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ORIGINAL ARTICLE

Fatty Acid Profile and Bioactive Compound Extraction in Purple Viper's Bugloss Seed Oil Extracted with Green Solvents

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Abstract Oil extraction from seeds of purple viper's bugloss (*Echium plantagineum*) was carried out using different solvents (chloroform:methanol, n-hexane, ethanol, 2-propanol and ethyl acetate) at room temperature and also using Randall extraction. Extraction yields were calculated and oils were analyzed in terms of fatty acid profiles and distribution among lipid classes, total polyphenol content, oxygen radical absorbance capacity (ORAC) and phytosterol content. No considerable differences were found on fatty acid profiles and distribution in oils regardless of the solvent and temperature used for the extraction. However, ethanol combined with Randall extraction (85 °C for 1 hour) offered the best results in terms of total polyphenol content (20.9 mg GAE/100 g oil), ORAC (468.0 μmol TE/100 g oil), and phytosterol amount (437.2 mg identified phytosterols/100 g oil) among all assayed extraction methods. A higher extraction temperature led to significantly higher concentrations of bioactive compounds and ORAC values in the oil when ethanol or 2-propanol were used as extracting solvent, but that was not the case using n-hexane except for the concentrations of β-sitosterol and stigmasterol, which were significantly higher using Randall extraction than room temperature extraction with n-hexane. Ethanol is classified as a "green solvent," and it could be considered a suitable option to produce oil from *E. plantagineum* seeds with a higher antioxidant capacity and bioactive compound concentration than the current commercial oil, which is usually extracted with n-hexane.

Keywords *Echium plantagineum* · Green solvents · Gamma-linolenic acid · Stearidonic acid · Bioactive compounds

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Introduction

Purple viper's bugloss (*Echium plantagineum* L.) is a plant species from Mediterranean origin that can be currently found throughout Europe, North America, and Australia. This plant is grown to extract and commercialize its seed oil, which has two main distinguishing features from other vegetable oils: it is naturally rich in stearidonic acid (SDA, 18:4n-3) and gamma-linolenic acid (GLA, 18:3n-6), which makes this oil highly appreciated in the nutrition and cosmetic industries (Rincón-Cervera and Guil-Guerrero, 2010). Several healthy properties have been reported for both polyunsaturated fatty acids (PUFA). GLA exhibits anti-inflammatory, antithrombotic, and lipid-lowering potential, and it enhances smooth muscle relaxation and vasodilatation (López-Martinez et al., 2006). SDA is effective for the treatment of rheumatoid arthritis, skin disorders, and hypertriglyceridemia (Guil-Guerrero, 2007). SDA is metabolized into longer-chain PUFA from the n-3 family such as eicosapentaenoic acid (EPA, 20:5n-3) and docosapentaenoic acid (DPA, 22:5n-3), more efficiently than alpha-linolenic acid (ALA, 18:3n-3) (Walker et al., 2013), which is the more abundant n-3 PUFA found in plant seed oils.

There are several methods available for seed oil extraction such as pressing, solvent extraction, supercritical fluids,

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enzymatic processing, ultrasounds, and microwaves (Chemat et al., 2017; Ixtaina et al., 2011; Koubaa et al., 2016; Yusoff et al., 2015). However, on industrial scale, seed oils are commonly extracted by cold pressing or solvent extraction (De Oliveira et al., 2013; Ixtaina et al., 2011). Cold pressing is not as efficient as solvent extraction to release the oil from the seed meal; thus, an appreciable amount of oil remains unextracted in the meal after cold pressing extraction, particularly when the oil content of seeds is lower than 30% (Azadmard-Damirchi et al., 2010). This way, solvent extraction is the current choice in most industries. Hexane is the most widely used solvent for oil extraction from seeds because of its advantages in terms of oil solubility and ease of recovery (Kumar et al., 2017). However, hexane is a petroleum-derived solvent and several health concerns (dizziness, slight nausea, headache, and fatigue for both acute and long-term exposure) have been described for this compound (Patnaik, 2007). Therefore, there is a need to look for alternative solvents, which are less detrimental for human health than n-hexane and with a more reduced environmental impact. Solvents aimed to develop an environmentally friendly process for oil extraction with simultaneous reduction of pollutants are called "green solvents". Ethanol, which is obtained by the fermentation of sugar-rich materials such as sugar beet and cereals, is one of the most common green solvents. Ethanol is suitable to be used on a large scale because it is easily available in high purity, has a low price, and is completely biodegradable (Chemat et al., 2012). Ethanol, 2-propanol, 1-butanol, or ethyl acetate is preferred over other solvents such as acetone, t-butanol, or ethylene glycol (Byrne et al., 2016).

