Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

OPEN ACCESS

Phenological phases of development of *Cornus florida* L. under conditions of introduction in Ukraine

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Article Details:
Received: 2023-08-08
Accepted: 2023-11-11
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0015

Phenological studies do not lose their relevance and are carried out in very different aspects. They are important for monitoring the response of plants to climate changes, the selection of new cultivars of plants, the introduction of new non-traditional fruit crops, agricultural production, etc. According to the BBCH scale, the phenological phases of development of *Cornus florida* L. (flowering dogwood), an ornamental and fruiting plant from the family Cornaceae Bercht. & J. Presl., with useful medicinal, antioxidant, technical, and ecological properties, are codified. In the climatic conditions of Ukraine (M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine, Kyiv (NBG)), *C. florida* plants go through a full cycle of seasonal development. *Cornus florida* biotypes, according to the BBCH scale, are characterized by eight of the ten principal stages of seasonal development, in particular: the development of buds (BBCH 0), leaves (BBCH 1), shoots (BBCH 3), inflorescence (BBCH 5), flowering (BBCH 6), fruit development (BBCH 7), fruit ripening (BBCH 8) and senescence, beginning of dormancy (BBCH 9). *Cornus florida* (subgenus *Cynoxylon* (Raf.) Raf.) differs from species of the subgenus *Cornus* by the descriptive and diagnostic features of phenophases. The development of *C. florida* in the NBG in dry 2021 confirmed its adaptive tolerance to seasonal water deficit, both in the region of the native range of the species (central Missouri) and in the experimental conditions of growing introduced plants in China. *Cornus florida* deserves a wide introduction on the territory of Ukraine.

Keywords: flowering dogwood, phenology, BBCH-scale, plant introduction

Introduction

Phenology is a key feature of plants of all species, as it determines their season and duration of growth and reproduction, as well as their ability to capture variable resources (Chuine and Régnière, 2017). Seasonal phases of plant development are determined by a complex of factors, including taxonomic affiliation, origin, ecological timing, geographical distribution of the species, as well as the growth form and duration of the life cycle of plants (Stuble et al., 2021). The course of vegetative development of plants is also affected by climatic changes (Piao et al., 2019; Fuccillo et al., 2022). Native and introduced or invasive species differ in phenological sensitivity (Willis et al., 2010; Calinger et al., 2013; Zettlemoyer et al., 2019; Giejsztowt et al., 2020; Calinger and Curtis, 2023), as well as various

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phases of plant development (Gallinat et al., 2015; Zohner and Renner, 2019; Zani et al., 2020).

Cornus florida L. (flowering dogwood, Cornaceae Bercht. & J. Presl), based on the results of the analysis of morphological features and molecular biological studies of the genus, is included in the subgenus *Cynoxylon* (Raf.) Raf., which together with the subgenus Syncarpea (Nakai) Q.Y.Xiang forms a separate evolutionary clade (group BB), species of which have large petaloid bracts (Eyde, 1988; Murrell, 1993, 1996; Fan and Xiang, 2001; Xiang et al., 2006; 2008; Xiang and Thomas, 2008; Wadl et al., 2010; Feng et al., 2012; Murrell and Poindexter, 2016; Thomas et al., 2021). The species is distributed in the Southeast of Canada, in the eastern and south-central parts of the USA, and the Northeast of Mexico (Murrell and Poindexter, 2016), which may indicate its significant adaptive plasticity and tolerance to drought and some other extreme environmental factors (Hinckley et al., 1979; Lu et al., 2020). In Canada, however, *C. florida* is an endangered species (Mitchell, 2021). In the region of the native range of the species, the amount of precipitation varies from 760 mm in the north to 2030 mm in the southern Appalachians; soils can be light (sandy), medium, or heavy (clay), according to pH – acidic, neutral or alkaline; according to the hydrothermal regime – dry, raw or well-moisturized; biotopes – shaded, semi-shaded or illuminated. The average annual temperature is +21 °C (in the south) and +7 °C (in the north), with extreme temperatures from +46 °C to -34 °C (McLemore, 1990).

Cornus florida is a very popular species in the USA and is the state tree and symbolic flower of the Commonwealth of Virginia, the state tree of Missouri, and the state flower of North Carolina (https://www.inaturalist.org/ taxa/54777-*Cornus*-florida). *Cornus florida* was selected as the 2018 Flower of the Year by the Virginia Native Plant Society (Hayden, 2018). This species is a desirable ornamental tree in urban and suburban landscapes, especially in temperate regions of North America. The aesthetic value of plants was an incentive for the selection of more than 80 cultivars and 24 varieties (Nowicki et al., 2018). *Cornus florida* has edible fruits that are characterized by a significant (up to 35% dry matter) lipid content (Stiles, 1984). The bark of plants has long been used for medicinal purposes by the Native Americans of North America, who especially valued it for its astringent, diaphoretic, mild stimulant, and tonic effects (Weiner and Weiner, 1980; Moerman, 1998; Diggs et al., 1999). Iridoids, flavonoids, and anthocyanins, which show high antioxidant activity, were found in the leaves and fruits (Graziose et al., 2012; He et al., 2014; Truba et al., 2020). *Cornus florida* plants improve the ecological cycle of calcium (Ca) due to its high concentration in the leaves and rapid decomposition of the latter, and therefore availability in the soil (Borer et al., 2013).

Cornus florida is introduced on all continents except Antarctica, in particular in South America (Colombia, Brazil), in 14 European countries, in Asia (Korean Peninsula, Japan, Philippines), Oceania (New Zealand), and in Australia, and is also listed as invasive species for South Africa (*Cornus florida*, 2022).

In Ukraine, *C. florida* is not a very common introduced species. In 1924, it was introduced into culture in Yalta (Kokhno and Trofymenko, 2005). Now it grows in the collections of botanical gardens and arboretums of Kyiv, Bila Tserkva, Kharkiv, Donetsk, and Askania-Nova (Teslyuk, 2011, 2016). In M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (NBG) *C. florida* has been cultivated since 2008 (Teslyuk, 2012).

For more than 100 years, flowering dogwood has been actively studied, especially in North America, in very diverse aspects: morphological, anatomical and biological (Shank, 1938; Gunatilleke and Gunatilleke, 1984; Dietrich et al., 1990), functional and physiological (Roberts, 1979; Borer et al., 2013; Lu et al., 2020), biochemical (Fernandez et al., 1996; Vareed et al., 2006; Truba et al., 2020; Al-Khafaji et al., 2023), structurally-population (Pais et al., 2017, 2020), molecular and genetic (Xiang et al., 2006; Hadziabdic et al., 2010, 2012; Zhang et al., 2013; Nowicki et al., 2018), etc.

Phenological observations of *C. florida* were carried out in native locations from different points of view and using various methods. It is the object of the project "The USA National Phenology Network" (*Cornus*, 2016; Crimmins et al., 2022). The greatest attention was paid to the initial, spring phases of plant development. They have been studied and used to determine the effect of air temperature on the initiation of dogwood flowering, to develop spring indices (Schwartz et al., 2013), to analyze trends in spring onset in the temperate regions of the Northern Hemisphere, and long-term changes in plant phenology in the Northeastern United States, and also to clarify the dynamics of the concentration of organic substances in plants during the growing season, etc. (Lieth and Radford, 1971; Reader, 1975; De La Pascua, 2020; Fuccillo Battle et al., 2022).

In modern studies of the seasonal development of various plants, the use of the BBCH scale (Biologische

Bundesanstalt, Bundessortenamt, und Chemische Industrie) is becoming increasingly popular, the main principles of which provide a unified code for similar phases of the seasonal development of each plant species, as well as the recognition of each phenophase by visual morphological features (Hack et al., 1992; Meier et al., 1994; 2009). Codification according to the BBCH scale is used to determine the phases of seasonal development of fruit plants (Larue et al., 2021; Ferrer-Blanco et al., 2022; Paradinas et al., 2022; Ramírez, 2023).

In Ukraine, the phenological features of many local and newly introduced species and cultivars of fruit plants, in particular *Cornus* L. (Klimenko, 1990), *Cydonia* Mill. (Klimenko, 1993), *Chaenomeles* Lindl. (Klimenko and Nedviha, 1999), *Persica* Mill. (is now called genus *Prunus* L.) (Holubkova, 2016), *Cynoxylon* (Raf.) Small (Teslyuk, 2016), *Malus* Mill. (Honcharovska, 2019), *Lycium* L. (Zhurba, 2021). According to the extended BBCH scale are codified phenophases of *Pseudocydonia* (C.K.Schneid.) C.K.Schneid. (Grygorieva et al., 2018), *Elaeagnus multiflora* Thunb. (Grygorieva et al., 2022), Californian endemic *Cornus sessilis* Torr. ex Durand (Klymenko et al., 2021), species of the *Cornus* subgenus (Klymenko and Ilyinska, 2021), as well as the new cultivar *C. mas* L. (Klymenko and Ilyinska, 2023).

The purpose of this study is to codify, following the BBCH scale, phenophases and to find out the course of seasonal plant development of the North American species *C. florida*, introduced in the NBG, in the climatic conditions of Ukraine.

Material and methodology

Study region, weather, and climate conditions

The study was conducted in 2021 in the NBG, located on the right bank of the Dnipro river in the southeastern part of Kyiv on the low Pechersky slopes of the Kyiv Upland in the Zvirynets tract (50° 27‘ N; 30° 31‘ E; 197 m. n. r. m.). The main climatic and edaphic factors of NBG are in the range of variation of similar indicators of the native range of *C. florida* (Table 1) (McLemore 1990; https://en.climate-data.org/; https://rp5.ua/Weather_archive_in_Kiev, (airport) [accessed January 29, 2022]. The only exceptions are the minimum amount of average annual precipitation (less in Kyiv) and the absolute maximum temperature: +46.0 °C within the native range of the species versus +39.4 °C in Kyiv. The year of the study (2021) was dry with a very warm spring and hot summer (Figure 1). For five months (March, June, July, September, and especially October), precipitation was significantly lower than normal, and the average monthly temperature in January and March and throughout the summer higher than normal. In particular, the first month of spring seemed dry and quite hot (Figure 1). On the fourth of March, the average daily temperature was equal to the biological minimum (+5 °C), and in the last six days of the month, it exceeded it.

Research objects

Genotypes of *C. florida* were obtained by seedlings from the state of Oregon (USA) in 2008 and introduced to NBG (Teslyuk, 2012). Two genotypes of *C. florida* were used in the studies.

Table 1 Climatic and edaphic factors of Kyiv and the region of native distribution of *Cornus florida* L.

Indicators	Kyiv (NBG)	Region of native distribution *C. florida*
Climate[1]	Dfb	Dfb, Dfa, Cfa, Bsh, Bsk
Biotopes	semi-shaded	shaded, semi-shaded, illuminated
Precipitation[2]**, mm**	649 (540)	760–2,030
Soils	medium (dark gray podzolic, low-humus black soil)	light (sand), medium, heavy (clay)
pH	slightly acidic, neutral	acidic, neutral, alkaline
hydrothermal regime	sufficiently hydrated	dry, raw, well hydrated
Temperature, °C		
average annual	+8.4 (+9.1)	+21 (south) +7 [north]
absolute maximum	+39.4 (+34.7)	+46.0
absolute minimum	-32.2 (-20.0)	–34.0

1 – the climate is given according to the Köppen-Geiger classification (Peel et al., 2007); 2 – precipitation and temperature indicators in 2021 are given in round brackets; Dfb – warm humid continental climate; Dfa – hot humid continental climate; Cfa – humid subtropical climate; Bsh – hot semi-arid climates; Bsk – cold semi-arid climates

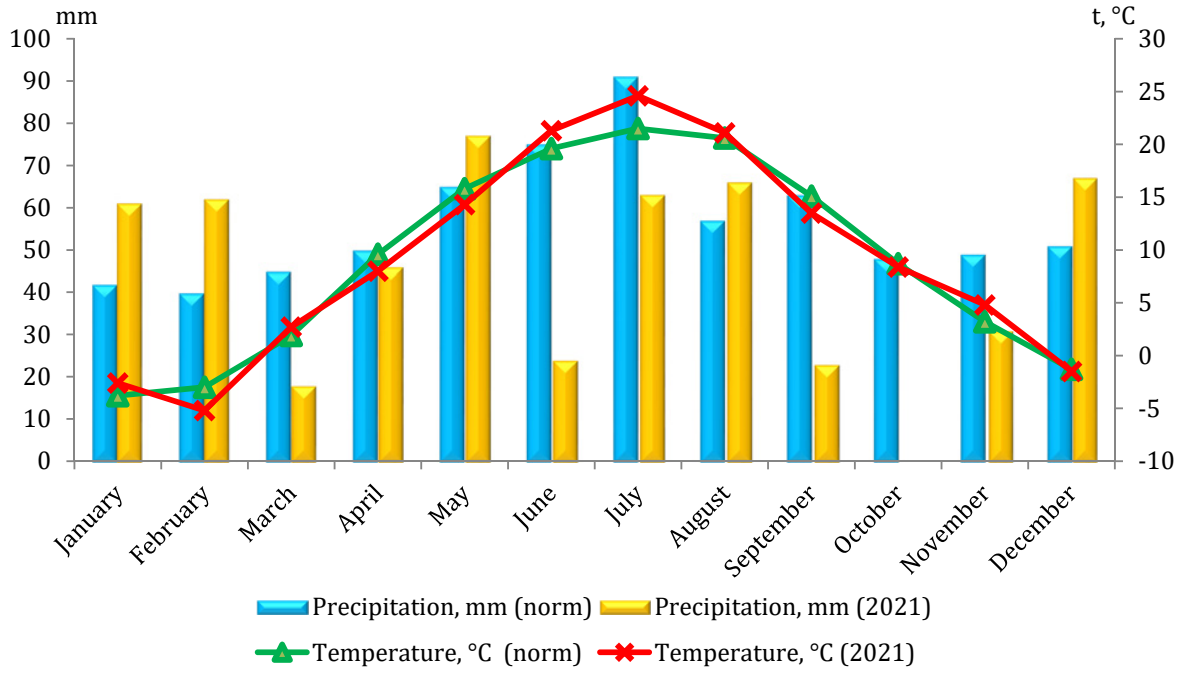

Figure 1 Average monthly precipitation and temperature in Kyiv in 2021

Phenological monitoring

The phenological study began on March 1, 2021. Observations were conducted 2–3 times per week through textual and photographic documentation. The course of seasonal development of *C. florida* genotypes was codified according to the extended BBCH scale (Hack et al., 1992; Meier et al., 1994, 2009; Klymenko and Ilyinska, 2021). For the beginning and end of each phenophase, the active and effective temperatures (t_{act} °C and t_{eff} °C respectively) were determined.

Results and discussion

Cornus florida is characterized by vegetative and complex renewal buds, which are initiated in mid-June and continue to develop until the onset of winter dormancy (Morse, 1907; Feng et al., 2012). Vegetative buds (Figure 2) are formed on shortened and elongated shoots. The formed complex buds consist of two vegetative and one generative bud and have one pair of common cataphylls, which can often be damaged and broken off during the rest period (Figure 2). Each vegetative bud of a complex bud has its own cataphyll, which initially partially covers the generative bud, and later only its axis (Figure 2). The inflorescence is surrounded by four bracts and has an elongated axis, which is formed during the rest period (Figure 2, 3). The development of an elongated axis of the generative bud distinguishes *C. florida* from species of the *Cornus* subgenus, the inflorescences of which are practically sessile (Klimenko, 1990; Klymenko and Ilyinska, 2021). Before the beginning of winter dormancy, the bracts increase in size, thicken, harden, and become scale-like structures surrounding the young flower buds. In the temperate climate of Ukraine (NBG, Kyiv), complex buds are laid in June and reach full development in November (Figure 3).

Figure 2 Vegetative (1) and complex (2) buds of *Cornus florida* L. during the rest period (January 6th, 2022)

Figure 3 Initiation of complex buds of *Cornus florida* L. in NBG (June – November 2021)

Under conditions of introduction, as well as in regions of native distribution (Lamb, 1915; Lieth and Radford, 1971), *C. florida* initiates vegetation with the simultaneous development of generative and vegetative buds, unlike *C. mas* and other species of the subgenus *Cornus*, which initiate seasonal development of flowering phenophase.

Principal growth stage 0: bud development

Vegetative buds of *C. florida* during the resting period are elongated-conical, covered with two scales with pubescent tips (Figure 2). In 2021, the period of rest continued until mid-March (Figure 2). Visually noticeable swelling of vegetative buds (phenophase 0) began in the second half of March (Figure 4, 5). The effective temperature at the beginning of phenophase 01 was insignificant, but at the end of February and in the second half of March, especially in the third decade, the maximum daytime temperatures were high – 10.6–15.0 °C, which contributed to the active development of vegetative buds. Light green areas of the first leaves (phenophase 07) appeared in the second decade of April, and at the beginning of the third decade of the month, the green tips of the leaves were clearly visible (phenophase 09) (Table 2). At that time, the total effective temperature was 89.8 °C. Therefore, in 2021, in the climatic conditions of NBG, the development of vegetative buds of *C. florida* lasted 45 days.

Principal growth stage 1: leaf development

Cornus florida has leaves with short petioles (3–20 mm long), ovate, elliptic, or obovate plates (5–12 × 2–7 cm), wedge-shaped or rounded base and sharply pointed apex. Their pubescence consists of Malpighian hairs. The adaxial surface is green, the abaxial surface is light green and whitish with tufts of truncated hairs in the corners of the secondary veins (Murrell and Poindexter, 2016).

In Kyiv, the opening of the first leaves of *C. florida* (phenophase 10) began almost simultaneously with the development of inflorescences at an effective temperature of 115.0 °C (Table 2, Figure 4, 5a–c). Further growth of the leaves occurred with a significant increase in the effective temperature. The first leaves fully unfolded (phenophase 11) in early May a few days after the beginning of flowering, and in mid-June they reached their final size (phenophase 19).

In a seasonal climate, the development of the leaves of woody plants, as well as the beginning of flowering, are determined by a complex of factors, among which temperature is the leading factor (Linkosalo et al., 2006; Parmesan, 2006; Polgar and Primack, 2011; Gerst et al., 2020; Denéchère et al., 2021). Funderburk and Skeen (1976) established a certain relationship between the dates of leaf opening and flowering of plants based on the results of phenological observations of 42 species of suburban forest near the city of Atlanta (Georgia, USA) during 1967–1971. Before flowering, leaves are formed in those species that begin to bloom after

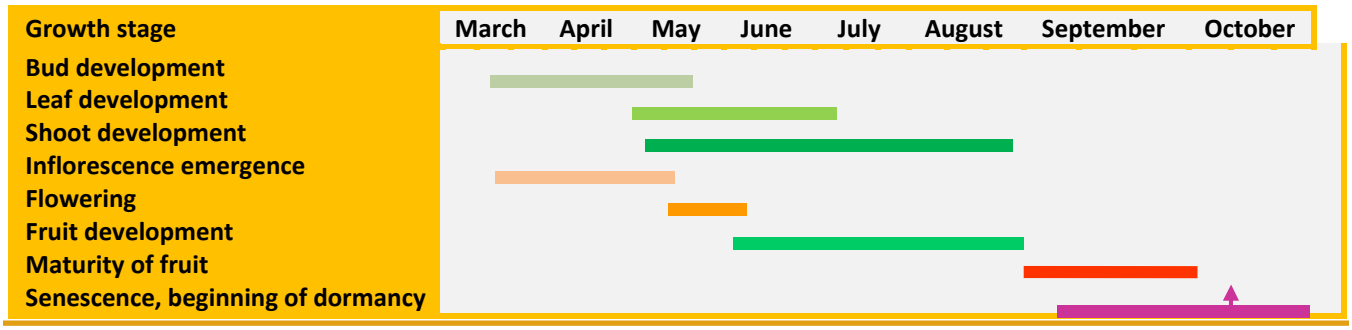

Figure 4 Phenological spectrum of seasonal development of *Cornus florida* L. in NBG, 2021
the arrow shows the beginning of the leaf fall

the 100th day of the year. After flowering, leaves develop in species that bloom until the 90th day of the year. More or less simultaneously with flowering period, the leaves of those species that bloom between 90 and 100 days of the year are formed. A similar sequence of seasonal phases of vegetation was also observed in some species of the genus *Cornus* introduced in NBG. In particular, the plants *C. mas* and *C. officinalis*

Figure 5a Phenological phases of development of *Cornus florida* L.

Figure 5b Phenological phases of development of *Cornus florida* L.

Figure 5c Phenological phases of development of *Cornus florida* L.

Table 2 Seasonal development of *Cornus florida* L. in NBG according to the BBCH scale, 2021

Scale	Characteristics	Date	t_{act}, °C	t_{eff}, °C
	Principal growth stage 0: bud development			
01	The beginning of swelling of the buds: the buds (vegetative) are noticeably enlarged, and the scales are elongated, with a light border	16.03	35.9	5.9
03	The end of swelling of the buds: the scales are separated, and light green areas of the buds are visible	29.03		
07	Budding begins: the green tips of the first leaves are visible	01.04		
09	The green tips of the leaves are about 5 mm long	22.04	246.9	89.8
	Principal growth stage 1: leaf development			
10	The green tips of the leaves are about 10 mm long; the first leaves begin to develop	30.04	312.0	115.0
11	The first leaves have unfolded, and others are still developing	11.05		
15	More leaves unfolded, and the first pair of leaves reached about half (≈7 cm) of their size	21.05		
19	The first leaves have reached the size typical for the species/variety (≈15 cm)	14.06	1,008.1	526.1
	Principal growth stage 3: shoot development			
31	The beginning of the growth of shoots: the axes of the developing shoots are visible; about 10% of the expected length	06.05	389.5	162.5
32	Shoots have reached about 20% of the expected typical length	25.06		
35	Shoots have reached about 50% of the expected typical length	15.07		
39	Shoots have reached about 90% of the expected typical length	27.08	2,739.3	1,963.6
	Principal growth stage 5: emergent inflorescence			
51	Bud swelling: bulbous inflorescence buds are closed, flat on top, surrounded by greenish-burgundy bracts	16.03	35.9	5.9
53	Budding: the outer bracts are open, the inner bracts are still closed	29.03		
55	All bracts are open, beginning to increase in size; the tops of individual densely arranged flower buds are visible	15.04		
56	Bracts are completely separated, enlarged, concave, outer – greenish-burgundy, inner – greenish-white; flower buds of the central part of the head are separated from each other; the petals clearly exceed the sepals	22.04		
57	Bracts are petaloid, deviated by almost 45°; flower buds in the “green bud“ stage	30.04		
59	Petaloid bracts are pinkish-white, horizontally oriented, and have reached their final size; flower buds in the “yellow bud“ stage	10.05	433.1	186.1
	Principal growth stage 6: flowering			
60	The first flower opened	11.05	450.2	198.2
61	Beginning of flowering: about 10% of the flowers are open	14.05		
65	Full bloom: at least 50% of the flowers are open, the petals of the first flowers have fallen	16.05		
67	Withering of most flowers: fertilization occurs, petals and stamens fall; senescence of bracts	20.05		
69	End of flowering: bracts, petals, and stamens of all flowers have fallen	27.05	696.7	364.7
	Principal growth stage 7: fruit development			
71	Fruit set: all ovaries are green, the same in shape and size	28.05	714.2	377.7
72	Fertilized ovaries are noticeably enlarged; the crown of the sepals of all ovaries is green	06.06		
73	Fertilized ovaries are almost twice as large as unfertilized ones	14.06		
75	The fruits have reached about half their final size; unfertilized ovaries are green and do not fall	15.07		
77	Fruits about 80% of the final size; the crown of the calyx and the column of the ovary begin to die, but remain on the fruit	12.08		
79	Fruits have reached their final size, green; unfertilized ovaries do not fall	20.08	2,606.8	1,866.1

Continuation of Table 2

Scale	Characteristics	Date	t_{act}, °C	t_{eff}, °C
	Principal growth stage 8: maturity of fruit			
81	The beginning of fruit ripening: a change in the colour of the fruit from light green to yellowish and to light pink; unfertilized ovaries are light green; the crown of the calyxes and the columns of the ovaries are brown	27.08	2739.3	1963.6
85	The color of the fruits progresses, becoming light red; unfertilized ovaries are yellowish-green; the crown of the cups and the column of fruits and ovaries acquire a dark anthocyanin color	01.09		
87	Increasing color intensity; 80% of the fruits have reached technical ripeness; the beginning of shedding of unfertilized ovaries	16.09		
89	All fruits are ripe for consumption: they have a typical taste and firmness; almost all unfertilized ovaries have fallen	04.10	3258.7	2293.0
	Principal growth stage 9: senescence, beginning of dormancy			
91	The growth of the shoots is complete, the leaves are still green	31.08	2815.0	2019.3
92	The leaves begin to change color	02.09		
93	The beginning of falling leaves	11.10		
95	Half of the leaves have changed color or fallen off	18.10		
97	All the leaves have fallen	29.10		
99	The beginning of the winter rest period	30.10	3466.3	2380.6

t_{act}, °C and t_{eff}, °C were calculated at the beginning and end of each phenophase

Siebold & Zucc. (subgenus *Cornus*) unfurl the leaves after flowering. According to our data, in 2021, their flowering began on March 30 – April 5, that is, 89–91 days from the beginning of the year. In *C. florida* (subgenus *Cynoxylon*) in DeKalb County (Georgia, USA), leaf development began on the 88th day of the year, and flowering was observed on the 101st day (Funderburk and Skeen, 1976). In NBG (Kyiv), the leaves of *C. florida* began to develop on the 121st day from the beginning of the year, and the first flowers opened on the 132nd day (Table 2). Therefore, in this species, the sequence of development phases is preserved regardless of the geographical location of the plants. While the date of the beginning of a separate phenophase varies and is determined, in a certain way, by the degree of influence of a complex of exogenous factors.

Principal growth stage 3: shoot development

After the unfolding of the first pair of leaves (stage 31), the first internode becomes visually visible, just as in species of the subgenus *Cornus* (Klymenko and Ilyinska, 2021). Formed vegetative shoots (phenophase 39) have from four to five to seven to eight internodes. The longest length (15–18 cm) is typical for the second internode, and the smallest (3–4 cm) for the last one. At the end of the growing season, the leaves of the distal node often remain underdeveloped. The main growth of shoots terminates at the end of August (Table 2).

Principal growth stage 5: inflorescence emergence

Cornus florida (subgenus *Cynoxylon*) has a cymoid head inflorescence, in contrast to species of the *Cornus* subgenus (*C. mas*, *C. officinalis*, etc.), which have botryoid umbel inflorescences (Feng et al., 2011, 2012), which is well demonstrated on the fifth stage (phenophase 5) of the seasonal development of plants.

In 2021, the dormant period of generative buds of *C. florida* continued until almost mid-March, just like vegetative buds. Visually noticeable swelling of the buds (phenophase 51) began with cracking of the outer bracts (Figure 5a–c). The inner bracts remained still closed (Table 2). After almost a month, all the bracts separated from each other and started to enlarge, resulting in the apices of several flower buds (phenophase 55). Phenophase 56 is characterized by intense growth and color change of bracts and the development of flower buds (rolled petals rise on the calyx). Distinct features of phenophase 57 are a deviation of enlarged bracts at an angle of about 45° and well-developed and separated flower buds (“green bud“ stage). The final stage of development of generative buds (phenophase 59) is characterized by horizontally oriented petaloid bracts that have reached the final size and shape (broadly obovate with a notch at the top) and typical (pinkish-white in our

plants) color and closed flower buds with yellow petals ("yellow bud" stage).

Cornus florida bracts complete development during phenophase 5. In NBG in 2021, their development and functioning lasted 52 days. In *C. florida* plants from the state of North Carolina (Raleigh, USA), bracts existed for a shorter time, about a month (Feng et al., 2012), which, we believe, reflects a certain dependence between the duration of their functioning and temperature and geographical factors. In our plants, a distinct synchronicity was also observed between the growth processes of bracts, vegetative buds, and typical leaves (Table 2, Figure 4, 5a–c). At the same time, practically, bracts developed (phenophase 53), vegetative buds bloomed, and light green areas of leaves appeared (phenophase 07). The full development of the bracts (phenophase 59) corresponded to the unfolding of the first pair of leaves (phenophase 11). Therefore, the vegetation of *C. florida* in NBG (2021), as well as in the region of its native range (Lamb, 1915; Reader, 1975; Funderburk and Skeen, 1976), begins with the simultaneous development of vegetative and generative buds.

Cornus florida has two features: intensive growth of bracts, as a result of which they acquire a petaloid shape, and lack of growth of peduncles, due to which the flowers are sessile. In *C. mas*, the bracts remain green and change little in size, and the peduncles are elongated at the beginning of flowering, as is characteristic of umbrellas (Gunatilleke and Gunatilleke, 1984; Klymenko and Ilyinska, 2021). Later, the bracts of *C. florida* fall off, and in *C. mas* they remain on the plant until the end of the vegetation period or even longer.

Principal growth stage 6: flowering

The flowers of *C. florida* are sessile, as is characteristic of a head-shaped inflorescence, and are very small. Hypanthium appressed-hairy, sepals 0.5–0.8 mm long, petals cream or greenish-yellow, 3.0–3.5 mm long (Murrell and Poindexter, 2016). From 15 to 35 flowers develop in one flower head (Gunatilleke and Gunatilleke, 1984). About 20 flowers were observed in the inflorescences of plants introduced in NBG. Corolla and stamens fall after flowering. In *C. florida*, in contrast to *C. mas*, anthers crack in mature flower buds, while in the latter after flower opening (Gunatilleke and Gunatilleke, 1984).

In Kyiv, as well as in the homeland of the species (Reader, 1975; Funderburk and Skeen, 1976), the flowering of plants begins simultaneously with the development of leaves. In 2021, in NBG, flowering of *C. florida* (phenophase 6) started on May 11 at an effective temperature of 198.2 °C (Table 2, Figure 4), which according to our data is six weeks later than *C. mas* varieties. The flower in the central part of the inflorescence was the first to open (phenophase 60), which is typical for cymoids. In the second half of flowering (phenophase 67), aging of the bracts was observed – the brightness of the color decreased, and brown spots appeared. Later they began to fall. At the end of flowering (phenophase 6), most of the bracts have fallen. In 2021, the flowering period lasted 17 days, which is close to similar data obtained in 2010–2012 (Teslyuk, 2012). Then, a flowering of *C. florida* in NBG continued for 12–18 days from May 5–12 to May 22–23. Therefore, the start date and duration of flowering of *C. florida* in different years in Kyiv (NBG) more or less coincide.

The flowering period of plants occupies a special place in the history of phenological observations. Perhaps this is the first phase of seasonal development, which began to be documented long before the emergence of phenology as a separate scientific discipline (Sparks and Menzel, 2002; Aono and Kazui, 2008). In floristic publications, from ancient to modern, flowering is one of two phenophases (the other is fruiting), which is necessarily given for all species. For example, according to "The Flora of North America North of Mexico", the flowering period of *C. florida* within its native range begins in March and ends in June, respectively, in its southern and northern parts (Murrell and Poindexter, 2016).

The onset of flowering, as well as other phenophases of *C. florida* in the region of native distribution, has been studied for more than 100 years (Smith, 1915; Wyman, 1950; Lieth and Radford, 1971; Funderburk and Skeen, 1976). It has been established that the date of the beginning of flowering varies geographically (Wyman, 1950) and is not constant in one locality from year to year (Smith, 1915). Plants in the southernmost part of the native range of the species, in particular in the state of Florida (Glen St. Mary, USA) bloom first (in mid-February or early March) (Wyman, 1950; Reader, 1975). Then the flowering of plants spreads to the north. In DeKalb County, Georgia (33° 51' N) in 1968, the first flowers opened on April 8, and the average date of onset of flowering during 1967–1971 ranged from April 6 to 10 (Funderburk and Skeen, 1976). In the capital of the USA (Washington DC; 38° 53' N), in 1914, flowering began in mid-April and lasted almost until the last decade of May (Lamb, 1915). According to Thomas Mikesell (Smith, 1915), in the city of Wauseon

(Fulton County, Ohio; 41° 33′ N) during 1883–1899 and in 1912, the flowering of the flowering plant began as early as last, and at the latest on May 30 and lasted 17 days. In the Arnold Arboretum of Harvard University (Boston, MA (42° 17′ N) during 1940–1950, flowering was observed in mid-May (Wyman, 1950). In the state of Maine (Portland, USA; 43° 40‘ N) and in the southern part of Ontario (Canada) *C. florida* begins to bloom in the second half of May (Wyman, 1950; Reader, 1975). Reader (1975) summarized previously known data and with the help of volunteers for four years (1970–1973) obtained indicators (1,200 observations) about the beginning of flowering of plants in almost the entire territory of the native range of *C. florida*. The author established that annual fluctuations in the date of flowering are usually caused by the sum of air temperatures above 2.2 °C in the 6 weeks before its onset, and geographical variations depend on environmental conditions, which confirms the previously established relationship between the date of dogwood flowering and air temperature (Reader, 1975).

The flowering period of *C. florida* in Kyiv (50° 27′ N) practically coincides with those in the northernmost part of its native range (about 47° N), according to Reader (1975). The duration of flowering of the species in Kyiv (12–18 days) and in the city of Wauseon, Ohio (17 days), according to the data of Mikesell (Smith, 1915), is also close, which confirms the conclusion of Funderburk and Skeen (1976) regarding the narrow range of dates flowering of this species. However, in Case Estates Botanical Garden (Weston, Massachusetts; 42° 21′ N) in 1984, flowering lasted five to six weeks (Gunatilleke and Gunatilleke, 1984), which is due, apparently, to very specific microclimatic growing conditions plants.

Principal growth stage 7: fruit development

Cornus florida is a cross-pollinated self-incompatible species, therefore, not all ovaries are fertilized (Gunatilleke and Gunatilleke, 1984). The development of the fruits of this species has been studied much less compared to the flowering phase. According to our data, after flowering (phenophase 71), all ovaries have almost the same shape, color (green), and size. In them, the crown of the calyx and the column of the ovary are also well-developed. In the future, unfertilized ovaries stop developing. A visually noticeable increase in the size of the fertilized ovaries (phenophase 72) is observed approximately one and a half weeks after the end of flowering, and the final size of the fruits after 12 weeks (phenophase 79).

According to Gunatilleke and Gunatilleke (1984) at the Case Estates Botanical Garden (27 km from Boston, Massachusetts, USA), differences in the size of fertilized and unfertilized ovaries of *C. florida* were noticeable four weeks after pollination, and fruit development lasted 16 weeks. The shorter duration of the period of fruit development in Kyiv is probably caused by a significant summer drought in 2021, especially in summer. The summer months of the Boston region, near which the Case Estates Botanical Garden is located, are characterized by higher average monthly temperatures (24. 2, 27. 8, 26. 9 °C, respectively, June, July, August) and average precipitation (90, 76, 90 mm, respectively, June, July, August). Therefore, *C. florida* plants are better adapted to high summer temperatures and react more sensitively to a low level of moisture supply.

Cornus florida differs from *C. mas* in some features of the biological development of fruits. In it, in particular, unfertilized ovaries do not fall off until the end of fruiting and the crown of the calyx and column of the ovary do not die. Whereas the “Fruit Development“ phenophase of *C. mas* and other species of the subgenus *Cornus* is known to be characterized by two waves of dying of unfertilized ovaries and gradual elimination of the crown of the calyx and column of the ovary (Klimenko, 1990).

Principal growth stage 8: maturity of fruit

Cornus florida drupes are ellipsoidal, usually red, very rarely yellow (13–18 × 6–9 mm), endocarps are also ellipsoidal (10–12 × 4–7 mm), smooth.

In Kyiv in 2021, fruit ripening (phenophase 81) began at the end of August (Table 2). Fruits began to gradually change color from light green and yellowish to light red, and unfertilized ovaries to yellowish (Figure 4, 5a–c). During phenophase 85, the color of the crown of the calyxes, fruit columns, and unfertilized ovaries changed. After reaching the stage of technical ripeness of fruits (phenophase 87), unfertilized ovaries began to fall. In heads with fully mature fruits, unfertilized ovaries are usually absent (phenophase 89).

So, in Kyiv in 2021, the fruit ripening process lasted about six weeks, which is twice as long (six weeks against three) as in 2010–2012 (Teslyuk, 2012). The difference in the duration of the fruit ripening process is probably due to the use of different observation methods, as well as the very hot and dry summer and autumn of 2021 (Table 1). Ripe fruits of *C. florida* plants introduced in NBG, as well as in the region of native distribution of the species, do not fall for a long time, just like *C. officinalis* and unlike *C. mas* and *C. sessilis*.

