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Research Article

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Comparative evaluation of oxidatively modified proteins in the equine plasma after treatment with extracts derived fromleaves of various *Camellia japonica* L. cultivars

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The main goal of the current study was to determine the antioxidant activity of leaf extracts of six *Camellia japonica* L. cultivars, using the biomarkers of protein oxidation [aldehydic and ketonic derivatives content] in the *in vitro* equine erythrocyte model. The leaves of *Camellia japonica* cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis plants cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (Kyiv, 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) for 2 min at room temperature. The extracts were then filtered and used for analysis after two weeks. A volume of 0.1 ml of the plant extracts was added to 1.9 ml of equine plasma. Phosphate buffer phosphate buffer was used for positive control. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged and plasma aliquots were used in the study. The aldehydic and ketonic derivatives content as a biomarker of protein oxidation was non-significantly altered after *in vitro* incubation with extracts obtained from the selected *Camellia japonica* cultivars. The percent of the increase had oscillated from 0.2 % (cv. Benikarako) to 2.4 % (cv. C.M. Wilson). Of the six plant extracts screened, *C. japonica* cv. La Pace exhibited the highest increase of the level of ketonic derivatives of oxidatively modified proteins (OMP) (by 15.3 %, $p > 0.05$). Cultivars C.M. Wilson, Kramer's Supreme, Benikarako, Mrs. Lyman Clarke, and Fanny Bolis exhibited a non-significant increase of ketonic derivatives' level by 10.8 %, 10.8 %, 7.6 %, 6.6 %, and 6.3 %, respectively. Some fluctuations in the protein oxidation profile in the plasma across different cultivars were found. Such differences could be related to the plant's metabolic state. Screening of *Camellia* species and their cultivars for other biological activities including antioxidant and anti-inflammatory activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

Keywords: *Camellia japonica*, cultivars, leaves, extracts, antioxidant activity, aldehydic and ketonic derivatives, protein damage, equine plasma

Introducion

Camellia genus belongs to the Theaceae family, found in southern and eastern Asia, from the Himalayas east to Japan and Indonesia. Green tea (*Camellia*) 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). The studies reveal that green tea possesses diverse pharmacological properties, in particular, to lower the incidence of metabolic syndromes, such as obesity, type II diabetes, and cardiovascular risk factors (Chacko et al., 2010). Long-term consumption of tea catechins could be beneficial against high-fat

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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* L. was found to be effective against Gram-positive, Gram-negative bacteria, and fungi (Khan et al., 2019). The most important finding of this study is that its aqueous extract shows inhibitory effect against drug-resistant microorganisms e.g. MRSA, *Pseudomonas aeruginosa*, and *Candida albicans* (Khan et al., 2019). The effects of green tea include anti-oxidative, anti-inflammatory, anti-arthritic, anti-stress, hypolipidaemic, hypocholesterolemic, skin/collagen protective, hepatoprotective, anti-diabetic, anti-microbial, anti-infective, anti-parasitic, anti-cancerous, inhibition of tumorigenesis and angiogenesis, anti-mutagenic, and memory and bone health-improving activities (Alagawany et al., 2020).

Camellia japonica, native to southern Asia (China, Taiwan, Korea, and Japan), is mainly used as an ornamental plant due to its colorful flowers presenting over 32000 recognized cultivars (Savige, 1993; Vela et al., 2013; Páscoa et al., 2019). A thorough literature survey carried out on *C. japonica* revealed that this species has been used traditionally in oriental ethnomedicine for health purposes, such as the treatment of stomach disorders, blood vomiting and bleeding due to internal and external injury, as well as a tonic and anti-inflammatory agent (Yoshikawa et al., 2007; Salinero et al., 2012). The fruits of this plant are used as traditional phytomedicine for the treatment of inflammatory and immunomodulatory diseases (Akanda and Park, 2017). The extract prepared from mature leaves of *C. japonica* has been widely used as an anti-aging material in foods and cosmetics (Mizutani and Masaki, 2014).

It is well known that the *C. japonica* leaf exhibits antioxidant activity due to its high content of polyphenolic compounds (Mizutani and Masaki, 2014). Some studies have already demonstrated that this plant possesses several biological benefits due to the presence of some phenolic compounds in the flowers (Nakajima et al., 1984) as well as aglycon flavonoids (quercetin, kaempferol, and apigenin) and glycosylated flavonoids (rutin and quercetin), and a mixture of saturated fatty acids in the leaves (Azuma et al., 2011). This composition of secondary metabolites seems to be responsible for its anti-plaque, anti-inflammatory, antioxidant activity, antimicrobial, anti-tumoral, anti-viral, anti-histaminic, anti-allergic properties, and skin healing activity (Azuma et al., 2011; Mizutani and Masaki, 2014; Jeong et al., 2010; Salinero et al., 2012). Moreover, *C. japonica* possesses a protective effect against oxidative stress-induced neurotoxicity and hypoglycemic potential (Jeong et al., 2010; Páscoa et al., 2019).

The study of Lee et al. (2017) demonstrated that *C. japonica* extracts promoted antioxidative protein expression and suppressed apoptosis in human corneal epithelial (HCE) cells. Piao et al. (2011) investigating the antioxidant properties of the ethanol extract of the flower of *C. japonica* (*Camellia* extract), revealed that *Camellia* extract exhibits antioxidant properties by scavenging reactive oxygen species (ROS) and enhancing antioxidant enzymes. *Camellia* extract contained quercetin, quercetin-3-O-glucoside, quercitrin, and kaempferol, which are antioxidant compounds. It exhibited 1,1-diphenyl-2-picrylhydrazyl radical and intracellular ROS scavenging activity in human HaCaT keratinocytes. Also, *Camellia* extracts scavenged superoxide anion generated by xanthine/xanthine oxidase and hydroxyl radical generated by the Fenton reaction. Furthermore, it increased the protein expressions and activity of cellular antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Piao et al., 2011).

Nevertheless, we can conclude from the bibliography analyzed that the antioxidant properties have not been comprehensively investigated among *C. japonica* cultivars. In the study of Páscoa et al. (2019), the antioxidant profile (total phenolic and flavonoid content and total antioxidant capacity) of 31 *C. japonica* cultivars leaves was determined and further assessed by near- and mid-infrared spectroscopy.

It is believed that the accumulation of oxidatively damaged proteins is associated with an age-related decline in cellular function. Recently, mammalian animal models have been used to evaluate the levels of these damaged proteins as biomarkers of oxidative stress (Friguet and Baraibar, 2019; Kämpf et al., 2019). The susceptibility of horses to oxidant-induced erythrocyte damage is demonstrated (Walter et al., 2014). Numerous studies using erythrocytes for the evaluation of the biological effects of medicinal plant extracts in cytotoxicity and toxicity assays have been published in the recent literature (Figueirêdo Júnior et al., 2019).

In this context, we have undertaken an attempt to determine the antioxidant activity of six cultivars of *Camellia japonica* i.e. Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis plants using the biomarker of oxidative modification of proteins [aldehydic and ketonic derivatives] in the

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in vitro equine plasma model. Investigations of this type are indicated and used for the preliminary *in vitro* toxicological evaluation of substances with the potential pharmacological application.

Material and methodology

Collection of plant materials and preparation of plant extracts

The leaves of *Camellia japonica* cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis plants cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (Kyiv, 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) for 2 min at room temperature. The extracts were then filtered and used for analysis after two weeks. 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 hr, 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 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 samples were processed for analysis less than 12 hr after blood withdrawal. Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 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 extracts was added to 1.9 ml of equine plasma. For positive control, phosphate buffer was used. After incubating the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Plasma aliquots were used in the study.

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

To evaluate the protective effects of the extract obtained from leaves of *Camellia* cultivars against free radical-induced protein damage in equine plasma, a carbonyl derivatives content of 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 and co-workers (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1M DNPH (dissolved in 2M HCl) was added to 0.1 ml of the sample after denaturation of proteins by 20 % trichloroacetic acid (TCA). After the addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 hr at 37 °C. The tubes were spun in a centrifuge for 20 min at 3000 g. After centrifugation, the supernatant was decanted and 1 mL of ethanol-ethylacetate solution was added to each tube. Following the mechanical disruption of the pellet, the tubes were allowed to stand for 10 min and then spun again (20 min at 3,000 g). The supernatant was decanted and the pellet washed thrice with ethanol-ethylacetate. After the final wash, the protein was solubilized in 2.5 mL of 8M urea solution. To speed up the solubilization process, the samples were incubated in a 90 °C water bath for 10–15 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient of 22000 $M^{-1}\cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the OMP level (significance level, p <0.05) was examined using the Kruskal-Wallis one-way analysis of variance (Zar, 1999). The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica software, version 8.0 (StatSoft, Poland) (Zar, 1999).

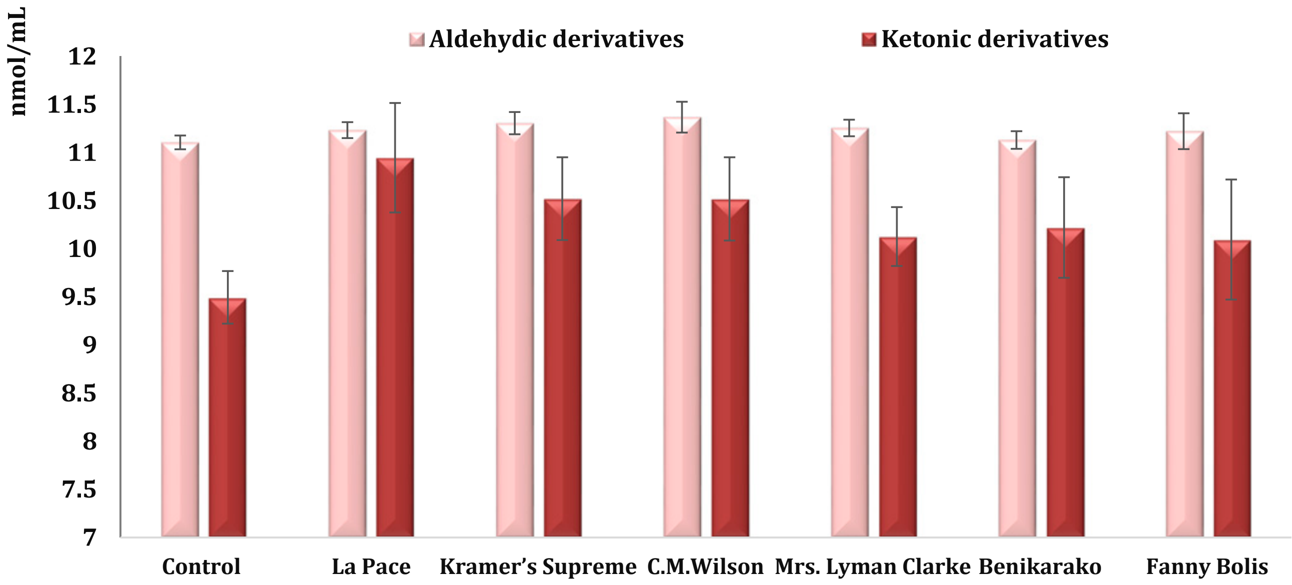

Figure 1 The content of aldehydic and ketonic derivatives as a biomarker of oxidatively modified proteins in the equine plasma after *in vitro* incubation with leaf extracts obtained *Camellia japonica* L. cultivars (M ±m, n = 18)

Results and discussion

The present study enabled the determination of the profile of oxidatively modified proteins in equine plasma after *in vitro* incubation with leaf extracts of several *C. japonica* cultivars, namely Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis, through standard biochemical methods as previously described.

The data on the content of aldehydic and ketonic derivatives as a biomarker of oxidatively modified proteins in the equine plasma after *in vitro* incubation with leaf extracts obtained *C. japonica* cultivars was demonstrated in Figure 1.

When equine plasma was incubated with extracts obtained from *C. japonica* cultivars, the content of aldehydic derivatives of OMP was non-significantly increased. The percent of the increase oscillated from 0.2 % (cv. Benikarako) to 2.4 % (cv. C.M. Wilson). Of the six plant extracts screened, *C. japonica* 'La Pace' exhibited the highest increase of the level of ketonic derivatives of OMP (by 15.3 %, $p > 0.05$). *Camellia* cultivars C.M. Wilson, Kramer's Supreme, Benikarako, Mrs. Lyman Clarke, and Fanny Bolis exhibited a non-significant increase of ketonic derivatives' level (by 10.8 %, 10.8 %, 7.6 %, 6.6 %, and 6.3 %, $p > 0.05$, respectively), as shown in Figure 1.

As seen from Figure 1, some fluctuations in the protein oxidation profile in the plasma across different cultivars were found. It would be reasonable to suggest that such differences could be related to the plant's metabolic state. Moreover, it could be explained by the various genetic background of various cultivars used in the current study. It is known, that cultivated *Camellia japonica* has been domesticated for centuries. In *C. japonica*, like most other ornamental flowers, the domestication process has resulted in several types of double flowers characterized by varying degrees and morphology of excessive petals (Sun et al., 2014). Its remarkable diversity of floral forms imparts a rich resource for understanding the genetic regulation of floral patterning and forms (Gao et al., 2005; Sun et al., 2014). Cultivated *Camellia* contains more than 5 types of double flowers with distinctive floral forms semi-double, formal-double, anemone, rose or peony doubles, mainly distinguished by number and arrangement of petals and stamens form (Li et al., 2017).

The *Camellia japonica* cultivars included in this study represent various double flowers types, i.e. "paeony" (Kramer's Supreme and Mrs. Lyman Clarke), "anemone" (C.M. Wilson and Benikarako), "formal double" (La Pace), and "semi-double" (Fanny Bolis).

Results obtained in our previous study showed that there is a possibility of using leaf extracts of various *C. japonica* cultivars as antioxidant agents in intensive 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 *C. japonica* cultivars was evaluated in our previous study (Kharchenko et

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al., 2017a). All extracts (except cultivars 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* cv. Kramer's Supreme' extract (by 52 %, $p = 0.004$), cv. C.M. Wilson (by 88 %, $p = 0.001$), cv. Mrs. Lyman Clarke (by 87.2 %, $p = 0.000$), and cv. Fanny Bolis (by 40.7 %, $p = 0.044$) compared to the control group. Likewise, the SOD activity in the muscle tissue after incubation with cv. La Pace and cv. Benikarako were also increased (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* cv. C.M. Wilson and cv. Benikarako. The levels of TAC were increased by 41.7 and 44.8 % ($p <0.05$) as compared with the control group. Leaf extracts of cv. La Pace and cv. Kramer's Supreme being incubated with muscle tissue have not changed the level of TAC, while the effect of the leaves extracts of cv. Mrs. Lyman Clarke and cv. Fanny Bolison the decreasing 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).

In our other study, designed to estimate the possible antioxidant potential of the leaf extracts of *C. japonica* cultivars incubating with equine erythrocytes suspension, the TBARS content as a biomarker of lipid peroxidation was non-significantly altered (accept cv. Mrs. Lyman Clarke). Of the six plant extracts screened, *C. japonica* cv. Mrs. Lyman Clarke exhibited the highest decrease of TBARS level (by 14.4 %, $p <0.05$). Similarly, cultivars C.M.Wilson, Kramer's Supreme, Benikarako and Fanny Bolis exhibited a non-significant decrease of TBARS level (by 8.8 %, 4.8 %, 3.6 %, and 3.1 %, $p >0.05$ respectively). TBARS level was non-significantly increased by 3.1 % ($p >0.05$) after incubation of erythrocyte suspension with leaf extract of 'Fanny Bolis' (Kharchenko et al., 2019). When equine erythrocytes were incubated with leaf extracts obtained from *C. japonica* cultivars, the TAC level was non-significantly altered (Tkachenko et al., 2020).

Recent investigations have associated plants belonging to the *Camellia* genus with anti-carcinogenic, immune-boosting, and antioxidative properties that may impact human health. For example, Higashi-Okai et al. (2001) have analyzed the antioxidant activity of the non-polyphenolic fraction of the residual green tea (*C. sinensis*) after hot water extraction. The non-polyphenolic fraction of residual green tea caused a significant suppression against hydroperoxide generation from oxidized linoleic acid in a dose-dependent manner. The ranks of suppressive activity against hydroperoxide generation were chlorophyll *a* > lutein > pheophytin a > chlorophyll *b* > β-carotene > pheophytin b. These results suggest that the non-polyphenolic fraction of residual green tea has potent suppressive activity against hydroperoxide generation from oxidized linoleic acid, which is derived from the antioxidant activities of chlorophylls *a* and *b*, pheophytins a and b, β-carotene, and lutein (Higashi-Okai et al., 2001).

The phenolic profiles, antioxidant and antiproliferative activities of 27 tea cultivars were determined by Zeng et al. (2017). Wide ranges of variation were found in analyzed cultivars for the contents of water-soluble phenolics (121.6–223.7 mg/g dry weight (DW)), total catechins (TC) (90.5–177.2 mg/g DW), antioxidant activities [peroxyl radical scavenging capacity (PSC) values 627.3–2332.3 μmol of vitamin C equiv./g DW, Oxygen radical absorbance capacity (ORAC) values (1865.1–3489.3 μmol of vitamin C equiv./g DW), cellular antioxidant activity (CAA) values (37.7–134.3 μmol of QE/g DW without PBS wash and 25.3–75.4 μmol of QE/g DW with PBS wash)] and antiproliferative activity (53.0–90.8 % at the concentration of 400 μg/mL extracts). The PSC, ORAC, and CAA values were significantly correlated with phenolics, epicatechin gallate (ECG), CC, and TC (Zeng et al., 2017). Ohmori et al. (2005) have assessed the antioxidant activity of six teas, including the aqueous extracts of green tea and oolong tea (*Camellia sinensis*), tochu (*Eucommia ulmoides*), *Gymnema sylvestre*, Japanese mugwort (*Artemisia princeps*), and barley (*Hordeum vulgare*), against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and LDL oxidation, and examined the association of LDL oxidizability with the plasma catechin levels in 10 healthy volunteers with a single dose of 5 g green tea powder. Green tea, therefore, showed the strongest antioxidant activity among the six different tea, and the inhibitory effects of green tea on LDL oxidation depended on the plasma catechin levels (Ohmori et al., 2005).

Many research papers have evidenced that the anti-inflammatory and gastroprotective mechanism of *C. japonica* is mediated by the modulation of oxidative stress, inflammatory cytokines, and enzymes via suppression of MAPK/NF-κB signaling pathways. Akanda and Park (2017) have investigated the immunopharmacological activities of *C. japonica* and have validated its pharmacological targets. These researchers found the production of NO and reactive oxygen species in RAW 246.7 cells were both suppressed by *C. japonica*. Moreover, *C. japonica* mitigated the HCl/EtOH-induced oxidative stress in gastric mucosa via the reduction of lipid peroxidation and elevation of NO production. Gastric mucosal damages were prominently improved by *C. japonica*, as confirmed by the histopathological evaluation. The gene expression of inflammatory cytokines and enzymes tumor necrosis factor α (TNF-α), interleukin 6 and 1β (IL-6, IL-1β), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) was notably downregulated by *C. japonica*. Also, *C. japonica* markedly attenuated the mitogen-activated protein kinases (ERK1/2, JNK, and p38) phosphorylation, COX-2 expression, and activation of transcription factor NF-κB and as well as phosphorylation and degradation of IκBα in the gastric mucosa (Akanda and Park, 2017).

Other species of *Camellia* plants also are a promising source of natural antioxidants and further studies might be a likely source of its use in remedy of different diseases. *C. sinensis* L. is traditionally used in many polyherbal preparations for the treatment of different diseases and infections. Its action has been associated with its antioxidant activities. As tea is a very popular beverage, tea polyphenols are expected to be a potent antioxidant and chemopreventive agents that can be taken with a normal diet and can be nontoxic due to their natural origin (Bag and Bag, 2020). Catechins are powerful antioxidants, and laboratory studies have suggested that these compounds may inhibit cancer cell proliferation. Some experimental and nonexperimental epidemiological studies have suggested that green tea may have cancer-preventative effects (Filippini et al., 2020).

Conclusions

The aldehydic and ketonic derivatives content as a biomarker of protein oxidation was non-significantly altered after *in vitro* incubation with extracts obtained from selected *Camellia japonica* cultivars. The percent of the increase had oscillated from 0.2 (cv. Benikarako) to 2.4 % (cv. C.M. Wilson). Of the six plant extracts screened, *C. japonica* cv. La Pace exhibited the highest increase of the level of ketonic derivatives of OMP (by 15.3 %, $p > 0.05$). Cultivars C.M. Wilson, Kramer's Supreme, Benikarako, Mrs. Lyman Clarke, and Fanny Bolis exhibited a non-significant increase of ketonic derivatives' level by 10.8 %, 10.8 %, 7.6 %, 6.6 %, and 6.3 %, $p > 0.05$ respectively. Some fluctuations in the protein oxidation profile in the plasma across different cultivars were found. We attribute the observed differences to the use of *C. japonica* cultivars with various genetic backgrounds. Moreover, such differences could be related to the plant's metabolic state. Overall, our analysis suggests that screening of *Camellia* species for other biological activities including antioxidant and anti-inflammatory activities is essential and may be effective for searching the preventive agents to be used in the pathogenesis of some metabolic diseases.

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Research Article OPEN ACCESS

Content of anthocyanins and flavonols in the fruits of *Cornus* spp.

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The genetic pool of the cornelian cherry (*Cornus mas* L.) at the M.M. Gryshko National Botanical Garden (NBG) of National Academy of Sciences of Ukraine includes more than 100 specimens collected from wild and cultivated plants in Ukraine, and cultivars of Bulgarian, Slovak, English, Austrian and Georgian selection. The genetic pool of cornelian cherry of the NBG presents a rich variety of biological and economic properties. Data on the content of anthocyanins and flavonols in the fruits of *Cornus mas* cultivars with early, medium and late fruit maturation periods are presented. The fruits of the *Cornus officinalis* Sieb. et Zucc. (CO-01, CO-02) and the hybrid *Cornus mas* × *Cornus officinalis* (cultivars Etude No 1 and No 2) were also studied. As a result of the study, it was found that the content of anthocyanins and flavonols in the fruits of *Cornus* has a significant difference in cultivars. Among *C. mas* cultivars with early fruit ripening, the most promising as a source of bioflavonoids preparations are 'Pervenets' and 'Volodimirskij', among *C. mas* cultivars with an average fruit ripening period, the most promising are the cultivars Mriya Shajdarovoi, Vydubetskyi and Titus, among the *C. mas* cultivars with a late fruit ripening period, the most promising are the cultivar Sokoline. Also promising are the hybrid *C. mas* × *C. officinalis* (cultivar Etude No. 1 and No. 2). These most promising cultivars and forms must commend for use in the food and medical sectors of the national economy.

Keywords: *Cornus mas*, *Cornus officinalis*, cultivar, fruits, anthocyanins, flavonols

Introduction

Subgenus *Cornus* L., which has a fragmented areal on the globe, is represented by four species: *Cornus mas* L. – in the west of the continent of Eurasia, *C. officinalis* Sieb. et Zucc. – in Japan, China and Korea, C. chinensis Wanger. – in the central region of China and *C. sessilis* Toor. – in North America (Browic, 1986).

C. mas (cornelian cherry) is a very ancient, cultivated plant, known in Ukraine as a culture since the time of Kievan Rus. The development of horticulture in Russia was associated with famous monasteries, especially Vydubetsky, Mezhyhirsky, Kyiv-Pechersk Lavra, it was here that many plants were introduced into culture, including cornelian cherry. The forms of these plants are very diverse, many of them can be directly introduced into the culture, and some can be converted into excellent cultivated plants by breeding. These plants include *C. mas* a very ancient fruit valuable food, medicinal, soil protection, ornamental plant, used in the Neolithic era. The modern range of cornelian cherry is the Pontic Mediterranean region: the southern Mediterranean regions of Europe, the southern foothills of the eastern Carpathians, as well as the Crimea, Caucasus and Asia Minor (Klymenko, 1990). Cornelian cherry is a culture that meets the standards of the time. According to the literature data and our research cornelian cherry yields are abundant and stable in culture (Klymenko, 2004; Klymenko et al., 2017b). The plant bares large juicy fruits, while not demanding thorough care. Its cultivation is very productive. Plants are usually not damaged by vermin and illnesses and do not need pest treatments.

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C. mas is recognized as a source of polyphenols, tannins, anthocyanins, and iridoids, all of which are present in both its fruits and leaves (Kucharska et al., 2015; Szczepaniak et al., 2019).

A high number of bioactive compounds have been identified in *C. mas* fruits (Klymenko et al., 2019), among which flavonoids exert favourable health effects especially by acting as potent antioxidants (Moldovan and David, 2017). Two iridoids (loganic acid and cornuside) and five anthocyanins (delphinidin, cyanidin, and pelargonidin glycosides) were identified in cornelian cherry fruits. The MS fragmentation pathways of the two iridoids were studied. The content of total iridoids in cornelian cherry fruits covered a wide range, from 86.91 to 493.69 mg/100 g fw. Loganic acid was the most dominant iridoid compound identified in cornelian cherry fruits and amounted to 88–96 % of total iridoids.

In most *C. mas* fruits, pelargonidin-3-galactopyranoside was dominant (Kucharskaa, et al., 2015). Cyanidin-3-rhamnosylgalactoside, a new anthocyanin, was isolated and identified from the berries of *C. mas*. Cyanidin-3-galactoside and delphinidin-3-galactoside were also identified (Du and Francis, 1973).

Bioactive compounds are present not only in flowers, but also in seeds, buds, shoots, leaves, pollen and bee pollen e.g. (Brindza et al., 2009; Krivoruchko etal., 2011; Hosseinpour-Jaghdani et al., 2017; Klymenko et al., 2017a; Levon et al., 2017; Brindza et al., 2018; Grygorieva et al., 2020; Przybylska et al., 2020).

The content of flavonoids in leaves of *C. mas* is 847.6 mg/100 g, in flowers – 1704.9 mg/100 g; in flowers of *C. officinalis* – 1448.9 mg/100 g (in terms of rutin). The content of acids in flowers of *C. mas* is 41.5 mg/100 g, in flowers of *C. officinalis* – 124.9 mg/100 g (in terms of chlorogenic acid). In leaves of *C. mas* rutin was identified, in flowers of *C. mas* and *C. officinalis* – chlorogenic and ellagic acids, rutin, kaempferol-3-O-glucoside and quercetin. The flavonoid rutin predominates in all studied samples (Krivoruchko, 2018). Based on HPLC–PDA–MS/MSn analysis eight compounds have been identified as quercetin, kaempferol, and aromadendrin glycosilated derivatives (Pawlowska, et al., 2010).

Juice, jam and compote from *C. mas* fruits are useful for anaemia, liver diseases, gout, stomach diseases (Sklyarevsky, 1975), Infusions of leaves and flowers are known as antipyretic and diuretic agents, and from the bark – as a tonic and stimulating (Chikov and Laptev, 1976). Medicines from fruits and leaves are used as astringents and disinfectants, selectively acting even on dysentery bacillus, typhoid pathogens. Cornelian cherry is also used for rheumatism, colds, fever and skin diseases (Blaze, 2000).

C. officinalis – native to Japan, Northeast China and Korea, is virtually unknown in Europe and grows only in the collections of Botanical Gardens. *C. officinalis* a relative of *C. mas*, native to northeastern China and Korea. It is cultivated in large areas in Japan – the local name – sandzaki. It is one of the important types of plant material used in Chinese traditional medicine. Is being quite common in Asia, and the UK, it is used even more widely than *C. mas*. In its homeland, *C. officinalis* is widely known as a medicinal plant. Studies conducted *in vitro* and *in vivo* have shown a multi-faceted protective effect of fruit extract against diabetes and its complications, in particular a diabetic nephropathy. Decoctions of fruits and leaves are used in folk medicine as a tonic, stimulating and astringent (Klymenko, 2002; Klymenko and Ilyinska, 2020).

Besides, we have researched cultivar Etude, which is an artificial hybrid from crossing *C. officinalis* × *C. mas*. Genotype obtained from grafting *C. officinalis* on *C. mas* (Klymenko and Ilyinska, 2020).

The presence of a large amount of biologically active substances in representatives of the Cornaceae family not only makes them a valuable medicinal raw material but also increases their resistance to many stress factors. Long-term observations have shown that representatives of the Cornaceae family have extremely high resistance to both pests and adverse weather conditions, plants bloom profusely and give birth (Klymenko, 2000).

All parts of plants of species of representatives of the Cornaceae family (fruits, leaves, bark, roots) are medicinal raw materials and have long been used in folk medicine for the treatment of anaemia, metabolic disorders, as an anti-scurvy, antidiabetic agent (Mamedov et al., 1990; Czerwińska and Melzig, 2018).

Very often medicinal cornel is used as an anti-diabetic medicinal plant, especially in East Asia. Fresh fruit treat diabetes, they reduce the level of glucose in the blood, increases the enzymatic activity of the pancreas, stimulate the processes of digestion. In Tibetan medicine, bark and leaves of medicinal cornel are used for the treatment of pleurisy, fever, powder of dried fruits to treat kidney illnesses (nephritis) (Yokozawa et al., 2009; Cao et al., 2011).

The purpose of this study was to determine the content of anthocyanins and flavonols in the fruits of plants of

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the genus *Cornus*. This will allow us to identify the most promising cultivars and forms for use in the food and medical sectors of the national economy.

Material and methodology

Biological material

Biochemical studies were carried out on fruits dried in a well-ventilated room in the absence of direct sunlight at a temperature of 20–25 °C and crushed in an electric mill. The fruits of *Cornus mas* plant cultivars were studied with early (Radist, Olena, Ekzotychnyi, Nespodivanyi, Elegantnyi, Nikolka, Nizhnyi, Pervenets, Volodymyrskyi, Samofertylnyi, Alyosha, Koralovyi Marka), intermediate (Koralovyi, Yantarnyi, Medok, Priorskyi, Yevgeniia, Svitliachok, Vyshgorodskyi, Lukianivskyi, Mriia Shaidarovoi, Starokyivskyi, Yoliko, Titus, Oryginalnyi, Kostia, Vydubetskyi) and late (Semen, Sokolyne, Yuvileinyi Klymenko, Kozerig, Kolisnyk), hybrid *Cornus mas* × *Cornus officinalis* (cultivars Etude No 1 and No 2) and two varieties of *C. officinalis* (CO-01 and CO-02). Plants collected from the collection of Department of Fruit Plants Acclimatization in M.M. Gryshko National Botanical Garden of the NAS of Ukraine (NBG) at the stage of fructification during 2019–2020. Biochemical analyses were carried out in the laboratory of Department of Fruit Plants Acclimatization of M.M. Gryshko National Botanical Garden.

Determination of total anthocyanins content

The quantity of anthocyanins was determined using a spectrophotometric method at a wavelength of 530 nm, using alcohol extraction from a homogenate of plant raw materials acidified with 3.5 % hydrochloric acid (Kriventsov, 1982).

The number of parallel determinations was 3. The accuracy of the method was in the range of 2.5–4.8 %. The data obtained were presented as mg/100 g dry matter (DM) in terms of cyanidin glycosides.

Determination of total flavonols content

To determine flavonols, an analytical sample of dried crushed fruits weighing 0.2–0.3 g was transferred to a flask, 3 ml of 80 % ethyl alcohol was added, and heated with a reflux coolbox for 45 minutes in a water bath. After that, the flask was cooled to room temperature and the suspension was filtered through a paper filter into a 100 ml volumetric flask. The resulting solution was brought to the mark with 80 % alcohol (solution A). 2 ml of solution A was placed in a 25 ml volumetric flask, 1 ml of a 2 % solution of aluminum chloride in 95 % ethanol was added, and the volume of the solution was brought to the mark with 95 % alcohol. The optical density of the solution was measured after 20 minutes at a wavelength of 390 nm in a cuvette with a layer thickness of 10 mm. The control was a mixture of solutions of aluminum chloride and acetic acid (Andreeva and Kalinkina, 2000).

The number of parallel measurements was 3. The accuracy of the method was in the range of 0.3–2.0 %. The obtained data were presented in mg/100 g DM.

The optical density of all the studied solutions was measured using a Zalimp KF 77 spectrophotometer (Poland).

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 26.0.0.1.

Results and discussion

According to our previous data (Ma et al., 2014; Biaggi et al., 2018; Bayram and Ozturkcan, 2020; Demir et al., 2020), representatives of the genus *Cornus* are rich in biologically active compounds. A major role in the formation of medicinal properties of cornelian cherry is due to the action of the secondary metabolites of the class of flavonoids – anthocyanins and flavonols (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 and Golubkova, 2016; Levon and Goncharovska, 2017).

Recently it was found that flavonoids affect signalise processes in living systems due to specific interactions with proteins, which perform regulatory functions (Stevenson and Hurst, 2007). Besides, flavonoids play an important role in the protection of plants against bacterial, viral and fungal infections, against the intrusion of vermin and insect damage. The biological role of flavonoids is their involvement in redox processes that occur in plants (Tarahovsky et al., 2013). Physical damage to plant tissues also leads to starting of the processes of oxidative degradation of flavonoids, preventing the development of wound infections (Walker, 1998).

The amount of anthocyanin in some cultivars of *C. mas* breeding of M.M. Gryshko National Botanical Garden of

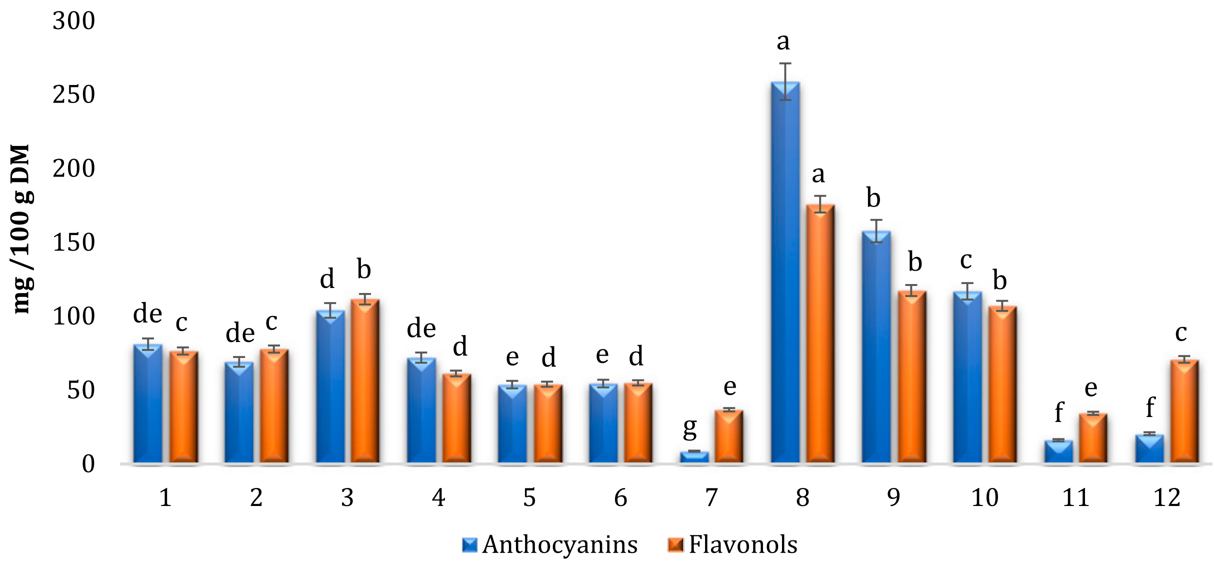

Figure 1 The content of anthocyanins and flavonols in the fruits of *Cornus mas* L. cultivars with an early ripening period: 1 – Radist; 2 – Olena; 3 – Ekzotychnyi; 4 – Nespodivanyi; 5 – Elegantnyi; 6 – Nikolka; 7 – Nizhnyi; 8 – Pervenets; 9 – Volodymyrskyi; 10 – Samofertylnyi; 11 – Alyosha; 12 – Koralovyi Marka (different superscripts in each column indicate the significant differences in the mean at p <0.05))

the NAS of Ukraine was in the range 477.1–850.0 mg% in the peel, 7.8–190.6 mg% the pulp (Klymenko, 2001). Studies of the content of anthocyanins in *Cornus mas* fruits were conducted in the Vlasina region (Serbia). The anthocyanin content was determined using spectrophotometric and high-performance liquid chromatography (HPLC) assays. The total anthocyanin content was 1383.2 mg/kg FW (Anđelković et al., 2015). This result is comparable to the results of our research.

New data for comparative analysis of different plant parts (leaf, flower and fruit) of *C. mas* are presented. Total phenolic content, flavonoid concentrations are analysed using *in vitro* standard spectrophotometric methods. Biological material of *C. mas* was collected from the region of Pčinja river gorge in south Serbia. Different solvents were used to extract flavonoids from the fruit: methanol, water, ethyl acetate, acetone, petroleum ether (Stankovic et al., 2014). Water extracts of *C. mas* fruits have indicators of 3.53 mg/g in terms of flavonoid content. These results have a higher result compared to our studies. *C. mas* fruit extracts with ethyl acetate show a very high result – 41.49 mg/g. The use of weakly polar solvents increases the completeness of the extraction of flavonoids from *C. mas* fruits.

As a result of our research, it was found that anthocyanin content in *C. mas* fruits with an early ripening period is in the range of 9–259 mg/100 g DM. The highest content of anthocyanins in fruits has the cv. Pervenets – 259 mg/100 g DM. Also, fruits of the cv. Volodymyrskyi have a fairly high anthocyanin content – 158 mg/100 g DM. The content of flavonols in the fruits of *C. mas* cultivars with an early ripening period is in the range of 34–176 mg/100 g DM. The highest content of flavonols in fruits also has the cv. Pervenets – 176 mg/100 g DM (Figure 1).

We conducted a correlation analysis between the content of anthocyanins and flavonols in the fruits of *C. mas* cultivars with an early ripening period. We found a very high correlation between the content of anthocyanins and flavonols in the fruits of *C. mas* plants with the early period of fruit maturation (r = 0.955).

The anthocyanin content in *C. mas* fruits with an intermediate ripening period is in the range of 10–166 mg/100 g DM. The highest content of anthocyanins in fruits has the cv. Mriia Shaidarovoi – 166 mg/100 g DM. Fruits of the cv. Vyshgorodskyi also has a fairly high anthocyanin content – 142 mg/100 g DM. The content of flavonols in the fruits of *C. mas* cultivars with an intermediate ripening period is in the range of 27–165 mg/100 g DM. The highest content of flavonols in fruits has the cv. Titus – 165 mg/100 g DM (Figure 2).

A correlation analysis between the content of anthocyanins and flavonols in the fruits of *C. mas* plants with an intermediate fruit ripening period showed a moderate correlation (r = 0.591).

The anthocyanin content in *Cornus mas* fruits with a late ripening period is in the range of 33–96 mg/100 g

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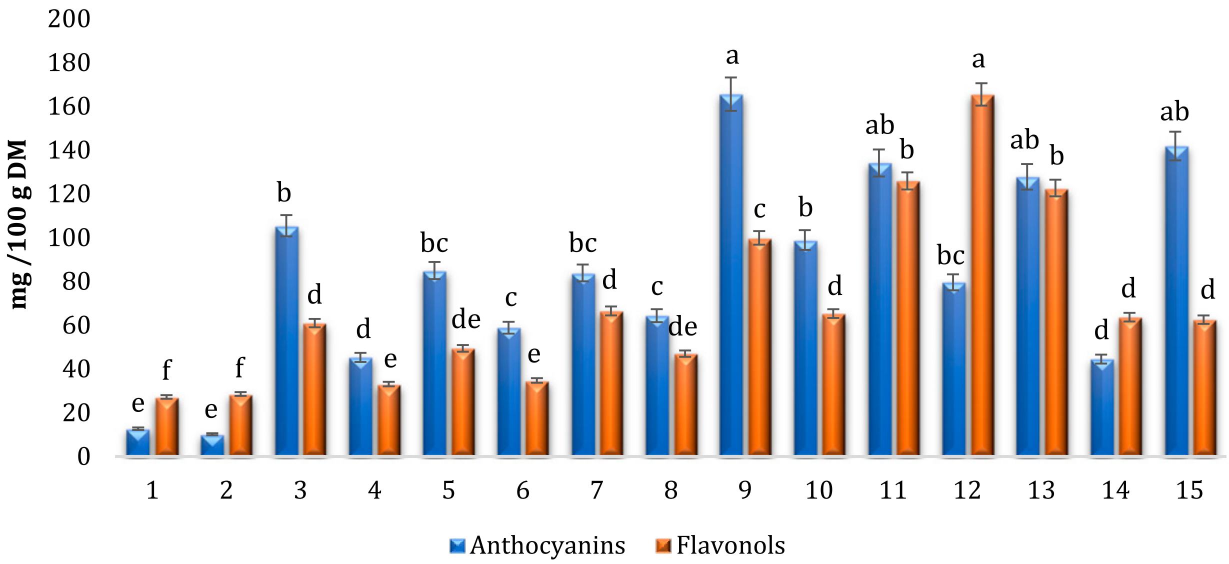

Figure 2 The content of anthocyanins and flavonols in the fruits of *Cornus mas* L. cultivars with an intermediate ripening period:
1 – Koralovyi; 2 – Yantarnyi; 3 – Medok; 4 – Priorskyi; 5 – Yevgeniia; 6 – Svitliachok; 7 – Vyshgorodskyi; 8 – Lukianivskyi; 9 – Mriia Shaidarovoi; 10 – Starokyivskyi; 11 – Yoliko; 12 – Titus; 13 – Oryginalnyi; 14 – Kostia; 15 – Vydubetskyi (different superscripts in each column indicate the significant differences in the mean at p <0.05))

DM. The highest content of anthocyanins in fruits has the cv. Sokolyne – 96 mg/100 g DM. The content of flavonols in the fruits of *C. mas* cultivars with a late ripening period is in the range of 36–88 mg/100 g DM. The highest content of flavonols in fruits also has the cv. Sokolyne – 88 mg/100 g DM (Figure 3).

Conducting a correlation analysis between the content of flavonols and anthocyanins for *C. mas* cultivars with a late ripening period showed a high level of correlation (r = 0.918).

We compared the average content of anthocyanins and flavonols in the fruits of *C. mas* cultivars with early, intermediate and late ripening. The diagram shows a decrease in the content of the studied compounds in cultivars with a late fruiting period. This may be due to a decrease in the length of daylight, which correlates with the quantitative content of anthocyanins and flavonols (Pan and Guo, 2016). *C. mas* cultivars with an early fruit ripening period were selected at the end of August, *C. mas* cultivars with an intermediate

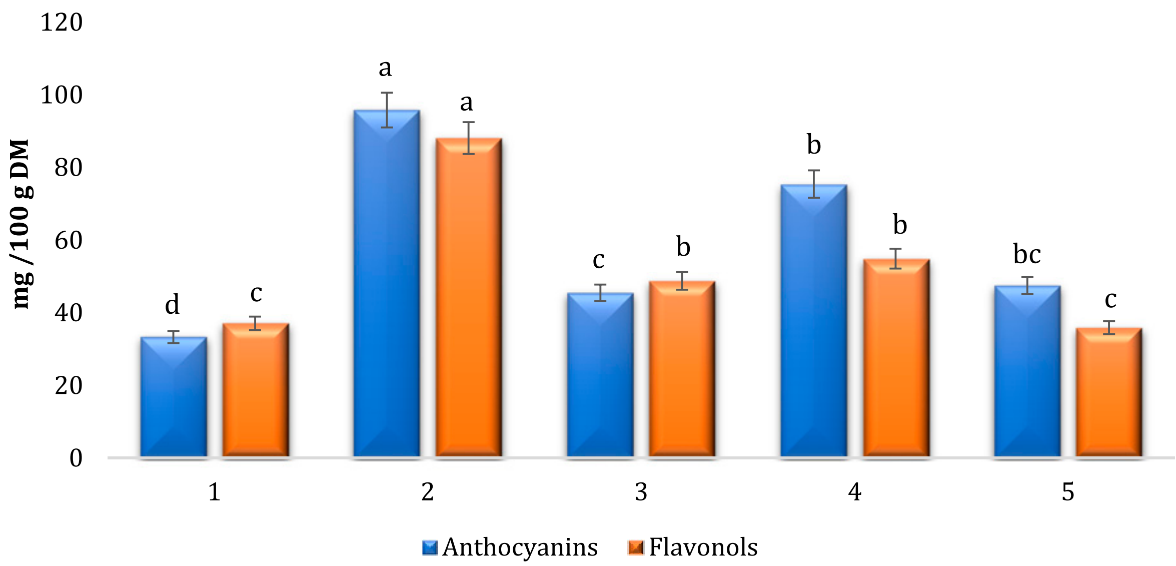

Figure 3 The content of anthocyanins and flavonols in the fruits of *Cornus mas* L. cultivars with a late ripening period:
1 – Semen; 2 – Sokolyne; 3 – Yuvileinyi Klymenko; 4 – Kozerig; 5 – Kolisnyk (different superscripts in each column indicate the significant differences in the mean at p <0.05))

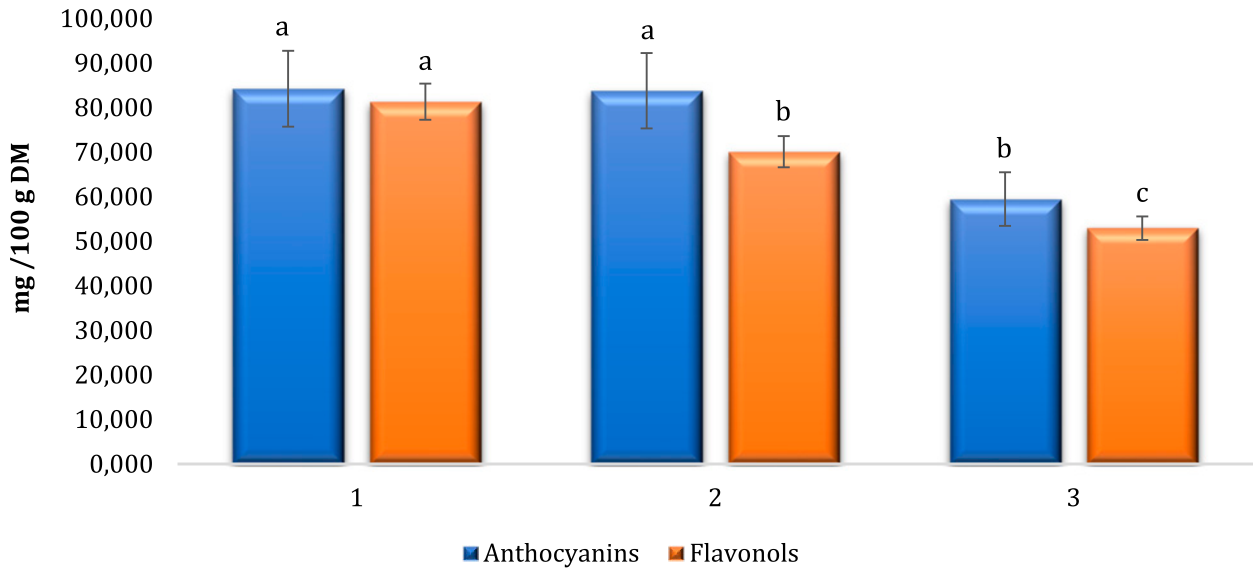

Figure 4 Comparison of the average anthocyanin and flavonol content in *Cornus mas* fruits with early, medium and late ripening periods:
1 – early fruiting; 2 – medium fruiting; 3 – late fruiting (different superscripts in each column indicate the significant differences in the mean at $p < 0.05$))

fruit ripening period were selected at the beginning of September. In this case, the length of daylight has not changed significantly. *C. mas* cultivars with late fruit ripening were selected in early October. During this period, the daylight hours became much shorter. This difference is visible on the histogram (Figure 4).

It was found that the content of anthocyanins in the fruits of hybrid *C. mas* × *C. officinalis* significantly exceeds their content in the fruits of *C. officinalis* and cultivars of *C. mas* and amounts to 99–117 mg/100 g DM. The anthocyanin content in *Cornus officinalis* fruits is in the range of 52–93 mg/100 g DM, in comparison with the content of anthocyanins in the fruits of *C. mas* cultivars with a late ripening period in the range of 33–96 mg/100 g DM.

The content of flavonols in the fruits of hybrid *C. mas* × *C. officinalis* significantly exceeds their content in the fruits of *Cornus mas*, but in comparison with the content

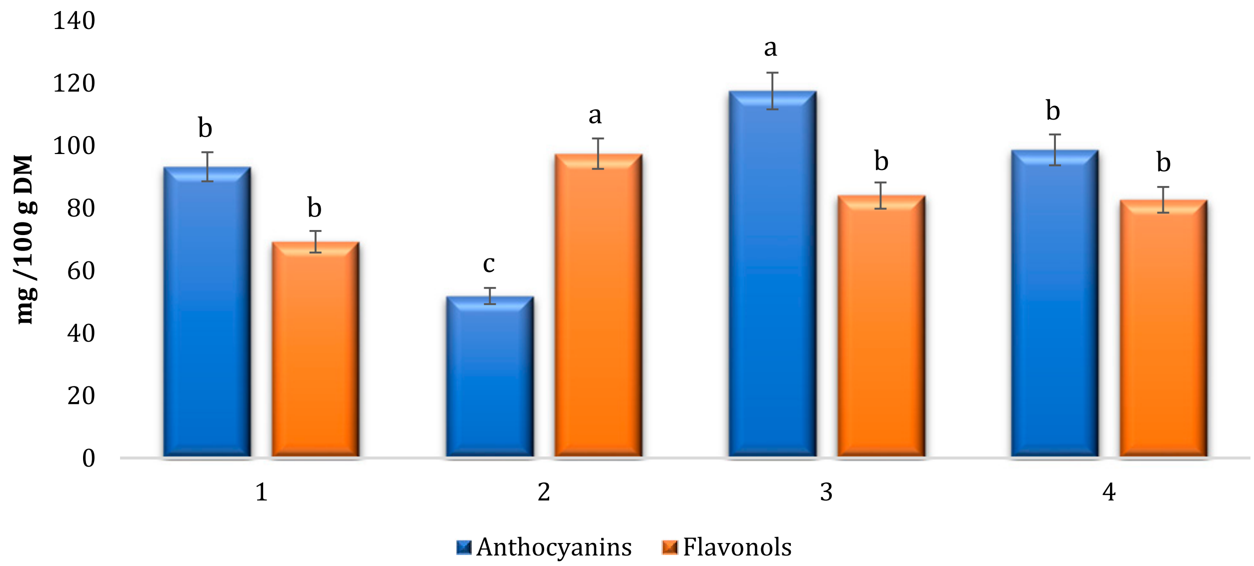

Figure 5 The content of anthocyanins and flavonols in the fruits of *Cornus officinalis* and *Cornus mas* × *Cornus officinalis* (cultivar Etude):
1 – *C. officinalis* (CO-01); 2 – *C. officinalis* (CO-02); 3 – cultivar Etude No 1; 4 – cultivar Etude No 2 (different superscripts in each column indicate the significant differences in the mean at $p < 0.05$)

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of flavonols in the fruits of *C. officinalis* is within the same limits. The content of flavonols in the fruits of hybrid *C. mas* × *C. officinalis* is 83–84 mg/100 g DM, in the fruits of *C. officinalis* – 69–97 mg/100 g DM, in the fruits of *C. mas* cultivars with a late ripening period – 36–88 mg/100 g DM (Figure 5).

Conclusions

The amplitude of variability in the content of anthocyanins and flavonols in the fruits of *Cornus* spp. genotypes were studied. It is shown that the differences in the content of these compounds are determined by the genetic properties of the varieties and the ripening period of the fruits. Cultivars Pervenets, Volodymyrskyi, Mriia Shaidarovoi, Vydubetskyi, Titus, Sokolyne (*C. mas*), as well as hybrid *C. mas* × *C. officinalis* (cultivars Etude No. 1 and No. 2) have the highest content of these compounds. A correlation was found between the content of flavonols and anthocyanins in the fruits of *C. mas* cultivars. The correlation coefficient ranges from moderate (r = 0.591) in cultivars with an average fruit ripening period, too high (r = 0.955 and r = 0.918) in cultivars with an early and late fruit ripening period. A decrease in the average content of flavonols and anthocyanins in the fruits of *C. mas* cultivars with a late fruit ripening period was found. We assume that this may be due to a reduction in the length of daylight hours. High content of biologically active substances in *Cornus* fruits, including anthocyanins and flavonols, allows using raw materials as an active prophylactic and therapeutic agent.

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Research Article

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Comparative assessment of the antibacterial efficacy of leaf extract obtained from *Ficus benjamina* L. (Moraceae) and its cultivars against *Aeromonas sobria* strain

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The present study is *in vitro* study to evaluate the antimicrobial activity of the ethanolic extracts derived from leaves of *Ficus benjamina* L. and its cultivars (Safari, Baroque, Amstel Gold, Reginald) against *Aeromonas sobria* to assess the possible use of this plant in preventing infections caused by this fish pathogen in aquaculture. Antimicrobial susceptibility of the tested *Aeromonas sobria* was performed by the Kirby-Bauer disc diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). Our results revealed, that *F. benjamina* and its cultivars possessed antibacterial properties against *Aeromonas sobria* strain. The ethanolic extract obtained from leaves of *F. benjamina* 'Safari' exhibited the maximum antimicrobial activity against *Aeromonas sobria* (the mean of inhibition zone diameter was 26.19 ±1.32 mm). *Aeromonas sobria* strain was susceptible to the *F. benjamina* 'Amstel Gold' (15.25 ±1.25 mm) and 'Reginald' (16.25 ±1.10 mm). *Aeromonas sobria* strain was the most resistant to *F. benjamina* (12.5 ±0.80 mm) and *F. benjamina* 'Baroque' (13.63 ±0.75 mm) leaf extracts. The results of this study provide a new perspective for the use of various *Ficus* species as medicinal plants to improve the antibacterial responses in aquaculture. Scanning electron microscopy has been employed to observe epicuticular wax structures which can be used to assure the correct identification of plant raw materials. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of their antioxidant effects on various tissues of salmonids are in progress.

Keywords: *Ficus benjamina* L., *Aeromonas sobria*, antimicrobial activity, disc diffusion technique, ethanolic extracts

Introduction

At the current time, there are intense and active investigations into natural products with biocidal activities for fish (Galina et al., 2009). Plant-derived compounds act as a better antibacterial, antiviral, immunostimulant, and antistress effect in fish and shellfish aquaculture. For that reason, there has been considerable interest in the use of medicinal plants in aquaculture to provide 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;

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Biller-Takahashi and Urbinati, 2014; Newaj-Fyzul and Austin, 2015; Vallejos-Vidal et al., 2016).

In this study, attention focused on the genus *Ficus* L., 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. 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 (Berg and Wiebes, 1992; Cook and Rasplus, 2003; Berg and Corner, 2005). Among the pharmacological properties demonstrated for the compounds present in the 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.

Ficus benjamina L. also referred to as a weeping fig tree, is a multipurpose tree found in a large area including India, southern China, Southeast Asia, Malaysia, the Philippines, northern Australia, and the islands of the South Pacific (Riffle, 1998). It grows as a large evergreen shrub, up to 8 m tall, with nearly 10 m wide-spreading crown and drooping shoots with young slender twigs (Imran et al., 2014). It is one of the most popular indoor ornamental plants worldwide. The plant is well known due to its medicinal potential. Its latex and some fruit extracts are used by indigenous communities to treat skin disorders, inflammation, piles, vomiting, leprosy, malaria, nose diseases, and cancer besides the use as a general tonic. The plant is also used as an antimicrobial, antinociceptive, antipyretic, hypotensive, and anti-dysentery remedy. The leaves and twigs are used as insect repellants (Imran et al., 2014). The leaves, bark, and fruits of *F. benjamina* contain various bioactive constituents like cinnamic acid, lactose, naringenin, quercetin, caffeic acid, and stigmasterol (Sirisha et al., 2010). *F. benjamina* wood uses in aerobic biofiltration as a support medium for the treatment of Tequila vinasses (Marco Antonio et al., 2018).

In this study, we evaluated the antimicrobial activity of the ethanolic extracts of *F. benjamina* and its cultivars, i.e. *F. benjamina* 'Safari', 'Baroque', 'Amstel Gold', 'Reginald' against *Aeromonas sobria* to evaluate the possible use of this plant in preventing infections caused by this fish pathogen in aquaculture. Given that standardization and quality control are essential analytical steps to assure the correct identification of plant raw materials to be used as plant-derived medicines, the micromorphology of *F. benjamina* leaf surfaces has been investigated with SEM procedure. The need for constant incorporation of leaf micromorphology in pharmacological investigations has been emphasized in some recent papers (Bilić et al., 2019; Khan et al., 2020).

The current study was conducted as a part of an ongoing project between the Institute of Biology and Earth Sciences (Pomeranian University in Słupsk, 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 and subtropical plants, cultivated *in vitro*.

Material and methodology

Collection of plant material and preparing plant extract

The leaves of *F. benjamina* and its cultivars (Safari, 'Baroque, Amstel Gold, Reginald) were sampled at National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine), and Botanic Garden of Ivan Franko National University in Lviv (Lviv, Ukraine). The sampled leaves were brought into the laboratory for antimicrobial studies. Freshly sampled leaves were washed, weighed, crushed, homogenized in 96 % ethanol (in proportion 1 : 10) at room temperature, and centrifuged at 3000 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

Aeromonas sobria (K825) strain, originated from freshwater fish species such as common carp (*Cyprinus* *carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), respectively, was isolated in the 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 Polska Sp. z o.o.) and incubated at 27 ±2 °C for 24 hr. 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 Polska Sp. z o.o.). 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 sobria* 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 hr at 28 ±2 °C. Seven drugs representing different antimicrobial classes as quinolones, tetracyclines, sulphonamides, and phenols were used. After incubation, the inhibition zones were measured. Interpretation criteria have been adopted from that available for *Aeromonas* salmonicida (CLSI, 2006).

Micromorphological leaf surface investigation

Visualization of leaf surfaces micromorphology of *Ficus benjamina* was undertaken with scanning electron microscopy (SEM) technique. The dried leaf samples were sputter-coated with carbon in a vacuum universal post (VUP-5M) and platinum in a JEOL JFC-1600 Auto Fine Coater. After then SEM analysis was carried out using a JEOL JSM-6700F scanning electron microscope at 15 kV acceleration voltage in the high vacuum mode.

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 ($p > 0.05$). 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) = 10–15 mm, and Resistant (R) ≤10 mm (Okoth et al., 2013).

Results and discussion

As can be seen from Figure 1, F. benjamina leaves are hypostomatic. Leaves possess paracytic stomata which are distributed regularly throughout the leaf surface between veins (Figure 1B). They are surrounded with a cuticular thickening that formed a rim (Figure 1C). The adaxial leaf surface is moderately undulate and pavement cells in the adaxial epidermis are difficult to recognize due to well-developed cuticle (Figure 1A). Nevertheless, epicuticular wax structures on the adaxial surface have not been observed. While the abaxial leaf surface has exhibited the deposition of small epicuticular wax plates (Figure 1C).

Results on *in vitro* antimicrobial activity assessment of ethanolic extracts derived from leaves of *F. benjamina* and its cultivars (Safari, Baroque, Amstel Gold, Reginald) against *Aeromonas sobria* strain expressed

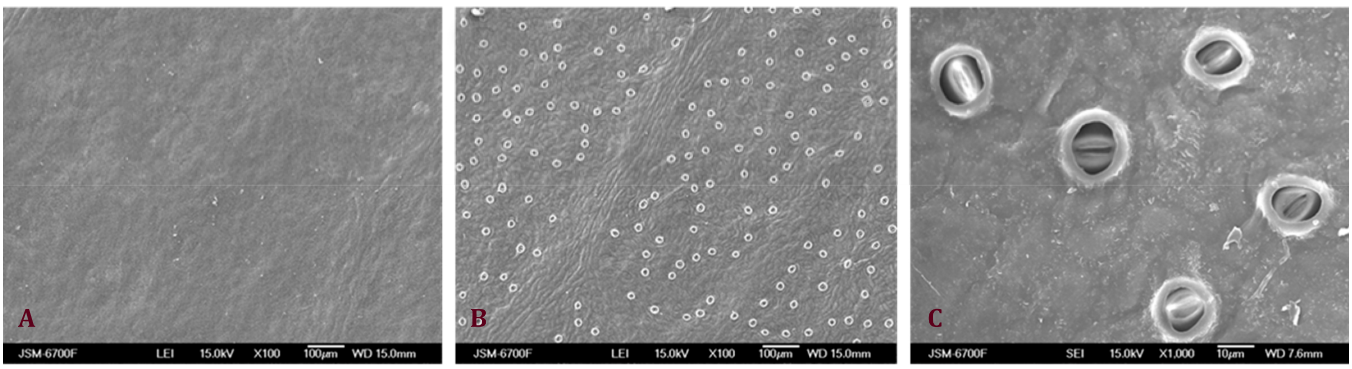

Figure 1 Scanning electron microscopy micrographs of adaxial (A) and abaxial (B, C) leaf surfaces of *Ficus benjamina* L.

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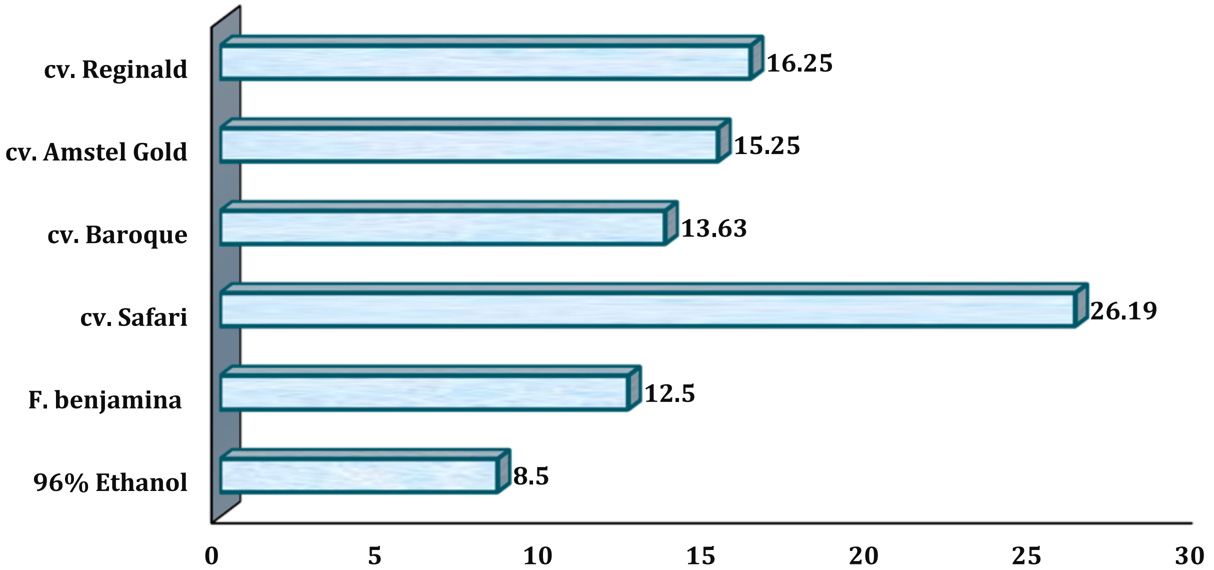

Figure 2 The mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *Ficus benjamina* L. and its cultivars (Safari, Baroque, Amstel Gold, Reginald) against *Aeromonas sobria* strain (1000 µL inoculum) (M ±m, n = 8)

as a mean of diameters of inhibition zone is presented in Figure 2.

Our results of the antimicrobial screening revealed, that *F. benjamina* and its cultivars possessed antibacterial properties against *Aeromonas sobria* strain. The ethanolic extract obtained from leaves of *F. benjamina* cv. Safari exhibited the maximum antimicrobial activity against *Aeromonas sobria* (the mean of inhibition zone diameters was 26.19 ±1.32 mm). *Aeromonas sobria* strain was susceptible to the *F. benjamina* cv. Amstel Gold (15.25 ±1.25 mm) and cv. Reginald (16.25 ±1.10 mm). *Aeromonas sobria* strain was the most resistant to *F. benjamina* (12.5 ±0.80 mm) and *F. benjamina* cv. Baroque (13.63 ±0.75 mm) leaf extracts (Figure 1).

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 vitro* study with promising results (Tkachenko et al., 2016a,b,d, 2017a,b, 2018, 2019). Most ethanolic extracts obtained from *Ficus* spp. in our 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* leaf extract. 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., 2016a,d). Additionally, among various species of the *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., 2016c). Antibacterial properties of plant extracts have been by far the most studied bioactivity with potential applications in aquaculture systems (Reverter et al., 2014).

Moreover, in our previous study (Buyun et al., 2018), we have evaluated the *in vitro* effect of extracts obtained from leaves of *F. benjamina* and its cultivars on the oxidative stress biomarkers (carbonyl content of the oxidatively modified proteins, total antioxidant capacity) in the muscle tissue of the rainbow trout. Our results have shown that extracts obtained from leaves of *F. benjamina* 'Safari' and 'Reginald' cultivars decreased non-significantly the lipid peroxidation biomarker and the ketonic derivatives of oxidatively modified proteins levels in the muscle tissue. Furthermore, our results showed that extracts obtained from leaves of *F. benjamina* and its cultivars increased substantially the total antioxidant capacity in muscle tissue by 76.9 % (*F. benjamina*), 66.9 % (*F. benjamina* cv. Safari), 70.5 % (*F. benjamina* cv. Baroque), 49.4 % (*F. benjamina* cv. Amstel Gold), and 42.8 % (*F. benjamina* cv. Reginald) ($p < 0.05$). The results of this study provide a new perspective on the use of various *Ficus* species as a medicinal plant to improve the antioxidant response of rainbow trout (Buyun et al., 2018).

It would be reasonable to suggest that these antimicrobial effects are determined by plant by-products, i.e. flavonoids. Indeed, the results of

Imran et al. (2014) indicated that *F. benjamina* is a good source of components with high antibacterial activity. The extracts and fractions of stem, root, and leaves exhibited considerable antimicrobial activity against four bacterial and two fungal strains. The range of antimicrobial activity expressed as diameters of inhibition zone for stem was 10.5 mm (n-hexane) – 22.83 mm (n-butanol). All the butanol fractions exhibited strong activity. The methanol extract (22.63 mm against *Pseudomonas aeruginosa*) and an n-butanolic fraction (22.83 against *Bacillus subtilis*) of stem showed substantial activity. The n-hexane, chloroform, and ethyl acetate sprouted a moderate value of diameters of inhibition zone, with maximum value disclosed by ethyl acetate (16.88 mm). The stem extract and fractions revealed the following order of antimicrobial potential against *Bacillus cereus*: methanolic > n-butanolic > ethyl acetate > chloroform > n-hexane (Imran et al., 2014).

F. benjamina also uses in the treatment of malaria which may be attributed to ursolic acid and lupeol. The study of Singh et al. (2020) emphasized the investigation of antiplasmodial activity of triterpenoids isolated from *F. benjamina* leaves. An unsaponified fraction of petroleum ether extract of plant leaves was subjected to silica gel column chromatography which led to the isolation of two known triterpenoids; namely ursolic acid and lupeol. These compounds were evaluated for antiplasmodial activity by schizont maturation inhibition assay using 3D7 *Plasmodium* strains. Both, ursolic acid and lupeol were found to exhibit significant antiplasmodial effect with an IC_{50} value of 18 and 3.8 µg/ml, respectively (Singh et al., 2020).

Wanderley et al. (2018) have evaluated the anthelmintic potential of a protease purified from the latex of *F. benjamina* against *Haemonchus contortus*, a gastrointestinal nematode that is responsible for high mortality rates in ruminant herds. A cysteine protease (FbP) inhibited both the development and escheatment of *H. contortus* larvae, with 50 % effective concentrations of 0.26 and 0.79 mg/mL, respectively. Thus, this cysteine protease from *F. benjamina* latex with anthelmintic activity against *H. contortus* could be a promising alternative for the development of products for use in parasite control programs (Wanderley et al., 2018).

Imran et al. (2014) showed that the HPLC analysis for the presence of phenolic acids permitted the identification of 5 phenolic acids, three in the stem, four in the root, and one in leaves. The total phenolic content (Folin-Ciocalteu) of the leaves of *F. benjamina* and *F. luschnathiana* were evaluated and screened by HPLC-DAD by Cruz et al. (2012). *F. luschnathiana* crude extract (CE) presented phenolic content higher than that of *F. benjamina* (149.92 ±3.65 versus 122.63 ±2.79 mg of GAE). Kaempferol (1.63 ±0.16 mg/g dry weight of CE) and chlorogenic acid (17.77 ±0.57 mg/g of butanolic fraction) were identified and quantified in *F. benjamina*. Additionally, rutin (15.55 ±1.92 mg/g) and quercetin (3.53 ±0.12 mg/g) were quantified in ethyl acetate and butanolic fractions, respectively. Sirisha et al. (2010) reported the presence of ursolic, α-hydroxy ursolic, protocatechuic, and maslinic acids in *Ficus* species, while cinnamic and caffeic acids and quercetin have been reported in leaves, bark, and fruits of *F. benjamina* (Almahyl et al., 2003). All the detected phenolic acids are known to have antioxidant properties (Imran et al., 2014). So these phenolic acids may be responsible for the antibacterial activities of *F. benjamina* and its cultivars. In addition to their antioxidant activity, flavonoids also show good antibacterial activity against both Gram-positive and Gram-negative isolates (Daglia, 2012; Coppo and Marchese, 2014; Barbieri et al., 2017). Flavonoids can be divided into six subfamilies based on differences in their molecular backbone structure: flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids (Barbieri et al., 2017). They can inhibit DNA gyrase, cell membrane function, and bacterial energy metabolism (Cushnie and Lamb, 2005; Safavi et al., 2015). In recent years, flavonoids have been studied for their ability to interact with DNA helicases, proteins essential for DNA replication, repair, and recombination (Lohman et al., 2008), and to prevent dNTPs binding. In particular, Chen and Huang (2011), studied 4 flavonoids (galangin, kaempferol, quercetin, and myricetin at 10 µM) revealed that they capable of inhibiting the interaction of *Klebsiella pneumoniae* DnaB helicase with dNTPs. Huang et al. (2015) have demonstrated that some flavonoids (kaempferol and myricetin, at 35 µM) inhibit the PriA helicase activity of *Staphylococcus aureus* (Barbieri et al., 2017).

There are increasing awareness and general acceptability of the use of plant-derived medicines in today's medical practice. Nevertheless, it is believed that one of the disadvantages of herbal medicine is the lack of standardization and quality control profiles (Kunle et al., 2012).

One of the methods of pharmacognostic studies is micromorphological analysis, which makes it possible to ensure the identity of the raw material of medicinal plants (Khan et al., 2020).

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The study of foliar epidermal anatomy of some ethnobotanically important species of genus *Ficus* has been undertaken by Khan et al. (2011). Comprehensive morphological studies of *Ficus* species by light and scanning electron microscopy had been undertaking by Klimko and Truchan (2006).

We believe that comparative anatomical, micromorphological studies and bio-elemental analysis could be considered as an important part of a pharmacognostic investigation to ensure the correct taxonomic identification of the plant screened based on micromorphological and anatomical features.

Conclusions

The present study was carried out to provide evidence of the antibacterial potency of the extracts obtained from leaves of *F. benjamina* and its cultivars as a potential source of natural antimicrobial agents. *F. benjamina* disclosed substantial bioactivity, and this plant can be regarded as a potential source of antibacterial agents. In conclusion, the results of this study provide a new perspective for the use of various *Ficus* species as medicinal plants to improve the antibacterial responses in aquaculture. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of their antioxidant effects on various tissues of salmonids are in progress.

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Research Article

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Studies of the chemical composition of fruits and seeds of pawpaw (*Asimina triloba* (L.) Dunal)

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An international team of scientists continues to study the resource potential of non-conventional or little-utilized plants. Pawpaw (*Asimina triloba* (L.) Dunal) fruit (pulp and peel) and seeds were analyzed for their nutritional compositions. Seeds exhibited significantly higher levels of crude protein, lipid, and vitamin E (11.82 %, 34.0 %, and 20.80 mg/kg, respectively) than those of the other parts. Sucrose in pulp was 501.40 g/kg, which was the highest among the samples. There is more fructose in the peel 111.90 g/kg. Results revealed that the total amino acids in the seeds, pulp, and peel of *A. triloba* were 144.6, 21.1, and 20.9 g/kg, respectively. Among the different plant parts used in this study, the seeds contained the most abundant essential amino acid and non-essential amino acid. The glutamic acid exhibited the highest concentration among the tested amino acids. Oleic and linoleic acids in seeds were 40.13 and 38.84 g/100 g, respectively, which were the highest among the pulp and peel. Potassium was the most abundant essential trace mineral element in different parts. This element is present in large ammounts in the peel (15487 mg/kg) and pulp (12198 mg/kg) compared to the seeds (3888 mg/kg). In the seeds, P, Ca and S were higher (1937, 1368, and 1322 mg/kg, respectively) than in pulp and peel (1046, 450, 499 and 831, 837, 646 mg/kg, respectively). The high content of beneficial substances makes it possible to include *Asimina triloba* in the list of species recommended for cultivation on a larger scale and to use its products more widely in dietary nutrition.

