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Research Article OPEN ACCESS

Micropropagation of *Monarda fistulosa* L. plants by axillary bud proliferation

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The procedure of micropropagation for efficient and stable conservation of *Monarda fistulosa* L., a medicinally important plant belonging to the Lamiaceae family, was developed. Our research consisted of the introduction *in vitro* culture of the precious genotype from a biochemical point of view, identified as a result of multiple analyzes carried out by the researchers of the Scientific Medicine Research Center of the 'Nicolae Testemitanu' State University of Medicine and Pharmacy. This genotype is characterized by an increased content of secondary metabolites (thymol and carvacrol). Axillary bud proliferation was initiated from nodal explants grown on ½ Murashige-Skoog (MS) nutrient medium supplemented with various cytokinins, such as 6-benzylaminopurine (BA), 6-(α, α-dimethylallylamino)-purine (2iP) and cytokinin-like growth regulators – thidiazuron (TDZ) with a concentration of 0.5 mg.L^{-1}. After 6 weeks of culture on a nutrient medium, containing 0.5 mg.L^{-1} BA was determined the maximum number of axillary shoots per explant (2.75 ±0.86) and the highest number of internodes per shoot (11.66 ±1.30). At the same time, the rhizogenesis was induced on that medium that allows *in vitro* micropropagation by a single stage. The advantages of the elaborated procedure are reducing reagents and energy expenses for *in vitro* cultivation by two times and increasing the coefficient of multiplication from 13 to 32.

Keywords: *Monarda fistulosa*, medicinal plant, cytokinin, micropropagation, axillary bud

Introduction

Monarda fistulosa L. (wild bergamot) from the Lamiaceae family is a newly introduced perennial aromatic, medicinal and decorative plant found in the wild flora of North America (Bodrug, 1993). Essential oils obtained from the aerial part of the respective species show bactericidal, fungicidal, anthelmintic, immunomodulatory and antiseborrheic activity (Ivankovic et al., 2006; Inouye et al., 2009; Zhilyakova et al., 2009; Grzeszczuk, 2020; Yezerska et al., 2021). The study of the effects of the ethanolic extracts and the essential oil also demonstrated ovicidal, insecticidal, and antifeedant properties (Elisovetcaia et al., 2018). For the extraction of essential oils, this species is cultivated in Canada, the USA, some European countries, the Caucasus, Crimea as well as in the Republic of Moldova (Bodrug, 1993). The research carried out on *M. fistulosa* plants cultivated in the Republic of Moldova

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demonstrated the presence of an increased content of thymol and carvacrol (Casian et al., 2013; Casian et al., 2017). For these reasons, the plants of this species deserve to be recognized as medicinal plants equally with the official species such as *Thymus vulgaris* L. and *Origanum vulgare* L. subsp. *hirtum* (Casian et al., 2017; Oparin et al., 2000). The presence of significant amounts of thymoquinone may determine a greater spectrum of its therapeutic activity (Casian et al., 2017). The ratio of the components and the yield of the essential oil from different organs of *M. fistulosa* plants may vary depending on genotype and geographic origin (Mazza et al., 1992). Research carried out within the Scientific Center for Drug Research (SCDR) of 'Nicolae Testemitanu' State University of Medicine and Pharmacy (Chisinau, Moldova) demonstrated that the maximum essential oil content was determined in leaves and inflorescences, and the lowest in stems (Casian et al., 2017). As a result of multiple analyses, the researchers of the Scientific Center identified a genotype characterized by an increased content of active principles. At the same time, in the active phase of development, the appearance of powdery mildew was also observed on the surface of the leaves of cultivated plants. The disease affects the leaves, causing them to drop, and respectively affects vigor, resistance to stress over time, and optimal performance. Being a perennial plant, the spores overwinter in buds and on debris, which is released in the spring to continue the disease. Similar problems have been observed by other researchers (Davidson, 2007; Xu et al., 2022). Taking into account that there are practically no plants free of viruses and pests, our research consisted of the introduction *in vitro* culture of the precious genotype from the biochemical point of view.

It is known that the multiplication of *M. fistulosa* plants by seeds remains the most frequently used technique. But, they may not produce identical clones, which is a disadvantage when the intention is to generate plants similar in their clonal fidelity.

On the other hand, plant multiplication through *in vitro* cultures maintains the plant genotype, to produce disease-free plants, increases biomass turnover rate, reduces growth duration, and uses limited space with controlled environmental conditions. Also, plant tissue culture techniques are an effective tool used to plant improvement and secondary metabolite production (George et al., 2008; Grigoriadou et al., 2019).

The number of papers describing the cloning of *M. fistulosa* plants is not so large and the data are fragmentary. Our research aimed to develop an *in vitro* micropropagation procedure for efficient and stable conservation of *M. fistulosa*. This allows us to continuously supply clone plants that will be used as standard material in the field of medicinal plant research.

Material and methodology

Plant material and sterilization methods

Young shoots of 5–6 cm, with several lateral buds, taken from a single plant of *Monarda fistulosa*, grown in the SCDR served as plant material for the research. After leaf removal, stem segments with accompanying lateral buds were washed first in running tap water, then in sterile water with a few drops of Tween-40 and bleach solution for 15 min. Finally, the plant material was rinsed three times with sterile distilled water.

Culture media and conditions

Aseptically prepared nodal segments of approximately 0.5–0.8 cm were vertically implanted in ½ Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.2% activated charcoal (used to reduce tissue oxidation due to phenolic compounds during growth explants), 3% (w/v) sucrose and 0.6% (w/v) agar. The culture media were adjusted to pH 5.8 before autoclaving at 121 °C for 22 min. Cultures were incubated at 25 ±2 °C under a 16/8-hour photoperiod (light/dark cycle) provided by cool white fluorescent lamps (approximately 20–40 $\mu mol.m^{-2}.s^{-1}$).

Shoot induction, multiplication and rooting

After the establishment of the *in vitro* culture of *M. fistulosa* on the ½ MS medium modified supplemented with 0.2% activated charcoal, there was used ½ MS medium absent of cytokinins (control) and supplemented with different cytokinins at concentrations of 0.5 $mg.L^{-1}$ such as 6-benzylaminopurine (BA), 6-(α, α-dimethylallylamino)-purine (2iP) and cytokinin-like growth regulators – thidiazuron (TDZ). The effects of different cytokinins in the modified ½ MS culture medium on the initiation of axillary bud development, multiple shoot induction, shoot elongation and rhizogenesis initiation were evaluated. The number of shoots and shoot length were recorded, after 6 weeks of culture. Multiplication coefficient, calculated as the number of new segments (for subculturing) obtained per explant.

Statistical analysis

Data analysis for the determination of standard deviation and significant differences was performed by Statgraphics Plus 5.0 software.

Results and discussion

The micropropagation methods have been widely applied for the rapid propagation of many other medicinal plant species (Debnath et al., 2006; Grigoriadou et al., 2019; Tsoktouridis et al., 2019). One of the most widely used micropropagation strategies is the proliferation and growth of axillary buds. Lateral shoots developed from axillary buds can be separated at the subculture stage and each can be cultivated as a separate explant, thereby increasing the proliferation rate. This method is also considered the most reliable way to produce plants genetically identical to the raw material. Shoot tips and axillary buds represent organized meristems, which are less prone to genetic changes, being more resistant than disorganized ones (Ngezahayo and Liu, 2014; Krishna et al., 2016).

The establishment of *in vitro* culture from *M. fistulosa* was a rather difficult procedure due to contaminations and the sensitivity of the plant tissue to high concentrations of NaOCl. The disinfected explants were placed on ½ MS medium lacking plant growth regulators. To ensure sterility the explants were cultivated for 2 weeks. The stabilization medium was excellent and produced shoots, which were later used in our experiments.

The next step was to test the effects of different plant growth regulators on the initiation and development of axillary buds from explants. One of the most important plant growth regulators are cytokinins that induce the development of axillary buds in a wide spectrum of plants (George et al., 2008; Shimizu-Sato et al., 2009; Aremu et al., 2014). The normally dormant axillary buds are induced to elongate by cytokinins (George et al., 2008; Aremu et al., 2014). Such treatment effectively removes the dominance of apical meristems so that they produce axillary shoots, often in large numbers. These shoots are used as miniature cuttings for further plant multiplication (George et al., 2008). The general pattern of response depends on the used cytokinins such as BA, 2iP, and TDZ shown in Figure 1.

After 3–6 days of inoculation of the explants on the cultivation media containing cytokinins, we observed the initiation of direct formation of single or multiple shoots depending on the absence or presence of the tested cytokinins.

The highest frequency of shoot induction was determined by the presence of BA (2.75 ±0.86 auxiliary shoots per explant) (Table 1).

The highest number of internodes per shoot (11.66 ±1.30) was also obtained on ½ MS medium supplemented with BA, while the maximum shoot length (18.42 ±2.65 cm) was determined on the medium supplemented with 2iP (Table 1). Therefore, BA in the medium significantly improved the frequency of axillary bud induction as well as shoot number compared to ½ MS medium without cytokinin (control). The higher frequency of bud induction and the number of shoots obtained from nodal segments exposed to BA was the result of the high number of bud induction and shoots induced from axillary buds on newly formed shoots. Our results confirm studies, where ½ MS medium

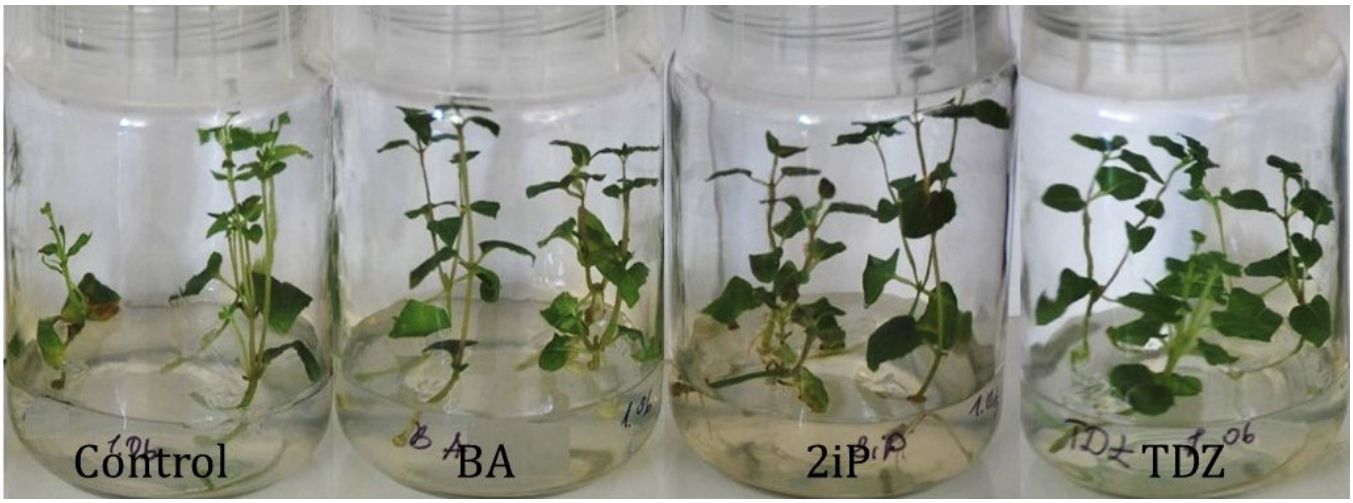

Figure 1 The plants of *Monarda fistulosa* L. grown in MS medium with different cytokinins
Control – absent of cytokinins; BA, 2iP and TDZ with a concentration of 0.5 mg.L^{-1}

Table 1 Effect of different cytokinins in MS medium on morphologic parameters of *Monarda fistulosa* L. plants

Variants	Control	BA	2iP	TDZ
Number of axillary shoots	1.8 ±0.42	2.75 ±0.86	2.18 ±0.60	1.5 ±0.52
Number of internodes	7.3 ±1.34	11.66 ±1.30	9.9 ±1.04	6.7 ±1.16
Length of shoots, cm	15.25 ±1.95	15.38 ±2.81	18.42 ±2.65	12.15 ±2.78
Multiplication coefficient	13	32	21	10
Number of roots	6.1 ±1.52	5.3 ±1.15	7.9 ±2.02	–
Length of roots, cm	4.4 ±2.01	3.2 ±1.13	2.7 ±0.95	–

BA – 6-benzylaminopurine; 2iP – 6-(α, α-dimethylallylamino)-purine; TDZ – thidiazuron

supplemented with BA, was the growth medium that stimulates multiple shoot formation in several plants, including species of the Lamiaceae family (Raja and Arockiasamy, 2008; Papafotiou and Martini, 2016; Islam et al., 2017).

The research carried out by Hrdlickov et al. (2014), demonstrated that the highest multiplication rate of the species *M. didyma* was obtained on MS supplemented with 0.5 mg.L^{-1} Kn (1.90 ±0.31 shoots per explant) and 1.5 mg.L^{-1} Kn (5.6 ±2.16 nodes per explant), practically 2 times less compared to our achievements. On the other hand, in the medium supplemented with TDZ, a retardation of the development of both the number of axillary shoots and their length was observed, which also influenced the number of internodes (Table 1).

In parallel, in three variants, including the control variant, the development of axillary buds and the elongation of shoots, as well as the initiation of rhizogenesis were established (Figure 2, Table 1).

Rooting of shoots is a very important part of any *in vitro* micropropagation scheme. A few plant species form adventitious roots on shoots at the stage of adventitious or axillary shoot elongation, but it is usually necessary to adopt a separate rooting procedure using special media or methods to induce root formation (George et al., 2008). Unlike the variant supplemented with TDZ, which provides for the initiation of direct shoot formation on a nutrient medium and the induction of rhizogenesis on another medium (two stages of *in vitro* seedling), the control variant, and those supplemented with BA and 2iP provide for *in vitro* cultivation only on a single nutrient medium (in a single stage) (Figure 2). Table 1 shows the effect of cytokinins on root formation. Except for TDZ, root induction occurred

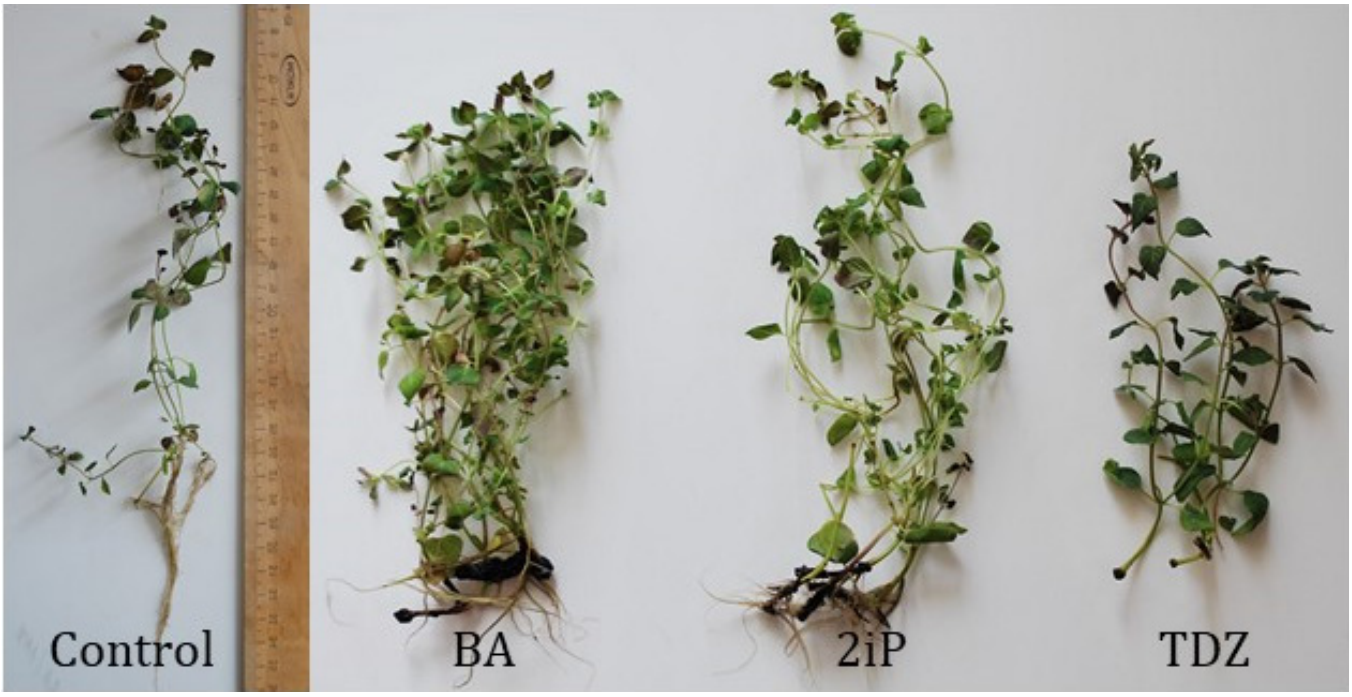

Figure 2 The plants of *Monarda fistulosa* L. at the final subcultivation stage, after 6 weeks of culture

in the rest of the variants. The addition of TDZ to the culture medium decreased not only shoot length but also root formation. The highest number of roots was observed in the presence of 2iP (7.9 ±2.02 roots per explant), followed by control (6.1 ±1.52 roots per explant). Instead, in the control variant, plants with the longest roots were obtained (4.4 ±2.01 cm). In this way, the elaborated micropropagation process allows for to reduction of two times the costs of reagents and energy resources for *in vitro* cultivation, increasing the multiplication coefficient from 13 to 32. Our further research will consist in presenting the results of the effects of the tested cytokinins on biomass accumulation and comparing the active principles accumulated in plants grown under artificial conditions with those grown under natural conditions.

Conclusions

Our research demonstrated that nodal explants of *Monarda fistulosa* possess a high potential for *in vitro* micropropagation through axillary bud proliferation. The procedure presented in this study is an efficient, rapid, and simple technique for axillary bud initiation and shoot proliferation on a relatively simple medium. The micropropagation of *Monarda fistulosa* plants using ½ MS nutrient medium containing 0.5 mg.L^{-1} BA, offers the possibility to reduce the procedure expenses and to increase the number of plants multiplied 3 times during a subcultivation.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Stimulation effect of alginite on *Rhodiola rosea* L. *in vitro* growth

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Stimulating the growth of plants, increasing their productivity, and resistance to stress factors are important tasks facing scientists and producers of agricultural products. Different compounds of natural origin can be used as biostimulants. Such chemicals as humic, fulvic, and salicylic acids, mineral elements, amino acids, chitosan, vitamins, poly-, and oligosaccharides are the typical components of biostimulants. They affected not only plant growth. Biostimulants also improve biosynthetic activity, including chlorophyll synthesis, and accumulation of plant metabolites. Alginite, founded in Slovakia in Maar near Pinciná village northeast of Lučenec, was studied as a plant growth stimulator. The application of biostimulants to medicinal plants allows increasing in their biomass and productivity. The effect of alginite on *in vitro* growth of *Rhodiola rosea* L. plants and their antioxidant activity has been analyzed in this study. Adding 1 or 10 mL.L^{-1} of MS medium alginite extract solution diluted to the concentration of 1% resulted in intensive plant growth. Shoots weight increased 4.48–6.64-fold compared to the control. Adding alginite extract solution also stimulated root growth (3.00–5.58-fold). Despite the decrease in the specific content of flavonoids in the plants, grown on media with alginite, the total content of flavonoids in these plants was higher than in the control ones due to a significant increase in biomass. The antioxidant activity of the samples grown on the alginite medium was higher than that of the control plants. Thus, alginite, a compound of natural origin, can stimulate the growth of *R. rosea* and increase the bioactivity of these plants.

Keywords: *Rhodiola rosea*, alginite, growth stimulation, flavonoids, antioxidant activity

Introduction

Producers of food products face the problem of increasing the productivity of crops by stimulating plant growth. Such stimulation is possible due to the use of inorganic and organic fertilizers, as well as due to specific plant growth regulators. Chemical compounds used in agriculture can pose environmental risks by contaminating soils and water. They can also negatively affect the soil microflora, changing its component composition. Besides traditional fertilizers, different natural biostimulants can promote plant growth. These compounds do not supply nutrients directly to the plants. At the same time, biostimulants facilitate plant metabolic processes improving nutrient availability

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(Drobek et al., 2019). Regulation (EU) 2019/1009 of the European Parliament and of the Council laid down rules on the making available on the market of EU fertilizing products.

Natural compounds and various extracts containing bioactive components can be regarded as biostimulants. Humic, fulvic and salicylic acids, mineral elements, amino acids, chitosan, vitamins, poly-, and oligosaccharides are the typical components of biostimulants of different natures (Bulgari et al., 2015; EL Arroussi et al., 2018). Some organisms (bacteria and fungi) in plants and soil can be considered biostimulants because they can induce changes in plant growth and biosynthetic activity (Bashan et al., 2014; Egamberdieva et al.; 2017; Park et al., 2017). Biostimulants can be applied via the soil or leaves by irrigation systems (Drobek et al., 2019).

Biostimulants affected not only plant growth. They improve biosynthetic activity, including synthesis of chlorophyll, stress resistance, and accumulation of metabolites (for instance, flavonoids well-known as powerful antioxidants) (De Pascale et al., 2017; Van Oosten et al., 2017; Yakhin et al., 2017; Fleming et al., 2019; Paul et al., 2019; Amjad Bashir, 2021). This is partially due to their effect on phytohormones synthesis and gene expression (De Pascale et al., 2017; Rouphael et al., 2020;).

In particular, chitosan based biostimulants positively influenced the quality of strawberry fruits and increased the concentration of phenolic compounds (Soppelsa et al., 2019). Seaweed extract altered the nutraceutical and antioxidative potential and improved the growth and yield of *Glycine max* L. (Kocira et al., 2019). Arbuscular mycorrhizal fungi increased the polyphenol content in *Crocus sativus* L. (Caser et al., 2019). Humates and lignosulfonates increase root growth, enhance photosynthesis and stimulate the Nitrogen metabolism of *Zea mays* L. (Ertani et al., 2019). Micronized and concentrated vermicompost, diatomaceous earth, and soy flour enhanced seedling growth and increased the integrity and compressive strength of seeds of *Trifolium pratense* L. and *Lolium perenne* L. (Qiu et al., 2020). Seaweed extract, legume-derived protein hydrolysate, and tropical plant extract increased leafy vegetable productivity in low-fertility soils, the physiological and biochemical status of *Lactuca sativa* L. plants (Mola et al., 2019). *Moringa oleifera* Lam. leaf extract, used as foliar spray or seed soaking improves the growth and yields of *Pisum sativum* L. plants by alleviating the inhibitory effects of soil salinity stress (Desoky et al., 2016).

More and more attention is paid to the design of preparations that stimulate plant growth and are natural and safe for the environment. Great attention should be paid to environmental sustainability in the case of biostimulant use (Le Mire et al., 2016). Alginite as a complex of compounds of natural origin can be named among other biostimulants. Alginite is an organic-bituminous rock with different organic and inorganic components that were sedimented together with the clays in post-volcanic outbursts during geological periods appropriate for algae occurrence (Kulich et al., 2001). Alginite was found in Slovakia in Maar near Pinciná village northeast of the town of Lučenec (Vass et al., 1997). A study of alginite and its effect on plant growth was conducted by several research groups (Barančíková et al., 2003; Gömöryová et al., 2009; Brindza et al., 2021a; 2021b; Kropp et al., 2021).

In this work, we studied the effect of alginite extract on the growth and bioactivity of *Rhodiola rosea* L. plants in *in vitro* culture. *R. rosea*, or Golden root, is well known medicinal plant. Traditionally these plants are used as adaptogens, antidepressants, and anti-inflammatory remedies (Kelly, 2001; Bawa and Khanum, 2009; Panossian et al., 2010). They are rich in polyphenols with antioxidant activity (Chen et al., 2012). Numerous bioactive compounds were studied in *R. rosea* (Chiang et al., 2015). Such chemicals as gossypetin-7-O-L-rhamnopyranoside, rhodioflavonoside, gallic acid, *trans-p*-hydroxycinnamic acid, and *p*-tyrosol were identified. The compounds were evaluated for their antibacterial and cancer cell activities (Ming et al., 2005).

The cultivation of golden roots under sterile conditions is a necessary element of the biotechnology process. In particular, such plants can be used to study the peculiarities of the synthesis of biologically active compounds that are characteristic of *R. rosea*. In addition, *in vitro* grown plants are necessary for the development of technologies for the genetic transformation technic. The use of the *in vitro* system when testing the effects of various compounds allows for completely standardizing the conditions (medium composition, temperature regime, humidification, etc) and avoiding possible additional and side effects caused by microorganisms in the soil.

Material and methodology

Plant cultivation and treatment

Alginite product ALGEXr-2 from natural alginite from the Pincina region (Figure 1a) was created by a research team from the Institute of Agronomic Sciences, Faculty of Agrobiology and Food Resources at the Slovak University of Agriculture in Nitra. The solution was prepared by the extraction using a mixture of sodium pyrophosphate decahydrate and sodium hydroxide (5 : 1) (Figure 1b). The resulting extract was diluted to obtain 1% concentration and sterilized through a filter with a pore diameter of 0.2 µm, Sartorius, Minisart (test solution).

Rhodiola rosea L. plants from the *in vitro* collection of the Laboratory of Adaptational Biotechnology, Institute of Cell Biology and Genetic Engineering, NAS of Ukraine, were used in the experiment. The apical parts of the shoots were separated and transferred to Petri dishes (100 mm) with the solidified half-strength Murashige and Skoog nutrient medium (Duchefa Biochemie, Netherlands) containing 2% sucrose. Sterile test solution in the concentrations of 1 mL.L^{-1} and 10 mL.L^{-1} was added to the medium. The plants grown on half-strength Murashige and Skoog medium were used as the control ones. In 30 days of cultivation at a temperature of +24 °C, the plants were removed from the medium and washed with distilled water. Morphometric and biosynthetic parameters of seedlings were determined (wet weight (WW) of the shoots and roots; total content of flavonoids; antioxidant activity).

Flavonoid content assay

Flavonoid content was studied by a modified method (Matvieieva et al., 2019). Before this study, the shoots and roots were homogenized in 70% ethanol. The extracts were centrifuged for 10 min at 14000 rpm (Eppendorf Centrifuge 5415C). Supernatants were used for flavonoid content assay. The absorbance of the samples was measured at 510 nm using the spectrophotometer Fluorat-02 Panorama. Specific flavonoid content was calculated by the calibration plot: $C_{(rutin)}$ = 1.7427D (R^2 = 0.9936) and expressed as milligrams per gram of wet weight in rutin equivalent (mg RE.g^{-1} WW). Total flavonoid content was calculated as a product of the specific flavonoid content and the weight of the "hairy" root sample and expressed as milligrams in rutin equivalent (mg RE).

Antioxidant activity assay

The plant extracts obtained for the total content of flavonoids study were used for antioxidant activity analysis using 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) by the method described in (Brand-Williams et al., 1995). The optical density of the mixture was determined at 515 nm on the Panorama Fluorate-2 spectrophotometer. The radical scavenging activity was determined by the formula: RSA = 100(A_0 – A_1)/A_0, where A_0 – absorbance of DPPH*; A_1 – absorbance of the sample in the reaction. Equivalent concentration (EC_{50}) was calculated as the corresponding weight of plant material required to obtain the extract with a 50% DPPH* inhibition level.

Figure 1 Natural alginite (a) and the extract used as the test solution (b)

Statistical analysis

All analyses were carried out in triplicate. Values were represented as mean and standard error (SE). The data were analyzed for statistical significance using ANOVA followed by the Tukey HSD test. P values less than 0.05 were considered significant. The linear regression method was applied to determine the antioxidant activities as EC_{50} by establishing the relationship between RSA and extract weight on linear intervals of curves (for the data where RSA <75%).

Results and discussion

The effect of alginite added to the culture medium on *R. rosea* was studied. Since the stimulating activity of alginite was determined by plants of various species (Brindza et al., 2021a, 2021b; Eftimová et al., 2021; Horčinová Sedláčková et al., 2021), it was possible to expect the presence of a similar effect when using golden root plants. We used an *in vitro* model, as this way of testing allows us to avoid any possible influence of alginite on the soil microbiome and, thus, on plant growth.

It was found that the addition of the test solution at a concentration of 1 $mL.L^{-1}$ significantly stimulated the growth of both shoots and roots (Figure 2).

In particular, the average weight of shoots was 6.64 times greater than the same parameter in the control plants, and the weight of roots was 5.58 times more. The addition of the test solution at a higher concentration (10 $mL.L^{-1}$) also increased the weight of plants (shoots by 4.48 times and roots by 3 times), although this parameter was lower than when the test solution was used at the lower concentration (Figure 3).

Cultivation of *Rhodiola rosea* plants on a medium to which the test solution was added in a lower concentration has led to a decrease in the specific content of flavonoids (Figure 4, bars 2). A similar effect was found when the concentration of the test solution in the medium increased (Figure 4, bars 3). This effect can be explained by the more active growth of plants in variants 2 and 3 compared to the control (variant 1), which requires more energy and synthetic resources and inhibits the process of accumulation of metabolites. A similar negative correlation between weight gain and the specific content of flavonoids was observed earlier (Matvieieva et al., 2019).

However, the total content of flavonoids synthesized in the shoots and roots of *Rhodiola rosea* plants in the experimental variants was significantly higher than in the control, due to the stimulation of growth in the presence of the test solution and the excess weight of the shoots and roots. As can be seen from Figure 5, the total content of flavonoids in the roots and shoots of variant No 2 was 1.48 and 3.35 times higher than the similar parameter of the control plants. The total content of flavonoids in the roots and shoots of the plants of variant No 3 also exceeded the parameters of the control plants and was 0.315 and 0.374 mg RE, respectively.

It is known that different biostimulants can affect not only plant growth but also secondary metabolism. For instance, the stimulator application increased the number of leaves, flowerheads, and dry root matter of *Calendula officinalis* plants and stimulated flavonoid synthesis in the plants (Oliveira Machado et al., 2014). These results correlate with the data obtained in our experiments.

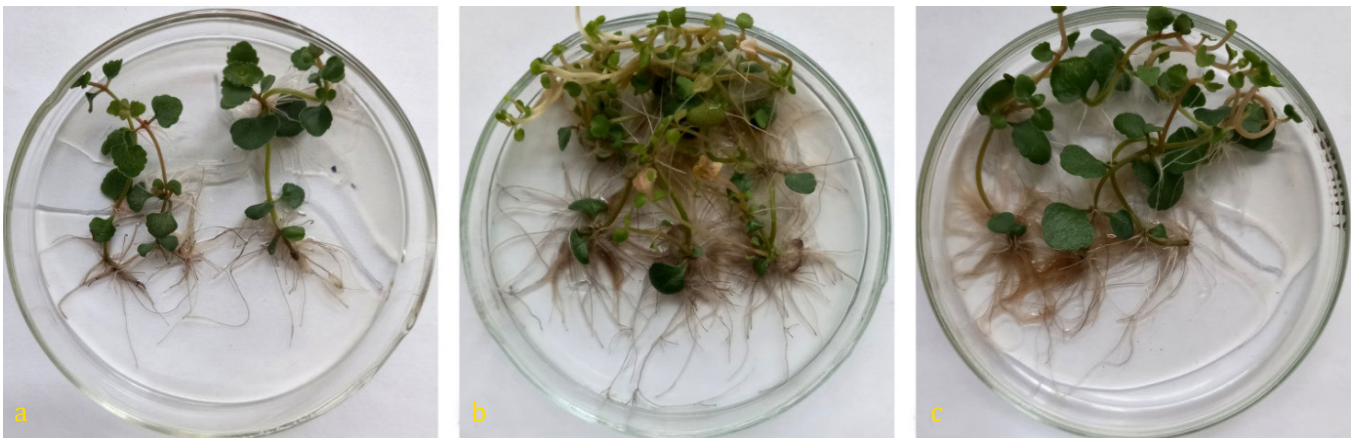

Figure 2 Growth of *Rhodiola rosea* L. plants on ½ MS medium
a – without test solution (the control); b – with the addition of the test solution, 1 $mL.L^{-1}$; c – with the addition of the test solution, 10 $mL.L^{-1}$

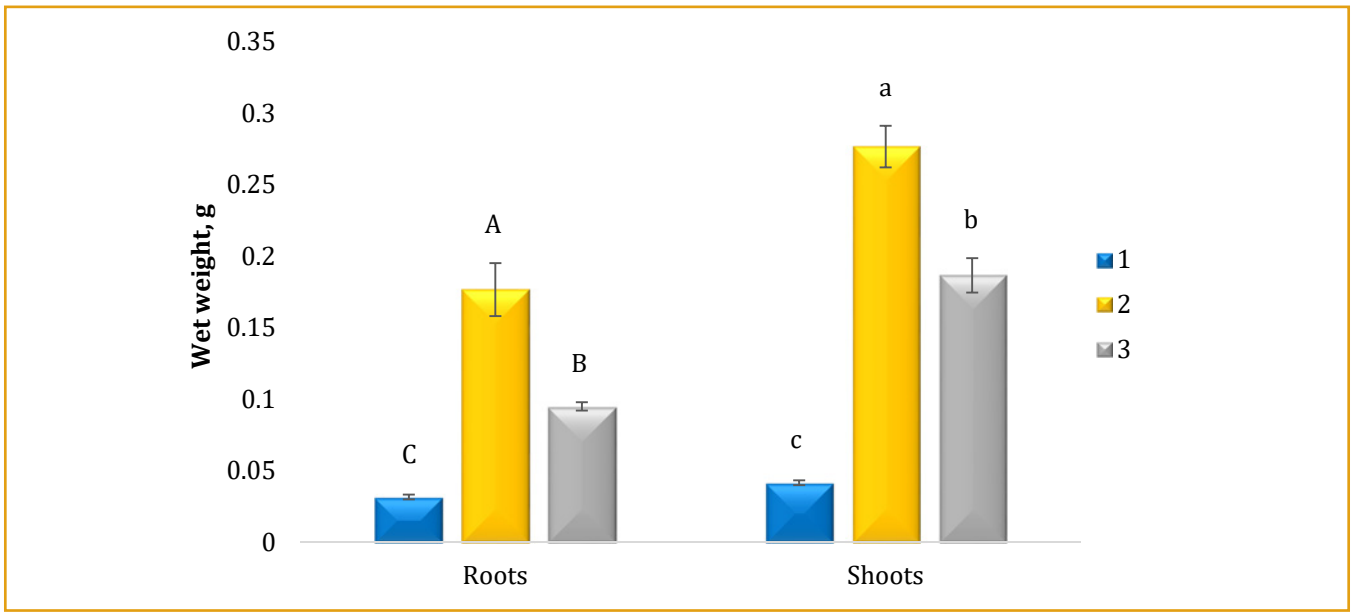

Figure 3 Weight of *Rhodiola rosea* L. plants (shoots and roots) grown on ½ MS medium
1 – the control; 2 – with the addition of the test solution, 1 mL.L^{-1}; 3 – with the addition of the test solution, 10 mL.L^{-1}. Upper and lower case letters belong to different comparisons

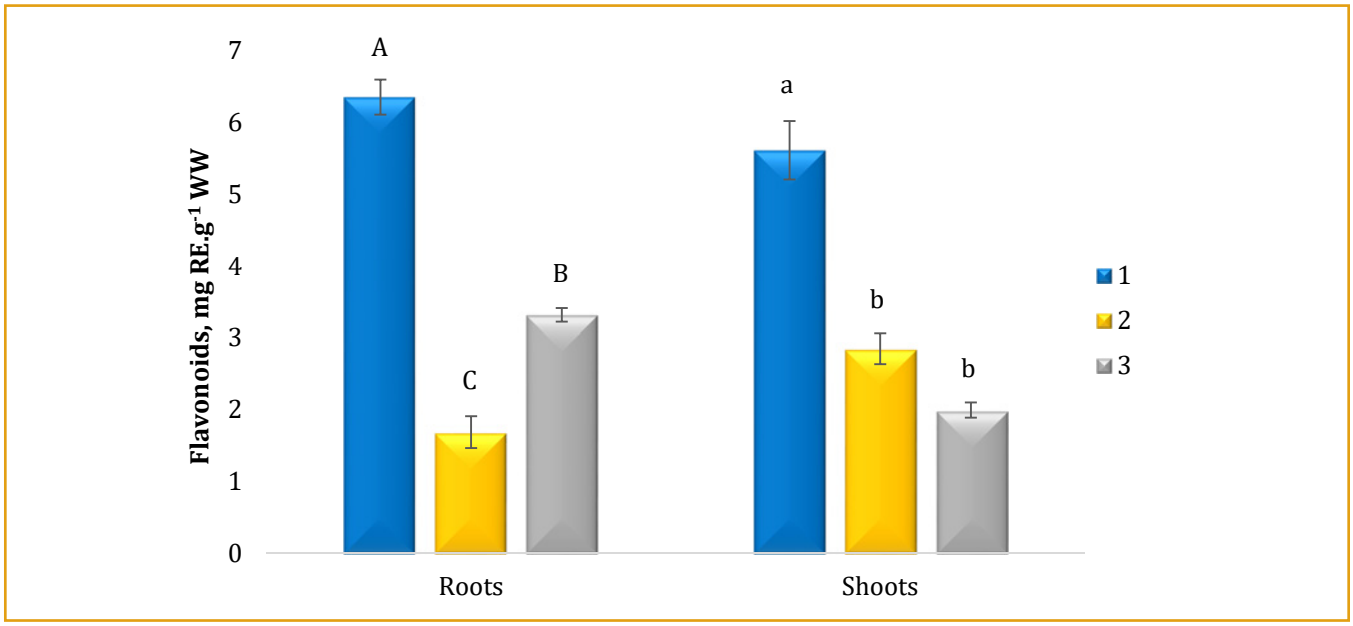

Figure 4 Specific content of flavonoids in shoots and roots of *Rhodiola rosea* L. plants on ½ MS medium
1 – control; 2 – with the addition of the test solution, 1 mL.L^{-1}; 3 – with the addition of the test solution, 10 mL.L^{-1}. Upper and lower case letters belong to different comparisons

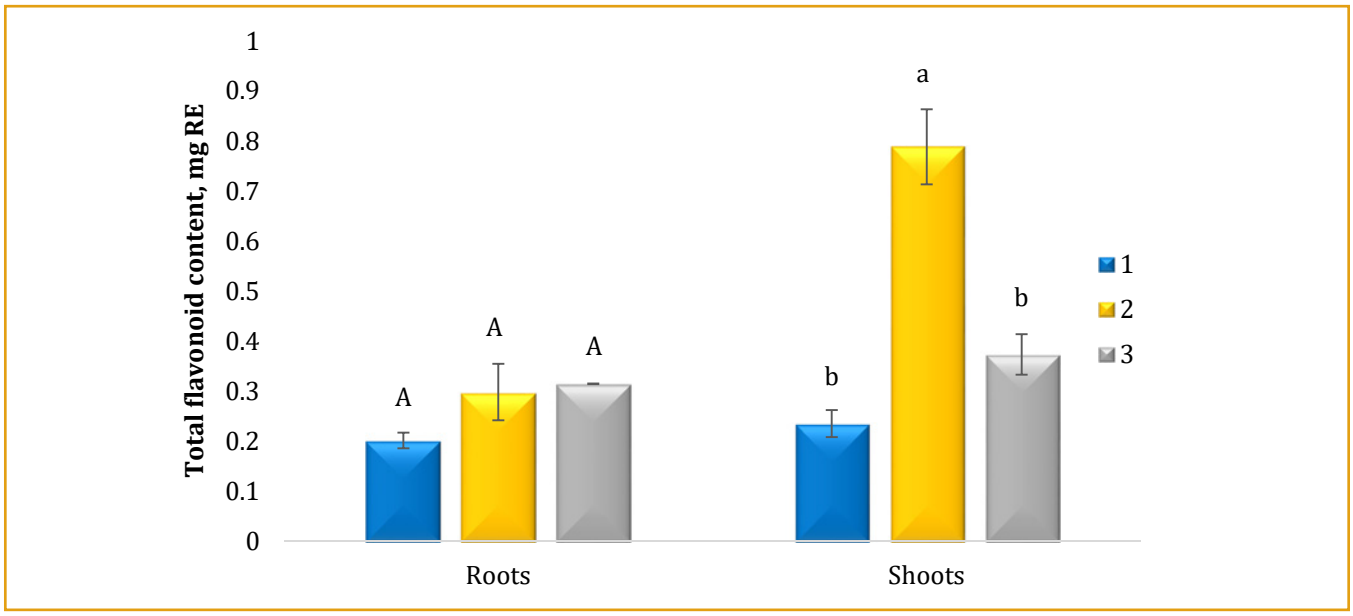

Figure 5 Total content of flavonoids in shoots and roots of *Rhodiola rosea* L. plants on ½ MS medium
1 – control; 2 – with the addition of the test solution, 1 mL.L^{-1}; 3 – with the addition of the test solution, 10 mL.L^{-1}. Upper and lower case letters belong to different comparisons

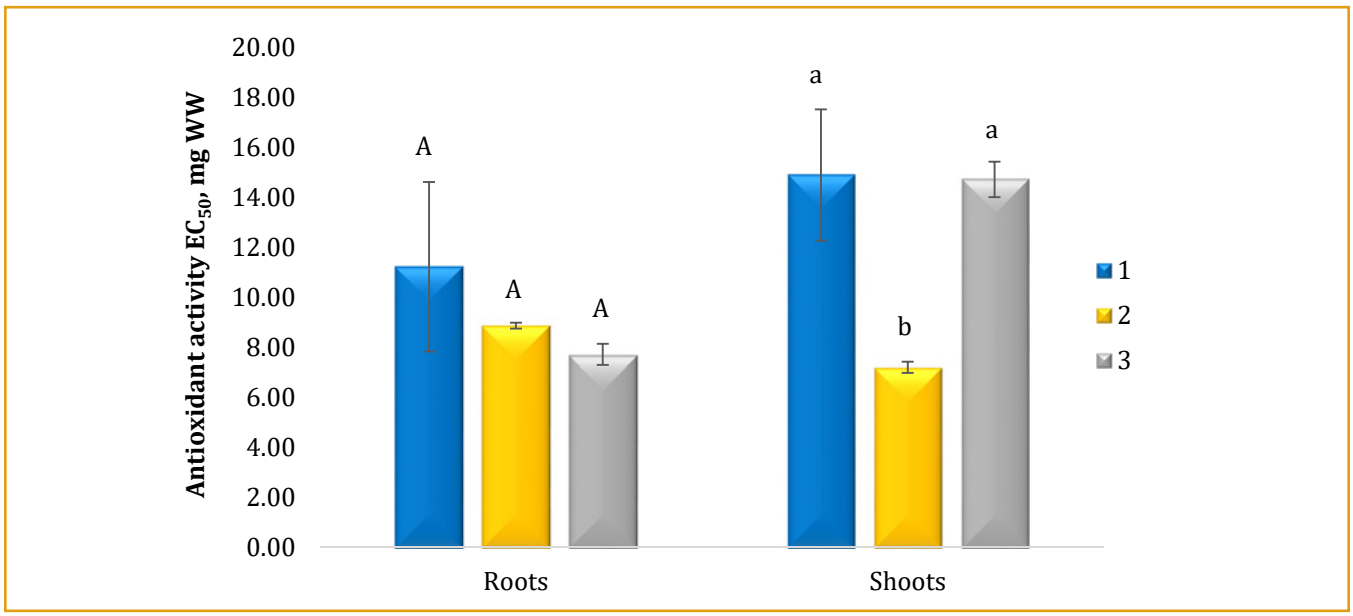

Figure 6 The antioxidant activity of root and shoot extracts of *Rhodiola rosea* L. plants
1 – control; 2 – with the addition of the test solution, 1 mL.L^{-1}; 3 – with the addition of the test solution, 10 mL.L^{-1}

The addition of alginite extract in a lower concentration has led to an increase in the level of antioxidant activity expressed in decreased equivalent concentration EC_{50} (Figure 6). When using the test solution in a lower concentration (1 mL.L^{-1}), a significant increase in the level of antioxidant activity in extracts from shoots was observed. However, using the test solution at a concentration of 10 mL.L^{-1}, the changes in antioxidant activity were within statistical error. The antioxidant activity of root extracts in the experimental variants also was not increased significantly.

The obtained results indicate significant stimulating activity of alginite extract. This effect was manifested both on roots and on shoots and was expressed in a 3.0–6.64-fold weight increase. The effect may be associated with humic acids in alginite (Barančíková et al., 2003), known as plant biostimulators. The stimulation effect

of these components was studied earlier (Chen et al., 2004). Authors have shown their positive influence on seed germination, root initiation, and total biomass growth of melon, ryegrass, and soybean plants. The humic acids improved Fe and possibly Zn nutrition in treated plants. Humic acids also promoted root growth (Jindo et al., 2020). It was studied that different biostimulants can affect root and shoot growth and increase plant biomass (Nardi et al., 2002; Jindo et al., 2012; Tužinský et al., 2015; Kim et al., 2019). They also affected the antioxidant and free radical-scavenging activities in treated plants (Zhu et al., 2006) and prevented plants from the negative effect of oxidative stress (Çimrin et al., 2010; Nephali et al., 2020; Omidbakhshfard et al., 2020). Alginite was also studied as a biostimulator that increases antioxidant activity in treated plants (Brindza et al., 2021a, 2021b; Eftimová et al., 2021; Horčinová Sedláčková et al., 2021).

Different aspects of alginite application as a possible biostimulant and its characteristics were studied in some institutions earlier (Beláček et al., 2002; Sarvašová, 2009; Nemcová et al., 2012–2015; Litavec and Barančíková, 2013; Barančíková and Litavec, 2016; Styková et al., 2016; Strompfová et al., 2018). Previously, according to the results obtained by scientists from the Slovak University of Agriculture in Nitra in 2021, the treatment of lawns (grass mixtures Liga, Midi, Renova, localization in Šajdíkove Humenice, Slovak Republic) with alginite extract containing humic acids led to increases in plant biomass. Such treatment also increased the seed and fruit weight of *Cucurbita pepo* L. var *oleifera* cultivated in Nitra in 2021. Leaves treatment increased the yield of *Triticum aestivum* L. var *aestivum* and *Avena sativa* L. (Vígľaš-Pstruša location) (Scientific report "Characteristics of treatment by alginite products in agriculture", Nitra, 2022, Slovak Agricultural University in Nitra).

Such results demonstrated the broad possibilities of using alginite extract to stimulate plant growth in field conditions. Our research showed that the *in vitro* addition of alginite extract solution also increased in biomass and accelerated the growth of the root system of *R. rosea* plants. This indicates that Alganite is a non-specific biostimulant of a vast spectrum of activity.

Conclusions

The addition of the test solution (1% alginite extract) at a concentration of 1 $mL.L^{-1}$ of the medium has led to a significant activation of the growth of both shoots and roots of Rhodiola plants in *in vitro* conditions. Such stimulation was accompanied by decreased specific flavonoid content in shoots and roots. However, due to the significantly greater weight of the plants, the total flavonoid content in the experimental variants was higher than in the control. An extract of alginite in a small concentration can be used both to stimulate the growth of *Rhodiola rosea* L. plants and to obtain an increased amount of flavonoids.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

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Analysis of the structure of flora of artificial phytocoenosis and assessment of its competitiveness against invasion of alien plants and their suitability for the creation introduction populations of rare species of the Caucasian flora

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The flora and its immigration groups in the plot "Caucasus" in M.M. Gryshko National Botanical Garden (Kyiv) were studied. Their compositions and stability of formed artificial phytocoenoses were analyzed. During 70 years of directed job on the phytogeographical plot "Caucasus" a number of flora complexes have been successfully formed, in which the vegetation of the Caucasus has been modeled. Those artificial phytocoenoses in which the share of plant species of the Caucasian flora is high and predominates over the share of other plant ˢpecies, the authors consider as mature. The prevalence of specially introduced species among the edificators is especially important for the full-fledged modeling of a plant group under *ex situ*. These in the studied plot "Caucasus" include forest stands, they were the most resistant to phytoinvasions. Artificial florocomplexes of sparse forests, steppes, and meadows also have a high species diversity of introduced species, but in this, they are significantly inferior to the corresponding phytocoenoses of the Caucasus. In general, there is a tendency that in the conditions of the botanical garden artificial phytocoenoses with the formed coenotic structure are resistant to the settlement of local plants and weeds and in their composition grows mainly a high proportion of rare plant species. Those artificial phytocoenoses in the plot "Caucasus", in which the share of introducers is less than 30%, should not be considered as formed, as such plantings are unstable to the spread of weeds and invasive plants. A tendency was found that mature artificial phytocoenoses are the most suitable for the successful formation of populations of rare species of Caucasian flora.

