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Optimisation of parameters, antioxidant activity, and component analysis of flavonoids from Ecliptae Herba

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Abstract

Ultrasonic probe extraction of total flavonoids from Ecliptae Herba (EH) was investigated using response surface methodology. The parameters based on Plackett-Burman design were optimised with the Box-Behnken design to improve the extraction yield. The optimal extraction conditions were as follows: extraction solvent of water, ultrasound frequency of 40 kHz, number of extractions of 3, extraction temperature of 60°C, and solvent-material ratio of 30:1 mL/g. Under these conditions, the experimental extraction yield was 42.62 ± 1.38 mg/g, which matched well with the predicted value of 43.74 mg/g. Conventional reflux extraction was compared to ultrasonic probe extraction. In addition, scanning electron microscopy (SEM) imaging of EH after extraction was carried out. The antioxidant capacity of total flavonoids was evaluated using DPPH, $ABTS^{+}$, superoxide anion, and reducing power assays, which suggested that the flavonoid extract from EH can be used as a potential antioxidant additive in the food and pharmaceutical industries. Furthermore, qualitative and quantitative analyses of the flavonoid extract were studied.

Introduction

Ecliptae Herba (EH), the dried aerial parts of *Eclipta prostrata* L., has been used in traditional Chinese folk medicine to nourish the liver and kidney, cool the blood, and promote haemostasis over several thousand years (State Pharmacopoeia Commission of the PRC, 2015). It is mainly distributed in Zhejiang, Jiangsu, and Guangdong Provinces of China (Han *et al.,* 2013). Phytochemical investigations have demonstrated that thiophene derivatives, steroids, triterpenes, and flavonoids are the major bioactive compounds of EH (Li, 2012). Pharmacological studies have shown that the flavonoids are responsible for the biological activity of EH, including its haemostatic (Wang *et al.,* 2005), immunomodulatory (Zhang *et al.,* 1997), anti-inflammatory, analgesic (Jo *et al.,* 2010), and antioxidant (Lin et al., 2005a; Wang *et al.,* 2012) effects. EH, when consumed alone or in combination with other Chinese herbs, is typically taken in the form of decoctions and infusions (State Pharmacopoeia Commission of the PRC, 2015). The most common solvent used for the herbal preparations and finished herbal products is water.

The extraction of flavonoids from EH can be performed using a variety of methods, including conventional reflux extraction (Lin *et al.,* 2005b) and new techniques such as microwave-assisted extraction (MAE) (Sun *et al.,* 2011), supercritical fluid extraction (SFE) (Han, 2013) and ultrasound-assisted extraction (UAE) (Wu *et al.,* 2014). In a previous report, an ultrasonic cleaning bath was employed for flavonoid extraction. The delivered intensity is low and highly attenuated by the water in the bath and the walls of the glassware used for the experiment (Chemat *et al.,* 2011). The second UAE is the ultrasonic probe or horn system (UPAE), which is much more powerful because the ultrasonic intensity is delivered on a small surface as compared to the ultrasonic bath. The highly localised intensity of the probe means that the sonication process is more efficient and effective than that in a bath. The obvious difference is that the probe is directly immersed into the reaction flask, so less attenuation can occur (Dey and Rathod, 2013). Furthermore, most ultrasonic baths are used without temperature control.

The parameters of the UPAE process, such as ultrasonic frequency, ultrasonic power, duty cycle, solvent, extraction time, number of extractions, extraction temperature, and liquid to material ratio associated with response surface methodology (RSM), were investigated. Although RSM has been widely used to optimise UAE of flavonoids in many reports (Şahin *et al.,* 2015; Wu *et al.,* 2017; Chen *et al.,* 2018), to the best of our knowledge, no paper has been published to investigate UPAE optimisation combined with RSM for the extraction of flavonoids from EH. Hence, the

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purpose of the present work was to optimise the variables of UPAE for the extraction of flavonoids from EH and provide a foundation for utilisation of this resource. The flavonoid extract was further evaluated for its antioxidant activity, and some components were identified, and their concentrations were determined using an HPLC system. Moreover, the microstructure of the sample from conventional reflux extraction and UPAE was also analysed using scanning electron microscopy.

