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Original Research Article

Ultrasonic-Assisted Extraction and Evaluation of Biological Activities of Flavonoids from *Flemingia philippinensis* Merr et Rolfe

Hua Li, Qiyu Lu and Ke Bian*
Department of Food Science, Henan University of Technology, Zhengzhou, 450001, Henan, China

For correspondence:* ***Email:* *lixian78101@163.com;* ***Tel/Fax:*** *+86-371-67758022*

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Abstract

Purpose: *To develop a simple and rapid method for extracting total flavonoids from the roots of Flemingia philippinensis and to investigate the antioxidant and anti-tumor activities of the extracts of the materials from various locations in China.*
Methods: *The total flavonoids in F. philippinensis were obtained by ultrasonic-assisted conventional solvent extraction method, and the extraction conditions were optimized by single factor and orthogonal test. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and anti-tumor activities, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, of the extract were evaluated. The contents of flemiphilippinin A, auriculasin, 5,7,3',4'-tetrahydroxy-6,8 –diprenylisoflavone and dorsmanins I were also determined.*
Results: *Optimal extraction conditions were as follows: extraction time, 40 min; methanol concentration, 85 %; and solvent to solid ratio, 40 mL/g; and number of extraction, once. Total flavonoid content varied greatly (3.7 - 14.35 %) among the 19 samples collected from different origins in China. Flemiphilippin A, 5,7,3',4'- tetrahydroxy-6,8 -diprenylisoflavone, auriculasin and dorsmanins I showed varying DPPH radical scavenging activities with effective half maximal concentration (EC_{50}) of 18.36, 23.59, 57.25 and 63.54 µg/mL, respectively. Flemiphilippinin A (5 µg/mL) also exhibited some level of antitumor activity against human hepatocellular carcinoma cell (BEL-7402), human lung epithelial (A-549) and human ileocecal adenocarcinoma cell (HCT-8) with inhibition of 91.13 ± 1.6, 91.22 ± 3.23, and 79.77 ± 3.57 %, respectively.*
Conclusion: *Total flavonoids can be extracted efficiently from F. philippinensis by ultrasonic-assisted extraction method. Flemiphilippinin A has a potential for use in medicine as an antioxidant and antitumor drug.*

Keywords: *Flemingia philippinensis, Flemiphilippinin A, Ultrasonic-assisted extraction, Flavonoids, Antioxidant, Antitumor*

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INTRODUCTION

Flemingia philippinensis Merr. et Rolfe, which belongs to the plant family Leguminosae, is mainly distributed in tropical areas of China. The traditional usage of the roots of *F. philippinensis* is for the treatment of rheumatism, arthropathy, leucorrhea, menalgia, menopausal syndrome, chronic nephritis, improving bone mineral density [1-3]. Modern pharmacological studies show that the extract from the roots of *F. philippinensis* exhibits anti-oxidative and anti-inflammatory activities due to its isoflavones [4]. The estrogenic and antiestrogenic bioactivities of the

methanol extract were confirmed by the effects on the proliferation of MCF-7 cells and induction of β-galactosidase activity [5]. Flavonoids are the main constituents of *F. philippinensis* [3,6-12], and many of them have pharmacological activities. Total flavonoids can be used to evaluate the quality of the herb [13,14].

To the best of our knowledge, there are no reports on total flavonoids content to evaluate the quality of the herb *F. philippinensis* and explore the antitumor activity. In this study, ultrasonic-assisted extraction of total flavonoids and antioxidant and antitumor activity of the flavonoids isolated from *F. philippinensis* were carried out.

EXPERIMENTAL

Chemicals and apparatus

Nineteen samples (numbered 1 - 19) of *F. philippinensis* were collected from different locations in China, mainly in Guangxi Province, in the spring of 2009. They were identified by Professor Xiaojun Ma, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. Voucher specimens (nos. FP001 - FP019) were deposited in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College. Standard genistein, flemiphilippinin A, auriculasin, 5,7,3',4'-tetrahydroxy-6,8 –diprenyl-isoflavone and dorsmanins I were purified by the first author to a level of ≥ 99 %, using a previously reported procedure [8-10].

All the cell lines were purchased from the Cell Culture Center of Institute of Basic Medical Science, Chinese Academy of Medical Sciences. 1,1-diphenyl-β-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide were purchased from Sigma Chemical Co. while Medium RPMI-1640 was purchased from Gibico Co. All other reagents were of analytical grade.

