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RESEARCH ARTICLE

Elaeagnus umbellata fruit - chemical composition, bioactive compounds, and kinetic of DPPH inhibition compared to standard antioxidants

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ABSTRACT

E. umbellata (Autumn Olive) is a medicinal plant used in traditional Asian medicine. In this study, we examine *E. umbellata,* which grows in central Europe (Poland). Despite significant climatic differences in both regions, we show that the fruits have a similar chemical composition and antioxidant activity to those observed in their native areas. For the first time, we examined the fatty acid composition of *E. umbellata*, showing that unsaturated fatty content for as much as 88.67% of total fat. Including the content of essential polyunsaturated fatty acids was over 50% of the total fat. We also examined proximate content, the content of fat-soluble vitamins, lycopene, beta carotene, and the mineral composition of the fruit. Moreover, using FT-IR spectrometry, we have shown that the fruits of the Autumn olive can change the distribution of chemical bonds when treated with hydrogen peroxide. The fruits of E. umbellata showed dose-dependent antioxidant properties with IC50 values of 76.27 μg/ml We also investigated the kinetic antioxidant activity of *E. umbellata* fruits based on DPPH radical inhibition compared to standard antioxidants (vitamin c and BHT). We have shown that using available protocols to spectrophotometrically study antioxidants' reaction with the DPPH radical may underestimate the effects of the Autumn Olive fruits.

Keywords: Antioxidants assays; Autumn olive; bioactive compounds; Elaeagnus umbellata; oxidative stress

INTRODUCTION

Elaeagnus umbellata (Autumn olive) is a plant that produces an abundance of oblong deep-red coloured fruits used as food and medicine in Asia. The Elaeagnus family's fruits are an excellent source of carotenoids, among which lycopene deserves special attention. Its content was 5-18 times (depends on the variety) higher than in tomatoes, which are the primary source of lycopene in the human diet (Fordham et al., 2001).

Berry is also a good source of unsaturated fatty acids. Fat from *E. angustifolia* seeds collected in China contained unsaturated fatty acids in a dominant proportion (more than 80% of all). The primary fatty acid was linoleic acid, followed by oleic acid (Kadir and Kuerbanjiang, 2011). To the best author knowledge, no such studies have been carried out for *E. umbellata*.

Mineral compounds are another essential ingredient in the fruit from the *Elaeagnus* family. (Ahmad et al., 2006) examined by spectrophotometry the content of calcium (Ca), magnesium (Mg), iron (Fe), phosphorus (P), potassium (K), and sodium (Na) of *E. umbellata* fruit.

Research indicates that *Elaeagnus* genus fruit may be useful in the treatment of diseases involving oxidative stress: rheumatoid arthritis, fever, asthma (Niknam et al., 2016), type 2 diabetes (Nazir et al., 2018), cardiovascular (Qayyum et al., 2019) and breast cancer (Jabeen et al., 2020). This is largely due to the antioxidant properties of *E. umbellata* fruits. The available research focuses on the

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effects of extracts (Uddin et al, 2014, Ishaq et al., 2015; Zglińska et al., 2021) or specific compounds isolated from E. umbellate f.e. catechins (Nazir et al., 2020) or essential oils (Nazir et al., 2021).

Such extracts can then be used, for example, as dietary supplements. Traditionally, in many Asian countries, these fruits are eaten as food (Khattak, 2012). In Western diets, it appears as a pro-health ingredient due to the high content of carotenoids (Pei et al., 2015).

This study intended to investigate the activity of the fruit as close as possible to how it would be eaten as food or feed.

Oxidative stress is the effect of the overactivity of reactive oxygen species (ROS) resulting from the imbalance between free oxygen radicals' secretion and their elimination from the cell by antioxidative systems. ROS are atoms or particles containing one or more unpaired electrons, which makes them highly reactive. As a result of the ROS activity, numerous multicellular damages occur, such as cell membrane lipid peroxidation, enzyme inactivation, DNA damage, and structural changes in the particles of carbohydrates and proteins. Numerous researches indicate that reactive forms of oxygen may develop many disorders such as cardiovascular disease, type 2 diabetes, neurodegenerative and psychiatric conditions (Gulcin, 2020; Shahidi, 2000).

