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Phenological stages of development of *Cornus* L. S. str. species (Cornaceae) according to BBCH scale

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Under conditions of pronounced dynamic climate change, the scope of phenological data is constantly expanding and increasingly extends beyond local regions. For the international integration of phenological research, a standardized description of the stages of plant development and their identical coding is used – the BBCH scale (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie). Phenophases of seasonal development, according to BBCH, are already unified for many different in origin, nature of ecology, and economic use of plants of common and little-known species and crops, including fruit. According to the BBCH scale, we described the seasonal stages of development of 23 cultivars of *Cornus mas* L. selection gene pool of the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine, genotypes of *C. officinalis* Siebold & Zucc., nine genotypes of *C. sessilis* Torr. ex Durand, as well as three genotypes of the artificial hybrid from crossing *C. officinalis* × *C. mas.* Phenological monitoring was carried out in NBG (Ukraine) two or three times a week for three seasons (2018–2020) from the beginning of vegetation (bud development) to November and the beginning of winter dormancy. In the studied species of the subgenus *Cornus*, as in some other stone fruit plants, there are eight of the ten main stages of seasonal development. In all studied species and cultivars of dogwood at the beginning of the growing season generative buds in their development significantly ahead of vegetative and plants begin seasonal development from the development of inflorescences and flowering (principal growth stage 5 and principal growth stage 6, respectively). Different species and varieties differ in the calendar dates of onset and duration of certain major stages of development. The obtained data are important for further studies of adaptive capabilities of *Cornus* species and cultivars in different climatic conditions, for practical use of genetic resources of studied *Cornus* species and cultivars, as well as for the introduction of little-known *Cornus* species and their use in agricultural production, pharmacology, ornamental and landscape gardens.

Keywords: *Cornus* spp., cultivars, phenology, BBCH-scale

Introduction

Seasonal reactions of plants – bud development, flowering, fruiting, dormancy, due to the hereditary genetic basis formed in the process of evolution, on the one hand, and on the other hand, largely depend on their sensitivity to environmental factors, including photoperiod, temperature, humidity, etc. (Parmesan, 2006; Wilczek et al., 2010; Zettlemoyer and Peterson, 2021). Under conditions of pronounced dynamic climate change, the scope of phenological data is

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constantly expanding and increasingly extends beyond local regions (Walther et al., 2002; Donnelly and Yu, 2017; Saxena and Rao, 2020).

The term "phenology" was used to describe the science that studies the periodic events of nature, proposed by Charles Morren. He first used it in a public lecture at the Royal Academy of Sciences in Brussels on December 16, 1849 (Demarée and Rutishauser, 2011), and in 1853 he used it in the title of an article on the observation of plants during the winter of 1852–1853 (Morren, 1853; Demarée and Rutishauser, 2011). However, accounting for phenological observations has a very long history. Perhaps the first documented phenological data were records of sakura blossoms in Kyoto, found in many diaries and chronicles from 812 to 1864 (Aono & Kazui, 2008). In Europe, some phenological observations began to be recorded in the early twelfth century. In particular, Ch. Morren, quoting information from Gabriel Peignot, said: "En 1172, l'hiver fut si doux que les arbres se couvrirent de verdure, et tout fut en pleine vegetation" (in 1172 the winter was so mild that the trees were covered greens, and everything was growing). For nearly five centuries, data on seasonal plant development have been fragmented or private (Sparks and Menzel, 2002). For example, the Marsham family of Norfolk (UK) recorded the flowering date of nearly 20 plant species for 154 years (1736–1958), including *Anemone nemorosa* L. (Sparks and Menzel, 2002). Phenology as a science that studies and systematizes seasonal events in plant life was substantiated by Carl Linnaeus. He organized a network of observation centers in Sweden, in "Philosophia botanica" (1751) set out the purpose and methods of phenological research, as an example, cited the results of his observations of many Uppsala plants during 1748–1749. Linnaeus identified the main phenophases of plant development: «Tempus Vigendi, Germinandi, Frondescendi, Effloresccndi, Vigilandi, Fructescendi, Defoliandi indicat Clima» (1751, p. 270) and even then he was convinced that the development of plants during the season is determined by the climate, and phenological calendars should be drawn up in each region in order to see the differences between them depending on the climate: «Calendaria Florae quotannis conficienda sunt in quavis Provincia, secundum Frondescentiam, Efflorescentiam, Fructescentiam, Defoliationem, observato simul Climate, ut inde constet diversitas Regionum inter se» (1751, p. 276). Later, phenological studies gained general recognition and in the early 19th century the period of flowering and fruiting was given for each species in many floristic publications (Bieberstein, 1808a, b; Rogovich, 1855).

For nearly 200 years (1751–1950) phenology developed in many countries and was usually coordinated with meteorological services (Schnelle, 1955). In Ukraine, instrumental meteorological and phenological observations were one of the first to be started by military doctor Lerhe in Kyiv in 1771. He reported, for example, that "on April 18, 1771, the trees began to bloom, but after three days snow fell, which did a lot of harm" (Lipinsky, 2011). Somewhat later, in the late 18th – early 19th centuries, the geographer, historian, and prominent public figure M.F. Berlinsky and the founder and rector of Kharkiv University V.N. Karazin began systematic hydrometeorological observations, an integral part of which were reports on the course of seasonal development of plants (Lipinsky, 2011).

Local phenological observations were carried out mainly according to various schemes, in which only the most important phases of development for plants of a particular species were most often described. For example, in Ukraine, in the vicinity of the city of Poltava, and other regions, S. Ilyichevsky conducted long-term observations of the flowering of more than 700 plant species since 1917 (Ilyichevsky, 1924a, 1934; Illichevs'kyy 1924b; Lipshits, 1950). The author emphasized that the dates of the beginning of flowering from year to year are very changeable, and the sums of the average daily temperatures, upon reaching which flowering begins, change much less. By analogy with the basic biogenetic law of E. Haeckel, S. Ilyichevsky (in parallel with Minio), employing statistical analysis of the number of flowering species during spring and summer, found that the general course of flowering of plants in its main features repeats the history of their development: at the beginning of the growing season, flowering begins predominantly species with primitively built flowers, and in the middle of summer, mainly those species that have a specialized flower structure bloom. This generalization in botanical literature is called the Minio-Ilyichevsky law (Lipshits, 1950; Samorodov and Khalimon, 2020).

In the middle of the 20th century, it was stated that the phenological scales used in some regions were not always suitable for use in others, and the phenological phases developed for some groups of plants did not correspond to the stages of development of other groups of them. As a result, standardized phenological scales began to be developed that reflected the stages of plant development, regardless of their taxonomic status or region of observation (Cautín and Agustí, 2005).

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In Eastern Europe in 1972, at a session of the Council of Botanical Gardens of the former USSR, a commission was created to develop a method of phenological research in botanical gardens, and in 1975 the Main Botanical Garden (GBS, Moscow), edited by P. Lapin, published a unified program of phenological observations of conifers, herbaceous and woody deciduous plants (1975). The developers separately considered the development of vegetative and generative organs of plants, used alphanumeric coding of the phenophase, and indicated the diagnostic features of each of them. This technique was applied by Zaitsev (1978, 1981) for statistical processing of data from long-term phenological observations, first for herbal perennials, and later for woody plants. Both techniques are popular and widely used in Eastern Europe.

In Western Europe, almost simultaneously, Zadoks et al. (1974) were among the first to propose 10 fundamental principles for constructing an integrated scale for determining the phenophases of plants, in particular, they emphasized that the phenophases should be recorded in the order of their ontogenetic manifestation, the main stages of plant growth should be indicated by symbols (for example, coded with numbers) so that they are available on all languages, the main phenophases must have unambiguous, and the secondary – two-digit codes and the like. Based on these principles, the authors developed a standardized phenological scale for cereals, in which they identified 10 coded numbers (from 0 to 9, respectively), the main phenological phases of development. Each such phase could include 10 or fewer secondary stages of development, which they also ciphered with numbers from 0 to 9. Principles of phenophase fixation, initiated by Zadoks et al. (1974), became more and more widespread and unified, in accordance with the consideration of certain groups of plants. In 1986–1987, in the Notes (Merkblatt) of the Federal Biological Institute for Agriculture and Forestry of Germany (Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundesrepublik Deutschland), several articles were published, which described the development stages (BBA-scheme) of first weeds, and then pome and stone fruit plants and strawberries using digital coding of the phenophases (Bleiholder et al., 1986; Berning et al., 1987a, b, c). The authors identified 10 macro- and 10 micro-stages of plant development, described the characteristic visual morphological features of each of them, and emphasized that in certain species different phenophases can be observed simultaneously, or e.g. in different stone fruits, the blooming of vegetative buds occurs before, during or after flowering.

Further development of the BBA-scheme was the BBCH scale (BBCH = Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie), proposed by Bleiholder et al. (1989), and the extended BBCH scale recommended by Hack et al. (1992) for the same encryption of phenological stages of development of monocotyledonous and dicotyledonous plants. The extended BBCH scale is the development of a joint project team of scientists from three institutions: the Federal Biological Institute for Agriculture and Forestry (der Biologischen Bundesanstalt für Land- und Forstwirtschaft (BBA), the Federal Bureau of Plant Varieties (des Bundessortenamtes (BSA) and the Association of Agricultural Industries (des Industrieverbandes Agrar (IV A).

The expanded BBCH scale is growing in popularity. Now it has become international and has already been adapted for many different in origin, ecology, and economic use of plants of common and little-known species and agricultural crops, including fruit crops (Danner et al., 2019; Vaidya, 2019; Liu et al., 2021).

According to the BBCH scale, unified stages of development of pome and stone fruits, currants and strawberries (Meier et al., 1994), *Punica granatum* L. (Melgarejo et al., 1997), *Olea europaea* L. (Sanz-Cortés et al., 2002), *Rosa* sp. (Meier et al., 2009), *Actinidia deliciosa* C.F.Liang & A.R.Ferguson (Salinero et al., 2009), *Diospyros virginiana* L. (Grygorieva et al., 2010), *Theobroma* sp. (Niemenak et al., 2010), *Mangifera indica* L. (Delgado et al., 2011), *Persea americana* Mill. (Alcaraz et al., 2013), *Mespilus germanica* L. (Atay, 2013), *Ziziphus jujuba* Mill. (Hernández et al., 2015), *Morus* sp. (Sánchez-Salcedo et al., 2017), *Pseudocydonia sinensis* C.K. Schneid. (Grygorieva et al., 2018), cultivar "Pero de Cehegín" *Malus domestica* Borkh (Martínez et al., 2019), *Diospyros kaki* Thunb. (García-Carbonell et al., 2001; Guan et al., 2021), *Juglans neotropica* Diels (Ramírez and Kallarackal, 2021) etc.

The genus *Cornus* L. (Cornaceae Bercht. Ex J. Presl) – dogwood, includes about 60 species of deciduous trees and shrubs, most common in the cold and temperate zones of Europe, North America, Asia, and Africa (Xiang et al., 2006; Murrell and Poindexter, 2016). All Dogwoods are characterized by opposite solid leaves, four-membered flowers, and drupes of different shapes and colors. Their generative structures are highly variable (Murrell, 1993; Xiang et al., 2006; Feng et al., 2011). According to the results of molecular studies, four clades are distinguished in the genus, which includes nine subgenera, including the subgenus *Cornus* or *Cornus* s. str. (Xiang et al., 2006, 2008; Murrell and Poindexter, 2016).

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Some species of the subgenus *Cornus* belong to ancient fruit and medicinal plants. In the northern Black Sea region (Moldova), the fruits of *C. mas* were used even in the Neolithic period (Yanushevich, 1986). In Ukraine, *C. mas* plants were cultivated already in Kievan Rus (Klymenko, 1990). Almost 2000 years old, the fruits of *C. officinalis* are used in traditional and official Chinese medicine for liver and kidney health, as well as for the treatment of diabetes and other diseases (Huang et al., 2018). *Cornus sessilis* has an edible fruit. According to Klymenko et al. (2021), they contain almost 6.3 % sugars, 66.0 mg/% ascorbic acid, 0.6 % tannins. In the United States, the plants of this California's endemic are grown mainly for use in horticulture, garden design, and landscaping (https://calscape.org/Cornus-sessilis-(Miner's-ogwwod)?srchcr=sc5f3b85d0f226d). *Cornus* species, especially *C. mas* (subgenus *Cornus*), have been the objects of phenological observations. Usually, attention was paid to the beginning of the flowering of plants of this species. For the first time, perhaps, data on the flowering of *C. mas* (= *C. mas*cula L.) were given by Ch. Morren (1853), describing the vegetation of plants in Liege, Kingdom of Belgium during the unusually warm winter of 1852–1853. The author noted that the plants in the hedge bloomed on January 12, while before that time the beginning of flowering came on January 31, later on April 2, and most often on March 4. In the Netherlands, in the period 1901–1968 average flowering date of *C. mas* fell on March 13, and during 2001–2005 – on February 15 (van Vliet, 2014). In the climatic conditions of Ukraine, S. Klymenko studied in detail all the main phases of the seasonal development of *C. mas* in the period 1976–1987, according to the GBS method (1990). Our study aims to describe, according to the BBCH scale, the phenological stages of development of species of the genus *Cornus* s. str. (subgenus *Cornus*), in particular *C. mas* (cultivars), *C. officinalis*, and *C. sessilis*, presented in the collection of the M.M. Gryshko National Botanical Garden of NAS of Ukraine (NBG).

Material and methods

Study region

The research was carried out in the NBG (southeastern part of Kyiv, the low Pechersk slopes of the Kyiv Upland, the Zverinets tract, coordinates 50° 22' N and 30° 33' E). The climate is moderately continental, winters are mild, summers are warm. The average monthly temperature in January is -3.5 °C, in July +20.5 °C. The average annual temperature is +7.7 °C. The average annual precipitation is 640 mm; fall out throughout the year.

Research objects

Genotypes of 23 cultivars of *C. mas* of the breeding gene pool of the NBG; genotypes *C. officinalis* (26-year-old maternal plant obtained in 1993). As biennial plant from "Northwoods Wholesale Nursery" Mollala (Oregon, USA), 8- and 16-year-old seedlings of the maternal plant), nine genotypes of *C. sessilis*, grown in NBG from seeds obtained from California (Sierra Seed Supply, USA), three hybrid genotypes from the crossing of *C. officinalis* and *C. mas* (cultivar Etude).

Phenological monitoring

The observation was carried out two to three times a week for three seasons (2018-2020) from the beginning of the growing season (bud development) to November and the beginning of winter dormancy. To fix and describe the phenological stages of growth, the extended BBCH scale for mono- and dicotyledonous plants (Hack et al., 1992), the adapted BBCH scale for pome and stone fruits, currants, and strawberries (Meier et al., 1994), as well as data on the seasonal development of *C. mas* in Ukraine, obtained by S. Klymenko in 1976–1987 (1990).

Results and discussion

In the climatic conditions of Ukraine (NBG) species and cultivars of dogwood (subgenus *Cornus*) go through a full development cycle. In accordance with the BBCH scale, eight of the ten main stages of seasonal development are clearly distinguished in them (Table 1, Figure 1A–C), in particular: development of buds (Principal growth stage 0), leaves (Principal growth stage 1), shoots (Principal growth stage 3), inflorescences (Principal growth stage 5), flowering (Principal growth stage 6), fruit development (Principal growth stage 7), ripening of fruits (Principal growth stage 8) and ageing and the beginning of dormancy (Principal growth stage 9). For dogwood, like other fruit plants (Meier et al., 1994), tillering (Principal growth stage 2, observed in cereals) and the development of organs of vegetative reproduction (Principal growth stage 4, characteristic, for example, of strawberries), are not characteristic. Plants begin their growing season by developing inflorescences and flowering (the fifth and sixth Principal growth stage of the BBCH scale, respectively).

Principal growth stage 5: inflorescence development. The buds of umbellate inflorescences are formed on dicyclic, usually shortened generative shoots, which during the first year form several metameres with opposite leaves (vegetative phase of development), including one or two elongated, others are shortened. On the generative shoots in mid-June,

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Table 1 Phenological stages of development of the species and the cultivars *Cornus* L. s. st. according to the BBCH scale

Scale	Characteristics
	Principal growth stage 5: inflorescence emergence
50	the buds of the inflorescence are closed and covered by brown bracts
51	rounded generative buds swelling, closed, surrounded by slightly isolated greyish-brown bracts
53	the buds of the inflorescence burst: the bracts are slightly separated; the top of a few flower buds is visible
55	all light brown-green bracts are separated and deviated from each other; flower buds are clearly visible, pressed against each other; pedicels are short
57	pedicels elongated, flower buds separated from each other, part of the buds raised above the bracts
59	the bracts are rejected almost at an angle of 45 °; a significant part of the flower buds rised above the bracts and form umbellate inflorescences
	Principal growth stage 6: flowering
60	first flower revealed
61	beginning of flowering: opened about 10 % of flowers
65	full flowering: at least 50 % of the flowers are open, the petals of the first flowers fall off
67	withering of most flowers: fertilization occurs; petals and stamens fall off
69	end of flowering: petals and stamens of all flowers fell off
	Principal growth stage 0: bud development
00	rest period: acute elongated and thin vegetative buds closed and covered with dark brown or reddish scales
01	the beginning of swelling of the buds: buds (vegetative) noticeably elongated and enlarged, scales with a light border
03	end of swelling of the buds: the scales are separated, visible light green areas of the buds
07	the beginning of bud burst: noticeable green first leaves tips
09	the green leaves tips are about 5 mm long
	Principal growth stage 1: leaf development
10	green leaf tips 10 mm above the bud scales; first leaves separating
11	the first leaves are unfolded; others are still unfolding
15	more leaves unfolded, but not yet at full size
19	the first leaves are fully developed: have reached the typical size for the species/cultivars size
	Principal growth stage 3: shoot development
31	beginning of shoot growth: axes of developing shoots visible; about 10% of the expected length
32	shoots (annuals) reached about 20 % of the expected length
35	shoots (annuals) reached about 50 % of the expected length
39	shoots (annuals) reached about 90 % of the expected length
	Principal growth stage 7: fruit development
71	fruit set: the ovaries increase in size; the beginning of the ovary falling off
72	the ovaries are green, surrounded by a dying crown of the calyx
73	the second fall of the ovaries
75	the fruits have reached about half of the final size
77	the fruits are about 70 % of the final size
79	the fruits have reached the final size, green
	Principal growth stage 8: maturity of fruit
81	the beginning of fruit ripening: change of color from green to light green
85	fruit color is progressing; the intensity of the color increases
87	the fruits acquire a color characteristic of the species or variety; 80 % of fruits have reached technical maturity
89	almost all fruits are ripe for consumption: they have a typical taste and hardness

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Continuation of table 1

	Principal growth stage 9: senescence, beginning of dormancy
91	shoot growth completed; terminal bud developed; foliage still fully green
92	leaves begin to discolour
93	the beginning of leaf fall
95	half of the leaves discoloured or fell off
97	all the leaves fell
99	the beginning of winter dormancy

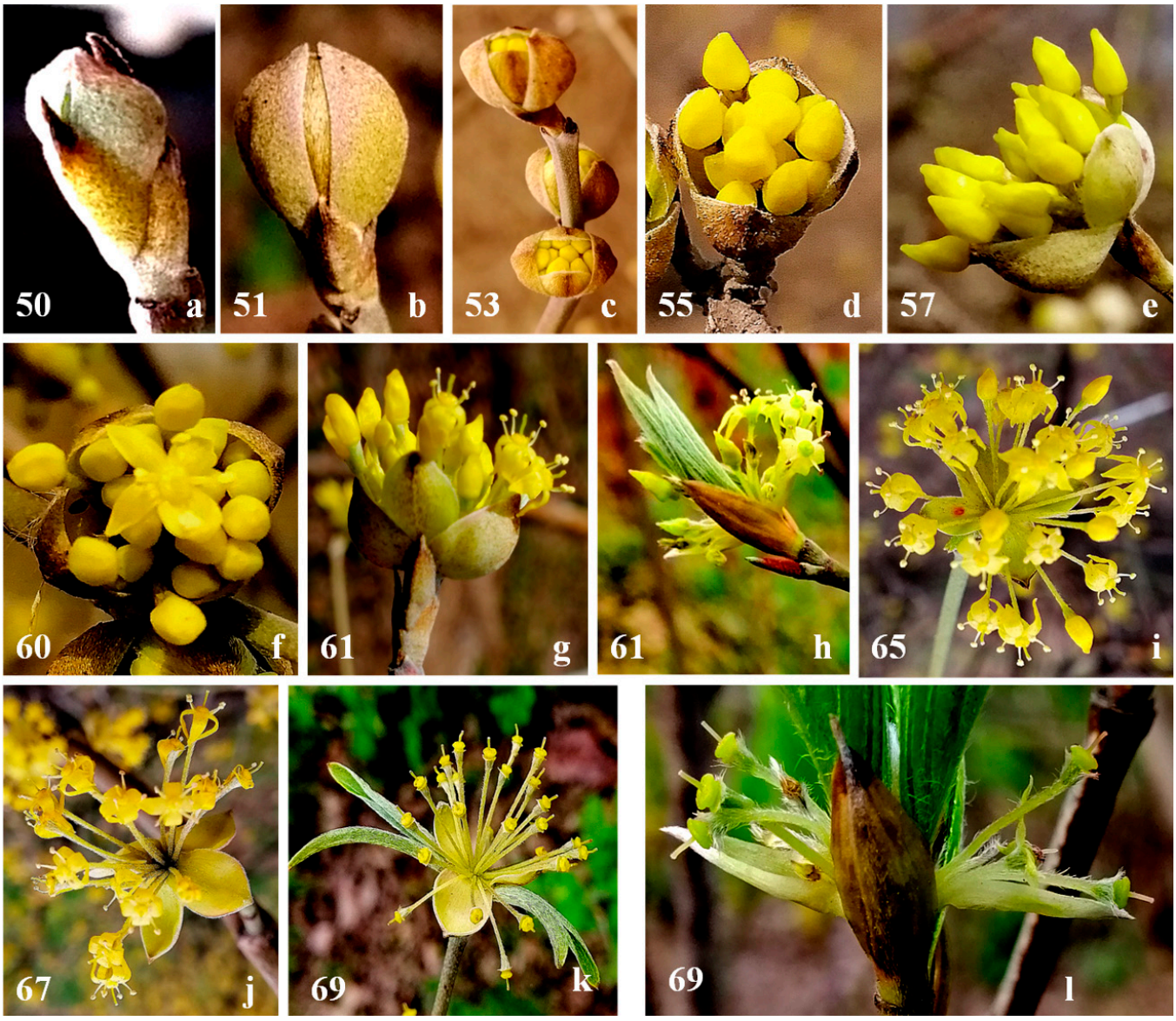

Figure 1A Phenological stages of *Cornus* species (subgenus *Cornus*)
a, ag – *C. mas*; b – *C. mas* 'Eugenia'; c – *C. mas* 'Alyosha'; d – *C. mas* 'Ekzotychnyy'; e, g, s, u, y, ae – *C. officinalis* × *C. mas* (cv. Etude); f – *C. mas* 'Nizhnyy'; h, l, m – *C. sessilis*; i, o, ab, ad, af – *C. officinalis*; j, k – *C. mas* 'Yuvileynyy Klymenko'; n, p, q, z – *C. mas* 'Kostya'; r, t – *C. mas* 'Starokyyivs'kyy'; v, ah – *C. mas* 'Elehantnyy'; w – *C. mas* 'Koralovyy'; x – *C. mas* 'Koralovyy Marka'; ac – *C. mas* 'Samofetyl'nyy'

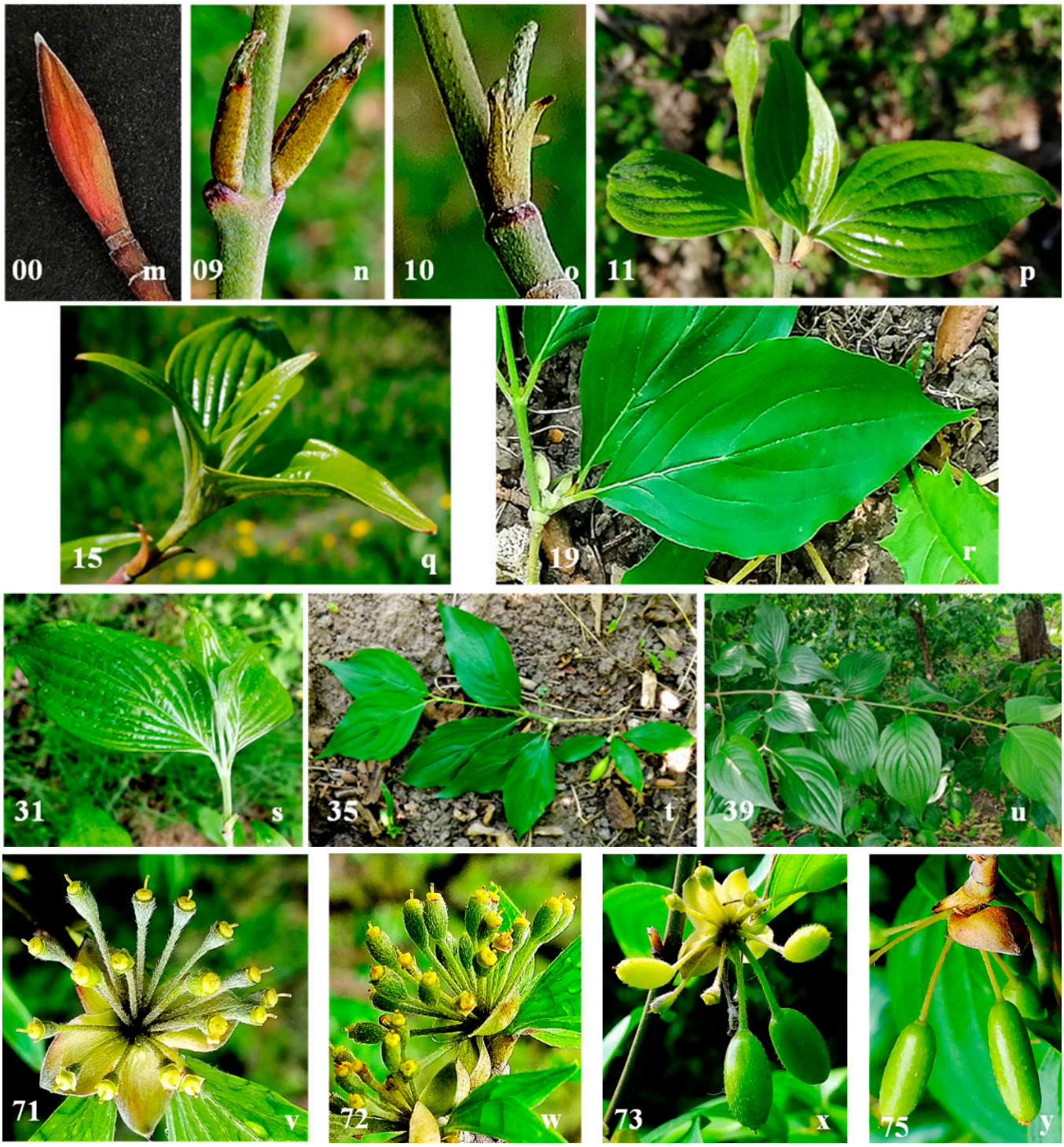

Figure 1B Phenological stages of *Cornus* species (subgenus *Cornus*)
a, ag – *C. mas*; b – *C. mas* 'Eugenia'; c – *C. mas* 'Alyosha'; d – *C. mas* 'Ekzotychnyy'; e, g, s, u, y, ae – *C. officinalis* × *C. mas* (cv. Etude); f – *C. mas* 'Nizhnyy'; h, l, m – *C. sessilis*; i, o, ab, ad, af – *C. officinalis*; j, k – *C. mas* 'Yuvileynyy Klymenko'; n, p, q, z – *C. mas* 'Kostya'; r, t – *C. mas* 'Starokyyivs'kyy'; v, ah – *C. mas* 'Elehantnyy'; w – *C. mas* 'Koralovyy'; x – *C. mas* 'Koralovyy Marka'; ac – *C. mas* 'Samofetyl'nyy'

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Figure 1C Phenological stages of *Cornus* species (subgenus *Cornus*)
a, ag – *C. mas*; b – *C. mas* 'Eugenia'; c – *C. mas* 'Alyosha'; d – *C. mas* 'Ekzotychnyy'; e, g, s, u, y, ae – *C. officinalis* × *C. mas* (cv. Etude); f – *C. mas* 'Nizhnyy'; h, l, m – *C. sessilis*; i, o, ab, ad, af – *C. officinalis*; j, k – *C. mas* 'Yuvileynyy Klymenko'; n, p, q, z – *C. mas* 'Kostya'; r, t – *C. mas* 'Starokyyivs'kyy'; v, ah – *C. mas* 'Elehantnyy'; w – *C. mas* 'Koralovyy'; x – *C. mas* 'Koralovyy Marka'; ac – *C. mas* 'Samofetyl'nyy'

the apical complex bud of the next year is already clearly visible. In the second year of development (generative phase of development), it consists of an inflorescence bud surrounded by four bracts, and two oppositely located vegetative buds, each of which has its cataphil. Almost half of the complex bud is wrapped by two buds scales fused at the base.

Principal growth stage 6: flowering. The flowers of the studied genotypes *C. sessilis*, *C. mas*, *C. officinalis*, and their hybrid (usually 15–27 in one inflorescence) are very small, actinomorphic, four-membered, bisexual; the calyx is very reduced, consists of four small teeth, the petals are yellow, lanceolate, about 4 mm long; stamens attached to the nectar disc, alternating with

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petals; gynoecium formed by two carpels, a simple column, and a cabbage stigma; the ovary is inferior, two-celled. After pollination, the petals are bent downward. The flowering of individual dogwood flowers occurs simultaneously. In umbrellas, it starts in the direction from its periphery to the center. The central flowers often remain underdeveloped and die off. Within the framework of the skeletal branch, flowering spreads from its base to the top, and on one plant, flowers are the first to open in its middle part – well lit and better protected from the north wind. Simultaneously, at least three secondary phenophases of flowering can be observed on the plant: 60, 61, and 65. The beginning and duration of the flowering period largely depend on weather conditions. Under favorable conditions, flowering lasts 10–14 days.

Principal growth stage 0: buds development. The development of vegetative buds begins when the plants reach the flowering phenophase (stage 61). Green leaf tips about 5 mm long (stage 09) can be observed during the full flowering of the plants (stage 65).

Principal growth stage 1: leaf development. Leaves begin to form with the deployment of the first pair of leaves. The unfolded the first pair of leaves (stage 11) is observed approximately four weeks after the end of flowering. These leaves reach their final development (stage 19) after a few weeks. New leaves on vegetative shoots form during a significant part of the growing season, and old ones can die off even before the beginning of November.

Principal growth stage 3: shoot development. Simultaneously with the deployment of the leaves of the first pair, the formation of vegetative shoots begins. The beginning of their growth (the axes of the shoots of stage 31 are visually noticeable), is observed after the complete deployment of the first pair of leaves. The studied species are characterized by monopodial continuation. Under optimal weather conditions, vegetative shoots at the end of their development form 4-8 elongated metameres with opposite leaves. The studied species are also characterized by the development of sylleptic shoots, which, in *C. sessilis*, for example, are formed in the leaf axils of the third node. In June, vegetative buds of the next year begin to form in the axils of mature leaves.

Principal growth stage 7: fruit development. In parallel with the growth of vegetative shoots, the development of fruits begins. In the first stages (stages 71, 72 and 73), the ovaries, surrounded by a dying crown of the calyx, increase in size and turn green. A significant part of the ovaries, and later of the fruits, albeit a smaller amount, fall off. The long process of fruit shedding is due to the extended period of flowering of the dogwood. Fruit growth is at first intense (stages 75, 77), and later – slower. Upon reaching the final size (stage 79), usually from one to two, to six, to seven fruits remain in one inflorescence.

Principal growth stage 8: fruit ripening. The onset of fruit ripening begins with a color change (stage 81). In all studied species, they first turn light green, and then gradually acquire a color characteristic of each species or cultivar. In the same inflorescence, as well as on the same tree, the fruits do not ripen simultaneously, which corresponds to a long flowering period of dogwood. The fruits are harvested for processing at the stage of technical ripeness (stage 87).

Principal growth stage 9: senescence, beginning of dormancy. The stage of ageing and the beginning of dormancy in the studied dogwood genotypes is extended in time and begins at the end of the period of mass ripening of fruits. First, the old leaves of the first and second metameres change colour and fall off (stages 92, 93); later, other leaves die off. As a result, under optimal weather conditions, leaf abscission (stages 93 and 95) in the genotypes of *C. sessilis*, *C. mas*, *C. officinalis*, and their hybrid is rather long and is not intense. Rapid, within a few days, leaf fall may be due to significant night frosts. All leaves (stage 97) in the studied species fall off infrequently. Usually, one or two pairs of them remain on the tops of the shoots for a very long time, sometimes until spring.

Conclusions

In this work, the phenological stages of development of genotypes of three species of the genus *Cornus* (*C. mas*, *C. officinalis*, *C. sessilis*), 23 cultivars of *C. mas* of the NBG breeding gene pool, and a hybrid from the crossing of *C. officinalis* and *C. mas* (cultivar Etude) are described for the first time according to the extended BBCH scale. In the studied species of the subgenus *Cornus*, as in some other stone fruit plants, there are eight out of ten main stages of seasonal development, in particular: development of buds (principal growth stage 0), leaves (principal growth stage 1), shoots (principal growth stage 3), inflorescences (principal growth stage 5), flowering (principal growth stage 6), fruit development (principal growth stage 7), ripening of fruits (principal growth stage 8) and senescence, beginning of dormancy (principal growth stage 9). In all studied species and cultivars of dogwood at the beginning of the growing season, generative buds in their development outstrip vegetative ones and plants

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begin seasonal development from the deployment of inflorescences and flowering (principal growth stage 5 and principal growth stage 6, respectively). Various species and cultivars differ among themselves in the calendar dates of the beginning and duration of specific principal growth stages of development. The data obtained are important for further studies of the adaptive capabilities of dogwood species and cultivars under different climatic conditions, for the practical use of the complex of genetic resources of the studied dogwood species and cultivars, as well as for the introduction of little-known species and their use in agricultural production, pharmacology, ornamental and landscape gardening.

Conflicts 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

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Effects of extracts derived from roots and stems of *Chelidonium majus* L. on oxidative stress biomarkers in the model of equine plasma

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Greater celandine (*Chelidonium majus* L., Papaveraceae) is a perennial herbaceous plant, with an upright and spreading stem, large leaves, and yellow flowers collected on the tops of the stems in rare umbel inflorescence. The main aim of the study was an assessment of the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification (OMP), total antioxidant capacity (TAC)] and also activity of antioxidant enzymes (catalase, ceruloplasmin) in the equine plasma after treatment by extracts derived from roots and stems of *C. majus* collected from rural and urban agglomerations. Plant materials were collected from natural habitats on the territory of the Kartuzy district in the Pomeranian province (northern part of Poland). Our results demonstrated that statistically significant reductions in lipid peroxidation byproducts were noted after incubation with extracts derived from roots of *C. majus* collected from both urban (by 35 %, $p < 0.05$) and rural (by 34 %, $p < 0.05$) agglomerations compared to the control samples. Stem extracts derived from *C. majus* also reduced TBARS levels, but only extracts derived from *C. majus* were collected from the rural areas; a statistically significant decrease (by 21 %, $p < 0.05$) was observed compared to the control samples. The lowest values in the content of the aldehydic derivatives of OMP were observed after incubation with extracts derived from roots of *C. majus* collected from both rural and urban areas. On the other hand, levels of ketonic derivatives of OMP were significantly increased after incubation with extracts derived from stems of *C. majus* collected from both rural and urban areas compared to the control samples, in contrast to extracts derived from roots of *C. majus* collected from urban areas, where there was a statistically significant reduction in ketonic derivatives of OMP (by 15 %, $p < 0.05$) compared to the control samples. A significant increase in the TAC levels was observed after incubation with root and stem extracts of *C. majus* collected from both urban and rural areas, but the highest values were observed after incubation with extracts derived from roots of *C. majus* collected from rural areas (by 66.7 %, $p < 0.05$) compared to the control samples. Stem extracts of *C. majus* collected from urban agglomerations were found to be most effective in increasing catalase activity (by 115 %, $p < 0.05$). Both root and stem extracts of *C. majus* collected from rural areas caused a statistically significant reduction in ceruloplasmin levels. These *in vitro* studies indicate that extracts from this plant are a significant source of natural antioxidants that could prevent the progression of various disorders caused by oxidative stress. However, the proportions of secondary metabolites responsible for the antioxidant activity of *C. majus* extracts are currently unclear. Therefore, further studies are needed to isolate and identify the antioxidant compounds present in the plant extracts. Screening of *C. majus* plant for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

Keywords: *Chelidonium majus*, root and stem extracts, equine plasma, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity, catalase, ceruloplasmin

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Introduction

Organismal life encounters reactive oxidants from internal metabolism and environmental toxicant exposure. Reactive oxygen and nitrogen species cause oxidative stress and are traditionally viewed as being harmful (Forrester et al., 2019). Reactive oxygen species (ROS) regulate cellular homeostasis and act as prime modulators of cellular dysfunction contributing to disease pathophysiology. ROS are byproducts of numerous enzymatic reactions in various cell compartments, including the cytoplasm, cell membrane, endoplasmic reticulum (ER), mitochondria, and peroxisome, as part of basal metabolic function (Allen and Bayraktutan, 2009; Goncharov et al., 2015; Forrester et al., 2019). Oxidative stress is now recognized to play a central role in the pathophysiology of many different disorders (Bedard and Krause, 2007).

Living cells are under constant oxidative attack from reactive oxygen species, which can cause, among other things, lipid peroxidation or increase the level of byproducts of excessive protein oxidation. The lipid peroxidation chain reactions products display high biological activity (Brieger et al., 2012; Paik et al., 2017). It destroys DNA, proteins, and enzyme activity as well as acts as a molecular to activate signaling pathways initiating cell death (Su et al., 2019). As proteins are highly abundant and react rapidly with many oxidants, they are highly susceptible to, and major targets of, oxidative damage. This can result in changes to protein structure, function, and turnover and loss or (occasional) gain of activity (del Río et al., 1992).

Accumulation of oxidatively-modified proteins, due to either increased generation or decreased removal, has been associated with both ageing and multiple diseases (Singh, 1996). Different oxidants generate a spectrum of broad, sometimes characteristic, post-translational modifications (Hawkings and Davies, 2019).

Equine erythrocytes are uniquely susceptible to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage (Harvey et al., 2003). Oxidants typically damage erythrocytes by oxidizing the heme iron in hemoglobin, reactive sulfhydryls, or unsaturated lipids in the membranes. The oxidation of the heme iron in hemoglobin to the ferric (Fe^{3+}) state generates methemoglobin, which is incapable of transporting oxygen (Baskurt and Meiselman, 1999; Boyer et al., 2002). Oxidation of sulfhydryl groups in the globin portion of hemoglobin can induce protein denaturation and the formation of Heinz body aggregates. The oxidation of sulfhydryl groups and unsaturated lipids can also compromise the erythrocyte membrane integrity (Wright et al., 1999). Reduced glutathione (GSH) can protect erythrocytes against oxidant injury, being oxidized itself to a disulfide; however, horses have a reduced ability to regenerate reduced GSH, compared with other mammals, likely due to the decreased activity of glutathione reductase in equine erythrocytes. Under normal conditions, equine erythrocytes have sufficient capacity to prevent oxidative damage (Robin and Harley, 1967; Medeiros et al., 1984).

A complex system of antioxidant defenses has evolved that generally holds this attack in balance. In recent years, an important increase in the attempts to find natural sources of molecules with biological potential has been noticed (Storz, 2005; Liou and Storz, 2010). Polyphenols from plants are such molecules, which have proven important antioxidant activity (Unuofin and Lebelo, 2020). Antioxidants are important agents involved in the protection against oxidative stress that has proven to be one of the most important causes of many diseases nowadays (Diniz do Nascimento et al., 2020). Therefore, in recent years, many researchers have focused on this direction, with important results in the curative or adjuvant treatment of some diseases (Ratnam et al., 2006; Pisoschi et al., 2016). Several medicinal plants have been proven to contain significant amounts of polyphenols, which added an important value to their use in the therapy of numerous diseases (Carocho and Ferreira, 2013).

Plants naturally produce many new metabolic compounds that have been an invaluable source of pharmacological discovery for centuries (Rahman, 2007). Plants, particularly those of the Papaveraceae family, produce many antioxidant factors including a wide range of natural defensive compounds such as phenols, terpenoids, alkaloids, polyacetylenes, lectins, and carotenoids (Păltinean et al., 2017). *Chelidonium majus* (CM) is a perennial herbaceous plant, with an upright and spreading stem, large leaves, and yellow flowers collected on the tops of the stems in rare umbel inflorescence. The plant is widely present in Europe and Asia, North America, and a part of Northwest Africa (Krahulcová, 1982). The plant contains, as major secondary metabolites, isoquinoline alkaloids, such as sanguinarine, chelidonine, chelerythrine, berberine, and coptisine. Other compounds structurally unrelated to the alkaloids have been isolated from the aerial parts: several flavonoids and phenolic acids (Colombo and Bosisio, 1996). Crude extracts of CM, as well as purified compounds derived from it, exhibit a broad spectrum of bioactive properties with a potential

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for the protection of human health, such as anti-inflammatory, antimicrobial, cytotoxic, analgesic, antioxidant, antiulcer, acetylcholinesterase- and butyrylcholinesterase-inhibitory, and hepatoprotective activities (Lee et al., 2007; Kuenzel et al., 2013). The high spectrum of antioxidant properties for CM that are increasingly being used suggests the necessity of further investigations regarding their influence on organs and tissue function, including the evaluation of molecular mechanisms involved to exploit them for potential therapeutic benefits (Lenfeld et al., 1981; Kokoska et al., 2002; Havelek et al., 2016).

The originality of this work is that it is a study on the antioxidant activity of CM using equine blood as an adequate model. Furthermore, to increase the rationale for the possible introduction of greater celandine extracts into phytotherapy, the present study also evaluated the level of ceruloplasmin and catalase activity after dosing of the greater celandine extracts. However, with the advent of modern and synthetic drugs and supplements, many of these natural plant-derived antioxidant compounds have remained unexplored. This is the main reason why characterization and testing the biological activity of extracts obtained from this plant is essential for their introduction in therapy as phytopharmaceuticals.

Therefore, in the present study, the oxidative stress biomarkers [2-thiobarbituric acid reactive substances, carbonyl derivatives of oxidative modification of proteins, total antioxidant capacity], as well as activity of antioxidant enzymes (catalase, ceruloplasmin) in the equine plasma, were used for assessing the antioxidant activity of root and stem extracts derived from *Chelidonium majus* collected in urban and rural agglomerations of Kartuzy district in the Pomeranian province (northern part of Poland).

Material and methodology

Collection of plant material

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

Preparation of plant extracts

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

Horses

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

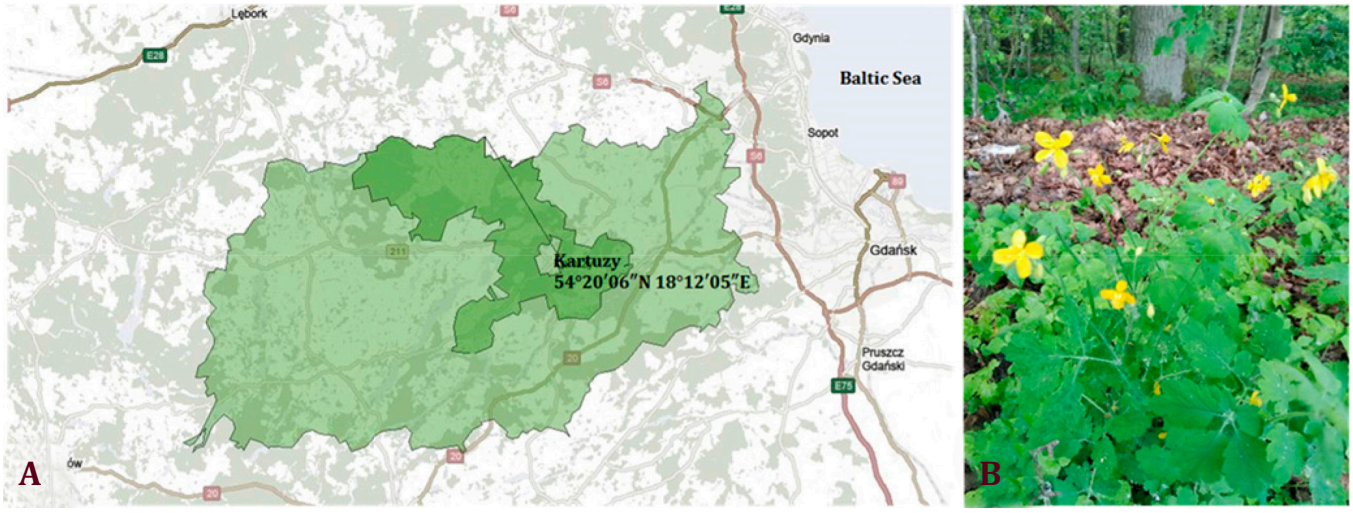

Figure 1 Location of Kartuzy in the map of northern Poland (A), where the greater celandine (B) was collected (Photo by Nataniel Stefanowski)

old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses, and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical, and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. The pellet of blood was re-suspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 mL of the plant extract was added to 1.9 mL of equine plasma. For positive control, 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 3,000 rpm for 5 min. Plasma aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances (TBARS) assay

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

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

To evaluate the protective effects of extracts derived from root and stem extracts derived from CM collected from urban and rural agglomerations against free radical-induced protein damage in equine erythrocytes and plasma, a content of carbonyl derivatives of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the plasma was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (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). 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 relatively the absorbance of the blank sample.

Assay of catalase activity

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al. (1988). One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 μmol H_2O_2 per min per mL of blood.

Assay of Ceruloplasmin level

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

Statistical analysis

The arithmetic means ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p > 0.05$). The significance of differences between the levels of oxidative stress biomarkers (significance level, $p < 0.05$) was examined using the Mann-Whitney *U* test (Zar, 1999). All statistical calculation was performed on separate data from

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each individual with Statistica 13.3 software (StatSoft, Krakow, Poland).

Results and discussion

The cellular components, that make up the cell membrane, the most exposed to the harmful action of free radicals are lipid structures, lipoproteins, and proteins. Damage of the abundant and thus most susceptible polyunsaturated fatty acid (PUFA) is termed lipid peroxidation (Srivastava and Shrivastava, 2016). The most widely used assay for lipid peroxidation is malonic dialdehyde (MDA) formation as a secondary lipid peroxidation product, with the 2-thiobarbituric acid reactive substances test (Xiong et al., 2020).

Protein oxidation reactions involve, among others, ROS free radicals and result in oxidative modification of amino acid side chains, peptide cleavage under the influence of reactive oxygen species, reactions of peptides with lipids and products of carbohydrate oxidation, and formation of carbonyl derivatives of proteins (Himmelfarb et al., 2000).

Figure 2 shows the TBARS levels obtained by incubating equine plasma in the presence of aqueous extracts derived from the root and stem of CM collected from rural and urban agglomerations. Statistically significant reductions in levels of lipid peroxidation byproducts were noted after incubation with root extracts of CM collected from both urban (by 35 %, $p < 0.05$) and rural (by 34 %, $p > 0.05$) agglomerations compared to the control samples. Stem extracts derived from CM also reduced TBARS levels, but only those collected in rural areas; there was a statistically significant decrease by 21 % ($p < 0.05$).

The aldehydic and ketonic derivatives of oxidatively modified proteins in the equine plasma after *in vitro* incubation with root and stem extracts derived from *C. majus* collected from rural and urban areas of Pomeranian region were present in Figure 3.

A similar result (Figure 3) was observed in the levels of aldehydic derivatives of oxidatively modified proteins, where the lowest value was observed after incubation with extracts derived from roots of CM collected from both rural and urban areas (by 7 and 8 %, respectively, $p > 0.05$) compared to the control samples. On the other hand, levels of ketonic derivatives of OMP showed that stem extracts of CM collected from both urban and rural areas, significantly increased levels of protein oxidation compared to the control samples (by 16 and 17 %, respectively, $p > 0.05$) in contrast to root extracts of CM collected from urban areas, where there was a statistically significant reduction in ketonic derivatives of oxidatively modified proteins by 15 % ($p < 0.05$) compared to the control samples.

Measurement of total antioxidant capacity (Figure 4) after incubation with CM extracts showed surprising results. Statistically significant increases in TAC levels were observed after incubation with root and stem extracts of CM collected from both urban and rural

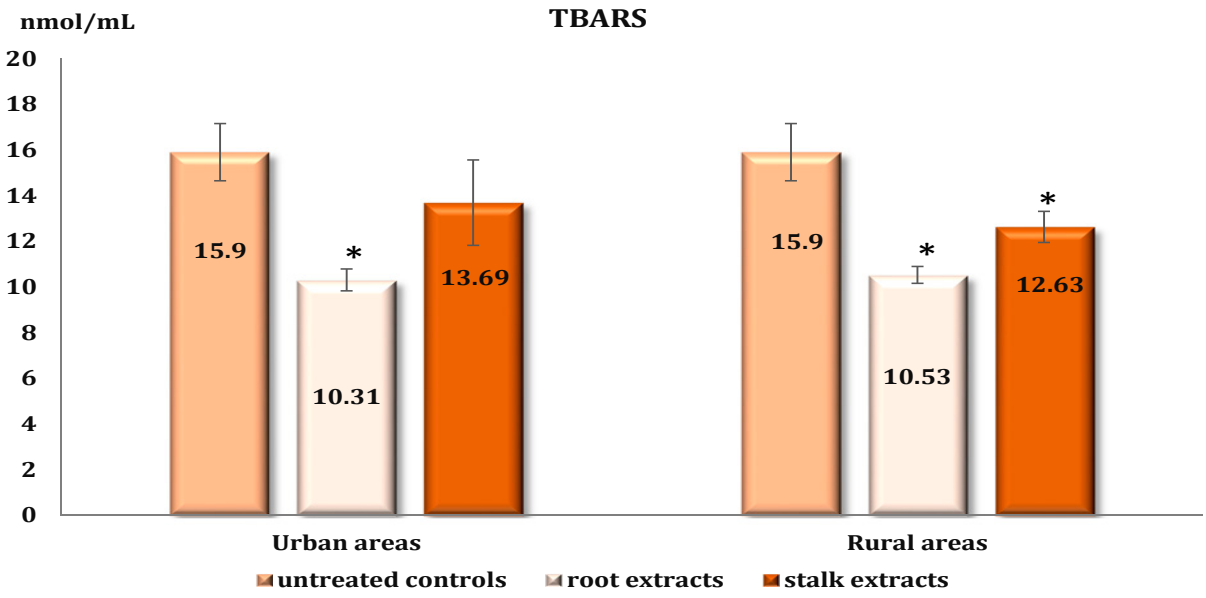

Figure 2 The TBARS content as a biomarker of lipid peroxidation in the equine plasma after *in vitro* incubation with root and stem extracts obtained from *Chelidonium majus* L. collected from rural and urban areas of Pomeranian region (M ±m, n = 8)
*– statistically significant differences ($p < 0.05$) compared to the control samples

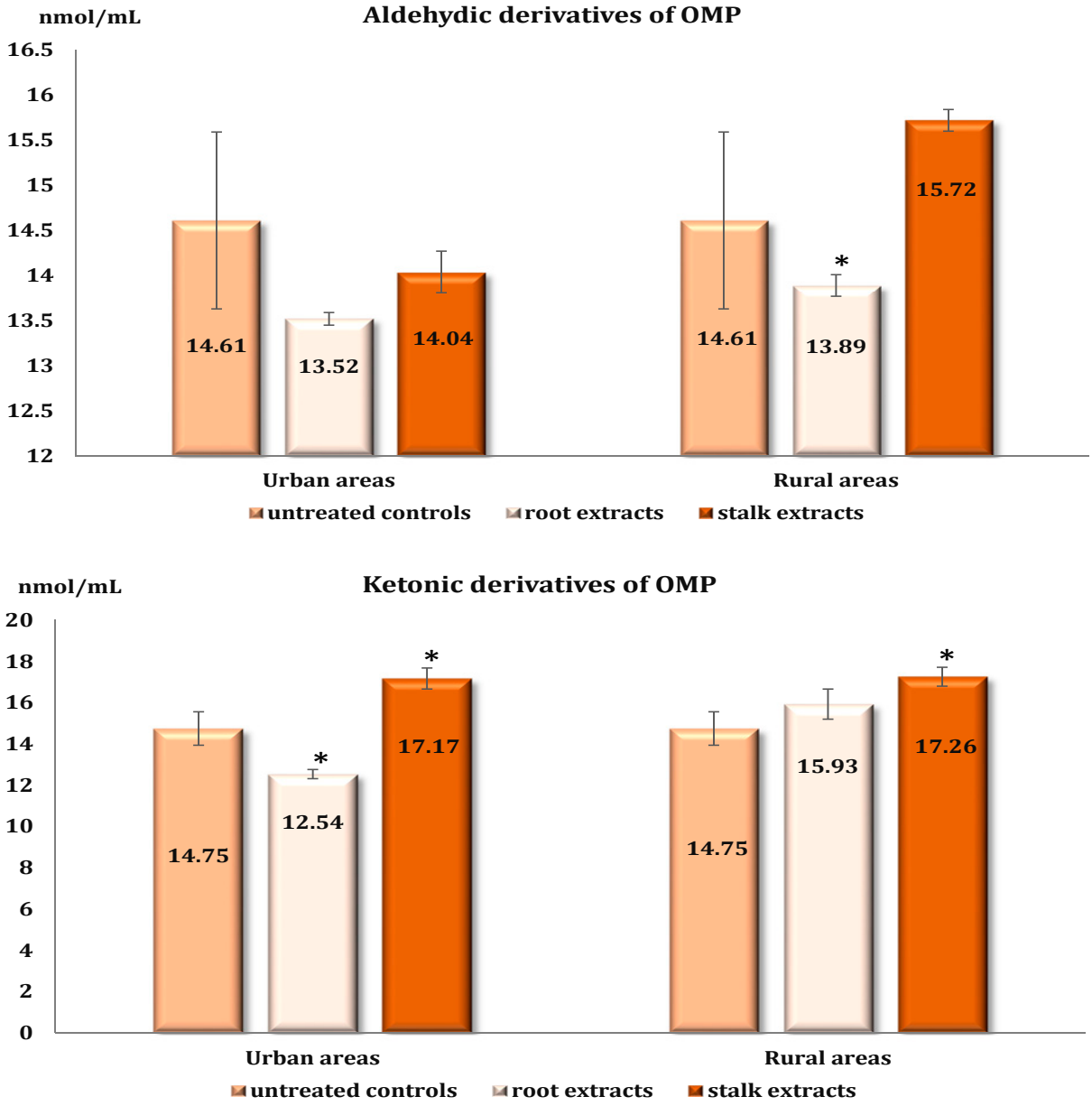

Figure 3 The aldehydic and ketonic derivatives of oxidatively modified proteins in the equine plasma after *in vitro* incubation with root and stem extracts obtained from *Chelidonium majus* L. collected from rural and urban areas of Pomeranian region (M ±m, n = 8)
*– statistically significant differences ($p < 0.05$) compared to the control samples

areas. Moreover, the highest value was observed after incubation with root extracts of *C. majus* collected from rural areas (increase by 67 %, $p < 0.05$ compared to the control samples).

Antioxidants are substances that prevent the oxidation of other compounds. Enzymatic antioxidants consist of superoxide dismutase and catalase. The enzymatic antioxidants have more effective protective effects against active and massive oxidative attacks due to the ability to decompose ROS (He et al., 2017). Therefore, this set of antioxidants play important role in diseases prevention. Catalase (CAT), is among the most important antioxidant enzyme against hydrogen peroxides (Muhlisin et al., 2016). In our study, stem extracts of CM collected from urban agglomerations were found to be most effective in increasing catalase activity (by 115 %, $p < 0.05$) (Figure 5). Root extracts of CM collected from rural agglomerations also significantly increased catalase levels by 65 % ($p < 0.05$). Probably, the increase in catalase activity has resulted in a 65 % increase in TAC level ($p < 0.05$) (Figure 4).

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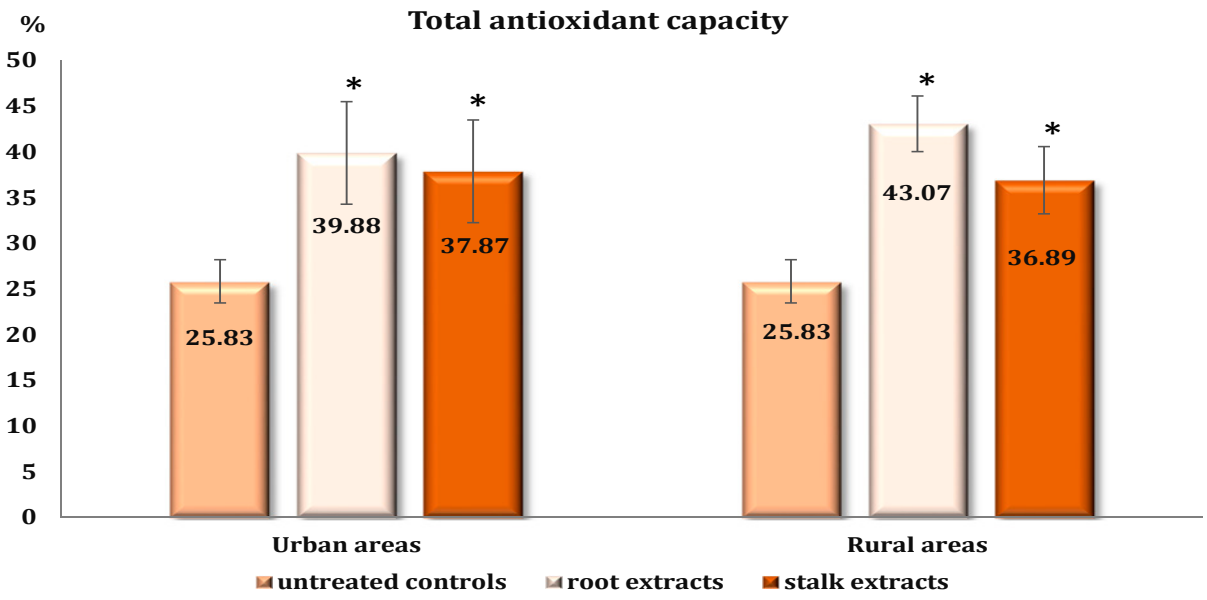

Figure 4 The total antioxidant capacity in the equine plasma after *in vitro* incubation with root and stem extracts obtained from *Chelidonium majus* L. collected from rural and urban areas of Pomeranian region (M ±m, n = 8)
*– statistically significant differences ($p < 0.05$) compared to the control samples

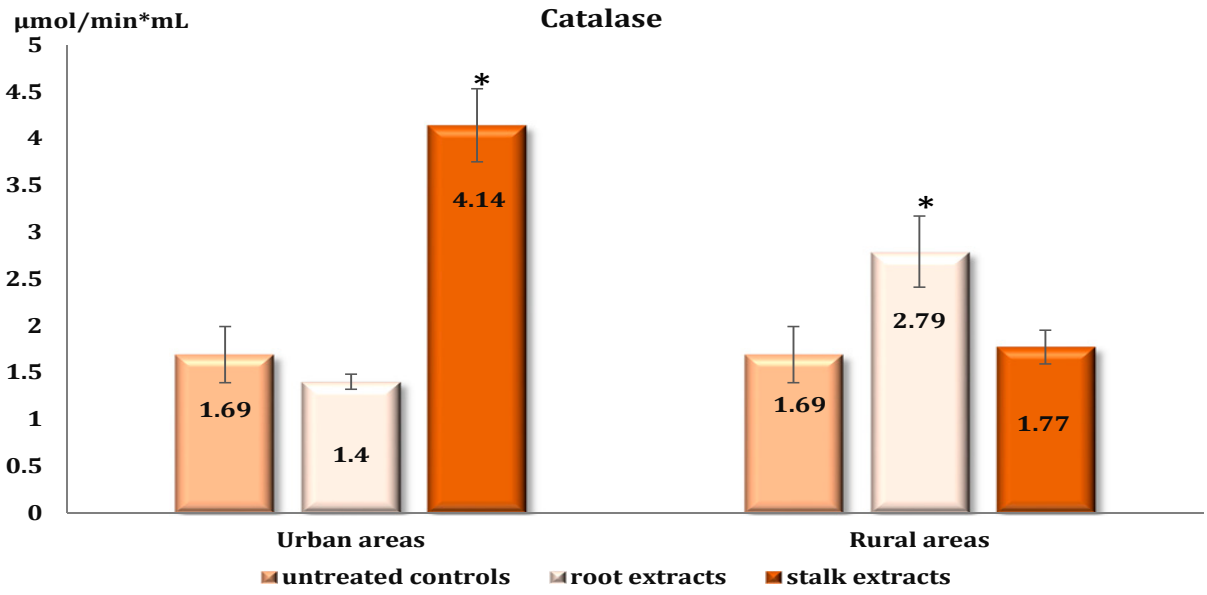

Figure 5 The catalase activity in the equine plasma after *in vitro* incubation with root and stem extracts obtained from *Chelidonium majus* L. collected from rural and urban areas of Pomeranian region (M ±m, n = 8)
*– statistically significant differences ($p < 0.05$) compared to the control samples

Ceruloplasmin (CP) is a copper-binding glycoprotein that is the major ferroxidase in liver-derived plasma (Arenas de Larriva et al., 2020). It is characterized as a copper (Cu)-containing protein that binds 40–70 % of the Cu in plasma and is mainly produced by the liver. This protein is a member of the multicopper oxidase family, an evolutionarily conserved group of proteins that use copper to couple substrate oxidation with the four-electron reduction of oxygen to water (Jeremy and Shukla, 2014). Apart from playing a role in copper and iron metabolism, CP is an acute-phase reactant that may work as an antioxidant but can also generate free radicals that may lead to several illnesses (Dadu et al., 2013). In our study, root and stem extracts of

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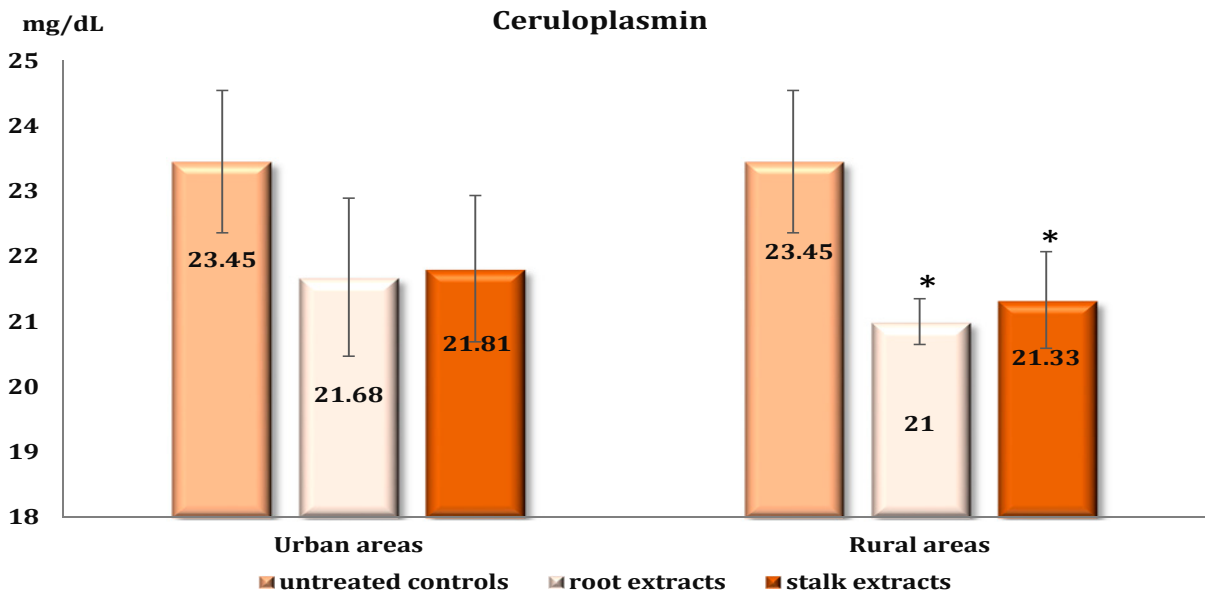

Figure 6 The ceruloplasmin level in the equine plasma after *in vitro* incubation with root and stem extracts obtained from *Chelidonium majus* L. collected from rural and urban areas of Pomeranian region (M ±m, n = 8)
*– statistically significant differences ($p < 0.05$) compared to the control samples

CM collected from rural areas caused a statistically significant reduction in ceruloplasmin levels by 10 and 9 %, respectively ($p < 0.05$) (Figure 6).

