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Carob Pulp Flour Extract Obtained by a Microwave-Assisted Extraction Technique: A Prospective Antioxidant and Antimicrobial Agent

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Abstract: In this study, carob pulp flour (CF) extract was characterized as a high-value antioxidant and antimicrobial agent. CF extracts were obtained using microwave-assisted extraction (MAE) and optimization of the MAE process was accomplished using response surface methodology. The studied processing parameters of MAE were the liquid/solid (L/S) ratio (10–30 mL/g), extraction time (15–35 min), and ethanol concentration (40–80% *w*/*w*). The efficiency of the extraction of valuable compounds from CF was evaluated by the determining extraction yield (Y), total phenolic(TP), total flavonoid (TF) content, and antioxidant activity (DPPH, FRAP, and ABTS). The optimized MAE parameters for maximizing the yields of target compounds and antioxidant activity were the L/S ratio 30 mL/g, extraction time 35 min, and ethanol concentration 40%. The experimentally obtained values for TP, TF, DPPH, FRAP, and ABTS were 1609.92 GAE/100 g, 271.92 CE/100 g, 99.02 µMTE/g, 50.45 µM Fe^{2+}/g, and 110.55 µMTE/g, respectively. The optimized CF extract was compared with the CF extracts obtained by conventional solid–liquid extraction (S/L) and ultrasound-assisted extraction (UAE) and was found to be more beneficial due to a 30% higher yield of TP and TF and 30–80% higher antioxidant activity. The phenolic profiles of the three extracts were quite similar. The microdilution method confirmed the antibacterial activity of MAE and S/L extracts while the antifungal effect was not observed.

Keywords: carob pulp flour; microwave-assisted extraction; response surface methodology; antioxidant activity; antimicrobial activity; phenolic profile

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1. Introduction

The carob tree (*Ceratonia siliqua* L.) is an evergreen tree widespread through the Mediterranean region and it is highly valued for its fruit. Among the various products that can be obtained from the carob fruit, the economic importance of the carob tree lies in the industrial use of carob gum, also known as locust bean gum [1]. On the other hand, the pulp is considered a by-product of the carob fruit industry and its processing was mainly focused on kibbling to various grades for animal feed due to its high nutritional value [2]. The organoleptic properties of carob pulp can be improved by roasting and subsequent milling to obtain carob flour, which can be used for human consumption. In addition to valuable nutrients, carob pulp and flour represent a source of other components; the secondary metabolites, such as phenolic acids, flavonoids, and tannins, have functional properties and

may provide health benefits to the human body (antioxidant, anti-inflammatory, or anti-aging properties). These non-nutrients are bioactive plant compounds that have antioxidant and antimicrobial activities, among others [3]. They also play an important role in the defense mechanism against plant pathogens and abiotic stress [4]. In addition, the high content of polyphenols in the carob pulp extracts, especially gallic acid and epigallocatechin gallate, has been associated with their potent antimicrobial activity [5]. Previously, various carob products and extracts have shown strong antimicrobial effects against different bacterial and fungal strains [6,7]. Therefore, carob pulp flour is an attractive raw material for obtaining extracts with high biological activity. However, the bioactive compounds to which the aforementioned activities are attributed are highly sensitive to different production methods, processes, and environmental conditions, so the extraction technique and processing conditions should be adapted to obtain high-quality antioxidant and/or antimicrobial agents.

In recent years, the extraction processes have shifted towards the application of "green" extraction methods. Microwave-assisted extraction (MAE) is one of the new emerging extraction techniques that has proven to be effective and economically justified for the extraction of bioactive compounds from various plant materials [8–10]. The simple manipulation and adjustment of process parameters, reduced extraction time, and lower cost are just some of the advantages of the MAE technique. Furthermore, the use of non-toxic solvents (water or ethanol in different concentrations) to perform MAE contributes to the importance of this technique in terms of environment protection and safety. Microwave radiation is generated by electric and magnetic fields. Microwave heating is induced by ionic conduction and dipolar rotation and is more intense the higher the dielectric constant of the solvent [11]. Unlike commonly used conventional extraction methods, microwaves heat the entire sample homogeneously and simultaneously [12]. Therefore, MAE can provide better results in terms of a higher extraction yield, higher bioactive compound content, and stronger antioxidant activity of plant extracts compared to conventional extraction [13]. Moreover, some of the bioactive compounds ubiquitously present in plants, such as coumarins, can be altered by microwave irradiation. Nowadays, microwave irradiation is rapidly recognized and widely used as a tool for the synthesis of coumarin derivates and coumarin metal complexes, which have been shown to exhibit significant antioxidant and antimicrobial activity [14–17]. In view of various process parameters that may affect the efficiency of extraction of the desired compounds by MAE, optimization of those dependent variables should be carried out and adopted for the particular plant material. For this purpose, the response surface methodology (RSM) is often used and can be successfully applied in conjunction with MAE to achieve the proposed objective.

Regarding the extraction of bioactive compounds from carob, Huma et al. [18] used RSM for the MAE optimization of carob kibble powder. The recovery of bioactive compounds was investigated in terms of the total phenolics and condensed tannins content; there are some inconsistencies between the presented results and the outcome of the optimization. Furthermore, MAE of carob bark was previously performed by Quiles-Carrillo et al. [19] while the total yield and DPPH antioxidant activity of the obtained extracts were studied and used as responses for the optimization of the extraction. Finally, Martić et al. [20] optimized the MAE of carob pulp flour based on the results of antioxidant activity determined by three in vitro methods (DPPH, FRAP, and ABTS) while the optimized extract was tested as a hepatoprotective agent. However, as far as the authors are aware, in the literature there is not a study of the thorough optimization of MAE where the means of the optimization include both multiple input and output factors to achieve the best overall extraction performance, as was recently performed in the case of ultrasound-assisted extraction (UAE) of carob pulp [21].

Therefore, in order to accomplish the set goal and to valorize carob pulp flour by obtaining high-quality extracts with high bioactivity, the conditions of MAE will be optimized using RSM. Obtaining carob pulp extracts with a maximum content of phenolic compounds, flavonoids, and antioxidant activity will be conducted by adjusting the MAE

process parameters: the L/S ratio, extraction time, and ethanol concentration. Additionally, the antimicrobial activity of the obtained extracts will be examined by determining the minimum inhibitory concentration (MIC) and minimum bactericidal and fungicidal concentration (MBC and MFC) for chosen representatives of bacteria and fungi. In this way, the bioactivity of optimized carob pulp flour extracts will be evaluated for further consideration of its application as a natural antioxidant agent and preservative.

2. Materials and Methods

2.1. Plant Material

The carob pulp flour (CF) used in this study originated from Croatia. It was purchased from a packaging service company Vega ADM (Senta, Serbia) as a product commercially available in Serbia (Figure 1).

Figure 1. Sample of carob pulp flour used for obtaining carob flour extracts.

2.2. Chemicals

Gallic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) while 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Sigma-Aldrich (Milano, Italy). 2,2′-azino-bis-(-3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from J&K Scientific GmbH (Pforzheim, Germany). The following reagents were obtained from Sigma-Aldrich (Steinheim, Germany): DPPH (1,1-diphenyl-2-picryl-hydrazyl-hydrate), TPZT (2,4,6-tris(2-pyridil)-s triazine), (±)-catechin, Folin–Ciocalteu reagent, iron(III)-chloride, iron(II)-sulfatheptahydrate, and potassium persulfate. In addition, the following standards were obtained: *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, ferulic acid, gallic acid, quercetin, rutin, and quercitrin. Other chemicals used in this study were of analytical grade.

