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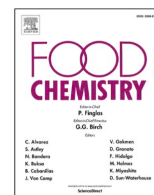

Chemical characterization of carob seeds (*Ceratonia siliqua* L.) and use of different extraction techniques to promote its bioactivity

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ABSTRACT

Nowadays, the use of carob (*Ceratonia siliqua* L.) is reduced to its seeds. In fact, the food additive E410, locust bean gum, is obtained from its endosperm. However, the available information regarding the bioactive potential of carob seeds is limited and, often, with poorly established terminologies, not allowing the reader to know if the studied samples included seedless or seeded samples. So, the present study intended to carry out a chemical characterization of carob seeds focused on their bioactive compounds and test their bioactive properties. Carob seeds proved to be a source of tocopherols and organic acids, including phenolic compounds. Its antioxidant potential was demonstrated *in vitro*, as well as its antimicrobial capacity. This work proves that carob seeds have other functions in addition to those associated with E410. Thus, another potential can be given to the locust bean seed, namely the function of food preservative.

1. Introduction

The carob tree (*Ceratonia siliqua* L.) is an evergreen plant that belongs to the Fabaceae (legume) family. The fruits from this species, native to the Mediterranean region and the Middle East, consist of elongated, straight, or curved compressed pods, of a bright dark brown color. So, the main parts of the carob are the pulp (90%), and seeds (8–10%) constituted by bark, endosperm, and germ (Mariod, 2019). *C. siliqua* has been used both in folk medicine (Li, Kunz, & Chemat, 2019) and as food (Li, Kunz, & Chemat, 2019). Carob pods present an average of 40–60% of carbohydrates, being essentially composed of sucrose (32–38%), fructose (5–7%), and glucose (5–6%). They also contain a considerable percentage of polyphenolic compounds, especially tannins (18–20%), dietary fibers (27–50%), and minerals (potassium, sodium, iron, copper, manganese, and zinc). On the other hand, they are low in protein (3–4%) and lipids (0.4–0.8%) (Rtibi et al., 2017). Although all the fruit can be used, today, the food application of carob pods is limited to its seeds (Nasar-Abbas et al., 2016). In fact, the so-called locust bean gum, obtained from the seed endosperm, is currently used as a food additive (E410), having a thickener, stabilizer, flavoring, and emulsifying function (Mortensen et al., 2017). Due to these properties, the seeds are also used in other applications for the pharmaceutical (Nayak, Hasnain, & Pal, 2019), textile (Abd-El-Thalouth, 2011), and cosmetic industries (Domloge, Portolan, Clement, & Botto, 2018). Although with potential application for several culinary options, the carob pulp has been used mainly for animal feed (Inserra et al., 2015).

According to FAO data (FAOSTAT, 2020), Portugal is the leading producer of carob (considering average values from 1994 to 2018), with an average annual production of 40,007.88 tonnes, followed by Italy (30,992.48 tonnes) and Morocco (22,462.72 tonnes), with Tunisia in ninth place with an average annual production of 938.4 tonnes.

Based on previously published results, the objective of this study was to carry out the chemical characterization (in terms of compounds with biological activity) of Tunisian locust bean seeds, as well as to study their bioactive properties, using different extraction techniques. For that, the seeds were submitted to a first screening, evaluating their content in tocopherols and organic acids. Afterward, the seeds were extracted using two different methods: a conventional methodology (maceration), and an innovative/greener technique (ultrasound-assisted extraction). For both methodologies, different proportions of solvents

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were used to obtain the best extraction conditions. The antioxidant, antimicrobial, cytotoxic and anti-inflammatory properties of the obtained extracts were assessed, and finally, the phenolic compounds profile from the most promising samples (those that showed better results in the bioactivity assays) was analyzed.

2. Materials and methods

2.1. *Ceratonia siliqua* L. samples

Ceratonia siliqua L. pods were collected in August 2019 in Téboulba, Monastir (Tunisia). The samples were washed to remove impurities. Afterward, the seeds were separated from the pulp and dried in an oven at 40 °C. Finally, the carob seeds were reduced to a fine dried powder (≈ 20 mesh), mixed to obtain homogeneous samples, and stored protected from light until further use.

2.2. Chemical characterization of *Ceratonia siliqua* L.

2.2.1. Tocopherols composition

The tocopherol composition was determined according to Heleno, Barros, Sousa, Martins, & Ferreira, 2010. Briefly, before the extraction procedure, butylhydroxytoluene (BHT; 10 mg/mL; 100 µL) and the internal standard tocol (2 mg/mL; 250 µL) were added to the samples. Then, the samples (≈ 500 mg) were extracted by vortex mixing with methanol (4 mL) and hexane (4 mL). A saturated NaCl aqueous solution (2 mL) was added and the mixture was homogenized. After centrifugation of the mixture (5 min, 4000 g), the upper layer, corresponding to the hexane fraction, was transferred to a 25 mL amber vial, and the samples were re-extracted twice with hexane. The combined extracts were dehydrated with anhydrous sodium sulfate, taken to dryness under a nitrogen stream, and stored at −20 °C. Tocopherol extracts were redissolved in 2 mL of *n*-hexane, filtered (0.22 µm disposable LC filter disk), and transferred to an amber injection *vial*.

The analysis was performed using a high-performance liquid chromatography (HPLC) system (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA). The tocopherol identification was performed by chromatographic comparisons with authentic standards and the quantification was based on the fluorescence signal response of each standard., using the internal standard method. The results were expressed in µg per 100 g (dry basis).

