Optimization Protocol and Bioactivity Assessment for the Microwave-assisted Extraction of Flavonoids from *Eucommia ulmoides* Oliver Seed Meal Using Response Surface Methodology

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Response surface methodology was utilized to optimize the microwave-assisted extraction of flavonoids from *Eucommia ulmoides* Oliver seed meal. In addition, the optimal processing conditions for the extraction of *E. ulmoides* seed meal flavonoids were as follows: a processing time of 30 min, a liquid to solid ratio of 54 to 1 (mL/g), an ethanol concentration of 77%, and a temperature of 69 °C. The total flavonoids extraction percentage was 0.6611%. Moreover, the total flavonoids extracted from *E. ulmoides* seed meal were good for scavenging diphenyl picryl hydrazinyl. The *E. ulmoides* seed meal total flavonoids exhibited an obvious dose-dependent inhibitory effect on α-glucosidase in the concentration range of 0.05 to 1.0 mg·mL^{-1}. The IC50 value of the *E. ulmoides* seed meal flavonoids was slightly lower than the IC50 value of acarbose. According to the results of the xanthine oxidase inhibitory activity test, the IC50 value of the *E. ulmoides* seed meal flavonoids was higher than the IC50 value of allopurinol.

Keywords: Eucommia ulmoides Oliver; Seed meal; Total flavonoids; Optimization; Response surface methodology; Box–Behnken rotatable design; DPPH scavenging activity; α-Glucosidase inhibitory activity; XO Inhibitory activity

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INTRODUCTION

Eucommia ulmoides Oliver, known as the Chinese rubber tree (Dù-zhòng) in China, or Tuchong in Japan (Anderson 1982; Wang *et al*. 2003; Ji and Su 2006), is used extensively as a commercial plant in China (Yao *et al*. 2012; Pan *et al*. 2014; Niu *et al*. 2016). In addition to its leaves and bark, *E. ulmoides* seeds are a major product of the plant. Because the seeds are rich in linolenic acids and linoleic acid oil, the seed oil has a high antioxidant capacity and biological activation, which can be used for the treatment of hypertension, rheumatoid arthritis, lumbago (Zhang *et al*. 2010; Wang *et al*. 2012). As such, it is used as a nutritious plant oil for functional foods (Zhu *et al*. 2020). The seed meal is a by-product from the production of seed oil, with *E. ulmoides* seed as the raw material. In a seed oil processing plant, the *E. ulmoides* seed meal (EUOSM) is often neglected and usually discarded as a waste product or fertilizer.

Correlation studies between the bioactivities and the total flavonoids content of various plant seeds have been undertaken (Farombi *et al*. 2002; Arimboor and Arumughan 2012; Orak *et al*. 2012; Yan *et al*. 2011; Xu *et al*. 2015; Ayoub *et al*. 2016). However, the flavonoids in defatted *E. ulmoides* seed meal, as well as its related activities, have few published studies.

Microwave assisted extraction (MAE) is a new green technology. Microwaves can quickly transfer energy, improve the extraction yield, and reduce the extraction time and solvent consumption (Eskilsson and Bjorklund 2000; Guo *et al*. 2001).

In the present study, *E. ulmoides* seed meal (EUOSM) was used as the research object. Then, through microwave-assisted extraction, the flavonoids were extracted from the seed meal (Guo *et al*. 2001; Fu *et al*. 2020). Through the preliminary screening using the single factor method, the significant factors that affect the yield of the EUOSM total flavonoids content will be further optimized *via* the response surface methodology (RSM) to obtain the optimal extraction conditions for flavonoids extraction from EUOSM, using a four factors RSM Box-Behnken design (BBD). Moreover, diphenyl picryl hydrazinyl (DPPH) scavenging, α-glucosidase inhibition, and xanthine oxidase (XO) inhibition were used to investigate the activity of the flavonoids from EUOSM.

EXPERIMENTAL

Materials

Eucommia ulmoides Oliver seeds were collected from the Cili, Zhangjiajie, and Hunan provinces in China. The seeds were pressed using supercritical fluid extraction technology. The seed meal was defatted with hexane (a 1 to 10 ratio, w/v) *via* extraction in a Soxhlet apparatus for 24 h. After air-drying to remove the residual solvent, the defatted product was obtained, which was kept in sealed plastic bags at a temperature of -4 °C prior to extraction.

