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Ultrasound-microwave assisted extraction of flavonoid compounds from *Eucommia ulmoides* leaves and an evaluation of their antioxidant and antibacterial activities

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Abstract: Ultrasound/microwave assisted extraction (UMAE) of flavonoid compounds from *Eucommia ulmoides* leaves was studied and the extraction conditions were optimized by the Plackett-Burman design (PBD) method combined with the Box-Behnken design (BBD). The antioxidant and antibacterial activities of the flavonoid extract were investigated. The results show that the optimal conditions were an ethanol concentration of 41%, microwave power of 178 W and an ultrasound extraction time of 26 min. Under these conditions, the yield of the flavonoid compounds was 2.454%±0.230%, which was higher than that after direct solvent extraction, ultrasound extraction and microwave extraction. The results of *in vitro* antioxidant assays showed that the flavonoid extract had scavenging capacity for DPPH, ABTS and hydroxyl radicals, with corresponding IC_{50} values of 30.76 mg/L, 21.09 mg/L, 248.4 mg/L, respectively. In addition, this extract exhibited strong antibacterial activity on *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium*.

Keywords: *Eucommia ulmoides* leaves; flavonoids; ultrasound-microwave-assisted extraction; antioxidant activity; antibacterial activity

Abbreviations: ultrasound/microwave assisted extraction (UMAE); Plackett-Burman design (PBD); Box-Behnken design (BBD)

INTRODUCTION

E. ulmoides Oliver (called Du-zhong, Si-xian, Si-zhong, etc.) is one of the oldest and precious restorative herbs traditionally used as medicine in China [1]. *E. ulmoides* leaves are rich in resources and easier to obtain than the bark. The active ingredients of the leaves are similar to those of the bark, and they include iridoids (aucubin), phenylpropanoids (chlorogenic acid), polysaccharides and flavonoids (such as rutin, kaempferol and quercetin) [2]. This allows the leaves to be used as medicine instead of the bark. Modern research has revealed that various pharmacological properties of *E. ulmoides* leaves were primarily attributable to these compounds [3]. Also, *E. ulmoides* leaves are richer in flavonoid compounds than the bark [4]. The flavonoid compounds have various biological functions, such as anticancer and antihypertensive activities, and in prevention of neurodegenerative diseases [5]. Their free radical scavenging ability and antibacterial activity make them a desired resource of natural antioxidants and bactericides. Due to the harmfulness of chemically synthesized antioxidants (such as butylated hydroxytoluene (BHT)), natural compounds replace synthesized antioxidants in food supplements [6].

Although flavonoid compounds have shown considerable promise in a range of applications, very few

extracts are used directly in food products and cosmetics. The reason for their limited application is because extracts do not produce as marked effects as pure compounds [7]. In fact, the extracts (for example, flavonoid compounds) are more cost-saving, easier to obtain and more efficacious when compared to pure compounds. In addition, higher doses of extracts that exhibit better antioxidant and antibacterial effects do not affect the sensory acceptability of food (flavors and odors).

The conventional extraction methods of flavonoid compounds from *E. ulmoides* leaves include solvent extraction and heating reflux extraction. However, both have some limitations and disadvantages, such as the high energy input, long extraction time and the use of a large number of different solvents. Moreover, the high heating temperature required for extraction may cause the degradation of flavonoid compounds [8]. To compensate for these deficiencies, novel technologies have been devised for the extraction of bioactive compounds, including ultrasound and microwaves [9]. High-frequency microwaves and the cavitation effect of ultrasound vibration can damage the plant's cell wall and membrane, increasing the release of bioactive components [10-13]. It was found that the separate use of cell-wall damage methods was less efficient than when the methods were used in combination. The UMAE system has emerged to meet the harsh demands of the extraction [14,15]. UMAE technology combines ultrasound vibration with open-ended microwaves. This system takes full advantage of the cavitation effect of ultrasound vibration and the high energy of microwaves to make up for the deficiencies of conventional extraction methods. Not only does this system create milder extraction conditions, a lower energy requirement and simplified manipulation, but also it avoids the decomposition of active ingredients under high temperature [16]. Moreover, it has been reported that the extraction technology has a high efficiency of extraction [17,18]. Therefore, the combination of ultrasound and microwave techniques can have more advantages than the use of single extraction methods [16,19]. In previous studies, flavonoid compounds were extracted by a single extraction method [20,21], and studies on UMAE have rarely been reported. Furthermore, no one has reported on the effect of UMAE on *E. ulmoides* leaves.

