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THE MORPHOLOGY OF POLLEN GRAINS OF SOME CULTIVARS *RUBUS FRUTICOSUS* L.

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The features of pollen grains sculpture of 8 cultivars *Rubus fruticosus* L. have been studied in details via the scanning electron microscope method. The pollen material picked up in the Central region of Russia and the Republic of Adygeya has been used in the research. The comparative data of morphometric factors are presented, the polymorphism of pollen grains – size and surface is determined. The complex sculptural types are formed by the different combination of 2 or 3 simple sculptural types. The complex structural type of exine is characteristic of the studied cultivars *Rubus fruticosus*. The average size of pollen grains polar axis is within 32.43–37.46 μm; the equatorial diameter is within 16.91–18.88 μm. The ratio of polar and equatorial axis (*P*/*E*) marks the degree of the pollen grains elongation (roundness). The research showed that the cultivation region gives a certain influence on the change of the pollen average dimensions, but it does not influence *P*/*E* ratio. The pollen grains form is oblong-ellipsoid. In the polar outline the grains are round. The pollen grains of the presented blackberry cultivars are 3- or 4-colporate, the apertures are long. The sculpture of the pollen grain exine is complex, it belongs to the two component sculptural type – rugulose, the picture has the species features. The morphometric and sculptural identical characteristics are separated: the form (the pollen grains elongation degree), the dimensions (the ratio of the polar axis to the equatorial axis (*P*/*E*), the number of apertures and the surface picture (microstructure).

Keywords: blackberry, cultivar, pollen morphology, scanning electron microscope

Introduction

Blackberry belongs to the absolutely polymorphic plant (Gruner, 2014). Lots of cultivars are complex hybrids and there are many cross-species hybrids among wild-growing forms that make difficult classification. That's why the possibility to identify the both forms, to determine the phylogenic connections between them using not only external characteristics, but also for

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microstructures applying modern equipment is great interest. Many authors are described pollen of different plant species by scanning electron microscope (Grygorieva et al., 2010, 2017; Brindza and Brovarskyi, 2013; Nikolaeva et al., 2014; Motyleva et al., 2015; Motyleva and Brindza, 2017).

Thereby the study of the pollen grains morphology – the dimensions, the form, the exine structure, the apertures number and place is more actual. The complex of morphological characteristics allows to determine the differences (or similarities) between the close species or cultivars, morphologic inhomogeneity, especially variability of its dimensions because of hybridization and polymorphism (Kupriyanova and Aleshina, 1978; Tzarenko, 2012). The comparative electron-microscopy researches of the blackberry different cultivars pollen growing in the Republic of Adygeya and the Central region of Russia have not been performed before; this fact has determined the purpose of our researches.

Material and methodology

Objects of research

The pollen material of *Rubus fruticosus* L. (subgenus *Eubatus* Focke) was picked up in the experimental plantations of Maikop OS VIR experimental station and in VNIISPK (Orel) in 2009–2011. Pollen was studied in 8 cultivars of *Rubus fruticosus* L. The pollen cultivars of Agavam and Thornfree were from different ecological – geographical zones – Russia and the Republic of Adygeya. Pollen cultivars of Darrow, Erie, Flint, Maxwell Early, Mc. Donald, Smoothstem are from Russia.

Scanning electron microscope (SEM)

Micropictures are taken on the scanning electron microscope JEOL JSM – 6390 in the laboratory of agroecology VNIISPK. Preliminary dried at T = 40–50 °C pollen was accurately pounded with a pestle to annual adhesion and was put on the special carbonic scotch placed on the object table of the scanning electron microscope with a thin metal spreading rod. 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 to perform 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.

Morphometric characteristics

The measurements of the pollen grains linear dimensions were performed via 200–300 times repeatability in about 30 field microscopy with the average value calculation. All the mentioned in the text dimensions are given in μm.

Statistical analysis

For statistical evaluation were used standard methods using statistical software Statgraphics Centurion XVII (StatPoint Inc.USA).

Results and discussion

The pollen grains morphometric values are given in Table 1. The average size of the polar axis for the *Rubus fruticosus* studied cultivars pollen grains is within 32.43–37.46 μm; the equatorial diameter is within 16.91–18.88 μm. The ratio of the polar axis to the equatorial one (*P*/*E*) marks the degree of the pollen grains elongation (roundness). The pollen of Agavam, Flint, Mc. Donald, Smoothstem and Thornfree cultivars is more elongate in comparison with other studying cultivars. Among these cultivars Smoothstem and Thornfree are closely related. It is possible that other 3 cultivars are phylogenically connected with one another. The comparison of the photometric parameters of Agavam and Thornfree cultivars pollen cultivated in different climatic zones (in the Caucasus and in the Central region) showed that the cultivation region gives a certain influence on the change of the pollen average dimensions, but it does not influence *P*/*E* ratio. The received results coincide with the pollen grains description and with the data received while studying *R. caesius* L. pollen from Latvia (Kupriyanova and Aleshina, 1978; Meng and Finn, 2002).

Table 1 The morphological characteristic of pollen grains of *Rubus fruticosus* L. representatives

Measurements	*n*	Min (μm)	Max (μm)	Average (μm)	*V* (%)	*P*/*E*
Agavam (Republic of Adygeya)						
P	250	36.49	39.78	37.17	2.76	2.08
E	250	14.35	19.31	17.85	6.48	
Agavam (Russia)						
P	300	27.91	39.62	36.82	6.83	2.06
E	300	15.28	22.01	18.55	5.62	
Darrow (Russia)						
P	245	30.59	39.61	34.83	6.71	1.76
E	245	12.91	19.81			
Erie (Russia)						
P	240	17.61	37.94	32.43	6.71	1.88
E	240	13.59	21.11	17.29	10.31	
Flint (Russia)						
P	250	33.45	39.77	37.46	3.17	2.09
E	250	15.67	19.17	17.85	5.63	
Maxwell Early (Russia)						
P	250	29.31	42.19	35.87	8.64	1.89
E	250	13.31	22.32	18.88	9.59	
Mc. Donald (Russia)						
P	250	22.52	38.97	33.05	8.24	2.02

Continue the Table 1

Measurements	*n*	Min (μm)	Max (μm)	Average (μm)	*V* (%)	*P*/*E*
Smoothstem (Russia)						
P	300	32.76	40.23	35.66	5.72	2.01
E	300	15.23	21.11	17.71	7.87	
Thornfree (The Republic of Adygeya)						
P	300	33.84	39.45	36.33	4.76	2.00
E	300	15.56	21.17	18.14	8.79	
Thornfree (Russia)						
P	240	30.52	39.34	34.92	6.83	1.99
E	240	14.64	23.11	17.58	9.14	

Notes: Min – minimum value; Max – maximum value; *V* – variation coefficient (%); *P* – polar axis; *E* – equatorial one; *P*/*E* – the ratio of the polar axis to the equatorial axis

The pollen grains form is oblong-ellipsoid, the apertures are long (Figure 1). In the pole outline the grains are round. In the received pictures it is clearly seen that the pollen grains of the presented blackberry cultivars are 3- or 4-colporate (Figure 1B, C), whereas according to the data given by Kupriyanova and Aleshina (1978) thet *Rubus fruticosus* pollen is only 3-colporate. Possibly it is connected with the fact that the authors used optical microscope: in the given pictures there is no position "polar view", but from the equatorial view it is difficult to count the number of apertures.

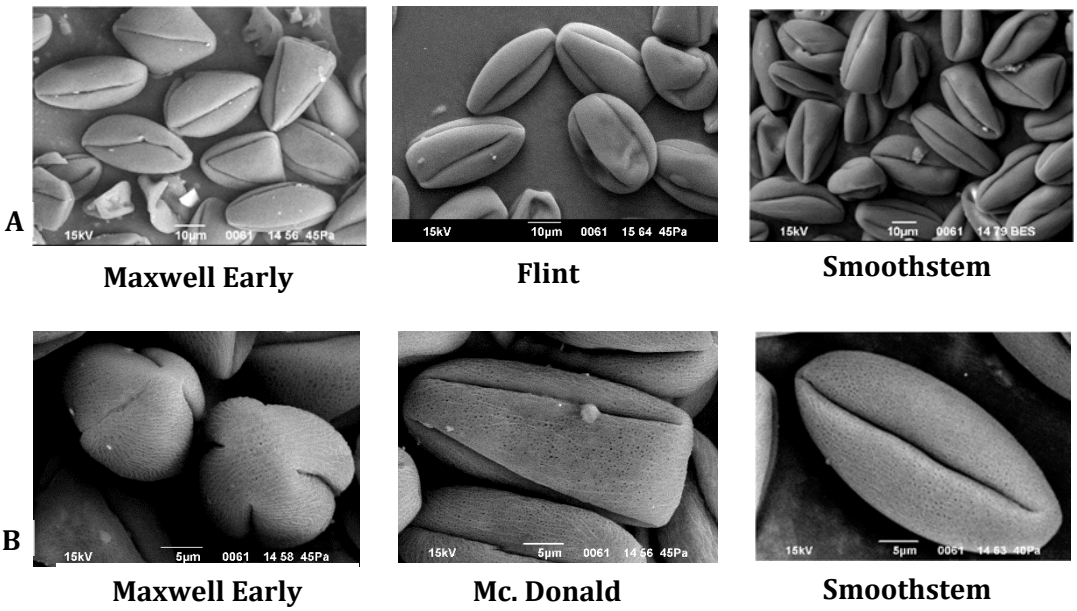

Figure 1A–B The pollen grains of some Rubus fruticosus L. cultivars. A – zoom.1000; B–zoom.3300; C–zoom. 5000; D-zoom. 10000

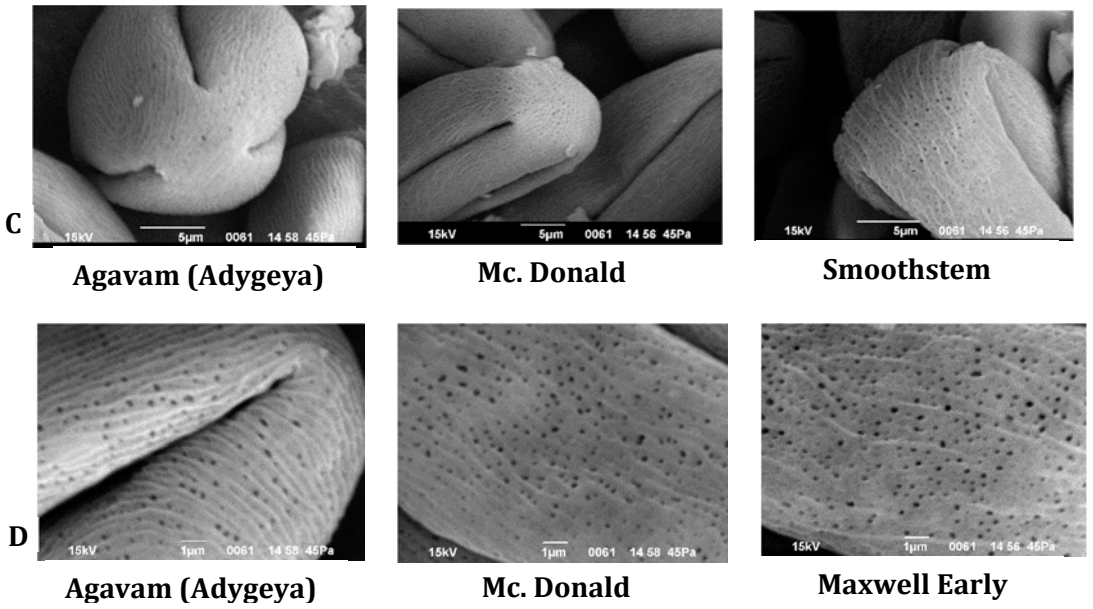

Figure 1A–B The pollen grains of some Rubus fruticosus L. cultivars. A – zoom.1000; B–zoom.3300; C–zoom. 5000; D-zoom. 10000

The exine pattern is tender-undulate, fine-mesh, the picture has the cultivars features (Figure 1D). The number of the misshapen pollen grains is up to 20–35%. This fact, in some ways, can be connected with the greenness of some pollen, but also with polyploid plants.

Conclusions

The studying of the blackberry pollen via scanning electron microscope allowed to separate the most important parameters which can be used to identify the representatives of *Rubus fruticosus*. They are the form (the pollen grains elongation, the length and the width ratio). Parameters such as the number of the apertures and the surface picture (microsculpture) are more specific for different cultivars. The certain influence of the blackberry cultivated region climatic conditions on the pollen dimensions was marked.

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THE PRODUCTION OF GRAFTED TOMATOES (*SOLANUM LYCOPERSICUM* L.) IN A MONOCULTURAL SYSTEM

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The possibility of applying different methods in grafting vegetable plants, give the possibility to improve the fruit quality. Genotypes of *Solanum lycopersicum* L. have been grafted on tomato rootstocks distinctively, and the impact of the rootstock on several important fruit quality parameters has been studied. The results obtained from the physicochemical analysis demonstrated that the maximum content of titratable acidity was observed in the control hybrids. The variants V-2 (Abellus + Emperador), V-5 (Lilos + Emperador), V-6 (Lilos + Maxifort) and hybrid V-7 (Beril) had a higher sugar and acid content. Results of evaluated were showed in many parameters in favour of variants. The following morphometric characteristics have been also assessed. The average number of clusters per plant of the non-grafted plant of the V-7 (Beril F1) hybrid was 12.6 compared with 14.3 (V-8) and 14.4 (V-9) pieces of variants. Overall, these results have shown the effectiveness of grafting in terms of plant productivity and the improvement of tomato fruit quality, which are of particular importance, because grafting is a quick and effective alternative to achieve these goals.

Keywords: graft, grafting, hybrid, scion, rootstock

Introduction

The production of vegetables on protected land using a monoculture system has a negative effect on the cultivation environment, the product quality and the productivity of vegetable species. Several authors signal the increase in the degree of aggression of diseases caused by pathogens that can be found in the soil (*Fusarium* spp., *Verticilium* spp., *Pythium* spp., *Phyttophthora* spp. etc.) of protected land, as the result of planting the same species on the same piece of land (Grimault and Prior, 1994; Oda, 1999; Miskovic et al., 2009). Repeated crop cultivation in greenhouse soil often leads to the spread of nematodes, which are difficult

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to fight using the thermal or chemical method (Roşca, 2009; Albacete et al., 2015). The grafting of vegetable plants is used to confer resistance against pathogens in the air and soil, to increase tolerance to abiotic stress factors, to improve water and nutrient absorption, and to increase the vigour of the graft (Lee, 1994; King et al., 2010). The grafting technology has been practiced with great success worldwide. Asia and Western Europe have been practicing this technology for a long time. Japan and Korea are the countries with the largest areas planted with grafted plants. The first scientific researches in the field of vegetable grafting were carried out at Kyusyu University and Korea University in Japan in 1920. They aimed at the establishing the technology of grafted seedlings production and the cultivation of grafted melons; the first rootstock used in research to graft watermelons was *Cucurbita moschata* Duch. Later on, during the second half of the 20th century, other vegetable species such as melons (1931), eggplants (1950), cucumbers (1960), tomatoes (1970) and bell peppers (1985) were commercially grafted (Bogoescu, 2015). Water melons accounts for 93–98% of the total area on which grafted plants grow, cucumber accounts for 72–95%, melons accounts for 35–90%, tomatoes accounts for 42–65% and the sweet peppers accounts for 15–35% of the total area. Greece, Turkey, the United States and Morocco are among the countries with a high share in the cultivation of grafted vegetables. In these countries some crops are cultivated on 100% of their total area; for example, the cultivation of melons in Greece (Taraka-Mavrona and Koutsika-Sotiriou, 2000). Recent studies have shown that the use of appropriate rootstocks helps to improve salinity and water stress in tomatoes (Schwarz et al., 2010; Keatinge et al., 2014). Turhan et al. (2011) reported that the grafting of tomato plants on rootstocks tolerant to abiotic stress had positively enhanced yields, especially of those grown in greenhouses. Also Oztekin et al. (2007) concluded that grafting could increase the tolerance of tomatoes to salinity, and promote the efficiency of water utilisation.

The research was conducted in order to study the growth, development and productivity of tomatoes in protected areas, according to the grafting method and the combination scion-rootstock.

Material and methodology

Biological material

Three hybrids of tomatoes (*Solanum lycopersicum* L.) were selected as the aim of the study and were used as scions, namely Abellus F1 (V-1), Lillos F1(V-4) and Beril F1 (V-7), which were cultivated in greenhouses. Two hybrids were used as rootstocks (Emperador F1 and Maxifort F1), which were characterized by a similar resistance / tolerance to abiotic stress (Rumbos et al., 2011). From them were grafted 6 variants Abellus + Emperador (V-2), Abellus + Maxifort (V-3), Lillos + Emperador (V-5), Lillos + Maxifort (V-6), Beril + Emperador (V-8) and Beril + Maxifort (V-9). Both the scion and the rootstock hybrids were indeterminate and they had round and red-coloured fruit. The phytometric indices of tomato plants were studied according to the combination scion-rootstock in a solar greenhouse (Figure 1). The quality of grafted tomato fruit according to the combination scion-rootstock, and the content of assimilating pigments (mg/g of fresh products) of the grafted tomato plants at the stage of organogenesis were also studied. Thirty days after sowing, when the seedlings reached the

stage of two or three leaves on the plant and the diameter of root neck was 2.5 mm, the plants were grafted by copulation and splitting.

Figure 1 Grafting through the method: a – grafting by copulation, 16 days after grafting; b – grating through displacement; c – 16 days after grafting

The experiments were carried out at the company the SRL "Ecoplantera", which deals with the production of flowers and vegetables seedlings, during the years 2013–2018.

Morphometric analysis

The following properties were measured by morphometric analysis: a) height of stem (cm), b) height of the first cluster (cm), c) thickness of stem (mm), d) number of leaves (pcs), e) length of internodes (cm), f) number of leaves between two clusters (pcs), g) average number of flowers cluster (pcs), h) number of clusters per plants (pcs). The 9 variants of *Solanum lycopersicum* L. were measured using a ruler (a, b, e) and digital calliper (c).

Physicochemical analysis

Chemical analyses were provided for all 9 variants of fresh tomato fruit. We determined following characters: a) dry matter content (%), b) amount of sugar (%), c) titratable acidity (pH), d) malic acid (%) per 100 g of fresh fruit, e) firmness of fruit (kgf/cm^2) comparing texture and structure between mature peeled fruits and fruits with peel.

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 descriptors of variability: average, the coefficient of variation (%), deviation from the mean. Data were analysed and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$).

Results and discussion

During the growth stage of the plants, a series of biochemical and morphometric measurements and phenological observations were performed at different stages of the plants' development. These indicators were better ninety days after the plants planting. The grafting of tomato plants had a significant effect on the vegetative growth (Table 1).

The results were similar to the results obtained by Khah et al. (2006), Gutul and Iliev (2017). They specified that grafted tomato plants were more vigorous and healthier than the control plants.

Table 1 Phytometric indices of tomato plants according to the combination scion-rootstock in a solar greenhouse

Parameters	Variant								
	V-1	**V-2**	**V-3**	**V-4**	**V-5**	**V-6**	**V-7**	**V-8**	**V-9**
Height stem (cm)	245	251	247	250	260	312	263	262	265
Height first cluster (cm)	22.3	27.4	23.4	38.2	33.7	34.4	26.4	19.5	19.3
Thickness stem (mm)	12.0	14.3	13.9	12.7	14.6	13.5	12.9	13.9	13.5
Number leaves (pcs)	32.6	33.6	33.0	33.0	33.0	30.5	29.1	32.5	35
Length internodes (cm)	7.4	7.3	6.4	6.3	6.0	7.5	6.7	7.8	7.7
Number leaves between two clusters (pcs)	3.1	2.5	2.8	2.7	3.1	2.9	3.4	2.8	2.4
Average number flowers cluster (pcs)	6.2	6.4	5.8	4.3	5.8	5.3	5.1	6.3	6.4
Number clusters (pcs)	10.4	14.9	12.4	10.7	13.6	15.1	12.6	14.3	14.4

During the growth period of the grafted plants, the morphometric parameters such as the average number of flowers per cluster, the number of clusters per plant, the thickness of stem, etc. increased significantly because of the influence of the rootstock, in comparison with the control group of plants (Figure 2). On all the rootstocks, the clusters of the grafted plants had a larger number of flowers compared to the plants which had not been grafted. For example, the average number of clusters per plant of the non-grafted plant of the V-7 (Beril F1) hybrid was 12.6 compared with 14.3 (V-8) and 14.4 (V-9) pieces, respectively, in the plants grafted on the Emperador F1 and Maxifort F1 rootstocks. These differences are due to the fact that they had been positively influenced by the vigorous growth of the rootstock. Due to a more developed root system of the selected rootstocks, a better absorption of nutrients and water happened (Mohammed et al., 2009), and as a result of it, an increase in fruit productivity and quality could be observed. Thus, plant grafting in an intensive system is a good alternative to common technologies. It helps to obtain cost-effective production.

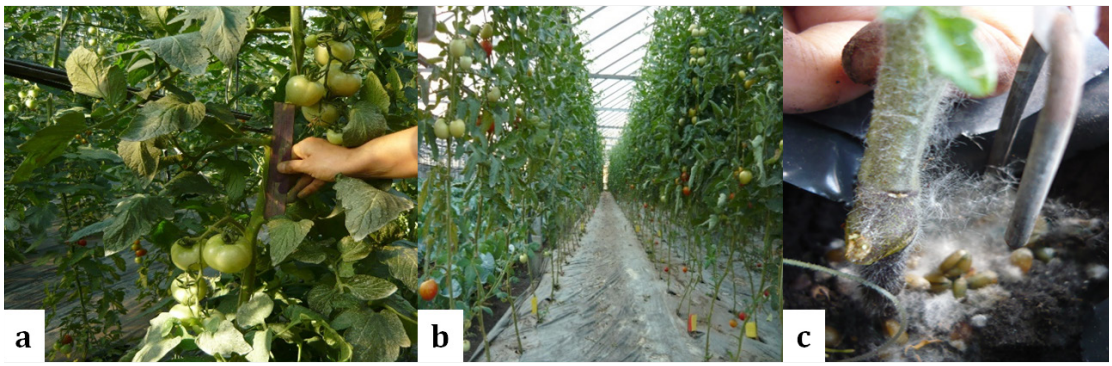

Figure 2 Measurement of the phytometric indications of tomato plants: a – length of internodes, b – diameter of the stem, c – number of leaves between two clusters

The increased number of tomatoes on the grafted plants could be attributed to the excessive growth of plants (Table 2).

Table 2 Quality of grafted tomatoes depending on the scion-rootstock combination

Variant	Dry matter content (%)	Amount of sugar (%)	Titratable acidity, pH	Malic acid (%)
Abellus	5.20±0.050	4.1±0.000	4.38±0.005	0.44±0.005
Abellus + Emperador	5.12±0.110	5.0±0.000	4.26±0.005	0.44±0.005
Abellus + Maxifort	4.50±0.000	4.6±0.110	4.36±0.010	0.43±0.005
Lilos	4.98±0.150	4.8±0.110	4.26±0.005	0.51±0.002
Lilos + Emperador	4.90±0.150	4.8±0.230	4.25±0.003	0.56±0.005
Lilos + Maxifort	4.90±0.150	4.9±0.110	4.26±0.013	0.41±0.005
Beril	5.20±0.000	5.0±0.000	4.29±0.010	0.46±0.001
Beril + Emperador	4.40±0.000	4.2±0.110	4.23±0.017	0.44±0.010
Beril + Maxifort	4.60±0.000	4.7±0.110	4.28±0.010	0.54±0.002

Table shows the value of the titratable acidity and dry matter content in the tomato fruit grafted on two different rootstocks. The results obtained from the physicochemical analysis demonstrated that the rootstocks had affected neither the pH value of the titratable acid nor the soluble dry matter content in tomato fruits, which falls within the value range of 4.38–4.23%; its maximum content was observed in the control hybrids. Similar results were reported by Arvanitoyannis (2005), who stated that grafting had not affected to the pH value of tomato fruit. Turhan et al. (2011), Echevarria et al. (2012) and Gajc-Wolska et al. (2010) observed that grafted tomato plants had improved the yield and components of the fruit. The pleasant taste is the result of a balanced ratio between acidity and sugar content, and the tomatoes which have a sweet-sour taste are the most appreciated.

On the basis of soluble dry matter content, and by reading the values, the content of carbohydrates and acidity (expressed in malic acid equivalent) in the tomatoes fruit was also

determined. Concerning the sugar-acidity balance, it can be seen that variants V-2 (Abellus + Emperador), V-5 (Lilos + Emperador), V-6 (Lilos + Maxifort) and hybrid V-7 (Beril) had a higher sugar and acid content (Table 2). The grafted plants had a more favourable balance between the content of sugar and acids. It is very important to know the firmness of the fruit which results from the interdependence between texture and structure, because it allows determining the time of harvest, the mode of harvesting, packing and transporting, as well as the quality and the storage life of tomatoes.

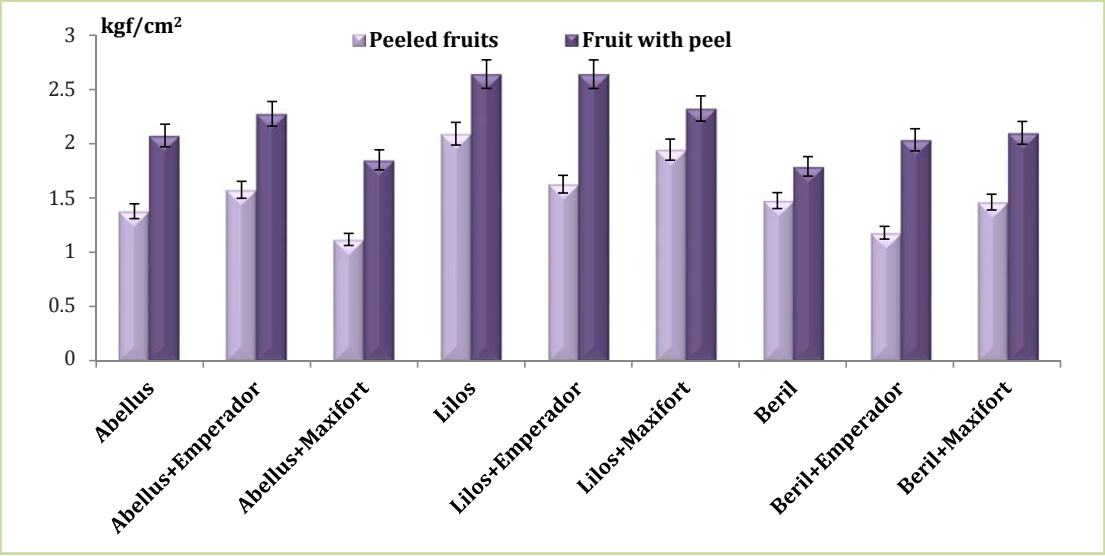

Figure 3 Structural and textural firmness of scion-rootstock tomato fruit

Concerning the "structural and textural firmness" index, all variants were resistant to transportation and storage, but variants V-3 (Abellus + Emperador), V-5 (Lilos + Emperador) and hybrid V-4 (Lilos) proved to be more resistant (Figure 3). Based on the results obtained in the experiments, it is obvious that the grafting of tomatoes on the Maxifort F1 and EmperadorF1 rootstocks has had a very important impact on the growth and development management of the plants. This technique, in most cases, hurries the flowering process which results in quantitative and qualitative production gains. The analysis of the biochemical and morphometric characteristics of the aerial parts of the plants indicates that all the grafted variants have the best indices compared to the control plants.

Conclusions

Our study and analyses of the morphometric and physicochemical characteristics of the aerial part of the plant, it was noticed that the grafted plants had the best results. In terms of sugar content and the balance between sugars and acidity in the fruit of the control plants, hybrid V-7 (Beril F1) was the best; among the grafted plants the variant V-2 (Abellus + Emperador), V-5 (Lilos + Emperador) and V-9 (Beril + Maxifort) were more remarkable. The obtained results represent a balanced sugar-acid ratio for all the variants; it has the key role in establishing the

taste. The research has been aimed at demonstrating that the use of rootstocks in tomatoes growing influences positively the biotic stress resistance, increases the productivity and enhances the ability to adapt to the environment.

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MICROBIOLOGIC, AGROCHEMICAL AND AIIELOPATHIC SOIL STATUS UNDER *LAVANDULA* L. PLANTS SPECIES IN FOREST-STEPPE ZONE

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The article reports the results of microbiological, agrochemical and allelopathic studies of the influence of *Lavandula angustifolia* Mill. and *Lavandula hybrida* Rev. introductive plants on the soil status. The aim of this study was to analyze the effect of hydrothermal and soil conditions of Forest-Steppe zone on the microbial cenosis formation and dynamics of taxonomic and ecology-trophic groups of microorganisms, the soil allelopathic activity and the ratio of biogenic elements. The analysis of the functional structure of microbial cenosis in the rhizosphere of lavender and lavandin shows a decreased quantity of micromicetes and an increase in the amount of spore-forming bacteria. The allelopathic activity of soil under lavender is characterized by a low level of toxicity. The processes of mineralization of organic matter in the soil were quite balanced. The influence of allelochemicals on the distribution of macro and microelements in the soil during lavender cultivation has been studied. The obtained results prove the positive effect of the studied plants on the microbiocenose and physical-chemical soil state that should be taken into account for their gardening.

Keywords: *Lavandula*, microbiocenosis, micromycetes, bacteria, soil allelopathic activity.

Introduction

Nowadays the disturbingly increasing anthropogenic effect on natural ecosystems leads to the reduction of the biological diversity of plant, animal and microbial world. The problem of preservation of phytobiodiversity can be solved out by means of sinecologic methods of plant introduction into the artificial phytocoenosis.

Aromatic plant species are characterized by polyfunctional properties as they are resistant to various anthropogenic pollutants and phytopathogenic microorganisms and do not accumulate phytotoxic substances in their environment, causing the phytosanitary effect on the ecosystem (Yurchak, 2005; Kovtun-Vodyanytska, 2017).

The species of *Lavandula* L. genus are known for their ability to influence the environment because of their high allelopathic activity. Moreover, the plants of *Lavandula* genus are popular

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horticultural species as they are characterized by long flowering period, high ionizing sanitary effect and do not require special growing conditions.

The role of biologically active substances and the associated microbiota of culturphytocoenosis of *Salvia sclarea* L., *Lavandula angustifolia* and *Mentha piperita* L. aromatic plants were studied by Yurchak (1999, 2005). We had earlier characterized the formation of the microbiocenosis in the soil samples under *Monarda didima* L., *Hyssopus officinalis* L. *Dracocephalum moldavicum* L., *Vitex agnus-castus* L. plant species of Lamiaceae family (Ellanska, 2010, 2013)

This way, the aim of this study was to characterize the main taxonomic and ecotrophic groups of microorganisms and to estimate the allelopathic activity of soil under the plants of *Lavandula* genus under the conditions of Forest-Steppe zone.

Material and methodology

Locating plants and data collection

This experimental study was carried out in the Department of Allelopathy of M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (Kyiv). *Lavandula angustifolia* Mill. and *Lavandula hybrida* Rev. plants were donated by the Botanical garden collection fund.

Microbiological researches

The bacterial abundance was quantified on meat-peptone agar (for ammonifiers), on ammonium starch agar (for inorganic nitrogen-immobilizers and actinomycetes), on Mishustin medium (spore-forming bacteria), on Hetchenson medium (cellulose decomposing bacteria), on Eshbi medium (for nitrogen-fixing bacteria by the application method), while the microscopic fungi were incubated on Capek medium. The ratio of certain ecological-trophic groups of microorganisms (mineralization-immobilization index) was calculated according to Andreyuk (2001), the index of organic matter transformation was analyzed by the method of Mucha (1980). We calculated the number of colony-forming units (GFU/g) of soil bacteria, taking into account the dilution of soil samples according to standard- accepted methods (Tepper, 2004).

Analysis of soil allelopathic activity

The estimation of soil allelopathic activity was carried out by direct bioassay method (Grodzinskij, 1990). Garden cress (*Lepidium sativum* L.) and winter wheat (*Triticum aestivum* L.) plants were used as test cultures. The soil samples with no influence of exometabolites of the studied plants were considered control ones.

Agrochemicals analysis

The content of macro- and trace elements in the root-soil soil was determined by the method of Rink's (1982) spectrometer with inductively coupled plasma ICAR 6300 DUO (USA). The obtained results were statistically processed using Microsoft Office 2007 software suite.

When presenting the results we have also taken into account the number of precipitations throughout the plant vegetation period (I group – the years characterized by the lower level of precipitations, II group – the years characterized by the higher level of precipitations).

Results and discussion

Microorganisms are considered an important consortium of agrophytocenoses and an indicator of fertility, biological activity and allelopathic state of soil (Yunosheva and Ellanskaya, 2015; Ellanskaya and Gorelov, 2017).

A distinctive feature of the aromatic plants is their production of essential oils. The lavender's soil microbiota forms under influence of its plant's excretions, in particular, essential oils, that have a specific effect on the functional structure of the microbiocenosis and organic matter decomposition (Vokou, 2002; Hassiotis, 2010).

Here we firstly analyzed the abundance and the ratio of basic taxonomic and eco-trophic microorganism groups of lavender rhizosphere microbial cenosis and inter-row soil under soil conditions of Forest- Step zone. The number of micromycetes was shown to decrease during the flowering period especially for the years characterized by the lower level of precipitations when comparing to the control samples (Figure 1).

At the same time, the abundance of these microorganisms was 1.5–2.0 time-lower in case of the rhizosphere samples of the studied plants than for the ones of the inter-row soil.

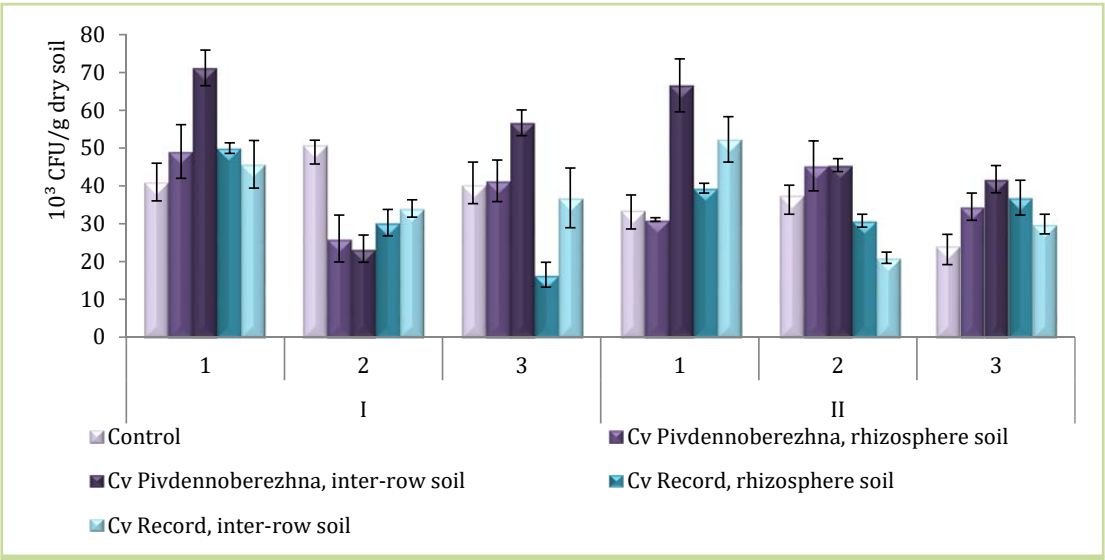

Figure 1 Micromycetes abundance in the soil samples under *Lavandula angustifolia* plants: 1 – growing stage; 2 – flowering stage; 3 – seed formation stage; I group – the years characterized by a lower level of precipitations; II group – the years characterized by higher level of precipitations

Lavender rhizosphere was characterized by a wider spectrum of micromycete species of *Penicillium* and *Trichoderma* genera comparing to the control ones (mostly the mucoral

forms). The noteworthy fact is that *Aspergillus* species were found in the studied soil samples probably due to the species-specific secretions of lavender plants. The quantitative parameters of spore bacteria growth were 1.4–2.4 times higher compared to control ones throughout all the phases of plant development. The abundance of ammonifiers, cellulose-destroying microorganisms and actinomycetes appeared to be increased in the studied soil samples. The development of nitrogen-fixing microorganisms was inhibited by up to 80% throughout the entire period of lavender plant vegetation during the years characterized by lower water availability, whereas such an inhibition did not exceed 16–19% for the years characterized by more precipitations.

Ammonifiers were shown to develop more intensively during the lavender emergence and flowering periods, their abundance declining by the end of vegetation period (Table 1).

The general abundance of nitrogen-fixing bacteria was descending throughout the vegetation period mostly in rhizosphere zone. During the years characterized with the higher water availability the abundance of microorganisms can be stated as relatively high, this parameter decreasing being observed only during the plant flowering period.

Basing the obtained results the processes of mineralization of organic matter in the soil appeared quite balanced. The mineralization-immobilization ratio during active plant vegetation period proved the accumulation of organic compounds, whereas the mineralization processes became more active at the end of the growing season.

The development of microorganisms in the soil samples under lavandin plants was characterized by analogical peculiarities to that of lavender ones (Figure 2). The representatives of *Penicillium*, *Trichoderma*, *Fusarium*, *Gliocladium* genera dominated. Spore-forming bacteria were developing much active during the flowering and seed formation periods. The actinomycete abundance prevailed in lavandin rhizosphere throughout all its vegetation periods. The positive development of nitrogen-fixing microorganisms was observed with only its slight inhibition during the flowering period for the years characterized by low precipitation level.

The results of our study correspond the developmental characteristics of the microbiocenosis of the other representatives of aromatic plant species of Lamiaceae family. The similar trends of the micromycete ratio was reported for the soil samples of *Monarda didima* та *Dracocephalum moldavicum*, their abundance declining in 2–5 times during germination and flowering periods comparing to the control ones. The decreasing of micromycete abundance in the rhizoshpere samples was also observed for the flowering period of *Salvia sclarea* L. and representatives of *Vitex* genera (Yurchak, 1997; Ellanska, 2013). The root and volatile exometabolites of lavender may inhibit this group of microorganisms. The increasing abundance of spore-forming bacteria, actinomycete, ammonifires was reported for microbcenosis of the soil under the *Salvia sclarea*, *Monarda didima* and *Mentha piperita* plants (Yurchak, 2005; Ellanska, 2010).

Consequently, the formation of microbiocenosis depends on several factors of climatic and soil conditions and plant species. The allelopathically active plant substances (essential oils, phenolcarboxylic acids) may become one of the microbe biomass regulators.

Table 1 Functional structure of microbiocenosis and mineralization indexes in the soil samples under *Lavandula angustifolia* Mill. Plants

Variant	Ammonifiers (106 CFU/g dry soil)			Inorganic nitrogen-immobilizers (106 CFU/g dry soil)			Mineralization / immobilization ratio			Organic matter transformation index		
	1*	2	3	1	2	3	1	2	3	1	2	3
Control	2.9 ±0.1	5.0 ±0.3	8.6 ±0.9	6.7 ±0.7	11.0 ±1.6	15.2 ±0.8	2.3	2.2	1.8	4.3	7.3	13.2
	6.0 ±0.1	2.8 ±0.3	9.6 ±1.1	7.9 ±0.8	9.3 ±0.6	4.7 ±0.7	1.3	3.3	0.5	0.7	3.7	28.6
Cultivar Pivdennoberezhna, rhyzosphere	6.0 ±0.7	13.4 ±0.4	4.2 ±0.1	3.5 ±0.6	14.2 ±1.5	12.6 ±1.6	0.6	1.1	3.0	15.8	25.1	5.6
	8.8 ±0.5	5.9 ±0.7	4.3 ±0.8	11.3 ±0.6	6.2 ±0.2	9.6 ±0.7	1.3	1.1	2.2	15.5	11.0	6.2
Cultivar Pivdennoberezhna, inter-row	6.5 ±0.7	7.2 ±0.8	3.4 ±0.2	3.9 ±0.6	11.7 ±0.2	10.7 ±0.6	0.6	1.6	3.1	17.3	11.8	4.6
	12.1 ±0.5	4.4 ±0.3	3.7 ±0.9	9.8 ±1.4	9.3 ±0.4	4.7 ±0.6	0.4	1.6	1.3	54.8	8.6	6.5
Cultivar Record, rhyzosphere	2.9 ±0.4	6.5 ±0.9	4.0 ±0.8	3.4 ±0.4	6.6 ±1.8	8.0 ±0.1	1.2	1.0	2.0	5.3	13.1	6.0
	6.1± 0.3	4.6 ±0.4	3.2 ±0.9	14.7 ±0.6	6.9 ±0.6	3.9 ±0.5	2.4	1.5	1.2	8.7	7.7	5.9
Cultivar Record, inter-row	4.2 ±0,6	6.1 ±0,3	4.9 ±0,5	4.5 ±0,5	8.1 ±1,7	10.9 ±1,3	1.1	1.3	2.2	8.0	10.9	7.2
	5.7 ±0.5	5.6 ±0.6	2.0 ±0.4	7.7 ±0.7	9.6 ±0.1	6.7 ±0.5	1.3	1.7	3.4	10.3	8.9	2.6

Note: * Sampling periods: 1 – growing stage; 2 – flowering stage; 3 – seeds formation stage; numerator – the years characterized by a lower level of precipitations; denominator – years characterized by higher level of precipitations.

The mineral substances are known for their key role in plant functioning and development as well as in the increasing of their resistance to abiotic and biotic environmental factors. The analysis of the content of biogenic elements in the soil samples under lavender and lavandin plants proved the tendency of decreasing of ammoniac nitrogen content starting from the blossoming period and increasing of nitrate-nitrogen concentration at the end of vegetation period. The phosphorus concentrations didn't significantly differ for the studied samples. The beginning and the end of the vegetation periods were characterized by rising of potassium amounts. The maximum magnesium concentration was detected for lavender rhizosphere during the seed formation period. The end of vegetation period was characterized by the 40% increase of magnesium concentration in the studied soil samples while its twice decreasing was shown for the control ones. The declining of iron content (up to 33%) was observed for all the rhizosphere soil samples of the studied plants during the growing season. The obtained results prove the positive effect of the studied plants on the physical-chemical soil state that should be taken into account for their gardening.

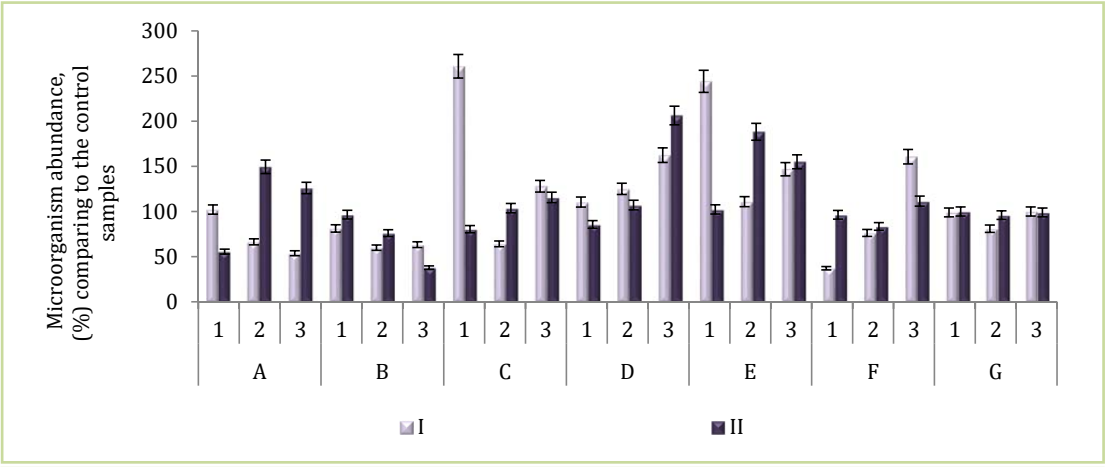

Figure 2 Microbial abundance in the soil samples under *Lavandula hybrida* Rev. plants: 1 – growing stage; 2 – flowering stage; 3 – seed formation stage; A – ammonifiers; B – inorganic nitrogen-immobilizers; C – micromycetes; D – spore-forming bacteria; E – actinomycetes; F – cellulosolytic; G – nitrogen-fixing; I group – the years characterized by the lower level of precipitations; II group – the years characterized by the higher level of precipitations

The toxicity of the lavender rhizosphere was considerably lower under the Forest-Steppe conditions compared to the conditions in the southern regions of Ukraine (Yurchak, 2005). The soil allelopathic activity depended on the water balance, as its effect appeared (basing the analyzed parameters) lower for the years characterized by high precipitation level and higher one for the arid years. The obtained data corresponding to the hypothesis that the level of soil toxicity depends on the intensity of essential oil adsorption, which is influenced by the precipitation level.

Conclusions

Therefore, the exometabolites of lavender and lavandin plants caused the 1.7–1.9 time decreasing of micromycete abundance in the rhizosphere. We also report the significant (in 1.4–2.4 times) increasing of spore-forming bacterium abundance and the improved development of actinomycetes, ammonifiers, cellulosolytic microorganisms. Basing the obtained results the processes of mineralization of organic matter in the soil appeared quite balanced. The allelopathic activity of the soil samples under lavender and lavandin plants was characterized by low toxicity level for the biotests. The soil allelopathic activity was proved to depend on the water availability: its characteristics were lower for the years of higher precipitation level and higher ones for the arid years. The microbiological analysis of the soil samples under the studied plants proved the variable microorganism biodynamics in the rhizosphere and inter-row samples of the aromatic plants as the periods of increasing or decreasing of microbiota abundance can be logically explained by the plant taxonomy, the geographical and climate conditions, the phases of plant development, the intensity of their physiological processes. Microbiologic, agrochemical and allelopathic soil status under *Lavandula angustifolia* Mill. and *Lavandula hybrida* Rev. plant species under the Forest-Steppe zone conditions was found out favourable for the successful introduction of these plants.

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DIFFERENTIATION OF SLOVAK AND TUNISIAN CASTOR GENOTYPES (*RICINUS COMMUNIS* L.) USING SCOT MARKERS

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The characterization of genetic diversity of genotypes is the basic prerequisite for the successful breeding programs of castor like other crops. In the present investigation 40 genotypes of castor were analysed using 10 start codon targeted (SCoT) markers. Ten primers produced 62 DNA fragments with an average of 6.20 bands per primer. From these ten primers, primer SCoT 65, were the most polymorphic, where 7 polymorphic amplification products were detected. The lowest number of amplified polymorphic fragments (3) was detected by primer SCoT 66. From the 62 amplified bands, 48 (85.94%) were polymorphic, with an average of 4.80 polymorphic bands per primer. To determine the level of polymorphism in the analysed group of castor genotypes, polymorphic information content (PIC) was calculated. The polymorphic information content (PIC) value ranged from 0.652 (ScoT 8) to 0.816 (SCoT 23) with an average of 0.738. The dendrogram of genetic relationships among 40 castor genotypes based on SCoT markers was constructed. The hierarchical cluster analysis showed that the castor genotypes were divided into 3 main clusters. Cluster 1 contained one Tunisian castor genotype KJ-2. Cluster 2 contained 2 castor genotypes RM-84 and RM-94. Cluster 3 was divided into subcluster 3A and subcluster 3B. Subcluster 3A contained 2 Tunisian castor genotypes- KJ-3 and KJ-4. Subcluster 3B contained 35 genotypes of castor. Two Tunisian castor genotypes of 3B subcluster (KJ-1 and KJ-5) were genetically the closest. We can assume that they have close genetic background. The present study shows effectiveness of employing SCoT markers in analysis of castor, and would be useful for further studies in population genetics, conservation genetics and genotypes improvement.

Keywords: Dendrogram, Castor, Molecular marker, SCoT analysis, Polymorphism

Introduction

Castor bean (*Ricinus communis* L.) has recently been highly rated as a source of raw material (oil) for biodiesel production, because beyond its high oil content (25–55%), it is a culture of great social appeal in Brazil by intensive use of workmanship in the field and allows for intercropping with other crops as beans, groundnuts or maize (Madail et al., 2007). In addition, castor bean cultivation is encouraged in areas of low water availability and is genetically improved to produce biofuel (Evangelista et al., 2004).

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These types of molecular techniques included random amplified polymorphic DNA (RAPD) (Štefúnová et al., 2015), amplified fragment length polymorphism (AFLP) (Molin et al., 2013), inter-simple sequence repeat (ISSR) (Žiarovská et al., 2013) and simple sequence repeats (SSRs) (Shehata et al., 2009). These marker systems are useful for biodiversity analyses, phylogenetic studies, germplasm management, cultivar identification, and other applications (Luo et al. 2010). Recently, a simple novel DNA marker technique namely start codon targeted (SCoT) polymorphism, was developed by Collard and Mackill (2009). Primers for SCoT marker analysis were designed from the conserved region surrounding the translation initiation codon, ATG (Sawant et al., 1999). Suitability of SCoT markers for the construction of genetic maps, fingerprinting and phylogenetic studies has been proved by many authors. In many crops, such as citrus (Mahjbi et al., 2015) and castor (Kallamadi et al., 2015).

The goals of this study were to examine the effectiveness of SCoT markers for analysis of genetic diversity of castor and to study genetic relationships among 40 castor accessions.

Material and methodology

Plant material and DNA extraction

Ricin lines (20) were obtained from the breeding station Zeainvent Trnava Ltd. (Slovakia) and next 20 ricin lines were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia. Regions of origin of analyzed genotypes of Tunisian ricin: KJ- Ksar jedid, K- Kebili, G- Gabes, M- Mornag, MD- Mednine. Genomic DNA was isolated from the 14 days leaves with GeneJET Plant Genomic DNA Purification Mini Kit according to the manufacturer's instructions. Castor genotypes were grown in a growth chamber on humus soil.

SCoT amplification and statistical analysis

A total of 10 SCoT primers developed by Collard and Mackill (2009) were selected for the present study (Table 1). Each 15-μL amplification reaction consisted of 1.5 μL (100 ng) template DNA, 7.5 μL Master Mix (Genei, Bangalore, India), 1.5 μL 10 pmol primer, and 4.5 μL distilled water. Amplification was performed in a programmed thermocycler (Biometra, Germany) using the following program: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; a final extension at 72 °C for 5 min. Amplified products were separated in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system Grab-It 1D pre Windows.

The SCoT bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The binary data generated were used to estimate the level of polymorphism by dividing the polymorphic bands by the total number of scored bands and to prepare a dendrogram. A dendrogram was constructed based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the help of SPSS professional statistics version 17 software package. For the assessment of the polymorphism between genotypes maize and usability SCoT markers in their differentiation we used polymorphic information content (PIC) (Weber, 1990).

Table 1 List of used 10 SCoT primers (Collard and Mackill, 2009)

SCoT Primers	Primer sequence (5´-3´)
SCoT 6	CAACAATGGCTACCACGC
SCoT 8	CAACAATGGCTACCACGT
SCoT 9	CAACAATGGCTACCAGCA
SCoT 12	ACGACATGGCGACCAACG
SCoT 23	CACCATGGCTACCACCAG
SCoT 66	ACCATGGCTACCAGCGAG
SCoT 65	ACCATGGCTACCACGGCA
SCoT 63	ACCATGGCTACCACGGGC
SCoT 62	ACCATGGCTACCACGGAG
SCoT 61	CAACAATGGCTACCACCG

Results and discussion

In this work, 10 primers were screened for PCR amplification of DNA and SCoT analysis in 40 castor genotypes. Table 1 and Table 2 shows sequences of these primers, total number of amplified fragments from 40 castor genotypes, the number of polymorphic bands and the polymorphic information content for each primer. Ten primers produced 62 DNA fragments (Table 2) with an average of 6.20 bands per primer. From these ten primers, primer SCoT 65, were the most polymorphic, where 7 polymorphic amplification products were detected. The lowest number of amplified polymorphic fragments (3) was detected by primer SCoT 66.

Table 2 The statistical characteristics of the10 SCoT markers used in castor

SCoT Primers	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	PIC
SCoT 6	5	4	80.00	0.729
SCoT 8	4	4	100.00	0.652
SCoT 9	6	4	66.66	0.780
SCoT 12	7	5	71.43	0.715
SCoT 23	7	5	71.43	0.816
SCoT 66	4	3	75.00	0.729
SCoT 65	9	7	77.78	0.651
SCoT 63	8	6	75.00	0.780
SCoT 62	5	4	80.00	0.715
SCoT 61	7	6	85.71	0.815
Average	6.20	4.80	85.94	0.738
Total	62	48	–	–

From the 62 amplified bands, 48 (85.94%) were polymorphic, with an average of 4.80 polymorphic bands per primer. To determine the level of polymorphism in the analysed group of castor genotypes, polymorphic information content (PIC) was calculated (Table 2). The polymorphic information content (PIC) value ranged from 0.652 (ScoT 8) to 0.816 (SCoT 23) with an average of 0.738.

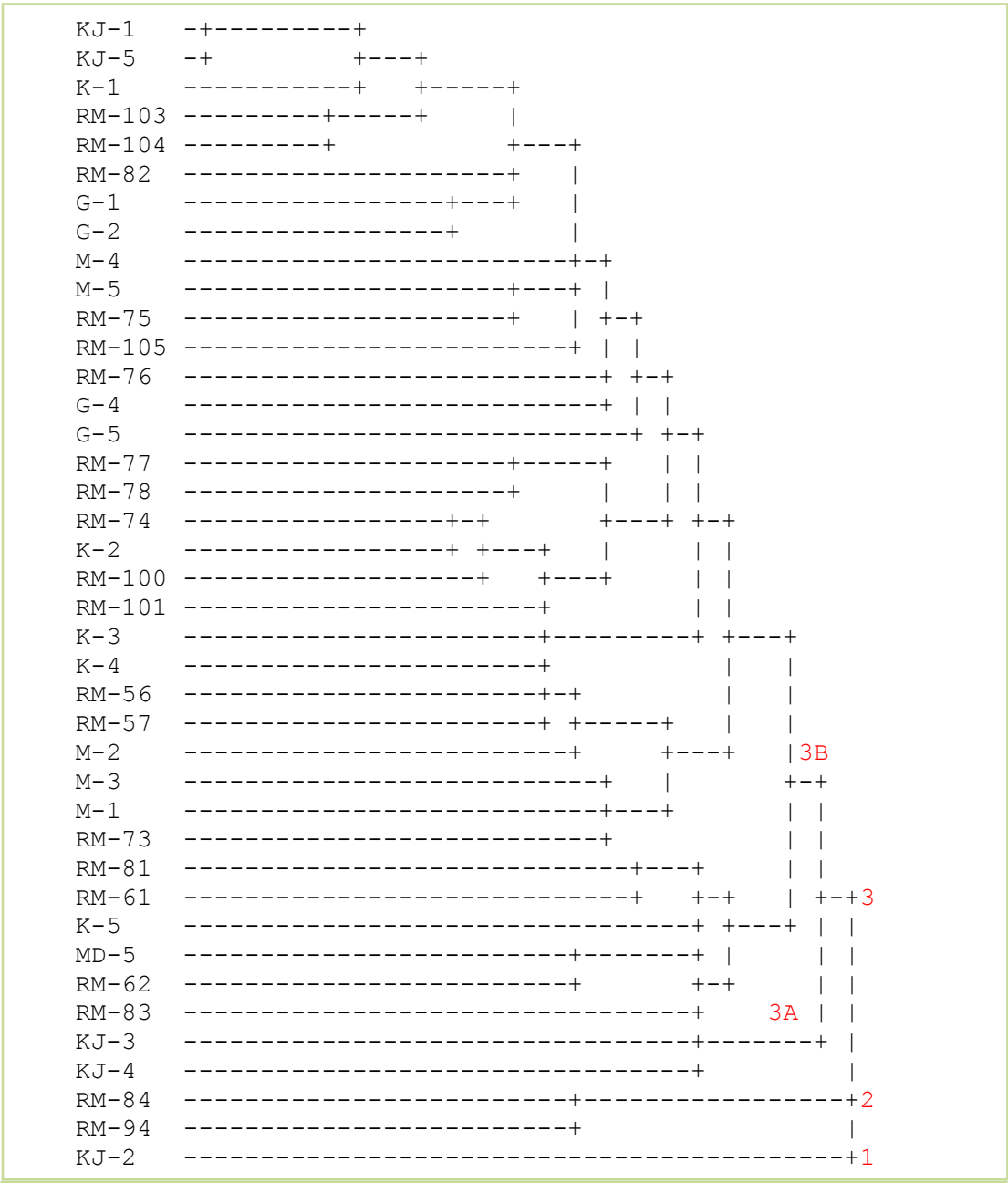

Figure 1 Dendrogram of 40 castor genotypes prepared based on 10 SCoT markers

The dendrogram of genetic relationships among 40 castor genotypes based on SCoT markers was constructed (Figure 1). The hierarchical cluster analysis showed that the castor genotypes were divided into 3 main clusters. Cluster 1 contained one Tunisian castor genotype KJ-2. Cluster 2 contained 2 castor genotypes RM-84 and RM-94. Cluster 3 was divided into subcluster 3A and subcluster 3B. Subcluster 3A contained 2 Tunisian castor genotypes- KJ-3 and KJ-4. Subcluster 3B contained 35 genotypes of castor. Two Tunisian castor genotypes of 3B subcluster (KJ-1 and KJ-5) were genetically the closest. We can assume that they have close genetic background (Figure 1).

CONCLUSION

In summary, SCoT marker analysis was successfully developed to evaluate the genetic relationships among the genus castor accessions originated from various area. The dendrogram of genetic relationships among 40 castor genotypes based on SCoT markers was constructed. The hierarchical cluster analysis showed that the castor genotypes were divided into 3 main clusters. Cluster 1 contained one Tunisian castor genotype KJ-2. Cluster 2 contained 2 castor genotypes RM-84 and RM-94. Cluster 3 was divided into subcluster 3A and subcluster 3B. Subcluster 3A contained 2 Tunisian castor genotypes- KJ-3 and KJ-4. Subcluster 3B contained 35 genotypes of castor. Two Tunisian castor genotypes of 3B subcluster (KJ-1 and KJ-5) were genetically the closest. Polymorphism revealed by SCoT technique was so abundant and could be used for molecular genetics study of the castor accessions, providing high-valued information for the management of germplasm, improvement of the current breeding strategies, and conservation of the genetic resources of castor species.

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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *BUNIAS ORIENTALIS* L. AND *SCORZONERA HISPANICA* L. ETHANOL EXTRACTS

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The current study was aimed to evaluate an accumulation in plant raw material of *Bunias orientalis* L. and *Scorzonera hispanica* L. the total content of phenolic compounds, phenolic acids, flavonoids, antioxidant activity (by DPPH-method) and reducing power of alcoholic extracts. Raw of investigated plants collected from experimental collections of M.M. Gryshko National Botanical Garden of the NAS of Ukraine (Kyiv) and dried for next investigation. Obtained results showed that total content of polyphenols in the above-ground parts of *B. orientalis* extracts was 52.88 mg g^{-1} GAE (gallic acid equivalent), phenolic acids – 11.29 mg g^{-1} CAE (caffeic acid equivalent), flavonoids – 39.91 mg g^{-1} QE (quercetin equivalent), antioxidant activity – 8.94 mg g^{-1} TE (Trolox equivalent) and reducing power of extracts – 184.59 mg g^{-1} TE (Trolox equivalent). Total content of polyphenols in the above-ground parts of *S. hispanica* plants was 60.16 mg g^{-1} GAE, phenolic acids – 20.71 mg g^{-1} CAE, flavonoids – 36.24 mg g^{-1} QE, antioxidant activity – 5.35 mg g^{-1} TE and reducing power of extracts – 125.40 mg g^{-1} TE. Also, total content of polyphenols in the roots of *B. orientalis* extracts was 9.75 mg g^{-1} GAE, phenolic acids – 1.73 mg g^{-1} CAE and reducing power of extracts – 138.70 mg g^{-1} TE. Root extracts of *S. hispanica* had total content of polyphenols of 10.87 mg g^{-1} GAE, phenolic acids – 2.62 mg g^{-1} CAE and reducing power of extracts – 110.96 mg g^{-1} TE. Flavonoids and antioxidant activity in the root extracts of both species weren't determined. The antimicrobial activity of alcoholic extracts of two investigated species was tested against 8 microorganisms by disc diffusion method. It was found that higher activity against microbial strains had alcoholic extracts of *B. orientalis* roots.

Keywords: *Bunias orientalis*, *Scorzonera hispanica*, antioxidant activity, polyphenols, flavonoids, phenolic acids, antimicrobial activity

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Introduction

Plants are potential sources of natural antioxidants with therapeutic properties that can be used in traditional and folk medicine (Chandra et al., 2014; Ivanišova et al., 2017). Representatives of Asteraceae Bercht. & J. Presl and Brassicaceae Burnett also are the good source of antioxidant compounds of different nature (Martinez-Sanchez et al., 2007; Vijaylakshmi et al., 2009; Sikora and Bodziarczyk, 2012). Cruciferous vegetables act as a good source of natural antioxidants due to the high levels of carotenoids, tocopherols and ascorbic acid (Ateya et al., 2016). *Bunias orientalis* L. (Turkish cabbage) is biennial or perennial hemicryptophyte that belongs to Brassicaceae. A recent study of *B. orientalis* connected with a high possibility to root because of powerful taproot leading to massive sprawl (Oliver et al., 2015). The complex of characteristics (biochemical toxicity, allelopathic effect, powerful taproot etc.) explained this plant as invasive (Dietz and Winterhalter, 1996; Dietz et al., 1999; Patamsitė et al., 2013). Also, it was studied nectar production and carbohydrate composition of this species (Denisow et al., 2016). Nevertheless, the accumulation of some biochemical compounds in different organs causes the useful properties of *B. orientalis* (Vinogradova and Kuklina, 2018). In the M.M. Gryshko Botanical Garden of the NAS of Ukraine have been studying these plants as forage and energetic culture and has been conducted selection work. Raw of this species is rich on protein, lipids, ash, ascorbic acid, carotene etc. (Uteush and Lobas, 1996).

Another species from Asteraceae family belongs to the genus of *Scorzonera* L. Some species of this genus have been used as traditional medicines with analgesic, antirheumatic, anthelmintic, stomachic and diuretic effects. These plants can be used for the treatment of the wound, hypertension, infertility, lung oedema, diarrhoea etc. (Sari et al., 2009). As reported Acikara Bahadir et al. (2013a), the plants of *Scorzonera* species used as medicinal and vegetable cultures possess promising antioxidant activity. One of the most effective compounds from antioxidants was found a chlorogenic acid. Extracts from some species of *Scorzonera* showed the activity on the wound healing that can be connected with the combined effect of the constituents (Küpeli et al., 2011). Useful properties of *Scorzonera* species for the food industry because of valuable biochemistry content were described (Mahjoub et al., 2009; Çitoğlu et al., 2010; Bashta et al., 2015). Some studies reported that raw of these plants contains flavonoids (apigenin, luteolin, kaempferol, rutin etc.) and triterpenoids (lupeol, daucosterol etc.) (Küpeli et al., 2011; Acikara et al., 2013; Benabdelaziz et al., 2014). Çetin et al. (2018) reported about seventeen triterpenoids in *S. veratrifolia*. However, the information about *Scorzonera* species is considerably limited in the existing literature (Erden and Kirbağ, 2015).

The aim of this study was to assess an antioxidant and an antimicrobial potential of *Bunias orientalis* L. and *Scorzonera hispanica* L. in the conditions of M.M. Gryshko National Botanical Garden of the NAS of Ukraine.

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 (NBG) in the Kiev city (50° 24' 55" N, 30° 33' 45" E).

Biological material

Observation on plants was conducted in the experimental collection of forage plants (*Bunias orientalis* L.) and vegetables crops (*Scorzonera hispanica* L.) of Cultural Flora Department of NBG. Plant raw material of investigated plants was collected in the stages of spring vegetation. In this study used above-ground part and roots of *B. orientalis* and above-ground part of *S. hispanica*.

Biochemical analysis

The biochemical analysis was done in 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.

Radical scavenging assay

The radical scavenging activity of samples was measured using 2.2-diphenyl-1-picrylhydrazyl (DPPH) (Sanchez-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 $mg.L^{-1}$; R^2 = 0.988) was used as the standard and the results were expressed in mg g^{-1} Trolox equivalents.

Reducing power

Reducing power of extracts was determined by the phosphomolybdenum method of Prieto et al. (1999) with slight modifications. The mixture of 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 $mg.L^{-1}$; R^2 = 0.998) was used as the standard and the results were expressed in mg g^{-1} Trolox equivalents.

Total polyphenol content

Total polyphenol content extracts was 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 $mg.L^{-1}$; R^2 = 0.996) was used as the standard and the results were expressed in mg g^{-1} gallic acid equivalents.

Total flavonoid content

Determination of total flavonoids content was conducted according to 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 aluminium 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^{-1}; R^2 = 0.997) was used as the standard and the results were expressed in μg g^{-1} quercetin equivalents.

Total phenolic acid content

Determination total phenolic acids content of extracts was carried out using 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^{-1}, R^2 = 0.999) was used as a standard and the results were expressed in mg g^{-1} caffeic acid equivalents.

Preparation of plant extracts for determination of antimicrobial activity

Plant raw material was dried, crushed and weighed out to 2 g and soaked in 20 mL of ethanol p.a. (Sigma, Germany) during two weeks at room temperature. After this ethanol plant extracts were filtered through the Whatman No. 1 filter paper. The obtained extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNFN838.1.2KT.45.18, KNF, Germany). For the antimicrobial assays, the crude plant extracts were dissolved in dimethylsulfoxide (Penta, Czech Republic) to 102.4 mg mL^{-1}.

Microbial strains

Eight strains of microorganisms were tested in this study, including *Bacillus cereus* CCM 869, *Candida albicans* CCM 8215, *Candida glabrata* CCM 8270, *Candida tropicalis* CCM 8264, *Clostridium perfingens* CCM 4435T, *Haemophilus influenza* CCM 4456, *Klebsiella pneumoniae* subsp. *pneumoniae* CCM 4415, *Salmonella enterica* subsp. *enterica* CCM 7189. All tested strains collected from the Czech Collection of microorganisms. The bacterial suspensions were cultured in the nutrient broth at 37 °C.

Disk diffusion method of determination of antibacterial activity

Antibacterial activity of ethanol extracts of *B. orientalis* and *S. hispanica* were determined by a disc diffusion method. Briefly, 100 μL of the test bacteria were grown in 10 mL of fresh media until they reached a count approximately 10^5 cells.mL^{-1}. Then 100 μL of the microbial suspension was spread onto Mueller Hinton agar plates. The extracts were tested using 6 mm sterilized filter paper discs. The diameters of the inhibition zones were measured in millimetres. All antimicrobial assays were performed in at least triplicate. Negative control was a filter disc impregnated with 10 μL of distilled water.

Statistical analysis

The statistically treated data are given in table as the arithmetical mean values and their standard errors.

Results and discussion

Phenolic compounds are a large group of the secondary metabolites widespread in plants. These compounds possess different biological activities, but the most important is antioxidant activity (Soobrattee et al., 2005; Podsędek, 2007). Study of Mahboubi et al. (2013) expected that ethanolic or ethyl acetate extracts with higher level of total phenolic content would exhibit higher antioxidant activity. However, for example, Kähkönen et al. (1999) reported that antioxidant activity does not necessarily correlates with high amounts of phenolics.

Our previous data concerning Brassicaceae showed that total antioxidant activity of selected species was 16.94–36.91% in methanol extracts and 26.53–65.66% in water extracts (Vergun and Rakhmetov, 2018). As shown in Tables 1 and 2, total content of polyphenols, phenolic acids and flavonoids was higher in the above-ground part of plants of *B. orientalis* than in roots. The considering of obtained results in details showed that content of polyphenols in above-ground part was 5.4 times more than in roots, content of phenolic acids – 6.5 times respectively. Similar results obtained for *S. hispanica* extracts.

The most widespread and diverse group of the polyphenols are the flavonoids. They have been suggested to play a preventive role in the development of some disease. They are highly effective scavengers of most oxidizing molecules (Baba and Malik, 2015; Hudz et al., 2017). The total content of flavonoids and antioxidant activity by DPPH-method weren't determined in the roots of the investigated plants.

Table 1 The content of phenolic compounds and antioxidant activity in above-ground parts of *Bunias orientalis* L. and *Scorzonera hispanica* L. in the period of spring vegetation

Parameter	*Bunias orientalis*	*Scorzonera hispanica*
Total content of polyphenols (mg g^{-1} GAE)	52.88 ±1.84	60.16 ±1.01
Total content of phenolic acids (mg g^{-1} CAE)	11.29 ±1.00	20.71 ±2.55
Total content of flavonoids (mg g^{-1} QE)	39.91 ±0.43	36.24 ±2.14
Antioxidant activity (mg g^{-1} TE)	8.94 ±0.07	5.35 ±0.12
Reducing power of extract (mg g^{-1} TE)	184.59 ±0.37	125.40 ±1.00

Notes: Means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of three independent experiments (±SD)

As reported Acikara Bahadir et al. (2013b), the chlorogenic acid is one of the major constituent of *Scorzonera* species, which probably responsible for the biological activities of these plants. As described by Erden and Kirbağ (2015), the total phenolic content of soil surface parts of different species of *Scorzonera* was from 14.92 to 40.19 mg g^{-1} GAE and flavonoids – from 30.47 to 110.08 mg g^{-1} QE. Granica et al. (2015) revealed that both subaerial and aerial parts of *S. hispanica* contain caffeoylquinic acid derivatives, which are the major phenolics in this plant. Also, in this study showed that presence of flavonoids was confirmed in aerial part of *S. hispanica*.

Table 2 The content of phenolic compounds and antioxidant activity in the roots of *Bunias orientalis* L. and *Scorzonera hispanica* L. in the period of spring vegetation

Parameter	*Bunias orientalis*	*Scorzonera hispanica*
Total content of polyphenols (mg g^{-1} GAE)	9.75 ±0.54	10.87 ±0.29
Total content of phenolic acids (mg g^{-1} CAE)	1.73 ±0.05	2.62 ±0.26
Total content of flavonoids (mg g^{-1} QE)	ND	ND
Antioxidant activity (mg g^{-1} TE)	ND	ND
Reducing power of extract (mg g^{-1} TE)	138.70 ±1.41	110.96 ±6.88

Notes: Means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of three independent experiments (±SD); ND – non determined.

The interest of natural plants products as antimicrobials is supported by the necessity to reduce the use of conventional antibiotics in food preservation (Ateya et al., 2016). Study of Prasad (2014) showed that different extracts of selected plants of Brassicaceae exhibited significant antimicrobial activity.

Investigation of the antimicrobial activity of root extracts of *B. orientalis* showed in the Figure 1. Our results of antibacterial testing with disc diffusion method demonstrate that *Candida albicans* and *Salmonella enterica* subsp. *enterica* were the most sensitive to *B. orientalis* root extract (5.00 and 4.33 mm respectively). Among tested bacteria, *Bacillus cereus* was the least sensitive (1.00 mm).

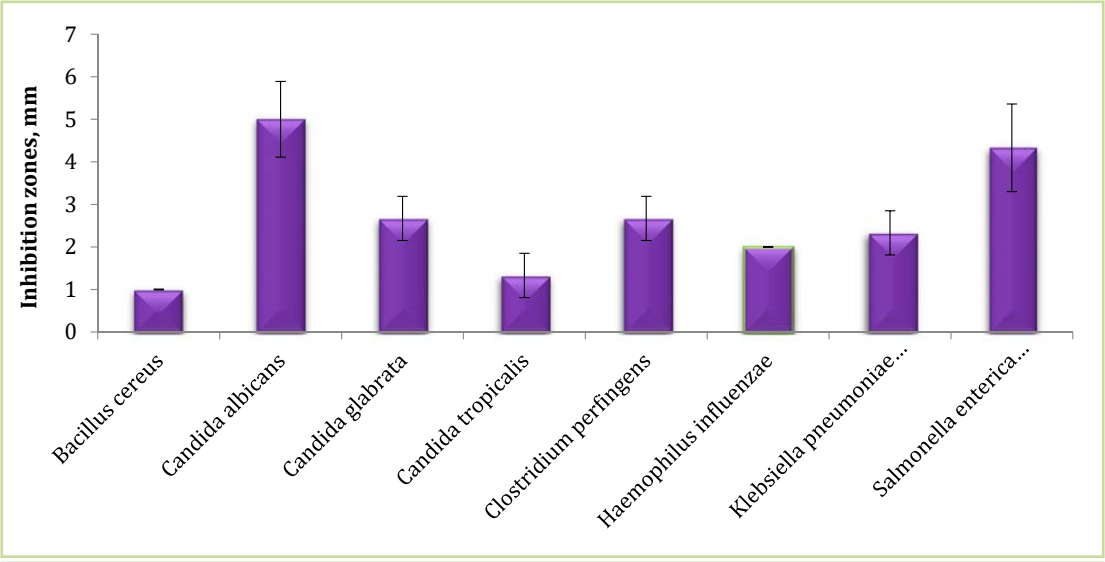

Figure 1 Antimicrobial activity of root plant extracts of *Bunias orientalis* L. (Means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of six independent experiments (±SD))

Our study showed also, that antimicrobial activity of above-ground extracts of *B. orientalis* wasn't determined. Ethanol root extracts weren't effective against all investigated microorganisms.

In the current research plant extracts (above-ground part) of *S. hispanica* showed antimicrobial activity against *Salmonella enterica* only. Inhibition zone in this case was 1.33 ±0.52 mm. Root extracts weren't effective against all microorganisms. Results of Sari et al. (2009) showed that *S. latifolia* and *S. veratrifolia* ethanol extracts were able to inhibit the growth of *Staphylococcus aureus*, *S. epidermidis*, *Shigella flexneri*, *Candida albicans*. Better results were found for petroleum ether fraction. Another study of *S. humilis* L. reported that no antibacterial activity against *Bacillus subtilis* and nor antifungal activity against *Candida albicans* wasn't found (Zidorn et al., 2000; Zidorn et al., 2002). As reported Erden and Kirbağ (2015), three species of genus of *Scorzonera* showed antimicrobial effect on *Escherichia coli* (17–27 mm of inhibition zone) and none of the plant extracts showed any effect against *Klebsiella pneumoniae*. Two species showed the antimicrobial activity against *Staphylococcus aureus* (8 mm of inhibition zone).

Conclusions

Thus, results of current study showed that ethanol extracts of *B. orientalis* and *S. hispanica* demonstrated a higher antioxidant activity of the above-ground parts of plants then of roots (content of polyphenols and phenolic acids). The content of flavonoids was observed in the above-ground parts of plants; however, in roots it wasn't determined. Reducing power was higher in the extracts of above-ground parts. Alcoholic extracts from the roots of *B. orientalis* demonstrated antimicrobial effect against all tested microorganisms. The highest antimicrobial activity was found against *Candida albicans* and *Salmonella enterica* subsp. *enterica*. Root extracts of *S. hispanica* weren't effective against all tested microbial strains but extracts of above-ground part was non-significant effective against *Salmonella enterica*. Authors considered that alcoholic extracts of investigated plants could be beneficial for new microbiological and pharmacological study.

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CYCADOPSIDA: HISTORY OF STUDY, VALUE, PERSPECTIVES OF USE

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САГОВНИКОВІ (CYCADOPSIDA): ІСТОРІЯ ВИВЧЕННЯ, ЗНАЧЕННЯ, ПЕРСПЕКТИВИ ВИКОРИСТАННЯ

Гайдаржи Ольга, Іванніков Роман

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Representatives of *Cycadopsida* – a group of ancient plants, that appeared in the Permian period of the Paleozoic era. During the Jurassic and Triassic periods of the Mesozoic, they were an indispensable component of the plant communities until the appearance and distribution of representatives of *Magnoliophyta*. Today, 307 taxa of cycads are known. Plants of some species are traditionally used by the local population in everyday life, for food or in folk medicine. For various reasons, populations of most species are endangered. Some cycads species are no longer found in nature. All species of *Ceratozamia*, *Chigua*, *Encephalartos* and representatives of *Cycas beddomei* Dyer, *Microcycas calocoma* Miq. A. DC., *Stangeria eriopus* (Kunze) Baill are listed in I appendix CITES list. The remaining species of Cycadaceae are listed in appendix II. Most representatives of *Cycadopsida* are listed in the Red List of International Union for the Conservation of Nature and Natural Resources. Recent studies have shown the presence in valuable herbs of *Cycadopsida* of valuable, and sometimes, unique compounds and phytocomposition that can be used in pharmacy and cosmetology. In the work, the scientific, practical and historical significance of the *Cycadopsida* is formulated, the traditional methods of their use and perspectives of study are indicated. Taking into account the main directions of scientific and educational work carried out in the NBG M.M. Gryshko National Academy of Sciences of Ukraine, with the collection of *Cycadopsida*, provides information on the history of the discovery and study of the representatives of this group of ancient gymnosperms. In the context of the formation of the modern taxonomic hierarchy of the *Cycadopsida* mentioned the most prominent scientists who were engaged in descriptions and study of this group of plants.

Keywords: *Cycadopsida*, *Cycas*, *Zamia*, gymnosperms

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Стійкість екологічних систем зокрема і біосфери в цілому значною мірою залежить від біологічного різноманіття. Зменшення різноманітності біоти – найбільш небезпечна серед змін довкілля, що відбуваються нині, бо це абсолютно незворотній процес. Зникнення в ході філогенезу окремих видів є частиною процесу еволюційного розвитку, проте, їм на заміну мають приходити нові види. Сьогодні, через споживацьке ставлення до природи (пряме чи опосередковане), через потужний вплив людини на природу цей процес критично порушено. Достатнє видове різноманіття, з одного боку, забезпечує гомеостатичний стан біогеоценозів, з іншого, для людини, при раціональному використанні, це невичерпне джерело природних ресурсів.

Саговники – це група унікальних, давніх рослин, які з'явилися вже в пермському періоді палеозойської ери. Вони панували в часи юрського та тріасового періодів мезозою і лише з появою та розповсюдженням представників *Magnoliophyta*, їх чисельність та різноманіття суттєво зменшились. До теперішнього часу описано 307 таксонів саговикових (IUSN, 2018). Сучасні представники саговникових широко розповсюджені в усьому світі (рис. 1), хоча основна частина видів (до 70%) походить з Китаю, В'єтнаму (рис. 2), Австралії, Південної Африки та Мексики (рис. 1). Близько 25% всіх видів класу знаходяться під загрозою зникнення, а 15% – вважаються вразливими внаслідок втрати середовища існування та неконтрольованого використання (Yang et al., 2017).

