Microwave-assisted extraction of phenolic compounds from *Morus nigra* leaves: optimization and characterization of the antioxidant activity and phenolic composition

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Abstract

BACKGROUND: *Morus nigra* (mulberry) fruit has been reported as a source of bioactive compounds, although information about their leaves is very limited. Usually, they are considered wastes and have been traditionally used only for tea preparation. The main objective of this work was to explore the valorization of mulberry leaves by polyphenols microwave-assisted extraction (MAE) and characterization of their antioxidant activity and phenolic composition. A 2^3 factorial design combined with response surface methodology were applied to characterize the effect of main microwave parameters on total phenolic content (TPC).

RESULTS: The optimized MAE conditions were 20 mL of ethanol:water (1:1; v/v), 120 °C, 28 min, 0.414 g and medium stirring speed. Under these conditions, TPC was 19.7 ± 2.0 mg gallic acid equivalents (GAE) g^{-1} dry plant (DP), and antioxidant activity was 15.3 ± 1.0 mg ascorbic acid (AA) g^{-1} DP (ferric reduction activity power – FRAP – assay), 18.6 ± 1.3 mg Trolox equivalents (TE) g^{-1} DP (2,2-diphenyl-1-picrylhydrazyl – DPPH – assay) and 186 ± 15 mg TE g^{-1} DP (oxygen radical absorbance capacity – ORAC – assay). When compared with ultrasound extraction and Pharmacopeia reference method, MAE was more efficient, representing a valuable technology. Of the 13 compounds identified by HPLC, the most abundant were rutin, chlorogenic, β-resorcylic and caffeic acids.

Keywords: *Morus nigra* leaves; microwave-assisted extraction; response surface methodology; antioxidant activity; phenolic compounds

ABBREVIATIONS

AA	ascorbic acid
AAPH	2,2'-azobis(2-methylpropionamide) dihydrochloride
CE	conventional extraction
CV	coefficient variation
DP	dry plant
DPPH-RSA	2,2-diphenyl-1-picrylhydrazyl radical scavenging activity
FRAP	ferric reduction activity power
GA	gallic acid
GAE	gallic acid equivalents
HPLC-PAD	high performance liquid chromatography with photodiode array detection
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave assisted extraction
ORAC	oxygen radical absorbance capacity
TE	trolox equivalents
TPC	total phenolic content
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid
UE	ultrasound extraction

INTRODUCTION

Plant foods are rich sources of phenolic compounds, which have been widely studied due to their potential health-promoting properties, namely antioxidant, anti-carcinogenic and anti-inflammatory activities.[1,2] Recently, the extraction of these compounds has been increasingly explored in order to satisfy food industry demands, which have shown a growing preoccupation in the replacement of synthetic antioxidants by natural ones.[3,4] *Morus nigra* L. is an important species from the *Moraceae* (mulberry) family. In most mulberry growing countries, plants from the *Moraceae* family are mostly appreciated for their fruits rather than for the foliage;[5] leaves are treated as waste or, in a few countries such as Serbia, have been traditionally used only for tea preparation. Still, almost all the parts of the tree have been used over thousands of years in folk medicine because of its multiple medicinal properties.[6] Several studies have demonstrated that mulberries are polyphenol-rich plants with a large variety of positive health effects such as hyperglycemic,[7] antinephritis,[8] and anti-inflammatory properties.[9] In fact, there is information about *M. nigra* fruits,[5,10–14] but very little about their leaves.[10,12,15,16] Thus, the need of new sources of antioxidants, as feasible, natural and inexpensive alternatives to synthetic antioxidants, associated with the recognized health benefits reported for mulberry plants, promote interest in the exploitation of mulberry leaves for the agro-food and other related industries.

Extraction is usually the limiting analytical step in the yield of bioactive compounds. The development of an optimum extraction process for phenolics can be challenging due to the different possible structures and their antioxidant activity that can lead to fast reaction with other matrix components.[17,18] Over the last decade, microwave-assisted extraction (MAE) has stood out due to its intrinsic benefits over traditional techniques, such as maceration, Soxhlet and ultrasound enhanced extraction, originating environment-friendly processes with energy-, time- and cost-savings and production of high quality extracts.[1,2,19–21] Also, extraction in closed vessels can be accomplished at high temperatures, which increases the mass transfer of the analyte from the sample matrix, allowing rapid processes without deterioration of thermally unstable compounds.[19] Supercritical CO_2 extraction is also a novel technique that has been explored for extraction of valuable compounds, which may be of importance for food and pharmaceutical industries, due to the several advantages (non-toxic and non-explosive properties, readily-available and easy removable from the products) of using CO_2 as solvent.[16] Recently, several groups have been investigating different extraction techniques to isolate bioactive components from mulberries.[5,10–12,22] Still, only one report concerning the application of MAE of polyphenols from mulberry fruits (from a different species, *M. alba*) has been published.[23] No study was found concerning the MAE of phenolic compounds, including their profile characterization, for mulberry leaves. Thus, the objectives of this work were to optimize the MAE of phenolic compounds from *M. nigra* leaves and to compare the yield and characteristics (concerning antioxidant activity and phenolic composition) of the optimum MAE extracts with those of ultrasound extraction (UE) and maceration (conventional extraction

(CE) methodology[24]). The phenolic composition (by high performance liquid chromatography with photodiode array detection, HPLC-PAD) and antioxidant activity (by Ferric Reduction Activity Power, FRAP; 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity, DPPH-RSA; and oxygen radical absorbance capacity, ORAC assays) of the extracts obtained under optimal conditions were characterized. A 2^3 factorial design,[25] combined with response surface methodology (RSM), was applied to characterize the effect of main MAE variables (extraction time, temperature, sample weight) on total phenolic content (TPC).

MATERIALS AND METHODS

Chemicals

Ultrapure water (18.2 MΩ cm; Milli-Q Simplicity 185 system, Millipore, Molsheim, France) was used in all the assays employed. The chemicals and reagents used in the present study, their CAS-Number, purity and suppliers are given in Table 1.