According to our data in NBG (2021), the fruits of *C. florida* began to ripen almost a month earlier, compared to the early cultivars of *C. mas*, and almost simultaneously with its late cultivars.

Forms with red fruits are most characteristic of *C. florida*. However, since the beginning of the last century (Rehder, 1921, 1927), the yellow color of fruits has been known. Rehder (1921) described a new yellow-fruited form (*C. florida* f. *xanthocarpa* Rehder) based on a specimen found in North Carolina (USA) in the herbarium of the Arnold Arboretum (Boston, Massachusetts, USA) and plants with yellow fruits from Long Island (New York state, USA). Later, in 1927, the author slightly changed the taxonomic rank of such plants and considered them not forms, but a variation of the flowering dogwood (*C. florida* var. *xanthocarpa* Rehder), that is, those of native origin. In the future, there will be a discussion about the status of these plants. Specimens described by Rehder (1921) and Howard (1961) were considered the variety. MacDonald (1968) found eight plants of *C. florida* with yellow fruits in the state of Tennessee (USA), of which five were spontaneous and three were found in commercial nurseries. The author concluded that the yellow-fruited varieties of *C. florida* come from plants of native flora. It was also established that two anthocyanins, peonidin, and petunidin, are absent in the exocarp of yellow fruits of *C. florida* (Chester and Stone, 1964). All *C. florida* biotypes introduced in the NBG have red fruits. So, for this species, as well as for *C. mas*, red-fruited and yellow-fruited forms are characteristic.

As already noted, fruiting is the second phenophase (the first is flowering) of the seasonal development of plants, which attracts the special attention of not only agrarians but also scientists of many botanical disciplines, including florists. For example, in the flora of North America (Flora of North America north of Mexico) it is indicated that the fruits (stones) of *C. florida* ripen during August – October (Murrell and Poindexter, 2016). According to Day and Monk (1977), the ripening of *C. florida* fruits begins when plant biomass stops increasing, that is, vegetative organs stop growing, and leaves begin to change color. For example, in the Southern Appalachians in southwestern North Carolina, fruit ripening and leaf color change began almost simultaneously, October 6–8 (Day and Monk, 1977). In the genotypes we studied, the main growth of shoots ended at the end of August. Almost at the same time, the first reddening fruits became noticeable, and the leaves began to change color on September 2 (Table 2, Figure 4, 5a–c). Therefore, it can be assumed that the processes of completion of vegetative growth of plants and ripening of fruits are interrelated and genetically determined, while the initial date of these processes, like other phenophases, is largely determined by a complex of exogenous abiotic factors, including geographical location.

Principal growth stage 9: senescence, beginning of dormancy

The last, autumn, phenophase of *C. florida* in Kyiv (2021) lasted almost two months (Table 2, Figure 4, 5a–c). The main growth of shoots was completed at the end of August almost simultaneously with the beginning of fruit ripening. The leaves began to change color in September (phenophase 92). Fall began in the second decade of October (phenophase 93) and within two weeks the leaves had completely fallen, probably due to very dry September and October (Figure 2). The rapid fall of *C. florida* confirms the known data on the negative effect of heat stress on the duration of the autumn phenophase and the vegetation period, in general.

Autumn phenology (phenophase 9), visually defined by the change in leaf color and fall, completes the growing season of woody plants of a seasonal climate. The date of onset of senescence and dormancy (phenophase 9) affects the duration of the growing season of plants, but this stage of their development has been little studied (Vitasse et al., 2009; Gallinat et al., 2015; Xie et al., 2015). However, it is known that in a seasonal climate, factors of autumn phenology include short day length, low temperatures, frost, drought or floods, and strong winds, which in a certain combination determine the beginning and duration of the rest period (Gallinat et al., 2015). Low temperatures, heavy rainfall, and severe heat stress lead to early dormancy, while moderate heat and rainfall slow leaf senescence and dormancy onset (Xie, Wang and Silander, 2015).

In the climatic conditions of Ukraine (NBG), plants of *C. florida* (subgenus *Cynoxylon*), as well as the studied genotypes of species and varieties of the subgenus *Cornus* (Klymenko, 1990; Klymenko et al., 2021; Klymenko and Ilyinska, 2021, 2023), undergo a full cycle of seasonal development. In 2021, the growing season of *C. florida*, from the beginning of swelling and budding to the end of October, lasted 230 days. In the territory of native distribution, the vegetation period of *C. florida* lasts from 160 (southern Michigan) to more than 300 days in the state of Florida, which reflects the influence of the geographical coordinates of the area on the specificity of the seasonal development of plants (Lieth and Radford, 1971; Winstead et al.,

1977; McLemore 1990; Murrell and Poindexter, 2016). The main climatic factors and edaphic conditions for growing plants in the NBG, according to most indicators, are within the range of their variability in the region of native distribution of the species (Table 1), which serves as a positive bioecological prerequisite for the widespread introduction of *C. florida* into culture as a valuable decorative and medicinal plant.

Conclusions

In the climatic conditions of Ukraine (NBG), plants of *C. florida* (subgenus *Cynoxylon*) go through a full cycle of seasonal development, just like biotypes of species and cultivars of the subgenus *Cornus*. In 2021, the growing season of *C. florida* lasted 230 days. The wide range of variation of climatic factors within the range of the species, its resistance to drought, as well as the seasonal development of *C. florida* in NBG in the dry year of 2021, testify to its adaptive tolerance to seasonal water deficit and serve as a positive bioecological prerequisite for the widespread introduction of *C. florida* into culture in the climatic conditions of Ukraine, as a decorative plant with high aesthetic value and a promising new source of plant raw materials.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

Funding

The co-authors is grateful to Bilateral Scholarship of the Ministry of Education, Science, Research and Sport and SAIA (Slovak Republic) for the scholarship for the research during which the presented knowledge was obtained.

Acknowledgements

The publication was prepared with the active participation of researchers in the International Network AgroBio*Net*, as a part of the international program "Agricultural Biodiversity to Improve Nutrition, Health and Quality of Life". The authors are indebted to the departmental colleagues for their support in the development of this manuscript.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

A Review

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Seasonal variety of selected indigenous plant raw materials and foodstuffs available for consumption in Namibia

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Article Details:
Received: 2023-10-04
Accepted: 2023-11-15
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0016

This review is based on the nutritional content of the available indigenous foods to identify area of future research. Most indigenous foods are affordable and has good source of micronutrients, but the consumption rate of these foods is declining. It is important to increase awareness of the need to consume indigenous foods to reduce food insecurity and malnutrition. Most indigenous foods are seasonal and range from cereal-based foods to beverage drinks. In Namibia, the most important part of the human diet are pearl millet, sorghum, and maize. These crops mostly are rich in complex carbohydrates (starch), protein, mineral compounds (potassium, calcium, iron and zinc), vitamins and polyphenols. From legumes crops are widely used cowpeas, groundnut and marama beans. Most legumes are a good source of protein, starch, dietary fibre, fats, and micronutrients. Legumes with high sources of protein can be used as meat alternatives, which is very actual and attractive in the food industry. Fruits and plant-based foods are very common in Nigeria are monkey oranges, marula fruits, bird plums, jackal berries, makalani palm, manketti and water lilies, tiger nuts, and roselle. These fruits contain carbohydrates, proteins and some micronutrients. Indigenous fruits have the potential to be used in medicine for treating diseases due to the high level of bioactive compounds, especially antioxidants. Some plants like roselle (*Hibiscus sabdariffa* L.) have the potential to control diseases like type 2 diabetes and hypertension. Most vegetables used in Namibia provide nutrients such as beta-carotene, ascorbic acid, mineral compounds (iron and calcium) protein, and phytochemicals. Traditional fermented beverages – oshikundu, omagongo, oshinwa, and mutete juice and known to provide a wide range of nutrients including vitamins A, C, B_{12}, iron, and calcium. Based on this review, we recommend sustainable promotion of these foods and regular consumption of indigenous foods as a solution to malnutrition. There is a great potential to grow indigenous foods in community nurseries and home gardens to increase the availability.

Keywords: traditional foods, crops, nutrients, Africa, bioactive compounds

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Introduction

The least populous and driest nation in the Sahara Desert is Namibia. It has a population of roughly 2.2 million and a total area of more than 824,000 km^2. Fortunately, Namibia has a diverse ecosystem that is home to over 690 uncommon, endemic, or nearly endemic plant species. Many of them are drought-tolerant plants that have real or potential agricultural significance (Maggs-Kölling et al., 2014; Rampa and Lovo, 2023). The country must import a considerable portion of its cereal needs, frequently well over half, even in the best years because total cereal production varies greatly according to rainfall. However, due to well-established commercial distribution networks, there is no national food security issue. In the past, the country has been able to easily and without government assistance meet its regular commercial import requirements. In years of drought, these are typically supplemented to some extent with food aid. Fish, cattle, and meat exports from Namibia are significant (Maggs-Kölling et al., 2014; Onwujekwe and Ezebma, 2021).

Indigenous foods can be defined as plant and animal-based foods that naturally found and produced in the specific place and eaten as part of traditional diets (Rampa and Lovo, 2023). Indigenous foods can be used to reduce hunger and malnutrition (Cheikhyoussef et al., 2013). Research shown that food and nutrition security can be improve by consuming indigenous foods as they are rich in nutrients (Rampa and Lovo, 2023). The availability and access of these foods may be declining due to increase of development, thus this causes the decrease in consumption (Mbhenyane, 2017). Information on nutritional value of most indigenous foods is scare in Namibia (Cheikhyoussef, Bille and Shikongo – Nambabi, 2013). There is a need in recognizing the benefits of indigenous foods in rural and urban areas in order to create consumption patterns along the nation (Mbhenyane, 2017).

Most of the traditional/indigenous foods are mainly grown or found in the locations of the cultures presented in northern, some central and southern parts of Namibia such as Oshiwambo, Rukwangali, Zambezi, Herero and Damara/Nama speaking groups. These indigenous foods are mostly domesticated, cultivated and grows in the wild at different part of the country. Cereal and legume crops like pearl millet, sorghums, maize, cowpeas, and bambara groundnuts are grown during rainy seasons in the northern part of Namibia while legumes crop like marama beans are wild – growing legumes.

Most fruits are known with several health benefits (Nyambe et al., 2019). Namibian depends on wild fruits for micronutrients sources, which provide most health benefits (Nyambe et al., 2019). Indigenous wild fruits harvested includes monkey orange, marula fruit tree, bird plum, makalani palm, manketti fruits. Wild plants and shrubs can be found in water ponds or grown on the land, this includes water lilies, tiger nuts, roselle and indigenous mushrooms. Majority of wild-growing plants/trees are found in the northern parts of Namibia. Indigenous cereals, fruits, vegetables, and beverages are mostly fermented and includes cereal based (oshikundu), fruits – based (omagongo, oshinwa and mutete). Indigenous fermented foods varies depending on the cultures in Namibia (Misihairabgwi and Cheikhyoussef, 2017).

This is a critical review on available information regarding nutritional value of some selected indigenous foods found in Namibia. There are a variety of indigenous foods available for consumption in Namibia that includes: indigenous cereals, legumes, fruits, plants, vegetables-based foods, and beverages. It is important to find solution to food security. Hence, the purpose of this review is to offer information about some indigenous foods available in Namibia.

Indigenous cereals and legume-based foods in Namibia

Pearl millet (*Pennisetum glaucum* L.), locally named mahangu in Oshiwambo is the traditional stable preferred crop in Namibia. Pearl millet is processed into flour, which is used to make porridge, bread and fermented drinks (Misihairabgwi and Cheikhyoussef, 2017). Traditional fermented foods and drinks made in Namibia mostly from milk, wild fruits, cereals, and cassava are sold to generate cash (Figure 1). They have social and nutritional value. Major drawbacks of spontaneous fermentation processes include their inefficiency, low yields of products, and variable product quality. Traditional fermented foods are primarily produced at the household level using largely uncontrolled spontaneous inoculation methods in which microorganisms associated with the raw food material and the processing environment serve as inoculants.

Oshikundu is prepared by mixing water, pearl millet flour, sorghum flour and pearl millet bran, a certain amount of previously fermented oshikundu is also added (Figure 2). The mixture ferments for several days at room temperature. The resulting drink has a brown colour and a thick texture, it is rich in lactic bacteria

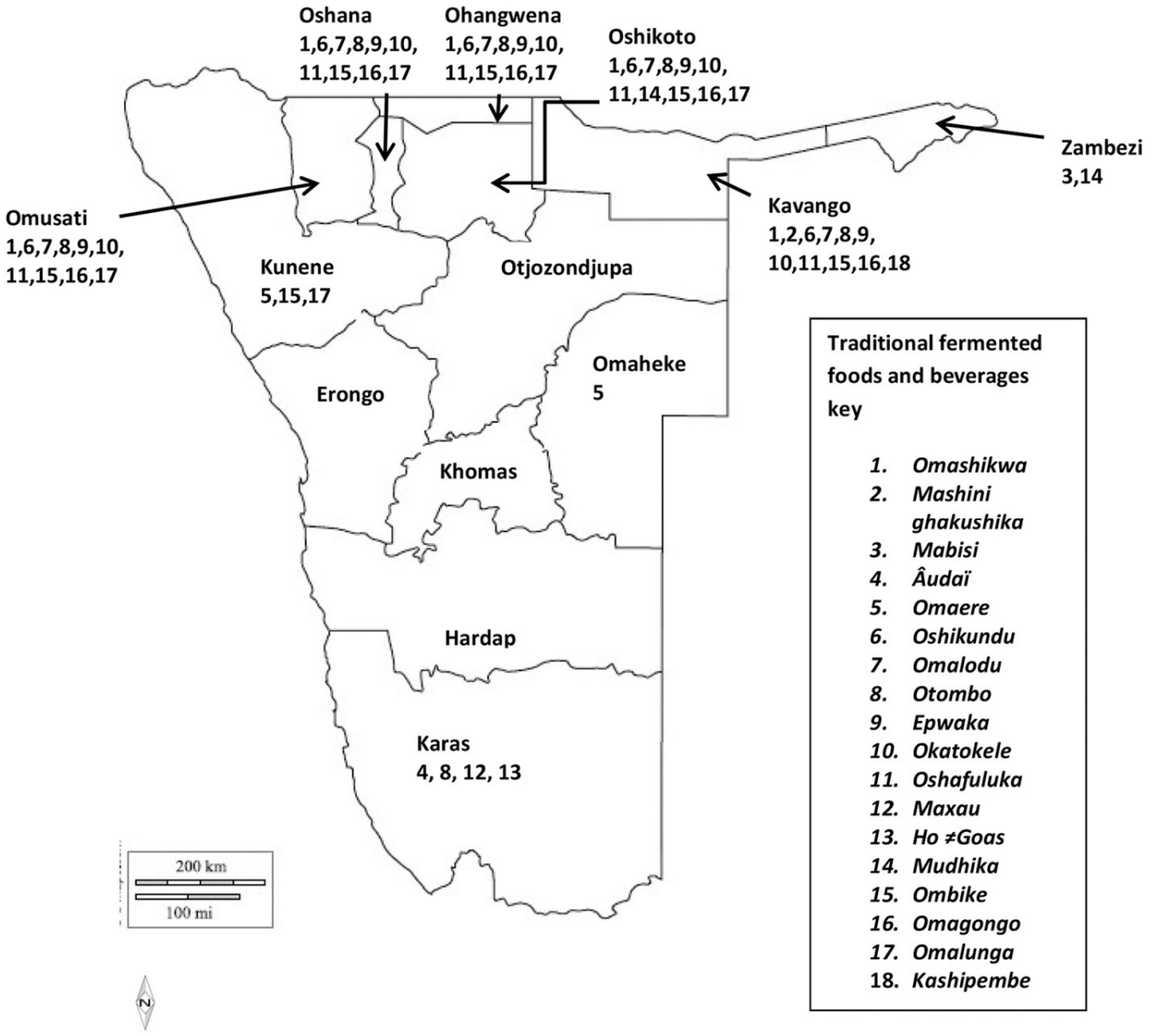

Figure 1 Namibian traditional fermented food and drink distribution
Source: Misihairabgwi and Cheikhyoussef, 2017

that increase the palatability of the drink. Oshikundu is usually cooked at home, playing an important role in social gatherings. Millet flour is rich in protein, carbohydrates, and minerals (calcium, zinc, iron and potassium) (Animasaun et al., 2019). Proteins are rich in albumin and globulin fractions, which contain a wide range of health-promoting amino acids (valine, leucine, isoleucine, threonine, methionine, and lysine). Millet is also an important source of vitamins B_1 and B_2. Millet does not contain any anti-nutritional substances. Polyphenols are dominated by gallic, protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, syringic, and ferulic acid (Satankar et al., 2020).

Sorghum (*Sorghum bicolor* L. Moench), local name iilyavala in Oshiwambo is a type of cereal crop cultivated in the Northern central regions of Namibia (Misihairabgwi and Cheikhyoussef, 2017). Sorghum can be eaten fresh as roasted grains, dried cooked grains, or processed into flour. Sorghum flour is used to make porridge and fermented beverage known Ontaku or oshikundu and Namibian opaque beer (Misihairabgwi and Cheikhyoussef, 2017; Embashu et al., 2019). Sorghum grains are rich in starch, mainly polysaccharides, protein and mineral compounds such as zinc, copper, manganese and iron, vitamin B_1, B_6 and beta-carotene (Cardoso et al., 2017). Sorghum has a high energy value and a low protein, fat and fibre content. The starch content is around 70% (amylase

Figure 2 Oshikundu – fermented drink based on cereals
Source: Misihairabgwi and Cheikhyoussef, 2017

content is 21–34% and amylopectin 65–80%), protein content ranged from 8–16%, fat content ~ 3.3%, ash 1.9%, and ~fibre 1.9%. The content of tannin (proanthocyanidin) and some other anti-nutritional substances, which can adversely affect digestibility, is listed as negative. However, this is more tied to varieties of sugar sorghum or varieties with coloured grains. Due to the low gluten content below 10 mg per 100 g of dry matter, it is advisable to use sorghum in a gluten-free diet. From polyphenols protocatechuic, hydroxybenzoic, vanillic, syringic, sinapic, *p*-coumaric, caffeic and ferulic acid were identified. These acids are important for their antioxidant properties (Kulamarva et al., 2009).

Figure 3 Maxau – fermented maize meal
Source: Misihairabgwi and Cheikhyoussef, 2017

Figure 4 Oshingali – traditional Namibian meal
Source: Misihairabgwi and Cheikhyoussef, 2017

Maize (*Zea mays* L.) is a cereal crop grown in Namibia. Maize contained two edible parts: kernel and germ. The kernel is consumed fresh by cooking or processed into products such as maize flour, starch, or breakfast cereals (Shah et al., 2016). Maize flour is used to make porridge (Shah et al., 2016) and fermented beverages known Maxau consumed by Damara/Nama people in the Karas region (Misihairabgwi and Cheikhyoussef, 2017). Maxau is a fermented maize meal product to which sugar is added for flavour (Figure 3). Water and maize meal are combined to create a slurry known as maxau. To create a thin mixture, water is added to the slurry after first being boiled separately. The maxau pot is then filled with sugar and wheat flour and let stand for 24 hours before being kept at room temperature. This combination is then boiled for 5 to 10 minutes. Maize kernels are good sources of starch, mineral compounds, especially potassium, magnesium and phosphor; vitamins that include ascorbic acid, pantothenic acid, niacin and thiamine while the germ parts provide mainly vitamin E benefits (Shah et al., 2016). Maize contains approximately 12% protein, but they are deficient in the content of essential amino acids, especially lysine and tryptophan.

Legumes are considered inexpensive meat alternatives, providing protein, complex carbohydrates and dietary fibre nutrients. Most legumes found in Namibia include cowpeas, groundnuts, and marama beans. Cowpeas (*Vigna unguiculata* L.) is a legume crop also known as black-eyed peas in Namibia. It can be eaten fresh or dried and can be processed into a soft product called Oshingali. A popular and nutrient-dense Oshiwambo traditional food is oshingali, or

black-eyed mashed beans (Figure 4). It is delicious and has a thick, creamy texture. Oshingali is served with pap and drizzled with marula oil. Cowpeas provide nutrients such as complex carbohydrates, fibre, protein, and micronutrients (iron, thiamine, and folate, copper (Enyiukwaet al., 2018; Jayathilake et al., 2018). Mostly, cowpea contain high-quality protein, but differs depending on the variety. Major protein, in cowpea are albumins and globulins (Jayathilake et al., 2018).

Groundnuts (*Arachis hypogea* L.) are a type of legume cultivated in Namibia. Groundnuts are consumed fresh by cooking or when dried can be roasted or cooked. It provides nutrients such as starch, oil, protein, and vitamin E. Groundnuts are a good source of mineral compounds such as potassium, magnesium, calcium, and phosphorus (Toomer, 2018). Raw, boiled, oil-extracted, roasted (as a snack), energy bars and sweets, and by blending peanut paste with other snack foods are all ways that people have consumed peanuts. With their nutrients (lipid profiles) and bioactive substances like phytosterols, phenolic compounds, stilbenes, lignans, and isoflavonoids, peanuts and peanut products have a good impact on human health. These bioactive substances offer defense against cancer, type 2 diabetes, and cardiovascular disease (Ciftci and Suan, 2022).

Marama bean (*Tylosema esculentum* Burch.) is a type of wild legume crop (Nepolo et al., 2009). It has two consumable parts – seed and tuber (Cullis et al., 2019). It has a high protein content (30–39%) and a low-cholesterol oil content (35–43%) that is rich in mono- and di-unsaturated fatty acids. It is also claimed to be a source of phytonutrients like isoflavones, tannins, trypsin inhibitors, phytates, and oligosaccharides. These nutrients have been linked to improved health, particularly the prevention of non-communicable diseases like cardiovascular disease, diabetes, and some cancers, and have been found in other foods. Marama bean seeds are good sources of complex carbohydrates, protein and oil (mono and poly-unsaturated fats). These beans contain micronutrients (vitamin A, E, B_3, B_6, B_{12}, folic acid, iron, zinc, and iodine) (Nepolo et al., 2009; Cheikhyoussef et al., 2010). The seed's protein level is on par with or slightly higher than that of soybeans. The oil content is close to that of peanuts and is two times that of soybeans. The immature tubers are more nutrient-dense than potatoes and yams and also contain protein (Maggs-Kölling et al., 2014).

Indigenous fruit-based foods in Namibia

Monkey orange (*Strychnos cocculoides* L.) is a type of indigenous wild fruit tree found in Namibia. Its fruits are eaten fresh straightaway after cracking because if exposed to air become inedible (Cheikhyoussef et al., 2013; Ngadze et al., 2017). This fruit provides carbohydrates, vitamins B, and C, iron, and zinc. To allow the fruit pulp to liquefy, monkey orange's fruit is buried in sand near homesteads. The ripe fruit can be consumed raw or sun-dried to make jam, fruit rolls, juice, and wines. The fruit is rich in vitamin C and B vitamins; however, the seeds of the monkey orange are toxic. In most cases, monkey oranges nutrient content varies depending on the plant species for instances *Strychnos innocua* reported to have a highest total carbohydrate content compared to others (Ngadze et al., 2017). Another species with brown colour such as *S. cocculoides*, *S. spinosa* and *S. pungens* has a good sources of phytochemicals (Ngadze et al., 2017).

Marula tree (*Sclerocarya birrea* L.) is a type of indigenous tree in Namibia (Cheikhyoussef et al., 2013). Marula fruits have two edible parts: fruits and nuts (kernels). These fruits can be eaten in fresh or fermented beverages (Mariod and Abdelwahab, 2012; Hiwilepo-van Hal et al., 2014) and processed into products like juice, jam and muffin (Cheikhyoussef et al., 2013). Marula fruits contain nutrients such as carbohydrates, monounsaturated fat, protein, micronutrients, and vitamin C. The nuts are consumed or used to extract oil (Martin, 2007). This nut is rich in protein, oil (monounsaturated fat), mineral compounds (Mg, P and K and phytochemical such as phenolic and antioxidant content. Marula tree (stem-bark ethanol extract) also have good medicinal properties by treating diabetics (Mariod and Abdelwahab, 2012; Hiwilepo-van Hal et al., 2014). Fresh or processed fruit is eaten. Dietary fibre, protein, vitamins (A, B_3, C, E, and carotene), mineral compounds, amino acids, and fatty acids are all present in significant levels in the fruit. Polyphenols, flavonoids, condensed tannins, and polysaccharides (pectin) are the main structural classes of marula fruit, and these substances can fend off chronic and degenerative illnesses. The marula fruit is a functional food because it contains substances that are good for health and can prevent sickness. Much research have shown that marula fruit is used in the production of juice, alcohol-based goods, jams and jellies, fruit leather, vinegar, and animal feed. When fully mature, the fruit's flavour is described to be pleasant rather than acidic and bitter. The marula is regarded as a multifunctional plant since the fruit kernels can be consumed or utilized to obtain oil (Mashau et al., 2022).

Cold-pressed oil from the seed kernels is a valued ingredient for skin-care products. It naturally softens, nourishes and revitalises the skin. It is absorbed easily and contain high levels of oleic and linoleic fatty acids, making it ideal for topical application. High in natural antioxidants, and one of the most stable oils available (ten times more resistant to oxidation and rancidity than olive oil), marula oil has been shown to use in cosmetic and for cooking (Maggs-Kölling et al., 2014).

Bird plum fruits (*Berchemia discolor* (Klotzsch) Hemsl) is an indigenous tree that grows in northern central Namibia. The fruits of this particular tree are eaten fresh or dried and processed in juice and jam. Bird plum fruits known as eembe in Oshiwambo are good sources of carbohydrates (sugar), vitamin C, mineral compounds (K, Ca, Mg, P), and phytochemicals such as flavonoid, total phenols, tannins, saponins, cardiac glycoside and tannins. To generate a potent indigenous alcoholic beverage like spirit or wine, eembe can be fermented. The fruit has a highly sweet flavor and can be used to flavour beer or porridge. The fruit juice is used to heal bleeding gums in traditional medicine (Cheikhyoussef et al., 2010). The fruit has a high sugar content in the pulp (30%), seeds that taste like walnuts, and 65 mg of vitamin C per 100 g of fruit. Eembe fruit harvesting takes place in northern Namibia between March and April. This occurs right at the end of the season when food crops are being cultivated at their peak. For the majority of farmers, picking wild fruits at this time of year is very convenient because it allows them to continue farming without interruption. In addition, after farmers have done their seasonal agricultural tasks, the bird plum fruits can be dried, stored, and processed (Nyambe et al., 2019).

Jackal berry (*Diospyros mespiliformis* L.), locally named Omwandi in Oshiwambo is an indigenous tree found mostly in the northern part of Namibia. Omwandi fruit trees bear fruits that are eaten when fresh or dried (Cheikhyoussef and Embashu, 2013). These fruits are rich in carbohydrates, protein, mineral compounds (calcium, copper, magnesium, sodium, iron, and potassium), and vitamin C (Magaji, 2019; Nyambe et al., 2019). Jackal berry fruits can be processed in jam and juice and when dried can be pounded and the powder is mixed with millet meal to make porridge called oshihenyandi (Danermark, 2019; Nyambe et al., 2019). The leaves are used for medicinal purposes, by treating conditions such as gingivitis, toothache, malaria, fever, wounds, sleeping sickness and helminths (Cheikhyoussef et al., 2011). The Diospyros species are widely used in traditional medicine in the tropical areas. As a tonic, powder, and poultice, leaves, barks, fruits, hardwoods, and roots have been used to treat a variety of ailments, including asthma, dermatitis, hypertension, atherosclerosis, lumbago, hemorrhage, sleeplessness, and biliousness, among others. Vermifuge, febrifuge, carminative, astringent, sedative, anti-hypertensive, constipation, and antidiuretic are among the common uses (Rauf et al., 2017).

Makalani palm (*Hyphaene petersiana* Klotzsch ex Mart) is an indigenous wild tree that grows in Namibia (Cheikhyoussef and Embashu, 2013). Makalani palm has two edible parts: fruits and the young trunk core eaten as a fresh vegetable. Makalani palm fruits are eaten when dried. This fruit is rich in protein, carbohydrates, micronutrients (potassium, zinc and iron), calcium and phosphorus are present in trace amounts. Intakes of 4,700 $mg.day^{-1}$ are approved to lower blood pressure level and decrease the risk of kidney stones (Nyambe et al., 2019). The trees may eventually die if the growth point is repeatedly cut off to extract sap for making palm wine. The stem pith can be consumed. A white endosperm core known as vegetable ivory, which is initially soft and edible and contains some liquid like coconut milk, lies beneath the fruit's outer fibrous husk. The Makalani palm's fruit, known as eendunga, is used to make ombike, the local alcoholic beverage (Cheikhyoussef and Embashu, 2013).

Manketti (*Schinziophyton rautanenii* Schinz) is another indigenous tree found in Namibia (Cheikhyoussef et al., 2019). Manketti has two edible parts nut or seed. The seed is eaten fresh or dried. The nuts can be milled and make products such as porridge and fermented juice, alcoholic drink and can be supplemented as food thickener when meat, fish and vegetable soaps and oil. This fruit is rich in carbohydrates, protein, and vitamins. Roasted seeds have a flavour akin to Brazil or cashew nuts. 60% of the seed's weight is oil. It is cooked using locally. The distinctive conjugated fatty acid, α-eleostearic acid, as well as other fatty acids including linoleic, oleic, and linolenic acids, are present in the edible yellow oil that is produced from the nut of the egg-shaped fruits (Maroyi, 2018).

Water lilies (*Nymphaea lotus* L.), the local name Omavo in Oshiwambo is found growing in ponds as a wild plant. The edible part is the seeds which are a good source of carbohydrates, lipids, and vitamins (Adelakuna et al., 2016). Water lilies roots can be made into products such as tea that can be used for treating sore throat and mounth irritation and the leaves can be used for lotion (soften skin). The flower stalks are consumed raw or cooked, the seeds are pickled or

roasted, the unripe fruits are consumed raw, the tubers are consumed raw or roasted for the starch, or they can be dried and processed into flour. The flour made from the mashed tubers can be kept in storage for several months (Wasagu et al., 2015).

Tiger nuts, scientific name *Cyperus esculentum* L. is a plant like glass with tubers. Tiger nuts have one edible part: tubers which can be eaten fresh, when dried or roasted, or baked as vegetable and can be processed into flour. Tiger nuts contain nutrients such as oil, protein, sugar, and fibre. Tiger nuts are rich in micronutrients (Cu, P, Zn, K, Na) (Wayah and Shehu, 2013; Adel et al., 2015; Nina et al., 2019). It also has bioactive components such as phenols, organic acids, and alkaloids. The tiger nut is a good source of monounsaturated fatty acid-rich edible oils. Tiger nut oil has a similar nutritional value to olive oil. Starch, a food element that is both renewable and inexpensive, is also widely present. Despite the relatively low protein content, it has been proven to be suitable for diabetic patients and people with digestive disorders, and after intake, it may help prevent heart disease. This tuber's dietary fibre helps to reduce gastrointestinal problems, colon cancer, and obesity. The tiger nut has strong antioxidant qualities and can be utilized as a source of natural antioxidants because flavonoids are present in it (Yu et al., 2022).

Hibiscus sabdariffa L. is herbal shrub plant local name in omutete in Oshiwambo and mutete in Rukwangali (Hilger, 2005; Ismail et al., 2008) The edible parts are calyxes and it is used to prepare herbal drink, beverages, and jam. *Hibiscus sabdariffa* calyxes are good sources of vitamin C, protein, beta-carotene, and total sugar. This nutrient content differs from leaves and seeds (Khan, 2017). Mutete is fermented beverages made from *Hibiscus sabdariffa*. Mutete is rich in vitamin A (beta carotene), iron, calcium, protein and organic acids (Anel et al., 2016; Khan, 2017). The safe medicinal plant known as *Hibiscus sabdariffa* is well known for its deliciousness as well as for its nutritional and therapeutic benefits. It contains a variety of medically significant substances known as phytochemicals. The use of plants in treating many medical conditions, such as cancer, inflammatory illnesses, and various cardiovascular problems, has been thoroughly studied by various academics in various contexts (Singhet al., 2017).

Indigenous vegetable-based foods in Namibia

There are several indigenous leaf vegetables consumed countrywide and known to have nutritional benefits (Hilger, 2005).

Amaranth (*Amaranthus thunbergia* Moq.) grows naturally in Namibia. This vegetable contains two edible

Figure 5 Nara (*Acanthosicyos horridus* Welw. Ex Hook.f.) processing in Namibia Desert
Source: Maggs-Kölling et al., 2014

parts: grains and leaves. This can be eaten when fresh or dried. The leaves are good sources of vitamin C, and dietary fibre. It can be used as spinach, combined with milk or fat and sorghum or maize, or eaten with pearl millet porridge. The grains contain protein nutrients. It is possible to cook the grains whole, and it turns very gelatinous in this form. However, because it is challenging to crush all of the little seeds in the mouth, some of the seeds will pass through the digestive system undigested (Venskutonis and Kraujalis, 2013; Maurya and Arya, 2018). Comparing 100 g of fresh amaranth leaves to 100 g of cabbage, the nutrients in amaranth are higher in protein (4.0 vs. 1.4%), calcium (480 vs. 44 mg), iron (10 vs. 0.8 mg), β-carotene (10.7 vs. 1.2 mg), and vitamin C (135 vs. 33 mg). *Amaranthus* leaves are a great source of antioxidant phytochemicals such as vitamin C, phenolic acids, and flavonoids as well as antioxidant pigments like betalain, β-xanthin, β-cyanin, anthocyanins, carotenoids, and chlorophyll. Amaranth contains antioxidants that act as a natural defence against a number some illnesses, including arthritis, emphysema, cancer, cataracts, atherosclerosis, retinopathy, cardiovascular diseases, and neurological diseases (Obianuju and Olubukola, 2022).

Nara (*Acanthosicyos horridus* Welw. Ex Hook.f.) is a melon-bearing bush that only grows in the Namib Desert and lacks leaves (Figure 5). The Namib Desert is the only place where plants may be found, and they are largely connected to the rivers that finish there or run through it, as well as the palaeochannels that they create. The stony desert plains are devoid of them. Nara resemble melons and typically weigh between 1 and 2.5 kg. Even when fully ripe, they have a pale green exterior and are spiky. It has a watery, orange-yellow flesh that is sweet and aromatic and has a taste similar to avocado or a cucumber-pineapple hybrid. Currently being studied in nara are cucurbitacins, which are bitter substances that are both toxic and potentially medicinal. The huge seeds have buttery kernels and are white or cream in color. The sweet, juicy flesh is eaten raw or cooked into a pulp. The pulp is used to make "nara chocolate" by drying it in the sun for several days straight on the sand or, more recently, on plastic sheets. The pulp is also consumed with oatmeal. Consumed alone or with cooked maize porridge, this fruit-roll product is high in vitamins, minerals, and trace elements. The dried seeds, which are incredibly tasty and healthy and contain 31% protein and 57% oil, are enjoyed as a snack. Both the chocolate and the seeds are easily transportable, retain their freshness for long periods, and are consumed frequently. The oil made from the seeds is either packaged or combined with other substances to make food or cosmetic items (Maggs-Kölling et al., 2014; Cheikhyoussef et al., 2017).

Figure 6 Ombidi (*Cleome gynandra* L.) – wild vegetable in Namibia
Source: West African plants, 2023

Ombidi (*Cleome gynandra* L.) is a wild vegetable in Namibia (Hilger, 2005) (Figure 6). It is eaten cooked as a vegetable mixed with amaranth. This vegetable is rich in protein, and micronutrients such as iron, calcium, vitamin A, fats, and vitamin C (Mishra et al., 2011). Some mashed foods have fresh leaves as an ingredient, and other weaning foods contain dried leaves that have been ground and added. Vitamin C concentration in leaves can be reduced by up to 81% when they are boiled, while it is reduced by 95% when they are dried (Heever and Venter, 2007). The tender leaves or young branches, and frequently the blooms, are boiled and eaten as a potherb, pleasant condiment, stew, or side dish throughout Africa. Other mashed foods have fresh leaves as an ingredient, other weaning foods contain dried leaves that have been ground and added. Due to their bitterness, the leaves are typically boiled with other green vegetables including cowpea, amaranth, and blackjack. The vegetable provides a great source of vitamins (A and C) and minerals (calcium and iron), in particular. Vitamin C concentration in leaves can be reduced by up to 81% when they are boiled,

while it is reduced by 95% when they are dried. The vegetable is a staple diet in rural parts of Namibia. This leafy vegetable, which in some nations is the only one available during the relish-gap period, is crucial to household food security during drought. To prepare a remedy that is consumed to treat illnesses like scurvy, leaves can be pulverized. In some cultures, leaves are cooked, marinated in sour milk for two to three days, and then consumed as a wholesome meal that is thought to enhance vision, give energy, and treat marasmus. It is a meal that is strongly advised for expectant and nursing mothers (Mishra et al., 2011).