In the current study, we investigated the effects of CM extracts on lipid peroxidation and biomarkers of oxidatively modified proteins, as well as on antioxidant defense in equine plasma. Our study suggests that the crude extracts obtained from CM roots exhibit effective antioxidant activity when incubated with equine plasma. The protective effect of CM extracts is evident from the improvement of antioxidant enzymes activity exemplified by catalase and increase in total antioxidant capacity. The antioxidant defense system was improved with the suppression of aldehydic and ketonic derivatives of oxidatively modified proteins after incubation with CM extracts. CM extracts have also shown anti-inflammatory activity expressed by decreasing plasma ceruloplasmin levels.

In our previous study (Stefanowski et al., 2021a, b) on muscle tissue of rainbow trout (*Oncorhynchus mykiss* Walbum), we also demonstrated the antioxidant activity of CM extracts. Our results showed that extracts of CM collected from both urban and rural areas statistically significantly reduced the level of aldehydic derivatives of OMB by 18.8 % ($p < 0.05$). The analysis of the levels of ketonic derivatives of OMP showed that extracts of CM collected from both urban and rural areas statistically significantly decreased the level of ketonic derivatives of OMP by 20.6 and 21.5 %, respectively (for urban areas), as well as 26.7 and 12.5 % (for rural areas). Lower levels of lipid peroxidation were observed after incubation with stem extracts, while those collected from rural areas showed the lowest result (by 11 %). Root extracts of CM collected from urban and rural areas increased TBARS levels. Analysis of oxidatively modified protein in the blood of rainbow trout after *in vitro* incubation with root and stem extracts showed that extracts can inhibit the production of oxidative carbonyls by scavenging free radicals.

Phytochemical constituents in the Papaveraceae family are known to be biologically active compounds and they are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer (Zielińska et al., 2018). CM contains, as major constituents, isoquinoline alkaloids (such as sanguinarine, chelidonine, chelerythrine, berberine, protopine, and coptisine), flavonoids, and phenolic acids (Gilca et al., 2010). Both crude extracts of CM and purified compounds derived from it exhibit a wide variety of biological activities (anti-inflammatory, antimicrobial, immunomodulatory, antitumoral, choleretic, hepatoprotective, analgesic, etc.) which are in concordance with the traditional uses of CM (Gilca et al., 2010; Zielińska et al., 2018).

Phenols are a large class of secondary metabolites. Phenolics (including flavonoids) are among the most active antioxidants, as well as the most important stabilization factors of the oxidative processes.

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Flavonoids include many pharmacological and biological properties. In phytopharmacy, flavonoids are the active ingredients, of plant origin, with strong biological effects, while in dietetics and food industries they present important phytonutrients, preservatives, spices, and aromatics substances (Stanković et al., 2010, 2011; Jakovljevic et al., 2013). The highest concentration of total phenolic compounds was observed by Jakovljevic et al. (2013) in the spring when the CM was in the rosette stage with well-formed, thick leaves, but when the whole plant is not yet sufficiently developed. When the plant enters the flowering stage, there is a lack of that concentration, which again increases when the plant begins with the formation of fruit. The concentration of flavonoids is the greatest just before flowering and before fruiting. During flowering, the concentrations of these secondary metabolites are the lowest. The antioxidant activity and total phenol concentrations are the highest in the spring months during the rosette stage (Jakovljevic et al., 2013).

Neither strong nor direct relations were found between the antioxidant activity of the plant and the concentrations of flavonoid content and phenolic compounds. This could be due to the complexity of the substances which cause the antioxidant activity of this plant. The composition evolution in two opposite ways simultaneously for two compounds characterized by high individual activities could lead to the compensatory effect of the final activity (Gourine et al., 2010; Jakovljevic et al., 2013).

The analysis conducted by Nawrot et al. (2016) confirmed the presence of the protein components of the antioxidant defense system in the CM latex. These proteins form the first line of defense against different stress conditions and help to prevent the attack of different pathogens, which are highly abundant in the milky sap. Peroxidase 12-like and isoflavone reductase homolog were present only in the milky sap. The presence of class III plant peroxidase, glyoxalase, quinone reductase, and ubiquitin in CM latex was previously reported by Nawrot et al. (2007a, b).

Lee et al. (2007) in animal studies showed, that CM methanol extract significantly suppressed the progression of collagen-induced arthritis in mice. This action was characterized by a decreased production of TNF-α, IL-6, Interferon(IFN)-γ, B cells, γδ-T cells (in spleen), and an increased proportion of CD4+CD25+ regulatory T cells. The serum levels of IgG and IgM RA factors were decreased. Song et al. (2002) in *in vitro* studies showed an interesting immunomodulatory potential exhibited by a protein-bound polysaccharide extracted from *C. majus* (CM-Ala), which showed mitogenic activity on spleen cells, bone marrow cells, and increased the number of granulocyte macrophage-colony forming cells (GM-CFC). When CM extract was used in combination with recombinant IFN-γ, there was a marked combined induction of NO and TNF-α production in mouse peritoneal macrophages.

Plants are an important origin of natural substances that are the raw material for various pharmaceutical and therapeutic applications due to the presence of phytochemicals, such as alkaloids. Alkaloids, which are found in different plant species, possess numerous biological activities. Some alkaloids have strong cytotoxic effects on various cancer cells (Deljanin et al., 2016). The CM root has higher all determined alkaloid contents as compared to extract that is obtained from herb (Petruczynik et al., 2019). The highest contents of chelerythrine, sanguinarine, and berberine possessing very high cytotoxic activity were found in the root extract. These isoquinoline alkaloids might have synergistic high cytotoxic activity on cancer cell lines. Extracts that were obtained from CM exhibit very strong cytotoxicity (Zielińska et al., 2020). The studies of Petruczynik and co-workers (2019) indicated the strong cytotoxicity of these extracts against Pancreatic Cell Lines (PANC-1, IC_{50}, 20.7 μg/mL) and Cell Line human Caucasian colon adenocarcinoma grade II (HT-29, IC_{50}, 20.6 μg/mL), and moderate cytotoxic activity against Cell Line human breast adenocarcinoma (MDA-MB-231, IC_{50}, 73.9 μg/mL), Cell Line human from human lung (carcinoma, A-549), HeLa, and Cellosaurus cell line SGC-7901 cell lines. Their studies have shown that the extracts from CM are also cytotoxic against other cell lines (FaDu, SCC-25, MCF-7, MDA-MB-231, and CRL1634). FaDu and SCC-25 cell lines belong to the so-called head and neck squamous cell carcinomas are often resistant to chemotherapy, even including targeted drug therapy.

Conclusions

The results showed that the extracts obtained from the roots and stems of *C. majus* exhibited effective antioxidant activity when incubated with equine plasma. The protective effect of CM root extracts is evident from the improvement of antioxidant defenses and increase in total antioxidant capacity. The antioxidant defense system was improved with the suppression of aldehydic and ketonic derivatives of oxidatively modified proteins after incubation with root and stem extracts. CM extracts have shown anti-inflammatory activity expressed as a decrease in plasma ceruloplasmin levels. The pronounced effect

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of CM root extract can be attributed to the secondary metabolites it contains, such as polyphenols and flavonoids. Finally, further studies are needed to reveal the exact cellular mechanisms of the effects of CM extract on erythrocyte and plasma function. These *in vitro* studies indicate that extracts from this plant are a significant source of natural antioxidants that could prevent the progression of oxidative stress. However, the proportions of secondary metabolites responsible for the antioxidant activity of CM extracts are currently unclear. Therefore, further studies are needed to isolate and identify the antioxidant compounds present in the plant extracts.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

OPEN ACCESS

Phytochemistry and inflorescences morphometry of Invasive *Solidago* L. (Goldenrods) species – valuable late autumn mellifers

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In Europe, two invasive North American species of *Solidago* L. have been detected: *S. canadensis* L. and *S. gigantea* Ait. Both species provide a stable late harvest and are valued by beekeepers for their ability to produce pollen and nectar in late fall. There is a significant correlation between the chemical components of flowers and goldenrod honey, so the study of the phytochemical composition of inflorescences (heads) seems to be very actual. The work aims to determine the total content of saccharides, phenolic compounds, and flavonoids in the heads of *S. canadensis* and *S. gigantea* for comparative evaluation of bee production quality, and also to specify morphometric differences in the heads of both species and to reveal the amplitude of their variability. The material was collected in the Moscow and Pskov Regions (Russia). The total content of the saccharides in *S. canadensis* heads was 27.33 ±0.54 %, with monosaccharides ~44–46 %. In *S. gigantea*'s heads total content of the saccharides was 1.5 times lower – 18.07 ±0.73 %, the content of monosaccharides was 7.39 ±0.15 %. The total content of phenolic compounds in the heads of *S. canadensis* was 105.36 ±1.45 mg GAE/100 g and in *S. gigantea*'s heads was 98.41 ±1.71 mg GAE/100 g. The total flavonoid content as quercetin equivalents was 58.23 ±0.17 mg QE/100 g in the heads of *S. canadensis* and 41.97 ±0.34 mg QE/100 g in *S. gigantea*'s heads. Thus, in *S. canadensis* heads the content of the total content of saccharides is 1.5 times higher, the content of phenolic compounds is 1.3 times higher, and the content of flavonoids is 1.4 times higher than in *S. gigantea*. A feature allowing the diagnosis of these species is the size of the heads: *S. canadensis* has smaller heads than *S. gigantea* (4.4 × 1.8 vs. 6.4 × 2.3 mm).

Keywords: *Solidago*, flowers, heads, saccharides, phenolic compounds, flavonoids

Introduction

In Europe, two alien North American species of *Solidago* L. of the Triplinervae section are detected: *S. canadensis* L. and *S. gigantea* Ait. (especially well when growing in homogeneous conditions of the experimental plot). The species differ in morphological characters as well. *S. canadensis* has pubescent shoots, short rhizomes, spreading panicles, and toothed leaves. *S. gigantea* has glabrous shoots (except for the panicle axis), long rhizomes, compact panicle, finely serrated or smooth-margin leaf blades (Figure 1). The species also differ in morphometric features of the heads, but no statistical

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analysis of these parameters has been carried out so far.

Both *Solidago* species are aggressive plants that actively disperse in ruderal habitats, meadows, pastures, fields, forests, roadsides, riverbanks, etc. (Vinogradova et al., 2010). Both species are recognized as invasive weeds that reduce natural biodiversity by displacing native plants (The most..., 2018). The ecological risks that both *Solidago* species pose to the environment are of great concern. However, there is another, positive value of *S. canadensis* and *S. gigantea*, manifesting themselves in their use as sources of valuable raw materials with high added value. Thus, both species provide a stable late harvest and are valued by beekeepers for their ability to produce pollen and nectar in the late fall. This variety of honey is rarely pumped because all of the nectar and pollen go to support the bee colonies and prepare them for wintering. According to the Canadian and Polish experience, goldenrod honey yields up to 150 kg per hectare – the same amount as sunflowers. Germacren D, which has not been identified in other monofloral kinds of honey, is present in goldenrod honey (Amtmann, 2009).

Although there is no complete similarity between the chemical components of *Solidago* flowers and goldenrod honey (Amtmann, 2009), there is a significant correlation. Honey absorbs the medicinal qualities of the plant from which it is collected, so the study of the phytochemical composition of inflorescences (heads) seems very actual.

The work aims to determine the total content of saccharides, phenolic compounds and flavonoids in heads of *S. canadensis* and *S. gigantea* for comparative evaluation of bee production quality, and also to specify morphometric differences in the structure of flowers and heads of both species and to reveal the amplitude of their variability.

Material and methodology

Biological material

Samples of *S. canadensis* and *S. gigantea* collected in local invasive populations in Moscow, Moscow Region, and Pskov Region were included in the study. Plants were collected during the beginning of flowering, and the collected samples were dried in bundles suspended in the air at room temperature in a shady place. Only inflorescences (heads), without panicle tips, were used in the phytochemical analysis. For comparative evaluation of morphometric traits, plants growing in the same agrophone were selected.

Studied samples:

1. *S. canadensis* (Moscow, Main Botanical garden, 55° 50‘ 21.3“ N 37° 35‘ 09.5“ E),
2. *S. gigantea* (Moscow, Main Botanical garden, 55° 50‘ 40.9“ N 37° 35‘ 31.8“ E),
3. *S. canadensis* (Moscow Region, Zvenigorod, 55° 42‘ 11.6“ N 36° 46‘ 29.6“ E),
4. *S. gigantea* (Moscow Region, Zvenigorod, 55° 42‘ 16.7“ N 36° 46‘ 29.6“ E),
5. *S. canadensis* (Pskov Region, Izborsk, 57° 43‘ 16.4“ N 27° 52‘ 11.3“ E).

Determination of total saccharides content and water-soluble carbohydrates

The content of water-soluble carbohydrates and total saccharides content were determined according to the methods “Determination of sugars by spectrophotometric method“ (OFS.1.2.3.0019.15 Gosudarstvennaya, 2015) and “Method for determining the content of water-soluble carbohydrates and starch from one sample“ (Patent RU 2406293 C1). The results were expressed in milligrams per 100 g of air-dry material.

Determination of total polyphenol content (TPC)

The total polyphenol content (TPC) was measured by the method “Method for determining the total content of phenolic compounds in plant samples“ (Patent RU 2700787 C1) using the Folin-Ciocalteu reagent. The samples were prepared and analysed as follows: 0.5 g of the plant sample was ground with 15 mL of 96 % ethanolic solution, extraction was carried out for 45 minutes in a water bath, followed by centrifugation. A quantity of 0.75 mL of each sample was mixed with 0.75 mL of the Folin-Ciocalteu reagent (diluted 5 times), 1.5 mL of 20 % (w/v) sodium carbonate, and 7 mL of distilled water. After 60 min in darkness, the absorbance at 725 nm was measured with the spectrophotometer Spekol (1300, Analytik Jena, Germany). Gallic acid (25–300 mg/L; R^2 = 0.998) was used as the standard. The results were expressed in mg/g DM gallic acid equivalent.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined by the method of the GF XIII ed., (p. 332), art. “Grass of *Persicaria hydropiper* (L.) Delarbre“. Plant samples (0.5 g) were extracted with an 80 % solution of ethanolic solution for 60 minutes in a water bath with a reverse refrigerator. An aliquot of 2 mL of the sample was mixed with 1mL of 1 % (w/v) ethanolic solution

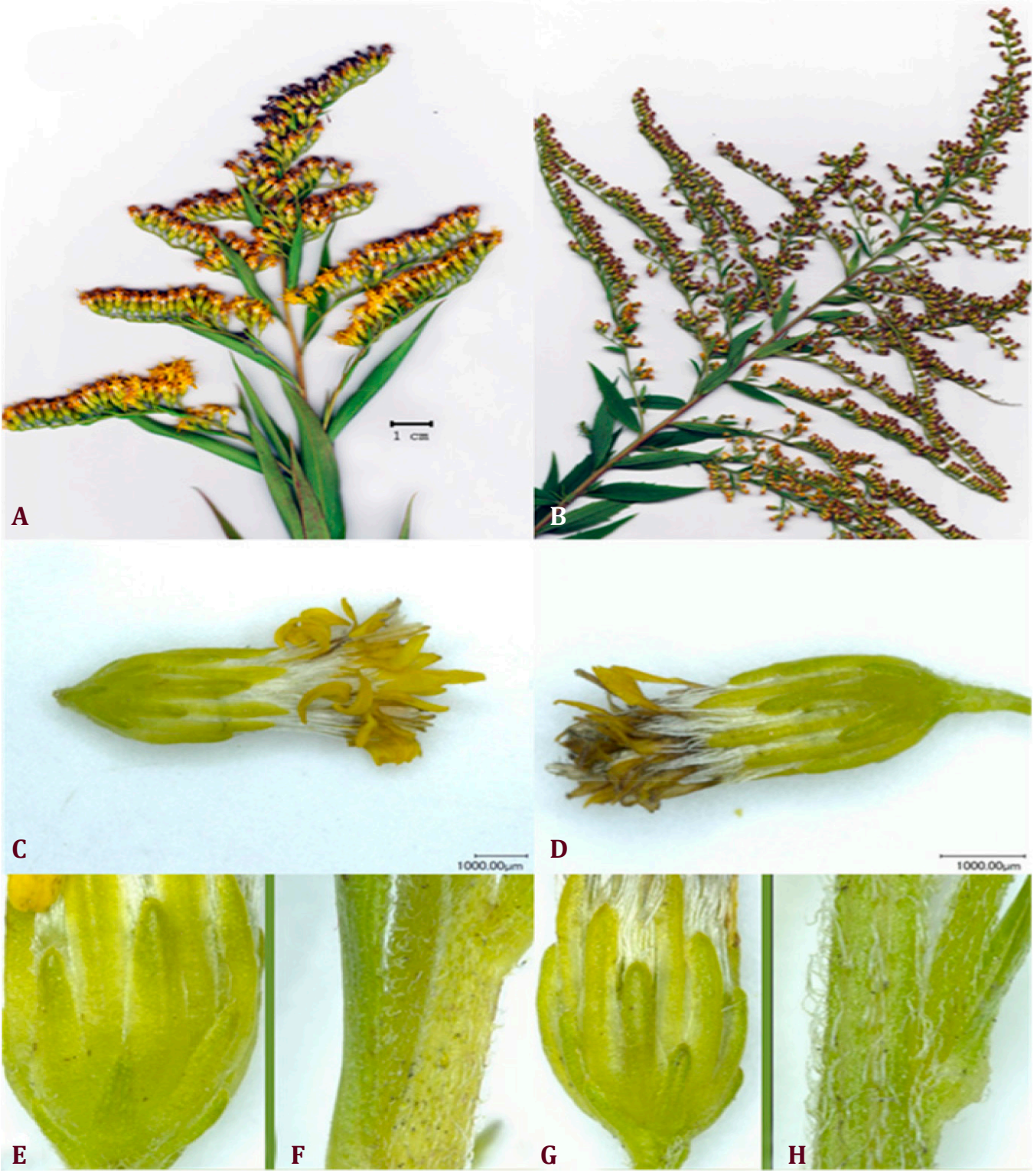

Figure 1 Morphology of generative organs of *Solidago* spp.
Panicles: A – *S. gigantea*, B – *S. canadensis*; heads: C – *S. gigantea*, D – *S. canadensis*; involucre: E – *S. gigantea*, G – *S. canadensis*; rachis: F – *S. gigantea*, H – *S. canadensis*

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of aluminum chloride and 3 mL of distilled water. After 20 min in darkness, the absorbance at 430 nm was measured using the Spekol (1300, Analytik Jena, Germany). Quercetin (1–400 mg/L; R^2 = 0.9977) was used as the standard. The results were expressed in mg/g DM quercetin equivalent.

Determination of morphometric characters

To evaluate the morphometric characters, plants growing in the same agricultural background were selected, the sample consists of 50 heads for each species; studied parameters (length and diameter of the head, length of the involucre) were measured using a digital electron microscope Keyence VHX 1000.

Statistical analysis

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

Results and discussion

The total content of the saccharides in *S. canadensis* heads vary from 21.66 ±0.21 to 31.07 ±0.68 and the average was 27.33 ±0.54 %, with monosaccharides slightly less than half of this parameter (the average 12.05 ±0.11 %). Previously, we found that in the inflorescences of *S. canadensis*, fructose dominates in the composition of monosaccharides, all other monosaccharides were present in trace amounts (Shelepova et al., 2019a, b). In *S. gigantea* heads total content of the saccharides was 1.5 times lower – 18.07 ±0.73 %, the content of monosaccharides was 7.39 ±0.15 %. According to the literature data, goldenrods are rich in acidic polysaccharides due to the presence of hexuronic acid and its derivatives, while the presence of acidic polysaccharides and polyphenols may be the result of the aggregation of polysaccharide chains with polyphenols (Liu et al., 2018). The resulting polyphenolic glycoconjugates have anticoagulant activity, which allows us to consider them as new sources of anticoagulant compounds (Pawlaczyk et al., 2009).

A wide range of phenolic compounds is present in the inflorescences and flowers of plants. These compounds are divided into two main groups: phenolic acids and flavonoids. Therefore, one of the goals of this study was to determine the content of phenolic compounds and flavonoids in the heads of two species of goldenrod.

The total content of phenolic compounds in the heads of *S. canadensis* varied from 101.17 to 115.38 mg GAE/100 g and the average was 105.36 ±1.45 mg GAE/100 g. In *S. gigantea* heads total content of phenolic compounds was 1.3–1.1 times lower ranging from 91.04 to 101.07 mg GAE/100 g and the average was 98.41 ±1.71 mg GAE/100 g. This is significantly lower than the levels of phenolic acids (440–1200 mg/100 g) in the inflorescences of goldenrod growing in the more southern regions of Europe. But significantly higher than the levels of phenolic compounds found in honey from goldenrod (Jasicka-Misiak et al., 2018).

In the heads of *Solidago*, according to literature data, glycosides are represented mainly by glycosides of quercetin, kaempferol, and isorhamnetin (Zekič et al., 2021).

The total flavonoid content as quercetin equivalents in the heads of *S. canadensis* ranged from 49.3 to 64.1 (on average 58.2 ±0.17) mg/100 g. The highest flavonoid content (64.1 ±0.07 mg/100 g) was observed in the sample collected in Moscow, while the lowest flavonoid content (49.3 ±0.01 mg/100 g) was found in the sample collected in the Pskov region. The flavonoid content in *S. gigantea* heads was slightly lower compared to *S. canadensis* heads and averaged 41.97 ±0.34 mg/100 g.

Our results are significantly lower than the flavonoid levels found by Jasicka-Misiak et al. (2018). Thus, according to their data, the concentration of flavonoids in *Solidago* flowers was from 850 to 1 380 mg/100 g. The authors noted that the flavonoid content depends on climatic conditions. The highest levels of flavonoids were found in herbs collected in places with the greatest amount of sunlight. The authors suggest that the concentration of flavonoids in plants is proportional to the intensity of sunlight (Rosłon et al., 2014) and high temperature combined with low humidity (Karlová, 2006).

Our data on the comparison of the two species confirm the results of other studies. Thus, the analysis of flavonoids in extracts of the invasive *S. canadensis* revealed a significant increase in the content of rutin (quercetin-3-O-beta-rutinoside) in its composition compared to *S. gigantea*. The authors hypothesized that it is the synthesis of flavonoids that may determine the invasion strategy of *S. canadensis*. When entering the rhizosphere with exudates, flavonoids (mainly quercetin glycosides) can interact with ammonium. As a result, new compounds, phenol-ammonia complex, are formed. At low concentrations (20 μg/mL), these substances stimulate the formation of lateral and

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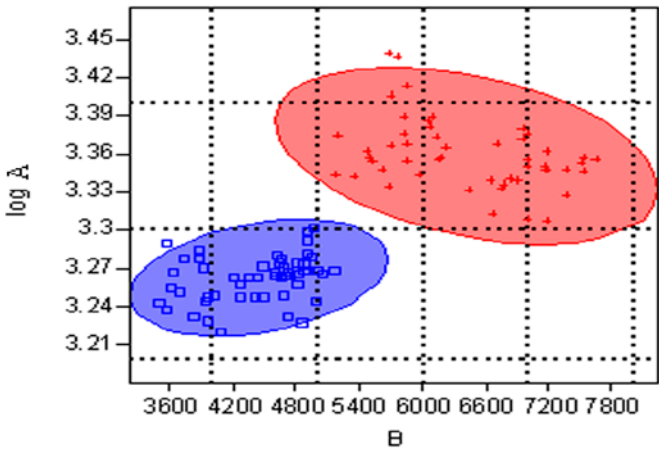

Figure 2 The head's size: *S. canadensis* (blue oval), and *S. gigantea* (red oval)
A – head's diameter; B – head's length

adventitious roots in plants, and in concentrations more than 100 µg/mL inhibit them. Enhanced synthesis and release of flavonoids, therefore, is an important element of the strategy for the biotransformation of a new habitat by alien plants species (Likhanov et al., 2021). The hypothesis about the determining influence of allelopathy on the invasive success of *S. canadensis* is also discussed by other scientists (Abhilasha et al., 2008; Li et al., 2011; Liao et al., 2011; Zhang et al., 2011; Baleţentiene, 2015; Wang et al., 2016; Mozdzen et al., 2020).

For *S. gigantea* the head's length was 5.2–7.7 mm (in average 6.4 ±0.1 mm; V = 11 %), the head's diameter 2.0–3.0 mm (2.3 ±0.0 mm; V = 9 %), the involucre's length was 2.9–4.4 mm (3.7 ±0.1 mm; V = 11 %). For *S. canadensis*, these parameters are significantly lower: the head's length was 3.5–5.2 mm (in average 4.4 ±0.1 mm; V = 11 %), the head's diameter 1.5–2.1 mm (1.8 ±0.0 mm; V = 6 %), the involucre's length was 2.6–3.9 mm (3.1 ±0.1 mm; V = 8 %). However, the relative size of the involucre, on the contrary, is higher in *S. canadensis* – it is 70 % of the head's length, while in *S. gigantea* the involucre is 60 % of the head's length.

In Central Europe, *S. canadensis* occurs more frequently than *S. gigantea* and prefers drier and warmer habitats (Kabuce and Priede, 2021). It is not advisable to intentionally cultivate *Solidago* species as melliferous plants near the apiary. In some European countries, there is even a fine for this. Both *S. canadensis* and *S. gigantea* are aggressive invasive species and can displace valuable native honey plants from natural plant communities. In this case, the bee production in the apiary will decrease during the spring and summer periods.

Conclusions

In *S. canadensis* heads the total content of saccharides is 1.5 times higher, the content of phenolic compounds is 1.3 times higher, and the content of flavonoids is 1.4 times higher than in *S. gigantea*. A feature allowing the diagnosis of these species is the size of the heads: *S. canadensis* has smaller heads than *S. gigantea* (4.4 × 1.8 vs. 6.4 × 2.3 mm).

Conflicts 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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Research Article

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Nutritional value, bioactive components and antioxidant activity of *Schisandra chinensis* (Turcz.) Baill. leaves

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As a part of the ongoing interest in the nutritional and antioxidative properties of a little-known East Asian plant species, the aim of the study was to determine the contents of macronutrients and selected elements, profiles of fatty and amino acids, the content of phenolic compounds, β-carotene, vitamin A and E, and antioxidant activity of *Schisandra chinensis* (Turcz.) Baill. edible leaves. *Schisandra chinensis* leaves contained a small amount of lipids – 4.36 % and 12.38 % of proteins. Sugars (fructose, maltose, sucrose, and lactose) were detected only in trace amounts (<0.5 g/kg). The β-carotene content was 17.70 mg/kg. The fatty acid profile of leaves was represented by palmitic C16:0 (44.6 g/100 g of oil), linoleic C18:2 *9c12c* (17.9 g/100 g of oil), and α-linolenic C18:3 (*9c12c15c* 10.6 g/100 g of oil) acids. Nine out of 18 amino acids detected in leaves were essential amino acids (68.80 g/kg of dry matter leaves). Glutamic acid was found to be the major component of non-essential amino acids (25 g/kg of dry matter), followed by aspartic acid (16.2 g/kg of dry matter) and leucine (14.2 g/kg of dry matter). The element composition of leaves demonstrated the presence of: macroelements (K, P, S, Ca, Mg, Na), microelements (Zn, Fe, Cu, Mn, Cr, Se), and metals (Al, As, Cd, Ni, Hg, Pb). Potassium was the most abundant element in *Schisandra chinensis* leaves (10209 mg/kg of dry matter of leaves), followed by Ca, P, and Mg. The spectrophotometric assays enabled detecting phenolic compounds from three categories: polyphenols (44.32 mg galic acid equivalents/g of dry matter of leaves), total flavonoids (29.16 mg quercetin equivalents/g of dry matter of leaves) and phenolic acids (6.12 mg caffeic acid equivalents/g of dry matter of leaves) in *Schisandra chinensis* leaves. The antioxidant activity of *S. chinensis* leaves, as determined

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by DPPH•, was at the level of 9.19 mg TEAC/g of dry matter of leaves, and 214 mg TEAC/g of dry matter of leaves (as determind by molybdenum reducing antioxidant power). The composition of *Schisandra chinensis* leaves suggest it to become an inexpensive novel plant source of functional foods, supplements, and as new ingredient in human diet.

Keywords: *Schisandra chinensis*, leaves, chemical composition

Introduction

Ample studies have shown that non-traditional, little-known, and underutilized edible leaves of plant species, also offer some nutritional value; being a good source of macroelements, minerals, polyphenols, with impressive antioxidant activity, thus gained interest as potential functional foods (Monka et al., 2014; Ivanišová et al., 2017a; Horčinová Sedláčková et al., 2018, 2019; Klymenko et al., 2017, 2019; Vinogradova et al., 2020; Grygorieva et al., 2017, 2018a, 2018b, 2018c, 2020a, 2020b). However, to the best of authors' knowledge, the data about nutrient composition and therapeutic properties of different morphological parts of plants is highly limited.

Schisandra chinensis (Turcz.) Baill. fruits proved to be a rich source of carbohydrates, vitamins, phytosterols, organic acids (Hancke et al., 1999; Wu et al., 2011; Tong et al., 2012; Ekiert et al., 2013; Szopa and Ekiert, 2014). The main bioactive components determined in fruits are lignans ("schisandra lignans"), which are also present in *Schisandra chinensis* leaves, but in lower amounts (Ekiert et al., 2013). Study of *Schisandra chinensis* leaves performed by Xia et al. (2015) allowed detection of 16,17-Seco-pre-schisanartanes (C_{29}), known as new triterpenoid from plant species of the family Schisandraceae. *Schisandra chinensis* leaves are known to be used in infusions or as spices (Ciorchină et al., 2011). The essential oil of *S. chinensis* also possess valuable properties (Merdzhanov et al., 2016).

The health beneficial properties of *Schisandra chinensis* fruits primarily refer to Traditional Chinese Medicine. Previous studies showed that *Schisandra chinensis* fruits possess hepatoprotective (Panossian and Wikman, 2008), anti-inflammatory (Hu et al., 2014), anticancer (Hwang et al., 2011) immunostimulant (Chen et al., 2012a; Zhao et al., 2013), anti-obesity (Park et al., 2012), antiviral (Xu et al., 2015), antibacterial (Chen et al., 2011; Hakala et al., 2015; Tvrdá et al., 2020), adaptogenic, ergogenic activity (Jeong et al., 2013; Sa et al., 2015), antioxidant and detoxifying properties (Chiu et al., 2008; Yim et al., 2009; Thandavarayan et al., 2015; Wang et al., 2018). Moreover, *S. chinensis* fruits extracts are used in cosmetic industry (Quirin, 2008; Dweck and Marshall, 2009; Chiu et al., 2011).

Despite the well-studied composition and properties of the fruits of *Schisandra chinensis*, the nutritional value and bioactive components of leaves remains to be studied. Thus, the aim of this study was to determine the contents of macronutrients and selected elements, profiles of fatty and amino acids, the content of phenolic compounds, β-carotene, vitamin A and E, and antioxidantive activity of *Schisandra chinensis* edible leaves.

Material and methodology

Sampling

The *Schisandra chinensis* leaves (Figure 1) were collected in July 2020 from the trees growing in the Botanical Garden (Slovak University of Agriculture in Nitra).

Chemicals and reagnets

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

Analysis of proximate composition

Dry matter, ash, and protein contents were determined according to CSN-EN 12145 procedures (1997). Total lipid content was determined according to ISO method (ISO 659, 1998).

Analysis of sugars

For the determination of sugars content, 1 g of leaves was vigorously shaken with 10 mL of water/ethanol mixture (4:1) on a vertical shake table (GFL, Germany). After 1 h of the extraction, the mixture was centrifuged at 6000 rpm for 4 min (EBA 21, Hettich, Germany). The supernatant was filtered through filter paper with 0.45 mm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with ultrapure water.

An HPLC analysis of sugars (fructose, maltose, sucrose, lactose) was performed using an Agilent Infinity 1260 instrument (Agilent Technologies, USA) equipped with an 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 comparison the relative retention times of sample

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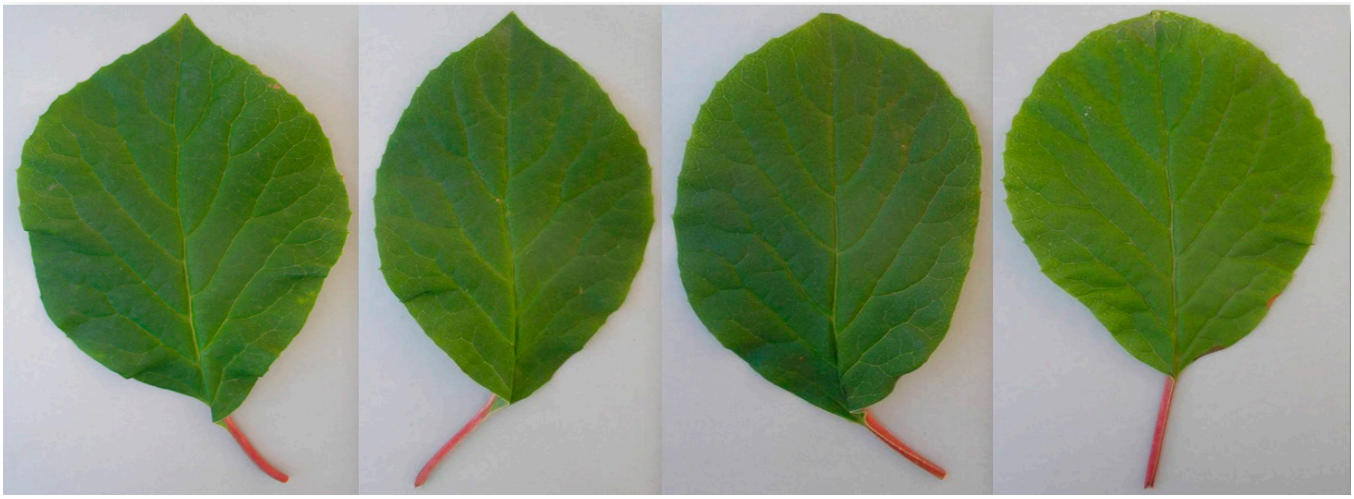

Figure 1 Leaves of *Schisandra chinensis* (Turcz.) Baill.

peaks with standards Sigma-Aldrich (Steinheim, Germany). The contents of sugars were expressed as g/kg of dry leaves.

Beta-carotene carotenoids content

Beta-carotene content was extracted following the method of Sarker and Oba (2019). 1 g of dry leaf sample was ground thoroughly in a mortar and pestle with 10 mL of 80 % acetone. After removing the supernatant in a volumetric flask, the extract was centrifuged at 10 000 × g for 3–4 min. The final volume was brought up to 20 mL. The absorbance was taken at 510 nm and 480 nm using a spectrophotometer (UV-VIS spectrophotometer, Jenway Model 6405, England). Data were expressed as mg beta-carotene per kg of dry leaves. The following formula was used to measure the beta-carotene content:

$$\text{Beta-carotene} = 7.6(\text{Abs. at } 480) - 1.49(\text{Abs. at } 510) \times \text{final volume}/1000$$

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). Leaves 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.

Determination of amino acids

Amino acid profile was determined by ion-exchange chromatography using an AAA-400 Amino Acid Analyzer (Ingos, Czech Republic) and post-column derivatization with ninhydrin and a VIS detector. Separation was provided on a glass column (length 350 mm, inner diameter 3.7 mm) filled with a strong cation exchanger in the LG ANB sodium cycle (Laboratory of Spolchemie) with average particles size of 12 µM and 8 % porosity. The column was heated within the range of 35–95 °C, with the elution of amino acids at 74 °C. A double-channel VIS detector with the inner cell volume of 5 µL was set to 440 and 570 nm. A solution of ninhydrin was prepared in 75 % v/v methyl cellosolve and in 2 % v/v 4 M acetic buffer (pH 5.5). $SnCl_2$ was used as a reducing agent. Solution of ninhydrin was stored in an inert atmosphere (N_2) without access of light at 4 °C. The flow rate was 0.25 mL/min, and the reactor temperature was 120 °C. Individual amino acids values were expressed as g/kg of dry leaves.

Fatty acid composition

Fatty acid (FA) composition of extracted fat from *Schisandra chinensis* leaves was determined as follows: the samples were prepared according to official methods Ce 2-66 (1997) to convert the oils into fatty acid methyl esters (FAME). The FAME profile was analyzed by gas chromatography (GC-6890-N, Agilent Technologies, Santa Clara, USA) equipped with capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Santa Clara, CA, USA) and FID detector (250 °C; constant flow, hydrogen 40 mL/min, air 450 mL/min.). A detailed description of the

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chromatography conditions is presented in the work Szabóová et al. (2020). Standards of a C4–C24 FAME mixture (Supelco, Bellefonte, PA, USA) were applied in order to identify FAME peaks. The evaluation was carried out by the ChemStation 10.1 software.

Spectrophotometric assays of phenolic compounds

Total phenolics content (TPC)

The TPC was determined spectrophotometrically at 700 nm (UV-VIS spectrophotometer, Jenway Model 6405, England) according to Singleton and Rossi (1965) using Folin-Ciocalteu's reagent. Briefly, 0.1 mL of leaves ethanolic extract was diluted with 8.8 mL of distilled water, mixed with 0.1 mL of the Folin-Ciocalteu's reagent and 1 mL of 20 % sodium carbonate. The mixture was kept in darkness for 30 min before measurement of absorbance. Gallic acid (25–300 mg/L; R^2 = 0.998) was used as the standard. The results were expressed as gallic acid equivalents (GAE; mg GAE/g of dry matter of leaves).

Total flavonoids content (TFC)

The TFC was determined spectrophotometrically at 415 nm (UV-VIS spectrophotometer, Jenway Model 6405, England) according to a modified method of Shafii et al. (2017). An aliquot of 0.5 mL of leaves ethanolic extract was mixed with 0.1mL of 10 % ethanolic solution of aluminum chloride, 0.1 mL of 1 M potassium acetate and 4.3 mL of distilled water. The mixture was kept in darkness for 30 min before measurement of absorbance. Quercetin (1–400 mg/L; R^2 = 0.9977) was used as the standard. The results were expressed as mg quercetin equivalents (QE)/g of dry matter of leaves.

Phenolic acid content (TPA)

The TPA was determined spectrophotometrically at 490 nm (UV-VIS spectrophotometer, Jenway Model 6405, England) according to a method described in Farmakopea Polska (1999). A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10 % $NaNO_2$ + 10 % Na2MoO4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of distilled water. Caffeic acid (1–200 mg/L, R^2 = 0.999) was used as a standard. The results were expressed as mg caffeic acid equivalents (CAE)/g of dry matter of leaves.

Determination of antioxidant activity

DPPH• radical scavenging activity

The free radical scavenging activity of leaves extract on DPPH• (2,2-diphenyl-1-picrylhydrazyl) was determined according to Sánches-Moreno et al. (1998). Briefly, 0.4 mL of the extract was mixed with 3.6 mL of DPPH• solution (0.025 g of DPPH• in 100 mL of methanol). The absorbance was measured at 515 nm using a UV-Vis spectrophotometer (Jenway Model 6405, England). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10–100 mg/L; R^2 = 0.989) was used as standard. The results were expressed as mg of Trolox equivalents (TEAC)/g of dry matter of leaves.

Molybdenum reducing antioxidant power (MRAP)

The MRAP of leaves extract was determined according to the method of Prieto et al. (1999) with slight modifications. The mixture of 1 mL of extract, monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then rapidly cooled. The absorbance was measured at 700 nm using a UV-Vis spectrophotometer (Jenway Model 6405, England). Trolox (10–1000 mg/L; R^2 = 0.998) was used as the standard. The results were expressed as mg of Trolox equivalents (TEAC)/g of dry matter of leaves.

Statistic analysis

The results were subjected to one-way ANOVA followed by Tukey-Kramer test, when the differences between mean values were considered significant at $p < 0.05$. The variability of all parameters was evaluated by descriptive statistics. The results were presented as means with standard error (SE). The PAST 2.17 software was used.

Results and discussion

The protein content of *Schisandra chinensis* leaves (12.38 %) was higher than in many cultivated fruits, up to 1%. Our results are in agreement with other studies; in generally, leaves are a poor lipid source. *Schisandra chinensis* leaves contained a small amount of lipids – 4.36 %. Sugars (fructose, maltose, sucrose, and lactose) were detected only in trace amounts (< 0.5 g/kg) (Table 1). Monosaccharides are involved in almost all major plant metabolic processes such as synthesis of organic acids, amino acids, polyphenols, pigments, and aromatic compounds (Halford et al., 2011). It has been

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observed that leaves accumulate monosaccharides in plants grown under stress conditions (Wind et al., 2010). Probably, lower levels of these macronutrients in leaves indicate optimal environmental conditions for plant growth.

Table 1 Proximate composition of *Schisandra chinensis* (Turcz.) Baill. leaves

Component	Mean ±SE
Proteins	12.38 ±0.16
Lipids (%)	4.36 ±0.09
Saturated fatty acids (g/100 g oil)	54.40 ±0.16
Monounsaturated fatty acids (g/100 g oil)	11.30 ±0.11
Polyunsaturated fatty acids (g/100 g oil)	28.50 ±1.07
Fructose (g/kg)	<0.5
Maltose (g/kg)	<0.5
Sucrose (g/kg)	<0.5
Lactose (g/kg)	<0.5
Dry matter (%)	92.34 ±2.44
Ash (%)	5.70 ±0.17
Vitamin A (retinyl acetate) (mg/kg)	<0.1
β-carotene (mg/kg)	17.70 ±0.10
Vitamin E (α-tocopherol) (mg/kg)	48.58 ±2.66

Schisandra chinensis leaves proved to be rich in carotenoids, mainly β-carotene (17.7 mg/kg). Substantial share of β-carotene has a great impact on the colour, it is well known that carotenoids are one of the most common natural pigments protecting plants against photo-oxidative reactions. They are also the most effective antioxidants trapping molecular singlet oxygen and peroxyl radicals. At the same time, carotenoids enhance the effect of other antioxidants (Stahl and Sies, 2003).

The content of α-tocopherol in leaves was assayed at the level of 48.58 mg/kg. Vitamin E includes four tocopherols and tocotrienols, which main biochemical function is thought to bind organic peroxyl radicals (Shahidi and Ambigaipalan, 2015). These reactions determine the antioxidant activity of vitamin E, protecting tissue lipids from free radical attack.

The application of gas chromatography enabled determination of 20 fatty acid in lipid fraction extracted from *Schisandra chinensis* leaves belonged to all groups (Mišurcová et al., 2011) (Table 1 and 2); saturated – SFAs (54.4 g/100 g of the oil), monounsaturated – MUFAs (11.3 g/100 g of the oil), and polyunsaturated – PUFAs (28.50 g/100 g of the oil). The FA profile of leaves was represented mainly by palmitic (C16:0) 44.6 g/100 g of oil, linoleic C18:2 *9c12c* 17.9 g/100 g of oil, and α-linolenic C18:3 *9c12c15c* 10.6 g/100 g of oil acids (Table 2). The amount of these three FAs was 76.44 % of the total FAs. The substancial presence of palmitic acid was confirmed in many previous studies; palmitic acid (27.7–60.0 %) dominated in the FA profile of leaf of many species from the Lamiaceae family (Cacan et al., 2018; Kilic, 2018); *Cassia tora* (L.) Roxb. (18.6–38.7 %) (Shukla et al., 2018); *Nicotiana* species (13.0–18.0 %) (Koiwai et al., 1983); *Cistus ladanifer* L. (13.6–17.5 %) (Jerónimo et al., 2020). In most cases, oleic acid is the second compound in the fatty acid profile (12.5 %). According to study of Kumar et al. (2009) oleic acid has insecticidal activity against *Aedesae gyptii* larvae. This FA was also detected in the FA profile of *S. chinensis* leaves (6.8 %).

Table 2 Fatty acid composition (g/100 g of oil) of lipids of *Schisandra chinensis* leaves

Fatty acid	Mean ±SE
SFAs	7.63
C14:0	1.61 ±0.08
C16:0	44.60 ±1.58
C17:0	0.51 ±0.03
C18:0	3.94 ±0.08
C20:0	1.27 ±0.03
C22:0	1.16 ±0.04
C24:0	1.26 ±0.02
MUFAs	11.27
C16:1	3.99 ±0.05
C18:1	6.79 ±0.11
C20:1	0.49 ±0.03
PUFAs	28.49
C18:2	17.90 ±0.63
C18:3	10.59 ±0.48

Note: Saturated fatty acids – SFAs; monounsaturated fatty acids – MUFAs; polyunsaturated fatty acids – PUFAs

Changes in FA composition of different morphological parts of the plants may be affected by abiotic stress such as extreme temperatures below and above zero and moisture deficiency (Gigon et al., 2004; Liu and Huang, 2004; Zhong et al., 2011; Li et al., 2017). Lipids play an important role in metabolic processes of organisms (Cakir, 2004). Also, it is well-known that FAs have antibacterial and antifungal properties (McGaw et al., 2002; Seidel and Taylor, 2004). Many studies indicated that a decreased risk of cardiovascular disease, coronary heart disease, cancer, hypertension, type 2 diabetes, kidney diseases, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and osteoporosis,

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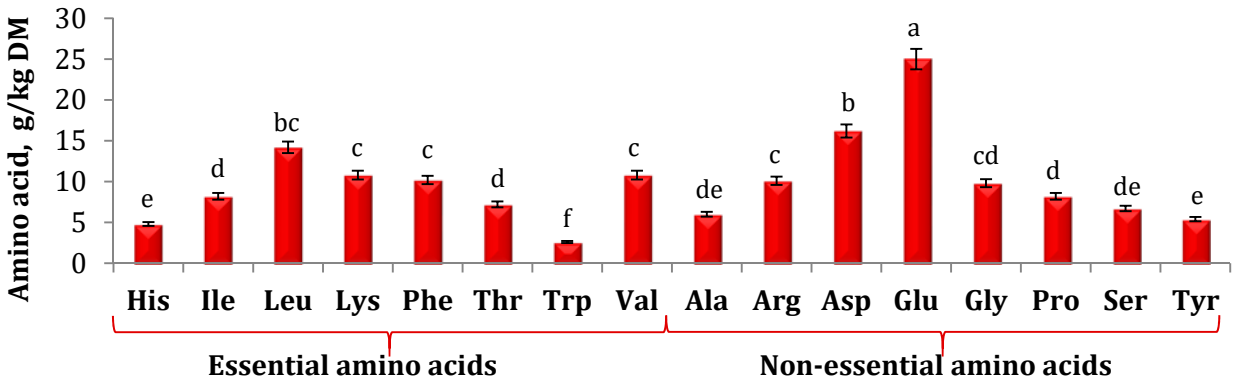

Figure 2 Amino acid composition (g/kg of dry matter; DM) of *Schisandra chinensis* leaves
a, b, c, d, e, f – different superscripts indicate the significant differences at p <0.05

with consumption of PUFAs, especially FAs from n-3 family (De Caterina et al., 2000; Mišurcová et al., 2011; Abedi and Sahari, 2014). Our results indicated that *Schisandra chinensis* leaves may represent a novel potential plant source of FAs important for nutritional reasons.

Eighteen amino acids were detected in *Schisandra chinensis* leaves, nine of them were essential amino acids and nine non-essential ones (Figure 2).

The content of amino acids in leaves was at the level of 156.20 g/kg of dry matter; while content of total essential amino acids was 68.80 g/kg of dry matter (amounted 44.05 %) and 87.40 g/kg of dry matter (55.95 %) for total non-essential amino acids. Glutamic acid was found to be the major component of non-essential amino acids (25 g/kg of dry matter), followed by aspartic acid (16.2 g/kg of dry matter) and leucine (14.2 g/kg of dry matter).

The contents of macroelements (K, P, S, Ca, Mg, Na), microelements (Zn, Fe, Cu, Mn, Cr, Se), and metals (Al, As, Cd, Ni, Hg, Pb) are presented in Table 3. Elements present in plants are responsible for their properties (including toxicity) since they are catalysts of most biochemical processes occurring in plants. Analysis of elements in leaves is an important guide to sustainable plant nutrition. The presence of trace elements and their content in plants are of considerable interest both from the theoretical point of view and of their medical application. In the etiology of many diseases, the imbalance of the content of trace elements in the human body plays an important role for mainatnance of good health (Penauelas et al., 2001; Erdal et al., 2006; Lipa, 2013; Yildirim et al., 2015).

Potassium (K) was the most abundant element in *Schisandra chinensis* leaves (10209 mg/kg of dry weight of leaves), followed by Ca, P, and Mg. It should be highlighted that potassium is an essential mineral, important mainly to maintain body water and to participate in transmitting nerve impulses to muscles, thus consumption of such leaves may have influence in covering daily required amount of this element. Regarding the presence of metals, the content of aluminium (Al; 32.1 mg/kg of dry weight of leaves) dominated among detected metals in *Schisandra chinensis* leaves. For maitanance of human health it is very important to study the contents of heavy metals, like for example Cd, Ni and Al, because some plants may have the tendency to accumulate toxic metals. Also, amounts of metals, especially toxic ones may provide useful information about environmental pollution levels.

Phenolic compounds are known as phytonutrients, secondary metabolites or bioactive compounds (Yoona et al., 2016). Phenolic compounds can prevent excessive free radicals and have positive health benefits such as anti-carcinogenic, anti-inflammatory activities, anti-bacterial, anti-diabetics, prevent neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, prion disease, and motor neurone disease (Siracusa et al., 2019), decrease the level of blood pressure, improvement of plasma lipid profile and endothelial function (Yoona et al., 2016).

The leaves of many non-traditional plants repersent promising source of antioxidants (Sakanaka et al., 2005; Priya and Nethaji, 2015; Ferlemi and Lamari, 2016; Klymenko et al., 2017; Urbanaviciute et al., 2019; Grygorieva et al., 2020b).

The spectrophotometric assays enabled detecting phenolic compounds belonging to; polyphenols, phenolic acids, and flavonoids, in *Schisandra chinensis* leaves. The content of polyphenols was 44.32 mg

Table 3 Elements composition of *Schisandra chinensis* leaves (mg/kg of dry weight)

Element	mean ±SE
Macroelements	
K	10 209 ±387
P	3 277 ±222
Ca	7 330 ±310
S	987 ±68
Mg	4 012 ±412
Na	28.0 ±0.9
Microelements	
Zn	32.0 ±1.2
Fe	54.0 ±1.2
Cu	9.10 ±0.8
Mn	47.4 ±1.8
Cr	1.20 ±0.06
Se	0.19 ±0.01
Metals	
Al	32.1 ±1.9
As	<0.3
Cd	0.114 ±0.002
Ni	0.49 ±0.02
Hg	0.017 ±0.002
Pb	0.66 ±0.020

GAE/g of dry leaves, total flavonoids – 29.16 mg QE/g of dry leaves and phenolic acids – 6.12 mg CAE/g of dry leaves (Figure 3).

According to study of Mocan et al. (2014), *S. chinensis* leaves contained more polyphenols (62.36 ±1.38 mg/g DM) and flavonoids (35.10 ±1.23 mg/g DM) compering with their fruits (9.20 ±0.43 and 7.65 ±0.95 mg/g d.w., respectively).

According to previously published data, leaves of cultivated and wild plants are a valuable source of polyphenols and flavonoids. Thus, the TPC in *Mangifera indica* L. leaves was at the level of 65 mg/g, *Anacardium occidentale* L. – 58.57 mg/g. Moreover, TPC determined in ethanolic extracts of *Cymbopogm citrates* leaves was 28.30 mg/g, *Carica papaya* L. leaves – 21.80 mg/g (Iyawe and Azih, 2011), *Euphorbia* spp. leaves – 19.10–20.30 mg/g (Gapuz and Besagas, 2018), *Azadirachta indica* Juss leaves 14.43 mg/g (Iyawe and Azih, 2011). According to Thi and Hwang (2014), the polyphenol content of *Aronia mitschurinii* leaves ranges from 139.3 to 250.8 mg GAE/g of DM. However, results of TPC of Shahin et al. (2019) were significantly higher – 765.63 mg GAE/g of dried leaves of *Aronia melanocarpa*. According to Męczarska et al. (2017), the leaves of *Amelanchier alnifolia* had a polyphenol content at the level of 185.23 mg GAE/g DM.

Research of Barreira et al. (2010) and Stankovic et al. (2014), who studied total flavonoid content of leaves of *Castanea sativa* Mill., indicated that the flavonoids content was up to 3-fold higher (73.31–90.39 mg/g) compering with our results of flavonoids of *Schisandra chinensis* leaves. It was proved that methanolic extracts were the richest in flavonoids: TFC was 90.28 mg/g of methanolic extracts of *Ziziphus jujuba* Mill. leaves, while in the ethanolic extract the TFC was only 22.18 mg/g (Al-Saeedi et al., 2016). Grygorieva et al. (2020b) studied the content of phenolic compounds in the leaves of several non-traditional plants. The *Lycium barbarum* leaves distinguished by the highest content of polyphenols and flavonoids (95.84 mg GAE/g and 54.61 mg QE/g, respectively).

The antioxidant activity of *S. chinensis* leaves, as determined by DPPH•, was at the level of 9.19 mg TEAC/g of DW, and 214 mg TEAC/g of DW (as determind by molybdenum reducing antioxidant power – MRAP; Figure 3). For the comparison, the radical scavenging activity (DPPH) of some other plant leaves is lower than assayed for *S. chinensis* leaves: *Aronia mitschurinii* leaves – 6.92 mg TEAC/g of DM; *Cornus mas* – 9 mg

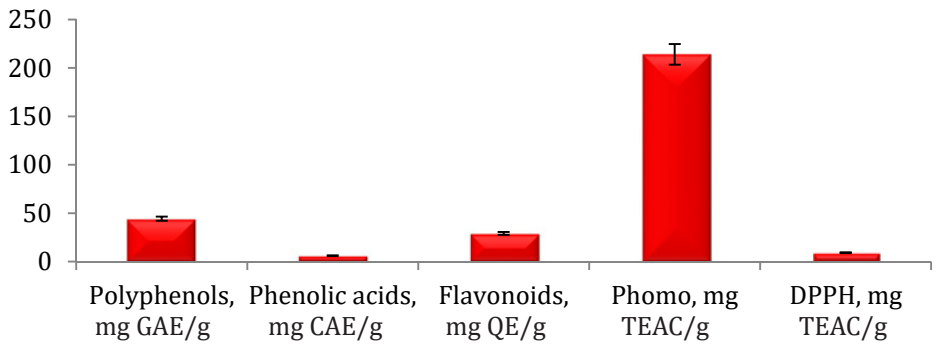

Figure 3 Bioactive compounds and antioxidant potential of *Schisandra chinensis* leaves

TEAC/g of DM. Antioxidant activity determined by molybdenum reducing antioxidant power ranged from 109.43 mg TEAC/g of DM (*A. mitschurinii* leaves) to 322.95 mg TEAC/g of DM (*C. mas* leaves) (Grygorieva et al., 2020b).

Some previous studies were also focused on the composition of *S. chinensis* plant and its products (Tvrdá et al., 2020). The antioxidant capacity of *S. chinensis* essential oil, measured by DPPH test (IC_{50}), was determined as 4.17 mg/mL (Chen et al., 2012b), while the free-radical scavenging activity of the extract was 5.93 mg TEAC/g of DM. According to the MRAP assay, the antioxidant activity of the extract was 140.52 mg TEAC/g of DM. The TPC of the extract was 16.52 mg GAE/g of DM, the TFC was 2.66 mg QE/g of DM and the carotenoids content was 0.15 mg β-carotene/g of DM (Tvrdá et al., 2020). Also, antioxidant activity of *S. chinensis* berries was studied previously by Ivanišová et al. (2017b), who determined DPPH• – 5.85 mg TEAC/g, MRAP – 148.87 mg TEAC/g, and TPC – 15.55 mg GAE/g of berries. Compering these findings with our results for antioxidant properties of *S. chinensis* leaves, it can be concluded that not only *S. chinensis* berries but also leaves posses fairy good antioxidant capacity.

Hamauzu et al. (2006) described that the fruits and leaves of *Schisandra chinensis* are a valuable natural source of caffeoylquinic acid and epicatechin. High levels of antioxidants and antiproliferative compounds make it possible to recommend this species for use in pharmaceutical and therapeutic nutrition, for the prevention and treatment of such human pathologies as cardiovascular disease and cancer.

Since the leaves of *Schisandra chinensis* are an inexpensive natural source of bioactive components, the extract from them can be used for the prevention and treatment of atherosclerosis, hypertrophic heart disease, hypertension, and diabetes.

Conclusions

This study demonstrates that *Schisandra chinensis* leaves may be regarded as a valuable source of minerals: K (10209 mg/kg of dry weight of leaves), Ca, P, Mg, and phenolic compounds (polyphenols – 44.32 mg GAE/g of dry leaves), with fairly good antioxidant activity (DPPH• test – 9.19 mg TEAC/g of dry leaves, and MRAP test – 214 mg TEAC/g of dry leaves). The β-carotene content was 17.7 mg/kg. The fatty acid profile of leaves was represented by palmitic C16:0 (44.6 g/100 g of oil), linoleic C18:2 *9c12c* (17.9 g/100 g of oil), and α-linolenic C18:3 (*9c12c15c* 10.6 g/100 g of oil) acids. Nine out of 18 amino acids detected in leaves were essential amino acids (68.80 g/kg of dry leaves), with significant share of glutamic acid (25 g/kg of dry weight), followed by aspartic acid (16.2 g/kg of dry weight) and leucine (14.2 g/kg of dry weight). The established composition of *Schisandra chinensis* leaves suggest it to become an inexpensive novel plant source of functional foods, supplements, and as a new ingredient in human diet.

