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BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY IN DIFFERENT EXTRACTS OF SEA BUCKTHORN (*Hippophae rhamnoides* L) BERRIES

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Abstract

Sea buckthorn fruits were used as raw material for the extraction of bioactive compounds as a first important step in phytochemicals further valorization in different industries using three extraction methods: ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and conventional extraction method. Both modern methods offer many advantages compared with the conventional solvent extraction, in terms of the solvent and parameters of extractions used. The results illustrated that the UAE gives comparable values for extraction efficiency. The process conditions for maximum phytochemical extraction were time of 45 min; temperature of 40 °C, frequency of 40 kHz, and power of 100 W. The extraction protocol revealed a total carotenoid content of 42.43±0.17µg/g DW, a β-carotene content of 35.35±0.06 µg/g DW, and a lycopene content of 9.82±0.03 µg/g DW when an solvent mixture of ethyl acetate:hexane (2:1) was used for extraction. Higher values of TPC (805.34±5.5 mg GAE/g DW), TFC, (211.65±0.75 mg CE/g DW) and antioxidant activity of 2.15±0.01 µg Trolox/ml extract (DPPH) and 3.47±0.03 µg Trolox/ml extract (ABTS) were obtained when using ethanol:acetone solvent mixture (4:3).

Keywords: *Hippophae rhamnoides L.;* bioactive compounds; carotenoids; antioxidant activity; ultrasound-assisted extraction (UAE); microwave-assisted extraction (MAE); maceration

Introduction

Sea buckthorn (*Hippophae rhamnoides L.*) also called river-sea buckthorn, is a part of the Order Eleagnales, family Elaeagnaceae, and it is a plant native to Europe and Asia, which is now widespread throughout the world (Górnaś *et al.*, 2014; Michel *et al.*, 2012). Sea buckthorn has a unique composition, forming a cocktail of

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ingredients that can usually be found separately. The plant fruits are an important source of bioactive compounds such as carotenoids: α-carotene, lycopene, lutein, and zeaxanthin (Pop *et al.*, 2014), flavonoids: isorhamnetin, quercetin, kaempferol (Guo *et al.*, 2017), vitamins C and E, organic acids, amino acids, micro- and macronutrients, being considered as fruits with high industrial potential (Asofiei *et al.*, 2019). Carotenoids in sea buckthorn are the main class of compounds with several biochemical and immunological functions (Milani *et al.*, 2017) due to their antioxidant, anti-inflammatory and anti-tumor activities (Pop *et al*. 2014). Also, the flavonoids are the compounds responsible for antioxidant effects, are associated with a positive influence on various diseases such as cancer, Alzheimer's, atherosclerosis (Lee *et al.*, 2009; Panche *et al.*, 2016).

Due to its functionality, white sea buckthorn can be used to manufacture juices, candies, jellies, jams, alcoholic and non-alcoholic beverages, pastry and confectionery, dairy products, natural flavors for food. The extraction of bioactive compounds from plants represents an important step in the valorization of these phytochemicals as a source of supplements, additives, and ingredients in dietary supplements such as capsules, and oral solutions in cosmetics, and pharmaceutical industry.

The most common method to obtain extracts from plants is solvent extraction. However, in recent years different extraction techniques were applied for the phytochemical extraction, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), either separate or simultaneous (Asofiei *et al.*, 2016; Chemat *et al.*, 2012; Isopencu *et al.*, 2019; Li *et al.*, 2018).

It was demonstrated that the ultrasound-assisted extraction with frequencies higher than 20 kHz has an essential role in the facilitation of the extraction of the organic compounds using different solvents (Roidaki *et al.*, 2015). UAE generates spheres that disrupt the cell walls of the plant matrix, thus releasing the contents of the cells (Chemat *et al.*, 2011; Vilkhu *et al.*, 2008)(Chemat et al., 2011; Vilkhu et al., 2008) and by adding a suitable solvent or a mixture of solvents under controlled parameters (temperature, time, and frequency) the efficiency of the extract increases.

Among modern extraction alternatives, the MAE is considered to be an alternative for conventional solvent extraction, which requires both a short extraction time and a reduced amount of solvent (Elez Garofulić *et al.*, 2013).

