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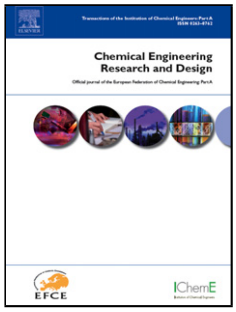

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PII: S0263-8762(20)30096-4

DOI: https://doi.org/10.1016/j.cherd.2020.03.002

Reference: CHERD 4022

To appear in: *Chemical Engineering Research and Design*

Received Date: 6 November 2019

Revised Date: 7 February 2020

Accepted Date: 2 March 2020

Please cite this article as: Rajković KM, Vasić M, Drobac M, Mutić J, Jeremić S, Simić V, Stanković J, Optimization of extraction yield and chemical characterization of optimal extract from *Juglans nigra* L. leaves, *Chemical Engineering Research and Design* (2020), doi: https://doi.org/10.1016/j.cherd.2020.03.002

Research paper

Optimization of extraction yield and chemical characterization of optimal extract from *Juglans nigra* L. leaves

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Abstract

The extraction yield of *Juglans nigra* L. leaves was assessed at different ethanol concentrations (0 – 96% (v/v)) and solvent-to-solid ratios (5 – 20 kg kg^{-1}). The response surface methodology (RSM) and artificial neuron network with genetic algorithms (ANN-GA) were developed to optimize the extraction variables. The RSM and ANN-GA models determined 50% (v/v) ethanol concentration and 20 kg kg^{-1} solvent-to-solid ratio as optimal conditions, ensuring an extraction yield of 27.69 and 27.19 g $100g^{-1}$ of dry leaves. The phenolic compounds in optimal extract were quantified: 3-O-caffeoylquinic acid (2.27 mg $g^{-1}$of dry leaves), quercetin-3-O-galactoside (10.99 mg g^{-1} of dry leaves) and quercetin-3-O-rhamnoside (15.07 mg $g^{-1}$of dry leaves) using high-performance liquid chromatography (HPLC). The minerals in optimal extract were quantified: macro-elements (the relative order by content was: K > Mg >Ca) using inductively coupled plasma optical emission spectrometry (ICP-OES) and micro-elements (the relative order by content was: Zn >Rb>Mn>I>Sr> Ni > Cu > Co > V > Ag > Se) using inductively coupled plasma mass spectrometry (ICP-MS). The extraction coefficients for minerals were determined and were highest for K (64.3%) and I (53.5%). Optimization of extraction process resulted in high extraction yield from *J. nigra* leaves and optimal extract containing different phytochemical compounds.

Abbreviations:

ANN - artificial neural network

GA - genetic algorithm

RSM - response surface methodology

HPLC - high-performance liquid chromatography

ICP-OES - inductively coupled plasma optical emission spectrometry

ICP-MS - inductively coupled plasma mass spectrometry

MSE - mean square error

MRPD - mean relative error percentage

R^2 - coefficient of determination

CV - coefficient of variation

Adeq. Prec. - adequate precision

y - extraction yield

x_1 - ethanol concentration

x_2 - solvent-to-solid ratio

F – Fisher statistic

3Cqa - 3-O-caffeoylquinic acid

Q3Gal - quercetin-3-O-galactoside

Q3Ram - quercetin-3-O-rhamnoside

LOQ - limit of quantification

LOD - limit of detection

Keywords: *Juglans nigra*; Artificial neural network; Response surface methodology; Phenolic constituents; Minerals

1. Introduction

The genus *Juglans* L. contains about 20 species (Nicese et al., 1998) and among them, *Juglans regia* L. is most commonly studied. However, Camara and Schlegel (2016) promoted that due to the chemical composition of its fruit, *J. nigra* (Black Walnut) has multiple biological significance similar to *J. regia* as well as other nuts. Additionally, some other studies highlighted the medical importance of *J. nigra* fruit (Amarowicz et al, 2008; Camara and Schlegel, 2016). Rorabaughet et al., (2011) have shown that *J. nigra* nuts protect low-density lipoprotein against oxidation *in vitro*, however, resistance to oxidation *in vivo* was not proved. Fitschen et al. (2011) have shown a relationship between nuts consumption and reduced risks for heart disease due to its effect on blood lipids. *J. nigra* nuts intake led to an approximately 1% reduction of total cholesterol. Effects of *J. nigra* nuts were gender-dependent: the total cholesterol decreased in men for 4.5% and increased in women for 1.8% (Fitschen et al., 2011). Thus the majority of chemical research was focused on the fresh kernel of *J. nigra* (Rorabaughet al., 2011; Nwosu et al., 2015). However, despite a very diverse phytochemical composition of *J. nigra* leaves (Gavrilović et al., 2018; Ponder Jr et al., 1979; 2005), there is no detailed chemical analysis.

Gavrilović and coworkers (2018) optimized total phenolic extraction from *J. nigra* leaves in order to examine their antioxidant potential. The use of mathematical modeling significantly improved extraction process optimization from *Juglans* species (Nour et al, 2016; Vieira et al, 2017). The models such as response surface methodology (RSM) and artificial neural network with genetic algorithm (ANN-GA) are often utilized for extraction processes modeling and optimization (Alara et al., 2018; Simić et al, 2016). RSM uses the model equation to represent the dependence of the extraction yield on the used operational

extraction parameters (Said and Amin, 2015). ANN works on the principle of biological neural networks, and its limitations in the optimization of various processes are overcome by combining with genetic algorithm (GA) (Rajković et al., 2015).

Nowadays, interest in functional food and herbal products is increasing. However, there are no available data on extraction optimization from *J. nigra* leaves, relative to total extractive substances. Accordingly, the aim of this research was to optimize variables for the extraction of total extractive substances from *J. nigra* leaves. Therefore, specific objectives included: (i) optimization of operational variables (ethanol concentration, solvent-to-solid ratio) using RSM and ANN–GA; ii) chemical characterization of *J. nigra* leaves extract obtained under optimal conditions. Within chemical characterization of extract, high-performance liquid chromatography (HPLC) analysis was used for the determination of phenolic composition, whereas inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS) were used for mineral composition assessment.

2. Materials and Methods

2.1 Sample collection and extraction of *Juglans nigra* leaves

Juglans nigra leaves were collected during summer at Aleksinac locality, the southeast region of Serbia (located at 43º 32᾽ 11″N/, 21º 42′ 11″E). The voucher specimen was deposited at the Herbarium of the Department of Botany, University of Belgrade-Faculty of Pharmacy (HFF) under the number 3906HFF. Plant material was identified by the botanist, prof. Branislava Lakušić (Department of Botany, University of Belgrade – Faculty of Pharmacy). The identification was made according to the Flora of Serbia (Jovanović, 1970), based on the morphological characteristics of herbarium specimens in the vegetative stage of development.