Conflicts 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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Research Article

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Influence of variety on total polyphenols content and antioxidant activity in apple fruits (*Malus domestica* Borkh.)

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Recently, an increasing interest in fruits and vegetable eating has been noticed in particular through epidemiological and biochemical studies pointing to their health benefits for human health. Specifically, apples (*Malus domestica* Borkh.) are one of the most frequently consumed fruits. Therefore, the current study aimed to compare the total polyphenols content (TPC) and antioxidant activity (AA) of apple fruits from different columnar apple trees growing in experimental conditions. For this purpose, fruits of 7 apple cultivars obtained from the Botanic Garden (Slovak University of Agriculture, Slovakia; vegetative season 2019/2020) were analysed. Among them, four green-yellow cultivars: Golden Delicious, Granny Smith, Goldcats, Rondo; one green cultivar with red spots: Kordona; one red cultivar: Redcats, and one pink cultivar: Pompink, were used. Method using the Folin-Ciocalteu reagent was applied to quantify TPC, and radical scavenging activity of the samples was measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavengers. Total polyphenols content (mg GAE/g dry weight) ranged from 2.92 ±0.31 (cv. Pompink) to 9.02 ±0.32 (cv. Granny Smith), whereas statistically significant differences ($p < 0.05$) were recorded between all analysed cultivars except for Rondo (5.07 ±0.44) and Kordona (4.18 ±0.35) cultivars as compared to Redcats (4.96 ±1.06) one. As with TPC, the lowest values for AA were observed in the Pompink cultivar (35.03 ±3.49 %) and, conversely, the highest ones in the cultivar Granny Smith (88.55 ±4.25 %). These results suggest that the values for TPC and AA in apple fruits are strongly influenced depending on the apple cultivars. From this aspect, upon achievement of this survey, and employing more samples, it will be possible to create a comprehensive overview of the antioxidant properties of apple fruits with the possibility of their application in the food industry to develop innovative products with added value.

Keywords: *Malus domestica*, cultivars, DPPH assay, Folin-Ciocalteau assay

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Introduction

Recently, there has been a growing interest in including fruits and vegetables in the human diet, as many scientific papers have confirmed their beneficial effect on their health (Wolfe et al., 2003). Apples (*Malus domestica* Borkh.) are widely cultivated throughout the world for their well-known nutrition and refreshing taste (Moscetti et al., 2018), and they are a highly researched crop within the scientific community (Havryliuk and Kondratenko, 2019; Horčinová Sedláčková et al., 2020; Horčinová Sedláčková et al., 2021). These fruits are not consumed just fresh; approximately one-third of them are processed into various goods, from which beverages belong to the most popular (Candrawinata et al., 2012). Indeed, due to their rich phytochemical profile indicating their health-promoting action on the health of the consumers, intake of apples and apple juice/products is still increasing around the world (Hyson, 2011).

Apple fruits are the main dietary sources of antioxidants, in particular phenolic compounds (Tsao et al., 2005), of which mainly flavanols, flavonols, hydroxycinnamic acids, anthocyanins, and dihydrochalcones are the major polyphenolic groups (Łata et al., 2009). The presence of polyphenols has gained special attention in apples because of their association with an improved antioxidant activity (AA) related to their ability to scavenge free radicals (Kalinowska et al., 2014; Li et al., 2014). The strong AA of apples is of great importance for human health because it can prevent various chronic diseases (Boyer and Liu, 2004). Furthermore, the anticancer and antiviral potential of apple fruits has been revealed by many *in vitro* studies (Suárez et al., 2010; Jakobek et al., 2013).

Generally, the variations in the phenolic profile of apples depend on many factors, such as apple cultivar, growth period, growing season (Awad et al., 2000; Tsao et al., 2003), maturity, geographical location (Awad and Jager, 2002), storage conditions (Golding et al., 2001) and also processing parameters (Candrawinata et al., 2013).

Therefore, our study aimed to evaluate the total polyphenols content (TPC) and the AA in different apple cultivars, and thus to select the cultivars with the highest content of the valuable natural antioxidants for their application in the production of enrichment food products.

Material and methodology

Plant material

Plant material used in the current study included 7 apple cultivars grown under precisely defined the same experimental condition, i.e., Golden Delicious, Granny Smith, Goldcats and Rondo, Kordona, Redcats, and Pompink (Table 1), which were obtained from the Botanic Garden (Slovak University of Agriculture, Slovakia; vegetative season 2019/2020). The samples were harvested at the full maturity stage, and whole fruits without seeds were used for analyses.

Chemicals

All the chemicals were obtained from Reachem (Bratislava, Slovakia) and Sigma Aldrich (Saint-Louis, Missouri, USA) and used without further purification.

Preparation of extracts

Ethanolic extracts were prepared from apple tree fruit samples dried at 45 °C for 24 h using a laboratory hot air dryer (Universal oven UF 160, Memmert GmbH + Co.KG, Büchenbach, Germany). For each extraction, 0.2 g of sample was extracted by 20 mL of 80 % ethanol for 2 h and centrifuged at 4000 × g for 10 min in Rotofix 32A (Hettich, Spenge, Germany). The supernatants were used for measurement of total polyphenols content (TPC) and detection of antioxidant activity (AA).

Table 1 Characterization of studied cultivar samples.

Samples	Colour of peel	Planting year	Country of origin	Ripening period
Goldes Delicious	green-yellow	2003	USA	late
Granny Smith	green-yellow	2003	Australia	late
Goldcats	green-yellow	2014	Germany	medium/late
Rondo	green-yellow	2014	Czechia	medium/late
Kordona	green with red spots	2014	Czechia	medium/late
Redcats	red	2014	Germany	medium
Pompink	pink	2014	Germany	medium/late

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Evaluation of total polyphenols content

TPC was measured by the method of Valková et al. (2021) using the Folin-Ciocalteu reagent. In this case, 0.1 mL of apple extracts was mixed with 0.1 mL of Folin-Ciocalteu reagent, 1.0 ml of 200 g/L sodium carbonate, and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured using Jenway 6405 UV/Vis spectrophotometer (Cole-Parmer, Stone, United Kingdom). Gallic acid was used as a standard, and the results were expressed in grams per kilogram of gallic acid equivalents (GAE) dry weight (dw).

Evaluation of antioxidant activity

AA of extract samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to previously described procedures (Valková et al., 2021). Volumes of 0.4 mL of apple extracts were added to 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the reaction mixture at 515 nm was determined using a spectrophotometer (Jenway 6405 UV/Vis, Cole-Parmer, Stone, United Kingdom). The AA was calculated as a percentage (%) and determined according to the following formula: AA% = [(A0 – AAT)/A0 × 100]; where A0 is the absorbance of the control reaction (DPPH radical); A1 is the absorbance of the tested sample. The AA values increased in the following manner: weak (0–29 %) < medium-strong (30–59 %) < strong (60 % and more).

Statistical analysis

Data from all analyses, which were performed in triplicates, were statistically evaluated using Prism 8.0.1 (GraphPad Software, San Diego, California, USA). One Way analysis of variance (ANOVA) followed by Tukey's test was used to assess the significance of differences between the analysed groups of samples. The level of significance was set at $p < 0.05$.

Results and discussion

Total polyphenols content of samples

In general, spectrophotometric methods for the determination of total polyphenols have been widely used in fruit analysis, as well as in pomology (Ivanova et al., 2010). In our study, the TPC in apple cultivars was assessed using Folin-Ciocalteu assay, which allows the estimation of all flavonoids, anthocyanins, and non-flavonoid phenolic compounds, i.e., all phenolics present in the samples (Benvenuti et al., 2004). Our results showed that TPC in analysed fruit extracts ranged from 2.92 ±0.31 (cv. Pompink) to 9.02 ±0.32 mg GAE/g (cv. Granny Smith), with an average of 5.80 ±0.32 mg GAE/g (Table 2). Thus, the presented data demonstrate that the green-yellow peel cultivar of apples had a higher average TPC (7.13 ±0.32 mg GAE/g) than the other cultivars suggesting a relationship between TPC content and the colour of apple peel. Interestingly, cultivars planted in 2003 with the late-ripening period (cvs. Golden Delicious and Granny Smith) had a significantly higher TPC content than other ones planted in 2014 with medium or medium-late ripening period. Indicating that both factors (planting year, ripening period) could also be associated with the TPC of cultivars. On the other hand, the country of origin does not seem to play a key role in the TPC between the cultivars. Moreover, statistically significant differences ($p < 0.05$) were recorded between all analysed cultivars, except for Rondo (5.07 ±0.44 mg GAE/g) and Kordona (4.18 ±0.35 mg GAE/g) as compared to Redcats (4.96 ±1.06 mg GAE/g).

Polyphenols are antioxidant ingredients present in apple fruits, and because of their free radical scavenging activities, they exert a beneficial impact on human health (Kschonsek et al., 2018). As a very good source of polyphenols, they are widely consumed and available in supermarkets worldwide throughout the year (Wojdyło et al., 2008). Moreover, polyphenols are also closely related to the flavor and colour of apple products, and their composition notably depends on the apple cultivars (Guo et al., 2013).

Regarding the Granny Smith cultivar, its TPC was estimated by Alberto et al. (2006) to be 6.80 ±0.15 mg GAE/g, which is a lower value as it was demonstrated by our result (9.02 ±0.15 mg GAE/g). Values for TPC in cv. Granny Smith were also evaluated in the researches by Lotito and Frei (2004), and Henríquez et al. (2010); however, the comparison between our finding and the published data could not be performed since the values for TPC were determined either by different methods or in different units. Further, the TPC of ethanolic extract obtained from our Golden Delicious (7.71 ±0.27 mg GAE/g) was consistent with a previous report by Massias et al. (2015) showing the TPC of the methanolic extract of this cultivar being 7.92 mg GAE/g. In contrast, the study by Junjian et al. (2013) revealed that the TPC of such methanolic extract was only 2.98 mg GAE/g. We assume that these differences (between our and other studies) can be attributed to biological factors (i.e., genotype and cultivar), as well as edaphic and environmental (i.e., temperature, light intensity, stress) conditions, and agricultural practices (fertilization, mulch colour, early forcing, and planting date). In addition, the solubility of polyphenols also depends on

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Table 2 Antioxidant activity and total content of polyphenols in samples of apple extracts

Colour	Cultivars	TPC (mg GAE/g)	AA (%)
Green-yellow	Golden Delicious	7.71 ±0.27a	88.49 ±0.73a
	Granny Smith	9.02 ±0.32b	88.55 ±4.25ad
	Goldcats	6.73 ±0.26c	65.66 ±1.66b
	Rondo	5.07 ±0.44d	61.92 ±4.74b
Green with red spots	Kordona	4.18 ±0.35e	41.69 ±2.07c
Red	Redcats	4.96 ±1.06def	84.38 ±1.31d
Pink	Pompink	2.92 ±0.31g	35.03 ±3.49e

Notes: means ± standard deviation. Values in the same column with different letters are significantly different ($p < 0.05$)

the solvent used, the degree of polymerization of the phenolic compounds, and their interaction (Anttonen et al., 2006; Markowski and Plocharski, 2006; Mureşan et al., 2012; Zhang et al., 2016).

Antioxidant activity of samples

The AA of apple cultivars was measured by the DPPH method in which the colour changes from purple DPPH radical to reduced yellow diamagnetic 2-diphenyl-1-picryl hydrazine are detected (Asale et al., 2021). The values for inhibition of DPPH (%) in apple extracts are summarized in Table 2. Depending on apple cultivars, antioxidant DPPH capacity in measured samples ranged from 35.03 ±3.49 (medium strong; cv. Pompink) to 88.55 ±4.25 % (strong; cv. Granny Smith). Demonstrable differences ($p < 0.05$) were recorded among all analysed cultivars, except for between Granny Smith, Golden Delicious, and Redcast extract samples. Considering of the peel colour, the AA of the samples increased in the following manner: 35.03 ±3.49 % (pink cv.) <41.69 ±2.07 % (green with red spots cv.) <76.16 ±14.36 % (green-yellow cv.) <84.38 ±1.31 % (red cv.). A similar trend of the relationship between planting year and ripening period and TPC was also observed in the case of AA.

Being an important source of natural antioxidants, apples possess one of the highest levels of radical scavenging capacity from the most consumed fruits and vegetables (Chinnici et al., 2004). Similar to our findings (88.55 ±4.25 and 88.49 ±0.73 %, respectively), strong values for AA in the methanolic extracts of Granny Smith (92.71 ±0.33 %) and Golden Delicious (70.73 ±1.49 %) were detected by Asale et al. (2021). Moreover, our results showed that values for AA increased in proportion to their TPC. This fact was also confirmed by the studies of Loots et al. (2006), and Tsao et al. (2005) who reported a positive correlation between both parameters investigated.

The current research revealed that apple samples analysed contained a relatively high TPC and exerted good antioxidant properties. In this context, their consumption may reduce the prevalence of various diseases including cardiovascular disorders and cancer (Knekt et al., 1997; Reagan-Shaw et al., 2010), thereby having a beneficial effect on human health. Taking into account this aspect, the World Health Organization (WHO) recommends eating 400 g of fruits and vegetables per day (approx. 5 pieces) (Dhandevi and Jeewon, 2015). Hence, the consumption of apples and their incorporation into developed food products may be considered one of the appropriate tools for improving the global health of the human population.

Conclusions

The goal of this study was to screen the TPC and AA in ethanolic extracts from seven different *Malus domestica* cultivars. The obtained data clearly demonstrates that the TPC in extracts was cultivar-dependent ranging from 2.92 ±0.31 (cv. Pompink) to 9.02 ±0.32 mg GAE/g (cv. Granny Smith). In effect, the higher values for TPC were observed in green-yellow peel cv. of apples as compared to the other ones. Since growing experimental conditions were the same, our results suggested that TPC content is associated with the colour of apple peel, ripening period, and also the age of apple trees. Moreover, the analysed samples did not display uniform DPPH radical scavenging activity. Indeed, its values ranged, similar to TPC, from 35.03 ±3.49 (cv. Pompink) to 88.55 ±4.25 % (cv. Granny Smith). From the analysed set of cultivars, our results showed that cv. Granny Smith and cv. Golden Delicious seems to be the most suitable for consumption in the daily diet and the development of enrichment food products to improve human health. In effect, both aspects (high amount of TPC and AA strong values) can help to increase the presence of biologically active substances within human nutrition.

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Conflicts 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

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Morphometric parameters of plants of *Crambe* spp. during vegetation

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Plants of *Crambe* spp. belong to Brassicaceae Burnett family and some of them are known as economically important species. Extracts of *Crambe* spp. exhibited numerous biological activities such as antioxidant, antimicrobial, antiproliferative, cytotoxic, etc. This study was aimed to research morphometric parameters of plants of five species of *Crambe* L. during vegetation season (from the start of vegetation, budding stage, flowering stage, and to fruitage): *C. cordifolia* Steven, *C. hispanica* subsp. *abyssinica* (Hochst. et R.E.Fr.) Prina, *C. koktebelica* (Junge) N. Busch, *C. maritima* L., *C. steveniana* Rupr. The plant height, leaf length, leaf width, petiole length, length of panicle measured in cm, flower length, flower width, corolla length, petal length, petal width, fruit length, and fruit diameter measured in mm. The increment of investigated plants at the period of vegetation start-budding was 47.7–128.9 cm and plant height increased 2.67 (*C. maritima*) – 5.57 (*C. koktebelica*) times, at the budding-flowering period 9.7–94.4 cm and plant height increased 1.08 (*C. cordifolia*) – 1.85 (*C. steveniana*) times, at the period flowering-fruiting 8.20–26.0 cm and plant height increased 1.06 (*C. cordifolia*) – 1.10 (*C. maritima*) times. The variability of morphometric features during vegetation was following: height of plant from 0.57 to 13.98 %, length of leaf from 1.25 to 13.65 %, width of leaf from 2.17 to 48.10 %, length of petiole from 3.34 to 18.01 %, length of panicle from 1.41 to 13.09 %, width of panicle from 1.62 to 17.37 %, and stem diameter from 2.89 to 23.47 %. The study of morphometric parameters of flowers showed that the length of flower was 5.03–16.46 mm, width of flower 2.01–5.54 mm, corolla length 2.94–10.58 mm, petal length 3.63–7.11 mm, and petal width 3.64–5.51 mm depending on species. The morphometric parameters of fruits were the following: fruit length 3.02–10.10 mm and fruit diameter 4.08–8.78 mm depending on species. The thousand-fruit weight was 7.30–12.50 g. Selected morphometric parameters showed Pearson's coefficients with high values (r = 0.843–0.994) during vegetation. A comparative study of the morphometric parameters of investigated species of *Crambe* in M.M. Gryshko National Botanical Garden had a variability of morphometric parameters depending on species, period of growth, and organ of a plant. These results can be useful for selective work and detecting the diagnostic signs.

Keywords: *Crambe*, morphometric parameters, correlation

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Introduction

Plants of the *Crambe* L. genus belong to Brassicaceae Burnett family and contain around 40 species (Francisco-Ortega et al., 2002; Prina, 2021). Some species from this genus are promising fodder, food, decorative and medicinal plants (Kalista, 2017). Plant raw is a rich source of nutrients (Vergun et al., 2018), especially ascorbic acid (Vergun et al., 2019). HPLC analysis of amino acid composition detected that *Crambe cordifolia* Steven and *C. koktebelica* (Junge) N.Busch contain the L-glutamic acid, glycine, L-arginine, L-leucine, which were predominant for both species (Slobodianiuk et al., 2021). Plant extracts of *Crambe* spp. exhibited cytotoxic, antiproliferative, allelopathic (Razavi and Nejad-Ebrahimi, 2009), antioxidant, and antimicrobial activities (Vergun et al., 2021). The essential oil of the leaves and flowering tops of C. orientalis includes nitriles, isothiocyanates, esters, fatty acids, alkanes, ketones, aldehydes, terpenes. The major components in the flowering top oil were 2-methyl-5-hexenenitrile and 3-butenyl isothiocyanate, in leaves octyl-acetate (Razavi and Nejad-Ebrahimi, 2009). The most well-known and economically important species among others is *C. hispanica* subsp. *abyssinica* (Hochst. et R.E.Fr.) that produces valuable vegetable oil with an erucic acid content of up to 50 % (Queiroz et al., 2019; Samarappuli et al., 2020). Through genetic engineering and cross, breeding was obtained transgenic crambe lines producing wax esters that are important industrial feedstock (Li et al., 2019). This species is used as an alternative renewable energy source for biodiesel production (Kurt et al., 2018).

The study of shoot's system of different species showed that these plants are chamaephytes (Sanyal and Decocq, 2015) or hemicryptophytes (Scherbakova and Kalistaya, 2013; Sanyal and Decocq, 2015). *C. abissinica* and *C. maritima* can grow on saline soils and be tolerant to salty water irrigation (Vos et al., 2010; Qi et al., 2017).

Considering the important value of these plants and the lack of information this work was aimed to study some morphometric peculiarities of five *Crambe* species growing in the Forest-Steppe of Ukraine. The study of morphometric parameters of plants is a very important aspect due to origin, condition of growth, especially concerning introduction in a new area, peculiarities of vegetative reproduction (Buckley et al., 1997; Brindza et al., 2019). The research of morphometric parameters is also valuable for the ecological estimation of plants (Meng et al., 2009).

Material and methodology

Biological material

In this study investigated species from *Crambe* L.: *C. cordifolia* Steven, *C. hispanica* subsp. *abyssinica* (Hochst. et R.E.Fr.) Prina, *C. koktebelica* (Junge) N. Busch, *C. maritima* L., *C. steveniana* Rupr. The plants were studied in 2018–2019 at the experimental fields of the M.M. Gryshko National Botanical Garden of the NAS of Ukraine in the Kyiv city (50° 24' 55" N, 30° 33' 45" E) during vegetation season. Photos of flowers taken with digital USB microscope Sigeta Expert.

Morphometric characteristics

Morphometric parameters fixed at the start of vegetation, budding, flowering, and fruitage. The measuring of plant height, length, and width of leaves, length of petioles, length of panicles were used. The length and width of flower, corolla length, length, and width of petal were used as morphometric parameters of flowers. The fruit length and diameter of fruits were used to measure the fruits. Thousand-fruit mass weighed on analytical scales Kern ACJ.

Statistical analysis

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

Results and discussion

The study of morphological, morphometric, physiological, etc., characteristics of plants due to climate change is becoming a global last time. The temperature fluctuations influence duration of the vegetation period and biomorphological peculiarities of plants (Aslam et al., 2021). The leaf is one of the most significant features for the identification of concrete species in plant morphology among other plant organs. Wherein have used different classifications of leaf features (Kumar et al., 2019). The morphometric parameters of leaves can be useful in ecophysiological study to estimate the tolerance of a plant to the environment, for example, in the case of *C. maritima* as a salt-tolerant crop (Vos et al., 2010).

Investigated plants of *Crambe* belong to the perennial (*C. cordifolia*, *C. koktebelica*, *C. maritima*, *C. steveniana*) and annual (*C. hispanica* subsp. *abyssinica*) plants. They originate from the Mediterranean and East African regions, species distributed from Macronesian archipelagoes to the West of China and from the

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Figure 1 Plants of *Grambe* L. genus at the start of vegetation
1 – *C. hispanica* subsp. *abyssinica* (Hochst. et R.E.Fr.) Prina; 2 – *C. cordifolia* Steven; 3 – *C. koktebelica* (Junge) N. Busch; 4 – *C. maritima* L.; 5 – *C. steveniana* Rupr.

Arctic Polar Circle to the Scandinavian Peninsula, etc. (Prina, 2009). In conditions of the Ukrainian Forest-Steppe, these plants pass a full cycle of growth and development. The duration of vegetation period for *C. cordifolia*, *C. koktebelica*, *C. maritima*, *C. steveniana* was 122, 121, 98, and 124 days, respectively. We observed that the average start of vegetation for all perennial plants was on 13th March–2nd April, budding started on 27th April–14th May, flowering started on 10th May–7th June, and fruiting on 3rd June–12th July. The start of growth at the beginning of vegetation depending on climate conditions of years that was also noticed in the study Fontana et al. (1998).

At the start of vegetation (spring growing), beginning from the second year, plants formed the rosette of leaves (Figure 1) after that formed following vegetative-generative plant shoots. This period continues to average 44–62 days.

We measured morphometric parameters during vegetation period, and plant height at the start of vegetation was from 15.00 to 28.40 cm (Table 1). Leaf length and width in this period were 9.00–20.20 cm and 5.03–15.36 cm, respectively. The length of petioles was from 2.96 to 16.37 cm. The variability of morphometric parameters (V %) at the period of start vegetation was following: plant height 2.71–13.98 %, leaf length 3.71–13.65 %, leaf width 2.55–11.44 %, length of petiole 4.88–11.94 %. The level of variability of plant morphometric parameters is often studied in the context of weather conditions such as a sum of effective temperatures, precipitations (Mikolaychuk, 2007), and changing of growth conditions (Gorlacheva and Kustova, 2013). The study of selected morpho-biometric parameters of plants is important to research differences between genotypes (Bella et al., 2020).

At the budding stage height of plants was from 63.80 to 157.10 cm, leaf length from 18.13 to 64.60 cm, leaf width from 7.13 to 39.00 cm, length of petiole from 8.31 to 30.10 cm, and length of panicles from 18.00 to 147.80 cm depending on species (Table 2). The increment of investigated plants was 47.7–128.9 cm and plant height increased 2.67 (*C. maritima*)–5.57

Table 1 Morphometric parameters of plants of *Crambe* spp. at the start of vegetation

Species	Height of plant (cm)	Leaf length (cm)	Leaf width (cm)	Length of petiole (cm)
C. cordifolia	27.80 ±0.24^{a}	17.62 ±0.21^{a}	15.36 ±0.13^{a}	16.37 ±0.28^{a}
C. hispanica* subsp. *abyssinica	15.00 ±0.67^{c}	11.29 ±0.49^{c}	5.03 ±0.18^{c}	2.96 ±0.08^{d}
C. koktebelica	28.20 ±0.65^{a}	13.25 ±0.20^{b}	9.17 ±0.08^{b}	6.35 ±0.10^{b}
C. maritima	28.40 ±1.11^{a}	9.00 ±0.30^{d}	6.80 ±0.20^{c}	4.54 ±0.17^{c}
C. steveniana	25.00 ±0.30^{b}	20.20 ±0.25^{a}	12.35 ±0.15^{a}	8.88 ±0.16^{b}

Notes: different superscripts in each column indicate the significant differences in the mean at $p < 0.05$

Table 2 Morphometric parameters of plants of *Crambe* spp. at the budding stage

Species	Height of plant (cm)	Leaf length (cm)	Leaf width (cm)	Length of petiole (cm)	Length of panicle (cm)
C. cordifolia	116.00 ±1.01^{b}	37.80 ±0.64^{b}	39.00 ±0.78^{a}	29.90 ±0.82^{a}	75.60 ±0.74^{b}
C. hispanica* subsp. *abyssinica	63.80 ±1.72^{c}	18.13 ±0.29^{c}	7.13 ±0.13^{c}	8.31 ±0.20^{c}	18.00 ±0.75
C. koktebelica	157.10 ±1.24^{a}	64.60 ±1.18^{a}	26.60 ±0.74^{b}	13.80 ±0.36^{b}	147.80 ±2.00^{a}
C. maritima	76.10 ±1.22^{c}	29.50 ±0.76^{b}	25.60 ±1.53^{b}	16.60 ±0.95^{b}	66.10 ±0.70^{b}
C. steveniana	80.60 ±2.56^{c}	35.00 ±0.80^{b}	27.00 ±0.78^{b}	30.10 ±1.43^{a}	79.70 ±0.58^{b}

Notes: different superscripts in each column indicate the significant differences in the mean at $p < 0.05$

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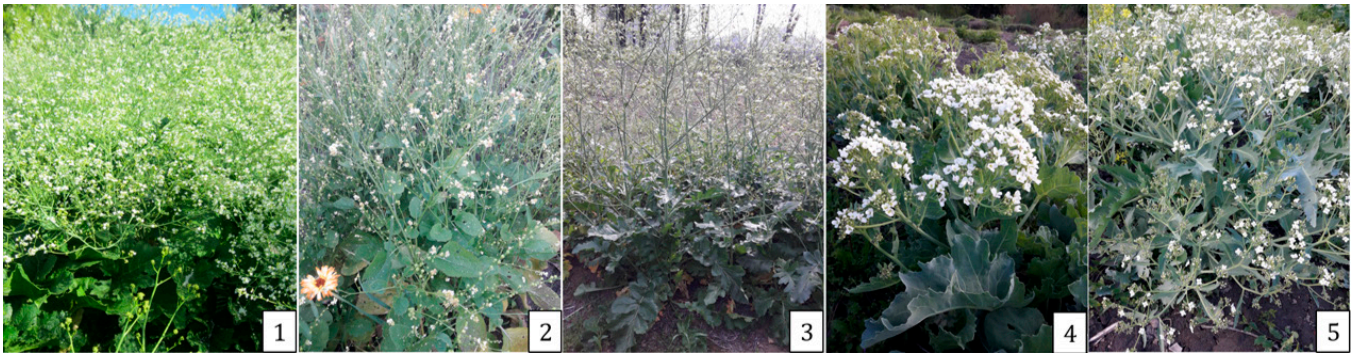

Figure 2 Plants of *Grambe* L. at the flowering stage
1 – *C. cordifolia* Steven; 2 – *C. hispanica* subsp. *abyssinica* (Hochst. et R.E.Fr.) Prina; 3 – *C. koktebelica* (Junge) N. Busch; 4 – *C. maritima* L.; 5 – *C. steveniana* Rupr.

Figure 3 Flowers of plants of *Grambe* L.
1 – *C. cordifolia* Steven; 2 – *C. hispanica* subsp. *abyssinica* (Hochst. et R.E.Fr.) Prina; 3 – *C. koktebelica* (Junge) N. Busch; 4 – *C. maritima* L.; 5 – *C. steveniana* Rupr.

(*C. koktebelica*) times. At the budding stage, the variability of morphometric parameters was 2.46–9.95 % for plant height, 5.04–8.03 % for leaf length, 5.53–18.71 % for leaf width, 7.70–18.01 % for length of petiole, 2.29–13.09 % for panicle length, 3.25–17.37 % for panicle width, and 6.02–23.47 % for stem diameter.

At the flowering stage (Figure 2) length of plants was from 73.50 to 251.50 cm, leaf length from 21.60 to 86.90cm, leaf width from 8.22 to 68.10 cm, length of petioles from 10.55 to 42.70 cm, and length of panicles from 25.60 to 160.90 cm depending on species (Table 3).

The height of plants of *C. hispanica* subsp. *abyssinica* was 51.9–90.7 cm, according to Kurt et al. (2018). The increment of studied plants at the budding-flowering period was 9.7–94.4 cm and plant height increased 1.08 (*C. cordifolia*)–1.85 (*C. steveniana*) times. The variability of morphometric parameters at the period of flowering was following: plant height 1.90–3.64 %, leaf length 2.81–10.28 %, leaf width 2.28–48.10 %, length of petiole 5.91–9.89 %, length of panicles 2.77–9.24 %, width of panicle 2.46–6.78 %, and 6.21–9.90 %.

Flowers of *Crambe* spp. are typical for Brassicaceae and consist of 4 petals, 6 stamens (Figure 3) and collected in the panicles.

Flower elements had the following values depending on species: flower length was 5.03–16.46 mm, flower width was 0.23–0.55 mm, corolla length was 2.94–7.14 mm, petal length 3.63–6.56 mm, petal width 3.64–5.51 mm (Table 4).

Table 3 Morphometric parameters of plants of *Crambe* spp. at the flowering

Species	Height of plant (cm)	Leaf length (cm)	Leaf width (cm)	Length of petiole (cm)	Length of panicle (cm)
C. cordifolia	126.30 ±0.77b	43.80 ±0.84b	42.30 ±0.60b	38.30 ±0.72a	84.80 ±0.75b
C. hispanica* subsp. *abyssinica	73.50 ±0.86c	21.60 ±0.71c	8.22 ±0.06c	10.55 ±0.20c	25.60 ±0.76c
C. koktebelica	251.50 ±2.84a	67.60 ±0.61a	29.80 ±0.36b	21.20 ±0.67b	160.90 ±1.97a
C. maritima	95.10 ±0.83c	48.00 ±0.90b	35.70 1.20b	28.80 ±0.62b	79.90 ±1.16b
C. steveniana	149.60 ±1.78b	86.90 ±0.99a	68.10 ±1.20a	42.70 ±0.84a	92.20 ±0.85b

Notes: different superscripts in each column indicate the significant differences in the mean at $p < 0.05$

At the fruiting stage length of plants was from 78.50 to 277.90 cm, leaf length from 25.60 to 96.10 cm, leaf width from 10.50 to 77.80 cm, length of petioles from 12.50 to 53.20 cm, and length of panicles from 33.80 to 186.50 cm (Table 5). At the period flowering-fruiting increment of investigated plants was 8.20–26.0 cm. According to Rakhmetov and Rakhmetova (2015), the increment of different genotypes of *Brassica rapa* L. × *B. campestris* f. *biennis* DC. at the period of flowering-ripening was in the range 10.1–20.6 cm. The variability of investigated parameters was 0.57–4.04 % for plant height, 1.25–6.39 % for leaf length, 2.17–6.73 % for leaf width, 3.34–6.37 % for length of petiole, 1.41–6.80 % for length of flowering, 1.62–4.93 % for the width of panicle, 2.89–8.00 % for stem diameter.

According to faceted classification, developed by Iljinska (2013), fruits of the *Crambe* genus are related to choriarticulate-pseudoseptate dimericarps. *Crambe* fruits consist of two segments, where the only top seed-containing segment is fully developed (Kalista et al., 2014). Fruit length in our study was 3.02–10.10 mm, diameter 3.41–8.78 mm (Table 6). One of the most important characteristics of fruits and seeds is the thousand-fruit weight. That is a significant parameter, among others, of seed and oil yield (Kwiatkowski et al., 2020). Thousand-fruit weight in our study was 7.30–12.50 g. Kwiatkowski et al. (2020) determined this parameter for *C. abyssinica* as 7 g on average that was 1.5 g less than in our study.

The study of correlations between morphometric parameters is widely used in botanical science for different plant parts such as fruits (Ivanišová et al., 2017; Grygorieva et al., 2018a; Mangino et al., 2021), leaves (Chitwood and Otoni, 2017; Grygorieva et al., 2018b), etc. Between studied morphometric parameters, we found a correlation with different levels. A very strong correlation was found between leaf width and petiole length (r = 0.959), leaf length and width (r = 0.844), a strong correlation was between leaf length and length of petiole (r = 0.708) at the start of vegetation period (Table 7).

Table 4 Morphometric parameters of *Crambe* spp. flowers

Species	Flower length (mm)	Flower width (mm)	Corolla length (mm)	Petal length (mm)	Petal width (mm)
C. cordifolia	14.91 ±0.21^{b}	4.52 ±0.01^{a}	6.35 ±0.05^{b}	5.61 ±0.08^{a}	4.54 ±0.08^{a}
C. hispanica* subsp. *abyssinica	12.04 ±0.18^{b}	2.01 ±0.06^{b}	10.58 ±0.07^{a}	7.11 ±0.26^{a}	4.22 ±0.14^{a}
C. koktebelica	5.03 ±0.09^{c}	2.32 ±0.01^{b}	2.94 ±0.07^{c}	3.63 ±0.04^{b}	3.64 ±0.07^{b}
C. maritima	16.14 ±0.72^{a}	4.27 ±0.04^{a}	6.23 ±0.21^{b}	5.49 ±0.24^{a}	4.71 ±0.10^{a}
C. steveniana	16.46 ±0.10^{a}	5.54 ±0.01^{a}	7.14 ±0.28^{b}	6.56 ±0.09^{a}	5.51 ±0.07^{a}

Notes: different superscripts in each column indicate the significant differences in the mean at p <0.05

Table 5 Morphometric parameters of plants of *Crambe* spp. at the fruiting

Species	Height of plant (cm)	Leaf length (cm)	Leaf width (cm)	Length of petiole (cm)	Length of panicle (cm)
C. cordifolia	134.70 ±0.48^{a}	53.70 ±1.10^{b}	47.20 ±0.42^{b}	44.70 ±0.48^{a}	94.70 ±0.71^{b}
C. hispanica* subsp. *abyssinica	78.50 ±1.01^{b}	25.60 ±0.50^{c}	10.50 ±0.23^{c}	12.50 ±0.23^{c}	33.80 ±0.73^{c}
C. koktebelica	277.90 ±0.51^{a}	76.70 ±0.71^{a}	37.30 ±0.48^{b}	25.00 ±0.40^{b}	186.50 ±0.84^{a}
C. maritima	104.40 ±0.80^{b}	53.70 ±0.58^{b}	43.80 ±0.47^{b}	36.30 ±0.74^{b}	87.80 ±0.56^{b}
C. steveniana	162.70 ±0.67^{a}	96.10 ±0.38^{a}	77.80 ±0.54^{a}	53.20 ±0.62^{a}	103.10 ±0.53^{b}

Notes: different superscripts in each column indicate the significant differences in the mean at p <0.05

Table 6 Morphometric parameters and mass of fruits of *Crambe* spp.

Species	Fruit length (mm)	Fruit diameter (mm)	Thousand-fruit weight (g)
C. cordifolia	3.20 ±0.13^{b}	4.08 ±0.06^{b}	7.30 ±0.15^{b}
C. hispanica* subsp. *abyssinica	3.02 ±0.12^{b}	3.41 ±0.09^{c}	8.50 ±0.17^{b}
C. koktebelica	4.10 ±0.10^{b}	4.30 ±0.10^{b}	8.70 ±0.15^{b}
C. maritima	10.10 ±0.35^{a}	8.78 ±0.15^{a}	12.50 ±0.23^{a}
C. steveniana	5.30 ±0.22^{b}	5.58 ±0.16^{b}	11.10 ±0.18^{a}

Notes: different superscripts in each column indicate the significant differences in the mean at p <0.05

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Table 7 Pearson's coefficients between morphometric parameters of *Crambe* spp. at the start of vegetation

Parameter	Height of plant	Length of leaf	Width of leaf	Length of petiole
Length of leaf	0.173	1		
Width of leaf	0.537*	0.843**	1	
Length of petiole	0.473	0.708*	0.959**	1

Notes: ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

Table 8 Pearson's coefficients between morphometric parameters of *Crambe* spp. at the budding stage

Parameter	Height of plant	Length of leaf	Width of leaf	Length of petiole	Length of panicle
Length of leaf	0.955**	1			
Width of leaf	0.524*	0.489	1		
Length of petiole	0.074	0.093	0.792*	1	
Length of panicle	0.904**	0.986**	0.539*	0.162	1

Notes: ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

Table 9 Pearson's coefficients between morphometric parameters of *Crambe* spp. at the flowering stage

Parameter	HP	LL	WL	LP	LPN	LF	WF	LC	LPT
LL	0.641*	1							
WL	0.232	0.843**	1						
LP	0.103	0.659*	0.945**	1					
LPN	0.953**	0.676*	0.334	0.246	1				
LF	-0.731*	-0.004	0.483	0.585	-0.588	1			
WF	-0.144	0.566*	0.911**	0.953**	0.017	0.780*	1		
LC	-0.858**	-0.577	-0.305	-0.280	-0.968**	0.495	-0.067	1	
LPT	-0.831**	-0.329	0.007	0.014	-0.910**	0.685*	0.232	0.949**	1
WP	-0.408	0.426	0.767*	0.733*	-0.324	0.873**	0.884**	0.332	0.605*

Notes: HP – height of plants; LL – length of leaf; WL – width of leaf; LP – length of petiole; LPN – length of panicles; LF – length of flower; WF – width of flower; LC – length of corolla; LPT – length of petal; WP – width of petal; ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

Table 10 Pearson's coefficients between morphometric parameters of *Crambe* spp. at the fruiting

Parameter	HP	LL	WL	LP	LPN	FL	FD
LL	0.659*	1					
WL	0.260	0.876**	1				
LP	0.072	0.701*	0.947**	1			
LPN	0.970**	0.673*	0.328	0.175	1		
FL	-0.195	0.136	0.272	0.260	0.001	1	
FD	-0.195	0.196	0.363	0.365	0.008	0.994**	1
TFW	-0.175	0.324	0.420	0.313	-0051	0.905**	0.901**

Notes: HP – height of plants; LL – length of leaf; WL – width of leaf; LP – length of petiole; LPN – length of panicles; FL – length of fruit; FD – fruit diameter; TFW – thousand-fruit weight; ** – correlation is significant at the level of 0.01; * – correlation is significant at the level of 0.05

A very strong correlation was determined between leaf length and petiole length (r = 0.986), plant height and leaf length (r = 0.955), the height of plant and panicle length (r = 0.905), and strong relation found between leaf width and petiole length (r = 0.792) at the budding period (Table 8).

At the flowering stage, a very strong correlation was detected between petiole length and plant height (r = 0.954), length of petiole and width of flower (r = 0.953), length of corolla and length of petal (r = 0.949), petiole length and leaf width (r = 0.945), width of flower and width of petal (r = 0.884), and between width and length of the leaf (r = 0.843) (Table 9).

At the fruiting period length of fruit strongly correlated with fruit diameter (r = 0.994), plant height and length of panicle (r = 0.970), plant height and petiole length (r = 0.969), leaf width and petiole length (r = 0.947), flower length and thousand-fruit weight (r = 0.905), length and width of leaf (r = 0.877), length of leaf and length of petiole (r = 0.701) (Table 10).

Conclusion

Thus, a study of the morphometric parameters of investigated species of *Crambe* genus in M.M. Gryshko National Botanical Garden can be useful for further biological investigations of these plants due to the previous partial lack of data. We found that the width of leaf for *C. steveniana* at the flowering was the most variable parameter and the plant height for *C. koktebelica* at the fruiting was the least variable one. Also, considering obtained data, the results can be used for selective work with plants of *Crambe* spp. In addition, assume that some results of our study may use for the determination of diagnostic signs.

Conflicts 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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Review

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Phytohormones

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During evolution in plants, a sophisticated system of receptors and signaling pathways has emerged that allows for appropriate responses and responses to these signals. One of such signals is the plant hormone – phytohormone. Abscisic acid is a sesquiterpene, which has important roles in seed development and maturation, in the synthesis of proteins and compatible osmolytes, which enable plants to tolerate stresses due to environmental or biotic factors, and as a general inhibitor of growth and metabolic activities. Abscisic acid is essential for the initiation and maintenance of tuber dormancy. Its main antagonist is gibberellic acid, which affects carbohydrate metabolism, indicating the budding of tubers. Gibberellic acid is a naturally occurring plant hormone that is used as a plant growth regulator to stimulate both cell division and elongation that affects leaves and stems. Salicylic acid belongs to a group of plant phenols that play an important role in the regulation of plant growth, development, and interaction with other organisms. Salicylic acid (SA), a phytohormone, is a promising compound that can reduce the sensitivity of plants to environmental stresses through regulation of the antioxidant defense system, transpiration rates, stomatal movement, and photosynthetic rate. Phytohormones are essential regulators of plants in two physiological processes that coordinate growth, reproduction, and stress resistance. These molecules also show biological activities on human cells and animal models.

Keywords: phytohormones, abscisic acid, gibberellic acid, salicylic acid

Introduction

The first mention of plant hormones (phytohormones) dates back to the 19th century when the German botanist Julius Von Sachs predicted the existence of a cell messenger responsible for the formation and growth of plant organs (Taiz et al., 2014). According to this theory, the messenger is influenced by external influences, such as gravity. Although these views were not supported by any known chemicals, they later led to the discovery of phytohormones. Around the same time, Charles Darwin, along with his son Francis, observed the effect of light on the growth of the *Phalaris canariensis* (Darwin, 1880). In the case of lighting the coleoptiles on one side, there was bending and growth of the plant behind the light. However, this phenomenon did not occur when the tip was covered with a cap. Based on these results, Darwin formulated a hypothesis that assumes the existence of a certain signal arising at the peak of the growth peak and propagating to the site of bending (Christie

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and Murphy, 2013). The term phytohormone was first defined in 1948 by the plant physiologist Thimann, to distinguish between animal and plant hormones (Went and Thimann, 1948).

Phytohormones (plant hormones) are chemical compounds produced in plants in very low concentrations, but able to regulate the most important developmental and growth processes of the plant cell. Phytohormone concentrations can vary considerably between organ types, for example in the roots compared to leaves (Ciura and Kruk, 2018). At the same time, they work as chemical messengers to communicate cellular processes in higher plants over longer distances, thereby mediating signaling between plant organs (Voß et al., 2014). Phytohormones play key roles and coordinate various signaling pathways during the response to abiotic stress. They regulate both external and internal stimuli (Kazan, 2015). Plant hormones determine the formation of flowers, stems, leaves, leaf fall, and the development and ripening of the fruit. They also shape the plant, affect seed growth, flowering time, sex of flowers, ageing of leaves and fruits. They are vital for plant growth, and without them, plants would only be a mass of undifferentiated cells (Procházka et al., 1998). The best-known groups of phytohormones include auxins, cytokinins (CK), gibberellins, ethylene, abscisic acid (ABA), and brassinosteroids (Kundan et al., 2015; Ciura and Kruk, 2018).

Abscisic acid

Abscisic acid (ABA) (Figure 1) is an optically active C15 carboxylic acid terpenoid. Its discovery dates back to the early 1960s when it was found to be involved in seed germination (Chen et al., 2019). According to the orientation of the carboxyl group on the second carbon, we can distinguish the cis and trans isomers of ABA. Thus, since ABA is optically active, it can exist as the R or S isomer. The S form is active naturally occurring, while the R form is inactive and occurs in plant vents (Taiz and Zeiger, 2010).

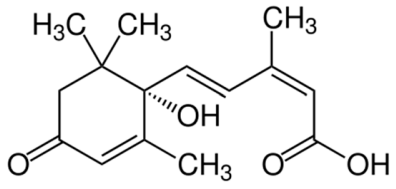

Figure 1 Abscisic acid (https://upload.wikimedia.org/wikipedia/commons/thumb/d/d9/Abscisic_acid_Structural_Formula_V1.svg/440px-Abscisic_acid_Structural_Formula_V1.svg.png)

The original name of abscisic acid was abscisin II and was first isolated from cotton (Humplík et al., 2017). ABA plays a vital role in many physiological processes during plant growth. Its important roles include fruit development, responses to biotic and abiotic stresses (Dong et al., 2015; Vishwakarma et al., 2017).

ABA can act as a promoter but also as an inhibitor. Its main effects and functions include regulating the water regime of plants, closing, and opening vents, inhibiting elongation growth, regulating dormancy (Vishwakarma et al., 2017). ABA reduces the growth rate of growing tissues and organs, so it is an auxin antagonist in this effect. This effect is accompanied by a reorientation of the microtubules longitudinally in the direction of growth. However, if the concentration of ABA is low, prolonged growth will not be affected (Procházka et al., 1998).

The most important function of the ABA is to regulate the water regime of plants. If the plant is deficient in water, the ABA will cause the vents to close. When there is a lack of water, there is a rapid increase in ABA in roots and leaves. In dehydration, if ABA levels are high, there is a positive stimulation of root growth and, conversely, inhibition of shoot growth (Vishwakarma et al., 2017). When there is a lack of water in the leaves, the ABA is transferred from the mesophyll to the epidermis, where it causes the airways to close and restrict transpiration. Thus, abscisic acid is considered to be a defense mechanism of the plant against stress, it reduces the negative effect not only in insufficient hydration of the plant but also at low temperature or salinity and can adapt the plant to these conditions (Procházka et al., 1998; Nambara, 2017).

Abscisic acid exists naturally in plants as both the anionic form (ABA–) and the protonated form (ABAH). ABAH can diffuse passively across the plasma membrane, and ABA diffusion decreases significantly with cytoplasmic alkalization, which increases during osmotic stress (Chen et al., 2019).

Biosynthesis of ABA

Two ABA biosynthesis pathways have been proposed. In the forward pathway (sesquiterpenoid pathway), ABA is derived from farnesyl diphosphate (FDP) (Chen et al., 2019). In the indirect pathway (carotenoid pathway), ABA is formed by the cleavage of carotenoids (Nambara, 2017). This pathway has been proposed due to the structural similarity between ABA and xanthoxin (a carotenoid degradation product). It has been confirmed by several biochemical studies and is therefore thought to be synthesized by ABA in higher

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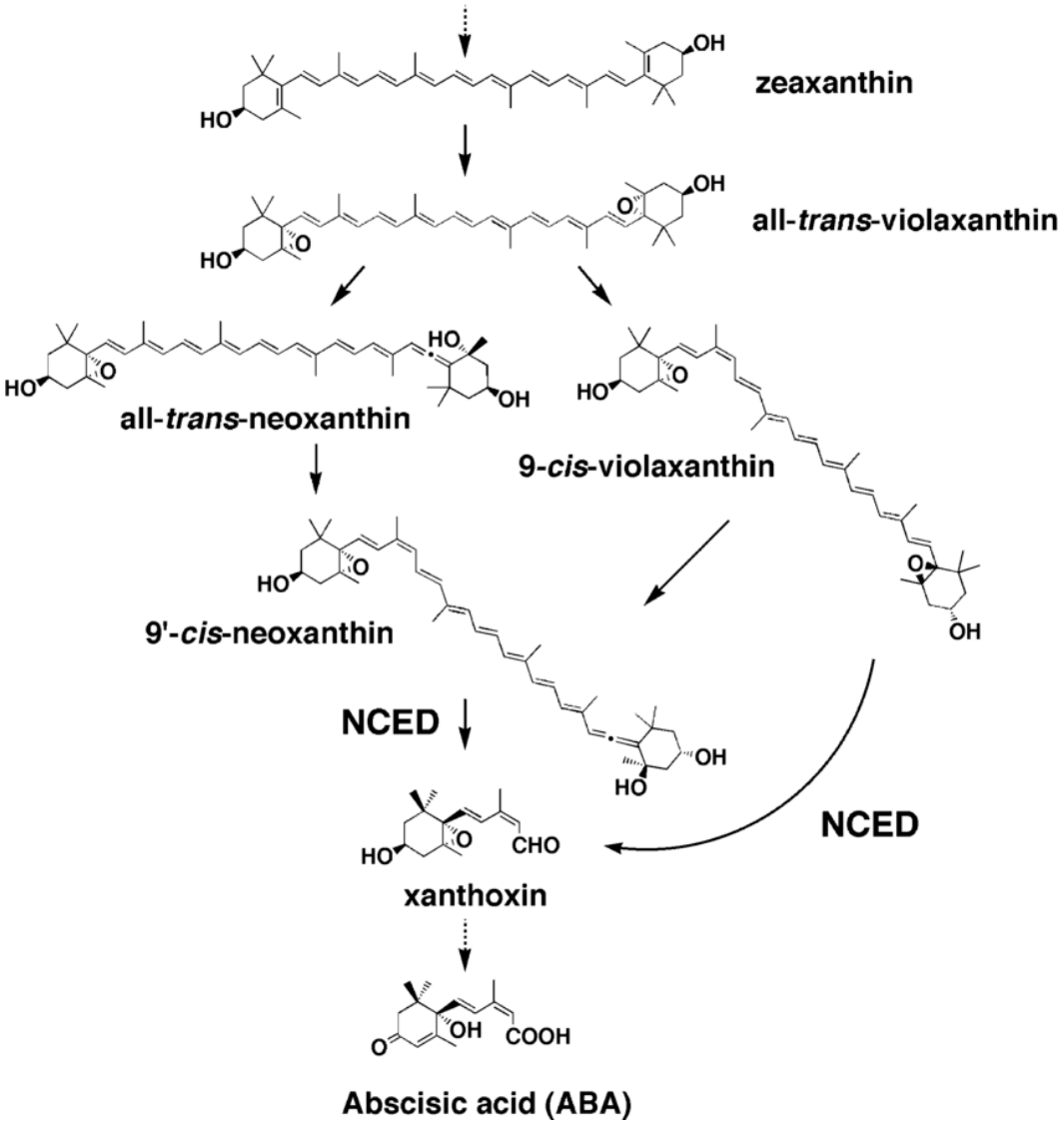

Figure 2 Biosynthesis pathway of ABA (https://www.researchgate.net/profile/Kazuo-Nakashima/publication/8462646/figure/fig1/AS:601693926486046@1520466419599/ABA-biosynthesis-pathway-in-higher-plants-ABA-is-derived-from-C-40-carotenoids-such-as.png)

plants through the carotenoid pathway (Ruiz-Sola and Rodrígeuz-Concepción, 2012).

This pathway begins from the precursor isopentenyl diphosphate (IPP), which is synthesized in plastids from glyceraldehyde-3-phosphate and pyruvate via methylerythritol phosphate (Nambara and Marion-Poll, 2005). This is followed by the synthesis of violaxanthin catalyzed by zeaxanthin epoxidase (ZEP). Violoxanthine is converted to 9-*cis*-neoxanthin, which is subsequently cleaved to xanthoxal by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED). The final steps in biosynthesis are the oxidation of xanthoxal to ABA-aldehyde and then to ABA catalyzed by aldehyde oxidases (Nambara, 2017).

Gibberellins

After the discovery of auxin, scientists attributed all regulation of developmental phenomena in plants to auxin for about 20 years, until the second group of hormones, gibberellins, was characterized in 1950 (Taiz and Zeiger, 2010). Gibberellins (GA) are phytohormones that primarily determine the height of plants. The first gibberellin was isolated in Japan from the fungus *Giberella fujikuroi*, after which they were named (Kwon and Paek, 2016; Binenbaum et al., 2018).

All gibberellins are native. They are isoprenoids from the group of diterpenes. Gibberellins are a large group of similar substances and some of them are precursors of active hormones (Gupta and Chakrabarty, 2013; Camara et al., 2018).

Unlike active auxin, gibberellins retain activity in the plant and also do not cause toxicity, i.e. they are not harmful to the plant even in higher concentrations. There is no particular enzymatic mechanism for degrading gibberellins in plants, but they can be converted to inactive forms that form conjugates with carbohydrates. The biogenesis of gibberellins is affected by light and controlled by phytochrome (Rodrigues et al., 2011).

It is proven that their synthesis takes place in the root tips or is transported to them, later distributed by basipetal movement, especially by the transpiration current (Brestič and Olšovská, 2001).

Transport takes place by wood and varnish. Transport can also take place acropetally, non-polar. Their movement is often oriented, regardless of polarity, behind the IAA source (Binenbaum et al., 2018).

Gibberellins are probably produced in all plant organs. The highest concentrations are in sites of active growth and emerging organs (Gupta and Chakrabarty, 2013). The regulatory effects of gibberellins include the enhancement of apical dominance along with abscisic acid. With ABA, they also induce parthenocarpy in seedless vine varieties (*Vitis vinifera*) and enlarge berries. They indicate the formation of flowers in photo periodically sensitive plants, thus accelerating their flowering and enlarging the flowers. They suppress the development of female flowers and support the development of male flowers. They also prevent the ageing of leaves and fruits and stimulate seed germination (Rodrigues et al., 2011; Cheng et al., 2017).

Gibberellic acid (GA3) (Figure 3) is a diterpenoid carboxylic acid that belongs to the gibberellin family and acts as a natural plant growth hormone. It is produced by plants and some microorganisms, such as fungi and bacteria. GA3 has a promising application in the agro-industrial sector due to its properties related to plant development. GA3 is applied to crops, orchards, and ornamental plants, where it plays a role in seed germination, response to abiotic stress, enhanced fruit growth, stem elongation, flowering, barley malting, and other physiological effects that occur in its interaction with other phytohormones (Camara et al., 2018).

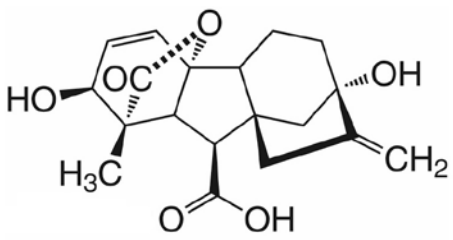

Figure 3 Gibberellic acid (https://img.p-lab.cz/userimages/product_main/1770/2007_656dde6c4fa997a1fce-c59a522ddeb5d_large.jpg)

Biosynthesis of gibberellins

The biosynthetic pathway occurs through certain intermediates. The synthesis of GA is carried out via terpenes from geranylgeranyl diphosphate by plants and fungi. The four isoprenoid molecules are linked together to form a linear molecule of 20 carbon atoms, known as geranylgeranyl diphosphate (GGPP). This molecule is transformed into *ent*-copalyl diphosphate by the action of *ent*-copalyl diphosphate synthase (CPS), which in turn is converted to a tetracyclic compound known as *ent*-kaurene by the action of *ent*-kaurene synthase (KS). *Ent*-kauren oxidase (KO) in plants and P450-4 in fungi catalyze the gradual oxidation of *ent*-kauren to C-19 to form *ent*-kaurenic acid, which is subsequently affected by kaurenic acid oxidase (KAO) in plants and P450-1 in fungi converts to GA12-aldehyde. In plants, GA12-aldehyde is first converted to GA12 and then converted to GA9 by the action of GA20-oxidase, which is responsible for the production of C19-GA. In a parallel pathway, GA12 is also 13-hydroxylated to produce GA53, which is converted to GA20 by C20-oxidase. Then, GA3-oxidase converts GA20 and GA9 by adding a 3β-hydroxyl group to GA1 and GA4, respectively. GA3 is synthesized by converting GA20 to GA5 using GA3-oxidase. This stage varies between species and depends on environmental conditions (Gupta and Chakrabarty, 2013; Camara et al., 2018; Hedden, 2020).

In fungi, GA12-aldehyde is 3β-hydroxylated to GA14-aldehyde, which is oxidized to form GA14. The latter is again converted to GA4 by oxidation with C20. GA4 is the first bioactive molecule to be formed and desaturated to form GA7, which is then converted to GA3 by 13-hydroxylation. GA1 is formed by 13-hydroxylation of GA4 (Camara et al., 2018; Hedden, 2020).

The pathways of biosynthesis in plants and fungi during the conversion of geranylgeranyl diphosphate to *ent*-kaurene and subsequent conversion to GA12-aldehyde are similar. The pathways differ from the stage at which GA12-aldehyde is converted to other GA, in the order in which the 3β-hydroxylation and 13-hydroxylation steps occur in plants and fungi. Plant and fungal GA biosynthetic pathways are described in Figure 4 (Camara et al., 2018).

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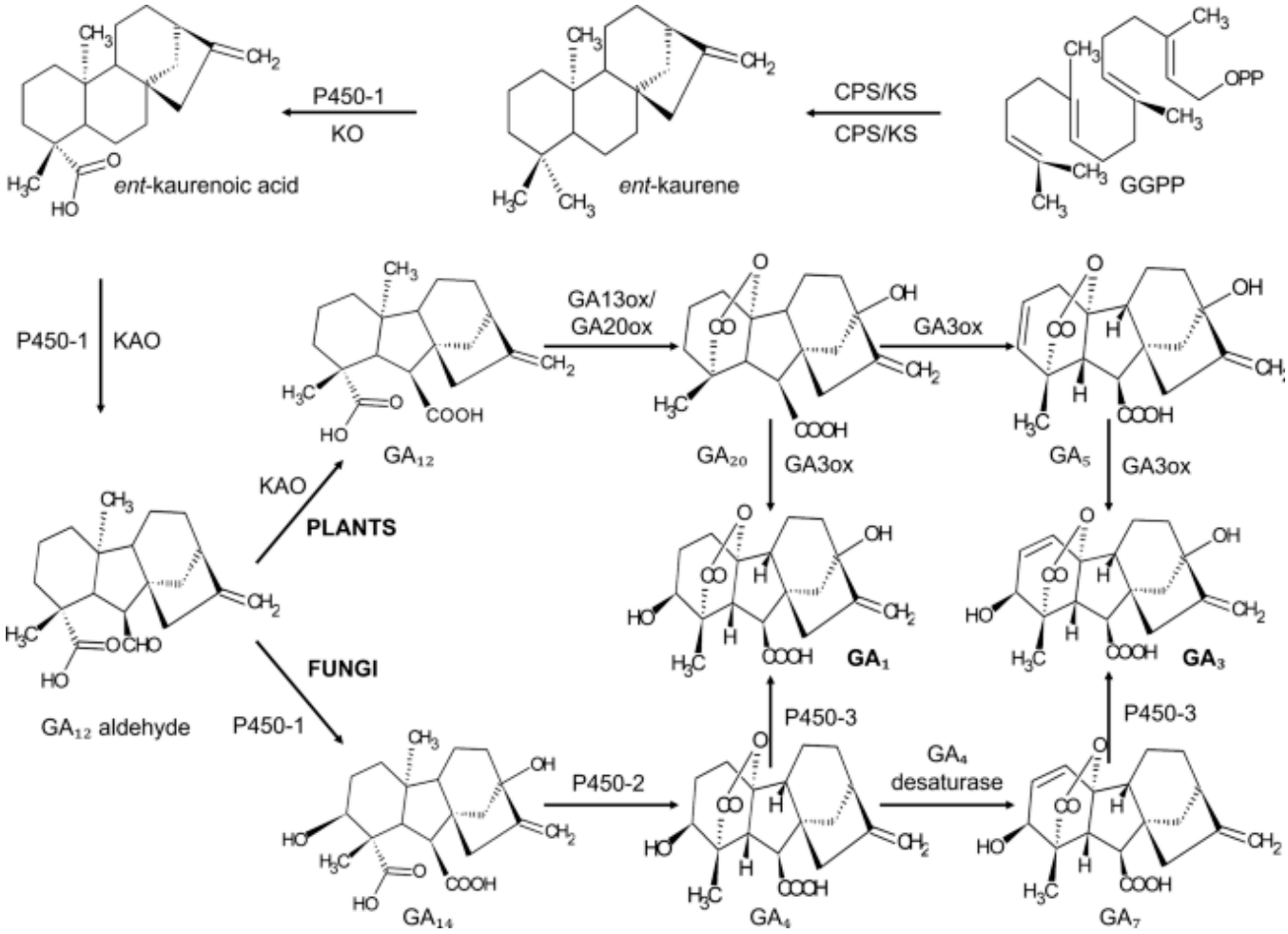

Figure 4 Biosynthesis of gibberellins in plants and fungi (https://media.springernature.com/lw685/springer-static/image/art%3A10.1007%2Fs00425-018-2959-x/MediaObjects/425_2018_2959_Fig3_HTML.png?as=webp)

Salicylic acid

Several centuries ago, the ancient Greeks used willow leaves and bark to alleviate pain and fever. Johann Buchner successfully isolated a small amount of salicin (glycoside salicyl alcohol) in 1828, which was identified as the major salicylate in willow bark (Maruri-López et al., 2019; Arif et al., 2020).

