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EXPERIMENTAL PAPER

Antioxidant potential of *Hippophae rhamnoides* L. extracts obtained with green extraction technique

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Summary

Introduction: Antioxidants, isolated from different plant parts, are widely used due to their ability to prevent the development of so-called oxidative stress. Sea buckthorn (*Hippophae rhamnoides* L.) is one of the plants with expected antioxidant properties.

Objective: The aim of the study was to evaluate the antioxidant activity of ethanolic, methanolic and acetonic extracts of *H. rhamnoides* leaves, ripe and unripe fruits obtained by ultrasound-assisted extraction.

Methods: To estimate the antioxidant potential of the extracts the DPPH, FRAP, ABTS and Folin-Ciocalteu methods were applied. Moreover, the influence of the extrahent, as well as extraction time, on this activity was evaluated.

Results: Sea buckthorn leaf extracts showed higher activity, contrary to the fruit extracts. Moreover, higher activity of ripe fruit extracts compared to unripe material extracts was found. To obtain the highest content of antioxidants in the extracts, ultrasound-assisted extraction for 60 min with methanol should be applied.

Conclusions: The presented *in vitro* results could lead to the conclusion that *H. rhamnoides* seems to be a valuable source of antioxidants to be applied in various branches of industry.

Key words: ***sea buckthorn, DPPH, FRAP, ABTS, Folin-Ciocalteu method, ultrasound-assisted extraction***

Słowa kluczowe: ***rokitnik zwyczajny, DPPH, FRAP, ABTS, metoda Folin-Ciocalteu, ekstrakcja ultradźwiękowa***

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INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L.) is a common plant in several countries, including Russia and Germany. Its health-promoting properties are highly valued. This herbal is used in many industry sectors, including food, pharmaceutics and cosmetics. Sea buckthorn had been used, among others, in ancient times as an agent to treat helminthiasis in horses. Its leaves and young sprouts led horses to gain weight quickly and to improve their fur shine. Treatises of Tibetan medicine from the 7th century BC recommended using, among others, sea buckthorn as an anti-diarrheal, antitussive agent, which also improves blood circulation. Moreover, some information on the application of sea buckthorn oil by Genghis Khan's army as a sedative and wound-healing agent can be found [1]. *H. rhamnoides* is a shrub about 5–8 m in height. It belongs to the oleaster family (*Elaeagnaceae*). The sea buckthorn shoots are covered with thorns, and their lanceolate, narrow leaves are greenish-grey on the top, while the undersides are white or light-brown. Its flowering period is at the end of April. Female flowers are yellow, whereas the male ones are greenish. The fruits are yellow to red-orange [2, 3]. The sea buckthorn leaves are rich in nutrients and bioactive substances such as polyphenols, the major compounds responsible for the antioxidant potential. In addition, the leaves contain carotenoids and chlorophyll with the highest concentrations found in fresh material. On the other hand, the dried leaves are rich in proteins and amino acids as well as folic acid, mineral salts and esterified sterols. Fruits contain vitamins, lipids, micronutrients, carotenoids, flavonoids, phospholipids, tannins, organic acids and sugars. Similarly, as in the leaves, phenolic compounds (mainly flavonoids and phenolic acids) are responsible for the antioxidant activity. It is worth mentioning that the fruit content of vitamin C, a known and valuable antioxidant, mostly depends on the quantity of flavonoid compounds. This is related to their ability to stabilise ascorbic acid. The composition of fruit depends on their maturity and size, the climate of the cultivation region as well as on their further processing [1, 2]. The content of valuable substances in the leaves and fruits of sea buckthorn provides the possibility for use in pharmaceutical and cosmetic products. This plant can be applied as an agent for strengthening immunity formulations, as well as those supporting the treatment of digestive, blood circulation and urogenital system diseases as well as in eye or skin disorders. It is also used in cosmetic products due to its antioxidant properties [3, 4]. The antioxidant potential is very important as the excess of free radicals in the body may contribute to the development of so-called oxidative stress. This phenomenon leads to damage of important body structures such as proteins, nucleic acids and lipids [5] and to the development of many diseases, i.e. neoplastic [6], neurodegenerative [7] and psychiatric disorders [8, 9] as well as a number of metabolic diseases including diabetes and its comorbid conditions [10]. Increasingly, it is suggested that some of the synthetic antioxidants can accumulate in the body, which may result in damage to internal organs occurring (e.g. liver) and initiate carcinogenesis [11]. So, searching for new natural sources of antioxidants seems to be important. Such compounds should protect organisms against the harmful effects of free radicals, without any adverse reactions.