The leaves were dried in the air and grounded thus obtaining plant material particles of average size of 0.75 mm.

The extraction was performed using an ultrasonic assisted extraction method. Indirect ultrasonication was performed using an ultrasonic bath with thermostat (Sonic, Niš, Serbia, power 120 W, frequency 40 kHz). The grounded plant material was mixed with solvent (ethanol at concentrations of 0; 50; 70; 96% (v/v)) at solvent-to-solid ratios of 5, 10, 15, 20 kg kg^{-1}, in a reflux appliance. Ultrasonic assisted extraction was performed during 80 min at 40 ° C and obtained suspension was vacuum filtered. The solvent was removed from the filtrate in a rotary vacuum evaporator and the extract was dried at 60 ° C to constant weight. The mass extracted from dry plant material defines the yield of total extractive substances known as extraction yield in g $100g^{-1}$of dry leaves. Each experiment was performed in duplicate.

2.2 Modeling and optimization

Two mathematical models were used for modeling and optimization of ultrasonic assisted extraction of *J. nigra* leaves: RSM model and ANN-GA model. A factorial experiment involving two factors (ethanol concentration and solvent-to-solid ratio) was used to optimize the extraction yield. Each factor consisted of four levels including ethanol concentration (0; 50; 70; 96% (v/v)) and solvent-to-solid ratio (5, 10, 15, 20 kg kg^{-1}).

2.2.1 RSM model

The 4^2 full factorial experiments with two replications were fitted to the second-order polynomial equation (1):

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ij} x_{ij}^2 + \sum_{i<1} \sum_{j=2}^{k} b_{ij} x_i x_j \quad (1)$$

where y is the dependent variable (experimental value), x is an independent variable, b_i and b_{ij} (i=0, 1, 2 and j=1, 2) are the coefficients of equation (1) and the terms $x_i x_j$ and x_i^2 represent the interaction and quadratic terms, respectively. The coefficients (b_i and b_{ij}) obtained by the multiple nonlinear regression method.

The performance of the developed RSM model was assessed following statistical parameters: the coefficient of determination (R^2), the coefficient of variation (*CV*), the adequate precision (*Adec. Prec.*) and the mean relative percentage deviation (*MRPD*) (Milić et al., 2013). Another way to evaluate model suitability is the lack-of-fit test. The statistical significance of models as well as independent variables and their interactions were estimated using ANOVA. Design Expert software 7.0 (Trialversion, Stat-EaseInc., Minneapolis, USA) was used for statistical analysis.

2.2.2 ANN-GA model

Based on obtained experimental data, ANN model with Levenberg–Marquardt (LM) algorithm was developed, using MATLAB2013a. Developed ANN model consisted of an input neurons layer made up of independent operating variables (ethanol concentrations and solvent-to-solid ratios), hidden neurons layer and output neurons layer made up of dependent operating variable (extraction yield). The heuristic method was used to obtain the optimal number of neurons in the hidden layer. The procedure was carried out as previously described (Simić et al., 2016). Basic performances of ANN model are given in Table 1. Statistical criteria used for assessing ANN model were: the mean square error (*MSE*), coefficient of determination (R^2) and the mean relative error percentage (*MRPD*) (Milić et al., 2013). Thereafter, genetic algorithm (GA) was applied to optimize the interior space of the developed ANN model. GA parameters were obtained as previously described (Milić et al., 2013).

Table 1.

2.3 HPLC analysis

Dried ethanol extract (obtained with ethanol concentration of 50% (v/v) and solvent-to-solid ratio of 20 kg kg^{-1}), dissolved in 50% (v/v) ethanol (5 mg mL^{-1}) and filtered through 0.45 µm filter (Captiva, Agilent Technologies), was analyzed by HPLC using an Agilent 1100 HPLC system with diode-array detection. Instrumental conditions for HPLC analysis were: 250 × 4.6 mm analytical column with 5 µm particle size (Zorbax EclipseXDB-C18), 0.8 ml/min flow rate, 25 °C temperature, 20 µl injection volume. Solvent A, 0.03% phosphoric acid, and solvent B, 10% of A in acetonitrile, were used for gradient elution as follows: initial 10% of B, raised to 25% in 5 min, then kept constant until 15 min, 15-20 min raised to 30%, 20-25 min increased to 50%, 25-30 min increased to 70% of B, returned to initial conditions until 35 min and kept constant for 3 min. The wavelengths on which chromatograms were recorded were 210, 250, 320 and 350 nm. Qualitative analysis was performed by comparing the UV spectra and the retention time of the detected components with those obtained for chlorogenic acid (Carl Roth), quercetin 3-O-galactoside (Carl Roth), quercetin 3-O-rhamnoside (Carl Roth) as well as by comparing with literature data (Kammerer et al., 2004; Wua et al., 2013). For the quantification of phenolic acid and flavonoids external standard method was used with standards of chlorogenic acid and quercetin 3-O-galactoside. Data on the obtained calibration curves and validation parameters are given in Table 2.

Table 2.

2.4. ICP-OES and ICP-QMS analysis

The content of minerals was determined in ethanol extract of the leaf as well as in dry leaf sample. For determination of mineral content, 5 mL of ethanol extract (obtained with

ethanol concentration of 50% (v/v) and solvent-to-solid ratio of 20 kg kg^{-1}) evaporated and diluted to 50 mL ultra-pure water and 0.3 g of leaf sample were subjected to microwave digestion with 7 mL of concentrated HNO_3 and 1 mL of concentrated H_2O_2 in a microwave oven Bergfhof (Speedwave, Berghof, Germany).

For the determination of major elements (Al, Ca, Fe, K, Mg and Na) Inductively Coupled Atomic Emission Spectrometer, ICP-OES (Thermo Scientific, United Kingdom), model 6500 Duo was used. Concentrations of trace elements (Ag, As, Ba, V, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sr, Se, Zn, Rb and I) were determined with ICP-QMS (iCAP Q, Thermo Scientific X series 2). Instrumental conditions for determination on ICP-OES and ICP-QMS are given in Table 3.

Table 3.

3. Results and discussion

3.1 Extraction optimization from *Juglans nigra* leaves

3.1.1RSM modeling and optimization

The experimental matrix of operating variables with extraction yield is represented in Table 4. The RSM model describing the relationship between the extraction yield (y) and two variables, namely ethanol concentration (x_1) and solvent-to-solid ratio (x_2), is presented by Eq. (2):

$$y = 9.635 + 0.214x_1 + 1.147x_2 + 0.002x_1x_2 - 0.003x_1^2 - 0.028x_2^2 \quad (2)$$

Table 4.

