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Antioxidant Activity of Carob Tree (*Ceratonia siliqua* L.) Leaf Extracts Obtained by Advanced Extraction Techniques

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Abstract: Carob (*Ceratonia siliqua* L.) is a widely spread Mediterranean evergreen tree whose plant parts are rich in bioactive compounds with potential for application in functional food production. Carob leaves are the least explored part of the plant, and the main compounds of interest are polyphenols. Advanced extraction techniques, such as pressurized liquid (PLE), microwave-assisted (MAE) and ultrasound-assisted (UAE) extraction have not been sufficiently explored for their potential in extracting these compounds. Therefore, the aim of this paper was to optimize the parameters (temperature and time) of PLE, MAE and UAE of carob leaf polyphenols, characterize the individual compounds by ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS^2) and determine the antioxidant activity of the extracts. The optimal temperature and time were 160 °C/5 min for PLE, resulting in a total phenolic content of 68.21 mg of gallic acid equivalents (GAE) g^{-1}. For MAE and UAE, the optimal conditions were 70 °C/10 min, under which total phenolic contents of 78.80 and 55.98 mg GAE g^{-1} were achieved, respectively. A total of 26 compounds, represented mainly by myricetin, quercetin-3-rhamnoside and gallic acid, were identified in extracts obtained by all three extraction techniques. All extracts showed high antioxidant activity (0.46–1.05 and 0.50–0.58 mmol TE g^{-1} for FRAP and DPPH, respectively), which correlated with the polyphenolic content and was affected by the concentration of ascorbic acid (0.03–0.52 mg mL^{-1}), confirming that carob leaf extracts obtained by advanced extraction possess high potential for application in functional food products.

Keywords: carob leaves; green extraction; pressurized liquid extraction; microwave-assisted extraction; ultrasound-assisted extraction; polyphenols; antioxidant activity

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1. Introduction

In light of growing consumer demand for functional products enhanced with natural elements, there has been a surge in interest to explore novel sources of natural ingredients. One of the plant species that proved to be an excellent basis for obtaining functional products, due to its rich chemical composition, is carob.

Carob (*Ceratonia siliqua* L.) is a perennial, evergreen tree from the Fabaceae family, native to the Mediterranean region, but now widely distributed across the world. It often grows in dry and rocky areas, preferring warm climates. Carob is resistant to drought and soil salinity and can tolerate high temperatures, enabling it to thrive in challenging environments, expanding agricultural possibilities and contributing to food security in areas prone to arid conditions [1,2]. Its significance is appreciated not only for its resilience and economic value, but also for the nutritional value of all parts of the plant (pods, pulp, seeds, leaves), which are used in the food, pharmaceutical and cosmetic industries due to their biological activities [3]. Carob pods consist of 90% pulp and 10% seeds. They contain large amounts of carbohydrates, fibers, proteins, minerals, fats and polyphenols and have

been used in human nutrition and as animal feed since ancient times [4,5]. The pulp is used to make molasses and syrup, while carob seed powder is commonly utilized as a cocoa substitute in various food applications, as it has a similar sweet flavor but contains less fat and caffeine [6]. In addition, the carob leaves, which are often overshadowed by prominent pods, are a rich source of valuable nutrients, such as carbohydrates, protein and fiber, and contain even more polyphenols, compared to pods [4,7]. Polyphenols have an antioxidant effect and can contribute to various health benefits [8]. As a result of the high content of bioactive molecules, carob is not only a source of powerful antioxidants, but also has antimicrobial, antiproliferative and anti-inflammatory effects, being used as a diuretic, an anti-diarrheal agent and for treating obesity [3,6,9].

In order to isolate highly valuable bioactive compounds such as polyphenols from the matrix, an extraction process is required. It is therefore important to apply and optimize a suitable extraction technique that enables a high yield and stability of the target components during extraction and storage [10,11]. Various techniques can be applied to obtain the extracts, and advanced extraction techniques, such as pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are increasingly being applied. These techniques, compared to conventional extraction, enable rapid and environmentally friendly extraction by improving solvent penetration into leaf sample pores and enhancing mass transport. This results in increased leachability of target components, leading to higher yields, while consuming a lower amount of energy and solvent, which is why they are considered as "green techniques" [12,13]. Optimizing multiple process parameters is crucial for the efficient extraction and isolation of bioactive molecules such as polyphenols having in mind optimal yield and superior quality of the extracts. The most important parameters include the selection of the suitable solvent, temperature and extraction time. Following the extraction process, the subsequent stage involves the identification and quantification of targeted compounds. Ultra-performance liquid chromatography, coupled with tandem mass spectrometry (UPLC-MS^2), has proven to be an excellent technique for the chemical characterization of polyphenols, as it integrates chromatographic and spectral techniques [14]. Overall, the combination of advanced extraction techniques with UPLC-MS^2 enables reliable and precise analysis of polyphenols in a wide range of samples.

The influence of advanced extraction techniques on polyphenols from carob leaves has not yet been sufficiently investigated and, to our knowledge, MAE and PLE have never been applied to carob leaves. Consequently, the aim of this work was to examine the influence of different extraction conditions of PLE, MAE and UAE on the polyphenol content in carob leaf extracts and to determine the optimal extraction conditions for their isolation. An additional aim was to characterize individual polyphenols from carob leaf extracts obtained by advanced techniques under optimal conditions using UPLC-MS^2 and to examine their antioxidant activity by DPPH and FRAP assays.

