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Optimization of ultrasound-assisted extraction of total polyphenolic compounds from *Juglans nigra* L. leaves

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Abstract: The ultrasound-assisted extraction of *Juglans nigra* L. leaves was optimized with respect to total phenolic content (*TP*) of the extracts by varying the concentration of aqueous ethanol solution (*E*) and different solvent-to-solid ratio (*S*). The influence and optimum of the operating parameters (*E* and *S*) was examined using response surface methodology (RSM). The statistical criteria indicated the adequacy, reliability and precision of the developed RSM model. RSM showed that maximum extraction yield of TP 28.59 mg g^{-1} of draw plant was achieved at the optimal values of 50% E and 20 kg kg^{-1} S. Using the modelled optimized conditions, the detected relative difference between the predicted and the experimental yield was ±2.3 %. The determined TP content in the extracts varied from 12.54 to 29.26 mg GAE g^{-1} of dry plant indicated that *J. nigra* is a valuable source of phenolic substances. The extracts of *J. nigra* leaves obtained under optimal conditions showed good antioxidant activity (IC_{50} = = 18.91±0.03 μg cm^{-3}) which was determined by the scavenging effect on 2,2--diphenyl-1-picrylhydrazyl radical. The optimization of the TP extraction process is the important step in improving techno-economics of the potential commercial preparation of *J. nigra* extracts, as natural source of antioxidants.

Keywords: black walnut; polyphenols; response surface methodology.

INTRODUCTION

Juglans nigra L. (Black walnut) is a medium to large tree, site-sensitive and grows best on deep, fertile, well-drained, nearly neutral pH soils. Besides its economic value, *J. nigra* is also used in traditional medicine.[1] There are studies showing anti-inflammatory, antimicrobial, anti-oxidative and antibacterial act-

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ivity of black walnut, as well as ability of black walnut to depress the tumour growth rate and to prevent lead induced testicular toxicity in rats.[2–5]

The polyphenolic compounds, important parameters in assessing the quality of numerous natural products, are responsible for beneficial health effect these products exhibit.[6] The polyphenolic profiles of bark, sapwood and heartwood, leaves and husks from *J. nigra* were characterized using different polyphenols (juglone, *α*-hydrojuglone-4-glucoside, myricetin, myricitrin, sakuranetin, sakuranin, neosakuranin and *o*-diphenols).[7–9] The extraction of polyphenolic compounds from *Juglans* species has been carried out with methanol and ethanol as solvents.[10–12]

In recent years two extraction methods such as ultrasound-assisted (US-AE) and microwave-assisted extractions have been used for the extraction of polyphenolic compounds from the leaves of *Juglans* species.[11,12] US-AE offers a series of advantages such as improved efficiency, reduced extraction time and temperature.[13,14] There several reports on the ultrasound-assisted extraction of polyphenols from *J. regia* leaves.[11,15] On the other hand, there is no data on US--AE of total phenolics (TP) from *J.nigra* leaves.

Many factors, such as solvent concentration, temperature and extraction time, have been determined to influence the US-AE efficacy of polyphenolic compounds from *J. regia* leaves.[11] Optimization of ultrasound extraction and simultaneous microwave/ultrasonic-assisted extraction of phenolic compounds from *J. regia* was assessed.[11,16] Despite the interesting properties of *J. nigra* and its extracts, there is lack of data regarding the optimal conditions for the polyphenols extraction. The TP compounds from *J. regia* attracted great attention because of their antioxidant potential.[10,11] Accordingly, the aims of this work are to extract TP compounds from *J. nigra* leaves using US-AE, to estimate the influence of the operational parameters (ethanol concentration, solvent-to-solid ratio), as well as to optimize operational parameters using response surface methodology (RSM) in order to obtain a maximal possible extraction yield of TP. In addition, the antioxidant activity of the extract, obtained under optimal conditions, is evaluated by the determination of radical scavenging effects on DPPH.

