Asian Journal of Chemistry; Vol. 26, No. 9 (2014), 2802-2808

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16962

Response Surface Optimization for Extraction of Flavonoids from *Artemisia lactiflora Wall. ex DC*. and Evaluation of Antioxidant Capacities *in vitro*

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Received: 30 December 2013; *Accepted*: 21 February 2014; *Published online*: 28 April 2014; AJC-15122

Response surface methodology was used to optimize ultrasonic-assisted extraction of total flavonoids from *Artemisia lactiflora Wall. ex DC.* with a Box-Behnken design. The results indicated that the experimental yield of 6.396 ± 0.013 % was in good agreement with the value predicted by the model at the optimum extraction condition of ethanol concentration 53.5 % (v/v), liquid-to-solid ratio 22.95:1 (mL/g) and extraction time 23.2 min. The antioxidant activities of the purified extract were evaluated *in vitro* by 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-*bis*(3-ethyl benzothiazoline-6-sulphonic acid) diammonium salt, ferric reducing antioxidant power and ferrous ion-chelating ability assays. In addition, nine flavonoids were isolated from *A. lactiflora.* for the first time. Among them, quercetin (compound **5**) had the highest 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity with an IC_{50} of 7.6 ± 0.3 μM, indicating an important role of 5 for the antioxidant activity of *A. lactiflora*. The results showed that the flavonoids in this plant can be used as a source of potential antioxidants.

Keywords: *Artemisia lactiflora*, Ultrasonic-assisted extraction, Response surface methodology, Flavonoids, Antioxidant activity.

INTRODUCTION

Artemisia lactiflora Wall. ex DC. (Chinese name "Bai-Bao-Hao"), a perennial herbaceous plant belonging to the *Compositae* family is mainly distributed in the middle and southwest part of China. It grows vigorously in summer and autumn and it is usually used for both food and medicine. The whole grass of *A. lactiflora* is a kind of vegetable in some districts of China and also commonly used in traditional Chinese medicine for the treatment of many diseases, such as toothache, acute hepatitis with jaundice, chronic bronchitis, stomatitis, pharyngitis, tonsillitis, otitis media, eczema, trauma, diarrhea and digestion disorders[1]. Previous phytochemical investigations led to the isolation of flavonoids, coumarins, sesquiterpene lactones, lignins and phenolic compounds from *A. lactiflora*[2-4]. However, little information is available about biological activities such as antioxidation from *A. lactiflora*.

Oxidative modifications of DNA, proteins, lipid and small cellular molecules by reactive oxygen species (ROS) play an important role in a wide range of diseases and age related degenerative disorders[5]. It is generally accepted that various degenerative disorders are caused by oxidative damage such as cardiovascular diseases, Parkinson's disease, Alzheimer's disease, atherosclerosis and cancer[6]. Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions[7]. Antioxidant supplements or antioxidant-containing foods may be used to help the human body to reduce singlet oxidative damage[8].

Flavonoids are among the most studied phytochemicals found in plants and include a large number of different molecules which may result in diverse biological activities such as antiinflammatory[9], antilipoperoxidant[10] and anticancer activities[11]. Flavonoids have also been found to inhibit a wide range of enzymes involved in oxidation systems such as 5-lipoxygenase, cyclooxygenase, monooxygenase, or xanthine oxidase[12]. It is important to determine the amount and composition of flavonoids in plants.

In recent years, various new techniques have been utilized for the extraction of natural products from plants, including ultrasound-assisted extraction[13], microwave-assisted extraction[14], supercritical fluid extraction[15] and accelerated solvent extraction[16]. Among these, ultrasound-assisted extraction is a simple, efficient and inexpensive alternative to conventional extraction techniques[17].

Response surface methodology (RSM) is a useful technique for the investigation of several input variables which influence the performance and quality characteristics of a product or process under investigation. The main advantage of RSM is the reduced number of experimental trials needed to evaluate

multiple parameters and their interactions. Therefore, it is less laborious and less time-consuming than other approaches required to optimize a process[18]. Box-Behnken design, a type of RSM, is more efficient, easier to execute and interpret the results in comparison with others and widely used by many researchers[19,20].

The purpose of the present study was to optimize the process of ultrasonic-assisted extraction of bioactive total flavonoids from *A. lactiflora* using response surface methodology. Four methodologies were employed for evaluating the antioxidant activities of the purified flavonoids *in vitro*, measurement of: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity, 2,2'-azino-*bis*(3-ethyl benzothiazoline-6-sulphonic acid) diammonium salt (ABTS) free radical-scavenging activity, ferric reducing antioxidant power (FRAP) and ferrous ion-chelating ability (FIC). The investigation into its chemical constituents was also carried out. Consequently, nine flavonoids [eupatilin (**1**), 5,7,8,4'-tetramethoxyflavone (**2**), luteolin (**3**), luteolin-7-O-β-D-glucopyranoside (**4**), quercetin (**5**), quercetin-3-O-α-L-pyran-rhamnside (**6**), tricin (**7**), 3'4'-dimethoxyluteolin (**8**) and rhamnetin (**9**)] were isolated (Fig. 1). Moreover, the antioxidant activities of compounds (**1-8**) were evaluated so as to test the potential use of *A. lactiflora* as a source of natural antioxidants.

