Romanian Biotechnological Letters *Vol. 17, No.3, 2012*

ORIGINAL PAPER

Determination of optimal extraction parameters of mulberry leaves using Response Surface Methodology (RSM)

Received for publication, June 10, 2010
Accepted, September 11, 2011

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Abstract

The effects of solvent concentration (ethanol/water, 40–80%, v/v), temperature (40 – 80°C), and liquid-solid ratio (10–30 ml/g, solvent volume per g of raw material) such as their interaction on extractability of total phenolics and total flavonoids, and antioxidant activity of mulberry leaves were studied. The optimal conditions for the phenolics and flavonoids extraction, and antioxidant activity were determined by response surface methodology. The Box–Behnken design showed that polynomial regression models were in good agreement with the experimental results with the correlation coefficients of 0.9850, 0.9004 and 0.9858 for total phenolics, total flavonoids content and antioxidant activity, respectively. The experimental values agreed with those predicted, thus indicating suitability of the used model and the success of response surface methodology in optimizing the extraction conditions of investigated system. This is the first report on optimizing of extraction technology of phenol compounds and antioxidant activity of mulberry plants grown in Serbia. This information will be of considerable value to the commercial producers of mulberry trees in the country.

Key words: *Morus alba*, mulberry leaves, extraction, response surface methodology, total phenolics, total flavonoids, antioxidant activity

1. Introduction

Natural plants have received a lot of attention as sources of biologically active substances including antioxidants, antimutagens and anticarcinogens [1]. However, scientific information on antioxidant properties of various plants, particularly those that are less widely used in culinary and medicine is still limited. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants, functional foods and nutraceuticals [2, 3].

Mulberry (*Morus alba* L) plant was originally grown in China and widely used in both the agricultural and medicinal fields. Mulberry leaves, bark and branches have been used in traditional medicine to alleviate fever, suppress atherosclerosis, alleviate hypertension, as well as for its diuretic and antihyperglycemic effects [4, 5]. Mulberry has been highlighted in various scientific researches, aiming to document evidence of its medicinal benefits [6]. Some of those scientific works have reported the antioxidant activity of mulberry leaves. Moreover, the ethanol extracts of mulberry leaf have manifested to stop atherosclerosis as they inhibit the oxidative modification of LDL such as flavonol glycoside [7]. Furthermore, Doi, Kojima, and Fujimoto [8] reported that 1-butanol extract of mulberry leaves scavenged the DPPH radical and inhibited the oxidative modification of rabbit and human LDL. In mulberry leaves there have been explored even five flavonol glycosides (rutin, isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin and kaempherol 3-(6-acetylglucoside)) [9, 10]. Mulberry leaf extracts have been discovered to significantly reduce blood glucose in alloxan-induced

diabetic mice [11]. Mulberry leaf powders have been reported to have hypoglycemic and hypolipidemic effects in type 2 diabetic patients [12]. Recently, the mechanism of their actions has been partly associated to their antioxidant activity, which is closely related to the rutin, quercetin, isoquercitrin and other flavonoids presented in mulberry leaves [4].

Phenolics possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic and anticarcinogenic properties, as well as the ability to modify gene expression [13, 14]. Mulberry leaves are good sources of phenols, including flavonoids, anthocyanins, mineral elements, etc. [4, 15, 16]. The extraction parameters may affect the quality and quantity of antioxidant activity in mulberry leaves, but this has never been clarified. Many factors, such as solvent composition, the extraction time, temperature, pH, liquid-solid ratio and particle size, may significantly influenced the liquid-solid extraction [17-23]. The extraction and purification of bioactive compounds from natural sources has become very important for the utilization of phytochemicals in the preparation of dietary supplements or nutraceuticals, functional food ingredients, and additives to food, pharmaceutical and cosmetic products [24].

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes, in which a response of interest is influenced by several factors (independent variables). RSM not only defines the effects of the independent variables, but also generates a mathematical model, which describes the chemical or biochemical processes [25]. Currently, RSM is being used in the extraction of phenolic compounds from various sources [26-28]. Response surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food systems including extraction of phenolic compounds from berries and evening primrose meal, anthocyanins from black currants and sunflower hull and vitamin E from wheat germ, among others [18-28].