ANOVA was used to analyze the statistical significance of the equation model, the operating variables, their interactions and the validity of the fitting (Table 5). The F-value and

p-value pointed out that developed model has statistical significance at the confidence level of 95%. The R^2, *CV* and *Adeq. Prec*. values showed adequacy, reliability and precision of the developed RSM model (Table 5). The lack of fit was not statistically significant ($p > 0.05$) which indicated that the model was adequate for predicting the extraction yield.

Table5.

Two operating variables (x_1 and x_2), their interaction and two square values ($x_1^2$and x_2^2) showed significant impact on y ($p < 0.05$) (Table 5). The effects of ethanol concentration and solvent-to-solid ratio on the extraction yield are shown in Fig.1. The extraction yield increased with the increase of ethanol concentration up to 50% (v/v) and further increase in ethanol concentration led to decrease the extract yield (Fig 1A). The highest yield was achieved when 50% (v/v) ethanol was used whilst the lowest one was obtained when 96% (v/v) ethanol and pure water were used. It is known that water causes swelling of the cell to increase the surface area, while elevated ethanol concentration could cause disruption of cell matrix, increase the solvent diffusivity and improve the solubility of substances (Simić et al., 2016). Therefore, ethanol concentration in the range from 0 to 50% (v/v) had a beneficial effect on extraction yield, however further excessive increase in ethanol percentage caused higher degradation rates and overall decreased the extraction yield. It is shown that solvent polarity plays an important role in solubility, especially when using ultrasonic assisted extraction, as in this study. Therefore, the addition of organic solvent in water reduces surface tension of the medium allowing thus easier induction of ultrasonic cavitation (Rostagno and Prado, 2013).

RSM model suggests that the solvent-to-solid ratio has a positive effect on the extraction yield (Eq. 2). In contrast, in this paper a smaller interval of solvent-to-solid ratio was investigated, hence providing a more accurate optimum ratio. Yield of extraction

increased rapidly when solvent-to-solid ratio ranged from 5 to 15 kg kg^{-1}. This is consistent with mass transfer principles, meaning that the concentration gradient between solid and liquid is higher when a higher solvent-to-solid ratio is used. The changes in solvent-to-solid ratio affect the solubility and equilibrium constant of compounds in the extraction solvent (Cacace and Mazza, 2003). Also, higher solvent-to-solid ratio reduces solution viscosity thereby reducing the level of the ultrasonic cavitation threshold (Rostagno and Prado, 2013). Therefore, the lowest extraction yield was obtained at the lowest solvent- to-solid ratio of 5 kg kg^{-1} leaving most of the extraction substance content in the plant matrix. Solvent-to-solid ratio ranging from 15 to 20 kg kg^{-1} gave a high extraction yield because of the increase in mass solubility and transfer rate from the plant material to the solvent. Also, interaction between these two operating variables was confirmed by the shape of the contours (Fig. 1B). The results of this study coincide with available literature data (Nour et al, 2016; Rajković et al., 2017), and they showed that the high extraction yield could be obtained for *J. nigra* leaves by using moderate ethanol concentration and solvent-to-solid ratio.

According to the RSM results, the optimal extraction conditions required for the highest extraction yield were x_1 of 50% (v/v) and x_2 of 20 kg kg^{-1}, respectively. The maximum predicted value of the extraction yield was 27.69 g 100 $g^{-1}$of dry leaves. This value coincided with the extraction yield obtained at x_1 of 50% (v/v) and x_2 of 20 kg kg^{-1} (27.78 g 100$g^{-1}$of dry leaves), verifying the developed model. Similar and higher values of extraction yield have also been reported for hydroalcoholic extraction from *J. regia* leaves (Vieira et al., 2017).

Figure 1.

3.1.2 ANN modeling and ANN-GA optimization

The developed ANN model consisted of two input neurons (x_1 and x_2), 10 hidden neurons and one output neuron (y) as shown in Fig. 2. The ANN model (2-10-1) showed good performances, the highest R^2-values (training = 0.986, testing = 0.911and validation = 0.964) and the lowest values of *MSE* (training = 0.41, testing= 3.19 and validation = 1.21). The best validation performance was determined to be 1.21 at epoch 7. R^2 value very close to 1 (0.953) indicated good correlation between predicted and experimental values of the extraction yield. Since 3D and contour plots for ANN model were similar to those of the RSM model, they were not shown in this paper.

Figure 2.

Application of GA over the ANN model determined the optimal set of independent variables $x_1$and x_2. Within the ranges of the independent variables applied during development of the model, the highest predicted extraction yield of 27.19g $100g^{-1}$ of dry leaves corresponded to x_1 of 50% (v/v), x_2 of 20 kg kg^{-1}. Predicted ANN-GA value of extraction yield coincided with yields obtained experimentally for the same optimal conditions (27.78g $100g^{-1}$ of dry leaves), thus verifying developed model.

3.1.3 Comparison of RSM and ANN models features

The accuracy of the RSM and ANN models was estimated by calculating the *MRPD*. The ANN showed a slight advantage over RSM due to lower *MRPD* value (± 4.3% and ± 2.7% for RSM and ANN, respectively). Experimental values of the extraction yield and predicted values obtained by using developed RSM and ANN models showed good agreement as illustrated in Fig. 3. Additionally, ANN-GA and RSM models determined same optimal conditions for extraction (x_1 of 50% (v/v), x_2 of 20 kg kg^{-1}), and extraction yields predicted for that conditions differ very little from the corresponding experimental values (the relative deviations between predicted and experimental values of extraction yield are ± 0.3%

and ± 0.5% for RSM and ANN, respectively). Low values of relative percentage deviations between predicted and experimental extraction yield values (<10%) indicated that both models are adequate for prediction of extraction yield under optimal conditions. Therefore, RSM and ANN-GA models, based on different working principles, can be used as mutual controls when determining optimal extraction conditions.

Figure 3.

3.2 Chemical characterization of optimal extract from *J. nigra* leaves

The optimal extract obtained with ethanol concentration 50% (v/v) and solvent-to-solid ratio 20 kg kg^{-1} contained different compounds, therefore their chemical characterization was necessary.

3.2.1 Phenolic constituents

In *J. nigra* leaf optimal extract obtained in his study, one phenolic acid, namely neochlorogenic acid (3-O-caffeoylquinic acid, 3Cqa), and two flavonols: hyperoside (quecetin-3-O-galactoside, Q3Gal) and quercitrin (quecetin-3-O-rhamnoside, Q3Ram) were identified. The same compounds were previously identified in the extracts of *J. nigra* fresh kernel (Rorabaugh et al., 2011) along with other phenolic components, such as 5-caffeoylquinic acid, 4-caffeoylquinic acid, and the flavonols quercetin-3-rutinoside, quercetin-3-pentoside and quercetin-3-arabinoside, which were not identified in the present study. In addition, these compounds were previously detected in leaf (Amaral et al., 2004; Jalili and Sadeghzade, 2012; Pereira et al. 2007; Zhao et al., 2014) and kernel (Rorabaugh et al., 2011) of *J. regia*. The phenolic profile of *J. nigra* leaves has not been studied prior to this investigation.