Total flavonoid extraction

The roots were dried at < 60 ^{o}C in an oven, powered to 40-mesh, weighed (0.5027 g) and placed in a 25 mL volumetric flask to which methanol was added to make up to volume. The flask was sealed, shaken well, and then extracted ultrasonically. The flavonoid content of the filtrate was determined spectrophotometrically at 258 nm (UV-1700 UV spectrophotometer, Shimadzu Corporation, Japan). The flavonoid content was calculated with reference to genistein standard. The flavonoid yield (Y) was defined as the ratio of total flavonoid in the filtrate to total flavonoid in raw material.

Extraction design

The effect of sonication time (A), methanol concentration (B), solvent volume (C) and extraction times (D) on the yield of total flavonoids were investigated.

A three-level, four-factor, orthogonal design was employed, in which 9 experiments ($L_9 3^4$) were involved, and the flavonoid yield (Y) was used as response in evaluating the extraction (Table 2). The factors and levels studied were determined based on the single factor experiments, such as A (20, 40 and 60 min), B (70, 85 and 100 %), C (10, 20 and 30 mL) and D (1, 2 and 3 times).

DPPH radical scavenging activity of extract

DPPH radical scavenging activity was measured according to previous studies method [15]. Methanol solution of DPPH 2 mL of 0.2 mM radicals was incubated with a series of different concentrations of the extract or flavonoids. The absorbance of the mixture was measured at 517 nm after 30 min of incubation at room temperature in the dark. Ascorbic acid (Vc) was used as the control and methanol as the blank.

The scavenging effect was calculated using equation 1.

$$\text{Scavenging effect \%} = (1 - AS/AB) \times 100 \quad \ldots\ldots (1)$$

where AS and AB stand for absorbance obtained for a sample (the extract, flavonoid or Vc) and the blank, respectively.

Table 1: Single factor experimental design

	Extraction time (A, min)	Methanol concentration (B, %)	Solvent volume (C, mL)	Extraction times (D, times)
1	10, 20, 30, 40, 50, 60	70	20	1
2	20	50, 60, 70, 85, 90, 100	20	1
3	20	70	5, 10, 15, 20, 25, 30	1
4	20	70	20	1, 2, 3

DPPH radical scavenging activity was plotted against sample concentration to obtain the EC_{50}, which represents concentration of extract required to scavenge 50 % of DPPH radicals.

Antitumor test

Human hepatoma cell line BEL-7402, human lung carcinoma cell line A-549 and human colorectal cancer cell line HCT-8 were cultured in RPMI-1640 medium with 10 % new-born bovine serum (NBS), and incubated at 37 ^{o}C in a humidified atmosphere that contained 5 % CO_2. For the experiment, cells were seeded into 96-well plates at a density of 5 × 10^3 cells/well. After 24 h, triplicate wells were treated with media and agents. Fluorouracil (5-FU) was used as positive control. After 72 h incubation at 37 ^{o}C in 5 % CO_2, the drug-containing medium was removed and replaced by 100 µL fresh medium with 0.5 mg/mL MTT solution. After 4 h incubation, the medium with MTT was removed and 200 µL DMSO was added to each well. The plates were gently agitated until the color reaction was uniform and the OD570 was determined using a microplate reader (Wellscan MK3, Lab systems Dragon).

Statistical analysis

Each experimental value is expressed as the mean ± standard deviation (n = 3). Statistical analysis was performed using Graphpad prism 5.0 and data analyzed using one way analysis of variance (ANOVA) for comparison between groups followed by Dunnett's multiple comparison test at a significant level of $p < 0.05$.

RESULTS

Optimization of extraction conditions

As shown in Figure 1, the optimal conditions were as follows: sonication time, 40 min; methanol concentration, 85 %; solvent volume, 20 mL and number of extraction, once for 0.5 g root powder.

The results of orthogonal design experiments were shown in Table 2. In order to keep consistent with single factor test, the extraction conditions were optimized as follows: sonication time, 40 min; methanol concentration 85 %; solvent volume was 20 mL and number of extraction, once, at room temperature for 0.5 g of sample no. 4. The effect of the factors was in the rank order: B > A > D > C in the ranges studied.