2. Materials and Methods

2.1. Chemicals and Reagents

Purified water was prepared utilizing the advanced Milli-Q system manufactured by Millipore Corp. (Bedford, NY, USA), ensuring the highest quality for experimental procedures. High-performance liquid chromatography (HPLC) grade acetonitrile (CH_3CN) and acetic acid (CH_3COOH) of exceptional purity were procured from J.T. Baker Chemicals in Deventer, The Netherlands. The Folin-Ciocalteu reagent was acquired from Merck (Darmstadt, Germany). Ethanol (C_2H_5OH, 96%) of analytical grade was sourced from Kemika d.d. (Zagreb, Croatia). Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ascorbic acid, sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$, 99%) and sodium carbonate (Na_2CO_3, 99.5%) were obtained from Kemika d.d. (Zagreb, Croatia). Antioxidants reference standards, such as Trolox ($C_{14}H_{18}O_4$) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), vital for assessing radical scavenging activity, were procured from Acros Organics (Geel, Belgium). High purity methanol (CH_3OH) was sourced from Honeywell (Riedel-de-Haën, Bucharest, Romania).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,6-dichlorophenolindophenol (DCPI, 97%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl, 37%) was sourced from Carlo Erba Reagents (Val-de-Reuil, France). Commercial HPLC standards, including rosmarinic acid ($C_{18}H_{16}O_8$), gallic acid ($C_7H_6O_5$), syringic acid ($C_9H_{10}O_5$), *p*-coumaric acid ($C_9H_8O_3$), caffeic acid ($C_9H_8O_4$), chlorogenic acid ($C_{16}H_{18}O_9$), protocatechuic acid ($C_7H_6O_4$), ferulic acid ($C_{10}H_{10}O_4$), myricetin ($C_{15}H_{10}O_8$) and quercetin-3-glucoside ($C_{21}H_{20}O_{12}$), were selected from Sigma–Aldrich (St. Louis, MO, USA) to serve as precise standards for analytical calibration. Additionally, supplemental compounds, such as procyanidin B2, catechin, epicatechin gallate, epigallocatechin gallate, rutin ($C_{27}H_{30}O_{16}$), kaempferol-3-glucoside and apigenin were obtained from Extrasynthese (Genay, France). Quercetin-3-rutinoside ($C_{27}H_{30}O_{16}$) was sourced from Thermo Fisher Scientific (Waltham, MA, USA). Standard stock solutions were produced in methanol. For the specific compound apigenin, dissolution in ethanol with dimethyl sulfoxide (0.5%) was carried out. Working standard solutions (10–200 mg L^{-1}) were then prepared by diluting the prepared stock solutions to achieve five determined concentrations, ensuring precise calibration for accurate analytical measurements.

2.2. Plant Material

Wild carob leaves were collected in Grgurići, Croatia (42.784227 N, 17.876950 E), in August 2023 and identified by the Laboratory for Biology and Microbial Genetics at the Faculty of Food Technology and Biotechnology, University of Zagreb. After harvest, the material was air-dried in shade at ambient temperature until dry matter content >95%, as determined by drying to constant mass at 103 ± 2 °C, was achieved. Before the extraction procedures, the leaves were ground into a coarse powder (particle size 0.45–1 mm) using the GT 1108 electric grinder (Tefal, Rumily, France).

2.3. Pressurized Liquid Extraction (PLE)

Polyphenols were extracted from carob leaf sample using PLE with a Dionex™ ASE™ 350 extractor from Thermo Fisher Scientific Inc. (Sunnyvale, CA, USA), employing 50% aqueous ethanol as the extraction solvent. Ground carob leaves (1 g) were combined with 2 g of Dionex™ ASE™ diatomaceous earth (Thermo Fisher Scientific Inc, Sunnyvale, CA, USA; lot no. 171) and placed into 34 mL stainless steel cells equipped with two cellulose filters. To optimize polyphenols isolation, variations in temperature (ranging from 60 to 160 °C) and static time (5 to 15 min) were applied (refer to Table 1), while maintaining other extraction parameters constant: one extraction cycle, pressure of 10.34 MPa, 30 s of nitrogen purge, and 30% volume flush. After the extraction, the obtained extract was filtered using Whatman No. 40 filter paper into a 50 mL volumetric flask and supplemented with the extraction solvent. The resulting extracts were stored in plastic Falcon tubes at −18 °C. All extractions were performed in duplicate (n = 2).

2.4. Microwave-Assisted Extraction (MAE)

The polyphenolic extracts from carob leaves were obtained by MAE in an Ethos Easy reactor (Milestone, Italy). The extraction was carried out with a constant power of 500 W, stirring at 50% and a post-extraction ventilation time of 1 min. The varied parameters were temperature (30–80 °C) and extraction time (5–15 min) (Table 1). The time required to achieve the extraction temperature was varied as follows: 1 min for temperatures of 30 and 40 °C, 2 min for temperatures of 50 and 60 °C and 3 min for temperatures of 70 and 80 °C. For every extraction run, 1 g of ground sample was blended with 40 mL of 50% aqueous ethanol in an extraction vessel equipped with a magnetic stirrer, which was placed in the microwave extractor. Subsequently, the process after extraction was the same as described in Section 2.3.

Table 1. Experimental design and values of total phenolic content in carob leaf extracts obtained by PLE, MAE and UAE.

Extraction Technique	Temperature (°C)	Extraction Time (min)	Total Phenols (mg GAE g^{-1} Leaf)
PLE	60	5	40.81 ± 0.63
		10	34.96 ± 0.70
		15	33.72 ± 0.29
	80	5	44.17 ± 0.22
		10	44.91 ± 0.31
		15	43.72 ± 0.50
	100	5	42.90 ± 0.49
		10	37.09 ± 0.52
		15	42.94 ± 0.98
	120	5	46.86 ± 0.42
		10	53.60 ± 0.18
		15	42.34 ± 0.83
	140	5	62.73 ± 0.28
		10	53.47 ± 0.13
		15	67.73 ± 0.46
	160	5	68.21 ± 0.31
		10	68.94 ± 0.54
		15	80.73 ± 0.20
MAE	30	5	60.78 ± 0.90
		10	56.81 ± 0.61
		15	62.84 ± 0.30
	40	5	51.25 ± 0.59
		10	69.20 ± 0.60
		15	61.60 ± 0.68
	50	5	61.74 ± 0.34
		10	63.18 ± 0.73
		15	71.00 ± 0.43
	60	5	63.27 ± 0.68
		10	71.58 ± 0.53
		15	73.93 ± 0.43
	70	5	65.50 ± 0.41
		10	78.80 ± 1.29
		15	90.67 ± 0.93
	80	5	75.88 ± 0.76
		10	83.02 ± 0.72
		15	82.41 ± 1.43
UAE	30	5	31.64 ± 0.22
		10	51.44 ± 0.85
		15	42.21 ± 0.57
	40	5	45.23 ± 0.29
		10	32.46 ± 0.62
		15	46.52 ± 0.59
	50	5	30.23 ± 0.35
		10	45.60 ± 0.09
		15	42.04 ± 0.24
	60	5	25.87 ± 0.23
		10	44.39 ± 0.79
		15	53.27 ± 0.95
	70	5	43.75 ± 1.41
		10	55.98 ± 0.53
		15	55.82 ± 0.48
	80	5	40.01 ± 0.13
		10	43.82 ± 0.51
		15	51.21 ± 1.23

PLE = pressurized liquid extraction. MAE = microwave-assisted extraction. UAE = ultrasound-assisted extraction. GAE = gallic acid equivalents. Values are expressed as mean ± SD.