An essential element of defence against the harmful effects of oxidative stress are dietary antioxidants. Antioxidants are a large group of chemical compounds inhibiting oxidative reactions in the organism. Different compounds can have various antioxidant effects. Food is a complex matrix in which many kinds of antioxidants can be found at once. The first group blocks the radicals' reaction by transmitting them hydrogen or electrons, leading to more considerable stability compounds. Such compounds are, among others: phenols, hydroquinones and tocopherols. The second common group are substances that demonstrate synergistic properties. They can chelate ions that take part in the creation of radicals. Their activity consists of transferring protons and electrons to phenoxy radicals, and this way, their original antioxidative activity is brought back to them. Substances that take up oxygen are, among others: ascorbic acid (vitamin C), ascorbyl palmitate, amino acids, flavonoids, vitamin A, carotenoids (Shahidi, 2000; Neha et al., 2019).

The study aimed to assess the antioxidant activity of *E. umbellata* fruit and characterize the bioactive ingredients that may affect it.

MATERIAL AND METHOD

Sample

Elaeagnus umbellata fruits were obtained from a farm located in central Poland in Europe. Fruits were harvested after ripening and were lyophilized immediately after arriving at the laboratory.

Proximate content

The representative samples of the berry were analyzed for contents of dry matter (on Oven Drying at 135°C for 2 Hours), crude protein (Kjeldahl method), crude fat (Soxhlet method) and crude ash (burning at 550°C for 6h), according to AOAC International (*CEU Repositorio Institucional: Official methods of analysis of AOAC International. Volume I, agricultural chemicals, contaminants, drugs/edited by William Horwitz.*, no date) Analyses were made in three replications.

Mineral content

The content of minerals was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES). Fruits were homogenized, dried at 60 °C and ground. The samples were mineralized in concentrated HNO_3 using the microwave method. The Cu, Fe, Mg, P, Se and Zn were determined, as we made before (Łozicki et al., 2020).

Lycopene content

Lycopene was extracted from fruits by a mixture of BHT in acetone, ethanol and hexane (1: 1: 2). Detection was performed by measuring absorbance at λ = 503 nm in the hexane layer using ELISA-reader. The lycopene content was calculated using the lycopene extinction coefficient in hexane of 3120 at this wavelength (Zglińska et al., 2021). N=3.

Total phenolic content

The ground fruit was placed in a mixture of methanol and water (1:1), shaken for 20 minutes and then centrifuged. The residue was treated with a mixture of acetone and water (7:3), shaken for 10 minutes and centrifuged again. The filtrates were combined and mixed thoroughly. The phenol content was determined by the method with the Folin-Ciocalteu reagent developed by Zhang et al. **β-carotene, and fat-soluble vitamins**

Extraction of fat from fruits and saponification was performed according to the AOAC procedure (1990) at room temperature. Analysis of β-carotene and fat-soluble vitamins was conducted in crude fat using an Agilent 1100 HPLC (Agilent, Waldbronn, Germany), as was reported earlier (Zglińska et al., 2021). The concentration was calculated based on a standard curve prepared from commercially available standards (Sigma-Aldrich). N=3.

Fat and fatty acids

Seeds from the lyophilized pulp of oleaster's fruits were used for fat and fatty acids analysis. The fat percentage content in the *E. umbellata* seeds was determined with the modified Folch's method The extraction was performed in triplicate. The total fat was extracted from the mixture of the chloroform/methanol (1:1). Then, to determine the percentage content of fat (% content of wet weight) in the seeds, the solvent was evaporated under vacuum and then under a nitrogen atmosphere and weighed. Obtained fat dissolved in mixed hexane and isopropanol. The sample was kept at -20°C until GC analysis.