Total flavonoid content

The total flavonoid content in 19 samples were determined by calibration curve of genistein (Y = 0.1726X−0.0756 with r^2=0.9996) and repeated three times for each under the optimal conditions. The results in Table 3 showed that the total flavonoid content were of great difference

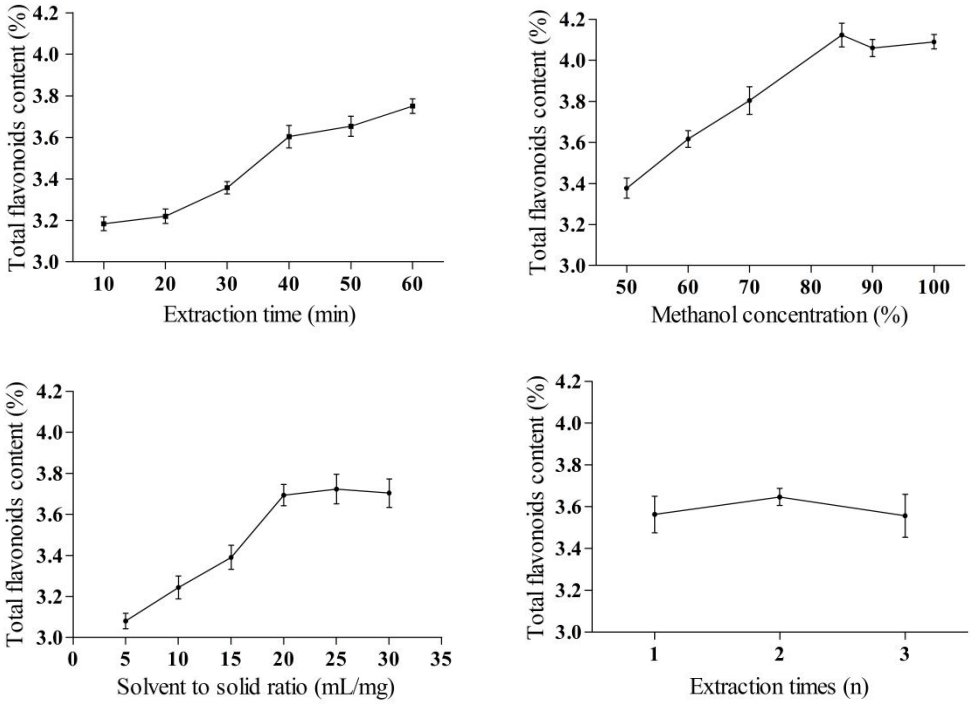

Figure 1: Effect of different levels of each factor on the yield of total flavonoids

Table 2: Analysis and results of orthogonal design ($L_9 3^4$)

Variable	Extraction time (A, min)	Methanol concentration (B, %)	Solution volume (C, mL)	Extraction times(D, times)	Total flavonoids content (y_{jk},%)
1	20	70	10	1	4.50
2	40	85	20	1	4.75
3	60	100	30	1	4.31
4	40	70	30	2	4.38
5	60	85	10	2	4.51
6	20	100	20	2	4.25
7	60	70	20	3	4.41
8	20	85	30	3	4.38
9	40	100	10	3	4.09
$\overline{y}_{j1}$	4.38	4.43	4.37	4.52	
$\overline{y}_{j2}$	4.41	4.55	4.47	4.38	
$\overline{y}_{j3}$	4.41	4.22	4.35	4.29	
R_j	0.03	0.24	0.12	0.23	
Optimized level	A2	B2	C2	D1	

between 19 samples in which the lowest was 3.75 % from Yunnan and the highest was 14.35 % from Daoxian Hunan. In addition, the content of total flavonoids in the xylem was different from that in phloem, the result showed that total flavonoids in xylem was much more than in phloem.

Antioxidant activity

The antioxidant activity of 85 % methanol extract of *F. philippinensis* (specimen nos. 1 - 19) and flavonoid compounds isolated from *Zhongshan guangxi* sample is reported in Figure 2.

The extracts of 19 samples exhibited DPPH radical scavenging activities in the range 12.20 ± 1.41 % to 42.69 ± 1.07 % at concentration of 25 µg dry matter/mL, which suggested that the activities remarkably consisted with the content of their total flavonoids extracted under the optimal condition.

Four flavonoids, flemiphilippinin A, auriculasin, 5,7,3',4'-tetrahydroxy-6,8- diprenylisoflavone and dorsmanins Ⅰ, exhibited DPPH radical scavenging activities in a concentration dependent manner, in the range of 5 to 50 mg/mL (Figure 2). Flemiphilippinin A was found to have the highest DPPH scavenging activity (86.76 %), followed by auriculasin (79.38 %), 5,7,3',4'-tetrahydroxy-6,8- diprenylisoflavone (51.67 %) and dorsmanins Ⅰ (48.41 %), compared to a standard concentration of 50 mg/mL (Figure 2). The EC_{50} values (µg/mL) in descending order were flemiphilippinin A (18.36) > auriculasin (23.59) > 5,7,3',4'-tetrahydroxy-6,8-diprenyl-isoflavone (57.25) > Dorsmanins (63.54), while the EC_{50} values of positive control Vc was 8.20 µg/mL.