2.5. Ultrasound-Assisted Extraction (UAE)

For the UAE of carob leaves polyphenols, an ultrasound bath (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany) equipped with a temperature control unit was used at the standard device frequency of 37 kHz. The temperature (30–80 °C) and the sonication time (5–15 min) were varied as shown in Table 1. Briefly, a glass beaker containing 1 g of ground sample was combined with 40 mL of 50% aqueous ethanol pre-heated at the selected temperatures and placed in the ultrasonic bath during the varied extraction times. The process after extraction was the same as described in Section 2.3.

2.6. Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined by a slightly modified method previously described [15]. A volume of 100 µL of appropriately diluted extract (50% aqueous ethanol for blank) was mixed with 200 µL of Folin-Ciocalteu reagent and 2000 µL of distilled water in a test tube. After 3 min, 1000 µL of 20% Na_2CO_3, w/v aqueous solution was added to the mixture, which was then tempered in a water bath at the temperature of 50 °C for 25 min. Absorbance was read at the wavelength of 765 nm using the VWR UV 1600-PC spectrophotometer (VWR International GmbH, Darmstadt, Germany). Working standard solutions of gallic acid with a concentration range of 50–500 mg L^{-1} were used to produce a calibration curve from which the TPC was calculated and expressed as mg gallic acid equivalents (GAE) g^{-1} of dry carob leaf. Each measurement was conducted in duplicate.

2.7. Antioxidant Activity

The determination of antioxidant activity in the extracts was carried out using two methodologies, as detailed by Dobroslavić et al. [16].

2.7.1. FRAP-Ferric Reducing Antioxidant Power Assay

To prepare the FRAP reagent, 20 mM aqueous iron (III) chloride solution, 10 mM TPTZ reagent in 40 mM hydrochloric acid and 300 mM acetate buffer (pH 3.8) were mixed in a ratio of 1:1:10. Additionally, 240 µL distilled water, 80 µL sample and 2080 µL FRAP reagent were added into the glass tubes, mixed well (with a vortex mixer) and incubated at 37 °C for 5 min. Afterwards, the absorbance was read at 593 nm on the spectrophotometer. The calibration curve was prepared using working standard solutions of Trolox in the concentration range of 25–1000 µmol L^{-1}. The results were expressed as mmol equivalents of Trolox (TE) per g of dry carob leaf. Each measurement was conducted in duplicate (n = 4).

2.7.2. DPPH Radical Scavenging Assay

Briefly, 750 µL of extract and 1500 µL of 0.2 mM DPPH solution in methanol were added into the test tube. The test tubes with the contents were kept for 20 min in the dark at room temperature, after which the absorbance was measured at 517 nm using the VWR UV 1600-PC spectrophotometer (VWR International GmbH, Darmstadt, Germany), with methanol as a blank. The calibration curve was prepared using working standard solutions of Trolox in the concentration range of 10–150 µmol L^{-1}. The results were expressed as mmol TE per g of dry carob leaf. Each measurement was conducted in duplicate.

2.8. Determination of Ascorbic Acid Content

The content of ascorbic acid was determined according to standard simple redox titration of ascorbic acid by 2,6-dichlorophenolindophenol (DCPIP) [17], which served as an oxidant and acid-base indicator, aiding in pinpointing the end point of titration, marked by the appearance of a pink hue in the solution, caused by the surplus of DCPIP reagent. Briefly, DCPIP reagent was standardized by titration of 5 mL of ascorbic acid solution (0.2 mg mL^{-1}), which was mixed with 150 mL distilled water and 5 mL of 10% (v/v) aqueous acetic acid, thus obtaining f_{AA}, which reflects the concentration of ascorbic acid responsive to 1 mL of DCPIP. After standardization, the same procedure was carried out with

blank (distilled water) and the extracts (in quadruplet), thus obtaining the mean volumes of DCPI spent for titration (V_B and V_E) and f_{DCPIP}, calculated as follows (Equation (1)):

$$f_{DCPIP} = \frac{5\ \text{mL}}{V_B} \quad (1)$$

where f_{DCPIP} is the DCPIP factor, and V_B is the volume of DCPIP spent for titration of blank.

The concentration of ascorbic acid in the extract was then calculated according to Equation (2):

$$\frac{(V_E - V_B) \times f_{DCPIP} \times f_{AA}}{V} \quad (2)$$

where V_E is the volume of DCPIP spent for titration of the extract, V_B is the volume of DCPIP spent for titration of blank, f_{DCPIP} is the DCPIP factor calculated from V_B, f_{AA} is the concentration of ascorbic acid responsive to 1 mL of DCPIP and V is the volume of extract used in titration.

2.9. Polyphenolic Characterization of the Extracts by UPLC-MS^2

The carob leaf extracts, obtained by PLE, MAE and UAE at optimal conditions, were filtered into glass vials through 0.45 µm syringe filters and analyzed by UPLC-MS^2 on the Agilent series 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) paired with an Agilent 6430 Triple Quad LC/MS mass spectrometer (Agilent, Santa Clara, CA, USA) and Agilent MassHunter Workstation Software (ver. B.04.01, Agilent, Santa Clara, CA, USA) for data processing and instrument control. The analytes were ionized by ESI ion source in positive and negative modes. Desolvation and collision were performed using nitrogen at a flow rate of 11 L h^{-1}, temperature of 300 °C, nebulizer pressure of 2.76 bar and capillary voltage of 4/−3.5 kV. Separation was carried out using a C18 column (Agilent Zorbax Eclipse Plus; 100 × 2.1 mm; particle size 1.8 µm, Agilent, Santa Clara, CA, USA) at the temperature 35 °C and an injection volume of 2.5 µL. The solvent composition, gradient conditions, collision energy for individual compounds and the analytical quality parameters, including detection and quantification limits, were described previously [18]. Compounds were identified based on the standard calibration or, for the compounds lacking reference standards, through comparison of mass fragmentation patterns with literature reports, as described in detail previously [18]. Concentrations of identified polyphenols were expressed as mg g^{-1} of dry carob leaf. All analyses were performed in duplicate.