2.2.2. Organic acids composition

The organic acids profile was assessed using the method previously described Pereira, Barros, Carvalho, & Ferreira, 2013. The samples (≈ 1.5 g) were extracted at room temperature by adding 25 mL of metaphosphoric acid, and placed under magnetic stirring (150 rpm) for 20 min. The extracts were filtered (Whatman No 4 paper), and before the analysis the samples were filtered through 0.22 µm nylon filters and transferred to HPLC *vials*. The analysis was carried out by ultra-fast liquid chromatography (UFLC) coupled to photodiode array detector (PDA). The organic acids were quantified by comparison of the peak area recorded at 215 nm (245 nm for ascorbic acid) with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g (dry basis).

2.3. *Ceratonia siliqua* L. extraction

Carob seeds were extracted by maceration (ME) and ultrasound-assisted extraction (UAE). For both extraction methods, water and ethanol were chosen as solvents, with four different proportions being used: i) ethanol:water (25:75; v/v); ii) ethanol:water (50:50; v/v); iii) ethanol:water (75:25; v/v); and iv) 100% water.

For ME the dried powdered samples (1 g) were placed in a beaker with 30 mL of each of the four solvents, under magnetic stirring (150 rpm) for 1 h at room temperature. After this period, the extracts were filtered (Whatman No 4 paper) and the extraction procedure was repeated with an additional portion of the solvent. The obtained extracts were combined, the ethanol was removed (rotary evaporator Büchi R-210, Flawil, Switzerland) and the residual aqueous phase was frozen and lyophilized (freeze 4.5 FreeZone model 7750031, Labconco, Kansas City, MO, USA).

The UAE was carried out in an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA), based on a methodology previously optimized by Rached et al. (2016). The dried powdered samples (3 g) were extracted with 100 mL of each of the four solvents by the ultrasonic device at 375 W for 10 min. The extracts obtained were filtered (Whatman No 4 paper) and, as for the ME, the ethanol was removed, and the residual aqueous phase was frozen and lyophilized.

2.4. Bioactive properties of *Ceratonia siliqua* L.

The lyophilized extracts previously obtained were re-dissolved in water for the antioxidant activity assay TBARS (stock solution 10 mg/mL); in 5% solution of DMSO in distilled water for the antimicrobial activity assays (stock solution100 mg/mL); and in distilled water to test the cytotoxic and anti-inflammatory activities (stock solution 8 mg/mL). Subsequently, these solutions were diluted successively to obtain the concentrations necessary to perform the experimental work.

2.4.1. Antioxidant activity

The antioxidant capacity of the extracts was evaluated following two *in vitro* assays: i) ability to inhibit the formation of thiobarbituric acid reactive substances (TBARS assay) in brain cell homogenates; and ii) oxidative hemolysis inhibition (OxHLIA assay).

TBARS assay. Porcine (*Sus domesticus*) brains were obtained from official slaughtered animals, dissected, and homogenised with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate that was centrifuged at 3000g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the obtained extracts (0.2 mL) at different concentrations in the presence of $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by TBA (2%, w/v, 0.38 mL), and the mixture was heated at 80 °C for 20 min. After centrifugation (3000 g, 10 min), the colour intensity of the supernatant was measured by its absorbance at 532 nm. Trolox (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control. The results were expressed as EC_{50} values (µg/mL), *i.e.*, extract concentration providing 50% of antioxidant activity. This concentration (EC_{50}) was calculated by interpolation from the graph of TBARS formation inhibition percentage against sample concentration (Barreira, Rodrigues, Carvalho, & Ferreira, 2013).

Antihaemolytic activity. The antihaemolytic activity of the extracts was evaluated as previously described by Lockowandt et al. (2019). Briefly, a solution of erythrocytes at 2.8% was prepared (v/v) and resuspending in PBS. In a 48-well microplate the erythrocyte solution was mixed with PBS solution (control), the studied samples dissolved in PBS, or water (for complete haemolysis). The optical density was measured at 690 nm and after that, the microplate was incubated under the same conditions and the optical density was measured every 10 min at the same wavelength for approximately 400 min. The percentage of the erythrocyte population that remained intact was calculated and the results were expressed as haemolysis delay time (Δt). The Δt values were then correlated to the antioxidant sample concentrations and, from the correlation obtained, the extract concentration able to promote a Δt haemolysis delay was calculated. The results were presented as EC_{50} values (µg/mL) at Δt 60 min (extract concentration required to keep 50% of the erythrocyte population intact for 60 min).

2.4.2. Antimicrobial activity

The antimicrobial potential of the extracts was evaluated against a

panel of different pathogenic bacterial and fungal strains, mainly food contaminants.

Antibacterial activity. The following Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Salmonella* Typhimurium (ATCC 13311), *Enterobacter cloacae* (ATCC 35030); and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (clinical isolate), and *Listeria monocytogenes* (NCTC 7973), were used to determine the potential antimicrobial activity of the samples. The microorganisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. The antibacterial activity evaluation was performed according to a previously described methodology (Reis et al., 2014). The results were expressed as MIC (minimum inhibitory concentration), and MBC (minimum bactericidal concentration), determined by the microdilution method. MICs, obtained from the susceptibility testing of various bacteria to tested extract, were determined also by a colorimetric microbial viability assay based on reduction of INT colour and compared with positive control for each bacterial strain. MBC was determined by serial sub-cultivation of 10 mL into microplates containing 100 mL of TSB. The lowest concentration that shows no growth after this subculturing was read as the MBC. The commercial food additives sodium benzoate (E211) and potassium metabisulphite (E224) were used as positive controls; DMSO (5%) was used as negative control.