Keywords: National Botanical Garden, biodiversity, artificial phytocoenoses

Introduction

At the current stage of the development of biological sciences, the invasion of alien organisms, which leads to phytopollution and a decrease in the diversity of native taxa, is an urgent problem (Mashhadi and Radosevich, 2004; Burda et al., 2015; Pyšek et al., 2020; Baquero et al., 2021; Szumańska et al., 2021; Vrabič-Brodnjak and Možina, 2022). It is important to develop management to reduce the negative impact of invasive organisms on local ecosystems and cultivated plants (Prabakaran et al., 2019; Baquero et al., 2021). In this connection, the issues of monitoring and studying the acclimatization and naturalization of alien plants in the scientific centers of their introduction – Botanical

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Gardens (Burda et al., 2015; Konaikova and Peregrym, 2023).

Currently, the importance of research on the spontaneous diversity of vascular plants in Botanical Gardens is growing (Galera and Sudnik-Wojcikowska, 2004; Atha et al., 2016). Due to significant work on the introduction of new plants, the spontaneous flora of vascular plants in botanical gardens are extremely diverse and dynamic; their inventory is often reduced to compiling lists of recorded taxa and draws attention to the need to monitor invasive species (Heywood and Sharrock, 2013). At the same time, the study of the structure of phytocoenoses in botanical gardens is little practiced, because artificial ecosystems in culture are rare, but the study of the resilience of ecosystems to the impact of invasive species is important (Burda et al., 2015; Vainoriene, 2021; Rakhmetov and Zaimenko, 2022). Therefore, a detailed study of the taxonomic composition of artificial phytocoenoses in botanical gardens and their resistance to biological invasion is relevant.

The leading and one of the largest scientific institutions of Ukraine, which studies the acclimatization of alien plants *ex situ*, is the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (NBG) (Zaimenko et al., 2018; Rakhmetov and Zaimenko, 2022; Zaimenko and Rakhmetov, 2022). Currently on the territory of this institution stable artificial phytocoenosis, which are integral landscape elements of the NBG and to some extent model the vegetation of several regions of the temperate zone of Eurasia are presented. The species composition of phytocoenoses requires constant monitoring in order to rationally care for the plots, project formation of plantings, and scientifically sound replenishment of collections. When inventorying the flora of collection plots, as a rule, the establishment of the full species composition is not targeted, and wild (native and alien) plant species are often ignored. Instead, the ratio of introduced plants in the artificial phytocoenosis can be an indicator of the success of its formation, maturity, and stability. It is well known that natural communities show the highest resistance to phytoinvasions, and their anthropogenic disturbance leads to the loss of horizontal and vertical coenotic connections, penetration of invasive species, and gradual transformation (Bulakh, 2010; Burda et al., 2015). Resistance to phytoinvasions is one of the integral features of a mature natural phytocoenosis.

The artificial phytocoenosis is an artificial ecosystem, but depending on the purpose of its formation, it may have the same characteristics as native phytocoenosis: tiered, complex structure, mosaic, etc. It is natural that in the case of successful selection of the species composition of the introduced artificial phytocoenosis will be quite resistant to the negative impact of alien species, and therefore will acquire the maturity inherent in the native phytocoenosis.

This study was aim to conduct a comprehensive inventory of the plant species composition both in individual exposition sections and the plot as a whole; to analyze the resistance of artificial phytocoenoses to the spread of invasive plants depending on the immigration structure of the species composition of their flora.

Material and methodology

Location of the study

The research was conducted during the last decade (2010–2020) based on the phytogeographical plot "Caucasus" of the M.M. Gryshko National Botanical Garden, National Academy of Sciences of Ukraine (NBG). Information on the species composition of the collection in the plot "Caucasus", the structure of individual exposition sections, as well as rare plant species in the collection and introductory populations, have already been given earlier (Didenko, 2016; Didenko, 2018; Shynder, 2015, 2019).

Objects of research

The plot "Caucasus" currently has a heterogeneous vegetation and consists of about 20 individual sections and dendrogroups. Artificial phytocoenoses representing different geographical regions of the Caucasus were formed in these sections. 8 main sections have the largest areas and high presentability: beech forests, deciduous forests, Talysh, woodland, maple grove, birch grove, meadows, and steppes (Figure 1 and 2). In 1950–1960, hardy plants imported from the Caucasus were specially planted on these flora complexes to create artificial phytocoenoses in Kyiv. Now these 8 sections are the models of vegetation of the Caucasian region. Also, the structure of artificial plantations of plane tree alley is compared – this artificial phytocoenosis is not a model of natural vegetation, but is an ornamental park plantation. An artificial phytocoenosis with the participation of Caucasian plants spontaneously formed in the alley of plane trees.

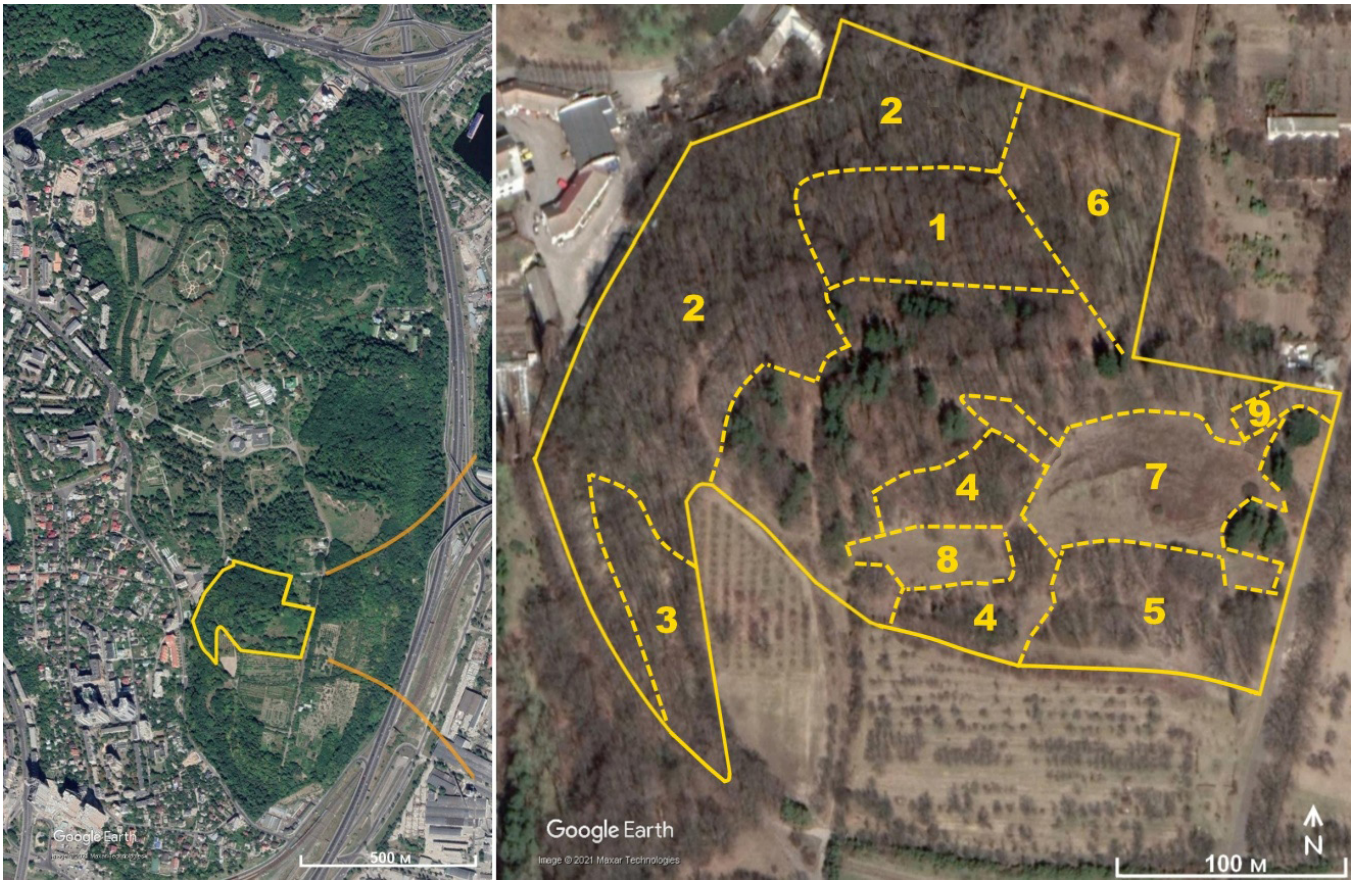

Figure 1 Map of the plot "Caucasus" on the territory of the National Botanical Garden (left) and the scheme of its division into individual sections (right)
1 – Beech Forests; 2 – Deciduous Forests of the Great Caucasus; 3 – Talysh; 4 – Woodland; 5 – Maple Grove; 6 – Birch Grove; 7 – Meadows; 8 – Steppe; 9 – Platans Alley

Immigration groups of plants

Plants in the artificial phytocoenoses of the plot "Caucasus" for analysis purposes were divided into 5 immigration groups:

1. species that are within the native flora of the Caucasus region;
2. other introduced species that are not native in the Caucasus but have been planted for various reasons;
3. native species, absent in the natural flora of the Caucasus;
4. alien species, xenophytes;
5. alien species, escaped plants (ergasiophygophytes).

Species of the first two groups are part of the cultivated plants of the NBG, although they often grow spontaneously. Species from other immigration groups form a spontaneous flora of the botanical plot and are not subject to collection monitoring. The structure and formation of the spontaneous flora of the NBG are revealed in detail in a special series of articles (Shynder, 2019).

Results and discussion

The first plants from the Caucasus were brought to the NBG in 1946. In that period, up to 1600 types of vascular plants of the Caucasian flora were introduced into the plantation. Most of them were first introduced in Ukraine. The florocomplexes formed on the plot had a wide ecological range – from coastal to semi-desert. The results of the introduction and acclimatization of many species of Caucasian flora are highlighted in the works of Kharkevych (1966). It should be noted that the author took into account the negative experience of predecessors in the establishment and formation of plant communities in terms of introduction outside their range. Kharkevych emphasized that it is practically impossible to protect artificial phytocoenosis of the phytogeographical plot from invasions of alien and native plant species (Kharkevych, 1966). But artificial plant communities in the botanical garden can be very close to natural phytocoenoses in species and coenotic structure (Shynder, 2015; Didenko & Shynder, 2020). Namely, a well-formed and complex structure of the

Figure 2 Artificial phytocoenosis on plot "Caucasus" of the M.M. Gryshko National Botanical Garden
1 – Beech Forests; 2 – Deciduous Forests; 3 – Talysh; 4 –Woodland; 5 – Birch Grove; 6 – Meadows; 7 – Steppe; 8 – Platans Alley

phytocoenosis is the best natural barrier against the penetration of invasive plants (Elton, 1958; Protopopova et al., 2002).

Much later, in the phytogeographical plots of Gryshko National Botanical Garden, sociological studies of rare plants and their populations became important. The inventory period began in 2003. 218 introduced species of flora of the Caucasus, which grow in the plot "Caucasus", were noted. In addition, the study of the status of introduced populations of rare species of Caucasian plants has been started. Little attention was paid to the study of artificial phytocoenosis and its structure. From 2010 to 2014, an inventory of the species composition of the flora and vegetation of this area was carried out (Shynder, 2014; Shynder and Kruglyak, 2015). According to the results, it was established that a total of 406 plant species from 89 families grow on this site; of them, there are 350 species of Caucasian flora from 83 families.

Since 2014 the collection of living plants in the plot has been significantly supplemented by newly introduced species from the Caucasus (Didenko, 2016, 2019), and many previously lost taxa have been restored. Given this, the species composition of the collection needs to the monitoring, and the database needs in the adjustment.

According to the results of the inventory of the collection and spontaneous flora of the main allotments in the plot "Caucasus", modern information on their species diversity was obtained (Table 1) (Didenko and Shynder, 2020).

Consider in more detail the taxonomic composition of plants in individual phytocoenoses in the plot "Caucasus". The floristic section "Beech forests" covers an area of 0.5 ha and has a fully formed structure of tree and grass tiers. The shrub layer is quite sparse due to significant shading. Within the allocation, 62 species of plants grow, of which 39 species are introduced from the Caucasus, that is the majority, including woody – 7, shrubby – 5, and herbaceous perennials – 25. The share of alien plants is very indicative, which is only 3.33% of xenophyte species (*Impatiens parviflora* DC. and *Oxalis stricta* L.) do not pose a threat to the formed populations of perennial plants of Caucasian origin. In this section, relatively few (10) native perennials are represented. Thus, in the composition of beech artificial phytocoenosis alien species play almost no role, which indicates its maturity and resistance to invasion.

The deciduous forests unite the tree stands of the former lowland non-flooded forests, tugai forests, and deciduous forests of the Greater Caucasus. During long-term acclimatization, some of the tree species that were planted as edificators of these allotments fell out of the plantations, so it is now advisable to combine these stands into one prefabricated forest allotment with an area of about 1.5 ha. Its stand is formed by *Carpinus betulus* L., *C. orientalis* Mill., *Fraxinus excelsior* L., and species of the genera *Acer* and *Ulmus* with the participation of many other tree species. In total, 114 species of plants were recorded in this floristic section, 67 of which were introduced from the Caucasus. The shrub layer is sparse and represented by only 6 Caucasian introducers, and there are 46 introducers of the flora of Caucasus in the composition of the herbage.

The share of alien plants is slightly more than 5% and currently, they do not pose a significant threat

Table 1 The correlation of immigration groups of the species in individual sections of the plot "Caucasus"

Sections	Immigration groups of the species					Total
	cultivated plants of the Caucasian flora	cultivated plants of other floras	native plants	xenophytes	escaped plants from other plots of the Botanical Garden	
Beech Forests	39 (62.9%)	3 (4.8%)	18 (29.0%)	2 (3.2%)	–*	62 (100.0)
Deciduous Forests	67 (58.8%)	10 (8.8%)	31 (27.2%)	3 (2.6%)	3 (2.6%)	114 (100.0)
Talysh	36 (49.3%)	6 (8.2%)	21 (28.8%)	4 (5.5%)	6 (8.2%)	73 (100.0)
Woodland	62 (43.4%)	3 (2.1%)	54 (37.8%)	12 (8.4%)	12 (8.4%)	143 (100.0)
Maple Grove	22 (36.7%)	5 (8.3%)	21 (35.0%)	4 (6.7%)	8 (13.3%)	60 (100.0)
Birch Grove	14 (30.4%)	–*	25 (54.3%)	4 (8.7%)	3 (6.5%)	46 (100.0)
Meadows	63 (37.7%)	2 (1.2%)	70 (41.9%)	17 (10.2%)	15 (9.0%)	167 (100.0)
Steppe	34 (39.1%)	1 (1.1%)	43 (49.4%)	6 (6.9%)	3 (3.4%)	87 (100.0)
Platans Alley	19 (31.1%)	2 (3.3%)	30 (49.2%)	8 (13.1%)	2 (3.3%)	61 (100.0)

to introductory populations. In contrast to the beech floristic section, the phytocoenose of which is actually represented by one forest group, the stand of deciduous forests is polydominant. According to the ratio of immigration groups in its species composition, this floristic section is also stable and mature.

In the southern part of the plot "Caucasus" is a section of the Talysh relict forest. This section has great scientific value as a well-formed model of the Talysh forest and on quantity of rare and endemic species. In total, 73 species grow in this section; almost half of them (36 species) were introduced from Talysh. The share of native species is slightly more than a quarter, and alien – more than 13%. This immigration structure indicates that the vegetation cover on the plot is less stable compared to the other forest sections discussed above. This can be explained by the depleted structure of tree and shrub tiers in this section due to the mismatch of climatic conditions of Talysh and Kyiv. Therefore, the artificial model of forest coenosis from this remote Caucasus region now has a lot of free ecotones in the phytocoenotic structure, and therefore is quite prone to settlement by native and alien plants. Among the latter, there are a number of expansive, in particular: *Berberis aquifolium* Pursh, *Celtis occidentalis* L., *Lonicera tatarica* L., *Parthenocissus inserta* (A. Kern.) Fritsch, which increases the need for control of vegetation in this section.

Woodlands include the artificial phytocoenosis of oak crooked forest and arid sparse forest and are presented mainly in the form of ecotonic band groups around the steppe section. Under the tent of trees, a large number of shrubs and woods outskirt species of plants have found shelter here. Among them are 14 mostly low-growing trees, such as *Prunus mahaleb* L., *Crataegus pentagyna* Waldst. & Kit., *Juniperus foetidissima* Willd., *Quercus macranthera* Fisch. & C.A.Mey. etc., 12 shrubs, 34 herbaceous perennials. A significant share (37.76%) of the species composition belongs to native species, among which herbaceous perennials predominate, and a fairly significant share (16.78%) is occupied by alien plants. This artificial phytocoenosis was favorable for the settlement of aboriginal plant species, many of which in the conditions of NBG are confined to such habitats. The formed artificial floristic groups have rather steady and mature structures of wood and bush tiers, but the grass stand on the structure is heterogeneous. In general, the modeling of ecotone groups on the plot was quite successful, because the more long-lived tree and shrub tiers, which are a kind of phytocoenotic framework, were resistant to phytoinvasions.

The stands of the maple grove in the plot "Caucasus" were formed by species of the genus *Acer* with the participation of *Fraxinus angustifolia* Vahl subsp. *oxycarpa* (M.Bieb. ex Willd.) Franco & Rocha Afonso. High proportions of native (35.0%) and alien (20%) species of flora indicate that this phytocoenosis is far from mature and not resistant to phytoinvasions. Many years of experience in keeping the plot shows that the artificial phytocoenosis in the maple grove is under the constant influence of successions, in particular, due to the abundant undergrowth of *Acer* spp., *Cornus sanguinea* L. subsp. *australis* (C.A.Mey.) Jáv. C.A.Mey, *Celtis occidentalis* L., etc. Therefore, its existence in a state of a grouping of the park type, most suitable for the successful growth of introductory populations of rare species here, is possible only with the periodic clearing of undergrowth.

The basis of the birch grove is the stand *Betula pubescens* Ehrh. var. *litwinowii* (Doluch.) Ashburner & McAll. and *B. pendula* Roth. This section presents only 14 species that were introduced from the Caucasus, ie less than a third of the total species composition, and native flora species predominate in the floristic composition (54.35%). Currently, this artificial phytocoenosis is in a state of degradation and needs artificial support and rejuvenation. The meadow section presents various groups of meadow vegetation of the Caucasus, for example: mountain tall grass and steppe-meadow grass. In total, there are 167 species in the area of 0.6 ha, including 63 introduced from the Caucasus. The shares of native (41.92%) and alien (19.16%) species of flora are relatively high here. It should be noted that among perennial grass species in the area, Caucasian introducers make up about 46% and are slightly inferior to native species. Therefore, this artificial phytocoenosis, although marked by high representativeness, but is not mature enough and not sufficiently resistant to the influence of primarily native and alien plant species of flora. However, among the latter, there are almost no edificators and dominants, and high phytocoenotic positions in this species have introducers from the Caucasus. Of the undesirable species, only the invasive alien species *Solidago canadensis* L. has a significant effect in the late aspect.

The Caucasian steppe section with an area of 0.2 ha is located on the slope of the southern exposition. Vegetation is represented by meadow-steppe phytocoenosis. Of the 87 plant species, only 34 species were introduced from the Caucasus. Interestingly, the flora of this section is almost twice as rich as the meadow section, and the migratory structures of the

flora in both these sections are very similar. The share of local plant species in the steppe section (49.4%) is significantly higher, and alien plants (10.4%) is much lower, which indicates a higher overall resistance of the heterogeneous phytocoenoses formed here to invasions of alien species. As part of the coenotic framework – a group of perennial grasses as in the meadow section, the shares of introducers from the Caucasus (46.8%) and native species (50.0%) are almost equal. Thus, the steppe artificial phytocoenosis turned out to be quite mature and resistant to the influence of alien plants, and to a large extent, this resistance is provided by a successful combination of populations of native and introduced meadow-steppe plant species.

As part of the artificial phytocoenosis of the dendrogroup – Platans Alley 61 species of plants were noted, of which only 19 were introduced. Half (49.18%) are native forest species of flora and a fairly high share of alien plants (over 16%). This artificial phytocoenosis is not a model of aboriginal phytocoenosis, so the migratory structure of its flora can be compared with other artificial phytocoenoses that have been specially formed.

Based on the comparison of the share of rare species in the composition of phytocoenoses, the sozological value of individual sections in the plot "Caucasus" was established. The largest number of rare species of flora of the Caucasus is represented in the artificial phytocoenosis in the Beech Forests 58.1% (Table 2). The species structure of this phytocoenosis is characterized by the largest share of Caucasian plants, and the share of weeds and native species is minimal. Thus, the section on Beech Forests is an example of the successful formation of an artificial coenosis in the Botanical Garden, which is resistant to alien species and has great sozological value. This became possible due to the successful selection and acclimatization in the Botanical Garden of the main tree species of beech forests, which have now formed the coenotic structure of natural communities.

In other sections, where the share species of flora of the Caucasus in the structure of the phytocoenosis is high, the share of rare species is 17.8–26.7% (Table 2). On this sections predominate in a small proportion of weeds and this also indicates the high phytocoenotic stability of artificial phytocoenoses in the botanical garden and their high sozological value. In the section Steppe, the share of species of flora of the Caucasus is only 40.2%, but more than half of them are rare. Dominant in this artificial phytocoenosis is mainly local meadow and steppe plants, therefore, it is not stable enough in the Botanical Garden, but its sozological value is high.

Finally, the vegetation in Birch Grove, Meadows, and Platans Alley was the least resistant to native plant species and weeds. The share of species of flora of the Caucasus here is 30.4–38.9%, and rare species among them are very few – only 4.3–8.2% (Table 2). Thus, in general, there is a tendency that fully formed phytocoenoses in the Botanical Garden are more resistant to the settlement of local plants and weeds and in their composition grow the mostly high proportion of rare plant species. Instead, unformed artificial phytocoenosis are not resistant to the penetration of local plants and weeds, and their sozological value is low.

Such a regularity is characteristic of natural ecosystems. It is widely known that invasive plant species are the fastest to expand on ruderal and disturbed phytocoenoses, and in native phytocoenoses alien plants are mostly unable to successfully take root and compete with a large number of native plants and their grouping (Elton, 1958; Protopopova et al., 2002, 2014; Burda et al., 2014; Miroshnik, 2016). Empty

Table 2 Rare plants in individual sections of the plot "Caucasus"

Section	Total species	Caucasian flora species	%	Rare species	%
Beech forests	62	42	67.7	36	58.1
Deciduous forests	114	77	67.5	25	21.9
Talysh	73	42	57.5	13	17.8
Woodland	143	65	45.5	28	19.6
Maple grove	60	27	45.0	16	26.7
Birch grove	46	14	30.4	2	4.3
Meadows	167	65	38.9	11	6.6
Steppe	87	35	40.2	19	21.8
Platans alley	61	21	34.4	5	8.2

Niche Hypothesis and Species Richness Hypothesis – both hypotheses are among the main theories that explain the phenomenon of phytoinvasions (Mosyakin, 2009). However, the taxonomic richness of vegetation of specific phytocoenoses is not always a guarantee of their resistance to the spread of foreign plants (Stohlgren et al., 2001, 2003). To date, all aspects of this process have not been fully explored. But, obviously, at different stages of development of phytocoenoses their stability is provided by different levels of species richness (Mosyakin, 2009).

In Botanical Gardens and Arboretums, the impact of invasive plant species is very noticeable, as most of the plantations are artificial, and therefore they are not resistant to the impact of aggressive alien plants. For example, in the M.M. Gryshko National Botanical Garden conducted experiments on the effect of invasive alien plants on artificial steppe phytocoenoses and it was confirmed that the greatest resistance to the spread of some foreign plants has formed phytocoenoses of perennial steppe grasses (Maryushkina, 2002). To date, in Botanical Gardens and Arboretums, the most invasive activity is mainly naturalized wood escaped plants, in particular, vines. They often begin to spread uncontrolled in ruderal areas where there is no natural vegetation (Kovtoniuk, 2019; Doiko et al., 2021). The experience of studying invasive plants in the M.M. Gryshko National Botanical Garden and Syretsky Dendrological Park of national importance shows that most foreign plants are found in open grassy areas, and in shaded forest areas such species are few (Shynder, 2019; Glukhova et al., 2020). 55 invasive plants have been marked in the In the park-monument of landscape art "Feofania" (Kyiv) and most of them are spreading uncontrollably in artificial plantations and ruderal phytocoenoses (Hubar and Konyakin, 2020). Monodominant plantations of introduced shrub and perennial herbaceous plants also have high resistance to the influence of invasive plants (Didenko, 2014, 2017; Shynder and Kruglyak, 2014). This is largely due to the fact that the indigenous vegetation in the city of Kyiv is forest and therefore plantations of trees and shrubs are generally more resistant, especially to the influence of herbaceous alien plants.

Thus, the phytogeographical plot "Caucasus" is represented by a whole complex of sections, which simulated the Caucasian vegetation. Currently, the most mature and resistant to phytoinvasions were forest phytocoenoses, primarily beech and deciduous. They are dominated by introducers, and the participation of alien plants is minimal. Thus, these artificial phytocoenosis are successful models of forest ecosystems of the Caucasus. Instead, birch and maple groves have proved to be unstable and require constant intervention to maintain their condition. Artificial phytocoenoses of woodlands, meadows and steppes also proved to be stable.

In those sections where the share of native and alien plant species is high, it is necessary to conduct a purposeful introduction of Caucasian flora species to fill the habitats and increase the overall competitiveness of the artificial phytocoenosis. For those formed by artificial phytocoenosis that model natural ecosystems, the desired share of introducers in the total species composition should be at least 40–50%. This is especially important for the main tier (sub-tier) – wood tier in forest phytocoenoses, shrub-tree tier – in shrub vegetation, and dominant sub-tier of perennial grasses in meadows and steppes. That artificial phytocoenosis in the plot «Caucasus», in which the share of introducers is less than 30%, should not be considered as formed. The identified patterns of stability of phytocoenoses depending on the immigration structure of their species composition can be successfully used not only in the introduction of plants into botanical gardens but also in predicting the consequences of the introduction process.

Conclusions

According to the results of the inventory of the taxonomic composition in the plot "Caucasus" in M.M. Gryshko National Botanical Garden, the artificial phytocoenoses that model the forest vegetation of the Caucasus region are mature. Artificial phytocoenoses of sparse forests, steppes, and meadows also have a great variety of introduced plants of the Caucasian flora, but in this, they are significantly inferior to the natural plant communities of the Caucasus region. Those artificial phytocoenoses, in which the share of introduced species is less than 30%, it is inexpedient to call formed. In general, there is a tendency that in the conditions of the botanical garden mature artificial phytocoenoses are the most resistant to the penetration of unwanted native plants and weeds. In the composition of such plant communities are usually found many rare plants that form stable populations.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Lavandula spp. diversity assessment by molecular markers as a tool for growers

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Lavender is one of the most important medicinal plants. The quality and the therapeutic properties of the essential oil of lavender are determined by the quantity and the biological activity of the individual components. Different lavender varieties often have a characteristic profile, differing in their individual substances, with small differences affecting the aroma and properties of the essential oil. Genetic variation between species and cultivars, as well as environmental conditions, nutrition, season, and type of tissue, all influence the chemical composition of lavender essential oil. The collection of twelve genotypes of *Lavandula* spp., which includes species of *L. angustifolia* (Mill.), and *Lavandula* × *intermedia* Emeric ex Loisel. were grown in the locality of Malé Leváre (Slovakia). These species, without further specification of varieties, were screened for DNA polymorphism by random amplified polymorphic DNA (RAPD) markers. Leaf and flower samples were analyzed using 4 universal random decamers. Two decamers (OPB 11 and OPB 18) provided efficient and reproducible amplification profiles. Individual genotypes and leaf and flower samples were characterized using DNA fingerprint cards containing digital electrophoretic profiles generated by random decamer primers and the corresponding image of the lavender genotype. This low-cost and effective approach to genetic diversity screening can provide useful documentation for lavender growers and additional genotype specifications.

Keywords: Lavender, RAPD, DNA fingerprinting, DNA polymorphism, genetic diversity

Introduction

One of the most commercially exploited species is *Lavandula angustifolia* Mill., which originated in the Mediterranean region, but today the cultivation of this species and its cultivars is widespread all over the world, including Slovakia. Species belonging to the genus Lavender are rich in secondary metabolites such as phenolic acids, flavonoids, coumarins, terpenes,

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and tannins (Hawrył et al., 2019). Lavender is one of the most important medicinal plants, and due to its calming effects, this species is popularly used as part of adjunctive treatment for anxiety conditions, to relieve psychological stress, or to induce sleep (Ghavami et al., 2022). Lavender is mainly cultivated for the production of lavender essential oil, which is used in perfumes, cosmetics, gastronomy, and aromatherapy (Lis-Balchin, 2002). The genus Lavender includes approximately 47 species, dozens of subspecies, and hundreds of hybrids (Prusinowska and Śmigielski, 2014). The most important and economically valuable species include: *L. angustifolia* Mill., *L. latifolia* Medik., *L. stoechas* L., and *L. intermedia* Emeric ex Loisel.

Different lavender species often have a characteristic profile, differing in the representation of individual compounds, with even small differences affecting the aroma and properties of the essential oil (Gonçalves and Romano, 2013; Demasi et al., 2018). Thus, the chemical composition of lavender essential oil is largely influenced by genetic variation among species and cultivars, but also by external factors such as, for example, temperature conditions, water quantity, altitude and geographical location, fertilizers, season, or the type of weed from which it was obtained (Lis-Balchin, 2002; Demissie et al., 2011; Hassiotis et al., 2014).

The morphological, agronomic, and other characteristics required to classify and identify plant genotypes within species are effectively complemented by molecular markers. The importance and usefulness of these characteristics are considerable, both for research and breeding as well as for practical use (Gálová et al., 2013, 2016). Studying and comparing the molecular information of individual organisms involves searching for DNA polymorphisms (Malik et al., 2017). Although random amplified polymorphic DNA (RAPD) has several limitations, it is still an effective tool for DNA polymorphism detection. It is low-cost method, detecting a large number of polymorphic loci and revealing genomic variation in coding and non-coding regions of the genome (Zhang et al., 2007; Hnia et al., 2013). RAPD uses a polymerase chain reaction to amplify random DNA fragments using short oligonucleotides, most commonly decamers. The nucleotide order of the primers is random, with 50-80% guanine content and cytosine. After propagation, the fragments are resolved in an agarose gel. In the PCR reaction, a single random primer species binds to genomic DNA at two different positions on the complementary strands of the template DNA. If these binding sites are within the amplifiable range, the specific DNA fragment is amplified. The presence of RAPD fragments in the electrophoretic profile corresponds to the dominant allele and the absence of the recessive allele. Amplified fragments range in size from 0.5–5.0 kb (Bežo et al., 2015).

The work aimed to apply a universal random molecular marker RAPD to obtain genotype-specific DNA fingerprints of lavender genotypes, specifically for leaves and flowers samples, and design for each variety a "DNA fingerprint card" documentation providing the grower with a closer specification of cultivated genotypes.

Material and methodology

A number of different genotypes of lavender plants have been made available by the lavender grower and owner of the company "Levanduland, Ltd." in the town of Malé Leváre (https://levanduland.sk/). Except for the area of 1 hectare, where are cultivated 13 000 seedlings of *L. angustifolia* Mill., the grower collected various species of lavender cultivated on a small experimental area. This collection includes species of *L. angustifolia*, and *Lavandula* × *intermedia* (Figure 1), without further variety specification.

Sampling

Three plants per genotype were collected, marked by the position code (designation of the line/the order of the plant in the row) for matching the results to individual genotypes (Figure 1). At the time of sampling, a single genotype (Figure 2 F) had not flowered yet, so the analyses come only from leaf samples. Immediately after collection, the samples were placed in a labeled bag and stored in a portable refrigerated container containing the frozen plates. Upon arrival at the laboratory, photo documentation (Figure 2) was made with close-ups of the flowers, and the plants were briefly stored at 20 °C.

Genomic DNA isolation

Genomic DNA from leaves and flowers of individual genotypes was isolated by NucleoSpin Plant II DNA isolation Kit (Macherey Nagel™) according to the manufacturer's instructions, with subsequent DNA quality and quantity control by Nanophotometer (Implen P360). Since the purity of the DNA obtained was not satisfactory due to the high abundance of secondary metabolites, the samples had to be purified.

Figure 1 View of the experimental area with different genotypes of lavender in the company "Levanduland, Ltd."

DNA purification

Samples were purified by adding 1/10 of a sample volume of 3 mol.dm^{-3} NaOAc pH 5.2 and 2.5 sample volumes of 100% EtOH. Samples were incubated overnight at -20 °C followed by centrifugation at 12000 rpm, for 20 minutes. After pipetting the supernatant, 400 µl of 70% EtOH was added to the DNA pellet and then centrifuged for 5 minutes at 12000 rpm. The precipitate was dissolved in 30 µl of ultrapure H2O.

RAPD-PCR Assay

RAPD primers sequences originated from a decameric oligonucleotides database: OPB-05 (5´ TGC GCC CTTC 3´), OPB-11 (5´ GTA GAC CCGT 3´), OPB-18 (5´ CCA CAG CAGT 3´) and OPB-20 (5´ GGA CCC TTAC 3´) were applied on leaf and flower samples of twelve lavender genotypes. Solis Dynazyme chemicals were used for RAPD-PCR assay realized by C1000 Thermal Cycler (BIO-RAD) as follows: 1 × Buffer B (0.8 mol.dm^{-3} Tris-HCl, 0.2 mol.dm^{-3} $(NH_4)_2SO_4$), 2.5 mmol.dm^{-3} $MgCl_2$, 0.2 mmol.dm^{-3} dNTP, 2U FIREPol DNA polymerase, 0.4 µmol.dm^{-3} primer and 30 ng of DNA. The amplification of each sample was repeated twice. The amplification protocol was as follows: initial denaturation at 94 °C for 2 minutes, followed by 45 cycles of denaturation at 94 °C for 1 minute, annealing at 36 °C for 1 minute, polymerization at 72 °C for 2 minutes, and the final polymerization at 72 °C for 7 minutes.

Electrophoresis of amplified products

The amplification assay was followed by electrophoresis analysis on a 1.5% agarose gel with a 1 kb size marker (Bioron, ready to use). A loading dye (6 × loading dye, Invitrogen) was added to the PCR samples and 10 µl of the sample was loaded onto the gel. Electrophoresis was performed at a constant voltage of 100 V for 45 minutes.

Statistical analysis

Amplification of each sample followed by electrophoretic separation of DNA fragments was repeated twice. The electrophoreograms were recorded by GeneSnap software (Syngene). The amplification results were processed based on digital recordings of electrophoreograms by GeneTool Analysis software (Syngene).

Figure 2 Lavender genotypes collected from the experimental area: A–L represent individual lavender genotypes

Results and discussion

Genomic DNA polymorphism of twelve different lavender genotypes of various species (*L. angustifolia*, and *Lavandula* × *intermedia*), was analyzed by four RAPD primers (OPB-05, OPB-11, OPB-18, and OPB-20). Two of them (OPB5 and OPB 20) did not provide efficient amplification profiles for all samples analyzed. DNA polymorphism of analyzed samples was observed with primers OPB-11 (5´ GTA GAC CCGT 3´) and OPB-18 (5´ CCA CAG CAGT 3´). By the primer, OPB-11 have amplified 127 DNA fragments in total, of which 64 in leaf samples and 63 in flower ones. For the primer OPB-18 has observed 151 DNA fragments in total, out of which 73 were for leaf and 78 were for flower samples.

The main output of this study was to provide growers with documentation in the form of 'DNA fingerprint cards' for each lavender genotype grown in the trial area. DNA fingerprint cards generated by RAPD marker OPB-11 are presented in Figures 3–5 and by marker OPB-18 in Figures 6–8. For each genotype, cards are provided separately for leaf and flower samples.

There are different types of molecular markers. They may differ in a number of ways – such as their technical requirements; time and price complexity (Žiarovská et al., 2011, 2016; Gálová et al., 2013, 2016). The presence of polymorphisms between individuals will lead to a different pattern of markers after electrophoresis has been performed. These patterns are comparable to a "fingerprint", therefore these techniques are

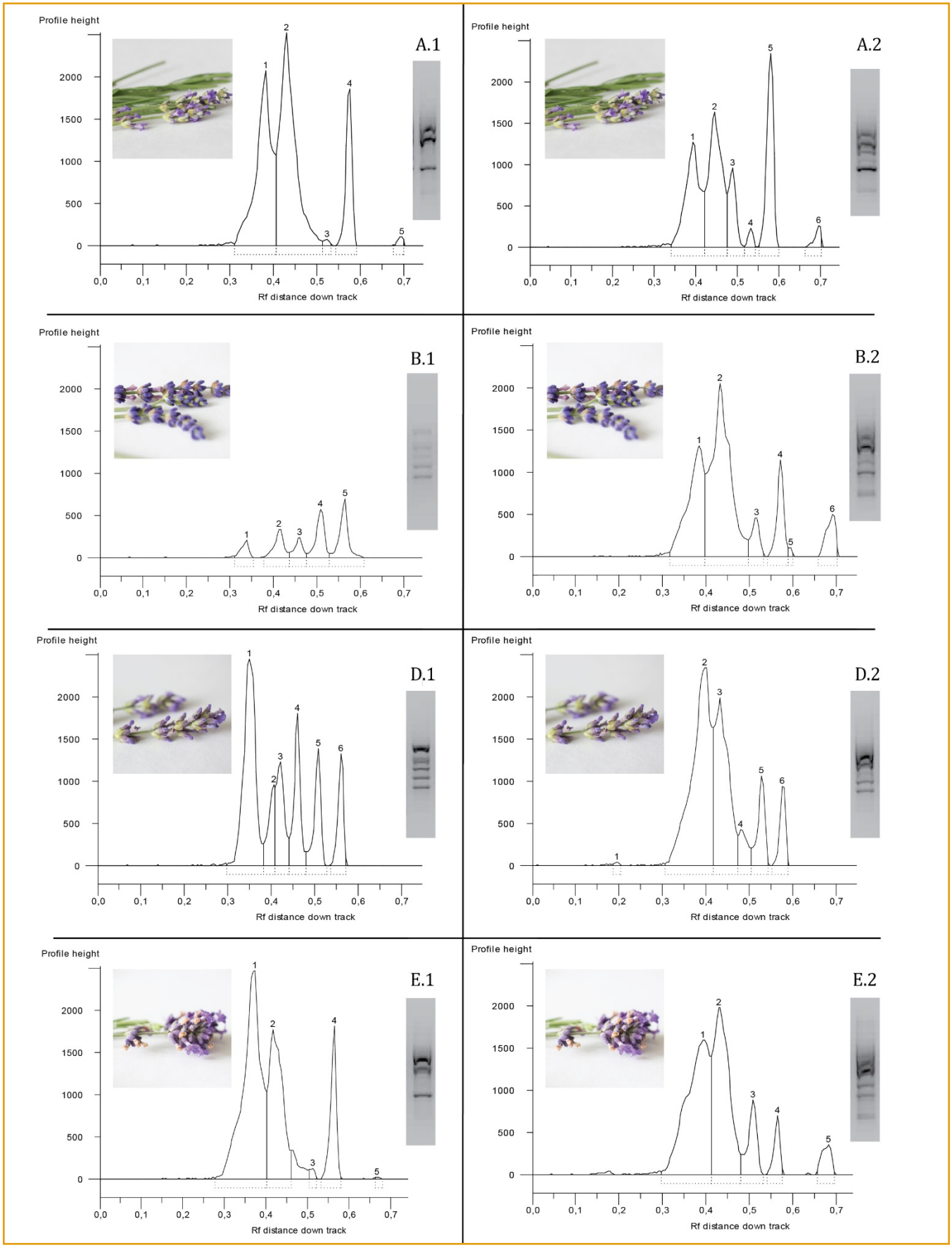

Figure 3 DNA fingerprint cards generated by OPB-11
A–D represent lavender genotypes; 1 – leaf sample; 2 – flower sample

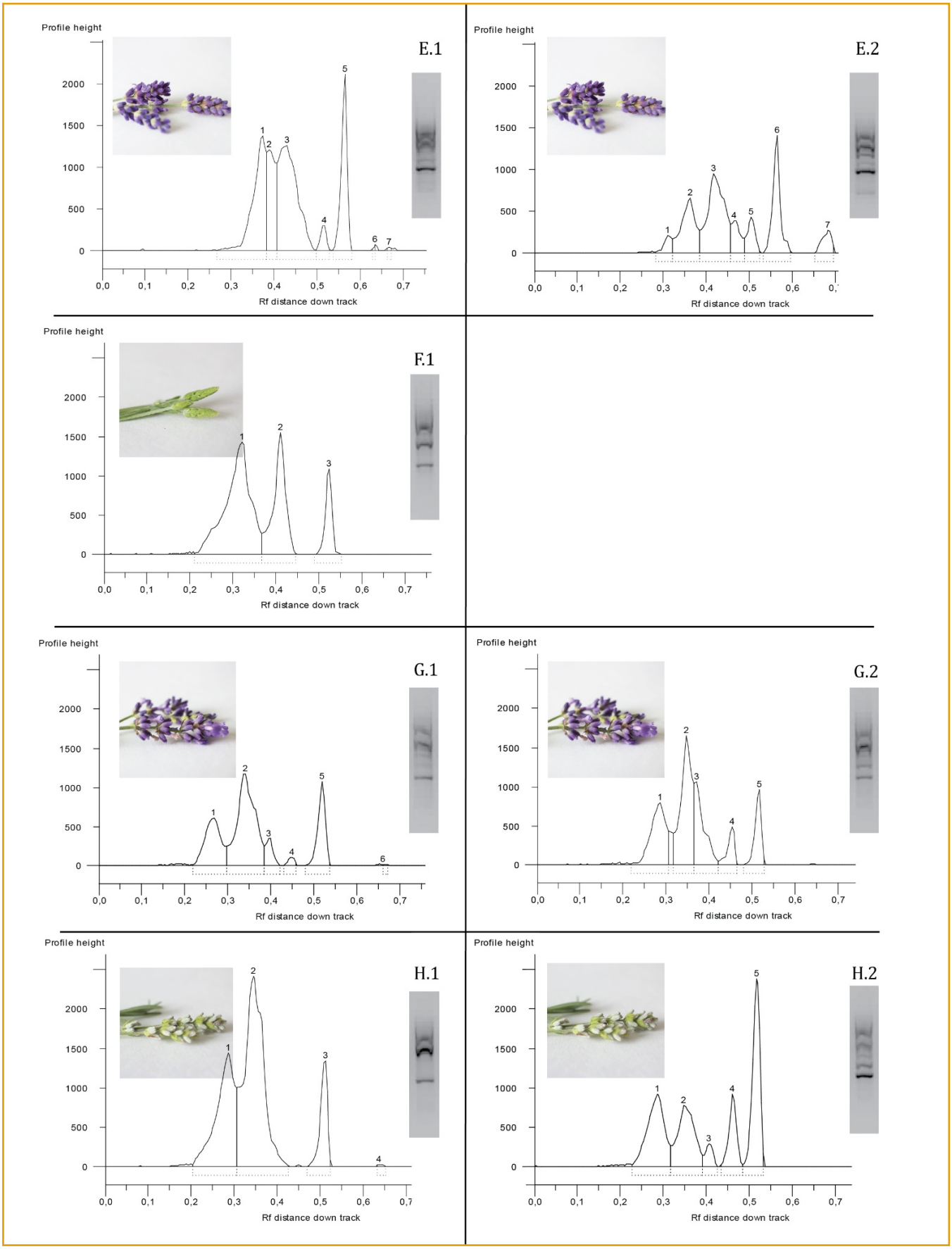

Figure 4 DNA fingerprint cards generated by OPB-11
E–H represent lavender genotypes. 1 – leaf sample; 2 – flower sample. Genotype F was the only one not yet flowering at the time of sampling

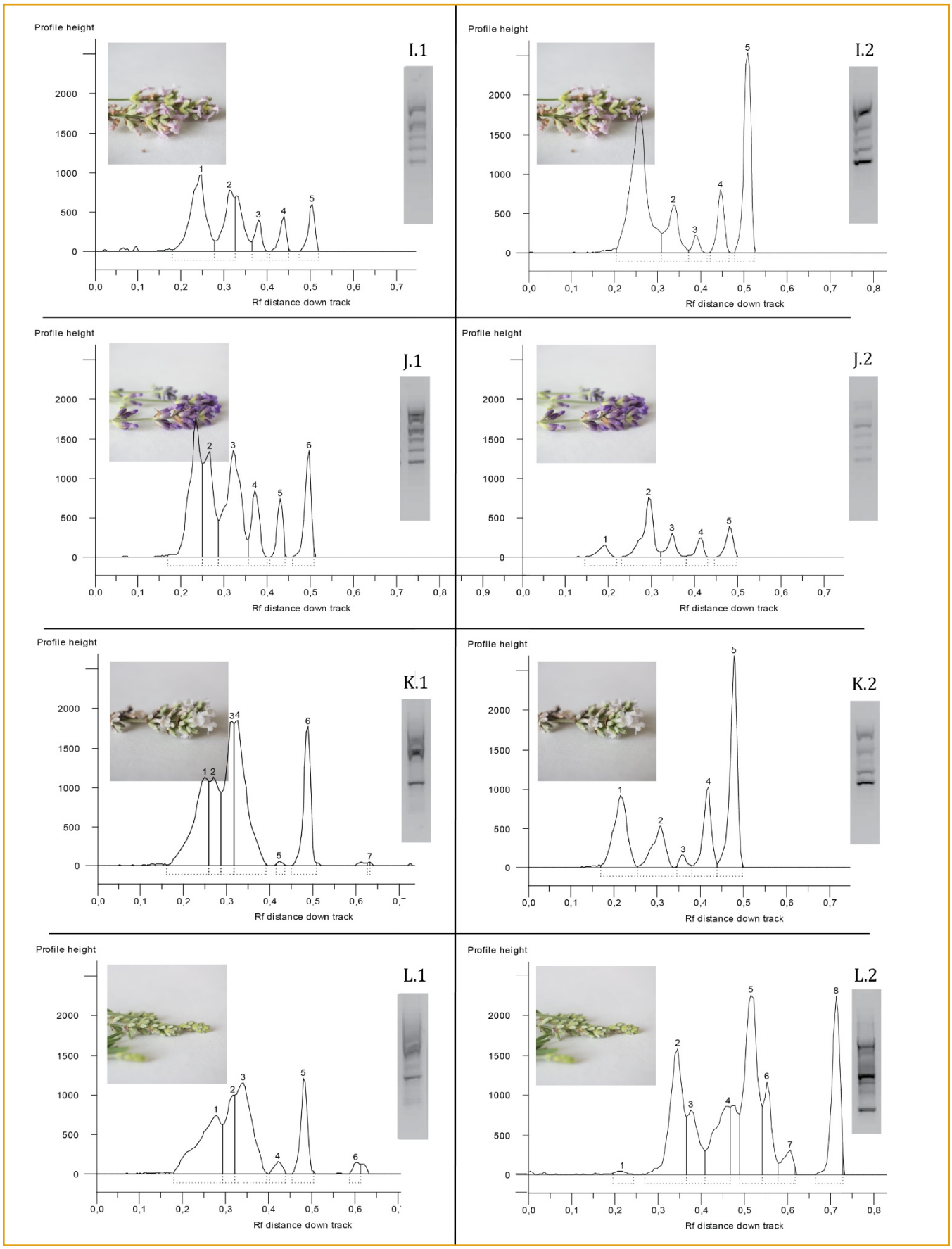

Figure 5 DNA fingerprint cards generated by OPB-11
I–L represent lavender genotypes. 1 – leaf sample; 2 – flower sample