Keywords: pawpaw, fruits, seeds, chemical compositions

Introduction

The search for new plant species, especially neglected and underutilized plant species that are a valuable source of biologically active compounds, and creation on its basis the new generation of nutritional supplements, has recently become an urgent branch of modern biological science, and one of the most important scientific directions (Brindza et al., 2006, 2016; Klymenko et al., 2017). Guided by these new requirements, an international team of scientists has been studying for several years the content of biologically active compounds in various organs of cultivated new plants to the region (Monka et al., 2014; Ivanišová et al., 2017; Klymenko et al., 2017, 2019; Grygorieva et al., 2018; Horčinová Sedláčková et al., 2018; Grygorieva et al., 2020a, b; Vinogradova et al., 2020). This article focuses on *Asimina triloba* (L.) Dunal.

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Asimina triloba (pawpaw, paw paw, paw-paw, common pawpaw) belongs to the family Annonaceae Juss. native to eastern North America and Canada (Layne, 1996). *A. triloba* fruits are rich in vitamins and minerals (Templeton et al., 2003; Pomper and Layne 2005), are a good source of potassium and several essential amino acids, and they contain significant amounts of riboflavin, niacin, calcium, phosphorus, and zinc (Galli et al., 2007), have a high polyphenolic (Harris and Brannan, 2009; Brannan et al., 2012, 2014) and antioxidant content (Kobayashi et al., 2008; Brindza et al., 2019).

A. triloba can be used as an alternative to bananas fruits in most recipes (Jones et al., 1995). Fruits of pawpaw are very fragrant and resemble a combination of aromas of banana and mango, and may be used commercially in cosmetics and skin products (Layne, 1996; Brannan et al., 2012). The extract of unripe *A. triloba* fruit has a value not only as a functional food, but has therapeutic potential for the treatment of cancer as a naturally derived substance that may be less toxic than conventional chemotherapy drugs (Nam et al., 2018b).

Biologically active compounds are not only in fruits, but in different parts of the plant: roots, bark, twigs, leaves, flowers, and seeds (Hui et al., 1989; Zhao et al., 1992, 1993, 1994; Alali et al., 1999; Goodrich et al., 2006; Cuendet et al., 2008; Farag, 2009; Pande and Akoh, 2010). The roots, twigs, flowers, and seeds of *A. triloba* contain acetogenins, which are strong inhibitors of cancer cells (Ratnayake et al., 1992; Woo et al., 1995; Ko et al., 2011; Sica et al., 2016). *A. triloba* leaf essential oil has strong activity against cancer cell lines (Alali et al., 1999; Farag, 2009).

A. triloba fruit, leaf, bark, and twig extract may be an effective insect feeding deterrent (Rupprecht et al., 1986; Ratnayake et al., 1992; Zhao et al., 1994; Gu et al., 1999; Sedlacek et al., 2010).

Despite the importance of *A. triloba* as a nutritional and medicinal plant in the conditions of Ukraine, this species is very little spread. Thus, the objective of the present study was to investigate and compare the nutritional compositions of pulp, peel, and seeds of *A. triloba*.

Material and methodology

Biological material

A. triloba (Figure 1) seeds and fruits (pulp and peel) (Figure 2) were collected in September 2020 from the trees growing in an M.M. Gryshko National Botanical Garden (Kyiv, Ukraine; 197 m a.s.l.).

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), and CentralChem (Slovakia).

Phytochemical analyses

Determination of dry matter, ash, and protein content

Total dry matter, ash, and protein content were determined according to EN method (CSN EN 12145, 1997). Total lipid content was determined according to the methods specified in ISO method (ISO 659:1998).

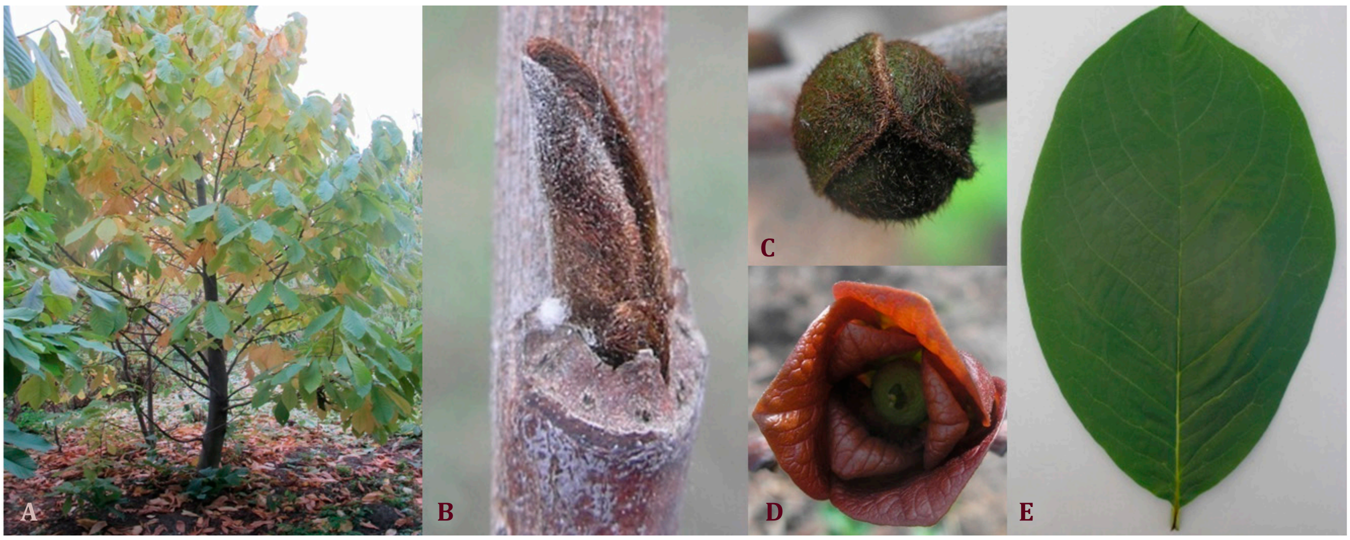

Figure 1 Tree (A), vegetative bud (B), generative bud (C), flower (D), and leaf (E) of *Asimina triloba* (L.) Dunal

Figure 2 Fruits and seeds of *Asimina triloba* (L.) Dunal

Determination of saccharides

For the determination of saccharides, 1 g of a sample was extracted with 10 mL of extraction the solution (ultrapure water and ethanol mixed in a ratio of 4 : 1) in a 50 mL centrifugation tube placed on a vertical shake table (GFL, Germany). After 1 hr of extraction, the samples were centrifuged for 4 min at 6000 rpm in a centrifuge (EBA 21, Hettich, Germany); the supernatant was filtered using a filter with 0.45 mm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with ultrapure water. An Agilent Infinity 1260 liquid chromatography (Agilent Technologies, USA) equipped with ELSD detector was used for the determination of saccharides. A Prevail Carbohydrates ES column (250/4.6 mm) was used as a stationary phase and acetonitrile (VWR) mixed with water in 75 : 25 volume ratio was used as the mobile phase.

Determination of carotenoid

Total carotenoid content expressed as beta-carotene was analyzed at a wavelength of 445 nm spectrophotometrically (VIS spectrophotometer UV Jenway Model 6405 UV/VIS). Sample (1 g) was disrupted with sea sand and extracted with acetone until complete discoloration. Petroleum-ether was added and then water with the purpose of the separation of phases. After the separation, the petroleum ether-carotenoid phase was obtained and the absorbance was measured (ČSN 560053, 1986).

Determination of mineral contents

Sample for elemental analysis was prepared using the wet ashing method in a microwave oven (Milestone 1200, Milestone, Italy). Total of 0.25 g sample matrix was decomposed in a mixture of nitric acid (6 mL) (Analytika Praha Ltd, Czech Republic) and hydrochloric acid (2 mL) (Analytika Praha Ltd, Czech Republic). Then decomposition sample was filtered using a filter with 0.45 mm pore size and filled up to 25 mL in a volumetric flask with ultrapure water. Elemental analysis was performed using ICP-OES (Ultima 2, Horiba Scientific, France) according to the procedure described by Divis et al. (2015).

Determination of amino acids

Amino acids were determined by ion-exchange liquid chromatography (Model AAA-400 amino acid analyzer, Ingos, Czech Republic) using post-column derivatization with ninhydrin and a VIS detector. A glass column (inner diameter 3.7 mm, length 350 mm) was filled manually with a strong cation exchanger in the LG ANB sodium cycle (Laboratory of Spolchemie) with average particles size 12 µM and 8 % porosity. The column was heated within the range of 35 to 95 °C. The elution of the studied amino acids took place at a column temperature set to 74 °C. A double-channel VIS detector with the inner cell volume of 5 µL was set to two wavelengths: 440 and 570 nm. A solution of ninhydrin (Ingos, Czech Republic) was prepared in 75 % v/v methyl cellosolve (Ingos, Czech Republic) and in 2 % v/v 4 M acetic buffer (pH 5.5). Tin chloride ($SnCl_2$) was used as a reducing agent. The prepared solution of ninhydrin was stored in an inert atmosphere (N_2) in darkness at 4 °C. The flow rate was 0.25 (mL/min) and the reactor temperature was 120 °C.

Statistical analysis

Basic statistical analyses were performed using PAST 2.17. Data were analyzed with ANOVA test and differences between means compared through the Tukey-Kramer test ($p < 0.05$). The variability of all these parameters was evaluated using descriptive statistics.

Results and discussion

In the course of the study of the nutritional properties of *A. triloba* for a recommendation to use it as a raw material for industry, it is crucial to identify the chemical composition of the different organs of the plant.

The total protein content was 11.82, 3.64, and 3.98 % in seeds, pulp, and peel, respectively (Table 1); the total lipid content was 34.0, 1.12, and 3.89 %, respectively. According to Nam et al. (2018a) *A. triloba* seeds also showed significantly higher levels of crude protein and crude lipid.

Chitturi et al. (2013) evaluated the protein levels and antioxidant potential of air-dried medicinally significant domestic fruit peels and their extracts.

Monosaccharide analysis of neutral carbohydrate part showed the presence of fructose (4.20, 87.10, and 111.90 g/kg, respectively) and sucrose (24.30, 501.40, and 227.50 g/kg, respectively) in seeds, pulp, and peel, respectively, while other saccharides, such as maltose and lactose were found only in low amounts only (<0.5 g/kg). Nam et al. (2018a) showed that fructose, glucose, and sucrose showed the highest levels in the fruit of *A. triloba* at 1691.35 mg%, 2148.20 mg%, and 9321.24 mg%, respectively.

The free sugar content, as has been demonstrated by Andersen (1986), depends on the following climatic characteristics: temperature, rainfall, relative humidity, and degree of light.

Therefore, the phytochemical composition of *A. triloba*, including free sugars is expected to vary according to cultivation conditions. At the same time, the intensity of sweetness from free sugars and their composition will differ depending on the plant organs.

Carotenoids are widely distributed in nature. They are diverse in structure and in their function for human health. Carotenoids are useful for the prevention of certain types of cancer. They can prevent photosensitization in some skin diseases and increase immune response in infections. Their anti-aging effects on the human body are also known (Kurahashi et al., 2009).

A. triloba contains beta carotene in seeds, pulp, and peel (4.80, 6.60, and 12.70 mg/kg, respectively). As shown from the results, beta carotene accumulated in peel 2 times more than in pulp and approximately 3 times more than in seeds. Differents fruits investigation on β-carotene content showed that in the peel this vitamin concentrated the most. Ghosh et al. (2019) believe the presence of β-carotene in fruit peel wastes might be a contributing factor for its antioxidant activities. *Citrus reticulata* Blanco (Ghosh et al., 2019), *Mangifera indica* L. (Ranganath et al., 2018; Ghosh et al., 2019), *Musa acuminata* Colla (Budhalakoti, 2018), *Malus domestica* Borkh. (Delgado-Pelayo et al., 2014) have a high in β-carotene content and they also show potential antioxidant activity. According to Arora et al. (2008), banana peel could be a potential source of carotenoids. The content of carotenoids and β-carotene depends

Table 1 Contents of some phytochemical compounds of *Asimina triloba* (L.) Dunal

Components	Seeds ($\bar{x} \pm S_x$)	Pulp ($\bar{x} \pm S_x$)	Peel ($\bar{x} \pm S_x$)
Total dry matter (%)	96.73 ±3.06	87.07 ±2.68	84.12 ±2.33
Total content of protein (%)	11.82 ±0.12	3.64 ±0.05	3.98 ±0.18
Total content of ash (%)	1.47 ±0.04	4.27 ±0.09	4.04 ±0.05
Total content of lipids (%)	34.0 ±1.02	1.12 ±0.06	3.89 ±0.07
Beta carotene (mg/kg)	4.80 ±0.05	6.60 ±0.09	12.0 ±0.09
Saturated fatty acids (g/100 g oil)	8.90 ±0.03	30.50 ±0.60	25.70 ±0.10
Monounsaturated fatty acids (g/100 g oil)	32.10 ±0.16	28.0 ±0.09	24.90 ±0.11
Polyunsaturated fatty acids (g/100 g oil)	46.60 ±1.13	18.20 ±0.13	23.70 ±0.09
Fructose (g/kg)	4.20 ±0.04	87.10 ±0.21	111.90 ±1.16
Maltose (g/kg)	<0.5	<0.5	<0.5
Sucrose (g/kg)	24.30 ±0.10	501.40 ±12.18	227.50 ±9.32
Lactose (g/kg)	<0.5	<0.5	<0.5
Vitamin A (retinyl acetate) (mg/kg)	<0.1	<0.1	<0.1
Vitamin E (α-tocopherol) (mg/kg)	20.80 ±0.08	8.80 ±0.11	19.10 ±1.09

Note: $\bar{x}$ – arithmetic mean; S_x – standard error of the mean on many factors such as degree of maturation, type of soil, climatic conditions, etc. (Dhandapani et al., 2017). Fruits and vegetable processing industries produce huge waste in the form of peels, seeds, liquid, and molasses which are a good source of carbohydrates, proteins, fibres, vitamins, and minerals (Kaur et al., 2018).

The major quantitative tocopherol in *A. triloba* seeds, pulp, and peel was α-tocopherol (20.80, 8.80, and 19.10 mg/kg DWP, respectively). The oil contents were 34.0 (seeds), 1.12 (pulp), and 3.89 % (peel) dry weight plant material. The majority of plant oils are accumulated in the seeds (Bates et al., 2013) and this parameter is controlled by genetic effects (embryonic, cytoplasmic, maternal) and interactions between the genotype and environment (Liu et al., 2014).

The composition and amount of free amino acids varied among the different parts (Figure 3). The taste, flavour, and quality of various foods are directly related to the quantity and quality of free amino acids that accumulate naturally in nutrition products (Kabelova et al., 2008; Sinesio et al., 2009). Amino acid analysis has shown that the studied *A. triloba* seeds, pulp, and peel contained 18 amino acids (9 essential and 9 non-essential). The contents of the total amino acid of the seeds were significantly higher than those of the pulp and peel, which is consistent with previous studies that found that most of the amino acids of the seeds of *A. triloba* was in higher levels than those in the fruit (Nam et al., 2018a). The total amino acids in the seeds, pulp, and peel of *A. triloba* were 144.6, 21.1, and 20.9 g/kg, respectively. The glutamic acid exhibited the highest concentration in seeds with the highest mean content among the tested amino acids, accounting for more than 22.68 % of the entire amino acid profile. Alanine, glutamic acid, and histidine were the major amino acids detected in the peel, and glutamic acid, and aspartic acid were the main amino acids found in the pulp.

Our data coincide with those of Nam et al. (2018a) that glutamic acid was the major amino acid measured in seeds having the highest level of 1396.27 mg%.

Fatty acids play an important role in human health. Thus, they are valuable in the prevention of cardiovascular diseases, coronary heart disease, and cancer. Their role is also important in the prevention of inflammatory, thrombotic and autoimmune diseases, hypertension, type 2 diabetes, kidney diseases, rheumatoid arthritis, and ulcerative colitis (De Caterina et al., 2000; Abedi and Sahari, 2014). Lauric, palmitic, linolenic, linoleic, oleic, stearic, and myristic fatty acids have antibacterial and antifungal properties (McGaw et al., 2002; Seidel and Taylor, 2004). In this regard, we determined the fatty acid content in various organs of *A. triloba*.

Total fatty acids varied in different parts of *A. triloba* (Figure 4, 5, 6). The unsaturated fatty acid content was higher than the saturated fatty acid content in all samples, and the difference between unsaturated fatty acid and saturated fatty acid was more pronounced in pawpaw seeds. In contrast, the difference between the contents in pulp and peel was negligible.

In the present study, *A. triloba* contained palmitic acid, oleic acid, linolenic acid, and linoleic acid. Oleic

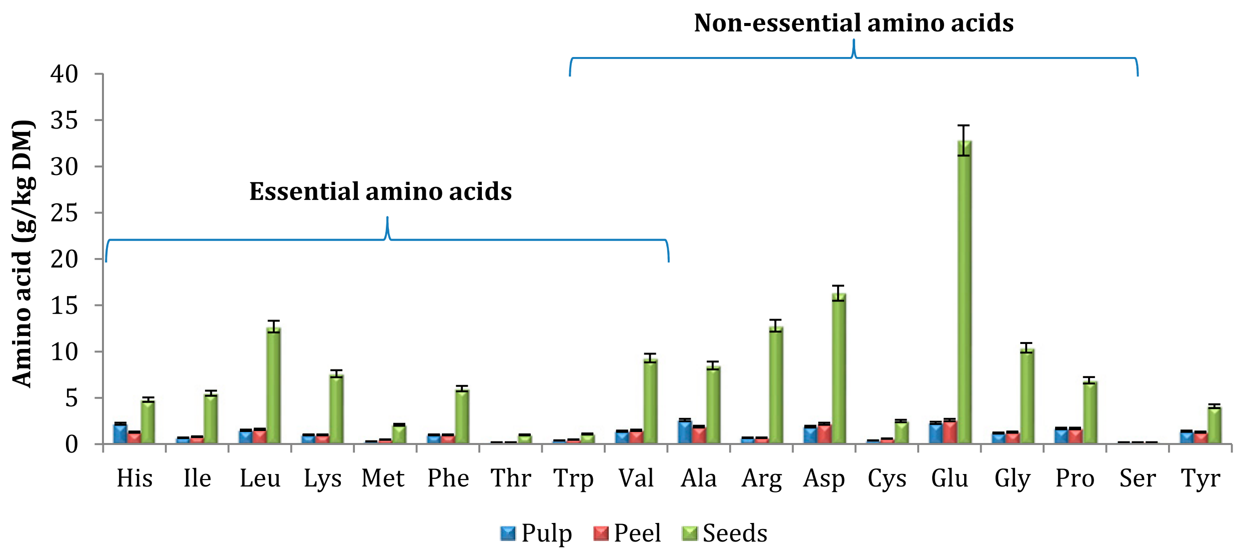

Figure 3 Amino acid composition of *Asimina triloba* (L.) Dunal seeds, pulp, and peel, g/kg DM (different superscripts in each column indicate the significant differences in the mean at $p < 0.05$)

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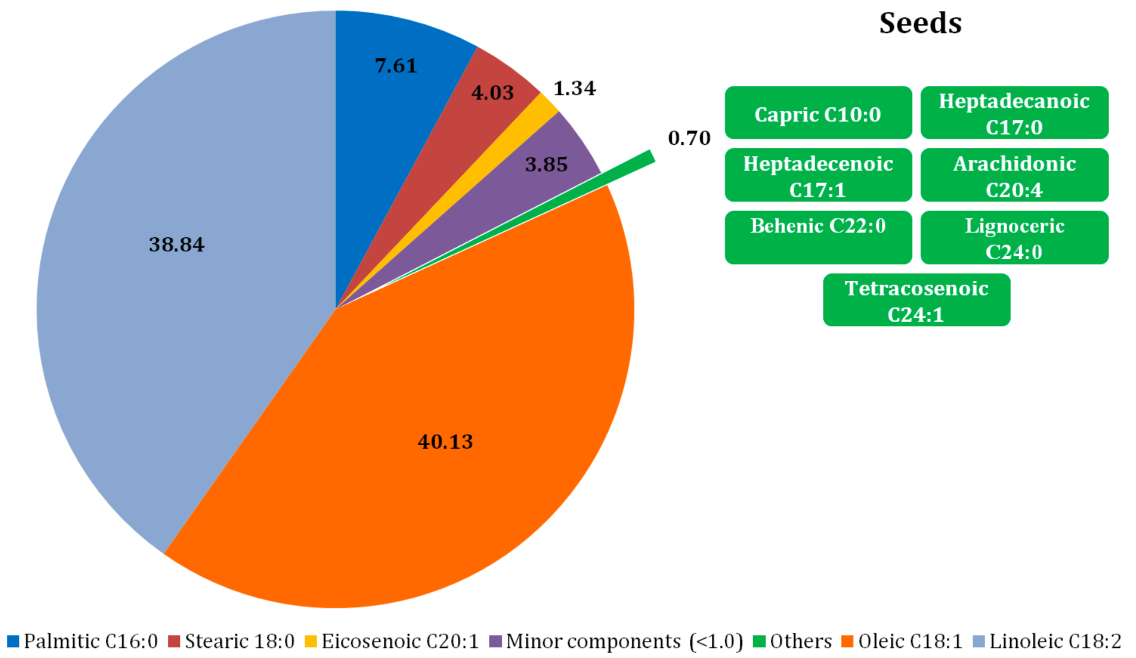

Figure 4 Fatty acid composition from seeds of *Asimina triloba* (L.) Dunal (g/100 g oil)
Minor components (<1.0): Caprylic C8:0 (0.32); Lauric C12:0 (0.30); Myristic C14:0 (0.26); Palmitoleic C16:1 (0.79); Linolenic C18:3 (0.80); Arachidic C20:0 (0.37); Erucic C22:1 (0.32); Docosadienoic C22:2 (0.69) are in the purple part, their total amount is 3.85 g/100 g oil

acid and linoleic acid in the seeds samples were 41.59 and 40.25 %, respectively (Figure 4). Palmitic acid and stearic acid were the minor fatty acids in leaves, accounting for 7.89 and 4.18 % of the total fatty acids, respectively. Unsaturated fatty acids were also predominant in seeds, which accounted for 86.23 % of total fatty acid while saturated fatty acids accounted for 13.77 %.

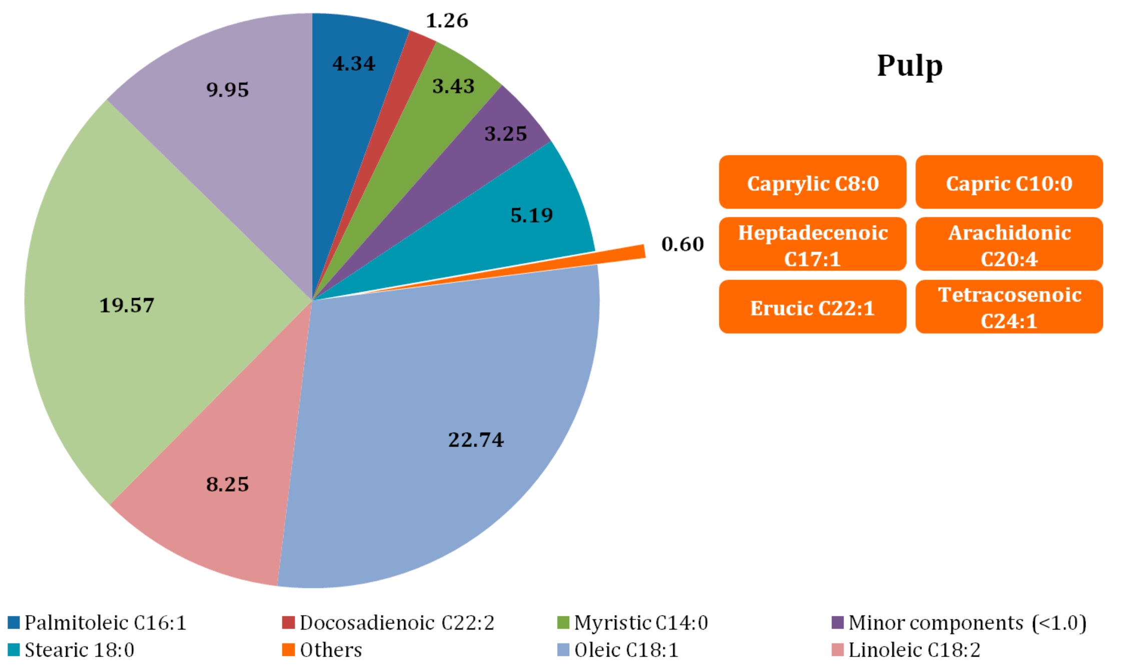

Figure 5 Fatty acid composition from pulp of *Asimina triloba* (L.) Dunal (g/100 g oil)
Minor components (<1.0): Lauric C12:0 (0.42); Heptadecanoic C17:0 (0.27); Arachidic C20:0 (0.30); Eicosenoic C20:1 (0.90); Behenic C22:0 (0.75); Lignoceric C24:0 (0.61) are in the right column: are in the right column, in the purple part, their total amount is 3.25 g/100 g oil

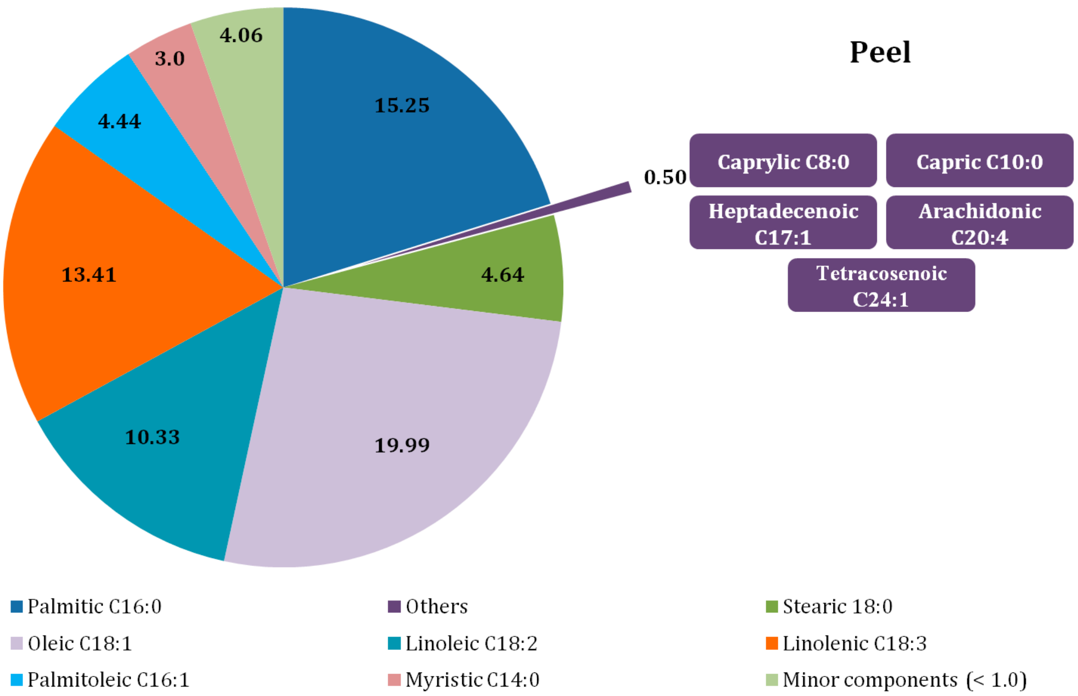

Figure 6 Fatty acid composition from peel of *Asimina triloba* (L.) Dunal (g/100 g oil)
Minor components (<1.0): Lauric C12:0 (0.39); Heptadecanoic C17:0 (0.24); Arachidic C20:0 (0.34); Eicosenoic C20:1 (0.30); Behenic C22:0 (0.98); Erucic C22:1 (0.13); Docosadienoic C22:2 (0.78); Lignoceric C24:0 (0.90) are in the right column, their total amount is 4.06 g/100 g oil

As reported by Nam et al. (2018a) the contents of oleic and linoleic acids contained in the seeds were 5905.11 and 8045.56 mg%, respectively, which was more than 120 times and 820 times the oleic and linoleic acids contained in the fruit, respectively.

Oleic acid and palmitic acid in pulp accounted for 28.66 and 24.67 % of total fatty acids, followed by linolenic acid and linoleic acid, accounting for 12.54 and 7.89 % of total fatty acids, respectively (Figure 5). Unsaturated fatty acids were also predominant in pulp, which accounted for 60.88 % of total fatty acid while saturated fatty acids accounted for 39.12 %.

In peel, oleic acid, palmitic acid, linolenic acid, and linoleic acid accounted for 26.23, 20.01, 17.59, and 13.55 % of total fatty acids, respectively (Figure 6). Stearic acid and palmitoleic acid were the minor fatty acids in the peel, accounting for 6.09 and 5.83 % of the total fatty acids, respectively. Unsaturated fatty acids were also predominant in the peel, which accounted for 65.70 % of total fatty acid while saturated fatty acids accounted for 34.30 %.

Oleic acid was present in the largest amount in the seeds, pulp, and peel. Oleic acid belongs to the omega-9 fatty acids. Omega-9 fatty acids (MUFAs) have one double bond per one molecule of a fatty acid. Oleic acid (18:1 n-9), as the major MUFAs, is the main characteristic of the Mediterranean Style Diet, which is high in olive oil (Rustan and Drevon, 2005). The monounsaturated fatty acids (MUFAs) at normal amounts in the diet do not affect blood cholesterol levels (Hegsted et al., 1993). When polyunsaturated fatty acids in the diet are replaced by monounsaturated fatty acids, such as oleic acid, they do not increase blood cholesterol levels (cholesterolemia). However, increased MUFAs content in the diet lower than LDL- and total cholesterol and increases HDL-cholesterol (Mattson and Grundy, 1985), and thus MUFAs reduce the risk of cardiovascular disease.

The palmitic acid, stearic acid, and myristic acid belong to the saturated fatty acids (SFAs). SFAs increase and are the primary determinants of serum cholesterol. The polyunsaturated fatty acids (PUFAs) actively lower serum cholesterol (Hegsted et al., 1993). Dietary omega-3 polyunsaturated fatty acids (ω-3 PUFAs), including alpha-linolenic acid, docosapentaenoic acid, and eicosapentaenoic acid, are most studied. The anti-inflammatory and hypotriglyceridemic effects of these fatty acids are well known, whereas pro-inflammatory properties have been recognized in their dietary counterparts, the ω-6 PUFAs (D'Angelo et al., 2020).

In order to comply with plant food safety requirements, the concentration of heavy metals in plants must be evaluated. Toxic elements including mercury, lead, and cadmium can be present in trace amounts. Major mineral elements such as iron, copper, zinc, and manganese should not exceed critical thresholds, as excessive concentrations are also toxic (Lekouch et al., 2001).

The average contents of the elements in the different parts of *A. triloba* are shown in Table 2. Macroelement and trace element concentrations in the leaf samples revealing the following trend: Ca > P > Mg > K > S > Fe > Zn > Al > Mn > Cu > Na > Se > Cr > Ni > Pb > As > Hg > Cd. These elements were also detected in seeds samples according to the following order: K > P > Ca > S > Mg > Fe > Zn > Cu > Na > Mn > Al > Ni > Se > As > Cr > Pb > Cd > Hg. In the pulp samples, the following concentrations were observed: K > P > S > Ca > Mg > Fe > Na, Cu > Al > Zn > Mn > As > Cr, Se, Ni > Pb > Cd > Hg. In the peel samples, the following result was obtained: K > Ca > P > S > Mg > Fe > Na > Cu > Al > Zn > As > Cr, Se, Ni > Mn > Pb > Cd > Hg.

For all of these elements, the required amount of the individual‘s daily intake (mg/day) has been determined (Anke et al., 2002). The average concentrations of Hg, As, and Pb analyzed in *A. triloba* were below the maximum allowable levels.

Table 2 shows that mineral elements are higher in the peel than in the other parts of *A. triloba*. K was the most abundant essential trace mineral element in different parts. There is more of this element in the peel (15487 tmg/kg) and pulp (12198.0 mg/kg) than in the seeds (3888.0 mg/kg). In the seeds, P, Ca and S were higher than in pulp and peel. Seeds and pulp contribute moderate trace elements because of their high water content. Nam et al. (2018a) report that seeds (289.17 mg% fresh weight) have more potassium content than fruits (239.36 mg% fresh weight).

The many reports proposed that the chemical composition content may differ according to several factors such as growing season, cultivars, and climatic conditions (Rohloff et al., 2005; Jang et al., 2011; Nwofia et al., 2012). Therefore, the present study suggests that the chemical composition content of *Asimina triloba* can depend on their different parts, as well as these other factors.

Our conclusion provides additional information to elucidate the medical functions and nutritional properties of *A. triloba*. The fruits of *A. triloba* can also be recommended for use as a potential functional food, nutraceutical, or dietary food supplement in processed form.

Table 2 Mineral composition of different parts of *Asimina triloba* (L.) Dunal (mg/kg)

Minerals	Seed ($\bar{x} \pm S_x$)	Pulp ($\bar{x} \pm S_x$)	Peel ($\bar{x} \pm S_x$)
P	1937.0 ±122	1046.0 ±75	831.0 ±48
K	3888.0 ±215	12198.0 ±334	15487.0 ±361
Ca	1368.0 ±96	450.0 ±28	837.0 ±31
S	1322.0 ±99	499.0 ±31	646.0 ±45
Fe	26.0 ±1.02	25.0 ±1.12	31.0 ±1.09
Mn	7.1 ±0.5	1.1 ±0.02	1.5 ±0.01
Mg	1021.0 ±65	396.0 ±35	573.0 ±47
Na	8.0 ±0.7	5.0 ±0.2	11.0 ±0.4
Al	1.1 ±0.01	4.4 ±0.2	3.7 ±0.2
Cr	<0.2	<0.2	<0.2
Cu	14.0 ±0.5	5.0 ±0.3	4.0 ±0.2
Zn	17.0 ±0.4	3.0 ±0.1	3.0 ±0.2
Se	0.25 ±0.05	<0.2	<0.2
As	<0.3	<0.3	<0.3
Cd	0.050 ±0.002	<0.01	<0.01
Ni	0.74 ±0.08	<0.2	<0.2
Hg	0.009 ±0.0001	0.007 ±0.0002	0.006 ±0.0001
Pb	<0.1	<0.1	<0.1

Note: $\bar{x}$ – arithmetic mean; S_x – standard error of the mean

Conclusion

Diverse amino acids, fatty acids, and mineral elements that are essential to human health were in *Asimina triloba*. The glutamic acid, oleic acid, linoleic acid, palmitic acid, K, P, Ca, and S were found in relatively high levels. High potassium content was found in all the plant parts of *A. triloba*. Considering these results, *A. triloba* fruits and seeds can contribute to the coverage of nutritional recommendations in human nutrition and some nutritional requirements in the human diet.

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Research Article

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Evaluation of antibacterial activity of the ethanolic extracts derived from leaves of *Coelogyne brachyptera* Rchb. f. (Orchidaceae)

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The present study was aimed to assess the antibacterial activity of ethanolic extract obtained from leaves of *Coelogyne brachyptera* Rchb. f. For the current study, a panel of organisms including *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) (mecA negative), *S. aureus* subsp. *aureus* Rosenbach (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* (Migula) Castellani and Chalmers (ATCC® 25922™), *E. coli* (Migula) Castellani and Chalmers (ATCC® 35218™), *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC® 27583™) were used. The antimicrobial susceptibility testing was done on Muller-Hinton agar by the disc diffusion method. The results of our study revealed the differential efficacy of ethanolic extract obtained from leaves of *C. brachyptera* on the test organisms. The ethanolic extract obtained from leaves of *C. brachyptera* revealed significant antibacterial activity against studied strains compared to control samples (96 % ethanol). A statistically significant increase ($p < 0.05$) in the inhibition zone diameters of strain growth was 47 % for *S. aureus* subsp. *aureus* ATCC® 25923™, 40 % for *S. aureus* subsp. *aureus* ATCC® 29213™, and 44 % for *S. aureus* NCTC 12493. A non-significantly increase in inhibition zone diameters of *E. coli* strains' growth was also observed. The plant can be a source material to the herbal drug industry since it has some important antimicrobial components in the extracts that can be used for the development of therapeutic phytomedicine and phytoveterinary.

Keywords: orchids, ethanolic leaf extract, antibacterial activity, disc diffusion technique, *Staphylococcus aureus* subsp. *aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*

Introduction

Orchidaceae is one of the largest and more diverse families of flowering plants with approximately 25,000 species in 736 genera currently recognized (Chase et al., 2015). Orchids widely distributed as epiphytes, lithophytes, or terrestrials, have been used all over the world in traditional healing and treatment systems of several diseases (Kong et al., 2003; Pant, 2013). It was found that medicinal orchids mainly are encompassed by the next genera: *Anoectochilus* Blume, *Bulbophyllum* Thouars, *Calanthe* R. Br., *Coelogyne* Lindl., *Cymbidium* Sw., *Cypripedium* L., *Dendrobium* Sw., Eria Lindl., *Galeola* Lour., *Gastrodia* R. Br., *Gymnadenia* R. Br., *Habenaria* Willd., *Ludisia* A. Rich., *Luisia* Gaudich., *Nervilia* Comm. ex Gaudich., and *Thunia* Rchb. f. (Szlachetko, 2001; Kovaćs et al., 2008; Pant, 2013). Orchids have been reported to possess useful therapeutic activities like antitumor, hypoglycaemic, antimicrobial, immunomodulatory, hepatoprotective, antioxidant, and neuroprotective activities (Prasad and Koch, 2014; Biswas et al., 2016; Bhatnagar and Ghosal, 2018). It is believed that these pharmaceutical properties are due

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to the activities of many phytochemicals, including alkaloids, flavonoids, phenanthrenes, terpenoids, steroids, and their derivatives, which are present in various parts of orchid plants (Zhang et al., 2015).

In recent years, the assessment of antibacterial properties of orchids has received considerable attention (Singh et al., 2012; Tkachenko et al., 2015, 2018; Buyun et al., 2016, 2017, 2018; Soumiya and Christudhas Williams, 2017). Some orchid species are used as potent inhibitors against Gram-positive and Gram-negative bacteria and also proved to be a potent antimicrobial agent (Singh et al., 2012).

The family Orchidaceae is not only one of the most numerous, ecologically, and morphologically diverse families of flowering plants, but also one of the most endangered plant taxa (Zhang et al., 2015). Orchids are widely and illegally harvested from the wild for local, regional, and international trade as ornamental and medicinal plants. The demand for medicinal orchids is drastically increasing since the international trade of medicinal plants is becoming a major force in the global economy (Hinsley et al., 2017). However, the natural source of these plants has been significantly reduced due to indiscriminate collection, global climate changes, the specificity of life-history strategies, including specialized pollination syndromes, and association with mycorrhizal fungi (Gravendeel et al., 2004). Therefore, to conserve orchid plants in the wild and to meet the demand for medicinal plant material, assessment of biological activity of plants maintained under glasshouse conditions and developing new biotechnologies for plant reproduction *in vitro* are urgently needed.

Thus, although the antimicrobial activity of many orchid species, including *Coelogyne* species, has been effectively established against a wide spectrum of microorganisms (Majumder et al., 1995, 2001, 2011; Kovács et al., 2008; Chen et al., 2018), bacterial drug resistance continues to be a worldwide public health issue in the treatment of infectious diseases, thereby stimulating the search for new alternatives with fewer side effects (Mambe et al., 2019).

Previously, we have given considerable attention to the 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 the antifungal potential of orchids 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 fungus strain, *Candida albicans* was conducted by Buyun et al. (2018). Marked antifungal efficacy was observed in the 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 profound inhibitory activity against test fungus (mean diameter of inhibition zones ranging from 16 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 to 23.5 mm). Moreover, it has been observed that ethanolic extract from pseudobulbs of *C. speciosa* revealed the highest antibacterial activity (21 mm as the diameter of the 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).

The present study was aimed to determine the antibacterial activity of *Coelogyne brachyptera* Rchb. f. against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains, clinically important bacteria, which are indicator organisms commonly used in various projects to monitor antibiotic resistance (Roser et al., 2016).

Given that standardization and quality control are essential analytical steps to assure the correct identification of plant raw materials to be used as plant-derived medicines, the micromorphological investigation of *Coelogyne brachyptera* leaf has been undertaken using light microscopy. The need for constant incorporation of leaf micromorphology in pharmacological investigations has been emphasized in some recent papers (Bilić et al., 2019; Khan et al., 2020). Additionally, this investigation was conducted as part of a conservation research program focusing on preventing the extinction of rare and endangered orchid species.

Material and methodology

Collection of plant material

The leaves of *C. brachyptera* plants cultivated under glasshouse conditions were sampled at M.M. Gryshko National Botanical Garden (NBG, 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, the NBG 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).

Various databases are available for searching collections of living plants, confirming the taxonomic identity of having been reviewed, e.g. World Checklist of Orchidaceae (Govaerts et al., 2016), International Plant Names Index, The Plant List, the IUCN Red List (IUCN, 2013).

Coelogyne brachyptera is found in Burma, Thailand, Cambodia, Laos, and Vietnam. It grows epiphytically in the primary mountain forest, the most frequent at an altitude of 1000 to 2500 meters above sea level (Averyanov et al., 2003). It is a sympodial orchid with pseudobulbs of one internode, narrowly conical, 4-angled, slightly grooved, pale green, carrying 2 leaves. The leaves are elliptic to elliptic-lanceolate, subacute, plicate, 7-nerved, with an undulate margin. The flowering of *C. brachyptera* under glasshouse condition at NBG was observed in March – April (Figure 1). The duration of anthesis of a single inflorescence did not exceed 2 weeks.

Figure 1 Vegetative shoot with inflorescence of *Coelogyne brachyptera* Rchb. f. plant, cultivated at NBG's glasshouses (Kyiv, Ukraine)

Preparation of plant extracts

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

Bacterial test strain and growth conditions

For this study, a panel of organisms including *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) (mecA negative), *S. aureus* subsp. *aureus* Rosenbach (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* (Migula) Castellani and Chalmers (ATCC® 25922™), *E. coli* (Migula) Castellani and Chalmers (ATCC® 35218™), *Pseudomonas aeruginosa* (Schroeter) Migula (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 hr 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 (CLSI, Performance Standards for Antimicrobial Susceptibility Testing, 2014).

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 (10^8 CFU/mL) of the bacterium and spread with sterile swabs (Bauer et al., 1966).

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.

LM investigations of leaf surface micromorphology

For the micromorphological investigation of the leaf surface epidermal cells, Clarke's method of making impressions technique of the leaf surface has been used (Clarke, 1960). To produce the leaf epidermal impressions (imprints or replicas) the clear nail polish was applied on both surfaces of *C. brachyptera* leaf and allowed to dry for several minutes. After then the thin film was gently peeled from the leaf surface using transparent tape and the peel subsequently was mounted in water. Stomatal type, density, size were determined using nail polish imprints taken from both leaf surfaces.

For viewing epidermal features imprints light microscope Primo Star (Carl Zeiss, Jena, Germany) has been employed. The light microscopic images were captured with a digital camera Canon PowerShot A640. For measuring the dimension of epidermal cells the AxioVs40 V 4.8.2.0 software has been used (Carl Zeiss, Jena, Germany).

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 the extract 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, v. 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

In Figure 2, we can see that the adaxial epidermis in *C. brachyptera* leaf is composed of mostly hexagonal cells, although polygonal or isodiametric cells have been observed. The number of epidermal cells on the adaxial leaf surface varied from 160 to 224 (198.30 ±6.12) per 1 mm^2; cells are from 51.18 to 158.10 (93.44 ±1.62) µm in length and from 40.31 to 94.35 (70.32 ±0.90) µm in width.

The cells of the abaxial epidermis of the leaves are mostly rectangular or irregularly shaped; walls are straight or curved. Angles within adjacent boundaries are straight or pointed. The number of epidermal cells on the abaxial leaf surface varied from 208 to 256 (226.33 ±4.93) per 1 mm^2; cells from 50.28 to 134.15 (88.27 ±2.21) µm in length and from 32.95 to 80.58 (59.43 ±0.72) µm in width. The density of trichomes on both surfaces varied within the range of 2–4 per 1 mm^2.

Stomata were located only on the lower leaf surface (hypostomatous leaves). The stomatal complex is recognizable allowing us to determine the stomatal types based on nail polish imprints. The most common stomatal type was tetracytic, occasionally anomocytic stomata occurred with 5 or 6 subsidiary cells. The stomata are rounded, usually scattered or distributed in small groups of 2–3 stomata. Subsidiary cells are located parallel to the guard cells and clearly differ from the main epidermal cells of the leaves both in shape and size.

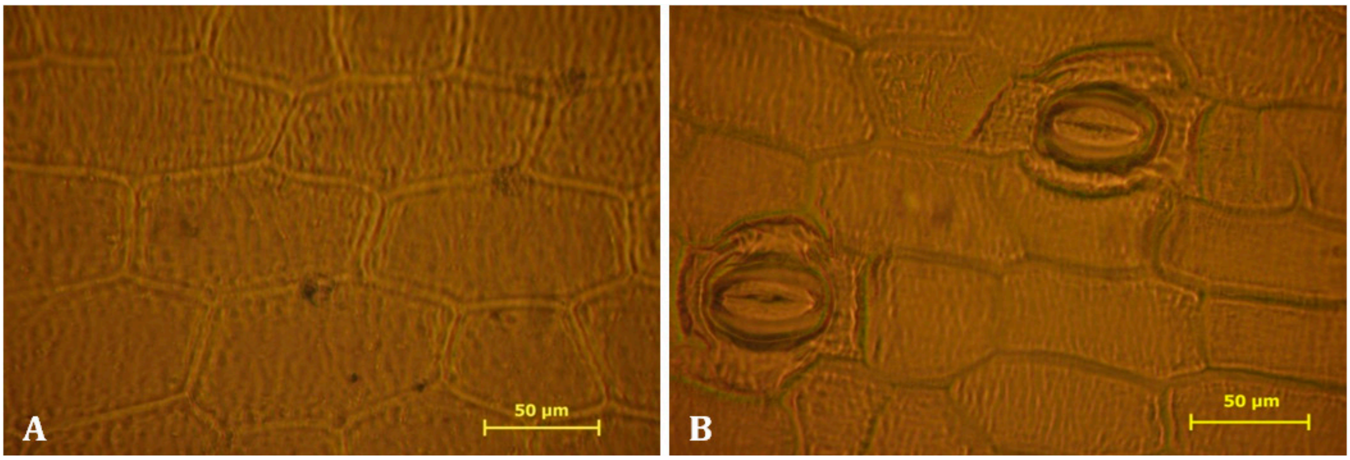

Figure 2 Adaxial (A) and abaxial (B) epidermal cells with stomata on *Coelogyne brachyptera* Rchb. f. leaf observed in nail polish imprints

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Stomata density varied from 10 mm^{-2} to 15 mm^{-2} (12.77 ±0.59). Stomatal size (guard cell length) ranged within range 47.55–68.19 µm (58.54 ±0.61 µm).

The ethanolic extract obtained from leaves of *C. brachyptera* resulted in considerable suppression of *Staphylococcus aureus* strains' growth. Moreover, the differential efficacy of ethanolic extract obtained from leaves of *C. brachyptera* on the test organisms was noted. Consequently, the extract displayed intermediate antibacterial potency against *S. aureus*, i.e. the mean of inhibition zone diameters was (12.45 ±1.18) mm, (12.51 ±0.99) mm, and (12.87 ±1.16) mm for *S. aureus* subsp. *aureus* (ATCC® 25923™), *S. aureus* subsp. *aureus* (ATCC® 29213™), and *S. aureus* NCTC 12493, respectively. On the other hand, *E. coli* exhibited lower susceptibility for the impact of the ethanolic extract obtained from leaves of *C. brachyptera*. The mean of inhibition zone diameters was (12.11 ±1.02) mm and (10.40 ±0.95) mm for *E. coli* (ATCC® 25922™) and *E. coli* (ATCC® 35218™), respectively. *P. aeruginosa* (ATCC® 27583™) strain was the most resistant to the impact of the ethanolic extract obtained from leaves of *C. brachyptera* with the mean of inhibition zone diameter (9.53 ±0.95) mm (Figure 3, 4).

Moreover, the ethanolic extract obtained from leaves of *C. brachyptera* revealed significant antibacterial activity against studied strains compared to control samples (96 % ethanol). A statistically significant increase ($p < 0.05$) in inhibition zone diameters of strain growth was 47 % (for *S. aureus* subsp. *aureus* ATCC® 25923™), 40 % (for *S. aureus* subsp. *aureus* ATCC® 29213™), and 44 % (for *S. aureus* NCTC 12493) (Figure 3, 4). A non-significantly increase ($p > 0.05$) in inhibition zone diameters of *E. coli* strains' growth was also observed (by 27 % for *E. coli* ATCC® 25922™ and by 31 % for *E. coli* ATCC® 35218™, respectively).

The present study has revealed that ethanolic extract derived from the leaves *Coelogyne brachyptera* exhibited intermediated antibacterial activity against different Gram-positive and Gram-negative strains studied (inhibition zone diameter were ranged from 8.5 to 15.5 mm) (Figure 3 and 4). Moreover, it has been observed that ethanolic extract obtained from the leaves *Coelogyne brachyptera* revealed the highest antibacterial activity against *S. aureus* strains (11.0–15.5 mm as the diameter of inhibition zone) compared to *E. coli* and *P. aeruginosa* strains (Figure 3 and 4).

In our previous study (Buyun et al., 2017), we have studied the antibacterial effects of the ethanolic extract obtained from *C. brachyptera* leaves against specific Gram-positive (*Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S. aureus* locally isolated) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, metallo-β-lactamases (MβL)-

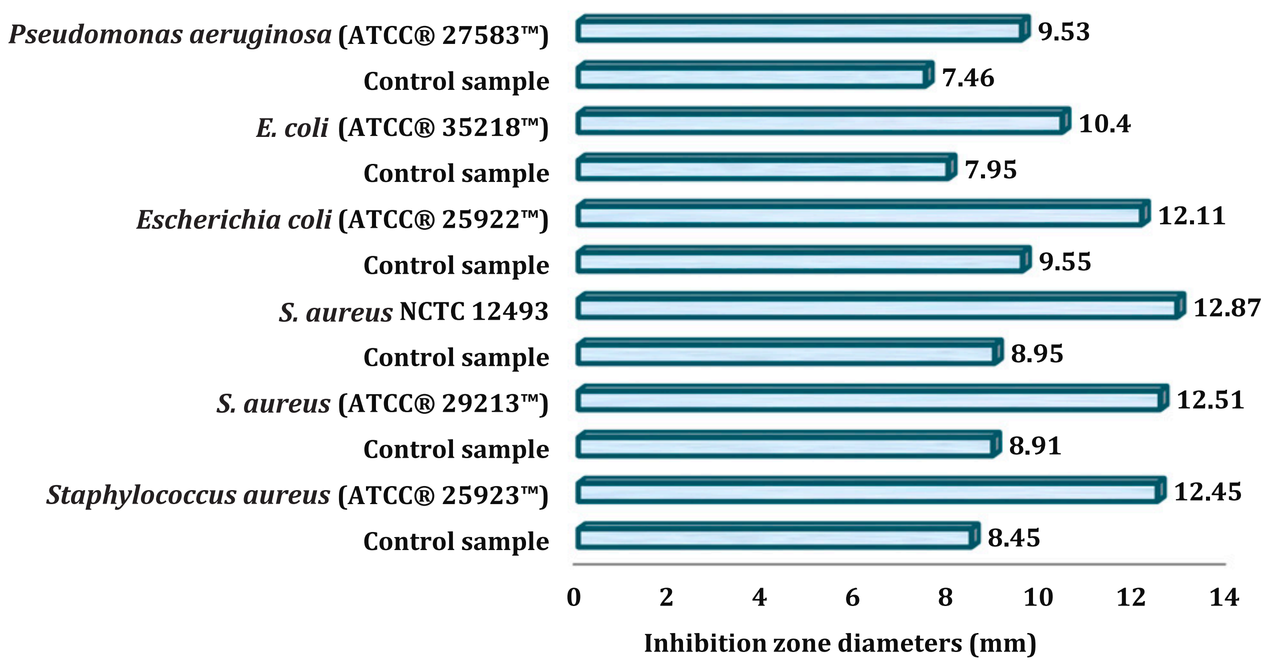

Figure 3 The mean of inhibition zone diameters achieved by an impact of the ethanolic extract obtained from leaves of *Coelogyne brachyptera* concerning *S. aureus* subsp. *aureus* (ATCC® 25923™), *S. aureus* subsp. *aureus* (ATCC® 29213™), and *S. aureus* NCTC 12493, *E. coli* ATCC® 25922™, *E. coli* ATCC® 35218™, and *P. aeruginosa* ATCC® 27583™ (n = 8)

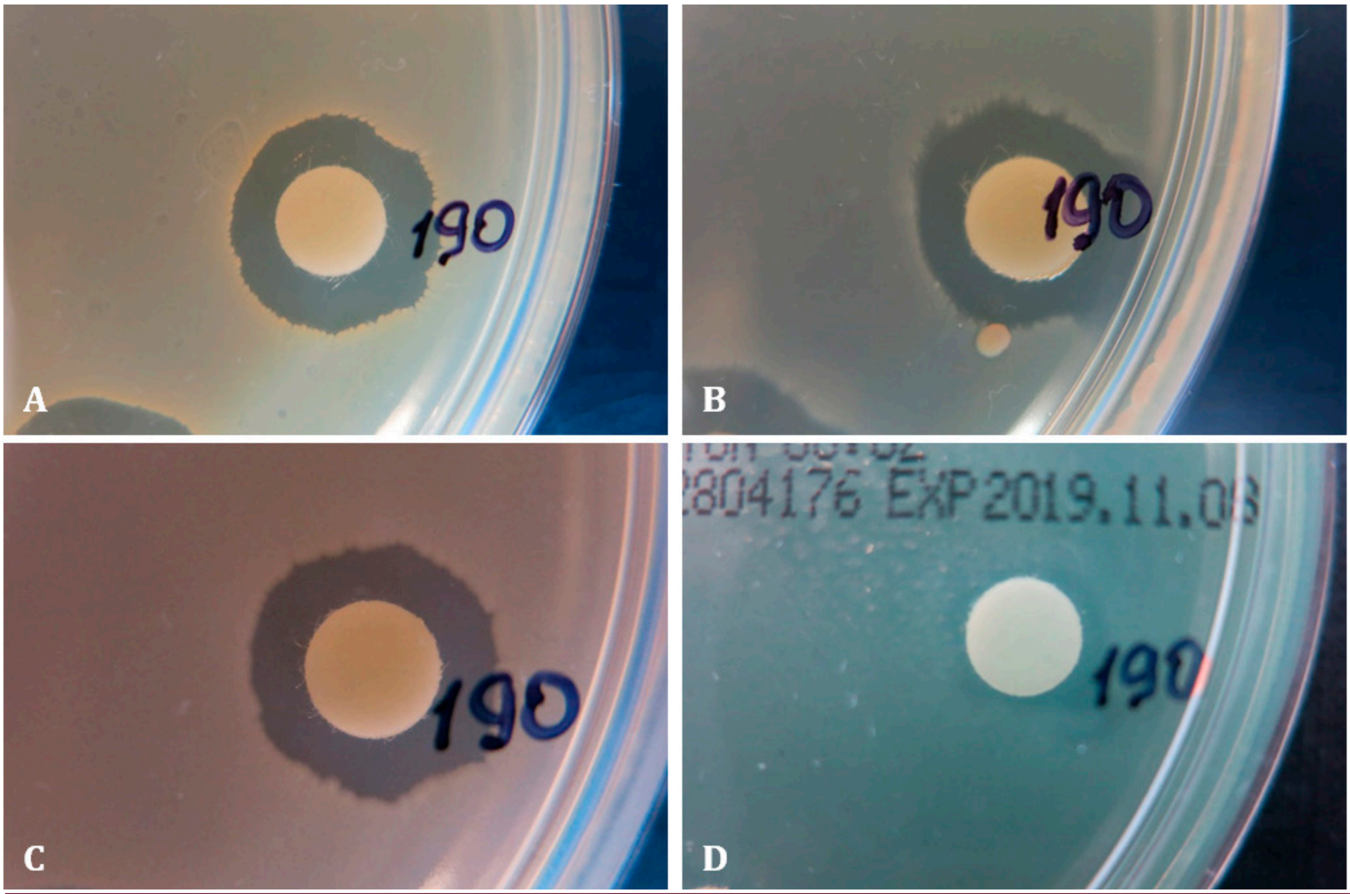

Figure 4 Examples of a disc diffusion assay plate showing the halos in the bacterial lawn resulting from the antibacterial activity of extract obtained from *C. brachyptera* leaves against *S. aureus* subsp. *aureus* ATCC® 29213™ (A), *S. aureus* NCTC 12493 (B), *E. coli* ATCC® 25922™ (C), and *P. aeruginosa* ATCC® 27583™ (D) (Photo: O. Gyrenko)

positive *Pseudomonas aeruginosa* locally isolated, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* locally isolated). Our results showed that the ethanolic extract of *C. brachyptera* leaves has displayed a strong inhibitory effect against the Gram-positive bacterial strains (20 mm diameter of inhibition zone for *S. aureus* and 26.5 mm for methicillin-resistant *S. aureus*), and moderate activity against Gram-negative bacteria (18.2 mm for *E. coli*, 16.5 mm for *P. aeruginosa* and 18.3 mm for (MβL+) *P. aeruginosa*, and 14.8 mm for *S. enteritidis*). Gram-positive strains (*S. aureus* and methicillin-resistant *S. aureus*) were more susceptible to the ethanolic leaf extracts of *C. brachyptera* as compared to Gram-negative bacteria (Buyun et al., 2017).

Literature data also suggest that some members of the orchid family are used as a potent inhibitor against Gram-positive and Gram-negative bacteria and also proved to be a potent antimicrobial agent. For example, Naganandaand Satishchandra (2013) have evaluated the antibacterial and antifungal activity of *Dendrobium nodosum* Dalzell (syn. *Flickingeria nodosa* (Dalzell) 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 the 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 *Trichophyton mentagrophytes* (Nagananda and Satishchandra, 2013).

Chemical analyses conducted by Majumder et al. (1995, 2001), revealed the presence of two phenanthrene derivatives in pseudobulbs of *C. cristata*: *coeloginanthridin* and *coeloginanthrin*. Phenanthrenes are the prototypical opioids that are presumably formed by oxidative coupling of the aromatic rings of stilbene precursors and possess several biological activities (Kovács et al., 2008). Phenanthrenes have been studied for cytotoxicity, antimicrobial, spasmolytic, anti-inflammatory, anti-platelet aggression, anti-allergic, immunomodulatory, anticancer, anti-aging, atherosclerosis properties (Majumder et al., 2001; Kovaćs et al., 2008; Chen et al., 2018). The anti-stress

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and antioxidant activity of similar herbs from the Orchidaceae family have also been reported (Habbu et al., 2012; Sing et al., 2012; Mishra et al., 2018).

Moreover, further investigation afforded two new stilbenoids, designated *coeloginone* and *coeloginanthrone* (Majumder et al., 2011). Stilbenoids are the major secondary metabolites reported in some orchids based on previous phytochemical studies, e.g. in *Arundina graminifolia* (D. Don) Hochr. (Auberon et al., 2016). These metabolites are also known to display a wide range of biological activities such as antioxidant, antiviral, cytotoxic, and antitumoral properties (Chen and Chen, 2005; Prasad and Koch, 2014; Biswas et al., 2016; Bungtongdee et al., 2018; Mishra et al., 2018).

Mishra et al. (2018) have investigated the antioxidant and antibacterial activities of 5 different extracts and derived fractions from the tubers of *Satyrium nepalense* D. Don, a high altitude medicinal orchid of the Indian Himalayan region. Identification of the most active fractions, phytochemical characterization, total phenolic, and flavonoid contents, and biological activities were also evaluated. Petroleum ether, chloroform, ethyl acetate, methanol, water extracts, and methanol fractions were screened for their antibacterial activity at various doses (10, 50, and 100 mg/mL) against ten Gram-negative and Gram-positive bacterial strains by disc diffusion method. Methanol extract exhibited the highest antioxidant and antibacterial activities in comparison with the other extracts. Levels of phenolics and flavonoids were also the highest in the same extract. Phytochemical investigation of the active fractions of the methanol extract led to the isolation of gallic acid (19.04 mg/g) and quercetin (23.4 mg/g). Therefore, methanol extract showed interesting potential for both antioxidant and antibacterial activities (Mishra et al., 2018).

Three orchids namely, *Rhynchostylis retusa* (L.) Blume, *Tropidia curculigoides* Lindl., and *Satyrium nepalense*, traditionally used in tuberculosis, asthma, and cold stage of malaria in folk medicine, were studied by Bhatnagar et al. (2017). The most significant antimycobacterial activity was observed with the n-hexane fraction of the flower of *Satyrium nepalense* with a minimum inhibitory concentration (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. The investigation of Bhatnagar et al. (2017) resulted in the identification of *S. nepalense* as the most promising plant, which possessed all three activities in a significant proportion.

Recently it has been shown that some plant-derived bioactive compounds produced by fungal endophytes of orchids have been proven to possess antimicrobial and antioxidant activities (Vaz et al., 2009; Jiang et al., 2015; Bungtongdee et al., 2018). For example, Vaz et al. (2009) have examined the antimicrobial activity of endophytic fungi isolated from the leaves, stems, and roots of 54 species of Orchidaceae collected in a Brazilian tropical ecosystem. In total, 382 filamentous fungi and 13 yeast isolates were obtained and cultured to examine the production of crude extracts. Thirty-three percent of the isolates displayed antimicrobial activity against at least one target microorganism. Furthermore, the endophytic fungi isolated from different *Dendrobium* species could be of the potential antibacterial or antifungal resources (Xing et al., 2011). Thus, 10 endophytic fungi in *Dendrobium devonianum* Paxton and 11 in *D. thyrsiflorum* B.S. Williams exhibited antimicrobial activity against at least one pathogenic bacterium or fungus among 6 pathogenic microbes (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*). Out of the fungal endophytes isolated from *D. devonianum* and *D. thyrsiflorum*, *Phoma* displayed strong inhibitory activity (inhibition zones in diameter >20 mm) against pathogens. *Epicoccum nigrum* from *D. thyrsiflorum* exhibited antibacterial activity even stronger than ampicillin sodium. Fusarium isolated from the two *Dendrobium* species was effective against the pathogenic bacterial as well as fungal pathogens (Xing et al., 2011).

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 previous studies (Buyun et al., 2016, 2017) The ethanolic extracts from leaves and pseudobulbs of *C. speciosa* exhibited strong activity against *S. aureus* (inhibition zone diameters were 21.5 mm and 19 mm, respectively), while the methanolic extract from leaves and pseudobulbs revealed mild activity (8.1 and 8 mm). Moreover, it has been observed that ethyl acetate, hexane, and dichloromethane extracts obtained from leaves and pseudobulbs of *C. speciosa* revealed 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), whereas other extracts from pseudobulbs revealed minimum activity (inhibition zone diameter was 12 mm) (Buyun et al., 2016, 2017). The ethanolic extracts obtained from leaves and pseudobulbs of five *Coelogyne* spp. were found to exhibit fairly strong antibacterial activity towards *Enterobacter cloacae* strain used, the diameter of inhibition zones varied within 8.0–25.5 mm (Buyun et al., 2019).

Leaf morphological traits play an important role in maintaining water balances in epiphytes (Zhang et al., 2015). It was evidenced, that the patterns of stomatal type in the studied group might represent a useful diagnostic characteristic. Data on the characteristics of the leaf surface will provide important information for the standardization of raw materials, and on the other hand is taxonomically important for the identification of samples (Song et al., 2020).

Micromorphological characteristics of leaves have been applied in systematic studies for different taxonomic groups. Recently, leaf micromorphology using microscopic analysis has also been used to facilitate accurate authentication and quality control of medicinal plants (Song et al., 2020), including orchids species such as *Dendrobium huoshanense* (Zhang et al., 2017). The results presented in the paper are potentially useful for advancing research on the assessment of medicinal properties, propagation, and conservation of *Coelogyne brachiptera*, as well as other *Coelogyne* species, under *ex situ* conservation.

Conclusions

We continued our investigations concerning the determination of the antibacterial activity of ethanolic extracts obtained from leaves and pseudobulbs of various plants belonging to the *Coelogyne* genus. In the current study, we aimed to determine the antibacterial activity of *Coelogyne brachyptera* against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains. The results of our study revealed the differential efficacy of ethanolic extract obtained from leaves of *C. brachyptera* on the test organisms. The extract displayed intermediate antibacterial potency against *S. aureus*. On the other hand, *E. coli* exhibited lower susceptibility for the impact of the ethanolic extract obtained from leaves of *C. brachyptera*. *P. aeruginosa* (ATCC®27583™) strain was the most resistant to the impact of the ethanolic extract obtained from leaves of *C. brachyptera*. Nevertheless, there is still room for an in-depth investigation, to make these plants best use in medicine and veterinary and to select them as an alternative to bacterial resistance. The promising results on medicinal plants screening for antibacterial activity could be considered as primary information for further phytochemical and pharmacological studies. In particular, the next step in our further investigation will be HPLC-profiling of the plant extract to find new bioactive compounds from a natural source.

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Antioxidant activity of extracts of wild *Humulus lupulus* L.

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Plants from natural flora (wild-growing) as well as cultural plants demonstrated numerous useful properties that play an important role in human life. They often can be used as medicinal, forage, food, etc. Searching for new sources of valuable capacities of wild plants still is actual. *Humulus lupulus* L. (hop) is well-known species from the small family Cannabaceae that is used as a bittering agent in the brewing industry and as a medicinal plant. *H. lupulus* raw is a valuable source of biologically active compounds and demonstrated different biological activities. This study was aimed to determine the antioxidant activity and accumulation of polyphenol compounds in raw wild plants of *H. lupulus*. Raw (leaves, stems, and female flowers) collected from the natural flora of M.M. Gryshko National Botanical Garden of the NAS of Ukraine. In ethanol extracts of leaves, flowers and stems determined 54.13, 44.69 and 23.76 mg GAE/g (gallic acid equivalent) of polyphenol content (TPC), respectively; 7.24, 4.92, 2.56 mg CAE/g (caffeic acid equivalent) of phenolic acids (TPAC), respectively; 45.48, 29.64 and 18.31 mg QE/g (quercetin equivalent) of flavonoid content (TFC), respectively. Molybdenum reducing power of leaf, flower, and stem extracts was 168.17, 236.45, and 97.57 mg TE/g (Trolox equivalent), respectively; antioxidant activity by DPPH method 8.64, 8.02, and 7.97 mg TE/g, respectively. This study showed that the highest values of polyphenol compounds found in the leaves and the lowest in the stem extracts. The strongest correlation found between TPAC and TFC (r = 0.995), TPC and TPAC (r = 0.978), TPC and TFC (r = 0.952), TFC and DPPH (r = 0.936). This investigation showed the high antioxidant potential of the wild *H. lupulus* plant that can be used in the further pharmacological study.

Keywords: *Humulus lupulus*, hop, polyphenol compounds, antioxidant activity, correlation

Introduction

Genus *Humulus* L. belongs to Cannabaceae Martynov and includes *Humulus lupulus* L., *H. scandens* (Lour.) Merr. and *H. yunnanensis* Hu. Species from this genus originated from China, were spread to America and Europe (Small, 1978; Small, 1980).

H. lupulus (hop) is a dioecious perennial plant, which regrows every spring from rhizomes of an underground rootstock. It is vine producing stems annual, slender, climbing, growing up to 6–9 m in length. The leaves are dark green colored, long petiolate, heart-shaped with 3–5 lobs, sharply toothed and they have a very rough surface. The male flowers are long racemes, 7.5–12.5 cm long, while the female inflorescences are cone-like catkins (strobiles), 2.5–5.0 cm long, made up of overlapping membranaceous bracts (Zanoli and Zavatti, 2008).

H. lupulus have been used for different medical purposes such as sleep disturbances, insomnia anxiety, excitability, for treating acne, dysmenorrhea, and amenorrhea (Arsene et al., 2015). Specific organoleptic properties of this plant allow to use in the beer industry

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(Zanoli and Zavatti, 2008). The genetic diversity of this plant an important direction in selection work in Ukraine (Melnychuk et al., 2008) and abroad (Mafakheri et al., 2020). This species primarily used for medicinal purposes than for beer brewing (Lin et al., 2019).

Different characters such as agronomic traits, diseases and pests resistance, chemical composition have been used for hop improvements (Skomra et al., 2013).

Cons (Liu et al., 2007; Karabín et al., 2016; Kobus-Cisowska et al., 2019) and seeds of hop (Alonso-Esteban et al., 2019) exhibited the antioxidant activity. An antimicrobial activity showed extracts of female inflorescences (Arsene et al., 2015; Bocquet et al., 2019) and seeds (Alonso-Esteban, 2019). Extracts also demonstrated the insecticidal, antiviral (Sotto et al., 2018), antitumor, antiproliferative, anticancer (Van Cleemput et al., 2009), antimutagenic (Wang et al., 2014), anti-inflammatory (Bohr et al., 2005), and detoxication activity. Insecticidal effects of cons extracts of *H. lupulus* caused by xanthohumol, which can act against storage insects (Aydin et al., 2017).

In methanol extracts of *H. lupulus* identified prenylated chalcones, prenylflavanones, 4-hydroxybenzaldehyde, sistosterol-3-O-β-glucopyranoside, humulinone, cohumulinone (Chadwick et al., 2004). The functional components of *H. lupulus* dried cons are α-acids (2–7 %), β-acids (2–10 %), essential oils (0.5–3.0 %), polyphenols (3–6 %), amino acids (0.1 %), protein (15 %) (Lin et al., 2019). Content α- and β-acids increased during vegetation (Kavalier et al., 2011). Among phenolic compounds identified flavan-3-ols, procyanidins, phenolic acids (Kavalier et al., 2011), catechine (13.7 mg/g), epicatechin (3.9 mg/g) (Alonso-Esteban et al., 2019), gallic acid, vanillic acid, caffeic acid, syringic acid, rutin, luteolin, *t*-cinnamic acid, etc. (Keskin et al., 2019). The study of ten hop strains with different content of α-acids showed that the boiling procedure increased the content of polyphenol compounds and total antioxidant activity (Elrod et al., 2019). The main phenolic compounds present in extracts of *H. lupulus* were flavonoids isoquercitrin and quercetin (Almeida et al., 2019).

This study was aimed to determine the antioxidant activity of ethanol extracts of different parts of wild *H. lupulus* plants as a potential source of polyphenol compounds.

Material and methodology

The plants were grown in 2017 at the experimental fields of the M.M. Gryshko National Botanical Garden of the NAS of Ukraine in the Kyiv city (50°24'55"N, 30°33'45"E).

Biological material

Plant raw material collected from natural flora of M. M. Gryshko National Botanical Garden of the NAS of Ukraine at the stage of flowering. The leaves, stems, and female inflorescences dried in a ventilated dryer at 60 °C, according to Almaguer et al. (2014).

Biochemical analysis

The biochemical analysis was done at the Slovak University of Agriculture in Nitra (Slovak Republic). For planned analyses, 0.2 g of milling fraction was extracted with 20 mL of 80 % ethanol for 24 hours. After centrifugation at 4000 g with Rotofix 32 A (Hettich, Germany) for 20 min, the supernatant was used for measurement of the total content of polyphenols.

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), and CentralChem (Slovakia).

Phytochemical analyses

Total polyphenol content (TPC)

Total polyphenol content extracts were measured by the method of Singleton and Rossi, (1965) using Folin-Chiocalteu reagent. 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Chiocalteu 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 using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25–250 mg/L; R^2 = 0.996) was used as the standard and the results were expressed in mg/g gallic acid equivalents.

Total flavonoid content (TFC)

Determination of total flavonoid content was conducted according to a procedure which was described by Shafii et al. (2017). 0.5 mL of sample extract was mixed with 0.1 mL of 10 % (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M sodium 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 (0.01–0.5 mg/l; R^2 = 0.997) was used as the standard and the results were expressed in μg/g quercetin equivalents.