Materials and methods

Plant materials, chemicals, and reagents

Eclipta prostrata L. was collected from Xiuwu County in Henan Province, China and identified by Prof. Yanfang Wu. The samples were air dried at room temperature and then ground to pass through a 60-mesh sieve. The powder was maintained in sealed polyethylene bags at 4°C before analysis. Rutin, isoquercitrin, luteoloside, and buddleoside were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)], and vitamin C were purchased from Sigma Co. (Shanghai, China). Apigenin-7-O-β-D-glucoside was isolated in our laboratory, and its purity was determined to be over 98%, normalised to the peak area detected using HPLC. Deionised water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other organic solvents used in the present work were purchased from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjing, China), and were of analytical grade.

Extraction procedure

Extraction was performed using a sonochemical reactor equipped with two ultrasonic probes operating at two frequencies, 25 and 40 kHz, with a rated power of 1,500 W and a temperature control device (YMNL-2008DE, Nanjing YMNL Instrument Co., Jiangsu, China). Sample powder (2 g) was placed into a 100 mL round-bottomed glass vessel, and mixed with solvent. Primary experiments were performed to select the optimal extraction solvent and ultrasonic frequency. Then, the extraction procedure was carried out based on variable permutations as designed by the RSM software. The mixtures were centrifuged at 4,000 rpm for 15 min, and the supernatants were collected for total flavonoid analysis.

Screening of significant factors

Plackett-Burman design (PBD) is highly effective in screening the most important factor because PBD does not take interactions between the parameters into account (Siva Kiran *et al.,* 2010). In the present work, a Plackett-Burman factorial design was applied using six parameters at two levels to determine the important variables affecting total flavonoid extraction yield.

The path of steepest ascent

Based on the PBD data, three variables affecting the total flavonoid extraction yield were identified namely the number of extractions, extraction temperature, and liquid to material ratio. The range in the vicinity of the optimal conditions was obtained by using the steepest ascent method. Here, experiments were carried out along the path of steepest ascent with a confirmed interval.

Box-Behnken design

The defined factors affecting extraction efficiency, such as the number of extractions (X_1), extraction temperature (°C, X_2), and liquid to material ratio (mL/g, X_3), were used as independent parameters that should be optimised for the extraction. Box-Behnken design (BBD) was applied to determine the optimal conditions. The complete design with five replications of the centre points, resulting in a total of 17 combinations, was carried out based on the matrix established by Design-Expert software (version 8.0.5, Stat-Ease Inc., Minneapolis, USA). The coded and actual values of the experimental variables and their levels for the BBD are shown in Table 1. The BBD data were analysed by multiple regression to fit the quadratic polynomial model in Eq. 1:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad \text{(Eq. 1)}$$

where, Y = predicted response, β_0 = constant, and β_1, β_2, and β_3 = linear coefficients of X_1, X_2, and X_3, respectively, β_{11}, β_{22}, and β_{33} = squared coefficients of X_1, X_2, and X_3, respectively, and β_{12}, β_{13}, and β_{23} = interactive coefficients of the model.

Determination of total flavonoid content

The total flavonoid content was determined following a previously reported method (Baba and Malik, 2015). Briefly, 1 mL of sample extraction was pipetted into a 10 mL volumetric flask. Then, 0.5 mL of $NaNO_2$ (5% w/v) was added and incubated for 6 min. Next, 0.5 mL of $AlCl_3$ (10% w/v) was mixed in. After 6 min, 4 mL of NaOH (1 M) was added, and the mixture was diluted to 10 mL using distilled water.

Table 1. Coded levels, runs, and responses used in experimental design for RSM.

Run	Extraction time (X_1)	Temperature (X_2, °C)	Solvent to material (X_3, mL/g)	Yield (mg/g)
1	0 (2)	0 (45)	0 (20)	31.34
2	0 (2)	-1 (30)	1 (30)	35.25
3	0 (2)	1 (60)	-1 (10)	27.53
4	-1 (1)	0 (45)	-1 (10)	17.64
5	0 (2)	0 (45)	0 (20)	31.11
6	0 (2)	0 (45)	0 (20)	31.23
7	0 (2)	0 (45)	0 (20)	32.12
8	1 (3)	0 (45)	-1 (10)	33.67
9	0 (2)	1 (60)	1 (30)	37.79
10	-1 (1)	1 (60)	0 (20)	23.61
11	1 (3)	-1 (30)	0 (20)	36.29
12	0 (2)	-1 (30)	-1 (10)	24.51
13	-1 (1)	0 (45)	1 (30)	28.90
14	-1 (1)	-1 (30)	0 (20)	21.53
15	1 (3)	0 (45)	1 (30)	42.06
16	0(2)	0 (45)	0 (20)	33.23
17	1 (3)	1 (60)	0 (20)	39.90

The solution reacted for 15 min, and the absorbance was measured against a blank at 510 nm. The calibration curve of a rutin standard solution was used to determine the total flavonoid content. The results were expressed as rutin equivalents in mg/g dry weight.