The fatty acids profile was determined with the gas chromatograph with FID detector according to PN – EN ISO: 5509, PN-EN ISO: 5508 as previously by Ciemniewska-Żytkiewicz (Ciemniewska-Żytkiewicz et al., 2015). The temperature of the injector was 225ºC, and that of the detector was 250ºC. A column that was used was 60m in length and 0,25mm in diameter. Methyl esters of fatty acids were identified using the model Supelco 37 component FAME Mix (Supelco, USA). N=2.

Distribution of fatty acids in sn-2 and sn-1,3 triacylglycerols

The work utilized the pancreatic lipase enzyme's ability to selectively hydrolyze ester bonds at the sn-1,3 positions of triacylglycerols, assuming their equivalence. The use of a regiospecific enzyme allowed the determination of the fatty acid composition in the sn-2 internal position of triacylglycerol molecules. The procedure described by Ciemniewska-Żytkiewicz (2015) was used in the study.

After the enzymatic reaction, thin-layer chromatography (TLC) was used to separate products. Tiles (20 × 20 cm) coated with Kieselgel G silica gel were used in a layer with a thickness of 0,5 mm. A mixture of hexane, diethyl ether, and acetic acid was used to develop the chromatogram in which the volume ratio of the components was 50: 50: 1, respectively. The separated mono- and diacylglycerol bands, free fatty acids, and triacylglycerols that had not reacted were identified using an iodine chamber. Isolated sn-2 monoacylglycerols were removed along with the gel from the plates and eluted with diethyl ether. Received in this way, fatty acids in sn-2 monoacylglycerols were identified using gas chromatography, as previously described.

Antioxidant activity

The freeze-dried fruit was ground and diluted in methanol to a concentration of 1000 μg/L (stock). The resulting solution was vortexed (2000 rpm, 5 min) and filtered through a Whatman filter.

a) The reaction of *E.umbellata* with hydrogen peroxide.

The solvent was evaporated on a rotary evaporator at a temperature of 40°C and a vacuum of 100 hPa. The extract prepared in this way was divided into two parts. One part was the group "*E.umbellata* alone". The second part was used to create the group "E.umbellata + H_2O_2." For each sample, 10 μl of 3% H_2O_2 was added to 10 mg of the sample and mixed.

FT-IR spectra were registered in the middle range, i.e. 4000 – 400 cm-1, using Perkin Elmer System 2000 spectrometer, as before (Zglińska et al., 2020). Powdered samples were mixed with spectrally pure KBr (potassium bromide) in the approximate mass ratio of 1:300; the mixture was powdered in a laboratory mill to obtain fine powder and then carefully placed in a pellet maker to cover the bottom disc entirely. The pellet maker containing a sample, closed with another disc, was then placed in a hydraulic press and pressed with a pressure of 8 tons for three minutes. Pallets obtained this way were taken off from the pallet maker and placed in a spectrometer-dedicated pallet-holder. In turn, it was slide into a slide located in the measuring chamber of the spectrometer. Registration started with collecting spectral data of background that is spectrum without any sample in measuring chamber. The resolution was set to 1 cm-1, and 25 scans were collected. The next working spectrum was automatically ratioed to the background spectrum to obtain the resultant spectrum finally saved. Spectral data, i.e. wavenumber and intensity of each band occurring in studied spectra, were visually compared to find differences. Grams IA software was used to operate the System 2000 spectrometer to register spectra and analyze spectral data.

b) DPPH inhibition

zA previously prepared stock of *E.umbellata* in methanol was diluted to obtain a series of dilutions ranging from 10-150 μg/ml. DPPH test was prepared, as before (Zglińska et al., 2021).

The IC50 value (the amount of antioxidant necessary to halve the initial DPPH concentration) was calculated based on the inhibition curve.