Although a recent work reported the use of ethanol and ethyl acetate to extract oil from *E. plantagineum* seeds (Castejón et al., 2018), these authors focused only on extraction yield and fatty acid profiles, but identity and quality parameters were not measured. In this work, oil extraction from *E. plantagineum* seeds was assayed using green solvents (ethanol, 2-propanol, ethyl acetate) and compared with the standard extraction using n-hexane. The effect of the temperature during extraction (room temperature or Randall extraction) on extraction yield and oil quality (fatty acid profile and distribution on lipid classes, total polyphenols content, oxygen radical absorbance capacity [ORAC] and phytosterol content) was also tested.

The hypothesis raised in this work is that alcohol-based extractions are as efficient as hexane-based extractions regarding oil yield, and that oils extracted from *E. plantagineum* seeds with alcohols such as ethanol contain higher amounts of phenolic compounds and higher values for ORAC. This fact may be due to the polarity affinity between the solvent (alcohol) and the polyphenol molecules compared with the conventional oil extracted with n-hexane. Additionally, Randall extraction using higher temperatures leads to an increase in the solvent fluidity thus allowing a deeper penetration of the solvent into the seeds biomass and a more efficient extraction of bioactive compounds in the oil.

Experimental Procedures

Solvents and Reagents

Unless otherwise stated, all solvents, reagents, and standards used in this work are from Merck KGaA (Darmstadt, Germany).

Samples

Plants from *E. plantagineum* were grown by researchers from the Faculty of Agronomy at the University of Concepción (VIII Region, Chile) and manually collected seeds (1.5 kg) were sent to the laboratory facilities. Once in the laboratory, seeds were cleaned and kept in paper bags in a dry and dark place until processing.

Oil Extraction

Seeds were kept in an oven at 45 °C overnight and then crushed with a mortar before extraction. An aliquot of ground seeds (5.0 g) was collected and placed in an oven at 110 °C for 4 hours to estimate the moisture content of seeds. The rest of the ground seeds were used for oil extraction using several food-grade solvents (n-hexane, absolute ethanol, 2-propanol and ethyl acetate). Folch extraction method (Folch et al., 1957) was used to compare the extraction yield with the other assayed methods. Ground seeds (5.0 g) were accurately weighed and then 50 mL of solvent (chloroform:methanol 2:1 v/v) was added. The mixture was magnetically stirred under an inert atmosphere (nitrogen) for 1 hour in the dark at room temperature and then vacuum filtered using a glass funnel with a fritted glass and a paper filter to remove the seed meal, which was then washed with fresh solvent (50 mL). The filtrate was transferred to a 250-mL separating funnel and 25 mL of an aqueous sodium chloride solution (0.9% w/v) was added. The funnel was manually shaken, and after two layers were formed, the lower one was collected and filtered through anhydrous sodium sulfate (to remove traces of water) and the solvent was removed in a rotary evaporator at 40 °C. The oil residue was weighed and stored at −20 °C under an inert atmosphere (nitrogen) until further use. Extraction at room temperature using n-hexane, absolute ethanol, 2-propanol, and ethyl acetate was carried out adding 50 mL of solvent to 5.0 g of ground seeds. The mixture was magnetically stirred under an inert atmosphere (nitrogen) for 1 hour in the dark at room temperature and then vacuum filtered using a

glass funnel with a fritted glass and a paper filter to remove the seed meal, which was washed with fresh solvent (50 mL). The solvent was removed in a rotary evaporator at 40 °C and the oils were weighed and stored at −20 °C under an inert atmosphere (nitrogen) until further use. Extractions using the same seed:solvent ratio (1:10 w/v) (Rosas-Mendoza et al., 2017; Samaram et al., 2013) were also carried using a Behr E4 Randall extractor (Behr Labor-Technik GmbH, Düsseldorf, Germany) at 75 °C with n-hexane and 85 °C with absolute ethanol and 2-propanol. Randall extraction with ethyl acetate was not carried out because this solvent can damage the rubber component of the equipment. The samples were immersed in the boiling solvent for 1 hour and then rinsed for 30 min. The resulting liquid phase (the solvent containing the oil) was filtered through anhydrous sodium sulfate and the solvent was then removed in a rotary evaporator at 40 °C. Oils were stored at −20 °C under inert atmosphere (nitrogen) for further analyses. Three replicates were carried out for each treatment.