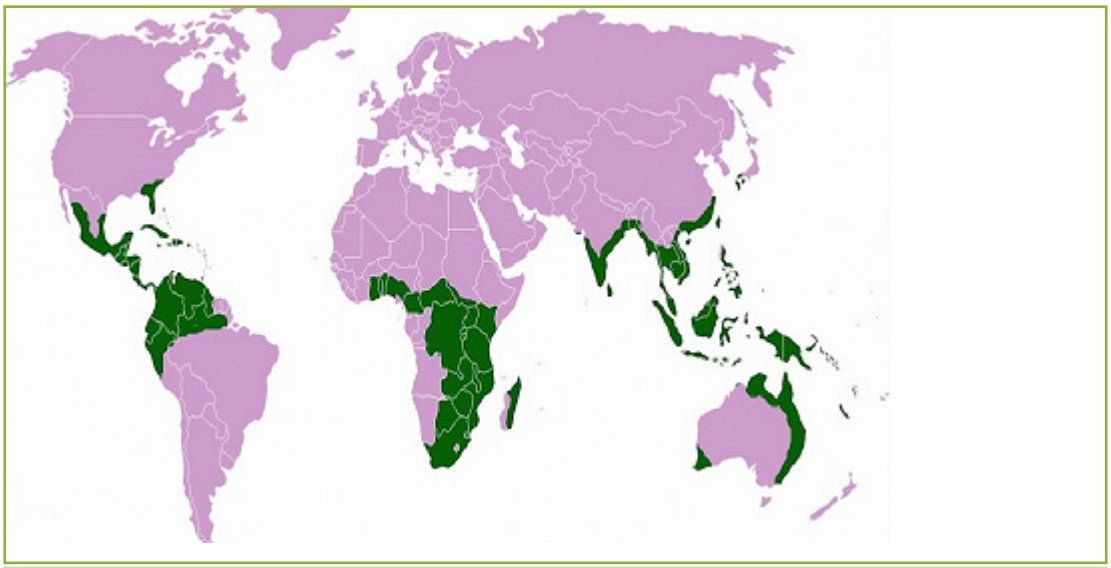

Рисунок 1 Розповсюдження представників Cycadopsida
Figure 1 Distribution of representatives of *Cycadopsida*

Деякі види саговникових відомі лише завдяки декільком екземплярам, що залишились в природі. Всі види родів *Ceratozamia* Brongn., *Chigua* D.W.Stev., *Encephalartos* Lehm. і представники *Cycas beddomei* Dyer, *Microcycas calocoma* Miq. A. DC., *Stangeria eriopus* (Kunze) Baill занесені у Додаток I списку CITEC (CITES, 1973). Інші види родини Cycadaceae Pers. внесені у Додаток II. Більшість представників цієї групи рослин

занесені у Червоний Список Міжнародного Союзу Охорони Природи і Природних ресурсів – МСОП (IUSN, 2018).

Саговникові – рідкісні рослини, в ботанічних садах України представлені зазвичай поодинокими екземплярами та тривіальними видами. Найбільші колекції утримуються в оранжереях Національного ботанічного саду ім. М.М. Гришка, у ботанічному саду ім. акад. О.В. Фоміна Київського Національного університету ім. Тараса Шевченка, ботанічному саду Львівського університету ім. І.Я. Франка та ботанічному саду Харківського Національного університету ім. В. Н. Каразіна.

У Європі не існує місць природного зростання саговникових, хоча до нас дійшли згадки про те, як більше чотирьох тисяч років тому давні етруски знайшли дивну скам'янілість (стовбур одного з представників *Cycadopsida*) (Wieland, 1916). Незвичні на вигляд рештки рослини, імовірно, привернули увагу, і вони встановили скам'янілий стовбур у некрополі Марзаботто (нині муніципалітет в Італії, у регіоні Емілія-Романья). Цей викопний екземпляр є зразком, з якого було описано *Cycadeoidea etrusca* Capellini & Solms. Його рештки і сьогодні знаходяться в Музеї Каппелі в Болоньї.

Рисунок 2 Південний В'єтнам, заповідник. *Cycas* spp. в місцях природного зростання, 2014 р.
Figure 2 South Vietnam, nature reserve. *Cucas* spp. in places of natural habitats, 2014

В місцях природного зростання *Cycadopsida* традиційно широко використовуються місцевим населенням у господарстві (в харчуванні, в народній медицині, для виготовлення різних побутових виробів, у садівництві і т.д.). В Азії їх застосовують для

прикрашання садів, низькорослі представники видів, що походять із Південного Китаю та Північного В'єтнаму популярні як рослини в стилі бонсай. У китайській та японських культурах саговники високо цінують як символи довголіття, про що свідчить широке застосування народної назви "Фен Вей Цао" ("трава-хвіст фенікса") або "Фен Вей Джао" ("пальма-хвіст фенікса"), як натяк на міфічного фенікса, легендарну істоту, що "відроджується з полум'я його похоронного багаття".

Як джерело харчових продуктів, саговникові застосовували з прадавніх часів в Індії, Південно-східній Азії, Японії, Австралії і інших країнах. Згадки про рослини цієї групи зустрічаються з IX століття. В записах двох арабських натуралістів йдеться про те, що здавна рослини роду *Cycas* L. використовували як джерело борошна в Індії.

Європейці вперше познайомилися з представниками цієї групи голонасінних ще у XIII ст., коли венеціанський купець Марко Поло привіз на батьківщину саго – крохмалевмісний продукт, що отримують зі стовбура саговникових. Існують записи про те, що пізніше, у XVIII ст., учасники експедиції Дж. Кука познайомилися зі звичаєм вживанням в їжу аборигенами Австралії насіння саговників (Miquel, 1842, 1862). Пізніше, у XVI столітті, А. Пігафетта, Ф. Кастанеда і Ф. Дрейк знайшли рослини *Cycas* на Молуккських островах (інд. Kepulauan Maluku), де, маючи інформацію про їх їстівні властивості, спробували їх вживати їжу.

З початком масштабного освоєння Нового Світу багато мандрівників-натуралістів робили у своїх щоденниках нотатки та рисунки цих незвичних рослин, повідомляли про відкриття нових видів. Живі саговники регулярно завозили в ботанічні сади та створювали гербарії.

Одні із перших нотаток про саговникові в Новому Світі були зроблені Дж. Леріо. У своїй подорожі до Бразилії, у 1576 році, він спостерігав використання рослин, які корінне населення називало "айрієм". Рослини цієї групи тепер відносять до роду *Zamia* L. Одними з перших натуралістів які зібрали цінний матеріал під час своїх досліджень у тропічній Америці були А. Гумбольдт і Е. Бонплан (1799–1804). Пізніше, Ф. Поепіг в 1830-х роках, повідомляв про збори зразків саговникових в Перу та Болівії.

Серед приватних колекцій відділялось свого часу зібрання англійця Дж. Етіса. Він був ботаніком-аматором, вивчав саговникові, спілкувався з багатьма вченими-ботаніками того часу. Його гербарій було пожертвувано Британському музею в Лондоні. Також в літературі (Osborne et al., 2006) згадується Ф. Гарбарі, лікар з Тренто (північ Італії), колекцію якого успадкував ботанічний сад Флоренції в 1907 році. Л. Каліфано, всесвітньо відомий патологоанатом, а також відомий колекціонер саговникових, заповів свою колекцію Неапольському ботанічному саду (1976 р.).

Особливий інтерес з ботанічної точки зору викликає робота датського дослідника Ф. Лібманна, який у 1840 році в Мексиці збирав матеріали для опису представників Dioon Lindl. та *Ceratozamia* Brongn. (Wendland, 1854). Водночас, В. Карвінскій, у 1841–1843 рр. очолював експедицію до Південної Америки, організовану Петербурзькою Академією наук. Певний час він досліджував флору Мексики. Після його подорожі до ботанічного саду в м. Санкт-Петербург було привезено живий матеріал, на

прикладі якого було описано новий вид, назва якого не збереглась. Впродовж XIX століття надходили данні від мандрівників-натуралістів про нові види, що знаходили в Південній Америці – у цей період було зібрано багато рослинного матеріалу як для ботанічних садів, так і для приватних колекцій.

Перший ботанічний опис рослини, що належить до порядку *Cycadales*, був здійснений ботаніком Х. Драакестейном, який описав саговникові під назвою Todda-panna у 1682 році. Згодом, К. Ліней, у 1753 році, використовуючи ілюстрації цього ботаніка, назвав рід *Cycas* (Linneaus, 1753).

Перші серйозні кроки у систематиці саговникових зробив Ф. Мікаель, голландський ботанік. Його робота (видана у 1842 р.) є прикладом наукової строгості та якості корисної інформації. У 1861 році він опублікував свої дописи за назвою: "Prodromus Systematis Cycadearum", в яких оприлюднив всю відому на той час інформацію з біології та систематики цих рослин. Ще одна вичерпна за інформацією монографія була видана 1868 році де Кандолем (de Candolle) "Cycadaceae. Prodromus systematis naturalis". Не менш важливою, з нашого погляду, є робота директора ботанічного саду у Санкт-Петербурзі Регеля (Regel, 1857) "Cycadearum generum specierumque revisio".

Тільки наприкінці XIX сторіччя роботи німецького ботаніка В. Гофмейстера разом з роботами японських ботаніків С. Хіразе та С. Ікено визначили місце саговникових серед вищих рослин як однієї з найдревніших груп голонасінних. Перші ботанічні описи родів *Cycas* і *Zamia*, як зазначалося, були зроблені К. Лінеєм у 1753 році. Пізніше стало відомо, що під родовою назвою "*Cycas*" К. Лінней описував як мінімум три різних види, які відомі і на сьогодні. Серед його записів частіше за все згадувався *Cycas circinalis* L., проте з восьми екземплярів, які були використані при описах К. Лінеєм лише два належать виду *C. circinalis*. Інші екземпляри, як стало відомо пізніше, належать видам *Cycas revoluta* Thunb., 1784, та *Cycas rumphii* Miq.,1839.

У 1842 році Ф. Мікаель описав австралійський рід *Macrozamia* Miq., в наступному році Д. Ліндлі описав новий північноамериканський рід, який він назвав Dioon. Пізніше (1861, 1868, 1870) цим автором було опубліковано низку досліджень по саговниковим, серед яких була робота під назвою "Nouveaux materiaux pour servir a la connaissance des Cycadees", в якій було оприлюднено всі відомі на той час знання з систематики та біології цих рослин. Рід *Zamia* вперше описав К. Лінней у 1763 (Linneaus, 1763).

Саговникові, що належать до роду *Encephalartos* Lehm., вперше описані Й. Леманном в 1834 році. Назва роду походить від грецьких слів "*en*", що означає "в", "*cephale*", що означає "голова" і "*artos*", що означає "хліб". За свідченнями автора, туземне населення отримувало муку з насіння верхньої частини стробілів рослин цього роду (Lehmann, 1834). За два роки, у 1846 р. А. Бронгніарт описав рід, який він назвав *Ceratozamia* Brongn.(з гр. «xeras» – «ріг»), щоб підкреслити своєрідну будову спорофілів (Brongniart, 1846).

У 1853 році Т. Мур описав невеликий південноафриканський рід, який він назвав *Stangeria* T. Moore на честь доктора Стангера, генерального начальника Натальської провінції, який в 1851 році відіслав з ним рослину в Англію з порту Натал (Moore,

1853). Вперше вид *Stangeria eriopus* (Kunze) Baill. описав О. Кунце в 1892 році. Першочергово цей вид було ідентифіковано як папороть роду *Lomaria* и тільки потім, беручи до уваги роботи Мура по опису стробілів, цей вид систематизували як той, що відноситься до порядку *Cycadales* (Vorster, 1985).

У 1857 році Е. Регель описав новий австралійскій рід, якому він дав назву *Lepidozamia* Lehm., додаючи до назви *Zamia* грецьку назву ("*lepis*" = шкала), тому що листкові рубці на стовбурі цієї рослини нагадують луску змії (Regel, 1857). Пізніше, у 1863 році, Дж. Хукер описав новий австралійський рід *Bowenia* Hook. f., на честь ботаніка і першого губернатора Квінсленда сера Дж. Ф. Боуена. Де Каудолле описав рід *Microcycas* (Miq.) A. DC. – ендем з о. Куба, додавши слово "*micro*" до назви *Cycas*, хоча це не зовсім коректно, оскільки за розмірами екземпляри Microcyas бувають більші, ніж рослини *Cycas* (Caudolle, 1868).

Одним із найвідоміших дослідників саговникових був американський ботанік К. Дж. Чемберліан, чиї праці викликали інтерес завдяки великій кількості наукових даних та новизні його підходу до вивчення предмету. Впродовж 15 років він подорожував Африкою, Америкою та Австралією, спостерігаючи саговникові в їх природному середовищі, що дало поштовх до написання у 1919 році монографічного зведення "The living cycads". Це інформативна та цікава монографія, яка і на сьогодні залишається актуальною стосовно таксономії, морфології та репродуктивної біології саговникових; більшість наведеної інформації отримано автором внаслідок проведення оригінальних досліджень (Chamberlain, 1919).

На початку 1940-х років К. Дж. Чемберлен написав ще одну монографію, присвячену рослинам порядка *Cycadales*, в якій він повідомив про певні неточності попередніх досліджень цієї групи. Приміром, він рішуче критикує практику описання нових видів з єдиного зразка гербарію або живої рослини сумнівного походження, без розуміння мінливості цих рослин протягом їх життєвого циклу. На жаль, ця робота ніколи не була опублікована. Копії рукопису Чемберлена зберігаються в Тропічному саду Ферріхільда в Майямі, Нью-Йоркському ботанічному саду та Ботанічному саду Неаполя (Chamberlain, 1943; Haynes, 2007).

Систематика цієї групи голонасінних впорядковується і донині. Майже половина визнаних на сьогодні день видів саговникових були таксономічно ідентифіковані в останні 20 років. Так, нові таксони саговників були описані і в останні десятиліття, зокрема, Д. Стівенсон і К. Ностог описали новий, ендемічний, розповсюджений на дуже обмеженій території східної частині Південної Америки рід роду *Cycas* – *C. chigua* (Stevenson, 1990).

Як було зазначено, за останніми офіційними даними на сьогодні відомо 307 видів саговникових, які відносяться до 11 родів. Сучасні рослини цієї групи розповсюджені в усіх частинах світу, крім Європи та Антарктиди. Станом на 2018 р. загальноприйняте систематичне зведення саговникових є результатом об'єднаної роботи Д. Хендрікса, К. Хілла, Р. Осборна та Д. Стівенсона, які працювали над ним упродовж останніх двох десятиліть. У коментарях до нього автори зазначають: "Наш перший "Світовий

список" був опублікований в "*Encephalartos*, Journal of the Cycad Society of South Africa" з незначними змінами в додатковому списку в наступному випуску того ж журналу. Кілька оновлень слідували за новими відкриттями та змінами в таксономії та в світогляді, особливо в родах *Cycas*, *Encephalartos*, *Macrozamia* і *Zamia*, що зробили попередні списки застарілими. Вони були представлені на різних міжнародних конференціях присвячених питанням біології саговникових" (Hill et al., 2004).

Широке використання представників *Cycadopsida* у народній медицині завжди наводило на думку проте, що ці рослини містять цінні, фізіологічно активні сполуки. Від початку, хімічні дослідження саговникових зосередилися на виділенні та ідентифікації токсичних складових. Перші дослідження були здійснені в Австралії наприкінці IXX ст. Вони були спрямовані на виділення та вивчення таких речовин як смоли, муцин і щавлева кислота. У 1900 роках голландський уряд в Ост-Індії на численні прохання щодо вивчення шкідливих компонентів насіння *C. circinalis*, що був джерелом харчового крохмалю на островах, започаткував його дослідження. Робота була здійснена в Голландії, було виділено "аморфний безазотистий глікозид", який було названо пакоеін. Пізніше проводились дослідження *C. revoluta* та *Macrozamia spiralis* (Salisb.) Miq., насіння яких було визнано отруйними (Whitelock, 2002).

Друга світова війна перервала дослідження саговникових, але відразу після війни вони були відновлені. У 1949 і 1951 рр., Б. Ланглей, Н. Ріггс, та Б. Лісгое, які працювали з австралійськими видами, виділили глікозид макрозамін. Пізніше він був виділений з *Cycas*, *Bowenia* та *Encephalartos*. У 1956 році Н. Ріггс отримав схожі, але не ідентичні речовини з насіння *C. circinalis* (Whiting, 1963).

Крім глікозидів у саговникових було виділено декілька інших сполук. К. Нішіда і М. Йошиміра виділили на ідентифікували інозитом з насіння *C. revoluta*. У 1949 році, працюючи з *Macrozamia riedlei* (Gaudich.) Ч. Гарднер, Е. Ріггс описали виділення та ідентифікацію сек'юітолу (sequoyitol), простого моноетилового ефіру мезо-інозиту. Одна з причин цікавості до вивчення сек'юітолу в тому, що він міститься у мозку і нервовій тканині.

Пошуки інших токсинів, крім циказіну, у саговникових увінчались успіхом у 1967 році, коли було виділено і описано β-метил-L-аланін (BMAA), доведено, що ця сполука продукується ціанобактеріями, що вступають у симбіотичні відносини з коренями деяких саговникових (Vega et al., 1967). У 1967 амінокислота β-метил-L-аланін (BMAA) була виділена з рослин – представників *Cycas*. Нейротоксична активність цього з'єднання була підтверджена шляхом введення BMAA курчатам і молодим щурам. Згідно досліджень, тільки L-ізомер цієї сполуки проявляє токсичні ефекти (Vega et al., 1968). Встановлено, що BMAA міститься в насінні саговникових. В результаті подальших досліджень було доведено, що це з'єднання відповідає за високий рівень захворюваності в Гуамі у місцевого населення БАС (боковий аміотрофічний склероз). Хоча, відомо, що при традиційному вживанні тієї кількості BMAA, яка надходить в організм не достатньо, щоб викликати неврологічне ушкодження. Проте було висловлено припущення, що BMAA може біологічно посилюватись шляхом біологічного ланцюга. При подальших

дослідженнях BMAA було встановлено, що сильний токсичний вплив на мозок можливий лише при дуже високих концентраціях. Епідеміологічні дані людини та вивчення тварин в природних умовах показують, що кількість BMAA, що надходить до організму з мукою, яку отримують при переробці саговникових, не достатньо, щоб викликати нейродегенеративне захворювання.

Упродовж останніх десятиліть широко вивчались антибактеріальні властивості саговникових, зокрема досліджувалась наявність алкалоїдів, сапоніни і вуглеводи у насінні *Cycas circinalis* (Kalpashree et al., 2013; Moawadetal, 2013). Саркотеста, склеротеста та ендотеста були досліджені на антибактеріальну активність проти трьох патогенних бактерій, зокрема *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*. Порівняння інгібуючих активність екстрактів з антибіотиками ванкоміцин і пеніцилін проти людських патогенних бактерій показало, що екстракт метанолу з насіння *Cycas circinalis* був більш дієвим, ніж у протестованих антибіотиків. Дослідження показали, що листя *Cycas revoluta* також володіють потужною антимікробною і антиоксидантною діями (Bissa et al., 2008). Також було досліджено пептид Ala-Trp-Lys-Leu-Phe-Asp-Asp-Gly-Val, що отримали насіння *Cycas revoluta* за допомогою обернено-фазової рідинної хроматографії. Цей пептид демонструє шкідливий вплив проти людського епідермоїдного раку і ракових клітин товстої кишки (HCT15). Пептид викликає гальмування проліферації ракових клітин і подальшого руйнування нуклеосомної структури, індукції апоптозу шляхом прямого зв'язування ДНК (Mandal, 2012). Апоптоз був виявлений в гістологічних зрізах тканини мозку і кишечника дорослих мишей, яких годували препаратами з насіння *Lepidozamia peroffskyana*. Ця форма клітинної смерті була також знайдена на високих рівнях в мозковій тканині мишей, народжених від дорослих мишей, яких годували препаратом (Gobe, 1994).

Результати досліджень *Cycas beddomei* показали наявність 23 фенольних сполук, 7 флавоноїдів і 5 антоціанідінових з'єднань у всіх частинах рослини. Ці сполуки є біологічно активними в якості антимікробних проти патогенних бактерій людини, що викликають шкірні захворювання, ревматизм і виразки (Alekhya et al., 2013).

Нами було досліджено листя представників *Ceratozamia kuesteriana* Regel, *Ceratozamia robusta* Mig., *Cycas circinalis* L., *Cycas micholitzii* Dyer, *Cycas revoluta* Thunb., *Cycas rumphii* Mig., *Zamia integrifolia* L.f., *Zamia loddigesii* Mig., *Zamia pumila* L.. Роботи проводились з рослинами колекцій Національного ботанічного саду ім. М.М. Гришка НАН України та ботанічного саду ім. акад. О.В. Фоміна КНУ ім. Тараса Шевченка. В результаті проведених досліджень було виявлено, що найбільш токсичними за вмістом аза-глакозидів є *Ceratozamia kuesteriana* Regel, *Zamia loddigesii* Mig. та *Cycas circinalis* L. не менш важливими є результати порівняльного біохімічний аналізу, що виявив незначні відмінності у різностатевих представників окремих видів (Грахов та ін., 2015; Гайдаржи та ін., 2018).

В останні десятиліття спостерігається все більший інтерес до біосистематики, порівняльної морфології, екології, фізіології та репродуктивної біології саговникових,

зокрема, не можна не згадати про професора К. Норстога, лідера у вивченні репродуктивної біології цієї групи (Norstog et al., 1997).

Свідоцтва токсичності саговникових надходять з багатьох джерел. На жаль, клінічні і лабораторні данні щодо захворювань, пов'язаних зі споживанням саговникових в їжу, майже відсутні. Експерименти показали, що глікозиди призводять до пошкодження печінки, але немає доказів, що споживання будь-яких компонентів рослин можуть призвести до паралічу.

Висновки

Таким чином, беручи до уваги літературні дані та отримані результати власних досліджень, можна стверджувати, що представники класу *Cycadales* є цінними джерелами біологічно активних сполук, та потребують подальшого, більш глибоко вивчення. Відкритими є питання щодо статевої ідентифікації представників цієї групи рослин, які ще не досягли репродуктивного віку, розмноження, *ex situ* та *in vitro*, раціонального використання та збереження in situ. Важливим є вивчення рослин в умовах *ex situ* з метою встановлення динаміки росту і розвитку рослин впродовж тривалого часу для формулювання оптимальних агротехнічних заходів з метою успішного утримання колекцій у ботанічних закладах та вивчення перспектив інтродукції. Нині більшість саговникових знаходяться під загрозою вимирання, оскільки вони походять з місць, які активно експлуатуються людиною. Деякі види відомі лише за кількома особинами. Однак, через інтерес до їх хімічного складу, декоративних властивостей, багато саговникових знайшли притулок у державних та приватних садах по всьому світу, проводяться роботи щодо збереження зникаючих видів, триває селекція.

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STUDY OF MORPHOLOGICAL CHARACTERISTICS OF POLLEN GRAINS OF *ARONIA MITSCHURINII* A.K. SKVORTSOV & MAITUL.

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Morphology of pollen grain was described for nine *Aronia mitschurinii* A.K. Skvortsov & Maitul. phenotypes (AM-01 – AM-09) at the laboratory of Department of Tropical and Subtropical plants of M.M. Gryshko National Botanical Garden of NAS of Ukraine (Kyiv) and Institute of Biodiversity Conservation and Biosafety at Slovak University of Agriculture in Nitra using an electron microscope Carl Zeiss LS 15. The measurement of morphometric parameters was carried out on 50 pollen grains from each phenotype using the AxioVision Rel. 4.8.2.0 program. The measurements were made in micrometer (μm). The length of 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 are radial-symmetrical, isopolar, oblong-ellipsoid and 3- and 4-colporate. Texture is sinuous-tuberculate in equatorial zone and finely bumpy in polar zone. The polar axis and equatorial diameter of pollen grains values were varied from 34.16 to 50.14 μm and from 16.10 μm to 25.71 μm, respectively. This study showed that there were differences among the phenotypes in all measured factors. It is known that phenotypic variability is an evolutionarily fixed response of any group of organisms with a constant genotype to changes in environmental conditions and it is adaptive. Therefore, our research suggests that all individuals forming the introduction population of *Aronia mitschurinii* are sufficiently adapted to the conditions of M.M. Gryshko National Botanical Garden of NAS of Ukraine.

Keywords: *Aronia mitschurinii*, phenotype, pollen, SEM, morphology

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Introduction

Research into the morphological characteristics of pollen grains by scanning electron microscopy (SEM) from specific genotypes and cultivars are important and useful for taxonomy, phylogeny, palaeobotany, breeding programmes, e.g., *Pyrus* spp. (Westwood and Challice, 1978; Motyleva et al., 2017), *Vitis vinifera* L. (Ahmedullah, 1983), *Prunus cerasus* L. (Miaja et al., 2000), *Olea europaea* L. (Javady and Arzani, 2001), *Prunus armeniaca* L. (Arzani et al., 2005), *Cornus mas* L. (Mert, C. 2009), *Diospyros* spp. (Grygorieva et al., 2010, 2013, 2017), *Corylus avellana* L. (Nikolaieva et al., 2014), *Ziziphus jujuba* Mill. (Rouhakhsh et al., 2014), *Castanea sativa* Mill. (Grygorieva et al., 2015), *Cydonia oblonga* Mill. (Radović et al., 2016), and *Cichorium intybus* L. (Adamchuk et al., 2017). Size, shape, surface morphology, and ultrastructure of pollen grains are of great importance in the characterization of the pollen grains (Erdtman, 1966; Fogle, 1977; Martens and Fretz, 1980; Brindza and Brovarskyi, 2013; Dyakova, 2014; Brovarskyi et al., 2017; Chlebo and Adamchuk, 2017).

The characteristics of pollen grains are often additional diagnostic features for taxa of various ranks, as shown for *Caragana arborescence* Lam. (Kuklina et al., 2015), *Lupinus polyphyllus* Lindl. (Vinogradova et al., 2012), *Robinia* spp. (Vinogradova et al., 2013), *Solidago* L. complex (Vinogradova, 2012).

The properties of the pollen grains of different phenotypes of *Aronia mitschurinii* have not been evaluated in detail yet.

The knowledge of pollen morphological characteristics can be an adequate method for identification phenotypes of *Aronia mitschurinii*.

Material and methodology

Locating trees and data collection

The pollen of 9 *Aronia mitschurinii* phenotypes (AM-01 – AM-09) from the collection of M.M. Gryshko National Botanical Garden of NAS of Ukraine (NBG) was investigated.

Pollen grains collection

Freshly flowers (not opened) were collected randomly from the different genotypes at the balloon stage (May 2018). Pollen samples released from dry flowers 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 Department of Tropical and Subtropical plants of NBG and Institute of Biodiversity Conservation and Biosafety of Slovak University of Agriculture in Nitra (IBS) 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 to perform 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, size and the dimensions of pollen grains for each *Aronia mitschurinii* genotypes were determined by using a scanning electron micrograph (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 micrometers (μm). The characterization of pollen grains was calculated by taking the following parameters: the polar axis (P – line connecting the proximal and distal pole), the equatorial axis (E – a line perpendicular to the polar axis and located in the equatorial plane).

Statistical analysis

Basic statistical analyses were performed using PAST 2.17; hierarchical cluster analyses of similarity between genotypes were computed on the basis of the Bray-Curtis similarity index; multi-dimensional scaling (MDS) analyses were performed in PRIMER (Clarke and Gorley, 2006). Variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by Stehlíková (1998).

Results and discussion

This study of pollen morphology of tested *Aronia mitschurinii* showed that pollen grains are radially symmetrical, isopolar and according to the localization of apertures are zono-tricolpate. Three compound apertures are according to distribution equidistant. The size, shape of pollen grains and number of apertures are documented on Figure 1.

Pollen grains oblong-ellipsoid, the apertures are long. In the received pictures it is clearly seen that the pollen grains of the presented blackberry breeds are 3 – and 4 – colporate. In polar view pollen grains are circular and triangular with straight or convex sides, in equatorial view – elliptical. Colpi, with uneven edges and pointed or blunted ends, almost converge at the poles. Colpi have granular membrane. Colpi are elliptic, longitudinal and elongated with straight or orbicular-dentate edges. Texture is sinuous-tuberculate in equatorial zone and finely bumpy in polar zone.

The polar axis (P), equatorial diameter (E) and polar axis to equatorial diameter (P/E) ratio of pollen grains of nine *Aronia mitschurinii* phenotypes were measured using scanning electron microscopy (SEM), and the results are displayed in Table 1. An important morphological characteristic is the size of pollen grains. The length of polar axis (P) varied from 34.16 to 50.14 μm and the width of the equatorial axis (E) was in the range of 16.10–25.24 μm. The values of variation coefficient were in the range of 4.20–5.84% for polar axes and in the range of 6.31–9.28% for equatorial axes.

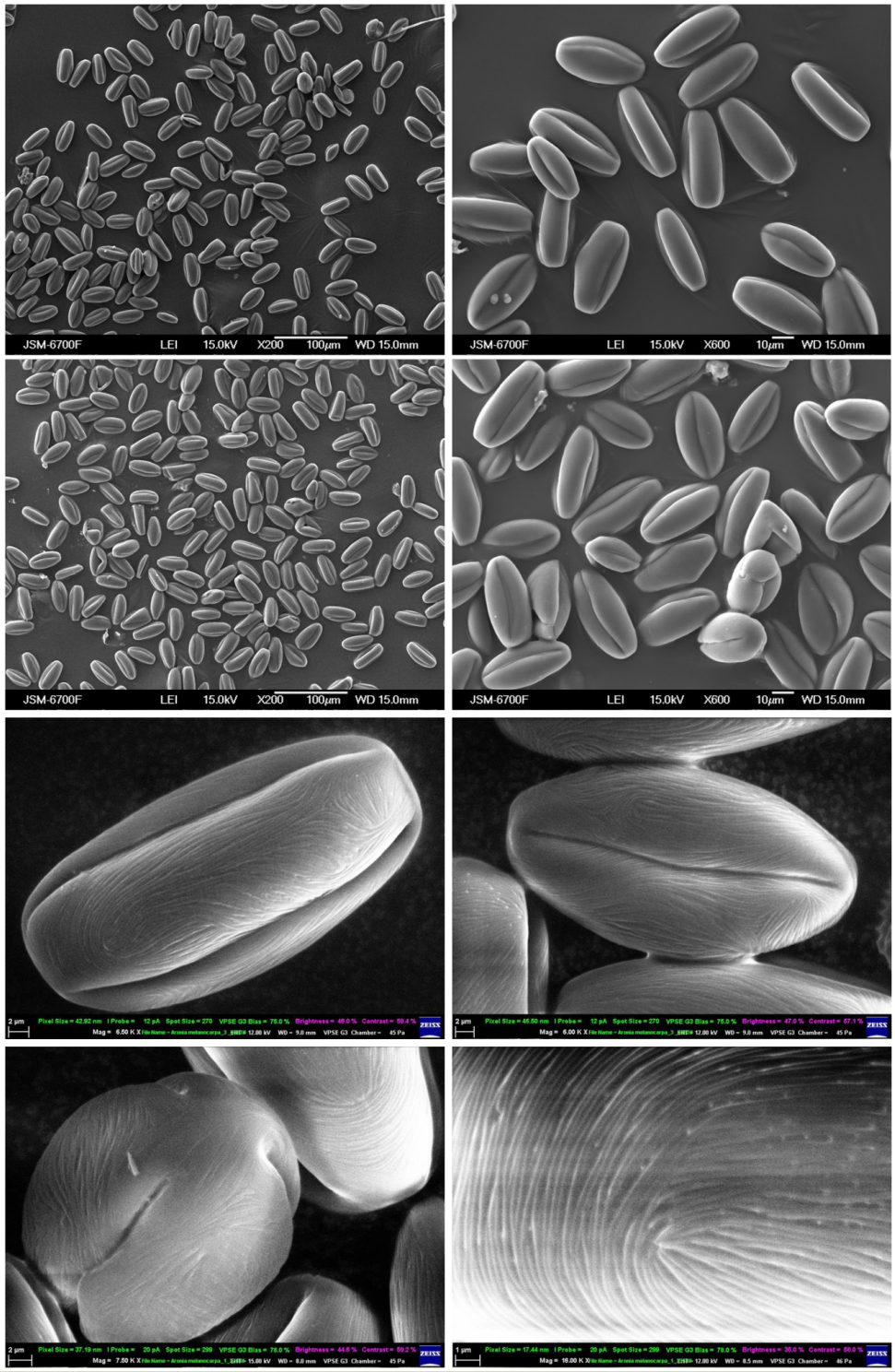

Figure 1 Pollen grains of *Aronia mitschurinii* A.K. Skvortsov & Maitul. species in different positions (Photo: Gurnenko, 2018; Motyleva, 2018)

Table 1 The measured pollen morphological traits of selected phenotypes of *Aronia mitschurinii* A.K.Skvortsov & Maitul.

Phenotypes	min	max	CV %	min	max	CV %	min	max	CV %
	P – Polar axis (µm)			E – Equatorial axis (µm)			SI – shape index (*P*/*E*)		
AM-01	39.78	47.39	5.10	17.47	23.58	8.02	1.68	2.53	10.41
AM-02	40.14	50.10	5.84	18.99	25.24	6.55	1.70	2.45	7.97
AM-03	39.38	49.12	4.65	19.35	23.96	6.71	1.80	2.43	8.82
AM-04	36.53	47.12	5.48	18.49	24.29	6.67	1.63	2.30	8.64
AM-05	38.95	50.14	5.56	19.10	25.00	6.31	1.74	2.32	7.83
AM-06	41.26	49.38	4.55	16.51	22.09	7.40	1.90	2.91	8.54
AM-07	40.69	49.50	4.20	16.94	22.35	7.34	1.93	2.59	7.50
AM-08	34.16	41.77	5.47	16.10	20.29	6.38	1.80	2.48	7.78
AM-09	39.04	47.92	5.15	16.23	24.28	9.28	1.83	2.85	10.99

Note: min – minimum value; max – maximum value; CV – variation coefficient (%).

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 1.63 to 2.91. Mean values of morphological parameters of pollen demonstrated on Figure 2. According to the average values, the phenotype AM-08 has the smallest pollen grains 38.00 × 18.22 µm (Figure 2A). On the dendrogram (Figure 2B), you can see that the phenotype AM-08 is really separated from the other samples. According to literary data, *Aronia mitschurinii* is a facultative apomict, and all her cultivars have only one genotype (Persson-Hovmalm et al., 2004; Vinogradova and Kuklina, 2014).

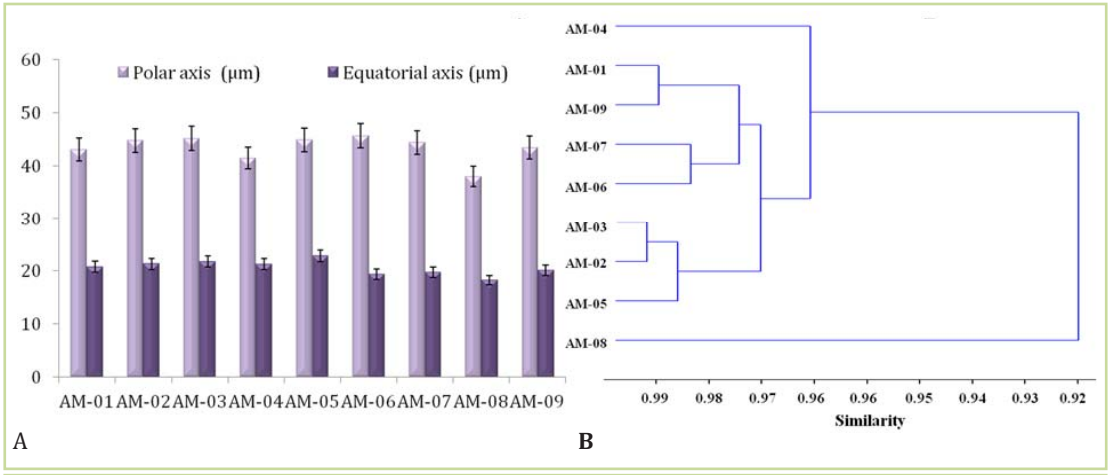

Figure 2A–B The average values and dendrogram of *Aronia mitschurinii* A.K. Skvortsov & Maitul. 9 phenotypes based on morphometric characteristics of pollen Conclusions

However, such a significant difference in the AM-08 phenotype compare with other samples still suggests the possibility of generation some genetic changes in the introduction population. Perhaps they are associated with hybridization or with accumulation of phenetic micromutations, which are not uncommon in botanical gardens, where samples of different geographical origin are cultivated close to each other. In the future, we propose to study the nature of these differences by molecular genetic methods.

Conclusions

Morphology characteristics of pollen grains of any genotypes and cultivars are important for breeding programmes and the studing of germplasm. Thus, the detailed pollen morphological and micro-sculptural characteristics of 9 phenotypes was investigated by using scanning electron microscopy. The analysis of morphological characteristics of pollen showed significant differences among *Aronia mitschurinii* phenotypes concerning the dimensions of pollen grain (length, width, and their ratio). Some of the pollen morphological parameters analysed be used for identification of *Aronia mitschurinii* phenotypes.

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CHARACTERIZATION OF TUNISIAN CASTOR GENOTYPES (*RICINUS COMMUNIS* L.) USING RAPD MARKERS

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The aim of this work was to detect genetic variability among the set of 20 Tunisian castor genotypes using 5 RAPD markers. Amplification of genomic DNA of 20 genotypes, using RAPD analysis, yielded 42 fragments, with an average of 7.00 polymorphic fragments per primer. Number of amplified fragments ranged from 5 to 9, with the size of amplicons ranging from 200 to 1400 bp. The polymorphic information content (PIC) value ranged from 0.662 to 0.855 with an average of 0.780 and diversity index (DI) value ranged from 0.669 to 0.857 with an average of 0.785. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. Knowledge on the genetic diversity of castor can be used for future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production.

Keywords: Castor, Genetic diversity, Molecular markers, RAPD technique

Introduction

Castor (*Ricinus communis* L., $2n = 2x = 20$, Euphorbiaceae), is industrially important non-edible oilseed crop widely cultivated in the arid and semi-arid regions of the world (Govaerts et al., 2000). The seed of castor contain more than 45% oil and this oil is rich (80–90%) in an unusual hydroxyl fatty acid, ricinoleic acid (Jeong and Park, 2009). Castor oil is the only vegetable oil soluble in alcohol, presenting high viscosity, and requiring less heating than others oils during the production of biodiesel (Jeong and Park, 2009). Due to its unique chemical and physical properties, the oil from castor seed is used as raw material for numerous and varied industrial applications, such as: manufacture of polymers, coatings, lubricants for aircrafts, cosmetics, etc., and for the production of biodiesel (Jeong and Park, 2009). With more than 95% of the world's castor production concentrated in limited parts of India, China, and Brazil (Sailaja et al., 2008), and because of the ever increasing world-wide demand of castor for industrial use, there is a pressing need to increase the hectarage and productivity of castor. Castor is a cross pollinated crop and is usually cultivated as a hybrid in India, as hybrids give significantly greater yields than pure lines or varieties (Birchler et al., 2003; Reif et al., 2007).

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Higher magnitude of heterosis and genetically superior hybrids can be obtained by combining diverse parents in hybrid development. Conventional diversity analysis methods, in the field, are time consuming, laborious, resource intensive and drastically affected by environmental factors, therefore, a technique that is rapid and not affected by environment is needed for assessment of genetic diversity and selection of parental lines for use in hybrid development programmes (Santalla et al., 1998). Genetic diversity assessment prior to developing hybrids can aid in better exploitation of heterosis. Assessment of genetic variation using molecular markers appears to be an attractive alternative to the conventional diversity analyses and can also aid in management and conservation of biodiversity. A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner (Santalla et al., 1998).

The aim of this study was to detect genetic variability among the set of 20 Tunisian castor genotypes using 5 RAPD markers.

Material and methodology

Plant material and DNA extraction

Ricin lines (20) were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia. DNA of 20 genotypes of castor was extracted from 10 day old leaves using the Gene JET Plant Genomic DNA Purification Mini Kit.

RAPD amplification

Amplification of RAPD fragments was performed according to Gajeraa et al. (2010) (Table 1) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA). Amplifications were performed in a 25 µl reaction volume containing 5 µl DNA (100 ng), 12.5 µl Master Mix (Genei, Bangalore, India), and 1 µl of 10 pmol of primer. Amplification was performed in a programmed thermocycler (Biometra, Germany) with initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, primer annealing at 38 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Amplified products were separated in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system Grab-It 1D pre Windows.

Table 1 List of RAPD primers

RAPD primers	Primer sequence (5´-3´)	Molecular weight range (bp)
OPA-03	AGTCAGCCAC	330–870
OPA-13	CAGCACCCAC	370–1800
OPB-08	GTCCACACGG	530–1550
OPD-02	GGACCCAACC	280–1850
SIGMA-D-14	TCTCGCTCCA	350–900

Data analysis

The RAPD bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed. For the assessment of the polymorphism between genotypes ricin and usability RAPD markers in their differentiation we used diversity index (DI), the probability of identity (PI) and polymorphic information content (PIC).

Results and discussion

PCR amplifications using 5 RAPD primers produced 42 DNA fragments that could be scored in all genotypes. The selected primers amplified DNA fragments across the 20 genotypes studied, with the number of amplified fragments varying from 5 (OPD-02) to 9 (OPB-08), and the amplicon size varying from 200 to 1400 bp. Of the 42 amplified bands, all 42 were polymorphic, with an average of 7.00 polymorphic bands per primer. The polymorphic information content (PIC) value varied from 0.662 (OPD-02) to 0.855 (OPB-08), with an average of 0.780 and index diversity (DI) value varied from 0.669 (OPD-02) to 0.857 (OPB-08) with an average of 0.785 (Table 2). A dendrogram based on UPGMA analysis separated 20 Tunisian castor genotypes into three groups. First cluster contained 5 genotypes from the region Souassi (S-1 – S-5). Second cluster of 13 ricin genotypes (2) was divided into two main clusters (2a and 2b). Main cluster 2a was divided into subclaster 2aa and 2bb. Subclaster 2aa contained four genotypes from the region Bouthay (BT-1, BT-2, BT-3, BT-5) and one genotypes from the region Matmata (MT-1). Subcluster 2ab contained 3 Tunisian ricin genotypes from the region Ghomrassen (GH-2, GH-4, GH-5). Main cluster 2b contained 2 ricin genotypes from the region Ghomrassen (GH-1 and GH-3) and 3 genotypes from the region Sidi bou ali (BA-2, BA-3, BA-4). Third cluster contained 2 ricin genotypes from the region Sidi bou ali (BA-1 and BA-5). We could not distinguish two genotypes, S-4 and S-5, which can be caused due the same genetic background (Figure 1).

Table 2 The statistical characteristics of the RAPD markers used in castor

Primers	Number of alleles	DI	PIC	PI
OPA-03	8	0,778	0,776	0,016
OPA-13	7	0,805	0,794	0,010
OPB-08	9	0,857	0,855	0,009
OPD-02	5	0,669	0,662	0,071
SIGMA-D-04	6	0,817	0,811	0,019
Average	7,00	0,785	0,780	0,025

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

Gajeraa et al. (2010) used 30 RAPD polymorphic primers for the analysis of 22 castor bean genotypes. RAPD analysis yielded 256 fragments, of which 205 were polymorphic, with an average of 6.83 polymorphic fragments per primer. Genetic diversity of 37 ricin genotypes

grown in China using RAPD markers was studied by Li et al. (2012). Using RAPD markers, together they detected 122 alleles, of which 71 were polymorphic, representing the percentage of polymorphism alleles 58.20%. In the study Machado et al. (2013) used 58 RAPD primers for the analysis of 15 castor bean cultivars. The genetic dissimilarity between cultivars was calculated by Jaccard's index, using the unweighted pair-group method with arithmetic mean (UPGMA). Pecina-Quintero et al. (2013) study the diversity and genetic relationships among accessions of *R. communis* from the state of Chiapas, México using AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) markers. Tomar Rukam et al. (2014) investigated the fingerprinting and phenotyping of 25 castor genotypes available in Gujarat and other States of India using RAPD and ISSR markers. One hundred thirty decamer RAPD primers from Operon series (OPA to OPZ – five from each series) were screened with the DNA of the 2 castor genotypes. Some researchers have considered RAPD markers to represent segments of DNA with noncoding regions and to be selectively neutral (Landergott et al., 2001), and some studies have shown that RAPD markers are distributed throughout the genome and may be associated with functionally important loci (Penner, 1996).

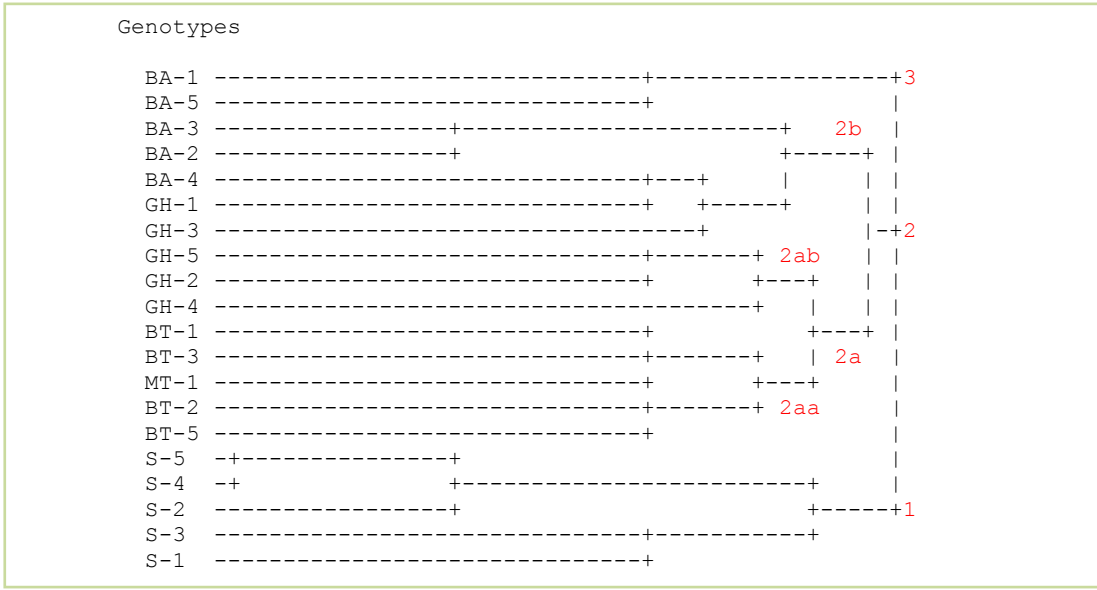

Figure 1;Dendrogram of 20 Tunisian castor genotypes prepared based on 5 RAPD markers. Regions of origin of analyzed genotypes of Tunisian ricin: S – Souassi; BT – Bouthay; GH – Ghomrassen; BA – Sidi bou ali; MT – Matmata

Conclusions

The analysis showed that the RAPD markers are very effective molecular markers for the assessment of the genetic diversity in castor bean. A dendrogram based on UPGMA analysis separated 20 Tunisian castor genotypes into three groups. Using 5 RAPD markers only two castor bean genotypes have not been distinguished. Our analysis proved utilization of RAPD markers for differentiation of used set of castor genotypes. For better discrimination of the analyzed ricin genotypes, it is necessary to use a higher number of RAPD markers.

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ALLELOPATHIC AND BIOCHEMICAL CHARACTERISTICS OF THE ROOT ENVIRONMENT OF *ASIMINA TRILOBA* (L.) DUNAL

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The paper is devoted to the study of the allelopathic and biochemical characteristics of the root environment of *Asimina triloba* (L.) Dunal (pawpaw) introduced from North America to M.M. Gryshko National Botanical Garden of NAS of Ukraine. The plants were divided into the following age groups: a) young plants (2–4 years old), b) plants of the middle age (5–7 years old), c) the old plants (14–16 years old) and d) the oldest plants (over 22 years old). Allelopathic and biochemical analyses were conducted in dynamics on phases of plant development during flowering, fruitage and the end of the growing season. Rhizosphere soil samples were collected at 0–20 cm layer. The fallow soil was used as a control. The presence of allelochemicals in root environment of *A. triloba* by modified Neubauer and Schneider method was established. As a result, the inhibition of the growth processes and accumulation of dry matter in the roots and shoots of acceptor plants with an increase in the age of pawpaw was observed. Biochemical state of the root environment was assessed by redox potential (*Eh*) values. The redox status varied from weakly to highly reducing soil conditions during the growing season. The lowest soil *Eh* level for the oldest plants was determined. The predominance of reduction processes in the rhizosphere soil of *A. triloba* indicates the accumulation of mobile organic compounds, which can function as allelochemicals. The content of phenolic compounds in the rhizosphere soil of *A. triloba* was 1.3–3.0 times higher than control. The concentration of phenolic allelochemicals increased with the age of plants, and also at the end of the growing season. Thus, *A. triloba* forms a powerful allelopathic regime of the root environment, which is due to the accumulation of free organic compounds, mainly phenolic nature.

Keywords: *Asimina triloba*, root environment, allelochemicals, phenolic compounds, redox potential

Introduction

Recently, in Ukraine, great attention is paid to the introduction of new and non-traditional plants into culture both for preserving biodiversity and for obtaining stable yields of high-

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quality production as a natural source of bioactive agents. *Asimina triloba* (L.) Dunal (pawpaw) is a promising new ornamental and fruit crop for dissemination on Ukraine territory, including botanical gardens, arboretums, farms, etc. Pawpaw is rich in various bioactive compounds, due to which it possesses valuable nutritional, antioxidant, insecticidal, medicinal, including anti-tumoral, properties, as well as high adaptive potential to adverse environmental factors (Cuendet et al., 2008; Farag, 2009; Pande and Akoh, 2010; Sedlacek et al., 2010; Ferreira et al., 2011; Brannan et al., 2015; Ortutu et al., 2015; Koul, 2016; Levon and Klymenko, 2016; Mangal et al., 2016; Avula et al., 2018; Nam et al., 2018a).

A. triloba belongs to the mainly tropical and subtropical family *Annona*ceae Juss. *A. triloba* is a native North American species. Pawpaw is widespread in the eastern United States, ranging from New York, and southern Michigan on the north, south to northern Florida, and west to eastern Texas, Nebraska, and Kansas; it is also present in Ontario, Canada (Hormaza, 2014). *A. triloba* is widely cultivated in Korea for its different parts, which contain inhibitors of cancer cells and antioxidant compounds (Nam et al., 2017; Nam et al., 2018a, b). The prerequisites for the successful introduction of new species are not only their adaptability and bioecological characteristics, but also to a large extent understanding the risks associated with allelopathic effects both in relation to other species and in monoculture (Zaimenko et al., 2017). One of the negative consequences of introduction may be the aggressive invasion of new species into natural areas. Therefore, the study of the allelopathic potential of new and non-traditional plants is actual and necessary both from a scientific and a practical standpoint. The allelopathic interactions of invasive shrub *Lonicera maackii* in comparison with native species *A. triloba* were investigated (McEwan et al., 2010). The morphometric parameters of introduced pawpaw seedlings in combination with various groups of ornamental species commonly used in Romania were studied (Szilagyi and Marian, 2011).

In view of the above mentioned, the purpose of the work was to analyse the allelopathic and biochemical characteristics of the root environment of *Asimina triloba* as a new fruit crop for Ukrainian horticulture.

Material and methodology

Plant material and soil source

The object of research was the root environment of *Asimina triloba* from orchard plots of M.M. Gryshko National Botanical Garden of Ukraine National Academy of Sciences. Plants were divided into the following age groups: a) young plants (2–4 years old), b) plants of the middle age (5–7 years old), c) the old plants (14–16 years old) and d) the oldest plants (over 22 years old). Rhizosphere soil samples were collected at 0–20 cm layer. The fallow soil was used as a control. The soil is dark grey podzolized.

Allelopathic and biochemical analyzes were conducted in dynamics on phases of plant development during flowering (I), fruitage (II) and the end of the growing season (III).

Allelopathic activity

Allelopathic activity of the soil was studied by modified Neubauer and Schneider method (Black, 1993). Winter wheat (*Triticum aestivum* L., Poliska 90 cultivar) was used as the test plant.

Biochemical analyses

The redox potential (*Eh*) was measured in soil suspension modelling soil solution at the soil to distilled water ratio as 1 : 1 by potentiometric technique (Labuda and Vetchinnikov, 2011; Fiedler et al., 2007). Phenolic compounds were extracted from the soil by desorption method using an ion exchanger KU-2-8 (H^+) (Pavliuchenko et al., 2014).

Data analysis

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

Results and discussion

The presence of allelochemicals in the root environment of *A. triloba* was established. As a result, the inhibition of the growth processes and accumulation of dry matter in the roots and shoots of acceptor plants (*Triticum aestivum*) with an increase in the age of pawpaw was observed. The allelopathic activity of the root environment was the largest at the end of the growing season (Figure 1, 2). It should be noted that the rhizosphere soil of young pawpaw plants caused an insignificant allelopathic effect on test plants throughout the growing season.

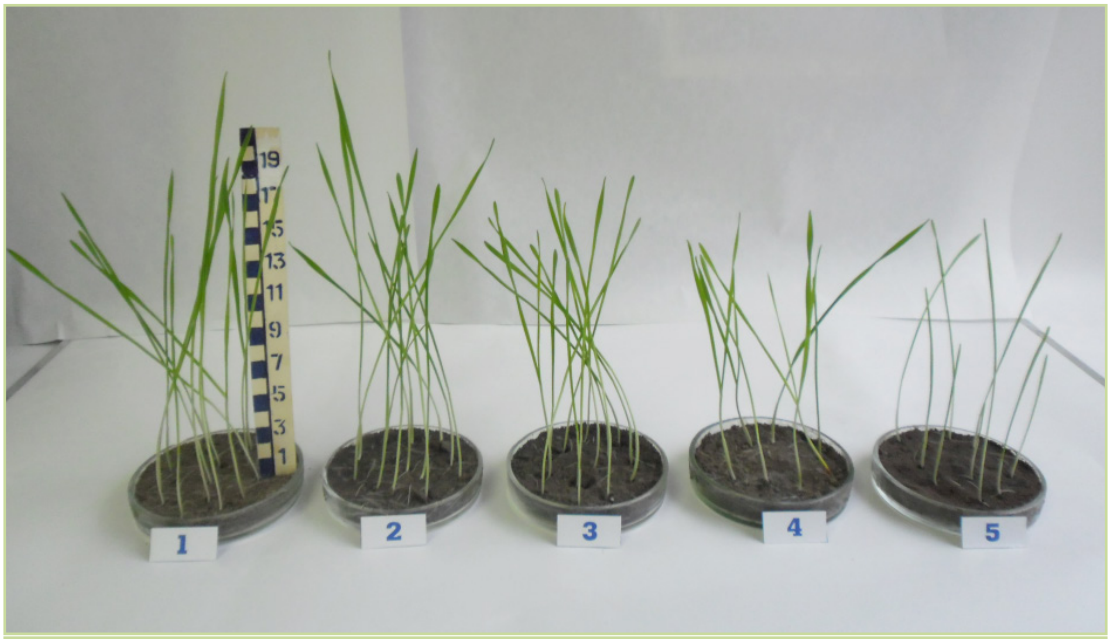

Figure 1 Allelopathic activity of root environment of *Asimina triloba* (test plant – *Triticum aestivum*): 1 – control; 2 – young plants; 3 – plants of the middle age; 4 – the old plants; 5 – the oldest plants

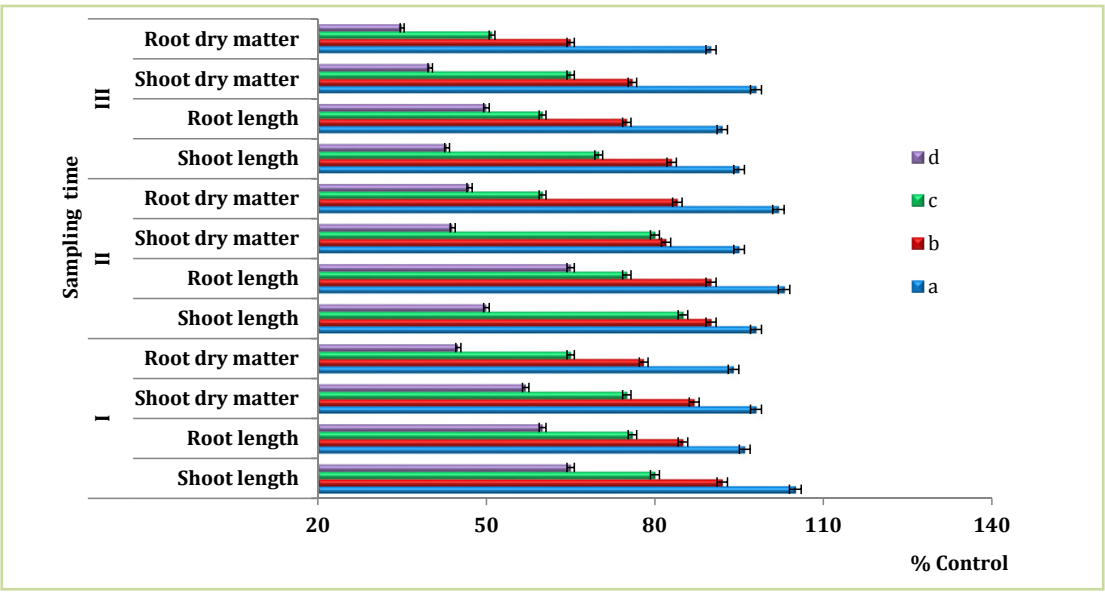

Figure 2 Allelopathic activity of root environment of *Asimina triloba* (test plant – *Triticum aestivum*),% control: a – young plants; b – plants of the middle age; c – the old plants; d – the oldest plants

Biochemical state of the root environment was assessed by redox potential (*Eh*) values. Redox potential is a measure of the ratio of oxidized to reduced forms in a solution (Tokarz and Urban, 2015). Oxidation–reduction (redox) reactions in soils are mainly controlled by microbial activity, the presence of oxygen and carbon supplies (Fiedler et al., 2007; Tokarz and Urban, 2015). *Eh* varies depending on many factors, such as temperature, humidity, aeration, the content of organic matter, soil horizon (Husson, 2013). Plants can significantly influence *Eh* in the soil environment through root exudates (Husson, 2013). The redox potential is used as an indicator of the oxygenation status and the content of biogenic forms and toxins in the soil environment (Tokarz and Urban, 2015). Therefore, *Eh* fluctuations are important for the detection of phytotoxic allelochemicals in the root environment.

Weakly and moderately reducing conditions prevailed in the root environment of young plants, the middle age and the old plants of *A. triloba*. The lowest soil *Eh* values for the oldest plants were established. In this case, the redox status varied from moderately to highly reducing soil conditions during the growing season. The predominance of reduction processes (*Eh* < 400 mV) in the rhizosphere soil of *A. triloba* indicates the accumulation of mobile organic compounds, which can function as allelochemicals.

Phenolic compounds are the most important and common plant allelochemicals in the ecosystems, as well as precursors of humic substances in soils (Macias et al., 2004; Li et al., 2010). The pawpaw different tissues are rich natural source of phenolic acids and flavonoids such as gallic acid, epigallocatechin, catechin, chlorogenic acid, caffeic acid, ellagic acid, epicatechin, epigallocatechin gallate, p-coumaric acid, gallocatechin gallate, ferulic acid, epicatechin gallate, rutin, catechin gallate, naringin,and quercetin (Pande and Akoh, 2010;

Brannan et al., 2015; Ortutu et al., 2015; Levon and Klymenko, 2016; Nam et al., 2017). These phenolic compounds may be released to the root environment from *A. triloba* different parts by means of root exudation, leaching and decay of plant residues in soil. Therefore, the next stage of our research was to determine the content of phenolic compounds in the root environment of pawpaw. The content of phenolic allelochemicals in the rhizosphere soil of *A. triloba* was 1.3–3.0 times higher than control (Figure 3). The concentration of phenolic compounds increased with the age of plants, and also at the end of the growing season.

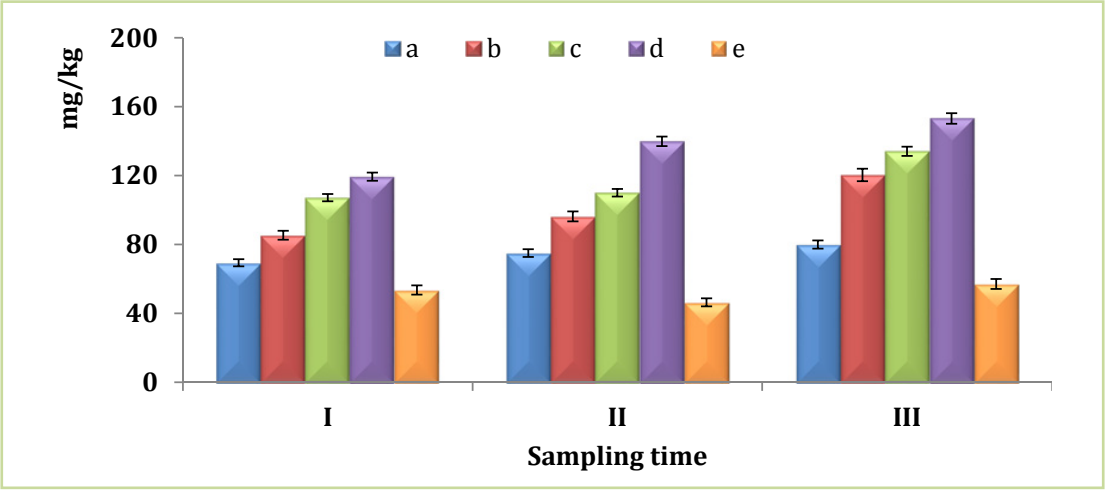

Figure 3 Phenolic compounds content in soil under *Asimina triloba*, mg.kg^{-1}: a – young plants; b – plants of the middle age; c – the old plants; d – the oldest plants; e – control

Conclusions

Thus, *Asimina triloba* forms a powerful allelopathic regime of the root environment, which is due to the accumulation of free organic compounds, mainly phenolic nature. Long-term cultivation of *A. triloba* enhances the intensity of soil reduction processes and its allelopathic effect on the root environment, which leads to an increase in phytotoxicity.

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A COMPARATIVE STUDY OF EFFECT OF VARIOUS *SANSEVIERIA* THUNB. LEAF EXTRACTS ON THE LIPID PEROXIDATION IN THE EQUINE ERYTHROCYTE SUSPENSION

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In this study, we continued our investigations concerning the interaction of leaf extracts obtained from various *Sansevieria* species with the equine erythrocytes. The purpose of the present study was to compare the *in vitro* effect of buffer extracts obtained from leaves of various species from *Sansevieria* genus against lipid peroxidation in equine erythrocytes. The succulent leaves of living *Sansevieria* plants were sampled for the study. The results of the study showed that the leaves of *S. francisii* and *S. forskaliana* led to a non-significantly decrease of 2-thiobarbituric acid reactive substances (TBARS) concentration in erythrocytes. However, *S. hyacinthoides* and *S. cylindrica* had a significant increase of TBARS level in the extract-treated erythrocytes (by 29.7 and 21%, p <0.05, respectively). The outcome of this study suggests that *Sansevieria* species has a promising antioxidant and prooxidant potential. Further studies involving bioassay-guided identification of the main compounds in plants is necessary to affirm and maximize the possible use of the plant as a therapeutic remedy for prevention of lipid peroxidation in erythrocytes. The antioxidative and prooxidative mechanism of various *Sansevieria* species in equine erythrocyte suspension will be further studied in detail. The obtained information may be useful in the clinical usage of plants in medicine and veterinary.

Keywords: *Sansevieria* Thunb., extracts, 2-thiobarbituric acid reactive substances (TBARS), equine erythrocytes

Introduction

Recently, tropical and subtropical plants containing antioxidants have become an area of scientific research because they have greater health benefits with various pharmacological activities. *Sansevieria* Thunb., a genus with diverse ethnobotanical uses in its geographical distribution range, has occupied an important place among plant genera applied for treatment of a broad spectrum of diseases and disorders (Khalumba et al., 2005; Staples and Herbst, 2005;

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Takawira-Nyenya et al., 2014). The use of these plants in folk medicinal remedies for treating various health problems has been reported. These plants have been tested in the treatment of hemorrhoids, pain, smallpox, chicken-pox, and measles, venereal diseases, malnutrition, paralysis, epilepsy, convulsions, and spasm, pulmonary troubles, and a vermifuge, as well as a remedy for parasitic infections. In studies carried out in Nakuru and Maragua districts of Kenya by Khalumba et al. (2005), they identified five use categories of *Sansevieria* plants, namely medicine (33% of the reports), fibers (24%), soil conservation (22%), fodder (18%), and other uses (14%) for four species, *Sansevieria ehrenbergii* Schweinf. ex Baker,*S. parva* N.E.Br., *S. raffillii* N.E. Br., and *S. suffruticosa* N.E. Br. Chhabra et al. (1987) mentioned the use of *S. bagamoyensis* N.E.Br. for the treatment of convulsive fever in Tanzania. Watt and Breyer-Brandwijk (1962) listed the use of *S. hyacinthoides* (L.) Druce in the treatment of a toothache and earache and the use of the rhizome decoction of *S. kirkii* as a purgative both reported from East Africa. Yet, Kiringe (2006) reported on the use of *S. volkensii* Gürke for the treatment of sexually transmitted diseases such as gonorrhea. In Kenya, Owuor and Kisangau (2006) included the use of *S. parva* leaf sap for treatment of snake bite wounds and *S. kirkii* Baker extracts for treatment of snake bite wounds. The ethanol and water extracts of *S. trifasciata* Prain leaves showed a dose-dependent and significant increase in pain threshold and possess mild analgesic properties (Anbu et al., 2009). This seems to provide a rationale for the use of this plant in fever and inflammatory disorders. In Africa, the plant is used as a protective charm against evil or bewitchment (Stafford et al., 2008). The use of the plant in folk medicine for the treatment of different ailments such as ear-ache, ulcer, jaundice, pharyngitis, skin itches, urinary diseases, analgesic and antipyretic is well known (Ighodaro et al., 2017). Moreover, the aqueous root extract of *S. liberica* Gerome and Labroy is used in African folklore medicine for ailments including chronic pain, inflammatory conditions, and convulsive disorders (Amida et al., 2007). The root part of *S. liberica* is used in ethnomedicine in the treatment of fever, headache and cold, as well as analgesic, antibiotic and anti-inflammatory (Watt and Breyer-Brandwijk, 1962). Preparations of the *S. liberica* are commonly used across Nigeria for the treatment of inflammatory conditions (Akindele et al., 2015). Nevertheless, in spite of these data, Takawira-Nyenya et al. (2014) reported that the documentation of ethnobotanical uses of genus *Sansevieria* is incomplete.

Hence, it has been demonstrated that *Sansevieria* spp. plant biomaterial is a superior biosorbent for trimethylamine (TMA) removal. Both living and non-living *Sansevieria* spp. can be effectively used for removal of trimethylamine (TMA). Boraphech et al. (2016) have studied cleanup of trimethylamine (fishy odor) from the contaminated air by various species of *Sansevieria* spp. and their leaf materials. The results showed that living *S. kirkii* plant was the most effective while *S. masoniana* was the least effective in TMA removal. Two major pathways were involved in stomata opening and epicuticular wax on the leaf surface. In the presence of TMA, the stomata opening in *Sansevieria* spp. was induced, which enhanced TMA removal under light conditions (Boraphech et al., 2016). Boraphech and Thiravetyan (2015) have investigated the possible mechanisms of trimethylamine (TMA) degradation and mineralization by highly efficient C3 and crassulacean acid metabolism (CAM) plants. From screening 23 plant species, *Pterocarpus indicus* (C3) and *S. trifasciata* (CAM metabolism) were the most effective in polar gaseous trimethylamine uptake, reaching up to 90% uptake of initial

TMA (100 ppm) within 8 h, and could remove TMA at cycles 1–4 without affecting photosystem II (PSII) photochemistry. Up to 55 and 45% of TMA was taken up by *S. trifasciata* stomata and leaf epicuticular wax, respectively. During cycles 1–4, interestingly, *S. trifasciata* changed its stomata apertures, which was directly induced by gaseous TMA and light treatments. Fatty acids, particularly tetradecanoic (C14) acid and octadecanoic (C18) acid, were found to be the main cuticular wax components in both plants and were associated with TMA removal ability. Moreover, the plants could degrade TMA via multiple metabolic pathways associated with carbon/nitrogen interactions (Boraphech and Thiravetyan, 2015).

In our previous study (Buyun et al., 2016; Tkachenko et al., 2017), we have evaluated the antibacterial capacity of ten species of *Sansevieria* genus against *Staphylococcus aureus* and *Escherichia coli* in order to validate scientifically the inhibitory activity for microbial growth attributed by their popular use and to propose new sources of antimicrobial agents (Buyun et al., 2016; Tkachenko et al., 2017). Although antimicrobial activities of extracts obtained from leaves of various species of *Sansevieria* genus were investigated (Buyun et al., 2016, 2017; Tkachenko et al., 2016, 2017), no previous works have demonstrated their protective effects against lipid peroxidation. Therefore, the aim of this study was to evaluate the *in vitro* effect of buffer extracts obtained from the leaves of various species from *Sansevieria* genus against lipid peroxidation in equine erythrocytes. Erythrocytes are the most common type of blood cells and are the vertebrate body's principal means of delivering oxygen from the lungs or gills to body tissues via the blood (Pandey, Rizvi, 2010). Red blood cell along with its membrane has always been an important medium for the study due to the important role it plays in varied physiological and metabolic processes (Jha et al., 2009; Karabulut et al., 2009; Pandey et al., 2009).

Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity (Webster and Toothill, 1987) and red cell damages by free radicals would probably be associated with hemolysis (Szweda-Lewandowska et al., 2003).

The erythrocytes of mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure (Pagano and Faggio, 2015). The erythrocyte could be isolated and handled easily so that they could provide a good model for many assays (Alagawany et al., 2016; Farag and Alagawany, 2018). Additionally, the high concentration of polyunsaturated fatty acids in RBCs membrane, the high oxygen tension, and redox active hemoglobin molecules [the source of reactive oxygen species in erythrocyte] make them a good biological lipid membrane model especially for screening the oxidative stress conditions induced by various substances (Farag and Alagawany, 2018).

Our present study is in agreement with results obtained in our previous study including assessment of antioxidant activity of extracts obtained from leaves of selected species from *Sansevieria* species (Tkachenko et al., 2017). When erythrocytes were incubated with leaf extracts of various species from *Sansevieria* genus, the aldehydic derivatives level was significantly reduced by 13.6% (p <0.05) for *S. forskaliana* extract. Moreover, all extracts (except *S. francisii* extract) reduced the formation of intracellular aldehydic derivatives of oxidatively modified proteins (OMP) in the extracts-treated erythrocytes, but these results were non-significant. Treatment by extracts of various *Sansevieria* species reduced the concentration of

ketonic derivatives of OMP when compared to untreated erythrocytes. The most potent effect was demonstrated by the *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* compared to control samples (phosphate buffer) (16.1, 14.7, 13.4, 12.9, 12.9, 12.7, 12.1%, respectively). However, there were no significant changes in other extracts. The experimental evidence obtained in our previous study indicated that various species of *Sansevieria* genus are a rich source of compounds that manifest antioxidant activity and can effectively protect erythrocytes against oxidative-induced damage. Thus, *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* may be a valuable source of natural antioxidants that may potentially be recommended for applications in medicine and veterinary practice. According to the above-mentioned antioxidant mechanisms, extracts of various species from *Sansevieria* genus may inhibit the formation of protein carbonyl by scavenging free radicals formed *in vitro*. According to many supporting documents, it can be assumed that secondary plant metabolites, i.e. polyphenolic compounds in extracts of various species from *Sansevieria* genus extract may contribute to the antioxidant activity (Tkachenko et al., 2017).

The main purpose of our present study was to evaluate the level of 2-thiobarbituric acid reactive substances (TBARS) as biomarkers of lipid peroxidation in equine erythrocyte suspension induced by treatment of leaf extracts obtained from various species of *Sansevieria* genus.

Material and methodology

Collection of plant material

The leaves of *Sansevieria* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Specifically, the leaves of *Sansevieria* francisii Chahin, *S. caulescens* N.E.Br., *S. suffruticosa* N.E.Br., *S. roxburghiana* Schult. & Schult.f., *S. metallica* Gérôme & Labroy, *S. gracilis* N.E.Br., *S. hyacinthoides* (L.) Druce, *S. cylindrica* Bojer ex Hook., *S. canaliculata* Carrière, *S. aethiopica* Thunb., *S. kirkii* Baker, *S. trifasciata* Prain, *S. forskaliana* (Schult. & Schult.f.) Hepper & J.R.I.Wood, *S. fischeri* (Baker) Marais, *S. dooneri* N.E.Br., *S. intermedia* N.E.Br., *S. parva* N.E.Br. were sampled for the study. Various databases available for searching collections of living plants, e.g. *World Checklist of Selected Plant Families* (WCSP, 2018), International Plant Names Index, The Plant List, have been used for the taxonomic identity of plants screened.

Preparation of plant extracts

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

Horses

Eighteen healthy adult horses from central Pomeranian region in Poland (village Strzelinko, N 54º 30’ 48.0” E 16º 57’ 44.9”), aged 8.9 ±1.3 years old, including 6 Hucul pony,

5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were taken simultaneously in all horses from the jugular vein in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Whole blood was stored in sterile tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min at 4 °C using a refrigerated centrifuge to remove plasma. The separated erythrocytes were washed three times in 4 mM phosphate buffer saline (PBS), pH 7.4. After centrifugation, the supernatant and the buffy coat were carefully removed with each wash. Washed erythrocytes were finally re-suspended to the desired hematocrit level in 4 mM PBS. The erythrocytes were stored at 4 °C and used within 2 h of sample preparation. A volume of 0.1 mL of the various extracts was added to 1.9 mL of clean equine erythrocyte suspension. For positive control, PBS was used. After incubation the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocytes aliquots were used in the study.

Quantitative estimation of lipid peroxidation by determination of the 2-thiobarbituric acid reactive substances (TBARS)

The most important product of lipid peroxidation reacting with thiobarbituric acid (TBA) is malondialdehyde (MDA) (Lykkesfeldt, 2007). Therefore, the lipid peroxidation was determined by quantifying the concentration of TBARS by Kamyshnikov (2004) for determining the malonic dialdehyde (MDA) concentration. Briefly, 0.1 mL of erythrocyte suspension was added to 1 mL of 20% of trichloroacetic acid (TCA) and 1 mL of 0.8% of 2-thiobarbituric acid (TBA). The mixture was heated in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The concentration of MDA (μmol per mL) was calculated using $1.56\ 10^5\ mM^{-1}\ cm^{-1}$ as the extinction coefficient.

Statistical analysis

The mean ± the standard error of the mean (S.E.M.) values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). In order to find significant differences (significance level, p <0.05) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

Figure 1 illustrates the level of 2-thiobarbituric acid reactive substances (TBARS) in equine erythrocyte suspension induced by treatment of leaf extracts obtained from various species of *Sansevieria* genus as compared with treatment by phosphate buffer (control).

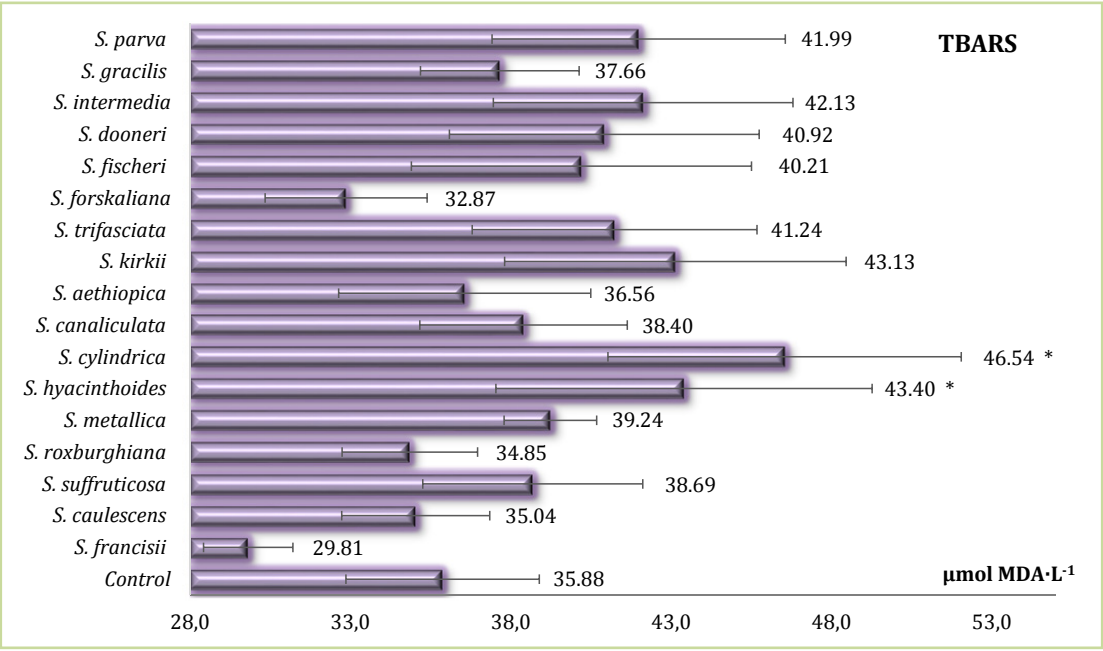

Figure 1 The level of 2-thiobarbituric acid reactive substances (TBARS) in equine erythrocyte suspension induced by treatment of leaf extracts obtained from various species of *Sansevieria* genus as compared with treatment by phosphate buffer (control). The data were presented as The mean ± the standard error of the mean (S.E.M.) and analyzed using one-way analysis on ranks (ANOVA) using Kruskal-Wallis test by ranks
* *P* value <0.05 was considered as significant (*n* = 18)

According to the results from the protective effect of extracts obtained from leaves of selected *Sansevieria* species, the incubation time of 1 h was chosen for assessing the concentration of TBARS in erythrocyte suspension. At 1 h of incubation, the TBARS concentration of untreated erythrocytes was 35.88 ±3.02 µmol/L. The results indicated that extracts of *S. francisii* and *S. forskaliana* led to a decrease of TBARS concentration in erythrocytes (by 16.9 and 8.4%, $p > 0.05$, respectively). These changes were statistically non-significant ($p > 0.05$). When erythrocytes were incubated with *S. aethiopica*, *S. caulescens*, *S. roxburghiana*, *S. gracilis*, the TBARS level was similar to that of the untreated erythrocytes. In the meantime, the treatment of *S. canaliculata*, *S. suffruticosa*, *S. metallica*, *S. fischeri*, *S. dooneri*, *S. trifasciata*, *S. parva*, *S. intermedia*, and *S. kirkii* non-significantly increase the formation of intracellular TBARS in the extract-treated erythrocytes by approximately 7–20%, respectively. However, *S. hyacinthoides* and *S. cylindrica* had a significant increase of TBARS level in the extract-treated erythrocytes (by 29.7 and 21%, $p < 0.05$, respectively) (Figure 1).

In this study, *S. hyacinthoides* and *S. cylindrica* caused a significant increase of TBARS level in the extract-treated erythrocytes (Figure 1). Despite the medicinal relevance of plants, our studies have suggested that these plants are potentially pro-oxidant in dose studied (50 mg per mL). The chemical compounds responsible for the toxic effects of plants are probably produced as part of the plant's defence mechanism against pest and herbivores or to gain an advantage over competing for plants (Ighodaro et al., 2017). According to the results obtained, we addressed the hypothesis that by-products in the extracts of various *Sansevieria* species can be responsible for their prooxidant activity. Phytochemical screening of the plants has shown the presence of carbohydrates, saponins, glycosides, flavonoids, steroids in the leaves (Mimaki et al., 1996, 1997). The interest in possible health benefits of flavonoids has increased owing to their potent antioxidant and free radical scavenging activities observed *in vitro*. Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented and their prooxidant properties have been actually described *in vivo* (Procházková et al., 2011). Studies evidently indicate that natural antioxidants, including polyphenols, flavonoids, anthocyanins, and carotenoids, can act as pro-oxidants, which produce reactive oxygen species and cause oxidative stress (Eghbaliferiz and Iranshahi, 2016). Due to their prooxidant properties, they are able to cause oxidative damage by reacting with various biomolecules, such as lipids, proteins, and DNA (Procházková et al., 2011). The prooxidant activity is typically catalyzed by metals, particularly transition metals such as Fe and Cu, present in biological systems (Eghbaliferiz and Iranshahi, 2016).