Sampling

Voucher specimens, *M. nigra* L. No 2-1753, were identified and placed at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad, Serbia. Samples of mulberry leaves were dried naturally (in the shade) for one month. Then, leaves were mechanically homogenized, at 9000 rpm for 0.5 min, in a blender (HotmixPRO Gastro, Modena, Italy) before being used in the subsequent experimental assays. Mean particle size, 0.307 ± 0.032 mm, was determined using sieve sets (Erweka, Germany).

Microwave-assisted extraction

A MARS-X 1500 W (CEM, Mathews, NC, USA), using 14 Teflon extraction vessels and temperature (Probe RTP-300 Plus, CEM, Mathews, NC, USA; ± 3 °C) and pressure (Digital Pressure Gauge ESP 1500 Plus, CEM, Mathews, NC, USA; ± 10 psi) control sensors, was used for MAE. Samples (0.08 – 0.92 g of dried and homogenized *M. nigra* leaves) were extracted using 20 mL of the diverse tested solvents (water, methanol and ethanol), and mixtures of these solvents in several ratios (1:4, 1:1, 4:1 v/v; organic solvent:water) at selected temperatures (66 – 134 °C) for 3 to 37 min with constant stirring (medium; 600 rpm) (Table 2). The MAE cavity floor has a rotating magnetic plate located below the floor, which contains four – 2800 gauss magnets. When the magnetic plate is spinning, it causes the magnetic stir bar placed in each extraction vessel to spin inside the vessel; this feature is programmed by the method. After filtration of the supernatant through cellulose filter (0.45 μm; Whatman, Clinton, NJ, USA), extracts proceeded for quantification by HPLC-PDA.

For MAE optimization, a 2^3 factorial design coupled with RSM was used. This model is a combination of mathematical and statistical techniques useful for process optimization, which enables evaluation of the effect of experimental variables (in this study: time (X_1, min.), sample weight (X_2, g) and extraction temperature (X_3, °C)) and their interactions on the response variable studied (TPC).[25] The main objective of RSM is to optimize this response or determine the region that satisfies the operating specifications,[19,21] while, for instance, the model-based experimental analysis (MEXA), enables the identification of kinetic models.[26] In comparison with other designs such as the Box–Behnken design, the applied model takes into account all points and offers better views on the impact of

Table 1. List of chemicals and reagents used in the study

Chemical	CAS-No.	Purity	Supplier
L(+)-ascorbic acid (AA)	50-81-7	≥99%	Merck (Darmstadt, Germany)
2,2′-Azobis(2-methylpropionamide)dihydrochloride (granular)	2997-92-4	97%	Sigma-Aldrich (Steinheim, Germany)
Caffeic acid	331-39-5	≥98%	Sigma-Aldrich (Steinheim, Germany)
(+)-Catechin	154-23-4	≥98%	Sigma-Aldrich (Steinheim, Germany)
Chlorogenic acid	327-97-9	>95%	Sigma-Aldrich (Steinheim, Germany)
p-Coumaric acid	501-98-4	≥98%	Sigma-Aldrich (Steinheim, Germany)
2,2-Diphenyl-1-picrylhydrazyl (free radical; DPPH)	1898-66-4	97%	Sigma-Aldrich (Steinheim, Germany)
(−)-Epicatechin	490-46-0	≥97%	Sigma-Aldrich (Steinheim, Germany)
Ethanol absolute anhydrous	64-17-5	99.9%	Carlo Erba (Peypin, France)
Ferulic acid	1135-24-6	≥99%	Sigma-Aldrich (Steinheim, Germany)
Fluorescein sodium salt (for fluorescent tracers)	518-47-8	≥98.5%	Sigma-Aldrich (Steinheim, Germany)
Folin and Ciocalteau's phenol reagent	not applicable	p.a.	Sigma-Aldrich (Steinheim, Germany)
Formic acid	64-18-6	≥99%	Merck, Darmstadt, Germany
Gallic acid (GA)	149-91-7	≥99%	Sigma-Aldrich (Steinheim, Germany)
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox)	53188-07-1	97%	Sigma-Aldrich (Steinheim, Germany)
Iron (III) chloride hexahydrate	10025-77-1	≥99%	Sigma-Aldrich (Steinheim, Germany)
Kaempferol	520-18-3	≥98%	Sigma-Aldrich (Steinheim, Germany)
Methanol	67-56-1	99.9%	Merck, Darmstadt, Germany
Naringenin	67604-48-2	98%	Sigma-Aldrich (Steinheim, Germany)
Naringin	10236-47-2	≥95%	Sigma-Aldrich (Steinheim, Germany)
di-Potassium hydrogen phosphate anhydrous	7758-11-4	≥ 99.0%	Merck (Darmstadt, Germany)
Protocatechuic acid	99-50-3	99.63%	Sigma-Aldrich (Steinheim, Germany)
Quercetin	6151-25-3	95%	Sigma-Aldrich (Steinheim, Germany)
β-Resorcylic acid	89-86-1	≥97%	Sigma-Aldrich (Steinheim, Germany)
Rutin hydrate	207671-50-9	≥94%	Sigma-Aldrich (Steinheim, Germany)
Sinapic acid	530-59-6	≥99%	Sigma-Aldrich (Steinheim, Germany)
Sodium acetate trihydrate	6131-90-4	≥99.5%	PanReac AppliChem (Barcelona, Spain).
Sodium carbonate	497-19-8	≥99%	Sigma-Aldrich (Steinheim, Germany)
Sodium dihydrogen phosphate monohydrate	10049-21-5	≥98%	Merck (Darmstadt, Germany)
Syringic acid	530-57-4	≥98%	Sigma-Aldrich (Steinheim, Germany)
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	3682-35-7	≥98%	Sigma-Aldrich (Steinheim, Germany)
Vanillic acid	121-34-6	≥97%	Sigma-Aldrich (Steinheim, Germany)

inputs on responses. The 2^3 factorial design provides high quality predictions over the entire design space and requires factor settings outside the range of the factors in the factorial part. The Box–Behnken design is rotatable but it contains regions of poor prediction quality. 2^3 full factorial design also exhibits orthogonality, which is a very desirable property in the design of experiments and is the main reason why two-level factorials are so popular and successful. The real operational values and the respective coded ones applied for the 2^3 design are summarized in Table 2.