Antitumor activity

The results for antitumor activities are presented in Table 3.

Flemiphilippinin A exhibited significant cytotoxic activity to three human cancer cell lines (HCT-8, A-549 and BEL-7402) *in vitro*, while three others showed no inhibitory activity against the three cancer cell lines. The antitumor activity of flemiphilippinin A against the three human cancer cell lines were quantitatively assessed further which was indicated in terms of IC_{50} (ug/mL) values, which tested concentration were 0.005, 0.05, 0.5, 5 ug/mL. The results showed that flemiphilippinin A exhibited a potent inhibitory effects on the indicated cell lines, especially for HCT-8, with an IC_{50} value of 1.17, which higher than the positive control 5-FU with IC_{50} value 1.4. The IC_{50} values of flemiphilippinin A inhibiting on A-549 and BEL-7402 were more than 5.0, which were higher than those of 5-FU, respectively, with IC_{50} value of 1.8 and 0.5.

DISCUSSION

In the present study, total flavonoid was extracted and analyzed antioxidant activity using scavenging DPPH radical activity. The antioxidant and antitumor activities of the four

Table 3: Total flavonoid content and DPPH radical scavenging activity of 19 samples from different locations in China

No.	Sample	Total flavonoids (%)	Summary[a] ($p < 0.05$)	DPPH radical scavenging activity[b] (%)	Summary[a] ($p < 0.05$)
FP001	Lipu Guangxi	5.29 ± 0.15	ns	14.29 ± 2.70	ns
FP002	Ningming Guangxi	10.57 ± 0.35	***	26.17 ± 0.68	***
FP003	Zhongshan Guangxi	11.54 ± 0.08	***	32.50 ± 1.39	***
FP004	Zhongshan Guangxi	5.00 ± 0.56	--	16.78 ± 1.67	--
FP005	Laitu Guangxi	9.38 ± 0.06	***	24.65 ± 2.38	***
FP006	Jingxi Guangxi	9.24 ± 0.08	***	24.30 ± 2.72	***
FP007	Fuzhou Guangxi	8.32 ± 0.20	***	20.85 ± 0.49	ns
FP008	Pingle Guangxi	4.52 ± 0.14	ns	20.00 ± 2.23	ns
FP009	Gongcheng Guangxi	12.71 ± 0.11	***	40.11 ± 2.50	***
FP010	Longsheng Guangxi	9.29 ± 0.04	***	25.52 ± 1.15	***
FP011	Shangsi Guangxi	10.73 ± 0.11	***	29.10 ± 2.39	***
FP012	Rongshui Guangxi	5.37 ± 0.50	ns	18.03 ± 2.19	ns
FP013	Jinxiu Guangxi	4.50 ± 0.13	ns	15.60 ± 1.42	ns
FP014	Tianlin Guangxi	5.10 ± 0.07	ns	18.48 ± 2.31	ns
FP015	Daoxian Hunan	14.35 ± 0.06	***	42.69 ± 1.07	***
FP016	Chengdou Sichuan	4.48 ± 0.09	ns	18.90 ± 2.55	ns
FP017	Sichuan	4.81 ± 0.13	ns	21.27 ± 1.29	*
FP018	Guangdong	4.20 ± 0.14	**	16.53 ± 0.59	ns
FP019	Yunnan	3.74 ± 0.36	***	12.20 ± 1.41	*

*[a]Statistical significance (no. 4 sample acted as control, p < 0.05); *significant; **very significant; ***highly significant; ns = not significant; [b]DPPH radical scavenging activity was at concentration of 25 µg dry matter/mL*