EXPERIMENTAL

A. lactiflora was obtained from a local store in Anhui, China. *A. lactiflora* was cleaned and air dried in an oven at 40 °C overnight, ground to powder using an electric grinder (YK-400A, Shandong Yikang Experimental Instrument Co., Ltd., Shandong, China) for 10 min and then passed through a 80-mesh sieve.

An ultrasonic cell disintegrator (JY92-II, Xinzhi Bio-technology and Science Inc., Zhejiang, China) was used for ultrasonic extraction of flavonoids, a UV-2450 spectrophotometer (Shimadzu Corporation, Japan) for total flavonoids analysis of sample.

Rutin, vitamin C, butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 1,3,5-tri(2-pyridyl)-2,4,6-triazine (TPTZ), 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ferrozine and ethylene-diaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium nitrite, aluminum nitrate, sodium hydroxide, iron(III) chloride, iron(II) chloride, aluminum chloride, chloride acid, sodium carbonate and dibasic sodium phosphate were obtained from Fluka (Buchs, Switzerland). Other solvents for extraction and separation were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Flavonoids extraction of *A. lactiflora* with ultrasonic treatment: The total flavonoids extraction from *A. lactiflora* by ultrasonic treatment was performed in an ultrasonic cell disintegrator. *A. lactiflora* powders (2 g) were placed in a conical flask with aqueous ethanol at the desired concentrations, then the conical flask was held in the ultrasonic cell disintegrator and exposed to the extract for different time at varied liquid-to-solid ratios as dictated by the experimental design. Ice bathing was used to ensure the temperature of solution was below 50 °C during the entire extraction process.

Experimental design: A three level, three variable Box-Behnken factorial design (Design Expert software, Trial Version 7.1.6, Stat-Ease Inc., Minneapolis, MN) was applied to determine the best combination of extraction variables for the yield of *A. lactiflora* total flavonoids. Three extraction variables considered in this research were X_1 (ethanol concentration), X_2 (liquid-to-solid ratio) and X_3 (extraction time) and the proper range of the three variables were determined on the basis of a single-factor experiment for the total flavonoids yield (Table-1). Table-1 lists the range of independent variables and their levels. As from Table-2, the whole design consisted of 17 experimental points carried out in random order, five replicates at the center of the design were used to allow for estimation of a pure error sum of squares. The response value in each trial was the average of triplicates.

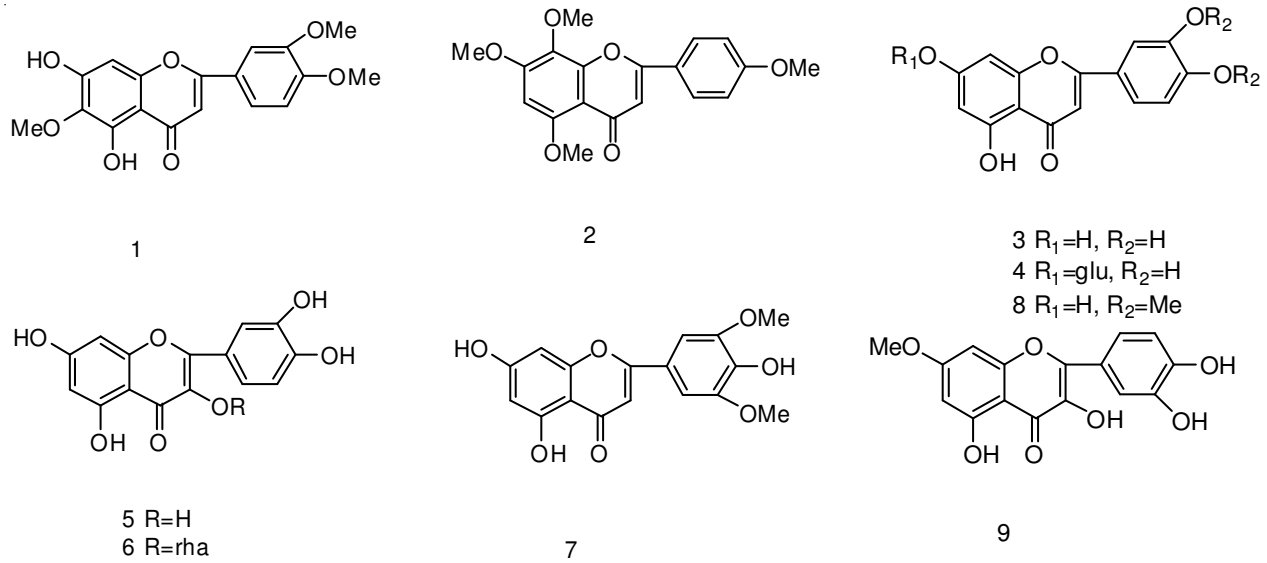

Fig. 1. Structures of compounds **1-9**: eupatilin (**1**), 5,7,8,4'-tetramethoxyflavone (**2**), luteolin (**3**), luteolin-7-O-β-D-glucopyranoside (**4**), quercetin (**5**), quercetin-3-O-α-L-pyranrhamnside (**6**), tricin (**7**), 3'4'-dimethoxy-luteolin (**8**), rhamnetin (**9**)

