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Comparison of Conventional and Green Extraction Techniques for the Isolation of Phenolic Antioxidants from Sea Fennel

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Abstract: In this study, different extraction methods were compared for the isolation of bioactive phenolic compounds from sea fennel: microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and conventional solvent extraction (CSE). Total phenolics, flavonoids, and tannins were determined spectrophotometrically. In contrast, the determination of individual phenolics was performed by high-performance liquid chromatography coupled with an ultraviolet/visible detector (HPLC-UV/VIS). Two in vitro assays (ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryl-hydrazyl (DPPH)) were performed to determine antioxidant activity. The maximum extraction of phenolic compounds was achieved with 50% ethanol, while the MAE method showed the highest extraction efficiency, with a total phenolic content of more than 25 mg of gallic acid equivalents (GAE)/mg and a chlorogenic acid content of more than 10 mg/g, respectively. The highest antioxidant potential was also observed in the samples from MAE (the FRAP value ranged from 173 to 185 µmol Fe^{2+}/g, and the DPPH inhibition ranged from 55 to 59%), which was consistent with the extraction results. Although the phenolic antioxidants of sea fennel have been extensively studied recently, to the authors' knowledge, this study was the first to evaluate the application of the new techniques for their isolation and to show the advantages of MAE compared to the other techniques used.

Keywords: antioxidants; *Crithmum maritimum*; halophyte; microwave-assisted extraction (MAE); phenolic compounds; ultrasound-assisted extraction (UAE)

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1. Introduction

The Mediterranean halophyte *Crithmum maritimum* L. (Apiaceae), known as sea fennel, rock sorrel, or sea crest, is one of the edible halophytes that is also known for its medicinal and cosmetic uses [1–4]. It is rich in biologically highly active compounds, of which phenolics are the most widely known [5–9]. Phenolic compounds, molecules containing at least one phenolic unit in their structure, are plant secondary metabolites widely known for their great protective biological properties such as antioxidant, anticarcinogenic, antimutagenic, antimicrobial, antiangiogenic, and anti-inflammatory activities [9–12]. Recently, due to the multiple health benefits of phenolics, which have been confirmed by various studies, there has been a great interest in the isolation of phenolic compounds from various natural sources, especially from edible medicinal and aromatic plants. Various pretreatments of the plant material, such as the drying method or the particle size of the powder after homogenization, have a significant influence on the yield of the isolated compounds. In addition, extraction conditions such as solvent selection, solvent-to-plant material ratio, extraction time and temperature, and the application of novel techniques also have a significant effect on the quality and quantity of phenolic compounds extracted from different plant samples [13]. Among these parameters, the choice of solvent is one of the crucial steps for the efficient extraction of the desired compounds. Solvent extraction should be

designed and optimized to obtain the highest yield of isolated target compounds, since different materials and chemical compounds require different approaches. For food and pharmaceutical applications, in most cases, aqueous and alcoholic solvents (mainly ethanolic) of different polarities are combined and optimized to extract the highest concentration of bioactive compounds, especially phenolics [14]. Studies have shown that water content increases the extraction efficiency of polyphenolics, because the contact area between the substance and the solvent increases due to the swelling effect of water on plant tissue [15]. Recently, scientists have also investigated the application of unconventional extraction techniques such as microwave-assisted extraction (MAE) [16–18], ultrasound-assisted extraction (UAE) [19,20], pressure-assisted liquid extraction (PLE) [21,22], supercritical CO_2 extraction (SC-CO_2) [23,24], pulsed electric field (PEF)-assisted extraction [25,26], and enzyme-assisted extraction (EAE) [27–29] compared to conventional solvent extraction (CSE) methods, such as Soxhlet extraction, decoction, maceration, infusion, percolation, digestion, serial exhaustive extraction, and simple mixing, due to their numerous advantages [13]. These new techniques are considered to be environmentally friendly extraction methods and are therefore suitable for application.

In recent years, interest in sea fennel and its bioactive compounds has increased due to their potential use in various industries such as the food, cosmetic, and pharmaceutical industries. Among the various phytochemicals of sea fennel, special attention has been paid to phenolics, especially since most of them are phenolic acids, chlorogenic acid, and their derivatives, which have numerous beneficial effects on health. However, the authors are aware that the use of "green" extraction techniques such as USE and MAE has not yet been evaluated compared to CSE for their isolation. Therefore, the aim of this study was to determine the extraction method that yielded the highest content of phenolic compounds in sea fennel extracts. This was achieved by (a) selecting the best extraction solvent mixture; (b) comparing three different extraction methods (CSE with stirring, MAE, and UAE); (c) the ultraviolet–visible (UV-VIS) analysis of the total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC); (d) the determination of the isolated individual phenolic compounds by high-performance liquid chromatography–ultraviolet/visible (HPLC-UV/VIS) analysis; and (e) the determination of the antioxidant activity of the samples by two in vitro assays (ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assays).

2. Materials and Methods

2.1. Chemicals

All reagents and solvents used were of analytical grade. Acetonitrile and methanol were HPLC grade and were purchased from Sigma (Sigma–Aldrich GmbH, Steinheim, Germany), as were the phenolic HPLC standards used (chlorogenic acid (3-O-caffeoylquinic acid), cryptochlorogenic acid (4-O-caffeoylquinic acid), neochlorogenic acid (5-O-caffeoylquinic acid), caffeic acid, gallic acid, *p*-hydroxybenzoic acid, sinapic acid, ferulic acid, protocatechuic acid, and rutin).

2.2. Plant Material

The aerial parts of sea fennel plants (*Crithmum maritimum* L.) collected in May 2022 on the island of Čiovo (Central Dalmatia, Croatia) were used as plant material (Figure 1). Young and healthy green leaves and shoots, about one kilogram of fresh material from the same population, were selected for the study, while the woody parts of the plant were discarded. The plant material was frozen and dried by lyophilization, and the dry plant material was homogenized in a hand mill and sieved through a 1 mm diameter sieve for 20 min.

Figure 1. Sea fennel plant.

2.3. Extractions

To prepare the extracts, 10 g of the powdered plant material was mixed with 100 mL of solvent. Different water–ethanol mixtures were used for the preliminary experiments: 0%, 25%, 50%, and 75% aqueous ethanol (v/v).