2.10. Experimental Design and Statistical Analysis

Statistica version 10.0 software (Statsoft Inc., Tulsa, OK, USA) was employed for comprehensive data analysis. To optimize each extraction technique, a full factorial design, as outlined in Table 1, was conducted. This design involved 18 experimental trials, each performed in duplicate, to evaluate how extraction parameters influenced the TPC of the resulting extracts. The independent variables (X), which included temperature and extraction time, were varied across six and three levels, respectively, while TPC was designated as the dependent variable (Y). To ensure the robustness of the analysis, assessments for normality and homogeneity of data and residuals were carried out utilizing Shapiro–Wilk's and Levene's tests, respectively. For datasets meeting normality and homogeneity criteria, a one-way analysis of variance (ANOVA) was employed, followed by Tukey's Honest Significant Difference (HSD) multiple comparison test. Conversely, for datasets not meeting these criteria, the Kruskal–Wallis one-way ANOVA, coupled with multiple comparisons of mean ranks, was utilized. Moreover, the values obtained from UPLC-MS^2 analysis and antioxidant activity assays were compared using a one-way ANOVA. All statistical analyses considered significance at $p \leq 0.05$.

3. Results and Discussion

This paper studied the influence of temperature and time during the PLE, MAE and UAE of polyphenols from carob leaves. The raw data were statistically analyzed, and the results are shown in Table 2. The extracts, which were chosen as optimal based on the results of the statistical analysis, were further analyzed by UPLC-MS^2 in order to determine the individual polyphenolic content, while the antioxidant activity in the selected extracts was determined by the DPPH and FRAP methods. As shown in Table 1, the TPC of carob leaf extracts ranged from 25.87 to 90.67 mg GAE g^{-1}, which is in the range reported in the literature for carob leaves. The few existing studies reported TPC values of 6.28 [19], 21.41 [20], 45.26 [7] and up to 680 mg GAE g^{-1}, achieved through successive extraction and fractionation with different solvents [21]. As for differences between the techniques, the obtained ranges overlapped (PLE 33.72–80.73 mg GAE g^{-1}, MAE 51.25–90.67 mg GAE g^{-1} and UAE 25.87–55.98 mg GAE g^{-1}), with UAE yielding the lowest value and MAE yielding the highest value. Since similar values were achieved by all three techniques, the differences were likely a result of the applied extraction parameters, the effects of which are discussed further in text.

Table 2. Influence of temperature and time on the phenolic content of carob leaf extracts obtained by PLE, MAE and UAE.

	Source of Variation	N	Total Phenols (mg GAE g^{-1})
PLE	Temperature (°C)		$p < 0.01$ *
	60	12	36.50 ± 0.94^{a}
	80	12	44.27 ± 0.18^{b}
	100	12	$40.98 \pm 0.85^{a,b}$
	120	12	47.60 ± 1.40^{b}
	140	12	61.31 ± 1.78^{c}
	160	12	72.63 ± 1.73^{d}
	Static time (min)		$p = 0.49$
	5	24	50.95 ± 2.20^{a}
	10	24	48.83 ± 2.40^{a}
	15	24	51.87 ± 3.46^{a}
MAE	Temperature (°C)		$p < 0.01$ *
	30	12	60.14 ± 0.77^{a}
	40	12	60.68 ± 2.22^{a}
	50	12	$65.31 \pm 1.23^{a,b}$
	60	12	69.59 ± 1.39^{b}
	70	12	78.32 ± 3.11^{c}
	80	12	80.43 ± 1.01^{c}
	Time (min)		$p < 0.01$ *
	5	24	63.07 ± 1.52^{a}
	10	24	70.43 ± 1.85^{b}
	15	24	73.74 ± 2.15^{b}
UAE	Temperature (°C)		$p < 0.01$ *
	30	12	41.76 ± 2.44^{a}
	40	12	41.40 ± 1.92^{a}
	50	12	39.29 ± 1.98^{a}
	60	12	41.18 ± 3.44^{a}
	70	12	51.85 ± 1.74^{b}
	80	12	45.01 ± 1.42^{a}
	Time (min)		$p < 0.01$ *
	5	24	36.12 ± 1.52^{a}
	10	24	45.61 ± 1.52^{b}
	15	24	48.51 ± 1.11^{b}

PLE = pressurized liquid extraction. MAE = microwave-assisted extraction. UAE = ultrasound-assisted extraction. N = number of trials. GAE = gallic acid equivalents. Values are expressed as mean ± SE. Values within group marked with different letters are statistically different at $p \leq 0.05$. * Statistically significant at $p < 0.05$.

3.1. Influence of Temperature on the Total Phenolic Content of Carob Leaf Extracts

Temperature plays a vital role in the extraction process, as it directly impacts the rate of mass transfer and the solubility of targeted compounds. Typically, higher temperatures are anticipated to augment the diffusion of solvents into the plant matrix, consequently increasing the solubility of the targeted compounds and leading to their greater desorption [22]. However, high temperatures pose a risk of degradation thermosensitive compounds, which is why optimization is crucial. In the present research, temperatures applied for MAE and UAE were in the range of 30–80 °C, since the boiling point of 50% of aqueous ethanol is around 80 °C [23]. In the case of PLE, elevated temperatures were employed, due to the capability of high pressure to enable the utilization of solvents at temperatures surpassing their boiling points [24].

The results of PLE showed that higher applied temperatures had a statistically significant effect on the TPC of carob leaves, with the highest temperature of 160 °C emerging as the optimal one. In this study, the yield obtained at the highest applied temperature was 50% higher than that at the lowest temperature during PLE. According to the literature, PLE at temperatures above 150 °C leads to a significant increase in TPC yield due to the release of polyphenols from the plant material and the degradation of lignin, while at higher temperatures (above 180 °C), thermal degradation may occur [22,25]. Other studies on the leaves of *Urtica dioica* L. [26], *Elaeagnus rhamnoides* L. [27] and *Laurus nobilis* L. [16] also concluded that increasing the temperature in PLE increased the yield of TPC.

As for MAE, an increase of the TPC was observed at temperatures above 50 °C, with the highest value obtained at 70 °C. Further increase of temperature to 80 °C did not yield a higher TPC. Therefore, 70 °C emerged as optimal for the MAE of carob leaf polyphenols. Dobrinčić et al. [28] also observed a rise in the TPC of *Olea europaea* L. leaf extracts with the increase in temperature from 45 °C to 80 °C, which is consistent with the results of this study. The results were also partially in line with the results achieved during MAE *Salvia officinalis* L. using 30% ethanol, where a steady increase of TPC was observed with the increase in temperature from 30 to 80 °C [28,29], with the difference of the optimal temperature in the mentioned study being 80 °C. The differences could be a result of varying chemical composition of plant materials, namely the presence of other constituents, such as proteins or carbohydrates, which influence the yield of extraction and therefore require different extraction conditions. In the case of UAE, results were similar to MAE, with no differences observed in the range of 30–60 °C, while the highest TPC was achieved at 70 °C. As opposed to MAE, further increasing the temperature to 80 °C resulted in a lower TPC, indicating thermal degradation. In addition, it was shown that during UAE, higher temperatures increase the solvent's vapor pressure, resulting in a lower cavitation force and a consequent lower yield of the targeted compounds [30].

