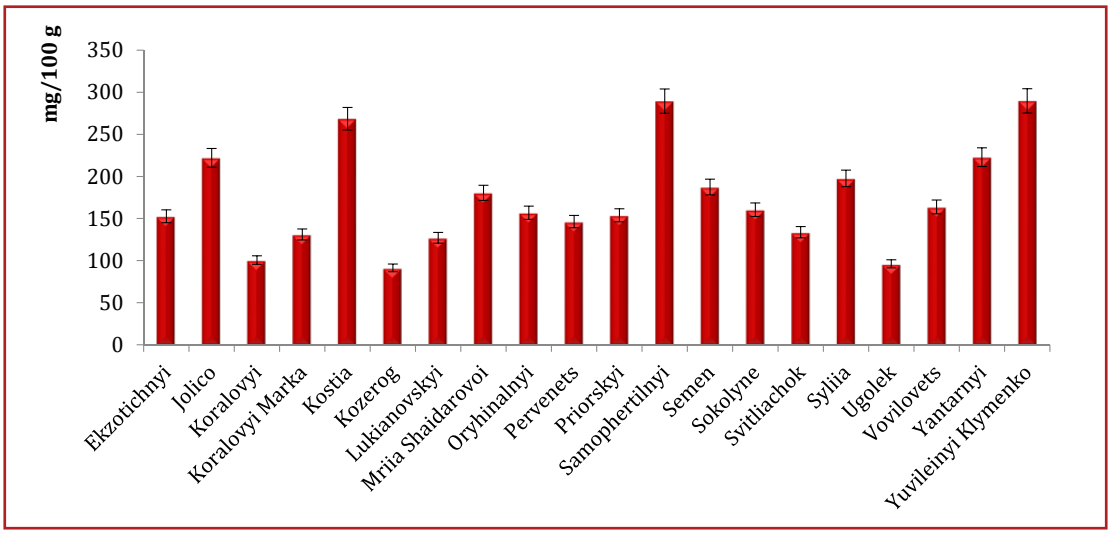


Figure 6 Total polyphenol content of cultivars of *Cornus mas* L. fruits extracts based on gallic acid equivalents (GAE, Line) (mg/100 g)

Polyphenol content can be linked to the amount of juice and peel color (Demir and Kalyoncu, 2003; Bijelić et al., 2011; Cosmulescu et al., 2018). Cultivar Szafer possessed the most intense purple color of all cultivars, and it was also the richest in TPC among the cultivars tested by Kucharska et al. (2011). Milenkovic-Andjelkovic et al. (2015) indicated in their study that the polyphenol content slightly depended on the cultivation conditions which differ from year to year (90 mg GAE/g DW in 1 year and 91 mg GAE/g DW in the following one).

The differences in total polyphenol content may be caused by the different extraction methods used in that study compared to the other studies.

Results on the basis of polyphenolic compounds are distributed into four basic groups by cluster analysis as we can see at Figure 7: Group 1: Yantarnyi, Jolico, Sylia, Semen, and Mriia Shaidarovoi; Group 2: Pervenets, Vovilovets, Sokolyne, Priorskyi, Ekzotychnyi, Oryginalinyi, Lukianivskyi, Svitliachok and Koralovyi Marka; Group 3: Kozerog, Ugolek and Koralovyi; Group 4: Bolgarskyi, Samophertilnyi and Kostia.

The fourth group can be distinguished by the highest content polyphenols. As for the representatives of the third group, they have the lowest content of polyphenols.

