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Seed oil and seed oil byproducts of common purslane (*Portulaca oleracea* L.): A new insight to plant-based sources rich in omega-3 fatty acids

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ABSTRACT

In the present study, nutritional value, chemical composition and bioactive properties of purslane seeds, seed oils and seedcakes were examined. Data were analyzed by a one-way ANOVA, while means were compared with Tukey's HSD test. For seed oil extraction mechanical and ultrasound assisted methods were tested. Cold extraction methods (CE1 and CE2) resulted in higher oil yield (increased by 33.7% and 38.1%, respectively) comparing to hot extraction (HE) method. Seeds contained the highest amount of fats and energy (15.03 ± 0.06 g/100 g dry basis (db) and 459 ± 1 kcal/100 g db, respectively), while seedcakes from CE2 had the highest content in proteins and ash (31.20 ± 0.03 and 4.27 ± 0.06 g/100 g db, respectively). Seeds and seedcakes contained a balanced content of linoleic and α-linolenic acids (33.80–34.74% and 32.83–34.64%, respectively). HE and CE1 oils had slightly higher amounts of α-linolenic (39.67% and 39.57%, respectively) than linoleic acid (35.44% and 35.13%, respectively), whereas CE2 oils contained twice as much linoleic as α-linolenic acid (49.77% and 24.18%, respectively). In conclusion, the tested materials are good sources of omega-6 and omega-3 fatty acids and proteins, while extraction method affected oil yield and fatty acids composition of seed oils.

1. Introduction

The beneficial role of omega-3 fatty acids to human health has increased market needs for functional foods and dietary supplements enriched with this specific type of fatty acids (Calder, 2018; Gheysen et al., 2019). The main omega-3 fatty acids associated with health beneficial properties are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are commonly found in seafood and microalgae, and α-linolenic acid (ALA), which is most commonly found in plants (Perona, Garcia-Rodrigue, & Castellano, 2018). Nowadays, the recommended daily dose for omega-3 fatty acids (250–500 mg/day) is met through seafood consumption or dietary supplements containing fish oils, which are rich in EPA and DHA, whereas a very small proportion of daily intake is attributed to plant-based dietary sources (Andre et al., 2019; Tocher, 2015). Despite the well-known beneficial effect of long and very long fatty acids (LCFA and VLCFA, respectively), consumption in many countries does not meet daily requirements due to particularities of specific consumer groups which are reluctant to consumption of fish products (*e.g.* tuna, mackerel, salmon, herring and sardines) or due to inability of world fish production to meet global population needs in LCFA and VLCFA (Salem & Eggersdorfer, 2015; Tocher, Betancor, Sprague, Olsen, & Napier, 2019). Therefore, there is an urgent need to find alternative sources of such fatty acids taking into special consideration that terrestrial plants are sources of ALA which is also associated with beneficial effects and can be metabolized in human body to EPA and DHA (Dubois, Breton, Linder, Fanni, & Parmentier, 2007; Perona et al., 2018).

Various plants have been indicated as rich sources of omega-3 fatty acids, some of which are currently being used in the food and pharmaceutical industry (Dubois et al., 2007). Vegetable oils from species such as sacha inchi (*Plukenetia volubilis*), linseed (*Linum usitatissimum*) and perilla (*Perilla frutescens*) are rich in ALA (45–50%, 52–55%, and 47–64% for sacha ichi, linseed and perilla respectively; Kim et al., 2019; Tavarini et al., 2019; Wang, Zhu, & Kakuda, 2018) and

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monounsaturated fatty acids (MUFA), whereas chia (*Salvia hispanica*) and purslane (*Portulaca oleracea*) contain ALA (61.3% and 32.4% for chia and purslane, respectively) and linoleic acid (LA) in amounts that account to omega-6/omega-3 ratios with values lower than 4, indicating high nutritional value (Dubois et al., 2007; Guil-Guerrero & Rodríguez-García, 1999). Genetically modified and transgenic plants have been also suggested as potential sources of EPA and DHA in aquaculture to replace direct use of fish oils for fish feeding (Tocher, 2015; Tocher et al., 2019). However, climate change and competition of weeds with conventional crops under unfavorable conditions should be also considered and alternative crops resilient to environmental constraints could be an option for plant sources rich in omega-3 fatty acids (Karkanis, Ntatsi, Alemardan, Petropoulos, & Bilalis, 2019).

Purslane is a wild edible species with wide distribution throughout the world, which is traditionally consumed raw as salad or cooked in many dishes of the broader Mediterranean region (Gonnella et al., 2010; Petropoulos, Karkanis, Martins, & Ferreira, 2016). Its medicinal and therapeutic properties are well known since centuries ago and several scripts describe its cultivation for medicinal purposes (Gonnella et al., 2010). The main edible parts are the aerial parts of the plant (leaves and stems), which have been characterized in terms of chemical composition and nutritional value in numerous reports (Oliveira et al., 2009; Palaniswamy, McAvoy, & Bible, 2004; Petropoulos et al., 2015). Aerial plant parts have a high nutritional value due to the presence of high amounts of ALA, however the high oxalic acid and nitrates content hampers the wide acceptance of the species in human diet (Gonnella et al., 2010). According to ethnobotanical studies, seeds can also be consumed by humans and animals, while they also possess medicinal properties similar to aerial parts (Bosi, Guarrera, Rinaldi, & Mazzanti, 2009). Seed extracts have been associated with antibacterial activities against *Staphylococcus aureus* (Tayel et al., 2018), while Nazeam, El-Hefnawy, Omran, and Singab (2018) and Jalali Mousavi, Niazmand, and Shahidi Noghabi (2015) have highlighted the antihyperlipidemic and antioxidant properties of purslane seeds. Seed oils are very nutritious since they are rich in PUFA consisting mainly of LA, ALA and oleic acid (OA), while they also contain phenolic compounds (protocatechuic and *p*-hydroxybenzoic acids) and phenolic lipids (alkylresorcinols) (Gunenc, Rowland, Xu, Marangoni, & Hosseinian, 2019; Kavosi, Mohammadi, Shojaee-Aliabadi, Khaksar, & Hosseini, 2018). However, considering.