Microwave-assisted Extraction

A multifunctional pulverizer was used to pulverize the dried EUOSM. It was then passed through a 60-mesh sieve. The sieved product was used to conduct experiments according to the four factors, *i.e.*, microwave time (min), ethanol concentration (%), extraction temperature (°C), and liquid to solid ratio (mL/g). In addition, the total flavonoids content was determined and calculated after extraction.

Determination of the Total Flavonoids Content

The total flavonoids content, expressed as rutin equivalents (RE), was determined by a colorimetric method using aluminum nitrate, as described by Moreno *et al.* (2000), with slight modifications. First, 1.44 mL of the sample solution was diluted with 0.64 mL of a 70% ethanol solution. Then, 0.16 mL of a 5g / 100g $NaNO_2$ solution was added to the mixture, and after 6 min, 0.16 mL of a 10g / 100g $AL(NO_3)_3$ solution was added. The mixture was allowed to stand for 6 min, and then 1.6 mL of a 4 g / 100 g NaOH in an aqueous solution was added. The solution was mixed well, and immediately afterwards the absorbance was measured against a blank at 510 nm using a UV-VIS spectrophotometer after standing for 12 min. The results were expressed as mg of rutin equivalents.

Diphenyl Picryl Hydrazinyl (DPPH) Radical Scavenging Assay

The DPPH free radical scavenging capacity was calculated according to Zhu *et al.* (2020), with slight modifications. A total of 2 mL of freshly prepared 0.246 mg/mL DPPH solution (the blank solution was equivalent ethanol) was mixed with an appropriate amount of the determinant solution in a water bath at a temperature of 25 °C in a dark place. After 30 min, the absorbance was measured at least three times per blend *via* a UV-VIS spectrophotometer at 510 nm.

α-Glucosidase Inhibitory Activity Assay

The α-glucosidase inhibition activity of the EUOSM flavonoids were measured according to Asghari *et al.* (2015), with slight modifications. First, 160 µL of phosphate buffer (a pH of 6.9), 20 µL of α-glucosidase, and 20 µL of the sample solution in different concentrations were added into 96-well plate in sequence. Then, the mixture was stored at a temperature of 37 °C for 15 min, using acarbose as the positive control. Next, 40 µL of PNPG (4-nitrophenyl-β-D-glucopyranoside) was added and incubating at a temperature of 37 °C for 30 min. The OD value was measured at 405 nm using a microplate reader (SPECTRA Max-Plus 384, Molecular Devices, Sunnyvale, CA, USA).

Xanthine Oxidase (XO) Inhibitory Activity Assay

The XO inhibitory activity of the EUOSM flavonoids were measured according to Nguyen *et al.* (2004) by using 96-well plates. The OD value was measured at 290 nm using a microplate reader.

Statistical Analysis

Statistical analysis was carried out using SPSS software (Version 17.0, SPSS Inc., Chicago, IL). Origin software (Version 8.5, OriginLab Co., Northampton, MA, USA) was used for data interpolation, fitting, and curve plotting. The design of the experiments, analysis of the results, and prediction of the responses were carried out using Design-Expert software (Version 11, Stat-Ease, Inc., Minneapolis, MN, USA).

RESULTS AND DISCUSSION

Effects of the Single Factor Extraction Conditions

Microwave irradiation time

The introduction of a microwave-assisted method for the extraction of flavonoids can effectively reduce the extraction time and energy consumption; as such, it is an efficient method (Jing *et al.* 2019; Memarzadeh *et al.* 2020). Figure 1A shows that when the microwave irradiation time ranged from 15 to 35 min, the total flavonoids yield markedly increased and reached its maximum value after 30 min. When the extraction time was greater than 30 min, the flavonoids extraction rate began to significantly decrease. In contrast, when the microwave irradiation time continued longer than 35 min, the flavonoids extraction rate showed a slight decrease, which may be related to the loss of flavonoids components. According to the results shown in Fig. 1A, an appropriate extraction time for the extraction of flavonoids should be limited to within 25 to 35 min.