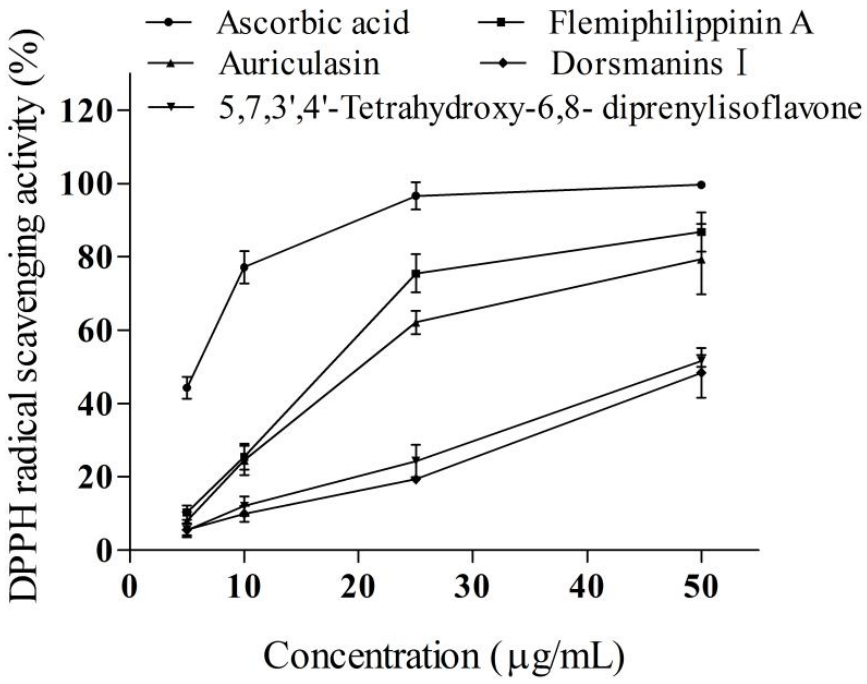

Figure 2: DPPH radical scavenging activity of flavonoids isolated from *F. philippinensis*

Table 3: Anti-tumor activity of the extract and flavonoids from *F. philippinensis* (sample no. 4) used in preliminary screening

Sample *	Inhibition rate (%)		
	BEL-7402[a]	A-549[b]	HCT-8[c]
Methanol extract	39.67	44.84	47.91
Flemiphilippinin A	91.13	91.22	79.77
Auriculasin	64.98	3.33	47.35
5,7,3',4'-Tetrahydroxy-6,8 – diprenylisoflavone	0.68	5.15	9.65
Dorsmanins I	0.15	0.43	13.84

**Anti-tumor activity determined at a concentration of 5 µg/mL for each samples; [a]BEL-7402 = human hepatocellular carcinoma cell; [b]A-549 = human lung epithelial; [c]HCT-8 = human ileocecal adenocarcinoma cell*

flavonoids isolated from *F. philippinensis* was also evaluated.

Ultrasonic-assisted extraction is a common and valid method to extract phytochemical compounds, including volatile constituents [16], flavonoid [17] and polysaccharides [18], etc. The orthogonal test design is a method that utilizes the orthogonal experiment tables to analyze experiment results scientifically. It provides a reasonable amount of information for testing the optimal combination from a fewer number of assays, therefore reducing more experiments associated with the analysis [19]. Under the optical extraction condition optimized with orthogonal design ($L_9 3^4$), total flavonoids concentration was in agreement with those evaluated by HPLC [20].

In the main peaks of the fingerprints, 14 peaks were identified by comparison of their retention times and UV absorption spectra with those of standard compounds which were separated from *F. philippinensis*. The identified peaks belonged to flavonoid. From the HPLC results, the most flavonoid compounds in this material were extracted under these extraction conditions. The total content of each flavonoid in the fingerprint was consistent with the result for total flavonoids. Therefore, using this method to extract and analyze total flavonoids in *F. philippinensis* is rational and feasible.

A preliminary study on antioxidant and antitumor activities of twelve flavonoids, isolated from *F. philippinensis*, suggested that there are only four flavonoids with antioxidant activities and one with antitumor activities. The results showed that the prenylated flavonoids (eg. flemiphilippinin A) had higher antioxidant activity than the prenylated ones (e.g., erythrinin B and genistin), this may be related to the hydroxyl number and three-dimensional structure of the molecule, which can easily capture the free radicals caused by steric hindrance effect due to different prenyl groups. The underlying molecular phenomena of the radical scavenging activity of flavonoids can be explained by the ease of hydrogen atom abstraction and the ease of the termination of the flavonoid aroxyl radicals [21].

CONCLUSION

Among the materials tested, the plant material from Daoxian Hunan demonstrated the strongest radical scavenging, as well as the highest flavonoid contents. Flemiphilippinin A has high antioxidant and antitumor activities at low concentrations. The findings of this work also indicate that *F. philippinensis* is a potential new source of natural antioxidants and that flemiphilippinin A can be developed as an antioxidant and antitumor agent with great commercial prospects in the pharmaceuticals industry.

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