Food Chemistry 186 (2015) 97–105

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Extraction, chemical composition and antioxidant activity of flavonoids from *Cyclocarya paliurus* (Batal.) Iljinskaja leaves

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ARTICLE INFO

Article history:
Available online 15 July 2014

Keywords:
Cyclocarya paliurus
Microwave-assisted extraction
Flavonoids
Antioxidant activity
Chemical composition
Response surface methodology

ABSTRACT

Microwave-assisted extraction of flavonoids from *Cyclocarya paliurus* (Batal.) Iljinskaja leaves, its chemical composition and antioxidant activity were investigated in this study. The influences of parameters including temperature, extraction time, ratio of material to solvent and solvents on the yield of flavonoids were studied. The optimal conditions were determined and the quadratic response surfaces were drawn from the mathematical models. The maximum extraction yield of 15.64 mg/g was achieved at temperature of 76.8 °C, extraction time of 15 min, alcohol concentration of 63.2% and ratio of solvent to material of 21.4:1. Five main constituents in the extract including quercetin-3-O-β-D-glucuronide, quercetin, kaempferol-3-O-β-D-glucuronide, kaempferol-7-O-α-L-rhamnoside and kaempferol were identified by LC–MS. *In vitro* antioxidant assays showed that the extract exhibited a strong DPPH radical-scavenging ability with IC_{50} value of 0.146 mg/mL. Results indicated that MAE was a suitable approach for the selective extraction of flavonoids from *C. paliurus* (Batal.) Iljinskaja leaves.

1. Introduction

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*) which belongs to the genus *Cyclocarya* Iljinskaja (Juglangdaceae), is the sole species in its genus, and mainly found in the mountainous regions in the tropics and subtropics (Xie, Li, Nie, Wang, & Lee, 2006). It grows mainly in the south of China, and is called "sweet tea tree" in Chinese (Xie, Shen et al., 2013). The leaves of *C. paliurus* are used in folk medicine for the treatment of diabetes mellitus, hypertension, hyperliposis, etc. (Li et al., 2000; Kurihara et al., 2003; Xie, Xie, Nie et al., 2010; Xie, Liu et al., 2013). The leaves of this plant have been widely used in China, which may be related to abundant organic compounds, especially flavonoids. Flavonoids are found to be the main active compounds in *C. paliurus* (Xie, Wang, Yi, & Wang, 2004), which have many biological activities, such as preventing hyperglycemia, diabetes mellitus, hypertension and coronary heart disease. Many flavonoids were isolated from *C. paliurus*, such as cyclocarioside, cyclocaric acid, kaempferol, quercetin and isoquercitrin. (Shu, Xu, & Li, 1996; Xie et al., 2004; Zhang et al., 2010).

Conventional techniques to obtain flavonoids, such as heating, boiling, or refluxing, usually require several hours or even days for the extraction process and a large volume of solvent, and may result in a loss of flavonoids due to hydrolysis, ionisation and oxidation during extraction (Li, Chen, & Yao, 2005). Microwave irradiation, which has proved to be a clean, efficient and convenient energy source, has been widely utilised in natural products extraction (Jiao et al., 2014; Xie et al., 2012; Zhang et al., 2013). Microwave-assisted extraction (MAE) is known to be a fast and efficient method for the extraction of flavonoids from plants. Compared with the traditional methods, MAE has many advantages, such as higher extraction rate, shorter extraction time, use of less solvent, better productivity and higher quality products (Chen, Xie, & Gong, 2007; Zhang, Yang, & Wang, 2011). Recently, MAE has been applied to extract various bioactive compounds from plants, such as terpenes from caraway seeds (Chemat, Aït-Amar, Lagha, & Esveld, 2005), quercetin from *Flos Sophorae* (Li et al., 2004) and flavonoids from cultivated *Epimedium sagittatum* (Zhang et al., 2013). A dynamic MAE system designed by Chen et al. (2008) markedly enhanced extraction yield of flavonoids from *Herba Epimedii*. To our knowledge, the optimised conditions of

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http://dx.doi.org/10.1016/j.foodchem.2014.06.106
0308-8146/

MAE used in extraction of flavonoids from *C. paliurus* have not been reported yet.

Response surface methodology (RSM), an important tool in process and product improvement, is a collection of experimental design and optimisation techniques that enables the researcher to determine the relationship between the response and the independent variables (Eren & Kaymak-Ertekin, 2007). To the best of our knowledge, there has been no report about the application of RSM in the optimisation of MAE conditions for the extraction of flavonoids from the leaves of *C. paliurus*.

The objective of this study was to optimise the MAE conditions for the extraction of flavonoids from *C. paliurus*. RSM was employed to study the optimal temperature, extraction time, solid–liquid ratio and concentration of alcohol, which could maximise the yield of flavonoids from *C. paliurus*. Then, the colorimetric method with $AlCl_3$/methanol system was used to quantify total flavonoids, which has been reported to be a simple, quick and accurate method (Zhao, Xu, & Liu, 2004). In addition, the antioxidant activity of flavonoids from *C. paliurus*, obtained under optimised MAE extraction conditions was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. The chemical composition of the MAE extract was also evaluated by liquid chromatography–mass spectrometry (LC–MS) analysis.

2. Materials and methods

2.1. Plant materials

The leaves of *C. paliurus* were collected in Xiushui County, Jiangxi Province, China. A voucher specimen was deposited at the State Key Laboratory of Food Science and Technology, Nanchang University, China. The leaves were air dried and ground into a fine powder (40–60 mesh) in a mill.