Market needs for alternative sources of omega-3 fatty acids and the limited world production of fish oils justify the use of terrestrial sources of PUFA. Purslane could be a potential candidate species for this purpose which is already consumed for its edible leaves and stems, whereas no common use of seeds and seed oils is registered. The high content of purslane aerial parts in antinutritional factors (oxalic acid and nitrates) has hampered so far the commercial exploitation of the species as an alternative vegetable crop. Therefore, the aim of the present study was to evaluate chemical composition and any cytotoxic effects of other plant parts and by-products such as raw seeds, seed oils and seedcakes in order to suggest them as potential alternative/complementary matrices for the pharmaceuticals and cosmetics industry, as well as for the food industry. Moreover, the effect of extraction methods on seed oil yield and oil chemical composition was also tested.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of high-performance liquid chromatography (HPLC) grade from Lab-Scan (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, MO, USA), as also other individual fatty acid isomers, tocoferols standards (α-, β-, δ-, and λ-isoforms, > 98% HPLC purity), sugars standards (D(−)-fructose, D(+)-glucose, D(+)-melezitose, D(+)-sucrose and D(+)-trehalose; > 98% HPLC purity), gallic acid (≥99% HPLC purity), and organic acids standards (oxalic and macic acids; ≥99% HPLC purity). Racemic tocol (98% HPLC purity), 50 mg/mL, was purchased from Matreya (PA, USA). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO) was perched by Merck KGaA, Germany. Fetal bovine serum (FBS), Hank's balanced salt solution, non-essential amino acid solution (2 mmol/L), penicillin/streptomycin solution (100 U/mL and 100 μg/mL, respectively) and Dulbecco's Modified Eagle's Medium (DMEM) were from Hyclone (Logan, UT, USA). Acetic acid, ellipticine and sulforhodamine B (SRB) were from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade and were obtained from common sources. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Plant material and growing conditions

The experiment was carried out at the experimental field of the University of Thessaly, in Velestino (Greece) during the summer growing period of 2017. Purslane seeds were purchased from Hortus Sementi Srl. (Budrio, Italy) and sown directly in soil (May 10, 2017) with a distance of 30 cm and 5 cm between rows and within rows, respectively. The experimental included three plots of 5 × 5 m (25 × 3 = 75 m^2 in total). Before sowing, base dressing was applied at a rate of 100 kg/ha (10-10-10; N–P–K). A sprinkler irrigation was implemented for irrigation at regular intervals (once every week and after sowing and until 10 days before harvesting). Soil was sandy clay loam (38% sand, 36% silt, and 26% clay), with pH = 7.4 (1:1 soil/H_20) and organic matter content = 1.3%, while no pesticides or other agrochemicals were used. Harvest of seeds took place on July 11, 2017 by cutting plants with a harvesting knife, while for seed yield assessment samples from 3 random squares of 1 m^2 in each plot were considered. A second harvest was applied at the last fortnight of August 2017 from resprouting plants and seed yield was estimated as mentioned before.

After each harvest, batch samples of seeds were used for oil extraction, while seeds and the obtained seed-cakes from each extraction method (see section 2.3) were ground with an electric ball mill (PX-MFC 90 D, Kinematica AG, Switzerland), stored at −20 °C, and then were lyophilized. The lyophilized samples were put in plastic air-sealed bags and stored at −80 °C until further analysis.

2.3. Seed oil extraction methods

For seed oil extraction, seeds from both harvests were used according to the method previously described by the authors (Petropoulos et al., 2018). Briefly, two mechanical methods were implemented (hot and cold extraction). Oil extraction with mechanical pressing is widely used in vegetable oil industry and implements screw presses that force oil out of plant material. The main difference between hot and cold extraction methods is the use of a cooling module in the latter that allows oil extraction without increasing its temperature due to pressing forces. Hot extraction (HE) was performed with the small type screw oil press TäbyPressen Type 40 (Skeppsta Maskin AB, Örebro, Sweden), where oil temperature during the extraction ranged between 53 and 55 °C. The details of oil extraction procedure have been previously described by Petropoulos et al. (2018) (nozzle diameter: 6 mm; seed feeding: 5 kg/h; rotational speed: 78 rpm). Cold extraction was carried out with CE1 and CE2 methods. The CE1 method was carried out by Amygdalea S.A. (Volos, Greece) with the use of a Komet DD 85G twin screw vegetable oil expeller equipped with a jacketed cooling system (IBG Monforts Oekotec GmbH & Co.KG; Mönchengladbach, Germany) and according to the conditions previously described by Petropoulos et al. (2018) (nozzle diameter: 4 mm; seed feeding rate: 20 kg/h; rotational speed: 65 rpm). The CE2 method was performed by Giachanas - Cold Pressed Seed Oils S.A. (Evros, Greece) with the use of a Henan

6 YL-160 screw oil press (Henan VIC Machinery Co. Ltd; Henan, China). Oil temperatures during cold extraction 2 (CE2) were retained between 22 and 24 °C with the use of a jacketed cooling system, while seedcake dimensions were between 0.8 and 1.5 mm (this expeller is not provided with a nozzle for seedcake output), and seed feeding rate and rotational speed were adjusted at 33 kg/h and 130 rpm, respectively. All extraction methods were carried out in triplicate. Before analyses, oils from all methods were subjected to centrifugation twice ($3500 \times g$ for 10 min) and supernatants were put in dark vials for storage (room temperature and dark conditions) until analysis.

Extraction yield (%) of each method was calculated as the amount of oil (g) per total amount of seeds (g of dry basis (db)). Moreover, Soxhlet and ultrasonic assisted extractions were used to estimate the total amount of oil contained in ground seeds and seeds cakes and further to evaluate the oil recovery (%) for each of the tested mechanical methods (Arampatzis, Karkanis, & Tsiropoulos, 2019). In particular, ground raw seeds and seedcake samples (5.0 g) were extracted with 200 mL of hexane by refluxing in a Soxhlet apparatus, for 4–8 h (~32 cycles, using a Soxhlet extractor with the capacity of 250 mL) to assess oil recovery. The ultrasonic-assisted extraction of oil from seeds and seedcakes was carried out at room temperature by putting 1 g of powdered samples in 30 mL of hexane and then applying direct sonication (35 kHz probe, 285 W, and 29 °C) for 30 min (Elma TRANSSONIC T 460/H, Auckland, New Zealand) (Saleh et al., 2015). In both methods (ultrasonic-assisted and Soxhlet), the solvent was removed under reduced pressure for 1 h at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland), in order to estimate the oil content. The dried extracts were reconstituted in 25 mL methanol and stored until the analysis for fatty acids composition.