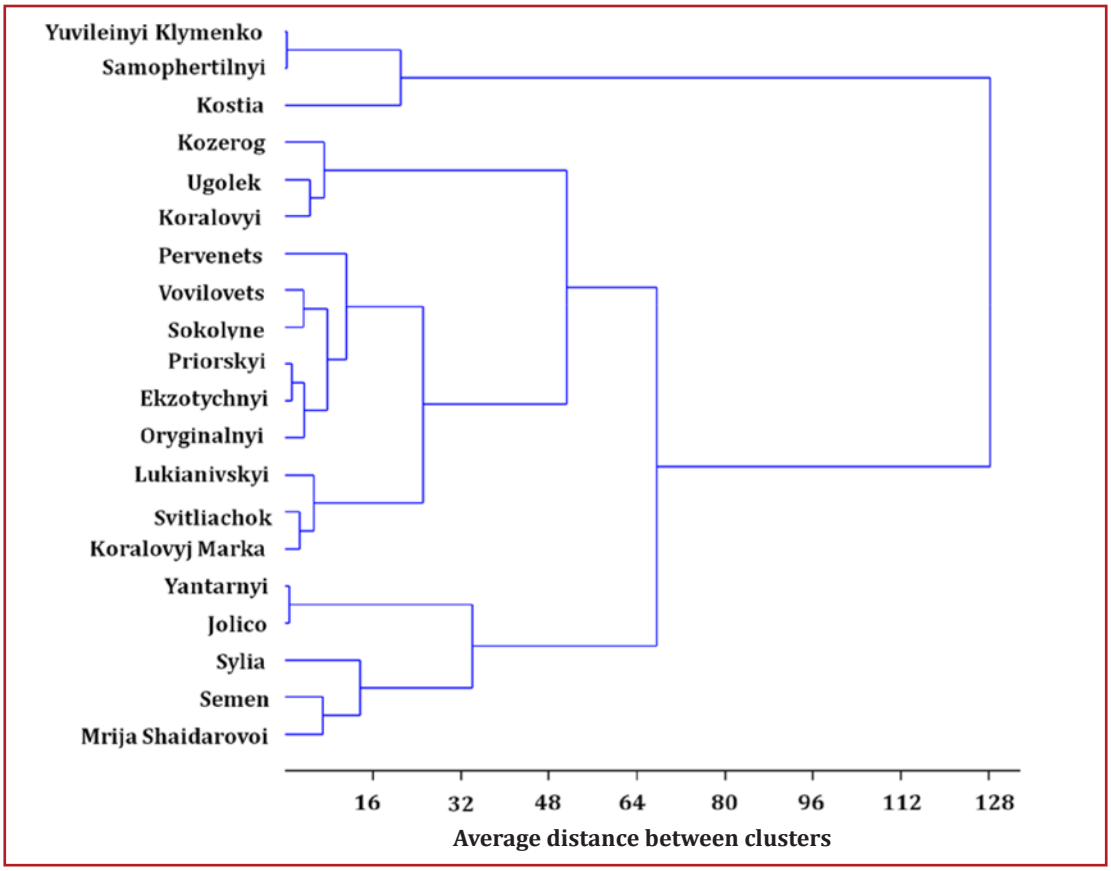


Figure 7 Cluster diagram of polyphenolic compounds of 20 cultivars *Cornus mas* L.

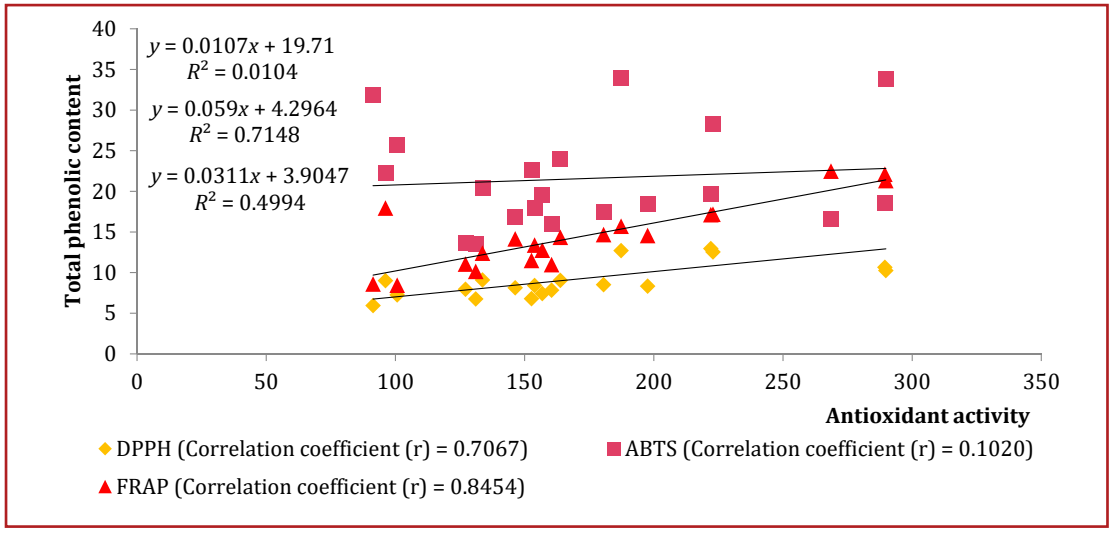


Figure 8 Linear correlation between the total phenolic content and antioxidant activity

Correlation analysis was used to explore the relationships between the individual phenolic compounds and antioxidant capacities (ABTS, DPPH, and FRAP) measured for all fruit extracts from twenty *Cornus mas* cultivars (Figure 8).

The findings of this study indicate that TPC present high and positive correlations with FRAP scavenging capacity and DPPH (r = 0.845, p <0.05; r = 0.706, p <0.05, respectively). Between the TPC and ABTS there is a very weak correlation (r = 0.102).

The correlation analysis between radical scavenging and polyphenolic composition has been additionally proven in many studies (Pantelidis et al., 2007; Ersoy et al., 2011; Milenkovic-Andjelkovic et al., 2015).

Conclusions

The antioxidant activities and total phenol contents of fruit extracts from twenty *Cornus mas* cultivars were studied. Interest in new sources of anti-inflammatory and antioxidant compounds has recently become a major research issue. Cornelian cherry receiving particular attention for its significant amounts of phenolic compounds and vitamins, which exhibit a wide range of biological and pharmacological properties. Obtained data showed that investigated cultivars have a potent antioxidant activity that can be used for further investigation and utilization of cornelian cherry. The results of our study also showed that cornelian cherry is an important traditional plant that deserves special attention for widespread growth in Ukraine due to its high biochemical characteristics, as well as useful nutritional and medicinal properties. Cluster analysis allows us to determine the possible relationships between the analyzed cultivars based on the observed parameters. The amount of these compounds depends on the plant genotype and the ripeness of fruits. Currently, this plant is less known and this new source can be potential as a functional food or value-added ingredient in the future in our dietary system.

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