EXPERIMENTAL

Sample collection

Juglans nigra L. leaves were collected during summer at Aleksinac locality (located at 43° 32′ 11″N/, 21° 42′ 11″E), southeast region of Serbia (Voucher No. 3906HFF; Herbarium of the Department of Botany, University of Belgrade-Faculty of Pharmacy). The leaves were dried in shadow and ground immediately prior to extraction, thus obtaining the plant particles of average size 0.75 mm.

Ultrasound-assisted extraction

An ultrasonic bath (Sonic, Niš, Serbia, power 120 W, frequency 40 kHz) was used for indirect ultrasonication. Temperature was kept constant using thermostated water that circul-

ated through the ultrasonic bath. The ultrasonic power input was 7.3±0.3 W.[13] The ground plant material was mixed with solvent (ethanol at concentrations of 0, 50, 70 and 98 vol. %) in an Erlenmeyer flask, equipped with a reflux condenser.

The ultrasound-assisted extraction was performed for 80 min at 40 °C and at solvent-to-solid (*S*) values of 5, 10, 15 and 20 kg kg^{-1}. After 80 min, the suspension of plant particles in solvent was cooled to the room temperature, taken from the flask and filtered under vacuum to separate the liquid extract from the solid residue. Each experiment was performed in a duplicate. The solvent was then evaporated using a rotary vacuum evaporator until a half-solid residue was obtained, which was then dried at 60 °C to constant weight, then it was measured. The yield of extract was in range from 6.5 to 34.5 mg cm^{-3}.

Total polyphenolic content determination

The total phenolic content (*TP*) in the extracts was determined with a spectrophotometer (LKB Biochrom Ultrospec II UV/VIS) according to the Folin–Ciocalteu method using gallic acid as a standard.[17] The tested extract (20×10^{-6} dm^3) and Folin–Ciocalteu reagent (1×10^{-3} dm^3) were placed in a 10×10^{-3} dm^3 volumetric flask. Aliquots (0.8×10^{-3} dm^3) of 7.5 % aqueous Na_2CO_3 solution were added to the solution and the reaction mixture was increased up to 10×10^{-3} dm^3 with distilled water. The absorbance of the mixture was measured after 30 min at 765 nm. *TP* was expressed as gallic acid equivalents (GAE) in mg per 1 g of dry weight of plant (mg g^{-1} dw).

Optimization of the extraction procedure

In order to obtain the maximum TP yield from *J. nigra* the optimization of extraction parameters was carried out by RSM. The independent process variables were ethanol concentration (*E*) and solvent-to-solid ratio (*S*). RSM models were developed in the form of a second-order polynomial equation:

$$y = b_0 + b_1E + b_2S + b_{12}ES + b_{11}E^2 + b_{22}S^2 \quad (1)$$

where *y* is the yield of total polyphenolic compounds, *E* (%) is ethanol concentration, *S* solvent-to-solid ratio, b_i and b_{ij} (i = 0, 1, 2 and j = 1, 2) are the parameters of Eq. (1) obtained by the multiple nonlinear regression method.

The performance of developed models was assessed with various statistical criteria such as: coefficient of determination (R^2), coefficient of variation (*CV*), adequate precision (*Adec. Prec.*) and the mean relative percentage deviation (*MRPD*).[18,19] The statistical significance of models as well as independent variables and their interactions were estimated by ANOVA (analysis of variance). Using developed models, the maximum predicted TP yields and the optimal extraction conditions were determined through the optimization with the target goal "maximum" using the Design-Expert 7.0.0 trial software (Stat-Ease Inc., Minneapolis, MN).