Surface structural observations of EH

Scanning electron microscopy (SEM) was performed to obtain images of EH using a Flex SEM 1000 (Hitachi, Japan). The dried samples were sputtered with a thin gold film using a magnetron ion sputter metal coating device (IXRF Systems, Inc., Japan). To obtain optimum quality SEM images, a high vacuum was achieved by accelerating the potential of 20 kV under a high vacuum of 10 Pa.

Preparation of the sample for antioxidant capacity evaluation

Twenty grams of EH powder was extracted using water under the optimal conditions obtained in the present work. The solvent was evaporated under reduced pressure by rotary evaporation at 60°C. The infusions were lyophilised, and an extract of 0.4 g was obtained. The lyophilised extract was dissolved in methanol and kept at 4°C until use.

DPPH radical scavenging assay

The DPPH test was performed following our previous studies (Wang *et al.,* 2012; Wu *et al.,* 2017). Briefly, an aliquot of 1.7 mL of 0.2 mM DPPH in ethanol solution and 1.3 mL of various concentrations of sample were mixed and shaken vigorously, and then the mixture was allowed to reach equilibrium at room temperature for 1 h. The absorbance was determined at 517 nm using a Tianmei UV2000 spectrophotometer. The DPPH radical scavenging activity was calculated using Eq. 2:

$$\text{Scavenging rate} = [\frac{(A_{-A})}{A}] \times 100 \quad \text{(Eq. 2)}$$

where, A_s = absorbance of pure DPPH, A_i = absorbance of DPPH in the presence of sample, with vitamin C as the reference standard.

ABTS radical scavenging assay

The capacity to scavenge the ABTS radical cation ($ABTS^+$) was evaluated following the method described by Re *et al.* (1999) and Jagtap *et al.* (2019). The $ABTS^+$ solution was produced by the reaction of 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature in the dark for 12 – 16 h. The solution was diluted using ethanol to reach an absorbance of 0.700 ± 0.020 at 734 nm. Then, 0.4 mL of each test sample was added to 3.6 mL of $ABTS^+$ solution. The mixture incubated for 30 min at 30°C, and the absorbance was immediately measured at 734 nm. Vitamin C was used as a reference. The scavenging rate was also calculated using Eq. 2.

Superoxide anion scavenging capacity

The method described by Mu *et al.* (2012) was used to estimate the scavenging capability of each sample. Briefly, each mixture consisted of 2.5 mL of Tris-HCl buffer (pH 7.8, 50 mM) containing 1 mM EDTA and 0.1 mL of various concentrations of sample. The mixed solutions were kept for 20 min at ambient temperature. Next, 6 mM pyrogallol (0.4 mL) was added, and the mixture was shaken rapidly. The absorbance of the mixed solution was measured at 420 nm every 30 s for 3 min, and the slope was calculated as *k* (abs/min). Vitamin C was used as a reference. The superoxide anion scavenging activity was calculated using Eq. 3:

$$\text{Scavenging activity (\%)} = 1 - \frac{k(sample) - k(blank)}{k(control)} \times 100 \quad \text{(Eq. 3)}$$

where, control = mixed solution without sample, and blank = mixed solution without pyrogallol.

Reducing power assay

Slight modifications were made to the method of Berrabah *et al.* (2019) to determine the reducing power of each sample. Briefly, 0.2 mL of the tested sample was mixed with 1.0 mL of phosphate buffer (0.2 M, pH 6.6) and 1.0 mL of potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 min. Then, 1 mL of TCA (10% w/v) was added, and the mixed solution was centrifuged at 3,000 rpm for 10 min. Next, 2 mL of the supernatant was mixed with 2 mL of distilled water and 0.5 mL of ferric chloride solution (0.1% w/v). The absorbance at 700 nm was measured to determine the reducing power. Vitamin C was used for comparative purposes.