Fatty Acid Profiles

Extracted seed oils were derivatized to fatty acid methyl esters (FAME) according to a previous work (Rincón-Cervera and Guil-Guerrero, 2010). Fatty acid profiles were obtained by gas–liquid chromatography coupled with flame ionization detection (GC-FID) using an Agilent 6890 N equipment and a 7683B autosampler (Agilent Technologies, Santa Clara, CA, USA). Oven temperature was initially set at 140 °C for 5 min, then increased at 4 °C min^{-1} to 190 °C, then at 1 °C min^{-1} to 220 °C and then at 4 °C min^{-1} to 240 °C, being temperature kept at 240 °C for 5 min. Nitrogen was used as carrier gas and the split ratio was set at 1:100. Temperature of injector and detector were set at 270 and 260 °C, respectively. A Supelco SP-2560 capillary column (100 m x 0.25 mm x 0.2 μm film) (Sigma-Aldrich, St. Louis, MO, USA) was used to carry out the analysis. FAME were identified according to their respective retention times compared with a mix of analytical standards (37 components FAME Mix from Supelco, Sigma-Aldrich, St. Louis, MO, USA). FAME which were not available in the mix of standards, such as SDA, were identified after derivatization and GC injection of a commercial *E. plantagineum* seed oil (15200 NEWmega Echium oil from DeWit Specialty Oils, Texel, The Netherlands) whose fatty acid profile is known (Rincón-Cervera et al., 2018). Analyses were carried out by triplicate and results are reported as mean value ± SD.

Lipid Profiles

Extracted seed oils were fractionated by thin-layer chromatography (TLC) to separate lipid classes according to a previous work (Rincón-Cervera et al., 2013). Each oil (15 mg) was dissolved in 0.2 mL diethyl ether and then applied into a previously activated (105 °C, 60 min) silica gel plate (20 × 20 cm) (SIL G-25 from Macherey-Nagel GmbH, Düren, Germany). Mobile phase was n-hexane:diethylether:acetic acid 70:30:1 (v/v/v). After development, plates were dried at room temperature and sprayed under an iodine stream in nitrogen. Five different classes were fractionated by TLC: sterol esters (SE), triacylglylcerols (TAG), free fatty acids (FFA), diacylglycerols (DAG), and lipids with a higher polarity than DAG (such as monoacylglycerols and phospholipids) which were classified as "polar lipids" (Table 1). Bands corresponding to the different lipid classes were scraped off and placed into test tubes capped with PTFE-lined caps. Derivatization to FAME and GC-FID analyses were carried out as mentioned above. Heptadecanoic acid (17:0) from Sigma-Aldrich (Schnelldorf, Germany) was used as internal standard for quantification purposes. Analyses were carried out by triplicate and results are reported as mean ± SD.

Total Phenolic Content and ORAC

Phenolic compounds were extracted from the seed oils by liquid–liquid extraction by adding 0.25 mL of n-hexane to 500 mg oil and vortexing for 1 min. Then, 0.25 mL of methanol/ultrapure distilled water (60:40 v/v) was added. After vortexing again, the resulting mixture was centrifuged at 2500*g* during 5 min at 20 °C. The hydro-alcoholic phase was removed from the supernatant, and the remaining n-hexane plus the extracted oil fraction was subjected to two additional extractions using 0.25 mL of the methanol/water mixture each time. The hydro-alcoholic extracts thus obtained were pooled and subsequently used to assay the total phenolic contents (TPC), employing the Folin–Ciocalteu assay as described by (Wu et al., 2004). Briefly, 15 μL of the extract was mixed with 200 μL of a solution containing the Folin–Ciocalteu reagent, 40 μL of an aqueous sodium carbonate solution (20% w/v) and 45 μL distilled water. After incubation of the resulting solution at 37 °C for 30 min, the optical density was measured at 765 nm in a 96-well plate using a quadratic regression equation obtained between the Trolox concentration Microplate Reader (Synergy HT, Winooski, VT, USA). TPC were estimated based on a standard curve of gallic acid and expressed as mg of gallic acid equivalents (GAE) per 100 g of oil sample.