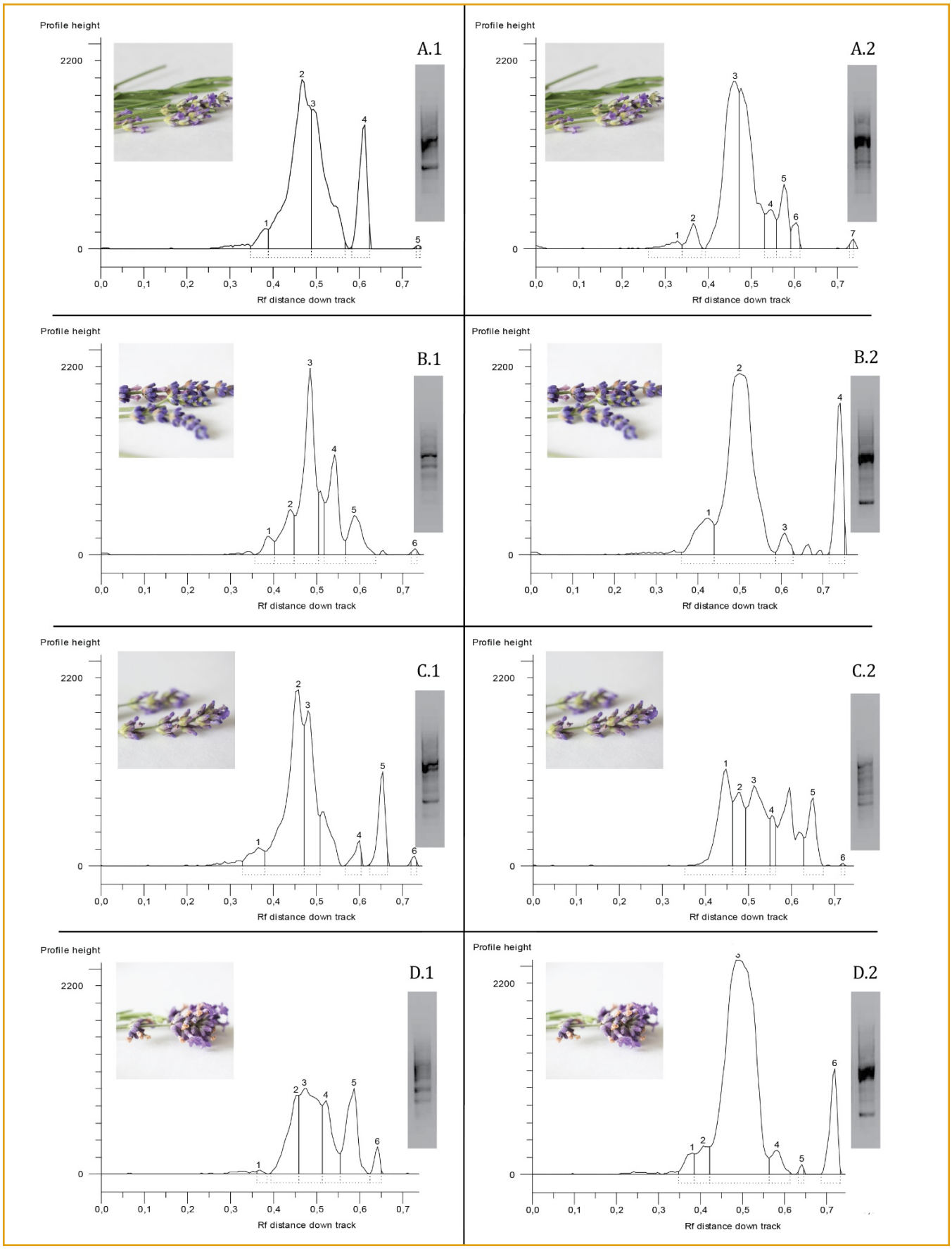

Figure 6 DNA fingerprint cards generated by OPB-18
A–D represent lavender genotypes. 1 – leaf sample; 2 – flower sample

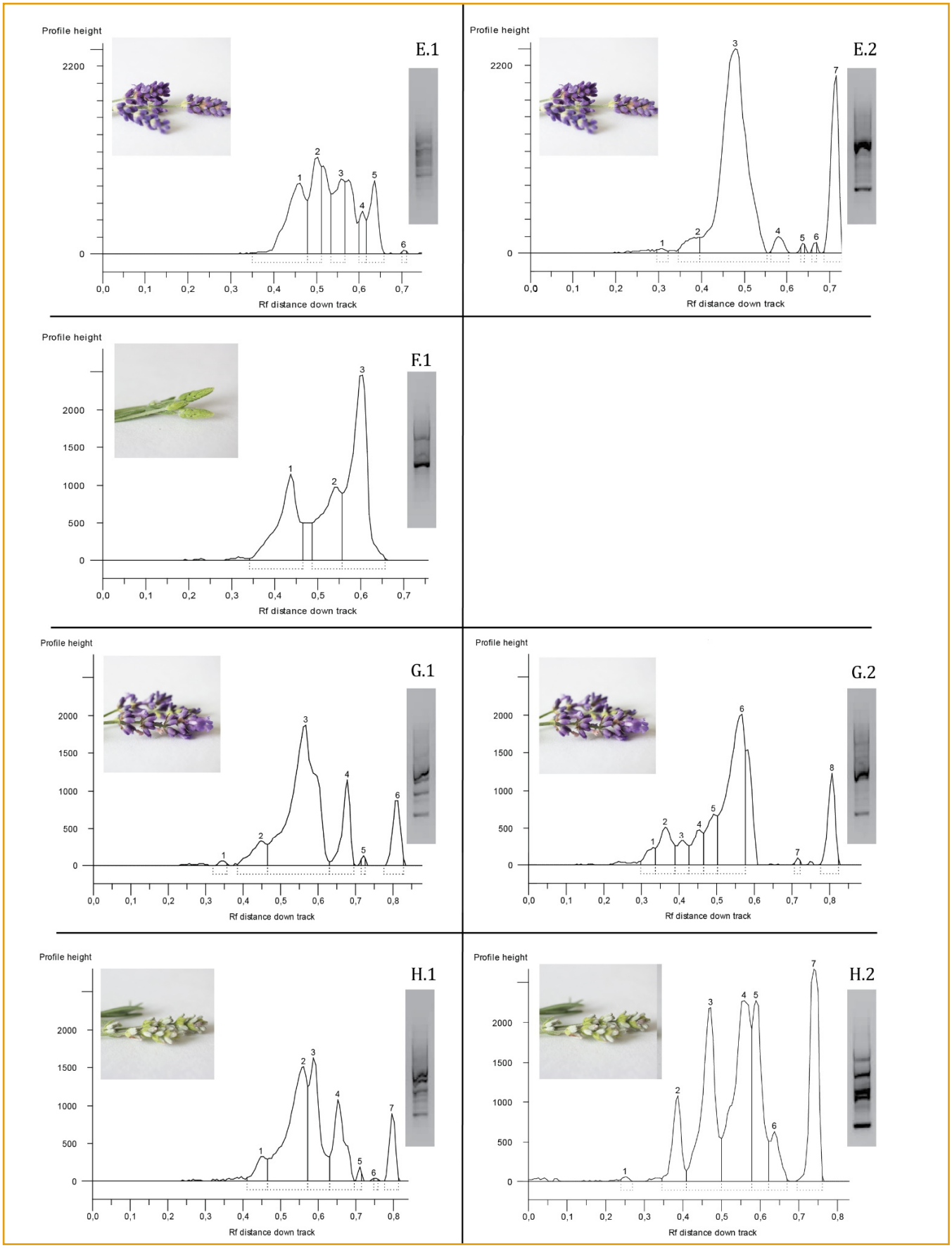

Figure 7 DNA fingerprint cards generated by OPB-18
E–H represent lavender genotypes. 1 – leaf sample; 2 – flower sample. Genotype F was the only one not yet flowering at the time of sampling

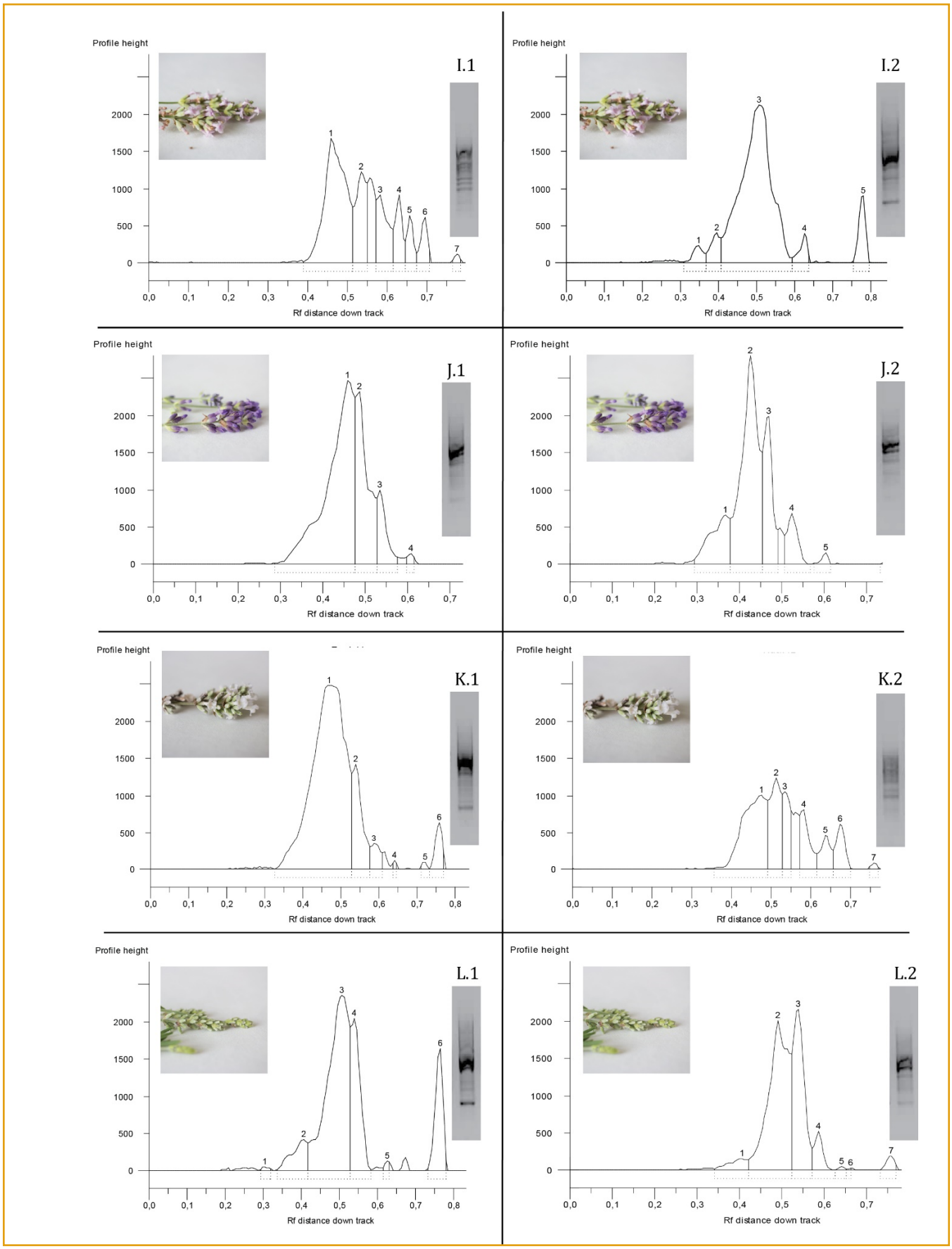

Figure 8 DNA fingerprint cards generated by OPB-18
I–L represent lavender genotypes. 1 – leaf sample; 2 – flower sample

sometimes referred to as fingerprinting techniques. The DNA polymorphisms between the individuals tested are revealed by these patterns (Poczai et al., 2013; Batley, 2015).

However, the quality of the isolated genomic DNA is also crucial to ensure efficient amplification. Emphasis is placed on the removal of residual secondary metabolites (Bulavin et al., 2020), as evidenced by our DNA isolation and purification procedure. For RAPD amplification was successful even isolation from dried leaves using the CTAB protocol (Ștefan et al., 2019).

The genetic diversity of seven *Lavandula* multifida L. populations from three bioclimates in Tunisia was estimated by RAPDs and allozymes (Hnia et al., 2017). By seven random decamer primers were amplified 97 RAPD markers, while in our case we observed a higher number of RAPD markers by one primer (127 by OPB-11 and 151 by OPB 18) from 12 samples. The effectiveness and sensitivity of RAPD markers are confirmed by the fact that relatively higher polymorphism was found in populations from the lower semi-arid bioclimate (Hnia et al., 2017).

The analysis of homology among 10 introduced lavender species was performed by RAPD. Fifteenth selected primers amplified 364 bands and the average number of bands amplified by a primer was 20.8 (Yanling et al., 2007). The collection of genotypes included species *L. angustifolia*, *L.* × *intermedia*, *L. stoechas*, *L. stoechas pedunculata*, *L. dentata* and *L. lannata* × *L. dentata*.

A similar study was performed with different populations of dentate lavender collected in the Algerian littoral (Gadouche et al., 2019). Despite the fact that the populations were sampled in different ecoregions, the dendrogram obtained by cluster analysis showed two clusters with a similarity index higher than 68%, demonstrating that the populations are genetically close despite their geographical distance.

Despite the sequence random origin of RAPD primers, not all types of decamers provide successful amplification, which has been demonstrated in our and other studies. We tested four random decametres out of which only two provided effective and reproducible amplification. To determine the intra- and interspecific genetic diversity of two *Lavandula* species: *L. angustifolia* Mill. (7 different varieties) and *L. stoechas* L., 14 RAPD primers were evaluated, of which 4 were selected according to the number of amplified polymorphic sites (Ștefan et al., 2019).

The rationale for the use of RAPD molecular markers for Lamiaceae species is supported by several current studies (Chowdhury et al., 2017; Ahmed and Al-Sodany, 2019; Saha et al., 2020; Sunar et al., 2020; Zhend et al., 2021; Ahmed et al., 2022).

Conclusions

This study aimed to provide an effective and low-cost approach for genetic diversity assessment useful for lavender growers to characterize individual genotypes. Obviously, a large-scale experiment applying a larger number of tested RAPD markers would be needed to complete the DNA fingerprinting identification cards. However, our analyses confirmed the suitability of this type of marker for such a complementary service for growers and practical use.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Early ripening and marketability of the products in different types of the sweet cherry orchards in the Ukrainian Forest-Steppe

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The considerable vigour of the sweet cherry (*Prunus avium* L.) trees' growth and their late fruit-bearing beginning complicate the industrial highly efficient orchard creation. Besides, the requirements of the global trade networks increased to the fruit marketable quality as well. Therefore, the purpose of our research was the selection of large-fruited cultivars bred in Ukraine as well as clonal rootstocks including Gisela 5, Gisela 6, and Studenykivska as well as cvs Krupnoplidna, Etyka, and Annushka appeared to influence positively the beginning of the orchards fruit-bearing, the acceleration of the early maturity, providing the constantly high fruits productivity and marketable quality. The field, laboratory, and comparative methods of the investigations were applied as well as the statistical ones. The highest productivity was ensured by the semi-vigorous rootstocks (like Gisela 6). Their average yield per tree in the five-year age was 14.4–18.5 kg.tree^{-1} depending on the variety. The maximum number of trees loaded with fruits was provided by Krupnoplidna (28.9 kg.tree^{-1}). On account of the surface unit was 25.7 t.ha^{-1}. It was noted that irrespective of this index the trees at a young age were able to ensure the highest fruits marketable quality. The highest qualitative indices were provided in the orchards where the cultivar of the middle and middle-late ripening terms were used in which products unidimensional job lots were formed (74.8–100%) with the fruit diameter 29.8–35 mm. At the same time more rapid rates of fruits diminution were noted in the orchards on the semi-dwarfing rootstocks 'Gisela 5' and 'Studenykivska' where the average fruit mass in the eight-year age reduced almost by two times as compared to those which were formed in the 5–6 year orchards. Therefore, it is most expedient to create cherry plantations on semi-vigorous rootstock Gisela 6, which provides 1.8–2.1 times higher productivity of trees, as well as the high marketable quality of fruits during the period of productive use of plantations.

Keywords: *Prunus avium*, orchard constructions, clonal rootstocks, marketability, products quality

Introduction

Among the fruit crops sweet cherry (*Prunus avium* L.) is the least favorable for intensive horticulture. The trees of this crop are characterized by great vigour and late fruit-bearing beginning that complicates the creation of early ripening low-holed orchards. One of the optimum methods of solving this problem is utilizing clonal rootstocks (Balmer, 2015; Musacchi et al., 2015; Grandi and Lugli, 2017; Bujdoso and Hrotko, 2019).

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Under modern conditions the requirements of the global trade networks for sweet cherry fruits marketable quality increased considerably the diameter of which should exceed 28 mm that demands the selection of large-fruited cultivars (Kappel et al., 2012; Quero-Garcia et al., 2017; Szpadzik et al., 2019). According to the FAOSTAT data (2021). Ukraine was included among the ten leading sweet cherry fruit producers with a total crop of 61.8 thousand t and a yield of 6 $t.ha^{-1}$.

Today, there are more than 2000 varieties of this culture in the world. Ukraine is the world leader concerning the creation of this crop cultivar. Only in the last 30 years, about 500 new cherry cultivars were created, of which more than 100 were created by Ukrainian breeders (Milatović and Nikolić, 2011).

Nowadays, in the State Register of Varieties of Ukraine suitable for distribution in Ukraine, among the 28 registered cultivars, domestic cultivars predominate and only four (Regina, Summit, Cordia, Bigarreau Burlat) are foreign selected (State Register, 2022). This ensures self-sufficiency according to this indicator and makes it possible to choose adapted varietal composition of plantings for various reasonable and climatic conditions.

Ukrainian breeders have created large-fruited cherry varieties with high taste qualities with an average fruit weight of 10–12 g, which can exceed 18 g under the conditions of irrigation (Turovtsev and Turovtseva, 2002). Therefore, they meet the requirements of global trade networks.

In this connection, it is actually to select inland large-fruited cultivars as well as dwarf rootstocks that would contribute to the decrease of the tree's unproductive period and ensure the products stable high productivity and marketable quality.

Material and methodology

Plant material and setting up the experiment

The experiments were conducted in the orchards of the Institute of Horticulture of the National Academy of Agrarian Sciences (IH NAAS) of Ukraine (Kyiv) in two field experiments.

In the first experiment (the elaboration of effective sweet cherry (*Prunus avium* L.) orchards types to obtain competitive fruits, planting in 2015) the cultivars Melitopolska Myrna, Krupnoplidna, Etyka, and Annushka were studied in the orchards on the four clonal rootstocks, in particular, vigorous were researched as wells –vigorous Colt (5 × 3 m), semi-vigorous Gisela 6 (4.5 × 2.5 m) and semi-dwarfing Gisela 5 and Studenykivska (4.5 × 2.0 m).

In the second experiment (selection of competitive strains for industrial orchards, planting in 2018) 25 large-fruited inland cultivars of different ripening rates were researched as well as two foreign ones on the semi-vigorous rootstock Gisela 6 (4.5 × 2.5 m). Control cultivars were Valery Chkalov (early), Talisman (middle), and Lyubava (middle and late), which are included in the State register of the cultivars of the plants valuable for the spread in Ukraine for 2022 in accordance with the terms of their maturity (Table 1).

Research methodology

There were 9 estimated trees in each variant with the orbicular crown and lowered fruit-bearing zone. The repetition was three-fold. The soil of the experimental plots (dark-grey, podzol, light loamy, on the carbonate loess) was managed on bare fallow without irrigation.

Conducted a study on early ripening, fruit productivity, and marketable quality. Estimates and observations in conformity with the main indicators of growth and fruit-bearing were carried out by applying the accepted methods (Karpenchuk and Melnyk, 1987; Syedov and Ogoltsova, 1999; Metodica..., 2005), in particular, early fruiting was assessed by counting flowers and fruits on 9 typical trees of each variant. After removing these fruits, the yield from each tree was recorded using the weight method. The average weight of the fruit was determined by weighing 100 fruits from each variant of the experiment. The marketability of the collected products was determined according to GSTU 01.1-37-165:2004 and by dividing them into fractions according to the transverse diameter of the fruit: 19.0–22.9 mm, 23.0–27.9 mm, 28.0–29.7 mm, 29.8–31.3 mm and 31.4 mm and more.

Statistical analysis

For processing the experimental research results statistically and determining the reliability and substantiality of the obtained data the dispersing method of the statistical analysis was used with the application of the computer program AGROSTAT. The value will be presented as the mean value of the standard deviation (SD). The data were analyzed for statistical significance with the help of the Student's *t*-test. *P* values less than 0.05 were considered significant.

Table 1 List of cultivars of cherry (*Prunus avium* L.) that are being studied in experiment 2, rootstock Gisela 6 (4.5 × 2.5 m) (planting in 2018)

Cultivars of the early and middle early ripening term	Cultivars of the middle maturity term	Strains of the middle-late and late ripening term
Valery Chkalov (control)	Talisman (control)	Lyubava (control)
Skazka	Krupnoplidna	Temporion
Dzherelo	Dilema	Zodiak
Valeriya	Vasylisa Prekrasna	Udivityelna
	Elektra	Anonce
	Melitopolska Myrna	Nizhnist
	Yaroslavna	Anshlag
		Novynka Turovtseva
		Etyka
		Annushka
		Donetska Krasunya
		Donchanka
		Bigarreau Hatif Burlat
		Regina

Results and discussion

To ensure rapid fruiting of cherry plantations in the practice of global industrial horticulture, weakly growing clonal rootstocks are used (Santos et al., 2006; Cantin et al., 2010; Long et al., 2017; Vignati et al., 2022).

Our observations of the plant's development in the orchard showed that their early maturity and productivity depended to a considerable degree both on a cultivars and rootstock (Figure 1). For example, in the third year after planting already in the orchards on cultivars Gisela 5, Gisela 6, and Studenykivska nearly all the trees (83–100% of their total amount in these variants) the establishment of fruit bud) was noted while on the rootstock Colt, they were absent altogether. The highest extent of the generative bud establishment on the mentioned rootstocks contributed to their early fruit-bearing beginning. The individual strain peculiarities were established for flower creation on the three-year trees. For instance, cultivars Krupnoplidna, Etyka, and Annushka had 180–250 individuals while cultivar Donchanka had their largest number of 320 which testified to these cultivars' early ripening on the low-holed clonal rootstocks. The trees of the above-mentioned cultivars on Gisela 6 proved the most productive ones at the beginning of the fruit-bearing. Their yield in the fourth year after planting with one-year planting trees was 3.2–4.1 kg.tree^{-1}, whilst in the fifth 12.7–15.5 kg.tree^{-1}, which is 1.6–2.6 times higher than on rootstocks Gisela 5 and Studenykivska. On the rootstock Colt the self-contained fruit-bearing was noted in the fourth year after planting and the next year the yield did not exceed 0.3–0.4 kg.tree^{-1}.

On average during five fruit-bearings on Gisela 6 (the cultivars Krupnoplidna, Annushka, and Etyka) was 10.3–13.1 kg.tree^{-1}, that is by 1.8–2.1 times higher than on the rootstocks Gisela 5 and Studenykivska and on the Colt did not exceed 0.7–5.7 kg.tree^{-1}. The increase in tree productivity by year and their marketability are presented in Figure 1 by the cultivar Krupnoplidna. A similar regularity was observed in the other studied cultivars of Annushka, Etyka, and Melitopolska Myrna.

The analogous results as regards the positive effect of Gisela 6 as well as Egervar on the sweet cherry fruits' productivity and marketability were achieved by Hungarian scientists (Bujdoso and Hrotko, 2016).

During the research years, the marketability of the product depended on strain peculiarities, rootstock, and orchard age. As for as the age rose the fruit size on rootstocks of different vigour reduced gradually. It achieved 19.3–24.3% on the middle rootstocks in the eight-year age. At the same time on the vigorous rootstock Colt, the fruit size decrease was not observed (Report, 2022).

More rapid rates of the physical indexes decrease were noted on cultivars Gisela 5 and Studenykivska. Their fruit's average mass was reduced nearly by two times in the eight-year age as compared to those

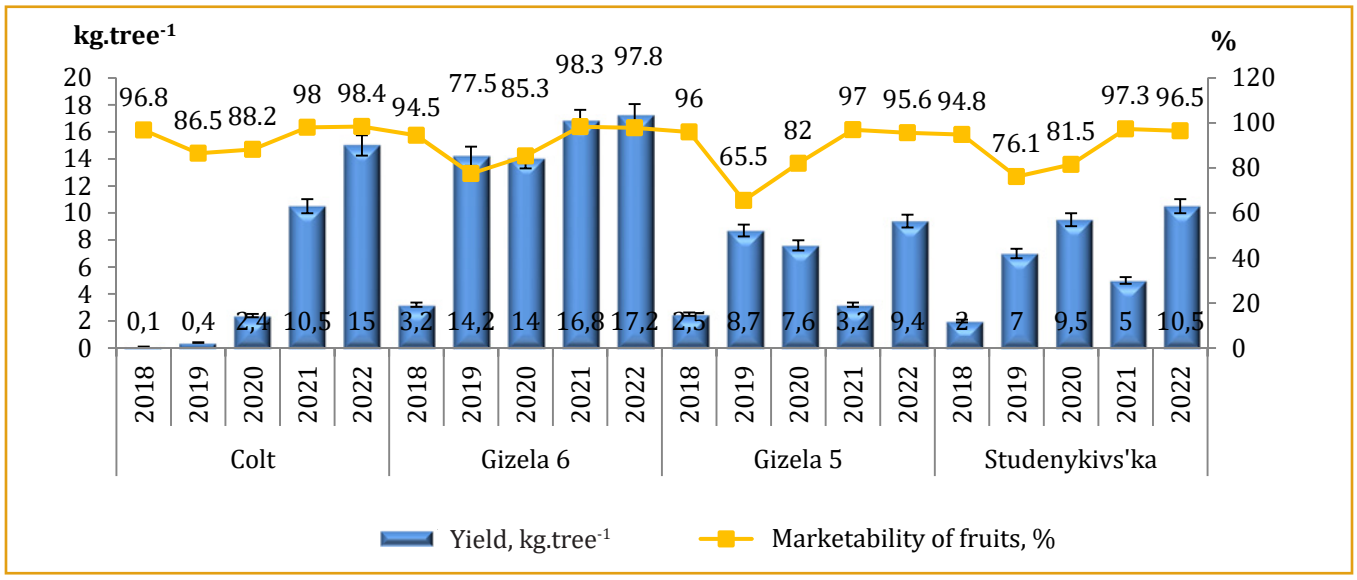

Figure 1 Sweet cherry cultivar Krupnoplidna productivity and marketability of fruits on different rootstocks, 2018–2022

which were formed in 5–6 year orchards (9.1–9.8 g). The description is presented of the sweet cherry large-fruited cultivars bred in Ukraine on the highly productive middle rootstock Gisela 6.

The world's market tendencies testify that today fruits with a diameter of more than 28 mm are considered they have dark coloration and dense flesh. That contributes to their high transportability (Yue et al., 2014; Meland et al., 2017; Blanko et al., 2019). Leading countries-exporters of sweet cherry conduct active searches of large-fruited strains with transportable fruits to ensure high profitableness of this business (Naranjo, 2013; Long et al., 2021).

The main indicator that regulates the commercial quality of cherry fruits is their transverse diameter. In EU countries, the requirements for the quality of fruits of this breed are regulated by the standard of the UN European Economic Commission, where cherry fruits of the highest grade must have a diameter of at least 20 mm, and fruits of the first and second grade at least 17 mm (Standart FFV-13, 2017).

The US State Standard (Washington standards, 2005) has the highest level of requirements for the commercial quality of cherry fruits, by which it is normatively established that the minimum size of fruits in terms of the largest diameter should be at least 21.4 mm. With this in mind, active efforts are being made to breed and select large-fruited varieties in the leading cherry fruit-producing countries (Campoy et al., 2015; Dong et al., 2022; Szilágyi et al., 2022).

In our experiments, the fruits calibration as concerns the largest cross diameter showed that their highest indexes of linear and marketability were provided on middle rootstock in the first years after the tree's fruit-bearing beginning. For example, in experiment 2 in the fifth year after planting among early cultivars unidimensional job lots with a fruit diameter of 23–28 mm were formed by cultivars Valery Chkalov, Kazka, and Dzherelo (83.7–100%).

Among the cultivars of the middle maturation rate, it is Dzherelo that belonged to this group (92%), and such as Talisman and Electra ensured linear job lots at a level of 74.2–93.8% with the diameter of the fruit 28–29.7 mm. This met the global trade network requirements and testified to the high inland strains competing (Table 2).

The highest marketability indices were provided by the cultivars of the middle-late ripening rate. Bigarreau Hatif Burlat (74.8%), Vasylisa Prekrasna (96.8%), and Temporion (99.3%) are among them and formed job lots with the fruit diameter 29.8–31.3 mm and such as Krupnoplidna and Etyka had the highest level (100%) with the fruit diameter 34–35 mm.

Talisman (14.4 kg.tree^{-1}), Etyka (14.8 kg.tree^{-1}), Krupnoplidna (17.3 kg.tree^{-1}), Anonce (18.3 kg.tree^{-1}), Anshlag (18.5 kg.tree^{-1}) and Donchanka (20 kg.tree^{-1}) distinguishing themselves for the highest average yield in the five-year age (Report, 2022). The maximum load of the trees with fruits was ensured by cv Krupnoplidna – 28.9 kg.tree^{-1}. In the account the surface unit was 25.7 t.ha^{-1}. The largest fruits of this variety formed a mass of 18.4 g with the largest cross diameter of 35 mm and the one-dimensionality of the products was 100% which testified that irrespective of

Table 2 Structure of the sweet cherry fruit marketable quality concerning their cross diameter (%)

Cultivars	General marketability of fruits (100%)				
	including share of the fruits by fractions with the diameter (%)				
	19.0–22.9 mm	23.0–27.9 mm	28.0–29.7 mm	29.8–31.3 mm	31.4 mm and >
Cultivars of the early and middle early ripening term					
Valery Chkalov (control)	3.6 ±0.9	96.4 ±0.9	0	0	0
Skazka	0	100	0	0	0
Dzherelo	16.3 ±0.6	83.7 ±0.6	0	0	0
Valeriya	5.6 ±0.6	94.4 ±0.6	0	0	0
Cultivars of the middle maturity term					
Talisman (control)	6.8 ±0.9	19.0 ±1.3	74.2 ±1.3	0	0
Krupnoplidna	0	0	0	0	100
Dilema	8.0 ±1.5	92.0 ±1.5	0	0	0
Vasylisa Prekrasna	0	0	3.2 ±0.5	96.8 ±0.5	0
Elektra	0	6.2 ±0.6	93.8 ±0.6	0	0
Melitopolska Myrna	5.8 ±0.4	35.3 ±2.2	58.9 ±2.3	0	0
Yaroslavna	7.8 ±1.3	92.2 ±1.3	0	0	0
Strains of the middle-late and late-ripening term					
Lyubava (control)	0	8.5 ±0.6	91.5 ±0.6	0	0
Temporion	0	0.72 ±0.7	0	99.3 ±0.7	0
Zodiak	2.6 ±0.6	2.7 ±0.8	94.7 ±1.4	0	0
Udivityelna	0	14.4 ±1.2	85.6 ±1.2	0	0
Anonce	0	11.9 ±0.4	88.1 ±0.4	0	0
Nizhnist	0	85.3 ±0.6	14.7 ±0.6	0	0
Anshlag	0.4 ±0.3	22.6 ±2.0	77 ±1.7	0	0
Novynka Turovtseva	9.3 ±0.7	90.7 ±0.7	0	0	0
Etyka	0	0	0	0	100
Annushka	0	5.9 ±0.6	94.1 ±0.6	0	0
Donetska Krasunya	0	5.7 ±0.6	94.3 ±0.6	0	0
Donchanka	4.2 ±0.9	95.1 ±0.9	0	0	0
Bigarreau Hatif Burlat	0	0	25.2 ±0.9	74.8 ±0.9	0
Regina	0	4.3 ±1.1	95.7 ±1.1	0	0

within rows, means differ ($p < 0.05$)

the load degree the trees in the young age were able to provide the highest indicators of the fruit marketable quality.

It should be noted that Valeriy Chkalov and Krupnoplidna varieties are donors of high fertility and are widely used in industrial plantations of various countries and breeding programs (Turovtsev and Turovtseva, 2002; Kishchak and Kishchak, 2015; Kishchak, 2017). Today, China has the world's largest fresh cherry fruit market, and Hongdeng and Krupnoplidna are the most popular varieties in industrial orchards in this country (Zhang et al., 2019). In Latvia, which is located between 55–56° north latitude, the basis of the industrial assortment is the Ukrainian varieties Krupnoplidna and Valery Chkalov and the Canadian variety Lapins (Ruisa, 2008). In the Research and Breeding Institute of Pomology Holovousy (Czech Republic) sweet cherry cultivar Tamara with fruits is rather large bred from crossing cherry varieties Krupnoplidna × Van. This variety has become widespread in Europe, is being studied in the USA, and is even successfully exported from Australia to Hong Kong at high prices (Vávra et al., 2021).

The description is presented of the sweet cherry large-fruited cultivars bred in Ukraine on the highly productive middle rootstock Gisela 6. These cultivars stood out during our research.

Krupnoplidna

A middle ripening cultivar that ripens at the beginning of the third decade of June created a the Melitopol Research Station of Horticulture as a result of the pollination of the cultivar Napoleon Bila with the mixture of the pollen of the strains Valery Chkalov + Elton + Jabule (Figure 2).

The tree is vigorous, the crown is orbicular with middle density and begins fruit-bearing in the third year after planting ensuring a yield of 17.3 kg.tree^{-1} (maximum 28.9 kg.tree^{-1}) on the middle clonal rootstock in the five-year age and the fruit-bearing age 45–50 kg.tree^{-1} (Report, 2022). The fruits are very large, linear, and wide-orbicular, with a diameter of 28–29 mm, and an average mass of 10.0 g (maximum 18.4 g with a diameter of 34–35 mm). The colour is dark-red. The skin is thin, dense, and shining, and the tear-off from the fruit is dry. The flesh is dark-red, cartilaginous, and juicy. The taste is sour-sweet. The degustation valuation is 8.8 points (in accordance with the 9-point scale). The fruits contain 14.6% of dry soluble substances, 12.1% of sugars, and 0.7% of acids. The stone is large and round, it separates from the flesh not completely (Report, 2022).

The cultivar is resistant to monilia (*Monilia cinerea*) and affectioned weakly with cocomycosis (*Cocomyces hiemalis*), high winter-hardy. Plants of this cultivar are partially self-fertile. The best pollinators are Surprise, Franz Josef, Donchanka, and Bigarreau Hatif Burlat. Since 1983 cultivars is spread in the Ukrainian Forest-Steppe and Steppe.

Figure 2 Fruit-bearing of the cultivar Krupnoplidna

Etyka

Middle-late cultivar ripes the third decade of June created at the Donetsk Branch of IH NAAN of Ukraine as a result of crossing the cultivar Donchanka × Valery Chkalov (Figure 3).

Figure 3 Fruit-bearing of the cultivar Etyka

The tree is middle-growing with the orbicular branchy ramified well crown, and begins fruit-bearing in the third-four years after planting providing an average yield of 14.8 kg.tree^{-1} (maximum 21.7 kg.tree^{-1}) and in the fruit-bearing age of 45–60 kg.tree^{-1}(Report, 2022). The fruits are rather large, unidimensional, and wide-orbicular, with an average mass of 10–11 g and a diameter of 29–30 mm (maximum 18.1 g with a diameter of 34–35 mm). The skin is thin, dense, and shining, and the tear-off from the fruit is dry. The flesh is dark-red, juicy, cartilaginous, its taste is pleasant sour-sweet. The degustation valuation is 8.7 points. The fruits contain 18.6% of dry soluble substances, 13.6% of sugars, and 0.6% of acids. The stone is small, it separates easily from the flesh (Report, 2022). Etyka is resistant to major diseases, and high winter-hardy. The plants of this cultivar are self-fertile. The best pollinators are Donetskyi Ugolyok, Donchanka, Yaroslavna, and Annushka. The cultivar has been recommended for growing in the Forest-Steppe and Steppe of Ukraine.

Temporion

Middle-late cultivar ripens at the end of the third decade of June created at the Melitopol Research Station of Horticulture as a result of the pollinating Drogana

Zhovta with the mixture of the pollen of cultivar Valery Chkalov + Sonyachna Kulya (Figure 4).

Figure 4 Fruit-bearing of the cultivar Temporion

The tree is vigorous with the branchy crown of the middle density, and begins fruit-bearing in the third-fourth year after planting ensuring a yield of 5.7–7.0 kg.tree^{-1} in the fifth-year age and 40–50 kg.tree^{-1} in the fruit-bearing one (Report, 2022). The fruits are fairly large, linear, and wide-orbicular, with an average mass of 11.0 g and diameter of 29 mm (maximum 13.5 g and diameter 30–31 mm). The tear-off from the fruit stem is dry. The fruit's colour is dark-red. The skin is of middle thickness dense shining, and the tear-off from the fruit is dry. The flesh is dark-red, cartilaginous, and juicy. The taste is perfectly pleasant sour-sweet. The degustation valuation is 8.8–9.0 points. The fruits contain 16.4% of dry soluble substances, 13.0% of sugars, and 0.7% of acids. The stone is small orbicular and separates easily from the flesh (Report, 2022). The cultivar is resistant to monilia (*Monilia cinerea*), cocomycosis (*Cocomyces hiemalis*), and high winter-hardy. Plants of this cultivar are self-sterile. The best pollinators are Donchanka and Bigarreau Hatif Burlat. The cultivar has been recommended for cultivation in the Ukrainian Forest-Steppe and Steppe.

Conclusions

Thus, the investigation showed that semi-vigorous rootstock Gisela 6, in contrast to vigorous rootstock Colt and semi-dwarf ones (Gisela 5 and Studenykivska), contributes to stable high productivity of plantings and the formation of high marketable fruit quality during the period of operation of the garden (conclusion by of experiment 1). Middle, middle-late, and late-ripening term cherry varieties, in particular Krupnoplidna, Etyka, and Temporion, provide early fruiting and obtain 99.3–100% of fruits with a diameter of more than 29.8 mm, which meets the modern requirements of global trade networks (conclusion of experiment 2).

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

A comparative study of *Silphium* spp. antioxidant activity

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This study aimed to evaluate the parameters of antioxidant activity of different parts of *Silphium* spp. during budding and flowering periods. The four species of *Silphium* L. genus from the experimental collection of the Cultural Flora Department of M.M. Gryshko National Botanical Garden of the NAS of Ukraine were prepared and used in this research. Total polyphenol content was from 12.32 (*S. asperrimum*, stems at the budding) to 95.21 (*S. laciniatum*, leaves at the flowering stage) mg GAE.g^{-1} DW depending on the organ and stage of vegetation. The total content of phenolic acids in ethanol extracts of investigated plants was from 3.52 (*S. asperrimum*, buds at the budding) to 34.58 (*S. laciniatum*, leaves at the flowering) mg CAE.g^{-1} DW. The total flavonoid content in investigated extracts was from 3.67 (*S. perfoliatum*, stems at the budding) to 57.31 (*S. laciniatum*, leaves at the budding) mg QE.g^{-1} DW. Antioxidant activity was determined by two assays namely the DPPH method and molybdenum-reducing power of extracts and was from 3.33 (*S. trifoliatum*, stems at the budding) to 9.11 (*S. laciniatum*, flowers at flowering) mg TE.g^{-1} and from 29.11 (*S. trifoliatum*, stems at the budding) to 185.22 (*S. laciniatum*, leaves at the flowering) mg TE.g^{-1} DW, respectively. It was found a very strong correlation between total polyphenol compounds, flavonoids, phenolic acids, and molybdenum reducing power r = 0.715–0.905. A weak or moderate correlation was found between DPPH scavenging activity and investigated phenolic compounds (r = 0.110–0.522). Obtained data can be useful for further deep biochemical investigation of plant raw of *Silphium* spp. and in livestock nutrition branch.

Keywords: *Silphium* spp., polyphenols, flavonoids, phenolic acids, correlation

Introduction

Genus *Silphium* L., commonly known as rosin-weed, belongs to Asteraceae Bercht. & J. Presl family includes 33 species and originated from Northern America (Clevinger and Panero, 2000; Peni et al., 2020). Representatives from this plant family are well-known as medicinal plants with different biological activities such as antioxidant (Shymanska et al., 2020), antifungal, and immunosuppressive (Wolski and Kędzia, 2018).

S. perfoliatum L. is a high-productive crop (Šiaudinis et al., 2012; Ţîţei et al., 2013; Peni et al., 2020) that can grow for over 15 years (Bury et al., 2020) and be harvested for different purposes such as fodder

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(Pichard, 2012; Shalyuta and Kostitskaya, 2018), honey, ornamental (Jucsor and Sumalan, 2018), medicinal (Kowalska et al., 2022), energetic (Mueller et al., 2019; Šiaudinis et al., 2019; Cossel et al., 2020; Cumpido-Marin et al., 2020; Rakhmetov et al., 2020) plants. The high productivity and calorific value of *S. perfoliatum* allow using plant raw on biogas (Gansberger et al., 2015) and solid (Jasinskas et al., 2014) production. According to Rakhmetov et al. (2020), the yield of energy of plant raw of various *Silphium* genotypes in the conditions of Forest-Steppe of Ukraine was from 43.81 (*S. asperrimum* Hook) to 149.27 (*S. integrifolium* Michx.) Gcal.ha^{-1} at the budding-flowering stage. It is reported about phytoremediation uses *S. perfoliatum* (Peni et al., 2020).

The chemical composition of raw *Silphium* plants is protein, amino acids, fat, cellulose, water-soluble sugars, and minerals (especially potassium, calcium, magnesium, iron, and manganese) (Kowalska et al., 2020). The biochemical compound content of *S. trifoliatum* L. decreased with plant development and the stage of growth before the flowering was the best for harvesting (Kowalski, 2007). Seeds of *Silphium* spp. contained 33.5% of protein, 24.1% of fat, 9.58% of water-soluble sugars, and 25.4% of cellulose. Among the amino acids of seeds, glutamic acid (up to 23%) and leucine (7.76%) were predominant. Main seed fatty acids are determined linolic (44.4%) and oleic (13.2%) (Kowalski & Wiersiński, 2004). In the petroleum ether extracts of leaves and inflorescences identified α-amyrine, heptacosane, stigmasterol, γ-sitosterol, β-marine, etc. The labdane type diterpene and sesquiterpene dominated in rhizome extracts (Kowalski, 2005). According to Ţîţei (2014), the raw protein content of *S. perfoliatum* was 16.33%, and in amino acid composition prevailed glutamine, leucine, and asparagine. Investigation of the above-ground part of *Silphium* spp. raw showed a content of dry matter 21.14–29.02%, water-soluble sugars 3.54–12.17%, crude fiber 29.46–48.24%, ascorbic acid 77.12–296.35 mg%, β-carotene 0.23–1.54 mg% (Rakhmetov et al., 2019).

Leaf, inflorescence, and rhizome extracts of *S. perfoliatum* exhibited activity against some gram-positive and gram-negative bacteria, and rhizome alcohol extracts had the highest antibacterial effect (Kowalski and Kędzia, 2007). Investigations showed that ethanol extract of *S. perfoliatum* inhibited the growth of fungi species *Alternaria alternata* and *Colletotrichum coccodes* that can be used as biological preparation for the management of plant diseases (Jamiołkowska and Kowalski, 2012).

The extracts of these plants and their components such as polysaccharides (Shang et al., 2017) exhibited antioxidant activity (Borchardt et al., 2009; Kowalska et al., 2022). Kowalski and Wolski (2003a, 2003b) identified in phenolic acid fractions caffeic, *p*-coumaric, *p*-hydroxybenzoic, ferulic, and vanillic acids and the most predominant was caffeic acid. The study of the phenolic acid complex of *S. trifoliatum* extracts showed the presence of protocatechuic and salicylic acids besides the above. The phenolic acid complex is found both in free and bounded forms in the leaves, inflorescences, and rhizomes of *S. trifoliatum* (Kowalski, 2007). The flavonoid fraction of *S. trifoliatum* leaf and inflorescences extracts were flavonoid glycosides and kaempferol (Kowalski, 2007), the total content of flavonoids was 0.87 and 1.05% for *S. perfoliatum* and *S. integrifolium* (Kowalski and Wolski, 2003a; Kowalski, 2004). Also, among polyphenol compounds determined 7.34–11.24% of tannins (Kowalska et al., 2020).

This study dwells on the investigation of the polyphenol content and antioxidant activity of ethanol extracts of different organs of *Silphium* spp. as a source of antioxidants.

Biological material

In this study investigated plants of *Silphium* L. (Figure 1) growing in the M.M. Gryshko National Botanical Garden of NAS of Ukraine in Kyiv (NBG). Some species were investigated in an experimental study in 2019–2020, including *S. asperrimum* Hook., *S. laciniatum* L., *S. perfoliatum* L., and *S. trifoliatum* L.

Figure 1 *Silphium* spp. at the flowering stage
1 – *S. asperrimum* Hook.; 2 – *S. laciniatum* L.; 3 – *S. perfoliatum* L.; 4 – *S. trifoliatum* L.

All biochemical analyses were conducted at the Slovak University of Agriculture in Nitra (Slovak Republic).

Chemicals

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and CentralChem (Slovakia).

Preparations of extracts

An amount of 0.25 g of each sample was extracted with 20 mL of 80% ethanol for 2 h in a laboratory shaker GFL 3005 (GFL, Burgwedel, Germany). Then, the samples were centrifuged at 4605 RCF (Rotofix 32 A, Hettich, Germany) for 10 min and the supernatant was used for measurement of FRSA (antiradical activity) using DPPH, MRAP (antioxidant activity) using phosphomolybdenum method and measurement of other antioxidant properties (detection of total polyphenol, total flavonoid, and phenolic acid content).

Total polyphenol content of extracts

The total polyphenol content (TPC) was measured by the method of Singleton and Rossi (1965) using the Folin-Ciocalteu reagent. A quantity of 0.1 mL of each sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate, and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured with the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25–300 $mg.L^{-1}$; R^2 = 0.998) was used as the standard. The results were expressed as $mg.g^{-1}$ DW gallic acid equivalent.

Total phenolic acid content

The content of phenolic acids (TPAC) was determined using 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. The results were expressed in $mg.g^{-1}$ caffeic acid equivalents (CAE).

Total flavonoid content of extracts

The total flavonoid content (TFC) was determined by the modified method described by Shafii et al. (2017). An aliquot of 0.5 mL of the sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M potassium acetate, and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (1–400 $mg.L^{-1}$; R^2 = 0.9977) was used as the standard. The results were expressed in $mg.g^{-1}$ DW quercetin equivalent.

Free radical scavenging activity

Free radical scavenging activity (FRSA) of samples (antiradical activity) was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sánchéz-Moreno et al., 1998). An amount of 0.4 mL of sample was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the reaction mixture was determined with the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10–100 $mg.L^{-1}$; R^2 = 0.989) was used as the standard and the results were expressed in $mg.g^{-1}$ DM Trolox equivalents.

Molybdenum-reducing power of extracts

The molybdenum-reducing power (MRP) of samples was determined by the method of Prieto et al. (1999) with slight modifications. The mixture of the sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M), and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then cooled to room temperature. The absorbance at 700 nm was detected with 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 $mg.g^{-1}$ DM Trolox equivalent.

Statistical analysis

The results are expressed as mean values of three replications ± standard deviation (SD); Data were analyzed with the ANOVA test and differences between means were compared through the Tukey-Kramer test ($p < 0.05$).

Results and discussion

The phytochemical investigation of plant raw materials demonstrated the therapeutical properties of various plant products. Some plants from Asteraceae, as reported in previous reviews, have medicinal applications (Bessada et al., 2015). Last time, numerous studies showed that plants from this family are a rich source of biologically active compounds that caused the antioxidant activity of plant raw (Vijaylakshmi et

al., 2009; Bakar et al., 2015; Kumar et al., 2019a), and their preparations (Rolnik et al., 2021). The antioxidant activity of plant extracts has been correlated with polyphenol compounds of different natures (Piluzza and Bullitta, 2009; Spiridon et al., 2011; Terpinc et al., 2012). It has been determined diversity of polyphenol compounds of different Asteraceae species has pharmacological importance and systematic value (Williams et al., 2009; Pavlenko-Badnaoui et al., 2021).

Polyphenols are a widely distributed group of biologically active compounds in plant raw, products and plant waste that includes flavonoids and phenolic acids (Abbas et al., 2017; Mourtzinos and Goula, 2019). Some authors divided polyphenols into flavonoids and non-flavonoids (Stagos, 2020). These compounds possess various functions among which are antioxidant and health-promoting (Maqsood et al., 2014). Nutritionists' attention to plant polyphenols is explained by their health effects of it such as anticancer (Dai and Mumper, 2010). Phenolic compounds can be used as chemical markers in botanical chemosystematics studies on different taxonomic levels (Míka et al., 2005). It is found a negative correlation between polyphenol consumption with cardiovascular diseases, cancer, and diabetes (Abbas et al., 2017).

The polyphenol, flavonoids, and phenolic acids content of four species of *Silphium* changed during vegetation. The accumulation of total polyphenol content in our study was from 12.32 (*S. asperrimum*, stems at the budding) to 95.21 (*S. laciniatum*, leaves at the flowering stage) mg GAE.g^{-1} DW depending on the organ and stage of vegetation (Figure 2). Leaves of investigated *Silphium* spp. accumulated polyphenols from 38.58 to 83.32 mg GAE.g^{-1} DW at the budding stage and from 69.13 to 95.21 mg GAE.g^{-1} DW at the flowering stage. TPC in the buds was from 34.87 to 46.58 mg GAE.g^{-1} DW at the budding and in flowers from 42.11 to 52.67 mg GAE.g^{-1}. This parameter in stems at the budding and flowering stage was 12.32–38.36 and 18.23–41.22 mg GAE.g^{-1}, respectively. As shown in Figure 2, at the budding and flowering stages the total content of polyphenol compounds was the least accumulated in the stems of all investigated plants except plants of *S. perfoliatum* buds at the stage of budding. At both budding and flowering stages, the most polyphenols accumulated in the leaves.