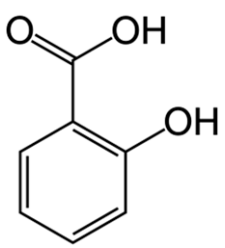

Figure 5 Salicylic acid (https://upload.wikimedia.org/wikipedia/commons/thumb/8/8e/Salicylic-acid-skeletal.svg/220px-Salicylic-acid-skeletal.svg.png)

Salicylic acid (Figure 5) or ortho-hydroxybenzoic acid belongs to a diverse group of plant phenols, which are substances bearing an aromatic ring to which a hydroxyl group or a functional derivative thereof is attached. Plant phenols are often classified as secondary metabolites, which play an important role in the regulation of plant growth, development, and interaction with other organisms (Maruri-López et al., 2019).

Free salicylic acid is a crystalline white powder having a melting point of about 157–159 °C. It is moderately soluble in water and very soluble in polar organic solvents. The saturated aqueous salicylic acid solution has a pH of 2.4. Salicylic acid fluoresces at 412 nm, while its excitation wavelength is at 301 nm, allowing it to be detected using a more sensitive fluorescence detector (Hayat and Ahmad, 2007).

Salicylic acid is produced by plants as protection against stress and disease (Metwaly and El-Shatoury, 2017).

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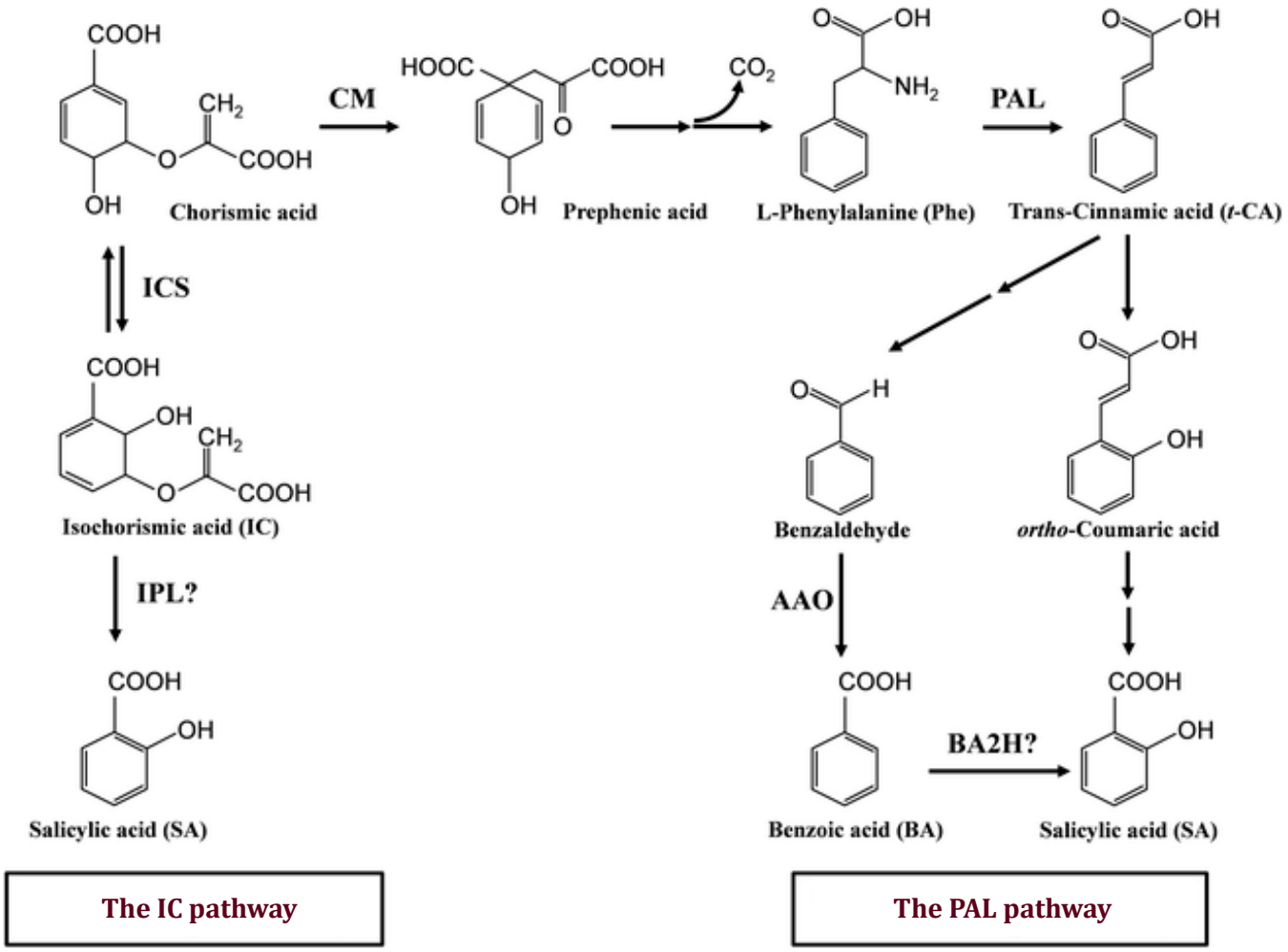

Figure 6 Biosynthesis pathways of salicylic acid (https://media.springernature.com/full/springer-static/image/art%3A10.1186%2Fs12915-017-0364-8/MediaObjects/12915_2017_364_Fig1_HTML.gif)

This can then serve as an explanation for its higher content in organically grown vegetables without the use of pesticides. John Paterson and his team from the University of Strathclyde found that soups made from organically grown vegetables contained six times more salicylic acid than soups made from vegetables grown in a conventional way (Klimeková and Lehocká, 2021).

Salicylic acid (SA) is a naturally occurring hormone that functions as an important signaling molecule. It affects various biochemical and physiological functions and has a different effect on tolerance to both biotic and abiotic stress (Li et al., 2019; Maruri-López et al., 2019). Thus, it is a key phytohormone that regulates defense against both biotrophic and hemibiotrophic pathogens and is often the primary chemical signal induced during the resistant response. This small molecule plays a crucial role in stimulating the plant's immune response. Usually, SA affects seed germination, seedling establishment, cell growth, respiration, ventricular closure, gene expression associated with ageing, responses to abiotic stress, basal thermotolerance, nodulation in legumes, and crop fertility (Vlot et al., 2009; Maruri-López et al., 2019). It further activates universal transcriptional reprogramming and resistance to a wide range of pathogens (Fu et al., 2012).

Biosynthesis of salicylic acid

Salicylic acid is a natural derivative of cinnamic acid, an intermediate of the shikimate metabolic pathway, and it is a starting material for the synthesis of many phenolic compounds. Its biosynthesis probably proceeds in two different ways (Figure 6) (Zhang and Li, 2019).

The first synthetic route proposes the formation of salicylic acid by decarboxylation of the cinnamic acid side chain to benzoic acid, which subsequently undergoes hydroxylation at the C-2 position. An enzyme catalyzing the β-oxidation of cinnamic acid to

benzoic acid has been identified in the summer oak (*Quercus robur*), but other enzymes responsible for the conversion of benzoic acid to salicylic acid have not yet been identified. The second route describes the hydroxylation of cinnamic acid to o-coumaric acid, from which salicylic acid is formed by subsequent decarboxylation. The conversion of cinnamic acid to o-coumaric acid is catalyzed by the enzyme *trans*-cinnamate-4-hydroxylase, which was first discovered in pea seedlings, but enzymes that activate the conversion of o-coumaric acid to salicylic acid have not yet been characterized. When radioactive 14C-benzoic acid or 14C-cinnamic acid was involved, labeled salicylic acid was formed in *Gaultheria procumbens*, which supported the theory of salicylic acid synthesis from cinnamic acid via benzoic acid as an intermediate. In higher plants, both synthetic pathways are involved in salicylic acid formation (Zhang and Li, 2019; Arif et al., 2020).

Conclusions

Phytohormones are essential regulators of plants in two physiological processes that coordinate growth, reproduction, and stress resistance. These molecules also show biological activities on human cells and animal models. Importantly, these phytohormones are not plant-specific and have even been shown to be endogenously produced in the human body or human cell cultures. When we are constantly exposed to this molecular, they are no strangers to human physiology. This means that they are likely to be involved in various physiological processes. In addition, several phytohormones may also be produced by microbes, and such compounds produced in our intestines are likely to have physiological effects (Kim et al., 2020).

Some phytohormones are anti-inflammatory compounds that inhibit several inflammatory diseases. For example, the treatment of ABA in humans and animal models requires beneficial effects against a wide range of inflammatory diseases such as type 2 diabetes, colitis, atherosclerosis, glioma, and depression. Salicylates have pharmacological properties in cardiovascular disease, colon cancer, and diabetes. Administered citokinins or its derivatives lead to attenuated oxidative stress in mammalian cells and anti-cytotoxicity in neoplastic cells. High IAA production has been shown to attenuate liver damage caused by a high-fat diet, relying on the aryl hydrogen receptor. Phytohormones that are not endogenously produced can also have physiological effects and have anti-inflammatory effect. For example, gibberellins (GA) induce the anti-inflammatory protein A20 in filter epithelial cells that could protect against asthma (Chanclud and Lacombe, 2017; Kim et al., 2020).

Conflicts 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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Methodological approach to the elaboration of the analytical procedure of the antioxidant activity determination of the *Schisandra chinensis* (Turcz) Baill. extracts

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The DPPH assay provides an easy and rapid way to evaluate the antioxidant activity of herbal preparations. The most important stage of the elaboration of the analytical procedure of total antioxidant activity measured by the DPPH test is to select the volume of an extract or its appropriate dilution for the determination of the reliable total antioxidant activity of the extract. If the concentration of antioxidants is too high in an extract, the kinetic curves of antioxidant activity changes on time are more parallel to axis *X* (time) and what is more, the total antioxidant activity does not depend on the volume of the extract. The analytical procedure of the antioxidant activity determination of the *Schisandra chinensis* (Turcz) Bail. extracts was elaborated, namely a volume and dilution of the extract was selected, rutin was chosen for the calibration curve. The calibration curve was plotted in the concentration range of 95–305 mg/L ($y = 0.228x+7.0992$, $R^2 = 0.9945$). The results suggest that the leaves of *S. chinensis* are a valuable source of antioxidant compounds with significant antioxidant activity. The antioxidant activity was evaluated by the DPPH test. It was equal to 227.2–443.6 mg/L in the extracts or 1.14–2.22 mg/g in the leaves of rutin-equivalents depending on the particle size. Additionally, it was established that particle size in the range of 2 to 3 mm was optimal for the preparation of *Schisandra chinensis* extracts as the antioxidant activity was the highest. The ethanol absorption coefficient is a main technological parameter in the pharmaceutical manufacture of extracts. The absorption coefficient of the *Schisandra chinensis* leaves for 70 % ethanol was in the range of 3.4 to 6.5 ml/g and depended on the particle size.

Keywords: *Schisandra chinensis*, ethanolic extracts, antioxidant activity, DPPH test

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Introduction

Schisandra chinensis (Turcz) Bail., Chinese magnolia vine belongs to the family of Magnoliaceae. *Schisandra chinensis* is widely distributed in China, Japan, Korea, and other countries due to its cultivation (Panossian and Wikman 2008; Lai et al., 2015; Qui et al., 2018; Hu et al., 2020). This plant is described in many Pharmacopeias. Among them are the Chinese Pharmacopeia, the Japanese one, the Pharmacopeia of the United State of America, and the State Pharmacopeia of Ukraine (Szopa et al., 2018; Qui et al., 2018; Hu et al., 2020).

The principal active substances of *Schisandra chinensis* are lignans and triterpenes (Song et al., 2016; Szopa et al., 2018; Qui et al., 2018; Hu et al., 2020). Fruits are rich in organic acids, especially citric acid (Hu et al., 2020). According to Hu et al., there existed a strong correlation between the total phenolic content of extracts of fruits and antioxidant activity determined by the DPPH test (Hu et al., 2020).

Modern medical researches prove that *S. chinensis* contains multiple active components which can protect liver cells (Ip and Ko, 1996; Ip et al., 2007; Teraoka et al., 2012), restrain oxidation (Jung et al., 2000; Sheng et al., 2011), prevent senility (Nishiyama et al., 1996; Hsieh et al., 1999; Hsieh et al., 2001; Kang et al., 2005), improve human body immunity ability (Mizoguchi et al., 1991). It can have a positive influence on the pulmonary system, kidney, liver, skin, central nervous system, etc. (Kim et al., 2004; Fu et al., 2008; Lai et al., 2015; Gao et al., 2016; Huang et al., 2017; Li et al., 2019; Liu et al., 2019). The renoprotective effects of the *Schisandra chinensis* extract are related to its antiapoptotic and antioxidant abilities, which induced the attenuation of CsA-induced autophagic cell death (Lai et al., 2015). Schizandrin B reduces UVB-irradiation damages of skin fibroblasts and epidermal keratinocytes (Gao et al., 2016).

There are some studies on the anti-inflammatory activity of *Schisandra chinensis* extracts and some individual lignans (Song et al., 2016; Szopa et al., 2018; Qui et al., 2018). The leaves of *Schisandra chinensis* are richer in polyphenolic compounds (Mocan et al., 2014). However, there are few studies related to *Schisandra chinensis* leaves extracts. Some authors indicate that *Schisandra chinensis* leaves are a valuable source of flavonoids with important antioxidants (Yu et al., 2017) and antimicrobial activities (Mocan et al., 2014). The *Schisandra chinensis* leaves extract showed stronger antimicrobial activity compared to the fruit extract (Mocan et al., 2014).

To widen the potential use of *S. chinensis* in antioxidant biomedicine, the present study was carried out to study antioxidant activities of extracts from the leaves of *S. chinensis* of different particle sizes, using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Therefore, the purpose of our studies was to evaluate the antioxidant activity of the extracts prepared from the powdered leaves of *Schisandra chinensis* of the different sizes: 1–2 mm, 2–3 mm, 3–4 mm, and 5–7 mm. For the correct evaluation of antioxidant activity by the DPPH method, it is necessary to select an appropriate ratio of a solution of DPPH to an extract. For that reason, one more aim was to elaborate an appropriate analytical procedure for the evaluation of the antioxidant activity of the extracts by the DPPH method.

Material and methodology

While carrying out this research, the following methods were used: analysis, synthesis, systematization, and comparison for processing published scientific data; technological method (remaceration); spectrophotometric method for the elaboration of the analytical procedure of the determination of the total antioxidant activity by DPPH test.

Plant material

Schisandra chinensis leaves were collected in the Arboretum Mlynany (Slovakia) in the middle of July of 2021. The specimen was stored in the herbarium of Institute of Plant and Environmental Sciences, Slovak University of Agriculture in Nitra. The voucher specimen number is Sch-2.

Reagents

The following reagents were used: ethanol 96 % (manufacturer "Centrachem" (Slovakia)), DPPH ("Sigma Aldrich"), and rutin hydrate ("Sigma Aldrich").

Extraction

The dry leaves were ground into powder and fractionated through different sieves (1, 2, 3, 5, and 7 mm) before the preparation of extracts.

Four extracts were prepared, using the different fractions of the powdered leaves. The remaceration consisted of maceration for 24 hours and the following two macerations for a period of 3 h for each one. The filtration was performed after each maceration and the obtained extracts were combined. Therefore, the total maceration accounted for 30 hours (24 hours +

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Table 1 Characterization of the obtained extracts

Number of the extracts	Particle size (mm)	Mass of the leaves (g)	Added volume of ethanol/obtained volume of an extract		Total volume of the extract (µL)
			main maceration	additional two macerations	
Extract 1	1–2	1.54	10/0	5.0/4.2 5.0/3.3	7.5
Extract 2	3–4	2.50	15/3.3	5.0/4.5 5.0/5.0	12.5
Extract 3	2–3	2.50	15/2.4	5.1/3.6 7.0/7.0	12.5
Extract 4	5–7	2.50	15/6.6	3.2/2.4 5.0/2.8	12.5

2 macerations × 3 hours = 30 hours). Features of the preparation of the extracts are provided in Table 1.

DPPH free radical scavenging activity assay

The two described analytical procedures were put in base of the evaluation of the extracts of *Schisandra chinensis* (Hudz et al., 2017; Hu et al., 2020), where authors used ascorbic acid as a positive control. In our study we used rutin. Additionally, Mocan et al. (2014) used quercetin as a positive control.

DPPH was dissolved in 96 % ethanol to a final approximate concentration of 0.003 %. In the free radical scavenging activity assay, 1 950 µL of the DPPH solution was added to 2 000 µL tubes, followed by 50 µL of the extract or its dilution. The solutions were mixed and incubated in the dark at room temperature. The absorbance was recorded at a wavelength of 515 nm (As). The mixture of 1 950 µL of 96 % ethanol solution with 50 µL of the extract was used as a blank for the extract. The value A0 was recorded when 1 950 µL of DPPH solution was mixed with 50 µL of ethanol after incubation under the former conditions. 96 % ethanol solution was used as a blank for the DPPH solution.

To compare the free radical scavenging activity of the four extracts, the rutin equivalents of each sample were calculated. Rutin was dissolved in 50 % ethanol solution and diluted to different five concentrations. Then 50 µL of each solution was mixed with DPPH solution. The reaction mixtures were incubated for 40 min. Their absorbance was recorded at a wavelength of 515 nm each 10 min. The mixture of 1 950 µL of 96 % ethanol solution with 50 µL of each rutin solution was used as a blank for each rutin solution.

In addition, we studied the stability of reaction mixtures or, in other words, we studied the kinetics of the reaction of DPPH with the extracts and rutin depending on the time.

Statistical analysis

All the analyses of the DPPH test for each extract, its dilution, and solutions of rutin were carried out in triplicate and the results were expressed as a mean value ± standard deviation (SD).

Results and discussion

Herbal preparations are used as an alternative source of medicines to mitigate the diseases associated with oxidative stress (Priya and Nethaji, 2015). The free-radical scavenging activity of the extracts was evaluated using the widely used 2,2-diphenyl-1-picrylhydrazyl (DPPH) test (Sheng et al., 2011; Mocan et al., 2014; Ivanišová et al., 2017; Vergun et al., 2018, 2021; Grygorieva et al, 2020; Shelepova et al., 2020; Tvrdá et al., 2020; Mňahončaková et al., Vinogradova et al., 2021). The DPPH assay provides an easy and fast mode to estimate antioxidant activity. This test is based on electron-transfer. DPPH produces a violet solution in ethanol or methanol. The reduction of DPPH in the presence of an antioxidant or mixture of antioxidants leads to the formation of non-radical form DPPH-H of yellow or yellowish colour (Sheng et al., 2011; Mocan et al., 2014; Rachman et al., 2015). According to our studies, the colour of final mixtures can be light purple or yellow depending on the concentration of antioxidants.

Various modifications and optimizations of DPPH assay are described for their adaptation to tested extracts or are the invention of researchers (Sheng et al., 2011; Mocan et al., 2014; Hu et al., 2020).

The most important stage of the development of the analytical procedure of the total antioxidant activity measured by the DPPH test is to select the volume of an extract or its appropriate dilution for the determination of the reliable total antioxidant activity of the extract. From our experience, if the concentration of antioxidants is too high in an extract,

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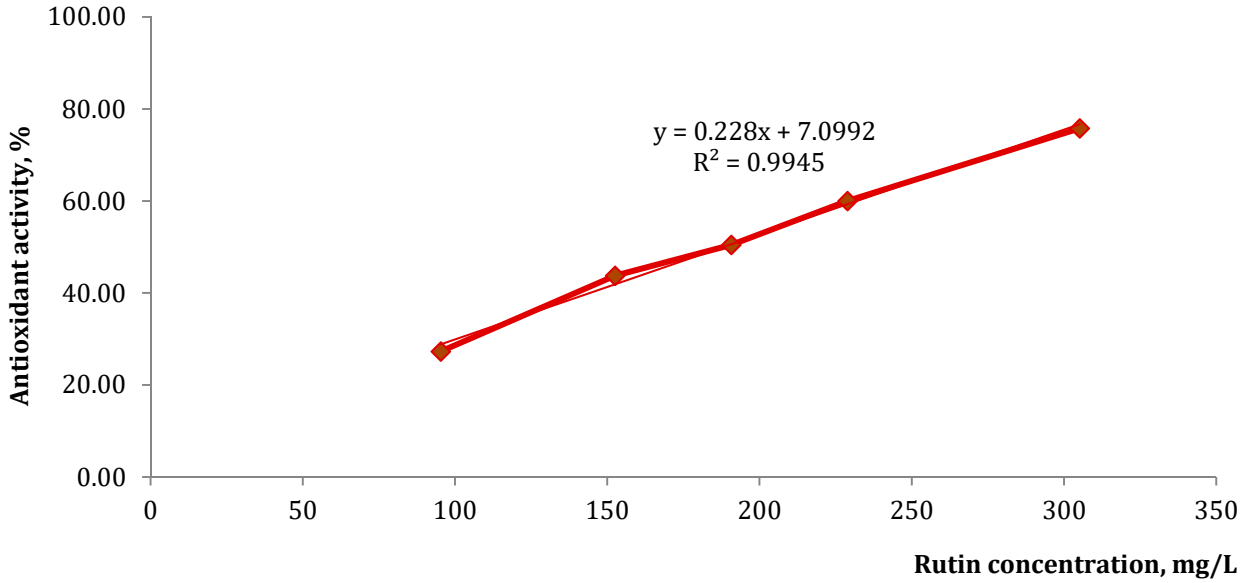

Figure 1 Calibration curve of rutin

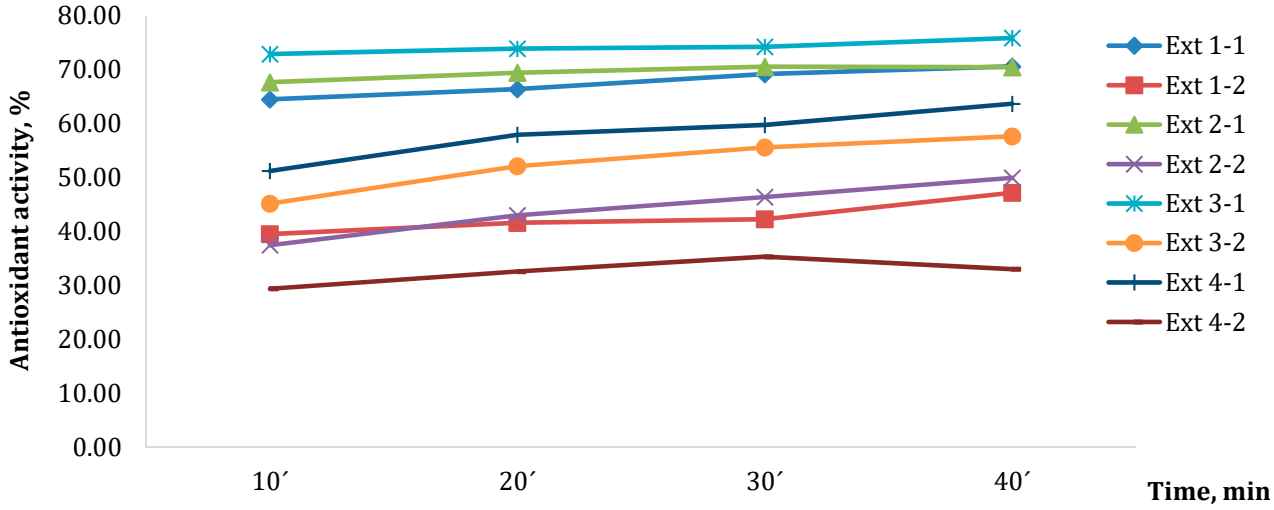

Figure 2 Dependence of the antioxidant activity of the extracts of *Schisandra chinensis* (Turcz) Bail. on time
Ext 1-1, Ext 2-1, Ext 3-1, Ext 4-1 – extracts 1, 2, 3 and 4 without dilution and Ext 1-2, Ext 2-2, Ext 3-2, Ext 4-2 – extracts 1, 2, 3 and 4 diluted twice by 70 % ethanol

namely if the content of biologically active substances is very large and an appropriate dilution was not selected, then DPPH is reduced very quickly without dependence on dilution and final mixtures get yellow. In that case, the kinetic curves of antioxidant activity changes on time are more parallel to axis *X* (time), and, what is more, the total antioxidant activity is equal to 80–85 % and did not depend on the volume of the extract of beebread (20–50 µl) (Hudz et al., 2017). In other words, the higher the rate of DPPH consumption is, the more powerful the antioxidant potential (Mocan et al., 2014). Therefore, it is necessary to decrease the volume of the sample or dilute it.

According to the literature, DPPH has an absorption maximum at a wavelength of 514–517 nm (Ivanišová et al., 2017; Shelepova et al., 2019; Vergun et al., 2019, 2021; Grygorieva et al, 2020; Hu et al., 2020; Tvrdá et al., 2020). Reducing a colour intensity of a reaction mixture is carried out by various authors at the time range of 4–15 minutes to 1 hour (Sheng et al., 2011; Mocan et al., 2014; Hu et al., 2020).

The results obtained by the DPPH test were presented as rutin equivalents, mg/L and mg/g (Table 2). The percentage of DPPH consumption was converted to rutin equivalents by using a calibration curve ($y = 0.228x + 7.0992$, $R^2 = 0.9945$) with the rutin

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Table 2 Antioxidant activity of the *Schisandra chinensis* (Turcz) Bail. extracts expressed in %, mg/L and mg/g of rutin-equivalents

№	Antioxidant activity (%), mean ± SD		Concentration of rutin-equivalents (mg/L) in the extract*	Concentration of rutin-equivalents (mg/g), in the leaves*
	native extracts	diluted extract twice		
1	70.6 ±0.1	47.2 ±0.8	351.8 ±6.0	1.76
2	70.5 ±1.3	50.0 ±1.3	376.3 ±9.8	1.88
3	75.9 ±0.9	57.7 ±0.9	443.6 ±6.9	2.22
4	63.7 ±1.5	33.0 ±0.6	227.2 ±4.1	1.14

Notes: * – calculations were performed on the values of AA of the diluted extracts

Table 3 Calculations of the experimental determination of the absorption coefficient of 70 % ethanol

№	Particle size (mm)	Calculations
1	1–2	X1 = (10–0) : 1.54 = 6.5 ml/g
2	3–4	X2 = (15–3.3) : 2.5 = 4.7 ml/g
3	2–3	X3 = (15–2.4) : 2.5 = 5.0 ml/g
4	5–7	X4 = (15–6.6) : 2.5 = 3.4 ml/g

solutions in the concentration range of 95–305 mg/L (Figure 1).

From Figure 1 we can observe that the higher the rate of DPPH consumption is, the more powerful is the antioxidant potential. For undiluted *Schisandra chinensis* extracts the antioxidant activity was in the range of 70–76 % independing on particle size in the case of extracts 1, 2, and 3. This pointed to an inappropriate ratio of antioxidants content and DPPH. Therefore, firstly we diluted our extracts twice and measured the antioxidant activity.

In Figure 2 we presented the dependence of the antioxidant activity of the undiluted and diluted extract on the time. The ratio of an extract to a final dilution was 1 to 2.

Our studies are in line with studies performed by Sheng et al. (2011) and Rachman et al. (2015). Increasing the concentration of compounds with antioxidant activities enhances the antioxidant activity of a reaction mixture (Sheng et al., 2011; Rachman et al., 2015). Moreover, such an increase in the concentration of compounds with antioxidant activities leads to that the kinetic curves of antioxidant activity changes on concentration are parallel to axis *X* (Sheng et al., 2011; Rachman et al., 2015).

From Table 2 we can observe that the total antioxidant activity of the native extracts is equal to 63.7–75.9 %. This antioxidant activity did not correlate with the values of the antioxidant activity of the same diluted extracts except for extract 4. We can suppose that it is necessary to select the appropriate dilution of an extract of the total antioxidant activity of this undiluted extract exceeds 64 %. Moreover, the kinetic curves of the dependence of antioxidant activity on time are more parallel to axis *X* for extracts 1–3 that is in line with the studies performed for the extracts of beebread (Hudz et al., 2017).

Furthermore, it was established that particle size in the range of 2–3 mm is optimal for the preparation of *Schisandra chinensis* extracts. We observed in our study that an increase in the surface area available for molecular transport contributes to a more extensive mass transfer of compounds with antioxidant activity into an extract if not considering extract 1.

Additionally, rutin was selected as a marker for the DPPH test as such flavonoid glycosides as rutin, hyperoside, quercitrin, and isoquercitrin and flavonoid aglycones as myricetin, kaempferol, and quercetin were identified in the extract of the *Schisandra chinensis* fruits Bail (Mocan et al., 2014; Tvrdá et al., 2020). Moreover, rutin was dominated flavonoid among glycosides in the extract (Tvrdá et al., 2020).

The DPPH assay showed that the free-radical scavenging activity of the extract from fruits was 5.93 mg of Trolox equivalents/g d.w. (Tvrdá et al., 2020). The antioxidant activity of *S. chinensis* leaves was 26.87 ±0.84 mg QE/g of plant material, while the antioxidant activity of *S. chinensis* fruits was 7.80 ±0.55 mg QE/g of plant material (Mocan et al., 2014).

We cannot compare our results with published data as antioxidant activity was expressed in rutin equivalents in our studies.

The ethanol absorption coefficient is a main technological parameter in the pharmaceutical manufacture of extracts (Yezerska et al., 2021). The results of the technological studies are provided in Table 3. It was revealed that the coefficient of alcohol absorption of the crushed leaves depended on the size of particles.

We observed such regularity: the more particle size, the less was the absorption coefficient (Table 3).

Conclusion

The analytical procedure of the antioxidant activity determination of the *Schisandra chinensis* extracts by the DPPH test was developed from a point of view of choosing a volume and dilution of the extracts, marker for the calculation of the antioxidant activity of the extracts. The calibration curve was plotted in the concentration range of 95 to 305 mg/L of rutin ($y = 0.228x + 7.0992$, $R^2 = 0.9945$). The results suggest that the leaves of *Schisandra chinensis* are a valuable source of antioxidant compounds with significant antioxidant activity. The antioxidant activity of the *Schisandra chinensis* extracts (1 : 5) was equal to 227.2–443.6 mg/L rutin-equivalents depending on the particle size. Additionally, it was established that particle size in the range of 2–3 mm was optimal for the preparation of *Schisandra chinensis* extracts as the antioxidant activity was the highest. This study established the basis for future research into the elaboration of the *Schisandra chinensis* extracts from leaves.

Conflicts 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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Research Article

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Cultural extension of *Ginkgo biloba* L. in Slovakia

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Ginkgo biloba L. is the oldest species of tree on our planet. It is a dioecious species characterized by exceptional resistance to climate change and natural influences, which predetermines it as a suitable species for urban planting. Not to be overlooked is the ginkgo benefit in terms of the medical effects of leaf metabolites. Due to its aesthetic value ginkgo is becoming popular in family gardens. The aim of our research was the evidence and description of ginkgo trees in Slovakia. The study presents the completion of data on the cultural distribution of this tree. We confirmed the occurrence on more than one hundred localities (103 localities). Some previously registered localities (18) were not found, or trees were felled for various reasons (5). We evaluated the basic dendrometric and growth parameters of the oldest ginkgo trees (aged 242 – 111 years), found in 35 localities of Slovakia. The presented research results bring several new information concerning the tree gender determination, tree habitus, phenological rhythm of development and others. Morphologically interesting solitary trees, including trees with shoots known as lignotubers or "basal chichi" (locality Hajná Nová Ves), or the occurrence of fruits on the leaves, referred to as cv. Ohatsuki (Lučenec) are described and documented by photos.

Keywords: maidenhair tree, locality, occurrence, Slovak Republic

Introduction

Ginkgo biloba L. is a dioecious species endemic to China (Li et al., 2009). It is a famous living fossil and is the only known extant representative of *Ginkgophyta* (He et al., 2015; Šmarda et al., 2016). It contains several different biologically active compounds which play a role in defense mechanisms against insects, bacteria and fungi (Singh et al., 2008). The leaves of ginkgo are a rich source of compounds with antioxidant activity (van Beek, 2000) and are commonly used as phytomedicines in the treatment of atherosclerosis and cerebrovascular insufficiency (Kleijnen and Knipschild, 1992; Xie et al., 2003) depression, memory loss, headaches, and vertigo (Diamont et al., 2000). A recent study of extracts from the leaves of *Ginkgo biloba* L. from some Slovakian localities showed that these extracts can be used as antimicrobial and antioxidant additives due to their significant antioxidant and antimicrobial activity which was sample-specific (Ražná et al., 2020).

In Slovakia, *Ginkgo biloba* L. is relatively little known, although in historical parks and gardens it grows in several places, from plantings from the late 18th and 19th centuries (e. g. Bratislava, Topoľčianky, Nová Ves nad Žitavou, Beladice, Lučenec, Betliar, Jasov, Košice,

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Humenné, etc.) (Tokár, 1968; Tokár, 1970; Ražná et al., 2014). Current horticultural practice confirms that ginkgo belongs to a small group of species capable of resisting urban exhaustion, salinization, and water scarcity (Raček et al., 2009, 2010, 2011), and at the same time, with its aesthetic value, effectively beautifies the urban environment. From the horticultural point of view, ginkgo is becoming more and more popular, and its coloured and shaped cultivars are gaining more and more space in family gardens (Tomaško, 2004; Hrubík et al., 2011).

We began research into the cultural distribution and occurrence of *Ginkgo biloba* L. in 2007. We focused first on the districts of Nitra and Zlaté Moravce, and later the entire Nitra self-governing region. The most suitable conditions for research were provided by fruit trees in the State Forest Park in Topoľčianky, the Arborétum Mlyňany of the Slovak Academy of Sciences, historical parks in Nová Ves nad Žitavou, Beladice, and trees planted in the Nitra City Park in Sihoť. Later, we extended the research to the districts of Topoľčany, Trnava, Levice, Nové Zámky and Komárno. Intensive field research continued in 2010–2011 in other districts of Slovakia (former districts of the West and Central Slovakia regions). We presented a summary of the acquired knowledge and more comprehensive research in two monographs (Ražná et al., 2014; Ražná and Hrubík, 2016). The mentioned publications contain a comprehensive overview of the determined taxation values, complete biological data, horticultural values, and an evaluation of the health status of ginkgo trees that grow in Slovakia. The publication also includes a complete list of trees growing in historical parks, street plantings (mostly as tree lines and alley plantings), and other objects of public (and accessible private) greenery in towns and villages in Slovakia. In addition to the results of field research, considerable attention was paid to a genomic screening of polymorphism using molecular markers, as well as antioxidant and antimicrobial parameters of selected individuals of ginkgo (Ražná et al., 2019; Ražná et al., 2020).

The impetus for concluding and completing the research on the cultural distribution of *Ginkgo biloba* in Slovakia was, on the one hand, the processing of scientific knowledge acquired so far, but especially the commitment to conclude current and interesting issues of spreading one of the rare cultural trees – *Ginkgo biloba* in Slovakia. The first findings on the distribution of ginkgo in Slovakia were obtained as early as 1965–1975, as part of cooperation on extensive and comprehensive research in the Arborétum Mlyňany – Institute of Dendrobiology of the Slovak Academy of Sciences. When selecting research sites, we therefore, accepted the published results of the cultural distribution of foreign trees in Slovakia (including *Ginkgo biloba*), (Benčať, 1982). It contains a total of 80 sites (75 historic parks, 5 other greenery). From the available literature, internet sources, other sources, as well as from our own findings, we supplemented and evaluated the ginkgo trees in other (new) localities. For the purposes of this publication, we selected a list of the oldest trees of *Ginkgo biloba*, growing in Slovakia. The locality contains the name and registration number of the historical park and garden (P – 44), district – according to the current territorial division of the territory (TO – Topoľčany), period of the foundation of the dendrological building in the relevant century (e. g. 19/1 – first half of the 19th century, with the designation of the years – 1850). For this purpose, we selected several figures of interesting solitary trees and conclusions based on our long-term research, concerning tree habitus and gender differentiation features.

Material and methodology

Biological material

In research of cultural extension of *Ginkgo biloba*, we followed methodologies and working procedures. As already mentioned, we have fully accepted the well-known and published list of identified localities (Benčať, 1982) (Figure 1), including other, new localities identified from other unpublished, internet sources, as well as information on previously registered or otherwise registered trees (e. g. state-protected trees, where old and memorial ginkgo trees were included). At each locality, which we personally evaluated, we surveyed the existing ginkgo trees, on which we obtained basic taxation data (trunk circumference, trunk diameter 1.3 m above the ground; tree height, crown width, health status, horticultural value – according to the 5-point scale, in the case of larger trees, we also determined the circumference of the trunk at the ground) (Hrubík et al., 2011) and made an up – to – date photo documentation of the trees at a specific locality.

When determining the main dendrometric dimensions of ginkgo trees, we used a specialized textile band, which measures, on one side of the band, in units of cm, and on the other side of the band, are measured units converted to the values of the average. During the field research, we used this textile band to measure the circumference of the tree trunk, thus also obtaining the measured value of the trunk diameter. We measured

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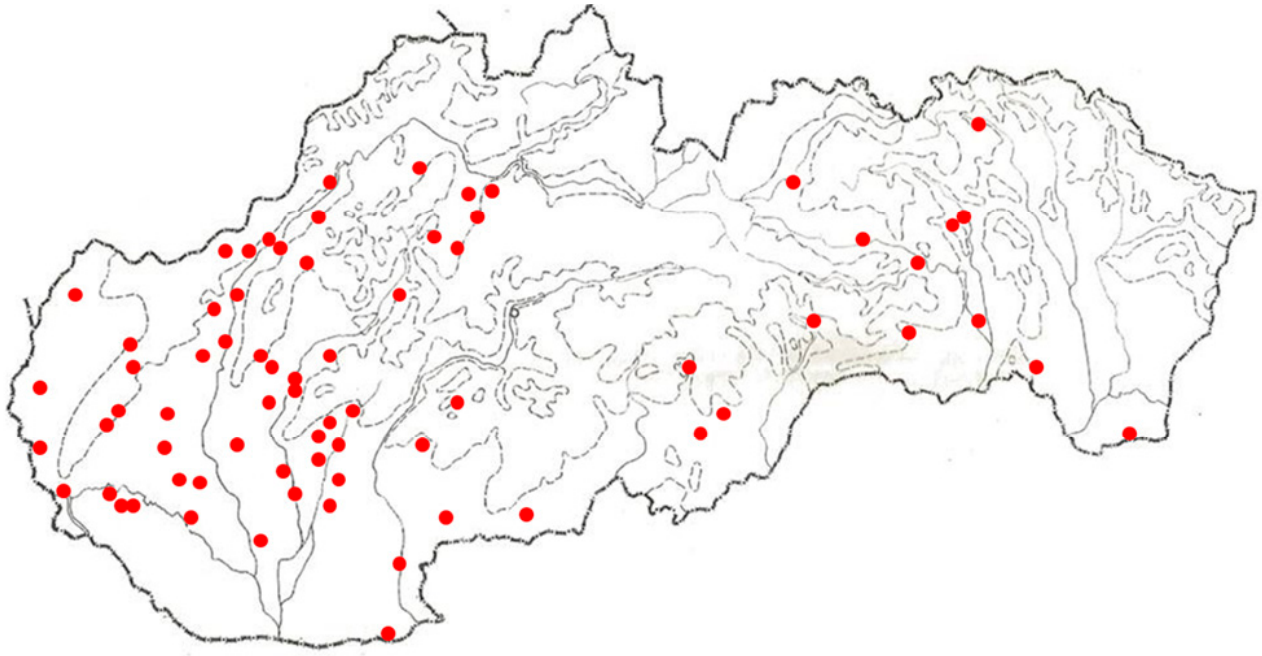

Figure 1 Localities of occurrence of *Ginkgo biloba* L. identified by Benčať (1982)

the height of the tree with a SUUNTO altimeter; we estimated the width of the crown by a step in two intersecting directions according to the shape of the crown (however, most of them were solitary trees with a regular crown). We identified the sex of the trees. The blossoms are dioecious, male and female blossoms occurring separately on two different trees. There are some additional tree gender differentiation features as crown habitual marks, and the angle of protruding lateral branches.

Determining the age of *Ginkgo biloba* L. trees was, in our experience, the most problematic. For some trees, especially younger (juvenile) individuals, the data of the tree age or year of planting are relatively accurate (a value must be added to this data, it is 5–10 years from the cultivation of seedlings from seed in the nursery until the planting of seedlings in a specific location). In addition, we used the Pressler drill (number of annual rings on a dendrometric borehole) to determine the age, but the evaluation of the obtained results was time and money consuming (we obtained a total of 25 boreholes), so we did not continue in this approach. However, we used a mathematical formula $V = \left(5/[\pi \times RL]\right) \times d$, where d is the diameter of the trunk (in cm) and RL is the width of the annual ring (in cm) (Kolařík, 2005).

Another way of determining the age of trees works with information about the period of origin, respectively the establishment of a specific historical park (dendrological building), the most often it is the 18th and 19th to 20th centuries (1901–2000). Determining the age of trees according to the circumference of the trunk (d 1.3 m) is another option, but the most accurate is the year of planting of a particular tree.

Research area

All obtained data on individual trees were processed in a tabular overview by locality, including data on a specific historical park or object of other greenery. The tabular overview includes findings on the tree gender (male – m; and female – f), juvenile age (until the period of occurrence of seeds, or male and female generative organs), and the occurrence of seed propagation (seedlings under fruiting female trees). For completeness, we present a table of localities of cultural extension of *Ginkgo biloba* in Slovakia according to Benčať (1982).

Description of a model tree, *Ginkgo biloba* L.

Ginkgo is a long-lived deciduous tree. It can reach more than a thousand years old. Depending on the growing conditions, mature trees reach a height from 20 to 40 m. The crown is somewhat ovoid to obovoid, tending to be asymmetric, primary branches ascending at ca. 45° from trunk (Flora of North America). In males' trees, it is usually slimmer, in females' ones bushier (Figure 2). Ginkgo trees produce two types of shoots: long shoots with widely spaced leaves that subtend axillary buds; and short shoots with clustered leaves that lack both internodes and axillary buds. Under stressful growing

Table 1 Localities of cultural extension of *Ginkgo biloba* L. in Slovakia according to Benčať (1982)

District	Localities	Registration number of localities
Bardejov	Bardejov spa	P-328
Bratislava-city	Bratislava	P-103, 105,108,110
	Častá – Červený Kameň	P-424
	Malacky	SG
Bratislava-city surroundings	Modra	P-90
	Stupava	P-96
	Tomášov	P-100
	Hubice	P-192
Dunajská Streda	Sládkovičovo	P-145
	Tomášikovo	P-148
Komárno	Kravany nad Dunajom	P-189
Košice	Jasov	P-396
	Košice	P-382, 388, 390, 392
	Bohunice	P-149
Levice	Horné Semerovce	P-158
	Pohronský Ruskov	SG
Lučenec	Lučenec – Ipeľské brickfield	SG
	Nenince	P-276
	Abramová	SG
	Martin	P-228
Martin	Mošovce	P-231
	Necpaly	P-230
	Turčianska Štiavnička	P-225
	Báb	P-133
	Beladice	P-124
	Ivanka pri Nitre	SG
	Mlyňany Arborétum	P-129
Nitra	Nová Ves nad Žitavou	P-135
	Sľažany	P-118
	Šurianky	P-114
	Telince	SG
	Topoľčianky	P-115
	Komjatice	P-166
Nové Zámky	Obid	SG
	Palárikovo	P-174
	Trávnica	P-169
Poprad	Kežmarok	SG
Považská Bystrica	Klobušice	P-217
	Horenická Hôrka – Medné	P-205
Prešov	Malý Šariš	P-348
	Župčany	P-347
Prievidza	Prievidza	SG
	Bojnice	park by the castle

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Table 1 Localities of cultural extension of *Ginkgo biloba* L. in Slovakia according to Benčať (1982)

District	Localities	Registration number of localities
Rimavská Sobota	Hnúšťa	SG
	Rimavská Sobota	P-292
	Veľký Blh	P-286
Rožňava	Betliar	P-407
Senica nad Myjavou	Cerová-Lieskové	P-30
	Gbely	P-28
Spišská Nová Ves	Bystrany	P-357
	Jaklovce	P-359
Topoľčany	Hajná Nová Ves	P-44
	Horné Obdokovce	P-50
	Janova Ves	P-42
	Oponice	P-52
Trebišov	Kovarce	P-48
	Kazimír	P-416
	Pribeník	P-418
Trenčín	Adamovské Kochanovce	P-8
	Částkovce	P-21
	Kočovce	P-17
	Motešice	P-13
	Trenčín	P-7, SG
	Záblatie	P-4
	Zemianske Podhradie	P-12
Trnava	Piešťany	P-58
	Rakovice	P-66
	Smolenice	P-70
	Trnava	P-78, SG
	Voderady	P-84
Žiar nad Hronom	Banská Štiavnica	P-246
Žilina	Rajecké Teplice	P-223

Note: SG – surrounding greenery, that is, the tree is in a territory other than a historic park or garden

conditions, Ginkgo can produce secondary trunks at or just below ground level. These secondary stems originate from root-like, positively geotropic shoots known as lignotubers or "basal chichi" (van Beek, 2000) (Figure 3). The bark is smooth, gray, relatively quickly turns into a brown-gray bark, which is cracked in longitudinal irregular plates (Figure 4c).

Buds are brown, globose, scales imbricate, margins scarious (Flora of North America) (Figure 4e). Leaves are 30–60 × 40–100 mm fan-shaped (with long stalks) with dichotomously branched veins (Figure 4a). They are flat, firm, leathery, light green, golden yellow in autumn before falling (Figure 4d). It blooms in May (Figure 4b). The flowers are dioecious, growing only on shortened shoots (Pagan and Randuška. 1988). Microsporophylls occur in small, conelike clusters (are pendulous). In females' trees, an upright 25–40 mm long stalk grows in the armpits of scales or leaves, which is widened at the end. Ovules occur in pairs at the ends of a short stalk. The ovule is surrounded by a cup-shaped cushion called a collar. Seeds obovoid to ellipsoid, yellow to orange, 2.3–2.7 × 1.9–2.3 cm, mostly 1.1–1.2 times longer than broad, glaucous, rugose, with an apical scar, maturing in a single season, usually 1 per peduncle, occasionally polyembryonic, outer coat foul-smelling; peduncles orange, glaucous, ridged, 3–9.5 cm, collar broadly elliptic, 7.2–8.6 mm broad (Vreštiak and Osvald, 1994; Klečková, 2010;

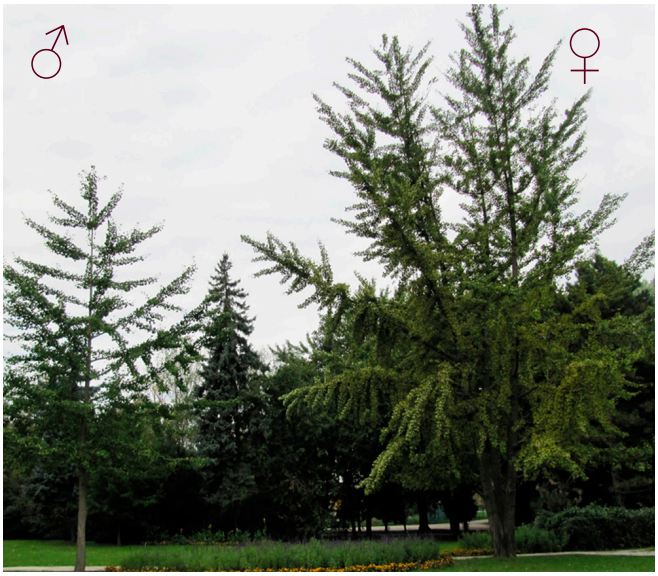

Figure 2 Male (left) and female (right) tree of ginkgo grown in city park in Nitra
Photo by Pavel Hrubík

Figure 3 Shoots known as lignotubers or "basal chichi". Ginkgo grows in historical park near the manor house, Hajná Nová Ves
Photo by Pavel Hrubík

Zvolen (3 pregenerative individual trees). In other localities we recorded from 1 to 2 trees. The total number of localities (towns and villages) with the occurrence of *Ginkgo biloba* was 103. In addition, we

Figure 4 Fan-shaped leaves with dichotomously branched veins (a), scaly stamens in short spikes on male tree (b), bark (c), golden-yellow leaves in autumn (d), buds (e)
Photos by Pavel Hrubík

Figure 5 Fruits on female tree (a), seeds (b) and seed of irregular shape with stalks
Photos by Pavel Hrubík

Flora of North America). Figure 5a shows fruits on the female tree, seeds after removal of the flesh (Figure 5b), and irregular shape of seeds from the ginkgo grown in Lučenec.

Ginkgo seeds are dormant when they fall from the tree because the embryo is not fully developed, being only about 4 to 5 mm in length. If seeds are collected shortly after dispersal, are cleaned, and placed in a warm greenhouse, the embryo will grow to its full size, 10 to 12 mm in length and germinate within eight to ten weeks. Ginkgo shows a long juvenile period, typically not reaching sexual maturity until 20 to 30 years of age (van Beek, 2000). The foul odour associated with mature seeds is result of the presence of butanoic and hexanoic acids (Zhou and Wang, 2020).

Results and discussion

Main dendrometric values of ginkgo trees

In Slovakia, we found the occurrence and cultural distribution of *Ginkgo biloba* in 103 localities (Table 2). The total number of trees reached 292 individuals (including new plantings in Bratislava – Košická street, tree line of 29 trees in the middle dividing grass strip, *Ginkgo biloba* cv. Fastigiata; group plantings at the level crossing with Bajkalská street, 30 pieces; in Senica – 25 trees in a two-sided alley on Hurbanova street, of which 4 in the tree line on Hviezdoslavova street, in Žilina above the shopping center, *Ginkgo biloba* (cv. Fastigiata – 5 pregenerative individuals). Some *Ginkgo biloba* trees were felled (despite protests from the civil public) at the site of the programmed construction of a hotel in Trenčín (one male tree in 2008); for hygienic and safety reasons (Košice, Rázusova street; Rimavská Sobota, Hviezdoslavova street no. 25); technical damage to the adjacent building (Košice, campus of the University of Veterinary Medicine and Pharmacy); tree damaged and destroyed during lawn mowing (Kežmarok, Gymnázium P.O. Hviezdoslava); a total of 5 Ginkgo trees.

The most numerous localities (apart from the already mentioned pregenerative individuals in tree lines and alley plantings) were recorded in the Mlyňany Arborétum SAS, 17 trees (of which one is a male tree, three females, fruiting trees, 13 pregenerative trees); In Bratislava, 14 trees (of which 5 in the Botanical Garden of the Charles University in Prague, 2 in Petržalka, Janko Kráľ Park; 2 on Dunajská Street in the courtyard of the fitness centre), the tree line on Košická Street is mentioned separately; 9 trees in Košice (of which 5 trees in the premises of the L. Pasteur University Hospital; 7 trees in Piešťany (3 Parks on the Island, 4 Parks at the Secondary Vocational Horticultural School), 5 trees in Palárikovo (3 males and 2 females trees, 3 trees in Nitra (of which 2 trees – male and female tree, planted in 1963 in the Nitra City Park in Sihoť); more cultivars and pregenerative individuals are still growing in the Botanical Garden of the Slovak University of Agriculture, which were not included in the research. In Topoľčianky we register 4 trees (of which one male and 3 female trees); in Nová Ves nad Žitavou 4 trees (female trees); we recorded 3 trees in the following localities: Bojnice (male individuals); Topoľčany (one female tree, 2 pregenerative individuals); Trenčín (2 female and 1 male tree); Trnava (2 male, 1 female tree); Banská Štiavnica (2 male and 1 female tree);

Table 2 Characteristics of *Ginkgo biloba* L. trees in Slovakia. The data come from the research period in 2014–2016

Locality	Trunk circumference (cm)	Trunk diameter (cm)	Tree height (m)	Crown width (m)	Gender (♂, ♀)	Age
Abramová (SG), TR	238	76	14	18 × 14	♀	121
Arborétum Mlyňany SAV, (P-129), 1892, ZM (11)	92	29,3	16	10 × 4	♂	47
Arborétum Mlyňany SAV, (P-129), 1964, ZM (1)	30	9,6	10	3 × 3	juv.	15
Arborétum Mlyňany SAV, (P-129), 1964, ZM (10)	18	6,0	7	3 × 3	♀	10
Arborétum Mlyňany SAV, (P-129), 1964, ZM (12)	60	19,2	11	5 × 6	juv.	31
Arborétum Mlyňany SAV, (P-129), 1964, ZM (13)	88	28,2	14	8 × 8	juv.	38
Arborétum Mlyňany SAV, (P-129), 1964, ZM (14)	90	28,8	16	8 × 8	juv.	44
Arborétum Mlyňany SAV, (P-129), 1964, ZM (15)	53	16,9	14	4 × 4	juv.	26
Arborétum Mlyňany SAV, (P-129), 1964, ZM (16)	43	13,7	14	3 × 3	juv.	22
Arborétum Mlyňany SAV, (P-129), 1964, ZM (2)	17	5,4	6	3 × 3	juv.	12
Arborétum Mlyňany SAV, (P-129), 1964, ZM (3)	23	7,4	8	3 × 3	juv.	9
Arborétum Mlyňany SAV, (P-129), 1964, ZM (4)	82	26,2	16	6 × 6	♀	41
Arborétum Mlyňany SAV, (P-129), 1964, ZM (5)	31	9,9	8	5 × 6	juv.	16
Arborétum Mlyňany SAV, (P-129), 1964, ZM (6)	64	20,4	10	5 × 5	juv.	32
Arborétum Mlyňany SAV, (P-129), 1964, ZM (8)	66	20,7	12	6 × 8	juv.	33
Arborétum Mlyňany SAV, (P-129), 1964, ZM (9)	70	22,4	12	8 × 10	♀	36
Arborétum Mlyňany SAV, (P-129), KD, ZM	18	5,8	5	2 × 2	juv.	9
Arborétum Mlyňany SAV, (P-129), 1964. ZM (17)	49	15,7	12	3 × 3	juv.	24
Báb (P-133), NR	187	59,7	16	10 × 12	♂	95
Bánovce nad Bebravou, City park, BN	101	32,3	16	12 × 6	♂	51
Banská Štiavnica (P-246), Botanical garden, ZH	178	56,8	20	3 × 8	♀	90
Banská Štiavnica (P-246), Botanical garden, ZH	191	69,9	18	12 × 10	♂	111
Banská Štiavnica (P-246), Botanical garden, ZH	112	36,0	16	8 × 10	♂	57
Beladice (P-124), ZM	259	82,5	18	16 × 16	♂	131
Beladice (P-124), ZM	237	75,2	20	16 × 16	♀	119
Betliar (P-407), RV	233	74,3	19	19 × 14	♂	118
Betliar (P-407), RV	12	4,0	3	1,5 × 1,5	juv.	6

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First continuation of table 1

Locality	Trunk circumference (cm)	Trunk diameter (cm)	Tree height (m)	Crown width (m)	Gender (♂, ♀)	Age
Bojnice (P-1, 2, 3), PD	276	87,9	21	10 × 10	♂	140
Bojnice (P-1, 2, 3), PD	218	69,5	16	10 × 10	♂	110
Bojnice (P-1, 2, 3), PD, 415 cm	290	92,4	26	12 × 12	♂	147
Bratislava (P-103), Botanical garden UK, BA	168	53,5	14	15 × 15	♀	85
Bratislava (P-103), Botanical garden UK, BA	222	70,7	16	8 × 8	♀	112
Bratislava (P-103), Botanical garden UK, BA	141	45,0	14	14 × 14	♀	72
Bratislava (P-103), Botanical garden UK, BA	125	37,0	12	6 × 5	♀	58
Bratislava (P-103), Botanical garden UK, BA	118	37,2	17	15 × 15	♀	53
Bratislava (P-105), Mountain park, BA	42	13,4	8	5 × 3	juv.	21
Bratislava (P-108), PKO, statue of D. Jurkoviča, BA	105	33,5	14	8 × 8	♂	53
Bratislava, Bohúňova St. 4, BA	114	36,4	18	6 × 6	♀	58
Bratislava, Dunajská St., BA, 168 cm	124	39,5	14	7 × 6	♀	63
Bratislava, Dunajská St., BA, park near FITNES	60	19,0	8	4 × 3	♂	30
Bratislava, Godrova St. 8, BA	215	65,0	16	12 × 12	♀	103
Bratislava, Košická St., alley, BA, 2008, 29 pcs.	18-35	5,5-11,0	4-5	2 × 2	juv.	18
Bratislava, At the Habán mill, BA	29	9,3	6	2 × 2	juv.	15
Bratislava, J. Kráľ Gardens, 18/2-1751, BA	165	52,5	16	14 × 13	♀	83
Bratislava, J. Kráľ Gardens, BA, 540 cm	467	148,8	20	28 × 24	♀	236
Bratislava, Slovanet Ltd., group planting. Bajkal St.	17-21(30 ks)	5-7	3-5-6	1 × 2	juv.	10
Brezová pod Bradlom, PS, SE	168	53,6	16	15 × 15	♂	65
Brusno Spa (P-242)	9	2,9	3,5	1 × 2	juv.	10
Budimír, at the chapel, (P-378), KE	155	49,4	22	7 × 7	♂	79
Bystrany (P-357), SN	179	57,0	22	8 × 8	♀	91
Bystrany (P-357), SN	57	18,2	3	3 × 3	♀	29
Cerová-Lieskové (P-30), SE	183	58,3	26	12 × 14	♂	93
Častá (Červený Kameň), PK, 447/142,4 cm	375	122,2	14	12 × 14	♀	194
Častkovce (P-21), NM	172	54,5	19	12 × 8	♀	87
Červený Hrádok, ZM, 1964	26	8,3	9	2 × 2	juv.	13
Dubnica nad Váhom, IL	123	39,3	18	8 × 8	♂	62
Fiľakovo,(P-272) City park	191	60,9	18	14 × 14	♂	97
Fiľakovo, (P-272) City park	173	55,2	20	16 × 8	♂	114
Fričovce (P-345), PO	171	54,5	16	6 × 5	♂	87
Galanta (P-146), GA	285	90,5	20	18 × 20	♂	144

Second continuation of table 1

Locality	Trunk circumference (cm)	Trunk diameter (cm)	Tree height (m)	Crown width (m)	Gender (♂, ♀)	Age
Gbely, Art School	80	25,5	8	4 × 4	juv.	41
Hájna Nová Ves (P-44), TO	478	152,4	22	30 × 30	♀	242
Hnúšťa (SG), RS, 320 cm	263	83,8	26	12 × 15	♂	133
Hokovce – historical park	166	52,8	16	14 × 12	♀	84
Hokovce – historical park	35	11	8	8 × 4	juv.	18
Hokovce – historical park	36	12	8	8 × 6	juv.	18
Hokovce, private garden	220	70,1	25	12 × 14	♂	111
Horenická Hôrka-Medné (P-205), PU	345	110,2	25	20 × 20	♀	175
Horné Obdokovce (P-50), TO	192	61,2	25	12 × 12	♂	97
Horné Semerovce (P-158), LV	325	103,7	19	14 × 10	♂	165
Hubice, historical park	184	58,7	18	16 × 12	♂	93
Humenné (P-340), HE,	275	87,6	22	15 × 20	♀	139
Jaklovce (P-359), GL, 230 cm	203	64,7	21	8 × 8	♀	103
Janova Ves (P-42), TO	373	111,2	22	20 × 20	♂	197
Jasov, monastery garden (P-396), KE	220	70,2	14	14 × 14	♂	112
Kalinovo, park PS	61	19,5	12	3 × 3	juv.	31
Kazimír (P-416),TV, 260 cm, double	229;170	73;54,2	17	22 × 20	♀	116
Klobušice (P-217), IL	212	67,5	22	15 × 15	♀	107
Klobušice (P-217), IL	179	57,0	22	14 × 14	♂	91
Klobušice (P-217), historical park	165	52,6	13	10 × 5	♀	84
Kočovce (P-17), NM	206	65,6	25	10 × 10	♀	104
Komárno (P-187), KN	76	24,2	12	10 × 12	♀	38
Komjatice (P-166), NZ, 365 cm	327	104,2	25	22 × 22	♀	165
Košice (P-382), Botanical garden UPJŠ, KE	69	22,0	12	5 × 4	juv.	35
Košice (P-388), Park J. A. Komenského, KE	312	99,7	20	20 × 20	♂	158
Košice (P-390), FN, KE Rastislavova St. (1)	197	62,7	22	14 × 16	♀	100
Košice (P-390), FN, KE Rastislavova St. (2)	131	41,8	19	4 × 0,5	♀	66
Košice (P-390), FN, KE, Rastislavova St., (4)	121	38,5	12	11 × 19	♀	61
Košice (P-390), FN, KE, Rastislavova St. (3)	151	48,1	18	10 × 12	♂	46
Košice (P-390), FN, KE, Rastislavova St. (5)	133	42,5	11	10 × 9	♀	68
Košice, Masarykova St. 3, ZŠ	381	121,7	27	24 × 12	♂	193
Košice, Town square MMM, KE	15.5	5.0	5	2 × 2	juv.	8
Kovarce (P-48), TO	167	53.2	19	12 × 12	♂	85