In our previous study we have evaluated the lipid peroxidation biomarkers and total antioxidant capacity in the muscle tissue of rainbow trout (*Oncorhynchus mykiss* Walbaum) under incubation with extracts derived from the leaves of various *Sansevieria* species, aimed at the further improving methods for preventing and treating fish diseases by increasing the natural resistance of fish organism using antibacterial and antioxidant agents in aquaculture (Maryniuk et al., 2017). The most potent antioxidant effect was demonstrated for the extracts of *S. caulescens*, *S. suffruticosa*, *S. hyacinthoides*, *S. canaliculata*, *S. aethiopica*, *S. gracilis*, and *S. parva* as compared to phosphate buffer control (46.6, 66.8, 77.3, 49.8, 71.1, 63.4, 39.4%, respectively). The results showed that extracts of *S. hyacinthoides* and *S. aethiopica* efficiently increased the total antioxidant capacity in rainbow trout muscle tissue (Maryniuk et al., 2017). Among plant extracts screened for *in vitro* antioxidant properties in rainbow trout muscle tissue, the strongest toxicity responses were exhibited by *S. cylindrica*, *S. canaliculata*, *S. trifasciata*, *S. metallica* extracts (Maryniuk et al., 2017).

On the other hand, many studies clearly demonstrate that various plants of *Sansevieria* genus are effective agents in the treatment and prevention of many diseases and disorders. For instance, the results obtained in a study of Adeyemi et al. (2009) suggest that the aqueous root extract of *S. liberica* possesses antidiarrhoeal property due to inhibition of gastrointestinal propulsion and fluid secretion, possibly mediated through inhibition of the nitric oxide pathway. This justifies the use of the plant extract for the treatment of diarrhea.

Moreover, Akindele et al. (2015) have evaluated the anticancer activity of root extracts of *S. liberica* using a combination of *in vitro* and *in vivo* models. Sulforhodamine B (SRB) *in vitro* cytotoxicity assay, Sarcoma-180 (S-180) ascites and solid tumor, and L1210 lymphoid leukemia

in vivo models were used in their study. The hydroethanolic extract of the root of *S. liberica* (SL-A002) was significantly active (IC_{50} ≤30 μg.mL^{-1}) against HeLa cancer cell line (Mothana et al., 2009), the aqueous extract (SL-A003) against HCT-116, and the dichloromethane : methanol (DCM : MeOH; 70 : 30) extract (SL-A004) against THP-1 and A549 human cancer cell lines. Only SL-A002 showed significant activity in the Sarcoma-180 ascites model with peak tumor growth inhibition of 89.36% produced at the dose of 120 mg.kg^{-1} relative to 97.96% for standard drug 5-FU at 20 mg.kg^{-1}. The hydroethanolic extract of *S. liberica* was subsequently found to be active in the Sarcoma-180 solid tumor model eliciting 47.40% tumor growth inhibition at the dose of 100 mg.kg^{-1} compared to 50.18% for standard drug 5-FU at the dose of 20 mg.kg^{-1}. This extract was also found to be significantly active in the L1210 lymphoid leukemia model (Akindele et al., 2015).

Amida et al. (2007) have investigated the acute and subchronic toxicity patterns of the *S. liberica*. Acute toxicity tests were carried out in mice, and the median lethal dose was estimated. Subchronic (52 days) studies were conducted in rats with oral daily doses of 80, 400 and 2000 mg.kg^{-1}. Parameters observed for at the end of chronic tests included changes in body and vital organ weights, mortality, hematological, biochemical, hepatic and male reproductive effects. *S. liberica* did not produce any visible toxicities or mortality with oral doses up to 20 g.kg^{-1} within 14 days of single treatment, but i.p. administration caused mortalities with LD50 of 668.3 ±47.6 mg.kg^{-1}. In the chronic tests, neither mortality nor visible signs of lethality were seen in rats. No significant change in the weight of the kidney, liver, heart, and spleen, but at 400 mg.kg^{-1}, a significant reduction in weight of the lungs was recorded. Significant increases in the weight of testes, sperm count, and motility were produced. There were no changes in the sperm head and tail abnormalities, but significant increases in the percentage of normal sperm cells. Biochemical parameters like the aminotransferases were not affected, but a significant increase in alkaline phosphatase and uric acid levels, at 2 g.kg^{-1}, was detected. Significant increase and decrease in red blood cells and white blood cells were recorded, respectively, but no changes in levels of platelet volume and hemoglobin level. Results of Amida et al. (2007) indicated that the aqueous root extract of *S. liberica* shows that it is relatively safe when given orally, and there is a pointer toward possible usefulness to boost red blood cells and increase sperm quality, but findings indicate potential to affect hepatic cells at high doses, when administered chronically (Amida et al., 2007).

The treatment with the plant extracts of the rhizomes of *S. liberica* protects the liver against carbon tetrachloride-induced hepatotoxicity in Wistar albino rats (Ikewuchi et al., 2011). On gas chromatographic analysis of the extract, twenty nine known flavonoids were detected, consisting mainly of 31.94% apigenin, 20.66% quercetin, 11.28% kaempferol, 5.99% naringenin, 5.83% (-)-epicatechin, 3.69% biochanin, 3.58% (+)-catechin, 2.72% daidzein, 2.20% ellagic acid, 2.04% butein. The extract showed very significant hepatoprotection against carbon tetrachloride-induced hepatotoxicity in the rats, by reducing plasma total bilirubin and protein, plasma alkaline phosphatase, alanine and aspartate transaminases levels. The reduction towards the normal value, of the levels of these plasma indices of liver integrity and function, is an indication of the ability of the extract to protect normal structural and functional integrity of the poisoned liver, and also to protect against subsequent carbon

tetrachloride hepatotoxicity, enabling regeneration process. A fact that was confirmed by histopathological studies on liver sections: that revealed that the treated animals had normal hepatic cells. This hepatoprotective activity may have been produced via inhibiting lipid peroxidation by exerting a membrane-stabilizing action or inhibiting cytochrome P_{450} aromatase (Ikewuchi et al., 2011).

The antioxidant and antiproliferative activities of *S. roxburghiana* methanol extract and its fractions have been explored by Maheshwari et al. (2017). Anti-proliferative effect of the extract and fractions were evaluated in HCT-116, HeLa, MCF-7, HepG2, and A-549 cancer cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sulforhodamine B (SRB) assay methods. High-performance liquid chromatography (HPLC) and high-performance thin layer chromatography (HPTLC) fingerprint profiling were carried out for extract and different fractions. Significant antioxidant and anti-proliferate activity were detected in ethyl acetate fraction. Ethyl acetate fraction showed prominent scavenging activity in 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and nitric oxide antioxidant assays with a concentration yielding 50% inhibition (IC_{50}). Cytotoxicity of ethyl acetate fraction of *S. roxburghiana* was the highest among other fractions against HCT-116, HeLa, and MCF-7cancer cell lines with IC_{50} values, by MTT and by SRB assay. The presence of gallic acid in the ethyl acetate fraction of *S. roxburghiana* rhizomes was confirmed by HPLC and HPTLC analysis. Results of Maheshwari et al. (2017) suggested that the ethyl acetate fraction of *S. roxburghiana* exhibited effective antioxidant and antiproliferative activities. The phenolic compounds identified in ethyl acetate fraction could be responsible for the activities (Maheshwari et al. 2017).

In a study by Bhattacharjee et al. (2017), the therapeutic potential of protocatechuic acid isolated from the *S. roxburghiana* rhizomes against DC employing a rodent model of type 2 diabetes (T2D) was examined. T2D was induced by high-fat diet + a low-single dose of streptozotocin (35 $mg.kg^{-1}$, i.p.). T2D rats exhibited significantly (p <0.01) high fasting blood glucose level. Alteration in serum lipid profile (p <0.01) and increased levels of lactate dehydrogenase (p <0.01) and creatine kinase (p <0.01) in the sera of T2D rats revealed the occurrence of hyperlipidemia and diabetic pathophysiology. A significantly (p <0.01) high levels of serum C-reactive protein and pro-inflammatory mediators revealed the establishment of inflammatory occurrence in T2D rats. Besides, significantly high levels of troponins in the sera revealed the establishment of cardiac dysfunctions in T2D rats. However, protocatechuic acid (50 and 100 $mg.kg^{-1}$, p.o.) treatment could significantly reverse the changes in serum biochemical parameters related to cardiac dysfunctions. Molecular mechanism studies demonstrated impairment of signalling cascade, IRS1/PI3K/Akt/AMPK/p 38/GLUT4, in glucose metabolism in the skeletal muscle of T2D rats. Significant (p <0.01) activation of polyol pathway, enhanced production of AGEs, oxidative stress and up-regulation of inflammatory signalling cascades (PKC/NF-κB/PARP) were observed in the myocardial tissue of T2D rats. However, protocatechuic acid (50 and 100 $mg.kg^{-1}$, p.o.) treatment could significantly (p <0.05–0.01) stimulate glucose metabolism in skeletal muscle, regulated glycemic and lipid status, reduced the secretion of pro-inflammatory cytokines, and restored the myocardial physiology in T2D rats near to normalcy. Histological assessments were also in agreement

with the above findings. In silico molecular docking study again supported the interactions of protocatechuic acid with different signalling molecules, PI3K, IRS, Akt, AMPK PKC, NF-κB, and PARP, involved in glucose utilization and inflammatory pathophysiology. In silico ADME study predicted that protocatechuic acid would support the drug-likeness character and to be a new therapeutic agent for DC in future (Bhattacharjee et al., 2017).

Conclusions

The results of the study showed that the leaf extracts of *S. francisii* and *S. forskaliana* led to a non-significantly decrease of TBARS concentration in the equine erythrocytes. When erythrocytes were incubated with *S. aethiopica*, *S. caulescens*, *S. roxburghiana*, *S. gracilis*, the TBARS level was similar to that of the untreated erythrocytes. In the meantime, the treatment of *S. canaliculata*, *S. suffruticosa*, *S. metallica*, *S. fischeri*, *S. dooneri*, *S. trifasciata*, *S. parva*, *S. intermedia*, and *S. kirkii* non-significantly increase the formation of intracellular TBARS in the extract-treated erythrocytes by approximately 7–20%, respectively. However, *S. hyacinthoides* and *S. cylindrica* had significant increased TBARS level in the extract-treated erythrocytes (by 29.7 and 21%, $p < 0.05$, respectively). It can be inferred from this study, that *Sansevieria* species have a promising antioxidant and prooxidant potential. Further studies involving bioassay-guided identification of the main compounds in plants is necessary to affirm and maximize the possible use of the plant as a therapeutic remedy for prevention of lipid peroxidation in erythrocytes. The antioxidative and prooxidative mechanism of various *Sansevieria* species in equine erythrocyte suspension will be further studied in detail. The obtained information may be useful in the clinical usage of plants in medicine and veterinary. Finally, these findings justify the traditional uses of *Sansevieria* plants for therapeutic purposes.

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A PROMISING ALTERNATIVE FOR TREATMENT OF BACTERIAL INFECTIONS BY *SANSEVIERIA CYLINDRICA* BOJER EX HOOK LEAF EXTRACT

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The aim of this study was to assess the *in vitro* antibacterial activity of ethanolic extract prepared from *Sansevieria cylindrica* Bojer ex Hook leaves against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains, clinically important bacteria, which are indicator organisms commonly used in programs to monitor antibiotic resistance. For this study, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* NCTC 12493, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27583 were used. The disc diffusion assay (Kirby–Bauer method) was used to screen for antibacterial activity of leaf extract. The results of antibacterial activity clearly showed that the extract has shown antibacterial activity against the entire tested organisms. The extract has shown better activity against *S. aureus* and *P. aeruginosa* strains compared to the *E. coli* strains. The diameters of inhibition zones were (22.5 ±1.24) mm, (20.5 ±1.3) mm, and (16.4 ±0.95) mm for *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, and *S. aureus* NCTC 12493, respectively. The extract has shown less antimicrobial activities against *P. aeruginosa*. Finally, the ethanolic extract exhibited mild antibacterial activity against *E. coli*. Further chemical analysis of the aforementioned plant extract should be performed to determinate their chemical composition and identify the exact secondary metabolites responsible for the antimicrobial activity. In addition, the extract should be subjected to pharmacological evaluations with the aim of assessing its efficacy and toxicity, interactions and contraindications.

Keywords: *Sansevieria cylindrica* Bojer ex Hook, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, leaves, ethanolic extract, antimicrobial activity, agar disk diffusion assay

Introduction

The emergence of human pathogenic microorganisms that are resistant to major classes of antibiotics has increased in recent years, due to the indiscriminate use of commercially available antimicrobial agents. It was suggested that resistance mechanisms probably have evolved from genes present in organisms that produce antibiotics (Hawkey, 1998). The presence of antibiotic-resistant strains among food derived microorganisms suggests that

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it may play a much more important role in transferring the antibiotic resistance encoding genes than previously thought (Chajęcka-Wierzchowska et al., 2016; Bielikova et al., 2017). Considering, that antibiotics are used for prophylactic purposes in livestock and poultry production, in vegetable and fruit cultivation and as well as in beekeeping (Ding and He, 2010), it was suggested that the meat of farm animals, fruit, vegetables, and water may be a source of resistant strains (Chajęcka-Wierzchowska et al., 2017).

Sansevieria Thunb., a genus with diverse ethnobotanical uses in its geographical distribution range, has occupied an important place among plant genera applied for treatment of a broad spectrum of diseases and disorders (Khalumba and Mbugua, 2005; Staples and Herbst, 2005; Takawira-Nyenya et al., 2014). Comprehensive information concerning ethnobotanical uses of various *Sansevieria* species in Kenya was presented and critically evaluated by Takawira-Nyenya and coauthors (2014). These authors reported that ethnobotanical data on various *Sansevieria* species have been well documented in various locations in East Africa. For example, Bally (1937) reported that *S. kirkii* Baker roots are used for the treatment of foot sores (cited by Takawira-Nyenya et al., 2014). As previously described, *S. trifasciata* Prain has been used for the treatment of inflammatory ailments and snakebite (Morton, 1981). Moreover, *S. trifasciata* are used in folk medicine for treating bronchitis, asthma, food poisoning, toxemia, cough, snake bite, insect bite etc. (Seth, 2005). The *S. trifasciata* extracts possess mild analgesic properties and elicit analgesic-, anti-inflammatory and antipyretic activity in mice (Anbu et al., 2009).

The other documented folk medicinal uses of *Sansevieria* species include treatment for abdominal pains, diarrhea, and hemorrhoids (Andhare et al., 2012). In traditional health care practice, the leaves of *S. liberica* Gérôme and Labroy are used as painkillers, and in the treatment of smallpox, chicken pox, measles and most venereal diseases (Chigozie and Chidinma, 2013). It was reported that preparations of *S. liberica* plant are used in the treatment of ear and eye infections, inflammation (leaf juice); tooth-ache (fruit juice together with fluid from snails); fever, headache, and cold (fume from burning leaves inhaled); cough, pain, inflammation, infections, convulsion, diarrhoea, and as stimulating tonic (root decoction) (Watt and Breyer-Brandwijk, 1962). The hydroethanolic extract of *S. liberica* possesses also significant anticancer activity (Akindele et al., 2015). The pressed juice of the *S. liberica* leaves is dropped in the eyes and ears for the treatment of infections and inflammations (Chigozie and Chidinma, 2013). Additionally, the *S. liberica* roots are used for the treatment of convulsion, epilepsy, paralysis, malnutrition, pulmonary troubles, vermifuges, cough and debility (Ikewuchi et al., 2011). In Nigeria, the leaves and roots of *S. liberica* are used in traditional health-care practice for the treatment of asthma, abdominal pains, colic, diarrhea, eczema, gonorrhea, hemorrhoids, hypertension, diabetes mellitus, menorrhagia, piles, sexual weakness, wounds of the foot, and alleviating the effects of snake bites (Chigozie and Chidinma, 2013). The anti-anemic and sedative and anticonvulsant activities of the *S. liberica* leaves and roots have been reported (Ikewuchi et al., 2010; Adeyemi et al., 2007).

The roots and rhizomes of *S. roxburghiana* Schult. & Schult. f. are used in the traditional medicine as the remedies for diabetes, inflammation, pains, fever, asthma, wound,

hypertension, oxidative stress and rheumatism (Dhiman, 2006; Pulliah, 2006; Haldar et al., 2010). *S. roxburghiana* could offer an overall protective effect through attenuating hyperglycemia and arresting inflammation in type 2 diabetes and its associated cardiomyopathy (Bhattacharjee et al., 2016). *S. senegambica* Baker is used in traditional health practice in southern Nigeria for treating bronchitis, inflammation coughs, boils and hypertension. It is also used in arresting the effects of snake bites, as well as in compounding solutions used as hair tonics. A dose-dependent hypocholesterolemic effect of the extract suggesting a likely protective role of the extract against dyslipidemia and the development of cardiovascular diseases was also revealed (Ikewuchi, 2012). African *S. ehrenbergii* were shown to possess anticancer activity against the P388 lymphocytic leukemia cell line and a panel of human cancer cell lines. Sansevistatin 2 and other saponins isolated from the same source, exhibited antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (Pettit et al., 2005). From the leaves of *S. cylindrica*l Bojer ex Hook, a new steroidal saponin was isolated and showed inhibition of the capillary permeability activity (Da Silva Antunes et al., 2003). Watt and Breyer-Brandwijk (1962) listed the use of *S. hyacinthoides* (L.) Druce in the treatment of a toothache and earache and the use of the rhizome decoction of *S. kirkii* as a purgative both reported from East Africa. Yet, Kiringe (2006) reported on the use of *S. volkensii* Gürke for the treatment of sexually transmitted diseases such as gonorrhea. In Kenya, Owuor and Kisangau (2006) included the use of *S. parva* N.E.Br. leaf sap and *S. kirkii* Baker extracts for treatment of snake bite wounds. Nevertheless, in spite of these data, Takawira-Nyenya et al. (2014) reported that the documentation of ethnobotanical uses of genus *Sansevieria* is incomplete.

Sansevieria species also showed antimicrobial activity (Onah et al., 1994; Aliero et al., 2008; Philip Deepa et al., 2011; Sheela et al., 2012). In our previous study, we have evaluated the antibacterial capacity of ten species of *Sansevieria* genus against *Staphylococcus aureus* in order to validate scientifically the inhibitory activity for microbial growth attributed by their popular use and to propose new sources of antimicrobial agents (Buyun et al., 2016). The selected bacterial strain *S. aureus* is widespread and causes serious problems due to their pathogenicities and high levels of drug resistance. This has caused many clinical problems in the treatment of infectious diseases because the commercially available antibiotics commonly used are sometimes associated with adverse effects such as hypersensitivity, allergic reaction, and immunosuppression in the host. Thus, the search for the discovery of new antimicrobial agents is an urgent need. The results proved that the inhibition zones ranged between 16 and 34 mm. *S. fischeri* and *S. francisii* extracts were particularly active against tested strain (diameters of inhibition zones were 34 mm). This was followed by the activities of *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* leaf extracts (diameters of inhibition zones ranged from 25 to 31 mm). The ethanolic extracts of *S. canaliculata* and *S. trifasciata* showed less antimicrobial activities (16 to 16.5 mm). The results proved that the ethanolic extracts of *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* exhibited a favorable antibacterial activity against *S. aureus*. By the agar diffusion method, the ethanolic extracts of *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, and *S. metallica* leaves showed anti-*S. aureus* activity, evidencing that ethanol is an efficient organic solvent to be used for the extraction of bioactive plant materials (Buyun et al., 2016). As previously

mentioned, our results also revealed that the ethanolic extracts obtained from leaves of *S. kirkii*, *S. arborescens*, *S. roxburghiana*, *S. francisii*, *S. forskaliana*, *S. cylindrica*, *S. trifasciata*, *S. canaliculata*, *S. caulescens*, *S. metallica*, *S. aethiopica* possess antibacterial potency against *Escherichia coli* isolates and may be used as natural antiseptics and antimicrobial agents in medicine (Tkachenko et al., 2017).

Consequently, the results of the present study confirm the importance of the studied plants of *Sansevieria* species as a source of bioactive compounds for the treatment of infectious diseases. Therefore, the current study was designed to test the efficacy of ethanolic extract prepared from *S. cylindrica* Bojer ex Hook leaves against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains, clinically important bacteria, which are indicator organisms commonly used in various projects in order to monitor antibiotic resistance (Boss et al., 2016).

Materials and methodology

Collection of plant material and preparation of plant extract

Totally expanded leaves of *Sansevieria cylindrica* plants were sampled for study (Figure 1A). *S. cylindrica* is a most distinct stemless succulent plant that grows fan-shaped, with stiff leaves growing from a basal rosette. It forms in time a colony of solid cylindrical leaves (Figure 1B). The species is interesting in having cylindrical instead of strap-shaped leaves. It spreads by rhizomes – roots that travel under the soil surface and develop offshoots some distance from the original plant. Rosette is formed a few leaves distichous rosettes with 3–4 leaves (or more) from underground rhizomes. Leaves are round in cross-section, leathery, rigid, erect to arching, channelled only at the base, dark-green with thin dark green vertical stripes and horizontal grey-green bands about (0.4)1–1,5(-2) m in height and about 2–2,5(-4) cm thick. The 2.5–4 cm flowers are tubular, delicate greenish-white tinged with pink and lightly fragrant. Blooming season: It blooms once a year in Winter to Spring (or summer too) (http://www.llifle.com).

Freshly collected leaves were washed, weighted, crushed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity. All extracts were stored at 4 °C until use.

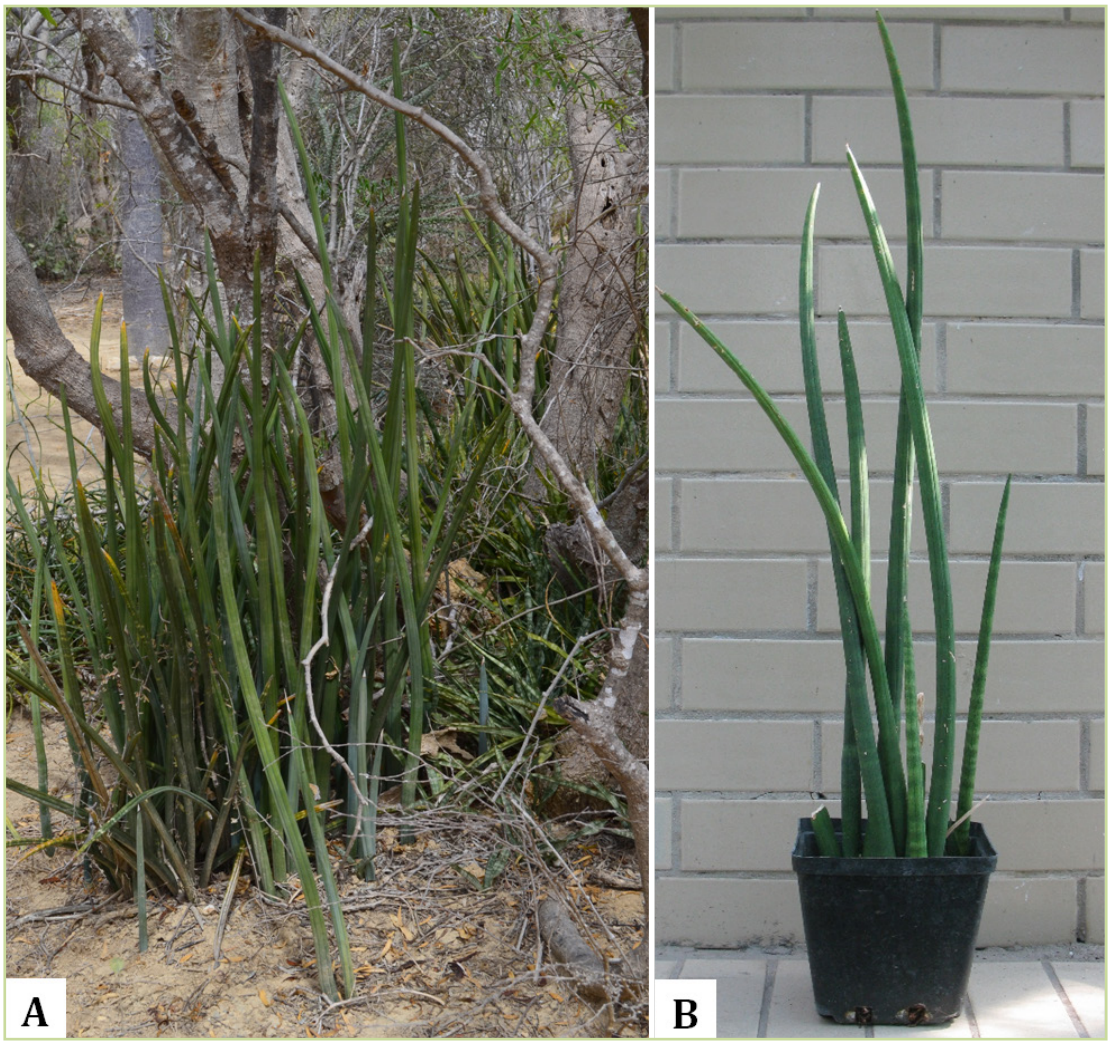

Figure 1 *Sansevieria cylindrica* Bojer ex Hook: A – the growth habit of *S. cylindrica* in semi-natural habitat, Madagascar, Toliara, d'Antsokay Arboretum (photo by Lyudmyla Buyun); B – a specimen of *S. cylindrica* cultivated under glasshouse conditions at NBG (Kyiv, Ukraine) (photo by Denis Krupoderov)

Bacterial test strain and growth conditions

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

inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration.

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

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

Statistical analysis

All statistical calculation was performed on separate data from each species with STATISTICA 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*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

In line with the growing interest in the antibacterial potential of different plants, we examined the antibacterial properties of *Sansevieria cylindrica* leaves against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* strains. The results of antibacterial activity screening are given in the Figures 2 and 3, which clearly indicate that the extract has shown antibacterial activity against the entire tested organisms. The extract has shown better activity against *S. aureus* and *P. aeruginosa* strains compared to the *E. coli* strains. The diameters of inhibition zones were (22.5 ±1.24) mm, (20.5 ±1.3) mm, and (16.4 ±0.95) mm for *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, and *S. aureus* NCTC 12493, respectively. The extract has shown less antimicrobial activities against *P. aeruginosa*. The mean of the inhibition zone was (17.8 ±1.25) mm. Finally, the ethanolic extract exhibited mild antibacterial activity against *E. coli* [mean of inhibition zone ranged (16.8 ±0.85) mm for *E. coli* ATCC 25922 and (15.1 ±1.1) mm for *E. coli* ATCC 35218] (Figures 2 and 3).

The *Sansevieria* species are an important source of potentially useful structures for the development of new chemotherapeutic agents (Adeyemi et al., 2007; Ikewuchi et al., 2010; Andhare et al., 2012; Ikewuchi, 2012; Bhattacharjee et al., 2016). Antimicrobial screening of plant extracts with the *in vitro* antibacterial activity assay, usually, represents a starting point for antimicrobial drug discovery (Amenu, 2014). Consequently, in this study, the antibacterial activity of *S. cylindrica* leaf extract (Figures 2 and 3) was investigated against

the standard Gram-positive strains: *Staphylococcus aureus* (ATCC 25923, ATCC 29213, NCTC 12493) and Gram-negative strains: *Pseudomonas aeruginosa* (ATCC 27583) and *Escherichia coli* (ATCC 25922, ATCC 35218) by the disc diffusion method. This was carried out by placing discs impregnated with test material on the surface of inoculated MH agar plates. The plates were then kept in an incubator at 37 °C for 24 hours and diameters of zones of inhibition were measured. Clear inhibition zones unravelled that the compounds showed the antibacterial activity of the antibiotic disc against bacterial strains. It was observed that controlled strain of both Gram-positive and Gram-negative strains: *E. coli*, *P. aeruginosa* and *S. aureus* were sensitive against *S. cylindrica* extract. It is concluded that plant extract possesses antibacterial activity against tested organisms. The zone of inhibition varied suggesting the varied degree of efficacy and different substances of the extract on the target strains. The antibacterial activity of the *S. cylindrica* extract may be due to the presence of various active metabolites.

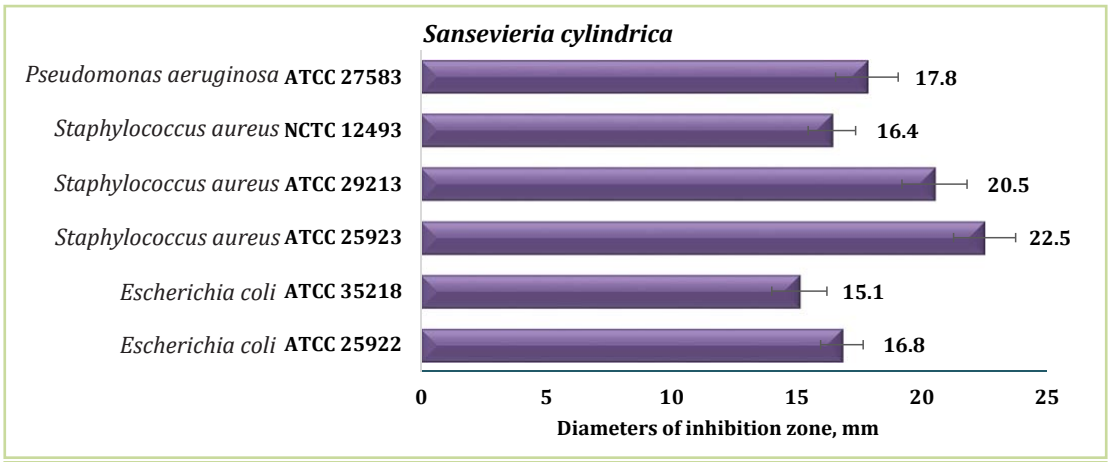

Figure 2 The mean of inhibition zone diameters of ethanolic extracts obtained from leaves of *Sansevieria cylindrica* against *S. aureus*, *E. coli*, and P. eaeruginosa (n = 8)

The results of the present study reinforce the importance of the analyzed plants as a source of bioactive compounds for the treatment of *S. aureus*, *P. aeruginosa*, and *E. coli* related infectious diseases. Similar results were described for other species of *Sansevieria* genus. For example, Deepa Philip et al. (2011) have carried out phytochemical analysis and antimicrobial investigation of different solvent and aqueous extracts of the leaves and rhizome of *S. roxburghiana* against a panel of clinically significant bacterial and fungal strains (*Salmonella paratyphi*, *Shigella sonnie*, *Salmonella typhi*, *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus luteus*, *Enterococcus* spp., *Klebsiella pneumoniae*, *Proteus vulgaris* and *Cryptococcus neoformans*, *Candida albicans* and standard strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853). Susceptibility testing by disc diffusion assay revealed significant antimicrobial activity of methanolic and acetone extracts of leaves against Gram-positive bacteria such as *M. luteus*, *B. cereus*, *Enterococcus* spp., *S. aureus*, Gram-negative bacteria such as *P. vulgaris*, *P. aeruginosa*, *P. fluorescence*, *S. typhi*, *S. paratyphi*, *K. pneumoniae*,

S. sonnei and *E. coli*, fungal strains *Cryptococcus* spp. and *C. albicans*. Ethyl acetate extracts of rhizomes also exhibited appreciable antimicrobial activity against most of the pathogens tested. The minimum inhibitory concentrations (MIC) of the various extracts by agar dilution method ranged from 1.0 to 8.0 $mg.ml^{-1}$. The leaf extracts exhibited better antimicrobial activity than rhizomes (Deepa Philip et al., 2011). The diethyl ether, alcohol, and acetone extracts of *S. roxburghiana* rhizome showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (Sheela et al., 2012). The antibacterial activity of ethanolic extract of the rhizome of *S. roxburghiana* against the four pathogenic bacteria, *S. typhi*, *P. fluorescens*, *P. aeruginosa* and *E. coli* was assessed by a zone of inhibition in the study of Poonam Sethi (2013). All the microbes were sensitive to the ethanolic extract of the plant and showed a potential activity. Maximum activity was seen in the case of *P. fluorescens* where the zone diameter was 32 mm (300 $\mu g.ml^{-1}$). The minimum inhibitory concentration study revealed that the value for the *S. typhi* and *E. coli* as 80 and 60 $\mu g.ml^{-1}$ for *P. fluorescens* and *P. aeruginosa* (Poonam Sethi, 2013).

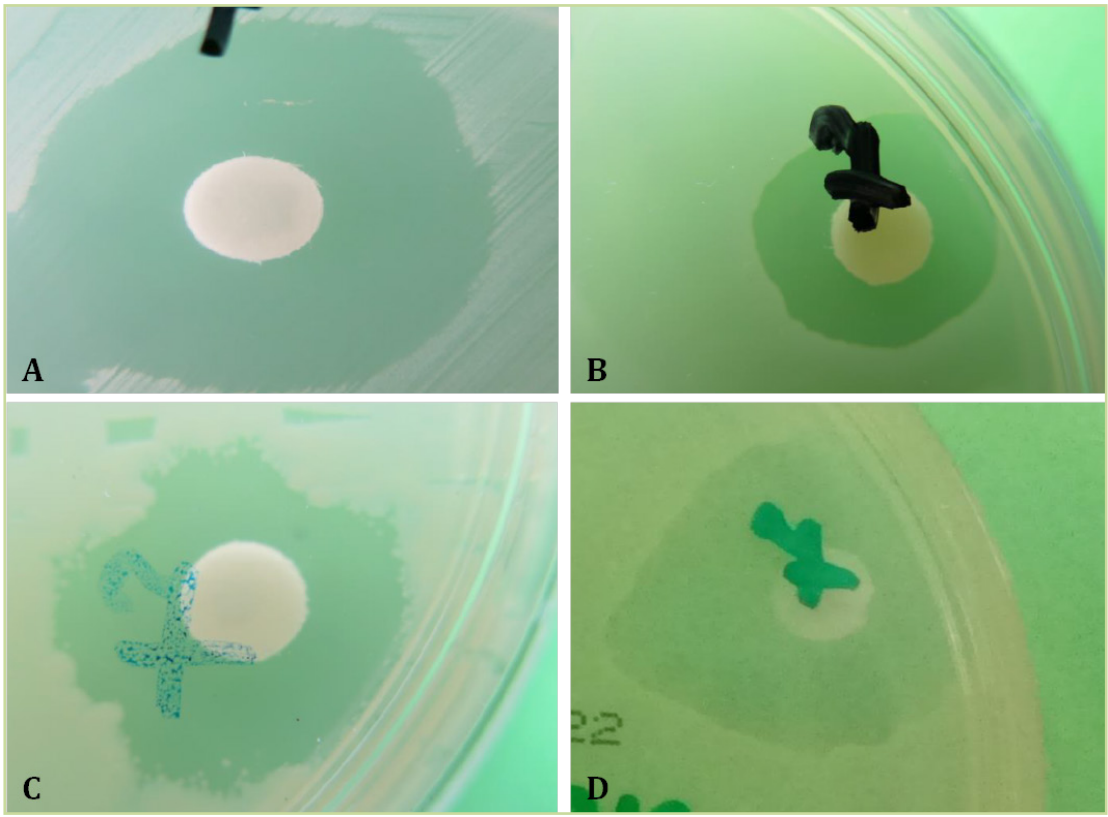

Figure 3 Antimicrobial activity of ethanolic extract obtained from leaves of *S. cylindrica* against *E. coli* ATCC 25922 (A), *E. coli* ATCC 35218 (B), *P. aeruginosa* ATCC 27583 (C), and *S. aureus* ATCC 29213 (D) measured as inhibition zone diameter

By the agar diffusion method, the ethanolic extracts from *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, and *S. metallica* showed anti-*S. aureus* activity, evidencing

that ethanol is an efficient organic solvent to be used for the extraction of bioactive plant materials. The microbial growth inhibition capacity relies on the rich variety of phytochemicals including carbohydrates, saponin, flavonoids, phenols, alkaloid, anthocyanin and cyanine, glycosides, proteins, and phytosterols (Deepa Philip et al., 2011). The phytochemical screening revealed the high presence of alkaloids in the methanolic extract of *S. roxburghiana* compared to acetone, chloroform, and ether. Flavonoids were present in ethanolic and ether extracts in moderate proportions; saponins were present in ethanolic and methanolic extracts in moderate proportions. Steroids were shown in higher proportions in methanol, chloroform and ether and moderate in acetone; terpenoids presence were was shown in chloroform and absent in all rest of the extracts. Tannins were high in acetone and methanol and moderate in ethanol and chloroform. Phenols were only in methanol fractions, while quinones were presented in methanol, chloroform, and ether in moderate levels (Kumar and Kumari, 2015).

Saponins are a diverse family of secondary metabolites (glycosylated phytoanticipins) that are found in a wide range of plant species and can be divided into three major groups, triterpenoid, steroid or steroidal glycoalkaloid, depending on the structure of their aglycones. Because they have potent antimicrobial activities it is proposed that the natural role of these molecules in plants is to confer protection against potential pathogens (Osbourn, 2003; González-Lamothe et al., 2009). Flavonoids are also well known as antibacterial agents against a wide range of pathogenic microorganism (Cushnie and Lamb, 2011; Coppo and Marchese, 2014; Xie et al., 2015). Recent advances in understanding the antibacterial properties of flavonoids were described by Cushnie and Lamb (2011). Several mechanisms of actions have been proposed for the synergistic and antibiotic resistance-modulating activity of flavonoids. For the galloyl flavan-3-ols, it has been suggested that these modulate β-lactam resistance by reducing d-alanylation of cell wall teichoic acid [resulting in inactivation of penicillin-binding protein 2a (PBP2a)], or by intercalating into the cytoplasmic membrane and inducing structural changes that result in delocalization of PBP2a. For less-studied compounds in the flavone, isoflavone, flavonol and flavolan classes, it has been suggested these increase antibiotic efficacy via multiple mechanisms of actions, i.g. through β-lactamase inhibition, efflux pump inactivation, cytoplasmic membrane destabilization, disruption of PBP2a synthesis and topoisomerase inhibition (Cushnie and Lamb, 2011).

The specific function of many phytochemicals is still unclear; however, a considerable number of studies have shown that they are involved in the interaction of plants/pests/diseases (Amenu, 2014). It was suggested, that in order to defend themselves, plants are armed with constitutive, pre-existing defence types such as cell wall barriers or pre-formed and stored antimicrobial toxins (Balmer et al., 2013).

To our mind, the scope of our current study is not limited only by searching plant-derived agents to be applied to medicine. Furthermore, we believe, that our findings are linked with the fundamental understanding of factors and mechanisms underlying both basal immunity and induced systemic resistance in angiosperms, in monocots, particularly.

Conclusions

The results proved that the extract from *S. cylindrica* exhibits a favorable antibacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa*. Further chemical analysis of the aforementioned plant extract of *S. cylindrica* should be performed to determinate their chemical composition and identify the exact secondary metabolites responsible for the antimicrobial activity. In addition, the extract should be subjected to pharmacological evaluations with the aim of assessing its efficacy and toxicity, interactions and contraindications. Thus, the preliminary screening assay indicated that the leaves of *S. cylindrica* with antibacterial properties may offer alternative therapeutic agents against bacterial infections.

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IN VITRO ASSESSMENT OF ANTIOXIDANT EFFECT OF *BEGONIA REX* PUTZ. LEAF EXTRACT ON OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTES MODEL

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The main goal of our study was to assess the antioxidant effect of leaf extract obtained from *Begonia rex* Putz. on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification] and antioxidant defences [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity] using the equine erythrocytes model. Freshly collected *B. rex* leaves were washed, weighted, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in ratio 1: 19, w/w) at room temperature. The extracts were then filtered and used for analysis. A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (phosphate buffer) was used. After incubation, the mixture at 37 °C for 60 min with continuous stirring, erythrocytes, and plasma aliquots was used in the study. The extract during incubation of erythrocyte suspension caused a non-considerable TBARS formation (by 18%, p >0.05), while the content of aldehydic and ketonic derivatives of oxidatively modified proteins was decreased (by 7 and 8%, p >0.05, respectively) compared to control. The aqueous leaf extract of *B. rex* has proven the most effective to increase the catalase and GPx activity (by 44%, p >0.05 and 62%, p <0.05). The increase of the catalase and GPx activity was induced by TAC enhancement by 34% (p >0.05). SOD activity was non-significantly decreased by 17% (p >0.05). *B. rex* extract caused the statistically significant decrease in ceruloplasmin level by 64% (p <0.05). These *in vitro* assays indicate that *B. rex* leaf extract screened is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity of *B. rex* extract is currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract.

Keywords: *Begonia rex*, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, antioxidant defence

Introduction

Begonia L. is a mega-diverse genus containing more than 1800 species, with a very high proportion of microendemics and hotspots of diversity in the Andes and Southeast Asia

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(Hughes et al., 2018). The first living plant in *Begonia* was introduced to Europe during the eighteenth century, and thereafter over 400 natural species have been introduced for horticulture and many cultivars have been developed (Tebbitt, 2005). *Begonia*s are among the most popular ornamental plants in the world thanks to their large, showy, and long-lasting multicolor flowers, ranging from white to pink, red, and yellow (Sakhanokho et al., 2013; Twyford et al., 2014). They are used as garden plants and potted plants, in hanging baskets, and as greenhouse flowers, as well as potherbs or leaf vegetables in many parts of the world. The roots and tubers of some species have been reported to possess antimicrobial activities and are used to treat various ailments (Sakhanokho et al., 2013).

In our previous study (Tkachenko et al., 2016; Buyun et al., 2017), we have assessed the anti-*Escherichia coli* activity of the ethanolic extracts from the leaves of *Begonia* species, i.e. *B. solimutata* L.B. Sm. & Wassh., *B. goegoensis* N.E.Br., *B. foliosa* Kunth, *Begonia* × *bunchii* L.H. Bailey (syn. *Begonia* × erythrophylla Hérincq), *B. thiemei* C.DC., *B. peltata* Otto & Dietr., *B. heracleifolia* Cham. & Schltdl., *B. dregei* Otto & Dietr., *B. mexicana* G. Karst. ex Fotsch. In our study, ethanolic extracts obtained from leaves of *Begonia* species had an average activity against *E. coli*. The inhibition zone diameter observed for *B. solimutata* was 14 mm, 11.5 mm for *B. goegoensis*, 13 mm for *B. foliosa*, 13.5 mm for *Begonia* × *bunchii*, 15 mm for *B. thiemei*, 19 mm for *B. peltata*, 12 mm for *B. heracleifolia*, 11.5 mm for *B. dregei*, and 16 mm for *B. mexicana*. The highest antimicrobial effect was recorded for *B. peltata*, *B. mexicana*, and *B. thiemei*. The most antimicrobial effective plant against *E. coli* was *B. peltata*, being highly active with the ethanolic extract (inhibition zone diameter 19 mm). The obtained results highlighted the interesting antimicrobial potency of various *Begonia* species and provided a scientific basis for the traditional use of these plants in the treatment of microbial infections (Tkachenko et al., 2016; Buyun et al., 2017). Moreover, the highly active antimicrobial effects of various *Begonia* species against *Candida albicans* and *Pseudomonas aeruginosa* isolates are worthy of highlighting (Buyun et al., 2016; Tkachenko et al., 2017). Furthermore, the antimicrobial activity showed by *Begonia* species screened is in agreement with previous findings on the antimicrobial effects produced by numerous *Begonia* species.

We also have assessed the percentage of equine erythrocyte hemolysis induced by treatment with extracts of various species of *Begonia* genus to exemplify their further potential development and use as a drug against metabolic diseases in medicine and veterinary (Tkachenko et al., 2017). Our study demonstrated that among 30 species of *Begonia* genus, the most species of plants investigated possessed anti-hemolytic activity. The results of these biological assays demonstrated that compounds present in *B. glabra*, *B. aconitifolia*, *B. sanguinea*, *B. thiemei*, *B. masoniana*, *B.* × *credneri*, *B. oxyphylla*, *B. subvillosa*, *B. ulmifolia*, *B. convolvulaceae* can prevent the formation of methemoglobin and reduce hemolysis, while *B. erythrophylla*, *B. psilophylla*, and *B. arborescens* var. *oxyphylla* extracts can facilitate the formation of methemoglobin and hemolysis in healthy equine blood. Extracts from leaves of *B. foliosa*, *B. rex*, *B. solimutata*, *B. mexicana*, *B. goegoensis*, *B. imperialis* var. *smaragdina*, *B. pustulata*, *B. peltata*, *B. cucullata*, *B. angularis*, *B. boisiana*, *B. venosa* exhibited the decrease of percentage hemolysis of equine erythrocytes, but these alterations were non-significant (Tkachenko et al., 2017).

In erythrocytes, interactions between biomolecules and the components of plant extract take place. As a result, alterations in oxidative balance, as well as changes in cellular membrane properties, may appear. A disturbance in pro-oxidative-antioxidative balance (the increase in methemoglobin content and reactive oxygen species formation) leads to erythrocyte damage, including changes in cytoskeleton and cell's membrane such as formation and tearing off the bubbles. In the consequence of these processes, the erythrocytes are excessively eliminated from blood (Bors et al., 2012). Equine erythrocytes are more sensitive to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage, i.e. methemoglobin formation, alteration of aggregation, and reduction of cellular deformability (Baskurt and Meiselman, 1999). Therefore, the high susceptibility of equine erythrocytes to oxidant damage and the resulting hemorheologic alterations may have important consequences for tissue perfusion and cardiovascular adequacy in horses (Baskurt and Meiselman, 1999; Walter et al., 2014).

Oxidants typically damage erythrocytes by oxidizing the heme iron in hemoglobin, reactive sulfhydryls, or unsaturated lipids in the membranes. The oxidation of the heme iron in hemoglobin to the ferric (Fe^{3+}) state generates methemoglobin, which is incapable of transporting oxygen. Methemoglobin can be enzymatically reduced back to the functional ferrous (Fe^{2+}) state, primarily by nicotinamide adenine dinucleotide (NADH)-dependent methemoglobin reductase (Wright et al., 1999; Walter et al., 2014). Sulfhydryl groups in proteins and unsaturated lipids in erythrocyte membranes are especially susceptible to oxidation. Oxidative denaturation and the precipitation of the globin portion of hemoglobin into large aggregates result in the formation of Heinz bodies that can bind to and alter membranes. Membrane structure also is altered by the oxidation of sulfhydryl groups and by lipid peroxidation (Harvey, 1997). NADPH is produced by the initial enzyme reactions of the pentose phosphate pathway, and erythrocytes increase pentose phosphate–pathway metabolism in response to oxidants to provide the NADPH necessary for the regeneration of reduced glutathione (GSH). In healthy humans and animals, most glutathione in erythrocytes is maintained as GSH, with low concentrations of oxidizing glutathione (GSSG) being present (Harvey, 1997; Harvey et al., 2003). Erythrocytes from horses are slower than erythrocytes from other species studied in their ability to regenerate GSH after it has been oxidized *in vitro* (Agar et al., 1974; Harvey et al., 2003). Horse erythrocytes also appear less able to protect themselves against oxidative injury induced by incubation with high levels of ascorbate, which stimulates the GR reaction by the oxidation of GSH (Harvey and Kaneko, 1977; Harvey et al., 2003). Therefore, though many model systems are frequently used to study the biochemical alterations under the condition of oxidative stress including the tissues from various parts of the body, erythrocytes, as the most common type of blood cells, get superiority amongst them (Pandey and Rizvi, 2010). Red blood cell along with its membrane has always been an important medium for the study due to the important role it plays in varied physiological and metabolic processes (Karabulut et al., 2009).

In this study, we have focused on the antioxidant effect of leaf extract obtained from *Begonia rex* Putz. on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification] and antioxidant defences [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity,

ceruloplasmin level, and total antioxidant capacity (TAC)] using the equine erythrocytes model. Thus, equine erythrocytes were proved to be a good tool for analyzing the oxidative stress biomarkers as a mechanism of antioxidant action of *B. rex* leaf extract.

Material and methodology

Collection of plant material

The leaves of *Begonia rex* Putz., cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. The biochemical screening of *Begonia* leaf extracts has been carried out in the laboratory of Institute of Biology and Environmental Protection, Pomeranian University in Slupsk (Poland). Our current scientific project has been undertaken in the frame of cooperation programme between the Institute of Biology and Environmental Protection (Pomeranian University in Slupsk, Poland) and NBG, aimed at assessment of medicinal properties of tropical plants has encompassed some tropical mega-diverse genera, including genus *Begonia* with a near pantropical distribution.

Preparation of plant extracts

The *B. rex* has striking metallic patterns with variations of red and silver over a green background on a rippled surface (Zhang et al., 2009). Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in ratio 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Horses

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

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min to remove plasma. The pellet of blood was re-suspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (phosphate buffer) was used. After incubation the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocytes and plasma aliquots were used in the study.

2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 mL of sample (blood, plasma, and erythrocytes' suspension) was added to 2 mL of distilled water, 1 mL of 20% TCA and 1 mL of 0.8% TBA. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3.000 g for 10 minutes. The μmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as the extinction coefficient.

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

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1M DNPH (dissolved in 2 M HCl) was added to 0.1 mL of the sample after denaturation of proteins by 20% trichloroacetic acid (TCA). After addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 h at 37 °C. The tubes were spun in a centrifuge for 20 min at 3.000 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 22.000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Assay of superoxide dismutase activity

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0) by Kostiuk et al. (1990) method. Briefly, 1.0 mL of C reagent was mixed with 0.1 mL of a blood sample (dilution in water 1 : 1000). C reagent was made *ex tempore* (a mixture of equal volumes of 0.1 M K, Na-phosphate buffer, pH 7.8 and 80 mM EDTA solution); pH of C reagent was adjusted to 10.0 by adding TEMED. Distilled water (0.1 mL) was added to blank vials instead of the blood sample. The total volume of all samples was brought up to 2.4 mL using distilled water. The reaction was initiated by adding 0.1 mL of quercetin (1.4 μM dissolved

in dimethyl sulfoxide). Absorbance at 406 nm was measured immediately and after 20 min addition of quercetin solution. Activity is expressed in units of SOD per mL of blood.

Assay of catalase activity

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al. (1988). The reaction was initialized by adding 0.1 mL of plasma into the incubation medium (2 mL of 0.03% H_2O_2 solution) and to 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution (blank sample). The duration of the reaction was 10 min at room temperature. The reaction was terminated by rapid adding 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution to incubation medium and 1 mL of 125 mM H_2SO_4 to all samples. All samples were centrifuged at 3.000 g for 5 min. The absorbance of the obtained solution was measured at 410 nm and compared with that of the blank. One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 mmol H_2O_2 per min per L of blood.

Assay of glutathione peroxidase activity

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by detecting the non-enzymatic utilization of GSH (the reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to by the method of Moin (1986). The assay mixture contained 0.8 mL of 0.1 M Tris-HCl buffer with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 mL of 4.8 mM GSH, 0.2 mL of hemolyzed erythrocytes (1 : 20), 1 mL of 20 mM t-butyl hydroperoxide, and 0.1 mL of 0.01 M 5,5-dithiobis-2-nitrobenzoic acid. The rate of GSH reduction was followed spectrophotometrically at 412 nm. GPx activity is expressed as μmol GSH per min per mL of blood.

Assay of ceruloplasmin level

The ceruloplasmin (CP, EC 1.16.3.1) level in the plasma was measured spectrophotometrically at 540 nm, as described by Ravin (1961). The assay mixture contained 0.1 mL of plasma, 0.4 M sodium acetate buffer (pH 5.5), and 0.5% p-phenylenediamine. The mixture was incubated at 37 °C for 60 min. Before cooling at 4 °C for 30 min, the mixture was added to 3% sodium fluoride for inhibition. Ceruloplasmin was expressed in mg per L of plasma.

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37 °C. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3.000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed.

The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

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

Results and discussion

In this study, the investigation of the effect of *B. rex* extract on the lipid peroxidation and oxidatively modified protein biomarkers, as well as antioxidant defences in the equine erythrocyte suspension was undertaken. In relation to blood cells, circulating erythrocytes are regularly exposed to stress conditions and are especially vulnerable as they have no membrane repair mechanism or regenerative capacity (Savignone and Palacios, 2017)

Figure 1A summarizes the results obtained by incubating equine erythrocyte suspension in the presence of the aqueous extract of *B. rex*. As seen, the presence of the extract during incubation of erythrocyte suspension caused a non-considerable TBARS formation (by 18%, p >0.05), while the content of aldehydic and ketonic derivatives of oxidatively modified proteins was decreased (by 7 and 8%, p >0.05, respectively) compared to control.

It is generally assumed, that membrane phospholipids of aerobic organisms are continually subjected to oxidant challenges from endogenous and exogenous sources, while peroxidized membranes and lipid peroxidation products represent constant threats to aerobic cells. The most widely used assay for lipid peroxidation is malondialdehyde (MDA) formation as a secondary lipid peroxidation product, with the thiobarbituric acid reactive substances test (Draper et al., 1993; Valavanidis et al., 2006). Protein oxidation reactions involve various propagating radicals and ROS and the results are oxidative modifications of amino acid side chains, reactive-oxygen-species-mediated peptide cleavage, reactions of peptides with lipids and carbohydrate oxidation products, and formation of carbonyl derivatives of proteins (Valavanidis et al., 2006). Of the various indices of protein oxidation, protein carbonyl formation is the best studied with increases in tissues and organs of organisms (Stadtman and Berlett, 1998).

Antioxidants help prevent cellular damage caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide anion radical ($O_2^{\cdot-}$) (Halliwell and Gutteridge, 1989). Antioxidants can be enzymes or molecules such as vitamins E and C, urea, glutathione etc. Antioxidant enzymes include superoxide dismutase (SOD), which catalyzes the dismutation

of $O_2^{\cdot-}$ to water and oxygen, catalase (CAT), which reduces H_2O_2 to water and oxygen, and glutathione reductase (GR), which regenerates reduced glutathione (GSSG) used as a direct scavenger of ROS or as a substrate for the antioxidant enzyme glutathione peroxidase (GPx) (Halliwell and Gutteridge, 1989). In our study, the aqueous leaf extract of *B. rex* has proven the most effective to increase the catalase and GPx activity (by 44%, p >0.05 and 62%, p <0.05) (Figure 1A). The increase of the catalase and GPx activity was induced by TAC enhancement by 34% (p >0.05) (Figure 1B). SOD activity was non-significantly decreased by 17% (p >0.05) (Figure 1B).

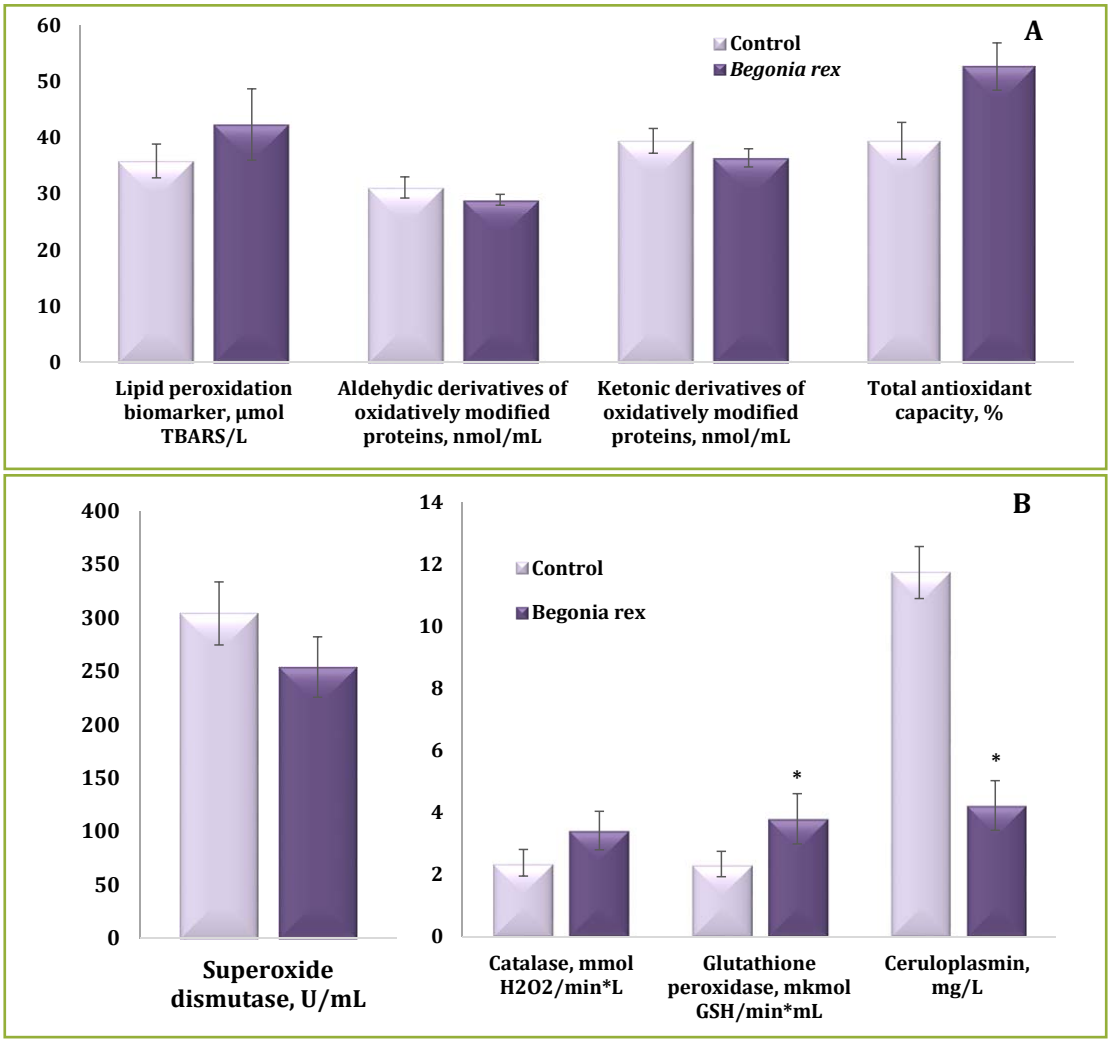


Figure 1 The TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity (A), superoxide dismutase, catalase, glutathione peroxidase, and ceruloplasmin activity (B) in the equine erythrocytes suspension after *in vitro* incubation with *Begonia rex* leaf extract (M ±m, n = 18)

Ceruloplasmin is a serum ferroxidase that contains greater than 95% of the copper found in plasma. This protein is a member of the multicopper oxidase family, an evolutionarily conserved group of proteins that utilize copper to couple substrate oxidation with the four-electron reduction of oxygen to water (Hellman and Gitlin, 2002). It has been proposed to ceruloplasmin function in copper transport, oxidation of organic amines, Fe^{2+}-oxidation and the regulation of cellular iron levels, and regulation of catechols metabolism, radical scavenging and other antioxidant processes (Healy and Tipton, 2007). In our study, *B. rex* extract caused the statistically significant decrease in ceruloplasmin level by 64% (p <0.05) (Figure 1B).

Based on the collected data, positive trends were observed in the regressions of TBARS level against ketonic derivatives of OMP level (r = 0.477, p = 0.045), and SOD activity (r = 0.553, p = 0.017) for in the equine erythrocyte suspension after *in vitro* incubation with *B. rex* leaf extract (Figure 2A); in the case of SOD activity *vs.* ketonic derivatives of OMP level the regression was positive and significant (r = 0.540, p = 0.021) (Figure 2B). The SOD activity *vs.* TAC level regression was significant and reversible, which showed the relationship between increased TAC level and decreased SOD activity (r = -0.547, p = 0.019) (Figure 2B). The same results were obtained when regressions were performed on GPx activity (r = -0.489, p = 0.039) (Figure 2C), except in case the GPx activity *vs.* catalase activity regression was significant and positive (r = 0.802, p = 0.000) (Figure 2C).

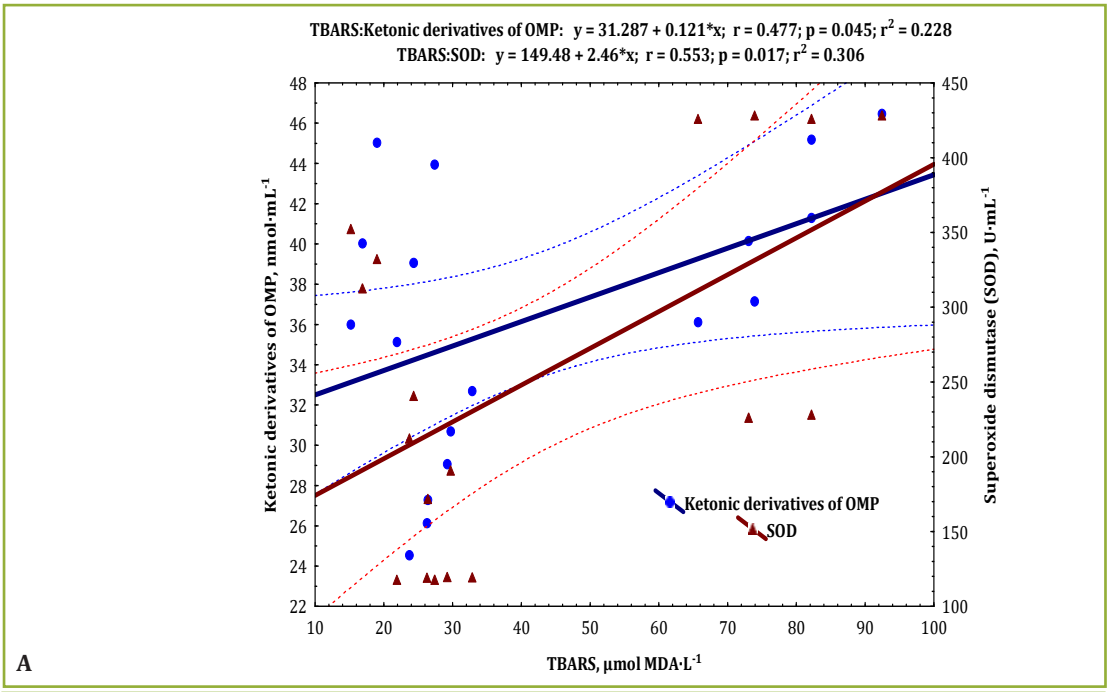
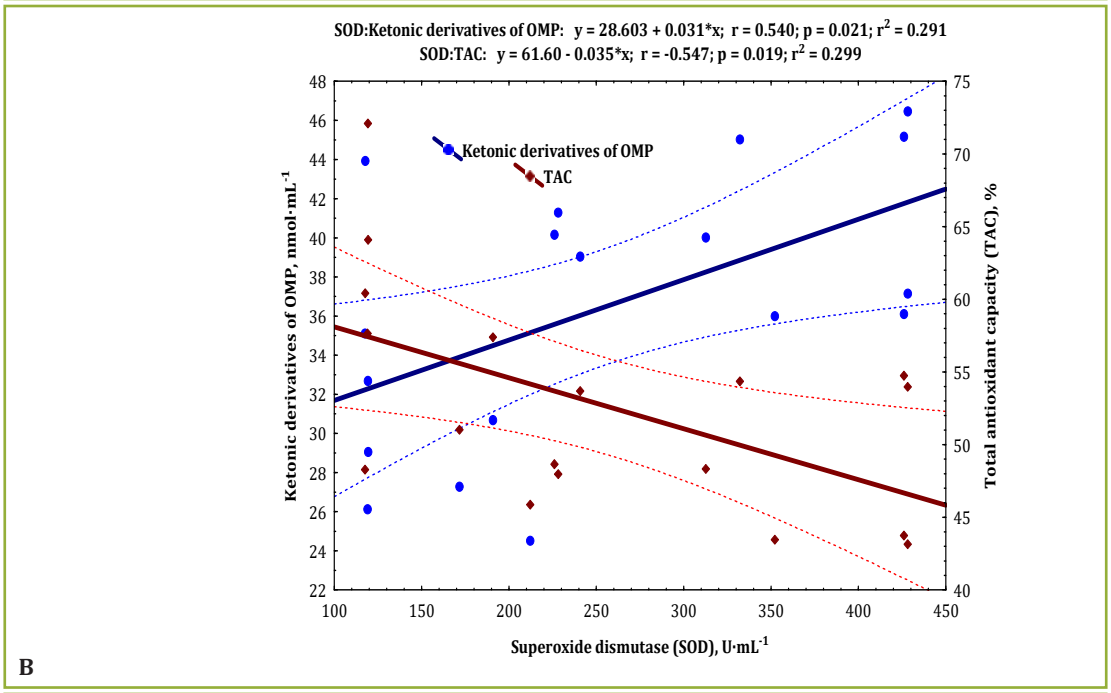


Figure 2A Correlations between oxidative stress biomarkers TBARS level, ketonic derivatives of OMP level, and SOD activity (A), SOD activity, ketonic derivatives of OMP level, and TAC activity (B), TAC level, catalase and GPx activity (C) in the equine erythrocyte suspension after *in vitro* incubation with *Begonia rex* leaf extract



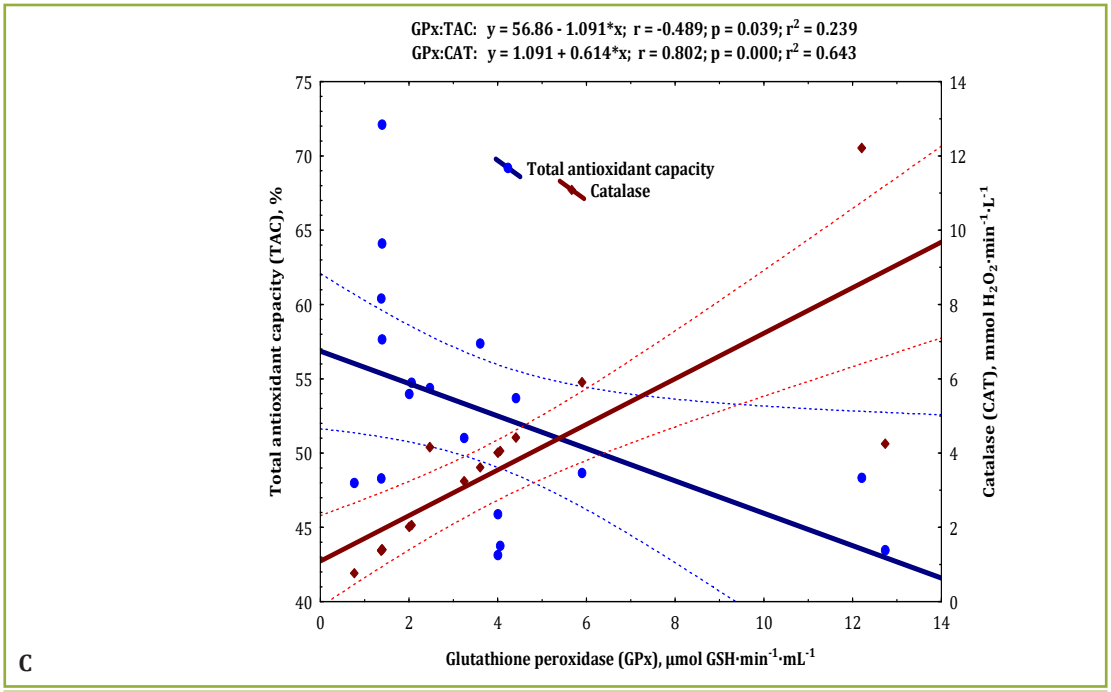


Figure 2B–C Correlations between oxidative stress biomarkers TBARS level, ketonic derivatives of OMP level, and SOD activity (A), SOD activity, ketonic derivatives of OMP level, and TAC activity (B), TAC level, catalase and GPx activity (C) in the equine erythrocyte suspension after *in vitro* incubation with *Begonia rex* leaf extract

This paper demonstrates changes in the oxidative stress biomarkers of equine erythrocytes incubated with *B. rex* leaf extract (Figures 1 and 2). Accordingly, our study suggests that crude extract obtained from *B. rex* leaves has the effective antioxidant effect after treatment of equine erythrocytes. Protective effects of *B. rex* leaf extracts became apparent by amelioration in antioxidant enzymes' activities and the increase of total antioxidant capacity. The antioxidant defence system was improved concurrence with suppression of aldehydic and ketonic derivatives of oxidatively modified proteins by treatment of *B. rex* extract. *B. rex* extract showed anti-inflammation effect represented as decreasing on ceruloplasmin level in the plasma. The pronounced effect of leaf *B. rex* extract could be attributed to its secondary metabolites, e.g. polyphenols and flavonoids contents.

Phytochemical constituents in *Begonia* species are known to be biologically active compounds and they are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer (Suresh and Nagarajan, 2009). It has become increasingly clear that all secondary metabolite components displayed antioxidant and antimicrobial properties through different biological mechanisms (Hossain and Nagooru, 2011). Variation in the chemical profile of extracts could influence their biological activities. Therefore, it was important to know the chemical composition of extracts to correlate with their antioxidant activities. A study conducted by Kalpanadevi and Mohan (2012) has shown that the extracts of *B. malabarica* Lam. and *B. floccifera* Bedd. contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. Kalpanadevi and Mohan (2012) have evaluated the total phenolic, flavonoid contents and *in vitro* antioxidant activity of methanol extracts of *B. malabarica* and *B. floccifera* whole plant. The methanol extracts of whole plants of *B. malabarica* and *B. floccifera* showed potent *in vitro* antioxidant activities using various models, i.e. DPPH, hydroxyl, superoxide and ABTS radical scavenging activity. *B. malabarica* and *B. floccifera* whole plant extracts (methanol) exhibited potent *in vitro* antioxidant activity in DPPH radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging, ABTS radical cation scavenging and reducing power in comparison to the known antioxidants, such as ascorbic acid and Trolox. It was observed that methanol extracts of the whole plant of *B. malabarica* had higher activity than that of the whole plant extract of *B. floccifera*. At a concentration of 1 $mg.mL^{-1}$, the scavenging activity of methanol extract of the whole plant of *B. malabarica* reached 96.14% while at the same concentration, that of the *B. floccifera* was 63.51%. At a concentration of 1 $mg.mL^{-1}$, the scavenging activity of methanol extract of the whole plant of *B. malabarica* exhibited higher activity than ascorbic acid. Superoxide radical scavenging activity of *B. malabarica* and *B. floccifera* whole plant extracts was studied and compared with ascorbic acid. It was observed, that the superoxide radical scavenging activity of *B. malabarica* and *B. floccifera* extracts increased with increasing concentration. At a concentration of 1 $mg.mL^{-1}$, the superoxide radical scavenging activity of methanol extracts of *B. malabarica* and *B. floccifera* were found to be 81.55 and 62.56%, respectively. Among the studied plant extracts, *B. malabarica* exhibited higher activity (79.11%) at a concentration of 1 $mg.mL^{-1}$ than Trolox. The reducing power of extracts increased with increase in concentration. Nevertheless, the reducing power values of the methanol extracts of *B. malabarica* whole plant was slightly higher than that of ascorbic acid (Kalpanadevi and Mohan, 2012).

Preliminary phytochemical screening of *B. floccifera* and *B. malabarica* conducted by Ariharan et al. (2012) showed the presence of a number of bioactive constituents, i.e. vitamin C. The contents of flavonoids (including glycosides of quercetin and kaempferol), anthocyanins and ascorbic acid in overground part of plants of 7 species and cultivars of *Begonia* genus (*B. bahiensis*, *B. bowerae*, *B. carolineifolia*, *B. fischeri*, *B. heracleifolia*, *B.* '*Erythrophylla*', *B.* '*Helen Teupel*') were determined by Karpova et al. (2009). The contents of flavonoids were 24–650 mg% of dry weight, including glycosides of quercetin – 3–76 mg%. Kaempferol and glycosides were detected only in species of section Gireoudia (1.2–5.7 mg%). The contents of anthocyanins were between 60 and 157 mg%, ascorbic acid – 5–43 mg% of fresh weight. Studied plants of *Begonia* can be considered as the sources of biologically active compounds with antioxidant and antimicrobial activity (Karpova et al. 2009). The results of the phytochemical screening of the methanolic flower extracts of *B. floccifera* revealed that phenol, tannins, xanthoproteins, steroids, tannins, phytosterols, triterpenoids, sapogenins, coumarins and carbohydrates (Jeeva and Marimuthu Antonisamy, 2012).

Many studies have suggested that plant secondary metabolites obtained from *Begonia*ceae representatives are responsible for their antioxidant activity. Literature data confirmed that extracts from various parts of the *Begonia* plants exhibited strong antioxidant properties, effectively deactivating the stable, synthetic DPPH radical (1,1-diphenyl-2-picrylhydrazyl). For example, Indrakumar et al. (2014) have evaluated the antimicrobial and *in vitro* antioxidant potential of extracts of *B. dipetala*. Antimicrobial activity, DPPH free radical scavenging activity, Superoxide anion scavenging activity, Nitric oxide scavenging activity, and Ferric reducing antioxidant power assay were carried out on different concentration of the extracts. The reducing power assay of ethanolic extract showed a reduction at various concentrations similar to that of standard ascorbic acid. DPPH scavenging activity of the ethanolic extract showed the IC_{50} value of 32.34 when compared to that of standard BHT which was 20.3. Scavenging activity showed IC_{50} Value of 165.45, nitric oxide scavenging activity showed the IC_{50} value of 134.20 when compared to that of standard ascorbic acid which was 32.14. The DPPH radical scavenging activity was higher (93.3%) when the concentration was increased. The reducing power of the extract increased with the increasing concentration. The *in vitro* antioxidant studies clearly indicate that the ethanolic extract of *B. dipetala* has significant antioxidant activity (Indrakumar et al., 2014).

The results of the Aswathy et al. (2016) study suggested the health-promoting properties of anthocyanin in *Begonia* cultivars (*B. heracleifolia* Cham. & Schltdl. and *B. malabarica* Lam. and three cultivars of *B. rex* (*B. rex* 'Baby Rainbow' L.H.Bailey, *B. rex* 'Black Beauty' & *B. rex* 'Sir Percy') in terms of their antioxidant activity, stable over time. Morphologically the cultivars showed variation among each other and showed variation in anthocyanin content, with the *B. rex* 'Baby Rainbow' and *B. rex* 'Black Beauty' possessing highest anthocyanin content which is morphologically distinguishable. Anthocyanin was found to be an effective antioxidant in different *in vitro* assays when compared to the standard antioxidants. The extracts of *Begonia* may have excellent potential as functional ingredients representing the potential source of natural antioxidant (Aswathy et al., 2016).

Methanol and ethyl acetate extract of *B. trichocarpa* has shown a marked dose-dependent antioxidant activity in both DPPH free radical scavenging method and Nitric acid scavenging method in the study of Sindhu et al. (2016). DPPH assay of methanol extract and ethyl acetate extract shows maximum% inhibition 53.0 and 50.93% at the concentration of 400 $\mu g.mL^{-1}$ respectively, whereas ascorbic acid exhibit 70.36% and IC_{50} values were 335.23, 370.74 and 16.84 $\mu g.mL^{-1}$ respectively. Nitric acid scavenging activity of methanol extract and ethyl acetate extract shows maximum% inhibition, i.e. 46.53% 27.36% respectively, while ascorbic acid exhibit 53.34%, IC_{25} values were found 150.87, 509.16 and ascorbic acid 0.633 $\mu g.mL^{-1}$. Total phenol content of different extracts of *B. trichocarpa* was estimated; out of this methanol extracts contain 49.96% of phenol content and 23.71% anthocyanin content present in the leaf. The antioxidant activity of *B. trichocarpa* may be due to the high phenol content and the presence of anthocyanin in the leaf give a supporting evidence for this (Sindhu et al., 2016).

The components derived from other species belonging to the Cucurbitaceae family also exert significant antioxidant activity both *in vivo* and *in vitro* study. For example, Arawwawala et al. (2011) have determined whether aerial parts of *Trichosanthes cucumerina* extracts can exert significant antioxidant activity in CCl_4-induced toxicity model in rats. The antioxidant activity of a hot water extract and a cold ethanolic extract of *T. cucumerina* aerial parts was evaluated by assessing its (a) radical scavenging ability and prevention effect of lipid peroxidation *in vitro*, and (b) effects on lipid peroxidation and antioxidant enzyme activities, *in vivo*. *In vitro* antioxidant assays (DPPH, TBARS, and carotene-linoleic acid assays) clearly demonstrated the antioxidant potential of *T. cucumerina* aerial parts extract. Moreover, hot water extract increased SOD activity (by 91.2%) and GPx activity (by 104.4%), while cold ethanolic extract increased SOD activity (by 115.5%) and GPx (by 96.4%) in CCl_4-induced toxicity model in rats. Treatments with hot water extract and cold ethanolic extract prevented the accumulation of lipid peroxidation products by 30.5 and 33.8%, respectively, in liver tissues compared to the rats exposed only to CCl_4 (Arawwawala et al., 2011).

Melon (*Cucumis melo* L.) concentrate exhibits an antioxidant capacity partly due to its high level of SOD activity, maybe in conjunction with other antioxidant compounds present in this melon extract associated with a relevant angiotensin 1-converting enzyme-inhibitory activity. Carillon et al. (2012) have assayed *in vitro* the antioxidant capacity and angiotensin 1-converting enzyme (ACE) inhibitory activity of a melon concentrate rich in superoxide dismutase. The total antioxidant capacity (TAC) was measured by the Trolox equivalent antioxidant capacity assay (TEAC), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay, and the ferric reducing antioxidant power assay (FRAP). The ability of the extract to scavenge three specific reactive oxygen species [superoxide radical anion ($O_2^{\cdot-}$), hydroxyl radical (HO·) and hydrogen peroxide (H_2O_2)] was also investigated in order to better evaluate its antioxidant properties. Even if the measures of TAC were relatively low, results clearly established an antioxidant potential of SOD-Melon concentrate that exhibited the highest radical-scavenging activity towards, with an IC_{50} 12-fold lower than that of H_2O_2 or HO·. This lets the hypothesis that the antioxidant potential of SOD-Melon concentrates could be mainly due to its high level of SOD (Carillon et al., 2012).

Chen et al. (2014) have delineated the antioxidant activity of Radix Trichosanthis (RT), the dry root tuber of *Trichosanthis kirilowii* Maxim (Cucurbitaceae) both *in vitro* and *in vivo* models by using ethyl acetate (EtOAc), n-butanol, and the mixture of n-butanol and EtOAc fractions. The *in vitro* antioxidant activity was detected by using DPPH free radical, hydrogen peroxide scavenging, and reducing power assays. After pretreatment with different fractions saponins at 2 and 3 $mg.kg^{-1}$/d of crude drug, respectively, an established CCl_4 induced acute cytotoxicity model was used to evaluate the *in vivo* antioxidant potential by detection of superoxide dismutase (SOD), malonaldehyde (MDA), lactate dehydrogenase (LDH), and total antioxidant capacity (T-AOC) levels. The *in vitro* assay showed that the antioxidant activity of all the three fractions was promising. The reducing power of the EtOAc and the mixture of n-butanol and EtOAc extracts increased in a dose-dependent manner. As for hydrogen peroxide scavenging capability, the n-butanol fraction mainly demonstrated a time-dependent manner, whereas the EtOAc fraction showed a dose-dependent manner. However, in case of *in vivo* assay, an increase of SOD and T-AOC and the decrease of MDA and LDH levels were only observed in n-butanol (2 $mg.kg^{-1}$/d of crude drug) extracts pretreatment group. RT saponins in n-butanol fraction might be a potential antioxidant candidate, as CCl_4-induced oxidative stress has been found to be alleviated, which may be associated with the time-dependent manner of n-butanol saponins in a low dose (Chen et al., 2014).