Due to limited information about *Silphium* spp. polyphenol content comparison with other results is difficult. Existing data about such a large group as Asteraceae, on the whole, showed that polyphenol content significantly varied. For example, *Aster scaber* leaf extracts were investigated in different solvents, and the maximal value of polyphenol content found in ethyl acetate (322.43 mg GAE.g^{-1}), minimal in water (23.67 mg GAE.g^{-1}) (Thiruvengadam et al., 2014). The polyphenol content of *Chrysanthemum parthenium* was 3.48 mg GAE 100 g^{-1} and the flavonoid content was 1.27 mg RE 100 g^{-1} (Hanganu et al., 2016). As reported Azzouzi et al. (2016), this parameter in *Centaurea choulettiana* Pomel extracts detected 325.81 mg GAE.g^{-1}. As resulted by Indradi et al. (2017), the TPC of four species from Asteraceae was from 3.19 to 16.48 mg GAE 100 g^{-1} depending on extracts. The TPC in ethanol extracts of *Cichorium itybus* L. was 33.91 mg GAE.g^{-1} (Vergun et al., 2019). A study of other Asteraceae representatives showed that the TPC of *Helianthus tuberosus* was 7.9–11.1 mg GAE.g^{-1} in leaf

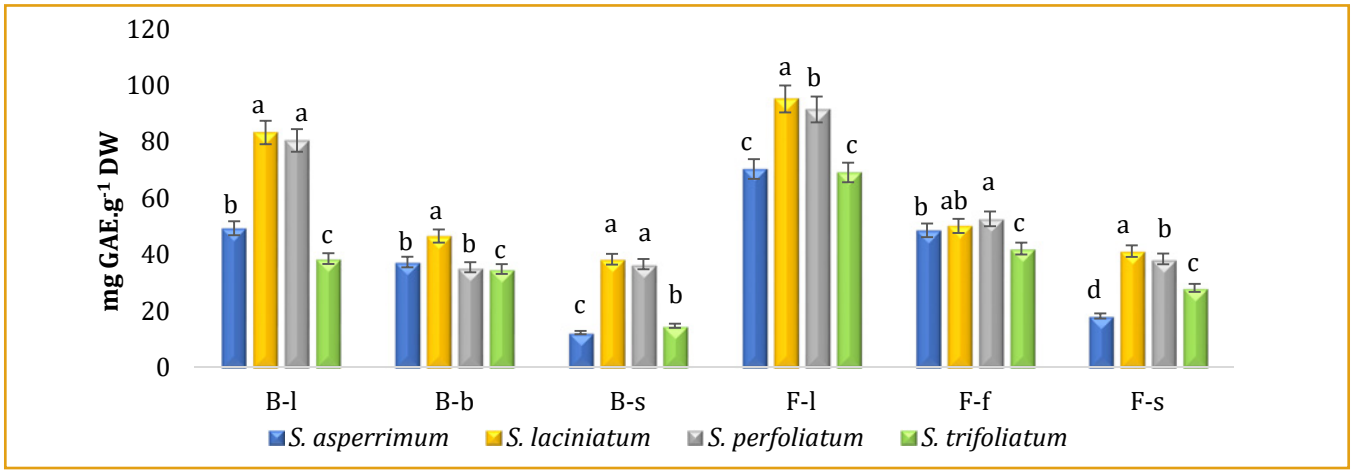

Figure 2 The total content of polyphenol compounds of ethanol extracts of *Silphium* spp.
GAE – gallic acid equivalent, B-l – leaves at the budding; B-b – buds at the budding; B-s – stems at the budding; F-l – leaves at the flowering; F-f – flowers at the flowering; F-s – stems at the flowering. Means in each column followed by different letters are significantly different ($p < 0.05$)

extracts, 4.0–5.3 mg GAE.g^{-1} in flower extracts, and 0.9–1.7 mg GAE.g^{-1} in stem extracts (Showkat et al., 2019). In *Matricaria recutita* L. extracts determined 21.4 mg GAE.g^{-1} of polyphenol compounds (Al-Dabbagh et al., 2019). In extracts of *Helianthus annuus* L. determined the TPC during stem extension was 21.9 mg GAE.g^{-1}, visible bud period 17.7 mg GAE.g^{-1}, at the early, mid-, and late flowering 20.4, 29.3, and 21.7 mg GAE.g^{-1}, respectively (Gai et al., 2020). *Matricaria chamomilla* L. extracts showed a TPC of 3.72–7.94 mg GAE.g^{-1} (Hassanpour et al., 2020). As reported by Muhtadi (2021), the TPC of *Helianthus annuus* leaves, inflorescences, and bark extracts was 35.14, 24.88, and 17.46 mg GAE.g^{-1}, respectively. Thus, the total content of polyphenols and antioxidant activity depended on the solvent, for example, as shown in a study with *Vernonia blumeoides* (Asteraceae) where in n-butanol fraction determined the total polyphenol content was approximately 4 times more than in ethanol crude extracts (Aliyu et al., 2011).

One of the most important classes of polyphenols is phenolic acids and their derivates (Mourtzinos and Goula, 2019). They play an important role in the human diet and demonstrated antimicrobial and strong antioxidant activity (Heleno et al., 2015). Among phenolic acids of *Silphium* spp. leaves identified complexes of benzoic and cinnamic acids (Williams et al., 2009). Among benzoic acids identified *p*-hydroxybenzoic, protocatechuic, isovanilic, gallic, ellagic, vanillic, syringic, and salicylic; among cinnamic acids found chlorogenic, rosmarinic, *p*-coumaric, caffeic, hydrocaffeic, ferulic, isoferulic, *m*-coumaric (Kowalska et al., 2022).

The total content of phenolic acids in ethanol extracts of investigated plants was from 3.52 (*S. asperrimum*, buds at the budding) to 34.58 (*S. laciniatum*, leaves at the flowering) mg CAE.g^{-1} DW depending on the species and part of the plant (Figure 3). At the budding stage, TPAC in the leaves was from 6.8 to 23.89 mg CAE.g^{-1} DW, and at the flowering from 13.36 to 34.58 mg CAE.g^{-1} DW. Buds accumulated TPAC from 3.52 to 13.68 mg CAE.g^{-1} DW and flowers from 9.08 to 21.14 mg CAE.g^{-1} DW. In stem extracts, TPAC was 3.72–8.57 mg CAE.g^{-1} DW at the budding and 5.86–14.12 mg CAE.g^{-1} DW at the flowering. Stem extracts showed TFC from 3.72 to 8.57 mg QE.g^{-1} at the budding stage and from 5.86 to 14.12 mg QE.g^{-1} DW at the flowering. Most contents of flavonoids for all investigated species accumulated in the leaves, the least in the stems. However, exclusion was found for *S. asperrimum*, stem ethanol extracts of which demonstrated higher TPAC than bud extracts.

According to Kowalski and Wolski (2003b), the content of phenolic acids in dry extracts of *S. perfoliatum* leaves and inflorescences were 23.2 and 25.5 mg 100 g^{-1}, respectively. The leaf fraction of *S. trifoliatum* contained up to 21.15 mg 100 g^{-1}, inflorescences up to 52.73 mg 100 g^{-1}, and rhizomes up to 10.33 mg 100 g^{-1} of phenolic acids, among which caffeic acid was predominant (Kowalski, 2007). It was determined of 4.56 mg CAE.g^{-1} of TPAC in the ethanol extracts of *Cichorium intybus* (Vergun et al., 2019).

Flavonoids are a group of secondary metabolites with numerous functions such as survival and reproductive fitness (Williams et al., 2009). As many authors emphasize, flavonoid action is not clear full, however, they may regulate the molecular and cellular processes (Spencer et al., 2009; Kumar et al., 2019b). Asteraceae representatives are characterized by the presence of flavonoid aglycons such as kaempferol, apigenin, gekwanin, acacetin, luteolin, nepetin, eupaletin, etc.

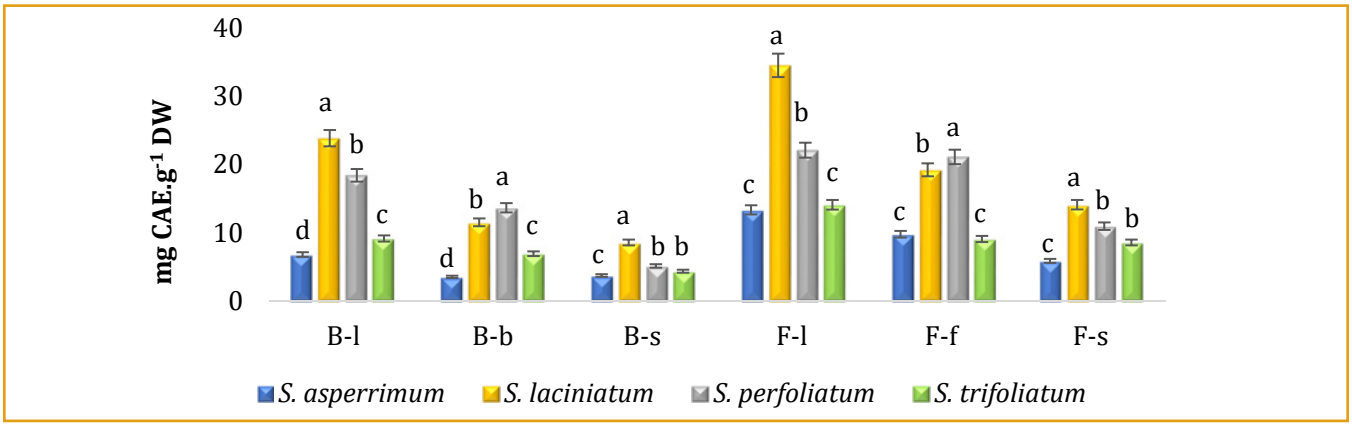

Figure 3 The total content of phenolic acids in ethanol extracts of *Silphium* spp.
CAE – caffeic acid equivalent, B-l – leaves at the budding; B-b – buds at the budding; B-s – stems at the budding; F-l – leaves at the flowering; F-f – flowers at the flowering; F-s – stems at the flowering. Means in each column followed by different letters are significantly different ($p < 0.05$)

Considering the results of the genus *Silphium* should be noted that *S. terebinthinaceum* does not contain aglycones that are quite incoming among Asteraceae (Valant-Vetschera and Wollenweber, 2007). The flavonoid composition of *Silphium* spp. are derivates of the flavonols quercetin, isorhamnetin, and kaempferol (Williams et al., 2009).

TFC in investigated extracts was from 3.67 (*S. perfoliatum*, stems at the budding) to 57.31 (*S. laciniatum*, leaves at the budding) mg QE.g^{-1} DW depending on species and period of growth (Figure 4). Leaf extracts at the budding and flowering stage had TFC 29.31–57.31 and 36.15–51.78 mg QE.g^{-1} DW, respectively. In the buds and flowers, this parameter was 11.12–25.45 and 24.29–32.2 mg QE.g^{-1} DW, respectively. TFC in the stems at the budding and flowering stage was 3.67–22.53 and 11.07–20.96 mg QE.g^{-1} DW, respectively. TFC in the leaf extracts depends on the period of growth and species was maximal and in stem extracts was minimal.

According to Azzouzi et al. (2016), the TFC of *Centaurea choulettiana* Pomel extracts was 236.73 mg QE.g^{-1}. Extracts of *Matricaria recutita* L. showed a TFC of 157.9 mg QE.g^{-1}. As reported by Indradi et al. (2017), different representatives of Asteraceae had the TFC from 0.83 to 23.49 mg QE 100 g^{-1} depending on species and extracts. The TFC in the extracts of *Cichorium intybus* was 26.29 mg QE.g^{-1} (Vergun et al., 2019). According to Hassanpour et al. (2020), in extracts of *M. chamomilla* L. the TFC was 1.37–2.98 mg RE.g^{-1} (rutin equivalent). Muhtadi (2021) determined 10.91, 4.58, and 2.59 mg QE.g^{-1} of TFC in the leaf, inflorescence, and bark extracts of *Helianthus annuus*, respectively.

This study demonstrated that leaves of all investigated *Silphium* species accumulated the highest content of total polyphenols, flavonoids, and phenolic acids. The comparable analysis of reviews about different Asteraceae representatives and obtained data showed that the content of polyphenol compounds, flavonoids, and phenolic acids depends on many factors such as species, extracts, conditions of growth, methods of detection, etc. Due to the lack of sufficient data on a concrete plant species, it is difficult to compare obtained results, however, this gives a general idea about an accumulation of certain groups of polyphenol compounds and their antioxidant activity.

Exist numerous antioxidant activity methods (Alam et al., 2013; Pisoschi et al., 2016; Romulo, 2020) that can indicate that polyphenol compounds are present (Mourtzinos and Goula, 2019). According to Alam et al. (2013), ethanol extracts are most commonly used for antioxidant capacity studies. Antioxidant activity depends on species, populations (Hassanpour et al., 2020), and extracts (Chatha et al., 2006; Wong et al., 2006; Borah et al., 2012). Among antioxidant activity methods, the most widely used is the DPPH (α,α-diphenyl-β-picrylhydrazyl) method based on electron donation and the reaction of discoloration of DPPH radical (Brand-Williams et al., 1995; Alam et al., 2013; Shahidi and Zhong, 2015).

Antioxidant activity by the DPPH method of extracts was from 3.33 (*S. trifoliatum*, stems at the budding)

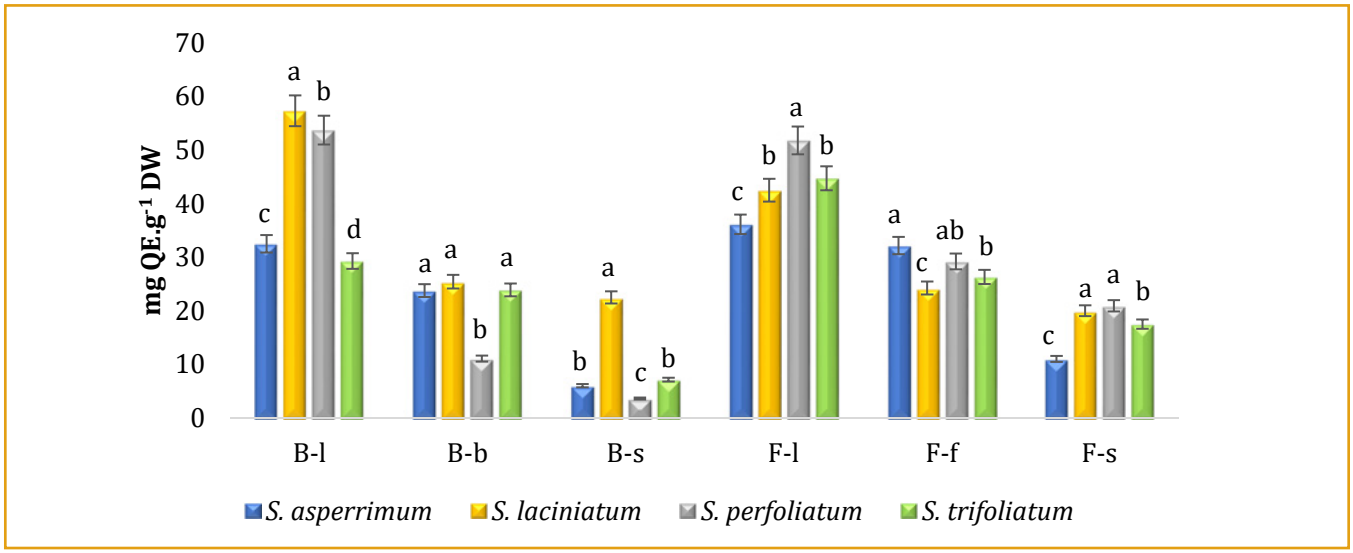

Figure 4 Total content of flavonoids of ethanol extracts of *Silphium* spp.
QE – quercetin equivalent, B-l – leaves at the budding; B-b – buds at the budding; B-s – stems at the budding; F-l – leaves at the flowering; F-f – flowers at the flowering; F-s – stems at the flowering. Means in each column followed by different letters are significantly different ($p < 0.05$)

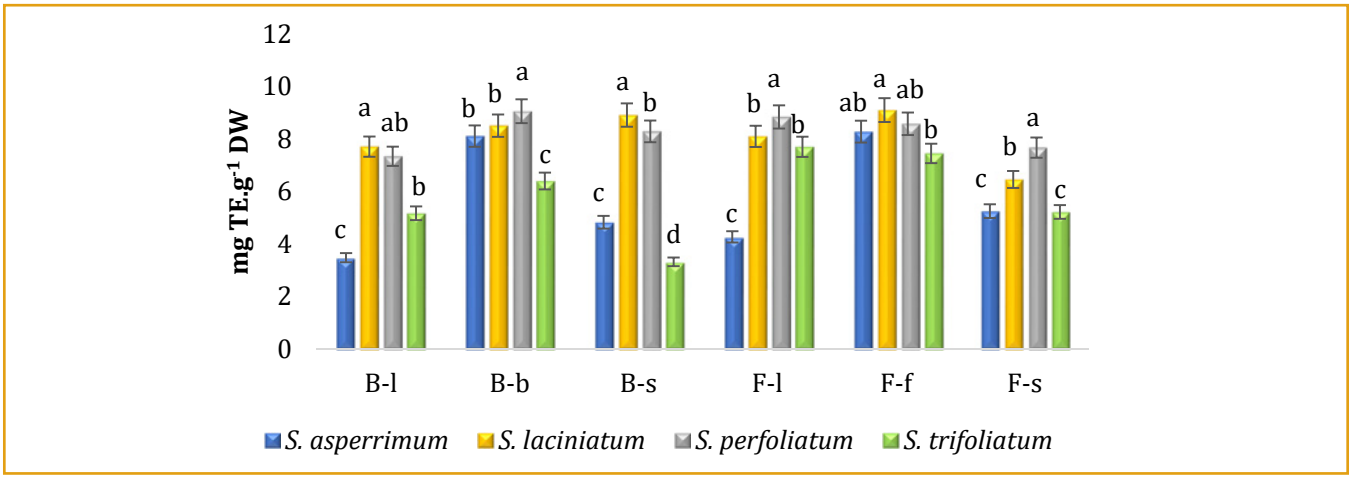

Figure 5 DPPH-radical scavenging activity of ethanol extracts of *Silphium* spp. by DPPH method
TE – Trolox equivalent, B-l – leaves at the budding; B-b – buds at the budding; B-s – stems at the budding; F-l – leaves at the flowering; F-f – flowers at the flowering; F-s – stems at the flowering. Means in each column followed by different letters are significantly different ($p < 0.05$)

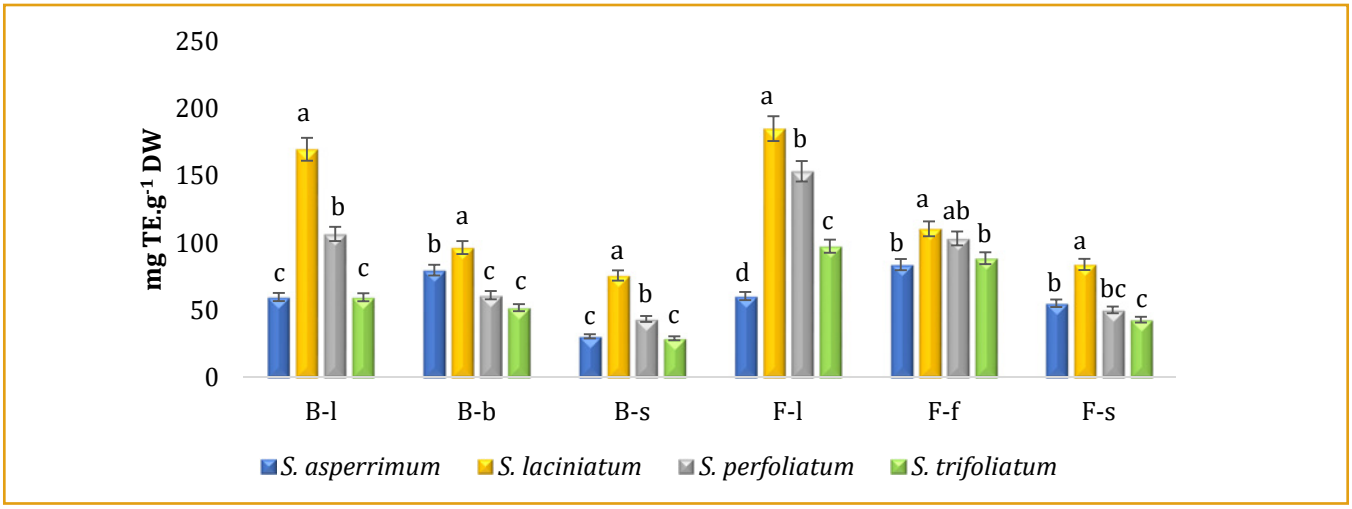

Figure 6 Molybdenum reducing power of ethanol extracts of *Silphium* spp.
TE – Trolox equivalent, B-l – leaves at the budding; B-b – buds at the budding; B-s – stems at the budding; F-l – leaves at the flowering; F-f – flowers at the flowering; F-s – stems at the flowering. Means in each column followed by different letters are significantly different ($p < 0.05$)

to 9.11 (*S. laciniatum*, flowers at flowering) mg TE.g^{-1} DW depending on stage and organ (Figure 5). RP of leaf extracts at the budding and flowering was 3.49–7.72 and 4.28–8.85 mg TE.g^{-1} DW, respectively. Bud and flower extracts showed the RP of 6.41–9.07 and 7.46–9.11 mg TE.g^{-1} DW, respectively. Stem extracts exhibited RP from 3.33 to 8.92 mg TE.g^{-1} DW at the budding and from 5.23 to 7.68 mg TE.g^{-1} DW at the flowering.

Extracts of *Elephantopus scaber* L., *Eclipta alba* (L.) Hassk., *Pluchea indica* (L.) Less, and *Taraxacum officinale* Weber ex F. H. Wigg had DPPH scavenging activity from 16.66 to 16.48 µg.ml^{-1} (Indradi et al., 2017). DPPH-radical scavenging activity of ethanol extracts of *Cichorium intybus* was 8.35 mg TE.g^{-1} (Vergun et al., 2019). Antiradical activity of different extracts of *Silphium* spp. showed that methanol extracts of four investigated species stem had less values, the maximum values demonstrated water extracts of leaves, buds, or inflorescences (Shymanska et al., 2020).

Antioxidant capacity by phosphomolybdenum method based on the reduction of Mo (VI) to Mo (V) by sample extract and described by Prieto et al. (1999). Use of different temperatures (from 40 to 100 °C) at the leaf's extraction of *Gynura divaricate* the highest antioxidant activity by phosphomolybdenum method was at 90 and 100 °C (Wan et al., 2011). In total, the antioxidant activity by phosphomolybdenum method of extracts was from 29.11 (*S. trifoliatum*, stems at the budding) to 185.22 (*S. laciniatum*, leaves at the flowering) mg TE.g^{-1} DW depending on the species and

stage of vegetation (Figure 6). MRP of leaf extracts at the budding and flowering stage was 59.65–169.85 and 60.53–185.22 mg TE.g^{-1} DW, respectively. Extracts of *S. asperrimum* showed the highest MRP in the generative organs, whereas, all other species had the highest values in the leaves at both periods. In this case, the lowest MRP was in the stem extracts of all investigated plants.

Plants of *Scorzonera hispanica* L. at the start of spring vegetation had antioxidant activity by phosphomolybdenum method 125.46 mg TE.g^{-1} (Vergun et al., 2018). Extracts of *Cichorium intybus* at the flowering stage showed 93.01 mg TE.g^{-1} of MRP (Vergun et al., 2019). According to Alper et al. (2021), ethanol extracts of *Centaurea solstitiales* and *Urospermum picroides*, other species of Asteraceae, showed antioxidant activity by this method of 49.23 and 42.13 mg TE.g^{-1}, respectively. In this study, different parts of *Silphium* spp. at the flowering stage showed activity by this method from 42.97 to 185.22 mg TE.g^{-1}. This study showed that extracts of *S. laciniatum* exhibited the highest molybdenum-reducing power depending on the plant part and stage of growth.

Numerous studies demonstrated a positive correlation between total polyphenol content and antioxidant activity. However, this depends on the assay of determination and plant species. A strong correlation between antioxidant activity by the phosphomolybdenum method of plant extracts and total phenolic content and total flavonoid content was found in some studies (Khan et al., 2012). A very strong correlation between investigated parameters at the budding stage was found between TPC and TFC (r = 0.924), TPC and MRP (r = 0.890), TPAC and MRP (r = 0.869), TPC and TPAC (r = 0.864), TFC and MRP (r = 0.858), TPAC and TFC (r = 0.793) (Table 1).

At the flowering stage, a very strong correlation was found between TPC and TFC (r = 0.941), TPAC and MRP (r = 0.905), TPC and MRP (r = 0.825), TPC and TPAC (r = 0.810), TFC and MRP (r = 0.715) (Table 2).

The study of different Asteraceae species showed the existence of a correlation between parameters of antioxidant activity. Aktumsek et al. (2013) found for *Centaurea* L. taxa a strong correlation between antioxidant activity and total phenolic content and flavonoid content (r = 0.84–0.96). The reducing power of extracts in that study also showed a strong correlation with total phenolic content. Muhtadi (2021) found a very strong correlation between DPPH antioxidant activity and TPC (r = 1.000) and with TFC (r = 0.962).

Opposite, the study of *Helichrysum* spp. didn't show a correlation between total phenolic content and antioxidant activity by the phoshomolybdenum method and DPPH method (Albayrak et al., 2010). Negative correlations between DPPH scavenging activity and TPC (r = -0.180–0.952) were found in extracts of Asteraceae species (Indradi et al., 2017). The study of *Cichorium intybus* extracts demonstrated

Table 1 Correlation between antioxidant parameters of *Silphium* spp. at the budding stage

Parameter	TPC	TPAC	TFC	DPPH	MRP
TPC	1.000				
TPAC	0.864**	1.000			
TFC	0.924**	0.793**	1.000		
DPPH	0.342*	0.378*	0.110*	1.000	
MRP	0.890**	0.869**	0.858**	0.451*	1.000

TPC – total phenolic content; TPAC – total phenolic acid content; TFC – total flavonoid content; DPPH – DPPH-radical scavenging activity; MRP – molybdenum reducing power of extracts; ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

Table 2 Correlation between antioxidant parameters of *Silphium* spp. at the flowering stage

Parameter	TPC	TPAC	TFC	DPPH	MRP
TPC	1.000				
TPAC	0.810**	1.000			
TFC	0.941**	0.617*	1.000		
DPPH	0.397*	0.522*	0.428*	1.000	
MRP	0.825**	0.905**	0.715**	0.648*	1.000

TPC – total phenolic content; TPAC – total phenolic acid content; TFC – total flavonoid content; DPPH – DPPH-radical scavenging activity; MRP – molybdenum reducing power of extracts; ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

a strong positive correlation between antioxidant activities and polyphenol compound groups: between MRP and TFC (r = 0.997), between DPPH and TPC (r = 0.996), between DPPH and TFC (r = 0.976), between DPPH and TPAC (r = 0.971), between MRP and TPC (r = 0.971), between MRP and TPAC (r = 0.884) (Vergun et al., 2019).

According to this study's results, between DPPH scavenging activity of extracts and different polyphenol compounds existed weak or moderate relation depending on the stage of growth (r = 0.110–0.378 at the budding and r = 0.397–0.522 at the flowering) whereas between molybdenum reducing power and polyphenol compounds found a strong correlation (r = 0.859–0.890 at the budding and r = 0.715–0.905 at the flowering). Between the two methods of antioxidant activity, determination found a moderate correlation. It should be concluded from these data that total phenolic compounds contribute highly to the antioxidant activity by the phosphomolybdenum method and contributes slightly to the antioxidant activity by the DPPH method of investigated species of *Silphium*.

Conclusions

Obtained data on antioxidant parameters of *Silphium* spp. showed that its promising crops with antioxidant activity. The content of polyphenol compounds and phenolic acids in ethanol extracts of all investigated plants was the highest in the leaves and the maximal value had plants *S. laciniatum* (95.21 and 34.58 mg CAE.g^{-1}, respectively). The accumulation of flavonoids was uneven. In this case, plants *S. asperrimum* and *S. trifoliatum* accumulated maximal total flavonoid content in the leaves at the flowering stage, and in its turn, *S. laciniatum* and *S. perfoliatum* in the leaves at the budding stage. The highest content of flavonoids was determined in raw *S. laciniatum* (57.31 mg QE.g^{-1}). The highest antioxidant activity by the DPPH method was found at the budding stage for *S. perfoliatum* (buds) and flowering stage for *S. asperrimum* (stems), *S. laciniatum* (flowers), *S. trifoliatum* (leaves). Ethanol extracts of all investigated plants demonstrated maximal values of molybdenum-reducing power at the flowering stage but *S. asperrimum* in the flowers and *S. laciniatum*, *S. perfoliatum*, and *S. trifoliatum* in the leaves. This research can be useful for further deep biochemical investigation of plant raw of *Silphium* spp. and in livestock nutrition branch.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

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Facets of the elaboration of the *Salvia sclarea* L. extracts

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In this article we presented some facets of the development of liquid ethanolic extracts of *Salvia sclarea* L. The primary purpose was to elaborate the analytical procedure of the quantitative determination of the total flavonoid content with the establishing dominating group of flavonoids in the extracts of *Salvia sclarea* by colorimetric aluminum chloride method. The second purpose of the work was to study the influence of pharmaceutical factors like temperature of extraction, particle size of the herbal raw material and ultrasound on the extraction of flavonoids. It was studied that the higher temperatures and ultrasound induced higher extraction of flavonoids from the herb of *Salvia sclarea*. The TFC was in the range of 6.4 to 14.7 mg rutin-equivalents per one gram of the herb and depended on temperature of extraction, particle size of herbal raw material and ultrasound on the extraction of flavonoids. Rutin was chosen as a commercially available marker. The calibration curve was plotted in the concentration range of 10 to 102 mg.L^{-1} of rutin. The wavelength of maximum absorption of four extracts was in the range of 390–391 nm that indicated that flavons were dominating group of flavonoids in these extracts. These studies can be basis for the development of extracts of *Salvia sclarea* for pharmaceutical and cosmetic industries.

Keywords: *Salvia sclarea*, ethanolic extracts, total flavonoid content

Introduction

Currently, the role of medicinal plants as a source of herbal medicinal preparations has become a topic of global importance (Zengin et al., 2018, Kivrak et al., 2019). Plants from the Lamiaceae family containing terpenes and polyphenols have interesting biological activities (Valant-Vetschera et al., 2003; Tavakkoli et al., 2014, Kivrak et al., 2019; Yezerska et al., 2021). This family consists of about 200 genera and from 3200 to 6500 species and is known as a family of aromatic plants (Valant-Vetschera et al., 2003; Schmiderer et al., 2008; Asadi et al., 2010; Hanganu et al., 2018;

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Hudz et al., 2021). Most genera of Lamiaceae are rich in terpenoids, iridoid glycosides and flavonoids (Valant-Vetschera et al., 2003; Asadi et al., 2010; Aćimović et al., 2018; Zengin et al., 2018; Yezerska et al., 2021). The characteristic feature of plants from the Lamiaceae family is good antioxidant capacity, which is connected to many medicinal properties. Among these activities are anti-inflammatory, antidiabetic, antiviral and antitumor ones (Asadi et al., 2010; González-Chávez et al., 2017). It was stated that the dichlorometane extract of *Salvia connivens* had an anti-inflammatory effect as it reduced the levels of the proinflammatory cytokines IL-1b, Il-6 and TNF-a and increased the level of the antiinflammatory cytokine IL-10 in the culture medium of macrophages stimulated with lipopolysaccharides (González-Chávez et al., 2017).

Clary sage (*Salvia sclarea* L.) belongs to the most popular species in the genus *Salvia*, which includes more than 900 species (Kintzios, 2000; Valant-Vetschera et al., 2003; Asadi et al., 2010; Šućur et al., 2016b; González-Chávez et al., 2017; Zengin et al., 2018; Kivrak et al., 2019; Afonso et al., 2021; Svydenko et al., 2022). *Salvia sclarea* is an aromatic biennial or perennial plant, 20 to 120 cm tall with a thick square stem (Angelova et al., 2016). It comes from Southern Europe, but it is bred all over the world for the health-promoting properties of the essential oil and as an ornamental plant often used in perfumery (Schmiderer et al., 2008; Goncerariuc et al., 2016; Angelova et al., 2016; Aćimović et al., 2018). The essential oil is obtained from the fresh parts of the sage which is in full flowering stage. The content of essential oil in fresh inflorescences ranges from 0.15 to 0.20% (Kuzma et al., 2009; Angelova et al., 2016).

Clary sage is well known for its high value essential oil, widely used in perfumery. This oil possesses high biological activity and it can be used for the treatment of patients with stress, tension, depression, insomnia (Kamatou et al., 2008; Yaseen et al., 2014, Hao et al., 2015). Linalool (18%), linalyl acetate (63%) and sclareol (6%) are among the major components of the essential oil from leaves and inflorescences of *Salvia sclarea* (Schmiderer et al., 2008). The chemical composition of essential oil significantly depends on the part of the plant (leaf, corola, or calyx) and type of glandular trichomes (Schmiderer et al., 2008).

The aqueous extract of *Salvia sclarea* exhibits allelopathic and insecticidal properties valued in agriculture because allelochemicals are attractive as new classes of herbicides (Šućur et al., 2015; Šućur et al., 2016a; Šućur et al., 2016b). Clary sage seeds are rich in fatty acids and have high levels of antioxidant and antiradical activities making them suitable for use as nutraceuticals (Aćimović et al., 2018). *S. sclarea* is used not only in medicine, but also as additive in the food industry, where the interest in this plant is constantly growing due to the search for healthy and natural food (Goncerariuc et al., 2016). This species is used widely as an ornamental and landscape plant (Çetinkale Demirkan and Akat, 2018).

Recent studies demonstrate analgesic, antimicrobial (Kuzma et al., 2009; Hristova et al., 2013), anti-anxiety (Gross et al., 2013; Yang et al., 2014), antidiabetic (Raafat and Habib, 2018; Afonso et al., 2021) and cytotoxic effects (Kuzma et al., 2009; Gulcin et al., 2004), insecticidal activity (Šućur et al., 2015) of essential oil and extracts of *S. sclarea*. The inhalation of clary oil may be useful for inducing relaxation in female urinary incontinence patients, as well as in menstrual and digestive problems (Szentmihalyi et al., 2009; Verma, 2010; Seol et al., 2013).

Polyphenols are secondary metabolites that are broadly spread in the plans. They are known for their antioxidative capacity as they neutralize free radicals which are responsible for cell damage. Phenolic acids are largely responsible for antioxidant properties of the extracts of *Salvia sclarea*. Caffeic acid, compared to other species of sage (*S. aetiopsis*, *S. austriaca*, *S. nutans*, *S. verticillatta*, *S. nemorosa*), is most abundant in *Salvia sclarea*. Apart from caffeic acid, HPLC analysis showed that compounds such as *p*-coumaric or ferulic acids can be isolated from clary sage. Rosmarinic acid was previously isolated from the methanolic extracts of some *Salvia* species leaves (*S. officinalis*, *Salvia glutinosa*, *Salvia aethiopis* and *S. sclarea*) (Bandoniene et al., 2005, Kostic et al., 2017).

Kharazian (2013) identificated flavonoids in leaves of seven wild growing *Salvia* species from Iran. Flavons were the most frequent flavonoid in seven *Salvia* species (35.7%) and dihydroflavonoles were in the least concentrations (5.3%). The highest flavonoid content was identified in *S. multicaulis* and *S. hydrangea*. It can be concluded that the flavonoid constituents seem to be a suitable indicator in chemotaxonomic studies in *Salvia* genus. Hanganu et al. identified such flavonoids like isoquercitrin (2208 $\mu g.g^{-1}$ of the plant dry weight), luteolin (780 $\mu g.g^{-1}$ the plant dry weight), luteolin (293 $\mu g.g^{-1}$ the plant dry weight) (Hanganu et al., 2018).

In general, there are a few studies directed at the approach of the elaboration of the standardized procedure of the determination of the total flavonoid content with the justification of choosing a marker for

the calculation the content of flavonoids, establishing the dominating group of flavonoids on the base of the absorption maximum and repeatability of results in different time of the reaction with aluminum chloride with the simultaneous study of the influence of pharmaceutical factors on the extraction of flavonoid (Hudz et al., 2017). Therefore, the goal of the work was to provide the justified approach to the elaboration of the standardized procedure of the determination of the total flavonoid content (TFC) for the fluid extract of *S. sclarea*.

Material and methodology

While carrying out this study, such methods were used: analysis, synthesis, systematization, and comparison for processing published scientific data; technological method (maceration); spectrophotometric method for the elaboration of the analytical procedure of the determination of the TFC by aluminum spectrophotometric method.

Plant material

The aerial parts of *Salvia sclarea* L. were collected at late flowering stage in August 2017 in the Sector of mobilization and saving herbal resources of the Rice Institute of the National Agrarian Academy of Sciences of Ukraine located in Plodove of Kherson region of Ukraine (latitude: 46° 39‘ 20.92“ N, longitude: 32° 37‘ 4.08“ E).

Reagents

The following reagents were used: ethanol 96% (manufacturer “Centrachem“ (Slovakia)), aluminium chloride (’Sigma Aldrich‘), and rutin hydrate (’Sigma Aldrich‘).

Extraction

The plant material was dried at room temperature, then crushed (to particle size 0.5–5.0 mm) and subjected to extraction by ethanol of different concentrations (65 and 70%). The crushed particles of *Salvia sclarea* were extracted with 65 and 70% ethanol. The characteristic of the extracts used in this study is provided in Table 1.

Total flavonoid content (TFC)

TFC was determined using the slightly modified analytical procedures of differential spectrometry provided by Hudz et al. (2017). This procedure is considered a main procedure for the measuring the TFC in herbal preparations and beekeeing products (Pękal and Pyrzynska, 2014; Hudz et al., 2017). Rutin trihydrate was used to build the calibration curve in the concentration range of 10 to 102 mg.L^{-1}. Rutin trihydrate dissolution in 50% ethanol was carried out with the aid of ultrasound. The results were expressed as rutin equivalents: mg eq-rutin.L^{-1} of an extract and mg eq-rutin.g^{-1} of the *S. sclarea* herb.

The TFC was calculated as follows. 10, 30, 50, 70 and 100 µL of the stock solution of rutin trihydrate (1016 mg.L^{-1}) were diluted with 50% ethanol up to 1.0 mL. The obtained dilutions of rutin trihydrate were mixed with 1.0 ml of 2% aluminum chloride hexahydrate in 50% ethanol. After the incubation at room temperature for 75 ±15 min the spectra of the reaction mixtures were measured in the range of 360 nm to 460 nm with the spectrophotometer «Photometry Hitachi U-2810». The volume of 2% aluminum chloride hexahydrate in 50% ethanol was substituted by the same volume of 50% ethanol in the blank for each dilution of rutin trihydrate. In a like manner, 50 or 100 µL of the developed extracts of the *S. sclarea* herb were mixed with 1.0 ml of aluminum chloride hexahydrate. The mixture was mixed by vortex and incubation was performed at room temperature for 75 ±15 minutes. The volume of 2% solution of aluminum chloride was substituted by the same amount of 50% ethanol in blank. The test was carried out for each extract in triplicate. The TFC was calculated

Table 1 Characteristics of the *Salvia sclarea* L. extracts

Identification number of extract	Particle size	Ratio of raw material to the solvent	Maceration time and conditions	Yield of an extract, ml
E-1	0.5–5.0 mm	5.0 g to 110 ml of 70% ethanol	200 min at ultrasound and a temperature of (40–46 °C) plus 21 hour of maceration at room temperature	89.5
E-2	0.5–5.0 mm	5.0 g to 108 ml of 65% ethanol	6 days at room temperature	82.5
E-3	0.5–5.0 mm	5.0 g to 108 ml of 65% ethanol	100 min at (36–41 °C) plus 6 days at room temperature	81.5
E-4	2.0–5.0 mm	5.05 g to 50 ml of 70% ethanol	7 days at room temperature	32.0

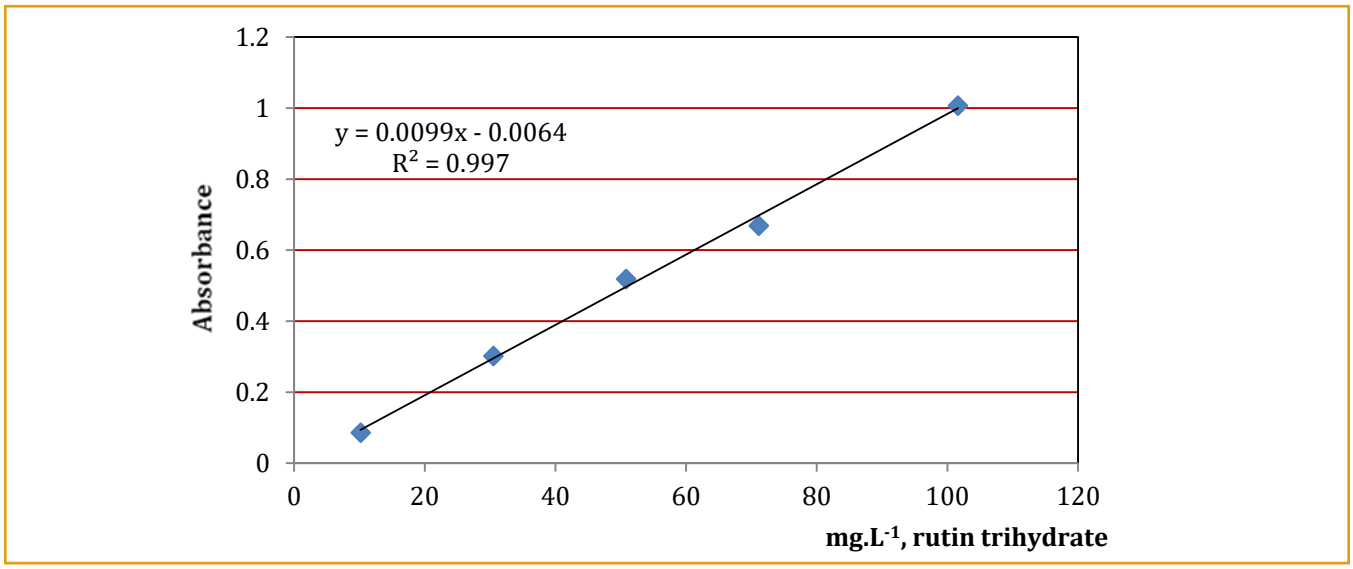

Figure 1 Calibration curve of rutin trihydrate

using expression C = c × 20(10) × k, where C is TFC of the tested extract, c is TPC taken from the calibration curve, 20 for 50 µL of an extract or 10 for 100 µL of an extract is coefficient of dilution of the extracts for their testing E-4, E-3, E-1 and E-2 respectively, k is coefficient for the recalculation of rutin trihydtate into rutin (0.917).

Results and discussion

Flavonoids are considered as principal substances in plants. In this paper, quantitative determination of TFC was carried out by colorimetric aluminum chloride method. The principle of aluminum chloride colorimetric method is that aluminum chloride forms complexes with flavones and flavonols wherein it reacts with the C-4 keto group and hydroxyl groups of the ring C, and/or ring A and/or ring B (Pękal and Pyrzynska, 2014; Hudz et al., 2017). In this work four extracts of *Salvia sclarea* were investigated. The TFC in the tested extracts was in the range of 596 to 1150 mg.L^{-1} depending on the particle size of the herbal raw material, heating and ultrasound in the extraction. The TFC in the herbal raw material was in the range of 6.6 to 14.7 mg per one gram. Our study confirmed that complex of rutin with aluminum chloride had the maximum absorption at 413 nm in differential spectra in the range of rutin concentrations of 10.0–102.0 mg.L^{-1} (50% ethanol was as a solvent). The wavelength of maximum absorption of four extracts was in the range of 390–391 nm that indicated that flavons were dominating group of flavonoids in these extracts. According to literature data, flavones (chrysin, apigenin, and luteolin) and glycosides of flavonols have maximum absorption less 415 nm (Hudz et al., 2017). In our studies published earlier the solutions of complexes aluminum chloride with quercetin (20 mg.L^{-1}), rutin (50.2 mg.L^{-1}), and chrysin (80 mg.L^{-1}) had the maximum absorption at the wavelengths of 425.9 ±0.3 nm at 77 min of the reaction, 412.3 ±0.3 nm at 82 min, 388.4 ±0.7 nm at 81 min, respectively. The tinctures of *Monarda fistulosa*, *Satureja hortensis*, *Thymus vulgaris*, and *Mentha piperita* had the maximum absorption at 391.2 ±0.5 nm at 91 min, 389.9 ±0.5 nm at 76 min, 391.8 nm at 83 min, 394.9 ±1.1 nm at 78 min, respectively (Yezerska at al., 2021). Repeatability of the position of an absorption maximum of the extracts is very good at performing analyses in the different times of the reaction of forming complexes flavonoids with aluminum chloride or in different days of performing analysis (Table 2). We also can conclude that all the four extracts had the absorption maximum about 390 nm.

In addition, we used 50 or 100 µL of the extracts in order to obtain the values of absorbance of the solutions of the extracts or rutin with aluminum chloride not more 0.80 according to rules ofspectrophotometry. In general, if the absorbance of a reaction mixture is less than 0.05 or significantly higher 1.0, it is necessary to correct the volume of a sample for the determination of TFC (Hudz et al., 2017). Moreover, we selected rutin as a commercially available marker for constructing its calibration curve (Hudz et al., 2021). Furthermore, it was detected as a phenolic compounds in some specis of *Salvia* (*S. potentillifolia*, *S. albimaculata* and *S. nydeggeri*) (Kivrak et al., 2019). Hanganu et al. employed also rutin for measuring TFC in some *Salvia* species, including *S. sclarea*.

According to literature data flavons are identified in many Lamiaceae species, including *Salvia* genus. Veličkovič et al. (2007) analyzed extracts from garden (*Salvia officinalis* L.) and glutinous (*Salvia glutinosa* L.) sage by ultrasonic and classical maceration. The flavonoids were also detected in considerable quantities in the plant material from which the essential oils had been already removed. Apigenin and its derivatives (e.g., apigenin 4'-methyl ether), scutellarein 6-methyl ether, isoscutellarein 8-methyl ether, luteolin and 6-OH-luteolin-6-methyl ether were distinctive for *S. officinalis*. Apigenin, luteolin, 6-OH-luteolin-6-methyl ether, kaempherol 3-methyl ether, kaempherol 3,7-dimethyl ether, quercetin 3,7,3'-trimethyl ether and quercetin 3,7,3',4'-tetramethyl ether were distinctive for *S. glutinosa*.

Valant-Vetschera et al. (2003) studied chemodiversity of various species of Lamiaceae family, in particular flavonoid profile, and they recognized for *Salvia sclarea* some flavonoids such as apigenin (5,7,4'-triOH flavone), genkwanin (5,4'-diOH-7-OMe flavone), 5-OH-7,4'-diOMe flavone, salvigenin (5-OH-6,7,4'-triOMe flavone).

Flavonoids display a wide range of pharmacological activities, including neuroprotective, anti-inflamatory one (Asadi et al., 2010; Tavakkoli et al., 2014). In study performed by Asadi et al. (2010), the methanolic extracts of *S. hydrangea* and *S. sclarea* (≤50 μg.ml^{-1}) were shown to fight DNA oxidative damage of PC12 neural cells in rats induced by Fe(II)–H_2O_2. Additionally, the neuroprotective effects of *Salvia* extracts from *S. hydrangea* and *S. sclarea* against oxidative stress (using H_2O_2 as oxidative agent), were observed in PC12 neural cells, for which pretreatment with 100 μg.mL^{-1} significantly protected cell survival (76 to 93%) with respect to the control. This order *Salvia hydrangea*, *Salvia macilenta* > *Salvia multicalis*, *Salvia sclarea* > *Salvia xanthocheila* > *Salvia lachnocalyx* has shown the scavenging ability of six species methanolic extracts reported by Asadi et al. (2010). Asadi et al. consider above mentioned plants as a source of preparations for treating neurodegenerative diseases (Asadi et al., 2010). Tavakkoli et al. (2014) studied the methanolic extracts of *Salvia santolinifolia* Boiss. and *S. sclarea* L. (100 μg.mL^{-1}). These extracts reduced the H_2O_2-stimulated ROS production in neuronal PC12 cells by 61.9 and 61.4%, and showed significant neuroprotection in the MTT assay by 34.7 and 39.5%, respectively. In the same study, the *S. santolinifolia* extract significantly reduced H_2O_2-induced apoptosis (Tavakkoli et al., 2014). Recent studies demonstrate anti-inflamatory activity of the ethanolic extract of *S. sclarea*. The treatment with the extract, compared to the untreated group of the rats, significantly decreased the inflammation diminishing the levels of IL-1β, IL-6 and TNF-α, reducing the gingival tissue lesions and preserving bone alveolar resorption (Kostic et al., 2017).

