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Original Research Article

Response Surface Optimized Extraction of Total Triterpene Acids from *Eriobotrya japonica (Thunb) Lindl* (Loquat) Leaf and Evaluation of their *In vitro* Antioxidant Activities

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Abstract

Purpose: *To optimize extraction of total triterpene acids from loquat leaf and evaluate their in vitro antioxidant activities.*
Methods: *The independent variables were ethanol concentration, extraction time, and solvent ratio, while the dependent variable was content of total triterpene acids. Composite design and response surface method were used to optimize the extraction process, while antioxidant activity was evaluated in vitro using α,α-diphenyl-β-picrylhydrazyl (DPPH) and pyrogallol autoxidation methods.*
Results: *The optimum extraction conditions were as follows: 81 % ethanol, extraction time 160 min, and extraction cycles 2, ratio of solvent to sample 14mL/g. Under these conditions, the yield of total triterpene acids reached 3.41 %. Furthermore, total triterpene acids exhibited strong reducing power, as well as radical scavenging activity against DPPH (IC_{50}, 203.41 mg/L) and superoxide anion free radical; when the concentration was > 274.64 mg/L, scavenging ability increased significantly.*
Conclusion: *Total triterpene acids from loquat leaf possess an antioxidant activity in vitro, but further studies are required to develop them into effective drugs and health foods for human applications.*

Keywords: *Eriobotrya japonica (Thunb.) Lindl.,, Triterpene acids, Extraction optimization, Response surface methodology, Antioxidant*

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INTRODUCTION

In recent years, increasing numbers of plant total triterpene acids (TTA) are being used in medicine. Total triterpene acids possess various pharmacological activities, including anti-inflammatory [1], anti-atherosclerotic and anti-diabetic activity [2], anti-proliferative [3], and antifibrotic [4]. At the same time, research has shown that triterpene acids possess antioxidant activity [5].

Eriobotrya japonica Lindl. is cultivated in Zhangtan region, Huangshan City, China. The leaf is a traditional medicine in China, and several studies suggest that the leaf contains a variety of active ingredients, such as volatile oil, flavonoids, phenolics, triterpene acids, etc [6]. Up to now, no detailed investigation has been conducted on optimizing extraction of total triterpene acids from loquat leaves. Furthermore, to the best of our knowledge, no study has been conducted to explore the antioxidant activities of TTA.

Response surface methodology is increasingly being employed in optimizing processes in pharmaceutical research [7,8]. Therefore, the objective of this study was to use a central composite design to optimize the effects of

extraction solvent, solvent/sample ratio, and extraction time on the yield of TTA. Furthermore, the antioxidant activity of TTA was evaluated.

EXPERMENTAL

Plant material

Loquat leaves were collected from Huangshan City in China, during the month of May 2013 and authenticated by Mrs LM Pan, Department of Pharmacy, Anhui University of TCM, China. A voucher specimen (no. HSSX 130504) was deposited in the Department of Pharmaceutical Engineering, Huangshan University, Hungshan, China for future reference. The fresh leaves were dried at room temperature, crushed into a fine powder and stored in an air tight container until further use. Ursolic acid was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) while DPPH and pyrogallic acid were supplied by Duly Biotech (Nanjing, China). All other chemical reagents used in the experiments were of analytical grade while double-distilled water was used throughout the study. T6 UV-Vis Spectrophotometer (New Century, Beijing, China) was used for TTA analysis at 545 nm.

Extraction and evaluation of TTA yield

Ten grams of loquat leaf powder were placed in a round flask and mixed with ethanol. The extraction process was carried out using a reflux apparatus. After reflux extraction, the filtrate was condensed under reduced pressure, degreased with petroleum benzin, extracted with water saturated butanol (1:1), and dried by water bath. After dilution with methanol appropriately, spectrophotometric analysis was performed at 545 nm.

The yield (%) of TTA was calculated by dividing the weight of TTA extracted from loquat leaf by the weight of dried raw leaf, and expressed as a percentage.

Determination of TTA

Ursolic acid was used as a standard and a range of concentrations (9.4, 18.8, 28.2, 37.6 and 47 mg/L) was used to create a standard curve. Perchloric acid (800 µL) and vanillin-glacial acetic acid reagent (5%, 400 µL) was added to the extract sample. The reaction mixture was incubated at 60 ^{o}C for 15 min. Glacial acetic acid (5 mL) was then mixed with it and the absorbance of the mixture measured spectrophotometrically at 545 nm. The content of total triterpene acids was calculated from the standard curve. All of determinations were made in triplicate.