The PBD method, as an efficient test design method, can screen out significant factors with less test times from various factors [22]. It greatly reduces the workload of screening out significant factors influencing the extraction of flavonoid compounds. Based on PBD experiments, response surface methodology was used to obtain the optimum experimental conditions [23,24]. BBD, one of response surface design methods, is a powerful experimental design tool that has been used to optimize and elucidate the complicated functional relationships between factors and response values. Nowadays, the method is widely used in extraction, drug screening and other optimization processes due to its high accuracy and good predictability [25]. However, only a limited number of studies have reported on the extraction of flavonoid compounds from *E. ulmoides* leaves by the combined use of PBD and BBD.

In this study, UMAE for the flavonoid-enriched extract from *E. ulmoides* leaves was investigated and the operational conditions were optimized by the PBD method combined with BBD. In addition, the result of UMAE was compared with the results of other single method extractions, such as ultrasound-assisted, microwave-assisted extraction and solvent extraction. The antioxidant and antibacterial activities of the flavonoid extract were also measured in multi-test systems *in vitro*. This aim of the study was to establish the optimal conditions of UMAE for the extraction of flavonoid components from *E. ulmoides* leaves, and to provide bioactivity information about the flavonoid extract of *E. ulmoides* leaves.

MATERIALS AND METHODS

Experimental materials

The *E. ulmoides* leaves were collected in August 2017 from the arboretum of Zhangjiajie Jishou University campus (Zhangjiajie, China), (29°20′ W; 110°47′ E); Rutin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, butylated hydroxytoluene (BHT), ascorbic acid (Vc) and dimethyl sulfoxide (DMSO) were purchased from Aladdin Chemicals (Shanghai, China). A hydroxyl radical determination kit was purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Mueller-Hinton Agar was purchased from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd (Guangzhou, China). Other

chemical agents were purchased from Macklin Biochemical Co., Ltd (Shanghai, China). *Staphylococcus aureus* ATCC 29523, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028 were provided by the Microbiology Laboratory, Guangdong Institute of Analysis (Guangzhou, China).

Methods

An UMAE method for a flavonoid-enriched extract from *E. ulmoides* leaves was established by combining the PBD method with BBD. The antioxidant and antibacterial activities of the flavonoid extract obtained by an optimal extraction procedure were also evaluated. The schematic representation of the processes involved in the whole experimental setup is presented in Supplementary Fig. S1.

Extraction of flavonoid compounds from E. ulmoides leaves

The leaves were dried to constant weight at 50°C and broken up in a disintegrator (DYF-1000C, Wenling Lina Machinery Co., Ltd, Wenling, China). The leaf powder was sieved through a mesh (0.3 mm) and stored at room temperature in dark plastic bags.

Three g of powdered *E. ulmoides* leaves were added to an Erlenmeyer flask with an ethanol solution and soaked at room temperature for 20 min. After swelling, it was extracted twice under the preset conditions by the UMAE system (Ultrasonic Microwave Reaction System, Nanjing Shunliu instruments Co., Ltd, Nanjing, China). An SL-SM200 Ultrasonic-Microwave Cooperative Extractor/Reactor was operated with ultrasound and microwave power of 1200 W at a frequency of 2450 MHz, as shown in Supplementary Fig. S2. The filtrates were combined in a 200-mL volumetric flask.

Quantitative analysis of flavonoid compounds

The yield of flavonoid compounds was estimated according to the method of Li et al. [26] with some modifications, and calculated using rutin as standard. Briefly, 5 mg of rutin standard substance were dried to a constant weight at 105°C, and dissolved in 100 mL methanol. Then, 1.0, 2.0, 3.0, 4.0 and 5.0 mL of standard rutin solution were added to 10-mL volumetric flasks and diluted with methanol. Absorbance was measured at 360 nm and the standard curve of rutin was obtained to calculate the concentration of the flavonoid compounds. The yield of the flavonoid compounds was expressed as flavonoid compounds equivalents in percentages of dry material.

The measurement of samples was as follows: 1 mL of *E. ulmoides* leaf extract solution was added to an evaporating dish with 1 g of preprocessed polyamide powder. The polyamide powder was volatilized with ethanol in a water bath; after adsorption it was transferred to the chromatographic column. The system was eluted by benzene and methanol to obtained the pure flavonoid compound solution. The absorbance was measured at 360 nm against a blank sample (methanol). The yield of flavonoid compounds was calculated as follows:

$$Y(\%)=A_e/A_p\times 100\%$$

where A_e and A_p are the qualities of the flavonoid compounds and of the *E. ulmoides* leaf powder, respectively.

