Tropical Journal of Pharmaceutical Research December 2013; 12 (6): 1045-1051
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v12i6.27

Original Research Article

Ultrasonic-Assisted Extraction and Antioxidant Activity of Flavonoids from *Adinandra nitida* Leaves

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Received: 5 September 2013 *Revised accepted: 11 October 2013*

Abstract

***Purpose**: To identify the main flavonoid in Adinandra nitida leaf, employ response surface methodology to optimize its ultrasonic-assisted extraction, and determine the antioxidant activity of the obtained extract.*
***Methods**: The main flavonoid in Adinandra nitida leaf was obtained by traditional solvent extraction and recrystallization methods, and identified by ultraviolet-visible spectroscopy (UV), Fourier transform infrared spectroscopy (FT-IR), electrospray ionization-mass spectrometry (ESI-MS), nuclear magnetic resonance spectroscopy (NMR). By using response surface methodology, the effects of extraction time, ethanol concentration and ultrasonic frequency on flavonoid yield were investigated and optimal conditions determined. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and reducing power of the obtained extract was also examined.*
***Results**: The main flavonoid in Adinandra nitida leaf was identified as camellianin A. Optimal extraction conditions were as follows: extraction time, 30.25 min; ethanol concentration, 63.84 %; and ultrasonic frequency, 45 KHz. The mean experimental flavonoid yield under optimum conditions was 84.52 ±1.65 %, which agreed with the predicted value of 83.02 %. The obtained flavonoid extract was an effective scavenger of DPPH radicals with IC_{50} of 0.02 mg/mL. A linear correlation between concentration of the flavonoid extract and reducing power was observed with a coefficient of $r^2 = 0.9867$.*
***Conclusion**: By using ultrasonic-assisted extraction, the main flavonoid in Adinandra nitida leaf can be obtained at a high yield. The flavonoid displays a strong DPPH radical scavenging activity and reducing power, which makes it potentially useful in the food and pharmaceuticals industries.*

***Keywords:** Adinandra nitida, Ultrasonic-assisted extraction, Response surface methodology, Flavonoid, Antioxidant.*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Adinandra nitida, a particular wild plant in South China, is a kind of flavonoid-rich plant source. Its leaves have been consumed as health tea (Shiyacha) and herbal medicine for hundreds of years. It is reported to have many curative effects, such as reduction of blood pressure, antibacterial, antitumor, anti-toxicity, and analgesic activities. It has been reported that the flavonoid content of its leaves could be > 20 % [1-3].

The classical techniques for the solvent extraction of materials from plants are based upon the correct choice of solvent coupled with the use of heat and/or agitation. The extraction of organic compounds contained

within the body of plants and seeds by a solvent is significantly improved by the use of power ultrasound. The mechanical effects of ultrasound provide a greater penetration of solvent into cellular materials and improves mass transfer. There is an additional benefit for the use of power ultrasound in extractive processes which results from the disruption of biological cell walls to facilitate the release of contents [4].

Response surface methodology (RSM) with appropriate experimental designs, e.g., central composite design (CCD), has been effectively applied to optimize the intended parameters in the extraction and modification of bioactive compounds [5,6]. The objective of this study was to identify the main flavonoid in *Adinandra nitida* leaves, employ response surface methodology to optimize its ultrasonic-assisted extraction, and determine the antioxidant activity of the obtained

EXPERIMENTAL

Materials and chemicals

Leaves of *Adinandra nitida*, collected in Pingle city, Guangxi Province, China, and was identified by Dr. Guihua Xu of Henan Institute of Science and Technology. A voucher specimen (NO. LBG-1) was deposited in the Food Chemistry Lab of Henan Institute of Science and Technology. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and butylated hydroxyanisole (BHA) were purchased from Sigma. All other reagents were of analytical grade.

Preparation of the main flavonoid in *Adinandra nitida* leaf

About 20.00g of the powdered leaves of *Adinandra nitida* was extracted with 300 ml of 60 % ethanol in a water bath at 80 °C for 5 h and then filtered. The filtrate was concentrated under vacuum at 45 °C and freeze-dried (Alpha 1-4, Christ, Germany), about 7.67 g of the extract was obtained. The obtained extract was purified to get the crystal by recrystallizing for 8 times from water. After drying at 60 °C, 0.3959 g of a light yellow product was obtained.