Then we examined the kinetics of the *E. umbellata* reaction with the DPPH radical compared to standard antioxidants. Solutions of 100 μg/L *E. umbellata* and standard antioxidants were prepared. BHT (Sigma-Aldrich) was diluted in methanol and vitamin C (Sigma-Aldrich) in deionized water. Next, 290 μL 0.2 mM $DPPH^{\cdot}$ solution in absolute methanol was combined with 5 μL each solution on a 96-well plate.

Absorbance was measured immediately with an interval of 0 to 90 min (for up to 1 h, every 1 min, above every 5 min.) N = 6.

The statistical significance of the differences observed between *E. umbellata* fruit and standard antioxidants was examined using the ANOVA test.

RESULT AND DISCUSSION

Proximate composition of fresh *Elaeagnus umbellata* fruit.

The plant's fresh berry contained 30,94% dry matter, 4,97% crude protein, 2,13% crude fat and 4,88% crude fibre. The values were similar to those observed in an earlier study in Turkey (Khattak, 2012). That shows that, despite significant differences in the climate, the basic chemical composition of fruit grown in Europe is similar to the fruit grown in Asia, where this plant comes from (Table 1).

Carotenoids and vitamins

The content of each carotenoid changes during the fruit ripening. Lycopene is the main carotenoid in ripe red-pigmented *E. umbellata* fruit. According to Fordham (2001) constitutes 72-82% of total carotenoids in these fruits. The lycopene contents in naturalized fruits of autumn olive analyzed in a Fordham study (western USA) were 38,23 mg/100 g wet matter compared with 41,31mg/100g wet matter in the current study. Beta carotene were 0,38 and 0,87 mg/100g wet matter, respectively.

E. umbellata fruits from Poland were characterized by a high vitamin E content (alpha-tocopherol). It was 10.17mg/100g compared to 1,16-2,82 mg/100g wet matter marked in the USA study (Pei et al., 2015).

That confirms the high dietary value of fruit harvested in Poland. Carotenoids, phenols and vitamin E are strong antioxidants. Also, lycopene is known as an anti-cancer agent. Earlier studies indicate that lycopene biological activity is synergistic with vitamin E, f. e. lycopene and α-tocopherol could synergistically inhibit prostate carcinoma proliferation in vitro (Pastori et al., 1998) or to prevent LDL oxidation (Müller et al., 2016).

Table 1: The proximate composition of *E. umbellata* fruits harvested in Europe (this study) and Asia (Khanzadi, 2012) expressed as a percentage in wet weight

Parameter	Value in the current study. (Poland, Europe)	Value in Khattak et al. (2012). (Turkey, Asia)
Dry matter (%)	30.94	28.6
Protein (%)	4.97	4.0
Fibre (%)	4.88	5.9
Fat (%)	2.13	2.3

Mineral composition

The contents of some of the mineral elements: iron, magnesium, phosphorus, selenium, calcium and zinc are presented in Table 3. The content of individual mineral compounds differs in the works of authors from other countries. In the Khattak (2012) study Fe and Zn content was higher (53.1 and 232.1 mg/kg d.m.) than in this study, but Mg and P content was lower (162.4 and 183.3 mg/kg d.m.). In turn, fruits from Pakistan contained 150.0-154.1, 189.0-192.0 and 63.7-67.0 mg/kg d.m. of magnesium, phosphorus and calcium, respectively.

Fat and fatty acids

The fatty acid composition of studied *E. umbellata* seeds oil is given in Table 4.