ORAC was assayed as previously described (Wu et al., 2004) using 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) as a source of peroxyl radicals and fluorescein as oxidizable probe. In brief, 45 μL of the abovementioned hydro-methanolic extract pool (diluted in phosphate buffer 75 mM pH 7.0) was transferred to a 96-well microplate containing each well 50 μL of AAPH (18 mM) and 175 μL of

Table 1 Data regarding oils extracted from *E. plantagineum* seeds by different methods: lipid extraction yield (g oil/100 g seeds), fatty acid profiles (% individual FA of total FA) and fatty acid distribution among lipid classes (g FA in each lipid class/100 g FA in the oil)

	Room temperature					Randall extraction		
	Folch	n-hexane	Ethanol	2-Propanol	Ethyl acetate	n-hexane	Ethanol	2-Propanol
Oil yield (g/100 g seeds)	24.1 ± 1.0a	23.0 ± 0.5ab	22.2 ± 0.3abc	21.9 ± 0.7abc	20.1 ± 1.3c	22.7 ± 0.9abc	21.6 ± 1.3bc	23.3 ± 1.4ab
FA profiles (% of total FA)								
16:0	7.05 ± 0.01a	6.89 ± 0.01c	6.96 ± 0.02bc	7.14 ± 0.08d	6.99 ± 0.02ab	6.97 ± 0.00ab	7.00 ± 0.01ab	7.06 ± 0.01a
18:0	2.89 ± 0.01a	2.84 ± 0.01c	2.88 ± 0.00a	2.92 ± 0.02b	2.88 ± 0.01a	2.90 ± 0.00ab	2.88 ± 0.00a	2.90 ± 0.01a
18:1n-7	0.61 ± 0.01a	0.60 ± 0.00a	0.61 ± 0.00a	0.65 ± 0.02b	0.61 ± 0.01a	0.60 ± 0.01a	0.61 ± 0.00a	0.61 ± 0.00a
18:1n-9	16.02 ± 0.01a	15.95 ± 0.02a	16.04 ± 0.03a	16.40 ± 0.28b	16.05 ± 0.06a	15.96 ± 0.01a	15.99 ± 0.01a	15.89 ± 0.02a
20:1n-9	0.78 ± 0.00ab	0.78 ± 0.01a	0.78 ± 0.01a	0.80 ± 0.02b	0.78 ± 0.01ab	0.79 ± 0.01ab	0.78 ± 0.00ab	0.79 ± 0.00ab
18:2n-6	17.62 ± 0.01a	17.64 ± 0.02ab	17.57 ± 0.03a	17.75 ± 0.12b	17.58 ± 0.02a	17.63 ± 0.01ab	17.53 ± 0.01a	17.57 ± 0.01a
18:3n-6	9.58 ± 0.01a	9.58 ± 0.02a	9.50 ± 0.01a	9.37 ± 0.12b	9.56 ± 0.02a	9.57 ± 0.02a	9.53 ± 0.00a	9.61 ± 0.01a
18:3n-3	34.48 ± 0.03a	34.72 ± 0.01b	34.68 ± 0.03ab	34.21 ± 0.21c	34.56 ± 0.07ab	34.63 ± 0.03ab	34.68 ± 0.02ab	34.56 ± 0.03ab
18:4n-3	10.97 ± 0.01ab	11.00 ± 0.02ab	10.98 ± 0.01ab	10.76 ± 0.07c	10.99 ± 0.03ab	10.95 ± 0.00a	11.00 ± 0.01ab	11.03 ± 0.01b
FA distribution among lipid classes (g FA/100 g FA in the oil)								
Triacylglycerols[a]	93.46 ± 0.45	93.71 ± 0.31	92.53 ± 0.60	92.31 ± 0.94	92.59 ± 0.22	93.12 ± 0.15	92.72 ± 0.60	92.75 ± 0.61
Diacylglycerols[a]	2.10 ± 0.16	2.59 ± 0.20	2.92 ± 0.29	2.46 ± 0.42	3.12 ± 0.02	2.83 ± 0.02	3.05 ± 0.48	2.69 ± 0.15
Free fatty acids	2.66 ± 0.31a	2.85 ± 0.05ab	3.12 ± 0.17ab	3.21 ± 0.01ab	3.40 ± 0.18ab	3.29 ± 0.18ab	3.15 ± 0.14ab	3.49 ± 0.28b
Polar lipids	1.29 ± 0.19ab	0.42 ± 0.12c	1.01 ± 0.18abc	1.65 ± 0.40b	0.51 ± 0.01c	0.41 ± 0.04c	0.73 ± 0.03bc	0.73 ± 0.12bc
Sterol esters[a]	0.49 ± 0.18	0.43 ± 0.06	0.42 ± 0.04	0.38 ± 0.04	0.38 ± 0.02	0.35 ± 0.03	0.31 ± 0.01	0.34 ± 0.06

Results are expressed as mean values ± SD ($n = 3$). In each row, values with different letters indicate that they are significantly different ($P < 0.05$).