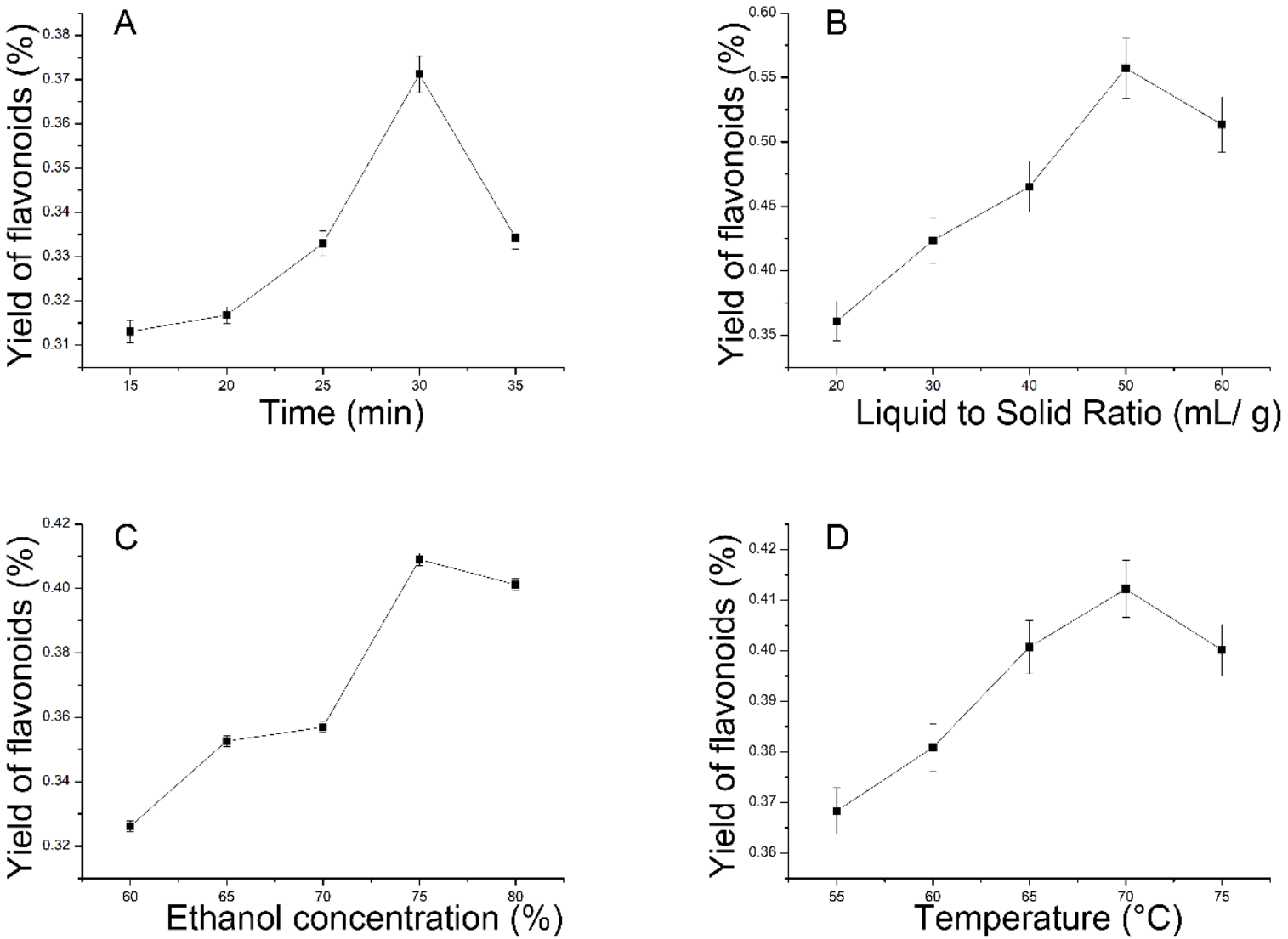

Fig. 1. Effects of the different parameters on the flavonoids yield: A) Time; B) Liquid to solid ratio; C) Ethanol concentration; and D) Temperature

Liquid to solid ratio

As shown in Fig. 1B, the flavonoids extraction rate significantly increased as the liquid to solid ratio was increased from 20:1(mL/g) to 50:1(mL/g) and reached the maximum extraction rate at a liquid to solid ratio of 50:1(mL/g). Moreover, the results shown in Fig. 1B indicate that the optimal liquid to solid ratio for flavonoids extraction may appear in the ratio range of 40:1 through 60:1. Additional information is needed for further optimization.