PBD

The following factors were used to evaluate the significance for the yield of flavonoid compounds: X_1 ethanol concentration (35%, 55%); X_2 solid-liquid ratio (1:15 g/mL, 1:25 g/mL); X_3 microwave power (100 W, 200 W) and X_4 microwave extraction time (140 s, 210 s); X_5 ultrasound power (200 W, 400 W) and X_6 ultrasound extraction time (20 min, 40 min). The experiment design included 12 runs of different combinations of independent variables coded as X_1-X_6 and conducted at high (+1) or low (-1) levels, as described in Supplementary Tables S1 and S2.

BBD

The Box-Behnken design is a new type of analytical procedure for the optimization of the experimental processes. It reveals the relationship between factors and response. Based on the PBD experiments, three important independent variables were investigated by BBD, including ethanol concentration (X_1), microwave power (X_3) and ultrasound extraction time (X_6). In addition, low and high levels for each variable were selected: X_1 (35%, 45%), X_3 (150 W, 200 W) and (20

min, 30 min); 17 experiments were examined according to a three-variable-three-level BBD to optimize the extraction process and the yield of flavonoid compounds was regarded as the evaluation metric of the experiments. The factors and levels are presented in Supplementary Table S3, and the formulations and results of BBD are shown in Supplementary Table S4. Design-Expert (version 8.0.5) statistical software (Stat-Ease, Minneapolis, MN, USA) was applied to analyze the experiment data.

Antioxidant assays

DPPH free radical scavenging activity

The DPPH free radical scavenging activity was measured according to the described method [27] with some modifications. The flavonoid extract solutions (20, 40, 60, 80, 100 mg/L), Vc (5, 10, 15, 20, 25 mg/L) and BHT (20, 40, 60, 80, 100 mg/L) were prepared before the test. Two mL of the DPPH solution (0.20 mmol/L) were commixed with 2 mL of the determinant solution (the blank solution was ethanol) in a water bath at 25°C in the dark. The absorbance of each mixture solution was determined three times at λ=517 nm after 30 min.

ABTS free radical scavenging activity

The ABTS free radical scavenging activity was measured according to the described method [28] with some modifications. Working solutions of ABTS (7.40 mmol/L) and potassium persulfate (2.60 mmol/L) were mixed in the dark for 12 h to produce oxidized (ABTS$^{\bullet}$). The stock solution was then diluted with absolute ethanol to obtain an absorbance of 0.70±0.02 units at λ=734 nm. After that, 3.6 mL of the ABTS$^{\bullet}$ solution was mixed with 0.4 mL of the determinant solution (the blank solution was absolute ethanol), and with the flavonoid extract solution (10, 20, 30, 40, 50 mg/L), Vc (2, 4, 6, 8, 10 mg/L) and BHT (10, 20, 30, 40, 50 mg/L)) at room temperature. The absorbance of the mixtures was determined three times at λ= 734 nm after 6 min.

Hydroxyl radical free radical scavenging activity

The hydroxyl radical free radical scavenging activity was measured using the hydroxyl radical determination kit according to the described method [29] with some modifications. The determinant solutions contained the flavonoid extract solution (100, 200, 300, 400, 500 mg/L), BHT (100, 200, 300, 400, 500 mg/L) and Vc (100, 200, 300, 400, 500 mg/L), respectively. The experiment was performed as per the manufacturer's specification and the absorbance of each mixture was measured three times at λ=550 nm.

Calculation of clearance rate and IC_{50} value

The antioxidant ability was evaluated by the clearance rate and the IC_{50} values. A higher clearance rate reveals stronger antioxidant activity and vice versa. The clearance rate was determined as follows:

$$Y(\%)=(1-A_1/A_0)\times 100\%$$

where A_0, A_1 are the absorbances of the blank sample and the test samples, respectively. The IC_{50} value denoted the concentration of the antioxidant required for a 50% clearance rate. In the study, Origin 8.0 was used to fit the curve and to calculate the value of IC_{50}.