3.2. Influence of Time on the Total Phenolic Content of Carob Leaf Extracts

Time is another important parameter during extraction of bioactive compounds. Prolonged exposure of the plant material to solvent is expected to enhance the extraction yield by promoting the diffusion of the compounds from the plant matrix [16]. However, prolonged exposure to the solvent might cause oxidation, or in case of higher temperatures, thermal degradation of the targeted compounds [31], therefore finding optimal time for each plant material is essential for a successful extraction process.

Regarding PLE, time had no statistically significant effect on the extraction of TPC from carob leaves, so the optimal extraction time was 5 min, offering both time and energy savings. Dobroslavić et al. [16] also concluded that time had no significant effect in optimizing the PLE conditions for the isolation of phenols from *L. nobilis* L. leaves. Similarly, other studies [32–34] have confirmed that a static time of 5 min during PLE proved to be effective for the isolation of polyphenols from various plant materials. Briefly, a short extraction time at a high temperature was optimal for efficient isolation and no degradation of the target components occurred.

In the case of both MAE and UAE, the statistical analysis showed an increase of TPC between 5 and 10 min, while further prolongation to 15 min had no significant influence indicating that, following the Fick's law of diffusion, equilibrium was achieved in that time [35]. Therefore, 10 min can be considered the optimal time for both techniques. This is consistent with the literature data on other plant materials. For example, 10 min was the optimal extraction time during the MAE of *Sideritis scardica*, *Sideritis raeseri* and *Origanum vulgare* L. [36], as well as *L. nobilis* L. polyphenols [11]. The same was observed during the UAE of polyphenols from *Mesembryanthemum edule* L. and *L. nobilis* L. [11,37].

Based on the results of the statistical analysis, the optimal temperature and time were defined for the three advanced techniques as follows: 160 °C and 5 min for PLE, 70 °C and 10 min for both MAE and UAE.

3.3. Individual Polyphenolic Content of the Carob Leaf Extracts

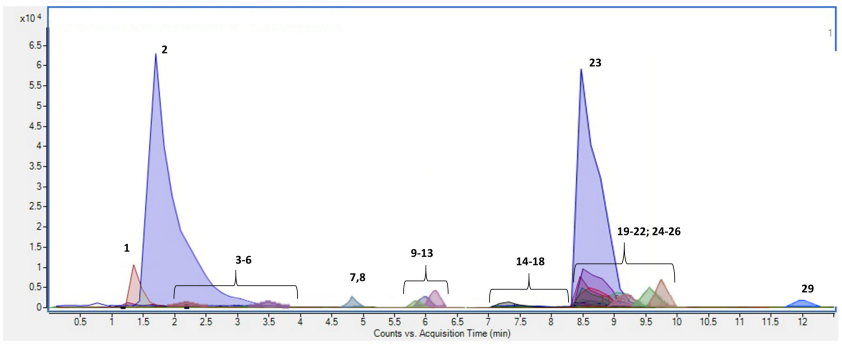
Once the optimal temperature and extraction time were established for each of the three extraction techniques used, the resulting optimal extracts underwent UPLC-MS^2 analysis (Table 3). A total of 26 compounds, comprising flavonols, phenolic acids, flavones, flavan-3-ols and proanthocyanidins, were identified in the extracts obtained through all three techniques. Their identification was based on comparisons with authentic standards or by matching fragmentation patterns described in prior literature [10]. Chromatograms are shown in Figure 1.

Flavonols were the predominant group of carob leaf polyphenols, represented mostly by myricetin and quercetin-3-rhamnoside, which are known to usually be the most abundant flavonoids in carob [6,38,39]. Phenolic acids were the second most significant group of polyphenols, with gallic acid making up approximately 90% of them, which is consistent with the literature, since gallic acid was shown to be the main representative of phenolic acids in carob leaves [3,6]. As for the influence of the extraction techniques, UAE yielded the lowest concentration of both flavonols and phenolic acids even though the optimal temperature and time were the same as in MAE. It is possible that the degradation of the compounds occurred due to application of a frequency higher than 20 kHz (37 kHz), which can increase the generation of hydroxyl radicals that promote the decomposition of polyphenols [40]. The content of flavonols was the same in the extracts obtained by PLE and MAE, while PLE was more successful in yielding phenolic acids, possibly due to the application of high temperature, above 150 °C, which was shown to result in the release of bound phenolic acids due to the breakage of lignin bonds [22]. The content of other groups was significantly lower than that of flavonols and phenolic acids. Flavan-3-ols were mainly represented by epigallocatechin gallate, which was found to be a significant flavan-3-ol in carob leaves [6], while the flavones whose content is expected to be low in carob leaves were represented by apigenin and luteolin [3]. Unlike the proanthocyanidin content in carob pods, which can reach up to 20% of dry weight, the content of proanthocyanidins in carob leaves was reported to be only 0.7% of dry weight [41]. Therefore, the low content of the detected procyanidin trimer type B is consistent with literature data. A slightly higher concentration of proanthocyanidins was observed in the extract obtained by UAE, likely due to the cavitation-induced mechanochemical breakage of polymer units [42]. The TPC determined by UPLC-MS^2 was lower than the one determined by spectrophotometric analysis and showed different trends between techniques where MAE was not dominant over PLE, likely due to the presence of other extracted non-phenolic compounds, such as ascorbic acid, carbohydrates, minerals, other organic acids, or pigments such as chlorophyll, which can all be detected by spectrophotometer [43,44] and cause an apparently higher result.

