

ARTICLE

Optimization of Ultrasonic-assisted Extraction of Flavonoid from *Portulaca oleracea* L. by Response Surface Methodology and Chemical Composition Analysis

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Abstract Optimization of ultrasonic extraction of *Portulaca oleracea* L. flavonoids (POF) was investigated using single-factor experimentation combined with response surface methodology. The optimal conditions for the highest yield (16.25 mg RE/g DW) of POF was 39.01% ethanol, 55.25°C extraction temperature, 15 min extraction time, and 23.92 (v/m) liquid-to-solid ratio. The crude extract of POF was purified on the polyamide resin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole assays of the flavonoids were evaluated, which suggested the concentrations of the flavonoids (0–1 mg/mL) and quercetin (0–1 mg/mL). Quercetin was identified in the extract by comparing relative retention time of the reference standard.

Keywords cytotoxicity · flavonoids · portulaca oleracea L · response surface methodology · ultrasonic extraction

Introduction

Portulaca oleracea L., belonging to the family of the Portulacaceae, has been commonly used as an edible vegetable and folk medicine in many areas of the world. In China, the plant is also called “Chang-ming-cai” and has been used in traditional Chinese medicine for the treatment of various diseases including inflammatory, analgesics, diabetes, and cancer (Chan et al., 2000; Gong et al., 2009; Dong et al., 2010; Shen et al., 2013). These beneficial effects are presently believed to be linked to the fact that *Portulaca oleracea* L. is rich in phytochemicals. Recently, several bioactive compounds such as betacyanins, flavonoids,

polysaccharides, and alkaloids have been extracted, isolated, and identified from *Portulaca oleracea* L. (Gong et al., 2009; Yang et al., 2009; Dong et al., 2010; Wang and Yang, 2010; Zhu et al., 2010; Kokubun et al., 2012; Shen et al., 2013).

Extraction is a very important stage in the recovery of bioactive compounds from natural samples, where extraction procedures must be versatile, relatively simple, inexpensive, as well as able to both preserve and extract most of the bioactive compounds present in a plant matrix. The content of flavonoids is influenced by many factors, including the genus, the place of the plant growth, the extraction conditions, and technology (Mao et al., 2008; Liu et al., 2010). However, little information is available concerning the optimization of extraction of the flavonoids compounds in *Portulaca oleracea* L. Thus, the economic feasibility of industrial processing of *Portulaca oleracea* L. requires more investigation to optimize the extraction process to increase the yield of extracted active substances. In optimization study, single-factor experiments and response-surface methodology (RSM) are two most common experimental designs. It has been successfully demonstrated that RSM can be used to optimize the total flavonoids compound from many medicine plants. In the present study, single-factor experiments were used to provide data regarding extraction factors (ethanol concentration, extraction temperature, liquid-solid ratio, and extraction time) on the extraction yield of *Portulaca oleracea* L. flavonoids (POF). Subsequently, these factors were analyzed by RSM to attain more precisely determined optimal extraction conditions for maximum POF yield.

Ultrasonic-assisted extraction (UAE) has been used to extract functional components from different plants. This process is more rapid than traditional methods. UAE creates shear forces that break cell walls and facilitates the release of cellular constituents of plant material into the extraction solvent (Teng et al., 2010).

The cytotoxicities of flavonoids were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow

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tetrazole (MTT) Assays. The aim of our work was to provide scientific basis on the effects of extraction factors from *Portulaca oleracea* L., explore the concentrations of flavonoids, and offer scientific reference for utilization of the POF.

Materials and Methods

Materials and Chemicals. Wild *Portulaca oleracea* L. were collected in July of 2012 from a cultivation located in Nanchang, Jiangxi province, China, and identified by School of Medicine, Jiangxi Science & Technology Normal University. Fresh and intact *Portulaca oleracea* L. was selected, washed, and then dried using a hot air oven at 60°C. Dried *Portulaca oleracea* L. were ground in a traditional medicine disintegrator (HK-20B, Hangzhou Saixu Machine Company, China) to obtain a fine powder as experimental material. Auto Science DL-800B Ultrasonic Cleaner (China) was the solvent for extraction.