The predominant fatty acid was oleic acid (C18:1 n-9), followed by linoleic acid (C18:2 n-6) and alpha-linolenic acid (C18:3, n-3). The content of all unsaturated acids was exceptionally high and was equal to 88,67%. It should be noted that the particularly important polyunsaturated acids (PUFA) content was over half of all fatty acids, and Ω 3/Ω 6 ratio was ½. According to Kusova oil from *E. augustiflora* seeds consists mainly of unsaturated acids: linoleic acids (52,2%), oleic acid (23,4%) and α-linolenic acid (12,2%). Compared to this, *E. umbellata* determined in this study is characterized by a more favourable Ω 3/Ω 6, which is close to ½ compared to ¼ detected in *E. augustiflora fruits* (Kusova and Luk'yanchikov, 1990).

Fats from plants contain linoleic and alpha-linoleic acids - precursors of omega 6 and omega 3 families. Under oxidative stress conditions, lipid mediators derived from polyunsaturated fatty acids, such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are synthesized. Fatty acid derivatives from the omega 6 families: eicosanoids and isoprostanes, are highly pro-inflammatory and affect many disease processes.

Table 2: Content of selected carotenoids and fat-soluble vitamins in *E. umbellata* fruits

component	lycopene	β-carotene	vit. E	Vit. K	Total phenols
mg/100g (dry matter)	133.520	2.800	32.900	0.006	828.53

Table 3: Content of selected minerals in fruits of *E. umbellata*

Ca [mg/kg dry matter]	Fe [mg/kg dry matter]	Mg [mg/kg dry matter]	P [mg/kg dry matter]	Se [mg/kg dry matter]	Zn [mg/kg dry matter]
4.65	28.17	604.33	2874.67	<2.00	18.37

Table 4: Fatty acid composition (g/100 g of total fatty acids) of *Elaeagnus umbellata* seeds oil

Fatty acid	Content
14:0	0.12
15:0	0.07
16:0	7.97
16:1	0.87
17:0	0.07
17:1	0.08
18:0	2.66
18:1 Ω 9	37.13
18:2 Ω 6	34.12
18:3 Ω 3	16.22
20:0	0.44
20:1	0.25
∑SFA	11.33
∑MUFA	38.33
∑PUFA	50.34

Contrary, EPA and DHA resolvins have anti-inflammatory effects. The omega 3 fatty acids replace fatty acids from the omega 6 family, limiting oxidative stress's negative effects (Simopoulos, 2002; Calder, 2017; Innes and Calder, 2018).

The properties of fats depend not only on the composition of fatty acids but also on the structure of triacylglycerols. Significantly, palmitic acid is favourably absorbed from the sn-2 position as a monoacylglycerol rather than a free fatty acid from the sn-1 or sn-3 position. Free palmitic acid forms insoluble complexes with calcium ions that cannot be absorbed in the small intestine and are thus excreted in the faeces (Bryś et al., 2017). Autumn Olive fat contains 12,2% of palmitic acids in the sn-2 position (Table 5). That is in line with the fact that most animal and vegetable fats have this fatty acid primarily in the sn-1 and sn-3 positions.

Goncharova and Glushenkova (1990) showed differences in 3 morphological forms of *Elaeagnus angustifolia* fruit collected in 3 regions of Uzbekistan. They differed in the content of fatty acids and composition of the classes of lipids. Differences in climatic conditions explain these discrepancies. In light of these results, comparing fruit from Asia and Europe seems particularly interesting.

Antioxidant potential

1) *E. umbellata* fruits react with hydrogen peroxide, changing the arrangement of its chemical bonds

Among free oxygen radicals, the superoxide anion (O_2^-) and its conversion products, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$), are of significant importance. H_2O_2, in particular, is often used as a model to study oxidative stress.

This section states about chemical bonds changes that occur if any chemical reaction, including redox reaction happens. To evidence the antioxidant properties of *E. umbellata* fruits, their influence on the chemical structure of H_2O_2 was investigated. The FT-IR spectra of *E. umbellata* (EU) alone and the EU treated with H_2O_2 (EU +H_2O_2) were examined. They are presented on Fig. 2. Clear differernces listed beneath confirm changes occurring in bonds of studied materials.

