

Research Article

Optimized Ultrasonic-assisted Extraction of Flavonoids from *Pyracantha fortuneana* Fruit and Evaluation of Antibacterial Activities

¹Yun Zhang, ^{1,2}Wei Wu, ¹Xin Yu, ¹Anju Xiao and ¹Jing Lin

¹School of Medicine, Hubei Polytechnic University,

²Hubei Key Laboratory of Kidney Disease Pathogenesis and Intervention, Huangshi CN-435003, P.R. China

Abstract: Ultrasonic-Assisted Extraction (UAE) method was developed for extraction of total flavonoids from *Pyracantha fortuneana* fruit. The UAE parameters and the antioxidant activities of flavonoids extract were investigated. Important variables and their levels were obtained using single factor analysis method and central composite design. Through Response Surface Methodology (RSM) design experiments, the processing conditions were optimized as follows: ethanol concentration, 81.15%; liquid-solid ratio, 30.00 mL/g; extraction time, 3.14 h; and temperature, 69.55°C. Under the optimum condition, the extraction yield was 1.261%, which was well matched with the predicted values of 1.263%. The antibacterial activities of extract against four bacterial strains were determined by agar well diffusion method and the results indicated that the extract showed potent antibacterial activities, which concludes its application as natural antibacterial agent.

Keywords: Antibacterial activities, central composite design, flavonoids, *Pyracantha fortuneana*, response surface methodology

INTRODUCTION

Pyracantha fortuneana is an Asian species of firethorn of family Rosaceae, locally known as Huo-ji. It is abundantly distributed in China and Japan, where it is used as a traditional Chinese medicine for treatment of indigestion (Deng *et al.*, 1990). Pharmacologic studies and clinical practice have demonstrated that the methanol or water extract of *P. fortuneana* fruits exhibits many of the activities including tyrosinase inhibition (Van Gelder *et al.*, 1997), antioxidant activities (Hou *et al.*, 2003, 2002), antibacterial activity (Turker *et al.*, 2012). Since *P. fortuneana* has a promising application in pharmaceutical, cosmetic and nutraceutical food industries, it is important to develop research on extraction procedures for the extraction of these health-promoting compounds from *P. fortuneana*.

Traditional extraction techniques, including heating, boiling, refluxing and soxhlet extraction, have been used to extract flavonoids from vegetal materials, however, the disadvantages are the loss of flavonoids owing to hydrolysis, oxidation and ionization during extraction as well as the long extraction time (Zhang *et al.*, 2011; Bimkr *et al.*, 2011; Grigonis *et al.*, 2005). Recently, much wider attention has been given to applications of Ultrasound-Assisted Extraction (UAE), which can more easily be scaled up for commercial production (Ma *et al.*, 2012). The UAE not only lead to

a decrease in solvent consumption and a shorter extraction time, but also support an enhancement in extraction efficiency owing to the effects of acoustic cavitations produced in the solvent by the passage of an ultrasonic wave.

Response Surface Methodology (RSM) is a very useful statistical tool, which was first introduced by Box and Wilson (1951). It uses sequential experimental techniques to evaluate the effects of multiple factors and their interaction on one or more response variables (Myers and Montgomery, 2002) and build an empirical model. It has been reported that RSM can be used to optimize the total flavonoid compound from many medicinal plants (Liu *et al.*, 2010). The objective of the work is to establish the optimal condition of UAE of flavonoids from *P. fortuneana* for the development and application of the resource. In this study, UAE of flavonoids from *P. fortuneana* was investigated and the operational parameters were optimized using central composite design with RSM.

MATERIALS AND METHODS

Materials: *P. fortuneana* fruits were provided by Jointown Pharmaceutical Group (Wuhan, China). The dried *P. fortuneana* fruits were milled to about 250 µm and stored at a desiccator at room temperature (20±3°C)

Corresponding Author: Wei Wu, School of Medicine, Hubei Polytechnic University, Huangshi CN-435003, P.R. China, Tel./Fax: +86 715 6348622

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until used. Rutin was purchased from Sigma Chemicals Co. All other chemical reagents used in experiments were of analytical grade and purchased by Sinopharm Chemical Reagent Co., Ltd. The water used in the experiments was double distilled.

Ultrasound-assisted extraction of flavonoids: In this study, ultrasound-assisted extraction was performed in an ultrasonic device.