DPPH radical scavenging capacity

In order to evaluate antioxidant activity of extract from *J. nigra* leaves the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) scavenging capacity was measured. Extracted solution (3 cm^3, 29.1–1.1 μg cm^{-3}) was incubated with DPPH solution (2 cm^3, 90 μmol dm^{-3}) for 30 min in the dark and afterwards the absorbance was measured at 517 nm using LKB Biochrom Ultrospec II UV/VIS spectrophotometer. A blank control of ethanol/water mixture was run in each assay. Inhibition of DPPH radical was calculated as a percentage (%) using the following equation:

$$\text{Scavenging effect, \%} = 100\frac{A_{\text{DPPH}} - A_{\text{S}}}{A_{\text{DPPH}}} \quad (2)$$

where A_S is the absorbance of the solution when the sample extract was added and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50 % inhibition (IC_{50}) was calculated from the graph representing dependence of scavenging effect on concentration of *J. nigra* leaf extracts. IC_{50} was expressed in μg cm^{-3}. The antioxidant measurements were performed in duplicate, and the data were presented as average ± standard deviations (*SD*).

RESULTS AND DISCUSSION

RSM modelling

The *TP* range for used *E* and *S* values was from 12.54 to 29.26 mg GAE g^{-1} dw. The changes in the TP yield (y) along with two operating variables (*E* and *S*) were analyzed using RSM model. The 4^2 full factorial experiments with two replications were carried out in random order to study the effect of different variables on the yields of TP. The experimental matrix with actual factors levels of investigated process variables along with the achieved *TP* are presented in Table I. A multiple nonlinear regression analysis of the experimental data was used to generate a second-order polynomial model that predicts the yield of TP from *J. nigra* leaves:

$$y = 6.28 + 0.356E + 1.74S - 0.002ES - 0.004E^2 - 0.048S^2 \quad (2)$$

TABLE I. The matrix of 4^2 full factorial experiment with two replications and the total polyphenolic compounds; *SD* – standard deviation

Exp. No.	Experimental matrix		Response			
	Factors		*TP* / mg GAE g^{-1} dw			
	E	*S* / kg kg^{-1}	Series 1	Series 2	Mean	*SD*
1	0	5	13.46	14.00	13.73	0.38
2	50	5	21.33	21.33	21.33	0.00
3	70	5	19.12	18.73	18.93	0.27
4	96	5	11.68	10.64	11.16	0.74
5	0	10	18.57	19.02	18.80	0.32
6	50	10	25.85	25.95	25.90	0.07
7	70	10	23.41	23.19	23.30	0.16
8	96	10	15.67	14.89	15.28	0.55
9	0	15	21.41	21.56	21.49	0.11
10	50	15	28.09	27.73	27.91	0.25
11	70	15	25.43	25.16	25.30	0.19
12	96	15	17.38	16.65	17.02	0.52
13	0	20	21.98	21.62	21.80	0.26
14	50	20	28.21	28.98	28.59	0.02
15	70	20	25.18	24.65	24.92	0.38
16	96	20	16.83	15.92	16.38	0.64

The summarized results of ANOVA for the second-order polynomial model, which indicate the significance and adequacy of the chosen model, are given in Table II. The *F*-value and *p*-value (Table II) demonstrated that the developed model has statistical significance at the confidence level of 95 %. The R^2 values proved a good fit by the second-order polynomial equation, while relatively low

values of the *CV* indicated the remarkable precision and reliability of the model. The values of *Adec. Prec.* which measures the signal to noise ratio, were much higher than 4, so the model can be used to navigate the design space. The low values of *SD* (Table II) also implied the reliability and the accuracy of the developed model. Diagnostic plots are shown in Fig. 1. The residuals were close to the straight line and evenly distributed around it, confirming the normal distribution of residuals and validating the ANOVA results (Fig. 1A). The values of Cook's distance, shown in Fig. 1B, were far below the limit of 0.8, indicating that there were no outliers in the experimental data sets. Random scattering of the dots around diagonal could be observed by comparing the predicted and actual values of y (Eq. (1)) in Fig. 1C.