Conclusions

The results of this research indicated that crude extract obtained from *B. rex* leaves has the effective antioxidant effect after treatment of a suspension of equine erythrocytes lysed. Protective effect of *B. rex* extract is evident by amelioration in antioxidant enzymes' activities and the increase of total antioxidant capacity. The antioxidant defencse system was improved concurrence with suppression of aldehydic and ketonic derivatives of oxidatively modified proteins by treatment of *B. rex* extract. *B. rex* extract showed anti-inflammation effect exhibited as decreasing of ceruloplasmin level in the plasma. The pronounced effect of *B. rex* leaf extract, probably, could be attributed to its secondary metabolites content, e.g. polyphenols and flavonoids contents. Finally, further investigation is necessary to reveal the exact cellular mechanisms of the effect of *B. rex* extract on the erythrocyte function. These *in vitro* assays indicate that plant extract screened is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. In addition, suspension of equine erythrocytes lysed is a sensitive assay applied to detect the antioxidant effect of leaf extract obtained from *Begonia rex* Putz. on oxidative stress biomarkers. However, the components responsible for the antioxidative activity of *B. rex* extract is currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract.

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OXIDATIVE STRESS BIOMARKERS IN THE MUSCLE TISSUE OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) AFTER *IN VITRO* TREATMENT OF *SANSEVIERIA CAULESCENS* N.E.BR. EXTRACT

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The goal of this study was to assess *in vitro* the effect of buffer extract obtained from leaves of *Sansevieria caulescens* N.E.Br. on the 2-thiobarbituric acid reactive substances (TBARS) as lipid peroxidation biomarker, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity (TAC) in the muscle tissue of the rainbow trout. Our study suggests that the leaf *S. caulescens* extract have shown good antioxidant potential *in vitro* study after incubation with muscle tissue homogenate of rainbow trout. There were no significant changes for TBARS level as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins between values in control group and in the muscle tissue of rainbow trout after incubation with extracts from leaves of *S. caulescens*. Our results showed that extract of *S. caulescens* efficiently increased the total antioxidant capacity in muscle tissue by 46.6% (p <0.05) due to inhibited the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Taking into account existing experimental evidence, it is reasonable to assume that secondary plant metabolites, i.e. polyphenolic compounds in the extract of *S. caulescens* may contribute to the antioxidant activity. Further studies including both the use of other medicinal plants as food additives in aquaculture and the assessment of its antioxidant effects on various tissues are in progress.

Keywords: rainbow trout (*Oncorhynchus mykiss*), *Sansevieria caulescens* N.E.Br., 2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins, total antioxidant capacity

Introduction

The fishery is a source of income and social development, particularly in developing countries, playing a great role in food security and livelihood (FAO, 2016). Huge loss of production in aquaculture is occurring because of many reasons. Studies showed that almost fifty percent of

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production loss is because of diseases which are more severe in developing countries (Assefa et al., 2018). In recent years, to develop the alternative practices for disease management in aquaculture, attention was diverted to find novel drugs. Biological and chemical disease control strategies such as using probiotics, prebiotics, and medicinal plants are widely in use. Plant product application in aquaculture for disease control is one of the promising alternatives to antibiotics (Assefa et al., 2018). Plant-derived compounds act as a better antibacterial, antiviral, immunostimulant and antistress effect in fish and shellfish aquaculture (Anusha et al., 2014). Use of herbal therapy within animal production, as well as in the diet of commercial fishes has shown promise, in that it is natural and biodegradable and has antimicrobial activity against various pathogens, including those relating to fish (Valladão et al., 2015). The herbals having the characteristics of immunostimulants been able to increase the survival and reduce the pathogenic load against pathogenic challenge by improving the immune system in fishes (Anusha et al., 2014). On the other hand, many plant-derived compounds and medicinal plant products have been found to have non-specific immunostimulating properties in animals, of which more than a dozen have been evaluated in fin and shellfishes and, specifically, to prevent and control fish diseases (Galina et al., 2009). However, applying a new component as a drug in the fish diet requires more research on the effects on the physiological and health status of animals. Undoubtedly, a healthy diet is an important factor in the prevention of widespread various diseases in aquaculture. Therefore, the study of diet components such as dietary supplements, particularly drugs, is essential (Banaee et al., 2011).

Considering that almost all fish produced from aquaculture is for human consumption, residual drugs in fish products can affect people who consume them, and antimicrobials released into aquatic environments can select for resistant bacteria. Moreover, these antimicrobial-resistant bacteria, or their resistance genes, can be transferred to humans (Park et al., 2012).

In this study, attention was focused on *Sansevieria* Thunb., a genus with diverse ethnobotanical uses in its geographical distribution range, which occupies an important place among plant genera applied for treatment of a broad spectrum of diseases and disorders (Watt and Breyer-Brandwijk, 1962; Chhabra et al., 1987; Khalumba et al., 2005; Staples and Herbst, 2005; Kiringe, 2006; Owuor and Kisangau, 2006; Takawira-Nyenya et al., 2014). Our previous study (Buyun et al., 2016; Tkachenko et al., 2017) have highlighted the antibacterial capacity of ten species of *Sansevieria* genus against *Staphylococcus aureus*. These plants have been screened in order to validate scientifically the inhibitory activity for microbial growth attributed to their popular use and to propose new sources of antimicrobial agents. The leaves of *Sansevieria canaliculata* Carrière, *S. trifasciata* Prain, *S. cylindrica* Bojer ex Hook., *S. parva* N.E.Br. (syn. *S. dooneri* N.E.Br.), *S. fischeri* (Baker) Marais, *S. kirkii* Baker, *S. aethiopica* Thunb., *S. metallica* Gérôme & Labroy, *S. caulescens* N.E.Br., *S. francisii* Chahin were used. Our results proved that the zones of inhibition ranged from 16 to 34 mm. Extracts from the leaves of *S. fischeri* and *S. francisii* were particularly active against tested organism (inhibition zones comprise up to 34 mm in diameter). This was followed by the activities of extracts from the *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* leaves (diameters of inhibition zones ranged from 25 to 31 mm). The ethanolic extracts of *S. canaliculata* and *S. trifasciata* showed less antimicrobial activities (diameters of inhibition zones ranged between 16 and 16.5 mm). The results

proved that the ethanolic extracts from *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* exhibit a favorable antibacterial activity against *S. aureus* (Buyun et al., 2016; Tkachenko et al., 2017).

In our previous study, we also studied the antioxidant activity of extracts obtained from leaves of selected species from *Sansevieria* species against oxidative stress using equine erythrocyte suspension (Tkachenko et al., 2017). When erythrocytes were incubated with leaf extracts of various species from *Sansevieria* genus, the aldehydic derivatives level was significantly reduced by 13.6% (p <0.05) for *S. forskaliana* extract. Moreover, all extracts (except *S. francisii* extract) reduced the formation of intracellular aldehydic derivatives of oxidatively modified proteins (OMP) in the extracts-treated erythrocytes, but these results were non-significant. Treatment by extracts of various *Sansevieria* species reduced the concentration of ketonic derivatives of OMP when compared to untreated erythrocytes. The most potent effect was demonstrated by the *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* compared to control samples (phosphate buffer) (16.1, 14.7, 13.4, 12.9, 12.9, 12.7, 12.1%, respectively). However, there were no significant changes in other extracts. The experimental evidence obtained in our previous study indicated that various species of *Sansevieria* genus are a rich source of compounds that manifest antioxidant activity and can effectively protect erythrocytes against oxidative-induced damage. Thus, *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* may be a valuable source of natural antioxidants that may potentially be recommended for applications in medicine and veterinary practice. According to the above-mentioned antioxidant mechanisms, extracts of various species from *Sansevieria* genus may inhibit the formation of protein carbonyl by scavenging free radicals formed *in vitro*. According to many supporting documents, it can be assumed that secondary plant metabolites, i.e. polyphenolic compounds in extracts of various species from *Sansevieria* genus extract may contribute to the antioxidant activity (Tkachenko et al., 2017).

Although antimicrobial activities of extracts obtained from the leaves of various species of *Sansevieria* genus were investigated so far (Aliero et al., 2008; Sheela et al., 2012; Kingsley et al., 2013; Buyun et al., 2016, 2017; Tkachenko et al., 2017), there is still much work to do, because studies regarding their total antioxidant defences as well as assessment of marker of lipid peroxidation under *in vitro* incubation with the muscle tissue of the rainbow trout (*Oncorhynchus mykiss* Walbaum) have not been undertaken yet.

Consequently, the aim of this study was to evaluate *in vitro* the effect of buffer extract obtained from leaves of *Sansevieria caulescens* on the 2-thiobarbituric acid reactive substances (TBARS) as lipid peroxidation biomarker, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the muscle tissue of the rainbow trout.

Materials and methodology

Collection of plant material

The leaves of *Sansevieria caulescens* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine (Figure 1).

Figure 1 General view of *Sansevieria caulescens* N.E.Br. plant (Photo by Myroslava Maryniuk)

Preparation of plant extract

Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extract was then filtered and investigated for its antioxidant capacity. The extract was stored at -20 °C until use.

Experimental fish

Clinically healthy rainbow trout with a mean body mass of 80–120 g were used in the experiments. The experiments were performed in water at 14.5 ±0.5 °C and pH 7.2–7.4. The dissolved oxygen level was about 9 ppm with additional oxygen supply, with a water flow of 25 $L.min^{-1}$, and a photoperiod of 12 h per day. The same experimental conditions were used during the whole research. The water parameters were maintained under constant surveillance. The fish were held in square tanks (150 fish per tank) and fed commercial pelleted diet.

Muscle tissue samples

The muscle tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2) using a glass homogenizer immersed in an ice water bath. Homogenates were centrifuged at 3000 g for 15 min at 4 °C. After centrifugation, the supernatant was collected and frozen at -20 °C until analyzed. All enzymatic assays were carried out at 22 ±0.5 °C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate. The reactions were started by adding the tissue supernatant.

Experimental design

The supernatant of the muscle tissue was used to incubate with *S. caulescens* extract (in a ratio of 19 : 1) at room temperature. The control group (trout muscle tissue) was incubated with 100 mM Tris-HCl buffer (pH 7.2) (in a ratio 19:1). The incubation time was 2 hours. Oxidative stress biomarkers were studied in the incubated homogenate (control group and in samples with extract).

Determination of 2-thiobarbituric acid reactive substances (TBARS)

The level of lipid peroxidation was determined by quantifying the concentration of TBARS by Kamyshnikov (2004) for determining the malonic dialdehyde (MDA) concentration. Briefly, 2.1 mL of sample homogenate was added to 1 mL of 0.8% of 2-thiobarbituric acid (TBA), and 1 mL of 20% of trichloroacetic acid (TCA). The mixture was heated in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The concentration of MDA (nmol.mg^{-1} of protein) was calculated using 1.56 10^5 mM^{-1} cm^{-1} as the extinction coefficient.

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

To evaluate the protective effects of the extract against free radical-induced protein damage, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the samples was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. 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 addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 h 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 3000 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 22.000 M^{-1}·cm^{-1}. Carbonyl groups (nmol per mg of protein) were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/

ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37 °C. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences (significance level, p <0.05) was examined using the Mann-Whitney U test (Zar, 1999). All statistical calculation was performed on separate data from each individual with STATISTICA 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

In a present study, we have studied the influence of extracts derived from leaves of *S. caulescens* on the TBARS level as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins and the total antioxidant capacity in the muscle tissue of rainbow trout after incubation with extract obtained from leaves of *S. caulescens* in *in vitro* culture. There were no significant changes for TBARS level between the value in the control group and in the muscle tissue of rainbow trout after incubation with extracts derived from leaves of *S. caulescens* (Figure 2).

It is considered that disturbed levels of reactive oxygen species (ROS) contribute to the pathology of many disorders and diseases. ROS and oxidative stress may cause cellular damage by directly and irreversibly damaging macromolecules such as proteins, membrane lipids, and DNA; another major cellular consequence of reactive oxygen species is the reversible modification of protein thiol side chains that may affect many aspects of molecular function (Terrill et al., 2013). The redox dysregulation and ROS do not just beget further reactive species but also drive antioxidant protein expression. The redox-regulated antioxidant response offers the possibility of controlling the extent of ROS available to damage proteins and to restore the intracellular redox potential (Griffiths et al., 2014). In our study, the level of aldehydic and ketonic derivatives of oxidatively modified proteins was non-significantly changed in muscle samples incubated with an extract obtained from the leaves of *S. caulescens* (Figure 2).

The total antioxidant capacity (TAC) is determines the ability of a tested material to neutralize oxygen-free radical specific form, irrespectively to the specific antioxidant activity of present antioxidants (Wang et al., 1997). Our results showed that extract of *S. caulescens* efficiently

increased the TAC level in muscle tissue by 46.6% (p <0.05) due to inhibited the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level (Figure 2).

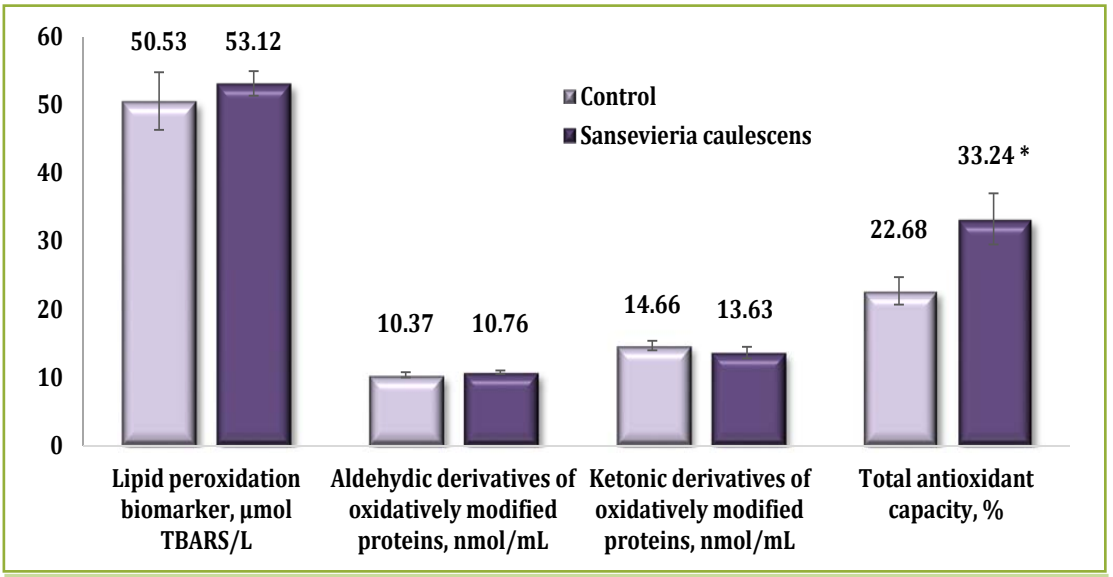

Figure 2 The level of 2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins and total antioxidant capacity in the muscle tissue of rainbow trout after incubation with extracts from leaves of *Sansevieria caulescens* (M ±m, n = 8)
* the changes are statistically significant (p <0.05) compared to the control group

We suggested that high TAC value of muscle tissue is the result of a high content of by-products, i.e. alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, proteins, carbohydrates etc. in *S. caulescens*. The antioxidant activities of most plant extracts can be traced to these bioactive constituents. For instance, various compounds belonging to the terpenoid and flavonoid groups are known to be biologically active (Grassmann, 2005; González-Burgos and Gómez-Serranillos, 2012; Bartikova et al., 2014). Due to their antioxidant behaviour terpenes have been shown to provide relevant protection under oxidative stress conditions in different diseases including liver, renal, neurodegenerative and cardiovascular diseases, cancer, diabetes as well as in ageing processes (González-Burgos and Gómez-Serranillos, 2012). The existing data indicate that monoterpenes and diterpenes, which are the main components of essential oils, act as allelopathic agents, attractants in plant-plant or plant–pathogen/herbivore interactions or repellants (Grassmann, 2005). Moreover, many sesquiterpenes biological activities (anti-inflammatory, antiparasitic and anti-carcinogenic activities) are based on antioxidant or pro-oxidant actions of sesquiterpenes. Structure, concentration, metabolism as well as the type of cells determine if sesquiterpene acts as anti-oxidant or pro-oxidant (Bartikova et al., 2014). On the other hand, the natural flavones, as well as some of their synthetic derivatives, have been shown to exhibit several biological activities, including antioxidant, anti-inflammatory, antitumor, anti-allergic, neuroprotective, cardioprotective and antimicrobial (Catarino et al., 2015). Also, flavonoids are found to have an effect on several

mammalian enzymes like protein kinases that regulate multiple cell signalling pathways and alterations in multiple cellular signalling pathways are frequently found in many diseases (Singh et al., 2014). Some flavones interfere in distinct oxidative-stress related events by directly reducing the levels of intracellular free radicals (hydroxyl, superoxide and nitric oxide) and/or of reactive species (e.g. hydrogen peroxide, peroxynitrite and hypochlorous acid) thus preventing their amplification and the consequent damage of other biomolecules such as lipids, proteins and DNA (Catarino et al., 2015). Flavones and flavonols re-establish the redox regulation of proteins, transcription factors and signalling cascades that are otherwise inhibited by elevated oxidative stress (Dajas et al., 2013). Flavones can also hinder the activity of central free radical-producing enzymes, such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) or inducible nitric oxide synthase (iNOS) and can even modulate the intracellular levels of pro-oxidant and/or antioxidant enzymes (Catarino et al., 2015).

Indeed, the study of *S. roxburghiana* and *S. trifasciata* has revealed the presence of important compounds which were separated by thin layer chromatography (Kingsley et al., 2013). Preliminary phytochemical screening of the extracts of *S. trifasciata* plant showed the presence of alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, proteins and carbohydrates (Anbu et al., 2009). Additionally, the methanolic extract of the whole plant of *S. trifasciata* has yielded 12 steroidal saponins, 10 of which are new constituents (Mimaki et al., 1996). Phytochemical analysis of the whole plant of *S. trifasciata* has resulted in the isolation of four new pregnane glycosides (Mimaki et al., 1997). Gas chromatographic analysis of the leaves revealed the presence of alkaloids, allicins, glycosides, and saponins (Ikewuchi et al., 2011). Pettit et al. (2005) have isolated three new spirostanol saponins designated sansevierin A (1), sansevistatin 1 (2), and sansevistatin 2 (3) (10^{-5}% yield) from the CH_3OH-CH_2Cl_2 extract of *S. ehrenbergii*, accompanied by three known steroidal saponins (4–6), using bioactivity-directed isolation procedures. Each of the saponins was evaluated against the P388 lymphocytic leukemia cell line and a panel of human cancer cell lines. Except for 1, all were found to cause inhibition of cancer cell growth. In addition, most of the saponins exhibited antimicrobial activity, particularly against the pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* (Pettit et al., 2005). In addition, a new steroidal saponin from the leaves of *S. cylindrica* was isolated by Da Silva Antunes et al. (2003). The steroidal saponin showed no hemolytic effects in the *in vitro* assays and demonstrated inhibition of the capillary permeability activity (Da Silva Antunes et al., 2003). Accumulated evidence suggests that saponins have significant neuroprotective effects on attenuation of central nervous system disorders, such as stroke, Alzheimer‘s disease, Parkinson‘s disease, and Huntington‘s disease due to mechanisms of their neuroprotective function including antioxidant, modulation of neurotransmitters, anti-apoptosis, anti-inflammation, attenuating Ca^{2+} influx, modulating neurotrophic factors, inhibiting tau phosphorylation, and regeneration of neural networks (Sun et al., 2015).

Our study is in agreement with results obtained by other researchers. They have revealed hypoglycemic, hypolipidemic, immune-modulating, anti-inflammatory, ocular-, hepato-renal and cardio-protective potentials of other species of *Sansevieria* genus. Chigozie and Chidinma

(2013) have investigated the ability of an aqueous extract of the leaves of *S. liberica* to alter the hematology, plasma biochemistry and ocular indices of oxidative stress in alloxan-induced diabetic rats. Diabetes mellitus was induced by injection of alloxan (80 $mg.kg^{-1}$ body weight), via the tail vein. The extract was administered orally at 100, 200 and 300 $mg.kg^{-1}$ body weight (both to normal and diabetic rats), and metformin at 50 $mg.kg^{-1}$ body weight. Compared to test control, the treatment dose-dependently, significantly lowered (p <0.05) ocular malondialdehyde content, atherogenic indices, red cell, total white cell and lymphocyte counts, mean cell hemoglobin concentration; and plasma levels of glucose, triglyceride, total-, very low density lipoprotein-, low density lipoprotein- and non-high density lipoprotein cholesterols, total, conjugated and unconjugated bilirubin, sodium, urea, blood urea nitrogen, as well as plasma activities of alkaline phosphatase, alanine and aspartate transaminases. However, the treatment significantly increased (p <0.05) hematocrit, hemoglobin concentration, mean cell hemoglobin, and mean cell volume, neutrophil and monocyte counts, and plasma levels of high density lipoprotein cholesterol, potassium, chloride, calcium, bicarbonate and total protein, ocular ascorbic acid content and ocular activities of catalase and superoxide dismutase (Chigozie and Chidinma, 2013).

The leaves of *S. liberica* also possess anti-inflammatory effects. Chinasa et al. (2011) have evaluated the anti-inflammatory property of the leaves of *S. liberica* and to ascertain the toxicity and phytochemical profiles of the extract of the leaves. The crude extract was then fractionated into n-hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF) and methanol fraction (MF). The crude extract and the fractions were screened for anti-inflammatory activity using egg albumen-induced paw (systemic) edema in rats as a measure of acute inflammation. The crude extract and the fractions significantly (p <0.05) inhibited the development of paw edema induced by egg albumen in rats. The potency/activity of the crude extract and the fractions increased in the order HF > CE > MF > CF > EF, with the crude extract and HF at 400 $mg.kg^{-1}$ exhibiting inhibition comparable to that obtained with 5 $mg.kg^{-1}$ diclofenac sodium. Phytochemical screening of the crude extract and the fractions showed the presence of various bioactive substances such as alkaloids, saponins, flavonoids, terpenoids, steroids, glycosides, reducing sugars, tannins, resins, carbohydrates, proteins, acidic compounds, fats and oils (Chinasa et al., 2011).

The extract of the whole plant part of *S. roxburghiana* might possess some chemical constituents that are responsible for analgesic, cytotoxic and antioxidant activities. Roy et al. (2012) have investigated the crude methanolic extract of the whole plant part of *S. roxburghiana* and the possibility of analgesic, cytotoxic and antioxidant activities. The analgesic activity was assessed by acetic acid induced writhing test in mice. The cytotoxic activity was evaluated by brine shrimp lethality bioassay while the antioxidant effect was measured by 1, 1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) free radical scavenging assay. The ethyl acetate soluble fraction of the crude extract was found to have significant (p <0.001) analgesic activity at the oral dose of 100 $mg.kg^{-1}$ body weight. The crude methanolic extract along with its all partitions revealed mild to moderate free radical scavenging activity (Roy et al., 2012).

Oxidative stress is considered to be an important component of various diseases. Identifying markers of oxidative stress has been the focus of many researchers as they have the potential

to act as an "integrator" of a multitude of processes that drive various kind of pathobiology (Ho et al., 2013). Therefore, in our opinion, any research assessing the biomarkers of oxidative stress has fundamental importance for the treatment of various diseases and disorders. On the other hand, plants with antioxidant potential may offer alternative therapeutic agents in the aquaculture industry.

Conclusions

Our study suggests that the *S. caulescens* leaf extract has shown good antioxidant potential *in vitro* study after incubation with muscle tissue homogenate of rainbow trout. There were no significant changes for TBARS level as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins between values in control group and in the muscle tissue of rainbow trout after incubation with extracts from leaves of *S. caulescens*. Nevertheless, our results showed that extract of *S. caulescens* efficiently increased the total antioxidant capacity in muscle tissue by 46.6% (p <0.05) due to inhibited the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Taking into account existing experimental evidence, it is reasonable to assume that secondary plant metabolites, i.e. polyphenolic compounds in the extract of *S. caulescens* may contribute to the antioxidant activity. In conclusion, the results of this study provide a new perspective on the use of various *Sansevieria* species as a medicinal plant to improve the antioxidant response of rainbow trout. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of its antioxidant effects on various tissues are in progress. Finally, research needs to be focused on subjecting fish to these compounds to determine their effectiveness, stability, and impact both on the host and on the environment.

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CHARACTERISTIC OF LEAF PELTATE GLANDULAR TRICHOMES AND THEIR VARIABILITY OF SOME LAMIACEAE MARTINOV FAMILY SPECIES

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Glandular trichomes are important taxonomic characters in the Lamiaceae family. The objects of our investigations were plants of Lamiaceae family collected from the experimental base of Institute of Rice of the National Academy of Agrarian Sciences (Plodove, Ukraine). Fresh leaves were collected for the morphological analyse. The dimensions of glands (diameter in μm) were measured using of macroscope Discovery V12 Carl Zeiss at the Institute of Biodiversity Conservation and Biosafety of the Slovak University of Agriculture in Nitra (Slovakia). Peltate glandular trichomes with essential oil are localized on the adaxial and abaxial leaf surfaces. Their size on the adaxial and abaxial leaf sides is different. The diameter of trichomes on the adaxial and abaxial side of leaves was 65.70 and 61.41 (*Nepeta transcaucasica* Grossh.), 70.10 and 74.54 (*Salvia officinalis* L.), 72.02 and 67.39 (*Monarda fistulosa* L.), 80.53 and 85.11 (*Ocimum basilicum* L.), 94.35 and 105.12 (*Satureja montana* L.), 95.11 and 60.37 (*Thymus serpyllum* L.), 96.48 and 90.57 (*Thymus vulgaris* L.), 106.88 and 107.69 (*Hyssopus officinalis* L.), 110.61 and 88.22 (*Hyssopus angustifolius* Bieb.), respectively. It was noted that the leaf adaxial side of *Salvia sclarea* L. had no glandular trichomes of lamiaceous type at all but on an abaxial side of leaves only with the diameter 96.47 μm. The tested plant species showed significant differences in size and colour of peltate glandular trichomes producing essential oils. This difference can be used during macroscopic analysis of herbal substances. Aromatic plants that were grown in Steppe zone of South Ukraine are perspective plants because of presence a well-generated excretory system which contains essential oils.

Keywords: Lamiaceae, leaf, glandular trichome, morphometric characteristic

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Introduction

Medicinal herbs producing essential oils are economically important for food, pharmacy and cosmetics industry (Mashanov et al., 1988, Shanaida et al., 2018). Therefore, there are many teams oriented on the experimental study of these species (Nesterenko et al., 1937; Muhortova et al., 1973; Simonov et al., 1987; Hlypenko et al., 1987; Rabotyagov and Korsakova, 2000; Kovtun-Vodyanytska, 2017). Species investigation is often focused on cultivation environment and plant ontogenesis effects on physiological/biochemical processes leading to synthesis and production of essential oils (Korsakova, 1998; Mishurova, 1991; Scharapov et al. 1987; Svidenko a Rabotyagov, 2000). Many medicinal plants differ in amounts of essential oil produced by stalks, leaves, inflorescences and in structure, localization and density of glandular trichomes. Inflorescences and leaves produce the highest amount of ether oils (Svidenko, 1999). It is generally accepted that essential oils are formed in unicellular or multicellular glandular trichomes (peltate and capitate ones) of some members of Lamiaceae Martinov (Baran et al., 2010). According to the European Pharmacopeia, peltate glandular trichomes in members of the Lamiaceae family are also named as secretory trichomes of lamiaceous type or glandular trichomes of the lamiaceous type consisting of epidermal cells, a short stalk cell and a multicellular head (European pharmacopoeia, 2016). In the Lamiaceae family, the morphology, distribution and frequency of glandular trichomes are employed as discriminative characters at subfamily level (Baran et al., 2010, Atalay et al., 2016,). Most of the aromatic genera are in the subfamily Nepetoidae (Baran et al., 2010). As a rule, peltate glandular trichomes have a small, rounded, unicellular stalk and a globular or ovoid head composed of 4, 8 or 12 radiating cells with a raised common cuticle (Baran et al., 2010; Kahraman, 2010; European pharmacopoeia, 2016). Sometimes, a head can even contain 4–8 or 14–18 cells (Marin et al., 2012; Atalay et al., 2016; Shanaida, 2016). A large secretory head usually comprises 1, 2, 4 central cells and 4, 6, 8–10 or 6–14 peripheral cells arranged in one-two concentric circles (Baran et al., 2010; Kahraman, 2010; Atalay et al., 2016). There is a confirmation of the relation between the number and size of glands and amount of produced oils by given plant part. Currently, it is proved that the synthesis and accumulation of essential oils basically take place in glandular cells (Malankina, 2007). There are number of studies on trichome morphology of the Lamiaceae family (Serrato-Valenti et al., 1997; Corsi and Bottega, 1999; Kaya et al., 2003; Krstic et al., 2006; Kamatou et al., 2007; Bagherpour et al., 2010; Kahraman et al., 2010; Seyedi and Salmaki, 2015; Atalay et al., 2016), which confirmed, that the presence of trichomes, their structure and localization character are diagnostic features of taxons and have a significant value in pharmacognosy practice (Shanaida, 2016). Kondratenko (1975) determined the direct correlation between the specialized glands number and the essential oils production in the experiments with the species *Thymus vulgaris* L. adaptability and mutation variability. Based on breeding activities, Rabotyagov (1983) elaborated a model of lavender productivity, documenting the dependence of essential oils production by plant on the number of flowers and amount of glands on plant parts. In spite of the above-mentioned information, there is still a lack of the knowledge of inter-species and intra-species differences as well as the numbers and size of plant glands in some species of the Lamiaceae family. Hence, our experimental activities were oriented on variability study of glandular trichomes dimensions of the lamiaceous type some species of Lamiaceae family.

Material and methodology

Locating plants and data collection

Plant material collected from the experimental base of Institute of Rice of the National Academy of Agrarian Sciences (Plodove, Ukraine) and fresh leaves were collected for the morphological analyses. Microscopic study of localization glandular trichomes producing essential oil was conducted at the Institute of Biodiversity Conservation and Biosafety at Slovak University of Agriculture in Nitra (Slovakia). The size of glands occurring on leaves was experimentally evaluated for 10 selected species: *Thymus vulgaris* L., *Thymus serpyllum* L., *Hyssopus angustifolius* Bieb., *Hyssopus officinalis* L., *Satureja montana* L., *Salvia officinalis* L., *Salvia sclarea* L., *Monarda fistulosa* L., *Ocimum basilicum* L., and *Nepeta transcaucasica* Grossh. Plant samples were collected between 2015–2016.

Morphometric characters

The dimensions of glands (diameter in μm) were measured on leaves using of macroscope Discovery V12 Carl Zeiss. 5 plant leaves were investigated from every species. 25 measuring were conducted on every side of the leaf. Summarizing whole study, 1250 measurements were conducted.

Statistical analyses

Basic statistical analyses were performed using PAST 2.17; hierarchical cluster analyses of similarity between species were computed on the basis of the Bray-Curtis similarity index.

Results and discussion

Glandular trichomes are important taxonomic characters in the Lamiaceae family (Xiang et al., 2010). Significant differences were detected in numbers, shape and colour of peltate glandular trichomes (Figure 1). The colour of these trichomes of different plant species varied from transparent to light-yellow or from transparent to red-brown, which depends on the stage of trichome growth. The colour of secretory trichomes of lamiaceous type could be used as an additional tool at carrying out macroscopic pharmacognostic analysis.

Our study confirmed that size and distribution of peltate glandular trichomes have valuable taxonomical significance at the species level (Xiang et al., 2010; Marin et al., 2012; Atalay et al., 2016). Our study established that the size of peltate glandular trichomes depends on species of plants that conforms with conclusions of Baran et al. (2010). Peltate glandular trichomes with essential oil are localized on the adaxial and abaxial leaf surfaces. Their size on the adaxial and abaxial leaf sides is not the same. These trichomes were found of minimum and maximum dimensions from 55.30 (*Nepeta transcaucasica*) to 91.45 μm (*Hyssopus officinalis*) and from 80.19 (*Monarda fistulosa*) to 142.89 μm (*Thymus serpyllum*), respectively (Table 1). On the abaxial side of leaves, peltate glandular trichomes were found and had minimum dimensions from 45.36 (*Nepeta transcaucasica*) to 93.19 μm (*Hyssopus officinalis*) and maximum dimensions from 72.74 (*Thymus serpyllum*) to 123.08 μm (*Hyssopus officinalis*).

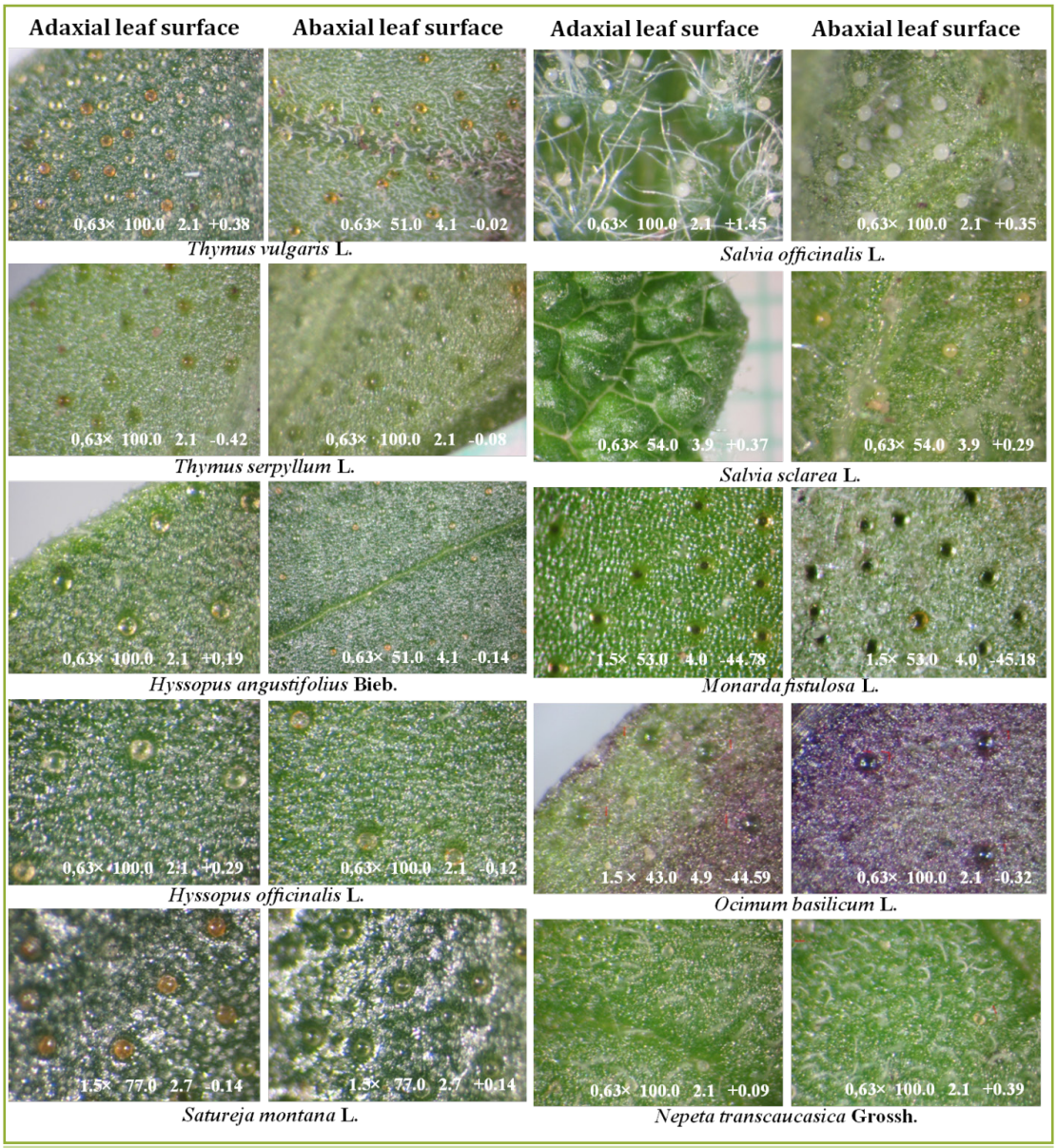

Figure 1 Size, shape and colour of peltate glandular trichome on the adaxial and abaxial leaf surfaces for the selected species of Lamiaceae Martinov family

Table 1 Variability of the peltate glandular trichome average size on the adaxial and abaxial leaf surface for selected species of Lamiaceae Martinov (μm)

Plant species	Leaf side	min	max	Sx	V%
***Thymus vulgaris* L.**	adaxial	85.61	113.02	7.47	7.74
	abaxial	77.96	105.97	8.08	8.92
***Thymus serpyllum* L.**	adaxial	59.13	142.89	28.49	29.95
	abaxial	49.96	72.74	5.65	9.35
***Hyssopus officinalis* L.**	adaxial	91.45	173.9	16.71	15.64
	abaxial	93.19	123.08	8.41	7.81
***Hyssopus angustifolius* Bieb.**	adaxial	90.09	133.89	13.44	12.15
	abaxial	78.72	97.43	4.40	4.99
***Satureja montana* L.**	adaxial	79.91	109.09	7.56	8.01
	abaxial	73.67	120.04	11.07	10.53
***Salvia sclarea* L.**	adaxial	not detected			
	abaxial	80.93	107.83	6.98	7.23
***Salvia officinalis* L.**	adaxial	60.33	85.21	6.09	8.69
	abaxial	65.19	83.95	4.83	6.48
Monarda ***fistulosa* L.**	adaxial	64.55	80.19	4.41	6.13
	abaxial	58.61	77.63	5.88	8.73
***Ocimum basilicum* L.**	adaxial	68.79	92.96	7.25	9.00
	abaxial	67.70	99.09	8.66	10.18
***Nepeta transcaucasica* Grossh.**	adaxial	55.30	103.25	10.53	16.02
	abaxial	45.36	81.48	6.77	11.03

Note: min, max – minimal and maximal measured values; Sx – standard deviation; V – coefficient of variation (%)

According to average values, the least trichomes on the adaxial and abaxial sides of the leaves were found for *Nepeta transcaucasica* – 65.70 and 61.41 μm, respectively (Figure 2).

The biggest size of peltate glandular trichomes on the adaxial side of the leaves was found for *Hyssopus angustifolius* (110.61 μm) and on the abaxial side of the leaves of *Hyssopus officinalis* (107.69 μm). Seven species formed larger glands on the adaxial side of the leaves compared to the abaxial ones. Larger peltate glandular trichomes on the upper side of leaves are characteristic for the species *Satureja montana*, *Ocimum basilicum* and *Salvia officinalis*. *Hyssopus officinalis* formed uniformly large glands on both adaxial and abaxial sides of the leaf.

The large peltate glandular trichomes are located in deepenings on the adaxial and abaxial leaf surfaces and are densely distributed on the adaxial and abaxial leaves surface of *Satureja montana* that is in line with studies of Marin et al. (2012).

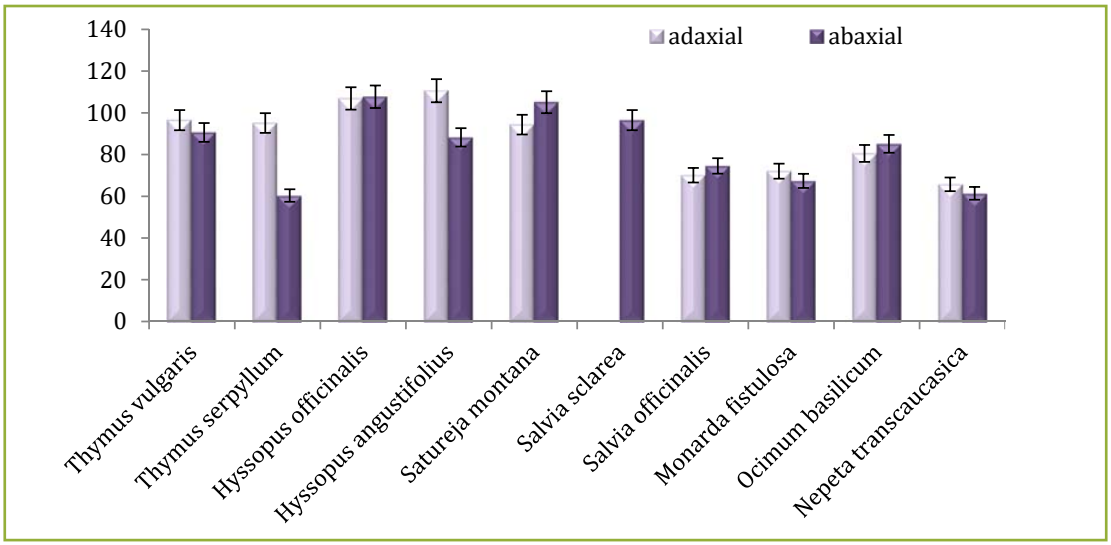

Figure 2: Mean value glandular trichome on the adaxial and abaxial side of leaves for selected species of family Lamiaceae Martinov

High degree of variability has been found with peltate glandular trichomes size of *Thymus serpyllum* species, where the variability degree exceeded 20%. A moderate variability degree (from 10 to 20%) of glands size showed the species *Hyssopus angustifolius*, *Hyssopus officinalis*, *Satureja montana*, *Ocimum basilicum* and *Nepeta transcaucasica*. Low degree of variability (up to 10%) of glands size has been detected by species *Thymus vulgaris*, *Salvia officinalis*, and *Monarda fistulosa*.

The adaxial surface of *Thymus vulgaris* leaves has more glandular trichomes of lamiaceous type compared to the abaxial surface of ones. The two surfaces *Thymus serpyllum* leaves contain approximately the same quantity of these glandular trichomes. The numerous glandular trichomes of the lamiaceous type of amber or reddish brown colour had a shiny spherical shape. However, it was noted that *Thymus serpyllum* had significantly less of these glandular trichomes with yellow or brown colour compared to *Thymus vulgaris* that could be explained by less yield of essential oil (minimum 12 $mL.kg^{-1}$) for *Thymus vulgaris* (anhydrous drug) and minimum 3.0 $mL.kg^{-1}$ of essential oil for whole or cut, dried, flowering aerial parts of *Thymus serpyllum* (European pharmacopoeia, 2016).

Our study also demonstrated that two surfaces of *Salvia officinalis* leaves had approximately the same quantity of these glandular trichomes. However, it was noted that the leaf adaxial side of *Salvia sclarea* had no glandular trichomes of lamiaceous type at all. Such a difference could be explained by less yield of essential oil (0.19–0.38%) for *Salvia sclarea* and minimum 15 $mL.kg^{-1}$ of essential oil for the whole dried drug or minimum 10 $mL.kg^{-1}$ of essential oil for the cut drug (anhydrous drug) of *Salvia officinalis* (European pharmacopoeia, 2016).

The feature of the glandular trichomes of lamiaceae type of *Monarda fistulosa* L. is their location. They are located below of epidermis surface in the mesophyll that conforms with studies conducted by Shanaida et al. (2016).

The feature of the glandular trichomes of lamiaceae type of *Hyssopus angustifolius* Bieb. is a distinction in their sizes on both leaf sides. These glandular trichomes are significantly larger on the adaxial side. While glandular trichomes of lamiaceae type of *Hyssopus officinalis* are the same size on both leaf sides.

Based on cluster analyze was done a dendrogram (Figure 3). In this study, 10 species were grouped into three main clusters based on the highest similarities.

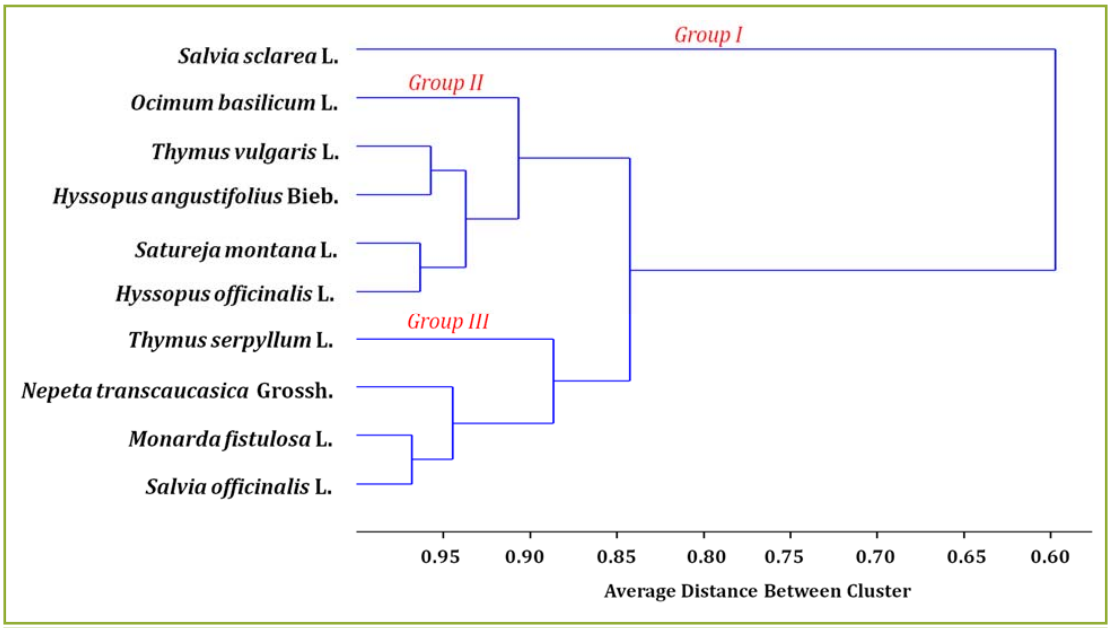

Figure 3 Cluster dendrogram based on morphometrics parameters adaxial and abaxial surfaces of leaves for selected species of family Lamiaceae Martinov

On the dendrogram, it can be seen that *Salvia sclarea* (Group I) really differs from the other samples because peltate glandular trichomes on the adaxial leaf surface have not been determined. Only simple trichomes were found. *Salvia sclarea* formed peltate glandular trichomes only on the abaxial leaf surface. Group II included the species which had the least signs comparing with Group III.

Conclusion

The tested plant species showed significant differences in size and colour of peltate glandular trichomes producing essential oils. This difference can be used during macroscopic analysis of herbal substances. Aromatic plants that were grown in Steppe zone of South Ukraine are perspective plants because of presence a well-generated excretory system which contains essential oils. It is planned to continue the investigation of morphological and anatomical properties of peltate glandular trichomes with essential oils and to conduct a study of chemical analysis of the essential oils.

Acknowledgement

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ANTIOXIDANT POTENTIAL OF SELECTED OIL PLANTS OF BRASSICACEAE BURNETT

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The economically important species of genus *Brassica* L. were investigated in this study. Alcohol and water plant extracts of cultivars and varieties of *Brassica campestris* and *B. rapa* were inspected on antiradical activity by DPPH-method (with 2,2-diphenyl-2-picrylhydrazyl). Obtained extracts were measured on a spectrophotometer at wavelength 515 nm. Antiradical activity of methanol extracts was in the range from 34.29 (*B. rapa* subsp. *rapifera* Metzger (f. *biennis*), f. EOTRFO) to 58.09% (*B. campestris* f. *biennis* D.C., cv. Oriana). Water extracts demonstrated this activity in range from 58.18 (*B. campestris* f. *biennis* D.C., cv. Oriana) to 84.25% (*B. campestris* f. *biennis* D.C. × *B. rapa* L., f. EOTFVS). Antioxidant activity (AA) of extracts of investigated plants was expressed in ascorbic acid equivalent (mg g^{-1} AAE). The highest AA of methanol extracts was found for *B. campestris* f. *biennis* D.C. × *B. rapa* L., cv. Fitopal (86.07 mg g^{-1} AAE), the lowest one – for *B. rapa* subsp. *rapifera* Metzger (f. *biennis*), f. EOTRFO (52.78 mg g^{-1} AAE). Maximal AA of water extracts was registered for *B. campestris* f. *biennis* D.C. × *B. napus* f. *biennis* D.C., cv. Innovacia (121.77 mg g^{-1} AAE), minimal – for *B. campestris* f. *biennis* D.C. × *B. rapa* L., f. EOTFVS (85.18 mg g^{-1} AAE).

Keywords: oil plants, *Brassica*, antioxidant activity

Introduction

The Brassicaceae Burnett one is the most important group of plants in the food industry that includes a wide range of horticultural crops with economic significance. The high content of lipids in the seeds (more than 40%) makes this group of crops very valuable among other plants (Hodur et al., 2012; Chen et al., 2015). The last study concerning of the Brassicaceae has demonstrated results about their human health benefits such as reduced risk for generative diseases. It is a good source of carotenoids (Bjorkman et al., 2011; Kumar and Andy, 2012).

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The genus *Brassica* L. is the most important from Brassicaceae, which includes some crops and species of great worldwide economic importance such as *Brassica oleracea* L., *Brassica napus* L., *Brassica rapa* L. (Cartea et al., 2011). Plants from Brassicaceae such as *Brassica*, *Camelina* species are not only known for their high fat and protein contents for human and animal consumption, but recognized as a rich source of nutrients such as vitamins, minerals, carbohydrates, amino acids, and different groups of phytochemicals: phenolics, glucosinolates, fatty acids etc. (Jensen et al., 1996; Goffman et al., 1999; Jahangir et al., 2009; El-Beltagi et al., 2010; Cartea et al., 2011; Rakhmetov et al., 2014; Vergun et al., 2017b). The main fatty acid composition of *B. campestris* var. *oleifera* during growth was α-linoleic acid, linoleic acid, cis-10-heptadecenoic acid, and palmitic acid. The most abundant was oleic acid content (Peiretti et al., 2012). *B. campestris* seeds, also, rich in lipid composition (Sharma et al., 2003). Some results support the beneficial effects of turnip (*B. rapa*) in the management of metabolic syndrome (An et al., 2010; Abo-youssef, Mohammed, 2013). These plants characterized by different pharmaceutical effects such as antioxidant, anti-inflammatory, antiepileptic, anti-diabetic, immunological, cardiovascular etc. (Al-Snafi, 2015). As reported Rajamurugan et al. (2012), methanol extracts of *B. nigra* leaves demonstrated the protective effect at the hepatic and renal injury because of anti-inflammatory and antioxidant effect.

Plants from genus *Brassica* is a source of antioxidants of different nature such as flavonoids, tannins and other phenolic compounds (Ryu et al., 2012; Gul et al., 2013; Routray et al., 2013). Plant raw material of these plants contains phenolic acids such as caffeic, sinapic, *p*-coumaric, ferulic etc. (Seong et al., 2016). On the basis of the experimental work of Behman and Sani Mohamadi (2017), extracts from leaves and roots of *B. rapa* possess antibacterial properties. It could be used as possible food antimicrobial preservative in the food industry. These plants also demonstrated the quick tolerance to salt stress in some investigations (Jan et al., 2016; Jan et al., 2017).

The aim of this study was to determine an antioxidant potential of some oils plants of Brassicaceae.

Material and methodology

Biological material

The plants were grown in 2017 in 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). Plant material was collected from the experimental collections of oil plants of the Department of the Cultural flora of M.M. Gryshko National Botanical Garden of NAS of Ukraine in the stage of flowering and analyzed in the laboratory of a department.

Biochemical analysis

Biochemical analyze of antioxidant activity detection was conducted according to Brand-Williams et al. (1995). Plant extracts were prepared in two solvents – methanol and distilled water. 1 g of dry plant raw material was mixed with 25 ml of each solvent. Extraction was carried out during 12 hours at continuous stirring. Preparing of the radical solution was

following: 25 mg of DPPH-radical (2,2-diphenyl-2-picrylhydrazyl) was solved in methanol (in 100 ml volumetric flask) and used for following dilution (1 : 10). 0.1 ml of investigated plant extract was added to 3.9 ml of radical solution. The optical density of the radical solution was measured immediately and after 10 min of incubation in the dark after adding a sample. The measurement was conducted at 515 nm on the spectrophotometer (Unico 2800 UV/VIS). Obtained data calculated using a formula:

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

Statistical analysis

Obtained data were expressed in mg g^{-1} AAE (ascorbic acid equivalent). The statistically treated data are given in the table as the arithmetical mean values and their standard errors.

Results and discussion

According to our previous data concerning to biochemical properties of Brassicaceae, seeds of these plants are the rich source of lipids (17.72–37.61%). Also, plant raw material characterized by an energetic value of 5,039.33–6,108.00 KcaL.kg^{-1} (Vergun et al., 2017a). Results obtained by Fernandes et al. (2007) indicated that turnip is an easily accessible dietary source of biologically active compounds. The antioxidant potential exhibited by the different turnip edible parts is obviously determined by their composition.

Antioxidant activity has been assessed in many ways (Antolovich et al., 2002). The DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) free radical method has been widely applied for estimating antioxidant activity in recent years (Molyneux, 2004). It is an antioxidant assay based on electron-transfer that produces a violet solution in an alcohol solvent. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to a colorless ethanol solution (Brand-Williams et al., 1995).

It was studied methanol and water extracts to evaluate the antioxidant potential of investigated plants (Table 1). As shown on the table, methanol extracts exhibited antiradical activity from 34.29 (*B. rapa* subsp. *rapifera* Metzger (f. *biennis*), f. EOTRFO) to 58.09% (*B. campestris* f. *biennis* D.C., cv. Oriana). Antiradical activity of water extracts was from 58.18 (*B. campestris* f. *biennis* D.C., cv. Oriana) to 84.25% (*B. campestris* f. *biennis* D.C.× *B. rapa* L., f. EOTFVS).

According to Fernandes et al. (2007), the antioxidant capacity of different extracts of *B. rapa* correlated with both total phenolic and organic acids amounts. Differences in antioxidant activity of *Brassica* crops were related to differences in total phenolic content but also to differences in phenolic composition for most samples (Soengas et al., 2012). Our previous data showed that *B. campestris* f. *biennis* D.C.× *B. napus* f. *biennis* D.C., cv. Innovacia had an antiradical activity of methanol extracts 21.54 and water extracts – 50.62% that was less than in current research (Vergun and Rakhmetov, 2018). Also, methanol extracts of *B. rapa* and *B. rapa* subsp. *rapifera* Metzger demonstrated 19.86 and 42.42% of inhibition and water

extracts – 62.96 and 63.57% respectively. Data obtained for *B. rapa* subsp. *rapifera* Metzger was similar. Also, Sun et al. (2009) reported that the antioxidant activity of methanol extracts was higher than in acetone and water extracts by DPPH-method in some Brassicaceae. This is consistent with our obtained data where only *B. campestris* f. *biennis* D.C., cv. Oriana had the same results in both methanol and water extracts.

Table 1 The antiradical activity of plant extracts of selected oil crops (%)

Name of sample	Methanol extracts	Water extracts
***B. campestris* f. *biennis* D.C., cv. Horlytsia-FHO**	47.70 ±0.91	58.26 ±0.81
***B. campestris* f. *biennis* D.C., cv. Oriana**	58.09 ±0.52	58.18 ±1.78
***B. campestris* f. *biennis* D.C., f. EOSOF-2**	36.39 ±0.20	67.04 ±0.49
***B. campestris* f. *biennis* D.C.× *B. napus* f. *biennis* D.C., cv. Innovacia**	39.09 ±0.72	78.60 ±0.93
***B. campestris* f. *biennis* D.C.× *B. rapa* L., cv. Fitopal**	41.29 ±1.83	83.55 ±2.59
***B. campestris* f. *biennis* D.C.× *B. rapa* L., cv. Obrii**	35.38 ±1.16	61.18 ±0.47
***B. campestris* f. *biennis* D.C.× *B. rapa* L., f. EOTFVS**	50.45 ±1.65	84.25 ±0.76
***B. campestris* f. *biennis* D.C.× B.*rapa* L.×B.*napus* f. *biennis* D.C., f. EOHBFTRO-2**	41.67 ±0.67	77.67 ±0.48
***B. rapa* subsp. *rapifera* Metzger**	42.43 ±0.81	63.57 ±1.25
***B. rapa* subsp. *rapifera* Metzger (f. *biennis*), f. EOTRFO**	34.29 ±0.99	65.67 ±0.38

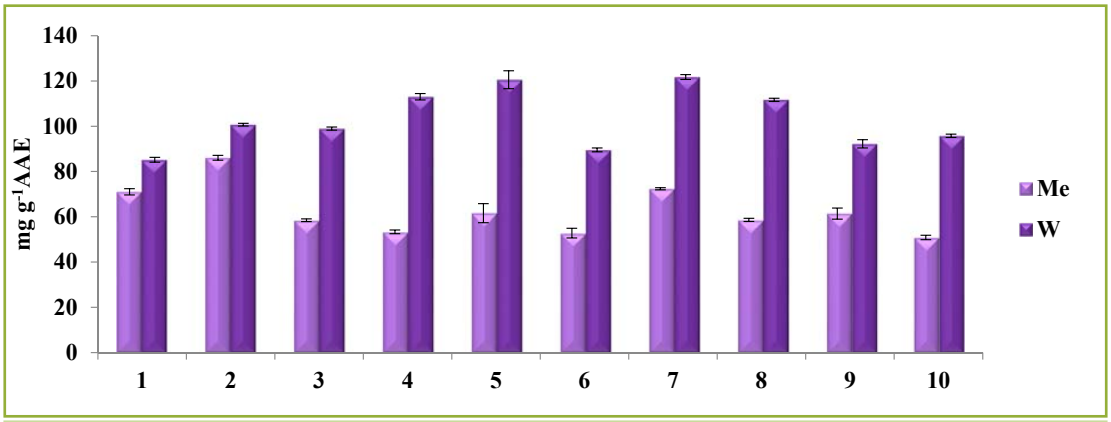

Figure 1 Antioxidant activity of plant extracts of selected oil plants (mg g^{-1} AAE): 1 – *B. campestris* f. *biennis* D.C.× *B. rapa* L., f. EOTFVS; 2 – *B. campestris* f. *biennis* D.C.× *B. rapa* L., cv. Fitopal; 3 – *B. rapa* subsp. *rapifera* Metzger; 4 – *B. campestris* f. *biennis* D.C. × *B. rapa* L. × *B. napus* f. *biennis* D.C., f. EOHBFTRO-2; 5 – *B. campestris* f. *biennis* D.C. × *B. rapa* L., cv. Obrii; 6 – *B. rapa* subsp. *rapifera* Metzger (f. *biennis*), f. EOTRFO; 7 – *B. campestris* f. *biennis* D.C.× *B. napus* f. *biennis* D.C., cv. Innovacia; 8 – *B. campestris* f. *biennis* D.C., cv. Horlytsia-FHO; 9 – *B. campestris* f. *biennis* D.C., cv. Oriana; 10 – *B. campestris* f. *biennis* D.C. × *B. rapa* L. × B. *napus* f. *biennis* D.C., f. EOHBFTRO-2

Antioxidant activity expressed in ascorbic acid equivalent represented in Figure 1. Methanol extracts showed activity from 50.87 (*B. campestris* f. *biennis* D.C. × *B. rapa* L. × *B. napus* f. *biennis* D.C., f. EOHBFTRO-2) to 86.07 mg g^{-1} AAE (*B. campestris* f. *biennis* D.C. × *B. rapa* L., cv. Fitopal). Water extracts exhibited antioxidant activity from 85.18 (*B. campestris* f. *biennis* D.C. × *B. rapa* L., f. EOTFVS) to 121.77 mg g^{-1} AAE (*B. campestris* f. *biennis* D.C. × *B. napus* f. *biennis* D.C., cv. Innovacia).

Conclusions

The cultivars and varieties of *Brassica campestris*, *B. rapa* have the high antioxidant potential in the conditions of M.M. Gryshko National Botanical Garden of the NAS of Ukraine. Study of methanol extracts showed that minimal antiradical activity was found for *B. rapa* subsp. *rapifera* Metzger (f. *biennis*), f. EOTRFO and maximal – for *B. campestris* f. *biennis* D.C., cv. Oriana. The most investigated plants had an antiradical activity of water extracts more than 60%. Antioxidant activity of water extracts of investigated plants was more than in methanol extracts.

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TOTAL CONTENT OF PHENOLIC COMPOUNDS IN THE ETHANOL EXTRACTS OF *GALEGA OFFICINALIS* L. AND *G. ORIENTALIS* LAM.

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This study represents results of determination of total phenolic compounds in raw of two species of *Galega* L. It was investigated plant extracts of *Galega officinalis* L. (goat's-rue) and *Galega orientalis* Lam. (fodder galega) which are known as fodder crops with high productivity of green mass and medicine plants (Hasani-Ranjbar et al., 2009; Peiretti, 2009; Shojaee et al., 2015; Teleută et al., 2015). The current study was aimed to evaluate an accumulation in selected plants the total content of phenolics as compounds with antioxidant activity. Raw of investigated plants collected from experimental collections of M.M. Gryshko National Botanical Garden of the NAS of Ukraine (Kyiv) during vegetation. Biochemical analysis conducted in the Slovak University of Agriculture in Nitra. The phenolic content in ethanol extracts was measured using Folin-Chocalteu reagent. The procedure for preparing extracts was done according to Singleton and Rossi, 1965 and measuring conducted using spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid was used as the standard and the results were expressed in mg g^{-1} gallic acid equivalent (GAE). Study of *G. officinalis* extracts showed that accumulation of total phenolic content in different organs was in the range from 9.13 to 32.76 mg g^{-1} GAE. In extracts of *G. orientalis* was identified total phenolics content from 6.73 to 26.77 mg g^{-1} GAE. It was established that less concentration of studied compounds found in the stems for both species.

Keywords: *Galega officinalis*, *Galega orientalis*, phenolics

Introduction

Leguminous plants (Fabaceae Lindl.) are a perspective group of crops, which ecological and economic function is important in agriculture. It is one of the most important plant families in the production of food for humans and livestock, as well as in the production of industrial products. These crops have provided interesting as forage grasses with high productivity and play an important role as N fixators (Peiretti, 2009; Teleută et al., 2015). Plants from Fabaceae family are of interest in relation to biologically active compounds, especially individual, in different organs (Danilčenko et al., 2017).

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Among economically important leguminous plants can be highlight goat's rue (*Galega officinalis* L.) and fodder galega (*Galega orientalis* Lam.). Plants of species of *Galega* L. are valuable perennial and productive crops with the protein-rich chemical composition of plant raw material (Baležentienė, 2008). Results obtained by Peiretti (2009) showed that *G. officinalis* has the potential for large-scale ensiling if plants are harvested at the budding stage or during regrowth. These species cultivated as medicinal plants due to the biochemical composition of plant raw material and as garden plants (Baležentienė and Spruogis, 2011; Kumar et al., 2012). As described in some reports, *G. officinalis* uses in traditional phytotherapy due to hypoglycemic, diuretic properties, and weight-reducing ability (Hasani-Ranjbar et al., 2009; Shojaee et al., 2015). Biochemical composition of *Galega* species is ascorbic acid, carotene, soluble sugars, lipids, protein, ash, alkaloids, macroelements, etc. (Symanowicz and Kalembasa, 2012; Vergun et al., 2012; Shymanska et al., 2017). Also, the phytochemicals screening revealed that in aqueous, methanolic, ethanolic and acetone extracts were found flavonoids, tannins, cardiac glycosides, terpenes and steroids. Methanolic extracts of goat's rue significantly improved the lipid profile in a clinical study (Luka et al., 2017).

The aim of this study was to determine the content of polyphenol compounds in different organs of *G. officinalis* and *G. orientalis* during vegetation in the conditions of M.M. Gryshko National Botanical Garden of the NAS of Ukraine (NBG).

Material and methodology

The plants were grown in 2017 in 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). The total area of the experimental field for each species was 25 m^2. For this study, the average weight of ten plants selected by randomized method was used. In 2017, the active plant growth started on 21th of March for *G. officinalis* and the 5th of April for *G. orientalis*. Plant growth was ended in November for both species of investigated plants. Many years observations showed that the end of vegetation depends on early frosts. Monthly average temperatures in the vegetation period of year of study were following: March – +3.9 °C, April – +10.7 °C, May – +16.2 °C, June – +19.1 °C, July – +21.2 °C, August – +21.2 °C, September – +15.8 °C, October – +6.9 °C, November – +2.6 °C.

Biological material

Observation on plants was conducted in the experimental collection of Cultural Flora Department of NBG. Plant raw material of two species *Galega officinalis* and *G. orientalis* were collected in the stages according to Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie (BBCH) coding system. According to BBCH scale, plant samples were taken at the phenological growth stages described for faba bean (*Vicia faba* L.) (Meier, 2001). Four principal growth stages were assigned: leaf development (19 – nine or more leaves infolded), inflorescence emergence (50 – flower buds present, still enclosed by leaves), flowering (65 – full flowering: flowers open on 5 racemes per plant), and ripening (80 – beginning of ripening: seed green, filling pod cavity). For chemical analyses plant raw material was dried at 35 °C for three days. After this, the samples were milled in the powder condition.

Biochemical analysis

The biochemical analysis was done in 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. The total phenolic content of extracts was measured using Folin-Chocalteu reagent (Singleton, Rossi, 1965). 0.1 ml of each sample was mixed with 0.1 ml of the Folin-Ciocalteu reagent, 1 ml of 20% (w/v) sodium carbonate, and 8.8 ml of distilled water. After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid was used as the standard and the results were expressed in mg g^{-1} gallic acid equivalents (GAE) (25–300 mg l^{-1}; R^2 = 0.998).

Statistical analysis

The statistically treated data are given in the table as the arithmetical mean values and their standard errors. Data were submitted ANOVA and differences between means compared through the Tukey-Kramer test (α = 0.05).

Results and discussion

The therapeutic potential of natural medicinal plants as antioxidants in reducing free radicals suggests that most of the plants have a high antioxidant potential, which can be useful therapeutically (Veeru et al., 2009). Plant secondary metabolites such as polyphenol compounds play an important role in the defence against free radicals. Polyphenols exhibit a wide range of biological effects including antibacterial, anti-inflammatory, hepatoprotective, antiviral, anticancerogenic etc. (Soobratee et al., 2005; Piluzza and Bullitta, 2011). Numerous studies described the good or high linear correlation between the concentration of phenolic compounds and antioxidant capacity determined by the DPPH-method (Mareček et al., 2016; Ivanišova et al., 2017). This suggests that phenolic content could be used as an indicator of antioxidant properties of the plants (Piluzza and Bullitta, 2011).

Polyphenols can form several hydrogen bonds and even ionic bonds with most proteins. They modulate the activity of many proteins, involving enzymes, ion channels etc. As a consequence many polyphenols are pharmacologically active, being among antioxidants, anti-inflammatory, antibacterial, antifungal, and antiviral (Wink, 2013). The previous study of *G. officinalis* extracts showed the presence of phenolics as flavonol triglycosides, kaempferol, galegin, medicagol, quercetin etc. (Kahkeshani et al., 2015).

The content of phenolic compounds in different parts of *G. officinalis* parts ranged from 9.13 to 32.76 mg g^{-1} GAE (Table 1). It should be noticed, that most of the results about the content of phenolic compounds concern to the *G. officinalis* plants. According to Tusevski et al. (2014), total phenolic content for plants of *G. officinalis* was 32.53 ±2.80 mg g^{-1} GAE. Pehlivan Karakas et al. (2016) obtained twenty phenolics compounds from methanol leaves extracts of *G. officinalis*. Total phenolic content in them was 36.69 mg g^{-1} of dry extract.

Table 1 The content of total phenolic compounds in plant raw material of *Galega officinalis* L. and *G. orientalis* Lam., mg g^{-1} GAE

Stage of growing	Organ of plan	*Galega officinalis* L.	*Galega orientalis* Lam.
Leaf development	aerial part	17.06 ±0.89 b	19.96 ±0.62 ab
Inflorescence emergence	leaves	19.50 ±1.14 b	25.21 ±0.17 a
	stems	9.61 ±1.45 b	8.09 ±0.44 d
	buds	31.51 ±1.11 a	23.43 ±1.53 a
Flowering	leaves	22.58 ±0.67 b	15.43 ±0.28 c
	stems	9.13 ±0.30 d	6.73 ±0.23 e
	flowers	32.76 ±1.26 a	19.58 ±0.85 ab
Ripening	leaves	18.61 ±0.95 b	26.77 ±0.51 a
	stems	15.28 ±0.67 c	10.87 ±0.99 d
	fruits	19.61 ±1.33 b	13.58 ±0.62 c

Notes: Means in columns followed by different letters are different at $p = 0.05$; each value represents the mean of three independent experiments (±SD)

The total content of phenolics of *G. orientalis* plant raw material ranged from 6.73 to 26.77 mg g^{-1} GAE during vegetation. According to Baležentienė (2009) report, the highest total content of phenols was determined at the budding stage which was characterized as the most intensive growth period of the plant shoot. Moreover, the study of Kahkeshani et al. (2015) demonstrated that mixture of *G. officinalis* and *Nigella sativa* L. (4 : 1 w/w) had shown milk stimulating activity and the total phenol content 77.72 μg mg^{-1} GAE. Also, we detected in the previous study the total antioxidant activity of methanol, ethanol and water extracts (Shymanska et al., 2018). According to obtained data, antiradical activity of *G. officinalis* and *G. orientalis* plant extracts was minimal in stems for both species (11.24 and 11.74% respectively). Likewise, high antiradical activity was identified in methanol and ethanol extracts of generative organs (up to 90%). These data consistent with the obtained results as interrelated. Further research should be focused on the study of more species of the investigated genus and detailed investigation of their polyphenol compounds.

Conclusions

Thus, in conditions of M.M. Gryshko National Botanical Garden of the NAS of Ukraine, plants of *Galega officinalis* and *G. orientalis* accumulated uneven quantity of phenolic compounds during vegetation. Comparing the analysis of two investigated species showed that the most content of total phenolics was determined in the *G. officinalis* flowers extracts in the stage of flowering and the least – in the stems extracts of *G. orientalis*. Both *G. officinalis* and *G. orientalis* accumulated the least content of phenolic compounds in stems. Generative organs of *G. officinalis* had higher total content of phenolics than vegetative.

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LEAF CHARACTERISTICS AS IMPORTANT MORPHOMETRIC DISCRIMINATORS FOR CHESTNUT (*CASTANEA SATIVA* MILL.) GENOTYPES

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This study was carried out in order to determine the leaf characteristics of some chestnut (*Castanea sativa* Mill.) genotypes in the M.M. Gryshko National Botanical Garden (Kyiv, Ukraine) and also to determine whether the leaf morphometric characteristics could be used for differentiation of genotypes. In this study, 9 chestnut genotypes (CS-01 – CS-09) were used. Some leaf parameters such as lamina length, lamina width, petiole length, petiole width, petiole thickness, teeth length, teeth width, stomata length, stomata width were studied. Morphometric parameters were following: leaves length from 85.0 to 250.0 mm, leaves width from 26.0 to 100.0 mm, petiole length from 8.07 to 36.18 mm, petiole width from 0.91 to 2.60 mm, petiole thickness from 0.81 to 2.44 mm, teeth length from 1.27 to 5.05 mm, teeth width 0.87 to 2.82 mm. The shape indexes of leaves were found ranging from 2.45 to 4.26. Analysis of coefficient of variation (CV) showed a high variability in morphometric characteristics between *Castanea sativa* samples. Data showed that the teeth width is the most variable signs (from 15.30 to 22.18%). Other studied characteristics have an average level of variability. Collected quantitative data were subjected to principal hierarchical cluster analysis. The cluster analysis of morphometric parameters exhibited that broad morphologic diversity was found in *Castanea sativa* genotypes examined in this study. Most of the chestnut genotypes could be differentiated easily by using leaf morphometric characteristics.

Keywords: *Castanea sativa*, leaf, morphometric characteristic, discrimination, genotype

Introduction

The study of morphological traits of leaf have been used frequently in practice by scientists to study genetic variability (Dickinson et al., 1986; Aravanopoulos et al., 2001; Kremer et al.,

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2002; Ertan, 2007; Neophytou et al., 2007), since the leaf location, size, shape and anatomical characteristics of leaf plates vary widely depending on environmental conditions. These signs reflect the degree of plant resistance, because the leaves are the most important organs of photosynthesis and transpiration (Sahin and Soylu, 1991; Bruschi et al., 2003; Pinto et al., 2011; Mensah, 2012). These characteristics are important for genotypes identification base on the observation of morphological characteristics of which expressions are largely influenced by developmental, environmental and cultivation factors (Serdar and Kurt, 2011; Poljak et al., 2014). Many authors use morphological analyses to estimate the variability of *Castanea sativa* and which confirm that the leaf parameters can be appropriate variables for establishing the level of genotype variability (Aravanopoulos et al., 2001; Aravanopoulos, 2005; Bolvansky and Uzik, 2005; Álvarez-Álvarez et al., 2006; Ertan, 2007; Zarafshar et al., 2010; Serdar and Kurt, 2011; Mujagić-Pašić and Ballian, 2012; Poljak et al., 2014; Atefe et al., 2015).

Identification of genotypes based on morphological traits allows us to find valuable specimens for further selection, which is very valuable, especially for woody plants (Skvortsov et al., 2005; Brindza et al., 2007; Monka et al., 2014; Grygorieva et al., 2014, 2018; Kucelova et al., 2016; Vinogradova et al., 2017).

The objective of this research was to evaluate the leaf characteristics of seven *Castanea sativa* Mill. genotypes and also to determine whether leaf morphometric characteristics could be used for differentiation of genotypes.

Material and methodology

Locating trees and data collection

The objects of the research were 45-year-old plants of *Castanea sativa* from seed origin, which are growing in Forest-Steppe of Ukraine in M.M. Gryshko National Botanical Garden of NAS of Ukraine (NBG). Seeds were brought from Czech, Carpathians, Kyrgyzstan. They are well adapted to the climatic and soil conditions (Grygorieva et al., 2017; Klymenko et al., 2017). Field studies were carried out in August, 2017 on mature leaves of 9 chestnut genotypes (CS-01 – CS-09). In the study, chestnut genotypes with superior fruit characteristics were preferred. Trees in the same exposure were selected.

Morphometric characteristics

Leaf morphological parameters were determined on the 1-year-old shoots (middle part of shoot). It was selected from the tree of each genotype 30 leaves from the four sides of crown. The total number of selected leaves was 1080. For each leaf, seven characteristics were measured. In this study, leaf length (LL) in cm; leaf width (LW) in cm; petiole length (PL) in mm; petiole width (PW) in mm; petiole thickness (PT) in mm; teeth length (TL) in mm; teeth width (TW) in mm. The measurements were made in each leaf element as shown in Figure 1.

Statistical analyses

Basic statistical analyses were performed using PAST 2.17; hierarchical cluster analyses of similarity between phenotypes were computed on the basis of the Bray-Curtis similarity

index; multi-dimensional scaling (MDS) analyses were performed in PRIMER (Clarke and Gorley, 2006). Variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by Stehlíková (1998).

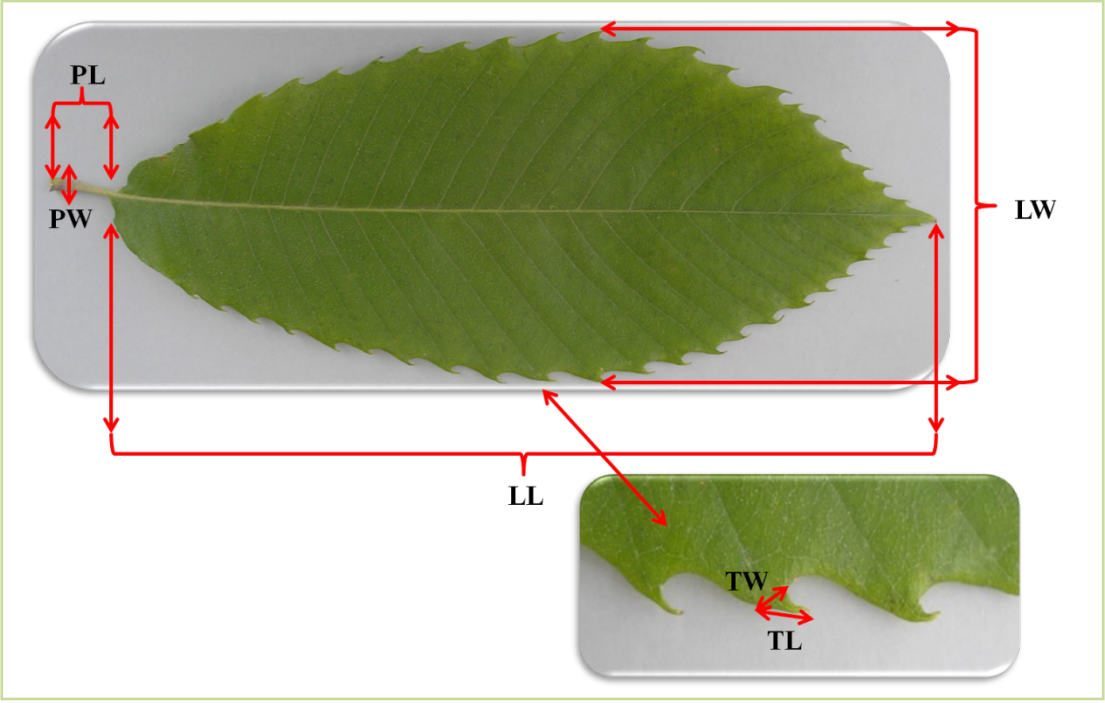

Figure 1 Illustration of measuring process: leaf length, leaf width, petiole length, petiole width, teeth length, teeth width

Results and discussion

Castanea sativa leaves arranged alternately, are simple, shiny in appearance and dark green in color. The leaf coloration changes within the chestnut genotypes. However, there is a clear distinction among the upper surface coloration (green-dark) and the lower surface (light green) coloration. The margins of leaf are toothed. In the collection several types of leaves could be found lanceolate, narrow elliptic, broad elliptic. The shape of apex narrow acuminate, broad acuminate, acute. The shape of base acute, obtuse, cordate. The incisions of margin mucronate and dentate. The petiole colour is yellowed or red.

The images of sweet chestnut leaves of various genotypes are shown on Figures 2, 3, 4, 5 and 6.