Antibacterial assays

Preparation of bacterial suspension

Staphylococcus aureus, *Escherichia coli* and *Salmonella typhimurium* strains were inoculated on nutrient medium and incubated in aseptic conditions at 37°C for 24 h. A fraction of the activated bacteria was cultured in a shaker incubator at 37°C for 24 h. The concentration of the bacteria was adjusted to 10^7-10^8 CFU/mL.

Determination of inhibition zone

The prepared bacterial suspensions (300 μL) were pipetted onto Mueller-Hinton agar. After the bacterial suspension fully penetrated, an Oxford cup was placed in a Petri dish using sterile forceps. Then, 200 μL of extract solutions with different concentrations (10, 30, 50, 70 and 90 g/L) were taken into the Oxford cup. Gentamicin sulfate (positive control, 0.2 g/L) was used as a positive control and 5% DMSO solution was the negative control. The Petri dishes were incubated at a constant temperature of 37°C. The inhibition zone of each sample was measured by a Vernier caliper in triplicate and experiments were repeated three times.

Evaluation of minimum inhibitory concentration and minimum bactericidal concentration

The flavonoid extract was dissolved in 5% DMSO at a concentration of 100 g/L. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were obtained by the two-fold dilution method [30] with some modifications. The starting inoculum was 1.0×10^6-1.0×10^7 CFU/mL and the concentration of the flavonoid extract was tested in the range 0.04883 g/L-50 g/L. The MIC is the minimum concentration of the flavonoid extract solution required to prevent microorganism growth. The MBC is the lowest flavonoid extract solution concentration, resulting in >99.9% reduction of the initial inoculum after 24 h incubation at 37 °C. All experiments were performed in triplicate.

RESULTS

UMAE

The effect of variables on the yield of flavonoid compounds was determined by conducting 12 experiments given by the PBD model (Table 1). $P<0.05$ was considered significant in results of analysis of variance (ANOVA). The determination coefficient (R^2=0.9324) and the adjusted determination coefficient (R^2_{Adj}= 0.8512) indicated a high degree of fit for the model. We found that the ethanol concentration (X_1) had a highly significant effect on the yield of flavonoid compounds, while the microwave power (X_3) and ultrasound extraction time (X_6) had a significant effect. The effects of solid-liquid ratio (X_2), microwave extraction time (X_4) and ultrasound power (X_5) were not significant on the yield. Therefore, the ethanol concentration (X_1), microwave power (X_3) and ultrasound extraction time (X_6) should be further optimized by BBD, and the remaining value brought to a low level (solid-liquid ratio 1:15 g/mL, microwave extraction time of 140 s and ultrasound power 200 W).

Optimization of UMAE

Multivariate regression analysis was performed on the results of the BBD to obtain a second order polynomial equation:

$$Y=2.440+0.052X_1+0.033X_3+0.039X_6+0.032X_1X_3+0.043X_1X_6+0.0045X_3X_6-0.072X_1^2-0.110X_3^2-0.066X_6^2,$$

where Y, X_1, X_3 and X_6 represent the yield of the flavonoid compounds (%), the ethanol concentration, microwave power and ultrasound extraction time, respectively.

The statistical parameters obtained from ANOVA of the optimization model are shown in Table 2. The F-value of 76.55 and $P<0.0001$ indicated that the model is of great significance and could predict real experimental data. The R^2=0.9899 and R^2_{Adj}=0.9770 confirmed that the model was significant in the statistics. The pure error ($P>0.05$) indicated that the calculated values agree well with the experimental results. The signal-to-noise ratio was revealed by the "Adeq Precision", and a ratio greater than 4 was acceptable [31]. Thus, the "Adeq Precision" value of 21.072 for the yield of flavonoid compounds demonstrated an adequate signal. In conclusion, the model was functional for the experiment.

Table 1. Variance analysis of PBD for the yield of flavonoid compounds.

Source	Sum of square	df	Mean square	*F*-Value	*P*-Value	Coefficient estimate	Significance
Model	0.060	6	0.010	11.49	0.0085	2.20	**
X_1	0.035	1	0.035	40.19	0.0014	0.054	**
X_2	0.005677	1	0.005677	6.50	0.0513	0.022	
X_3	0.008911	1	0.008911	10.20	0.0242	-0.027	*
X_4	0.002791	1	0.002791	3.20	0.1339	-0.015	
X_5	0.00007008	1	0.00007008	0.08	0.7883	-0.002417	
X_6	0.007651	1	0.007651	8.76	0.0315	0.025	*
Residual	0.004367	5	0.0008734				
Cor Total	0.065	11					
R^2=0.9324	R^2_{Adj}=0.8512	$R^2_{Adeq\ Precision}$=10.485					

* represents $P<0.05$, **represents $P<0.01$; $P<0.05$ indicates that the factor has a significant effect, whereas $P<0.01$ indicates that the factor has a highly significant effect.