Component analysis of total flavonoids

Component analysis was performed using Waters e2695 Series equipment coupled to a 2489 detector (Waters, USA). Separation was conducted using a Waters Sunfire C_{18} column (250 × 4.6 mm, 5 μm) at a constant temperature of 30°C. The mobile phase consisted of water with 0.1% acid (A) and acetonitrile (B). The flow rate was 1.0 mL/min. The elution conditions were as follows: (1) 0 min, 10% B; (2) 15 min, 25% B; and (3) 25 min, 55% B. The injection volume was 10 μL, and the analytes were detected at 350 nm.

Results and discussion

The choice of solvent

The type of solvent is vital to extract target compounds. A suitable solvent for extracting objective components should be investigated carefully. Based on our previous results, different concentrations of ethanol were applied to extract the total flavonoids. It was obvious that water was the optimal solvent for extracting flavonoids. This is understandable because the extracted compounds possessed hydroxyl groups and glycosides (Liu *et al.,* 2012). The flavonoids were, therefore, extracted in higher amounts in more polar solvents. Furthermore, water is inarguably the safest, most inexpensive, and most environmentally friendly and accessible polar extraction solvent. It is traditionally used for Chinese medicine bioactive extractions in the form of decoctions and infusions (Ma *et al.,* 2017).

The choice of ultrasonic frequency

Ultrasonic frequency plays a crucial role in target compound extraction, which influences cavitational activity and treatment performance (Wang *et al.,* 2017). To evaluate the effects of ultrasonic frequency, different frequencies of the ultrasound probe, including 25 and 40 kHz, were investigated. A high extraction yield was obtained with the 40 kHz treatment. This is in agreement with the report by Vetal *et al.* (2013) which indicated that the ursolic acid extraction yield from *Ocimum sanctum* at 40 kHz was higher than that at 25 kHz.

The results of PBD

PBD was conducted to evaluate the influence of the extraction variables. The effects of six parameters, namely, temperature, time, liquid-solid ratio, number of extractions, ultrasound power, and duty ratio, were determined in 12 runs. It was observed that the three main factors, including extraction time, extraction temperature, and ratio of liquid to solid had high contributions to the response, hence selected for further studies.

The results of steepest ascent

Increasing the number of extractions, temperature, and ratio of liquid to material positively affected the total flavonoid yield. Moreover, the extraction yield reached a maximum value when the parameters were 3 extraction times, 60°C, and 30 mL/g, respectively. Furthermore, an increase in the variables had no significant impact on the total flavonoid content.

Model fitting and analysis

The total flavonoid extraction yields obtained from 17 experiments are given in Table 1. The data from the BBD were fitted to second-order polynomial equations. Analysis of variance (ANOVA) was conducted to assess the significance of the coefficients of the model using Design-Expert software. Table 2 illustrates the ANOVA results, which suggests that all the linear coefficients and the quadratic coefficient of the extraction time had a significant effect on the response ($p < 0.05$). However, no significant effects of the quadratic coefficients of temperature and ratio of solvent to material were observed, but all the interactive coefficients were observed in the response. The quadratic regression model was significant ($p < 0.01$), indicating that the model obtained in the present work fitted well with the experimental data of extraction yield. The value of R^2 is considered an important parameter to evaluate the model, and R^2 value very close to 1 indicates a good correlation. The regression coefficient (R^2 = 0.9927) obtained in the present work revealed a good

fit between the responses and independent variables. In addition, the lack of fit test for the response was not significant ($p > 0.05$), which indicates that the model fitted the experimental data.

Table 2. The results of ANOVA for response surface quadratic model.

Source	Sum of Squares	dF	Mean Square	F-value	p-value
Model	684.72	9	76.08	139.34	< 0.0001
X_1	453.61	1	453.61	830.76	< 0.0001
X_2	15.82	1	15.82	28.97	0.0010
X_3	206.55	1	206.55	378.29	< 0.0001
$X_1 X_2$	0.59	1	0.59	1.07	0.3350
$X_1 X_3$	2.06	1	2.06	3.77	0.0933
$X_2 X_3$	0.058	1	0.058	0.11	0.7548
X^2	4.98	1	4.98	9.13	0.0194
X^2	0.63	1	0.63	1.15	0.3199
X^2	0.095	1	0.095	0.17	0.6885
Residual	3.82	7	0.55		
Lack of Fit	0.66	3	0.22	0.28	0.8384
Pure Error	3.16	4	0.79		
Cor Total	688.85	16			

The coefficient of determination (R^2) of the model was 0.9944.