TABLE-1
INDEPENDENT VARIABLES AND THEIR LEVELS USED IN THE RESPONSE SURFACE DESIGN

Independent variables	Factor level		
	-1	0	1
X_1: ethanol concentration (%)	50	60	70
X_2: liquid-to-solid ratio (mL/g)	15:1	20:1	25:1
X_3: extraction time (min)	20	30	40

TABLE-2
BOX-BEHNKEN DESIGN MATRIX OF THREE VARIABLES AND THE EXPERIMENTALLY OBSERVED RESPONSES

No.	X_1: ethanol concentration (%)	X_2: liquid-to-solid ratio (mL/g)	X_3: extraction time	Y: Yield of *A. lactiflora* TF (%)
1	0(60)	0(20:1)	0(30)	6.304
2	1(70)	-1(15:1)	0(30)	5.962
3	-1(50)	1(25:1)	0(30)	5.352
4	-1(50)	-1(15:1)	0(30)	4.551
5	1(70)	1(25:1)	0(30)	5.934
6	0(60)	0(20:1)	0(30)	6.221
7	0(60)	1(25:1)	1(40)	6.104
8	0(60)	-1(15:1)	-1(20)	5.684
9	1(70)	0(20:1)	1(40)	5.861
10	1(70)	0(20:1)	-1(20)	6.201
11	0(60)	0(20:1)	0(30)	6.271
12	0(60)	0(20:1)	0(30)	6.248
13	0(60)	-1(15:1)	1(40)	5.968
14	-1(50)	0(20:1)	-1(20)	4.998
15	-1(50)	0(20:1)	1(40)	5.021
16	0(60)	1(25:1)	-1(20)	6.259
17	0(60)	0(20:1)	0(30)	6.211

Experimental data were fitted to a quadratic polynomial model and regression coefficients were obtained. The non-linear computer generated quadratic model used in the response surface was as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i+1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j+1+1}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y is the dependent variable (extraction yield), β_0 is the model constant, β_i, β_{ii} and β_{ij} are the model coefficients and ε is the error. They represent the linear, quadratic and interaction effects of the variables, respectively.

Determination of total flavonoid content: For the total flavonoid content, the method based on Blasa *et al.*[21] with some modifications was used. One milliliter of properly diluted *A. lactiflora* total flavonoids sample was mixed with 4 mL of deionized water in a 10 mL volumetric flask and 0.3 mL of $NaNO_2$ (5 %, w/v) was added. Five minutes later, 0.3 mL of $AlCl_3$ (10 %, w/v) was added. Then, after incubation for 6 min, the sample was neutralized with 2 mL NaOH solution (1 mol/L), followed by the addition of 2.4 mL of deionized water. The solution was mixed and shaken vigorously. The absorbance was read at 510 nm and the quantification was carried out using a calibration curve.

Different concentrations of rutin (8.5-170 µg/mL) were used for calibration, giving a linearity of 0.9999 (R^2). The results were expressed as rutin equivalents (µg rutin/g sample) as a mean of three replicates.

Evaluation of flavonoid antioxidant activities

Radical scavenging activity by the 2,2-diphenyl-1-picryl-hydrazyl method: The antioxidant activity of purified *A. lactiflora* total flavonoids (purified) was measured in terms of hydrogen donating or radical scavenging ability using the stable radical 2,2-diphenyl-1-picrylhydrazyl[22]. A volume of 50 µL of purified *A. lactiflora* total flavonoids at different concentrations was placed in a cuvette and 2 mL methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (6×10^{-5} mol/L) was added. The mixtures were well shaken in a Vortex (2500 rpm) for 1 min and then placed in a dark room. The decrease in absorbance at 517 nm was determined after 0.5 h for all samples. Ethanol was used to zero the spectrophotometer. Absorbance of the radical without antioxidant was used as the control. The inhibition percentage of the 2,2-diphenyl-1-picrylhydrazyl radical was calculated from the following equation:

$$I\ \% = \left[\frac{(A_{blank} - A_{sample})}{A_{blank}}\right] \times 100\ \%$$

where A_{sample} is the absorbance of a sample solution and A_{blank} is the absorbance of the blank solution. IC_{50} value is the effective concentration that could scavenge 50 % of the 2,2-diphenyl-1-picrylhydrazyl radicals.