2.3. Microwave-Assisted Extraction Process

Microwave-assisted extraction (MAE) was performed using a homemade MAE setup which consisted of a remodeled microwave oven (NN-E201W, Panasonic, Osaka, Japan), 500 mL round flask, and condenser. In total, 50 mL of the appropriate concentration of extraction solvent (40, 60 and 80% *w*/*w*) and the amount of plant material needed for achieving the desired L/S ratio (10, 20, and 30 mL/g) were mixed in the round flask, connected to the condenser, and positioned into the microwave oven. The irradiation power was set to a constant 600 W and the extraction time for each sample was customized (15, 25, and 35 min) according to the experimental plan (Table 1). Obtained extracts were cooled, centrifuged, and the supernatant was collected into dark glass flasks and stored at 4 °C until further analyses (Figure 1).

Table 1. Experimental domain with coded and natural values of independent variables used in the experimental design.

Independent Variables	Coded Levels		
	−1	0	1
	Natural Levels		
L/S ratio (mL/g)	10	20	30
Extraction time (min)	15	25	35
Ethanol concentration (%)	40	60	80

2.4. Total Extraction Yield

In order to determine the total extraction yield (Y), vacuum evaporation and further drying of 5 mL of the obtained extracts were applied. The results were expressed as a percentage of the total extractable solids per 100 g of dry CF (%, *w*/*w*).

2.5. Total Polyphenols Content

The total phenols (TP) content in the obtained extracts was determined using the Folin–Ciocalteu procedure [22]. For the preparation of the standard calibration curve, gallic acid was used (y = 87.060x + 0.0176, R^2 = 0.996) and the absorbance of the samples was measured at 750 nm (6300 Spectrophotometer, Jenway, Stone, UK). The content of phenolic compounds was expressed as g of gallic acid equivalents (GAE) per 100 g dry weight (DW).

The total flavonoid (TF) content was determined using an aluminum chloride colorimetric assay [23]. For the preparation of the standard calibration curve, catechin was used (y = 7.988x + 0.0098, R^2 = 0.988) and the absorbance was measured at 510 nm. The results were expressed as g of catechin equivalents (CE) per 100 g DW. All measurements for total polyphenols content were performed in three replicates and the results were expressed as the mean value.

2.6. Antioxidant Activity

The free radical scavenging capacity of CF extracts was investigated using 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH•) and following the procedure of Espín et al. [24]. Properly diluted CF extracts (0.1 mL) were mixed in 10 mL cuvettes with 2.9 mL of freshly prepared solution of DPPH reagent in methanol (65 µM and adjusted to achieve absorbance of 0.70 ± 0.02). The reaction mixture was incubated at ambient temperature in the dark for 60 min. The absorbance was then measured at 517 nm (6300 Spectrophotometer, Jenway, Stone, UK). The set of freshly prepared Trolox aqueous solutions were used to obtain the calibration curve (0–0.80 mM, y = −91.032x + 0.6679, R^2 = 0.999) and the results were reported as µM of Trolox equivalents per g dry weight (DW).

According to the assay described by Oyaizu [25], the reducing power of CF extracts towards Fe^{3+} was determined. The FRAP reagent was prepared from 40 mM HCl solution and 20 mM $FeCl_3$ aqueous solution as well as 10 mM 2,4,6-tris(2-pyridil)-s triazine (TPZT) and 300 mM acetate buffer (pH = 3.6) which were mixed in a 1:1:10 (*v*/*v*/*v*) ratio. In total, 0.1 mL of properly diluted extracts and 1.9 mL of FRAP reagent were mixed and incubated in the dark for 10 min at 37 °C. The absorbance was measured with a UV–VIS spectrophotometer at 593 nm (6300 Spectrophotometer, Jenway, Stone, UK). The series of freshly prepared Fe^{2+} (Fe_2SO_4) aqueous solutions was used for obtaining the calibration curve (0–0.23 mM, y = 362.280x + 0.0966, R^2 = 0.998) and the results were expressed as µM of Fe^{2+} equivalents per g DW.

The ABTS free radical scavenging capacity of CF extracts was measured using a modified method originally reported by Re et al. [26]. ABTS stock solution was prepared from a mixture (1:1, *v*/*v*) of 7 mM 2,2′-azino-bis-(-3-ethylbenzothiazoline-6-sulfonic acid) of diammonium salt (ABTS) aqueous solution and 2.45 mM potassium persulfate aqueous solution and left at ambient temperature in the dark for 16 h. A prepared stock solution was then diluted with 300 mM acetate buffer (pH = 3.6) in order to reach an absorbance of

0.70 ± 0.02. 0.1 mL of properly diluted extracts and 2.9 mL of ABTS reagent were mixed and left at ambient temperature in the dark for 300 min. Measurements were carried out with a UV–VIS spectrophotometer (6300 Spectrophotometer, Jenway, Stone, UK) at 734 nm. Calibration was performed using freshly prepared Trolox aqueous solutions (0–0.80 mM, $y = -99.053x + 0.6381$, $R^2 = 0.989$) and the results were expressed as μM of Trolox equivalents per g DW.

All the measurements of antioxidant activity were performed in three replicates and the results were expressed as mean values.

2.7. Experimental Plan and Statistical Analysis

The experimental plan was based on face-centered Central Composite experimental design which implies the investigation of three parameters on three levels. By the application of mentioned experimental design, 20 experimental runs with 6 central points were generated. The influence of the liquid–solid ratio (X_1, mL/mg), extraction time (X_2, min), and ethanol concentration (X_3, % *w*/*w*) on the extraction yield, total polyphenols content, and antioxidant activity was assessed using response surface methodology responses (RSM). Targeted responses were fitted to the second-order polynomial model using the following Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the corresponding response variable, X_i and X_j are the variables affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept, linear, quadratic, and interaction terms, respectively. The coefficient of determination (R^2), coefficient of variance (CV), and *p*-values for the model and lack of fit were used for the estimation of the chosen mathematical model. Process optimization was performed based on the experimental results for all investigated responses and the selection of optimal MAE conditions was conducted according to the obtained desirability function (D). The software used for the experimental planning and statistical analysis was Design-Expert v.11 Trial (Stat-Ease, Minneapolis, MN, USA).

2.8. Conventional and Ultrasound-Assisted Extraction

In order to compare the efficiency of the MAE of bioactive compounds from carob flour, conventional solid–liquid extraction (S/L) and ultrasound-assisted extraction (UAE) of bioactive compounds from CF were performed. In both cases, 1.67 g of carob flour was mixed with 50 mL of extraction solvent (aqueous ethanol 40%, *w*/*w*). S/L extraction was performed by stirring the extraction mixture with 137 rpm shaking speed for 24 h at 28 °C. UAE was performed in sonication water bath equipment (EUP540A, EUinstruments, Paris, France) working at a fixed frequency (40 kHz) and the process parameters were 35 °C, 15 min, and 60 W/L. After extraction, liquid extracts were immediately centrifuged at 4000 rpm for 10 min and the supernatant was collected into dark glass flasks and stored at 4 °C until further analyses.

2.9. Phenolic Profile and HPLC Analysis

The obtained extracts were analyzed using HPLC-DAD Agilent Technologies 1100 liquid chromatographer (Agilent Technologies, Santa Clara, CA, USA) according to the previously described method [27]. Analytes of interest were separated using a Nucleosil C18 column (250 mm, i.d. 4.6 mm, 5 μm particle size; Macherey Nagel, Düren, Germany). A 1% solution of formic acid (A) and methanol (B) was used as the mobile phase. In order to make the separation more efficient, a gradient elution program was used (0 min—10% B; 10 min—25% B; 20 min—45% B; 35 min—70% B; 40 min—100% B; and 46 min—10 % B) with a variable flow rate (0–10 min, 1 mL/min; 10–20 min, 0.8 mL/min; 20–30 min, 0.7 mL/min; and 30–46 min, 1 mL/min). The volume of the injection was set at 10 μL.