Table 5: Distribution of the selected fatty acids in triacylglycerols in *Elaeagnus umbellata* seeds oil

FA	TAG	Fatty acid composition [%] in positions:		The share of the fatty acid in the sn-2 position [%]
		sn-2	sn-1,3	
C16:0	7.9	2.87	10.4	12.2
C18:0	2.7	3.8	2.1	47.0
C18:1	37.1	23.1	44.1	20.8
C18:2	34.2	47.5	27.6	46.3
C18:3	16.3	22.1	13.4	45.2

In the EU spectrum, two weak bands at 522 cm^{-1} and 528 cm^{-1} occur, while in spectrum EU + H_2O_2, only one band at 507 is present. The latter is wide and more intense than each of the bands at the spectrum of EU. The band at 590 cm^{-1} occurs in spectra of both EU and EU+$H_2O_{2\ sample}$s, although the band in the spectrum of EU alone is significantly less intense. The difference in wavenumber of two bands with similar middle intensity, present at the 635 cm^{-1} and 643 cm^{-1} for EU + H_2O_2 and EU alone, respectively, and supports significant difference in bonding or interactions in studied samples. The middle intense band at 874 cm^{-1} in the spectrum of the EU alone, is shifted by 6 cm^{-1} (located at 868 in spectrum of EU treated sample).

Although, it is hard to assign each band to the appropriate vibrations of atoms, the differences observed evidence different chemical structures present in each sample. This differences result from the reaction of EU with H_2O_2 most probably redox reaction. The spectral range of 1600 - 1800 cm^{-1} (Fig. 3) contains bands generated by vibrations of C=O group involved in various bonds, e.g. ester, carboxylic or carbonyl (Coates, 2006).

Both samples contain C=O group located at 1730cm^{-1}. However very weak additional band at 1681 cm^{-1} is present in the sample before H_2O_2 treatment while absent in the sample after H_2O_2 treatment. The spectra in the region 1687 – 1631 cm-1 are slightly different, indicating again chemical changes that occurred. They can associated with redox reactions that occur between H_2O_2 and EU. However, it is difficult to state what exactly reaction occurs as H_2O_2 can be both oxidized or reduced depending on the redox potential of the redox system it reacts with. Differences in spectra are shown in Fig. 2.

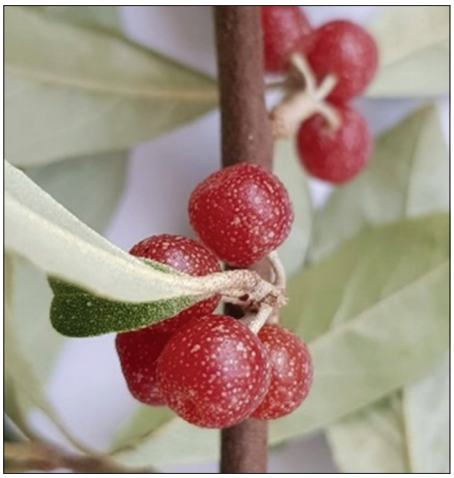

Fig 1. Eleagnus umbellata fruits immediately before harvest.

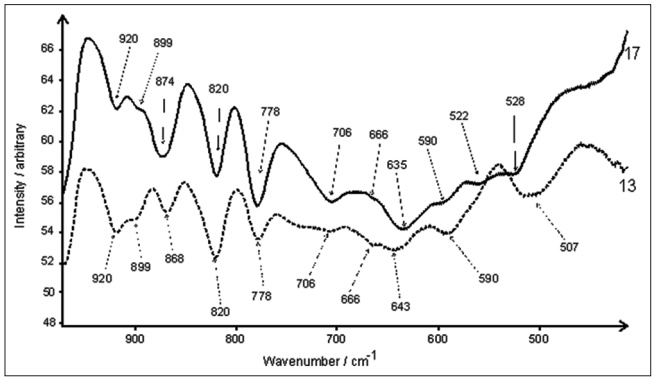

Fig 2. Spectra of two samples in the region 1000 – 500 cm-1. The solid line upper spectrum is E. umbellata alone (EU – sample 17), while the dotted lower line is for E. umbellata treated with H2O2 (EU + H2O2 – sample 13).