To compare different extraction methods, the CSE, MAE, and UAE methods were performed. MAE was carried out using ETHOS X (Milestone Srl, Bergamo, Italy), while for the UAE, a Transsonic 310/H ultrasonic bath (Elma, Singen, Germany) was used.

Nine samples were extracted with water–ethanol at a 1:1 ratio (v/v) for 30 min. For each extraction method, three samples were extracted under different conditions (Table 1). Different temperatures (room temperature (RT), 40, and 60 °C) were used for the CSE method, which involved stirring, and for the UAE method, while different microwave powers (300 W, 500 W, and 700 W) were used for the MAE method. After each extraction, the suspensions were filtered through folded filter paper, and the resulting filtrates were stored at a temperature of +4 °C for 24 h, then filtered again and used for further analyses.

Table 1. The parameters of the applied microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and conventional solvent extraction (CSE) of the samples.

Sample	Extraction Method	Time	Conditions	
A	MAE	30 min	Power	300 W
B				500 W
C				700 W
D	UAE		Temperature	RT
E				40 °C
F				60 °C
G	CSE		Temperature	RT
H				40 °C
I				60 °C

RT—room temperature.

2.4. Spectrophotometric Measurements of Total Phenolic, Flavonoid, and Tannin Content

Spectrophotometric measurements (UV-VIS) were performed using a SPECORD 200 Plus, Edition 2010 (Analytik Jena AG, Jena, Germany).

The TPC in all sea fennel extracts was determined by the Folin–Ciocalteu method [30]. Folin–Ciocalteu phenol reagent (125 µL) was added to a cuvette containing a sample (25 µL) and distilled water (1.975 mL), and after 5 min, Na_2CO_3 solution (10%, *w*/*v*) (375 µL) was added. The absorbance of the mixture was measured after 2 h at 765 nm. The results were calculated using the calibration curve for gallic acid and expressed as milligrams of gallic acid equivalents (GAEs) per gram of dry plant material (d.p.m.) (mg GAE/g d.p.m.).

The TFC was determined by the colorimetric method using aluminium chloride [31]. The studied extracts (250 µL) were mixed with distilled water (1.25 mL) and sodium nitrite (5%, *w*/*v*) (75 µL). After 5 min, aluminium chloride (10%, *w*/*v*) (150 µL) was added and, after another 5 min, sodium hydroxide (1 M) (0.5 mL) was added. Distilled water was used to bring the final volume to 3 mL. The absorbance was measured at 510 nm. The results were calculated using the calibration curve for rutin and expressed as milligrams of rutin equivalents per gram of dry plant material (mg RE/g d.p.m.).

The TTC was determined using the vanillin–HCl method by measuring the absorbance at 500 nm [32]. To the vanillin solution (4%, *w*/*v*) in methanol was added the sample aliquot, and concentrated HCl (750 µL) was pipetted after shaking. The absorbances of the reaction mixtures were measured at 500 nm after standing for 20 min. The results were calculated using the calibration curve for catechin and expressed as milligrams of catechin equivalents per gram of dry plant material (mg CE/g d.p.m.). All measurements were performed in triplicate for each sample.

2.5. High-Performance Liquid Chromatography–Ultraviolet/Visible (HPLC-UV/VIS) Analysis of Individual Phenolic Compounds

A Shimadzu Nexera HPLC system LC-40 (Shimadzu, Kyoto, Japan) equipped with a UV/VIS detector was used for the analysis of phenols. Phenolic compounds were separated in a Phenomenex C18 (250 mm × 4.6 mm, 5 µm; Torrance, CA, USA) reverse-phase column. The temperature was maintained at 35 °C, and the flow rate was 1.0 mL/min. We used 0.2% phosphoric acid in water as the aqueous mobile phase (A), and methanol–acetonitrile was used as the organic mobile phase (B) at a ratio of 1:1 (*v*/*v*). The elution started isocratically with 4% B, and then the gradient program was set as follows: 0–16 min (linear gradient up to 15% B), 16–50 min (linear gradient up to 35% B), 50–62 min (linear gradient up to 4% B), and 62–65 min (4% B). The initial conditions were established in 2 min and maintained for 10 min to equilibrate the column. The peaks were identified by comparing the retention times and absorbance spectra at 220 and 320 nm with the peaks obtained under the same conditions with phenolic standards. Quantification was performed using an external standard calibration curve, and the results are expressed as milligrams of compound per gram of dry extract (mg/g). Each sample was injected twice.

2.6. Antioxidant Activity

The determination of antioxidant activity was performed using two different metrics: the reducing power of the samples, obtained via the ferric-reducing antioxidant power (FRAP) method [13], and the free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals [10].

In the FRAP method, the activity of the antioxidants reduced iron(III)-tripyridyltriazine (FeIII-TPTZ) complexes to iron(II) complexes. An aliquot of the samples (10 µL) was added to 300 µL of FRAP reagent. The change in absorbance was measured at 593 nm, and the results of the FRAP assay are expressed in micromoles of Fe^{2+} equivalents per gram of extract (µM Fe^{2+}/g).

The DPPH assay results are expressed as the percentage of DPPH radical inhibition (% inhibition). The free-radical working solution was prepared by dissolving DPPH in ethanol

to reach an initial absorbance of 1.2 ± 0.02. A 50 µL aliquot of the samples was added to 200 µL of the DPPH solution, the mixture was shaken, and after 60 min the decrease in absorbance was measured.

All measurements were performed in triplicate in 96-well plates using a UV/Vis microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA).

2.7. Statistical Analysis

All results are expressed as mean ± statistical deviation (SD). The relations between the data examined are described using the Pearson product-moment correlation coefficient (r), calculated using the program GraphPad (Version 8.0.1, San Diego, CA, USA).

2.8. Greenness Assessment Method

The greenness of the extraction techniques was assessed using the Analytical GREEnness (AGREE) tool for sample preparation, which was introduced by Pena-Pereira et al. [33]. A metric system wherein input criteria referred to the 12 significance principles was applied. The default weights were set.