Identification of the main flavonoid in *Adinandra nitida* leaf

UV analysis was performed on a TU-1810PC spectrophotometer (Purkinje, China) and the spectra of the flavonoids in methanol were recorded and processed by UV Win 5.0.5 software (Purkinje, China). IR analysis was performed on a TENSOR 27 infrared spectrophotometer (Bruker, Germany) and the data were recorded and processed by OPUS 4.0 software. ESI-MS^2 analyses were carried out on a LCQ Deca XP MAX electrospray ionization mass spectrometer (Finnigan, USA) in the negative ion mode. ^{13}C-and ^{1}H-NMR spectra were recorded in DMSO-d_6 using a DRX-400 NMR spectrometer (Bruker, Germany) at 400 MHz.

Ultrasonic-assisted extraction

The ultrasonic-assisted extraction was performed using a KQ-200VDE ultrasonic bath (Kunshan Ultrasonic Instrument Co. Ltd., Kunshan City, Jiangsu Province, China) with the output power 200 W, which could work at three ultrasonic frequency of 45, 80, 100 KHz. The sample of 1 g of the dried powder was placed in a flask and extracted with 40 mL of ethanol at different concentration at different ultrasonic frequencies for different times and then filtered under vacuum. The filtrate was diluted to 100 mL for determining the flavonoid content.

Flavonoid yield

The flavonoid content in the filtrate was estimated by UV spectrometry. The absorbance of the suitably diluted sample at 330 nm was determined by using a TU-1810PC UV spectrophotometer (Purkinje, Beijing, China). The flavonoid content was calculated with reference to camellianin A standard.
The dried powdered leaves (1.0056 g) were placed in a Soxhlet extractor and refluxed at 80 °C for 10 h with 150 mL methanol, and then the extract was collected and diluted to 250 mL with methanol to determine the flavonoid content in raw material.

The flavonoid yield (Y) was defined as the ratio of total flavonoid in the filtrate to total flavonoid in raw material.

Experimental design

A three-level, three-factor, central composite design (CCD) was employed, in which 39 experiments were involved, and the flavonoid yield (Y) was used as response in evaluating the extraction (Table 1). The factors and levels studied were determined on the basis of the factorial experiments, such as extraction time (5, 20, and 35 min), ethanol concentration (40, 70, and 100 %), and ultrasonic frequency (45, 80, and 100 KHz). The CCD combined the vertices of a hypercube whose coordinates are given by the 2^n factorial design with the “star” points. The star points were added to the factorial design to

provide for estimation of curvature of the model. Six replicates (No. 2, 6, 15, 27, 30 and 34) at the center of the design were used to allow for estimation of "pure error" sum of squares. Experiments were randomized in order to minimize the effects of unexplained variability in the observed response due to extraneous factors.

Preparation of *Adinandra nitida* leaf extract

The powdered leaves of *Adinandra nitida* (2 g) were extracted with 80 mL of 63 % ethanol at 45 KHz for 30 min and then filtered under vacuum. The filtrate was collected and freeze-dried (Four-Ring Science Instrument Plant Beijing Co, Ltd, China), The obtained extract was gained for the following antioxidant assays.

DPPH radical scavenging assay

DPPH radical scavenging assay was done according to a previously published method [7]. Briefly, 2 mL of DPPH solution (0.2 mmol/L, in ethanol) was incubated with different concentrations of the extract, BHA. The reaction mixture was shaken and incubated in the dark for 30 min, at room temperature. And the absorbance was read at 517 nm against ethanol. Controls containing ethanol instead of the antioxidant solution, and blanks containing ethanol instead of DPPH solution were also made. The inhibition of DPPH radical by the samples, i.e., DPPH activity, was calculated according to Eq 1.

DPPH activity (%) = 100{Ac – (As – Ab)}/Ac …… (1)

where Ac, As and Ab and the absorbance of control, sample and blank, respectively.

DPPH radical scavenging activity was plotted against sample concentration to obtain the IC_{50}, defined as the concentration of sample required to cause 50 % inhibition.