Standard rutin (95%) and quercetin (95%) were purchased from Sigma Chemical Company (USA). NaNO₂, Al(NO₃)₃, NaOH, ethanol, and all other chemicals used were of analytical grade and obtained from Guoyao Pure Chemical Industries (China).

The mouse β -cell line HIT-T15 was provided by Sichuan University. In brief, cells were maintained in 1640 medium (GIBCO, USA) supplemented with 15% fetal bovine serum (Sijiqing, China), 100 units/mL penicillin, and 50 μ g/mL streptomycin and incubated at 37°C in a 5% CO₂/95% air, humidified incubator. (Thermo Forma Series, USA)

Solvent extraction of flavonoids. The single factors for solvent extraction procedures were set as follows: dried powder of *Portulaca oleracea* L. (0.5 g) was placed in a 50-mL round bottomed flask. Firstly, the influence of the concentrations of extract solvent on POF yield was studied. Ten milliliters of different concentrations (30–100%) of ethanol were added, and the extraction was performed for 20 min at 60°C in the ultrasonic cleaning bath. Secondly, the impact of the liquid-to-solid ratio on POF yield in the range of 10 to 40 v/m was studied. Different volumes of 40% ethanol (5–20 mL) were added, and the extraction was performed for 20 min at 60°C in the ultrasonic cleaning bath. Thirdly, the effect of extraction temperature was investigated in the range from 30 to 80°C. Ten milliliters of 40% ethanol was added, and the extraction performed for 20 min at different temperatures (30–80°C) in the ultrasonic cleaning bath. Lastly, the effect of extraction time on the extraction was investigated. Ten milliliters of 40% ethanol was added, and extraction performed was at different times (5–50 min) at 50°C in the ultrasonic cleaning bath. All extracts were centrifuged at 4000 rpm for 10 min. The supernatant was collected for the determination of POF content.

RSM experimental design. The extraction parameters of flavonoid compounds from *Portulaca oleracea* L. were optimized using RSM. The Box-Behnken was used to investigate the effects of four independent variables (the extraction variables) on the

Table 1 Coded and actual levels of three variables in Box-Behnken design

Factor	Levels used, actual (coded)		
	Low (-1)	Medium (0)	High (+1)
X ₁ : Ethanol concentration (%)	30	40	50
X ₂ : liquid-to-solid ratio (v:m)	15	20	25
X ₃ : extraction temperature (°C)	40	50	60
X ₄ : extraction time (min)	5	15	25

Table 2 Experimental design using Box-Behnken and corresponding results

Run No.	X ₁	X ₂	X ₃	X ₄	Actual Value/ %	Predicted Value/%
1	-1	-1	0	0	10.72	10.50
2	1	-1	0	0	12.62	12.61
3	-1	1	0	0	13.53	13.54
4	1	1	0	0	13.07	13.29
5	0	0	-1	-1	14.58	14.68
6	0	0	1	-1	14.63	14.50
7	0	0	-1	1	13.10	13.23
8	0	0	1	1	14.37	14.26
9	-1	0	0	-1	11.41	11.53
10	1	0	0	-1	14.95	14.87
11	-1	0	0	1	13.10	13.09
12	1	0	0	1	11.83	11.61
13	0	-1	-1	0	13.94	13.71
14	0	1	-1	0	13.73	13.61
15	0	-1	1	0	12.15	12.18
16	0	1	1	0	15.85	15.99
17	-1	0	-1	0	10.77	10.84
18	1	0	-1	0	13.52	13.59
19	-1	0	1	0	13.05	13.08
20	1	0	1	0	12.15	12.18
21	0	-1	0	-1	13.73	13.90
22	0	1	0	-1	15.58	15.40
23	0	-1	0	1	12.41	12.69
24	0	1	0	1	14.99	14.92
25	0	0	0	0	15.85	15.85
26	0	0	0	0	15.79	15.85
27	0	0	0	0	15.95	15.85
28	0	0	0	0	15.69	15.85
29	0	0	0	0	15.95	15.85

response variables (Y_n) of the crude extracts (Table 1). A three-level, four-variable central composite rotatable design was developed to determine the best combinations of extraction conditions for POF. Ethanol concentration (X₁), liquid-to-solid ratio (X₂), extraction temperature (X₃), and extraction time (X₄) were chosen as independent variables.