Chemical standards of caffeic acid (CA), gallic acid (GA), *p*-coumaric acid (pQA), *trans*-cinnamic acid (CNA); rosmarinic acid (RA); chlorogenic acid (CHA); ferulic acid (FA); quercetin (Qe); rutin (R); and quercitrin (Qt) were analyzed under the same conditions in order to obtain the calibration curves necessary for analytes quantification (Table S1). The eluted analytes were monitored at three wavelengths: gallic acid, caffeic acid, and *trans*-cinnamic acid at 280 nm; *p*-coumaric acid, chlorogenic acid, rosmarinic acid, ferulic acid, and quercetin at 330 nm; and rutin and quercitrin at 350 nm while the UV spectra required for analytes identification were recorded in the 190–400 nm range. The number of analytes of interest was expressed in µg per g of dry extract.

2.10. Antimicrobial Activity: The Broth Microdilution Method

The antimicrobial activity of carob pulp extracts was analyzed using the broth microdilution method (Figure 2) according to Kocić-Tanackov et al. [28]. Antibacterial activity was analyzed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6598, and *Bacillus cereus* ATCC 11778 while antifungal activity was determined against *Saccharomyces cerevisiae*, *Aspergillus flavus*, and *Penicillium aurantiogriseum*. *A. flavus* was isolated from corn flour, a product of the Serbian market, while *Penicillium aurantiogriseum* was isolated from whole wheat flour, also a product of the Serbian market. Isolated and identified isolates were kept on Sabouraud Maltose Agar (SMA) (Merck, Darmstadt, Germany) at 4 °C as part of the collection of the Laboratory for Food Microbiology at the Faculty of Technology Novi Sad, University of Novi Sad, Serbia, while tested bacteria were maintained on Mueller–Hinton agar (MHA) (Merck, Darmstadt, Germany) slants at 4 °C.

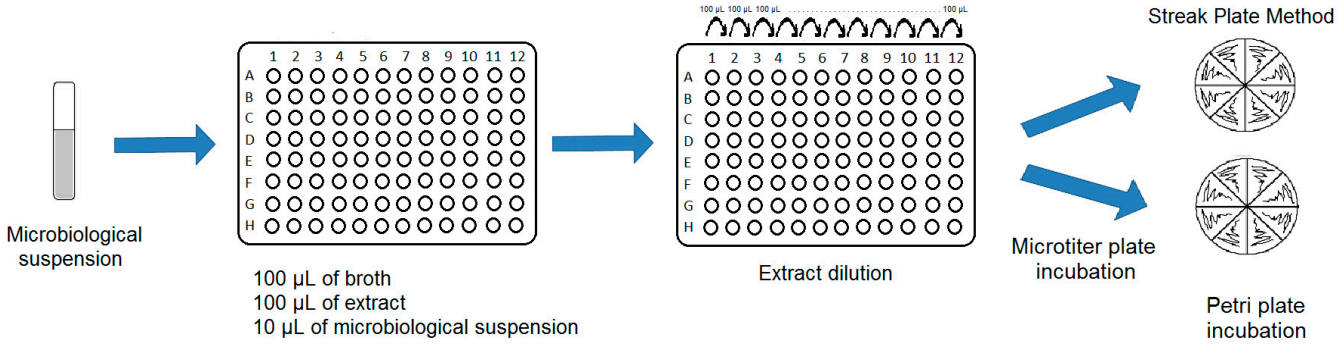

Figure 2. Broth microdilution method.

The bacteria and yeast suspensions were prepared in 0.85% sterile saline with bacterial strains that were cultured on an MHA for 24 h at 37 °C (for *B. cereus* at 30 °C) and with yeast and mold cultures grown on SMA at 25 °C for 48 h or 7 days, respectively. The final concentration of the suspension was 10^8 CFU/mL which was measured using a McFarland Standard (bioMérieux SA, Marcy I'Etoile, France) and McFarland densitometer (Biosan SIA, Riga, Latvia). The suspensions of mold were prepared in 10 mL of sterile saline containing 0.5% Tween 80 and the obtained suspension was adjusted with a hemocytometer (Improved Neubauer Counting Chambers, Blaubrand®, Wertheim, Germany) to 10^8 conidia/mL. All tests were performed in duplicate for each extract.

3. Results and Discussion

3.1. Model Fitting

The influence of MAE process parameters on the extraction yield, yield of polyphenols, and antioxidant activity of CF was investigated by three varying input factors which were selected and ranged based on the results of our previous study [20]. Since the irradiation power had previously shown no significant effect on the antioxidant activity of CF extracts, the mentioned parameter was kept constant at 600 W throughout the experiments. The range of extraction time and ethanol concentration was adjusted to higher values (15–35 min and 40–80%, respectively) in order to extend the experimental domain to determine more

precisely the extent of their impact and to implement the optimization of CF extraction in the best possible way. The liquid–solid (L/S) ratio in the range of 10–30 mL/g was added to the experimental design due to its known influence on the extraction processes [29]. Table 2 shows the experimentally obtained values and the range of results for each individual investigated responses.

Table 2. Experimental plan with input factors and experimentally obtained values for investigated responses.

Run	Input Factors *			Responses					
	X_1 [mL/g]	X_2 [min]	X_3 [%]	Y [%]	TP [mg GAE/100 g]	TF [mg CE/100 g]	DPPH [μM TE/g]	FRAP [μM Fe^{2+}/g]	ABTS [μM TE/g]
1	20	25	60	42.28	960.56	205.52	75.67	31.66	110.78
2	20	25	80	36.94	635.88	177.34	61.27	22.93	56.24
3	20	25	60	42.62	1055.52	196.45	79.00	38.11	125.30
4	20	25	60	42.88	946.78	192.63	75.67	35.55	113.04
5	10	15	40	41.42	875.41	184.45	74.00	37.76	124.12
6	30	15	40	59.48	1603.80	309.18	139.74	48.33	171.22
7	20	25	60	38.06	966.78	178.30	79.67	33.55	114.04
8	30	35	40	58.48	1801.36	326.86	155.19	57.68	200.26
9	20	15	60	42.58	926.87	175.91	83.57	35.02	110.46
10	10	35	80	29.77	545.37	113.27	49.68	24.21	65.71
11	30	15	80	51.80	948.31	247.56	101.82	28.57	96.35
12	10	15	80	36.23	568.34	116.85	52.32	22.85	71.85
13	20	35	60	43.74	986.60	182.59	81.46	37.84	112.07
14	10	25	60	41.40	833.30	158.89	68.91	34.23	101.05
15	20	25	60	40.98	922.28	174.47	90.24	39.52	112.39
16	10	35	40	37.90	881.54	159.61	65.75	34.01	102.34
17	30	25	60	57.08	1429.21	269.53	133.78	44.98	142.82
18	20	25	40	42.32	1124.44	209.35	102.70	48.06	138.85
19	30	35	80	45.04	899.30	234.66	96.91	29.27	89.90
20	20	25	60	41.70	785.97	182.59	88.13	37.93	116.26

* X_1—liquid–solid ratio; X_2—extraction time; X_3—ethanol concentration.

The observed results for Y, TP, TF, DPPH, FRAP, and ABTS were fitted to the second-order polynomial model and model testing was performed considering several descriptive parameters. The coefficient of determination (R^2) was used as an indicator of the model fitting. Very high values of R^2 for Y, TP, TF, DPPH, FRAP, and ABTS were determined as 0.9617, 0.9739, 0.9678, 0.9754, 0.9334, and 0.9685, respectively. Therefore, all applied models were considered to be in accordance with obtained experimental results. To determine the significance of the models, Fisher's test and lack of fit testing were performed as the part of the analysis of variance (ANOVA) (Table 3). The statistically significant p-values (<0.05) for all investigated responses in model testing and the non-significant ($p > 0.05$) lack of fit for all studied responses (Table 3) indicated that the applied model can adequately represent the experimental results.