The purpose of the study was to extract the bioactive compounds using an ultrasonic treatment, microwave extraction, and convention solvent extraction to determine the content of biologically active compounds in sea buckthorn, such as total carotenoids, β carotene, lycopene, total polyphenols, total flavonoids and the antioxidant capacity of the extracts in order to establish the more suitable extraction protocol.

Materials and methods

Chemicals

Hexane, acetone, ethyl acetate, ethanol and methanol HPLC-grade, and sodium carbonate (Na_2CO_3) were purchased from Sigma Aldrich Steinheim, Germany. ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, Folin-Ciocalteu reagent, gallic acid solution, potassium chloride (KCl), hydrochloric acid (HCl), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), Trolox solution were obtained from Merck, Darmstadt, Germany.

Materials

The sea buckthorn was purchased from a local market from Galați area, in early September 2018. The sea buckthorn berries were washed, frozen, and lyophilized at -42 °C at a pressure of 0.1mBar for 48 hours (CHRIST Alpha 1-4 LD Plus lyophilizer, Germany).

Extractions

The compounds were extracted using different solvents and solvent mixtures, and the extraction techniques used were ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and conventional extraction method. The polar solvents used were ethanol, acetone, ethyl acetate, formic acid, and water. In contrast, the hexane was used as non-polar solvent and sunflower oil as a non-polar and lipophilic system (Table 1).

Ultrasound-assisted extraction (UAE)

UAE was performed using an ultrasonic bath system (MRC Scientific Instruments). 5 g of lyophilized of sea buckthorn was mixed with 20 ml of solvents and introduced in an ultrasonic bath (MRC Scientific Instruments) equipped with a digital control system of sonication time, temperature, and frequency. The following ultrasound-assisted extracts using different organic solvents or solvent mixtures: extraction in ethanol, extraction in ethanol:acetone (4:3), extraction in ethanol:hexane:acetone (4:3:1), ethyl acetate:hexane (2:1), have been tested for carotenoid extraction (Olives Barba *et al.*, 2006). The extraction was performed at a constant frequency of 40 kHz, at a constant power of 100 W for 20 min. Coldwater was added to maintain a constant temperature of 40 ± 1°C in the ultrasonic bath. Then, the supernatant was separated by centrifugation at 5000 rpm for 10 min. After the centrifugation, the extraction was repeated 4 times, and the collected supernatants were concentrated at 40°C in a vacuum rotary evaporator (RVC 2-25 Martin Christ, United Kingdom) to obtain the dried extracts. The dried extracts were stored at 4°C before subsequent analysis.

Several sequential extractions with different solvent mixtures were carried out using the same lyophilized matrix. The residue obtained after first extraction in ethyl acetate:hexane (2:1) mixture, was further extracted consecutively with 20 ml solvent mixture ethanol: hexane (4: 3), 20 ml mixture formic acid: acetone: water (0.35: 20: 80) and 20 ml extraction with water. After solvent addition over the

initial matrix, the UEA extraction was applied each time, using the parameters from Table 2 and Figure 1. The residues obtained from each extraction step were collected and concentrated under vacuum condition, for phytochemical and antioxidant analysis. In order to intensify the compound extraction, a separate extraction was also performed, and the extraction was repeated four times, using the same conditions.

Table 1. The characteristics of the extraction methods

Code sample	Extraction system	Parameters Time, min (sec, h) / Temperature, °C / Frequency, KHz / Power, W
	UAE	
UAE1	ethanol 96%	45 min / 40 °C/ 40 KHz / 100 W
UAE2	ethanol:acetone (4:3)	
UAE3	ethanol:hexane:acetone (4:3:1)	
UAE4	ethyl acetate:hexane (2:1)	
UAE5	ethyl acetate:hexane (1:2)	
UAE6	hexane:acetone (2:1)	
UAE7	acetone 80%:ethanol 70% (1:1)	
UAE8	ethanol (70%)	
UAE9	Sunflower oil	15 min / 40 °C/ 40KHz / 100 W
UAE10		30 min / 40 °C/ 40KHz / 100 W
UAE11		45 min / 40 °C/ 40KHz / 100 W
	MAE	
MAE1	ethanol 70%	15 sec / 47÷61 °C / 420W
MAE2	Sunflower oil	30 sec / 70.6 °C / 1050 W
MAE3		40 sec / 47.5 °C / 735 W
MAE4		30 sec / 54.2 °C / 525 W
	MAE - UAE	
MAE - UAE1	Sunflower oil	UAE - 15 min / 40-50 °C / 40 KHz / 100 W MAE - 30 sec / 42.1°C / 525 W
	CSE	
CSE1	ethanol 70%	48h / 40°C