Optimization design

Central composite design (CCD) was selected for the optimization of extraction process and a three-variable, five-level CCD was used. The main factors affecting extraction efficiency, including the ethanol concentration, ratio of solvent to sample and extraction time, were selected as independent variables that should be optimized for the extraction of total triterpene acids. The range of independent variables and their levels are shown in Table 1. The yield of TTA was the dependent variable.

DPPH radical scavenging test

A slight modification was made to a previous method [9] used to measure the scavenging activity of DPPH free radicals. Ten milliliters of 24 mg/mL DPPH solution in ethanol and TTA at various concentrations in 70 % ethanol (1 mL) were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. The absorbance of the solution was spectrophotometrically measured at 517 nm. DPPH radical scavenging activity was calculated according to Eq 1.

Scavenging rate (%) = $(1 - A_i/A_0)100$ ……. (1)

where A_0 is the absorbance of pure DPPH and A_i the absorbance of DPPH in the sample.

Superoxide anion radical scavenging test

Superoxide radicals were generated by pyrogallic acid method [10], but with a slight modification. The system contained 6.0 mL Tris–HCl buffer (0.05 M, pH 8.2), 0.5 mL sample solution of different concentrations, 0.5 mL pyrogallic acid (7.0 mM), and 0.5 mL thick hydrochloric acid for termination the reaction. The solution was

Table 1: Independent variables and their levels used in the response surface design

Independent variable	Factor level				
	-1.732	-1	0	1	1.732
X_1 ethanol concentration(%)	50	58.45	70	81.55	90
X_2 ratio of solvent to sample(mL/g,)	8	9.69	12	14.31	16
X_3 extraction time (h)	1	1.42	2	2.58	3

incubated at 25 °C and determined spectrophotometrically at 322 nm. In the blank, the sample solution was substituted with Tris–HCl buffer. The superoxide radical scavenging ability was calculated as in Eq 2.

Scavenging effect (%) = $\{(A_0 - A_i)/A_0\}100$ ……. (2)

where A_0 is the absorbance without sample and A_i the absorbance with sample.

Statistical analysis

Design-Expert software (version 7.1.4, State-Ease Inc, Minneapolis, MN, USA) was used to analyze the experimental data. Statistical comparison within groups was carried out by one-way analysis of variance (ANOVA). If the absolute F-value is greater and *p*-value is samller, the corresponding variables would be more significant. Values representing the concentrations of the investigated extracts that cause 50 % inhibition (EC_{50}) were determined by linear regression analysis.

RESULTS

Fitting the eesponse surface model

Table 2 shows the yield of total triterpene acids (*Y*) obtained from all the experiments. A multiple linear regression was established based on the data in Table 1 by a quadratic polynomial model (Eq 3).

$$Y = \gamma_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i \neq j=1}^{3} \alpha_{ij} X_i X_j \quad (3)$$

where *Y* is the predicted response, γ_0 is a constant, α_i, α_{ii} and α_{ij} are the linear, quadratic and interactive coefficients of the model, respectively. Accordingly, X_i and X_j represent the levels of the independent variables, respectively [11,12]. The response variable and three independent variables are related by second-order polynomial equation (Eq 4).

$$Y = 3.15 + 0.18 + 0.13\ X_2 + 0.052X_3 - 0.005\ X_1X_2 - 0.0025X_1X_3 + 0.025X_2\ X_3 - 0.04X_1^2 - 0.05X_2^2 - 0.0037X_3^2 \quad (4)$$

where Y is the yield of TTA (%), X_1 is the ethanol concentration (%), X_2 is the ratio of solvent to sample (mL/g), X_3 is the extraction time (h).

The regression coefficient and ANOVA data are listed in Table 3. It was clear that the model fit well with the response variables, considering the model could explain the variability of the responses. The coefficient of multiple determination (*R*2) was 0.9887, proving that a high correlation was obtained [13].