The aim of the study was to evaluate and compare the antioxidant potential of ethanolic, methanolic and acetonic extracts of *H. rhamnoides* leaves as well as ripe and unripe fruits, obtained by the green technique, i.e. ultrasound-assisted extraction. Four methods, based on different mechanisms of action, were applied to evaluate the antioxidant capacity of the extracts. Moreover, the influence of applied solvent, extraction time and maturity of raw material was also assessed.

MATERIAL AND METHODS

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), (ABTS) 2,4,6-tripyridyl-S-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Sigma-Aldrich, USA; Folin-Ciocalteu reagent and iron(III) chloride hexahydrate from Merck, Darmstadt, Germany; whereas the sodium acetate anhydrous, sodium carbonate anhydrous, potassium persulfate, 36% hydrochloric acid, acetone, methanol and 99.5% acetic acid, all of analytical grade, were from Chempur, Piekary Śląskie, Poland.

Plant material

The plant material consisted of the leaves, ripe and unripe fruits (including pulp with peel) of

H. rhamnoides obtained in 2015 which we cultivated in Świnoujście (West Pomeranian region, Poland). Leaves were harvested in June, the unripe fruit in the next month, whereas the ripe fruit was picked in September. Fresh material (5%) was extracted with acetone, methanol and 70%, as well as 96%, (*v/v*) ethanol, using ultrasound-assisted extraction at a frequency of 40 kHz, for 15, 30 and 60 minutes.

Antioxidant activity determination

Four methods: DPPH, FRAP, ABTS and Folin-Ciocalteu (F-C) were applied to evaluate the antioxidant properties of the extracts. These techniques are commonly used to determine *in vitro* the antioxidant potential of plant extracts.

Evaluations of antioxidant capacities were performed as previously described by Muzykiewicz *et al.*, Zielonka-Brzezicka *et al.* and Nowak *et al.* [12–14]. To determine the DPPH radical scavenging activity, 0.3 mM ethanolic DPPH solution with an absorbance of 1.000±0.020 at 517 nm was used. An aliquot of 2850 μl of this solution was mixed with 150 μl of the extract. After 10 minutes of incubation at room temperature, the spectrophotometric measurements of absorbance at 517 nm were taken [12-14].

To evaluate the reducing power of the extracts, the FRAP method was applied. The working solution was obtained by mixing 1 volume of 20 mM $FeCl_3$, 1 volume of 10 mM TPTZ (in 40 mM HCl), and 10 volumes of acetate buffer (pH 3.6). Then, 80 μl of the extract was mixed with 2320 μl of working solution and incubated at room temperature for 15 minutes. The absorbance was taken at 593 nm [12-14].

For ABTS analysis, a 7 mM ABTS solution in 2.45 mM aqueous $K_2S_2O_8$ solution was prepared and allowed to stand in the dark at room temperature for 24 hours, then diluted to an absorbance of 0.700±0.005 at 734 nm. An aliquot of 25 μl of extract was added to 2500 μl of working solution. The absorbance measurements at 734 nm were performed after 6 minutes of incubation [12-14].