For the routine extraction of flavonoids, the powder (1 g) of *P. fortuneana* fruits was placed into a 100-mL flask, soaked with ethanol solvent at different concentrations (varying from 70 to 95%) with a ratio of solid-liquid (g/mL) (ranging from 1:10 to 1:35) for 30 min, then placed in the temperature-controlled ultrasonic device (SY360, Ninson Ultrasonic Instrument Company, Shanghai, China) at 40 kHz for a given time (extraction time ranging from 1.5 to 4.0 h), while the temperature of the water bath ranged from 55 to 80°C. The extract was filtered and the filtrate was collected for the determination of the flavonoids.

Experimental design: In this study, the extraction parameters were optimized using Response Surface Methodology (RSM). A central composite design was employed in this optimization study based on the results of the single factor experimental data. Four independent variables, ethanol concentration (X_1), solid-liquid ratio (X_2), extraction time (X_3) and temperature (X_4) were the independent variables selected to investigate the effects on the Yield of flavonoids (Y). The independent variables were coded at five levels (-2, -1, 0, 1 and 2) and the complete design consisted of 27 experimental points including six replications of the centre points. In detail, ethanol concentration (75.0, 75.5, 80.0, 82.5 and 85.0%, respectively), liquid-solid ratio (25.0, 27.5, 30.0, 32.5 and 35.0 mL/g, respectively), extraction time (2.50, 2.75, 3.00, 3.25 and 3.50 h, respectively) and temperature (65.0, 67.5, 70.0, 72.5 and 75.0°C, respectively) were investigated. The Design Expert software (Version 8.0, Stat-Ease, Inc., Minneapolis, MN, USA) was used to analyze the experimental design data and calculate predicted responses.

Determination of total flavonoids content: The total flavonoids content was determined using a colorimetric method (Li *et al.*, 2011). Briefly, 1.0 mL diluted sample was transferred to 25-mL volumetric flask and then 3 mL 0.1 mol/L Aluminium Chloride ($AlCl_3$) solution was added. The final volume was adjusted to 25 mL with 95% (v/v) ethanol. The mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 410 nm against a blank solution. The amount of flavonoids was expressed as rutin equivalents through the calibration curve of rutin, which presented a linear response within 10-50 $\mu\text{g/mL}$. The regression equation was $y = 45.605x - 0.9189$, $R^2 = 0.9995$.

Antimicrobial assay: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumonia* were chosen as test microorganisms. The flavonoid extract was dissolved in sterile water and diluted to different concentrations (15, 30, 45 and 60 mg/mL, respectively). Gentamicin (10 $\mu\text{g/mL}$) was used as positive control. The antimicrobial assay was carried out in agreement with the method of Deans and Ritchie (1987). Briefly, the test bacterial strains were incubated overnight at 25°C (10^5 mL^{-1} final concentration). One mL amounts of each culture were pipetted into separate sterile Petri dishes to which 20 mL amounts of molten Iso-Sensitest agar (45°C) were added. Next, wells of 4 mm diameter were made in the centre of each agar plate and then 15 mL test extracts were added. The plates were incubated inverted in dark at 25°C for 48 h and zones of growth inhibition were measured by Vernier calipers.

RESULTS AND DISCUSSION

Single factor analysis method: To determine the single factor experiment condition, the effect of four main factors including ethanol concentration, liquid-solid ratio, extraction time and temperature were studied.

Extraction of flavonoids from *P. fortuneana* fruits was performed at different ethanol concentrations (70, 75, 80, 85, 90 and 95%, respectively). The processes were carried out at 65°C for 2.5 h at the solid-liquid ratio of 1:25 and the results were shown in Fig. 1a. It can be seen from Fig. 1a that extraction yields started to increase with increasing ethanol solution concentrations and reached a maximum at the ethanol solution concentration of 80%, followed by a decrease with further increase in ethanol concentration. Therefore, the optimal ethanol concentration of flavonoids from *P. fortuneana* fruits was 75~85%.

The effects of the solid-liquid ratios from 1:10 to 1:35 on the extraction yield were investigated. The processes were carried out at 65°C for 2.5 h at ethanol concentration of 80%. As depicted in Fig. 1b, the effect of various liquid-solid ratios on the extraction yields of flavonoids were prominent. When the solid-liquid ratio increased from 1:10 to 1:30, the extraction yield of flavonoids was improved rapidly and then decreased. Therefore, solid-liquid ratio of 1:30 was used in the subsequent experiments.