Leaf rape (*Brassica napus* L.) is a traditional leafy vegetable widely grown Zambezi region. This vegetable consists of high-quality micronutrients (β-carotene and vitamin C), oil, proteins, and mineral compounds. The leaves can be used as a potherb, added to salads, or eaten raw or cooked. Also, the leaves are fermented for later use (Batista et al., 2011).

In Namibia are also used indigenous mushroom as a seasonal food. These mushrooms are a good source of protein, crude fibre, mineral compounds, and vitamins and are low in lipids. It also possesses good properties of anticancer, cardiovascular, and antibacterial effects (Olusegun, 2007).

Conclusion

Food and nutrition security can be improved by increasing indigenous foods intake. Better knowledge of the nutritional value needs to be provided to consumers, to increase the consumption of these foods. People in urban towns are willing to purchase indigenous and traditional products, particularly if they are proven to be nutritious and of health benefits. The majority of native foods are inexpensive sources of fibre, phytochemicals, micronutrients, unsaturated fat, complex carbohydrates, and protein. Traditional foods are crucial to include in our diets since they promote healthy lifestyles and lessen the risk of non-communicable diseases, including diabetes and hypertension. Native cuisine may also serve as a starting point for the creation of numerous therapeutic foods. Through international trade with developed markets, sustainable wild gathering and selling of indigenous goods have the potential to make a significant contribution to the reduction of rural poverty and the preservation of natural resources.

Conflicts of interest

The authors have no conflicts to declare.

Ethical statement

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

Funding

This publication was supported by the project NITT SK II "Národná infraštruktúra pre podporu transferu technológií na Slovensku".

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Silver nanoparticles initiation using *Calendula officinalis* L. hairy root extracts

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Article Details:
Received: 2023-10-05
Accepted: 2023-11-16
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0017

Nanoparticles (NPs) of various metals, in particular, copper, silver, gold, zinc, and titanium, are now considered promising and multifunctional components for biomedical applications. For the formation of nanoparticles, a "green" synthesis method has been developed based on the use of plant extracts to initiate nanoparticles. This method is considered simple and safe as it involves the use of extracts from well-studied plants. The main condition for the possibility of "green" synthesis of metal nanoparticles is the presence of the similar activity in the extracts. The possibilities of using *Calendula officinalis* L. hairy root extracts for the synthesis of silver nanoparticles (AgNPs) and its dependence on flavonoid concentration in the extracts were studied in this work. The extracts obtained with the use of 70% ethanol had the highest reducing activity in comparison with the aqueous extracts. Reducing activity of the extracts correlated with the concentration of flavonoids. The presence of nanoparticles of different sizes was confirmed by using transmission electron microscopy. Colloid solutions of AgNPs obtained using the extract with a higher content of flavonoids had significantly higher absorption values in the range of 420–440 nm, which is characteristic of AgNPs. An increase in absorption over time (up to two weeks) indicates long-term preservation of reducing activity in the mixture with $AgNO_3$. The smallest AgNPs (0.33… 7 nm) were formed when an aqueous extract was used, and the largest ones (up to 41.83 nm) with an extract obtained with 96% ethanol. Thus, it is the aqueous *C. officinalis* extract that should be chosen if it is necessary to obtain silver nanoparticles of small size.

Keywords: *Calendula officinalis*, rol genes, hairy roots, flavonoids, bioactivity, AgNPs

Introduction

Nanotechnology has made significant progress in the 21st century. Nanoparticles (NPs) of various metals, in particular, copper, silver, gold, zinc, and titanium, are now considered as promising and multifunctional components for biomedical applications (Dos Santos et al., 2014; Adebayo et al., 2019; Akinola et al., 2020). In the recent years, the interest highly increased

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in the use of nanoparticles in the biomedical field for diagnostics and treatment of diseases, in particular, for the targeted delivery of drugs, vaccines, and genes. Currently, more than 40 plant species have been tested to use plant extracts for the synthesis of silver nanoparticles (Rafique et al., 2017; Vanlalveni et al., 2021; Nie et al., 2023).

"Green" synthesis is a simple and safe method for metal nanoparticle initiation. It is based on the possibility that plant-derived compounds reduce the ions of metals accompanied by NP formation. It is known that plants synthesize a wide spectrum of bioactive metabolites (polyphenols, tannins, polysaccharides, proteins, amino acids, alkaloids, etc.). Due to their chemical activity, these compounds can reduce metal ions, resulting in the formation of metal nanoparticles. In addition, these plant compounds can also act as NP stabilizers, thus providing stability to the newly formed nanoparticles (Asif et al., 2022). In particular, Giri et al. (2022) studied AgNPs synthesized by *Eugenia roxburghii* DC. They evaluated spectrophotometric characteristics, their structure (by X-ray diffraction), together with morphology and size (by high-resolution transmission electron microscope). Liaqat et al. (2022) obtained stable silver nanoparticles using *Eucalyptus camaldulensis* and *Terminalia arjuna* extracts, as well as their combinations.

According to the published data, different plant extracts were used for silver nanoparticle (AgNPs) initiation. Such interest in the development of methods of "green" synthesis of nanoparticles is promoted by their bioactivity and possible practical application. For example, it was shown that AgNPs possessed antibacterial, antifungal (Jalal et al., 2018; Khan et al., 2021; Marinescu et al., 2022), and anticancer (Hemlata et al., 2020; Wang et al., 2021) activities.

Calendula officinalis L. is a medicinal plant with a sufficiently well-studied chemical composition. Secondary metabolites synthesized by the plants are used to treat a number of diseases (Butnariu and Coradini, 2012; Ashwlayan et al., 2018; Ak et al., 2020). In recent years a number of metal nanoparticles were obtained using *C. officinalis* extracts, in particular, titanium (Wei et al., 2021), gold (Zhao et al., 2022), and silver (Zhangabay and Berillo, 2023), and their possible practical application in pharmacology and medicine was studied. Earlier *C. officinalis* hairy roots were used for the production of secondary metabolites, as well as for studying the effect of various factors on their growth and biosynthetic activity (Długosz et al., 2013; Al-Abasi et al., 2022).

In our work, we investigated the possibility of using *C. officinalis* hairy root extracts that differed in flavonoid content for AgNPs synthesis. Special interest in the use of extracts from the hairy roots of medicinal plants is associated with a possible increase in their content of bioactive compounds with reducing properties.

Material and methodology

Preparation of extracts

To prepare the extracts, *C. officinalis* hairy roots from the *in vitro* collection of the Institute of Cell Biology and Genetic Engineering, NAS of Ukraine(Figure 1a) were cultured on solidified Murashige and Skoog medium (Duchefa, Netherland) for 4 weeks. After that, the roots were removed from the medium, washed with deionized water, homogenized in the solvents (deionized water, 40%, 70%, and 96% ethanol), and centrifugated at 14,000 rpm for 10 minutes (Eppendorf Centrifuge 5415C). The supernatants were collected and used for the further studies.

Total flavonoid content assay

To determine the total content of flavonoids, we have used a reaction with aluminum chloride (details in Matvieieva et al., 2019). The optical density of the samples was measured using a Fluorat-02 Panorama spectrofluorimeter at λ = 510 nm. The content of flavonoids was estimated in $mg.g^{-1}$ wet weight (ww) in routine equivalent (RE) using the calibration graph C = 1.9575*x*, R^2 = 0.9723.

Nanoparticle initiation

The extracts were added to the silver nitrate solution at a concentration of 1 mM (200 µl of the extract was added to 4 ml of the silver nitrate solution), mixed, and incubated in a water bath at a temperature of 80 °C for 60 min. The presence of reductive activity was determined visually by the formation of a colloidal solution of AgNPs (a change in the color of the solution to yellow-brown). The presence of the formed nanoparticles was confirmed using transmission electron microscopy.

UV-Vis spectra assay

The surface plasmon resonance of the samples (spectra of the obtained AgNPs colloidal solutions) was measured in 1, 7, and 14 days in the wavelength range of 300… 700 nm by a Fluorat-02 Panorama spectrophotometer.

Transmission electron microscopy

The transmission electron microscope (TEM) analysis was performed with a JEM-I230 microscope (JEOL, Tokyo, Japan), with an accelerating voltage of 80 kV. For the sample preparation, 0.05 µl of AgNP colloid solutions were applied to a grid and air-dried. AgNPs spectrometry was performed using an X-MAX N 80T detector (Oxford's instruments, Oxford, UK) integrated with a TEM 1230 transmission electron microscope (Jeol, Tokyo, Japan) operating at 15 kV accelerating voltage. Nanoparticle sizing was conducted using the ImageJ software (https://imagej.net/ij/download.html).

Statistical analysis

All analyses were performed in triplicate. Data were analyzed with ANOVA followed by Tukey's test using R version 4.2.2 software. Differences at the level of $p < 0.05$ were considered statistically significant.

Results and discussion

The samples obtained by the extraction with different solvents were studied for the total flavonoid content. This content differed depending on the used solvent. It was the lowest in the case of using water for the extraction (3.40 ±0.12 mg RE.g^{-1} ww) and increased when ethanol in the concentrations of 40%, 70%, and 96% was used (5.75 ±0.22, 6.68 ±0.48, and 5.12 ±0.26 mg RE.g^{-1} ww, respectively).

After adding the extracts to the silver nitrate solution and heating the mixture, the appearance of a yellow-brown color was observed. This is an evidence of the formation of silver nanoparticles. Analysis of the UV-Vis spectra of the obtained colored solutions carried out in the range of λ = 300… 600 nm, revealed a characteristic peak at a wavelength of about 420–430 nm that confirms the possessed reducing activity of the extracts. According to the spectra shown in Figure 1, the extract obtained with the use of 70% ethanol had the highest activity (the optical density changed from 1.60 units in one day to 2.43 units in 14 days). The lowest activity was detected in the case of aqueous extract (0.50 units in one day and 1.25 units in 14 days). Therefore, the extract, which had the highest concentration of flavonoids, showed the highest reducing activity in the reaction with silver nitrate. It should be noted that the height of the peaks increased during two weeks (Figure 1A–B a, b, c). This indicates the stability of the reducing activity of the extracts in the reaction mixture during this period.

Earlier (Bohdanovych and Matvieieva, 2022) we suggested using the process of obtaining a colloidal

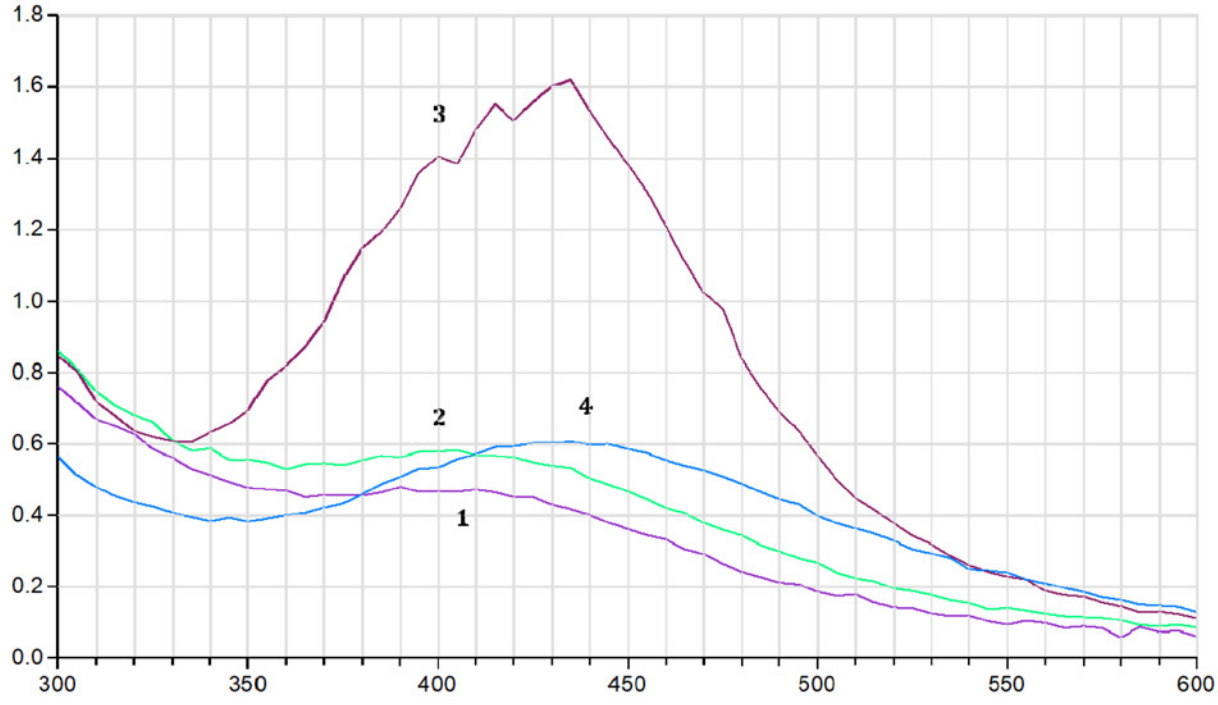
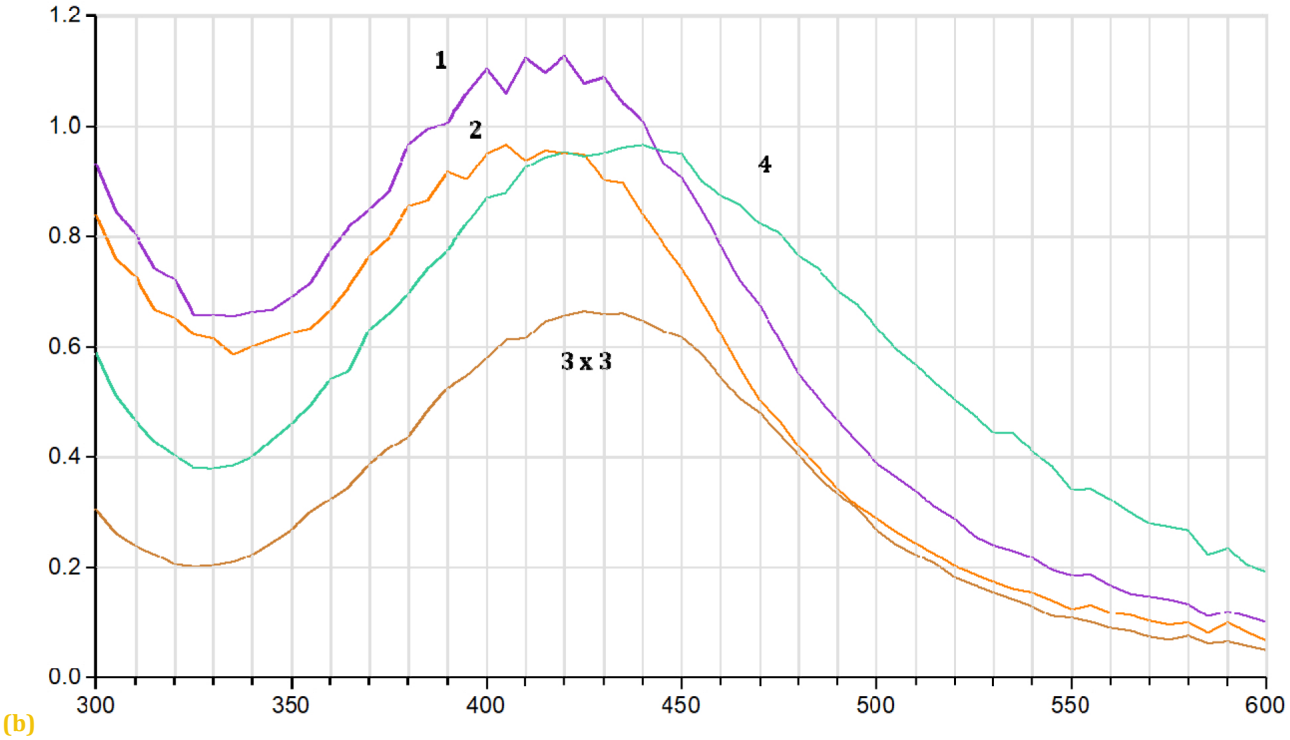
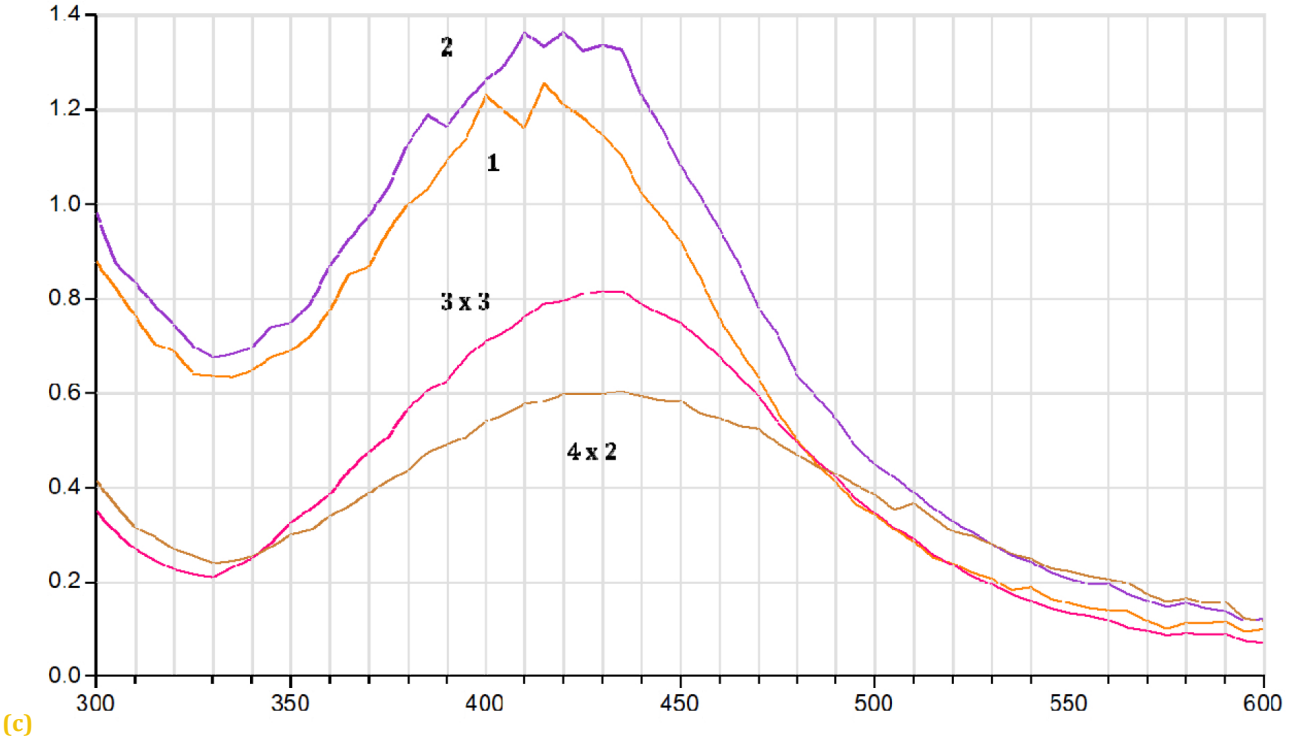


Figure 1A UV-Vis absorption spectra of colloidal solutions of silver nanoparticles in one (a), 7 (b), and 14 days (c) days after the initiation of their formation
aqueous (1) and ethanol (2 – 40%, 3 – 70%, 4 – 96%) extracts from *Calendula officinalis* L. hairy roots; x3 and x2 – the sample were diluted three or two times



(b)



(c)

Figure 1B UV-Vis absorption spectra of colloidal solutions of silver nanoparticles in one (a), 7 (b), and 14 days (c) days after the initiation of their formation
aqueous (1) and ethanol (2 – 40%, 3 – 70%, 4 – 96%) extracts from *Calendula officinalis* L. hairy roots; x3 and x2 – the sample were diluted three or two times

solution of silver nanoparticles for a comparative assessment of the reducing power of plant extracts added to the $AgNO_3$ solution. This conclusion was based on the analyses of the UV-Vis spectra of colloid solutions obtained by various extracts of hairy wormwood roots. The studied reducing activity correlated with the content of flavonoids in the used extracts. According to these data, the obtained results indicate that the spectrophotometric analysis can indeed be used as an indicator of reducing activity since a correlation of the height of the peaks at $\lambda = 440$ nm with the total content of flavonoids in extracts from wormwood was found. Alim and coauthors (Alim et al., 2020) approved the possibility of using spectra in the UV-Vis range to characterize nanoparticle solutions. Similar results were obtained by us in the study of four extracts from *C. officinalis* hairy roots. Colloid solutions of AgNPs obtained using the extract with a higher content of flavonoids (No 3) had significantly higher absorption values in the range of 420–440 nm, which is characteristic of AgNPs. An increase in absorption over time (up to two weeks) indicates long-term preservation of reducing activity in the mixture with $AgNO_3$.

The analysis using transmission electron microscopy confirmed the presence of nanoparticles (Figure 2). Nanoparticles were mostly round, but varied in size. According to the data obtained, the smallest AgNPs (0.33… 7.0 nm) were formed when an aqueous extract was used, the largest ones (up to 41.83 nm) – when an extract obtained with 96% ethanol was used for NP initiation (Figure 3). Such differences are obviously caused by the different chemical composition of the extracts used for NPs formation (higher content

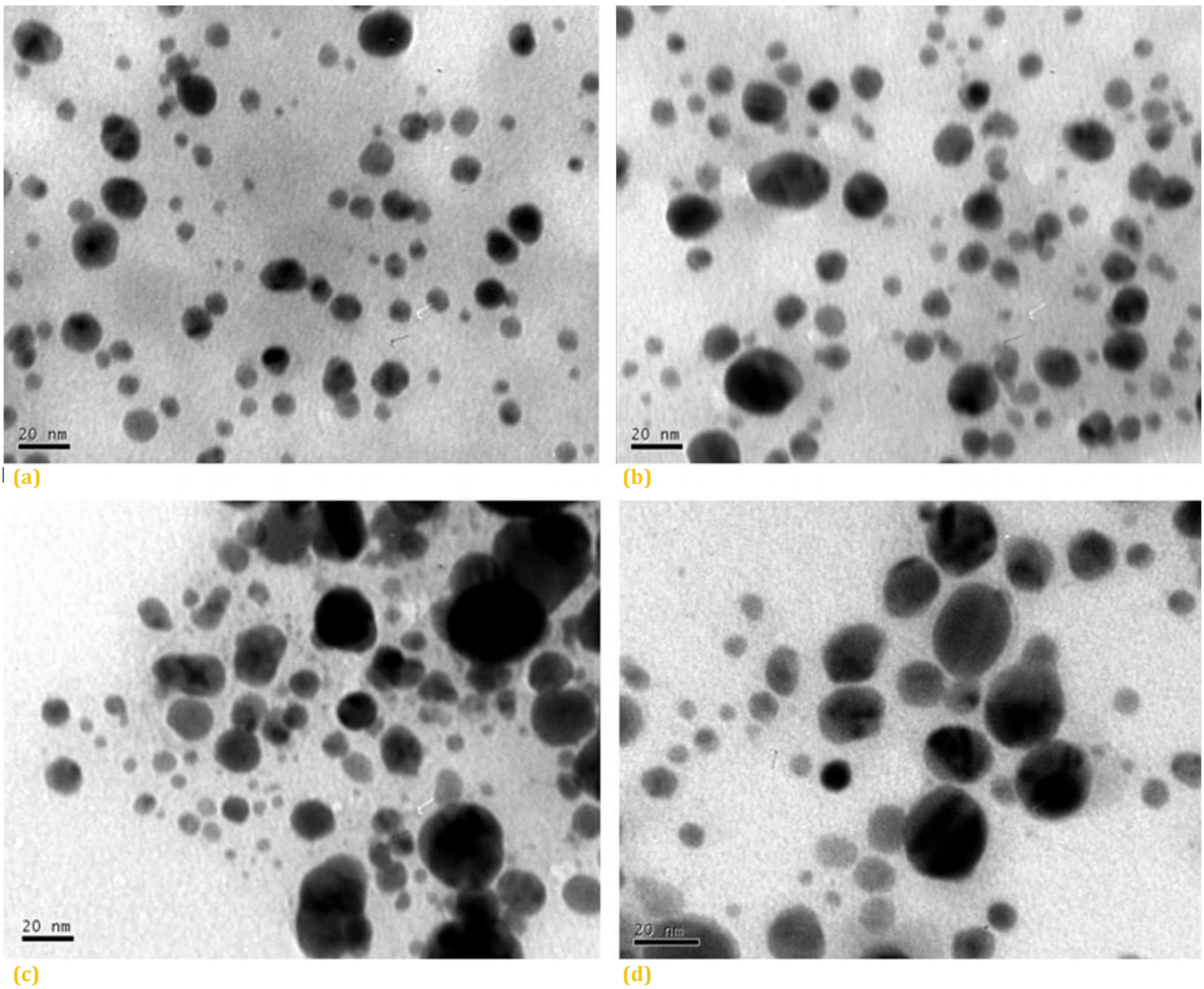


Figure 2 Microphotographs (TEM, JEM-I230) of silver nanoparticles obtained using *Calendula officinalis* L. hairy roots extracts
a – aqueous; b – 40%; c – 0%; d – 96% ethanol

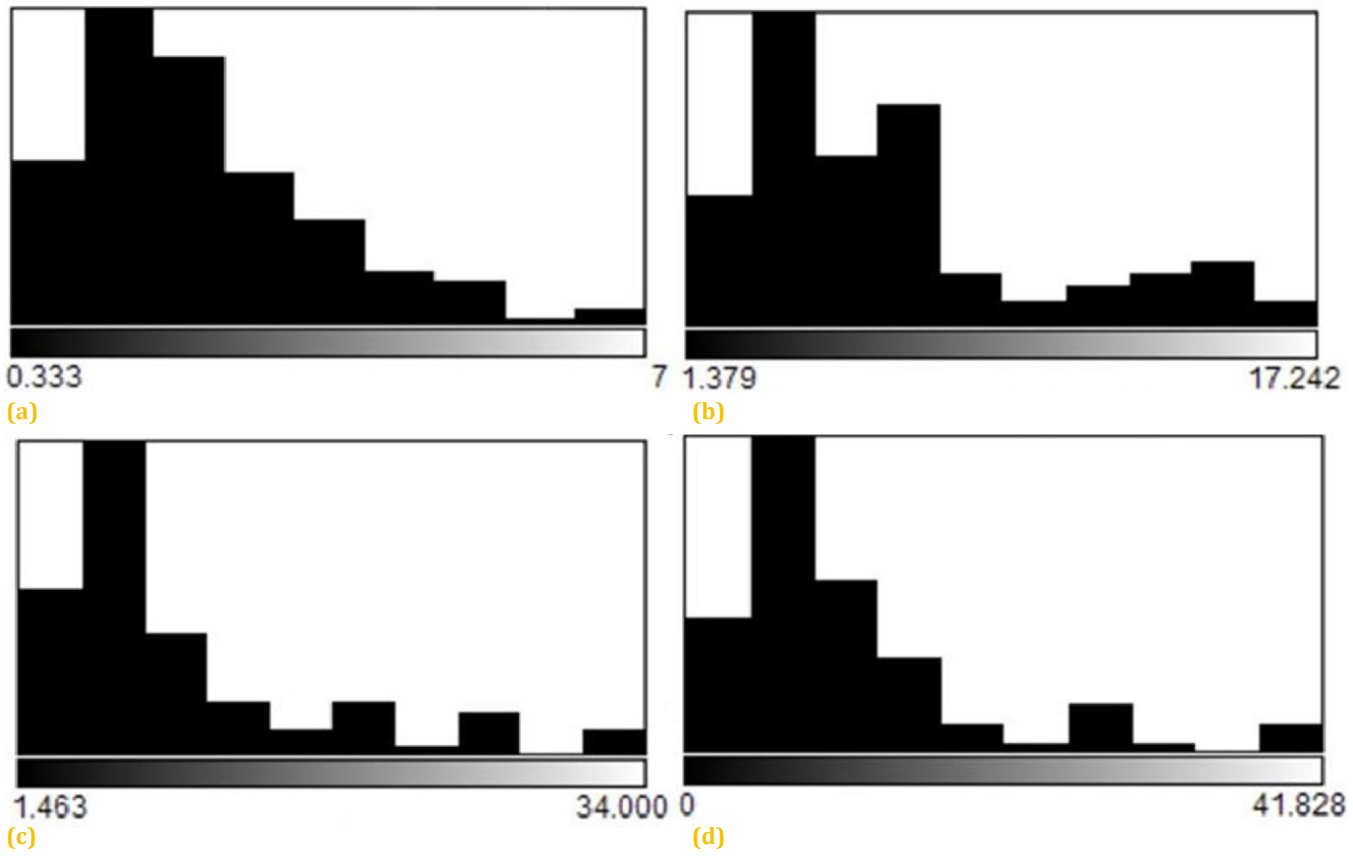


Figure 3 AgNPs size distribution
a – aqueous; b – 40%; c – 0%; d – 96% ethanol extracts were used for nanoparticle initiation

of glycosylated flavonoids in the aqueous extract and higher concentration of aglycones in the extract obtained using high concentration ethanol).

Silver nanoparticles were previously obtained by different groups of researchers using extracts from *C. officinalis* plants. Silver nanoparticles are usually obtained by reacting plant extracts with silver nitrate solution (El-Kemary et al., 2016; Xu et al., 2021; Asif et al., 2022; Liaqat et al., 2022). However, it was shown that such NPs can also be obtained using a solution of silver sulfate (Olfati et al., 2021). Dried leaves or flowers (El-Kemary et al., 2016; Olfati et al., 2021; Xu et al., 2021; Balciunaitiene et al., 2022) were used for this purpose. At the same time, until now, the hairy roots of these plants have not been used to obtain silver nanoparticles. However, hairy roots obtained by genetic transformation may have a changed chemical composition. Therefore, nanoparticles obtained using them may also have changed characteristics.

In various studies, the authors present the characteristics of the NPs. Such AgNPs differed in size and other parameters (TEM, Zeta potential, fluorescence spectroscopy, FT-IR spectrum, UV-visible absorption spectra analyses). These features are probably related to differences in the qualitative and quantitative chemical composition of the used extracts, extract/silver nitrate solution ratios, or the use of silver sulfate in the reaction mixture. Unfortunately, it is impossible to make a correlation between the composition of the extracts and the characteristics of the nanoparticles, since the publications lack data on the chemical analysis of the used extracts. At the same time, it is possible to compare the sizes of nanoparticles obtained in different studies. In particular, El-Kemary et al. (2016) mixed dried *C. officinalis* plants with 32% EtOH for extract obtaining. This extract was used for AgNPs initiation after the incubation with $AgNO_3$ solution. Biosynthesized AgNPs ranged from 30 to 50 nm. Analysis of surface plasmon resonance in the UV-visible absorption spectrum of a colloidal solution of NPs revealed the maximum absorption peak at 435 nm. In the experiments of Xu et al. (2021), dried leaves of *C. officinalis* were ground and extracted by boiling water. The nanoparticles were formed in a spherical shape in the range of 38.05 to 75.41 nm. SPR bands were detected at the wavelength of 452 nm. Balciunaitiene et al. (2022) studied the process of NPs initiation by *C. officinalis* aqueous inflorescences

extract as a reducing agent. The size of NPs was up to 35 nm and the particles do not tend to form large agglomerates.

A comparison of the results of the above studies shows that, in general, the use of *C. officinalis* water extracts or using of ethanol in lower concentrations for the extraction can result in the production of smaller AgNPs. Such data are consistent with the results of our experiments since nanoparticles of a smaller size were also obtained using the aqueous extract (up to 7 nm). The use of 70% or 96% ethanol to obtain extracts has led to a significant increase in the size of nanoparticles (up to 40 nm). Probably, this may be related to the different chemical compositions of such extracts and the specifics of extracting compounds from *C. officinalis* plants. These results should be taken into account when it is necessary to obtain nanoparticles of different sizes.

In our work, we did not study the bioactivity of the obtained AgNPs, but such work is of interest and will be conducted. The expediency of such an analysis emerges from the results of a number of publications regarding the presence, for example, of antimicrobial (Olfati et al., 2021; Asif et al., 2022; Liaqat et al., 2022; Giri et al., 2022) and antitumor activity (Xu et al., 2021) of silver nanoparticles obtained using *C. officinalis* plants.

Conclusions

The possibilities of using *C. officinalis* hairy root extracts for AgNP synthesis and its dependence on flavonoid concentration in the extracts were studied. The extracts obtained with the use of 70% ethanol had the highest activity in comparison with the aqueous extracts. The reducing activity of the extracts correlated with the concentration of flavonoids. The presence of nanoparticles of different sizes was confirmed by using transmission electron microscopy. The smallest AgNPs (0.33… 7.0 nm) were formed when an aqueous extract was used, and the largest ones (up to 41.83 nm) with an extract obtained with 96% ethanol.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

Funding

The work was financially supported by the National Academy of Sciences of Ukraine (research project 0123U101081 “Synthesis of recombinant pharmaceutical proteins and increasing the content of biologically active natural compounds in plants”).

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article

OPEN ACCESS

In vitro antibacterial efficacy of different natural linden honey against some gram-positive and gram-negative strains

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Article Details:
Received: 2023-10-07
Accepted: 2023-11-16
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0018

Linden honey is rich in organic acids, such as formic acid and malic acid, which have antibacterial and anti-inflammatory properties. This, in turn, helps prevent bacterial infections and inflammatory diseases that can lead to serious cardiovascular disorders. These bactericidal and antiviral properties are due to the high content of inhibin, lysozyme, and apidicin. These are bactericidal and antiviral enzymes that are responsible for the strong antibiotic properties of linden honey. The aim of the current study was *in vitro* antimicrobial profiling of different natural linden honey produced by Polish manufacturers, exhibiting inhibitory activity against Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™. The different natural linden honey produced by Polish manufacturers such as Beekeeping farm "Pszczółka" (Ustka, Poland), Beekeeping farm "Mazurskie Miody Bogdan Piasecki" (Tomaszkowo, Poland), Beekeeping farm "Sądecki Bartnik" (Stróże, Poland), Beekeeping farm "Zaczarowany Ogród", and Beekeeping farm "Karolczak Cezary" (Sławno, Poland) were used in the current study. The samples were stored in resalable vials at 5 °C in the dark but were allowed to adjust to room temperature before investigation. The testing of the antibacterial activity of different natural linden honey was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. This study demonstrated that all samples of natural linden honey produced by Polish manufacturers demonstrated mild antibacterial activity against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains. More sensitive to all samples of natural linden honey studied was *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain following *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain. *Pseudomonas aeruginosa*

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(Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey. The presented results revealed the antibacterial activities of different samples of linden honey produced by Polish manufacturers, which require further study to be correctly understood and explained.

Keywords: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* strains, Kirby-Bauer disc diffusion technique

Introduction

Linden honey is a variety of honey produced by bees from the nectar of linden (*Tilia* L.) flowers. *T. cordata* Mill. and *T. platyphyllos* Scop. are native to much of Europe, with their ranges extending from southern Finland to southern Italy and the Caucasus. *T. cordata* is the more abundant of the two species and its core region is central and eastern Europe (Eaton et al., 2016). Linden flowers contain many beneficial compounds that can also be found in linden honey, including tannins, organic acids, flavonoids, and pectins (Naef et al., 2004; Qiao et al., 2020; Bodor et al., 2020). Linden trees were among the most sacred trees in the Slavic tradition, perhaps that is why now linden honey is one of the most popular species in Poland (Ţenche-Constantinescu et al., 2015).

The visual and taste properties of linden honey make it quite distinctive. Linden honey has an intense and very "honey" flavor, sweet but sour. Some people even claim that the taste of linden honey is a bit sharp and sometimes it can even be slightly bitter. In turn, the color of linden honey is light yellow and straw-colored, but it can also be slightly greenish depending on the linden variety and the harvesting period (Konopleva, 2011; da Silva et al., 2016). Linden honey contains 74% glucose and fructose, about 1.5–3.0% sucrose and water. Micronutrients present in linden honey include essential oils, flavonoids, the glycoside tiliacin, the triterpene taraxerol, tannins, bitter compounds, and the enzyme lysozyme. The main mineral is potassium. Honey contains vitamins C, H, PP, and B vitamins (Dżugan et al., 2018). The glycemic index of this product, due to the content of simple sugars, is relatively high (although not the highest among honey). It is approximately 55, depending on the origin of linden honey, which places it among kinds of honey with an average glycemic index. It can therefore be included in the diet of diabetics in a very small and controlled amount (Atayoğlu et al., 2016).