Total polyphenol content was evaluated by the F-C method by mixing 2700 μl of 5 mM Na_2CO_3 and 150 μl of extract with 150 μl of 10%(*v/v*) F-C reagent aqueous solution. The spectrophotometric measurements were performed at 750 nm after 15 min incubation at room temperature [12-14].

Antioxidant activity has been expressed as trolox equivalents [mg trolox/g raw material] in all the applied methods.

Statistical analysis

Data are presented as the arithmetical mean±standard deviation (SD). ANOVA one-way analysis of variance at a significance p=0.05 was employed. The mean values were grouped taking into account the part of the plant used to obtain extracts (leaves, unripe or ripe fruit) as well as the method of antioxidant activity evaluation (DPPH, FRAP, F-C, ABTS). Tuckey's multiple range test (n=3) was applied for comparison of means. The Pearson correlations (r) between the antioxidant activity values obtained with different methods were also calculated. Statistical calculations were done using Statistica 12 PL Software (StatSoft, Polska).

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

Table 1 presents the antioxidant activity of *H. rhamnoides* extracts (expressed as trolox equivalents) obtained using the DPPH, FRAP, ABTS and F-C methods. All the evaluated extracts showed antioxidant potential. The highest activity, regardless of the applied method, was found for leaf extracts, whereas it was significantly lower for fruit extracts.

The leaf extract in 70% (*v/v*) ethanol (15 min extraction) and in acetone (30 min) showed the highest activity – both 4.48±0.02 mg trolox/g raw material. On the contrary, the lowest potential of 0.44±0.01 mg trolox/g raw material was found for 70% (*v/v*) ethanolic extract of unripe fruit, extraction time 15 min.

Among the extracts evaluated with the FRAP method, the highest antioxidant activity was found for methanolic extract from leaves obtained during one-hour ultrasound-assisted extraction (58.06±0.42 mg trolox/g raw material), while the lowest was for ripe fruit extract in 70% (*v/v*) ethanol, also extracted for one hour (1.01±0.14 mg trolox/g raw material).

The highest total polyphenol content, determined by the F-C method, was observed for leaves extracted for 60 min and the lowest for ripe fruit (extraction time 15 min), both prepared in 70% (*v/v*) ethanol – 28.37±0.29 and 1.01±0.14 mg trolox/g raw material, respectively.

The antioxidant activity was also estimated by the ABTS method. The highest potential of 20.47±0.02 mg trolox/g raw material was observed for acetonic extract from leaves. On the contrary, the ripe fruits extract prepared using the same

Table 1.

Antioxidant properties of sea buckthorn leaves, ripe and unripe fruit extracts, evaluated using DPPH, FRAP, Folin-Ciocalteu and ABTS methods, expressed as trolox equivalents (mg trolox/g raw material) (mean±SD)