The purpose of the present study was to investigate the effects of temperature, liquid-solid ratio and solvent concentration on the antioxidant capacity and levels of antioxidant phenolic compounds in extracts of mulberry leaves using response surface methodology.

2. Materials ande Methods

2.1. Chemicals and Reagents

1,1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), Folin–Ciocalteu reagent were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Chlorogenic acid and rutine were purchased from Sigma (Sigma, St. Luis, MO, USA). Aluminium chloride hexahydrate, anhydrous sodium carbonate, and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical reagent grade.

2.2. Simple preparation

In this research dried plant material was used. Voucher specimens (*Morus alba* L. N^{o} 2-1794, Kać, UTM 34TDR211, 25.06.2010. det.: Goran Anačkov) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad [29]. The samples of mulberry leaves were dried naturally (in the shade, the draft) for a month. Samples were grounded in the blender before the extraction. The mean particle size (d=0.3092±0.16 mm) was determined using sieve sets (Erweka, Germany).

Plant samples (10.0 g) were extracted by different solvent (ethanol: water, 40–80%, v/v), at temperature (40–80°C) and liquid-solid ratio (10–30 ml/g, solvent volume per g of raw material). The extraction process was carried out using bath termostate

(Laborgerateborse, Germany). After filtration, extracts were stored in the flask at 4°C to prevent oxidative damage until analysis.

2.3. Determination of Antioxidant Compounds

The total phenolics content (TP) in the extracts was determined by the Folin–Ciocalteu method [30, 31], and was expressed as mass (mg) of chlorogenic acid equivalents (ECA) per mass (g) of dry mulberry leaves. Triplicate tests were conducted for each sample.

The total flavonoids content (TF) content has been determined by aluminium chloride colorimetric assay [32], using rutine as a standard. It has been expressed as mass (mg) of rutine equivalents (ER) per mass (g) of dry mulberry leaves. Triplicate tests were done for each sample.

2.4. DPPH Assay

The free radical scavenging activity of mulberry leaves extract was determined as described by Espin [33]. Briefly, the mulberry extract was mixed with methanol (96%) and 90 μM DPPH to give final concentration of 0.05, 0.075, 0.1 and 0.2 mg/ml of extract. After 60 min at room temperature, the absorbance was measured at 517 nm and expressed as radical scavenging capacity (%RSC). %RSC was calculated using familiar equation [34]. This activity was also expressed as the inhibition concentration at 50% (IC_{50}), the concentration of test solution required to obtain 50% of radical scavenging capacity. The values are presented as the mean of three measurements.

2.5. Experimental design

Response surface methodology (RSM) with Box–Behnken design was applied for determining optimal solvent concentration, extraction temperature and liquid-solid ratio for extracts of mulberry leaves [35, 36]. The solvent concentration (X_1), extraction temperature (X_2) and liquid-solid ratio (X_3) were independent variables studied to optimize total phenols, total flavonoids and antioxidant activity of mulberry leaves extracts. Investigated factors and levels tested are reported in Table 1.

Table 1. The uncoded and coded levels of independent variables used in the RSM design

Independent variables	Symbols	Levels		
		Low (-1)	Middle (0)	High (+1)
Solvent (%)	X_1	40	60	80
Temperature (°C)	X_2	40	60	80
Liquid-solid ratio (ml/g)	X_3	10	20	30

The experimental data were fitted with second order RSM with the following form:

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

where y is response (the total phenolics content, the total flavonoids content, and the IC_{50} value, respectively), β_0, β_j, β_{jj}, β_{ij} are constant coefficients of intercept, linear, quadratic, and interaction terms, respectively. X_i and X_j are coded independent variables (solvent concentration, temperature, liquid-solid ratio). Analysis was performed using commercial software *Design-Expert*®, ver. 7. (Stat Ease, USA).

The analysis of variance (ANOVA) was also used to evaluate the quality of the fitted model. The test of statistical difference was based on the total error criteria with a confidence level of 95.0%.