2.4. Chemical analyses

2.4.1. Nutritional value

The proximate composition (moisture, proteins, fat and ash) were analytically determined in lyophilized purslane samples (Zirbus GmbH Sublimator 4 × 5 × 6 freeze dryer) following the Official Methods of Analysis (AOAC, 2016) while total carbohydrates were calculated by difference. The lyophilization procedure was applied as follows: a) cooling of the product to a final temperature of – 55 °C at atmospheric pressure (1000 mbar) for 4 h, b) heating to a shelf temperature of 0 °C under a vacuum of 0.15 mbar for 18 h, c) heating to a shelf temperature of 25 °C under a vacuum of 0.15 mbar for 24 h. The crude protein content of the samples was determined following the macro-Kjeldahl method ($N \times 6.25$), the total fat using a Soxhlet apparatus with petroleum ether as the extraction solvent, and the ash content by sample incineration at 550 ± 15 °C. Total carbohydrates were calculated by its difference, using the following equation: Total carbohydrates (g/100 g db) = 100 − (g fat + g protein + g ash) (FAO, 1998a). Total energy was calculated according to the following equation: Energy (kcal/100 g db) = 4 × (g of proteins + g of carbohydrates) + 9 × (g of fat) or kJ/100 g db = 17 × (g of proteins + g of carbohydrates) + 37 × (g of fat) (FAO, 1998b).

2.4.2. Tocopherols

Tocopherols were determined in lyophilized purslane samples following a procedure previously described by Dias et al. (2013) and analyzed by high-performance liquid chromatography (HPLC) (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA). The fluorescence detector was programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards and tocol was used as the internal standard (IS). Quantification was based on calibration curves obtained from commercial standards of each compound using the internal standard (IS) methodology. The results were expressed in mg per 100 g of dry basis (db).

2.4.3. Free sugars

The composition of the free sugars was determined according to a methodology previously described by Barros et al. (2013b). HPLC mentioned above coupled to a refractive index detector (Knauer Smartline 2300) was the chosen methodology, the data were analyzed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). Identification was carried out by comparing authentic standard retention times, while quantification (DataApex, Podohradska, Czech Republic) was achieved using the IS method (melezitose as IS), by applying calibration curves constructed from authentic standards. Free sugars were further expressed in g/100 g db.

2.4.4. Organic acids

Samples (~1.5 g) were extracted by stirring with 25 mL of *meta*-phosphoric acid (25 °C at 150 rpm) for 45 min and subsequently filtered through Whatman no. 4 paper. After, the samples were filtered through 0.2 μm nylon filters and the analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) coupled to photodiode array detector (PDA) (Barros, Pereira, & Ferreira, 2013). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm × 4.6 mm i.d. - internal diameter.) thermostatted at 35 °C. Detection was carried out in a PDA using 215 nm as preferred wavelengths. For the quantitative analysis, calibration curves with known concentrations of commercial standards were constructed, and the organic acids present in the samples were determined by peak area comparison at 215 nm. The results were expressed in g/100 g db.

2.4.5. Fatty acids

Fatty acid content was determined after *trans*-esterification of the lipid fraction obtained through Soxhlet extraction as previously described by Obodai et al. (2017). Briefly, fatty acids were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (*v:v:v*), during at least 12 h in a bath at 50 °C and 160 rpm; then 3 mL of deionized water were added, to obtain phase separation; the FAME were recovered with 3 mL of diethyl ether by shaking in *vortex*, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a *vial* with Teflon, and before injection the sample was filtered with 0.2 μm nylon filter from Merck Millipore (Oeiras, Portugal). The analysis was carried out using a DANI model GC 1000 instrument equipped with a *split/splitless* injector set at 250 °C with a *split* ratio of 1:50, a flame ionization detector (FID) (GC-FID; DANI1000, Contone, Switzerland) set at 260 °C and a Zebron-Kame column (30 m × 0.25 mm ID × 0.20 μm *df*, Phenomenex, Lisbon, Portugal). The following oven temperature program was used: initial temperature of 100 °C, held for 2 min, then a 10 °C/min ramp to 140 °C, 3 °C/min ramp to 190 °C, 30 °C/min ramp to 260 °C and held for 2 min. Hydrogen was used as the carrier gas with a flow-rate of 1.1 mL/min, measured at 100 °C. Fatty acid identification and quantification was performed by comparing the relative retention times of FAME peaks from samples with those of standards (reference standard mixture 47,885-U, Sigma, St. Louis, MO, USA). The results were recorded and processed using the Software Clarity DataApex 4.0 Software (Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

2.5. Bioactive properties

2.5.1. Extraction methodology

A hydroethanolic extract was obtained by stirring the raw material (1 g) in an aqueous ethanolic solution (80% ethanol, *v/v*; 30 mL) at room temperature (25 °C) for 60 min. After filtration (Whatman no. 4), the extraction procedure was repeated once. Then, the solvent was recovered in order to obtain a dry extract: first, evaporation at 40 °C and reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland), and then freeze-drying (FreeZone 4.5, Labconco, Kansas

City, MO, USA).

2.5.2. Total phenolic compounds (TPC)

The total phenolic compounds were determined in the extracts (seeds and seedcakes) and in the oils, by applying the *Folin-Ciocalteu* methodology with some modification performed by the authors (Sarmento, Barros, Fernandes, Carvalho, & Ferreira, 2015). The absorbance was measured at 765 nm (AnalytikJena 200 spectrophotometer, Jena, Germany) and gallic acid was used as a reference compound to obtain the calibration curve (0.05–0.8 mM; $Y = 1.7967X + 0.0274$; $R^2 = 0.9997$). The results were expressed as mg of gallic acid equivalents (GAE) per gram of extract or mg GAE per g of oil.

2.5.3. Antimicrobial assays

To determine the antimicrobial activity, a panel of Gram-positive bacteria [*Bacillus cereus* (ATCC 11632), *Mariniluteicoccus flavus* (ATCC 10240), *Bacillus cereus* (food isolate) and *Listeria monocytogenes* (NCTC 7973)], Gram-negative bacteria [*Escherichia coli* (food isolate)] and fungi [*Aspergillus fumigatus* (ATCC 9197), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839) and *Trichoderma viride* (IAM 5061)] were used. The antimicrobial assay was carried out by the microdilution methodology as previously described by Petropoulos et al. (2019). The concentrations that completely inhibited bacterial growth (minimum inhibitory concentration: MICs) were determined by a colorimetric microbial viability assay, and MBC and MFC (minimum bactericidal concentration and minimum fungicidal concentration, respectively) were also calculated. Streptomycin, ampicillin, ketoconazole and bifonazole (Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls and 5% dimethyl sulfoxide (DMSO) was used as a negative control.