As can be seen from Table 2, the TFC was in the ramge of 6.4 to 14.7 mg per one gram of the herb. The highest flavonoid content was observed for extracts 1 and 3 in reference to one gram of the herb. For their preparation heating and ultrasond were used. Our data are in line with results of Jasicka-Misiak et al. (2018). These authors studied phenolic compounds in *S. officinalis* and *S. sclarea* growing in different habitats and determined the total phenolic content in the range of 63.9 (*S. officinalis*) to 134.4 (*S. sclarea*) mg.GAE.g^{-1}. The yield of flavonoids from herb of *Salvia sclarea* was the highest in extracts for the preparation of which higher temperature (36–46 °C) and ultrasonic were used. The TFC was the highest in the extract in which the solvent-herb ratio was the least (10 : 1) and particle size was in the range of 2 to 5 mm, however the extraction of flavonoids from the herb (depletion degree of the raw material) was the least.

Hanganu et al. (2019) determined only 2.0 mg rutin eqvivalets per one gram of the herb of *Salvia sclarea*. Hovewer, these cauthors used shortlasted extraction. The material was extracted at a temperature of 60 °C (on a water bath) with 70% ethanol for 30 min.

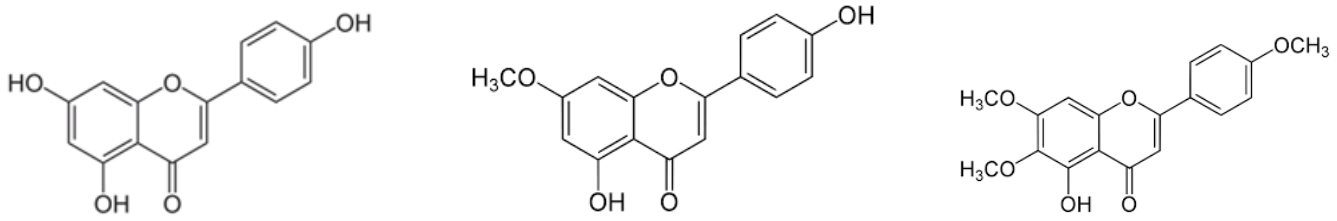

Figure 2 Structures of some identified flavonoids in *Salvia sclarea* L.

Table 2 Calculations of the TFC in the developed tinctures and precision of the analytical procedure

Identification of extracts	Absorption maximum/mean value ± SD (nm)	Volume of the extract (µL)	Absorbance/mean absorbance ± SD	TFC ±SD (extract)	TFC (1 g of the herb)	Time of the reaction
E-1	390.2, 390.2, 390.0/390.1 ±0.1	50	0.404, 0.382, 0.428/0.405 ±0.023	831.1 ±59.4 mg.L^{-1}	14.7 mg.g^{-1}	62 min
			in 57 min			
	390.1, 389.1, 389.7/389.6 ±0.5	50	0.365, 0.404 0.422/0.405 ±0.023	815.0 ±71.5 mg.L^{-1}	14.6 mg.g^{-1}	119 min
E-2	391.9, 392.3, 391.1/391.8 ±0.6	100	0.587, 0.587, 0.577/0.584 ±0.006	596.4 ±12.5 mg.L^{-1}	9.8 mg.g^{-1}	68 min
			in 16 min			
	391.9, 392.1, 391.1/391.7 ±0.5	100	0.592, 0.596, 0.585/0.591 ±0.006	603.4 ±12.5 mg.L^{-1}	10.0 mg.g^{-1}	84 min
E-3	391.3, 391.0, 391.1/391.1 ±0.2	50	0.306, 0.329, 0.318/0.318 ±0.012	655.4 ±37.2 mg.L^{-1}	10.7 mg.g^{-1}	80 min
			in 5 days			
	391.1, 391.1, 390.7/391.0 ±0.2	50	0.330, 0.305, 0.336/0.324 ±0.016	667.5 ±45.3 mg.L^{-1}	10.9 mg.g^{-1}	88 min
E-4	391.0, 391.1, 390.5/390.9 ±0.3	50	0.566, 0.523, 0.599/0.563 ±0.038	1150.3 ±89.7 mg.L^{-1}	7.4 mg.g^{-1}	86 min
			in 1 day			
	391.4, 391.0, 391.1/391.2 ±0.2	50	0.481, 0.520, 0.524/0.508 ±0.024	1039.2 ±61.4 mg.L^{-1} 90.34% from initial value	6.6 mg.g^{-1}	78 min

Conclusion

The analytical procedure of the TFC determination of the *Salvia sclarea* extracts by the colometric aluminum chloride method was developed from a point of view of choosing a volume and dilution of the extracts (50 or 100 µL), marker for the calculation of the antioxidant activity of the extracts. The calibration curve was plotted in the concentration range of 10 to 102 mg.L^{-1} of rutin. The results suggest that the herb of *Salvia sclarea* is a valuable source of flavonoids. The wavelength of maximum absorption of four extracts ranged from 390 nm to 391 nm. Such an absorption maximum indicated that flavons were dominating group of flavonoids in the extracts of *Salvia sclarea*. The influence of pharmaceutical factors like temperature, particle size of herbal raw material and ultrasound on the extraction of flavonoids was established. It was revealed that the higher temperatures induced higher extraction of flavonoids. These studies can be basis for the development of extract of *Salvia sclarea* for pharmaceutical and cosmetic industries

Conflict of interest

The authors declare no conflict of interest.

Ethical Statement

This article does not contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Antibacterial activity of extracts derived from leaves of *Ficus elastica* Roxb. ex Hornem. (Moraceae) and its cultivars against three *Aeromonas* spp. strains

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The present study aimed to evaluate the antimicrobial activity of the ethanolic extracts derived from the leaves of *Ficus elastica* Roxb. ex Hornem. and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas sobria*, *Aeromonas hydrophila*, and *Aeromonas salmonicida* subsp. *salmonicida* to evaluate the possible use of this plant in preventing infections caused by this fish pathogen in aquaculture. The leaves of *F. elastica* and its cultivars, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine. Specifically, the leaves of *F. elastica* and its cultivars were sampled for our study. Three *Aeromonas* strains: *Aeromonas sobria* (K825) and *Aeromonas hydrophila* (K886), as well as *Aeromonas salmonicida* subsp. *salmonicida* (St30), originated from freshwater fish species such as common carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), respectively, were isolated in Department of Fish Diseases, National Veterinary Research Institute in Puławy (Poland). Antimicrobial susceptibility of the tested *Aeromonas* strains was performed by the Kirby-Bauer disc diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2014), with some modifications. Our results of the antimicrobial screening revealed, that *F. elastica* and its cultivars possessed mild antibacterial properties against the *A. sobria* and *A. hydrophila* strains. The ethanolic extract derived from leaves of *F. elastica* 'Variegata' exhibited the maximum antimicrobial activity against *A. sobria*, while the ethanolic extract derived from leaves of *F. elastica* exhibited the maximum antimicrobial activity against *A. hydrophila* and *Aeromonas salmonicida* subsp. *salmonicida* strains. The results of this study provide baseline information on the potential validity of extracts derived from leaves of *F. elastica* and its cultivars in the treatment of infections associated with fish pathogen *Aeromonas* spp..

Keywords: *Ficus elastica*, antimicrobial efficacy, Kirby-Bauer disk diffusion technique, fish pathogens, susceptibility, resistance

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Introduction

Bacterial and viral diseases are the most serious type of disease affecting aquatic animals and a serious obstacle to the development of the aquaculture industry (Liao et al., 2022). Antibiotics and chemicals are common means to prevent and control fish diseases, but their use is currently limited or even banned due to serious issues such as drug residues, pathogen resistance, and environmental pollution (Zhang et al., 2022). In aquaculture, medicinal herbs, and extracts are increasingly promising supplements and alternatives due to their effectiveness, safety, environmental friendliness, and less drug resistance (Valladão et al., 2015). Herbal essential oils contain many bioactive components with powerful antibacterial, antioxidant, and immune-boosting properties, suggesting their use in aquatic animals (Dawood et al., 2022). Medicinal herbs and their extracts can affect growth performance and stimulate the immune system when used in a fish diet. In addition, the use of herbal medicines and their extracts can reduce oxidative stress caused by several stressors in fish farming (Ahmadifar et al., 2021). A wide range of medicinal plants such as herbs, seeds, and spices in various forms such as raw, extracts, mixed, and active compounds are used as immunostimulants and result in a marked boost in the immune system of fish to prevent and control microbial diseases (Awad and Awaad, 2017). Some of these herbs are *Ficus* species that have a long history of use as a food source, in medicine, planting, and other industries and fields of human activity, partly owing to their great diversity and wide distribution range. Among popular ethnomedicinal uses of *Ficus* are treatments of skin damage, 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 elastica Roxb. ex Hornem. is a large monoecious evergreen (rarely deciduous) tree up to 30 m tall. The species is considered to naturally originate from NE India, Myanmar, Malay Peninsula, Sumatra, and Java, but is also commonly cultivated in that areas and throughout the world. It belongs to those species known as hemi-epiphytes, which start life as an epiphyte in the crown of another tree and then send roots down to the ground enveloping the trunk of the host tree. Although usually occurring in forests, this species can also grow as a terrestrial tree or shrub in dry habitats such as cliffs and limestone hills. Its glabrous coriaceous spirally arranged leaves reach 10–40 cm in length and 5–22 cm in width; they are elliptic to oblong with an acuminate apex and cuneate to obtuse or rounded base. The pedunculate glabrous figs of 1.0–1.5 cm in diameter are born axillary or just below the leaves, in pairs or solitary, and turn yellow at maturity (Berg and Corner, 2005).

Standardized extracts of *F. elastica* could be used in traditional medicine for the treatment of wounds and other topical infections (Mbosso et al., 2012). Also, *F. elastica* extracts revealed significant *Schistosoma mansoni* worm reductions and exhibited antischistosomal activity (Seif el-Din et al., 2014). Mbosso Teinkela et al. (2018) revealed *in vitro* cell-growth inhibition activities by methanolic extract of *F. elastica* against *Plasmodium falciparum* strain 3D7 and *Trypanosoma brucei brucei*, as well as against HeLa human cervical carcinoma cells. At the 25 $\mu g.mL^{-1}$ concentration, the extract of *F. elastica* exhibited plasmodiacidal activity (IC_{50} value of 9.5 $\mu g.mL^{-1}$) and trypanocidal (IC_{50} value of 0.9 $\mu g.mL^{-1}$) activity. Extract presented low cytotoxic effects on the HeLa cancer cell line (Mbosso Teinkela et al., 2018). Leaf extract of *F. elastica* is employed as a diuretic agent besides treating skin infections and allergies (Phan et al., 2012).

In the current study, we studied the antimicrobial activity of the ethanolic extracts derived from the leaves of *F. elastica* and its cultivars (*F. elastica* 'Rubra', 'Robusta', 'Burgundy', 'Variegata') against *Aeromonas sobria*, *Aeromonas hydrophila*, and *Aeromonas salmonicida* subsp. *salmonicida* to evaluate the possible use of this plant in preventing infections caused by this fish pathogen in aquaculture.

Material and methodology

Collection of plant materials and preparing plant extract

The leaves of *F. elastica* and its cultivars (Figure 1), cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine (Kyiv). Specifically, the leaves of *F. elastica* and its cultivars, i.e. Rubra, Robusta, Burgundy, Variegata were sampled for our study.

The sampled leaves were brought into the laboratory for antimicrobial studies. Freshly sampled leaves were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1 : 10) at room temperature, and centrifuged at 3000 g for 5 minutes. Supernatants were stored at -20 °C in bottles protected with laminated paper until required.

Figure 1 General view of Ficus elastica Roxb. ex Hornem. plant (A) and a leaf of this plant (B)
Photo: Yevhen Sosnovsky

The current study was conducted as a part of an ongoing project between the Institute of Biology and Earth Sciences (Pomeranian University in Słupsk, Poland), Faculty of Veterinary Medicine and Animal Sciences, University of Life Sciences (Poznań, Poland), M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine (Kyiv, Ukraine), and Ivan Franko National University in Lviv (Lviv, Ukraine) undertaken in the frame of cooperation program aimed at assessment of medicinal properties of tropical and subtropical plants, cultivated *in vitro*.

Bacterial strains for antimicrobial activity assay

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

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

Antimicrobial susceptibility of the tested *Aeromonas* strains was performed by the Kirby-Bauer disc diffusion method (1966) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (2014), with some modifications. Each inoculum of particular bacteria species in the density

of 0.5 McFarland was cultured on Mueller-Hinton agar. After inoculation of bacteria, a maximum of 5 wells per Petri dish with a diameter of 6 mm each was cut into the medium, and plant extracts were added to them. Plates were incubated for 24 h at 28 ±2 °C and the inhibition zones for each well were measured. For each extract, eight replicates were assayed. The plates were observed and photographs were taken. Zone diameters were determined and averaged. Ethanol (at 96% strength, POCH, Poland) as used to prepare the extracts was also used as the negative control for the microbiological study.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). To find significant differences (significance level, $p < 0.05$) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 13.3 software (TIBCO Software Inc.). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥15 mm, Intermediate (I) = 10–15 mm, and Resistant (R) ≤10 mm (Okoth et al., 2013).

Results and discussion

The genus *Aeromonas* includes a collection of ubiquitous Gram-negative rods widely distributed in the aquatic environment (Colwell et al., 1986). The genus *Aeromonas* can be divided into motile and non-motile species (Janda and Abbott, 2010). Currently, 31 species are described in the genus (Fernández-Bravo and Figueras, 2020). Several motile species of *Aeromonas* are known to be pathogens of aquatic animals, and interest in this genus has recently increased due to its zoonotic potential (Janda and Abbott, 2010; Park et al., 2020). *Aeromonas sobria* is a Gram-negative, uniflagellate, rod-shaped, motile, facultative anaerobic bacterium of the genus *Aeromonas* (Taslimi et al., 2018). It is widely distributed in natural environments, including water, soil, feces, etc., and is an opportunistic bacterium for humans, aquatic animals, livestock, and poultry (Zhang et al., 2021). Results on *in vitro* antimicrobial activity assessment of ethanolic extracts derived from leaves of *F. elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas sobria* strain expressed as a mean of diameters of inhibition zone is presented in Figure 2.

Our results of the antimicrobial screening revealed, that *F. elastica* and its cultivars possessed mild antibacterial properties against the *A. sobria* strain. The ethanolic extract obtained from leaves of *F. elastica* 'Variegata'

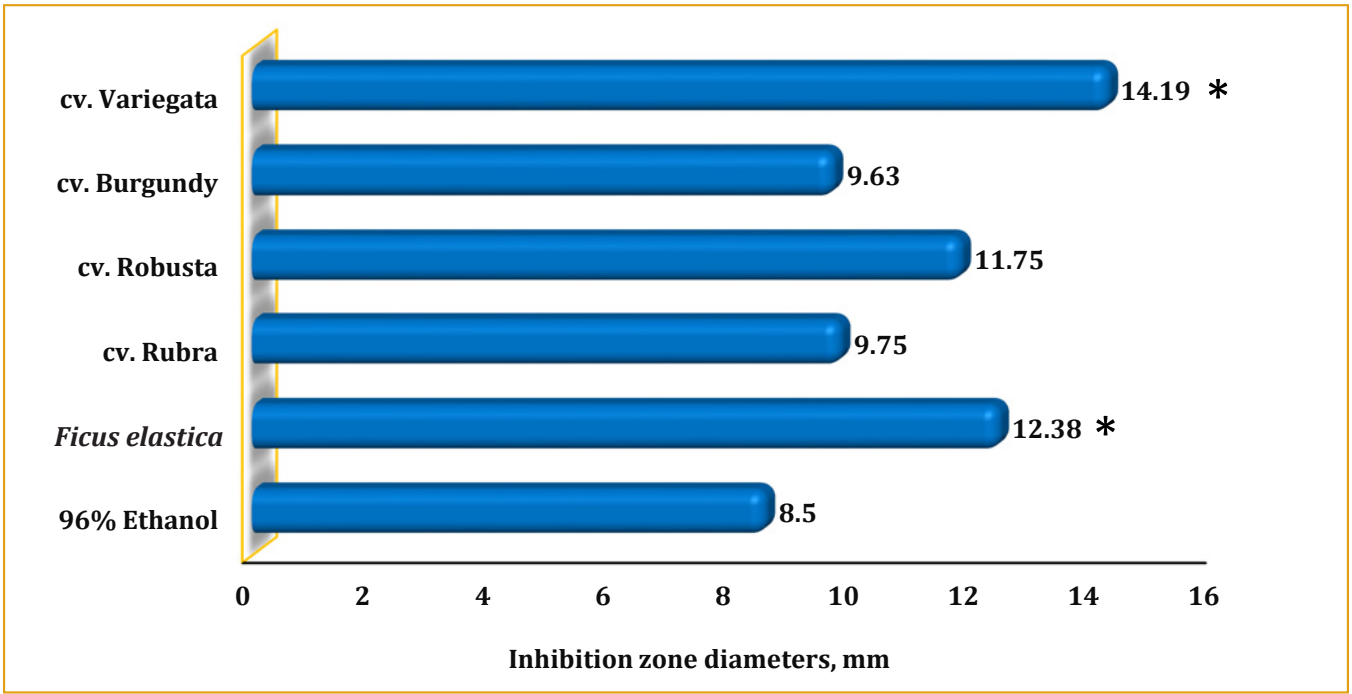

Figure 2 The mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *Ficus elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas sobria* strain (1000 µL inoculum) (M ±m, n = 8)
*– changes are statistically significant compared to the 96% ethanol

exhibited the maximum antimicrobial activity against *A. sobria* (the mean of inhibition zone diameters was 14.19 ±0.73 mm). *A. sobria* strain was susceptible to the *F. elastica* (12.38 ±0.82 mm) and 'Robusta' (11.75 ±0.53 mm). *A. sobria* strain was the most resistant to *F. elastica* 'Rubra' (9.75 ±0.41 mm) and *F. elastica* 'Burgundy' (9.63 ±0.38 mm) leaf extracts. Statistically significant increase in the mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *F. elastica* and its cultivars was demonstrated for *F. elastica* (by 45.6%, $p < 0.05$) and *F. elastica* 'Variegata' (by 66.9%, $p < 0.05$) (Figure 2).

Aeromonas hydrophila is a Gram-negative bacterium that is widely distributed in the aquatic environment and can cause septicemia in both fish and humans (Ji et al., 2015). The disease affects many aquaculture sectors potentially requiring antimicrobial treatments (Gieseker et al., 2022). Results on *in vitro* antimicrobial activity assessment of ethanolic extracts derived from leaves of *F. elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas hydrophila* strain expressed as a mean of diameters of inhibition zone is presented in Figure 3.

Our results of the antimicrobial screening revealed, that *F. elastica* and its cultivars possessed mild antibacterial properties against the *A. hydrophila* strain. The ethanolic extract obtained from leaves of *F. elastica* exhibited the maximum antimicrobial activity against *A. hydrophila* (the mean of inhibition zone diameters was 12.38 ±0.82 mm). *A. hydrophila* strain was susceptible to the *F. elastica* (12.38 ±0.82 mm) and 'Robusta' (10.31 ±0.49 mm). *A. hydrophila* strain was the most resistant to leaf extracts derived from *F. elastica* 'Rubra' (9.25 ±0.59 mm), *F. elastica* 'Variegata' (9.69 ±0.62 mm), and *F. elastica* 'Burgundy' (9.50 ±0.50 mm). A statistically significant increase in the mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *F. elastica* and its cultivars was demonstrated for *F. elastica* (by 43.1%, $p < 0.05$) (Figure 3).

Aeromonas salmonicida, which is known as the only non-motile species in the genus *Aeromonas*, is an important pathogen in salmonid aquaculture and is responsible for typical furunculosis (Vanden Bergh and Frey, 2014). Furunculosis is a ubiquitous disease that affects aquaculture operations worldwide and is characterized by high mortality and morbidity (Dallaire-Dufresne et al., 2014). Results on *in vitro* antimicrobial activity assessment of ethanolic extracts derived from leaves of *F. elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas salmonicida* subsp. *salmonicida* strain expressed as a mean of diameters of the inhibition zone is presented in Figure 4.

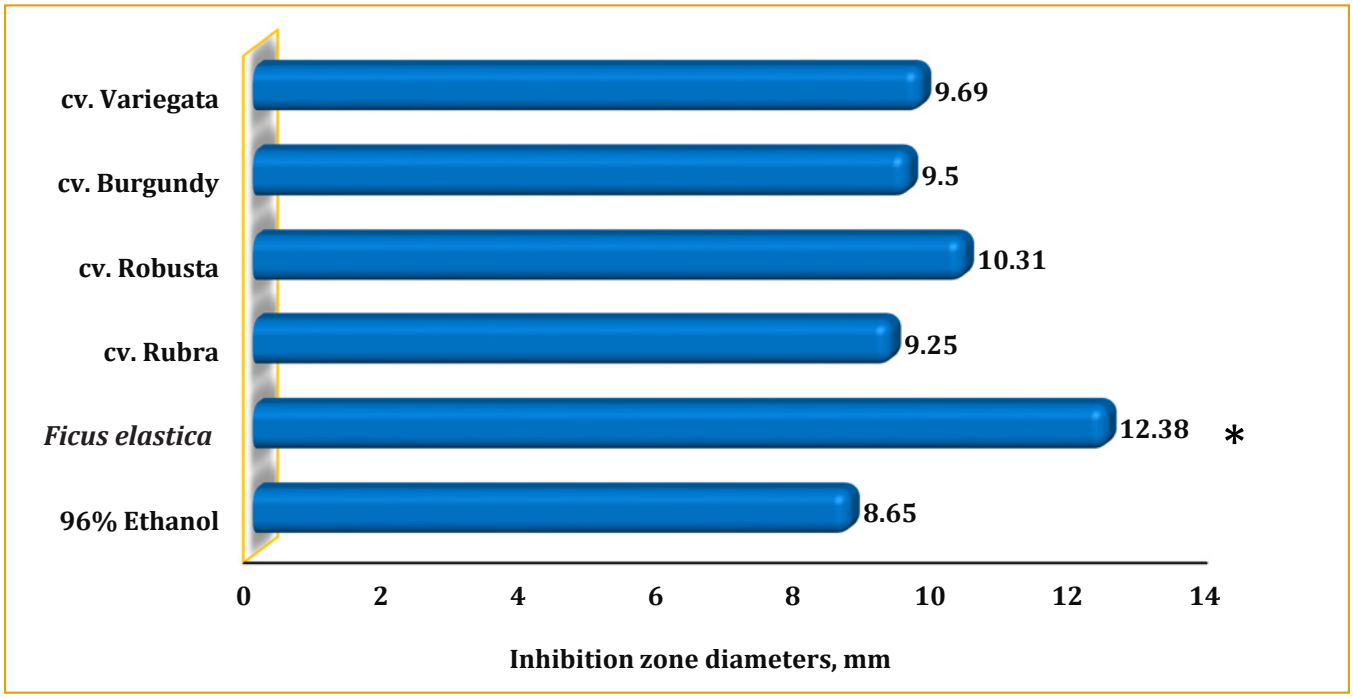

Figure 3 The mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *Ficus elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas hydrophila* strain (1000 µL inoculum) (M ±m, n = 8)
*– changes are statistically significant compared to the 96% ethanol

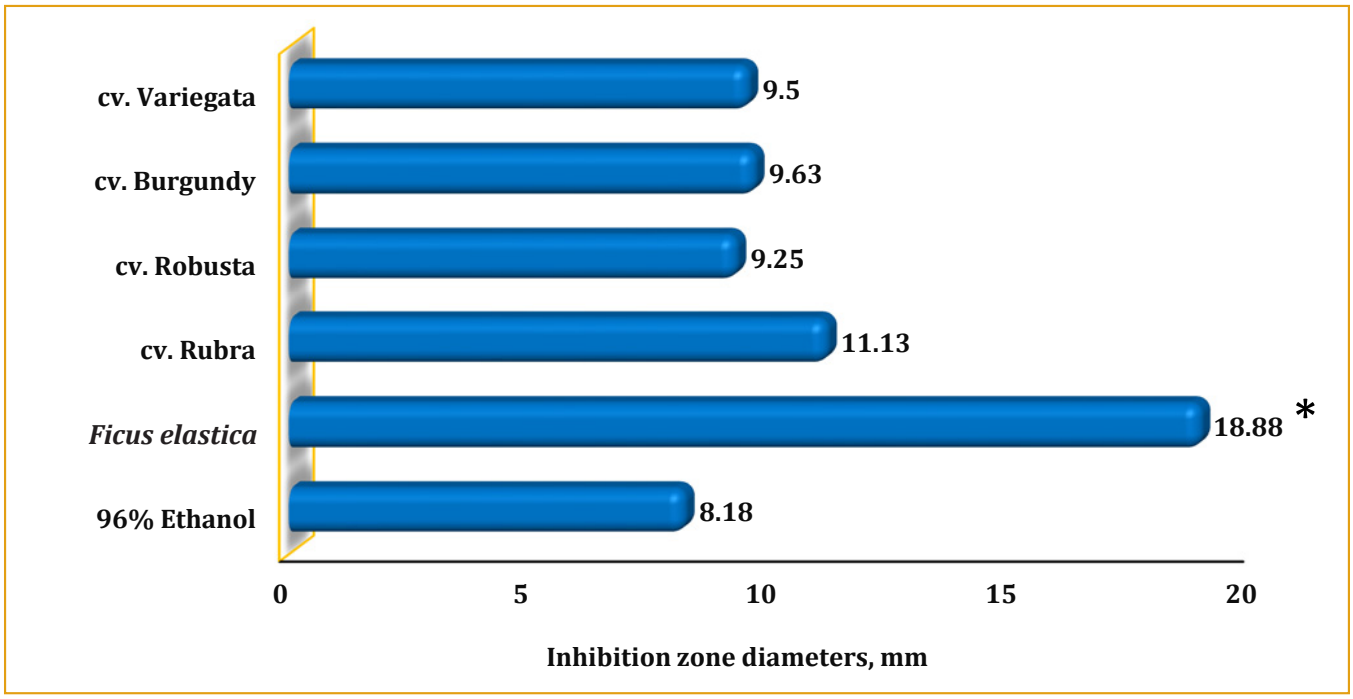

Figure 4 The mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *Ficus elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas salmonicida* subsp. salmonicida strain (1000 µL inoculum) (M ±m, n = 8)
*– changes are statistically significant compared to the 96% ethanol

Our results of the antimicrobial screening revealed, that *F. elastica* and its cultivars possessed mild antibacterial properties against the A. *salmonicida* strain. The ethanolic extract derived from leaves of *F. elastica* exhibited the maximum antimicrobial activity against A. *salmonicida* (the mean of inhibition zone diameters was 18.88 ±0.48 mm). A. *salmonicida* strain was susceptible to *F. elastica* 'Rubra' (11.13 ±0.74 mm). A. *salmonicida* strain was the most resistant to leaf extracts derived from *F. elastica* 'Robusta' (9.25 ±0.56 mm), *F. elastica* 'Variegata' (9.50 ±0.33 mm), and *F. elastica* 'Burgundy' (9.63 ±0.46 mm). A statistically significant increase in the mean inhibition zone diameters induced by ethanolic extracts derived from leaves of *F. elastica* and its cultivars was demonstrated for *F. elastica* (by 130.8%, $p < 0.05$) (Figure 4).

Moreover, in our previous study (Opryshko et al., 2020), we evaluated the *in vitro* possible antioxidant effects of extracts derived from leaves of *F. elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) using oxidative stress biomarker [2-thiobarbituric acid reactive substances (TBARS) as a biomarker of lipid peroxidation] using of human erythrocytes as a cell model after incubation with plant extracts in two doses (5.0 and 0.5 $mg.mL^{-1}$). Our results revealed that treatment of human erythrocytes by extracts derived from leaves of *F. elastica* and its cultivars 'Rubra' and 'Burgundy' in the dose of 0.5 $mg.mL^{-1}$ caused a statistically significant decrease of TBARS level by 27.3% ($p < 0.05$), 32.4% ($p < 0.05$), and 33.5% ($p < 0.05$), respectively. The increase in TBARS level was observed after the treatment of human erythrocytes by extracts derived from leaves of *F. elastica* 'Robusta' and 'Variegata' (by 12.3 and 9.3%, $p > 0.05$, respectively) compared to untreated controls. After treatment of human erythrocytes by extracts derived from leaves of *F. elastica* and its cultivars (Rubra, Burgundy, and Robusta) in the dose 5 $mg.mL^{-1}$, the increase of TBARS level (by 5.7%, 39.5%, 82%, and 87.5%, $p < 0.05$) was observed. Only extract derived from leaves of *F. elastica* 'Variegata' (5 $mg.mL^{-1}$) caused the decrease in TBARS level (by 29.2% $p < 0.05$) compared to untreated controls. Among extracts studied (0.5 $mg.mL^{-1}$), *F. elastica* 'Burgundy' exhibited the lowest TBARS level (decreased by 33.5%, $p < 0.05$) while in dose 5 $mg.mL^{-1}$, *F. elastica* 'Variegata' decreased TBARS level by 29.2% ($p < 0.05$) (Opryshko et al., 2020).

We also evaluated the *in vitro* effect of extracts obtained from leaves of *Ficus elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) on the levels of aldehydic and ketonic derivatives of oxidatively modified proteins in the muscle tissue of the rainbow trout (*Oncorhynchus mykiss* Walbaum) (Tkachenko et al., 2022). Our results revealed that the incubation

of muscle tissue of rainbow trout with extracts derived from the leaves of *F. elastica* and its cultivars resulted in the same levels of aldehydic derivatives of OMP compared to the untreated samples. On the other hand, the levels of ketonic derivatives of OMP were statistically non-significant decreased to the values 12.83 ±1.0 nmol.mg^{-1} protein for *F. elastica* extract, 12.03 ±1.26 nmol.mg^{-1} protein for *F. elastica* 'Rubra' extract, 12.89 ±1.25 nmol.mg^{-1} protein for *F. elastica* 'Robusta' extract, 11.81 ±1.21 nmol.mg^{-1} protein for *F. elastica* 'Burgundy' extract, 12.39 ±1.35 nmol.mg^{-1} protein for *F. elastica* 'Variegata' extract compared to the untreated samples (14.16 ±1.02 nmol.mg^{-1} protein). The percentage of decreased levels of ketonic derivatives of OMP in the muscle tissue of rainbow trout after incubation with extracts derived from leaves of *F. elastica* and its cultivars compared to the values of untreated controls was as follows: 9.4% for *F. elastica* extract, 15% for *F. elastica* 'Rubra' extract, 9% for *F. elastica* 'Robusta' extract, 16.6% for *F. elastica* 'Burgundy' extract, 12.5% for *F. elastica* 'Variegata' extract, respectively. Thus, two extracts derived from leaves of *F. elastica* 'Burgundy' and *F. elastica* 'Rubra' after incubation with muscle tissue of rainbow trout resulted in the maximum decrease in the levels of ketonic derivatives of OMP. The present study ascertained the antioxidant potency of the extracts derived from the leaves of *F. elastica* and its cultivars as a potential source of natural antioxidants (Tkachenko et al., 2022).

Many of our studies confirmed the antioxidant properties of *Ficus* plants against fish pathogens (Pękala-Safińska et al., 2021; Tkachenko et al., 2016a-e, 2022). In our previous study, we evaluated the antimicrobial activity of ethanolic extracts of *Ficus* plant species against *Aeromonas* strains (Pękala-Safińska et al., 2021). As the average over the three *Aeromonas* species, the highest antimicrobial activity among all the tested ethanolic extracts was observed in *F. binnendijkii* leaves with inhibition zone diameters (IZD) of 23.75 ±1.64 mm against *A. sobria*, 20.63 ±1.45 mm against *A. hydrophila*, and 15.75 ±0.80 mm against A. *salmonicida*. *F. craterostoma* extract was effective against *A. sobria* with an IZD of 15.25 ±0.90 mm and against A. *salmonicida* with a zone of 15.25 ±1.15 mm, while *F. deltoidea* extract was effective against *A. sobria* across 18.81 ±1.25 mm and A. *salmonicida* across 20.13 ±0.79 mm diameters. *F. hispida* extract inhibited *A. sobria* the best and showed an IZD of 25.56 ±1.63 mm followed by the extracts of *F. binnendijkii* presenting an IZD of 23.75 ±1.64 mm and *F. tinctoria giving* one of 22.5 ±1.20 mm. The IZD results also showed that isolates of *A. sobria* revealed intermediate susceptibility to ethanolic extracts of *F. aspera*, *F. benjamina*, *F. elastica*, *F. formosana*, *F. johannis* subsp. *afghanistanica*, *F. natalensis* subsp. *leprieurii*, *F. religiosa*, *F. villosa*, and *F. virens*, which created mean IZDs ranging from 10 to 15 mm. The isolates appeared to be resistant to extracts of 18 *Ficus* species (43.9%), which only restricted growth in mean IZDs of less than 10 mm (Pękala-Safińska et al., 2021).

Therapeutic potential for the use of various plants of the *Ficus* genus in the control of bacterial diseases was evaluated against fish pathogens in *in vitro* study with promising results (Tkachenko et al., 2016a-e, 2022). 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 from *F. drupacea*, *F. septica*, *F. deltoidea*, as well as *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma*, exhibit 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* possess considerably sufficient antibacterial potential against *C. freundii* (Tkachenko et al., 2016b). Among various species of *Ficus* 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 derived from *Ficus* spp. proved effective against the bacterial strain of Gram-negative *A. hydrophila* tested, with 10–12 mm zones of inhibition being observed. *A. hydrophila* demonstrated the highest susceptibility to *F. pumila*. The highest antibacterial activity against *A. hydrophila* (200 µL of standardized inoculum) was displayed by *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* leaf extracts (Tkachenko et al., 2016c). 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., 2016d).

It is generally assumed that the antibacterial activity of various *Ficus* species can be explained due to the presence of secondary metabolites that are probably responsible for the test organism's susceptibility to them. The main chemical classes of the phytochemical compounds occurring in the extracts, obtained from the plants belonging to the genus *Ficus*, are alkaloids, anthocyanins, balsams, carbohydrates, flavonoids, free anthraquinones, tannins, glycosides, amino acids, organic acids, fatty acids, terpenes, resins, phytosterols, aliphatic alcohols, volatile components and saponins (Ali and Chaudhary, 2011; Ashraf et al., 2021; Murugesu et al., 2021). The presence of alkaloids and flavonoids both reveals their activity against pathogenic bacteria and suggests a role in the limitation of fungal infection, given that many flavonoids exhibit antifungal activity (Wan et al., 2017). Among polyphenols, flavan-3-ols, flavonols, and tannins received the most attention due to their wide spectrum and higher antimicrobial activity in comparison with other polyphenols, and to the fact that most of them are able to suppress a number of microbial virulence factors (such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins) and show synergism with antibiotics (Coppo and Marchese, 2014).

Conclusions

In the current study, we investigated the antimicrobial activity of the ethanolic extracts derived from the leaves of *F. elastica* and its cultivars (Rubra, Robusta, Burgundy, Variegata) against *Aeromonas sobria*, *Aeromonas hydrophila*, and *Aeromonas salmonicida* subsp. *salmonicida* to evaluate the possible use of this plant in preventing infections caused by this fish pathogen in aquaculture. Our results of the antimicrobial screening revealed, that *F. elastica* and its cultivars possessed mild antibacterial properties against the *A. sobria* and *A. hydrophila* strains. The ethanolic extract derived from leaves of *F. elastica* 'Variegata' exhibited the maximum antimicrobial activity against *A. sobria*, while the ethanolic extract derived from leaves of *F. elastica* exhibited the maximum antimicrobial activity against *A. hydrophila* and *Aeromonas salmonicida* subsp. *salmonicida* strains. The results of this study provide baseline information on the potential validity of extracts derived from leaves of *F. elastica* and its cultivars in the treatment of infections associated with fish pathogen *Aeromonas* spp.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Morphological characteristics of selected flower parts of *Cucurbita pepo* L. Styriaca Group

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The study aimed to determine the variability of some morphological characters on flowers of selected plants within the population of ESO variety of Styrian oil pumpkin (*Cucurbita pepo* L. Styriaca Group). For experimental evaluation, we used two years (2020–2021) measurements of 1792 selected pistillate and staminate flowers from individual plants grown in the field conditions in the Kolíňany settlement (the Slovak Republic). We determined for staminate flowers the range for the length of petals (25.00–134.00 mm), width of petals (14.00–92.00 mm), width of sepals (3.00–31.00 mm), height of sepals (3.00–34.00 mm), length of anther (4.00–24.00 mm), weight of flowers (0.97–11.81 g), weight of petals (0.27–7.14 g), weight of sepals (0.07–2.70 g) and weight of stamens (0.05–6.21 g). High degrees of variability were confirmed for all characters. We determined for pistillate flowers the range for the length of petals (41.00–121.00 mm), width of petals (10.00–100.00 mm), length of the pistil (5.70–15.72 mm), weight of flower (2.91–33.59 g), weight of petals (0.90–9.92 g), weight of sepals (0.61–2.93 g), and weight of pistil (0.07–1.72 g). Variability for traits confirmed moderate to high degree of variability. Our obtained results can serve as a basis for the taxonomy of the species (morphometric approach), for its selection, but also culinary purposes, where edible flowers are still used to a greater extent in gastronomy in the preparation of meals. For this purpose, genotypes with the production of larger flowers could be particularly interesting.

Keywords: Styrian oil pumpkin, staminate flower, pistillate flower, morphometric analysis, variability

Introduction

Pumpkin (*Cucurbita* spp.), which belongs to the family Cucurbitaceae Juss., is a worldwide important agricultural crop produced and consumed all over the world. Numerous agricultural products are major sources of functional components consisting of nutrients and bioactive phytochemicals which may provide substantial health benefits and contribute to the good status of human health. Pumpkin fruits are an extremely healthful agricultural crop with a balanced mixture of beneficial nutrients for human health and nutrition (Dhiman et al., 2009; Dar et al., 2017; El Khatib and Muhieddine, 2019). Recently, an increasing interest has been given not only to the pumpkin flesh and its by-products mainly seeds and peels (Aruah et al., 2010; Lebeda et al., 2017; Tripti et

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al., 2019; Horčinová et al., 2022; Öztürk et al., 2022), but also to its flowers and some vegetative parts (e.g. buds, tender stem tips, leaves, tendrils), which are also used as vegetables (Nee, 1990; Merrick, 1991; Lira, Andres and Nee, 1995; Ghosh and Rana, 2021). Flower buds and flowers of *C. pepo* are also edible and several products gaining its importance as a functional food (Gutierrez, 2016; Ghosh and Rana, 2021).

Flores De Calabaza in Mexico, Classic stuffed peppers in West America, Pakoda or Vajji in India are dishes prepared from pumpkin flowers. In general, the flowers are used as raw in salads, cooked with other vegetables, and steamed in soups. Canned blossoms are also available in the local Mexican markets but always fresh blossoms are preferred for consumption (Ghosh and Rana, 2021). The delicate texture and slightly sweet flavour of *C. pepo* flowers have made them one of the favourite ingredients. These flowers are a rich source of healthy constituents such as phenols and flavonoids (hesperidin, quercetin-3-O-glucoside, catechin, epicatechin, rutin, and syringic acid as dominant compounds), carotenoids (Morittu et al., 2019; Ghosh and Rana, 2021), and are used in traditional medicine (Lu et al., 2016; Dar et al., 2017; Morittu et al., 2019).

The hypoglycaemic effects of *C. pepo* flower extract obtained *in vitro* are confirmed *in vivo*. The extract also affected *in vivo* lipid metabolism but did not reveal benefits on ROS production. Finally, obtained results highlighted *C. pepo* flowers as a food aimed at satisfying both taste and health (Morittu et al., 2019).

Few people grow *Cucurbita* flowers for decorative purposes as a flower lasts only one morning. Only one species (*C. maxima*) is known to have very aromatic flowers (Lira et al., 1995).

Most members of the Cucurbitaceae are monoecious, which means each plant has flowers with only stamens along with other flowers which are only pistillate at the same plant. These are commonly called male (staminate) and female (pistillate) flowers (Whitaker and Robinson, 1986; Lira et al., 1995; Rzedowski and Rzedowski, 2001).

Cucurbita flowers are large, gamopetalous with tubular-campanulated corollas, and showy, with a cream-coloured or light yellow or bright-yellow orange corolla. Flowers grow from the axil of a leaf. Staminate flowers have column-like stamens, with free or more or less connivent filaments, and the yellowish anthers are joined together forming a cylindrical or narrowly pyramidal structure. Pollen is orange coloured. Pistillate flowers have an inferior ovary with numerous horizontally positioned ovules, the styles are fused in almost their entire length or are only shortly free in the apex. The pistil consists of three carpels fused in one ovary with 3 short styles partially fused at their base, each of which ends with a bilobed stigma. Stigmas are large, fleshy or sunken, or lobulated, and slight modifications can be seen in the structure of the perianth regarding the staminate ones, mainly corresponding to differences in the size of one or some of its parts (Agbagwa and Ndukwu, 2004; Caracuel et al., 2012; OECD, 2016).

Both the staminate and pistillate flowers are pollinated by various wild bees (Hurd et al., 1971; Lobo and Bravo Méndez, 2021), and produce large amounts of abundant nectar (Teppner, 2000). Nectaries are found in a chamber at the base of the stamens in staminate flowers and a lower ring at the base of the pistil in pistillate flowers (Nepi and Paccini, 1993; Nepi et al., 2001; Vidal et al., 2006).

The experiment aimed to determine the production and variability of some morphological characters on flowers taken from selected individual plants within the ESO variety of oil pumpkin (*Cucurbita pepo* Styriaca Group).

Material and methodology

Biological material

In the experiments, 1792 flowers from randomly selected plants from the cultivated population of *Cucurbita pepo* Styriaca Group on an area of 150 ha situated near the Kolíňany settlement (SW part of the Slovak Republic) were evaluated. Flowers were taken in July and August 2020, and 2021 and analyzed in the laboratory at the Institute of Plant and Environmental Sciences in Nitra (the Slovak Republic). The nomenclature of plant names is according to Danihelka et al. (2012).

Morphometric analysis

A total of 7 quantitative characters were evaluated in the pistillate flowers and 9 quantitative characters in the staminate flowers:

1. Pistillate flowers (female) – 53 flowers were evaluated in the 2020 year:
   - ❒ length of petals (g), width of petals (mm), length of pistil (mm), weight of whole flower – fertile and sterile flower parts (g), weight of petals (g), weight of sepal (g), weight of pistil (g).

2. Staminate flowers (male) – 591 flowers were evaluated in 2020 and 1148 flowers in 2021:
   - length of petals (g), width of petals (mm), length of sepal (mm), width of sepal (mm), length of anther (mm), weight of whole flower – fertile and sterile flower parts (g), weight of petals (g), weight of sepal (g), weight of stamens (g).

The flowers and their parts were measured in the fresh weight by digital scale (Kern ADB-A01S05, Germany; KERN DS – type D-72336, Kern and Sohn GmbH, Germany), accurate to 0.01 g. Flowers were measured by ruler and digital calliper (METRICA 111 – 012, Czech Republic) accurately to 0.02 mm.

Image analysis

Shape and colour of staminate and pistillate flowers and details of individual parts.

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

Statistical analysis

It was evaluated the variability of each character using descriptive statistics. For the characteristics, it was used the basic descriptors of variability: average, minimum measured value, maximum measured value, and the coefficient of variation (%). The degree of variability was determined by the coefficient of variation values. The given parameter is independent of the unit of the evaluated character. Theoretically, they can acquire different values (Stehlíková, 1998). We used analysis of variance (ANOVA) in the program STATISTICA 1.10 to determine the dependence between individual characters.

Results and discussion

Variability of flower characters

In pumpkins, we distinguish between staminate and pistillate flowers on the same plant. The flowers of *Cucurbita* are monoecious, large, yellow, solitary; corolla sympetalous; stamens 3; filaments free but anthers confluent into a head, one 1-locular, the others 2-locular, loculi elongated, sigmoid-flexuous; female flowers with 3 rudimentary stamens at the bottom of the calyx (Hutchinson, 1969; Teppner, 2004; Baranec et al., 2009).

Genetic and environmental conditions play a role in influencing the sex expression of flowers, and their development into male or female states. *Cucurbita* can exhibit wide variation in the proportion of male to female flowers on a plant (OECD, 2016). Many authors reported that the production of female flowers is frequently less than that of male flowers (Zomlefer, 1994; Janick and Paull, 2007; Kiramana and Isutsa, 2017). Temperature and day length influence how long a plant remains in the male phase, as well as the ratio of male to female flowers. High temperatures, high light intensity, and long-days favour the production of male flowers and a longer male phase. Low temperatures, low light intensity, and short days favour the development of female flowers. The number of developing fruits already present in a plant also affects the flower ratio (Robinson and Decker-Walters, 1997; McCormack, 2010).

In *C. pepo*, Nepi and Pacini (1993) found a 16.5 : 1 relation between the number of male and female flowers. The first flowers are staminate (male), after which three or four pistillate (female) flowers appear. Although pistillate flowers differentiate later in plant development, females develop faster than males, resulting in near synchronization at the anthesis of the flowers of both generative organs (Janick and Paull, 2007).

Sex expression in cucurbits is also influenced by hormones produced within the plant. Maynard (2007) studied the effect of gibberellins and ethylene on the development of staminate and pistillate flowers, respectively. Natural and synthetic auxins promote pistillate flower development.

Staminate flowers

The staminate flowers variability was evaluated with a total of the following nine quantitative characters: the length of petals, the width of petals, the width of sepals, the height of sepals, the length of an anther, the weight of the whole flower, the weight of petals, the weight of sepals and the weight of stamens. Statistical indicators of the variability of quantitative traits are presented in Table 1.

Length and width of petals

Male flowers are on pedunculate raceme with 6–10 flower heads or sometimes solitary with very long peduncles, more numerous and earlier than the female flower (Agbagwa, and Ndukwu, 2004; Vidal et al., 2010).

The results of the statistical analysis show that the tested genotypes in 2020 reached the length of the petals in the range from 40 mm to 132 mm.

Table 1 Main statistical indicators of the variability of staminate flowers in the experimental population of *Cucurbita pepo* L. Styriaca Group

Characters	Year	n	min	max	x̄	V %
Length of petals (mm)	2020	591	40.00	132.00	80.08	21.26
	2021	1148	25.00	134.00	90.98	16.08
Width of petals (mm)	2020	591	14.00	90.00	27.18	18.96
	2021	1148	25.00	92.00	54.09	19.14
Width of sepals (mm)	2020	591	3.00	31.00	17.01	20.38
	2021	1148	5.00	25.00	14.14	25.56
Height of sepals (mm)	2020	591	6.00	34.00	10.23	29.38
	2021	1148	3.00	23.00	9.99	23.35
Length of the anther (mm)	2020	591	4.00	24.00	12.07	22.39
	2021	1148	5.00	24.00	13.88	20.92
Weight of whole flower (g)	2020	422	1.20	11.81	3.63	32.98
	2021	1151	0.97	9.99	3.25	35.47
Weight of petals (g)	2020	1151	0.40	7.14	2.29	37.98
	2021	422	0.27	6.32	2.29	37.64
Weight of sepals (g)	2020	422	0.15	2.70	0.69	43.52
	2021	1149	0.07	2.40	0.24	55.82
Weight of stamens (g)	2020	422	0.10	6.21	1.00	44.69
	2021	1149	0.05	1.00	0.20	36.01

n – the number of measurements; min, max – minimal and maximal measured values; x̄ – arithmetic mean; V – coefficient of variation (%)

The value of the coefficient of variation indicates a high degree of variability (21.26%). In 2021, genotypes reached values ranging from 25 to 134 mm, and a moderate degree of variability was determined (16.08%). In terms of width, in 2020 a range of 14 to 90 mm was determined. In 2021, the value of the trait was determined in the range from 25 to 92 mm. The average value of the coefficient of variation was found for both years (18.96–19.14%).

Peniašteková (2008) mentioned diameter of *C. pepo* flowers in the interval 70–110(-180) mm and Chrtková (Chrtková, 1990) reported in her study diameter of *C. pepo* flowers in the range (50-)70–110(-200) mm without differentiation between male and female flowers. Teppner (2004) recorded *Cucurbita* flowers more or less bigger, in cultivated species up to 100–130 mm long and up to 200(-220) mm in diameter.