Table 2. Analysis of variance and regression coefficients of the calculated surface quadratic model for the yield of flavonoid compounds.

Source	Sum of square	df	Mean square	*F*-Value	*P*-Value	Significance
Model	0.15	9	0.017	76.55	<0.0001	**
$_X1$	0.022	1	0.022	98.91	<0.0001	**
X_3	0.008581	1	0.008581	39.42	0.0004	**
X_6	0.012	1	0.012	55.55	0.0001	**
X_1X_3	0.004096	1	0.004096	18.82	0.0034	**
X_1X_6	0.007310	1	0.007310	33.59	0.0007	**
X_3X_6	0.000081	1	0.000081	0.37	0.5611	
X_1^2	0.022	1	0.022	98.97	<0.0001	**
X_3^2	0.047	1	0.047	216.44	<0.0001	**
X_6^2	0.018	1	0.018	83.06	<0.0001	**
Residual	0.001524	7	0.0002177			
Lack of fit	0.00009075	3	0.00003025	0.084	0.9650	
Pure error	0.001433	4	0.0003582			
Sum	0.15	16				
R^2=0.9899	R^2_{Adj}=0.9770		$R^2_{Adeq\ Precision}$=21.072 Pred R^2=0.9756			

* represents P<0.05, **represents P<0.01; P<0.05 indicates that the factor has a significant effect, whereas P<0.01 indicates that the factor has a highly significant effect.

The P-value was applied to ascertain the significance of each coefficient and demonstrated the intensity of the interaction between each independent variable. It can be seen that the coefficients of X_1, X_3, X_6, X_1X_3, X_1X_6, X_1^2, X_3^2 and X_6^2 were highly significant (P<0.01). In addition, X_3X_6 was not statistically significant (P>0.05). For the X_1X_3 and X_1X_6 interaction, the value of P<0.01 indicated that the ethanol concentration was significantly related to the microwave power and ultrasound extraction time.

The three-dimensional (3D) response surface plots and the contour plots were obtained by Design-Expert software, and are shown in Fig.1. Fig. 1A shows the interactive effects of ethanol concentration and microwave power on the yield of flavonoid compounds, with the ultrasound extraction time (at zero level). The yield of flavonoid compounds first rose and then fell with increasing ethanol concentration from 35% to 45% and microwave power from 150 W to 200 W. The maximum yield was obtained when the ethanol concentration and microwave power were 41% and 180 W, respectively. Fig. 1B proved the interactive effects of the ultrasound extraction time and ethanol concentration on the yield of flavonoid compounds when keeping microwave power at zero level. The maximum yield of flavonoid compounds was obtained when the ethanol concentration was 41% and the ultrasound extraction time was 26 min. Fig. 1C revealed that the interactive effects between the ultrasound extraction time and microwave power presented non-significant effects.

Verification experiment

The optimal extraction parameters for obtaining the maximum yield of flavonoid compounds were as follows: ethanol concentration 40.67%, microwave power 178.33 W, and an ultrasound extraction time of 25.67

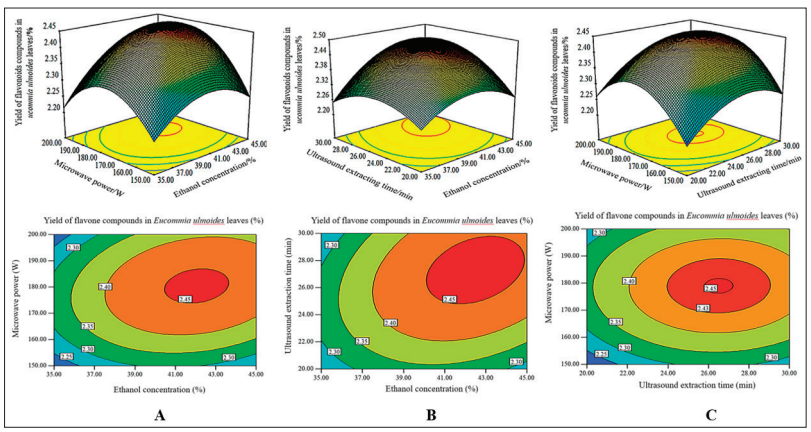

Fig. 1. The response surface diagram and contour map of ethanol concentration (X_1), microwave power (X_3) and ultrasound extraction time (X_6) to the extraction yield of flavonoid compounds. **A** – The interactive effects of the ethanol concentration (X_1) and microwave power (X_3). **B** – The interactive effects of the ethanol concentration (X_1) and ultrasound extraction time (X_6). **C** – The interactive effects of microwave power (X_3) and ultrasound extraction time (X_6).