[a] No significant differences were found for FA distributed as triacylglycerols within oils extracted with different procedures. The same fact was observed for the FA amounts found in diacylglycerols and sterol esters.

fluorescein (108 nM). The microplate was placed in a Multi-Mode Microplate Reader (Synergy HT) and incubated for 60 min at 37 °C with shaking every 3 min. During the incubation, the fluorescence (485 nm Ex/520 nm Em) was monitored continuously. ORAC activity was estimated based on a standard curve of Trolox®, using the net area under the fluorescence decay curve. ORAC activity was expressed as micromoles of Trolox equivalents (μmol TE) per 100 g of the oil sample. Analyses were carried out by triplicate and results are reported as mean ± SD.

Analysis of Phytosterols

Phytosterol content in Echium oils was achieved through oil saponification at 80 °C for 80 min under reflux using a 50% KOH aqueous solution and further extraction of the unsaponifiable fraction with toluene, sterol derivatization to trimethylsilyl ethers using hexamethyldisilane and trimethylchlorosilane as derivatizing reagents, and GC-FID analysis according to a modified AOAC official method 994.10 as described in (Sorenson and Sullivan, 2006). An amount of 0.45 g oil was used for phytosterol analysis. Identification and quantification were carried out using standards of β-sitosterol (≥95%), campesterol (~65%) and stigmasterol (~95%) (Sigma-Aldrich, Schnelldorf, Germany). Purity of each standard was considered for phytosterol quantification in oil samples. Analyses were carried out by triplicate and results are reported as mean ± SD.

Statistical Analysis

The Shapiro–Wilk test was carried out to assess normality within data. Two-way ANOVA and Tukey's post hoc test were used to evaluate statistical significance ($P < 0.05$). The IBM SPSS Statistics for Windows software package version 21.0 was used to perform statistical analysis.

Results and Discussion

Oil Extraction Yield

Moisture content of seeds used for extractions was 5.6%. Folch extraction yielded 24.1 g oil/100 g seeds (Table 1), which is in agreement with previous evidence pointing out that oil content is in the range 20–27% for *E. plantagineum* seeds (Gray et al., 2010). Considering the effect of temperature on extraction yield, no significant differences were found between Randall and room temperature extraction when the same solvent was used in any case (Table 1). At room temperature, no significant differences regarding yield were observed between extractions using Folch and other solvents, except for ethyl acetate whose extraction yield was significantly lower (20.1%). Randall extraction yields ranged between 23.3% (2-propanol) and 21.6% (ethanol), but no significant differences were detected among them (Table 1). Considering that ethanol is more sustainable and cheaper than n-hexane, it may become a suitable alternative in terms of oil extraction yield. Furthermore, ethanol (ignition temperature of 425 °C) is recognized as nontoxic and has less handling risks than n-hexane (ignition temperature of 225 °C). Boiling point of ethanol (78.2 °C) is only nine degrees Celsius higher than that of n-hexane (69.0 °C) (Smallwood, 2012), which means that solvent removal after oil extraction does not need very high temperatures using either ethanol or n-hexane.

In a recent work, it was shown that the temperature had an effect on oil extraction yield from *E. plantagineum* seeds when using pressurized liquid extraction (PLE) with different solvents: oil extraction yield ranged from 26.4% at 60 °C to 31.1% at 150 °C using n-hexane as solvent, and from 16.9% at 60 °C to 31.9% at 200 °C using ethanol (Castejón et al., 2018). When ultrasound-assisted extraction (UAE) was used with different solvents (30 min extraction time), ethanol was more efficient than n-hexane and ethyl acetate to extract the oil, especially when extraction was carried out at 55 °C (29.1% yield). Authors assayed PLE, UAE, and microwave-assisted extraction (MAE) and the highest yield (31.2%) was achieved using PLE with ethanol at 150 °C and 10 min extraction time, which was comparable with the classical Soxhlet extraction with n-hexane (8 hours extraction time) (31.3%) (Castejón et al., 2018). Although PLE, UAE, and MAE are emerging techniques with promising potential for seed oil extraction, they are not yet suitable for large-scale oil production due to their high cost. In contrast, the solvent extractions assayed in the current work are more easily scalable, do not use temperatures higher than 85 °C, and extraction times (1 hour) are much lower than the classical Soxhlet extraction (8 hours).