Linden honey is worth using for colds, flu, upper respiratory tract infections, bronchitis, and pneumonia. It has diaphoretic, antitussive, expectorant, and antispasmodic properties. It has unique healing properties, supports the nervous and immune systems, and contains lots of antioxidants. It soothes inflammation and has an antipyretic effect. Due to its diuretic effect, reducing swelling and gently lowering blood pressure, linden honey is recommended for circulatory system diseases. For people with cardiovascular disorders, its antispasmodic and calming effects, which result from the presence of essential oils, are also important. For this reason, it is recommended to use honey in cases of insomnia, chronic stress, nervous hyperactivity, and even neurosis (Khalil, 2023).

Linden honey has strong antibiotic, diaphoretic, antitussive, expectorant, and antispasmodic properties. Linden honey is recommended for urinary and genital system infections, digestive system disorders due to its antibiotic activity, and rheumatic diseases due to its anti-inflammatory effect. Honey eliminates pathogenic microorganisms such as staphylococci, streptococci, yeast fungi, *Klebsiella pneumonia*, *Escherichia coli*, and *Candida albicans* (Lusby et al., 2005; Irish et al., 2006; Kuncic et al., 2012; Almasaudi, 2021). The effectiveness of honey against microorganisms depends on the type of honey produced, which depends on its botanical origin, the health of the bees, its origin and the processing method (Almasaudi, 2021).

In the current study, *in vitro* antimicrobial profiling of different natural linden honey produced by Polish manufacturers was performed, exhibiting inhibitory activity against Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™.

Materials and methodology

Natural linden honey

The different natural linden honey produced by Polish manufacturers such as Beekeeping farm "Pszczółka" (Ustka, Poland; 54° 34′ 43″ N, 16° 52′ 09″ E), Beekeeping farm "Mazurskie Miody Bogdan Piasecki" (Tomaszkowo, Poland; 53° 43′ 04″ N 20° 24′ 32″ E), Beekeeping farm "Sądecki Bartnik" (Stróże, Poland; 49° 39′ 24″ N, 20° 58′ 48″ E), Beekeeping farm "Zaczarowany Ogród", and Beekeeping farm "Karolczak Cezary" (Sławno, Poland; 54° 21′ 44″ N, 16° 40′ 49″ E) were used in the current study. The samples were stored in resalable vials at 5 °C in the

dark but were allowed to adjust to room temperature before investigation.

Determination of the antibacterial activity of honey samples by the disk diffusion method

The testing of the antibacterial activity of linden honey was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). In the current study, Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ were used. All strains used in the current study originated from ATCC (American Type Culture Collection), a nonprofit, global biological resource center and standards organization and the leading developer and supplier of authenticated cell lines and microorganisms. Strains were purchased in Pol-Aura Sp. z o.o. (Zabrze, Poland).

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

Statistical analysis

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

Results and discussion

Figures 1 and 2 summarize the results obtained by the mean diameters of the inhibition zone around the growth of *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains induced by different natural linden honey produced by Polish manufacturers.

After applying different natural linden honey to *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain, we noted a statistically non-significant increase in the zone of growth inhibition by 17.72% ($p > 0.05$) for linden honey ("Zaczarowany Ogród"), by 13.95% ($p > 0.05$) for linden honey ("Sądecki Bartnik") and by 4.83% ($p > 0.05$) for linden honey ("Mazurskie Miody Bogdan Piasecki") compared to the control samples (9.54 ±0.85 mm). Linden honey from Beekeeping farm "Pszczółka" and Beekeeping farm "Karolczak Cezary" caused the decreased inhibition zone diameters after application on culture dishes with *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain (by 14.89% and 14.15%, $p > 0.05$, respectively) compared to the control samples (Figure 1).

We observed similar trends after *in vitro* application of different natural linden honey against *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain, where we also observed a statistically significant increase in the zone of growth inhibition by 40.14% ($p < 0.05$) for linden honey from Beekeeping farm "Pszczółka", by 41.98% ($p < 0.05$) for linden honey from Beekeeping farm "Mazurskie Miody Bogdan Piasecki". A statistically non-significant increase in the zone of growth inhibition of growth of *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain after *in vitro* application of linden honey was observed, i.e. by 13.38% ($p > 0.05$) for linden honey from Beekeeping farm "Sądecki Bartnik", by 10.29% ($p > 0.05$) for linden honey from Beekeeping farm "Zaczarowany Ogród", and by 15.07% ($p > 0.05$) for linden honey from Beekeeping farm "Karolczak Cezary" against the control samples (7.10 ±0.56 mm) (Figure 1).

Pseudomonas aeruginosa (Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey. After applying linden honey samples to *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain, a statistically non-significant

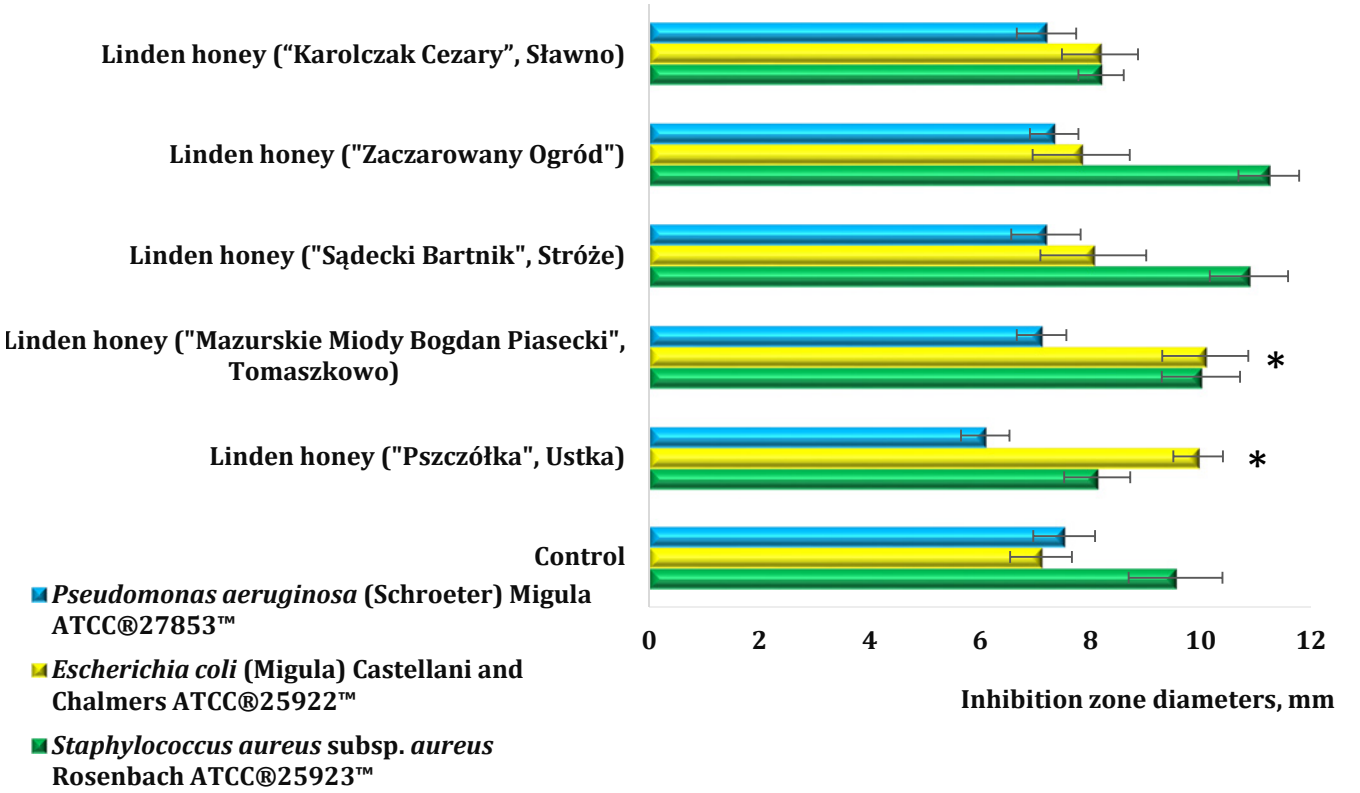

Figure 1 The mean inhibition zone diameters induced by different natural linden honey produced by Polish manufacturers against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ (M ±m, n = 8)
*– changes were statistically significant compared to the 96% ethanol

decrease in the zone of growth inhibition was noted, i.e. by 19.02% (p >0.05) for linden honey from Beekeeping farm "Pszczółka", by 5.46% (p >0.05) for linden honey from Beekeeping farm "Mazurskie Miody Bogdan Piasecki", by 4.39% (p >0.05) for linden honey from Beekeeping farm "Sądecki Bartnik", by 2.40% (p >0.05) for linden honey from Beekeeping farm "Zaczarowany Ogród", and by 4.26% (p >0.05) for linden honey from Beekeeping farm "Karolczak Cezary" compared to the control samples (7.52 ±0.56 mm) (Figure 1).

The results of the current study revealed that all samples of natural linden honey produced by Polish manufacturers demonstrated mild antibacterial activity against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains. More sensitive to all samples of natural linden honey studied was *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain following to *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain. *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey.

Among the features of linden honey, it is worth highlighting its antibacterial, expectorant, diaphoretic, and antipyretic properties. It may have a calming and slightly soporific effect. In practice, it has an antitussive, warming, soothing effect and regulates blood pressure. Also, linden honey has a mild diuretic effect on the human body. Due to the above-mentioned features of linden honey, it is certainly worth emphasizing its help in the fight against colds, flu symptoms, and diseases of the respiratory, circulatory, and nervous systems (Khalil, 2023). Apart from colds and periods of reduced immunity, linden honey is worth using due to its beneficial effect on the nervous system in the face of insomnia or exhaustion of the body. In states of nervousness or increased stress, linden honey can also be great. Moreover, it can be a great ingredient in the diet of athletes due to the content of easily digestible carbohydrates. Convalescents can also benefit from its properties, because not only is it a source of easily accessible energy (which they need in excess), but also due to its ingredients it supports the immune system. Linden honey can be successfully used, for example, in peptic ulcer disease; it alleviates the symptoms associated with it (Khalil, 2023).

The factors influencing the antibiotic effect of honey are quite complex. They can be divided into three groups: physical, chemical, and biological (Kędzia and Hołderna-Kędzia, 2017). Physical factors include high osmotic pressure resulting from the high sugar content in honey, as well as low pH caused by the presence of organic acids. Chemical factors include primarily hydrogen peroxide produced as a result of an enzymatic reaction (glucose oxidase), as well as methylglyoxal found in manuka honey and, in some varieties of honey, high content of phenolic compounds, including phenolic acids and flavonoid compounds. However, biological factors include peptides – lysozyme and defensin-1, probably the same substance, only named differently (Patton et al., 2006; Al-Waili et al., 2011; Kwakman and Zaat, 2012; Almasaudi, 2021). Other phytochemical factors such as tetracycline, peroxides, amylase, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohols, and benzoic acid make honey active against pathogenic bacteria and have either bacteriostatic or bactericidal effects. All of these factors vary depending on the nectar source and storage conditions (Almasaudi, 2021).

Gram-positive target strains were most susceptible to honey samples. In contrast, according to previous observations, Gram-negative microbes were less sensitive to all honey samples (Kwakman et al., 2011; Mandal and Mandal, 2011). The difference in susceptibility to honey and other antibacterial agents between Gram-positive and Gram-negative microbes may be due to cell wall composition. Gram-positive bacteria do not have an outer membrane protecting the peptidoglycan layer, unlike Gram-negative bacteria, which makes it easier for antimicrobial agents to penetrate and cause damage (Matzen et al., 2018).

As demonstrated in the literature, honey has antibacterial activity (bacteriostatic and bactericidal effect), similar to antibiotics, against test organisms and provides alternative therapy against certain bacteria (Mohapatra et al., 2011). The antibacterial activity of honey likely depends on the pasture in which the bees were raised, climatic conditions, as well as the natural composition of the flower nectar (Abd-El Aal et al., 2007). Many studies revealed that honey was effective against methicillin-resistant *Staphylococcus aureus*, hemolytic streptococci, and vancomycin-resistant enterococci (Lusby et al., 2005).

The antioxidant and antibacterial activity of 21 ypes of honey derived from Mount Olympus (Mt. Olympus), a region with great plant biodiversity, were studied by Stagos et al. (2018). The antibacterial activity was examined against the growth of S. *aureus* and *P. aeruginosa* by the agar well diffusion assay and the determination of the minimum inhibitory concentration (MIC). The MIC of the tested honey types against S. *aureus* ranged from 3.125 to 12.5% (v/v), while the MIC of Manuka honey was determined to be 6.25% (v/v). The MIC values of the tested honey

Figure 2 The mean diameters of the inhibition zone around the growth of *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ (A) and *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ (B) induced by different natural linden honey produced by Polish manufacturers

types against *P. aeruginosa* ranged from 6.25 to 12.5% (v/v) and the MIC of Manuka honey was determined at 12.5% (v/v). Moreover, the results suggested that the presence of hydrogen peroxide and proteinaceous compounds in the honey types accounted, at least in part, for the antibacterial activity. Better free radical scavenging efficiency has been demonstrated in these types of honey compared to Manuka honey. Moreover, antioxidant activity was observed in four types of honey tested that were converted into powder by freeze-drying. Data showed that the three powdered honey retained their antioxidant properties after lyophilization, making them suitable for further bioactivity assessment and application (Stagos et al., 2018).

The physicochemical characteristics and antioxidative, antibacterial and antiproliferative effects of nineteen samples of different honey types (acacia, linden, heather, sunflower, phacelia, basil, anise, sage, chestnut, hawthorn, lavender and meadow) collected from different locations in the Western Balkans (Republic of Serbia, Kosovo, Bosnia and Herzegovina, and Northern Macedonia) were evaluated by Sakač et al. (2022). Antibacterial activity was estimated *in vitro* using agar diffusion tests and measuring minimal inhibitory concentration (MIC). Among investigated bacterial strains following resistant potencies were determined: *Escherichia coli* > *Escherichia coli* ATCC 8739 > *Enterococcus faecalis* > *Proteus mirabilis* > *Staphylococcus aureus* > *Staphylococcus epidermidis*. The linden honey from Fruška Gora (MIC values of 3.12% and 6.25% against *Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively) and phacelia honey (MIC values of 6.25% and 3.12% against *Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively) showed the strongest antibacterial activity (Sakač et al., 2022). The antibacterial potential of linden honey could be related to methyl syringate, which was found to be the most abundant component besides lindenin in linden honey and known to act as an antibacterial agent (Almasaudi et al., 2017; Sakač et al., 2022). As mentioned by Qiao et al. (2020), methyl syringate and lindenin were the most abundant components in rape and linden honey; moreover, their average contents reached 10.44 and 21.25 $mg.kg^{-1}$, respectively. The presence of two identified terpene acids, i.e., 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid (1) and 4-(1-methylethenyl) cyclohexa-1,3-diene-1-carboxylic acid (2), was confirmed in (Swiss) linden honey after solid-phase extraction and HPLC purification were identified by Frérot et al. (2006).

Hulea et al. (2022) investigated the antioxidant profile and the antimicrobial activity of four different types of monofloral honey (manuka, brassica rapeseed, acacia, and linden honey) against some bacterial/fungal ATCC strains and some multidrug-resistant strains isolated from chronic otitis in dogs. The antioxidant characterization of the analyzed honey samples showed the highest antioxidant activity and concentrations of total polyphenols and total flavonoids in Manuka honey, followed by linden honey. Manuka honey was proven to be the most effective on most clinical isolates concerning antimicrobial activity in comparison with brassica rapeseed, acacia, and linden honey. Except for *B. cepacia* and *P. vulgaris*, all the clinical isolates were sensitive to the antibacterial activity of honey. Regarding the ATCC strains, Manuka honey 10% was the most effective in inhibiting all the strains tested except for *P. aeruginosa*. The efficacy classification was Manuka honey > brassica rapeseed honey > acacia honey > linden honey (Hulea et al., 2022).

Correlation establishing between factors affecting the antibacterial nature of honey (osmolarity, acidity, content of hydrogen peroxide and non-peroxide components) and the honey harvest area environmental parameters as well as harvesting, processing and storage conditions is necessary to clarify the reasons for minor differences of the antibacterial efficacy of the same concentrations linden honey different manufacturers.

Conclusions

In the current study, we assessed *in vitro* antimicrobial profiling of different natural linden honey produced by Polish manufacturers was performed, exhibiting inhibitory activity against Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™. This study demonstrated that all samples of natural linden honey produced by Polish manufacturers demonstrated mild antibacterial activity against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains. More sensitive to all samples of natural linden honey studied was *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain following to *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain. *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain was resistant

to different natural linden honey. The presented results revealed the antibacterial activities of different samples of linden honey produced by Polish manufacturers, which, to be correctly understood and explained, require further study.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

Funding

This work was supported by The International Visegrad Fund, and the authors are cordially grateful for this.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Variability of flavonoid content, reducing and antioxidant activity in *Althaea officinalis* L. hairy roots

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Article Details:
Received: 2023-10-14
Accepted: 2023-11-17
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0019

Althaea officinalis L. is widely used as a medicinal plant due to its antiseptic, antioxidant, antimicrobial, anti-inflammatory, and gastroprotective properties. *A. officinalis* roots contain a great number of secondary metabolites including flavonoids which exert antioxidant and chelating abilities. Flavonoids possess protective effects against several chronic diseases, in particular neurodegeneration and cancer; they have also neuroprotective, hepatoprotective, anti-bacterial, anti-inflammatory, anti-viral, and anti-cancer effects. Tissue cultures of different plant species are a promising source of secondary metabolites with pharmacological activities, and hairy roots are one of the types. Hairy roots are known as fast-growing, genetically stable cultures, effective producers of both biomass and specialized plant metabolites including flavonoids. *A. officinalis* hairy roots and roots of *in vitro* cultured control (initial) plants were used in this research to study flavonoid content and some biochemical characteristics (antioxidant activity and ability to reduce iron ions Fe^{3+} to Fe^{2+}) of their ethanolic extracts. Two groups of hairy root lines were studied. Hairy roots of one group were obtained as the result of transformation with A4 wild *Agrobacterium rhizogenes* strain while the second group was initiated by transformation with *A. rhizogenes* strain carrying human interferon-α2b gene under the control of the sugarbeet root-specific Mll promoter. Among the two groups of hairy root lines no significant differences were detected that could suggest the role of additional genes in the antioxidant status of the hairy roots: in both groups, there were lines with low, medium and high values of the studied parameters. The total flavonoid content correlated with DPPH scavenging activity and reducing capacity. The results of study confirm flavonoid participation in antiradical reactions in *A. officinalis* hairy root cells.

Keywords: *Althaea officinalis*, hairy roots culture, bioactivity, rol genes, flavonoids

Introduction

Althaea officinalis L. is a perennial medicinal plant of the family Malvaceae Juss. It originates from the temperate regions of India (Ross, 2001), but is now widespread in temperate and subtropical regions of Europe, America, Asia, and North Africa. In Ukraine, it grows near lakes and rivers.

A. officinalis has been used for a long time as a medicinal plant because of its antiseptic, antioxidant, antimicrobial, anti-inflammatory, and gastroprotective properties (Xue et al., 2023). Marshmallow preparations (powder, aqueous infusion, liquid extract, syrup) are used as an expectorant for catarrhal conditions of the respiratory tract, as well as for diarrhea, acute gastritis

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and enterocolitis. It is also used for treating asthma and diseases of the upper respiratory tract. Marshmallow roots contain up to 35% of mucilagious substances, which determine the healing properties of the plant, as well as starch (up to 37%), sucrose (10.2%), betaine (up to 4%) and fatty oil (up to 1.7%), astragalin, mucopolysaccharides, arabinofuranan, caffeic acid, chicorin, coumarin and coumarin acid, diosmetin, kaempferol, luteolin, quercetin, and scopolin (Xue et al., 2022). Polysaccharides are well-known bioactive compounds naturally synthesized in these plants (Karimi et al., 2021). Flavonoid content correlated with this parameter of antioxidant activity of the plants was studied (Sadighara et al., 2012).

Great attention is paid now to the study of bioactivity of such plant-derived chemicals as flavonoids. Their chemical structure, classification and therapeutic properties are deeply analyzed in some publications (Heim et al., 2002; Kumar and Pandy, 2013; Ahn-Jarvis et al., 2019; Atala et al., 2017; Hussain et al., 2020).

Flavonoids are plant secondary metabolites, polyphenol compounds of low molecular weight that have a three-ring structure in the C6–C3–C6 form. Over 4,000 flavonoids have been identified by now (Heim et al., 2002). They originate from various plant sources (fruits, vegetables, wines, teas and cocoa) and can be sub-classified into six different types (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, and isoflavones) (Heim et al., 2002; Shahidi and Yeo, 2018; Hussain et al., 2020).

The mechanisms of flavonoid action are diverse and multiple. Flavonoids possess health-promoting properties either directly or indirectly. Most of their health effects are attributed to the flavonoid antioxidant and chelating abilities (Heim et al., 2002). The bioavailability, metabolism, and biological activity of flavonoids depend on the specific chemical structure, total number of hydroxyl groups, and substitution of functional groups about their nuclear structure (Heim et al., 2002; Kumar and Pandy, 2013). Flavonoids possess protective effects against several chronic diseases, including neurodegeneration and cancer; neuroprotective and hepatoprotective activities; anti-bacterial, anti-inflammation and anti-virus effects as well as reducing cardiovascular diseases and type-2 diabetes (Kumar and Pandy, 2013; Rodriguez-Mateos et al., 2014; Costa et al., 2016; Shahidi and Yeo, 2018; Hussain et al., 2020).

Plant hairy roots can be a promising source of flavonoids. They are known as fast-growing, genetically stable cultures, and effective producers of both biomass and specialized plant metabolites. Detailed analysis of the published data concerning the use of hairy root cultures as a source of polyphenolic antioxidants has been summarized by Malarz et al. (2022). The number of plant species of different families studied for this purpose include *Lotus corniculatus* L., *Fagopyrum tataricum* (L.) Gaertn., *Leontopodium alpinum* Cass, *Ipomea batatas* (L.) Lam., *Antirrhinum majus* L., *Medicago truncatula* Gaertn., *Vitis vinifera* L., *Camellia sinensis* (L.) Kuntze, *Panax ginseng* C.A. Meyer, *Isatis tinctoria* L., *Scutellaria baicalensis* Georgi, *Psoralea corylifolia* L., *Raphanus sativus* L., *Cichorium intybus* L. and a number of others (Park et al., 2016; Balasubramanian et al., 2018; Malarz et al., 2022, Matvieieva et al., 2023). Different types of elicitation were studied to increase flavonoid production in hairy root cultures (Park et al., 2016).

In this work, we compared the content of flavonoids and bioactivity in extracts from *A. officinalis* hairy roots obtained as the result of genetic transformation with different strains of *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) (NCBI Taxonomy Database, 2023). They carried only transferred *A. rhizogenes* genes (obtained by transformation with *Agrobacterium rhizogenes* A4 wild strain) and those that had an additional human interferon-α2b gene (transformed with *A. rhizogenes* carrying human interferon-α2b target gene under the control of the sugar beet root-specific Mll promoter, pCB161).

Material and methodology

Plant material

Althaea officinalis hairy roots and the control plants from the collection of the Laboratory of Adaptional Biotechnology of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used in the work (Figure 1). Hairy roots were obtained earlier after cocultivation of *A. officinalis* leaves cocultivation with suspension of *Agrobacterium rhizogenes* A4 according to the method described in the article (Matvieieva et al., 2013). Plants and roots were cultivated in Petri dishes on 1/2 Murashige and Skoog (Duchefa, Netherland) solidified medium at 24 °C. Two groups of hairy roots were studied. The roots from the first one were obtained by transformation with *Agrobacterium rhizogenes* A4 wild strain and carried only transferred *A. rhizogenes* genes. The roots of the second group were transformed with *A. rhizogenes* carrying the human interferon-α2b target gene under the control

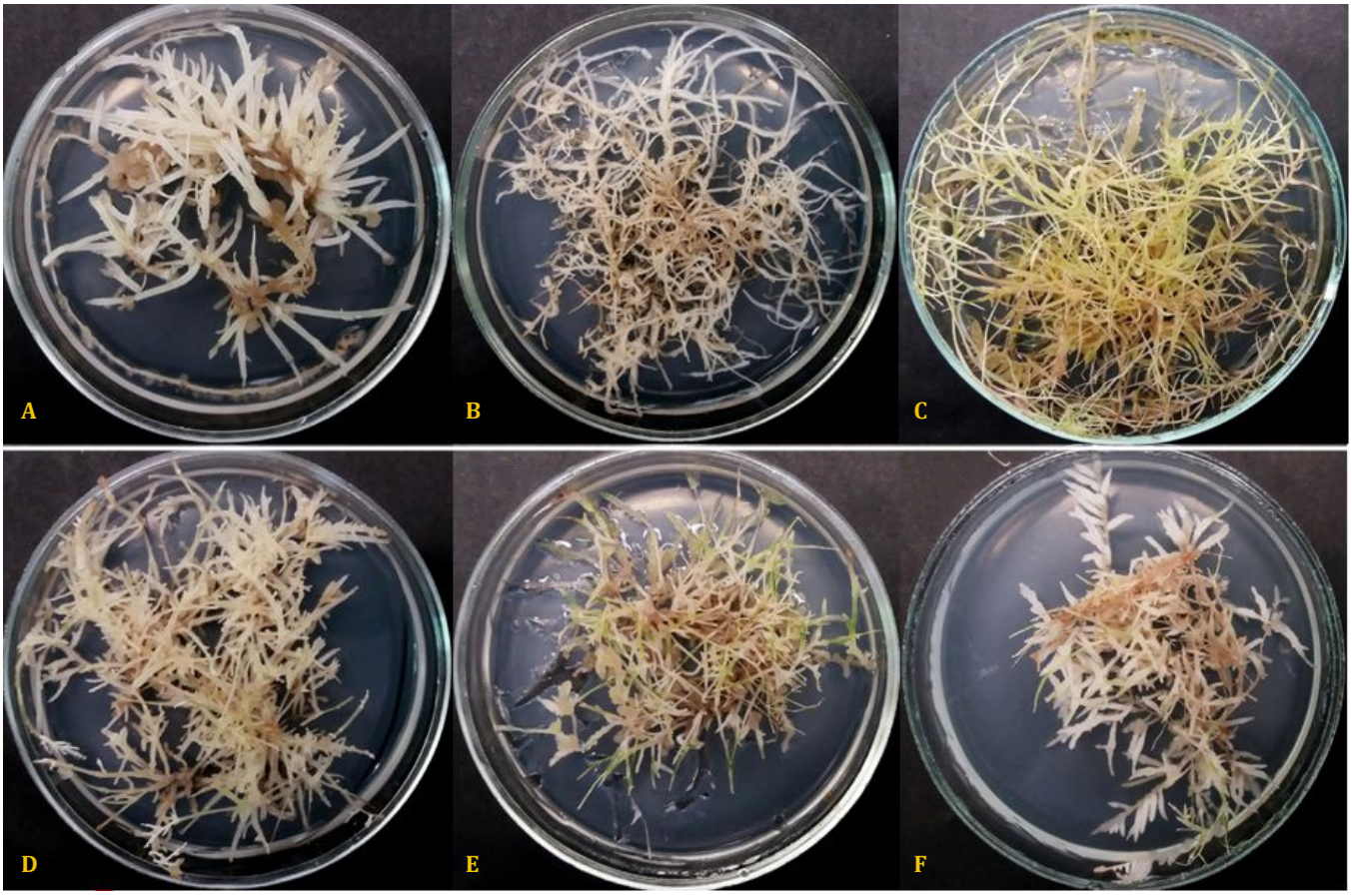

Figure 1 *Althaea officinalis* L. hairy root lines from the collection of the Laboratory of Adaptation Biotechnology in the Institute of Cell Biology and Genetic Engineering National Academy of Sciences of Ukraine
A – line 1; B – line 2; C – line 3; D – line 4; E – line 5; F – line 6; lines 1–3 were obtained as a result of transformation with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 – with *A. rhizogenes* strain carrying human interferon-α2b target gene under the control of the sugar beet root-specific Mll promoter (pCB161)

of the sugar beet root-specific Mll promoter, pCB161 and had an additional human interferon-α2b gene.

Total flavonoid content assay

The content of flavonoids was determined spectrophotometrically (Pekal and Pyrzynska, 2014). To prepare the extracts, the roots were separated from the medium, washed with distilled water, dried with filter paper, weighed (0.3 g) and homogenized in 3 ml of 70% ethanol. The resulting homogenate was transferred to test tubes and centrifuged in an EppendorfCentrifuge 5415 C microcentrifuge for 10 min. The reaction mixture in the cuvette contained 0.25 ml of extract supernatant, 1 ml of deionized water, 0.075 ml of 5% $NaNO_2$ solution. After 5 minutes, 0.075 ml of 10% $AlCl_3$ solution was added, then 0.5 ml of 1 M NaOH and 0.6 ml of deionised water were added. Absorption was determined at λ = 510 nm. The content of flavonoids was calculated using the formula $y = 0.8842x$ ($R^2 = 0.9988$).

DPPH scavenging activity assay

For this study, the root samples were homogenized in 70% ethanol, and centrifuged, and the supernatants were used for the analysis. The activity of the hairy root extracts was studied spectrophotometrically on a Fluorate-02-Panorama spectrofluorimeter using the DPPH test (Brand-Williams et al., 1995).

The reaction was carried out in cuvettes with the addition of the extract (0.62, 0.12, 0.25 and 0.5 ml) to the DPPH solution. The cuvettes were kept for 20 minutes in the dark. The optical density of the mixtures was determined at a wavelength of λ = 550 nm. The level of the activity (%) was calculated according to the following formula:

$$AOA = \left[(OD_1 - OD_2)/OD1\right] \times 100\%$$

where: OD_1 was the optical density of the control sample; OD_2 – optical density of the reaction mixture (extract with DPPH)

The ability to radical scavenging was determined by the effective concentration parameter EC_{50}. The effective concentration was calculated as the extract concentration (root wet weight) required to remove 50% DPPH in the sample, expressed as mg FW in rutin equivalent.

Reducing power assay

A study of the ability of root extracts to reduce iron ions Fe^{3+} to Fe^{2+} was carried out spectrophotometrically on a Fluorate-02-Panorama spectrofluorimeter according to the method described in the article (Zhao et al., 2008) with some modifications. The reaction mixture contained 0.312 ml of 0.2 M phosphate buffer (pH 6.6); 0.312 ml of 1% potassium hexacyanoferrate (III) and the root extract. The cuvettes were incubated in a water bath at 50 °C for 30 min. After that, 0.312 ml of 10% trichloroacetic acid, 1.25 ml of deionized water and 0.25 ml of 0.1% iron (III) chloride were added to the reaction mixture. The comparison solution was prepared using the same method, but instead of the extract, 0.25 ml of deionized water was added. The optical density was measured at a wavelength of λ = 700 nm. The activity was evaluated by the effective concentration parameter ($EC_{0.5}$), which corresponds to the amount of wet root mass (mg FW) required to obtain OD = 0.5.

Statistical analysis

All analyses were carried out in triplicate. Three replications of all analyses were carried, and results were processed by Statistical Analysis Software (SAS) (2004) Version 9.2. SAS Institute Inc., Cary. The data were analyzed for statistical significance using Student's *t*-test and Tukey's method. *P* values less than 0.05 were considered significant. The linear regression method was applied and the coefficient of determination (R^2) was calculated for establishing the relationship between the values.

Results and discussion

Some differences were detected in the total flavonoid content in hairy root lines. It is worth noting that variations in this parameter were observed among the lines obtained by the same method (using a wild strain of bacteria or bacteria with an additional plasmid, Figure 2). In particular, the roots of one line obtained by transformation using the wild strain A4 (column 1, Figure 2) contained significantly fewer flavonoids compared to the roots of two other lines obtained with the same method (columns 2 and 3, Figure 2) – 0.16 ±0.04 mg RE.g^{-1} FW, 1.75 ±0.37 mg RE.g^{-1} FW, and 2.29 ±0.21 mg RE.g^{-1} FW, respectively. Differences were also found among the root lines of the second group, but they were not significant. These roots contained flavonoids in concentrations of 1.29 ±0.19 mg RE.g^{-1} FW, 1.56 ±0.02 mg RE.g^{-1} FW, and 1.90 ±0.10 mg RE.g^{-1} FW (columns 4–6, Figure 2). Thus, the variability of flavonoid content in the roots of different lines was revealed. This, perhaps, can be explained by the peculiarities of the plant's transformation by

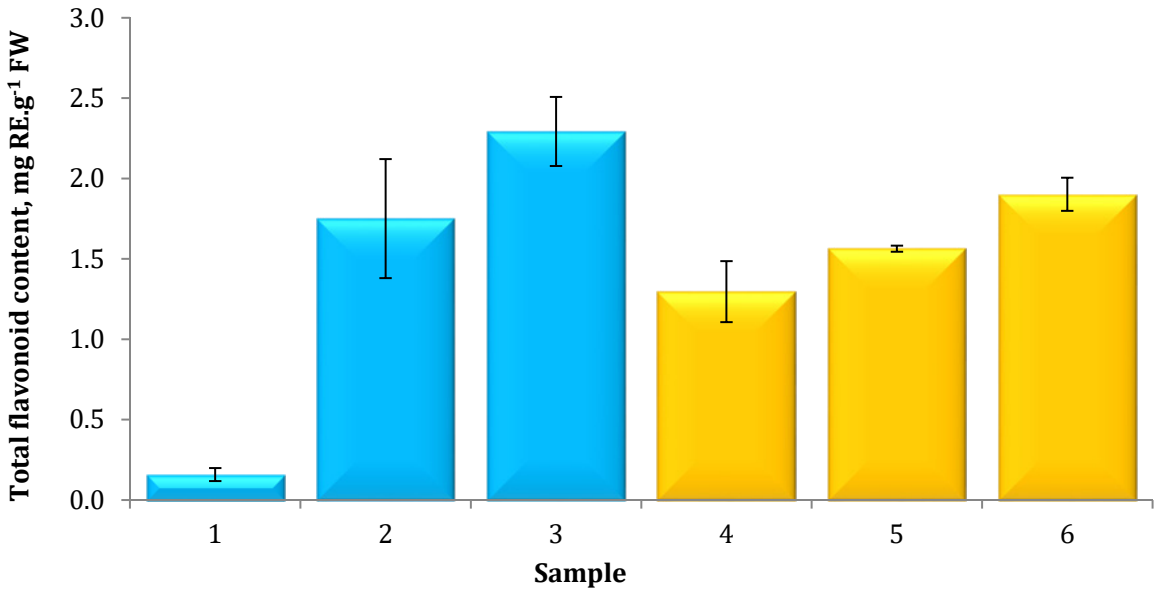

Figure 2 The total content of flavonoids in *Althaea officinalis* L. hairy root lines
lines 1–3 were transformed with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 were transformed with *A. rhizogenes* strain carrying human interferon-α2b target gene

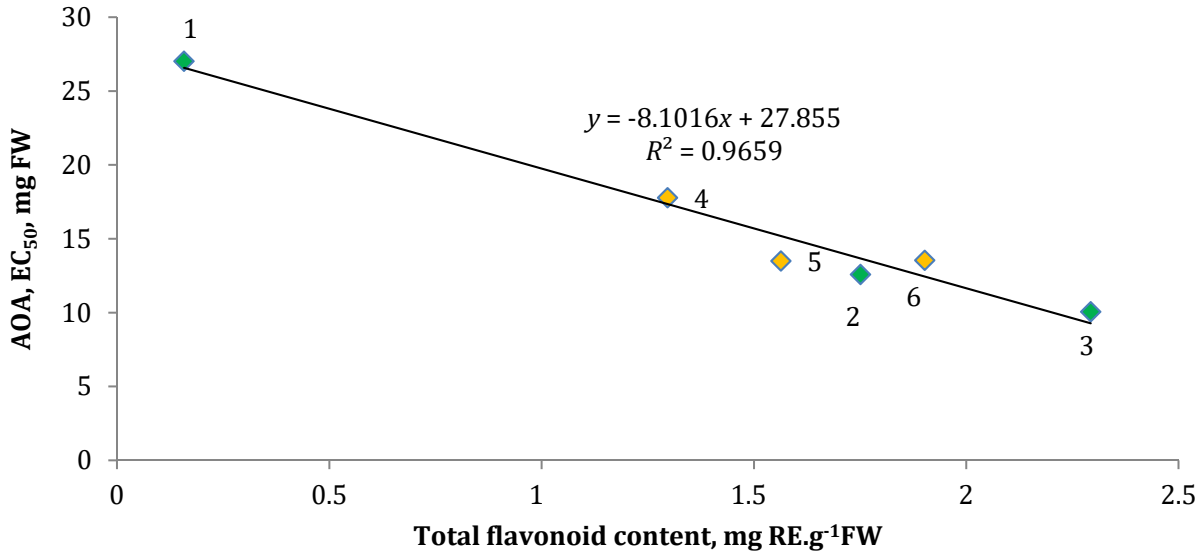

Figure 3 Correlation between the total flavonoid content and DPPH scavenging activity (AOA) in *A. officinalis* hairy root lines
lines 1–3 are the result of transformation with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 – with *A. rhizogenes* strain carrying human interferon-α2b target gene

A. rhizogenes and the transfer of bacterial rol genes to the plant genome.

DPPH scavenging activity of hairy root extracts initiated via transformation by *A. rhizogenes* wild strain varied from EC_{50} = 27.02 mg (the lowest activity in root line no 1) to the highest activity in root line No3 (EC_{50} = 10.06 mg). The activities of the hairy root extracts obtained using *A. rhizogenes* with an additional *ifn*-α2b gene also differed from each other. Effective concentrations in these samples Nos 4, 5, and 6 were 17.79 mg, 13.51 and 13.55, respectively. A correlation was observed between the total content of flavonoids and the level of DPPH scavenging activity, which is shown in Figure 3. Such a correlation can be explained by the fact that flavonoids are powerful antioxidants and can participate in radical reduction in the test reaction. The antioxidant activity of flavonoids has been studied in some publications. Mechanisms of the reactions with ROS (reactive oxygen species) as well as the activity of flavonoid's metabolites were detected (Hotta et al., 2002; Galleano et al., 2010; Dueñas et al., 2010; Amić et al., 2014; Alov et al., 2015; Atala et al., 2017).