Table 6.

In the optimized extract of *J. nigra* leaf, the contents of flavonols, Q3Gal and Q3Ram, expressed as hyperoside were 39.7 mg g^{-1} of dry extract and 54.4 mg g^{-1} of dry extract, respectively. The content of 3Cqa (neochlorogenic acid), expressed as chlorogenic acid, was 8.2 mg g^{-1} of dry extract. The content of these compounds is also calculated and expressed on dry plant material (dry leaves) (Table 6). Two flavonols, Q3Ram and Q3Gal were more abundant than phenolic acid, neochlorogenic acid. A literature survey on the content of 3Cqa, Q3Gal and Q3Ram in the extracts obtained from kernel and leaf of *Juglans* species by different extraction techniques and conditions is given in Table 6. Compared to this study, acetone and methanol extracts from *J. nigra* fresh kernel contained reduced amounts of 3Cqa, Q3Ram and Q3Gal (Rorabaugh et al., 2011). The lower amounts of these compounds may reflect differences in structure of plant parts as well as different polarity of used solvents and extraction technique. The quantification of individual phenolic compounds among *Juglans* species was most frequently studied in *J. regia* (Jalili and Sadeghzade, 2012; Pereira et al., 2007; Zhao et al., 2014; Amaral et al., 2004). If the impact of extraction parameters on the extraction yield is neglected, it could be noted that the ethanol extract from leaves of *J. nigra* studied in this paper has higher 3Cqa and Q3Gal content than ethanol (60% (v/v)) extract from *J. regia* leaves (Zhao et al., 2014), lower than water extract from *J. regia* leaves (Pereira et al., 2007), and similar to methanol extract content (Jalili and Sadeghzade, 2012; Amaral et al., 2004) (Table 6). Also, this work showed that Q3Ram was the most abundant flavonoid in *J. nigra* leaves extract while this compound was found in the lower amounts in the extracts from *J. regia* leaves (Table 6).

In this study phenolic composition of *J. nigra* leaves extract was reported for the first time. The quantification of individual phenolic compounds is quite important considering multiple biological effects these molecules exhibit (Shui and Leong, 2002). Hyperoside and

quercitrin present in the *J. nigra* leaf extract are considered as good antioxidants due to their structure, namely the presence of *ortho*-dihydroxy group on the aromatic ring (Jalili and Sadeghzade, 2012). The obtained *J. nigra* leaf extract is rich in valuable antioxidant compounds, and therefore could find potential application in food, cosmetics and pharmaceuticals industry.

3.2.2 Minerals content

The minerals content in the *J. nigra* leaf optimal extract obtained in this study were determined by ICP-OES and ICP-QMS (Table 7). The K, Mg and Ca contents of *J. nigra* leaf extract were found very high (Table 7). The relative order of macro-elements by content was: K > Mg > Ca. In addition, Zn, Rb, Mn, J, Sr, Ni, Cu, Co, V, Ag and Se contents of *J. nigra* leaf extract were very low (Table 7). The relative order of micro-elements by content was: Zn >Rb>Mn> J >Sr> Ni > Cu > Co > V > Ag > Se. Also, in *J. nigra* leaf extract toxic elements were found in the following order As > Cd >Pb.

Table 7.

The ethanol extraction coefficients of minerals were determined based on their contents in the leaves sample (Section 2.4). The macro- and micro-element contents in leaves sample of *J. nigra*, together with the coefficients of ethanol extraction, are presented in Table 7. Results obtained in this study were different from mineral values which were previously reported in *J. nigra* leaves (Ponder Jr. et al., 1979; 2005). These differences between cultivars are probably the result of different cultivation conditions (land composition, geographical variations), as well as the application of different analytical procedures and methods (Ponder Jr. et al., 2005).

The most abundant major-element in leaves sample was Ca (Table 7), however, we found a low extraction coefficient for Ca in ethanol extract of leaves (only 0.5%). K levels were higher than Mg, with a very high extraction coefficient (up to 64.3%), while for Mg, it was moderate (up to 28.1%). The most abundant micro-element in leaves was Rb followed by Sr. We found a low extraction coefficient for Sr in our extract (only 0.8% was extracted). The content of Mn in leaves was 49.41 µg g^{-1} of dry leaves and the extraction coefficient was 12.0%. The content of Zn in leaves was 36.25 µg g^{-1} of dry leaves, but high extraction coefficient was calculated (37.0%). Iodine was second element with very high extraction coefficient in 50% (v/v) ethanol (53.5%). Toxic metals were also present in both leaves sample and leaves extract. The content of As in leaves was low (7.74 µg g^{-1} of dry leaves), while its extraction coefficient was moderate (25.3%). We measured high percentages of Pb from leaves transferred into 50% (v/v) ethanol (extraction coefficient 50.1%). Determined moderate extraction coefficient for Cd (26.4%) could, however, be considered insignificant, since our research also revealed that this metal did not accumulate in leaves (0.24µg g^{-1} of dry leaves, Table 7). Hg was not extracted with 50% (v/v) ethanol (Table 7).

This study points to the importance of knowing the coefficients of extraction for bioactive elements with recognized health benefits as well as the coefficients of extraction for toxic elements from plants. Under optimal extraction conditions determined in this paper, the obtained ethanol extraction coefficient was highest for bioactive element K, essential element I, and toxic element Pb. Therefore, during optimization of extraction process from plants, it is necessary to focus not only on obtaining the maximum yield, but also on higher coefficients of extraction for bioactive elements, and lower extraction coefficients for toxic elements.

Results obtained for *J. nigra* leaf extract in this study were similar compared to mineral values reported for *J. nigra* walnut kernels by Camara and Schlegel (2016). The

nutritional value of walnuts mainly comes from the presence of some bioelements present in the traces (Trandafir et al., 2016). The high quantity of K, Mg, as well as the presence of some essential elements such as I, Mn, Cu and Zn enables *J. nigra* leaf extract as well as walnut kernels to be considered as a good source of bioelements.

In conclusion, chemical characterization of the extract obtained under optimal conditions determined by RSM and ANN-GA models contributes to the valorization of *J. nigra* leaves extract as valuable source of compounds potentially applicable as ingredients for dietary supplements or functional food. The optimal conditions based on the general extraction yield can vary depending on whether polyphenols or minerals are extracted. Obtained results represent an adequate starting point for further experiments regarding the optimization of extraction process of target compounds from *J. nigra* leaves with potential biological significance. It is essential to investigate impact of different extraction variables such as solvent composition, extraction temperature, extraction time and solvent-to-solid ratio on yield of these target compounds. Therefore, the future research will be focused on determining optimal conditions for extraction of compounds potentially beneficial to human health from *J. nigra* leaves.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technology Development of the Republic of Serbia (Grant Nos. 175034, 173020 and 173021).We thank

Zoran Paunovic for kindly providing us leaf sample and prof. Branislava Lakušić for identification of plant material.