Ethanol concentration

The changing trend of the total flavonoids yield with relation to the ethanol concentration is shown in Fig. 1C; the total flavonoids yield increased as the ethanol concentration increased. The total flavonoids yield was the highest when the ethanol concentration reached 75%. When the ethanol concentration ranged between 75% and 80%, the total flavonoids yield showed a downward trend. Therefore, it was advisable to keep the ethanol concentration between 70% to 80%.

Temperature

Figure 1D shows that the highest flavonoids extraction occurred when the temperature was increased to 70 °C. The flavonoids extraction extent decreased when the temperature ranged from 70 to 75 °C. Considering that a high temperature might affect the structure and biological activity of the flavonoids, an excessively high temperature would reduce the extent of flavonoids extraction. Therefore, it was advisable to keep the temperature between 65 °C and 75 °C.

Optimal Extraction Conditions *via* Box-Behnken Design (BBD)

Based on single factor screening, the effects of the time, liquid to solid ratio, ethanol concentration, and temperature on the flavonoids yield were optimized *via* the BBD method. The actual value and the predicted value of the total flavonoids yield, the analysis of variance of the model, and the reliability test of the regression equation are listed in Tables 1 and 2.

The results of the ANOVA are shown in Table 2, and the multivariate regression-fitting model was used to obtain the quadratic regression equation of the total flavonoids yield for four independent variables, as shown in Eq. 1,

$$Y = -8.76506 + 0.11409 \cdot A + 0.041514 \cdot B + 0.057058 \cdot C + 0.12646 \cdot D + 4.50000E\text{-}005 \cdot A \cdot B - 3.43000E\text{-}004 \cdot A \cdot C - 7.09000E\text{-}004 \cdot A \cdot D - 2.15000E\text{-}005 \cdot B \cdot C + 3.00000E\text{-}005 \cdot B \cdot D - 2.83000E\text{-}004 \cdot C \cdot D - 6.81933E\text{-}004 \cdot A^2 - 3.97358E\text{-}004 \cdot B^2 - 1.68433E\text{-}004 \cdot C^2 - 6.13933E\text{-}004 \cdot D^2 \quad (1)$$

where A is the extraction time (min), B is the liquid to solid ratio (mL/g), C is ethanol concentration (%), D is temperature (°C), and Y is the total flavonoids yield (%).

The ANOVA analysis of the total flavonoids yield is presented in Table 2.

The linear coefficients (B, C, and D) were significant, which indicated that the liquid to solid ratio, ethanol concentration, and temperature greatly influenced the total flavonoids yield.

The interaction terms (AC and AD) and the quadratic terms (A^2, B^2, and D^2) were significant (a *p*-value less than 0.001). The obtained F-value (231.1873) and the corresponding *p*-value (lower than 0.0001) implied that the model was highly significant. The lack of fit F-value (1.74) implied the lack of fit was not significant relative to the pure

error. There was a 31.19% chance that a lack of fit F-value this large could occur due to noise.

The R^2 equaled 0.9957, which indicated that the model could explain 99.57% variation of the total flavonoids yield. The regression equation was significant, and the relationship between each factor and response value could be accurately described. Therefore, the equation could determine the optimal flavonoids extraction process parameters.

Table 1. Results of the Response Surface Analysis

Run	Factor					
	A	B	C	D	Flavonoids Yield (%)	
	Time (min)	Liquid to Solid Ratio (mL/ g)	Ethanol Concentration (%)	Temperature (°C)	Experimental	Predicted
1	30	40	75	75	0.5558	0.5524
2	30	60	80	70	0.6380	0.6417
3	30	40	70	70	0.5636	0.5633
4	25	50	75	65	0.5968	0.5995
5	30	50	70	65	0.6201	0.6202
6	30	40	80	70	0.5675	0.5716
7	30	50	75	70	0.6437	0.6475
8	25	40	75	70	0.5562	0.5565
9	35	60	75	70	0.6284	0.6295
10	30	50	70	75	0.6272	0.6295
11	30	60	70	70	0.6384	0.6377
12	30	50	80	65	0.6415	0.6406
13	30	50	75	70	0.6462	0.6475
14	35	50	70	70	0.6316	0.6321
15	30	50	75	70	0.6478	0.6475
16	25	60	75	70	0.6247	0.6243
17	25	50	75	75	0.6269	0.6301
18	35	40	75	70	0.5509	0.5527
19	30	50	80	75	0.6203	0.6216
20	30	60	75	75	0.6301	0.6277
21	30	40	75	65	0.5611	0.5602
22	30	50	75	70	0.6499	0.6475
23	25	50	70	70	0.6147	0.6143
24	30	60	75	65	0.6294	0.6295
25	35	50	75	65	0.6354	0.6356
26	25	50	80	70	0.6414	0.6376
27	30	50	75	70	0.6488	0.6475
28	35	50	80	70	0.624	0.6211
29	35	50	75	75	0.5946	0.5953