Raw material	Extraction time [min]	Ethanol 96% (*v/v*) [mg trolox/g raw material]	Ethanol 70% (*v/v*) [mg trolox/g raw material]	Methanol [mg trolox/g raw material]	Acetone [mg trolox/g raw material]
			DPPH		
Leaves	15	4.28±0.03 b	4.48±0.02 a	3.89±0.01 c	3.71±0.02 d
	30	4.27±0.02 b	3.51±0.01 e	3.86±0.01 c	4.48±0.02 a
	60	4.21±0.01 b	3.41±0.06 f	3.77±0.04 d	4.47±0.02 a
Unripe fruit	15	1.34±0.08 g	0.44±0.01 i	2.72±0.09 c	1.35±0.06 g
	30	1.73±0.03 e	0.93±0.03 h	3.05±0.04 b	1.55±0.04 f
	60	1.98±0.08 d	2.07±0.05 d	3.66±0.05 a	1.38±0.06 g
Ripe fruit	15	0.97±0.01 c	0.78±0.02 de	1.29±0.08 a	0.77±0.03 de
	30	0.97±0.05 c	0.75±0.03 e	1.20±0.06 ab	0.68±0.05 e
	60	1.12±0.04 b	1.16±0.05 ab	1.25±0.05 ab	0.89±0.05 cd
			FRAP		
Leaves	15	18.52±0.61 i	24.89±0.28 f	39.70±0.58 d	42.46±0.47 c
	30	25.71±0.36 f	46.08±0.55 b	45.47±0.16 b	20.37±0.19 h
	60	29.92±0.40 e	45.32±0.19 b	58.06±0.42 a	22.10±0.45 g
Unripe fruit	15	4.22±0.01 e	2.43±0.14 f	10.41±0.24 b	4.36±0.26 e
	30	6.61±0.02 cd	2.70±0.11 f	11.40±0.43 b	5.26±0.21 de
	60	7.43±0.04 c	7.23±0.24 c	14.03±0.27 a	5.17±0.25 e
Ripe fruit	15	2.22±0.03 ef	4.33±0.26 a	3.11±0.04 cd	1.90±0.05 fg
	30	2.62±0.06 de	1.62±0.11 fg	3.27±0.06 bc	1.61±0.03 fg
	60	1.58±0.13 g	2.94±0.29 cd	3.82±0.29 ab	2.00±0.08 fg
			Folin-Ciocalteu		
Leaves	15	15.98±0.38 g	17.83±0.38 f	24.93±0.30 c	25.11±0.27 c
	30	19.70±0.35 e	26.19±0.22 bc	25.15±0.37 c	16.01±0.22 g
	60	22.07±0.36 d	28.37±0.29 a	27.35±0.29 ab	18.40±0.18 ef
Unripe fruit	15	3.30±0.18 ef	1.69±0.25 h	4.99±0.23 b	2.79±0.20 fg
	30	3.71±0.13 de	2.37±0.08 gh	5.93±0.28 a	4.59±0.19 bcd
	60	4.88±0.27 bc	4.00±0.23 cde	6.42±0.08 a	4.17±0.25 bcde
Ripe fruit	15	2.20±0.12 f	1.01±0.14 g	2.79±0.14 def	1.89±0.00 fg
	30	4.22±0.20 c	3.69±0.23 cd	2.64±0.22 ef	1.97±0.12 f
	60	9.98±0.28 a	7.14±0.21 b	3.23±0.14 de	2.39±0.25 ef
			ABTS		
Leaves	15	12.37±0.06 g	16.25±0.20 e	17.41±0.08 d	20.47±0.02 a
	30	17.46±0.23 d	19.03±0.50 b	20.25±0.53 a	13.85±0.21 f
	60	18.46±0.08 bc	17.75±0.26 cd	20.00±0.19 a	17.20±0.17 d
Unripe fruit	15	2.20±0.24 de	0.61±0.15 f	3.92±0.49 b	1.03±0.19 f
	30	3.40±0.45 bc	1.51±0.45 ef	2.71±0.27 cd	1.44±0.10 ef
	60	2.84±0.35 cd	4.16±0.56 b	8.15±0.25 a	1.44±0.21 ef
Ripe fruit	15	0.21±0.02 de	0.60±0.02 b	0.31±0.03 bcde	0.13±0.04 e
	30	0.54±0.04 bc	1.18±0.30 a	0.51± 0.12 bcd	0.21±0.04 de
	60	0.25±0.06 cde	0.34±0.05 bcde	0.33±0.07 bcde	0.19±0.05 e

Mean values marked by different letters differ significantly taking into account the particular raw material and the applied method of antioxidant activity evaluation. Significance level p=0.05; n=3.

extrahent, showed the lowest antioxidant capacity – 0.13±0.04 mg trolox/g raw material. In both cases extraction time was 15 min.

The correlations (r>0.900; p<0.0001) between activities of the extracts of the same plant part, determined with different methods, are presented in figure 1. In the group of leaf extracts the highest correlation coefficient was observed for FRAP *vs.* F-C methods (r=0.949; p<0.0001). For unripe fruit extracts, high correlations were found for the methods: FRAP *vs.* DPPH (r=0.991; p<0.0001), F-C *vs.* DPPH (r=0.932; p<0.0001) and F-C *vs.* FRAP (r=0.923; p<0.0001). The correlation coefficients in the activity of ripe fruit extracts determined with different methods were significant.