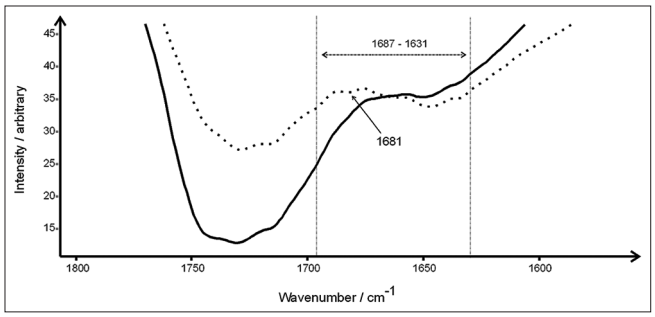

Fig 3. Spectra of the *E. umbellate* (EU) alone (solid line), and *E. umbellata* treated with H_2O_2 (dotted line) in the spectral range of 1800 – 1600 cm^{-1}. Band located at 1730 cm^{-1} is generated by C=O oscylations.

2) The fruit of E. umbellate has strong antioxidant properties.

Autumn olive fruits exhibited high free radical scavenging activities against DPPH radicals with IC50 values of 76.27 μg/ml. This is a value similar to that achieved by other authors. However, there are some discrepancies which are most likely due to the way the sample was prepared. Most of the work available is based on testing the antioxidant activity of extracts from various parts of the *E. umbellata*. The team of Ishaq reports the IC50 value for Autumn olive fruits in the range 45.4-49 μg/mL depending on the reagent used for the extraction. The highest value was obtained for methanol and n-hexane (Ishaq, Habib and Rathore, 2015). The antioxidant activity higher by approx. 36% compared to the current study, probably results from the higher content of phenols (1630-2000 vs 828.53 mg/100g). Similarly, Uddin et al. showed the activity of methanol extract from the aerial parts (berries and leaves) of *E. umbellata* an IC level of 50.5 μg/ml (Uddin et al., 2014). On the other hand, Nazir et al. Obtained an IC50 of 70 μg/ml for essential oil (Nazir et al., 2021) and 65 μg/ml for catechins (Nazir et al., 2020) obtained from *E. umbellata*, which is very similar to the result obtained in this study.

The value for *E. umbellata* is comparable to that of other berries. The concentration of the extract, which inhibits 50% of the DPPH radical, ranges from 55.38 to 59.13 μg/mL for various species of berry of *Ribes nigrum* (Paunović and Mašković, 2020), 62.0 μg/mL for *Phytolacca americana* (Nabavi et al., 2009), 54.5 μg/mL for *Punica granatum* (Mathew and Subramanian, 2014) and 279 ug/ml for *Phoebe cooperiana* (Payum et al., 2013) fruits. Fruit consumed popularly in Europe is characterized by a much lower antioxidant activity: 0.7 mg/mL for blueberry, 0.8 mg/mL for raspberry, 1.4 mg/mL for blackberry, 5.6 mg/mL for strawberry (Hangun-Balkir and McKenney, 2012).

The results of our research indicate that the fruit of *E. umbellata* fruit is a good source of antioxidants, and their action is much stronger than that of typical berries consumed in Europe.