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Total phenolic acid content (TPAC)

Determination of total phenolic acid content of extracts was carried out using a method of Farmakopea Polska (1999). 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent, 0.5 mL of 1 M sodium hydroxide (w/v), and 0.5 mL of distilled 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 caffeic acid equivalents.

DPPH radical scavenging assay (DPPH)

The radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sánchéz-Moreno et al., 1998). The extracts (0.5 mL) were mixed with 3.6 mL of radical solution (0.025 g of DPPH in 100 mL ethanol). The absorbance of the sample extract was determined using 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 = 0.988) was used as the standard and the results were expressed in mg/g Trolox equivalents.

Molybdenum reducing power of extracts (MRP)

The reducing power of extracts was determined by the phosphomolybdenum 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) incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10–1000 mg/L; R^2 = 0.998) was used as the standard and the results were expressed in mg/g Trolox equivalents.

Statistical analysis

Basic statistical analyses were performed using PAST 2.17. Data were analyzed with ANOVA test and differences between means compared through the Tukey-Kramer test ($p < 0.05$). The variability of all these parameters was evaluated using descriptive statistics.

Results and discussion

Wild plants had importance in human history from old times and use as a potential source of biologically active compounds and individual components. They are used not as medicinal plants only but as food and forage also (Pinela et al., 2017). Last year's reports showed that searching for natural plant antioxidants has some advantages along with obtaining synthesized antioxidants (Antal, 2010).

The most common polyphenol compounds from *H. lupulus* are catechins, phenolic acids, flavonol glycosides, and procyanidins (Kavalier et al., 2011).

The content of polyphenol compounds at the flowering stage in *H. lupulus* ethanol extracts was from 23.76 to 54.13 mg GAE/g depending on the plant part (Figure 1). As shown from Figure 1, the maximal level of TPC determined in leaves, minimal in stems.

Inflorescence extracts showed 151.42 mg GAE/g of TPC that was 3.3 times more than in our experiment (Arsene et al., 2015). Research of *H. lupulus* cons found 3.5 mg/100 g of caffeic acid equivalent and 4.8 mg/100 g of the chlorogenic acid equivalent of TPC (Bubueanu et al., 2015). Almeida et al. (2019) found that TPC in extracts of *H. lupulus* was from 27.31 to 33.93 mg GAE/g. According to Sotto et al. (2018), the TPC of inflorescence extracts was 7.1 µg/g of tannic acid equivalent. As reported Keskin et al. (2019), methanol extracts of cons contained 7.12 mg GAE/g and leaf's 6.86 mg GAE/g of TPC. Rheay et al.

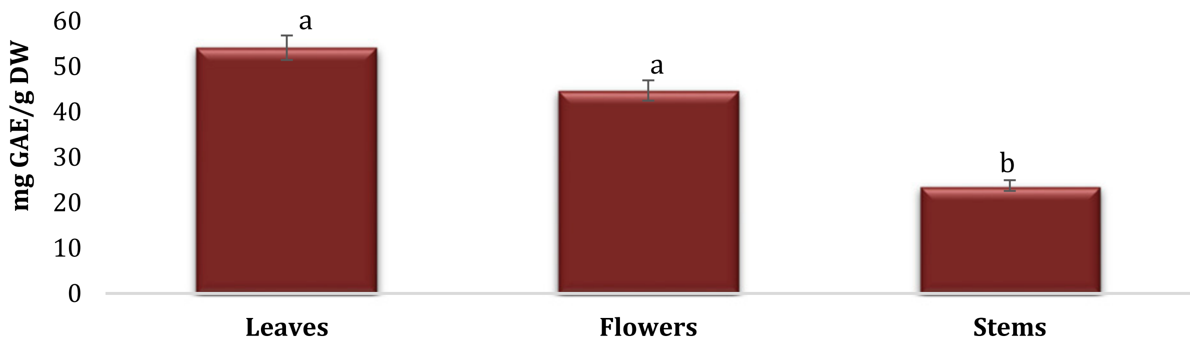

Figure 1 Content of total polyphenol content of *Humulus lupulus* L. extracts at the stage of flowering:
GAE – gallic acid equivalent; different superscripts in each column indicate the significant differences in the mean at $p < 0.05$

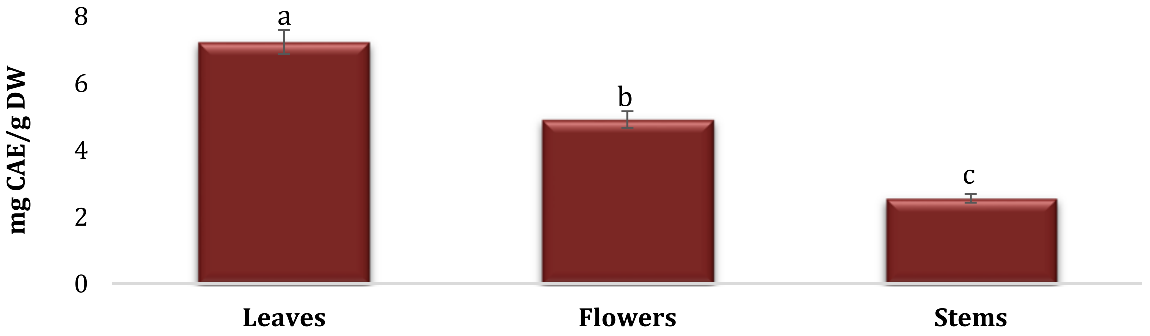

Figure 2 Content of phenolic acids of *Humulus lupulus* L. extracts at the stage of flowering:
CAE – caffeic acid equivalent; different superscripts in each column indicate the significant differences in the mean at $p < 0.05$

(2020) determined TPC from 50.08 to 92.41 mg GAE/g depending on *H. lupulus* cultivar.

Phenolic acids are secondary metabolites from plants that are divided into two major groups – hydroxycinnamic and hydroxybenzoic acids (Tanase et al., 2019). This group of polyphenol compounds well-known antioxidants that also exhibited cardioprotective, antidiabetic, antiulcer, anticancer, anti-inflammatory, neuroprotective, hepatoprotective activities (Saibabu et al., 2015). Leaves of vegetables contain phenolic acids in the highest concentration, as reported Kumar and Goel (2019). Among phenolic acids of *H. lupulus*, the most widespread is ferulic acid (Ahmed et al., 2019).

The total content of phenolic acids in *H. lupulus* extracts was from 2.56 to 7.24 mg CAE/g DW (Figure 2).

It should be noted that the least content of phenolic acids found in the stems and the most in the flowers. The same results were found in different organ extracts of *Scutellaria baicalensis* and *Galega* spp., where the lowest content of phenolic acids found in stems at the flowering (Vergun et al., 2019, 2020).

One of the most widespread polyphenols are flavonoids. This a group of natural substances found in fruits, vegetables, grains, herbs, stems, roots, flowers, etc. The main groups of flavonoids are flavones, flavanones, catechins, and anthocyanins that act as antioxidants (Nijveldt et al., 2001). Flavonoids exhibited numerous health-promoting effects and biological activities such as antioxidant, antiviral (Tapas et al., 2008), anti-inflammatory, anti-cancerogenic, anti-mutagenic. They found abundantly in plant raw that makes plants valuable source of these compounds (Panche et al., 2016). Flavonoids also demonstrated antimicrobial and antifungal activity (Saleem et al., 2017). The total content of flavonoids in the ethanol extracts was from 18.31 to 45.48 mg QE/g DW (Figure 3).

TFC of inflorescence extracts, according to Arsene et al. (2015), was 26.46 mg RE/g. According to Sotto et al. (2018), the TFC of inflorescence extracts was 3.8 µg QE/g. Almeida et al. (2019) found that TFC in *H. lupulus* extracts was from 52.94 to 54.47 mg QE/g. Ahmed et al. (2019) found in the ethanol leaf extracts 56 mg QE/g of TFC.

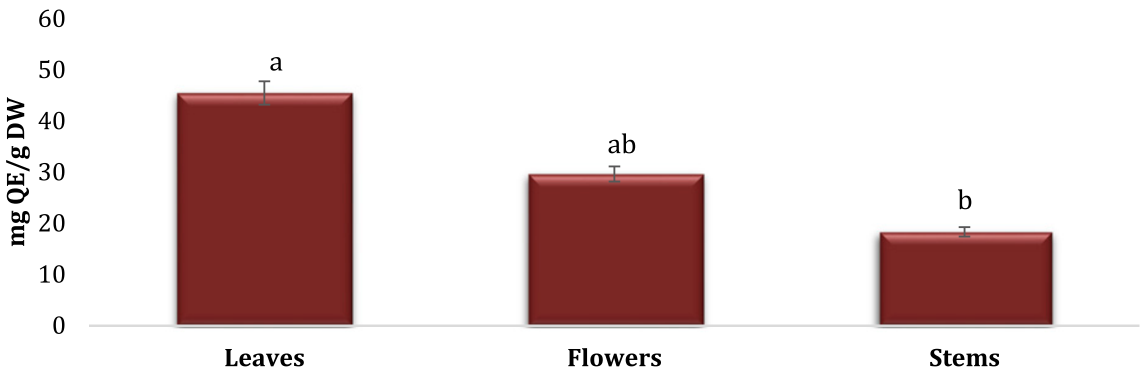

Figure 3 Content of flavonoids of *Humulus lupulus* L. extracts at the stage of flowering:
QE – quercetin equivalent; different superscripts in each column indicate the significant differences in the mean at $p < 0.05$

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Table 1 Antioxidant activity of *Humulus lupulus* L. extracts at the stage of flowering

Plant extract	Molybdenum reducing power of extract (mg TE/g DW)	DPPH radical scavenging assay (mg TE/g DW)
Leaves	168.17 ±9.17ab	8.64 ±0.03a
Flowers	236.45 ±10.72a	8.02 ±0.05a
Stems	97.57 ±3.28b	7.97 ±0.27b

Note: TE – trolox equivalent; different superscripts in each column indicate the significant differences in the mean at p <0.05

Table 2 Pearson's coefficients between antioxidant parameters of *Humulus lupulus* L. extracts at the stage of flowering

Parameter	TPC	TPAC	TFC	MRP	DPPH
TPAC	0.978**	1			
TFC	0.952**	0.995**	1		
MRP	0.680*	0.513*	0.424*	1	
DPPH	0.783*	0.895**	0.936**	0.077*	1

Note: TPC – total polyphenol content, TPAC – total phenolic acids content, TFC – total flavonoid content, MRP – molybdenum reducing power, DPPH – antioxidant activity by DPPH method; ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

In this study, the antioxidant activity was determined by both the phosphomolybdenum and DPPH methods. MRP of investigated extracts decreased in the following order: flowers > leaves > stems (Table 1). Concerning the antioxidant activity by the DPPH method, results decreased by the following order: leaves > flowers > stems.

There are not enough data about antioxidant activity by the phosphomolybdenum and DPPH methods but some authors confirmed exhibiting the antioxidant activity by different extracts of plants from Cannabaceae and found a correlation between TPC and reducing power of extracts (Mkpenie et al., 2012; Niknejad et al., 2014).

The correlation analysis showed a very strong relations between TPAC and TFC (r = 0.995), TPAC and TPC (r = 0.978), TPC and TFC (r = 0.952), antioxidant activity by DPPH method and TFC (r = 0.936) and TPAC (r = 0.895) (Table 2). Strong correlation found between total content of polyphenols and antioxidant activity by DPPH method (r = 0.783) and molybdenum reducing power (r = 0.680).

According to Gorjanović et al. (2013), the TPC of *H. lupulus* extracts correlated with antioxidant activity by the DPPH method (r = 0.986). In our experiment, this parameter was lower. Our results of antioxidant parameters relations showed that TPC was positively correlated with all investigated parameters.

Conclusions

This study demonstrated that ethanol extracts of wild plants of *H. lupulus* are a valuable source of polyphenol compounds with high antioxidant activity. The most content of polyphenols, flavonoids, and phenolic acids found in the leaf's extracts, the least content detected in the stem extracts. Values of correlation between investigated groups of polyphenol compounds and antioxidant activity by DPPH method was higher than with antioxidant activity by phosphomolybdenum method but all groups of polyphenols showed a strong correlation with antioxidant activities (by two methods). The results of this study could be used in further pharmacological studies.

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Research Article OPEN ACCESS

Comparison of old and local apple varieties and seedlings (*Malus domestica* Borkh.) in the variability of some morphological characters of fruits and seeds

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Old and local varieties of cultivated plant species selected from natural populations adapted to long-term cultivation, which represent a rich genetic potential for the development of agroecosystems and agriculture under specific conditions, resources for an environment aestheticization, landscaping and development of cultural traditions. The research focused on determining the economic value of a selected collection of old and local varieties of apple tree (*Malus domestica* Borkh.), widespread in Slovakia for their practical use in organic farming or as genetic resources for breeding new varieties for organic food production. For experimental evaluation, we used two collections: 1) 73 old and local varieties of apple trees concentrated and preserved *ex situ* in a clone repository in the village Bacúch; 2) 77 self-sown seedlings, that spontaneously emerged as a result of free pollination and are growing *in situ* around Nitra, Levice, Nové Zámky, Šaľa, Galanta, Hlohovec, Piešťany, Prievidza, Partizánske, Zlaté Moravce. We determined for all specimens the range for the weight of fruits 53.63–207.40/16.13–197.59 (g), height of fruits 41.47–72.93/29.55–74.04 (mm), diameter of fruits 51.46–84.66/36.85–78.43 (mm), length of core/13.16–27.36/11.24–25.86 (mm), diameter of core 18.26–33.46/13.72–30.86 (mm), weight of 10 seeds 0.38–0.77/0.29–0.98 (g), height of seeds 6.68–9.90/6.16–9.83 (mm), diameter of seeds 3.73–5.71/3.51–5.27 (mm). The results document that in both collections there are genotypes suitable for organic cultivation, and further selective improvement.

Keywords: *Malus domestica*, genetic resources, clone repository, morphometric analysis, variability

Introducion

The native range of apple tree (*Malus domestica* Borkh.) is difficult to determine, as the species is a product of domestication and multiple hybridizations across the world over thousands of years. In Slovakia, fruit growing has a long tradition. Apple trees have a dominant position in fruit growing. *Malus domestica* from the genus *Malus* from the family Rosaceae and the subfamily Pomoideae is an example of the most important, the most widespread and best adapted fruit tree of temperate zone in terms of production. *Malus occupies* a central place in the folklore, culture and art (Robinson et al., 2001; Harris et al., 2002; Juniper and Mabberley, 2006; Velasco et al., 2010).

A local variety is a domesticated, locally adapted, traditional variety of a species of plant that has developed over time, through adaptation to its natural and cultural environment of agriculture, and due to isolation from other populations of the species. Local varieties are generally distinguished from cultivars.

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They have been selected from natural populations and grown for nutritional use or other purposes. Due to their long-term cultivation in different areas, they have adapted to certain specific growing conditions, thus acquiring a high degree of tolerance against adverse environmental factors. Old cultivars and varieties are highly disease resistant to apple scab, powdery mildew, green apple aphid, apple codling moth in general (Militaru et al., 2015; Papp et al., 2015). Cultivation the less susceptible varieties is the most obvious way to reduce problems with pests and diseases; therefore, the choice of apple varieties for organic farming is extremely important. Great effort has been put into developing breeding programmes to create scab resistant varieties. However, older varieties that originated before the appearance of pesticides might be less susceptible than newer varieties and would thereby be a better choice for organic farming (Kühn et al., 2003; Militaru et al., 2015; Papp et al., 2015).

Regarding polyphenols, it is known that old and local apple varieties were characterized by a higher content of polyphenols and stronger antioxidant properties than commercial varieties, which enjoys a high growth rate, but unfortunately, these new varieties are characterized by a very low content of bioactive compounds, including polyphenolic compounds (Kuznetsova et al., 2017; Oszmiański et al., 2019). The consumption of such apple varieties may reduce the polyphenolic compounds in the dietary supply (Iacopini et al., 2010; Donno et al., 2012). Some studies presented the amounts of biologically active substances in old and new varieties were similar (Wojdyło et al., 2008). In the study of Feliciano et al. (2010), both traditional and exotic apple varieties from Portugal showed high amounts of polyphenols. It should be noted that environmental conditions can influence on the polyphenol amounts.

Local varieties represent the means of production for the development of agroecosystems and agriculture in specific conditions, resources for the aestheticization of the environment, landscaping and the development of cultural traditions (Brindza, 2001; Tóth et al., 2004; Ganopoulos et al., 2017). One of the largest collections of old apple varieties is located in a neighbouring Poland and Ukraine and spread over the territory of the then ancient Eastern Galicia in Central Europe (Dovbysh and Borodai, 2011; Żygala et al., 2011).

It is generally known that many local varieties, as well as cultivars, were selected from local self-sown individuals – seedlings (Boček, 2008a, 2008b; Hulin et al., 2012; Posolda et al., 2019). The establishment of clone repositories to save the endangered gene pool of plants has an application in our country for many fruit species such as pear, cherry, plum, chestnut, etc. (Bolvanský and Užík, 2012; Paprstein et al., 2013; Benediková et al., 2016). It is necessary to identify and evaluate genotypes based on the morphometrical and biochemical traits in various conditions, as evidenced by the many authors (Ivanišová et al., 2017; Vinogradova et al., 2017; Grygorieva et al., 2017a,b, 2018a,b; Fatrcová Šramková et al., 2019; Levon and Golubkova, 2019; Vergun et al., 2020).

This study aimed to evaluate the genetic resources of apple tree for organic farming in the collection of old and local varieties of *Malus domestica* Borkh. as well as self-sown seedlings widespread in Slovakia.

Material and methodology

Biological material

Two collections of biological material were used as genetic resources for the study:

1. Old and local varieties from different areas of Slovakia which are kept *ex situ* in a clone repository in the village Bacúch – 73 selected genotypes. In the experiments, samples were marked as R and the appropriate number.
2. Wild self-sown individuals – fruit-beraing seedlings from different localities (Nitra, Levice, Nové Zámky, Šaľa, Galanta, Hlohovec, Piešťany, Prievidza, Partizánske, Zlaté Moravce) in the form *in situ* – 77 selected genotypes. In the experiments, samples were marked as S and the appropriate number.

The total number of evaluated genotypes were 150.

Fruits with peduncle were taken from trees in September and October 2010 and analysed in the morphometric laboratory at the Institute of Biodiversity Conservation and Biosafety in Nitra (Slovakia).

Morphometrical analysis

They were evaluated the following characters:

a) fruits – 30 fruits were evaluated from each genotype (n = 30), weight of fruit (g), height of fruit (mm), diameter of fruit (mm), length of core (mm), diameter of core (mm), depth of stalk cavity (mm), depth of eye basin (mm);
b) seeds – 30 seeds were evaluated from each genotype (n = 30), weight of 10 seeds (g), height of seed (mm), diameter of seed (mm).

The weights were determined by digital scale (Kern ADB-A01S05, Germany; KERN DS – type D-72336,

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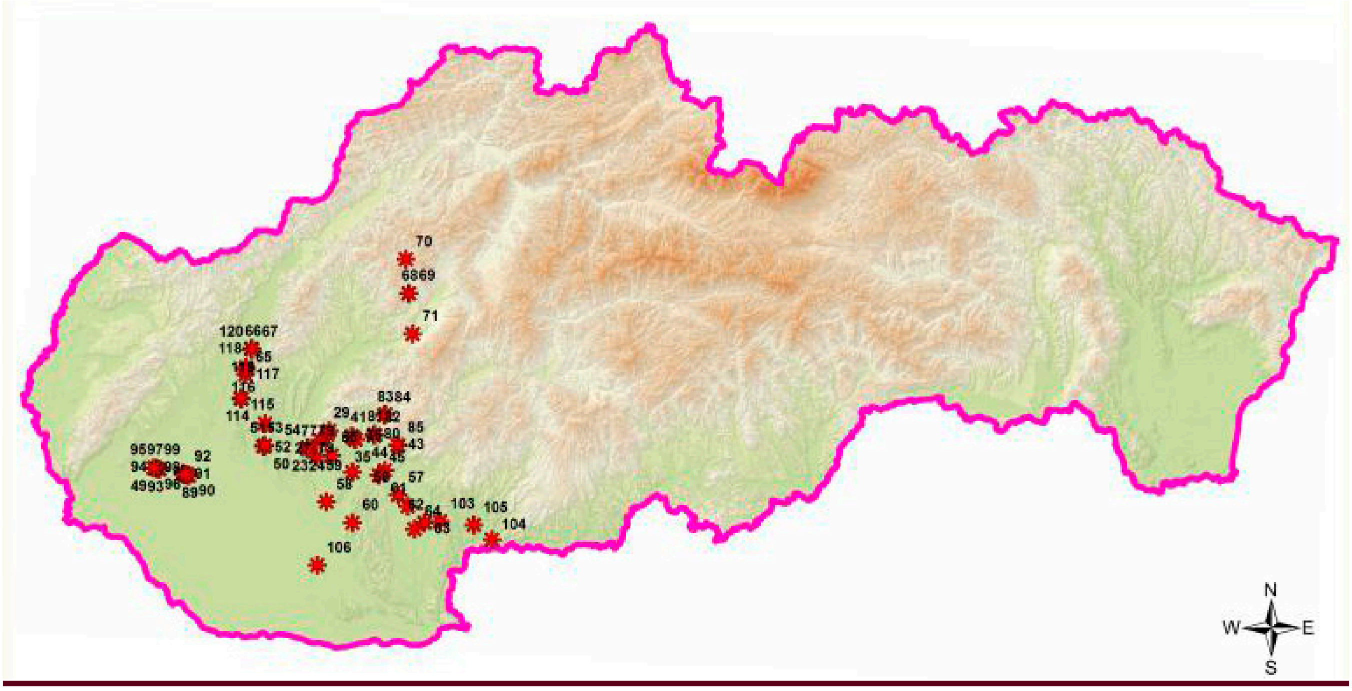

Figure 1 Localization of local varieties and seedlings of *Malus domestica* Borkh. within Slovakia using GPS: the detailed data can be found in Table 1

Table 1 Localities of varieties and seedlings *Malus domestica* Borkh. in Slovakia and their altitude

Genotype	Locality	Region of Slovakia	Altitude, m a.s.l.
R01-R77	Bacúch	central Slovakia	590–630
S01-S10	Nitra	western Slovakia	167
S11-S20	Levice	south-western Slovakia	165
S21-S30	Nové Zámky	south-western Slovakia	114
S31-S40	Šaľa	south-western Slovakia	116
S41-S50	Galanta	south-western Slovakia	119
S51-S60	Hlohovec	western Slovakia	146
S61-S70	Piešťany	western Slovakia	160
S71-S80	Prievidza	western Slovakia	309
S81-S90	Partizánske	western Slovakia	190
S91-S99	Zlaté Moravce	western Slovakia	192

Note: altitude – meters above sea level

Kern and Sohn GmbH, Germany), accurate to 0.01 g. Fruits and seeds were measured by a digital calliper (METRICA 111 – 012, Czech Republic) accurate to 0.02 mm.

Image analysis

1. Fruit: the shape of the fruit, the shape of the apical part of the fruit (at the stalk), depth of stalk cavity, depth of eye basin, the shape of the basal part of the fruit, basic colour of the skin at the full maturity, the colour of the pulp of ripe fruit.
2. Seeds: the shape of seeds.

Images were obtained using the stereomicroscope ZEISS SteREO Discovery.V20 (MicroImaging GmbH 37081 Göttingen, Germany), and Fuji FinePix S 7000 and Panasonic DMC FZ50 digital cameras.

Statistical analysis

It was evaluated the variability of each character using descriptive statistics. For the characteristics it was used the basic descriptors of variability: average, minimum measured value, maximum measured value, the coefficient of variation (%). The degree of variability was determined by the coefficient of variation values.

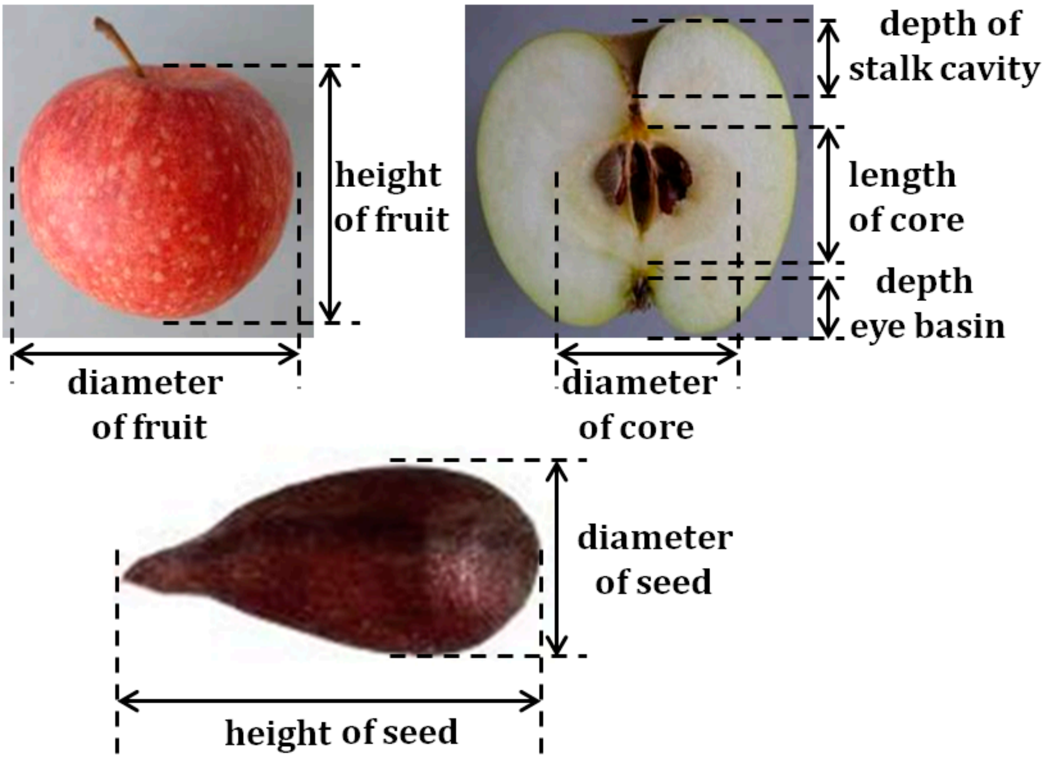

Figure 2 Illustration of measuring process: fruit height and diameter, core length and diameter, depth of stalk cavity and depth of eye basin

The given parameter is independent of the unit of the evaluated character. Theoretically, they can acquire different values (Stehlíková, 1998). We used analysis of variance (ANOVA) in the program STATISTICA 1.10 to determine the dependence between individual characters.

Results and discussion

Evaluation and identification of genotypes based on morphological traits are important for the detection and selection of individuals that are suitable genetic material for hybridization and breeding program of new varieties, which contributes to the global conservation of biological diversity (Monka et al., 2014; Grygorieva et al., 2017a,b, 2018a,b; Motyleva et al., 2017, 2018; Ivanišová et al., 2017; Vinogradova et al., 2017; Brindza et al., 2018, 2019; Fatrcová-Šramková et al., 2019; Horčinová Sedláčková et al., 2020).

Morphometrical analysis of fruits

When evaluating the genotypes under study (Table 2), the average weight of the fruits has been determined in the range of 3.63 g (R18/9) – 207.40 g (R30/7)/16.13 g (S12) – 197.59 g (S03). The coefficients of variation were determined in the range of 4.18 (R33/12) – 17.02 (R18/7) %/11.08 (S38) – 40.61 (S22) %. These data demonstrate that the characters are from the low to very high degree of variability. The above comparisons show that it is possible to search for genotypes with the required fruit size in the populations of wild seedlings.

The differences in the weight of tested varieties were significant, and that is in full compliance with the studies assortment of old apple varieties from Denmark 77–205 g (Kühn et al., 2003), Montenegro 62.23–182.34 g (Božović et al., 2013), Croatia 26–325 g (Jakobek et al., 2020), Romania 117.0–186.5 g (Mitre et al., 2015), Bosnia and Herzegovina 63.77–208.97 g (Stanivuković et al., 2017).

Dvořák et al. (1976) classified fruits according to 3-years-old average weight as extremely small (below 15 g); very small (16–48 g); small (49–70 g); smaller (71–110 g); medium (111–150 g); larger (151–200 g); large (201–250 g); very large (251–350 g) and extremely large (above 351 g). Michálek (2003) divides apple varieties according to the size of the fruit while declaring the size of the fruit according to the dimensions – height and diameter of the fruit at the place of the largest diameter. According to the given descriptor, it recognizes smaller fruits – the average transverse diameter is up to 55 mm, medium-sized (55–70 mm), large fruits (71–85 mm) and very large fruits (more than 85 mm). According to the above

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Table 2 Variability of fruits of old and local varieties of *Malus domestica* Borkh.

Weight of fruits (g)												
	Seedlings						**Genotypes from repository Bacúch**					
	n	**min**	**max**	**x̄**	**V**	**H**		**n**	**min**	**max**	**x̄**	**V**	**H**
Genotypes with low values													
S12	30	12.20	20.50	16.13	18.83	k	**R18/9**	30	47.7	59.7	53.63	7.59	g
S69	30	30.00	45.00	36.45	11.95	j	**R35/10**	30	50.7	70.1	59.36	11.13	f
Genotypes with high values													
S03	30	142.90	331.00	197.59	28.25	a	**R30/7**	30	170.5	241.5	207.40	11.76	a
S20	30	86.90	215.50	185.28	20.75	a	**R16/12**	30	168.4	205.4	187.39	6.53	ab
Height of fruits (mm)													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S12	30	27.40	32.30	29.55	5.60	e	**R33/12**	30	38.70	43.70	41.47	3.33	d
S69	30	33.60	39.30	36.38	4.70	ed	**R1/4**	30	41.5	44.6	42.86	1.86	d
Genotypes with high values													
S22	30	59.30	87.90	74.04	14.08	a	**R30/7**	30	66.70	78.50	72.93	5.70	a
S03	30	58.60	83.40	67.91	12.03	a	**R30/8**	30	62.20	80.50	71.32	9.03	a
Diameter of fruits (mm)													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S12	30	33.50	40.00	36.85	6.03	f	**R18/9**	30	48.90	54.20	51.46	3.71	e
V69	30	43.10	50.10	45.67	5.57	e	**R22/3**	30	49.00	53.80	51.58	2.94	e
Genotypes with high values													
S03	30	72.60	95.10	78.43	8.12	a	**R30/8**	30	76.30	90.90	84.66	6.56	a
S20	30	59.30	83.50	77.86	8.86	a	**R16/12**	30	80.20	84.30	82.41	1.75	a
Depth of stalk cavity (mm)													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S24	30	0.00	5.30	2.04	83.00	cd	**R22/3**	30	0.00	3.60	1.67	67.63	e
S12	30	1.00	4.40	2.96	34.28	cd	**R1/12**	30	4.20	6.40	5.36	16.29	d
Genotypes with high values													
S20	30	11.30	18.80	16.11	12.66	a	**R14/11**	30	13.20	16.30	14.82	6.83	a
S30	30	8.90	17.80	14.44	17.50	a	**R27/11**	30	9.20	16.60	13.77	18.17	b
Depth of eye basin (mm)													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S38	30	0.70	2.60	1.22	52.24	d	**R18/9**	30	1.20	3.20	2.10	33.14	c
S08	30	0.50	2.90	1.23	56.33	d	**R33/12**	30	1.10	3.10	2.17	26.16	c
Genotypes with high values													
S22	30	8.10	18.10	11.69	29.09	a	**R3/16**	30	7.20	10.00	8.86	9.77	a
S20	30	4.80	19.20	9.58	39.18	b	**R16/14**	30	7.60	9.50	8.60	6.71	a

Table 2 continued

Length of core (mm)													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S10	30	10.10	12.70	11.24	9.24	d	**R31/2**	30	10.90	15.70	13.16	14.61	dc
S30	30	10.80	13.70	12.26	8.41	d	**R33/12**	30	11.40	14.50	13.42	8.84	dc
Genotypes with high values													
S03	30	20.00	29.50	25.86	14.11	a	**R3/2**	30	26.40	28.50	27.36	3.31	a
S47	30	18.50	25.60	22.06	11.67	a	**R41/4**	30	21.70	33.60	26.80	18.47	a
Diameter of core (mm)													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S22	30	0.94	1.08	1.01	4.35	c	**R1/4**	30	17.50	19.00	18.26	3.60	d
S76	30	0.98	1.03	1.00	1.54	c	**R3/2**	30	17.10	20.10	18.52	6.89	d
Genotypes with high values													
S75	30	12.70	14.40	13.72	4.79	a	**R20/8**	30	30.90	35.80	33.46	6.49	a
S47	30	13.20	16.30	14.98	7.70	a	**R18/7**	30	26.20	30.30	28.42	5.46	b
Index of fruit shape													
	Seedlings						**Genotypes from repository Bacúch**						
Genotypes with low values													
S02	30	0.69	0.79	0.74	4.48	ab	**R1/4**	30	0.65	0.74	0.69	4.17	b
S67	30	0.69	0.78	0.76	3.56	ab	**R7/7**	30	0.68	0.72	0.70	1.49	b
Genotypes with high values													
S22	30	0.94	1.08	1.01	4.35	a	**R41/4**	30	1.15	1.35	1.26	4.21	a
S76	30	0.98	1.03	1.00	1.54	a	**R5/4**	30	0.97	1.25	1.09	6.60	ab

Note: n – the number of measurements; min, max – minimal and maximal measured values; $\bar{x}$ – arithmetic mean; V – coefficient of variation (%); H – LSD homogeneity test at $P_{0.05}$

descriptor, the fruits of seedlings can be characterized as small to large.

The average height of fruits of the genotypes under the study was in the range 41.43 mm (R33/12) – 72.93 mm (R30/7)/29.55 mm (S12) – 74.04 mm (S22) and the diameter of fruits was in the intervals of 51.46 mm (R18/9) – 84.66 mm (R30/8)/36.85 mm (S12) – 78.43 mm (S03). The collection of self-sown seedlings showed a significantly higher variation range in both evaluated traits. The coefficients of variation confirm the low or the medium degree of variability of the characters. Parameters are shown in table 2.

Jakobek et al. (2020) recorded heights (34–79 mm) and diameters (41–89 mm) of old varieties. The average height and diameter of old apple varieties cultivated in Bosnia and Herzegovina (Stanivuković et al., 2017) were recorded in the interval 50.08–67.21 mm and 53.52–80.23 mm, respectively. Results showed by Božović et al. (2013) in Montenegro, where the intervals of evaluated traits were 42.29–64.70 mm and 54.08–78.27 mm respectively, are similar to the data shown. Michálek (2003) states that from the market point of view, mainly varieties with medium to large fruits are in demand. Small or too large fruits are commercially unattractive. This customer requirement must be taken into account at assessing genotypes as a potential gene pool in breeding programs, as there are 19.5 % of samples with small fruits (below 55 mm) in our research collection.

An important diagnostic feature is the depth of stalk cavity and depth of eye basin, because the measured features may have a specific range for each variety and genotype. We determined the average depth of the stalk cavity in the collection of old and local varieties/wild seedlings in the range of 1.67 mm (R22/3) – 14.82 mm (R14/11)/2.04 mm (S24) – 16.11 mm (S20). The results show that some fruits did not have stalk cavity (Table 2). We determined the average depth of eye basin in the collection of old and local varieties/wild seedlings in the range of 2.10 mm (R18/9) – 8.86 mm (R3/16)/1.22 mm (S38) – 11.69 mm (S22). We

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Figure 3 Variability in the shape and the colour of fruits of evaluated genotypes of seedlings of *Malus domestica* Borkh.

did not find any significant differences between the collections. The values of the coefficients of variation confirm the low or extremely high degree of variability of the traits.

Michálek (2003) distinguishes the shapes of the stalk cavity as narrow and shallow, wide and shallow, wide and deep, narrow and deep. In some varieties, a characteristic swollen formation is formed, which often overgrows and tilts the stalk to one side. We recorded a relatively large variability of the pomological feature (Figure 3). In the calyx part of the fruit, the shape, size and eye basin are important features. The depth of eye basin and its shape can be important because they are a little variable (Figure 4). According to Michálek (2003), we know the following eye basin: the small eye basin, the spacious eye basin, the short eye basin, the funnel eye basin, plumpness eye basin.

The average length of core of the genotypes under the study was in the range of 13.16 mm (R31/2) – 27.36 mm (R3/2)/11.24 mm (S10) – 25.86 mm (S03), and diameter of core was in the range of 18.26 mm (R1/4) – 33.46 mm (R20/8)/13.72 mm (S75) – 30.86 mm (S3). We did not find any significant differences between the collections in the length and diameter of core, but diameters were relatively lower in the collection of wild seedlings. Coefficients of variation confirm the low or medium degree of variability of both characters.

Figure 4 Variability in the characters of the depth of stalk cavity and depth of eye basin of the evaluated genotypes of the apple tree (*Malus domestica* Borkh.)

We determined the average value of the fruit shape index in the collection of old and local varieties and in the collection of wild seedlings. It is in the range from 0.69 (R1/4) to 1.26 (R41/4) and from 0.74 (S02) to 1.01 (S22). The comparison of genotypes with low and high values of the trait and variation ranges of the evaluated trait shows that genotypes with different values of the fruit shape index were determined in both collections. We did not find any significant differences between the collections. The coefficients of variation confirm the low degree of variability of the trait in both collections. Jakobek et al. (2020) recorded fruit shape index values (0.7–1.2) of old varieties.

The results from the analysis of variance of the evaluated traits (Table 3, Table 4) confirm the statistically significant differences between the evaluated genotypes.

Iqbal et al. (2011) described analytical methods tested in a laboratory for estimation of volume of axi-symmetric fruits like apples based on single view fruit images and the shape-based analytical models. The fruits are categorized into spherical, ellipsoid and paraboloid shapes with appropriate analytical models for their volume estimation. In both our collections of genotypes, spherical, elliptical and parabolic fruits are

Table 3 Analysis of variance of evaluated fruit traits of genotypes of old and local varieties of *Malus domestica* Borkh. from the repository Bacúch

Factors	f	S	MS	F	H	LSD	
Weight of fruit (g)							
Between genotypes	9	124512.500	13 834.720	137.226	0.000	0.05	14.447
Within genotypes	90	9073.531	100.817			0.01	17.018
Total	99	133586.031					
Height of fruit (mm)							
Between genotypes	9	4463.000	495.888	70.057	0.000	0.05	3.828
Within genotypes	90	637.052	7.078			0.01	4.509
Total	99	5100.052					
Diameter of fruit (mm)							
Between genotypes	9	5470.781	607.864	73.086	0.000	0.05	4.149
Within genotypes	90	748 535	8.317			0.01	4.888
Total	99	6219.316					
Depth of stalk cavity (mm)							
Between genotypes	9	652.568	72.507	81.934	0.000	0.05	1.353
Within genotypes	90	79.645	0.884			0.01	1.594
Total	99	732.213					
Depth of eye basin (mm)							
Between genotypes	9	321.236	35.693	54.705	0.000	0.05	1.162
Within genotypes	90	58.721	0.652			0.01	1.369
Total	99	379.958					
Length of core (mm)							
Between genotypes	9	494.136	54.904	81.520	0.000	0.05	1.180
Within genotypes	90	60.615	0.673			0.01	1.391
Total	99	554.752					
Diameter of core (mm)							
Between genotypes	9	502.640	55.849	29.630	0.000	0.05	1.975
Within genotypes	90	169.636	1.884			0.01	2.327
Total	99	672.277					

Note: f – number of degrees of freedom; S – the sum of squares; MS – average square; F – Fischer test value; P – statistical significance by Fischer test; H – homogeneity; LSD – a least significant difference

Table 4 Analysis of variance of evaluated fruit traits of seedlings of *Malus domestica* Borkh.

Factors	f	S	MS	F	H	LSD	
Weight of fruit (g)							
Between genotypes	9	247224.800	27469.420	50.756	0.000	0.05	33.472
Within genotypes	90	48708.177	541.202			0.01	39.431
Total	99	295932.990					
Height of fruit (mm)							
Between genotypes	9	11953.730	1328.193	65.014	0.000	0.05	6.503
Within genotypes	90	1838.614	20.429			0.01	7.661
Total	99	13792.348					
Diameter of fruit (mm)							
Between genotypes	9	12673.470	1408.163	83.695	-0.000	0.05	5.901
Within genotypes	90	1514.241	16.824			0.01	6.952
Total	99	14187.709					
Depth of stalk cavity (mm)							
Between genotypes	9	827.314	91.923	33.774	-0.000	0.05	2.373
Within genotypes	90	244.951	2.721			0.01	2.796
Total	99	1072.266					
Depth of eye basin (mm)							
Between genotypes	9	575.351	63.928	46.523	-0.000	0.05	1.686
Within genotypes	90	123.668	1.374			0.01	1.986
Total	99	699.019					
Length of core (mm)							
Between genotypes	9	1722.531	191.392	39.174	0.000	0.05	3.180
Within genotypes	90	439.705	4.885			0.01	3.746
Total	99	2162.237					
Diameter of core (mm)							
Between genotypes	9	1096.727	121.858	34.799	-0.000	0.05	2.692
Within genotypes	90	315 156	3.501			0.01	3.171
Total	99	1411.883					

Note: f – number of degrees of freedom; S – the sum of squares; MS – average square; F – Fischer test value; P – statistical significance by Fischer test; H – homogeneity; LSD – a least significant difference

most represented (Figure 3), which is in accordance with the literature data.

Apples may vary in colour, from uniformly dark-red, red, reddish, green, orange, yellow, white, or bi-coloured, such as striped or blushed red on a yellow or green background. Results have shown high variability of shapes and colours in both collections of *Malus domestica*.

The core of the fruit usually consists of five seed carpels pockets or carpels. Sometimes some fruits have only four or three carpels. Each pocket contains seeds. The number of seeds per carpel is determined by the vigour and health of the plant. Different varieties of apples will have a different number of seeds. Each carpel generally contains two seeds. Seeds are smooth, shiny, and chestnut brown (Jackson, 2003; Huff, 2012–2013).

The individual varieties are characterized not only by the shape of the core but also by its size and its location (Michálek, 2003). Figure 5 documents some differences in the shape of the core. On the cross-section, we can see 10 vascular bundles in a circle around the core. They seem like darker or lighter dots. In total, we can observe 10 vascular bundles, of which 5 are located directly opposite the tops of the carpels, the other 5 are between them. The vascular bundles determine the angularity of the fruit. If they are in a circle and evenly

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Figure 5 Comparison of selected genotypes from the evaluated collection of seedlings of *Malus domestica* Borkh. in the number of vascular bundles in the longitudinal and cross section

developed, the fruit is uniformly rotund in cross-section. If they are in two circles, the outer ones tend to be more developed and the fruit is thus become slightly angular (Kohout, 1960; Dvořák et al., 1976; Michálek, 2003). The examples on the presented photo (Figure 5) document that in the evaluated collection of genotypes has a relatively large variability of this pomological feature.

Morphometrical analysis of seeds

On the seeds, we evaluated the characteristics of the weight of 10 seeds (g), the height of seeds (mm) and the diameter of seeds (mm). We determined the average weight of seeds in the genotypes under the study in the range from 0.38 g (R15/5) to 0.77 g (R41/1) and from 0.29 g (S28) to 0.98 g (S92). In the collection of wild seedlings, we recorded a higher range of variation in the evaluated trait. The coefficients of variation confirm the low or medium degree of variability of the trait. The average height of seeds for the collection of old and local varieties was in the range from 6.67 mm (R18/9) to 9.89 mm (R16/14) and for the collection of wild seedlings from 6.16 mm (S40) to 9.83 mm (S67). We did not find any significant differences between the collections. The coefficients of variation confirm the low degree of variability of the trait. We determined the

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Table 5 Variability of seeds of old and local varieties and of wild seedlings of *Malus domestica* Borkh.

Weight of seeds (g)													
	Seedlings						Genotypes from repository Bacúch						
	n	min	max	x̄	V	TH		n	min	max	x̄	V	TH
Genotypes with low values													
S28	30	0.24	0.40	0.29	15.38	c	**R15/5**	30	0.34	0.41	0.38	5.79	b
S12	30	0.25	0.36	0.30	13.20	c	**R23/3**	30	0.36	0.42	0.39	5.17	b
Genotypes with high values													
S92	30	0.88	1.00	0.98	5.18	a	**R41/1**	30	0.74	0.81	0.77	2.99	a
S89	30	0.85	1.00	0.93	7.14	a	**R29/5**	30	0.72	0.76	0.74	1.78	a
Height of seeds (mm)													
	Seedlings						Genotypes from repository Bacúch						
Genotypes with low values													
S40	30	5.81	6.72	6.16	4.66	c	**R18/9**	30	6.19	7.05	6.67	4.19	b
S62	30	5.44	6.71	6.25	6.07	c	**R31/10**	30	6.15	7.42	6.68	5.24	b
Genotypes with high values													
S67	30	9.06	10.63	9.83	5.58	a	**R16/14**	30	9.24	10.56	9.89	4.85	a
S38	30	8.10	10.22	9.34	6.30	a	**R27/11**	30	9.35	10.05	9.66	2.44	a
Diameter of seeds (mm)													
	Seedlings						Genotypes from repository Bacúch						
Genotypes with low values													
S82	30	3.02	3.96	3.51	7.88	c	**R23/3**	30	3.23	4.05	3.73	7.55	c
S07	30	3.19	3.99	3.57	6.77	c	**R41/4**	30	3.24	4.14	3.75	6.94	c
Genotypes with high values													
S72	30	4.85	5.48	5.26	3.64	a	**R5/4**	30	4.48	9.87	5.71	27.05	a
S89	30	4.66	5.47	5.08	4.37	a	**R19/12**	30	4.51	7.79	5.63	20.93	a
Index of seed shape													
	Seedlings						Genotypes from repository Bacúch						
Genotypes with low values													
S62	30	1.17	1.41	1.35	5.26	bc	**R19/12**	30	0.66	1.62	1.36	25.59	b
S40	30	1.44	1.68	1.54	4.69	b	**R7/6**	30	0.55	1.88	1.45	32.58	b
Genotypes with high values													
S49	30	2.07	2.84	2.45	10.60	a	**R27/11**	30	2.32	2.83	2.52	7.06	a
S19	30	1.88	2.62	2.28	10.57	a	**R16/12**	30	2.11	2.71	2.43	7.77	a

Note: n – the number of measurements; min, max – minimal and maximal measured values; x̄ – arithmetic mean; V – coefficient of variation (%); H – LSD homogeneity test at $P_{0.05}$

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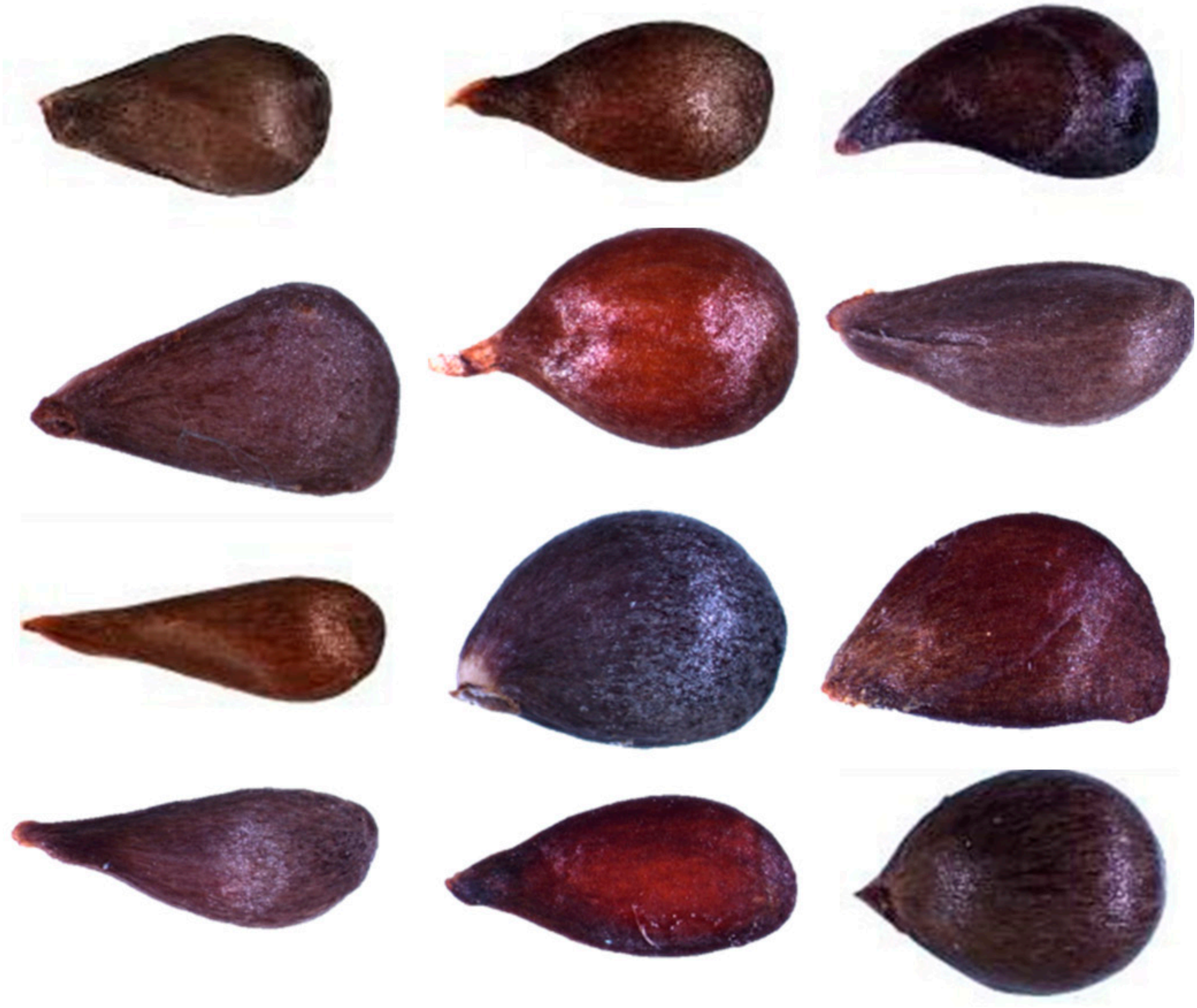

Figure 6 Comparison of selected genotypes from the evaluated collection of seedlings of *Malus domestica* Borkh. in the shape of seeds

average diameter of seeds in the collection of old and local varieties in the range 3.73 mm (R23/2) – 5.71 mm (R5/4) and for the collection of wild seedlings 3.51 mm (S82) – 5.26 mm (S72). We did not find any significant differences between the collections. The coefficients of variation show that the degree of variability of this trait within both collections varies from low to high (Table 5). Our results do not diverge from the data of Jacobek et al. (2020), who estimated the weight of seeds in fruits from 0.07 to 0.53 g, and the weight of a single seed from 0.03 to 0.08 g.

We determined the average value of the seed shape index in the collection of old and local varieties in the range 1.36 (R19/12) – 2.52 (R27/11) and in the collection of wild seedlings 1.35 (S62) – 2.45 (S49), respectively. A comparison of genotypes shows that genotypes with different seed shape indices were identified in both collections. We did not find any significant differences between the collections. The coefficients of variation show that the degree of variability of this trait within both collections varies from low to high.

Figure 6 shows a comparison of selected genotypes from the evaluated collection of the natural seedlings of the apple tree (*Malus domestica*) in seed shapes.

The analysis of variance of the evaluated traits (Table 6) confirmed the statistically significant differences between the evaluated genotypes.

Table 6 Analysis of variance of evaluated seed traits of genotypes from two collections of *Malus domestica* Borkh.

Factors	f	S	MS	F	H	LSD	
Genotypes from repository Bacúch							
Weight of 10 seeds (g)							
Between genotypes	9	0.888	0.098	42.074	0.000	0.05	0.069
Within genotypes	90	0.211	0.002			0.01	0.082
Total	99	1.099					
Height of seeds (mm)							
Between genotypes	9	78.918	8.768	24.898	0.000	0.05	0.853
Within genotypes	90	31.696	0.352			0.01	1.005
Total	99	110.614					
Diameter of seeds (mm)							
Between genotypes	9	16.238	1.804	10.330	0.000	0.05	0.601
Within genotypes	90	15.718	0.174			0.01	0.708
Total	99	31.957					
Seedlings							
Weight of 10 seeds (g)							
Between genotypes	9	0.724	0.080	268.465	0.000	0.05	0.024
Within genotypes	90	0.027	0.000			0.01	0.029
Total	99	0.751					
Height of seeds (mm)							
Between genotypes	9	43.955	4.884	25.377	0.000	0.05	0.024
Within genotypes	90	17.320	0.192			0.01	0.743
Total	99	61.276					
Diameter of seeds (mm)							
Between genotypes	9	10.109	1.123	15.295	0.000	0.05	0.389
Within genotypes	90	6.609	0.073			0.01	0.459
Total	99	16.719					

Note: f – number of degrees of freedom; S – the sum of squares; MS – average square; F – Fischer test value; P – statistical significance by Fischer test; H – homogeneity; LSD – a least significant difference

Conclusions

Based on morphometric analysis of fruits and seeds of both collection:

1. of old and local varieties,
2. of spontaneous seedlings from free pollination, we determined the range of phenotypic variability for all traits and combinations of traits in both groups of evaluated genotypes.

When comparing the ranges of variability for all evaluated traits, we found a significant degree of agreement. The results confirm that some individuals that grow wild and represent spontaneous seedlings from free pollination have a set of economically important traits and are ready to be used as potential genetic resources for a breeding program. Future efforts focused on "wild forms" should focus on preserving all unique genotypes to maintain both cultural heritage and biological genetic diversity.

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Research Article

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Prospects of the application of some species of the Lamiaceae family and some features of the development of their tinctures

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The search for new powerful herbal products with anti-inflammatory, antimicrobial and antinociceptive activities presents an important area of pharmaceutical research. Some plants of the Lamiaceae family are well-known for their significant antimicrobial, anti-inflammatory, and pain-relieving activities. Species of the Lamiaceae attract a great scientific interest mainly due to the diversity of terpenes and phenolic compounds, including phenolic acids and flavonoids. Essential oils possess certain antimicrobial activity. For experimental studies, we selected four herbs. Among them were *Monarda fistulosa* L., *Satureja hortensis* L., *Thymus vulgaris* L., and *Mentha piperita* L. Four tinctures of the above-mentioned herbs were elaborated and partly phytochemically evaluated. We established the coefficients of alcohol absorption for the tested raw materials and the maximum absorption for active markers and ticntures after adding aluminum chloride that is needed for the development and standardization of tictures. The solutions of complexes aluminum chloride with quercetin (20 mg/L), rutin (50.2 mg/L), and chrysin (80 mg/L) had the maximum absorption at the wavelengths of 425.9 ±0.3 nm at 77 min of the reaction, 412.3 ±0.3 nm at 82 min, 388.4 ±0.7 nm at 81 min, respectively. The tinctures of *Monarda fistulosa*, *Satureja hortensis*, *Thymus vulgaris*, and *Mentha piperita* had the maximum absorption at 391.2 ±0.5 nm at 91 min, 389.9 ±0.5 nm at 76 min, 391.8 nm at 83 min, 394.9 ±1.1 nm at 78 min, respectively. The carried out spectrophotometric studies confirmed the prevalence of flavones in the tested tinctures, considering the proximity of the maximum absorption of the tested tinctures and chrysin. The next studies will be continued at the standardization of the developed tinctures and the establishment of their antimicrobial activity.

Keywords: *Monarda fistulosa*, *Satureja hortensis*, *Thymus vulgaris*, *Mentha piperita*, ethanolic extracts, essential oil

Introduction

The search for new powerful anti-inflammatory and antimicrobial herbal products with antinociceptive potential presents an important area of pharmaceutical research (Casian et al., 2020). Some plants of the family of Lamiaceae Lindl. are well-known for their significant antimicrobial, anti-inflammatory and pain-relieving properties (Ben-Arye et al., 2011; Hamidpour et al., 2014; Honcarenko et al., 2019; Casian et al., 2020; Shanaida et al., 2021a, b). In this context, *Monarda fistulosa* L., *Satureja hortensis* L., *Thymus vulgaris* L., and *Mentha × piperita* L. are the subject of several studies in the field of pharmaceutical technology and pharmacology (Thompson et al., 2003; Hamidpour et al., 2014; Liu et al., 2014; Marwa et al., 2017; Fierascu et al., 2018; Shanaida, 2018; Casian et al., 2020; Shanaida et al., 2021a, b).

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Species of the Lamiaceae attract great attention mainly due to the diversity of monoterpenes (Thompson et al., 2003; Honcharenko et al., 2019; Hudz et al., 2020). It has been revealed that essential oils possess certain antimicrobial activity (Ben-Arye et al., 2011; Rezvanpanah et al., 2011; Honcharenko et al., 2019). Therefore, they could be considered as active substances of potential antimicrobial herbal preparations (Rezvanpanah et al., 2011). The essential oil of *Satureja hortensis* contains significant amounts of two phenolic ketones: carvacrol and thymol. They are isomeric compounds and contain a phenol group in their structures (Hamidpour et al., 2014). The thymol chemotype of *Thymus vulgaris* produces thymol (22.4–72.9 %) and carvacrol (0.8–26.8 %) in glandular trichomes on the surface of leaves (Thompson et al., 2003). Carvacrol and thymol have a strong inhibitory effect on the growth of a wide range of microorganisms, including fungi and bacteria (Hamidpour et al., 2014).

Rosmarinic acid is the major compound of the aqueous and ethanolic extracts of *Dracocephalum moldavica*, *Ocimum americanum*, *Satureja hortensis* and other herbs of the Lamiaceae family (Shanaida et al., 2018; Shanaida et al., 2021a). Rosmarinic acid is known for its antiviral, antioxidant, anti-inflammatory, and immunostimulating activities (Hamidpour et al., 2014; Shanaida et al., 2021a). It was established that administration of pure rosmarinic acid at a dose of 25 mg/kg decreased the carrageenin-induced paw oedema in rats at 6 h by over 60 %. The effect of rosmarinic acid (25 mg/kg) can be comparable with Trolox (30 mg/kg) and indomethacin (10 mg/kg), which are known as a strong antioxidant and anti-inflammatory substances, respectively (Rocha et al., 2015).

Herbal preparations of some species of the Lamiaceae family (thyme, mint, and oregano) are stated as safe and efficient for the symptomatic treatment of discomfort conditions related to strep throat (Ben-Arye et al., 2011; Wijesundara et al., 2019).

Therefore, the development of herbal preparations with antioxidant, anti-inflammation and antimicrobial activity for the prevention and treatment of inflammatory and infectious diseases of the oral cavity is a topical question of modern medicine and pharmaceutical technology.

Material and methodology

While carrying out the research, the following methods were used: analysis, synthesis, systematization and comparison for processing published scientific data; technological method (maceration); spectrophotometric method for the development of the analytical procedure of the determination of the total flavonoid content.

Plant material

Aerial parts of *Monarda fistulosa* (wild bergamot, bee balm, or horse mint) were collected in 2019 and *Satureja hortensis* (summer savory), *Thymus vulgaris* (thyme), and *Mentha piperita* (peppermint, balm mint, lamb mint) were collected in 2017. All the raw materials were picked up in the flowering stage in the Kherson region (Ukraine). The voucher specimens were deposited at the Herbarium of the Sector of Mobilization and Conservation of Plant Resources of the Rice Institute of the NAAS (Plodove, Kherson region, Ukraine) and at the Department of Analytical and Ecological Chemistry of University of Opole (Poland). The aerial parts of the four herbs were dried and kept at room temperature (15–25 °C) in a dark place before the preparation of the tinctures.

Extraction

All the tinctures were obtained in a ratio of the herbal substance to a final product as approximately 1 to 10. As a solvent, 70 % ethanol was used. The herbal substance was reduced to pieces. The crushed herbal substance was sieved through suitable sieves with the size of holes of 0.5 and 5.0 mm. Then the ground herbal substance of the size in the range of 0.5–5.0 mm was mixed with 70 % ethanol. The mixtures stood in closed containers. Maceration was performed at room temperature for 7 days. After this period the residue was separated from the extraction solvent by means of filtration through a paper filter.

Determination of the maximum absorption in differential spectra

For the determination of the maximum absortption we used the analytical procedure of differential spectrometry provided by Hudz et al. (2017a) for the estimation of the TFC in bee bread and by Hudz et al. (2020) for *Satureja montana*. 50 μL of the developed tinctures were diluted with 50 % ethanol up to 1.0 mL and was mixed with 1.0 of 2 % solution of aluminum chloride hexahydrate. The mixture was mixed by vortex and incubation was done at room temperature for 70–90 min. The volume of 2 % solution of aluminum chloride was replaced by the same amount of 50 % ethanol in the blank. The measures of all the spectra were carried out for each tincture in triplicate in the range of 360–440 nm. Instead of 50 μL of a tincture,

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we used the stock solutions of rutin trihydrate (1000 mg/L), quercetin dihydrate (400 mg/L) and 200 µL of chrysin (400 mg/L).

Results and discussion

Lamiaceae is a large plant family of mostly shrubs and herbs (Hamidpour et al., 2014; Hassanzadeh et al., 2016; Karpova et al., 2020). These plants are popular due to various biological activities, including antioxidant and antimicrobial ones (Rezvanpanah et al., 2011; Li et al., 2014; Karpova et al., 2020). These properties are closely related to a variety of secondary metabolites. Species of the Lamiaceae family are used in folk medicine for many years (Ben-Arye et al., 2011; Karpova et al., 2020). Currently, anti-inflammatory, antitussive, diuretic, anti-asthmatic, antiseptic, antispasmodic, and antipyretic activities of the herbs were revealed. Some species demonstrated even antiviral properties (Karpova et al., 2020; Shanaida et al., 2021a).

Monarda is a genus endemic to North America. This genus embraces annual and perennial flowering plants. Many species are grown as ornamentals in different countries because the flower color ranges from red to pink or light purple. *Monarda* plants produce a high quantity of essential oil. *Monarda fistulosa* and *M. didyma* (oswego tea) have a long history of use as medicinal plants by Native Americans (Francati and Gualandi, 2017).

Monarda fistulosa is commonly known as an annual or perennial medicinal plant. It produces monoterpenes in trichomes located on leaves, calyces, and even flower petals. When these trichomes are broken, the scent of escaping monoterpenes appears almost immediately. Monoterpenes have been used for thousands of years as fragrances and flavors. However, plants use them for a variety of functions, including suppression of plant competitors, repelling herbivores, or attracting pollinators and seed dispersers (Harborne, 1993).

Monarda fistulosa is known for its strong therapeutic effects. Its essential oil is characterized by high antibacterial, antimycotic, and anti-inflammatory activities (Zhilyakova et al., 2009). Wild bergamot is mentioned among plants with a high content of thymol and carvacrol up to 60–70 % in the essential oil. At the same time, it also produces significant amounts of thymoquinone – a substance with antimycotic, anticancerous and antituberculous activity (Casian et al., 2020). For this reason, it could be proposed for the treatment of the throat. Shanaida et al. showed that the major constituent of the methylene chloride extract of *Monarda fistulosa* was thymol (23.73 %), followed by carvacrol (10.09 %), *p*-cymene (9.74 %), and thymoquinone (8.52 %) (Shanaida et al., 2021b). Casian et al. (2020) stated about a yield of 12.5–14.5 g from 1 kg of the dried plant material of *Monarda fistulosa* and content of 20–32 % of thymoquinone and 23–32 % of thymol and carvacrol.

The genus *Satureja* L. (savory) embraces about 200 species of herbs and shrubs which are grown mostly in the Europe, Mediterranean region, North Africa, the Canary Islands, South America, and West Asia (Hamidpour et al., 2014). *Satureja hortensis* is an annual herbaceous crop species, strongly branched, with linear leaves.

Dried summer savory contains approximately 0.2–3.0 % of volatile oil (Hamidpour et al., 2014; Hassanzadeh et al., 2016). The main compounds found in extracts and essential oils of *Satureja hortensis* are terpenoids, phenolic compounds, flavonoids, tannins, steroids, acids, gums, mucilage, and pyrocatechols (Hamidpour et al., 2014). According to different studies, the main components of the volatile oil of *Satureja hortensis* are thymol (0.3–28.2 %), γ-terpinene (15.30–39 %), carvacrol (11–67 %), *p*-cymene (3.5–19.6 %), α-pinene (2.91 %) (Hamidpour et al., 2014; Fierascu et al., 2018). For instance, 18 compounds were identified in summer savory collected in Timis County (western region of Romania) during the growing season of the year 2017. Among them were: γ-terpinene (37.862 %), *o*-cymene (15.113 %), thymol (13.491 %), carvacrol (13.225 %), (+)-*4*-carene (6.086 %), β-myrcene (3.931 %), α-thujene (3.695 %), β-caryophyllene (1.496 %), β-pinene (1.374 %), isothymol (0.645 %), D-limonene (0.558 %), α-thujone (0.546 %) and camphor (0.521 %) (Popovici et al., 2019). Rezvanpanah et al. (2011) identified 31 compounds in summer savory of Iranian origin. Among them were: γ-terpinene (31.95 %), *p*-cymene (2.69 %), thymol (1.11 %), carvacrol (48.69 %), (+)-4-carene (6.086 %), β-myrcene (1.78 %) (Rezvanpanah et al., 2011).

The leaves of summer savory are rich in phenolic compounds, particularly rosmarinic acid and flavonoids, which provide a high antioxidant capacity of the leaves (Hamidpour et al., 2014; Shanaida et al., 2018).

The methanolic extract obtained by maceration contained rosmarinic acid (24.9 mg/g), caffeic acid (1.3 mg/g), naringenin (1.1 mg/g), isoferulic acid (220 µg/g), and apigenin (165 µg/g) (Fierascu et al., 2018). The high pressure liquid chromatography analysis confirmed the presence of gallic acid, caffeic

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acid, chlorogenic acid, ferulic acid, rosmarinic acid, and flavonoids (rutin, hyperoside, quercitrin, apigenin, quercetin, catechin, and apigenin-7-glucoside) in aqueous extracts of *Satureja hortensis* herb of Ukrainian origin (Shanaida et al., 2018).

The herbal products obtained from summer savory have antioxidant, antimicrobial, antiparasitic, pesticidal, anti-inflammatory, analgesic, hepatoprotective and anticancer properties (Hamidpour et al., 2014; Fierascu et al., 2018).

Carvacrol, cymene and thymol in the essential oil provide antimicrobial activities against food, plants, and human pathogens (Hassanzadeh et al., 2016). The evaluation of the essential oil obtained from Iranian plants showed good antimicrobial activity against several types of microorganisms, with minimum inhibitory concentration values ranging from 0.06 µL/mL for *Candida glabrata* to 8 µL/mL for *Pseudomonas aeruginosa* and minimal lethal concentration values ranging from 0.06 µL/mL for *Candida glabrata* to 16 µL/mL for *Pseudomonas aeruginosa*. The results were superior to those obtained for the used reference substances (vancomycin, gentamicin and amphotericin) for all the studied microorganisms, with exception of *Pseudomonas aeruginosa* (Fierascu et al., 2018).

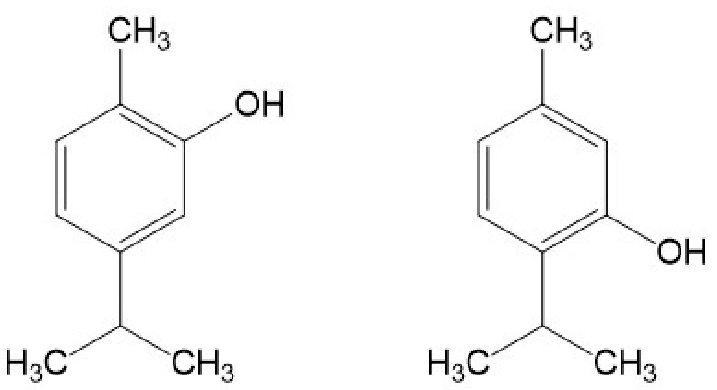
The antimicrobial mechanism is related to damage in membrane integrity, causing leakage of ions and other cell compounds and eventually death of a microbial cell (Fierascu et al., 2018).

Thymus vulgaris is an aromatic plant, which is used for medicinal and spice purposes almost everywhere in the world (Morales, 2002; Honcharenko et al., 2019). *Thymus vulgaris* shows a polymorphic variation in monoterpene production, the presence of intraspecific chemotype variation being common in the genus *Thymus*. The wild thymus grown in southern France had six chemotypes (geraniol, α-terpineol, thuyanol-4, linalool, carvacrol, and thymol). Each of these six chemotypes is named after its dominant monoterpene (Thompson et al., 2003).

The chemical structure of the most important compounds of the essential oil of *Satureja hortensis* and *Thymus vulgaris* (thymol, carvacrol and *p*-cymene) is presented in Figure 1.

Mentha piperita is a hybrid mint – a cross-species between watermint and spearmint. It is one of the most economically important medicinal and aromatic plants (Shah and Mello, 2004; Liu et al., 2014). The essential oil of this plant possesses antimicrobial, anti-inflammatory, antitussive, local anesthetic activities (Shah and Mello, 2004; Ben-Arye et al., 2011).

Marwa et al. (2017) established that the essential oil of *Mentha piperita* contained menthol (46.32 %), menthofuran (13.18 %), menthyl acetate (12.10 %), menthone (7.42 %), and 1,8-cineole (6.06 %) as the principal constituents. The tested essential oil demonstrated strong inhibitory activity against the tested microorganisms (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Micrococcus luteus* ATCC 14452, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium*, *Bacillus cereus*, *Candida albicans*, and *Candida tropicalis*. The minimum inhibitory concentrations ranged from 0.062 to 0.5 % (v/v), except for *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* was the least sensitive and was only inhibited by concentrations as high as 0.5 % (v/v) (Marwa et al., 2017).



carvacrol thymol p-cymene

Figure 1 Chemical structure of carvacrol, thymol, and p-cymene

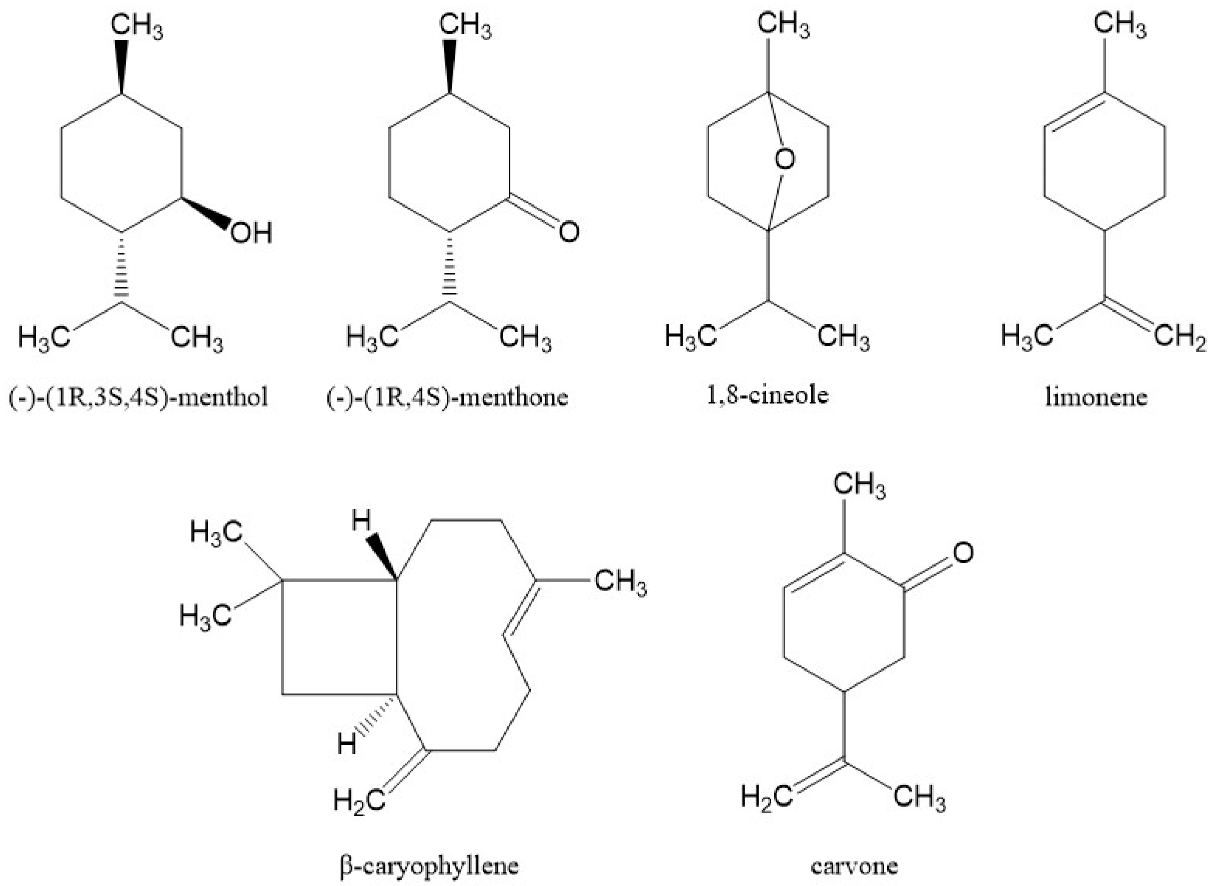


Figure 2 Active compounds of peppermint essential oil

The chemical structures of the major compounds of the essential oil of *Mentha piperita* are depicted in Figure 2.