Radical scavenging activity by the ABTS method: The ABTS assay was carried out according to the method described by Gursoy *et al.*[23] with some modifications. The $ABTS^{+\bullet}$ solution was produced by mixing ABTS and $K_2S_2O_8$ at final concentrations of 7 and 2.45 mmol/L, respectively. The $ABTS^{+\bullet}$ solution was stored at room temperature for 12-16 h in the dark before use. Furthermore, the solution was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm and equilibrated at 30 °C. For the spectrophotometric assay, 3 mL of the $ABTS^{+\bullet}$ solution and 0.1 mL of different concentrations of *A. lactiflora* purified were mixed and the absorbance was measured immediately after 0.5 h at 734 nm with ethanol as the blank solvent. The inhibition percentage of the ABTS radical was calculated from the following equation:

$$I\ \% = \left[\frac{(A_{blank} - A_{sample})}{A_{blank}}\right] \times 100\ \%$$

where A_{sample} is the absorbance of a sample solution and A_{blank} is the absorbance of a blank solution. IC_{50} value is the concentration at which $ABTS^{+\bullet}$ radicals are scavenged by 50 %.

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay was carried out according to the procedure of Suárez *et al.*[24] with slight modifications. The working FRAP reagent was prepared with 10 mmol/L TPTZ solution in 40 mmol HCl, 0.3 M sodium acetate buffer (pH 3.6) and 20 mmol/L iron(III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was freshly prepared before analysis and warmed to 37°C prior to use. 3 mL of the working FRAP reagent was mixed with 0.1 mL sample solution of different concentrations and 0.3 mL of deionized water. The reaction mixture was incubated for 0.5 h at 37 °C in a waterbath. The absorbance was then recorded at 593 nm. Trolox was used as the standard and the total antioxidant capacity of *A. lactiflora* purified was estimated in terms of Trolox equivalent antioxidant capacity (TEAC) in µmol/L of Trolox.

Ferrous ion-chelating ability assay: The ferrous ion-chelating (FIC) assay was carried out according to the method of Singh and Rajini[25] with some modifications. Solutions of 2 mmol/L $FeCl_2 \cdot 4H_2O$ and 5 mmol/L ferrozine were diluted 10 times. Briefly, a solution (1 mL) of different concentrations of *A. lactiflora* purified was mixed with 1 mL $FeCl_2 \cdot 4\ H_2O$. After 5 min incubation, the reaction was initiated by the addition of ferrozine (1 mL). The mixture was shaken vigorously and after a further 10 min incubation period, the absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Chelating effect (\%)} = \left[\frac{(1 - A_{sample})}{A_{blank}}\right] \times 100\ \%$$

where A_{sample} is the absorbance of a sample solution and A_{blank} is the absorbance of a blank solution. To determine the concentration needed to obtain a 50 % chelating effect (EC_{50}), the percentage of the chelating effect was plotted against the sample concentration.

Isolation of flavonoid compounds from *A. lactiflora*: The air-dried and powdered whole glass of *A. lactiflora* (3 kg) was extracted using the optimal extraction condition obtained from response surface methodology (RSM). The extract was decanted, filtered under vacuum and concentrated in a rotary evaporator. The crude extract (189.6 g) was then used for chemical component analysis.

The resulting crude extract was separated by macroporous adsorption resin D101 [H_2O:ethanol, (100:0-5:95)] to give six fractions (Fr·A1-Fr·A6). Five grams of the mixture of Fr·A3 and Fr·A4 were set aside for the antioxidant experiments. Fr·A3 was loaded onto the normal-phase silica gel column chromatography [petroleum ether:acetone, (5:1-1:2)] to give five fractions (Fr·B1-Fr·B5). Fr·B3 was purified by semi-preparative HPLC (MeOH-H_2O, 70:30) to afford compounds **1** (20.5 mg) and **2** (18 mg). Fr·B4 was purified by semi-preparative HPLC (MeOH-H_2O, 40:60) to afford compounds **3** (12.2 mg), **4** (16 mg) and **5** (13.5 mg). Fr·A4 was separated using a silica gel column [chloroform: methanol, 100:1-5:1] to give eight fractions (Fr·C1-Fr·C8). Fr·C4 and Fr·C5 were repeatedly subjected to RP_{18} [MeOH : H_2O, 4:6-9:1] and purified by Sephadex LH-20 (methanol) column and semi-preparative HPLC to yield compound **6** (21 mg), compound **7** (15.5 mg), compound **8** (25 mg) and compound **9** (6 mg), respectively.

Statistical analysis: Results were expressed as the mean ± SD for at least three replicates. One-way analysis of variance (ANOVA) was used to compare the means. The responses obtained from each set of experimental design (Table-2) were subjected to multiple non-linear regressions using the Design-Expert 7.1.6 software package. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and the significances of the regression coefficient were checked by a *F*-test and *P*-value.