Furthermore, the lack of fit test was used to verify the adequacy of the fit. As shown in Table 3, in the case of TTA, the lack of fit was not significant ($p = 0.1933$). This indicates that the trial data fitted well with the model. Additionally, the *p* values were used to assess the significance of each coefficient, which in turn might indicate the pattern of the interactions between the variables. In this case, a smaller *p* value indicates a more significant corresponding coefficient. In brief, the linear coefficients (X_1, X_2 and X_3) , cross product coefficient (X_2X_3) and quadratic term coefficients(X_1^2, X_2^2) were significant, with a very small *p* value ($p < 0.05$), while the other term coefficients were not significant ($p > 0.05$).

Optimization of extraction conditions of TTA

Based on the regression equation obtained by Design-Expert 7.1.4, the graphical presentations of the yield of TTA affected by X_1, X_2, and X_3 are presented in Figure 1. The independent variables and maximum predicted values corresponded with the optimum values of the dependent variables obtained by the equation 5. The contour plots can reflect the strength of the interaction effects (X_1X_2, X_1X_3, X_2X_3). According to Table 3, the interaction between ratio of solvent to sample and extraction time was significant ($p < 0.05$). As shown in Figure 1c, it can be concluded that the maximum total flavonoids extraction could be achieved when the ratio of solvent to sample and extraction time were 11.82 % and 1.92 h, respectively.The optimal extraction conditions were obtained from response surface analysis as follows: ethanol concentration 80.94 %, ratio of solvent to sample 14.24 mL/g, extraction time 2.56 h, and the predicted maximum yield of TTA was 3.45 %.

Verification of predictive model

It is desirable to investigate the accuracy and reliability of the model equation for predicting an optimum conditions. Taking into account industry operating convenience [14], the confirmatory experiment was tested under the conditions: ethanol concentration 81%, ratio of solvent to sample 14 mL/g, extraction time 160min. A mean value of yields of TTA (3.41 ± 0.07 %, n = 3) was obtained, and the difference between the real value and the predicted value was not significant ($p > 0.05$). It indicated that the model was fit for the extraction process.

DPPH scavenging activity

The α,α-diphenyl-β-picrylhydrazyl (DPPH) radical is widely used to evaluate the radical-scavenging properties of antioxidants. A lower absorbance of

Table 2: Central composite design and results for extraction yield of TTA

Run	X_1/Ethanol concentration (%)	X_2/Ratio of solvent to sample (mL/g)	X_3/Extraction time (h)	Y/Yield of TTA (%)
1	70.00	12.00	1.00	3.06
2	81.55	14.31	2.58	3.42
3	70.00	12.00	3.00	3.25
4	90.00	12.00	2.00	3.36
5	70.00	12.00	2.00	3.14
6	70.00	12.00	2.00	3.12
7	58.45	9.69	2.58	2.79
8	70.00	16.00	2.00	3.28
9	58.45	14.31	1.42	2.93
10	58.45	9.69	1.42	2.72
11	70.00	12.00	2.00	3.18
12	81.55	14.31	1.42	3.26
13	70.00	12.00	2.00	3.18
14	70.00	12.00	2.00	3.16
15	81.55	9.69	1.42	3.10
16	70.00	12.00	2.00	3.15
17	50.00	12.00	2.00	2.73
18	70.00	8.00	2.00	2.75
19	58.45	14.31	2.58	3.07
20	81.55	9.69	2.58	3.13

Table 3: Regression coefficients and ANOVA.data

Source	Sum of squares	Df	Mean square	*F*-value	*P*-value
Model	0.79	9	8.8×10^{-2}	97.56	< 0.0001
X_1	0.44	1	0.44	491.34	< 0.0001
X_2	0.25	1	0.25	273.31	< 0.0001
X_3	3.8×10^{-2}	1	3.8×10^{-2}	42.09	< 0.0001
X_1X_2	2.0×10^{-4}	1	2.0×10^{-4}	0.22	0.6479
X_1X_3	5.0×10^{-5}	1	5.0×10^{-5}	0.055	0.8186
X_2X_3	5.0×10^{-3}	1	5.0×10^{-3}	5.54	0.0404
X_1^2	2.6×10^{-3} 4.0×10^{-3}	1	2.6×10^{-3}	28.38	0.0003
X_2^2	2.1×10^{-4}	1	4.0×10^{-3}	44.20	< 0.0001
X_3^2	9.02×10^{-3}	1	2.1×10^{-4}	0.23	0.6399
Residual	6.27×10^{-3}	10	9.02×10^{-4}		
Lack of Fit	2.75×10^{-3}	5	1.25×10^{-3}	2.28	0.1933
Pure Error	0.80	5	5.5×10^{-4}		
Total		19			