Peppermint is famous for its flavoring and medicinal properties and is used in food, cosmetics, and medicines. It is helpful in symptomatic relief from illnesses such as colds, cramps, indigestion, nausea, sore throat, toothache, or even cancer. Many pharmacologic studies also have shown that peppermint possesses antioxidant, cytotoxic, antiallergenic, antiviral, and antibacterial activities with few side effects (Shah and Mello, 2014).

For experimental studies, we selected four herbs. Among them were *Monarda fistulosa*, *Satureja hortensis*, *Thymus vulgaris*, and *Mentha piperita*. Four tinctures of the *Thymus vulgaris*, *Satureja hortensis*, *Mentha piperita*, and *Monarda fistulosa* herb were elaborated. Ethanol absorption coefficient is an important technological parameter in the tincture manufacture. The results of the technological research are presented in Table 1.

It was established that the coefficient of alcohol absorption of the crushed raw material with the size of particles in the range of 0.5 to 5.0 mm was 2.8 ml/g,

Table 1 Calculations of the experimental determination of the absorption coefficient of 70 % ethanol

Name of herb, year of the collection	Mass of a crushed raw material (g)	Volume of 70 % ethanol for the extraction (ml)	The amount of the tincture obtained after absorption (ml)	Calculations
***Thymus vulgaris*, 2017**	7.5	100	79.0	X_1 = (100–79):7.5 = 2.80 ml/g
***Satureja hortensis*, 2017**	4.1	58	40.5	X_2 = (58–40,5):4.1 = 4.30 ml/g
	5.5	78	55.0	X_2 = (78–55): 5.5 = 4.20 ml/g
***Mentha piperita*, 2017**	6.6	93	66.5	X_3 = (93–66.5):6.6 = 4.00 ml/g
***Monarda fistulosa*, 2019**	4.2	59	39.0	X_4 = (59–39):4.2 = 4.80 ml/g

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4.0 ml/g, 4.25 ml/g, and 4.8 ml/g, respectively, for the herb of *Thymus vulgaris*, *Satureja montana*, *Mentha piperita* and *Monarda fistulosa* for 70 % ethanol.

In our studies, we also elaborated the analytical procedure for the determination of the total flavonoid content in the tinctures. We adopted the elaborated analytical procedure for the determination of the total flavonoid content in bee bread tinctures for the tinctures of the tested four herbs (Hudz et al., 2017a,b; Hudz et al., 2020).

The study of the total flavonoid content should be carried out by identifying the dominant group of flavonoids by determining the maximum absorption in the differential spectrum after adding aluminum chloride. The maximum absorption is necessary to select an analytical marker with identical or close maximum absorption of its complex with aluminum chloride in an identical solvent. Spectrometric studies should be supplemented by further chromatographic ones to confirm the correct choice of analytical marker to recalculate the amount of flavonoids (Hudz et al., 2017a; Hudz 2020).

As a result of our study we established that the solutions of quercetin (20 mg/L), rutin (50.2 mg/L), and chrysin (80 mg/L) had the maximum absorption at the wavelengths of 425.9 ±0.3 nm at 77 min, 412.3 ±0.3 nm at 82 min, 388.4 ±0.7 nm at 81 min, respectively.

The tinctures of *Monarda fistulosa*, *Satureja hortensis*, *Thymus vulgaris*, and *Mentha piperita* had one maximum absorption at 391.2 ±0.5 nm at 91 min, 389.9 ±0.5 nm at 76 min, 391.8 ±0.3 nm at 83 min, 394.9 ±1.1 nm at 78 min, respectively, in the range of 360–440 nm. The differential spectra are provided in Figure 3–5.

Therefore, the conducted spectrophotometric studies confirmed the domination of flavones in the tested tinctures. Considering the proximity of the maximum absorption of the tested tinctures and chrysin, this reference substance could be used for the determination and calculations of the total flavonoid content in tinctures.

Conclusions

The present study sets the basis for future research into the development of antimicrobial and anti-inflammatory herbal products. The knowledge of the performed review about the chemical profile of essential oil and extracts of the studied species will aid in explaining the observed biological activity. The aqueous and ethanolic extracts and essential oil could be considered as cheap, easily accessible, and a potential source of

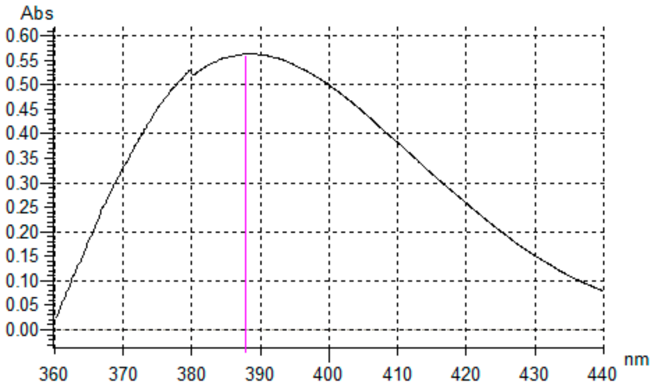

Figure 3 Differential spectrum of chrysin, concentration of chrysin 80 mg/L, A = 0.604 ±0.038, time of the reaction 81 min, λ_{max} = 388.4 ±0.7 nm

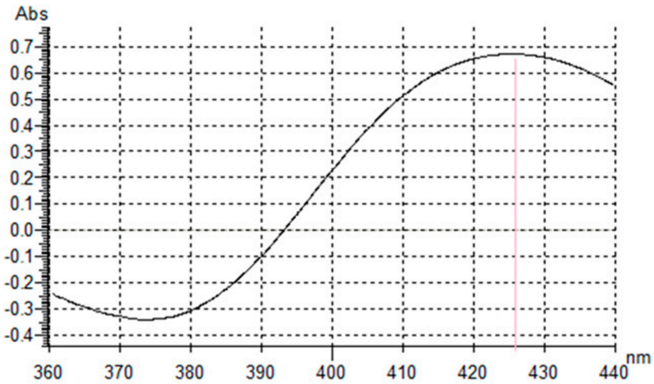

Figure 4 Differential spectrum of quercetin dihydrate, concentration of quercetin dihydrate 20 mg/L, A = 0.619 ±0.045, time of the reaction 77 min, λ_{max} = 425.9 ±0.3 nm

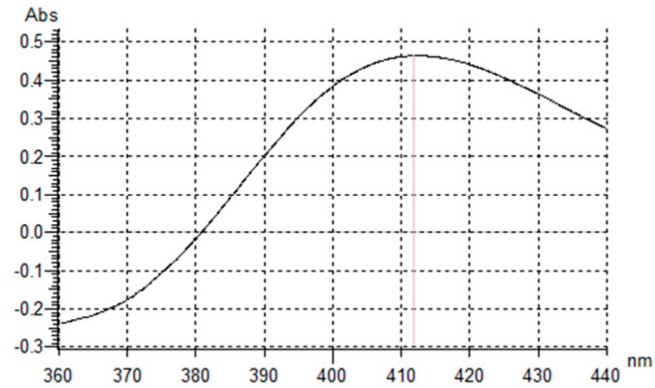

Figure 5 Differential spectrum of rutin trihydrate, concentration of rutin trihydrate 50.2 mg/L, A = 0.458 ±0.010, time of the reaction 82 min, λ_{max} = 412.3 ±0.3 nm

natural antioxidants and antimicrobial compounds for the food and pharmaceutical industry. We established the coefficients of alcohol absorption as important technological parameters for the tested raw material with the purpose of the preparation of tinctures. The coefficient of alcohol absorption of the crushed raw material with the size of particles in the range of 0.5 to 5.0 mm was 2.8 ml/g, 4.0 ml/g, 4.25 ml/g, and 4.8 ml/g, respectively, for the herb of *Thymus vulgaris*, *Mentha piperita*, *Satureja hortensis*, and *Monarda fistulosa* for 70 % ethanol. The solutions of quercetin (20 mg/L), rutin (50.2 mg/L), and chrysin (80 mg/L) had the maximum absorption at the wavelengths of 425.9 ±0.3 nm at 77 min, 412.3 ±0.3 nm at 82 min, 388.4 ±0.7 nm at 81 min, respectively, after adding aluminum chloride. The tinctures of *Monarda fistulosa*, *Satureja hortensis*, *Thymus vulgaris*, and *Mentha piperita* had the maximum absorption at 391.2 ±0.5 nm at 91 min, 389.9 ±0.5 nm at 76 min, 391.8 nm at 83 min, 394.9 ±1.1 nm at 78 min, respectively. Therefore, the performed spectrophotometric studies confirmed the domination of flavones in the tested tinctures, considering the proximity of the maximum absorption of the tested tinctures and chrysin. Future studies will be directed at the standardization of the developed tinctures and the establishment of their antimicrobial activity.

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Research Article OPEN ACCESS

Effect of alginite in the form of ALGEX$_r$ 6 preparation on the biomass formation and antioxidant activity of some medicinal plants

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The aim of the research is to determine the effect of application of the developed product (extract) called ALGEX$_r$ 6 from natural mineral rock alginite in two different watering periods on the formation of root biomass and above-ground plant biomass of a selected group of medicinal plants (*Melissa officinalis* L., *Malva verticillata* L. and *Ocimum* × *citriodorum* Vis.) and determining the antioxidant activity in dried leaves and whole plants in aqueous and methyl alcohol extracts by DPPH method. The experiment was established in the Botanical Garden at the Slovak University of Agriculture in Nitra throughout 2020. ALGEX$_r$ 6 was prepared by a research team at the Slovak University of Agriculture in Nitra in the form of an extract from the natural mineral rock alginite with an application of thermal and chemical treatment. In the experiment, ALGEX$_r$ 6 was applied in the form of a watering in two variants with the same concentration of 3 % solution in 2 decilitres of water, but various application in terms of days in the pre-harvest stage of the above-ground plant biomass of 30 individual plants from each species. There are two diametrically opposite trends of ALGEX$_r$ 6 application that are manifesting themselves in *M. officinalis* and *M. verticillata* by reducing the root and above-ground part biomass compared to the control variant. The percentage proportionality of root/above-ground part biomass in *M. officinalis* decreased from 62.48/30.31 % (control), to 45.57/18.85 % (variant 1) and to 36.07/17.27 % (variant 2), as well as in *M. verticillata* the root/above-ground part biomass decreased from 16.03/13.93 % (control), to 14.97/9.42 % (variant 1) and to 11.61/10.14 % (variant 2). In the species *Ocimum* × *citriodorum* Vis. the opposite trend manifested. The application of ALGEX$_r$ 6 watering resulted in increasing the antioxidant activity on the tested plant parts, especially in aqueous extracts in *M. officinalis* (from 19.30/control to 33.61 %/variant 1, 2), also in *O. citriodorum* (from 26.56/control to 44.16 %/variant 1), while in methyl alcohol extracts, the antioxidant activity showed a slight increase in all tested species.

Keywords: ALGEX$_r$ 6, *Melissa officinalis*, *Malva verticillata*, *Ocimum* × *citriodorum*, root, weight of above-ground part, antioxidant activity, aqueous extract, methyl alcohol extracta

Introduction

Medicinal herbs are used due to their health benefits, special aroma, taste and are considered one of the richest sources of bioactive compounds (Shanayda and Korablova, 2015; Shymanska et al., 2018; Mňahončáková et al., 2019; Ivanišová et al., 2017, 2020). The commercial development of plants as sources of antioxidants to enhance health and food

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preservation is of current interest (Rice-Evans et al., 1997). Epidemiological studies have suggested positive associations between the consumption of phenolic-rich foods or beverages and the prevention of diseases (Scalbert and Williamson, 2000; Martins et al., 2012). These effects have been attributed to antioxidant components such as plant phenolics, including flavonoids and phenylpropanoids, phenolic acids, lignan precursors, terpenes, mixed tocopherols, phospholipids, polyfunctional organic acids, other essential antioxidants such as β-carotene, vitamins C and E, selenium, zinc and others (Rice-Evans et al., 1996; Phippen, 1998, 2000; Svidenko et al., 2015a,b; Miraj et al., 2016). Thus, natural antioxidants have gained popularity in recent years. Antioxidants are primarily secondary metabolites contained in higher plants that eliminate free radicals that are harmful to the human body (Prugar, 2008). Antioxidants interfere with the process of oxidation by free radical reactions or reduction of hydroperoxides formed and the elimination of oxygen present. Natural antioxidants are obtained from plant material mainly as extracts, including medicinal and spicy plants such as Salvia spp. (Skybitska et al., 2015), *Origanum vulgare* L., *Melissa officinalis* L. (Rasmussen, 2011), *Ocimum basilicum* L., *Mentha piperita* L. (Svidenko et al., 2015a,b), *Rosmarinus officinalis* L. (Mňahončáková et al., 2019), *Allium ursinum* L. (Balaeva-Tichomirova and Leonovich, 2017), *Malva verticillata* L. (Mikhailova and Ebel, 2015), *Symphytum* spp. (Vergun et al., 2017) and other ones (Vergun et al., 2018, 2019, 2021; Shymanska et al., 2020).

Increasing or decreasing antioxidant activity in plants is conditioned by many factors. One of the factors is the growing environment. The amendment of organic or inorganic material to topsoil is considered a way to improve the physical properties of soil. Such materials affect the living space of soil microorganisms and thus also their activity (Dlapa et al., 2004; Fernandez et al., 2007; Ismail and Ozawa, 2007). The implementation of suitable natural materials that are non-toxic and their effect is almost in short time after application could be a chance for the future agriculture. One such material would be alginite. Alginite is a sedimentary laminated rock – an oil shale (Jámbor and Solti, 1975), which originated in basalt maar lakes. The name alginite originally belonged to petrographic constituent of coal residues consisting of algae (Solti, 1987). Dark laminae are rich in amorphous organic matter and well-preserved cells of green alga *Botryococcus braunii* (Ognjanova-Rumenova and Vass, 1998; Vass et al., 2003). The grey laminae are composed of clay minerals derived from weathered basaltic tuff. In addition to organic matter, the rock contains considerable amounts of macronutrients such as P, K, Ca, Mg, as well as numerous trace elements. Alginite is quite rich in nutrients, except nitrogen. Release of phosphorus, potassium and microelements (Gregor and Bublinec, 1999) could significantly contribute to supplying the demands of the microbial population (Ognjanova-Rumenova and Vass, 1998; Motyleva et al., 2014). Moreover, both the clay minerals and organic matter contained in alginite have high cation-exchange capacities (in contrast to quartz sand), thus regulating cation concentration in the soil solution (Schachtschabel et al., 1984). The content of heavy metals lies below toxicity limits. A large specific surface area ranging from 300 to 650 m^2/g results in a water retention capacity of approx. 110 % (Russell, 1990; Vass et al., 1997; Kulich et al., 2001). Tests of alginite from the deposits in Pula and Gerce (Hungary) showed that it can be used in agriculture and forestry to improve soil quality, soil water dynamics and nutrient content, to increase organic matter content, colloid content and to protect soil against acidification, desiccation and leakage of nutrients (Vass et al., 2003). No negative side effects for the environment have been observed (Kulich et al., 2001). In agriculture, alginite is also able to improve the water and nutrients regime and increasing of soil colloids (Beláček, 2006). Organic matter of alginite is a component of some types of kerogen with a predominance of type II alongside amorphous organic matter (Vass et al., 1997).

The aim of the work was to determine the effect of application of the developed product (extract) called $ALGEX_r$ 6 from natural mineral rock alginite in two different watering periods on the formation of root and above-ground plant biomass of three species of medicinal plants with a determination of antioxidant activity in dried leaves and dried whole plants in aqueous and methyl alcohol extracts by DPPH.

Material and methodology

Environment of plants cultivation

From each tested species *Melissa officinalis* L. (MO), *Malva verticillate* L. (MV) and *Ocimum citriodorum* Vis. (OC), 30 individual plants were planted in containers with a diameter of 210 mm. Garden soil was used as a cultivation medium. The experiments were established in the Botanical Garden at the Slovak University of Agriculture in Nitra in 2020 at an altitude of 167 m a. s. level (Figure 1).

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Figure 1 Demonstration of above-ground parts of medicinal plants: A – *Ocimum* × *citriodorum* Vis.; B – *Melissa officinalis* L.; C – *Malva verticillata* L. (Photo: Mňahončáková, 2020)

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Application of ALGEX$_r$ 6 preparation

ALGEX$_r$ 6 was prepared by a research team at the Slovak University of Agriculture in Nitra in the form of an extract from a natural mineral rock with the application of thermal and chemical treatment. The product is not registered yet.

In the experiment, ALGEX$_r$ 6 was applied in the form of a watering:

1. in variant 1 – only one application in the concentration of 3 % solution in 200 mL of water applied 10 days in the pre-harvest stage of the above-ground plant biomass,
2. in variant 2 – the first application in the concentration of 3 % solution in 200 mL of water applied 20 days in the pre-harvest stage of the above-ground plant biomass and the second application in the concentration of 3 % solution in 200 mL of water applied 10 days in the pre-harvest stage of the above-ground plant biomass,
3. control variant (marked C) – without application of ALGEX$_r$ 6 was implemented in each plant species (Table 1).

At the end of our experiment, the plants were removed from the containers. The roots of the plants were washed from the soil under running water. After drying in an unheated greenhouse, the weight of the roots and above-ground plant biomass was determined individually for each plant.

Free radical scavenging activity

The antiradical activity of dried leaves and dried above-ground plant biomass of medicinal plants were determined in methanolic (ME) and aqueous extract (AE). The samples 1 g in 25 mL water/methyl alcohol were mixed for 12 hours and antiradical activity was determined after filtration of samples. In the frame of antiradical activity (ability to eliminate the free radicals) was tested the capacity of medicinal plants to remove DPPH• radicals (2,2-diphenyl-1-picrylhydrazyl) using methods of Brand-Williams et al. (1995). Absorbance at 515 nm has been registered in regular time intervals until the reaction equilibrium was reached – using the GENESYS 20 Vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). First was measured the DPPH• (Sigma Aldrich, USA) absorbance without antioxidant substance (control). The inhibition of DPPH• radicals was calculated in percent of free DPPH• radicals in the samples using the method of Von Gadow et al. (1997):

$$\% \text{ Inh} = \frac{A_0 - A_1}{A_0} \cdot 100$$

where: A_0 is the absorbance of control in time t = 0 min (DPPH• solution), A_1 is the absorbance in the presence of antioxidant in time t (min), the result is in % of DPPH• radical inhibition

Statistical analysis

It was evaluated the variability of the test files in each character using descriptive statistics. For the characteristics of the files, it was used the basic

Table 1 Determination of antioxidant activity in plant parts in aqueous extracts (AE) and methyl alcohol extracts (ME) in tested plant species grown in variants

	Variants	*Melissa officinalis* L. (MO)	*Malva verticillata* L. (MV)	*Ocimum* × *citriodorum* Vis. (OC)
Dried leaves – DL	Control – C – AE	MOC-DLAE	MVC-DLAE	OCC-DLAE
	Variant 1 – AE	MO1-DLAE	MV1-DLAE	OC1-DLAE
	Variant 2 – AE	MO2-DLAE	*	*
	Control – C – ME	MOC-DLME	MVC-DLME	OCC-DLME
	Variant 1 – ME	MO1-DLME	MV1-DLME	OC1-DLME
	Variant 2 – ME	MO2-DLME	*	*
Dried herbs – DH	Control – C – AE	MOC-DHAE	MVC-DHAE	OCC-DHAE
	Variant 1 – AE	MO1-DHME	MV1-DHME	OC1-DHME
	Variant 2 – AE	MO2-DHME	*	*
	Control – C – ME	MOC-DHME	MVC-DHME	OCC-DHME
	Variant 1 – ME	MO1-DHME	MV1-DHME	OC1-DHME
	Variant 2 – ME	MO2-DHME	*	*

Note: * – untested variants; DL – dried leaves; DH – dried herbs; AE – aqueous extract; ME – methyl alcohol extract

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descriptors of variability: average, minimum measured value, maximum measured value, the coefficient of variation (%). Data were analyzed with ANOVA test and differences between means compared through the Fisher test (α = 0.05). The degree of variability was determined by the coefficient of variation values. The given parameter is independent of the unit of the evaluated character. Theoretically, they can acquire different values (Stehlíková, 1998).

Results and discussion

Melissa officinalis (MO)

In our experiment with the application of an innovated preparation of alginite (ALGEX$_r$ 6) in the form of watering for selected medicinal herbs grown in containers in two different variants, we determined the following effects on the evaluated parts of plants. The average weight of the root was determined in the range from 36.70 g (MO2) to 62.48 g (MOC), the average weight of the above-ground plant biomass in the range 17.27 g (MO2) – 30.31 g (MOC). The values of coefficients of variation indicate a medium to a high degree of variability (15.43 %/MOC – 34.44 %/MO2). After application of ALGEX$_r$ 6, we recorded a higher proportion of roots (67.33 % in MOC) between the control variant (MOC) and the other two variants (MO1 and MO2) in comparison with above-ground plant biomass (32.67 %/MOC). After application of ALGEX$_r$ 6 watering, we recorded approximately the same proportion of the weight of roots (70.73 %/MO1) and above-ground plant biomass (29.27 %/MO1) compared to the control variant (Figure 2). Analysis of variance confirmed the differences between the

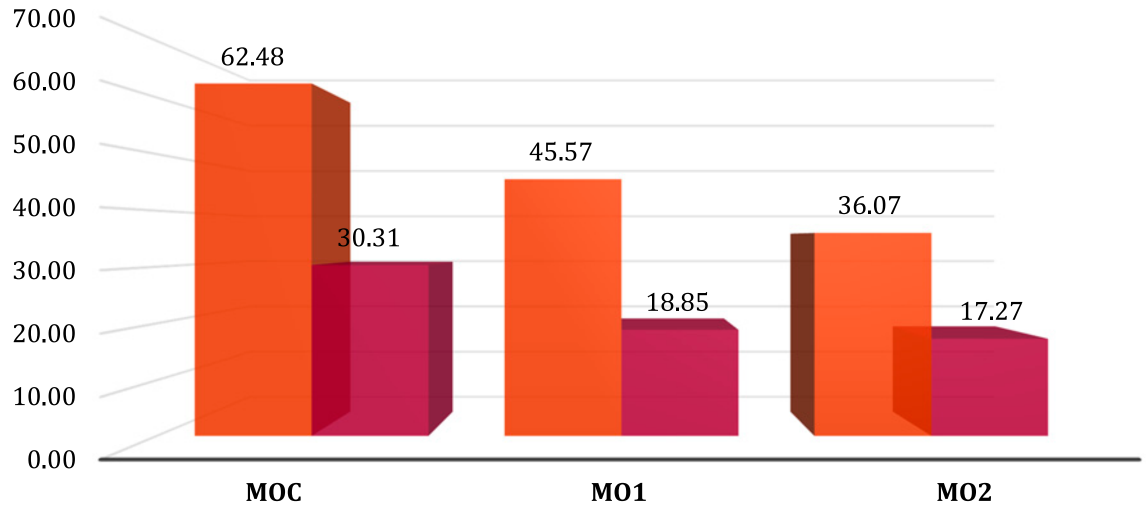

Figure 2 Comparison of the weight of *Melissa officinalis* L. root to the total weight of the above-ground plant biomass in the control variant and in the variants after application of ALGEX$_r$ 6:
MOC – control; MO1 – variant 1; MO2 – variant 2

Table 2 Analysis of variance to evaluate the effect of application of ALGEX$_r$ 6 on the weight of roots and the weight of above-ground plant biomass of *Melissa officinalis* L. plants with statistical differences between the evaluated variants

Source of variation	SS	df	MS	F	p-value	F crit	Differences between variants				
	Weight of root (g)						Variants	x̄	V %	MO2	MO1
Between variants	3579.19	2	1789.59	13.71	0.00	3.35	MOC	62.48	20.72	+++	++
Within variants	3522.66	27	130.46				MO1	45.57	23.41	–	
Total variability	7101.86	29					MO2	36.07	29.07		
Weight of above-ground plant biomass (g)											
Between variants	1013.50	2	506.75	11.63	0.00	3.35	MOC	30.31	29.22	+++	+++
Within variants	1175.92	27	43.55				MO1	18.85	21.75	–	
Total variability	2189.43	29					MO2	17.27	34.44		

Note: SS – sum of squares; df – degrees of freedom; MS – mean square; F – F statistic; p-value – probability (α = 0.05); F crit – F-critical value; x̄ – arithmetic mean; V % – coefficient of variation (%); MO1 – variant 1; MO2 – variant 2

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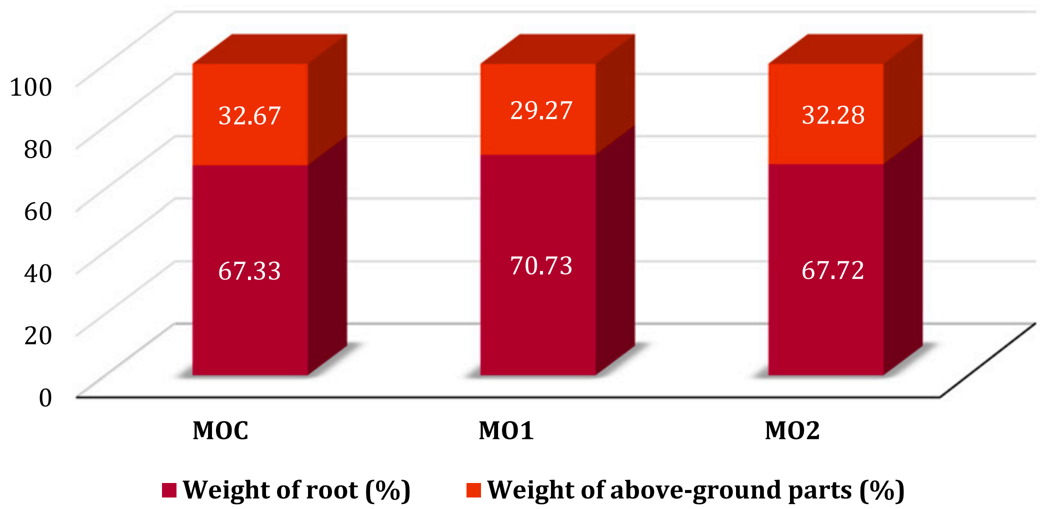

Figure 3 Comparison of the ratio of the weight of *Melissa officinalis* L. root (%) and the weight of above-ground plant biomass (%) of the total weight of the above-ground mass in the control variant and after application of ALGEX$_r$ 6 in both variants:
MOC – control; MO1 – variant 1; MO2 – variant 2

evaluated control variant and the two variants with different period ALGEX$_r$ 6 watering. We determined a statistically highly significant effect on the reduction of root weight and weight of above-ground plant biomass in both MO1 and MO2 variants compared to the control variant (Table 2 and Figure 2). The results from the analysis of variance confirmed the statistically significant differences between the evaluated traits (Table 2).

In experiments, we applied ALGEX$_r$ 6 two times (Variant 2 – MO2). In the aqueous extract, the smallest values of antioxidant activity were in the dried leaves of the control variant (19.30 %) and variant 1 (20.56 %). The highest values of antioxidant activity were achieved (MO2) dried leaves (77.74 %) and samples of whole plants (MO2) in methyl alcohol extracts (76.27 %).

After the first application of ALGEX$_r$ 6 the antioxidant activity increased especially in aqueous extracts in dried herbs (33.61 %). The effect on the increase of antioxidant activity in the evaluated traits was recorded after the second application of ALGEX$_r$ 6 in both aqueous and methyl alcohol extracts (Figure 4). Results from the analysis of variance (ANOVA) of the evaluated traits (Table 3) confirm the statistically significant differences between aqueous and methyl alcohol extracts.

Table 4 and Figure 4 showed statistically significant differences between dried leaves and dried herbs in

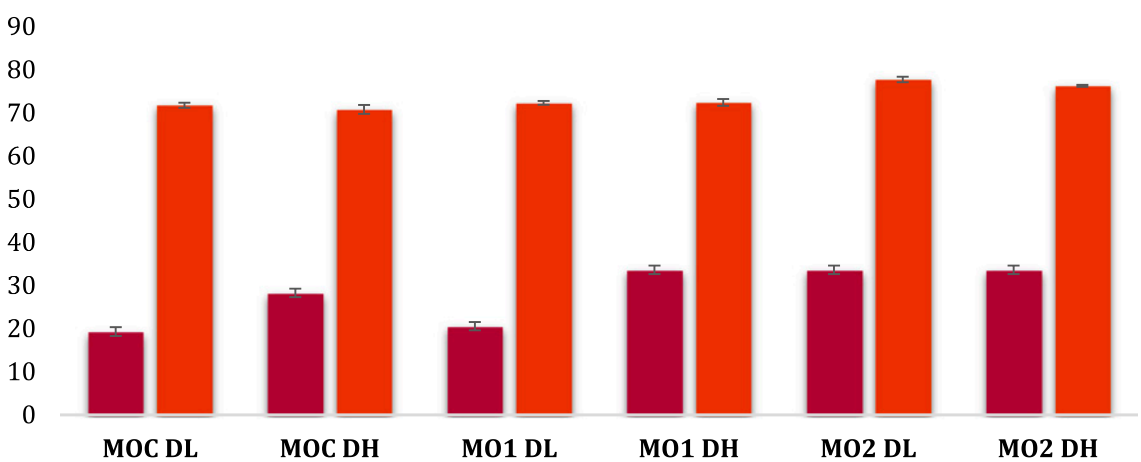

Figure 4 Comparison of antioxidant activity (%) in dried leaves (DL) and whole plants (DH) in aqueous and methanol extracts of *Melissa officinalis* L. grown in control variant (MOC) and after applications of ALGEX$_r$ 6 in variants MO1 and MO2

Table 3 Analysis of variance to evaluate the antioxidant activity in aqueous and methanol extracts of *Melissa officinalis* L. grown in control variant (MOC) and after applications of ALGEX$_r$ 6 in variants MO1 and MO2

Effect	SS	df	MS	F	p-value
AA – Aqueous Extract					
Absolute Member	10958.30	1	10958.30	454.10	0.00
Variant	508.62	5	101.72	4.22	0.02
Statistical Error	289.58	12	24.13		
AA – Methanol Extract					
Absolute Member	97368.54	1	97368.54	74412.82	0.00
Variant	115.91	5	23.18	17.72	0.00
Statistical Error	15.70	12	1.31		

Note: AA – antioxidant activity; SS – sum of squares; df – degrees of freedom; MS – mean square; F – F statistic; p-value – probability (α = 0.05)

Table 4 Statistical differences in antioxidant activity between dried leaves (DL) and dried whole plants (DH) of *Melissa officinalis* (MO) determined in aqueous (AE) and methyl alcohol extracts (ME) by Fischer test (LSD)

Variants	$\bar{x}$	V%	MOC-DLAE	MOC-DHAE	MO1-DLAE	MO1-DHAE	MO2-DLAE	MO2-DHAE	MOC-DLME	MOC-DHME	MO1-DLME	MO1-DHME	MO2-DLME
MOC-dried leaves aqueous extract	19.30	14.80											
MOC-dried herbs aqueous extract	28.24	1.10	+										
MO1-dried leaves aqueous extract	20.55	4.26	–	+									
MO1-dried herbs aqueous extract	33.61	31.47	+	–	+								
MO2-dried leaves aqueous extract	19.51	6.17	–	+	–	+							
MO2-dried herbs aqueous extract	26.80	17.66	+	+	+	+	+						
MOC-dried leaves methanol extract	71.77	1.41	+++	+++	+++	+++	+++	+++					
MOC-dried herbs methanol extract	70.78	2.46	+++	+++	+++	+++	+++	+++	–				
MO1-dried leaves methanol extract	72.30	1.02	+++	+++	+++	+++	+++	+++	–	–			
MO1-dried herbs methanol extract	72.41	1.87	+++	+++	+++	+++	+++	+++	–	–	–		
MO2-dried leaves methanol extract	77.74	1.42	+++	+++	+++	+++	+++	+++	–	–	–	–	
MO2-dried herbs methanol extract	76.27	0.53	+++	+++	+++	+++	+++	+++	–	–	–	–	–

Note: - – differences are disproven; + – differences are statistically significant; +++ – statistical significance with even smaller differences; arithmetic mean; V % – coefficient of variation (%)

methyl alcohol extracts in comparison of dried samples in aqueous extracts.

The concentration of an aqueous extract of *M. officinalis* capable to inhibit 50 % DPPH radical formation (IC_{50} value) was found to be 309 µg dry leaves per extract mL, whereas for CAF 80 µg/mL. Considering that a cup (250 mL) of a 2 % *M. officinalis* infusion or decoction contains according to findings ~1700–3300 mg dry extract it can be safely said that its consumption may effectively contribute to daily radical inhibitors intake (Papoti et al., 2019). Antioxidant extract yield from raw material *M. officinalis* leaves was 0.4 g extract/100 g plant material used rancimat method (Ribeiro et al., 2001). *M. officinalis* ethanolic extracts showed a very good antioxidant activity in the DPPH test, correlated with the content in total phenols: higher in the case of *M. officinalis* leaves extract (32.76 mg GAE/g) and lower for *M. officinalis* stems extract (8.4 mg GAE/g) (Moacă et al., 2018).

Malva verticillata (MV)

The average weight of the root in *M. verticillata* was determined in the range from 11.60 (MV2) to 16.03 g (MVC), the average weight of the above-ground plant biomass in the range 9.42 (MV1) – 13.93 g (MVC). The values of the coefficients of variation indicate a medium

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Table 5 Analysis of variance to evaluate the effect of application of ALGEX$_r$ 6 on the weight of roots and the weight of above-ground plant biomass of *Malva verticillata* L. plants with statistical differences between the evaluated variants

Source of variation	SS	df	MS	F	p-value	F crit	Differences between variants				
	Weight of root (g)						Variants	x̄	V %	MV2	MV1
Between variants	106.94	2	53.47	2.33	0.11	3.35	MVC	16.03	30.77	++	–
Within variants	617.37	27	22.86				MV1	14.97	39.00	–	
Total variability	724.32	29					MV2	11.61	27.47		
Weight of above-ground plant biomass (g)											
Between variants	117.35	2	58.67	4.00	0.02	3.35	MVC	13.93	35.20	++	+++
Within variants	395.27	27	14.63				MV1	9.42	26.73	–	
Total variability	512.62	29					MV2	10.14	36.25		

Note: SS – sum of squares; df – degrees of freedom; MS – mean square; F – F statistic; p-value – probability (α = 0.05); F crit – F-critical value; x̄ – arithmetic mean; V % – coefficient of variation (%); MV1 – variant 1; MV2 – variant 2

to a high degree of variability (14.47 %/MV2) – 38.99 %/MV1). Analysis of variance determined the differences between the evaluated control variant and the two variants with different ALGEX$_r$ 6 watering. We determined a statistically highly significant effect on the reduction of root weight in the MV2 variant compared to the control variant (Table 5 and Figure 5). The application of ALGEX$_r$ 6 resulted in a statistically significant reduction in the formation of above-ground plant biomass in both variants MV1 and MV2 in comparison with the control variant (Table 6 and Figure 4). After application of ALGEX$_r$ 6, we recorded the same proportion of roots (53 %) and above-ground plant biomass (46 %) between the control variant (MVC) and the MV2 variant. After application of ALGEX$_r$6 watering, we recorded an increase in the proportion of roots (61 %) compared to the proportion of above-ground plant biomass (38 %/MV1). This is documented in Figure 5. The results from the analysis of variance (Table 7) confirm the statistically significant differences between the evaluated traits.

DPPH provides a rapid, simple and sensitive method of evaluating the antioxidant activity of natural antioxidants. Application of ALGEX$_r$ 6 in the experiment had the effect of increasing the antioxidant activity in aqueous and methyl alcohol extract. Mutual comparison of controls (MVC) and MV1 variants showed that in general we recorded higher values of antioxidant activity in methanol and aqueous extracts compared to control variants. It is evidenced that the effect of ALGEX$_r$ 6 application determines the antioxidant activity (Figure 7).

Results from the analysis of variance (Table 6) confirmed the statistically significant differences in

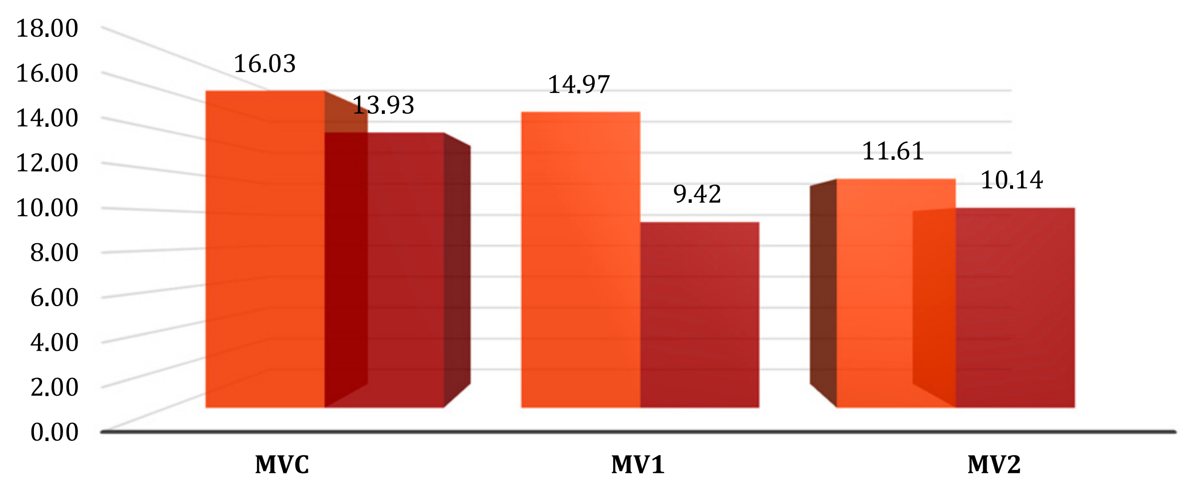

Figure 5 Comparison of the weight of *Malva verticillata* L. root to the total weight of the above-ground plant biomass in the control variant and in the variants after application of ALGEX$_r$ 6:
MVC – control; MV1 – variant 1; MV2 – variant 2

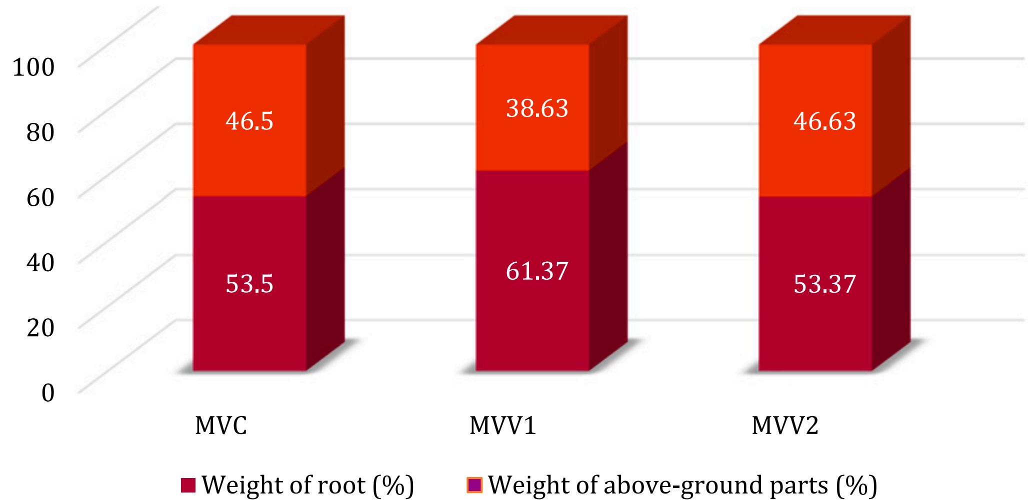

Figure 6 Comparison of the ratio of the weight of root (%) and the weight of above-ground plant biomass (%) of the total weight of the above-ground mass in the control variant and after application of ALGEX$_r$ 6 in both variants: MVC – control; MV1 – variant 1; MV2 – variant 2

aqueous and methyl alcohol extracts after application of ALGEX$_r$ 6.

Table 7 showed statistically significant differences between dried leaves and dried herbs after application of alginate preparation.

Bao et al. (2018) studied the antiradical scavenging activity of fresh leaves, stems and dried seeds of *M. verticillata* by three methods. The results showed that *M. verticillata* seeds had the highest ability to scavenge DPPH free radicals (22.14 ±0.59 mg AAE/g extract), followed by the leaves (12.62 ±0.41 mg AAE/g extract) and stems (5.15 ±0.19 mg AAE/g extract), in that order. In addition, the seeds had higher levels of antioxidants than fatsia (19.08 ±1.08 mg AAE/g extract), sesame seeds (11.09 ±0.57 mgAAE/g extract), bok choy (5.83 ±0.44 mg AAE/g extract), and broccoli (3.96 ±0.21 mg AAE/g extract) (Loizzo et al., 2016). The extracts of *M. verticillata* showed a strong ability to remove ABTS free radicals; the leaves had the strongest effect, at 363.83 ±4.22 mg Trolox/g extract, followed by the seeds at 76.47 ±5.37 mg Trolox/g extract and the stems, with the weakest effect of 46.72 ±5.07 mg Trolox/g extract. The activity of each part of *M. verticillata* was stronger than those found in vegetables such as Chinese chives (30.63 ±0.34 mg Trolox/g extract) and broccoli (45.17 ±2.41 mg Trolox/g extract) (Loizzo et al., 2016). These results are different from the ability to scavenge DPPH free radicals. The leaves of *M. verticillata* exhibited the best ability to scavenge ABTS free radicals. The activity

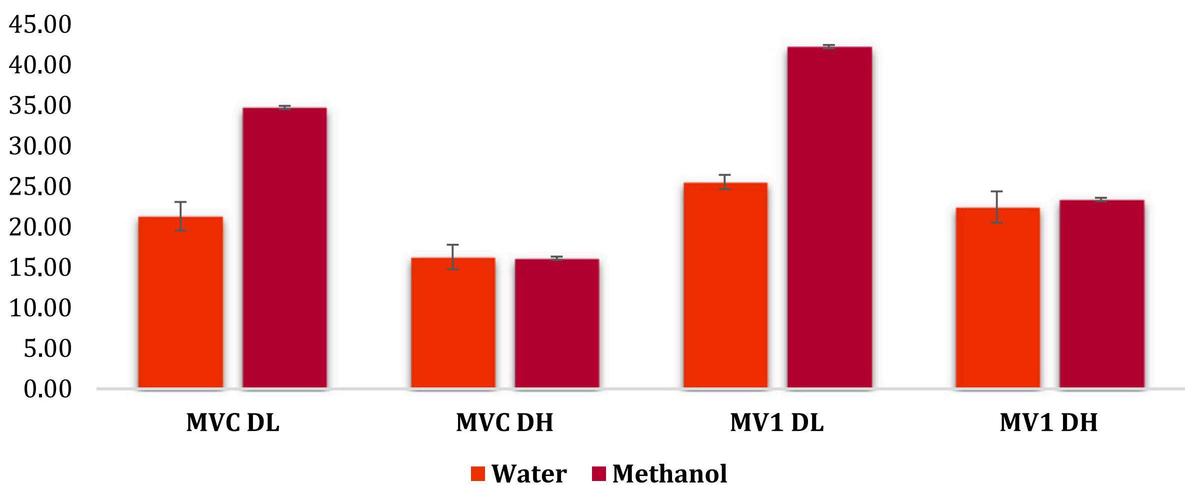

Figure 7 Comparison of antioxidant activity (%) in dried leaves (DL) and whole plants (DH) in aqueous and methanol extracts of *Malva verticillata* L. grown in control variant (MVC) and after applications of ALGEX$_r$ 6 in variants MV1 and MV2

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Table 6 Analysis of variance to evaluate the antioxidant activity in aqueous and methanol extracts of *Malva verticillata* L. grown in control variant (MVC) and after applications of ALGEX$_r$ 6 in variants MV1 and MV2

Effect	SS	df	MS	F	p-value
AA – Aqueous Extract					
Absolute Member	5491.17	1	5491.17	735.65	0.00
Variant	133.40	3	44.47	5.9573	0.02
Statistical Error	59.72	8	7.46		
AA – Methanol Extract					
Absolute Member	10178.67	1	10178.67	23088.43	0.00
Variant	1217.33	3	405.78	920.43	0.00
Statistical Error	3.53	8	0.44		

Note: AA – antioxidant activity; SS – sum of squares; df – degrees of freedom; MS – mean square; F – F statistic; p-value – probability (α = 0.05)

Table 7 Statistical differences in antioxidant activity between dried leaves (DL) and dried whole plants (DH) of *Malva verticillata* L. (MV) determined in aqueous (AE) and methyl alcohol extracts (ME) by Fischer test (LSD)

Variants	x	V %	MVC-DLAE	MVC-DHAE	MV1-DLAE	MV1-DHAE	MVC-DLME	MVC-DHME	MV1-DLME	MV1-DHME
MVC-dried leaves aqueous extract	21.31	14.25								
MVC-dried herbs aqueous extract	16.27	16.27	+							
MV1-dried leaves aqueous extract	25.53	6.01	–	+						
MV1-dried herbs aqueous extract	22.42	14.94	–	+	–					
MVC-dried leaves methanol extract	37.73	2.38	+++	+++	+++	+++				
MVC-dried herbs methanol extract	16.12	2.67	+	-	+	+	+++			
MV1-dried leaves methanol extract	42.25	2.06	+++	+++	+++	+++	+	+++		
MV1-dried herbs methanol extract	23.38	1.51	–	+	–	–	+++	+	+++	

Note: - – differences are disproven; + – differences are statistically significant; +++ – statistical significance with even smaller differences; arithmetic mean; V % – coefficient of variation (%)

of *M. verticillata* leaves was stronger than that of the seeds.

Ocimum × *citriodorum* (OC)

We determined the average weight of the root in the range from 3.08 g (OCC) to 8.20 g (OC1), the average weight of the above-ground plant biomass in the range 8.61 g (OC2) – 9.95 g (OCC). The values of the coefficients of variation indicate a low to the high degree of variability (7.70 %/OCC) – 43.53 %/OC2). The differences between the evaluated control variant and the two variants with different watering of ALGEX$_r$ 6 indicated a statistically highly significant effect on the increased root weight in the OC1 variant compared to the control variant and the demonstrable difference between OC1 and OC2 variant (Table 8 and Figure 8). After application of ALGEX$_r$ 6, we recorded a different proportion of roots and above-ground plant biomass between the control variant (OCC) and other variants (OC1 and OC2) where ALGEX$_r$ 6 was applied 10 or 20 days in the pre-harvest stage of the above-ground plant biomass. We recorded a decrease in the proportion of above-ground plant biomass (51.56 %/OC1 and 62.60 %/OC2) compared to the proportion of above-ground plant biomass of the control variant (76.36 %). This is documented in Figure 9. The results from the analysis of variance (Table 8) confirm the statistically significant differences only for root weights.

The basil flowers are irrelevant in terms of their usage. It is scientifically proven (Majdi et al., 2020) that *O.* × *citriodorum* is used as a natural source of bioactive substances when consumed in the form of food or extract. In general, the dried leaves and whole herbs showed higher antioxidant activity especially in the methyl alcohol extracts (Figure 10). In dried leaves and dried whole herbs, we determined significantly increased antioxidant activity in aqueous extracts after

Table 8 Analysis of variance to evaluate the effect of application of $ALGEX_r$ 6 on the weight of roots and the weight of above-ground plant biomass of *Ocimum* × *citriodorum* Vis. plants with statistical differences between the evaluated variants.

Source of variation	SS	df	MS	F	p-value	F crit	Differences between variants				
	Weight of root (g)						Variants	x̄	V %	OBV2	OBV1
Between variants	132.68	2	66.34	12.58	0.00	3.35	OCC	3.08	27.88	–	+++
Within variants	142.29	27	5.27				OC1	8.20	38.73	++	
Total variability	274.97	29					OC2	5.14	43.53		
Weight of above-ground plant biomass (g)											
Between variants	11.12	2	5.56	0.68	0.51	3.35	OCC	9.95	24.27	–	–
Within variants	218.50	27	8.09				OC1	8.73	35.11	–	
Total variability	229.62	29					OC2	8.61	34.96		

Note: SS – sum of squares; df – degrees of freedom; MS – mean square; F – F statistic; P-value – probability (α = 0.05); F crit – F-critical value; x̄ – arithmetic mean; V % – coefficient of variation (%); OC1 – variant 1; OC2 – variant 2

alginate application, which results in increasing some biologically active substances soluble in water.

Results from the analysis of variance (Table 9) confirm the statistically significant differences between aqueous and methyl alcohol extracts.

Table 10 showed statistically significant differences between dried leaves and dried herbs in methyl alcohol extracts and aqueous extracts.

Juliani and Simon (2002) researched the antioxidant potential of dried leaves of five green and four purple basil cultivars and breeding lines by ABTS and FRAP assay showed that essential oils were a very low antioxidant activity varying from 0.05 % 'Purples Ruffles' > 0.7 % *O. sanctum* > > 2.0 *O. bassilicum* to 5.9 % in 'Sweet' basil (FRAP) and from 0.1 % in 'Purples Ruffles' > 0.2 % *O. citriodorum* > 0.4 % *O. sanctum* > 1.2 % *O. bassilicum* to 4.1 % in 'Sweet' basil (ABTS).

Hakkim et al. (2008) studied the radical scavenging ability of antioxidants of eight *Ocimum* species in dried form. From the percentage scavenging values the *O. gratissimum* extract was the most potent scavenger (81.1 ±2.1 %) followed by *O. americanum* (77.4 ±1.4 %) > *O. minimum* (70.1 ±2.2 %) > *O. citriodorum* (60.6 ±2.5 %) > *O. kilimandscharicum* (56.2 ±2.1 %) > *O. grandiflorum* (51.3 ±2.3 %) > *O. lamiifolium* (46.2 ±2.2 %) > *O. selloi* (42.4 ±2.4 %).

Kovár et al. (2021) studied the influence of alginite (powder, crushed alginite) and extracts from it (sodium solution, potassium solution) on parameters as germination dynamics, average germination, germination rate and mean germination time of Kentucky bluegrass (*Poa pratensis* L.). The positive

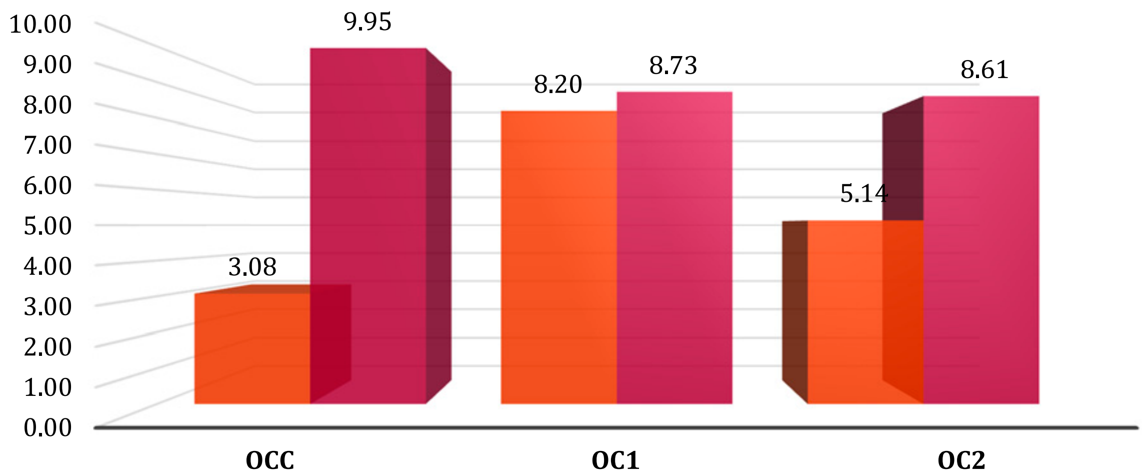

Figure 8 Comparison of the weight of *Ocimum* × *citriodorum* Vis. root to the total weight of the above-ground plant biomass in the control variant and in the variants after application of $ALGEX_r$ 6:
OCC – control; OC1 – variant 1; OC2 – variant 2

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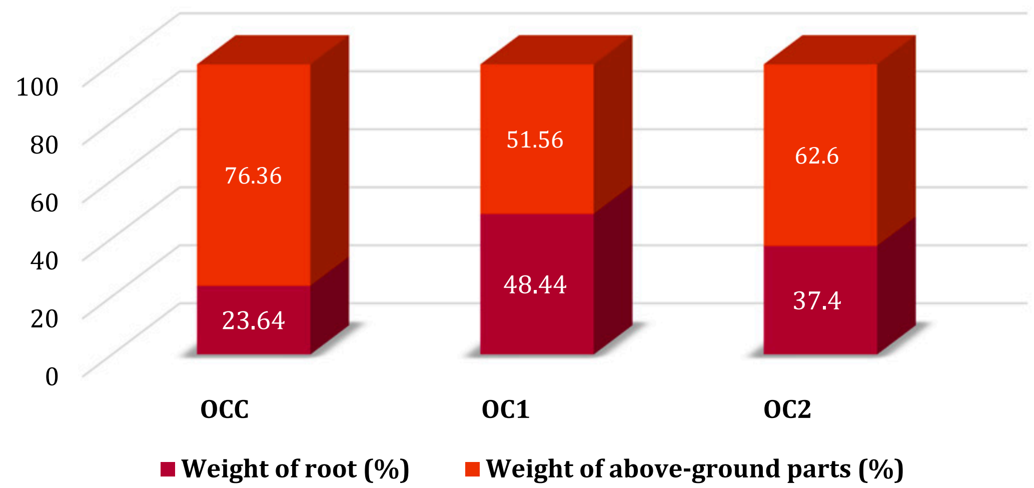

Figure 9 Comparison of the ratio of the weight of root (%) and the weight of above-ground plant biomass (%) of the total weight of the above-ground mass in the control variant and after application of ALGEX$_r$ 6 in both variants: OCC – control; OC1 – variant 1; OC2 – variant 2

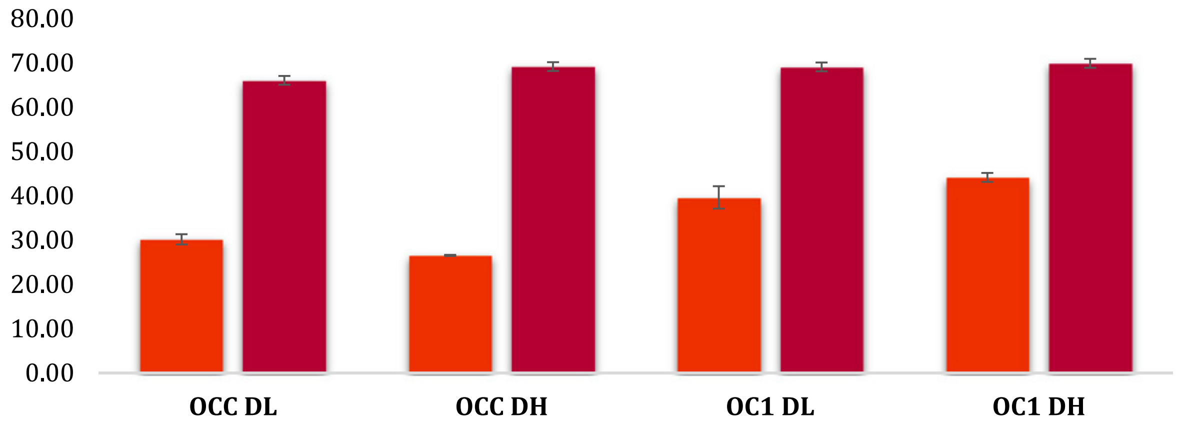

Figure 10 Comparison of antioxidant activity (%) in dried leaves (DL) and whole plants (DH) in aqueous and methanol extracts of *Ocimum* × *citriodorum* Vis. grown in control variant (OCC) and after applications of ALGEX$_r$ 6 in variants OC1 and OC2

Table 9 Analysis of variance to evaluate the antioxidant activity in aqueous and methanol extracts of *Ocimum* × *citriodorum* Vis.) grown in control variant (OCC) and after applications of ALGEX$_r$ 6 in variants OC1 and OC2

Effect	SS	df	MS	F	p-value
AA – Aqueous Extract					
Absolute Member	14811.51	1	14811.51	2253.82	0.00
Variant	599.79	3	199.93	30.422	0.00
Statistical Error	52.57	8	6.57		
AA – Methanol Extracts					
Absolute Member	56435.06	1	56435.06	13218.96	0.00
Variant	26.15	3	8.72	2.04	0.19
Statistical Error	34.15	8	4.27		

Note: AA – antioxidant activity; SS – sum of squares; df – degrees of freedom; MS – mean square; F – F statistic; p-value – probability (α = 0.05)

Table 10 Statistical differences in antioxidant activity between dried leaves (DL) and dried whole plants (DH) of *Ocimum* × *citriodorum* (OC) determined in aqueous (AE) and methyl alcohol extracts (ME) by Fischer test (LSD).

Variants	x	V %	OCC-DLAE	OCC-DHAE	OC1-DLAE	OC1-DHAE	OCC-DLME	OCC-DHME	OC1-DLME	OC1-DHME
OCC-dried leaves aqueous extract	30.16	6.66								
OCC-dried herbs aqueous extract	26.55	0.83	–							
OC1-dried leaves aqueous extract	39.65	11.02	+	+						
OC1-dried herbs aqueous extract	44.14	3.96	+++	+++	+					
OCC-dried leaves methanol extract	66.08	4.94	+++	+++	+++	+++				
OCC-dried herbs methanol extract	69.18	1.14	+++	+++	+++	+++	–			
OC1-dried leaves methanol extract	69.11	1.82	+++	+++	+++	+++	–	–		
OC1-dried herbs methanol extract	69.92	2.92	+++	+++	+++	+++	–	–	–	

Note: - – differences are disproven; + – differences are statistically significant; +++ – statistical significance with even smaller differences; arithmetic mean; V % – coefficient of variation (%)

and at the same time significant effect of alginite (crushed from) and its extracts was manifested especially in increasing of germination by 340.00 % (alginite extract), increasing average germination by 201.70 % (crushed alginate) and by 334.20 % (alginite extract), values of the mean germination time showed a shortening with using by 4.95 days (alginite extract), by 3 days (crushed alginite), by 2.82 days (powder application) compared to the controls.

Conclusions

Alginite as a bituminous rock is specific to the components of some types of kerogen with predominating kerogen type II. In addition to organic matter, the rock contains a spectrum of micronutrients – macroelements, mainly P, K, Ca and Mg, as well as a large number of microelements. Many plant species responded to the application of alginite with various effects during germination, growth, development, production of seeds, fruits, but also by increasing many biologically active substances, which also contribute to the quality of plant parts. We did not evaluate the content of their specific biologically active substances in the tested medicinal plant species. Reducing the weight of roots and above-ground parts of plants generally has a positive effect on the increase of biologically active substances in medicinal plant species. This trend was reflected by increasing antioxidant activity in the evaluated species.

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Research Article

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Antimicrobial efficacy of ethanolic extracts obtained from leaves of *Camellia japonica* L. cultivars against *Escherichia coli* strain

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The present study was aimed to determine the antibacterial activity of six plants, i.e. *Camellia japonica* L. (cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis) against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain. Ethanolic extracts were prepared by freshly crushed leaves and evaluated for their antimicrobial activity against *Escherichia coli* ATCC® 25922™ strain using disc diffusion assay. The increase of the mean of the diameters of the inhibition zone was 58.4 % for cv. Kramer's Supreme, 29.2 % for cv. La Pace and cv. Mrs. Lyman Clarke, 22.5 % for cv. Fanny Bolis, 19.1% for cv. Benikarako, and 18 % for cv. C.M. Wilson compared to the control samples (96 % ethanol). Among the six plant extracts, *C. japonica* 'Kramer's Supreme' exhibited the highest inhibitory zones against the tested strain (the mean of the zone of inhibitions was 14.1 ±1.1 mm). The intermediate activity was presented by other cultivars studied. 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. These results could provide a theoretical basis for making full use of *Camellia japonica* and its cultivars. Moreover, their antibacterial activities can play an important role in medicine, veterinary, food preservation, and other aspects. Mechanisms of antibacterial activities remain to be studied.

Keywords: *Camellia japonica*, cultivars, leaves, *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™), antibacterial efficacy, disc diffusion technique, ethanolic extracts

Introduction

The emergence of new infectious diseases and the development of drug resistance in pathogenic microorganisms prompts scientists to discover novel plant-derived bioactive compounds (Jeyaseelan and Jashothan, 2012). Therefore, medicinal plants are nowadays widely screened to determine their bioactivity and to isolate novel bioactive compounds (Khan et al., 2011). Antimicrobial properties of medicinal herbs are being increasingly reported from different parts of the world.

Camellia japonica L. is one of the best-known species of the genus *Camellia* that belongs to the Theaceae family and is widely grown in Korea and Japan (Lee et al., 2017; Jeon et al., 2018). As the ornamental plant, *Camellia japonica* is widely distributed worldwide. Previous studies have demonstrated that *Camellia japonica* has antioxidant activity. For example, Piao et al. (2011) investigating the antioxidant properties of the ethanol extract of the flower of *C. japonica* (*Camellia* extract), revealed that *Camellia* extract exhibits antioxidant properties by scavenging ROS and enhancing antioxidant enzymes. *Camellia* extract

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contained quercetin, quercetin-3-O-glucoside, quercetin, and kaempferol, which are antioxidant compounds. *Camellia* extract exhibited 1,1-diphenyl-2-picrylhydrazyl radical and intracellular reactive oxygen species (ROS) scavenging activity in human HaCaT keratinocytes. Also, *Camellia* extracts scavenged superoxide anion generated by xanthine/xanthine oxidase and hydroxyl radical generated by the Fenton reaction in a cell-free system. Furthermore, *Camellia* extract increased the protein expressions and activity of cellular antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Piao et al., 2011). Results of Kim et al. (2012) indicate that *C. japonica* oil exerts anti-inflammatory effects by downregulating the expression of the inducible isoform of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes through inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) signaling. A daily oral administration of camellia oil distillate fraction effectively inhibited spontaneous lung metastasis of BL6 cells (Miura et al., 2007).

Previous studies reported the antimicrobial activity of green tea leaves to Gram-positive organisms (Hamilton-Miller, 1995; Hamilton-Miller and Shah, 2000); however, discrepancies were found regarding its activity concerning Gram-negative rods (Shetty et al., 1994; Gordon and Wareham, 2010). The role of non-polymeric phenolic and polymeric tannin constituents in the antioxidant and antibacterial properties of six brands of green, black, and herbal teas of *Camellia sinensis* were investigated by Chan et al. (2011). Minimum inhibitory dose against Gram-positive *Micrococcus luteus*, *Staphylococcus aureus*, and *Bacillus cereus*, and Gram-negative *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa* were evaluated using the disc diffusion method. The susceptibility of the same strains to plant extracts was assessed using the disc diffusion method. Extracts and fractions of all six teas showed no activity against the three Gram-negative bacteria. Green tea inhibited all three Gram-positive bacteria with *S. aureus* being the least susceptible. Black and herbal teas inhibited the growth of *M. luteus* and *B. cereus*, but not *S. aureus* (Chan et al., 2011). Antibacterial activity of tea (*C. sinensis*) and coffee (*Coffee arabica*) with special reference to *Salmonella typhimurium* was assessed by Shetty et al. (1994). Extracts of Black tea, Japanese green tea, China tea, or Coffee inhibited the growth of various bacteria causing diarrhoeal diseases. Tea or coffee also showed bactericidal activity against *Vibrio cholerae*, *S. typhimurium*, and *S. typhi* (Shetty et al., 1994). Antimicrobial activity of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against clinical isolates of *Stenotrophomonas maltophilia* was studied by Gordon and Wareham (2010). EGCG has promising *in vitro* antimicrobial activity against *S. maltophilia*.

The results of Lee et al. (2008) strongly suggest that the anti-allergic activity of leaf extract of *C. japonica* is mediated through inhibiting degranulation and allergic cytokine secretion in mast cells. Moreover, an aqueous extract derived from petals of *C. japonica* at a concentration of 100 mg/ml was bacteriostatic against all the foodborne pathogens, i.e. *Salmonella typhimurium* DT104, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* (Kim et al., 2001). The findings of Park et al. (2015) indicated that *C. japonica* fruit extract could be a valuable candidate for herbal medicine for cardiovascular diseases associated with endothelial dysfunction and atherosclerosis. The extracts of *C. japonica* fruits exhibit a strong cardiovascular protective effect, inducing endothelium-dependent nitric oxide (NO)-mediated relaxation *via* the redox-sensitive PI_3-kinase pathway (Park et al., 2015). *C. japonica* fruits could be a potent herbal therapeutic option and source of functional food for the prevention and treatment of atherosclerosis and other diseases associated with hypercholesterolemia (Lee et al., 2016). Anti-inflammatory and gastroprotective mechanisms of *C. japonica* fruits are mediated by modulation of oxidative stress, inflammatory cytokines, and enzymes via suppression of mitogen-activated protein kinases (MAPK)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways (Akanda and Park, 2017). *C. japonica* oil may be considered as possible wrinkle-reducing candidates for topical applications (Jung et al., 2007) and exerts anti-inflammatory effects (Kim et al., 2012).

Synergistic antimicrobial activity of *C. sinensis* and *Juglans regia* against multidrug-resistant bacteria (350 Gram-positive and Gram-negative strains belonging to 10 different bacterial species) was investigated by Farooqui et al. (2015). *C. sinensis* showed higher antibacterial activity against MDR *S. typhi* than to other Gram-negative isolates (Farooqui et al., 2015).

The domestication of double flower in *Camellia japonica* and other related species has resulted in different types of double flower patterns (Vainstein, 2002). The typical *Camellia japonica* flower was defined as a single flower, with one row of overlapping petals (usually less than 8), and a columnar stamen cluster, and one normal pistil in the center. In general, within cultivated

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Camellia five major types of the double flower were identified by morphological characterizations of flower organ number, organ shape, and compositions (Gao, 2005), suggesting various diversifications of molecular mechanisms underlying the control of double flower development (Li et al., 2017).

In our previous study, the *in vitro* antimicrobial activity of ethanolic extracts of leaves derived from *Camellia japonica* (cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis) against clinical cefuroxime-resistant *Enterobacter cloacae* strain was evaluated (Kharchenko et al., 2019). It was revealed that *C. japonica* and its cultivars possess a mild antibacterial efficacy. The current study is a continuous line of our investigations directed towards the assessment of antibacterial potentials of *Camellia* plants.

Escherichia coli is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, diarrhea, urinary tract infection, and other clinical infections such as neonatal meningitis and pneumonia (Orskov and Orskov, 1985; Krogfelt, 1991). Although most genetic subtypes of *E. coli* can be harmless residents of the gastrointestinal tract, it also has the pathogenic capacity to cause severe diarrheal and extraintestinal diseases (Croxen et al., 2013). Moreover, *E. coli* is regarded among clinically important bacteria, which are indicator organisms commonly used in various projects to monitor antibiotic resistance (Boss et al., 2016). *E. coli* is a Gram-negative, oxidase-negative, rod-shaped bacterium from the family Enterobacteriaceae. It can grow both aerobically and anaerobically, preferably at 37 °C, and can either be nonmotile or motile, with peritrichous flagella. Pathogenic variants of *E. coli* (pathovars or pathotypes) cause much morbidity and mortality worldwide (Croxen et al., 2013). Also, the development of bacterial resistance to presently available antibiotics has necessitated the search for new antimicrobial agents.

Thus, the present study was aimed to determine the antibacterial activity of six plant *Camellia japonica* cultivars against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain.

Material and methodology

Collection of plant material

The leaves of *Camellia japonica* (cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis) plants cultivated at glasshouses under natural light, were sampled at M.M. Gryshko National Botanical Garden (Kyiv, Ukraine). The leaves were sampled in September 2018.

Figure 1 *Camellia japonica* L. cultivars with various double flower types maintained at M.M. Gryshko National Botanic Garden, NAS of Ukraine

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The *Camellia japonica* cultivars included in this study represent four various double flowers types, i.e. "paeony" ('Kramer's Supreme' and 'Benikarako'), "rose" ('C.M. Wilson' and 'La Pace'), "semi-double" ('Mrs. Lyman Clarke'), and "formal double" ('Fanny Bolis') (Figure 1).

Preparation of plant extracts

The collected leaves were brought into the laboratory for antimicrobial studies. Freshly washed leaves were crushed, weighed, and homogenized in 96% ethanol at room temperature to obtain the final concentration of extract 50 mg per 1 mL. The extracts were then filtered and investigated for their antimicrobial activity. The storage of extracts was in dark glass firmly sealed bottles at temperature +4 °C.

The disk diffusion method for evaluation of antibacterial activity of plant extracts

The *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain was used in the current study. 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 disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) (Bauer et al., 1966). 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 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 (CLSI, 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).

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 antibacterial activity of tested extracts. All statistical calculation was performed on separate data from each extract. The data were analyzed using 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 study was conducted to evaluate the *in vitro* antimicrobial activity of leaf extracts of 6 *C. japonica* cultivars. The data on zones of inhibition of bacterial growth of plant extracts against the *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain is demonstrated in Figure 2 and 3.

The crude extracts were analyzed for their antibacterial effect by the determination of their inhibitory zones against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain. Among the six plant extracts screened, 'Kramer's Supreme' exhibited the highest inhibitory zones against the tested strain (the mean of the zone of inhibitions was 14.1 ±1.1 mm). The intermediate activity was presented by cultivars La Pace and Mrs. Lyman Clarke (11.5 ±0.9 mm and 11.5 ±1.1 mm), cv. Fanny Bolis (10.9 ±1.2 mm), cv. Benikarako (10.6 ±0.9 mm), and cv. C.M. Wilson (10.5 ±1.0 mm) (Figure 2 and 3). The antibacterial effect of positive control was also recorded (the mean value of the inhibition zone was 9.1 ±0.5 mm). The increase of the mean of the diameters of the inhibition zone was 58.4 % for cv. Kramer's 29.2 % for cv. La Pace and cv. Mrs. Lyman Clarke, 22.5 % for cv. Fanny Bolis, 19.1% for cv. Benikarako, and 18 % for cv. C.M. Wilson compared to the control samples (96 % ethanol).

In this study, we investigated the antimicrobial activity of plant extracts by agar well diffusion. In the current study, cultivars Kramer's Supreme, C.M. Wilson, La Pace, Mrs. Lyman Clarke, Benikarako, Fanny Bolis were less potent against the test bacterium due to the observed zone of growth inhibitions.

It is noteworthy to mention that this slight effect on the *E. coli* growth, Gram-negative organisms, is most likely due to the protective nature of the outer membrane of their cell walls. The comparison of our data, with those

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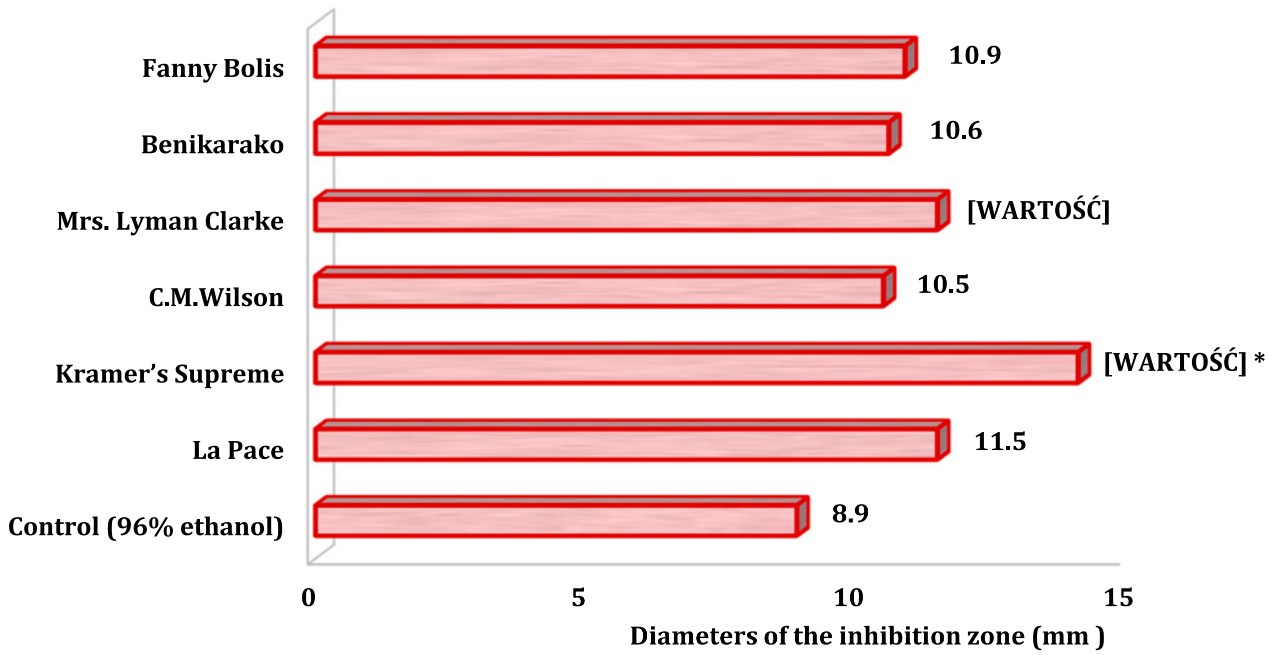

Figure 2 Zone of growth inhibitions of *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain induced by the extracts obtained from leaves of various *Camellia japonica* L. cultivars in millimeter (n = 8)
* changes are statistically significant (p <0.05) compared to 96 % ethanol (control sample)

published by other authors, reveals the findings of other researchers. Greater resistance of Gram-negative bacteria to plant extracts has been reported (Joshi et al., 2009; Koohsari et al., 2015) and this could be attributed to the differences in their cell wall structure (Ikigai et al., 1993).