Figure 2 Variability in the shape of *Castanea sativa* Mill. leaves

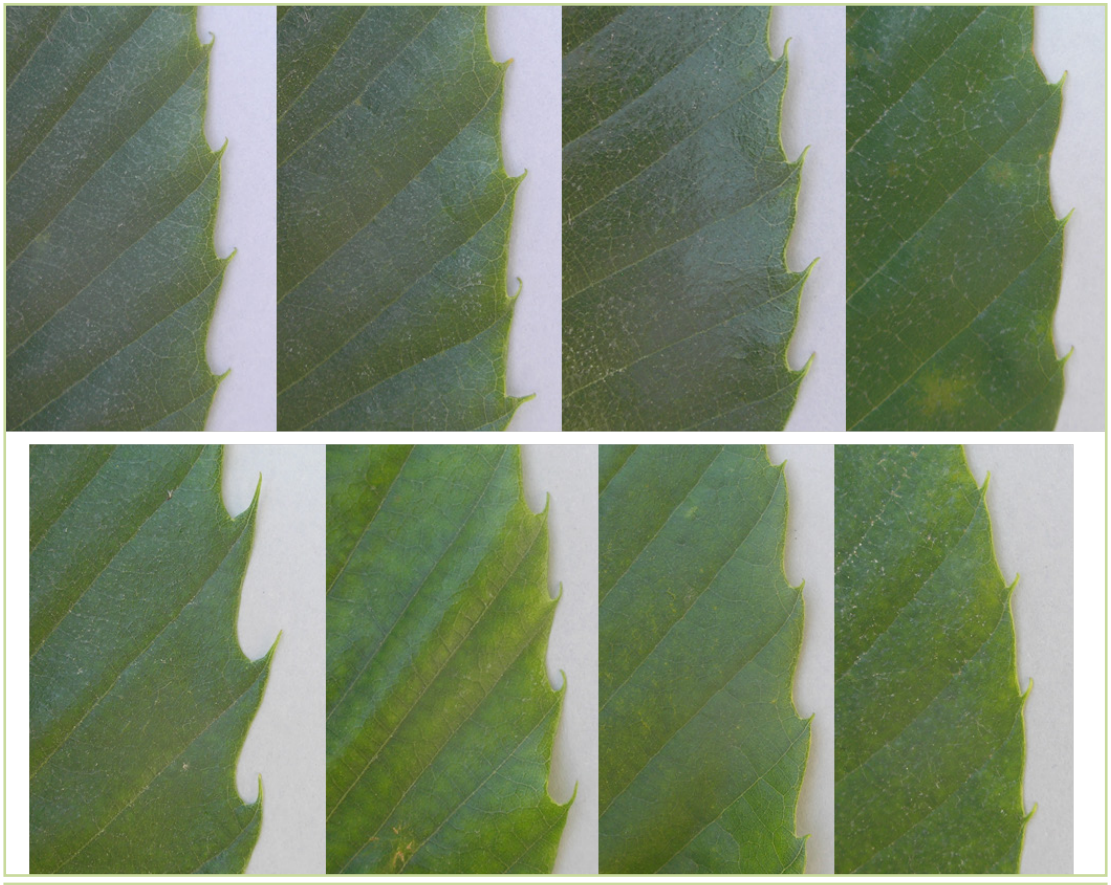

Figure 3 Variability in the teeth of *Castanea sativa* Mill.

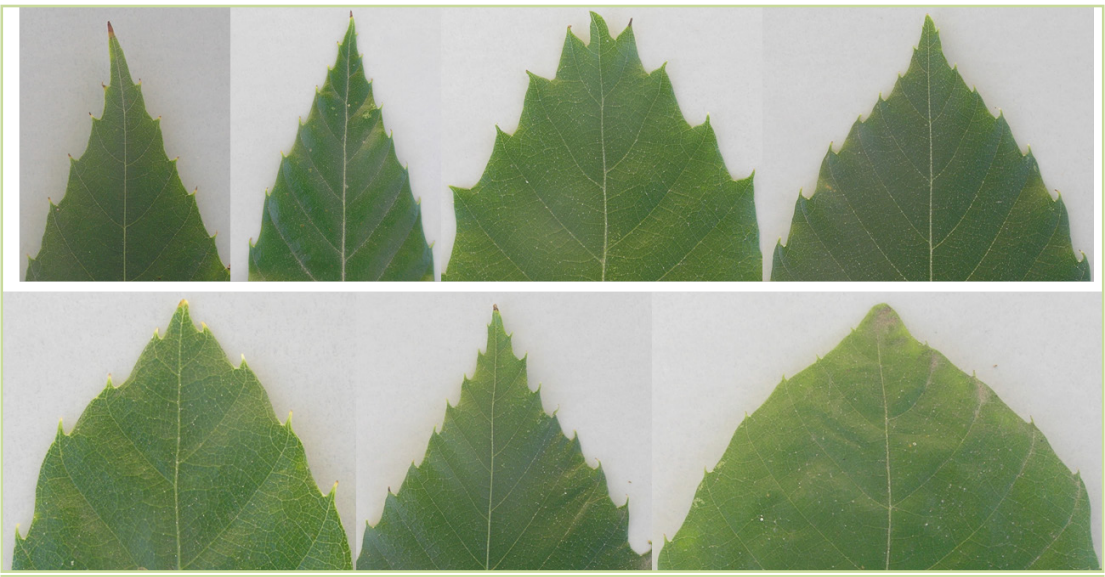

Figure 4 Shape of leaf apex of *Castanea sativa* Mill.

Figure 5 Shape of leaf blade bases of *Castanea sativa* Mill.

Figure 6: Variability in the petiole of *Castanea sativa* Mill.

The length of sweet chestnut leaves of present study was in the range of 85.0 (CS-08) to 250.0 (CS-06) mm (Table 1). Significant differences in leaves were reaffirmed a lot of authors from different countries (Table 2). The leaves length was determined in range from 9.20 to 30.80 cm by Mujagić-Pašić and Ballian Dalibor (2012), from 14.21 to 24.08 cm by Álvarez-Álvarez et al. (2006), from 14.50 to 21.20 cm by Poljak et al. (2014), from 17.70 to 23.40 cm by Serdar and Kurt (2011), from 21.30 to 23.40 cm by Zarafshar et al. (2010), from 22.44 to 25.05 cm by Atefe et al. (2015). Data comparison shows a high consistency with our results.

Table 1 The variability of some morphometric parameters of leaves of *Castanea sativa* Mill. genotypes

Genotypes	min	max	S_x	CV%	Genotypes	min	max	S_x	CV%
Leaf length					**Petiole width**				
CS-01	108	189	14.85	10.76	**CS-01**	0.91	1.42	0.11	10.55
CS-02	119	229	23.47	13.37	**CS-02**	1.13	1.56	0.11	8.20
CS-03	148	215	14.90	8.44	**CS-03**	1.63	2.24	0.14	7.62
CS-04	103	167	17.15	13.14	**CS-04**	1.02	1.38	0.10	8.44
CS-05	98	187	18.60	12.11	**CS-05**	1.25	2.20	0.20	12.54
CS-06	155	250	23.83	11.75	**CS-06**	1.61	2.60	0.23	11.74
CS-07	148	206	15.78	9.00	**CS-07**	1.34	2.17	0.20	10.94
CS-08	85	210	23.30	13.91	**CS-08**	1.41	2.08	0.18	10.42
CS-09	114	237	28.02	16.63	**CS-09**	1.33	2.25	0.20	12.37
Leaf width					**Petiole thickness**				
CS-01	28	47	4.76	12.89	**CS-01**	0.81	1.20	0.12	12.16
CS-02	30	57	7.00	16.85	**CS-02**	1.05	1.66	0.14	11.12
CS-03	52	77	5.14	7.88	**CS-03**	1.19	1.87	0.16	10.64
CS-04	26	42	4.76	13.84	**CS-04**	0.85	1.39	0.14	13.07
CS-05	28	54	6.73	15.75	**CS-05**	1.00	1.62	0.15	11.51
CS-06	54	100	10.40	12.58	**CS-06**	1.46	2.44	0.24	13.09

Continue the Table 1

Genotypes	min	max	S_x	CV%	Genotypes	min	max	S_x	CV%
	Leaf width					**Petiole thickness**			
CS-07	59	75	4.24	6.37	**CS-07**	1.03	2.15	0.25	15.60
CS-08	35	65	7.32	14.18	**CS-08**	1.20	1.90	0.19	12.84
CS-09	38	63	6.18	12.54	**CS-09**	1.13	2.11	0.21	14.12
	Petiole length					**Teeth length**			
CS-01	18.24	28.06	2.44	10.22	**CS-01**	1.27	2.93	0.35	16.76
CS-02	18.31	34.56	4.55	17.80	**CS-02**	1.61	3.15	0.43	18.95
CS-03	10.03	19.96	2.81	19.49	**CS-03**	1.76	4.19	0.60	19.90
CS-04	19.32	35.85	3.89	13.86	**CS-04**	1.62	3.67	0.56	20.56
CS-05	16.15	23.92	2.26	11.10	**CS-05**	2.03	3.88	0.49	16.82
CS-06	14.03	24.89	2.95	15.73	**CS-06**	2.07	4.41	0.52	16.77
CS-07	8.07	13.97	1.66	15.08	**CS-07**	1.86	3.96	0.53	17.60
CS-08	17.91	36.18	4.92	17.97	**CS-08**	1.80	5.05	0.69	20.68
CS-09	13.02	19.07	1.58	10.28	**CS-09**	1.49	3.83	0.48	18.12

Teeth width				
Genotypes	**min**	**max**	S_x	**CV%**
CS-01	0.87	2.03	0.22	16.07
CS-02	1.04	2.12	0.23	16.83
CS-03	1.10	2.38	0.32	18.61
CS-04	1.01	2.12	0.27	18.76
CS-05	1.02	2.30	0.30	17.42
CS-06	1.20	2.45	0.36	21.69
CS-07	1.39	2.82	0.33	15.30
CS-08	1.13	2.65	0.39	22.18
CS-09	1.03	2.71	0.33	18.04

Note: min, max – minimal and maximal measured values; S_x – standard deviation; CV – coefficient of variation (%)

The leaves width in our analyses was determined in the range of 26.0 (CS-04) to 100.0 (CS-06) mm (Table 1). The leaves width was determined in range from 2.50 cm by Mujagić-Pašić and Ballian Dalibor (2012) to 14.80 g by Zarafshar et al. (2010) (Table 2).

The petiole length, width and thickness in our analyses was determined in the range of 8.07 (CS-07) to 36.18 (CS-08) mm, from 0.91 (CS-01) to 2.60 (CS-06) mm and from 0.81 (CS-01) to 2.44 (CS-06) mm, respectively (Table 1). Maximal signs of petiole length were 6.70 cm (Table 2) found in study Mujagić-Pašić and Ballian Dalibor (2012).

Table 2 Variability of some morphometric characteristics on *Castanea sativa* Mill. leaves according to the authors from different countries

Authors	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)
Álvarez-Álvarez et al. (2006)	14.21–24.08	4.46–8.68	1.02–2.21
Zarafshar et al. (2010)	21.30–23.40	14.10–14.80	1.30–1.50
Serdar and Kurt (2011)	17.70–23.40	4.10–6.77	1.90–2.90
Mujagić-Pašić and Ballian Dalibor (2012)	9.20–30.80	2.50–12.0	0.50–6.70
Poljak et al. (2014)	14.50–21.20	4.70–8.50	1.90–2.80
Atefe et al. (2015)	22.44–25.05	6.98–8.66	1.33–1.92

The teeth length and width in our analyses were determined in the range of 1.27 (CS-01) to 5.05 (CS-08) mm and of 0.87 (CS-01) to 2.82 (CS-07) mm, respectively (Table 1). Mean values of morphological parameters of leaves demonstrated on Figure 7. There is a significant difference between the morphological parameters of leaves size that confirmed by results from multi-dimensional scaling visual distribution of studied genotypes (Figure 8). The sample CS-04 (green ellipse) with the smallest leaves size and the sample CS-06 (light green ellipse) with the largest leaves size differ each another with the probability of 95% (the ellipses in the figure do not overlap).

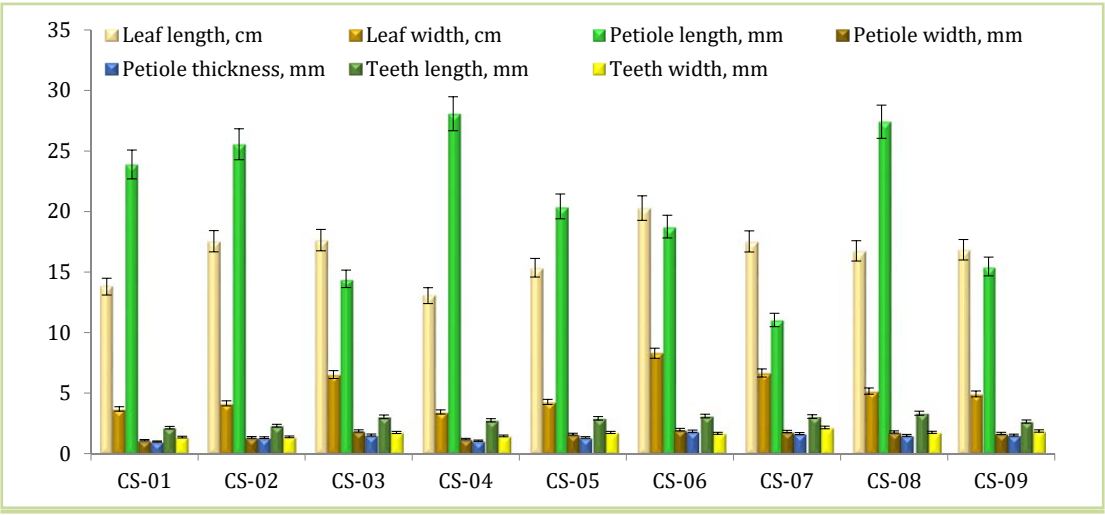

Figure 7: Mean values for leaf of *Castanea sativa* Mill. genotypes

The coefficient of variation showed the difference in variability of morphological signs between *Castanea sativa* samples (Table 1). Data showed that the most variable signs are the teeth width from 15.30 to 22.18%. Other studied characteristics have an average degree of variability.

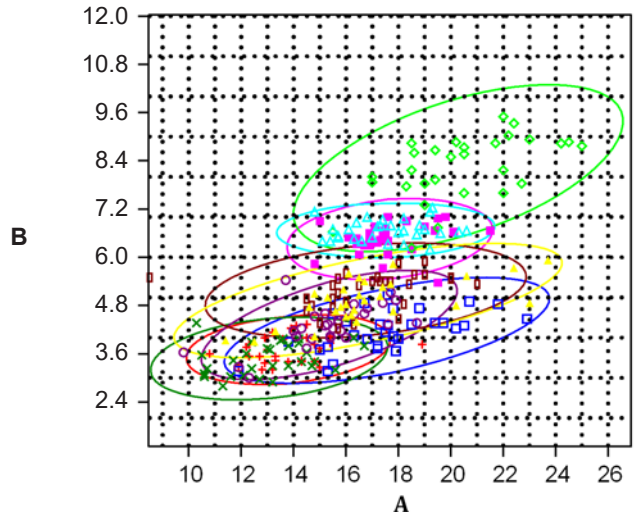

Figure 8: The scatter plot of leaf resulted from PCA in space coordinate axes based on the two components

The shape of each object can be characterized by the shape index, i.e. the length to width ratio. Figure 9 represents the shape indexes of leaves. The shape index of the leaves was found in the range from 2.45 (CS-06) to 4.26 (CS-02), so the genotypes collection demonstrates significant variability in the shape of the leaves, as seen in Figure 2. These parameters can be used for the identification of the genotypes.

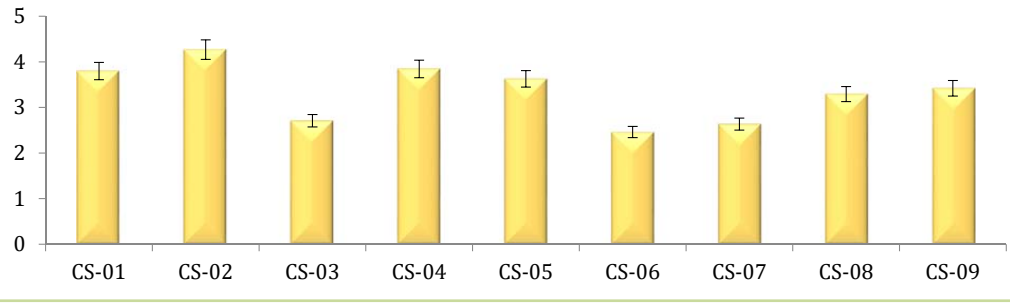

Figure 9: Comparison of the tested *Castanea sativa* Mill. genotypes in the shape index of leaves

The bivariate Pearson correlations revealed very strong correlation between PW/PT ($r = 0.92$), LW/PT and LL/PT ($r = 0.92$), strong correlation revealed between LW/PW ($r = 0.89$), LL/LW ($r = 0.87$), PW/TL ($r = 0.82$), LL/PW ($r = 0.81$), PW/TW ($r = 0.74$). Moderate positive correlations were between the PT/TL, PT/TW, TL/TW, LW/TL, and LW/TW.

Table 3 The matrix of Pearson correlation coefficients for 21 pairs of variables leaf *Castanea sativa* Mill.

Parameters	Lamina length	Lamina width	Petiole length	Petiole width	Petiole thickness	Teeth length
Lamina width	0.87*					
Petiole length	-0.49	-0.65				
Petiole width	0.81*	0.89*	-0.62			
Petiole thickness	0.92*	0.92*	-0.62	0.95*		
Teeth length	0.45*	0.64	-0.26*	0.82	0.68*	
Teeth width	0.42	0.58*	-0.77	0.75*	0.69	0.66*

Note: Significant according to the *t*-test (p <0.05)

The cluster analysis was performed according to the hierarchical cluster analysis method using the mean value to distinguish similar groups among the various leaves morphometric parameters.

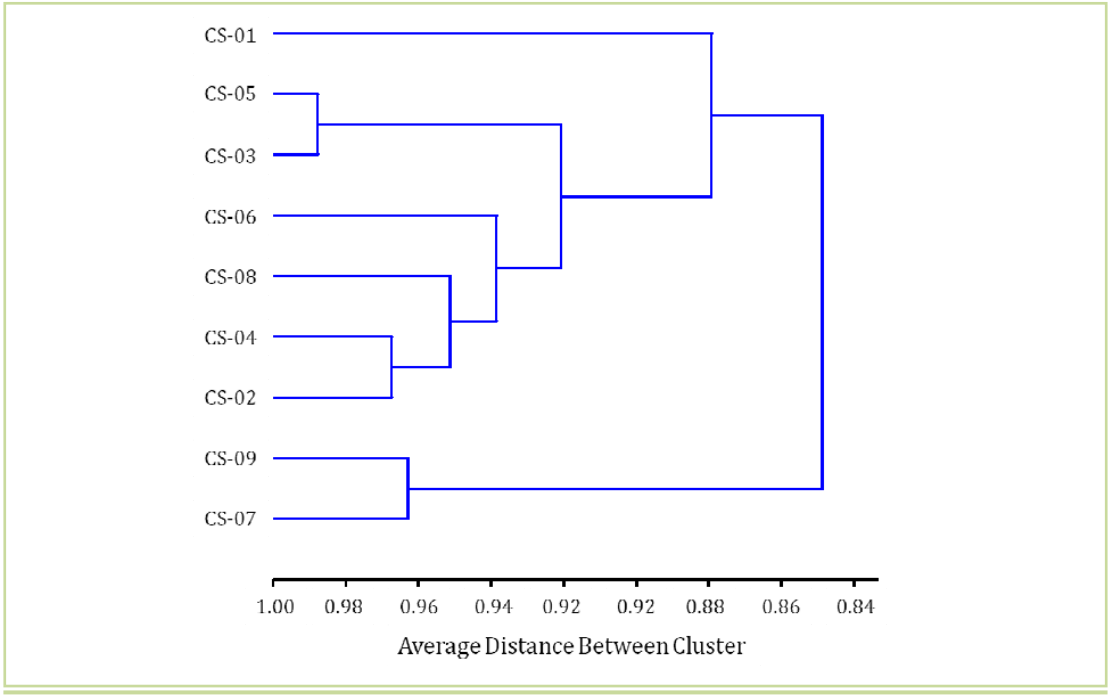

Figure 10: Cluster dendrogram based on morphometrics parameters of *Castanea sativa* Mill. leaves genotypes

The Figure 10 clearly identified significant differences between tested *Castanea sativa* genotypes. In this study, nine genotypes were grouped into four main clusters based on highest similarities. Cluster I contained the genotype (CS-01) only, which differs from other

genotypes of collection by lamina length and width. Cluster II contained only two (CS-03 and CS-05) *Castanea sativa* genotypes. In the cluster III included 4 genotypes and in the cluster IV – 2 genotypes, which had similar mean values of leaves morphological parameters. Figure 10 confirms results from the evaluated variability of morphometric characteristics (Table 1).

Conclusions

In general, our results indicate considerable leaf variation in the collections studied. The leaf parameters are suitable variables to detect levels of variability. The high diversity observed in the introduction population *Castanea sativa* studied is very important for the conservation of the species genetic resources. The high variability of the chestnut collection, created in the Grishka Botanical Garden, is a guarantee of its stability in the conditions of introduction (Skvortsov et al., 2005). In the course of further breeding work with this crop, it will be possible, based on this study, to select specimens with the most economically valuable traits in the first year of life of the seed offspring.

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IN VITRO EVALUATION OF OXIDATIVE STRESS BIOMARKERS IN THE MUSCLE TISSUE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) EXPOSED TO LEAF EXTRACT OF *FICUS BENJAMINA* L. AND ITS CULTIVARS

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The aim of this study was to evaluate the *in vitro* effect of extracts obtained from leaves of *Ficus benjamina* L. 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. The leaves of *F. benjamina* and its cultivars, i.e. *F. benjamina* 'Safari', 'Baroque', 'Amstel Gold', 'Reginald' were sampled for our study. Our results showed that extracts obtained from leaves of *F. benjamina* 'Safari' and *F. benjamina* 'Reginald' decreased non-significantly the lipid peroxidation biomarker in the muscle tissue. Extracts obtained from leaves of *F. benjamina* and its cultivars decreased the ketonic derivatives of oxidatively modified proteins in the muscle tissue. Our results showed that extracts obtained from leaves of *F. benjamina* and its cultivars increased efficiently the total antioxidant capacity in muscle tissue by 76.9% (*F. benjamina*), 66.9% (*F. benjamina* 'Safari'), 70.5% (*F. benjamina* 'Baroque'), 49.4% (*F. benjamina* 'Amstel Gold'), and 42.8% (*F. benjamina* '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. 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., rainbow trout (*Oncorhynchus mykiss* Walbaum), muscle tissue, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

Introduction

Herbs are currently used in commercial aquaculture as growth-promoting substances, antimicrobial agents, nutrients as well as many other applications. Their potential to prevent

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and control fish diseases is also being studied (Galina et al., 2009). The use of immunostimulants, as dietary supplements, can improve the innate defence of animals providing resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer and vaccination (Bricknell and Dalmo, 2005). During the functioning of the immune system, such as in phagocytosis, reactive oxygen and nitrogen species are generated. If they are left unchecked they can affect the components of the immune system by inducing oxidative damage. Natural compounds from medicinal plants having antioxidant and immunomodulatory activities have potential as therapeutic agents in this regard (Devasagayam and Sainis, 2002).

Many studies have proved that plant-derived additives enhanced the growth of fishes and protected from diseases. The non-specific immune system of fish is considered to be the first line of defence against invading pathogens (Ahilan et al., 2010).

In this study, attention was focused on *Ficus* L., a genus with diverse ethnobotanical uses in its geographical distribution range. The genus has occupied an important place among plant genera applied for 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 (Lansky and Paavilainen, 2011).

Ficus benjamina L. (Moraceae Gaudich.) 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. 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). 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 an insect repellant (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). The ability of plant extracts to inhibit the activity of bacteria having potential interest as fish pathogens have been well documented. Nevertheless, although antimicrobial activities of extracts obtained from the leaves of various species of *Ficus* genus were investigated (Solomon-Wisdom et al., 2011; Olusesan et al., 2010; Namita and Mukesh, 2012; Tkachenko et al., 2016–2018), studies regarding their antioxidant properties *in vitro* model with the muscle tissue of the rainbow trout (*Oncorhynchus mykiss* Walbaum) have not been undertaken. To estimate oxidative stress using animal models, many markers of oxidative stress are used. One of the oldest but still widely used assays for the determination of oxidative stress in serum is the TBARS (thiobarbituric acid reactive substances) assay (Dasgupta, Klein, 2014). TBARS are a common way to measure lipid peroxidation products in cells, tissues, and body fluids. TBARS is probably

the oldest and one of the most widely used assays for measuring lipid peroxidation end product malondialdehyde, a reactive aldehyde produced by lipid peroxidation of polyunsaturated fatty acids (Marrocco et al., 2017).

Therefore, the purpose of this study was to evaluate the *in vitro* effect of extracts obtained from leaves of *Ficus 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 current scientific project undertaken in the frame of cooperation programme between Institute of Biology and Environmental Protection (Pomeranian University in Slupsk, Poland), M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine (Kyiv, Ukraine), and Ivan Franko Lviv National University (Lviv, Ukraine) directed to assessment of medicinal properties of tropical plants.

Figure 1 The growth habit of *Ficus benjamina* L. (A) and twigs with syconia (B)

Material and methodology

Collection of plant material

The leaves of plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Specifically, the leaves of *F. benjamina* and its cultivars, i.e. *F. benjamina* 'Safari', 'Baroque', 'Amstel Gold', 'Reginald' were sampled for our study.

Preparation of plant extracts

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

Experimental fish

Clinically healthy rainbow trout (*Oncorhynchus mykiss* Walbaum) with a mean body mass of 80–120 g were used in the experiments. The study was carried out in a Department of Salmonid Research, Inland Fisheries Institute (Rutki, Poland). The experiments were performed in water at 14.5 ±0.5 °C and pH 7.2–7.4. The dissolved oxygen level was about 9 ppm with additional oxygen supply, with a water flow of 25 $L.min^{-1}$, and a photoperiod of 12 h per day. The same experimental conditions were used during the whole research. The water parameters were maintained under constant surveillance. The fish were held in square tanks (150 fish per tank) and fed commercial pelleted diet.

Muscle tissue samples

The trout muscle tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2). The minced muscle tissue was rinsed clear of blood with cold isolation buffer and homogenized in a homogenizer H500 with a motor-driven pestle on ice. Homogenates were centrifuged at 3,000 g for 15 min at 4 °C. After centrifugation, the supernatant was collected and frozen at -20 °C until analyzed. Protein contents were determined with the method described by Bradford (1976) with bovine serum albumin as a standard. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ±0.5 °C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) (n = 8). The enzymatic reactions were started by adding the tissue supernatant.

Experimental design

The supernatant of the muscle tissue was used to incubate with extracts obtained from leaves of *F. benjamina* and its cultivars (in a ratio 19 : 1) at room temperature. The control group (trout muscle tissue) was incubated with 100 mM Tris-HCl buffer (pH 7.2) (in a ratio 19 : 1). The incubation time was 2 hours. Oxidative stress biomarkers were studied in the incubated homogenate (control group and in samples with extracts obtained from leaves of *F. benjamina* and its cultivars).

The 2-thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was evaluated by the production of 2-thiobarbituric acid-reactive substances (TBARS). An aliquot of the homogenate was used to determine the lipid peroxidation status of the sample by measuring the concentration of 2-thiobarbituric-acid-reacting substances (TBARS), according to the method of Kamyshnikov (2004). Reaction mixture contained sample homogenate (2.1 mL, 10% w/v) in tris-HCl buffer (100 mM, pH 7.2), 2-thiobarbituric acid (TBA; 0.8%, 1.0 mL), and trichloracetic acid (TCA; 20%, 1.0 mL). The total volume was kept in a water bath at 100 °C for 10 min. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. TBARS values were reported as nmoles malonic dialdehyde (MDA) per mg protein.

Carbonyl groups of the oxidatively modified proteins assay

Carbonyl groups were measured as an indication of oxidative damage to proteins according to the method of Levine and co-workers (1990) in the modification of Dubinina and

co-workers (1998). Samples were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2M HCl. Blanks were run without DNTP. Afterwards, proteins were precipitated with 20% TCA and centrifuged for 20 min at 3,000 g. The protein pellet was washed three times with ethanol: ethyl acetate (1 : 1) and incubated at 37 °C until complete resuspension. The carbonyl content was measured spectrophotometrically at 370 nm (aldehydic derivatives, OMP_{370}) and at 430 nm (ketonic derivatives, OMP_{430}) (molar extinction coefficient 22,000 $M^{-1} \cdot cm^{-1}$) and expressed as nmol per mg protein.

Total antioxidant capacity (TAC) assay

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was incubated for 48 hrs at 37 °C. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 1 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

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

Results and discussion

In a present study, we have studied the influence of extracts derived from leaves of *F. benjamina* and its cultivars, grown under glasshouse conditions, on the lipid peroxidation measured by the quantity of TBARS level in the muscle tissue of rainbow trout after incubation with extracts *in vitro*. As presented in Figure 2, our results showed that extracts obtained from leaves of *F. benjamina* 'Safari' and *F. benjamina* 'Reginald' decreased non-significantly the TBARS level in muscle tissue by 21.4% and by 9% (p >0.05), respectively. On the other hand, extracts obtained from leaves of *F. benjamina* 'Baroque' and 'Amstel Gold' increased non-significantly the TBARS level in muscle tissue by 11.6% and by 10.4% (p >0.05), respectively (Figure 2).

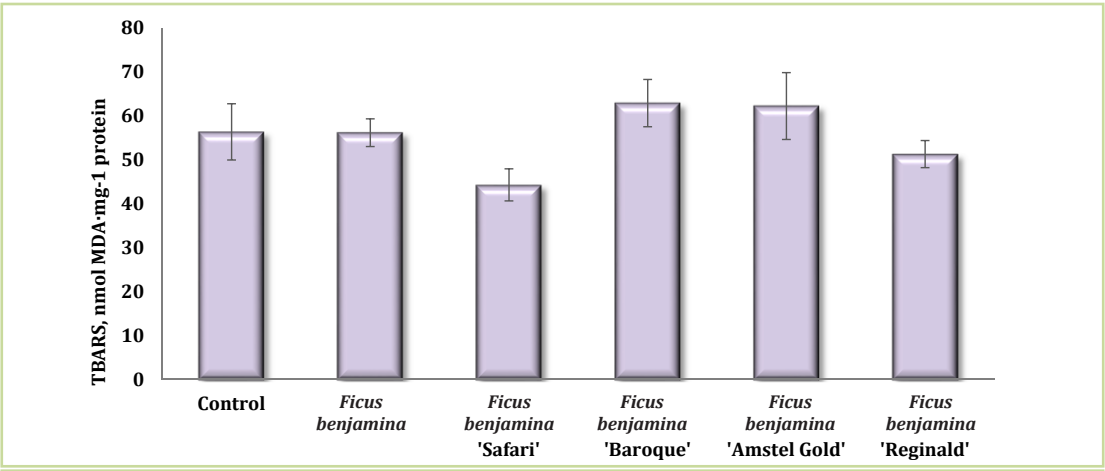

Figure 2 Lipid peroxidation measured by the quantity of TBARS level (nmol MDA·mg^{-1} protein) in the muscle tissue of rainbow trout after incubation with buffer extracts obtained from leaves of *Ficus benjamina* and its cultivars (M ±m, *n* = 8)

Our results also showed that extracts obtained from leaves of *F. benjamina* and its cultivars decreased the ketonic derivatives of oxidatively modified proteins in the muscle tissue by 5.1% (*F. benjamina*), 5.6% (*F. benjamina* 'Safari'), 13.1% (*F. benjamina* 'Baroque'), 5.9% (*F. benjamina* 'Amstel Gold'), and 1.8% (*F. benjamina* 'Reginald'). This decrease was non-statistically significant (p >0.05). Aldehydic derivatives content was ranged on the control group level (Figure 3).

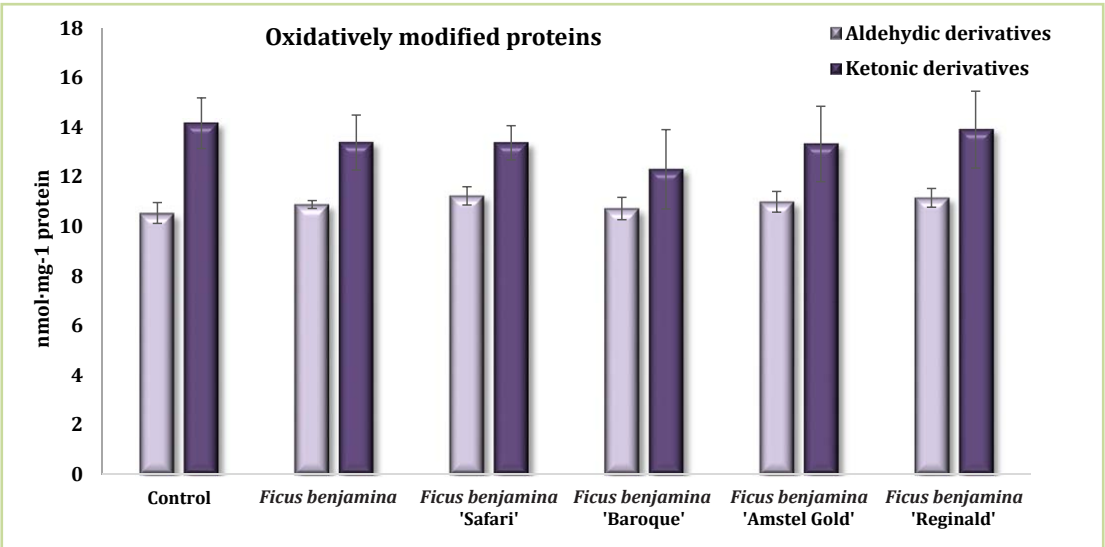

Figure 3 Level of the aldehydic and ketonic derivatives (nmol·mg^{-1} protein) in the muscle tissue of rainbow trout after incubation with buffer extracts obtained from leaves of *Ficus benjamina* and its cultivars (*M* ±m, n = 8)

In a present study, we also have investigated the influence of extracts derived from leaves of *F. benjamina* and its cultivars on the total antioxidant capacity in the muscle tissue of rainbow trout after incubation with extracts *in vitro*. Our results showed that extracts obtained from leaves of *F. benjamina* and its cultivars increased efficiently the TAC level in muscle tissue by 76.9% (*F. benjamina*), 66.9% (*F. benjamina* 'Safari'), 70.5% (*F. benjamina* 'Baroque'), 49.4% (*F. benjamina* 'Amstel Gold'), and 42.8% (*F. benjamina* 'Reginald') (p <0.05) (Figure 4).

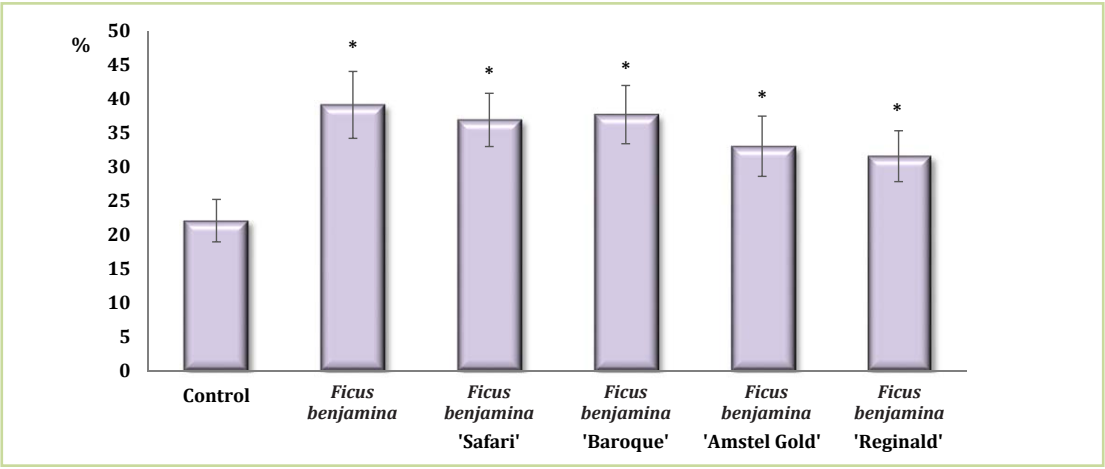

Figure 4 The total antioxidant capacity in the muscle tissue of rainbow trout after incubation with buffer extracts obtained from leaves of *Ficus benjamina* and its cultivars (M ±m, n = 8).* – the changes are statistically significant (p <0.05) compared to the control group

Therefore, the results suggested that the extracts screened could be a potential source of natural antioxidants. Supplementation of extracts obtained from leaves of *F. benjamina* and its cultivars caused to increase of antioxidant responses in muscle tissue of trout. It would be reasonable to suggest that these antioxidant effects are determined by their by-products, i.e. flavonoids. Indeed, the results of Imran et al. (2014) indicated that *F. benjamina* is a good source of antioxidants with high reducing power. *F. benjamina* disclosed substantial bioactivity, and this plant can be regarded as a potential source of antioxidant agents. The root and leaves showed good antioxidant activity, whereas stem extract and fractions revealed good antimicrobial activity. *F. benjamina* disclosed substantial bioactivity, being root extract and fractions the most active. This plant can be regarded as a potential source of antioxidant and antimicrobial agents. This investigation is in line with our previous works which have revealed a great potential of *Ficus* species as plants with potent antimicrobial properties. In our previous study, the *in vitro* antimicrobial activity of the ethanolic leaf extracts of various *Ficus* species against fish pathogens was evaluated (Tkachenko et al., 2016–2017).

Imran et al. (2014) in their study revealed that the methanol extract and n-butanol fraction showed greater percent inhibition of linoleic acid system, compared to other fractions. The percent inhibition in the linoleic acid system for stem was in the range of 16.94–78.16, in root 20.57–85.87 and leaves 26.82–69.81%. The maximum percent inhibition was determined

by methanol extract (85.87) and butanol fraction (81.48) of the root. The results of Imran et al. (2014) experiments revealed that the antioxidant potential of plant increased linearly with the increase in concentration. The methanol extract as well as fractions of root exhibited a linear rise in absorbance value for various concentrations 0.56 nm: 2.5 ($mg.mL^{-1}$), 0.87 nm: 5 ($mg.mL^{-1}$), 1.03 nm: 7.5 ($mg.mL^{-1}$) and 1.49 nm: 10 ($mg.mL^{-1}$). The presence of phenolic compounds might be the reason for reducing power. The results of this assay indicated that the plant is a good source of antioxidants with high reducing power. Methanol extracts of stem, root, and leaves exhibited IC_{50} values of 50.1, 58.81 and 49.86 $\mu g.mL^{-1}$, respectively. The maximum value of IC_{50} was demonstrated by root's fraction of n-hexane (580.75 $\mu g.mL^{-1}$), indicating that this fraction showed minimum free radical scavenging activity. Unlike the n-hexane fraction, chloroform and ethyl acetate fractions exhibited lower values of IC_{50}. The methanol extract and *n*-butanolic fractions showed maximum free radical scavenging activity. The *n*-hexane fractions of root revealed the maximum value of IC_{50} (580.75 $\mu g.mL^{-1}$). Methanol and n-butanol fractions exhibited the lowest IC_{50} values, showing a maximum value (158.34 $\mu g.mL^{-1}$) for root (Imran et al., 2014).

Literature reports indicate that the power of bioactive compounds is associated with the antioxidant activity of *F. benjamina* (Mousa et al., 1994; Parveen et al., 2009; Ogunwande et al., 2012; Yarmolinsky et al., 2012; Imran et al., 2014). Essential oils obtained by hydrodistillation of leaves of *F. benjamina* were analyzed for their constituents by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) by Ogunwande et al. (2012). The leaf oil of *F. benjamina*, collected during the day, contained high contents of alpha-pinene (13.9%), abietadiene (9.7%), cis-alpha-bisabolene (8.2%) and germacrene-D-4-ol (8.4%), while the night sample was dominated by germacrene-D-4-ol (31.5%), 1,10-di-epi-cubenol (8.8%) and hexahydro farnesyl acetone (8.3%). This could be a possible indication of differences in emissions of volatiles by *F. benjamina* during the day and night (Ogunwande et al., 2012). 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 root and one in leaves. *F. benjamina* fruit extracts also showed antitumor and antibacterial activity (Mousa et al., 1994), while aqueous and alcoholic leaf extracts had significant antinociceptive activity (reducing sensitivity to painful stimuli) in analgesia test (Parveen et al., 2009). The fruit extracts of *F. sycomorus*, *F. benjamina*, *F. benghalensis*, and *F. religiosa* were screened for bioactivity. *F. bengalensis* and *F. religiosa* demonstrated activity in the brine shrimp test (*Artemia salina*) which indicates toxicity, whereas *F. sycomorus* and *F. benjamina* showed no activity. All the fruit extracts exhibited antitumor activity in the potato disc bioassay. None of the tested extracts showed any marked inhibition of the uptake of calcium into rat pituitary cells GH4C1. The extracts of the four tested *Ficus* species had significant antibacterial activity, but no antifungal activity (Mousa et al., 1994). The aqueous and alcoholic extracts of leaves of F. bejamina showed significant antinociceptive activity in an analgesiometer test (Parveen et al., 2009). The presence of polyphenols in *F. benjamina* glandular epithelium has been reported (Pennisi et al., 1999). A new triterpene, named serrate-3-one, along with phytoconstituents pentacontanyl decanoate, friedelin and beta-sitosterol have been detected in *F. benjamina* (var. *comosa*) benzenoid extracts (Parveen et al., 2009). All flavone glycosides quercetin 3-O-rutinoside (1), kaempferol 3-O-rutinoside (2) and kaempferol 3-O-robinobioside (3)

from *F. benjamina* showed high antiviral activity against Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) without any significant activity against Varicella Zoster Virus (VZV) in the study of Yarmolinsky et al. (2012). Kaempferol 3-O-robinobioside showed the highest antiherpetic activity, similar to that of acyclovir (ACV). The highest antiviral activity of all these flavone glycosides was obtained when the infected cells were treated during and after infection (Yarmolinsky et al., 2012).

Flavonoids and phenolic acids may be responsible for antioxidant activities of *Ficus benjamina* and its cultivars in the muscle tissue of rainbow trout after *in vitro* incubation. Indeed, flavonoids act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and light screening. Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce the free radical formation and to scavenge free radicals (Pietta, 2000). Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in a free radical generation; (2) scavenging ROS; and (3) upregulation or protection of antioxidant defences (Kumar and Pandey, 2013). Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth (Brown et al., 1998).

Antioxidant activity usually means the ability of a compound to delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and reducing oxidative stress (Bhanwase and Alagawadi, 2016). Therefore, the antioxidant containing in *F. benjamina* leaf extracts may offer resistance to rainbow trout against the oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevents diseases by elevating the specific immune response.

Conclusions

Present study ascertained the antioxidant potency of the extracts obtained from leaves of *F. benjamina* and its cultivars as a potential source of natural antioxidants. *Ficus benjamina* disclosed substantial bioactivity, and this plant can be regarded as a potential source of antioxidant agents. Thus, 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. 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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PRELIMINARY *IN VITRO* SCREENING OF ANTIBACTERIAL ACTIVITY OF LEAF EXTRACT FROM *FICUS NATALENSIS* SUBSP. *NATALENSIS* HOCHST. (MORACEAE) AGAINST FISH PATHOGENS

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The aim of this study was to screen the antibacterial efficacy of ethanolic extract obtained from the leaves of *Ficus natalensis* subsp. *natalensis* Hochst. against fish pathogens, *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri* and to evaluate the possible use of this plant in preventing infections caused by these bacteria in aquaculture. The bacterial growth inhibition tests were carried out using agar well diffusion method with the use of crude ethanolic extract. Muller-Hinton agar plates were inoculated with 200 and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium. In our study, the *A. hydrophila* strain (200 and 400 µl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract (inhibition zone diameters ranged from 10.0 to 11.8 mm). *C. freundii* (200 and 400 µl) was resistant to ethanolic extract (7.5 and 11.7 mm). The extract exhibited intermediate antibacterial activity against *P. fluorescens* causing a zone of inhibition, comprising at least 10.2–12.8 mm for 200 µL and 7.4–11.2 mm for 400 µL of bacterium strain. *Y. ruckeri* (200 and 400 µl) revealed intermediate susceptibility (8.0 and 16.5 mm). This may be because the herbs have more than one extractable active compound that provides antimicrobial activity. Further chemical analysis of the aforementioned plant extract should be performed to determinate chemical composition and identify the exact phytocompounds responsible for the antimicrobial activity.

Keywords: *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri*, antimicrobial activity, disc diffusion technique, ethanolic extract

Introduction

Currently, outbreaks of parasitic, bacterial, and fungal diseases act as major limiting factors for fish farming, meaning that producers have to make use of massive amounts of antibiotics,

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disinfectants, and pesticides in order to control mortality and avoid huge economic losses (Valladão et al., 2015). In this context, there is an urgent need for the development of alternative therapies against bacterial pathogens in aquaculture production. Consequently, several alternatives to the use of antibiotics have been applied successfully in aquaculture (Romero et al., 2012). The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment (Balcázar et al., 2006). Other sources of alternative treatment are essential oils and plant-derived extracts, which have been used *in vivo* as antibacterial agents to control bacterial infections (Cowan, 1999). There is documented evidence for the development of resistance of fish pathogens to antimicrobial agents (McPhearson et al., 1991; Founou et al., 2016; Gabriel et al., 2017). After the emergence of multi-drug resistant pathogens in aquaculture, the research for new remedy alternatives has led to the recognition of the potential of plant extracts for treating the infections associated to fish pathogens (McPhearson et al., 1991). These compounds may constitute alternative prophylactic and therapeutic agents in aquaculture because of their antibacterial properties (Turker and Yıldırım, 2015). Moreover, large numbers of plants have been proven to be rich sources of cheaper immune-enhancing and growth promoter substances, with a wide therapeutic and preventive spectrum of activity, potentially useful in solving the multiple health problems that characterize aquaculture (Dügenci et al., 2003; De Vico et al., 2018).

In our previous studies, therapeutic potential for the use of various plants of *Ficus* L. genus in the control of bacterial diseases was evaluated against fish pathogens in *in vitro* study with promising results (Tkachenko et al., 2016-2018). Antibacterial properties of plant extracts have been by far the most studied bioactivity with potential application in aquaculture systems (Reverter et al., 2014). Castro and co-workers (2008) have revealed by agar diffusion assay that 31 methanolic extracts of Brazilian plants presented antibacterial activity against the fish pathogenic bacteria, i.e. *Streptococcus agalactiae*, *Flavobacterium columnare*, and *A. hydrophila*. *F. columnare* being the most susceptible microorganism to most of the tested extracts. Similarly, Wei and Musa (2008) also studied the susceptibility by assay of minimum inhibitory concentration (MIC) of two Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*), four Gram-negative bacteria (*C. freundii*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*) and 18 isolates of Edwardsiella tarda to aqueous extract of garlic (500, 250, 125, 62.5 $mg.mL^{-1}$), and found that all garlic extracts were effective against the tested pathogenic bacteria.

The angiosperm family Moraceae Gaudich., or mulberry family, is a diverse group of nearly 1100 species, predominantly woody and with milky latex in all parts of their body, which are distributed throughout the tropics and subtropics and rarely extend to the temperate zone. They are represented by a variety of growth forms, such as terrestrial and hemi-epiphytic trees, shrubs, lianas, subshrubs, and herbs, with small unisexual flowers assembled into various, often peculiar inflorescences (Datwyler and Weiblen, 2004; Clement and Weiblen, 2009). The pantropical genus *Ficus*, with its approximately 750 species, is the largest within the family and one of the most speciose genera of flowering plants. Among all Moraceae, it is characterized by the presence of waxy glands on vegetative organs, heterostyly, and prolonged protogyny, that is the anthesis of staminate flowers in already mature fruits. These

features are functionally linked to the unique pollination mode in *Ficus* involving mutualistic relationships with agaonid wasps (order *Hymenoptera*). The closed urceolate inflorescences provide a shelter for the development of wasps, which, in turn, are the only pollinators of these plants ensuring their reproductive propagation (Cook and Rasplus, 2003; Berg and Corner, 2005). *Ficus* trees have a number of uses in various industries and fields of human activity. The reported biological activities of *Ficus* plants include antioxidant (Mohan et al., 2015), antiplasmodial (Muregi et al., 2003), anticancer (Mbosso et al., 2016a), antimicrobial (Awolola et al., 2017), antiulcer (Galati et al., 2001), antidiarrhoeal (Mandal and Kumar, 2002), anti-pyretic (Rao et al., 2002), and gastroprotective (Rao et al., 2008) properties. Virtually all parts of their body are utilized in ethnomedicine to cure disorders of digestive and respiratory systems, skin diseases, parasitic infections, etc. Some species have been cited to have analgesic, tonic, and ecbolic effects (Lansky and Paavilainen, 2011).

Ficus natalensis subsp. *natalensis* Hochst. is a monoecious evergreen tree up to 30 m tall or shrub, hemi-epiphytic or terrestrial, which naturally occurs in southern and eastern Africa. The leaves are 2.5–10.0 cm long and 1–5 cm across (sub)coriaceous and glabrous, elliptic to oblong or obovate, with plane margin and acuminate to the rounded apex. Figs are born in the leaf axils or just below the leaves, pedunculate, globose to ellipsoid to obovoid, 1.5–2.0 cm in diameter, glabrous, at maturity reddish, orange or yellowish to brown (Berg and Wiebes, 1992).

Figure 1 Leaves of *Ficus natalensis* subsp. *natalensis* Hochst.

Therefore, the aim of this study was to assess the antibacterial efficacy of ethanolic extract obtained from the leaves of *Ficus natalensis* subsp. *natalensis* against fish pathogens, *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri* and to evaluate the possible use of this plant in preventing infections caused by these bacteria in aquaculture.

The current investigation was conducted as a part of ongoing project between Institute of Biology and Environmental Protection (Pomeranian University in Slupsk, Poland), M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine (Kyiv,

Ukraine), and Ivan Franko Lviv National University (Lviv, Ukraine) undertaken in the frame of cooperation program aimed at assessment of medicinal properties of tropical plants, cultivated *in vitro*.

Material and methodology

Collection of plant material and preparing plant extract

The leaves of *F. natalensis* subsp. *natalensis* were sampled at National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine) and Botanic Garden of Ivan Franko Lviv National University (Lviv, Ukraine). The sampled leaves of *F. natalensis* subsp. *natalensis* were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 10) at room temperature, and centrifuged at 3.000 g for 5 minutes. Supernatants were stored at -20 °C in bottles protected with the laminated paper until required.

Method of culturing pathological sample and identification method of the bacteria

Aeromonas hydrophila (strain E 2/7/15) and *Pseudomonas fluorescens* (strain E 1/7/15) isolated locally from internal organs of rainbow trout (*Oncorhynchus mykiss* Walbaum) with clinical features of furunculosis (kidneys were grey, the liver was pale and fragile, enlarged spleen with exudate in the body cavity), as well as *Citrobacter freundii* isolated locally from gill of eel (*Anguilla anguilla* L.) with clinical features of disease were used as test organisms. Samples of internal organs (kidneys, spleen, liver) weighing 2 g were taken and homogenized before preincubation in TSB broth (Trypticase Soy Broth, Oxoid®) for 24 hrs. After preincubation, bacterial culture was transferred to two different cultivation media: TSA (Trypticase Soy Agar, Oxoid®) and BHIA (Brain Heart Infusion Agar, Oxoid®) supplemented with 5% of sheep blood (OIE Fish Diseases Commission, 2000). After 48 hrs of incubation at 27 °C, characteristic pink colonies were selected for further examination.

The isolates of *Y. ruckeri* were collected from clinically healthy fish and fish with clinical symptoms of yersiniosis. Internal tissues (predominantly pronephros and gills), as well as intestinal swabs, were sampled. Tissue samples were homogenized and inoculated on nutritional agar with 5% blood (Columbia Blood Agar, Oxoid®). Following a 24 h incubation period at 25 ±2 °C, distinctive colonies were transferred onto TSA. Round, elevated, shining and whitish colonies without hemolytic properties were considered typical of *Y. ruckeri*. After 24h-incubation at 25 ±2 °C, an oxidase test and Gram-staining were performed. Gram-negative and oxidase-negative isolates were cultured on TSA medium and incubated for 24 h at 25 ±2 °C.

Preliminary characterization of isolates

Bacterial species were identified with the use of the oxidase test and API E test kit (BioMérieux, France). The results of the test were interpreted in accordance with the manufacturer‘s protocol, after 24 hrs of incubation at 27 °C. Codes + + V-V--- + V + + + --- + -VV + in API E test were identified as *A. hydrophila*. The strain was obtained from Diagnostics Laboratory of Fish and Crayfish Diseases, Department of Veterinary Hygiene, Provincial Veterinary Inspectorate in Olsztyn (Poland).

For characterization of *Y. ruckeri* isolates, bacteria were Gram-stained and then morphologically evaluated. The 24h bacterial culture was wet-mounted and a microscopic smear on the slide was prepared. Following fixation over the flame, the slide was Gram-stained with a Gram color set (Merck) according to the manufacturer's instructions. The shape of the bacteria was determined by observing the microorganisms under a light microscope at 1000x with immersion oil (Kocwowa, 1981; Whitman and MacNair, 2004). Motility was examined on a wet mount. A drop of distilled water was put on a coverslip and bacteria were mounted on it with drops of distilled water put on the corners of a slip. The slip was then covered with a special microscopic slide with an indentation and the whole set was vigorously turned. The motility of the bacteria was evaluated under a light microscope at 400× (Kocwowa, 1981; Whitman and MacNair, 2004).

Oxidase test was performed according to the manufacturer's instruction (Merck). Biochemical properties of individual *Y. ruckeri* isolates were investigated with the API 20E system (BioMérieux, France). Tests were performed according to the manufacturer's instructions. The results, namely, the presence or a lack of reaction, were read based on the key featured in the operating procedure provided by the manufacturer of the assay. The results were analyzed with the Apiweb software (BioMérieux, France) to identify the investigated bacterium.

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

Strains tested were plated on TSA medium (Tryptone Soya Agar) and incubated for 24 hr at 25 °C. Subsequently, the microorganisms were suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibacterial activity (Bauer et al., 1966). Muller-Hinton agar plates were inoculated with 200 and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) 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. The antimicrobial susceptibility testing was done on Muller-Hinton agar by disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). A negative control disc impregnated by sterile ethanol was used in each experiment. The sensitivity of strain was also studied to the commercial preparation with extracts of garlic (in dilution 1 : 10, 1 : 100 and 1 : 1000). After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hrs at 25 °C. The diameters of the inhibition zones were measured in millimetres and compared with those of the control and standard susceptibility disks. Activity was evidenced by the presence of a zone of inhibition surrounding the well.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test (p >0.05). In order to find significant differences (significance level, p <0.05) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Poland). The

following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Data on antimicrobial activities of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* against *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri* isolated from fish expressed as mean of diameters of inhibition zone are presented in Figs 2–6. The mean inhibition zone diameter induced by 96% ethanol was (8.4 ±0.6) mm. The ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* increased the zone of *Aeromonas hydrophila* growth inhibition to (11.5 ±0.9) mm (200 μL of standardized inoculum) and (11.3 ±1.2) mm (400 μL), respectively. The extract also increased the inhibition zone for *Citrobacter freundii* growth – (9.6 ±0.95) mm for 200 μL of standardized inoculum and (10.7 ±0.98) mm for 400 μL of standardized inoculum. Similarly, the growth of *Pseudomonas fluorescens* was inhibited by application of the ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis*. The inhibition zone was (11.1 ±1.5) mm for 200 μL of standardized inoculum and (9.8 ±1.1) mm for 400 μL of standardized inoculum. The highest degree of growth inhibition zone (14.4 ±1.4) mm was observed for 200 μL of *Yersinia ruckeri* isolated (increased by 71%, p <0.05, compared to control sample), while 400 μL of standardized inoculum was resistant to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (Figure 2).

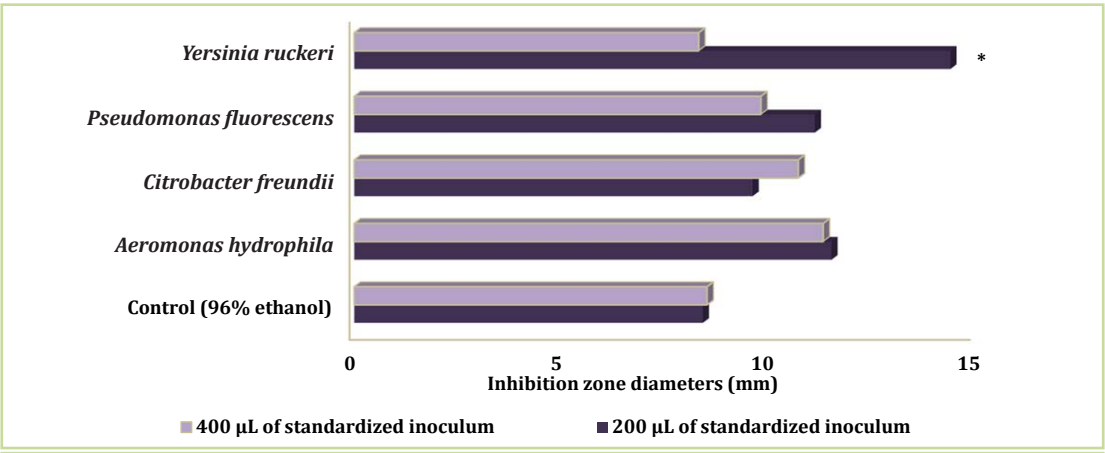

Figure 2 The mean inhibition zone diameters induced by ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* against *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri*. Muller-Hinton agar plates were inoculated with 200 and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of bacteria
* changes were statistically significant (p <0.05) compared to control sample (96% ethanol)

In our study, the *A. hydrophila* strain (200 and 400 μl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters ranged from 10.0 to 11.8 mm) (Figures 2, 3).

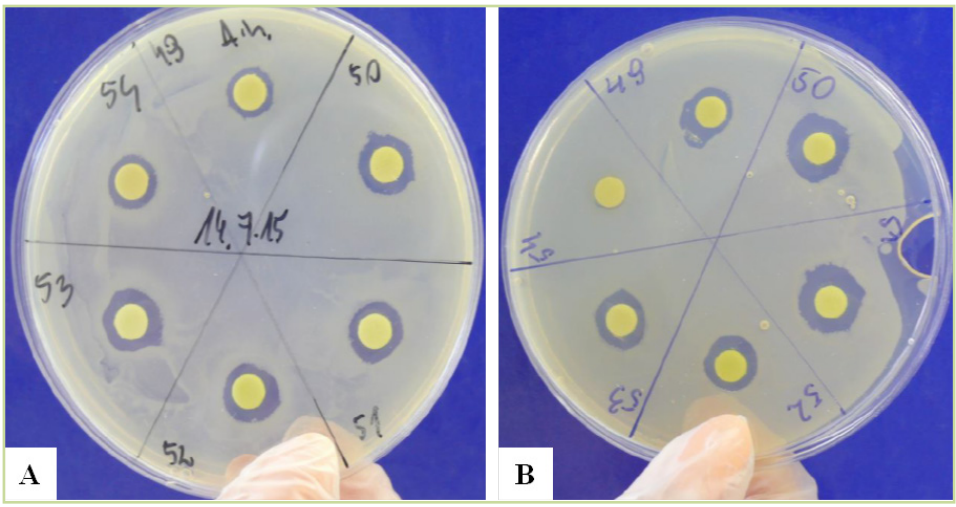

Figure 3 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Aeromonas hydrophila*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

Our results also demonstrated that the *C. freundii* (200 and 400 µl of standardized inoculum) was resistant to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters ranged between 7.5 and 11.7 mm) (Figure 4).

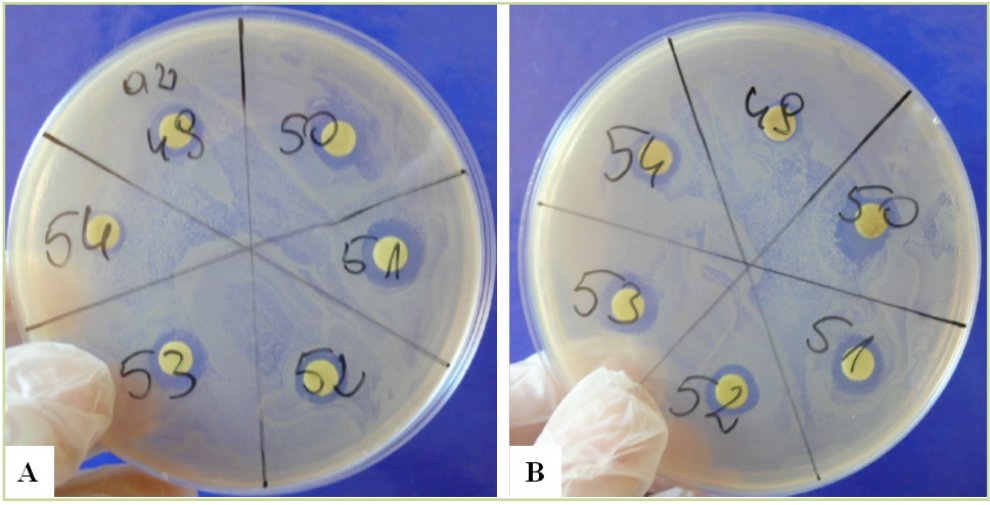

Figure 4 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Citrobacter freundii*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

The ethanolic extract derived from *F. natalensis* subsp. *natalensis* leaves exhibited intermediate antibacterial activity against *Pseudomonas fluorescens* causing a zone of

inhibition, comprising at least 10.2–12.8 mm for 200 μL and 7.4–11.2 mm for 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of bacterium strain (Figures 2, 5).

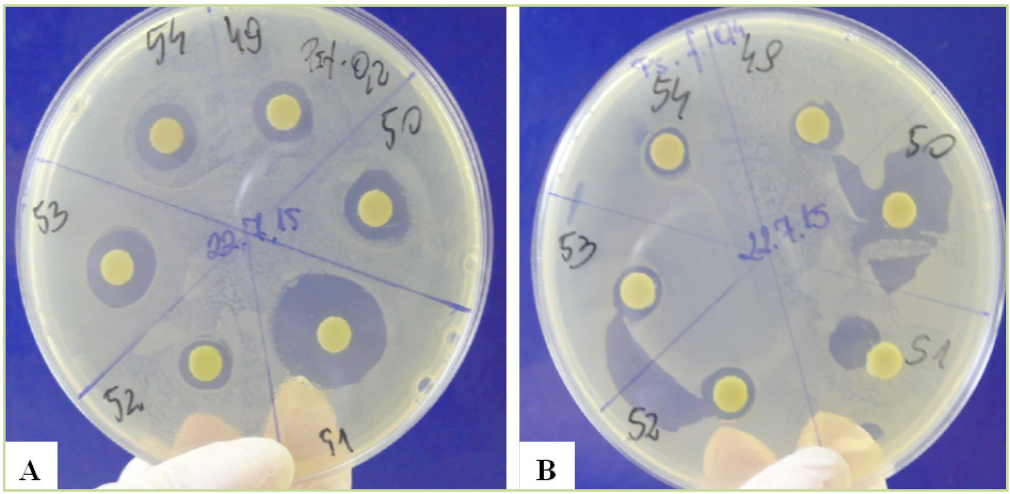

Figure 5 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Pseudomonas fluorescens*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

Y. ruckeri (200 and 400 μl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters were ranged between 8.0 and 16.5 mm) (Figure 6). Moreover, statistically significant increase (by 71%, p <0.05) of inhibition zone diameters induced by ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* compared to control (96% ethanol) was observed.

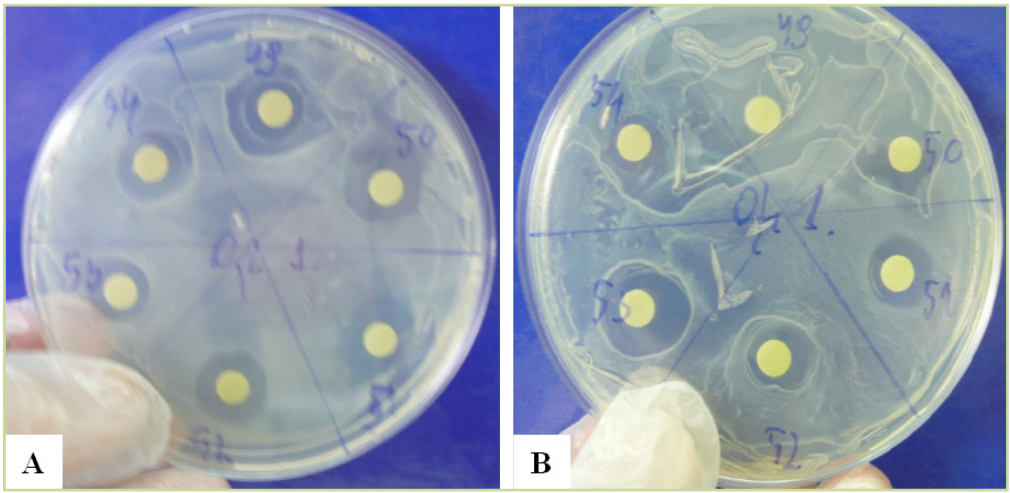

Figure 6 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Yersinia ruckeri*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

This investigation is in line with our previous works which have revealed a great potential of *Ficus* species as plants with potent antimicrobial properties. In our previous study, the *in vitro* antimicrobial activity of the ethanolic leaf extracts of various *Ficus* species against *Citrobacter freundii* was evaluated. The results proved that the extracts derived from *F. drupacea*, *F. septica*, *F. deltoidea* as well as *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma* leaves exhibit a favorable antibacterial activity against *C. freundii* (200 μL of standardized inoculum) (Tkachenko et al. 2016b). Our results also proved that the ethanolic extracts obtained from *F. pumila*, *F. binnendijkii* 'Amstel Gold', *F. carica*, *F. erecta*, *F. hispida*, *F. mucuso*, *F. palmeri*, *F. religiosa* lan eaves possess considerably sufficient antibacterial potential against *C. freundii* (Tkachenko et al., 2017c). Among various species of *Ficus* plants screened ethanolic extracts of the leaves of ten *Ficus* species: *F. hispida*, *F. binnendijkii*, *F. pumila*, *F. rubiginosa*, *F. erecta*, *F. erecta* var. *sieboldii*, *F. sur*, *F. benjamina*, *F. craterostoma*, *F. lyrata*, *F. palmeri* (the species are listed in the order of effectiveness against pathogen tested) were the most effective against *P. fluorescens* (200 μL of standardized inoculum) (Tkachenko et al., 2016a). Moreover, previous investigation has shown that the most effective against *P. fluorescens* (400 μL of standardized inoculum) were the ethanolic extracts obtained from leaves of ten *Ficus* species: *F. craterostoma*, *F. cyathistipula*, *F. drupacea* 'Black Velvet', *F. hispida*, *F. macrophylla*, *F. mucuso*, *F. pumila*, *F. villosa* (Tkachenko et al., 2016e). In our study, most ethanolic extracts obtained from *Ficus* spp. proved effective against the bacterial strain of Gram-negative *A. hydrophila* tested, with 10–12 mm zones of inhibition being observed. Interestingly, *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., 2016c,d,f). Among various species of *Ficus* genus exhibiting moderate activity against *A. hydrophila* (400 μL of standardized inoculum), the highest antibacterial activity was displayed by *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* leaf extracts (Tkachenko et al., 2016c,d,f).

Our results also demonstrated that the *C. freundii* revealed intermediate susceptibility to *F. hispida* (inhibition zone diameters ranged between 11 and 15 mm). *A. hydrophila* revealed intermediate susceptibility concerning to ethanolic extract obtained from leaves of *F. hispida* (inhibition zone diameters were ranged from 8 to 12 mm). The most effective at least causing a zone of inhibition within 14–16 mm was an ethanolic extract from *F. hispida* leaves against *P. fluorescens* both in 200 μL of standardized inoculum of the bacterium (inhibition zone ranged from 15 to 16 mm in diameter) and 400 μL (14–15 mm) (Tkachenko et al., 2017d). Our results also indicated that extract obtained from *F. mucuso* offer a promising alternative to the use of antibiotics in controlling of infection caused by *A. hydrophila*, *C. freundii*, *P. fluorescens*, *Y. ruckeri*. In our study, ethanolic extracts obtained from *F. mucuso* proved effective against bacteria tested, with 10–15 mm zones of inhibition being observed. *F. mucuso* demonstrated the highest antibacterial activity against *C. freundii* and *P. fluorescens*. Among various bacteria tested, the highest susceptibility for 400 μL of standardized inoculum of *C. freundii* and *P. fluorescens* was noted (Tkachenko et al., 2017e). Moreover, the *A. hydrophila* (200 and 400 μl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. benghalensis* (inhibition zone diameters ranged from 8 to 12 mm). The *C. freundii* strain (200 and 400 μl of standardized inoculum) displayed mild susceptibility to an ethanolic

extract obtained from leaves of *F. benghalensis* (inhibition zone ranged between 8 and 10 mm in diameter). The ethanolic extract derived from *F. benghalensis* leaves exhibited the highest antibacterial activity against *Pseudomonas fluorescens* causing a zone of inhibition, comprising at least 8–14 mm, both in 200 µL and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of bacterium strain. *Y. ruckeri* isolate (200 and 400 µl of standardized inoculum) revealed good susceptibility to an ethanolic extract obtained from leaves of *F. benghalensis* (inhibition zone diameters ranged between 8 and 15 mm) (Tkachenko et al., 2017b). The antimicrobial activity profile of ethanolic extract obtained from leaves of *F. pumila* against the tested pathogen strains indicated that *Y. ruckeri* was the most susceptible bacterium (200 and 400 µl of standardized inoculum) among all the bacterial test strains. Similarly, *P. fluorescens* was found to be a sensitive strain (13–14 mm for 200 µL and 9–10 mm for 400 µL of standardized inoculum of bacterium strain) although *A. hydrophila* and *C. freundii* was found to be least susceptible to an ethanolic extract obtained from leaves of *F. pumila*. Of all the bacterial strains included in the test, *Y. ruckeri* (200 and 400 µl of standardized inoculum dilution) and *P. fluorescens* (200 µl) were found to be the most susceptible and *C. freundii*, which is an isolate from gills of eel, was found to be the least inhibited bacterium (Tkachenko et al., 2018).

Ajaib et al. (2016) also have concluded that methanolic extract of *F. natalensis* bark was potentially a promising candidate to be used as a natural source of antifungal medicine. The antimicrobial potential assessment was carried out by using four bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative), *Staphylococcus aureus* and *Bacillus subtilis* (Gram-positive) and two fungal strains *Aspergillus niger* and *Aspergillus oryzae*. The maximum antibacterial activity against *S. aureus* was produced by petroleum ether extract of leaves and bark with a zone of inhibition of 50 ±0.51 mm and 55.7 ±1.15 mm, respectively. The chloroform leaves extract also revealed an inhibition zone of 50 ±2 mm against *S. aureus*. Similarly, the petroleum ether extract of leaves showed significant activity with an inhibition zone of 40 ±0.4 mm against *B. subtilis*. The petroleum ether extract of leaves showed an inhibition zone of 44.7 ±0.57 mm against *E. coli*. The petroleum ether extract of bark and distilled water extract of leaves also exhibited good inhibition of 30 ±0.57 mm and 30 ±0.26 mm respectively against *E. coli*. The results of antibacterial activity evaluation against *P. aeruginosa* were reported by the petroleum ether extract of bark (47 ±0.4 mm). Chloroform and methanol extracts of bark and petroleum ether and methanolic extracts of leaves gave promising results. The MIC value of the methanol extracts of bark and leaves at concentration 1.25 mg.mL^{-1} and 5 mg.mL^{-1} against *E. coli* showed less effective, whereas methanolic extracts of bark against *P. aeruginosa* inhibited the growth up to concentration of 0.625 mg.mL^{-1} but the leaves extract of methanol against *P. aeruginosa* lost its activity beyond at concentration of 5 mg.mL^{-1}. The methanolic extracts of bark and leaves reduced the growth of *S. aureus* up to 0.625 mg.mL^{-1} and *B. subtilis* was grown only below the concentration of 0.625 mg.mL^{-1}. Assessment of antifungal activity revealed that *A. niger* was inhibited by all the extracts with variable potency. The methanol extract of bark was found to be most potent against *A. niger* with a zone of inhibition 43.7 ±1.527 mm. The petroleum ether extract of leaves was least effective against fungal strain (9 ±0 mm). The petroleum ether extract of bark showed a considerably high activity with a zone of inhibition 37 ±0.577 mm. All extracts showed a moderate level of inhibition against *A. oryzae* between 23 ±2.645 mm to 34.8 ±1.607 mm. The estimation of MIC also supported

the activity of methanol extract of bark. The methanol extract of bark showed inhibition of *A. niger* and *A. oryzae* up to the concentration of 0.625 mg.mL^{-1}. The methanol extract of leaves showed low inhibition towards both fungal strains (10 mg.mL^{-1} against *A. niger* and 5 mg.mL^{-1} against *A. oryzae*). *A. niger* was more susceptible to the extracts of the plant, whereas *A. oryzae*, was somewhat resistant to the tested extracts (Ajaib et al., 2016).

The crude stem bark and fruit extracts of *F. natalensis* subsp. *natalensis* were tested for their antibacterial activity against five Gram-negative and seven Gram-positive strains and for their potential anti-biofilm activity was evaluated by Awolola et al. (2017). As the result, the dichloromethane-soluble fruit extract was active against sensitive and resistant *Staphylococcus aureus* strains, *Enterococcus faecalis*, and *Staphylococcus xylosus*. In the anti-biofilm assay, exposure to ethyl acetate, methanol and aqueous methanol leaf, stem bark and fruit extracts decreased adhesion with a biofilm reduction of ≥100% for all three tested organisms: *E. coli*, *P. aeruginosa*, and *S. aureus*. The methanol leaf extract demonstrated the most potent anti-adhesion potential against *E. coli* (218% biofilm reduction) (Awolola et al., 2017).

Consequently, the antimicrobial property of *F. benghalensis* leaf extract may be manifested due to its constituents. As was shown, the phytochemical screening of the leaf, stem bark and fruit extracts of *F. natalensis* subsp. *natalensis* (Awolola et al., 2017) detected the presence of four triterpenoids, ergosta-4,6,8(14),22-tetraene-3-one (1), stigma-4-ene-3-one (2), 3β-hydroxy-21β-H-hop-22(29)-ene (3), sitosterol and a quinone, tectoquinone (4). These compounds (2, 3 and 4) demonstrated broad-spectrum antibiotic effects against eight of the twelve bacterial strains tested in a study by Awolola et al. (2017). The isolated compounds exhibited strain-specific anti-adhesion potential, with biofilm reduction against *P. aeruginosa*, but not *E. coli* or *S. aureus* (Awolola et al., 2017). The finding that quinone in terpenoids is an essential substructure for anti-Gram-positive-bacteria activity has been reported previously (Saruul et al., 2015). Menaquinone is a component of electron transport chains in a majority of anaerobic bacteria and Gram-positive bacteria. Due to its exclusivity in bacteria, menaquinone is thought to be a potential target for the development of therapeutically effective antibacterial agents without side effects (Paudel et al., 2016).

Conclusions

An *in vitro* test for antibacterial activity revealed that 96% ethanolic leaf extract of *F. natalensis* subsp. *natalensis* used in this study was able to inhibit the growth of fish pathogens (*Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri*). In our study, the *A. hydrophila* strain (200 and 400 µl of standardized inoculum) displayed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters ranged from 10 to 11.8 mm). *C. freundii* (200 and 400 µl of standardized inoculum) was resistant to ethanolic extract (inhibition zone diameters ranged between 7.5 and 11.7 mm). The ethanolic extract derived from *F. natalensis* subsp. *natalensis* leaves exhibited intermediate antibacterial activity against *Pseudomonas fluorescens* causing a zone of inhibition, comprising at least 10.2–12.8 mm for 200 µL and 7.4–11.2 mm for 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of bacterium strain. *Y. ruckeri* (200 and 400 µl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained

from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters were ranged between 8.0 and 16.5 mm). This may be because the herbs have more than one extractable active compound that provides antimicrobial activity. Overall, our findings provide support that further chemical analysis of the aforementioned plant extracts should be performed to determinate their chemical composition and identify the exact phytocompounds responsible for the antimicrobial activity. In addition, they should be subjected to pharmacological evaluations with the aim of assessing theirs *in vivo* efficacy, toxicity, potential adverse effects, interactions, and contraindications.

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OXIDATIVE STRESS BIOMARKERS IN THE EQUINE PLASMA AND ERYTHROCYTES TREATED *IN VITRO* BY LEAF EXTRACT OBTAINED FROM *FICUS RELIGIOSA* L. (MORACEAE)

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In the present study, we highlight the antioxidant potential of aqueous extract of *Ficus religiosa* L. leaves in equine plasma and erythrocyte suspension. In this study, we have focused on the antioxidant effect of leaf extract obtained from *F. religiosa* on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification (OMP), total antioxidant capacity (TAC)] using the equine erythrocytes model. Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w). The equine erythrocyte aliquots and plasma were used in the study. A volume of 0.1 ml of the *F. religiosa* extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (blank), phosphate buffer was used. Treatment by extract reduced the erythrocytes TBARS level by 25.3% (p = 0.009), while plasma TBARS level was increased by 75.6% (p = 0.000), as compared to untreated erythrocytes. When plasma was incubated with extract, the ketonic derivatives level was significantly increased by 22.8% (p = 0.000), while non-significantly decrease both aldehydic and ketonic derivatives of OMP was observed. Treatment by *F. religiosa* extract caused the increase of TAC in plasma and erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant. All these data suggest that *F. religiosa* could be explored for its antioxidant potential in equine erythrocyte suspension.

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

Introduction

Increase in prevalence of disease-related oxidative stress disorders has been on the rise in the entire world since the past decades. Oxidative stress has been implicated in numerous chronic degenerative diseases such as cardiovascular diseases, cancers, type 2 diabetes,

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neurodegenerative diseases, obesity, and hypertension. However, reactive oxygen species (ROS) may have dual roles in many pathologies (Paur et al., 2011). Oxidative stress, characterized by an imbalance between oxidants and antioxidants in favor of oxidants, leads to disruption of redox signaling and physiological function (Sies, 1986, 2015).

It is clear that a beneficial effect of a large intake of one single antioxidant (such as high-dose vitamin C, vitamin E, or β-carotene supplement) would not be expected. An alternative and much more likely strategy would be to test the potential beneficial effects of antioxidant-rich foods, since such foods typically contain a large combination of different antioxidants, which are selected through plant evolution to protect every part of the plant cells against oxidative damage (Blomhoff, 2005; Paur et al., 2011). Significant positive effects with few antioxidant properties in the modern drugs pave for the alternative medicines in managing the disease (Lee et al., 2014).

Ficus religiosa L. a large deciduous tree up to 35 m in height known by more than 150 names, is known to possess high antidiabetic, anticonvulsant, antiamnesic, wound healing, anti-inflammatory and antibacterial property (Singh et al., 2011). It is native of the sub-Himalayan tract, Bengal, and central India. *F. religiosa* tree begins its life epiphytically and then strangle the host by its far-growing roots that extend to the ground, establishing it as an independent tree. The therapeutic utilities of *F. religiosa* have been indicated in traditional systems of medicine like Ayurveda, Unani, etc. It has been used to cure the disorders of the central nervous system (epilepsy, migraine, etc.), endocrine system (diabetes, etc.), gastrointestinal tract (vomiting, ulcers, stomatitis, constipation, liver diseases, etc.), reproductive system (menstrual irregularities, etc.), respiratory system (asthma, cough, etc.) and infectious diseases (chickenpox, elephantiasis, leprosy, tuberculosis, gonorrhea, scabies, etc.) (Singh et al., 2011).

Fresh plant materials, crude extracts and isolated components of *F. religiosa* showed a wide spectrum of *in vitro* and *in vivo* pharmacological activities, i.e. antidiabetic, cognitive enhancer, wound healing, anticonvulsant, anti-inflammatory, analgesic, antimicrobial, antiviral, hypolipidemic, antioxidant, immunomodulatory, antiasthmatic, parasympathetic modulatory, estrogenic, antitumor, antiulcer, antianxiety, antihelmintic, endothelin-receptor antagonistic, apoptosis inducer and hypotensive activity (Singh et al., 2011). Moreover, different extracts of *F. religiosa* showed high antimicrobial activity (Tkachenko et al., 2016). Medicinal importance of this plant encouraged us to carry out the antimicrobial investigation of the ethanolic extract of *F. religiosa* leaves against Gram-negative bacteria *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922), as well as Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 49619). We noted in preliminary experiments, that ethanolic extract from *F. religiosa* leaves showed potent antibacterial activity against *S. aureus* (diameter of growth of inhibition zones was 20.5 mm), *E. coli* (16.5 mm), and *P. aeruginosa* (14.0 mm), while antibacterial activity against *K. pneumonia* and *S. pneumoniae* was less profound (Tkachenko et al., 2016).