3. Results and discussion

Effects of solvent concentration (ethanol: water, 40–80%, v/v), temperature (40–80°C) and liquid-solid ratio (10–30 ml/g, solvent volume per g of raw material) on antioxidant (phenolics and flavonoids) contents, and antioxidant activities of mulberry extracts were investigated. Research papers who are reporting on extraction of compounds/extracts with antioxidant activity dealing with different extraction solvents: water, methanol, ethanol, petroleum ether, n-hexane, dichloromethane and ethyl acetate. Correlation between antioxidant activity and total phenolic content has been established; mostly target compounds for obtaining extract with antioxidant activity are phenolics. Considering polarity of phenolic compounds they are extracted by polar solvents. The impact of applying different proportions of water-ethanol mixture (30-70% ethanol-water) was studied by Liyana Pathirana and Shahidi [27]. Tables 2 and 3 show the experimental data and the regression coefficients obtained by fitting experimental data to the second order response models for total phenolics content, total flavonoids content, and IC_{50} value, respectively. The experimental design has seventeen simplified experimental sets with five replication performed at central values of the independent variables (Table 2). The effect of the linear, quadratic or interaction coefficients on the response was tested for significance by analysis of variance (ANOVA). Regression coefficients of intercept, linear, quadratic, and interaction terms of the model were calculated using least square method. The degree of significance of each factor is represented by its p – value. The fitted model represent the experimental data well with high correlation coefficients, R^2, varying from 0.9858 to 0.9004 (Table 3). Analysis of variance (Table 4) also shows that the regression models for total phenolics content, total flavonoids content, and IC_{50} value were statistically relevant with a significance level ranging from $p < 0.0001$ (for total phenolics content and IC_{50} value) to $p = 0.0088$ (for total flavonoids content), and the models had no significant lack of fit ($p > 0.05$). Thus, well-fitting models for total phenolics content, total flavonoids content and IC_{50} value were successfully established. Some representative response surfaces are given in Figs. 1–3.

Table 2. Experimental matrix and values of observed response

Run	Solvent concentration (%)	Temperature (°C)	Liquid-solid ratio (ml/g)	Phenolics content (mg ECA/g)	Flavonoids content (mg ER/g)	IC_{50} (mg/ml)	Coded solvent concentration variable	Coded temperature variable	Coded liquid-solid ratio variable
1	40	40	20	12.843	4.6444	0.1380	-1	-1	0
2	40	60	10	9.915	5.6872	0.1350	-1	0	-1
3	40	80	20	15.124	5.7354	0.1100	-1	1	0
4	40	60	30	18.472	5.2813	0.0823	-1	0	1
5	60	40	10	9.487	4.6444	0.1570	0	-1	-1
6	60	80	10	13.693	6.0309	0.0753	0	1	-1
7	80	60	10	13.652	5.1207	0.0741	1	0	-1
8	80	80	20	20.664	5.4908	0.0710	1	1	0
9	80	60	30	22.576	4.4621	0.0740	1	0	1
10	60	80	30	23.070	4.5791	0.0706	0	1	1
11	60	60	20	19.361	6.0605	0.0759	0	0	0
12	60	60	20	19.981	6.0159	0.0758	0	0	0
13	60	60	20	19.035	6.4011	0.0821	0	0	0
14	60	60	20	19.860	6.0105	0.0762	0	0	0
15	60	60	20	19.084	6.0005	0.0809	0	0	0
16	80	40	20	14.903	5.3420	0.1197	1	-1	0
17	60	40	30	14.865	5.2869	0.0874	0	-1	1

Table 3. Regression coefficients of polynomial function of response surface for total phenolic content, total flavonoid content and IC_{50} value