2.5.4. Hepatotoxic activity

Hepatotoxic activity was evaluated following the methodology previously described by the authors (Abreu et al., 2011), using a primary cell culture (PLP2) prepared from a porcine liver. The results were measured through the Sulforhodamine B method and results were expressed as GI_{50} values (concentration that inhibits 50% of cell growth). Ellipticine was used as positive control.

2.6. Statistical analysis

For each experimental plot, three samples of seeds were collected for seed yield evaluation (n = 3), while for chemical composition analyses and oil extraction methods three batch samples from the all collected seeds were also used (n = 3). For statistical analysis of chemical composition and bioactivity assays, three batch samples were analyzed for each extraction method, while all the performed assays were carried out in triplicate. Statistical analysis was performed with Statgraphics 5.1.plus (Statpoint Technologies, Inc., VA, USA). Since the outcome variables displayed normal distributions, data were evaluated by one-way ANOVA, while the means of values were compared with Tukey's HSD test. All significance level was set at less than 0.05.

3. Results and discussion

The results regarding nutritional value and the content of sugars, tocopherols and organic acids are presented in Table 1. Seeds and seedcakes obtained from CE2 method contained the highest moisture content, while seeds had also the highest fat content and energy. Similar results have been reported by Vioque, Pastor, and Vioque (1994) who studied seed oils from several wild species and obtained yields within the same range of our study (18.2%). Seedcakes obtained from hot extraction (HE) had the highest fat content, which also indicates the lower oil extraction yield of hot extraction method comparing to cold extraction methods (45.7%, 61.1% and 63.1% for HE, cold extraction 1 (CE1) and cold extraction 2 (CE2) methods, respectively) since more oil remained in seedcakes after the extraction procedure (Fig. 1a). Protein and ash contents were the highest for seedcakes obtained from CE2 method, whereas CE1 had the highest carbohydrates content. Extraction method has a significant effect ($p \leq 0.05$) on seed oil extraction yield from oleaginous plants and all the processing steps have to be optimized depending on seed characteristics in order to achieve the highest extraction efficiency. According to Ionescu et al. (2014), mechanical extraction or pressing methods for oil extraction are commonly used in oil the production industry as the most cost effective methods; however, several parameters have to be considered for higher oil extraction efficiency, including screw or rotational speed, restriction size or nozzle diameter, seed morphology (size, shape), seed moisture content, temperature and pressure applied during the pressing process. The differences in pressing parameters between the methods tested in our study could explain the observed differences in oil extraction yield and consequently in the fat and protein content of the obtained seedcakes. Therefore, customized screw presses with specifications optimized for very small seeds such as purslane seed should be considered to increase the efficiency of oil extraction (Ionescu et al., 2015).

Sucrose was the only detected sugar and the highest content was detected in seeds, followed by seedcakes from CE2, CE1 and HE methods (Table 1). To the best of our knowledge, this is the first report for free sugars composition in purslane seeds and seedcakes. However, sucrose has been reported as the main detected soluble sugar in cardoon and rapeseed seeds (Hill, Morley-Smith, & Rawsthorne, 2003; Petropoulos et al., 2019), since hexose sugars content decreases and sucrose content increases during seed development to provide the adequate energy for the developing embryo through the inversion of sucrose to glucose and fructose (Hill et al., 2003). Regarding the tocopherols content, only two vitamin E isomers were detected in the tested samples, namely α- and γ-tocopherol, with the latter being the most abundant, especially in seeds where the highest content was observed (Table 1). Similar results have been reported for okra seeds where α- and γ-tocopherol were also the only vitamin E isomers detected (Petropoulos et al., 2017), whereas to the best of our knowledge no previous studies for tocopherol content in purslane seeds and seedcakes have been reported. Oxalic acid was the only organic acid detected in seeds and seedcakes obtained from different extraction methods, while traces of malic acid were also recorded (Table 1). The highest content of oxalic acid was observed in seedcakes obtained from CE1, followed by seedcakes from HE, whereas seedcakes from CE2 and seeds contained low amounts of oxalic acid and total organic acids content. To the best of our knowledge, this is the first report for organic acid composition in purslane seeds and seedcakes.

Seed yield was 1100 kg/ha for the 1st harvest and 125.3 kg/ha for the 2nd harvest, resulting in a total of 1225.3 kg/ha (**data not shown**). A previous study by Ghamari, Shafagh Kolvanagh, Sabaghpour, and Dabbagh Mohammadi Nassab (2016) reported significantly lower seed yield of purslane comparing to our study (355.3–605.1 kg/ha), which could be attributed partly to lower plant densities and/or differences in the genotypes tested (wild ecotypes comparing to improved cultivars). Oil seed content in the 2nd harvest was considerably low (17.6% and 10.6% in the 1st and 2nd harvest, respectively) suggesting that extending the growing period by performing a 2nd harvest would not result in a significant increase of total oil yield per hectare. However, taking into account the short growing period from sowing to seed harvesting (60–75 days after sowing) it worth's investigating the application of two consecutive growing seasons, especially in warmer regions where high temperatures early in the spring allow for early sowing providing the adequate time for one more late sowing.

Fatty acids composition of the studied seeds, seed oils and seedcakes is presented in Table 2. In all the tested materials, the main detected fatty acids were two PUFA, namely linoleic and α-linolenic acids, followed by palmitic, oleic and stearic acids although significant ($p \leq 0.05$) differences in fatty acids were observed. In particular, seed oils obtained with CE2 contained the highest amounts of linoleic acid

Table 1
Nutritional value (g/100 g db), energetic value (kcal/100 g db), and composition in sugars (g/100 g db), tocopherols (mg/100 g db) and organic acids (g/100 g db) of the studied purslane seeds and seedcakes (mean ± SD).