Fatty Acid Profiles of Extracted Oils

Seed oil extracted by the Folch method showed a fatty acid profile with nearly 10% saturated fatty acids (SFA), 17.4% MUFA, 27.2% n-6 PUFA, and 45.5% n-3 PUFA (Table 1). Among PUFA, GLA accounted for 9.58% of all fatty acids, whereas ALA and SDA percentages were 34.48 and 10.97, respectively. This fact is in agreement with the range in which these PUFA are found in Echium seed oils (Guil-Guerrero et al., 2015). Regarding GLA and SDA percentages in extracted oils, values were not significantly different among the assayed extraction processes (Table 1), and only using 2-propanol at room temperature decreased significantly GLA and SDA percentages in the oil. However, such values are not really much lower than those

found in the other extracted oils, and the statistical significance is due to the small SD among measurements. This way, no relevant differences in fatty acid profiles were found in oils extracted with other solvents and different temperatures in this work (Table 1), showing that solvent type or temperature extraction had no significant effect on fatty acid profiles. These results are in agreement with a recent work where fatty acid profiles of extracted Echium oils were not largely affected by solvent type, extraction temperature, or extraction mode (Soxhlet, PLE, UAE, and MAE) (Castejón et al., 2018).

Fatty Acid Distribution in Lipid Classes

The distribution of fatty acids within lipid classes was assessed in this work. Fatty acids in oils were mostly found esterified as TAG, which is in agreement with existing evidence pointing out that fatty acids in crude vegetable oils are mainly esterified as TAG (Pighinelli and Gambetta, 2012). Fatty acids found as TAG ranged between 92.31% of total fatty acids when 2-propanol was used at room temperature and 93.71% with n-hexane at room temperature, although no significant differences were detected in any group. No significant differences were found regarding fatty acids found either as DAG or SE. FFA ranged between 2.66% of total fatty acids in the oil extracted by Folch and 3.49% in the oil extracted with 2-propanol in Randall mode. Although Chilean food regulation set the maximum FFA level at 0.25% in edible oils (Food Health Regulation), it must be considered that Echium seed oil obtained in this work is unrefined, and that through neutralization, FFA level can be decreased to lower limits than that established for edible oils in the Chilean regulation (Pal et al., 2015).

Total Phenolic Content and ORAC

TPC in extracted Echium oils was strongly affected by both solvent type and temperature extraction (Table 2). The lowest TPC were found when using n-hexane as extracting agent with no significant influence of the temperature used in the extraction (1.35 and 1.30 mg GAE/100 g oil for extraction at room temperature and Randall, respectively). A value of 9.95 mg GAE/100 g oil was found when carrying out the Folch extraction; this value was significantly higher than that obtained with any of the three other tested solvents at room temperature. However, the highest TPC were obtained when using alcohols as extract solvents in the Randall mode, especially when ethanol was used (20.9 mg GAE/100 g oil) versus 2-propanol (17.1 mg GAE/100 g oil) (Table 2). The extraction with n-hexane yielded the lowest ORAC values regardless of whether the extraction was carried out at room temperature or in the

Table 2 Data regarding total phenolic content (TPC), oxygen radical absorbance capacity (ORAC) and phytosterol content of crude oils extracted from *E. plantagineum* seeds by different methods