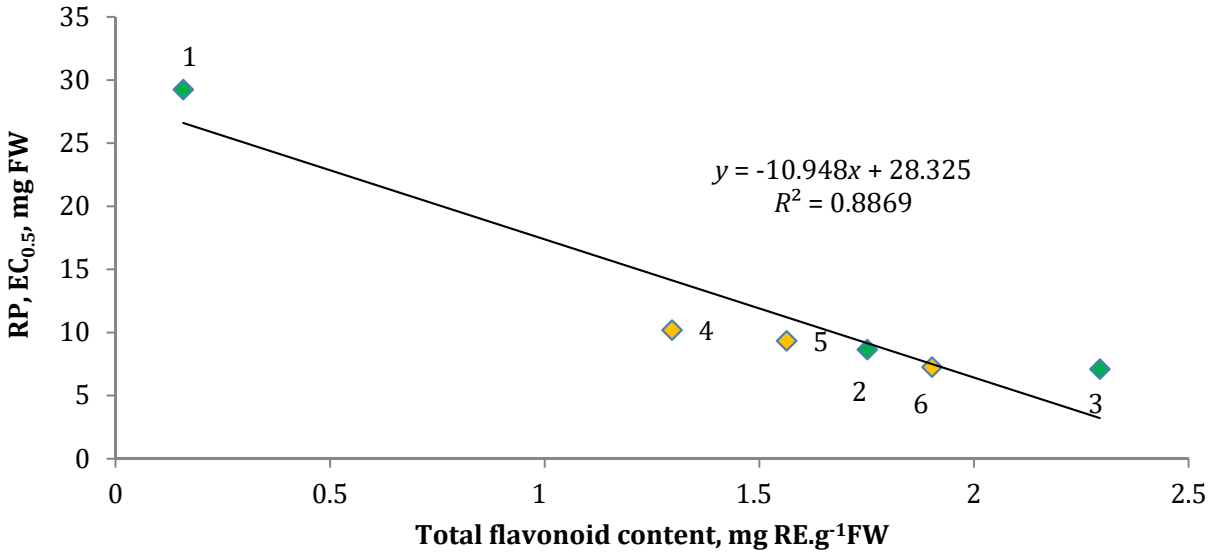

Figure 4 Correlation between the total flavonoid content and reducing power (RP) in *A. officinalis* hairy root lines
lines 1–3 are the result of transformation with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 – with *A. rhizogenes* strain carrying human interferon-α2b target gene

The revealed antiradical activity of the studied extracts is of considerable practical interest. ROS that can be formed in the human body pose a threat to human health. Oxidative stress is considered a basis for the initiation of many diseases including inflammation-related diseases, neurodegeneration, and cancer. It can be initiated when there is an imbalance between ROS and the activity of the antioxidant defense system of cells. In this case, the presence of a sufficient amount of flavonoids can prevent the negative effect of ROS. So, flavonoids are considered now as dietary supplements with a wide range of bioactivity (Ross and Kasum, 2002; Ahn-Jarvis et al., 2019; Rana et al., 2022).

The reducing power of the extracts varied from $EC_{0.5}$ = 7.10 mg (the highest activity, No 3) to $EC_{0.5}$ = 29.24 mg (the lowest activity, No 1). It also correlated with total flavonoid content (Figure 4). Since a correlation was found between the total content of flavonoids and the reducing activity of extracts of *A. officinalis* hairy roots it can be assumed that this activity is largely due to the presence of flavonoids in the plants of this species.

The effect of the genetic transformation on flavonoid synthesis and bioactivity of different plants was studied earlier. Transformation-induced changes in total phenol contents in transgenic tobacco plants (Seong et al., 2012). Antioxidant activity increased in transgenic *Perilla frutescens* plants which overexpressed the γ-tocopherol methyltransferase gene (Ghimire et al., 2015). Increased antioxidant levels were detected in pRi-transformed *Rehmannia glutinosa* (Piątczak et al., 2016). Variations in flavonoid content and antioxidant activity of *Artemisia vulgaris* hairy roots were studied by us earlier (Matvieieva et al., 2019). Wang et al. (2006) studied a great increase of phenolic compounds in transformed with *Agrobacterium rhizogenes Echinacea purpurea*.

Tavassoli and Safipour Afshar (2018) compared the effect of different *A. rhizogenes* strains (A4, A13, ATCC15834, and ATCC15834$_{(GUS)}$) on the total content of flavonoids and phenols in marshmallow hairy roots in the hormone-free liquid medium after 50 days of cultivation. The highest total phenolic compounds were detected in the roots transformed by A13 strain. At the same time, the highest flavonoid content was studied in hairy roots transformed by A4 strain. The authors concluded that the secondary metabolite of the studied plants depended on the bacterial strain used for transformation.

Conclusions

Studies of *Althaea officinalis* hairy roots have shown that root lines differ both in flavonoid content and in the level of antiradical and reducing activity. This may be due to the specificity of the transformation by *Agrobacterium rhizogenes* when the foreign genes are incorporate in different loci which can lead to the differences in the functions of plant cells. In *A. officinalis* hairy root lines obtained via genetic transformation with *A. rhizogenes* A4 wild type or with *A. rhizogenes* with the additional gene of interest, some variations were found in all studied parameters. This fact suggests the impact of bacterial rol genes presented in all hairy root lines on the antioxidant status of the hairy roots: in both groups regardless of the presence/absence of the interferon-α2b gene there were lines with low, medium and high values of studied parameters. According to the results of the experiments, the total content of flavonoids correlated with DPPH absorption activity and reducing capacity, which confirms the participation of flavonoids in antiradical reactions in hairy root cells.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

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

Funding

The work was partially supported by Visegrad Fund grant No 52300061 (Slovakia).

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

In vitro antioxidant response of the equine blood treated by extract derived from leaves of *Ficus sagittata* Vahl (Moraceae)

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Article Details:
Received: 2023-10-15
Accepted: 2023-11-18
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0020

The current study aimed to investigate the oxidative stress biomarkers, such as 2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity, as well as antioxidant defenses (activity of superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin) in the equine erythrocytes and plasma to evaluate the antioxidant activities of the aqueous extract derived from leaves of *Ficus sagittata* Vahl collected at two Botanic Gardens, i.e. M.M. Gryshko National Botanic Garden (Kyiv, Ukraine) and the Botanic Garden of Ivan Franko National University in Lviv (Lviv, Ukraine). Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in the proportion of 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. A volume of 0.1 mL of the plant extracts was added to 1.9 mL of clean equine erythrocytes or plasma (the final concentration of the extract was 5 $mg.mL^{-1}$). For positive control, 0.1 mL of phosphate buffer (pH 7.4) was used. The treatment of equine plasma and erythrocytes by extracts derived from leaves of *F. sagittata* resulted in reduced carbonyl derivatives of the oxidatively modified protein. When equine erythrocytes were incubated with the extract derived from leaves of *F. sagittata* collected in NBG (Kyiv), the TBARS levels were significantly increased compared to the untreated samples. The incubation of equine plasma with an extract derived from leaves of *F. sagittata* resulted in an increase in the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase with a simultaneous decrease of ceruloplasmin level. The level of total antioxidant capacity was significantly increased after the treatment by extract derived from leaves of *F. sagittata* collected in NBG. However, further detailed investigation, especially *in vivo* and *in vitro* antioxidant studies is needed to justify the use of extract derived from leaves of *F. sagittata* as a natural source of antioxidants.

Keywords: lipid peroxidation, oxidatively modified proteins, superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin, total antioxidant capacity

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Introduction

The mulberry family (Moraceae) is represented by mainly woody tropical or (more rarely) temperate species with specialized canals within their body containing milky latex, the feature most obviously distinguishing the family from other members of the order Urticales in which it is currently placed. The family comprises 37 genera and 1,050–1,100 species with a diversity of growth forms, including terrestrial trees, shrubs, climbers, hemi-epiphytes, subshrubs, and herbs. High variation is also observed in the morphology and arrangement of leaves. The small unisexual flowers of these plants are assembled into inflorescences varying considerably in structure and position on a plant among the species, although this variety can be reduced to their two basic types:

1. largely bisexual and circular in outline,
2. unisexual and elongate, racemose or spicate.

These features partly account for the subdivision of the family into five tribes: Moreae, Artocarpeae, Dorstenieae, Castilleae, and Ficeae (Berg, 2001; Datwyler and Weiblen, 2004; Clement and Weiblen, 2009).

Ficus L. is the only genus of the tribe Ficeae and the largest within the family, containing ca 750 species distributed in the tropics and subtropics worldwide. Despite the exceptionally large species diversity of *Ficus* unproportional to that of other moraceous taxa, its consideration as a single entity is well-grounded on several specific features, among which are the presence of waxy glands on vegetative plant parts, heterostyly, and anthesis of staminate flowers when the fruits are mature (Berg, 2001; Cook and Rasplus, 2003; Berg and Corner, 2005).

Ficus sagittata Vahl is a climbing shrub when young, often starting life as an epiphyte. As it grows older it can become a tree. It often starts life as an epiphyte in the branch of a tree and can eventually send down aerial roots that, once they reach the ground, provide extra nutrients that help the plant grow more vigorously. These aerial roots can completely encircle the trunk of the host tree, constricting its growth – this, coupled with the more vigorous top growth, can lead to the fig outcompeting and killing the tree in which it is growing. The plant is sometimes harvested from the wild for local medicinal use. It is cultivated for its ornamental value (https://tropical.theferns.info/viewtropical.php?id=Ficus+sagittata).

Ficus plants have a lot of pharmacological effects, being used both in traditional medicines and contemporary treatment of different disorders. Recent studies showed the therapeutic efficacy of *Ficus* spp., especially in respiratory, cardiovascular, and central nervous system disorders (Cagno et al., 2015; Alamgeer et al., 2017; Salehi et al., 2021). Also, *Ficus* plants are used for the treatment of diabetes (Deepa et al., 2018).

In our previous study (Tkachenko et al., 2018, 2019), we highlighted the antioxidant potential of an aqueous extract derived from leaves of other *Ficus* species using an equine erythrocyte suspension. In the study (Tkachenko et al., 2018), we have focused on the antioxidant effect of an extract derived from leaves of *F. religiosa* L. on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives of protein oxidative modification (OMP), total antioxidant capacity (TAC)] using the model of equine erythrocytes. Treatment by extract reduced the erythrocyte's TBARS level by 25.3% ($p = 0.009$), while plasma TBARS level was increased by 75.6% ($p = 0.000$), as compared to untreated erythrocytes. When equine plasma was incubated with extract, the level of ketonic derivatives was significantly increased by 22.8% ($p = 0.000$), while a non-significantly decrease in both aldehydic and ketonic derivatives of OMP was observed (by 1.6% and 8.9%, $p > 0.05$). Treatment by *F. religiosa* extract caused the increase of TAC in plasma and erythrocyte suspension when compared to untreated erythrocytes. However, these changes were statistically non-significant. All these data suggest that *F. religiosa* could be explored for its antioxidant potential using an equine erythrocyte suspension (Tkachenko et al., 2018).

Later, we investigated the *in vitro* antioxidant activity of aqueous extracts derived from the leaves developed on the shoots of various developmental stages (juvenile and mature/generative) of *F. pumila* L. using the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives of protein oxidative modification, total antioxidant capacity] on the model of equine erythrocyte suspension (Tkachenko et al., 2019). The treatment with the extract derived from leaves of mature shoots reduced the erythrocyte's TBARS level by 22% ($p = 0.029$), while the TBARS level was increased by 15.5% ($p > 0.05$) when incubated with an extract derived from leaves of juvenile shoots as compared to untreated erythrocytes. When equine erythrocytes were incubated with the extract obtained from leaves of mature shoots, the ketonic derivatives level was significantly decreased by 6.9% ($p = 0.040$), while a non-significantly decrease in both aldehydic and ketonic derivatives of OMP was observed after incubation with an extract derived from juvenile shoots (by 8.18 and 12.5%, $p > 0.05$).

The treatment by *F. pumila* leaf extract (from juvenile and mature shoots) caused the increase of TAC in erythrocyte suspension as compared to untreated erythrocytes. Thus, extracts derived from both juvenile and mature shoots increased the total antioxidant capacity of equine erythrocytes (Tkachenko et al., 2019).

The current study aimed to investigate the oxidative stress biomarkers, such as 2-thiobarbituric acid reactive substances, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity, as well as antioxidant defenses (activity of superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin) in the equine erythrocytes and plasma to evaluate the antioxidant activities of the aqueous extract derived from leaves of *Ficus sagittata*.

Materials and methodology

Collection of plant materials

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

Preparation of plant extracts

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in the proportion of 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -25 °C until use.

Horses

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

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. 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 prepared from the leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) was added to 1.9 mL of clean equine erythrocytes or plasma (the final concentration of the extract was 5 $mg.mL^{-1}$). For positive control, 0.1 mL of phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, biochemical assays were done. Erythrocytes and plasma aliquots were used in the study.

The 2-Thiobarbituric acid reactive substances 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 and described in the paper by Tkachenko et al. (2022). This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The nmol per 1 mL was calculated using $1.56 \cdot 10^5$ $mM^{-1}. cm^{-1}$ as the extinction coefficient.

The carbonyl derivatives of oxidatively modified proteins assay

To evaluate the protective effects of the extract against free radical-induced protein damage in samples, a content of carbonyl derivatives of oxidatively modified proteins (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the samples was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina et al. (1995) and described in the paper by Tkachenko et al. (2022). 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

The total antioxidant capacity (TAC) level in 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 by Galaktionova et al. (1998) and described in the paper by Tkachenko et al. (2022). The sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated the absorbance of the blank sample.

Superoxide dismutase activity assay

The activity of superoxide dismutase (SOD, E.C. 1.15.1.1) was assessed by its ability to dismutate superoxide generated in the process of quercetin auto-oxidation in an alkaline medium (pH 10.0), as proposed by Kostiuk et al. (1990) and described in the paper by Tkachenko et al. (2022). The activity was expressed in units of SOD per mL.

Catalase activity assay

The activity of catalase (CAT, E.C. 1.11.1.6) was determined by measurement of the decrease in H_2O_2 in the reaction mixture, using a spectrophotometer at the wavelength of 410 nm and the method described by Koroliuk and co-workers (1988) and described in the paper by Tkachenko et al. (2022). One unit of catalase activity was defined as the amount of enzyme necessary to decompose 1 μmol H_2O_2 per min per mL.

Glutathione peroxidase activity assay

The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined by detecting the nonenzymatic utilization of GSH (reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB), as proposed by Moin (1986) and described in the paper by Tkachenko et al. (2022). GPx activity is expressed as μmol GSH per min per mL.

Ceruloplasmin level assay

Ceruloplasmin (CP, E.C. 1.16.3.1) level in the plasma was measured spectrophotometrically at the wavelength of 540 nm as described by Ravin (1961) and described in the paper by Tkachenko et al. (2022). The assay mixture contained 0.1 mL of plasma, 5 mL of 0.4 M sodium acetate buffer (pH 5.5), and 0.1 mL of 0.5% *p*-phenilendiamine. 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 is expressed as milligrams per dL of plasma.

Statistical analysis

The mean ±S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p > 0.05$). The significance of differences (significance level, $p < 0.05$) was examined using the Kruskal-Wallis test by ranks (Zar, 1999). All statistical calculations were performed on separate data from each individual with Statistica 13.3 software (TIBCO Software Inc., USA).

Results and discussion

The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and the total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) in was assessed and shown in Figure 1.

As can be seen in Figure 1, treatment by extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) resulted in non-significant changes in the TBARS level of (33.65 ±1.86 nmol.mL^{-1}) and significantly increased TBARS levels to (41.69 ±3.81 nmol.mL^{-1}) compared to the untreated samples (35.88 ±3.02 nmol.mL^{-1}) (Figure 1). When equine erythrocytes were incubated with the extract derived from leaves of *F. sagittata* collected in NBG (Kyiv), the TBARS levels were significantly increased by 16.2% ($p < 0.05$) compared to the untreated samples.

The levels of aldehydic derivatives of oxidatively modified proteins were not changed after treatment by extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv). When equine erythrocytes were incubated with the extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv), the levels of ketonic derivatives (34.82 ±1.66 nmol. mL^{-1} and 35.67 ±1.82 nmol.mL^{-1}) were significantly decreased by 11.8% ($p < 0.05$) and 9.6% ($p < 0.05$) compared to the untreated samples (39.47 ±2.20 nmol.mL^{-1}). Additionally, a non-significantly increased TAC level was observed after incubation with an extract derived from leaves of *F. sagittata* collected in BG (Lviv) (by 3.1%, $p > 0.05$). The TAC levels were

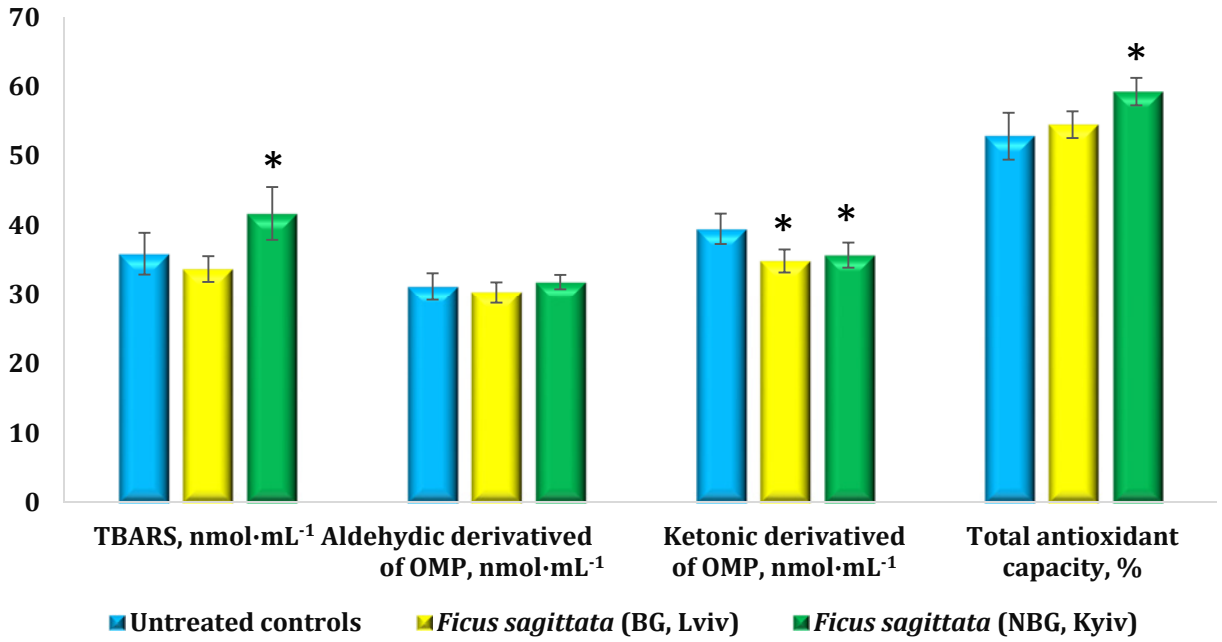

Figure 1 The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes after *in vitro* treatment by extracts derived from leaves of *Ficus sagittata* Vahl collected in BG (Lviv) or NBG (Kyiv) (M ±m, n = 18)
*– statistically significant differences between treated and untreated samples (p <0.05)

increased after treatment by extracts derived from leaves of *F. sagittata* collected in NBG (Kyiv) (by 12.2%, p <0.05) (Figure 1).

Activities of catalase and glutathione peroxidase, as well as ceruloplasmin levels in the equine plasma after *in vitro* incubation with extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) represented in Figure 2.

All cells have a complex antioxidant defense system, consisting of interacting low- and high-molecular

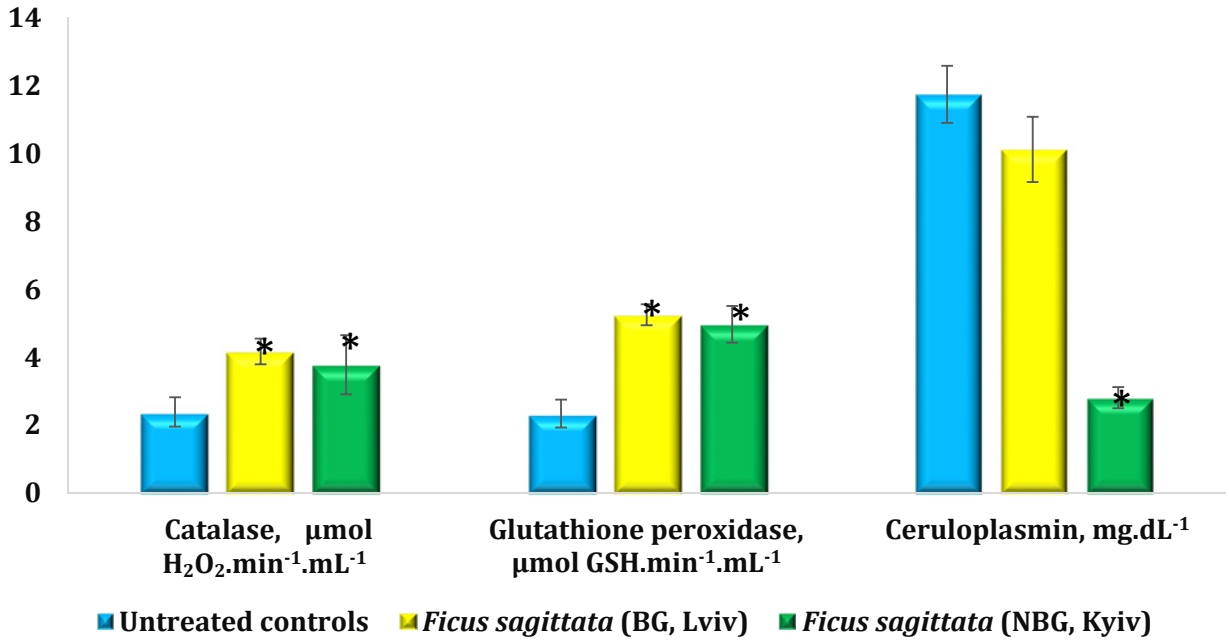

Figure 2 Activities of catalase and glutathione peroxidase, as well as ceruloplasmin level in the equine plasma after *in vitro* incubation with extracts derived from leaves of *Ficus sagittata* Vahl collected in BG (Lviv) or NBG (Kyiv) (M ± m, n = 18)
*– statistically significant differences between treated and untreated samples (p <0.05)

components. Among them, superoxide dismutase (SOD), glutathione peroxidases (GPx), and catalase (CAT) play a central role (Cerutti et al., 1994). In the current study, SOD activity was increased to (363.92 ±37.48 U.mL^{-1} and 365.31 ±37.78 U.mL^{-1}) in the equine plasma after *in vitro* incubation with extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) compared to the untreated samples (303.96 ±29.51 U.mL^{-1}). This was a 19.7% ($p > 0.05$) and 20.2% ($p > 0.05$) increase in SOD activity compared to the untreated samples. Catalase activity was non-significantly increased to values (4.17 ±0.38 μmol H_2O_2.min^{-1}.mL^{-1} and 3.78 ±0.87 μmol H_2O_2.min^{-1}.mL^{-1}) in the equine plasma after *in vitro* incubation with extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) compared to the untreated samples (2.38 ±0.43 μmol H_2O_2.min^{-1}.mL^{-1}). This was a 74.5% ($p < 0.05$) and 58.2% ($p < 0.05$) increase in CAT activity compared to the untreated samples (Figure 2).

Glutathione peroxidases are thiol-based enzymes that catalyze the reduction of H_2O_2 and hydroperoxides to H_2O or alcohols, they mitigate the toxicity of these compounds to the cell (Passaia and Margis-Pinheiro, 2015). Similarly to SOD and CAT activity, GPx activity was also increased to (5.25 ±0.31 μmol GSH.min^{-1}.mL^{-1} and 4.97 ±0.54 μmol GSH.min^{-1}.mL^{-1}) in the equine plasma after *in vitro* incubation with an extract derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) compared to the untreated samples (2.34 ±0.41 μmol GSH.min^{-1}.mL^{-1}). This was a 124.4% ($p < 0.05$) and 112.4% ($p < 0.05$) increase in GPx activity compared to the untreated samples (Figure 2).

Ceruloplasmin (CP) is a copper-containing multifunctional oxidase of plasma, an antioxidant, an acute-phase protein, and a free radical scavenger (Samygina et al., 2017). It has been proposed to function in copper transport, oxidation of organic amines, iron(II) oxidation, and the regulation of cellular iron levels, catechols, radical scavenging, and other antioxidant processes (Healy and Tipton, 2007). In the current study, the CP level was decreased to (10.12 ±0.96 mg.dL^{-1} and 2.81 ±0.31 mg.dL^{-1}) in the equine plasma after *in vitro* incubation with extracts derived from leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv) compared to the untreated samples (11.74 ±0.84 mg.dL^{-1}). This was a 13.8% ($p > 0.05$) and 76.1% ($p < 0.05$) decrease in CP levels compared to the untreated samples (Figure 2).

In the current study, we used an *in vitro* model of equine plasma and erythrocytes to assess the antioxidant properties of an aqueous extract derived from the leaves of *F. sagittata* collected in BG (Lviv) or NBG (Kyiv). Many results also clearly suggest that treatment by herbal extracts *in vivo* and *in vitro* studies prevents organ damage through a decrease of lipid peroxidation and protection of the antioxidant defense system. On this basis, the current study was conducted to evaluate the antioxidant properties of an extract derived from the leaves of *F. sagittata*. The main finding of the current study was that this extract was able to decrease both aldehydic and ketonic derivatives of OMP, with a simultaneous increase in the activity of antioxidant enzymes (SOD, CAT, and GPx) in the equine erythrocytes and plasma after *in vitro* treatment.

Many studies *in vivo* revealed the antioxidant properties of extracts derived from different *Ficus* plants. For example, the study of Ahmed et al. (2013) evaluated the protective effects of sequential acetone extract of *Ficus racemosa* L. bark at two doses (FR250; 250 mg per kg and FR500; 500 mg per kg p.o.) against doxorubicin-induced renal and testicular toxicity in rats. Extract pretreatment (500 mg per kg) decreased TBARS and increased glutathione levels in the kidney and testis to control levels. These observations were substantiated by histopathological studies, wherein normal renal and testicular architecture was restored in FR500 group. Thus, administration of *F. racemosa* stem bark extract offers significant renal and testicular protection by inhibiting lipid peroxidation-mediated through scavenging free radicals (Ahmed et al., 2013). Also, *F. racemosa* extract was a potent chemopreventive agent and suppresses potassium bromate-induced nephrotoxicity in rats (Khan and Sultana, 2005). The results of Ahmed and Urooj (2010) indicate that *F. racemosa* possesses potent hepatoprotective effects against carbon tetrachloride-induced hepatic damage in albino rats. The acetone extract of *F. racemosa* bark possesses potential cardioprotective activity against doxorubicin-induced cardiotoxicity in rats by scavenging free radicals generated by the administration of the drug (Ahmed and Urooj, 2012).

Antidiabetic activity of *Ficus amplissima* Smith. bark extract in streptozotocin-induced diabetic rats was demonstrated by Arunachalam and Parimelazhagan (2013). Similarly, the antidiabetic effect of *Ficus religiosa* L. extract in streptozotocin-induced diabetic rats was revealed by Pandit et al. (2010). *F. religiosa* bark extract showed a significant anti-lipid peroxidative effect in the pancreas of streptozotocin-induced diabetic rats. The phenolic constituents of the aqueous-ethanolic extract of Tunisian *Ficus carica* L. fruit (FE) and its antihyperlipidemic and antioxidant

activities in high-fat diet-induced hyperlipidemic rats (HFD) were evaluated by Belguith-Hadriche et al. (2016). The FE has a significant hypocholesterolemic effect and antioxidant activity in HFD-fed rats. This beneficial effect may be partly due to phenolic constituents, especially vitexin, dihydroxybenzoic acid di-pentoside as well as rutin. The results indicate that properly dried figs can be used as a good source of phenolic compounds (Slatnar et al., 2011). Phytochemical studies on fruits and leaves of fig plants have explored that they are rich in phenolics, organic acids, and volatile compounds. Owing to the rich and diversified presence of biologically active compounds, they possess various biological activities such as antioxidant, anti-inflammatory, antibacterial, anticancer, hepatoprotective, antidiabetic, antifungal, antiviral, antimutagenic, antipyretic, antituberculosis, anti-angiogenic, antiparasitic, hematostatic, anticonstipation, and antiwarts activities (Hajam and Saleem, 2022).

Conclusions

In the current study, we investigated the changes in the levels of oxidative stress biomarkers and antioxidant defenses using the model of equine erythrocytes and plasma aimed to assess the antioxidant activities of the aqueous extract derived from the leaves of *Ficus sagittata* collected in BG (Lviv) or NBG (Kyiv). The treatment of equine plasma and erythrocytes by extracts derived from leaves of *F. sagittata* resulted in reduced carbonyl derivatives of the oxidatively modified protein. When equine erythrocytes were incubated with the extract derived from leaves of *F. sagittata* collected in NBG (Kyiv), the TBARS levels were significantly increased compared to the untreated samples. The incubation of equine plasma with an extract derived from leaves of *F. sagittata* resulted in an increase in the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase with a simultaneous decrease of ceruloplasmin level. The level of total antioxidant capacity was significantly increased after the treatment by extract derived from leaves of *F. sagittata* collected in NBG (Kyiv). However, further detailed investigation, especially *in vivo* and *in vitro* antioxidant studies is needed to justify the use of extract derived from leaves of *F. sagittata* as a natural source of antioxidants.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

Funding

This work was supported by The International Visegrad Fund, and the authors are cordially grateful for this.

Acknowledgements

The current study is a continuation of our cooperation with M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine) and Botanic Garden of Ivan Franko National University in Lviv (Lviv, Ukraine) concerning investigations of the antibacterial and antioxidant properties of extracts derived from leaves of some tropical and subtropical plants.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Seasonal variation of antioxidant activity of *Nigella* spp. in Ukraine

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Article Details:
Received: 2023-10-10
Accepted: 2023-11-19
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0021

This study aimed to evaluate the antioxidant potential of ethanol extracts of *Nigella* L. species from the Kherson Oblast of Ukraine. The extracts of seeds and above-ground parts of *N. damascena* L., *N. hispanica* L., *N. orientalis* L., and *N. sativa* L. cv. Diana were used to determine the total phenolic content (TPC), total flavonoid content (TFC), total phenolic acid content (TPAC), free radical scavenging activity (FRSA), and molybdenum reducing power (MRP). The plant raw material taken for the experiment in sprouting, budding, flowering, and ripening. The TPC of seed extracts was from 8.11 to 35.12 mg GAE.g^{-1}, TFC from 3.21 to 12.65 mg QE.g^{-1}, TPAC from 2.1 to 8.21 mg CAE.g^{-1}, FRSA from 3.56 to 7.32 mg TE.g^{-1}, and MRP from 23.47 to 68.34 mg TE.g^{-1}. The study of TPC investigated *Nigella* spp. during vegetation showed accumulation of them from 33.65 to 54.11 mg GAE.g^{-1} in the sprouting, from 54.89 to 65.76 mg GAE.g^{-1} in the budding, from 43.18 to 88.43 mg GAE.g^{-1} in the flowering, and from 49.21 to 71.45 mg GAE.g^{-1} in the ripening depending on species. The TFC was from 17.87 to 27.18 mg QE.g^{-1} at the sprouting, from 31.87 to 43.54 mg QE.g^{-1} in the budding, from 29.11 to 57.34 mg QE.g^{-1} in the flowering, and from 23.98 to 50.32 mg QE.g^{-1} in the ripening, depending on species. The TPAC was 12.11–17.32 mg CAE.g^{-1} in the sprouting, 11.17–18.43 mg CAE.g^{-1} in the budding, 10.09–28.45 mg CAE.g^{-1} in the flowering, and 17.86–22.43 mg CAE.g^{-1} in the ripening. FRSA was in the sprouting 4.56–7.77 mg TE.g^{-1}, in the budding 6.38–8.11 mg TE.g^{-1}, in the flowering 7.12–9.67 mg TE.g^{-1}, and in the ripening 5.12–9.54 tmg TE.g^{-1} depending on species. The MRP in the sprouting was 27.14–65.29 mg TE.g^{-1}, in the budding 45.48–77.5 mg TE.g^{-1}, in the flowering 77.89–94.32 mg TE.g^{-1}, and in the ripening 96.11–110.87 mg TE.g^{-1}. A strong correlation found between MRP and TPC, TFC, TPAC (r = 0.824–0.965) in the seed extracts. The results obtained in this study can be used for further biochemical and farmaceutical research.

Keywords: Black cumin, total polyphenol content, total flavonoid content, total phenolic acid content, correlation

Introduction

Species of *Nigella* L. genus belong to Ranunculaceae Juss. and well-known in many countries in the world as medicinal and culinary plants. The center of species origin and diversity is the Western-Irano-Turanian region (Zohary, 1983). The species quantity of this genus is approximately 20. The most known species are *N. damascena* L. (also named lady-in-a-mist or

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ragged lady) and *N. sativa* L. (also named black cumin or black seeds) which are used for ornamental and medicinal purposes and have a high commercial interest (Salehi et al., 2021). From a therapeutic point of view, *N. sativa* showed the possibility of treating respiratory, gastrointestinal, and rheumatic disorders and is used as a spice in foodstuffs in many countries (Tiji et al., 2021).

Most research on *Nigella* spp. raw concerning of seed or essential oil seed (Bessedik, 2016; Busari et al., 2020) composition and biological activities (Boudiaf et al., 2010). In most cases, it relates to *N. sativa* raw. *N. sativa* seed extracts are used in the cosmeceutical branch as soaps, creams, oils, shampoos, and capsules (Eid et al., 2017).