3. Results and Discussion

3.1. Solvent Optimization

Preliminary experiments were performed to select the best solvent in terms of extraction yield. Aqueous solvents are known to be suitable for the extraction of compounds with strong polarity [34]. The addition of ethanol, a generally recognized as safe (GRAS) solvent according to the US Food and Drug Administration (FDA), could extend the polarity range. In order to use solvents that are as environmentally and health-friendly as possible, samples were extracted in different mixtures of water and ethanol (0%, 25%, 50%, 75%, and 100% ethanol) at room temperature for 30 min. The TPC, TFC, and TTC were selected as the main parameters for the evaluation of extraction efficiency. The highest TPC and TFC were obtained for extraction with 50% ethanol, while the highest TTC was obtained for extracts prepared with 100% ethanol (Figure 2). However, the lowest TPC and TFC were observed when this solvent was used. The TPC was similar when extraction was performed with 25% (77.48%) or 75% (77.58%) ethanol, and the TFC was higher (93.50%) for extraction with 75% ethanol. Alemán et al. [35] reported the highest extraction yield of phenolics in ethanol:water extracts (70%, *v*/*v*) compared to pure water extracts of sea fennel prepared by UAE.

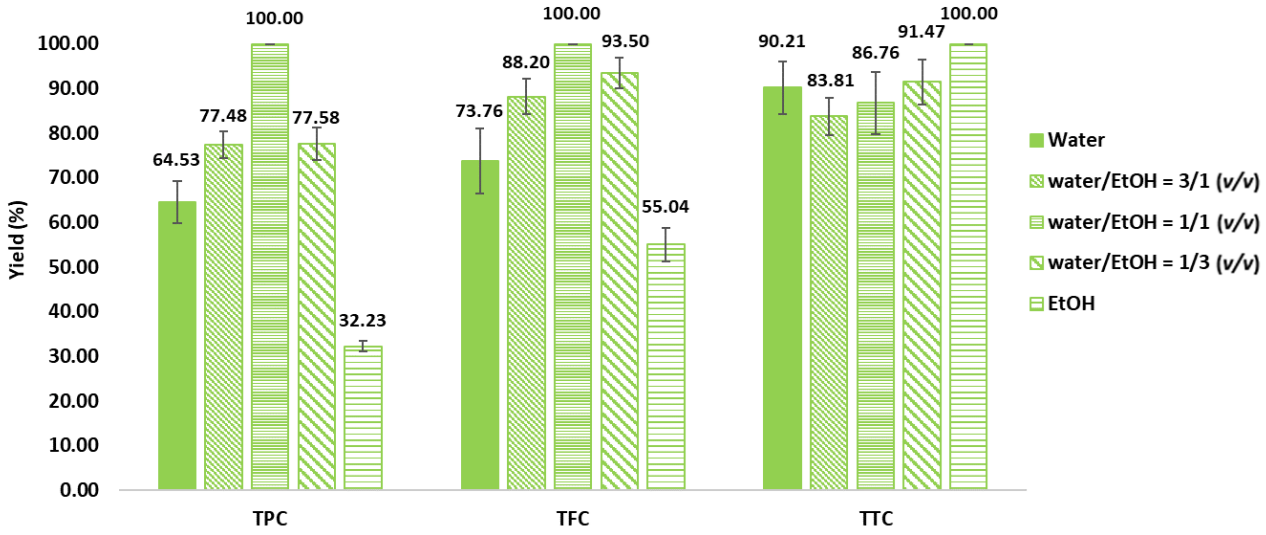

Figure 2. Total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) yield for each water–ethanol combination during preliminary experiments.

Osmic et al. [36] studied the influence of the solvent and extraction conditions (time and temperature) on phenolic antioxidants in sage (*Salvia officinalis* L.) extracts obtained by CSE and found that 40% ethanolic extracts produced the highest TPC and TFC. The optimal extraction time was 50 min, and the highest extraction yield was obtained at RT. Jiménez-Moreno et al. [37] concluded in their study that the selection of the most suitable extraction conditions depended on the target compounds to be extracted. The authors tested aqueous ethanolic mixtures, as in our study, and reported that the maximum polyphenolic content of grape stem extracts was obtained using 50% ethanol at 40 °C. The highest TPC and TFC extraction yields were obtained by Urías-Orona et al. [38] with 50% ethanol from *Moringa oleifera* leaves, as well as in the study of Fatiha et al. [39] on spearmint. Lohvina et al. [40] studied the effect of different mixtures of water and ethanol on the extraction of phenolic compounds from fenugreek seeds (*Trigonella foenum-graecum* L.). Their results showed that 70% ethanol was the best extraction solvent when CSE was used as the extraction method.

3.2. Ultraviolet–Visible (UV-VIS) Analysis of Total Phenolic Content, Total Flavonoid Content, and Total Tannin Content

Lyophilized and homogenized material from the aerial parts of sea fennel was subject to extraction for 30 min using CSE, MAE, or UAE. For CSE and UAE, room temperature and temperatures of 40 °C and 60 °C were used, respectively, while for MAE, the microwave power was 300 W, 500 W, or 700 W. UV-VIS analyses were performed for TPC, TFC, and TTC (Figure 3).