RESULTS AND DISCUSSION

Table-2 shows the experimental conditions and the results of yield of *A. lactiflora* total flavonoids. Table-3 presents the results of fitting the model to the data. The results of analysis of variance (ANOVA) indicate that the contribution of the quadratic model was significant for response of *A. lactiflora* total flavonoids extraction yield. The fitted quadratic model is given in eqn. (2). The significance of each coefficient was determined using the *F*-test and *P*-value as shown in Table-3. The corresponding variables become more significant if the absolute *F*-value becomes greater and the *P*-value becomes smaller[26]. Lack of fit is also provided in Table-3 and was used to check the quality of the fitted models.

$$Y = 6.25 + 0.50X_1 + 0.19X_2 - 0.024X_3 - 0.21X_1X_2 - 0.091X_1X_3 - 0.11X_2X_3 - 0.64X_1^2 - 0.16X_2^2 - 0.088X_3^2 \quad (2)$$

as shown by the equation, the second-order polynomial response surface model was fitted to the response variable. However, the exploration and optimization of a fitted response surface model such as this might produce poor or misleading results[27]. Thus, it was necessary to check if the model exhibited a good fit or not. Regression analysis and ANOVA were used for fitting the model and examining the statistical significance of the terms. The results of the ANOVA are given in Table-3. The model had a high model *F*-value (F = 112.49) and a low *p*-value ($p < 0.0001$), indicating the model was highly significant. The corresponding coefficient of determination (R^2) value was 0.9931, indicating that only 0.69 % of the total variations was not explained by the model. The value of the adjusted determination coefficient (Adj $R^2 = 0.9843$) also confirmed that the model was highly significant. At the same time, a low value of coefficient of variation (C.V. = 1.15 %) indicated a high degree of precision and a good deal of reliability of the experimental values. There was no significance in the lack of fit $p > 0.05$), indicating that the model could be used to predict the response. The regression coefficient values of eqn. 2 are listed in Table-3. It can be seen that the linear coefficients (X_1 and X_2), quadratic term coefficients (X_1^2, X_2^2 and X_3^2) and the interaction terms (X_1X_2, X_1X_3 and X_2X_3) were significant, with a low *p*-value ($p < 0.05$). The only term which was not significant ($p > 0.05$) was the linear coefficient X_3. The full model was used to make the 3-D response surface plots to predict the relationships between the independent and dependent variables.

TABLE-3
ANALYSIS OF VARIANCE FOR RESPONSE SURFACE QUADRATIC MODEL OBTAINED FROM EXPERIMENTAL RESULTS

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F
Model	4.536	9	0.504	112.489	< 0.0001
X_1	2.036	1	2.036	454.27	< 0.0001
X_2	0.275	1	0.275	61.412	0.0001
X_3	0.00442	1	0.00442	0.986	0.354
X_1X_2	0.172	1	0.171	38.332	0.0004
X_1X_3	0.0329	1	0.0329	7.350	0.0302
X_2X_3	0.0482	1	0.0482	10.749	0.0135
X_1^2	1.737	1	1.737	387.633	< 0.0001
X_2^2	0.106	1	0.106	23.711	0.0018
X_3^2	0.0329	1	0.0329	7.337	0.0303
Residual	0.0314	7	0.00448		
Lack of Fit	0.0257	3	0.00855	5.982	0.0584
Pure Error	0.00572	4	0.00143		
Cor Total	4.569	16			

The 3-D response surface plots were employed to determine the interaction of the extraction conditions and optimal levels that have the most significant effect on the yield. The response surfaces plots based on the model are shown in Fig. 2. Fig. 2a shows that both ethanol concentration and liquid-to-solid ratio have a quadratic effect on the yield. It was clear from Fig. 2a that the minimum response of yield (4.551 %) occurred when the ethanol concentration was at the lowest level. The yield increased considerably as the ethanol concentration increased, indicating that ethanol concentration had a significant effect on the response (yield of *A. lactiflora* total flavonoids). As the ethanol concentration increased, the responses were maximal at approximately the middle of the liquid-to-solid ratio. Fig. 2b shows the quadratic effects of extraction time and ethanol concentration on the yield. Similarly, the effects of extraction time and liquid-to-solid ratio are shown in Fig. 2c. Considering all the responses, it is evident that ethanol concentration, liquid-to-solid ratio and the square effects had a significant effect on the yield of *A. lactiflora* total flavonoids while the effect of extraction time was more limited. The results agreed well with the analysis of variance as shown in Table-3.

