ANALYSIS OF FLAVONOIDS FROM LEAVES OF *ACANTHOPANAX BRACHYPUS* HARMS

HAO-BIN HU*, XU-DONG ZHENG, HUAI-SHENG HU

College of Chemistry and Chemical Engineering, Longdong University, Qingyang 745000, China

(Received: March 1, 2012 - Accepted: November 27, 2012)

ABSTRACT

Flavonoids were extracted from the leaves of *Acanthopanax brachypus* Harms by microwave-assisted extraction (MAE), and four known flavonoids were isolated from the extracts by column chromatography. On the basis of physico-chemical constants and spectral data, their structures were elucidated as quercetin, quercitrin, rutin and hyperin. The influences of experimental conditions, such as solvent concentration, solution-solid ratio, microwave irradiation time and power on the extraction yields were discussed in orthogonal experiments. The four flavonoids content in the leaves was determined by HPLC analysis. The analytical results reveal that *A. brachypus* leaves are abundant in flavonoids, the contents of quercetin, quercitrin, rutin and hyperin are 0.86, 1.65, 2.19 and 2.68 mg·g⁻¹, respectively.

Key Words: *Acanthopanax brachypus* Harms, Araliaceae, Flavonoids, Microwave-assisted extraction, HPLC.

1. INTRODUCTION

Acanthopanax genus belonging to the Araliaceae family includes 37 species (excluding variety) around the world, it is widely distributed in Korea, Japan, China and the far-eastern region of Russia. About 26 species and 18 varieties grow in mainland China.¹ The roots and stem barks of these species have traditionally been used as a health drink, tonic and sedative,² as well as for the treatment of rheumatism, diabetes, chronic, bronchitis, hypertension, gastric ulcer, antistress and ischemic heart disease,³ and are popularly used as a health supplement in Korea.⁴ *Acanthopanax brachypus* Harms is the shrubs of *Eleutherococcus senticosus* Maxim in *Acanthopanax* genus, which is narrowly distributed in Northwest Plateau area, and it is an endemic and endangered species in China.⁵ Research indicates that its fruits can relax women's menopause syndrome and exhibit immunostimulatory and anticancer activities, and its rhizomatic extracts has also been successfully used for inhibition of various allergic responses in China and Korea.⁶ Nowadays, the root, leaves and flower are employed for various therapeutic purposes.⁷

Flavonoids are present in widespread medical plants and show many bioactivities,⁸ and are widely used as remedies because of their spasmolytic, antiphlogistic, and diuretic properties.⁹ It was also indicated in epidemiological studies that their consumption always assured a reduced risk of cancer and cardiovascular disease.^{10,11} Preliminary TLC investigations revealed that flavonoids are abundant in *A. brachypus* leaves. So it is significant to develop an efficient extraction and analysis method of flavonoids from *A. brachypus*.

It is well known that extraction is the key step of the separation and determination of the effective constituents in medicinal plants, so it is very important to develop a fast and effective extraction method. Up to now, many conventional extraction techniques have been performed, such as the room temperature extraction, the reflux extraction, Soxhlet extraction, ultrasonication extraction, and so on, which are labor-intensive, time-consuming and sample amount-requiring. More recently, microwave-assisted extraction (MAE) has been extensively investigated in the extraction of the effective constituents in medicinal plant¹² because the extraction time, solvent and energy exhausting can be reduced and extraction yield and selectivity can be improved.¹³ MAE is a process in which solvent in contact with the sample is heated by microwave energy in order to extract some chemical components from the sample matrix into the solvent. The usage of microwave energy as a heat source causes the selective heating of the plant matrix over the extractant.¹⁴ The highly localized temperature and pressure can cause the selective migration of target compounds from the material to the surroundings at a more rapid rate and gain similar or better recoveries compared with the conventional extractions.¹⁵ In recent years, it has been reported that many effective constituents in medicinal plants are extracted by MAE, such as ginsenosides from *Ginseng* roots,¹⁶ flavonols from *Flos Sophorae*¹⁷ and *Herba Epimedii*.¹⁸ In this paper, flavonoids were extracted from *A. brachypus* leaves by MAE method, and isolated by column chromatography. Their structures were elucidated as quercetin, quercitrin, rutin and hyperin, on the basis of physico-chemical constants and spectral data. The influences of experimental conditions on the extraction yields were discussed in orthogonal experiments. The four flavonoids content in the leaves was determined by HPLC analysis. We hope that our contribution will provide

scientific basis for further development and utilization of the leaves of *A. brachypus*.