TABLE II. Analysis of variance for the second-order polynomial model

Source of variance	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	779.31	5	155.86	48.66	< 0.0001[a]
E / %	67.31	1	67.31	21.01	0.0001[a]
S	190.73	1	190.73	59.55	< 0.0001[a]
E×*S*	4.87	1	4.87	1.52	0.2284[b]
E^2	516.64	1	516.64	161.30	<0.0001[a]
S^2	45.20	1	45.20	14.11	0.0009[a]
Residual	83.28	10	3.20		
Lack of fit	21.565.63	5	7.56	15.83	< 0.0001[a]
Pure error	3.58.65	8	0.48		< 0.0001[a]
Cor Total	432.3862.58	15			0.0001[a]
R^2	0.904				
CV	8.64				
Adec. Prec.	21.85				
MRPD / %	±6.9				

[a]Statistically significant at the confidence level of 99 %; [b]statistically not significant at the confidence level of 95 %

The effects of two independent variables (*E* and *S*) on the yield of TP were reported through the significance ($p < 0.05$) coefficient of the second-order polynomial model (Table II). The *p*-values lower than 0.05 indicated that the first--order linear effect of two analyzed variables, as well as the second-order quadratic effect of *E* and *S* had a significant influence on polyphenols extraction.

The non-significant factor was interaction of two analyzed variables (*E*×*S*). Linear effects of *E* and *S* showed a positive effect on the TP yield. The positive effects of independent variables revealed that their rise can also cause an increase in the response value.[20] As expected, the *E* and *S* coefficient suggested that the extraction yield of TP is higher at high solvent-to-solid ratio and the ethanol concentration in the range of tested *S* (0–20 kg kg^{-1}) and *E* (0–96 %).

The three-dimensional (3D) response surfaces, which are the graphical representations of the quadratic polynomial regression equation, are illustrated in Fig 2A.

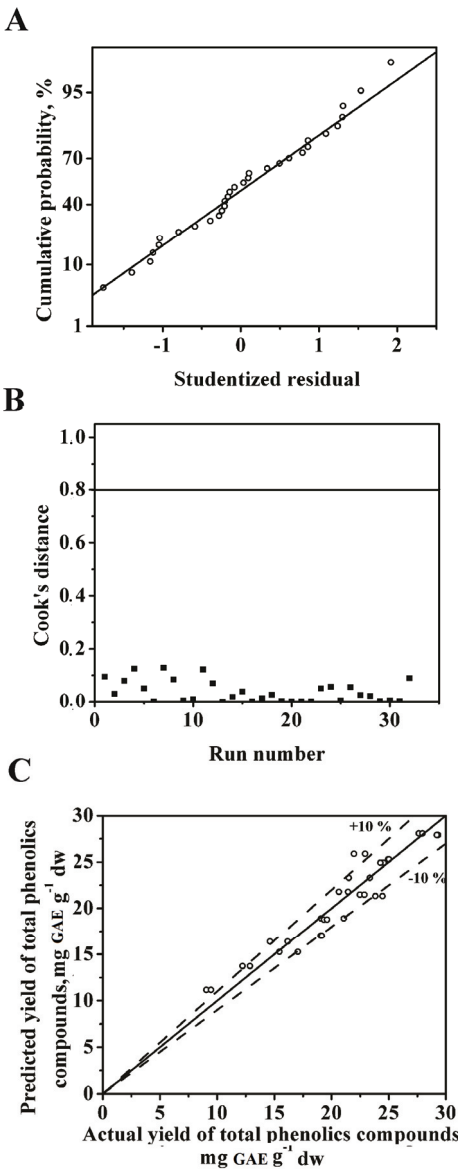

Fig 1. Diagnostic plots of: A) normal probability plot of residuals, B) Cook's distance and C) predicted and actual values of total phenolics.