Umiel et al. (2007) studied some characteristics of pumpkin male and female flowers, such as the number produced per plant of each, male and female, corolla length, and corolla texture for suitability for the production and marketing of the squash flowers as culinary item, which is known for centuries (Paris and Janick, 2005).

The accessions of *C. pepo* subsp. texana produced smaller corollas than those of *C. pepo* subsp. pepo and overall, the corolla length of male flowers was larger than that of female flowers (Umiel et al., 2007).

Width and height of sepals

In the first year, the range of width of sepals was determined from 3 to 31 mm. In the second experimental year, the value of the value of the trait was determined in the interval from 5 to 25 mm with medium and high degrees of variability for both years (20.38–25.56%).

For the height of the sepals, we recorded a range from 6 mm to 34 mm, and in the next year of evaluation, the value of the trait was determined in the range from 3 to 23 mm. A high degree of variability was determined in both evaluated years (23.35–29.38%) (Table 2).

Length of anther

In the first experimental year we determined values in the interval from 4 to 24 mm for the length of anther with a high coefficient of variation (22.39%) and the next year we determined an interval from 5 to 24 mm with a high degree of variability (20.92%), which points to some differences between genotypes.

Weight of the whole flower

We recorded a relatively high variability for the weight of whole flowers (32.98–35.47%) in both years with the following intervals 1.20–11.81 and 0.97–9.99 g.

Weight of petals and sepals

When we compared the first and second experimental years and intervals of values 0.40–7.14 and 0.27–6.32 g for the weight of petals, results point to a very high coefficient of variation (37.64–37.98%) and thus very significant differences between genotypes. The same differences we recorded for the weight of sepals with a significantly high degree of variability (43.52–55.82%) and values for a character in the range 0.15–2.70 g and 0.07–2.40 g in both experimental years.

Weight of stamens

In the last evaluated character, we recorded the weight of stamens in the intervals 0.10–6.21 and 0.05–1.00 g, respectively for the first and second experimental year. The medium and high degree of variability between genotypes confirmed the values of the coefficient of variation (36.01–44.69%).

Pistillate flowers

The pistillate flowers variability was evaluated with a total of the following seven quantitative characters: the length of petals, the width of petals, the length of the pistil, the weight of the whole flower, the weight of petals, the weight of sepals and the weight of pistil. Statistical indicators of the variability of quantitative traits are presented in Table 2.

The basic indicators of the variability of quantitative traits of pistillate flowers are presented in Table 2.

Length and width of petals

In the experimental year 2020, the length of petals was determined in the range from 41 mm to 121 mm (Table 2), and the width of petals from 10 to 100 mm. Coefficients of variation indicated a moderate and high degree of variability in both characters (19.30–26.16%).

Weight of whole flower, petals, and sepals

The weight of the whole flower was determined in the range from 2.91 to 33.59 g. The experimental evaluation determined the weights of petals and the weight of sepals in the intervals from 0.90 to 9.92 g and from 0.61 to 2.93 g, respectively. The values of the coefficients of variation indicated a high degree of variability between genotypes (29.84–44.64%) for all evaluated traits.

Length and weight of pistil

Another characteristic of female flowers was the length of the pistil with values from 5.70 to 15.72 mm. The coefficient of variation indicated a moderate degree of variability between genotypes (17.45%). The weight of the pistil was determined in the range of 0.07 to 1.72 g. The coefficient of variation pointed to a high degree of variability between genotypes (42.32%) for all evaluated genotypes.

The weight ratio of individual parts of the flowers was also evaluated during the years 2020 and 2021 (Figure 1). From the obtained experimental data, the ratio of the basic parts of the flowers was determined, represented by 65–71% petals, 21–29% sepals, and 6 to 8% is the economically less used part of the flowers – stamens (contained pollen grains).

Other floral characteristics were measured by Ezin et al. (2022). They observed positive correlations between the length of female flower peduncles and the

Table 2 Main statistical indicators of the variability of evaluated pistillate flower traits in the experimental population of *Cucurbita pepo* L. Styriaca Group

Characters	n	min	max	$\bar{x}$	V %
Length of petals (mm)	53	41.00	121.00	69.05	19.30
Width of petals (mm)	53	10.00	100.00	61.74	26.16
Length of pistil (mm)	53	5.70	15.72	11.70	17.45
Weight of whole flower (g)	53	2.91	33.59	12.54	44.64
Weight of petals (g)	53	0.90	9.92	4.42	30.63
Weight of sepals (g)	53	0.61	2.93	1.43	29.84
Weight of pistil (g)	53	0.07	1.72	0.74	42.32

n – the number of measurements; min, max – minimal and maximal measured values; $\bar{x}$ – arithmetic mean; V – coefficient of variation (%)

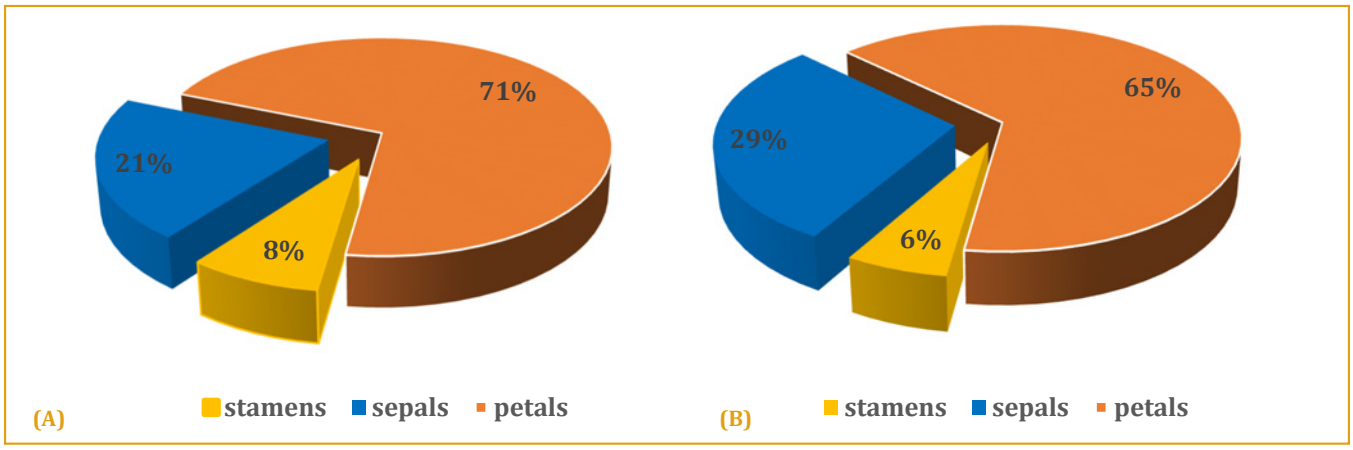

Figure 1 Average weight ratio of the individual basic anatomical parts of the flowers of *Cucurbita pepo* L. Styriaca Group from the total average weight of the fresh flowers (A) 2020 and B (2021) (%)
*– changes are statistically significant compared to the 96% ethanol

male flower peduncles (r = 0.60) as well as between the number of female flowers and the number of male flowers (r = 0.74) cultivated pumpkin landraces.

Lima et al. (2022) recorded positivive association between the petal length and the corolla diameter, and between access to the nectar and the anther size, with a difference between pumpkin cultivars (*C. moschata* Duch.) and cultivation conditions.

Umiel et al. (2007) confirm that *C. pepo* subsp. pepo produced large and firm flowers, which are more suitable for culinary usage than those of the *C. pepo* subsp. texana which are softer, noticeably less firm.

Figure 2 Variability in the shape and colour of staminate and pistillate flowers of *Cucurbita pepo* L. Styriaca Group
A–B – pistillate flowers; C–D – staminate flowers; E – detail of pistil; F–G – detail of stamens; H – detail of staminate flower bud

Conclusions

For the first time are described in detail the weight and size of sepals, petals, stamens, and pistils of *C. pepo* Styriaca Group species. Literary data on this topic is limited. The knowledge obtained can serve as a basis for the taxonomy of the species, for its selection, but also culinary purposes, where edible flowers are still used to a greater extent in gastronomy in the preparation of meals. For this purpose, genotypes with the production of larger flowers could be particularly interesting.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statements

This article does not contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with extracts derived from leaves and pseudobulbs of *Coelogyne pandurata* Lindl. (Orchidaceae) plants

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The current study was conducted to investigate the antioxidant properties of extracts derived from leaves and pseudobulbs of *Coelogyne pandurata* Lindl. using biomarkers of oxidative stress (2-thiobarbituric acid reactive substances (TBARS) as biomarkers of lipid peroxidation, aldehydic and ketonic derivatives of oxidative modification of proteins (OMP), total antioxidant capacity TAC)) in the equine erythrocytes after *in vitro* treatment with the extracts. The obtained results demonstrated the prooxidative activity of *C. pandurata* extract used in the studied dose (5 mg.mL^{-1}) on the equine erythrocytes. Our results also showed that extract derived from the leaves and pseudobulbs of *C. pandurata* after incubation with erythrocyte samples caused to remaining the TAC level at a high level as compared to the group treated by phosphate buffer (controls), while levels of aldehydic and ketonic derivatives of OMP were unchanged. Our results also revealed that extracts derived from the leaves and pseudobulbs of *C. pandurata* after incubation with equine erythrocyte samples caused to increase in the TBARS level compared to untreated samples. Future studies will be conducted to evaluate dose-dependent changes in the levels of oxidative stress biomarkers after incubation with extracts derived from *C. pandurata* using various cell models. Moreover, the plant compound profile characteristics and antioxidant activity of different *Coelogyne* plants may encourage the wider use of these orchids in the development of new medicinal substances in medicine and veterinary.

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

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Introduction

Orchids constitute one of the largest and most highly developed families of angiosperms and form an extremely peculiar group of flowering plants that are of great value for ornamental, medicinal, conservation, and evolutionary research (Zhang et al., 2018; Wang et al., 2019). Epiphytic orchids are often characterized by succulent leaves with thick cell walls, cuticles, and depressed stomata, while terrestrial orchids have rhizomes, corms, or tubers (Zhang et al., 2018). Various orchids are available through traditional breeding and micropropagation as they are valuable as pot plants and cut flowers in horticultural markets (Wang et al., 2019). Experiments with alkaloids, terpenes, stilbenoids, bibenzyls, phenanthrenes, flavonoids, and polysaccharides isolated from Orchidaceae Juss. have shown their potential medicinal utility (Sut et al., 2017). To date, several classes of phytocomponents have been isolated from therapeutically used orchids, demonstrating great chemical diversity (Sut et al., 2017). Among them, phenol derivatives have been studied for their biological activity, especially anticancer properties (Wang et al., 2021; Śliwiński et al., 2022), anti-inflammation (Jiang et al., 2019; Zhang et al., 2021), and anti-neurodegeneration properties (Li et al., 2017; Zhang et al., 2022).

Coelogyne Lindl. is a genus of about 200 species, distributed from Southeast Asia to the southwestern Pacific Islands (Aung et al., 2017; Zhou et al., 2018; Jiang et al., 2020). *Coelogyne* plants are characterized by a free, never-saccate lip, with high lateral lobes over the entire length of the hypochile and smooth, papillose, toothed, or warty keels on the epichile (Zhou et al., 2018). Most species grow in tropical montane and lowland forest areas (Jiang et al., 2020). The *Coelogyne* genus belongs to the group of orchids that possesses medical properties (Pérez Gutiérrez, 2010; Pant, 2013).

The interesting species within the genus *Coelogyne*, comprising considerable interest for screening of biological activity of various parts of the plants, is *Coelogyne pandurata* Lindl. *Coelogyne pandurata* is found in Malaysia, Sumatra, Borneo, and the Philippines as a large-sized, hot-growing epiphyte found on large trees near rivers or terrestrial with well-spaced, strongly compressed, oblong or suborbicular, sulcate pseudobulb carrying 2, apical, plicate, elliptic-lanceolate, leaves with a stout petiole that blooms in late spring-summer out of the center of newly emerging growths with up to 15 flowers on a terminal, arched to pendant, 15 to 30 cm long, racemose inflorescence. The simultaneously opening flowers are highly fragrant of honey to cinnamon but are short-lived (http://www.orchidspecies.com/).

The current study was conducted to investigate the antioxidant properties of extracts derived from leaves and pseudobulbs of *C. pandurata* using biomarkers of oxidative stress [2-thiobarbituric acid reactive substances (TBARS) as biomarkers of lipid peroxidation, aldehydic and ketonic derivatives of oxidative modification of proteins (OMP), total antioxidant capacity TAC)] in the equine erythrocytes after *in vitro* treatment with the extracts.

Material and methodology

Collection of plant materials and preparation of plant extracts

The leaves and pseudobulbs of *C. pandurata* plants (Figure 1) cultivated under glasshouse conditions were sampled at M.M. Gryshko National Botanic Garden (NBG, Kyiv, Ukraine). Since 1999 the whole collection of tropical and subtropical plants (including orchids) has had the status of a National Heritage Collection of Ukraine and is supported through State Funding. Besides, the NBG collection of tropical orchids was registered at the Administrative Organ of CITES in Ukraine (Ministry of Environment Protection, registration No. 6939/19/1-10 of 23 June 2004). Freshly collected leaves and pseudobulbs were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in the proportion of 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. The extract was stored at -25 °C until use.

Our current scientific project was undertaken in the frame of the cooperation program between the Institute of Biology and Earth Sciences (Pomeranian University in Słupsk, Poland) and M.M. Gryshko National Botanic Gardens of the National Academy of Sciences of Ukraine, directed to assessment of medicinal properties of tropical plants has encompassed some tropical mega-diverse genera, including Orchidaceae.

Horses and collection of blood samples

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

Figure 1 Vegetative shoot with inflorescence of *Coelogyne pandurata* Lindl. plant, cultivated at NBG's glasshouses (Kyiv, Ukraine)
Photo: Lyudmyla Buyun

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.

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood samples were processed for analysis less than 12 h after blood withdrawal. Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3000 rpm for 5 min to remove plasma. The pellet of erythrocytes was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extracts was added to 1.9 ml of equine erythrocytes. For positive control, incubation of equine erythrocytes with 4 mM phosphate buffer (pH 7.4) was used. After incubating the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocyte aliquots were used in the current study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The nmol of MDA per mL was calculated using $1.56 \cdot 10^5$ $mM^{-1}.cm^{-1}$ as the extinction coefficient.

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

To evaluate the protective effects of the extracts derived from leaves and pseudobulbs of *C. pandurata* against free radical-induced protein damage in equine plasma, a carbonyl derivatives content of protein

oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocyte suspension and plasma was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient of 22000 $M^{-1}\cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

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). The sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated concerning the absorbance of the blank sample.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p > 0.05$). The significance of differences between the OMP level (significance level, $p < 0.05$) was examined using the Kruskal-Wallis one-way analysis of variance (Zar, 1999). The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 13.3 software (TIBCO Software Inc., Krakow, Poland) (Zar, 1999).

Results and discussion

Levels of TBARS, aldehydic and ketonic derivatives of OMP, and total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with an extract derived from leaves of *Coelogyne* pandurate were presented in Figure 2.

Our results revealed that extract derived from the leaves of *C. pandurata* after incubation with equine erythrocyte samples caused to increase in the TBARS level (55.20 ±6.68 $nmol\cdot mL^{-1}$) (by 53.8%, $p < 0.05$) compared to untreated samples (35.88 ±3.02 $nmol\cdot mL^{-1}$). On the other hand, the content of aldehydic derivatives of OMP in the erythrocyte samples after incubation with an extract derived from the leaves of *C. pandurata* was not altered (31.27 ±1.56 $nmol\cdot mL^{-1}$) compared to the untreated samples (31.16 ±1.89 $nmol\cdot mL^{-1}$). Moreover, the content of ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from the leaves of *C. pandurata* was non-significantly decreased (by 2.2%, $p > 0.05$). A non-significant increase in the TAC level of the tested samples incubated with an extract derived from the leaves of *C. pandurata* was observed (58.55 ±2.75%

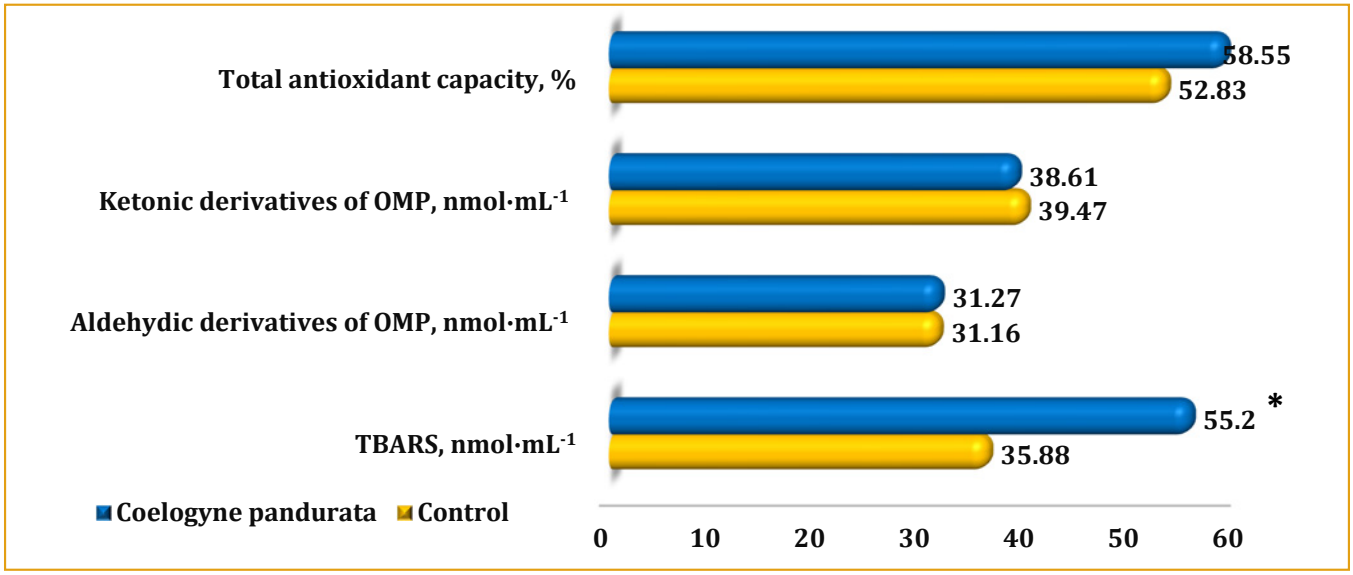

Figure 2 Levels of TBARS, aldehydic and ketonic derivatives of OMP, total antioxidant capacity in the equine erythrocytes after *in vitro* incubation with an extract derived from leaves of *Coelogyne pandurata* Lindl.
*– changes were statistically significant ($p < 0.05$) compared to untreated control

compared to the untreated samples 52.83 ±3.38%). A percentage of non-significant increase was 10.8% (p >0.05) (Figure 2).

Levels of TBARS, aldehydic and ketonic derivatives of OMP, and total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with an extract derived from pseudobulbs of *Coelogyne* pandurate were presented in Figure 3.

Our results revealed that extract derived from the pseudobulbs of *C. pandurata* after incubation with erythrocyte samples caused to increase in the TBARS level (45.05 ±4.74 nmol.mL^{-1}) (by 25.6%, p <0.05) compared to untreated samples (35.88 ±3.02 nmol.mL^{-1}). On the other hand, the content of aldehydic derivatives of OMP in the erythrocyte samples after incubation with an extract derived from the pseudobulbs of *C. pandurata* was not altered (30.97 ±1.23 nmol.mL^{-1}) compared to the untreated samples (31.16 ±1.89 nmol·mL^{-1}). Moreover, the content of ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from the pseudobulbs of *C. pandurata* was non-significantly decreased (by 10.9%, p >0.05). A non-significant increase in the TAC level of the tested samples incubated with an extract derived from the pseudobulbs of *C. pandurata* was observed (54.68 ±2.69% compared to the untreated samples 52.83 ±3.38%) (Figure 3).

The antioxidant efficacy of some orchids was reported by some researchers using *in vitro* and *in vivo* models. For example, *in vitro* free radical scavenging, LCMS-based metabolic profiling, and anti-inflammatory activity of *Dendrobium macrostachyum* Lindl. were studied by Sukumaran and Yadav (2016). The results showed a relatively high concentration of phenolics, high scavenger activity, and high anti-inflammatory activity of the stem extract compared to the leaf extract (Sukumaran and Yadav, 2016). Paudel et al. (2019) assessed of antioxidant and cytotoxic activities of stem extracts of *Dendrobium crepidatum* Lindl. & Paxton. The above extracts showed antioxidant and cytotoxic properties using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, due to the presence of tetracosane, triacontane, stigmasterol, and some phenol derivatives (2-methoxy-4-vinylphenol, 2-methoxy-5-(1-propenyl)-phenol, *p*-mesyloxyphenol, and 2,6-dimethoxy-4-(2-propenyl)-phenol) (Paudel et al., 2019). Also, *Dendrobium moniliforme* (L.) Sw. extracts contain a number of bioactive compounds which exhibit both antioxidant and cytotoxic activities against free radicals and cancer cell lines respectively (Paudel et al., 2018). The volatile fractions from fresh inflorescences of naturally growing orchids *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp. fragrans (Pollini), *Anacamptis pyrimidalis* (L.) R., *Ophrys holosericea* (Burm.) Greuter and *Serapias vomeracea* (Burm. f.) B. were isolated and analyzed in the study of Robustelli Della Cuna et al. (2019). These volatile compounds may represent a particular feature of these plant species, playing a critical role in the interaction with pollinators. DPPH assay evaluating the antioxidant activity of the essential oils was carried

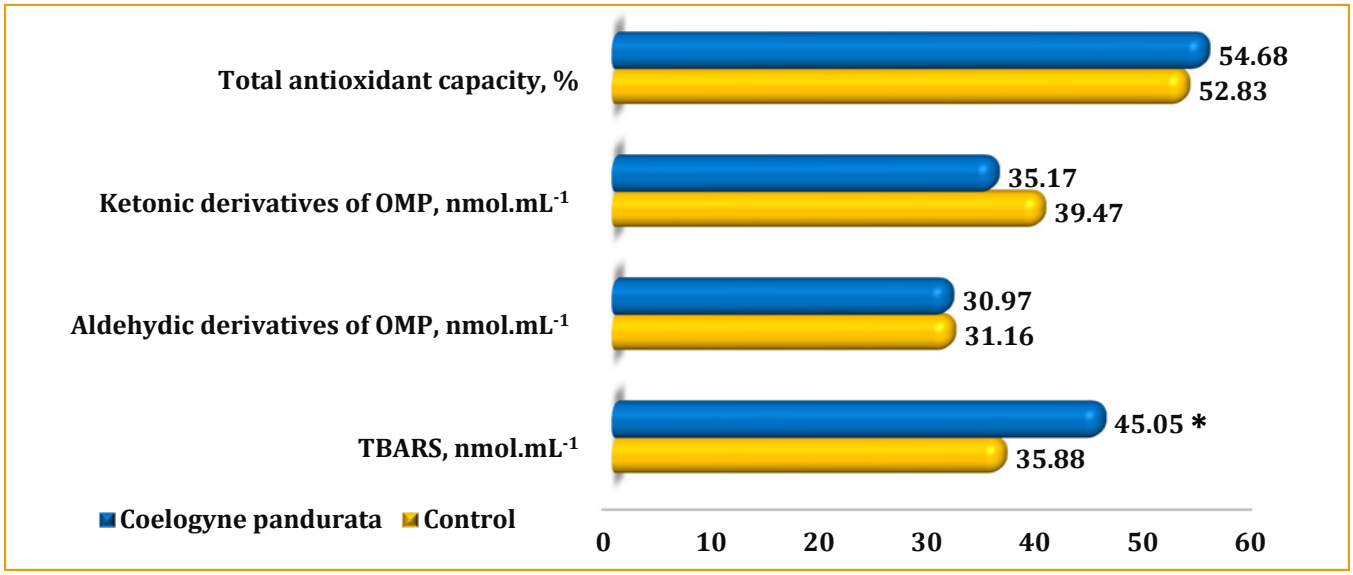

Figure 3 Levels of TBARS, aldehydic and ketonic derivatives of OMP, total antioxidant capacity in the equine erythrocytes after *in vitro* incubation with an extract derived from pseudobulbs of *Coelogyne pandurata* Lindl.
*– changes were statistically significant (p <0.05) compared to untreated control

out, showing a dose-dependent antioxidant activity (Robustelli Della Cuna et al., 2019).

The antioxidative properties of flowers and the aboveground part of *Anacamptis pyrimidalis* L. from Vojvodina were studied by Stajner et al. (2010). Activities of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and glutathione peroxidase), quantities of malondialdehyde, superoxide, hydroxyl radicals, and reduced glutathione and also the contents of chlorophylls a and b, carotenoids and soluble proteins were determined. The results of these researchers indicated that the aboveground part of the plant exhibited higher antioxidant activity due to low MDA and lipofuscin pigment accumulation, higher scavenging activity, and antioxidant capacity (Stajner et al., 2010). *In vitro* antidiabetic, antioxidant activities, and GC-MS analysis of *Rhynchostylis retusa* (L.) Blume and *Euphorbia neriifolia* L. leaf extracts were revealed by Kumar et al. (2021). This study revealed significant inhibition of α-amylase activity and retardation in glucose diffusion with *E. neriifolia* and *R. retusa* extract in a dose-dependent manner, depending on the extraction solvent. In addition, GC–MS analysis of methanolic, aqueous, and petroleum ether extracts suggested the presence of diverse phytochemical entities with known anti-inflammatory, and antioxidant properties, possibly implicated for use in diabetic conditions (Kumar et al., 2021).

We also investigated the changes in the oxidative stress biomarkers using the model of equine erythrocytes and plasma to evaluate the antioxidant activities of the aqueous extract derived from leaves of *Coelogyne brachyptera* Rchb.f. (Buyun et al., 2022). Results of our study revealed that erythrocytes were more sensitive to the action of an extract derived from leaves of *C. brachyptera*. The levels of aldehydic and ketonic derivatives of oxidatively modified proteins in the treated erythrocytes were significantly decreased, while these parameters were no-changed in the equine plasma. The treatment of equine erythrocytes by extract derived from leaves of *C. brachyptera* increased lipid peroxidation. On the other hand, plasma TBARS level after treatment by extract derived from leaves of *C. brachyptera* was at the same level as in untreated controls. The level of total antioxidant capacity was not-significantly changed after treatment both in equine plasma and erythrocytes (Buyun et al., 2022).

Conclusions

The aim of the current study was to investigate the antioxidant properties of extracts derived from leaves and pseudobulbs of *C. pandurata* using biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with the extracts. The obtained results demonstrated the prooxidative activity of *C. pandurata* extract used in the studied dose (5 $mg.mL^{-1}$) on the equine erythrocytes. Extracts derived from the leaves and pseudobulbs of *C. pandurata* after incubation with equine erythrocyte samples caused to increase in the TBARS level compared to untreated samples remaining with the TAC level at a high level as compared to the group treated by phosphate buffer (controls), while levels of aldehydic and ketonic derivatives of OMP were unchanged. Future studies will be conducted to evaluate dose-dependent changes in the levels of oxidative stress biomarkers after incubation with extracts derived from *C. pandurata* using various cell models. Moreover, the plant compound profile characteristics and antioxidant activity of different *Coelogyne* plants may encourage the wider use of these orchids in the development of new medicinal substances in medicine and veterinary.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Nutritional composition of *Phacelia tanacetifolia* Benth. bee pollen and inflorescences

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The interest in natural products, namely, bee products is actual nowadays. The Bee pollen is a rich source of nutrients and biologically active compounds with numerous biological activities such as antioxidant, antimicrobial, anticancer, anti-inflammatory, etc. The search for new plant raw as a source of natural products has a practical meaning. The goal of this study was to evaluate the biochemical composition of bee pollen and inflorescences of *Phacelia tanacetifolia* Benth. Grown in Slovakia. The plant raw material was collected from the experimental plots of Slovak Agricultural University in Nitra. There were conducted following biochemical analyses: dry matter, protein, ash, lipid, β-carotene, fatty acid, amino acid, and saccharide content. *Ph. tanacetifolia* bee pollen had 73.3% of dry matter, 27.44% of protein, 2.77% of ash, 5.35% of lipids, 3.0 mg.kg^{-1} of β-carotene, 32.2 g.100 g^{-1} of saturated fatty acids, 5.7 g.100 g^{-1} of monounsaturated fatty acids, and 55.7 g.100 g^{-1} of polyunsaturated fatty acids. The nutritional composition of *Ph. tanacetifolia* inflorescences was 91.05% of dry matter, 18.37% of protein, 15.49% of ash, 4.5% of lipids, 50.4 mg.kg^{-1} of β-carotene, 34.0 g.100 g^{-1} of saturated fatty acids, 8.8 g.100 g^{-1} of monounsaturated fatty acids, and 45.5 g.100 g^{-1} of polyunsaturated fatty acids. The prevailing amino acids investigated raw were glutamic, aspartic acid, proline, and leucine. The content of bee pollen fructose higher 44 times than inflorescences fructose. The content of maltose and lactose in both raw was less than 0.5 g.kg^{-1}. Among saturated fatty acids, the most prevailed for both bee pollen and inflorescences was palmitic acid (28.42 and 27.93 g.100 g^{-1} of fat, respectively), oleic acid (4.99 and 8.06 g.100 g^{-1} of fat, respectively) prevailed among monounsaturated fatty acids and linolenic acid (45.47 and 23.27 g.100 g^{-1} of fat, respectively) among polyunsaturated fatty acids. Bee pollen of investigated samples had the highest content of potassium (6239 mg.kg^{-1}), phosphorus (6039 mg.kg^{-1}), and sulfur (2403 mg.kg^{-1}). The obtained data can be useful in the food industry and further pharmaceutical and apicultural research.

Keywords: lacy phacelia, nutrients, fatty acids, amino acids

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Introduction

Phacelia tanacetifolia Benth. an annual herbal widely cultivated as ornamental and medicine species from Hydrophyllaceae R. Brown ex Edwards originated from North America (Pinke et al., 2022). This species is well adapted to different conditions of growth (Kizilsimsek and Ates, 2004). This is an ecological benefit plant and is well-known as a honey plant with the best quality of honey, use for mulch due to its high absorption of calcium and phosphorus in the soil, as a forage plant, prevents erosion, soil bio cleaner from nematodes (Jaramaz and Jaramaz, 2009). *Ph. tanacetifolia* is used for forage purposes as monoculture and in mixtures with for example *Pisum arvense* L. (Ates et al., 2014). The content of nutrients in different combinations of feed mixtures of *Pisum arvense* with *Ph. tanacetifolia* (75% + 25%, 50% + 50%, etc.) increased compared with the monoculture of *Ph. tanacetifolia* (Ates, 2012). Due to its numerous useful properties, *Ph. tanacetifolia* can be recommended as a multipurpose crop in the farming system (Ozkan, 2020).

Genç Lermi and Palta (2014) investigated that yield parameters and nutritive composition of raw depended on the sowing period and were higher during the autumn period. This species can be potent material for cosmetic and pharmaceutical properties due to the content of biologically active compounds (Kruk et al., 2018).

Another study demonstrated the allelopathic potential of *Ph. tanacetifolia*, however, a negative allelopathic effect was found at high concentrations, and the most effective were leaf extracts (Kliszcz et al., 2023). Also, seed meal extracts from *Ph. tanacetifolia* were not affected at 1% concentration in the soil (Restuccia and Scavo, 2023).

Bee products, namely, bee pollen has been used since ancient time and demonstrated therapeutic properties (Denisow and Denisow-Pietrzyk, 2016). Bee pollen exhibited numerous pharmacological activities such as anti-inflammatory, antioxidant, antifungal, antiviral, immunostimulant, anti-allergic, and analgesic (Saisavoey et al., 2020; Khalifa et al., 2021).

Ph. tanacetifolia is an essential source of quality nectar and pollen (Ardalani et al., 2021). Bee pollen contains flower pollen with nectar and bee secretions, is used as a health food supplement, and is a source of lipids, sugars, vitamins, proteins, amino acids, carotenoids, flavonoids, etc. (Qian et al., 2008).

Taking into account the useful properties of *Ph. tanacetifolia* raw, this study aimed to evaluate the biochemical composition of bee pollen and inflorescences of this species from the Slovak Republic that can be useful for further pharmaceutical and apicultural studies.

Material and methodology

Biological material

The plant raw material of *Phacelia tanacetifolia* Benth. collected from the experimental plots of Slovak Agricultural University in Nitra (Slovak Republic). It was collected inflorescences and bee pollen during the period of mass flowering in 2022.

Biochemical analyses

All biochemical analyses were conducted at the Slovak University of Agriculture in Nitra (Slovak Republic). All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and CentralChem (Slovakia).

The total dry matter, protein, ash, and lipid content determination

The total dry matter, protein, and ash were determined according to Hrytsajenko et al. (2003). Plant samples were dried in a drying oven at 105 °C till constant weight in aluminum boxes. The protein content was determined by the Kjeldahl method. The total ash content was conducted by combustion at 550 °C in the oven till constant weight. Results are given in percentages. The detailed procedures are described in Vergun et al. (2022a, 2022b). The total lipid content is conducted by the Soxhlet method with petroleum ether extraction (Hewavithrana et al., 2020). The low-boiling petroleum ether (40 °C) was used as an extractor. The difference in masses before and after the extraction process is used to calculate the total lipid content.

Analysis of sugars

Sample preparation: cornelian cherry samples of 1 g with 10 mL of water/ethanol (4 : 1) were vigorously mixed (vertical shake table; GFL, Germany). After 60 min of extraction, the mixture was centrifuged at 6000 rpm for 4 min (EBA 21, Hettich, Germany). The supernatant was filtered through the filter paper with 0.45 mm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with water. An HPLC analysis of sugars (fructose, maltose, sucrose, lactose) was performed using an Agilent 1260 Infinity instrument (Agilent Technologies, Santa Clara, USA)

coupled to an evaporative light scattering (ELSD) detector. Separation of sugars was conducted on a Prevail Carbohydrates ES column (250 × 4.6 mm). Acetonitrile/water (75 : 25, v/v) was used as the mobile phase. The identification of sugars was made by comparing the relative retention times of sample peaks with standards (Sigma-Aldrich, Steinheim, Germany). The contents of sugars were expressed as $g.kg^{-1}$ of the dry sample.

The total carotenoid content

The total carotenoid content expressed as β-carotene was analyzed spectrophotometrically at the wavelength 440 nm (VIS spectrophotometer UV Jenway Model 6405 UV/VIS). Sample (1 g) was disrupted with sea sand and extracted with acetone until complete discoloration. Petroleum-ether was added and then water, in purpose to the separation of phases. After the separation, the petroleum ether-carotenoid phase was obtained, and the absorbance was measured (ČSN 560053, 1986).

Analysis of amino acid profile

Amino acids were determined according to the standard procedure of AOAC (1990). The bee pollen samples were analyzed in the following way: were undergone a hydrolysis process in 6 N hydrochloric acid (HCl) at 110 °C for 24 h followed by the reconstitution of the samples with physiological buffer (0.12 N, pH 2.2) and analyzed by the amino acid analyzer (Model AAA-400, Ingos, Czech Republic)) associated with LCA K07/Li (PEEK column 4.6 × 150 mm) column.

Analysis of fatty acid composition

Lipid fraction extracted from each morphological part of *Cornus mas* was determined as follows: the samples were prepared according to official methods Ce 2-66 (1997) to convert triacylglycerols into methyl esters of fatty acid (FAMEs). The FAMEs were analyzed by gas chromatography using an Agilent 6890N instrument (Agilent Technologies, Santa Clara, USA) equipped with a flame ionization detector (FID; 250 °C; constant flow, hydrogen 40 $mL.min^{-1}$, air 450 $mL.min^{-1}$), a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Santa Clara, CA, USA). A detailed description of the chromatography conditions is presented in the work of Szabóová et al. (2020). Standards of a C4-C24 FAME mixture (Supelco, Bellefonte, PA, USA) were applied to identify FAME peaks. The evaluation was carried out by the ChemStation 10.1 software. The contents of FAs were expressed as $g.100\ g^{-1}$ of lipids.

Elemental analysis

The contents of macroelements, microelements and trace metals were determined by the inductively coupled plasma optical emission spectroscopy (ICP-OES) according to Divis et al. (2015) by using an ICP-OES instrument (Ultima 2, Horiba Scientific, France). Samples were prepared for analysis after microwave digestion (Milestone 1200, Milestone, Italy), 0.25 g of sample was decomposed in a mixture of nitric acid (6 mL) (Analytika Praha Ltd, Czech Republic) and hydrochloric acid (2 mL) (Analytika Praha Ltd, Czech Republic). After the decomposition sample was filtered through filter paper (0.45 mm pore size) and filled up to 25 mL in a volumetric flask with pure water.

Statistical analysis

The results are expressed as mean values of three replications ± standard deviation (SD). Data were analyzed with the ANOVA test and differences between means were compared through the Tukey-Kramer test ($p < 0.05$).

Results and discussion

The biochemical composition is one of the most important parameters in the evaluation of plant raw material that depends on the stage of growth, conditions of development, species, genotypes, etc. (Vergun et al., 2022a). The content of dry matter, protein, ash, lipids, β-carotene, ascorbic acid, fatty acid, and others is a basic nutritive composition evaluation of plant raw material (Sharma and Kaushik, 2021; Vergun et al., 2022b). Plants produce the phytochemicals that protect them from diseases. The soul food source for many insects is floral nectar and pollen (Chlebo and Adamchuk, 2017; Palmer-Young and Thursfield, 2017). The nutritional composition of bee pollen is carbohydrates, proteins, lipids, vitamins, minerals, and polyphenols, that exhibit numerous biological activities (Arruda et al., 2013; Li et al., 2018; Ghosh and Jung, 2020). The biochemical composition of bee pollen depends on plant origin, ecological conditions, and conditions after collecting (Denisow and Denisow-Pietrzyk, 2016). Thakur and Nanda (2020) summarized data from numerous studies of the biochemical composition of bee pollen and reviewed the content of carbohydrates from 18.50 to 84.25%, proteins from 4.50 to 40.70%, lipids from 0.41 to 13.50%, fibre from 0.15 to 31.26%, ash from 0.50 to 7.75%, phenolic compounds from 0.69 to 213.0 mg $GAE.g^{-1}$, etc., depended on plant species.

In this study, *Ph. tanacetifolia* bee pollen had 73.3% of dry matter, 27.44% of protein, 2.77% of ash, 5.35%

of lipids, 3.0 mg.kg^{-1} of β-carotene, 32.2 g.100 g^{-1} of saturated fatty acids, 5.7 g.100 g^{-1} of monounsaturated fatty acids, and 55.7 g.100 g^{-1} of polyunsaturated fatty acids (Figure 1). The nutritive composition of *Ph. tanacetifolia* inflorescences was 91.05% of dry matter, 18.37% of protein, 15.49% of ash, 4.5% of lipids, 50.4 mg.kg^{-1} of β-carotene, 34.0 g.100 g^{-1} of saturated fatty acids, 8.8 g.100 g^{-1} of monounsaturated fatty acids, and 45.5 g.100 g^{-1} of polyunsaturated fatty acids.

As reported Singh et al. (1999), the lipid content of *Brassica campestris* L., *Cosmos bipinnatus* Cav., and *Raphanus sativum* L. pollen was 20.3, 19.4, and 17.8%, respectively. Human and Nicolson (2006), found that the bee pollen composition of *Aloe greatheadii* var. *davyana* depended on condition after collection. In this case, fresh pollen, bee-collected pollen, and stored pollen had different protein, lipid, ash, and carbohydrate content. The most content of protein, ash, and lipids had samples of fresh pollen and carbohydrates the samples of storage pollen. According to Addi and Lamessa (2009), the study of the nutritive composition of numerous bee pollen of different plant species from twelve families showed the accumulation of 7.87–28.68% of crude protein. The experiment with the use of *Ph. tanacetifolia* as feed culture showed that cows produced milk with 598 g.kg^{-1} of saturated fatty acids, 350 g.kg^{-1} of monounsaturated fatty acids, and 52.1 g.kg^{-1} of polyunsaturated fatty acids, whereas feed phacelia contained saturated, monounsaturated, and polyunsaturated fatty acids 263. 97, and 641 g.kg^{-1}, respectively (Käbler et al., 2011). The study of different species showed that fatty acid content varied from 0.52 to 8.21%. Fatty acids account for 3% of the total lipid content of pollen grains (Gercek et al., 2022). As reported Denisow and Denisow-Pietrzyk (2016), the β-carotene content of bee pollen can be from 0.01 to 0.20 g.kg^{-1} (10–200 mg.kg^{-1}). Bee pollen of *Taraxacum officinale* L. had a lipid content 19.04% (Prđun et al., 2021).The study of selected plant species showed that geographical origin affected the fatty acid composition of bee pollen (Liolios et al., 2022).

Amino acids are one of the important biochemical components of bee pollen. According to Bayram et al. (2021), studied bee pollen samples showed the predominant content of proline, asparagine, and aspartic acid. A high concentration of proline, in this case, can be the parameter of sample freshness. The quantitive and qualitative amino acid content of bee pollen depends on numerous factors among which is harvesting season (Al-Kahtani et al., 2020).

As shown in Figure 2, the amino acid content of bee pollen from *Ph. tanacetifolia* decreased in the following order: glutamic acid > aspartic acid > proline > lysine > leucine > glycine > alanine > arginine> phenylalanine. The rest amino acids had a concentration of less than 10 g.kg^{-1}.

Silva et al. (2014) determined the amino acid composition of *Senna* spp. and detected serine and proline as prevailed in pollen. According to Ghosh et al. (2020), bee pollen of selected plant species accumulated the most glutamic acid (2.65 g.100 g^{-1} for *Trifolium repens* L. and 1.29 g.100 g^{-1} for *Coreopsis drummondii* (D.Don) Torr & A. Gray) and lysine (1.17 g.100 g^{-1} for *Erigeron annus* (L.) Pers. and 1.12 g.100 g^{-1} for *Oenothera biennis* L.).

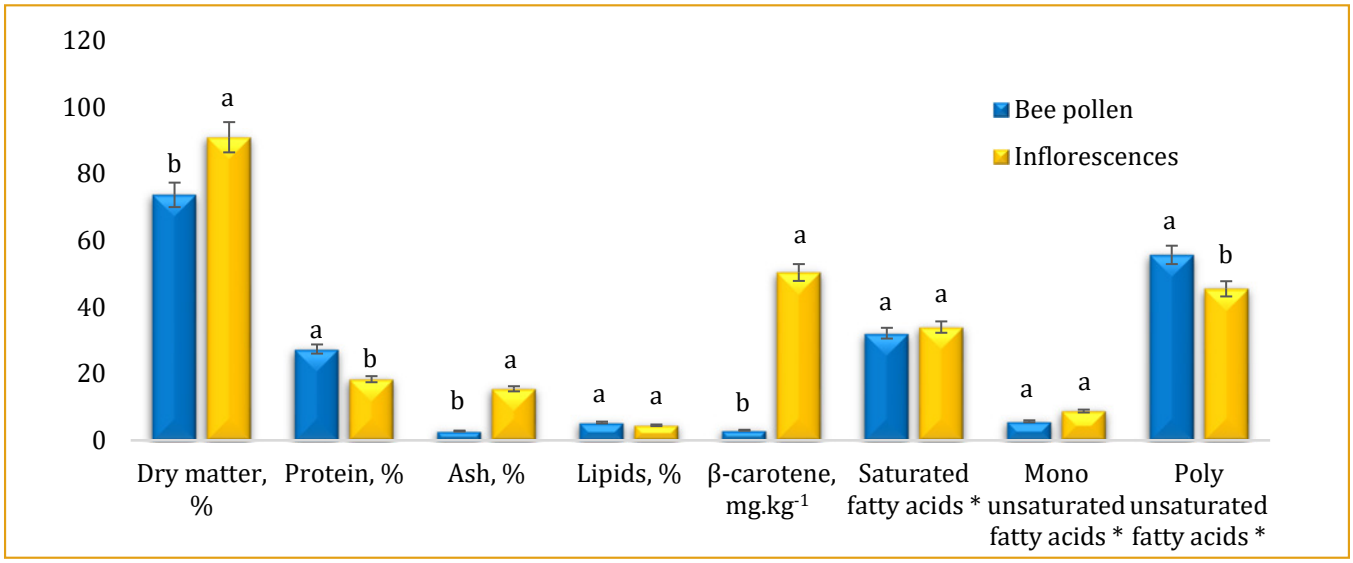

Figure 1 The nutritive composition of bee pollen and inflorescences of *Phacelia tanacetifolia* Benth.
* – g.100 g^{-1} of fat. Means in each column followed by different letters are significantly different (p <0.05)

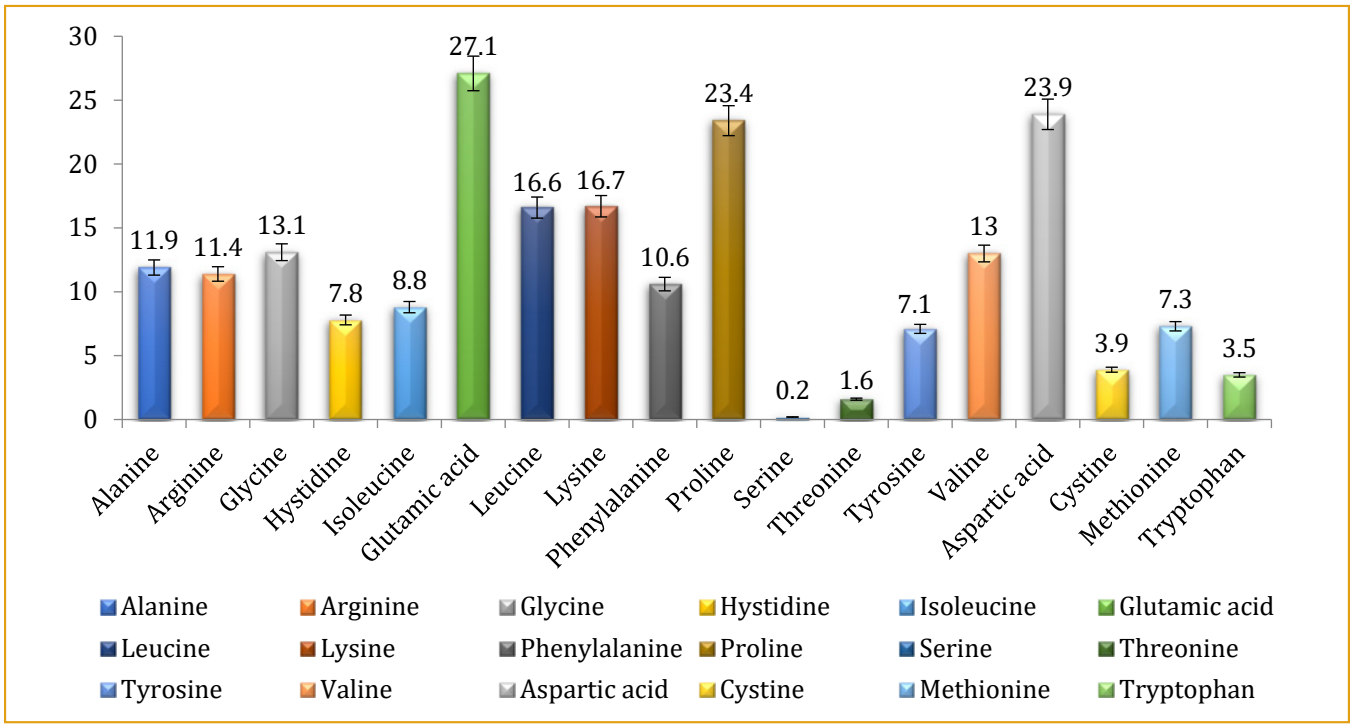

Figure 2 The protein composition of bee pollen of *Phacelia tanacetifolia* Benth., g.kg^{-1}

Figure 3 demonstrated that the content of amino acids in flower extracts decreased in followed order: glutamic acid > aspartic acid > leucine > phenylalanine > lysine > valine > glycine, proline > isoleucine. The rest of the amino acids accumulated in quantity less than 7.0 g.kg^{-1}.

The content of proline in the three honey compositions with *Ph. tanacetifolia*, as reported by Horčinová Sedlačková et al. (2022), was 224.38–296.21 mg.kg^{-1}.