Table 2. Analysis of Variance for the Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	0.029018	14	0.002073	231.1873	< 0.0001
A	1.47E-06	1	1.47E-06	0.163962	0.6917
B	0.015689	1	0.015689	1749.939	< 0.0001
C	0.000115	1	0.000115	12.79356	0.0030
D	7.2E-05	1	7.2E-05	8.03412	0.0132
AB	2.03E-05	1	2.03E-05	2.258655	0.1551
AC	0.000294	1	0.000294	32.80599	< 0.0001
AD	0.001257	1	0.001257	140.1707	< 0.0001
BC	4.62E-06	1	4.62E-06	0.515587	0.4845
BD	9E-06	1	9E-06	1.003847	0.3334
CD	0.0002	1	0.0002	22.33252	0.0003
A^2	0.001885	1	0.001885	210.2802	< 0.0001
B^2	0.010242	1	0.010242	1142.35	< 0.0001
C^2	0.000115	1	0.000115	12.82834	0.0030
D^2	0.001528	1	0.001528	170.4342	< 0.0001
Residual	0.000126	14	8.97E-06		
Lack of fit	0.000102	10	1.02E-05	1.743028	0.3119
Pure error	2.34E-05	4	5.86E-06		
Corrected total	0.029143	28			
R^2	0.9957				
R^2adj	0.9914				
C.V.%	0.49				

Note: Df: degrees of freedom; R2adj: R2 adjusted; C.V.%: coefficient of variation

Plotted Results of the Response Surface Analysis

From the regression equation, the response surface and contour maps of the ratio of liquid to time, the liquid to solid ratio, the ethanol concentration, and the temperature affecting the total flavonoids yield were obtained, as shown in Figs. 2A through 2F.

Figure 2A shows that the effect of the time and liquid to solid ratio on the total flavonoids yield was parabolic. The total flavonoids yield first increased and then decreased as the time and liquid to solid ratio increased. According to Fig. 2B, the total flavonoids yield first increased and then tended to plateau as the time and ethanol concentration increased. As shown in Fig. 2C, as the time and temperature increased, the total flavonoids yield gradually increased and then tended to plateau. Figure 2D shows that the effect of the liquid to solid ratio and ethanol concentration on the total flavonoids yield was parabolic. The total flavonoids yield first increased and then decreased as the time and liquid to solid ratio increased. Figure 2E shows that as the liquid to solid ratio and temperature increased, the total flavonoids yield first increased and then decreased. From plots of the response surface and contours, the total flavonoids yield did not increase after a certain value when the ethanol concentration and temperature increased, as shown in Fig. 2F.

In this study, within the limits of the factors affecting the total flavonoids yield, the optimum conditions were predicted as follows: a time of 30.1 min, a liquid to solid ratio of 54.5 to 1 (mL/g), an ethanol concentration of 77.2%, and a temperature of 69.2 °C, with a

total flavonoids yield of 0.656%. Considering the actual laboratory extraction conditions, the optimal extraction conditions for flavonoids extraction were adjusted to a time of 30 min, a liquid to solid ratio of 54 to 1 (mL/g), an ethanol concentration of 77%, and a temperature (D) of 69 ℃. To verify and confirm the optimal extraction conditions, the flavonoids extraction process was performed in triplicate, and the average flavonoids extraction rate was calculated as 0.661%, which was highly similar to the predicted value (0.656%). This showed that the optimized process was reliable and reproducible in terms of flavonoids extraction.