2.2. Chemicals and reagents

Methanol and formic acid (HPLC grade) were obtained from Merck (Darmstadt, Germany). Ethanol was purchased from Shanghai Chemicals and Reagents Co. (Shanghai, China). Quercetin was supplied by China Institute for Drugs and Biological Products Identification (Beijing, China). Butylated hydroxytoluene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid (V_C) were purchased from Sigma (St. Louis, MO, USA). All the other reagents were of analytical grade. Ultrapure water was prepared by Milli-Q50 (Millipore Corp., Bedford, MA, USA) water purification system. All solvents used for HPLC measurements were filtered (0.45 μm) before being used.

2.3. MAE

The MAE method was performed in a closed vessel unit MDS-2002AT (Shanghai Sineo Microwave Chemical Technology Co., Ltd., Shanghai, China). The system was equipped with a temperature sensor for monitoring and regulating the internal temperature of the extraction vessels. Maximum oven power for this system was 800 W. The pressure was pre-established at a safe limit about 1.5 MP which could not go beyond during the extraction process. Dry samples (2 g) were placed into 100 mL PFTE (CEM) extraction vessels and the solvent added. The container was capped, and the system was started-up. The exact temperature was detected by a sensor. When the desired temperature was reached, the heating device would automatically shut down for a while until the temperature dropped. The extraction temperature and time were set at different degrees according to different conditions. After extraction, the vessels were left for several minutes to cool down to room temperature. Then each extract was filtered and ethanol was added in order to make the total volume of 100 mL (Chen et al., (2007)).

2.4. Experimental design

RSM was used to design the MAE of total flavonoids from the leaves of *C. paliurus*. To explore the effect of independent variables on the response within the range of investigation, a central composite rotate design with four independent variables (X_1, temperature, X_2, extraction time, X_3, solid–liquid ratio and X_4, alcohol concentration) at five levels was performed. Each independent variable had coded levels of −2, −1, 0, 1 and 2. The experimental designs of the coded (x) and actual (X) levels of variables are shown in Table 1. The variables were coded according to Eq. (1):

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \tag{1}$$

where x_i is the coded value of an independent variable, X_i is the real value of the independent variable, X_0 is the real value of an independent variable at the centre point, and ΔX is the step change value.

The yield of flavonoids was considered as the dependent variable or response. For a central composite rotate design with four independent variables at five levels, 31 experimental runs are required. The actual design of experiments is given in Table 1.

The experimental results were fitted to a second-order polynomial model, and the regression coefficients were determined. The quadratic model for predicting the optimal point was expressed according to Eq. (2):

$$Y_k = b_{k0} + \sum_{i=1}^{4} b_{ki}x_i + \sum_{i=1}^{4} b_{kii}x_i^2 + \sum_{j=1}^{4} b_{kij}x_i x_j \tag{2}$$

where b_{k0}, b_{ki}, b_{kii} and b_{kij} are constant regression coefficients of the model, while x_i, x_j are the independent variables.

2.5. Determination of flavonoids content and yield of flavonoids

The content of flavonoids was determined by spectrophotometry using the aluminium chloride colorimetric method (Amir et al., 2012) with some modifications. Briefly, 0.5 mL diluted solution containing flavonoids was added to a 10 mL test tube, and 0.1 mL of 5% (w/w) $NaNO_2$ and 4 mL of 80% (v/v) ethanol were mixed for 5 min, and then 0.1 mL of 10% $AlCl_3$ (w/w) was added and mixed, 6 min later, 3 mL of 1 mol/L NaOH was added. After 15 min, the absorbance of the solution at 410 nm was measured with a double beam uv/vis spectrophotometer (TU-1900, PGENENAL, Beijing, China) against the same mixture, without the sample as a blank. The calibration curve was prepared by preparing rutin solutions at concentrations from 10 to 100 μg/mL in methanol. The concentration of total flavonoids in extract was expressed as mg of rutin equivalents per gram dry weight of extract. The calibration curve was determined to be as the following: $y = 0.0269x + 0.0054$, where y is absorbance value of sample, x is sample concentration (10–300 μg/mL) ($R^2 = 0.9996$).

In the present work, the percentage extraction of flavonoids was expressed according to Eq. (3):

$$\text{Percentage extraction (w/w)} = \frac{\text{Mass of flavonoids(in extracted solution)}}{\text{Mass of material(samples)}} \times 100\% \tag{3}$$

2.6. Determination of antioxidant activity

The total flavonoids obtained were subjected to screening for its possible antioxidant activity. The scavenging activity of flavonoids

Table 1
Experimental design runs in Statistical Analysis System (SAS version 9.0) and the observed responses value of extraction yield with different combinations of temperature (X_1), extraction time (X_2), solid–liquid ratio (X_3), and alcohol concentration (X_4).