References

Alara, R.O., Abdurahman, N.H., Afolabi, H.K., Olalere, O.A., 2018. Efficient extraction of antioxidants from Vernonia cinerea leaves: Comparing response surface methodology and artificial neural network. Beni-Suef Univ. J. Basic Appl. Sci. 7, 276–285. https://doi.org/10.1016/j.bjbas.2018.03.007.

Amaral, J.S., Seabra, R.M., Andrade, P.B., Valentao, P., Pereira, J.A., Ferreres, F., 2004. Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves. Food Chem. 88, 373–379. https://doi:10.1016/j.foodchem.2004.01.055.

Amarowicz, R., Dykes, G.A., Pegg, R. B., 2008. Antibacterial activity of tannin constituents from *Phaseolus vulgaris*, *Fagopyrum esculentum*, *Corylus avellana* and *Juglans nigra*. Fitoterapia 79, 217-219. https://doi.org/10.1016/j.fitote.2007.11.019.

Cacace, J., Mazza, G., 2003. Mass transfer process during extraction of phenolic compounds from milled berries. J. Food Eng. 59, 379–389. http://dx.doi.org/10.1016/S0260-8774(02)00497-1.

Camara, C.R.S., Schlegel, V., 2016. A Review on the Potential Human Health Benefits of the Black Walnut: A Comparison with the English Walnuts and Other Tree Nuts. Int. J. Food Prop. 19, 2175-2189. https://doi.org/10.1080/10942912.2015.1114951.

Fitschen, P.J., Rolfhus, K.R., Winfrey, M.R., Allen, B.K., Manzy, M.,. Mahe, M.A., 2011. Cardiovascular Effects of Consumption of Black Versus English Walnuts. J. Med. Food 14, 890–898. https://doi.org/10.1089/jmf.2010.0169

Jalili, A., Sadeghzade, A., 2012. Total phenolics content and Phenolic profile of Walnut (*Juglans regia* L.) Leaves in different Cultivars Grown In Iran. Int. Res J. Biotechnol. 3, 010-017.

Jovanović, B., 1970. Juglans L. In Flora SR Srbije, Vol II, Josifovic M. (ed.), Srpska akademija nauka i umetnosti, Beograd.

Gavrilović, M., Rajković, K.M., Simić, V., Jeremić, S., Mirković, S., Jevtić, A.S.J., 2018. Optimization of ultrasound-assisted extraction of total polyphenolic compounds from *Juglans nigra* L. leaves. J. Serb. Chem. Soc. 83, 1273-1284. https://doi.org/10.2298/JSC180321069G.

Kammerer, D., Carle R., Schieber, A., 2004. Characterization of phenolic acids in black carrots (Daucus carota ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid. Commun. Mass. Spectrom. 18, 1331-1340.

Milić, P.S., Rajković, K.M., Stamenković, O.S., Veljković, V.B., 2013. Kinetic modeling and optimization of maceration and ultrasound-extraction of resinoid from the aerial parts of white lady's bedstraw (*Gallium mollugo* L.). Ultrason. Sonochem. 20, 525–534. http://dx.doi.org/10.1016/j.ultsonch.2012.07.017.

Nicese, F.P., Hormaza, J.I., Mc Granahan, G.H., 1998. Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers. Euphytica, 101, 199-206.

Nour, V., Trandafir, I., Cosmulescu, S., 2016. Optimization of ultrasound-assisted hydroalcoholic extraction of phenolic compounds from walnut leaves using response surface methodology. Pharm. Biol. 54, 2176-2187. https://doi.org/10.3109/13880209.2016.1150303.

Nwosu, J.N, Ndukauba, O.E., Olawuni, I.A.,Okafor, D.C., Enwereuzoh, R.O., Ojukwu, M., 2015. The Mineral and Vitamin Compositions of Black Walnuts using Roasting Toasting and Cooking. Int. J. Life. Sci. 4, 58-65. https://doi: 10.13140/RG.2.2.27483.67360.

Pereira, J.A., Oliveira, I., Sousa, A., Valentao, P., Andrade, P.B., Ferreira, I.C.F.R., Ferreres, F. Bento, A., Seabra, R., Estevinho L., 2007. Walnut (*Juglans regia* L.) leaves:

Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. Food Chem. Toxicol. 45, 2287–2295. https://doi:10.1016/j.fct.2007.06.004.

Ponder Jr, F., Phare, R.E., Schlesinger, R.C., 1979. Seasonal variation in foliar composition of black walnut trees. Commun. Soil Sci. Plan. 10, 1513-1521. http://dx.doi.org/10.1080/00103627909367006.

Ponder Jr, F., Jones J.E., Mueller R., 2005. Using Poultry Litter in Black Walnut Nutrient Management. J. Plant Nutr. 28, 1355-1364. http://dx.doi.org/10.1081/PLN-200067454.

Rajković, K., Pekmezović, M., Barac, A., Nikodinović- Runić, J., Arsić- Arsenijevic, V., 2015. Ihibitory effect of thyme and cinnamon essential oils on *Aspergillus flavus*: Optimization and activity prediction model development. Ind. Crop. Prod. 65, 7–13. http://dx.doi.org/10.1016/j.indcrop.2014.11.039.

Rajković, K.M., Jeremić, S., Milić, P.S., Kostić, M., Arsić-Arsenijević, V., Gavrilović, M., Krstić, B., 2017. Period. Polytech. Chem. Eng. 61, 200-206. https://doi.org/10.3311/PPch.9580.

Rorabaugh, J.M., Singh, A.P., Sherrell, I.M., Freeman, M.R., Vorsa, N., Fitschen, P., Malone, C., Maher, M.A., Wilson, T., 2011. English and Black Walnut Phenolic Antioxidant Activity *in Vitro* and Following Human Nut Consumption. Food Nutri. Sci. 2, 193 -200. https://doi.org/10.4236/fns.2011.23026.

Rostagno, M.A., Prado, J.M., 2013. Natural Product Extraction: Principles and Applications. https://doi.org/10.1039/9781849737579.

Said, K.A.M., Amin M.A.M., 2015. Overview on the Response Surface Methodology (RSM) in Extraction Processes. J. Appl. Sci. Pro. Eng. 2, 8-17. https://www.researchgate.net/publication/281943340.

Shui, G., Leong L.P., 2002. Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography. J. Chromatogr A, 977, 89–96.