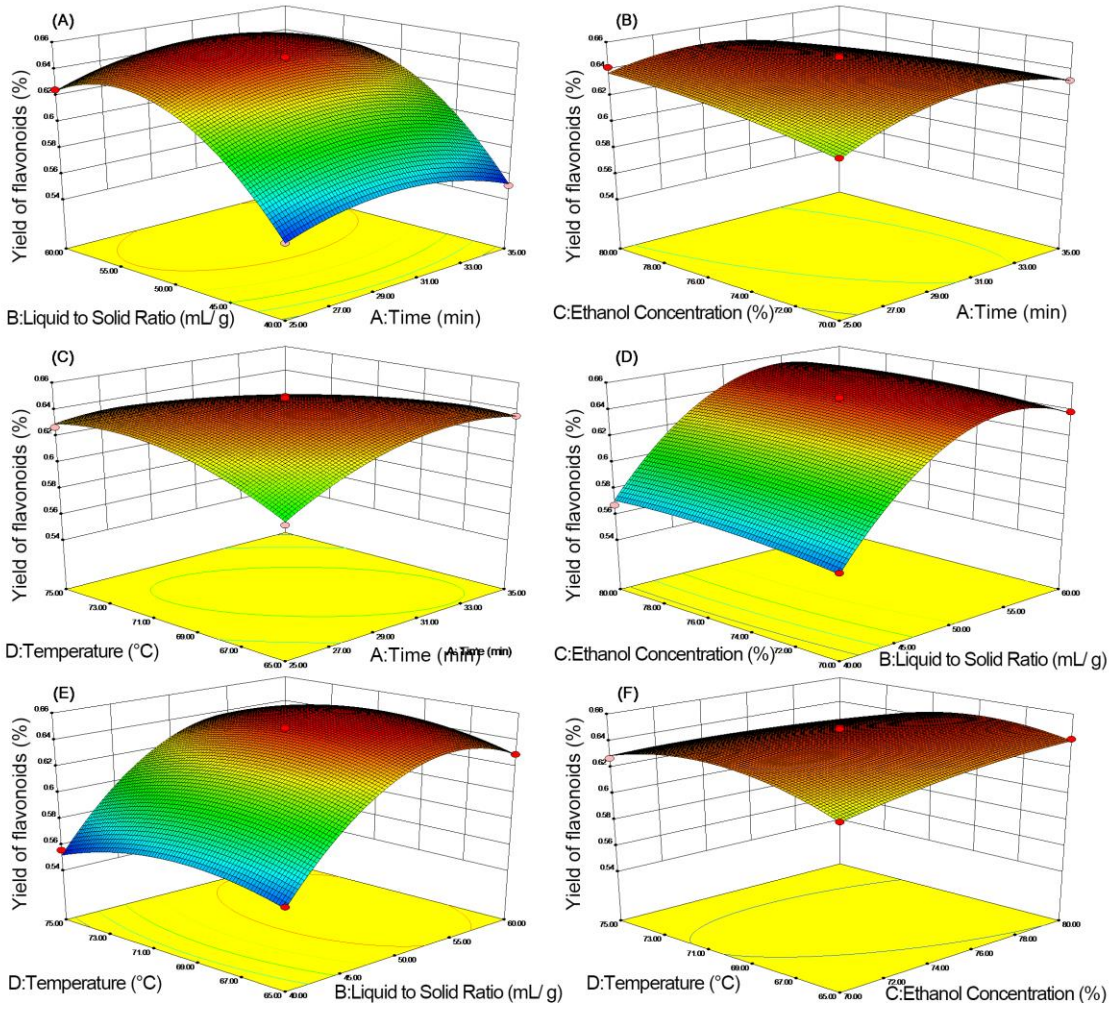

Fig. 2. Response surface (3D) showing the optimization of the flavonoid yield *via* the Box-Behnken design (BBD) method

Diphenyl Picryl Hydrazinyl (DPPH) Radical Scavenging Analysis

Diphenyl picryl hydrazinyl (DPPH) free radicals, as one of the most harmful reactive oxygen species (ROS), is usually associated with oxidative injury, resulting in tissue damage and even cell death. As shown in Fig. 3, *E. ulmoides* seed flavonoids and Vitamin C (VC) exhibited an obvious dose-dependent inhibitory effect on DPPH free radicals. The DPPH scavenging ability of *E. ulmoides* seed flavonoids was lower than that of VC, ranging from 0.005 mg/mL to 0.01 mg/mL. The results showed that the flavonoids extracted from *E. ulmoides* seed meal were good for scavenging DPPH.