Microwave-assisted extraction (MAE)

Microwave-assisted extraction was carried out in the microwave field of power 420W, 525 W, 735 W, and 1050 W. Ethanol was used in a solid-liquid ratio of 1:20 and 420W power for 15 seconds. The sunflower oil extraction was carried out varying the temperature from 47 to 70.6 °C by variating the power from 420W to 1050 W, for 15-40 sec at atmospheric pressure. The supernatant was separated by centrifugation at 5000 rpm for 10 minutes, and extraction was repeated four times. The collected supernatants were concentrated under vacuum condition at 40 °C in a rotary vacuum evaporator (RVC 2-25 Martin Christ, United Kingdom) to obtain dry extracts and were stored at 4 °C before further analysis.

Table 2. The sequential and separate ultrasound-assisted extraction methods used

Code	Extraction system	Parameters Time, min / Temperature, °C / Frequency, KHz
SqUAE1 SeUAE1	ethyl acetate:hexane (2:1)	20 min / 40 °C / 40 KHz
SqUAE2 SeUAE2	ethanol:hexane (4:3)	20 min / 40 °C / 40 KHz
SqUAE3 SeUAE3	formic acid:acetone:water (0.35:20:80)	20 min / 40 °C / 40 KHz
SqUAE4 SeUAE4	water	20 min / 50 °C / 40 KHz

Sequential UAE (SqUAE) - the same matrix for every extraction, Separate UAE (SeUAE) - new matrix for each extraction

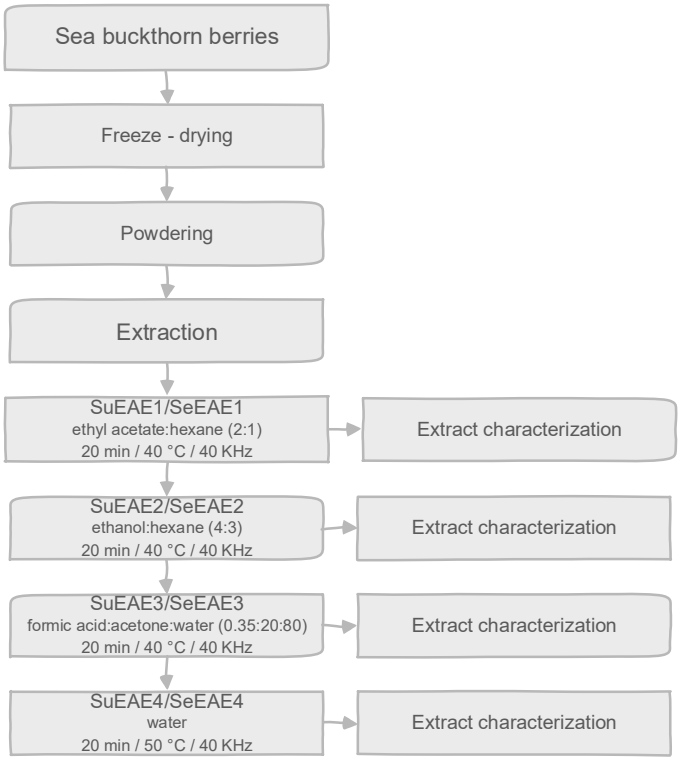

Figure 1. Scheme of the sample preparation process. Sequential UAE (SqUAE) - the same matrix for every extraction, Separate UAE (SeUAE) - new matrix for each extraction

Conventional solvent extraction (CSE)

The extract was obtained by mixing 5 g of the lyophilized vegetable powder with 20 ml of ethyl alcohol 70% solution at 40 °C for 48 hours in the thermostat chamber. The obtained mixture was centrifuged at 5000 rpm, at temperature of 10

°C, for 10 minutes, the supernatant was collected and concentrated. The supernatant was collected, and the extraction was repeated four times. After extraction, the collected supernatants were concentrated under vacuum condition at 40 °C in a rotary vacuum evaporator (RVC 2-25 Martin Christ, United Kingdom) and stored at 4 °C before further analysis.

Determination of phytochemicals and the antioxidant activity

Total carotenoids content

The extracts are diluted in the extraction solvent and the absorbance is read at λ = 470 nm for total carotenoids, λ = 450 nm for ß carotene and λ = 503 nm for lycopene (Rodriguez-Amaya and Kimura, 2004).