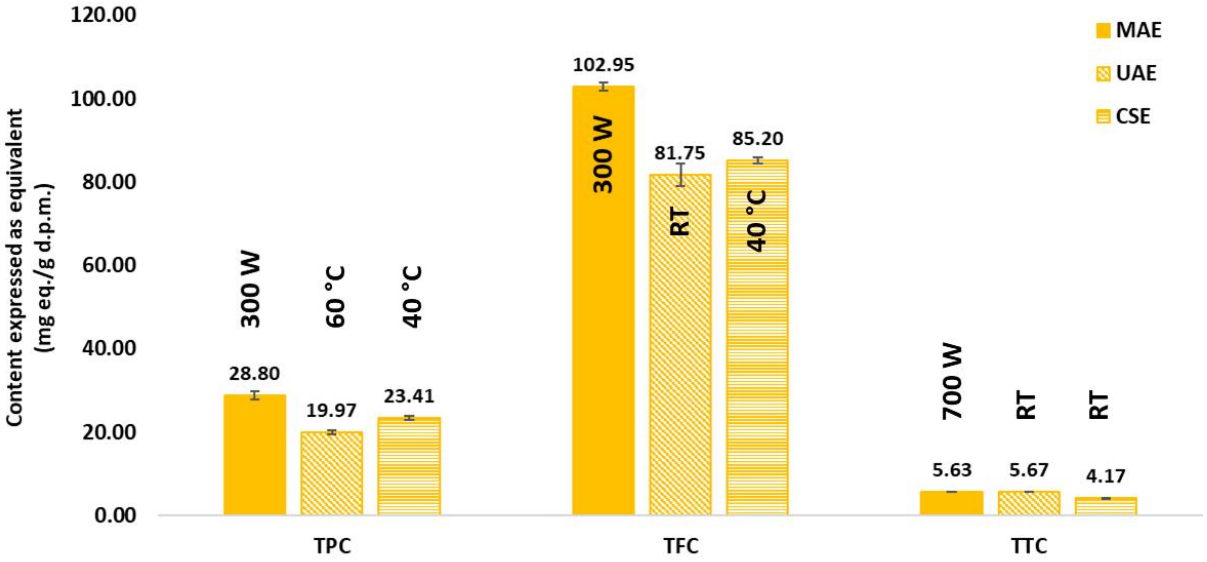

Figure 3. Total phenolic content (TPC) expressed in mg GAE/g d.p.m., total flavonoid content (TFC) expressed in mg RE/g d.p.m., and total tannin content (TTC) expressed in mg CE/g d.p.m. in the optimal extracts obtained by each extraction method. GAEs—gallic acid equivalents; REs—rutin equivalents; CEs—catechin equivalents; d.p.m.—dry plant material; RT—room temperature; MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; CSE—conventional solvent extraction.

The highest TPC was obtained with MAE (from 25.91 mg GAE/g at 700 W to 28.80 mg GAE/g at 300 W), while UAE appeared to be the extraction method that achieved the lowest content of extracted phenols (from 18.46 mg GAE/g at 40 °C to 19.97 mg GAE/g at 60 °C) (Table 2). Recent scientific reports on different plant samples have shown that MAE achieved a higher TPC than other extraction methods [41,42]. Cristina et al. [43] compared the UAE and SFE of sea fennel and marsh samphire and reported that UAE was a more efficient extraction method for ethanol extracts of both halophytes. The CSE method resulted in a TPC of 20.61 mg GAE/g at RT and 23.41 mg GAE/g at 60 °C. Meot-Duros and Magné [44] reported a TPC of 23–33 mg GAE/g for methanolic extracts from the aerial

parts of sea fennel obtained by the CSE method, while Houta et al. [10] determined a range of 9.42 to 17.11 mg GAE/g of phenolic compounds in methanolic extracts of sea fennel (various plant parts). In our previous study, ethanolic leaf extracts, obtained by UAE at 50 °C, had a TPC content of 35.1 mg GAE/L [5]. Hayta and İşçimen [45] compared CSE (2 h), MAE (300 s), and UAE (10 min) in terms of the methanolic and aqueous extracts of vine leaves and concluded that MAE was the most effective method, mainly because of its significantly shorter duration. Goltz et al. [46] investigated the influence of the solvent and extraction technique on the TPC of *Achyrocline satureioides*. UAE with 70% ethanol as the extraction solvent resulted in a higher TPC compared to CSE. Gharekhani et al. [47] studied different methods and conditions for the extraction of phenolics from eucalyptus leaves. In their study, MAE provided a higher extraction yield and selectivity compared to CSE and UAE.

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC), expressed as equivalents (mg eq./g d.p.m.), in sea fennel extracts obtained by different extraction methods.

Type of Extraction	Condition	TPC (mg GAE/g d.p.m.)	TFC (mg RE/g d.p.m.)	TTC (mg CE/g d.p.m.)
MAE	300 W	28.80 ± 0.88	102.95 ± 1.01	3.68 ± 0.10
	500 W	27.17 ± 0.42	97.83 ± 1.29	3.14 ± 0.08
	700 W	25.91 ± 0.45	89.61 ± 1.04	5.63 ± 0.06
UAE	RT	19.96 ± 0.52	81.75 ± 2.62	5.67 ± 0.09
	40 °C	18.46 ± 0.83	80.53 ± 0.35	3.87 ± 0.09
	60 °C	19.97 ± 0.58	77.69 ± 0.56	3.34 ± 0.02
CSE	RT	20.61 ± 077	79.56 ± 0.84	4.17 ± 0.13
	40 °C	23.41 ± 0.57	85.20 ± 0.76	3.35 ± 0.14
	60 °C	20.87 ± 0.53	82.96 ± 2.05	3.40 ± 0.09

GAEs—gallic acid equivalents; RE—rutin equivalents; CE—catechin equivalents; d.p.m.—dry plant material; RT—room temperature; MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; CSE—conventional solvent extraction.

From the results shown in Figure 2, it can be seen that the extraction method used had an effect on the amount of flavonoids extracted from the samples. Similar to the TPC, the highest TFC was obtained using MAE (from 89.61 mg RE/g d.p.m. at 700 W to 102.95 mg RE/g d.p.m. at 300 W) (Table 2). The other two extraction methods, UAE and CSE, showed similar results, with slightly higher values observed for CSE. The amount of total flavonoids extracted ranged from 77.69 mg RE/g d.p.m. at 60 °C to 81.75 mg RE/g d.p.m. at RT when UAE was used, and from 76.56 mg RE/g d.p.m. at RT to 85.20 mg RE/g d.p.m. at 40 °C when the CSE method was used. Similar to the TPC, the TFC increased more than ten-fold in two halophytes tested when UAE was used compared to the SFE method [43].

The TTC was approximately equal for MAE at 700 W (5.63 mg CE/g d.p.m.) and UAE at RT (5.67 mg CE/g d.p.m.) (Figure 3 and Table 2). Huma et al. [42] compared unconventional extraction methods, MAE and UAE, with CSE by maceration in the extraction of tannins from *Ceratonia siliqua*. They used 45% ethanol as the solvent, and with approximately equal TTC values, the MAE method was the most efficient, taking only 4.5 min, while UAE required 30 min and CSE 120 min. RT (4.71 mg CE/g d.p.m.) appeared to be the most beneficial when CSE was used compared to elevated temperatures (3.35 mg CE/g d.p.m. at 40 °C; 3.40 mg CE/g d.p.m. at 60 °C). To obtain a similar yield of polyphenols in 50% ethanolic extracts of green tea leaves, the duration of the MAE method at 20 °C was shorter (4 min) compared to that of the UAE method (90 min) at the same temperature and heat-reflux extraction (45 min) at 85 °C [48].