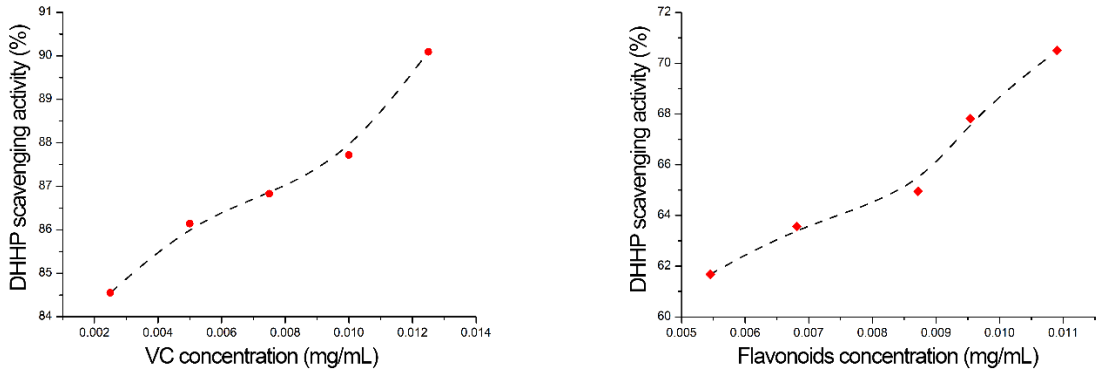

Fig. 3. Linear regression plots for the DPPH radical scavenging (Flavonoids and VC)

α-Glucosidase Inhibitory Activity Analysis

Slowing carbohydrate digestion and glucose absorption by inhibiting α-glucosidase activity has been widely used to control postprandial blood sugar in order to treat diabetes.

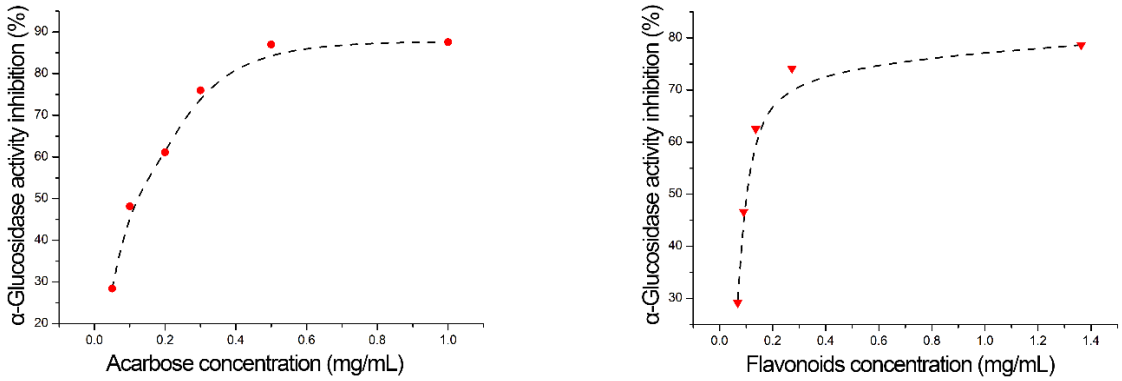

Fig. 4. α-Glucosidase inhibitory activity of flavonoids

As shown in Fig. 4, the EUOSM total flavonoids content and acarbose exhibited an obvious dose-dependent inhibitory effect on α-glucosidase in the concentration range of 0.05 to 1.0 mg·mL^{-1}. The IC50 values of the EUOSM total flavonoids content and acarbose were 0.1122 and 0.1134 mg·mL^{-1}, respectively (Table 3). The IC50 value of the total EUOSM flavonoids content was slightly smaller than the IC50 value of acarbose.