The carotenoid, ß carotene and lycopene concentrations were calculated using equation 1 (Britton, 2009):

$$\text{Total carotenoid content } (\mu g/g) \text{ / ß carotene / lycopene } = \frac{A \times V \times 10^4}{A_1 \times W}$$

where:

A - absorbance of the analyzed sample at λ = 470, 450 and 503 nm

V - the volume of extract used for analysis, ml

W - the weight of the sample, g

A_1 - absorption coefficient for carotene (2500), ß carotene (2592), and lycopene (3450).

Total phenolic content (TPC)

The method described by Turturica *et al.* (2016) was used for TPC determination with some modifications. The 0.20 mL of extract was mixed with 15.8 mL of distilled water and 1 mL of Folin-Ciocalteu reagent, diluted 1:2. The mixture was stirred vigorously using Vortex mixer V1 plus (Boeco, Germany) and allowed to stand for 10 minutes, after which 3 mL of 20% Na_2CO_3 was added. The mixture was incubated for 60 minutes at room temperature, and then the absorbance at a wavelength λ = 765 nm compared to the blank, was read. Gallic acid was used as a standard for the calibration curve, and the results obtained were expressed as mg gallic acid equivalent (GAE) / g DW.

Total flavonoid content (TFC)

The total flavonoid content was determined using a spectrophotometric method *based* on the reaction between aluminum chloride ($AlCl_3$) and phenolic compounds, described by Turturica *et al.* (2015). A volume of 0.25 ml of the extract was mixed with 1.25 mL of distilled water and 0.075 mL of 5% sodium nitrite. The mixture was allowed to react for 5 minutes, after which 0.15 mL 10% aluminum chloride was added. After a 6-minutes, 0.5 mL of NaOH 1M was added, and the resultant absorbance of the mixture was read at the wavelength of λ = 510 nm against the blank. The total flavonoid content was determined using the standard catechin curve and was expressed as mg catechin equivalents (CE)/g DW.

Antioxidant activity

ABTS radical cation scavenging activity was assessed by applying the method of Re *et al.* (1999). The measured volume of 5mL of 7mM ABTS was mixed with 5mL of 2.45mM $K_2S_2O_8$ and kept overnight. The solution was diluted with 50% ethanol to obtain an absorbance of 0.7 at λ = 734 nm±0.020. Then 1mL of diluted ABTS was mixed with 10μL of extract, and absorbance was measured at λ = 734 nm.

The percentage of ABTS inhibition was assessed against blank using the equation 2:

$$\text{Inhibition ABTS (\%)} = \frac{A - B}{A} \times 100$$

where,

A is the absorbance of blank;

B is the absorbance of the sample.

A calibration curve was plotted with % ABTS scavenged against the concentration of Trolox, and the results were expressed in mM Trolox / g DW.

DPPH assay. The method using DPPH (2,2-diphenyl-1-picrylhydrazyl) described by Turturica *et al.* (2016), was used to determine the antioxidant activity. DPPH solution was added to the extract, and after a 1 hour and 30 minutes on the dark conditions, the absorbance at λ = 515 nm was read (Turturică *et al.*, 2015). The percentage of DPPH inhibition was assessed against blank using the equation 3:

$$\text{Inhibition DPPH (\%)} = \frac{A - B}{A} \times 100$$

where,

A is the absorbance of blank.

B is the absorbance of the sample.

A calibration curve using as standard Trolox was used, and the results were expressed in mM Trolox / g DW.

Statistical analysis

Statistical data analysis was performed using Minitab 17. Experimental data were expressed as mean ± standard deviation. The significance of the difference between varieties was analyzed using one-way ANOVA and Tukey's test ($p < 0.05$).