The biochemical composition of *N. sativa* seeds from Yemen, Iran, and Malaysia, according to Haron et al. (2014), was the following: crude fat from 20.63 to 28.71%, crude protein from 11.35 to 14.04%, total moisture from 5.37 to 7.93%, total ash from 4.15 to 4.51%, total carbohydrates from 48.69 to 57.18%, calcium 2,242 $mg.kg^{-1}$, potassium 6,393 $mg.kg^{-1}$, magnesium 2,234 $mg.kg^{-1}$, saturated fatty acids 1.42%, polyunsaturated fatty acids 65.13%. The nutrient content of seeds of *N. damascena* and *N. sativa* from Algeria, according to Benazzouz-Smail et al. (2023), was the following: 3.41 and 4.71% of moisture, 34.3 and 42.3% of crude fat, 25.10 and 21.60% of crude protein, 32.47 and 27.23% of total carbohydrates, 5.62 and 6.90% of reducing sugars, 4.72 and 4.16% of ash, 0.56 and 0.33% of sodium, respectively.

There exist numerous reports about the biological activities of *N. sativa* plant parts. As reported Bourgou et al. (2008), the shoot and root methanol extracts of *N. sativa* demonstrated significant antimutagenic activity. The investigation of biological activities of *N. sativa* seeds found the antioxidant (Ashraf et al., 2011; Alenzi et al., 2013; Akinwumi et al., 2020; Adam and Shuiab, 2022), antiviral, antimicrobial, anti-inflammatory (Eid et al., 2017), anti-toxic, anticancer, immunomodulatory (Ciesielska-Figlon et al., 2023) and antidiabetic (Houcher et al., 2007; Alenzi et al., 2013) activities of its extracts. The seed extracts of *N. sativa* were effective against *Salmonella typhi*, *Bacillus cereus*, *Klebsiella pneumonia*, *Escherichia coli*, etc. (Hassan et al., 2016).

The existence of different seed components can be used in cryobiological investigations (Awan et al., 2018). Also, the antioxidant, antiproliferative, and antiangiogenic activities of *N. sativa* pulp were found (Tan, 2018).

The essential oil composition is the most studied topic of these plants study. The main components of essential oil of *N. sativa* seeds are thymoquinone (57.9%), *p*-cymene (28.9%), alpha-phellandrene (6.3%), alpha-terpineol (2.7%), limonene (1.3%), etc. (Ibrahim et al., 2022). The thymoquinone also demonstrated an important antioxidant (Alenzi et al., 2013; Chung et al., 2023), cytotoxic, antifungal, and anticancer activities (Elsharkawy et al., 2021).

Also, these species are promising honey plants. The *Nigella honey* contains 14.3% of moisture, 73% of total soluble solid, 0.99% of protein, 0.18% of ash, 84.4% of total carbohydrates, 350.4 Kcal of energy (per 100 g), 558 ppm of sodium, 1063 ppm of potassium, 75.4 ppm of calcium, 58.8 ppm of magnesium, 344.2 ppm of iron, 5.6 ppm of lead, 95.5 mg of gallic acid equivalent of total polyphenols (per 50 ml), 2.66 mg of catechin equivalent of total flavonoid content (per 50 ml), and 0.69 mg of ascorbic acid (per 100 ml) (Linkon et al., 2015).

This study aimed to evaluate the antioxidant activity of the above-ground part and seeds of *Nigella* spp. from South Ukraine as a potential source of antioxidants depending on the stage of growth.

Material and methodology

Plant material

The species of *Nigella* L. from the South of Ukraine (Kherson Oblast) were used in this study: *N. damascena* L., *N. hispanica* L., *N. orientalis* L., and *N. sativa* L. cv. Diana (Figure 1). The seeds were planted and above-ground parts (herb) were taken for analysis at the sprouting, budding, flowering, and ripening stages during 2020–2021 from the experimental collection of the Institute of Climate Smart Agriculture of the National Academy of Agrarian Sciences of Ukraine (Kherson region, v. Plodove). Also, the seeds of the investigated species were analyzed.

Biochemical analyses

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

Chemicals

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

Figure 1 Plants of *Nigella* spp. in the flowering
1 – *Nigella* hispanica L.; 2 – *N. orientalis* L.; 3 – *N. damascena* L.; 4 – *N. sativa* L. cv. Diana is in the flowering stage

Preparations of extracts

An amount of 0.25 g of each sample was extracted with 20 mL of 80% ethanol for 2 h in a laboratory shaker GFL 3005 (GFL, Burgwedel, Germany). Then, the samples were centrifuged at 4,605 RCF (Rotofix 32 A, Hettich, Germany) for 10 min and the supernatant was used for measurement of FRSA (antiradical activity) using DPPH, MRAP (antioxidant activity) using phosphomolybdenum method and measurement of other antioxidant properties (detection of total polyphenol, total flavonoid, and phenolic acid content).

Total polyphenol content of extracts

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

Total phenolic acid content

The content of phenolic acids (TPAC) was determined using the procedure described by Árvay et al. (2017). 0.5 ml of sample extract was mixed with 0.5 ml of 0.5 M hydrochloric acid, 0.5 ml Arnova reagent, 0.5 ml of 1 M sodium hydroxide (w/v), and 0.5 ml of distilled water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid 1–200 $mg.L^{-1}$ (R^2 = 0.999) was used as a standard. The results were expressed in $mg.g^{-1}$ caffeic acid equivalents (CAE).

Total flavonoid content of extracts

The total flavonoid content (TFC) was determined by the modified method described by Shafii et al. (2017). An aliquot of 0.5 mL of the sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M potassium acetate, and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (1–400 $mg.L^{-1}$; R^2 = 0.997) was used as the standard. The results were expressed in $mg.g^{-1}$ DW quercetin equivalent.

Free radical scavenging activity

Free radical scavenging activity (FRSA) of samples (antiradical activity) was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sánchéz-Moreno et al., 1998). An amount of 0.4 mL of sample was mixed with 3.6 mL of DPPH solution (0.025 g DPPH

in 100 mL ethanol). The absorbance of the reaction mixture was determined with the spectrophotometer Jenway (6,405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10–100 mg.L^{-1}; R^2 = 0.989) was used as the standard and the results were expressed in mg.g^{-1} DM Trolox equivalents.

Molybdenum-reducing power of extracts

The molybdenum-reducing power (MRP) of samples was determined by the method of Prieto et al. (1999) with slight modifications. The mixture of the sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M), and distilled water (0.8 mL) was incubated at 90 °C for 120 min, then cooled to room temperature. The absorbance at 700 nm was detected with the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10–1,000 mg.L^{-1}; R^2 = 0.998) was used as the standard and the results were expressed in mg.g^{-1} DM Trolox equivalent.

Statistical analysis

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

Results and discussion

Polyphenol compounds are plant-produced second metabolites with numerous biological activities and health benefits (Rana et al., 2022). The total content of polyphenol compounds depends on numerous factors such as period of growth, ecological conditions of growth (Rini et al., 2023), species and genotypes, plant raw, extracts, etc. (Vergun et al., 2023).

The study of *Nigella* spp. antioxidant activity and antioxidant compounds as a rule related to *N. sativa* seeds as the most popular raw among other species (Neha et al., 2014; Pop et al., 2020; Muzolf-Panek and Gliszczyńska-Świgło, 2022).

This study showed that the TPC of seed extracts of investigated plants was from 8.11 to 35.12 mg GAE.g^{-1}, TFC from 3.21 to 12.65 mg QE.g^{-1}, TPAC from 2.1 to 8.21 mg CAE.g^{-1}, FRSA from 3.56 to 7.32 mg TE.g^{-1}, and MRP from 23.47 to 68.34 mg TE.g^{-1} (Figure 2). It should be noted that minimal values were found for seed extracts of *N. sativa* cv. Diana and maximal for *N. hispanica* except for FRSA. In this case maximal value found for *N. orientalis*.

It was found 31.15 mg GAE.g^{-1} of TPC and 16.34 mg GAE.g^{-1} of the total flavonoid content of seed extracts obtained by Soxhlet extraction (Goga et al., 2012). Kaushik and Barmanray (2012) determined in ethanol extracts of *N. sativa* seeds 138.59 mg GAE.100 g^{-1} of TPC. The study of different seed extracts of *N. sativa* demonstrated the most TPC (81.31 mg GAE.mg^{-1})

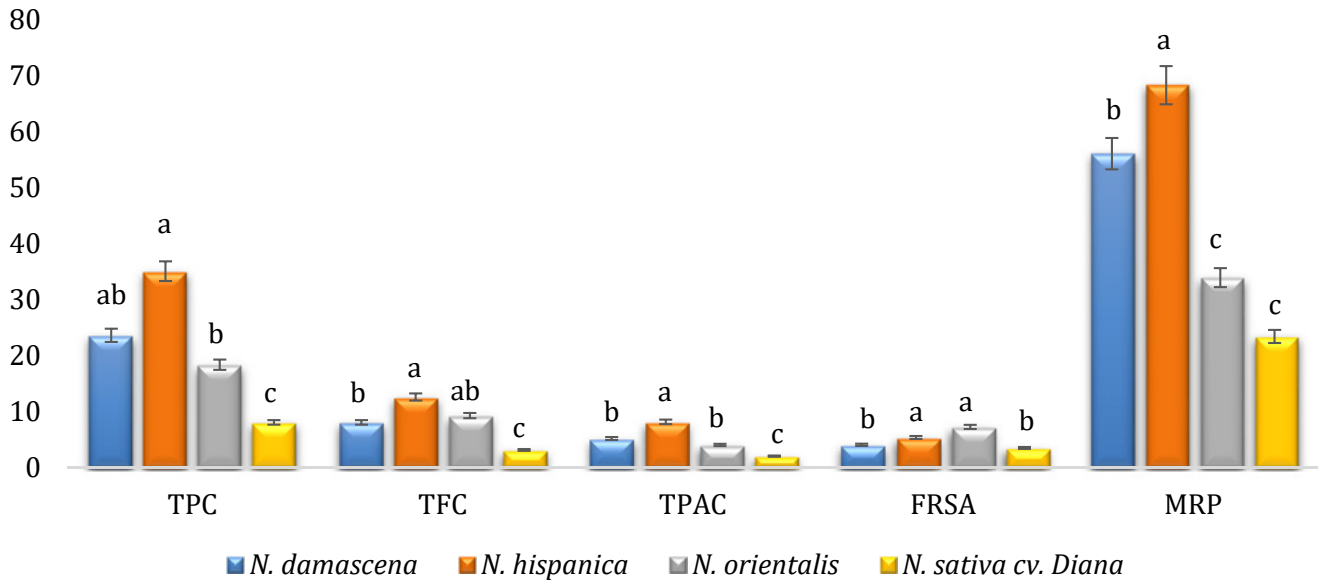

Figure 2 The antioxidant parameters of *Nigella* spp. seed extracts
TPC – total phenolic compounds, mg of gallic acid equivalent per gram; TFC – total flavonoid content, mg of quercetin equivalent per gram; TPAC – total phenolic acid compounds, mg of caffeic acid equivalent per gram; FRSA – free radical scavenging activity, mg of Trolox equivalent per gram; MRP – molybdenum reducing power, mg of Trolox equivalent per gram. Means in each column followed by different letters are significantly different ($p < 0.05$)

and TFC (5.20 mg QE.mg^{-1}) in the chloroform extracts (Meziti et al., 2012). Mazandarani (2015) determined 121.3 mg GAE.g^{-1} of TPC and 194.04 mg QE.g^{-1} of TFC in seed extracts of *N. sativa*. Guergouri et al. (2017) determined 16.67 µg GAE.g^{-1} of TPC and 3.83 µg QE.g^{-1} of TFC in the seed oil of *N. sativa*. Sadik et al. (2017) detected 19.9 mg GAE.g^{-1} DW of TPC in seed extracts of this species. The study of different extracts of *N. sativa* seeds showed that TPC in water, methanol, and ethanol extracts was 51.63, 31.16, and 5.47 µg GAE.g^{-1}, respectively. The chloroform and dichloromethane extracts demonstrated the absence of TPC (Dalli et al., 2021). The TFC in the same study was 10.11, 18.4, and 39.82 µg QE.g^{-1} in water, methanol, and ethanol extracts, respectively. It should be noted that chloroform and dichloromethane extracts showed 14.11 and 16.4 µg QE.g^{-1} of TFC, respectively (Dalli et al., 2021). The study of Alrashidi et al. (2022) demonstrated that TPC of oil extracts from seeds *N. sativa* was 116.39 mg GAE.g^{-1} in methanol extracts, 106.94 mg GAE.g^{-1} in ethanol extracts, and 238.80 mg GAE.g^{-1} in mixed extracts (methanol-water (1 : 1)).

Benazzous-Smail et al. (2023) determined in *N. sativa* and *N. damascena* seed extracts 6.28 and 18.41 mg GAE.g^{-1} of TPC, respectively, and 0.59 and 1.38 mg QE.g^{-1} of TFC, respectively.

Flavonoids are biochemical substances widely presented in plant raw and demonstrated antimicrobial, antioxidant, anticancer, and anti-inflammatory activities (Tungmunnithum et al., 2018).

The study of TPC of investigated *Nigella* spp. herb extracts during vegetation showed accumulation of them from 33.65 to 54.11 mg GAE.g^{-1} in the sprouting, from 54.89 to 65.76 mg GAE.g^{-1} in the budding, from 43.18 to 88.43 mg GAE.g^{-1} in the flowering, and from 49.21 to 71.45 mg GAE.g^{-1} in the ripening depending on species (Figure 3). At all, the TPC accumulation was found in a range of 33.65–88.43 mg GAE.g^{-1} depending on species and stage of growth.

As reported Bourgou et al. (2008), the TPC of shoots and root extracts of *N. sativa* was 10.04 and 4.01 mg GAE.g^{-1}, respectively. *Consolida regalis* Gray (Ranunculaceae) ethanol extracts of leaves, stems, and flowers demonstrated 12.41, 5.15, and 16.29 mg GAE.g^{-1} of TPC, respectively (Ucar, 2018). Cuce (2023) determined 19.17, 18.59, and 21.24 mg GAE.g^{-1} of the TPC in flower, root, and leaf extracts of *Adonis paryadrica* (Boiss.) Kandemir & Autaç (Ranunculaceae), respectively.

We determined TFC from 17.87 to 27.18 mg QE.g^{-1} at the sprouting, from 31.87 to 43.54 mg QE.g^{-1} in the budding, from 29.11 to 57.34 mg QE.g^{-1} in the flowering, and from 23.98 to 50.32 mg QE.g^{-1} in the ripening, depending on species (Figure 4). At all TFC of investigated plants was from 17.87 to 57.34 mg QE.g^{-1} depending on species and period of growth.

The study of different solvents of *N. sativa* seeds demonstrated 413.33 mg QE.100 g^{-1} of TFC (Kaushik and Barmanray, 2012). In the flower, root, and leaf extracts of *Adonis paryadrica* detected 32.42, 0.54, and 54.97 mg RE.g^{-1} (rutin equivalent) of TFC, respectively (Cuce, 2023).

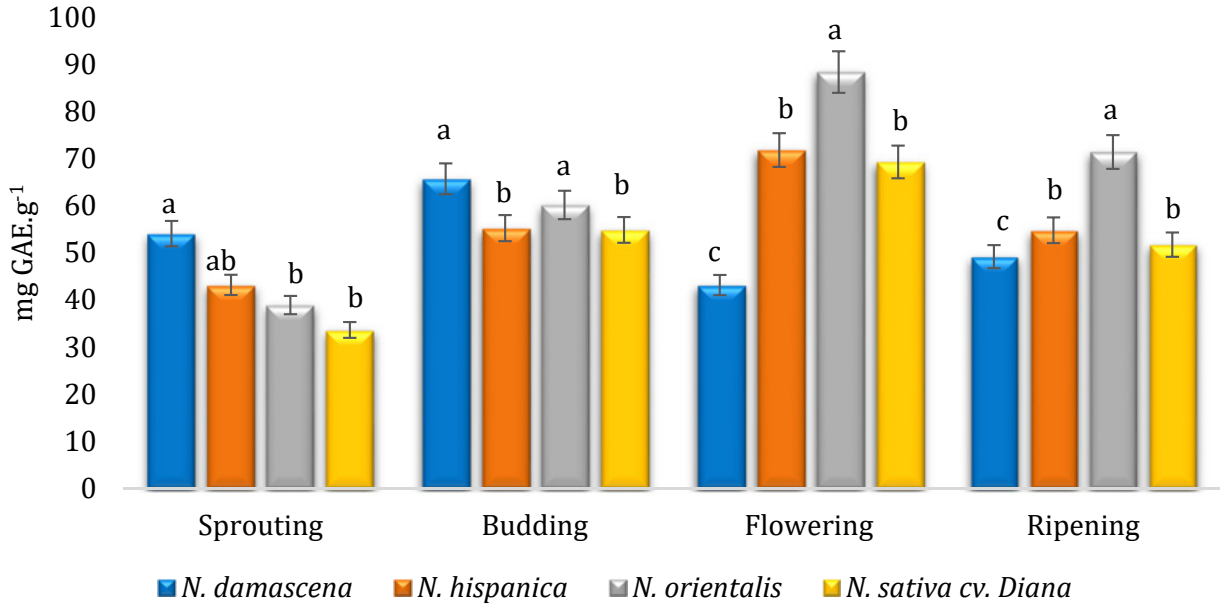

Figure 3 The seasonal variation of the total polyphenol content of *Nigella* spp. herb extracts
GAE – gallic acid equivalent. Means in each column followed by different letters are significantly different ($p < 0.05$)

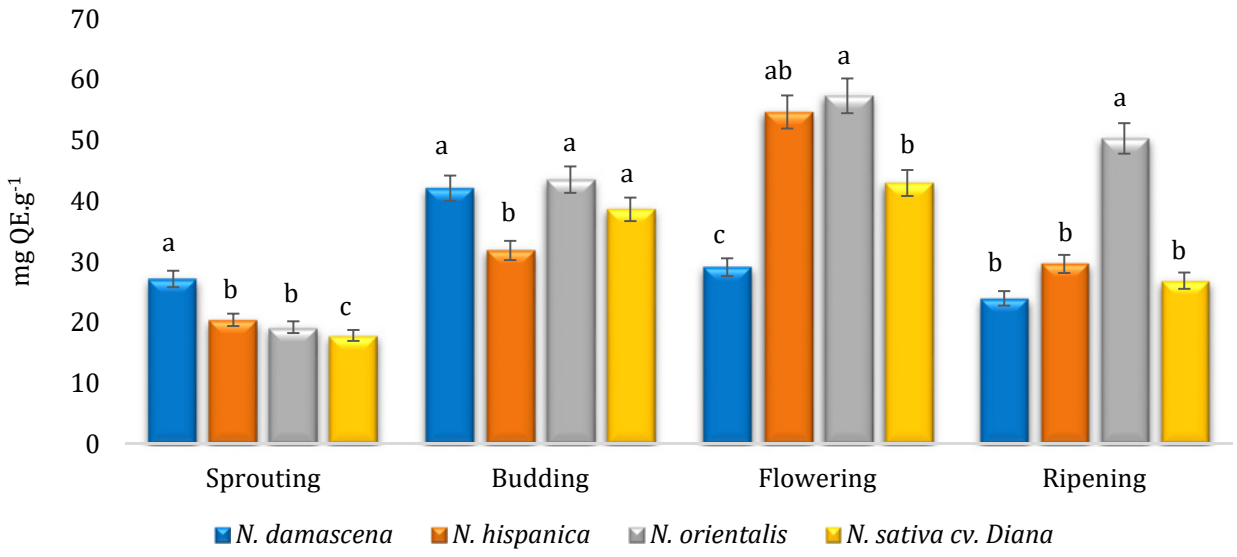

Figure 4 The seasonal variation of the total flavonoid content of *Nigella* spp. herb extracts
QE – quercetin equivalent. Means in each column followed by different letters are significantly different ($p < 0.05$)

The TPAC of investigated extracts was from 10.09 to 28.45 mg CAE.g^{-1} depending on species and stage of growth (Figure 5). This parameter was 12.11–17.32 mg CAE.g^{-1} in the sprouting, 11.17–18.43 mg CAE.g^{-1} in the budding, 10.09–28.45 mg CAE.g^{-1} in the flowering, and 17.86–22.43 mg CAE.g^{-1} in the ripening. The minimal values of TPAC were found in the sprouting and budding stage for *N. sativa* cv. Diana plants, in the flowering stage for *N. damascena*, and the ripening stage for *N. orientalis*. The maximum values of TPAC in the sprouting were determined for *N. damascena*, in the budding and ripening for *N. hispanica*, and the flowering period for *N. orientalis*.

The gallic, hydroxybenzoic, syringic, vanillic, caffeic, coumaric, and cinnamic acids were identified in extracts of *N. damascena* and *N. sativa* (Benazzous-Smail et al., 2023).

The most widely used method of antioxidant activity determination is the DPPH method which changes the extract coloring from purpure to yellow or green depending on raw. Kaushik and Barmanray (2012) studied different solvents and found high DPPH scavenging ability in methanol extracts (58.08%) and low in water extracts (20.81%) of *N. sativa* seeds. The study of *N. sativa* seed extracts from antioxidant potential by the DPPH method showed an increase in the

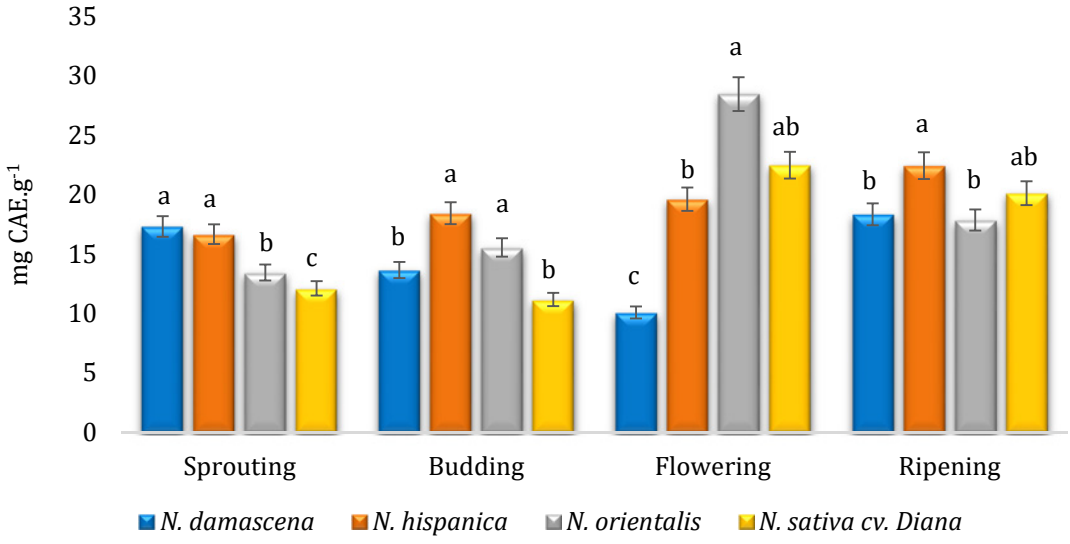

Figure 5 The seasonal variation of the total phenolic acid content of *Nigella* spp. herb extracts
CAE – caffeic acid equivalent. Means in each column followed by different letters are significantly different ($p < 0.05$)

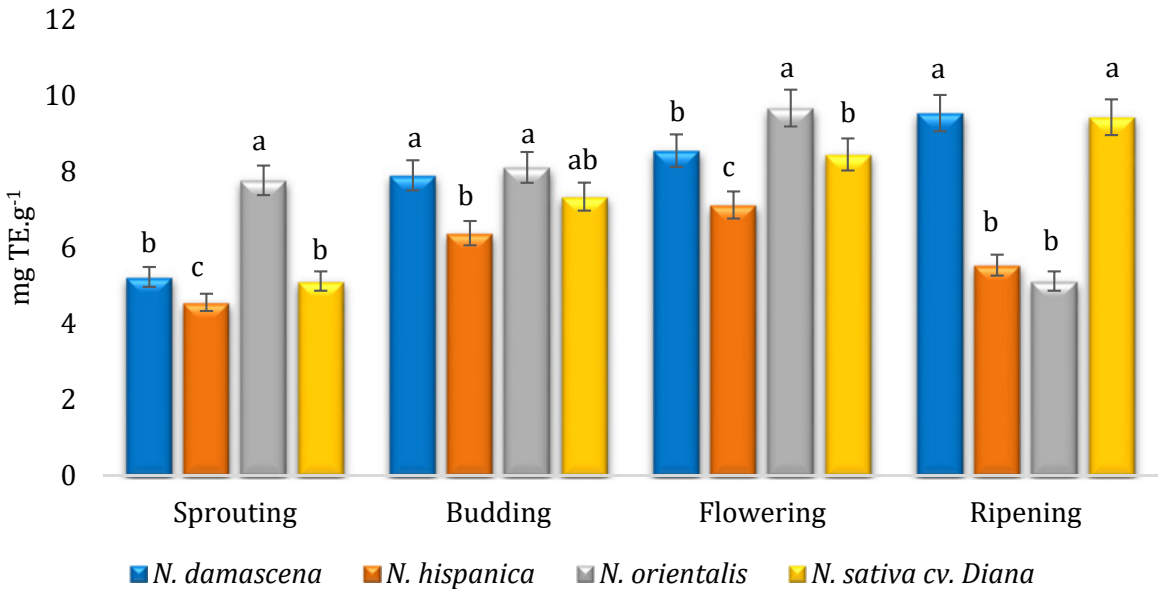

Figure 6 The seasonal variation of antioxidant activity by DPPH method of *Nigella* spp. herb extracts
TE – Trolox equivalent. Means in each column followed by different letters are significantly different ($p < 0.05$)

radical scavenging ability depending on concentration (Hassan et al., 2016). Mammad et al. (2017) found that methanol seed extracts of *N. sativa* had moderate antioxidant activity by the DPPH method.

FRSA of ethanol extracts of *Nigella* species showed that in the sprouting it was 4.56–7.77 mg TE.g^{-1}, in the budding it was 6.38–8.11 mg TE.g^{-1}, in the flowering it was 7.12–9.67 mg TE.g^{-1}, and in the ripening, it was 5.12–9.54 mg TE.g^{-1} depending on species (Figure 6). It should be noted that highest values of this parameter were determined for *N. orientalis* in the sprouting, budding, and flowering, and for *N. damascena* cv. Diana in the ripening.

The study by Malik et al. (2017) demonstrated *in vitro* antioxidant activity by the DPPH method of different Ranunculaceae (root extracts) from 0.016 to 0.251 g TE.g^{-1} DW (or from 16 to 251 mg TE.g^{-1} DW). Among these species are representatives of Aconimum L., Anemone L., and Pulsatilla L. genera. The study concerning *Adonis paryadrica* flower, root, and leaf

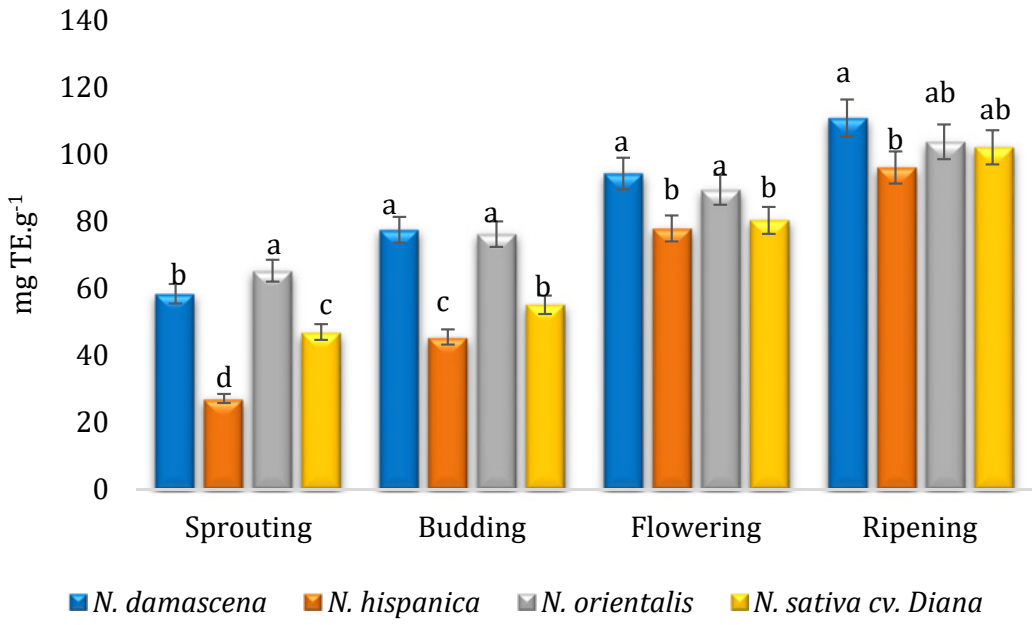

Figure 7 The seasonal variation of reducing power of *Nigella* spp. herb extracts by phosphomolybdenum method
TE – Trolox equivalent. Means in each column followed by different letters are significantly different ($p < 0.05$)

extracts showed that antioxidant activity by the DPPH method was 30.94, 12.58, and 38.26 μmol TE.g^{-1}, respectively (Cuce, 2023).

The MRP of investigated plant extracts depended on the stage of growth and species. In the sprouting, it was 27.14–65.29 mg TE.g^{-1}, in the budding it was 45.48–77.5 mg TE.g^{-1}, in the flowering 77.89–94.32 mg TE.g^{-1}, and in the ripening it was 96.11–110.87 mg TE.g^{-1} (Figure 7).

The study of relations between antioxidant activity by various methods and numerous groups of polyphenol compounds in plant extracts demonstrated a strong correlation between them (Kędzierska-Matysek et al., 2021; Szabo et al., 2021). A strong positive correlation can observed between different polyphenol compounds and antioxidant activity considering also conditions such as different times and temperatures of conducting (Lim et al., 2019).

The correlation analysis of obtained data concerning antioxidant parameters of investigated species showed that between two methods of antioxidant activity of herb extracts was a moderate correlation (r = 0.568) (Table 1). However, both FRSA and MRP had a weak correlation with TPC (r = 0.298 and r = 0.373, respectively), TFC (r = 0.263 and r = 0.336, respectively), and TPAC (r = 0.228 and r = 0.340, respectively).

Also, between accumulated compounds and the molybdenum-reducing power of seed extracts existed a strong correlation (r = 0.824–0.965). However, between two assays of antioxidant activity was a very weak correlation (r = 0.051). Between FRSA and TFC of seed extracts, a moderate correlation (r = 0.597) was found whereas with TPC (r = 0.285) and TPAC (r = 0.251) a correlation was weak.

According to Dalli et al. (2021), a strong correlation was found between the total flavonoid compounds and antioxidant activity by the DPPH method in seed extracts (r = 0.986). In our study, the TFC of seed extracts demonstrated a moderate correlation with FRSA (r = 0.597). Khiya et al. (2021) in the study of *Salvia officinalis* extracts found a strong correlation between TPC and FRSA by the DPPH method (r = 0.771) and between TPC and MRP (r = 0.932). In our study, between TPC and MRP of seed extracts was found a trong correlation, and in the rest extracts observed weak or moderate correlation.

Conclusions

The obtained data demonstrate the high antioxidant potential of different extracts of *N. damascena*, *N. hispanica*, *N. orientalis*, and *N. sativa* cv. Diana from the Kherson Oblast (Ukraine) during four stages of growth. The higher values of TPC, TFC, TPAC, and MRP were determined in seed extracts of *N. hispanica*, and FRSA in extracts of *N. orientalis*. The lowest values of all parameters demonstrated the study of seed extracts of *N. sativa* cv. Diana. The seasonal variation of TPC was the following: in the sprouting and budding maximal values found in extracts *N. damascena*, in the flowering and ripening in extracts of *N. orientalis*. The extracts of *N. damascena* showed high values of TFC in the sprouting and *N. orientalis* in the budding, flowering, and ripening. The maximal values of TPAC

Table 1 Pearson's coefficients of correlation of antioxidant parameters of *Nigella* species

Parameters	TPC	TFC	TPAC	FRSA	MRP
Herb					
TPC	1.000	0.941**	0.685*	0.298	0.373
TFC	0.941**	1.000	0.461	0.263	0.336
TPAC	0.685*	0.461	1.000	0.228	0.340
FRSA	0.298	0.263	0.228	1.000	0.568*
MRP	0.373	0.336	0.340	0.568*	1.000
Seeds					
TPC	1.000	0.939**	0.997**	0.285	0.965**
TFC	0.939**	1.000	0.925**	0.597*	0.824**
TPAC	0.997**	0.925**	1.000	0.251	0.960**
FRSA	0.285	0.597*	0.251	1.000	0.051
MRP	0.965**	0.824**	0.960**	0.051	1.000

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

were found in sprouting in *N. damascena* extracts, in the budding and ripening in *N. hispanica* extracts, and in the flowering in *N. orientalis* extracts. FRSA and MRP were maximal in the sprouting in *N. orientalis* extracts, in the ripening in *N. damascena* extracts. The maximal values of FRSA in the budding and flowering were determined in *N. orientalis* extracts, and MRP in the same period in *N. damascena* extracts. This study can be helpful for the identification of more needed periods of growth of *Nigella* spp. for obtained plant raw with high antioxidant activity. Also, these results can be useful for further structural biochemical investigations.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

Funding

The authors are thankful to the International Visegrad Fund and Bilateral Scholarship Programme for supporting this research.

Acknowledgements

The publication was prepared with the active participation of researchers in the International Network AgroBio*Net*, as a part of the international program "Agricultural Biodiversity to Improve Nutrition, Health and Quality of Life". The authors are indebted to the departmental colleagues for their support in the development of this manuscript.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with extracts derived from different pseudobulbs of *Dendrobium parishii* Rchb.f. (Orchidaceae) plants

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Article Details:
Received: 2023-10-15
Accepted: 2023-11-19
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0022

The current study was conducted to investigate the antioxidant properties of extracts derived from different pseudobulbs of *Dendrobium parishii* Rchb.f. using biomarkers of oxidative stress (2-thiobarbituric acid reactive substances as biomarkers of lipid peroxidation, aldehydic and ketonic derivatives of oxidative modification of proteins (OMP), total antioxidant capacity (TAC)) in the equine erythrocytes after *in vitro* treatment with the extracts. The current study is a continuation of our cooperation with M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine) concerning investigations of antibacterial and antioxidant properties of extracts derived from leaves and pseudobulbs of some species belonging to the Orchidaceae family. The antioxidant properties of extracts derived from different pseudobulbs of *D. parishii* using biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with the extracts revealed that extracts derived from different pseudobulbs of *D. parishii* exhibited varying activity. Extracts derived from the first, second, and third pseudobulbs of *D. parishii* increased lipid peroxidation after *in vitro* treatment of equine erythrocytes. Extracts derived from the first, sixth, and seventh pseudobulbs of *D. parishii* caused to decrease in the levels of aldehydic derivatives of OMP. On the other hand, ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from all parts of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) were decreased. Moreover, extracts derived from the first and sixth parts of pseudobulbs of *D. parishii* after incubation with erythrocyte samples caused to increase in the TAC levels. The study of extracts derived from *D. parishii* supports its favorable biological activities and lays a strong foundation for further exploration of its structure-activity relationships and activity development, providing experimental data for the development and utilization of extracts of *D. parishii*.

Keywords: *Dendrobium parishii*, pseudobulb extract, equine erythrocytes, biomarkers, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

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Introduction

Orchids (Orchidaceae) are a family of monocotyledonous perennial herbs; over 750 genera and 20 thousand species (according to other sources 35 thousand), in both hemispheres; most abundant and diverse in the tropics of America and South Asia. Some orchids are used in industry (vanilla) and medicine (orchid); many are grown in greenhouses. About 100 species, are in the tropics; Several species are cultivated there for their fruits containing vanillin (Zhang et al., 2018; Wang et al., 2019). In traditional Chinese medicine, orchids are still used for medicinal purposes. According to the studies, the uses of dried orchids range from improving vision to treating cancer. Information about the healing properties of orchids and their use as food moves from West to East more than from East to West. Many good discoveries in the East are kept secret from the West even today, with the development of information technology. Additionally, many cultures that use orchids for food do not document it as well as we would like (Bulpitt et al., 2007; Rokaya et al., 2014; Shang et al., 2017; Jiang et al., 2021).

Experiments with phytocomponents such as alkaloids, terpenes, stilbenoids, bibenzyls, phenanthrenes, flavonoids, and polysaccharides isolated from Orchidaceae have shown their potential medicinal utility (Sut et al., 2017). To date, several classes of phytocomponents have been isolated from therapeutically used orchids, demonstrating great chemical diversity (Sut et al., 2017). Among them, phenol derivatives have been studied for their biological activity, especially anticancer properties (Wang et al., 2021; Śliwiński et al., 2022), anti-inflammation (Jiang et al., 2019; Zhang et al., 2021), and anti-neurodegeneration properties (Li et al., 2017; Zhang et al., 2022).