Term [a]	Coefficients	Standard error	F - value	*p* - value [b]
Total phenolic content				
Intercept	19.46	0.34		
X_1	1.93	0.27	51.07	0.0002
X_2	2.56	0.27	89.60	<0.0001
X_3	4.03	0.27	222.57	<0.0001
X_1^2	-1.35	0.37	13.20	0.0084
X_2^2	-2.23	0.37	35.81	0.0006
X_3^2	-1.96	0.37	27.65	0.0012
X_1X_2	0.87	0.38	5.19	0.0568
X_1X_3	0.092	0.38	0.058	0.8171
X_2X_3	1.00	0.38	6.85	0.0345
$R^2 = 0.9850$				
Total flavonoid content				
Intercept	6.10	0.13		
X_1	-0.12	0.10	1.30	0.2924
X_2	0.24	0.10	5.48	0.0517
X_3	-0.23	0.10	5.23	0.0560
X_1^2	-0.40	0.14	7.87	0.0263
X_2^2	-0.40	0.14	7.97	0.0257
X_3^2	-0.56	0.14	15.96	0.0052
X_1X_2	-0.24	0.14	2.65	0.1479
X_1X_3	-0.063	0.14	0.19	0.6758
X_2X_3	-0.52	0.14	13.07	0.0086
$R^2 = 0.9004$				
IC_{50} value				
Intercept	0.078	0.0022		
X_1	-0.016	0.0018	81.20	<0.0001
X_2	-0.022	0.0018	155.76	<0.0001
X_3	-0.016	0.0018	81.97	<0.0001
X_1^2	0.013	0.0024	27.29	0.0012
X_2^2	0.019	0.0024	60.80	0.0001
X_3^2	0.0005	0.0024	0.049	0.8313
X_1X_2	-0.0005	0.0025	4.35	0.0755
X_1X_3	0.013	0.0025	28.08	0.0011
X_2X_3	0.016	0.0025	42.75	0.0003
$R^2 = 0.9858$				

[a] X_1: solvent; X_2: temperature; X_3: solid-liquid ratio.

[b] $p < 0.01$ highly significant; $0.01 \leq p < 0.05$ significant; $p \geq 0.05$ not significant.

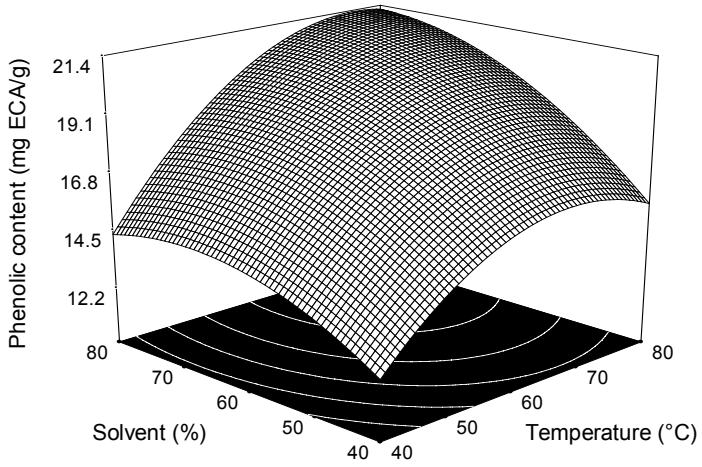
Table 4. Analysis of variance (ANOVA) for the response surface quadratic model for total phenolic content, total flavonoid content and IC_{50} value

Source	Sum of squares	Degree of freedom	Mean square	F-value	*p*-value
Total phenolic content					
The recovery					
Model	268.77	9	29.86	51.17	< 0.0001
Residual	4.09	7	0.58		
Lack of fit	3.32	3	1.11	5.80	0.0612
Pure error	0.76	4	0.19		
Total	272.85	16			
Total flavonoid content					
The recovery					
Model	5.31	9	0.59	7.03	0.0088
Residual	0.59	7	0.084		
Lack of fit	0.47	3	0.16	5.35	0.0695
Pure error	0.12	4	0.029		
Total	5.90	16			
IC_{50} value					
The recovery					
Model	0.012	9	0.0013	54.18	<0.0001
Residual	0.0002	7	0.00002		
Lack of fit	0.0001	3	0.00004	4.79	0.0822
Pure error	0.00004	4	0.000009		
Total	0.012	16			

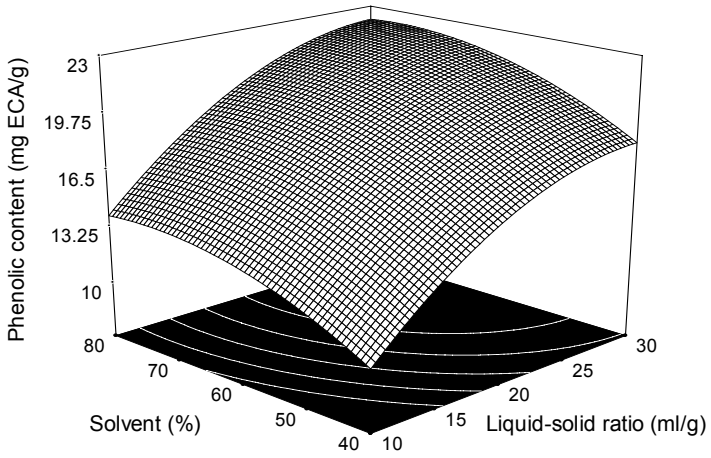
3.1. Total phenolics content of mulberry extract