Hence, these extracts would not be good candidates as drugs lead to an antibacterial agent 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.

The results obtained in the current study are in line with early reports. The potential presence of naturally occurring antimicrobials in petals of *C. japonica* active against foodborne pathogens in microbiological media and food was studied by Kim et al. (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. Similarly, 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).

Similar results were obtained also for other species of *Camellia* plants. For example, Zihadi et al. (2019) have investigated the antibacterial potential of ethanolic extract of Green tea (*Camellia sinensis*) and Neem (*Azadirachta indica*) leaves on methicillin-resistant *Staphylococcus aureus* (MRSA) and Shiga-toxigenic *Escherichia coli* (STEC). Results obtained by Zihadi et al. (2019) revealed that the maximum zone diameter of inhibition value was observed for green tea against MRSA (7.5 mm) and minimum for neem (4.9 mm). Moreover, the highest zone diameter of inhibition against STEC was also for green tea and the combination of green tea and neem (4.5 mm). The minimum inhibitory concentration (MIC) values of green tea extract were 15.625 and 31.25 mg/ml against MRSA and STEC, respectively. The combination had a similar MIC (46.87 mg/ml) against both organisms. Green tea showed the lowest minimum bactericidal concentration (MBC) values, 31.25 and 62.5 mg/ml, against MRSA and STEC, respectively. Thus, green tea and neem leaves showed good antimicrobial effects and can be used to explore novel antimicrobial compounds against MRSA and STEC (Zihadi et al., 2019).

Also, Hafiz et al. (2018) have characterized the *in vitro* antibacterial and antioxidant potential of winged

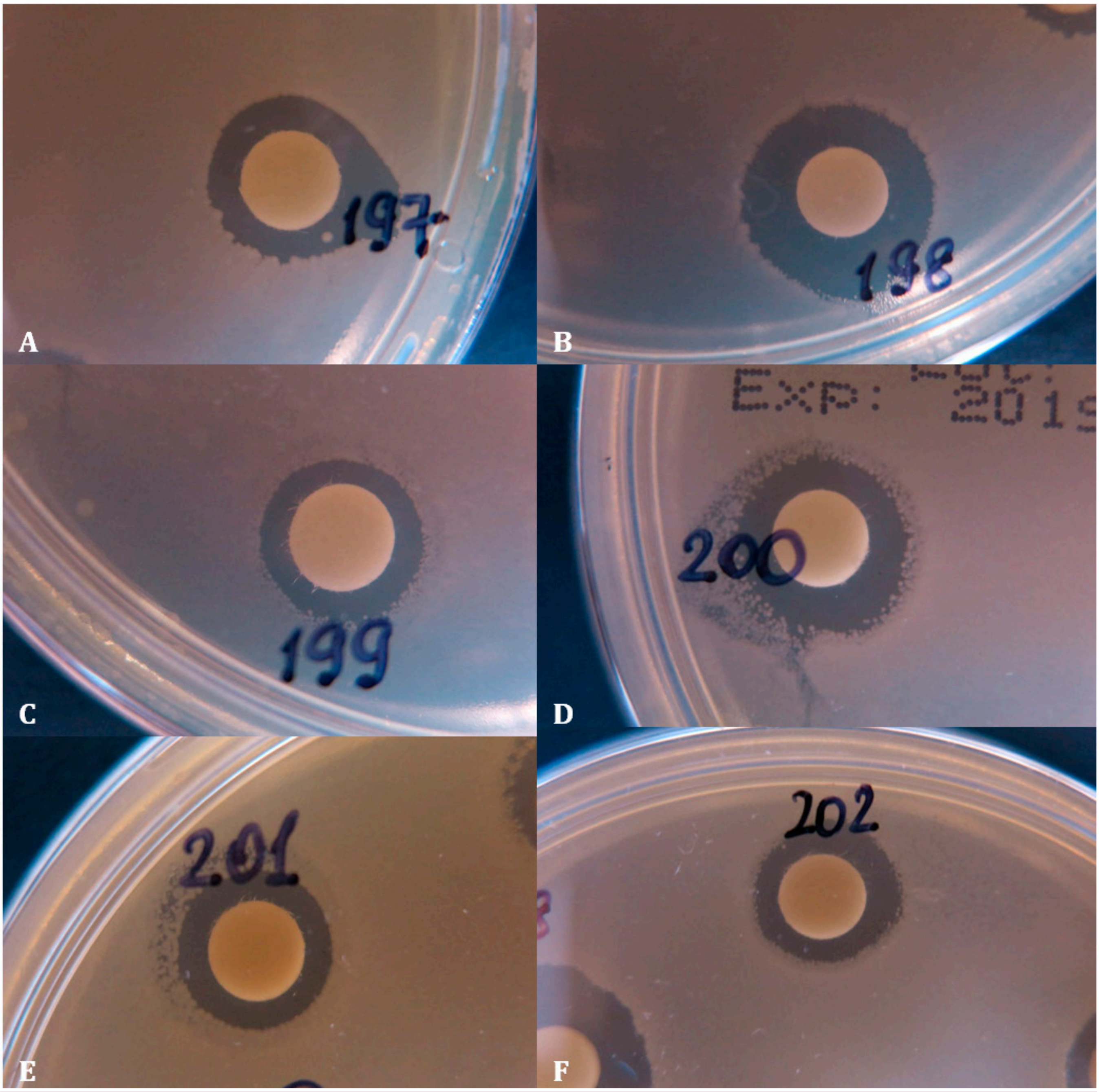

Figure 3 Antimicrobial activity of ethanolic extracts of various *Camellia japonica* L. cultivars analyzed by Kirby-Bauer disc-diffusion assay:
La Pace (A), Kramer's Supreme (B), C.M. Wilson (C), Mrs. Lyman Clarke (D), Benikarako (E), and Fanny Bolis (F)

prickly ash, green tea, and thyme. The antibacterial potential of extracts showed a significant extent of the activity against *Bacillus subtilis* and *E. coli*. Antioxidant potential exhibited the highest phenolic and flavonoid content in *C. sinensis*. The total phenolic content was significantly higher (1456.26 ±12.05 mg gallic acid) in an 80 % ethanolic fraction of *C. sinensis*. The flavonoid content in different plant extracts ranged from 8.17 ±2.02 to 376.29 ±7.11 mg/g. The radical scavenging DPPH assay also showed the significant antioxidant capacity of selected plants with the methanolic (50 %) extract of *C. sinensis* found to be the most potent (78.95 ±7.12 %) (Hafiz et al., 2018).

Extracts of green tea strongly inhibited *Escherichia coli*, *Streptococcus salivarius*, and *Streptococcus mutans*. The antibacterial effect of green and black tea extracts was compared with those of amoxicillin, cephradine, and eugenol (Rasheed and Haide, 1998). Anita et

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al. (2014) have evaluated the *in vitro* antimicrobial activity of *C. sinensis* extract on *Streptococcus mutans* and *Lactobacillus acidophilus*, the representative microbes of dental caries. MIC of green tea extract on *S. mutans* and *L. acidophilus* was found to be 0.2 and 0.3 % respectively, MBC was found to be 0.8 and 0.9 %, respectively. The mean zone of inhibition for 30 µl containing 300 µg of ethanolic extract of green tea and control against *S. mutans* was 18.33 mm and 14.67 mm, respectively. The mean zone of inhibition for 30 µl containing 300 µg of ethanolic extract of green tea and control against *L. acidophilus* was 12.67 mm and 7.33 mm, respectively. Thus, green tea has antibacterial activity against predominant cariogenic bacteria namely *S. mutans* and *L. acidophilus* (Anita et al., 2014).

On the other hand, an aqueous extract of *C. sinensis* was found to be effective against Gram-positive, Gram-negative, and fungi, as well as against drug-resistant microorganisms e.g. MRSA and *P. aeruginosa* and *Candida albicans*. Khan et al. (2019) have investigated the antibacterial and antifungal potential of aqueous extract of *C. sinensis*. Antibacterial activity was determined by disc and well diffusion assay. MIC and MBC were calculated by the broth dilution method. Miles and Misra technique was used to find out the colony-forming unit per/ml. All the test organisms revealed a diverse range of vulnerability against aqueous extract. Among Gram-positive, MRSA showed to be the most sensitive with the least MIC and MBC while Gram-negative *Pseudomonas aeruginosa* exhibited the highest sensitivity. In Miles and Misra, a progressive decline in the log of CFU/ml was observed. In the time-kill assay, a decline was noted in the viable count of *S. aureus* after exposure to 18 % aqueous extract of *C. sinensis* (Khan et al., 2019).

From a survey of the literature on this subject, it was noticed that *Camellia* species have been a subject of intense phytochemical investigation. In particular, it was shown that *C. sinensis* is the potential source of bioactive phenolic compounds with high antimicrobial and antioxidant properties. The phytochemical screening, antimicrobial, antioxidant, and cytotoxic properties of *C. sinensis* were evaluated in the study of Shah et al. (2018). The phytochemical screening revealed the presence of an applicable amount of lycopene, β-carotenes, flavonoids, and tannins in *C. sinensis*. Among the phytochemicals, tannin was found to be significantly higher in the tea plants. The results showed that the stem part of *C. sinensis* presented greater antimicrobial potential than the leaf and root. Antioxidant activity (assessed through % inhibition of linoleic acid peroxidation test) was the highest (89.22 %) in n-hexane extract of root part as compared to other extracts. Finally, the cytotoxicity analysis (hemolytic activity against human erythrocytes) of plant extract showed the negligible (%) lysis of RBCs ranging from 1.73 to 4.01 % (Shah et al., 2018). Camargo et al. (2016) have investigated the antioxidant and anticandidal activities of leaves obtained from *C. sinensis* by non-fermentation (green and white teas), semi-fermentation (red tea), and fermentation method (black tea). The results showed that non-fermented teas have a higher concentration of phenolic compounds, and then presented the best inhibitory activity of hemolysis, the best inhibition of conjugated diene formation, and more pronounced antioxidant activity in all tests. The highest anticandidal activity was obtained from fermented tea, followed by non-fermented tea (Camargo et al., 2016). Xiang et al. (2018) have determined the disinfectant efficacy of ozonated camellia oil on *Staphylococcus aureus*. According to the plate count method and turbidimetry, the bacterial concentration in the ozonated camellia oil group was lower than that in the negative control group and base oil (camellia oil) group (Xiang et al., 2018).

The crude extracts of six different plants of green tea *C. assamica* and *C. 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. 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).

In a separate study, we also 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. 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., 2017a,b; 2018).

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). 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 the regulation of 17 individual genes. 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).

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) (Gradisar 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-Martínez et al., 2005). EGCG also acts as an antifolate compound on *Candida albicans*, disturbing its folic acid metabolism (Navarro-Martínez 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

The alcoholic extracts of *Camellia japonica* and its cultivars revealed mild antibacterial activity against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain. The antimicrobial ability of various samples of these plants might be due to a wide variety of compounds. 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 chemical study as natural antibiotics to identify the secondary metabolites. These results could provide a theoretical basis for making full use of *C. japonica* and its cultivars. Moreover, their antibacterial activities can play an important role in medicine, veterinary, food preservation, and other aspects. Mechanisms of antibacterial activities remain to be studied.

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Research Article OPEN ACCESS

Comparison of budding methods of two cultivars of cornelian cherry (*Cornus mas* L.)

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Cornelian cherry (*Cornus mas* L.) is a little-known fruit species that deserves attention because of the possibility of increasing the biodiversity of crops and making the human diet more attractive. The fruit of the cornelian cherry, due to its health-promoting properties, can be widely used in the food, pharmaceutical and cosmetic industries. Increasing the cultivation area of this species depends on the availability of high-quality planting material. The reproduction of valuable varieties is possible only with the use of vegetative reproduction. One of the most effective methods of cornelian cherry reproduction is budding on cornelian cherry rootstocks. In the experiment carried out in the conditions of South-Eastern Poland, the efficiency of T-budding and chip-budding of two cultivars of cornelian cherry (Nikolka and Koralovyi Marka) was compared depending on the intensity of fertilization. Unusual weather conditions in spring, especially low temperature, delay the growth of rootstocks. However, foliar fertilization resulted in obtaining maiden trees with a larger diameter than those fertilized in the soil. The efficiency of budding under the experimental conditions was on average 40 %, and it was higher for the cultivar Koralovyi Marka and the chip-budding method. The variety and method of fertilization did not affect the efficiency of budding. In contrast, chip-budding budding was higher than that of T-budding multiplied. In this experiment a strong positive correlation was proved between the trunk diameter of maiden trees and their height.

Keywords: Cornelian cherry, cultivars, chip-budding, T-budding, sleeping eye, budding efficiency

Introduction

Cornelian cherry (*Cornus mas* L.) is a valuable fruit plant belonging to the Cornaceae family. Cornelian cherry was known already in antiquity, where its wood was used to make tools, as well as in everyday life when consuming fruit (Bieniek et al., 2017). Cornelian cherry is a widespread species in Eurasia, as its range extends from Central and Southern Europe to the Caucasus and Central Asia. Prefers sunny positions, mountain dry, rocky slopes. The range of its occurrence is up to 1511 m above sea level (Hassanpour et al., 2012). It also withstands heavily shaded positions. In the forest it grows with hornbeam (*Carpinus*) and Hungarian oak (*Quercus frainetto*). Larger concentrations of these plants can be found in the Aegean, Mediterranean, Black Sea basins and in the North-Eastern Anatolia in Turkey (Šilić, 2005). In natural sites, cornelian cherry occur most often in the form of multi-stem shrubs growing up to 3–7 m tall. However, when formed, they produce a trunk and a crown. First, they form lofty crowns, and at a later age they become spherical-flattened (Czerwińska and Melzig, 2018). Cornelian cherry fruits are associated with cherries or plums due to their juicy fruit, which are also drupes with a tart-sour, sometimes sweet-sour taste and a specific aroma. It grows slowly and the first tiny fruitlet can be seen in late April when the leaves unfold. The shape of the fruit depends on the variety. It can be round, oval, oblong, pear-shaped, bottle-shaped, elongated. The length of the fruit varies between 1–4 cm and the diameter is about 2 cm. The

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colour of the fruit can range from cream, yellow, pink, red, cherry to almost black. The peel of the fruit is elastic and shiny. The weight of the fruit ranges from 1.2 g to 6 g and even up to 10 g (Ukrainian cultivars) (Klymenko, 2017). Fruits from natural areas are small, sour and relatively little juicy with a large stone, especially in prolonged drought. Moreover, the yields from such sites are generally small: 3–5 kg of fruit per bush. They rarely reach 10–15 kg per bush, with very optimal humidity and temperature conditions. In the case of cornelian cherry, breeding selection is carried out in terms of the following criteria: fruit size, colour and shape, share of the stone in the total weight of the fruit, period of ripening and harvesting, uniformity of fruit ripening, yielding size and quality, frost resistance, growth strength and self-fertility (Hassanpour and Ali Shiri, 2014).

Cornelian cherry is still a little-known fruit species and obtaining high-quality planting material has not yet been properly elaborated. There are attempts to reproduce this species by seed and vegetatively, however, with each method, difficulties are encountered. Unfortunately, cornelian cherry, when propagated vegetatively by softwood and semi-wood cuttings, develops roots very poorly (Pirlak, 2000; Korszun and Kolasiński, 2001; Korszun and Kolasiński, 2002; Jagła and Król, 2011). The commercial cultivation of popular fruit plants such as apple, pear, plum and cherry trees is based on the use of trees obtained by such the methods like budding or grafting on rootstocks. Rootstocks control the growth force of the plant, enabling the cultivation of trees in a smaller space when growing on their own roots, and above all, they accelerate the fruiting period. It is heterovegetative reproduction, which involves the joining of two plants, usually closely related, to create a new organism. The detached part of the plant, usually a short section of the shoot, is called a scion, and that part of the plant that constitutes the root system on which the scion is grafted is called the rootstock. Such a method in which a scion consisting of an internode or several internodes and one to several buds is transferred onto the rootstock is called grafting. However, if only the bud is transferred together with a small portion of the stem with an axillary bud, it is the so-called budding. Cornelian cherry can be propagated by budding, performed in summer with a sleeping eyes on cornelian cherry seedlings, and also by grafting in winter on cornelian cherry rootstocks, rushed in a greenhouse. In addition to the faithful reproduction of the mother plant's features, it guarantees a much faster start of the fruiting period. About 50 % of maiden trees bloom and bear fruit in the second year after budding, and almost 100 % in the third year.

The aim of this study was to investigate the effectiveness of budding methods (chip-budding and T-budding) with the sleeping eyes of two Ukrainian cultivars: Nikolka and Koralovyi Marka on cornelian cherry seedlings using foliar fertilization of rootstocks.

Material and methodology

Material

The experiment was carried out in 2019–2020, in a private nursery in Czesławice, 22.268333 °E, 51.306944 °N, near Nałęczów (Poland), on fertile loess soil, rich in humus.

The average air temperature in the years 2019 and 2020 (Table 1) exceeded the long-term average, but

Table 1 Mean monthly air temperature and precipitation in the study years against the multi-annual mean/total (1951–2012)

Month	Air temperature (°C)			Precipitation (mm)		
	monthly mean			monthly total		
	2019	2020	multi-annual mean (1951–2012)	2019	2020	multi-annual total (1951–2012)
April	8.4	9.3	7.4	48.6	39.0	39.0
May	10.9	12.3	13.0	71.5	60.7	60.7
June	18.3	21.3	16.3	79.9	65.9	65.9
July	18.5	18.4	18.0	71.1	82.0	82.0
August	20.0	19.7	17.2	68.1	70.7	70.7
September	15.0	14.1	12.6	78.7	53.7	53.7
October	10.3	10.5	7.6	89.2	40.1	40.1
Mean/total for the year	9.6	9.8	7.3	861.9	558.9	558.9

in May, the two seasons marked by a decline in the average temperature. Precipitation in the 2020 season were similar to the sum of multi-year, while the 2019 season was much more precipitation, especially in September and October.

200 pieces of two-year-old generative cornelian cherry rootstocks were used for the experiment. The seedlings were planted on April 16, 2019, spaced every 25–30 cm, in two rows. Before planting the plants, fertilizers such as urea in the amount of 200 kg/ha and hydrocomplex in the amount of 400 kg/ha were applied to the whole experiment. In each row, 50 rootstocks were separated, which from May were fertilized 8 times with foliar preparations such as: Yara Tera Kristalon Orange 0.05 g/10 l, Asahi SL 10 ml/10 l, Basfoliar Aktiv SL 10 ml/10 l, Yaraliva calcinit flakes 0.05 kg/10 l.

Yara Tera Kristalon Orange is a multi-component mineral fertilizer, perfect for foliar feeding of plants, it is fully water-soluble and it is a chloride-free fertilizer. It contains NPK 6 + 12 + 36 and MgO with microelements (boron, copper, iron, manganese, molybdenum and zinc).

In contrast, Asahi SL is a growth regulator in liquid form, it has an influence on growth and better quality of the crop. It is used when the conditions are unfavorable and stressful for plant growth, e.g. drought, low temperature.

Basfoliar Aktiv SL foliar fertilizer based on sea algae extract, is an organic and mineral solution containing NPK 3 + 27 + 18 with the addition of boron, copper, iron, manganese, zinc and molybdenum, and also contains plant hormones, amino acids, vitamins and micronutrients.

Yaratera calcinit flakes is a calcium saltpetre in flakes to be dissolved in water, it improves the condition and development of plants, and also reduces the susceptibility of plants to fungal diseases and possible damage.

Methods

Adult cornelian cherry trees are not susceptible to disease and pests, however seedlings can be susceptible to diseases, particularly fungal diseases. Therefore, the following preparations were used in the experiment: Topsin M 500 S.C. (thiophanate – methyl) in the amount of 1.5 l/ha, Mospilan 200 SP (acetamiprid) in the dose of 125 mg/ha. Topsin is a contact fungicide, used against tree cancer, bark rot and brown rot of stone trees. On the other hand, Mospilan is systemic insecticide that protects the plant against pests such as aphids or adults cockchafer.

Budding began on August 17, 2019. Before budding, maintenance works were carried out, such as: manual weeding, removing the sprouds from the trunk area (at a height of 25 cm from the ground) and wiping the trunk with a clean cloth to remove soil dirt on the rootstock in order to be able to perform the budding procedure in a clean manner.

Figure 1 Methods of budding of cornelian cherry with the "sleeping eye":
A – T-budding; B – Chip-budding

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Two cultivars of sleeping eyes were used for budding: Nikolka and Koralovyi Marka. In one row the rootstocks were budded with buds (quiescent) of the cv. Nikolka, and in the next – cv. Koralovyi Marka. In each row, both in the part fertilized only with soil and in the foliar fertilization, two budding methods were performed: T-budding and chip-budding.

In the next season on March 19, 2020, the effectiveness of budding was assessed on the basis of the percentage of successful unions.

On August 21, 2020, the diameter of the maiden stem (at the base of the stem) was measured with an electronic calliper and the height of the maiden trees was determined with a ruler.

Statistical analysis

Statistical calculations were performed in STATISTICA for Windows Version 5.5A. The obtained results were subject to statistical analysis by means of analysis of variance (ANOVA). The significance of the differences among the characteristics was assessed based on confidence intervals calculated by means of a Tukey's test, with a significance level of 5 %.

Results and discussion

Cornelian cherry, due to its very valuable fruit, is cultivated in many countries, incl. in Ukraine, the Czech Republic, Slovakia, Iran, Turkey, Serbia, France, Austria and Poland. The cultivars differ in fruit ripening, shape, colour, taste, the share of seeds in the fruit, as well as in chemical properties, e.g. soluble solids content, vitamin C, anthocyanins (Kucharska et al., 2011), antioxidant activities and phenolic compounds (Klymenko et al., 2019). Obtaining valuable varieties is possible only through vegetative reproduction. An important factor in intensifying the production is high-quality nursery material. On a small scale, dogwood can be reproduced by layering (Ivanicka and Cvopa, 1977; Klymenko, 2004; Fedosova and D'Antuano, 2012; Klymenko et al., 2017). Another way of vegetative reproduction is cuttings. Herb seedlings are very delicate, sensitive and it is necessary to use fogging in their production (Ivanicka, 1989). It is an effective method, but to a large extent depends on the date of harvesting the cuttings and the type of rooting preparation (Stepanowa et al., 1986; Smykov et al., 1987; Pirlak, 2000; Gąstoł, 2007). Jagła and Król (2011), comparing the efficiency of reproduction of several less known fruit species, including dogwood, with the use of cuttings collected at the turn of July and August, supported by rooting Himal and Rhizopon, concluded that the dogwood took root the least. At both dates of picking the cuttings, the degree of rooting ranged from 0 % (seedlings without rooting preparation) to 33 % of seedlings treated with Rhizopon rooting preparation. Cornelian cherry can also be propagated *in vitro*. It is a species that is relatively easy to replicate, but in tissue cultures, as in the case of cuttings, rooting is very poor.

There are not many experiments comparing the effect of planting material on the growth and yield of cornelian cherry in an orchard. However, when assessing the rate at which fruiting begins, the best method seems to be grafting or budding on cornel rootstocks *Cornus mas* (Klymenko, 2004; Klymenko et al., 2017) or *Cornus amomum* (Ochmian et al., 2019). In addition to repeating the characteristics of the mother plant, it guarantees a much faster start of fruiting, even compared to plants obtained by cuttings. About 50 % of maiden trees bloom and yield in the second year after budding and almost 100 % in the third year (Klymenko, 1990). On the other hand, cornelian cherry seedlings obtained from seeds, bear their first fruit 8 years after planting at the earliest. Ochmian et al. (2019) found that the cornelian cherries varieties obtained by budding *Cornus amomum* showed weaker tree growth, but the fruit quality did not deteriorate compared to trees on their own roots. The fruits of dark-skinned cultivars (Jolico, Schönbrunner and Shumen) from budded trees had significantly more anthocyanins. Ciltivar Jolico mainden tree was characterized by a much larger diameter and weight.

The rootstock is an important component of the tree as it determines its growth strength, adaptation to soil conditions, disease resistance, size and quality of crops, tolerance to salinity and drought. In the case of cornelian cherry, seedlings of cornelian cherry is a good rootstock in terms of physiological compatibility. Difficulties with vegetative reproduction necessitate the use of generative rootstocks, i.e. rootstocks propagated from seeds. This type of reproduction does not ensure uniform quality, but in the case of stone trees (cherries, cherries and plums), generative rootstocks are also used in nursery technology. When comparing the method of producing dogwood trees, one can refer to the production of maiden trees with a 2-year-old-crown (Sadowski et al., 2006). The root system of such trees is three years old. Trees must be well developed and branched. The quality characteristics of trees depend on the species. For apple trees, budding 20 cm above the ground are most desirable, on dwarf rootstocks with a highly formed crown, at least 60–80 cm high, with not very long side shoots (up to 60 cm long). In the case of cornelian cherry, the budding site cannot be too high, it is practically the area of the second internode (oral information). On the basis of the research by Bielicki et al. (2008) assessing the quality of young apple, pear, cherry, plum and quince trees obtained by the ecological method, the largest number of trees in relation to seed stocks was obtained in the case of apple trees (80.3–91.2 %) and pear trees (42.9–87 %), and the lowest for cherries (8.4–17.3 %). Klimenko (2004) and Fedosova and D'Antuano (2012) confirmed that the most effective way to reproduce cornelian cherry is budding (90–98 % yield) and grafting (75–78 % yield). In the work of Szot et al. (2020), the efficiency of budding was very high (from 92.91 to 97.95 %), which indicates the usefulness of this method for cornelian cherry reproducing. The quality of the rootstocks has a great influence on the buds acceptance. In organic farming, it is difficult to protect against diseases and pests, thanks to which the rootstock is less overgrown and the duration of cambium activity is limited. Cornelian cherry, devoid of monocultures, is quite resistant to diseases and dangerous pests such as aphids and mites. The growth of maiden trees also depends to a large extent on the date of budding. According to nurserymen, the best term for budding in the T-budding is the period of intensive cambium division. In Central, Southern and Eastern Poland, cornelian cherry budding should start from late July to mid-August (Hołubowicz et al., 1993). In the experiment of Szot et al. (2020), the budding was performed in mid-August, and the high effectiveness of the treatment proves the good activity of the cornelian cherry cambium during this period. The cultivar Szafer had the highest budding efficiency (97.95 %), while the weakest genotypes: Roch, Okazały, Gruszkowy, Za bankkiem S1 and cultivar Paczoski (from 92.91 to 93.65 %). This confirms the observations of Bijelić et al. (2016), who proved in Serbia that August budding is a more effective method of reproduction (from 56.67 to 83.62 %) than grafting in the April (from 21.67 to 30.33 %). In this experiment, the effectiveness of budding was investigated (Table 2, Figure 2).

When assessing the influence of the variety, the method of budding and fertilization on the effectiveness of budding, it was found that the participation of successful unions (50 %) was in the case of the cv. Koralovyi Marka, with the budding in the T-budding and without the application of foliar fertilizers. The poorest efficiency (22 %) was that of budding in the T-budding with the buds of the cv. Nikolka, without the application of foliar fertilizers. Chip-budding was found to be more effective with both cultivars. The buds of the cv. Koralovyi Marka were better taken than by cv. Nikolka. The climatic conditions, which determine the quality of the rootstocks, have a significant influence on the budding effect.

The growth of maiden trees in a nursery depends on the genetic characteristics of the species and the variety. Bielicki et al. (2008) found that among the studied species, plums grew best on seedlings, and the cultivar had a decisive influence on the quality of the obtained maiden trees. Stachowiak and Świerczyński (2004) proved that the growth of cherry maiden trees in a nursery depends mainly on the rootstock, but among the studied cultivars, the cultivars 'Hardy Giant' and 'Sumit' had the highest trunk diameter and total height. Jacyna (2004) found that in the case of pear trees, the variety has a greater influence on the branching of maiden trees than the rootstock. Jacyna (2004) and Łanczont (2004) proved that there is a strong correlation between the trunk diameter and the number of shoots of apple, pear and plum maiden trees, which makes the trunk diameter an important quality feature of young trees. Lipecki et al. (2015) found a strong correlation between the diameter of 'Łutówka' maiden trees and the total length of

Table 2 The influence of *Cornus mas* L. cultivar, budding method and fertilization on efficiency of budding determined as participation of successful unions (%)

Cultivar	Budding method	Fertilization	Participation of successful unions (%)		
Koralovyi Marka	T-budding	application of foliar fertilizers	34	42	43.5
		without the foliar fertilizers	50		
	Chip-budding	application of foliar fertilizers	42	45	
		without the foliar fertilizers	48		
Nikolka	T-budding	application of foliar fertilizers	34	28	35.0
		without the foliar fertilizers	22		
	Chip-budding	application of foliar fertilizers	46	42	
		without the foliar fertilizers	38		

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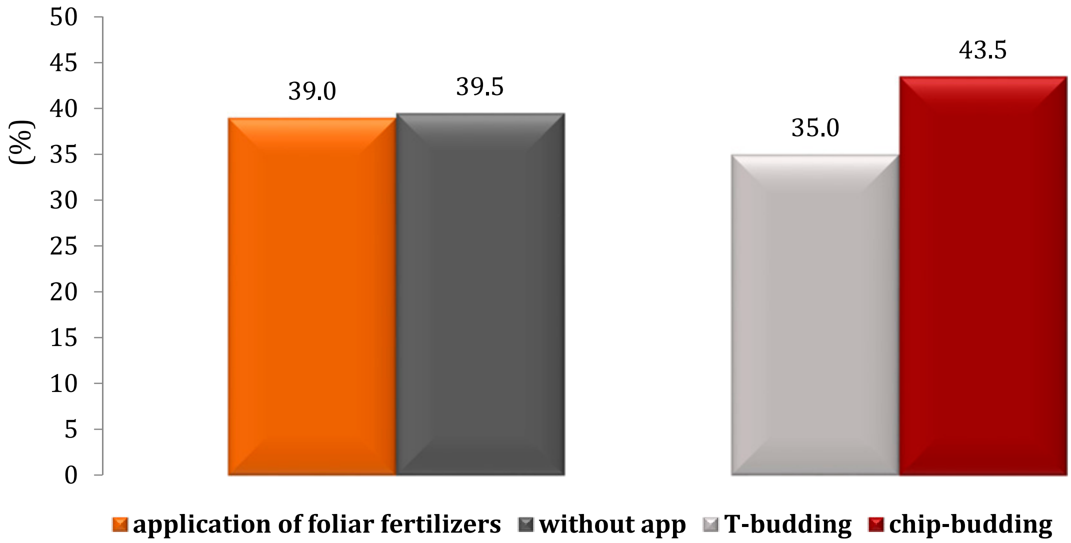

Figure 2 Participation of successful unions (%) depending on fertilization (first two bars) and methods of budding (another 3 and 4 bars)

side shoots. In the experiment of Szot et al. (2020) showed that the stem diameter was strongly positively correlated with the height of the maiden trees and the number of branches. In this experiment a strong positive correlation was proved between the trunk diameter of maiden trees and their height (r = 0.66, p <0.05) In the experiment of Szot et al. (2020), plant height was influenced by genetic features, i.e. cultivar and genotype. The highest values were found for the cultivar Bolestraszycki, the genotype Okazały and the cultivar Dublany (respectively: 1493, 1461 and 1493 mm), and the shortest ones for the Za bankiem S1 and Roch genotypes (1013 and 1050 mm). The height of the maiden trees determined on the basis of Ekaterina (2008) is similar, as in the first year of vegetation the maiden trees reach 120–150 cm. On the other hand, Bijelić et al. (2016) obtained significantly lower trees (from 73.7 to 90.7 cm) as a result of budding in August and grafting in April. In this experiment, the maiden cultivars of individual cultivars did not differ significantly in height (Table 3).

The maiden trees of cv. Nikolka were 89.54 cm high, while cv. Koralovyi Marka height was 90.68 cm. When assessing the influence of the variety, method of budding and fertilization, it was found that the highest were cv. Koralovyi Marka at chip-budding budding after foliar fertilization (93.12 cm), while the lowest cv. Nikolka, budding in T-budding, after foliar fertilization (88.34 cm). Maiden trees reproduced by chip-budding were found to be higher than T-budding.

An important feature that proves the quality of maiden trees is the diameter of the trunk. In the experiment of Szot et al. (2020) the diameter of the stem was similar for the cultivars and genotypes tested. However, the largest diameter was achieved by the cv. Dublany (16.84 mm), while the cv. Raciborski (12.93 mm) was the smallest. The obtained results confirmed the

Table 3 The influence of *Cornus mas* L. cultivar, budding method and fertilization on height of maiden

Cultivar	Budding method	Fertilization	Height of maiden (cm)		
Koralovyi Marka	T-budding	application of foliar fertilizers	88.60ab*	89.51ab	90.68a
		without the foliar fertilizers	90.41ab		
	Chip-budding	application of foliar fertilizers	93.12b	91.85b	
		without the foliar fertilizers	90.58ab		
Nikolka	T-budding	application of foliar fertilizers	88.34a	88.76a	89.54a
		without the foliar fertilizers	89.18ab		
	Chip-budding	application of foliar fertilizers	92.25ab	90.32ab	
		without the foliar fertilizers	88.40ab		

Notes: * – values in the same column with different letters are significantly different at p <0.05

Table 4 The influence of *Cornus mas* L. cultivar, budding method and fertilization on diameter of maiden stem (mm)

Cultivar	Budding method	Fertilization	Diameter of maiden stem (mm)		
Koralovyi Marka	T-budding	application of foliar fertilizers	8.03a*	8.06a	8.18a
		without the foliar fertilizers	8.10a		
	Chip-budding	application of foliar fertilizers	8.47ab	8.30a	
		without the foliar fertilizers	8.13a		
Nikolka	T-budding	application of foliar fertilizers	9.46c	9.23b	9.25b
		without the foliar fertilizers	9.00bc		
	Chip-budding	application of foliar fertilizers	9.29c	9.26b	
		without the foliar fertilizers	9.23c		

Notes: * – values in the same column with different letters are significantly different at p <0.05

research of Klymenko (1990), which in the case of the trunk diameter of cornelian cherry maiden trees was from 13 to 15 mm and is slightly higher than in the case of Bijelić et al. (2016), where the maiden trees of the studied genotypes had a diameter of 10 to 13.61 mm.

In this experiment, the maiden tree of cv. Nikolka had a significantly larger diameter (9.25 mm) than that of cv. Koralovyi Marka (8.18 mm) (Table 4).

When assessing the influence of the variety, method of budding and fertilization on the diameter of the trunk, it was found that the highest values of the mentioned feature were characteristic of the Nikolka maiden trees with T-budding, after application of foliar fertilizers (9.46 mm) and chip-budding, both after fertilization and without fertilization (9.29 and 9.23 mm).

The lower values of the described features in relation to the data from the literature should be explained by the unfavourable weather conditions in the 2019 season, which had a significant impact both on the quality of rootstocks, and then on the buds takes and the development of maiden trees. The two-year-old seedlings after planting had very unfavourable growth conditions due to the low temperatures in May, so that at the time of budding they had a diameter of about 8.7 mm. The literature lacks data on the optimal diameter of the rootstock at which the cornelian cherry should be budding with a quiescent. When compared to other species, e.g. hazel (*Corylus colurna*), it is about 8–12 mm (Ninic-Todorovic et al., 2009). Thus, in the present experiment, the rootstocks reached the minimum optimal diameter for budding.

Cornelian cherry clearly responds favourably to fertilization with greater fruit mass and more vigorous growth. It is best if it is fertilized organically, but fertilization with artificial fertilizers is equally effective and has no negative side effects (Jaćimović et al., 2020). There is still no long-term experience concerning the

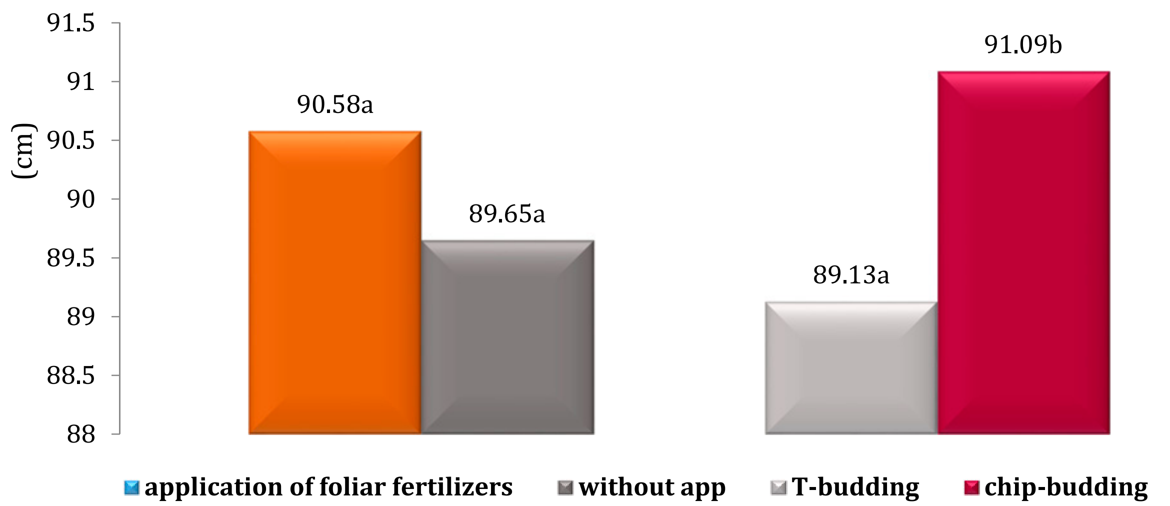

Figure 3 Height of maiden of *Cornus mas* L. (cm) depending on fertilization (first two bars) and methods of budding (another 3 and 4 bars)

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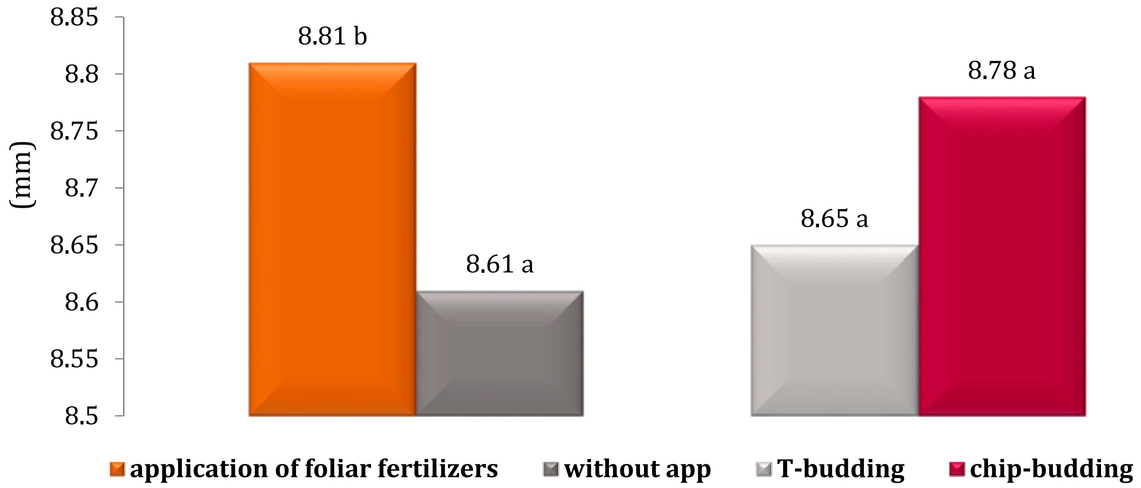

Figure 4 Diameter of maiden stem of *Cornus mas* L. (mm) depending on fertilization (first two bars) and methods of budding (another 3 and 4 bars)

influence of cornelian cherry fertilization on its growth and yielding. However, based on the preliminary experiments of Krakow researchers (Domagała-Świątkiewicz et al., 2013), it can be concluded that the plant adapts to the different availability of macronutrients such as P, K and Mg, showing no clear symptoms of their lack. A strict correlation was found between the content of P and K in the soil and the amount of these components in the leaves. Further detailed research in this direction is necessary because the content of macro- and microelements in plant tissues depends to a large extent on the conditions prevailing in a given growing season.

In this experiment, the use of foliar fertilizers significantly increased the diameter of the trunk compared to maiden fertilizers fertilized only in the soil. However, no effect of foliar fertilization on the effectiveness of budding and the height of maiden trees was found (Figure 2 and Figure 3).

On the other hand, the diameter of the fertilized rootstocks was significantly larger than those without fertilizers (Figure 4).

The production of high-quality nursery material enables cornelian cherry to quickly enter the fruiting period. However, the production of a cornelian cherry tree is a very laborious and time-consuming period. In order to obtain a seedling as a rootstock, it is necessary to cool down twice for breaking seed dormancy. Then a minimum of two or three years for the thickening of the rootstock, i.e. obtaining a diameter of 8–12 mm, optimal for budding, and only the next year after budding, the trees are ready to be planted permanently. Therefore, including the period of obtaining the rootstock, the production of trees lasts 5–6 years. This period can be shortened to 2–3 years by producing rootstocks each year.

Conclusions

Unusual weather conditions in spring, especially low temperature, delay the growth of rootstocks. However, foliar fertilization resulted in obtaining maiden trees with a larger diameter than those fertilized in the soil. The efficiency of budding under the experimental conditions was on average 40 %, and it was higher for the cv. Koralovyi Marka and the chip-budding method. The variety and method of fertilization did not affect the efficiency of budding. In contrast, chip-budding budding was higher than that of T-budding multiplied. In this experiment a strong positive correlation was proved between the trunk diameter of maiden trees and their height.

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Research Article

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Study of morphological characters of pollen grains sweet chestnut (*Castanea sativa* Mill.) by scanning electron microscopy

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The studying of *Castanea sativa* Mill. pollen allows us to determine the details of morphological characteristics and described the most important parameters and pollen sculpture that can be used to identify representatives of species. The general characteristics and significant morphological traits of pollen grains of 16 genotypes *Castanea sativa* Mill. collected from M.M. Gryshko National Botanical Garden of Ukraine(Kyiv) were observed via a scanning electron microscope (SEM). The measurement of morphometric parameters was carried out on 50 pollen grains from each genotype using the AxioVision Rel. 4.8.2.0 program. The length of the polar axis (P) and the equatorial diameter (E) of grain, P/E ratio were measured and their variation was compared among studied genotypes. SEM investigations showed that the pollen grains of *Castanea sativa* are small-sized, the equatorial view is elliptic, the polar view is angular, with convex mesocolpia, more or less circular. This study showed the small differences between the genotypes in all measured factors. The average polar axis and equatorial diameter of pollen grains values varied in the interval 19.10 ±0.21–20.53 ±0.21 and 8.86 ±0.05–9.85 ±0.08, respectively. We determined the variation coefficient in the range of 3.37–8.93 %. It was noted that diversity of surface sculpturing of pollen grains in combination with shape and sizes enables to use of a complex of thin morphological signs for *Castanea sativa* pollen identifications.

Keywords: *Castanea sativa*, genotype, pollen, SEM, morphology

Introduction

Castanea sativa Mill. (Fagaceae Dumort.) is a species of chestnut native grown in South-Eastern Europe and Asia Minor. Sweet chestnut (*Castanea sativa* Mill.) is a multipurpose species that is cultivated for its nuts and contributes positively to the forestry landscape. It is distributed mainly in the Northern Hemisphere, in Asia mostly in China, Korea and Japan, in Southern Europe from Turkey to the Atlantic Islands and in the United States. Southern Europe and Turkey is the main area where *Castanea sativa* is predominant (Silici et al., 2007).

Flowering chestnuts occurs over a relatively wide period, depending on climatic conditions, especially temperature. The opening of male flowers and the release of pollen occurs from mid-June in early flowering forms, until mid-July in late flowering forms. However, the successively flowering of male flowers can also be observed within the tree at various levels. At the level of the shoot, first, the male catkins bloom at the base and later the catkins closer to the top and finally the male part of the bisexual catkins. At the level of the catkin, the flowers in the lower part bloom first and later the flowers in the upper part of the catkin. At the level of the glomerulus, flowers bloom at the perimeter and then in the centre of the glomerulus. Pollen from bisexual catkins is released at a time when unisexual catkins have already bloomed. This pollen has the same viability as pollen from unisexual catkins and the ability to fertilize female flowers. Pollen releases from the end of August to the beginning

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of September from the secondary catkins forming on summer shoots. The formation of summer shoots can be caused by an unusual course of weather during vegetation, but it can also be a permanent feature of some genotypes (Benčať, 1967; Bolvanský et al., 2008; Bakay and Pástor, 2015).

Pollen grains adapted to different strategies of pollination and also have anatomical-morphological differences. These male haploid organisms usually have variable parameters: the pollen shape and size, the number, type, and position of apertures, and the pollen wall with its diverse structure and sculpture. The characters of these parameters in comparative pollen (and spore) morphology are at least as important as any other morphological character of the diploid generation (Halbritter et al., 2018).

Pollen morphological studies proved to be indispensable for the understanding of evolutionary processes and systematics (Persson et al., 1996; Carlo and Paula, 2004).The ability to identify plants from their pollen has enabled botanists and ecologists to reconstruct past assemblages of plants and identify periods of environmental change (e.g., Fægri and Iversen, 1989; Moore et al., 1991). Research into the morphological characteristics of pollen grains by scanning electron microscopy (SEM) of specific genotypes and cultivars are important and useful for taxonomy, palaeobotany, phylogeny, breeding programmes, e.g. (Giveyrel et al., 2000; Radice et al., 2003).

Taxonomists and paleobotanists considered the importance of pollen development and morphology in clarifying the classification and identity of many plant species, e.g., *Cornus mas* L. (Mert, 2009), *Crataegus* spp. (Wrońska-Pilarek et al., 2013), *Diospyros virginiana* L. (Grygorieva et al., 2017), *Pyrus communis* L. (Motyleva et al., 2017), *Sambucus nigra* L. (Horčinová Sedláčková et al., 2020; Wrońska-Pilarek et al., 2020), *Aronia mitschurinii* A.K. Skvortsov & Maitul. (Grygorieva et al., 2018), *Prunus persica* (L.) Batsch (Radice et al., 2003), *Malus domestica* Borkh (Motyleva et al., 2017, 2018), *Rubus fruticosus* L. (Motyleva et al., 2018).

Data about Fagaceae pollen morphology were obtained due to the use of a light microscope (LM) and scanning electron microscope (SEM) by authors Bergamini (1975) in Italy, Solignat and Chapa (1975) in France, Mert et al. (2007), Tüylü and Sorkun (2007), Evrenosoglu and Misirli (2009) and Çelemli et al. (2016) in Turkey, Grygorieva et al. (2015, 2016) in Slovakia and Ukraine. The complex of morphological characteristics and ultrastructure allows determining the differences (or similarities) between the *Castanea* species (van Benthem et al., 1984; Grygorieva et al., 2015; Xioung et al., 2020).

The knowledge of pollen morphological characteristics can be an adequate method for identification genotypes of *Castanea sativa*.

Material and methodology

Locating trees and data collection

The pollen of 16 *Castanea sativa* genotypes (CS-01 – CS-16) from the collection of M.M. Gryshko National Botanical Garden of NAS of Ukraine (NBG) was investigated at the laboratory of the Department of Tropical and Subtropical plants of NBG. Collected pollen from 30-year-old *Castanea sativa* plants (Figure 1).

Pollen grains collection

Freshly flowers (not opened) were collected randomly from the different genotypes at the balloon stage (June 2020). Pollen samples released from male flowers (catkins) were further dried under laboratory conditions. The dry pollen was used for a microscopic study of morphological characteristics. The samples of pollen grains were applied to double-tape, fastened to metal object tables with 10 mm diameter.

Scanning electron microscopy (SEM)

The pollen grains were studied at the laboratory of the Department of Tropical and Subtropical plants of NBG using an electron microscope Carl Zeiss LS 15, and the microphotographs were taken. The comparative morphological studying of the pollen grains was performed according to the working rules on the SEM JEOL JSM-6390 in the conditions of low vacuum (P = 60 Pa) with the following zooming: 500 times – during the measurements; 1000–10000 times – while taking the pictures of the exine sculpture features. Using the regime of low vacuum allows performing the pollen studying without its preliminary chemical treatment and to receive undistorted data about the research object that makes the process of the probe preparation easier. Typical exine patterns, shape, sizes and dimensions of pollen grains for *Castanea sativa* individuals were determined by using a scanning electron microscope (SEM).

Morphometric characteristics

The measurement of morphometric parameters was carried out on 50 pollen grains from each genotype using the AxioVision Rel. 4.8.2.0 program. The measurements were made in micrometres (μm). The

Figure 1 *Castanea sativa* Mill. in M.M. Gryshko National Botanical Garden of Ukraine, Kyiv (Photo: Grygorieva, 2019)

characterization of pollen grains was calculated by taking the following parameters: the polar axis (P – the line connecting the proximal and distal pole), the equatorial axis (E – the line perpendicular to the polar axis and located in the equatorial plane).

Statistical analysis

Statistical analysis was performed using SAS®9.2 software; hierarchical cluster analyses of similarity between genotypes were computed on the basis of the Bray-Curtis similarity index; BiPlot analysis was performed in SAS® 9.2 software. The variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by Stehlíková (1998).

Results and discussion

A study of 16 evaluated individuals of *Castanea sativa* pollen morphology showed that pollen grains are very small-sized. Using optical microscopy the pollen grain of *Castanea sativa* type is 3-zonocolporate, isopolar, P/E ratio is prolate, outline in the polar view is lobate, the grain is 3 apertures. Ectoaperture is colpus, long, narrow and sunken; margins are distinct, regular; ends acute; colpus membrane occasionally with irregular granules; fastigium absent; sexine extensions above endoaperture usually present; costae colpi absent. Endoaperture is colpus, lalongate, elliptic in outline; margins are distinct, straight; ends acute or irregularly diffuse, sometimes with horns; costae distinct, narrow. Exine is thin. Sexine equally thick, or slightly thicker than nexine. Sexine subdivisions weakly developed. Outlines: Equatorial view is elliptic, the polar view is angular, with convex mesocolpia, more or less circular. Ornamentation under the scanning electron microscope is rugulate, perforate, tectum is eutectate, infratectum columellate, columellae is very short, endexine is compact-continuous, intine monolayered, pollen coatings absent, Ubisch bodies are present. We can confirm theis knowledge with some authors (van Benthem et al., 1984; Mert et al., 2007; Grygorieva et al., 2015, 2016; Çelemli et al., 2016; PalDat, 2021).

This type is easily distinguished from other types in the Fagaceae family by its much smaller size, its endocolpus and its semi-erect to erect P/E ratio.

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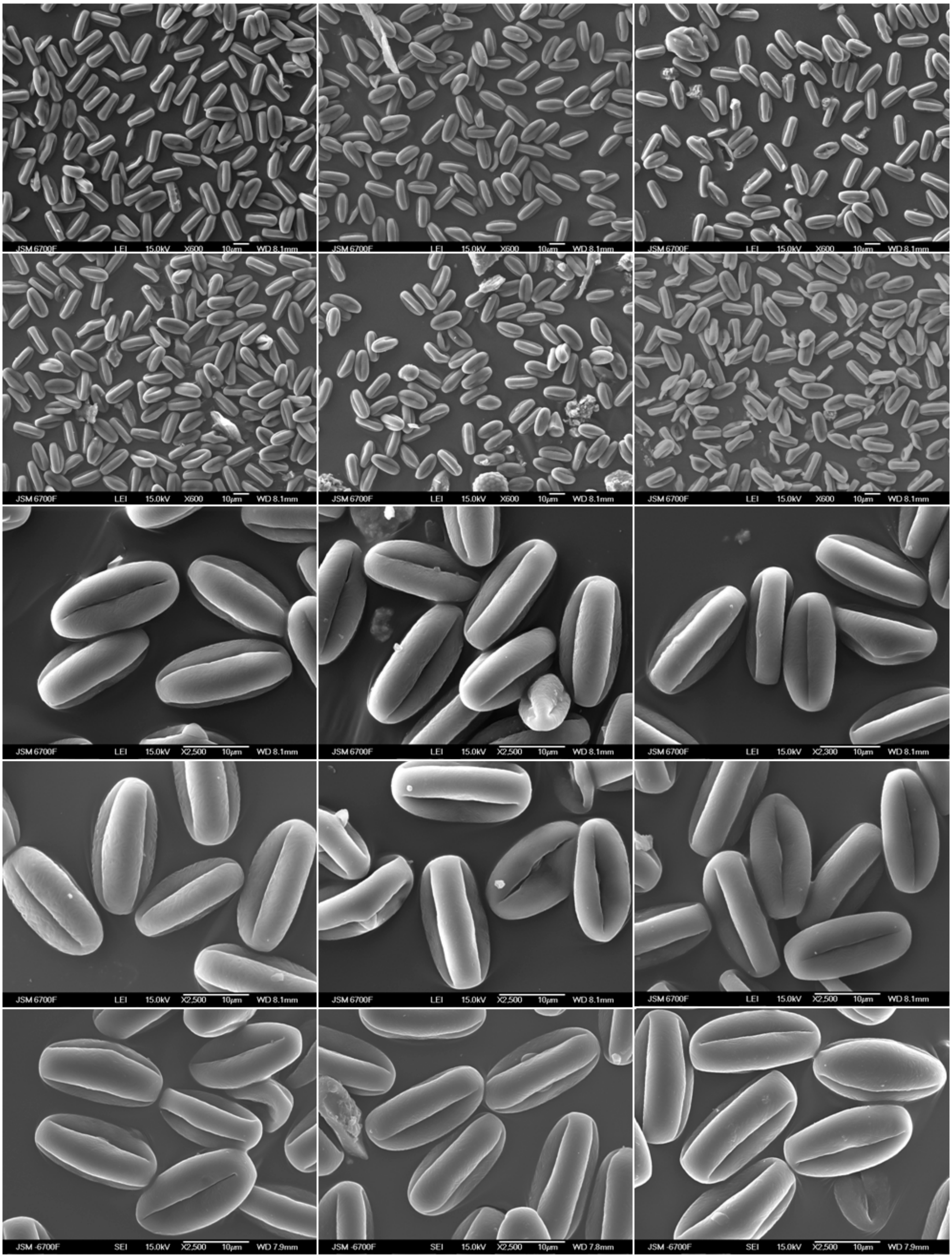

Figure 2 Pollen grains of *Castanea sativa* Mill. species in different positions (Photo: Gurnenko, 2020)

Furthermore, this type differs in the thickness of the exine and the rather smooth appearance of the grains in LM, in contrast with the sculptured grains of the *Fagus* and *Quercus* types. Its endocolpus, especially at the ends, is less regular than the endoaperture of *Fagus sylvatica* (van Benthem et al., 1984).

The polar axis (P), equatorial diameter (E) and polar axis to equatorial diameter (P/E) ratio of pollen grains of sixteen *Castanea sativa* individuals were measured using scanning electron microscopy (SEM), and the results are displayed in Table 1. An important morphological trait is the size of pollen grains. The length of the polar axis (P) varied from 13.13 (CS-14) to 23.75 (CS-16) µm and the width of the equatorial axis (E) was in the range from 6.57 (CS-14) to 11.84 (CS-10) µm. The average length of the polar axis was in the interval 19.10 ±0.21 (CS-04, CS-15) – 20.53 ±0.21 (CS-14), the average width of the equatorial diameter was in the interval 8.86 ±0.05 (CS-08) – 9.85 ±0.08 (CS-10).

According to the average values, the genotype CS-10, CS-12 and CS-14 have the largest pollen grains. The values of variation coefficient were in the range from3.37(CS-102) to 8.93(CS-14) % for polar axes and from 5.13 (CS-08) to 7.90 (CS-11) % for equatorial axes. Shape index (SI) of pollen grain depends on parameters of polar (P) and equatorial (E) axis. Shape index (the P/E ratio) of tested species varied from 2.05 (CS-10) to 2.22 (CS-03, CS-08, CS-13). The sizes of pollen *Castanea sativa* are similar with results of Grygorieva et al. (2015, 2016) and Mert et al. (2007), whereas in comparison sizes of pollen grains and P/E ratio (2.05–2.22) with authors Çelemli et al. (2016) (1.37) or Bergaminy (1975) (1.2–1.6) our studied genotypes *Castanea sativa* are being greater (Table 2).

The obtained results are confirmed by the data of Haragsim (2004), who states that the chestnut belongs to the species with the smallest pollen grains. Solignat and Chapa (1975) determined the pollen length in the interval from 14 to 18 µm and a pollen width in the range from 10 to 14 µm. Modification of length may not be positively correlated with the modification of pollen width. Grygorieva et al. (2015) in their study of evaluation of Ukrainian edible chestnuts reported a pollen length from 12.58 to 24.39 µm and width from 7.10 to 13.92 µm. Thus, the length to width ratio (shape index) can vary from 1.4 to 2.0, and pollen grains can have a short-elliptical to elongated-elliptical shape.

In the years 2013/2015 Grygorieva et al. (2016) determined and observed the length of pollen grains in the range 17.04–22.65 µm/16.78–22.08 µm and width

Table 1 The measured pollen morphological traits of selected genotypes of *Castanea sativa* Mill.

Genotypes	min	max	x	V%	min	max	x	V%	min	max	x	V%
	P – polar axis (µm)				E – equatorial axis (µm)				SI – shape index (P/E)			
CS-01	17.20	20.73	19.14	4.04	8.09	10.05	9.05	5.26	1.82	2.40	2.12	6.30
CS-02	17.36	21.73	19.48	5.67	7.75	10.68	8.95	7.41	1.89	2.55	2.18	6.14
CS-03	17.13	21.53	19.78	4.60	7.20	10.65	8.94	7.12	1.91	2.56	2.22	6.47
CS-04	16.01	21.13	19.10	7.35	7.69	10.28	9.15	5.86	1.67	2.41	2.09	8.15
CS-05	14.17	21.92	19.29	6.46	7.69	10.59	9.21	5.94	1.57	2.44	2.10	7.13
CS-06	14.44	21.65	19.51	6.07	7.04	10.28	9.03	6.12	1.83	2.59	2.16	6.81
CS-07	17.42	20.76	19.36	4.21	8.06	10.43	9.19	6.09	1.89	2.41	2.11	5.84
CS-08	17.50	21.29	19.68	3.66	7.97	9.98	8.86	5.13	1.94	2.61	2.22	4.68
CS-09	14.38	21.74	19.70	6.09	8.50	10.99	9.46	5.77	1.62	2.43	2.09	7.86
CS-10	18.36	21.66	20.12	3.37	8.42	11.84	9.85	6.07	1.60	2.31	2.05	6.37
CS-11	15.27	22.13	19.53	6.00	7.88	11.81	9.47	7.90	1.70	2.38	2.07	7.10
CS-12	14.31	22.67	20.16	7.07	7.94	10.50	9.33	6.25	1.76	2.41	2.17	7.90
CS-13	16.92	22.58	19.84	5.27	7.99	10.43	8.94	5.49	1.89	2.49	2.22	5.56
CS-14	13.13	22.94	20.53	8.93	6.57	10.54	9.39	7.87	1.71	2.51	2.19	5.57
CS-15	14.07	21.12	19.10	5.82	7.40	10.28	8.98	6.43	1.84	2.41	2.13	5.90
CS-16	16.95	23.75	19.84	5.00	7.88	10.94	9.16	5.85	1.77	2.58	2.17	7.07

Note: min – minimum value; max – maximum value; x- average; V – variation coefficient (%)

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Table 2 Literature data on pollen morphological traits in the *Castanea sativa* Mill.

Characteristic	Value	Authors	Country
Polar axis (µm)	10–15	Bergaminy, 1975	Italy
	14–18	Solignat&Chapa, 1975	France
	13.33–21.30	Mert et al., 2007	Turkey
	14.48	Tüylü&Sorkun, 2007	Turkey
	16.25 (11.3–18.3)	Evrenosoglu & Misirli, 2009	Turkey
	12.58–24.39	Grygorieva et al., 2015	Ukraine
	16.78–22.65	Grygorieva et al., 2016	Slovakia
	12–16	Çelemli et al., 2016	Turkey
Equatorial axis (µm)	8–12	Bergaminy, 1975	Italy
	10–14	Solignat & Chapa, 1975	France
	–	Mert et al., 2007	Turkey
	12.46	Tüylü&Sorkun, 2007	Turkey
	8.15 (6.5–9.2)	Evrenosoglu & Misirli, 2009	Turkey
	7.10–13.92	Grygorieva et al., 2015	Ukraine
	7.21–12.07	Grygorieva et al., 2016	Slovakia
	10–13	Çelemli et al., 2016	Turkey
SI – shape index	1.2–1.6	Bergaminy, 1975	Italy
	–	Solignat & Chapa, 1975	France
	–	Mert et al., 2007	Turkey
	1.16	Tüylü & Sorkun, 2007	Turkey
	2.02 (1.75–2.27)	Evrenosoglu & Misirli, 2009	Turkey
	1.4–2.0	Grygorieva et al., 2015	Ukraine
	–	Grygorieva et al., 2016	Slovakia
	1.37	Çelemli et al., 2016	Turkey

of pollen grains in the range 7.21–12.07 µm/7.32–11.22 µm in the conditions of Slovakia. Bergamini (1975) detected for various staminal types a pollen length of 10–15 µm, pollen width of 8–12 µm and a shape index of 1.2–1.6. It has been found that the size of the pollen grains, expressed by the length of their polar axis, is positively correlated with the length of the stamens. Individuals of the brachystaminate type have smaller pollen grains than individuals of the mesostaminate type and those, in turn, smaller grains than individuals of the longistaminate type. However, variability in the size and shape of pollen grains also occurs within an individual genotype (Bolvanský and Salaj, 1988).

In our work, we confirmed that pollen grains have a short-elliptical to elongated-elliptical shape, which is documented in Figures 1.

Mert et al. (2007) described pollen grains of both male-fertile ('Firdola', 'Karamehmet', 'Sarialama', and 'Haciomer') and male-sterile ('Osmanoglu' and 'VakitKestanesi') chestnut (*Castanea sativa* Mill.) cultivars like tricolporate, the germinal furrow extending almost the full length of the grain axis. Pollen grains have a slightly reticulate exine. Pollen grain length varied from 13.33–21.30 µm and decreased significantly in the male-sterile cultivars. Three different pollen shapes were observed among the cultivars: prolate, perprolate, and sub-prolate. The ultrastructure of the pollen grains did not differ between male-fertile and male-sterile cultivars. Intine, exine and total wall thickness (exine + intine) of pollen grains were determined as 83.2–153.1 nm, 432.8–520.0 nm, and 516.0–651.6 nm, respectively; and variations were significant ($p < 0.05$) among cultivars.

Based on the cluster analysis of all 16 studied pollen characteristics, a dendrogram for the genotypes of *Castanea sativa* was made (Figure 3). On the

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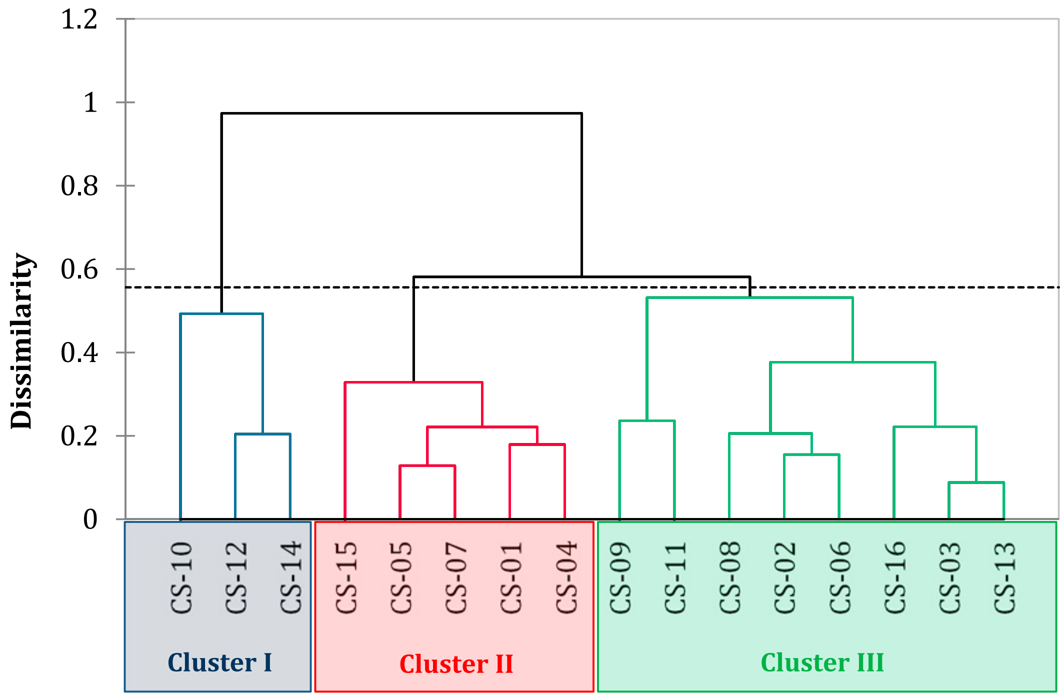

Figure 3 Cluster dendrogram of morphometric parameters pollen of *Castanea sativa* Mill. genotypes

dendrogram, you can see three main clusters, while cluster I (CS-10, CS-12, CS-14) has the highest values, cluster II (CS-01, CS-04, CS-05, CS-07 and CS-15) has the lowest values.

More detailed relationships between genotypes were revealed by Principal component analysis (PCA). The PCA used in our work showed that 99.98 % of the variability observed was explained by the first two components (Table 3). PC1, PC2, and PC3 accounted for 55.77, 44.21 and 0.02%, respectively. PC1 was positively correlated with the polar and equatorial axis, whereas the shape index showed a low negative correlation. PC2 was positively correlated with the polar and equatorial axis and shape index. Positive values for PC1 correspond to the genotypes with a higher polar axis as shown in Figure 4. Genotypes CS-10, CS-12 and CS-14 were included in this group.

The highest negative values for PC1 indicate the genotypes with the smallest polar axis. This group includes genotypes CS-01and CS-15 (Figure 4). The genotypes CS-03 and CS-13 have the highest PC2 due to the highest equatorial axis. The positive PC3 value indicates the largest shape index. These characteristics were observed in genotypesCS-03, CS-08, CS-13.

Table 3 Eigenvalues and proportion of total variability, eigenvectors of the three principal components (PC), and component scores for *Castanea sativa* Mill. pollen 16 genotypes

Selection	Component scores		
	PC1	**PC2**	**PC3**
Eigenvalue	1. 673	1. 326	0.001
Variance (%)	55.77	44.20	0.024
Cumulative	55.77	99.98	100.000
Variable	**Component loadings**		
	PC1, λ = 55.77	**PC2, λ = 44.20**	**PC3, λ = 0.02**
Polar axis (μm)	0.167	0.848	-0.503
Equatorial axis (μm)	0.747	0.225	0.625
SI – shape index	-0.644	0.481	0.595

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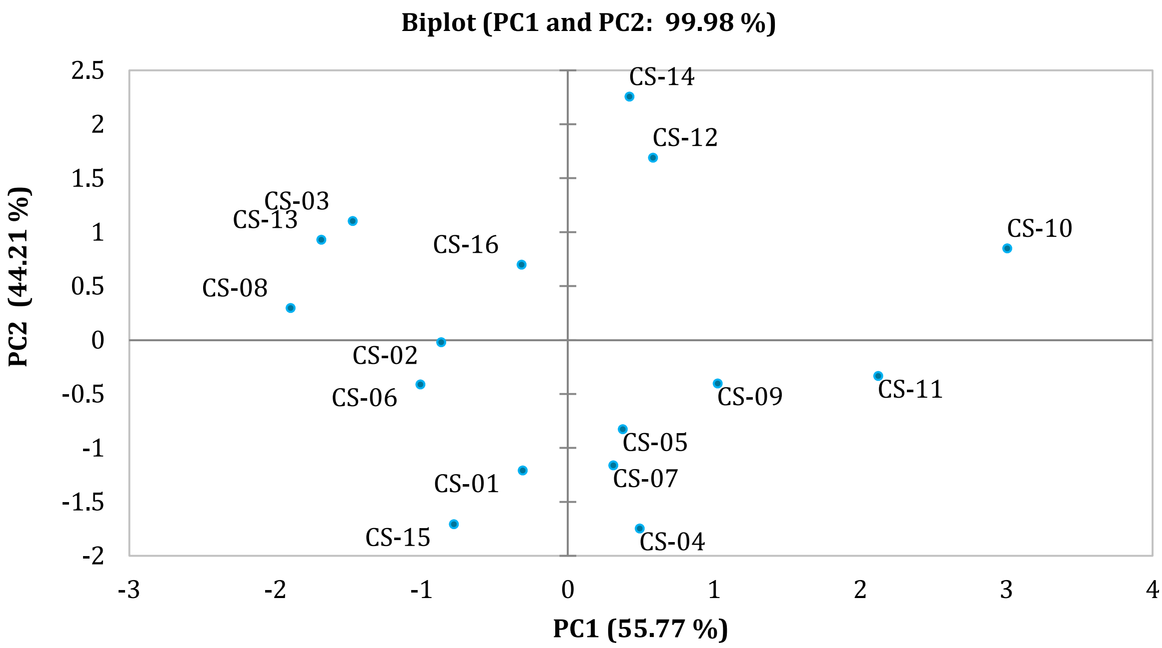

Figure 4 Biplot based on principal components analysis (PCA) for pollen morphometric parameters of *Castanea sativa* Mill. 16 genotypes

Conclusions

The studying of the *Castanea sativa* pollen via scanning electron microscope allowed us to determine the most important parameters which can be used to identify the representatives of species. The detailed pollen morphological and micro-sculptural characteristics were investigated, described and analyzed by using hierarchical cluster analysis dendrogram and BiPlot. The main parameters such as the form (the pollen grains elongation, the length and the width ratio) are specific for different *Castanea* species. Results from our analyses showed small differences among *Castanea sativa* phenotypes from Ukraine. Some of these pollen morphological parameters can be used for identification and comparison with the following analyses of *Castanea* species phenotypes.

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Research Article

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Evaluation of the antibacterial activity of ethanolic extracts obtained from roots and stalks of *Chelidonium majus* L. against *Escherichia coli* strains

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Nowadays, antibiotic resistance among pathogenic bacteria is increasingly common. The *Chelidonium majus* L. is one of the most widely used genera in folk medicine, where it is popular for its antibacterial activity. The purpose of this study was to evaluate the antimicrobial effects of five ethanolic extracts obtained from stalks and roots of *C. majus* collected from the rural and urban agglomerations on the territory of the Kartuzy district in the Pomeranian province (northern part of Poland) against *Escherichia coli* strains. The *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) and *Escherichia coli* Castellani and Chalmers (ATCC® 25922™) strains were used in the current study. It has been observed that ethanolic extracts derived from stalks and roots of *C. majus* collected from rural and urban areas revealed weak antibacterial activity (7.95–8.25 mm as the diameters of inhibition zone) compared to the control samples. Our results revealed that the extracts derived from stalks and roots of *C. majus* have shown weak antibacterial activity against the tested strains. The detailed chemistry of the active compounds and possible mechanism(s) of actions of the bio-molecules responsible for the observed activity was not addressed in the current study. Thus, further evaluation for the nature of active compounds (bio-molecules) and detailed mechanism(s) of their interaction with microbial strains are recommended.