The experimental design consists of 29 factorial experiments and three replicates of the central point. A total of 29 runs were performed to optimize the process parameters, namely ethanol concentration, liquid-solid ratio, extraction temperature, and

extraction time. The level and code of variables considered in the present study are shown in Table 2. Experimental runs were randomized, to minimize the effects of unexpected variability in the observed responses.

The software Design-Expert 8.0 was used for the experimental design, data analysis, quadratic model buildings, and graph plotting. The responses function (polysaccharides yield Y) was partitioned into linear, quadratic and interactive components as follows:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i>j}^k \beta_{ij} X_i X_j$$

where β_0 is defined as the constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, and β_{ij} the cross-product coefficient, X_i and X_j are the levels of the independent variables whereas k denotes the number of the tested factors ($k=4$). The analysis of variance (ANOVA) tables were generated, and the effect and regression coefficients of individual linear, quadratic, and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value at a probability (p) of 0.001, 0.01, and 0.05. The regression coefficients were then used to determine statistical calculations to generate contour maps from the regression models (Bashi et al., 2012).

Determination of POF content. Total POF contents in the extracts were determined by using the colorimetric method with some modifications (Zhu et al., 2009). The reaction mixture contained 0.5 mL of extract, 4 mL of 40% ethanol, and 0.3 mL of 5% NaNO_2 . After 5 min, 0.3 mL of 10% $\text{Al}(\text{NO}_3)_3$ was added. After 6 min, 4 mL of 4% NaOH solution were added. The mixed solution was then immediately diluted to volume of 10 mL with 40% ethanol, mixed thoroughly, and left standing at room temperature for 15 min. The absorbance of the reaction mixture was measured at 510 nm by a UV spectrophotometer (Lambda 35 PerkinElmer, USA). Total POF contents were calculated by calibration curve using rutin as a standard. $Y = 7.57x - 0.0419$ ($R^2 = 0.9986$). POF content was expressed as rutin equivalent (RE), in mg RE/g dry weight (DW).

Purification of Flavonoids from *Portulaca oleracea* L. by Polyamide Resin. The extract of flavonoids from *Portulaca oleracea* L. obtained under the optimized condition was purified using a column packed with polyamide resin. The 95% (v/v) ethanol was used for desorption solvent (Wang et al., 2012). The 70% ethanol component of flavonoids was collected. The component was lyophilized and the resulting dry powder was stored at 4°C. **High performance liquid chromatography (HPLC) Analysis of Flavonoids from *Portulaca oleracea* L.** HPLC analysis were carried out on a Agilent 1100 Series using a VWD detector and a discovery C18 reversed-phase column (5.0 μm , 250 mm \times 4.6 mm). The flow rate was 1.0 mL/min and the column was operated at 30°C. The mobile phase consisted of a gradient elution of acetonitrile (solvent A) and 0.2% acetic acid (solvent B). The gradient program was: 20% of A, 80% of B. The injected volume

was 10 μL and detection wavelength was 360 nm.

In Vitro Cytotoxicity Activity. The cytotoxicity *in vitro* was measured using the MTT Assay (Chen et al., 2004; Jana et al., 2012) on the HIT-T15. The cytotoxic effect of each treatment was expressed as percentage of cell viability relative to the untreated control cells. The cells were diluted to 100,000 cells per mL using complete media to dilute cells. One hundred microliters of cells (10,000 total cells) were added into a 96-well plate and incubate overnight. Subsequently, cells were treated with flavonoids or quercetin, followed by addition of 20 μL of 5 mg/mL MTT to each well and incubated for 4 h at 37°C in a culture hood. DMSO (150 μL) solvent then was added, the wells were covered with tinfoil, and cells were agitated on orbital shaker for 10 min. The absorbance was read at 590 nm.