min. The predicted yield of flavonoid compounds was 2.456% by BBD. In view of the feasibility of the experiment, the optimal parameters were modified to an ethanol concentration of 41%, microwave power of 178 W, and an ultrasound extraction time of 26 min. Under the selected optimal conditions, the validity of the model equation to predict the optimal response value was verified. The actual yield of flavonoid compounds was 2.454%, which almost coincided with the predicted yield (2.456%), as shown in Supplementary Table S5. There is a strong correlation between the actual results and the predicted results, indicating the response surface model can better reflect the expected optimization.

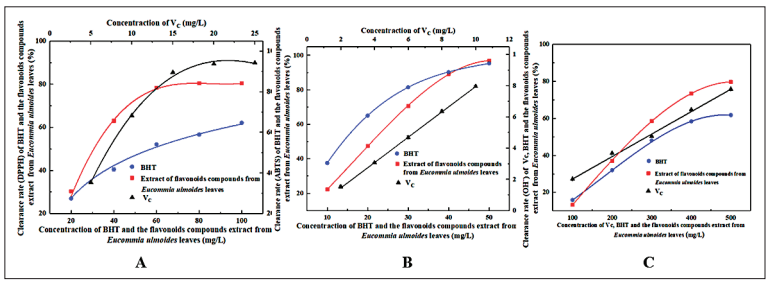

Fig. 2. Scavenging effects of the flavonoid extract from *E. ulmoides* leaves on DPPH radical, ABTS radical and OH radical. **A** – Effect of the flavonoid extract from *E. ulmoides* leaves on the removal of the DPPH radical. **B** – Effect of the flavonoid extract from *E. ulmoides* leaves on the removal of the ABTS radical. **C** – Effect of the flavonoid extract from *E. ulmoides* leaves on the removal of the hydroxyl radical.

Comparison of the effect on the flavonoid compound yield of *E. ulmoides* leaves by different extraction methods

According to the optimal conditions of the previous results, ultrasound extraction [32], microwave assisted extraction [33], UMAE, and solvent extraction in the terms of yield of flavonoid compounds were compared. The yield of flavonoid compounds extracted by ultrasound-assisted extraction, microwave-assisted extraction, UMAE and solvent extraction were 2.041%, 1.731%, 2.454% and 1.568%, respectively. We found that the yield of flavonoid compounds extracted by UMAE was higher than those of the other three methods. Compared with the ultrasonic-assisted extraction method, the microwave-assisted extraction method and the solvent-extraction method, the yield obtained by UMAE was 20.24%, 41.77% and 56.51% higher, respectively.

Antioxidant activity analysis

DPPH free radical scavenging activity

The pairing of the single electron of •DPPH occurred due to the presence of free radical scavengers, resulting in a decrease of the maximum absorbance value at 517 nm. The lower absorbance demonstrates a better scavenging capacity. Fig. 2A shows the scavenging capacity of the flavonoid extract, with BHT and Vc as positive controls. The scavenging rate of •DPPH increased significantly with the increase in concentration in the range of measured concentrations. At the concentration of 100 mg/L, the clearance rate of the extract was 80.37%. The results demonstrated that the antioxidation efficiency of the extract (IC_{50}=30.76 mg/L) for DPPH radical scavenging capacity was better than for BHT (IC_{50}=55.33 mg/L), and worse than for Vc (IC_{50}=6.914 mg/L).

ABTS free radical scavenging activity

The scavenging mechanism of the scavenging capacity for ABTS• was as follows: with the presentence of oxidizing agents, the ABTS is oxidized to ABTS•; however, antioxidants could inhibit the production of ABTS• [31]. As shown in Fig. 2B, the scavenging capacity for ABTS• of the extract was significantly increased in a concentration-dependent manner in the range of measured concentrations. At the concentration of 50 mg/L, the clearance rate of the flavonoid extract was 96.88%. We observed that the scavenging capacity of the flavonoid extract (IC_{50}=21.09 mg/L) was worse than for BHT (IC_{50}=13.74 mg/L) and Vc (IC_{50}=6.349 mg/L).