Experimental results were adjusted with a second-order regression (Equation (1)):[25]

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where y is TPC, β_0, β_j, β_{jj}, β_{ij} are the intercept, linear, quadratic, and interaction constant coefficients, respectively; X_i and X_j are coded independent factors (extraction time, sample weight and extraction temperature), and ε is the experimental error.

Statistical treatment was with RSM software Design-Expert version 7 (Stat-Ease, Minneapolis, *Minnesota*, USA). Analysis of variance (ANOVA) was performed at $P = 0.05$. The acceptability of the proposed model was assessed by the correlation coefficient (R^2) and the model *P*-value.

Ultrasound extraction and conventional extraction

For UE and CE, approximately 0.414 g (accurately weighed) of dried *M. nigra* leaves were extracted with 20 mL of ethanol:water (1:1; v/v). Sonication was performed in Erlenmayer flasks for 1 h in an ultrasonic bath (Raypa® Trade, Terrassa, Spain). The temperature was maintained at 60 °C (± 1 °C).[27] CE was performed in Erlenmayer flasks for 24 h at room temperature, in the dark, in a shaker (KS 4000i, IKA®-Werke GmbH & Co. KG, Germany) at 150 rpm.[12,24] The supernatants attained were filtered through cellulose filter (0.45 µm; Whatman, Clinton, NJ, USA), and then they were analysed.

Total phenolic content

TPC values were quantified applying a colorimetric assay using Folin–Ciocalteu reagent in accordance with Barroso *et al.*[28] Measurements were determined at 765 nm using 96-well plates in a multi-mode microplate reader (BioTek Instruments, USA). Calibration plots were constructed using GA as standard antioxidant and values were presented as GA equivalents per weight of dry plant (mg GAE g^{-1} DP). Triplicate absorbance readings were made for each sample, and the uncertainty associated to TPC assay was 0.072 mg GAE g^{-1} DP. All the presented results were expressed as the mean ± standard deviation (SD).

Table 2. Real values and coded levels for the experimental design 2^3 (X_1 – extraction time, min; X_2 – sample weight, g; X_3 – temperature, °C) and results (mean of three replications for each run except for the center point, which corresponds to six experiments) for the total phenolic content (TPC, expressed as mg GAE g^{-1} dry plant, mean $\pm$ standard deviation, n = 3). Conditions: 20 mL of ethanol:water (1:1; v/v) and medium stirring speed

Run	X_1 (min)	X_2 (g)	X_3 (°C)	TPC $\pm$ SD(mg GAE g^{-1} dry plant)
1	10 (-)	0.25 (-)	80 (-)	12.3 $\pm$ 1.3
2	30 (+)	0.25 (-)	80 (-)	14.0 $\pm$ 1.3
3	10 (-)	0.75 (+)	80 (-)	13.4 $\pm$ 1.1
4	30 (+)	0.75 (+)	80 (-)	15.8 $\pm$ 1.8
5	10 (-)	0.25 (-)	120 (+)	15.9 $\pm$ 1.7
6	30 (+)	0.25 (-)	120 (+)	16.4 $\pm$ 1.1
7	10 (-)	0.75 (+)	120 (+)	16.4 $\pm$ 0.4
8	30 (+)	0.75 (+)	120 (+)	16.6 $\pm$ 0.6
9	3 (-1.682)	0.50 (0)	100 (0)	11.4 $\pm$ 0.9
10	37 (+1.682)	0.50 (0)	100 (0)	17.3 $\pm$ 0.9
11	20 (0)	0.08 (-1.682)	100 (0)	16.8 $\pm$ 0.9
12	20 (0)	0.92 (+1.682)	100 (0)	14.5 $\pm$ 1.4
13	20 (0)	0.50 (0)	66 (-1.682)	11.9 $\pm$ 0.8
14	20 (0)	0.50 (0)	134 (+1.682)	15.7 $\pm$ 1.1
15	20 (0)	0.50 (0)	100 (0)	17.2 $\pm$ 1.3
16	20 (0)	0.50 (0)	100 (0)	17.1 $\pm$ 0.9
17	20 (0)	0.50 (0)	100 (0)	17.2 $\pm$ 0.5
18	20 (0)	0.50 (0)	100 (0)	17.2 $\pm$ 1.5

Ferric reduction activity power

FRAP assay was executed with some modifications as described by Barroso *et al.*[28] Briefly, FRAP reagent (10 mL of 300 mmol L^{-1} acetate buffer (pH 3.6) plus 1 mL of 10 mmoL TPTZ in 40 mmol L^{-1} HCl and 1 mL of 20 mmol L^{-1} $FeCl_3$) was mixed with acetate buffer (1:3, v/v ratio). Then, 180 μL of this mixture and 20 μL of sample (or ethanol for the blank assay) were introduced in a microplate well. A calibration curve was prepared with AA and absorbance readings were registered in a microplate reader (black 96-well plates, Nunc™ black microwell, Denmark) at 593 nm at 37 °C. Triplicate absorbance measurements were made for each sample, and the uncertainty associated with the FRAP assay was 0.043 mg AAE g^{-1} DP. All the presented results were expressed as the mean $\pm$ SD.

DPPH radical scavenging activity

DPPH-RSA measurements were performed at 517 nm, against the stable radical 2,2-diphenyl-1-picrylhydrazyl, using trolox for construction of the calibration curves.[28,29] Higher absorbance results for the reactive solution correspond to lower free radical scavenging activity. Triplicate absorbance readings were made for each sample, and the uncertainty associated with DPPH-RSA assay was 0.167 mg TE g^{-1} DP. All the results presented were expressed as the mean $\pm$ SD.