Total phenolics content was significantly influenced by temperature and liquid-solid ratio independent variables ($p < 0.0001$, Table 3). Furthermore, the interaction between solvent concentration and temperature (X_1X_2) and solvent concentration and liquid-solid ratio (X_1X_3) didn't have a significant effect on total phenolic content ($p = 0.0568$ and $p = 0.8175$).

Fig. 1a shows that total phenolics content of the extracts increased with increasing of solvent concentration. Furthermore, the total phenolics content increased also with increasing of extraction temperature up to about 65°C where further increase in temperature did not cause a significant change in total phenolics content. From Fig. 1b it can be seen that the total phenolics content increased with increasing liquid-solid ratio from 10 to 30 ml/g. The total phenolics content of mulberry extracts varied from 9.915 to 23.070 mg ECA/ g of dry leaves, according to different investigated parameter levels.



(a)



(b)

Fig. 1 (a) Effects of solvent concentration and temperature (liquid-solid ratio, 20 ml/g), and (b) effects of solvent concentration and liquid-solid ratio (temperature, 60°C) on total phenolic content of the mulberry extracts

The influence of the solvent concentration (50-80% of ethanol) and temperature (25-80°C) on the extractability of total phenols from milled soybeans has been published by Jokić [37]. The authors reported that 50% ethanol was the most effective solvent, what is the opposite of the results that we obtained in this study. Rostango [38] found that it is necessary to add a certain amount of water in the extraction solvent in order to improve the extraction of phenolic compounds. The water content higher than 60% resulted in a reduction of the extraction yield of the same components. The extraction efficiency of the phenolic compounds is reduced by using pure ethanol as the solvent since the phenols, due to a number of hydroxyl groups (such as flavonoids, especially those with sugars in molecule), are hydrophilic, and as such generally more soluble in water-ethanol solutions than in pure alcohol. In research of Cacace and Mazza [24] the maximum yield of total phenol compounds from berries were achieved with 67% ethanol at temperature of 40°C. In 2002, the same

authors [39], optimized the extraction of phenolics from black currants using different temperatures (6, 20, 40, 60, and 74°C), and solvent to solid ratios (6, 20, 40, 60, and 74 ml/g). Maximum yield of total phenolics was obtained at 19 ml of solvent per g of milled frozen berries, which is lower than our values. In the same study, authors reported that the temperature can affect the extraction of a given compound by modifying its diffusion coefficient and the solubility in the solvent. Thus, an increase in the temperature would increase the diffusion coefficient and hence the rate of diffusion and increase total phenolics content, like in our study. The same conclusion was confirmed by the study of Jokić [37].

The second order polynomial model used to express the total phenolic content (y) as a function of independent variables (in terms of coded values) is shown below:

$$y = 19.36 + 1.93X_1 + 2.56X_2 + 4.03X_3 - 1.30X_1^2 - 2.182X_2^2 - 1.91X_3^2 + 0.87X_1X_2 + 0.092X_1X_3 + 1.00X_2X_3 \quad (2)$$

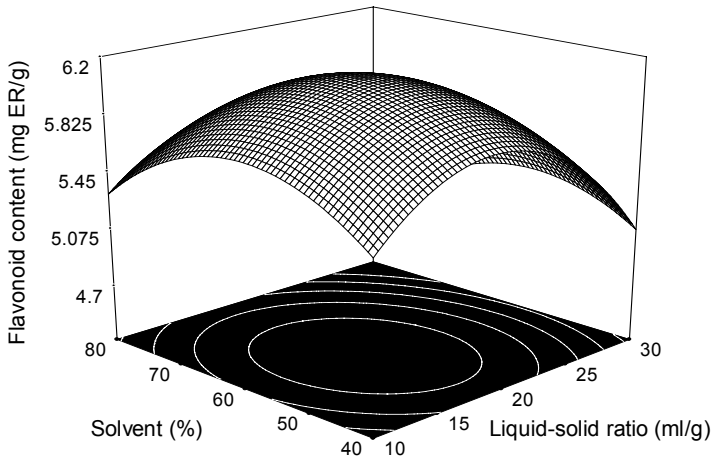
where y is the total phenolics content, X_1 is solvent concentration, X_2 is temperature and X_3 is liquid-solid ratio. By computation, the optimal conditions for total phenolics content were determined at liquid-solid ratio of 29.19 ml/g, temperature of 68.15°C and ethanol concentration of 68.30% solvent, and the predicted total phenolics content was 23.3096 mg ECA/ g of dry mulberry leaves. Under these optimal conditions, the experimental value was 23.3864 mg ECA/ g dry leaves, which agreed with those predicted.