3) Kinetic of antiradical activity

It was observed that some compounds react very quickly with DPPH, but for the majority of the compounds tested, the reactions are slower, and the mechanisms seem to be more complex result (Bondet, Brand-Williams and Berset, 1997). Vitamin C is known to react very quickly. As shown in Fig. 4, the vitamin C solution achieved a very rapid increase in reduction strength in the first 5 minutes. did not notice changes in the strength of DPPH inhibition by vitamin C during 1-60 min. That was probably because vitamin C reached its maximum reaction strength before first measuring absorbance. Most tests are currently carried out in plate readers that allow measuring absorbance much faster and at much smaller intervals. Synthetic antioxidant, BHT, reacted much more slowly, reached the maximum level of vitamin C in 20 minutes. Bondet et al. (1997) also assessed that BHT reacts slowly with DPPH. A mixture

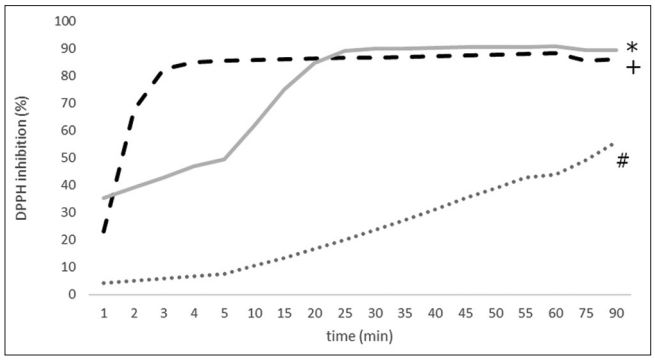

Fig 4. Kinetics of DPPH radical inhibition by *E. umbellata* fruits (grey dotted line), vitamin c (black dotted line) and BHT (grey line). Different symbols (*,+,#) mean that the results for the groups differ statistically at p <0.05.

of 0.5ml BHT with 3.5ml of DPPH solution reaches a plateau after 5 hours.

The concentrations of tested antioxidants are usually much lower. Therefore, the plateau is achieved much faster, as in the current study. Capturing the dynamics of vitamin C reaction with DPPH is difficult. Especially in traditional protocols, absorbance is measured after a 30-minute incubation of tested antioxidant and DPPH. In this case, only the final reaction effect will be observed for both vitamin C and BHT. Antioxidant activity is more complicated to establish when testing food products**.** *E. umbellata* fruits are a source of various antioxidants that can work differently. As seen in Fig. 4, the *E. umbellata* fruit solution has a slower growth rate than the above antioxidants. As mentioned earlier, the antioxidant activity of fruits will result from various antioxidants' content and the interaction between antioxidants or antioxidants and other fruit ingredients. In Liu study, lycopene reacted very slowly with DPPH and did not reach maximum levels for 60 minutes of the experiment (Liu et al., 2008). In turn, vitamin E's reaction achieved a relatively rapid increase in 10 minutes, after which it significantly slowed down. The strength of DPPH inhibition by *E. umbellata* increases until the last measurement in 90 min. It can be assumed that the slower reaction time of free radicals *in vitro* will allow for a longer duration of antioxidant activity *in vivo*.

CONCLUSION

E. umbellata fruits growing in central Europe are a valuable source of numerous bioactive ingredients, making them a useful component of human and animal diets. Vitamins, phenols, carotenoids, minerals and unsaturated fatty acids contribute to the high antioxidant power of the fruit. Characterization with FT-IR spectroscopy showed that changes occur during the fruit extract reaction with hydrogen peroxide, indicating an antioxidant effect. Kinetic studies have shown that a typical protocol for testing antioxidants' reaction with the DPPH radical may give erroneous results because the fruit in methanol does not reach the maximum antioxidant power within the traditionally used time of 20-30 minutes. Further research is needed to understand the exact steps involved in this reaction.

Author contribution

Conceptualization, K.Z. and T.N.; methodology, J.B., K.Z., P.K., validation, T.N; A. R-G., K.B.; formal analysis, K.Z, J.B, A.R-G., P.K., M.R., K.B.; data curation, A.R.-G. and K.Z.; writing—original draft preparation, K.Z., A.R-G; writing—review and editing, T.N. and K.Z., P.K.; visualization, A.R.-G.;

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