An increasing number of studies are published on markers of oxidative stress in a whole range of human diseases (Frijhoff et al., 2015). The World Health Organization has defined

a biomarker as any substance, structure, or process that can be measured in the body or its products and influence or predicts the incidence of outcome or disease (WHO, 2001). The oxidant and antioxidant equilibrium are known to play an important role in equine medicine and equine exercise physiology. Moreover, interest in the role of oxidative stress (OS) status in equine medicine and exercise physiology has increased the need for the development of reliable methods to assess the biomarkers related to OS (Kusano et al., 2016). Nevertheless, not many studies have been conducted to quantify the antioxidant effect of leaf extract various plants on oxidative stress biomarkers in horses (Tkachenko et al., 2016, 2017).

Consequently, in this study, we have focused on the antioxidant effect of leaf extract obtained from *F. religiosa* on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] using the equine plasma and erythrocytes model. Equine erythrocytes are more sensitive to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage, i.e. methemoglobin formation, alteration of aggregation, and reduction of cellular deformability (Baskurt and Meiselman, 1999). The erythrocytes represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure and cytotoxicity assay (Pagano and Faggio, 2015). Exposure of erythrocytes to oxidative stress lead to lipid peroxidation that could alter the membranes of RBCs inducing membrane protein conformation and protein cross-linking by decreasing membrane protein content and consequently lead to abnormal cell morphology and hemolysis that could disturb the microcirculation (Asha Devi et al., 2005; Farag and Alagawany, 2018). Thus, equine erythrocytes were proved to be a good tool for analyzing the oxidative stress biomarkers as a mechanism of antioxidant action of *F. religiosa* leaf extract.

Material and methodology

Collection of plant materials

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

Preparation of plant extracts

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

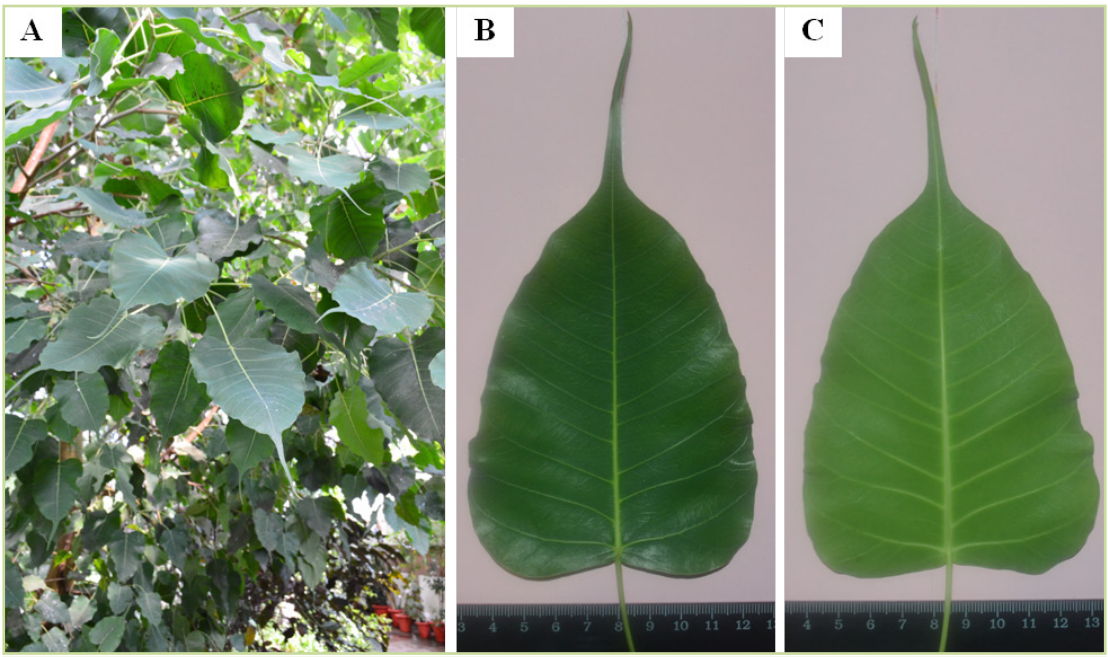

Figure 1 Leaf morphology of *F. religiosa*: A – general view; B – adaxial leaf surface; C – abaxial leaf surface

Horses

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

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min to remove plasma Blood was stored into The pellet of blood was re-suspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (phosphate buffer) was used. After incubation the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

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

determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 mL of sample (blood, plasma, and erythrocytes' suspension) was added to 2 mL of distilled water, 1 mL of 20% TCA and 1 mL of 0.8% TBA. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3,000 g for 10 minutes. The μmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as the extinction coefficient.

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

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1 M DNPH (dissolved in 2M HCl) was added to 0.1 mL of the sample after denaturation of proteins by 20% trichloroacetic acid (TCA). After addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 h at 37 °C. The tubes were spun in a centrifuge for 20 min at 3,000 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 22,000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37ºC. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The

level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

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

Results and discussion

Recently, a vast number of methods have been developed and used to measure the extent and nature of oxidative stress, ranging from the oxidation of DNA to proteins, lipids, and free amino acids (Frijhoff et al., 2015). As we know, lipid peroxidation is one of the consequences of oxidative damage, and it is one of the chief mechanism for cell injury and death (Çimen Burak, 2008). It is well documented that lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids and its occurrence in biological membranes causes impaired membrane function, impaired structural integrity (Halliwell and Gutteridge, 1985), decreased fluidity, and inactivation of a number of membrane-bound enzymes.

Malondialdehyde (MDA), the well-characterized product of the lipid peroxidation of erythrocytes, is a highly reactive bifunctional molecule, that could impair various membrane functions by cross-linking the erythrocytes' proteins and phospholipids leading to diminished survival and induce hemolysis (Farag and Alagawany, 2018). Moreover, lipid peroxidation of erythrocytes may be implicated in cell aging and variable pathological conditions. The determination of MDA level provides a good measure of lipid peroxidation. The most common method used to assess the MDA level is the 2-thiobarbituric acid reactive substances (TBARS) assay. The TBARS content as a biomarker of lipid peroxidation in the equine erythrocytes suspension after *in vitro* incubation with *F. religiosa* leaf extract and shown in Figure 2A. As shown in Figure 2A, treatment by extract reduced the erythrocytes TBARS level by 25.3% (p = 0.009), while plasma TBARS level was increased by 75.6% (p = 0.000) when compared to untreated erythrocytes.

Nevertheless, it was noted that despite their widespread use, all methods that detect both MDA and TBARS have their pitfalls (Spickett et al., 2010). Specifically, Moore and Roberts (1998) have found that in the TBARS assay, up to 98% of the measured MDA can be formed by the high-temperature conditions during the procedure itself.

Oxidative damage of proteins can occur directly by the interaction of the protein with ROS or indirectly by the interaction of the protein with a secondary product (resulting from the interaction of radical with lipid or sugar molecule). Modification of a protein under oxidative stress can occur *via* peptide backbone cleavage, cross-linking, and/or modification of the side

chain of virtually every amino acid (Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009). Moreover, most protein damage is irreparable and oxidative modification of the protein structure can lead to loss of enzymatic, contractile, or structural function in the affected proteins, thus making them increasingly susceptible to proteolytic degradation (Levine and Stadtman, 2001; Fisher-Wellman and Bloomer, 2009). Albumin is the main (48–76% of total proteins) and the most osmotically active protein fraction of a horse serum (Winnicka, 2011) whereas a globulin fraction is a heterogeneous group of blood proteins including carrier proteins, enzymes, immunoglobulins and other inflammatory molecules (Abeni et al., 2013). ROS induced oxidation of arginine, lysine, threonine, or proline amino acid residues generates reactive carbonyl derivatives (RCD), which can be readily measured by reaction with 2,4-dinitrophenylhydrazine (Radák et al., 2000, 2002). Protein RCD is used very often as a marker of oxidative modification of proteins (Radák et al., 2000, 2002, 2008). When equine plasma was incubated with extract, the ketonic derivatives level was significantly increased by 22.8% (p = 0.000) (Fig. 1B), while non-significantly decrease both aldehydic and ketonic derivatives of OMP was observed (by 1.6 and 8.9%, p >0.05).

The total antioxidant capacity (TAC) includes an enzymatic antioxidant such as superoxide dismutase, catalase, glutathione peroxidase, as well as some macromolecules (albumin, ceruloplasmin, and ferritin), and its assessment may contain more information than a single review of its constituent parts (Gad et al., 2011). As shown in Figure 2C, treatment by *F. religiosa* extract caused the increase of TAC in plasma and erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant.

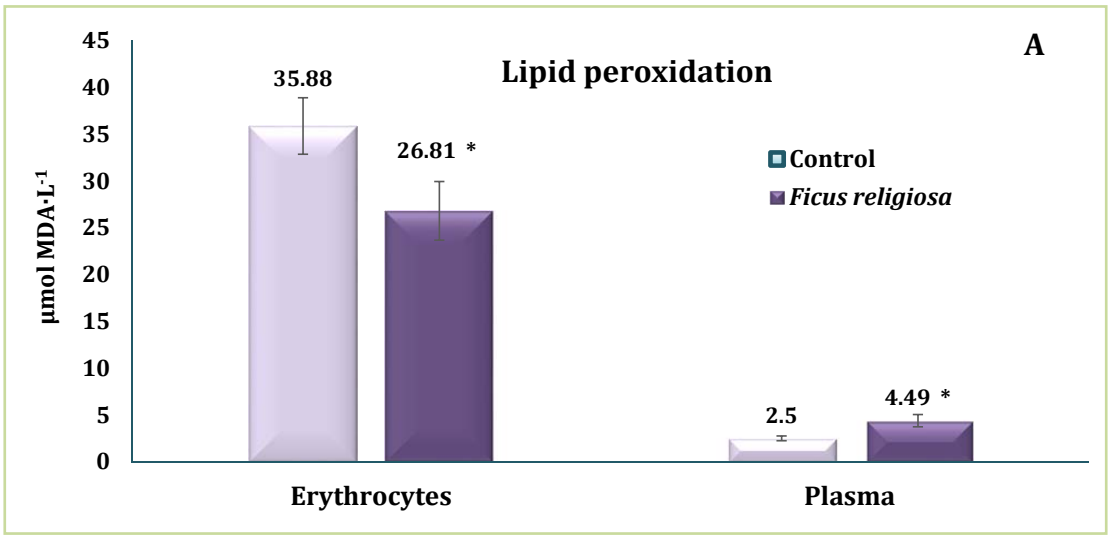

Figure 2A The TBARS content as a biomarker of lipid peroxidation (A), aldehydic and ketonic derivatives of oxidatively modified proteins (B), and total antioxidant capacity (C) in the equine erythrocytes suspension after *in vitro* incubation with *Ficus religiosa* leaf extract (M ±m, n = 18)

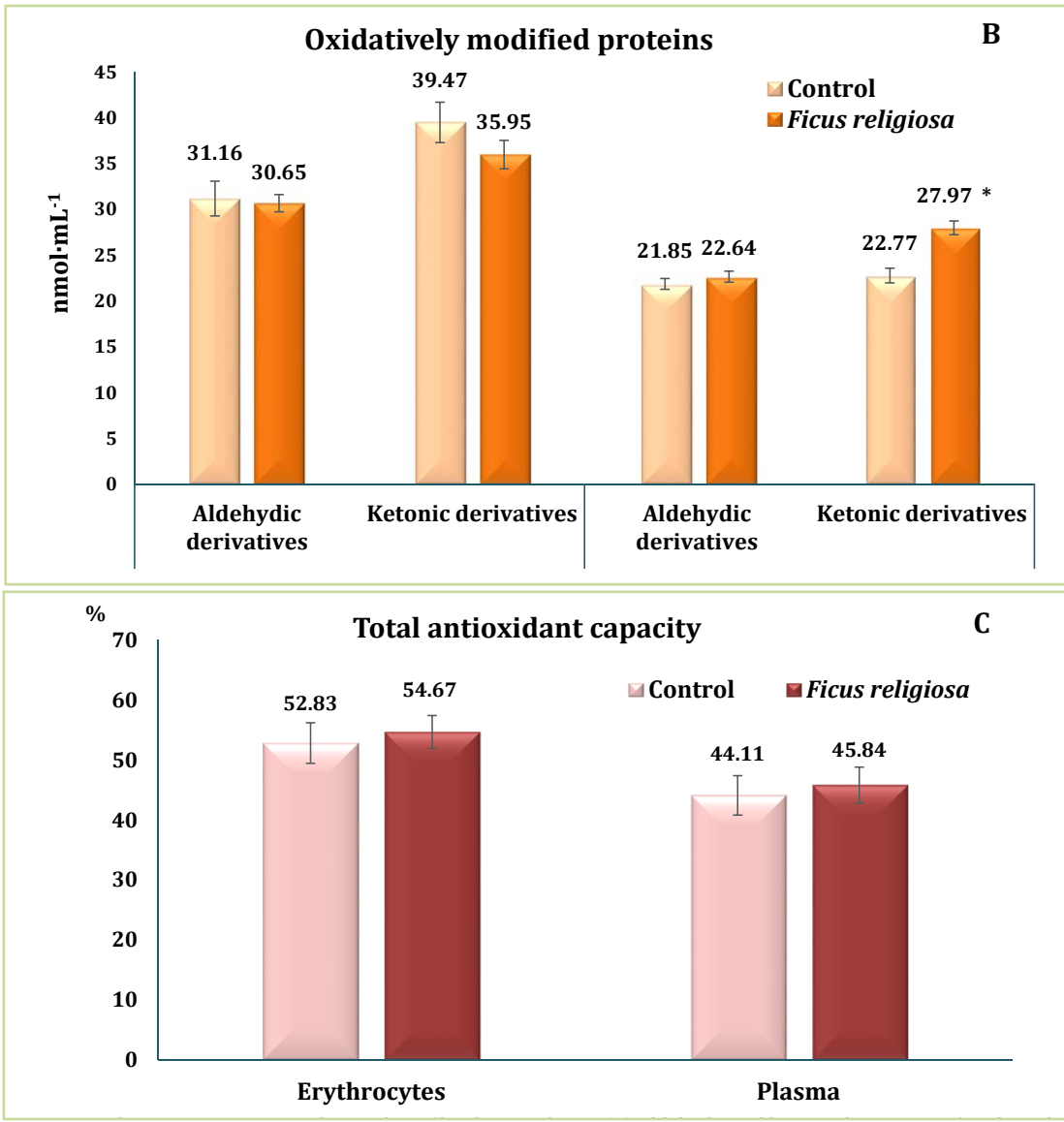

Figure 2B–C The TBARS content as a biomarker of lipid peroxidation (A), aldehydic and ketonic derivatives of oxidatively modified proteins (B), and total antioxidant capacity (C) in the equine erythrocytes suspension after *in vitro* incubation with *Ficus religiosa* leaf extract (M ±m, n = 18)

Several correlations between checked parameters were found (Figure 3). Erythrocyte TBARS level correlated inversely with plasma TBARS level (r = -0.532, p = 0.023) and plasma TAC level (r = -0.525, p = 0.025) (Figure 3A). Decreased erythrocyte TBARS level induced the increase of plasma TBARS level and TAC level. The erythrocyte TAC level correlated inversely with plasma TBARS level (r = -0.742, p = 0.000) and correlated positively with aldehydic derivatives of OMP in plasma (r = 0.645, p = 0.004). High level of TAC in erythrocyte suspension after

incubation with leaf extract obtained from *F. religiosa* induced reduce plasma TBARS level, while aldehydic derivatives of OMP positively correlated with plasma TAC level (Figure 3).

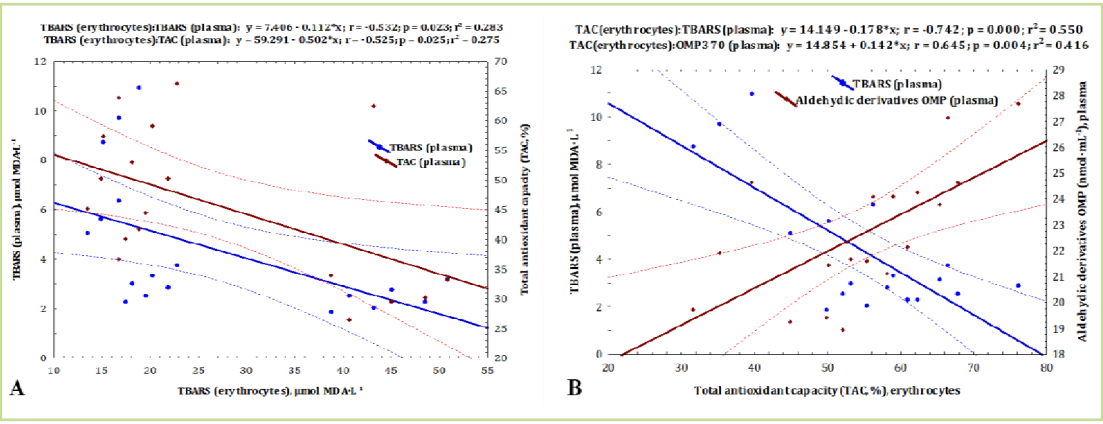

Figure 3 Correlations between oxidative stress biomarkers – plasma and erythrocyte TBARS, plasma TAC level (A), erythrocyte TAC level, plasma TBARS level and aldehydic derivatives of OMP (B) in the equine erythrocytes suspension after *in vitro* incubation with *Ficus religiosa* leaf extract

Many results also clearly suggest that treatment by herbal extracts *in vivo* and *in vitro* study prevents organ damage by a decrease of lipid peroxidation and protection of the antioxidant defense system. Several methods have been developed to measure the free radical scavenging capacity, regardless of the individual compounds, which contribute towards the total capacity of a plant product in scavenging free radicals (Lo Shu-Fung et al., 2004). For instance, the *in vitro* antioxidant effect of the ethyl acetate root extract of *F. religiosa* using diphenyl picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging, reducing capacity and hydrogen peroxide scavenging assay was investigated by Sharma and Gupta (2007). The extract showed reducing potential, scavenged DPPH radical (87.61%) at 250 $\mu g.mL^{-1}$ and hydrogen peroxide (70.25%) at 1000 $\mu g.mL^{-1}$. The investigators suggested the role of polyphenolic components (determined by the Folin-Ciocalteu's phenolic reagent method) for the observed antioxidant effect (Sharma and Gupta, 2007).

The effects of four extracting solvents [absolute ethanol, absolute methanol, aqueous ethanol (ethanol: water, 80 : 20 v/v) and aqueous methanol (methanol: water, 80 : 20 v/v)] and two extraction techniques (shaking and reflux) on the antioxidant activity of extracts of barks of *Azadirachta indica* A. Juss. (Meliaceae), *Acacia nilotica* (L.) Delile (Leguminosae), *Syzygium cumini* (L.) Skeels (syn. *Eugenia jambolana* Lam.) (Myrtaceae), *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn. (Combretaceae), leaves and roots of *Moringa oleifera* Lam. (Moringaceae), the fruit of *F. religiosa*, and leaves of *Aloe vera* (L.) Burm. f. (syn. *Aloe barbadensis* Mill.) (Asparagaceae) were investigated by Sultana and co-workers (2009). The fruit powder of *F. religiosa* was subjected to shaking and refluxing with absolute ethanol, absolute methanol, 80% hydro-ethanol and 80% hydro-methanol to get various extracts. Total phenolic components and flavonoids were quantified in all the extracts. The extracts were subjected

to DPPH scavenging and percent inhibition of linoleic acid assay. The investigators found that the 80% hydro-methanolic extract obtained by refluxing contained the highest amount of antioxidant components (phenols and flavonoids) and showed maximum antioxidant effect. The study suggested that the extracting solvent and extraction technique affects the antioxidant activity of the plant extracts (Sultana et al., 2009). Apart from the bark, roots, and fruits, the antioxidant effect of the aqueous, methanolic and ethanolic leaf extracts at 35–36 μg.100 mg $^{-1}$ concentration, in similar *in vitro* DPPH assay has also been reported (Preethi et al., 2010).

Anandjiwala et al. (2008) have also reported the free radical scavenging activity of an Ayurvedic preparation *Panchvalkala* and its individual components (stem bark of *Ficus benghalensis*, *F. racemosa* L. (syn. *F. glomerata* Roxb.), *F. religiosa*, *F. virens* Aiton and *Thespesia populnea* (L.) Sol. ex Corrêa (Malvaceae). Being stem barks, these samples contained phenolics (ranging from 3.5 to 10.8% w/w) and tannins (1.6 to 7.0% w/w). This prompted researchers to study the free radical scavenging activity of *Panchvalkala* and its components which were evaluated in three *in vitro* models viz. 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, superoxide radical scavenging activity and reducing power assay. *Panchvalkala* and its individual components showed significant antiradical activity by bleaching 1,1-diphenyl-2-picrylhydrazyl radical (EC_{50} ranging from 7.27 to 12.08 μg) which was comparable to pyrogallol (EC_{50} 4.85 μg). Thin layer chromatography of the methanol extracts when sprayed with 0.2% 1,1-diphenyl-2-picrylhydrazyl in methanol revealed several bands with antiradical activity as seen by bleaching of 1,1-diphenyl-2-picrylhydrazyl. All the samples showed good superoxide scavenging potential (EC_{50} ranging from 41.55 to 73.56 μg) comparable to ascorbic acid (EC_{50} 45.39 μg) in a dose-dependent manner. The reduction ability, Fe^{3+} to Fe^{2+} transformation was found to increase with increasing concentrations of all the sample extracts. *Panchvalkala* and its components showed good free radical scavenging activity which can be attributed to tannins and phenolics along with other compounds. Free radical scavenging activity could be one of the mechanisms of action of *Panchvalkala*, including its anti-inflammatory activity (Anandjiwala et al., 2008).

Since oxidative stress as a consequence of free radicals generated during body‘s cellular respiration has been implicated in the pathogenesis of many human diseases, the use of antioxidants has been suggested as a common treatment approach for these disorders. The antioxidant potential of different parts of *F. religiosa* can, therefore, be further explored in ameliorating the oxidative stress-related disorders (Singh et al., 2011). *F. religiosa* bark possesses also significant antidiabetic activity. The antidiabetic effect of aqueous extract of *F. religiosa* bark in normal, glucose-loaded hyperglycemic and streptozotocin-induced diabetic rats was investigated in a study by Pandit et al. (2010). The aqueous extract of *F. religiosa* bark also showed a significant increase in serum insulin, body weight and glycogen content in liver and skeletal muscle of streptozotocin-induced diabetic rats while there was a significant reduction in the levels of serum triglyceride and total cholesterol. The aqueous extract of *F. religiosa* bark also showed a significant anti-lipid peroxidative effect in the pancreas of streptozotocin-induced diabetic rats (Pandit et al., 2010).

Kapoor et al. (2011) have demonstrated the phytopharmacological potential and anti-asthmatic activity of *F. religiosa*. Histamine and acetylcholine were used to guinea pigs to establish bronchospasm model. In *in vivo* study, the aqueous extract of *F. religiosa* leaves at doses of 150 and 300 mg.kg^{-1} was administrated to guinea pigs, and the broncho-protective activity of the aqueous extract of *F. religiosa* leaves was compared with aminophylline at 25 mg.kg^{-1}. While in *in vitro* study, and 10, 20, 30 g.mL^{-1} of the aqueous extract of *F. religiosa* leaves was administrated to guinea pigs, respectively, and mast cell stabilizing activity of the aqueous extract of *F. religiosa* leaves was compared with ketotifen at 10 g.mL^{-1}. Administration of the aqueous extract of *F. religiosa* leaves (150 and 300 mg.kg^{-1}, ip.) produced a significant effect on latency to develop histamine and acetylcholine-induced pre-convulsive dyspnea. In the mast cell stabilizing model, the aqueous extract of *F. religiosa* leaves at 10, 20 and 30 μg.mL^{-1} could significantly increase the number of intact cells. It can be concluded that the aqueous extract of *F. religiosa* leaves is effective on histamine and acetylcholine-induced bronchospasm in guinea pigs (Kapoor et al., 2011).

F. religiosa latex and constituents have excellent nephroprotective and curative activities and thus have great potential as a source for natural health products. The possible nephroprotective and curative effects of *F. religiosa* latex methanol extract against cisplatin-induced acute renal failure was determined by Yadav and Srivastava (2013). The anti-ulcer activity and acute toxicity of *F. religiosa* leaf ethanolic extract in animal models were evaluated by Gregory and co-workers (2013). Anti-ulcer activity of *F. religiosa* ethanolic extract (250 and 500 mg.kg^{-1} body weight) was studied on stress-induced ulcer animal models. Results showed that the extract treatments prevented ulcer area and gastric secretion in a dose-dependent manner. Administration of 2,000 mg.kg^{-1} extract did not show any acute toxicity in albino mice. The preliminary phytochemical analysis identified the presence of flavonoids in the ethanolic extract of *F. religiosa*. The anti-ulcer activity is probably due to the presence of flavonoids (Gregory et al., 2013).

An orally administered aqueous root extract of *F. religiosa* has dose-dependent and potent anticonvulsant activities against strychnine- and pentylenetetrazole-induced seizures in mice, as described in the study of Patil et al. (2011). The anticonvulsant activity of the extract (25, 50 and 100 mg.kg^{-1}, p.o.) was investigated in strychnine-, pentylenetetrazole-, picrotoxin- and isoniazid-induced seizures in mice. Rat ileum and fundus strip preparations were used to study the effect of the extract on acetylcholine (Ach)- and serotonin (5-HT)-induced contractions, respectively (Patil et al., 2011). The hydroethanolic extract of adventitious roots of *F. religiosa* has anticonvulsant activity. Retention of anticonvulsant effect in the saponins-rich fraction-treated animals indicated the role of saponins for the activity (Singh et al., 2012).

The petroleum ether extract of *F. religiosa* plant showed to be an antioxidant and showed a promising effect in animals with Parkinson‘s disease, significantly attenuating the motor defects and also protecting the brain from oxidative stress. In study of Bhangale and Acharya (2016), effects of *F. religiosa* (100, 200, and 400 mg.kg^{-1}, p.o.) were evaluated using *in vivo* behavioral parameters like catalepsy, muscle rigidity, and locomotor activity and its effects on neurochemical parameters (malonic dialdehyde, catalase, superoxide dismutase, and glutathione) in rats. The experiment was designed by giving haloperidol to induce catalepsy

and 6-hydroxydopamine to induce Parkinson's disease-like symptoms. The increased cataleptic scores (induced by haloperidol) were significantly (p <0.001) found to be reduced, with the petroleum ether extract of *F. religiosa* at a dose of 200 and 400 $mg.kg^{-1}$ (p.o.). 6-Hydroxydopamine significantly induced motor dysfunction (muscle rigidity and hypolocomotion). The 6-Hydroxydopamine administration showed a significant increase in lipid peroxidation level and depleted superoxide dismutase, catalase, and reduced glutathione level. Daily administration of petroleum ether extract of *F. religiosa* (400 $mg.kg^{-1}$) significantly improved motor performance and also significantly attenuated oxidative damage (Bhangale and Acharya, 2016). The effect of flavonoid-rich ethyl acetate fraction of the crude fig extract of *F. religiosa* in combination with phenytoin on seizure severity, depressive behavior, and cognitive deficit in pentylenetetrazol (PTZ)-kindled mice was investigated in a study of Singh et al. (2014). The flavonoid-rich ethyl acetate fraction of the crude fig extract was found to show significant antioxidant potential in various *in vitro* free radical scavenging assays. Biochemical investigations of the brain tissue showed amelioration of TBARS, reduced glutathione (GSH) levels, and reduced catalase and acetylcholinesterase activities, thereby indicating suppression of oxidative stress (Singh et al., 2014).

The antiviral activity of *F. religiosa* extracts against herpes simplex virus type 2 (HSV-2), the main causative agent of genital ulcers and sores was investigated in a study of Ghosh et al. (2016). Water and chloroform bark extracts were the most active against HSV-2, and also against an acyclovir-resistant strain. The water extract has a direct virus-inactivating activity. By contrast, the chloroform extract inhibits viral attachment and entry and limits the production of viral progeny. The chloroform extract of *F. religiosa* did not inactivate extracellular virus particles but targeted early steps of the viral replicative cycle such as virus attachment and/or entry. Moreover, a significant reduction in the number of viral plaques was also observed when the extract was added to the methylcellulose medium after infection. This finding suggests that the virus was blocked in some of the initial infections (Ghosh et al., 2016). The antiviral activity of *F. religiosa* extracts against respiratory viruses such as a human respiratory syncytial virus (RSV) and human rhinovirus (HRV) was demonstrated by Cagno et al. (2015). The antiviral activity of *F. religiosa* was tested *in vitro* by plaque reduction and virus yield assays and the major mechanism of action was investigated by virus inactivation and time-of-addition assays. *F. religiosa* methanol bark extract was the most active against HRV with an EC_{50} of 5.52 $\mu g.mL^{-1}$. This extract likely inhibited the late steps of the replicative cycle. Water bark extract was the most active against RSV with an EC_{50} between 2.23 and 4.37 $\mu g.mL^{-1}$. Partial virus inactivation and interference with virus attachment were both found to contribute to the anti-RSV activity. Replication of both viruses was inhibited in viral yield reduction assays (Cagno et al., 2015).

F. religiosa has been shown to exert diverse biological activities including apoptosis in breast cancer cell lines and it could be explored for its chemopreventive potential in cervical cancer. The anti-neoplastic potential of aqueous extract of *F. religiosa* bark in human cervical cancer cell lines, SiHa and HeLa were demonstrated by Choudhari et al. (2013). The aqueous extract of *F. religiosa* bark altered the growth kinetics of SiHa (HPV-16 positive) and HeLa (HPV-18 positive) cells in a dose-dependent manner. It blocked the cell cycle progression at a G1/S

phase in SiHa that was characterized by an increase in the expression of p53, p21 and pRb proteins with a simultaneous decrease in the expression of phospho Rb (ppRb) protein. On the other hand, in HeLa, FRaq induced apoptosis through an increase in intracellular Ca^{2+} leading to loss of mitochondrial membrane potential, the release of cytochrome-c and an increase in the expression of caspase-3. Moreover, aqueous extract of *F. religiosa* bark reduced the migration as well as invasion capability of both the cervical cancer cell lines accompanied with downregulation of MMP-2 and Her-2 expression. Interestingly, aqueous extract of *F. religiosa* bark reduced the expression of viral oncoproteins E6 and E7 in both the cervical cancer cell lines (Choudhari et al., 2013).

The antioxidant role of *F. religiosa* after *in vitro* incubation with equine erythrocytes suspension might be due to its chemical constituents like flavonoids and phenolic compounds. Furthermore, flavonoids traditionally expose antioxidant activity. Chemical analysis conducted by Suryawanshi et al. (2011) found that leaves of *F. religiosa* contained appreciable amounts of campestrol, stigmasterol, isofucosterol, α-amyrin, lupeol, tannic acid, arginine, serine, aspartic acid, glycine, threonine, alanine, proline, tryptophan, tyrosine, methionine, valine, isoleucine, leucine, nonacosane, *n*-hentricontanen, hexacosanol, and *n*-octacosan. On the other hand, the findings of Taskeen and coworkers (2009) showed, that quercetin was the most abundant among flavonols. Phytosterols (2.8%) like campesterol, stigmasterol, sitosterol and 28-isofucosterol, and triterpene alcohols (28.5%) like α-amyrin, β-amyrin, and lupeol have been isolated from the non-saponifiable fraction of light petroleum leaf extract of *F. religiosa* (Singh et al., 2011). Along with phytosterols and triterpene, 7.1% of long-chain hydrocarbons [*n*-nonacosane and *n*-hentriacontane] and 7.9% of aliphatic alcohols [*n*-hexacosanol and *n*-octacosanol] have also been isolated from the same fraction (Behari et al., 1984; Williamson and Hooper, 2002). The leaves of *F. religiosa* contain a high amount of l-cystine, l-lysine, l-arginine, dl-serine, dl-aspartic acid, glycine, dl-threonine, dl-∞-alanine, l-proline, tryptophan, l-tyrosine, dl-methionine, dl-valine, dl-isoleucine and l-leucine (Verma and Bhatia, 1986). The leaves contain around 1.5% of total tannin content, which comprises a tannic acid and condensed tannins (Singh, 1977; Niranjan et al., 2007; Singh et al., 2011). The leaves are rich in minerals like calcium, phosphorous, iron, copper, manganese, magnesium, zinc, potassium and sodium (Williamson and Hooper, 2002; Singh et al., 2011).

Conclusions

In conclusion, the results obtained from the present studies revealed that leaf extract of *Ficus religiosa* exhibited antioxidant activity after *in vitro* cultivation with equine erythrocytes. These findings suggest that the extensive use of this herbal in treating various types of disorders might, therefore, be justified by its antioxidant activities. The results also indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results.

Nevertheless, in order to avoid any misinterpretation of the results obtained in this study, another alternative marker for evaluating lipid peroxidation level in equine erythrocyte suspension and assessing free radical scavenging potency of leaf extracts of plant extracts screened could be employed.

Additionally, further studies aimed at the isolation and identification of active substances from the extract obtained from *Ficus religiosa* leaves could also disclose compounds with better therapeutic value. It is believed that screening of all the investigated plants for other biological activities including anti-inflammatory, wound healing and antioxidant activities are essential in medicine and veterinary.

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OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTE SUSPENSION AFTER *IN VITRO* INCUBATION WITH LEAF EXTRACT OBTAINED FROM *THYMUS SERPYLLUM* L. EMEND. MILL. (LAMIACEAE)

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The main aim of the study was an assessment of the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] in the equine erythrocytes after treatment with *Thymus serpyllum* L. emend. Mill. extract. Leaves of *Th. serpyllum* were collected among grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine). Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w). The equine erythrocyte aliquots were used in the study. The pellet of blood was re-suspended in phosphate buffer (pH 7.4). A volume of 0.1 ml of the *Th. serpyllum* extract was added to 1.9 ml of clean equine erythrocytes. For positive control (blank), phosphate buffer was used. After incubation the mixture at 37 °C for 60 min with continuous stirring, samples were used for the biochemical assays. Lipid peroxidation biomarker, aldehydic and ketonic derivatives of oxidatively modified proteins, total antioxidant capacity was non-significantly altered after *in vitro* incubation with an extract obtained from *Th. serpyllum*. Screening of *Thymus* species for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

Keywords: *Thymus*, leaf extract, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

Introduction

Thymus serpyllum L. emend. Mill., known as Breckland thyme, wild thyme, or creeping thyme, is a perennial shrub, native to regions of northern and central Europe. It has a long stem; leaves are oval (rounded at the top, tapered at the base), and glabrous on the face and underside,

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while at the base along the edge they have long trichomes, a prominent central vein, and less prominent lateral veins. Inflorescences are 4–7 cm tall and form in a series along a low-lying stem, with a uniform layer of trichomes on all sides. Flowers are located at the top of the stems and form spherical (or more rarely elongated) verticillaster. Wild thyme grows best on dry, stony ground, open sandy heaths, and grasslands (Diklić, 1974; Jarić et al., 2015). *Th. serpyllum* is a medicinal plant with antioxidant, antimicrobial, antitumor, and cytotoxic properties with effective medicinal application in pharmaceutical, food, and cosmetic industries as an anthelmintic, a strong antiseptic, an antispasmodic, a carminative, deodorant, diaphoretic, disinfectant, expectorant, sedative, tonic, anticholesterolemic and immunostimulant plant (Jarić et al., 2015).

The chief component of the essential oil of *Th. serpyllum* is carvacrol, while it also contains borneol, isobutyl acetate, caryophyllene, 1,8-cineole, citral, citronellal, citronellol, *p*-cymene, geraniol, linalool, α-pinene, γ-terpinene, α-terpineol, terpinyl acetate, and thymol in relatively high concentrations (Thomson, 2004). In addition to essential oil, wild thyme also contains flavonoids, phenol carboxylic acids, and their derivatives, triterpenes, and tannins (Thomson, 2004). The interest in the formulation of pharmaceuticals, nutraceuticals, and cosmeceuticals based on thymol is due to several studies that have evaluated the potential therapeutic uses of this compound for the treatment of disorders affecting the respiratory, nervous, and cardiovascular systems. Moreover, this compound also exhibits antimicrobial, antioxidant, anticarcinogenesis, anti-inflammatory, and antispasmodic activities, as well as a potential as a growth enhancer and immunomodulator (Salehi et al., 2018). The noteworthy effects of thymol are largely attributed to its anti-inflammatory (*via* inhibiting recruitment of cytokines and chemokines), antioxidant (*via* scavenging of free radicals, enhancing the endogenous enzymatic and non-enzymatic antioxidants and chelation of metal ions), antihyperlipidemic (*via* increasing the levels of high density lipoprotein cholesterol and decreasing the levels of low density lipoprotein cholesterol and low density lipoprotein cholesterol in the circulation and membrane stabilization) (*via* maintaining ionic homeostasis) effects (Nagoor Meeran et al., 2017).

Thymol exhibits *in vitro* antioxidant activity on high-fat-diet-induced hyperlipidemia and atherosclerosis. The antioxidant properties may be related to its phenolic structure, which may adsorb and neutralize free radicals and exhibit redox properties (Yu et al., 2016). Moreover, it may suppress the progression of high-fat-diet-induced hyperlipidemia and atherosclerosis by reducing aortic intimal lipid lesion, lowering serum lipids and oxidative stress, and alleviating inflammation-related responses (Yu et al., 2016). This compound scavenges hydroxyl free radicals and produces phenoxyl radicals, major transient species (Nagoor Meeran et al., 2017).

An earlier study in our laboratory showed no toxic effects in terms of hemolysis or increased methemoglobin content linked with leaf extracts of various *Thymus* species on equine erythrocytes. Moreover, Nagoor Meeran and Prince (2012) also confirmed the antioxidative effect of thymol *via* increase of the activity of endogenous antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and the level of other non-enzymatic antioxidants such as vitamin C, vitamin E, and reduced glutathione (Nagoor Meeran and Prince, 2012), and thereby the total antioxidant status *in*

vivo (Youdim and Deans, 2000). In our study, the extracts actually reduced hemolysis and hemoglobin oxidation. Ethanol-based extracts obtained from various *Thymus* species revealed pronounced antibacterial ability. A number of reports concerning thymol revealed its antioxidant activity *via* scavenging of free radicals, enhancing the endogenous enzymatic and non-enzymatic antioxidants and chelation of metal ions (Youdim and Deans, 2000; Nagoor Meeran and Prince, 2012; Jarić et al., 2015; Nagoor Meeran et al., 2017).

Equine erythrocytes are more sensitive to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage, i.e. methemoglobin formation, alteration of aggregation, and reduction of cellular deformability (Baskurt and Meiselman, 1999). It was shown, that horses have a greater risk than other mammalian species of developing methemoglobinemia and hemolytic anemia following ingestion of oxidizing toxins, due to deficiencies in the mechanisms that protect against oxidative damage in erythrocytes. Erythrocytes from horses are slower than erythrocytes from other species studied in their ability to regenerate GSH after it has been oxidized *in vitro* (Harvey et al., 2003). These reduced abilities may be related to the fact that horse erythrocytes have lower glutathione reductase (GR) activities than erythrocytes from humans and most domestic animal species, and the Michaelis-Menton constant (Km) of GSSG for GR is higher in horses than in three other species measured. Moreover, sulfhydryl groups in proteins and unsaturated lipids in membranes are especially susceptible to oxidation. Oxidative denaturation and the precipitation of the globin portion of hemoglobin into large aggregates result in the formation of Heinz bodies that can bind to and alter membranes. Membrane structure also is altered by the oxidation of sulfhydryl groups and by lipid peroxidation (Harvey, 1997).

Erythrocytes were proved to be a good tool for analyzing the oxidative stress and lipid peroxidation as a mechanism of toxic action in various studies (Baskurt and Meiselman, 1999). Erythrocytes help in assessing the toxicity of various extracts as well. Oxidative damage to erythrocytes after exposure to extracts induced alterations in the morphology of cells, membrane protein conformation, protein cross-linking, lipid peroxidation and consequently hemolysis of erythrocytes (Farag and Alagawany, 2018). Therefore, in the present study, the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity] in the equine erythrocytes was used for assessing the antioxidant activity of *Th. serpyllum* extract.

Material and methodology

Collection of plant materials

Plants were harvested in June-August, 2016. Leaves of *Th. serpyllum* were collected among grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 03´ 58,9´´, E 26° 13´ 37,5´´, 257 m a.s.l.).

Identification of this species was made according to Nachychko (2015). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all attached material and used to prepare extracts.

Figure 1 Plant of *Thymus serpyllum* used in our study. Photo by Viktor Nachychko

Preparation of plant extracts

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

Horses

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

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored

in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min to remove plasma. The pellet of blood was re-suspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control (phosphate buffer) was used. After incubation the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocytes aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malondialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 mL of sample (blood, plasma, and erythrocytes' suspension) was added to 2 mL of distilled water, 1 mL of 20% TCA and 1 mL of 0.8% TBA. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3.000 g for 10 minutes. The μmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as the extinction coefficient.

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

To evaluate the protective effects of extracts obtained from leaves of *Th. serpyllum* against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1 M DNPH (dissolved in 2M HCl) was added to 0.1 mL of the sample after denaturation of proteins by 20% trichloroacetic acid (TCA). After addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 h at 37 °C. The tubes were spun in a centrifuge for 20 min at 3,000 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 22.000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37 °C. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

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

Results and discussion

Many lipid peroxidation (LPO) products exert cytotoxicity, but sublethal concentrations of LPO products induce cellular adaptive responses and enhance tolerance against subsequent oxidative stress through upregulation of antioxidant compounds the and enzymes (Niki, 2009). This adaptive response is observed not only for chemically reactive carbonyl compounds but also for chemically stable compounds. On the other hand, LPO, as well as reactive oxygen and nitrogen species, have been shown to play an important role as a regulator of gene expression and cellular signaling messenger (Niki, 2009).

When equine erythrocytes were incubated with an extract obtained from *Th. serpyllum*, the TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered. The *Th. serpyllum* extract reduced the formation of intracellular aldehydic and ketonic derivatives of OMP in the extract-treated erythrocytes (by 8.8 and 6.3%, p >0.05), but these results were non-significant. Total antioxidant capacity was non-significantly increased by 8.1% (p >0.05) (Figure 2).

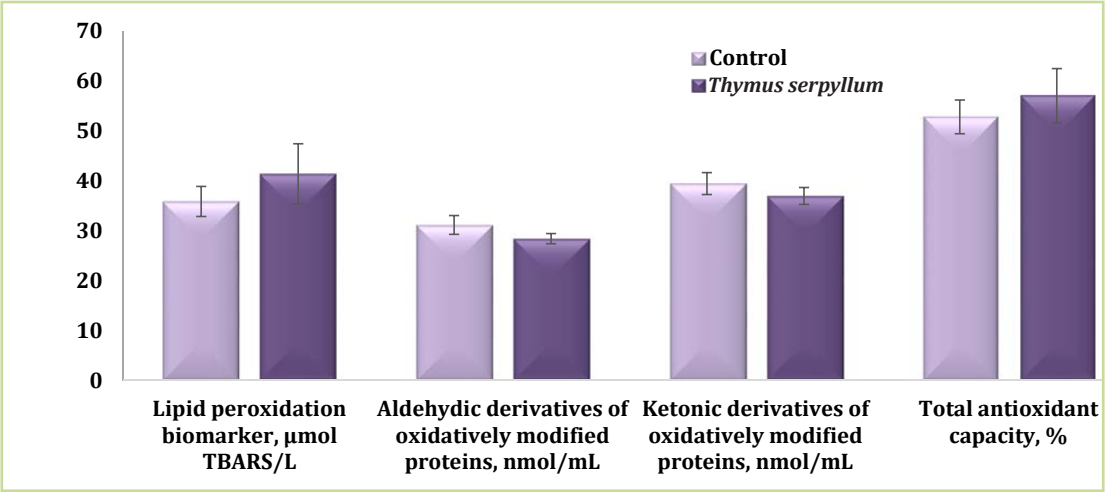

Figure 2 The TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes suspension after *in vitro* incubation with leaf extract obtained from *Thymus serpyllum* (*M* ±m, *n* = 18)

Many *in vitro* studies confirmed antioxidant properties of thyme extracts. Many results also clearly suggest that treatment by *Thymus* extracts *in vivo* and *in vitro* prevents organ damage *via* protection of the antioxidant defense system and scavenge of hydroxyl free radicals by producing of phenoxyl radicals, major transient species (Nagoor Meeran et al., 2017). For example, Petrović et al. (2014) studied the antioxidant capacity of wild thyme essential oil in terms of its ability to neutralize DPPH (1,1-diphenyl-the 2-picrylhydrazyl) free radicals, that is, the ability of the components of the essential oil to donate hydrogen atoms and transform DPPH into its reduced form DPPH-H. Their results showed that the essential oil exhibited significantly better antioxidant activity when compared to synthetic antioxidants like butylated hydroxyanisole (BHA) and in particular butylated hydroxytoluene (BHT) (Petrović et al., 2014). The essential oil of *Th. serpyllum* growing in Croatia revealed poorer ability to neutralise DPPH radicals than BHA, BHT, tocopherol, ascorbic acid compared to the essential oil of *Th. vulgaris* L. Hussain et al. (2013) also demonstrated that the essential oil of *Th. serpyllum* exhibited less ability to neutralize DPPH radicals than BHT and thymol.

Six different assays were employed in the study of Kindl et al. (2015) in order to evaluate the antioxidant properties of the ethanolic extracts of selected *Thymus* species growing in Croatia (*Th. longicaulis* C. Presl., *Th. praecox* Opiz subsp. *polytrichus* (A.Kern. ex Borbás) Jalas, *Th. pulegioides* L., *Th. serpyllum* subsp. *serpyllum*, *Th. striatus* Vahl, and *Th. vulgaris*) as well as elucidate its mode of action. The tested *Thymus* extracts and pure compounds at different concentrations (0.4–25 $\mu g.mL^{-1}$) significantly inhibited DPPH• in a concentration-dependent manner. The activities of plant extracts were 11–28, 23–52, and 52–85% at 1.56 , 3.13, and 6.25 $\mu g.mL^{-1}$, respectively. At the mentioned concentrations, *Th. serpyllum* subsp. *serpyllum* as well as a commercial sample of *Th. vulgaris* were the least effective. Rosmarinic acid and luteolin at concentrations up to 3.13 $\mu g.mL^{-1}$ showed the highest radical

scavenging effectiveness (56 and 50% at 0.8 μg.mL^{-1}, resp.). Interestingly, at concentrations ≥12.5 μg.mL^{-1}, activities of most *Thymus* species were comparable to that of luteolin. DPPH radical scavenging activities of the tested samples were assessed using IC_{50} values which are inversely related to their antioxidant abilities. The obtained IC_{50} values of studied *Thymus* extracts were in the range 3.01–6.01 μg.mL^{-1}. The scavenging effects of the extracts decreased in the order of *Th. longicaulis* > *Th. praecox* subsp. *polytrichus* > *Th. pulegioides*, *Th. striatus* > *Th. vulgaris* > *Th. serpyllum* subsp. *serpyllum* (Kindl et al., 2015). Moreover, all tested *Thymus* extracts inhibited nitrite formation in a concentration-dependent manner. They scavenged NO• by 16–45, 37–58, and 54–72% at 50 , 100 , and 200 μg.mL^{-1}, respectively. At these concentrations, *Th. longicaulis* and *Th. pulegioides* showed the highest activity, while *Th. serpyllum* subsp. *serpyllum* demonstrated the weakest effect. Rosmarinic acid and luteolin inhibited the formation of NO• by 58% already at 25 μg.mL^{-1}. Comparing obtained IC_{50} values, the effectiveness of plant extracts as NO• scavengers were in the following descending order: *Th. longicaulis*, *Th. pulegioides* > *Th. striatus*, and *Th. vulgaris* > *Th. praecox* subsp. *polytrichus* > *Th. serpyllum* subsp. *serpyllum*. The IC_{50} values of the most potent *Th. longicaulis* and *Th. pulegioides* were 71.57 and 69.77 μg.mL^{-1}, respectively. Rosmarinic acid (IC_{50} = 15.67 μg.mL^{-1}) and luteolin (IC_{50} = 18.31 μg.mL^{-1}) demonstrated the greatest NO• scavenging activity, even significantly higher (p <0.001) than Trolox (IC_{50} = 53.91 μg.mL^{-1}), and these results were in accordance with the findings obtained by DPPH assay (Kindl et al., 2015). All investigated *Thymus* extracts inhibited lipid peroxidation in a concentration-dependent manner. The activities of plant extracts at concentrations of 10 μg.mL^{-1} and 100 μg.mL^{-1} were in the ranges 32–40 and 56–76%, respectively. *Th. longicaulis* (IC_{50} = 34.30 μg.mL^{-1}) and *Th. pulegioides* (IC_{50} = 34.83 μg.mL^{-1}) exhibited once again the most powerful antioxidant effect, comparable to that of rosmarinic acid (IC_{50} = 21.07 μg.mL^{-1}). The IC_{50} values obtained for the other four extracts were in the range 63.01–80.00 μg.mL^{-1}, without significant difference between them. All investigated plant samples were active in a concentration-dependent manner with total antioxidant capacities ranging between 238.16 and 293.82 mg equivalents of ascorbic acid (AAE)/g. Their effectiveness decreased in the following order: *Th. longicaulis*, *Th. praecox* subsp. *polytrichus* ≥ *Th. striatus* > *Th. pulegioides*, *Th. vulgaris* ≥ *Th. serpyllum* subsp. *serpyllum*. The activity of *Th. longicaulis* was comparable to that of Trolox. Rosmarinic acid was found to have the much higher total antioxidant capacity (598.34 mg AAE.g^{-1}) than Trolox (307.89 mg AAE.g^{-1}). Luteolin showed the lowest activity in comparison to all tested samples (67.72 mg AAE.g^{-1}) (Kindl et al., 2015).

Promising results in terms of the antihypertensive effect of thyme have been observed in Mihailovic-Stanojevic et al. (2013) study. They have evaluated total phenol and flavonoid contents, antioxidant capacity, free radical scavenging activity and potential antihypertensive effect of aqueous extract obtained from *Th. serpyllum* in spontaneously hypertensive rats and in normotensive Wistar rats. Total phenol content of *Th. serpyllum* was 2008.33 ±10.6 mg.L^{-1} gallic acid equivalents, and rosmarinic and caffeic acids were predominant phenolic compounds. The rosmarinic and caffeic acids as predominant phenols presented in the *Th. serpyllum*. The ferric reducing/antioxidant power and antioxidant capacity analysis revealed strong antioxidative properties of *Th. serpyllum*. *In vitro* nitric oxide-scavenging activity of 1 mg.L^{-1} *Th. serpyllum* was 63.43% with the IC_{50} value of 122.36 μg.mL^{-1}. Because

the spontaneously hypertensive rats are a useful model to investigate human essential hypertension, and compounds which lower blood pressure in rats also lower blood pressure in hypertensive humans, results obtained in a study of Mihailovic-Stanojevic et al. (2013) could be promising in using *Th. serpyllum* in hypertensive patients.

Moreover, *Th. serpyllum* may be a promising candidate in the development of novel therapeutic drugs for breast cancer treatment. Bozkurt et al. (2012) have evaluated the effects of *Th. serpyllum* on apoptosis and epigenetic events in breast cancer cells. XTT cell viability assay was used to determine cytotoxicity, while the DNA fragmentation and caspase 3/7 activity assays were used in the assessment of apoptosis. DNA methyltransferase (DNMT) and histone deacetylase (HDAC) activities were evaluated by ELISA and verified by qRT-PCR. *Th. serpyllum* extract induced significant cytotoxicity in breast cancer cells (MCF-7 and MDA-MB-231) but not in normal cells. It also induced apoptosis is and inhibited the DNMT and HDAC activities in MDA-MB-231 cells (Bozkurt et al., 2012).

Essential oil of *Th. serpyllum* also showed the strongest inhibitory effect on the growth and mycotoxin production of *Aspergillus ochraceus*, *A. carbonarius*, and *A. niger* which may have been related to the synergistic or cumulative effects of its components. Minimal inhibitory concentration (MIC) determined for the essential oil and thymol, and selected concentration of the total phenolic content in extract inhibited fungal growth and ochratoxin A biosynthesis by more than 60%, depending on the conditions and duration of incubation with the fungi (Sokolić-Mihalak et al., 2012).

The aqueous extract of *Th. serpyllum* also might be used alone or in combination with insulin to manage diabetes and its associated complications. Alamgeer and et al. (2016) have evaluated and compare the hypoglycemic activity of different solvents extracts of *Th. serpyllum* in rabbits. Diabetes was induced with a single intravenous injection of alloxan monohydrate (150 $mg.kg^{-1}$). The crude powder of *Th. serpyllum* (500 $mg.kg^{-1}$ b.w.) significantly reduced the blood glucose level in both normal and diabetic rabbits. Ether and aqueous extracts of *Th. serpyllum* significantly reduced the blood glucose level with maximum effect (p <0.001) produced by aqueous extract, which was selected for further study. Aqueous extract significantly inhibited the rise in glucose level in oral glucose tolerance test. The extract showed a synergistic effect with different doses of insulin; however serum insulin level of the diabetic rabbits was not significantly increased by the extract. The HbA1c level was significantly (p <0.05) reduced whereas hemoglobin level was significantly increased in three months of study. Phytochemical screening of the aqueous extract showed the presence of alkaloids, flavonoids, tannins, terpenoids, reducing sugar and cardiac glycosides (Alamgeer et al., 2016). According to many supporting documents, it can be assumed that secondary plant metabolites, i.e. polyphenolic compounds in extracts of various species from *Thymus* genus extract may contribute to the antioxidant activity.

Conclusions

The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered after *in vitro* incubation with an extract obtained from *Th. serpyllum*. The *Th. serpyllum* extract reduced the formation

of intracellular aldehydic and ketonic derivatives of OMP in the extract-treated erythrocytes, but these results were non-significant. Total antioxidant capacity was non-significantly increased. The lack of clinical safety and toxicity data for thyme, and many other herbs that are increasingly being used, suggests the necessity of further investigations regarding their influence on organs and tissues function, including the evaluation of molecular mechanisms involved in order to exploit them for potential therapeutic benefits (Rašković et al., 2015). Screening of *Thymus* species for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

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THE ANTIBACTERIAL ACTIVITIES OF SOME *THYMUS* (LAMIACEAE) REPRESENTATIVES AGAINST *SALMONELLA ENTERIDITIS* STRAIN LOCALLY ISOLATED

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Some of the plants of the *Thymus* genus were previously reported for their antimicrobial activities. Therefore, the aim of this study was to evaluate the antimicrobial effects of five ethanolic extracts obtained from leaves of some *Thymus* representatives (*Thymus serpyllum* L. emend. Mill., *Th. pannonicus* All., *Th.* × *porcii* Borbás, *Th. pulegioides* L., *Th. alpestris* Tausch ex A. Kern.) against *Salmonella enteritidis* strain. Freshly leaves were washed, weighted, crushed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity. Antimicrobial activity was determined using the agar disk diffusion assay. The ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *Salmonella enteritidis* studied in this work. The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* (13.1 ±0.85 mm) followed by *Th. alpestris* (12.6 ±0.25 mm), *Th.* × *porcii* (12.2 ±0.55 mm), and then by *Th. serpyllum* (10.5 ±0.23 mm). These plant extracts could be a potential source of new antibacterial agents. Further and more specific studies, *in vivo*, are recommended to determine the efficacy of these plants in the treatment of *Salmonella*-induced bacterial infections.

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

Introduction

Salmonella infection represents a considerable burden in both developing and developed countries and to cause 93 million enteric infections and 155,000 diarrheal deaths each year (Majowicz et al., 2010; Wierup et al., 2017). The majority of clinical disease in animals and humans is caused by serovars within the *Salmonella enterica* subspecies (Pham and McSorley, 2015). Over 50% of the isolates proved resistant to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole, the primary treatments of choice for salmonellosis

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(Chu and Chiu, 2006). Use of antibiotics might result in elevation the risk of spawning more resistant strains (O'Brien, 2002). Utilizing the various herbal substitutes in order to replace antibiotics might offer an alternative for the prevention and/or control of this disease (Snow Setzer et al., 2016).

Medicinal herbs have many potential clinical and therapeutic applications in a modern medical setting because they have been reported to contain bioactive components (Kwon et al., 2008; Bielikova et al., 2017). *Thymus* genus is attracting considerable attention from many researchers, and many such herbs have a long history of medicinal use (Viuda-Martos et al., 2011). The analysis and identification of the constituents of the active principals in these herbs has increased our understanding of their individual pharmacological actions as tonics, carminatives, antitussives, aromatic, expectorant, stomachic, antispasmodic, bronchospasmolytic, diuretic, sedative, diaphoretic, and antiseptics, as well as anti-inflammatory, antioxidant, anthelmintic, hepatoprotective and antitumor agents (Khan and Abourashed, 2010; Nabavi et al., 2015). Antimicrobial agents can also be derived from *Thymus* species (Rota et al., 2008; Xu et al., 2008; Palaniappan and Holley, 2010; Mathela et al., 2010; Rivas et al., 2010; Pemmaraju et al., 2013; Kavoosi et al., 2013; de Morais et al., 2014; Marchese et al., 2016). These versatile pharmacological effects can be attributed to the secondary plant metabolites, especially to essential oil and polyphenols. Plants from the genus *Thymus* are rich in different active substances such as thymol, carvacrol, *p*-cymene and terpinene (Nabavi et al., 2015).

However, many species from *Thymus* genus are yet to be explored scientifically and moreover, the need to find a lasting solution to the problem of infectious diseases caused by *Salmonella* strains necessitated further exploration of plants exhibiting antimicrobial activity. This study screened and evaluated selected representatives of the *Thymus* genus for their antibacterial activities against *Salmonella enteritidis* strain locally isolated. This study evaluated the antimicrobial potentials of the four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine against *Salmonella enteritidis* strain locally isolated.

Material and methodology

Collection of plant materials

Samples were harvested in June-August, 2016. Leaves of *Thymus serpyllum* L. emend. Mill. were collected among grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 03′ 58.9′′, E 26° 13′ 37.5′′, 257 m a.s.l.). Leaves of *Th. pannonicus* All. were harvested among grass in the roadside between the two cultivated fields (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 09.6′′, E 26° 13′ 19.2′′, 283 m a.s.l.). Leaves of *Th. pulegioides* L. were collected among grass nearby land parcels (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 02.8′′, E 26° 14′ 13.9′′, 306 m a.s.l.). Leaves of *Th.* × *porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*) were sampled in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv, Ukraine; N 49° 49' 15.1", E 24° 05' 12.5", 348 m a.s.l.). Leaves of *Th. alpestris* Tausch ex A. Kern. were harvested on the side of the road below the stream, in

mountain valley Shumneska (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 32.3“, E 24° 21‘ 26.4“, 1259 m a.s.l.). Identification of these five taxa was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all attached material and used to prepare ethanolic extracts.

Preparation of plant extracts

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

Bacterial strain and Agar diffusion susceptibility testing

Antimicrobial activity was determined using the agar disk diffusion technique (Bauer et al., 1966). The clinical isolates of *S. enteritidis* were obtained from the Department of Bacteriology, Regional Hospital in Koszalin (West-Pomeranian Voivodeship, Poland). The strain was grown in a test tube containing 45 mL of sterile nutrient broth (Oxoid™ Ltd.) at 37 °C for 24 hours. The purity of the inoculum was confirmed by plating on appropriate selective media and microscopic examination of the Gram-stained smear. A loopful of inoculum was transferred by streaking onto a Xylose Lysine Desoxycholate Agar (XLD agar) (Oxoid™ Ltd.). Plates were incubated for 24 hours at 37 °C. Bacterial morphology was confirmed by optical microscopy. Several colonies were collected with a sterile inoculating loop, transferred into a sterile saline solution, and adjusted to the desired concentration using the McFarland nephelometer standards

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

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999).

All statistical analyses were performed using Statistica 8.0 software (StatSoft, Poland). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

In order to identify *Thymus* species with antibiotic properties against salmonellosis, the four species and one interspecific hybrid of *Thymus* genus were tested against *Salmonella enteritidis* using the disk-agar method (Figure 1). Ethanol (96%) as the negative control showed inhibition zones of the test strain (8.5 ±0.49 mm). Of the herbal extracts tested, all leaf extracts obtained from *Thymus* species were found to have antibacterial activity against *S. enteritidis* strain tested; inhibition zones ranged from 8 to 16 mm. Moreover, both *Th. alpestris* and *Th. pulegioides* extracts exhibited intermediate antibacterial activity against *S. enteritidis* with statistically significant diameters of inhibition zones (12.6 ±0.25 mm and 14.3 ±0.59 mm, respectively). The inhibition zones produced by leaf extracts obtained from *Th. pannonicus* and *Th.* × *porcii* indicated that both showed effective antimicrobial activities, although these extracts showed slightly higher activity, based on inhibition zone sizes (13.1 ±0.85 mm and 12.2 ±0.55, respectively), these results were non-significantly (p >0.05) (Figure 1).

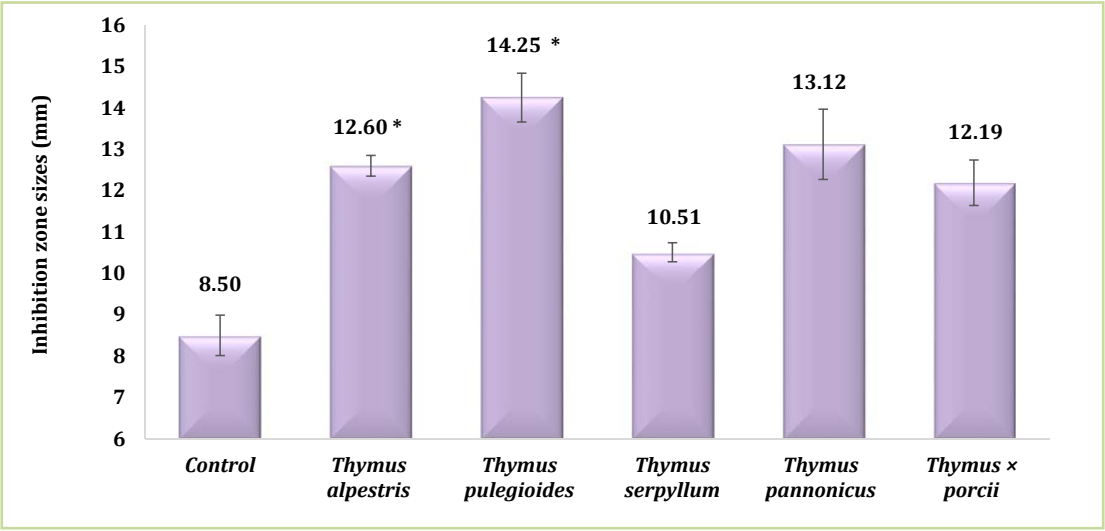

Figure 1 The mean of inhibition zone diameters of ethanolic extracts obtained from leaves of various *Thymus* plants against *Salmonella enteritidis* strain locally isolated (*M* ±m, *n* = 8)
* the changes are statistically significant (p <0.05) compared to the control group (96% ethanol)

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

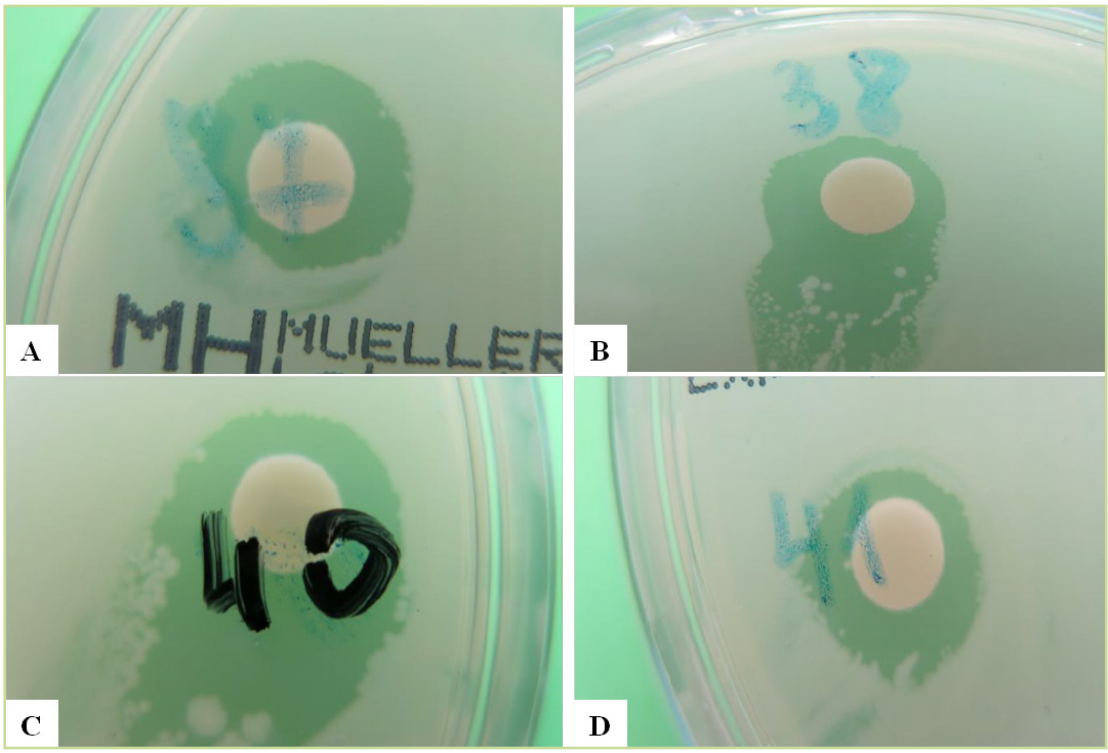

Figure 2 Antimicrobial activity of various ethanolic extracts obtained from leaves of *Th. pannonicus* (A), *Th.* × *porcii* (B), *Th. pulegioides* (C), *Th. alpestris* (D) against *Salmonella enteritidis* strain measured as inhibition zone diameter

It should be noted that the most antimicrobial effective plant against *Salmonella enteritidis* strain was *Th. pulegioides*, being highly active with the mean diameter of inhibition zone 14.3 ±0.59 mm. The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* (13.1 ±0.85 mm) followed by *Th. alpestris* (12.6 ±0.25 mm), *Th.* × *porcii* (12.2 ±0.55 mm), and then by *Th. serpyllum* (10.5 ±0.23 mm) (Figure 1). Since the antibacterial effectiveness of medicinal plants varies dramatically depending on the phytochemical characteristics of plant families and subfamilies, it is not surprising to note the difference in this efficacy even when using samples taken from the same plant, but from two different regions.

Variation in the chemical profile of extracts could influence their biological activities. Therefore, it was important to know the chemical composition of extracts to correlate with their antimicrobial activities. Most of the antimicrobial activity in essential oils from *Thymus* genus appears to be associated with high amounts of monoterpenoid phenols (thymol and carvacrol) or monoterpenic alcohols (geraniol and linalool) (Petrović et al., 2016). The thymol is responsible for antimicrobial activity (Marchese et al., 2016). Results of this study are in agreement with other research showing that thyme essential oil (especially thymol chemotype) possesses high activity against both Gram-positive and Gram-negative bacteria (Karaman et al., 2001; Rasooli and Mirmostafa, 2002; Rota et al., 2008; Maksimović et al., 2008;

Nejad Ebrahimi et al., 2008; Ruiz-Navajas et al., 2012; Ballester-Costa et al., 2013; Moghimi et al., 2016; Fadli et al., 2012, 2016, 2018; Schött et al., 2017; Semeniuc et al., 2017; Vitali et al., 2017). However, it should be noted that ethanolic extracts have a complex composition and their antimicrobial activities were due to a synergist effect between a large number of components present in small amounts in the extracts.

The two main bioactive compounds in thyme essential oil are thymol and carvacrol responsible for most therapeutic aspects of the thyme extracts, i.e. antibacterial, antifungal, anti-inflammatory, and antioxidant activities (Petrović et al., 2016). In a study by Rota et al. (2008), most of the antimicrobial activity in essential oils from *Thymus* genus appears to be associated with phenolic compounds (thymol and carvacrol). In *Th. hyemalis* Lange oil (thymol, thymol/linalool, and carvacrol chemotypes) 49, 51 and 51 components were identified representing about 97, 98.4 and 86.7% of the total detected constituents. Major components quantified for the thymol chemotype were: thymol (43%) followed by *p*-cymene (16.0%) and γ-terpinene (8.4%); for *Th. hyemalis* thymol/linalool chemotype: linalool (16.6%), thymol (16.0%), γ-terpinene (9.8%), 1-8-cineole (5.4%), borneol (4.7%), verbenone (4.8%); and for *Th. hyemalis* carvacrol chemotype were: carvacrol (40.1%), *p*-cymene (19.8%), borneol (5.0%) and thymol (2.9%). Attending to the volatile profile of the essential oils, a richer relative concentration of terpenic hydrocarbons (γ-terpinene), alcohols (linalool, (Z)-verbenol, terpinen-4-ol, α(alpha)-terpineol, geraniol, spathulenol) ketones (camphor, verbenone) and thymol oxygenated derivates (thymol methyl ether) were quantified in *Th. hyemalis* (thymol ch.) when compared to *Th. zygis* L. (thymol ch.) and *Th. vulgaris* L. (thymol ch.). Results of these authors suggest that it could be a synergistic action among phenolic components and these compounds. Carvacrol is another phenolic component that described the chemotype of *Th. hyemalis* essential oil. The assays using this essential oil (40% carvacrol) showed bactericidal and bacteriostatic activities similar to *Th. hyemalis* (43% thymol) since concentrations under 0.2 $\mu l.mL^{-1}$ were enough to achieve the MIC and MBC for 9 of the 10 microorganisms assayed in the study of Rota et al. (2008). The bacteriostatic properties of this oil are suspected to be associated with the carvacrol content (Rota et al., 2008).

The antimicrobial activity of *Thymus* species has been well studied. Since the 1960's, and especially over the past 20 years, a large number of *Thymus* essential oils, extracts, and their isolated compounds have been studied for the antimicrobial activity. These products are of particular interest because no bacterial resistance or adaptation has been described, and low or insignificant side effects have been found both for the essential oils and whole extracts (Nabavi et al., 2015). Semeniuc et al. (2017) have compared the antibacterial effects of several essential oils alone and in combination against different Gram-positive and Gram-negative bacteria associated with food products. Parsley, lovage, basil, and thyme essential oils, as well as their mixtures (1 : 1, v/v), were tested against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium*. Thyme essential oil exhibited the best inhibitory activity against all bacteria evaluated by the Kirby-Bauer disk diffusion test (range 12.16–36.41 mm), followed by basil, lovage, and parsley essential oils. Its zone of inhibition is larger (for *E. coli* and *S. typhi*murium) or similar (for *B. cereus*) to the size of gentamicin zone (the antibiotic used as positive control). Among the tested

microorganisms, it produces the largest zone of inhibition against *E. coli* (strong inhibitory effect), followed by *S. typhi*murium (moderate inhibitory effect), *B. cereus* (moderate inhibitory effect), *P. aeruginosa* (mild inhibitory effect), and *S. aureus* (mild inhibitory effect). *P. aeruginosa* is the most susceptible to all essential oils and their combinations. Three essential oils combinations show antagonistic effects against *P. aeruginosa* (parsley/thyme, lovage/thyme, and basil/thyme essential oils), and the other three combinations indifferent effects (parsley/lovage, parsley/basil, and lovage/basil essential oils). Parsley/lovage, parsley/basil, and parsley/thyme essential oil mixtures display significantly higher antibacterial activities than the parsley essential oil. Basil essential oil does not significantly affect the antibacterial activity of lovage/basil essential oil mixture. Thyme essential oil significantly contributes to the antibacterial activity of parsley/thyme and lovage/thyme essential oil mixtures but does not significantly influence the antibacterial activity of basil/thyme essential oil mixture. All pairwise combinations exhibit lower antibacterial activities than the thyme essential oil against all five bacteria. Considering that thyme essential oil has the highest percentage yield and antibacterial potential from all tested formulations, it is therefore recommended to be used alone as the antimicrobial agent (Semeniuc et al., 2017).

Cutillas et al. (2018) have supported the potential use of *Thymus* sp. essential oils as natural preservatives of natural food, cosmetic and pharmaceutical ingredients. They have analyzed by gas chromatography coupled with mass spectrometry detection six samples of red thyme (*Th. zygis*) and two samples of winter thyme (*Th. hyemalis*) essential oils obtained from plants cultivated in south-eastern Spain and extracted by steam distillation. Thymol (30–54%), *p*-cymene (14–27%) and γ-terpinene (8–28%) were the most abundant components of *Th. zygis* essential oil, while 1,8-Cineole (3–37%), *p*-cymene (1–29%), linalool (8–13%) and thymol (0–19%) were the most abundant components in the case of *Th. hyemalis* essential oil. Enantioselective gas chromatography identified (-)-linalool, (-)-borneol and (+)-limonene as the main enantiomers. Several methods to evaluate antioxidant capacities were applied to the essential oils, concluding that their activities were mainly due to thymol and linalool. The inhibition of lipoxygenase activity, mainly due to thymol, *p*-cymene, and linalool, suggested their possible use as anti-inflammatories. The high antibacterial and antifungal activities determined for the essential oils means that they can be used as natural preservatives (Cutillas et al., 2018). Moreover, the antimicrobial activity of five plant extracts against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* using agar disc diffusion technique was investigated by Mostafa et al. (2018). Ethanolic extracts of *Punica granatum* L., *Syzygium aromaticum* (L.) Merr. & L. M. Perry, *Zingiber officinale* Roscoe, and *Thymus vulgaris* were potentially effective with variable efficiency against the tested bacterial strains at a concentration of 10 $mg.mL^{-1}$. These plant extracts which proved to be potentially effective can be used as natural alternative preventives to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial agent applications (Mostafa et al., 2018).

In research of Safarpoor et al. (2018), *Thymus daenensis* Čelak. and *Silybum marianum* (L.) Gaertn. extracts with and without the presence of silver nanoparticles (Ag-NPs) were prepared and used against some pathogenic bacteria and fungi to detect new sources of

antimicrobial agents. The antimicrobial activities of *Th. daenensis* and *S. marianum* extract with and without the presence of Ag-NPs were investigated at concentrations from 12.5 to 50 $mg.mL^{-1}$ against *Staphylococcus aureus* (Gram-positive organism) and *Escherichia coli* (Gram-negative organism), and fungal strains were *Aspergillus oryzae* and *Candida albicans*. Antimicrobial activity determined using agar disc diffusion method revealed that the activities of Ag-NPs/*Th. daenensis* were superior to Ag-NPs/*S. marianum* and extracts (*Th. daenensis* and *S. marianum*). The medicinal plant extract can be used to synthesize the Ag-NPs as an eco-friendly and inexpensive method in large scale. The results of Safarpoor et al. (2018) showed that the prepared Ag-NPs/extracts as good antibacterial and antifungal agents can be potentially applied against rapidly increasing antibiotic resistance.

To increase the sensibility of *Salmonella typhimurium* strain, a mixture of *Thymus vulgaris*, *Rosmarinus officinalis* L. and *Myrtus communis* L. essential oils were used in combined treatment by experimental design methodology (mixture design) in the study of Fadil et al. (2018). The chemical composition of essential oils was firstly identified by gas chromatography and gas chromatography/mass spectrometry and their antibacterial activity were evaluated. The results of this first step have shown that thymol and borneol were the major compounds in *Th. vulgaris* and *M. communis* L. essential oils, respectively, while 1,8-cineole and α-pinene were found as major compounds in *R. officinalis*. The same results have shown a strong antibacterial activity of *Th. vulgaris* essential oil followed by an important power of *M. communis* essential oil against a moderate activity of *R. officinalis* essential oil. The optimization of mixtures antibacterial activities has highlighted the synergistic effect between *Th. vulgaris* and *M. communis* essential oils. A formulation comprising 55% of *Th. vulgaris* and 45% of *M. communis* essential oils, respectively, can be considered for the increase of *Salmonella typhimurium* sensibility (Fadil et al., 2018).

Conclusions

The ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against the *Salmonella enteritidis* studied in this work. The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* (13.1 ±0.85 mm) followed by *Th. alpestris* (12.6 ±0.25 mm), *Th.* × *porcii* (12.2 ±0.55 mm), and then by *Th. serpyllum* (10.5 ±0.23 mm). These plant extracts could be a potential source of new antibacterial agents. Further and more specific studies, *in vivo* as well are recommended to determine the efficacy of these plants in the treatment of *Salmonella*-induced bacterial infections.

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TOTAL ANTIOXIDANT CAPACITY IN THE MUSCLE TISSUE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) UNDER *IN VITRO* INCUBATION WITH LEAF EXTRACTS OF SOME *THYMUS* (LAMIACEAE) REPRESENTATIVES

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The aim of this study was to evaluate the *in vitro* effect of buffer extracts obtained from leaves of various representatives of *Thymus* genus on the total antioxidant capacity (TAC) of the muscle tissue of the rainbow trout (*Oncorhynchus mykiss* Walbaum). Leaves of *Thymus serpyllum* L. emend. Mill., *Th. pannonicus* All., *Th. pulegioides* L., *Th.* × *porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*), *Th. alpestris* Tausch ex A. Kern. were harvested to study. Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at ambient temperature. The supernatant of the rainbow trout muscle tissue was used to incubate with leaf extracts of various representatives from *Thymus* genus (in a ratio 19:1) at room temperature. The most potent antioxidant effect was demonstrated for the extracts of *Th. alpestris*, *Th.* × *porcii*, *Th. pannonicus*, *Th. serpyllum*, and *Th. pulegioides* compared to phosphate buffer control (59.5, 48, 45.9, 43.9, and 38.8%, p <0.05, respectively). The results of this study provide a new perspective on the use of various *Thymus* species as a medicinal plant to improve the antioxidant response of rainbow trout. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of its antioxidant effects on various tissues are in progress.

Keywords: *Thymus*, leaf extracts, rainbow trout (*Oncorhynchus mykiss* Walbaum), muscle tissue, total antioxidant capacity

Introduction

Recently, medicinal plants came as a promising and substitute method for the control of fish disease in aquaculture that offers an alternative because of their immunomodulatory effects, to the drugs, chemicals, and antibiotics currently used in aquaculture to control diseases (Galina et al., 2009). Additionally, medicinal plants are used in aquaculture not only as

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chemotherapeutics but also as feed additives, as they contain a wide variety of nutrients and chemical compounds with many biological activities including growth promotion, appetite stimulation, immune stimulation, antimicrobial, and anti-stress in fish (Chang, 2000; Galina et al., 2009; Citarasu, 2010). The mechanisms of action of plants and their derivatives are attributed to the presence of many active second metabolites such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids (Galina et al., 2009; Citarasu, 2010; Harikrishnan et al., 2011). Therefore, the development of new additives for aquaculture still attracts the attention of many researchers and fish farmers comprising the eco-friendly approach for the control of fish pathogens.