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Third continuation of table 1

Locality	Trunk circumference (cm)	Trunk diameter (cm)	Tree height (m)	Crown width (m)	Gender (♂, ♀)	Age
Krakovany, Park in the courtyard of the company, PN	40	12.8	7	3 × 3	juv.	20
Kravany nad Dunajom (P-189), KN	189	60.5	16	13 × 14	♂	96
Lučenec (SG), Ipeľské tehelne Ltd., LC, cv. ´Ohatsuki´	267	85.1	22	16 × 16	♀!!	135
Lučenec, City park, LC	40	12.0	12	8 × 8	♂	19
Malacky (SG), MA	16	5.3	5	3 × 3	juv.	10
Malacky (SG), MA	18	6.0	7	3 × 1	juv.	10
Malacky, private garden (SG), MA	59	18.8	12	7 × 7	♀	20
Malinovo,(P-99) SC	38	12.5	8	4 × 4	♂	20
Malý Šariš (P-348), PO	285;315	91;100.7	20	20 × 20	♂	160
Martin (P-228), MT	180	57.0	18	14 × 10	♂	91
Michalovce, park in front of the bank VÚB, MI	190	60.5	17	10 × 16	♂	96
Modra (P-90), PK	193	61.5	14	6 × 6	♀	98
Mošovce (P-231), TR	125	39.8	12	15 × 14	♂	63
Motešice (P-13), TN	110	35.0	15	12 × 10	♂	56
Necpaly (P-230), MT	195	62.2	27	8 × 8	♀	99
Nedožery-Brezany,168/120	48	15.3	10	5 × 4	juv.	24
Nenince (P-276), VK, 240 cm	195	62.0	16	8 × 8	♂	99
Nitra, Ďurčanského St. 12	60	19.2	5	6 × 6	juv.	31
Nitra, Faculty of Natur. Sci.UKF, NR	25	8.0	6	3 × 3	juv.	13
Nitra, Kupecká ulica, alley	6 pcs.	-	4 – 5	2 × 2; 3 × 3	juv.	10
Nitra, Nitra city park, 1963, NR, triple trunk (234 cm/74,5 cm)	120;119;96	38.2;30.7	18	18 × 18	♀	60
Nitra, Nitra city park, 1963. NR	81	26.0	12	14 × 12	♂	41
Nitra, Penzión LU×	15	4.8	6	3 × 3	juv.	10
Nitra, rest. MALIBU	107	34.2	15	9 × 9	♀	54
Nitra, Špitálska St.1, NR	102	32.5	12	9 × 9	♂	52
Nová Dubnica, TN	21;33	6.7;10.8	3.5;4.5	1 × 1;2 × 2	juv.	17
Nová Ves nad Žitavou (P-135), NR (1)	228	72.7	16	18 × 18	♀	116
Nová Ves nad Žitavou (P-135), NR (2)	277	88.3	17	18 × 18	♀	140
Nová Ves nad Žitavou (P-135), NR (4)	172	54.8	20	2 × 9	♀	87
Nová Ves nad Žitavou (P-135), NR	202	64.4	22	16 × 16	♀	102
Nový Život-Tonkovce, (P-191), DS	97	31.0	15	3 × 3	♂	49
Oponice (P-52), TO	185	59.0	13	10 × 8	♂	94
Palárikovo (P-174), NZ (4)	247	78.6	20	14 × 14	♂	125
Palárikovo (P-174), NZ (1)	316	101.0	20	19 × 19	♂	160
Palárikovo (P-174), NZ (2)	62	19.7	10	6 × 7	♀	31
Palárikovo (P-174), NZ (3)	60	19.2	12	7 × 5	♀	31
Palárikovo (P-174), NZ (5)	100	31.9	10	0 × 9	♂	51
Piešťany (SG), private garden, PN	280	89.2	25	20 × 20	♂	142

Fourth continuation of table 1

Locality	Trunk circumference (cm)	Trunk diameter (cm)	Tree height (m)	Crown width (m)	Gender (♂, ♀)	Age
Piešťany (P-58), Spa park near bath "Eva", PN	152	48.5	16	12 × 10	♂	97
Piešťany (P-58), Spa park, PN	202	64.9	23	16 × 14	♀	120
Piešťany (P-58), Spa park near outdoor bath, PN	186	59.3	25	16 × 14	♀	120
Piešťany, Park at High school, (118) PN	76;71;27	24.3;22.7; 8	15	6 × 6	juv.	37
Piešťany, Park at high school,(116) PN	82	26.2	15	8 × 8	juv.	41
Piešťany, Park at high school,PN	35	11.2;12;12.8	10	4 × 4	juv.	17
Pohronský Ruskov (SG), LV, 300 cm	261	83.2	14	23 × 20	♂	132
Považ. Bystrica, at the cinema MIER	48	15.5	4	4 × 4	juv.	50
Prešov, Art Garden, PO	265	84.5	18	15 × 15	♂	134
Pribeník (P-418),TV	330	105.5	25	16 × 22	♂	168
Prievidza (SG), PD	128	40.8	15	8 × 8	♀	65
Rajecké Teplice (P-223), Spa park, ZA	15	5.0	3	0,5 × 0,5	juv.	8
Rakovice (P-66), PN	237	75.5	20	18 × 15	♀	120
Rimavská Sobota (P-292), RS	126	40.2	16	12 × 12	♀	64
Rimavská Sobota, Športová St. č.4, RS	135	43.0	10	8 × 8	♀	68
Rimavská Sobota, Športová St. č.4, RS	155	49.5	18	6 × 10	♂	79
Ružomberok, Military hospital.	120;57	38.2;18.2	14;7	8 × 8	♂	95
Senica (SG), SE, alley-25 ks	7-10-12	4.8-9.8	3-6	2 × 2	juv.	8-16
Senica, Hviezdoslavova St., 4 pcs, row of trees SE	26-31	8.4-9.8	7	2 × 2	juv.	16
Senné (SG), private garden, 1964, VK	42	13.5	12	3 × 4	juv.	22
Sereď, private garden	59	18.8	12	7 × 7	juv.	30
Slanec, Park around PS and KG, KE, 362 cm	278	88.5	18	16 × 14	♂	141
Sľažany (P-118), ZM,	118	37.7	16	10 × 8	♂	60
Slovenské Pravno, PS	3	1.0	1	0,5 × 0,5	juv.	10
Spišská Belá, KK	198	63.0	16	8 × 9	♂	100
Stupava (P-96), MA	195	62.2	20	12 × 14	♂	99
Súdovce (SG), KA	302	96.5	14	12 × 10	♂	153
Šamorín, private garden	169	53.8	14	12 × 12	♂	40
Šišov (P-34), BN	160	51.0	28	8 × 8	♂	81
Šurianky (P-114), NR, 120 cm	87	27.8	12	6 × 0	♂	44
Tomášikovo (P-148), GA, 430 cm	315	100.4	25	16 × 16	♀	162
Tomášov (P-100), 362 cm	290	90.7	20	20 × 19	♂	144
Topoľčany (P-40), TO	110	35.0	17	6 × 6	♀	56
Topoľčianky (P-115), ZM (1)	225	71.7	20	16 × 16	♀	114
Topoľčianky (P-115), ZM (2)	305	97.5	12	14 × 14	♂	155
Topoľčianky (P-115), ZM (3)	261	83.2	18	16 × 16	♀	132
Topoľčianky (P-115), ZM (4)	92	29.3	12	6 × 6	♀	47
Tovarníky, (P-40), hist. park ObÚ	157	50	18	3 × 4	♀	79

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Fifth continuation of table 1

Locality	Trunk circumference (cm)	Trunk diameter (cm)	Tree height (m)	Crown width (m)	Gender (♂, ♀)	Age
Trávnica (P-169), NZ	239	76.2	22	10 × 8	♀	121
Trenčianske Teplice – Spa park (35 cm)	19	6.2	4	0,5 × 1	juv.	10
Trenčianske Teplice – Spa park (54 cm)	31	9.5	6	5 × 5	juv.	15
Trenčianske Teplice – Spa park (95 cm)	64	20.4	12	8 × 8	juv.	32
Trenčín (P-7), TN	290	92.7	16	10 × 10	♀	147
Trenčín (P-7), TN	365	116.7	16	12 × 12	♂	185
Trenčín (P-7), TN	210	67.0	17	10 × 10	♀	106
Trnava (P-78), TT	136	43.3	16	14 × 12	♂	72
Trnava (P-78), TT	116	36.9	14	6 × 6	♂	69
Trnava, (SG), Kalinčiakova St. 17, TT	151	48.2	12	16 × 14	♀	77
Turčianska Štiavnička (P-225), MT	233	74.2	24	9 × 9	♀	118
Veľký Blh (P-286), RS	190	60.5	14	0 × 14	♀	96
Vištuk, Park OÚ, PK	22	7.0	5	5 × 3	juv.	11
Voderady (P-84) historical park, TT	292	93	18	18 × 18	♂	148
Voderady (P-84), TT, 480 cm	213	68.0	16	15 × 15	♂	108
Vráble, private garden	110	35.0	16	10 × 10	♂	40
Záblatie (P-4),TN	178	56.7	15	10 × 12	♀	90
Zemianske Podhradie (P-12), NM	219	70.0	24	16 × 14	♂	111
Zvolen, at the factory Bučina	51	16.3	12	2 × 2	juv.	26
Zvolen, at the factory Bučina	45	14.0	10	2 × 2	juv.	22
Zvolen, at the factory Bučina	25	8.0	6	2 × 1	juv.	13
Želiezovce (P-162), LV	195	62.0	16	15 × 10	♂	98
Žilina, at the Department Store MIRAGE, 5 pcs ZA	25-29	8.0-9.3	4-5-6	1 × 1	juv.	13
Župčany (P-347), PO	136	43.3	16	9 × 10	♂	69

Notes: P-44 – registration number of the historical park and garden, TO – Topoľčany – district – according to the current territorial division, 19/1 – first half of the 19th century, with the designation of the years – 1850 of foundation of the dendrological building, SG – surrounding greenery, that is, the tree is in a territory other than a historical park or garden, PS – primary school, KG – kindergarten, juv. – an individual in juvenile growth

recorded 20 localities where no trees were found, but previously were identified by Benčať (1992) or these are private and religious buildings, unavailable at the time of our research.

We included 45 ginkgo trees that reached their maximum dimensions. The group of trees with a trunk circumference of more than 400 cm included individuals in Hájná Nová Ves – 477 cm; Bratislava – Petržalka, Sady J. Kráľa 467 cm. More numerous was the group of trees with trunk circumference over 300 cm (13 trees) and over 200 cm (33 trees).

Some new findings on the cultivation and growth of *Ginkgo biloba*

The number of localities with the intentional introduction of *Ginkgo biloba* in Slovakia cannot be complete, because in recent years, at the beginning of the 21st century, other new plantings were added in various categories of urban greenery, but also in private gardens and parks.

During our research, we occasionally recorded the nesting of birds (garden turtle, gray crow, common magpie) on the trees of the two-lobed ginkgo. We found nesting cavities on tree trunks and skeletal branches, which cut down woodpeckers in soft wood (even healthy trees), or in the cracks of tree bark we found collected seeds of yew (*Taxus baccata* L.). We first discovered the natural rejuvenation and occurrence of ginkgo seedlings in Trenčín (2007), and later in Palárikovo (2011); Nitra – Nitra City Park (2011); Tomášikovo (2011), Bratislava – Botanical Garden (2011), Godrova St. no. 8 (2014); Košice – park around

L. Pasteur Hospital (2014); Nová Ves nad Žitavou (2014). The best conditions for seed germination are in the fallen and accumulated leaves under the tree. The natural seed propagation (Figure 6) and occurrence of seedlings of foreign wood can be considered as the peak phase in the process of introduction, and in the case of ginkgo it was rare and rare.

When assessing the horticultural value and health of *Ginkgo biloba* trees, we did not find any major differences from typical habitual features (except for deformation and suppression of tree crowns, in a group or under the crowns of other surrounding trees), so we rated the trees with the highest number of points (5th grade). We did not record the occurrence of animal pests (especially leaf-eating, sucking, and wood-destroying insect pests) or fungal diseases on ginkgo trees during this research, and we also evaluated them at the highest level 5. The only disadvantage that will probably limit the cultivation of trees in our conditions is the unpleasant smell (after rotting meat) of ripening and falling fruit from female individuals. There have already been cases of tree felling for hygienic and safety reasons if the fruit pollutes busy streets, but especially pedestrian sidewalks, and there is a risk of slipping and possible fall of pedestrians. As a precautionary measure, we recommend covering the sidewalks under the fruiting trees, making structures covered with foil or tarpaulin with a tendency to the fruiting trees, permanent removal of deciduous fruit from the sidewalks, especially in parks, front gardens, and street plantings.

Figure 6 Natural seed propagation under the ginkgo tree
Photo by Pavel Hrubík

Figure 7 Large fruits observed on female ginkgo tree grown in the city park in Nitra
Photo by Pavel Hrubík

During our field research, we also recorded certain morphological differences (habitual features on trees –

Figure 8 Distinctive golden-yellow colour of leaves in autumn in the locality Bratislava – Gardens of J. Kráľ
Photo by Pavel Hrubík

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Figure 9 The variability of fruits stalks
Photo by Pavel Hrubík

branching and shape of the crown; angle of protrusion and growth of lateral branches from the main trunk; leaf fall time; tree sex differences). Large fruits on long stems, we recorded on trees in Kočovce, Rimavská Sobota, Nová Ves nad Žitavou, Topoľčianky, Nitra, Bratislava – Botanical Garden (Figure 7). Distinctive golden-yellow colour of leaves in autumn in localities Kočovce, Nová Ves nad Žitavou, Topoľčianky, Trenčín, Bratislava – Gardens of J. Kráľ in Petržalka, Dunajská

Figure 10 Epicormic sprout on the ginkgo tree trunk
Photo by Pavel Hrubík

Figure 11 Root stalks growing on the trunk of a felled ginkgo tree in Trenčín
Photo by Pavel Hrubík

street (Figure 8). A special peculiarity was the autumn in 2014, when during our research on September, October, and mid-November, the ginkgo trees kept the green leaves in the entire crown of the tree. At the same time, there are also differences between localities

Figure 12 A lateral branch formed after mechanical damage to the ginkgo strain in the locality Trávnica
Photo by Pavel Hrubík

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Figure 13 Heavily branched ginkgo tree with trunk circumference 478 cm in the locality Hajná Nová Ves. The oldest tree of *Ginkgo biloba* in Slovakia
Photo by Pavel Hrubík

in Slovakia from one year to another. The prevailing finding was that the leaves on the male trees fall off earlier than on the female fruiting trees (preservation of the leaves during fruit ripening is also more logical). In 2014, this did not manifest itself in Nitra – Nitra City Park, because in mid-November, the leaves on the female tree were fallen, while on the neighboring male tree, all the leaves remained golden-yellow on the tree.

The size of the leaves is very variable, sometimes the length of the leaf stalk is extreme (6.2–7.6 cm in Kočovce) (Figure 9), the leaves are deeply lobed, large, fleshy, they are on the stem and roots samplings of the trees (Figure 10), (15.5 × 13.5 cm, leaf lobe depth up to 8 cm, leaf size 12 × 14.8, 11 × 13.5 cm, length of samplings 100–150–180 cm, more than 220 pieces of root stalks around the trunk circumference of a felled tree in Trenčín).

The occurrence of samplings on the stems and roots of *Ginkgo biloba* trees is extremely strong. This was most pronounced in Trenčín, after the felling of a male tree on the construction site of the future hotel in 2008. The following spring, we found massive 100–150–180 long shoots on the trunk of the tree, more than 220 pieces around the entire circumference of the trunk. The leaves

Figure 14 Ginkgo tree in Janova Ves with unique branching and trunk circumference 373 cm
Photo by Pavel Hrubík

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Figure 15 Observation of the occurrence of fruits on the leaves, referred to as cv. Ohatsuki of ginkgo growing in Lučenec Photos by Pavel Hrubík and Katarína Ražná

on the samplings were large, fleshy, deeply lobed; bark on young shoots cinnamon brown, with pronounced brown lenticules, shoots 1–2 years old (Figure 11). Stump youth also occurred on felled trees in Košice; on the sawed branches and on the trunk of the *Ginkgo biloba* in Horné Semerovce (the budding shoots were also on the cut pieces of the trunk lying on the ground under the tree). It also regenerates branches broken by the wind, snow, or the weight of ripening fruits (Topoľčianky). It does not tolerate shading, limiting the growth of the crown by climbing trees (*Hedera helix* L. – Arborétum Mlyňany, Piešťany, Senné).

Also important is the rapid healing of wounds after mechanical damage to the trunk and branches, sawing of branches in the crown of the tree, sawing of the dry terminal top of the tree (then a beautiful, fan-shaped crown and top of the side branches – Trávnica); very good and fast is "hardening" after trimming thinner branches and healing larger wounds on the tree trunk (Figure 12).

In terms of the vertical distribution of *Ginkgo biloba* in Slovakia, we consider the highest-lying locality *Ginkgo biloba*, trees growing in the Botanical Garden of the Secondary Vocational Forestry School in Banská Štiavnica (three trees, one of them fruiting, female tree); the lowest localities included the ginkgo trees in Kravany pri Dunaji, Komárno, Pribeník, and Kazimír.

It is worth mentioning some of the particularly special trees, specific to twin habits, trunk dimensions, and fruits on leaves (Figures 13–15).

In the end, we present a list of the oldest trees of *Ginkgo biloba* in Slovakia (Table 3). The data on trees dimensions and age come from the period of our research in 2016.

On the importance of recognizing genetic diversity of *Ginkgo biloba* populations pointed out several studies focused on applying different molecular markers as genomic microsatellites (Yan et al., 2006; Yan et al., 2009; Li et al., 2009; Xie et al., 2013), RAPD (Fan et al., 2004; Li et al., 2013; Mei et al., 2014), ISSR (Mei et al., 2014), RFLP (Shen et al., 2005) and microsatellites (Xu et al., 2015) markers based on chloroplast DNA and microRNA-based markers (Ražná et al., 2020). Attention is also paid to the gender differentiation of ginkgo trees at the molecular level (Jiang et al., 2003; Liao et al., 2009; Milewicz and Sawicki, 2013).

In order to establish a molecular base to understand the evolution of ginkgo and to resolve the ambiguous phylogenetic relationship of ginkgo among the gymnosperm, several studies have been performed (Brenner et al., 2005; Lin et al., 2011; Lin et al., 2012; Šmarda et al., 2016).

Whereas ginkgo leaves contain a quantity of medicinally valuable compounds, extensive studies are carried on the identification and characterization of genes and molecules connected to these biosynthetic processes (Tekeľová et al., 2006; Zittlau, 2007; Wang et al., 2010; Han et al., 2015; He et al., 2015; Wang et al., 2015).

The genetic background of ginkgo resistance to a wide spectrum of biotic and abiotic stress conditions was also analysed (Mohanta, 2012). *In vitro* approaches of ginkgo micropropagation are known from the literature (Tommasi and Scaramuzzi, 2004; Mantovani et al., 2013).

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Table 3 The oldest *Ginkgo biloba* L. trees in Slovakia (2016)

Locality	Trunk circumference (cm)	Trunk diameter (cm)	The height of the tree (m)	Crown width (m)	Gender (M, F)	Age
Hajná Nová Ves (P-44), TO, 19/1-1850	478	152.4	22	30 × 30	F	242
Bratislava, J. Kráľ Gardens, BA, (P-110), 18/2 -1751	467	148.8	20	28 × 24	F	236
Janova Ves (P-42), TO,19/2-1851	373	111.2	22	20 × 20	M	197
Košice, Masarykova St.3, PS, KE, 19/1-1850	381	121.7	27	24 × 12	M	193
Častá (Červený Kameň) (P-424, PK,18/2-1771	375	122.2	14	12 × 14	F	194
Pribeník (P-418), TV, 19/1-1850	330	105.5	25	16 × 22	M	168
Komjatice (P-166), NZ, 18/2-1751 (365 cm)	327	104.2	25	22 × 22	F	165
Horné Semerovce (P-158), LV, 20/1-1950	325	103.7	19	14 × 10	M	165
Tomášikovo (P-148), GA, 8/2-1751, (430 cm)	315	100.4	25	16 × 16	F	162
Palárikovo (P-174), NZ, 19/1- 1850	316	101.0	20	19 × 19	M	160
Malý Šariš (P-348, PO, 19/1 - 1850	315;285	100.7;91.0	20	20 × 20	M	160
Horenická Hôrka-Medné (P-205, PU, 19/2-1851	345	110.2	25	20 × 20	F	175
Košice, Park J. A. Komenský (P-388),KE	312	99.7	20	20 × 20	M	158
Voderady (P-84), TT, 19/2-1851 (480 cm)	292	93.0	18	18 × 18	M	148
Súdovce, KA, 20/1 – 1950	302	96.5	14	12 × 10	M	153
Topoľčianky (P-115). ZM, 18/2 - 1751	305	97.5	12	14 × 14	M	155
Bojnice (P-1; 2; 3), PD, 19/1-1850, (415 cm)	290	92.4	26	12 × 12	M	147
Tomášov (P-100), SC, 18/2-1751 (362 cm)	290	90.7	20	20 × 19	M	144
Galanta (P-146), GA, 19/2-1851	285	90.5	20	18 × 20	M	144
Piešťany, PN, 19/1-1850 (SG)	280	89.2	25	20 × 20	M	142
Slanec, PS, KG, KE, 19/1-1850 (362 cm)	278	88.5	18	16 × 14	M	141
Nová Ves nad Žitavou (P-135), NR, 19/1-1850	277	88.3	18	22 × 24	F	140
Bojnice, PD, 19/1-1850	276	87.9	21	10 × 10	M	140
Humenné (P-340), HN, 18/2	275	87.6	22	15 × 20	F	139
Lučenec, Ipeľské tehelne, (SG), LC, cv.´Ohatsuki´	267	85.1	22	16 × 16	F	135
Topoľčianky (P-115), ZM, 18/2-1751 (293 cm)	261	83.2	18	16 × 16	F	132
Pohronský Ruskov (SG), LV, 20/1-1950 (300 cm)	261	83.2	14	23 × 20	M	132

Continuation of table 3

Locality	Trunk circumference (cm)	Trunk diameter (cm)	The height of the tree (m)	Crown width (m)	Gender (M, F)	Age
Beladice (P-124), ZM, 19/2-1851	259	82.5	18	16 × 16	M	131
Hnúšťa (SG), RS, 20/1-1950 (320 cm)	263	83.8	26	12 × 15	M	133
Palárikovo (P-174), NZ, 19/1 - 1850	247	78.6	20	14 × 14	M	125
Trávnica (P-174), NZ, 18/2- 1751	239	76.2	22	10 × 8	F	121
Rakovice (P-66), PN, 19/2 - 1851	237	75.5	20	18 × 15	F	120
Beladice (P-124), ZM, r. – 130 r.	237	75.2	20	16 × 16	F	130
Abramová (SG), TR, 18/2-1751	238	76.0	14	18 × 14	F	121
Betliar (P-407), RV, 19/1 - 1850	233	74.3	19	19 × 14	M	118
Trenčín (P-7), TN, 19/1 - 1850	365	116.7	16	12 × 12	M	185
Turčianska Štiavnička (P-225). MT, 18/2-1751	233	74.2	24	9 × 9	F	118
Kazimír (P-416), TV, 19/1-1850 (260 cm)	229;170	73.0;54.2	17	22 × 20	F	116
Nová Ves nad Žitavou (P-135), NR, 19/1-1850	228	72.7	16	23 × 20	F	115
Topoľčianky (P-115), ZM, 18/2-1751	225	71.7	20	16 × 16	F	113
Trenčín (P-7), TN, 19/1-1850	290	92.7	16	10 × 10	F	147
Prešov, Garden of Art (SG)	265	84.5	18	15 × 15	M	134
Jasov, Monastery Garden	220	70.2	14	14 × 14	M	112
Hokovce, private garden	220	70.1	25	12 × 14	M	180
Bratislava, Bot. garden	222	70.7	16	8 × 8	F	112

Notes: P-44 – registration number of the historical park and garden, TO - Topoľčany – district – according to the current territorial division, 19/1 – first half of the 19th century, with the designation of the years – 1850 of foundation of the dendrological building, SG – surrounding greenery, that is, the tree is in a territory other than a historical park or garden, PS – primary school, KG – kindergarten

During our long-term research of *Ginkgo biloba* and its cultural distribution in Slovakia, we verified or confirmed the knowledge about the growth, cultivation, use of fruits and seeds of this rare foreign tree.

Ginkgo biloba is a dioecious tree. Determining and differentiating the sex of trees is difficult, until the time of flowering and fruiting trees, is practically impossible. Several published findings concerning the gender differentiation, morphological and physiological features are known (Benčasť, 1982; Pagan and Randuška, 1988; Tomaško, 2004; Benčať, 2009; van Beek, 2000; Begovic, 2011; Kwant, 2011; Zhang et al., 2015). However, based on our long-term research and experiences obtained from three dendrological expeditions in the Democratic People's Republic of Korea (1983 and 1985) and China (1998), we have also come to conflicting views and experiences:

- The male and female trees occur in a ratio 1 : 1. Based on our research and practical experience, we cannot confirm this, and we observed that males predominate in the population.
- Habitus of the tree: male tree – side branches protruding at an acute angle from the main trunk; female tree – horizontally – horizontally projecting lateral branches from the trunk of the tree (almost at right angles). To our knowledge, it is exactly the opposite. The male tree has branches protruding horizontally (almost at right angles). The female tree has branches protruding from the main trunk at an acute angle.
- Number of grooves (ribs) per seed: seeds from which male trees grow have 3 ribs; those producing female tree – 2 ribs. To our knowledge, this phenomenon has the opposite character. The seeds from which male trees grow have 2 ribs on the seed (these are in absolute predominance); the ones producing female tree has 3 ribs per seed (a rare occurrence).

- The male trees bloom 2–3 weeks earlier than female ones. Due to the distances between the research localities of ginkgo trees, we did not perform regular phenological monitoring, so we cannot confirm the previous thesis, but we can agree with the data.
- Gender differentiation of *Ginkgo biloba* trees can be determined by the depth of cuts on the leaf blade: male trees have a deep cut on the leaf blade; female trees have a shallow notch at the leaves, up to entire leaves. We cannot confirm this thesis with certainty, moreover, the variability of the leaves on the one tree is very great.
- Fallen leaves in autumn: male trees tend to fall earlier; female trees have a later date of leaf fall. We can agree with this thesis, but in 2014, in Nitra City Park, the leaves of the male tree were kept two weeks longer (and fell later, while the female tree had been without leaves for a long time).

Conclusions

The final analysis of the research results on the cultigenous area of *Ginkgo biloba* in Slovakia confirmed the occurrence of this rare tree in more than one hundred localities (103 localities). No trees were found in the 18 previously registered localities of the occurrence of ginkgo, 5 trees were felled for various reasons (hygienic, safety, other reasons). The number of trees in solitary plantings (or in groups of three trees) reached 203 trees. 89 trees were planted in tree lines and alley plantings (especially in the city streets). There were 73 male trees, 65 female and 154 pregenerative individuals, a total of 292 trees of *Ginkgo biloba*. The number of ginkgo trees decreases in the following row: Bratislava – 42 trees; Senica – 27; Zlaté Moravce – 26; Nitra – 10; Košice – 9; Piešťany – 9; Topoľčany – 8; Žilina. 7; Nové Zámky – 7; in other districts (32) only 1–3 trees grow; we did not detect *Ginkgo biloba* trees in 31 districts. We evaluated the basic dendrometric and growth parameters of the oldest ginkgo trees, found in Slovakia. We evaluated 42 trees (aged 242 – 111 years) in 35 localities.

Conflict of interests

Authors declare no conflict of interests.

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

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Storage-dependent effects of oregano essential oil on lipid peroxidation and total antioxidant capacity in the grapeseed oil

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Natural preservatives such as essential oils can be used as alternatives to chemical additives which could extend the shelf life of various food products. The knowledge about them can have important economic responsiveness by a decrease of spoilage-induced losses. The purpose of the current study was to investigate the content of 2-thiobarbituric acid reactive substances (TBARS) and total antioxidant capacity (TAC) in the grapeseed oil with the use of a commercial oregano essential oil (Etja, Elblag, Poland) as an antioxidant agent by monitoring the lipid peroxidation and antioxidant capacity. The effect of the oregano essential oil on the oxidative stability of the grapeseed oil was evaluated throughout 120 days of storage. The current results demonstrated that administration of oregano essential oil, exhibiting free radical scavenging activity determined by TBARS assay, exerts beneficial effects on preventing lipid peroxidation in grapeseed oil by limiting the TBARS levels and simultaneously increase of total antioxidant capacity, especially at 15–60 days of storage. At 60–120-day periods of storage, the TBARS levels were significantly lowered from control samples. The highest level of total antioxidant capacity was observed on 30-days compared to the control samples. Thus, edible adding containing essential oils have potential application in the plant oils to maintain their characteristics during the different shelf life.

Keywords: Grapeseed oil, oregano essential oil, 2-thiobarbituric acid reactive substances, lipid peroxidation, total antioxidant capacity, storage

Introduction

Oregano is known as the name used to refer to a great variety of plants, at least 61 species, and 17 genera belonging to six different botanical families. Verbenaceae and Lamiaceae are the most conspicuous families. Within the Lamiaceae family are the plants belonging to the genera *Origanum* and *Hedeoma*; while the genera *Lippia* and *Lantana* belong to the Verbenaceae family. The other families are Rubiaceae, Apiaceae, and Asteraceae (Kintzios, 2012; Baser and Buchbauer, 2015; Leyva-López et al., 2017). *Hedeoma patens*, *Lippia graveolens*, *Lippia palmeri*, *Lippia alba*, *Origanum dictamnus*, *Origanum hirtum*, *Origanum onites*, *Origanum vulgare* are some plants of oregano

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species producing essential oils (EOs) (Economou et al., 2011; Baser and Buchbauer, 2015; Leyva-López et al., 2016, 2017).

Oregano is a plant that has been used as a food seasoning since ancient times. The main compounds identified in the different oregano EOs are carvacrol and thymol, which are responsible for the characteristic odor, antimicrobial, and antioxidant activity (Rodriguez-Garcia et al., 2016). However, their content may vary according to the species, harvesting season, and geographical sources. For example, the qualitative and quantitative composition of EO compounds of European *Origanum vulgare* L. was analysed by Lukas and co-workers (2015). The content of EO compounds of European *O. vulgare* ranged between 0.03 and 4.6 %. The monoterpenes were primarily made up of sabinene, myrcene, *p*-cymene, 1,8-cineole, β-ocimene, γ-terpinene, sabinene hydrate, linalool, α-terpineol, carvacrol methyl ether, linalyl acetate, thymol, and carvacrol. Among the sesquiterpenes β-caryophyllene, germacrene D, germacrene D-4-ol, spathulenol, caryophyllene oxide, and oplopanone were often present in higher amounts. According to the proportions of cymyl-compounds, sabinyl-compounds, and the acyclic linalool/linalyl acetate, three different main monoterpene chemotypes were defined. The cymyl- and the acyclic pathway were usually active in plants from the Mediterranean climate whereas an active sabinyl-pathway was a characteristic of plants from the Continental climate (Lukas et al., 2015). On the other hand, the comparative results of *O. vulgare* collected from four different regions in the Kumaon region (Uttarakhand, India) also showed differences in the chemical constituents of the EOs (Pande et al., 2012). The oil of *O. vulgare* collected from Dhoulchina and Champawat (chemotype I) shows *p*-cymene (6.7–9.8 %), γ-terpinene (12.4–14.0 %), thymol (29.7–35.1 %), and carvacrol (12.4–20.9 %) as major constituents while the oil from Kilbury and Rushi village (chemotype II) shows linalool (6.7–9.7 %), bornyl acetate (12.6–16.8 %), β-caryophyllene (10.5–13.8 %) and germacrene D (6.3–11.3 %) as the major constituents (Pande et al., 2012).

The study of Verma et al. (2012) also showed that the plant stage had a significant effect on the EO content and composition of *O. vulgare*. A total of 38 constituents, representing 97.4–99.7 % of the total oil composition, were identified. Major components of oils were thymol (40.9–63.4 %), *p*-cymene, (5.1–25.9 %), γ-terpinene (1.4–20.1 %), bicyclogermacrene (0.2–6.1 %), terpinen-4-ol (3.5–5.9 %), α-pinene (1.6–3.1 %), 1-octen-3-ol (1.4–2.7 %), α-terpinene (1.0–2.2 %), carvacrol (<0.1–2.1 %), β-caryophyllene (0.5–2.0 %) and β-myrcene (1.2–1.9 %). Thymol, terpinen-4-ol, 3-octanol, α-pinene, β-pinene, 1,8-cineole, α-cubebene, and (E)-β-ocimene were observed to be higher during the full flowering stage (Verma et al., 2012). These substances as antibacterial agents make the cell membrane permeable due to its impregnation in the hydrophobic domains, this effect is higher against gram-positive bacteria (Rodriguez-Garcia et al., 2016; Sakkas et al., 2017).

Several studies have been conducted to determine and evaluate the biological properties of oregano EO. Most of the studies are focused on antimicrobial activity, such as antifungal, bactericidal, and antiviral (Leyva-López et al., 2017). Also, recently other properties of EO compounds have come to the attention of researchers. The oregano EO can be used as alternatives to chemical additives which could extend the food products' shelf life. The knowledge about them can have important economic feedback by reducing losses attributed to spoilage and by allowing the products to reach distant and new markets (Pavelková et al., 2014). The oregano EO has antioxidant properties effective in retarding the process of lipid peroxidation in fatty foods and scavenging free radicals (Rodriguez-Garcia et al., 2016).

Therefore, it is interesting to study the progress of lipid oxidation in plant oils with the addition of oregano EO as an antioxidant. We hypothesized that oregano EO would inhibit or reduce the level of lipid oxidation in plant oils due to the antioxidative properties of the EO. Also, contents of the lipid peroxidation marker in the plant oils were monitored during the storage period to investigate if lipid oxidation can have effects on the fate of bioactive compounds in the plant oils during storage. Thus, the purpose of the current study was to investigate the content of 2-thiobarbituric acid reactive substances (TBARS) and total antioxidant capacity (TAC) in the grapeseed oil with the use of a commercial oregano EO (Etja, Elblag, Poland) as an antioxidant agent by monitoring the lipid peroxidation for 120-days storage period.

Materials and methodology

Preparation of samples

The grapeseed oil was obtained from a local shop. Grapeseed Oil (Monini, Italy) is a product that contains polyunsaturated fatty acids. The energy value of 100 mL is 3 404 kJ/828 kcal, fat 92 g, including 11 g saturated fatty acids, 24 g monounsaturated fatty acids, and 57 g polyunsaturated fatty acids. The grapeseed oil sample (5 mL) was incubated with 0.1 mL of oregano EO (Etja,

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Elblag, Poland) (final concentration was 20 µg/mL) at 25 °C for 240 days. This reaction mixture was shaken gently while being incubated for a fixed interval at 25 °C. Samples were removed at 0, 8, 15, 30, 60, and 120 days of storage for analysis. The grapeseed oil was used as the control samples.

Assay of 2-thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was evaluated by TBARS according to the method described by Kamyshnikov (2004) with some modifications. Briefly, 0.1 mL of sample was added with 2 mL of distilled water, 1 mL of 20 % trichloroacetic acid (TCA), and 1 mL of 2-thiobarbituric acid (TBA) in a test tube and, the tube content was immediately vortexed. Following water bath treatment at 100 °C for 15 min, the tube content was cooled rapidly down to room temperature and centrifuged at 1 000 × g for 10 min. Then, absorbance was measured at 540 nm with a spectrophotometer (Specol 11, Carl Zeiss Jena, Germany) against blind (2.1 mL distilled water and 2 mL TCA-TBA solution). TBARS were calculated as µmoles malonic dialdehyde (MDA) per L of the sample.

Measurement of total antioxidant capacity (TAC)

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

Statistical analysis

Results are expressed as the mean. All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). Significance of differences in the lipid peroxidation biomarker in the samples (significance level at $p < 0.05$) was examined using the Mann-Whitney test according to Zar (1999). All statistical calculations were performed on separate data from each sample with STATISTICA 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

Lipid oxidation is a very complex process initiated by peroxidation of the unsaturated fatty acid in phospholipid membranes to form primary oxidation products, hydroperoxides. The hydroperoxides decompose into further secondary oxidation products, such as aldehydes, ketones, alkenes, and alcohols that cause off-flavours and odors in food products (Kumar et al., 2015). The effect of the oregano EO on oxidative stability of the grapeseed oil was evaluated throughout 120 days of storage. The inclusion of the oregano EO

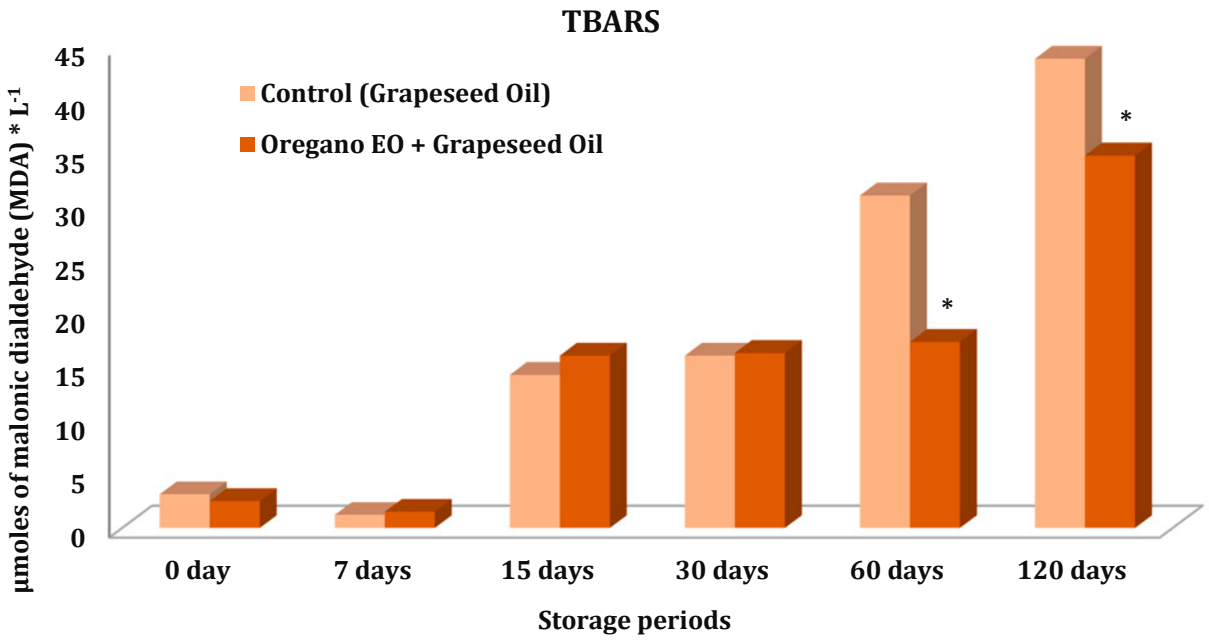

Figure 1 The effect of the addition of oregano essential oil and storage time on TBARS value (a biomarker of lipid peroxidation) in the grapeseed oil at 7, 15, 30, 60, and 120 days of storage
* – means are significantly different ($p < 0.05$, $n = 6$)

in plant oil and storage time significantly affected TBARS values at 60 and 120 days (Figure 1). The effect of the interaction of the addition of oregano EO and storage time on TBARS value in the grapeseed oil was presented in Figure 1.

The oregano EO decreased lipid oxidation significantly ($p < 0.05$) during storage, which showed the highest decrease at 60 days (by 44 %, $p < 0.05$) and at 120 days (by 20.5 %, $p > 0.05$) compared to the control sample. The oregano EO decreased lipid oxidation by 20 % ($p > 0.05$) at the start of the study (0 days), but this change was non-significant. At 15 and 30 days, the TBARS values reached approximately 14.36 and 16.16 µmols/L, corresponding to an increase in TBARS levels to 16.15 and 16.41 µmols/L for samples enriched by oregano EO (Figure 1). Thus, oregano EO incubated with grapeseed oil caused the maximum decrease of lipid peroxidation on 60 and 120 days.

Total antioxidant capacity (TAC) is an analyte frequently used to assess the antioxidant status of biological samples and can evaluate the antioxidant response against the free radicals produced in a given condition (Rubio et al., 2016). The effect of the addition of oregano EO and storage time on total antioxidant capacity in the grapeseed oil at 7, 15, 30, 60, and 120 days of storage was demonstrated in Figure 2.

Total antioxidant capacity in the grapeseed oil at 7, 15, 30, and 60 days of storage was decreased by 6.2 %, 12.9 %, 19.4 %, and 36.1 %, respectively compared to the start of the study (0 days). The adding of oregano EO increased the TAC level in the grapeseed oil at 15, 30, and 60 days of storage compared to the control samples (grapeseed oil) by 17.4 %, 30.7 %, and 16.2 % ($p < 0.05$), respectively (Figure 2). On 7 and 120 days, the non-significantly increase of TAC level compared to the control samples was also observed (by 9.2 and 9.3 %, $p > 0.05$) (Figure 2).

The potent antioxidant properties of oregano EOs are of potential interest to the food, cosmetic and pharmaceutical industries (Leyva-López et al., 2017). The potent antioxidant properties of oregano EOs are of potential interest to the food, cosmetic and pharmaceutical industries (Leyva-López et al., 2017). For example, the application of oregano EOs as natural preservatives is recommended in meat products, especially in chicken and fish meats. The effect of ethylenediaminetetraacetate (EDTA), oregano (*Origanum vulgare*), and thyme (*Thymus vulgaris*) oils, on the chicken breast fillets, was examined by Pavelková and co-workers (2014). The chicken breast fillets were stored under vacuum packaging (VP), at 4 ±0.5 °C for 18 days. There were used the following treatments of chicken breast fillets: Air-packaged (AC,

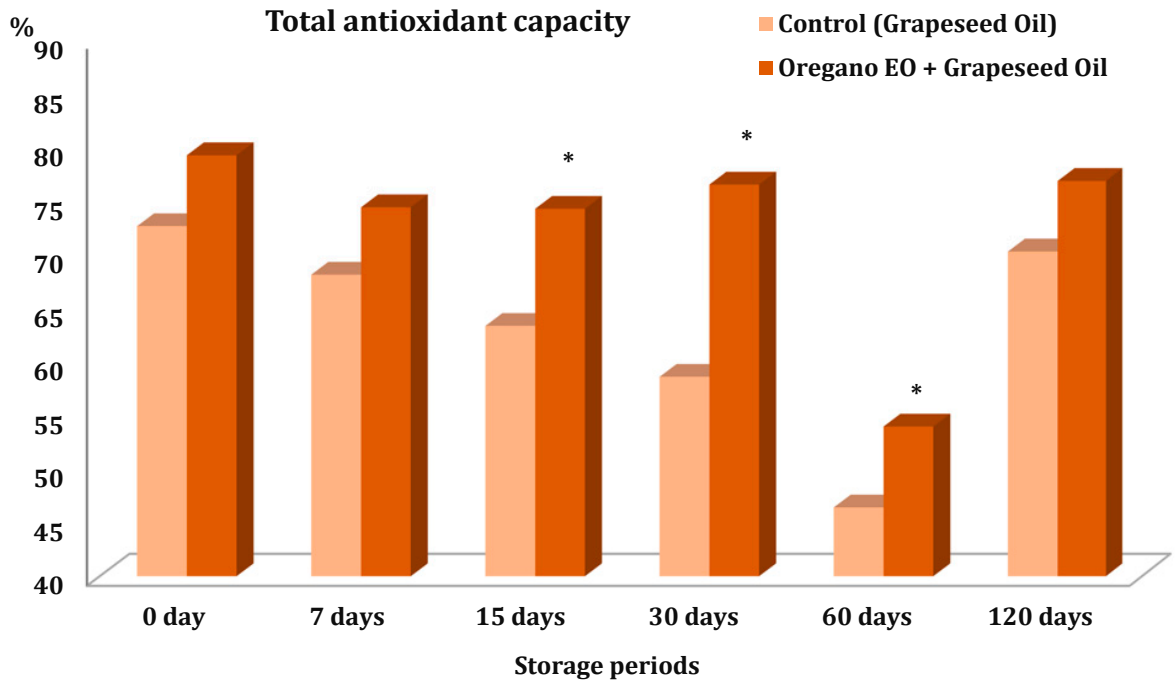

Figure 2 The effect of the addition of oregano essential oil and storage time on total antioxidant capacity (TAC, %) in the grapeseed oil at 7, 15, 30, 60, and 120 days of storage
* – means are significantly different ($p < 0.05$, $n = 6$)

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control samples), vacuum-packaged (VPC, control samples), VP with EDTA solution 1.50 % w/w (VPEC, control samples), VP with oregano EO 0.20 % v/w (VP + O) and VP with thyme EO 0.20 % v/w, (VP + T). The use of oregano, thyme EOs, and EDTA with a combination of vacuum packaging has significant effects on the reduction of all followed groups of microorganisms compared with a control group without vacuum packaging and untreated control group (Pavelková et al., 2014).

A similar study was conducted using lamb and beef meats. The effect of thyme and oregano EOs, as well as modified atmosphere packaging (MAP) in extending the shelf life of fresh lamb meat stored at 4 °C, was investigated by Karabagias et al. (2011). In a preliminary experiment, thyme EO and oregano EO were used at concentrations 0.1 and 0.3 % v/w. Based primarily on the sensory analysis (odor) but also on microbiological data, the shelf life of lamb meat was 7 days for air packaged samples, 9–10 days for samples containing 0.1 % of thyme EO and 21–22 days for MAP packaged samples containing 0.1 % thyme EO. Tsigarida et al. (2000) reported a reduction in initial microflora of beef meat fillets by 2–3 log cfu/g with the addition of 0.8% of oregano EO with lactic acid bacteria and *Listeria monocytogenes* indicating the most apparent decrease in all gaseous environments. The addition of 0.8 % oregano EO resulted in limited growth aerobically and survival/death of *L. monocytogenes* in MAP/VP, regardless of film permeability. These results are also in agreement with those of Skandamis and Nychas (2001) who reported an immediate suppression of TVC in minced beef meat by 1 log cfu/g when oregano EO was added at a concentration of 1 %. Oregano EO delayed microbial growth and suppressed the final counts of the spoilage microorganisms. It also caused a pronounced alteration in the physicochemical properties of the minced meat.

Also, the effect of thyme and oregano EOs (0.05 %, v/w) on the shelf life of salmon and seaweed burgers was assayed by Dolea et al. (2018). Three types of salmon and seaweed burgers were prepared: without EO, burgers with red thyme EO (0.05 %, v/w), and burgers with oregano EO (0.05 %, v/w), which were vacuum packaged and stored at 4 °C for 17 days. Physicochemical and microbiological analyses were carried out periodically throughout storage. The addition of both EOs did not have any effect on the evolution of the pH, the moisture content, or texture parameters. Only the thyme EO managed to slightly slow down the increase of total volatile basic nitrogen and trimethylamine nitrogen. The samples with oregano EO and especially those with thyme EO showed minor oxidation. The salmon and seaweed burgers without EOs and those which contained oregano EO showed a faster increase of mesophilic counts than those which had thyme EO, but no noticeable improvement was observed in the shelf life of the burgers with thyme EO. To improve the shelf life of the fish and seaweed burgers, it would be necessary to increase the concentration of both EOs (Dolea et al., 2018).

The effects of quince seed mucilage film (QSMF) containing oregano or thyme EOs on shelf life extension of rainbow trout (*Oncorhynchus mykiss*) fillets during refrigerated storage (4 °C) were evaluated by Jouki et al. (2014) over 18 days. Films were prepared in four different concentrations of EOs, including 0, 1, 1.5, and 2 %. The control and the wrapped fillet samples were analysed periodically for microbiological (aerobic and psychrotrophic count, *Pseudomonas* spp., H_2S-producing bacteria, lactic acid bacteria, and Enterobacteriaceae), chemical (TBA, TVB-N, TMA-N), and sensory characteristics. Bacteria grew most quickly in trout fillets stored in air, followed by those wrapped with QSMF and the lowest counts were in wrapped samples with QSMF + 2 % thyme EO. *Pseudomonas* spp., Enterobacteriaceae, and LAB counts were significantly lower in samples wrapped with QSMF + 2 % thyme EO. The lowest TBA value was obtained in fillets wrapped in QSMF containing 2 % oregano EO. The strong antioxidant activity of QSMF + 2 % oregano EO was related to the composition of oregano EO. The GC analysis of EO components revealed that carvacrol (81.85 %) was the major component of oregano EO. TBA value varied for all treatments and remained lower than 2 mg MDA/kg throughout storage. QSMF extended the microbial shelf life of rainbow trout fillets by 2 days, whereas the QSMF + 1 % oregano EO, QSMF + 1.5 % oregano EO, QSMF + 2 % oregano EO, QSMF + 1 % thyme EO, QSMF + 1.5 % thyme EO and QSMF + 2 % thyme EO resulted in a significant shelf life extension of the trout fillets by 3, 5, 9, 6, 10 and 11 days, respectively, as compared to the control samples (Jouki et al., 2014).

Badia et al. (2020) have evaluated the influence of oregano and rosemary EOs on the growth of lactic acid bacteria and the physicochemical properties of the refrigerated vacuum-packed Tuscan sausage. After the addition of 0.05 wt% and 0.1 wt% of EO to the sausage, the rosemary EO provided a higher extension of the shelf life of the sausages (approximately 3 and 5 days, respectively) than the oregano EO (approximately 1 and 3 days, respectively). After adding 0.2 wt% and 0.4 wt% of EO, the oregano EO resulted in a larger increase

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of the shelf life of the samples (about 8 and 14 days, respectively) when compared with the rosemary EO (about 7 to 11 days, respectively). All the treatments slowed the growth of the lactic acid bacteria but they did not change the maximum bacterial population (Badia et al., 2020).

The antioxidant effects of oregano EO and tannic acid combinations on ground chicken breast and thigh meats were studied by Al-Hijazeen et al. (2018). Six treatments, including 1) control (none added), 2) 100 ppm oregano EO + 5 ppm tannic acid, 3) 100 ppm oregano EO + 10 ppm tannic acid, 4) 200 ppm oregano EO + 5 ppm tannic acid, 5) 200 ppm oregano EO + 10 ppm tannic acid, and 6) 5 ppm butylated hydroxyanisole (BHA) for breast or 14 ppm for thigh meat, were prepared. Thigh meat patties showed higher 2-thiobarbituric acid reactive substances (TBARS), total carbonyl, and volatiles content compared to the breast meat during storage. A combination of 200 ppm oregano EO with 10 ppm tannic acid showed the most significant effects ($p < 0.05$) on TBARS, total carbonyl, and off-odor volatile formation for both breast and thigh meats. Oregano EO (200 ppm) and 10 ppm tannic acid combination also showed positive effects on the sensory scores of chicken thigh meat. Thus, the combination of 200 ppm oregano EO and 10 ppm tannic acid could be a good replacement for the synthetic antioxidants in-ground cooked chicken meat (Al-Hijazeen et al., 2018).

Essential oils from caraway (Carum carvi) seeds and oregano (*Origanum vulgare*) plants were included in dairy cow diets to study the effects on terpene composition and sensory properties of the produced milk, as well as feed consumption, production levels of milk, and methane emissions (Lejonklev et al., 2016). Two levels of EOs, 0.2 and 1.0 g of oil/kg of dry matter were added to the feed of lactating cows for 24 d. No effects on feed consumption, milk production, and methane emissions were observed. The amount and composition of volatile terpenes were altered in the produced milk based on the terpene content of the EOs used, with the total amount of terpenes increasing when EOs were added to the diet. Sensory properties of the produced milk were altered as well, and milk samples from animals receiving EO treatment were perceived as having a fresher aroma and lower stored aroma and flavour (Lejonklev et al., 2016).

Conclusions

The current results demonstrated that administration of oregano EO, exhibiting free radical scavenging activity determined by TBARS assay, exerts beneficial effects on preventing lipid peroxidation in grapeseed oil by limiting the TBARS levels and simultaneously increase of total antioxidant capacity, especially at 15–60 days of storage. At 60–120-day periods of storage, the TBARS levels were significantly lowered from control samples. The highest level of total antioxidant capacity was observed on 30-days compared to the control samples. Thus, edible adding containing essential oils have potential application in the plant oils to maintain their characteristics during the different shelf life.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

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

Acknowledgments

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Research Article

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Biological properties of honeysuckle (*Lonicera caerulea* L.): a review

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The honeysuckle (*Lonicera caerulea* L.) belonging to the Caprifoliaceae family, has been used for a long time in Chinese, Japanese and Russian folk medicine. Nowadays, the fruits of honeysuckle are becoming more and more popular also in Europe – mainly in Poland, Slovenia, the Czech Republic, and Slovakia because of valuable medicinal properties and they are popularly used as an ingredient of dietary supplements and medicinal preparations. The fruits of *Lonicera caerulea* are rich in phenolics, especially anthocyanins and vitamin C. The major bioactive anthocyanin of haskap is cyanidin-3-*O*-glucoside (C3G). Consumption of high amounts of an antioxidant substance may have a positive impact on human health, particularly the prevention of cancer and inflammatory diseases. The berries of blue honeysuckle containing a significant amount of biologically active substances can be included into the group of so-called "superfruits". Consumers are constantly seeking better alternatives, healthier products of plant origin, to rule out negative aspects, and this will be an alternative to widely existing food products. In addition, the growing interest of producers in new products rich in health-promoting properties makes them more attractive to the consumer. The content and health properties of the fruit were identified to be dependent on the cultivar, genotype, and the place of harvesting. This paper reviews and highlights the limited nutritional and therapeutic information currently available on the honeysuckle.

Keywords: *Lonicera caerulea*, honeyberry, cultivars, biochemical composition, health benefits

Introduction

Species belonging to the genus *Lonicera* such as *Lonicera caerulea* var. edulis, *L. caerulea* var. *kamtschatica*, *L. caerulea* var. *altaica*, *L. caerulea* var. *byarnikovae*, and *L. caerulea* var. *emphyllocalyx*, as well as their hybrids, collectively known as *Lonicera caerulea* L., also known as blue honeysuckle, haskap, honeyberry, sweet berry honeysuckle or edible honeysuckle, are representative of such plants (Chmiel et al., 2014; Chang et al., 2018; Gawroński et al., 2020). The berries of blue honeysuckle contain a significant amount of biologically active substances. These berries can be included into the group of so-called "superfruits" (Bojarska et al., 2019). Haskap is a deciduous berry shrub, growing to 1.5–2.0 m tall. Its flowers are pale yellow, melliferous, and have a delicate, pleasant aroma

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(Grobelna et al., 2020). The berries have an elongated elliptic or cylindrical shape, are dark purple (Figure 1).

They can reach approximately 2 cm in length and 1 cm in width (Bieniek et al., 2005; Hummer et al., 2012; Yamamoto et al., 2014; Auzanneau et al., 2018; Gołba et al., 2020). The fruits have thin skin with a characteristic waxy coating. Their weight ranges from 0.3 to 2.0 g. The taste can be characterized as bitter to sour-sweet, varying among cultivars (Hummer et al., 2012; Gołba et al., 2020). Blue honeysuckle plants have been used for ages in Asia for their medicinal properties (Thompson and Chaovanalikit, 2006; Ochmian et al., 2012). Blue honeysuckle has a lot of positive features: early ripening (even two weeks before strawberry), exceptional hardiness, no specific demands for soil and climatic conditions, or low susceptibility to pests and diseases (Szot and Wieniarska, 2012). In the literature, the first mention of this plant originates from Russia in the 17th century. At present, honeyberry is cultivated across Japan, China, Russia, Central, and Eastern Europe – Poland, the Czech Republic, Slovenia, Slovakia, North America-Canada, and the USA (Svarcovaa et al., 2007; Senica et al., 2018; Becker and Szakiel, 2019; Grobelna et al., 2019). According to the available literature, the fruits of honeyberry are a valuable source of vitamins, minerals, and secondary metabolites with properties that are important for maintaining proper human health (Grobelna et al., 2020). Currently, in many countries, there are two serious problems: diseases of civilization and ageing of the population. They can be limited, among others through a proper diet, rich in fruit, especially those with both high antioxidant activity and content of polyphenols (Korczyński et al., 2015). A high total polyphenolic content and antioxidant activity are typical for blue honeysuckle berries (Rupasinghe et al., 2018; Grobelna et al., 2019) and determine the edible value and health benefits of this plant. The chemical composition of berries varies depending on genetic factors (cultivar), climate, weather conditions, as well as agronomic practices (Szot and Wieniarska, 2012). Fully ripened fruits contain between 12.4 and 20.3 % of dry matter, with a predominance of fructose and glucose (Rupasinghe et al., 2018; Grobelna et al., 2020). The noteworthy among bioactive compounds are anthocyanins (Rupasinghe et al., 2018; Grobelna et al., 2019). The most abundant anthocyanin is cyanidin-3-glucoside (79–92 %), whereas cyanidin-3,5-diglucoside, peonidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-rutinoside, and pelargonidin-3-glucoside occur in smaller amounts (Wang et al., 2016; Grobelna et al., 2020). Cyanidin-3-*O*-glucoside (C3G) comprises over 60 % of the total polyphenols. There is evidence of significant antioxidant, cardio-protective, anti-inflammatory, neuroprotective, anticancer, and anti-diabetic properties of C3G-rich haskap preparations and C3G alone both *in vitro* and *in vivo* (Wang et al.,

Figure 1 The berries of blue honeysuckle before harvesting
Foto B. Markuszewski and O. Grygorieva

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2016; Rupasinghe et al., 2018; Grobelna et al., 2020). The other group of chemical compounds identified in the fruits of blue honeysuckle is phenolic acids, flavonoids, including flavan-3-ols, flavons, flavanols, and organic acids, iridoids (Kucharska et al., 2017; Oszmiański and Kucharska, 2018; Becker and Szakiel, 2019). Blue honeysuckle berries are characterized by a high content of vitamin C, which can reach up to 187 mg/100 g fresh weight (FW) (Jurikova et al., 2012; Caprioli et al., 2016). In addition, they also contain the mineral components, potassium is dominant, followed by phosphorus and calcium, magnesium and iron in smaller amounts, and trace amounts of manganese, copper, and zinc (Rupasinghe et al., 2018; Grobelna et al., 2019; Grobelna et al., 2020). They are characterized by a high content of organic acids, among which citric acid is the most dominant and constituted 47 % of all organic acids. Among other organic acids are malic, phytic, oxalic, quinic, and shikimic acids were also present. At the same time, oxalic, quinic, and shikimic acids were present in the lowest amounts and constituted, respectively, 5; 4, and 1 % (Grobelna et al., 2020, Wojdyło et al., 2013). Health-promoting properties of the haskap berries include protective effects against cardiovascular and neurodegenerative diseases, osteoporosis, type 2 diabetes, anaemia, as well as antimicrobial, anticarcinogenic, and anti-inflammatory activity (Park et al., 2005; Kula et al., 2013; Celli et al., 2014; Caprioli et al., 2016; Wang et al., 2016; Cory et al., 2018; Grobelna et al., 2019; Gawroński et al., 2020).

Selection of cultivars and characteristics of the cultivation

The honeyberry is a quite new orchard species that at the turn of the XXI century went to commodity production in Poland. Then Zofia‘s Łukaszewska‘s cultivars are called ‘Wojtek‘ (Figure 2), ‘Jolanta‘ and number ‘46‘ that is colloquially called ‘Zojka‘ (Figure 3), and number ‘44‘ appeared in crops. They turned out to be attractive for producers because of tasty big fruits that does not fall off. Another advantage of these species is that they bloom at the same time, which favours their good pollination. Currently, Polish, Russian and Canadian varieties are offered on the market, but they still require careful checking in the Polish climate and specific growing conditions (Podymiak, 2015). Many new varieties, Russian and Canadian breeding, have appeared in Polish nurseries. They are characterized by attractive large fruits, high fertility, and valuable pro-health properties (Bieniasz et al., 2015).

The cultivars in Poland include Wojtek, Jolanta, Atut, Duet, Brązowa, Czarna, and Warszawa (Ochmian et al., 2008; Ochmian et al., 2012; Kaczmarska et al., 2015; Becker and Szakiel, 2019; Grobelna et al., 2020). The most popular Canadian cultivars are Blue Velvet, Tundra, Aurora, Borealis, Indigo Gem, and Honeybee (Rupasinghe et al., 2012; Rupasinghe et al., 2018; Becker and Szakiel, 2019; Grobelna et al., 2020). The previous observations showed that so far the cv. Wojtek, planted with cv. Zojka as a pollinator, has proved its best in commercial plantations (Figure 4). Both cultivars bloom at a similar time and are a well-suited pair for large plantations. It is best to plant them in a proportion of 3:1, that is for 3 rows of the cv. Wojtek there should be 1 row of cv. Zojka (Podymiak, 2015). It is estimated that the cultivation area of hascap in Poland may amount to as much as 2000–2500 ha. These are both small, several-hectare plantations as well as large, specialized (Podymiak, 2020).