Results and Discussion

The results of a single factor for solvent extraction of flavonoids. The concentration of extraction solvent influences the efficiency of flavonoids: Generally lower concentration of ethanol is suitable for extraction of polar flavonoids compounds and higher concentration ethanol is suitable for extraction of non-polar flavonoids compounds. The effect of the concentration of ethanol on extraction yield of POF is shown in Fig. 1. Extraction yield of POF was greatly influenced by ethanol concentration. The POF significantly increased from 15.12 to 15.83 mg RE/g dry weight (DW) when the concentration of ethanol increased from 30 to 40%. POF recovery was parabolic with a maximum value at 40% ethanol. However, as the concentration of ethanol continued to increase up to 100%, the POF decreased to 9.44 mg RE/g DW. The reason may be because the flavonoid glycosides were the major constituents of the plant, higher concentration of ethanol was adverse to extract the flavonoid glycosides. Thus, 40% ethanol was used for subsequent RSM to optimize extraction conditions.

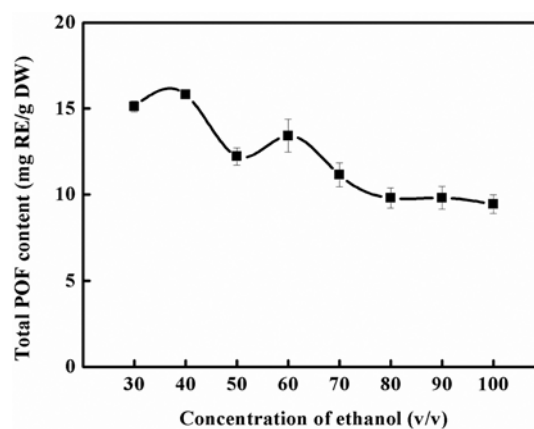


Fig. 1 Effect of ethanol concentration on POF yield. Other extract conditions were 20:1 liquid-to-solid ratio, 60°C extraction temperature, and 20 min extraction time.

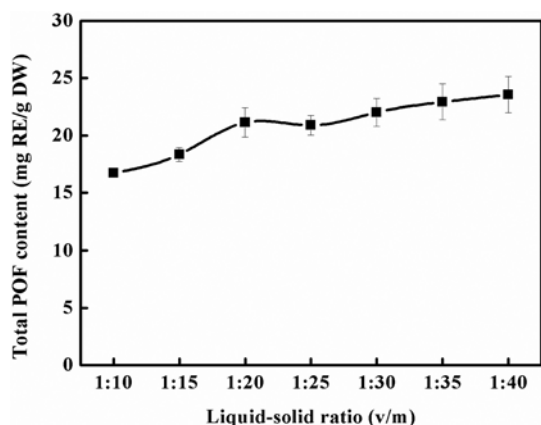


Fig. 2 Effect of liquid-to-solid ratio on POF yield. Other extract conditions were 40% ethanol, 60°C extraction temperature, and 20 min extraction time.

The effect of liquid-to-solid ratio on the extraction yield of POF is shown in Fig. 2. Extraction was carried out at different liquid-to-solid ratios (10–40, v/w), whereas other extraction parameters were constant (40% ethanol, 60°C extraction temperature and 20 min extraction time). The extraction yields of POF significantly increased from 14.22 to 16.37 mg RE/g DW as the liquid-to-solid ratio increased within the range of 10–20 (v/m). However, the extraction yields did not significantly change when the liquid-to-solid ratio continued to increase.

Increased temperature led to increases of cavitation number, surface contact area, and decreases of solvent media viscosity and density. These factors favored the release of bioactive compounds from plant material and plant cell decomposition, enhancing solubility and diffusion coefficients. Extraction was carried out at different extraction temperatures (30–80°C), whereas other extraction parameters were constant (40% ethanol, 20:1 liquid-to-solid ratio, and 20 min extraction time). The effect of extraction temperature on the extraction yield of POF is shown in Fig. 3. The POF extraction yields significantly increased from 11.71 to 15.18 mg RE/g DW as the extraction temperature increased from 30 to 50°C. The POF recovery was maximized at 50°C and then declined moderately with further increases in temperature.