Oxygen radical absorbance capacity

ORAC was evaluated based on Prior *et al.*[29] with slight modifications. The reaction mixture was executed in 75 mmol L^{-1} phosphate buffer (pH 7.4); the final volume of the solution was 0.200 mL. Antioxidant standard as trolox solutions (six solutions between 12.5 and 125 μmol L^{-1}) or blank as PBS buffer or samples (25 μL), and fluorescein solutions (150 μL; 70 nmol L^{-1} final concentration) were placed in the microplate well. Solutions were pre-incubated for 30 min at 37 °C. AAPH solution (25 μL; 12 mmol L^{-1} final concentration) was joined and fluorescence was registered continuously over 100 min at 37 °C. Excitation and emission filters were

485 nm and 528 nm, respectively. Triplicate absorbance readings were made for each sample, and the uncertainty associated with the ORAC assay was 0.166 mg TE g^{-1} DP. All the presented results were expressed as the mean $\pm$ SD.

HPLC-PDA analysis

An aliquot of 1.0 mL of *M. nigra* leaves extract was filtered through a 0.20 μm PTFE syringe filter (Teknokroma, Barcelona, Spain) before injection (20 μL). The HPLC system (Shimadzu Corporation, Kyoto, Japan) used was equipped with a LC-20 AD prominence pump, a DGU-20AS prominence degasser, a CTO-10AS VP column oven, a SIL-20A HT prominence autosampler, and a SPD-M20A photodiode array (PDA) detector. Separation of polyphenols was achieved on a *Phenomenex* Gemini C_{18} column (250 mm $\times$ 4.6 mm, 5 μm) using a mobile phase composed of methanol (A) and water (B) both with 0.1% formic acid and a gradient program based on the method described by Rubilar *et al.*[30] The flow rate was 1.0 mL min^{-1} and column temperature was kept at 25 °C. The gradient program starts with 15% A and 85% B; 0–20 min, 15–30% A; 20–40 min, 30–45% A; 40–45 min, 45–50% A; 45–50 min, 50–55% A; 50–65 min, 55–70% A; 65–75 min, 70–100% A which was maintained for 5 min and returned to initial conditions in 10 min. Monomeric flavan-3-ols ((+)-catechin and (-)-epicatechin), hydroxibenzoic acids (gallic, vanillic, protocatechuic, syringic and β-resorcylic), naringin, naringenin and cinnamic acid were monitored at 280 nm, hydroxycinnamic acid derivatives (caffeic, chlorogenic, *p*-coumaric, ferulic and sinapic) at 320 nm, and rutin, quercetin and kaempferol at 360 nm. Phenolic compounds identification on extracts was made comparing the retention times and UV–Vis spectra with those obtained for their pure standards under the same HPLC-PDA analysis. Individual stock solutions (2000 mg L^{-1}) of the 18 selected polyphenols were prepared in methanol, and their mixtures to plot calibration curves ranging from 1 to 50 mg L^{-1} were made in methanol–water (50:50, v/v). Limit of detection (LOD) and quantification (LOQ, mg L^{-1}), and the coefficient of correlation (R^2) are shown in Table 3. Triplicate

injections were made for each sample, and the uncertainty associated with HPLC-PDA analysis ranged from 0.001 (cinnamic acid and naringin) to 0.14 (rutin) mg g^{-1} DP. All the presented results are expressed as the mean $\pm$ SD.

RESULTS AND DISCUSSION
Preliminary MAE tests
Before applying RSM, preliminary trials were performed to select the experimental range for the MAE factors that are more influential in the recovery of phenolic compounds. One of the most important parameters in MAE, owing to the properties of microwave electromagnetic radiation, is the extraction solvent. It should be selected based on the polarity (similar as much as possible to that of the analytes), the partition-equilibrium constant, boiling temperature, toxicity (as low as possible in order to attain an environment-friendly process), the type of solute–matrix bonds, and MAE solvents must as well present preferentially high dissipation factor (tan δ).[1,31] This physical–chemical characteristic of the solvent expresses its capability to absorb microwave radiation and convert it to thermal energy.[31] Bearing this in mind, and based also on previous studies,[10,12,15,16,21] different pure solvents, i.e. water, methanol and ethanol, and mixtures of these solvents in several ratios (1:4, 1:1, 4:1 v/v; organic solvent:water) were tested using a sample weight of 0.50 g and a solvent volume of 20 mL at 100 °C for 20 min. The highest outcomes were achieved with mixtures of methanol:water (1:1; v/v) (19 $\pm$ 2 mg GAE g^{-1} DP) and ethanol:water (1:1; v/v) (17 $\pm$ 1 mg GAE g^{-1} DP), and the less interesting were reached with the pure organic solvents (9 $\pm$ 1 and 7 $\pm$ 1 mg GAE g^{-1} DP for methanol and ethanol, respectively). Even though methanol has been described as one of the most efficient solvents for phenolic compounds extraction,[1,2,32] ethanol:water (1:1; v/v) was selected as the optimum one for subsequent RSM optimization studies because the yields attained with both mixtures were not significantly different, and ethanol is a food grade solvent. It is also a solvent that can be produced from different bioresources (biosolvent) by fermentation, and can contribute to the sustainability of industrial processes.[33] The reported proximate analysis of *M. nigra* leaves revealed that their protein content ranged from 20.9 to 29.1% (DP) depending on the genotype studied.[34] Polyphenols can be linked to proteins either by reversible or irreversible mechanisms through hydrogen binding and hydrophobic interactions.[35] These interactions affect the structure, content of free polyphenols, antioxidant capacity and bioavailability of phenolic compounds in food.[36,37] Therefore, when polyphenols are linked to proteins, the total antioxidant capacity of extracts can decrease, it being necessary to destroy the bonding between polyphenols and proteins. This can be achieved by using organic solvents mixed with water (or increasing the temperature of the extraction), which will decrease the hydrogen bonding and increase the hydrophobic binding between proteins and free polyphenols.[35] However, it is necessary that the extraction conditions do not promote precipitation of the proteins,[36] and consequently the co-precipitation of polyphenols. The results obtained also showed that the TPC yield improved with growing ethanol content up to 50%. Both water and ethanol in low concentration readily enter into the cells and interact with proteins, however a large content of ethanol can denature proteins,[36] avoiding the dissolution of polyphenols and thereafter affecting the recovery.[37] By augmentation with water, the polarity of the prepared extraction mixture will rise and, since phenolics are also polar, their recovery should also increase.[38] However, when