One of the most important functions of plant sugars is the regulatory role during growth and development and it depends on numerous factors such as cold or drought stress, pathogens, phosphorus deficiency, and peculiarities of growth namely increased sugar demand in different plant tissues (Ciereszko, 2018). The plant sugars also with gene expression translate the nutrient status at the different periods of growth (Stephen et al., 2021). Sugars are distributed in the different

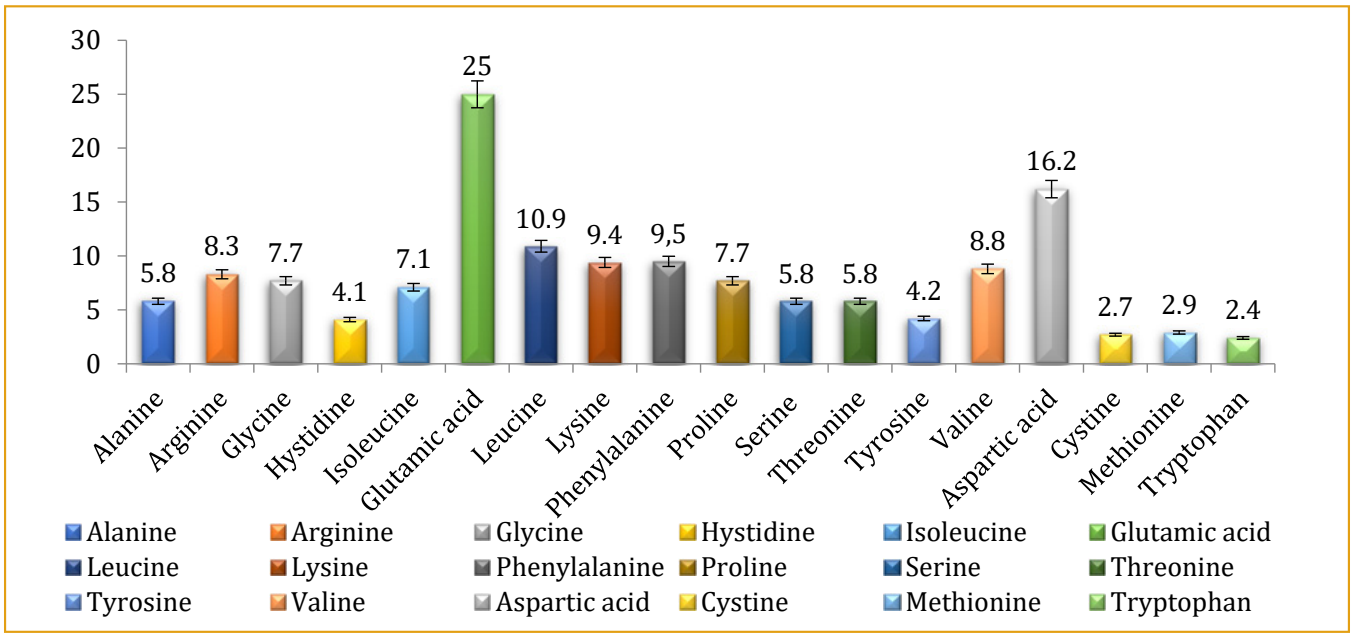

Figure 3 The protein composition of inflorescences of *Phacelia tanacetifolia* Benth., g.kg^{-1}

Table 1 The content of saccharides in bee pollen and inflorescences of *Phacelia tanacetifolia* Benth. (g.kg^{-1} of dry weight; mean ±SE)

Saccharide	Bee pollen	Inflorescences
Fructose	174.7 ±3.12	3.9 ±0.21
Maltose	<0.5	<0.5
Saccharose	<0.5	4.2 ±0.11
Lactose	<0.5	<0.5

plant parts, including inflorescences and flowers that produce nectar which mainly includes fructose, glucose, sucrose, etc. (Adamchuk et al., 2017; Gardana et al., 2018).

The content of saccharides of *Ph. tanacetifolia* bee pollen is represented in Table 1. The content of bee pollen fructose higher 44 times than inflorescences fructose. The content of maltose and lactose in both raw was less than 0.5 g.kg^{-1}.

Thakur and Nanda (2020) reviewed that different bee pollen glucose content was from 2.77 to 28.49 g.100 g^{-1}, fructose from 4.9 to 33.48 g.100 g^{-1}, sucrose from 0.05 to 9.02 g.100 g^{-1}, etc, depended on plant species. According to Horčinová Sedlačková et al. (2022), the sucrose content of honey composition with *Ph. tanacetifolia* was 0.84–7.37%. In this case, the highest sucrose content was found in composition with *Aesculus hippocastanum* L. and *Robinia pseudoacacia* L.

The fatty acid composition of pollen is one of the most important biochemical parameters. Among saturated fatty acids, the most prevailed for both bee pollen and inflorescences was palmitic acid (Table 2). Oleic acid prevailed among monounsaturated fatty acids and linolenic acid among polyunsaturated fatty acids.

It should be noted that fatty acids such as C8:0, C20:4, C22:1, and C24:1 are determined as nondominant and were in quantity less than 0.1 mg.kg^{-1}. Hsu et al. (2021) studied 11 different bee pollen samples and resulted also the prevailed palmitic and linoleic acids. Al-Kahtani et al. (2021) determined that the lipid and fatty acid content of *Brassica napus* L., *Medicago sativa* L., *Helianthus annuus* L., etc., depended on

Table 2 The fatty acid composition of bee pollen and inflorescences of *Phacelia tanacetifolia* Benth. (mg.kg^{-1} of dry weight; mean ±SE)

Fatty acid	Bee pollen	Inflorescences
Saturated fatty acid		
Capric acid (C10:0)	0.34 ±0.02	<0,1
Lauric acid (C12:0)	0.23 ±0.01	0.19 ±0.01
Myristic acid (C14:0)	1.37 ±0.01	1.86 ±0.12
Palmitic acid (C16:0)	28.42 ±1.13	27.93 ±1.24
Heptadecanoic acid (C17:0)	<0.1	0.21 ±0.01
Stearic acid (C18:0)	0.93 ±0.05	2.79 ±0.21
Arachidic acid (C20:0)	0.25 ±0.02	0.64 ±0.05
Behenic acid (C22:0)	0.23 ±0.01	0.37 ±0.01
Monounsaturated fatty acid		
Palmitoleic acid (C16:1)	0.66 ±0.02	0.35 ±0.01
Heptadecenoic acid (C17:1)	<0.1	0.77 ±0.04
Oleic acid (C18:1)	4.99 ±0.62	8.06 ±0.35
Eicosenoic acid (C20:1)	<0.1	0.36 ±0.02
Polyunsaturated fatty acid		
Linoleic acid (C18:2)	10.19 ±0.54	22.26 ±0.78
Docosadienoic acid (C22:2)	1.47 ±0.21	<0.1
Linolenic acid (C18:3)	45.47 ±1.23	23.27 ±0.92

Table 3 Element composition of bee pollen and inflorescences of *Phacelia tanacetifolia* Benth. (mg.kg^{-1} of dry weight; mean ± SE)

Element	Bee pollen	Inflorescences
	Macroelements	
K	6239 ±350	27038 ±546
P	6039 ±267	6624 ±234
Ca	1067±76	33282 ±215
S	2403 ±123	4068 ±156
Mg	553±32	4513 ±173
Na	18.02 ±0.21	601 ±31.12
	Microelements	
Zn	55.01 ±2.12	45.13 ±2.51
Fe	56.21 ±1.54	45.25 ±1.76
Cu	9.03 ±0.23	12.02 ±0.65
Mn	21.9 ±0.56	36.8 ±0.43
Cr	<0.2	0.44 ±0.02
	Metals	
Al	2.42 ±0.11	8.05±0.22
As	<0.3	<0.3
Cd	0.045 ±0.001	0.210 ±0.06
Hg	0.003 ±0.0001	0.008 ±0.0001
Pb	0.10 ±0.001	0.75 ±0.002

seasonal collection. The palmitic, stearic, oleic, and linolenic acids have prevailed in this case.

Bioelements including macro- and micronutrients present in the bee pollen in quantity of 1.6% (Komosinska-Vassev et al., 2015). The composition of macro-, microelements, and metals in the bee pollen and inflorescences of *Phacelia tanacetifolia* Benth. were significantly different mostly depending on the sample (Table 3). Bee pollen of investigated samples had the highest content of potassium, phosphorus, and sulfur. Investigated inflorescences had a higher content of most elements than bee pollen, especially K, Ca, Na, and Mg.

Matuszewska et al. (2021) determined that investigated bee pollen and royal jelly contained a high content of potassium, phosphorus, and sulfur which is similar to our study. According to Valverde et al. (2023), the study of seventy-one samples showed that terms of the apiary and harvesting had no affected on the elemental composition of bee pollen. Phosphorus and potassium have prevailed elements in this case, the same as in the present study. Potassium and phosphorus are two of the most essential elements of bee pollen which are contained in the most quantity along with other elements (Ghouizi et al., 2023).

Conclusions

Taking into account obtained data, it should be noted that bee pollen and inflorescences of *Ph. tanacetifolia* from the Slovak Republic are rich sources of nutrients and have a useful biochemical composition. The raw bee pollen and inflorescences had a high content of dry matter, ash, lipids, β-carotene, saturated, unsaturated fatty acids, and macro- and microelements. Additionally, the proteins of bee pollen of *Ph. tanacetifolia* and its inflorescences had high concentrations of glutamic, aspartic acid, proline, and leucine. The content of bee pollen fructose higher 44 times than inflorescences fructose. Among saturated fatty acids, the most prevailed for both bee pollen and inflorescences was palmitic acid. Oleic acid prevailed among monounsaturated fatty acids and linolenic acid among polyunsaturated fatty acids. Bee pollen of investigated samples had the highest content of potassium, phosphorus, and sulfur, while inflorescences had a higher content of potassium, calcium, sodium, and magnesium. The obtained data concerning the biochemical composition of bee pollen and inflorescences of *Ph. tanacetifolia* can be useful in the pharmaceutical, food, apicultural, and cosmetic industries.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statements

This article does not contain any studies that would require an ethical statement.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

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Assessment of physical, chemical, microbiological, and sensory characteristics of global and local cola carbonated soft drink brands in Egypt

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This study aimed to estimate the physical, chemical, microbiological, and sensory characteristics of commercial cola-carbonated soft drink brands in the Egyptian market to evaluate the safety of these drinks. Two commercial drinks brands, global and local cola, were used in the study. Physico-chemical, microbiological, and sensory characteristics of cola soft drinks were affected ($p \leq 0.05$) by the cola brand. The global cola soft drink had higher ($p \leq 0.05$) physico-chemical characteristics than the local cola soft drink after production and for 6 months storage period. In addition, the Global cola soft drink brand had a higher percentage of taste, odour, appearance, and overall acceptability than the local cola soft drink brand by 20%, 22.53%, 12.42%, and 18.55%, respectively, after production. Gradual ($p \leq 0.05$) decline in all sensory scores was detected for 6-month storage period. The global cola soft drink had a much lower total bacterial count (2.3 CFU.100 ml^{-1}) than a local cola soft drink (123 CFU.100 ml^{-1}). Thus, local brands of cola consumption after production could have public health risks due to high microbial load and deteriorated product shelf life. Also, quality control during processing and public health awareness could mitigate risk. During the 6-month storage period, the total bacterial count was completely absent after 3 and 4 months for global and local cola soft drinks, respectively. The global and local cola soft drink brands during the storage period at room temperature for 6 months were completely free of aciduric bacteria, coliform bacteria, and *Escherichia coli*, yeast, and mould as well. The results indicate that the physico-chemical attributes of all tested carbonated soft drinks samples (global & local) are within the European guidelines of carbonated soft drinks but are not compatible with the label of the reported bottle. Also, local brands of cola consumption after production could have public health risks due to high microbial load and deteriorated product shelf life.

Keywords: cola, physico-chemical, microbiological, sensory, *Escherichia coli*

Introduction

A soft drink is a beverage that does not contain alcohol; carbonated soft drinks are commonly known as soda, POP, or soda POP in parts of the United States and Canada, or Fizzy drinks in the U.K. In 2013, sales of carbonated soft drinks reached an annual volume of 196 billion litre, representing 12% of the global drink's volume. Despite the issue of sugar content

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surrounding carbonated soft drinks linked with the obesity epidemic, carbonated soft drinks have still managed to achieve an average annual growth rate of 2.6% (Ashurst, 2016). Also, approximately 5.3 billion litres of carbonates were consumed in the UK. The total volume of carbonated soft drinks consumed in the European Union per capita was 243.6 litres (Garavaglia et al., 2019).

Soft drinks are produced by mixing treated water, carbonated under pressure, with sugar (sucrose or fructose), acids, colouring agents, and preservatives. It contains around 8–12% (w/v) of sugars, 0.05–0.3% (w/v) of acidulant, 3.0–4.5% (w/v) carbon dioxide, and 0.1–0.5% (w/v) of flavouring agent (Sharma, 2018). In many cases, soft drinks contain caffeine, a central nervous system and metabolic stimulant derived from the kola (cola) nut extract, which is added as a flavouring agent, even if the amount which is usually present is less than that which is found in tea and coffee, except for energy drinks. In cola-type beverages, caffeine is considered generally recognized as safe (GRAS) up to a maximum use level of 0.02% (Preedy, 2014). Phosphoric acid is present abundantly in cola soft drinks. There are reports that phosphoric acid concentration in cola soft drinks ranged between 175–200 ppm. WHO/FDA phosphoric acid daily recommended dosage is 1000 ppm.day^{-1} (Helal, 2020). These beverages contain caramel, fruit juice, or caffeine with the addition of carbon dioxide, which contributes to their thirst-quenching effect: they can all be therefore considered under the denomination of 'soft drinks.' All these beverages are also characterized by the absence of ethyl alcohol, and they can be freely consumed by children (Brenna, 2014).

Physical, chemical, and microbiological criteria of treated water used in the production of carbonated soft drinks must comply with drinking water specifications according to World Health Organization (WHO). Also, principal constituent levels such as Brix (TSS), titratable acidity, pH, carbonation ratio, caffeine, and phosphoric acids must be monitored and controlled during processing, and must be confirmed with standard specifications of the global and local authorities (Sarwar, 2016).

The shelf life of carbonated soft drinks is varied, with a low possibility of deterioration due to low pH, carbonation levels, acids regulators, and the presence of natural and/or artificial preservatives. On the other hand, due to the nutrient content and composition, the majority of soft drinks are subjected to microbial spoilage (Hiko and Muktar, 2020).

Soft drinks have been consumed regularly; sugar has a high-calorie content that will give the body energy that it lacks. However, all that energy is short-lived, and it can only give short bust of increased productivity (Lobo and Satish, 2018). Sugar can preserve and enhance the flavour of a drink and gives a satisfying sensation (Kregiel, 2015). Meanwhile, the problem arises due to high consumption of sugary drinks which leads to various health hazards (obesity, diabetes mellitus, or non-alcoholic fatty liver diseases). Sugar-sweetened beverages have contributed to an increase in obesity, hypertension, type 2 diabetes, and other metabolic disorders (Malik et al., 2011). Soft drinks have long been blamed for causing damage to teeth, especially among children. They have the potential to cause erosion. However, there are mitigating factors serving to reduce greatly the damage that soft drinks might at first be thought to cause. Sugar-free drinks are widely available, and are targeted at all age ranges, rather than just at slimmer (a reduction in sugar content would have little effect; is total absence that is necessary) (Ashurst, 2016).

The main objective of this study was to estimate the major constituents, microbial load in commercial brands (global and local) of carbonated cola to reveal the safest cola soft drink brand in the Egyptian market.

Material and methodology

Two different cola soft drinks, namely global and local brands, packaged in 1.0 litre polyethylene terephthalate (PET) bottles were collected after production during the winter season (2016). Global and local cola carbonated soft drinks brands were obtained from local markets of El-Sadat City, Menoufia Governorate, and 6th of October City, Giza Governorate, Egypt, respectively.

Storage of cola soft drink brands: global and local cola carbonated soft drink brands were analyzed after production immediately and during six months storage periods at laboratory temperature (22 °C ±2) to compare their physical, chemical, microbiological, and sensory properties.

Physico-chemical methods

The degassing of cola soft drink brands (global and local) was accomplished according to the method described by (Sagharizade, et al., 2019) using Commercial Somex Degassing Unit Somex Soft Drink Degasser (Bally Vourney CO. Cork, Ireland). The pH was measured using a pH meter (Jenway 3510 pH Meter, England) as described by (Rangana 1977); according to the

manufacture manual; the Anton Paar Carbo Qc (DMA 48/DMA 58, Austira) measuring system for monitoring and measuring CO_2 & O_2 was used (Anton Paar Manual, 2010). Reducing sugars were measured according to Miller (1959). Density was measured as described by (Steinbach et al., 2014). Caffeine was estimated as described by (Amos-Tautua et al., 2014); phosphoric acid was measured as described by (Lozano-Caleroand and Martín-Palomeque, 1996); the titratable acidity, and sugars (ºBrix, refractometer; ATAGO Model 5000 DCX, Research Analytical, Japan) measurements were performed in triplicate (AOAC 2005).

Microbiological methods

A membrane filter procedure for enumerating total bacterial, yeast, mould and aciduric bacteria, total coliform, and *E. coli* counts was developed and evaluated with some modifications as follows: Appropriate volumes (100 mL) of global and local cola carbonated soft drink brands samples were passed through 0.45 µm gridded membrane filters (MCE) using vacuum funnel assembly. Then, samples were allowed to be drawn completely via a vacuum pump through the filter, and the filters then were placed on the selected medium, incubated at the proper temperature and for the appropriate period, then counted to confirm the colonies.

Total bacterial, yeast, mould, and aciduric bacteria viable counts were carried out according to (Braux et al., 1997). The total coliform bacterium was detected with some modifications according to AOAC (2005). *Escherichia coli* detection was carried out according to Downes and Ito (2001).

Sensory method

Global and local cola carbonated soft drink brands were subjected to sensory evaluation directly after production and every month during six months storage periods for appearance, taste, and overall acceptability by a trained panel consisting of ten members (average age mid-30 s) selected from laboratory staff and a team of the sensory test; using Hedonic scale rating 1–9 points (1 = dislike very much; 9 = like very much) to assess the differences. Experts evaluated soft drink samples offered at the same time in a specific area of sensory test in the soft drink samples plant quality assurance laboratory without special lighting. Water was provided for rinsing purposes.

Statistical analysis

Global and local cola-carbonated soft drinks were determined as the mean of ten replicates, while the physicochemical properties of global and local cola-carbonated soft drink brands were determined as the mean of three replications. Two-way Factorial Design analysis of variance was used for global and local cola carbonated soft drinks' physicochemical and sensory properties. The LSD was used for comparison among means, considering significance at 0.05% level, using Costas version 6.311 (Copyright 1998–2005, CoHort software).

Results and discussion

Physicochemical properties of global and local cola carbonated soft drinks brands during 6 months storage period

The CO_2, pH, Density, O_2, TSS, reducing sugars, titratable acidity, phosphoric acid, and caffeine were evaluated, and the data was shown in Table 1. Initially, all parameters were within acceptable quality limits for tested global and local cola brands. Meanwhile, the physicochemical characteristics of cola soft drink brands were affected ($p \leq 0.05$) by the type of cola brand. The global cola soft drink brand had higher ($p \leq 0.05$) physico-chemical characteristics than the local cola soft drink.

After production, physico-chemical characteristics of cola soft drinks brands had carbonation levels of 3.99 ±0.05 (v/v) and 2.83 ±0.20 (v/v), respectively. After 6 months of storage (Table 1), carbonation volume decreased gradually for both brands 1.81 ±0.02 (v/v) and 1.60 ±0.02 (v/v). A value of 4.0 (V/V) of CO_2 in Coca-Cola PET bottles is usually used to guarantee the original characteristics quality and extend the shelf-life of Coca-Cola (Licciardello et al., 2011). After production, the hydrogen ion (2.14 ±0.0) of the local cola soft drink brand recorded an acidic value more than the global cola brand (2.80 ±0.0). During the storage period, pH values were significantly ($p \leq 0.05$) decreased. The decrease in pH values may be due that the interaction between the weak carbonic acid and the strong phosphoric acid. The density of the global cola soft drink (1.0422 $gm.cm^{-3}$) is similar to values reported by Charrondiere et al. (2012) and Jayeola (2001). The density of cola soft drinks was not affected ($p \geq 0.05$) during the storage period. The stability of the density during storage is good because the density increase involves the danger of the increase of the maximum internal gas pressure. Global cola soft drink brands had a higher ($p \leq 0.05$) O_2 value in bottled packages (1.8 ppm) than the local brand (0.5 ppm). This difference is due to O_2 ingress rates which imparted

Table 1 Physical characteristics of cola soft drink brands during storage at room temperature for 6 months

Physical characteristics	Brands	Storage period (months)						
		0	1	2	3	4	5	6
CO_2 (V/V)	global	3.99 ±0.05	3.74 ±0.01	3.47 ±0.05	3.17 ±0.02	2.8 ±1.20	2.63 ±0.34	1.81 ±0.02
	local	2.83 ±0.20	2.39 ±0.16	1.50 ±0.02	1.65 ±0.02	1.54 ±0.02	1.17 ±0.14	1.60 ±0.02
pH	global	2.80 ±0.0	2.70 ±0.02	2.51 ±0.04	2.44 ±0.04	2.33 ±0.03	2.25 ±0.02	2.12 ±0.04
	local	2.14 ±0.0	2.05 ±0.02	1.96 ±0.02	2.02 ±0.03	1.9 ±0.13	1.8 ±0.02	1.63 ±0.04
Density ($gm.cm^{-3}$)	global	1.0483 ±0.0007	1.0450 ±0.0003	1.0432 ±0.0002	1.0411 ±0.0004	1.0401 ±0.0002	1.0395 ±0.0005	1.0382 ±0.0003
	local	1.0224 ±0.0004	1.0221 ±0.0001	1.0222 ±0.0001	1.0222 ±0.0000	1.0222 ±0.0000	1.0222 ±0.0002	1.0222 ±0.0001
O_2 (ppm)	global	1.80 ±0.0	2.00 ±0.0	7.0 ±0.0	10.60 ±1.15	13.60 ±0.57	17.30 ±1.15	26.60 ±1.52
	local	0.5 ±0.2	2.00 ±0.0	11.0 ±0.0	7.3 ±1.15	8.0 ±0.0	10.3 ±0.5	17.0 ±1.7

Values are expressed as the mean of triplicate measurements

Table 2 Statistical analysis of physical characteristics of cola soft drink brands during storage at room temperature for 6 months

Physical characteristics	Brands		Storage period (months)						
	global	local	0	1	2	3	4	5	6
CO_2 (V/V)	3.99^{a}	2.83^{b}	3.41^{a}	3.06^{b}	2.41^{c}	2.21^{d}	1.90^{e}	1.80^{ef}	1.70^{f}
LSD	0.08					0.15			
pH	2.80^{a}	2.14^{b}	2.47^{a}	2.36^{b}	2.24^{c}	2.25^{c}	2.11^{d}	2.02^{e}	1.86^{f}
LSD	0.01					0.03			
Density ($gm.cm^{-3}$)	1.0422^{a}	1.0285^{b}	1.0353^{a}	1.0335^{a}	1.0326^{a}	1.0315^{a}	1.0310^{a}	1.0307^{a}	1.0301^{a}
LSD	0.01					0.01			
O_2 (ppm)	1.80^{a}	0.50^{b}	1.15^{e}	2.00^{e}	9.00^{d}	9.00^{d}	10.83^{c}	13.83^{b}	21.83^{a}
LSD	0.12					1.01			

Means with different letter in the same row are significantly different ($p \leq 0.05$). LSD: least significant difference

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Table 3 Chemical characteristics of cola soft drink brands during storage at room temperature for 6 months

Chemical characteristics	Brands	Storage period (months)						
		0	1	2	3	4	5	6
Total soluble solids (%)	global	10.87 ±0.03	10.84 ±0.05	10.65 ±0.0	10.51 ±0.01	10.45 ±0.02	10.40 ±0.05	10.30 ±0.01
	local	6.0 ±0.06	5.96 ±0.05	6.06 ±0.03	6.04 ±0.01	6.03 ±0.02	6.0 ±0.05	5.99 ±0.02
Reducing sugars (%)	global	0.035 ±0.05	0.058 ±0.01	0.068 ±0.03	0.084 ±1.15	0.10 ±0.02	0.116 ±0.04	0.128 ±0.01
	local	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Titratable acidity (%)	global	13.5 ±0.6	11.72 ±0.2	11.76 ±0.38	12.38 ±0.13	12.95 ±0.21	13.50 ±0.07	13.72 ±0.06
	local	12.0 ±0.18	14.08 ±0.16	14.26 ±0.11	14.84 ±0.05	15.20 ±0.0	15.30 ±0.0	15.56 ±0.02
Phosphoric acid (ppm)	global	15.85 ±0.02	15.87 ±0.06	15.80 ±0.01	15.80 ±0.01	15.82 ±0.04	15.83 ±0.05	15.81 ±0.02
	local	15.79 ±0.05	15.80 ±0.5	15.8 ±0.0	15.81 ±0.05	15.80 ±0.0	15.78 ±0.09	15.74 ±0.08
Caffeine (ppm)	global	30.33 ±0.01	28.3 ±0.02	14.42 ±0.39	22.80 ±0.04	17.81 ±0.17	14.94 ±0.02	11.58 ±0.02
	local	15.52 ±0.04	5.15 ±0.06	9.56 ±0.05	3.76 ±0.42	3.20 ±0.06	2.86 ±0.12	1.68 ±0.51

Values are expressed as the mean of triplicate measurements

Table 4 Statistical analysis of chemical characteristics of cola soft drink brands during storage at room temperature for 6 months

Chemical characteristics	Brands		Storage period (months)						
	global	local	0.0	1	2	3	4	5	6
Total soluble solids (%)	10.87^{a}	6.00^{b}	8.43^{a}	8.38^{b}	8.34^{c}	8.26^{d}	8.23^{d}	8.19^{e}	8.14^{f}
LSD	0.05					0.03			
Reducing sugars (%)	0.035^{a}	0.00^{b}	0.017^{g}	0.029^{f}	0.034^{e}	0.042^{d}	0.050^{c}	0.058^{b}	0.064^{a}
LSD	0.01					0.002			
Titratable acidity (%)	13.5^{a}	12.00^{b}	12.75^{g}	12.90^{f}	13.07^{e}	13.61^{d}	14.07^{c}	14.4^{b}	14.64^{a}
LSD	0.11					0.05			
Phosphoric acid (ppm)	15.84^{a}	15.79^{b}	15.81^{a}	15.83^{a}	15.80^{a}	15.81^{a}	15.81^{a}	15.79^{a}	15.79^{a}
LSD	0.02					0.31			
Caffeine (ppm)	20.34^{a}	15.52^{b}	17.93^{a}	16.72^{b}	13.28^{c}	11.99^{d}	10.50^{e}	8.90^{f}	8.63^{f}
LSD	0.14					0.27			

Means with different letter in the same row are significantly different ($p \leq 0.05$). LSD: least significant difference

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to the packaging (bottle weight reduction, cap change, new bottle shape, etc.).

Total soluble solids and caffeine of cola soft drinks decreased ($p \leq 0.05$) by increasing the storage period. Reducing sugars and titratable acidity of cola soft drinks had an opposite trend. However, the phosphoric acid of cola soft drinks was not affected ($p \geq 0.05$) during the storage period. Regarding the global cola brand, the hydrolysis of sucrose during the storage period resulted in a decrease in TSS and increased reducing sugar. A similar reduction in TSS of cola soft drinks was reported by Idris et al. (2016) during 10 months of storage. Bubnik et al. (1995) revealed that the increase in the storage period caused an increase in the inversion of sucrose in fresh and stored soft drinks. Birkhed (1984) reported that the TSS of cola soft drinks ranged from 9.8 to 9.3% during the storage period. Sharma (2018) found that the concentration of reducing sugar was found in all the sets of Pepsi-cola containing 0.023%. Results of TSS and reducing sugars of local cola brand didn't show inversion. This could be explained by the fact that the local cola soft drink brand contains sweeteners without using sucrose in the manufacturing. Also, this is due to variations in recipes and formulations. Local cola soft drink TSS was 6.00% hence, it could be categorized as a calorie-reduced soft drink that contains less than 50% of the total sugars in the corresponding regular beverages (4.41–5.91%), mainly as fructose. The titratable acidity of cola soft drinks increased from 12.75 to 14.64% after 6 months of storage. The increase in titratable acidity could be explained by the formation of weak acids in cola soft drinks. Also, variation in titratable acidity during the storage period could be explained by hydrolysis, oxidation, and fermentation processes (Nilugin and Mahendran, 2010).

Phosphoric acid levels were found in global and local cola soft drink brands at concentrations of 15.84 ppm and 15.79 ppm, respectively. These values represented about 8% of the values (175–200 ppm) reported by Grenby et al. (1989). Caffeine contents of global and local cola soft drink brands were 20.34 and 15.52 ppm, respectively. These values are less than those reported by Walker et al. (1997), who recorded that caffeinated cola contains 33.0 ppm caffeine. Amos-Tautua and Diepreye (2013) revealed that caffeine content in soft drinks varies from 10 : 50 mg per serving, however, the US Food and Drug Administration (FDA 2006) limits the maximum amount in carbonated soft drinks to 6 $mg.oz^{-1}$. Therefore, the allowed caffeine content in soft drinks may be ranged between 30 : 72 $mg.355\ mL^{-1}$. The caffeine of cola soft drinks decreased from 17.93 to 8.63 ppm after 6 months of storage. Thus, the present results of phosphoric acid and caffeine concentrations follow USA soft drinks standards that can be used for formulating health policy.

The decreasing caffeine content during the storage period could be explained by using caffeine as a source of antioxidants, and antimicrobials against a broad range of foodborne pathogens, microorganisms and could be used as alternative preservative, with the potential of enhancing the safety and quality of drinks. Also, caffeine could be affected by O_2 permeability during the PET-packages storage period (Helal, 2020).

Microbial analysis

Bacterial counts of global and local cola soft drink brands during the storage period at room temperature for 6 months are shown in Table 5. The microbial contaminations in carbonated soft drinks are prevented by the combined influences of high sugar levels, acidity, carbonation, and good facilities and sanitation procedures (Ayres et al., 1980). The global cola soft drink had a much lower total bacterial count (2.3 $CFU.100\ ml^{-1}$) than a local cola soft drink brand (123 $CFU.100\ ml^{-1}$). The total bacterial count of global cola soft drinks was comparable with the value (less than 50 $CFU.100\ ml^{-1}$) reported by the Saint Lucia Bureau of Standards (2004) for carbonated beverages. However, the total bacterial count of local cola soft drinks was much higher than global cola brands where bacterial growth can tolerate lower pH due to poor quality control and bad manufacturing practices and non-conformities during processing. Oranusi et al. (1994) reported that the total bacterial count of the cola soft drink brand was 26 $CFU.100\ ml^{-1}$. The total bacterial counts of global and local cola soft drinks increased after one month of storage period. The total bacterial counts of global and local cola soft drinks decreased after two and three months of the storage period, respectively. However, the total bacterial count was absent after three and four months for global and local cola soft drinks, respectively. This effect is due to the effectiveness of the acidic pH of cola soft drinks on microorganism colonies. The global and local cola soft drink brands during the storage period at room temperature for 6 months were completely free from aciduric bacteria, coliform bacteria, and *E. coli*. The coliform bacteria count should be less than 1.0 $CFU.100\ ml^{-1}$ and *E. coli* count must be absent (ISO, 2004). Yeast and mould of global and local cola soft drinks during the storage period at room temperature for 6 months are shown in Table 5. Yeast and mould were not detected in global and local cola soft drinks

Table 5 Bacterial, yeast and mould counts (CFU.100 ml^{-1}) of cola soft drink brands during storage at room temperature for 6 months

Storage periods	Control		1		2		3		4		5		6	
Brand	**Pepsi**	**Sina**	**Pepsi**	**Sina**	**Pepsi**	**Sina**	**Pepsi**	**Sina**	**Pepsi**	**Sina**	**Pepsi**	**Sina**	**Pepsi**	**Sina**
Total count	2.3	123	3	158	1	110	0.0	205	1.6	TNTC	0.0	0.0	0.0	0.0
Yeast	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mould	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acid. Bacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Coliform	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
E. coli	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Blank	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

TNTC: too numerous to count

Table 6 Sensory properties of cola soft drink brands during storage at room temperature for 6 months

Sensory characteristics	Brands	Storage period						
		0	1	2	3	4	5	6
Taste	global	8.52 ±1.24	7.7 ±2.13	7.2 ±1.89	5.6 ±1.11	4.8 ±1.99	4.00 ±2.15	3.50 ±2.10
	local	7.10 ±0.05	6.75 ±1.29	6.50 ±2.15	5.25 ±1.18	4.80 ±2.22	3.90 ±1.95	3.20 ±0.94
Odour	global	8.7 ±0.33	7.7 ±0.48	7.6 ±0.92	7.00 ±0.94	6.20 ±1.39	5.90 ±0.87	5.30 ±1.05
	local	7.10 ±0.84	6.80 ±0.84	6.10 ±1.28	5.30 ±1.11	5.10 ±1.51	4.60 ±0.98	3.80 ±1.22
Appearance	global	8.87 ±0.31	8.7 ±0.52	8.9 ±0.31	8.2 ±0.47	8.10 ±0.81	7.50 ±0.46	7.00 ±0.24
	local	7.89 ±0.99	7.80 ±1.14	7.20 ±0.97	7.30 ±1.25	7.20 ±1.03	7.00 ±0.60	5.90 ±3.30
Overall acceptability	global	8.69 ±0.62	8.03 ±1.04	7.90 ±1.03	6.93 ±0.84	6.36 ±1.39	5.80 ±1.16	5.26 ±1.13
	local	7.33 ±0.64	7.11 ±0.90	6.60 ±1.46	5.95 ±1.18	5.70 ±1.58	5.16 ±1.17	4.30 ±1.82

Values are expressed as the mean of triplicate measurements

Table 7 Statistical analysis of sensory properties of cola soft drink brands during storage at room temperature for 6 months

Sensory properties	Brands		Storage period (months)						
	global	local	0.0	1	2	3	4	5	6
Taste	8.52^{a}	7.10^{b}	7.80^{a}	7.20^{ab}	6.85^{b}	5.40^{c}	4.80^{cd}	3.95^{de}	3.35^{e}
LSD	0.40					0.87			
Odour	8.70^{a}	7.10^{b}	7.90^{a}	7.25^{ab}	6.85^{bc}	6.15^{cd}	5.65^{d}	5.25^{de}	4.55^{e}
LSD	0.51					0.95			
Appearance	8.87^{a}	7.89^{b}	8.40^{a}	8.25^{a}	8.05^{ab}	7.75^{ab}	7.65^{ab}	7.25^{bc}	6.45^{c}
LSD	0.62					0.86			
Overall acceptability	8.69^{a}	7.33^{b}	8.01^{a}	7.55^{a}	7.35^{a}	6.45^{b}	6.05^{bc}	5.50^{cd}	4.75^{d}
LSD	0.43					0.86			

Means with different letter in the same row are significantly different ($p \leq 0.05$). LSD: least significant difference

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during the storage period at room temperature for 6 months. Cola soft drinks had a low pH value and a high carbonation and low levels of nutrients, these conditions are sufficient to inhibit the low levels of organisms (Ashurst and Hargitt, 2009).

Sensory properties

Sensory properties of global and local cola soft drinks during the storage period at room temperature for 6 months are shown in Table 6 and 7. The sensory properties of cola soft drinks were affected ($p \leq 0.05$) by the type of cola brand and storage period. The global cola soft drink had higher ($p \leq 0.05$) sensory properties than the local cola soft drink. Jayeola (2001) reported that no significant differences were observed between global and local cola soft drinks in sensory properties. In general, the sensory properties were not affected ($p \leq 0.05$) up to the second month of the storage period followed by a gradual decreased ($p \leq 0.05$) up to the sixth month of storage. Although the sensory properties of cola soft drinks gradually decreased from the second month to the end of the storage period, and still acceptable. These results agreed with those reported by Abeker (2009), who reported that carbonated soft drinks' sensory characteristics start to decline by increasing the storage period.

Conclusion

Global cola soft drink brands had higher percentage contents of carbon dioxide, pH, density, and total soluble solids, reducing sugars, titratable acidity, phosphoric acid, and caffeine values than local cola soft drink brands after production. Also, global cola soft drinks revealed a low total bacterial count compared with local cola soft drinks after production. Although, both cola brands are free from aciduric bacteria, coliform, and *E. coli* as well as yeast and moulds. Global cola soft drink brands had a higher percentage of taste, odour, appearance, and overall acceptability than local cola soft drink brands by 20%, 22.53%, 12.42%, and 18.55%, respectively after production. Gradual ($p \leq 0.05$) decline in the all-sensory scores can be seen for the 6-month storage period. Both cola brands were acceptable in terms of taste, odour, appearance, and overall acceptability up to 3rd, 5th, 6th, and 5th, respectively. The variation present in major constituents, microbial load, and sensory properties among tested commercial cola carbonated soft drink brands gives it the characteristics that determine its selection by the customers. Hence, the global cola soft drink brand samples were in the complaint with the standard limit present by USA soft drinks standards.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

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In vitro antibacterial efficacy of the commercial wintergreen (*Gaultheria procumbens* L.) essential oil against some Gram-positive and Gram-negative strains

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The aim of the current study was *in vitro* antimicrobial profiling of commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens* (Natural essential oil – Wintergreen oil Bamer®) against Gram-positive strains such as *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) (resistant to vancomycin; sensitive to teicoplanin) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®29212™), *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®29213™, *Staphylococcus aureus* NCTC12493™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®35218™. The testing of the antibacterial activity of wintergreen EO was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. This study demonstrated that commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens* (Natural essential oil – Wintergreen oil Bamer®) possesses significant antimicrobial activity against Gram-positive bacteria, such as *Enterococcus faecalis* strains. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* strains were resistant to commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens*. This study showed that commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens* could be a potential preparation as a source of natural antibacterial properties. Future pharmacological studies and development in other areas are thus warranted.

Keywords: *Gaultheria procumbens*, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* strains

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Introduction

Many publications prove the antibacterial properties of essential oils from medicinal plants (Vergnes et al., 2014). *Gaultheria procumbens* L. (American wintergreen, Ericaceae) is an aromatic, evergreen shrub native to north-eastern North America and cultivated worldwide in regions of temperate climate as an ornamental and medicinal plant (Kiran and Prakash, 2015; Michel et al., 2019; Lawson et al., 2021). Many studies have revealed that the extracts and compounds derived from *Gaultheria* plants exhibit a wide spectrum of pharmacological activities *in vitro* and *in vivo*, covering anti-inflammatory, analgesic, anti-oxidative, and antibacterial properties (Liu et al., 2013; Luo et al., 2018). It is known that *Gaultheria* plants accumulate a wide variety of polyphenols (Middleton, 1992), including methyl salicylate as the main component of the essential oil (Magiera et al., 2019), as well as nonvolatile compounds such as salicylate glycosides, procyanidins, and flavonoids (Liu et al., 2013; Magiera et al., 2019; Michel et al., 2020). Traditionally, plants rich in polyphenols, especially salicylates, have been used worldwide in the form of extracts, tinctures, infusions, and decoctions to treat a number of inflammatory diseases that are cross-related with oxidative stress (Michel et al., 2022).

In traditional medicine, the aerial parts (stems and leaves) of *G. procumbens* and other *Gaultheria* species, as well as methyl salicylate-rich essential oils distilled from the plants, are used (both externally and internally) in the treatment of disorders connected with inflammation, pain, and infection, including rheumatoid arthritis, influenza, the common cold, tracheitis, pharyngitis, pleurisy, fever, prostatitis, swelling and muscular pain, and some skin and periodontal problems (Olszewska et al., 2021). Essential oil from *G. procumbens* is mainly composed of methylsalicylate (MeSA) (>96%), a compound that can be metabolized in plant tissues to salicylic acid, a phytohormone inducing plant immunity against microbial pathogens (Mullen et al., 2014; Olszewska et al., 2021).

In the current study, *in vitro* antimicrobial profiling of commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens* (Natural essential oil – Wintergreen oil Bamer®) was performed, exhibiting inhibitory activity against Gram-positive and Gram-negative strains.

Material and methodology

Wintergreen essential oil

The wintergreen EO was provided by Polish essential oil manufacturers (Bamer®, Włocławek, Poland). The investigated sample did not contain additives or solvents and was confirmed to be natural by the manufacturers. Product description: Natural essential oil – Wintergreen oil Bamer®. The highest quality, pure, natural essential oil, is obtained from fresh leaves of the wintergreen (*Gaultheria procumbens* Leaf Oil). Laboratory tested.

About the manufacturer: Bamer® has been offering 100% natural, pure essential oils and fragrance compositions since 1993. Application studies on patients conducted at the Medical Academy confirmed the effectiveness of Bamer® oils in aromatherapy and cosmetics. The products are not tested on animals. Safety assessments, numerous certificates and approvals, compliance with the latest pharmacopoeia Ph.Eur. and IFRA, positive opinion of the National Institute of Hygiene guarantee the highest pharmaceutical and cosmetic quality of oils. Bamer® oils have been submitted to the European Commission via CPNP (Cosmetic Products Notification Portal). Bamer® essential oils are certified by the National Institute of Hygiene, IFRA, and laboratory analyses.

The samples were stored in resalable vials at 5°C in the dark but were allowed to adjust to room temperature before investigation. Geographical origins were excluded as information was mostly not available.

Determination of the antibacterial activity of essential oils by the disk diffusion method

The testing of the antibacterial activity of wintergreen EO was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). In the current study, Gram-positive strains such as *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) (resistant to vancomycin; sensitive to teicoplanin), and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®29212™), *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®29213™, *Staphylococcus aureus* NCTC12493™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®35218™ were used.

The strains were inoculated onto Mueller-Hinton (MH) agar dishes. Sterile filter paper discs impregnated with wintergreen EO were applied over each of the culture dishes. Isolates of bacteria with wintergreen EO were then incubated at 37 °C for 24 h. The Petri dishes were then observed for the zone of inhibition produced by the antibacterial activity of wintergreen EO. A control disc impregnated with 96% ethanol was used in each experiment. At the end of the 24 h period, the inhibition zones formed were measured in millimetres using the vernier. For each strain, eight replicates were assayed (n = 8). The Petri dishes were observed and photographs were taken. The susceptibility of the test organisms to the wintergreen EO was indicated by a clear zone of inhibition around the discs containing the wintergreen EO and the diameter of the clear zone was taken as an indicator of susceptibility. Zone diameters were determined and averaged. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥15 mm, Intermediate (I) = 10–15 mm, and Resistant (R) ≤10 mm (Okoth et al., 2013; Tkachenko et al., 2022).

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of the wintergreen EO tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 13.3 software (TIBCO Software Inc., Krakow, Poland) (Zar, 1999).

Results and discussion

Figures 1 and 2 summarize the results obtained by the mean diameters of the inhibition zone around the growth of Gram-positive strains such as *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) (resistant to vancomycin; sensitive to teicoplanin), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®29212™), *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®29213™, *Staphylococcus aureus* NCTC12493™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula

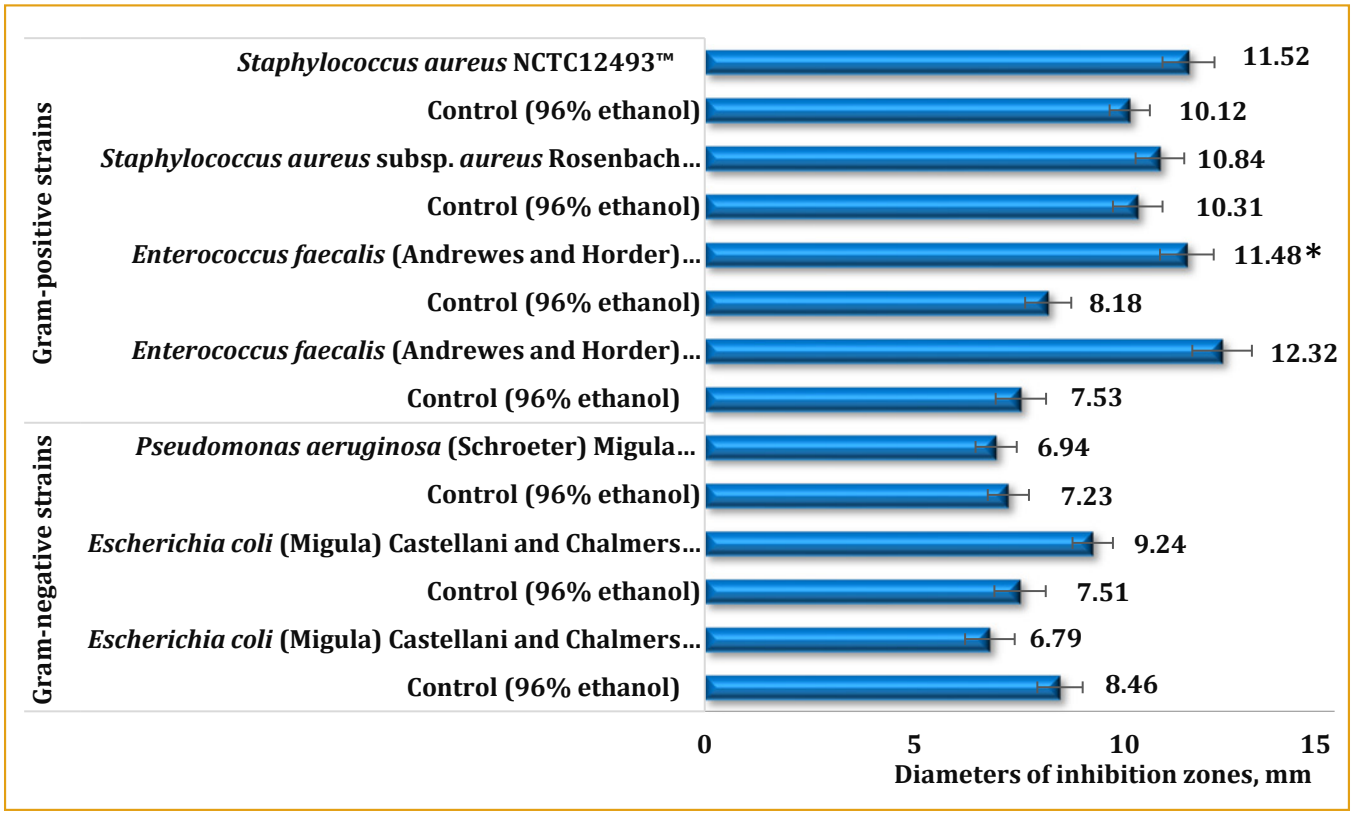

Figure 1 The mean inhibition zone diameters induced by commercial wintergreen essential oil against Gram-positive and Gram-negative strains (M ± m, n = 8)
*– changes are statistically significant compared to the 96% ethanol

ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®35218™ strains induced by wintergreen EO.

After applying wintergreen EO to *E. faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) strain, we noted a statistically significant increase in the zone of growth inhibition by 40.3% ($p < 0.05$) compared to the control samples (11.48 ±0.64 vs. 8.18 ±0.55 mm). We observed similar trends after *in vitro* application of wintergreen EO against *E. faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®29212™) strain, where we also observed a statistically significant increase in the zone of growth inhibition by 63.6% ($p < 0.05$) against the control samples (12.32 ±0.71 vs. 7.53 ±0.6 mm). *Staphylococcus aureus* strains were resistant to wintergreen EO. After applying wintergreen EO to *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®29213™ and *Staphylococcus aureus* NCTC12493™ strains, a statistically non-significant increase in the zone of growth inhibition by 5.1% ($p > 0.05$) and by 13.8% ($p > 0.05$) compared to the control samples (10.84 ±0.58 vs. 10.31 ±0.59 mm) and (11.52 ±0.62 vs. 10.12 ±0.48 mm), respectively (Figure 1).

Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli*

Figure 2 The diameters of the inhibition zone around the growth of Gram-positive strains such as *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®29212™) (A), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) (B), *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®29213™ (C), *Staphylococcus aureus* NCTC12493™ (D), and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ (E), and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®35218™ (F) strains induced by wintergreen EO

(Migula) Castellani and Chalmers ATCC®25922™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®35218™ were resistant to wintergreen EO. Adding wintergreen EO to *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®35218™ strains resulted in statistically non-significant changes in the zone of growth inhibition (decrease by 19.7% and increase by 23%, p >0.05) compared to the control samples (6.79 ±0.59 vs. 8.46 ±0.54 mm) and (9.24 ±0.48 vs. 7.51 ±0.61 mm), respectively. Similarly, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain was also resistant to wintergreen EO. The diameter of the zone of growth inhibition was (6.94 ±0.49 mm) compared to the control (7.23 ±0.49 mm) (Figure 1).