3.3. HPLC-UV/VIS Analysis of Individual Phenolic Compounds

HPLC-UV/VIS analysis was performed for the qualitative and quantitative detection of phenolic compounds. Of the ten compounds detected, nine were non-flavonoids (phenolic acids), and only one flavonoid compound was detected (rutin). This finding was in agreement with the results of Siracusa et al. [1], who found that chlorogenic acid (CGA), together with its isomers and higher derivatives, represented the only class of phenolics in sea fennel. The influence of the extraction method on the content of each analyte was determined (Table 3). CGA was the dominant phenolic constituent in all samples, with the highest content achieved when using MAE (from 10.10 mg/g at 700 W to 10.67 mg/g at 500 W) (Figures 4 and 5). CSE, as the second most effective extraction method, achieved rates from 81% at 60 °C to 92% at 40 °C, and UAE demonstrated rates from 78% at 60 °C to 81% at RT, the most effective temperature for MAE. A strong positive correlation was observed between the TPC results and the chlorogenic acid content of the samples (r = 0.9692). Several other reports have classified CGA as the major phenolic component in sea fennel extracts. In our previous study [5] on the different parts of the sea fennel plant, similar concentrations of chlorogenic acid were found in the samples. The extracts were prepared in 80% ethanol (*v*/*v*) by UAE at 50 °C, and the CGA concentration in the leaves was 8.1 mg/g. Lower concentrations were detected in the flowers, while the concentrations in the stems were significantly lower (0.7 mg/g), indicating the importance of the plant part used for the study. In our later study on the influence of the harvest period on the phenolic composition of sea fennel, we reported that the samples collected in April using the method described above had the highest TPC and CGA concentration, while the CGA concentration in the other extracts ranged from 5.65 to 7.48 mg/g [6]. Alemán et al. [35] analyzed ethanol/water (70/30, *v*/*v*) and water extracts of sea fennel leaves and stems heated at 80 °C and sonicated. CGA was more abundant in the ethanolic extract (58.48 mg/g) than in the aqueous extract (42.61 mg/g). High concentrations of CGA, as well as its derivatives nCGA and cCGA, were detected by Pereira et al. [7] in sea fennel decoctions and infusions prepared using different parts of sea fennel plants. High concentrations of CGA in the methanolic extract mixed at 4 °C were found in sea fennel growing in sand (18.8–27.9 mg/g) and cliffs (3–10 mg/g) [44]. Routray and Orsat [49] compared the yield of CGA in blueberry leaves obtained after 1 h UAE and 24 h RT MAE (4, 14, and 24 min) at different power levels. The MAE of CGA from blueberry leaves was more effective than UAE for 24 h at RT, regardless of the duration and power level.

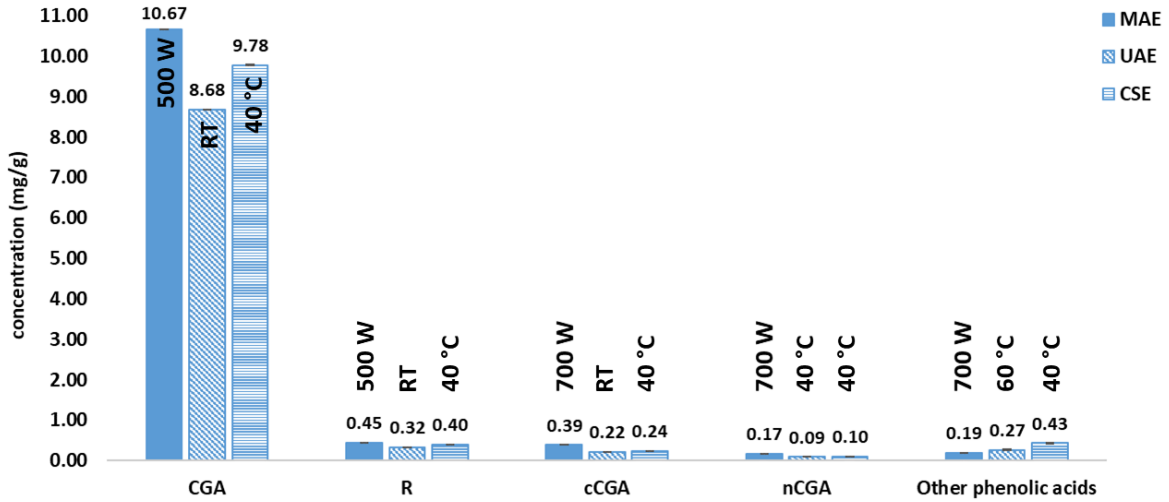

Figure 4. Comparison of the detected concentrations of phenolic compounds expressed in mg/g under the most effective conditions for each extraction method analyzed by HPLC-UV/VIS: chlorogenic acid (CGA); rutin (R); cryptochlorogenic acid (cCGA); neochlorogenic acid (nCGA); other phenolic acids—*p*-hydroxybenzoic acid (PHBA), sinapic acid (SA), ferulic acid (FA), caffeic acid (CA), gallic acid (GA), protocatechuic acid (PCA).

Table 3. Concentrations of phenolic compounds in sea fennel extracts obtained by different extraction methods expressed in mg/g analyzed by HPLC-UV/VIS.