The degree of data dispersion was described by the coefficient of variance (CV) whose values for Y, TP, TF, DPPH, FRAP, and ABTS were 4.71, 7.08, 6.81, 6.88, 8.76, and 7.16, respectively. Relatively low values of CV indicate the good reproducibility of experimental results. ANOVA was also used to calculate the regression coefficients of the second-order polynomial model and the corresponding p-values of the linear, interaction, and quadratic terms (Table 4).

Table 3. Analysis of variance for the quadratic polynomial model for Y, TP, TF, DPPH, FRAP, and ABTS.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Total extraction yield					
Model	1061.39	9	117.93	27.87	<0.0001
Residual	42.31	10	4.23		
Lack of Fit	26.44	5	5.29	1.67	0.2946
Pure Error	15.87	5	3.17		
Cor Total	1103.70	19			
Total phenols content					
Model	1,814,965.24	9	201,661.80	41.46	<0.0001
Residual	48,640.46	10	4864.05		
Lack of Fit	10,071.24	5	2014.25	0.26	0.9166
Pure Error	38,569.22	5	7713.84		
Cor Total	1,863,596.70	19			
Total flavonoids content					
Model	55,465.88	9	6162.88	33.29	<0.0001
Residual	1851.45	10	185.14		
Lack of Fit	1145.89	5	229.18	1.62	0.3038
Pure Error	705.57	5	141.11		
Cor Total	57,317.33	19			
DPPH					
Model	14,441.65	9	1604.63	44.03	<0.0001
Residual	364.47	10	36.45		
Lack of Fit	166.62	5	33.32	0.84	0.5725
Pure Error	197.86	5	39.57		
Cor Total	14,806.13	19			
FRAP					
Model	1404.33	9	156.04	15.58	<0.0001
Residual	100.18	10	10.02		
Lack of Fit	54.59	5	10.92	1.20	0.4241
Pure Error	45.59	5	9.12		
Cor Total	1504.51	19			
ABTS					
Model	20,404.96	9	2267.22	34.20	<0.0001
Residual	662.96	10	66.30		
Lack of Fit	526.44	5	105.29	3.86	0.0824
Pure Error	136.51	5	27.03		
Cor Total	21,067.91	19			

Table 4. Corresponding *p*-values of each regression coefficients (mean, linear, cross products, and quadratic) for all investigated responses.

	Response					
	Y	**TP**	**TF**	**DPPH**	**FRAP**	**ABTS**
β_0	<0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *
Linear						
β_1	<0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *	0.0002 *	<0.0001 *
β_2	0.0289 *	0.4057	0.7017	0.9000	0.3197	0.8880
β_3	0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *	<0.0001 *
Cross product						
β_{12}	0.7108	0.4214	0.4085	0.2380	0.1948	0.0530
β_{13}	0.2097	0.0009 *	0.3245	0.0065 *	0.0256 *	0.0019 *
β_{23}	0.1657	0.1925	0.8135	0.4079	0.7008	0.4089
Quadratic						
β_{11}	0.0005 *	0.0029 *	0.0092 *	0.0014 *	0.3271	0.0332 *
β_{22}	0.8616	0.8238	0.3211	0.4400	0.5409	0.7744
β_{33}	0.0236 *	0.0676	0.5157	0.3647	0.3495	0.0314 *

* Significant at 0.05 level.

Predictive model equations with neglected insignificant coefficients for all investigated responses (Equations (2)–(7)) were given as follows:

$$Y = 42.03 + 8.52 \cdot X_1 - 1.66 \cdot X_2 - 3.98 \cdot X_3 + 6.30 \cdot X_1^2 - 3.31 \cdot X_3^2 \quad (2)$$

$$TP = 950.33 + 297.80 \cdot X_1 - 268.94 \cdot X_3 - 144.29 \cdot X_1 \cdot X_3 + 164.91 \cdot X_1^2 \quad (3)$$

$$TF = 188.12 + 65.47 \cdot X_1 - 29.98 \cdot X_3 + 26.40 \cdot X_1^2 \quad (4)$$

$$DPPH = 83.01 + 31.68 \cdot X_1 - 17.54 \cdot X_3 - 7.31 \cdot X_1 \cdot X_3 + 15.90 \cdot X_1^2 \quad (5)$$

$$FRAP = 36.69 + 5.58 \cdot X_1 - 9.86 \cdot X_3 - 2.93 \cdot X_1 \cdot X_3 \quad (6)$$

$$ABTS = 113.11 + 23.55 \cdot X_1 - 35.67 \cdot X_3 - 12.04 \cdot X_1 \cdot X_3 + 12.12 \cdot X_1^2 - 12.27 \cdot X_3^2 \quad (7)$$

where X_1, X_2, and X_3 are the L/S ratio, extraction time, and ethanol concentration, respectively. As a result of the aforementioned predictive model equations, 3D surface plots for the investigated responses were created.

3.2. Yield of Targeted Compounds

From the experimentally obtained results in Table 2, it can be seen that the maximum values of all investigated responses were obtained under the same experimental conditions: L/S ratio 30 mL/g, extraction time 35, and ethanol concentration 40% (Run 8). The only exception is the total extraction yield which showed the second highest experimentally determined value under the mentioned process conditions. The highest extraction yield (59.48%) is obtained at similar process parameters with only the extraction time (15 min) differing from the previously mentioned conditions. It is worth noticing that the process parameters with which the highest extraction yield is obtained are also responsible for achieving the second highest experimental values for all the other responses (Run 6). With

the decrease in the L/S ratio and increase in the ethanol concentration, lowest values for Y, TP, TF, and DPPH (Run 10) and the lowest values for FRAP and ABTS (Run 12 and 2, respectively) are obtained (Table 2). The main difference between the aforementioned experimental runs is the extraction time, which, together with other previous observations, leads to the assumption that the extraction time in the observed experimental range does not have as pronounced of an effect on the investigated responses as the other input factors.

A more detailed presentation of the effects of input factors on the total phenolic and flavonoids yields is shown in Figure 3. The trend of the interaction of the process parameters is very similar for the observed responses. With the increase in the L/S ratio, Y, TP, and TF in carob extracts increased (Figure 3) which was also confirmed by the significance of the corresponding linear regression coefficients (Table 4). Increasing the volume of the solvent in an extraction process promotes solubility by increasing the contact area of the plant material with the solvent and accelerates mass transfer [30].