ethanol:water reached 20:80 (v/v), TPC diminished by about 15% probably due to differences in dielectric characteristics (i.e. in the dissipation factor of the solvent mixture regarding microwave energy, since it affects heat distribution in the sample).[1,31,37] Song et al.[4] and Pan et al.[39] also explored the effect of ethanol concentration on extraction yield of polyphenols by MAE from sweet potato and green tea leaves, respectively. In both reports it was demonstrated that an equal mixture of ethanol:water (1:1; v/v) originated the higher extraction yield, which is in accordance with the obtained results.

In preliminary experiments, the efficiency of MAE of phenolic compounds by ethanol:water (1:1; v/v) using different sample weights (0.50–2.0 g) and temperatures (60, 100, and 120 °C) was also assessed. The additional MAE conditions were kept constant, namely, 20 min extraction time, 20 mL of extraction solvent, and medium stirring. The selection of these conditions was established based on our previous experience with MAE and on data from the literature.[19–21,40] For the extraction temperature, the range tested in the present study was chosen bearing in mind that an increase in temperature results in higher desorption of analytes from the sample and in their easier solubilization within the solvent, improving the extraction efficiency. However, when extracting thermolabile compounds, such as phenolic compounds, high temperatures may lead to their degradation decreasing the extraction yield. Liazid et al.[40] evaluated the stability of several polyphenols and reported no degradation at temperatures up to 125 °C for extraction times of 20 min. Thus, the selected temperature range for this study was 60–120 °C. The MAE extraction durations are typically inferior (3–30 min) than those used in conventional techniques because of the inherent characteristics of microwave heating.[31] Yet, this operational factor needs to be reliably optimized to avoid/reduce compounds degradation and method expenses. Based on the data obtained, the selected input variables of the 2^3 factorial design were temperature, sample weight and extraction time; the experimental values for the center point were established as being 0.5 g of dried leaves, 20 mL of ethanol:water (1:1; v/v), 100 °C, 20 min of extraction and medium stirring (Table 2).

Optimization of MAE
Previous information about MAE optimization of phenolics from *M. nigra* plants was not found, and in particular not for those grown in Serbia and the Balkan region. It is well known that depending on the plant origin, crop growing conditions, environmental factors, and extraction technique, the content of biologically active compounds may vary, as well as the level of interfering substances. Thus, the influence of main MAE parameters, namely temperature, sample weight and extraction time, on TPC was evaluated. The coefficient estimates of the regression second-order polynomial model (Equation (1)) for TPC values were obtained based on experimental values (Table 2) and results are exhibited in Table 1S (Supporting information). Response surface regression originated the subsequent model equation (Equation ((2))) for the coded variables (± 1, 0, $\pm \alpha$):

$$Y = 17.15 + 1.08X_1 - 0.020X_2 + 1.17X_3 + 0.043X_1X_2$$
$$-0.43X_1X_3 - 0.29X_2X_3 - 0.85X_1^2 - 0.41\ X_2^2 - 1.06\ X_3^2 \quad (2)$$

Due to the high quality predictions over the entire design space usually exhibited by the applied 2^3 factorial design coupled with RSM, the non-significant factors ($P > 0.05$, Table 1S) were

Table 3. Analytical parameters of the calibration curves used for HPLC-PDA quantification of *Morus nigra* extracts

Compound	$(m \pm \Delta m)$[a]	$(b \pm \Delta b)$[b]	R^2	LOD[c] (mg L^{-1})	LOQ[d] (mg L^{-1})
gallic acid	50706 ± 214	-6217 ± 5928	0.9999	0.55	1.83
protocatechuic acid	33664 ± 661	-21592 ± 19863	0.998	2.77	4.24
catechin	13105 ± 138	2406 ± 3573	0.9997	1.28	4.27
chlorogenic acid	56066 ± 556	-32375 ± 14629	0.9997	1.23	4.09
vanillic acid	35314 ± 321	-931 ± 777	0.9998	1.03	3.45
caffeic acid	106608 ± 566	-16156 ± 13030	0.9999	0.57	1.91
epicatechin	69040 ± 491	-25251 ± 12141	0.9998	0.83	2.75
syringic acid	21490 ± 574	16716 ± 14077	0.998	3.08	5.26
β-resorcylic acid	12308 ± 49	-423 ± 136	0.9999	0.52	1.74
p-coumaric acid	127784 ± 937	-3060 ± 2341	0.9998	0.86	2.87
ferulic acid	107272 ± 902	-9853 ± 2276	0.9998	1.00	3.32
sinapic acid	95532 ± 1417	-8888 ± 3683	0.9993	1.81	6.04
naringin	33168 ± 49	616 ± 124	0.9999	0.18	0.59
rutin	31543 ± 79	1460 ± 197	0.9999	0.29	0.98
cinnamic acid	163683 ± 393	19094 ± 11379	0.9999	0.33	1.09
naringenin	62404 ± 494	12483 ± 12394	0.9998	0.93	3.11
quercetin	64868 ± 1312	-42618 ± 31783	0.9988	2.30	5.67
kaempferol	73460 ± 1244	14914 ± 3030	0.9991	1.94	6.46

[a] m: slope $\pm$ standard deviation (n = 5) expressed in μV min/mg L;
[b] b: intercept $\pm$ standard deviation (n = 5) expressed in μV min;
[c] LOD: limit of detection;
[d] LOQ: limit of quantification.