Plants of the mint family (Lamiaceae) produce many metabolites with antioxidant properties, that are present as secondary metabolites which are synthesized as a defence mechanism. Particularly, *Thymus* spp. also produce secondary metabolites with antimicrobial, antioxidant, antimicrobials, expectorants, antitussives, and antiplatelet drugs (Kulevanova et al., 2000; Figueiredo et al., 2008; Costa et al., 2012). The genus *Thymus* L. consists approximately 215 species currently recognized (Morales, 2002). These herbaceous perennials and sub-shrubs distributed in Europe, Northwest Africa, Ethiopia, Asia and Greenland (Morales, 2002; Bartolucci et al., 2013). It is one of the most widely used genera in folk medicine, where it is popular for its stimulatory action on all organism functions (Viuda-Martos et al., 2011). Many species of this genus are used in traditional medicine as tonics, carminatives, antitussives, aromatic, expectorant, stomachic, antispasmodic, bronchospasmolytic, diuretic, sedative, diaphoretic, and antiseptics, as well as anti-inflammatory, antioxidant, anthelmintic, hepatoprotective and antitumor agents (Khan and Abourashed, 2010; Nabavi et al., 2015). Internally, thyme is used for treatment of acute bronchitis, laryngitis, whooping cough, chronic gastritis, diarrhea, and lack of appetite, while externally in baths to treat rheumatic and skin problems (bruises, sprains, fungal infections) as well as for minor arthritis, gum disease, tonsillitis, etc. (Khan and Abourashed, 2010). Moreover, *Thymus* species have wide application nowadays in food as a culinary herb and food flavoring, as well as in cosmetics and pharmaceutics in toothpaste, soaps, detergents, creams, lotions, and perfumes. *Thymus* i.e. leaves of *Th. vulgaris* L. and *Th. zygis* L. is used as a spice in several foods.

With the increasing interest in herbal medicine, environmental protection, and natural products, numerous research in herbal or natural product studies has been published in recent years (Kuklina et al., 2017; Vergun et al., 2017). However, literature data on the antioxidant activity of *Thymus* species in the aquacultural application is practically unknown.

In the last decades, more attention is received the plants possessed antioxidative properties as important inputs in organic fish farming. Sönmez et al. (2015) have evaluated effects of dietary supplementation of sage (*Salvia officinalis*), mint (*Mentha spicata*) and thyme (*Thymus vulgaris*) oils on growth performance, lipid peroxidation level (malondialdehyde, MDA) and liver antioxidant enzyme activities (superoxide dismutase, SOD; catalase, CAT; glucose-6-phosphate dehydrogenase, G6PD; glutathione reductase, GR; glutathione-S-transferase, GST and glutathione peroxidase, GPx) in rainbow trout juveniles. Their work demonstrated changes in oxidative stress indices and antioxidant defense systems in the liver of rainbow trout after long-term exposure to different plant oils. Sage and thyme oils

have been determined as convenient and useful antioxidant enzyme stimulators and growth promoters. Although, mint oil generally caused an increase in almost all antioxidant enzyme levels, all growth parameters, feed conversion ratio, and survival were negatively affected by mint diets. Therefore, it is suggested that mint has some undesirable effects on rainbow trout physiology and is not a suitable feed additive. Overall, dietary inclusion of sage and thyme oils is effective in enhancing rainbow trout growth, reduction in MDA and least changing antioxidant enzyme activities at a low level of 500 mg per kg diet, and they can be used as important feed supplements for rainbow trout production (Sönmez et al., 2015). To evaluate the effect of different antioxidant and antimicrobial sources, semi-fried mullet fish fillets were dipped into an edible coating solution containing thyme and marjoram at 2.5 and 5.0% and stored at 4 °C. Samples coated with 5% thyme showed the lowest rate of peroxide formation (Yasin and Abou-Taleb, 2007). Moreover, coated samples with thyme at 2.5 or 5% showed the lowest incremental pattern in psychrophilic bacterial counts at any point of time during the cold storage (Kostaki et al., 2009). Therefore, these results indicate the potential use of these plant oils.

Therefore, the objective of the present study was to evaluate the *in vitro* the effect of buffer extracts obtained from leaves of various representatives from *Thymus* genus on the total antioxidant capacity (TAC) in the muscle tissue of the rainbow trout (*Oncorhynchus mykiss* Walbaum). TAC is an indicator frequently used to assess the antioxidant status of biological samples and can evaluate the antioxidant response against the free radicals produced in a given disease (Rubio et al., 2016).

Material and methodology

Collection of plant materials

Samples were harvested in June-August, 2016. Leaves of *Thymus serpyllum* L. emend. Mill. were collected among grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 03′ 58,9′′, E 26° 13′ 37,5′′, 257 m a.s.l.). Leaves of *Th. pannonicus* All. were harvested among grass in the roadside between the two cultivated fields (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 09.6′′, E 26° 13′ 19.2′′, 283 m a.s.l.). Leaves of *Th. pulegioides* L. were collected among grass nearby land parcels (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02′ 02.8′′, E 26° 14′ 13.9′′, 306 m a.s.l.). Leaves of *Th.× porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*) were sampled in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv, Ukraine; N 49° 49‘ 15.1“, E 24° 05‘ 12.5“, 348 m a.s.l.). Leaves of *Th. alpestris* Tausch ex A. Kern. were harvested on the side of the road below the stream, in mountain valley Shumneska (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 32.3“, E 24° 21‘ 26.4“, 1259 m a.s.l.) (Figure 1).

Identification of these five taxa was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all attached material and used to prepare extracts.

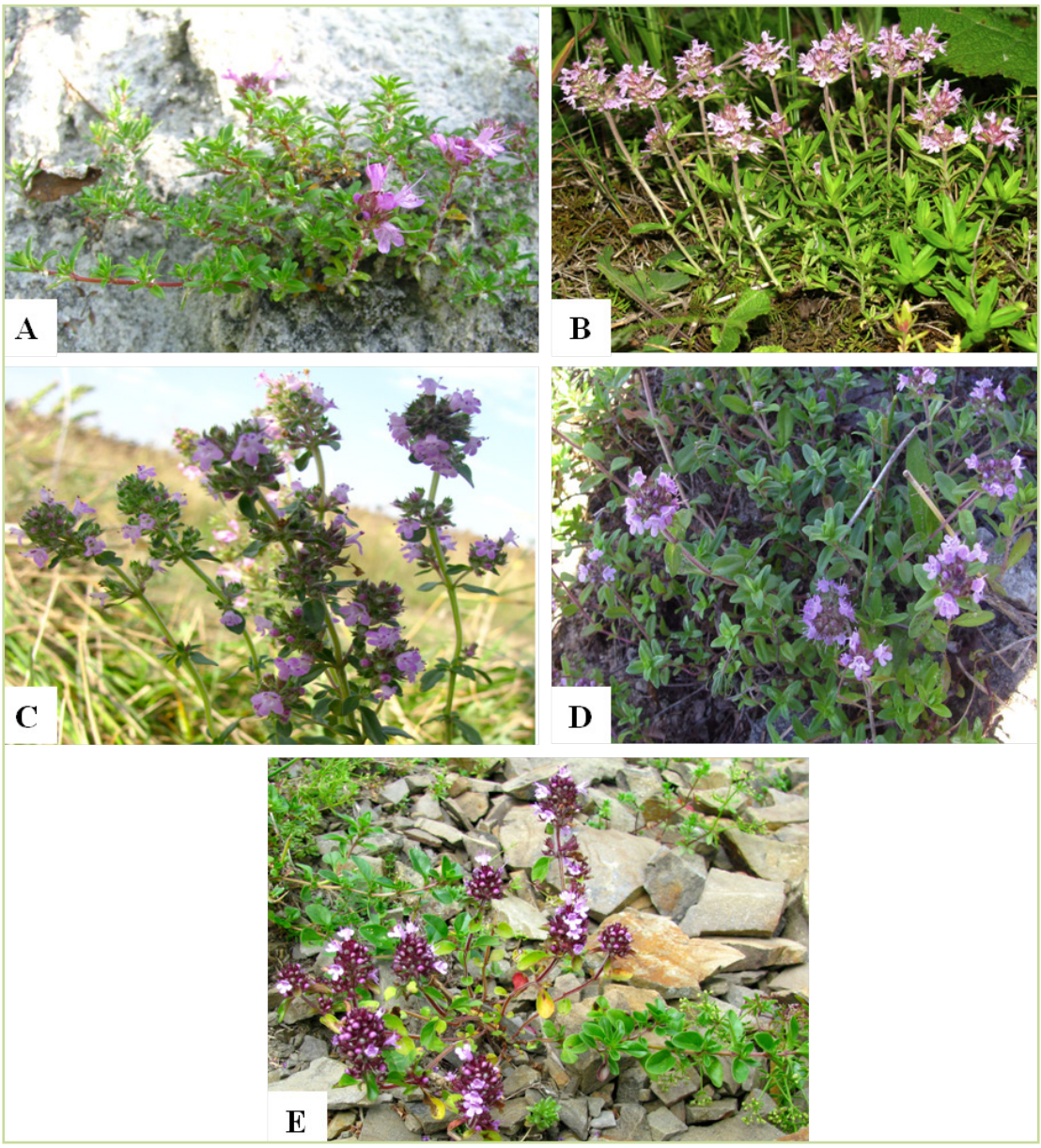

Figure 1 Plants of *Thymus serpyllum* (A), *Th. pannonicus* (B), *Th. pulegioides* (C), *Th.* × *porcii* (D), *Th. alpestris* (E) used in our study. Photos by Viktor Nachychko

Preparation of plant extracts

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

Experimental fish

Clinically healthy rainbow trout with a mean body mass of 80–120 g were used in the experiments. The experiments were performed in water at 14.5 ±0.5 °C and pH 7.2–7.4. The dissolved oxygen level was about 9 ppm with additional oxygen supply, with a water flow of 25 $L.min^{-1}$, and a photoperiod of 12 h per day. The same experimental conditions were used during the whole research. The water parameters were maintained under constant surveillance. The fish were held in square tanks (150 fish per tank) and fed commercial pelleted diet.

Muscle tissue samples

The muscle tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2) using a glass homogenizer immersed in an ice water bath. Homogenates were centrifuged at 3000 g for 15 min at 4 °C. After centrifugation, the supernatant was collected and frozen at -20 °C until analyzed. All enzymatic assays were carried out at 22 ±0.5 °C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate. The reactions were started by adding the tissue supernatant.

Experimental design

The supernatant of the muscle tissue was used to incubate with extracts of various species of *Thymus* (in a ratio 19 : 1) at room temperature. The control group (trout muscle tissue) was incubated with 100 mM Tris-HCl buffer (pH 7.2) (in a ratio 19:1). The incubation time was 2 hours. Total antioxidant capacity was studied in the incubated homogenate (control group and in samples with extracts of various *Thymus* species).

Measurement of total antioxidant capacity (TAC)

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37 ºC. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the

total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney U test (Zar, 1999). All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

In the present study, we investigated the influence of leaf extracts obtained from various *Thymus* representatives on the total antioxidant capacity in the muscle tissue of rainbow trout after incubation with extracts under *in vitro* conditions. The most potent antioxidant effect was demonstrated for the extracts of *Th. alpestris*, *Th.* × *porcii*, *Th. pannonicus*, *Th. serpyllum*, and *Th. pulegioides* compared to phosphate buffer control (59.5, 48, 45.9, 43.9, and 38.8%, p <0.05, respectively). The results showed that the leaf extract of *Th. alpestris* efficiently increased the TAC level in muscle tissue (Figure 2).

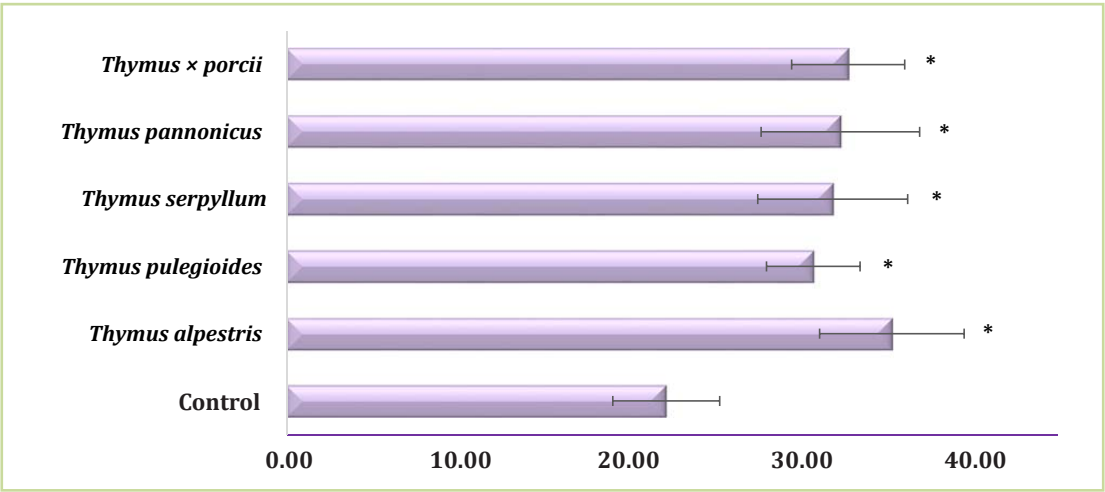

Figure 2 The total antioxidant capacity in the muscle tissue of rainbow trout after *in vitro* incubation with leaf extracts obtained from various *Thymus* representatives (M ±m, n = 8)
* the changes are statistically significant (p <0.05) compared to the control group

Treatment of muscle tissue by leaf extracts obtained from various *Thymus* representatives caused an increase of total antioxidant capacity in muscle tissue of trout. Therefore, it would be reasonable to suggest that this enhancement of antioxidant effects is determined by their by-products. There are different chemotypes, depending on the dominant component of the essential oil such as thymol, carvacrol, linalool, geraniol, thujanol, α-terpineol, borneol, and *p*-cymene (Nabavi et al., 2015). High antioxidant activities have been reported for the thymol and carvacrol present in the *Thymus* plant. Thymol, carvacrol, γ-terpinene, and *p*-cymene are the major compounds of genus *Thymus* plants which are responsible for its biological and pharmacological properties such as anti-mutagenic, antitumor, antioxidant, anti-inflammatory, etc. (Bhalla et al., 2013). In addition, results from *in vitro* and *in vivo* studies show that carvacrol possess a variety of biological and pharmacological properties including antioxidant, antibacterial, antifungal, anticancer, antimutagenic, antigenotoxic, analgesic,

antispasmodic, anti-inflammatory, angiogenic, anti-parasitic, antiplatelet, spasmolytic, and vasorelaxant, insecticidal, antihepatotoxic and hepatoprotective activities and uses such as feed additive, in honeybee breeding and in gastrointestinal ailments (Baser, 2008). In recent years, considerable research has been undertaken in an effort to establish the biological actions of carvacrol for its potential use in clinical applications (Suntres et al., 2015). Areas for future research are also covered are inhibitions of microbial and fungal toxin production and the anti-inflammatory, analgesic, anti-arthritic, anti-allergic, anti-carcinogenic, anti-diabetic, cardioprotective, gastroprotective, hepatoprotective, and neuroprotective properties of carvacrol as well as metabolic, synergistic, and mechanistic aspects (Friedman, 2014).

Phenolic compounds act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals. As an alternative antioxidant property, some phenolic compounds with dihydroxy groups can conjugate transition metals, preventing metal-induced free radical formation. The redox active metal ions such as Cu^{+} or Fe^{2+} interact with hydrogen peroxide (H_2O_2) through Fenton reaction to form hydroxyl radicals ($^{\cdot}OH$), which is the most reactive ROS known, being able to initiate free radical chain reactions by abstracting hydrogen from almost any molecule. Phenolic compounds with catecholate and gallate groups can inhibit metal-induced oxygen radical formation either by coordination with Fe^{2+} and enhance autoxidation of Fe^{2+} , or the formation of an inactive complex with Cu^{2+}, Fe^{2+} , or Cu^{+} with relatively weaker interaction. These two antioxidant actions can cause a reduction of the steady-state concentrations of free radicals and oxidant species. As a result, the subsequent oxidation of target molecules such as lipids, proteins, and nucleic acids is diminished (Dai and Mumper, 2010).

Pronounced antioxidant and free radical scavenging activity of the various *Thymus* species *in vitro* and *in vivo* was demonstrated in many studies. Mihailovic-Stanojevic et al. (2013) have indicated that aqueous extract obtained from *Th. serpyllum* (wild thyme) may protect against hypertension in an experimental model of essential hypertension. They have evaluated total phenol and flavonoid contents, antioxidant capacity, free radical scavenging activity and potential antihypertensive effect of aqueous extract obtained from *Th. serpyllum* in spontaneously hypertensive rats and in normotensive Wistar rats. Total phenol content of *Th. serpyllum* was 2008.33 ±10.6 $mg.L^{-1}$ of gallic acid equivalents (GAE), and rosmarinic and caffeic acids were predominant phenolic compounds. The ferric reducing/antioxidant power and antioxidant capacity analysis revealed strong antioxidative properties of *Th. serpyllum*. *In vitro* nitric oxide-scavenging activity of 1 $mg.L^{-1}$ *Th. serpyllum* extract was 63.43% with the IC_{50} value of 122.36 $\mu g.mL^{-1}$. Bolus injection of *Th. serpyllum* extract (100 $mg.kg^{-1}$ body weight i.v.) induced significant decrease of systolic and diastolic blood pressure and total peripheral resistance in spontaneously hypertensive rats, without effects on these parameters in normotensive Wistar rats. The cardiac index remained unchanged after *Th. serpyllum* extract treatment in all experimental rats. Given a dose of *Th. serpyllum* did not show significant nitric oxide-scavenging activity *in vivo* (Mihailovic-Stanojevic et al., 2013).

Chemopreventive efficacy of the *Th. longicaulis* C. Presl. extracts, by means of their anti-inflammatory, cytotoxic and antioxidant activities were assessed in a study of Galasso et al. (2014). To this purpose, each extract underwent an extensive screening towards five human

cell lines: CCRF-CEM (leukemia); U251 (glioblastoma); MDA-MB-231 (breast cancer); HCT-116 (colon cancer) and MRC-5 (lung fibroblasts) through XTT [2,3bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H tetrazolium hydroxide] test. The ability of the extracts to counteract cyclooxygenase-2 (COX-2) expression was also evaluated by COX-2 expression assay in human THP-1 monocyte-derived macrophages. COX-2 inhibition could represent a valuable anticancer strategy as it is associated with carcinogenesis and over-expressed in a variety of human malignancies. *Th. longicaulis* extract sampled in the autumn season, which was particularly rich in rosmarinic acid and methylapigenin, exhibited a strong antioxidant and anti-inflammatory effectiveness (Galasso et al., 2014).

Natural antioxidants such as phenolic compounds, flavonoids, and other phytochemicals can act as free radical scavengers (Tohidi et al., 2017). They also delay the lipid oxidation process and might be valuable antibacterial and antioxidant natural sources and seem to be applicable in both medicine and food industry (Baharfar et al., 2015). In a study by Baharfar et al. (2015), flavonoid-, polyphenol- and anthocyanin-rich extracts of *Th. kotschyanus* Boiss. & Hohen. aerial parts were evaluated for their antioxidant capacity, phenolic and flavonoid contents, and antibacterial activity. The results indicated that all of the samples possess high potent free radical scavenging and antioxidant activity. They were also rich in phenolic and flavonoid compounds. Interestingly, the antioxidant activity of various extracts positively correlated with their phenolic and flavonoid contents. Water extract which had the highest phenolic and flavonoid content, showed an appreciable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity as well as reducing power as compared with other extracts. On the basis of the obtained results from the antibacterial test, the extracts contained moderate to good growth inhibitory effect against both Gram-negative and Gram-positive bacteria. Therefore, the tested *Thymus* extracts might be valuable antibacterial and antioxidant natural sources and seem to be applicable in both medicine and food industry (Baharfar et al., 2015). Saidi et al. (2016) have investigated the antibacterial activity of *Th. daenensis* Čelak. extract against extended-spectrum β-lactamases (ESBL), metallo-β-lactamase-producing Gram-negative bacteria and methicillin-resistant *Staphylococcus aureus* (MRSA). Subsequently, the antioxidant activity of *Th. daenensis* extract was assessed. The association between phenolic compound and antioxidant activity was found for the ABTS• + method (43.52%) in the lowest level, while, for FRAD and DPPH• methods other trends occurred (70.5% correlation for DPPH• and 50.9% for FRAD) (Saidi et al., 2016).

Conclusions

According to the results of this study, we addressed the hypothesis that by-products in the leaf extracts obtained from various *Thymus* representatives may be a major contributor to increase of the antioxidant capacity of muscle tissue of rainbow trout after incubation *in vitro*. To prove this hypothesis, separation and characterization of secondary metabolites compound in plant extracts are required for further study. Moreover, it should be noted that measurement only TAC can provide limited information about the antioxidant status because TAC assays do not measure all antioxidant components (Rubio et al., 2016). Special attention should be given to the evaluation of the effects of plant extracts/products on fish growth,

hematological profiles, immune responses and resistance to infectious diseases (Bulfon et al., 2015). The present results suggest that the future herbal supplementation feeds responsible for returning antioxidant capacity of various tissue and, therefore, the triggering the immune system of the specific and innate immunity of salmonids. However, the exact mechanisms of inducing the biochemical and immunological parameter recoveries using modern molecular techniques should be applied to ensure that the species of herbal supplementation feeds used in aquaculture are correctly identified, for quality assurance as well as safety.

In conclusion, the obtained data provide a new perspective on the use of various *Thymus* species as a medicinal plant to improve the antioxidant response of rainbow trout. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of its antioxidant effects on various tissues are in progress.

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COMPARATIVE ANALYSIS OF PROTEOLYTIC ENZYMES OF HUMAN AND PULMONARY FRESHWATER MOLLUSCS

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ ПРОТЕОЛИТИЧЕСКИХ ФЕРМЕНТОВ ЧЕЛОВЕКА И ЛЕГОЧНЫХ ПРЕСНОВОДНЫХ МОЛЛЮСКОВ

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The article is devoted to substantiating the feasibility of obtaining proteolytic enzymes from the tissues of pulmonary freshwater molluscs for use in scientific and practical purposes. The article presents the results of the two phases of the study. Initially, the authors conducted a comparative analysis of the biochemical parameters of human, rat tissues and molluscs, and then they evaluated the degree of proteolytic enzymes homology in humans and molluscs using methods of bioinformatics. The results of studies of the first stage showed that the tissues of pulmonary freshwater molluscs can serve as the starting material for the production of proteins, including enzymes. The results of the second stage of research showed that in humans and molluscs the homology of the enzymes of unregulated proteolysis is 66–69% and that of the proteolytic enzymes of the ubiquitin-proteasome pathway is 72–76%. The obtained results substantiate the possibility of forming aquaculture of molluscs in order to obtain from their tissues proteolytic enzymes for use in biopharmaceutics, cosmetology and the food industry.

Keywords: proteolytic enzymes, *Lymnaea stagnalis*, *Planorbarius corneus*, *Biomphalaria glabrata*, docking

Введение

Легочные пресноводные моллюски прудовик (*Lymnaea stagnalis* L.) и роговая катушка (*Planorbarius corneus* L.) распространены в естественных водоемах Европы. Первый из них признан модельным организмом для исследования действия

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водорастворимых химических агентов в ЕЭС в 2010 году. Разработаны детальные требования к проведению строго контролируемых исследований в течение всей или части жизни моллюска (Series on Testing and Assessment. No. 121. Detailed review paper on molluscs life-cycle toxicity testing. JT03284405. Environment Directorate. Paris 2010). В этом фундаментальном документе приведены данные о достаточной идентичности различных метаболических путей, например, путей биосинтеза гормонов стероидной природы у *Lymnaea stagnalis*, а также у млекопитающих животных и человека. В большинстве сообщений, описывающих практическое использование этих видов пресноводных моллюсков, указывается на их участие в пищевых цепях экосистем, их роль как промежуточных переносчиков возбудителей некоторых заболеваний, использование в качестве модельных организмов для изучения физиологических процессов (размножение, нервная регуляция, клеточный метаболизм, генетика и др.). Однако нам не удалось найти систематизированных исследований, в которых бы обсуждался вопрос о возможности использования пресноводных легочных моллюсков для получения ферментов, продуктов питания, биологически активных веществ (Чиркин и др., 2012).

Ранее было показано, что в гемолимфе и гепатопанкреасе *Lymnaea stagnalis* и *Planorbarius corneus* имеются трипсиноподобные протеиназы, а также антитрипсиновые ингибиторы протеолиза и α2-макроглобулин (Chirkin and Dolmatova, 2017). Учитывая, что структуры протеолитических ферментов консервативны, была сформулирована задача предварительной оценки гомологии белков системы протеолиза у человека и легочных пресноводных моллюсков. В обращении к участникам Gordon Research Conference «Understanding Proteases and Their Roles as Regulators of Health and Disease» (3–8 июня 2018 года) указано, что протеазы играют разнообразную роль в регулировании биологии практически всех организмов. Фактически они составляют примерно 5% всех генов в любом данном геноме. Раньше протеазы были молекулярными единицами удаления «мусора», которые просто деградировали отработанные белки для поддержания общего гомеостаза. Однако детальные исследования продемонстрировали, что функции протеолитических ферментов намного сложнее, поскольку они играют ключевые роли в качестве регуляторов важных клеточных процессов, таких как клеточный сигналинг, деление клеток, их гибель и обмен веществ. Внеклеточные белки и некоторые белки поверхности клетки поглощаются эндоцитозом и деградируют в лизосомах. Эти органеллы содержат ряд кислых протеаз, включая катепсины B, H и D, а также многие другие гидролазы. Некоторые цитозольные белки деградируют в лизосомах после поглощения в аутофагических везикулах, которые сливаются с лизосомами. Учитывая не совпадение сроков деления клетки и ее митохондрий, некоторые исследователи выделяют понятие аутофагии митохондрий как тип запрограммированной гибели. Известна также шаперон-зависимая аутофагия, при которой происходит направленный транспорт частично денатурированных белков из цитоплазмы сквозь мембрану лизосомы в её полость. При разрушении мембраны лизосом происходит автолиз клетки. Однако во всех тканях живых организмов большинство внутриклеточных белков деградируют с помощью убиквитин (ubiquitin,

Ub) – протеасомного пути (UPP). Этот регулируемый тип протеолиза играет наиболее важную роль в сигналинге клетки (McCarthy et al., 2017). Уже бактерии используют протеолиз для разрушения или активации регуляторных белков и для получения сигналов для временного и пространственного контроля процессов морфологического развития (Lai and Caplan, 2011). В эпителиальных и нервных клетках за счет кальпаинов и убиквитин-зависимых протеасом формируются компартменты с определенным составом белков, в том числе ферментов, для выполнения специальных клеточных функций (Burger and Seth, 2004). Внутримембранный протеолиз, осуществляемый мембраносвязанными протеазами и комплексами γ-секретазы, является фундаментальным процессом трансдукции сигналов от рецепторов цитокинов, факторов роста, рецепторов смерти (фактора некроза опухоли типа I, FasR и TRAIL-R1/2). Протеолиз позволяет высвобождать растворимые рецепторные эктодомены и генерировать фрагменты цитоплазматических доменов рецептора. Этот процесс способствует образованию внутриклеточных посредников и формированию расходящихся внутриклеточных сигнальных путей (Lecker et al., 2006; Salvesen et al., 2016). Регулируемый протеолиз (сайт-специфический протеолиз) является важным биологическим механизмом регуляции экспрессии генов, клеточной сигнализации, развития и гибели клеток. Центральный компонент этой системы – протеасома может быть определена и как «протеаза клеточной смерти» (Vogel and Kristie, 2000). Деградация белков по АТФ-зависимому убиквитин-протеасомному пути включает в себя два этапа – ковалентное присоединение к субстрату полиубиквитиновой цепочки и деградацию помеченного белка 26S-протеасомой. Реакция убиквитирования осуществляется каскадом ферментов: E1 (Ubiquitin activating enzyme) – E2 (Ubiquitin conjugating enzyme) – E3 (Ubiquitin ligase). В протеасомах млекопитающих каталитически активными являются β1-, β2- и β5-субъединицы, причём все эти субъединицы обладают разными ферментативными активностями (каспазоподобной, трипсиноподобной и химотрипсиноподобной, соответственно). Протеасомное разрушение белков является быстрым процессом, который обеспечивает переключение важнейших механизмов экспрессии генов, клеточного цикла и апоптоза путем разрушения регуляторных белков p19, p21, p27, p53 и других, ряда транскрипционных факторов (Borquez and Gonzalez-Billault, 2011; Konovalova et al., 2014).

Целью настоящей работы было обоснование целесообразности получения протеолитических ферментов из тканей легочных пресноводных моллюсков для их использования в практических целях.

Материал и методика

На первом этапе работы был проведен сравнительный анализ биохимических показателей тканей легочных пресноводных моллюсков, плазмы крови человека и печени крыс линии Вистар. В плазме крови и гемолимфе определяли содержание общего белка, глюкозы, общего холестерола (ОХС), холестерола липопротеинов высокой плотности (ХС ЛПВП), триглицеридов (ТГ), мочевой кислоты и активность гамма-глутамилтрансферазы (ГГТ) с помощью наборов НТПК «Анализ Х» (Беларусь).

В тканях печени крысы и гепатопанкреаса моллюсков определяли содержание белка (Lowry et al., 1951), ДНК, РНК (Blober and Potter, 1968; Данченко, 2013) и гликогена (Krisman, 1962; Данченко и Чиркин, 2010). Средняя величина каждого показателя определялась в 8–10 повторностях, и сравнительный анализ производился методом параметрической статистики с использованием t-критерия Стьюдента.

На втором этапе работы поиск и отбор нуклеотидных последовательностей, кодирующих белки человека, осуществлялся на сервере https://www.ensembl.org; поиск гомологичных последовательностей для моллюсков осуществлялся на сервере https://www.ncbi.nlm.nih.gov при помощи ресурса BLAST; описание белков для человека было взято с ресурса https://www.uniprot.org; парное выравнивание и сравнение последовательностей человека и моллюсков выполнено в программе MEGA5.2; построение 3D-структур ферментов для моллюсков выполнялось на сервере https://swissmodel.expasy.org по шаблону 3D-структуры ферментов человека, найденных в банке данных трёхмерных структур белков и нуклеиновых кислот http://www.rcsb.org. В работе использован следующий алгоритм: поиск нуклеотидной последовательности → построение аминокислотных последовательностей сравниваемых белков → их парное выравнивание и оценка степени гомологии первичных структур – построение 3D-структур по шаблону структуры сравниваемого белка человека → оценка третичной структуры по архитектуре молекул и их доменной организации. Исследование мотивов и строения активных центров ферментов не входило в задачи данной работы. Ранее при сравнении результатов 2-х докингов между собой было выяснено, что 6 аминокислот трипсина у *Homo sapiens* и у *Biomphalaria glabrata* связываются с этионином в близких локусах молекул фермента. Аминокислоты для *Homo sapiens*: Asp 189, Ser 190, Gln 192, Ser 195, Val 213, Cys 220, аминокислоты для *Biomphalaria glabrata*: Asp 224, Ser 225, Gln 227, Ser 230, Val 248, Cys 254. Гомология молекул трипсина человека и моллюска составила 26,6% (Chirkin et al., 2017).

Результаты и дискуссия

В таблице 1 представлены биохимические показатели плазмы крови человека и гемолимфы моллюсков, а также ткани печени крысы и гепатопанкреаса моллюсков.

Таблица 1 Сравнение биохимических показателей плазмы крови и печени млекопитающих с аналогичными показателями гемолимфы и гепатопанкреаса моллюсков

Table 1 Comparison of biochemical parameters of blood plasma and liver of mammals with similar indicators of hemolymph and hepatopancreas of mollusks

Показатель	**Млекопитающие**	***Lymnaea stagnalis***	***Planorbarius corneus***
Исследуемая жидкость	**Плазма (*Homo sapiens*)**	**Гемолимфа**	
Общий белок (г.л$^{-1}$)	74,4±2, 35	14,9±0,24**	36,3±1,62*,**
Мочевая к-та (мкмоль.л$^{-1}$)	301±12,6	30,4±0,76**	89,1±2,00*,**
Глюкоза (ммоль.л$^{-1}$)	4,99±0,37	0,54±0,04**	1,15±0,08*,**
ОХС (ммоль.л$^{-1}$)	5,19 ±0,52	0,49 ±0,01**	0,33 ±0,01*,**
ХС ЛПВП (ммоль.л$^{-1}$)	1,22 ±0,12	0,06 ±0,01**	0,11 ±0,01*,**
ТГ (ммоль.л$^{-1}$)	1,47 ±0,14	0,35 ±0,01**	0,23 ±0,01*,**
ГГТ (Ед.л$^{-1}$)	40,1 ±4,25	187 ±9,42**	178 ±7,70**
Исследуемая ткань	**Печень (*Rattus*)**	**Гепатопанкреас**	
Общий белок (мг.г$^{-1}$)	225 ±9,82	203 ±4,30	205 ±7,50
ДНК (мг.г$^{-1}$)	3,12 ±0,42	2,44 ±0,08	2,73 ±0,29
РНК (мг.г$^{-1}$)	8,53 ±0,82	7,46 ±0,28	6,79 ±0,58
Гликоген (мг.г$^{-1}$)	42,5 ±3,10	27,0 ±0,36**	21,1 ±0,11*,**

Примечание: * P <0,05 при сравнении показателей *Planorbarius corneus* с *Lymnaea stagnalis*; ** P <0,05 при сравнении показателей *Homo sapiens* и *Rattus* с *Lymnaea stagnalis* и *Planorbarius corneus*

Установлено, что большинство биохимических показателей в плазме крови человека выше по сравнению с гемолимфой прудовиков и роговых катушек, вероятно, из-за незамкнутого кровообращения: общий белок в 4,99 и 2,05 раза, мочевая кислота в 9,90 и 3,38 раза, глюкоза в 9,24 и 4,34 раза, общий холестерол в 10,6 и 15,7 раза, холестерол липопротеинов высокой плотности в 20,3 и 12,2 раза, триглицериды в 4,20 и 6,39 раза, соответственно. Только один показатель – активность гамма-глутамилтрансферазы оказалась существенно выше в гемолимфе прудовиков и роговых катушек в 4,66 и 4,62 раза, по сравнению с активностью этого фермента в плазме крови человека. Вероятно, это результат большего контакта мембран паренхиматозных клеток с гемолимфой, омывающей эти клетки при отсутствии сосудистых стенок (Чиркин и др., 2018). Содержание белков, ДНК, РНК, гликогена в гепатопанкреасе легочных пресноводных моллюсков достаточно близко к уровню этих биополимеров в печени крысы. Результаты этих исследований позволили предположить, что ткани легочных пресноводных моллюсков могут служить исходным материалом для получения белков, в том числе ферментов, как это реализуется в промышленном масштабе при использовании тканей ряда морских гидробионтов.

Сравнительный биоинформатический анализ протеолитических ферментов человека и моллюска *Biomphalaria glabrata*, являющегося родственным организмом с *Planorbarius corneus*, представлен в таблице 2.

Таблица 2 Оценка гомологии первичных и третичных структур молекул внутриклеточных протеолитических ферментов человека и моллюска

Table 2 Evaluation of the primary and tertiary structures homology of the of the humans and mollusks molecules of intracellular proteolytic enzymes

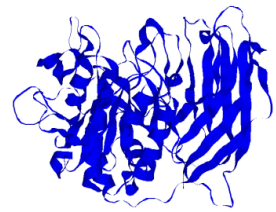
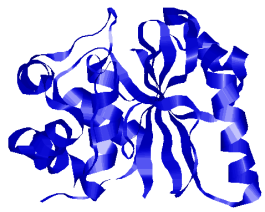
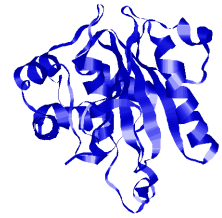
Фермент	Гомология	
	Последовательности аминокислот (%)	Третичные структуры, однотипные участки
Prolyl oligopeptidase (КФ 3.4.21.26)	66	альфа-спирализованный и бета-складчатый домены
ATP-dependent Clp protease proteolytic subunit (КФ 3.4.21.92)	68	признаки структуры типа семилучевой звезды
Furin - КФ 3.4.21.75	69	альфа-спирализованный и бета-складчатый домены
Signal Peptide Peptidase (КФ 3.4.11.2)	66	повторяющиеся 4 типа альфа-спиральных структур
Leucyl aminopeptidases (cytosol aminopeptidase, КФ 3.4.11.1)	66	аналогичная гексамерная структура
Thimet oligopeptidases (КФ 3.4.24.15)	66	преобладание альфа-спирализованных участков
Ubiquitin conjugating factor E4 B-like (КФ 6.3.2.19)	72	однотипные альфа-спирализованные фрагменты
Ubiquitin conjugating factor E2 W-like	75	одинаково чередуются альфа-спиральные, бета-структурные и неупорядочные фрагменты
Ubiquitin carboxyl-terminal hydrolase L5	72	сходные каталитические домены
Ubiquitin-like modifier-activating enzyme 5	76	построение домена, состоящего из альфа-спиральных и бета структурных участков
Amidophosphoribosyl-transferase (phosphoribosyl pyrophosphate amidotransferase) (КФ 2.4.2.14)	64	**ч**етыре однотипных домена
Adenylosuccinate lyase (adenylosuccinase, КФ 4.3.2.2)	68	мономеры имеют по три идентичных домена

Первые шесть ферментов, представленных в таблице 2, являются ферментами, которые можно отнести к группе строго нерегулируемых протеолитических ферментов: 1 – Prolyl oligopeptidase представляет собой цитозольную сериновую пептидазу, которая расщепляет пептидную связь С-концевого пролина; 2 – ATP-dependent Clp protease proteolytic subunit входит в состав высокоактивной сериновой эндопептидазы Clp; 3 – Furin является сериновой протеазой клеток животных, расположенной в аппарате Гольджи и напоминает бактериальный протеолитический фермент субтилизин; 4 – Signal Peptide Peptidase – это внутримембранная аспартил-протеаза, расщепляющая остаточные сигнальные пептиды, оставшиеся в мембране после действием

сигнальной пептидазы; 5 – Leucyl aminopeptidases (cytosol aminopeptidase) относится к ферментам, которые преимущественно катализируют гидролиз лейциновых остатков на N-конце пептидов и белков; 6 – Thimet oligopeptidases, известные как TOPs, являются металлопептидазами и у животных они участвуют в деградации пептидов – брадикинина, нейротензина, ангиотензина I и пептида Aβ. Первичные структуры этих ферментов человека и моллюска *Biomphalaria glabrata* после парного выравнивания демонстрируют гомологию в интервале 66–69%. 3D-структуры этих ферментов у человека и моллюска близки по архитектуре и наличию доменных структур. Следующие четыре фермента относятся к управляемому убиквитин-протеасомному пути протеолиза; 7 – Ubiquitin conjugating factor E4 B-like, конъюгирующие убиквитин ферменты, также известные как ферменты E2; 8 – Ubiquitin conjugating factor E2 W-like; 9 – Ubiquitin carboxyl-terminal hydrolase L5; 10) Ubiquitin-like modifier-activating enzyme 5.

Нуклеотидные последовательности этих ферментов для моллюсков оказались неполными, но, тем не менее, проведенный анализ фрагментов первичных структур показал более высокую степень гомологии при парном выравнивании 72–76%, а фрагменты 3D-структур позволили выявит сходство третичных структур на уровне общей архитектуры молекул и их доменного строения. Для сравнения биоинформатическому анализу были подвергнуты два фермента пуринового обмена, важного для синтеза нуклеотидов: 11 – Amidophosphoribosyl-transferase (phosphoribosyl pyrophosphate amidotransferase), который содержит аналог каталитической триады цистеиновыз протеаз, и 12 – Adenylosuccinate lyase (adenylosuccinase), который превращает аденилосукцинат в AMP и фумарат. Гомология этих ферментов у человека и моллюска такая же, как и у ферментов нерегулируемого протеолиза. В таблице 3 в качестве примера приведены 3D-структуры нерегулируемого и регулируемого ферментов протеолиза человека и моллюска *Biomphalaria glabrata*.

Таблица 3 3D-структуры клеточных протеолитических ферментов человека и моллюска
Table 3 3D structures of cellular proteolytic enzymes of the human and molluscs

Фермент	3D-структура фермента человека	3D-структура фермента моллюска
Furin		
Ubiquitin carboxyl-terminal hydrolase L5 (каталитический домен)		

Предсказание структуры белков *in silico* является эффективным способом получения моделей белков, структура которых еще не определена экспериментально. В данной статье использован метод гомологичного моделирования, который опирается на существующую "шаблонную" структуру, сходную по аминокислотной последовательности с моделируемым белком. Этот успешный метод получил достаточно широкое распространение при сравнительном анализе белков живых систем методами биоинформатики (Xiang, 2006; Zhang, 2008). Представленные данные показывают, что высокая степень гомологии ферментов протеолиза у человека и моллюска сопряжена с формированием близких третичных структур белков. Это свидетельствует в пользу предположения, что сравниваемые протеолитические ферменты человека и моллюсков выполняют однотипные функции. Можно также предположить, что более высокая степень гомологии первичных структур у протеолитических ферментов убиквитин-протеасомного пути может быть связана с более высокой степенью консервативности этого регулируемого пути внутриклеточного протеолиза.

Практическая значимость полученных данных о высокой степени гомологии протеолитических ферментов у человека и легочных пресноводных моллюсков обосновывает формирование аквакультуры моллюсков с целью получения из их тканей белковых ферментативных препаратов протеолитического действия в рамках задач биофармации, для совершенствования косметических средств и применения в пищевой промышленности.

Выводы

Содержание белков, ДНК, РНК, гликогена в гепатопанкреасе легочных пресноводных моллюсков близко к уровню этих биополимеров в печени млекопитающих.

Гомология ферментов нерегулируемого протеолиза у человека и легочных пресноводных моллюсков находится в пределах 66–69%, а убиквитин-протеасомного пути – 72–76%. Практическое значение высокой степени гомологии протеолитических ферментов у людей и пресноводных легочных моллюсков обосновывает возможность получения из тканей последних белковых ферментативных препаратов протеолитического действия для практического применения.

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BIOCHEMICAL COMPOSITION *POLYMNIA SONCHIFOLIA* POEPP. AT THE DOUBLE CUTTING

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Cultivation of *Polymnia sonchifolia* Poepp. in Ukraine does not guarantee a stable crop of root tubers, therefore, we are studied the possibility of using aboveground biomass of plants. Both tubers and extract of leaves reveal hypoglycemic effect (Kim et al., 2010). Plants of *P. sonchifolia* cv. Yudinka were used in this study. Leaves mass was cut twice on season to obtain maximal quantitative of plant raw material. The absolutely dry matter was determined by drying of plant raw material to constant mass; total content of sugars – by Bertrand method; concentration of ascorbic acid (AA) – by a 2.6-dichlorophenol-indophenol method; concentration of total carotene – using extraction with rubber solvent (petrol); the total lipid content was extracted with petroleum ether in Soxhlet extractor. The productivity of plant raw material at the double cutting was 22 $t.ha^{-1}$, whereas at the cutting once on season was obtained 12 $t.ha^{-1}$. Cutting promotes rejuvenation of plants and stimulates synthesis of biologically active compounds. In this case was observed increasing of yield of plants, content of dry matter and basic biochemical compounds of plants. Potential yield of content of dry mass of two cuttings were 3.08 $t.ha^{-1}$, while one cutting 2.16 $t.ha^{-1}$. Total content of sugars was higher in the young organs of shoot. Content of AA was higher in young leaves in the August than in October – 302.68 and 201.37 mg% respectively. The content of carotene decreased from 4.01 to 0.62 mg% during summer-autumn growing period. Quantity of lipids wasn't significant (1.25–3.60%), but the most content was found in the leaves.

Keywords: *Polymnia sonchifolia*, dry matter, total content of sugars, ascorbic acid, carotene

Introduction

Modern methods of glycaemia phytotherapy provide the use of plants with sugar-reducing, cytoprotective and immunostimulating properties (Kikhtyak, 1998; Jia et al., 2003). *Polymnia sonchifolia* Poepp., that originates from South America and belongs to Asteraceae family, is characterized the above properties. Numerous scientific investigations proved the hypoglycemic action of *P. sonchifolia* (Khokhla et al., 2015, 2016). Traditionally, the root tubers were known as medicinal plant raw material but it's also proved the effectiveness of use of

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leaves extracts for the decreasing of sugar level in the blood (Aybar and Riera, 2001; Kim et al., 2010; Mishchenko et al., 2012). *P. sonchifolia* leafe tea is used as medicinal remedy by locals in Latin America (Manrique et al., 2005). Aboveground part of this plant included to content of hypoglycemic phytotea that was produced in Ukraine (Pat. No 86475, 2013). Moreover, as plant with high physiological activity *P. sonchifolia* has valuable content of biogenic elements, flavonoids, phytosterols, polysaccharides, polyunsaturated fatty acids etc. (Lachman et al., 2003; Dashchenko et al., 2016). It is known, that leaves of these plants content high quantitative of phenolic compounds that determines the antioxidant properties of *P. sonchifolia* and makes possible using of plant raw material in human ration as protective and preventive remedies at the chronic illness such as atherosclerosis (Valentova and Ulrichova, 2003). From *P. sonchifolia* it is possible to make products for special medical purposes: extracts and powders (Motuzka, 2017).

At the place of origin the high of plants is 1.5–2.5 m (Manrique et al., 2005). Although the most favorable is subtropical climate, plants able to grow in the temperate climate. Cultivation of *P. sonchifolia* in conditions of Ukraine possible with cutting at the greenhouse conditions (Mishchenko et al., 2018). Obtaining high yield of tubers in the drought conditions can be problematic but aboveground part of plant is a valuable raw also.

There is a need of study of productive plant potential and their biochemical composition in the perspective of industrial use.

Material and methodology

Material

Plant material of *P. sonchifolia* cv. Yudinka was used in this study.

Experiment organization

Study of plants were conducted on experimental collection of M.M. Gryshko National Botanical Garden of the NAS (NBG) of Ukraine (Right Bank Forest-Steppe). The soil is dark-grey, podzolized, eroded. Planting scheme was 50 × 50 sm. First yield of biomass was conducted at 3th August with cutting of apex of stem on the heigh of 20 sm from the surface of soil; second yield – 1st October. Analyses were performed at the biochemical laboratory of Cultural Flora department of NBG.

Methodology of biochemical substances content

The absolutely dry matter determined by drying of plant raw material for constant mass at the 105 °C according to Yermakov et al. (1972). Total content of sugars was investigated by Bertrand method in water extracts; concentration of ascorbic acid (AA) of the acid extracts was determined by a 2.6-dichlorophenol-indophenol method that based on the reduction properties of AA. Both analyses carried out according to Krishchenko (1983). Concentration of total carotene determined according to Pleshkov (1985) using extraction with rubber solvent (petrol). The procedure of determination of total lipid level was performed using Soxhlet

extractor. Plant raw material was extracted with petroleum ether (boiling temperature is 40–60 °C) according to Yermakov et al. (1972).

Results and discussion

P. sonchifolia can grow without the formation of root tubers under conditions of industrial cultivation in the forest-steppe zone of Ukraine. We did an experiment in which the plants first used apical leaves, and then all biomass: leaves, stems and rhizomes. Since in August it is possible to use only apical leaves, the analysis of stems and rhizomes was not performed at this time.

The biochemical composition of *P. sonchifolia* in literary notes is mainly given at the time of harvesting of tubers. As a rule, it is investigated tubers. It is given that in yacon roots contains 69–93% water (King, 1987; Grau and Rea, 1997; Valentova and Ulrichova, 2003). According to our data, most of the water found in young leaves and stems (86 and 88% respectively) (Table 1). Most authors report the sugar content in tubers, which range from 12.5 (Valentova and Ulrichova, 2003) to 29% of total dry weigth (Asami et al., 1989). According to our data, the content of sugars increases in sequence: leaves < stems < rhizomes (Table 2). This is also confirm by studies Fukai et al. (1993). The content of ascorbic acid and carotene was rarely given in the literary notes by researchers. Grau and Rea (1997) report data on ascorbic acid and carotene content in the roots (13 and 0.08 $mg.g^{-1}$ respectively). Valentova and Ulrichova (2003) found carotene in tubers of 0.02 $mg.100\ g^{-1}$. The content of lipids in tubers significantly different depending on the variety (191–311 $mg.100\ g^{-1}$) according to Manrique et al. (2005). Valentova and Ulrichova (2003) reported that most lipids are in leaves (4.20%) less than in the stems (1.98%) and tubers (0.10–0.30%).

In our experiments, cutting of *P. sonchifolia* leaves mass was used twice on season to obtain maximal quantitative of plant raw material. After deleting of shoot leading of experimental plants on the main stems were grown lateral shoots, that formed new leaves mass. Second time the yield was collected 1st October. The comparative analyze conducted with plants which didn't cut at all (control). It was found that productivity of plant raw material at the double cutting was significantly high and was 22 $t.ha^{-1}$, whereas at the cutting once on year was obtained 12 $t.ha^{-1}$.

Qualitative content of plant raw material was determined by biochemical composition. It was noticed that content of dry mass accumulated more in all organs of plants that were cut once. This fact possible to explain, that the experimental plant above-ground organs were younger, than control. In the leaves of first and second cutting content of dry matter was approximately 14.00%, in the control plants – 18.03 % (Table 1). Evidence, double cutting not stimulates the accumulation of dry mass in under-ground part of investigated plants because in the end of vegetation season in rhizomes it was 3% less. Potential yield of content of dry mass of two cutting were 3.08 $t.ha^{-1}$, whereas, after one cutting 2.16 $t.ha^{-1}$ only.

Table 1 The content of dry matter in plant raw material of *Polymnia sonchifolia* Poepp. (%)

Part of plant	First cutting*		Second cutting**		Non-cutting plants**	
	M ±*m*	*V* (%)	*M* ±*m*	*V* (%)	*M* ±*m*	*V* (%)
Leaves	13.88 ±0.43	3.08	14.40 ±0.30	2.10	18.03 ±0.73	4.07
Stems			11.68 ±0.67	5.76	14.48 ±0.30	2.07
Rhizomes			15.89 ±1.17	7.37	19.05 ±0.94	4.94

Notes: *M* – arithmetic mean; *m* – standard error of the mean; *V* (%) – variation coefficient; * Cut date – 3th August; ** Cut date – 1th October

Total content of sugars was higher in young organs of shoot, that formed during two months. In the leave of experimental plants after second cutting was found 2% more sugars than in control plants (Table 2). In the stems and rhizomes level of total content of sugars increased. This trend saves in both – plants that were cut twice and plants without cutting. Root system of experimental and control plants, despite on organs of above-ground mass, had the same level of total content of sugars.

Table 2 The total content of sugar of plant raw material of *Polymnia sonchifolia* Poepp. (%)

Part of plant	First cutting*		Second cutting**		Non-cutting plants**	
	M ±*m*	*V* (%)	*M* ±*m*	*V* (%)	*M* ±*m*	*V* (%)
Leaves	9.34 ±0.42	6.37	12.43 ±0.51	5.84	10.31 ±0.73	10.13
Stems			29.33 ±0.29	1.41	24.83 ±2.12	12.08
Rhizomes			48.51 ±1.24	3.62	48.73 ±0.34	0.97

Notes: *M* – arithmetic mean; *m* – standard error of the mean; *V* (%) – variation coefficient; * Cut date – 3th August; ** Cut date – 1th October

Content of ascorbic acid, the same as total content of sugars, had higher signs in the tissues of younger organs. The most difference found in plant raw material that collected 3th August and in control plants during autumn period – 302.68 and 201.37 mg% respectively (Table 3). Evidence, that collecting twice can give plant raw material with higher quality. Comparing under-ground and above-ground organs of plants showed that leaves prevailed by ascorbic acid content.

Table 3 Content of ascorbic acid in plant raw material of *Polymnia sonchifolia* Poepp. (mg%)

Part of plant	First cutting*		Second cutting**		Non-cutting plants**	
	M ±*m*	*V* (%)	*M* ±*m*	*V* (%)	*M* ±*m*	*V* (%)
Leaves	302.68 ±3.06	1.43	263.52 ±2.70	1.45	201.37 ±2.16	1.52
Stems			51.00 ±2.54	7.05	44.94 ±2.05	6.45
Rhizomes			46.14 ±1.87	3.73	32.72 ±2.57	11.10

Notes: *M* – arithmetic mean; *m* – standard error of the mean; *V* (%) – variation coefficient; * Cut date – 3th August; ** Cut date – 1th October

It is found less content of carotene accumulated in the tissues of plants of *P. sonchifolia*. It should be noticed that in autumn period content of that in the leaves significantly decreased (from 4.01 to 0.62 mg%) if compare with value of 3th August (Table 4). In young leaves, that formed during August-September content of carotenoids also was less (0.24 mg%). Evaluation of plant raw material by this parameter showed that fraction of summer collecting is especially qualitative.

Table 4 Content of carotene in plant raw material of *Polymnia sonchifolia* Poepp. (mg%)

Part of plant	First cutting*		Second cutting**		Non-cutting plants**	
	M ±*m*	*V* (%)	*M* ±*m*	*V* (%)	*M* ±*m*	*V* (%)
Leaves	4.01 ±0.01	0.45	0.24 ±0.02	10.74	0.62 ±0.03	7.08
Stems			0.26 ±0.02	7.95	0.25 ±0.02	10.92
Rhizomes			0.14 ±0.01	12.44	0.29 ±0.02	7.29

Notes: M – arithmetic mean; m – standard error of the mean; V,% – variation coefficient; * Cut date – 3th August; ** Cut date – 1th October

Content of compounds of lipophilic fraction in the leaves of *P. sonchifolia* also was higher in plants that were cut twice in season. Quantity of lipids wasn't significant (1.25–3.60 %), but the most content was found in the leaves (Table 5).

Table 5 Content of lipids in plant raw material of *Polymnia sonchifolia* Poepp. (%)

Part of plant	First cutting*		Second cutting**		Non-cutting plants**	
	M ±*m*	*V* (%)	*M* ±*m*	*V* (%)	*M* ±*m*	*V* (%)
Leaves	3.60 ±0.14	3.75	2.63 ±0.12	4.69	1.99 ±0.02	0.86
Stems			1.68 ±0.03	1.85	1.25 ±0.02	1.37
Rhizomes			1.63 ±0.03	2.09	1.07 ±0.08	7.63

Notes: M – arithmetic mean; m – standard error of the mean; V,% – variation coefficient; * Cut date – 3th August; ** Cut date – 1th October

Conclusions

Cultivation of *P. sonchifolia* in conditions of Forest-Steppe of Ukraine for use as plant raw material for remedy and preventive products can be recommended to mowing it twice on season: in the beginning of August and in October. Cutting promotes rejuvenation of plants and stimulates synthesis of biologically active compounds. In this case was observed increasing of yield, content of dry matter and basic biochemical compounds of plants.

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MORPHOMETRIC CHARACTERISTIC OF WILD-GROWING GENOTYPES OF ELDERBERRY (*SAMBUCUS NIGRA* L.) WITH DARK AND GREEN FRUITS

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In the year 2018 has assigned retrieval and selection of genomes from natural populations Elderberry (*Sambucus nigra* L.) in Slovakia and Ukraine. As select criterion was used to period of maturation, measurement and form of fruits, amount and weight of drupes and other marks on habitus. For assessment of agricultural value these select genomes were qualified fruits and drupes. In the experiment 20 genomes were qualified. On basis of morphometric analysis in collection was determined the average weight of fresh infructescences in the interval 4.75–101.90 g. On basis of production analysis in collection genomes were determined the average weight of drupes in infructescences in fresh condition in the range of 2.64–93.18 g, the average amount of drupes in infructescences in the range of 37.2–839.4 pieces. In the research of genomes were assessed significant differences also in the form of fruits and in the colour of drupes. From the assessment collection of the genomes we selected some genomes with very positive production characteristics.

Keywords: *Sambucus nigra*, natural population, morphological characteristic, infructescence, drupe

Úvod

Sambucus nigra L. rastie v Európe, západnej a strednej Ázii a severnej Afrike. Už v dávnych časoch bola baza čierna obľúbená ako živá domáca lekáreň. Existujú záznamy z 5. a 4. storočia pred n. l., kde je baza opísaná ako prostriedok na liečenie mnohých ochorení, pričom sa využívajú všetky časti stromu t. j. kôra, čerstvé a sušené listy, čerstvé a sušené kvety, čerstvé a sušené plody a sušené korene (Hejný, 2001). Plody sú zaujímavé vysokým obsahom sambuciózy (8 %), organických kyselín (jablčná, citrónová, chinová, chlorogénová, askorbová, valérová) (Grieve, 1931; Hejný, 2001), antokyánových farbív (Wu et al., 2004; Jordheim et al., 2007), vitamínu C (Kaack and Austed, 1998), flavonoidov a iných polyfenolických zlúčenín (Wu et al., 2004; Thole et al., 2006). Antokyaníny sú v bazových plodoch obsiahnuté viac ako v 1 % suchej hmotnosti. Boli identifikované štyri základné antokyaníny: cyanidín-3-galaktozid (idaeín)

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alebo cyanidín-3,5-diglukozid (Cy-3,5-dG), cyanidín-3-sambubiozid (Cy-3-Sa), cyanidín-3-sambubiozid-5-glukozid (Cy-3-Sa-5-G), cyanidín-3-glukozid (Cy-3-G) (Šinková et al., 1995; Thole et al., 2006; Drábek, 2007). Množstvo týchto pigmentov sa pohybuje v rozmedzí 2 až 10 $mg.g^{-1}$ čerstvých plodov (Bridle and Garcia Viguera, 1996). Polyfenolický profil ovocných štiav, vrátane bazovej šťavy môže byť považovaný za akýsi komplex obsahujúci veľké množstvo antokyánov (Schwarz et al., 2001; Sanchez-Moreno et al., 2003; Bermúdez-Soto and Tomás-Barberán, 2004; Proestos et al., 2005), fenolov, flavónov a ďalších významných biologicky aktívnych komponentov (Oszmiański and Lama-Zarawska, 1995; Moszczyński, 1996; Abuja et al., 1998; Moszczyński, 1996; Obidowska, 1998; Oszmiański and et al., 1998; Obidowska, 1998; Taran et al., 2017). Všetky tieto chemické zlúčeniny vysoko korelujú s ich antioxidačnou kapacitou (Bermúdez-Soto and Tomás-Barberán, 2004; Lozova et al., 2017). Vrchotová et al. (2007) determinovali obsah rutínu a celkového kvercetínu v troch endogénnych druhoch *Sambucus* L. (*S. nigra* L., *S. racemosa* L., *S. ebulus* L.) rastúcich v strednej Európe (Atkinson and Atkinson, 2002). Čerstvé zrelé plody obsahujú tyrozín (Farçasanu et al., 2006). Bolo študované bioaktívne zloženie (flavonoidy a vitamín C) a stabilita farieb, ako aj antioxidačná kapacita citrónového nápoja (*Citrus limon* (L.) Burm. f.) s 5 % koncentrátom z plodov *Sambucus nigra* počas 56 dní skladovania, kde sa preukázala ochranná úloha antokyanínov pri stabilite kyseliny askorbovej, prítomný obsah dôležitých fytozlúčenín, antioxidačná kapacita a stálosť farbív v podmienkach *in vitro* (González-Molina et al., 2012). Je známe, že plody majú tiež antivírusovú, imunostimulačnú a antikarcinogénnu aktivitu (Mumcuoglu et al., 2010). Z uvedeného dôvodu sú plody *Sambucus nigra* hospodársky využívané predovšetkým v potravinárstve, farmácií a kozmetike. Šťavou z plodov sa prifarbujú červené vína alebo sa z nej vyrába bazové víno, používajú sa aj na prifarbovanie sirupov, nátierok, na prípravu a farbenie džemov, rôsolov, marmelád; cukroviniek a výrobkov mliekarenského priemyslu, cestovín, pekárenských a cukrárenských výrobkov. Tieto výrobky sú zároveň obohatené o cenné látky, nachádzajúce sa v jej plodoch (Börngen, 1990; Drábek et al., 2007). Z uvedeného dôvodu sa v mnohých európskych krajinách začína rozširovať pestovanie bazy čiernej aj v monokultúre (Ważbińska and Puczel, 2002) ako aj v krajinotvorbe (Ważbińska, 2000; Grygorieva et al., 2015). Často sa vyskytuje ako súčasť záhrad a verejných parkov.

Materiál a metodika

Biologický materiál

Cieľom práce sa stal prieskum a výber genotypov z prírodných populácií bazy čiernej (*Sambucus nigra* L.) s hodnotením variability ich produkčných znakov súplodí pre využitie v agropotravinárstve. Experimentálne sme zhodnotili 20 genotypov v podmienkach Slovenska (Sn-S-01, Sn-S-04, Sn-S-05, Sn-S-07, Sn-S-10, Sn-S-11, Sn-S-12, Sn-S-16, Sn-S-19, Sn-S-21, Sn-S-23, Sn-S-25, Sn-S-26) a Ukrajiny (Sn-U-31, Sn-U-33, Sn-U-34, Sn-U-41, Sn-U-48, Sn-U-50, Sn-U-51) v roku 2018. Súplodia boli zberané zo stromov alebo krov so stopkami; fotodokumentácia pochádza z exteriéru aj interiéru a ostatné hospodárske znaky sa hodnotili v laboratóriu na Inštitúte ochrany biodiverzity a biologickej bezpečnosti v Nitre. Vzorky z morfometrických analýz boli označené ako SN (*Sambucus nigra*) s priradením čísla genotypu.

Morfometrická analýza

Nasledujúce znaky sú merané pomocou morfometrických analýz:

a) celková hmotnosť zrelých súplodí so stopkou v g, *n* = 5 – 15 merané na analytických váhach (Kern PLS 360-3, Germany);
b) hmotnosť kôstkovičiek bez strapiny v g, *n* = 5 – 15 merané na analytických váhach (BOSCH SAE 200, Germany);
c) hmotnosť strapiny v g, *n* = 5 – 15 merané na analytických váhach (BOSCH SAE 200, Germany);
d) priemer kôstkovičiek v g, *n* = 50 merané posuvným meradlom s presnosťou na 0,01 mm;
e) celkový počet kôstkovičiek a počet nezrelých kôstkovičiek v jednom súplodí;
f) kompaktnosť a jednotnosť dozrievania plodov v každom genotype;
g) farba strapiny súplodí.

Výsledky a diskusia

Z hospodárskeho hľadiska sú pri baze čiernej najviac využívané súplodia ako zdroj biologicky aktívnych látok a prírodných farbív pre farmaceutické, potravinárske a kozmetické využitie. Z uvedeného dôvodu sme sa orientovali na hodnotenie niektorých znakov rastlinných častí.

Hmotnosť súplodí (g)

Pri hodnotení hmotnosti súplodí sme určili rozsah daného znaku od 4,75 (Sn-U-31) do 101, 90 g (Sn-U-51) s hodnotami variačných koeficientov v intervale od 12,88 (Sn-S-01) až do 68,43 % (Sn-U-50). Dokumentujú nízky až vysoký stupeň variability hodnoteného znaku medzi jednotlivými genotypmi, aj v rámci samotných genotypov (Tabuľka 1). Genotypy s vysokou hmotnosťou súkvetia sa vyznačovali aj vysokou hmotnosťou súplodia (Horčinová Sedláčková et al., 2018; Brindza et al., 2007). Takéto genotypy sú vhodné pre potravinársky, farmaceutický priemysel a pre lekárske účely. Uvedený výsledok dokumentuje, že aj v prírodných populáciách je možné vyselektovať genotypy vyznačujúce sa súplodím o požadovanej hmotnosti. Waźbińska and Puczel (2002) testovaním štyroch dánskych odrôd (Alleso, Korsør, Sampo a Samyl) a voľne rastúcich genotypov z prírodných populácií Poľska určili za trojročné obdobie štúdia priemernú hmotnosť súplodí v rozsahu od 29,9 do 67,4 g, Porpaczy and Laszlo (1984) detekovali súplodia s hmotnosťou 32,1 až 186,2 g a Kaack (1997) 51 – 112 g. Z porovnania údajov našich experimentov s uvedenými autormi sme určili len určitú zhodu. Niektorí autori určili aj vyššiu priemernú hmotnosť súplodí.

Hmotnosť kôstkovičiek (g)

V hodnotenej kolekcii voľne rastúcich genotypov bazy čiernej sme určili priemernú celkovú hmotnosť kôstkovičiek v čerstvom stave v rozsahu od 2,64 (Sn-U-31) do 93,18 g (Sn-U-51). Hodnoty variačných koeficientov pre uvedený znak sme určili v rozsahu od 11,13 (Sn-U-33) do 84,24 % (Sn-U-50). Hodnoty dokumentujú nízky až vysoký stupeň vnútrodruhovej variability daného znaku (Tabuľka 1). Waźbińska and Puczel (2002) pri meraniach štyroch dánskych odrôd a voľne rastúcich genotypov z prírodných populácií Poľska určili hmotnosť

100 kôstkovičiek (33 g) práve pri odrodách Sampo a Samyl. Kaack (1989) determinoval hmotnosť pri 100 kôstkovičkách kultivarov v intervale od 15 do 31 g, zatiaľ čo Porpaczy and Laszlo (1984) zaznamenali pri meraní tohto znaku značné kolísanie od 9 do 45 g. Vo všeobecnosti platí, že hmotnosť plodov je vyššia pri šľachtených odrodách v porovnaní s voľne rastúcimi formami. V hodnotenej kolekcii sme detekovali genotypy, pri ktorých sme určili hmotnosť kôstkovičiek nad 80 g, čo dokumentuje, že aj v prírode je možné vybrať produkčné typy bazy čiernej pre praktické využitie.

Tabuľka 1 Variabilita hmotnosti súplodí a hmotnosti kôstkovičiek v testovanej kolekcii vybraných genotypov *Sambucus nigra* L. z voľne rastúcich populácií na Slovensku a Ukrajine
Table 1 Variability of infructescences weight and drupes weight in the tested collection of selected genotypes *Sambucus nigra* L. from a wild-growing populations in Slovakia and Ukraine

	Hmotnosť súplodí (g)						Hmotnosť kôstkovičiek na strapci (g)				
	n	**min**	**max**	$\bar{x}$	***V***		***n***	**min**	**max**	$\bar{x}$	***V***
Genotypy s nízkymi hodnotami znaku											
Sn-U-31	5	2,25	8,92	4,75	57,90	**Sn-U-31**	5	1,40	4,52	2,64	48,79
Sn-S-19	5	4,63	8,75	7,38	22,95	**Sn-S-19**	5	4,08	7,46	5,98	22,77
Genotypy s vysokými hodnotami znaku											
Sn-S-16	5	63,21	152,37	90,26	39,71	**Sn-U-51**	5	77,06	124,07	93,18	20,60
Sn-U-51	5	82,79	136,54	101,90	21,24	**Sn-S-16**	5	61,64	137,38	85,10	35,95
Genotyp so zelenými plodmi											
Sn-U-50	15	0,81	15,19	4,96	68,43	**Sn-U-50**	15	0,37	11,84	3,32	84,24

Poznámky: *n* – počet meraní; min, max – minimálna a maximálna nameraná hodnota; $\bar{x}$ – aritmetický priemer; *V* – variačný koeficient (%)

Hmotnosť strapiny (g)

Strapina je časť súplodia, ktorá sa v podstate prakticky nevyužíva a pri technologickom spracovaní sa považuje za odpad. Pri hodnotení hmotnosti strapiny sme určili rozsah daného znaku od 1,13 (Sn-U-34) do 6,85 g (Sn-S-10, Sn-S-12). Hodnoty variačných koeficientov v rozmedzí 5,91 (Sn-S-01) – 82,92 % (Sn-S-23) dokumentujú nízky až vysoký stupeň variability hodnoteného znaku medzi jednotlivými genotypmi, ale aj v rámci samotných genotypov (Tabuľka 2).

Priemer kôstkovičiek (mm)

Pri hodnotení priemeru 50 kôstkovičiek z každého genotypu sme určili rozsah daného znaku od 4,97 (Sn-S-11) do 6,39 mm (Sn-S-26). Hodnoty variačných koeficientov v intervale 5,03 – 14,73 % dokumentujú nízky stupeň variability medzi jednotlivými genotypmi (Tabuľka 2).

V štúdií Waźbińska and Puczel (2002) udávajú hodnoty priemerov pri kôstkovičkách v rozsahu od 4,92 mm do 6,46 mm pri kultivaroch, zatiaľ čo pri divorastúcich genotypoch určili priemer kôstkovičiek 3,31 mm. Tutin et al. (1976) a Cinovskis (1997) určili priemer

kôstkovičiek v rozsahu 6 – 8 mm, Kaďarová (1986) udáva priemer jednotlivých kôstkovičiek v intervale 4,70 – 7,54 mm. Klymenko et al. (2018) určili priemer kôstkovičiek v rozsahu 2,95 – 4,36 mm.

Tieto odlišnosti pri tomto znaku môžu mať súvislosť s geografickou polohou, prípadne klimatickými, či pôdnymi podmienkami.

Tabuľka 2 Variabilita hmotnosti strapiny a priemeru kôstkovičiek v testovanej kolekcii vybraných genotypov *Sambucus nigra* L. z voľne rastúcich populácií na Slovensku a Ukrajine
Table 2 Variability of stem weight and drupes average in the tested collection of selected genotypes *Sambucus nigra* L. from a wild-growing populations in Slovakia and Ukraine

Hmotnosť strapiny (g)						Priemer kôstkovičiek (mm)					
	n	**min**	**max**	$\bar{x}$	***V***		***n***	**min**	**max**	$\bar{x}$	***V***
Genotypy s nízkymi hodnotami znaku											
Sn-U-34	5	0,99	1,41	1,13	17,18	**Sn-S-11**	50	4,39	5,57	4,97	9,11
Sn-S-19	5	0,55	2,01	1,48	38,49	**Sn-S-21**	50	3,85	5,28	4,67	9,02
Genotypy s vysokými hodnotami znaku											
Sn-S-10	5	3,87	13,54	6,85	55,64	**Sn-S-26**	50	5,43	7,20	6,39	6,54
Sn-S-12	5	5,30	10,50	6,85	32,28	**Sn-U-48**	50	5,61	7,16	6,23	7,45
Genotyp so zelenými plodmi											
Sn-U-50	15	0,44	3,35	1,64	41,07	**Sn-U-50**	50	5,14	6,47	5,77	5,03

Poznámky: *n* – počet meraní; min, max – minimálna a maximálna nameraná hodnota; $\bar{x}$ – aritmetický priemer; *V* – variačný koeficient (%)

Počet zrelých a nezrelých kôstkovičiek

V súplodiach bazy čiernej sa tvoria červené, neskôr tmavomodré až čierno-fialové, zriedka zelenkasté (Atkinson and Atkinson, 2002) guľovité, lesklé kôstkovičky s malým embryom v olejnatom endosperme. Sú približne 4–8 mm veľké (Hejný, 2001), väčšinou s tromi (5) semenami (Atkinson and Atkinson, 2002), ktoré sú 2 – 4 × 2 mm dlhé, vajcovito hrotité, stlačené, hnedej farby (Bolliger, 1999; Paganová, 2001) a plody obsahujú silne farbiacu červenú šťavu. Pri hodnotení celkového počtu kôstkovičiek na strapine sme určili rozsah daného znaku od 37,2 bobúľ (Sn-U-31) do 839,4 bobúľ (Sn-S-05). Hodnoty variačných koeficientov pre uvedený znak sme určili v rozsahu od 8,52 (Sn-S-04) do 73,08 % (Sn-U-50). Hodnoty variačných koeficientov dokumentujú nízky až vysoký stupeň variability hodnoteného znaku (Tabuľka 3).

Ważbińska and Puczel (2002) určili priemerný počet kôstkovičiek v súplodí v rozsahu 100–300, Porpaczy and Laszlo (1984) v rozsahu 207 – 925 a Mratinić and Fotirić (2007) v rozsahu 131 – 280. V porovnaní s uvedenými literárnymi údajmi sme detekovali v populácií voľne rastúcich genotypov bazy čiernej genotyp Sn-S-16, pri ktorom sme určili počet kôstkovičiek v rozsahu 569 – 1 203. Dosiahnuté výsledky z hodnotenia kolekcie korešpondujú s údajmi autorov.

Tabuľka 3 Variabilita celkového počtu kôstkovičiek a počtu nezrelých kôstkovičiek v testovanej kolekcii vybraných genotypov *Sambucus nigra* L. z voľne rastúcich populácií na Slovensku a Ukrajine

Table 3 Variability of the total amount of drupes and immature drupes in the tested collection of selected genotypes *Sambucus nigra* L. from a wild-growing populations in Slovakia and Ukraine

	Celkový počet kôstkovičiek						Počet nezrelých kôstkovičiek				
	n	**min**	**max**	$\bar{x}$	***V***		***n***	**min**	**max**	$\bar{x}$	***V***
Genotypy s vysokými hodnotami znaku											
Sn-S-25	5	627	1025	781,4	19,35	**Sn-S-10**	5	0	444	187,6	86,63
Sn-S-05	5	455	1199	839,4	39,44	**Sn-S-07**	5	115	424	213,6	57,31
Genotypy s nízkymi hodnotami znaku											
Sn-U-31	5	18	60	37,2	46,19						
Sn-U-41	5	42	152	69,8	67,16						
Genotyp so zelenými plodmi											
Sn-U-50	15	7	157	49,33	73,08						

Poznámky: *n* – počet meraní; min, max – minimálna a maximálna nameraná hodnota; $\bar{x}$ – aritmetický priemer; *V* – variačný koeficient (%)

Pri hodnotení počtu nezrelých kôstkovičiek na strapine sme určili rozsah daného znaku od 0 bobúľ do 213,6 bobúľ (Sn-S-07). Hodnoty variačných koeficientov dokumentujú stupeň variability od najnižších nulových hodnôt až po vysoký stupeň variability (Tabuľka 3). Súplodia v rámci jedného genotypu boli od úplne zrelých až po polozrelé s ešte nazelenalými kôstkovičkami. V podmienkach Ukrajiny sme určili genotyp (Sn-U-50) so zelenými plodmi a svetlo-bordovou strapinou v období plnej zrelosti v porovnaní s ostatnými genotypmi. Absencia antokyánov v plodoch by mohla byť dôsledkom chybných/chýbajúcich enzýmov, ktoré tieto farbivá nemohli vytvoriť (Obrázok 1). Nerovnomernosť dozrievania pri genotypoch je nevýhodná. Pre praktické využitie odrôd je jednoznačne žiadúce rovnomerné dozrievanie plodov.

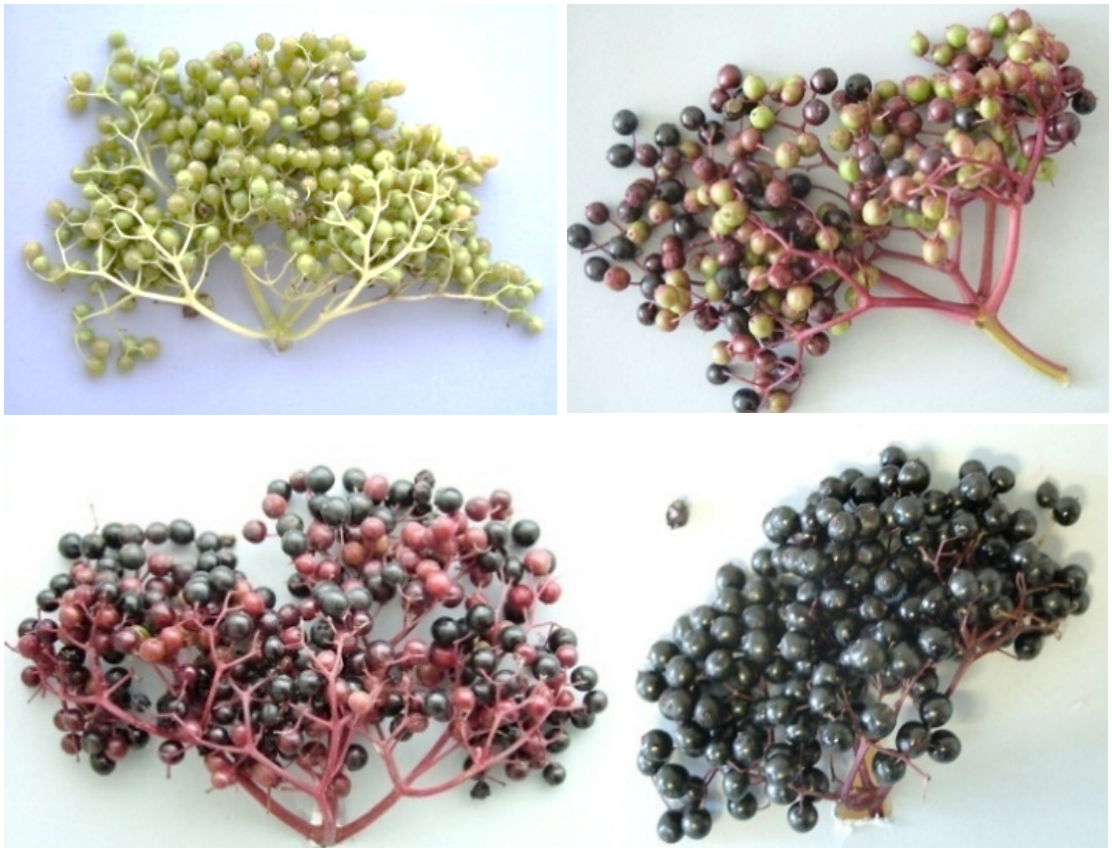
Obrázok 1 Detaily genotypu so zelenými súplodiami v kolekcií *Sambucus nigra* L. vo voľne rastúcej populácii na Ukrajine

Figure 1 Details of genotype with green fruits in the selected collection of *Sambucus nigra* L. from a wild-growing population in Ukraine

Spôsob dozrievania súplodí

Kompaktnosť a rovnomernosť dozrievania kôstkovičiek na súplodí sú významné hospodárske znaky najmä pri zbere súplodí a následnom praktickom využití v potravinárskom, farmaceutickom, či kozmetickom priemysle.

Pri prevažnej väčšine súplodí sme určili tmavobordovú až bordovočiernu farbu kôstkovičiek (Obrázok 2). Výnimkou bol genotyp (Sn-U-50) z Ukrajiny so zelenými kôstkovičkami (Obrázok 1). Rovnomerná forma dozrievania kôstkovičiek v rámci jedného strapca bola pozorovaná pri solitéroch rastúcich na slnečných stanovištiach. Genotypy vo vyššej nadmorskej výške dozrievali o niečo neskôr ako tie v nižších polohách.



Obrázok 2 Forma dozrievania vybraných genotypov *Sambucus nigra* L. vo voľne rastúcej populácii na Slovensku a Ukrajine

Figure 2 Form of maturation of selected genotypes *Sambucus nigra* L. in a wild-growing population in Slovakia and Ukraine

Farba stopky súplodia

V období dozrievania plodov, sa sfarbujú na červeno aj stopky, na ktorých sú umiestnené. V hodnotenej kolekcii voľne rastúcich genotypov bazy čiernej sme určili niekoľko odtieňov zafarbenia stopky súplodí: svetlo-zelenú, výrazne zelenú, žltozelenú, žlto-ružovú, ružovo-červenú, sýto bordovú, hnedo-bordovú (Obrázok 3).

Pri odrode Sambo je súkvetie v čase vytvárania plodu visiace, stopky v čase zrelosti sú purpurovo-fialové (Hričovský, 2001). Zdá sa, že tento znak je špecifický pre jednotlivé genotypy.