Extraction time is a factor that significantly influences the extraction efficiency of flavonoids from medicine plants or edible vegetables. The effect of different times on extraction yield of POF is shown in Fig. 4. Extraction was carried out at different times (5–50 min), whereas other extraction parameters were kept constant (40% ethanol, 20:1 liquid-to-solid ratio, and 50°C extraction temperature). When extraction time varied from 5 to 10 min, the yield of POF increased from 15.00 to 15.74 mg RE/g DW. When time was increased to 50 min, the yield of POF was decreased to 14.15 mg RE/g DW. It may be that the increase in extraction time led to the degradation of flavonoid compounds, which indicated that 10 min was sufficient to obtain the POF from *Portulaca oleracea* L. Thus, 10 min was determined as the

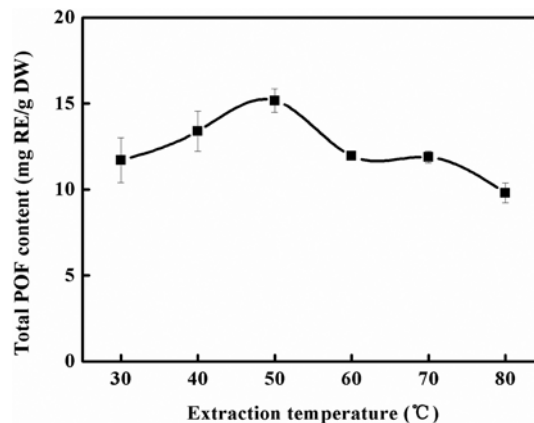


Fig. 3 Effect of extraction temperature on POF yield. Other extract conditions were 40% ethanol, 20:1 liquid-to-solid ratio, and 20 min extraction time.

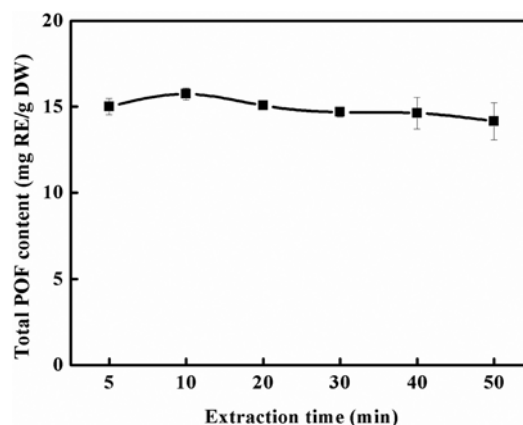


Fig. 4 Effect of extraction time on POF yield. Other extraction conditions were 40% ethanol, 20:1 liquid-to-solid ratio and 50°C extraction temperature.

favorable extraction time.

Optimization of the extraction Yield of POF by RSM. Total POF extracted from *Portulaca oleracea* L. was further optimized through the RSM approach. Ethanol concentration of 40% (v/v), extraction time of 10 min, liquid-to-solid ratio of 20, and extraction temperature of 50°C were chosen from previous single-factor experiments. The response values of POF of extracts obtained under various experimental conditions are shown in Table 2. Twenty-nine trials were performed to determine optimum conditions for extraction of POF. The experiments were carried out in random order as required in many design procedures. In the experimental design, the optimum condition evaluated was the operating conditions for maximizing percent extraction yield of POF. Table 2 shows the treatments with coded levels and the experimental results of POF. The POF yield ranged from 10.72 to 15.95 mg RE/g DW. The maximum yield of POF was recorded under the experimental conditions of $X_1=40\%$, $X_2=20$ (v/m) $X_3=50^\circ\text{C}$, and $X_4=15$ min. Maximum recovery of POF (15.95 mg