Experiment number	Coded variables				Actual variables				Observed yield (mg/g)[a]
	Temperature (°C) x_1	Time (min) x_2	Solid–liquid ratio (w/v) x_3	Ethanol concentration (%) x_4	Temperature (°C) X_1	Time (min) X_2	Solid–liquid ratio (w/v) X_3	Ethanol concentration (%) X_4	
1	−1	−1	−1	−1	60	10	1:15	60	14.84
2	−1	−1	−1	1	60	10	1:15	80	14.47
3	−1	−1	1	−1	60	10	1:25	60	14.22
4	−1	−1	1	1	60	10	1:25	80	12.70
5	−1	1	−1	−1	60	20	1:15	60	15.22
6	−1	1	−1	1	60	20	1:15	80	13.99
7	−1	1	1	−1	60	20	1:25	60	14.36
8	−1	1	1	1	60	20	1:25	80	12.99
9	1	−1	−1	−1	80	10	1:15	60	15.55
10	1	−1	−1	1	80	10	1:15	80	14.47
11	1	−1	1	−1	80	10	1:25	60	16.12
12	1	−1	1	1	80	10	1:25	80	14.25
13	1	1	−1	−1	80	20	1:15	60	15.33
14	1	1	−1	1	80	20	1:15	80	14.77
15	1	1	1	−1	80	20	1:25	60	15.37
16	1	1	1	1	80	20	1:25	80	15.17
17	−2	0	0	0	50	15	1:20	70	12.85
18	2	0	0	0	90	15	1:20	70	14.96
19	0	−2	0	0	70	5	1:20	70	14.52
20	0	2	0	0	70	25	1:20	70	14.92
21	0	0	−2	0	70	15	1:10	70	14.55
22	0	0	2	0	70	15	1:30	70	14.55
23	0	0	0	−2	70	15	1:20	50	15.26
24	0	0	0	2	70	15	1:20	90	12.61
25	0	0	0	0	70	15	1:20	70	14.73
26	0	0	0	0	70	15	1:20	70	14.88
27	0	0	0	0	70	15	1:20	70	14.82
28	0	0	0	0	70	15	1:20	70	14.77
29	0	0	0	0	70	15	1:20	70	14.95
30	0	0	0	0	70	15	1:20	70	14.89
31	0	0	0	0	70	15	1:20	70	14.89

[a] Mean of duplicate runs.

from *C. paliurus* towards DPPH-radical was measured according to the method by Xie, Xie, Nie et al. (2010) with some modifications. Briefly, 0.2 mmol/L solution of DPPH in ethanol was prepared daily before measurements, 2 mL of various concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/g) of the sample solutions were thoroughly mixed with 2 mL of freshly prepared DPPH and 1 mL ethanol. The mixture was shaken vigorously and allowed to stand for 30 min in the dark, and the absorbance at 517 nm was then measured against a blank with a double beam uv/vis spectrophotometer (TU-1900, PGENENAL, Beijing, China). A lower absorbance value of the reaction mixture indicates a higher free radical scavenging activity. Ethanol was used as the blank control, ascorbic acid and BHT were used as positive controls. All tests were carried out in triplicate. The capability to scavenge the free radical DPPH in percentage of sample ($I\%$) was calculated according to the following equation:

$$I\% = \frac{A_0 - (A_2 - A_1)}{A_0} \times 100 \quad (4)$$

where A_0 is the absorbance of the incubation DPPH solution without addition of the sample or positive controls, A_1 is the absorbance of the sample without DPPH solution and A_2 is the absorbance of the incubation mixture containing both the test sample and DPPH solution. The concentration of sample producing a 50% reduction of the radical absorbance (IC_{50}) was used as an index to compare the antioxidant activity.

2.7. Liquid chromatography–mass spectrometry (LC–MS) analysis

Chemical composition of flavonoids extracted from the leaves of *C. paliurus* was analysed by LC–MS method (Coppin et al., 2013). Methanol and 0.1% (v/v) formic acid were used as the mobile phase. The elution program was as follows: 0–40 min (30–60% methanol), 40–60 min (60–80% methanol), 60–70 min (80–30% methanol). The flow rate was 0.8 mL/min and the injection volume was 5 μL. The UV detector was set at the wavelength of 270 nm and the column temperature was maintained at 30 °C. In order to reduce the contamination flow into the ion source, the eluent was discharged to waste for the first 2 min.

The ESI interface operated in the negative mode was used. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode and scanned from m/z 100 to 600. ESI was conducted by using a needle voltage of 3.5 kV under optimum collision energy level of 60%. Pure nitrogen (99.999%) was used as a dry gas and at a flow rate of 250 L/h and capillary temperature at 350 °C. Nitrogen was used as nebulizer at 60 psi. Drying nitrogen was heated to 150 °C. The chemical composition of flavonoids extracted from the leaves of *C. paliurus* were separated and identified by the retention time in HPLC and mass spectral data. The ion spray voltage was held at 3000 V in positive-ion mode.

2.8. Statistical analysis

All data collected from MAE extraction experiments were centred by using three parallel measurements of mean ± SD. The

data of RSM were analysed using Statistical Analysis System (SAS version 9.0, SAS Institute Inc., Cary, North Carolina, USA) and used to design central composite rotatable design and analyse the experimental data. Data from the quadratic general rotary design were analysed by multiple regressions. Fischer's test was used to determine the type of model equation, while the student's *t*-test was performed for the determination of statistical significance of regression coefficients. *P* value <0.05 was regarded as significant, and *P* value <0.001 was regarded as very significant.

3. Results and discussion

3.1. Effect of temperature, ratio of solvent to material, duration of microwave radiation and solvents

3.1.1. Effect of temperature

Experiments were conducted to study the effect of temperature on the yield of flavonoids from *C. paliurus*. The extraction was performed at 40, 50, 60, 70, 80 and 90 °C, respectively, the extraction time was fixed to 5 min, the ratio of solvent to material was fixed to 20:1, 90% ethanol was selected as solvent, and the other conditions were kept the same. Generally, when MAE is conducted in closed vessels, the temperature could be increased above the boiling point of the solvent. These elevated extraction temperatures reportedly resulted in improved extraction efficiencies, since desorption of analytes from active sites in the matrix increased (Xie, Xie, Shen et al., 2010). Additionally, the solubility of total flavonoids could greatly be enhanced by increased temperatures, while surface tension and solvent viscosity may decrease, which may improve sample wetting and matrix penetration, respectively (Chen et al., 2007).