Antifungal activity. For the antifungal bioassays, six micromycetes were used: *Aspergillus fumigatus* (human isolate), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC11730), *Penicillium funiculosum* (ATCC 36839), *Trichoderma viride* (IAM 5061) and *Penicillium verrucosum* var. *cyclopium* (food isolate). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The antifungal activity evaluation was performed according to a previously described methodology (Reis et al., 2014). The results were expressed as MIC and MFC (minimum fungicidal concentration). MICs were determined by a serial dilution technique using 96-well microtiter plates. The fractions/extracts were dissolved in 5% solution of DMSO and added to broth malt medium with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 2 mL in microtiter plates containing 100 mL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. Sodium benzoate (E211) and potassium metabisulphite (E224) were used as positive controls; DMSO (5%) was used as negative control.

2.4.3. Cytotoxicity and anti-inflammatory activity

The potential hepatotoxicity of the lyophilized extracts from *C. siliqua* L. was tested in a primary cell culture, designed as PLP2 (Abreu et al., 2011). The cell culture was plated in a 96-well plate, at an appropriate density and the sulforhodamine B (SRB) assay was performed as previously reported by Reis et al. (2014). The cell growth inhibition was calculated after measurement of the absorbance of the control and the sample solutions at 540 nm. The results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Moreover, the cytotoxic activity of carob seeds extracts was assessed against four human tumour cell lines, namely HeLa (cervical adenocarcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines were plated in a 96-well plate, at an appropriate density and the same procedure described above for the SRB assay was performed for the growth inhibition. The results were also expressed as GI_{50} values.

The anti-inflammatory activity was evaluated in a RAW 264.7 cell line. The assay was performed following a procedure described by Taofiq et al. (2015). The nitric oxide produced by the cells in the presence or absence of the tested extracts was determined by measuring the absorbance at 540 nm and comparing with the standard calibration curve.

2.5. Phenolic compounds profile of the most promising extracts of Ceratonia siliqua L.

After obtaining the results from the bioactivity assays, especially the results for the antioxidant potential, two extracts were selected, one obtained by maceration and the other by ultrasound-assisted extraction. The phenolic profile of both extracts was determined. The lyophilized extracts previously obtained (to assess the bioactive properties) were redissolved at a concentration of 5 mg/mL in: i) EtOH:H_2O (75:25; v/v) for the extract obtained by ME; and ii) water for the extract obtained by UAE. The analysis was carried out using a LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) system, as previously described by Bessada et al. (2016). Double online detection was performed using 280, 330 and 370 nm as preferred wavelengths for DAD and in a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The identification of the phenolic compounds was performed based on their chromatographic behavior, UV–vis and mass spectra by comparison with standard compounds, when available, and using data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The peaks were quantified based on the area of the peak by using a manual quantification method, which is permitted by the software used. To integrate peaks a perpendicular line was drawn from the valley between the peaks to the baseline extended between the normal baseline before and after the group of peaks. The results were expressed as mg/g of dry weight (dw).

2.6. Statistical analysis

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 25 (IBM Corporation, New York, USA). Three samples were used for each preparation and all the assays were carried out in triplicate. The results were expressed as mean values ± standard deviation (SD). Whenever possible, an analysis of variance (ANOVA) was applied to compare differences among different samples. The typical requirements, homoscedasticity by the Levene test and normal distribution by the Shapiro Wilk's test, were preliminarily performed. The Welch test was applied to verify the existence of statistically significant differences. The ANOVA results were classified using the Tukey HSD test or Tamhane's T2, when homoscedasticity was verified or not, respectively. When a specific factor was studied using only two levels, a simple student's *t*-test was used to classify the results.

3. Results and discussion

3.1. Chemical characterization of Ceratonia siliqua L.

Carob seeds were chemically characterized for their content in some biologically active compounds, namely tocopherols and organic acids. The results are presented in Table 1. The seeds shown a high content specially of γ-tocopherol (20.2 μg/g dw), which has greatly contributed for a total tocopherol content of 30 μg/g dw. Fidan et al. (2020) found the four vitamers (α-, β-, γ-, and δ-tocopherol) in commercial carob seeds from Turkey. The authors reported the tocopherol values as percentage based on absolute dry weight, obtaining also higher quantities of γ-tocopherol (53.1%), followed by α-tocopherol (41.1%). Matthaus and Özcan (2011) reported the tocopherols profile of Turkey carob seeds oils, comparing wild and cultivated species. For the analyzed oils,

Table 1
Chemical characterization of *Ceratonia siliqua* L. seeds in terms of tocopherols (μg/g dw) and organic acids (mg/g dw). Values are presented as mean ± standard deviation (SD).

Compounds	*C. siliqua* L. seeds
α-Tocopherol	9.0 ± 0.6
γ-Tocopherol	20.2 ± 0.7
δ-Tocopherol	1.18 ± 0.04
Total tocopherols (μg/g dw)	30 ± 1
Oxalic	0.7 ± 0.2
Malic	3.9 ± 0.5
Citric	5.1 ± 0.9
Fumaric	tr
Total organic acids (mg/g dw)	10 ± 2

Tr - trace amounts.

γ-tocopherol prevailed (101.15 – 114.29 mg/100 g) over the other isoforms (1.85 – 70.39 mg/100 g). Thus, although the quantities may vary due to differences between samples and types of extraction, we conclude that carob seeds are effectively a source of vitamin E vitamers, namely tocopherols, which may contribute to their bioactive potential.