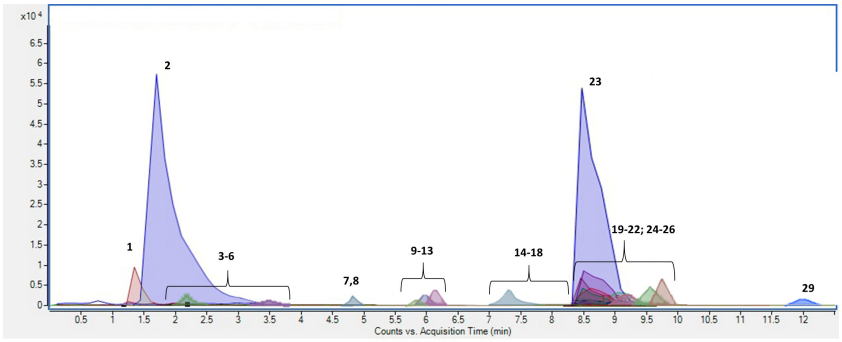
3.4. Antioxidant Activity of the Carob Leaf Extracts

The antioxidant activity of carob leaf extracts obtained at the optimal extraction parameters was evaluated using two in vitro model systems: FRAP and DPPH assays. The results, presented in Figure 2a,b, demonstrated that the antioxidant activity of extracts

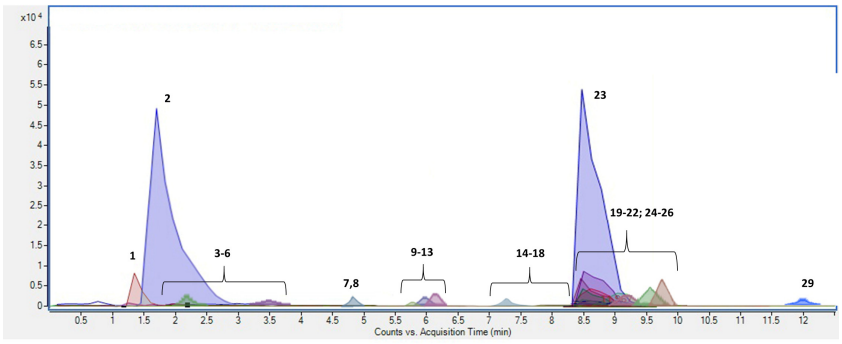
obtained by PLE, MAE, and UAE ranged between 0.46–1.02 mmol TE g^{-1} for the FRAP assay and 0.50–0.58 mmol TE g^{-1} for the DPPH assay. Custodio et al. [45] investigated the antioxidant activity of the extracts obtained by the decoction of various parts of the carob tree and found that the leaves showed the highest activity according to the FRAP and DPPH test, which can be attributed to the high polyphenolic content. Other studies [46,47] that compared the antioxidant activity of plant extracts using the above-mentioned methods also concluded that the highest antioxidant activity was shown by the extracts with the highest level of polyphenols. In order to investigate the effect of polyphenolic content in the optimal extracts obtained in this study and antioxidant activity, Pearson's correlation factors were calculated (Table 4).



(a)



(b)



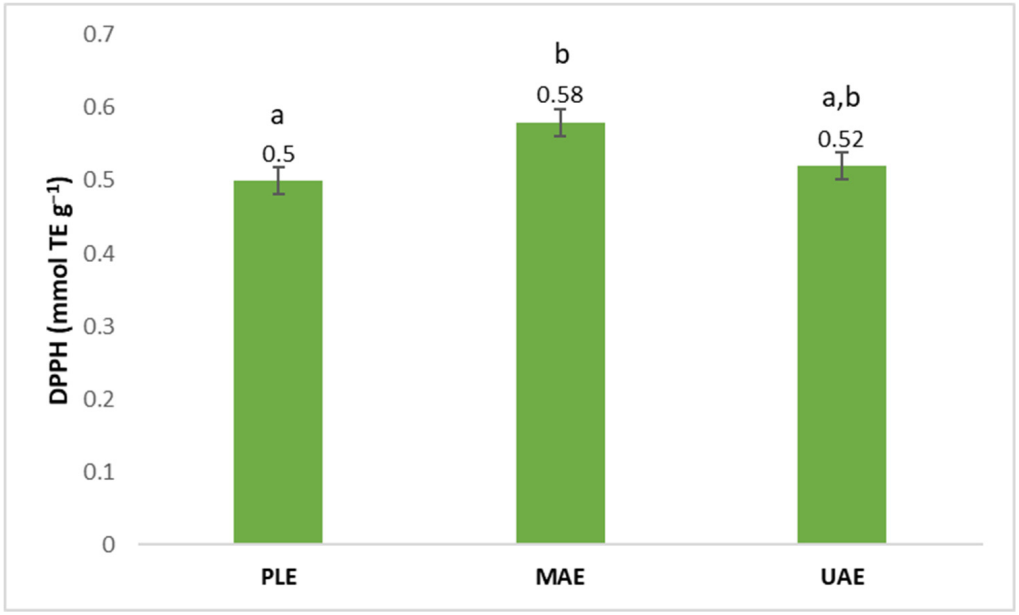
(c)

Figure 1. UPLC MS^2 chromatogram of optimal carob leaf extracts for (**a**) PLE, (**b**) MAE and (**c**) UAE in MRM acquisition mode. (1) Myricetin, (2) gallic acid, (3) 3,4-dihidrobenzoic acid hexoside, (4) apigenin-6-C(O-deoxyhexosyl)-hexoside, (5) syringic acid, (6) protocatechuic acid, (7) rosmarinic acid, (8) p-hydroxybenzoic acid, (9) chlorogenic acid, (10) caffeic acid, (11) catechin, (12) epicatechin, (13) epigallocatechin gallate, (14) procyanidin trimer, (15) luteolin-6-C glucoside, (16) p-coumaric acid, (17) rutin, (18) ferulic acid, (19) quercetin-3-glucoside, (20) apigenin, (21) kaempferol-3-rutinoside, (22) kaempferol-3-O-hexoside, (23) quercetin-3-pentoside, (24) isorhamnetin-3-hexoside, (25) quercetin-3-rhamnoside, (26) kaempferol-3-O-pentoside, (27) kaempferol-3-O-deoxyhexoside, (28) luteolin, (29) epigallocatechin gallate.