Fig. 1a shows the effect of different extraction temperatures on extraction of total flavonoids. The results showed that the yield increased rapidly at first, then slowed down to reach an equilibrium concentration. As shown in Fig. 1a, increasing the temperature of the solvent from 40 to 80 °C significantly increased the extraction efficiency (from 6.17 ± 0.32 to 12.99 ± 0.25 mg/g). This may be because higher temperature causes intermolecular interactions within the solvent to decrease, giving rise to higher molecular motion, and increasing the solubility (Kassama, Shi, & Mittal, 2008). However, higher extraction temperatures beyond 80 °C did not show any significant improvement in the extraction yield (Fig. 1a). High extraction temperatures above 80 °C were not investigated because higher temperatures may not have further effects or have negative effects resulting from degradation or conversion of the analytes (Xie, Xie, Shen et al., 2010). Therefore, 80 °C was considered as the optimal temperature for MAE with the highest yield.

3.1.2. Effect of ratio of solvent to material

The effect of the ratio of solvent to material on the MAE process was carried out at 10:1, 15:1, 20:1, 25:1, 30:1, 35:1 and 40:1, respectively, while the other experimental conditions were as follows: extraction temperature of 80 °C, extraction time of 5 min, and solvent of 70% (v/v) ethanol. The results showed that the yield of flavonoids tended to increase (from 14.56 ± 0.22 to 15.42 ± 0.45 mg/g) as the ratio of solvent to material increased from 10:1 to 20:1 (Fig. 1b). It was probably due to the fact that more solvent can enter cells while more flavonoids can permeate into the solvent under the higher ratio of solvent to material condition (Shan, Xie, Zhu, & Peng, 2012). However, the extraction percentage of total flavonoids decreased rapidly at the ratios of solvent to material above 20:1. This was probably due to an inadequate stirring of the solvent when a large volume of solvent was heated in the microwave (Chen et al., 2007). So the ratio of solvent to material of 20:1 was optimal for the extraction of total flavonoids.

3.1.3. Effect of duration of microwave radiation

In order to determine the optimum duration of microwave radiation, approximately 2 g of dried material sample was extracted

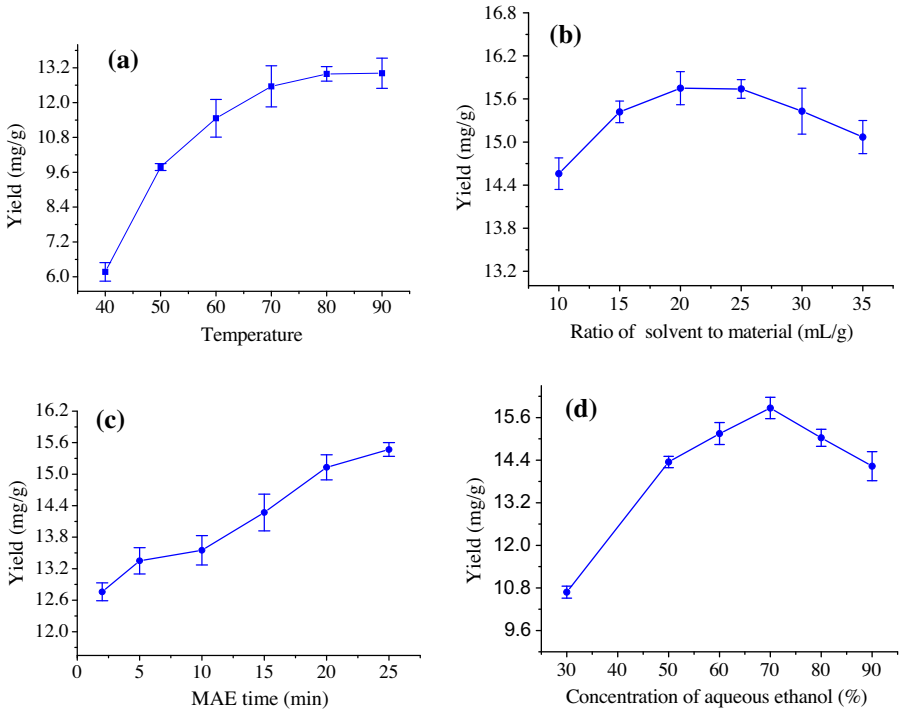

Fig. 1. The effect of temperature, ratio of solvent to material, duration of microwave radiation and the concentration of aqueous ethanol on the extraction yields: (a) temperature, (b) ratio of solvent to material, (c) extraction time, and (d) concentration of aqueous ethanol. Data represent means ± SD of three independent experiments.

with 40 mL of 90% (v/v) ethanol at 80 °C. The duration of microwave radiation was 3, 5, 10, 15, 20, 25 and 30 min, respectively.

Fig. 1c showed the effect of MAE time on the yield of flavonoids from the leaves of *C. paliurus*. It can be seen that the flavonoids yield increased from 12.76 ± 0.32 mg/g to 15.47 ± 0.23 mg/g as the extraction time was increased from 3 to 25 min. Further increases in extraction time resulted in little change in the yield of flavonoids. Therefore, the ranges of 5–25 min were used for further optimisation.