	Room temperature					Randall extraction		
	Folch	n-hexane	Ethanol	2-Propanol	Ethyl acetate	n-hexane	Ethanol	2-Propanol
TPC (mg GAE/100 g oil)	9.95 ± 0.21a	1.35 ± 0.07b	7.10 ± 0.01c	5.35 ± 0.07d	8.05 ± 0.07e	1.30 ± 0.14b	20.90 ± 0.01f	17.10 ± 0.14g
ORAC (μmol TE/100 g oil)	166.5 ± 0.7a	70.0 ± 1.4b	153.0 ± 4.2c	122.5 ± 0.7d	168.0 ± 1.4a	13.0 ± 0.0e	468.0 ± 0.4f	364.0 ± 1.4g
Phytosterol content (mg/100 g oil)								
Campesterol	147.21 ± 10.09ab	131.33 ± 0.73ac	125.38 ± 2.93c	125.41 ± 4.71c	151.55 ± 4.54ab	143.22 ± 5.85abc	190.55 ± 2.33d	156.76 ± 2.54b
β-sitosterol	106.64 ± 3.74a	106.09 ± 0.53a	101.96 ± 3.04a	104.86 ± 4.81a	122.51 ± 4.09b	140.38 ± 7.83c	194.07 ± 0.90d	150.66 ± 3.80c
Stigmasterol	11.94 ± 2.42a	9.75 ± 0.01a	12.38 ± 0.69a	14.70 ± 0.42ab	12.11 ± 1.56a	20.55 ± 1.57b	52.61 ± 1.15c	32.92 ± 3.28d

Results are expressed as mean values ± SD (n = 3). In each row, values with different letter/s indicate that they are significantly different ($P < 0.05$).

Randall mode. Ethanol extraction gave higher ORAC values than 2-propanol regardless of the extraction temperature. Considering that the polarity of ethanol is slightly higher than that of 2-propanol, the higher yields of TPC and ORAC associated with the use of ethanol in the Randall mode suggest that the phenolic compounds which are susceptible to be extracted from the *E. plantagineum* seeds have a high polarity. The fact that solvents with high polarity are more efficient to extract polyphenols is in agreement with previous evidence showing that alcohols are more suitable solvents than nonpolar solvents such as n-hexane for polyphenol extraction from plant material (Roby et al., 2013), and that among alcohols, those with a higher relative polarity such as methanol (relative polarity = 0.762) are more efficient than those with a lower relative polarity such as ethanol (relative polarity = 0.654) (Reichardt and Welton, 2010) (Roby et al., 2013). However, ethanol is more suitable than methanol to be used in the food industry.

The fact that the higher temperature used for the Randall extraction promotes a more efficient extraction of phenolic compounds using ethanol as solvent but not n-hexane can be partially explained due to the different viscosity of solvents at different temperatures. The viscosity of a given solvent decreases when the temperature raises, and the fluidity is consequently increased. The solvent is then more able to penetrate the seed material to extract bioactive compounds. Hexane viscosities at room temperature (20 °C) and at the temperature used for Randall extraction (75 °C) are 0.31 and 0.19 mPa·s, respectively (Anton Paar, 2019b), whereas for ethanol, viscosities at room temperature and Randall extraction temperature (85 °C) are 1.17 and 0.38 mPa·s, respectively (Anton Paar, 2019a). It means that viscosity of ethanol is more greatly reduced (67.5%) using Randall extraction versus room temperature extraction when compared with n-hexane (38.7% reduction using Randall extraction versus room temperature). This fact, considered together with the higher polarity of ethanol versus n-hexane, can explain the higher efficiency of ethanol regarding TPC values.

It has been shown that the degradation rate of natural polyphenols exposed at temperatures lower than 100 °C for 1 hour are low (<7.5% at 100 °C and < 5% at 80 °C) (Volf et al., 2014). It means that when Randall extractions were carried out (1 hour, 75 or 85 °C depending on the solvent used), polyphenols naturally occurring in seed oils should remain mostly stable. Longer extraction times or higher temperatures would lead to an increased degradation rate of these bioactive compounds and they are therefore discouraged.

Interestingly, when the TPC and ORAC values for the oils were correlated, an extremely high correlation coefficient ($R^2 = 0.9714$) was obtained (Fig. 1). Such high correlation arises from TPC and ORAC values that result from the analysis of oils obtained after using five different extraction solvents and two distinctive extraction conditions. Thus, it seems that, in each case, the efficiency with which phenolic compounds are extracted from the Echium seeds is comparable to the extraction efficiency of all compounds that account for the corresponding ORAC value. Former studies, conducted by us (Speisky et al., 2012) in antioxidant-containing plant foods have also revealed the existence of such high correlations, suggesting that the ORAC values obtained here for the oils extracted from the Echium seeds strictly depend on the contribution of phenolic compounds.