Reducing power assay

The reducing power of the sample was determined according to a published method [8]. 0.5 mL of the extract in ethanol was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbance was measured at 700 nm. Increased absorbance of reaction mixture indicated reducing power.

Data analysis

Experimental data from CCD were analyzed by means of RSM to fit the quadratic polynomial equation with the Design Expert software (version 8.0, State-Ease, Inc, Statistics Made Easy, Minneapolis, MN). The quadratic polynomial equation is shown in Eq 2.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \sum \beta_{ij} x_i x_j \ldots\ldots\ldots\ldots \quad (2)$$

where β_0 was the value of the fitted response at the center point of the design, which is point (0, 0, 0). β_0, β_i, β_{ii}, and β_{ij} were the constant, linear, quadratic and cross-product regression terms, respectively.

RESULTS

Main flavonoid in *Adinandra nitida* leaf

Based on UV, IR, ESI-MS and NMR, the flavonoid obtained in this study was identified as camellianin A (Figure 1).

UV, λ_{max}(nm) (MeOH) 263, 330; IR bands (KBr disc): 3384 (-OH), 1733 (Ester bond), 1630 (-C=O), 1579, 1516, 1495, 1454 (-Ar) cm^{-1}; ESI-MS^2 negative ion *m/z*: 681.92 ($[M+NO_3]^-$), 654.87 ($[M+Cl]^-$), 619.39 ($[M-H]^-$), 578.08 ([M-Acetyl-H]$^-$), 474.02 ([M-Rham-H]$^-$), 269.12 ([M-Acetyl-Rham-Glu-H]$^-$); ^{13}C-NMR: 176.31 (C-4), 170.70 (C-7″), 162.82 (C-2), 161.37 (C-4′), 160.90 (C-7), 159.36 (C-9), 157.68 (C-5), 128.46 (C-2′ and C-6′), 121.98 (C-1′), 116.51 (C-3′ and C-5′), 107.95 (C-10), 106.43 (C-3), 100.28 (C-1″), 99.75 (C-6), 97.79 (C-1‴), 96.94 (C-8), 77.47 (C-4″), 77.11 (C-3″), 74.03 (C-5″), 72.83 (C-2″), 71.11 (C-3‴), 71.02 (C-4‴), 70.55 (C-2‴), 69.25 (C-5‴), 63.50 (C-6″), 20.90 (C-8″), 18.52 (C-6‴); ^{1}H-NMR: 10.73 (1H, s, 7-OH), 10.20 (1H, s, 4′-OH), 7.86 (2H, d, J = 8.6 Hz, 2′- and 6′-H), 6.92 (2H, d, J = 8.6 Hz, 3′- and 5′-H), 6.62 (1H, s, 8-H), 6.53 (1H, s, 3-H), 6.50 (1H, s, 6-H), 5.51 (1H, d, J = 6.0 Hz, 1″-H), 5.20 (1H, s, 1‴-H), 3.16-4.64 (10H, m, Hs in sugar), 1.87 (3H, s, 8″-H), 1.09 (3H, d, J = 6 Hz, 6‴-H).

Diagnostic checking of the fitted model

The result of the central composite design is shown in Table 1. Multiple regression analysis of the experimental data yielded the second-order polynomial stepwise equations shown in Eqs 3 - 5.

When X_3 was 45 KHz, the model was as following:

$Y=20.07809+1.06062X_1+1.59667X_2+7.59259\times10^{-5}\times X_1X_2-0.017479\times X_1^2-0.013549\times X_2^2$ …… (3)

When X_3 was 80 KHz, the model was as following:

$Y=15.93643+1.10706\times X_1+1.58428\times X_2+7.59259\times10^{-5}\times X_1X_2-0.017479\times X_1^2-0.013549\times X_2^2$ …… (4)

When X_3 was 100 KHz, the model was as following:

$Y=24.11173+0.89695\times X_1+1.58428\times X_2+7.59259\times10^{-5}\times X_1X_2-0.017479\times X_1^2-0.013549\times X_2^2$ ……(5)