	Seeds	Seedcake (HE)[a]	Seedcake (CE1)	Seedcake (CE2)
Energy (kj/100 g db)	1920 ± 4a	1779 ± 6b	1734 ± 2c	1718 ± 1d
Energy (kcal/100 g db)	459 ± 1a	425.1 ± 0.1b	414.5 ± 0.5c	410.6 ± 0.2d
Moisture content (%, wb)	9.65 ± 0.03a	6.58 ± 0.03c	8.22 ± 0.11b	9.56 ± 0.04a
Fat (g/100 g db)	15.03 ± 0.06a	8.16 ± 0.06b	5.85 ± 0.09c	5.55 ± 0.01d
Proteins (g/100 g db)	27.58 ± 0.01d	30.8 ± 0.3c	31.0 ± 0.2b	31.20 ± 0.03a
Ash (g/100 g db)	4.0 ± 0.1b	3.92 ± 0.05c	3.68 ± 0.05d	4.27 ± 0.06a
Carbohydrates (g/100 g db)	53.43 ± 0.04d	57.2 ± 0.3c	59.4 ± 0.1a	58.98 ± 0.01b
Energy (kcal/100 g db)	459 ± 1a	425.1 ± 0.1b	414.5 ± 0.5c	410.6 ± 0.2d
Sucrose (g/100 g db)	6.48 ± 0.06a	3.85 ± 0.01d	4.0 ± 0.1c	6.36 ± 0.02b
Total Sugars (g/100 g db)	6.48 ± 0.06a	3.85 ± 0.01d	4.0 ± 0.1c	6.36 ± 0.02b
α-Tocopherol (mg/100 g db)	0.016 ± 0.001b	0.026 ± 0.002a	0.012 ± 0.001c	0.011 ± 0.002c
γ-Tocopherol (mg/100 g db)	3.7 ± 0.2a	2.78 ± 0.05b	1.96 ± 0.02c	1.99 ± 0.09c
Total Tocopherols (mg/100 g db)	3.7 ± 0.2a	2.81 ± 0.05b	1.97 ± 0.01d	2.00 ± 0.08c
Oxalic acid (g/100 g db)	0.520 ± 0.005d	0.740 ± 0.006b	0.760 ± 0.001a	0.530 ± 0.001c
Malic acid (g/100 g db)	tr	tr	tr	tr
Total organic acids (g/100 g db)	0.520 ± 0.005d	0.740 ± 0.006b	0.760 ± 0.001a	0.530 ± 0.001c

Different Latin letters in the same row refer to significant differences between seedcakes and seeds according to Tukey's HSD test ($p \leq 0.05$).
[a] HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2; tr: traces; db: dry basis; wb: wet basis.

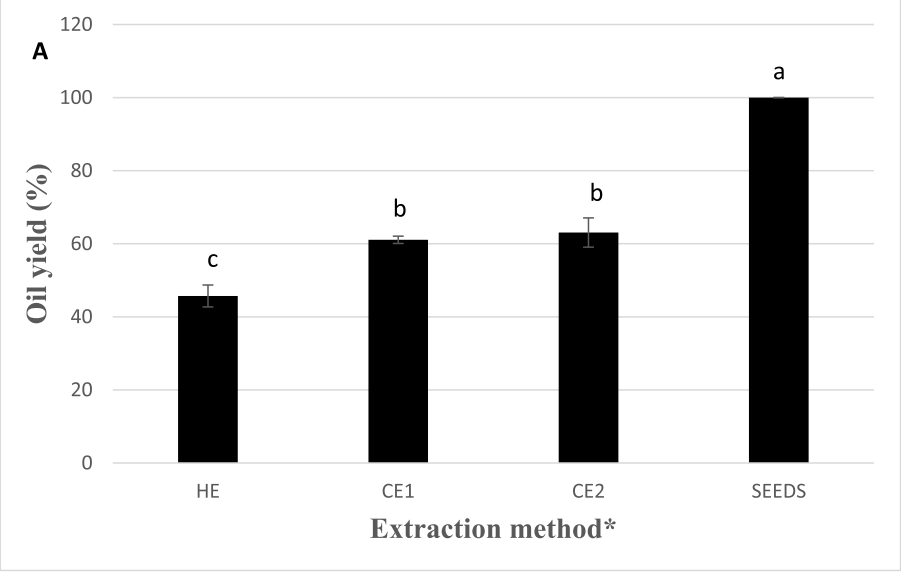

Fig. 1A. Oil yield (%) in relation to extraction method. Each extraction methods is compared with raw seeds which is considered to contain 100% of oil. Different Latin letters indicate differences between the tested extraction methods according to Tukey's HSD test ($p \leq 0.05$).
*HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2.

(49.77%), whereas seed oils obtained with HE were the most abundant in α-linolenic acid. On the other hand seedcakes obtained with HE, CE1 and CE2 were the most abundant in palmitic, oleic and stearic acids, respectively. Similar results have been reported by Stroescu, Stoica-Guzun, Ghergu, Chira, and Jipa (2013), Mousavi and Niazmand (2017), and Kavosi et al. (2018) who also detected higher amounts of linoleic than α-linolenic acid in oils obtained with solvent extraction regardless of the solvent used. Moreover, Delfan-Hosseini, Nayebzadeh, Mirmoghtadaie, Kavosi, and Hosseini (2017) evaluated three procedures for purslane seed oil extraction, namely solvent, cold press and microwave assisted cold press extraction and also observed a higher amount of linolenic acid than α-linolenic acid. In contrast, Vioque et al. (1994) reported a different fatty acids profile in seed oils with palmitic and stearic acid being the most abundant fatty acids, while Sodeifian, Ardestani, Sajadian, and Moghadamian (2018) detected higher amounts of α-linolenic than linoleic acid in oils obtained with supercritical fluid extraction. These differences in the literature reports indicate that extraction method has a significant effect on fatty acids profile and optimization of extraction protocols is needed to obtain oils of the desired quality (Stroescu et al., 2013). PUFA were the most abundant class of fatty acids in all the tested materials, mainly due to the high content of linoleic and α-linolenic acids, followed by saturated (SFA) and MUFA (Table 2). Moreover, the ratio of PUFA/SFA and n6/n3 were higher than 0.45 and lower than 4.0, respectively for all the tested materials indicating a high nutritional value (Guil, Torija, Giménez, & Rodriguez, 1996). Seed oils obtained with HE and CE1 methods showed lower n6/n3 ratio values due to the higher content in α-linolenic acid comparing to CE2 method.