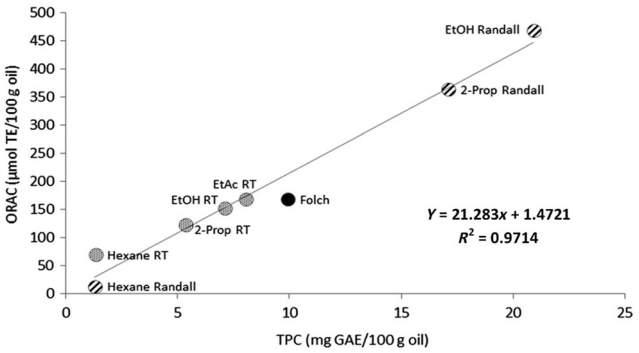

Fig. 1 Relationship between ORAC and TPC in extracted oils from Echium plantagineum seeds. EtOH, ethanol; 2-Prop, 2-propanol; EtAc, ethyl acetate; RT, room temperature

Beyond the health benefits associated with a higher consumption of phenol rich foods (Del Rio et al., 2013), the importance of producing oils with a high TPC and ORAC relates also to the fact that oils rich in PUFA are very susceptible to oxidative degradation.

Commercially available *E. plantagineum* seed oil, which is usually extracted with n-hexane, is very prone to undergo oxidation due to its high PUFA content (70–75% of total fatty acids in the oil) and tocopherols must be added as antioxidants to increase the shelf life of the product. The above-referred comparatively higher natural antioxidant capacity of the oil obtained with ethanol instead of hexane suggests that using the former solvent to extract Echium seed oil may demand the addition of much lower amounts of antioxidants to secure its oxidative stability. Even considering that refining usually reduces the concentration of phenolic compounds, higher TPC and ORAC than those of oil conventionally extracted with n-hexane could be expected, although this fact should be confirmed by further research.

Phytosterols

Campesterol, β-sitosterol and stigmasterol are the most common phytosterols found in seed oils. They have an inhibitory effect on the intestinal absorption of dietary cholesterol,

thereby lowering total cholesterol and low-density lipoprotein (LDL-cholesterol) levels in plasma (Ryan et al., 2007). All of them were identified and quantified in oils extracted from *E. plantagineum* seeds by different solvents and temperatures (Table 2). Campesterol was the most abundant phytosterol found in all cases followed by β-sitosterol and stigmasterol in lower concentrations, except in the oil extracted with ethanol in Randall mode where β-sitosterol (194.07 mg/100 g oil) and campesterol (190.55 mg/100 g oil) were the two main phytosterols followed by stigmasterol in a minor amount (52.61 mg/100 g oil).

β-sitosterol is usually more abundant than campesterol in seed oils (Fernández-Cuesta et al., 2012; Zarrouk et al., 2019), which is not in agreement with our results. However, a recent study reported higher amounts of campesterol than β-sitosterol in seed oils from some Boraginaceae species (Fabrikov et al., 2019). These data can support the results found in this study, considering that *E. plantagineum* belongs also to the Boraginaceae family.

As in the case of TPC, it was shown that the oil extracted with ethanol in Randall mode contained a significantly higher amount of phytosterols than other oils, reaching a concentration of 437.23 mg/100 g oil (campesterol + β-sitosterol + stigmasterol). Interestingly, ethanol at room temperature was the less effective solvent to extract phytosterols among all tested extractions (239.72 mg/100 g oil). When comparing oil extractions with n-hexane and 2-propanol at both temperatures, this trend was also found (Table 2). It means that extraction temperature plays a relevant role regarding phytosterol recovery in the oil. This may be also explained considering the higher fluidity of solvents when temperature rises as exposed above (Total Phenolic Content and ORAC section), allowing the solvent to penetrate deeper into the biomass and thus improving the extraction of these bioactive compounds.

Conclusion

Extraction of oil from *E. plantagineum* seeds using ethanol by Randall method is more efficient in terms of extraction yield of bioactive compounds (TPC and phytosterols) than the commonly industrial process with n-hexane. The ORAC value found in the oil is also the highest using this extraction mode compared with the other assayed processes, which is an important fact to be considered as *E. plantagineum* seed oil is rich in PUFA (GLA, ALA, and SDA) and therefore highly susceptible to oxidative degradation. This way, Randall extraction with ethanol might provide the oil with a superior resistance to oxidation agents. The fact that ethanol is not only classified as a "green solvent" but also cheaper than n-hexane can make of this solvent a convenient choice to replace n-hexane for seed oil extraction at industrial scale.

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Conflict of Interest The authors declare that they have no conflict of interest.

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