DISCUSSION

As already mentioned, the use of low molecular antioxidants is one way to protect the body against so-called oxidative stress. Polyphenols are the major group of plant antioxidants and are widely used in the cosmetic industry, for instance as ingredients of anti-ageing or whitening formulations as well as sunscreens. These substances are also used in pharmaceutical and food products and can be used as agents to help balance a healthy diet [15, 16].

Tian *et al.* [17], in their studies on composition of sea buckthorn leaves and fruits, stat that leaves contained such compounds as ellagitannins, (+)-catechin and flavonol glycosides, whereas flavonol glycosides, isorhamnetin glycosides and quercetin glycosides were found in the fruits. Moreover, they compared the concentration of phenolic compounds in the extracts of the leaves and fruits collected from various plants and found lower phenolic compound concentration in sea buckthorn extracts compared to extracts of other common plants, i.e. different varieties of blueberries, currants, hawthorn or chokeberries. In our study, higher antioxidant activity of leaf extracts compared to both ripe and

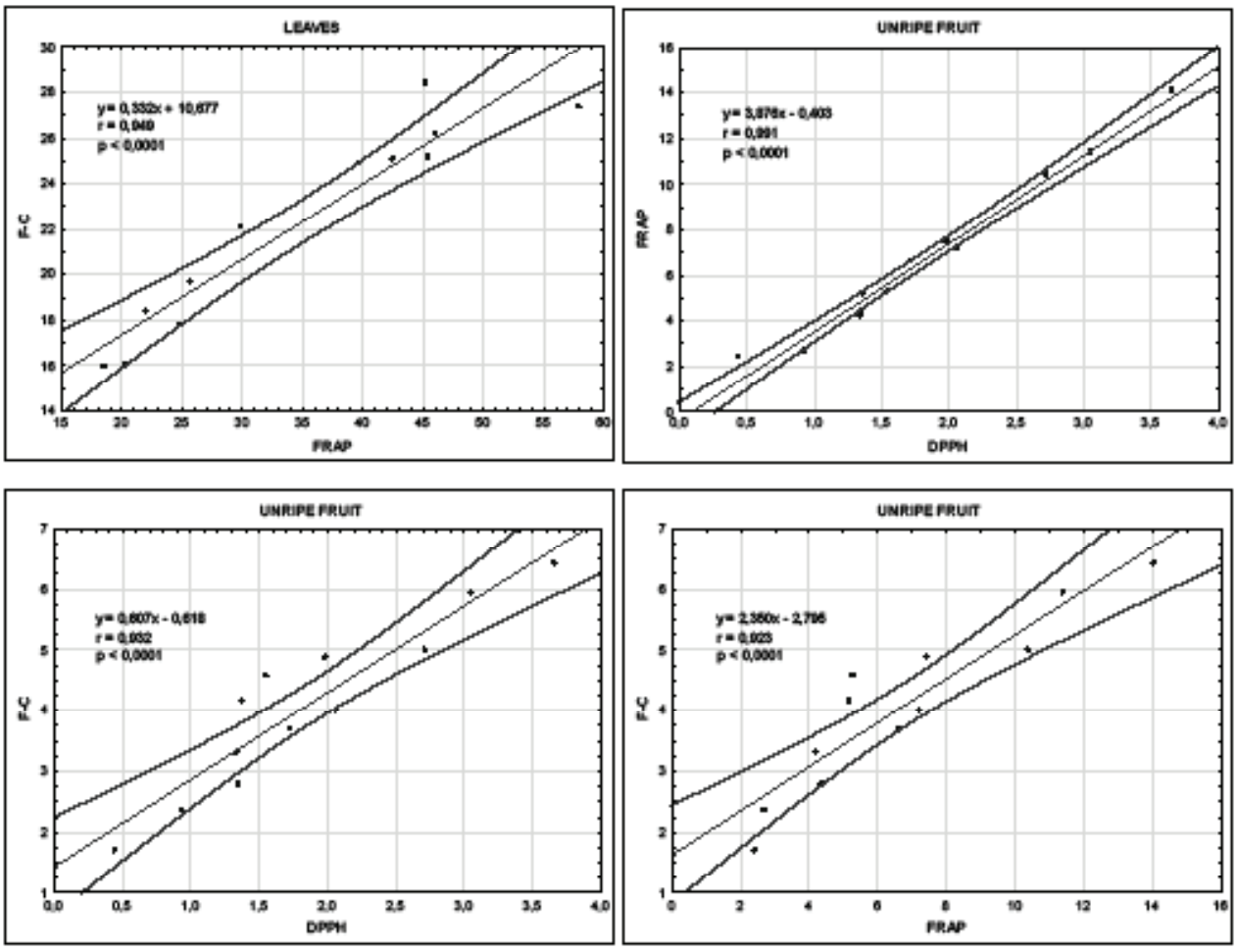

Figure 1.

Correlations between antioxidant activity expressed as mg trolox/g raw material evaluated using different methods (r – correlation coefficient, p – probability value)

unripe fruits extracts was observed. Similar results were obtained by others. Górnaś *et al.* [18] evaluated the content of lipophilic antioxidants in sea buckthorn leaf, fruit and shoot extracts and found the highest content of these compounds in the leaves. Fatima *et al.* [19] also analysed the concentration of flavonoids, vitamin C and tocopherol in sea buckthorn leaves and fruits. They found higher concentrations of antioxidants in leaf extracts. Also, Kumar *et al.* [20] observed that the concentration of prohealth phenolic compounds in sea buckthorn leaves was higher compared to their fruits. Kiewlicz *et al.