All yield data obtained in the present work were performed by multiple regressions to fit the second-order polynomial equation as follows:

$$Y=+31.81+7.53X_1+1.41X_2+5.08X_3+0.38X_1X_2-0.72X_1X_3-1.09X_2X_3-1.09X_1^2-0.39X_2^2-0.15X_3^2 \quad \text{(Eq. 4)}$$

As shown in Table 2, the ANOVA results indicated that there was a strong positive correlation ($R^2 = 0.9944$), which suggests that the model had a significant effect on the total flavonoid extraction yield ($p < 0.0001$). The lack of fit test examines the adequacy of the selected model describing the effect of the variables on the response. The *p*-value of 0.8384 demonstrated that the lack of fit was insignificant, thus implying that the model adequately described the data.

Three-dimensional response surfaces were plotted for the total flavonoid yield results presented in Figure 1, which shows the interactions between the different variables. As clearly observed from Figure 1A, at 30 mL/g solvent to material ratio, the extraction yield of total flavonoids increased with extraction time and ultrasound temperature. High temperature can increase the solubility and diffusivity of flavonoids. As a result, total flavonoids can be easily extracted into the solvent. The extraction yield increased with extraction time because the new extraction solvent is beneficial for maintaining the concentration difference between the inside and outside of the cell. The effect of extraction time and ratio of solvent to material is shown in Figure 1B, which demonstrates that the maximum extraction yield was obtained when the number of extraction times and ratio of solvent to material were 3 and 30 mL/g, respectively. The extraction yield increased with the increase in the ratio of solvent to material from 10:1 to 30:1. This is understandable because an increase in the extraction solvent volume could decrease the difference in solution concentration from the inside and outside of the plant cells, which consequently accelerates diffusion of the target compounds and causes more flavonoids to transfer into the solution. It is evident that the total flavonoid yield increased and reached a maximum value with an increase in the ratio of solvent to material and ultrasound temperature with two extraction times (Figure 1C).

The optimal extraction parameters obtained in the present work were as follows: number of extraction times of 3, extraction temperature of 60°C, and solvent-material ratio of 30:1 mL/g. The conditions were validated using three parallel experiments whose values were consistent with the predicted results. In fact, with the optimal variables, the predicted value of total flavonoids was 43.74 mg/g, which was close to the measured value of 42.62 ± 1.38 mg/g. Moreover, UPAE was more effective in terms of total flavonoid yield as compared to conventional reflux extraction (35.26 ± 1.82 mg/g).

SEM of the reflux extraction and UPAE methods

In order to evaluate the impact of ultrasound treatment, the microstructure of the sample residues of reflux extraction and UPAE are compared in Figure 2. It is notable that a small amount of damage was observed on the surface of the conventional reflux extraction sample (Figure 2A). Thus, the extraction yield of total flavonoids was lower using only the conventional reflux method. After applying ultrasonic probe treatment, many open pores could be seen on the sample surface, as shown in Figure 2B, which allowed flavonoid release and enhanced the mass transfer of the target compounds from inside the cells to the outside. This was due to the ultrasonic wave-generated acoustic cavitation that caused disruption of the cell wall (Mason *et al.,* 1996).