Figure 2 The fruits of cultivar Wojtek
Foto A. Bieniek

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Figure 3 The fruits of cultivar Zojka
Foto A. Bieniek

Blue honeysuckle starts bearing fruits in the second year after planting, and the full yield (3–5 kg) can be harvested in 8–15 years after planting (Grobelna et al., 2020). Honeyberry is long-lived and can bear fruit for up to 30 years. Shrubs that are 20- to 25-year-old can die out or yield less, but treatments such as pruning and removing older stems and branches can help the plant grow afresh (Becker and Szakiel, 2019; Grobelna et al., 2020). The soil and climatic conditions for hascap growing are relatively minimal. It tolerates a wide range of soil pH and the most favourable pH range is 5.5–8.0 (Pluta, 2015; Grobelna et al., 2020). The shrubs can grow in sandy and clay soils as well as in peaty and slightly acid soils (Dawson, 2017). Soil and foliar

Figure 4 The plantation of *Lonicera caerulea* located in the north-eastern part of Poland
Foto B. Markuszewski

Figure 5 The fruits of cultivars Wojtek and Zojka after mechanical harvesting
Foto A. Bieniek

fertilization can improve the size and quality of crops (Szot and Lipa, 2012). Moreover, the quality of the fruit can be modified by the climatic conditions prevailing in a given growing season (Szot and Wieniarska, 2012). *Lonicera caerulea* demonstrates very high frost resistance, shrubs can withstand temperatures down to -40 °C and flowers down to -8 °C (Ochmian et al., 2008; Pluta, 2015; Grobelna et al., 2020). The berries of honeysuckle ripen at the end of May are one of the first dessert fruits on the market. It is very rarely pest-attacked and therefore does not require special protection against fungal diseases and other pathogens. They can be cultivated using the organic method (Szot and Wieniarska, 2012; Celli et al., 2014; Pluta, 2015). New varieties are adapted to mechanical harvesting (Figure 5). Fruits can be sold fresh or developed by the processing and pharmaceutical industries (Pluta, 2015). The selection of a cultivar is also very important for health reasons as the amount of bioactive ingredients varies between cultivars (Rop et al., 2011). Szot and Wieniarska (2012) observed that the fruits of the cv. Duet in relation to cv. Atut is characterized by a higher weight and sugar content, but on the other hand, they have a lower dry matter, anthocyanin, vitamin C, and acidity content.

Health properties of honeysuckle

Blue honeysuckle belongs to fruit species with unique biological and chemical properties. They are a valuable source of vitamins, minerals, and secondary metabolites with properties that are important for maintaining proper human health (Grobelna et al., 2020). For a long time berries have been harvested from wild plants in the regions of Russia, Chine, and Japan (Gawroński and Kaczmarska, 2018). This plant was even called the "elixir of life" by the indigenous Ainu family living on the island of Hokkaido (Celli et al., 2014). The raw material was used to treat fever, headaches, and urinary tract diseases (Kaczmarska et al., 2015). In Tibetan medicine, honeyberry bark was used to obtain an analgesic preparation for chronic arthritis and headaches. In the Far East, anti-rheumatic baths were prepared from young buds, and a decoction of shoots was administered to stimulate the appetite (Skwarcow and Kuklina, 2002; Bieniek et al, 2005). It has been shown that the infusion of arachnid flowers is very helpful in the treatment of the bladder (Kawecki et al., 2007). Decoction of fruit and leaves can be used to treat eye diseases, angina, and periodontal disease (Isaczkin et al., 2003; Bieniek et al., 2005). Additionally, such a decoction inhibited ocular inflammation, in particular uveitis (Jin et al., 2006).

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Honeyberry is widely used in the treatment of viral and bacterial infections. The raw material can inhibit the growth of pathogenic bacteria strains such as *Candida parapsilosis*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Streptococcus mutans* and food-borne bacteria: *Listeria monocytogenes*, *Escherichia coli*, and *Campylobacter jejuni* (Palíková et al., 2008; Raudsepp et al., 2013). This property is particularly important due to the ability of the haskap berry to counteract diseases of the oral cavity and gastrointestinal tract. In addition, due to their detoxifying properties, fruits are used in poisoning with heavy metals, medications, and in the treatment of cardiovascular diseases. It also exhibits soothing properties in case of food allergic ailments (Li and Li, 2005). Fruit juice can treat ulcers and impetigo (Kawecki et al., 2007; Szot et al., 2014).

It was found that extracts from blue honeysuckle berries have anticancer properties. This anticancer property is related to the induction of antioxidant defence enzymes, inhibition of cancer cell proliferation, and factors causing metastases (Rupasinghe et al., 2018; Zhou et al., 2018). Iridoids have been recently identified in fruits of blue honeysuckle (Kucharska et al., 2016). Iridoids rarely occur in fruits, except for cornelian cherry, cranberry, lingonberry, and bilberry (Heffels et al., 2017; Kucharska et al., 2017). Recent studies have shown that longanic acid is the most abundant iridoid in blue honeysuckle berries (Kucharska et al., 2017). However, iridoids such as loganin, sweroside, secologanin, secoxyloganin, pentosides of loganin, and pentosyl-sweroside have also been identified (Kucharska and Fecka, 2016; Kucharska et al., 2017; Oszmiański and Kucharska, 2018; Grobelna et al., 2020). Loganin helped to alleviate *diabetes mellitus* by improving liver function and reducing nephropathy (Tundis et al., 2008; Park et al., 2011). Iridoids are biologically active compounds with anti-inflammatory, neuroprotective, hepatoprotective, hypotensive, and antibiotic properties (Heffels et al., 2017; Kucharska et al., 2017; Oszmiański and Kucharska, 2018). Prevalent antioxidants are able to reduce reactive oxygen species, counteracting ageing processes (Duthie, 2007; Gołba et al., 2020). Research shows that blueberry extract protects DNA from damage, preventing carcinogenesis (Duthie, 2007). Fruits reduce the negative effects of oxidative stress, caused by UV radiation, inhibiting the formation of free radicals. Kamchatka berry is also linked to its anti-inflammatory potential. Long-term exposure to inflammation may consequently lead to arteriosclerosis, neurodegenerative diseases, diabetes, and even cancer. It has been shown that the effect of using blueberries is comparable to that of diclofenac, a popularly used anti-inflammatory drug substance (Rupasinghe et al., 2018). The research has also shown the positive impact of honeysuckle berries on inhibiting melanogenesis, resulting in a whitening effect (Jurikova et al., 2012; Celli et al., 2014). The fruits of honeysuckle also have strong antidiabetic properties. In Podsędek et al. (2014) studies, the hascap berries showed the strongest α-glucosidase inhibitory activity among fruits such as blackcurrant, highbush blueberry, bilberry, red gooseberry, and sweet cherry. According to Johnson et al. (2011), inhibition of α-glucosidase and β-fructosidase allows delaying disaccharide digestion, which is important for postprandial hyperglycemia control in patients with diabetes. In addition, it has been shown that the plant has a positive effect on hyperthyroidism by reducing the level of thyroid stimulating hormone in the body (Park et al., 2016). The berries of honeysuckle also play an important role in fighting urinary tract symptoms and digestive problems (Kontiokari et al., 2003; Del Rio et al., 2010).

Conclusion

The main advantage of *Lonicera caerulea* is high content of bioactive compounds and, therefore, it can be used as a very good component of functional food, dietary supplements, and even herbal medicinal products. It is worth mentioning that not only fruits have a beneficial effect on health. Flowers have been proven to fight colds; bark has a diuretic effect and leaves help with throat infections. According to conducted studies, regular consumption of berries could reduce cancer and insulin resistance. The climatic and soil requirements of *Lonicera caerulea* enable its cultivation in the European countries, so the area under cultivation continues to increase. Therefore, to fully utilize these fruit, it is important to look for new ways to process them.

Conflicts 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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Research Article

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Antibacterial activity of ethanolic extracts obtained from roots and stems of *Chelidonium majus* L. against *Enterococcus faecalis* strains

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The present study is *in vitro* research aimed to evaluate the antimicrobial activity of the ethanolic extracts derived from roots and stems of *Chelidonium majus* L. against two *Enterococcus faecalis* strains to assess the possible use of this plant in preventing infections caused by this pathogen. Plant materials were harvested from natural habitats on the territory of the Kartuzy district in the Pomeranian province (northern part of Poland). The collected roots and stems were brought into the laboratory for antimicrobial studies. Freshly washed samples were weighed, crushed, and homogenized in 96 % ethanol (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity. The *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) and linezolid-resistant *Enterococcus faecalis* strain locally isolated were used in the current study. The antimicrobial susceptibility testing was done on Muller-Hinton agar by Kirby-Bauer disk diffusion susceptibility test protocol. The results of the current study showed that *C. majus* possess weak antimicrobial properties against the tested *Enterococcus faecalis* strains. The ethanolic extracts derived from roots of *C. majus* collected from rural areas exhibited the maximum antimicrobial activity against linezolid-resistant *E. faecalis* strain (the mean of inhibition zone diameters was 8.85±0.42 mm) compared to the control samples (7.1 ±0.91 mm). Stem extracts derived from *C. majus* collected from rural areas showed similar properties against the *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) strain (8.77 ±1.21 mm) compared to the control samples. Root extracts derived from *C. majus* collected from urban and rural areas exhibited weak antibacterial ability against linezolid-resistant *E. faecalis* strains (6.46 ±0.32 mm and 7.78 ±0.34 mm, respectively), as well as weak antibacterial ability against *E. faecalis* ATCC®51299™strains (7.9 ±1.08 mm and 7.97±0.85 mm, respectively) compared to the control sample (7.1 ±0.99 mm). The results of this study can induce to provide a new perspective for the use of various Papaveraceae families as medicinal plants to improve the antibacterial responses using other strains. Identification of precise molecular mechanisms responsible for inhibition of bacterial growth by these extracts requires further research.

Keywords: *Chelidonium majus*, linezolid-resistant *Enterococcus faecalis* strain, *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™), antibacterial efficacy, disc diffusion technique, ethanolic extracts

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Introduction

Enterococcus faecalis is a Gram-positive bacterium that commonly inhabits the gastrointestinal tract of mammals (Huycke et al., 1998; Brewer et al., 2020). Enterococcal species are core constituents of the intestinal flora of many animal species ranging from humans to flies (Macovei et al., 2006; McBride et al., 2007). When they enter a wound, bloodstream, or urinary tract, however, *E. faecalis* cells can cause serious infections. In immunocompromised individuals, however, it can cause a variety of complications, including surgical wound and urinary infections, endocarditis, and bacteremia (Macovei et al., 2006; Fisher and Phillips, 2009; García-Solache, 2019). *E. faecalis* is also resilient when exposed to a variety of stressors, allowing it to survive outside the body for extended periods of time, which likely increases transfer to patients in a hospital setting (Shepard and Gilmore, 2002). The ability of *E. faecalis* isolates to cause serious infections has been linked to the intrinsic ruggedness of the bacterium, which allows the organism to persist in the hospital environment and survive many host defenses, compounded by the acquisition of a variety of variable virulence traits by horizontal transfer from other organisms (Bonten et al., 1993; Shepard and Gilmore, 2002). Further, *E. faecalis* is resistant to a variety of antibiotics, complicating treatment strategies. Bacteria can survive antibiotic treatment through both resistance and tolerance (Murray, 1990). Resistance is defined as the inherited ability of microorganisms to grow in the presence of an antibiotic even at high concentrations, as indicated by high MIC values. The threat of *E. faecalis* to human health is further emphasized by its intrinsic low-level susceptibility to most of the available antibiotics, making treatment challenging (Murray, 1990; Bonten et al., 1993; Fisher and Phillips, 2009).

Many medical plants, for example, the Papaveraceae family, possessed significant antibacterial and antifungal activities against bacteria and other pathogens (Gerenčer et al., 2016). *Chelidonium majus* L., also known as greater celandine, is a plant of the family Papaveraceae, which grows wild in part of Asia, Central and Southern Europe, in the Azores and North America (Pantano et al., 2017). *Chelidonium majus* is a short-lived hemicryptophyte. It has up to 1 m high stem, branched and sparsely pubescent. The alternately placed leaves are light bluish at the bottom and green at the top. The basal leaves are long-petioled, with the obovate in contour, pinnatisect leaflets with 5–7 lobed segments. From April to October, the plant produces umbellate inflorescences with 2–6 flowers, which have 4 bright yellow petals and two whitish, early dropping sepals. The phytochemical and pharmacological properties, including the antibacterial activity of *C. majus*, have been reviewed, which is of great interest for its use in Chinese, Asian, and European herbal medicines The antimicrobial activity of *C. majus* is attributed mostly to the alkaloids and flavonoids (Zuo et al., 2008; Zielińska et al., 2018). The plant contains, first of all, alkaloids: benzylisoquinolic compounds (0.01–1.0 %), such as sanguinarine, chelidonine, chelerythrine, protoberberine, and berberine. According to many researchers, the antibacterial effect ascribed to *C. majus* herb depends first of all on quaternary ammonium groups of isoquinoline alkaloids (Colombo and Bosisio, 1995; Lenfeld et al., 2007; Zuo et al., 2008; Zielińska et al., 2018).

Considering the points previous results obtained in our laboratory, the current study aimed to find out *in vitro* the possible antimicrobial activity of the ethanolic extracts derived from roots and stems of *C. majus* collected in urban and rural agglomerations of Kartuzy district in the Pomeranian province (northern part of Poland) against two *Enterococcus faecalis* strains, i.e. *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) and linezolid-resistant *Enterococcus faecalis* strain locally isolated.

Material and methodology

Collection of plant material

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

Preparation of plant extracts

The collected roots and stems of *C. majus* were brought into the laboratory for antimicrobial studies. Freshly washed samples were weighed, crushed, and homogenized in 96 % ethanol (in proportion 1 : 19, w/w) at room temperature. Then the extracts were filtered and investigated for their antimicrobial activity.

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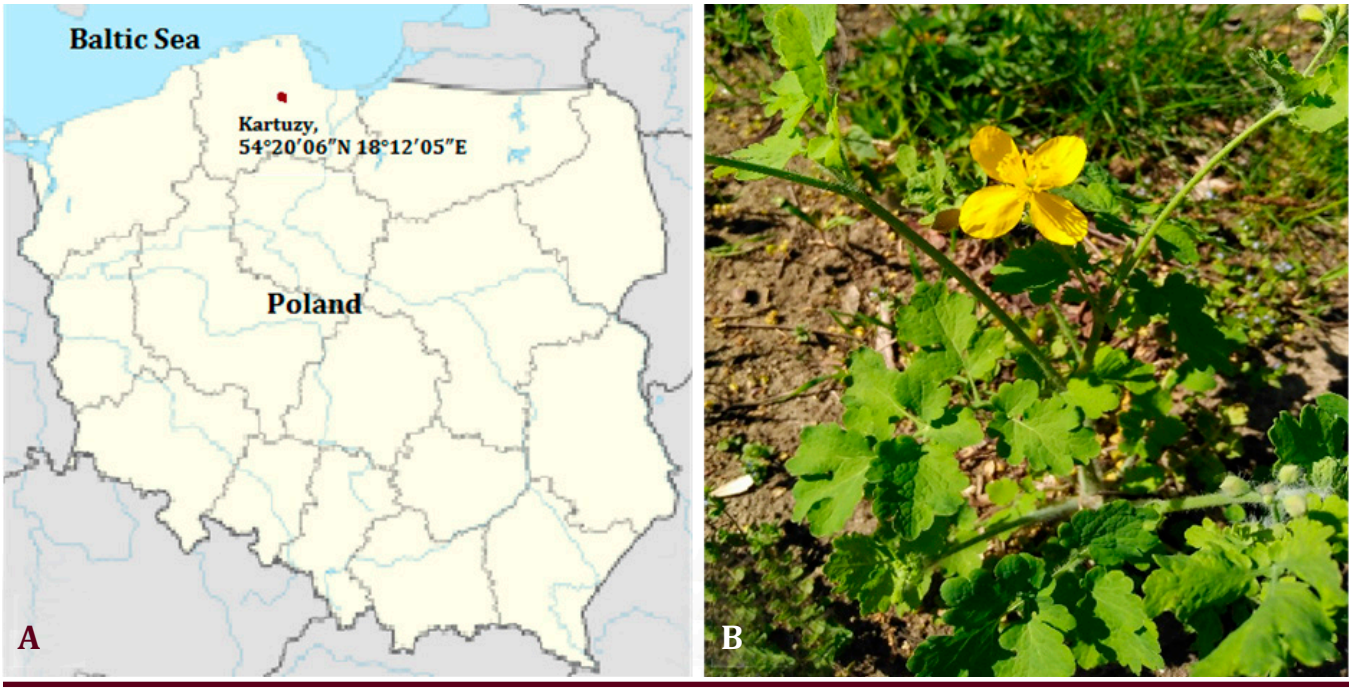

Figure 1 Location of Kartuzy in the map of Poland (A), where the *Chelidonium majus* L. (B) was collected
Photo by Nataniel Stefanowski

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

The *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) and linezolid-resistant *Enterococcus faecalis* strain locally isolated were used in the current study. Strains tested were plated on TSA medium (Tryptone Soy Agar) and incubated for 24 h at 37 °C. Then the suspension of microorganisms was suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was done on Muller-Hinton agar by Kirby-Bauer disk diffusion susceptibility test protocol (Bauer et al., 1966). Muller-Hinton agar plates were inoculated with 200 µl of standardized inoculum (10^8 CFU/mL) of the bacterium and spread with sterile swabs.

Sterile filter paper discs impregnated by extracts were applied over each of the culture plates, 15 min after bacteria suspension was placed. A negative control disc impregnated by sterile 96 % ethanol was used in each experiment. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 h at 37 °C. The assessment of antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the disks. The diameters of the inhibition zones were measured in millimeters and compared with those of the control and standard susceptibility disks. The activity was evidenced by the presence of a zone of inhibition surrounding the well (CLSI, 2014). The results of the disk diffusion test are "qualitative", in that a category of susceptibility (i.e., susceptible, intermediate, or resistant) is derived from the test rather than a MIC (Jorgensen and Ferraro, 2009).

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the arithmetic means ±S.E.M. All variables were randomized according to the antibacterial activity of tested extracts. All statistical calculation was performed on separate data from each extract. The data were analyzed using one-way analysis of variance (ANOVA) using Statistica software, version 13.3 (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥15 mm, Intermediate (I) = 10–15 mm, and Resistant (R) ≤10 mm (Okoth et al., 2013).

Results and discussion

Our study aimed to examine the antibacterial properties of *C. majus* roots and stems against two *Enterococcus faecalis* strains: *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) and linezolid-resistant *Enterococcus faecalis* strain locally isolated. The extracts of stems and roots of *C. majus* collected from rural and urban agglomerations

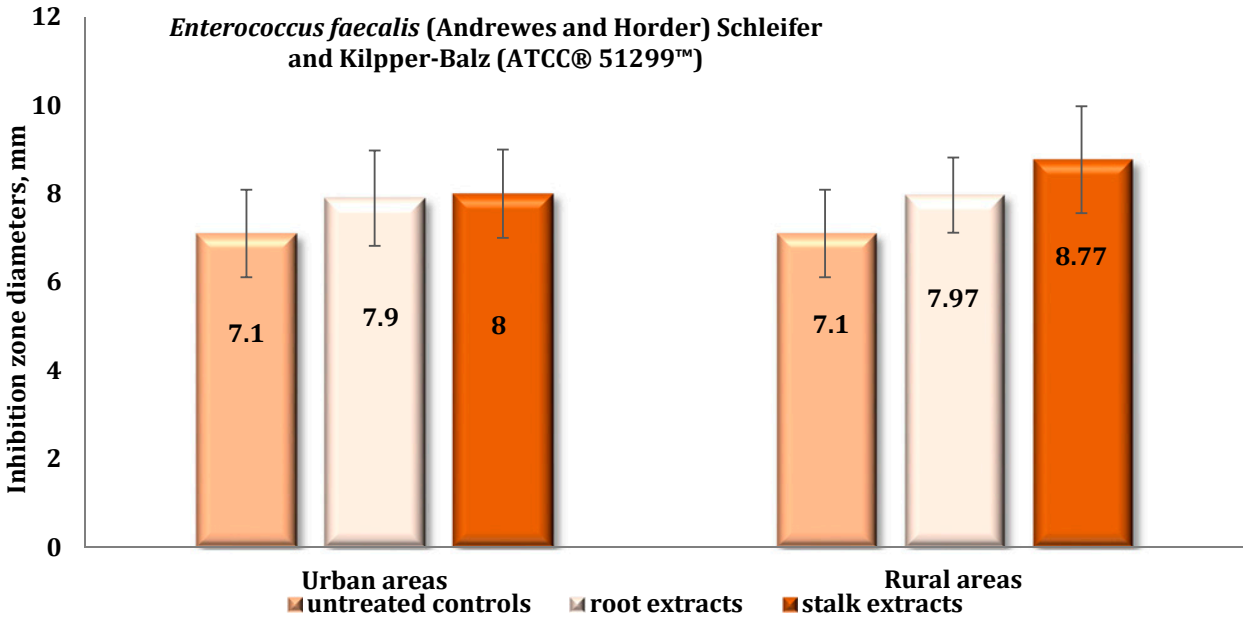

Figure 2 Zones of growth inhibitions of *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) strain induced by the extracts obtained from stems and roots of *Chelidonium majus* L. collected from the rural and urban agglomerations in millimeter (M ±m, n = 8)

did not show antimicrobial activity against the tested strains. The results of antibacterial activity screening are given in Figure 2–5. The data on diameters of zone inhibition of bacterial growth of plant extracts against the *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) strain is demonstrated in Figures 2 and 3. Figures 4 and 5 show data on the diameters of the zones of bacterial inhibition in plant extracts against linezolid-resistant *Enterococcus faecalis* strain locally isolated.

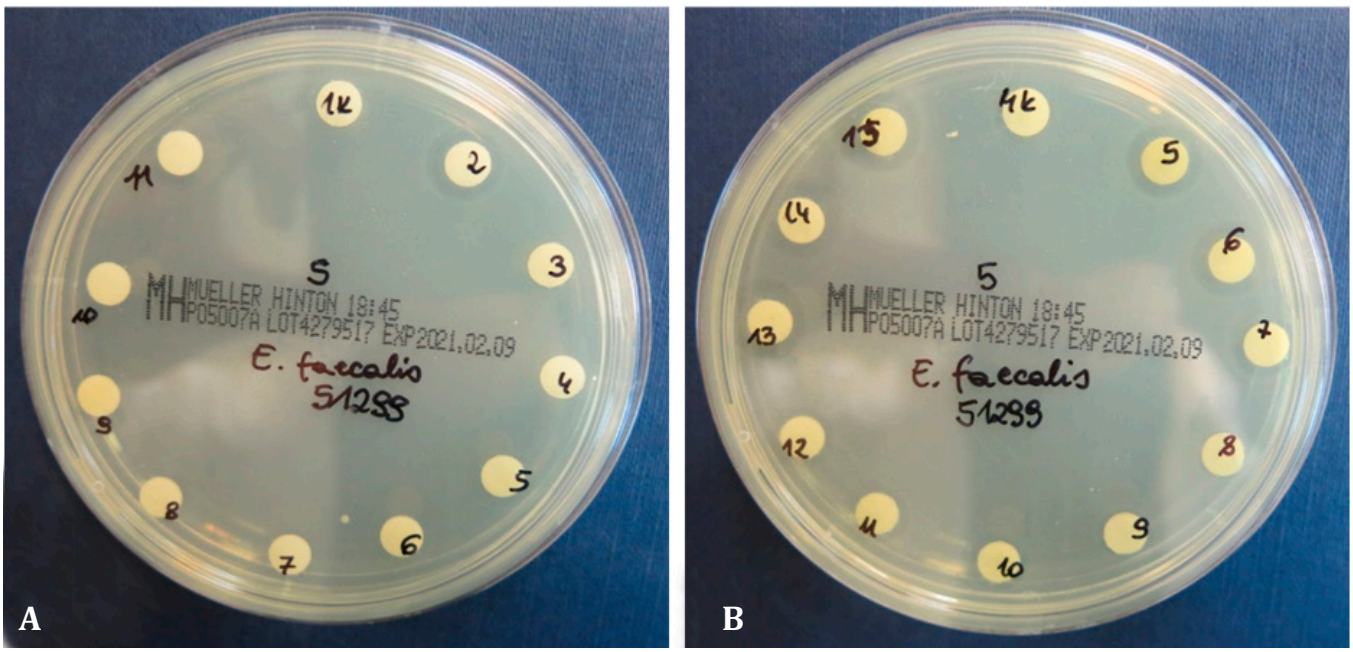

Figure 3 Example of plates in a disc diffusion assay showing the halos in the bacterial growth resulting from the antibacterial activity of extracts derived from roots (A) and stems of *Chelidonium majus* L. (B) collected from the rural and urban agglomerations against *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) strain

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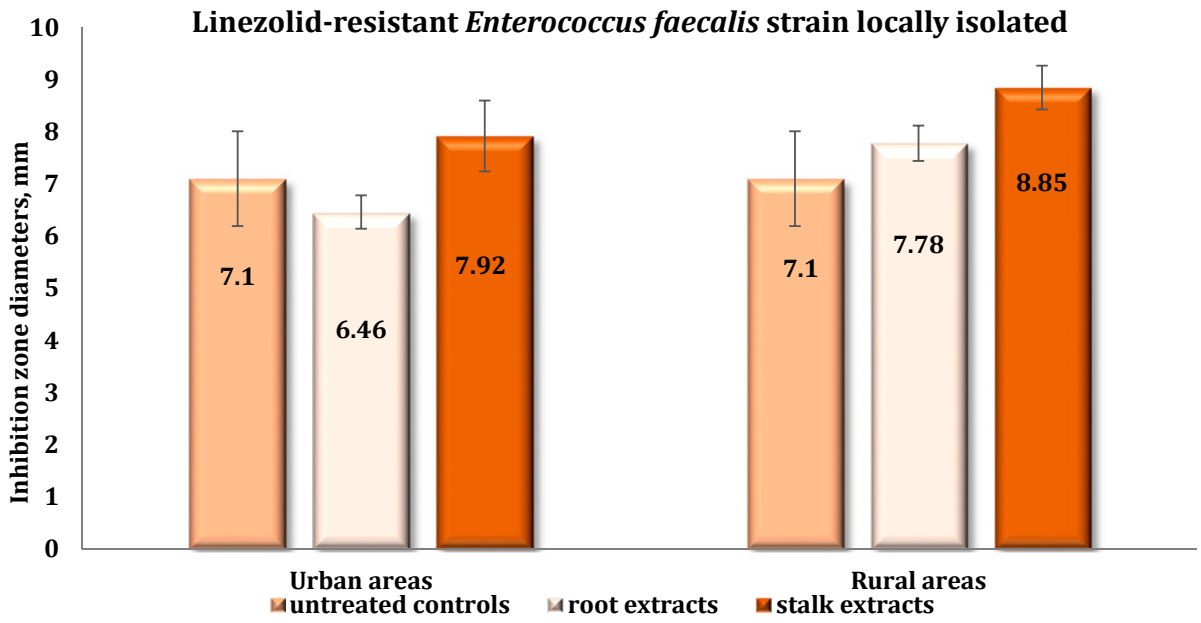

Figure 4 Zones of growth inhibitions of linezolid-resistant *Enterococcus faecalis* strain locally isolated induced by the extracts obtained from stems and roots of *Chelidonium majus* L. collected from the rural and urban agglomerations in millimeter (M ±m, n = 8)

Our results evaluating the antibacterial properties against *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™) strain are as follows (Figure 2). The ethanolic extract of *C. majus* derived from roots collected from rural agglomerations exhibited the maximum antimicrobial activity against *E. faecalis* (the mean of inhibition zone diameters was 8.77 ±1.21 mm) compared to the control samples (7.1 ±0.99 mm). There was a 25 % ($p > 0.05$) increase in the zone of inhibition compared to the control samples. Stem extracts of CM collected from urban areas also exhibited antibacterial ability against *E. faecalis* strain (7.9 ±1.08 mm) compared to the control samples (7.1 ±0.99 mm). There was

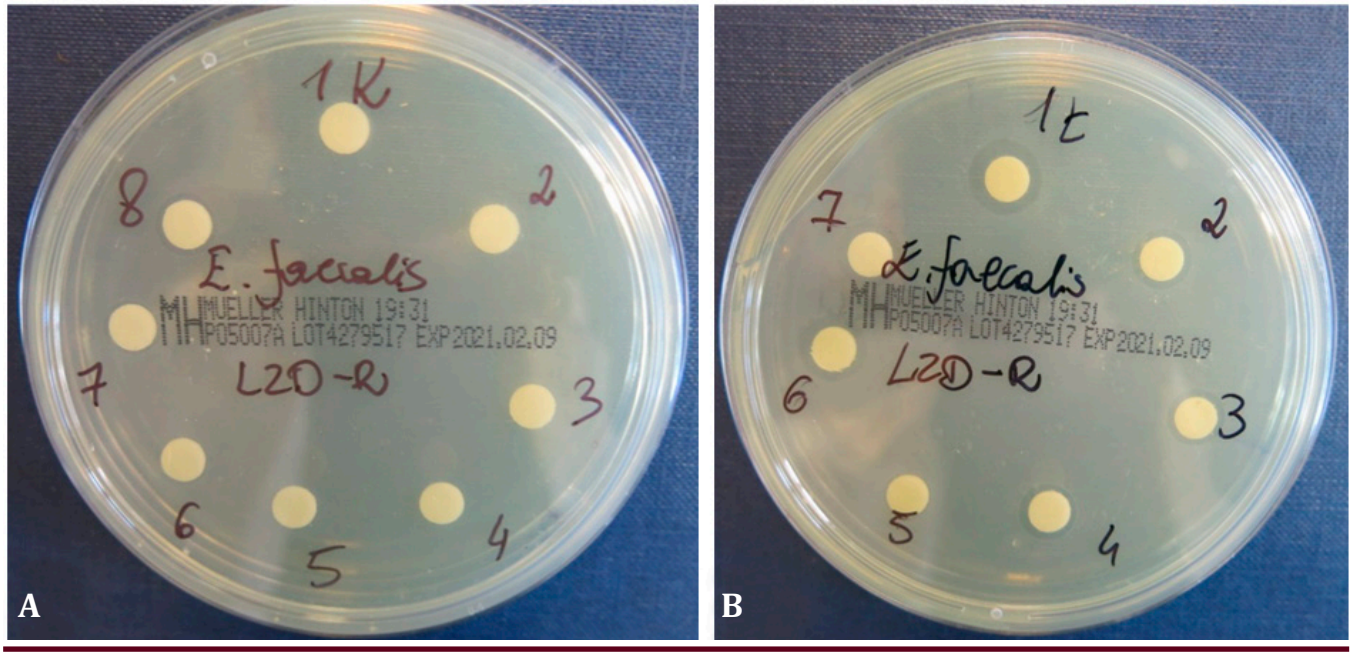

Figure 5 Example of plates in a disc diffusion assay showing the halos in the bacterial growth resulting from the antibacterial activity of extracts derived from roots (A) and stems of *Chelidonium majus* L. (B) collected from the rural and urban agglomerations against linezolid-resistant *Enterococcus faecalis* strain locally isolated

a non-significant increase in the zone of inhibition by 11 % ($p > 0.05$) compared to the control samples. Stem extracts also showed antibacterial properties against the *E. faecalis* strain, but a larger diameter of the zone of inhibition was observed in stem extracts of *C. majus* collected from rural areas (7.97 ± 0.85 mm) compared to the control samples (7.1 ±0.99 mm). *E. faecalis* strain was also susceptible to the stem extracts of *C. majus* collected from urban agglomerations (7.9 ±1.08 mm) (Figures 2, 3).

Similar results were obtained after analysis of the zones of growth inhibition against linezolid-resistant *Enterococcus faecalis* strain locally isolated. The present study showed that the highest zone of growth inhibition against *E. faecalis* strain was exhibited by ethanolic stem extracts of *C. majus* collected from rural agglomerations as compared to the control samples (inhibition zone diameter were ranged from 7.1 to 9.35 mm, 8.85 ±0.42 mm) (Figure 4). Moreover, it has been observed that ethanolic extracts derived from stems of *C. majus* collected from urban areas also revealed similar activity (7.0–8.75 mm as the diameters of inhibition zone, 7.92 ±0.68 mm) compared to the control samples (Figures 4 and 5).

The extracts derived from roots of *C. majus* collected in urban agglomerations have shown weak activity against the *E. faecalis* strain (Figure 4). The mean diameters of inhibition zones were (6.46 ±0.32 mm) compared to the control samples (7.1 ±0.91 mm). The extracts taken from the *C. majus* collected from the rural agglomerations showed no significant changes compared to the control samples. The above-presented results confirm the weak antibacterial activity of *C. majus* extracts against the tested *E. faecalis* strains (Figure 5).

In the present study, the antimicrobial activity of plant extracts was investigated by the agar disc diffusion method. In the current study, extracts from roots and stems of *C. majus* collected from rural and urban agglomerations were not effective against the tested bacteria due to the observed zone of growth inhibition. In our previous studies (Stefanowski et al., 2021a, b, c), we obtained different results against the tested strains. Regarding a strain of *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC®29213™), we demonstrated high antimicrobial activity of both stem and root extracts of *C. majus* collected from urban areas by measuring zones of growth inhibition (for root extracts 16.9 mm, for stems extracts 13.6 mm) compared to the control samples (8.8 mm). By investigating the antibacterial activity of *C. majus* extracts against *Staphylococcus aureus* NCTC 12493 strain, we demonstrated that stem extracts of *C. majus* collected from both urban and rural agglomerations exhibited high antibacterial activity (15.3 and 13.1 mm, respectively) compared to the control samples (9.1 mm), and increasing of the zones of growth inhibition was statistically significant. Our subsequent studies against *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC®51299™), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC®25922™), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC®35218™) strains showed that stem extracts of *C. majus* exerted significant antibacterial effects assessing the zone of growth inhibition (Stefanowski et al., 2021a, b, c).

Our results are not similar to results obtained by other researchers. According to Kokoška et al. (2002), root ethanolic extract derived from *C. majus* was found to be effective against Gram-positive bacteria (*S. aureus*, *Bacillus cereus*) (MIC 15.63 and 62.5 mg of dry plant material/mL, respectively), but was inactive against Gram-negative (*P. aeruginosa*). Also, the concentration of 62.5 mg of dry plant material/mL effectively inhibited *C. candida* in these experiments. The aerial parts of *C. majus* used in the study of Kokoška et al. (2002) were inactive against any of the test microbes. The antibacterial activity of sanguinarine (SAG), one of the several alkaloids of *C. majus*, was studied by Zhang et al. (2020) against *Providencia rettgeri*. All tested plant extracts manifested antimicrobial activity, related to different chemical structures of the alkaloids. SAG demonstrated strong activity against *P. rettgeri* biofilms. SAG not only inhibited biofilm formation but also destroyed the intact and viable biofilm. At 1/16 MIC, SAG inhibited biofilm formation by approximately 68 %, whereas at 1/4 MIC, more than 95 % of the biofilm was inhibited, thus an outstanding antibacterial effect of SAG was observed on *P. rettgeri* (Zhang et al., 2020).

Other *in vitro* studies have shown that berberine (alkaloid of Papaveraceae family) is effective against *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis* (Kaneda et al., 1991), *Helicobacter pylori* (MIC_{50} =12.5 µg/mL) (Mahady et al., 2003) and *Leishmania donovani* (Ghosh et al., 1985). Berberine displayed a significant antibacterial and antifungal activity against *Staphylococcus aureus* and different *Candida* spp. (MIC = 64 µg/mL for *Candida albicans*) (Freile et al., 2003). Berberine also inhibits *T. vaginalis* and its effect is comparable to metronidazole as regards potency (Soffar et al., 2001). In the mechanistic aspect, one study has shown that berberine has potent inhibitory activity against sortase A (SrtA) and sortase B (SrtB). The inhibition of sortase enzymes results

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in a marked reduction in the virulence and infection potential of *S. aureus*, so it may be an important mechanism in the antibacterial activity of berberine (Oh et al., 2006).

Conclusions

Ethanolic extracts of *C. majus* showed no antimicrobial activity against *Enterococcus faecalis* strains. However, the results obtained may suggest that the extracts obtained from different parts of *C. majus* need to be subjected to further microbiological and chemical tests for natural antibiotics to evaluate the antibacterial activity and identify secondary metabolites that may be responsible for the above properties. This study lays the foundation for further research to test the feasibility of using *C. majus* as an alternative method in antibiotic therapy. Knowledge of the phytochemical profile of *C. majus* will help in explaining the observed activity and designing activity fractionation experiments to isolate the active substances. Identification of precise molecular mechanisms responsible for inhibition of bacterial growth by these extracts requires further research.

Conflicts 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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Research Article

OPEN ACCESS

Study of phenotypes variability of pollen grains *Malus domestica* Borkh. by scanning electron microscopy

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The studying of *Malus domestica* Borkh. pollen allows us to determine the details of morphological characteristics and describe the most important parameters and pollen sculpture that can be used to identify representatives of species. We analysed pollen morphology of seven *Malus domestica* genotypes from old and local varieties from the territory of Slovakia via a scanning electron microscope. We used principal components analysis to explore variability in pollen grain size (polar and equatorial diameter), shape, aperture type, and exine ornamentation. The complexity of these morphological characteristics and ultrastructure allows determining the differences or similarities between the same and various species and genotypes, which may be a useful tool for systematics with significant diagnostic value. Findings confirmed small differences among the genotypes in measured traits with polar and equatorial diameters in the range from 31.85 to 42.85 µm and from 21.23 to 23.93 µm, respectively. Shape index (P/E ratio) depending on elongation or roundness of pollen grains varied from 1.44 to 1.87. Hierarchical cluster analysis and principal component analysis of morphological data helped to compare evaluated morphometric parameters and identified five closely related groups. It was noted that diversity of surface sculpturing of pollen grains in combination with shape and size enables to use of a complex of thin morphologic signs for *M. domestica* pollen identifications. Pollen data combined with other morphological evidence (e.g., floral characters) have more recently become an important indicator of which genotypes may be the best representatives of species.

Keywords: *Malus* domestica, pollen, SEM, sculpture, morphology

Introduction

Pollen morphology has developed throughout long-term evolution and shows species-specific characteristics (Erdtman, 1969; Blackmore et al., 2007; Katifori et al. 2010. The complex morphological characteristics and ultrastructure of pollen grains allow determining the differences or similarities between the species of fruit trees.

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Quantitative (dimensions) and qualitative (ornamentation, colour) data of pollen have significant value in botanical (Smitha et al., 2018) and taxonomic classification, due to preserved palynological features in many plants (Dogan and Baysal, 2019).

The exine sculpturing, aperture and aperture zone structure, grain shape, and grain size are all useful characters to distinguish genera and even species of Rosaceous pollen (Hebda et al., 1988; Motyleva et al., 2017).

External variables such as extreme climatic changes, but also mineral nutrition and many internal factors (e. i. number of chromosomes) may contribute to the variability of pollen morphological features, especially pollen grain size (Benčať et al., 1988; Ostrolucká and Križo, 1989). Fogle (1977a, b) used the length and width of the pollen grain, depth of exine ridges and prominence of pits in the exine to distinguish peach, nectarine, plum, cherry, apricot, apple and pear. Pollen ornamentation arrangement patterns are important to the exploration of plant genetic evolution and systematic taxonomy (Zhang et al. 2017; Elysiane et al. 2018). However, arrangement patterns are normally difficult to quantify (Zhang et al., 2017). Walker investigated pollen ornamentation characteristics in 1000 species from 35 families and found that pollen grain exine ornamentation evolution to exhibit an overall exhibit trend from regular to irregular and from simple to complicated (Walker, 1974). He and Hsu investigated the pollen morphology of 26 species and 5 hybrids of the genus *Malus* (He and Hsu, 1991). They pointed out that the major evolutionary trend of striae arrangement was from regular and parallel to irregular, dense and interlocking.

Previously, the pollen of some varieties of *M. domestica* Borkhausen was identified by some authors in America (Fogle, 1977a, b), New Zealand (Currie et al., 1997), Iran (Joneghani, 2008), Slovakia (Motyleva et al., 2017), Russia (Motyleva et al., 2018), Czech Republic (Pospiech et al., 2019).

The aim of the study was general characteristics and significant morphological traits of pollen grains of the domesticated apple (*Malus domestica* Borkh.) genotypes cultivated in Slovakia. We assumed that in the gene pool it is possible to detect the variability of quantitative and qualitative traits on the pollen grains and certain specific differences between the genotypes as intraspecific variability.

Material and methodology

Pollen samples and localization

Pollen samples of *M. domestica* (Md) were collected in the territory of Slovakia. As genetic resources were used old and local varieties from different areas of Slovakia which are kept *ex situ* in a clone repository in the village Bacúch (590–630 altitude, m. above s. l.). The whole territory of Slovakia belongs to the temperate climate zone. As the altitude rises, temperature decreases, precipitation increases, soil type changes, and the growing season is shorter.

Fresh flowers (not opened) were collected randomly from the different genotypes at the balloon stage (May 2011). Pollen samples released from dry flowers were further dried under laboratory conditions. The dry pollen was used for a microscopic study of morphological characteristics. The samples of pollen grains were applied to double-tape, fastened to metal object tables with a 10 mm diameter.

Analyses were studied at the All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery (Moscow, Russian Federation) using scanning and transmission electron microscopy. The morphometric parameters were carried out on 50 pollen grains from each genotype using the AxioVision Rel. 4.8.2.0. The length of the polar axis (P) and the equatorial diameter (E) of grains, P/E ratio were measured and compared among studied samples, the structure of pollen grain surface was described.

Morphometric analysis

The measurement of morphometric parameters was carried out on 50 pollen grains from each genotype using the AxioVision Rel. 4.8.2.0 program. The measurements were made in micrometres (μm). The characterization of pollen grains was calculated by taking the following parameters: the polar axis (P – the line connecting the proximal and distal pole), the equatorial axis (E – the line perpendicular to the polar axis and located in the equatorial plane), proximal/equatorial ratio (P/E). The pollen grains were studied at the laboratory of the Institute of Biodiversity Conservation and Biosafety using an electron microscope Carl Zeiss LS 15. The description terminology has been established with regards to Fogle (1977a, b), Martens and Fretz (1980), Marcucci et al. (1984), He and Hsu (1991), Halbritter et al. (2018), Auer (2021). The comparative morphological study of the pollen grains was performed according to the working rules on the SEM JEOL JSM-6390 in the conditions of low vacuum (P = 60 Pa) with the following zooming: 500 times – during the measurements;

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2 500–10 000 times – while taking the pictures of the exine sculpture features. Using the regime of low vacuum allows the pollen to be studied without its preliminary chemical treatment and undistorted data to receive about the research object, which makes the process of the probe preparation easier. Typical exine patterns, shape, size, and dimensions of pollen grains for each *M. domestica* genotype were determined by using a scanning electron micrograph (SEM).

Statistical analyses

Basic statistical analyses – the minimal and maximal values of the traits, arithmetic means, and coefficient of variation (CV, %) were performed using PAST 2.17. Results of the morphometric analysis were determined by mean ± standard deviation (SD) and statistical significance was estimated. The level of variability was determined by Stehlíková (1998). Pearson's correlation coefficient was used to depict the relationship between the two traits. Hierarchical cluster analyses of similarity between phenotypes were computed by the Bray-Curtis similarity index and were performed using PAST 2.17. Principal component analysis (PCA) was performed to evaluate relationships among variables and some possible genotype groupings based on similar properties by using XLSTAT procedure (XLSTAT 7.5, Addinsoft, USA). All the observed traits were shown in graphic form.

Results and discussion

Research of morphological features of pollen grains from specific genotypes by scanning electron microscopy are important and useful for systematic botany and taxonomy, palaeobotany, phylogeny, and breeding programmes, e.g. *Prunus* spp. (Miaja et al., 2000; Arzani et al., 2005), *Cornus mas* L. (Mert, 2009), *Diospyros* spp. (Grygorieva et al., 2010, 2017), *Corylus avellana* L. (Nikolaieva et al., 2014), *Castanea sativa* Mill. (Grygorieva et al., 2015; Horčinová et al., 2021), *Cydonia oblonga* Mill. (Radović et al., 2016), *Pyrus* spp. (Motyleva et al., 2017), *Aronia mitschurinii* A.K. Skvortsov & Maitul. (Grygorieva et al., 2018), *Sambucus nigra* L. (Horčinová et al., 2018, 2020).

We evaluated the variability of morphological characters of pollen grains (sporoform) and assessed their specific differences between selected genotypes of the species *Malus domestica* Borkh. Evaluation of the morphological features of pollen grains in seven randomly selected Slovakian genotypes is recorded in Table 1.

The pollen grains are medium-sized, as we can classify them in the 25–50 µm category. The mean size of the pollen grains ranges from 31.85 µm to 42.85 µm in length and from 21.23 µm to 23.93 µm in width. The smallest pollen grains from the tested genotypes were recorded at the Md-1/4 genotype (31.85 µm) and the largest at the Md-11/2 genotype (42.85 µm). The coefficient of variation indicates a certain degree of variability of the mentioned traits – the average length of the polar axis (6.05–10.68 %) and the equatorial axis (4.05–10.18 %). The genotype with the smallest pollen grains was characterized by the greatest variability in the mentioned traits. The shape index varied from 1.44 to 1.87. The dimensions of the pollen grains of the species examined are presented in Table 1.

The outline of dried apple pollen grains is elliptical, and the polar view is circular. Based on the type and number of apertures pollen grains of *Malus domestica* are tricolpate (tricolporate – fresh pollen) with three germinal furrows without visible pores, each furrow extending almost the full length of the pollen grain. According to the location of apertures, pollens are zonocolpate. This means that the elongated apertures –

Table 1 Variability of the average length of the polar and equatorial axis of pollen grains of selected genotypes of the species *Malus domestica* Borkh.

Genotypes	P – polar axis (µm)				E – equatorial axis (µm)				SI (P/E)
	min	max	x	V%	min	max	x	V%	
Md-1/4	26.57	37.96	31.85	10.68	16.89	23.82	21.23	10.18	1.51
Md-1/8	29.69	42.82	33.64	8.30	19.35	27.91	23.54	9.65	1.44
Md-2/10	34.47	43.75	38.54	7.07	17.94	26.69	22.96	9.17	1.69
Md-5/12	32.78	40.01	36.14	6.05	22.90	26.02	23.87	4.05	1.52
Md-9/5	34.21	52.11	40.99	12.12	20.45	27.09	23.93	8.10	1.72
Md-11/2	36.13	46.86	42.85	6.30	19.69	29.93	23.09	9.08	1.87
Md-33/9	31.03	42.12	36.07	8.59	20.19	25.75	23.37	6.23	1.55

Notes: min – minimal values; max - maximal values; x – arithmetic value; V% - coefficient of variation; SI – shape index (P/E)

Table 2 Literature data on pollen morphometric parameters in the *Malus domestica* Borkh.

Characteristic	Value	Authors	Country
Polar axis (µm)	32.17	Pospiech et al., 2019	Czech Republic
	27.91–36.62	Motyleva et al., 2017	Slovakia
	46.0–48.3	Joneghani, 2008	Iran
	40.1–43.8	Currie et al., 1997	New Zealand
Equatorial axis (µm)	22.78	Pospiech et al., 2019	Czech Republic
	13.28–17.17	Motyleva et al., 2017	Slovakia
	18.8–21.2	Joneghani, 2008	Iran
	20.9–23.2	Currie et al., 1997	New Zealand
SI – shape index	0.97	Pospiech et al., 2019	Czech Republic
	2.23–2.45	Joneghani, 2008	Iran
	1.89–1.98	Currie et al., 1997	New Zealand

colps are distributed equidistantly – at the same distance on the surface of pollen grains with the centre in the equatorial plane. They are traditionally tapered towards poles. The place of narrowing of the ridges at the pole (apocolpium) has an average of 7.2–8.0 µm. The mesocolpium or intercolpium is widest at the equator, i. e. at the same distance from both poles. The average width of the mesocolpium at the equator ranged from 15.0 to 25.2 µm. In the middle of the mesocolpium, we can observe the ridge of exina above the surface of the pollen grain.

The colour of pollen grains is also a taxonomic feature and tends to be different, mostly yellow in different shades. The colour of the pollen depends on the content of the type of plant dyes. The selected pollen grains were yellow with a shade of brown.

Based on the modern scanning electron microscope, we can compare *Malus domestica* pollen collected from various countries with our data (Table 2). Joneghami (2008) evaluated nineteen *Malus* species from botanical gardens and determined the mean size of the pollen grains ranges from 38.75 µm (*M. prattii*) to 57.69 µm (*M. glaucescens*) in length and from 19.15 µm (*M. kansuenses*) to 28.74 (*M. platycarpa*) in width. The author described the surface of *M. domestica* pollen as rough, striate exine sculpture, long ridges, straight and parallel to the furrows, slightly curved at the ends; exine surface perforated and pits sparsely distributed. The spaces between the longitudinal ridges are wide for *M. domestica* (Joneghami, 2008).

Motyleva et al. (2017) studied pollen grains *Malus domestica* and *Pyrus communis* of Slovakia genotypes. Pollen of *Malus domestica* rocks is more elongated compared with the pollen of *Pyrus communis* (P/E is 14 % more). The shape of the pollen grains is oblong-ellipsoidal. In terms of poles, the grain is round, the holes are long. A complex type of exine is characteristic of the studied species of Rosaceae.

Differentiation based on morphology has become difficult because of the increasing number of cultivars that have similar phenotypes (Whitmore, 1992). Methods of identification need to address also between sports arising from identical ancestors, because biochemical identification methods (DAN or isozyme "fingerprinting") have not differentiated sport derived from the same cultivar (Nybom, 1990; Marquard and Chan, 1995; Matsumoto et al., 1995; Sharon et al., 1995). Currie et al. (1997) characterized the apple sports of Red Delicious (Aversang and Ultrared), and Gala (Galalea and Splenola) genotypes from New Zealand according to quantitative data on pollen dimensions, ridge patterns and pore dimension with multivariate analyses (univariate analysis, multiple analysis of variance, canonical variate analysis, and discriminant analysis). Authors by these methods demonstrated differentiation between genetically and phenotypically similar apple genotypes and sports derived from the same cultivars.

Because pollen grain exine ornamentation is highly conserved and genetically stable (Sarwar and Takhashi, 2012), it has been often used to investigate plant origin, genetic evolution and systematic taxonomy (Sarwar et al., 2010; Sarwar and Takhashi, 2012; Qaiser et al., 2015). Zhang et al. (2017) evaluated exine ornamentation of 131 flowering crabapples – a group of small landscape trees or shrubs with reach germplasm of charming flowers, colourful fruits and many tree shapes. Authors examined parental and progeny population and described five types of flowering crabapple pollen exine ornamentation (a) Wholly

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Table 3 Evidence of differences in the average length of the polar and equatorial axis of pollen grains between selected genotypes of the species *Malus domestica* Borkh.

Genotypes	Md-1/4	Md-1/8	Md-2/10	Md-5/12	Md-9/5	Md-11/2	Md-33/9
Md-1/4	P	0.0818	0.0000**	0.0013**	0.0000**	0.0000**	0.0004**
	E	0.0005**	0.0083**	0.0017**	0.0003**	0.0045**	0.0039**
Md-1/8	0.0818	P	0.0000**	0.0326*	0.0000**	0.0000**	0.0146*
	0.0004**	E	0.2579	0.6584	0.5314	0.3825	0.7698
Md-2/10	0.0000**	0.0000**	P	0.0381*	0.0114*	0.0000**	0.0121*
	0.0082**	0.2579	E	0.2116	0.1106	0.7936	0.5099
Md-5/12	0.0013**	0.0326*	0.0381*	P	0.0002**	0.0000**	0.9572
	0.0017**	0.6584	0.2116	E	0.9417	0.2866	0.5300
Md-9/5	0.0000**	0.0000**	0.0114*	0.0002**	P	0.0532	0.0001**
	0.0003**	0.5314	0.1106	0.9418	E	0.1690	0.4180
Md-11/2	0.0000**	0.0000**	0.0000**	0.0000**	0.0532	P	0.0000**
	0.1690**	0.4180	0.1690	0.4180	0.1690	E	0.6592
Md-33/9	0.0004**	0.0146*	0.0121*	0.9572	0.0002*	0.0000*	P
	0.0039**	0.7698	0.5099	0.5300	0.4180	0.6592	E

Notes: * – p <0.05; ** – p <0.01; P – polar axis; E – equatorial axis

Regular Single-pattern Type (e.g. *M. robusta*), (b) Wholly Regular Multi-pattern Type (e.g. *M. halliana* cultivar Pink Double), (c) Partially Regular Single-pattern Type (e.g. M. cultivar Red Baron), (d) Partially Regular Multi-pattern Type (e.g. *M.* cultivar Everest), (e) Irregular Type (e.g. *M.* cultivar Velvet Pillar).

Table 3 illustrates the results of the significance of the differences observed in the tested morphological traits of pollen grains between specific genotypes. Statistical analysis (ANOVA) confirmed the significance of the difference (at a significance level p <0.05) in the average length of the polar axis (x = 38.54) of the Md-2/10 genotype from the other genotypes tested. We noted that a close relationship (alpha 0.05) between Md-1/4 and Md-1/8 genotype pairs and between Md-11/2, Md-33/9 and Md-5/12. In the case of the equatorial axis, the genotype Md-1/4 (x = 21.23) differed significantly from other genotypes, which did not differ significantly.

Data could be considered that hierarchical cluster analysis separates pollen selections into three closely related groups – main clusters. Other authors have also used cluster analyses to evaluate the morphological data of pollen, including pollen characteristics enabling a better understanding of taxonomic classification, the genus or subgenus relationships or phylogenetic lineages (Grygorieva et al., 2015, 2016, 2018; Horčinová et al., 2018, 2020, 2021; Baldemir et al., 2018; Soares et al., 2018).

The results of the cluster analysis simply illustrate groupings of genotypes with similar morphological characters.

Based on cluster analysis, the relationships of the tested genotypes according to morphological features are graphically displayed on a dendrogram (Figure 1).

The dendrogram demonstrates that genotypes represent a more heterogeneous group. Based on the evaluated morphological features, two genotypes Md-1/4 and Md-11/2 are the most separated from the set of genotypes (Figure 2), different from the other five, which form the second group of mutually closer genotypes.