* [21] compared the antioxidant activity of aqueous-alcoholic plant extracts, of absinthe wormwood, yellow melilot, common knotgrass and sea buckthorn and assessed the lowest antioxidant capacity was for sea buckthorn fruits. It should be added that Guo *et al.* [22] suggested that the phenolic compounds (for instance, flavonoids) in sea buckthorn fruit may differ depending on the subspecies of the plant used for the extraction. Moreover, in our previous study evaluating the antioxidant capacity of different parts of rowan, higher activity was also found in leaf extracts compared to those of fruits [12]. Malinowska [23] evaluated the antiradical activity of 10 plant extracts used in the cosmetic industry. She found one of the lowest potentials in *H. rhamnoides* extracts. In contrary, a higher flavonoid concentration was found in arnica, hawthorn, lungwort and burdock, as well as knotgrass extracts.

Also, the time taken for ultrasound-assisted extraction seems to be another important parameter in obtaining a high yield of antioxidant recovery from plant material. The majority with the highest antioxidant potential were extracted for 60 min, whereas for those with the lowest capacity this was only 15 min. Ghitescu *et al.* [24] worked on increasing the polyphenol content in spruce wood extracts and came to similar conclusions. They found that one-hour ultrasound-assisted extraction was the most effective. Moreover, Bimakr *et al.* [25] also confirmed that such an extraction method is effective in obtaining plant extracts with high antioxidant potential, especially the extracts containing phenolic compounds. Chemat *et al.* [26], in their comprehensive study on ultrasound-assisted extraction, stated that both physical factors (i.e. the frequency of ultrasound used) as well as indirect parameters (i.e. temperature or solvent used) may influence the effectiveness of extraction. Tiwari [27] emphasised the impact of factors such as the duration of the extraction, the ultrasound frequency and solvent applied as an extrahent on the obtained extracts and came to the conclusion that, depending on the plants as well as the type of raw material to be extracted, the above-mentioned extraction parameters should be taken into account.