3.2 Total flavonoids content of mulberry extract

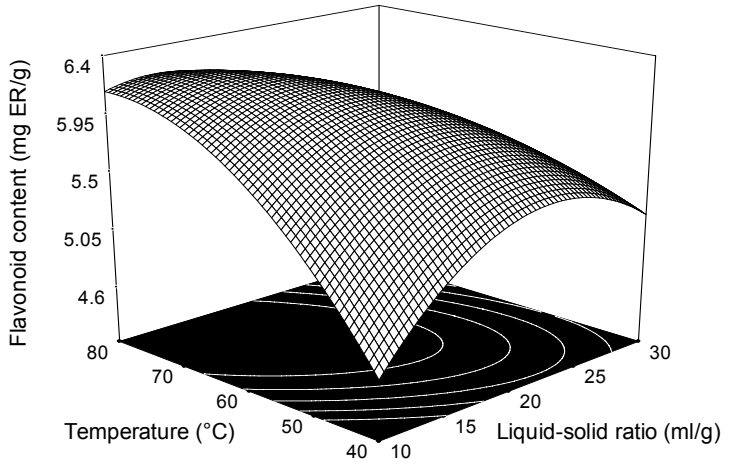
Flavonoids are considering as phenolic compounds with highest antioxidant activity due to their chemical structure [40]. Plant flavonoids are an important part of the diet because of their effect on human nutrition [4]. Our research work confirmed that effect of different parameters on the yield of flavonoids was similar to the effects on the yield of phenolic content. The polarity of the solvent played an important role in the selective extraction of different flavonoid families [41]. The ethanol, a polar solvent, effectively can extract flavonoids and their glycosides, catechols and tannins from raw plant materials [42], but solubility of these compounds can be enhanced using a mixed solvent over a limited compositional range [24], like in our study. The increase of total phenols and flavonoids yield with the increase of the solvent-to-solid ratio is consistent with mass transfer principles. The driving force during mass transfer within the solid is considered to be the concentration gradient, which was greater when a higher solvent-to-solid ratio was used, resulting in an increase of the diffusion rate [24]. Many authors agree in the fact that an increase in the extraction temperature favours extraction process enhancing both the solubility of solute and the diffusion coefficient, but also that beyond a certain value of temperature, phenolic compounds can be denaturated [19, 20]. Spigno and De Faveri [20] were concluded that phenols yield at 60°C was higher than at 28°C.

Table 3 shows that the quadratic term all three investigated parameters had the significant effect on the total flavonoids content of the extracts. The analysis of the results showed that the interaction between the temperature and liquid-solid ratio (X_2X_3) was the only highly significant factor affecting the total flavonoids content ($p = 0.0086$).

Fig. 2a shows that total flavonoids content in extracts increased with increasing of ethanol concentration from 40 to 60%. Further increase of ethanol concentration leaded to the decreasing the flavonoids content. The total flavonoids content increased with increasing liquid-solid ratio up to about 18 ml/g, as in the research Cacace and Mazza [39]. From Fig. 2b it can be seen that the total flavonoids content increased with increasing extraction temperature (from 40 to 70°C). According to different investigated parameters, the total flavonoids content of mulberry extracts varied from 4.4621 to 6.4011 mg ER/ g of dry mulberry leaves.