Seed pre-treatments may have a beneficial effect on oil extraction yield and extraction process efficiency, although special attention should also be given on the composition of the obtained oils (Uquiche, Jeréz, & Ortíz, 2008; Yoshida, Tomiyama, Hirakawa, & Mizushina, 2006). In our study, the oil extraction efficiency and the effect of ultrasound-assisted and Soxhlet extraction methods on fatty acid composition of seeds and seedcakes were tested. The extraction for 8 h with a Soxhlet apparatus using hexane as a solvent gave the best results in terms of oil extraction yield for all the tested materials, except for the case of seedcakes obtained with CE2 method where no differences between the defatting methods were observed (Fig. 1b). Fatty acid composition was not significantly ($p > 0.05$) affected ($p \leq 0.05$) by the applied defatting methods, following similar trends as shown in Table 2 for the same tested materials, and only slight fluctuations in fatty acids

Table 2
Fatty acids composition (relative percentage, %) of the studied purslane seeds, seedcakes and seed oils (mean ± SD).

	Seeds	Seedcake (HE)[a]	Seedcake (CE1)	Seedcake (CE2)	Seed oils (HE)	Seed oils (CE1)	Seed oils (CE2)
C6:0	0.011 ± 0.001c	0.015 ± 0.001b	0.025 ± 0.001a	0.009 ± 0.001d	nd	nd	nd
C8:0	0.003 ± 0.001d	0.008 ± 0.001c	0.011 ± 0.001b	0.012 ± 0.001a	nd	nd	nd
C10:0	0.002 ± 0.001d	0.004 ± 0.001c	0.006 ± 0.001a	0.005 ± 0.001b	nd	nd	nd
C12:0	0.014 ± 0.001d	0.022 ± 0.001c	0.025 ± 0.001a	0.024 ± 0.001b	nd	nd	nd
C14:0	0.056 ± 0.002e	0.064 ± 0.003d	0.067 ± 0.001c	0.079 ± 0.001a	0.048 ± 0.001g	0.053 ± 0.001f	0.071 ± 0.002b
C15:0	0.033 ± 0.001d	0.036 ± 0.001b	0.035 ± 0.001c	0.041 ± 0.001a	nd	nd	nd
C16:0	13.73 ± 0.08e	14.6 ± 0.1a	14.2 ± 0.1b	14.2 ± 0.2b	14.13 ± 0.03d	14.18 ± 0.04c	12.26 ± 0.09f
C16:1	0.102 ± 0.001d	0.104 ± 0.008b	0.102 ± 0.001d	0.103 ± 0.002c	0.085 ± 0.003e	0.085 ± 0.002e	0.11 ± 0.01a
C17:0	0.110 ± 0.001d	0.119 ± 0.001b	0.120 ± 0.004a	0.113 ± 0.005c	0.098 ± 0.001e	0.097 ± 0.003f	0.089 ± 0.001g
C18:0	3.12 ± 0.04d	3.14 ± 0.01c	3.23 ± 0.01b	3.25 ± 0.02a	3.078 ± 0.006f	3.10 ± 0.01e	3.07 ± 0.01f
C18:1n9	12.63 ± 0.05b	12.19 ± 0.07d	12.84 ± 0.02a	12.4 ± 0.2c	5.78 ± 0.01f	5.78 ± 0.01f	8.85 ± 0.02e
C18:2n6	33.80 ± 0.01g	34.51 ± 0.05f	34.61 ± 0.06e	34.74 ± 0.02d	35.44 ± 0.01b	35.13 ± 0.03c	49.77 ± 0.08a
C18:3n3	34.64 ± 0.01c	33.4 ± 0.1d	32.83 ± 0.06f	33.02 ± 0.01e	39.67 ± 0.03a	39.57 ± 0.05b	24.18 ± 0.05g
C20:0	0.69 ± 0.01c	0.69 ± 0.03c	0.74 ± 0.01b	0.75 ± 0.01a	0.631 ± 0.001e	0.645 ± 0.005d	0.454 ± 0.005f
C20:1	0.124 ± 0.004c	0.15 ± 0.01a	0.109 ± 0.003e	0.136 ± 0.004b	0.094 ± 0.003g	0.096 ± 0.001f	0.112 ± 0.001d
C20:3n3 + C21:0	0.037 ± 0.001b	0.018 ± 0.001d	0.051 ± 0.001a	0.021 ± 0.001c	nd	nd	nd
C20:5n3	0.004 ± 0.001c	0.004 ± 0.001c	0.006 ± 0.001b	0.008 ± 0.001a	nd	nd	nd
C22:0	0.78 ± 0.01d	0.85 ± 0.02c	0.91 ± 0.02b	0.94 ± 0.01a	0.257 ± 0.001e	0.26 ± 0.01e	0.138 ± 0.002f
C22:2	nd	nd	nd	nd	0.43 ± 0.03c	0.44 ± 0.02b	0.690 ± 0.003a
C23:0	0.034 ± 0.002b	0.029 ± 0.001c	0.028 ± 0.001d	0.035 ± 0.001a	0.26 ± 0.01f	0.27 ± 0.02e	0.218 ± 0.009g
C24:0	0.089 ± 0.004c	0.091 ± 0.001b	0.108 ± 0.001a	0.089 ± 0.001c	nd	nd	nd
Total SFA (% of total FA)	18.66 ± 0.05d	19.62 ± 0.09a	19.5 ± 0.1b	19.6 ± 0.2a	18.51 ± 0.03e	18.71 ± 0.07c	16.3 ± 0.1f
Total MUFA (% of total FA)	12.86 ± 0.06b	12.45 ± 0.09d	13.05 ± 0.02a	12.7 ± 0.2c	5.96 ± 0.01f	5.96 ± 0.01f	9.07 ± 0.03e
Total PUFA (% of total FA)	68.49 ± 0.01d	67.9 ± 0.2e	67.5 ± 0.1f	67.78 ± 0.02	75.53 ± 0.02a	75.33 ± 0.06b	74.6 ± 0.1c
PUFA/SFA	3.67 ± 0.03d	3.46 ± 0.05e	3.46 ± 0.1e	3.46 ± 0.01	4.09 ± 0.02b	4.03 ± 0.06c	4.58 ± 0.1a
n6/n3**	0.97 ± 0.01d	1.03 ± 0.05c	1.05 ± 0.06b	1.05 ± 0.02b	0.90 ± 0.01e	0.90 ± 0.03e	2.09 ± 0.08a

[a] HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2; nd: not detected.