3.1.4. Selection of solvents

In general, solvent is considered an important parameter for MAE because it may affect the absorption of microwave energy and the solubility of the target ingredients (Zhang et al., 2011). Methanol and ethanol are the most hackneyed solvent to the extraction of flavonoids. But ethanol has several advantages to methanol, like higher extraction efficiency, environmental compatibility and lower toxicity and cost. So ethanol was used as the best solvent in this study.

In this study, the effects of different concentrations of ethanol (30–100%, v/v) on extraction efficiency were determined. As shown in Fig. 1d, the yield of flavonoids increased from 10.68 ± 0.17 to 15.87 ± 0.29 mg/g when the concentrations of aqueous ethanol varied from 50% to 70%. These results suggest that the addition of some amount of water can improve the extraction efficiency. This may be because water has a potential of increasing the polarity of aqueous ethanol. Another possible reason may be due to the increase in swelling of plant material by water, which increased the contact surface area between the plant matrix and the solvent (Hemwimon, Pavasant, & Shotipruk, 2007). However, the yield of total flavonoids decreased rapidly when ethanol concentration was above 70%. In other words, the highest extraction efficiency was achieved at 70% ethanol. Similar results were reported by Zhang et al. (2013) in the extraction of flavonoids from cultivated *E. sagittatum*. Therefore, 70% ethanol was selected as optimal solvent.

3.2. Optimisation of MAE operating parameters by RSM

3.2.1. Model fitting

The values of responses (yield of flavonoids) at different experimental combinations were given in Table 1. The significance of each coefficient was determined using the *p*-value (Table 2). The corresponding variables would be more significant if the *p*-value becomes smaller. It was found that the variables with the largest effect were the linear terms of extraction temperature (x_1), ethanol concentration (x_4) and the quadratic term of extraction temperature (x_1^2) and extraction time (x_2^2), followed by the quadratic term of ethanol concentration (x_4^2). These results showed that the effect on the yield of total flavonoids was decreased in the following order when MAE was used to extract total flavonoids from *C. paliurus*: extraction temperature ($p < 0.0001$), ethanol concentration ($p < 0.0001$) and the solid–liquid ratio ($p = 0.0585$). After analysis, the regression model can be described by the following quadratic polynomial in terms of coded values.

$$\begin{aligned} Y = {} & 14.84589 + 0.519321x_1 + 0.058069x_2 - 0.144003x_3 \\ & - 0.561876x_4 - 0.179501x_1^2 - 0.004643x_1x_2 \\ & + 0.314814x_1x_3 + 0.049813x_1x_4 + 0.024836x_2^2 \\ & + 0.040804x_2x_3 + 0.093083x_2x_4 - 0.018421x_3^2 \\ & - 0.107039x_3x_4 - 0.172299x_4^2 \end{aligned} \quad (5)$$

In the quadratic, there were some nonsignificant coefficients, but they were not neglected optionally, because they had relativity between each other (invariable coefficient and quadratic coefficient, quadratic coefficient and quadratic coefficient) (Lu, Song, & Guo, 2002). In order to make it more intuitionistic, Eq. (1) was taken into Eq. (5) and Eq. (6) obtained as follows (in terms of uncoded values). The model filled Eq. (6) was made response surface and contour plots to predict the relationships between the independent variables and the dependent variables as follows:

$$\begin{aligned} Y = {} & 8.841573 + 0.143832X_1 - 0.174649X_2 - 0.314694X_3 \\ & + 0.165053X_4 - 0.001795X_1^2 - 0.000093X_1X_2 \\ & + 0.006296X_1X_3 + 0.000498X_1X_4 + 0.000993X_2^2 \\ & + 0.001632X_2X_3 + 0.001862X_2X_4 - 0.000737X_3^2 \\ & - 0.002141X_3X_4 - 0.001723X_4^2 \end{aligned} \quad (6)$$

Table 2
Regression coefficient, standard error, and ANOVA for the regression model of MAE extraction conditions.

Parameter	DF[a]	SS[b]	Mean square	*F*-value	Pr > F
x_1	1	6.472659	6.472659	53.96844	0.0001
x_2	1	0.080929	0.080929	0.674776	0.4235
x_3	1	0.497687	0.497687	4.149669	0.0585
x_4	1	7.576907	7.576907	63.17556	0.0001
x_1x_1	1	0.921374	0.921374	7.682334	0.0136
x_2x_1	1	0.000345	0.000345	0.002875	0.9579
x_2x_2	1	1.585723	1.585723	13.22161	0.0022
x_3x_1	1	0.039701	0.039701	0.33102	0.5731
x_3x_2	1	0.017639	0.017639	0.147073	0.7064
x_3x_3	1	0.026639	0.026639	0.222115	0.6438
x_4x_1	1	0.13863	0.13863	1.155881	0.2983
x_4x_2	1	0.009704	0.009704	0.080908	0.7797
x_4x_3	1	0.183317	0.183317	1.528478	0.2342
x_4x_4	1	0.848917	0.848917	7.078194	0.0171
Linear	4	14.62818	3.657045	30.49211	0.0001
Quadratic	4	1.703792	0.425948	3.551515	0.0295
Crossproduct	6	1.974354	0.329059	2.743664	0.0499
Model	14	18.30633	1.307595	10.90261	0.0001
Lack of fit	10	1.885254	0.188525	33.57298	
Error	16	1.918946	0.119934		
Pure error	6	0.033692	0.005615		
Total model	30	20.22527			
$R^2 = 0.991$					
Adj. $R^2 = 0.962$					

[a] DF, degrees of freedom.
[b] SS, sum of squares.