Keywords: *Chelidonium majus* L., *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™), disc diffusion techniquey

Introduction

Escherichia coli, a rod-shaped Gram-negative bacteria and a member of the bacterial family of Enterobacteriaceae, is the most prevalent commensal inhabitant of the gastrointestinal tracts of humans and warm-blooded animals, as well as one of the most important pathogens (Kaper, 2005). As a commensal, it lives in a mutually beneficial association with hosts and rarely causes diseases. It is, however, also one of the most common human and animal pathogens as it is responsible for a broad spectrum of diseases such as diarrhea, hemolytic uremic syndrome, and hemorrhagic colitis leading to acute renal failure and often death (Allocati et al., 2013; Jang et al., 2017). *E. coli* also includes pathogens of global significance that are responsible for epidemic dysentery (e.g. *Shigella*), neonatal meningitis (associated with the K1 capsular polysaccharide), hemolytic uremic syndrome (O157:H7), as well as a variety of other diseases (Wirt et al., 2006). The prevalence of multidrug-resistant *E. coli* strains is increasing worldwide principally due to the spread of mobile genetic elements, such as plasmids (Allocati et al., 2013). The genetic structure of commensal *E. coli* is shaped by multiple host and environmental factors. The determinants involved in the virulence of the bacteria may reflect adaptation to commensal habitats (Tenaillon et al., 2010). The increase of multidrug-resistant strains of *E. coli* also occurs in Europe and the spread of resistance in *E. coli*

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is an increasing public health concern in European countries (Allocati et al., 2013).

In the face of the rapidly increasing antibiotic resistance of microorganisms including bacteria, we tried to find alternative solutions to this actual problem. In the common knowledge of an ethnomedicine, it is known that plants contain a wide range of compounds including polyphenols, alkaloids, carotenoids, tannins that exhibit antibacterial properties. One of them is a representative of the Papaveraceae family, the greater celandine (*Chelidonium majus* L.), which we have taken a detailed interest in.

C. majus is a perennial plant growing in regions of moderate climate, on the continents of Europe, Asia, North America, and a part of Northwest Africa. In Poland, it is found across the entire country (Zielińska et al., 2018). The greater celandine herb is rich in medically valuable natural compounds. Their highest amounts are contained in immature fruits (2.4 %) and roots (4 %), while the aerial parts of the plant contain around 0.5 % of active compounds. The plant contains, first of all, alkaloids benzylisoquinoline compounds (0.01–1.0 %), such as sanguinarine, chelidonine, chelerythrine, and protoberberines, berberine, coptisine (Táborská et al., 1995; Shafiee and Jafarabadi, 1998; Kopytko et al., 2005; Pan et al., 2017; Warowicka et al., 2019). Crude extracts of several alkaloids extracted from *C. majus* exhibited antimicrobial, antiviral, and antifungal properties (Nawrot et al., 2020). Other compounds structurally unrelated to the alkaloids have been isolated from the aerial parts: several flavonoids and phenolic acids. *C. majus* extracts and their purified compounds exhibit interesting antiviral, antitumor, and antimicrobial properties both *in vitro* and *in vivo* (Colombo and Bosisio, 1996).

Considering the points highlighted above and based on previous results obtained in our laboratory, the current study aimed to find out *in vitro* possible antimicrobial activity of the ethanolic extracts derived from roots and stalks of *Chelidonium majus* against two *Escherichia coli* strains.

Material and methodology

Collection of plant material

Plant materials (Figure 1B) were harvested from natural habitats on the territory of the Kartuzy district (54°20′N 18°12′E) in the Pomeranian province (northern part of Poland) (Figure 1A). Kartuzy is located about 32 kilometers (20 miles) west of Gdańsk and 35 km (22 miles) South-East of the town of Lębork on a plateau at an altitude of approximately 200 meters (656 feet) above sea level on average. The plateau, which is divided by the Radaune lake, comprises the highest parts of the Baltic Sea Plate (http://www.kartuzy.pl/). Plants were collected from urban (n = 5) and rural agglomerations (n = 15) on the territory of the Kartuzy district.

Preparation of plant extracts

The collected roots and stalks were brought into the laboratory for antimicrobial studies. Freshly washed samples were weighed, crushed, 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.

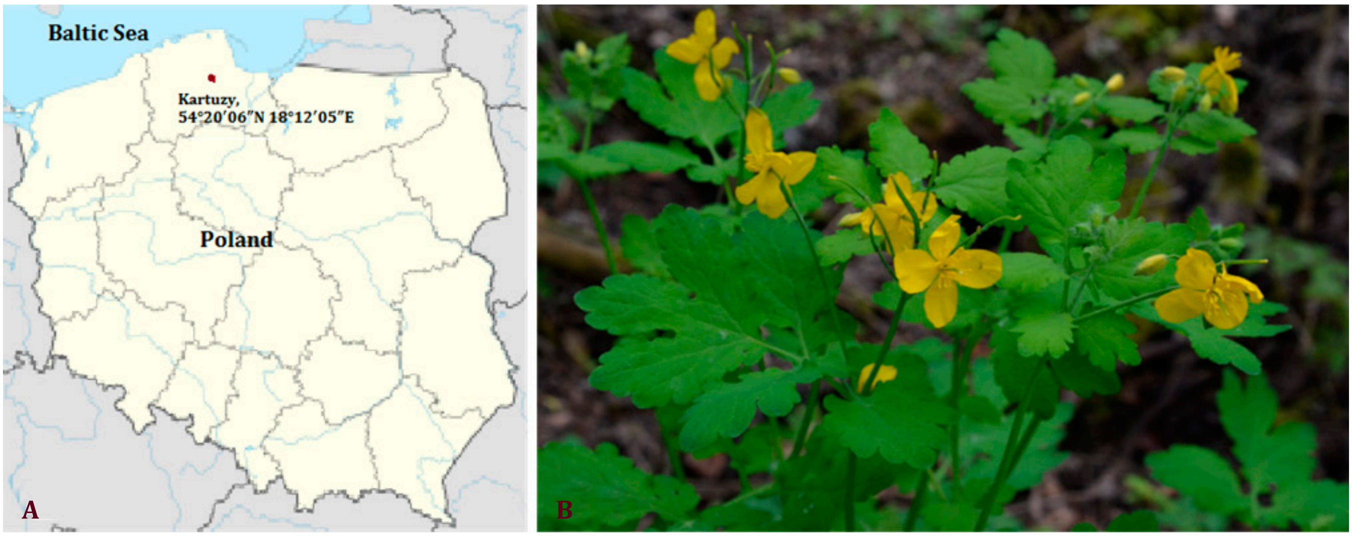

Figure 1 Location of Kartuzy in the map of Poland (A), where the *Chelidonium majus* L. (B) was collected

The disk diffusion method for evaluation of antibacterial activity of plant extracts

The *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) and *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strains were used in the current study. Strains tested were plated on TSA medium (Tryptone Soy Agar) and incubated for 24 h 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 disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) (Bauer et al., 1966). 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 extracts 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 (CLSI, 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).

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 antibacterial activity of tested extracts. All statistical calculation was performed on separate data from each extract. The data were analyzed using 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

Our study aimed to examine the antibacterial properties of *C. majus* roots and stalks against two *Escherichia coli* strains. The extracts derived from stalks and roots of *C. majus* collected from the rural and urban agglomerations have shown weak antibacterial activity against the tested strains. The results of antibacterial activity screening are given in Figure 2–5, which indicate that the extracts have shown weak antibacterial activity against the two *E. coli* strains. The data on diameters of zone inhibition of bacterial growth of plant extracts against the *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain is demonstrated in Figure 2 and 3.

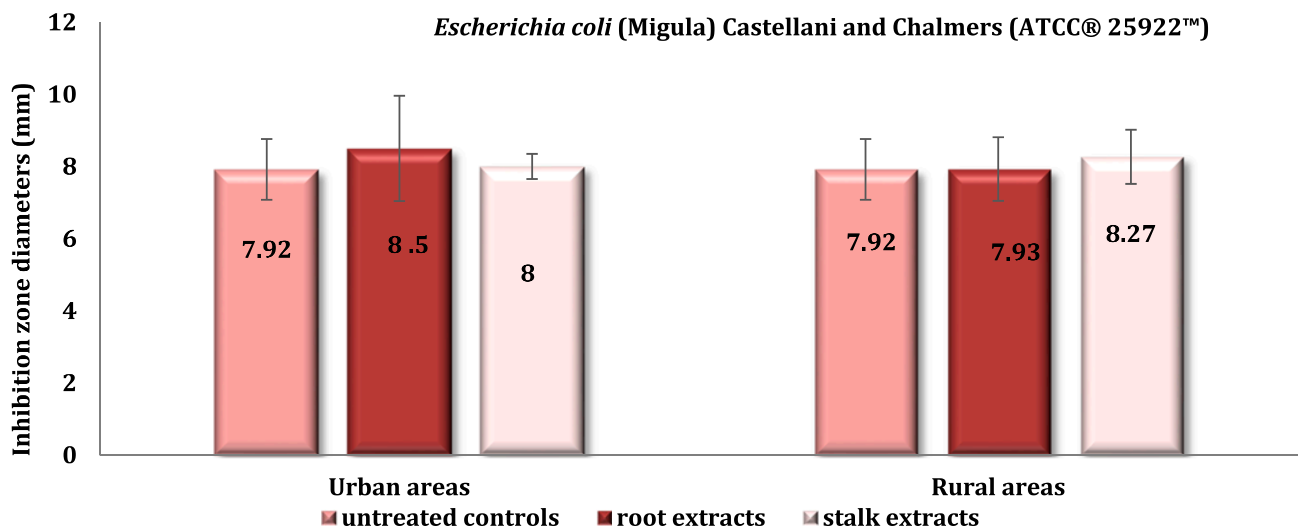

Figure 2 Zones of growth inhibitions of *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain induced by the extracts obtained from stalks and roots of *Chelidonium majus* L. collected from the rural and urban agglomerations in millimeter (M ±m, n = 8)

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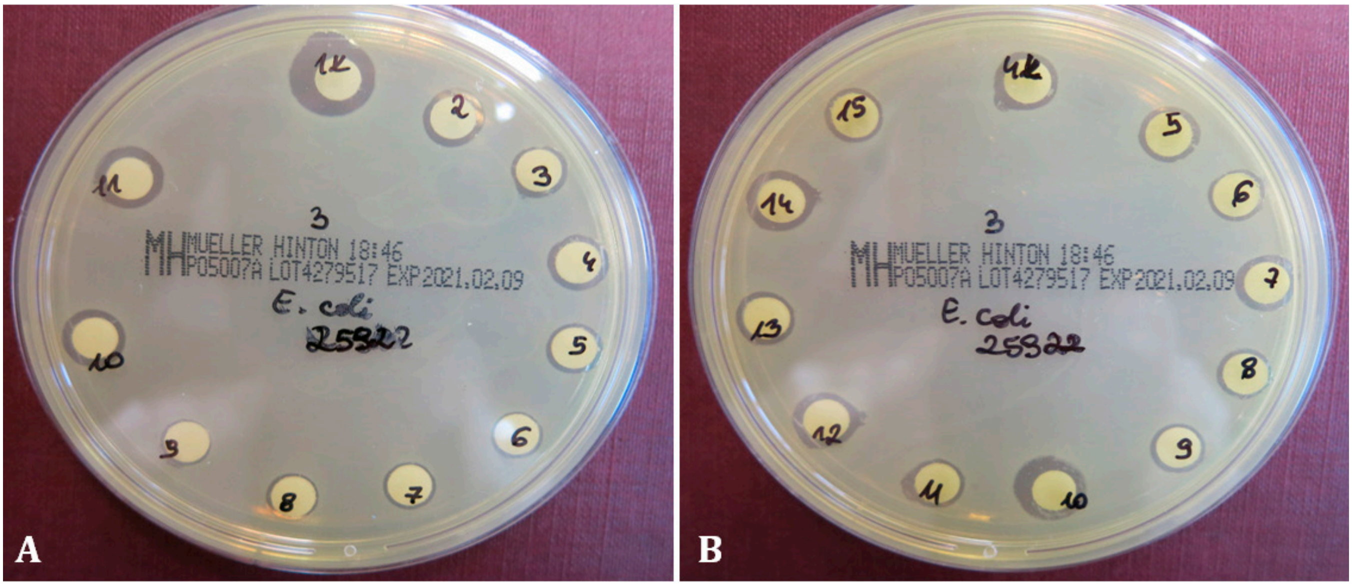

Figure 3 Example of plates in a disc diffusion assay showing the halos in the bacterial growth resulting from the antibacterial activity of extracts derived from roots (A) and stalks of *Chelidonium majus* L. (B) collected from the rural and urban agglomerations against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain

The extracts derived from roots collected in urban agglomeration have shown more considerable activity against the *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) strain. The mean diameters of inhibition zones were (8.5 ±0.65) mm compared to the control sample (8.1 ±0.75) mm. Similar results were obtained from the extracts derived from stalks of *C. majus* collected in rural agglomerations, i.e. (8.27 ±0.19) mm compared to the control samples (7.92 ±0.84). The stalk extracts have exhibited fewer antimicrobial activities against *E. coli*. The mean of the inhibition zone diameters was (8.0 ±0.16) mm. Finally, the ethanolic extracts derived from roots of *C. majus* collected from rural agglomerations exhibited weak antibacterial activity against *E. coli* (mean of inhibition zone ranged 7.93 ±0.23 mm).

The present study has shown that ethanolic extracts derived from the roots of *C. majus* from urban agglomerations also exhibited weak antibacterial activity against *Escherichia coli* (inhibition zone diameter was ranged from 7.95 to 8.5 mm) (Figure 4). Moreover, it has been observed that ethanolic extracts derived from stalks of *C. majus* collected from rural areas also revealed similar antibacterial activity

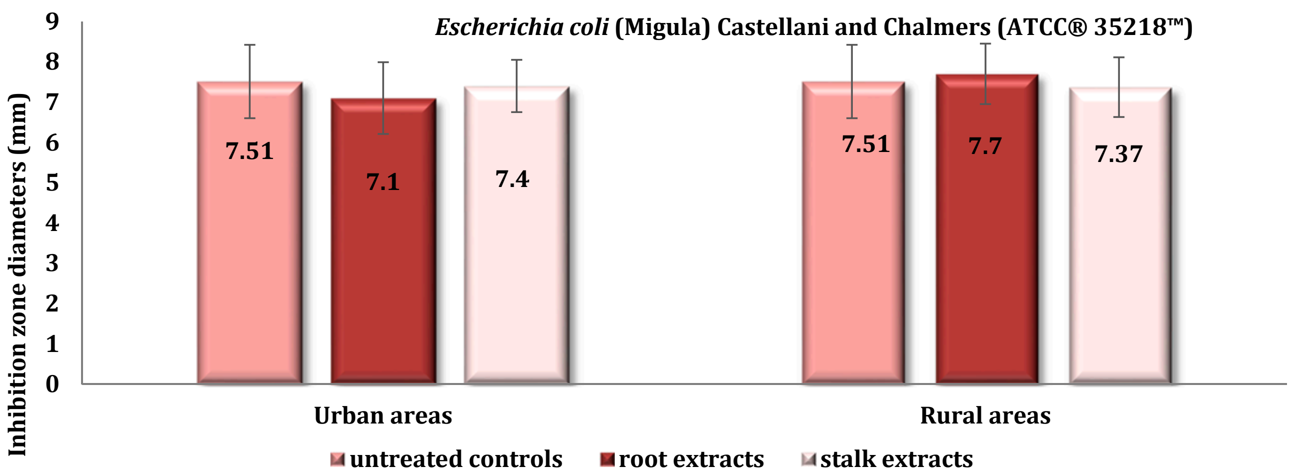

Figure 4 Zones of growth inhibitions of *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™) strain induced by the extracts obtained from stalks and roots of *Chelidonium majus* L. collected from the rural and urban agglomerations in millimeter (M ±m, n = 8)

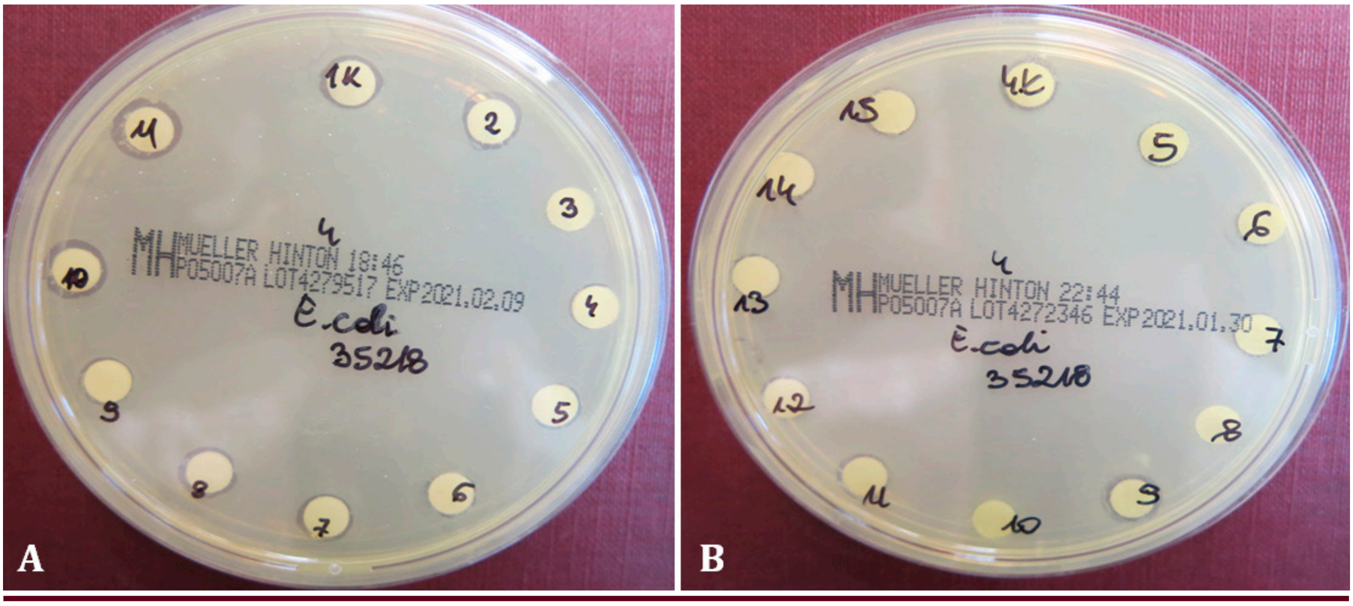

Figure 5 Example of plates in a disc diffusion assay showing the halos in the bacterial growth resulting from the antibacterial activity of extracts derived from roots (A) and stalks of *Chelidonium majus* L. (B) collected from the rural and urban agglomerations against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218™) strain

(7.95–8.25 mm as the diameters of inhibition zone) compared to the control samples (Figures 4 and 5).

The extracts derived from roots collected in rural agglomeration have shown weak activity against the *E. coli* strain (Figure 5). The mean diameters of inhibition zones were 7.7 ±0.65 mm compared to the control sample 7.51 ±0.75 mm. The extracts taken from the urban agglomeration showed no significant changes compared to the control sample. The above results show that only root extracts collected from rural areas can have weak antibacterial properties against this strain (Figure 5).

In this study, we investigated the antimicrobial activity of plant extracts by agar disc diffusion assay. In the current study, extracts derived from roots and stalks of *C. majus* collected from the rural and urban agglomerations were less potent against the test bacterium due to the observed zone of growth inhibitions.

Our results are not similar in comparison to another. *C. majus* is listed among one of the most active antimicrobial plants in a screening study by Kokoska et al. (2002). Crude extracts and several alkaloids isolated from *C. majus* exhibited antibacterial, antiviral, and antifungal properties. In experiments with multidrug-resistant bacteria existing in surgical wounds and infections of critically ill patients, *C. majus* ethanol extract affected Gram-positive bacteria. Ethanolic extracts of *C. majus* also showed antimicrobial activity against *Bacillus cereus*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* (Kokoska et al., 2002). Zielińska et al. (2019) have evaluated the antimicrobial activity of seven alkaloids and *C. majus* extracts from plants derived from natural habitats and *in vitro* cultures. A comparison of the alkaloid profile of extracts obtained from aerial parts and roots of plants collected from different habitats was also performed using chromatographic techniques. Moreover, the antimicrobial activity of seven major alkaloids was tested and the results were correlated with alkaloid content. All tested plant extracts manifested antimicrobial activity, related to different chemical structures of the alkaloids. Root extract used at 31.25–62.5 mg/L strongly reduced bacterial biomass. From the seven individually tested alkaloids, chelerythrine was the most effective against *Pseudomonas aeruginosa* (MIC at 1.9 mg/L), while sanguinarine against *S. aureus* (MIC at 1.9 mg/L). Strong antifungal activity was observed against *C. albicans* when chelerythrine, chelidonine, and aerial parts extract were used (Zielińska et al., 2019). The antibacterial effect of extracts and compounds isolated from the aerial part of *C. majus* acting against clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) was evaluated in the study of Zuo et al. (2008). The selective antibacterial activity against MRSA for 8-hydroxylated benzo[c]phenanthridine-type alkaloids isolated from *C. majus* was revealed.

The complex composition of alkaloids can manifest a wide spectrum of antimicrobial activity, arising from different chemical structures of the compounds.

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Hence, the antimicrobial activity of *C. majus* was also tested with use of various solvent extraction by Cirić et al. (2008). Antibacterial and antifungal tests were performed using 96 % methanol extracts from leaves and petioles of plants grown in nature as well as *in vitro* shoots and embryos (Cirić et al., 2008). The following Gram-positive bacteria were used: *Bacillus subtilis* ATCC 10707, *Micrococcus luteus* ATCC 9341, *Sarcinia lutea* ATCC 9391, and *Staphylococcus aureus* ATCC 6538, in addition to the Gram-negative bacteria *Agrobacterium rhizogenes* A4M70GUS, *A. tumefacians* A281, *Escherichia coli* ATCC 35218, *Proteus mirabilis* (clinical isolates), and *Salmonella enteritidis* ATCC 13076; and the yeast *Candida albicans* (clinical isolates). Methanol extracts of all samples showed significant activity against Gram-positive and Gram-negative bacteria and *C. albicans*. The extracts were not effective only against *P. mirabilis*. Extracts from various explants had higher antimicrobial activity against the tested microorganisms than that recorded for extracts from leaves and petioles of plants growing outdoors. When the results were compared with the antimicrobial activities of positive controls (streptomycin or bifonazole), some extracts showed equal antimicrobial activity against *E. coli*, *S. enteritidis*, and *C. albicans* (Cirić et al., 2008).

Singly from plant extracts, as it was previously summarized by Kędzia et al. (2013), Kędzia and Hołderna-Kedzia (2013), individually tested compounds showed different antimicrobial activity. Chelerythrine and sanguinarine were significantly more potent than chelidonine against Gram-positive (*Staphylococcus aureus*, *S. epidermidis*, *B. subtilis*, *B. anthracis*), Gram-negative (*Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Salmonella gallinarum*, *S. typhi*, *S. paratyphi*, *Proteus vulgaris*, *Shigella flexneri*) and acid-fast mycobacteria (*Mycobacterium tuberculosis*, *M. smegmatis*). Pan et al. (2017) have investigated an efficient method to purify chelidonine from the extract of *C. majus* using macroporous adsorption resins and evaluated the antifungal activity of chelidonine against *Botryosphaeria dothidea* as a model strain. The antifungal activity of enriched chelidonine products was studied with *B. dothidea*. The results showed that the EC_{50} of crude extracts, enriched chelidonine products, and the chelidonine standard against *B. dothidea* was 3.24 mg/mL, 0.43 mg/mL, and 0.7 mg/mL, respectively. The result of the antifungal activity test showed that chelidonine had the potential to be a useful antifungal agent (Pan et al., 2017).

According to Migas and Heyka (2011), an effective and safe to use antimicrobial compound is berberine. This is supported by studies on adult patients with acute diarrhea caused by enterotoxin *Escherichia coli* and *Vibrio cholerae*. Berberine reduces the hypersecretion of water and electrolytes induced by cholera toxin, as well as shortens the passage time of gastrointestinal contents through the small intestine. The *in vitro* studies of Migas and Heyka (2011) have shown that berberine sulfate inhibits *E. coli* cell adhesion to the mucosal epithelium, which may be related to blocking and preventing the formation of fimbriae on the bacterial cell surface. Enzymes synthesized by buttercup also exhibit antimicrobial activity. The plant synthesizes biologically active proteins: glycoproteins with lectin and DNA-ase activity, as well as two extracellular peroxidases (isolated from milk sap latex), which participate in the formation of hydrogen peroxide and other reactive oxygen species, protecting the plant from pathogenic microorganisms.

Conclusions

The ethanolic extracts of *Chelidonium majus* revealed weak antibacterial activity against both *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922™) and *E. coli* (Migula) Castellani and Chalmers (ATCC® 35218™) strains. The findings reported herein suggest that extracts derived from the different parts of *C. majus* merit further microbiological and chemical study as natural antibiotics to the assessment of antibacterial activity and identify secondary metabolites. The present study lays the basis for future research, to validate the possible use of *C. majus* 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.

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Research Article

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Structural diversity of trichomes and pubescence of Brassicaceae Burnett of the flora of Ukraine with an emphasis on ecology

Структурна різноманітність трихом і опушення Brassicaceae Burnett флори України з акцентом на екологію

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Species of the Brassicaceae family (crucifers), which are of great scientific and practical importance, are inherent by a wide variety of trichome structures. Trichomes – specialized cells of the epidermis, modulate the response of plants to biotic and abiotic stresses. We investigated the structural diversity of trichomes and the features of pubescence of all (252 species, 73 genera) Brassicaceae species of the flora of Ukraine. Trichomes were studied on living plants and based on materials from seven herbaria, as well as on the basis of analysis of literature data. Stereoscopic and electronic scanning (JSM-6060 LA) microscopes were used in this investigation. To describe the structural diversity of trichomes, an original four-level hierarchical classification was developed, taking into account a complex of features, including functional specificity (covering or glandular), the nature and degree of hair branching, as well as the features of the orientation of their rays. The pubescence of plants was divided into isomorphic (consists of one type of trichomes) and heteromorphic (formed by two or more types of hairs with or without glandular structures). Hairs are completely absent in 23 species of 15 genera of the flora of Ukraine. Isomorphic pubescence is characteristic of the most (170 species) cruciferous plants and is represented by simple (120 species), Malpighian (23 species), stellate (23 species), dendroid (3) and fork-shaped (1 species) trichomes. The composition of heteromorphic pubescence is dominated by a combination of simple and fork-shaped hairs: it is observed in 44 species. There are glandular structures in the pubescence of 14 species. The structural diversity of Brassicaceae trichomes correlates, in general, with the temperate continental climate of Ukraine. Species without hairs or pubescent with simple trichomes, including those in combination with fork-shaped hairs, are observed in biotopes with sufficient moisture, optimal insolation, and satisfactory soil quality. Species with dense branched trichomes inhabit arid biotopes with intense insolation, insufficient moisture, and poor substrate. In the flora of Ukraine, the evolutionary-phylogenetic line LII included non-pubescent species or those with simple hairs. The cruciferous line LI is characterized by isomorphic and heteromorphic pubescence. In species LIV, heteromorphic pubescence predominates. Glandular structures are observed in the heteromorphic pubescence of taxa of line LIII.

Keywords: Brassicaceae, trichomes structure, ecology of species, flora of Ukraine

Вступ

Родина Brassicaceae Burnett включає близько 4000 видів, які входять до складу 52 монофілетичних триб (Al-Shehbaz, 2012) або шести молекулярно-філогенетичних клад (Huang et al., 2015; Nikolov et al., 2019). Хрестоцвіті поширені на всіх континентах, за винятком Антарктики.

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У світовій економіці хрестоцвіті займають третє місце після злакових та бобових. Серед них багато важливих харчових, кормових, олійних, медоносних, вітамінних, лікарських, а також декоративних рослин (Sokolov, 1985; Ilyinska et al., 2007; Warwick, 2011; Anjum et al., 2018; Rakhmetov et al., 2018; Vergun et al., 2019, 2020, 2021; Jabeen, 2020). В агропромисловому комплексі та приватному секторі України найчастіше використовують види майже 20 родів, зокрема *Armoracia* G.Gaertn., B.Mey. & Scherb., *Brassica* L., *Camelina* Crantz, *Eruca* Mill., *Hesperis* L., *Lepidium* L., *Matthiola* L., *Raphanus* L. та ін.

До Brassicaceae належать декілька модельних об'єктів сучасної біології, зокрема: *Arabidopsis thaliana* (L.) Heynh. – відомий біологам усього світу (Mitchell-Olds, 2001; Van Norman and Benfey, 2009; Sivasubramanian et al., 2015); комплекс *Brassica oleracea* L. – дослідження перспектив використання видів для інновацій агросистем і виробництва продуктів харчування (Branca and Maggioni, 2020; Żyła et al., 2021); види *Capsella* Medik. – стандарт для дослідження способів запилення рослин (Hurka et al., 2012); види Aethionema W.T.Aiton та *Lepidium* – еталони для вивчення структурних і функціональних аспектів диморфізму плодів та особливостей їхнього еволюційного розвитку (Mummenhoff et al., 2009; Arshad et al., 2020; Walden et al., 2020b). Більше 100 видів із 11 родів Brassicaceae толерантні до важких металів і здатні гіперакумулювати Cd, Ni, Pb, Se, Sr, Zn (Warwick, 2011; Godé et al., 2012; Dar et al., 2018; Agnihotri and Seth, 2019; Nikolić and Tomašević, 2020). *Arabidopsis halleri* (L.) O'Kane et Al-Shehbaz та *Noccaea caerulescens* (J.Presl et C.Presl) F.K.Mey. – зразки для вивчення адаптації рослин до ґрунтів, забруднених важкими металами, зокрема цинком, кадмієм та свинцем, і для досліджень толерантності рослин до важких металів (Assunção et al., 2003; Bothe and Słomka, 2017). Ці види пропонують використовувати для фіторемедіації ділянок, забруднених важкими металами. Brassicaceae служать також моделлю для вивчення особливостей історичного розвитку рослин, завдяки біологічній, морфологічній, фізіологічній, генетичній та геномній різноманітності в поєднанні з широкою екологічною пластичністю (Bomblies and Weigel, 2007; Koenig and Weigel, 2015; Nikolov and Tsiantis, 2017; Nikolov et al., 2019; Mattila et al., 2020; Walden et al., 2020b).

У складі родини є рідкісні та ендемічні види, занесені до Червоних книг різних країн та континентів. У флорі України, наприклад, є 30 таких видів (Didukh, 2009). Великим числом представлені бур'янові рослини, здатні розповсюджуватися далеко за межі свого природного ареалу. На території України чужорідні види входять до складу родів *Capsella*, *Chorispora* R.Br. ex DC., *Descurainia* Webb et Berthel., *Diplotaxis* DC., *Draba* L., *Lepidium*, *Sisymbrium* L. тощо (Protopopova, 1991; Ilyinska et al., 2007; Dorofeev, 2012).

Brassicaceae властива велика морфологічна одноманітність будови квіток (див. Nikolov, 2019) і, навпаки, значна структурна різноманітність листків, плодів та трихом, що було відомо ще з часів Ліннея і відображено у флористичних публікаціях, у тому числі сучасних, що охоплюють різні за площею території, наприклад «*Flora Europaea*» (Ball et al., 1993), «Конспект флоры Восточной Европы» (Dorofeev, 2012), «*Flora of Pan-Himalaya*»(Al-Shehbaz, 2015) тощо.

Трихоми – спеціалізовані клітини епідерми, відіграють важливу роль в еволюції рослин. Вони сприяють їхньому нормальному розвитку за надзвичайно високих або низьких температур, посухи чи ультрафіолетового опромінення, слугують їм захисним бар'єром від біотичних і абіотичних стресів, регулюють газообмін, енергетичний та водний баланс (Xiao et al., 2017; Karabourniotis et al., 2020).

Прості (покривні) трихоми Brassicaceae одноклітинні, а залозисті – одноклітинні або багатоклітинні. Їх здавна описували і використовували в систематиці родини як важливі ознаки таксонів видового, інфрародового або родового рангів (Linné, 1753; Candolle de, 1821, 1824; Hayek, 1911; Bush, 1939; Janchen, 1942; Kotov, 1953, 1979, 1987; Dvořák, 1965, 1971, 1973; Ball et al., 1993; Polatschek, 2010, 2011). Перша спроба класифікації трихом хрестоцвітих належить Dennert (1885). Автор, залежно від наявності або відсутності та форми волосків, розділив 96 видів на три групи: з відсутніми трихомами; опушені простими нерозгалуженими і розгалуженими волосками; опушені тільки зірчастими трихомами. Prantl (1891) був й досі залишається єдиним систематиком хрестоцвітих, хто розглядав структурні особливості трихом важливими діагностичними та філогенетичними ознаками таксонів вищих рангів – триб і підтриб. Автор виділив п'ять типів простих волосків (нерозгалужені, безладно розгалужені, зірчасті, двороздільні і лускоподібні) і два типи

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залозистих структур (багатоклітинні з довгою ніжкою і одноклітинною залозистою голівкою та багатоклітинні сидячі) і використав їх для розробки власної системи родини. У подальших працях для родини вказували від чотирьох до 16 типів волосків (Theobald et al., 1979; Abdel, 2005; Beilstein et al., 2006, 2008).

Удосконалення методів сканувальної електронної мікроскопії та інтенсивний розвиток молекулярно-біологічних досліджень стали потужним стимулом для конкретизації будови трихом і розширення таксономічної й діагностичної інтерпретації їхніх структурних особливостей. Як результат – описані нові типи волосків, деталізована структура трихом багатьох видів, родів та триб в повному обсязі чи в межах окремих регіонів (Ilyinska, 2003; Ilyinska and Shevera, 2003a, b; Abdel, 2005; Ančev and Goranova, 2006; Beilstein et al., 2006, 2008; Spaniel et al., 2012; Fuentes-Soriano and Al-Shehbaz, 2013; Magauer et al., 2014; Mousavi and Sharifi-Rad, 2014; Mirzadeh et al., 2015). Тепер велику увагу приділяють дослідженню розвитку трихом Brassicaceae на молекулярному та генетичному рівнях (Pattanaik et al., 2014; Mazie and Baum, 2016; Doroshkov et al., 2019; Chopra et al., 2019; Hülskamp, 2019; Walden et al., 2020a). Встановлено, що процес розвитку трихом контролюється багатьма генами (Schellmann and Hulskamp, 2005). Широко аналізують також вплив біотичних та абіотичних чинників довкілля на формування, структуру та функціонування трихом (Broadhurst et al., 2004; Mershon et al., 2015; Prats-Mateu et al., 2016; Liu et al., 2017; Weigend et al., 2018; Fukuda et al., 2020; Karabourniotis et al., 2020). Актуальним залишається вивчення видового розмаїття волосків. Характеристику трихом відносять до числа обовʻязкових дескрипторів морфологічного опису нових таксонів (Brock et al., 2019; Koch and Lemmel, 2019; Al-Shehbaz, 2020; Salariato et al., 2020). Все ж в повному обсязі будова та функції трихом Brassicaceae ще не вивчені й досі.

Адаптивну важливість трихом у пристосуванні видів до чинників довкілля, особливо до посушливих умов існування, встановили ще в 20-му столітті (Schimper 1903; Palladin, 1912; Vasilevskaya, 1954; Johnson, 1975). Всебічні дослідження останніх десятиліть показали, що роль опушення у взаємодії рослин і довкілля недооцінювали (Steets et al., 2010; Bickford, 2016; Xiao et al., 2017; Karabourniotis et al., 2020). Трихоми модулюють енергетичний, водний та вуглецевий баланс рослин (Bickford, 2016). Існує взаємозвʻязок між щільністю трихом і захисною здатністю опушення. Спектральні властивості листків із незначним опушенням практично не відрізняються від таких неопушених листків (Mershon et al., 2015). Дефіцит ґрунтової води, висока температура повітря та недостатність тиску водяного пару збільшують щільність трихом (Bickford, 2016). Густе опушення захищає рослини від інтенсивної інсоляції (Yang et al., 2008), а УФ-випромінювання стимулює розвиток трихом у мутацій *Arabidopsis thaliana* (Yan et al., 2012). Суттєвий захист від світлового стресу забезпечують також кутикула та кутикулярний віск (Koch et al., 2008; Koch and Barthlott, 2009). Значно менше досліджень стосувалося захисної ролі тих чи інших структурних типів трихом. Роль морфологічної конструкції трихом листка в модуляції оптичних властивостей рослин родини Brassicaceae в альпійських біотопах досліджена на прикладі близькоспоріднених видів роду *Pachycladon* Hook.f. (Mershon et al., 2015). Автори показали, що саме структурний тип волосків, а не їхня щільність, є основним елементом листкової поверхні, що регулює відбивну і поглинаючу здатності листка. Найнижчий коефіцієнт відбиття був у не опушеного *P. fastigiatum* (Hook.f.) Heenan et A.D. Mitch., дещо вищий – у *P. enysii* (Cheeseman ex Kirk) Heenan et A.D. Mitch., на листках якого переважали фуркатні й прості волоски, і найвищий – у *P. stellatum* (Allan) Heenan et A.D. Mitch., що мав дуже розгалужені (дендроїдні – за визначенням авторів) трихоми.

Мета нашої роботи – зʼясувати структурну різноманітність трихом та специфіку опушения видів Brassicaceae флори України з особливим наголосом на їхню екологію.

Матеріали та методи

Методологія дослідження

Використано концепцію монотипного стандарту виду.

Обʻєкти та матеріал дослідження

Досліджено 252 види 73 родів родини Brassicaceae флори України. Морфологічну будову трихом вивчали на гербарних зразках, депонованих в гербаріях YALT, KW, LWKS, LWS, KWHA, LE, MW; акроніми гербаріїв наведені згідно Thiers (2020). Також було взято до уваги літературні дані. Для деяких видів волоски досліджували на живих рослинах.

Методи дослідження

Опушення всієї рослини аналізували за допомогою стереоскопічного мікроскопа. Структуру трихом листка і квітконіжки досліджували на сканувальному електронному мікроскопі JSM-6060 LA, для чого невеликі їхні фрагменти були приклеєні до металевого столика і напилені золотом, відповідно до загальноприйнятої методики. Для опису структурної різноманітності трихом ми розробили оригінальну чотирирівневу ієрархічну класифікацію трихом, на основі аналізу їхньої функціональної специфіки (покривні та залозисті), характеру і ступеня галуження, особливостей орієнтації їхніх променів, а також числа клітин в залозистому волоску (Рисунок 1). Класифікація включає п‘ять основних типів покривних волосків та два типи залозистих структур.

Прості (нерозгалужені) волоски в хрестоцвітих флори України дуже різноманітні за розміром, товщиною клітинної стінки і орієнтацією, що здавна використовували в систематиці родини для діагностики таксонів різних рангів.

Мальпігієві або фіксовані посередині (medifixed) трихоми не мають ніжки й складаються з двох первинних променів (розгалужень), які щільно притиснуті й паралельні до поверхні епідерми. Первинні промені (один або обидва) можуть дихотомічно галузитися. Залежно від особливостей галуження, спстерігаються дво-, три- (один з двох променів дихотомічно галузиться), чотири- (обидва первинні промені дихотомічно галузяться) й п‘яти-семипроменеві (один або обидва промені двічі дихотомічно галузяться) мальпігієві волоски. Прийнятий розподіл мальпігієвих трихом відповідає класифікації Polatschek (2010–2012, 2013a,b.) і включає волоски, описані різними авторами як: «T-shaped» (Metcalfe and Chalk, 1950), «2-armed, T-shaped», (Theobald et al., 1979), «medifixed» (Beilstein et al., 2006), «kompassnadelförmige» (Polatschek, 2010–2012, 2013a,b; Mutlu, 2010). Різноманітність (якісну

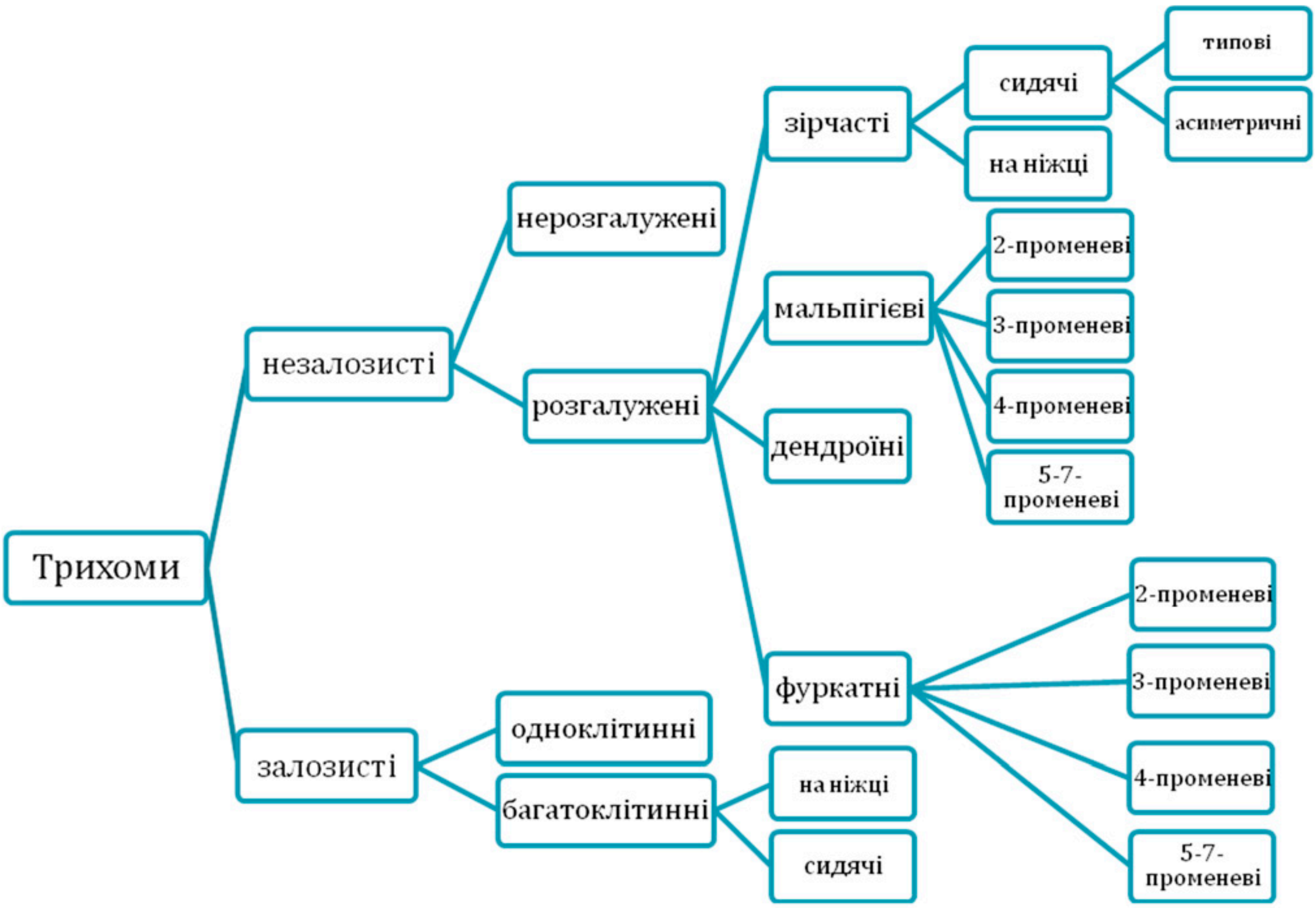

Рисунок 1 Класифікація трихом видів Brassicaceae Burnett флори України
Figure 1 Classification of trichomes of Brassicaceae Burnett species of Ukrainian flora

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і кількісну) мальпігієвих трихом практично всіх видів *Erysimum* L. s. l., в тому числі тих, що поширені на території України, дуже ретельно вивчив і описав A. Polatschek (2010–2012, 2013a,b). Автор підкреслив також, що «зірчасті» волоски, які вказують деякі автори для роду *Erysimum*, не тотожні тим зірчастим трихомам, що характерні для роду *Alyssum* L. (Polatschek and Rechinger, 1968).

Зірчасті трихоми сидячі або мають дуже короткі ніжки і декілька дихотомічно розгалужених первинних променів, які відгалужуються практично від одного центру і орієнтовані паралельно до поверхні епідерми. Кожний промінь зазвичай дихотомічно галузиться один або декілька разів й тоді формується багато (до кількох десятків) кінцевих розгалужень. Зірчасті волоски бувають симетричні, коли всі промені майже однаково розвинені, або асиметричні, якщо частина променів значно коротша, порівняно з іншими, або деформована чи недорозвинена. Асиметричні зірчасті волоски тільки з двома добре розвиненими променями й одним або декількома недорозвиненими іноді називають фуркатними, з чим не можна погодитися. У деяких видів, які відсутні у флорі України – промені з‘єднуються дуже розвиненою кутикулою і в таких випадках утворюються зірчасто-лускаті трихоми, наприклад в окремих видів триби *Physarieae* B.L. Rob. in A. Gray et S. Watson (Fuentes-Soriano and Al-Shehbaz, 2013). Для мальпігієвих та зірчастих трихом дуже характерна біомінералізація, особливо карбонатом кальцію (Hopewell et al., 2021).

Фуркатні (вилчасті) волоски складаються з двох частин – виразної проксимальної (ніжки) та дистальної, яка дихотомічно розділена на два нерозгалужені або розгалужені промені. Промені завжди спрямовані під кутом до поверхні листка чи іншого органу рослини, що відрізняє фуркатні волоски від мальпігієвих та зірчастих трихом із горизонтально орієнтованими променями. Найчастіше спостерігаються дво-, три- (один з двох променів дихотомічно галузиться), чотири- (обидва первинні промені дихотомічно галузяться) й п‘яти-семипроменеві (один або обидва промені двічі дихотомічно галузяться) фуркатні трихоми. Окремі види або їх групи можуть відрізнятися за довжиною ніжки та ступенем галузистості дистальної частини вилчастих волосків. Унаслідок розвитку дуже короткої ніжки формуються модифіковані фуркатні волоски, які часто називають розгалуженими, зірчастими або дендроїдними.

Дендроїдні трихоми мають виразну, але різної довжини, ніжку та безладно розгалужені, неоднакові за розміром і по-різному орієнтовані промені. Metcalfe (1950). Спочатку їх взагалі не наводили для хрестоцвітих, а потім автори вказували для родини три їхні модифікації: dendritic (branching), dendritic-many branched та dendritic-branching terminal (Theobold et al., 1979). Дефініція дендроїдних трихом дуже лаконічна (Payne, 1978). Можливо тому трактування цього типу трихом у хрестоцвітих, на наш погляд, дуже неоднозначе.

Залозисті волоски і залозки прийняті нами в загальновизнаному розумінні (наприклад, Theobald et al., 1979). Секреторні структури хрестоцвітих бувають багатоклітинними (на ніжці або сидячі) або одноклітинними (залозки).

Сукупність усіх трихом на рослині формує її опушення. Типи опушення можна виділяти за різними ознаками – залежно від розміру, форми чи орієнтації в просторі волосків, ступеня їхнього розвитку, особливостей та щільності розташування на органах рослини. Для видів Brassicaceae флори України ми виділяємо два типи опушення, зумовлені якісним складом трихом: ізоморфне (складається з одного будь-якого типу покривних волосків) і гетероморфне (включає два або декілька типів волосків із залозистими структурами або без них).

Статистичний аналіз

Для опрацювання отриманих даних використано програму PAST 2.17 (Норвегія, 2001).

Результати та обговорення

Рослини видів Brassicaceae можуть бути голими або опушеними. У флорі України відсутні волоски в 23 видів 15 родів (Рисунок 2). Неопушені рослини характерні для видів декількох моно- чи оліготипних родів, наприклад, *Armoracia*, *Calepina* Adans., *Myagrum* L. В деяких полі- чи оліготипних родах, наприклад *Noccaea* Moench, *Subularia* Ray ex L., та *Cakile* Mill., домінують неопушені рослини, тоді як в інших – види з відсутніми трихомами поодинокі (*Brassica*, *Cardamine* L., *Crambe* L., *Lepidium*, *Rorippa* Scop., *Cochlearia* L.). Більша половина видів (15) – це однорічники або дворічники, інші – багаторічники. Неопушені хрестоцвіті входять зазвичай до складу гумідних, аридних чи антропогенно трансформованих біотопів і показують досить високу толерантність до засолення й підвищеної

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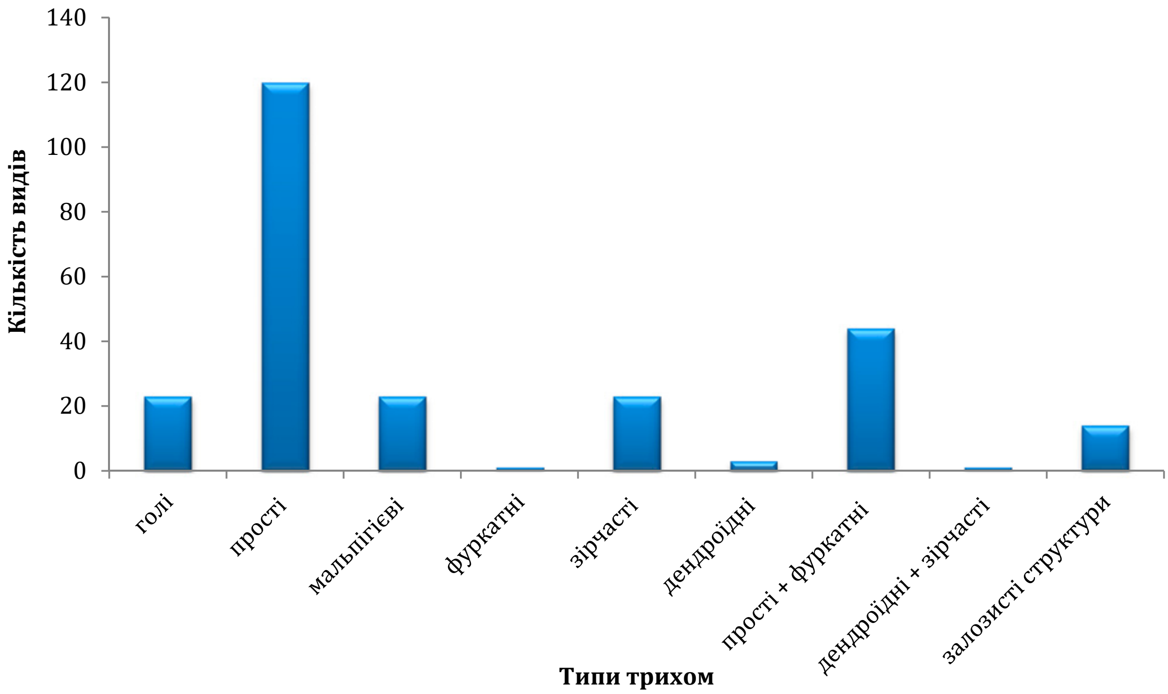

Рисунок 2 Кількісний розподіл трихом родини Brassicaceae Burnett флори України
Figure 2 Quantitative distribution of trichomes of Brassicaceae Burnett family of the flora of Ukraine

кислотності субстрату. Багато з них характерні для узбережних чи приморських екотопів (*Rorippa prolifera* Simonk., *R. dogadovae* Tzvelev, *Cakile euxina* Pobed., *Crambe maritima* L. тощо). Зазвичай таким видам властива добре розвинена кутикула з восковими структурами, як наприклад у *Myagrum perfoliatum* L. (Ilyinska et al., 1998). Деякі з них (наприклад, види *Cakile*) є сукулентами.

Опушення хрестоцвітих флори України сформоване дуже різноманітними за будовою волосками. Воно складається тільки з одного типу трихом (ізоморфне) або формується різними типами волосків (гетероморфне). Може бути факультативним – спостерігатися не на всіх рослинах одного й того ж виду чи не в усіх видів того самого роду або облігатним – характерним для всіх рослин виду чи для всіх видів роду.

Ізоморфне опушення досліджених видів флори України формується простими (нерозгалуженими), мальпігієвими, зірчастими, фуркатними чи дендроїдними волосками. Залозисті трихоми в його складі відсутні.

Прості (нерозгалужені) волоски – найпоширеніший тип трихом у хрестоцвітих флори України (Рисунок 2). Такі волоски характерні для 164 видів 44 родів. На рослинах 120 видів 24 родів вони формують ізоморфне опушення, а в інших – входять до складу гетероморфного. У політипних родах прості волоски спостерігаються не в усіх видів. Наприклад, види з наявними чи відсутніми волосками характерні для родів *Brassica*, *Cardamine* та *Lepidium*. Голі або незначно опушені рослини спостерігаються в *Rorippa austriaca* (Crantz) Besser, *R.* × *anceps* (Wahlenb.) Rchb., *R. brachycarpa* (C.A.Mey.) Hayek, а також *Diplotaxis tenuifolia* (L.) DC., *Barbarea arcuata* (Opiz ex J.Presl et C.Presl) Rchb., *B. vulgaris* W.T.Aiton та *B. verna* (Mill.) Aschers. Щільність та топографія простих трихом також варіюють. У *Sobolewskia sibirica* (Willd.) P.W.Ball і *Goldbachia laevigata* DC. такі волоски розвинені тільки по краю листків. У *Cakile monosperma* Lange та *C. baltica* (Jord. ex Rouy et Fouc.) Pobed. (відсутні в Україні) вони є лише на молодих квіткових бруньках (Pobedimova, 1953). Решта органів рослин цих видів голі. Дуже зрідка спостерігаються прості волоски у видів *Noccaea*. Вони можуть формуватися в *N. dacicum* (Heuff.) F.K. Mey. (відсутній в Україні) тільки на стеблах, а в *N. sarmatica* F.K.Mey. – на листках (папіли).

Екологічний діапазон видів, що мають облігатне або факультативне опушення із простих трихом, включає річкові або морські береги, луки, узлісся, лісові галявини, а також гірські скелі, осипища та відслонення. До складу лісових, прибережних та лучних біотопів входять види з родів *Lunaria* L., *Dentaria* L. (*Cardamine* s.l.), *Alliaria* Heist. ex Fabr., *Rorippa* тощо. Є також рослини степів, гір і оголень,

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наприклад, види *Crambe*, *Isatis* L., *Sisymbrium*, *Iberis saxatilis* L., *Hirschfeldia incana* (L.) Lagr.-Foss., *Teesdalia coronopifolia* (J.P.Bergeret) Thell. тощо. Частина видів, наприклад *Rapistrum rugosum* (L.) All., *Raphanus raphanistrum* L., *Sinapis arvensis* L. та ін., входять до складу антропогенно трансформованих біотопів. Дещо більше десяти видів (*Iberis amara* L., *I. umbellata* L., *I. pinnata* L., *Lunaria annua* L., *Eruca sativa* Mill., *Sinapis alba* L., *Raphanus sativus* L. та ін.) в Україні відомі в культурі й можуть дичавіти. Більшість з них вимоглива до зволоження й толерантна до бідних ґрунтів і засолення субстрату, частина – адаптована до інтенсивної інсоляції. Серед них багато кальцефілів, але є й ацидофіли.

Зірчасті трихоми дуже характерні для видів ірано-туранської за походженням триби Alysseae DC. і для частини видів (рід *Physaria* A.Gray) триби *Physarieae* із представниками здебільшого в Північній Америці (Oran, 1996; Fuentes-Soriano and Al-Shehbaz, 2013). У флорі України зірчасті трихоми спостерігаються в 23 видів п'яти родів (Рисунок 2). З них два, *Alyssum* та *Odontarrhena* C.A.Mey., багатовидові, а *Clypeola* L., *Berteroa* DC. і *Meniocus* Desv. – представлені одним видом кожний. Зірчасте опушення щільне, внаслідок чого рослини мають сіро-зелений колір. Окремі види або їхні групи розрізняються за діаметром волосків, характером галуження, товщиною і довжиною променів, формою центральної частини волоска, наявністю та ступенем розвитку інкрустації, а також щільністю опушення квітконіжок. Для *Odontarrhena borzaeana* (Nyár.) D.A.German характерне дуже щільне опушення листків і квітконіжок (Рисунок 3). Подібні волоски на листках і дуже розсіяно опушені квітконіжки

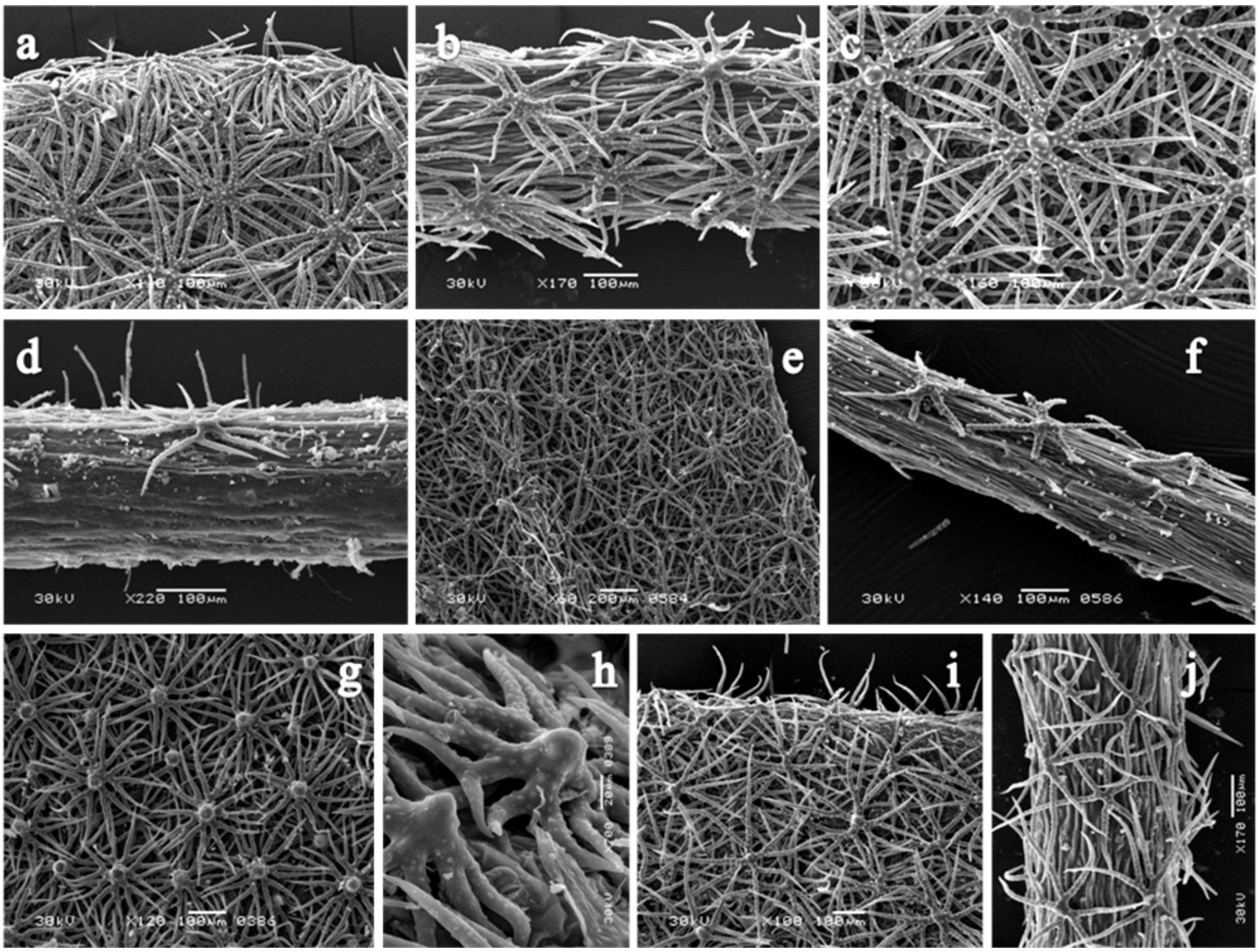

Рисунок 3 Трихоми листків і квітконіжок видів роду *Odontarrhena* C.A.Mey.:
a, b – *O. borzaeana* (Nyár.) D.A.German; c, d – *O. savranica* (Andrz. ex Besser) D.A.German; e, f – *O. muralis* (Waldst. & Kit.) Endl.; g, h – *O. gymnopoda* (P.A.Smirn.) D.A.German; i, j – *O. subalpina* (M.Bieb.) D.A.German; b, d, f, j – квітконіжка

Figure 3 Trichomes of leaves and peduncles of *Odontarrhena* C.A.Mey. genus species:
a, b – *O. borzaeana* (Nyár.) D.A.German; c, d – *O. savranica* (Andrz. ex Besser) D.A.German; e, f – *O. muralis* (Waldst. & Kit.) Endl.; g, h – *O. gymnopoda* (P.A.Smirn.) D.A.German; i, j – *O. subalpina* (M.Bieb.) D.A.German; b, d, f, j – peduncle

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мають *O. savranica* (Andrz.) D.A.German та *O. muralis* (Waldst. & Kit.) Endl. У *O. gymnopoda* (P.A.Smirn.) D.A.German спостерігаються 15–20 променеві трихоми з дуже опуклою центральною частиною, а в *O. subalpina* (M. Bieb.) D.A.German опушення листків густе, а квітконіжок – розсіяне (Рисунок 3).

У роді *Alyssum*, поряд із симетричними, спостерігаються асиметричні зірчасті трихоми. Вони часто позбавлені інкрустації і варіюють за кількістю та ступенем редукції променів (Рисунки 4–6). На листках і плодоніжках *A. trichostachyum* Rupr., *A. umbellatum* Desv. і *A. parviflorum* M.Bieb., наприклад, асиметричні зірчасті волоски особливо численні, а в *A. rostratum* Steven та *A. calycocarpum* Rupr. – дуже рідкісні. Інші види роду також різняться між собою за структурою трихом (Рисунки 4–6). Виразна своєрідність зірчастих трихом – досить довгі первинні промені і чотири-п‘ятипроменеві волоски на квітконіжках, властива рослинам *Meniocus linifolius* (Willd.) DC. *Berteroa incana* (L.) DC. має розсіяне зірчасте опушення, що складається із симетричних і асиметричних трихом. Останні крупніші й особливо характерні для плодоніжок (Рисунок 7). Для *Clypeola jonthlaspi* L. характерні зірчасті (інкрустовані та ні) і асиметричні зірчасті трихоми із розширеною основою і дуже довгими двома та короткими кількома променями (Рисунок 7).

Екологічний спектр хрестоцвітих (триба Alysseae), опушених зірчастими трихомами, відображає їхнє походження – середземноморсько-ірано-туранське. Рослини цієї групи історично

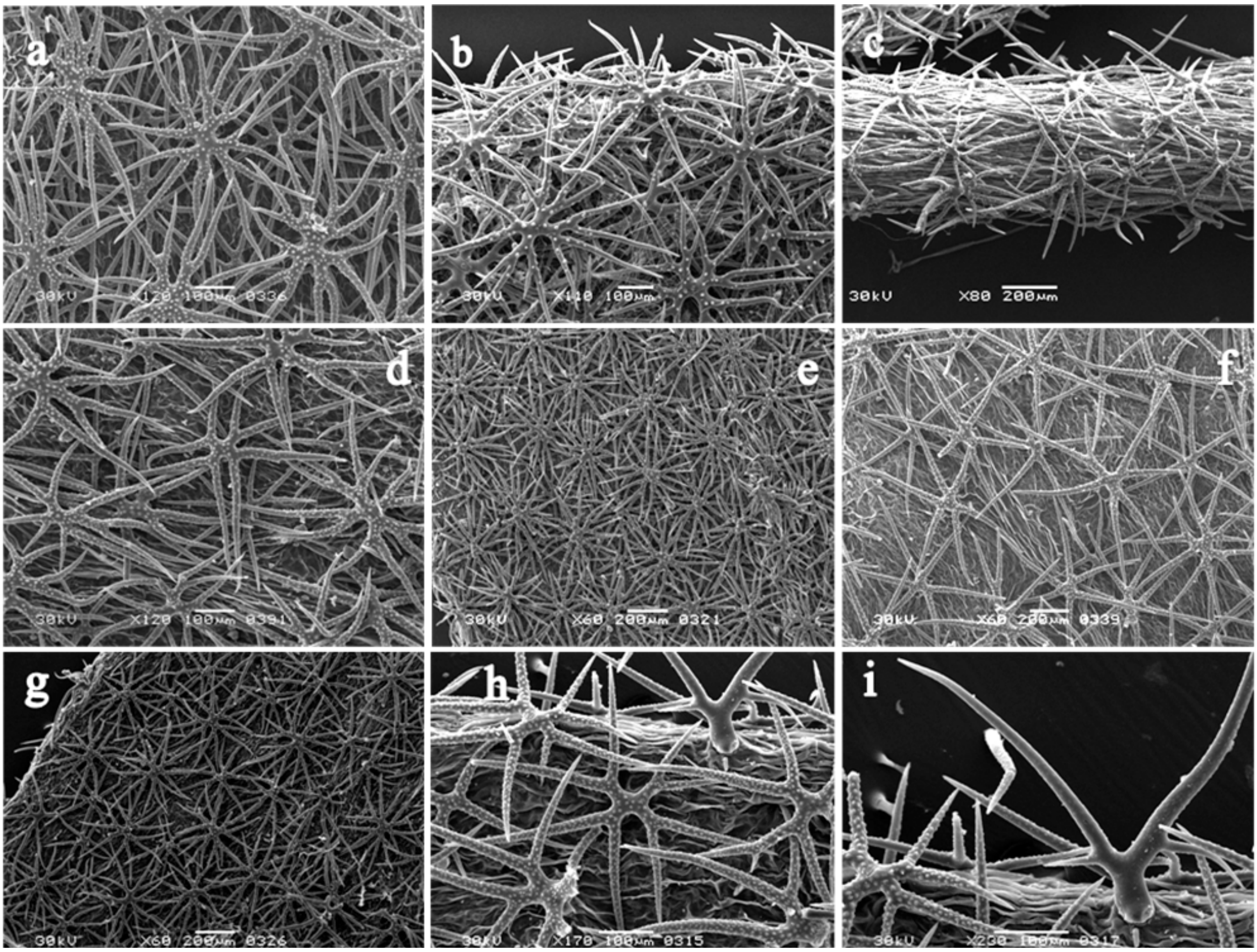

Рисунок 4 Трихоми листків і квітконіжок видів триби Alysseae DC. (*Alyssum* L.):
a – *A. alyssoides* L.; b, c – *A. calycocarpum* Rupr.; d – *A. desertorum* Stapf; e – *A. gmelinii* Jord. et Fourr.; f – *A. hirsutum* M.Bieb.; g – *A. iljinskajae* V.I.Dorof.; h, i – *A. minutum* Schlecht. ex DC.; c, h, i – квітконіжка

Figure 4 Trichomes of leaves and peduncles of Alysseae DC. tribe species (*Alyssum* L.):
a – *A. alyssoides* L.; b, c – *A. calycocarpum* Rupr.; d – *A. desertorum* Stapf; e – *A. gmelinii* Jord. et Fourr.; f – *A. hirsutum* M.Bieb.; g – *A. iljinskajae* V.I.Dorof.; h, i – *A. minutum* Schlecht. ex DC.; c, h, i – peduncle

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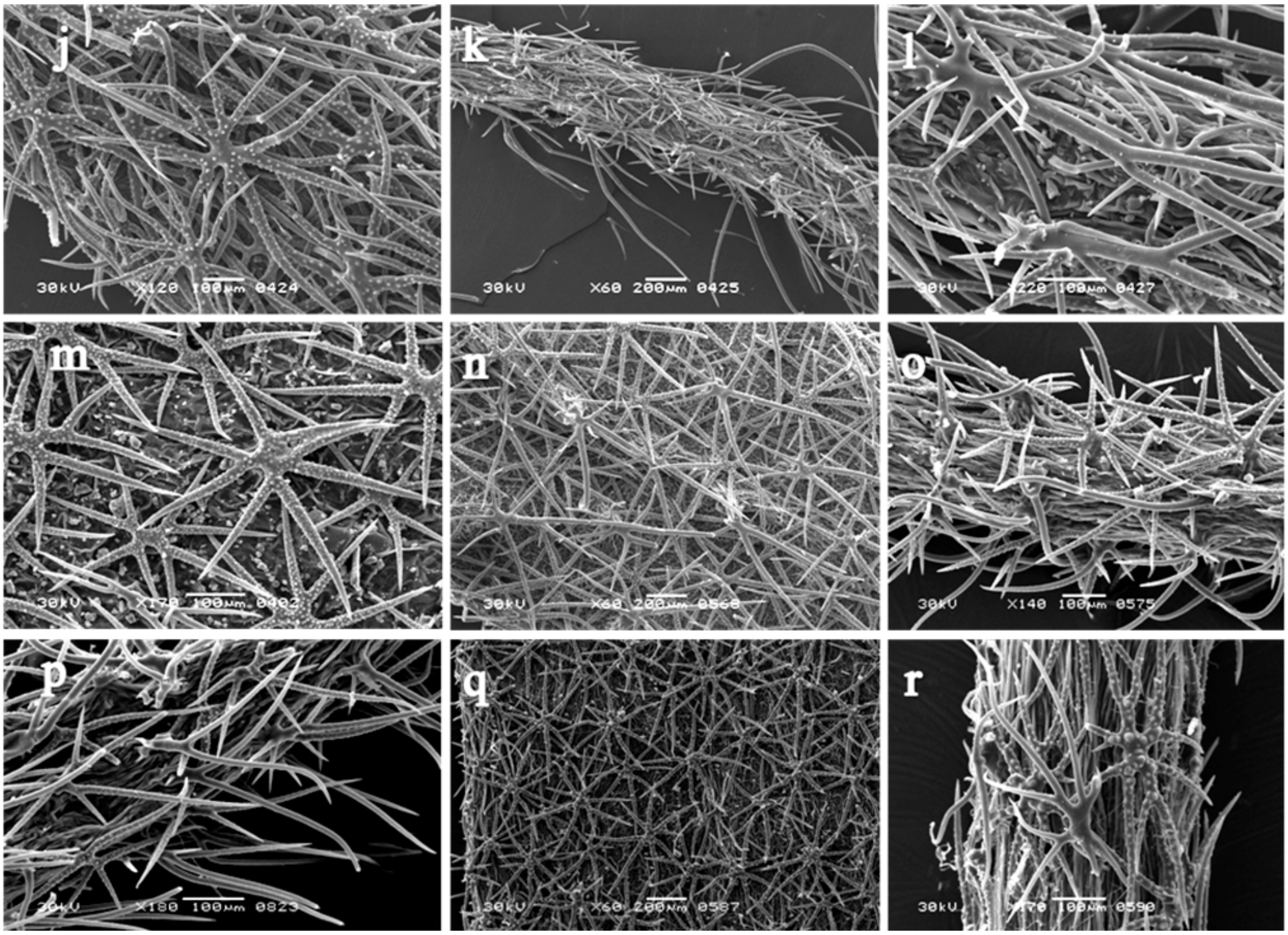

Рисунок 5 Трихоми листків і квітконіжок видів роду *Alyssum* L.:
j, k, l – *A. lenense* Adams; m – *A. smyrnaeum* C.A.Mey.; n, o, p – *A. parviflorum* M.Bieb.; q, r – *A. rostratum* Steven; k, l, o, p, r – квітконіжка

Figure 5 Trichomes of leaves and peduncles of *Alyssum* L. species:
j, k, l – *A. lenense* Adams; m – *A. smyrnaeum* C.A.Mey.; n, o, p – *A. parviflorum* M.Bieb.; q, r – *A. rostratum* Steven; k, l, o, p, r – peduncle

адаптовані до гідротермального стресу, обумовленому спекотним та сухим літом і холодною та вологою зимою й толерантні до бідного субстрату. Такі особливості характерні для клімату Середземноморської та Ірано-Туранської флористичних областей (Takhtajyan, 1978; Rotondi et al., 2003). На території України вони приурочені до височин, осипищ і оголень, в тому числі крейдяних, кам'янистих гірських схилів різних висот, вапняків, гранітів (зрідка), пішаників та пісків. Усі біотопи, до складу яких входять види із зірчастим опушенням, об'єднує ще дві особливості – наявність інтенсивної інсоляції та бідність субстрату. Формування густого опушення на рослинах є адаптацією до екстремальних умов існування, в тому числі до інтенсивної сонячної радіації. Виразна здатність існувати в суворих умовах довкілля дозволяє багатьом з них, особливо однорічникам, активно освоювати антропогенно трансформовані екотопи.

Дендроїдні трихоми у флорі України характерні для трьох видів двох родів: *Aurinia* Desv. (Alysseae) і *Schivereckia* Andrz. ex DC. (Arabideae DC.) (Рисунки 7, 8).