eliminated and the model equation was redefined as:

$$Y = 17.15 + 1.08X_1 + 1.17X_3 - 0.85X_1^2 - 1.06\,X_3^2 \qquad (3)$$

Then, the suitability of the model was analysed through analysis of variance (Table 2S in Supporting information, which includes the value of pure error as well as the sum of squares, degree of freedom and mean of squares). As wanted, high statistical significance ($P < 0.05$) was attained for the yield of second-order model (Equations (2) and (3)). Also, the high *F*-value (4.87) reached revealed the significant effect of the variables in the recovery (Table 2S, supporting information). The quadratic correlation coefficient of the established second order equation, 0.920, was clearly above the lowest recommended value for chemical data (> 0.8),[25] proving the acceptable relationship among experimental and expected values; sample variations of 92.0% for the extraction efficiency were ascribed to the independent parameters and on 8.0% of the total deviations could not be explained by the proposed model. Furthermore, the reliability of the model was also confirmed via the percentage variation coefficient (CV%); a CV value of 7.3% was reached which is clearly lower than 10% while the experimental and analytical uncertainty ranged from 2.4 to 11.4%.[41]

Regarding the linear, quadratic and interaction effects on the response (Table 1S, Supplementary information), time (X_1) and temperature (X_3) were the most influential parameters; both respective linear and quadratic factors were highly significant ($P < 0.05$). The highest TPC (17.3 ± 0.9 mg GAE g^{-1} DP) was achieved for experimental run number 10 (0.5 g, 100 °C, 37 min) while the lowest (run 9; 11.4 ± 0.9 mg GAE g^{-1} DP) was obtained when the time was drastically reduced to 3 min. 3D surface plots (Fig. 1) corroborated these findings. Concerning X_3, it can be perceived that TPC recovery increased for temperatures up to 130 °C. Raising the extraction temperature (that is directly related to the rise of pressure) accelerated the mass transfer of the analytes from the sample to the extraction solvent due to augmentation of the diffusion rate and solubility, and by the decrease of viscosity, surface tension, and of the strength of the bonds among the compounds and the matrix.[20] Nevertheless, the application of severe conditions (high temperature and pressure) may have a negative impact on the selectivity and may promote degradation of thermo-sensitive compounds. According to Liazid *et al.*,[40] most phenolic compounds were stable up to 125 °C, whereas a significant degradation of only epicatechin, resveratrol and myricetin was reported. Concerning sample weight, no linear or quadratic significant effects on TPC were found, but obviously experimental limitations exist. Sample weight is directly related to the solvent volume, and higher solvent volumes may not induce a rise in recovery. By 3D plot examination (Fig. 1), it can be concluded that sample weight has a positive influence on TPC recovery up to *c*. 0.6 g. Also, interaction between factors (X_1X_2, X_1X_3, and X_2X_3) had no statistical relevance in the responses evaluated. The software predicted a maximum TPC yield of 18.7 mg GAE g^{-1} DP at critical values $X_1 = 28.3$ min, $X_2 = 0.414$ g and $X_3 = 121.8$ °C. These conditions were tested experimentally but the temperature was adjusted to 120 °C to have a 5 °C safety margin to avoid thermal degradation of epicatechin. A mean value of 19.7 ± 2.0 mg GAE g^{-1} DP (n = 3) was achieved, which is in accordance with the predicted result. Therefore, the optimal MAE conditions were ethanol:water (1:1; v/v), 120 °C, 28 min, 0.414 g, 20 mL of solvent, and medium stirring speed.

Comparison with other extraction techniques
Characterization of antioxidant activity
Considering an environmental protection perspective, MAE and UE are reported as being promising alternatives for bioactive compounds extraction, thus MAE was compared with UE in addition to the traditional methodology of maceration.[12,16] Results are summarized in Table 4.
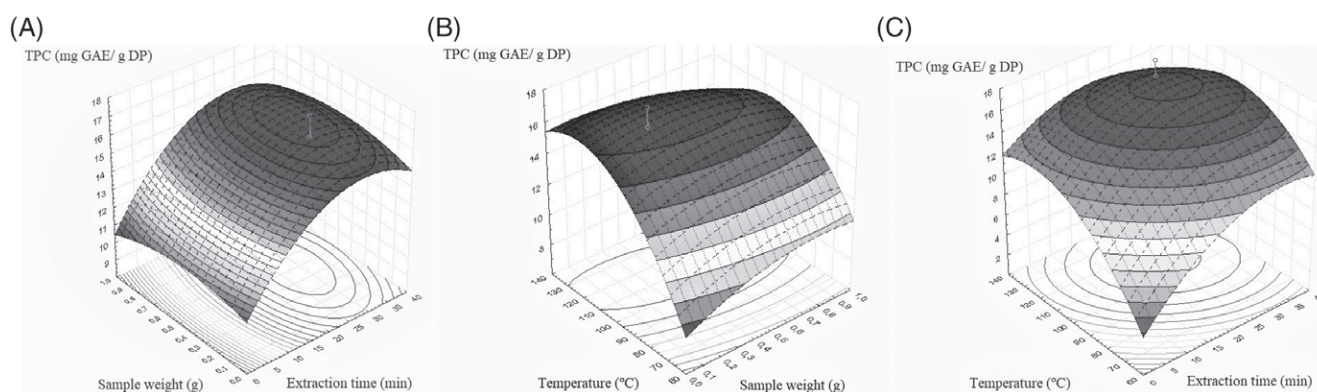
Figure 1. Response surface plots of TPC (mg GAE g^{-1} dry plant) as a function of: (a) extraction time (X_1, min) and sample weight (X_2, g); (b) extraction time (X_1, min) and temperature (X_3, °C); (c) sample weight (X_2, g) and temperature (X_3, °C).