2. EXPERIMENTAL

2.1 Apparatus and Materials

Melting points were determined on X-4 digital micro-melting point apparatus and are uncorrected. UV spectra were recorded on a Shimadzu UV-300 spectrophotometer, IR spectra on a Perkin-Elmer 577 spectro-meter with KBr pellets, and NMR spectra were recorded with a Bruker AMX-400 spectrometer (400 MHz for ¹H-NMR and 100 MHz for ¹³C-NMR). ESI-MS were performed using a Finnigan LCQ ion trap spectrometer equipped with a Finnigan electrospray source. An Agilent 1100 liquid chromatograph was used for HPLC analysis. Extraction experiments were carried out in a MSP-100E microwave extraction apparatus. Column chromatography was performed with Silica-gel H and Sephadex LH-20.

Ethanol of analytical grade was obtained from Beijing Chemical Factory (Beijing, China). Methanol and acetic acid used in the HPLC analysis were all of HPLC grade and purchased from Fisher (UK). The standards of hyperin, rutin, quercitrin and quercetin were purchased from the Chinese Authenticating Institute of Material and Biological Products (Beijing, China).

The leaves of *A. brachypus* Harms were collected from the Ziwlung mountains in Gansu Province (China) in June 2010, and identified by Prof. Xiao-qiang Guo from Life-Science College of Longdong University. A voucher specimen (No. 20070810732) was deposited in the Herbarium of Life-Science College, Longdong University, China. The dried leaves were crushed to powder to be studied.

2.2 Extraction and Isolation

The leaves of *A. brachypus* (1 kg) were ground and extracted with 50% ethanol under microwave power 300 W and irradiation time 8 min. The concentrated extract was suspended in H₂O and partitioned with petroleum ether, EtOAc and *n*-BuOH, successively. The EtOAc-soluble fraction was concentrated and chromatographed over silica gel using CHCl₃-MeOH (25:1→1:20, V/V), and combined after TLC detection to give nine fractions. Fraction 4 was repeatedly chromatographed on silica gel with CHCl₃-MeOH (5:1→1:4, V/V) to provide ten sub-fractions. Subfractions 4-7 were further purified by CC on Sephadex LH-20 (MeOH) and preparative TLC to get 1 (6 mg), 2 (10 mg), 3 (12 mg) and 4 (8 mg), respectively.

2.3 Method of Content Determination

2.3.1 Preparation of Sample

The sample powder of 1.000 g was weighed accurately, and then put in a 250 mL distillation flask, to which 30 mL of 50% ethanol was added. The mixture was heated by microwave irradiation time 8 min and power 300 W. Meanwhile, a reflux condenser was installed. The extract was filtered and then transferred into a 100 mL volumetric flask. The distillation flask and the sediment were rinsed three times. The rinsed solvent was also added into the flask, and then more solvent was added to the mark, this solution was used for HPLC analysis.

2.3.2 Chromatographic Conditions

HPLC analyses were performed with an Agilent 1100 liquid chromatograph

equipped with a Zorbax ODS C_{18} column (4.6 m $\times$ 250 mm, 5 μ m). The mobile phase was composed of methanol and water (0.2% acetic acid). A gradient procedure was used: 0-30 min, 15-50% methanol, 85-50% water. The temperature of the oven was adjusted at 32°C. The flow rate was kept at 1.0 mL/min and the fraction eluted was monitored by using DAD at 254 nm.

All of standard and extractive solutions obtained were centrifuged at 3500 r/min (10 min) and filtered through a 0.45 μ m Millipore membrane and injected into the HPLC system.

2.3.3 Preparation of Standard Solutions and Calibration Curves

Peak areas from the HPLC chromatogram were plotted against the known concentrations of standard solutions of varying concentrations to establish the calibration equations $y = ax + b$, where y represents the response value of the analyte in the sample (peak area), x the mass concentration of each analyte,

a slope value, and b intercept value. The standard solutions of quercetin, quercitrin, hyperin and rutin, were prepared by accurately weighing 0.010 g of the standard and dissolving in 10 mL of acetic acid/methanol (90:10, pH=3) solution. For the calibration curves, the standard solutions were diluted with water/methanol (90:10, pH=3) solution to get the required concentrations. The linear range and the linear regression equations were obtained through a sequence of 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 μ g/mL concentrations, and each determination was carried out in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were defined by relative standard deviation (RSD > 5%) and by a signal-to-noise of 3 and 10 times, respectively. The mean correlation coefficient, slope and intercept values for all four standards tested are indicated in Table 1.

Table 1. Standard calibration curves for flavonoids

Standard	Retention time (min)	Regression equation	Correlation coefficient (r)	Linear range (μ g/mL)	LOD ^b (μ g/mL)	LOQ ^b (μ g/mL)
Quercetin	28.9	$y = 54.89x + 25.51$	0.99975	0.10-20.0	0.023	0.037
Quercitrin	21.4	$y = 25.02x + 13.79$	0.99845	0.20-20.0	0.035	0.052
Rutin	18.3	$y = 21.83x + 23.61$	0.99923	0.20-30.0	0.019	0.045
Hyperin	16.8	$y = 18.67x + 21.39$	0.99964	0.50-40.0	0.044	0.089

The quantification showed a good linear relationship between peak area and concentration ($r > 0.99845$) for all standard solutions.