Sculpture (structure) of exina, e.i. the outer sporopollenin layer of sporoderma (pollen grain membranes) is an important morphological feature that is used to identify species in recent and fossil pollen, as it is specific to the species (Benčať et al., 1988; Ostrolucká, and Križo, 1989; Bolvanský and Ostrolucká, 1998). Exine sculpturing is not homogenous. Specific for the sculpture of the surface of the exine pollen of genotypes of the species *Malus domestica* Borkh. there are different thicknesses, densities, different arrangements, even plastically elevated structural elements in different directions looking like free-standing elements (baculum), which would correspond to the type of baculate sculpture. Between the scattered baculum elements, perforations are visible in places, mostly in the shape of round holes or even directly on their surface (Figure 3b/6B). According to literary data

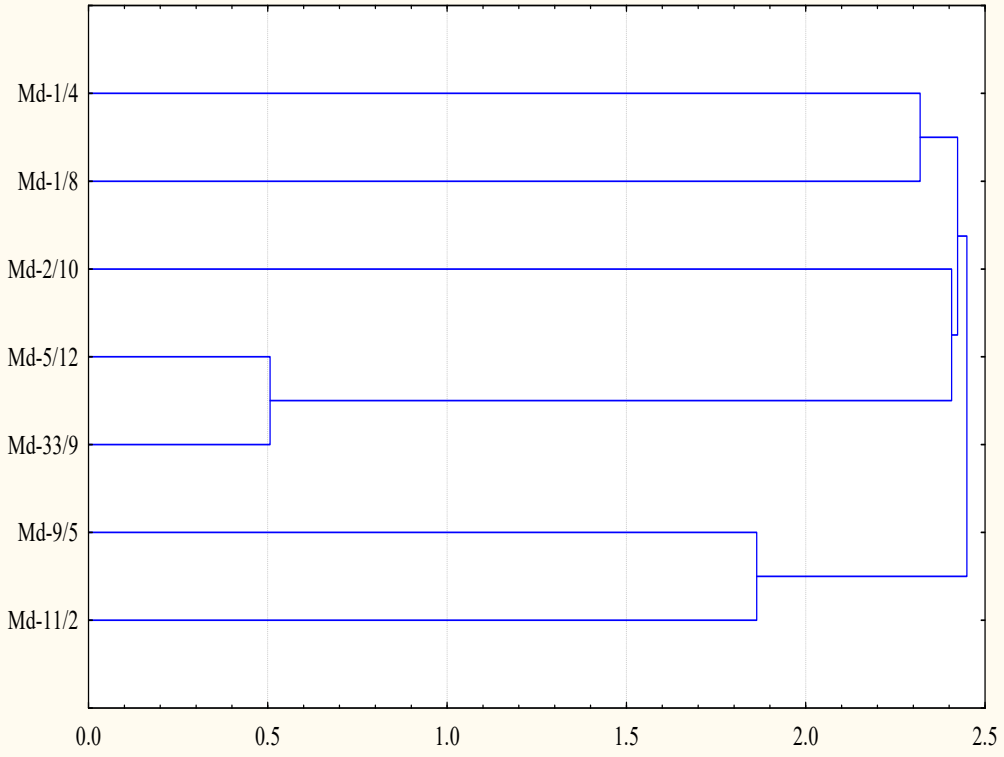

Figure 1 Cluster dendrogram of the relationship of evaluated genotypes of *Malus domestica* Borkh. according to morphometric features of pollen grains

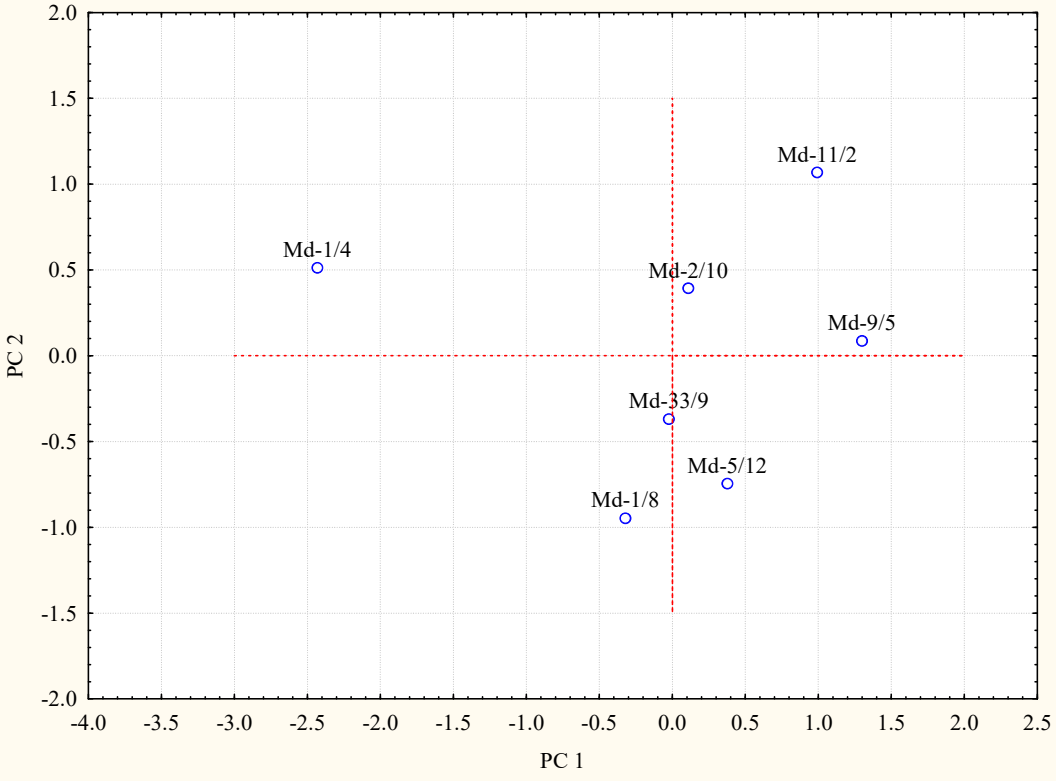

Figure 2 Biplot based on principal components analysis (PCA) for pollen quantitative and qualitative characteristics parameters of *Malus domestica* Borkh. 7 genotypes

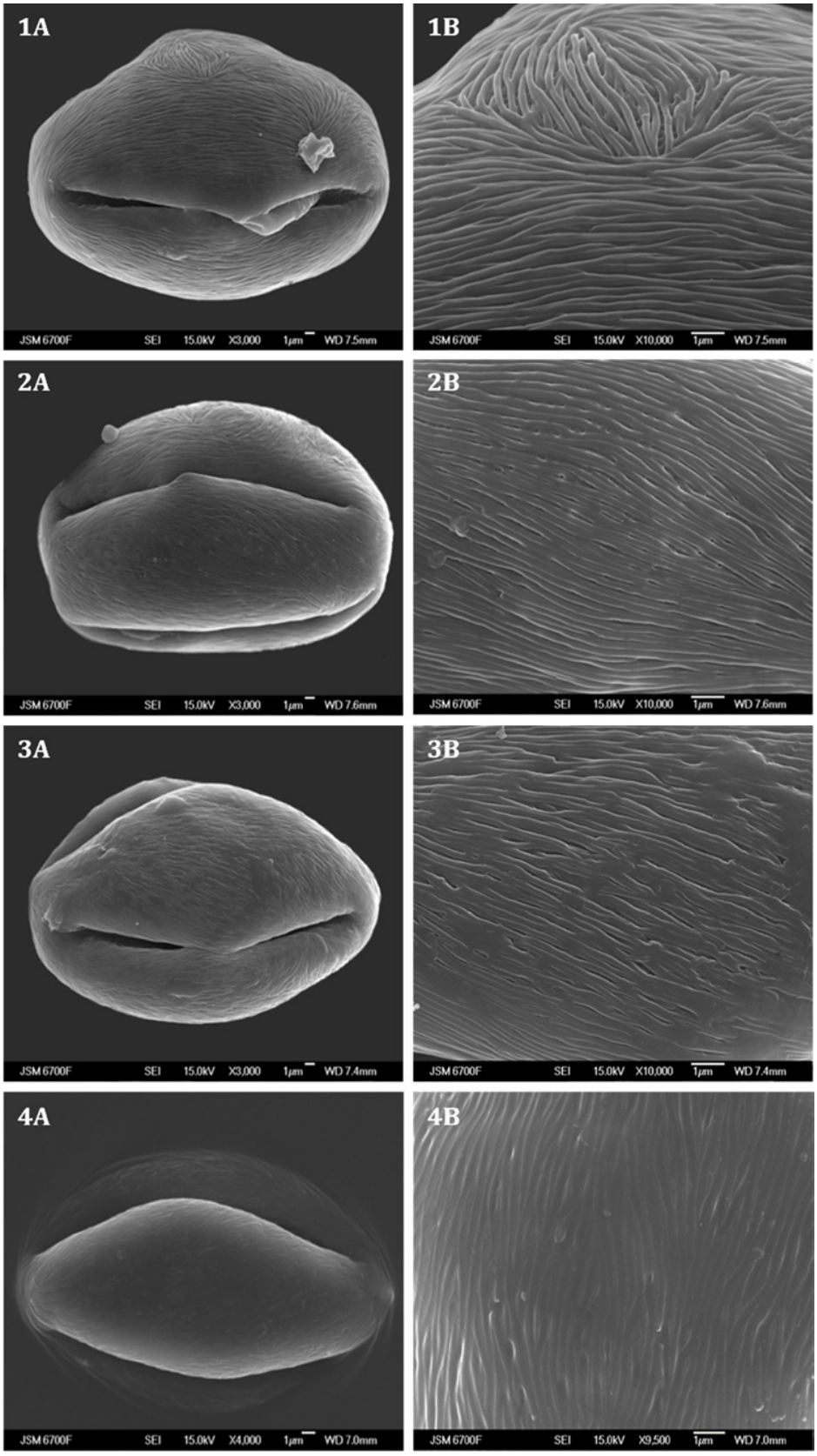

Figure 3a Pollen grain of the species *Malus domestica* Borkh.
1 – MD-1/4; 2 – MD-1/8; 3 – MD-2/10; 4 – MD-5/12; 5 – MD-9/5; 6 – MD-11/2; 7 – MD-33/9; 1A, 4A, 5A, 6A, 7A – pollen grain in equatorial position with one aperture; 2A, 3A – pollen grain in equatorial position with two apertures; A figures (Scale bar = 1μm × 3 000), B figures – detail of the sculpture of the exina surface in the place of the mesocolpium (Scale bar = 1 μm × 10 000)
Photo by Svetlana Motyleva, 2011

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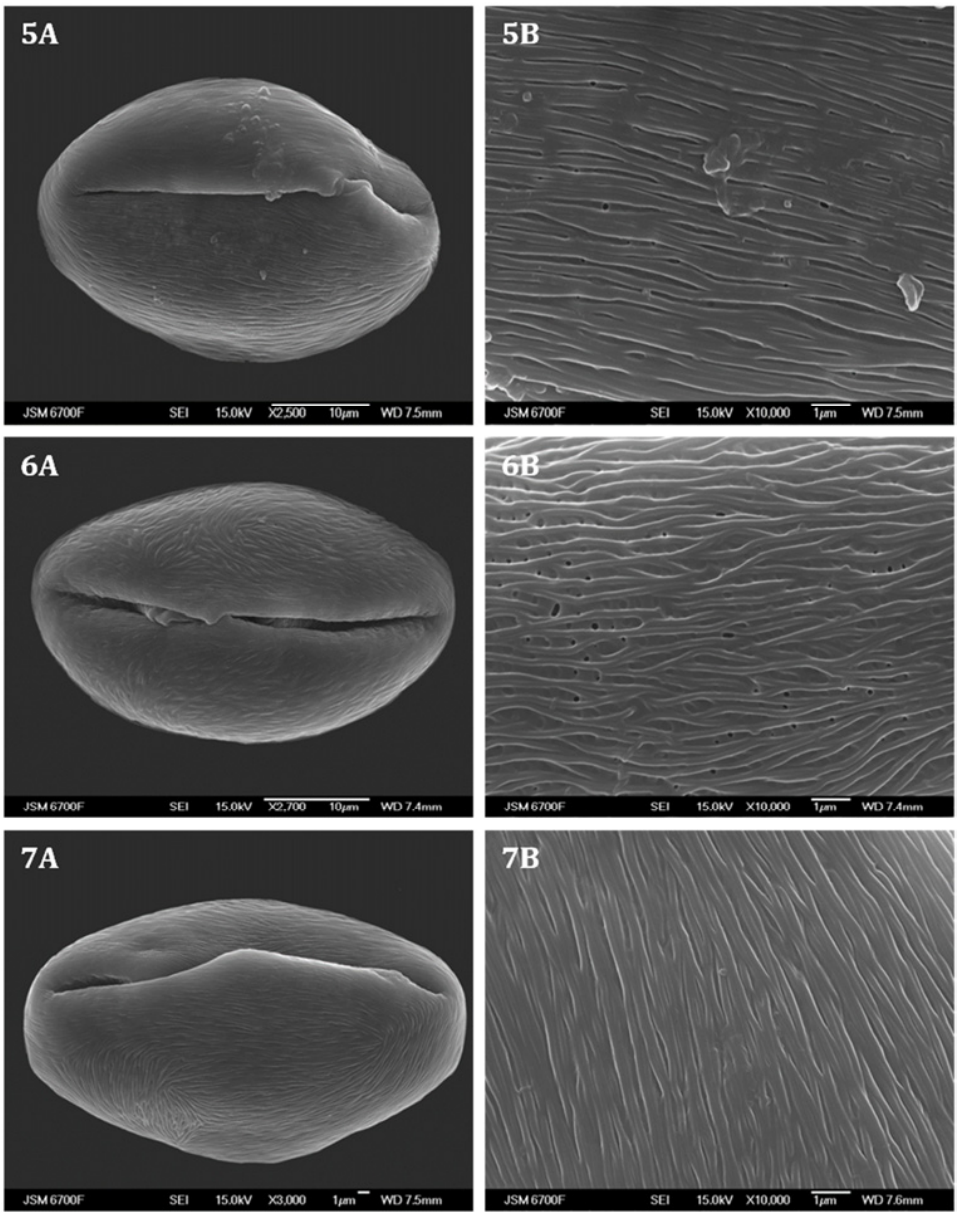

Figure 3b Pollen grain of the species *Malus domestica* Borkh.
1 – MD-1/4; 2 – MD-1/8; 3 – MD-2/10; 4 – MD-5/12; 5 – MD-9/5; 6 – MD-11/2; 7 – MD-33/9; 1A, 4A, 5A, 6A, 7A – pollen grain in equatorial position with one aperture; 2A, 3A – pollen grain in equatorial position with two apertures; A figures (Scale bar = 1μm × 3 000), B figures – detail of the sculpture of the exina surface in the place of the mesocolpium (Scale bar = 1 μm × 10 000)
Photo by Svetlana Motyleva, 2011

(Currie et al., 1997; Joneghani, 2008), exine sculpturing is variable but mostly striate, species may differ in the degree of density of ridges and their orientation. The ridges may be widely spaced resulting in reticuloid patterns, or they may be densely packed with obscured perforations. Ridges also vary from long and parallel to short and irregular in *Malus* L. species. The detail of the microstructure of exine pollen in individual genotypes, even with some differences, is documented in Figure 3a, b.

One criterion for identifying genotype differentiation was the perforation of the surface of the pollen grain exine, its presence (Figure 3a/2B, 5B, 6B) or absence (Figure 3a/1B, 3B, 4B, 7B) and the number of perforations per unit area (mm^2): less than 400 thousand/mm^2 (Figure 3a/2B, 5B) and more than 400 thousand/mm^2 (Figure 3b/6B). Another criterion appears to be the character of the sculptural elements of the exina of pollen grains and especially the presence of bonding rod-shaped or striped elements (Figure 3ab/3B, 5B, 7B) or their absence (Figure 3ab/1B,

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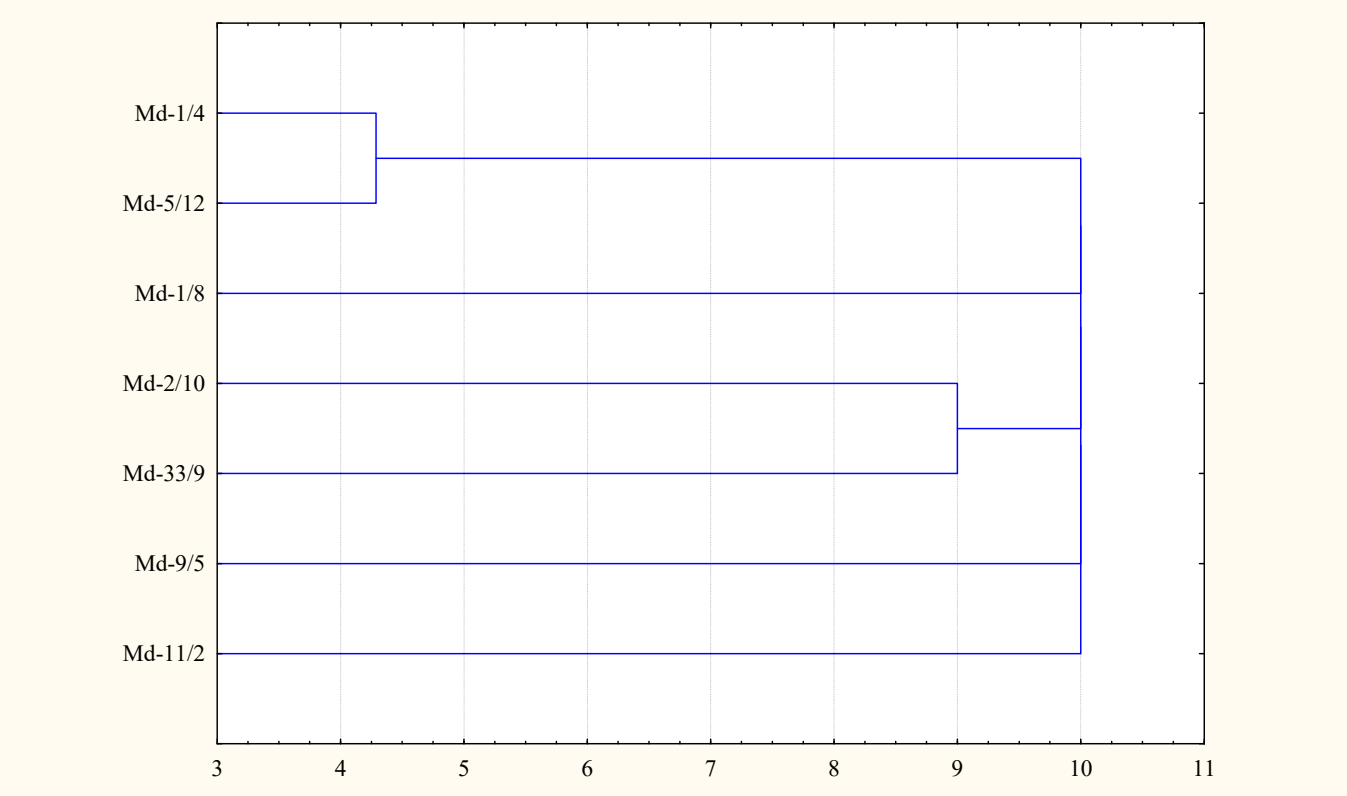

Figure 4 Cluster dendrogram of morphometric parameters pollen of *Malus domestica* Borkh. genotypes

2B, 4B, 5B), and their tortuousness (Figure 3ab/6B) or straightness (other figures). Based on the above mentioned, we classified the genotypes according to dendrogram (Figure 4):

Type I (genotype Md-11/2) – the presence of irregular striped sculptural elements, unconnected and the presence of perforations.

Type II (Md-9/5) – the presence of straight striped sculptural elements, connected and the presence of perforations.

Type III (Md-2/10 and Md-33/9) – the presence of straight striped sculptural elements, connected and absence of perforations.

Type IV (Md-1/8) – the presence of straight striped sculptural elements, unconnected and presence of perforations.

Type V (Md-1/4 and Md-5/12) – the presence of straight striped sculptural elements, connected and the absence of perforations.

Conclusions

The studying of the *Malus domestica* pollen via scanning electron microscope allowed us to determine the most important parameters which can be used to identify the representatives of species. The detailed pollen morphological and micro-sculptural characteristics were investigated, described and analysed by using hierarchical cluster analyses dendrograms and BiPlot (PC1 and PC2). The main parameters such as the form (the pollen grains elongation, P/E ratio) are specific for different *Malus* species. Results from our analyses showed differences among *Malus domestica* genotypes. Some of these pollen morphological parameters can be used for identification and comparison with the following analyses of *Malus* species.

Conflict of interests

The authors declare that they have no competing interests.

Ethical statement

This article complies with all ethical standards.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

OPEN ACCESS

Effect of alginite on some antioxidant indexes in extracts of two variants of *Mentha* and their toxicity

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The experimental work aimed to determine the effect of different applications of alginite on some antioxidant indexes in the extracts of the *Mentha* × *piperita* L. (cultivars Strawberry Mint and Chocolate Mint), and the acute toxicity of water extracts of the studied mint plants to freshwater invertebrates of the genus *Daphnia* sp. The content of total polyphenols, phenolic acids, and antioxidant activity were determined by spectrophotometric methods. The OECD manual No. 202 was used for the determination of EC_{50} 48 h in the water extracts of both variants of mint. The content of total polyphenols in all groups of the cv. Chocolate Mint ranged from 3.05 to 3.37 mg CAE/100 g DW. On the other hand, the significantly lower ($p < 0.001$) content of total polyphenols (from 1.01 to 1.38 mg CAE/100 g DW) was found in all groups with the cv. Strawberry Mint. The antioxidant activity determined by the DPPH method was from 115.7 to 119.5 mg TAEC/100 g DW in the cv. Chocolate Mint, but was 2 times lower (from 48.8 to 65.7 mg TAEC/100 g DW) in the cv. Strawberry Mint. The content of phenolic acids in the cv. Chocolate Mint was significantly higher (but only 1.1-fold), than in the cv. Strawberry Mint. The mean acute toxicity after 48 h exposure of the cv. Chocolate Mint to daphnids was nearly 40 times higher than the mean acute toxicity of the cv. Strawberry Mint (EC_{50} 0.25 mg/L vs. EC_{50} 9.62 mg/L, respectively). We can conclude, that the cv. Chocolate Mint is a more potent source of substances with antioxidant properties, such as polyphenols and phenolic acids, which is reflected in the significantly increased DPPH radical scavenging activity as compared to the cv. Strawberry Mint. We can only speculate that the higher amounts of the aforementioned active substances in the cv. Chocolate Mint were associated with an increased level of acute toxicity to the tested water invertebrates as compared to the cv. Strawberry Mint. The impacts of alginite application on all studied antioxidant indexes as well on the acute toxicity of the extracts of both variants of mint were not confirmed.

Keywords: mint, alginite, antioxidant, invertebrate, immobilization

Introduction

The current research is focused on the field of organic farming "bioagriculture" production of feedstuffs, which can contribute essentially to animal and human health and are environmentally friendly. One of the main requirements in the bioagricultural production is the usage of organic fertilizers instead of synthetic substances. Alginite is a complex soil aggregate of algae-based biomass from an organo-mineral origin (Kúšik et al., 2017; Brindza et al., 2021a, b) with high water absorption capacity (Valla et al., 1980), thus can be used as a natural organic fertilizer. The most effective usage of alginite is attributed to agricultural production as a natural fertilizer (Elečko et al., 1998). Slovakia has in Princiná, Lučenec district, one of the largest alginite deposits in Europe suitable for mining (Kulich et al., 2002).

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The plants of the genus *Mentha* belong to the important representatives of the Lamiaceae family. The genus *Mentha* consists of eighteen species and eleven hybrids. Several aromatic species of mint contain significant amounts of essential oil thus are economically important (Tucker and Naczi, 2006). The taxonomic and phylogenetic classification of the genus *Mentha* is very complicated and unclear due to the hyperdiverse of this group of plants (Heylen et al., 2021). Jedrzejczyk and Rewers (2018) summarize the problems with the taxonomic assignment of *Mentha* species to the following points: a) the existence of variation in basic chromosome number, b) frequent interspecific hybridization, c) cytomixis, d) polymorphism in morphology, e) essential oil composition under different environmental conditions, f) colonial mutant propagation, and g) the occurrence of polyploids, aneuploids, and nothomorphs.

The genus *Mentha* is characterized by the presence of surface glandular trichomes containing relatively huge amounts of essential oils (Bacílková and Paulusová, 2012). Phenolic acids (rosmarinic and caffeic acids), flavones (luteolin glycoside), and flavanones (eriodictyol glycoside) are probably the main antioxidants in mint (Riachi and De Maria, 2015). The active substances presented in mint are responsible for a broad range of biological activities including digestive, choleretic, carminative, antiseptic, antibacterial, antiviral, antispasmodic, antioxidant, anti-inflammatory, myorelaxant, expectorant, analgesic, tonic, and vasodilatation (McKay and Blumberg, 2006). The plants of the genus *Mentha* are known for their strong free radical scavenging and antioxidant activities that lead to the decrease of oxidative stress in the biological system (Nickavar et al., 2008). In the Slovak republic, *Mentha* × *piperita* L. is widely used as a medicinal plant, fresh leaves of mint are a popular spice and culinary drug, and are used for the preparation of hot drinks mostly in combination with ginger (Eftimová and Habán, 2012).

The cultivar Strawberry Mint (*Mentha* × *piperita*) is characterized by its shorter stature and small bright green leaves with purple lilac flowers, which have a distinctive sweet aroma that resembles the smell of strawberries. On the other hand, the cultivar Chocolate Mint is a variety of mint that has nuances of chocolate in fragrance and flavour and features rounded to lance-shaped dark green leaves and terminal spikes of small lavender flowers. Both varieties of mint require moist soils that are slightly acidic or neutral in pH and places with a balanced sun/shade regime.

This experimental work aimed to assess the antioxidant activity, the content of polyphenols and phenolic acids in the extracts of the *Mentha* × *piperita* (cultivars Chocolate Mint and Strawberry Mint) growing in the soil treated by different applications of alginite, as well as to determine the acute toxicity of the water extract of the dried plant samples to daphnids after 48 h exposure.

Material and methodology

In the experiment, the plants of the *Mentha* × *piperita* (cultivars Chocolate Mint and Strawberry Mint) were grown on artificially prepared 1 m^2 plots, on chernozem soil in the village of Hažín, Michalovce district. At the beginning of the experiment, the soil was planted with the seeds of different variants of mint and treated by various applications of alginite: a) control – the plots without the application of alginite, b) group 1 – the plots were treated with the application of powdered alginite in the dose of 0.1 kg/l of water per 1 m^2 (in total 1 kg of alginate), and group 2 – the plots were treated with the application of powdered alginite mixed into the soil at the dose 1 kg per 1 m^2. Except for alginite, the soil was not fertilized and no plant protection products were used. Rainwater from a reservoir was used for watering the plants once a day in the morning. At the end of the experiment, the samples of the plants (only leaves) were dried, cut by a mixer, and stored until analysis.

Total polyphenol content

The total polyphenol content in the aqueous plant extracts was measured using Folin-Ciocalteu reagent by the spectrophotometric method according to Singleton and Rossi (1965) modified by Suchý et al. (2013). Briefly, 0.1 mL of each extract was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20 % (w/v) sodium carbonate, and 8.8 mL of distilled water. After 30 min. in darkness, the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). The total polyphenol content in the aqueous extracts (as caffeic acid equivalent) was calculated from the caffeic acid calibration curve (10–100 µg caffeic acid equivalents (CAE)/L; $R^2 = 0.98$) and is expressed in mg CAE/100g DW.

Antioxidant activity (DPPH radical scavenging assay)

The radical scavenging activity in the methanol plant extracts of the samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Ahmad et al., 2014). The extracts (0.5 mL) were mixed with 3.6 mL of DPPH medium (0.025 g of DPPH in 100 mL of ethanol). The absorbance of the sample extract was determined

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using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (10–100 mg Trolox/L; R^2 = 0.99) was used as the standard for the calibration and the results were expressed in mg Trolox equivalent antioxidant capacity (TEAC)/100g DW.

Total phenolic acid content

Determination of total phenolic acid content in the ethanol plant extracts was carried out using a method of Farmakopea Polska (1999). Briefly, 0.5 mL of extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL of 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 was used as a standard for the calibration curve (0.01–1.0 mg caffeic acid equivalents (CAE)/L, R^2 = 0.99) and the results are expressed in mg CAE/100 g DW.

Acute toxicity on *Daphnia* sp.

The acute toxicity in the aquatic extracts of dried plant samples of both variants of mint was evaluated by the OECD manual No. 202: *Daphnia* sp.: Acute immobilisation test (OECD, 2004). The mobility of freshwater invertebrates exposed to the different concentrations of the substance is monitored during a 48-hour ecotoxicological test. Determination of the half-maximal effective concentration (EC_{50}) at 24 hours and/or 48 hours is recommended. The experiment was performed with some deviations from the manual, which do not affect the endpoints of the test. Briefly, young forms of daphnids were transferred to each test vessel from inert material. A volume of 10 mL of tested concentration for 5 daphnids per test vessel was used (20 daphnids per group). The dried samples of mint leaves were diluted in a hot water for 15 min. (the form of decoction) to the concentration of 0.5 mg/L, 1.0 mg/L and 5.0 mg/L. The control group was only with natural spring water.

Statistical analysis

All data were analyzed by the one-way analysis of variance (ANOVA) with the Tukey post-hoc test ($p < 0.05$). The ecotoxicological results were analyzed by the Probit statistical analysis and the Interpolation statistical method. Association between reported total polyphenols and antioxidant activity were examined by Spearman´s correlation.

Results and discussion

The genus *Mentha* is a rich source of different biologically active substances, such as essential oils. The main group are monoterpenes with the largest proportion up to 60 % presented by menthol, followed by isomenthone, neomenthyl acetate, and menthone only up to 5 % (Makkar et al., 2018). The amounts of active substances in the plants of peppermint differ widely from variety to variety. The wild mint contains up to 33 % of menthol, up to 29 % of menthone, to 18 % of pulegone, up to 11 % of 1,8-cineole, and less than 5 % of terpineol-4 and piperitone (Hajlaoui et al., 2008; Mkaddem et al., 2009).

Flavonoids, gentians, bitter tastants, triterpenes, and tannins are present in the leaves of mint. Sesquiterpenes, such as cadinene and β-caryophyllene, and other compounds (e.g. isoamyl alcohol, amyl alcohol, hexenol, acetaldehyde, and isovaleraldehyde) are also present in these plants but in small amounts (Bulkova, 2011). Growing evidence indicates that these polyphenols can act as potent natural antioxidants (Kanatt et al., 2007; Rita et al., 2016). The contents of total polyphenols in the aqueous extract of the *Mentha* × *piperita* (cultivars Chocolate Mint and Strawberry Mint) treated by the different applications of alginite are presented in Table 1.

Different laboratory methods for the determination of polyphenols from plants have been applied to study the antioxidant properties in these herbal matrixes. For example, Abootalebian et al. (2016) expressed the amounts of polyphenolic compounds in methanol extracts as tannic acid equivalents (TAE) which ranged from 50.0 to 67.0 mg TAE/g DW. The total phenolic content in *M. spicata* extract was nearly 26.0 mg of catechin equivalent per g DW as was reported by Kanatt et al. (2007). Scherer et al. (2013) used gallic acid equivalents (GAE) and reported that the methanolic extracts of *M. spicata* contained around

Table 1 Content of total polyphenols in the samples of mint treated by the different application of alginite

Cultivar chocolate mint	mg CAE/100 g DW	Cultivar strawberry mint	mg CAE/100 g DW
Control	3.37 ABCDE	Control	1.23 CFIX
Group 1	3.08 bIJK	Group 1	1.38 EGKM
Group 2	3.05 aFGH	Group 2	1.01 DHJxM

Notes: the same letters indicate statistical significance between the two respective groups: xx = $p < 0.05$; Xx = $p < 0.01$; XX = $p < 0.001$

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76.0 mg GAE per gram of DW. Benabdallah et al. (2016) analyzed the amounts of total polyphenols in five members of the genus *Mentha* spp. The authors found that *M. aquatica*, *M. arvensis*, and *M. piperita* are rich sources of the total polyphenols, while *M. pulegium*, *M. rotudifolia*, and *M. villosa* have low total polyphenol contents. The total phenolic content in Medina mint and Hasawi mint was 2.64 mg GAE/g DW and 1.54 mg GAE/g DW, respectively (Brown et al., 2019). Brahmi et al. (2015) compared the phenolic composition in three Algerian *Mentha* species: *M. spicata* L., *M. pulegium* L., and *M. rotundifolia* L., and the highest content of polyphenols (12.0 mg GAE/g DW) was revealed in *M. spicata*. On the other hand, the same authors (Brahmi et al., 2014) published the phenolic content in *Mentha* pulegium to be much higher around 55.0 mg GAE/g DW.

In our experiment, the highest content of total polyphenols (3.37 mg/100 g DW) was determined in the aqueous extract of the cv. Chocolate Mint in the control group. In addition, the amounts of total polyphenols in all samples of the cv. Chocolate Mint were significantly higher as compared to all samples in the cv. Strawberry Mint. We can state that the cv. Chocolate Mint is a richer source of polyphenols (nearly 3 times higher amount) than the cv. Strawberry Mint. On the other hand, we can conclude that the application of alginite did not affect the total polyphenol content in the plants of mint. Nevertheless, the cv. Strawberry Mint treated with the application of alginite mixed into the soil had the lowest amount of total polyphenol, only 1.01 mg CAE/100g DW, but this phenomenon was not revealed in the cv. Chocolate Mint (Table 1).

The usage of peppermint plants in traditional "folk" medicine is attributed to the high amount of biologically active compounds. The polyphenols and lipophilic flavonoids were found to possess a strong antioxidant potential and are the most important pharmaceutically active secondary metabolites in mint (Mimica-Dukic and Bozin, 2008). Growing evidence indicates that the biotic and abiotic factors can influence the composition of biologically active substances in the mint plants thus designating the antioxidant properties in the extracts (Gouvea et al., 2012; Rahimi et al., 2018; Brahmi et al., 2020).

In our study, a Spearman correlation analysis was performed to identify the association of the amount of total polyphenols with the antioxidant activity determined by the DPPH method. The radical scavenging ability (the antioxidant activity determined by the DPPH radical production) was positively correlated with a higher content of total polyphenols in the extract ($r = 0.75$, $^*p < 0.255$). These results are in accordance with the previous articles published by Benabdallah et al. (2016). The antioxidant activity was determined by the inhibition % of DPPH in the ethanol extracts of the cultivars Chocolate Mint and Strawberry Mint (Table 2).

Similarly, the statistical differences in the antioxidant activity between the groups of both variants of mint (Control, Group 1 and Group 2) were not found, but the antioxidant activity in all groups of the cv. Chocolate mint was 2 times higher in comparison to the groups with the cv. Strawberry mint (Table 2). The antioxidant activities in the methanol extracts of the *Mentha* × *piperita*, L. and *Mentha spicata*, L. were 84.81 and 52.66 %, respectively (Martiš and Eftimová, 2019). In this study, around 40 % of antioxidant activity by the DPPH method was found in the cv. Chocolate Mint and

Table 2 Antioxidant activity in the samples of mint treated by the different applications of alginite

Cultivar chocolate mint	mg TEAC/100 g DW	Inhibition % of DPPH	Cultivar strawberry mint	mg TEAC/100 g DW	Inhibition % of DPPH
Control	115.7ABC	39.2 %	Control	65.7ADG	21.9 %
Group 1	119.5DEF	40.5 %	Group 1	48.8BEH	16.1 %
Group 2	117.6GHI	39.8 %	Group 2	55.7CFI	18.5 %

Notes: the same letters indicate statistical significance between the two respective groups: XX = $p < 0.001$

Table 3 Content of phenolic acids in the samples of mint treated by the different applications of alginite

Cultivar chocolate mint	mg CAE/100 g DW	Cultivar strawberry mint	mg CAE/100 g DW
Control	1.57AbC	**Control**	1.49adg
Group 1	1.57DeF	**Group 1**	1.51beh
Group 2	1.53GhI	**Group 2**	1.49cfi

Notes: the same letters indicate statistical significance between the two respective groups: xx = $p < 0.05$; Xx = $p < 0.01$; XX = $p < 0.001$

Table 4 Effect of water extracts of the *Mentha* × *piperita* L. (cv. Chocolate Mint) on daphnids mobility in the control group

Parameter	Concentration (mg/L)			
	0.0	0.5	1.0	5.0
Affected after 24 h. (%)	0	20	35	70
Affected after 48 h. (%)	0	70	90	95
Endpoint (mg per liter)				
EC_{50} after 24 h &	2.16 mg/L (CI: 0.97–4.81 mg/L)			
EC_{50} after 48 h *	0.25 mg/L (CI: 0.26–2.32 mg/L)			

Notes: CI – confidential interval, & probit statistical analysis. * Interpolation statistical method, EC_{50} – half-maximal effective concentration

Table 5 Effect of water extracts of the *Mentha* × *piperita* L. (cv. Strawberry Mint) on daphnids mobility in the control group

Parameter	Concentration (mg/L)			
	0.0	0.5	1.0	5.0
Affected after 24 h. (%)	0	10	10	35
Affected after 48 h. (%)	0	15	10	40
Endpoint (mg per liter)				
EC_{50} after 24 h &	25.18 mg/L (CI: 5.31–119.52 mg/L)			
EC_{50} after 48 h &	9.62 mg/L (CI: 3.13–29.57 mg/L)			

Notes: CI – confidential interval, & probit statistical analysis, EC_{50} – half-maximal effective concentration

lower antioxidant activity of only up to 22 % in the cv. Strawberry Mint.

Hydroxycinnamic and hydroxybenzoic acids are the main groups of phenolic acids in these plants (Tanase et al., 2019). The study performed by Tahira et al. (2011) revealed that the major polyphenolic acids in the genus *Mentha* are rosmarinic acid, caffeic acid, ferulic acid, and eugenol. The content of phenolic acids in the ethanolic extracts of the cultivars Chocolate Mint and Strawberry Mint is presented in Table 3. All ethanol extracts of the cv. Chocolate Mint, had a higher content of phenolic acids as compared to the ethanol extracts of the cv. Strawberry Mint. No statistical differences in this index were observed between the groups within the same variant of mint.

It is difficult to compare the acute toxicity of mint extracts with the literature because only a very limited number of ecotoxicological studies concerning the acute toxicity of various substances present in the *Mentha* species were published so far. Recently, Miura et al. (2021) evaluated the toxicological effects of essential oils from different plants, including *Mentha* arvensis L. and *Mentha piperita* L. The authors revealed that the half-maximal effective concentration (EC_{50}) in daphnids after 48 hours of exposure (48 h) was 43.7 mg of *Mentha piperita* essential oil per litre of water medium and *Mentha* arvensis essential oil was less toxic. On the other hand, the EC_{50} 48 h for menthol, the predominant monoterpene presented in the *Mentha* species (Eftekhari et al., 2021), is estimated to be 26.6 mg/L (OECD, 2003).

In our study, only the leaves were used for the preparation of water extracts. Based on our findings, we could presume that the daphnids included in our experiment were affected by different substances (only the amounts of polyphenols and phenolic acids were determined in this study) which are naturally presented in the plant of mint and were extracted into the water extracts during their preparation. Overall, the water extract of the cv. Chocolate Mint was nearly 40 times more toxic to daphnids after 48 h of exposure than the water extract of the cv. Strawberry Mint (Table 4 and 5). Within the groups of the same variant of mint, the effect of alginate application on the toxicity of water extracts was not revealed. According to the acute toxicity rating scale for aquatic organisms provided by the U.S. Fish and Wildlife Service as published by El-Harbawi (2014), the water extract of the cv. Chocolate Mint is highly toxic, whereas the water extract of the cv. Strawberry Mint belongs to the group of moderately toxic substances.

Conclusion

We can conclude, that the *Mentha* × *piperita* (cv. Chocolate mint) possesses more potent antioxidant properties, due to the higher content of total polyphenols and

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phenolic acids, as compared to the cv. Strawberry Mint. Furthermore, the toxicity of the cv. Chocolate Mint is much higher than the toxicity of the cv. Strawberry Mint. This phenomenon is probably due to a higher amount of some active substances in the cv. Chocolate Mint as was confirmed by the statistically increased amount of all studied biologically active substances as compared to the cv. Strawberry Mint. The beneficial effect of alginite application on all studied parameters has not been demonstrated in this experiment, but the highest content of total polyphenols (3.37 mg/100g DW) was determined in the aqueous extract of the cv. Chocolate Mint in the control group, and the lowest amount of total polyphenol, only 1.01 mg CAE/100 g DW, was found in the water extract of the cv. Strawberry Mint treated with the application of 1 kg of alginite mixed directly into the soil.

Conflicts 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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Research Article

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Response and ability of the vines of cultivar Storgozia to recover after hail damage

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A study was performed to determine the response and the ability to recover cultivar Storgozia after the extreme meteorological phenomenon mesocyclone registered in May 2018, accompanied by intense hail and hurricane wind. The hail damage impact on the actual fertility elements, the growing strength and the degree of shoots ripening, the yield quantitative and qualitative indicators, and the grape's chemical composition had been determined for the achieving of the objective. The study found that the damages by hail on the green parts of the vine in 2018 did not have a negative impact on the process of formation of inflorescences in the winter eyes and did not affect the vine productivity of cv. Storgozia in the following year. The damages caused by hail at the beginning of the growing season had little effect on the growing strength and the degree of shoot ripening. The leaves and leaf area (1.36 m^2) of the main shoots are proven to be smaller in size in the year with hail, compared to the other two years, which reflects on the size of the leaves (3.38 m^2) and leaf area of the lateral shoots, which are proven to be larger after the registered hail. The total leaf area per vine had remained almost the same in all three years, as the difference was only in the leaf area ratio of the main and lateral shoots in 2018 that did not result in disruption of the vital for the vine plant physiological and biochemical processes in the leaves. Hail had a considerable negative effect on the structure of the cluster, the berry size, the yield per vine. The overall assessment of the quality of the grapes from the three harvests shows that with varying degrees of evidence for the individual indicators, hail has a significant adverse effect on its chemical composition in terms of sugar, titratable acids, anthocyanins, and phenolic compounds.

Keywords: vine, cultivar, response, ability to recover, hail

Introduction

The increased heat in the air, higher temperatures near the earth's surface, and decreasing snow cover registered in recent years have been among the ten key indicators showing global warming (MEW, 2012; IPCC, 2013). Higher temperatures and more intense and extreme meteorological phenomena would lead to very serious negative impacts on agriculture, natural resources, human health, etc., mainly in terms of reduced crop yields and productivity and hampering the ability of the countries to produce food (Hannah et al., 2013; Koleva-Lizama, 2017; Lazoglou et al., 2017; Santos et al., 2020).

Over the last 30 years in Bulgaria there had also been a steady trend towards global warming, compared to previous periods, as well as a rise in the frequency

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of extreme meteorological and climatic phenomena (Vlaskov, 2014; Rachev and Dimitrova, 2016; Rachev and Asenova, 2017; Vlaskov, 2017).

Due to these climatic changes, in recent years the vine as a crop had increasingly been adversely affected by some unpredictable meteorological phenomena such as late spring frosts and hail storms. Hail was usually associated with spring thunderstorms, but it might occur at any time of the year. It could cause serious damage to the vineyard, not only in terms of quantity and quality of grape production but also in terms of vineyard survival and long-term development (Sioutas et al., 2007; Papagiannaki et al., 2013). Most often, hail would cause significant damage to leaves, shoots, inflorescences, clusters, and berries, but at higher intensities, it might harm also the grapevine stems and cordons (Dry, 1986).

Depending on the weather and the hail intensity, as well as the clusters' density and their location on the vine, the damage might vary from different leaf ruptures to complete defoliation of the vine and large damage to the shoots and complete loss of harvest (Fiola and De Marsay, 2013; Krstic et al., 2014; Gourieroux, 2019).

Hail, through the damage they caused to the vine plant, most often affected its total leaf area, resulting in looser and smaller size and weight clusters and lower sugar content and total phenolic reserves of grapes. At the same time, hail-damaged vines had a larger leaf area of the lateral shoots and showed a greater accumulation of total soluble solids (TSS), but no adverse effects on photosynthesis, berry mass, grape acidity, and fertility the following year (Petoumenou et al., 2019).

The recovery rate of the vine plant depended entirely on the damage intensity and was individual for each vine and vineyard. Although very often hail damage could seem extreme, vine had the unique ability to recover very successfully through the development of shoots from different vine buds (Dry, 1986).

The objective of the study was to determine the response and the ability to recover the intraspecific red wine cultivar Storgozia after the hail.

Material and methodology

Plant material

The research work was carried out in the period 2017–2019 in an experimental plantation of the intraspecific red wine cv. Storgozia (Roychev, 2012), in the Experimental base of the Institute of Viticulture and Enology (IVE) – Pleven.

The planting distance was 2.50 × 1.30 m, and the vines were grown on a semi-high training system with a stem height of 1 m. The vines were spur-pruned, with a loading of 18 eyes (9 × 2) per vine.

The objective of the study was to determine the response and the ability to recover Storgozia variety grapevines after the registered on May 15, 2018, intense hail and hurricane wind. The indicators reported for the same variety in the previous year 2017 and the following one – 2019 were used as controls.

Influence of hail on the vegetative and generative abilities of the cultivar

For the implementation of the set goals and objectives, the following groups of indicators had been reported:

- To determine the impact of the damage caused by hail on the elements of actual fertility, the following indicators were recorded: ratio of developed eyes; ratio of fruit shoots; fertility rate per developed shoot and fruit shoot. The indicators characterizing the actual fertility were reported annually at the end of May for 10 vines (Katerov et al., 1990).
- To find out the effect of damage caused by hail on the vine growth strength, the maturation of the shoots was taken into account. The degree of maturation of the shoots was reported visually in late October and early November. After the shoots ripening, the following indicators were determined: average length per shoot (cm) – total mature and green part; the average length of the mature part of the shoot (cm); ratio of the mature part compared to the total length of the shoot; average thickness of the shoot in the 5th internode zone (mm) (Katerov et al., 1990).
- To determine the influence of damage caused by hail on some physiological processes occurring in the vine, the following indicators were reported: leaf area – the surface per leaf (cm^2), determined by linear parameters of the leaf blade and mathematical factors according to advanced Carbonneau method (Belberova and Tsvetanov, 2014; Belberova and Tsvetanov, 2017) and leaf area per vine (m^2) – determined by the defoliation per vine before harvest.
- To determine the effect of damage caused by hail on the quantitative yield indicators, it was accounted the average mass per cluster (g), the average mass per 100 berries, (g), the average yield per vine (kg) (Katerov et al., 1990).

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Chemical analysis of grapes

To determine the effect of damage caused by hail on the qualities and chemical composition of grapes, the generally accepted methods in winemaking were used (Ivanov et al., 1979): sugars (g/dm^3) – hydrometer of Dujardin; glucose and fructose (g/dm^3) – iodometric method; titratable acids (g/dm^3) – titration with NaOH; tartaric and malic acid (g/dm^3) – Pochinok's method; pH – pH meter. The phenolic availability in the grape skins was also determined concerning the content of anthocyanins (mg/dm^3) – method of Ribéreau-Gayon et Stonestreet and total phenolic compounds (TPC) – method of Singleton and Rossi (1965).

Statistical analysis

The statistical processing of the results was performed by analysis of variance (Dimova and Marinkov, 1999).

Results and discussion

The natural disasters that have become more frequent in recent years are attracting in the increasing interest due to their great social and economic significance and the growing financial losses they have caused (Messner and Meyer, 2006; MunichRe, 2012; Papagiannaki et al., 2014). Due to the effects of climate change (such as heat waves, drought, frost, flood, wind), grape growers face problems in managing vineyards (Takahashi et al., 1976; Flexas et al., 2002; Webb et al., 2010; Morales et al. al., 2014; Mosedale et al., 2015).

Although rare, hail can cause significant damage to vineyards in a short time, having a direct impact on vine growth, grape harvest, and quality during the year with registered hail and the following year (Bora et al., 2014; Bora et al., 2016; Theodore, 2018; Staffne and Carol, 2019).

There are a number of other studies in the literature on the effect of hail damage on various crops, such as apple (Tartachnyk and Blanke, 2002), cranberry (Wells and MacManus, 2013), potato (Jalali, 2013), maize (Miya et al., 2017).

On May 15, 2018, at about 5.00 p.m., a very rare Bulgaria meteorological phenomenon called mesocyclone was registered in the region of Pleven. In its nature, that was a thunderstorm, characterized by the presence of a mesocyclone – a powerful and constantly rotating upward air current. The element was accompanied by hurricane winds (gusts of over 150 km/h) and intense hail (Figure 1).

A large part (over 80 %) of the main shoots are in the experimental plantation of cv. Storgozia were broken from the base or at different heights, the leaf mass was torn, most of the inflorescences were destroyed or severely damaged.

In determining the productive potential of cv. Storgozia is characterized by high fertility, which varied within narrow limits during the individual years (Table 1). In 2017, the ratio of developed eyes (96.94 %) and fruit shoots (88.03 %) was high but shoots with 1 cluster predominated that initiated slightly lower rates of developed shoots (1.38) and fruit shoots (1.58). In 2018, before the registration of the hail storm, the rates of the recorded indicators were high. The developed eyes per vine were almost the same as those of the previous year (96.11 %), while the fruit shoots were less (83.03 %), but the shoots with 2 clusters predominated, which gave higher fertility rates – 1.81 clusters per developed shoot and 2.13 clusters per fruit shoot. After the hail and the green pruning, 87.78 % of the buds developed, 53.91 % of them were fruit-bearing, and the ratio of shoots with 1 and 2 clusters was almost the same. In 2019, the fertility of cv. Storgozia was very high, compared to the previous two years, which clearly showed that despite the significant damage caused by hail in 2018 on the green

Figure 1 Mesocyclone and hail in the Pleven region (May 15, 2018)

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parts of the vine, the formation of inflorescences in the winter eyes during the same growing season occurred normally and a phenomenon of this type and during that period of vegetation did not affect the fertility of cv. Storgozia for the following year.

Table 2 presented the results determining the influence of hail and the damage caused by it on the growing strength and the degree of shoot ripening of cv. Storgozia vines. The data revealed that the extreme meteorological phenomenon of hail had a direct impact only on the indicator of average shoot length, which was smaller (83.10 cm) compared to the other two years of the study (90.45 cm and 127.35 cm). By the end of the vegetation season, all shoots were fully mature, accompanied by the accumulation of the maximum amount of plastic substances in their tissues that determined the vine cold resistance. There was also a significantly increased growth in the year after the hail (2019), which showed the positive recovery response of cv. Storgozia, determined by its genetically enhanced resistance to stress factors.

From the statistical analysis of the data determining the actual fertility of the vines of cv. Storgozia, it was found that in 2018, before hail, the values of the number of inflorescences per vine, the fertility rate of developed and fertility rate of fruiting shoots are higher, compared to the control year 2017, after which, with varying degrees of evidence, hail has an adverse effect on the studied productivity indicators. The high regenerative abilities of cv. Storgozia are fully manifested in 2019, when the values of all studied indicators are proven to be higher than the period after the hail in 2018 and the control year 2017, except for the number of developed vine eyes in the control period.

The results in determining the hail impact on some physiological processes occurring in the vine were similar (Table 3). When measuring the average size of the leaves of cv. Storgozia vines, it was found that after the hail the sizes of the main shoots' leaves were considerably smaller. In 2018, they were respectively 27.5 and 21.6 % smaller compared to 2019 and 2017. The reduced size of the main shoots' leaves reflected the size of the lateral shoots' leaves that were larger after the hail. The average rates showed an insignificant decrease in the total size of leaf per vine after the hail, which would not have a negative impact on the course of the various physiological and biochemical processes in them.

In determining the leaf area per vine, it was found that for cv. Storgozia, in the year with the registered hail, the total leaf area was almost the same compared to both control years. The main difference was that in 2018, 28.7 % of it was formed by the leaves of the main shoots and 71.3 % from the lateral shoots' leaves, while in both years without hail ratio was respectively 81.5 and 18.5 % (2017) and 82.3 and 17.4 % (2019).

Similar results were obtained by Petoumenou et al. (2019), according to which lateral shoots can provide assimilation to support their growth and send the surplus to the main shoot, which contributes to the ripening of the crop. Based on the results by Baniță et al. (2020), the natural hailstorm caused an alteration in vegetative growth grapevines due to shoot damage induced by the hailstorm.

Table 1 Actual fertility of cultivar Storgozia vines for the period 2017–2019

Year		Developed eyes per vine (%)	Fruit shoots per vine (%)	Number of inflorescences per vine	Fertility rate per developed shoot	Fertility rate per fruit shoot
2017 control		96.94	88.03	23.75	1.38	1.58
2018	before hail	96.11 n. s	83.03 n. s	31.30 +	1.81 ++	2.13 ++
2018	after hail	87.78 --	53.91 ---	12.80 ---	0.81 ---	1.30 --
2019		95.00 n. s	93.06 +	30.90 +	1.65 +	1.78 +

Notes: n. s. – not significant; + significant; ++ well insured; +++ very well insured

Table 2 Growth strength and degree of shoot ripening of cultivar Storgozia in the period 2017–2019

Year	Average shoot length (cm)	Average internode length (cm)	Average length of the shoot mature part (cm)	% of the mature part compared to the total shoot length	Average shoot thickness in the 5th internode zone (mm)
2017 control	90.45	4.96	89.80	98.75	5.72
2018	83.10 --	6.25 +++	83.10 -	100.00 n. s	6.46 +
2019	127.35 +++	6.16 +++	111.25 +++	90.34 n. s	7.01 ++

Note: n. s. – not significant; + significant; ++ well insured; +++ very well insured

Mathematical evaluation of the data determining the strength of growth and the degree of maturation of shoots shows that hail has a proven negative impact only on the total length of shoots and the length of their mature part. With the indicators length and thickness of the internodes, the phenomenon initiates the formation of proven longer and thicker internodes of the individual shoots. The positive reaction of the vines of the studied cv. Storgozia is confirmed by the data in 2019, when for all indicators of growth and maturation of shoots, higher values are reported, compared to the control year 2017.

In determining the impact of hail damage on the quantitative and qualitative yield parameters and the chemical composition of the must, it was found that hail most clearly had a negative effect on this group of indicators (Table 4). The smaller number of inflorescences in the year 2018 with hail had a positive influence on the average mass per cluster that was higher than the other two years. The clusters were larger in size, but looser in structure. Despite the higher average mass per cluster, in 2018 the lowest average yield per vine was reported, due to the less number of clusters and the smaller berries in them.

Statistical analysis of the data shows that, as a result of the hail, the size of the main leaves and the leaf area formed by them are smaller, and those of the side shoots are larger, compared to the control. The summary indicator leaf area of 1 vine has almost the same values and the differences are unproven. The positive reaction and high recovery potential of cv. Storgozia, after hail, are confirmed by the results obtained in 2019, when all indicators of the leaf show proven positive differences compared to 2017, and in terms of leaf area, the differences between the two studied periods are minimal and mathematically unproven.

Regarding the quality characteristics of grape yield, it was found that all indicators showed a significant negative impact of hail on its chemical composition and the opportunities for the production of quality wines from the 2018 harvest. Due to the later cluster formation, the sugar content in grapes was considerably lower than in 2017 and 2019, while the acids are higher. However, glucose and fructose rates were within the

Table 3 Leaf size and leaf area of vines of the Storgozia variety for the period 2017–2019

Year	Average size per leaf (cm2)			Leaf area per 1 vine (m2)			
	main leaves	lateral leaves	average per leaf	main leaves	lateral leaves	total (cm^2)	leaf area per vine
2017 control	76.20	25.50	50.85	38 698.10	8 752.54	47 450.64	4.75
2018	59.75---	33.53++	46.64-	13 639.80---	33 799.12+++	47 438.94 n. s	4.74 n. s
2019	82.40+	30.11+	56.26+	37 228.68 n. s	7 863.58-	45 092.26 n. s	4.51 n. s

Notes: n. s. – not significant; + significant; ++ well insured; +++ very well insured

Table 4 Quantitative and qualitative characteristics of grape yield from cultivar Storgozia for the period 2017–2019

Indicators	Year		
	2017 control	2018	2019
Average yield per vine (kg)	3.450	2.667 --	4.606 +++
Average mass per cluster (g)	145.14	169.43 +	140.92 n. s
Average mass per 100 berries (g)	212.06	182.31 --	188.46 -
Sugars (g/dm^3)	237.00	204.00 ---	234.00 n. s
Titratable acids (g/dm^3)	5.88	6.62 ++	5.39 n. s
Tartaric acid (g/dm^3)	2.54	4.61 +++	1.05 --
Malic acid (g/dm^3)	4.20	4.99 +	4.20 n. s
Glucose (g/dm^3)	96.30	72.00 ---	103.50 n. s
Fructose (g/dm^3)	147.70	132.00 -	130.50 n. s
pH	3.43	3.58 n. s	3.50 n. s
Anthocyanins in skins (mg/dm^3)	439.48	285.65 ---	473.46 +
TPC (g/dm^3)	0.85	0.38 ---	0.90 n. s

Notes: n. s. – not significant; + significant; ++ well insured; +++ very well insured

normal ranges with a predominance of fructose, which was observed in all three years. The anthocyanins and TPC content in the grape skins from 2018 were significantly lower, respectively 285.65 mg/dm^3 and 0.38 g/dm^3. Their quantity was almost twice less than their content in the grapes from 2017 and 2019 harvests. The negative impact of hail on the analysed indicators, however, was completely overcome by cv. Storgozia the following year and the vintage had a chemical composition, without deviations from the variety typical specification.

Statistical analysis of the data determining the quantitative indicators of yield shows that hail has a proven negative effect on grain mass and yield of 1 vine. Due to the smaller number of bunches, their mass is proven to be higher than in 2017. In 2019, the mass of 1 bunch is approximately the same as in 2017, but most of the formed inflorescences initiate a higher yield of grapes and smaller grains in the bunches compared to the control period.

The results of the analysis of the quality of obtained grapes confirm the significant negative impact of the hail phenomenon established so far. In all examined indicators, with different degrees of evidence, compared to the control year 2017, lower (sugars, anthocyanins, TPC) or higher values (acids) are reported, which determine the quality of the obtained raw material from grapes as unsatisfactory. In 2019, the quality of the obtained grapes of the cv. Storgozia is identical to that in 2017 (control), which shows the high recovery potential of the studied variety after the hail.

From the above data, it could be argued that with the hail, registered at the beginning of the growing season, the yield and its quality from cv. Storgozia were significantly reduced, however the obtained grapes had satisfactory quantity and quality, minimizing the losses from this extreme meteorological phenomenon.

Conclusions

The damages caused by hail on the green parts of the vine in 2018 did not have a negative impact on the process of formation of inflorescences in the winter eyes and did not affect the vine productivity of cv. Storgozia in the following year. The damages caused by hail at the beginning of the growing season had little effect on the growing strength and the degree of shoot ripening. The leaves and leaf area of the main shoots are proven to be smaller in size in the year with hail, compared to the other two years, which reflects on the size of the leaves and leaf area of the lateral shoots, which are proven to be larger after the registered hail. The total leaf area per vine had remained almost the same in all three years, as the difference was only in the leaf area ratio of the main and lateral shoots in 2018 that did not result in disruption of the vital for the vine plant physiological and biochemical processes in the leaves. Hail had a considerable negative effect on the structure of the cluster, the berry size, the yield per vine. The overall assessment of the quality of the grapes from the three harvests shows that with varying degrees of evidence for the individual indicators, hail has a significant adverse effect on its chemical composition in terms of sugar, titratable acids, anthocyanins, and phenolic compounds.

Conflicts 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

Agrobiodiversity documentation in Puranchaur, Pokhara through four cell analysis: biodiversity of rice

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Agrobiodiversity indicates variety and variability among living organisms present in the farming area. The climatic variability of Nepal has supported the maximum diversity of agricultural crops and animal species. The number of cultivated food crops in Nepal is 577 including forage/pasture species among which 484 are indigenous and 93 are introduced/exotic species. This study was carried out to assess the biodiversity of rice in Puranchaur, Pokhara, Nepal. A field survey was conducted from 60 farmers who represent the total farming population. The collected data from the questionnaire survey were further analysed using SPSS Statistics 23.0. Out of the average total land (0.50 hectares), the average total cultivated land was only 0.39 hectares. The average productivity of rice in the study area was 4.035 m/ha. The different rice varieties cultivated by farmers in the study area were documented and analysed through four cell analysis method since it is a popular analysis tool to manage the local level agricultural diversity. From the study three rice landraces Pahele, Gurdi, and Jhinuwa were found to be the most preferred, and Anadi is the least preferred in the area. Based on this study, possible conservation strategies for threatened and endangered species were also identified. The possible interventions include on-farm conservation, *in situ* conservation, *ex situ* conservation, and conservation plant breeding.

Keywords: rice, diversity, landraces, four-cell analysis

Introduction

Agrobiodiversity is the combination of environment, biological diversity, and monitoring systems employed by a varying group of people and thereby exploitation of water and land resources to maintain the architecture of agro-ecosystem for food security and agricultural production (FAO, 1999). Nepal's geographical variety is immense, ranging from tropical flatlands on the southern side to the rocky, delicate, and snow-covered Himalayas on the northern side (MOFSC, 2014). Moving from the terai to the mountainous area of Nepal revealed a total of 118 ecosystems. A broad range of climate and topography has benefitted agricultural methods, their wild counterparts, and biological types (Khanal and Dangol, 2016). Nepal ranks tenth in Asia and thirty-first worldwide in terms of flowering plant diversity. There are 790 plant species important from the nutritional point of view and 577 cultivated plant species, including pasture species. There are 484 indigenous species and 93 exotic species among the

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577 species (Joshi et al., 2017). In order to conserve biodiversity along with agricultural biodiversity, Nepal joined the CBD (Convention on Biological Diversity) and ITPGRFA (International Treaty on Plant Genetic Resources for Food and Agriculture) in 1992 and 2009, respectively, Protocol of Cartagena on Biosafety and the Nagoya in 2001 and 2019, respectively; possess Agrobiodiversity Policy-2007 (updated on 2014) and various seed related acts, visions and policies (Joshi et al., 2020).

Cereals are the most pivotal food crops, accounting for 49.41 percent of the country's GDP. Rice is the dominant cereal grain, accounting for 20.75 percent of total AGDP (Bista et al., 2013). Rice production in Nepal lifted from 2.3 million tons to 5.61 million tons (1970–2019), climbing at a yearly pace of 2.78 % (World Data Atlas, 2019). Rice production has grown at an average annual rate of 0.16 % over the past 50 years, which is more or less than the level of the growing population. Traditional agricultural techniques, shortage of experienced labour force, environmental degradation, and other obstacles contribute to the yield gap of 45 to 55 % in rice (IRRI, n.d.). So, to improve crop productivity, the genetic resources must be conserved since they facilitate to development high yielding breeding lines and qualitative cultivars.

Nepal is one of the important centres for rice genetic resources as more than 1 700 rice genetic resources have been identified, ranging in altitude from 60 to 3,050 meters (MoAD, 2015). The ecological condition of terai, mid-hills, and high hills distinguish the prevalence of landraces of rice (Bajracharya et al., 2010)in traditional rice farming systems many diverse landraces are grown in all of the rice agro-ecosystems from low to high altitude. Three case study sites were selected to represent the major rice agro-ecozones: Bara (100-150 m found that high hills have the lowest rice diversity due to the chilling temperature and are dominated by the cold-tolerant Marshi rice while mid-hills have the highest rice diversity due to varying agroecosystems with a wide range of environmental variability. Similarly, terai (grain store of the country) has low diversity due to the landraces being phased out in favour of new varieties, but not due to ecology.

Diverse genetic resources are the foundation for sustainable development in agriculture. The availability of agricultural genetic resources is the fundamental requirement for achieving a further increase in productivity and maintaining food security in the country. However, genetic erosion is increasingly becoming the main issue in the most of the crop species around the world. Among the various methods used in agrobiodiversity documentation within the farming community, four-cell analysis is one of the most suitable and rapid methods for determining the amount and distribution of crop diversity within agricultural communities. It considers the richness and evenness of inter-specific and intra-specific biodiversity (Sthapit et al., 2014).

Material and methodology

Broad objective

The main objective of the study was to assess the diversity in the rice landraces being grown in the study area.

Specific objectives of the study:

- Record-keeping the rice landraces being grown in the study area.
- Analysis of the varietal selection and potential conservation approaches.

Site selection

For the documentation of agrobiodiversity, an assessment of rice landraces that are being grown in Puranchaur, Pokhara was done. Puranchaur is a small village at a distance of 17 km from Pokhara city. Puranchaur is situated at an altitude of 940 m. a. s. l. with a mean annual temperature of 20.6. It falls at a latitude of 28.32 degrees north and a longitude of 83.99 degrees east (Wikipedia, 2020).

Figure 1 Map of Kaski district, Nepal showing Puranchaur village (NEKSAP, 2013)

Method of data collection

The questionnaire was designed to collect data from the farmers. The pre-tested questionnaire was presented for direct interaction with a group of local farmers in the form of a field survey. Altogether 60 farmers were asked a series of questions as a representative of the total farming population in this area.

Four cell analysis

For four-cell analysis, rice varieties that are being cultivated by the farmers in the study area were documented and were classified into four different groups. The four groups were arranged based on the area (large and small) and household (many and few) as shown in the figure below. Then the discussion was carried out on the reasons the farmers have preferred some varieties over others. Based on the discussion with the farmers' methods for conservation of those varieties that are in threat and endangered were also identified. Generally, for this purpose, we prefer focus group discussion.

Statistical analysis

The data were collected, classified, and analyzed using various statistical procedures. The collected data were analyzed using SPSS Statistics 23.0 through descriptive statistics. Various bar diagrams and charts are used for the interpretation of the analyzed data.

Result and discussion

Rice which is among the three major leading crops in the world; together with wheat and mays they supply more than 50 % of all calories consumed by the entire population of the world (FAO, 2001).

According to FAOSTAT, in 2018–2019 rice was planted in 118 countries on an area of 167 million hectares, the annual grain production in the world is about 782 million tons. The main rice producers in the world are China (over 214 million tons), India (over 172 million tons), Indonesia (83 million tons), Bangladesh (56 million tons), Vietnam (44 million tons), Thailand (32 million tons) and Myanmar (25 million tons) (FAOSTAT, 2018–19).

Rice is majorly grown in Asian, African and Latin American countries, it is the chief and cheapest source of carbohydrate in majority of the developing nations. There has been a rise in global consumption of rice with subject to global population growth, raising the need to increase production with improvement in technology (Färe et al., 1994, 2001; Hassen et al., 2017; Bagirov et al., 2020).

Demographic information

The demography includes informations about age, gender, ethnicity, educational status, economically active population, main occupation, type of family of the respondents, food sufficiency (months), land distribution, and amount of cereal production in quintals.

Age of respondents

During the field survey, the majority of the respondents (38.3 %) were 35 to 45 years old while only 6.7 % of respondents were 25 to 35 years old (Table 1).