In order to determine the extraction kinetics, powdered samples were used for the reflux extraction with extraction times ranging from 1.5 to 4.0 h and the processes were carried out at 65°C, ethanol concentration of 80% and solid-liquid ratio of 1:30. As shown in Fig. 1c, the extract yield increased with the increase of extraction time from 1.5 to 3.0 h and

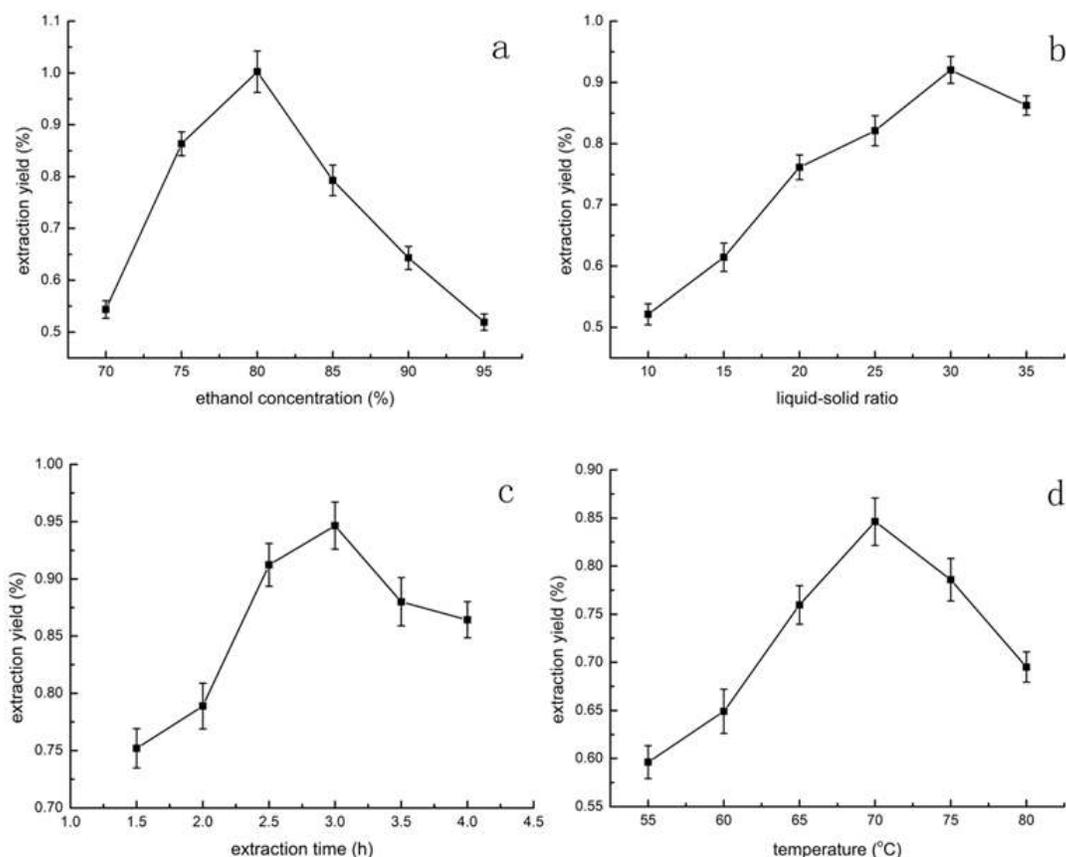


Fig. 1: Effect of ethanol concentration (a) liquid-solid ratio, (b) extraction time, (c) and temperature, (d) on the extraction yield of flavonoids from *P. fortuneana* fruits

Table 1: Factors and levels in the response surface central composite design arrangement and experimental results and results

Run	X ₁	X ₂	X ₃	X ₄	Extraction yield (%)
1	-1	-1	-1	1	1.169
2	0	0	2	0	1.223
3	-2	0	0	0	1.120
4	1	-1	-1	1	1.180
5	-1	-1	1	-1	1.173
6	1	-1	-1	-1	1.190
7	0	0	0	0	1.259
8	0	0	0	2	1.206
9	-1	1	1	-1	1.175
10	0	0	0	0	1.262
11	1	-1	1	-1	1.209
12	1	1	-1	1	1.174
13	0	2	0	0	1.175
14	0	0	-2	0	1.189
15	1	1	1	1	1.195
16	1	-1	1	1	1.193
17	2	0	0	0	1.174
18	0	0	0	0	1.260
19	0	0	0	0	1.263
20	-1	-1	-1	-1	1.171
21	0	0	0	0	1.256
22	-1	1	-1	1	1.168
23	0	-2	0	0	1.170
24	1	1	-1	-1	1.184
25	1	1	1	-1	1.208
26	0	0	0	0	1.264
27	-1	1	-1	-1	1.166

reached a peak at 3.0 h, then significantly decreased. An adequate extraction time not only leads to the dissolve of target materials, but also might cause loss of activities. Therefore, 2.5-3.0 h was chosen for the further optimization of extraction conditions.