Phenolic Compound		Concentration (mg/g) ± SD								
		MAE			UAE			CSE		
		300 W	500 W	700 W	RT	40 °C	60 °C	RT	40 °C	60 °C
CGA		10.50 ± 0.00	10.67 ± 0.00	10.10 ± 0.01	8.68 ± 0.00	8.38 ± 0.00	8.33 ± 0.01	8.91 ± 0.00	9.78 ± 0.01	8.66 ± 0.00
R		0.43 ± 0.01	0.45 ± 0.00	0.42 ± 0.00	0.32 ± 0.00	0.12 ± 0.00	0.32 ± 0.00	0.34 ± 0.00	0.40 ± 0.00	0.34 ± 0.01
cCGA		0.37 ± 0.00	0.38 ± 0.00	0.39 ± 0.00	0.22 ± 0.00	0.21 ± 0.00	0.21 ± 0.00	0.20 ± 2 0.00	0.24 ± 0.00	0.21 ± 0.00
nCGA		0.16 ± 0.00	0.17 ± 0.00	0.17 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.00	0.09 ± 0.00
Other phenolic acids		0.72 ± 0.02	0.74 ± 0.01	0.75 ± 0.00	0.55 ± 0.00	0.53 ± 0.01	0.57 ± 0.00	0.67 ± 0.11	0.78 ± 0.01	0.69 ± 0.01
Other phenolic acids	PHBA	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.11 ± 0.07	0.20 ± 0.00	0.19 ± 0.00
	SA	0.05 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.15 ± 0.01	0.11 ± 0.01	0.05 ± 0.00	0.04 ± 0.00
	FA	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
	CA	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.07 ± 0.00
	GA	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	PCA	0.01 ± 0.00	0.01 ± 0.00	<0.01	<0.01	<0.01	<0.01	0.05 ± 0.00	0.06 ± 0.00	0.05 ± 0.00

Chlorogenic acid (CGA); rutin (R); cryptochlorogenic acid (cCGA); neochlorogenic acid (nCGA); other phenolic acids—*p*-hydroxybenzoic acid (PHBA), sinapic acid (SA), ferulic acid (FA), caffeic acid (CA), gallic acid (GA), protocatechuic acid (PCA); microwave-assisted extraction (MAE); ultrasound-assisted extraction (UAE); conventional solvent extraction (CSE); standard deviation (SD) (n = 2).

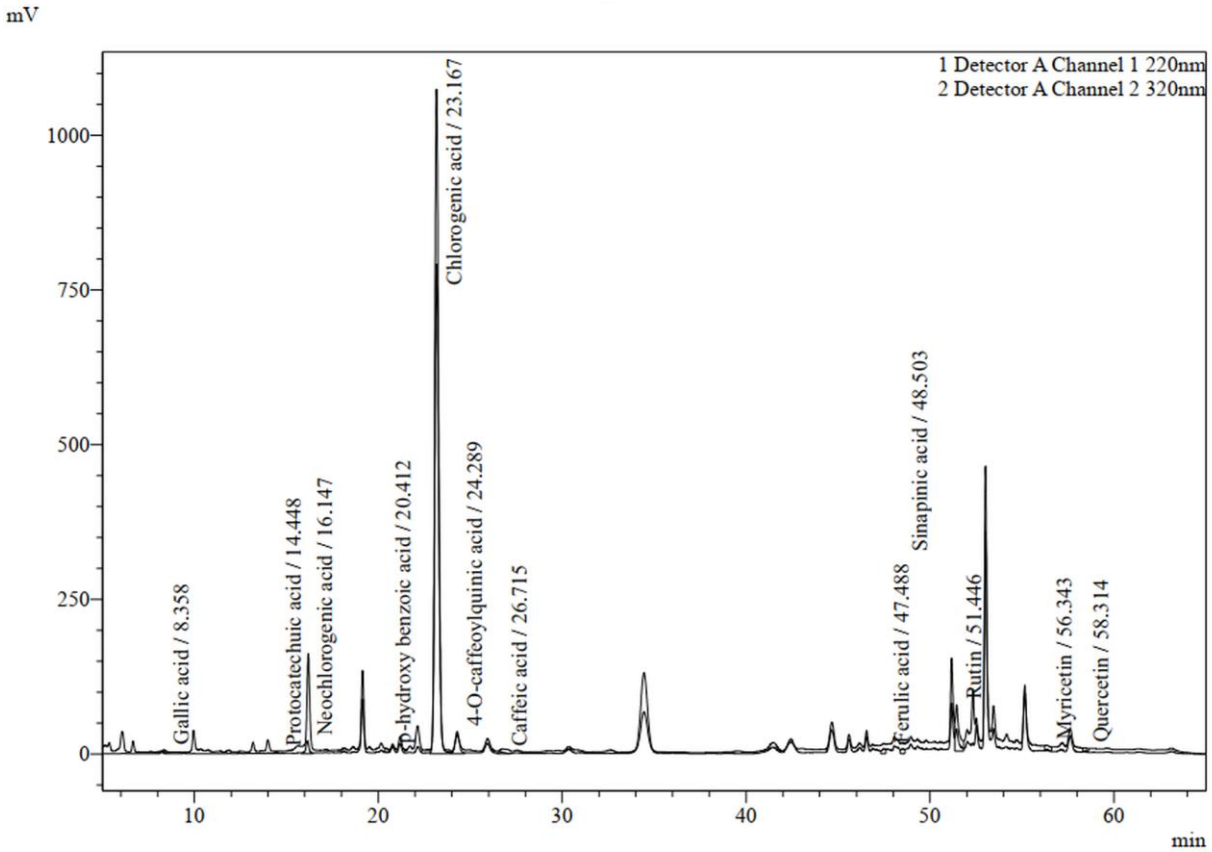

Figure 5. Chromatogram of the extract obtained under the optimum extraction conditions (MAE method; 500 W) for chlorogenic acid (the most abundant component).

Rutin (R), the only flavonoid detected and the second most abundant phenolic compound, was maximally extracted using MAE (0.42 mg/g at 700 W to 0.45 mg/g at 500 W) (Table 3, Figure 5). As for CGA, the CSE method was more effective than the UAE method, with a yield of 88% at 40 °C (Figure 4). In the study by Alemán et al. [35], rutin (4.52 mg/g) was detected only in the ethanol/water extracts (70/30, *v*/*v*) of sea fennel leaves. Chahyadi and Elfahmi [50] investigated the influence of CSE methods (maceration, water boiling, and reflux) and advanced extraction methods (UAE and MAE) on the rutin yield of cassava leaves. The highest yield was obtained with 60% aqueous ethanol via UAE at 50 °C for 90 and 120 min. MAE reduced the extraction time to 5 min at 540 W with 60% aqueous ethanol.

The two phenolic acids, cryptochlorogenic acid (cCGA) and neochlorogenic acid (nCGA), were extracted most effectively by the MAE method (Figure 4). The concentration values for cCGA ranged from 0.37 mg/g at 300 W to 0.39 mg/g at 700 W, and those for nCGA ranged from 0.16 mg/g at 300 W to 0.17 mg/g at 700 W. The second-best conditions for the extraction of cCGA and nCGA were CSE at 40 °C (Table 3). Again, the results for cCGA and nCGA were highly correlated with the TPC, with r values of 0.9267 and 0.9317, respectively.