Optimal extraction condition obtained using RSM model: Ethanol concentration, 53.5 % (v/v); liquid-to-solid ratio, 22.95:1 (mL/g); and extraction time, 23.2 min. To compare the predicted result (6.401 %) with the experimental value, the experiments were performed using the predicted conditions. The yield of 6.396 ± 0.013 % (n = 3) obtained from the real experiment demonstrated the validity of the RSM model, since there was no significant ($p > 0.05$) difference between the predicted value and the real value. The authentic experiments confirmed that the response model was adequate to reflect the expected optimization.

Antioxidant activity of purified *A. lactiflora*

Radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl method: As shown in Fig. 3, purified *A. lactiflora* could inhibit the 2,2-diphenyl-1-picrylhydrazyl radical better than BHT and slightly less than vitamin C. A moderate increase in radical scavenging activity was observed when the concentration of purified *A. lactiflora* increased. The 2,2-diphenyl-1-picrylhydrazyl assay measures the ability of the extract to donate hydrogen to the 2,2-diphenyl-1-picrylhydrazyl radical, which results in bleaching of the 2,2-diphenyl-1-picrylhydrazyl solution. The greater the bleaching action, the higher the antioxidant activity and the lower the IC_{50}. The 2,2-diphenyl-1-picrylhydrazyl scavenging data suggested that the sample was capable of scavenging free radicals, thus preventing the initiation and propagation of free-radical-mediated chain reactions. This could be beneficial in the preservation of foodstuffs, drug products and cosmetics where free-radical -mediated chain reactions result in lipid oxidation and subsequent deterioration of the products[28].

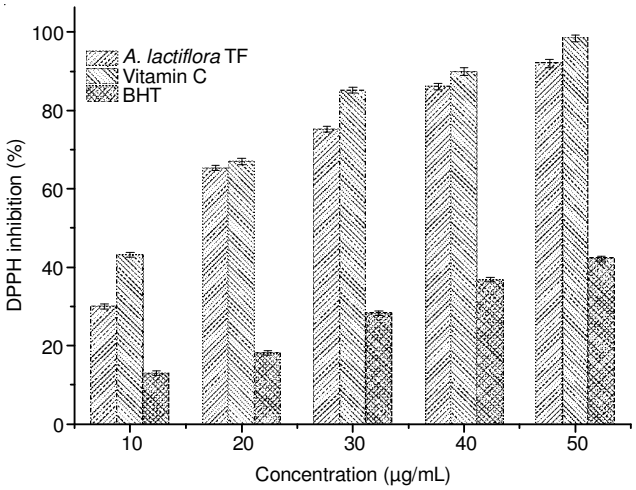

Fig. 3. 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activities of *A. lactiflora* purified and control standards (mean ± SD, n = 3)

Radical scavenging activity by ABTS method: The $ABTS^{+\bullet}$ radical formed from the reaction ABTS-e → $ABTS^{+\bullet}$ reacts quickly with the electron/hydrogen donors to form colorless ABTS. The ABTS assay has been used to quantitate different antioxidants such as vitamin C, tocopherol, glutathione, ursolic acid, protein, bilirubin, BHT, phenolic acid, flavonoids and catechin, among others[11]. *A. lactiflora* purified samples were examined for their ability to act as radical scavenging agents in the ABTS assay (Fig. 4). Similar to the 2,2-diphenyl-1-picrylhydrazyl assay, a concentration- dependent radical scavenging ability was apparent in the samples. *A. lactiflora* purified samples were found to be better scavengers than the well-known standard BHT, but not vitamin C with the IC_{50} values of 14.31, 17.53 and 7.18 µg/mL, respectively.