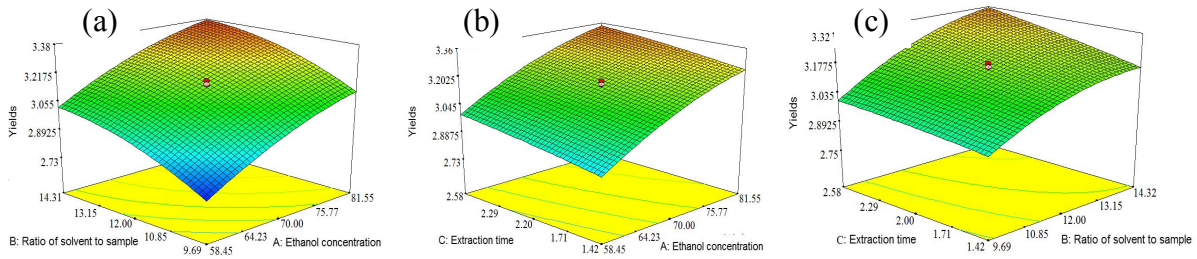

Figure 1: Response surface for the effect of independent variables on extraction of the TTA: (**a**) ethanol concentration and Ratio of solvent to sample; (**b**) ethanol concentration and extraction time; (**c**) ratio of solvent to sample and extraction time.

sample indicates a higher DPPH radical-scavenging activity. In Figure 2, TTA exhibited a steady increase in scavenging DPPH free radical with concentration increase, TTA concentration (X), and the DPPH removal rate (Y). There was a significant linear correlation, and the regression equation is shown in Eq 5.

$Y = 0.2039 X + 8.525$ ($r = 0.9957$), IC_{50} =203.41mg/L …. (5)

Therefore, the TTA have significant DPPH radical scavenging activity.

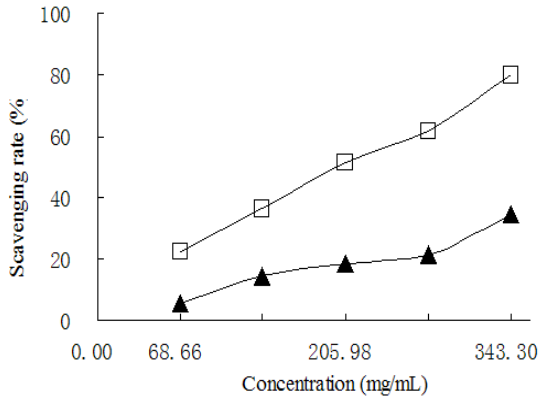

Figure 2: DPPH radical scavenging (□) and superoxide anion scavenging (▲) activities of TTA

Superoxide anion radical scavenging activity

Superoxide anion radical is known as an initial radical in the peroxidation of lipids [15]. The results of superoxide anion scavenging effect of TTA were given in Figure 2. As illustrated in the Figure 2, In the range of 68.66 - 274.64 mg/L, the scavenging effect increased slowly, and when the concentration is greater than 274.64 mg/L, with the increase of concentration, scavenging ability increased significantly. Therefore, TTA can be used to scavenge superoxide anion.

DISCUSSION

Triterpene acids, such as flavonoids, are widely distributed in plant, and they have been shown to possess significant antioxidant activities. However, insufficient studies have been conducted on the role of TTA on free radical management and antioxidant activity.

The extraction conditions for TTA were optimized by CCD, and a quadratic polynomial model was obtained from response surface methodology. The confirmatory experimental optimum conditions of TTA were as follows: ethanol concentration, 81 %; ratio of solvent to sample, 14 mL/g; extraction time, 160 min; and extraction cycles, 2. The actual optimal yield of (3.41 ± 0.07 %), which was obtained from confirmatory experiments, closely matched the predicted yield of 3.45 %.

Additionally, the antioxidant activities of TTA *in vitro* including DPPH radical scavenging activity, superoxide anion scavenging activity indicate that TTA possesses significant antioxidant activities. The antioxidant activity of TTA is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers [16].

CONCLUSION

The potential antioxidant capability of TTA has been established in this study. It is apparent that TTA can quench free radicals and convert them into stable products. Further studies are required to analyze the correlation of antioxidant activity of loquat leaf extract with a single constituent of the plant.

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