Powered of *P. fortuneana* fruits were used for the reflux extraction with 80% ethanol concentration and the processes were carried out respectively at 55, 60, 65, 70, 75 and 80°C, respectively for 3.0 h, with solid-liquid ratio of 1:30. It can be seen from Fig. 1d that the extraction yields started to increase with increasing temperature and reached a maximum at the 70°C, followed by a noticeable decrease with further increases in temperature. Thus, the optimal extraction temperature for flavonoids from *P. fortuneana* fruits was 65-75°C.

Optimization of flavonoids extraction: The operational parameters were optimized using central composite design combined with response surface methodology. And the four parameters, ethanol concentration, solid-liquid ratio, extraction time and temperature were chosen as independent variables for further optimization of the flavonoids extraction procedure. Experiments were randomized as detailed Table 1. By applying multiple regression analysis to the

Table 2: Analysis of Variance (ANOVA) for the regression equation

Source	S.S.	df	M.S.	F-value	p-value
Model	0.040	14	2.849E-003	193.56	<0.0001
X ₁	3.243E-003	1	3.243E-003	220.39	<0.0001
X ₂	4.167E-008	1	4.167E-008	2.831E-003	0.9583
X ₃	1.053E-003	1	1.053E-003	71.58	<0.0001
X ₄	1.654E-004	1	1.654E-004	11.24	0.0044
X ₁ X ₂	7.562E-006	1	7.562E-006	0.51	0.4845
X ₁ X ₃	2.481E-004	1	2.481E-004	16.86	0.0009
X ₁ X ₄	1.051E-004	1	1.051E-004	7.14	0.0174
X ₂ X ₃	3.906E-005	1	3.906E-005	2.65	0.1241
X ₂ X ₄	5.062E-006	1	5.062E-006	0.34	0.5663
X ₃ X ₄	1.806E-005	1	1.806E-005	1.23	0.2854
X ₁ ²	0.023	1	0.0230	1546.11	<0.0001
X ₂ ²	0.014	1	0.0140	937.43	<0.0001
X ₃ ²	5.416E-003	1	5.416E-003	368.02	<0.0001
X ₄ ²	5.131E-003	1	5.131E-003	348.64	<0.0001
Residual	2.208E-004	15	1.472E-005		
Lack of fit	1.774E-004	10	1.774E-005	2.05	0.2221
S.D.	3.836E-003	R ²	0.9945		
Mean	1.200	Adjusted R ²	0.9894		
C.V. %	0.320	Predicted R ²	0.9730		
PRESS	1.084E-003	Adequate precision	51.0420		

S.S.: Sum of square; M.S.: Mean square

experimental data, the response variable and the test variables are related by the following second-order polynomial stepwise equation:

$$Y = 1.26 + 0.012X_1 - 4.167E-005X_2 + 6.625E-003X_3 - 2.625E-003X_4 - 6.875E - 004X_1X_2 + 3.938E - 003X_1X_3 - 2.562E-003X_1X_4 + 1.563E-003X_2X_3 + 5.625E-004X_2X_4 - 1.062E-003X_3X_4 - 0.029X_1^2 - 0.022X_2^2 - 0.014X_3^2 - 0.014X_4^2$$

ANOVA was used to analyze the model for significance and suitability and a statistical summary was given in Table 2. The model *F* value of 193.56 with a low probability *p*-value shows high significance of the model. R² value for the model was 0.9945 which indicated that 99.45% of variation observed in the extraction yield of flavonoids could be attributed to ethanol concentration, solid-liquid ratio, extraction time and temperature among these variables. A signal to noise ratio of 51.042 as estimated by adequate precision measure indicated an adequate signal for the model. The predicted R² value of 0.9730 was in reasonable agreement with the adjusted R² value of 0.9894, which indicated a good agreement between the experimental and predicted values of extraction yield. The linear variables X₁ and X₂, the quadratic variables X₁², X₂², X₃² and X₄² were statistically very significant at *p*<0.0001; the linear variable X₄ and the two-variable interaction X₁X₃ and X₁X₄ had significant influences (*p*<0.05) on the extraction yield of flavonoids, whereas the linear variable X₃ and the two-variable interaction X₁X₂, X₂X₃, X₂X₄ and X₃X₄ had no significant influence (*p*>0.1) on the extraction yield of flavonoids. The lack of fit *F* value of 2.05 showed that lack of fit was insignificant. A low value of Coefficient of the Variance (C.V.%) (0.32) clearly indicated a high degree of precision and reliability of the experimental values.