Table 1: Central composite design arrangement and results

Experiment	Coded level			Flavonoid yield (%)
	Extraction time (min)	*Ethanol concentration (%)*	*Ultrasonic frequency (KHz)*	
	X_1	X_2	X_3	Y
1	0 (20)	1 (100)	0 (80)	53.71
2	0 (20)	0 (70)	0 (80)	75.94
3	0 (20)	-1 (40)	1 (100)	75.54
4	0 (20)	0 (70)	1 (100)	78.95
5	1 (35)	-1 (40)	0 (80)	75.21
6	0 (20)	0 (70)	1 (100)	77.28
7	-1 (5)	1 (100)	0 (80)	45.23
8	0 (20)	1 (100)	1 (100)	50.87
9	1 (35)	-1 (40)	-1 (45)	77.11
10	0 (20)	0 (70)	-1 (45)	79.73
11	1 (35)	1 (100)	1 (100)	50.53
12	1 (35)	0 (70)	-1 (45)	79.73
13	0 (20)	0 (70)	-1 (45)	77.22
14	-1 (5)	0 (70)	-1 (45)	73.87
15	0 (20)	0 (70)	0 (80)	73.76
16	0 (20)	-1 (40)	-1 (45)	76.05
17	1 (35)	0 (70)	1 (100)	72.08
18	1 (35)	0 (70)	0 (80)	81.74
19	1 (35)	1 (100)	0 (80)	54.22
20	-1 (5)	1 (100)	1 (100)	49.11
21	-1 (5)	-1 (40)	1 (100)	67.17
22	-1 (5)	-1 (40)	-1 (45)	68.17
23	-1 (5)	0 (70)	1 (100)	62.65
24	0 (20)	0 (70)	1 (100)	78.11
25	1 (35)	-1 (40)	1 (100)	74.60
26	0 (20)	0 (70)	-1 (45)	78.82
27	0 (20)	0 (70)	0 (80)	75.21
28	-1 (5)	0 (70)	0 (80)	65.38
29	-1 (5)	-1 (40)	0 (80)	63.37
30	0 (20)	0 (70)	0 (80)	76.27
31	-1 (5)	1 (100)	-1 (45)	45.11
32	0 (20)	0 (70)	1 (100)	71.96
33	0 (20)	-1 (40)	0 (80)	70.58
34	0 (20)	0 (70)	0 (80)	76.72
35	1 (35)	1 (100)	-1 (45)	63.32
36	0 (20)	0 (70)	-1 (45)	83.00
37	0 (20)	1 (100)	-1 (45)	59.13
38	0 (20)	0 (70)	-1 (45)	79.12
39	0 (20)	0 (70)	1 (100)	75.54

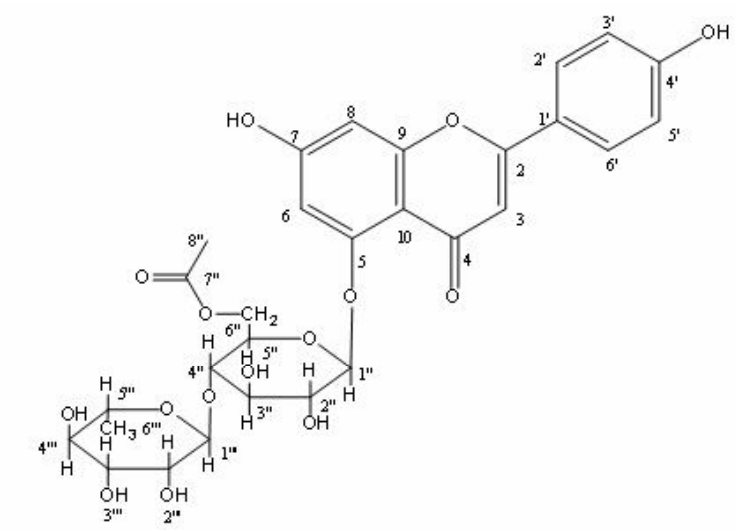

Figure 1: Chemical structure of camellianin A

Table 2: ANOVA for the fitted model

Source	Sum of squares	d_f	Mean square	F value	Prob>F
Model	4314.16	11	392.20	55.89	<0.0001
X_1	434.93	1	434.93	61.98	<0.0001
X_2	1732.05	1	1732.05	246.83	<0.0001
X_3	152.74	2	76.37	10.88	0.0003
X_1X_2	0.014	1	0.014	1.996×10^{-3}	0.9647
X_1X_3	32.89	2	16.45	2.34	0.1152
X_2X_3	16.19	2	8.09	1.15	0.3306
X_1^2	128.15	1	128.15	18.26	0.0002
X_2^2	1232.12	1	1232.12	175.58	<0.0001
Residual	189.47	27	7.02		
Lack of Fit	135.39	15	9.03	2.00	0.1156
Pure Error	54.08	12	4.51		
Cor Total	4503.62	38			