Results and discussion

Phytochemicals

For the phytochemical extraction, a combination of three methods was used: conventional solvent extraction, ultrasound-assisted extraction, and microwave-assisted extraction, with a variety of extraction parameters and solvents used. Phytochemical concentration values from sea buckthorn analyzed by different extraction methods are presented in Table 3. Statistical differences were observed

among most of the extraction methods used in the level of all selected phytochemicals ($p < 0.05$). The extraction protocols with the higher values of phytochemicals were UEA3-UEA6. The results can be due to the hexane presence in the extraction mixture. The combination of a polar solvent (ethanol, acetone in UAE3; ethyl acetate in UEA4 and UEA5; acetone in UAE6) with a nonpolar one (hexane) in different ratios leads to enhancing the solubilization of the nonpolar carotenoids (lycopene and ß-carotene) that possess a more hydrophobic nature and limited solubility in water (Strati and Oreopoulou, 2011a). The results are following the study realized by Strati and Oreopoulou (2011b) for the carotenoids extraction from tomato waste by using a mixture of polar and non-polar solvents. The results obtained revealed the highest yield in non-polar carotenoids (lycopene and β-carotene) extraction compared to the other mixtures.

Table 3. Phytochemicals from sea buckthorn analyzed by different extraction methods

Sample Code	Total carotenoids, µg/g DW	β-carotene, µg/g DW	Lycopene, µg/g DW	TPC, mg GAE/g DW	TFC, mg CE/g DW
UAE1	4.57±0.01^{e}	3.84±0.01^{e}	1.15±0.00^{f}	148.19±0.38^{f}	182.44±0.62^{a}
UAE2	25.08±0.03^{b}	21.01±0.01^{b}	5.97±0.02^{b}	805.34±5.5^{a}	211.65±0.75^{a}
UAE3	18.14±0.03^{d}	15.12±0.04^{d}	4.17±0.01^{e}	484.67±6.9^{b}	NA
UAE4	42.43±0.17^{a}	35.35±0.06^{a}	9.82±0.03^{a}	61.57±1.76^{g}	NA
UAE5	20.89±0.09^{c}	17.33±0.08^{c}	4.32±0.06^{d}	178.61±9.3^{e}	NA
UAE6	25.20±0.08^{b}	21.17±0.29^{b}	5.52±0.05^{c}	258.03±6.7^{d}	NA
UAE7	0.91±0.01^{f}	0.71±0.02^{f}	0.35±0.01^{g}	339.4±1.05^{c}	72.27±0.62^{b}
UAE8	1.09±0.01^{f}	0.87±0.01^{f}	0.43±0.00^{g}	182.67±0.85^{e}	87.98±4.2^{b}
UAE9	0.23±0.02^{g}	0.20±0.02^{g}	0.05±0.00^{h}	NA	NA
UAE10	0.24±0.03^{g}	0.20±0.03^{g}	0.05±0.01^{h}	NA	NA
UAE11	0.24±0.01^{g}	0.21±0.01^{g}	0.06±0.00^{h}	NA	NA
MAE1	0.97±0.02^{f}	0.77±0.015^{f}	0.38±0.01^{g}	145.04±1.35^{f}	100.16±16.14^{b}
MAE2	0.27±0.02^{g}	0.23±0.019^{g}	0.07±0.01^{h}	NA	NA
MAE3	0.27±0.01^{g}	0.23±0.01^{g}	0.06±0.00^{h}	NA	NA
MAE4	0.22±0.00^{g}	0.18±0.01^{g}	0.05±0.00^{h}	NA	NA
MAE - UAE1	0.24±0.01^{g}	0.20±0.01^{g}	0.05±0.00^{h}	NA	NA
CSE1	1.09±0.02^{f}	0.84±0.02^{f}	0.43±0.01^{g}	149.80±0.86^{f}	79.12±0.51^{b}

Means on each column that do not share a superscript letter are significantly different according to Tukey's test, $p < 0.05$; NA - not analyzed

The best results for the total carotenoid content, β carotene, and lycopene were obtained for UEA4 with the solvent mixture ethyl acetate: hexane (2: 1). The results were probably due to the polarity of the solvent mixture and the influence of the temperature value, which allowed the solubilization of the nonpolar carotenoids. At the opposite was the extraction in sunflower oil. For both MAE and UAE extraction, the results using sunflower oil as an extraction system showed very low extraction efficiency, which shows that vegetable oils as solvents for lipophilic compounds are not suitable. Despite the lower efficacy in the extraction

of carotenes in our study, the vegetable oils may still be considered a green substitute being non-volatile, safe, and economically solvent (Yara-Varón *et al*., 2017).