content were observed (Table S1; Supplementary Material). Similarly to this study, Samaram, Mirhosseini, Tan, and Ghazali (2013) reported differences in oil extraction yield for papaya seeds when ultrasound-assisted and solvent extraction were implemented without fatty acids composition being affected. In addition, in the same study it was suggested that prolonging extraction time up to 12 h increased oil extraction yield for solvent extraction, whereas ultrasound-assisted extraction resulted in similar to solvent extraction yields in only 30 min (Samaram et al., 2013). Moreover, Gutte, Sahoo, and Ranveer (2015) studied the effect of ultrasounds treatment on flaxseed oil and reported an increase in oil extraction yield by 11.5% without significant effects on the content of α-linolenic acid. However, they mentioned that optimization of the extraction process parameters is needed in order to increase oil extraction yields (Gutte et al., 2015). This efficiency of ultrasound-assisted extraction could be attributed to acoustic cavitation which may disrupt cell walls as well as to agitation of solvent allowing the better penetration of solvents into seeds (Chemat, Vian, & Cravotto, 2012).

Total phenolic compounds (TPC) content of the studied materials is presented in Table 3. Seedcakes contained higher amounts of TPC comparing to seeds, especially seedcakes obtained from CE2 method which had the highest TPC content (32.63 mg GAE/g extract), indicating the potential use of purslane seed oil byproducts as sources of bioactive compounds. TPC content in seed oils was also varied with HE

Table 3
Total phenolic compounds of the studied purslane seeds, seedcakes and seed oils expressed as mg of Gallic acid equivalents (mean ± SD).

	Folin-ciocalteu assay (mg GAE/g extract)
Seedcake (HE)[a]	25.9 ± 0.6b
Seedcake (CE1)	23.8 ± 0.4c
Seedcake (CE2)	32.63 ± 0.02a
Seeds	21.0 ± 0.5d
	Folin-ciocalteu assay (mg GAE/g oil)
Seed oil (HE)	0.23 ± 0.01a
Seed oil (CE1)	0.15 ± 0.01b
Seed oil (CE2)	0.22 ± 0.01a

Different Latin letters in the same column refer to significant differences according to Tukey's HSD test ($p \leq 0.05$).
[a] HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2.

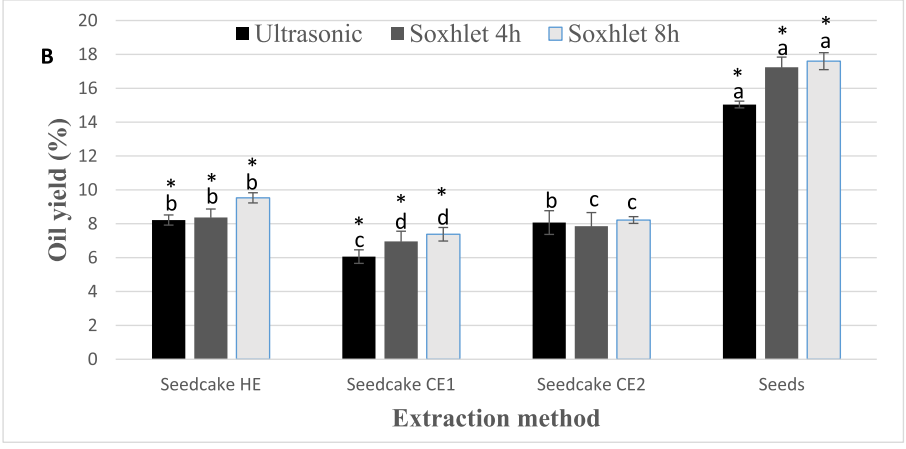

Fig. 1B. Oil yield (%) in relation to extraction and defatting method. Each extraction and defatting method is compared with raw seeds which is considered to contain 100% of oil. Different Latin letters indicate differences between the tested materials for the same defatting method according to Tukey's HSD test ($p \leq 0.05$). The asterisk symbol indicates differences between the defatting methods for the same material according to Tukey's HSD test ($p \leq 0.05$).
¥HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2.

Table 4
Antibacterial activity of the studied purslane seeds, seedcakes and seed oils (MIC and MBC mg/mL).

Compounds	*S. aureus* (ATCC 11632)		*E. coli* (food isolate)		*M. flavus* (ATCC 10240)		*B. cereus* (food isolate)		*L. monocytogenes* (NCTC 7973)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Streptomycin (positive control)	0.006	0.012	0.10	0.20	0.20	0.30	0.10	0.20	0.20	0.30
Ampicillin (positive control)	0.012	0.025	0.20	–	0.30	0.30	0.25	0.40	0.40	0.50
Seedcake (HE)[a]	2.00	4.00	4.00	8.00	2.00	4.00	1.00	2.00	4.00	8.00
Seedcake (CE1)	4.00	8.00	4.00	8.00	2.00	4.00	1.00	2.00	4.00	8.00
Seedcake (CE2)	2.00	4.00	4.00	8.00	2.00	4.00	1.00	2.00	4.00	8.00
Seeds	1.00	2.00	1.00	2.00	0.25	0.50	0.50	1.00	1.00	2.00
Seed oil (HE)	4.00	8.00	2.00	4.00	2.00	4.00	2.00	4.00	2.00	4.00
Seed oil (CE1)	4.00	8.00	2.00	4.00	2.00	4.00	2.00	4.00	4.00	8.00
Seed oil (CE2)	2.00	4.00	1.00	2.00	2.00	4.00	1.00	2.00	2.00	4.00

-: No activity; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration.
[a] HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2.

and CE2 methods having the highest content (0.23 and 0.22 mg GAE/g oil). Sodeifian et al. (2018) reported a higher TPC content in purslane seed oil obtained with supercritical fluid and solvent extraction (1.73 and 1.67 mg GAE/g oil, respectively), whereas Delfan-Hosseini et al. (2017) found TPC values similar to our study for solvent-extracted seed oils of purslane (0.19 mg GAE/g oil). Moreover, in a previous studies of our team with cardoon seed oils obtained with two methods corresponding to HE and CE1 methods of the present study, TPC content of HE method was also higher than that of CE1 method (Petropoulos et al., 2018). These differences in the literature reports could be attributed to different extraction parameters, as well as to genotypic differences of the studied materials.