Hydroxyl radical scavenging activity

The results of the scavenging capacity for the hydroxyl radical for the flavonoid extract are shown in Fig. 2C. The flavonoid extract exhibited good scavenging capacity at concentrations from 100 to 500 mg/L, and the scavenging capacity of the extract was increased at the tested concentration range. At the concentration of 500 mg/L, the clearance rate of the flavonoid extract was 79.67%. The IC_{50} of the flavonoid extract was 248.4

Fig. 3. Antibacterial activity of the flavonoid extract from *E. ulmoides* leaves. **A** – Antibacterial activity of the flavonoid extract from *E. ulmoides* leaves against *E. coli*. **B** – Antibacterial activity of the flavonoid extract from *E. ulmoides* leaves against *S. aureus*. **C** – Antibacterial activity of the flavonoid extract from *E. ulmoides* leaves against *S. typhimurium*. **D** – Negative control. **E** – Inhibition zone of the positive control (0.2 g/L). **F** – Inhibition zone of the flavonoid extract (90 g/L).

mg/L, which was superior to BHT (IC_{50}=306.5 mg/L) and Vc (IC_{50}=294.4 mg/L).

Antibacterial test analysis

Determination of the inhibition zone

As shown in Fig. 3, the inhibition zone of the control was not detected in the negative group. At the same time, clear inhibition zones can be observed in the positive control group and the experimental group around the Oxford cup. The values of the inhibition zones are presented in Table 3. It can be seen that the flavonoid extract from *E. ulmoides* leaves exhibited potent antibacterial activity to gram-positive and gram-negative bacteria. In addition, the antibacterial effect of the samples increased markedly in a concentration-dependent manner at the tested concentrations.

Determination of MIC and MBC

The MIC values of flavonoid extract against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* were 0.7813 g/L, 0.7813 g/L and 1.563 g/L, respectively. The MBC values were 1.563 g/L, 1.563 g/L, 3.125 g/L, respectively.

DISCUSSION

The traditional methods for flavonoid extraction are reflux and leaching extraction. These methods have many problems such as low extraction efficiency and high energy consumption [34]. The UMAE, as a complementary extraction method with both advantages of UAE and MAE, has recently received increasing attention [35], but only a few reports describe the application of UMAE methods in the extraction of flavonoids from *E. ulmoides* leaves. Moreover, factors affecting the yield of active ingredients increase as a result of the introduction of this extraction technique. For multi-factor and multi-level extractions, if orthogonal design or BBD is used to optimize the extraction conditions directly, the workload of experiments will be increased and precision will be reduced. Therefore, it is necessary to find possible solutions to reduce the experimental workload and improve yield. The PBD can screen out the factors that significantly affect the results through fewer experiments, thus greatly improving efficiency. In this study, UMAE was used to extract flavonoid compounds from *E. ulmoides* leaves in order to improve the yield. PBD combined with BBD was applied to optimize the extraction conditions. The results showed that the yield of flavonoid compounds by UMAE was higher than obtained by a single extraction (such as ultrasonic-assisted, microwave-assisted and solvent extraction methods). The reason that the higher yield of flavonoid compounds was obtained by UMAE could partly be attributed to the mutually reinforcing effects generated by ultrasound and microwaves [36]. The cavitation of ultrasound could break down

Table 3. Inhibition zone values of the flavonoid extract from *E. ulmoides* leaves at different concentrations on bacteria.

Test bacteria	Inhibition zone diameter (cm) ±RSD					
	Mass concentration of flavonoid compounds from *E. ulmoides* leaves /g•L^{-1}					Positive control (gentamicin sulfate) /g•L^{-1}
	10	30	50	70	90	0.2
Escherichia coli	2.005±0.027	2.816±0.053	3.177±0.043	3.290±0.077	3.430±0.001	2.428±0.102
Staphylococcus aureus	2.145±0.093	2.870±0.055	3.120±0.110	3.330±0.001	3.415±0.169	2.294±0.088
Salmonella typhimurium	1.925±0.005	2.782±0.020	2.935±0.005	3.180±0.001	3.313±0.074	2.333±0.101