Regarding the extraction of carotenoids by the conventional solvent extraction with 70% ethanol, low carotenoid content of 1.09±0.02 µg/g DW was obtained. The total carotenoids content of the extracts obtained by MAE2-MAE4 methods had similar values. Although microwave-assisted extraction (MAE) is considered a simple, rapid, and economical method for the carotenoids extraction, involving a short extraction time with a low amount of solvents (Hiranvarachat and Devahastin, 2014), the extraction oil system used was not comparable with UAE solvent used.

Pop *et al.* (2014) found a ß-carotene content between 19 and 74 µg/g DW and lycopene content of 14 - 23 µg /g DW, in six varieties of Romanian sea buckthorn, using solvent mixture - methanol: ethyl acetate: petroleum ether (1: 1: 1). In another study, Andersson *et al.* (2009) reported a contained of ß-carotene varied from 33.3 to 248.9 µg/g DW, corresponding to 40.0% of the carotenes extracted by a mix of solvent ethanol: n-hexane (4:3). Korekar *et al.* (2014) sampled 187 plants across the primary distribution site from Indian trans-Himalaya. They identified the total carotenoid content ranged from 10 to 144 µg /g FW, and thus, 1-206-fold variation was observed between plants. The samples were defatted with hexane, followed by two cycles of extraction with methanol.

The content of the phenolic compound of different extracts is found in Table 3. As expected, the extracts or mixture extract that are using ethanol as a solvent were generally rich in phenolic compounds. The UAE2 protocol with the solvents mixture of ethanol: acetone (4: 3) was the best suited for the extraction of total polyphenols from sea buckthorn. High values of 805.34±5.5 mg GAE/g DW were obtained, probably because both solvents are polar and miscible with water At the opposite is situated UAE4 with a mixture of ethyl acetate and hexane (2: 1) with the content of TPC 61.57 ± 1.76 mg GAE/g DW. Research conducted by Guo *et al.* (2017) on four subspecies of white sea buckthorn yielded an average of between 27.6 - 38.7 mg GAE / g DW. TPC in sea buckthorn ranged from 964 to 10704 mg GAE/100 g between individuals in the studied population by Korekar *et al.* (2014). Ursache *et al.* (2017) revealed TPC of 140.14±6.64 mg GAE/g DW at sea buckthorn extract.

The higher value for the total flavonoid (211.65±0.75 mg CE/g DW) content is by ultrasonic-assisted extraction combined with a solvent mixture ethanol: acetone (UAE2). The lowest value of 72.27 ± 0.62 mg CE/g DW was obtained with the ethanol acetone solvent mixture (UAE7) and represented 66.0% less than the most successful flavonoid extraction in this series of extractions. Ma *et al.* (2016) reported the contents of flavonols glycosides with ranging from 23 to 250mg/100g in fresh berries extracted with methanol. Ursache *et al.* (2017) reported a content of 5.04 ± 0.05 mg EC / g DW using an extraction mixture of ethanol:hexane (4:3).

Sequential extractions were also carried out on the same matrix by applying different solvent mixtures, and the results obtained were compared with separate extractions. The results obtained are presented in Table 4.

As expected, the total carotenoid, β carotene, and lycopene contents were decreasing at both subsequent and separate extraction, due to the decrease in the polarity of the solvents. The results are in agreement with the study conducted by Nawaz *et al.* (2020) in which the phytochemical content and also the antioxidant properties of *Phaseolus vulgaris* seeds was significantly influenced by polarity increasing of solvents.

Table 4. Phytochemicals from sea buckthorn analyzed by sequential and separate extraction methods

Sample Code	Total carotenoids, µg/g DW	β-carotene, µg/g DW	Lycopene, µg/g	TPC, mg GAE/g DW	TFC, mg CE/g DW
SeUAE1	57.81±0.06^{a}	47.96±0.04^{a}	11.97±0.01^{a}	352.14±11.32^{d}	NA
SeUAE2	25.94±0.03^{c}	21.54±0.03^{c}	5.30±0.03^{c}	146.42±2.11^{f}	NA
SeUAE3	3.85±0.06^{e}	3.33±0.04^{e}	2.14±0.02^{g}	765.27±8.11^{b}	127.80±2.41^{c}
SeUAE4	3.73±0.05^{e}	3.36±0.04^{e}	2.31±0.03^{e}	411.71±4.21^{c}	142.44±1.60^{c}
SqUAE1	29.05±0.04^{b}	24.19±0.03^{b}	6.41±0.01^{b}	196.27±9.32^{e}	NA
SqUAE2	12.80±0.05^{d}	10.62±0.02^{d}	2.59±0.02^{d}	143.49±3.20^{f}	NA
SqUAE3	3.30±0.03^{f}	2.80±0.02^{f}	1.70±0.01^{g}	1023.51±5.51^{a}	310.06±6.00^{a}
SqUAE4	1.61±0.02^{g}	1.39±0.02^{g}	0.87±0.02^{h}	368.12±5.37^{d}	259.69±5.40^{b}