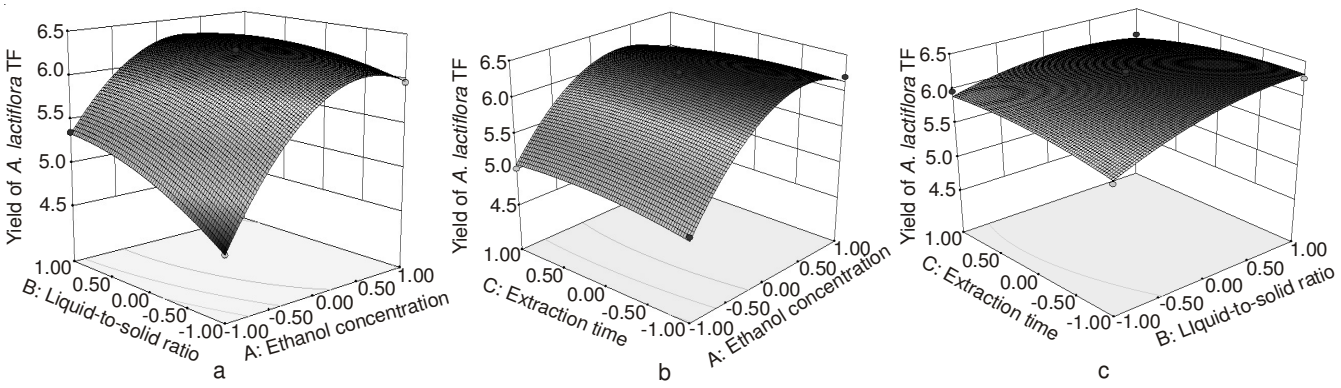

Fig. 2. Surface plots showing the yield of *A. lactiflora* total flavonoids; (a) Effect of ethanol concentration and liquid-to-solid ratio on the yield of *A. lactiflora* total flavonoids with extraction time of 23.2 min; (b) Effect of ethanol concentration and extraction time on the yield of *A. lactiflora* total flavonoids with liquid-to-solid ratio 20 mL/g; (c) Effect of liquid-to-solid ratio and extraction time on the yield of *A. lactiflora* total flavonoids with ethanol concentration 50 % (v/v)

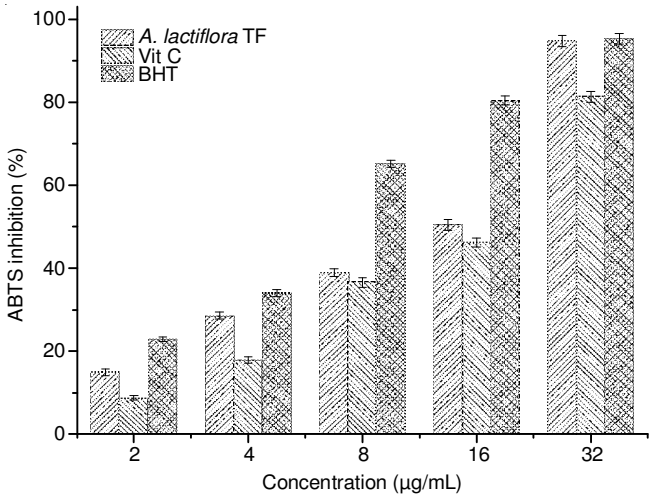

Fig. 4. ABTS radical-scavenging activities of *A. lactiflora* purified and control standards (mean ± SD, n = 3)

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay is based on the ability of the antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. The reaction is pH-dependent and the absorbance decrease is proportional to the antioxidant content[29]. In the present study, the trend for ferric ion reducing activities of *A. lactiflora* purified, vitamin C and BHT are shown in Fig. 5. A dose-dependent reducing capacity was found for all concentrations of the *A. lactiflora* purified samples which showed a higher ferric reducing capacity than the positive controls BHT and vitamin C.

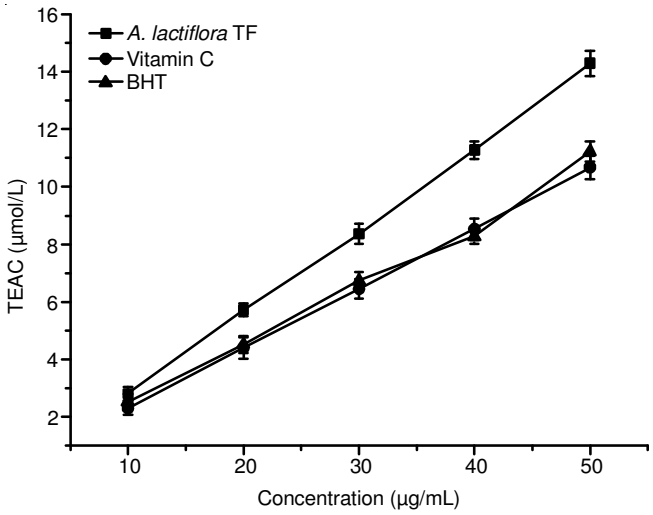

Fig. 5. TEAC of *A. lactiflora* purified and control standards of the FRAP assay (mean ± SD, n = 3)

Ferrous ion-chelating ability assay: As shown in Fig. 6, analysis of the metal ion-chelating properties indicated that all the concentrations of purified *A. lactiflora* tested were capable of chelating iron (II) and did so in a concentration-dependent manner with an EC_{50} of 10.38 μg/mL. Purified *A. lactiflora* showed a higher ion-chelating capacity than the positive control EDTA with an EC_{50} of 17.86 μg/mL. In general, it was concluded that purified *A. lactiflora* had high chelating activity which may prove useful because the chelation of transition metals is of great potential interest in the food industry[30].