Table 4. Comparison of microwave-assisted extraction (MAE) with conventional extraction (CE; maceration) and ultrasound extraction (UE). Conditions: 20 mL of ethanol:water (1:1; v/v) and 0.414 g dried leaves; results are expressed as mean ± standard deviation, n = 3

| | Extraction technique | | |
Parameter	MAE	CE	UE
Extraction time	28 min	24 h	1 h
Temperature (°C)	120	20	60
TPC (mg GAE g^{-1} DP)[a]	19.7 ± 2.0	17.7 ± 4.3	12.4 ± 0.9
FRAP (mg AAE g^{-1} DP)[b]	15.3 ± 1.0	12.4 ± 1.4	7.5 ± 0.1
DPPH-RSA (mg TE g^{-1} DP)[c]	18.6 ± 1.3	11.1 ± 0.6	10.9 ± 0.7
ORAC (mg TE g^{-1} DP)[d]	186 ± 15	106 ± 9	94 ± 5

[a] TPC: total phenolic content, expressed as mg gallic acid equivalents g^{-1} dry plant,
[b] FRAP, ferric reduction activity power, expressed as mg ascorbic acid g^{-1} dry plant;
[c] DPPH-RSA, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, expressed as mg trolox equivalents g^{-1} dry plant;
[d] ORAC, oxygen radical absorbance capacity, expressed as g trolox equivalents g^{-1} dry plant.

MAE was more efficient, producing richer extracts in terms of TPC (19.7 ± 2.0 mg GAE g^{-1} DP vs 17.7 ± 4.3 mg GAE g^{-1} DP for CE, and 12.4 ± 0.9 mg GAE g^{-1} DP for UE), and antioxidant capacities, i.e. FRAP (15.3 ± 1.0 mg AA g^{-1} DP vs 12.4 ± 1.4 mg AA g^{-1} DP for CE, and 7.5 ± 0.1 mg AA g^{-1} DP for UE), DPPR-RSA (18.6 ± 1.3 mg trolox g^{-1} DP vs 11.1 ± 0.6 mg trolox g^{-1} DP for CE, and 10.9 ± 0.7 mg trolox g^{-1} DP for UE) and ORAC (186 ± 15 mg TE g^{-1} DP vs 106 ± 9 TE g^{-1} DP for CE, and 94 ± 5 g TE g^{-1} DP for UE). These results can be explained by faster and greater damage to the structure of the cell and separation of membrane associated with polyphenols due to a combination of microwave irradiation (it is transformed into heat through ionic conduction and/or dipole rotation, so heat is directly generated within the material, volumetric heating) with high temperatures.[1,2,20] The capability to quickly heat the sample–solvent mixture is an intrinsic characteristic of MAE and the key benefit of this extraction technique. The lowest values were obtained with UE which may be caused by the induction of free radicals generation in the fluid, hence promoting oxidation and significant degradation of polyphenols.[2,27] Comparing the TPC for *M. nigra* leaves obtained by MAE with those found in the literature obtained by other techniques, a higher value was achieved. Memon *et al.*[10] reached a TPC of 13.8 mg g^{-1} DP using

sonication; this value is in close agreement with that reached in this study using the same methodology (12.4 ± 0.9 mg GAE g^{-1} DP for UE). These authors used methanol:water (4:1) as extracting solvent mixture, but methanol is a toxic solvent which reduced the applicability of the extracts produced. Also, Zadernowski *et al.*[22] and Sanchez-Salcedo *et al.*[5] investigated the TPC in fruits from *M. nigra* species, and values attained were lower than those from leaves, 11.5 and 13.6 mg g^{-1} DP, respectively. Therefore, the present work shows the potential usage of mulberry leaves as a promising antioxidant source in the agro-food and pharmaceutical industries.

Characterization of the phenolic composition
Phenolic compounds existing in *M. nigra* leaf extracts obtained by MAE, CE and UE were also characterized by HPLC-PDA analysis (Table 3). A representative HPLC-PDA chromatogram at 280 nm of MAE *M. nigra* extract obtained by application of the optimum conditions is presented in Fig. 2, whereas Table 5 shows the content of individual phenolics identified in *M. nigra* extracts attained using the different tested extraction techniques.

Based on the results achieved (Table 5), these compounds can be allocated to three different groups, namely phenolic acids, cinnamic acid derivatives and flavonoids.

Though no dissimilarities were noticed in the phenolic profile from *M. nigra* leaves in the extracts obtained with the several extractions tested, significant differences were observed in the amount of the individual phenolic compounds. The maximum yield of phenolic compounds identified and quantified was obtained for samples extracted by MAE (9.30 ± 0.93 mg g^{-1} DP). The total amount of individual phenolic compounds extracted from *M. nigra* leaves by UE was reduced by 42%. Regarding the CE, a 25% decrease in yield of individual polyphenols were reached compared with MAE procedure. Memon *et al.*[10] investigated the influence of different experimental methods, namely UE, stirring and homogenization, on the phenolic profile of *M. nigra L.* grown in Pakistan. They reported no differences in the phenolic profile; however, the quantity of phenolics extracted was different, in accordance with the information herein described.