Regarding the levels of organic acids, it was possible to detect oxalic acid, malic acid, citric acid, and trace amounts of fumaric acid (Table 1). The largest amounts were registered to citric and malic acids (5.1 and 3.9 mg/100 g dw, respectively), with a total amount of 10 mg/100 g dw of organic acids being recorded. To the authors best knowledge, there are no reports on organic acids present in carob seeds. There is a study reporting the organic acids profile, but for all carob fruit (pods and seeds; Arribas et al., 2019a). In this matrix, oxalic acid (0.161 g/100 g dw), quinic acid (0.42 g/100 g dw), malic acid (0.184 g/100 g dw), citric acid (0.40 g/100 g dw), and trace amounts of fumaric acid were detected.

3.2. Bioactive properties of Ceratonia siliqua L.

3.2.1. Antioxidant activity

After this first screening for some bioactive compounds, carob seeds were submitted to two extraction techniques, maceration (ME) and ultrasound-assisted extraction (UAE). As abovementioned (section 2.3), for both methodologies, water and ethanol were chosen as solvents, with four different proportions being used: i) ethanol:water (25:75; v/v); ii) ethanol:water (50:50; v/v); iii) ethanol:water (75:25; v/v); and iv) 100% water. The bioactive properties of all the obtained extracts were evaluated. The results for the antioxidant activity are presented in Table 2. Two different assays were selected to evaluate the antioxidant activity of carob seeds, TBARS and oxHLIA assays, avoiding methods like DPPH-radical scavenging activity or reducing power assays. This is because more accurate methodologies, using cells and therefore closer to the *in vivo* should be preferred, instead of relying only on chemical reactions. The lowest EC_{50} values were obtained for the TBARS assay (1.19 – 2.7 μg/mL), comparing with the oxHLIA assay (22 – 128 μg/mL). Comparing the different extractions performed, the best results for the ME were obtained using EtOH:H_2O (25:75; and 50:50; v/v) for the TBARS assay, and using EtOH:H_2O (25:75; v/v) and just water for the oxHLIA assay (Table 2). Regarding the UAE, the best results were obtained using EtOH:H_2O (75:25; v/v) and just water for the TBARS assay, as well as for the oxHLIA assay. Thus, and as would be expected, both extraction techniques and solvents influence the results obtained. However, and although the results have different orders of magnitude, some linearity was found, being possible to affirm that the ME using EtOH:H_2O (25:75; v/v) and the UAE using H_2O were the most effective extraction methodologies for promoting the antioxidant activity of the samples. The antioxidant potential of natural products is usually associated with the presence of bioactive compounds, such as tocopherols (previously determined) or polyphenols. There are some studies regarding the presence of polyphenols in carob samples; however, these are more focused on carob fruits in general (not exclusively on seeds) (Stavrou, Christou, & Kapnissi-Christodoulou, 2018). Today, and given that locust bean gum is already an authorized food additive, there is a greater curiosity about the chemical constitution of locust bean seeds, but the results are still scarce. About its antioxidant potential, the works found are mainly focused on leaves (Hajaji et al., 2010), on carob pods, considering all fruit (pods and seeds; Arribas, Cabellos, Cuadrado, Guillamón, & Pedrosa, 2019b; Goulas and Hadjisolomou, 2019) or after removing the seeds (Benchikh, Louaileche, George, & Merlin, 2014), on carob tree barks (Hajaji et al., 2011). In addition, the results published regarding the antioxidant potential of *C. siliqua* are mainly based on assays like DPPH and ABTS (Stavrou et al., 2018). Considering the works found in the literature in which there is a certainty that only carob seeds were used as the object of study, these are rare. Lakkab et al. (2019) performed the antioxidant activity of carob seed peels extracted with ethyl acetate, acetone, and methanol. The bioactivity was evaluated through three *in vitro* assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging activity assay, Ferric Reducing Antioxidant Power (FRAP) assay, and phosphomolybdenum assay. Therefore, the results cannot be directly related and compared, since extraction solvents and methodologies, as well as performed assays were different. The only conclusion that can be drawn is that due to the presence of bioactive compounds, which may be located on the peel of carob seeds, this type of samples reveals antioxidant potential. Rico et al. (2019), specifying results for the germ and the seed peel, showed that these matrices reveal antioxidant potential through the Oxygen Radical Absorbance Capacity (ORAC) assay; FRAP assay, DPPH-radical scavenging activity assay, and Trolox Equivalent Antioxidant Capacity (TEAC) assay. Overall, and having made a general review regarding the *in vitro* methodologies used to determine the antioxidant potential of carob seed samples, the present work adds not only information related to this specific matrix but proves this potential through more sensitive and precise methodologies that do not exist in the literature.

Table 2
Antioxidant activity of *Ceratonia siliqua* L. seeds. Values are presented as EC_{50} values (μg/mL; mean ± SD).