Means on each column that do not share a superscript letter are significantly different, according to Tukey's test, $p < 0.05$; NA - not analyzed

From Table 4, it also can be observed an increase of total polyphenols content upon sequential extraction, namely with the use of the acidified solvent mixture. Moreover, the highest polyphenols content of 1023.51 ± 5.5 mg GAE / g DW, was obtained for the aqueous extraction with acetone acidified with formic acid (SqUAE3), after 20 minutes of extraction at 35-40°C. The lowest polyphenol content with 86% less of the sequential extraction series was obtained with the solvent mixture ethanol:hexane (SqUAE2), with values of 143.49 ± 3.2 mg GAE / g DW. In contrast, SqUAE2 proved to be the best extraction of flavonoids with an aqueous mixture with acidified acetone, resulted a value of 310.06 ± 6.00 mg EC/g DW.

Antioxidant activity

The antioxidant activity determinations were performed using the ABTS and DPPH radical methods. The antioxidant activity of the extracts obtained by mixing the solvent and extraction procedure is important due to the beneficial effects they offer to the body. Antioxidant activity in this study was defined according to several parameters, such as operating conditions: hydrophilic/lipophilic environment, reaction time, etc. Table 5 summarizes the results of the antioxidant activity of the obtained extracts. The results revealed manly that most of the extracts were free radical inhibitors and acted as primary antioxidants.

Ultrasonic assisted extraction has a greater inhibition than other extraction techniques. The best results were obtained by ultrasonic-assisted extraction in combination with the solvent mixture: ethanol: acetone. The lowest result was obtained with the mixture ethyl acetate: hexane (1: 2) and represented an inhibition of 1.07 ± 0.05% by the DPPH free radical method.

Ursache *et al.* (2017) showed in the extract values obtained by the DPPH RSA method of 33.7 ± 0.29%, corresponding to 2.50 ± 0.02 µmol Trolox equiv./g DW. Saini and Keum (2018) reported values ranging from 86.35 ± 2.93 mg Trolox equiv./g for the conventional extraction method, to 383.86 ± 7.7193mg Trolox equiv./g for the extraction of subcritical water at 200 °C.

Table 5. Antioxidant activity of the samples analyzed by different extraction methods

Sample Code	µg Trolox/ml extract (DPPH)	I (%)	µg Trolox/ml extract (ABTS)	I (%)
UAE1	1.81±0.01^{b}	76.80±0.47^{b}	2.77±0.01^{b}	67.87±0.17^{cd}
UAE2	2.15±0.01^{a}	90.75±0.22^{a}	3.47±0.03^{a}	85.01±0.68^{b}
UAE3	1.09±0.01^{d}	46.66±0.33^{c}	1.57±0.03^{hi}	38.78±0.83^{g}
UAE4	0.59±0.01^{e}	22.21±0.28^{d}	0.80±0.03^{j}	19.63±0.83^{h}
UAE5	0.01±0.00^{f}	1.07±0.05^{e}	1.59±0.04^{hi}	37.32±1.04^{g}
UAE6	0.03±0.01^{f}	2.01±0.26^{e}	1.41±0.10^{i}	33.97±2.45^{g}
UAE7	1.84±0.00^{b}	89.56±0.16^{a}	2.57±0.01^{bc}	98.67±0.36^{a}
UAE8	1.56±0.01^{c}	75.97±0.61^{b}	2.82±0.03^{b}	85.03±1.03^{b}
UAE9	NA	NA	1.93±0.056^{fg}	52.82±1.26^{f}
UAE10	NA	NA	2.00±0.06^{ef}	57.01±1.76^{ef}
UAE11	NA	NA	2.27±0.12^{cde}	62.77±3.21^{de}
MAE1	1.56±0.00^{c}	76.24±0.19^{b}	2.44±0.06^{a}	74.80±1.89^{c}
MAE2	NA	NA	2.08±0.03^{ef}	57.36±0.68^{ef}
MAE3	NA	NA	2.19±0.04^{def}	57.93±0.97^{ef}
MAE4	NA	NA	0.70±0.04^{j}	19.18±1.10^{h}
MAE - UAE1	NA	NA	1.74±0.02^{gh}	42.39±0.45^{g}
CSE1	1.54±0.01^{c}	75.21±0.38^{b}	2.21±0.08^{def}	67.73±2.48^{cd}