Table 3 Analysis of variance(ANOVA) for the regression model

Source	Sum of Squares	df	Mean Square	F-value	P-value	significance
model	71.39	14	5.10	137.20	<0.0001	***
Residual	0.52	14	0.037			
Lack of Fit	0.47	10	0.047	3.74	0.1076	
Pure Error	0.050	4	0.013			
Cor Total	71.91	28				

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4 Test results of significance for regression coefficient

Factor	Coefficient Estimate	df	Standard Error	95%CI Low High		P value	
	15.85	1	0.086	15.66	16.03	<0.0001	***
X_1	0.46	1	0.056	0.34	0.58	<0.0001	***
X_2	0.93	1	0.056	0.81	1.05	<0.0001	***
X_3	0.21	1	0.056	0.092	0.33	0.0020	**
X_4	-0.42	1	0.056	-0.54	-0.30	<0.0001	***
X_1X_2	-0.59	1	0.096	-0.80	-0.38	<0.0001	***
X_1X_3	-0.91	1	0.096	-1.12	-0.70	<0.0001	***
X_1X_4	-1.20	1	0.096	-1.41	-1.00	<0.0001	***
X_2X_3	0.98	1	0.096	0.77	1.18	<0.0001	***
X_2X_4	0.18	1	0.096	-0.025	0.39	0.0805	
X_3X_4	0.30	1	0.096	0.097	0.51	0.0071	**
X_1^2	-2.41	1	0.076	-2.57	-2.24	<0.0001	***
X_2^2	-0.96	1	0.076	-1.12	-0.79	<0.0001	***
X_3^2	-1.02	1	0.076	-1.18	-0.86	<0.0001	***
X_4^2	-0.66	1	0.076	-0.83	-0.50	<0.0001	***

$R^2=0.9928$, $R_{Adj}^2=0.9855$.

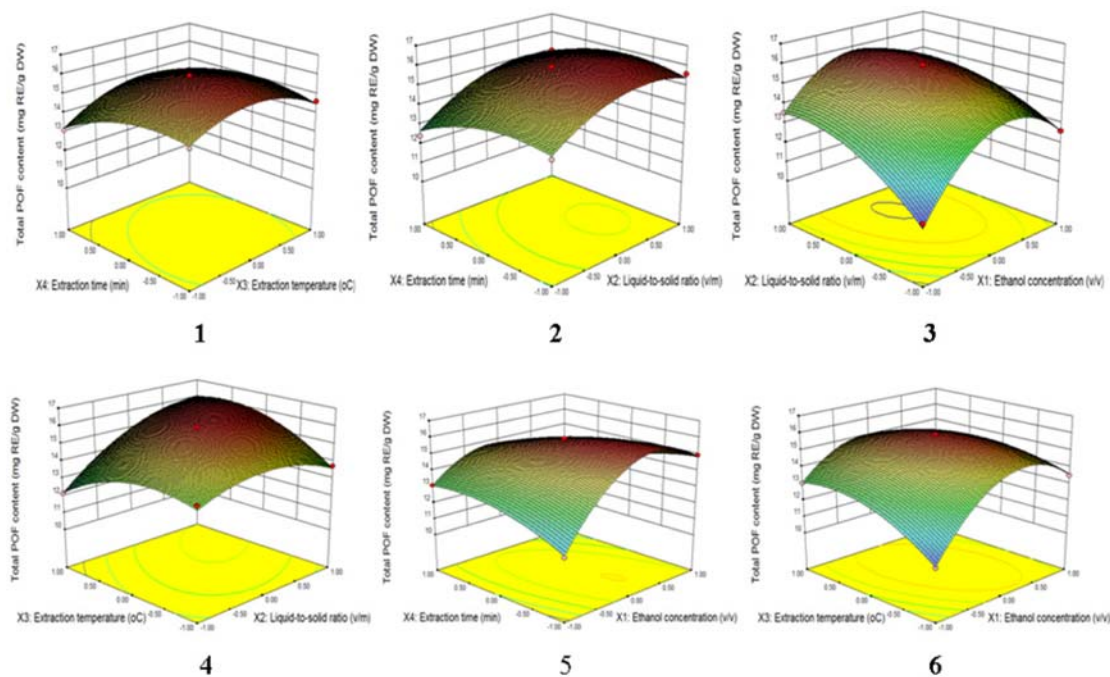


Fig. 5 Response surface and contour plots for the effect of independent variables on extraction of the flavonoids.