3.2.2. Analysis of variance

The coefficients of the above Eq. (6) were calculated, and the linearity and quadratic effect of the treatment variables, their interactions and coefficients on the response variables were obtained by analysis of variance (ANOVA) (Table 2). The results suggested a good fit with the Eq. (6) because the model was acceptable at $P = 0.0001$ and adequate with satisfactory coefficient of determination (R^2) of 96.2%. The predicted model seemed to reasonably represent the observed values. Thus, the response was sufficiently explained by the model. The factor *F*-test value (53.968) and *p*-value ($P < 0.001$) correspond to temperature (x_1), and *F*-test value and *p*-value corresponding to ethanol concentration (x_4) were 63.176 and 0.0001 ($P < 0.001$), while the *F*-test values for x_2 and x_3 were smaller (0.6748 and 4.1497, respectively). These results suggested that the temperature and ethanol concentration were directly related to the flavonoids yield. Furthermore, the model *F*-value of 10.903 also showed that the model was significant (Table 2). In addition, the value of R^2 (0.991) implied that the sample variations of 99.1% for the yield of flavonoids was attributable to the independent variables, and the adjusted R^2 n (AdjR^2) of the equation was 0.962 (Table 2), suggesting an excellent correlation between the independent variables. The values of R^2 and *P* ($P < 0.005$ when very significant) were 0.991 and 0.0001,

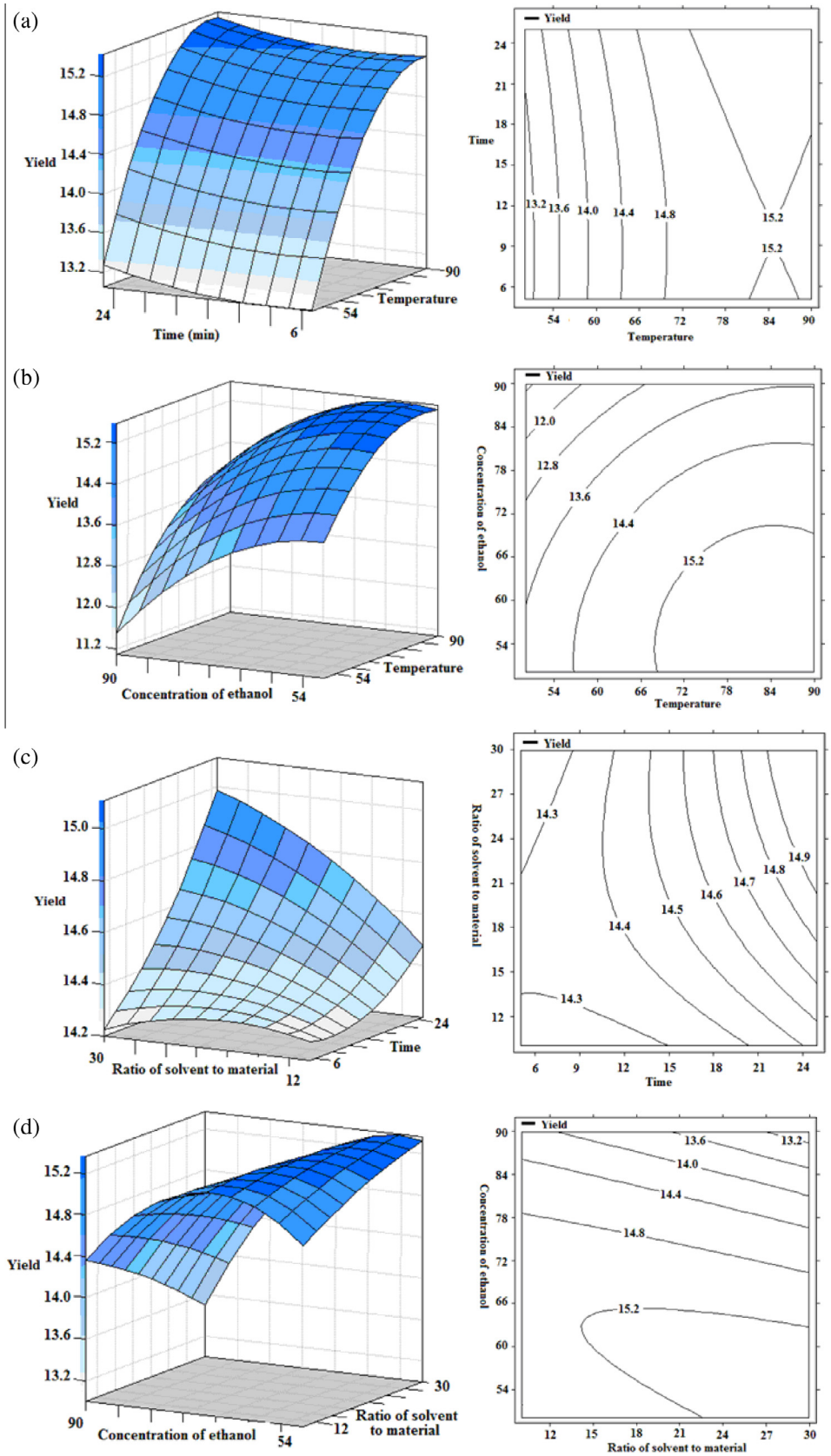

Fig. 2. Response surfaces and contours plots for flavonoids extracted from *C. paliurus* leaves using MAE. (a) extraction temperature (X_1) and extraction time (X_2), (b) extraction temperature (X_1) and ethanol concentration (X_4), (c) extraction time (X_2) and ratio of solvent to material (X_3), and (d) ratio of solvent to material (X_3) and ethanol concentration (X_4).

respectively (Table 2), which indicated a good agreement between the experimental and predicted values of the flavonoids yield. These results also suggested that the extraction yield was primarily determined by the linear and quadratic terms. Eq. (6) also showed that the linear effect was positive and quadratic effect was negative, and the coefficients of interaction were insignificant. Furthermore, the predicted results, according to the model for flavonoids yield, were close to the observed experimental responses, indicating that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters.