The pharmacological activities of pure compounds and crude extract from the *Gaultheria* genus were mainly focused on anti-inflammatory and analgesic properties (Liu et al., 2013). Some studies were carried out revealing the antibacterial properties of plants belonging to the *Gaultheria* genus. For example, Ma et al. (2001) screened for the anti-bacterial activity of extracts derived from *Gaultheria leucocarpa* var. *yunnanensis* (Franch.) T.Z.Hsu & R.C.Fang. Anti-bacterial tests with extracts derived from water, acetic ester, and n-butanol exhibited that 3 extracts from 22 samples possessed anti-*Staphylococcus aureus* activity, and the extracts from roots and stems showed the same result. Two extracts inhibited the growth of *Escherichia coli* and *Pseudomonas aeruginosa* in a dose-dependent manner. These results suggested that not only essential oil but other ingredients from *G. leucocarpa* var. *yunnanensis* have anti-bacterial activity. Anti-fungal tests of the same extracts didn't indicate remarkable action (Ma et al., 2001).

The chemical constituents and biological activities of essential oil and crude methanol extract of *Artemisia vulgaris* L. and *Gaultheria fragrantissima* Wall. were identified by Pandey et al. (2017). Gas chromatography-mass spectroscopy analysis revealed that leaves of *G. fragrantissima* contained methyl salicylate (95%) and asarone (4.64%). Furthermore, methanol extracts from leaves of *A. vulgaris* and *G. fragrantissima* were found rich in total flavonoids and phenolic content. HPLC analysis revealed the presence of rutin as a major flavonoid compound in the leaves of *G. fragrantissima*. Further, methanol extract of the *A. vulgaris* and *G. fragrantissima* showed the highest antioxidant and antibacterial properties compared to the essential oil (Pandey et al., 2017). The least antibacterial activity was observed with *G. fragrantissima* oil against Gram-positive and negative pathogenic strains. Although the oil of *G. fragrantissima* showed the least activity, the methanol extract revealed comparatively higher antibacterial activities with a zone of inhibition in the range of 11–14 mm (Pandey et al., 2017).

Studies on the antimicrobial activity of essential oil from the leaves of *Gaultheria yunnanensis* (Franch.) Rehder was carried out by Wang et al. (2005). The essential oil from the leaves of *G. yunnanens* presented similar antibacterial effects as methyl salicylate. It has antibacterial activity against *E. coli* and *S. aureus*, but the essential oil is superior to methyl salicylate, and the lowest antimicrobial concentration is 0.3125 and 5%, respectively (Wang et al., 2005). Klūga et al. (2021) have detected the antimicrobial activity of the essential oils on pathogenic microorganisms found in freshwater fish. Essential oil of *Gaultheria procumbens* showed strong antibacterial activity against *Yersinia* spp. and *Vacococcus* spp. (6.25 μL.mL^{-1}) (Klūga et al., 2021). Ojha et al. (2022) carried out a comparative analysis of *Gaultheria fragrantissima* essential oils based on geographical location, distillation time, and varying distillation conditions. Three samples showed notable antibacterial activity against *Staphylococcus epidermidis*, with a minimum inhibitory concentration (MIC) value of 156.3 μg.mL^{-1}. Similarly, one sample showed effectiveness against *Aspergillus niger* (MIC = 78.1 μg.mL^{-1}) (Ojha et al., 2022). Essential oil of *G. procumbens* exhibited the strongest antimicrobial activity against one strain of *S. aureus* with the disc diffusion test (7.33 mm) in the study of Kačániová et al. (2020). A MIC value of 12.50 μL.mL^{-1} was found for *S. aureus*, *S. capitis*, and one strain of *S. haemoliticus*, determined with the broth microdilution method (Kačániová et al., 2020). Higher activity of essential oil of *G. procumbens* against Gram-negative bacteria (*Acinetobacter baumanii*, *Aeromonas sobria*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, and *Serratia marcescens*) in comparison to Gram-positive microorganisms (*Staphylococcus aureus* and *Enterococcus faecalis*) was observed in the study of Hammer et al. (1999). Higher resistance of Gram-positive bacteria against essential oil of *G. procumbens* was shown by Nikolić et al. (2013).

The essential oils from the root, stem, and leaf of *Gaultheria longibracteolata* R.C.Fang were investigated by anti-bacterial assays by Luo et al. (2021). Oil extracts from *G. longibracteolata* root, stem, and leaf, as well as methyl salicylate, were tested on four bacterial species: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*,

E. coli, and *S. aureus*. According to the literature (Jia and Li, 2005), *G. yunanensis* can be used to treat skin infections. However, according to Luo et al. (2021), the oil extracts did not inhibit the growth of *P. aeruginosa*. In contrast, the oil did show dose-dependent inhibitory activity against the other three bacteria species. The 80% dilution of leaf oil extract showed the strongest inhibitory activity against *K. pneumoniae*; the other extracts also showed inhibitory effects against *K. pneumoniae*, which may explain the use of branches and roots in traditional medicine. For *E. coli*, the 80% dilutions of leaf oil, stem oil, and methyl salicylate provided the strongest inhibitory activities. The *G. yunannensis* was also used traditionally for treating skin infections (Jia and Li, 2005). All oil samples showed relatively weak antibacterial activities against *S. aureus* in the study of Luo et al. (2021). Additionally, methyl salicylate is the most abundant bioactive component of *G. longibracteolata* oil in this study. Nevertheless, the result showed that pure methyl salicylate had relatively weaker activity than those of mixed oil at the same dosage, which raised the possibility that other components also contributed to the observed activity (Luo et al., 2021). Neither essential oil nor methanol extract of *G. longibracteolata* showed any antibacterial activity against *P. aeruginosa*. The study on *G. longibracteolata* essential oil showed a similar result on *E. coli* and *S. aureus*, which could be because the oil of the two species shared the same main component, methyl salicylate (Luo et al., 2021).

Gaultherin, 2-[(6-O-beta-D-Xylopyranosyl-beta-D-glucopyranosyl)oxy] benzoic acid methyl ester, a natural salicylate derivative extracted from *Gaultheria yunnanensis*, has been shown to have analgesic and anti-inflammatory effects and lack gastric ulcerogenic effect compared to aspirin in the primary study of Zhang et al. (2007). Earlier, these researchers investigated the mechanism of action of gaultherin, which may rely on its active metabolite, and the mechanism responsible for the non-ulcerogenic property. The results showed that gaultherin (200 $mg.kg^{-1}$) significantly inhibited the abdominal contractions in the acetic acid-induced writhing test in mice. The anti-inflammatory effect of gaultherin was demonstrated in the croton oil-induced ear edema model in mice. The results showed that gaultherin and equimolar dose of aspirin produced comparable inhibitory effects. The study of the metabolism characteristics of gaultherin in mice and rats indicated that gaultherin could be metabolically converted to salicylate, which produced pharmacological effects and provided effective concentrations for an extended period (Zhang et al., 2006). Also, the extract and salicylate-rich fraction obtained from *Gaultheria trichophylla* Royle showed significant analgesic, anti-inflammatory, and antipyretic effects *in vivo*, *in vitro*, and in silico assays that support its use in traditional medicine (Alam et al., 2023).

Conclusions

In the current study, we assessed *in vitro* antimicrobial profiling of commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens* (Natural essential oil – Wintergreen oil Bamer®) against Gram-positive and Gram-negative strains. This study demonstrated that commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens* (Natural essential oil – Wintergreen oil Bamer®) possesses potential antimicrobial properties against Gram-positive bacteria, such as *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®29212™) strains. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* strains were resistant to commercial wintergreen essential oil derived from leaves of *Gaultheria procumbens*. This study showed that commercial wintergreen essential oil could be a potential preparation as a source of natural antibacterial properties. Future pharmacological studies and development in other areas are thus warranted.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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Research Article OPEN ACCESS

Variability of morphological parameters of *Diospyros lotus* L. flowers

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The morphological variability of the male and female flowers of 23 date plum (*Diospyros lotus* L.) genotypes found together at date plum collection in Arboretum Mlynany (Slovak Republic) was investigated. The collection consists of seed-propagated genotypes, which were established in 1970 with introduced seeds from different countries. Results showed that male and female flowers of *D. lotus* genotypes differed from each other in terms of shape and size. According to the morphological analysis, several features were found variable both within and between different plants. Moreover, significant differences in the colour of their corolla were detected. Female and male flowers of *D. lotus* genotypes were grouped according to the absolute parameters of flowers, and five (male flowers) and three (female flowers) clusters were identified. The degree of affinity between species was assessed using values of Euclidean distance. Using the principal component analysis, female and male flowers were separated into groups with similar morphological parameters. Our investigation has extended the understanding of the morphological features of *D. lotus* flowers as compared to the previously published data, which were limited only to a short description.

Keywords: date plum, flowers, morphometrical variability

Introduction

Discussing the negative impact of alien invasive species on natural biological diversity, we ask the primary question: what features of the introduced species contribute to its invasion? One of the hypotheses explaining the successful invasion of plants into the natural plant communities is one of high genetic variability of the initially introduced populations (Allard, 1965; Lavergne and Molofsky, 2007). The individual variation is a key determinant of the spread rate of species through landscapes (Sakai et al., 2001; Suda and Pyšek, 2010; Jongejans et al., 2011; Parker et al., 2013; Feulner et al., 2017). This hypothesis is supported by the example of a number of plant species including *Amaranthus* albus L., *Ribes aureum* Pursh, *Acer negundo* L., *Impatiens glandulifera* Royle,

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I. parviflora DC, *Bidens frondosa* L., *Conyza canadensis* L. In the present study, we test this explanation on the example of *Diospyros lotus* L., which is currently considered a potentially invasive species that invade natural plant communities of Southern Europe and Asia Minor.

D. lotus (Ebenaceae) is a deciduous tree, which grows naturally in China and other Asian countries (Yonemori et al., 2000; Bellini and Giordani, 2005). In cultural form, this species is spread in Korea, Pakistan, Afghanistan, Turkey, Albania, Spain, France, and Poland (Yonemori et al., 1998; Ayaz and Kadioglu, 1999; Ercisli and Akbulut, 2009).

D. lotus is a relict of the tertiary flora and one of the most ancient of modern deciduous moderately thermophilic species, which arose at the later stages of the evolution of the genus *Diospyros*. Under natural conditions, the height of this tree is up to 15 m (Ayaz and Kadioglu, 1999; Ercisli and Akbulut, 2009). It has been cultivated in several countries for its edible fruits and has also been used as a rootstock source for *D. kaki* L. (Onur, 1995; Ercisli and Akbulut, 2009).

D. lotus which is produced for its fruits has become invasive in Black Sea forests, adapting to the Mediterranean forest ecosystem, and damage to natural lands. This plant species has been acquired as invasive and today, it is devastating natural forest ecosystems and biological diversity (Necmi, 2011). *D. lotus* is been considered in Mediterranean Regions as one of the 20 transformers that are declared emerging invaders. This group of future major invaders, their "transformer behaviour" appears as early as the first establishment in primary biotopes and the further spreading of the species is then predictable (Brunel and Tison, 2005). There is evidence that *D. lotus* occurs on road embankments in Hyrcanian forests (Parsakhoo et al., 2009).

In Central Europe, the species has just begun naturalization. *Diospyros lotus* was found outside the Buda Arboretum of the Corvinus University of Budapest and also in many urban spaces in Hungary (Sütöri-Diószegi and Schmidt, 2010). However, in Switzerland, *Diospyros lotus* was included in the group of unsuccessful exotic plant species (Weber and Gut, 2004). The settlement of this species needs to be monitored, especially in connection with climate warming. This species may become the same "escaping from cultivation", as some ornamental plants (Pergl et al., 2016).

One of the real ways of detecting the adaptive potential of any species under introduction is by researching its variability. It is well known that the intraspecific diversity of the plant greatly increases during long-term breeding (Kohno and Kuznetsov, 2005) whereas in natural optimal conditions of growth, its variability is much less. The variability of features observed in species, which are grown up in different habitats, is caused by differences in their ecological conditions (Mamaev, 1972). Such data give the possibility to find out the adaptive mechanisms, which preserve the viability of species in certain environmental conditions (Mikovski et al., 2021).

To test the hypothesis of the high variability of successfully colonizing species, it is necessary to estimate the variability of their phenetic and genetic traits. We have previously described the degree of the variability of *Diospyros lotus* fruits (Grygorieva et al., 2009c). In this study, we estimate the variability of the morphometric parameters of the male and female flowers of this species in the introduction population of Slovakia.

Material and methodology

Locating trees and data collection

The studies were conducted in 2017 in the Arboretum Mlynany (Slovakia). The objects of the investigation were male (DLm-01–13) and female (DLf-01–10) plants of 23 *Diospyros lotus* genotypes grown from seeds, which have been brought from different habitats (South Korea, China, and Japan). The plants were planted at two years of age in 1970. They are well adapted to the climatic and soil conditions.

Morphometrical characters

13 male (staminate) and 10 female (pistillate) genotypes were investigated. From each tree, we sampled 30 flowers during the blossoming. Generally, we have analyzed 390 female and 300 male flowers. The morphological variability was defined by morphometric investigations of the following characteristics (Figure 1): length of corolla (CL), width of corolla (CW), mm; length of petals (PL), width of petals (PW), mm; number of petals (PN), pcs; length of sepals (SL), width of sepals (SW), mm; number of sepals (SN), pcs; amount of stamens of epipetalous whorl (NS1), amount of stamens of episepalous whorl (NS2), pcs; length of stamens of epipetalous whorl (LS1), length of stamens of episepalous whorl (LS2), mm: amount of staminodes (NS), pcs; length of staminodes (LS), mm;

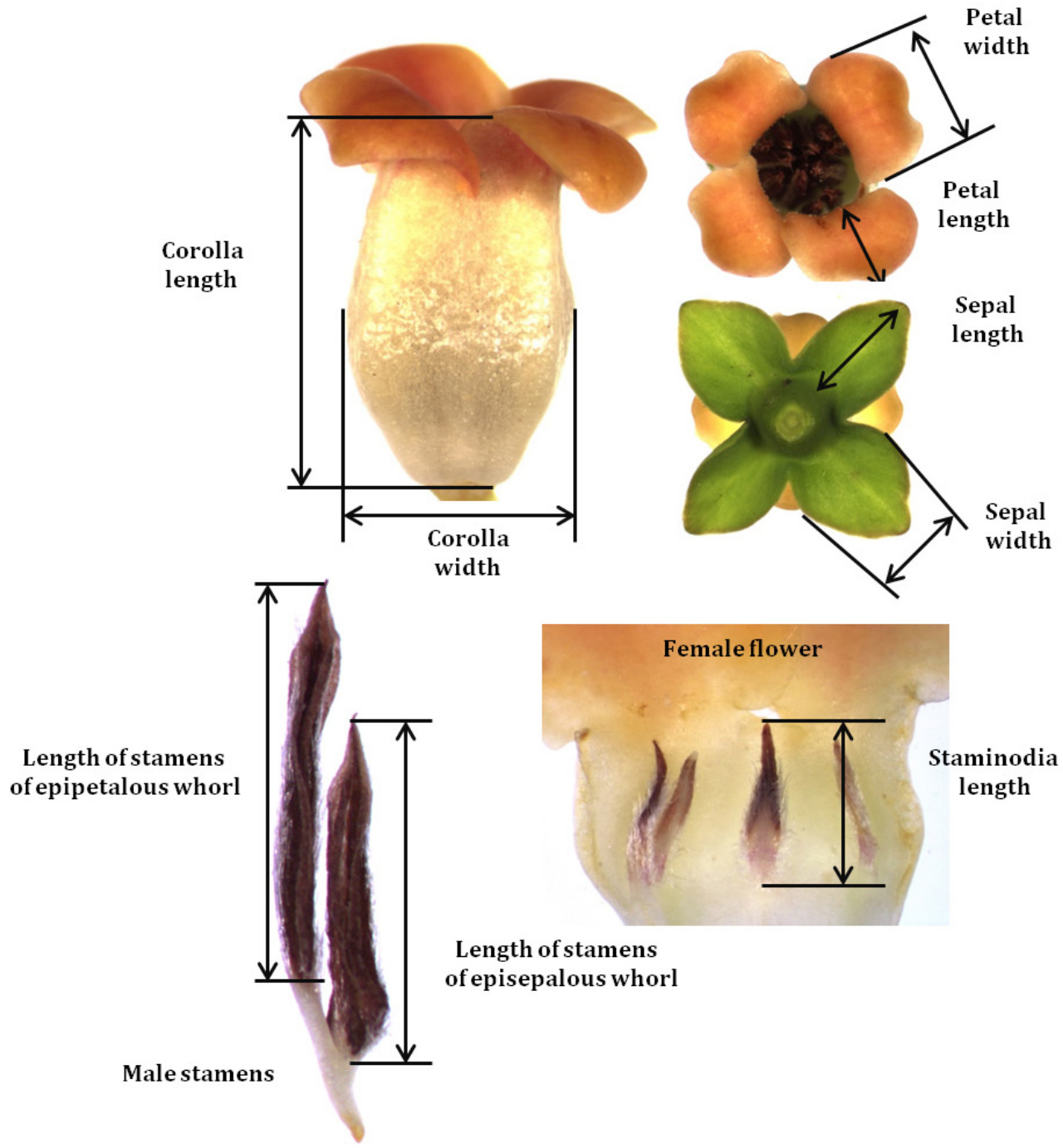

Figure 1 Flowers of *Diospyros lotus* L. showing the floral characteristics measured

shape index (CL/CW). The measurements were carried out on photo macrographs. The image analysis was carried out using the software AxioVs40 V 4.8.2.0.

Statistical analysis

Basic statistical analyses were performed using PAST 2.17 to calculate numerical characteristics such as sample size (n), range (minimum and maximum), mean value, standard deviation (SD), and coefficient of variation (CV) of a trait; hierarchical cluster analyses of similarity between genotypes were computed based on the Euclidean distance similarity index. The level of variability was determined by Stehlíková (1998). Principal component analysis (PCA) was performed to evaluate relationships among variables and any possible genotype groupings based on similar properties by using an XLSTAT procedure (XLSTAT 7.5, Addinsoft, USA).

Results and discussion

Population growth plants and climate change pose a serious threat to many ecosystems. Therefore, identifying new or rare species is becoming increasingly important. The geographical assignment of species is also of great relevance to broad biodiversity conservation projects. Morphometrics, the study of shapes of leaves, petals, and whole plants, has been applied to plants for many years and is of great importance to science, as it can help to distinguish between different species, to measure plant health, and even to model climate change (Cope et al., 2012).

The study of flower morphology is of particular interest because flowers are the most complex structures of the plant organism. In addition, the identification of morphometric parameters of flowers is necessary for the reliable detection of interpopulation changes (Rozov and Deineko, 2018). It is also very important studying from the interaction of a plant with a pollinator to plasticity and response to biotic and abiotic stresses (Jolles, 2015; Carleial et al., 2017; Sauquet et al., 2017; Spencer and Kim, 2018; Mikovski et al., 2021).

Descriptions of the morphology of the flowers of *D. lotus* are presented in Hiern (1873), Shu-Kang et al. (1996), and Sponberg (1977) works. But they are confined only by a brief description: female flowers subsessile, solitary on short, pubescent, bracteate pedicels 2–3 mm long; calyces green, 7–8 mm long, densely rufous-pubescent on the adaxial surface of the calyx tube, accrescent in fruit and sometimes persistent on the branchlets, the 4 or 5 lobes foliaceous, 6–7 mm long at anthesis; corolla reddish-brown, broadly urceolate, ca. 5 mm long, the lobes 2–3 mm long, more or less recurved; staminodes 8, curved over the surface of the ovary, pubescent with long, silvery hairs. Male flowers produced on the current year's growth, held ± nodding beneath the leaves. Staminate flowers are 6–7 mm. Long at anthesis, short-pedicellate, 3–5 together (or fewer through abortion) in rufous-pubescent, short-pedunculate, the bracts caducous; calyces green, finely pubescent, the 4 (or 5) deltoid lobes 1.5–2 mm long; corolla 4.5–6 mm long, white, ± campanulate and weakly 4- (5-) ribbed, the lobes pinkish or yellowish, recurved, ca. 2 mm long; stamens 16, rarely fewer, epipetalcus in 2 whorls, the largest stamens ca. 4 mm long; gynoecia abortive or rudimentary (Sponberg, 1977).

The investigation of *D. lotus* under introduction conditions shows evident polymorphism, which affects (nearly) all morphological features of this species (Grygorieva et al., 2009a, 2009b). We can find the varieties of all features which fluctuate in highly visible limits. In this case, not only the special features of the vegetative sphere of the plant are exposed to changeability, and also the constant features of generative organs (flowers), which are usually considered the most conservative.

Figure 2 The variability of size and shape of calyx
1 – male flowers (a – from one plant; b – from different studied plants); 2 – female flowers

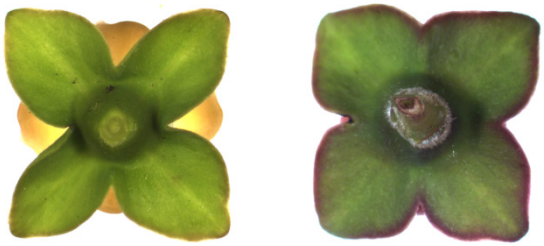

Figure 3 The colour of the edges of sepals of female flowers

As a result of our investigation, it was ascertained that flowers of *D. lotus* are strongly differentiated in shape, size, and colour of the corolla of both the male and female flowers (Figure 1–5).

According to our observations, the sepals of male and also female flowers are quite different in shape. There is a highly variable form of sepals of the same plant (Figure 2). Female flowers have bigger sepals than male flowers. Sepals of female flowers unlike male flowers are much larger than the corolla. The inner side of the sepal of both female and male flowers is always extremely densely pubescent. The hairs are colourless and pointed.

Usually, sepals are green and have different shapes. During the investigation, we found one plant, which had different coloured edges of sepals (Figure 3). Sepals of female flowers are longer and wider than the sepals of male flowers.

The corolla of female flowers is longer and wider than the corolla of male flowers. The corolla length of female and male flowers in our analyses was determined

Figure 5 The variability of colour of petals of corolla
1 – female flowers; 2 – male flowers

in the range from 3.29 to 10.02 mm and from 2.66 to 7.15 mm, respectively. Petals of female and male flowers are thick, and waxy, 2.37–7.46 mm and 1.07–4.92 mm length, 2.16–7.40 mm and 1.89–4.92 mm width, respectively (Table 1). They are usually 4, but there are flowers with 2 (male flowers) and 7 (female flowers)

Figure 4 The number of petals of corolla
1 – female flowers; 2 – male flowers

Table 1 Morphological characteristics of features of flowers of *Diospyros lotus* L.

Morphological parameters	min	max	mean	CV%	min	max	mean	CV%
	male flowers				female flowers			
Length of corolla (mm)	2.66	7.15	4.95	13.76	3.29	10.02	6.40	18.52
Width of corolla (mm)	2.10	6.77	4.53	11.93	4.0	8.09	5.28	12.48
Petals length (mm)	1.07	4.92	3.06	21.64	2.37	7.46	4.76	16.39
Petals width (mm)	1.89	4.92	3.40	13.79	2.16	7.40	4.48	23.61
Petals number	2.0	6.0	4.14	12.08	3.0	7.0	4.14	12.36
Sepals length (mm)	1.03	5.10	2.27	29.53	2.57	8.52	5.96	22.90
Sepals width (mm)	1.49	4.90	2.38	22.54	1.94	8.34	5.42	21.07
Number of sepals	3.0	6.0	4.13	11.78	3.0	6.0	4.11	10.61
Amount stamens of epipetalous whorl	7.0	9.0	7.97	4.21	–	–	–	–
Amount stamens of episepalous whorl	7.0	9.0	7.96	4.16	–	–	–	–
Length of stamens of the epipetalous whorl (mm)	2.12	4.72	3.45	8.92	–	–	–	–
Length of stamens of the episepalous whorl (mm)	2.09	3.84	2.95	11.69	–	–	–	–
Staminodes amount	–	–	–	–	7.0	10.0	7.99	4.53
Staminodes length	–	–	–	–	1.48	3.68	2.29	17.20

min, max – minimal and maximal measured values; mean – arithmetic mean; CV – coefficient of variation, %

petals (Figure 4). The length and width of *Diospyros lotus* of flowers of the present study were in the range from 2.57 to 8.52 mm and from 1.94 to 8.34 mm (female flowers), from 1.03 to 5.10 mm and from 1.49 to 4.90 mm (male flowers), respectively.

The stamens are often different in size. The length of stamens of the epipetalous whorl is from 2.12 to 4.72 mm and the episepalous whorl is from 2.09 to 3.84 mm (Table 1). Stamens of female flowers are reduced (staminodes). They are from 7 to 10. The staminodes are with short staminal threads, which are attached to the base of the corolla just two per one petal 1.48 to 3.68 mm long.

According to the morphological analysis, a lot of features vary both within a single plant (Figures 6 and 7) and between different plants (Table 1). This is confirmed by other studies on the same plant species (Armbruster, 1991; Williams and Conner, 2001; Ishii and Morinaga, 2005; Zhao et al., 2010), which allows us to estimate the individual and interpopulation variability of the species (Arteaga et al., 2015).

In conducting of statistical data the coefficients of variability of each morphological feature of generative organs of *D. lotus* were calculated. As a result, the fact of the existence of a certain degree of variability was confirmed.

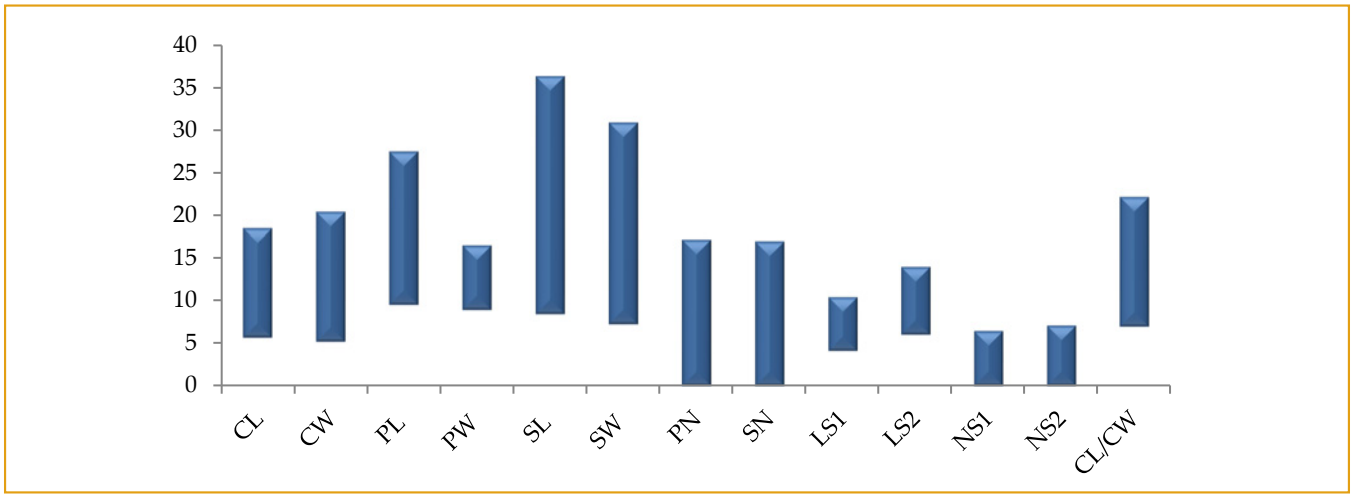

Figure 6 Level of the variability of morphometric parameters of male flowers of *Diospyros lotus* L. , %

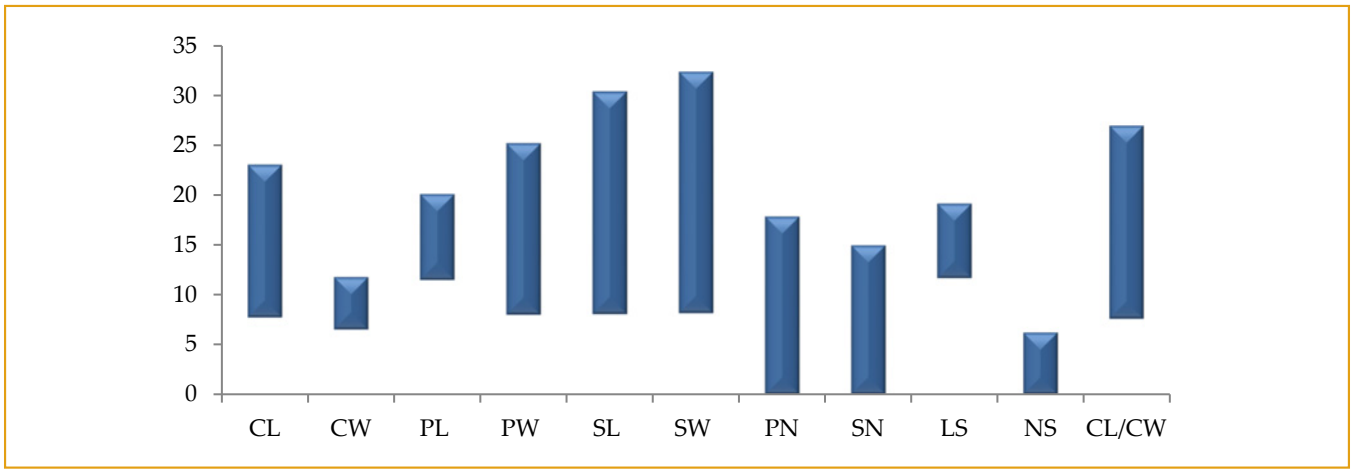

Figure 7 Level of the variability of morphometric parameters of female flowers of *Diospyros lotus* L., %

The range of variation of morphological features of male flowers is 4.11–36.31% (Figure 6). The high level of variability is determined for the length and width of sepals, length of petals with coefficients of variation 36.31, 30.88, and 27.45%, respectively. The least variable parameters are the length of stamens of epipetalous whorl and episepalous whorl (6.31 and 7.96%), the number of stamens of epipetalous whorl and episepalous whorl (6.45 and 7.10%), respectively. The other morphological features have an average degree of variability. Such a result is not surprising, because it is well known that the internal organs of the flower such as pistils and stamens are the least variable, so the classification of plant life is based on these features.

The range of variation of morphological features of female flowers is 8.0–32.38% (Figure 7). A high level of variability was found for the width and length of the sepals, shape index of the corolla, width of petals, and length of corolla width value of the coefficient of variation 32.38, 30.38, 26.94, 25.18 and 23.02%, respectively. These data are consistent with the results of other researchers who have compared the variability of perianth and internal flower structures (Herrera et al., 2008).

An acceptable solution for principal component analysis was reached when two dimensions of the model were found to be significant and explained 54.68% of the total variance of the original variables set (Table 2). The first component (PC1), accounting

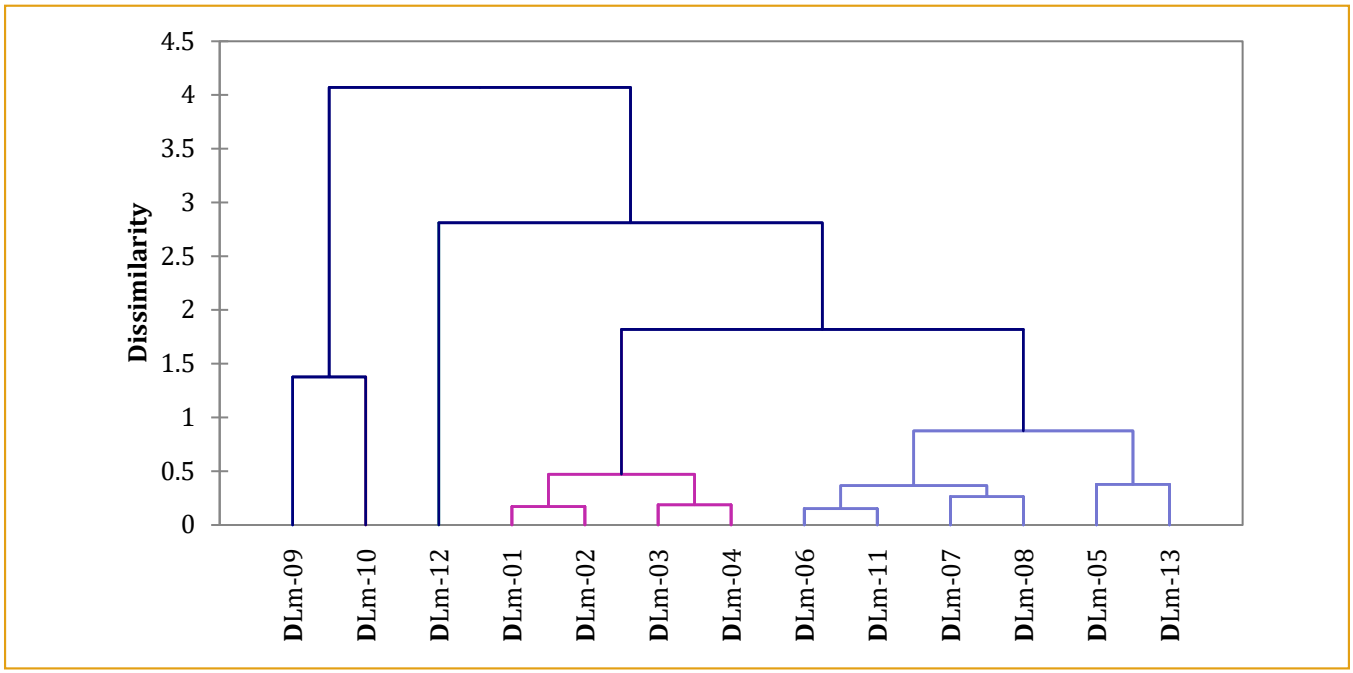

Figure 8 Cluster dendrogram based on morphometrics parameters of *Diospyros lotus* male flowers genotypes

Table 2 Eigenvalues, the proportion of total variability, and correlation between the original variables and the first four principal components for *Diospyros lotus* male flowers genotypes

Variable	Male flowers		Female flowers	
	PC1	PC2	PC1	PC2
CL	0.92	-0.15	0.77	-0.47
CW	0.68	-0.16	-0.17	-0.06
PL	-0.35	-0.12	-0.42	-0.44
PW	-0.19	0.41	0.88	0.15
SL	0.76	0.24	0.65	0.55
SW	0.63	0.57	0.74	0.12
PN	0.56	-0.38	-0.10	0.06
SN	0.55	-0.38	-0.39	-0.72
LS1	-0.53	-0.60	–	–
LS2	-0.75	-0.34	–	–
LS	–	–	-0.47	0.68
NS1	-0.45	0.76	–	–
NS2	-0.02	0.86	–	–
NS	–	–	-0.07	0.74
CL/CW	0.43	-0.07	0.81	-0.40
Eigenvalue	4.35	2.75	3.62	2.51
Variability (%)	33.47	21.21	32.93	22.82
Cumulative (%)	33.47	54.68	32.93	55.74

for 33.47% of the total variance, is dominated by male flower characters, namely length of corolla (CL) and length of sepals (SL). In the second component (PC2) dominated the number of stamens of the epipetalous whorl (NS1), the amount of stamens of the episepalous whorl (NS2) were the main contributors and explained 21.22% of the total variance.

The PCA used in our work in researching female flower genotypes showed that more than 73.74% of the variability observed was explained by the two components (Table 2). Positive values for PC1, accounting for 32.93% of the total variance, correspond to the genotypes with a higher length of corolla (CL), petals width (PW), and shape index (CL/CW). In the second component (PC2) dominated of the length of staminodes (LS) and amount of staminodes (NS) were the main contributors and explained 22.82% of the total variance.

The cluster and principal component analysis on the morphological characters have been carried out earlier for studying the variability of some other plant species (Vijayan et al., 2006; Al-Ruqaie et al., 2016; Krishnapillai and Wijeratnam, 2016; Yilmaz et al., 2017) and he may also be used as useful tools for screening the accessions (Bellini et al., 2003; Ercisli, 2004; Yildirim et al., 2010; Horčinová Sedláčková et al., 2021) and for effective management of genetic resources that is the first step toward any domestication process (Metougui et al., 2017).

By applying cluster analysis to variables retained by the PCA dendrogram in Figure 8, five groups of genotypes of male flowers were differentiated at a dissimilarity level of 1.0. Cluster I consisted of four genotypes (DLm-01, DLm-02, DLm-03, DLm-04). Cluster II included the largest number of genotypes (DLm-05, DLm-06, DLm-07, DLm-08, DLm-11, and DLm-13). Each cluster III, IV, and V included only one genotype.

The morphological similarity among 10 genotypes based on morphometrical characteristics of female flowers was examined by cluster analysis too (Figure 9). Dendrogram had shown three main clusters. Three of the 10 genotypes were included in Cluster I, 3 genotypes in Cluster II, and 4 genotypes in Cluster III.

Principal component and cluster analysis discriminated the sampled genotypes into five clusters (Figure 10) using the first two principal components and accounted for about 54.68% of the total variability among the *Diospyros lotus* genotypes, based on male flower traits, respectively.

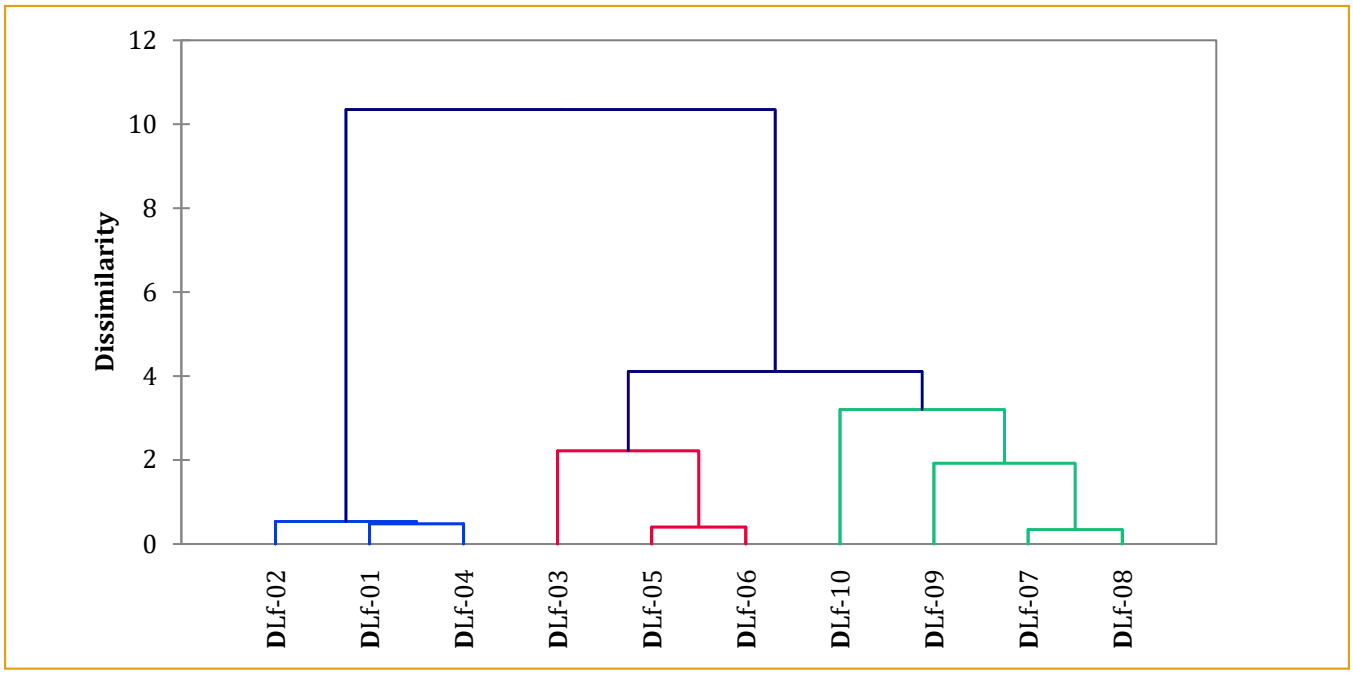

Figure 9 Cluster dendrogram based on morphometrics parameters of *Diospyros lotus* female flowers genotypes

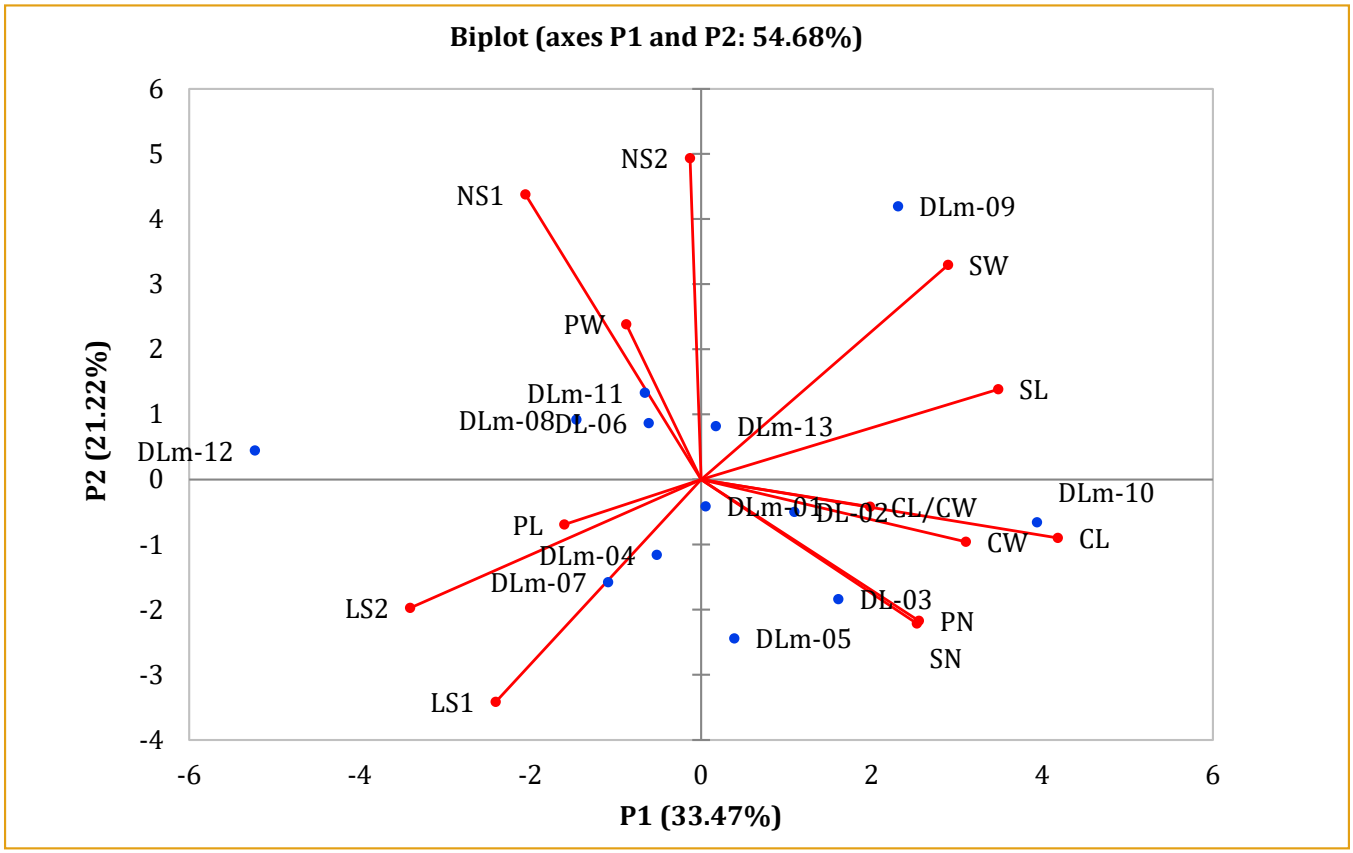

Figure 10 Biplot based on principal components analysis (PCA) for male flowers characteristics in *Diospyros lotus* L. genotypes

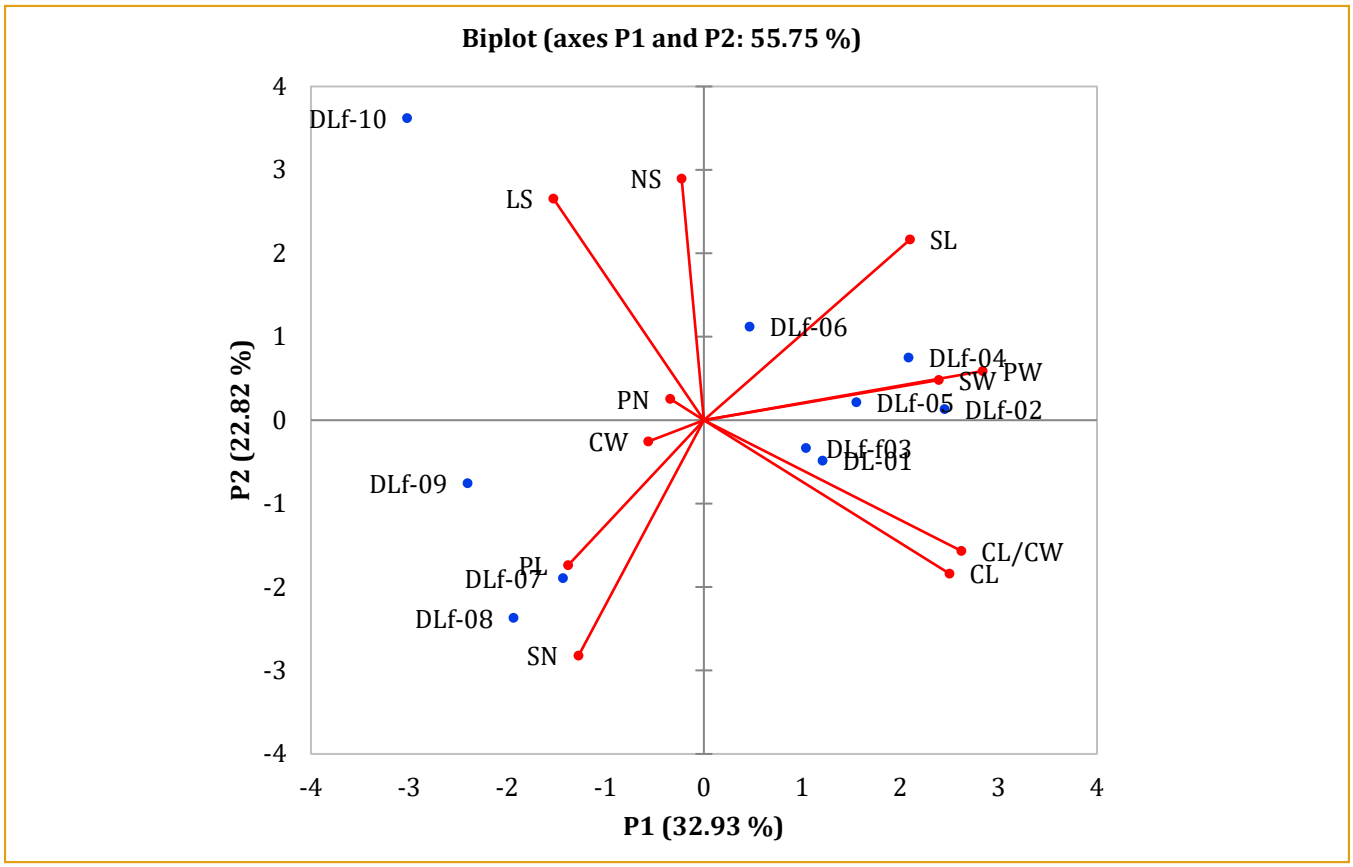

Figure 11 Biplot based on principal components analysis (PCA) for female flowers characteristics in *Diospyros lotus* L. genotypes

Regarding the flowers of female plants principal component and cluster analysis discriminated the sampled genotypes in three clusters (Figure 11) using the first two principal components and accounted for about 55.75% of the total variability among the *Diospyros lotus* genotypes, based on female flowers traits, respectively.

Conclusions

As we mentioned earlier, only a short description of the flower morphology of *Diospyros lotus* has already been studied. With these data, we contribute to the knowledge about this species. As a result of our research was ascertained that flowers of *Diospyros lotus* are very different in shape, size, and colour of both male and female flowers. The coefficient of variation of morphological features of female flowers ranges from 8.0 to 32.38% and male flowers – from 4.11 to 36.31%. The fluctuations of the values of investigated features characterize the plasticity of this species; which can be considered as some kind of adaptive reaction to changing ecological conditions. The high values of the coefficient of variability among the features of the generative sphere of *Diospyros lotus* can be explained by optimal conditions for this species. In the future, obtained data on the variability can be used as a theoretical and practical base for a whole series of problems in general biology. Thus, the introduction population of *Diospyros lotus* in the Mlynany Arboretum (Slovakia) is represented by numerous varieties. Its phenetic and genetic variability is quite large. On the one hand, this determines the stability of the species in the culture, and on the other, it can, because of the cross-pollination, contribute to the emergence of new genotypes that are more resistant to the soil and climatic conditions of Central Europe. We do not exclude the possibility of further expansion of the invasive distribution range of *Diospyros lotus*. Our data do not contradict the hypothesis of a relatively high level of genotype variation of alien species becoming invasive.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statements

This article does not contain any studies that would require an ethical statement.

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