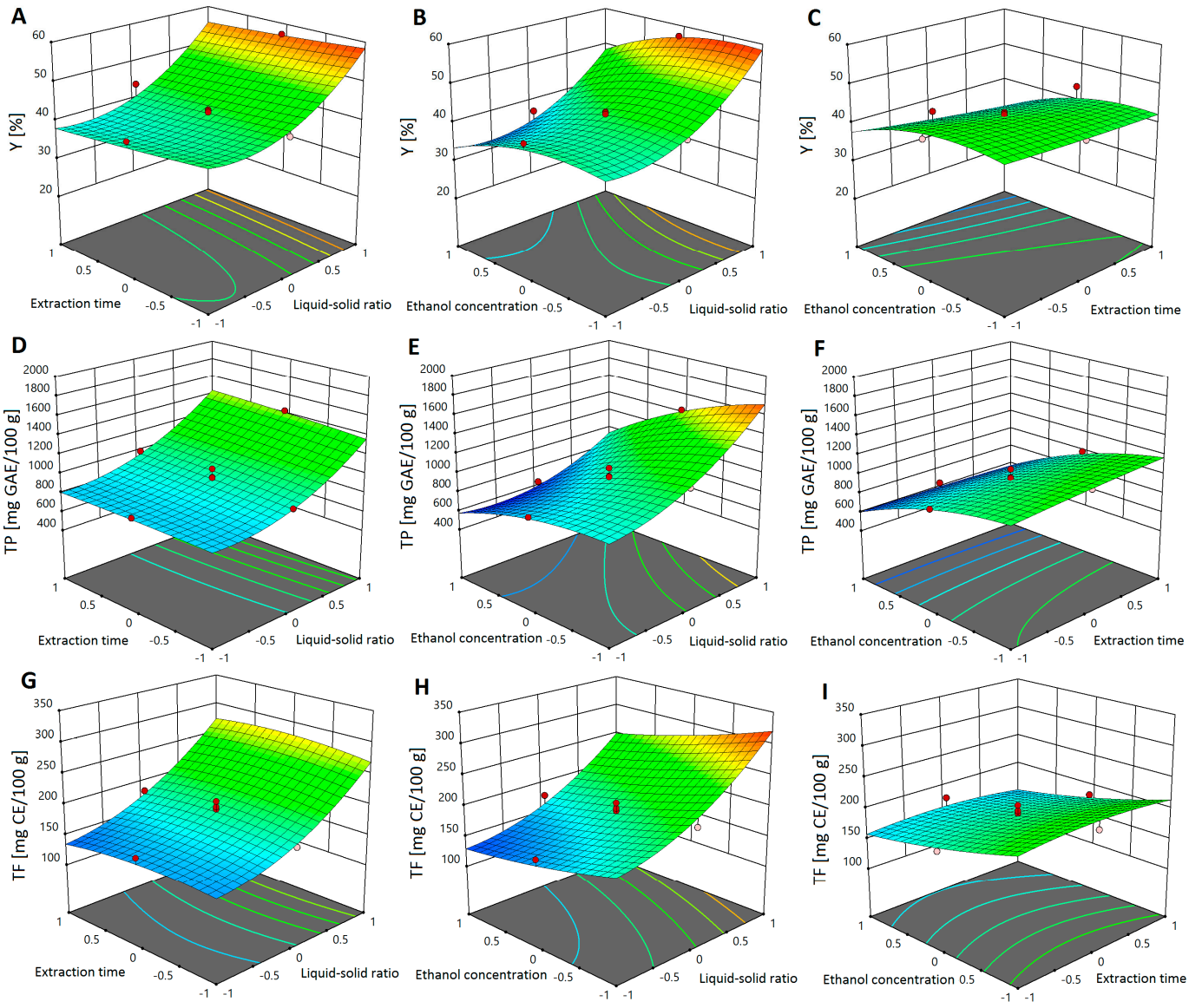

Figure 3. Influence of input factors on the total extraction yield (**A**–**C**), total phenols (**D**–**F**), and total flavonoid content (**G**–**I**).

In addition to the L/S ratio, ethanol concentration had a statistically significant effect on Y, TP, and TF (Figure 3, Table 4) but its effect was negative. Decreasing the ethanol

concentration decreases the dielectric constant and thus increases the selectivity towards hydrophilic compounds, resulting in an improved yield [31]. It is also evident that carob pulp contains a larger amount of soluble carbohydrates and sugars [19,32] which are recovered in higher quantities with more polar solvents. Among all of the investigated responses, the Y was the only response significantly affected by the extraction time (Table 4).

3.3. Antioxidant Activity

The results of the in vitro antioxidant activity of CF extracts are shown in Table 2. The scavenging capacity of CF extracts against DPPH radicals was measured in the range of 49.68 to 155.19 μM TE/g. The minimum value for DPPH was obtained at the following processing conditions: L/S ratio 10 mL/mg, extraction time 35 min, and 80% ethanol concentration. The scavenging capacity of CF extracts against $ABTS^+$ radicals ranged from 56.24 to 200.26 μM TE/g. The lowest value for the mentioned parameter was obtained at an L/S ratio 10 mL/mg, extraction time of 15 min, and ethanol concentration of 80%. The range of experimentally determined values for the reducing power of CF extracts was 22.85–57.68 μM Fe^{2+}/g. The minimum value determined by the FRAP assay was reached when the L/S ratio, extraction time, and ethanol concentration were 30 mL/mg, 15 min, and 80%, respectively. When it comes to the minimum values for DPPH, ABTS, and FRAP, it can be seen that the common feature is a high ethanol concentration and a partially lower L/S ratio considering the low values for antioxidant activity in general. On the other hand, for all three in vitro antioxidant methods used in this experiment, the highest values were obtained when the same MAE operating conditions were applied: the maximum L/S ratio (30 mL/mg), maximum extraction time (35 min), and minimum ethanol concentration (40%).

From Table 4, it can be seen that the most influential independent variables in the MAE process for obtaining CF extracts with high antioxidant activity were the L/S ratio and ethanol concentration. Single effects of mentioned variables had the strongest influence ($p < 0.0002$) but the influences of their cross-product ($p < 0.05$), quadratic terms of the L/S ratio (for DPPH and ABTS), and extraction time (only for ABTS) were also significant. The L/S ratio had a positive effect on DPPH, FRAP, and ABTS whereas ethanol concentration had a negative effect (Figure 4), which is similar to the effects found for Y, TP, and TF (Figure 3). Since the same MAE parameters affected both the yield of targeted compounds and antioxidant activity in the same way, this implies that the extracted bioactive compounds under specified conditions are responsible for the antioxidant activity of CF extracts. The literature data confirmed that TP and various parameters of antioxidant activity are strongly positively correlated in some other plant materials [33,34].

The maximum values achieved in this study for the antioxidant activity of CF extract were higher compared to the results of our previous research where the mentioned values reached 106.85 μM TE/g, 185.64 μM TE/g, and 51.45 μM Fe^{2+}/g for DPPH, ABTS, and FRAP, respectively [20]. In the same literature data, the optimized CF extract also showed effective in vivo antioxidant activity with a paracetamol-induced hepatotoxicity model in mice. This speaks in favor of the new, more elaborate setting of MAE parameters for the recovery of bioactive compounds from carob flour and suggests that with higher in vitro antioxidant activity, higher in vivo effects and bioactivity of the obtained CF extracts can also be expected.