Antioxidant activity properties of *C. siliqua* seeds			
Type of extraction	**Solvent of extraction**	**TBARS assay**	**oxHLIA assay**
ME	EtOH:H_2O (25:75; v/v)	1.45 ± 0.06^{de}	40.9 ± 0.4^{d}
	EtOH:H_2O (50:50; v/v)	1.51 ± 0.03^{de}	90 ± 3^{b}
	EtOH:H_2O (75:25; v/v)	1.59 ± 0.08^{d}	128 ± 2^{a}
	H_2O	2.2 ± 0.2^{b}	34.0 ± 0.6^{e}
UAE	EtOH:H_2O (25:75; v/v)	2.7 ± 0.2^{a}	93 ± 3^{b}
	EtOH:H_2O (50:50; v/v)	1.887 ± 0.009^{c}	74 ± 3^{c}
	EtOH:H_2O (75:25; v/v)	1.19 ± 0.02^{f}	23 ± 5^{f}
	H_2O	1.3 ± 0.1^{ef}	22 ± 2^{f}

The antioxidant activity was expressed as EC_{50} values, which means that higher values correspond to lower antioxidant potential. EC50: extract concentration corresponding to 50% of the antioxidant activity. Statistical classification was done using Tukey's HSD test. Different letters in each row indicate statistically different values between the samples ($p < 0.05$).

3.2.2. Antimicrobial activity

Regarding the antibacterial activity of *C. siliqua* seeds, the extracts obtained by ME inhibited the bacterial growth particularly of *Bacillus cereus* and *Escherichia coli* (Table 3). For these samples the MICs ranged between 1.5 and 6 mg/mL, and MBCs ranged between 2 – >8 mg/mL. For the other bacteria (*Staphylococcus aureus*, *Listeria monocytogenes, Salmonella* Typhimurium*, and Enterobacter cloacae*), MIC values ranged between 6 – >8, and MBC values between 8 – >8 mg/mL. Regarding the antibacterial activity of the extracts obtained by UAE, these were particularly bioactive against *B. cereus*, *L. monocytogenes*, and *E. coli* (Table 3). For these bacteria, the MICs obtained ranged from 0.5 to 6 mg/mL, and the MBCs between 1 and 8 mg/mL. For the other bacteria, MICs varied from 1 – >8, and MBCs between 4 – >8 mg/mL. Since the antimicrobial potential of the extracts was evaluated against a panel of

Table 3
Antibacterial activity of *Ceratonia siliqua* L. seeds. Values are presented as MIC and MBC values (mg/mL).

Antibacterial properties of *C. siliqua* seeds													
Type of extraction	Solvent of extraction	*S. aureus*		*B. cereus*		*L. monocytogenes*		*E. coli*		*S.* Typhimurium		*E. cloacae*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ME	EtOH:H_2O (25:75; v/v)	>8	>8	3	4	8	>8	1.5	2	>8	>8	>8	>8
	EtOH:H_2O (50:50; v/v)	8	>8	2	4	>8	>8	3	4	>8	>8	>8	>8
	EtOH:H_2O (75:25; v/v)	6	8	2	8	4	8	6	8	6	8	>8	>8
	H_2O	>8	>8	2	4	8	>8	4	>8	>8	>8	>8	>8
UAE	EtOH:H_2O (25:75; v/v)	8	>8	0.5	1	2	4	0.5	1	2	8	8	>8
	EtOH:H_2O (50:50; v/v)	6	8	0.5	2	3	4	1.5	2	4	8	6	8
	EtOH:H_2O (75:25; v/v)	2	4	2	4	6	8	2	4	8	>8	>8	>8
	H_2O	1	>8	3	4	4	8	3	4	6	8	6	8
Positive controls													
E211		4	4	0.5	0.5	1	2	1	2	1	2	2	4
E224		1	1	2	4	0.5	1	0.5	1	1	1	0.5	0.5

different pathogenic bacterial and fungal strains, mainly food contaminants, the positive controls used were commercial food additives, namely E211 and E224. Comparing our results with the positive controls, some extracts matched the MIC and MBC of the controls, and some extracts showed even lower values than the controls. This is the case of the extract obtained by UAE (EtOH:H_2O (25:75; v/v)), comparing the MIC and MBC with E224 for *B. cereus*, and comparing the MIC and MBC with E211 for *E. coli*; the extract obtained by UAE (EtOH:H_2O (50:50; v/v)), comparing the MIC and MBC with E224 for *B. cereus*; and the extract obtained by UAE (EtOH:H_2O (75:25; v/v)), comparing the MIC with E211 for *S. aureus* (Table 3).

Some of the main chemical antimicrobials used in food with *quantum satis status* include acetic acid (E260) or lactic acid lactic acid (E270). Moreira et al. (2019) tested both acids against *S. aureus*, *S. typhimurium*, *P. aeruginosa* and *E. coli*, obtaining MIC and MBC values between 6.25 and 12.50 μL/mL for lactic acid. For acetic acid the authors obtained MIC values of 0.78 μL/mL for all bacteria and MBC values ranging from 3.12 to 6.25 μL/mL. Regarding natural compounds, several extracts have been tested for their antibacterial capacity. For example, Jafarzadeh et al. (2020) evaluated the antimicrobial effects of aqueous and ethanolic extracts from three plants species including *Matricaria chamomilla* L., *Silybum marianum* (L.) Gaertn., and *Melissa officinalis* L., as well as the essential oil of *M. chamomilla* on 91 multidrug-resistant (MDR) *E. coli* isolates from patients with urinary tract infection. The authors reported the ethanolic extract from *M. chamomilla* as having the highest inhibitory activity (MIC = 0.11 μg/mL), and the lowest MIC values were reported for the aqueous extracts from *M. chamomilla* and *S. marianum* (MIC = 1113 μg/mL for both extracts). The highest bactericidal activity was also attributed to ethanolic extract from *M. chamomilla,* as well as the ethanolic extract from *S. marianum* (MBC = 1.11 μg/mL). Jafarzadeh et al. (2020) concluded that the ethanolic extract from *M. chamomilla* could substitute 18 studied antibiotics except for meropenem and piperacillin. It should be considered that after studying the chemical profile of the extracts, the authors assigned this bioactivity to some of the identified compounds such as flavonoids (*e.g.* catechin or quercetin).