Other phenolic acids included *p*-hydroxybenzoic acid (PHBA), sinapic acid (SA), ferulic acid (FA), caffeic acid (CA), gallic acid (GA), and protocatechuic acid (PCA). The highest content was obtained by the CSE method at 40 °C (0.43 mg/g) (Figure 4). For MAE, the most effective power level was 700 W (0.19 mg/g), and for UAE the most effective temperature was 60 °C (0.27 mg/g) (Table 3).

PHBA, FA, and coumaric acid were also detected in sea fennel infusions and decoctions by Pereira et al. [7].

When sea fennel by-products were extracted in 70% ethanol, most phenolic compounds were detected in the extract obtained by MAE (14 peaks) compared to UAE (11 peaks) and CSE (9 peaks). Vanillic acid (VA) and SA were more effectively extracted by CSE, while FA and CA were more effectively extracted by both UAE and MAE [51].

3.4. Antioxidant Activity

To analyze the antioxidant potential of the extracted samples and gain a better insight into their antioxidant properties, two different assays were used: reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results of the assays are shown in Figure 6. As expected, the highest antioxidant activity obtained by both the FRAP (173.50–185.01 µmol Fe^{2+}/g) and DPPH assays (55.74–58.46% inhibition of DPPH radicals) was observed in the samples extracted by the MAE method. The second-best antioxidant potential was observed for samples extracted by the CSE method and mixed at 40 °C, with 153.92 µmol Fe^{2+}/g (FRAP) and 51.06% inhibition of DPPH radicals (DPPH). This was consistent with the analysis of the phenolic compounds, as studies have confirmed that phenolic compounds have important antioxidant properties in plants [11], especially chlorogenic acid and its derivatives [52]. The beneficial antioxidant properties of chlorogenic acid, which have been demonstrated in a large number of studies, are due to the vicinal hydroxyl groups in its structure, which is why various studies have reported its significant role in the antioxidant properties of sea fennel extracts [5,11,44,53,54]. This was also confirmed by the results of our statistical analysis. A strong positive correlation was obtained between the results of the FRAP and DPPH assays and the TPC (r = 0.9603 and 0.9459, respectively), as well as between the FRAP and DPPH assays and the CGA (r = 0.9476 and 0.9187, respectively); nCGA (r = 0.9682 and 0.9526, respectively); and cCGA contents (r = 0.9639 and 0.9439, respectively). In agreement with this, in the study of Alemán et al. [35], the free-radical scavenging activity and reducing activity of sea fennel extracts were 1.4-fold and 2.1-fold higher in the 70% ethanolic extracts than in the water extracts. The free-radical scavenging activity of sea fennel from the Black Sea coast was studied by Cebi et al. [55], who reported very good activity against DPPH radicals, especially from methanolic extracts, which could be compared with the activity of commercial

antioxidants such as alpha-tocopherol and butylated hydroxytoluene (BHT). The beneficial antioxidant activity of sea fennel preparations against various free radicals, including DPPH, and their reducing activity, as shown by the FRAP method, were confirmed by Pereira et al. [7] in their study.

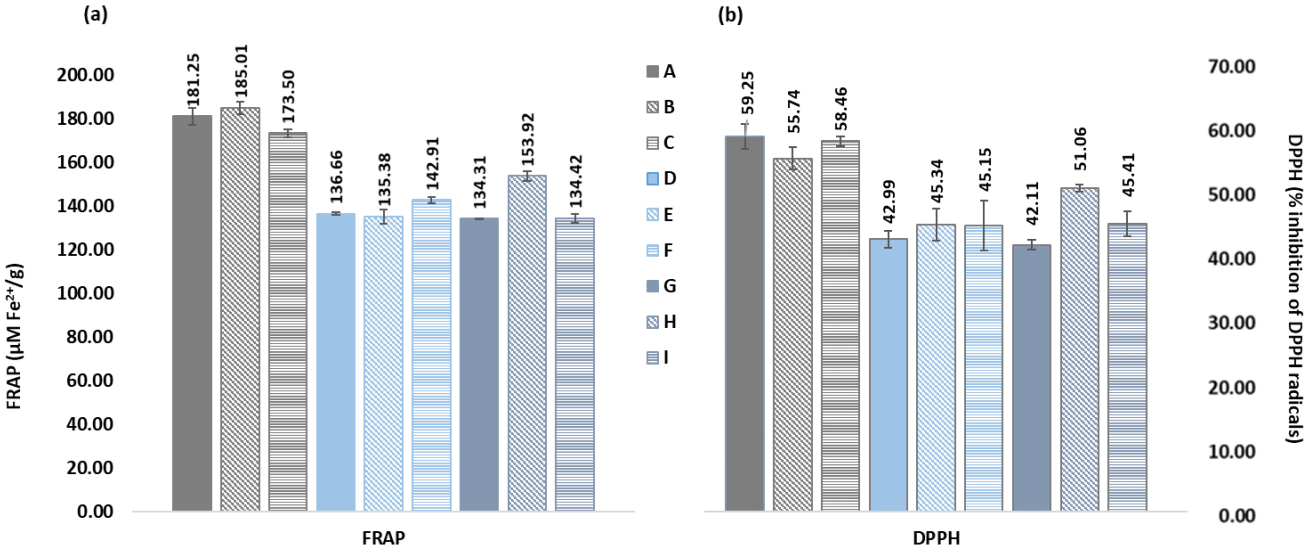

Figure 6. The free-radical scavenging activity of extracted samples obtained using (**a**) ferric reduction antioxidant power (FRAP) and (**b**) 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays (mean ± SD, n = 3). Extraction conditions: MAE (A—300 W, B—500 W, C—700 W); UAE (D—RT, E—40 °C, F—60 °C); CSE (G—RT, H—40 °C, I—60 °C).