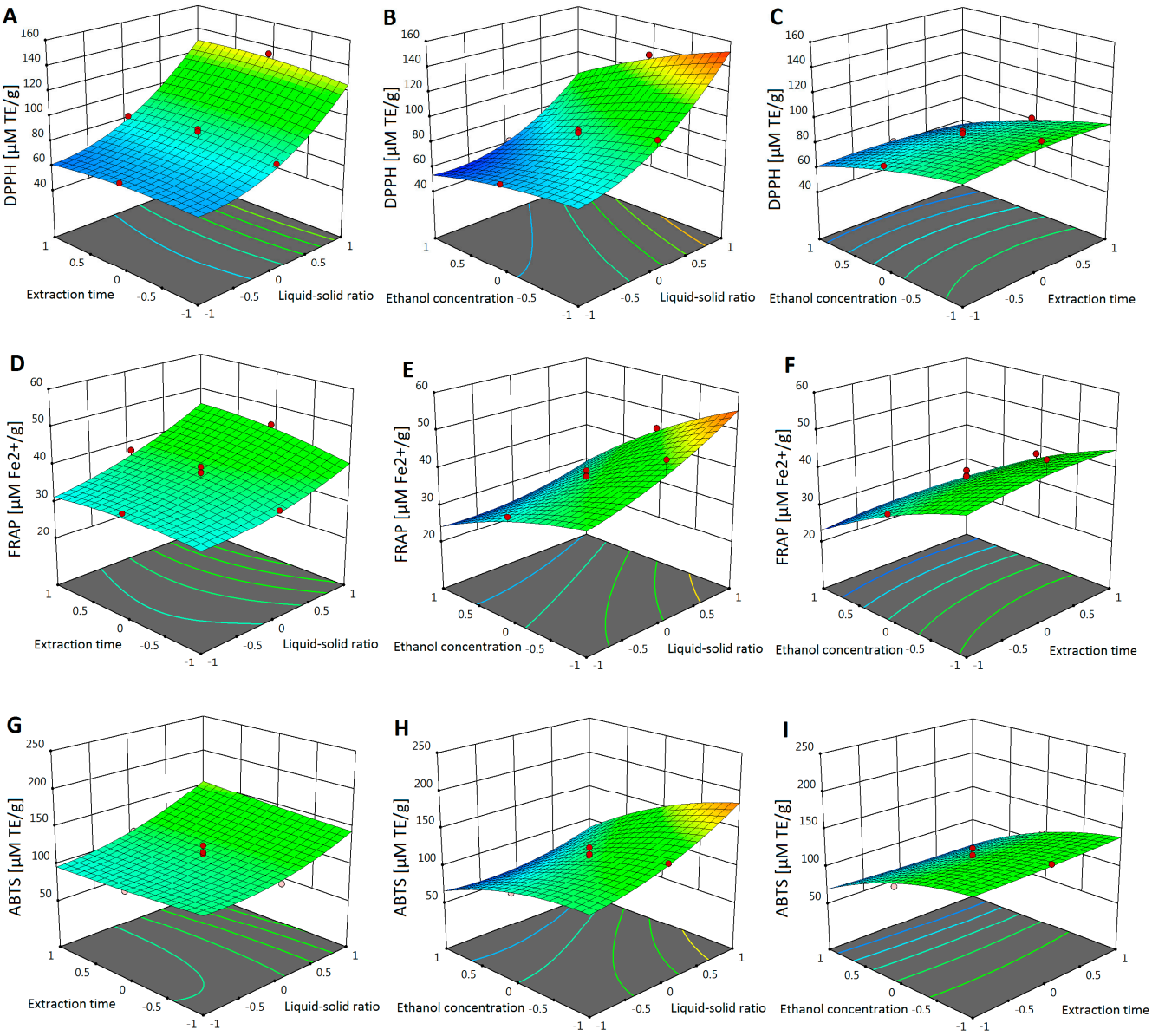

Figure 4. Influence of input factors on antioxidant activity determined by DPPH (**A**–**C**), FRAP (**D**–**F**), and ABTS (**G**–**I**).

3.4. Process Optimization and Comparison with Other Extraction Techniques

For the optimization purposes of carob flour extraction by MAE, response surface methodology was used. The aim of the optimization was to determine the appropriate MAE extraction parameters that would maximize the polyphenol yields and antioxidant activity of CF. The optimized process parameters were determined as follows: maximum L/S ratio (30 mL/g), maximum extraction time (35 min), and minimum ethanol concentration (40%). The comparison between the values predicted by the mathematical model and the experimental values obtained under the optimized MAE conditions is shown in Table 5. The verification of the optimization process was performed with a significant time distance and thus deviations from the expected results occurred. However, the comparison of predicted and experimental results for all investigated parameters was within the acceptable range. Therefore, the previously established suitability of the RSM prediction models for obtaining a valid insight about the influence and interactions of the extraction parameters and for the successful optimization of the MAE process was reconfirmed by the presented results.

Table 5. TP, TF, and in vitro antioxidant activity in predicted and experimentally observed optimized samples and in extracts obtained by UAE and S/L.

Sample *	TP [mg GAE/100 g]	TF [mg CE/100 g]	DPPH [μM TE/g]	FRAP [μM Fe^{2+}/g]	ABTS [μM TE/g]
$MAE_{predicted}$	1774.74 ± 88.74	315.53 ± 15.78	153.33 ± 7.67	56.98 ± 2.85	194.08 ± 9.70
$MAE_{experimental}$	1609.92 ± 56.15	271.92 ± 5.73	99.02 ± 6.41	50.45 ± 5.36	110.55 ± 9.74
S/L	1121.37 ± 32.27	190.72 ± 7.89	72.68 ± 4.21	10.39 ± 2.20	75.06 ± 7.33
UAE	1148.94 ± 50.61	182.59 ± 6.46	65.31 ± 9.18	9.33 ± 1.86	57.63 ± 21.33

* MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; S/L—solid–liquid extraction.

Moreover, carob flour was subjected to both conventional solid–liquid extraction (S/L) as well as to ultrasound-assisted extraction (UAE) as one of the modern techniques. A comparison of the results showed that both S/L and UAE extracts had 30% lower yields of TP and TF. An even greater decrease was observed in the antioxidant activity of the aforementioned extracts. It varied depending on the determination method and was most pronounced in the case of FRAP (80% lower values).

3.5. Quantification of Polyphenols

Photometric assays are a great tool for the fast screening of samples for the quantity and quality of polyphenols and antioxidant components; however there is always a tendency to under- or overestimate the phenolic content by cross-reactions with other reducing agents [21]. For this reason, more insight into phenolic profile of obtained extracts can be obtained by HPLC analysis, regardless of the fact that with HPLC quantification a number of minor compounds might not be taken into account if they are below their quantification limit [35].

The quantitative data obtained by HPLC are listed in Table S2 and the corresponding chromatograms are shown in Figure S1. Six phenolic compounds were identified for all three extracts, namely gallic acid, caffeic acid, rutin, quercetin, quercitrin, and chlorogenic acid. Quercetin was identified but its presence in studied CF extracts was below the limit of quantification (LOD = 1.50 μg/g of dry extract, LOQ = 10.00 μg/g of dry extract). Compared to our previous study [20], fewer compounds were detected in this work; however, those that were detected were present in a greater amount. For example, the predominant phenolic compound, gallic acid, was detected in this work in an amount of 2371.86 μg/g while in the previous study, it was 464.24 μg/g of dry extract. These differences can be attributed to the changes in extraction parameters and it can serve as an argument for the necessity of a comprehensive investigation of the different process parameters. Various process optimizations are also required for the extraction of bioactive compounds. The goal is to modify the range of extractable compounds and their amount to produce a product with the desired properties and composition.

A comparison of the phenolic profiles of carob extracts obtained by different extraction techniques showed only minor differences (Figure 5). It has already been mentioned that gallic acid was the predominant compound in carob flour extracts, which is in agreement with the literature data [19,36]. In our study, it ranged from 2318.70 to 2525.08 μg/g of dry extract. Similarly to our previous study, caffeic acid was the second most abundant compound identified (246.03–331.37 μg/g of dry extract) [20]. Other compounds were quantified with values < 180.00 μg/g. It was observed that the carob extract obtained by S/L had the highest yield of all the identified phenolic compounds except gallic acid. In fact, no significant difference was found as a result of the influence of the extraction techniques used for gallic acid (Figure 3, Table S1). For the other compounds, the difference between MAE and UAE is also negligible. The slightly higher amount of obtained phenolic compounds in the S/L extract could be due to the significantly longer extraction time (24 h) compared to MAE and UAE. The similarity of the phenolic profiles presented between extracts obtained by different extraction techniques may be a result of the otherwise similar extraction conditions (same extraction solvent and L/S ratio), which was intentional.