Means on each column that do not share a superscript letter are significantly different, according to Tukey's test, $p < 0.05$; NA - not analyzed

The series of sequential and separate extractions were analyzed, foresee which method for determining the antioxidant activity is suitable for the hydrophilic and lipophilic profile of sea buckthorn lyophilized. Table 6 shows the results of sequential and separate extraction (the DPPH radical method versus the ABTS radical).

The ABTS free radical method for sequential extractions provided extracts with higher antioxidant activity compared to the separate extraction protocols for both lipophilic and hydrophilic compounds. The increase of antioxidant activity in the sequential extraction (from the lipophilic to the hydrophilic ones) is probably due to the decrease of the aggressiveness of the solvents used. The results obtained for the antioxidant activity at the sequential extraction are slightly different from the

separate extraction. The lower inhibition values for ABTS (ABTS+) radical values of sequential and separate extracts (SqUAE2 and Se UAE2) with 70% ethanol indicates that ethanolic mixtures are better scavenger of ABTS (ABTS+) radical. The best result was obtained using the mixture of formic acid:acetone:water (0.35:20:80), with values of 4.01±0.01 µg Trolox/ml for ABTS method and with inhibition 99.29±0.23% was obtained for the mixture of ethyl acetate: hexane (2: 1), a result of 86.8% lower inhibition than for the best result.

Table 6. Antioxidant activity of the samples by sequential and separate extraction methods

Sample Code	µg Trolox/ml extract (DPPH)	I (%)	µg Trolox/ml extract (ABTS)	I (%)2
SeUAE1	0.07±0.01^{e}	4.07± 0.57^{e}	0.85±0.03^{d}	25.65±0.85^{c}
SeUAE2	0.64±0.01^{c}	35.29±0.41^{c}	0.78±0.01^{de}	19.28±0.23^{d}
SeUAE3	1.70±0.00^{a}	88.78±0.18^{a}	3.66±0.05^{b}	98.35±1.24^{a}
SeUAE4	1.13±0.01^{b}	57.21±0.23^{b}	2.64±0.04^{c}	73.50±1.04^{b}
SqUAE1	0.03±0.02^{e}	2,25±0.77^{e}	0.53±0.04^{f}	12.49±0.96^{e}
SqUAE2	0.17±0.01^{d}	9.84±0.75^{d}	0.623±0.03^{ef}	17.65±0.70^{d}
SqUAE3	1.72±0.01^{a}	89.00±0.40^{a}	4.01±0.01^{a}	99.29±0.23^{a}
SqUAE4	1.73±0.01^{a}	88.88±0.46^{a}	3.49±0.02^{b}	97.77±0.62^{a}

Means on each column that do not share a superscript letter are significantly different, according to Tukey's test, $p < 0.05$; NA - not analyzed

Conclusions

In this study, several extraction techniques have been tested to evaluate the phytochemical profile of sea buckthorn: conventional extraction using 70% ethanol and ultrasound, and microwave-assisted extractions using different organic solvents and vegetable oil as extractants. All extracts were characterized by the global phytochemical profile to establish the most efficient extraction procedure (carotenoid content, total polyphenol content, total flavonoid content, and antioxidant activity). The results showed that ultrasound-assisted extraction gives higher values for extraction efficiency for biological active compounds extraction by using the following parameters: time of 45 min; temperature of 40 °C, frequency of 40 kHz, and power of 100 W. Extraction with ethyl acetate:hexane (2:1) assisted by ultrasound resulted in the extraction of a larger quantity of carotenoids (total carotenoids, β carotene, and lycopene). The highest extraction yield of hydrophilic compounds (polyphenols and total flavonoids) was obtained with the mixture of formic acid: acetone: water (0.35:20: 0), assisted by ultrasounds, through sequential extractions of the compounds. The highest inhibition for antioxidant activity was obtained with mixtures of ethanol: acetone and formic acid: acetone: water, ultrasound-assisted.

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