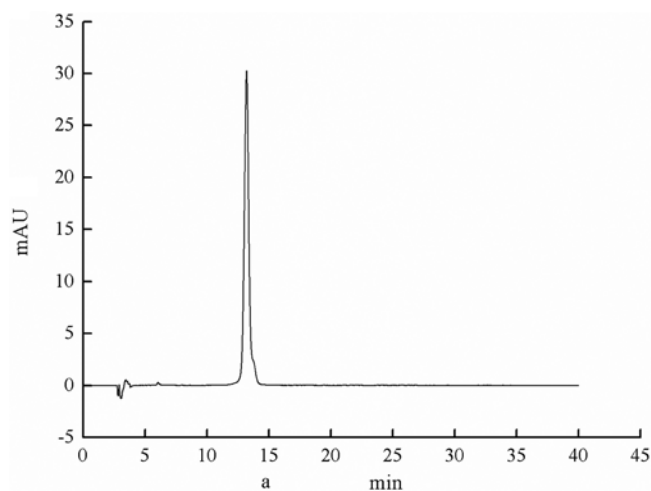


Fig. 6 The HPLC chromatograms of standard samples (Quercetin) (a) at 360 nm.

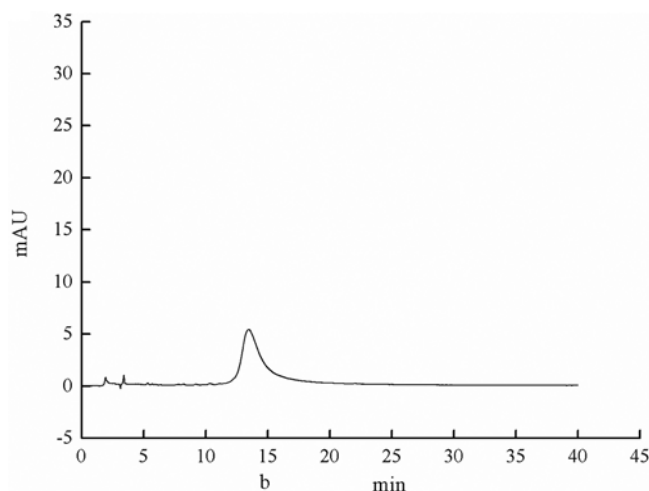


Fig. 7 The HPLC chromatograms of purified flavonoid (b) at 360 nm.

RE/g DW) was recorded during Run No. 27. The lowest POF (10.72 mg RE/g DW) was detected at Run No. 1. Consequently, a second-order polynomial equation can be obtained as the follows:

$$Y = 15.85 + 0.46X_1 + 0.93X_2 + 0.21X_3 - 0.42X_4 - 0.59X_1X_2 - 0.91X_1X_3 - 1.20X_1X_4 + 0.98X_2X_3 + 0.18X_2X_4 + 0.30X_3X_4 - 2.41X_1^2 - 0.96X_2^2 - 1.02X_3^2 - 0.66X_4^2$$

Y was the total POF content mg/g; X_1 was ethanol concentration/(v/v); X_2 was the liquid-to-solid ratio/(v/m); X_3 was the of temperature extraction/ $^{\circ}$ C; X_4 was the time of extraction/min.

Table 3 shows the analysis of variance (ANOVA) for the regression equation. The linear and quadratic terms were significantly different ($p < 0.05$), whereas the interaction between X_2X_4 was not. The lack of fit was used to verify the adequacy of the model. ANOVA for the lack of fit was not significant ($p > 0.05$) for the model, indicating that the model could adequately fit the experiment data.

According to the condition of each process, the yield of flavonoids was varied in the range from 10.72 to 15.95 mg/g. The value of R_{Adj}^2 (0.9855) for the equation is reasonably close to 1, indicating a high degree of correlation between the observed and predicted values, therefore the model is appropriate (Teng et al., 2010).