Three-Dimensional (3D) response surfaces were the graphical representations of regression function.

The Three Dimensional (3D) response surface curves (Fig. 2) show the relative effect of any two variables, while the other two variables are maintained constant. It can be seen from Fig. 2 that the response surface indicated that the extraction yield depended largely upon the ethanol concentration, extraction time and temperature, while no significant effect was observed in the solid-liquid ratio. This was in perfect accordance with the results of the ANOVA.

The optimal values of the selected three variables for maximizing extraction yield of flavonoids were determined by solving the model equation using the numerical optimization function in Design-Expert software. The model predicted a maximal extraction yield of 1.263%, which was under the optimal extraction conditions: ethanol concentration, 81.15%; liquid-solid ratio, 30.00 mL/g; extraction time, 3.14 h; and temperature, 69.55°C. To confirm these results, tests were performed in triplicate under the above optimized conditions and the extraction yield was 1.261%. This implied that there was a high fit degree between the value observed in experiment and the value predicted from the regression model. Therefore, the response surface modeling could be applied effectively to predict the extraction condition.

Antibacterial activity of flavonoids from *P. fortuneana*: The antibacterial activities of flavonoids from *P. fortuneana* presented in Table 3 and 4 are in general agreement with previously reported studies on Flavonoid rich plant extracts from species of *Hypericum* (Dall'Agnol *et al.*, 2003), *Capsella* (El-Abyad *et al.*, 1990) and *Chromolaena* (Aladesanmi *et al.*, 1986). All the bacterial strains demonstrated some degree of sensitivity to flavonoids extracts tested at different concentrations, but in dose dependent and showed maximum zone of inhibition at a dose of 60 mg/mL. As shown in Table 3, the extract exhibited