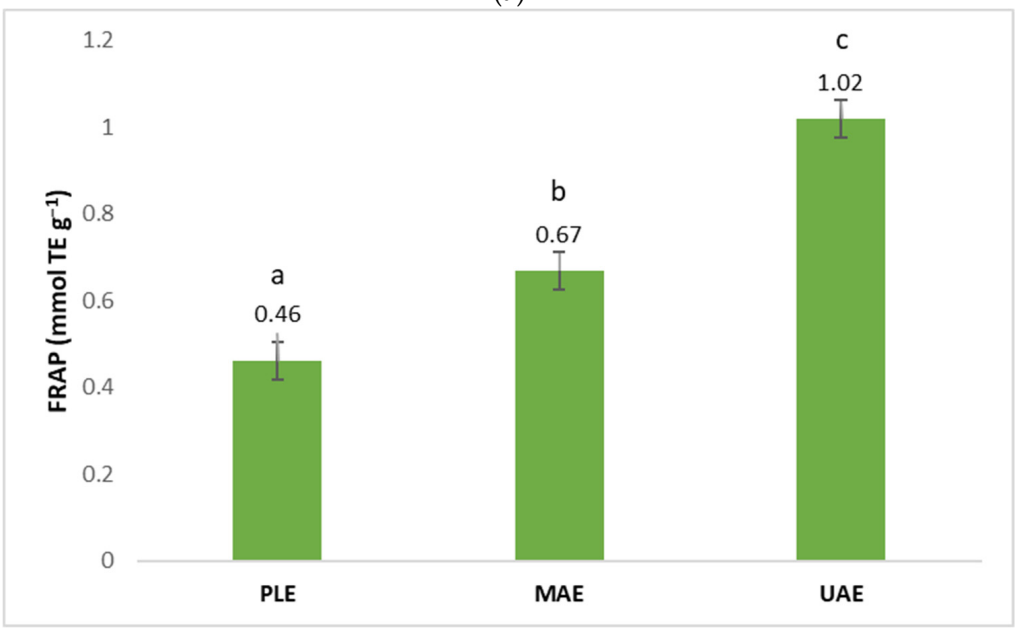
Table 3. UPLC-MS^2 analysis and ascorbic acid content of the carob leaf extracts obtained by PLE, MAE and UAE.

Compound Number	Retention Time	Tentative Identification	Concentration (mg g^{-1} Carob Leaf)		
			PLE	MAE	UAE
		Flavonols			
1	1.37	Myricetin *	9.57 ± 0.15 ^{b}	9.55 ± 0.19 ^{b}	8.66 ± 0.18 ^{a}
15	7.692	Rutin *	0.10 ± 0.00 ^{c}	0.06 ± 0.00 ^{a}	0.09 ± 0.00 ^{b}
17	7.969	Quercetin glucoside	0.76 ± 0.02 ^{a}	0.74 ± 0.02 ^{a}	0.90 ± 0.02 ^{b}
19	8.48	Kaempferol rutinoside	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{a}	0.01 ± 0.00 ^{c}
20	8.51	Kaempferol-*O*-hexoside	0.18 ± 0.00 ^{c}	0.17 ± 0.00 ^{b}	0.16 ± 0.00 ^{a}
21	8.52	Quercetin pentoside	0.49 ± 0.01 ^{b}	0.50 ± 0.01 ^{b}	0.44 ± 0.01 ^{a}
22	8.877	Isorhamnetin hexoside	0.29 ± 0.01 ^{a}	0.32 ± 0.01 ^{b}	0.32 ± 0.01 ^{b}
23	8.897	Quercetin rhamnoside	5.96 ± 0.12 ^{b}	6.17 ± 0.13 ^{b}	5.73 ± 0.12 ^{a}
24	9.178	Kaempferol-*O*-pentoside	0.03 ± 0.00 ^{a}	0.06 ± 0.00 ^{b}	0.03 ± 0.00 ^{a}
		Total flavonols	17.39 ± 0.35 ^{b}	17.58 ± 0.36 ^{b}	16.33 ± 0.33 ^{a}
		Phenolic acids			
2	1.679	Gallic acid *	9.09 ± 0.19 ^{b}	8.78 ± 0.18 ^{b}	6.61 ± 0.13 ^{a}
3	2.313	3.4-Dihidrobenzoic acid hexoside	0.01 ± 0.00 ^{b}	0.01 ± 0.00 ^{c}	0.00 ± 0.00 ^{a}
4	3.488	Syringic acid *	0.04 ± 0.00 ^{a}	0.04 ± 0.00 ^{a}	0.06 ± 0.00 ^{b}
5	3.508	Protocatehuic acid *	0.08 ± 0.00 ^{a}	0.08 ± 0.00 ^{a}	0.07 ± 0.00 ^{a}
6	4.259	Rosmarinic acid *	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{c}	0.01 ± 0.00 ^{b}
7	4.813	*p*-hydroxybenzoic acid	0.44 ± 0.01 ^{a}	0.41 ± 0.01 ^{a}	0.42 ± 0.01 ^{a}
8	5.043	Chlorogenic acid *	0.04 ± 0.00 ^{c}	0.01 ± 0.00 ^{a}	0.03 ± 0.00 ^{b}
9	5.711	Caffeic acid *	0.76 ± 0.02 ^{c}	0.03 ± 0.00 ^{b}	0.02 ± 0.00 ^{a}
14	7.28	*p*-coumaric acid *	0.28 ± 0.01 ^{b}	0.40 ± 0.01 ^{c}	0.23 ± 0.00 ^{a}
16	7.787	Ferulic acid *	0.01 ± 0.00 ^{a}	0.03 ± 0.00 ^{c}	0.02 ± 0.00 ^{b}
		Total phenolic acids	10.75 ± 0.22 ^{c}	9.8114 ± 0.2003 ^{b}	7.4583 ± 0.1522 ^{a}
		Flavones			
18	8.29	Apigenin *	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}
25	9.849	Luteolin *	0.08 ± 0.00 ^{a}	0.08 ± 0.00 ^{b}	0.09 ± 0.00 ^{b}
		Total flavones	0.13 ± 0.00 ^{a}	0.13 ± 0.00 ^{a}	0.14 ± 0.00 ^{b}
		Flavan-3-ols			
10	5.93	Catechin *	0.03 ± 0.00 ^{a}	0.05 ± 0.00 ^{c}	0.04 ± 0.00 ^{b}
11	5.937	Epicatechin	0.04 ± 0.00 ^{a}	0.07 ± 0.00 ^{b}	0.04 ± 0.00 ^{a}
12	6.02	Epigallocatechin gallate *	1.39 ± 0.03 ^{b}	1.25 ± 0.03 ^{b}	0.96 ± 0.02 ^{a}
26	12.159	Epicatechin gallate *	0.46 ± 0.01 ^{b}	0.37 ± 0.01 ^{a}	0.36 ± 0.01 ^{a}
		Total flavan-3-ols	1.93 ± 0.04 ^{c}	1.73 ± 0.04 ^{b}	1.39 ± 0.03 ^{a}
		Proanthocyanidins			
13	6.249	Procyandinin trimer type B	0.01 ± 0.00 ^{b}	0.01 ± 0.00 ^{a}	0.11 ± 0.00 ^{c}
		Total polyphenols	30.20 ± 0.62 ^{b}	29.25 ± 0.60 ^{b}	25.43 ± 0.52 ^{a}
		Ascorbic acid (mg/mL)	0.03 ± 0.00 ^{a}	0.44 ± 0.02 ^{b}	0.52 ± 0.02 ^{c}

PLE = pressurized liquid extraction. MAE = microwave-assisted extraction. UAE = ultrasound-assisted extraction. Values are expressed as mean ± SD. Values within row marked with different letters are statistically different at $p \leq 0.05$. * Identification confirmed by comparison with authentic standard.