3.2.3. Analysis of response surfaces

Through these 3D plots and their respective contour plots, it was very easy to understand the interactions between two variables and also to determine their optimum levels. The relationship between independent and dependent variables is illustrated in 3D representation of the response surfaces and 2D contour plots generated by the model for flavonoids (Fig. 2). Two variables were depicted in one 3D surface plot while the other two variables were kept constant.

As shown in Fig. 2a temperature exerted a quadratic effect on flavonoids production, but extraction time only had a slight effect to the total flavonoids yield, and the interactions between these two factors were insignificant (Table 2). The extraction yield was significantly time-dependant and increased with extended temperature, especially from 50 to 80 °C. The results indicated that the efficient extraction period for achieving maximum yield of flavonoids was about 80 °C. The results have shown that increasing extraction temperature in the extraction process was better than prolonging extraction time. Both temperature and ethanol concentration had a positive linear effect on the flavonoids yield (Fig. 2b). The total flavonoids yield rapidly increased with the increase of temperature, while decreased with the increase of ethanol concentration. A higher yield of total flavonoids can be obtained at a lower level of ethanol. This may be due to the presence of many glycosides in *C. paliurus*. A high yield of flavonoids can be obtained when lower ethanol concentration and higher extraction temperature were used.

Fig. 2c is the response surface and contour plots showing the effects of extraction time (X_2) and ratio of solvent to material (X_3) on the yield of flavonoids. The effect of extraction time on the flavonoids yield is shown in Fig. 2a. Again, these results indicated that both factors have slight effects on the total flavonoids yield, which is the same as the results in Table 2. The interaction effect was not significant (p value of $x_3x_2 = 0.71$). Fig. 2d described the interaction effects of the ratio of solvent to material (X_3) and ethanol concentration (X_4). The effect of ratio of solvent to material on the yield of total flavonoids displayed a linear increase when concentration of ethanol was lower while decrease when concentration of ethanol was higher. So a lower level of ethanol and higher ratio of solvent to material can result in a higher yield of total flavonoids.

3.2.4. Optimal processing conditions and verification of results

The optimum values of selected variables were selected on the basis of response surface. In summary, the optimal conditions of MAE process to obtain the highest flavonoids yield (15.64 ± 0.76 mg/g) from the leaves of *C. paliurus* were determined as follows: temperature of 76.8 °C, ratio of solvent to material of 21.4:1, concentration of alcohol of 63.2% and extraction time of 15 min.

The suitability of the model equation for predicting the optimum response values was tested by executing three experiments under five different conditions. The experimental yield of flavonoids from the leaves of *C. paliurus* was close to the predicted yield (Supplementary Table 1S). There was not statistically different at 3% level of significance between the experimental and predicted values. The results indicated that the experimental values were in good agreement with the predicted ones, and the regression model was accurate and adequate for the extraction process.

3.3. Antioxidant activity

Analysis of DPPH· scavenging ability is a widely used method to evaluate antioxidant activity in relatively short time compared to some other methods. The antioxidant activity of flavonoids from *C. paliurus* leaves was assessed with DPPH·-scavenging assay, in comparison with known antioxidants ascorbic acid and BHT. The DPPH radical-scavenging capacity of flavonoids from *C. paliurus* leaves is shown in Fig. 3.

It was observed that the flavonoids from *C. paliurus* leaves exhibited notable DPPH radical-scavenging activity, and the DPPH radical scavenging effects were increased with increasing concentrations. The antioxidant activity of flavonoids from *C. paliurus* leaves increased from 38.32 ± 0.32% to 94.68 ± 0.88% when the concentrations of the flavonoids increased from 0.1 to 0.8 mg/mL. The antioxidant activity (94.68 ± 0.88%) of flavonoids at 0.8 mg/mL was higher than that of BHT (68.52 ± 0.44%) (Fig. 3). These results indicated that flavonoids extracted by MAE had a strong DPPH radical-scavenging activity, with an IC_{50} value of 0.146 mg/mL. However, DPPH free radical-scavenging of CPP-1 was less than that of V_C, but, it was higher than that of BHT, a synthetic antioxidant, with an IC_{50} value of 0.667 mg/mL. Currently, little information is available on the antioxidant activity of flavonoids from *C. paliurus* leaves. It is already well established that there is a close linkage between oxidative stress caused by the action of free radicals and many forms of human diseases such as autoimmune disease, cancer, cardiovascular disease, senile dementia, and age-related functional declines. Antioxidant substances which scavenge free radicals play an important role in the prevention of free radical induced diseases. The most commonly used antioxidants at present time are butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA). However, these synthetic antioxidants have side effects such as liver damage and carcinogenesis. Therefore, it is more important to find the novel, effective and nontoxic compounds from natural sources. Recently, research interest has focused on flavonoids from various plants and herbs, which has the potential to become an useful complementary as it have putative health benefits, low cytotoxicity and do not cause significant side effect. Based on the