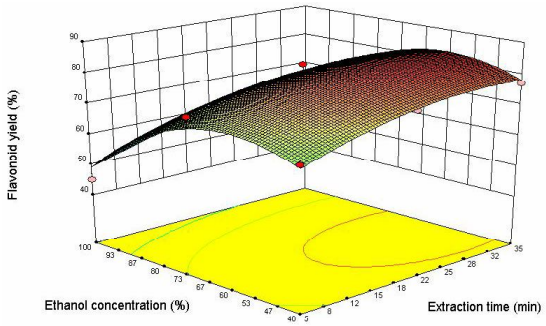

Figure 2: Effect of extraction time and ethanol concentration on flavonoid yield

The result of ANOVA is shown on Table 2. The Model F-value of 55.89 implies the model is significant. There is only a 0.01 % chance that a "Model F-Value" this large could occur due to noise. The Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X_1, X_2, X_3, X_1^2, X_2^2 are significant model terms. The Values greater than 0.1000 indicate the model terms are not significant. The “Lack of Fit F-value” of 2.00 implies the Lack of Fit is not significant relative to the pure error. There is an 11.56% chance that a "Lack of Fit F-value" this large could occur due to noise. The "Pred R-Squared" of 0.8907 is in reasonable agreement with the "Adj R-Squared" of 0.9408. The above diagnostic checking of the ftted model showed the models could be used to navigate the design space.

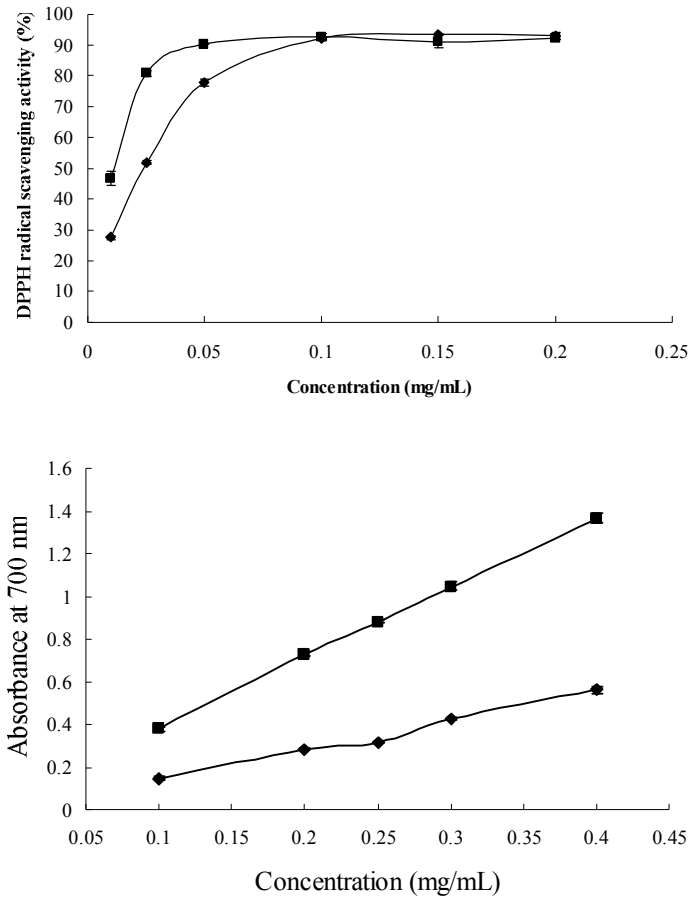

Figure 3: DPPH radical scavenging activity and reducing power of the extract (-♦-) and BHA (-■-)

Optimization of ultrasonic-assisted extraction

The effect of extraction time and ethanol concentration on flavonoid yield was shown in Figure 2. The optimum conditions were obtained by running the program of Design Expert software. The optimum conditions for independent variables and the predicted values of the responses also were presented as follows: extraction time 30.25 min, ethanol concentration 63.84 % and ultrasonic frequency 45 kHz. The estimated values for flavonoid yield, 83.02% was obtained at those conditions. A verification experiment at the optimum condition, consisting of 3 runs, was performed and the practical yield of 84.52 ± 1.65 % was obtained.