Simić, V.M., Rajković, K.M., Stojičević, S.S., Veličković, D.T., Nikolić, N.Č., Lazić, M.L., Karabegović, I.T., 2016. Optimization of microwave-assisted extraction of total polyphenolic compounds from chokeberries by response surface methodology and artificial neural network. Sep. Purif. Technol. 160, 89-97. https://doi.org/10.1016/j.seppur.2016.01.019.

Trandafir, I., Cosmulescu, S., Botu, M., Nour, V., 2016. Antioxidant activity, and phenolic and mineral contents of the walnut kernel (*Juglans regia* L.) as a function of the pellicle color. Fruits, 71, 177-184. https://doi: 10.1051/fruits/2016006.

Vieira, V., Prieto, M.A., Barros, L., Coutinho, J.A.P., Ferreira, O., Ferreira, I.C.F.R., 2017. Optimization and comparison of maceration and microwave extraction systems for the production of phenolic compounds from *Juglans regia* L. for the valorization of walnut leaves Ind. Crops Prod. 107, 341-352. http://dx.doi.org/10.1016/j.indcrop.2017.06.012.

Wua, J., Qiana, Y., Maoa, P., Chenb, L., Lub, Y., Wanga, H., 2013. Separation and identification of phenolic compounds in canned artichokeby LC/DAD/ESI-MS using core-shell C18 column: A comparative study. J. Chromatogr. B 927, 173-180. https://doi.org/10.1016/j.jchromb.2012.11.026.

Zhao, M-H., Jiang, Z-T., Liu, T., Li, R., 2014. Flavonoids in *Juglans regia* L. Leaves and Evaluation of *In Vitro* Antioxidant Activity via Intracellular and Chemical Methods. J. World Sci. ID 303878. http://dx.doi.org/10.1155/2014/303878.

Figure captions

Figure 1. Response surface plots (A) and contour (B) of extraction yield as a function of ethanol concentration and solvent-to-solid ratio.

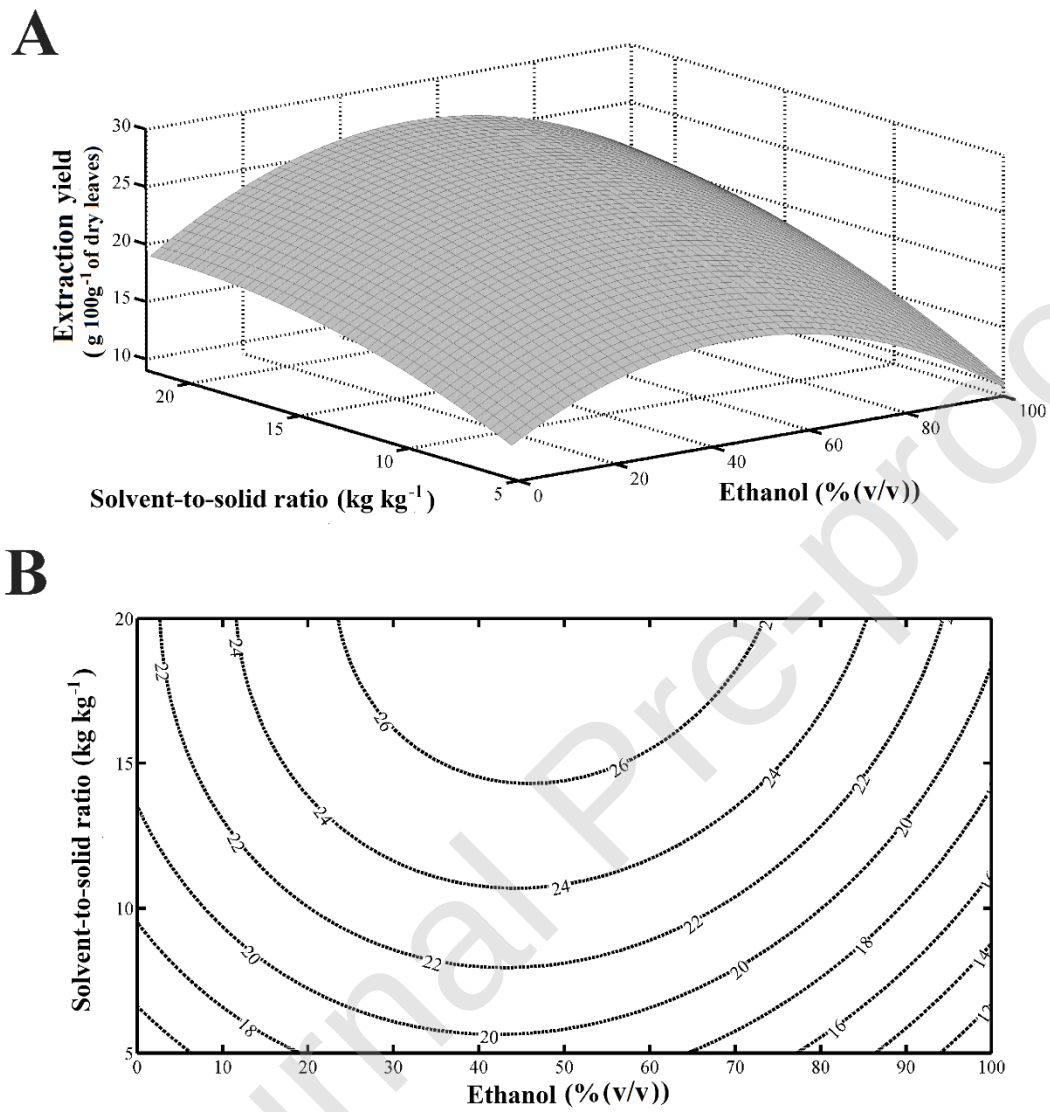

Figure 2. Artificial neural network (ANN) (x_1 - ethanol concentration; x_2 - solvent-to-solid ratio; y – extraction yield).