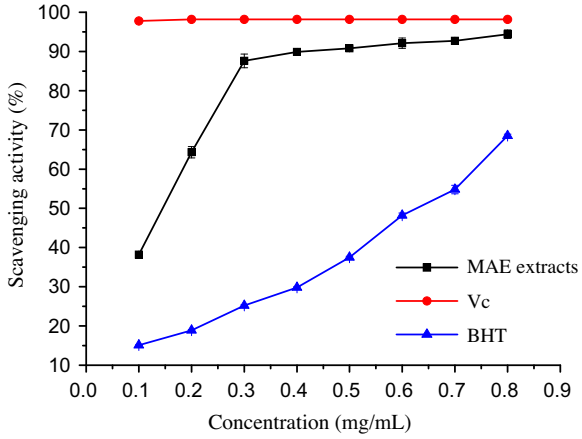

Fig. 3. Antioxidant activity of flavonoids from *C. paliurus* leaves assessed by the DPPH radical-scavenging assay. Data represent means ± SD of three independent experiments.

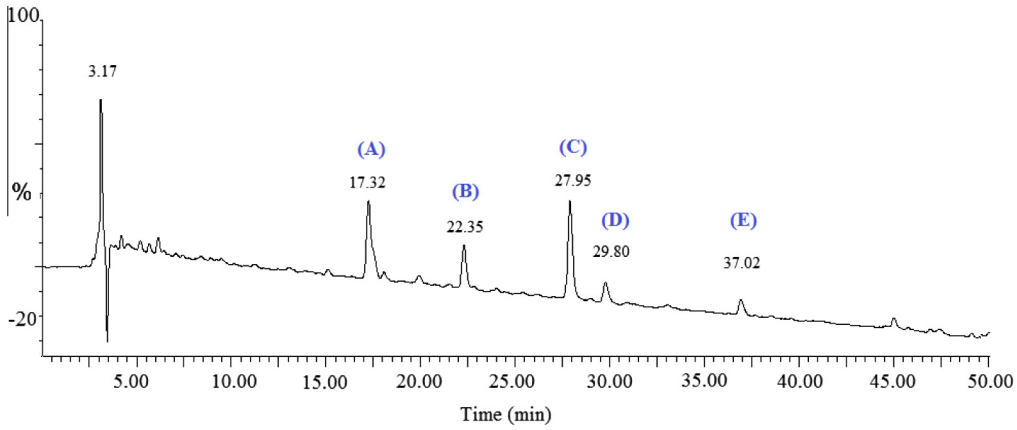

Fig. 4. Typical LC–MS chromatograms of flavonoids from *C. paliurus* leaves obtained by MAE. Peak A, quercetin-3-O-β-D-glucuronide; Peak B, kaempferol-3-O-β-D-glucuronide; Peak C, kaempferol-7-O-α-L-rhamnoside, Peak D, quercetin; and Peak E, kaempferol.

results obtained, we can conclude that the flavonoids from *C. paliurus* leaves may be potentially used as a health-promoting agent with antioxidant activity in human diets, as well as providing valuable natural antioxidants in functional foods or medicine.

3.4. Analysis of the flavonoids in C. paliurus by LC–MS

The LC–MS chromatograms of the flavonoids in the leaves of *C. paliurus* were given in Fig. 4. Five constituents were identified as quercetin-3-O-β-D-glucuronide, quercetin, kaempferol-3-O-β-D-glucuronide, kaempferol-7-O-α-L-rhamnoside and kaempferol, according to the data of quercetin standard and the literature (Fang et al., 2011; Li et al., 2005; Xie et al., 2004). The five flavonoids were further confirmed according to the MS chromatograms of the five compounds (Supplementary Fig. S1). Pharmacological and nutritional studies have found that these five compounds exhibited various activities such as antioxidant, antiosteoporotic, antitumor and immunoregulatory effects (Boots, Haenen, & Bast, 2008; Calderon-Montaño, Burgos-Moron, Perez-Guerrero, & Lopez-Lazaro, 2011; Xiao, Muzashvili, & Georgiev, 2014). Therefore, these five constituents in the extracts may be partly responsible for the antioxidant activity observed in flavonoids obtained by MAE.

4. Conclusions

Microwave irradiation, which has proved to be a clean, efficient and convenient energy source, has been utilised in the extraction of flavonoids from *C. paliurus*. RSM was successfully applied for optimisation of MAE of flavonoids from *C. paliurus* leaves. It was effective for estimating the effect of five independent variables. Both the temperature and concentration of ethanol had highly significant effects on the response value. The optimal flavonoids yield of 14.97 mg/g from 1 g dried *C. paliurus* leaves was obtained when the optimum conditions (temperature of 76.8 °C, extraction time of 15 min, concentration of alcohol of 63.2% and ratio of material to solvent of 1:21.4) were used. Application of MAE in the extraction of flavonoids from *C. paliurus* leaves dramatically reduced extraction time. Under optimised conditions the experimental yield agreed closely with the predicted yield. The flavonoids obtained by MAE exhibited good antioxidant activity with an IC_{50} value of 0.146 mg/mL. Thus, MAE was proposed as an alternative for the extraction of flavonoids from *C. paliurus* leaves, and RSM was an useful tool for the optimisation of MAE process of flavonoids from *C. paliurus* leaves.

Conflict of interest

Authors declare no conflicts of interest.

Acknowledgements

This work was supported by the research projects of Chinese National Natural Science Fund (No. 31201297), the Research Foundation for Objective-Oriented Project of State Key Laboratory of Food Science and Technology, Nanchang University, China (No. SKLF-ZZA-201301), and the Science Funds of Educational Commission of Jiangxi Province, China (No. GJJ14144).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.106.

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