Regarding the phenolic composition, among the identified and quantified compounds, rutin, chlorogenic, β-resorcylic and caffeic acids were the most abundant in all extracts. Concerning MAE, of the 12 identified and quantified compounds, these four correspond to 98% of the total quantified phenolic compounds in *M. nigra* extracts. These data are in accordance with previous work,[5,10–14,16,22] which reported that the main contributors to the
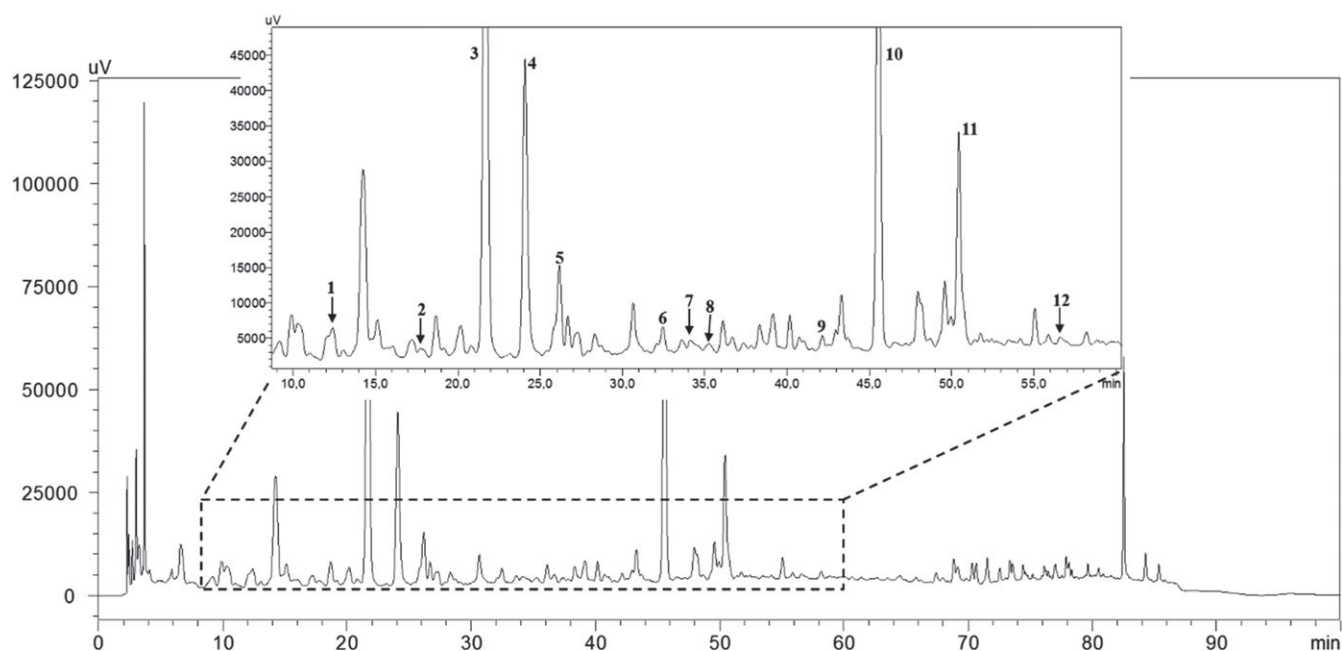
Figure 2. HPLC chromatogram at 280 nm for *Morus nigra* leaves extract obtained by applying optimized MAE conditions (20 mL of ethanol:water (1:1; v/v), 120 °C, 28 min, 0.414 g, and medium stirring speed); (1) protocatechuic acid, (2) (+)-catechin, (3) chlorogenic acid, (4) caffeic acid, (5) β-resorcylic acid, (6) *p*-coumaric acid, (7) ferulic acid, (8) sinapic acid, (9) naringin, (10) rutin, (11) cinnamic acid and (12) quercetin.

Table 5. Content of the identified phenolic compounds detected by HPLC-PDA in *Morus nigra* leaf extracts obtained by microwave-assisted extraction (MAE), conventional extraction (CE; maceration) and ultrasound extraction (UE)

Compound	MAE	CE	UE
	mean ± SD (mg g^{-1} dry plant; n = 3)		
Phenolic acids			
protocatechuic acid	<LOD[a]	<LOD	<LOD
β-resorcylic acid	0.99 ± 0.05	0.542 ± 0.009	0.31 ± 0.01
Cinnamic acids			
caffeic acid	0.84 ± 0.03	0.256 ± 0.008	0.197 ± 0.007
chlorogenic acid	2.79 ± 0.04	2.71 ± 0.02	2.61 ± 0.02
cinnamic acid	0.167 ± 0.002	0.088 ± 0.001	0.054 ± 0.001
p-coumaric acid	<LOD	<LOD	<LOD
ferulic acid	<LOD	<LOD	<LOD
sinapic acid	<LOD	<LOD	<LOD
Flavonoids			
catechin	<LOQ[b]	0.244 ± 0.004	<LOD
naringin	0.060 ± 0.004	0.053 ± 0.002	0.063 ± 0.001
quercetin	<LOD	0.40 ± 0.01	0.29 ± 0.01
rutin	4.45 ± 0.06	2.66 ± 0.14	1.91 ± 0.07
Phenolic content[c]	9.30 ± 0.93	6.95 ± 0.70	5.43 ± 0.54

[a] LOD: limit of detection;
[b] LOQ: limit of quantification;
[c] Phenolic content = sum of the quantified individual phenolic compounds.

phenolic content of *M. nigra* products were rutin and chlorogenic acid. Sánchez-Salcedo *et al.*[5] evaluated the phenolic content of white (*M. alba*) and black mulberry (*M. nigra*) fruits grown in Spain applying sonication with 80% aqueous methanol acidified with formic acid (1%) as extraction solvent at room temperature for 25 min. These authors reported that rutin content ranged from 0.13 ± 0.02 to 0.93 ± 0.06 mg g^{-1}, which is at least 5-fold lower than the value obtained for the MAE extract. Considering the chlorogenic acid content, values obtained by these authors ranged from 0.35 ± 0.05 to 3.18 ± 0.21 mg g^{-1} DP demonstrating a huge intra-species variability. These quantitative differences observed among the results obtained in this study and the ones reported in the literature may be attributed to the extraction technique employed, as well as to the extraction conditions tested (including the selected solvent).[5] Moreover, plant phenolic composition and content is also greatly influenced by the species and cultivation conditions.[12,13,42,43]

CONCLUSION

Overall, the results obtained demonstrated that *M. nigra* leaves can be exploited for the recovery of phenolic compounds, which could be important for new industrial uses. Adoption of MAE allows maximizing the extraction process, and simultaneously decreasing solvent consumption, cycle time and costs. MAE proved to be an interesting technique for phenolic compounds extraction, appropriate for current competitive industries with increasing requests for improved yield, and enhanced efficiency. Also, the present work revealed that *M. nigra* leaves are a rich source of polyphenols, mainly rutin, chlorogenic, β-resorcylic and caffeic acids, with high antioxidant activity.

Supporting Information

Supporting information may be found in the online version of this article.

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