Antibacterial and antifungal activity of purslane seeds, seed oils and seedcakes are presented in Tables 4 and 5. All the studied materials showed low efficacy against the tested bacteria in comparison to the positive controls (streptomycin and ampicillin) (Table 4). However, it worth's mentioning the higher antibacterial activity of seeds comparing to seedcakes and seed oils. Similarly, positive controls (bifonazole and ketoconazole) had a higher antifungal activity than the studied materials, while seeds and seedcakes were more efficient against *Aspergillus niger*, *A. versicolor*, and *Trichoderma viride* comparing to seed oils (Table 5). According to Tayel et al. (2018), purslane seed extracts showed moderate antibacterial activity against normal strains of *Staphylococcus aureus* and no activity against methicillin resistant strains, while Bakkiyaraj and Pandiyaraj (2011) reported the antibacterial activity of purslane leaf extracts against *Bacillus subtilis*, *S. aureus* and *Pseudomonas aeruginosa* attributed to the phytochemicals content of plant tissues.

Regarding the cytotoxicity of seeds, seed oil and seedcakes against PLP2 cells, the results showed no cytotoxic effects for seedcakes, whereas seed oils from CE1 had the lowest GI_{50} values (204.7 ± 13.7 μg/mL), followed by HE (204 ± 9 μg/mL), CE2 (221 ± 15 μg/mL) and seeds (259.7 ± 9.5 μg/mL) (Table 6). To the best of our knowledge, no reports regarding the cytotoxic effects of purslane seeds and seed products are available in the literature, since most of the existing reports refer to the aerial parts of the plant (Lei, Li, Liu, Zhang, & Liu, 2015; Lim & Quah, 2007). According to Eidi, Mortazavi, Moghadam, and Mardani (2015) and Farshori et al. (2014) who studied the cytotoxic effects of purslane aerial parts extract, the potency of extracts could be attributed to various phytochemicals present in plant tissues, including seeds, seed oils and seedcakes.

Table 6
Cytotoxicity of the studied purslane seeds, seedcakes and seed oils (GI_{50} values μg/mL).

	Hepatotoxicity PLP2 (non-tumor cells)
Purslane seeds	259.7 ± 9.5a
Seedcake (HE)[a]	> 400
Seedcake (CE 1)	> 400
Seedcake (CE 2)	> 400
Seed oil (HE)	204 ± 9c
Seed oil (CE1)	204.7 ± 13.7c
Seed oil (CE2)	221 ± 15b

Different Latin letters in the same column refer to significant differences between seedcakes and seeds according to Tukey's HSD test ($p \leq 0.05$).
[a] HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2.

4. Conclusion

In conclusion, the tested materials proved to be promising sources of linoleic and α-linolenic acid, whereas none of the tested materials

Table 5
Antifungal activity of the studied purslane seeds, seedcakes and seed oils (MIC and MFC mg/mL).

Compounds	*Aspergillus fumigatus* (ATCC 9197)		*Aspergillus niger* (ATCC 6275)		*Aspergillus versicolor* (ATCC 11730)		*Penicillium funiculosum* (ATCC 36839)		*Trichoderma viride* (IAM 5061)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bifonazole (positive control)	0.15	0.20	0.15	0.20	0.13	0.17	0.20	0.25	0.10	0.20
Ketoconazole (positive control)	0.20	0.50	0.20	0.50	0.20	0.47	0.20	0.50	0.20	0.30
Seedcake (HE)[a]	2.00	4.00	1.00	2.00	0.50	1.00	1.00	2.00	0.50	1.00
Seedcake (CE1)	1.00	2.00	1.00	2.00	0.50	1.00	0.50	1.00	0.50	1.00
Seedcake (CE2)	0.50	1.00	1.00	2.00	0.50	1.00	1.00	2.00	0.50	1.00
Seeds	0.50	1.00	1.00	2.00	0.50	1.00	1.00	2.00	0.50	1.00
Seed oil (HE)	2.00	4.00	4.00	8.00	2.00	4.00	2.00	4.00	4.00	8.00
Seed oil (CE1)	2.00	4.00	2.00	4.00	2.00	4.00	1.00	2.00	1.00	2.00
Seed oil (CE2)	2.00	4.00	2.00	4.00	4.00	8.00	2.00	4.00	4.00	8.00

-: No activity; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration; MFC: Minimal fungicidal concentration.
[a] HE: Hot extraction; CE1: Cold extraction 1; CE2: Cold extraction 2.

showed good results in terms of antibacterial and antifungal properties. In addition, the results of the present study demonstrated that the application of different mechanical extraction methods may have a significant effect ($p \leq 0.05$) on oil extraction yield from purslane seeds, while a significant effect ($p \leq 0.05$) of mechanical extraction methods on omega-6 and omega-3 fatty acids content of seed oils was also observed. Finally, considering the very small size of purslane seeds further research is needed to study the various parameters involved in the extraction process that could affect not only oil yield but also oil quality and chemical composition of the obtained oils and byproducts.

CRediT authorship contribution statement

Spyridon A. Petropoulos: Supervision, Writing - original draft, Writing - review & editing. **Ângela Fernandes:** Formal analysis, Data curation, Methodology. **Dimitrios A. Arampatzis:** Formal analysis. **Nikolaos G. Tsiropoulos:** Supervision. **Jovana Petrović:** Formal analysis. **Marina Soković:** Supervision. **Lillian Barros:** Writing - review & editing, Writing - original draft, Formal analysis, Data curation, Methodology. **Isabel C.F.R. Ferreira:** Funding acquisition, Supervision, Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109099.

Different Latin letters in the same row refer to significant differences between seeds, seedcakes, and seed oils according to Tukey's HSD test ($p \leq 0.05$).

C6:0 caproic acid; C8:0 caprylic acid; C10:0 capric acid; C12:0 lauric acid; C14:0 myristic acid; C15:0 pentadecanoic acid; C16:0 palmitic acid; C16:1 palmitoleic acid; C17:0 heptadecanoic acid; C18:0 stearic acid, C18:1n9c oleic acid, C18:2n6c linoleic acid, C18:3n3 α-linolenic acid, C20:0 arachidic acid; C20:1 eicosenoic acid; C20:3n3 eicosatrienoic acid; C20:5n3 eicosapentaenoic acid; C21:0 heneicosylic acid; C22:0 behenic acid; C22:2 docosadienoic acid; C23:0 tricosylic acid; C24:0 lignoceric acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n6/n3: ratio of omega-6/omega-3 fatty acids.

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