(**a**)



(**b**)

Figure 2. Antioxidant activity of carob leaves extract obtained by (**a**) DPPH assay and (**b**) FRAP assay at optimal PLE, MAE and UAE conditions. Values with different letter are statistically different at $p \leq 0.05$. PLE = pressurized liquid extraction. MAE = microwave assisted extraction. UAE = ultrasound assisted extraction. TE = trolox equivalents.

Considering the DPPH assay, the results were similar between the extracts obtained by all three techniques; however, MAE yielded a slightly higher DPPH value compared to the PLE extract (Figure 2a). As shown in Table 4, the correlation between antioxidant activity and the content of polyphenolic groups or their main representatives varied significantly. In the case of antioxidant activity determined by DPPH, only the content of myricetin, as the main representative of flavonols in the extracts, showed a statistically significant and highly positive correlation, indicating that myricetin has the most significant effect on the DPPH radical and significantly contributes to the antioxidant activity of carob leaf extracts. This was expected, since Gerhäuser [48] stated that flavonols such as myricetin are powerful scavengers of DPPH radicals, due to the presence of hydroxyl groups at

certain positions. In this study, all three optimal extracts contain high concentrations of myricetin, as shown by UPLC-MS^2 quantification, with the UAE extract having a slightly lower content of the mentioned compound (Table 3). Nevertheless, since the values were very similar, the consistent activity can be explained by the fact that all three extraction procedures were effective in the extraction of antioxidant polyphenols present in carob leaves. Other studies [49–51], which measured antioxidant activity in extracts obtained from different parts of carob, obtained lower DPPH values, which could be due to the use of different materials, solvents or extraction techniques.

Table 4. Correlation between antioxidant activity and the content of polyphenols in carob leaf extracts.

Group of Compounds	Pearson for DPPH	Pearson for FRAP
Flavonols	0.41	−0.87 *
Phenolic acids	0.00	−1.00 *
Flavones	0.54	0.89 *
Flavan-3-ols	−0.08	−1.00 *
Proanthocyanidins	−0.30	0.92 *
Gallic acid	0.17	−0.97 *
Myricetin	0.70 *	−0.64 *
Quercetin-3-rhamnoside	0.26	−0.93 *
Total phenols UPLC-MS^2	0.09	−0.98 *

* Statistically significant at $p < 0.05$.

In the case of FRAP, significant differences were observed between the three applied techniques, and, in this case, the extract obtained by UAE showed the highest antioxidant activity, while the PLE extract showed the lowest antioxidant activity (Figure 2b). All of the polyphenolic groups and individual representatives showed significant correlations with the antioxidant activity. However, only flavones and proanthocyanidins, whose content in the extracts is relatively low, showed a high positive correlation, indicating that their presence was followed by higher antioxidant activity. All of the other groups and individual representatives, including myricetin, quercetin, rhamnoside and gallic acid showed a high negative correlation, indicating that higher content was associated with lower antioxidant activity, as determined by the FRAP method. Since it is known that polyphenols effectively reduce the Fe^{3+} in the FRAP reagent [52], the negative correlation could potentially be explained by the fact that other constituents of carob leaf that were present in the extract had a stronger reaction with the Fe^{3+}. For example, ascorbic acid, which was shown to be an important factor in the antioxidant and antitumor activity of carob [53] and is well known for its ferric ion reducing properties [54], could have been the main contributor to the antioxidant activity determined by FRAP, therefore overshadowing the effects of polyphenols. Considering this, the differences in results obtained by different extraction techniques could also be explained by the content of ascorbic acid (Table 3), which was consistent with the results of the FRAP assay. The lowest content of ascorbic acid was determined in the extract obtained by PLE, followed by MAE and UAE, which had the highest content. The significantly lower content of ascorbic acid in the extract obtained by PLE could be a result of thermal degradation at the temperature of 160 °C applied during PLE, resulting in the lowest antioxidant activity in this extract [55]. The highest antioxidant activity of the extract obtained by UAE is supported by the content of flavones and proanthocyanidins, which was slightly higher in this extract, but it could also potentially be a result of the higher content of ascorbic acid and its oxidation protecting effects on polyphenols [56]. This is because oxidation often occurs during the application of ultrasound, leading to the generation of hydroxyl radicals and resulting in less efficient antioxidant activity [57]. Since the mechanisms of the FRAP and DPPH methods differ, it

is likely that the effect of ascorbic acid and other constituents was less significant in the DPPH method compared to the FRAP method.

4. Conclusions

This study optimized the temperature and time of PLE, MAE and UAE as advanced techniques for the efficient isolation of polyphenols from carob leaf, marking the first application of PLE and MAE for this purpose. The determined optimal temperature and time for MAE and UAE were 70 °C/10 min, and 160 °C/5 min for PLE. The phenolic profile of carob leaf extracts, acquired through all three techniques, encompassed 26 compounds, categorized into flavonols, phenolic acids, flavones, flavan-3-ols and proanthocyanidins groups. The predominant polyphenols were myricetin, gallic acid and quercetin-3-rhamnoside. The optimized PLE and MAE procedures showed slightly higher amounts of total and individual polyphenols compared to UAE. All examined extracts showed high antioxidant activity, as assessed by the FRAP and DPPH assays, indicating that carob leaf extracts obtained by advanced extraction techniques represent a rich source of polyphenols, which is supported by the content of ascorbic acid, making them promising ingredients for incorporation into functional foods. In order to achieve this, future research should focus on environmental growth factors and harvesting season during the cultivation of carob, all of which can contribute to obtaining higher yields of polyphenols. In addition, future research on micro- and nano-encapsulation, aimed at achieving maximum retention, quality and bioaccessibility of encapsulated carob leaf polyphenols, represents the next step toward the efficient utilization of this valuable plant source.

Author Contributions: Conceptualization, E.C. and E.D.; methodology, E.C., E.D. and Z.Z.; formal analysis, E.C., E.D. and Z.Z.; investigation, E.C. and E.D.; data curation, E.C. and E.D., writing—original draft preparation, E.C. and E.D.; writing—review and editing, M.R. and I.E.G.; supervision, I.E.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The dataset is available on request from the authors.

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