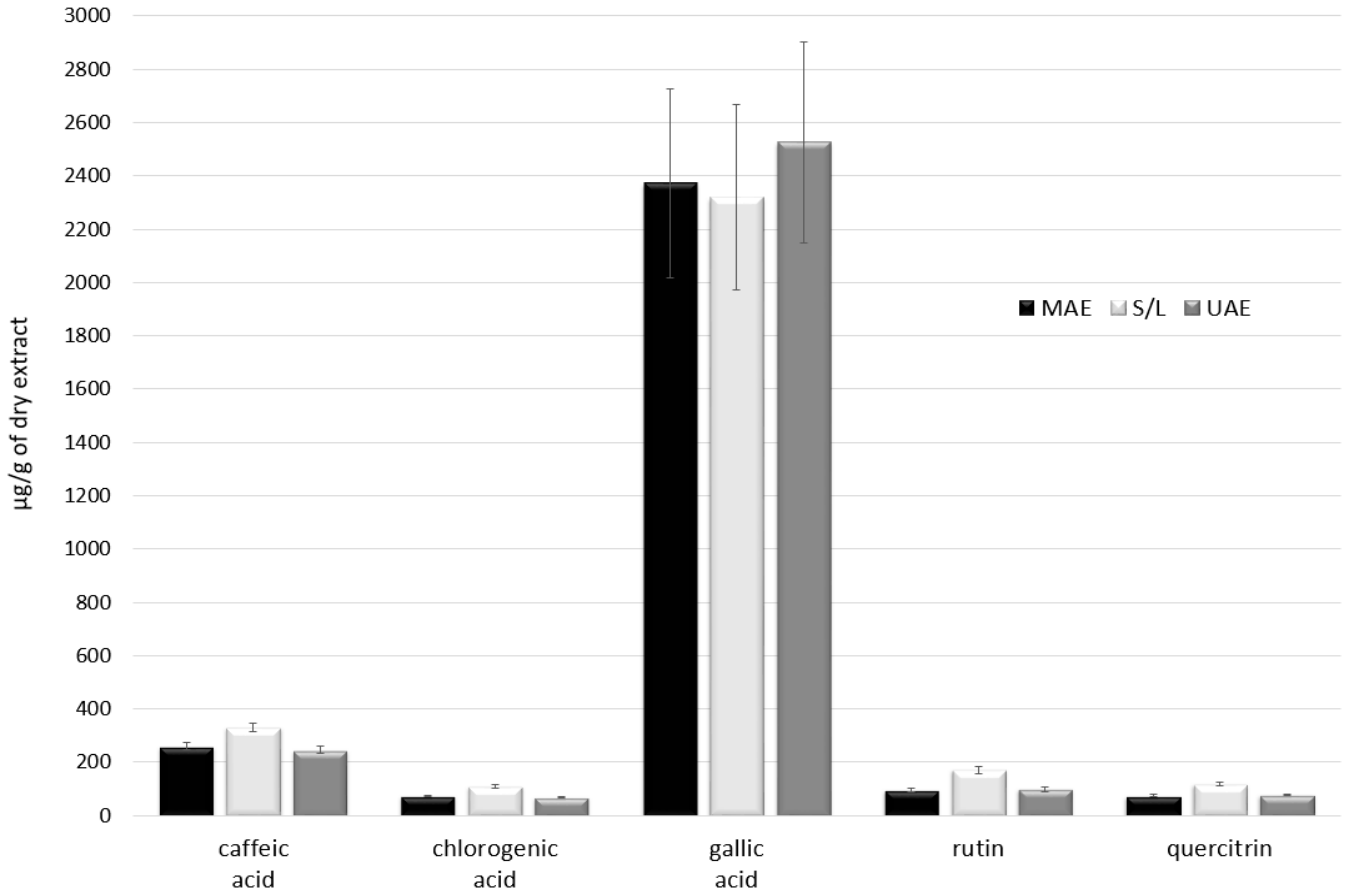

Figure 5. Influence of extraction techniques on the quantity of extractable phenolics from carob flour.

A review of the literature on the phenolic profile of carob extracts revealed significant differences in the literature data and one of the main obstacles was the impossibility to compare the results. The phenolic composition of carob-derived products is strongly influenced not only by the gender, cultivar, and geographical origin but also by the procedures used for sample preparation (e.g., roasting and sugar removal), extraction, and applied analysis [37].

3.6. Antimicrobial Activity

The in vitro antimicrobial activity of CF extracts against the tested microorganisms was quantitatively assessed using the microdilution method (Figure 6). Based on the results given in Table 6, CF extracts exhibited some antibacterial activity; however, antifungal activity was not observed. CF extract obtained by MAE and S/L showed somewhat higher antibacterial activity than CF extract obtained by UAE. MIC of MAE and S/L was 227.27 µL/mL while MBC was 454.54 µL/mL against all tested bacteria. Obtained results also indicate that CF extracts are not dependent on the type of bacteria and had the same MIC and MBC values against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) as well as sporogenous (*B. cereus*) bacteria.

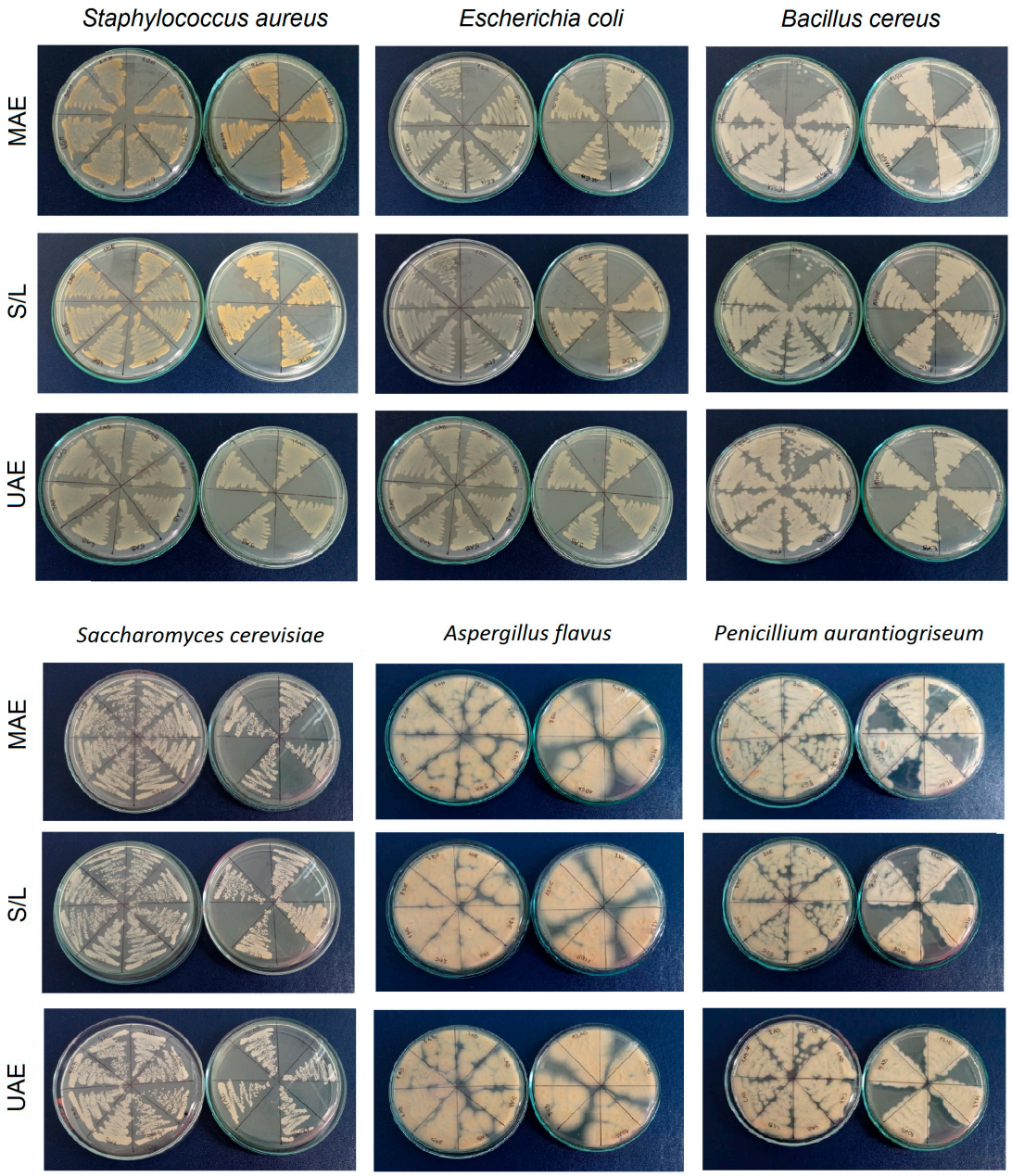

Figure 6. Antimicrobial activity of carob flour extracts obtained with different extraction techniques.