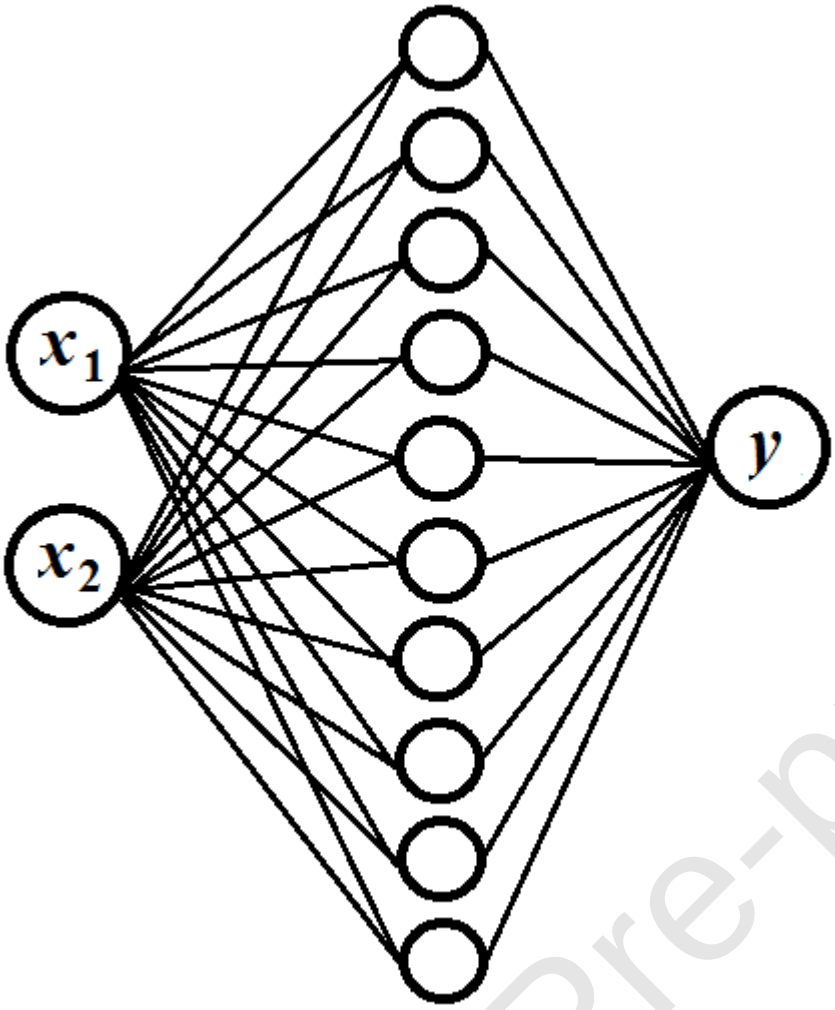

Figure 3. Diagnostic plots of predicted and actual values of extraction yield (○-RSM; Δ-ANN).

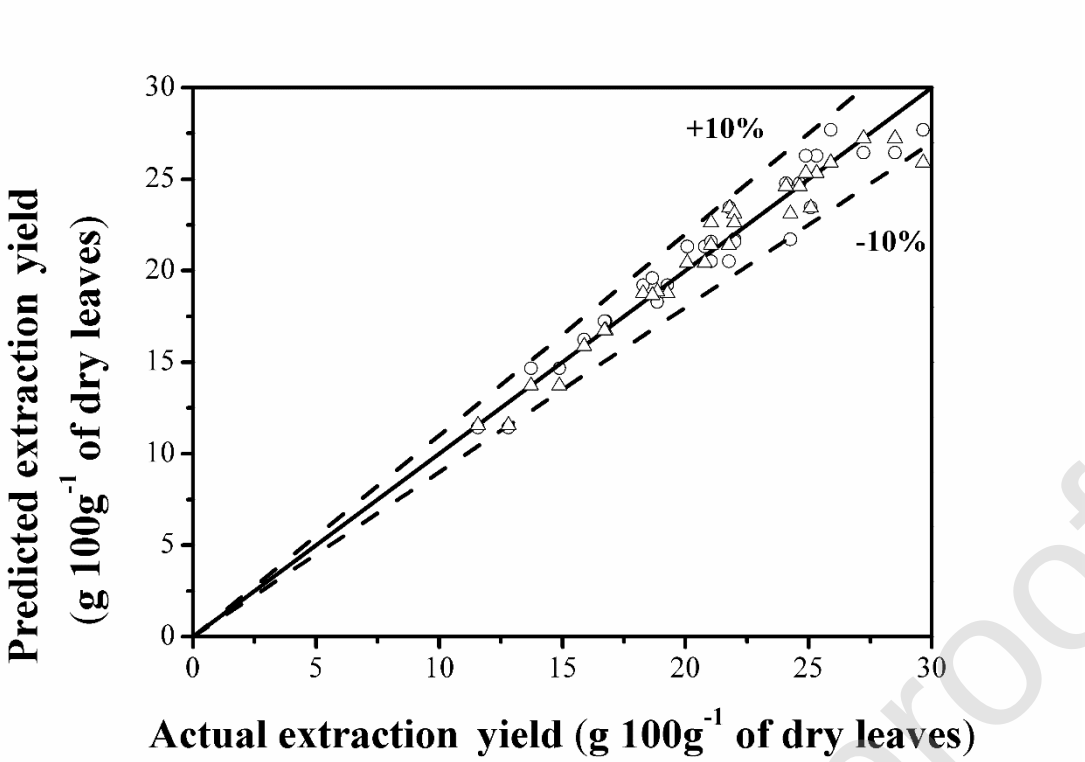

Table 1. Artificial neural network with genetic algorithm (ANN-GA) parameters.

Model	Property	Value/Comment
MANN	Algorithm	Levenberg-Marquardt back-propagation
	Learning	Supervised
	Input layer	No transfer function is used
	Hidden layer	Hyperbolic tangent transfer function
	Output layer	Hyperbolic tangent transfer function
	Number of training date	70% of the total data
	Number of validation date	15% of the total data
	Number of testing date	15% of the total data
	Number of training iterations	2-50
	Number of hidden neurons	1-20
GA	Population Size	10
	Generations	100
	Creation Function	Uniform
	Selection Function	Uniform
	Crossover Options:	Scattered
	Crossover Rate	0.8
	Mutation Probability	0.01

Table 2. Calibration curves of standard compounds and validation parameters

	Wavelenght, nm	Regression equation[a]	R^2	Linear range, mg cm^{-3}	LOQ[b], mg cm^{-3}	LOD[c], mg cm^{-3}
Chlorogenic acid	320	Y=56.019·X+21.271	0.997	0.020-0.500	0.020	0.005
Hyperoside	350	Y=20379·X+116.34	0.998	0.0028-0.3600	0.0028	0.001
	RSD Rt (%)	RSD (%)[d]				
Neochlorogenic acid	0.04	5.8				
Hyperoside	0.15	5.1				
Quercitrin	0.10	2.4				

[a] Y- peak area; X- concentration of compound (mg cm^{-3})
[b] LOQ - Limit of quantitation was determined as the lowest concentration of the standard at which the relative standard deviation of repeated injections is <20%.
[c] LOD - Limit of detection was estimated by visual evaluation of chromatograms of the standards and calculation of signal-to-noise ratio (S/N>3)
[d] Intraday precision is calculated as the relative standard deviation of response from six repeated injections of extract.

Table 3. Instrument operating conditions for ICP-OES and ICP-QMS.