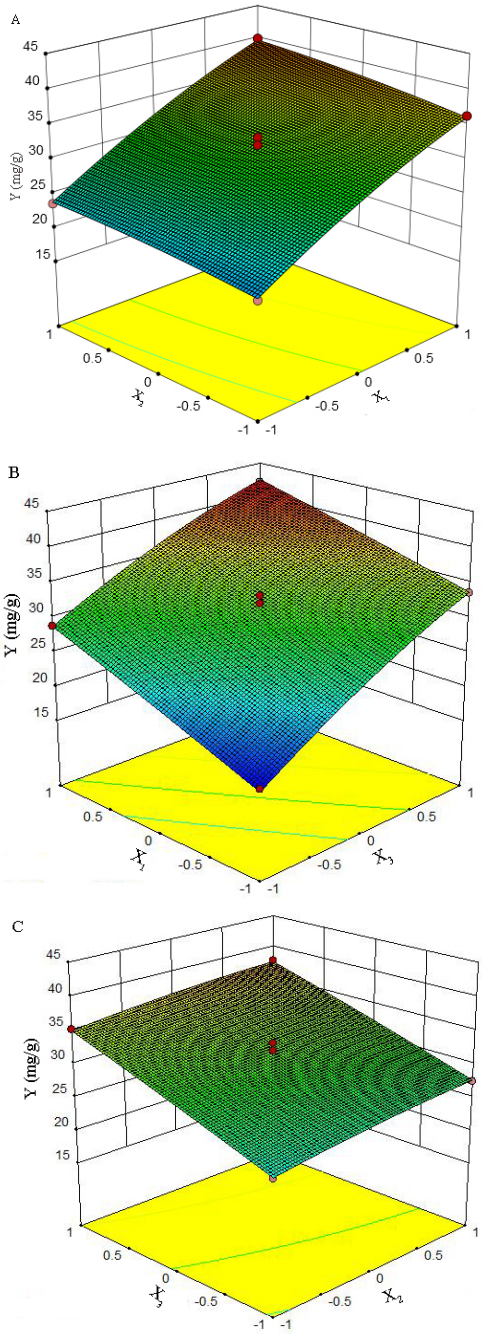

Figure 1. Response surface and contour plots for the effect of independent variables on extraction of the total flavonoids: (A) extraction times and temperature, (B) extraction times and solvent-material ratio, and (C) extraction temperature and solvent-material ratio.

DPPH radical scavenging activity

DPPH radical scavenging activity has been widely used to evaluate the antioxidant capacity of flavonoids from plant material *in vitro* (Lim *et al.,* 2019). The results of the DPPH assay are presented in Figure 3A. In Figure 3A, the flavonoid extract possessed notable scavenging ability and was concentration-dependent at concentrations less than 2.5 mg/mL. Vitamin C exhibited excellent antioxidant capacity. This result showed that the flavonoid

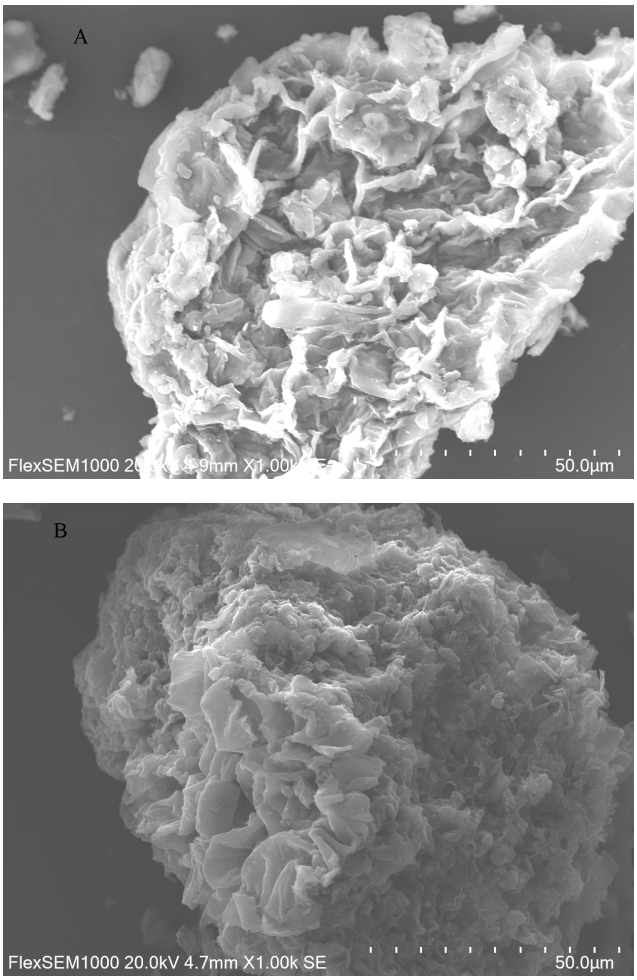

Figure 2. SEM of sample residue of conventional reflux extraction (A), and UPAE (B).

extract was effective in scavenging free DPPH radicals, but the extract was inferior to vitamin C.

$ABTS^{+}$ radical scavenging activity

The $ABTS^{+}$ assay has been extensively applied to evaluate the total antioxidant capacity of test samples (Loarca-Piña *et al.,* 2019). Figure 3B shows that the flavonoid extract was very effective in scavenging ABTS+ radicals, and the increase was concentration-dependent. At 0.25 mg/mL extract, the percent inhibition was 83.08%. However, the ABTS+ radical scavenging activity of the extract was weaker than that of vitamin C at the same concentration.