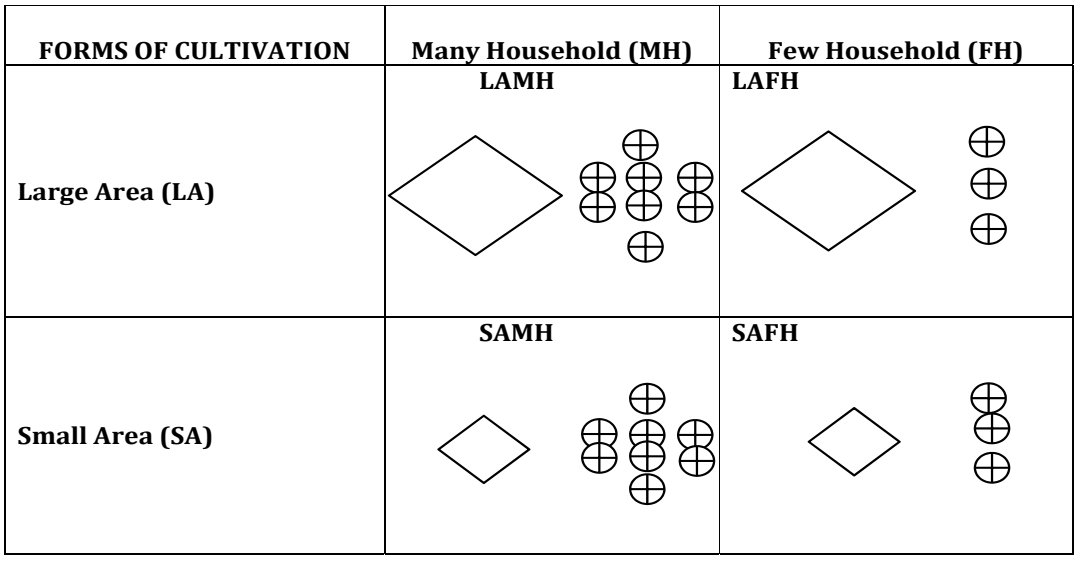

Figure 2 Four cell analysis showing the various forms of cultivation at a certain area and household (Shrestha et al., 2015)

Table 1 Age of the respondents

Age group	Frequency	Percent
25–35	4	6.7
35–45	23	38.3
45–55	18	30.0
55–65	10	16.7
65 and above	5	8.3
Total	60	100.0

Gender of respondents

Out of 60 respondents, the male respondents were 68.3 % and female respondents were 31.7 %; being selected for the survey (Figure 3).

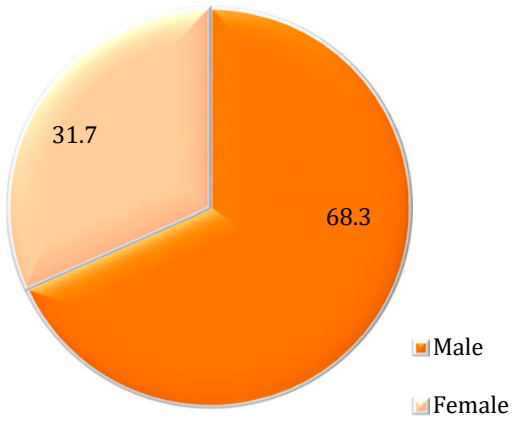

Figure 3 Gender of the respondents (%)

Table 2 Education of the respondents

Age group	Frequency	Percent
Illiterate	3	5
Literate	15	25
School Leaving Certificate	18	30
Intermediate/+2	15	25
Bachelors and above	9	15
Total	60	100.0

Table 3 Economically active population

Age group	Frequency	Percent
Below 15	71	23.13
15 to 59	201	65.47
Above 59	35	11.40
Total	307	100.0

Ethnicity of respondents

From the survey, the majority of the respondents were Brahmins (66.7 %), followed by Chhetris (28.3 %) and a few were Dalits (5 %) (Figure 4). This shows that the area is largely covered by the Brahmin community.

Education of respondents

Table 2 below shows that 30 % of the respondents had an School Leaving Certificate (SLC level of education in Nepal), 25 % were literate which means they can read and write, 25 % had intermediate/+2 level degree, 15 % respondents had bachelors and above degrees while only 5 % were illiterate.

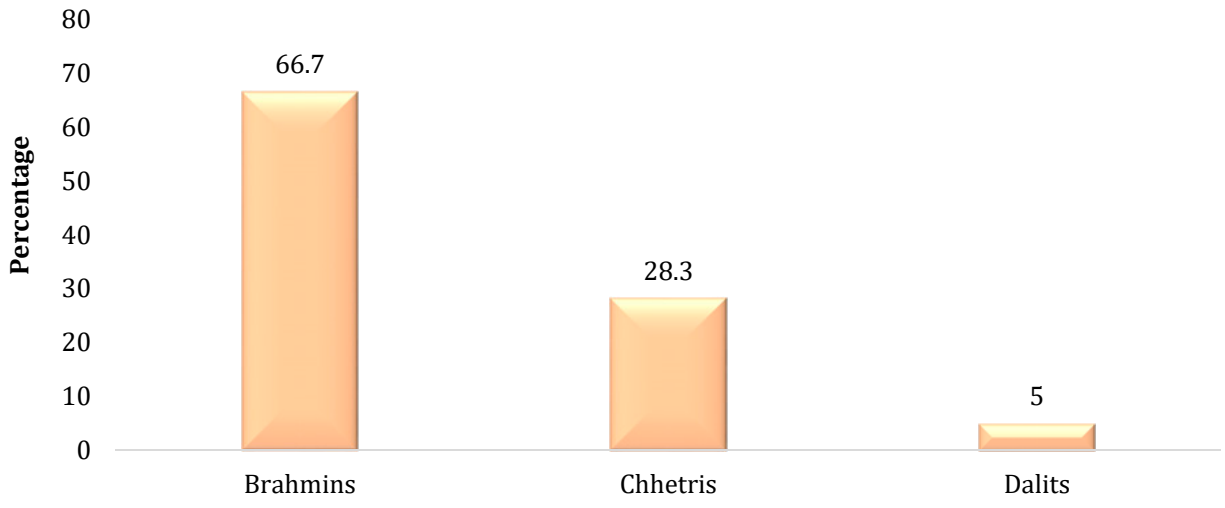

Figure 4 Ethnicity of the respondents

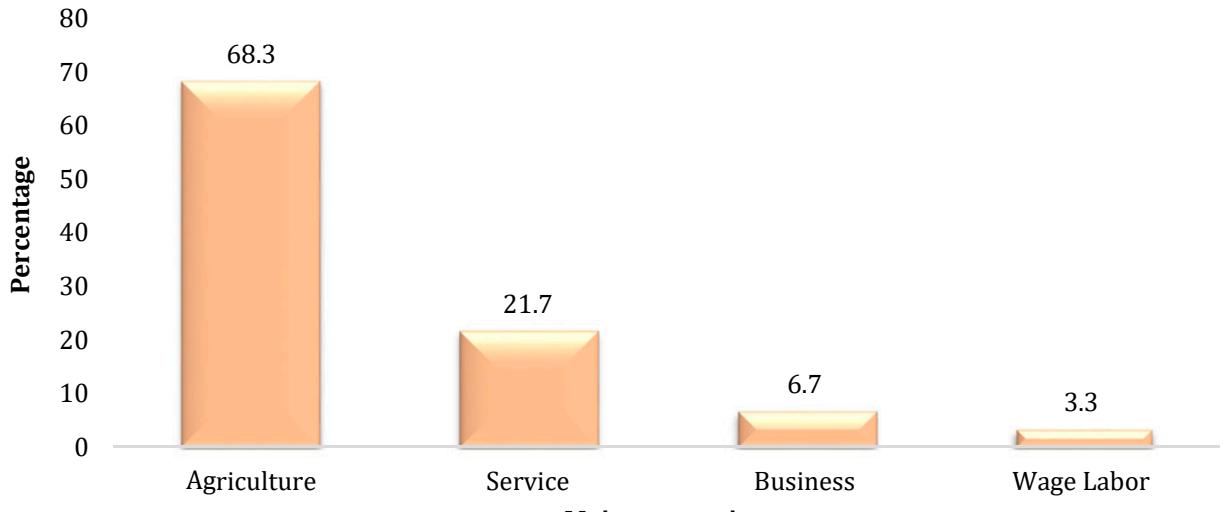

Figure 5 Main occupation of the respondents

Economically active population

There was a majority of economically active population aged 15 to 59 years old accounting 65.47 % followed by 23.13 % respondents below 15 years old and the respondents above 59 were the lowest with 11.40 % (Table 3).

Main occupation of respondents

The majority of the respondents were found to be engaged in agriculture (68.3 %) followed by 21.7 % on service, 6.7 % on business, and 3.3 % as wage labour (Figure 5).

Type of the family of respondents

Figure 6 below shows that the majority of the respondents have a joint type of family (61.7 %) while 38.3 % of the respondents have a nuclear family type. Join type family means a group in which descent through either the female or the male line is emphasized, live together with their spouses and offspring in one homestead and under the authority of one of the members and nuclear family consists of two parents and their children, but not including aunts, uncles, grandparents, etc.

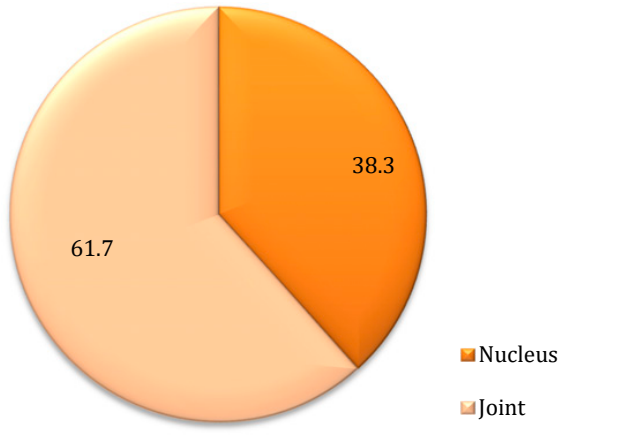

Figure 6 Type of family of the respondents

Food sufficiency from own production

The amount of food produced was sufficient for 6 to 12 months among 45 % of respondents followed by 43.3 % respondents for more than 12 months and only 11.7 % respondents for 3 to 6 months (Table 4).

Table 4 Food sufficiency from own production

Age group	Frequency	Percent
3–6 months	7	11.7
6–12 months	27	45.0
>12 Months	26	43.3
Total	60	100.0

Land distribution

Among the 60 surveyed respondents, the average total land was found to be 0.50 hectares (S.D. = 5.32). Out of total land, the average total cultivated land was only 0.39 hectares (Table 5).

Table 5 Land distribution

Land (in hectares)	Minimum	Maximum	Mean	Std. deviation
Total land	0.10	1.12	0.50	5.32
Total cultivated land	0.10	1.02	0.39	4.56

Productivity of rice in metric tons per hectare

Among the major cereals grown at Puranchaur, there is high production of rice in the study area. The average productivity of rice was 4.035 m/ha which is higher than the national productivity (3.81 m/ha) (MOALD, 2021).

India is the second largest producer of rice in the world after China, as the country produces 175.58 million tonnes of rice annually (FAOSTAT, 2018). Dey et al. (2020) studied variables area, production and productivity of rice during the period from 1950–1951 to 2015–2016. The total area under rice cultivation in India grew from 30 810 thousand hectares in 1950–1951 to 43 499 thousand hectares in 2015–2016. There has been not much increase in the area under rice cultivation in India. The growth trend shows that the area under rice grew at a compound growth rate of 0.52 %. The rice production of the country increased from 20 576 thousand tonnes in 1950–1951 to 104 408 thousand tonnes in 2015–2016. The production under rice grew at a compound growth rate of 2.5 %. Authors recorded also instability in rice area, production and productivity. During the entire period, highest variation was noticed for production in comparison to area and yield. The variation in rice production was 43.8 %, while the variation in area and yield were 11 % and 35.3 %, respectively.

In the Russian Federation, there has been an increase in sown areas, productivity and gross harvests. In 2019, the sown area of rice in Russia was brought to 173.8 thousand hectares, the yield was 5.5 t/ha and the gross harvest of paddy rice was 1098.7 thousand tons (Bagirov et al., 2020).

In Europe, where Japonica rice is cultivated, Italy is the leading rice producer, with around 227300 ha of rice-cultivated areas (Maclean et al., 2002). Additionally, a trend of continuous increase of the rice cultivation surface was observed during the last 30 years; also the area per farm has increased, moving from 20.9 ha of rice per farm in 1983 to 53 ha in 2012, with an increase of 3 to 5 % per year (Hassen et al., 2017).

Agrobiodiversity documentation of rice

List of rice varieties that are being cultivated in the study area:

- Pahele.
- Gurdi.
- Jethobudho.
- Jhinuwa.
- Dhitale Pahele.
- Hybrid.
- Anadi.

Farmers used to grow Sabitri, Gauri, Marsi, and Naule landraces of rice until a few years ago but currently, no farmer in the area is growing these landraces (Rijal et al., 1998) reported that Marsi is under threat and grown under stressful environmental conditions even at Seti river valley. Similarly, research at different ecological zones by Ghimire et al. (2018) found that Marsi is still grown in a limited area among limited households. But in the case of Puranchaur, Marsi is no longer grown in the area.

Flooded rice cultivation can provide important ecosystem services such as the preservation of wetland habitats for a range of aquatic and semi-aquatic wildlife, or of the local traditional landscapes (Hassen et al., 2017).

Four cell analysis

In four-cell analysis varieties cultivated in many households and large areas denote common varieties, those cultivated in few households and large areas and many households and small areas denote endangered varieties, and those cultivated in few households and small areas denote varieties in threat.

Based on the information collected, landraces Pahele, Gurdi, and Jhinuwa are preferred by most households. Landraces Gurdi and Pahele give high production, so they are the most preferred among the farmers in this area. On selling, Pahele fetches high prices than Gurdi. Jhinuwa variety is also preferred by many farmers as it gives satisfactory production and fetches a higher price on selling than those landraces Gurdi

Table 6 Result of four cell analysis done for rice in Puranchaur, Pokhara

Form of cultivation	Many household	Few household
Large Area	1. Pahele 2. Gurdi 3. Jhinuwa	1. Jethobudho 2. Dhitale pahele
Small Area	1. Hybrid	1. Anadi

and Pahele, because it is superior in terms of taste. But studies at Seti river valley found that Jhinuwa was under threat (Rijal et al., 1998). They cultivate these three landraces for home consumption, as well as for selling.

Jethobudho and Dhitale Pahele are cultivated by few farmers, and are less preferred landraces. According to the information given by the farmers, Jethobudho matures late in the season, so they do not prefer this landrace, and in the case of Dhitale Pahele, it does not give satisfactory production in this area. Hybrid rice is also a less preferred landrace because of its inferior taste when compared to other local varieties that are being cultivated in the study area.

Anadi is the least preferred landrace in the area. Only a few farmers cultivate in a very small area for home consumption only. Many farmers believe that it is the rice landrace with medicinal importance and superior taste, but they do not cultivate it on a commercial scale due to the very low production potential of this landrace.

But one of the studies by Rijal et al. (1998) at Seti river valley found that Jethobudho, Gurdi, Anadi, and Pahele are mostly grown by farmers; a different case than Puranchaur. Since the landraces Gurdi and Jethobudho were grown in a large area (Table 6), a similar type of wider adaptability was found at Seti river valley (Rijal et al., 1998).

Sawaneh et al. (2013) studied rice productivity of five countries in Southeast Asia for a period of three decades 1980–2010. From the result of the entire period, productivity increased on the average for Myanmar (2.5 %), Philippines (1.1 %), Thailand (1.1 %) and Vietnam (2.5 %) while Malaysia posted a regression in performance with -0.1%. Countries that sustained total factor productivity growth through technical change include Myanmar, Philippines and Vietnam with technical progress of 2.5 %, 0.6 % and 1.7 %, respectively. Though, Thailand being a major rice exporter in the world has sustained its growth through efficiency improvement (0.7 %) rather than technical progress (0.4 %) while Malaysia has shown technical regression (-0.1 %) over the entire time period. The results show that the average performance (growth) of the countries was sustained mainly by technical change rather than improvement in efficiency change, which is explained according to total factor productivity described by many authors (Caves et al., 1982; Färe, et al., 1994; Fulginiti and Perrin, 1998; Färe, et al., 2001; Pfeiffer, 2003; Coelli et al., 2005).

Research in Philippines analysed the rice production and consumption trends during 15 years (2000–2015) and found variety of factors (vulnerability of rice farming to weather and climate changes and natural disasters; harvest and postharvest techniques; limited rice production area; increasing population; smuggling, etc.) (Exconde, 2016).

Conservation strategy

The landraces that are the most preferred are common and on-farm conservation can be done easily. But the less preferred varieties are endangered and at threat of extinction from the region and therefore specific strategies are needed for their conservation. Local Initiatives for Biodiversity, Research and Development (LI-BIRD) has been actively participating in the conservation of local landraces using various biodiversity management community level practices and fairs, seed banks, and participatory plant breeding (Shrestha, 2007). But under our case, conservation strategies for the rice varieties in different categories are as follows:

1. For endangered landraces:
   - value addition and product diversification,
   - proper market channel,
   - varietal improvement through plant breeding tools,
   - improved market facilities,
   - *in situ* conservation,
   - launching germplasm conservation practices,
   - exchange of seed through farmer seed network system.
2. For the varieties in threat:
   - *ex situ* conservation,
   - collection and preservation of germplasm,
   - household gene bank improvements,
   - establishment of the seed bank and gene bank at the community level.

Conclusion

Nepal can be considered a biodiversity hotspot being highly diverse in biodiversity under human threat. Nepal is home to a wide range of plant and animal species. Being an agricultural country people grow different landraces of crops and different breeds of animal livestock. Though there exists variation in the use of the plant and animal species, use is not the same for all species. In the case of food crops, the varieties that are least preferred are more vulnerable to extinction. Common varieties can be the best

conserved on-farm whereas, for endangered and threatened ones conservation is more difficult. Thus, four-cell analysis can be the best method that helps in the documentation of agrobiodiversity and analysis of possible conservation strategies for different crops. Conservation strategies include on-farm conservation of common and more preferred varieties; value addition, strong market channel, and linkage, diversification, landraces enhancement through selection for endangered cultivars, and *ex situ* conservation through seed banks, gene banks, etc. for threatened species. Limited access to agricultural genetic resources can lead to food insecurity. Therefore, all relevant stakeholders should join hands and work together for biodiversity conservation through on-farm, *ex situ*, *in situ*, and conservation plant breeding.

Conflicts 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

Biological effects of alginite on tomato plants (*Lycopersicon esculentum*) and some insects (*Leptinotarsa decemlineata*, *Galleria mellonella* and *Halyomorpha halys*)

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The purpose of our work was to study the effect of Slovak origin alginite on germination of tomato seeds, growth and development of plants, as well as to evaluate biological activity of alginite against to insect pests. Tomato seeds with low germination capacity were treated by immersion in alginite solutions of concentration 0.0001–0.1 %. Alginite solutions with concentrations of 0.1–5.0 % were used for testing against insects. The application of alginite solutions significantly increases the germination of tomato seeds in laboratory conditions by 9.2–13.0 %. There was a significant increase in the length of seedlings and roots by 1.87-2.98 cm and 3.54–4.51 cm. In a greenhouse presowing treatment of tomato seeds with 0.001 % alginite solution significantly increased germination in comparison with the control (by 33.2 %) and 0.01 and 0.1 % alginite solutions (by 22.2 and 25.0 %). Monitoring of plant height showed that a month after the first seedlings emergence the average plant height was significantly higher in the variant 0.001 % alginite solution and reached to 11.41 cm. The yield of fruits in the variants treated with alginite solutions significantly exceeded the control by 316.53–327.71 g per one tomato bush. It was found that alginite solutions at a concentration of 0.1–1.0 % had low ovicidal (2.73–13.19 %) and insecticidal (5.0–33.3 %) effects against insects belonging to different orders – *Leptinotarsa decemlineata* (Coleoptera), *Galleria mellonella* (Lepidoptera) and *Halyomorpha halys* (Hemiptera). Alginite solutions did not have contact insecticidal activity; the death of insects was caused mainly by the consumption of treated feed. At the same time, a high antifeedant effect was revealed from 45.0 to 85.0 % against adults and larvae of 2–3 instars of *L. decemlineata* and larvae of 2–3 instars of *G. mellonella*. To conclude, the application of alginite contributed to an increase in seed germination and plant productivity, and it did not reveal significant biological activity against insects.

Keywords: alginite, seeds germination, plant growth, yield, insect biological activity

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Introduction

In modern conditions of climatic anomalies, it is extremely important to achieve stable production of high yields of significant agricultural crops. One of the leading places among vegetables belongs to tomatoes *Lycopersicon esculentum* Mill. Achieving high germination of seeds is possible with the integrated use of all agrotechnical methods, including the use of fertilizers, plant growth and development regulators. Alginite is considered one of the most promising materials with several of useful properties.

Alginite is an organic-bituminous rock, rich in macro- and microelements. Alginite arose as a result of the fossilization of accumulated organic (algae) and inorganic material, especially clay, carbonates, quartz and amorphous modification of silicic acid in the aqueous environment (Gancarčikova et al., 2019; Brindza et al., 2021a). In the Slovak Republic an alginite deposit (maar belonging to the Podrecany Basalt Formation, Pontian in age, approx. 6.5 m.y. B.C.), was discovered near Pinciná, close to the town of Lučenec, the center of the Novohrad region (Motyleva et al., 2014). Despite the fact that alginite contains trace elements, the toxicity of heavy metals is below the toxicity limit. The alginite has high water absorption capacity – up to 110 % and high specific surface (313–654 m^2 special features of the organic matter (kerogen type II) and by the presence of smectite (Brindza et al., 2021a). According to many authors, alginite, due to its exceptional chemical composition, improves the soil properties, thereby affecting the growth and development of plants, contributes to increase yields (Rauch and Földényi, 2012; Kádár et al., 2015; Benei and Rauch, 2016; Bednárová, 2019; Kropp et al., 2021).

The purpose of our work was to study the effect of Slovak origin alginite on germination of tomato seeds, growth and development of plants, as well as to evaluate biological activity of alginite against to insect pests.

Material and methodology

The experiments were carried out in laboratory conditions and in a greenhouse during 2020.

Preparation of alginite solutions

Alginite solutions at a concentration of 0.0001, 0.001, 0.01, 0.1, 1.0 and 5.0 % were prepared using distilled water in calibrated flasks. Alginite powder obtained from Slovak Republic was weighed on an analytical balance, and transferred quantitatively into a calibrated flask. Thereafter, the solution was thoroughly mixed and used immediately. The solution was thoroughly mixed before each use. Distilled water was used as a control.

Effect of alginite solution on seed germination

The influence of alginite on capacity of seed germination was studied in laboratory conditions using tomato seed with low germination rate. The seeds of tomatoes (*Lycopersicon esculentum* Mill.) were treated by immersion for 15 minutes in alginite solutions of concentration 0.0001, 0.001, 0.01, and 0.1 %. Then the seeds were germinated for 10 days in Petri dishes between moistened filter paper disks in thermostat at temperature of 25 °C (Rao et al., 2006, ISTA, 2017). Each variant consisted of four replicates, 100 seeds per replicate. Seeds treated with water served as a control. The total germination rate, the length of roots and seedlings were determined.

Determination of the effect of alginite solutions on plant growth

To determine the effect of alginite on growth of tomato plants, the tomato seeds were treated presowing by immersion for 24 hours in alginite solutions of concentration (0.1, 0.01 and 0.001 %). Treated seeds were sowed in greenhouse with drip irrigation in holes according to a randomized scheme. Each variant consisted of three replicates, 36 seeds per replicate. The indices of germination, plant height and yield from one tomatoes bush were analyzed.

Determination of the biological activity of alginite solutions in relation to insect test-objects from the orders Coleoptera, Lepidoptera and Hemiptera

The ovicidal, insecticidal and antifeedant properties of 0.1–5.0 % alginite solutions were determined according to standard methods in relation to insects belonging to different orders: *Leptinotarsa decemlineata* Say (Coleoptera), *Galleria mellonella* L. (Lepidoptera) and *Halyomorpha halys* Stal (Hemiptera) (Elisovetcaia et al., 2020). The contact effect of the extracts was determined by the method of topical application of 0.6 µl of alginite solutions to the dorsal surface of insects. The intestinal effect of the extracts was determined by immersion a nutritive substrate (potato leaves) into alginite solutions, as well as by introducing alginite solutions of the appropriate concentration into an artificial nutrient medium (ANM). Variants with treatment using distilled water served as control. The calculation of ovicidal activity was carried out according to the correction for the number of sterile eggs in the control.

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Statistical analysis

Data analysis for determination of standard deviation, significant differences and correlation coefficients were performed by Statgraphics Plus 5.0 programme.

Results and discussion

The treatment with alginite solutions significantly increased the germination of tomato seeds in laboratory conditions in comparison with the control by 9.2–13.0 % ($LSD_{0.05}$ = 8.82, p ≤0.05) (Table 1). The highest seed germination (81.3 %) was observed in the variant with the alginite concentration of 0.001 %, the lowest (77.5 %) – in the variant with the 0.1 % alginite concentration. There was no significant difference between the variants with alginite solutions treatment of various concentrations, but it was found that with a decrease in concentration from 0.1 to 0.01 and 0.001 %, the germination of tomato seeds insignificantly increases – by 1.0 and 3.8 %, respectively. Seed germination in the variant with the 0.0001 % alginite concentration is 1.3–2.3 % less than in the variants 0.001 and 0.01 %, respectively, but at the same time it is 1.5 % higher than in the 0.1 % alginite variant and significantly exceeds the control (by 10.7 %).

Kovár et al. (2021) found that powder, crushed alginite and alginite extracts (sodium solution, potassium solution) have a positive effect on seeds germination of of *Poa pratensis* L., increasing the average germination in comparison with the control by 33.33–334.20 %, as well as alginite and its products increase the germination rate by 0.04–1.52 seeds/day in *P. pratensis*. These data are also confirmed by our results obtained with tomato seeds. Thus, it is obvious that different preparative forms of alginite promote better germination of seeds of various species of cultivated plants.

In our experiments, the treatment of tomato seeds with alginite solutions led to an increase in the length of seedlings and roots in all variants in comparison with the control. At the same time, the most significant increase in the length of seedlings by 18.7 and 29.8 mm ($LSD_{0.05}$ = 16.1, p ≤0.05) was observed in two variants – at a concentration of alginite solutions of 0.01 and 0.1 % (Table 1). A significant increase in root length by 22.2, 35.4 and 45.1 mm was observed in three variants of treatment with alginite solutions – with a concentration of 0.001, 0.01 and 0.1 %, respectively ($LSD_{0.05}$ = 15.5, p ≤0.05) (Table 1).

Thus, it was found that 0.1, 0.01, and 0.001 % alginite solutions led to both a significant increase in the germination of tomato seeds and an increase in the length of seedlings and roots. Despite the fact that alginite at a concentration of 0.0001 % contributes to a significant increase in seed germination, the difference in the length of roots and seedlings is insignificant in comparison with the control.

Therefore, for the presowing treatment of tomato seeds, it were selected three of the four tested concentrations – 0.1, 0.01 and 0.001 % alginite solutions. The first seedlings emergence was noted on the 11th day after sowing, the last – on the 19th day after sowing.

The total germination of seeds in all variants of greenhouse experiments exceeded the control by 8.3–33.2 %. However, a significant difference ($LSD_{0.05}$ = 20.5, p ≤0.05) was noted only in the variant where the seeds were treated with 0.001 % alginite solution (Figure 1).

According to many authors (Ognjanova-Rumenova and Vaas, 1998; Vass, 1998; Kulich et al., 2001; Pichler et al., 2001; Brinza et al., 2021a), alginite contains a sufficient amount of humic acids, which, as heterogeneous organic compounds, are capable of improving soil fertility. Due to its water-saving properties, alginite is one of the best water-absorbing materials that can regulate the distribution of water towards the plant roots. Probably, these properties also play an important role in seed germination – supplying tomato seeds with nutrients, and retain and distribute moisture, thus creating optimal conditions for swelling and germination of seeds.

Table 1 Germination characteristics of tomato seeds treated with different concentrations of alginite

Variants	Germination (%)	Seedlings length (mm)	Roots length (mm)
Control	68.3	15.4	16.9
Alginite 0.0001 %	79.0	22.9	23.7
Alginite 0.001 %	81.3	30.8	39.1
Alginite 0.01 %	80.3	34.1	52.3
Alginite 0.1 %	77.5	45.2	61.9
$LSD_{0.05}$	8.82	16.1	15.5

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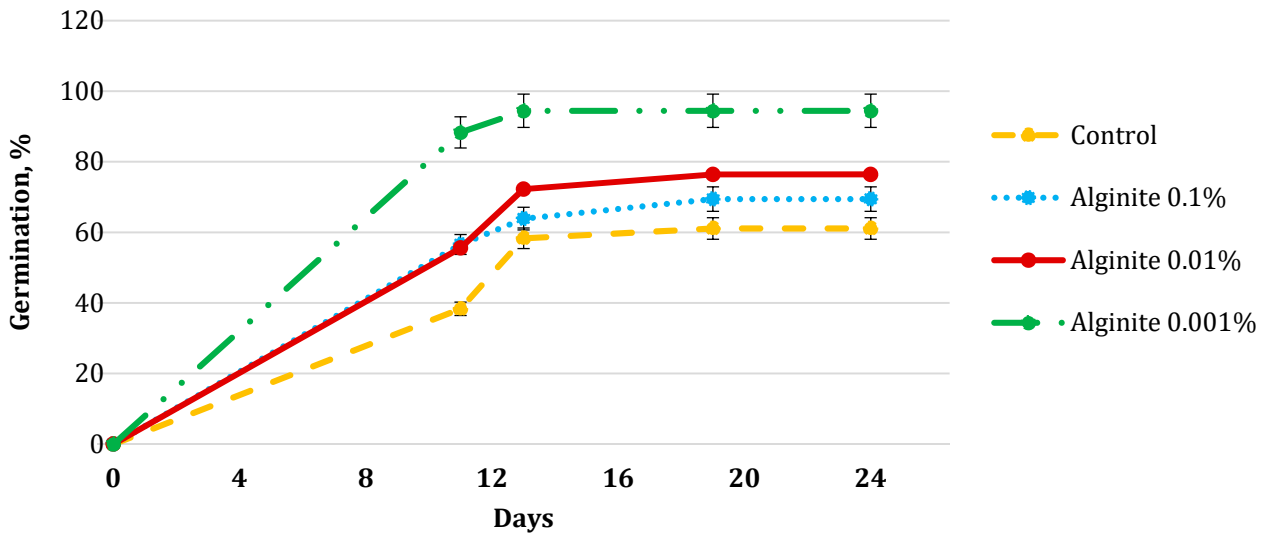

Figure 1 Germination rate of tomato seeds treated with alginite solutions in greenhouse conditions, 2020

Monitoring of plant height showed that a month after the first seedlings emergence the average of plant height was significantly higher in the variant 0.001 % alginite solution and reached to 11.41 cm ($LSD_{0.05}$ = 2.63, p ≤0.05). The plant heights in other tested variants were at the control level (8.21 cm) (Figure 2).

In the experiment, $ALGEX_r$ 6 was applied by Horčinová Sedláčková et al. (2021) in the form of a watering in two variants with the same concentration of 3 % solution in 2 deciliters of water, but various application in terms of days in the pre-harvest stage of the above-ground plant biomass of 30 individual plants from each species. There are two diametrically opposite trends of $ALGEX_r$ 6 application that are manifesting themselves in *Melissa officinalis* and *Malva verticillata* by reducing the root and above-ground part biomass compared to the control variant. The percentage proportionality of root/above-ground part biomass in *M. officinalis* decreased from 62.48/30.31 % (control), to 45.57/18.85 % (variant 1) and to 36.07/17.27 % (variant 2), as well as in *M. verticillata* the root/above-ground part biomass decreased from 16.03/13.93 % (control) to 14.97/9.42 % (variant 1) and to 11.61/10.14 % (variant 2). In the species *Ocimum* × *citriodorum* Vis. the opposite trend manifested (Horčinová Sedláčková et al., 2021).

In our experiments, it was found that in the variant with the treatment with 0.1 % alginite solution, the tomato plants looked depressed and lagged behind in growth from other variants by 2.51–5.71 cm. Also in this variant, within one and a half months after the appearance of the first seedlings, an average 30 % of plants was died. Therefore, the variant of presowing

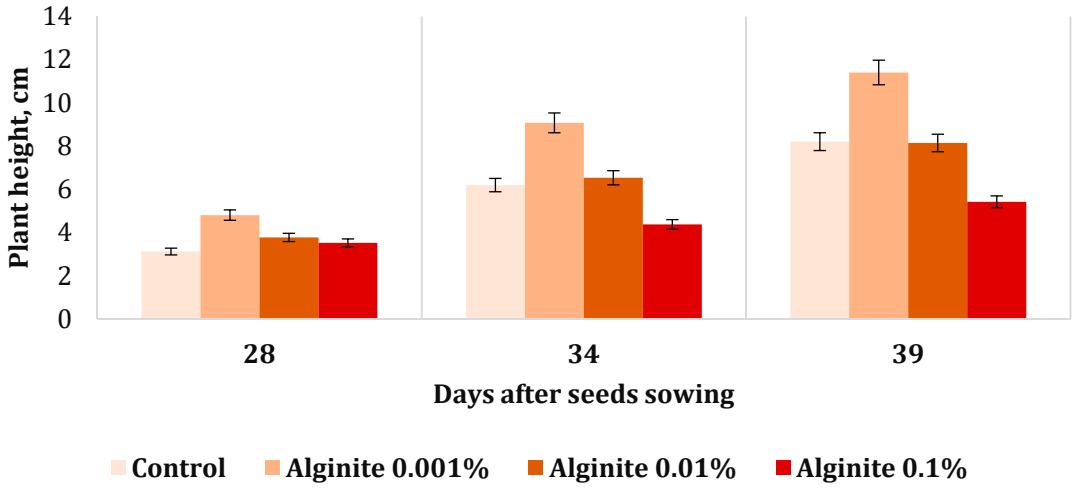

Figure 2 Height of tomato plants treated with alginite solutions in a greenhouse, 2020

seeds treatment with 0.1 % alginite solution was excluded from the experiment.

Cukor et al. (2017) studied growth parameters (height increment, mortality and foliar nutrient content) of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco), Scots pine (*Pinus sylvestris* L.) and a mixture of English oak (*Quercus robur* L.), red oak (*Quercus rubra* L.) and Norway maple (*Acer platanoides* L.) seedlings on former agricultural land in central Bohemia, Czech Republic. The results showed that alginite application had greater positive effect on height growth of seedlings than mortality, especially variant C. In most of the cases height increments were significantly positively affected ($p < 0.05$) by both variants of alginite application only in the third year after planting.

Bio-Algeen system (materials based on the marine algae, both of fossil and recent origin) was tested by Kupka et al. (2015) at planting stock of Norway spruce production in a forest nursery. In the plantation experiment, significantly lower mortality was documented in the first year since planting, as well as significantly faster growth for broad-leaved species. In the nursery experiment, considerably more favourable development of the root system was detected as well as better parameters of the above-ground part of the planting stock. As the most effective, the combination of root dipping to Bio-Algeen water solution with granulate application on bed surface and also spraying of aboveground part of seedlings after transplanting were documented. Applications of studied material thus represent important contribution for quality stock production and plantation success on the forested site.

Biochar application to soils is being considered as means to improve fertility while concurrently improving soil functions. Based on results, authors found that biochar increased the microbial biomass values even before the incubation. In single and combined biochar–alginite treatments, more bacterial biomass was adsorbed due to the higher adhesion capability and the increased surface area. The volume of the microbial adsorption is different from species to species and even strains (Kocsis et al., 2020).

The spring and summer seasons of 2020 were characterized by extreme temperatures. Thus, already in the first decade of June, the temperature reached 27–35 °C, and on some days 40-42 °C. In July, the average daily temperatures were even higher, and in August, the mark reached an extreme value 45 °C. In the greenhouse, even with the use of shading green textile and ventilation, the temperature exceeded 35 °C and often reached to 45 °C and more. These conditions significantly affected the ovary of plants. It is known that the pollination of tomato culture occurs mainly during the day, but the ripening of pollen – at night. In this case, the temperature at night should be in the range from +13 to +25 °C. During the day, the temperature indicators should not exceed +35 °C. At temperatures above, tomato pollen becomes sterile, the bush sheds flowers, and ovaries are not formed. Since the temperature conditions in 2020 during the summer season, which saw the flowering of tomatoes, significantly exceeded these indicators, the yield in 2020 was record low. In the control variant, the formation of fruits was sporadic. Plants treated with alginite formed significantly more fruits, which ultimately had a positive effect on the yield. The yield in the variants treated with alginite solutions significantly exceeded the control by 316.53–327.71 g per one tomato bush.

Earlier, Brindza et al. (2021b), investigating the effect of various of alginite preparations (ultrasound-treated alginite), found that they resulted in a reduction in plant weight as well as a reduction in the average fruit weight compared to the control variant. However, our research has shown a significant increase in yield. Probably, the discrepancy between the results can be associated with the use of completely different alginite preparations: we used aqueous solutions, while Brindza et al. (2021b) – solutions from alginite treated with ultrasound.

Thus, the results of our studies showed that alginite solutions at a concentration of 0.0001–0.1 % increase the germination of tomato seeds, promote an increase in seedlings and roots, and significantly increase the growth rate and productivity of plants. However, we did not add any form of alginite to the soil, but we believe that such research is worth doing in the future. It is also worth studying the effect of foliar treatments on plant growth and development. At the same time, it was reported (Gömöryová et al., 2009; Rauch and Földényi, 2012; Oravcová et al., 2018) that the introduction of alginite into the soil improves the condition of both the soil and plants. Therefore, the future research of alginite effects on the growth and development of plants by foliar treatments will be relevant. However, the treatment of plants during the growing season can affect the arthropod complex in the agrocenosis of tomatoes. This is due to the fact that, both in open field conditions and greenhouses, alginite solutions not only directly fall on insects in the agrocenosis of crops when processing plants, but can also dry out and remain on the surface of the leaf apparatus, getting inside phytophagous insects with food. Therefore,

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it is advisable to study the effect of alginite solutions on some common insects and phytophagous. Among pests at the moment in Europe, including the Republic of Moldova and the Slovak Republic, on Solanaceae crops such species as *Leptinotarsa decemlineata* Say (Coleoptera) and *Helicoverpa armigera* Hbn. (Lepidoptera: Noctuidae) are especially relevant (Elisovetcaia, 2010; Elisovetskaya and Nastas, 2012). In addition to these species, which have been known for a long time, a special role is played by the recently acclimatized, but already established themselves as extremely harmful species – the bugs *Nezara viridula* L. and *Halyomorpha halys* Stal. (Hemiptera) (Ivanova et al., 2020). Therefore, it was decided to select insect species belonging to these orders – Coleoptera, Lepidoptera, and Hemiptera – as test objects. Instead of the *Helicoverpa armigera* Hbn., the *Galleria mellonella* was selected. *G. mellonella* has a number of advantages over the cotton scoop: cultivation under laboratory conditions is more economical and less laborious, the degree of cannibalism in caterpillars of *G. mellonella* is significantly less pronounced than in caterpillars of cotton scoop. The marble bug was also selected based on the convenience of laboratory breeding.

It was revealed that alginite solutions at a concentration of 0.1–1.0 % had low ovicidal properties against *Halyomorpha halys* – 2.73 and 13.19 % in terms of the percentage of sterile eggs in the control (Table 2). The ovicidal effect is most likely due not to the toxicity of alginite itself, but to its physicochemical properties. Since alginite contains a clay fraction and is able to retain water, after processing the egg-laying, it covers the ovipositions like a slime film. After drying, such a film probably prevents both free access of air and the hatching of larvae from eggs.

The insecticidal activity of alginite solutions is low and is due to the intestinal action of the drug. Therefore, when treatment the feed, the death of imago and larvae of the Colorado potato beetle (*L. decemlineata*) was 10.0–20.0 and 20.0–30.0 %, respectively, and when feeding larvae of 2–3 ages *G. mellonella* with ANM with the addition of alginite solutions, the death of insects did not exceed 5.0–10.0 % (Table2). Probably, the insecticidal effect is due to the ingress of clay particles of alginite into the intestines of insects with feed, which subsequently leads to disruption of digestion processes and, as a consequence, to the death of insects. It was found that alginite solutions did not have contact activity with respect to insects – no death was observed with topical application of the solutions to the dorsal surface of insects (Table 2).

Despite the low insecticidal properties, alginite solutions against all species of insects showed a rather pronounced antifeedant effect (Table 2). Especially pronounced antifeedant properties were shown by 1.0–5.0 % solutions against imagoes *L. decemlineata* – 80.0–85.0 %. This is due to the fact that imagoes, unlike larvae, are able to tolerate longer hunger strikes. Therefore, the consumption of processed feed for imagoes decreased significantly during the experiment without significant damage to their survival. It was noted that, in comparison with the control, larvae of *G. mellonella* also significantly reduced feed consumption – by 51.24–68.92 %. We have previously

Table 2 Biological effects of alginite solutions on insects

Variants	*Haliomorpha halis*				*Leptinotarsa decemlineata*				*Galleria mellonella*	
	ovicidal activity (OA) (%)		insecticidal activity** (%)	antifeedant activity (%)	insecticidal activity*** (%)		antifeedant activity (%)		insecticidal activity*** (%)	antifeedant activity (%)
	proportion of non-hatching eggs	Ovicidal activity given sterile eggs in control	larvae 2 instars	larvae 2 instars	imago	larvae 2–3 instars	imago	larvae 2–3 instars	larvae 2–3 instars	larvae 2–3 instars
Control	8.3*		0	0	0	0	0	0	0	0
Alginite 0.1%	11.1	2.7	0	20.0	10.0	20.0	75.0	45.0	5.0	51.24
Alginite 1.0%	21.5	13.2	0	33.3	20.0	25.0	80.0	60.0	10.0	57.45
Alginite 5.0%	–	–	–	–	20.0	30.0	85.0	60.0	10.0	68.92

Notes: * proportion of sterile eggs, ** contact insecticidal activity, *** intestinal insecticidal activity

found that extracts from plants *Juniperus sabina* L., Cupressaceae and *Pinus sylvestris* L., Pinaceae are able to reduce the feed intake of *G. mellonella* by 47.4–84.5 % (Elisovetcaia and Brindza, 2018; Elisovetcaia et al., 2019). The data obtained by us on the effect of 0.1–5.0 % alginite solutions on insect nutrition revealed a sufficiently high level of antifeedant activity, comparable to the effect of plant extracts (Elisovetcaia et al., 2020).

Alginite as a bituminous rock contains a high content of silicon (Vass et al., 1997). The role of silicon (Si) on plant health has been tested under open field conditions, hydroponic cultures, and under greenhouse/glasshouse environment (Luyckx et al., 2017). Still, presently there are a limited number of studies, which demonstrate there are advantages of Si application for greenhouse crops.

Meeting the growing demand for vegetables under situations of biotic and abiotic stresses is a big challenge. Si application is considered as an eco-friendly approach for crop production; therefore, Si application is commonly recommended under package and practices for cereals. Likewise, in vegetables, Si application has been documented to reduce the attack of diseases (Bakhat et al., 2018). For example, potassium silicate treatment of pea seedlings was observed to increase chitinase and β-1,3-glucanase activity against the fungal pathogen *Mycosphaerella pinodes* and it is the causes of blight disease in pea (Dann et al., 2002). Similarly, Si application has considerably reduced the root rot and powdery mildew disease in cucumber and the rust disease of cowpea (Heath and Stumpf, 1986; Chérif et al., 1994; Liang et al., 2005). Moreover, nano-silicon application can prevent postharvest diseases of vegetables (James and Zikankuba, 2017; Barman et al., 2018). In this direction, studies have also demonstrated that higher Si content in plant tissues reduced the incidence of several insect pests (Reynolds et al., 2009). Correa et al. (2005) reported that soil or as a foliar spray of Si as calcium silicate to cucumber plants increases the mortality of the nymphs of *Bemisia tabaci*.

In our experiment, we did not evaluate all the effects of alginite on tomatoes presented by Brindza et al. (2021b). However, it can be assumed that in addition to better seed germination, plant growth and other effects that we have determined when applying alginite to tomato plants, other effects can be determined. Therefore, it is useful to continue the experiments with the application of alginite to tomatoes.

Conclusions

It was found that 0.0001–0.1 % alginite solutions contribute to a significant increase in the germination of tomato seeds, as well as an increase in length of seedlings and roots in comparison with the control by 1.4–3.7 times. In a greenhouse, the treatment with 0.01–0.001 % alginite solutions not only increased seedlings emergence and plant height, but also contributed to a significant increase in yield compared to control. Possessing low insecticidal properties against insects, alginite solutions, at the same time lead to a decrease in damage to treated plants due to a high antifeedant effect, thereby preserving quality of above-ground part of plant and yield.

Conflict of interests

The authors declare that they have no conflict interests.

Ethical statement

This article complies with all ethical standards.

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Research Article

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Toxicity of aqueous solutions of cosmetics in phytotest with *Lepidium sativum* L.

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Garden cress (*Lepidium sativum* L.) is a test-plant for studying the toxicity of substrates, which is the basis of phytotesting. Dangerous pollutants are surfactants contained in household chemicals, including cosmetics. The purpose of the work is to investigate the toxicity of cosmetics the micellar water on a phytotest with garden cress and to analyze it for possible effects on human health and the environment. Investigated available in the retail network of Ukraine means for removing makeup and cleansing the skin – micellar water. Seed germination energy (3rd day), seed germination, and biometric-morphometric parameters (length of roots and aboveground part of seedlings) (5th day) were determined. The results were processed statistically. It was found that the germination rates of garden cress seeds and biometric indicators of seedlings significantly decrease (by 14–100 %) with the increasing concentration of the studied micellar water. The phytotoxic effect ranged from 49.6 % to 100 %. It is established that the value of the total toxicity index of solutions is from 0.55 (concentration 6.25 %) to 0 (concentration 100 %), indicating an increase in the toxicity of the solution with increasing concentration. Determined that garden cress is a sensitive plant to the studied cosmetic. The obtained data confirm the high efficiency of this test plant for use in biotesting. The phytotest with *L. sativum* established the lethal effect of this cosmetic product at a concentration of 100 %. Phytotoxicity decreases when the solution is diluted. Given the results of phytotesting and the composition of the cosmetic product, it can be assumed that at a concentration of 100 % it can pose a potential danger to human health. Given the increase in the market of perfumes and cosmetics, the emergence of counterfeit products, we can expect an increase in the impact of cosmetics on the quality of the environment.

Keywords: *Lepidium sativum*, micellar water, phytotesting

Introduction

Garden cress (*Lepidium sativum* L.) is a recognized sensitive test-plant in bio testing the toxicity of various substrates. Current studies using garden cress reactions are concerned with assessing the toxicity of antibiotics (Tongur and Yildirim, 2015), analgesic drugs (Tongur et al., 2017a), beta-blocker drugs (Tongur et al., 2017b), new derivatives of heterocyclic compounds (Tsekhmister et al., 2012; Tkachuk et al., 2015), urban solid waste compost (Astaraei, 2009), nanoparticles (Joško et al., 2017; Mielcarz-Skalska and Smolińska, 2018), oil mill wastewater (Dehmani et al., 2020), some shampoos and dishwashing detergents

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(Ovsyannikova et al., 2015), heavy metals (Szaniszló and Demény, 2018), microplastics (Bosker et al., 2019; Pflugmacher, et al., 2020, 2021), in particular polyethylene terephthalate microplastics and acid rain (Pignattelli et al., 2021a, b). Most publications examine the level of seed germination, weight, and size of seedlings as test indicators.

Dangerous pollutants are surfactants (Rabosh and Kofanova, 2019). These petrochemical compounds are contained in household chemicals and can reduce the surface tension of water (Frolova et al., 2019). Once in the human body, detergents disrupt the physiological functions of the body due to exposure to enzyme activity (Grabovska et al., 2011). There is evidence that surfactants can affect the human body for a long time due to the properties of gradual accumulation in the brain, liver, heart, subcutaneous tissue (Yuan et al., 2014). That is, they affect the human body comprehensively, not just the skin. The problem is also complicated by the inability of the vast majority of treatment plants in our country to qualitatively remove surfactants and, as a result, their gradual accumulation in the environment (Frolova et al., 2019). Synthetic surfactants, among other organic compounds, are part of cosmetics, in particular micellar water. Therefore, this study aimed to investigate the toxicity of micellar water on a phytotest with garden cress and to analyze it for possible effects on human health and the environment.

Material and methodology

Test-plant

Garden cress (*L. sativum*) of the cultivar Aphrodite of the trademark GL SEEDS (consignment 1148, expiration date 10.2023) was used as a test-plant. The seeds of the test plant before the experiment did not succumb to negative effects and were stored under the same conditions. The seeds were washed with distilled water, then sterilized in 70 % alcohol for 60 s, then carefully washed with sterile distilled water, and then they were used. During the experiment, the energy of seed germination (3rd day), seed germination, and biometric-morphometric parameters (length of roots and aboveground part of seedlings) (5th day) were determined.

Investigated cosmetic product

We investigated a means available in the trade network of Ukraine for removing makeup and cleansing the skin – micellar water, which contained (according to the manufacturer): aqua, PEG-40 hydrogenated castor oil, glycerin, *Prunus* (*Amygdalus*) dulcis oil, panthenol, sorbitol, decyl glucoside, glyceryl glucoside, poloxamer 124, propylene glycol, disodium cocoyl glutamate, sodium chloride, trisodium EDTA, polyquaternium-10, 1,2-hexanediol, citric acid, sodium acetate, phenoxyethanol. We deliberately do not name the cosmetics used to prevent accusations of advertising or anti-advertising of certain brands.

Toxicity testing

Seeds of test plants (*L. sativum*) of 10 pieces were put in Petri dishes on filter paper moistened with distilled water (control) or a suitable aqueous solution of micellar water (experiment). The investigated concentrations of the micellar water were 6.25 %, 12.5 %, 25.0 %, 50.0 % and 100 %. The experiment was repeated three times. The incubation temperature of the Petri dishes was 23.0 ±2.0 °C. Seed germination energy (3rd day), seed germination, and biometric-morphometric parameters (length of roots and aboveground part of seedlings) (5th day) were determined.

The seed germination index (SGI) and the root length index (RLI) that exemplified phytotoxicity index were described in Eq. (1) and (2):

$$SGI = \frac{N_T(i) - N_C}{N_C} \quad (1)$$

$$RLI = \frac{LR_T(i) - LR_C}{LR_C} \quad (2)$$

where:

$N_T(i)$ and N_C represent the number of germinated seeds in test (i) and control, and $LR_T(i)$ and LR_C refer to the mean root length in test (i) and control respectively. Based on the published empirical value of risk assessment (Bagur-González et al., 2011; Mtisi and Gwenzi, 2019; Cai and Ostroumov, 2021), phytotoxicity can be sorted into four classes such as:

- slight (-0.25≤ SGI or RLI <0),
- moderate (-0.5≤ SGI or RLI <-0.25),
- high (-0.75≤ SGI or RLI <-0.5),
- extreme toxicity (-1≤ SGI or RLI <-0.75).

The phytotoxic effect (PhTE) and the toxicity index (TI) of the solutions were calculated (Eq. (3) and (4) (Bagdasaryan, 2005):

$$PhTE = \left(\frac{L_C - L_T(i)}{L_C} \right) \times 100 \quad (3)$$

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$$TI = \left(\frac{N_T(i)}{N_C} + \frac{LA_T(i)}{LA_C} + \frac{LR_T(i)}{LR_C} \right) \div 3 \quad (4)$$

where:
$N_T(i)$ and NC represent the number of germinated seeds in the test (i) and control, $LA_T(i)$ and LA_C refer to the mean length of the aboveground part in the test (i) and control, and $LR_T(i)$ and LR_C refer to the mean root length in the test (i) and control, respectively

Statistical analysis

Basic statistical analyses were performed using PAST 2.17 (Norway, 2001); the results are expressed as mean values of three replications ± standard deviation (SD) and differences between means compared through the Tukey-Kramer test ($p < 0.05$).

Results and discussions

Test-indicators of *L. sativum*

Various plants, including *Allium cepa* L. (Srivastava and Singh, 2020; Souza et al., 2020; Macar, 2021), *Lactuca sativa* L. (Lyu et al., 2018; Mtisi and Gwenzi, 2019; Gao et al., 2021), *L. sativum* (Szaniszló and Demény, 2018; Bosker et al., 2019; Pflugmacher et al., 2020, 2021; Pignattelli et al., 2021a, b), are used in phytotoxicity studies of malathion, tetraconazole, nanoparticles, heavy metals, phenol, effluents, receiving water, coal ash, polyethylene particles, microplastics. In the studies of testing solutions toxicity are used following parameters of these plants: germination and radicle elongation (Mtisi and Gwenzi, 2019), germination, the root, the shoot, and the overall seedlings length, the roots and shoots' fresh and dry weight, the pigment Chl a and Chl b content, catalase activity (Pflugmacher et al., 2021).

In our studies, we used germination energy, seed germination, aboveground part length, and seedling root length as the test indicators, due to their availability, ease of measurement, and sensitivity to toxic effects (Pflugmacher et al., 2020, 2021). The results of the study of test parameters of *L. sativum* under the influence of micellar water are shown in Table 1.

It was found that the germination energy and germination of garden cress seeds when watered with the studied solutions of micellar water significantly decreased compared to the control: by 12 % (at a concentration of 6.25 %), by 25 % (at a concentration of 12.5 %), by 18 % (at a concentration of 25.0 %), by 36 % (at a concentration of 50.0 %). Decreased germination of garden cress was also observed under the influence of a number of chemical compounds, in particular, heavy metals (Nouri and Haddioui, 2021), saline solutions (Uçarlı, 2020), some essential oils (Abd-ElGawad et al., 2021). In the latter case, researchers are even discussing potential herbicidal activity essential oils.

At the same time, there are reports of insensitivity of seeds germination to the action of toxicants, such as coal ash (Mtisi and Gwenzi, 2019) and nanomaterials (Bouguerra et al., 2016; Gavina et al., 2016; Soares et al., 2016).

In our study, when watering *L. sativum* seeds with a solution with the maximum test concentration (100 %), it did not germinate. This fact is probably related to both the osmotic stress for the plant (Uçarlı, 2020) and/or the chemical composition of the cosmetic product under study.

The biometric and morphometric parameters of garden cress also decreased with the increasing concentration of the studied cosmetic product. The

Table 1 Seed germination rates and biometric indicators of *Lepidium sativum* L. seedlings under the influence of aqueous solutions of micellar water

The concentration of the test compound (%)	Seed germination energy (%)	Seed germination (%)	Length of the aboveground part (mm)	Length of roots (mm)
0 (control)	93.3 ±3.3	93.3 ±3.3	23.8 ±0.5	10.0 ±0.3
6.25	80.0 ±0.0*	80.0 ±0.0*	12.0 ±0.3*	8.2 ±0.3*
12.5	70.0 ±0.0*	70.0 ±0.0*	***	6.1 ±0.3*
25.0	70.0 ±0.0*	76.7 ±3.3*	***	3.8 ±0.2*
50.0	60.0 ±0.0*	60.0 ±0.0*	***	3.0 ±0.3*
100	**	**	**	**

Note: * differences from control are significant at $p \leq 0.05$; ** the indicator was not measured because the seeds did not germinate; *** the indicator was not measured because the seedlings did not have the appropriate parts

length of the aboveground part of garden cress seedlings was determined only for control and variant with a concentration of micellar water of 6.25 %. In this case, the length of the aboveground part of the seedlings was 2 times significantly less than in the control. At concentrations greater than 6.25 %, the aboveground part of *L. sativum* seedlings was absent. A statistically significant decrease compared to the control was observed in the length of the roots: by 18 % (at a concentration of 6.25 %), by 39 % (at a concentration of 12.5 %), by 62 % (at a concentration of 25.0 %), by 70 % (at a concentration of 50 %). The decrease in the length of the roots/aboveground part of the seedlings of test plants with increasing concentration of solutions studied for their toxicity, noted by other researchers (Seneviratne et al., 2017; Nedjimi, 2020; Pflugmacher et al., 2020, 2021; Macar, 2021).

Phytotoxicity indices

In phytotests, various indices are calculated to evaluate the results. For example, calculate the percentages of relative seed germination, relative root growth, germination index (Pampuro et al., 2017), germination percentage, germination index, germination rate index, vigor index, coefficient of the velocity of germination and mean germination time (Nouri et al., 2020). Mtisi and Gwenzi (2019) note that positive seed germination index and root length index values indicate stimulation of germination or growth, while negative values denote phytotoxicity.

Based on the obtained data, we calculated the phytotoxicity indices of aqueous solutions of the studied micellar water, which are shown in Table 2.

According to the calculated phytotoxic indices, it was found that the toxicity of the studied solutions to garden cress increases with the increasing concentration of micellar water in them.

Micellar water is considered as the main biologically active compound of cosmetics (Korytniuk et. Al., 2019). At the same time, the composition of the studied cosmetic micellar water is alarming due to the content of surfactants, which toxic effect on living organisms is known. The molecular mechanisms of the biological effects of detergents include interaction with biological membranes (Cai and Ostroumov, 2021). Particularly dangerous to human health are PEG, propylene glycol (The "Dirty Dozen" ingredients..., 2010), trisodium EDTA (Safety assessment of EDTA..., 2019). PEGs (polyethylene glycols) are petroleum-based compounds that are widely used in cream bases for cosmetics as thickeners, solvents, softeners, and moisture carriers (The "Dirty Dozen" ingredients..., 2010). Some reports depending on manufacturing processes, PEGs may be contaminated with measurable amounts of 1,4-dioxane (a possible human carcinogen). (The "Dirty Dozen" ingredients..., 2010). It is indicated: "While carcinogenic contaminants are the primary concern, PEGs themselves show some evidence of genotoxicity and if used on broken skin can cause irritation and systemic toxicity. The industry panel that reviews the safety of cosmetics ingredients concluded that some PEGs are not safe for use on damaged skin (although the assessment generally approved the use of these chemicals in cosmetics). Also, PEGs function as "penetration enhancers", increasing the permeability of the skin to allow greater absorption of the

Table 2 Indices of phytotoxicity of aqueous solutions of micellar water

The concentration of the test compound (%)	SGI	RLI	PhTE	TI	Interpretation of the results of phytotest	Comments
0 (control)	0.00	0.00	0	1.00	no toxicity	no inhibition of growth
6.25	-0.14	-0.18	18	0.73	slight toxic effect	a slight inhibition of growth
12.5	-0.25	-0.39	39	0.45	a pronounced toxicity	inhibition of growth almost 50 %, no aboveground part growth observed
25.0	-0.18	-0.62	62	0.40	strong toxicity	inhibition of growth more than 50 %, no aboveground part growth observed
50.0	-0.36	-0.70	70	0.37	strong toxicity	inhibition of growth more than 50 %, no aboveground part growth observed
100	-1.00	-1.00	100	0.00	lethal effect, extreme toxicity	no seed germination, no aboveground part, and root growth observed

Note: SGI – the seed germination index; RLI – the root length index; PhTE – the phytotoxic effect; TI – the toxicity index

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product – including potentially harmful ingredients". (The "Dirty Dozen" ingredients..., 2010). Propylene glycol functions as a penetration enhancer can allow harmful ingredients to be absorbed more readily through the skin (The "Dirty Dozen" ingredients..., 2010). Lanigan and Yamarik (2002) based on the analysis of publications indicate that EDTA and its salts have been evaluated for the potential to cause chromosomal aberrations, semilethals, crossovers, forward mutations, replicative DNA synthesis, DNA strand breaks, dominant lethal, inhibition of metabolic cooperation and contact feeding, and sister-chromatid exchanges with mostly negative results. However, there are positive results, references to publications cited by these authors.

Regarding the impact of the components of the studied tool on the environment, the available scientific and methodological base contains only a few reports. Thus, it is reported that EDTA may contribute to aquatic toxicity at low concentrations and its release into natural waters should be minimized wherever possible (Sillanpää, 1997; Oviedo and Rodrígues, 2003). In the publications, the following components of cosmetics are considered as wastewater contaminants: phthalates, triclosan, bisphenol A (Water pollution..., 2007), microplastics, UV filters, some preservatives (parabens, triclosan) (Juliano and Magrini, 2017). But these chemical compounds are not specified in the composition. In general, aqueous solutions of investigated cosmetics at a concentration of 6.25 % had a weak toxic effect. However, in Ukraine and the world there is a steady increase in the market share of perfumes and cosmetics (Dobrovolskyi and Lohvynenko, 2018), the global micellar water market is projected to grow from 112.3 million U.S. dollars in 2017 to 184 million dollars in 2023 (Ridder, 2020). Currently, the Ukrainian market of cosmetic products is considered the second in the world after China in terms of sales of counterfeit products that do not meet sanitary and hygienic safety (Baitsar and Kordiiaka, 2015).

Conclusions

Thus, it was found that undiluted micellar water is extremely toxic to the test plant *L. sativum*. The phytotest established the lethal effect on phytotest of this cosmetic product at a concentration of 100 %. Phytotoxicity decreases when the solution is diluted. Given the results of phytotesting and the composition of the cosmetic product, it can be assumed that at a concentration of 100 % it can pose a potential danger to human health. Given the increase in the market of perfumes and cosmetics, the emergence of counterfeit products, we can expect an increase the impact of cosmetics on the quality of environment.

Conflicts 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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