ICP-OES		ICP-QMS	
Nebulizer	Concentric	Rf power (W)	1548
Spray chamber	Cyclonic	Gas flows (L/min)	13.9;1.09;0.8
Rf power (W)	1150	Acquisition time	3 x 50 s
Principal argon flow rate (L/min)	12	Points per peak	3
Auxiliary argon flow rate (L/min)	0.5	Dwell time (ns)	10
Nebulizer argon flow rate (L/min)	0.5	Detector mode	Pulse
Sample flow rate (mL/min)	1	Measured isotopes	^{107}Ag, ^{75}As, ^{138}Ba, ^{51}V, ^{111}Cd, ^{59}Co, ^{53}Cr, ^{63}Cu, ^{204}Hg, ^{55}Mn, ^{62}Ni, ^{208}Pb, ^{86}Sr, ^{82}Se, ^{68}Zn, ^{87}Rb, ^{127}I
Detector	CID86		
Selected wavelengths (nm)	Al (167.0); Ca (393.3); Fe (259.9); K (766.4); Mg (285.2); Na (589.5)		

Table 4. The matrix of 4^2 full factorial experimental design (with two replications) and the extraction yield.

Exp. number	Experimental matrix		Response			
	Factors		Extraction yield, g $100g^{-1}$ of dry leaves			
	x_1, % (v/v)	x_2, kg kg^{-1}	Series 1	Series 2	Mean	Stand. Dev.
1	0	5	15.05	14.29	14.67	0.54
2	50	5	18.63	19.79	19.21	0.82
3	70	5	16.64	17.83	17.24	0.84
4	96	5	11.13	11.73	11.43	0.42
5	0	10	18.48	18.11	18.29	0.26
6	50	10	22.86	24.03	23.44	0.83
7	70	10	21.19	22.23	21.71	0.74
8	96	10	16.10	16.35	16.22	0.18
9	0	15	20.35	20.67	20.51	0.23
10	50	15	25.53	27.01	26.27	1.05
11	70	15	24.18	25.38	24.78	0.85
12	96	15	19.50	19.71	19.61	0.15
13	0	20	20.66	21.98	21.32	0.94
14	50	20	26.64	28.73	27.69	1.48
15	70	20	25.61	27.27	26.44	1.18
16	96	20	21.35	21.82	21.59	0.33

x_1- ethanol concentration; x_2- solvent-to-solid ratio.

Table 5. 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	605.43	5	121.09	81.97	< 0.0001
x_1	9.29	1	9-29	6.29	0.0187
x_2	305.08	1	305.08	206.52	< 0.0001
x_1x_2	7.30	1	7.30	4.94	0.0351
x_{12}	250.99	1	250.99	169.90	< 0.0001
x_{22}	15.82	1	15.82	10.71	0.0030
Residual	38.41	26	1.48		
Lack of Fit	19.36	10	1.94	1.63	0.1859*
Pure Error	19.05	16	1.19		
Cor Total	643.84	31			
R^2	0.946				
CV	5.89				
Adeq Precision	30.89				

x_1 - ethanol concentration; x_2 - solvent-to-solid ratio; R^2 - coefficient of determination; *CV* - coefficient of variation.
*Statistically not significant at the confidence level of 95%.

Table 6. The content of 3-O-caffeoylquinic acid (3Cqa), quercetin-3-O-galactoside (Q3Gal) and quercetin-3-O-rhamnoside (Q3Ram) in the optimized extract of *J. nigra* leaves and literature survey of their contents in different *Juglans* species.

Plant material	Technique	Extraction conditions		Content, mg g^{-1} of dry plant leaves (mg g^{-1}dry extract)			References
		Solvent	*T* ,ºC	3Cqa	Q3Gal	Q3Ram	
J. nigra leaf	UEA	50% (v/v) EtOH	40	2.27 (8.2)	10.99 (39.7)	15.07 (54.4)	This work
J. nigra fresh kernel	MA	80% (v/v) C_3H_6O	25	0.67 10^{-3*}	1.39 10^{-3*}	0.23 10^{-3*}	Rorabaugh et al., 2011
	MA	80% (v/v) MeOH	25	1.5 10^{-3*}	0.05 10^{-3*}	0.14 10^{-3*}	
J. regia leaf	MA	MeOH	/	0.7-6.3	2.3-12.9	1.4-4.6	Jalili and Sadeghzade, 2012
	MA	H_2O	T_b	12.1-14.8	15.7-21.7	3.0-4.2	Pereira et al., 2007
	MAE	60% (v/v) EtOH	85	0.03	0.05	0.45	Zhao et al., 2014
	MA	MeOH	/	2.0-6.8	5.1-14.6	1.6-3.7	Amaral et al., 2004

T_b- boiling temperature; MA-Maceration; UEA-Ultrasonic assisted extraction; MAE-microwave assisted extraction; C_3H_6O - acetone; *mg g^{-1} wet wt

Table 7. The macro- and micro-element contents in optimal leaves extract and leaves sample of *J. nigra*, together with the coefficients of ethanol extraction.

	Content, mg g^{-1} of dry leaves		
Elements	Optimal extract of leaves	Leaves sample	% of extraction*
Macro-elements			
K	3.88	6.03	64.3
Mg	1.67	5.97	28.1
Ca	0.14	26.30	0.5
Micro-elements			
Zn	$13.40 \cdot 10^{-3}$	$36.25 \cdot 10^{-3}$	37.0
Rb	$7.99 \cdot 10^{-3}$	$125.70 \cdot 10^{-3}$	6.4
Mn	$5.94 \cdot 10^{-3}$	$49.41 \cdot 10^{-3}$	12.0
I	$0.90 \cdot 10^{-3}$	$1.68 \cdot 10^{-3}$	53.5
Sr	$0.86 \cdot 10^{-3}$	$108.40 \cdot 10^{-3}$	0.8
Ni	$0.85 \cdot 10^{-3}$	$2.03 \cdot 10^{-3}$	41.9
Cu	$0.66 \cdot 10^{-3}$	$6.44 \cdot 10^{-3}$	10.2
Co	$0.26 \cdot 10^{-3}$	$1.10 \cdot 10^{-3}$	23.3
V	$0.16 \cdot 10^{-3}$	$1.21 \cdot 10^{-3}$	13.2
Ag	$0.15 \cdot 10^{-3}$	$0.63 \cdot 10^{-3}$	24.4
Se	$0.09 \cdot 10^{-3}$	$0.34 \cdot 10^{-3}$	25.5
Cr	<LOD	$0.74 \cdot 10^{-3}$	
Toxic elements			
As	$1.96 \cdot 10^{-3}$	$7.74 \cdot 10^{-3}$	25.3
Cd	$0.06 \cdot 10^{-3}$	$0.24 \cdot 10^{-3}$	26.4
Pb	$0.52 \cdot 10^{-3}$	$1.04 \cdot 10^{-3}$	50.1
Hg	<LOD	$0.16 \cdot 10^{-3}$	

LOD-Limit of detection.
* % of extraction with 50% (v/v) ethanol