The graph in Fig. 2A shows the significant effects of independent variables on the yield of TP. The contour plots for TP yield as a function of *E* and *S* are presented in Fig. 2B. Their shape confirmed the interaction between two operating variables. Among the extraction operating variables, ethanol concentration and solvent-to-solid ratio were identified as the most significant variables influencing the TP yield in the case of *J. regia* leaves.[11] Now, in this work, we used

RSM model to examine the effect of *E* and *S* on the yield of TP from *J. nigra* leaves. The type and polarity of extracting solvents are significant factors affecting the extract yields and the resulting polyphenolic content.[14,17] TP yields increased correspondingly with the *E* in range from 0 to 50 % (Fig 2A), because the increase in ethanol concentration accelerates the damage of the cell membranes of the plant matrix. However, at *E* values above 50 % TP yield decreased (Fig 2A). This change is due to the fact that after a certain ethanol concentration and a change in the solvent polarity, impurities are extracted at a faster pace and the diffusion of bioactive suspensions become more difficult.[20]

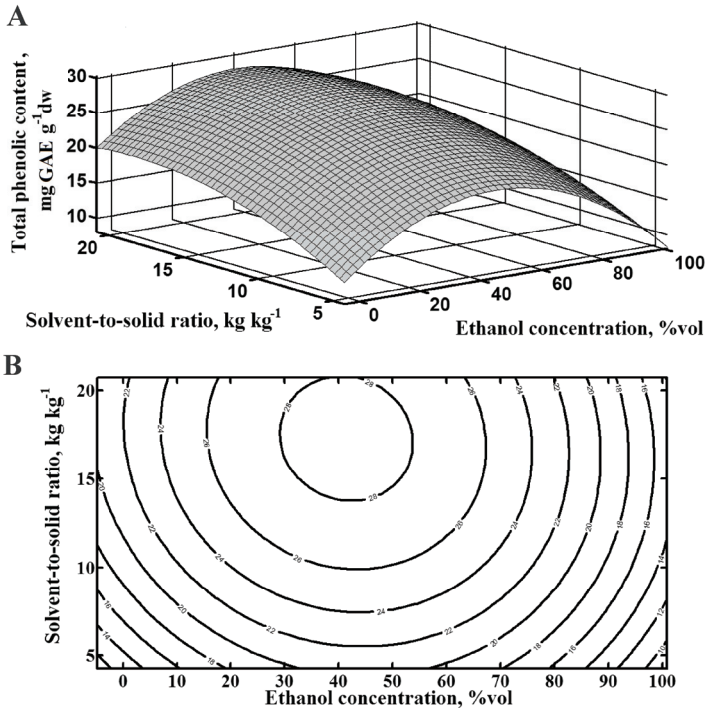

Fig 2. 3D (A) and contour (B) response surface plots for yield of total polyphenolic compounds as a function of ethanol concentration and solvent-to-solid ratio.

The solvent-to-solid ratio was also a vital factor for the increase of the yield of bioactive components using US-AE.[19] The TP yield increased with the increase of *S* (Fig 2A). This result is in accordance with earlier report which confirmed the positive effects of *S* on extraction efficiency of *J. regia* TP yield, due to an increased mass transfer which was achieved at higher *S* values.[6] The results obtained in the present study are generally in accordance with the previously published observances on the effects of extraction operating variables on TP yields from *Jouglas* species.[6,12]

RSM Optimization

Optimization of RSM model gave the maximum predicted value of TP yield 28.59 mgGAE g^{-1} dw, which corresponded to *E* of 50 % and *S* of 20 kg kg^{-1}. This value was in accordance with the TP yield (29.26 mgGAE g^{-1} dw), experimentally obtained at 50 % and 20 kg kg^{-1} thus verifying the developed model. The relative deviation between the predicted and the experimentally gained values of TP yield at the optimal model conditions is ±2.3 %. The relative percentage values of deviation between calculated and experimental values of TP yield at the optimal conditions for two series were < ±10 % which confirmed validity of the RSM under the range of tested *S* and *E*. The confirmed validity of the RSM under optimal conditions is in agreement with the previous studies.[11,12,17]