Comparing the results of the other authors with ours, we can conclude that although the extracts of the seeds of *C. siliqua* presented lower MIC and MBC values, some of them manage to be within the same order of magnitude as the others (*e.g.* the extracts obtained by UAE, EtOH:H_2O (25:75; v/v) and EtOH:H2O (50:50; v/v)). It should be highlighted that some of the plants presented in the Jafarzadeh et al. (2020) study as having bioactive properties, namely *M. officinalis*, is recognized for this potential, and it is a source of preservative extracts already studied for application as food additive (rosemary extract). Regarding the antifungal activity (Table 4), there was more variety in the values obtained. However, as for the antibacterial activity, some of them equaled or were lower than the positive controls. This was the case of the extract obtained by ME (EtOH:H_2O (25:75; v/v)), comparing the MIC with E211 for *Aspergillus versicolor*, and comparing the MIC and MFC with E211 for *Trichoderma viride*. The extracts obtained by ME (EtOH:H_2O (75:25; v/v) and 100% water) also shown lower MFC values comparing with E211 for *T. viride.* The extracts obtained by UAE (EtOH:H_2O (25:75; v/v)) revealed lower MIC comparing with E211 for *A. versicolor*, as well as lower MIC and MFC comparing with both positive controls for *T. viride*. The extracts obtained by UAE (EtOH:H_2O (50:50; and 75:25; v/v)) also showed lower MIC and MFC comparing with both positive controls for *T. viride*. Therefore, the extracts were more effective against the fungi *A. versicolor* and *T. viride* than against *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium funiculosum*, *Penicillium verrucosum* var. *cyclopium* (Table 4).

As for bacteria, different plant extracts with antimicrobial potential have been studied for their antifungal activity. For example, *Silene* species extracts exhibited strong antifungal activity (0.003 – 0.4 mg/mL) against 8 fungal strains food contaminants (*Aspergillus* and *Penicillium* genus), revealing higher bioactivity than bifonazole and

Table 4
Antifungal activity of *Ceratonia siliqua* L. seeds. Values are presented as MIC and MFC values (mg/mL).

Antifungal properties of *C. siliqua* seeds													
Type of extraction	Solvent of extraction	*A. fumigatus*		*A. niger*		*A. versicolor*		*P. funiculosum*		*T. viride*		*P. verrucosum* var. *cyclopium*	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ME	EtOH:H_2O (25:75; v/v)	6	8	6	8	1	2	2	4	1.5	2	2	4
	EtOH:H_2O (50:50; v/v)	4	8	8	>8	8	>8	8	>8	>8	>8	1.5	2
	EtOH:H_2O (75:25; v/v)	8	>8	4	8	2	4	1.5	2	1.5	2	1.5	2
	H_2O	2	4	2	4	1.5	2	2	4	1.5	2	3	4
UAE	EtOH:H_2O (25:75; v/v)	2	4	4	8	1.5	2	1.5	2	1	2	1	2
	EtOH:H_2O (50:50; v/v)	1	2	1	2	1	2	1.5	2	0.75	1	2	4
	EtOH:H_2O (75:25; v/v)	4	>8	8	>8	4	>8	2	>8	1.5	1	2	2
	H_2O	1.5	2	2	4	1	2	1.5	2	3	4	1	2
Positive controls													
E211		1	2	1	2	2	2	1	2	2	4	1	2
E224		1	1	1	1	1	1	0.5	0.5	1	1	0.5	0.5

ketoconazole. Also, the extracts obtained from the seeds of *Humulus lupulus* L. have been tested against fungi from the genus *Penicillium*, showing great antifungal properties (0.01 – 1.20 mg/mL) (Soković and Liaras, 2021).

As for the antioxidant activity, the studies found in the bibliography regarding the antimicrobial potential of *C. siliqua*, refer to studies carried out mainly on leaves or on pods without seeds (Meziani et al., 2015; Othmen, Elfalleh, Beltrán, Esteban, & Haddad, 2020). Even not using the exactly the same matrix (carob seeds), some of the studies, like this one, revealed that *C. siliqua* has activity against certain pathogenic microorganisms, namely *L. monocytogenes* (Aissani, Coroneo, Fattouch, & Caboni, 2012).

So, given the antioxidant properties of *C. siliqua* and its capacity to inhibit pathogenic microorganisms, especially food pathogens, it can be studied as a food preservative and help to extend the shelf-life of foodstuff. In fact, some authors have already linked this property to the ability of locust beans to preserve moisture in food, due to its fiber content (Nasar-Abbas et al., 2016). However, it is not yet used with this specific function. The results obtained in the present work on carob seeds antioxidant and antimicrobial capacity can be studied in more depth and, in the future, ally these properties with the already known functions of locust bean gum (E410).