Based on traditional folk uses, chemical composition, and pharmacological studies, *Dendrobium* is considered a promising medicinal and edible plant with multiple pharmacological activities (Li et al., 2023). *Dendrobium* was recorded in the Chinese Pharmacopoeia as an astringent, analgesic, tonic, and anti-inflammatory substance as early as around 200 AD (Li et al., 2023). Interestingly, *Dendrobium* stems and leaves have become a major part of research by Chinese and foreign researchers (Moretti et al., 2013; Prasad et al., 2017; Ke et al., 2020; Lou et al., 2020; Wang et al., 2022; Zhong et al., 2022; Li et al., 2023).

Wang (2021) in the review demonstrated that 131 compounds from *Dendrobium* plants have been reported to possess anti-inflammatory, antimicrobial, antioxidant, antiaging, anti-psoriasis, and tyrosinase-inhibitory activities, implying that *Dendrobium* plants are important resources for the discovery of active compounds and the development of new drugs and cosmetics. *Dendrobium crepidatum* Lindl. & Paxton, *Dendrobium denneanum* Kerr, *Dendrobium loddigesii* Rolfe, *Dendrobium nobile* Lindl., and *Dendrobium officinale* Kimura & Migo have been extensively studied. The major active compounds found in *Dendrobium* species are phenanthrenes, alkaloids, flavonoids, phenylpropanoids, and lignans. Several compounds, such as loddigesiinol A, (S)-5-methoxy-2,4,7,9-tetrahydroxy-9,10-dihydrophenanthrene, (S)-4-methoxy-2,5,7,9-tetrahydroxy-9,10-dihydrophenanthrene, 2,5-dihydroxy-4-methoxy-phenanthrene 2-O-β-D-glucopyranoside, (9R)-1,2,5,9-tetrahydroxy-9,10-dihydrophenanthrene 5-O-β-D-glucopyranoside, (+)-homocrepidine A, and vicenin 2, have significant anti-inflammatory activities and inhibit nitric oxide production (Wang, 2021).

The current study was conducted to investigate the antioxidant properties of extracts derived from different pseudobulbs of *Dendrobium parishii* Rchb.f. using biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with the extracts.

Material and methodology

Collection of plant materials and preparation of plant extracts

The pseudobulbs of *D. parishii* plants cultivated under glasshouse conditions were sampled at M.M. Gryshko National Botanic Garden (NBG), Kyiv, Ukraine. Since 1999, the whole collection of tropical and subtropical plants (including orchids) has had the status of a National Heritage Collection of Ukraine and is supported through State Funding. Besides, the NBG collection of tropical orchids was registered at the Administrative Organ of CITES in Ukraine (Ministry of Environment Protection, registration No. 6939/19/1-10 of 23 June 2004). Freshly collected pseudobulbs (seven parts beginning from the base of the growing tip of the rhizome, designated as numbers 1, 2, 3, 4, 5, 6, and 7) were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in the ratio of 1 : 9, w/w) at room temperature. The extracts were then filtered and used for analysis. The extract was stored at -25 °C until use.

Horses and collection of blood samples

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko,

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

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood samples were processed for analysis less than 12 hours after blood withdrawal. 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 erythrocytes was re-suspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extracts was added to 1.9 ml of equine erythrocytes. For positive control, incubation of equine erythrocytes with 4 mM phosphate buffer (pH 7.4) was used. After incubating the mixture at 37 °C for 60 min with continuous stirring, biomarkers of oxidative stress were assessed. Erythrocyte aliquots were used in the current study.

The 2-Thiobarbituric acid reactive substances 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 and described in the paper by Tkachenko et al. (2022). This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The nmol of MDA per mL was calculated using $1.56 \cdot 10^5$ $mM^{-1}.cm^{-1}$ as the extinction coefficient.

The carbonyl derivatives of oxidative modification of protein assay

To evaluate the protective effects of the extracts derived from pseudobulbs of *D. parishii* against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocyte suspension and plasma was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina et al. (1995) and described in the paper by Tkachenko et al. (2022). DNFH was used for determining carbonyl content in soluble and insoluble proteins. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient of 22,000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity

The total antioxidant capacity (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) and described in the paper by Tkachenko et al. (2022). The level of TAC in the sample (%) was calculated according to the absorbance of the blank samples.

Statistical analysis

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

Results and discussion

Levels of TBARS in the equine erythrocytes after *in vitro* incubation with extracts derived from seven parts of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) were presented in Figure 1.

Our results revealed that extracts derived from first three parts of pseudobulbs of *D. parishii* after incubation with equine erythrocyte samples caused to statistically significant increase in the TBARS levels (50.78 ±7.17 $nmol \cdot mL^{-1}$, 59.23 ±11.45 $nmol \cdot mL^{-1}$, and 52.77 ±9.59 $nmol \cdot mL^{-1}$, respectively) (by 41.5%, 65.1%,

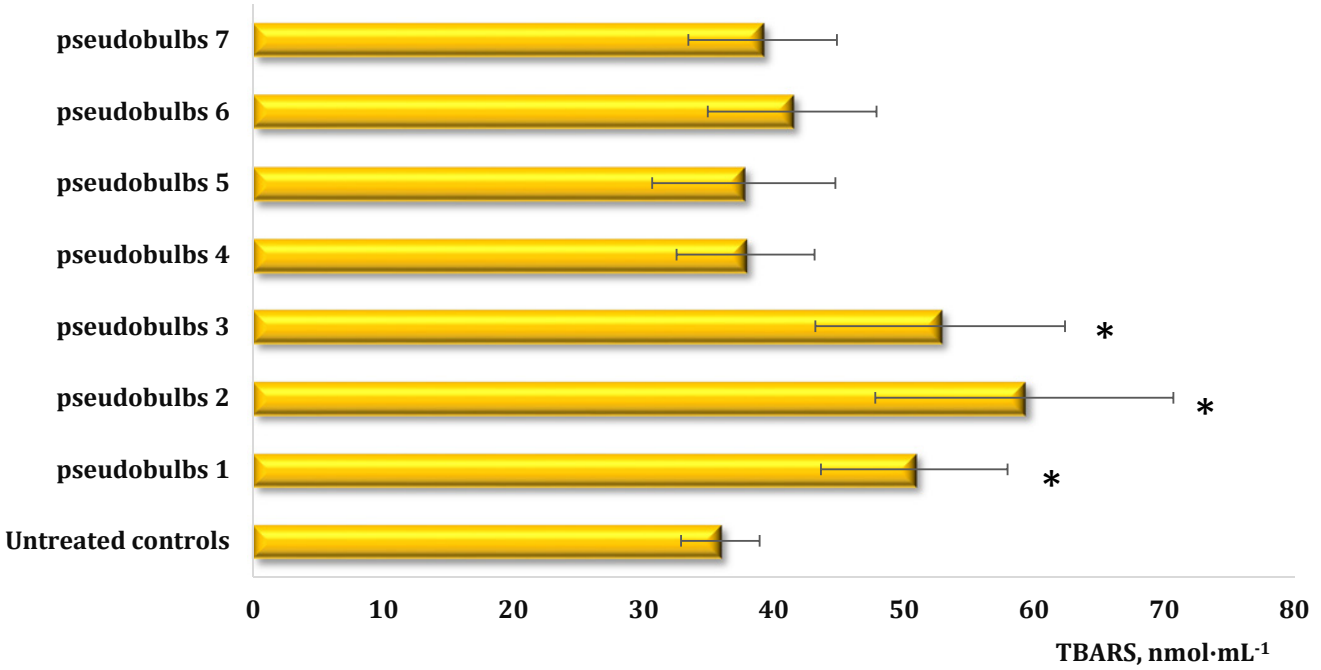

Figure 1 Levels of TBARS in the equine erythrocytes after *in vitro* incubation with extracts derived from seven parts of pseudobulbs of *Dendrobium parishii* Rchb.f. (beginning from the base of the growing tip of the rhizome)
*– changes were statistically significant (p <0.05) compared to untreated control

and 47.1%, p <0.05) compared to untreated samples (35.88 3.02 nmol·mL^{-1}). Extracts derived from last four parts of pseudobulbs of *D. parishii* after incubation with equine erythrocyte samples caused to statistically non-significant increase in the TBARS levels (37.82 ±5.30 nmol·mL^{-1}, 37.68 ±7.04 nmol·mL^{-1}, 41.40 ±6.48 nmol·mL^{-1}, and 39.13 ±5.71 nmol·mL^{-1}, respectively) (by 5.4%, 5%, 15.4%, and 9.1%, p >0.05) compared to untreated samples (35.88 ±3.02 nmol·mL^{-1}) (Figure 1).

Levels of aldehydic and ketonic derivatives of OMP in the equine erythrocytes after *in vitro* incubation with extracts derived from seven parts of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) were presented in Figure 2.

On the other hand, the contents of aldehydic derivatives of OMP in the erythrocyte samples after incubation with an extract derived from the first part of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) decreased to (28.14 ±1.08 nmol·mL^{-1}) compared to the untreated samples (31.16 ±1.89 nmol·mL^{-1}) (by 9.7%, P <0.05). Extracts derived from the last two parts of pseudobulbs of *D. parishii* after incubation with equine erythrocyte samples caused to statistically non-significant decrease in the levels of aldehydic derivatives of OMP to (29.30 ±1.35 nmol·mL^{-1}, and 29.03 ±1.08 nmol·mL^{-1}, respectively) (by 6%, and 6.8%, p >0.05) compared to untreated samples. Extracts derived from the second to fifth parts of pseudobulbs of *D. parishii* after incubation with equine erythrocyte samples caused to statistically non-significant increase in the levels of aldehydic derivatives of OMP by 3.9%, 2.6%, 5.6%, and 1% (p >0.05) for second, third, fourth, and fifth parts of pseudobulbs of *D. parishii*, respectively (Figure 2A).

Moreover, the contents of ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from all parts of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) decreased to (35.79 ±1.70 nmol·mL^{-1}, 37.61 ±2.01 nmol·mL^{-1}, 33.78 ±1.31 nmol·mL^{-1}, 33.53 ±1.49 mol·mL^{-1}, 36.56 ±2.03 nmol·mL^{-1}, 32.36 1.57 nmol·mL^{-1}, and 35.21 ±1.66 nmol·mL^{-1}) compared to the untreated samples (39.47 ±2.20 nmol·mL^{-1}) (by 9.3%, p >0.05 for extract from first pseudobulbs; by 4.7%, p >0.05 for extract from second pseudobulbs; by 14.4%, p <0.05 for extract from third pseudobulbs; by 15%, p <0.05 for extract from fourth pseudobulbs; by 7.4%, p >0.05 for extract from fifth pseudobulbs; by 18%, p <0.05 for extract from sixth pseudobulbs; by 10.8%, p >0.05 for extract from seventh pseudobulbs) (Figure 2B).

Levels of total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with extracts derived from pseudobulbs of *Coelogyne pandurate* were presented in Figure 3.

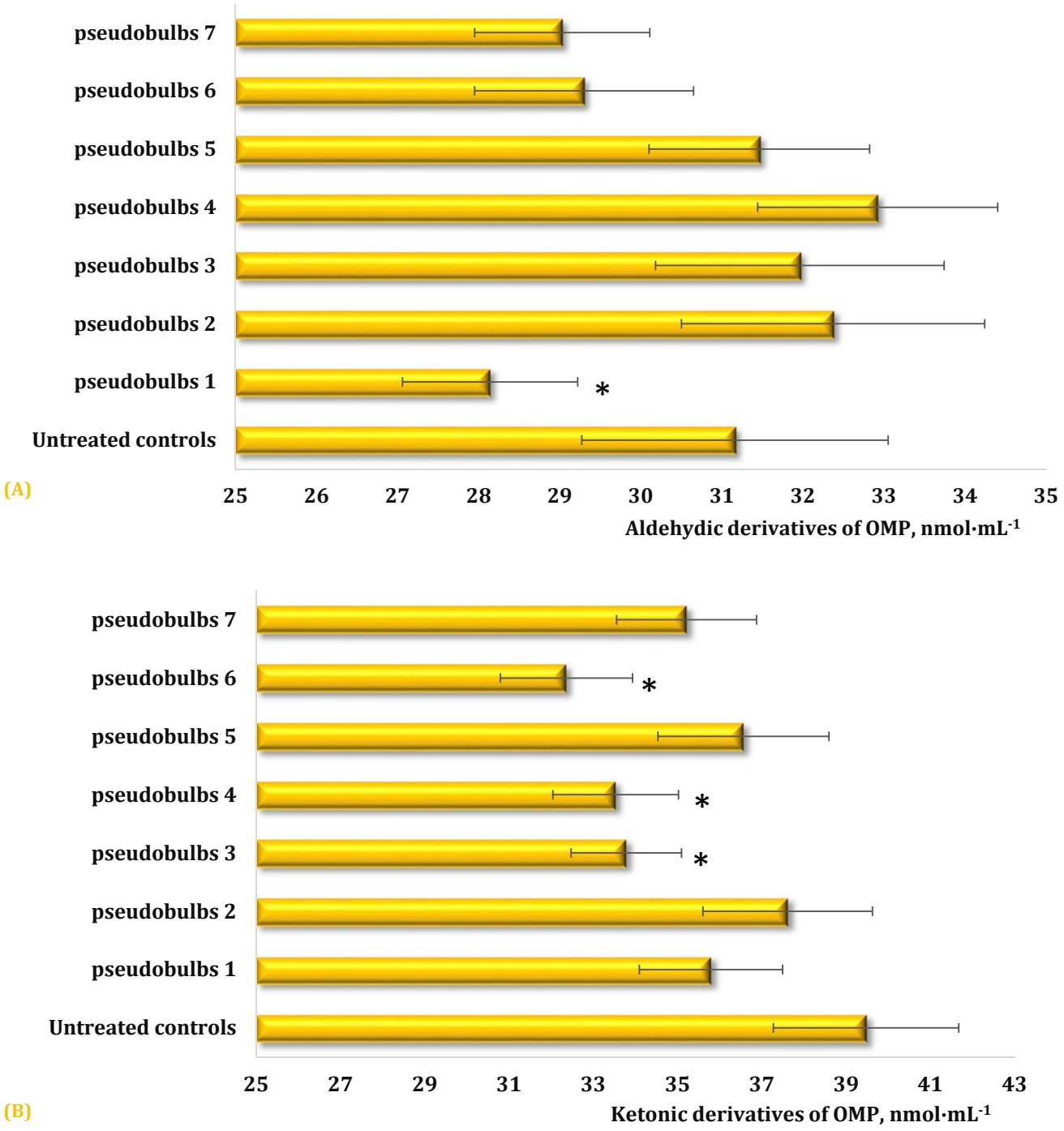

Figure 2 Levels of aldehydic and ketonic derivatives of OMP in the equine erythrocytes after *in vitro* incubation with extracts derived from seven parts of pseudobulbs of *Dendrobium parishii* Rchb.f. (beginning from the base of the growing tip of the rhizome)
*– changes were statistically significant ($p < 0.05$) compared to untreated control

Our results revealed that extracts derived from the first and sixth parts of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) after incubation with erythrocyte samples caused to increase in the TAC level (by 2.5% and 2.9%, p >0.05). On the other hand, extracts derived from the second, third, fourth, fifths, and seventh parts of pseudobulbs of *D. parishii* after incubation with erythrocyte samples caused to statistically non-significant decrease in the TAC level (by 1.9%, 8.4%, 13.4%, 8.1%, and 1.9%, p >0.05, respectively) (Figure 3).

Similarly, *in vitro* and *in vivo* studies reveal the antioxidant properties of *Dendrobium* plants. Polysaccharides of *Dendrobium* exhibit a variety of biological effects, including immunomodulatory, anti-tumor, gastro-protective, hypoglycemic, anti-inflammatory, hepatoprotective, and vasodilating effects (Chen et al., 2021). For example, *Dendrobium* potential effects with future perspectives for needed future research to maximize the use of bioactive compounds from *Dendrobium* for digestive tract disease treatment (Wu et al., 2023). Recent studies have shown that polysaccharide is one of the main biologically active components in *D. officinale* (He et al., 2022). Polysaccharides of *D. officinale* (DOP) can be considered an effective healthcare product for the treatment of precancerous lesions of gastric cancer and perhaps someday play a critical role in combatting gastric cancer. The research group of Zhao and co-workers has found that *D. officinale* extraction can prevent gastric carcinogenesis in rats through upregulating Bax and downregulating such factors as antiapoptotic B cell lymphoma 2 (Bcl-2), epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), and sphingosine-1-phosphate (S1P) (Zhao et al., 2015, 2017). Further analysis revealed that *D. officinale* extracts could regulate the levels of 8-hydroxy-deoxyguanosine (8-OHdG), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-PX) in plasma and cytokines related to carcinogenesis (Zhao et al., 2016). Polysaccharides, the main effective part of *D. officinale*, have been reported to harbor anticancer effects on gastric cancer cells and protective effects on experimental gastric ulcers in mice (Zeng et al., 2017; Zhang et al., 2018). *D. officinale* polysaccharides (DOP) prevent 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)-induced precancerous lesions of gastric cancer (PLGC) along with subsequent liver and kidney damage. The protective effects of DOP are associated with the reduction of 8-OHdG levels as well as the activation of the NRF2 pathway and its related antioxidant enzymes, heme oxygenase-1 (HO-1) and NADPH quinone oxidoreductase-1 (NQO-1) (Zhao et al., 2019).

The *Dendrobium nobile* Lindl polysaccharides as promising therapeutic candidates for UVB-induced

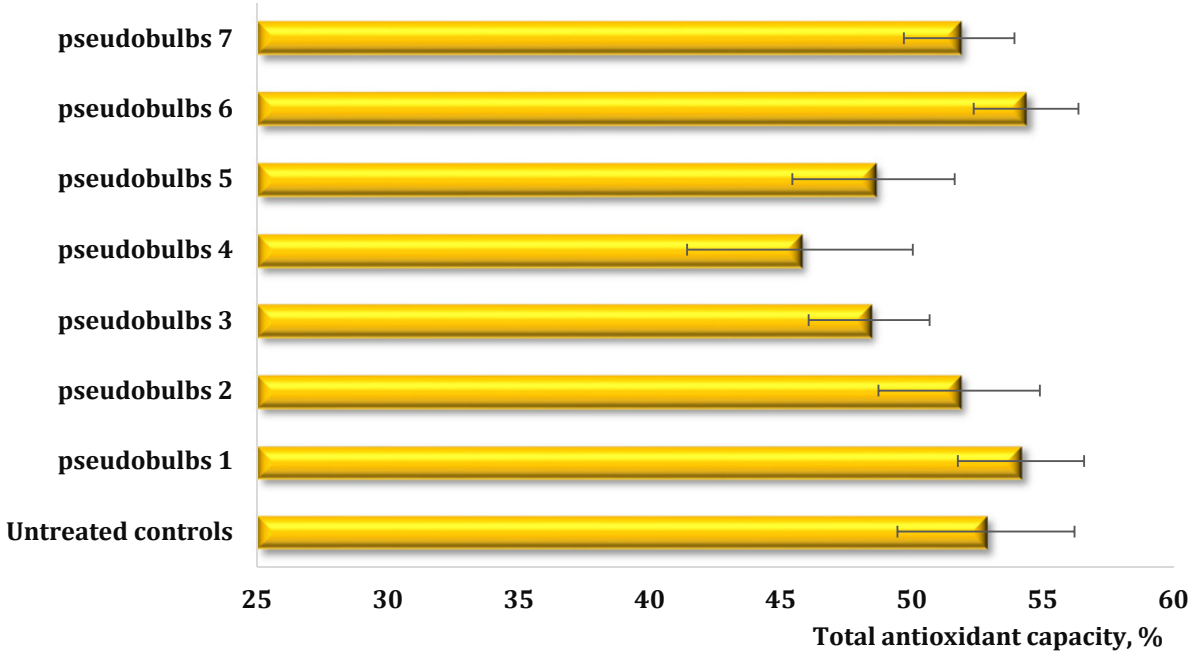

Figure 3 Levels of total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with extracts derived from seven parts of pseudobulbs of *Dendrobium parishii* Rchb.f. (beginning from the base of the growing tip of the rhizome)
*– changes were statistically significant (p <0.05) compared to untreated control

photodamage. Li et al. (2022) investigated the antagonistic effect of *Dendrobium nobile* Lindl. polysaccharides (DNLP) on UVA-induced photoaging of Human foreskin fibroblasts (HFF-1) and explore its possible anti-aging mechanisms. Results of these authors revealed that UVA irradiation reduced the viability, lifespan, and proliferation of HFF-1 cells, increased ROS and lipid peroxidation, and decreased the activities of free radical scavenging enzyme systems SOD, CAT, and GSH-Px. DNLP treatment can reverse UVA damage, reduce SA-β-Gal expression, reduce phosphorylation activation of the JNK/c-Fos/c-Jun pathway, and inhibit MMP-1, MMP-2 MMP-3, and MMP-9 protein expression (Li et al., 2022). DOP ameliorated UVB-induced oxidative damage and apoptosis in HaCaT cells via the regulation of MAPKs (Long et al., 2023).

The antioxidant activities of the polysaccharide *in vitro* assay indicate that DOP has a good scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, higher scavenging activity of hydroxyl radical and metal chelating activities (Luo et al., 2016). Zhang et al. (2022) compared the antioxidant activities and polysaccharide characterization of fresh and dry *D. officinale*. This study compared their antioxidant properties both *in vitro* and *in vivo*, and the molecular weight arrangement and monosaccharide composition of the fresh *D. officinale* polysaccharides (FDOPs) and the dried *D. officinale* polysaccharides (DDOPs). The results showed that the FDO and its polysaccharides had more significant effects on scavenging DPPH, ABTS, and hydroxyl radicals than the DDO. In addition, both the FDO and DDO significantly reduced lipid peroxidation levels and increased the SOD, T-AOC, CAT, and GSH levels in mice with acute liver damage caused by CCl_4, while the FDO and its polysaccharides were more effective. Histopathological analysis further verified the protective effect of the *Dendrobium* polysaccharides on CCl_4-induced liver injury (Zhang et al., 2022). The study of Lin et al. (2018) also revealed that DOP treatment exerted potentially hepatoprotective effects against APAP-induced liver injury. The decrease in alanine transaminase (ALT) and aspartate transaminase (AST) levels in the serum and reactive oxygen species (ROS), malondialdehyde (MDA), and myeloperoxidase (MPO) contents in the liver, as well as the increases in glutathione (GSH), catalase (CAT), and total antioxidant capacity (T-AOC) in the liver, were observed after DOP treatment. DOP treatment significantly induced the dissociation of Nrf2 from the Nrf2-Keap1 complex and promoted the Nrf2 nuclear translocation. Subsequently, DOP-mediated Nrf2 activation triggered the transcription and expressions of the glutamate-cysteine ligase catalytic (GCLC) subunit, glutamate-cysteine ligase regulatory subunit (GCLM), heme oxygenase-1 (HO-1), and NAD(P)H dehydrogenase quinone 1 (NQO1) in APAP-treated mice (Lin et al., 2018).

The purified polysaccharide from *D. officinale* presented significant immune-modulating activities involving ERK1/2 and NF-κB (He et al., 2016). *D. officinale* and its polysaccharides can significantly enhance cellular immunity and nonspecific immunity in mice (Liu et al., 2011). Humoral immunity was also enhanced after oral administration of *D. officinale*, but the polysaccharides had no influence. Both *D. officinale* and its polysaccharides markedly increased IFN-γ production by murine splenocytes (Liu et al., 2011). Also, *Dendrobium tosaense* Makino (syn. *D. officinale* Kimura & Migo) substantially boosted the population of splenic natural killer (NK) cells, NK cytotoxicity, macrophage phagocytosis, and cytokine induction in splenocytes (Yang et al., 2014). Bioassay using mouse macrophage cell line RAW264.7 indicated that DOP and its two subfractions enhance cell proliferation, TNF-α secretion, and phagocytosis in a dose-dependent manner. They also induced the proliferation of lymphocytes alone and with mitogens (Wei et al., 2016). DOP upregulated messenger RNA (mRNA) expression of anti-inflammatory/antioxidant proteins such as Nrf2 (nuclear factor erythroid 2-related factor), heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase (NQO1) in the liver (Chu et al., 2022).

We also investigated the changes in the oxidative stress biomarkers using the model of equine erythrocytes and plasma to evaluate the antioxidant activities of the aqueous extract derived from leaves of *Coelogyne brachyptera* Rchb.f. (Buyun et al., 2022). Results of our study revealed that erythrocytes were more sensitive to the action of an extract derived from leaves of *C. brachyptera*. The levels of aldehydic and ketonic derivatives of oxidatively modified proteins in the treated erythrocytes were significantly decreased, while these parameters were unchanged in the equine plasma. The treatment of equine erythrocytes by extract derived from leaves of *C. brachyptera* increased lipid peroxidation. On the other hand, plasma TBARS level after treatment by extract derived from leaves of *C. brachyptera* was at the same level as in untreated controls. The level of total antioxidant capacity was not-significantly changed after treatment both in equine plasma and erythrocytes (Buyun et al., 2022). Assessment of oxidative stress biomarkers

in the equine blood and the muscle tissue of rainbow trout (*Oncorhynchus mykiss* Walbaum) after *in vitro* incubation with leaf extract obtained from *D. parishii* was conducted in our previous study (Buyun et al., 2019, 2020).

Conclusions

The current study was conducted to investigate the antioxidant properties of extracts derived from different pseudobulbs of *Dendrobium parishii* Rchb.f. using biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with the extracts. The antioxidant properties of extracts derived from different pseudobulbs of *D. parishii* using biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with the extracts revealed that extracts derived from different pseudobulbs of *D. parishii* exhibited varying activity. Extracts derived from the first, second, and third pseudobulbs of *D. parishii* increased lipid peroxidation after *in vitro* treatment of equine erythrocytes. Extracts derived from the first, sixth, and seventh pseudobulbs of *D. parishii* caused to decrease in the levels of aldehydic derivatives of OMP. On the other hand, ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from all parts of pseudobulbs of *D. parishii* (beginning from the base of the growing tip of the rhizome) were decreased. Moreover, extracts derived from the first and sixth parts of pseudobulbs of *D. parishii* after incubation with erythrocyte samples caused to increase in the TAC levels. The study of extracts derived from *D. parishii* supports its favorable biological activities and lays a strong foundation for further exploration of its structure-activity relationships and activity development, providing experimental data for the development and utilization of extracts of *D. parishii*.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

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

Funding

The authors would like to extend their sincere appreciation to The International Visegrad Fund for supporting our study.

Acknowledgements

This work was supported by Pomeranian University in Słupsk (Poland) in cooperation with M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine).

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Chemical composition of *Castanea sativa* Mill. fruits

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Article Details:
Received: 2023-10-07
Accepted: 2023-11-20
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0023

Nowadays, especially important is the introduction of new plants into cultivation in connection with global climate change. Acclimatization of new fruit plants in Ukraine contributes to an increase in the biodiversity of flora. *Castanea sativa* Mill. (sweet chestnut) fruits belong to underutilized fruit plants of the forest-steppe of Ukraine, as a promising and economically profitable crop. The aim of the study, which focused on the nutritional value and composition of *C. sativa* fruits of Ukrainian origin, was to strengthen the knowledge about the contents of essential nutrients, fatty and amino acids profiles, and the content of selected elements. Chestnut fruits are distinguished by low lipids content (1.9%), a substantial share of proteins (14.9%) and fructose (19.3 $g.kg^{-1}$), and high β-carotene content (143.1 $mg.kg^{-1}$). The lipid fraction of chestnut fruits is strongly dominated by SFAs, namely palmitic acid (C16:0) 37.93 $g.100\ g^{-1}$ of oil, followed by the MUFA oleic acid (C18:1 *9c*) 9.07 $g.100\ g^{-1}$ of oil, and PUFA α-linolenic (C18:3 *9c12c15c*) 9.01 $g.100\ g^{-1}$ of oil. From 18 determined amino acids (128.1 $g.kg^{-1}$ of DW), glutamic acid was found to be the major component (17.2 $g.kg^{-1}$). Surprisingly essential amino acids/total amino acids ratio amounted to 44%, which according to FAO WHO may be regarded as high-quality protein plant food. Calcium and phosphorus were the most abundant elements (8,213 and 8,155 $mg.kg^{-1}$ of DW respectively), simultaneously with a low Na : K ratio (low amount of Na 9 $mg.kg^{-1}$). Summing up, presented composition and literature data regarding nutrients proved that *C. sativa* fruits are valuable ingredients in a healthy diet.

Keywords: sweet chestnut, nutritional value, fatty and amino acids, elements

Introduction

Nowadays, especially important is the introduction of new plants into cultivation in connection with global climate change (Klymenko et al., 2017; Raza et al., 2019). Acclimatization of new fruit plants in Ukraine contributes to an increase in the biodiversity of flora. *Castanea sativa* Mill. fruits belong to underutilized fruit plants in the forest-steppe of Ukraine, as a promising and economically profitable crop (Klymenko and Grygorieva, 2013; Grygorieva et al., 2017; Klymenko et al., 2017).

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Castanea sativa Mill. (chestnut) belongs to Fagaceae Dumort. family. In total, 13 Castanea species are recognized and are native to the temperate zone of the Northern Hemisphere; 5 in East Asia, 7 in North America, and one in Europe (Burnham et al., 1986). The most important of them are: *Castanea sativa* (Europe, Asia Minor, North Africa), *Castanea dentata* (Marsh.) Borkh. (USA), *Castanea mollissima* Blume and *C. crenata* Sieb. et Zucc. (Eastern Asia). However, *Castanea sativa* (sweet chestnut) is the most commonly consumed (Goulão et al., 2001).

Castanea sativa fruits have become very important in the human diet due to their nutritional composition and numerous health benefits. Fruits are rich in carbohydrates and can be regarded as a good source of essential fatty acids (Borges et al., 2006), vitamins C and E (Peña-Mendez et al., 2008; Barreira et al., 2009), organic acids (Ribeiro et al., 2007), polyphenols (Neri et al., 2010). *Castanea sativa* fruits are recognized as generally low in fat content, thus supporting a decrease in cholesterol levels. Moreover, fruits contain a high amount of macro- (K, P, Mg, Ca, Na) and micro-nutrients (Mn, Fe, Zn, and Cu) (Poljak et al., 2021). Nuts are predominantly consumed in roasted or boiled form, but also can be used as valuable ingredient in cake and candy manufacturing (Mert et al., 2007). It was stated that cooked *Castanea sativa* is a rich source of phenolic compounds, such as gallic and ellagic acids), and organic acids (mostly citric acid) (Gonçalves et al., 2010).

The study aims to strengthen the knowledge about the nutritional value of *Castanea sativa* Mill. fruits of Ukrainian origin. For that purpose, the contents of most essential nutrients, profiles of fatty and amino acids, and the content of selected elements of *Castanea sativa* were determined.

Material and methodology

Sampling

Fruits of *Castanea sativa* Mill. (Figure 1) were collected in July 2022 from trees growing in the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (Kyiv, Ukraine; 197 m a.s.l.). The biochemical composition of fruits was determined in dried matter.

Figure 1 *Castanea sativa* Mill.
(A) – tree; (B) – fruits

Chemicals and reagents

All the chemicals and reagents 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 content were determined according to CSN-EN 12145 procedures (1997). Total lipid content was determined according to the ISO method (ISO 659; 1998).

Analysis of sugars

For the determination of sugar content, 1 g of fruits was vigorously shaken with 10 mL of water/ethanol mixture (4 : 1) on a vertical shake table (GFL, Germany). After 1 h of extraction, the mixture was centrifuged at 6,000 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 1,260 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 comparing the relative retention times of sample peaks with standards Sigma-Aldrich (Steinheim, Germany). The contents of sugars were expressed as $g.kg^{-1}$ of dry weight.

β-carotene content

The extraction was performed by the method of Sarker and Oba (2019). Briefly, 1 g of dry chestnut fruits 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 measured at 480 and 510 nm using a spectrophotometer (UV-VIS spectrophotometer, Jenway Model 6405, England). The content of β-carotene was expressed as mg of β-carotene per kg of dry weight. The following formula was used to calculate β-carotene content:

$$\beta\text{-carotene} = 7.6(\text{abs. at } 480) - 1.49(\text{abs. at } 510) \times \text{final volume}/1{,}000$$

Elemental analysis

The contents of macro-, 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). Fruits 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. The results were expressed as $mg.kg^{-1}$ of dry weight.

Determination of amino acids

The 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 an average particle 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 an 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 2% v/v 4 M acetic buffer (pH 5.5). SnCl2 was used as a reducing agent. The solution of ninhydrin was stored in an inert atmosphere (N_2) without access of light at 4 °C. The flow rate was $0.25\ mL.min^{-1}$, and the reactor temperature was 120 °C. Individual amino acid values were expressed as $g.kg^{-1}$ of dry fruits.

Fatty acid composition

Lipids extracted from *Castanea sativa* fruits were converted to fatty acid methyl esters (FAME) to determine fatty acid (FA) composition according to the official method Ce 2-66 (1997). 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^{-1}, air 450 $mL.min^{-1}$). A detailed description of the chromatography conditions is presented in the work of Szabóová et al. (2020). Standards of a C4-C24 FAME mixture (Supelco, Bellefonte, PA, USA) were applied to

identify FAME peaks. The evaluation was carried out by the ChemStation 10.1 software. The contents of FAs were expressed as g.100.g^{-1} of oil.

Statistical analysis

The results were subjected to one-way ANOVA followed by the 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

Chestnut fruits contain various nutrients (proteins, lipids/fat, free sugars), vitamins (Table 1), and minerals (Table 3) that are important for human health. It was previously stated that chestnut fruits are mainly composed of carbohydrates: primarily starch, ranging from 38.6 up to 67.2 g.100 g^{-1} of dry weight (DW) (de Vasconcelos et al., 2010a). For the commercial quality of chestnut fruits, the presence of monosaccharides and disaccharides (glucose, fructose, sucrose, and maltose) is highly important. Interestingly, saccharose content can reach up to one-third of total sugars. The study of Ciucure et al. (2022) showed that sucrose was the most abundant sugar in chestnut fruits with values ranging from 20.34 up to 154.94 g.kg^{-1} of DW. The sucrose level of chestnut fruits cultivated in Romania was similar to those grown in Turkey (68.20–174.00 g.kg^{-1} of DW) (Mert et al., 2017), but lower than in chestnut fruits cultivated in Italy (2.98–245.09 g.kg^{-1} of DW) (Beccaro et al., 2020) and Portugal (40.30–233.00 g.kg^{-1} of DW) (Barreira et al., 2010), and higher compared with chestnut fruits cultivated in Tenerife (Spain) (31.10–99.40 g.kg^{-1} DW) (Suárez et al., 2012). In our study, the content of fructose was relatively high 19.3 g.kg^{-1} of DW in comparison with other analyzed sugars (Table 1). Research of Ciucure et al. (2022) revealed that fructose content ranged between 1.55–14.35 g.kg^{-1} of DW, and glucose 1.56–14.46 g.kg^{-1} of DW. However, data presented by Ciucure et al. (2022) was higher than those reported by other Authors who found glucose and fructose contents between not detected level and 3.1 g.kg^{-1} of DW for both monosaccharides (Míguelez et al., 2004) or between 0.56–2.40 g.kg^{-1} of DW for fructose and 0.49–1.90 g.kg^{-1} of DW for glucose (Suárez et al., 2012). Mert and Ertürk (2017) indicated fructose content between 1.5–8.0 g.kg^{-1} of DW and glucose between 4.0–11.3 g.kg^{-1} of DW – similar to Ciucure et al. (2022).