Nour *et al.*[11] showed, based on RSM optimization, that the liquid-to-solid ratio and the ethanol concentration are the limiting factors influencing the performance of extraction assisted by ultrasound. Our results are in good agreement with the earlier statement that water/ethanol mixtures (50–67 %) were preferred as solvent systems for phenolics extraction from walnut leaves using different techniques.[11,12] Similar results regarding the optimal settings of *S* for the extraction of phenolic compounds from *J. nigra* leaves were reported for *J. regia* by Vieira *et al.*[12] These results coincided with ones reported by Xi *et al.*[21] regarding concentration gradient between the leaves and the bulk of the solvent, which is greater when a higher solvent-to-solid ratio is used.

Previous studies indicated that the leaves of the *J. nigra* contained flavonoides, organic acids and ascorbic acids.[1] The TP yield from *J. nigra* leaves was determined for the first time in this study. Green walnuts (*J. regia*) flesh with the kernel and attached pellicle has greater phenolics and flavonols content than black walnuts (*J. nigra*).[7] However, there is no available data on direct comparisons between TP content of green walnuts and black walnuts leaves. Values for TP content of *J. nigra* leaves reported in this study (12.54–29.26 mg GAE g^{-1} dw) were within the range values (16–30 mg g^{-1} dw) previously reported by Amaral *et al.*[22] and Jalili *et al.*[23] (15.0–27.6 mg GAE g^{-1} dw) and lower than reported by Vieira *et al.*[12] (209 mg chlorogenic acid equivalents g^{-1} dw) for *J. regia* leaves extracts, all analyzed by liquid chromatography with a diode-array detector (HPLC/DAD) method. Besides the origin and the source of the walnuts leaves, there are other limiting factors affecting the content of TP, such as extraction technique, solvent and extraction procedure.[11,12,21,23] The ultrasonic and microwave extraction enable shorter processing time and solvent consumption, when compared to conventional maceration extraction.[24] However, one of the obstacles of the microwave extraction is the rapid increase in temperature of the extraction mixture that may quickly terminate the extraction process due to the boil of the solvent.[24] Therefore, in a number of previous studies[10,11] the authors chose US-AE method for extraction of TP from green walnut due to a range of

advantages such as lower extraction temperature, high reproducibility, simplified manipulation and lower energy input. However, the data on US-AE application on black walnut leaves has not been published in the literature so far. Also, the data on US-AE application for husk and flour of black walnut is also lacking, paving the way for future studies to address this issue.

Antioxidant activity of extracts J. nigra *leaves*

In this study, the capacity of scavenging DPPH free radical was used to estimate the antioxidant activity of extract obtained under optimum conditions from *J. nigra* leaves. The *J. nigra* leaf extracts displayed an effective concentration dependent scavenging capacity, for the concentrations ranging from 29.1 to 1.1 μg cm^{-3} (Fig. 3). The DPPH scavenging capacity of optimal extract of *J. nigra* expressed as IC_{50} was 18.91±0.03 μg cm^{-3}.

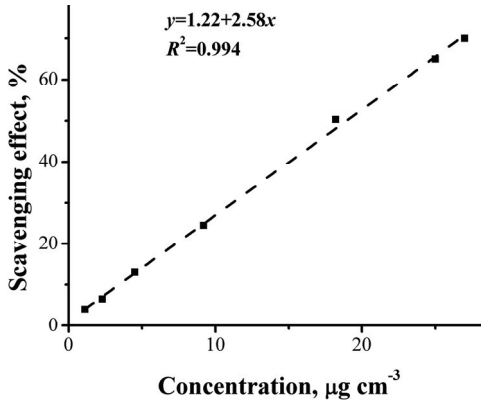

Fig 3. Scavenging effect of *J. nigra* leaf extracts on DPPH radical. The IC_{50} value was obtained by linear regression equation.