Obrázok 3 Porovnanie zafarbenia stopky súplodí *Sambucus nigra* L. vo voľne rastúcej populácie na Slovensku a Ukrajine

Figure 3 Comparison of stem coloration of *Sambucus nigra* L. in a wild-growing population in Slovakia and Ukraine

Závery

Na základe realizovaného prieskumu a hodnotenia 20 vybraných genotypov z voľne rastúcich populácií bazy čiernej (*Sambucus nigra* L.) v podmienkach Slovenska a Ukrajiny sme určili:

a) priemernú hmotnosť súplodí v rozsahu 4,75 g (Sn-U-31) do 101, 90 g (Sn-U-51);
b) priemernú celkovú hmotnosť kôstkovičiek v čerstvom stave v rozsahu od 2,64 (Sn-U-31) do 93,18 g (Sn-U-51);
c) priemernú hmotnosť strapiny v čerstvom stave v intervale od 1,13 (Sn-U-34) do 6,85 g (Sn-S-10, Sn-S-12);
d) priemer 50 kôstkovičiek z každého genotypu v rozsahu od 4,97 (Sn-S-11) do 6,39 mm (Sn-S-26);

e) priemerný počet kôstkovičiek v súplodí v intervale od 37,2 (Sn-U-31) do 839,4 bobúľ (Sn-S-05);
f) priemerný počet nezrelých kôstkovičiek pri nerovnomernom dozrievaní súplodí s vysokými hodnotami znaku až 213,6 (Sn-S-07);
g) rovnomernosť dozrievania na väčšine súplodí s výnimkou genotypu z Ukrajiny (Sn-U-50), ktorý mal začiatkom septembra všetky kôstkovičky zelené so svetlo-bordovou strapinou;
h) zafarbenia strapiny od svetlo-zelenej až po tmavo-bordové sfarbenie;
i) na základe výsledkov by bolo možné vybrať hospodársky významné genotypy pre praktické využitie.

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THE EFFECT OF HIGH TEMPERATURE ON THE FLAVONOID ACCUMULATION IN *ARTEMISIA* "HAIRY" ROOTS

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ВПЛИВ ПІДВИЩЕНОЇ ТЕМПЕРАТУРИ НА НАКОПИЧЕННЯ ФЛАВОНОЇДІВ У ТРАНСГЕННИХ КОРЕНЯХ РОСЛИН РОДУ *ARTEMISIA*

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The aim of the work was to investigate the short-term effects of high temperature on the flavonoid accumulation in *Artemisia vulgaris* L. and *A. dracunculus* L „hairy" roots. The roots were cultivated for one, two, and five days at +36 °C, then were grown at +24 °C for up to four weeks. The flavonoid content (expressed in rutin equivalent, RE) of ethanolic extracts was determined using a $AlCl_3$ method. "Hairy" root lines differed in their sensitivity to short-term high-temperature exposure. Both stimulation and inhibition of flavonoid accumulation, as well as no changes were observed in "hairy" root lines. Significant (1.7–6.4 times) decrease in the flavonoid content was observed in lines which characterized by a higher flavonoid content under standard conditions (+24 °C) without the temperature stress exposure. For both species, the average or strong negative correlation (R^2 = 0.37... 0.85) was observed for weight gain from the time of thermal stress. Thus, *A. vulgaris* and *A. dracunculus* "hairy" roots differed in the cellular metabolism activity. They differed in the ability of flavonoids synthesis and sensitivity to the short-term high temperature exposure. Such exposure can lead both to activation and inhibition of the synthesis of biologically active compounds, particularly, flavonoids.

Keywords: "hairy" roots, *Artemisia vulgaris* L., *Artemisia dracunculus* L., high temperature, flavonoid

Вступ

Генетична трансформація з використанням грунтових фітопатогенних бактерій *Agrobacterium rhizogenes*, яка є способом отримання «бородатих» коренів, приводить не тільки до зміни геному рослин, але й до значних змін у функціонуванні клітин

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та їх біосинтетичної активності. Доведено, що наслідком перенесення rol генів *A. rhizogenes*, які є у Т-ДНК агробактеріальної плазміди (Huffman, 1984) та які здатні інтегруватися в геном рослин, є активація вторинного метаболізму (Bonhomme, 2000; Komarovská, 2010). Виявлено, що ці гени стимулюють синтез фітоалексинів (Bulgakov, 2011), впливають на синтез вторинних метаболітів у клітинах рослин(Bulgakov, 2008a), а також зменшують кількість активних форм кисню (ROS) (Bulgakov, 2008b), хоча механізм цього явища досі недостатньо з'ясований (Bulgakov, 2018). Отже, трансформування приводить до численних змін у функціонуванні клітин, і, таким чином, може впливати на адаптацію до дії стресових факторів.

Результатом дії абіотичних стресів, таких як низька або висока температура, нестача вологи, підвищений вміст солей у грунті, дія ультрафіолетового випромінювання є зміни у процесах біосинтезу та накопичування ряду сполук, що є як відповіддю на дію стресу, так і захисною реакцією клітин, яка дозволяю рослинам вижити за критичних умов (Gupta, 1993). Крім того, оскільки за дії стресу відбувається накопичення активних форм кисню (Choudhury, 2017; Mittler, 2015; Ramel, 2012), рослини можуть реагувати шляхом активізації синтезу природних антиоксидантів, зокрема, флавоноїдів (Bandurska, 2013; Brunetti, 2013). У процесі відповіді на дію стресів задіяні сполуки білкової природи, у тому числі регуляторні та сигнальні молекули (Grativol, 2012; Krasensky, 2012; Umezawa, 2004; Walter, 2011; Wang, 2009; Zhu, 2010; Zhu, 2016), а також сполуки небілкової природи, зокрема, з антиоксидантними властивостями (Ahmad, 2010). Вивчення цих процесів дозволяє охарактеризувати метаболічну відповідь трансформованих організмів на дію стресового фактору, визначити молекулярно-біологічні аспекти цього процесу, зокрема, виявити гени, які беруть участь у регулюванні адаптації рослин до дії стресових факторів, та регуляцію їх активності.

«Бородаті» корені рослин, отримані після трансформування *A. rhizogenes*, відрізняються не тільки наявністю перенесених до їх геному генів, але й функціональними характеристиками. Так, вони характеризуються високою швидкістю росту, незалежністю від наявності у середовищі регуляторів росту, специфічним фенотипом, що зумовлено активністю агробактеріальних генів (Павлова, 2013). Разом з тим, наявність цих генів також може призводити до появи специфічної відповіді на дію стресових факторів. Тому метою цієї роботи було визначення особливостей росту культур «бородатих» коренів рослин полину *A. vulgaris* та *A. dracunculus* за дії короткотривалого високотемпературного стресу, а також оцінка впливу таких умов вирощування на накопичення флавоноїдів.

Матеріали та методи досліджень

«Бородаті» корені рослин *A. vulgaris* та *A. dracunculus*, отримані нами раніше (Дробот, 2015; Drobot, 2016.), вирощували у стерильних умовах в чашках Петрі на живильному середовищі Мурасіге-Скуга (Murashige, 1962) зі зниженою удвічі концентрацію макросолей. Вплив підвищеної температури досліджували культивуванням коренів протягом 1, 2 та 5 діб за температури +36 °С та подальшого вирощування (загальна

тривалість до 4 тижнів) при температурі +24 °С. Контролем слугували корені, які вирощували за стандартних умлв при температурі +24 °С протягом чотирьох тижнів. Приріст маси коренів визначали шляхом зважування. Концентрацію флавоноїдів (RE) визначали, використовуючи якісну реакцію з $AlCl_3$ (Pękal, 2014). Дані переставлені у вигляди середнього значення та довірчого інтервала на рівні значимості 95%. Порівняння середніх проводили за результатами дисперсійного аналізу та теста Тьюкі на рівні значимості 95%. Для з'ясування взаємозв'язку між величинами проводили кореляційно-регресійний аналіз і визначали коефіцієнт детермінації (R^2).

Результати та обговорення

Результати дослідження свідчать про наявність загальної тенденції до зменшення швидкості росту «бородатих» коренів двох видів полину при збільшенні часу культивування при +36 °С. Усі досліджувані зразки коренів *A. dracunculus* (№№1-3) були чутливими до підвищеної температури, оскільки вирощування за таких умов протягом 5 діб приводило до зменшення приросту маси відповідно у 3,1, 2,6 та 2,4 рази у порівнянні. Для *A. vulgaris* (№№1-3) це зменшення становило 1,8, 2,4 та 2,3 рази, проте однодобова теплова обробка приводида до підвищення приросту маси у ліній №1 та №2 у 1,2 та 1,9 рази. Для обох видів спостерігалася середня або сильна негативна кореляція (R^2 = 0.38... 0.85) приросту маси від часу теплового стресу.

Трансгенні корені двох видів полину при їх культивуванні за стандартних умов (+24 °С) відрізнялися за вмістом флавоноїдів (рис.1а, б). Зокрема, найбільшим вмістом сполук відрізнялися корені №2 *A. vulgaris* (12,45 мг РЕ.г$^{-1}$ СМ) та № 3 *A. dracunculus* (2,93 мг РЕ.г$^{-1}$ СМ). Найменше флавоноїдів накопичували корені №1 та № 2 *A. dracunculus* – відповідно 0,59 та 0,62 мг РЕ.г$^{-1}$ СМ. Вірогідно, причиною таких відмінностей для трансгенів утворених із одного батьківського генотипу є різне місце вбудовування перенесених генів і, відповідно, відмінності у функціонуванні змінених геномів.

Спостерігалося три типи реакції на тепловий стрес: толерантність *A. dracunculus* №2; стимулювання синтезу флавоноїдів внаслідок тієї чи іншої тривалості теплового стресу *A. dracunculus* №1 та *A. vulgaris* № 1, №3; зниження концентрації флавоноїдів у *A. dracunculus* №3 та *A. vulgaris* №2. Останній тип реакції помічено у ліній, що мали відносно високу концентрацію флавоноїдів за стандартних умов. Про лінійну залежність концентрації флафоноїдів у «бородатих» коренях можна говорити тільки у випадках №2 *A. vulgaris*, де спостерігалась середня негативна кореляція (R^2 = 0.64), та №1 A. dracunculus, середня позитивна кореляція (R^2 = 0.68).

Вирощування при підвищеній температурі приводило у ряді варіантів (лінії № 2 *A. vulgaris*, №3 *A. dracunculus*) до зменшення вмісту флавоноїдів. У той же час, такі умови активізували синтез флавоноїдів у коренях №1 *A. vulgaris*. Так, вміст досліджуваних сполук у цих коренях при дії підвищеної температури протягом однієї, двох та п'яти діб був більшим, ніж у контролі відповідно у 1.9 (p <0.05), 1.1 та 1.7 (p <0.05) рази. В інших випадках стимулюючий ефект спостерігався тільки в результаті певної тривалості дії фактору (p <0.05). Так, для лінії №3 *A. vulgaris* це була 1 доба (2.3 рази), а для №1

A. dracunculus – 5 діб (2.3 рази). Треба відмітити слабку позитивну кореляцію вмісту флавоноїдів від приросту маси як для *A. vulgaris* (R^2 = 0,27), так і для *A. dracunculus* (R^2 = 0.21).

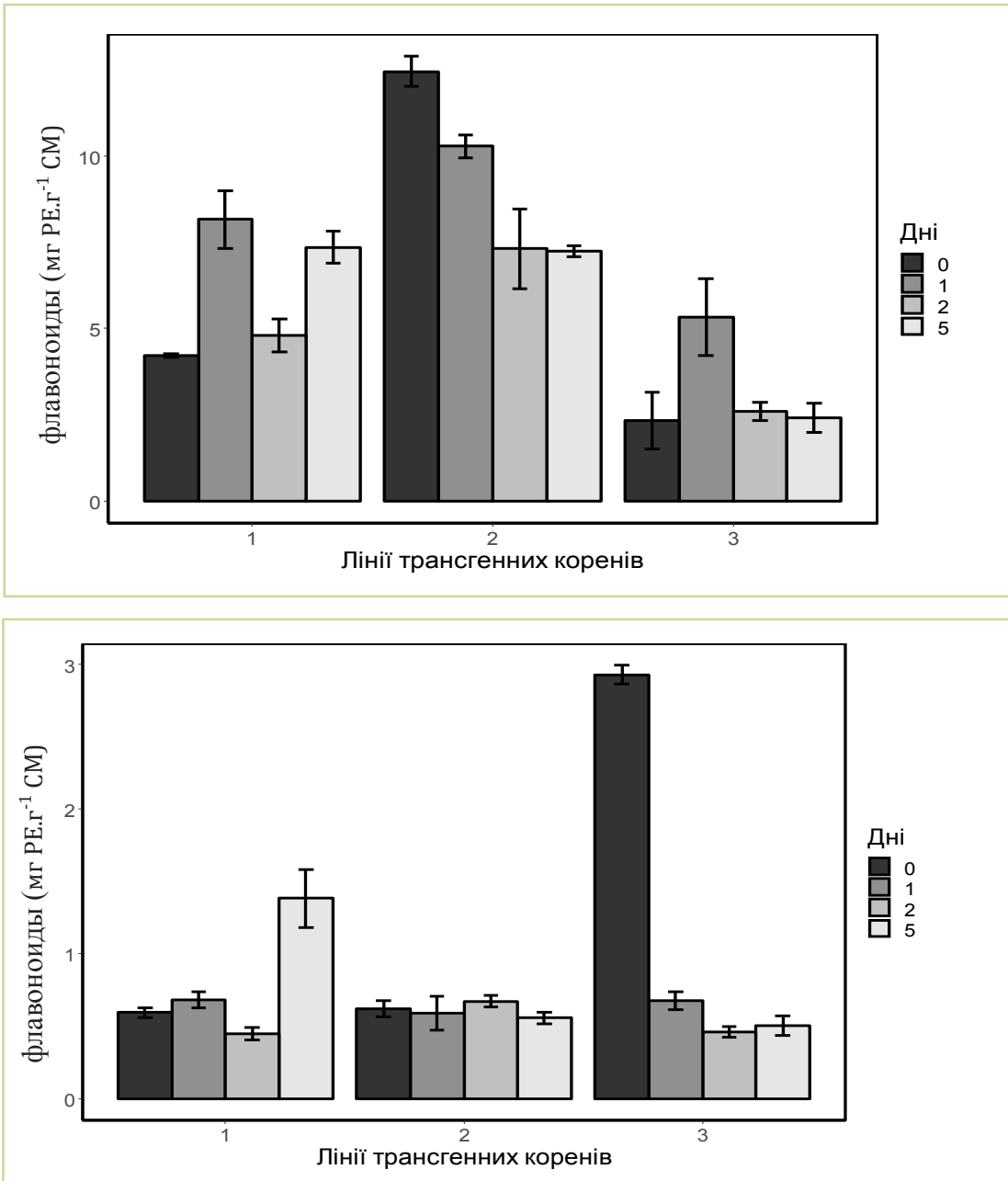

Рисунок 1 Вміст флавоноїдів у "бородатих» коренях *A. vulgaris* (а) та *A. dracunculus* (б) при їх вирощуванні за температури +36 °С протягом однієї, двох та п'яти діб

Отже, досліджувані зразки «бородатих» коренів рослин полину двох видів досить відрізнялися за їх чутливістю до короткотермінової дії підвищеної температури, що виражалося у зменшенні приросту біомаси та у більшості випадків вмісту флавоноїдів. Проте кількісні значення цих показників сильно різнилися як при вирощуванні «бородатих» коренів за нормальних умов, так і характером реакції на температурний стрес.

Висновки

Отже, генетична трансформація з використанням грунтових бактерій *Agrobacterium rhizogenes* веде до утворення «бородатих» коренів, що різняться за активністю клітинного метаболізму. Такі розбіжності виражаються у різній здатності коренів до синтезу флавоноїдів та різній чутливості трансформованих коренів до дії короткочасового температурного стресу. Результатом такої дії може бути як активізація, так і пригнічення метаболізму.

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STRUCTURE OF THE ROSES GENE POOL OF THE M.M. GRYSHKO NATIONAL BOTANICAL GARDEN OF NAS UKRAINE

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СТРУКТУРА ГЕНОФОНДУ ТРОЯНД НАЦІОНАЛЬНОГО БОТАНІЧНОГО САДУ ІМЕНІ М.М. ГРИШКА НАН УКРАЇНИ

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In article offered the characteristic of the gene pool of the M.M. Gryshko National Botanical Garden of NAS of Ukraine. Main sources of introduction of cultivars of *Rosa* L. are defined. Information on the beginning, methods and results of introduction is provided. The gene pool collection of roses, that comprises 27 species, 11 forms and 515 cultivars and has the status of the National property, is described by morphotypes, garden groups, countries of origin, the colour of flowers and by the years of breeding. As the result it was defined that the distribution of cultivars by the garden groups corresponds to the composition of the world collection of roses, the larger part of the collection is comprised of cultivars of foreign origin, the main part of the collection is composed by the tea-hybrid roses of pink colour. Main directions of further researches on introduction and selection of roses are outlined.

Keywords: gene pool, introduction, roses, new direction

Вступ

Троянди є однією з основних культур декоративного садівництва та промислового квітникарства. Велике значення вони також мають як ефіроолійні рослини. Світовий сортимент троянд нараховує нині близько 30 000 сортів.

Колекція троянд Національного ботанічного саду імені М.М. Гришка НАН України (НБС) почала створюватись у 50-ті роки XX ст. Основою її стали саджанці троянд,

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придбані у Німеччині у 1946 р. В подальшому колекція поповнювалася з ботанічних установ: Нікітського ботанічного саду (Ялта), Головного ботанічного саду РАН (Москва), Латвійського ботанічного саду (Саласпілс), а також ботанічного саду АН Польщі (Варшава), дендропарку „Софіївка" (Умань). Значна кількість нових сортів надходила з садових центрів та від аматорів (Рубцова, 2006; Мешкова, Рубцова, 2007; Tkachuk et al., 2017). Всього було випробувано близько 3 000 сортів, значна частина яких виявилася мало декоративними або не пристосованими до умов Києва, тому, що ріст і розвиток троянд у відкритому ґрунті України зумовлюється, перш за все, температурним режимом. У результаті було сформовано генофонд троянд, який нараховує 27 видів, 11 форм і 515 сортів.

Аналіз структури сформованого генофонду необхідний як для підведення підсумків інтродукційної роботи, так і для формування планів залучання перспективних інтродуцентів з особливо цікавих або слабо представлених у колекції груп. У зв'язку з ростом колекцій ботанічних садів необхідно постійно оновлювати відомості про колекційні фонди.

Матеріали та методи

Об'єктом дослідження була колекція троянд НБС, яка нараховує 27 видів, 11 форм і 515 сортів і має статус Національного надбання. Ця колекція була сформована в результаті тривалої інтродукційної роботи за методикою Ф.М. Русанова (1971). Дослідження структури генофонду троянд проведено за методикою В.М. Билова (1971), при плануванні інтродукційних робіт враховували Project of Core Collection of Roses for Their Preservation (2006).

Результати та їх обговорення

При вивченні великої кількості рослин одного роду в культурі доводиться працювати з неоднорідною групою рослин. Особливо це стосується великих поліморфних родів, зокрема роду *Rosa* L. Це ускладнює підведення підсумків інтродукційного експерименту. Тому є доцільним виділити в межах роду певні групи. Для роду *Rosa* такими групами є біоморфотипи (види та сорти, які виведено за їх участю) – для тих сортів, де чітко можна встановити належність до певного виду, а також садові групи за класифікацією American Rose Society (Modern Roses, 12, 2007) для всіх троянд, тому, що у більшості сортів у зв'язку із складним походженням не можна встановити видову належність. Вказані групи можна розглядати як самостійні одиниці в інтродукційному дослідженні. Такий підхід дає змогу краще вивчити механізм пристосування кожної групи до умов інтродукції.

В результаті скринінгу колекції роду *Rosa* НБС було виділено 15 біоморфотипів:

1. *R. rugosa* Thunb. та сорти: Abelzieds, Agnes, Buffalo Gal, Conrad Ferdinand Meyer, Delicia, Hansa, Hansaland, Kornik, Liga, Nova Zembla, Paksla, Pink Grootendorst, Pink Robusta, Purple Roadruner, Red Rugostar, Ritausma, Roibusta, Rose a Parfum de l'Hay, Rote Dagmar Hastrup, Rotes Phaenomen, Rugelda, Souvenir de Philemon

Cochet, Souvenir de Trelaze, Therese Bugnet, Tsaritsa Severa, White Grootendorst, Yellow Dagmar Hastrup, Henry Kelsey, F.J. Grootendorst, Martin Frobisher, White Roadruner, Snow Pavement.

2. *R. gallica* L. та сорти: Belle Hermine, Cardinal de Richelier, President de Sez, Versicolor, Violacea.
3. *R. alba* L. та сорти: Felicite Permantier, Maiden's Blush, Madam Plantier.
4. *R. spinosissima* L. та сорти: Red Nelly, White Scotch, Aicha, Karl Forster, Fruhlingsduft, Fruhlingsmorgen, Fruhlingsduft, Praire Dawn.
5. *R. beggeriana* Schrenk та її сорт Polstjarnan.
6. *R. moyesii* Hemsl. & E.H. Wilson та сорти: Margarita Hilling, Nevada,
7. *R. foetida* Herrm та сорти: Le Reve, Persian Yellow, Wildenfels Gelb.
8. *R. multiflora* Thunb. та сорти: Vltava, Wartburg, Velchenblau, Perennial Blue.
9. *R. centifolia* L. та сорти: Alain Blanchard, Village Maid, Chapeau de Napoleon, Mousseuse Rouge.
10. *R. eglanteria* L. та сорти: Alchemist, Ash Wednesday, Flammentanz, Kakhovka (Рубцова, Чижанькова, 2011).
11. *Hulthemia persica* Michx. ex J.F. Gmel. та сорти: Coral Babylon Eyes, Eyes for You, Eye of Tiger, Persian Autumn, Persian Mystery, Queen Babylon Eyes, Sunshine Babylon Eyes.
12. *R. nitida* Willd. та сорт Darts Defender.
13. *R. wichuraiana* Crep. та сорти: Dorothy Perkins, Excelsa, White Dorothy.
14. *Rosa damascena* Mill. та сорти: Festivalnaja, Iskra, Lada, Raduga, Stanwell Perpetual.
15. *R. helenae* Rehder & E.H. Wison та сорт Lykkefund.

Сорти з колекції троянд НБС належать до 16 садових груп (Таблиця 1). Виділені біоморфотипи входять до двох груп: гібриди шипшин та старовинні троянди.

Аналіз структури генофонду за садовими групами показав, що найчисельнішою є група чайно-гібридних троянд, яка складає 22,3 % всієї колекції.

Розподіл сортів за садовими групами відповідає структурі світової колекції троянд (Рубцова, 2009) і таким чином досліджений генофонд репрезентує світовий сортимет троянд.

Крім «класичних», або традиційних садових груп (чайно-гібридних, флорибунда, мініатюрних, витких, шрабів, ремонтантних, грандіфлора, ґрунтопокривних), генофонд троянд НБС представляє сорти досить рідкісних або малопоширених груп: старовинні, канадські, мускусні, ефіроолійні троянди, гібриди гультемії.

В НБС представлена найбільша в Україні (32 сорти) колекція старовинних троянд, створення деяких з них датується XVI ст. ('Versicolor'). В колекції є перший сорт української селекції Comtesse de Woronzoff, який був виведений 1833 р. другим директором Нікітського ботанічного саду М.А. Гартвісом (Клименко та ін., 2006,

2008). Старовинні троянди є дуже цінним матеріалом для селекції троянд. За генетичним різноманіттям вони наближаються до видів природної флори і є резервом генетичного матеріалу з важливими ознаками. Вони можуть сприяти вирішенню проблеми „генетичної ерозії“ (зменшення генетичної гетерогенності культивованих рослин, яка виникає при створенні сортів на основі близьких генотипів) (Рубцова, Чижанькова, 2016).

Таблиця 1 Розподіл сортів колекції троянд НБС за садовими групами
Table 1 Distribution of cultivars of rose collection of NBG by garden groups

Садова група	Кількість сортів	%
Чайно-гібридні	115	22,3
Флорибунда	71	13,7
Шраби	61	9,0
Виткі	57	11,1
Гібриди шипшин	46	10,1
Англійські	33	6,6
Старовинні	32	6,4
Ґрунтопокривні	28	5,6
Канадські	19	4,0
Мускусні	14	3,0
Мініатюрні	9	1,9
Ремонтантні	9	1,9
Грандіфлора	8	1,7
Гібриди гультемії	7	1,5
Дрифт	3	0,6
Ефіроолійні	3	0,6

Канадські троянди, як свідчить назва групи, виведені в Канаді, є дуже зимостійкими, що дуже важливо для умов Києва, де більшість троянд ростуть за межами свого екологічного оптімуму. Окремі сорти (наприклад, Hope for Humanity) за декоративністю не поступаються чайно-гібридним трояндам.

Мускусні троянди є складними гібридами *R. moschata* Mill., *R. multiflora* Thunb., *R. phoenica* Boiss., *R. sempervirens* L. та *R. arvensis* Huds. Вони мають розлогі кущі з дрібними квітками, зібраними у величезні суцвіття. Квітки мають специфічний «мускусний» аромат.

Сорти Фестивальна, Іскра, Лань репрезентують групу ефіроолійних троянд, які є промисловими культурами.

В останні роки колекція троянд НБС поповнилася сортами, які є гібридами між трояндами та *Hulthemia persica* Michx. ex J.F. Gmel. Цей напрямок інтродукції перспективний тому, що колекції троянд звичайно складаються з сортів, які відносяться тільки до підроду *Rosa* L. роду *Rosa* (Таблиця 2). *Hulthemia persica* є єдиним представником іншого підроду роду *Rosa* – *Hulthemia* (Dumort.) Focke.

Таблиця 2 Систематичний склад генофонду троянд НБС
Table 2 Systematic content of roses gene pool of NBG

Підрід	Кількість видів		Кількість сортів у колекції НБС
	існуючі	у колекції НБС	
***Hulthemia* (Dumort.) Focke**	1 (*Hulthemia persica* Michx. ex J.F. Gmel.)	0	7
***Hesperrhodos* Cockerell**	2	0	0
***Platyrhodon* (Hurst) Rehder**	1 (*Rosa* roxburghii Tratt.)	1	0
***Rosa* L.**	150	27	508

Hulthemia persica відрізняється від решти представників роду простими листками та темно-пурпуровою основою жовтих пелюсток. Поширена в країнах Середньої Азії, Казахстані, Ірані, Афганістані, Туреччині, на заході Китаю. Дуже посухостійка рослина.

В садах Західної Європи цей вид вирощується з 1836 р. Протягом 150 років селекціонери різних країн намагались схрестити *Hulthemia persica* з садовими трояндами з метою одержати гібриди з незвичайним забарвленням та темною плямою в основі пелюсток. Однак гібриди були одержані лише у 90-х роках XX ст. в Англії, а пізніше – в Нідерландах та США.

Зважаючи на посухостійкість *Hulthemia persica*, її гібриди з трояндами є перспективними як посухостійкі рослини з оригінальним забарвленням.

Rosa roxburghii була інтродукована нами у 2011 р. з ботанічного саду АН Польщі (в 2015 р. у інтродукованих рослин вперше зафіксовано цвітіння).

Генофонд троянд НБС постійно поповнюється сучасними сортами, які виведені в останнє десятиліття. Новинки світової селекції репрезентують сорти: Music Box, Princesse Clair, Queen Babylon Eyes, Roseromantic, Sunshine Babylone Eyes, William and Catherine, Anne de Kiev, Coral Babylon Eyes, Eye of Tiger та інш. Розподіл сортів троянд за роками виведення представлено у таблиці 3.

Забарвлення квітки є однією з основних декоративних ознак троянд. Сорти троянд колекції НБС мають різноманітне забарвлення. Найчисельніші рожеві та червоні сорти (Таблиця 4). Таке саме забарвлення переважає у природних видів. Сорти з квітками помаранчевих, бузкових, теракотових кольорів виведені відносно нещодавно, у дикорослих видів таке забарвлення відсутнє.

Таблиця 3 Розподіл сортів колекції троянд НБС за роками виведення
Table 3 Distribution of cultivars of rose collection of NBG by years of creation

Роки виведення сорту	Кількість сортів	%
До 1864 р.	33	6,5
1865–1900	12	2,3
1901–1921	17	3,3
1922–1942	25	4,8
1943–1963	81	15,7
1964–1984	114	22,1
1985–2005	189	36,7
2006–2016	39	7,6
Не визначено	5	1,0

Таблиця 4 Розподіл сортів колекції троянд НБС за забарвленням квіток
Table 4 Distribution of cultivars of rose collection of NBG by coloration of flowers

Забарвлення	Кількість сортів	%
Рожеві	155	30,2
Червоні	130	25,3
Жовті	107	20,8
Білі	42	8,1
Двоколірні	39	7,6
Помаранчеві	25	4,9
Бузкові	13	2,5
Теракотові	3	0,6

Нами також було встановлено, що більша частина колекції – сорти іноземного походження. Велика питома вага сортів, які походять із Західної Європи (72 %). (Таблиця 5).

Серед західно-європейських сортів найбільше тих, що виведені у Німеччині (фірма W. Kordes' Söhne) та у Франції (фірма Meilland International). У 2017 р. фірма W. Kordes' Söhne зробила суттєвий внесок у генофонд троянд НБС – подарувала 20 нових сортів власної селекції.

Таблиця 5 Розподіл сортів колекції троянд НБС за країнами походження
Table 5 Distribution of cultivars of rose collection of NBG by country of origin

Забарвлення	Кількість сортів	%
Німеччина	128	24,8
Франція	125	24,3
Великобританія	68	13,2
США	53	10,3
Україна	29	5,6
Нідерланди	25	4,9
Канада	20	3,9
Ірландія	12	2,3
Данія	10	1,9
Бельгія	10	1,9
Росія	3	0,6
Латвія	4	0,8
Швейцарія	2	0,4
Польща	2	0,4
Нова Зеландія	2	0,4
Японія	2	0,4
Фінляндія	2	0,4
Чехія	1	0,2
Люксембург	1	0,2
Китай	1	0,2
Іспанія	1	0,2
Казахстан	1	0,2
Швеція	1	0,2
Країна походження не визначена	12	2,6

Аналіз структури сформованого генофонду троянд є основою для формування планів залучання перспективних інтродуцентів з особливо цікавих або слабо представлених у колекції груп.

З урахуванням Project of Core Collection of Roses for Their Preservation (2006), нами визначено основні напрямки формування колекції троянд НБС, які можуть бути також використані фахівцями інших інтродукційних осередків троянд в Україні.

Основна увага у формуванні генофонду троянд приділяється інтродукції таких представників роду *Rosa*:

1. Види, які є цінними в систематичному відношенні, тобто репрезентують підроди, які відсутні в колекції НБС.

2. Види та сорти, які мають історичну або національну цінність (які мали важливе значення в історії троянд, а також сорти української селекції).
3. Види та сорти, які мають важливе значення для ландшафтного будівництва, зокрема для оптимізації урбанізованого середовища. Тут особлива увага приділяється трояндам канадської та американської селекції (які витримують зниження температури до -35 – 40 °C).
4. Види і сорти, які можуть бути використані в селекційних програмах.

Унікальна колекція, що зібрана в НБС, є базою для створення нових сортів троянд, найбільш пристосованих до умов Києва, які поповнюють генофонд. В селекційній роботі використовуються класичні методи селекції: висів насіння від вільного запилення, міжсортова та віддалена гібридизація, клонова селекція.

В результаті тривалої селекційної роботи було одержано значну кількість гібридних сіянців та спортових мутантів. На шість сортів: Хортиця (Заявка № 05246014), Граційний Танок (Заявка № 10246001), Враження (Заявка № 11246004), Акварель Роуз Парк (Заявка № 11246003), Вінтаж (заявка № 15339002), Карусель (Заявка № 14339001) одержано авторські свідоцтва. Сорти троянд, створені селекціонерами НБС, за комплексом показників відповідають міжнародним стандартам та занесені до Державного реєстру сортів рослин, придатних для поширення в Україні.

Висновки

В результаті тривалої інтродукційної роботи в НБС сформовано значний генофонд троянд з 27 видів, 11 форм і 515 сортів, він репрезентує 15 біоморфотипів та всі існуючі садові групи. В колекції представлено як старовинні, так і сорти сучасної селекції. Основну частину генофонду складають чайно-гібридні троянди рожевого кольору. Більша частина колекції – сорти іноземного походження, велика питома вага сортів, які походять із Західної Європи (72 %). Представлено стратегію подальшого формування генофонду.

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STUDY OF MORPHOLOGICAL CHARACTERISTICS OF POLLEN GRAINS OF *SAMBUCUS NIGRA* L.

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The aim of the work was to study the general characteristics and significant morphological traits of pollen grains, as the size, shape of pollen grains in *Sambucus nigra* L. The studies were performed on pollen obtained from genotypes cultivated in Ukraine (Kyiv). Morphology of pollen grain was described for thirteen *Sambucus nigra* genotypes (SN-1 – SN-13) at the laboratory of Department of Tropical and Subtropical plants of M.M. Gryshko National Botanical Garden. Pollen grains morphological traits were evaluated using the scanning and transmission electron microscopy. The measurement of morphometric parameters was carried out on 70 pollen grains from each genotype using the AxioVision Rel. 4.8.2.0 program. The measurements were made in micrometer (μm). The length of 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 *Sambucus* species are small to medium-sized, oblat-sphaeroidal-prolat shape, three-colporate and the exine adornments are of reticulate type without perpendicular thickness. This study showed that there were significant differences the genotypes in all measured factors. The polar axis and equatorial diameter of pollen grains values were varied from 22.11 to 29.07 μm and from 11.98 μm to 17.29 μm, respectively. This study confirmed small differences among the genotypes in all measured factors with variation coefficient in the range 2.87–6.02%. It was noted that diversity of surface sculpturing of pollen grains in combination with shape and sizes of them enables to use complex of thin morphologic signs for *Sambucus nigra* pollen identifications.

Keywords: *Sambucus nigra*, genotype, pollen, SEM, morphology, Ukraine

Introduction

The Caprifoliaceae family, which also includes *Sambucus*, *Viburnum*, *Lonicera* and other species has been an object of palynological interest for at least 60 years (Erdtman, 1952; Straka, 1952; Stachurska et al., 1963, 1970, 1971; Weberling, 1966; Kuprianova and Alyoshina, 1972). New information about Caprifoliaceae pollen morphology were obtained due to use light

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microscope (LM) and scanning electron microscope (SEM) by authors Böhnke-Gütlein and Weberling (1981) in Germany, Tank and Donoghue (1985, 2010) in USA, Accorsi et al. (1987) in Italy, Hu and He (1988) and Di Wei Zhong et al. (1993) in China, Maciejewska (1997) in Poland, Tarnavschi (1981) and Tamas et al. (1999, 2009) in Romania, Muccifora et al. (2003) in Italy, Tsymbalyuk and Bezusko (2017) in Ukraine.

The pollen grains have a definite shape, size, colour, structure for each species, genus and family and these characters are useful for systematic botany (Erdtman, 1952; Ciobanu, 1971; Brindza and Brovarskyi, 2013; Chlebo and Adamchuk, 2017; Grygorieva et al., 2015, 2017).

The complex of these morphological characteristics and ultrastructure allows determining the differences (or similarities) between the *Sambucus* species (Maciejewska, 2003; Brindza and Brovarskyi, 2013; Tsymbalyuk and Bezusko, 2017).

Using optical microscopy the pollen grain of Caprifoliaceae family, which includes *Sambucus* species is 3-colporate, porat, subsphaeroidal or oblatsphaeroidal, isopolar, tricellular (Charzyńska and Lewandowska, 1989; Muccifora et al., 2003; Brindza and Brovarskyi, 2013), 3-tetrasymetric, of small-middle or medium-sized and the exine without perpendicular thickness (Tamas et al., 2009). Authors Tarnavschi (1981), Maciejewska (1997), Tsymbalyuk and Bezusko (2017) studied pollen morphology of three species of the genus *Sambucus* (*S. nigra*, *S. ebulus* and *S. racemosa*) represented in the flora of Romania, Poland and Ukraine using light and scanning electron microscopy (SEM). Pollen grains of the studied species are 3-colporate; prolate, spheroidal or oblate-spheroidal in shape; small to medium-sized. Their outline in equatorial view is elliptical or circular, in polar view 3-lobed or slightly 3-lobed. Colpi are long, occasionally of medium length, with pointed and sometimes rounded ends. Pores are indistinct, covered by margins of colpi, or sometimes distinct. Sculpture exine macroreticulate (*S. ebulus*) and microreticulate (*S. racemosa* and *S. nigra*). Polar view is subcircular and equatorial view is suboblate – subcircular (PalDat).

The aim of this study was to obtain the images. The knowledge of pollen morphological characteristics can be an adequate method for identification genotypes of *Sambucus nigra*.

Materials and methodology

Locating trees and data collection

The pollen of 13 *Sambucus nigra* genotypes (SN-01 – SN-13) from the collection of M.M. Gryshko National Botanical Garden of NAS of Ukraine (NBG) was investigated.

Pollen grains collection

Freshly flowers (not opened) were collected randomly from the different genotypes at the balloon stage (June 2018). Pollen samples released from dry flowers 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 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, size and the dimensions of pollen grains for each *Sambucus nigra* genotypes were determined by using a scanning electron micrograph (SEM).

Morphometric characteristics

The measurement of morphometric parameters was carried out on 70 pollen grains from each genotype using the AxioVision Rel. 4.8.2.0 program. The measurements were made in micrometers (μm). The 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

Basic statistical analyses were performed using PAST 2.17; hierarchical cluster analyses of similarity between genotypes were computed on the basis of the Bray-Curtis similarity index; multi-dimensional scaling (MDS) analyses were performed in PRIMER (Clarke and Gorley, 2006). Variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by Stehlíková (1998).

Results and discussion

Study of 13 tested genotypes of *Sambucus nigra* pollen morphology showed that pollen grains are from small to medium-sized. In accordance with the ratio P/E (Tab. 1) the pollen tricolporate, oblate or spherical, sometimes oblate-spheroidal by the shape, in polar view pollen grain was 3-lobate and in equatorial view – elliptical or circular (Figure 1). Colpi were long, with more or less equal and clear edges with slightly pointed ends. Membranes of colpi were smooth. Mainly pores were blurred, covered with margins of colpi. But in very rare cases pores were distinct. Pollen wall was with tectum. Ectexine consisted of obvious, short and thin rod-shaped reinforcing elements. They were rarely located. The exsine surface had verrucate sculpturing with rounded cells by the shape. Sculpture of exine was microreticulate. Cells were small or medium size, circular, angled or circular-angled by the shape. Sometimes at the bottom of cells columns are observed. Knowledge from authors Tirnavschi (1981), Donoghue (1985), Maciejewska (1997), Tsymbalyuk and Bezusko (2017) confirmed our results.

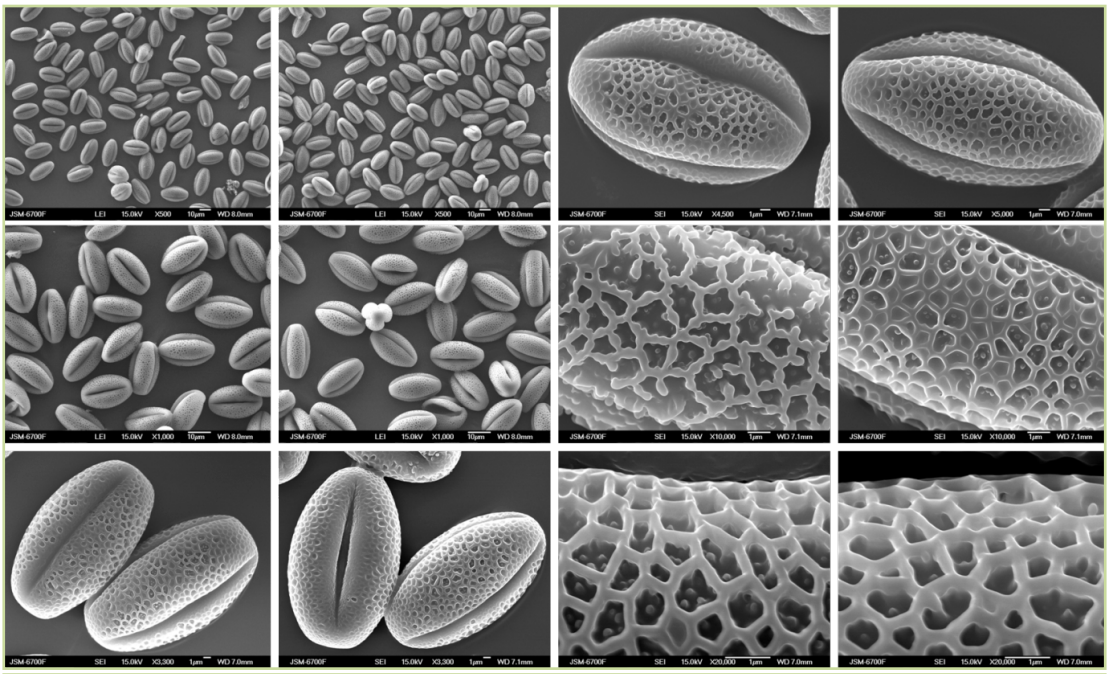

Figure 1 Pollen grains of *Sambucus nigra* L. species in different positions (Photo: Gurnenko, 2018)

Table 1 The measured pollen morphological traits of selected genotypes of *Sambucus nigra* L.

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*)			
SN-01	22.51	27.95	24.47	3.72	12.93	16.19	14.24	4.73	1.74	1.72	1.72	1.27
SN-02	22.99	26.00	24.51	2.87	12.58	16.21	14.39	5.29	1.83	1.60	1.70	1.84
SN-03	22.58	26.82	24.76	3.50	12.69	16.46	14.43	5.23	1.78	1.63	1.72	1.49
SN-04	22.30	26.15	24.36	3.51	12.61	16.09	14.11	6.02	1.77	1.63	1.73	1.72
SN-05	22.96	26.52	24.69	2.95	12.80	16.18	14.28	5.71	1.79	1.64	1.73	1.94
SN-06	24.07	29.07	26.10	5.49	13.22	17.29	15.19	6.55	1.82	1.68	1.72	1.19
SN-07	22.86	26.43	24.46	3.54	12.48	16.32	14.11	5.97	1.83	1.62	1.73	1.69
SN-08	22.74	26.02	24.43	3.15	12.60	16.52	14.12	4.92	1.80	1.58	1.73	1.56
SN-09	22.83	26.58	24.69	2.98	12.38	15.89	14.09	5.08	1.84	1.67	1.75	1.70
SN-10	22.86	26.45	24.58	2.95	12.31	15.43	13.95	5.13	1.86	1.71	1.76	1.74
SN-11	22.99	26.16	24.34	2.97	12.38	15.70	13.88	5.82	1.86	1.67	1.75	1.96
SN-12	22.11	27.07	24.41	4.33	11.98	16.01	14.12	5.18	1.85	1.69	1.73	1.20
SN-13	22.24	26.36	24.59	3.77	12.32	15.96	14.04	4.65	1.81	1.65	1.75	1.23

Note: min – minimum value; max – maximum value; *V* – variation coefficient (%)

The polar axis (*P*), equatorial diameter (*E*) and polar axis to equatorial diameter (*P*/*E*) ratio of pollen grains of thirteen *Sambucus nigra* phenotypes 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 polar axis (*P*) varied from 24.34 (SN-11) to 26.10 (SN-06) µm and the width of the equatorial axis (*E*) was in the range from 13.88 (SN-11) to 15.19 (SN-06) µm. According to the average values, the genotype SN-06 has the largest pollen grains 26.00 × 15.19 µm. The values of variation coefficient were in the range from 2.87 (SN-02) to 5.49 (SN-06) % for polar axes and in the range from 4.65 (SN-13) to 6.55 (SN-06) % 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 1.70 (SN-02) to 1.76 (SN-10). The sizes of pollen *Sambucus nigra* are very similar, the same for *P*/*E* ratio (1.70–1.76), whereas in comparison with authors Muccifora (0.89–1.42) or Tamas (1.88) are our studied genotypes *Sambucus nigra* the polar axis being greater (Table 2).

According to literary data, Tamas et al. (2009) and Muccifora et al. (2003) determined small size type (10–25 µm) in general, but our results have shown in one phenotype medium size type with average polar and equatorial axes with 26.10 and 17.29 µm, respectively. Tsymbalyuk and Bezusko (2017) analyzed and summarized data on participation of pollen grains of *Sambucus* spp. and *Sambucus nigra* in palynofloras in the plain part of Ukraine. Authors detected a length of polar axis and equatorial diameter of pollen grains in the interval from 15.9–21.3 to 13.3–18.6 µm, respectively (Table 2).

Table 2 Literature data on pollen morphological traits in the *Sambucus nigra* L.

Characteristic	Value	Autors
Polar axis (µm)	16.0–24.8	Maciejewska, 1997
	25.0	Muccifora et al., 2003
	24.25	Tamas et al., 2009
	15.9–21.3	Tsymbalyuk and Bezusko, 2017
Equatorial axis (µm)	16.0–22.0	Maciejewska, 1997
	12.5	Muccifora et al., 2003
	12.85	Tamas et al., 2009
	13.3–18.6	Tsymbalyuk and Bezusko, 2017
SI – shape index	0.89–1.42	Maciejewska, 1997
	1.88	Tamas et al., 2009

Results of multi-dimensional scaling are shown in Figure 2. In Figure, it is possible to see the visual distribution the size of the pollen of the studied genotypes. The sample SN-06 (green ellipse) is with the largest pollen size.

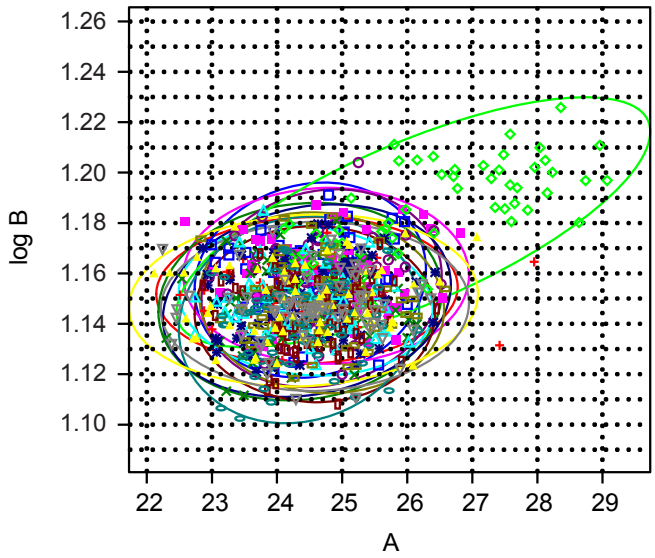

Figure 2 MDS plot of the similarity illustrating the length of the polar axis (A) and equatorial diameter (B) of pollen for studying samples of *Sambucus nigra* L. *Sambucus nigra* L.

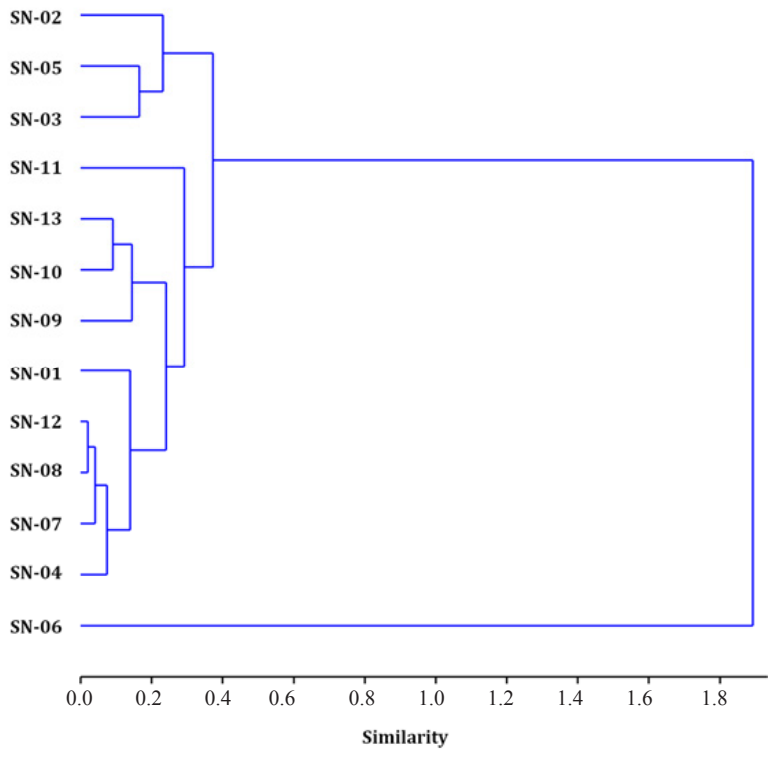

Figure 3 The dendrogram of *Sambucus nigra* L. 13 genotypes based on morphometric characteristics of pollen

Based on the cluster analysis of all 13 studied pollen characteristics, a dendrogram for the genotypes of *Sambucus nigra* was made (Figure 3). On the dendrogram, you can see that the sample SN-06 is really separated from other samples.

Conclusions

The studying of the *Sambucus nigra* pollen via scanning electron microscope allowed to determine the most important parameters which can be used to identify the representatives of species. The detailed pollen morphological and micro-sculptural characteristics of 13 phenotypes were investigated, described and analysed by using hierarchical cluster analysis dendrogram and MDS plot. The main parameters such as the form (the pollen grains elongation, the length and the width ratio) are specific for different *Sambucus* species. Results from our analyses showed small differences among *Sambucus nigra* phenotypes from Ukraine. Some of these pollen morphological parameters can be used for identification and comparison with following analyses of *Sambucus* species phenotypes.

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COMPARATIVE STUDY OF MORPHOMETRIC CHARACTERISTICS AND MINERAL COMPOSITION OF POLLEN *MALUS DOMESTICA* BORKH.

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Pollen apitherapy has value, because its composition is a mixture of valuable nutrients. Among them there are mineral substances that are the purpose of this study. In this work, macro (K, P, Mg and Ca) and oligo-mineral elements (Mo, S and Zn) were studied by energy dispersive spectrometry in ash *Malus domestica* Borkh. pollen samples from different ecological and geographical areas – Slovakia (Nitra) and Russia (Moscow). The mass fraction of elements in ashed pollen samples. The main elements in the composition of pollen are potassium (19.96–24.29) and phosphorus (5.54–6.81). Molybdenum and calcium are contained in approximately equal amounts (3.79–4.95) mass% in ash. The proportion of sulfur does not exceed 1.98, zinc 0.57% mass. In pollen samples from Slovakia, the proportion of potassium and phosphorus is higher by 18%. In pollen samples from Russia, the proportion of magnesium is higher by 5.8; calcium by 15; sulfur by 28 and zinc by 41%. The decreasing range of the content of mineral substances in the pollen of the *Malus domestica*. is determined: K > P > Mg > Mo ≥ Ca > S > Zn. The coefficient of variation of elements in the ash of pollen samples from Russia ranges from 19.6% (Mo) to 29.6% (P), which indicates the relative homogeneity of the data. The average values of the coefficients of variation of pollen collected in Slovakia were found in the elements Zn (23.5%), Ca (21%), S (20%) and K (15%). Low values of the coefficients of variation are noted for the elements Mg (10%), Mo (6.9%) and P (4.5%). Significant differences in the content of elements in ash are established only for K, Mg and S.The established differences in the coefficients of variation and correlation between the elements in pollen samples from Slovakia and Russia indicate the influence of the ecological-geographical conditions for the growth of apple plants. The results confirm that pollen *Malus domestica*. can be used as a natural source of minerals.

Keywords: pollen, *Malus domestica*, micromorphology, ash composition, analytical scanning electron microscopy

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Introduction

Plant pollen is a male gametophyte that develops in microsporangia from microspore and performs the function of pollination. Pollen is an important food for the bee colony and other insects and some animals. Pollen as part of the food chain in the animal world is a natural concentrate containing about 200 substances. Including mineral acids (triglycerides, phospholipids), flavonoids, vitamins and up to 1.6% of macro and micronutrients (Echigo et al., 1986; Villanueva et al., 2001, 2002; Somerville and Nicol, 2002; Almeida-Muradian et al., 2005; Szczesna, 2007; Yang et al., 2013), which are stimulators of biochemical and physiological processes of the body. And all this wealth is the most optimal ratio for the best assimilation. In official medicine, it is part of many pharmaceuticals. It is an effective remedy for the treatment and prevention of the absolute majority of diseases. Pollen is a natural biostimulator for the body. It has a very beneficial effect on higher nervous activity: the state of general satisfaction rises; working capacity, initiative, desire to act increases; optimism appears; decreased fatigue; speed of memorization and assimilation of new information increases. Pollen helps to produce its own body interferon, it boosts the body's immune defense; reduces the likelihood of disease, increases the elasticity of blood vessels, strengthens the capillaries, improves the adaptation of the body to weather changes, pressure drops in the atmosphere. With regular use of pollen, a qualitative improvement in the blood composition is noted, and the hematopoietic function of the body is activated. Pollen rejuvenates the entire body at the cellular level. The useful properties of pollen and the validity of its therapeutic use in various pathological conditions have been discussed in many scientific papers (Eschleman, 1996; Haro et al., 2000; Roulston and Cane, 2000; Almaraz-Abarca, 2004; Nogueira et al., 2012; Bogdanov, 2014). The chemical composition of pollen depends on the type of plant, its geographical origin, as well as other factors – climatic conditions, soil type, etc. The influence of the botanical origin of plants on the biochemical characteristics of pollen is shown (Chlebo et al., 2017). Comparative data of the mineral composition of pollen from different ecological-geographical zones little reflected in the literature.

Therefore, the purpose of this work was a comparative study of the mineral composition of the ash elements in the *Malus domestica* Borkh. pollen samples from different ecological-geographical areas.

Material and methodology

Plant Material

The objects of study were 5 samples of pollen *Malus domestica* from Nitra (Institute of Biodiversity Conservation and Biosafety at the Slovak University of Agriculture in Nitra) and 4 samples of pollen from the botanical garden in Moscow (Russia). The pollen was taken mechanically from the flowers in the "pink bud" state.

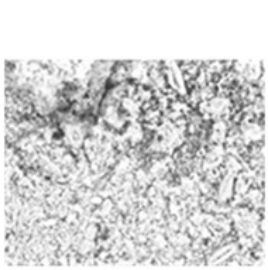
Preparing pollen for analysis

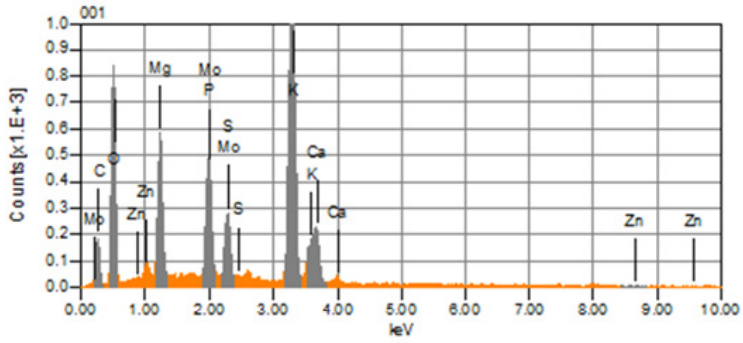
Preliminary dried at T = 40–50 °C pollen weighing with the mass of 10 g was mineralized in the muffle furnace Naberterm (Germany) at T = 400 °C. The received ash was dispergated by

ultrasound at 18 kHz frequency for 15 minutes. The dispergate even layer was applied on the object table covered with carbonic scotch.

Elemental Analysis

The ash composition was determined by the method of enodispersive spectrometry (EDS) based on analytical scanning electron microscope – JEOL JSM 6010 LA. The microscope resolution is 4 nm at accelerating voltage 20 kV (secondary electrons image), zooming is from 10× to 10 000×. While performing the elemental analysis the working distance (WD) is 10 mm. Energy-dispersive spectrometer allows to carry out the quantitative *X*-ray microanalysis with the desired analyzing area. *X*-ray microanalysis data are presented in the form of standard protocols which contain the microstructure picture of the sample under study, the table of the data in weighting and atomic correlation, spectra and histograms. The spectrum example is shown in Figure 1.





Chemical formula	mass%	Atom%	Sigma	K ratio	Line
C	64.93	71.39	0.02		K
O	25.69	21.20	0.05		K
Mg	6.39	5.21	0.11		K
P	5.73	4.31	0.11		K
S	2.20	1.08	0.17		K
K	26.77	14.26	0.01		K
Ca	5.49	2.16	0.11		K
Zn	0.13	0.04	0.21		K

Figure 1 Report of the results of the EMF analysis: the study area, the spectrogram and the table of results

Taking into consideration the spectrum lines intensity the concentration of the desired element can be determined. The fractional accuracy of the chemical analysis is spread in the following way: at the element concentration from 1 to 5% the accuracy is less than 10%; from 5 to 10% the accuracy is less than 5%; at the element concentration more than 10% the accuracy is less than 2%. The local analysis is 3 μm the scanned area is not less than 12 μm. 100 ash areas of each sample were studied.

Statistical analysis

For statistical evaluation were used standard methods using statistical software Statgraphics Centurion XVII (StatPoint Inc. USA).

Results and discussion

We analyzed potassium, phosphorus, magnesium, calcium, molybdenum, zinc and sulfur. Macro- and oligo-mineral elements from burned pollen are presented in Table 1.

Table 1 The mineral composition of pollen samples of *Malus domestica* Borkh,, mass.% in the ash

Samples	The studied elements						
	K	P	Mg	Mo	Ca	S	Zn
	Pollen *Malus domestica*, Moscow (Russia)						
1	14.27c	3.65b	4.01c	3.89b	3.10b	1.41b	0.34b
2	20.57b	6.34a	5.39b	5.37a	5.84a	1.81ab	0.32b
3	17.64c	4.81b	4.51bc	4.45b	3.71b	2.17a	0.31b
4	27.39a	7.37a	6.41a	6.07a	5.11a	2.53a	1.31a
The average	19.96b	5.54ab	5.07b	4.95b	4.44a	1.98a	0.57b
Variation coefficient (%)	27.90a	29.60b	20.80c	19.60c	28.30a	24.30b	28.90c
	Pollen *Malus domestica*, Nitra (Slovakia)						
1	29.01a	6.72a	3.95b	3.77a	3.15b	1.33a	0.28b
2	21.48b	6.54a	5.07a	3.41a	3.79b	1.31a	0.49a
3	23.35b	6.78a	4.81a	3.73a	3.15b	1.52a	0.38a
4	27.16a	7.33a	4.98a	4.01a	5.10a	1.08b	0.33a
5	20.44b	6.68a	5.11a	4.07a	3.77b	1.84a	0.48a
The average	24.29b	6.81a	4.78a	3.79a	3.79b	1.42a	0.39a
Variation coefficient (%)	15.10a	4.50b	10.0b	6.90b	21.0ab	20.0a	23.50a

Note: Means in columns followed by different letters are different at $p = 0.05$. Each value represents the mean of three independent experiments (±SD)

Potassium occurred at the highest concentrations in all tested pollen samples with average concentration in the range between 19.96–24.29% in the ash. Followed by phosphorus (5.54–6.81), magnesium (4.78–5.07), molybdenum (3.79–4.95) and calcium (3.79–4.44)% in the ash. The oligo-elements determined presented average values ranged between 1.42–1.98% for sulfur and 0.39–0.57% for zinc. The coefficient of variation of elements in the ash of pollen samples from Moscow ranges from 19.6% (Mo) to 29.6% (P), which indicates the relative homogeneity of the data. The average values of the coefficients of variation of pollen collected in Nitra were found in the elements Zn (23.5%), Ca (21%), S (20%) and K (15%). Low values of the coefficients of variation are noted for the elements Mg (10%), Mo (6.9%) and P (4.5%). Significant differences in the content of elements in ash are established only for K, Mg and S.

Calculated correlation coefficients between elements (Table 2). In pollen samples from Russia it is established that, there is a high correlation not only between macro-elements, for example, K and Mg (r = 0.99); K and P and K and Mo (r = 0.97); P and Mg and P and Mo (r = 0.99); Mg and Mo (r = 0.851) but also between oligo-mineral elements S and Zn (r = 0.75). Weak correlation found between Ca and S (r = 0.42) and Ca and Zn (r = 0.35). The mean correlation is between P and S (r = 0.76); P and Zn (r = 0.73); Mg and Ca (r = 0.79); Mg and S (r = 0.78); Mo and Zn (r = 0.77); S and Zn (0.75).

Table 2 Correlation matrix for the 6 elements in the ash pollen of *Malus domestica* Borkh in Moscow (Russia)

Elements	P	Mg	Mo	Ca	S	Zn
K	0.97*	0.99	0.97*	0.72*	0.84	0.87
P		0.98*	0.99*	0.87*	0.76*	0.73
Mg			0.99	0.79	0.78	0.83*
Mo				0.86	0.76*	0.76*
Ca					0.41*	0.34*
S						0.74

Note: *Significant according to the t-test (p <0.05)

Table 3 presents the correlation matrix between the elements in the pollen ash from Slovakia. High correlation is established by the elements P and Ca (r = 0.79), Mg and Zn (r = 0.78), P and Mg (r = 0.58), and P and K (r = 0.51). High negative correlations were found for the elements K and Zn (r = -0.91), K and Mg (r = -0.77), K and S (r = -0.67), and P and S (r = -0.57). Low correlations were found between P and Mg (r = 0.11), Mg and Mo (r = 0.10), Ca and Zn (r = -0.01).

Table 3 Correlation matrix for the 6 elements in the ash pollen of *Malus domestica* Borkh in Nitra (Slovakia)

Elements	P	Mg	Mo	Ca	S	Zn
K	0.53*	-0.77*	0.15	0.12*	-0.67	-0.97
P		0.11	0.58*	0.79	-0.57*	-0.53
Mg			0.10*	0.50*	0.21	0.78*
Mo				0.40*	0.27*	-0.24*
Ca					-0.51	-0.01
S						0.51*

Note: *Significant according to the t-test (p <0.05)

The correlation between elements in pollen samples from Nitra is very different from the correlation established between elements in pollen samples from Moscow. The established

differences in the coefficients of variation and correlation between the elements in pollen samples from Slovakia and Russia indicate the influence of the ecological-geographical conditions for the growth of apple plants.

The influence of ecological and geographical conditions on the mineral composition of pollen *Malus domestica* has been established. In pollen samples from Slovakia, the proportion of potassium and phosphorus is higher by 18%. In pollen samples from Moscow, the proportion of magnesium is higher by 5, 8; calcium by 1,5; sulfur by 28 and zinc by 41% respectively. Differences in the content of elements in pollen ashes may be related to the type of soil and the various ecological and geographical conditions of *Malus domestica* growing, this is confirmed by the study of honey (Felsner et al., 2004). Our results on the content of potassium, calcium in pollen are similar to the data reported in studies of the pollen of other plant species – sunflower, eucalyptus (Somerville and Nicol, 2002; Villanueva et al., 2001; Szczesna, 2007).

Modern nutritional research confirmed that minerals and trace elements are the vital importance to the life activities of the human body. For instance, potassium has function in the maintenance of water balance and distribution kidney and adrenal function, calcium plays an important role in building and maintaining the bones and teeth blood clotting, transmitting of the nerve impulses. Magnesium regulates nerve simulation and muscle contraction. The participation of zinc and sulfur in many kinds of enzymatic composition might play an important role in promoting the metabolism of organisms to strengthen immune ability (Avcin et al., 1991).

Conclusions

There is very little published data on the mineral composition of pollen from Rosaceae. This makes comparison with literature data difficult. Our studies have found that in the ash residue of pollen *Malus domestica*, regardless of ecological and geographical conditions, dominated by K and P, then Mg, Mo and Ca, S and Zn in smaller quantities, this indicates that the order of accumulation of elements in the pollen *Malus domestica* is determined genetically. The influence of ecological and geographical conditions on the content of potassium and calcium in the ash residue of pollen *Malus domestica* has been established. These results contribute to the mineral content information of pollens *Malus domestica* in Russia and Slovakia, and show that pollen can be considered good source of minerals.

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OPEN ACCESS

PHENOLOGICAL GROWTH STAGES OF CHINESE QUINCE (*PSEUDOCYDONIA SINENSIS* C.K. SCHNEID.): CODIFICATION AND DESCRIPTION ACCORDING TO THE BBCH SCALE

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The aim of the study was the determination of the main phenological growth stages less known species of Chinese quince in the conditions of Slovakia. For the study of the issue, we used 6 individuals produced in the Arboretum Mlyňany (Slovak Republic). The age of them will be estimated approximately 30 years. Experimental data gained in years 2016–2017 in phenological studies of Chinese quince (*Pseudocydonia sinensis* C.K. Schneid.) were utilized to describe phenological growth stages of given species. For the description of phenological growth stages, it was used BBCH Monograph (1997). Phenological observations and time data collection were provided at regular intervals in the text form and photo documentation. Complex phenological growth stages were processed based on the phenological records. Resulting data will be used for the list of descriptors preparation specified for the given species and oriented on the practical utilization in the research, breeding and genetic resources investigation. A feature of the system is that homologous stages of different crops are presented by the same codes.

Keywords: Chinese quince, BBCH-code, growth stage, adaptation

Introduction

Climate change and other adverse global factors in each region also have a significant effect on the cultivation of plant species to ensure food security and the provision of raw materials for other increasing needs for the population. It is, therefore, necessary to gradually introduce and test the adaptation of less-known and less-used plant species for different practical uses in each region. In Slovak conditions, the Chinese quince is being tested for a longer period of time. In order to recognize the adaptation of each species, it is necessary to know, inter alia the phenology of the species. This issue has become the main subject of the presented work.

Pseudocydonia sinensis Schneid. (Chinese quince) belongs to the family Rosaceae Juss., native to eastern Asia in China, and the only one species from *Pseudocydonia* C.K.Schneid. genus

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(USDA, 2013). It is closely related to the East Asian genus *Chaenomeles* Lindl. and to the European genus Cydonia Mill.

Fruit of the *Pseudocydonia sinensis* is yellow colored eatable pomes. It has elliptical (var. *ellipsoidea*) or ovoid (var. *ovoidea*) shape. Fruits are very big, the height of fruit 98.06–124.48 mm, the average diameter of fruit 62.33–88.64 mm, the average weight of the fruit in the range 197.85–466.38 g (Monka et al., 2014).

Fresh fruits of *Pseudocydonia sinensis* are sour and hard, and because of this used in the recycling form. From the fruits can be prepared spreads, marmalades, jams, fruit jellies, candied pulp, sweetened syrups and juices, wine, liqueurs (Monka et al., 2014; Klymenko et al., 2017). Fruits of the Chinese quince are used especially in traditional Chinese medicine as antitussives that central or peripheral suppress a cough. Fruits are used for the treatment of asthma, cold, sore throat, mastitis and tuberculosis in Korea (NPRI, 1998).

The Belgian botanist Charles Morren (1953) introduced the term phenology for the first time in 1853, but the history of phenology background is much older. Modern phenology is the study of the timing of recurring biological events in the animal and plant world, the causes of their timing with regard to biotic and abiotic forces, and the interrelation among phases of the same or different species (Lieth, 1974; Meier et al., 2009). The first known phenological network was installed by him in Sweden in the middle of the 18^{th} century. In his work Philosophia Botanica, he outlined methods for compiling annual plant calendars of leaf opening, flowering, fruiting and leaf fall, together with climatic observations so as to show how areas differ (Schnelle, 1955). Plant development, and thus phenological phases, show great inter-annual variability and also large spatial differences. Individual (e.g. genes, age) and environmental factors (weather and climate conditions in the micro and macro-scale, soil-conditions, water supply, diseases, competition, etc.) influence plants significantly. They can be viewed as integrative measurement devices for the environment. The seasonal cycle of plants, however, is influenced to the greatest extent by temperature, photoperiod and precipitation (Keatley, 2000; Morellato, 2000).

In order to gain comparable phenological data, it is necessary to define exactly the phases which are to be observed. The use of the so-called extended BBCH scale (BBCH Monograph, 1997) is recommended, based on Zadok et al. (1974) cereal code is a system for a uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant species. It is a general scale allowing their application to those plants for which no special scale is available. For the description of the main (longer-lasting) phenological development stages, called principal growth stages, clear and easily recognized external morphological characteristics are used. The secondary growth stages define a short step of development.

Bruns and van Vliet (2003) and Meier (2003) develop the relationship to modern agriculture, they recommend the BBCH system and the traditional meteorology and climatology.

Many researchers used the BBCH-scale for describing the growth stages of different fruit trees, for instance, *Malus domestica* Borkh., *Pyrus communis* L., *Prunus cerasus* L., *Prunus domestica* L., *Prunus persica* Batsch., *Prunus ameriaca* L. (Meier, 1994), *Citrus* spp. (Agustí et al., 1995), *Punica granatum* L. (Melgarejo et al., 1997), *Cydonia oblonga* Mill. (Martínez-Valero

et al., 2001), *Diospyros kaki* L. (García-Carbonell et al., 2001), *Olea europea* L. (Sanz-Cortés et al., 2002), *Actinidia deliciosa* C.F.Liang & A.R.Ferguson (Salinero et al., 2009), *Diospyros virginiana* L. (Grygorieva et al., 2010), *Mespilus germanica* L. (Atay, 2013), *Ziziphus jujuba* Mill. (Hernandez et al., 2015).

The objective of this study was to describe the phenological growth stages of *Pseudocydonia sinensis* genotypes based on the BBCH scale.

Materials and methods

Locating trees and data collection

In a phenological survey of *Pseudocydonia sinensis* were studied genotypes grown in arboretum Mlyňany (Figure 1). Measurements and observations were carried out over two growing seasons (2016–2017), from March to November. Measurements were made two to three times per week between March and June and once per week from June onwards. Representative plants were photographed to describe the phenological growth stages. The phenological growth stages of *Pseudocydonia sinensis* tree were described and defined between winter dormancy and leaf fall using the BBCH General scale.

Figure 1 Chinese quince (*Pseudocydonia sinensis* C.K. Schneid.)

Results and discussion

Pseudocydonia sinensis plants in the conditions of introduction characterized by a full cycle of seasonal growing which indicates that natural and climatic conditions are favorable for growing in this region. Plants are bloom and bear fruits. Chinese quince is promising as fruit, ornamental and medicinal plant and it can be widely grown in the conditions of Slovakia.

For fruit trees, the BBCH-scale uses eight of the 10 principal stages, starting with shoot growth (stage 0) and ending at the initiation of dormancy (stage 9). Three principal growth stages are assigned to vegetative growth, describing the bud development (stage 0), leaf development (stage 1) and shoot growth (stage 3), the latter being shared with flower development (stage 5). Flowering (stage 6), fruit growth (stage 7) and maturity of fruit (stage 8) complete the code.

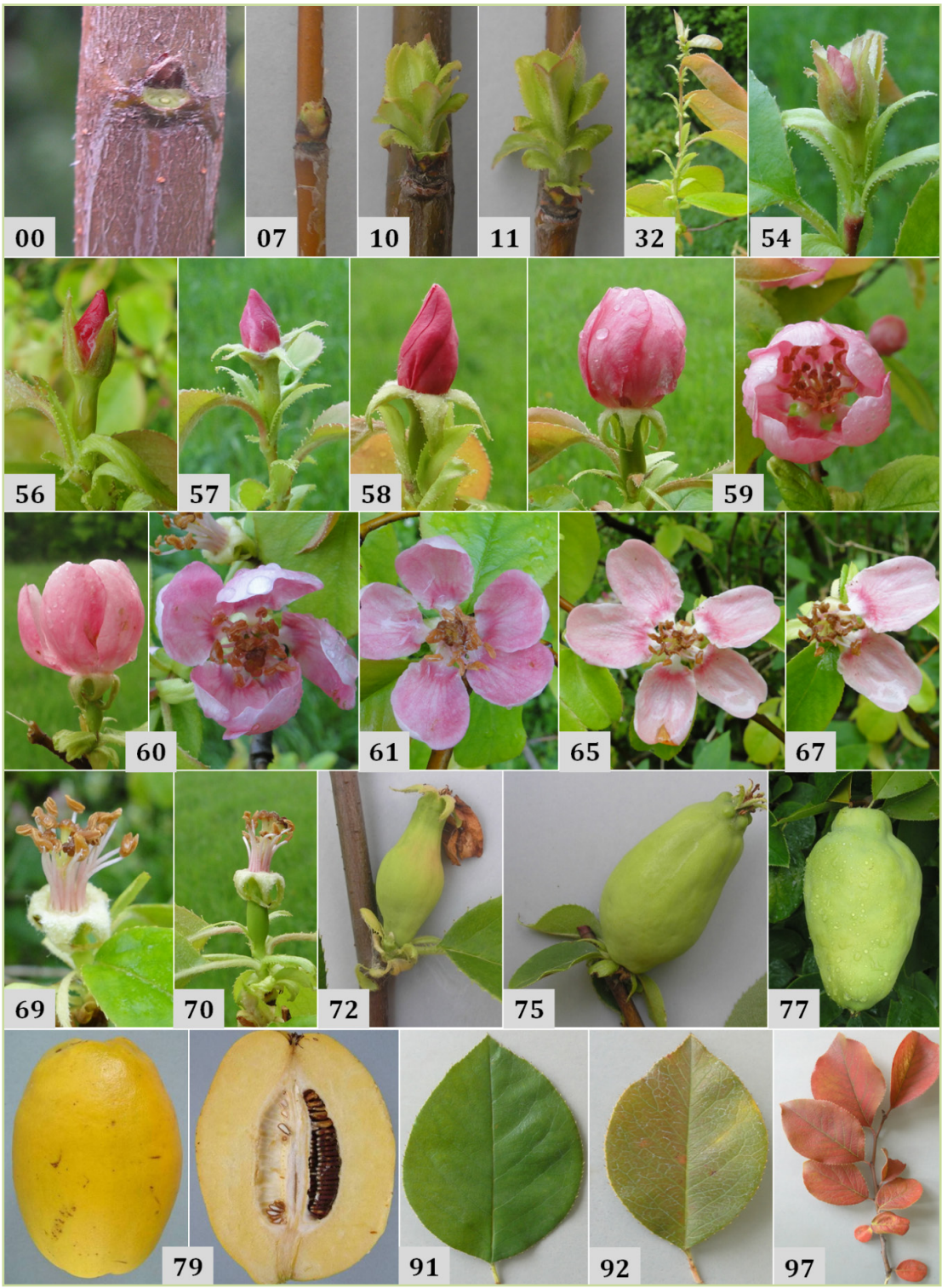

Figure 2 Phenological stages of the Chinese quince (*Pseudocydonia sinensis* C.K. Schneid.) tree

The secondary stages are also numbered from 0 to 9, is related to ordinal or percentile values of growth. Hence, value 1 of the principal stage of growth 6 (flowering) represents 10% of flowers in anthesis and its identification will be 61. Likewise, the value 5 of the principal stage 7 (fruit development) represents fruit at about 50% of the final size and will be defined, therefore, as 75. In other cases, values of secondary stages indicate qualitatively different stages within a given principal phenological stage; thus, within the flowering stage, the beginning of the anthesis (60) and flowers withered state (67) are identified.

Figure and Table 1 show the different phenological stages as well as the phenological codes and duration of *Pseudocydonia sinensis* tree phenology. The pattern of development of individual buds from selected trees generally matched that of all plants even though flowering was dependant on the position and orientation of the branches on the tree. There was no appreciable difference between buds for the duration of the successive phenological stages. The data in this study strictly refer to average values of monitored branches for every single experimental tree.

Table 1 Some of the primary and secondary phenological growth stages of Chinese quince (*Pseudocydonia sinensis* C.K. Schneid.) genotypes according to BBCH scale

Scale	Characteristics
	Principal growth stage 0: Bud development
00	Winter bud: the bud is dark brown, completely closed and very small (3-5 mm) in size.
01	Beginning of leaf bud swelling: buds visibly swollen, bud scales elongated, with light coloured patches.
03	End of leaf bud swelling: bud scales light coloured with some parts densely covered by hairs.
07	Beginning of bud break: first green leaf tips just visible.
09	Green leaf tips about 5 mm above bud scales
	Principal growth stage 1: Leaf development
10	Green leaf tips 10 mm above the bud scales; first leaves separating
11	First leaves unfolded (others still unfolding)
15	More leaves unfolded, but not yet at full size
19	First leaves fully expanded
	Principal growth stage 3: Shoot development
31	Beginning of shoot growth: axes of developing shoots visible; about 10% of final length
32	Shoots about 20% of final length
35	Shoots about 30% of final length
39	Shoots about 90% of final length
	Principal growth stage 5: Inflorescence emergence
51	Inflorescence buds swelling: calyx becomes visible; formed by five closed sepals protecting flower structure
53	Bud burst: scales begin to separate; beginning of peduncle elongation

Table 1

Scale	Characteristics
	Principal growth stage 5: Inflorescence emergence
54	Mouse-ear stage: green leaf tips 10 mm above bud scales; first leaves separating
55	Flowers still closed; sepals begin to separate
56	Flower petals elongating
57	Sepals open; petal tips visible; flowers with pink petals, still closed
59	Calyx opening: Sepals start opening showing the flower bud; apical leaf development
	Principal growth stage 6: Flowering
60	First flowers open
61	Beginning of flowering: about 10% of flowers open; anthers become visible; pollination begins
65	Full flowering: at least 50% of flowers open, first petals falling
67	Flowers fading: fecundation takes place; petals, stamens and pistils wither; petals fall
69	End of flowering: all petals fallen
	Principal growth stage 7: Fruit development
71	Fruit set: beginning of ovary growth; green ovary surrounded by dying petal crown, petals begin to fall; beginning of fruit let abscission; fruit fall after flowering
72	Immature fruit: fruit increases in size due to cell division (fruit size up to 20 mm)
73	Second fruit fall
75	Fruit about half of final size
76	Fruit about 60% of final size
77	Fruit about 70% of final size; light green fruit
79	Fruit growth: fruit reaches its final volume
	Principal growth stage 8: Maturity of fruit and seed
81	Maturation of the fruit: Change of color from green to light yellow; fruit gives off a pleasant aroma
87	Fruit ripe for picking; increase in color intensity
	Principal growth stage 9: Senescence. Beginning of dormancy
91	Shoot growth completed; terminal bud developed; foliage still fully green
92	Leaves begin to discolour
95	50% of leaves discoloured
97	Leaves fully discoloured
99	Winter rest period

Note: Description of the phenological growth stage of *Cydonia oblonga* and *Mespilus germanica* (Martínez-Valero et al., 2001; Atay, 2013)

In order to gain comparable phenological data, it is necessary to define exactly the phases which are to be observed. The use of the so-called extended BBCH scale (Meier, 1997) is recommended, based on Zadok et al. (1974) cereal code is a system for a uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant species.

Martínez-Valero et al. (2001) developed for the *Cydonia oblonga* and Atay (2013) for the *Mespilus germanica* description of phenological growth stages using the BBCH system and we have used it in the study of *Pseudocydonia sinensis*.

Conclusions

Chinese quince (*Pseudocydonia sinensis*) phenological development is described here for the first time using the BBCH scale. The use of extended BBCH scale for *Pseudocydonia sinensis* is important for successful implementation of orchard management practices including disease and pest control, a survey of genetic resources and further research purposes. The fruit species is potentially useful as a source of fruit for practical using in the food industry for the preparing of many products. Their biochemical composition provides great prospects for use as a raw material for therapeutic and pharmaceutical purposes, the development of biopesticides and cosmetic products. Trees can also be used in the landscaping and thus improve the environment. Therefore, this species is the perspective type for the practical use in Slovakia as well.

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