Antioxidant activities

In this study, a high DPPH radical scavenging activity was observed in both the extract and BHA in a concentration manner (Figure 3). The DPPH radical scavenging activity of the extract (IC_{50}, 0.02 mg/mL) was lower than that of BHA (IC_{50}, 0.01 mg/mL). Figure 3 also showed the reducing power of the extract and BHA. Both the samples showed some degree of reducing power but the reducing power of BHA was superior to that of the extract. The reducing power of the samples linearly increased with increasing concentration and the correlation coefficient (r^2) of the extract and BHA was 0.9867 and 0.9997, respectively.

DISCUSSION

In this study, by forming inclusion complex with HP-β-CD, the solubility of *Adinandra nitida* is potentially of great commercial interest in the food and phyto-pharmaceutical markets. In this study, the main flavonoid in *Adinandra nitida* leaves was identified as camellianin A, which supports the report of Yuan *et al* [9]. With camellianin A obtained by recrystallization 8 times from water as standard, the flavonoid content in the raw materials was17.91 %. To the best of our knowledge, there are few plant sources containing so much flavonoid as the leaf of *Adinandra nitida*.

When many factors and interactions affect desired responses, response surface methodology (RSM) is an effective tool for

optimizing the process. The basic principle behind response surface methodology (RSM) analysis is to relate the observed value (dependent variables) to process parameters (independent variables) using statistical methods, yielding a multivariate regression equation, often of second-order.

RSM takes interactions into consideration and optimizes the process parameters to reasonable range, with the advantage of less the number of replicates and the total time required to perform the experiments [10-11]. The relationship between the variables and responses can be better understood by examining the three-dimensional response surface plots, as shown in Figures 2, whose regression coefficients are generated from the predicted models. In this study, it was found that the performance of 45 KHz was superior to that of 80 or 100 KHz, which should attribute to the higher vibration intensity at 45 KHz. Figure 2 showed the effects of ethanol concentration, extraction time, and their mutual interaction on the flavonoid yield when the ultrasonic frequency was at 45 KHz. The maximum flavonoid yield appeared in the extraction time range of 28-35 min and the ethanol concentration range of 60-67%. Higher extraction time resulted in higher flavonoid yield at the same ultrasonic frequency, but the increase becomes smoother when the extraction time exceeded 28 min. And a suitable increment in the ethanol concentration (40-67%) increased the flavonoid by increasing the solubility of flavonoids in the solution. However, a higher extraction exceeding the optimal range of 60-67% had negative effects on flavonoid yield. Based on the models, the optimum condition could be determined as extraction time 30.25 min, ethanol concentration 63.84 % and ultrasonic frequency 45 kHz.

The antioxidant activity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals; therefore, it is important to employ commonly accepted assays to evaluate the antioxidant activity of plant extract. Numerous antioxidant methods have been developed to evaluate antioxidant activity and to explain how antioxidants function. Of these, reducing power, DPPH assay is the most commonly accepted assay to evaluate antioxidant activity [12]. It was found that the extract was an effective scavenger in quenching DPPH radicals and showed some degree of reducing power. Based on the above result, the flavonoid-rich extract could be widely used in could be used in food and pharmaceuticals industries.

CONCLUSION

The main flavonoid in plant leaf was identified as camellianin A. The optimum conditions of ultrasonic-assisted extraction of flavonoids from *Adinandra nitid* leaves were determined by using response surface methodology. The findings of this work indicate that the flavonoid-rich plant resource and its extract are a potential new source of natural antioxidant and health food with great commercial prospects in the food and pharmaceuticals industries.

ACKNOWLEDGEMENT

The financial support provided by the National Natural Science Foundation of China (31101232 and 21166024) is greatly appreciated.

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