3.2.3. Cytotoxicity and anti-inflammatory activity

The potential hepatotoxic effect of the extracts under study was evaluated in a cell culture obtained from porcine liver (PLP2). None of the extracts inhibited the cell growth at the concentrations tested ($GI_{50} > 400$ μg/mL), which, as expected, presupposes their safe use. However, the extracts also did not inhibit the growth of any of the four human tumor cell lines tested and did not reveal anti-inflammatory effects (data not shown). In this particular work, the antioxidant and antimicrobial activities seem to be the main and most promising bioactivities of carob seeds extracts, which do not appear particularly effective against tumor models from the cervix, liver, breast, and lung, nor do have anti-inflammatory activity *in vitro*. Custódio et al. (2011) provide the first evidence of *in vitro* cytotoxic activity of carob germ flour extracts on HeLa cells. They verified that the reduction on cell viability was dose-dependent and evident after a 24 h treatment. In the present work, it was not possible to verify any bioactivity in the cervical cell line or in any other, which may partly be due to the species itself (different origin, growth conditions, and/or chemical composition and bioactivities), or different extraction conditions. Rico et al. (2019), tested the viability of RAW 264.7 cells incubated for 24 h with different concentrations of carob extracts. The highest concentration tested (0.5 mg/mL) did not provoke changes in cell viability for the germ extract; however, this concentration significantly reduced cell viability in the case of seed peel. Nevertheless, the intermediate concentration tested (0.1 mg/mL) was significantly cytotoxic for the seed peel extract, resulting in 76.6% of remaining living cells. The lowest concentration tested (0.05 mg/mL), for both carob extracts, showed no significant changes, indicating no cell integrity damage. Comparing these results with our study, the differences may be explained by the different tested concentrations. In our case, the highest tested concentration was 400 μg/mL, which is below the concentrations for which activity was obtained for Rico et al. (2019).

3.3. Phenolic compounds profile of the most promising extracts of Ceratonia siliqua L.

Of the eight extracts under study and, after obtaining the results from the bioactivities, the two most promising extracts (one obtained by ME and other by UAE) were selected to carry out a detailed analysis of the profile in phenolic compounds. Since the results of the antimicrobial activity had more fluctuations, the results of the antioxidant activity were decisive for the choice of the extracts. So, the extracts obtained by ME and UAE with lower EC_{50} values for both performed assays (TBARS and oxHLIA) were selected. For ME, the extract obtained using EtOH:H_2O (25:75; v/v) was selected, and for UAE, the extract obtained using just water was selected (Table 2).

Polyphenols are a class of compounds well known for their bioactive potential and health benefits. Table 5 presents the data on the chromatographic characteristics, tentative identification and quantification of the phenolic compounds present in two different extracts of *C. siliqua* seeds. The table shows the compounds corresponding to the peaks that were possible to identify (through comparison with commercial standards or with the compound library). However, it should be highlighted that, given the chemical structure of phenolic compounds, their total extraction is often ineffective. In this way, some compounds may not have been extracted. In both samples, seven flavonoids were found, six *C*- glycosylated apigenin derivatives and one *O*-glycosylated quercetin derivative. Regarding the apigenin derivatives, the tentative identification of the six compounds was performed considering the previously described chromatographic identification of carob seeds germ flour by Picariello et al. (2017). Peaks **1** and **2** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 725 and characteristics MS^2 fragments that allowed the tentative identification as apigenin-6-*C*-(6″-*O*-glucoside)-arabinoside-8-*C*-glucoside and apigenin-6-*C*-glucoside-8-*C*-(6″-*O*-glucoside)-arabinoside, respectively (Picariello et al., 2017). Peaks **3**, **4**, and **5** also presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 563, that according the elution order and MS^2 fragments described by Picariello et al. (2017), allowed the tentative identification of the three peaks as apigenin-6-*C*-pentoside-7-*C*-hexoside (**3**), apigenin-6-*C*-arabinoside-8-*C*-glucoside (isoschaftoside, **4**), and apigenin 6-*C*-glucoside-8-*C*-arabinoside (schaftoside, **5**). Peak **6** ($[M-H]^-$ at *m/z* 739) was tentatively identified as apigenin-6-*C*-feryloyl-glucoside-8-*C*-arabinoside according to the previously described by Picariello et al. (2017). Finally, peak **7** presented a pseudomolecular ion at *m/z* 447 releasing a unique MS^2 fragment at *m/z* 301 (quercetin aglycone), corresponding to the loss of a rhamnosyl unit (146 u), being tentatively identified as quercetin-*O*-rhamnoside. This peak has been previously identified in carob kibbles by Rached et al. (2016).

According to the literature, seedless carob pods (kibbles or pulp) contain higher amounts of polyphenols compared to seeds or germs, the concentration depending heavily on genetic and environmental factors, and extraction conditions (Stavrou et al., 2018). However, in the study carried out by Rico et al. (2019), the authors compared the pulp, germ, and seed peel, obtaining quantities of total phenolic compounds of approximately 3065.71 μg/g dw for the pods, 7292.95 μg/g dw for the germ, and 4593.12 μg/g dw for seed peels. In addition, they concluded that flavones, like apigenin, were the most abundant class of phenolics present in carob germ, accounting for 38% of the total phenolic compounds, and flavonols (77%), like quercetin, was the major phenolic group detected in seed peel. Custódio et al. (2011) studied different Portuguese cultivars of carob trees, analysing the germ flour obtained from their seeds. The authors found different profiles in the studied species, and were able to identify different phenolic compounds, namely (+)-catechin; gentisic acid, chlorogenic acid; catechol; ferulic acid; gallic acid; myricetin; methyl gallate; quercetin; rutin; syringic acid; theophylline; and vanillin. The total values of quantified compounds were between 2.1 and 6.8 mg/g dw. It should be highlighted that the studied extracts were obtained after Soxhlet extraction, using methanol as a solvent, which may explain the difference in the profiles. Using ME and UAE it was possible to obtain higher levels of total phenolics, 22.1 and 27.0 mg/g dw, respectively. In another study, Albertos, Jaime, Diez, González-Arnáiz, & Rico (2015) stated that besides tannins, flavonoids accounted for most compounds identified in carob seed peel, mainly catechin, quercetin, and epicatechin. Picariello et al. (2017) performed a RP-HPLC-based semi-quantification of individual polyphenols in *C. siliqua* seed germ flour (SGF) extracts, obtaining total values around 8.3 mg/g SGF. Even with some differences in the profile, we found that the derivatives of apigenin and quercetin are effectively the predominant phenolic compounds in locust bean seeds, and the methods used in