In reviewing the literature data, it was not possible to find enough suitable literature in which the antimicrobial activity of carob pulp extract was considered. For activity testing, carob leaves and seeds were most often used as substrates and their essential oils were used more than extracts. However, Ait Ouahioune et al. [38] analyzed the antibacterial activity of macerates from kibbles, seeds, and leaves of *Ceratonia siliqua* L. and declared that obtained macerates showed weak antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The tested macerates promoted higher inhibition against *Staphylococcus aureus* compared to *Escherichia coli* and *Pseudomonas aeruginosa* suggesting that macerates have better antimicrobial activity against Gram-positive bacteria than against Gram-negative bacteria. Ben Hsouna et al. [39] analyzed the antimicrobial and antifungal activity of *Ceratonia siliqua* L. essential oil against 13 bacteria and 8 fungal strains. The obtained results showed that carob essential oil also had higher antimicrobial activity against Gram-positive than against Gram-negative bacteria. A more complex structure of the cell envelope, which contains a double membrane in Gram-negative bacteria that

restricts the diffusion of hydrophobic compounds compared to the single membrane of Gram-positive bacteria, is most likely responsible for this phenomenon [38–41]. Ben Hsouna et al. [39] attributed the antimicrobial activity of carob essential oil to the presence of a high percentage of hydrocarbons, monoterpenes, and oxygenated monoterpenes and their synergistic effect with minor constituents of the essential oil.

Table 6. Minimum inhibitory concentrations and minimum bactericidal/fungicidal concentrations (μL/mL) of carob flour extracts were obtained using different extraction techniques.

Microorganism	Parameter *	MAE	S/L	UAE
S. aureus	MIC (μL/mL)	227.27	227.27	>454.54
	MBC (μL/mL)	454.54	454.54	>454.54
B. cereus	MIC (μL/mL)	227.27	227.27	454.54
	MBC (μL/mL)	454.54	454.54	>454.54
E. coli	MIC (μL/mL)	227.27	227.27	>454.54
	MBC (μL/mL)	454.54	454.54	>454.54
S. cerevisiae	MIC (μL/mL)	>454.54	>454.54	>454.54
	MBC (μL/mL)	>454.54	>454.54	>454.54
A. flavus	MIC (μL/mL)	>454.54	>454.54	>454.54
	MFC (μL/mL)	>454.54	>454.54	>454.54
P. aurantiogriseum	MIC (μL/mL)	>454.54	>454.54	>454.54
	MFC (μL/mL)	>454.54	>454.54	>454.54

* MIC—minimal inhibitory concentration; MBC—minimal bactericidal concentration; MFC—minimal fungicidal concentration; MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; S/L—solid–liquid extraction.

However, the bioactive compounds in carob flour extracts tested in this work were gallic acid, caffeic acid, rutin, quercetin, quercitrin, and chlorogenic acid. Considering that gallic acid is the most abundant, antibacterial activity can be attributed mainly to it, of course with a synergistic effect with other less abundant components. Gallic acid is a member of the flavones (phenolic acid) family and belongs to the subclass of flavonoids [42,43]. The antimicrobial activity of gallic acid has been tested against various microorganisms. Pinho et al. [44] analyzed the antibacterial activity of different phenolic compounds (gallic acid, caffeic acid, rutin, and quercetin) and the disk diffusion assay showed that gallic acid was most effective against Gram-positive (*Staphylococcus epidermidis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Klebsiella pneumoniae*) at lower concentrations than the other compounds tested. In fact, gallic acid and caffeic acid, both present in carob flour extracts, were the only compounds tested that inhibited *K. pneumoniae*. The antibacterial capacity of flavonoids is based on their ability to create complexes with extracellular and soluble proteins and to destroy the cell wall of bacteria by interacting with essential enzymes responsible for maintaining the stability of this structure [44–46]. Pinho et al. [44] also pointed out that quercetin and rutin, also present in CF extracts tested in this work, had an effect on bacterial growth at much higher applied concentrations (more than 5 mg/mL).

According to the Khelouf et al. [47], carob pulp extract showed no activity against Gram-negative bacteria Salmonella spp; some activity against Gram-positive bacteria *S. aureus* and *B. cereus* (10 mm and 8 mm diameters of the inhibition zones, respectively), and yeast *Candida albicans* (6 mm diameter of the inhibition zones); and high antifungal activity against *Aspergillus niger* (25 mm diameter of the inhibition zones). In this work, CF extracts did not show activity against yeast S. cerevisiae which is good in some cases, such as when these extracts would be used as natural additives in the control of food products where this yeast plays a key role in fermentation, such as in beer or bakery products.

4. Conclusions

Relying on our previous study, in this work the advanced optimization of the bioactive compound extraction from carob pulp flour using MAE coupled with RSM was

performed. The dominant positive influence of the L/S ratio and negative influence of ethanol concentration as the input factors on the recovery of phenolic compounds led to the optimized CF extract with the following characteristics: TP, TF, DPPH, FRAP, and ABTS were 1609.92 GAE/100 g, 271.92 CE/100 g, 99.02 μM TE/g, 50.45 μM Fe^{2+}/g, and 110.55 μM TE/g, respectively. The achieved values for the investigated parameters in optimized MAE carob extract were higher in regard to both UAE and S/L extraction. Consequently, MAE can be considered a simple, efficient, and suitable technique for the extraction of the desired natural antioxidants from carob flour. Furthermore, five different phenolic compounds were identified using HPLC quantitative analysis in all three carob extracts with the predominance of gallic acid (2318.7–2525.08 μg/g of dry extract). Carob flour extracts obtained by MAE and S/L extraction exerted an antibacterial effect against *S. aureus*, *E. coli*, and *B. cereus* (MIC 227.27 μL/mL and MBC 454.54 μL/mL), somewhat higher than the UAE extract.

The presented conclusions indicate the broad value of the obtained CF extract and the wide possibility of its application in various industries. In food products, CF extract could be used not only for enrichment with bioactive substances but also as a replacement of chemical preservatives or additives for the enhancement of safety and sustainability food products. Another way to expand the use of this valuable extract could be pharmaceutical preparations for the treatment of various diseases. However, this field is yet to be thoroughly studied.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10090465/s1. Figure S1: Chromatograms of carob flour extracts obtained by (1) MAE, (2) S/L, and (3) UAE with detection at 280 nm, 330 nm, and 350 nm: 1-Gallic acid, 2-Quercetin, 3-Caffeic acid, 4-Chlorogenic acid, 5-Rutin, and 6-Quercitrin. Table S1: Validation parameters for HPLC analysis. Table S2: Phenolic profile of carob flour extracts obtained by different extraction techniques: (1) optimized microwave-assisted, (2) ultrasound-assisted, and (3) solid–liquid extraction.

Author Contributions: Conceptualization, B.P. and D.Š.-S.; methodology, S.K.-T. and B.P.; software, K.B.; validation, D.Š.-S. and S.K.-T.; formal analysis, J.Z., S.B. and D.B.; investigation, S.B., N.M., D.B. and K.B.; writing—original draft preparation, J.Z.; writing—review and editing, D.B., N.M. and B.P.; visualization, J.Z., S.B., N.M. and K.B.; supervision, D.Š.-S., S.K.-T. and B.P. All authors have read and agreed to the published version of the manuscript.

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