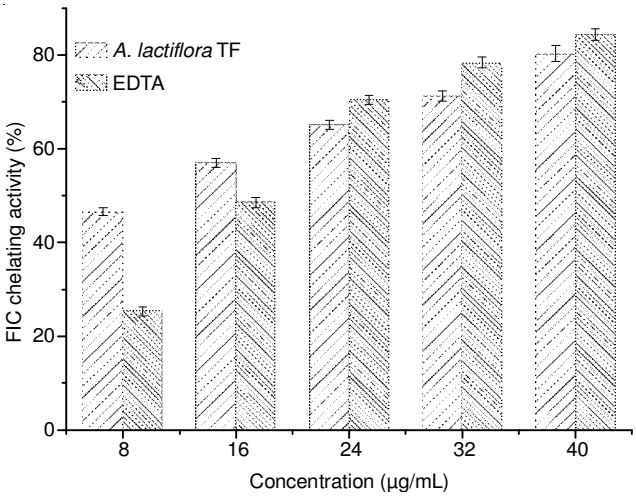

Fig. 6. Ferrous ion-chelating abilities of *A. lactiflora* purified and control standards (mean ± SD, n = 3)

However, BHT showed no ion-chelating activities in our experiment, the same as the results of Bounatirou *et al.*[31], but different from those of Viuda-Martos *et al*.[30]. As reported by Bounatirou *et al.*[31] BHT is a single-composition molecule which has no ion-chelating capacity.

Chemical constituents and their antioxidant capacities: The above investigation indicated that there are some antioxidants in the purified extract of the whole glass of *A. lactiflora*. In order to identify the compounds responsible for the high antioxidant activity, an investigation into the chemical constituents was also carried out. Nine compounds were isolated and their chemical structures were identified on the basis of their physicochemical properties and spectral data (MS, NMR and IR). Consequently, nine flavonoids [eupatilin (**1**), 5,7,8,4'-tetramethoxyflavone (**2**), luteolin (**3**), luteolin-7-O-β-D-glucopyranoside (**4**), quercetin (**5**), quercetin-3-O-α-L-pyranrhamnside (**6**), tricin (**7**), 3'4'-dimethoxyluteolin (**8**) and rhamnetin (**9**)] were isolated.

The antioxidant activities of eight flavonoids (**1-8**), which were all obtained in amounts higher than 12 mg, were determined by the 2,2-diphenyl-1-picrylhydrazyl assay with rutin as a positive control. The IC_{50} values of compounds **1**, **3**, **5** and **8** were calculated to be 21.5 ± 1.8, 9.8 ± 0.5, 7.6 ± 0.3and 18.2 ± 2.1 μM, respectively. This indicated that compounds **3** and **5** were more effective than rutin (IC_{50} = 10.6 ± 0.8 μM) with regard to their 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activities. The concentrations required for compounds **2**, **4**, **6** and **7** to produce 10 % radical inhibition were 55.5 ± 1.8, 188.2 ± 7.2, 79.8 ± 4.5 and 67.3 ± 2.5 μM, respectively. Their order of potency for 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity was: compound **5** > compound **3** > rutin > compound **8** > compound **1** > compound **2** > compound **7** > compound **6** > compound **4**. Compounds **3** and **5** were much more efficient than the other compounds. It was reported that the essential requirement for effective radical scavenging is the 3',4'-*ortho* dihydroxy configuration in ring B and the 4-carbonyl group in ring C of the flavonoid. The presence of the C_2-C_3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalization from ring B and it can increase the radical scavenging activity[27]. Compound

3 and **5** possess the 3',4'-*ortho* dihydroxy configuration in ring B of their flavonoid and hence show a strong 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity. Moreover, compound **5** has a hydroxyl group at position **3** and has the strongest 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, suggesting a structurally important role of the 3-OH group of the chroman ring for the enhancement of antioxidant activity.

Conclusion

The extraction conditions have significant effects on the yield of *A. lactiflora* total flavonoids. Using the surface and contour plots in the RSM was effective for estimating the effect of three independent variables (ethanol concentration, liquid-to-solid ratio and extraction time) on the yield. The optimum set of the independent variables obtained graphically was used to obtain the desired levels of total flavonoids during the practical extraction. The optimal experimental yield of 6.396 ± 0.013 % was obtained when the optimum conditions of the *A. lactiflora* total flavonoids extraction was ethanol concentration 53.5 %, liquid-to-solid ratio 22.95:1 (mL/g) and extraction time 23.2 min. Under this optimized condition, the experimental yield of *A. lactiflora* total flavonoids agreed closely with the predicted yield. The results obtained using four different methods to evaluate the *in vitro* antioxidant activity (2,2-diphenyl-1-picrylhydrazyl, ABTS, FRAP and FIC) showed that *A. lactiflora* purified can be considered a good source of natural compounds with significant antioxidant activity. In addition, 9 compounds were isolated from *A. lactiflora* total flavonoids for the first time. Among them, compound **5** (quercetin) with the highest 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, likely plays an important role in the antioxidant activity of this plant extract. This study suggests that *A. lactiflora* can provide valuable functional ingredients and have antioxidant potential for use in both medicine and food.

ACKNOWLEDGEMENTS

The authors gratefully acknowledged the financial support of this study by the Public Science and Technology Research Funds Projects of Ocean, China (No: 201305015-2), the Natural Science Foundation of Fujian Province (No: 2013J01380) and the Fundamental Research Funds for the Central Universities (No. JB-ZR1154 and JB-ZR1113).

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