The present study was designed to obtain the preliminary data regarding the antioxidant activity of extract from black walnut leaves. Rorabaugh and co-workers[7] reported *in vitro* oxidation inhibition of low density lipoproteins, by a black walnut extract, but so far no studies have been carried out to evaluate the antioxidant activity using DPPH assay. In large number of studies authors relied on this test when assessing antioxidant abilities of tested compounds.[10,11,23]

It is known that the leaves of green walnut,[25] kernels[26] and green husks[10] had high antioxidant activity. Basically, a higher the DPPH radical-scavenging activity is associated with a lower IC_{50} value. In this study, for the first time DPPH test showed that the extract of black walnut leaves also has high anti-oxidant activity (IC_{50} values lower than 1 mg cm^{-3}).

Phenolic compounds, including 5-caffeoylquinic acid, 3-caffeoylquinic acid, 4-caffeoylquinic acid, and the flavonols quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-pentoside, quercetin-3-arabinoside and quercetin-3-rhamnoside are reported to contribute to antioxidative activity of walnut leaves.[7,23] The

antioxidant properties of *J. nigra* leaves extracts were evaluated in order to identify a new natural source of antioxidants. However, previous research indicated that only about 80 % of the radical scavenging activity could be attributed to the phenolic compounds or flavonoids, signifying that other potent antioxidants are also present in the walnut leaves.[10] The future research will be addressed to the analyses of nonphenolic compounds from black walnut leaves.

CONCLUSION

In this work, for the first time, a statistical method based on RSM model was used to optimize the extraction of phenolic compounds from *J. nigra* leaves. The combined effects of ethanol concentration and solvent-to-solid ratio were assessed to maximize extraction yield of TP. The statistical criteria indicated the adequacy, reliability and precision of the developed RSM model. RSM showed that the optimal extraction parameters, which gave a maximum extraction yield of 28.59 mg g^{-1} dw, were obtained for 50 % (ethanol concentration) and 20 kg kg^{-1} (solvent-to-solid ratio). Using the modelled optimized conditions, the detected relative difference between predicted and experimental yield was ±2.3 %. Results of this work showed that RSM proved to be a useful tool for optimizing the extraction of total phenolics from *J. nigra* leaves. The significant radical scavenging effect of the *J. nigra* leaves extract, obtained under optimal condidtions on DPPH radicals, indicate a great potential for application in functional foods.

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ИЗВОД

ОПТИМИЗАЦИЈА УЛТРАЗВУЧНЕ ЕКСТРАКЦИЈЕ УКУПНИХ ПОЛИФЕНОЛНИХ ЈЕДИЊЕЊА ИЗ ЛИШЋА *Juglans nigra* L.

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Ултразвучна екстракција из лишћа *Juglans nigra* L. је била оптимизвана у односу на укупан садржај фенола (*TP*) у екстракату применом различитих концентрација водено–-етанолних раствора (*E*) и различитих односа чврстог и течног (*S*). Утицај и оптималност радних параметара (*E* и *S*) испитани су помоћу методологије одговора површине (response surface methodology, RSM). Статистички критеријуми указивали су на адекватност, поузданост и прецизност развијеног RSM модела. RSM је показао да је 28,59 mg еквивалента галне киселине (GAE) g^{-1} сувог биљног материјала максимални принос екстракције TP, постигнут при оптималним вредностима од 50 % *E* и 20 kg kg^{-1} *S*. Релативна разлика између предвиђеног и експерименталног приноса износила је ±2,3 % за одређене оптималне услове екстракције. Садржај TP у екстрактима варирао је од 12,54

до 29,26 mg GAE g^{-1} и показао да је *J. nigra* користан извор фенолних супстанци. Екстракт листа *J. nigra* добијен под оптималним условима показо је добру антиоксидативну активност (IC_{50} = 18,91±0,03 μg cm^{-3}), која је одређена ефектом чишћења 2,2-дифенил-1--пикрилхидразил радикала. Оптимизација екстракције ТР је важан корак у побољшању техно-економске потенцијалане комерцијалне примене *J. nigra* препарата као природног извора антиоксиданата.

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