Table 1 Proximate composition of *Castanea sativa* Mill. fruits (means ±SE)

Component	Content
Proteins (%)	14.60 ±0.67
Lipids (%)	1.90 ±0.09
Fructose (g.kg^{-1})	19.30 ±0.48
Maltose (g.kg^{-1})	<0.5
Sucrose (g.kg^{-1})	<0.5
Lactose (g.kg^{-1})	<0.5
Dry matter (%)	92.16 ±3.12
Ash (%)	4.38 ±0.11
β-carotene (mg.kg^{-1})	143.10 ±3.56
Vitamin A (retinyl acetate) (mg.kg^{-1})	<0.1
Vitamin E (α-tocopherol) (mg.kg^{-1})	74.50 ±2.19

It should be pointed out that *Castanea sativa* fruits contain a relatively low content of lipids (1.9%) and nutritionally important PUFAs (Tables 1 and 2). Lipids extracted from chestnut contained significant levels of saturated fatty acids (SFAs) 48.69 g.100 g^{-1} of oil (Table 1). Other Authors confirmed that chestnut fruits are distinguished by low crude fat content, ranging values between 0.66–3.60%, compared with hazelnuts or almonds (36–77%). Significant differences among the fat levels in chestnut fruits depend on their cultivar, country of cultivation, crop year, and environmental conditions (Borges et al., 2007). Our results support the view that generally fruits are known as poor lipid sources, such as Rosa rugosa pericarp (0.67–0.88%), red raspberries, blackberries, or strawberries (0.25–0.42%) (Tabaszewska et al., 2021). The protein content (14.6%) was recognized as high compared with other cultivated fruits, mostly up to 1%. A study of Ertürk et al. (2006) showed that chestnut fruits may contain only 0.49–2.01 g.100 g^{-1} of DW of lipids and 4.88–10.87 g.100 g^{-1} of DW of proteins. Moreover, chestnut fruits proved to be a rich source of carotenoids, mainly β-carotene (143.1 mg.kg^{-1}), whose content was similar to carrots or sweet potato cultivars known for their high amounts.

The application of gas chromatography enabled the detection of 14 fatty acids in lipid fraction extracted from chestnut fruits, which belonged to SFA, MUFAs, and PUFAs. The results of fatty acid composition are presented in Table 2. It was found that chestnut lipid fraction is strongly dominated by SFAs, namely palmitic acid (C16:0) 37.93 g.100 g^{-1} of oil, followed by the oleic acid (C18:1 *9c*) 9.07 g.100 g^{-1} of oil, and α-linolenic (C18:3 *9c12c15c*) 9.01 g.100 g^{-1} of oil. The fatty acid profile is dominated by SFAs, which provide

high stability of lipid fraction, however, these fatty acids are considered as not beneficial for the cardiovascular system (Barreira et al., 2009). The obtained results are in accordance with the findings of Zhou et al. (2021).

Table 2 Fatty acid composition (g.100 g^{-1} of oil) of lipids of *Castanea sativa* Mill. fruits (means ±SE)

Fatty acid	Content
SFAs	48.69 ±0.33
C12:0	0.57 ±0.02
C14:0	1.53 ±0.07
C16:0	37.93 ±1.34
C17:0	0.65 ±0.03
C18:0	5.53 ±0.07
C20:0	0.61 ±0.02
C22:0	1.00 ±0.02
C24:0	0.87 ±0.01
MUFAs	11.38 ±0.05
C16:1	0.76 ±0.02
C18:1	9.07 ±0.13
C20:1	0.69 ±0.02
C22:1	0.86 ±0.03
PUFAs	25.46 ±0.29
C18:2	7.44 ±0.17
C18:3	9.01 ±0.35

saturated fatty acids – SFAs; monounsaturated fatty acids – MUFAs; polyunsaturated fatty acids – PUFAs

Even though only two fatty acids from PUFAs were detected, their contents can be regarded as highly important due to their health-promoting properties and significant share in the whole fatty acid profile (25.46 g.100 g^{-1} of oil). Generally, chestnuts may be perceived as a valuable source of essential fatty acids, which are known as important in the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, neuronal development, and visual function (Benatti et al., 2004). Recently consumers have searched for plant sources of PUFAs, especially α-linolenic acid belonging to the n-3 family (a substrate for long-chain PUFAs EPA and DHA synthesis). The consumption of n-3 FAs can slow down the growth of cancer cells and markedly reduce the side effects of chemotherapy (Borges et al., 2007). In turn, oleic acid is the main component of the cell membrane and cell nucleus and also facilitates the dissolution and absorption of fat-soluble vitamins, such as vitamin E (Reboul, 2019). Together with oleic acid, α-linolenic acid is involved in the prevention and treatment of cardiovascular and cerebrovascular diseases, thanks to cholesterol elimination (Visioli and Poli, 2020).

Eighteen amino acids were detected in *Castanea sativa* fruits, nine of them belonged to essential amino acids and nine to non-essential ones (Figure 2). The content of amino acids in chestnut fruits was at the level of 128.1 g.kg^{-1} of DW, while the content of total essential amino acids was 56.3 g.kg^{-1} of DW (amounted to 44%) and 71.8 g.kg^{-1} of DW (56%) for total non-essential amino acids. It should be highlighted that glutamic acid was found to be the major component (17.2 g.kg^{-1}),

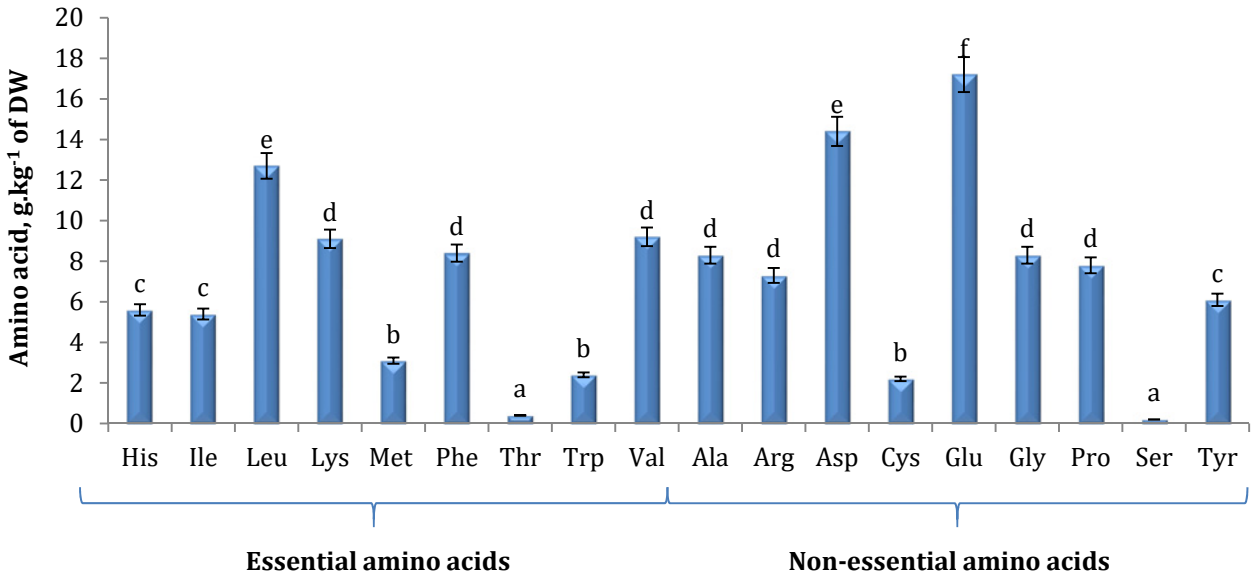

Figure 2 Amino acid composition (g.kg^{-1} of dry weight; DW) of *Castanea sativa* Mill. fruits
a, b, c, d, e, f – different superscripts indicate the significant differences at p <0.05

followed by aspartic acid (14.4 g.kg^{-1}) and leucine (12.7 g.kg^{-1}). Generally, chestnut fruits are not considered as a rich source of amino acids. However, it seems worth noting the fact that the essential amino acids/total amino acids ratio amounted to 44%, which suggests that chestnut fruits could be a source of high-quality protein in the human diet. FAO and WHO indicate that foods with a ratio above 40% are a perfect protein source, just like the composition of animal-origin products (Tabaszewska et al., 2021).

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) in studied samples of chestnut fruits are presented in Table 3. First of all, it should be highlighted that there is still considerable data lacking for the contents of minerals in chestnut fruits. In our study calcium (Ca) and phosphorus (P) were undoubtedly the most abundant elements in *Castanea sativa* fruits (8,213 and 8,155 mg.kg^{-1} of DW respectively), followed by Mg, K, and S. Ertürk et al. (2006) assayed 43–230 mg.100 g^{-1} of Ca, 107–191 mg.100 g^{-1} of P, and 70–160 mg.100 g^{-1} of Mg. Like most vegetables, the Na content in chestnut is very low (9 mg.kg^{-1} of DW). It should be noted that a high intake of Ca, Mg, and K together with a low Na intake is associated with protection against bone demineralization, arterial hypertension, insulin resistance, and cardiovascular risk (Segura et al., 2007). A low Na : K ratio makes chestnuts interesting for diets with a defined electrolytic balance (Borges et al., 2008). In the study of de Vasconcelos et al. (2010b) K and P were predominant and therefore these elements revealed the highest variability. The findings of Ciucure et al. (2022) revealed that K was the most abundant, ranging between 403.56 and 2,652.11 mg.100 g^{-1} DW, followed by Na which ranged between 4.63 and 17.20 mg.100 g^{-1} DW. Calcium contents ranged from 14.01 mg.100 g^{-1} in 'Marsol' to 29.58 mg.100 g^{-1} DW in 'Précoce Migoule', while Mg content varied between 48.62 mg.100 g^{-1} in 'Marsol' and 102.88 mg.100 g^{-1} DW in 'Précoce Migoule'.

Among microelements, the highest content was stated for Mn (712.5 mg.kg^{-1} of DW). For comparison, the content of Mn in different chestnut cultivars was within the range of 2–3.3 mg.100 g^{-1} of DW (de Vasconcelos et al., 2010b), and 0.7–5.5 mg.100 g^{-1} (Ertürk et al., 2006). Chestnut fruits also contained significant amounts of Fe (1.84–3.43 mg.100 g^{-1} of DW) and Cu (0.31–0.98 mg.100 g^{-1} of DW) which are similar to values found in other studies (Ertürk et al., 2006; Poljak et al., 2021)], but lower than those obtained by Borges et al. (2008) and de Vasconcelos et al. (2010b).

Table 3 Elements composition of *Castanea sativa* Mill. fruits (mg.kg^{-1} of DW) (mean ±SE)

Element	Content
Macroelements	
K	1,861 ±120
P	8,155 ±232
Ca	8,213 ±221
S	1,597 ±111
Mg	2,114 ±119
Na	9.0 ±0.9
Microelements	
Zn	26.0 ±1.7
Fe	51.0 ±1.2
Cu	8.0 ±0.5
Mn	712.5 ±67
Cr	4.70 ±0.07
Se	<0.2
Metals	
Al	12.5 ±0.9
As	<0.3
Cd	0.147 ±0.002
Ni	3.21 ±0.03
Hg	0.014 ±0.003
Pb	0.30 ±0.002

Regarding the presence of metals, the content of aluminum (Al; 12.5 mg.kg^{-1} of DW) dominated among all detected metals in *Castanea sativa* fruits. The content of Ni in chestnut fruits at the level of 3.21 mg.kg^{-1} of DW, does not exceed the maximum permissible value of 67.9 mg.kg^{-1} in vegetables for human consumption (Mensah et al., 2009). However, the content of metals was generally low. The accumulation of trace metals is a normal and essential process for the growth and nurturing of plants (Tabaszewska et al., 2021). Probably low amounts of trace elements resulted from low environmental contamination.

Conclusions

The presented study strengthens the knowledge about the nutritional value and composition of *Castanea sativa* Mill. fruits of Ukrainian origin. Chestnut fruits are characterized by low lipids content, substantial share of proteins and fructose, and high β-carotene content. The lipid fraction of chestnut fruits is strongly dominated by SFAs, namely palmitic acid, followed by the MUFA oleic acid, and PUFA α-linolenic. Chestnut fruits may be appreciated as plant sources of PUFAs,

especially α-linolenic acid belonging to the n-3 family. Among detected 18 amino acids, glutamic acid was found to be the major component. Surprisingly essential amino acids/total amino acids ratio amounted to 44%, which according to FAO WHO may be regarded as high-quality protein plant food. In response to limited information about minerals in chestnut fruits, our study showed that calcium and phosphorus were the most abundant elements, simultaneously with a low Na : K ratio. Summing up, presented composition and literature data regarding nutrients proved that chestnut fruits are valuable ingredients in a healthy diet. In addition, chestnuts are currently used in pediatrics for the treatment of gastroenteritis and as a gluten-free diet in cases of celiac disease.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

Funding

The authors are thankful to the International Visegrad Fund and Bilateral Scholarship Programme for supporting this research.

Acknowledgements

The publication was prepared with the active participation of researchers in the International Network AgroBio*Net*, as a part of the international program "Agricultural Biodiversity to Improve Nutrition, Health, and Quality of Life". The authors are indebted to the departmental colleagues for their support in the development of this manuscript.

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Agrobiodiversity for Improving Nutrition, Health and Life Quality

Research Article OPEN ACCESS

Pollen morphology of some species of the genus *Amelanchier* Medik.

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Article Details:
Received: 2023-11-20
Accepted: 2023-11-22
Available online: 2023-11-30

DOI: https://doi.org/10.15414/ainhlq.2023.0024

Pollen grain structure is one of the diagnostic taxonomic and phylogenetic parameters. Study of morphology and morphometry of pollen grains of *Amelanchier* spp. allows found new additional diagnostic parameters of species. We determined that pollen of *Amelanchier* spp. is variable both in size and morphology. SEM investigations showed that the pollen grains various species of *Amelanchier* are prolate and perprolate, the surface sculpture and shape index of the species vary. The average length of the polar axis varied from 27.38 to 47.14 µm and the width of the equatorial axis was in the range from 14.33 to 28.95 µm. Shape index (P/E) of tested species varied from 1.77 to 2.09. The most average length of pollen was *Amelanchier spicata* (43.24 µm) and the least length was *Amelanchier arborea* (13.69 µm). The most average width pollen was *Amelanchier canadensis* (23.04 µm), and the least width was *Amelanchier arborea* (16.07 µm). Cluster analysis showed that the relationships of the tested *Amelanchier* species according to morphological features are represented by two main groups. It is evidently that *A. arborea* which has the smallest parameters is really separated from other species. *A. lamarki*, *A. spicata* and *A. canadensis* are similar according to morphometric sizes. Studies via scanning electron microscope have established characteristic differences in the morphometric and microsculptural features of pollen for each of the studied *Amelanchier* spp. which can be used to identify the representatives of species. Differences in the size of pollen grains in comparison with other authors may be the result of many environmental factors specific to the geographical area, climatic conditions of the country such as constantly increasing atmospheric temperature, alternation of rainfall with a dry season and others, which could be the subject of further research.

Keywords: *Amelanchier* spp., pollen, SEM, morphology

Introduction

The science that focuses on the study of pollen grains produced by seed and gymnosperms and all other palynomorphs is called palynology. The word palynology comes from the Greek word “palunein“ meaning to sprinkle or scatter and the Latin word “pollen“ meaning flour or dust. This means that palynology is the “science of dispersed dust“ (Nughoro, 2018).

Palynology as a science creates many opportunities for its practical use. The study of symmetry, polarity, shape, size, structure, sculpture and apertures of spores can be very useful for many other sciences such as botany, oceanography, limnology, pedology,

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geology, paleontology, ecology, melitology, entomology, archeology, aerobiology, allergology, criminology and many others (Giveyrel et al., 2000; Radice et al., 2003; Shinwari and Khan 2004; Wrońska-Pilarek 2010; Ďurišová, 2018; Halbritter et al., 2018; Auer, 2021). Plant pollen is widely used in dietetic nutrition, in the production of medicines, bioadditives, vitamins, and in cosmetology (Hudz et al., 2017a; Hudz et al., 2017b; Nikolaieva et al., 2017). Pollen is an indicator that allows researchers to study past phytogeography, plant evolution, climate, rock and soil characteristics, air pollution levels, plant-insect relationships, and the botanical and geographic origins of bee products, among many other issues (Persson et al., 1996; Carlo and Paula, 2004; Hesse and Blackmore, 2013). The study of pollen found in sediments and sedimentary rocks allows us to obtain a lot of information about deep time, because the pollen grains are distinctive, and their outer covering known as the exine is very strong and therefore durable (De Vernal, 2009; Ďurišová, 2018; Halbtitter et al., 2018).

Amelanchier Medik. (family Rosaceae Juss.) commonly called saskatoon, saskatoonberry, sugarplum, serviceberry, juneberry, shadbush or northern serviceberry is native to temperate regions of the Northern Hemisphere, growing primarily in early successional habitats. It is most diverse taxonomically in North America, especially in the north-eastern United States and adjacent south-eastern Canada, and at least one species is native to every U.S. state except Hawaii and to every Canadian province and territory. Two species also occur in Asia, and one in Europe (Crompton and Wojtas, 1993).

Pollen morphology of selected *Amelanchier* species was described by some researchers in Texas (Weir, 1976), Pakistan (Perveen et al., 2014), Canada (Hebda et al., 1988; Hedba and Chinnappa, 1990a, b; 2014), Ukraine (Grygorieva et al., 2019), Armenia (Hayrapetyan, 2020), Poland (Hunt and Morawska, 2020).

The data of the authors on the size of the pollen grains of same plant species are variable and may vary, often depending on environmental factors or pollen treatment in the analyses. Various external factors such as air temperature, precipitation, desiccation, flood stress, drought stress, plant nutrition and effect of herbicides can influence on the size of pollen grains (Dajoz et al., 1991; Delph et al., 1997; Ejsmond et al., 2011, 2015; Yamburov et la., 2014; Mehmood et al., 2023; Wrońska-Pilarek et al., 2023).

The pollen grains adapted to different strategies have anatomical-morphological differences. The aim of this study was to compare the morphological parameters of pollen grains of the five species of genera *Amelanchier* Medik. and comparison their parameters, micromorphology and microsculpture.

Material and methodology

Pollen grains collection

Pollen grains studied on the species collected in Forest-Steppe of Ukraine in M.M. Gryshko National Botanical Garden of NAS of Ukraine at the laboratory of the Department of Tropical and Subtropical plants (NBG). The following species were analyzed: *Amelanchier alnifolia* (Nutt.) Nutt. ex M. Roem., *Amelanchier arborea* (F.Michx.) Fernald, *Amelanchier canadensis* (L.) Medik., *Amelanchier lamarckii* F.G. Schroed., *Amelanchier spicata* (Lam.) K. Koch. Freshly flowers (not opened) were collected randomly from the different *Amelanchier* spp. introduced in the condition of Kyiv at the balloon stage (May 2022). Pollen samples released from flowers were further dried under laboratory conditions. The dry pollen was used for a microscopic study of morphological characteristics. The samples of pollen grains were applied to double-tape, fastened to metal object tables with 10 mm diameter.

Scanning electron microscopy (SEM)

An investigation carried out at the laboratory of Department of Tropical and Subtropical plants of NBG using an electron microscope Carl Zeiss LS 15. The measurement of morphometric parameters was carried out on 50 pollen grains from each species using the AxioVision Rel. 4.8.2.0 program. The measurements were made in micrometre (μm). The length of a polar axis (P) and the equatorial diameter (E) of grain, P/E ratio were measured, and their variation was compared among studied species. The pollen shape classes (P/E ratio) were adopted according to the classification proposed by Erdtman (1952): oblate-spheroidal (0.89–0.99), spheroidal (1.00), prolate-spheroidal (1.01–1.14), subprolate (1.15–1.33), prolate (1.34–2.00) and perprolate (>2.01).

The comparative morphological studying of the pollen grains was performed according to the working rules on the SEM in the conditions of low vacuum (P = 60 Pa) with the following zooming: 500 times – during the measurements; 3000–10,000 times – while taking the pictures of the exine sculpture features. Using the regime of low vacuum allows performing the pollen studying without its preliminary chemical treatment and to receive undistorted data about the research object that makes the process of the probe

preparation easier. Typical exine patterns, shape, sizes, and dimensions of pollen grains for *Amelanchier* species were determined by using a scanning electron microscope (SEM).

Statistical analysis

Statistical analysis was performed using SAS®9.2 software; hierarchical cluster analyses of similarity between genotypes were computed based on the Bray-Curtis similarity index; Basic statistical analyses – the minimal and maximal values of the traits, arithmetic means, and coefficient of variation (CV, %) were performed using PAST 2.17. The variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by Stehlíková (1998). All the observed traits were shown in graphic form.

Results and discussion

The complex morphological characteristics and ultrastructure of pollen grains allow determining the differences or similarities between the species. Quantitative (dimensions) and qualitative (ornamentation, colour) data of pollen have significant value in botanical (Ďurišová, 2018) and taxonomic classification, due to preserved palynological features in many plants (Grygorieva et al., 2019; Motyleva et al., 2017, 2018a, b; Horčinová Sedláčková et al., 2020, 2021a, b).

SEM investigations showed that the pollen grains species of *Amelanchier* are prolate and perprolate, 3rd furrow is meridional, in outline from the pole are three furrow – the furrows are long, narrow, smooth. The surface sculpture and shape index of the species vary. The average length of the polar axis varied from 27.38 to 47.14 µm and the width of the equatorial axis was in the range from 14.33 to 28.95 µm. Shape index (P/E) of tested species varied from 1.77 to 2.09. The most average length of pollen was *Amelanchier spicata* (43.24 µm), *Amelanchier canadensis* (41.82 µm), the least length was *Amelanchier arborea* (31.35 µm). The most average width pollen was *Amelanchier canadensis* (23.04 µm), *Amelanchier spicata* (22.96 µm), and the least width was *Amelanchier arborea* (16.07 µm).

Pollen grains of *Amelanchier alnifolia* among other species the longest from the poles, slightly pointed. Ultra-sculpture of exine is finely trickled, often perforated on all surfaces. Pollen grains of *Amelanchier arborea* long from poles, rounded. Ultra-sculpture of exine is wide streaming and less perforated then *Amelanchier alnifolia*. Ultra-sculpture of *Amelanchier canadensis* exine is pronounced streaky, dense, relief, perforation not often, close to poles. Pollen grains of *Amelanchier lamarckii* and *Amelanchier spicata* had the most equatorial diameter and more rounded shape than other species. *Amelanchier lamarckii* ultra-sculpture width streaky, sometimes intermittent, perforated on all surfaces. Ultra-sculpture of exine of *Amelanchier spicata* not perforated, intermittently finely trickle. The shape of the pollen grains and the structure detail of their surface are illustrated micrographically (Figure 1).

Other SEM studies described pollen morphology of *Amelanchier* species. *A. alnifolia* grains tricolpate, subprolate in equatorial view; sculpturing striate; ektexine and endexine about 1.5 µm thick; structure tectate. SEM shows puncta in the striate spaces.

Hebda et al. (1988) studied *A. alnifolia* pollen grains in monads, isopolar, radially symmetrical, circular to elliptic in equatorial view, amb subtriangular with apertures at angles, spherical to prolate; pores angular, distension – predominant; non-3-colpate grains – common, equatorial bridge and/or pore flaps are present; grain highly susceptible to distension, so that most grains in silicone oil preparations are fully distended 3-colporate, occasionally appearing 3-colpate if not swollen, to syncolpate, and especially in some specimens 4-colporate or pericolporate. Size (PxE) is highly variable, shape circular to prolate.

Table 1 Variability of the basic statistic parameters of pollen grains of *Amelanchier* species

Genotypes	P – polar axis (µm)				E – equatorial axis (µm)				SI (P/E)
	min	max	s_x	V%	min	max	s_x	V%	
Amelanchier alnifolia	31.52	43.27	0.28	6.59	15.67	22.37	0.38	9.07	2.09
Amelanchier arborea	27.38	37.90	0.42	7.72	14.33	24.65	0.64	11.60	1.92
Amelanchier canadensis	35.36	44.73	0.29	5.31	19.57	28.95	0.56	10.18	1.83
Amelanchier lamarki	33.00	45.43	0.40	7.21	19.40	25.10	0.41	7.55	1.77
Amelanchier spicata	35.79	47.14	0.32	5.78	19.92	26.03	0.39	7.18	1.89

min – minimal values; max – maximal values; x – average s_x – standard error the mean; V% – coefficient of variation; SI – shape index (P/E)

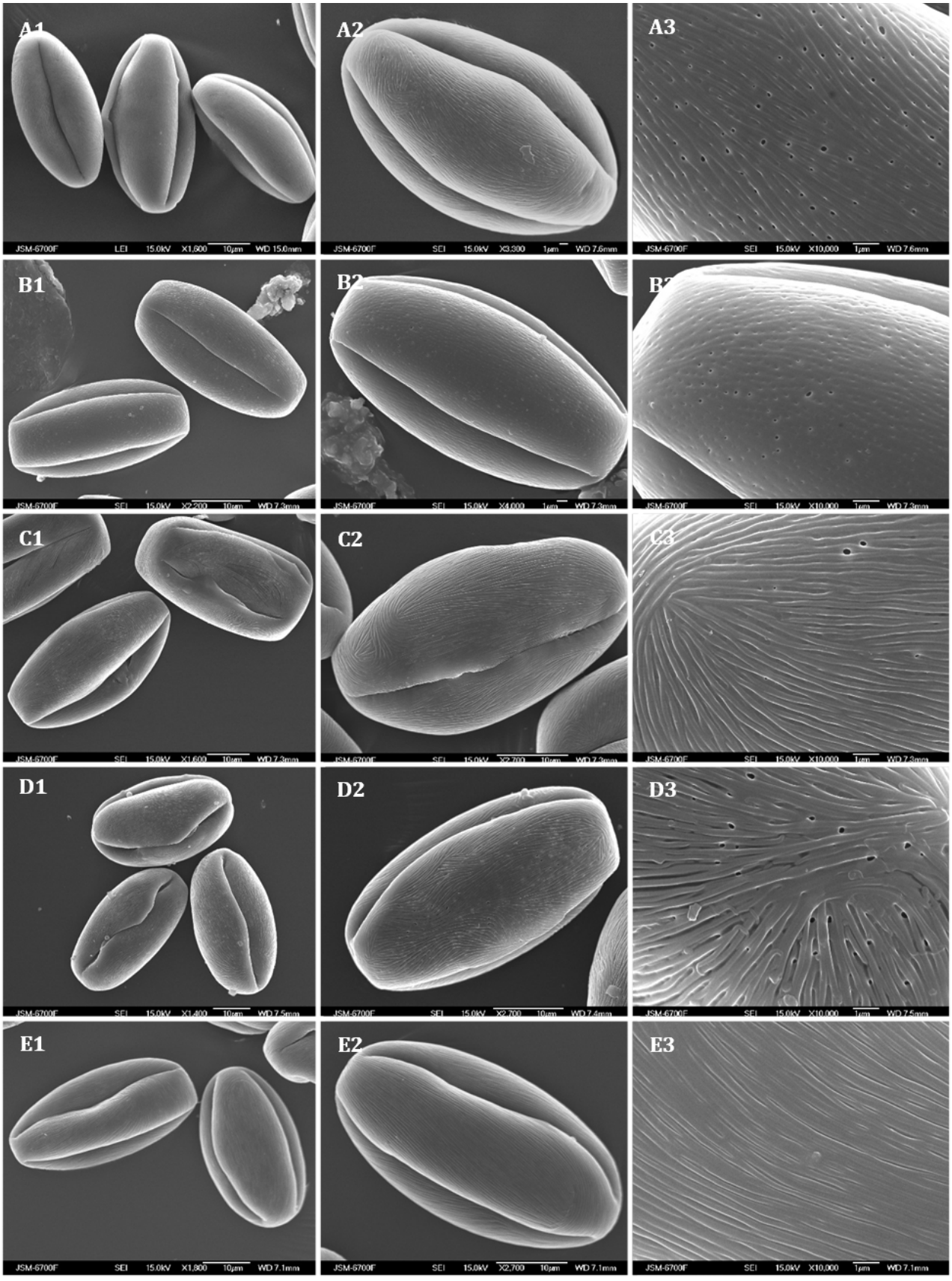

Figure 1 Pollen grains of *Amelanchier* Medik. species in different positions
Photo: Gurnenko, 2020
A – *A. alnifolia*; B – *A. arborea*; C – *A. canadensis*; D – *A. lamarki*; E – *A. spicata*; 1 figures (scale bar = 1 µm × 3,000): 2 figures (scale bar = 1µm × 5,000); 3 figures – detail of the sculpture of the exina surface in the place of the mesocolpium (scale bar = 1 µm × 10,000)

Based on cluster analysis, the relationships of the tested *Amelanchier* species according to morphological features are graphically displayed on a dendrogram (Figure 2). It is evidently that *A. arborea* which has the smallest parameters is really separated from other species. *A. lamarki*, *A. spicata* and *A. canadensis* are similar according to morphometric sizes.

Grygorieva et al. (2019) described *Amelanchier* pollen grains from Ukrainian conditions as oblong-spheroidal and small sizes with average length of the polar axis from 13.03 (*A. arborea*) to 21.37 µm (*A. spicata*) and the width of the equatorial axis from 6.55 (*A. arborea*) to 11.96 µm (*A. canadensis*). Shape index (P/E) of tested species varied from 1.85 to 2.11.

Hunt and Morawska (2020) studied pollen grains of *A. alnifolia* (Nutt.) Nutt. Ex M. Roem with following results of morphology: tricolporate, sculpture very finely striate to psilate; the striae were found to form patterns: scabrate to slightly striate (Perveen et al., 2014); sizes of polar axis 16(18.2)–25 µm, equatorial axis 16(17.7)–24 µm. Crompton and Wojtas, (1993) analysed sizes of polar 20.0–24.7 (22.5) µm and equatorial 17.1–20.9 (18.9) µm axes and authors Hebda and Chinnappa (1990a) concluded that *Amelanchier alnifolia* pollen grains exhibit systematic geographic variability and presented that dimensions is highly variable depending on source of collection and degree of swelling in silicone oil distended grains, P = (17.0) 21.0–28.0 (30.0) µm, E = (12.0) 19.0–28.0 (30.0) µm; undistended. P = (19.0) 21.0–29.0 (31.0) µm, E = (14.0) 15.0–19.0 (21.0) µm; glycerol, P =, distended 26.0–32.0 µm, E =27.5–33.0 µm; glycerol, P =, undistended 30.0–37.0 µm, E = 17.5–24.0 µm (Hebda et al., 1988).

Some authors also evaluated other *Amelanchier* species, e.g. *A. spicata* which has medium sized (26–50 µm), colporate, isopolar, oblate shape, outline in polar view is circular, dominant orientation is oblique, aperture type is colporus, aperture conditiom is colporate, tricolporate (Auer, 2021; PalDat, 2023); *A. ovalis* (= *A. rotundifolia* (Lam.) Dum.) pollen grains are 3-zonocolp-porate (poroidate), from oblong to oblate, outline in polar view rounded-triangular or rounded-3-lobed (Hayrapetyan, 2020); pollen has small sized (10–25 µm), size of hydrated pollen is 21–25 µm, shape is spheroidal, endexine very thin (Loos et al., 2021; PalDat, 2023), polar axis 14.5–22.5 µm, equatorial diameter 17.4–20.3 µm. Colpi is long, with thickened edges and with pointed, sometimes anastomosing ends (synaperturate); apocolpium diameter has 2.5–4.5 µm, mesocolpium width 12.4–14.5 µm; ornamentation of colpus membrane is granulate. Pores are not always clearly defined, elliptical, slightly elongated along the colpi, 5.1 µm × 3.5 µm. Exine 0.8–1.0 µm, columellae are not clearly defined. Exine ornamentation granulate (LM), exine ornamentation sinuosly finely striate (SEM) (Hayrapetyan, 2020); *A. utahensis* has size 21.4 (19–24) µm, shape is subprolate and spheroidal (Weir, 1976).

Various environmental factors such as geographical conditions and change of climate can influence on

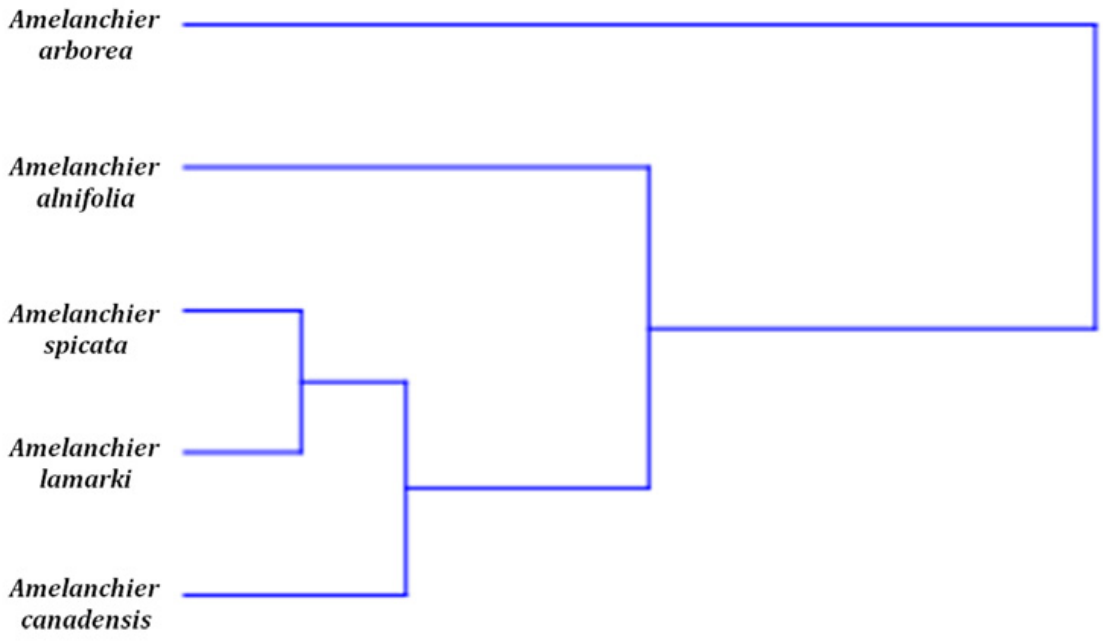

Figure 2 Dendrogram of *Amelanchier* species pollen according to morphometric features

the size of pollen grains (Dajoz et al., 1991; Delph et al., 1997; Ejsmond et al., 2011, 2015; Yamburov et al., 2014).

Ejsmond et al. (2011) studied influence of temperature increased on the pollen morphology, pollen size and pollen shape. Empirical results showed that pollen grains were larger when under intense desiccation stress, but without change pollen grain shape. Plants are known to produce fewer pollen grains but larger sizes to avoid desiccation stress during the flowering period which studied some authors (Ejsmond et al., 2011; Mehmood et al., 2023).

Yamburov et al. (2014) studied effects of drought and flood stress on pollen quality and quantity in *Clivia miniata* including pollen size, viability, germination, and number of pollen grains per anther while evaluating how stress influences these features. The study provides evidence that microsporogenesis of *Clivia miniata* is more sensitive to flooding than to drought. The studied pollen features can be ranked based on their degree of sensitivity to flooding in the following order: number of pollen grains per anther > pollen germination > pollen fertility > pollen size.

Effect of long-term herbicide influence on *Prunus serotina* pollen studied Wrońska-Pilarek et al. (2023). Pollen grains from the control variant had a longer equatorial axis, were more elongated in shape and had the largest range of exine thickness compared to the pollen from the herbicide-treated samples. The average exine thickness in the samples treated with herbicides were larger. There were differences in the P/E ranges of variability between the control and herbicide-treated samples. In the control sample the P/E ratio was 1.32–2.04 and elongated forms of pollen shapes prevailed, while in the herbicide-treated samples it ranged from 1.03 to 1.47. The share of deformed pollen grains in the herbicide-treated samples was lower than expected.

Differences in pollen grain size may also occur between different genotypes of the same species The size of the pollen grains can also vary within a single plant, the largest pollen grains are found in the best developed top inflorescences (Miter et al., 2016).

Conclusions

Studies via scanning electron microscope have established characteristic differences in the morphometric and microsculptural features of pollen for each of the studied species of *Amelanchier* spp. 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. The main parameters such as the form (the pollen grains elongation, P/E ratio) are specific for different *Amelanchier* species. Results from our analyses showed differences among various *Amelanchier* pollen grains. Some of these pollen morphological parameters can be used for identification and comparison with the following analyses of *Amelanchier* species. These species require detailed palynogeographic study. Differences in the size of pollen grains in comparison with other authors may be the result of many environmental factors specific to the geographical area, climatic conditions of the country such as constantly increasing atmospheric temperature, alternation of rainfall with a dry season and others, which could be the subject of further research.

Conflict of interests

The authors declare that they have no competing interests.

Ethical statement

This article complies with all ethical standards.

Funding

Co-authors are grateful to Visegrad Fund for the scholarship for the research during which the presented knowledge was obtained.

Acknowledgments

The publication was prepared with the active participation of researchers in international network AgroBio*Net*, as a part of the international program 'Agricultural Biodiversity to Improve Nutrition, Health and Quality of Life', within the project ITEBIO-ITMS 26220220115 'Support of Technologies Innovation for Special Bio-Food Products for Human Healthy Nutrition' and National Scholarship Programme of the Slovak Republic and Polish National Commission for UNESCO.

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