(a)



(b)

Fig. 2 (a) Effects of solvent concentration and liquid-solid ratio (temperature, 60°C), and (b) effects of temperature and liquid-solid ratio (solvent, 60%) on total flavonoid content of the mulberry extracts

The response surface was generated based on the second order equation:

$$y = 6.1 - 0.12X_1 + 0.24X_2 - 0.23X_3 - 0.40X_1^2 - 0.40X_2^2 - 0.56X_3^2 - 0.24X_1X_2 - 0.06X_1X_3 - 0.52X_2X_3 \quad (3)$$

where y is the flavonoids content, X_1 is the solvent concentration, X_2 is temperature and X_3 is liquid-solid ratio. The optimal conditions for flavonoids content were determined at liquid-solid ratio of 14.62 ml/g, temperature of 75.02°C and ethanol concentration 53.44%, and the predicted flavonoids content was 6.2699 mg ER/g. Under these optimal conditions, the experimental value was 6.1879 mg ER/g, which agreed with those predicted.

3.3 Antioxidant activity of mulberry extracts

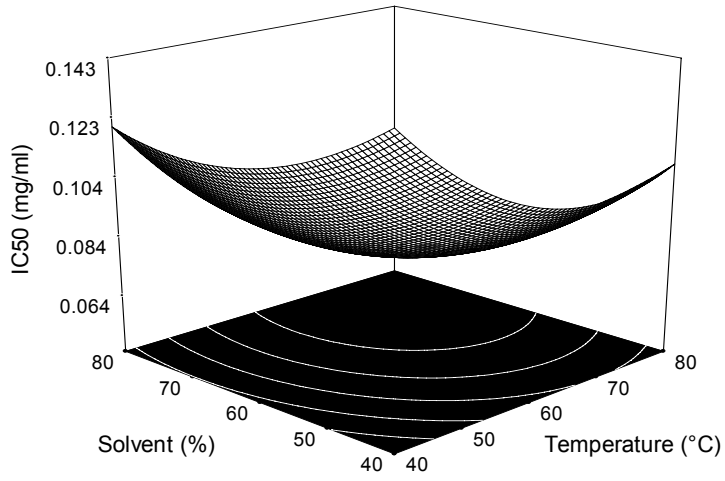
The IC_{50} values were used to report the $DPPH^{\cdot}$ scavenging capacity of mulberry extracts. The IC_{50} is the required initial concentration of a selected antioxidant sample to

quench 50% of the free radicals initially in the reaction system; therefore, a higher IC_{50} value corresponds to a lower antioxidant activity in the sample [33].

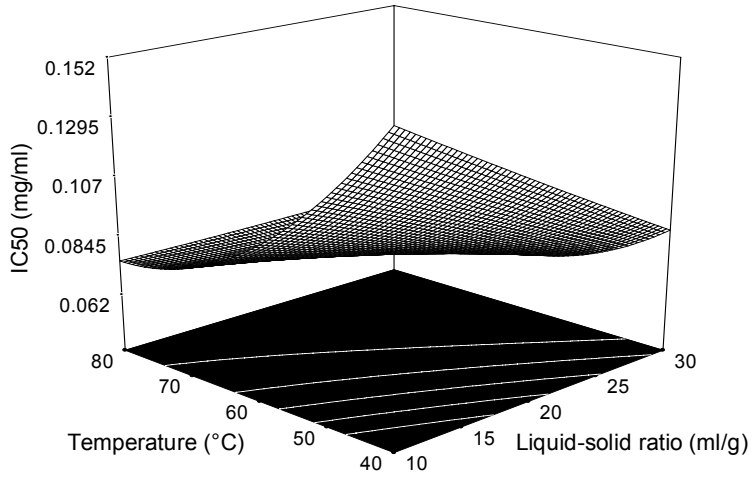
Some researchers reported that the antioxidant activity was correlated well with TP, and suggesting that these polyphenols have a major influence on the antioxidant activity [19, 20]. The antioxidant activity of mulberry extracts was in correlation with total phenolics content (Table 2). With increasing of total phenolics content, IC_{50} value decrease, what indicate higher antioxidant activity of extracts. Mulberry extracts with highest content of total phenolics compounds, have a lowest IC_{50} values, and because of that highest antioxidant activity. Maximum antioxidant activity, i.e. lowest IC_{50} value, indicates that the elevated temperature set aside some antioxidant components other than polyphenols. Shi [43] showed correlation between increased extraction temperature and concentration of gallic acid, epicatechin and catechin in the extract. Also, the study of Azizah [17] reported that the compounds of extract were stable at all temperatures (except higher at 90°C). Heating might soften the plant tissue and weaken the phenol-protein and phenol-polysaccharide interactions in seed meal, thus more polyphenols would migrate into the solvent. However, more proteins and polysaccharides could be extracted at higher temperature when water was used alone for extraction.