Table 3. The α-Glucosidase Inhibitory Activity of Flavonoids in Extracts Obtained from *E. ulmoides* Seed

Sample	Parameter	Estimate	Std. Error	Probit Regressive Equation	IC_{50} (mg•mL^{-1})	p	Sig.
Flavonoids	Intercept	1.521	0.241	Y = 1.521 + 1.601lg (X)	0.1122	< 0.0001	**
	X	1.601	0.214			< 0.0001	**
Acarbose	Intercept	2.304	0.207	Y = 2.304 + 2.437lg (X)	0.1134	< 0.0001	**
	X	2.437	0.247			< 0.0001	**

Xanthine Oxidase (XO) Inhibitory Activity Analysis

Figure 5 shows that the total EUOSM flavonoids content had a strong inhibitory effect on xanthine oxidase. The inhibitory activity increased as the flavonoids concentration increased. According to the results of the xanthine oxidase inhibitory activity test, the total EUOSM flavonoids content was positively correlated with the bacteriostasis effect. The IC50 values of the total EUOSM flavonoids content and allopurinol were 21.1 and 8.3 μg·mL^{-1}, respectively (Table 4). The IC50 value of the total EUOSM flavonoids content was higher than the IC50 value of allopurinol.

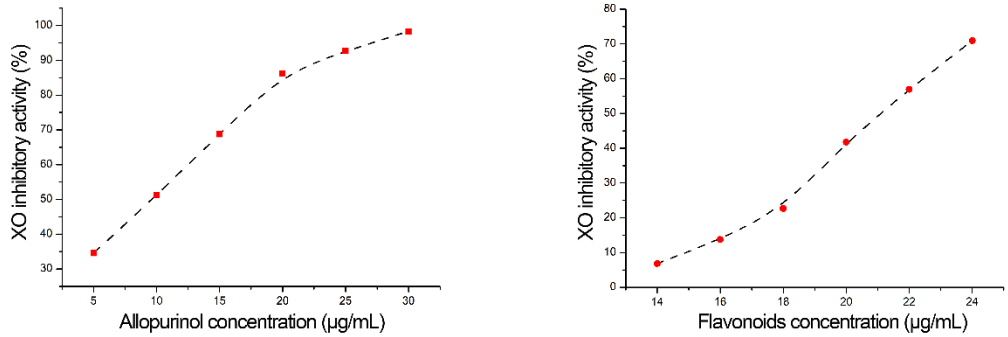

Fig. 5. The XO inhibitory activity of flavonoids

Table 4. Xanthine Oxidase (XO) Inhibitory Activity of Flavonoids in Extracts Obtained from *E. ulmoides* Seed

Sample	Parameter	Estimate	Std. Error	Probit Regressive Equation	IC_{50} (mg•mL^{-1})	p	Sig.
Flavonoids	Intercept	-20.539	1.483	Y = -20.539 + 15.506lg (X)	21.1140	< 0.0001	**
	X	15.506	1.929			< 0.0001	**
Allopurinol	Intercept	-4.225	0.467	Y = -4.225 + 4.608lg (X)	8.2581	< 0.0001	**
	X	4.608	0.419			< 0.0001	**

CONCLUSIONS

1. The optimal process for the extraction of EUOSM flavonoids was found to be as follows: a time of 30 min, a liquid to solid ratio of 54 to 1 (mL/g), an ethanol concentration of 77%, and a temperature of 69 ℃. The extraction yield of the EUOSM flavonoids was 0.6611%.
2. The EUOSM flavonoids were good for scavenging DPPH. The DPPH scavenging ability of the EUOSM flavonoids was lower than the DPPH scavenging ability of VC at a range of 0.005 mg/mL to 0.01 mg/mL.
3. The EUOSM flavonoids exhibited an obvious dose-dependent inhibitory effect on α-glucosidase at a concentration range from 0.05 mg·mL^{-1} to 1.0 mg·mL^{-1}. The IC50 value of the total EUOSM flavonoids content was slightly smaller than the IC50 value of acarbose.
4. According to the results of the xanthine oxidase inhibitory activity test, the IC50 value of the total EUOSM flavonoids content was higher than the IC50 value of allopurinol.

ACKNOWLEDGMENTS

This work was financially supported by the Science and Technology Innovation Program of the Hunan Province (Project no. 2018NK2042), the Science and Technology Program of the Xiangxi Autonomous Prefecture (Project no. 2019SF2005), the National Natural Science Foundation of China (Project no. 31560192), and the Technology Development Project of Hunan Gaoge Dairy Co., Ltd (Project no. 20431415000005).

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Article submitted: June 29, 2021; Peer review completed: September 5, 2021; Revised version received and accepted: September 13, 2021; Published: September 17, 2021.
DOI: 10.15376/biores.16.4.7367-7378