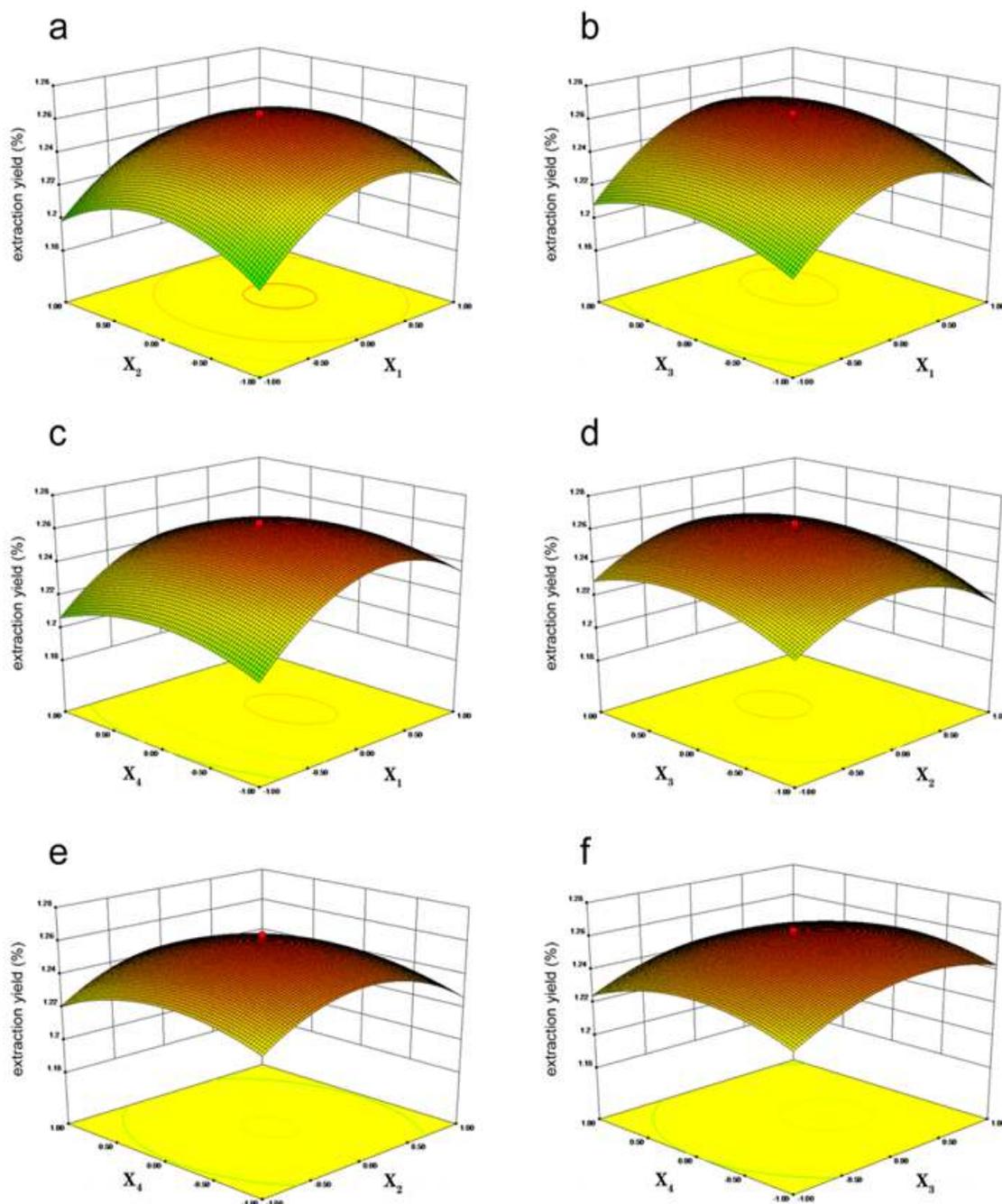


Fig. 2: Response surface plots showing effects of the extraction parameters on extraction yield of flavonoids, (a) at varying ethanol concentration (X_1) and liquid-solid ratio (X_2), (b) at varying ethanol concentration (X_1) and extraction time (X_3), (c) at varying ethanol concentration (X_1) and temperature (X_4), (d) at varying liquid to solid ratio (X_2) and extraction time (X_3), (e) at varying liquid-solid ratio (X_2) and temperature (X_4), (f) at varying extraction time (X_3) and temperature (X_4)

Table 3: Zones of growth inhibition (mm) showing antibacterial activity for flavonoids from *P. fortuneana*

Extract concentration (mg/mL)	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumonia</i>
15	12.4±0.85	11.3±0.59	7.8±0.65	9.10±0.35
30	16.8±0.71	14.5±0.61	9.0±0.73	11.30±0.60
45	19.1±0.76	17.1±0.96	12.3±0.52	13.75±0.77
60	21.3±1.00	19.9±0.27	15.8±0.98	16.30±0.88
Gentamicin (10 µg/mL)	27.7±1.10	25.1±1.04	20.8±0.89	21.50±0.92

Values for zone of growth inhibition are presented as mean±S.E.M.

Table 4: Minimum Inhibitory Concentration (MIC) of flavonoids from *P. fortuneana*

Bacteria	MIC of flavonoids Extract (mg/mL)	MIC of gentamicin (μ g/mL)
<i>Staphylococcus aureus</i>	3.0	0.9
<i>Bacillus subtilis</i>	4.0	2.2
<i>Escherichia coli</i>	3.0	1.3
<i>Klebsiella pneumonia</i>	6.0	3.6

maximum zone of inhibition against *Staphylococcus aureus* and minimum zone of inhibition against *Escherichia coli*. The results also showed that Gram-positive bacteria were more sensitive to the flavonoids extract than Gram-negative bacteria. The MIC of flavonoids extract and control (gentamicin) ranged between 3.0-6.0 mg/mL and 0.9-3.6 μ g/mL, respectively, which was shown in Table 4.

CONCLUSION

In this study, the ultrasonic-assistant extraction of flavonoids from *P. fortuneana* investigated with a central composite design based on response surface methodology in enhancing the flavonoids extraction yield. The optimum extraction parameters were obtained and the predicted yields of the flavonoids were well in agreement with the experimental ones. The antibacterial activities of the flavonoids were evaluated. Based on the results, it is revealed that the UAE is an effective method for *P. fortuneana* flavonoids extraction and the flavonoids exhibit antibacterial activities. As a functional compound in *P. fortuneana* fruits, perhaps this research will cause interest in utilizing flavonoids from *P. fortuneana* as an inexpensive source of health-promoting additives, e.g., natural preservatives. Thus, flavonoids from *P. fortuneana* have the potential to be developed into functional food ingredients and dietary supplements.

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