It is clear from the above data that it is an important challenge to find the proper solvent in order to find the most efficient extraction conditions for extracts of high antioxidant capacity. In our study, methanol seems to be the most effective extrahent because, among others, almost half of methanolic extracts of sea buckthorn showed the highest antioxidant properties. Moreover, methanolic extracts had not reached the lowest capacities, regardless of the raw material type nor evaluation method. Most of the lowest results were obtained for extracts in 70% (*v/v*) ethanol, however, in some cases such ethanolic extracts exhibited the highest antioxidant properties. With ethanol as the extrahent, it was found that several extracts prepared in this alcohol showed the lowest capacities, wherein only one of the other ethanolic extracts showed the highest activity. Some acetonic extracts also exhibited the lowest properties, however, quite rarely, the extracts made in this solvent showed the highest capacity. The effectiveness of methanol and 70% (*v/v*) ethanol as extrahents for isolating antioxidants was evaluated in our previous study on the antioxidant potential of *Ginkgo biloba* leaf extracts after the end of the growing season [14]. To obtain extracts, 40%, 70% and 96% (*v/v*) ethanol as well as 99.8% (*v/v*) methanol were used as solvents. Similar to the present study, high activity for extracts in 70% (*v/v*) ethanol and methanol was observed. Roby *et al.* [28] came to similar conclusions when comparing the antioxidant potential of sage, thyme and marjoram alcoholic extracts. They obtained the highest activities for methanolic extracts. Also, Hossain and Shah [29] evaluated the antioxidant activity of *Merremia borneensis* extracts and obtained the highest values for those prepared in 70% (*v/v*) ethanol. In their study, Jeszka-Skowron *et al.* [30] searched for the optimal extraction process to extract components with antioxidant potential from white mulberry leaves. They noted that extracts in 96% ethanol exhibit lower activity compared to those in diluted ethanol (<96%, >60%).

Taking into account the results obtained with different methods to determine antioxidant activity, it was found in this study that the highest activities of the same extracts, expressed as trolox equivalents, were observed if the FRAP method was used and the lowest if the DPPH method was applied. According to Alama *et al.* [31], the DPPH, FRAP, ABTS and C-F methods have been used quite often to evaluate such activities, however, among all

the procedures to evaluate *in vitro* antioxidant capacity, the DPPH method has been applied most frequently. According to Matysiak *et al.* [32], the lower values obtained using the DPPH method may partly be a result of its lower sensitivity compared to, for instance, the ABTS method. Apak *et al.* [33] claimed that the results obtained with any of the techniques are similar in terms of scavenging or deactivation of free radicals, however, they differ in terms of process kinetics, among others. Therefore, it is recommended to apply at least two analytical methods, based on different mechanisms to evaluate antioxidant activity and to perform at least three independent measurements for each extract, as none of the commonly used generally accepted procedures is sufficient to accurately evaluate the total antioxidant potential of the extracts.

To sum up, based on the results of this *in vitro* study, sea buckthorn extracts seem to be a valuable source of compounds with antioxidant potential and could be used as a substitute for synthetic antioxidants. Performed analyses may contribute to extending the application of *H. rhamnoides* extracts in several branches of industry.

CONCLUSIONS

1. All studied buckthorn extracts showed radical scavenging activity. The highest antioxidant capacities were found for leaf extracts. The potential of fruits, (lower than for leaves), in most cases, was significantly higher for unripe than for ripe fruit extracts.
2. One-hour extraction in methanol proved to be the most effective technique to obtain *H. rhamnoides* extracts of good antioxidant capacity.
3. The highest antioxidant activities were obtained with the FRAP method whereas the lowest were with the DPPH technique.
4 *H. rhamnoides* was proved to be a useful source of antioxidants and can be considered as a valuable raw material in various branches of industry.

Conflict of interest: Authors declare no conflict of interest

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