Superoxide anion scavenging capacity

Pyrogallol autoxidation is a reliable and inexpensive superoxide-scavenging assay method for antioxidants. In Figure 3C, the flavonoid extract possessed a strong and concentration-dependent superoxide anion scavenging capacity, which

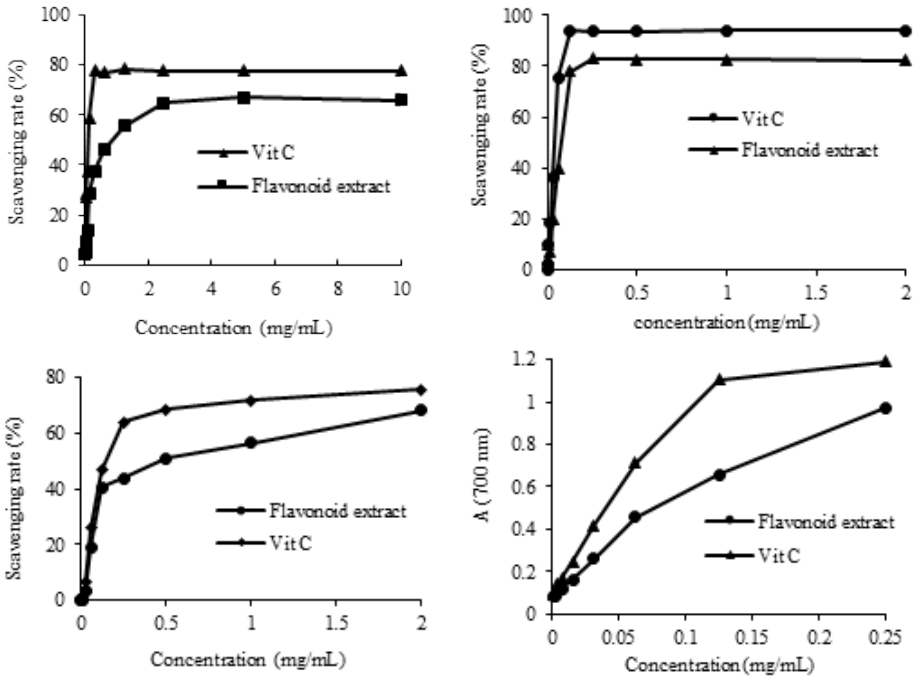

Figure 3. (A) DPPH radical scavenging activities of flavonoid extract of EH and vitamin C, (B) ABTS radical scavenging activities of flavonoid extract of EH and vitamin C, (C) superoxide anion scavenging activities of flavonoid extract of EH and vitamin C, and (D) reducing power of flavonoid extract of EH and Vitamin C.

suggested that the flavonoid extract could be used to scavenge superoxide anions. However, the superoxide scavenging capacity of the flavonoid extract was weaker than that of vitamin C at the same concentration.

Reducing power assay

The reducing power of the flavonoid extract was found to increase with increasing concentration. As shown in Figure 3D, a higher absorbance value presents a stronger reducing power of the samples. Clearly, the flavonoid extract indicated concentration-dependent reducing power. However, the reducing power of vitamin C was stronger than that of the sample.

The analysis of flavonoids

To clarify the material basis of antioxidant properties, further compound identification of the total flavonoids was carried out using HPLC. Four flavonoids, isoquercitrin, luteoloside, buddleoside, and apigenin-7-O-β-D-glucoside, were identified by comparing their retention times with those of standard substances. The contents of the four flavonoids were 0.82, 0.61, 1.86, and 0.55 mg/g, respectively. When compared with the total flavonoid content, the quantities of the four components were lower. This is because there are other compounds that need to be characterised. However, the other flavonoids could not be detected by HPLC at 350 nm. Further investigation is thus needed to identify other flavonoid profiles using liquid chromatography mass spectrometry technology.

Conclusion

In the present work, an ultrasonic probe system was successfully used to extract total flavonoids from EH with BBD. The optimal parameters were obtained using a second-order polynomial mathematical model. The conditions were validated, and the experimental value was 42.62 ± 1.38, which was very close to the predicted value of 43.74. In comparison with conventional reflux extraction, the total flavonoid yield was higher after UPAE. The SEM image of the residue was obtained. The ultrasound probe system disrupted the sample cells, thereby promoting release of the compounds inside the cells. The flavonoid extract exhibited notable antioxidant activity, and can be used as a potential antioxidant additive in the food industry. Qualitive and quantitative analyses of four compounds were investigated. Future studies on the identification of other compounds from the total flavonoid contents are in progress.

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