Aurinia saxatilis (L.) Desv. на території України характерний для гранітних, сланцевих і вапнякових відслонень та пісковиків. Крім того, рослини цього виду часто вирощують як декоративні, вони можуть дичавіти з культури. Обидва види *Schivereckia* стенотопні: *S. podolica* (Besser) Andrz. ex DC. росте здебільшого на берегових виходах вапняків басейну р. Дністер, а *S. mutabilis* (M.I.Alex.) M.I.Alex. – на крейдяних відслоненнях басейну р. С. Донець. Зауважимо, що таксономічний статус *Schivereckia*, в цілому, і другого виду, зокрема,

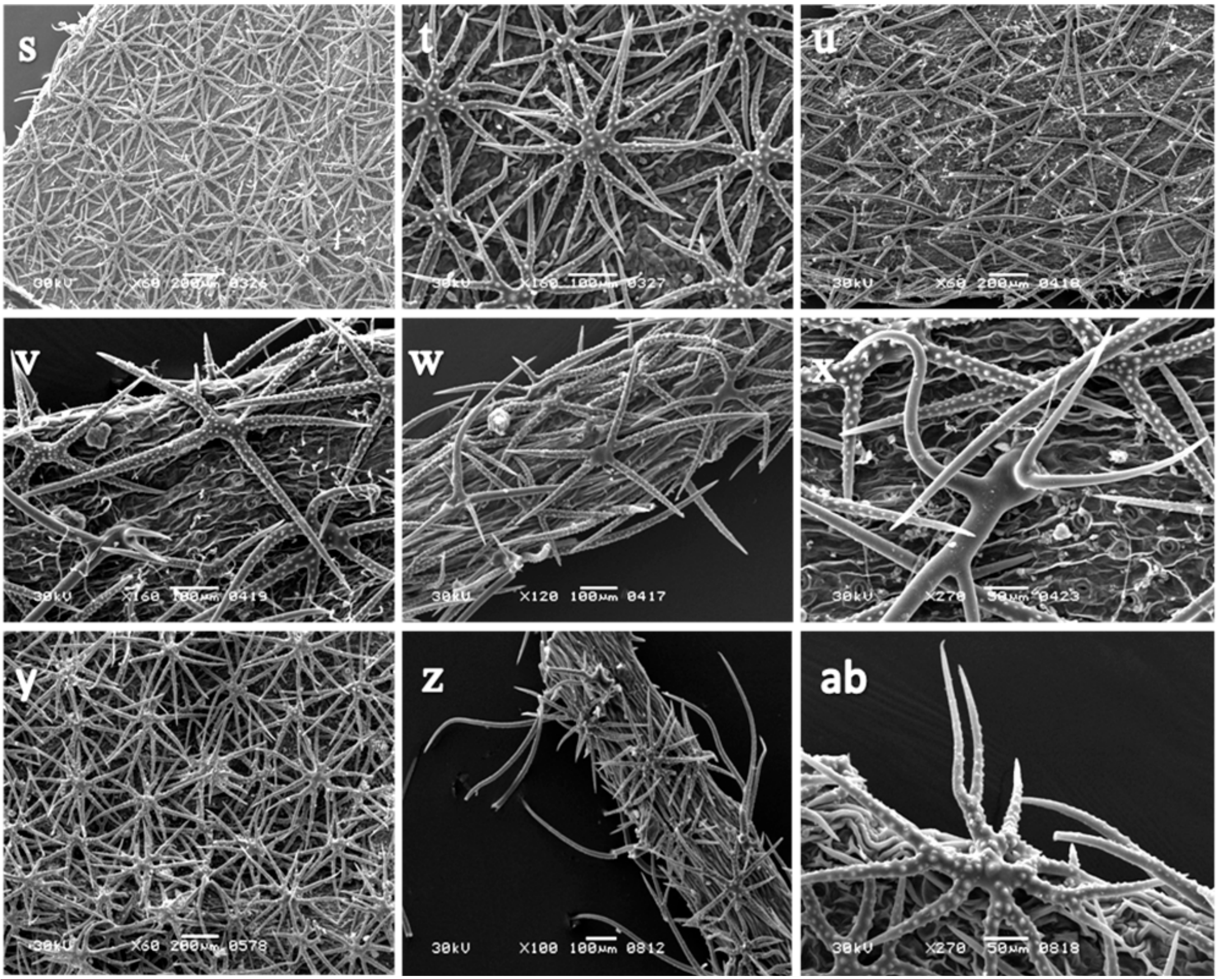

Рисунок 6 Опушення листків і квітконіжок видів *Alyssum* L.:
s, t – *A. kotovii* A.P.Iljinsk.; u, v, w, x – *A. umbellatum* Desv.; y, z, ab – *A. trichostachyum* Rupr.; w, x, z, ab – квітконіжка

Figure 6 Pubescence of leaves and peduncles of *Alyssum* L. species:
s, t – *A. kotovii* A.P.Iljinsk.; u, v, w, x – *A. umbellatum* Desv.; y, z, ab – *A. trichostachyum* Rupr.; w, x, z, ab – peduncle

остаточно ще не визначені (Jordon-Thaden et al., 2013). Види розрізняються за характером опушення та структурою трихом. Рослини *S. podolica* мають досить розсіяне опушення, яке складається із дендроїдних волосків із довгими ніжками. Опушення *S. mutabilis* значно щільніше, сформоване двома шарами волосків із ніжками різної довжини (Рисунок 8).

Мальпігієві трихоми властиві 23 видам трьох родів – *Lobularia* Desv., *Erysimum* та *Syrenia* Andrz. ex DC. (Рисунок 2). У *Lobularia maritima* (L.) Desv.) такі волоски двопроменеві (Рисунок 9). Рослини цього виду широко культивують, в тому числі в Україні, де вони можуть давати самосів і на півдні формувати спонтанні популяції, але на півночі страждають від морозів. Мальпігієві трихоми з двома, декількома або багатьма променями спостерігаються у видів *Erysimum* s. str. У багатьох із них вони формуються майже на всіх органах рослини в неоднакових комбінаціях і пропорціях, що здавна використовують в систематиці роду на різних рівнях таксономічної ієрархії. Тільки двопроменеві мальпігієві волоски характерні для *E. diffusum* Ehrh. Такі ж трихоми спостерігаються на стеблах *E. ucranicum* J. Gay та *E. krynkense* Lavrenko, а на їхніх листках є домішка трипроменевих волосків. Двопроменеві мальпігієві трихоми із домішкою трипроменевих характерні для *Erysimum* × *cheiri* (L.) Crantz (= *Cheiranthus cheiri* L.). Рослини цього виду широко відомі, в тому числі в Україні, як декоративні; вони можуть дичавіти з культури (наприклад, в Криму). Природний ареал *Erysimum* × *cheiri* включає Балкани

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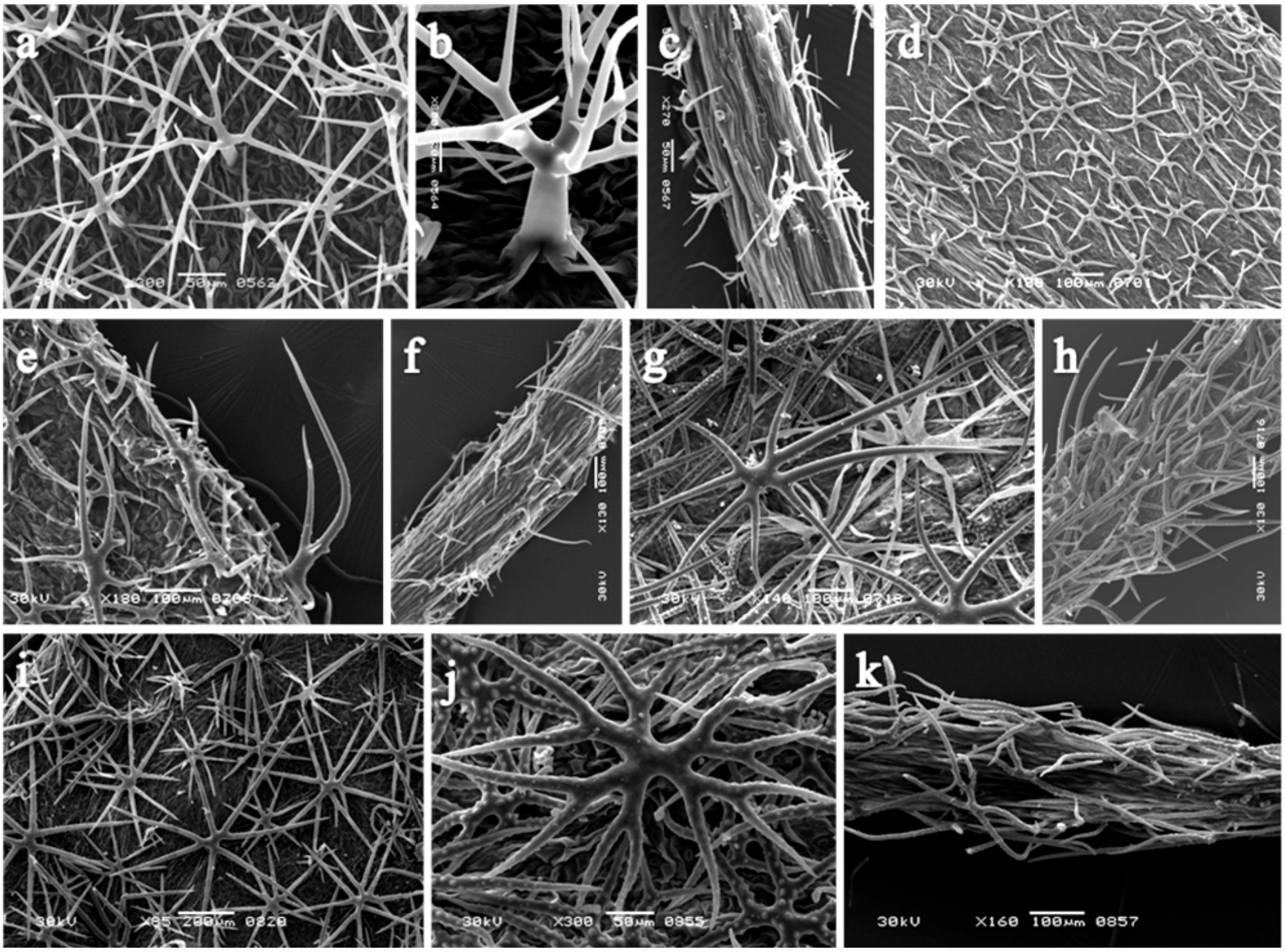

Рисунок 7 Трихоми листків і квітконіжок видів триби Alysseae DC.:
a, b, c – *Aurinia saxatilis* Desv.; d, e, f – *Berteroa incana* (L.) DC.; g, h – *Clypeola jonthlaspi* L.; i – *Fibigia clypeata* (L.) Medik.; j, k – *Meniocus linifolius* DC.; c, f, h, k – квітконіжка

Figure 7 Trichomes of leaves and peduncles of Alysseae DC. tribe species:
a, b, c – *Aurinia saxatilis* Desv.; d, e, f – *Berteroa incana* (L.) DC.; g, h – *Clypeola jonthlaspi* L.; i – *Fibigia clypeata* (L.) Medic.; j, k – *Meniocus linifolius* DC.; c, f, h, k – peduncle

і острови Егейського моря (Kotov, 1953; POWO, 2021b). На листках *E. leptostylum* DC. переважають чотири- і п'ятипроменеві трихоми, а інші форми складають домішку. Трипроменеві волоски з домішкою двопроменевих утворюють більшість на рослинах *E. witmannii* Zaw. (Gostin, 2009), а чотири- і п'ятипроменеві – спостерігаються дуже зрідка. Види *Syrenia* (часто включають в *Erysimum*) відрізняються від усіх видів *Erysimum* s. str. формуванням на плодах упоперек орієнтованих двопроменевих мальпігієвих волосків. Тоді як в роді *Erysimum* s. str. двопроменеві мальпігієві трихоми орієнтовані паралельно до довжини плоду. Для *Syrenia dolichostylos* Klokov та *S. ucrainica* Klokov характерні лише двопроменеві мальпігієві трихоми, тоді як на рослинах *S. talijevii* Klokov спостерігається домішка трироздільних волосків.

Екологічний діапазон досліджених видів *Erysimum*, як і роду в цілому, дуже широкий. На території України дев'ять видів із 19 поширені в дуже різноманітних екологічних умовах: від гумідних (лісових галявин, луків, річкових берегів) до аридизованих або аридних (степів, вапнякових, гіпсових, крейдяних, гранітних і піщаних оголень, піщаних та супіщаних напівпустель), а також входять до складу антропогенно змінених біотопів. Два з них, *E. ucranicum* і *E. krynkense*, – ендеміки крейдяних оголень. Три – *E. hungaricum* Zapał., *E. witmannii* і *E. froehneri* Polatschek, – монтанні рослини Карпат і Криму. Найбільш мезофільними є *E. aureum* M.Bieb. і *E. virgatum* Roth. Вони поширені в широколистяних лісах, на лісових галявинах, у чагарниках та на берегах водойм. Поширення *E. leucanthemum* (Stephan ex Willd.) B.Fedtsch. на території України обмежено

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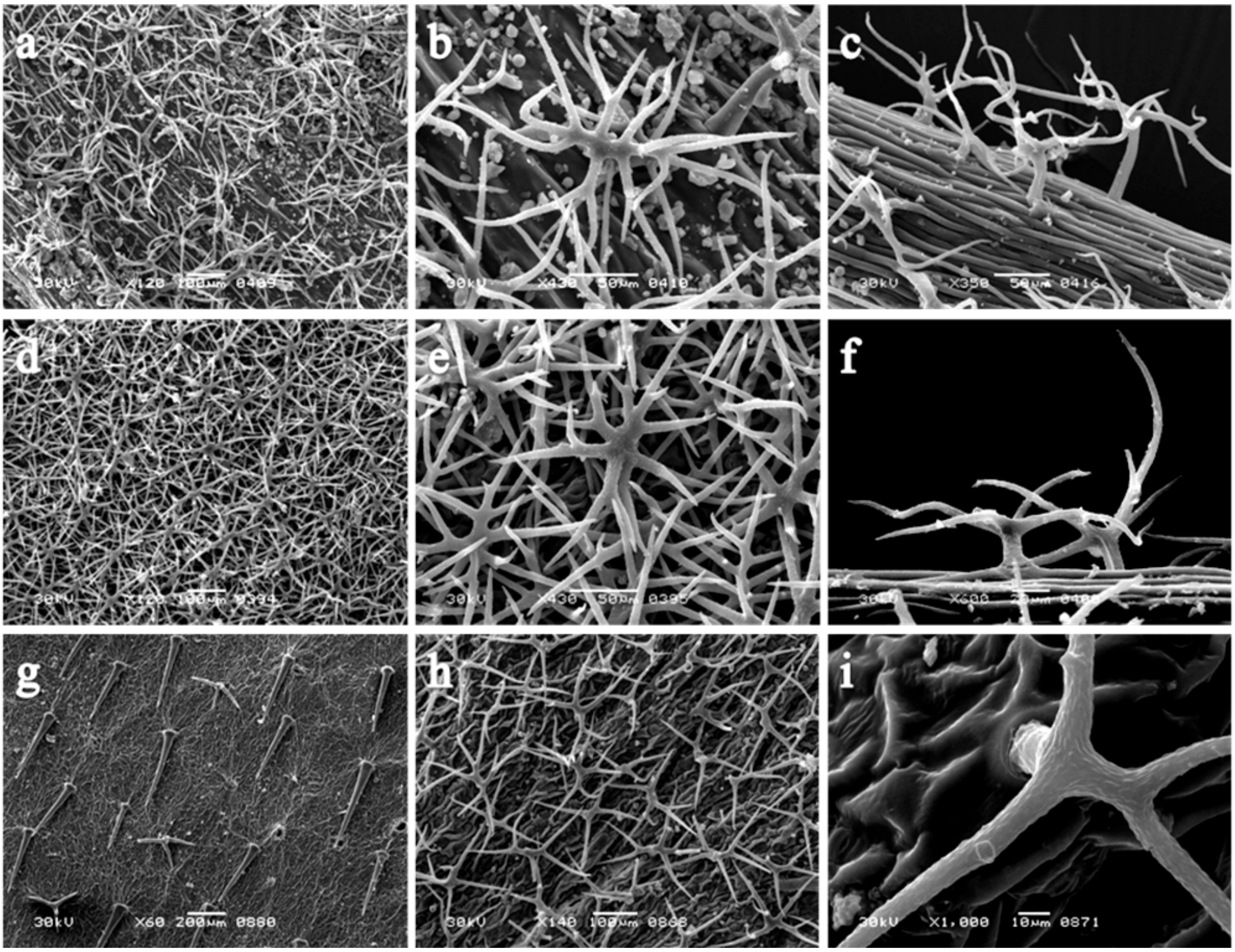

Рисунок 8 Трихоми листків і квітконіжок видів триб Arabideae DC. (*Schivereckia* Andrz. ex DC.: a, b, c – *S. podolica* (Besser) Andrz. ex DC.; d, e, f – *S. mutabilis* (M.I.Alex.) M.I.Alex.) та Camelineae DC. (g – *Catolobus pendulus* (L.) Al-Shehbaz; h, i – *Pseudoarabidopsis toxophylla* (M.Bieb.) Al-Shehbaz, O'Kane et R.A.Price); c, f – квітконіжки

Figure 8 Trichomes of leaves and peduncles of Arabideae DC. tribe species. (*Schivereckia* Andrz. ex DC.: a, b, c – *S. podolica* (Besser) Andrz. ex DC.; d, e, f – *S. mutabilis* (M.I.Alex.) M.I.Alex.) and Camelineae DC. (g – *Catolobus pendulus* (L.) Al-Shehbaz; h, i – *Pseudoarabidopsis toxophylla* (M.Bieb.) Al-Shehbaz, O'Kane et R.A.Price); c, f – peduncles

глинистими, кам'янистими і солонцюватими місцями в полинових степах. Тут проходить західна межа природного ареалу цього виду. Останній простягається від України до Північно-Західного Китаю (POWO, 2021c). Види роду *Syrenia* характерні для степових і північних пустельних регіонів від Угорщини (на заході) до Монголії (на сході) і до Дагестану (на півдні) (Kotov, 1953). На території України вони приурочені до пісків (річкових та морських) і оголень крейди (*S. talijevii*). Більшості вивчених видів з опушенням із мальпігієвих трихом характерний однаковий або дуже схожий комплекс адаптивних властивостей. Вони існують, як правило, в складі біотопів з розрідженою або майже відсутньою рослинністю, як і чимала частина хрестоцвітих (Ilyinska et al., 2007; Dorofeev, 2012; Polatschek, 2010–2012, 2013a,b; Turis, 2019). Багато з них пристосовані до інтенсивної інсоляції, бідного субстрату, недостатнього зволоження, а також до особливого теплового режиму в гірських та в напівпустельних регіонах з різкими перепадами добової температури. Чіткої кореляції між якісним складом мальпігієвих трихом і екологічними особливостями видів не спостерігається. Разом з тим виразно проявляється загальновідома закономірність: щільність опушення рослин зростає зі збільшенням ступеня аридності біотопів. Звертає на себе увагу і той факт, що у флорі України тільки *Erysimum* × *cheiri*, *E. froehneri* та *E. ucranicum* є багаторічниками. Решта – це монокарпіки (дворічники або однорічники).

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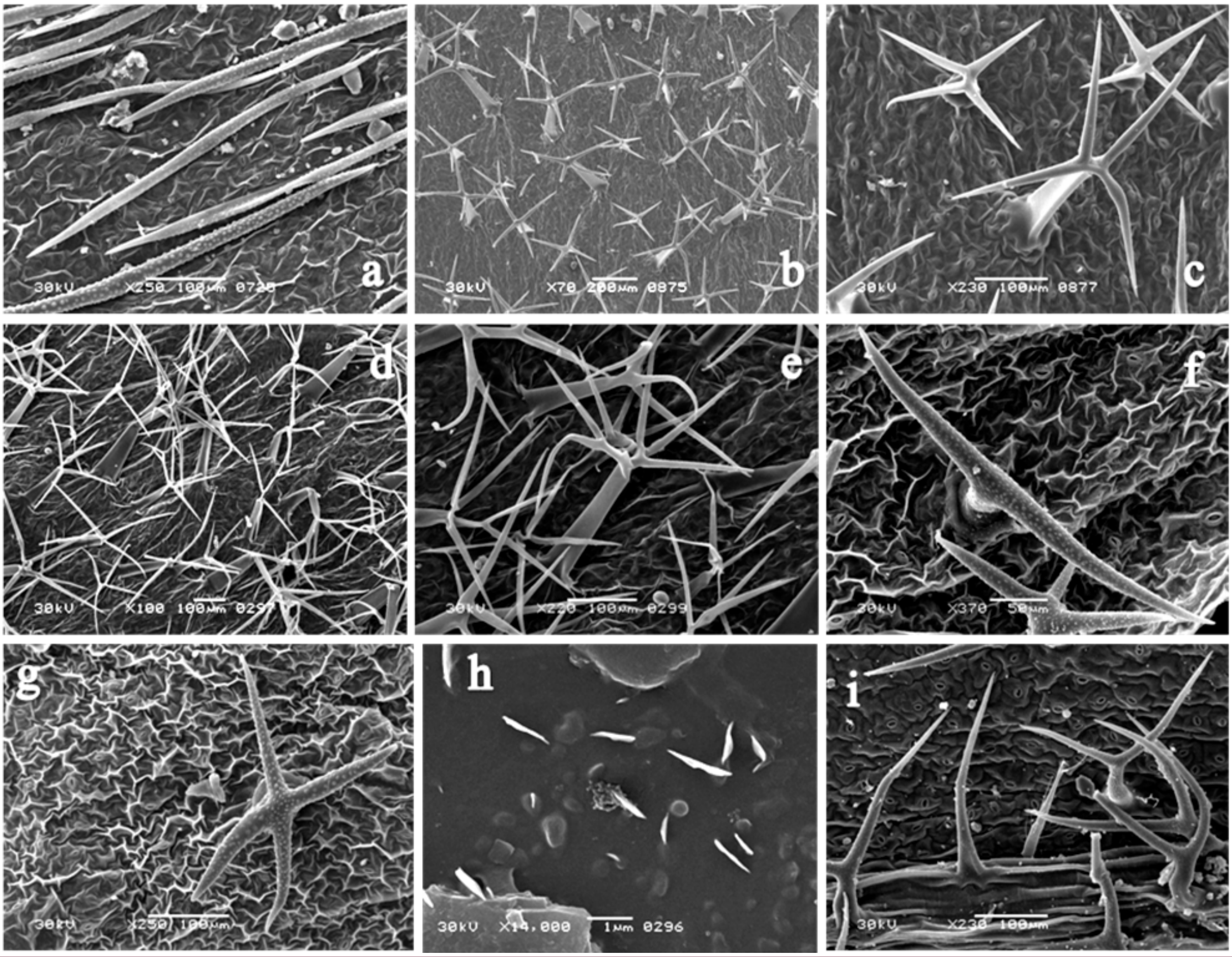

Рисунок 9 Трихоми листків видів триб Anastaticeae DC.:
a – *Lobularia maritima* (L.) Desv.) та Arabideae DC.; b, c – *Arabis alpina* L.; d, e – *A. caucasica* Willd.; f, g, h – *A. gerardii* Besser; i – *A. hirsuta* (L.) Scop.; h – епікутикулярний пластинчатий віск

Figure 9 Trichomes of leaves of Anastaticeae DC. tribe species:
a – *Lobularia maritima* (L.) Desv.) and Arabideae DC.;
b, c – *Arabis alpina* L.; d, e – *A. caucasica* Willd.; f, g, h – *A. gerardii* Besser; and – *A. hirsuta* (L.) Scop.; h – epicuticular lamellar wax

Фуркатні трихоми за літературними даними характерні для нещодавно знайденого в Криму *Olimarabidopsis pumila* (Stephan) Al-Shehbaz, O'Kane et Price (Fateryga et al., 2019). Abdel (2005) наводить для цього виду два типи волосків: trifixed і Y-shaped. Beilstein et al. (2008) такі трихоми відносять до типу «dendritic». Відповідно до нашої класифікації, вони відповідають фуркатним волоскам із двома, трьома чи чотирма променями. У класичних флористичних роботах для цього виду вказували короткі розгалужені трихоми (Bush, 1939; Kotov, 1979). Вид характерний здебільшого для солонцевих та солончакових біотопів.

Гетероморфне опушення Brassicaceae флори України формується двома або декількома типами покривних трихом (Рисунок 2). До його складу можуть входити залозисті волоски або залозки.

Гетероморфне опушення без залозистих структур характерно для 45 видів Brassicaceae флори України.

Комбінація простих і фуркатних трихом спостерігається в найбільшого числа видів (44) (Рисунки 9–11). Вони входять до складу 17 родів декількох триб: Arabideae DC. (*Arabis* L., *Aubrieta* Adans., *Draba*, *Drabella* (DC.) Fourr., *Erophila* DC., *Pseudoturritis* Al-Shehbaz), Camelineae DC. (*Capsella*, *Pseudoarabidopsis* Al-Shehbaz, O'Kane et R.A.Price, *Arabidopsis* (DC.) Heynh., Catolobus (C.A.Mey.) Al-Shehbaz, Neslia Desv.), Euclidieae DC. (*Euclidium* W.T.Aiton, *Neotorularia* Hedge

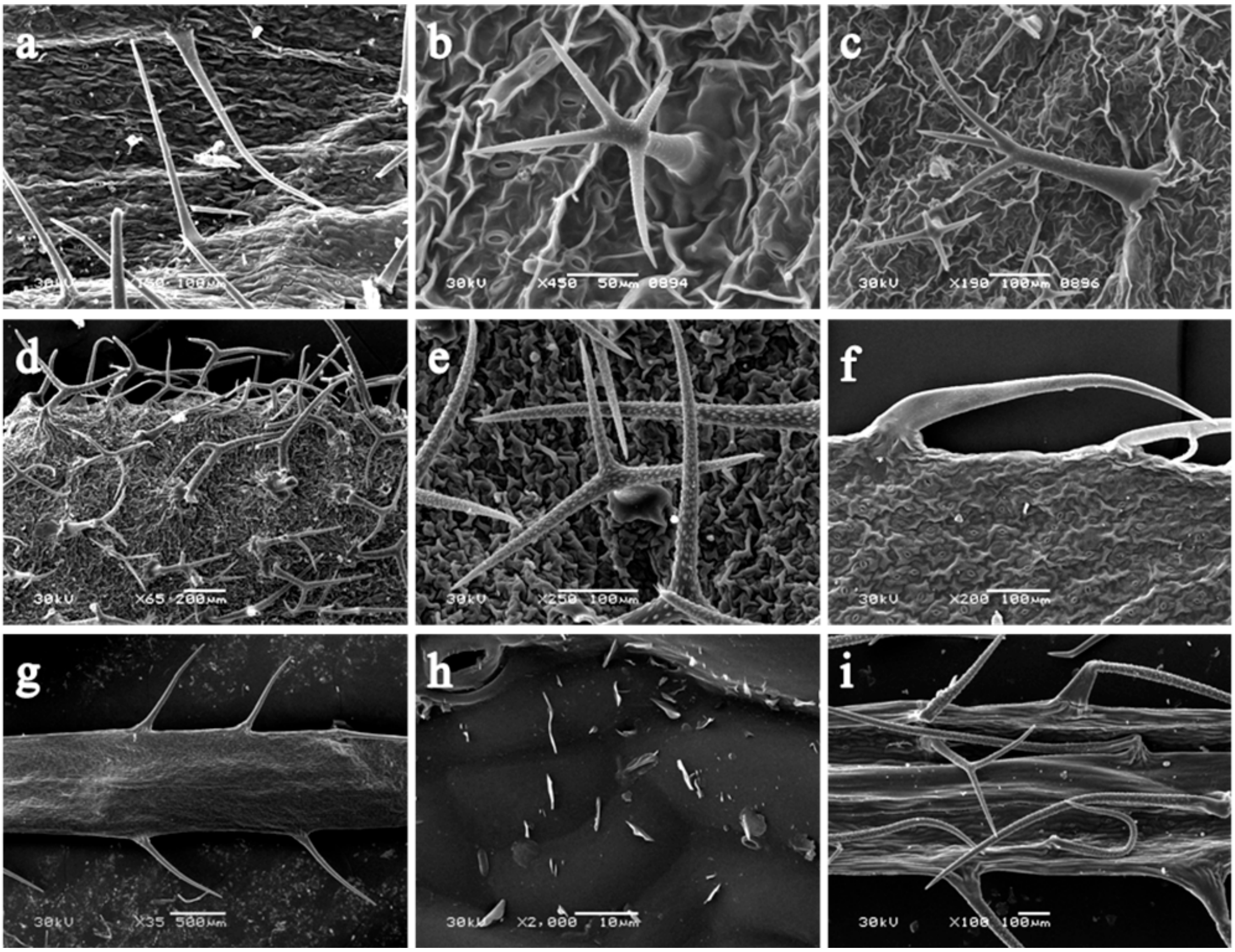

Рисунок 10 Трихоми листків видів триби Arabideae DC.:
a – *Arabis hornungiana* Schur; b, c – *A. recta* Vill.; d, e – *A. sagittata* (Bertol.) DC.; f – *A. sudetica* Tausch; g, h – *Draba aizoides* L.; i – *D. cuspidata* M.Bieb.; h – епікутикулярний пластинчатий віск

Figure 10 Trichomes of leaves of Arabideae DC. tribe species:
a – *Arabis hornungiana* Schur; b, c – *A. recta* Vill.; d, e – *A. sagittata* (Bertol.) DC.; f – *A. sudetica* Tausch; g, h – *Draba aizoides* L.; i – *D. cuspidata* M.Bieb.; h – epicuticular lamellar wax

et J.Léonard, *Strigosella* Boiss.), Descurainieae Al-Shehbaz, Beilstein et E.A.Kellogg (*Hornungia* Rchb., *Descurainia*) та Turritideae Buchenau (*Turritis* L.). У одних видів обидва типи трихом розвинені майже однаково, наприклад у *Pseudoturritis turrita* (L.) Al-Shebaz (Lang, 2020), в інших – зокрема в родах *Pseudoarabidopsis*, а також *Capsella* та *Descurainia* (Ilyinska and Shevera, 2003b) домінують фуркатні трихоми, особливо багатопроменеві, які часто називають зірчастими або дендроїдними. Прості волоски спостерігаються як домішка: у *P. toxophylla* – по краю і знизу по жилці листків (Ilyinska et al., 2007), а в *D. sophia* (L.) Webb ex Prantl – здебільшого на стеблах. Зауважимо, що іншим видам роду *Descurainia* (немає в Україні) властиве щільне опушення. Крім того, деяким (*D. incisa* (Engelm.) Britton, *D. longepedicellata* (E.Fourn.) O.E.Schulz, *D. sophioides* (Fisch. ex Hook.) O.E.Schulz), характерні залозисті папіли (Al-Shehbaz, 2010). Фуркатні трихоми можуть спостерігатися дуже зрідка або зовсім зникати, наприклад у гірського *Arabis hornungiana* Schur (Рисунок 10) і нещодавно описаного нового виду *Camelina neglecta* J.R.Brock, Mandáková, Lysak et Al-Shehbaz (Brock et al., 2019). Кількісне співвідношення простих і фуркатних трихом, їхня топографія на рослині, в цілому, і на листках, зокрема, щільність опушення, форма і розмір трихом, число і орієнтація променів, товщина клітинної стінки, а також характер інкрустації поверхні у видів роду *Arabis* дуже варіюють (Рисунки 9, 10), що використовують для діагностики видів. Дуже своєрідні чотирипроменеві фуркатні волоски на

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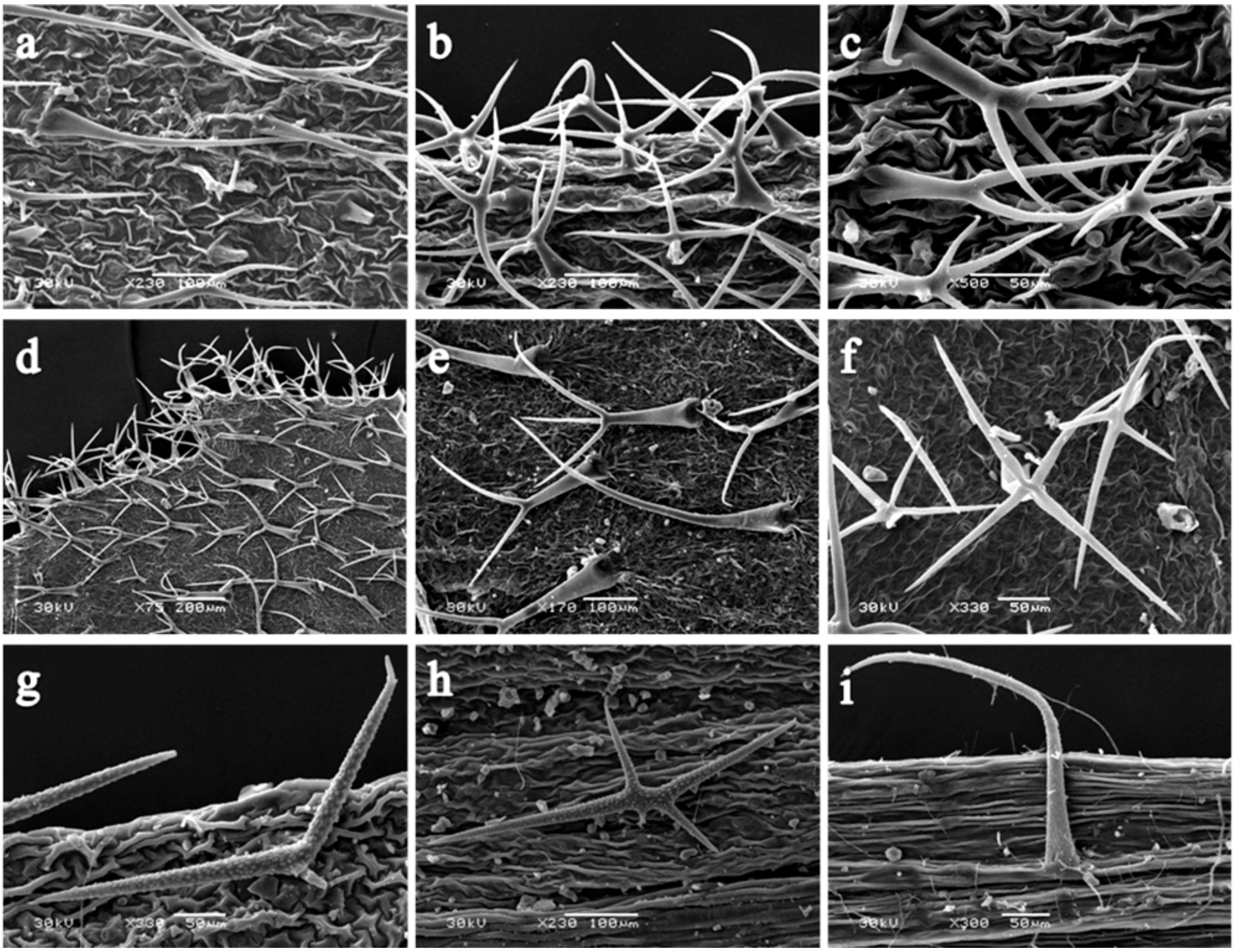

Рисунок 11 Трихоми видів *Draba* L.:
a, b, c – *D. carinthiaca* Hoppe; d, e, f – *D. nemorosa* L.; g, h, i – *D. sibirica* (Pall.) Thell.; i – генеративне стебло, інші – листки

Figure 11 Trichomes of *Draba* L. species:
a, b, c – *D. carinthiaca* Hoppe; d, e, f – *D. nemorosa* L.; g, h, i – *D. sibirica* (Pall.) Thell.; i – generative stem, others – leaves

довгих ніжках спостерігаються на листках *A. alpina* L. і *A. caucasica* Willd. (Рисунок 9).

Arabis sagittata (Bertol.) DC. відрізняється від інших видів роду наявністю на стеблі довгих простих, а на листках – дво-, три-, а іноді й чотирипроменевих фуркатних волосків (Рисунок 10). Для *Arabis sudetica* Tausch характерні неопушене стебло і прості й двопроменеві фуркатні волоски по краю листків (Рисунок 10). Різні комбінації простих і фуркатних трихом мають види *Draba* (Рисунки 10, 11). По краю листків *D. cuspidata* M.Bieb. формується війчасте опушення з довгих простих волосків з домішкою двопроменевих фуркатних. У *D. aizoides* L. війчасте опушення листків сформоване тільки простими волосками (Рисунок 12). Для листків *D. nemorosa* L. і *D. carinthiaca* Hoppe, характерні прості (нечисленні) та дво-, три- і чотирипроменеві фуркатні трихоми (Рисунок 11). У флорі України незначне гетерогенне опушення та пластинчатий епікутикулярний віск (інколи) мають види роду *Arabidopsis* (Рисунок 12).

Відповідно до екологічної приуроченості, всі види, що мають комбіноване опушення із фуркатних і простих волосків, можна розділити на дві групи. Першу – складають види рівнинних або/і гірських біотопів з достатньою чи навіть підвищеною вологістю й помірною інтенсивністю інсоляції. Це представники триб Arabideae, Turritideae та Camelineae (частково). Їхнє опушение сформоване, як правило, великими й дуже розсіяними трихомами, а на листках формуються іноді пластинчасті кристали воску, наприклад у *Arabis hornungiana*, *Arabidopsis ovirensis* (O'Kane et Al-Shehbaz) A.P.Iljinsk., *Draba aizoides* L. та ін.

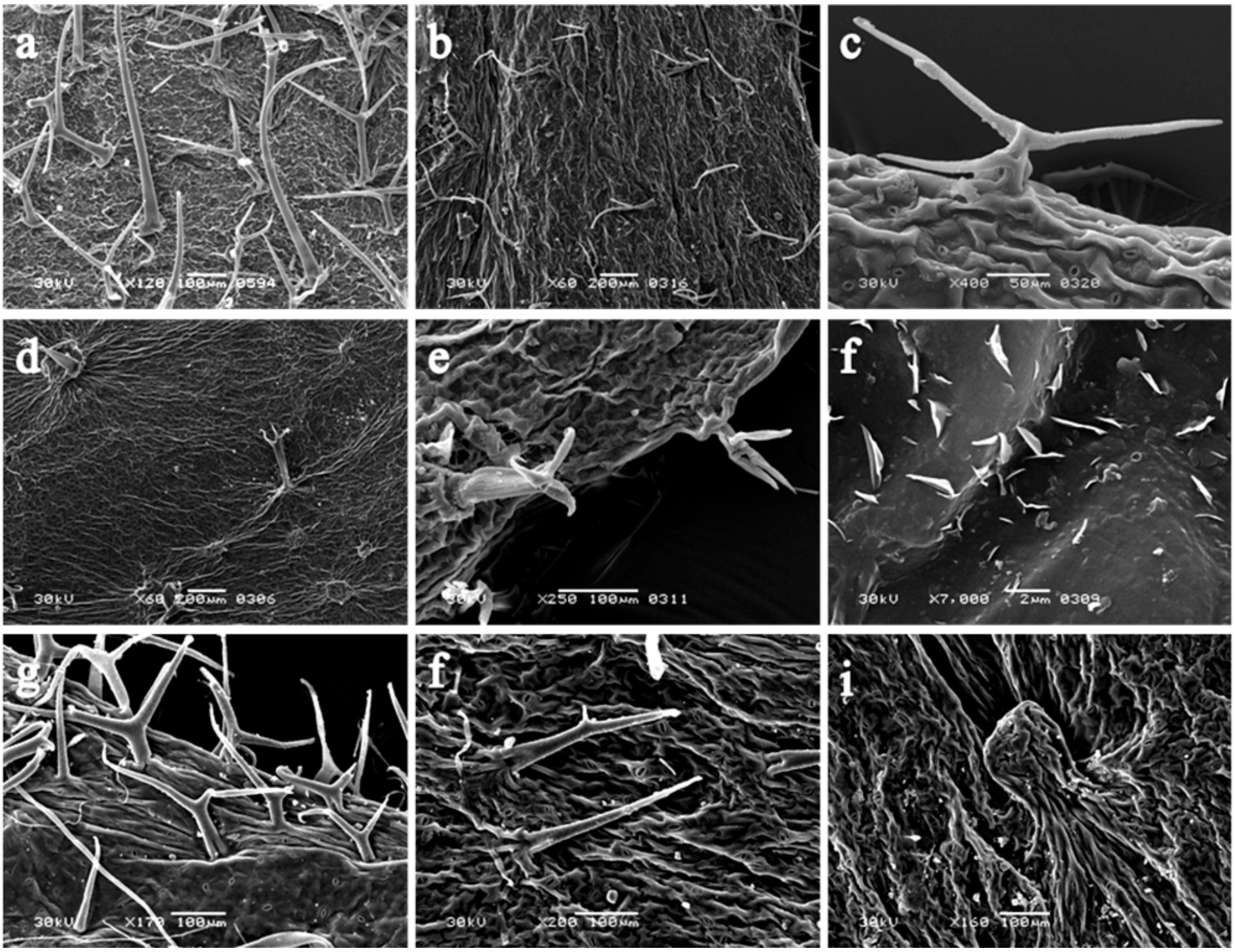

Рисунок 12 Трихоми листків видів *Arabidopsis* Heynh. (Camelineae DC.) та *Bunias* (Buniadeae DC.):
a – *Arabidopsis arenosa* (L.) Lawalrée; b, c – *A. multijuga* (Borbás) D.A.German; d, e, f – *A. ovirensis* (O'Kane & Al-Shehbaz) A.P.Iljinsk.; g, h, i – *Bunias orientalis* L.; f – епікутикулярний пластинчатий віск; i – багатоклітинна залозка

Figure 12 Trichomes of leaves of *Arabidopsis* Heynh. (Camelineae DC.) and *Bunias* (Buniadeae DC.):
a – *Arabidopsis arenosa* (L.) Lawalrée; b, c – *A. multijuga* (Borbás) D.A.German; d, e, f – *A. ovirensis* (O'Kane & Al-Shehbaz) A.P.Ilyinsk.; g, h, i – *Bunias orientalis* L.; f – epicuticular lamellar wax; i – multicellular gland

(Рисунки 10, 12). Другу групу склали представники триб Euclidieae (*Euclidium*, *Neotorularia* Hedge et J. Léonard, *Strigosella* Boiss.), Alyssopsideae Al-Shehbaz (*Olimarabidopsis pumila* (Stephan) Al-Shehbaz, O'Kane et Price) і Descurainieae Al-Shehbaz, Beilstein et E.A.Kellogg (*Descurainia*, *Hornungia* Rchb.). У флорі України всі види цієї групи – нещільно опушені однорічники. Їм характерні дуже дрібні фуркатні трихоми з розгалуженими променями і дуже короткими або навіть майже нерозвиненими ніжками, на відміну від волосків видів першої групи, внаслідок чого такі волоски часто відносять до дендроїдних чи до зірчастих. Ці види адаптовані до інтенсивної інсоляції й характерні для незадернованих степових схилів, кам'янистих осипищ, пісків, піщаних напівпустель, солонців або солончаків та інших аридних або аридизованих біотопів, в тому числі входять до складу сухих рудералізованих девастованих біотопів. До цієї групи належить *Capsella bursa-pastoris*, що поширений в дуже широкому діапазоні біотопів – від холодних пустельних, тундрових, тайгових, альпійських і субальпійських до спекотних пустельних і сухотропічних гавайських (CABI, 2021a; POWO, 2021a), а також у складі техногенних та інших порушених біотопів, особливо на легких піщаних ґрунтах. Решта видів мають досить локальний ареал, але так само адаптовані до нерегулярного зволоження субстрату, інтенсивного УФ-випромінювання, до ґрунтів різного типу й різного багатства, в тому числі солонцюватих і солончакових (*Pseudoarabidopsis toxophylla* (M.Bieb.) Al-Shehbaz, O'Kane et R.A.Price).

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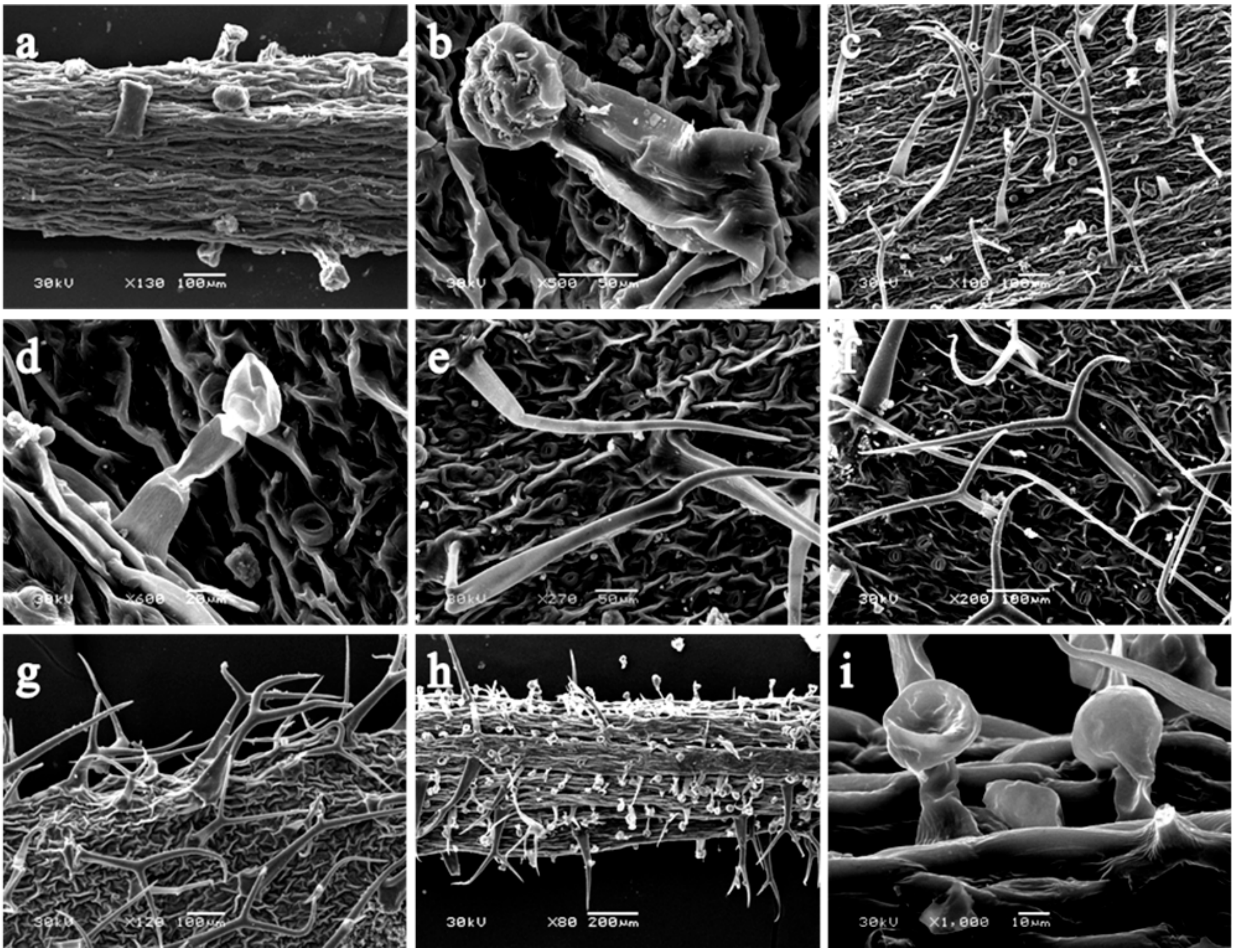

Рисунок 13 Трихоми листків і квітконіжок видів триб Chorisporeae C.A. Mey.:
a, b – *Chorispora tenella* (Pall.) DC.) та Hesperideae Prantl in Engler & Prantl (c, d, e – *Hesperis* tristis L.; f – *H. matronalis* L.; g – *H. pycnotricha* Borbás & Degen; h, i – *H. steveniana* DC.); a, h, i – квітконіжки; b, d, i – залозисті волоски

Figure 13 Trichomes of leaves and peduncles of Chorisporeae C.A.Mey.:
a, b – *Chorispora tenella* (Pall.) DC.) and Hesperideae Prantl in Engler & Prantl tribes species (c, d, e – *Hesperis* tristis L.; f – *H. matronalis* L.; g – *H. pycnotricha* Borbás & Degen; h, i – *H. steveniana* DC.); a, h, i – peduncles; b, d, i – glandular hairs

Комбінація зірчастих і дендроїдних трихом у флорі України спостерігається тільки в *Fibigia clypeata* (триба Alysseae) (Рисунок 7). Три видозміни зірчатих і одна – дендроїдних трихом наводять також для рослин цього виду болгарської флори (Ančev and Goranova, 2006).

Гетероморфне опушення із залозками або залозистими волосками властиве 14 видам п'яти родів п'яти триб: Chorisporeae C.A.Mey. (*Chorispora*), Dontostemoneae Al-Shehbaz et Warwick (*Clausia* Korn.-Trotzky), Hesperideae Prantl in Engler et Prantl (*Hesperis*), Anchonieae DC. (*Matthiola*), Buniadeae DC. (*Bunias*) (Рисунки 12, 13).

Комбінація простих і фуркатних волосків та багатоклітинних сидячих залозок спостерігається лише у видів оліготипного роду *Bunias* L. монотипної триби Buniadeae DC. (Рисунок 12). Середземноморський за походженням *B. erucago* L. у флорі України належить до числа адвентивних рослин, відомих тільки з кількох локалітетів (Kucherevs'kyy, 2004; Ilyinska et al., 2007; Yena and Svirin, 2011). Переднєазійський *B. orientalis* L. належить до числа високоінвазивних видів (відомий із близько 50-ти країн) (CABI, 2021b). Практично на всій території України він входить до складу антропогенно трансформованих, рудералізованних і рекреаційних біотопів (Ilyinska et al., 2007).

Комбінацію простих і залозистих багатоклітинних волосків мають рослини середньоазійського за походженням *Chorispora tenella* DC. (Рисунок 13) і джунгаро-монгольського *Clausia aprica* (Stephan ex Willd.) Korn.-Trotzky. Перший вид входить до складу солонцюватих і бур‘янових біотопів, особливо в Східній Україні. Другий – характерний для вапнякових і крейдяних схилів степів. Через північно-східну частину України, проходить західна межа його ареалу.

Комбінація простих, фуркатних і залозистих багатоклітинних волосків у флорі України властива лише видам роду *Hesperis* (Рисунок 13).

Характер розподілу на рослинах трихом кожного типу і щільність опушення в різних видів варіює. Густі довгі прості й рясні залозисті волоски спостерігаються на плодоніжках *H. sibirica* L., тільки фуркатні й залозисті – має *H. pycnotricha* Borbás et Degen. Для *H. steveniana* DC. характерні прості, фуркатні й рясні, зокрема на плодоніжках, залозисті трихоми. *Hesperis sylvestris* відзначається густим опушенням із коротких простих і залозистих волосків та фуркатних трихом (домішка, вгорі). Квітки, квітконіжки і вісь суцвіття *H. suaveolens* (Andrz.) Besser ex Steud. або розсіяно опушені залозистими і короткими простими волосками, або голі. Розсіяне опушення *H. matronalis* складається із довгих і коротких простих волосків із можливою домішкою фуркатних (вгорі) та залозистих. На рослинах *H. tristis* L. переважають розсіяні прості трихоми із домішкою (вгорі) фуркатних та залозистих (іноді).

У флорі України види *Hesperis* входять до складу лісових, гірських і степових біотопів. Деякі з них, наприклад *H. matronalis*, *H. sibirica* та *H. pycnotricha*, проявляють високу антропотолерантність. Вони розповсюдилися далеко за межі свого природного ареалу (Gudžinskas, 1997; Herloff, 1999; Ebel‘, 2002; Francis et al., 2009; Costache, 2011; Laz‘kov et al., 2011; Dorofeev, 2013; Mandáková et al., 2017; CABI, 2020). На території України ці види віддають перевагу біотопам із більш-менш оптимальним зволоженням. У регіоні сучасного формового різноманіття роду *Hesperis* (Балкани, Кавказ, Ірано-Туранська флористична область) переважають гірські види, зокрема субальпійські та альпійські, що поширені на схилах, скелях, кручах (Cullen, 1965; Takhtajyan, 1978; Duran, 2008; Duran and Çetin, 2016). Щільність опушения рослин цієї групи, як і попередніх, збільшується із посиленням ступеня аридности середовища їхнього існування.

Комбінація простих, фуркатних, зірчастих, дендроїдних і залозистих багатоклітинних волосків у флорі України характерна тільки для чотирьох видів роду *Matthiola* (Anchonieae DC.). У двох багаторічників (*M. odoratissima* (Pall. ex M.Bieb.) W.T.Aiton та *M. fragrans* Bunge) і двох інтродукованих однорічників (*M. incana* (L.) W.T.Aiton та *M. longipetala* (Vent.) DC.) вони формують дуже густе повстисте опушення. В цілому, всі інші види *Matthiola* також мають густе опушення й існують в кам‘янистих степах, на скелястих гірських схилах, крейдяних і вапнякових оголеннях. Вони адаптовані до теплового режиму з різкими перепадами добової температури, сильного УФ-випромінювання й толерантні до бідного субстрату та недостатнього вологозабезпечення в Макаронезії, Середземномор‘ї, Північно-Східній Африці, на Близькому Сході та в Південно-Західній Азії (Gowler, 1998; Zeraatkar and Assadi, 2018; Kaya et al., 2019).

Типи трихом у родах та трибах, які формують еволюційні лінії Brassicaceae (Nikolov et al., 2019), поширені неоднаково (Рисунок 14).

У флорі України тільки прості нерозгалужені волоски властиві рослинам 72 видів тих триб, що включені до складу еволюційної лінії LII (Sisymbrieae, Brassiceae, Thlaspideae, Calepineae, Coluteocarpeae). У видів деяких родів (*Crambe*, *Myagrum*, *Brassica* та інші) волоски можуть бути зовсім відсутні.

Голі рослини характерні для видів роду *Subularia*, що представляє окрему еволюційну лінію LV, а трибова належність цього роду, відповідно до молекулярних даних, ще не визначена.

До складу еволюційної лінії LI у флорі України увійшли 97 видів Brassicaceae із різноманітними типами трихом. У видів чотирьох триб (Camelineae, Turritideae, Alyssopsideae та Descurainieae) формується гетероморфне опушення, що складається із простих і фуркатних трихом. У останніх кількість променів варіює зазвичай від двох до п’яти-семи. Виразна ніжка й наявність на одному й тому ж листку волосків із різним числом променів дає підставу відносити їх до одного й того ж типу. Триба Erysimeae включає види із мальпігієвими волосками. Як і в фуркатних трихомах, кількість променів у них також мінлива. Тільки в окремих видів (наприклад *Erysimum diffusum* Ehrh.) спостерігаються типові мальпігієві трихоми із двома променями. В багатьох інших є більша чи менша домішка волосків із декількома променями, що використовують для діагностики видів (Polatschek, 2010–2012, 2013a,b). У трибах Lepidieae та *Cardamine*ae, опушення представлене простими волосками або відсутнє зовсім. Так само,

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LII	
Sisymbrieae	
Sisymbrium	H
Isatideae	
Isatis	H
Myagrum	A
Brassiceae	
Brassica	H
Cakile	A
Crambe	A, H
Diplotaxis	H
Eruca	H
Erucastrum	H
Hirschfeldia	H
Raphanus	H
Rapistrum	H
Sinapis	H
Thlaspideae	
Alliaria	H
Sobolewskia	H
Thlaspi	A
Calepineae	
Calepina	A
Goldbachia	H
Coluteocarpeae	
Noccaea	A, H
Microthlaspi	A
Conringieae	
Conringia	A
Anastaticeae	
Lobularia	M2
Cochlearieae	
Cochlearia	A
Iberideae	
Iberis	H
Teesdalia	H

Continuation 1

LV	
Триба невизначена	
Subularia	A
LI	
Camelineae	
Arabidopsis	H, F2,F3, F4
Camelina	H, F2,F3, F4
Capsella	H, F3, F4
Catolobus	H, F3, F4
Neslia	H, F3, F4, F5-7
Pseudoarabidopsis	H, F3, F4, F5-7
Turritideae	
Turritis	H, F2, F3, F4, F5-7
Alyssopsideae	
Olimarabidopsis	H, F2, F3, F4, F5-7
Erysimeae	
Erysimum	M2, M3, M4, M5-7
Syrenia	M2, M3
Descurainieae	
Descurainia	H, F2, F5-7
Hornungia	H, F2
Lepidieae	
Lepidium	H
Cardaria	A, H
Coronopus	H
Cardamineae	
Armoracia	A
Barbarea	H
Cardamine	A, H
Dentaria	H
Leavenworthia	A
Nasturtium	H
Rorippa	A, H
Biscutelleae	
Biscutella	H
Lunaria	H

Continuation 2

LIV	
Arabideae	
Arabis	H, F2,F3, F4, F5-7
Aubrieta	H, F2,F3, F4, F5-7
Draba	H, F2,F3, F4
Drabella	H, F2,F3, F4
Erophila	H, F2,F3, F4
Schivereckia	D
Stevenieae	
Pseudoturritis	H, F5-7
Alysseae	
Alyssum	S, AS
Aurinia	D
Berteroa	S, AS
Clypeola	S
Fibigia	S, D
Meniocus	S
Odontarrhena	S
LIII	
Anchonieae	
Matthiola	SS, D, GH
Buniadeae	
Bunias	H, F, G
Euclidieae	
Euclidium	H, F2, F3
Neotorularia	H, F2, F3, F4
Strigosella	H, F2, F3, F4, F5-7
Hesperideae	
Hesperis	H, F2, F3, GH
Dontostemoneae	
Clausia	H, GH
Chorisporeae	
Chorispora	H, GH

Рисунок 14 Різноманітність типів трихом родів Brassicaceae флори України: триби та роди еволюційних ліній (LI, LII, LIII, LIV, LV) і таксони, що не включені до жодної еволюційної лінії (не виділені кольором), розташовані згідно із філогеномним дослідженням Nikolov et al. (2019):
трибова належність роду *Subularia* ще не визначена, згідно із молекулярно-філогенетичними дослідженнями; A – трихоми відсутні; H – прості волоски; F2, F3, F4, F5-7 – фуркатні трихоми з двома, трьома, чотирма або п'ятьма – сімома променями; M2, M3, M4, M5-7 – мальпігієві волоски з двома, трьома, чотирма або п'ятьма – сімома променями; S – зірчасті трихоми сидячі, SA – зірчасті асиметричні, SS – зірчасті на ніжках, D – дендроїдні волоски

Figure 14 Diversity of trichomes types of Brassicaceae genera of the flora of Ukraine: tribes and genera of evolutionary lines (LI, LII, LIII, LIV, LV) and taxa not included in any evolutionary line (not highlighted by color) located according to the phylogenetic study of Nikolov et al. (2019):
Subularia genus is not yet been assigned to tribe, according to molecular phylogenetic studies; A – trichomes are absent; H – simple hairs; F2, F3, F4, F5-7 – forked trichomes with two, three, four or five – seven rays; M2, M3, M4, M5-7 – malpighian hairs with two, three, four or five – seven rays; S – appressed-stellate, SA – asymmetric-stellate, SS – stalked-stellate; D – dendritic hairs

як і в Biscutelleae, яка, відповідно до Nikolov et al. (2019), не була включена до жодної еволюційної лінії Brassicaceae, а пізніше віднесена до LII (Liu et al., 2020).

До еволюційної лінії LIV у флорі України належать 48 видів трьох триб: Arabideae, Stevenieae та Alysseae (Nikolov et al., 2019). Представники перших двох мають гетероморфне опушення, яке складається із простих та фуркатних трихом. На прикладі цих триб легко прослідковується залежність між ступенем розгалуженості трихом і щільністю опушення та екологічними чинниками оселищ видів. В роді *Draba* – одному з найбільших за видовою чисельністю, гірсько-альпійский

D. aizoides, відзначається дуже незначним опушенням із простих волосків по краю листка та епікутикулярними пластинчатими кристалами воску, що має гідрофобні властивості. В інших видів, що наявні у флорі України, опушення добре розвинене і складається із простих та фуркатних трихом із двома, трьома чи чотирма променями. Щільне опушення із дендроїдних волосків мають види *Schivereckia*. В екстремальних біотопах спостерігається збільшення ступеня галузистості фуркатних волосків або щільності опушення також у видів *Arabis*. У гірсько-альпійських *A. alpina* та *A. caucasica* домінують густі чотири- та п'яти-семипроменеві фуркатні волоски, тоді як у мезофільних *A. sagittata*, *A. gerardii* та *A. hirsuta* переважають розсіяні двопроменеві фуркатні трихоми або тільки прості нерозгалужені волоски. До складу інтенсивно інсольованих біотопів Середземномор'я входять густо опушені види *Aubrieta*, один з яких поширюється в Україні як інтродукована рослина (Illinska et al., 2019).

Аналогічна тенденція збільшення галузистості трихом і щільності опушення, спостерігається і в нещодавно описаній трибі Stevenieae (Al-Shehbaz et al., 2011), до якої включили *Pseudoturritis turrita*, що поширений на досліджуваній території. Дуже своєрідні за конструкцією трихоми характерні для *Ptilotrichum canescens* (DC.) C.A.Mey. (= *Stevenia canescens* (DC.) D.A.German = *Alyssum canescens* DC.), що розповсюджений від Південно-Західного Сибіру до Західних Гімалаїв (в Україні відсутній). Їх описували як зірчасті із пірчасто-розгалуженими променями (Bush, 1939) або як дендроїдні (Beilstein et al., 2008). Розвиток ніжки і двох первинних променів вказує на їхню належність до фуркатних трихом із пірчасто-розгалуженими променями. Отже, збільшення розгалуженості трихом є адаптивною реакцією рослин на екстремальні умови існування. Це підтверджує і густе опушення із зірчастих трихом, що властиве більшості видів ірано-туранської за походженням триби Alysseae. Трихоми *Aurinia saxatilis* визначали як зірчасті, але наше дослідження показало, що їх треба кваліфікувати як дендроїдні. Подібні волоски формуються, як домішка до зірчастих, і в *Fibigia clypeata*. Для рослин цього виду із Болгарії вказують зірчасті (сидячі та на ніжці) і дендроїдні трихоми (Ančev and Goranova, 2006). Зауважимо, що різноманітні за характером галуження зірчасті трихоми розвиваються також у видів північноамериканської, здебільшого, триби *Physarieae* (O'Kane, 2010; Fuentes-Soriano and Al-Shehbaz, 2013). Види обох триб подібні за характером екології біотопів, до складу яких вони входять – існують в біотопах кам'янистих відслонень зі слаборозвиненим рослинним покривом, інтенсивною інсоляцією та бідним субстратом. Отже, можна припустити, що в родині Brassicaceae дендроїдні волоски – це похідний тип трихом, що може формуватися в результаті збільшення ступеня галузистості інших їхніх типів, зокрема фуркатних чи зірчастих.

Еволіційну лінію LIII (або суперкладу «E») сформували види триб Chorisporeae, Dontostemoneae, Hesperideae, Anchonieae, Buniadeae, Shehbazieae та Euclidieae (Beilstein et al., 2006, 2008; Koch et al., 2007; Franzke et al., 2011; Huang et al., 2015; Guo et al., 2017; Mandáková et al., 2017; та ін.). Це єдина філогенетична група хрестоцвітих, до складу якої увійшли здебільшого ті види, що мають у своєму опушенні залозисті структури. У флорі України таких видів 14. Якщо розвиток покривних трихом є першою захисною реакцією епідермальної тканини рослин на екстремальні (особливо надмірну інсоляцію і високі або низькі температури) умови існування, то формування секреторних структур пов'язують зі специфікою обміну речовин тих чи інших груп рослин. У їхніх клітинах накопичуються, як правило, вторинні продукти метаболізму, ефірні олії (Huchelmann et al., 2017; Schuurink and Tissier, 2020). Секреторні утворення характерні для дуже багатьох видів сестринської родини Cleomaceae, що поширені здебільшого в тропіках та субтропіках Старого і Нового світу. Згідно з даними Angiosperm Phylogeny Group (APG IV, 2016), Cleomaceae є сестринською родиною Brassicaceae. У видів клеомових численні й дуже різноманітні за структурою залозисті волоски й рідкісні або зовсім відсутні прості трихоми (Pax, 1891; Iltis et al., 2011; Ilyinska, 2015). Отже, розвиток залозистих структур також вказує на існування близьких споріднених зв'зків між родинами Brassicaceae та Cleomaceae і є одним із свідчень паралельного розвитку цих двох родин (Hall et al., 2002; Iltis et al., 2011; APG-IV, 2016).

У таксонів флори України, що належать до LIII, просте опушення складається із нерозгалужених (*Clausia* та *Chorispora*) або із простих та фуркатних (триби Hesperideae, Euclidieae та Buniadeae) чи із зірчастих і дендроїдних (Anchonieae) трихом. Характерно, що більшість видів лінії LIII на досліджуваній території знаходяться на межі свого природного поширення (*Clausia aprica*, *Chorispora tenella*, *Strigosella africana*, *Neotorularia torulosa*,

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N. contortuplicata, *Bunias orientalis*, *Euclidium syriacum* (L.) W.T.Aiton) або належать до числа занесених (*Bunias erucago*), чи тих, що відомі в культурі (*Matthiola incana*, *M. longipetala*) і поширені здебільшого в трав'яних, синантропних біотопах або в інших, але із слабко розвиненим рослинним покривом. За нашими даними тільки рід *Hesperis* в Україні включає дев'ять видів, більшість яких є складовими ряду *Matronales* Tzvel. (Tzvelev, 1959). Результати нещодавніх молекулярно-біологічних досліджень близько 40 видів показали, що рід *Hesperis* сформувався в ірано-туранському флористичному регіоні в середньому міоцені під час редукції Тетіса і розширення знелісненних територій, а фуркатні й залозисті волоски належать до його анцесторних ознак (Eslami-Farouji et al., 2021). У видів *Hesperis*, що поширені в Південно-Західній Азії, наприклад, опушення значно густіше, ніж у тих, що характерні для флори України (Cullen, 1965; Dvořák, 1968a,b; Duran and Çetin, 2016; Eslami-Farouji, et al., 2018).

Висновок

Види Brassicaceae флори України мають ізоморфне (170 видів) або гетероморфне (59 видів) опушення. У 23 видів трихоми зовсім відсутні. Ізоморфне опушення в найбільшого числа (120) видів складається із простих волосків, у небагатьох – із мальпігієвих (23) або зірчастих (23) трихом; дуже незначна частка видів має дендроїдні (3) та фуркатні (1) волоски. Для гетероморфного опушення хрестоцвітих України найбільше характерна комбінація простих і фуркатних волосків (44). Поєднання дендроїдних та зірчастих трихом – спостерігається лише у одного виду, а залозисті структури властиві тільки 14 видам. На досліджуваній території за видовою чисельністю найкраще представлені хрестоцвіті еволюційно-філогенетичних ліній LI та LII. У цілому, структурна різноманітність трихом Brassicaceae відображає широку екологічну амплітуду видів і корелює, певною мірою, з помірно-континентальним кліматом України. У біотопах з достатньою вологістю, оптимальною інсоляцією та задовільною якістю ґрунту спостерігаються види без волосків або опушені простими трихомами, в тому числі в комбінації з фуркатними волосками. Види із щільним опушенням із розгалужених трихом населяють посушливі біотопи з інтенсивною інсоляцією, недостатнім зволоженням і бідним субстратом.

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Research Article

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Taxonomic characteristics of vegetative organs for invasive species of *Reynoutria* Hook.

Таксономические признаки вегетативных органов инвазионных видов рода *Reynoutria* Hook.

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Species of the genus *Reynoutria* Hook. were originally intentionally introduced from East Asia and in the twentieth century, three of them were successfully naturalized in Europe: *R. sachalinensis* (F. Schmidt) Nakai, *R. japonica* Houtt. and their cultigenous hybrid *R.* × *bohemica* Chrtek & Chrtková, which arose in the secondary distributional range of the parent species. As the taxa of the genus *Reynoutria* bloom very late, in late September-October, the characteristics of their leaves – shape, and type of pubescence – are mainly used as diagnostic ones. Identification of the species is often difficult, so this paper aims to identify diagnostic characters of their leaves in the secondary distribution range. Spontaneous invasive populations in Luxembourg, the Czech Republic, and Russia have been investigated. Comparisons have been made with plants growing in the natural range in Japan. Morphological characters were studied using a Keyence VHX-1000E digital microscope and an LEO 1430 VP scanning microscope. *R. sachalinensis* was found to be well distinguished from other species by its larger leaves with a heart-shaped base and a non-retracted apex. On the underside of the leaf, there are long uniseriate filiform trichomes along the lateral veins or outside the veins and peltate glands with 4-, 6- or 8-cell heads. For *R.* × *bohemica*, the presence of unicellular conical trichomes on the midrib was found to be a diagnostic feature in plants growing in Middle Russia (but not always) and is not applicable to plants from Czech populations, from which this species was actually described! In the taxonomic description of *R. japonica*, it has been suggested that the maximum height of the shoots should be significantly reduced (from 3 m to 1 m), as in the natural range this taxon has prostrate shoots. Plants of the secondary range with tall shoots are likely to be really *R.* × *bohemica* hybrids. It has been suggested that in Eastern Europe *R. japonica* grows generally very rare and predominantly in culture, and the information about most plants from invasive populations described in the literature as *R. japonica* should in fact be attributed to the hybrid complex *R.* × *bohemica*.

Keywords: *Reynoutria*, leaves, morphometric analysis, trichomes, variability

Введение

Исследование анатомии и микроморфологии вегетативных органов дает возможность выявить дополнительные диагностические признаки для систематики «трудных» таксонов или видов с позднеосенним цветением (Atalay et al., 2016; Svidenko et al., 2018; Hrytsyna et al., 2019; Vinogradova et al., 2021). Особенно важно получить эти сведения для чужеродных видов с тем, чтобы как можно более раньше диагностировать новый инвазионный таксон и принять меры по контролю его дальнейшего расселения.

Виды рода Рейнутрия *Reynoutria* Hook. (= *Fallopia* Adans.) семейства Polygonaceae – это многолетние травянистые растения с мощным ветвистым подземным корневищем. Стебли многочисленные,

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высотой 1–3 м, полые, прямостоячие, вверху ветвистые и часто дуговидно изогнутые, внизу обычно с красноватыми пятнами или штрихами (Vinogradova et al., 2010).

В естественном ареале в Корее *R. sachalinensis* (F. Schmidt) Nakai встречается в виде додекаплоидов с 2n = 132; у *R. japonica* Houtt. отмечены тетраплоиды (2n = 44), гексаплоиды (2n = 66) и октоплоиды (2n = 88); *R. forbesii* встречается как гексаплоид (2n = 66) и октоплоид (2n = 88). Очевидной корреляции между уровнями плоидности этих таксонов и морфологическими признаками не существует, за исключением того, что тетраплоиды *R. japonica* имеют несколько более толстые листья (Kim and Park, 2000).

В Европе натурализовались три вида: *R. sachalinensis*, *R. japonica* и их культигенный гибрид *R.* × *bohemica* Chrtek & Chrtková, возникший во вторичном ареале родительских видов. Гибридизация чужеродных видов привела в этом случае к формированию таксона с высоким инвазионным потенциалом, выше, чем у родительских видов (Buhk and Thielschb, 2015). *R. japonica* отмечен в Европе в 31 стране, в 12 из них этот вид рассматривается как инвазионный. *R. sachalinensis* отмечен в Европе в 29 странах, в 18 из них этот вид рассматривается как инвазионный. *R.* × *bohemica* отмечен в Европе в 26 странах, в 10 из них он включен в список инвазионных видов (CABI, 2021).

Несмотря на высокий инвазионный потенциал, рейнутрию до сих пор используют в декоративных целях. Молодые побеги готовят в пищу как спаржу. В традиционной китайской медицине *R. japonica* используется для лечения гнойных заболеваний, боли в горле, зубной боли, язвы, геморроя, хронического бронхита (Patocka et al., 2017; Zhang et al., 2019). Побеги и корни обладают антиоксидантными, антивирусными, противовоспалительными, кардиопротекторными и противоопухолевыми свойствами (Nawrot-Hadzik et al., 2018). В современной медицине рейнутрию используют для лечения нейро-кардиоваскулярных и воспалительных заболеваний, гриппа, гиперлипидемии и ожогов кожи (Saito et al., 1997; Nhiem et al., 2014). На основе рейнутрии выпускают препарат под торговой маркой Milsana*, который эффективен для борьбы с мучнистой росой томатов (Konstantinidou-Doltsinis et al., 2006). Имеются данные, что рейнутрию можно использовать в качестве фиторемедиатора для снижения содержания тяжелых металлов (Ibrahimpašić et al., 2020).