Both the surface and contour plots based on derived equation visualized the relationship between the response and the experimental levels of each factor, by which the optimum condition for the maximum yield could be deduced.

Three-dimensional response surface plots are presented in Fig. 5. An increase of liquid-to-solid ratio (X_2) results in an increase of POF to a maximum at certain levels, and an increase of ethanol concentration (X_1), result in the increase of POF, which then decrease when ethanol concentration continues to increase.

The ethanol concentration, liquid-to-solid ratio, and extraction

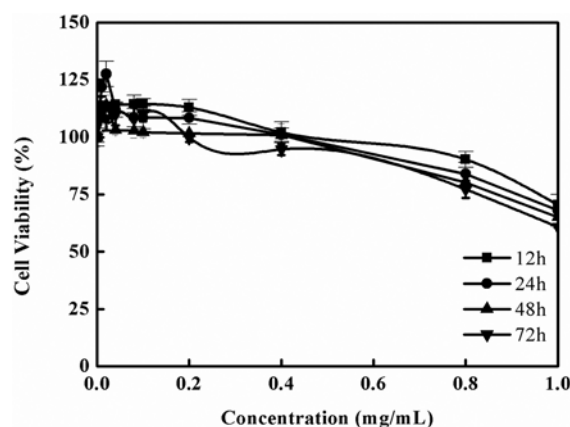


Fig. 8 Cell viability measured using the MTT assays at concentrations of flavonoids that increased from left to right: 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, and 1.0 mg/mL.

time were the most significant parameters. UAE is inexpensive, due to less instrumental requirements, simple, and efficient alternative to conventional extraction techniques. Our results are higher than the extraction of Zhu et al. (2009), confirming that the regional differences of *Portulaca oleracea* L., and conditions for the extraction are important for the samples. The ultrasound-assisted extraction methodology has lower cost than solvent reflux extraction and soxhlet extraction (Ghafoor et al., 2009).

The optimal values of the selected variables were obtained by solving the regression equation. Using RSM, the optimal conditions of POF were extraction of 39.01% ethanol, 55.25 $^{\circ}$ C extraction temperature, and 23.92 (v/m) liquid-to-solid ratio with 15 min extraction time. To confirm these results, three triplicate tests were performed under optimized conditions. The POF yield value was 16.20 \pm 0.12 ($n = 3$), which clearly showed that the model fitted the experimental data and therefore optimized the POF extraction procedure.

Flavonoid Compounds Identification. Figs 6 and 7 show the

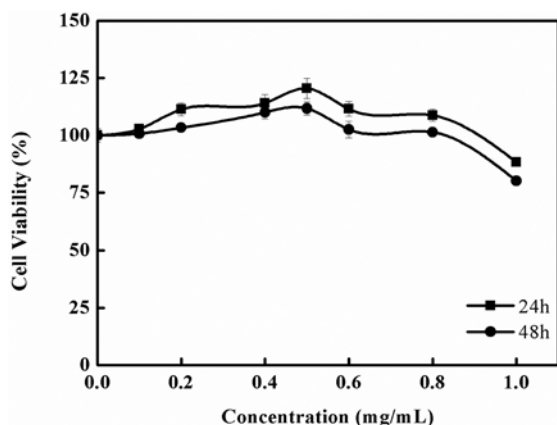


Fig. 9 Cell viability measured using the MTT assays at concentrations of quercetin that increased from left to right: 0, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, and 1.0 mg/mL.

HPLC chromatogram of standards and extract. By comparing relative retention time, quercetin was identified. Quercetin was shown to be the predominant flavonoid.

In Vitro Cytotoxicity Assay. Figs 8 and 9 show that the compounds were screened for any toxic effect they might have by adding the flavonoids or quercetin, and measuring the effect on cell viability using the MTT reduction assay in HIT-T15 cells. None of the compounds tested was toxic to the cells at low concentrations. They also provide experimental evidence for their toxicity at doses of 0–1mg/mL.

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