From the reported results and their comparison with the data of other authors, it could be concluded that the choice of the most suitable extraction conditions (solvent, time, temperature, the application of additional treatments such as ultrasound and microwaves, etc.) depended on both the target compounds to be extracted and the raw material used (plant and plant part). In the case of sea fennel, the phenolic compounds present in the samples were predominantly phenolic acids, while other phenolic subgroups were present in much lower proportions. In this study, the extraction temperatures for CSE and UAE were limited to 60 °C, since this value is usually reported as the temperature that does not affect the stability of phenolics and does not cause their degradation. However, for MAE, the temperature of the samples almost reached the boiling temperature, which could have been the reason for the high phenolic content of the samples obtained by this extraction method and their corresponding high antioxidant activity.

3.5. Greenness Assessment Method

In this study, AGREE [33], an analytical environmental performance metric for sample preparation, was used to evaluate the environmental performance of the extraction methods tested. In this way, the most appropriate extraction method for a given sample type could be determined. The results are presented in Figure 7, which shows the evaluation of the environmental performance of the extraction methods.

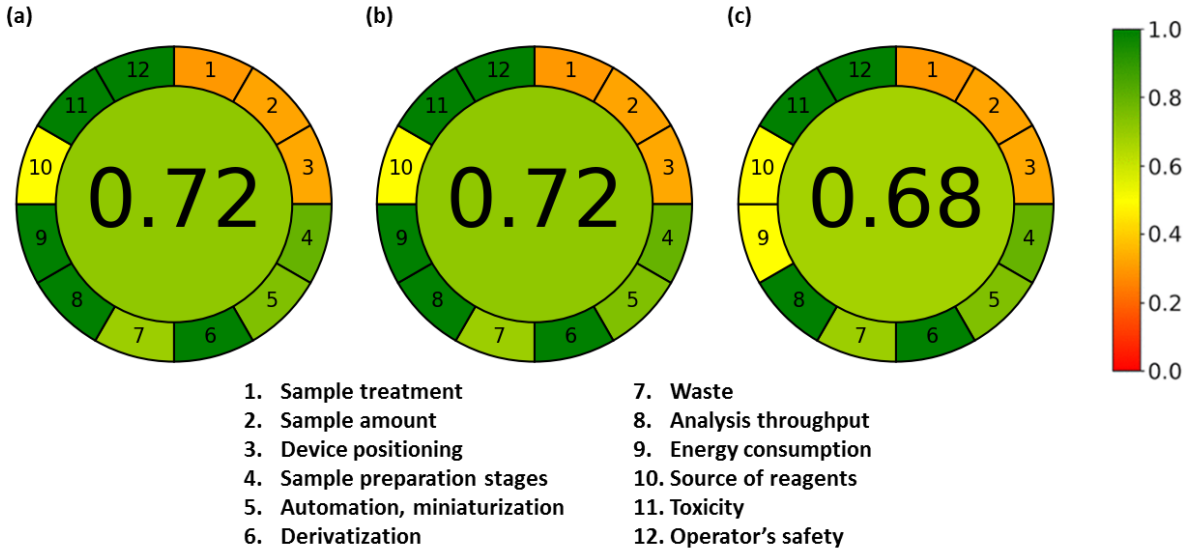

Figure 7. Results of the AGREE analysis for each extraction method performed: (**a**) CSE, (**b**) UAE, (**c**) MAE.

The comparison showed that the UAE and CSE methods were more environmentally friendly than the MAE method, with a final score of 0.72. The final score of the MAE method was 0.68, as it was more energy-intensive than the UAE and CSE methods but still competitive. Improving the efficiency of sample processing by minimizing the amount of sample used could improve the method overall.

4. Conclusions

In this study, the effects of different extraction methods on the extraction of phenolic compounds from sea fennel were investigated. The results showed that the maximum amount of extracted phenolic compounds was obtained with a mixture of water and ethanol at a ratio of 1:1 (v/v), while the lowest phenolic content was obtained in extracts prepared using pure water (65%) and pure ethanol (32%), which was consistent with previous findings that ethanol water mixtures were more efficient than the corresponding mono-component solvent systems in the extraction of phenolics. When comparing the extraction methods applied for the isolation of phenolics, microwave-assisted extraction showed the highest efficiency in phenolic extraction, followed by conventional solvent extraction with stirring at 40 °C. In the case of microwave-assisted extraction, a higher microwave power resulted in a lower phenolic content in the sea fennel extracts. A temperature effect was observed for conventional solvent extraction, and the highest content of phenolics was obtained in the extract prepared at 40 °C, while for ultrasound-assisted extraction this temperature produced the lowest amount of phenolics. High-performance liquid chromatography revealed chlorogenic acid as the dominant phenolic compound in all samples, followed by rutin as the only detectable flavonoid and the phenolic acids cryptochlorogenic acid and neochlorogenic acid. The antioxidant assays, ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryl-hydrazyl (DPPH), were performed following the acquisition of the extraction results and showed the highest antioxidant potential in the samples extracted by microwave-assisted extraction. The use of generally recognized as safe (GRAS) water and solvents such as ethanol during the extraction process could ensure a safe and high-quality extract, while replacing the conventional solvent extraction method with green extraction methods reduced energy and solvent consumption and increased the extraction yield.

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Abbreviations

AGREE	analytical greenness
BHT	butylated hydroxytoluene
CA	caffeic acid
CEs	catechin equivalents
cCGA	chlorogenic acid
nCGA	neochlorogenic acid
CSE	conventional solvent extraction
d.p.m.	dry plant material
DPPH	2,2-diphenyl-1-picryl-hydrazyl
EAE	enzyme-assisted extraction
EtOH	ethanol
FA	ferulic acid
FDA	US Food and Drug Administration
FRAP	ferric reducing antioxidant power
GA	gallic acid
GAEs	gallic acid equivalents
GRAS	generally recognized as safe
HPLC-UV/VIS	high-performance liquid chromatography coupled with an ultraviolet/visible detector
MAE	microwave-assisted extraction
PCA	protocatechuic acid
PEF	pulsed electric field
PLE	pressure-assisted liquid extraction
PHBA	*p*-hydroxybenzoic acid
R	rutin
REs	rutin equivalents
RT	room temperature
SA	sinapic acid
SC-CO_2	supercritical CO_2 extraction
SD	statistical deviation
TFC	total flavonoid content
TPC	total phenolic content
TTC	total tannin content
UAE	ultrasound-assisted extraction
UV-VIS	ultraviolet–visible spectrophotometric measurements

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