Поскольку таксоны рода *Reynoutria* зацветают очень поздно, в конце сентября-октябре, в качестве диагностических признаков, в основном, используются особенности их листовой пластинки – форма и характер опушения. Выявлены три основных типа трихом:

1. конические одноклеточные трихомы,
2. однорядные нитевидные трихомы, состоящие из 1–8 клеток,
3. пельтатные железистые трихомы.

Типы трихом и их распределение могут быть полезны для различения таксонов в секции (Moon et al., 2011). Отмечено, что *R. sachalinensis* характеризуется специфическими анатомическими особенностями: наличием в листьях друз оксалата кальция и крахмальных гранул (Cîrlig et al., 2016).

В нашей прошлой работе (Vinogradova et al., 2010) мы характеризовали по признакам вегетативных органов следующие различия между таксонами:

- *R. sachalinensis* (F. Schmidt) Nakai. Листья очень крупные, длиной до 20–45 см, с явственно сердцевидным основанием, нижняя сторона листовой пластинки с длинными (часто более 2 мм) многоклеточными волосками.
- *R. japonica* Houtt. var. *japonica*. Растения высотой 1–4 м. Листовая пластинка яйцевидная или овальная с оттянутой треугольной верхушкой, снизу голая или по жилкам папиллозная, основание усеченное или широко клиновидное, часто несколько неравнобокое. Самые крупные листья на главном побеге длиной до 20–25 см, на боковых побегах листья мельче.
- *R. japonica* var. compacta (Hook. f.) Moldenke. Растения высотой до 1 м. Листья довольно мелкие, длина самых крупных из них не превышает 10 см.
- *R.* × *bohemica* Chrtek & Chrtková. Листья с усеченным, реже сердцевидным или ширококлиновидным основанием. Листья снизу хотя бы по жилкам с густыми острыми коническими 1–3-клеточными волосками. У теневых растений листья иногда почти голые, но конические волоски сохраняются хотя бы в основании листовой пластинки. В начале мая при развертывании листовых пластинок, по крайней мере, у части растений с обеих сторон листа хорошо заметны более длинные простые волоски длиной 1–2 мм.

Рисунок 1 Стелющиеся побеги *Reynoutria japonica* Houtt. в естественном ареале
Figure 1 Prostrate shoots of *Reynoutria japonica* Houtt. in the native distribution range

Исследования, проведенные нами в последние годы, показали, что этот ключ не всегда применим и нуждается в корректировке. Так, в 2016 г. нам посчастливилось наблюдать *R. japonica* var. *japonica* в ее естественном ареале в окрестностях Токио и на острове Сикоку. На равнине растения имели

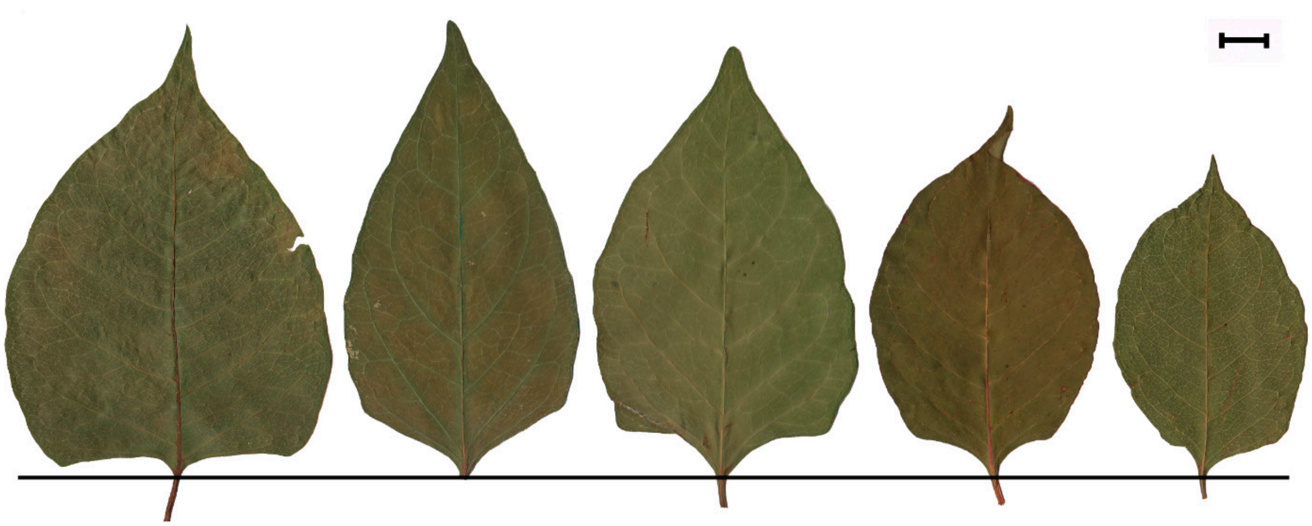

Рисунок 2 Форма листовой пластинки *Reynoutria japonica* Houtt. в естественном ареале (каждый лист собран в разных клонах)
Figure 2 Leaf blades of *Reynoutria japonica* Houtt. from the native distribution range (each leaf collected from a different clonas)

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стелющиеся побеги (Рисунок 1), а в нижнем поясе гор высота растений не превышала 1 м. Основание листовой пластинки у всех особей было клиновидным (Рисунок 2) и не очень схожим с теми листьями, которые мы наблюдаем у растений в Средней России. Это подтолкнуло нас к более детальному изучению признаков листа таксонов рода *Reynoutria* во вторичном ареале.

Материал и методика

Растительный материал

R. × *bohemica*: листья собраны в 5 локальных популяциях (или клонах) г. Москвы и в 9 популяциях Московской области: в Балашихинском, Дмитровском, Долгопрудном, Мытищинском, Одинцовском (2 местообитания), Орехово-Зуевском, Пушкинском и Раменском р-нах. В анализ были включены также листья, собранные авторами в г. Вьянден и в зарослях по реке Сюр (Люксембург), в г. Прага и в окрестностях г. Карловы Вары (Чехия) и культивируемые клоны в поселках Южного Берега Крыма.

R. japonica var. *japonica*: листья собраны авторами в естественном ареале в пос. Минами (о-в Сикоку, Япония) и в окрестностях г. Токио. Этот же таксон, согласно этикетке, содержится в коллекции Никитского ботанического сада (этот образец мы также включили в анализ).

R. japonica var. *compacta*: листья с растений, культивируемых в Главном ботаническом саду Российской академии наук (ГБС РАН, Москва, Россия) и в ботаническом саду Карлова университета (Прага, Чехия).

R. sachalinensis: листья собраны авторами в трех локальных популяциях (интродукционная популяция в ГБС РАН, г. Звенигород Московской области и район Новокосино г. Москвы) и получены из естественного ареала (окрестности г. Южно-Сахалинск).

Объем исследованного материала был увеличен за счет просмотра гербарных образцов таксонов рода *Reynoutria*, хранящихся в Гербарии ГБС РАН (MHA) и в Гербарии МГПУ (MOSP).

Морфометрический анализ

Из каждой локальной популяции (= клона) брали по 3 осевых побега и просматривали по 2 листа с каждого на цифровом микроскопе Keyence VHX-1000E при увеличении до x 200. Листья с боковых побегов в анализ не включали, поскольку их форма отличается от формы листьев главного побега. Форму основания листовой пластинки определяли по углу, сформированному средней жилкой и осью, проходящую от основания черешка к краю листовой пластинки. Для исследования ультраскульптуры листовой пластинки использовали сканирующий электронный микроскоп LEO 1430 VP.

Статистический анализ

Результаты обработаны методами математической статистики в программе PAST.

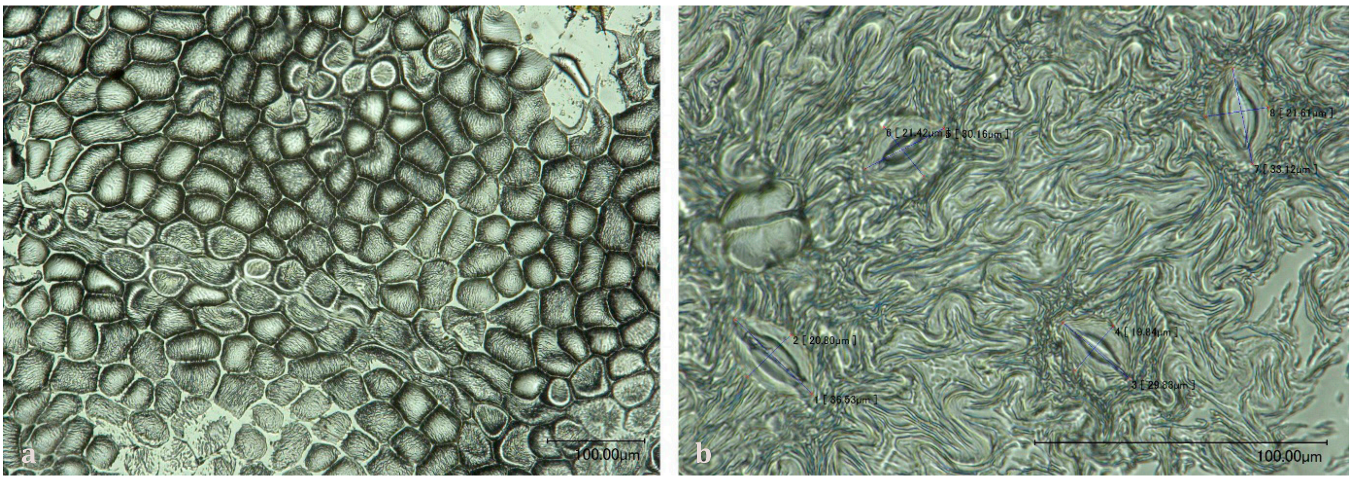

Рисунок 3 Листовая пластинка *Reynoutria* × *bohemica* Chrtek & Chrtková:
a – верхняя сторона без устьиц; b – нижняя сторона с аномоцитными устьицами и пельтатной железкой

Figure 3 Leaf blade of *Reynoutria* × *bohemica* Chrtek & Chrtková:
a – upper side without stomata; b – lower side with anomocytic stomata and peltate glandular trichome

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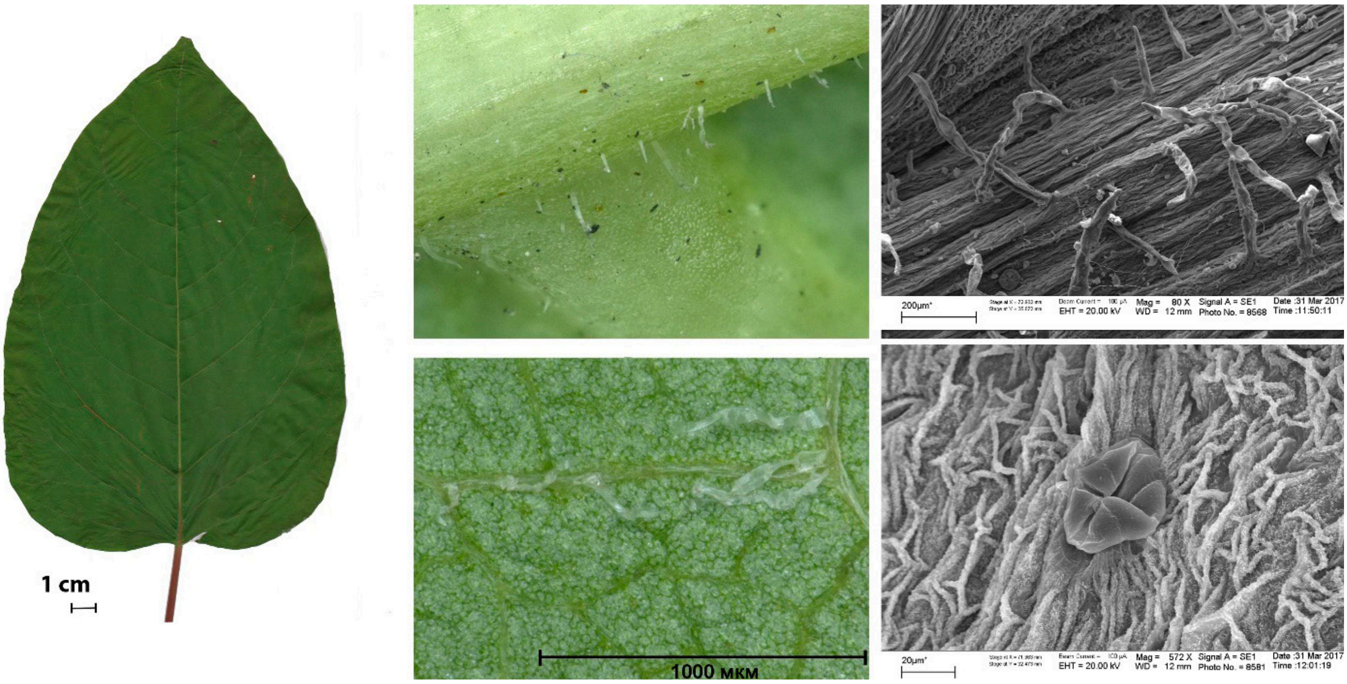

Рисунок 4 Лист *Reynoutria sachalinensis* (F. Schmidt) Nakai: сердцевидное основание, однорядные длинные трихомы и пельтатная железка с восьмиклеточной головкой на нижней стороне листа

Figure 4 Leaf blades of *Reynoutria sachalinensis* (F. Schmidt) Nakai: heart-shaped base, uniseriate filiform trichomes and peltate glandular trichome with 8–celled head on the lower side of leaves

Результаты и обсуждение

У всех видов листья черешковые, верхняя сторона листовой пластинки голая, листья гипостоматические, т.е. устьица у них располагаются только на нижней стороне листовой пластинки (Рисунок 3).

R. sachalinensis хорошо отличается по сердцевидному основанию листа (Рисунок 4): угол между средней жилкой и осью от основания черешка к краю листовой пластинки у всех образцов тупой, т.е. >90° и в среднем составляет 97.7 ±2.7° (от 95 до 103°). Верхушка листа не оттянутая.

Хорошо отличается таксон и по микроморфологическим признакам: на нижней стороне листовой пластинки имеется две группы трихом:

1. короткие по средней жилке,
2. длинные по второстепенным жилкам или вне жилок (Рисунок 4).

Средняя длина длинных трихом у звенигородского образца 256.6 (149–494) мкм, у новокосинского образца – 373.5 (210–494) мкм. Очень длинных трихом (до 2 мм), которые упоминаются в определителях, мы не находили. Пельтатные железки имеют чаще всего головку из 4 клеток, но встречаются железки и с 6-клеточной, и с 8-клеточной головкой. Вместе с характерными признаками генеративных органов – опушенными цветоножками и опушенными листочками околоцветника внешнего круга, наличием обильной пыльцы и способностью к активному плодоношению (Vinogradova et al., 2016) особи, принадлежащие к этому виду, определяются однозначно.

R. japonica var. *compacta* выделяется среди других таксонов по плотной текстуре и по форме листовой пластинки. Листья у этого растения кожистые и б/м округлые, а нередко ширина листа даже превышает его длину (Рисунок 5). На нижней стороне листа имеются пельтатные железки, но трихомы отсутствуют, в том числе и по жилкам.

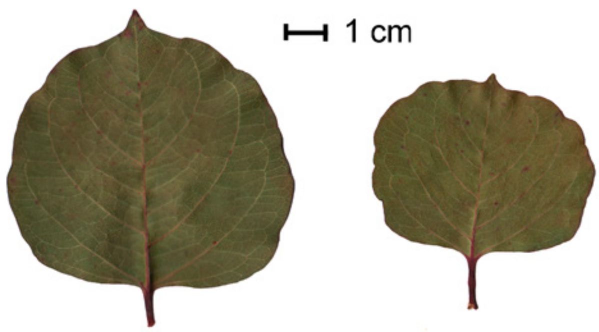

Рисунок 5 Листья *Reynoutria japonica* var. compacta (Hook. f.) Moldenke

Figure 5 Leave blades of *R. japonica* var. compacta (Hook. f.) Moldenke

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R. japonica var. *japonica* в естественном ареале (Рисунок 2) имеет клиновидное основание листа, и угол между средней жилкой и осью от основания черешка к краю листовой пластинки у всех образцов острый, т.е. <90°, и в среднем составляет 63.4 ±4.5° (от 54 до 79°). Единственный образец этого таксона из вторичного ареала, который, согласно документации, намеренно интродуцирован непосредственно из Японии, собран нами в Никитском ботаническом саду и также характеризуется острым углом (70°), однако имеет более округлую форму. На немногочисленных гербарных образцах, собранных непосредственно в Японии (MHA, MW, MOSP), этот признак также прослеживается.

Форма листьев всех остальных образцов варьирует настолько сильно, что по этому признаку разграничивать во вторичном ареале *R. japonica* var. *japonica* от *R.* × *bohemica* достаточно проблематично (Рисунок 6). Поэтому остальные исследованные нами образцы из европейской части вторичного ареала мы предварительно относим к гибридогенному комплексу *R.* × *bohemica*, включающему бэккроссы с родительскими видами.

R. × *bohemica*: форма основания листовой пластинки варьирует от слабо сердцевидной (угол 97°) до округло-клиновидной (угол 73°), в среднем 84.2 ±1.6°. Форма варьирует даже в пределах одного клона, что мы наблюдали в многокилометровой заросли в долине реки Сюр в Люксембурге (Рисунок 6 m–p). К тому же листья, формирующиеся к концу вегетационного сезона (на боковых побегах или на верхушках осевых побегов), нередко значительно отличаются по форме от листьев срединной формации на осевых побегах (Рисунок 6 i–l). Верхушка листа оттянутая.

Напротив, микроморфологические признаки листьев у всех образцов варьируют слабо. На верхней стороне листовой пластинки трихомы отсутствуют. На нижней стороне листа имеются пельтатные железки, расположенные вне жилок. Число их составляло от 3 до 5 в мм^2, а диаметр – от 40 до 50 мкм (Таблица 1). Острые конические одноклеточные трихомы по главной жилке отсутствуют у растений, собранных в Чехии, а также

Таблица 1 Признаки листьев *Reynoutria* × *bohemica* Chrtek & Chrtková из различных популяций Восточной Европы
Table 1 Leaf characteristics of *Reynoutria* × *bohemica* Chrtek & Chrtková from different populations in Eastern Europe

Исследованные популяции	Географические координаты	*Угол в основании листа, градусы	Конические трихомы по главной жилке	Пельтатные железки с 4-х клеточной головкой
Люксембург, река Сюр	N 49.9093 E 5.9272	95–103	нет	есть
Чехия, Прага, Ботанический институт	N 50.0717 E 14.4216	75–87	нет	есть
Чехия, Карловы Вары, у автобусного вокзала	N 50.2300 E 12.8626	77–89	нет	есть
Чехия, Карловы Вары, институт	N 50.2300 E 12.8724	82–88	нет	есть
Московская область, Орехово-Зуево, Хотеичи	N 55.5028 E 38.7936	76–90	нет	есть
Московская область, Реутов	N 55.7519 E 37.8628	75–86	есть	есть
Московская область, Орудьево	N 56.4474 E 37.5142	75–80	есть	есть
Московская область, Пушкино	N 56.0266 E 37.8328	77–85	есть	есть
Москва, Новокосино	N 55.7339 E 37.8473	70–82	есть	есть
Москва Восточное Бирюлево	N 55.5995 E 37.6746	87–90	есть	есть
Алушта, Крым	N 44.6711 E 34.3979	86–90	есть	есть

Примечание: * угол между средней жилкой и осью от основания черешка к краю листовой пластинки

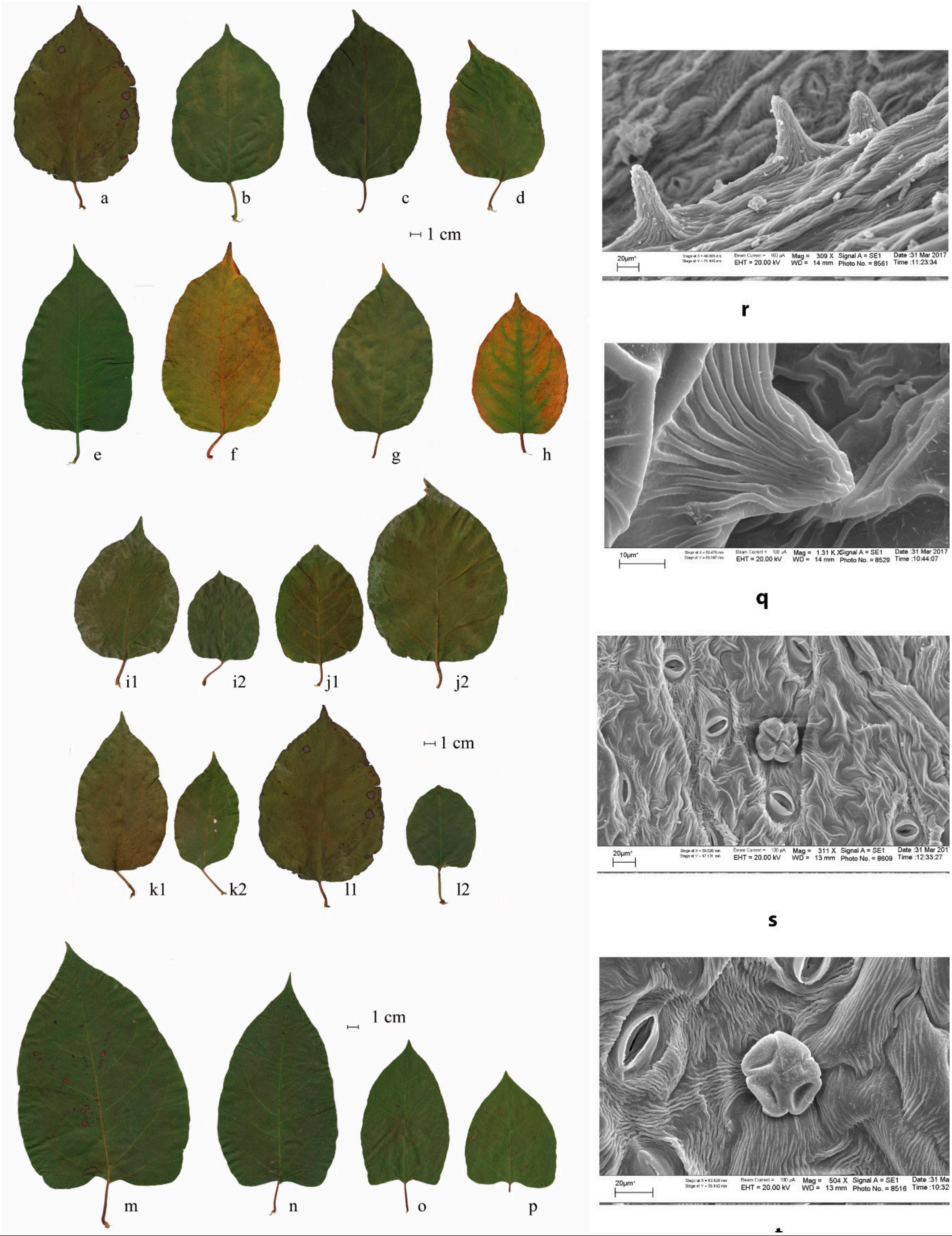

Рисунок 6 Листья *Reynoutria* × *bohemica* Chrtek & Chrtková:
a–h – разные клоны из Московской области; i–l – разные клоны из Средней России (с индексом 1 с осевых побегов, а с индексом 2 – с боковых побегов того же растения); m–p – одна прибрежная популяция по реке Сюр (Люксембург) с особей, произрастающих не менее, чем в 50 м друг от друга; r–q – конические одноклеточные трихомы по средней жилке; s–t – устьица и пельтатные железки с четырехклеточной головкой

Figure 6 Leaves of *Reynoutria* × *bohemica* Chrtek & Chrtková:
a–h – different clones from the Moscow Region; i–l – different clones from Central Russia (with index 1 from axial shoots and index 2 from lateral shoots of the same individual); m–p – one coastal population along the Syur River (Luxembourg) from individuals growing at least 50 m apart each other; r–q – conical unicellular trichomes along the middle vein; s–t – stomata and peltate glandular trichomes with 4–celled head

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в одной из популяций Московской области (из Орехово-Зуево). На гербарных листах, хранящихся в MHA, MW и MOSP, этот тип трихом отмечен лишь у 40 % образцов.

Полученные нами результаты позволяют предположить, что интродуцированная в Англию более 100 лет назад *R. japonica* var. *japonica* компенсировала отсутствие первоначальной генетической изменчивости обширной серией межвидовой гибридизации с интродуцированной позднее *R. sachalinensis* и образованием гибрида *R.* × *bohemica*, способного к обратному скрещиванию с родительскими видами и генерированию разнообразных генотипов. Это подтверждается как различным уровнем плоидности комплекса *R.* × *bohemica* – от тетраплоидов до октоплоидов, так и его генетическим разнообразием. В Чехии, например, в 88 изученных клонах выявлено 33 мультилокусных генотипа (Mandák et al., 2003, 2005; Bailey, 2012). Однако сведения по уровню плоидности рейнутрии в Словакии, в Криваньских горах, иные. С использованием метода проточной цитометрии показано, что заросли рейнутрии состоят из трех таксонов, каждый из которых представлен только одним цитотипом: гексаплоидным (2n = 6x ~ 66) у *R.* × *bohemica* (23 клона), октоплоидным (2n = 8x ~ 88) у *R. japonica* var. *japonica* (18 клонов) и тетраплоидным (2n = 4x ~ 44) у *R. sachalinensis* (2 клона). Морфометрия 23 признаков листьев продемонстрировала, что наиболее надежным диагностическим признаком *Reynoutria* является опушение листа. Признано, что R. ×*bohemica* очень часто путают с *R. japonica* var. *japonica*, присутствие которой вне культуры переоценивается (Mereďa et al., 2019).

В естественном ареале изучаемые таксоны еще более разнообразны: они представлены особями с различными уровнями плоидности и более разнообразными железками. Так, мы не обнаружили на листьях пельтатных железок с 2-клеточной и с 14-ти клеточной головкой, которые отмечены корейскими учеными (Moon et al., 2011). Различий между видами по этому признаку мы также не отметили: все таксоны имели железки с 4-х клеточной головкой, только у *R. sachalinensis*, как исключение, наблюдаются железки с с 6-ти и с 8-ми клеточными головками. Напротив, в естественном ареале в Корее у *R. japonica* обнаружены только восьмиклеточные пельтатные железки, а у *R. sachalinensis* – четырех- или восьмиклеточные железки (Khalil et al., 2020).

Выводы

Ключ для определения таксонов рода *Reynoutria* во вторичном ареале по признакам листовой пластинки нуждается в некоторой корректировке. Во-первых, необходимо отказаться от указания такой значительной высоты побегов (1–4 м) для *R. japonica* var. *japonica*, и определить ее высоту в 1,0–1,5 м. Во-вторых, в ключ следует включить отмеченные нами различия в строении пельтатных железок – у *R.* × *bohemica* они имеют 4-х клеточную головку, тогда как у *R. sachalinensis*, помимо 4-х клеточных головок, встречаются железки с 6-ти и с 8-ми клеточными головками. В-третьих, для *R.* × *bohemica* наличие конических волосков на средней жилке является диагностическим признаком у растений, произрастающих в Средней полосе России (но и то не всегда), однако не характерен для из чешских популяций, откуда этот вид, собственно, и был описан. Возможно, в Восточной Европе *R. japonica* вообще произрастает крайне редко, и преимущественно в культуре, а сведения по большинству растений, описываемых в литературных источниках как *R. japonica*, на самом деле следует относить к гибридогенному комплексу *R.* × *bohemica*.

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Review

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Unique effects of alginite as a bituminous rock on soil, water, plants and animal organisms

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The publication aims to present some knowledge about the less-known but at the same time very scarce bituminous rock alginite with comprehensive practical use in the form of a brief overview. Alginite is an organic-bituminous rock that was formed about six million years ago from dead fossil algae *Botryococcus braunii* Kütz and diatoms in the area of today's Pannonian Basin. In Europe, alginite deposits have been discovered in Hungary, Austria and Slovakia in Pinciná village. Alginite mining is mostly used in Hungary in the Gerce area. Alginite is grey to dark grey, in the wet state dark laminated, clayey with the form of disintegrating clay. Alginite has very valuable physical, mechanical and chemical properties. It is a natural bituminous rock with a favourable content of basic nutrients (P, K, Ca, Mg and S) for plants except for nitrogen content. Alginite contains a large number of microelements, which increase the agrochemical possibilities of its use. It can be applied in its natural form without chemical treatment. It is an ecological raw material that improves the soil and does not negatively affec the environment. Alginite has become the subject of research in many workplaces. The overview presents the basic physical, mechanical and chemical properties of alginite and selected knowledge and research results that have enabled the practical use of alginite in natural or technologically modified form in agriculture in the formation of growth, development and crop formation and quality of seeds and fruits of cultivated crops, forestry, remediation and improvement of soil and water properties, decomposition of herbicides, stabilization of beneficial microorganisms in animal organisms and other areas.

Keywords: alginite, bituminous rock, organic matter, properties, knowledge, use, agriculture, forestry, remediation waters, decomposition of herbicides

Introduction

The basic purpose of modern agriculture to provide nutritious and safe raw materials for food by economic processes and sustainable and environmentally responsible production thereof. The duality of the agricultural production namely the connection between the saving of environment and the profitability is the most important question recently which can be solved only by the maintaining of the fertility of the soil. The fertility of soil can be adjusted by added minerals and nutrients. In the soil, which is an independent living media the nutrients are exposed most diverse of chemical and biological transformations before they are utilized by plants. The cultivation and fertilizing should keep the intensive soil life because intensive soil fertility without intensive soil life is not possible. As

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a result of the agricultural practices of the last decades, the fertility of the soils is decreased considerably because the amount the organic materials which provide intensive soil life is reduced considerably. Our soils suffer from carbon-starvation which is enhanced by the fact that the produced primary and secondary organic products are transported from the production area for industrial use. Besides the lack of organic matters the microelement content of our soils also reduces.

For providing the proper agricultural functionality the organic content of the soil has to be at least 2 %. With this, the soil provides the life conditions of microorganisms that mineralize the organic materials and which activities determine basically the fertilizer capability of the soil. Soils having less organic content are unable to maintain the nutrients evolved from the mineralized organic compounds, thus these are washed out from the soil caused severe environmental problems e.g. the lack of microelements in food plants which cause severe health problems for humans at the end of the food chain. The forms of the disadvantageous changes of soils are the compactness of the soil, bad air and water management, the starting of the salinization on the watered areas, the erosion and deflation. The basic cause of these changes is the degradation of the organic-mineral colloids of soil which results that there are no reactants enough in suitable amount or composition for the chemical and biological reaction in the soil. In the soil, the mineral elements for the life of the microorganisms are provided by a so-called organic-mineral, in other words, organomineral complex. The restoration of the structure and the function of these organomineral complexes are the primary purpose of soil improvement and soil conditioning.

Bituminous rock alginite – a scarce natural raw material

Research institutes in many countries around the world are also looking for and testing several natural rocks. One of these mineral rocks is alginite.

Alginite as a bituminous rock is a scarce natural raw material. Hitherto, alginite has been discovered in Australia (Ozimic, 1982), Hungary (Solti, 1987), Austria (Solti et al., 1987), Slovakia (Vass et al., 1995, 1997a,b,c, 1998), USA (Stankiewicz et al., 1996) and China (Jia et al., 2013).

The first research and its practical use with bituminous rock in the Gerce region is in Hungary. Alginite from Hungary became the subject of follow-up research (Ravasz et al., 1994; Németh et al., 2008; Kádár et al., 2015; Vígh et al., 2016; Soós et al., 2018) and the search for practical possibilities of its use in Austria (Solti et al., 1987), the Czech Republic (Tužinský et al., 2015; Holzer et al., 2018), Spain (Mastalerz et al., 2013), Slovenia (Komac, 2016), Romania (Kentelki, 2010), Germany (Hippmann et al., 2016, 2018) and Russia (Motyleva et al., 2014).

An economically important deposit of alginite was also located in Slovakia in Maar near Pinciná village north-east of the town of Lučenec (Vass et al., 1997a). The exploitation of alginite from the deposit is only in the beginning stage. Nevertheless, alginite has become an important research object even in the conditions of Slovakia at the Geocomplex research workplace (Kovár et al., 2021), University of Veterinary Medicine and Pharmaceuticals in Košice (Nemcová et al., 2012–2015; Styková et al., 2016; Strompfová et al., 2018), Technical University of Zvolen (Bublinec and Gregor, 1997; Beláček, 1998, 2003, 2006; Beláček et al., 2002; Sarvašová, 2009), Slovak University of Agriculture (Kulich et al., 2001, 2002; Kovár et al., 2021), National Agriculture and Food Centre – Soil Science and Conservation Research Institute (Barančíková et al., 2003; Litavec and Barančíková, 2013; Barančíková and Litavec, 2016), State Geological Institute of Dionýz Štúr (Baláž et al., 2010), many research institutes (Elečko et al., 1998) and other universities (Bednárová, 2019).

Since 2016, significant research activities have been provided by several research teams at the Slovak University of Agriculture in Nitra. Research activities at the level of basic and applied research are focused on the development and application of various preparations developed from natural alginite on various plant species to recognize the effects of alginite on germination, plant growth and development, biomass formation, seed and fruit yields, tolerance of biotic and abiotic factor of the growing environment and the quality of plant parts.

Alginite, as an organic-bituminous rock, originates mainly from the bodies of dead algae (from the Latin algae). It formed as a rock during various geological periods, especially those that gave algae optimal conditions for growth and reproduction. Post-volcanic crater lakes or swamps were sites where alginite arising (Vass et al., 1995, 1997a,b,c, 1998).

The complex characterization of the alginite deposit in Pinciná in Slovakia was processed by Vass et al. (1995, 1997a,b,c) and other research teams (Kulich et al., 2001, 2002; Litavec and Barančíková, 2013).

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Basic characteristics of alginite

Alginite, which fills a former inland sea at Pinciná, is a grey to dark grey laminated, clayey rock rich in organic substances, weakly strengthened organogenic sedimentary rock with a form of disintegrating clay (Vass, 2005). It was formed from algae in the aquatic environment. In the rock, the dark and light laminae of 0.5 to 2.0 mm in thickness are alternating (Figures 1 and 2). Dark laminae are rich in *Botryococcus* remains whose funnel cell covers consist of organic material similar to sporopoleminium (Vass, 1998). The light lamina is formed from clay material and contains repositories of diatoms formed by opal (Vass, 2005).

In the wet state, there is a dark-coloured unconsolidated clayey rock. This is confirmed by the results from various boreholes in the alginite deposit in Pinciná, which drilled in 2020 by Geocomplex (Figure 3).

Alginite has the characteristic of organic soil. Organic substances significantly increase humidity and plasticity. The humidity determined by drying at 22–30 °C was in the range of 57.75–78.63 %. Samples from the top layer of the alginite showed 15 % higher humidity when dried at 60 °C than when open-air drying. When dried at 105 °C temperature, the humidity was

Figure 1 Plates of natural alginite (Photo: J. Vrábel, 2020)

Figure 2 Natural alginite in the Pinciná deposit, Slovakia (Photo: Š. Hajdu, 2017)

Figure 3 Colour of wet alginite samples obtained from different depths at the Pinciná deposit (Photo: A. Oravec, 2020)

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higher than 100 %. These data correspond well with the water absorption capacity (Vass et al., 1997a,b,c).

The specific weight of alginite was determined by Vass et al. (1997a,b,c) in the range of 2.06 to 2.35 $g.cm^{-3}$ and Kulich et al. (2002) 2.568 $g.cm^{-3}$. The specific weight of alginite compared to standard soil (2.65–2.75 $g.cm^{-3}$) is relatively low. The presence of organic matter affected the bulk density and specific weight of the dry rock.

Vass et al. (1997a,b,c) determined a porosity for alginite in the range of 62.44 to 68.45 % and Kulich et al. (2002) only 40.8 % and high absorbency of 82.0 % indicate a high sorption capacity, which originates in a polymineral composition of alginite containing clay minerals and organic matter.

The water absorption capacity of alginite (Aranyi coefficient) in alginite Pinciná was determined by Vass et al. (1997a,b,c) in the range of 80-108. This is more than the value of the water absorption capacity of alginite in natural soils. Heavy soil has the largest water absorption capacity, 61–80 (Vass et al., 1997a,b,c). The water balance of the soil depends on the content of the clay component in the soil and on its mineralogical composition. In the case of alginite, the absorption capacity also depends on the organic matter content.

Alginite from Pinciná is an important natural raw material for improving the absorption properties of the soil, as well as for increasing the water holding capacity. One kilogram of alginite from Pinciná can absorb and retain about 1 litre of water for a long time without expanding its volume. It also retains decay, similar to the unsaturated state. Therefore, alginite makes it possible to accelerate and improve the transport of water into plants according to the needs of individual plant species (Vass et al., 1997a,b,c).

Vass et al. (1995, 1997a,b,c) determined in alginite from Slovakia the pH values in the range 5.87–7.10, Litavec and Barančíková (2013) in the range of 5.50–7.20, which indicates a weakly acid to the neutral reaction of alginite. Only in the case of sampling point 11 at a depth of 8 m did they detect a weakly basic reaction of alginite (pH = 8.13), with a considerably high concentration of inorganic carbon of 4.6 %. Vass et al. (1997a,b,c) determined an average pH of 6.28 in alginite. The results of the authors agree with the results of analyzes in the Algalit laboratory (Hungary), according to which the pH in alginite from Hungary was determined in the range of 4.79–6.94 (Ravasz et al., 1994). The basic pH values of alginite allow the classification of alginite among neutral natural raw materials with a higher humus content in the range of 6.53–33.10 % (Kulich et al., 2002).

The relatively low content of $CaCO_3$ in alginite from Pinciná was caused by geological conditions. There are no carbonate rocks around the deposit. The authors (Vass et al., 1997a,b,c) determined the content of $CaCO_3$ in alginite in the range of 1.16–3.72 % (mean 2.17 %) by atomic absorption spectrometry. In the alginite from the borehole VPA-7, it determined the $CaCO_3$ content from 1.06 to 14.74 % (mean 2.61 ±3.03 %). Ravasz et al. (1994) determined a $CaCO_3$ content ranging from 0.0 to 14.0 % (mean 0.7 ±2.16 %).

Salt content is an important indicator in the evaluation of fertilizers and soil activators. Soils should contain as few salts as possible to avoid the salinity of the soil. According to the total salt content, the following are distinguished: soils with a trace salt content (<0.05 %), slightly saline soils (0.05–0.15 %), saline soils (0.15–0.40 %) and highly saline soils (>0.40 %). Plants sensitive to soil quality do not grow on weakly saline soil, on saline soil only the most resistant plants and on strongly saline soil no plants grow (Ravasz et al., 1994). The salt concentration was determined by the laboratory Algalit (borehole VPA-1 and 3) and GS SR (borehole VPA-7). The average salt content was determined to be 0.1, respectively 0.041 %. Kulich et al. (2001) determined the salt content in alginite samples in the range of 0.04–0.05 %. According to Vass et al. (1997a,b,c) alginite from the Pinciná deposit with salt content corresponds to weakly saline soil, respectively soil with a trace salt content.

Vass (1998) determined an average C_{org} content of about 9 % from analyzes of organic matter of alginite. The distribution of organic carbon is unequal. From the contents of C_{org} by multiplying a Welt coefficient of 1.72 (Valla et al., 1980), the humus content was calculated to be about 15 % (Vass et al., 1998).

Although alginite from the Pinciná deposit contains a higher content of organic carbon in the alginite, the content of humified organic matter is low. Type II kerogen predominates in organic matter of algal origin (Vass, 1998). The fractional composition of the humified organic matter has a higher content of fulvic acids than humic acids. This is evidenced by the low value of the carbon ratio of humic acids to fulvic acids (Litavec and Barančíková, 2013).

The proportion of the clay fraction in the alginite from the Pinciná deposit was determined to be in the range of 11–79 %. Of the clay minerals, illite and kaolinite predominate, the most represented mineral is

Table 1 Mineralogical composition of alginite according to the values of diffraction maximum values (Kulich et al., 2001)

Minerals	Diffraction max. values	Minerals	Diffraction max. values
Smectite	14–15 Ä	**Kaolin**	7.0–3.6 Ä
Quartz	4.2–3.3 Ä	**Dolomite**	2.8 Ä
Illit-mica	10.0–5.0–3.3 Ä	**Chlorite**	7.0–4.7–3.6 Ä
Calcite	3.8–3.0 Ä	**Feldspars**	3.7–3.2 Ä

smectite. The specific surface area of alginite confirms its high sorption capacity with a specified range of 313–654 $m^2.g^{-1}$ (Vass et al., 1997b). There is a direct linear relationship between the specific surface area and the smectite content as well as the organic matter content. Sorption tests confirmed the ability of alginite to bind lead from polluted waters, cadmium sorption is less efficient (Vass et al., 1997a).

Clay minerals (smectite, illite), carbonates (calcite, dolomite) and amorphous quartz and silica are the dominant mineral components of alginite, while gypsum, plagioclase, K-feldspar, siderite, goethite, pyrite and magnesite can also be found in smaller amounts. In 1974, within the framework of the mapping research programme of the Geological Institute of Hungary (MÁFI), Solti (1999) explored one-time volcanic craters buried in alginit and basalt bentonite. The mineralogical composition of alginite is also reported by the team of Kulich et al. (2001), which documents the data in Table 1.

Chemical composition of alginite

Concerning biogenic elements, in addition to the nitrogen content, the presence of P, K, Ca, Mg and S can be positively assessed for alginite (Table 2). A comparison of the content of macroelements shows significant variability between alginite samples. This is because alginite as a sedimentary rock is not homogeneous (Russell, 1990; Vass et al., 1997a,b,c; Kulich et al., 2001).

Alginite contains a large number of microelements that increase the agrochemical value of alginite (Table 3). Regarding the risk elements (As, Cd, Pb, Ag, Cr, Se) in no case were the values of the limit contents according to the standard valid in the territory of the Slovak Republic exceeded (Kulich et al., 2001). The comparison of the content of microelements shows significant variability between alginite samples.

Regarding the nutrients available to plants from natural alginite, the contents of phosphorus, potassium, calcium and magnesium are higher than the optimal contents of these elements in agricultural

Table 2 Basic chemical composition of samples of powdered alginite (% of dry matter) from the Pinciná deposit according to the results of the accredited laboratory Reg. No. 038 / S-025

Measured parameter	Evaluation of an alginite sample by authors´ results of this article			Results from literary data	
	alginite sample 3533	alginite sample 3548	alginite sample 3536	Vass et al. (1997) borehole VPA1,3,4,5	Kulich et al. (2001)
SiO_2	47.55	47.29	47.10	39.1–53.04	37.50–53.30
Al_2O_3	17.85	18.31	18.44	13.06–17.89	11.05–22.50
N	–	–	–	0.10–23.80	–
TiO_2	1.11	1.11	1.11	0.90–1.30	1.10–1.34
Fe	6.86	7.08	7.03	0.97–5.75	5.85–8.00
CaO	1.07	0.97	0.90	0.93–2.32	0.80–8.40
MgO	1.43	1.42	1.39	0.62–1.92	0.60–4.02
MnO	0.04	0.04	0.04	0.01–0.36	0.04–0.30
P_2O_5	0.12	0.13	0.13	0.42–0.92	0.15–1.64
Na_2O	0.30	0.45	0.43	0.33–0.81	0.33–0.74
K_2O	1.09	1.54	1.54	1.05–1.85	1.05–1.37
SO_2	–	–	–	0.42–2.47	–
Humidity	8.65	1.71	1.58	–	–

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Table 3 Content of microelements in powder alginite (mg/kg of dry matter) from the Pinciná deposit according to the results of an accredited laboratory Reg. No 038/S-025

Measured parameter	Alginite sample 3533	Alginite sample 3548	Alginite sample 3536	Vass (1997a)
Cr	138	183	145	18–68
Ag	<0.1	<0.1	<0.1	0.1–2.19
As	4.87	6.22	4.94	1.48–9.35
Ba	364.2	374.3	386.0	196–1109
Be	1.8	2.3	2.3	0.93–1.85
Bi	0.25	0.53	0.58	<10
Cd	<0.5	<0.5	<0.5	0.010–0.145
Co	25.8	34.4	32.6	4–29
Cs	<5	5.2	5.1	–
Cu	30.9	40.5	43.7	31–83
Ga	24	20	20	9–20
Ni	123	173	146	43–209
Pb	10.7	17.3	20.6	6–58
Sn	5	6	11	<3
Sr	59.8	58.9	59.4	51–123
V	121	124	121	30–75
Zn	85	109	119	76–120
Zr	137	130	132	135–321
Sb	0.92	0.51	3.56	0.04–7.80
Rb	27	88	86	45–98
Se	<0.1	<0.1	<0.1	0.01–0.05
Te	<0.5	<0.5	<0.5	–
La	34	34	35	–
Nb	23	26	26	–
Ce	64	64	64	–
Y	21	22	22	–

soils. Only the nitrogen content is insufficient (Kulich et al., 2001).

Organic matter of alginite

Ognjanova-Rumenova and Vaas (1998) found that the dark layers of alginite are rich in residues of the algae *Botryococcus braunii* Kütz. The finer, lighter layers of alginite are rich in diatoms. In the published study a total of 181 taxa are observed in the present study. They refer to 35 genera, 128 species, 37 varieties and 6 forms, and belong to 13 families, 4 orders and classes Centrophyceaea and Pennatophyceaea. Diatoms were identified to species whenever possible; however ten entities could be assigned to the genus only. The diatom flora mostly consists of modern species – 92.4 %, but the group of extinct species (7.6 %) is abundant in some levels (i.e. *Pliocaenicus omarensis* (Kütz.) Round & Hak., *Aulacoseira distans* var. *scala* (Ehr.) nov. comb, etc.). In general, pennate forms are the most varied (94.5 %). The species-rich genera *Navicula* Bory and *Cymbella* Ag. can be distinguished, they account for 26.5 % of the entire flora. These are followed by the genera *Pinnularia* Ehr. (8.8 %), *Fragilaria* Lyngb. (7.2 %), *Achnanthes* Bory (7.2 %) and *Gomphonema* Ehr. (6.1 %). The class Centrophyceae accounts for 5.5 % of the diatom flora. More of its representatives are widely spread, in some levels they are rock-forming and occur as dominants or subdominants.

The authors of the presented publication analyzed more than 200 samples of alginite from different parts of the Pinciná deposit and confirmed the results of the

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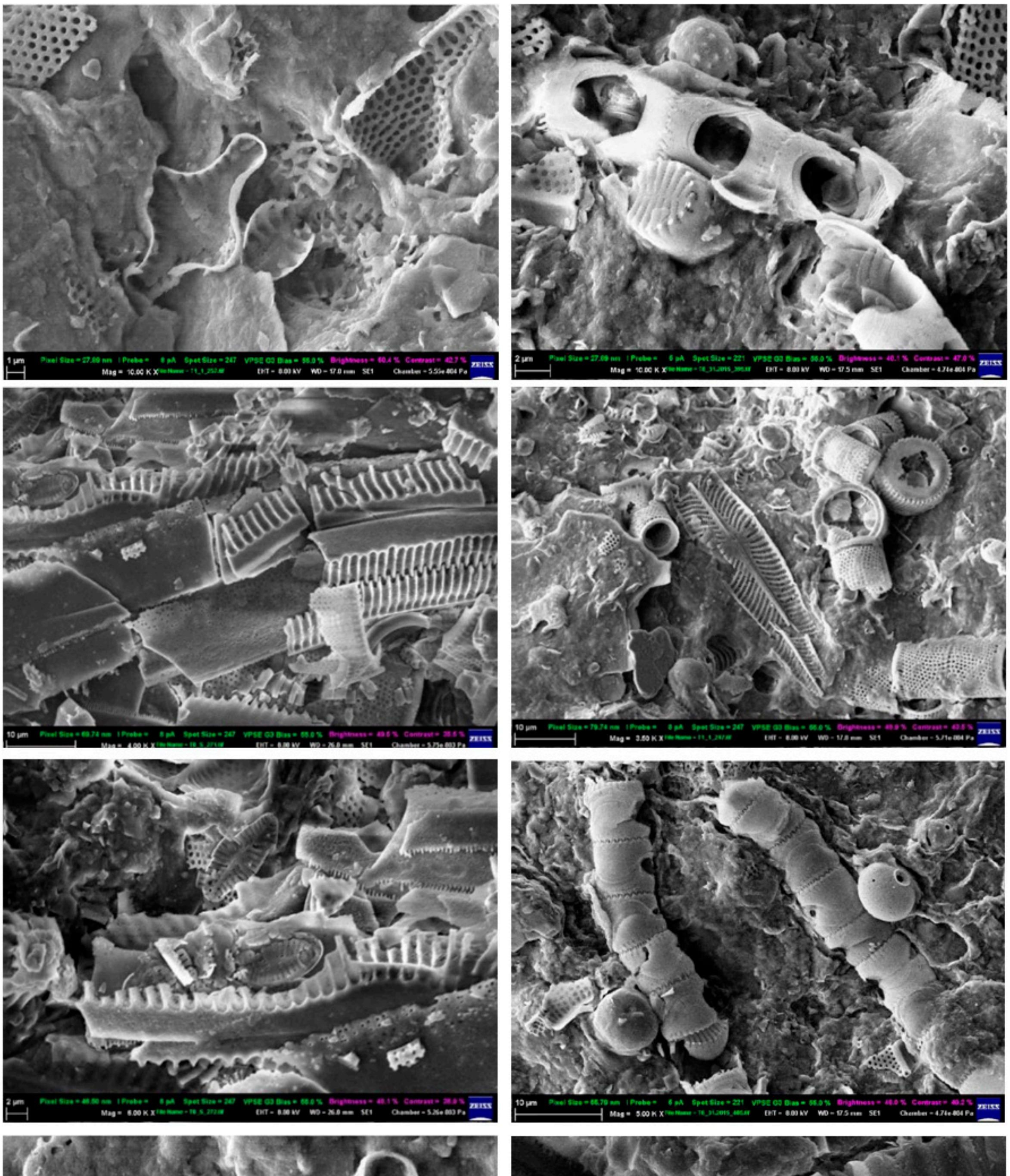

Figure 4 Demonstration from some species of diatoms found in alginite samples from the Pinciná deposit (Photo: R. Ostrovský, 2017)

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works of Ognjanova-Rumenova and Vaas (1998), which we also document in Figure 4.

In 74 samples of alginite, Vass (1998) determined a humus content in the range of 6.88–45.37 %. Kulich et al. (2001) determined the humus content in the range of 6.53–31.1 % and Pichler et al. (2001) document a humus content of 15.5 % in alginite.

In experiments, Litavec and Barančíková (2013) found that alginite, despite a sufficient content of organic carbon (OC = 5.5 %), contains relatively little humified organic matter. Its fractional composition has a higher content of fulvic acids than humic acids.

Vass (1998) determined an average C_{org} content of about 9 % from analyzes of organic matter of alginite. The distribution of organic carbon is unequal. From the contents of C_{org} by multiplying a Welt coefficient of 1.724 (Valla et al., 1980), the humus content was calculated to be about 15 %.

Organic matter in alginite is converted to kerogen. Kerogen (gr.) is a complex mixture of organic chemical compounds that make up the most abundant fraction of organic matter in sedimentary rocks. As kerogen is a mixture of organic materials, it is not defined by a single chemical formula. Its chemical composition varies substantially between and even within sedimentary formations. Kerogen is insoluble in normal organic solvents in part because of the high molecular weight of its component compounds. The soluble portion is known as bitumen. When heated to the right temperatures in the earth's crust, (oil window c. 50–150 °C, gas window c. 150–200 °C, both depending on how quickly the source rock is heated) some types of kerogen release crude oil or natural gas, collectively known as hydrocarbons (fossil fuels). When such kerogens are present in high concentration in rocks such as organic-rich mudrocks shale, they form possible source rocks. Shales that are rich in kerogen but have not been heated to the required temperature to generate hydrocarbons instead may form oil shale deposits (Vandenbroucke and Largeau, 2007).

Practical use of alginite

Alginit as a natural material and its organic-mineralogical composition to give a possibility to use in organic farming system management. As mineral-rich in high organic matter content, biogenic and trace elements have excellent preconditions for use mainly in agriculture as a natural fertilizer increasing fertility. Alginit is also very interesting for the high content of humic substances, which are an important component of natural organic matter in soil and sediment. Humic substances heterogeneous organic compounds are environmentally important substances that can improve soil fertility. With its hydro-saving properties, it is one of the excellent water absorbent material with the potential to regulate the distribution of water toward the plant roots.

In more than 40 years since the discovery of alginite as a bituminous rock and knowledge of its properties, significant research has gained significant knowledge of its comprehensive use. The next part of the review presents some results and findings from various areas of research, which documents (...research, which documents...) that alginite is a unique, but still little known bituminous rock.

Effects of alginite application on plant germination

The influence of alginite and extracts from it on germination of Kentucky bluegrass (*Poa pratensis* L.) researched Kovár et al. (2021). The effect of alginite (powder, crushed alginite) and extracts from it (ALGEX 2 – "sodium solution", ALGEX 4 – "potassium solution") was observed in a controlled condition of a climatic chamber (26 °C/15 °C; 12 h light/12 h dark; 70 % rh) in an experiment with *Poa pratensis* L. on parameters as germination dynamics, average germination, germination rate and mean germination time. There were 5 treatments in this experiment: V1 – control without alginite and extracts from it; V2 – alginite powder 10 % vol.; V3 – crushed alginite 10 % vol.; V4 – 1.5 % extract ALGEX 2 and V5 – 1.5 % extract ALGEX 4. A significant increase in the germination of *Poa pratensis* L. was recorded as early as the 7th day of the experiment, using the ALGEX 4 extract, where the germination reached 340.00 % compared to the control. From the overall point of view, the positive and at the same time significant ($p = 0.0000$) effect of alginite and its extracts were manifested. The application of the solid form of alginite increased the average germination by 51.70 % (powder) and 201.70 % (crushed alginite), while the use of alginite extracts increased by 33.33 % (ALGEX 2) and 334.20 % (ALGEX 4) compared to the control. Alginite and its products can also be positively evaluated in terms of their effect on the germination rate, which they increased by 0.04–1.52 seeds/day in *Poa pratensis* L. compared to the control. The most apparent acceleration of germination was achieved with the application of alginite in liquid form (ALGEX 4) – 1.57 seeds/day, respectively alginite in solid form (crushed alginite) – 0.75 seeds/day with significant ($p = 0.0052$) differences between treatments. The values of the mean germination time showed a shortening of the germination time in all treatments using alginite, namely by 2.82 days with powder application, by 3 days with crushed alginite, by 1.31 days with ALGEX 2 extract and by 4.95 days with extract ALGEX 4.

Effects of alginite application on soil improvement and crop production

Vass et al. (2002) in a three-year experiment, the authors tested the effect of alginite from the Pinciná deposit on the chemical properties of soils in three sites – light soil, agglomerated brown soil, sandy-clay soil with a comparison of unfertilized parcels and other fertilization combinations. At the end of the experiment, the decrease in pH of alginite-fertilized soils was the lowest of all fertilization combinations. The content of plant-acceptable P and K in the soil decreased significantly, which means that alginite facilitates nutrient uptake by plants. The humus content also decreased but in the alginite-treated soil the least. The content of exchangeable bases was higher than in unfertilized soil. The sorption capacity level at one site reached the fully saturated degree, decreased at other sites, but relatively least in alginite-treated soils. The contents of heavy metals in the soils changed slightly, with exception of the site in Piteľová, where the contents before and after the end of the experiment exceeded the indicator value, which is probably a consequence of the floods of the river Hron. Relatively higher concentrations of heavy metals in alginite-treated soil indicate a higher sorption capacity of alginite, which protects plants from increased uptake of toxic metals.

Gömöryová et al. (2009) tested the effect of the amendment with alginite, an organic rock originating from the biomass of fossilized unicellular algae, on microbial activity of forest soils using a pot experiment. Five variants of soil-alginite mixtures were tested in three replicates with two forest soils: a loose sandy soil and a sandy loam. Gravimetric moisture closely correlated with the dose of alginite in both soils. Basal respiration and catalase activity increased with the dose of alginite in the sandy soil, but not in the sandy loam, where the highest response was observed at intermediate doses of alginite. The correlations of microbial activity parameters with moisture in the sandy soil were also much closer than in the sandy loam. The amendment with alginite was thus effective in improving some of the selected microbial activity indicators, but the optimum dose of alginite strongly depended on soil texture.

Effects of alginite application on growth, development, crop formation and quality

Kádár et al. (2015) studied the effect of alginit on the soil and triticale over three years (2012–2014), in a long-term field experiment set up on acidic sandy soil in Nyirlugos. A 100 $t.ha^{-1}$ rate of alginit was applied in concentrations 0, 50, 100 and 150 $kg.ha^{-1}.year^{-1}$ N. On alginit-treated plots, the pH (KCl) increased from 3.9 to 6.2 Every year the application of alginite increased the yield of straw and grain triticale. Alginit treatment generally doubled the yields, especially on plots treated with 150 $kg.ha^{-1}.year^{-1}$ N, which became acidified and impoverished in Ca, Mg, K and P. Alginit treatment increased the Mg, Mo and Cd uptake and reduced that of Mn, Zn and Ba by triticale seeds. The Ca, Mg, S and Mo concentrations increased in the straw, while the incorporation of Mn, Zn, Ba, Cu, Ni and Co was inhibited by alginit. Alginit is a suitable mineral for the amelioration of similar acid sands, which may eliminate the acidity caused by excessive N supplies and improve the water holding capacity, colloidal and nutrient status and drought-tolerance, and thus their fertility.

Komac (2016) determined the application of alginite as an additive for improving water-retention properties of soil, and if its use improves the quality and increases crop yields. An experiment carried out with a Chinese cabbage (*Brassica rapa* subsp. *pekinensis* (Lour.) Hanelt) and a germination test with cress. Even the highest dose of alginit (A2 = 60 $t.ha^{-1}$) did not alter the soil water capacity. Germinating cress test showed that alginit is not phytotoxic but encourages the emergence and growth of seedlings. Alginit increased the yield of the fresh mass of cabbage compared with unfertilized control of 22–30 % and had a similar effect as the bovine farmyard manure.

Vígh et al. (2016) studied "P37N01" hybrid maize (*Zea mays* L.) in a large-plot fertilization experiment on brown forest soil. Two various nitrogen fertilizers (Nitrosol – 30 % N and Pétisó – 27 % N) were applied. Five various treatments were conducted with 130 $kg.ha^{-1}$, and one with 165 $kg.ha^{-1}$ specific amount of nitrogen active ingredient. At three treatments the "Nitro-sol 130 $kg.ha^{-1}$" was applied altogether with additives: Alginit 10 $l.ha^{-1}$, Dudarit 10 $l.ha^{-1}$ and Dudarit 10 $l.ha^{-1}$ + Alginit 10 $l.ha^{-1}$. It was found that the nitrogen uptake in the leaves was highest at "Nitrosol 130" treatment; the phosphorus uptake was the highest at "Nitrosol + Alginit + Dudarit" treatment. The highest boron concentration in leaves was observed at "Nitrosol 165" treatment; the copper and manganese concentrations were the highest at "Nitrosol 130"

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treatment; the molybdenum at "Nitrosol + Alginit + Dudarit" treatment, while zinc uptake was the highest at "Nitrosol + Alginit" culture. Dudarit and alginit mixed with Nitrosol facilitate primarily the incorporation of essential macro- and microelements in the seeds (not to leaves) of maize.

Oravcová et al. (2018) researched the content of mercury in soil and in plants grown in contaminated soil with the addition of Alginite. Samples of soil contaminated with mercury were taken from the locality of Malachov and then mixed with Alginite in a 1 : 3 and 1 : 1 ratio. These substrates were applied on *Brassica napus* L. var. *napus*. (rape). Plant and soil samples were analyzed on a dedicated spectrophotometer – AMA 254. In the samples of substrates used for growing rape, we found a mercury content from 0.0929 to 2.9085 mg/kg. The mercury content in the above – rape biomass from 0.0425 to 0.3302 mg/kg. The sorption properties of Alginite were most pronounced in the above-mentioned rape biomass when a drop-in mercury content of 0.2005 mg/kg was recorded. The resulting values were compared with the limit values that were exceeded in many cases. From the values, it has found, that Alginite has confirmed its sorption properties, which can be further used in the treatment of the physical-chemical properties of lighter soils, the decontamination of soils devastated by anthropogenic activity.

Effects of alginite application on rooting and growth of forest plant species

Tužinský et al. (2015) evaluated the effect of alginite on the growth parameters of seedlings of Douglas-fir, Scots pine and line mixture of pedunculate oak, red oak and Norway maple (broadleaves) on former agricultural land with an unfavourable hydrophysical regime. The following doses of alginite were tested in the experiment: control (variant A without alginite), 0.5 kg of alginite (B) and 1.5 kg of alginite (C) when planting both conifers and mixtures of broadleaves. The number of seedlings on the sub-plots was 400 individuals, only in the case of Douglas-fir, the number was 200 individuals. Therefore every combination of tree species and the amount of alginite had 4 replications. The parameters of growth and development of individual trees (height, increment and mortality) show that after 2 years, both doses of alginite had statistically positive effects on height increments.

Sarvašová (2009) tested the effects of the alginite on growth and development of 4-years-old European silver fir (*Abies alba* Mill.) and Norway spruce (*Picea abies* (L.) Karst.) plants. The best results as measured by the height of the aboveground part, length of the root system and root-collar diameter. The positive effect of alginite for silver fir was observed only at the height of the aboveground part and the length of the root system, for spruce as well as in thickness root neck. Alginite belongs to perspective natural soil conditioners.

Beláček et al. (2002) documented that alginite in forestry can be used not only as a fertilizer. Fundamental to the argument is the following observation that alginite improves; supports the entrance of water to plants that is very important mainly after forest planting. During the first years, the root system of plants is not able to gather water from deeper horizons of soil. Grounds for supposing that alginite can be used mainly for sandy types of soil (spread mostly in Záhorie and Cerová vrchovina highlands) are just because these types of soil are with underdeveloped structure. They are very light, permeable and without the ability of water retention for a longer time.

Effects of alginite application on water remediation

One class of contaminants gathering increasing concern is endocrine-disrupting chemicals (EDCs). This pollution stems from one of three sources: agriculture (pesticides), industry (plasticizers, etc.), and pharmaceutical products. With the multitude of chemical additives, nature-based solutions are in particular demand. Alginite, mined in Hungary, has been studied regarding its potential for EDC remediation in polluted water. Alginite is immature oil shale, consisting of clay minerals, feldspars, quartz, accessories, and an organic component. This organic component, mainly derived from ancient algae and pollen, has been diagenetically modified and is now harmless, rendering Alginite a safe material for treating water. Although the material is already approved and in use for soil amelioration, technologies for water treatment are still missing. Guhl et al. (2018) found the extensive capability of Alginite to immobilise EDCs, such as 17b- estradiol, diclofenac, and others. Adsorption isotherms have been determined for a multitude of compounds; the general capability of Alginite to remedy endocrine-disrupting contamination has thus been shown. However, the actual mechanism of EDC removal by Alginite remains elusive. Acknowledging the heterogeneous nature of this material, a fractionation of the material into clay-rich and organic-rich parts aims at understanding the individual component's action towards pollutants. Interestingly, ethinylestradiol- and carbamazepine-polluted waters react differently to Alginite components. However, it has already been published that organo-clay materials are suitable for treating diclofenac-polluted waters (diclofenac adsorbs poorly onto untreated clay).

The adsorptive removal of endocrine-disrupting chemicals (EDC) from municipal wastewater is a problem of wastewater treatment technology that has not yet been fully resolved (Kropp et al., 2021). Tröbs and Bertau (2017) examined alginite for its suitability as an adsorbent for water purification. Nevertheless, alginite (43 $m^2.g^{-1}$) compared to activated carbon (594 $m^2.g^{-1}$) has a lower specific surface area, the area-related loading capacity compared to activated carbon is high and is only below the values found for activated carbon in the case of EDC with a high degree of dissociation. This is the result of the interaction of kerogens as a nonpolar organic phase as well as basic seam-neutral crystal surfaces of the inorganic matrix. The adsorption of the EDC takes place primarily at the kerogen phase of the alginate. The differently realizable equilibrium loads can be explained well by the different lipophilia of the investigated EDC model substances. The adsorption behaviour on alginite is strongly influenced by the degree of dissociation dependent on the pH value. Considering the economic application of alginite in municipal wastewater treatment technology, the adsorbent must be assessed with regards to its area-related, weight-related loading capacity. This shows that alginate and activated carbon can complement each other extremely effectively and develop an improved cleaning effect in combination. From an economic point of view, the significantly lower price of alginite (approx. 50–100 €.t^{-1} vs. 1000–2000 €.t^{-1}) has a positive effect. For these reasons, alginite seems highly interesting for wastewater treatment. Due to its high carbonate content, alginite also shows a buffering effect, so that the addition of chalk (lime) to the revitalization basin could be minimized.

Frišták et al. (2015) used Alginite, as a component of some types of kerogen alongside amorphous organic matter for adsorptive separation and removal of Cd^{2+} ions from aqueous solutions. Alginite material was characterized by X-ray diffraction, ATR-FTIR, cation exchange-capacity and specific surface area analyses. Evaluation of alginite sorption properties showed the effect of solution pH value in the range from 2 to 6 on the sorption capacity of alginite. At slightly acidic conditions (pH 6.0–6.5), the alginite samples exhibited a sufficient sorption capacity and stability. The pseudo-second-order kinetic model described the sorption data better than the pseudo-first-order kinetic model. The equilibrium of cadmium sorption by alginite was reached within 120 min. Maximal sorption capacity (Q_{max}) calculated from experimental equilibrium data (Langmuir adsorption isotherm) was 23.62 $mg.g^{-1}$. Sorption energy of Cd^{2+} ions calculated from the Dubinin–Kaganer–Radushkevich model confirmed the ion-exchange mechanism of cadmium removal for alginite sorbent. The alginite from central European geological maar (Pula, Hungary) can be utilized for the production of new non-conventional sorbents or mineral filters for the removal of toxic metals.

Effects of alginite application on herbicide degradation

Rauch and Földényi (2012) studied the catalytic effect of an alginite was studied under laboratory conditions on the decomposition of herbicide propisochlor. The breakdown process was followed in four parallel experiments: buffered solution (pH = 7), in buffer solutions containing alginite or bentonite (a common rock), also in the solution obtained from the extraction of the alginite (alginate/buffer = 1/10). In the aqueous phase, the decomposition of the herbicide was followed by using HPLC–UV and the metabolites of degradation were identified by GC–MS techniques. For the phenomenological description of the experimental decay curves, two parallel reactions with first-order kinetics were taken into account. During the time of experiments, no significant decomposition was observed either in the pure buffer solution or in the presence of bentonite. When, however, alginite was added to the system the degradation of propisochlor was accelerated dramatically: after 5 days, its concentration dropped below 50 % of the initial value. The identified degradation products indicate both reductive as well as oxidative biological mechanisms operating under anoxic conditions. It was proved earlier that the sulfenic and sulfinic acid ester derivatives of the propisochlor appeared in the metabolic pathways of animals and plants, but they were not detected yet in the degradation process occurring in the soil environment. Among the degradation products, a dehydrochlorinated derivates is a new metabolite identified in experiments. Results corroborate the algae-related origin of the alginite and forecast its application as a soil ameliorating agent.

Benei and Rauch (2016) performed adsorption experiments with alginite with acetochlor, propisochlor and 2,4-dichlorophenol. The alginite has a high content of organic matter, but its composition differs significantly from the humic substances in the soils. In alginite, humic substances make up only a quarter of all organic matter, 75 % of which is kerogen, which, according to the test results, plays a significant role in the binding of organic pollutants. This is supported

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by the adsorption experiments performed on humus-depleted alginite and, as the main mineral constituent, bentonite. Humic substances on the surface have been shown to inhibit access to kerogen, so the tested substances bound in the highest amount in all cases of demineralized alginite. Studies have also shown that due to the excellent buffering capacity of alginite, media with different pH do not cause a significant change in the amount of material bound.

Humic acids of soils and alginite

Barančíková and Litavec (2016) examined the differences in chemical composition between humic acids (HA) from alginite and humic acids isolated from different soil types. The differences in elemental analysis and ash proportion in HA extracted by modified IHSS (International Humic Substances Society) method (C = 35.4, H = 43 atomic %, ash content = 0.08 %) and simplified extraction method (C = 31, H = 31 atomic %, ash content = 7.42 %) can be caused by different concentration of extraction solution, and also differences in the purification of HA. The differences in chemical structure between alginate HA and HA isolated from different soil types according to the data of elemental analysis (C content of alginite HA = 35.4 atomic %, C content in soils HA = 38.2–49.1 atomic %) and 13C nuclear magnetic resonance (NMR) spectra (degree of aromaticity of alginite HA = 24.4 % and soil HA= 35.9–53 %) were found. Results of 13C NMR show that the content of aromatic carbon was decreasing in the following order: Haplic Chernozem HA > Andic Cambisol HA > Haplic Cambisol HA > alginite HA. Based on the obtained results, it can be concluded that the differences in the chemical structure of alginite and soil HA can be explained by the difference in the origin of organic matter in alginite and soil samples. The source of organic matter in alginite is mainly type II kerogen from algae and that of soil is lignin and cellulose (type III kerogen) of higher plants.

Effects of application of alginite on stabilization of beneficial microorganisms in animal organisms

Strompfová et al. (2018) investigated the effects of dietary supplementation with canine-derived probiotic strain *Lactobacillus fermentum* CCM 7421 in combination with alginite in dogs. Alginite is a loam-like material of volcanic origin composed of clay minerals and fossilised unicellular algae. The effects of these additives on faecal microbiota, faecal characteristics, short-chain fatty acid profile, haematology, serum biochemistry and cellular immunity parameters were monitored. The results of this straightforward experiment showed beneficial effects in the combined Alginite + *L. fermentum* group. In detail, a decrease in faecal coliforms and clostridia and an increase in lactic acid bacteria, haemoglobin and serum magnesium levels compared to baseline were observed in the A + LF group ($p < 0.05$). In contrast, sole application of alginite (A group) led to several unexpected effects such as an increase in clostridial population and serum alanine aminotransferase and a decrease in haemoglobin concentration ($p < 0.05$). The addition of alginite prevented a decrease in faecal pH and serum mineral content observed in the LF group. This indicates the possibility of applying alginite in dogs' nutrition as a combinative additive with probiotic bacteria for restoring optimal acid-alkali balance without affecting positive probiotic effects.

Nemcová et al. (2012–2015) investigated the potential utilization of alginite and its humin extracts for stabilisation of beneficial microorganisms intended for the development of new application forms of these microorganisms. Procedures for preparation of laboratory extracts of alginite suitable for preparation of optimum alginite skeleton for solid substrate fermentation of beneficial bacteria and production of alginate containing cultivation media were developed and validated. A patent proposal was prepared to consist of the description of the way of stabilisation of beneficial micro-organisms by cultivation on alginite grains. Binding to an alginite carrier saturated with a suitable cultivation medium stabilizes probiotic micro-organisms during production and their application. The probiotic microorganisms capable of producing biofilm on alginite grains are in a dormant (quiescent) state with minimal metabolic processes, exhibit resistance to stress and multiply in the body after passaging in the body intestinal or skin microenvironment with a positive biological effect.

Conclusions

The report presents only very brief information and knowledge about alginite. Alginite is a natural and non-toxic organic-bituminous rock, with a significant content of basic nutrients (P, K, Ca, Mg and S) for plants except for nitrogen and content of more than 60 microelements, which increase its agrochemical value. Alginite can be practically used in its natural and technologically modified form. The application of alginite does not damage the soil or the environment. The physical, mechanical, chemical, pedological, agrochemical and other properties of alginite confirm that alginite as a bituminous rock has unique possibilities for practical use in agriculture, forestry, remediation and soil and water improvement, pesticide decomposition and stabilization of microorganisms in animal organisms and various other fields. Alginite applied to the soil can retain water and nutrients dissolved in it, which it gradually releases to the roots of plants during periods of precipitation deficit. When applied correctly, it significantly affects the yield and quality of seeds and fruits of crops. It actively affects the biological, physiological and biochemical processes of plants. Despite many findings, alginite is still a less-known bituminous rock. The least knowledge is about the chemical composition and thus about the effects of the organic part of alginite, which is referred to as type II kerogen.

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Conflict of interest

The authors declare no conflict of interest.

Ethical statement

This article does not contain any studies that would require an ethical statement.

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