The statistical analysis showed that the linear term of solvent concentration, temperature, and liquid-solid ratio, the quadratics of solvent concentration and temperature, as well as the interactions between solvent concentration and liquid-solid ratio, and between temperature and liquid-solid ratio, showed significant effects on the antioxidant activity of extracts (Table 3).

Fig. 3a shows that IC_{50} value of the extracts decreased with increasing ethanol concentration and temperature, which means that the antioxidant activity of extracts were higher at this conditions. Liyana-Pathirana and Shahidi [27] presented that solvent composition displayed a quadratic effect on the response yielding maximum between 50% and 60% ethanol concentration. That ethanol concentration was the opposite from the results that we obtained in this study. From Fig. 3b it can be seen that IC_{50} value slightly decreased with increasing liquid-solid ratio from 10 to 30 ml/g, which means that chosen liquid-solid ratio did not show significant effect on the antioxidant activity.



(a)



(b)

Fig. 3 (a) Effects of solvent concentration and temperature (liquid-solid ratio, 20 ml/g), and (b) effects of temperature and liquid-solid ratio (solvent, 60%) on IC_{50} value of the mulberry extracts

The second order polynomial model used to express the IC_{50} value (y) as a function of independent variables (in terms of coded values) is shown below:

$$y = 0.078 - 0.016X_1 - 0.022X_2 - 0.016X_3 + 0.013X_1^2 + 0.019X_2^2 + 0.0005X_3^2 - 0.005X_1X_2 + 0.013X_1X_3 + 0.016X_2X_3 \quad (4)$$

where y is IC_{50} value, X_1 is the solvent concentration, X_2 is temperature and X_3 is liquid-solid ratio. The minimum conditions from IC_{50} value were determined at liquid-solid ratio of 20 ml/g, temperature of 80°C and ethanol concentration 80%, and the predicted IC_{50} value was 0.068 mg/ml. Under these optimal conditions, the experimental value was 0.071 mg/ml, similar with those predicted.

4. Conclusions

Response surface methodology was successfully applied for extraction of total phenolics, total flavonoids, and antioxidant activity of mulberry leaves. The high correlation of the mathematical model indicated that a quadratic polynomial model could be used to optimize total phenolic and flavonoid content, as well as the antioxidant activity of mulberry extracts. The analysis of variance (ANOVA) showed that the regression models was statistically good with a significance level of $p < 0.0001$ for total phenolics content and IC_{50} value, and of $p = 0.0088$ for total flavonoids content. Considering the maximum amount of extracted total phenolics, the optimal conditions were: liquid-solid ratio (29.19 ml/g), temperature (68.15°C) and solvent concentration (68.30%). The optimal conditions to obtain the highest flavonoids content were determined to be at liquid-solid ratio of 14.62 ml/g, temperature of 75.02°C and 53.44% ethanol. The optimal conditions for antioxidant activity were obtained at liquid-solid ratio of 20 ml/g, temperature of 80°C and 80% ethanol. Under these optimal conditions, the predicted amount of total phenolics content was 23.3096 mg ECA/ g of dry leaves, total flavonoids content was 6.2699 mg ER/ g of dry leaves and IC_{50}

value was 0.068 mg/ml. The experimental values were shown to be in agreement with those predicted, thus indicating the adequacy of the fitted model. This is the first report on optimizing of extraction technology of phenol and flavonoids compounds and antioxidant activity of mulberry plants grown in Serbia and Balkan region. This information will be of considerable value to the commercial producers of mulberry trees cultivation or pharmaceutical industry for potential new mulberry supplement production.

5. Acknowledgements

Financial support of this work by the Serbian Ministry of Education and Science, Project No. TR 31013, is gratefully acknowledged. We are grateful to dr. Goran Anačkov, Department of Biology and Ecology, Faculty of Natural Sciences, University of Novi Sad, for support in term of confirmed and deposited *Morus alba* L. at the BUNS Herbarium.

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