the cytoarchitecture of *E. ulmoides* leaves and increase the release of flavonoid compounds. Simultaneously, this action imparted a larger contact area between the flavonoid compounds and the extraction solvent [37,38]. Auxiliary rapid heating by microwaves ruptured leaf tissue and released more compounds [39]. Similar results were reported when sequential UMAE was used for the extraction of pectin from pomelo peel [40]. The results proved that the combined extraction techniques had greater potential and a higher efficiency of 154.95% and a 31.39% improvement of yield. However, in their experimental design, the factors affecting the yield were not optimized, resulting in some of them having insignificant effects on the yield, which can increase workload and make the results less precise. Similarly, it was demonstrated that the yield of natural dye from sorghum husk via UMAE was 3.6-fold higher than the yield of 4.6% obtained by the conventional shaking method [41], however, in this study, the experimental conditions were optimized by several single factor experiments and the significance of these factors was not evaluated. These may result in lower accuracy of the experimental results, notably of the yield.

Excessive reactive oxygen/nitrogen species (ROS-RNS) production disturbs the antioxidant/pro-antioxidant system and contributes to oxidative stress, which contributes towards development in humans of diabetes mellitus, neurodegenerative disorders, cardiovascular diseases, cancer, etc. [42,43], with studies pointing to a strong correlation between bioactive compounds and antioxidant activities [44,45]. Consequently, in this study the flavonoid extract was screened for antioxidant activities by different assays (•DPPH, ABTS$^{\bullet}$ and hydroxyl radicals). Our results revealed that the flavonoid extract had a strong scavenging effect on •DPPH, ABTS$^{\bullet}$ and hydroxyl radicals, and that the scavenging rate was concentration-dependent. The antioxidant capacity of the flavonoid extract can be explained by the high number of active hydroxyl groups, which donate hydrogen for binding with the radical to terminate the free radical oxidation chain reaction [46,47]; these results were in accordance with those reported previously [48]. We observed that the antioxidant activity increased significantly (P<0.05) with the increase of flavonoid concentration, which was in agreement with a previous report [49], however, it should be pointed out that our results for the scavenging capacity for •DPPH (IC_{50}=30.76 mg/L) and hydroxyl radicals (IC_{50}=248.4 mg/L) were better. The reason for this might be that more active ingredients, such as phenolic compounds, were extracted from *E. ulmoides* leaves through UMAE, resulting in increased scavenging capacity.

Plant extracts have shown considerable promise in a range of applications in industrial production, and several extracts due to their phytochemical constituents possess antimicrobial activity. Moreover, plant extracts do not produce the numerous side effects that are frequently related with synthetic antimicrobials [50]. In this study, the flavonoid extract from *E. ulmoides* leaves exhibited potent antibacterial activity to gram-positive and gram-negative bacteria. A previous study showed similar results, with both bacteria and fungi being inhibited by the extract from *E. ulmoides* leaves [51]. Moreover, the results of MIC and MBC for *Staphylococcus aureus* and *Salmonella typhimurium* indicated that the contained flavonoid exerted stronger antimicrobial effects on gram-positive bacterial than on gram-negative bacteria. This phenomenon was due to gram-positive bacteria being generally more sensitive to antibacterial agents than gram-negative bacteria [52], which may be because of the significant difference between the outer layers of gram-negative bacteria and gram-positive bacteria [53]. Surprisingly, the results of MIC and MBC for *Escherichia coli* (gram-negative bacteria) were the same as for *Staphylococcus aureus* (gram-positive bacteria). The reason for this inconsistency could be because other antibacterial compounds were also extracted via UMAE, including iridoids, phenylpropanoids, etc. Some of these compounds may display a better inhibitory effect on *Escherichia coli*, however, the specific reason for this needs further clarification.

CONCLUSIONS

UMAE and BBD combined with PBD provide an effective approach in the preparation and optimization of the extraction of flavonoid compounds. The experimental yield of flavonoid compounds was 2.454% (with a relative standard deviation (RSD)=0.23%) under optimized conditions of an ethanol concentration of 41%, microwave power of 178 W and an ultrasound extraction time of 26 min. The result of the yield was higher than that obtained after direct solvent extraction. The flavonoid extract showed a strong scavenging effect on

DPPH, ABTS and hydroxyl radicals and an antibacterial effect against *E. coli* and *S. aureus*. The flavonoid extract of *E. ulmoides* leaves has considerable potential in the development of cosmetics and functional foods.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Wang%20et%20al_Supplementary%20Material_4921.pdf