Table 5
Chromatographic characteristics (retention time (Rt), wavelengths of maximum absorption (λmax), and mass spectral data), tentative identification, and quantification (mg/g dw) of the phenolic compounds in the extracts of *Ceratonia siliqua* L. seeds obtained by maceration and ultrasound-assisted extractions (mean ± SD).

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification ME	UAE	*p*-Value
1	5.63	322	725	665(9), 563(100), 473(33), 383(6), 353(6)	Apigenin-6-*C*-(6″-*O*-glucoside)-arabinoside-8-*C*-glucoside	1.9945 ± 0.0006	3.03 ± 0.04	<0.001
2	6.52	324	725	665(13), 563(100), 473(14), 383(5), 353(5)	Apigenin-6-*C*-glucoside-8-*C*-(6″-*O*-glucoside)-arabinoside	3.76 ± 0.08	5.6 ± 0.1	<0.001
3	13.12	335	563	473(100), 443(78), 383(30), 353(30), 311(5)	Apigenin 6-*C*-pentoside-7-*C*-hexoside	2.64 ± 0.06	3.7 ± 0.1	<0.001
4	13.44	336	563	473(65), 443(100), 383(16), 353(21), 311(5)	Apigenin-6-*C*-arabinoside-8-*C*-glucoside (isoschaftoside)	7.991 ± 0.006	10.8 ± 0.2	<0.001
5	14.98	334	563	473(93), 443(100), 383(21), 353(27), 311(5)	Apigenin-6-*C*-glucoside-8-*C*-arabinoside (schaftoside)	0.64 ± 0.01	0.78 ± 0.02	<0.001
6	21.15	392	739	545(98), 425(100)	Apigenin-6-*C*-feryloyl glucoside-8-*C*-arabinoside	0.901 ± 0.003	0.867 ± 0.002	<0.001
7	21.85	347	447	301(100)	Quercetin-*O*-rhamnoside	4.2 ± 0.1	2.18 ± 0.04	<0.001
					Total phenolic compounds	**22.1 ± 0.1**	**27.0 ± 0.5**	<0.001

Standard compounds used to quantify each compound: apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0.998$; LOD = 0.19 μg/mL; LOQ = 0.63 μg/mL, peaks 1, 2, 3, 4, 5, and 6); and quercetin-3-*O*-rutinoside ($y = 13343x + 76751$, $R^2 = 0.999$; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL, peak 7). Statistical classification was done using *t*-Student test.

this work may be more efficient in terms of extracting higher concentrations of these compounds.

4. Conclusion

Carob (*Ceratonia siliqua* L.) has been used in traditional medicine in many countries, such as Tunisia. Its fruits, in the form of pods, have been recognized as a source of bioactive compounds with co-adjuvant effects in several disorders. However, carob pods are mostly used for animal feed, and there is little information regarding their chemical constitution and bioactive potential.

With the accomplishment of this work, we verified that this natural matrix is a source of tocopherols, mainly γ-tocopherol, as well as organic acids, mainly malic and citric acids. Applying two different extraction techniques, we verified that the maceration process (ME) with EtOH: H_2O (25:75; v/v), and ultrasound-assisted extraction (UAE) using only water, are the most efficient methods to obtain better results for the antioxidant activity. The obtained extracts also revealed antimicrobial properties, mainly against *B. cereus*, *E. coli*, *A. versicolor*, and *T. viride*. In some cases, the MIC and MBC/MFC values obtained were similar or even better than those of the commercial additives used as controls. The profile of phenolic compounds shown by the most bioactive samples proved that these extracts are especially rich in flavonoids, namely *C*-glycosylated apigenin derivatives and *O*-glycosylated quercetin derivatives.

Although the results obtained can be promising, further and in-depth studies are needed to confirm them since they were based mostly on *in vitro* tests (major work limitation). In the future, the extracts that showed the greatest potential here could be improved and standardized, being subsequently tested not only in cell models *in vitro* but also *in vivo*. Moreover, the extracts can be tested as food additives with preservative capacity.

This study adds information to that available in the literature, associating functions to a matrix that seems to be under-valued and that may have other useful applications for the food industry (*e.g.* potential food preservative) or others, such as the cosmetic industry (*e.g.* associated with its antioxidant / preservative function).

CRediT authorship contribution statement

Siwar Ben Ayache: Investigation. **Filipa S. Reis:** Investigation, Conceptualization, Data curation. **Maria Inês Dias:** Investigation. **Carla Pereira:** Investigation. **Jasmina Glamočlija:** Investigation. **Marina Soković:** Writing - review & editing. **Emna Behija Saafi:** Writing - review & editing. **Isabel C. F. R. Ferreira:** Project administration, Conceptualization, Writing - review & editing. **Lillian Barros:** Project administration, Investigation, Conceptualization, Writing - review & editing. **Lotfi Achour:** Writing - review & editing.

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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