2.3.4 Determination of Flavonoid Content

The four flavonoids were separated from other compounds. The analytical operation can be completed in 30 min. The chromatograms of standards and extract are shown in Figure 1. In the present work, the extraction yield of flavonoids was defined as follows: extraction yield (mg/g) = mass of flavonoid/mass of material.

Fig. 1. HPLC chromatograms of flavonoid standards (A), extract obtained by MAE (B) from *A. brachypus* leaves

Peaks are indicated as follows: (1) hyperin (2) rutin (3) quercitrin (4) quercetin

3. RESULTS AND DISCUSSION

3.1 Structural Elucidation

By extensive spectroscopic analysis and by comparing the physico-chemical constants and spectral data (MS, ^1H -, ^{13}C -NMR and DEPT) with those previously described in literature, four flavonoids were identified as quercetin (1),¹⁹ quercitrin (2),²⁰ rutin (3)²¹ and hyperin (4)²² (Figure 2).

1 R = OH 2 R = ORha 3 R = OGlu-(6 $\leftarrow$ 1)-Rha 4 R = OGal

Fig. 2. Structure of compounds 1-4

Quercetin (1), yellowish needles (MeOH), mp 315-317°C. ESI-MS (m/z): 301 [M-H]⁻. UV $\lambda_{\text{max}}^{\text{MeOH}}$ (log ϵ): 370 (4.42), 302 sh, 253 (3.71) nm. IR $\nu_{\text{max}}^{\text{KBr}}$: 3409 (-OH), 1664 (-C=O), 1613, 1564, 1521 (-Ar) cm^{-1} . ^1H -NMR (DMSO- d_6 , 400 Hz) δ_{H} : 12.48 (1H, s, HO-5), 10.75 (1H, s, HO-7), 9.57 (1H, s, HO-3), 9.31 (1H, s, HO-3'), 9.26 (1H, s, HO-4'), 7.66 (1H, d, $J = 2.2$ Hz, H-2'), 7.54 (1H, dd, $J = 2.2, 8.7$ Hz, H-6'), 6.78 (1H, d, $J = 8.7$ Hz, H-5'), 6.41 (1H, d, $J = 2.0$ Hz, H-8), 6.21 (1H, d, $J = 2.0$ Hz, H-6). ^{13}C -NMR (DMSO- d_6 , 100 Hz) (DEPT) δ_{C} : 176.1 (C, C-4), 164.6 (C, C-7), 161.0 (C, C-9), 156.3 (C, C-5), 147.9 (C, C-2), 146.5 (C, C-4'), 145.4 (C, C-3'), 136.3 (C, C-3), 123.3 (C, C-1'), 120.9 (CH, C-6'), 115.7 (CH, C-2'), 115.3 (CH, C-5'), 103.6 (C, C-10), 98.3 (CH, C-6), 93.7 (CH, C-8).

Quercitrin (2), yellowish crystal (MeOH), mp 183-185°C. ESI-MS (m/z): 447 [M-H]⁻. 301 [M-H-146]⁻. UV $\lambda_{\text{max}}^{\text{MeOH}}$ (log ϵ): 350 (4.10), 256 (4.30) nm. IR $\nu_{\text{max}}^{\text{KBr}}$: 3410 (-OH), 1658 (-C=O), 1600, 1551, 1463 (-Ar) cm^{-1} . ^1H -NMR (DMSO- d_6 , 400 Hz) δ_{H} : 12.55 (1H, s, HO-5), 10.68 (1H, s, HO-7), 9.45 (1H, s, HO-3'), 9.25 (1H, s, HO-4'), 7.42 (1H, d, $J = 2.1$ Hz, H-2'), 7.37 (1H, dd, $J = 2.1, 8.5$ Hz, H-6'), 6.83 (1H, d, $J = 8.5$ Hz, H-5'), 6.42 (1H, d, $J = 2.0$ Hz, H-8), 6.21 (1H, d, $J = 2.0$ Hz, H-6), 5.23 (1H, d, $J = 1.5$ Hz, H-1'), 1.48 (3H, d, $J = 5.5$ Hz, H-6''), 3.25-3.76 (4H, m, H-2''-5''). ^{13}C -NMR (DMSO- d_6 , 100 Hz) (DEPT) δ_{C} : 177.9 (C, C-4), 164.2 (C, C-7), 161.4 (C, C-5), 158.4 (C, C-2),

157.3 (C, C-9), 148.4 (C, C-4'), 145.3 (C, C-3'), 134.3 (C, C-3), 121.9 (CH, C-6'), 121.2 (C, C-1'), 116.6 (CH, C-2'), 115.5 (CH, C-5'), 109.1 (CH, C-1''), 104.2 (C, C-10), 98.8 (CH, C-6), 93.7 (CH, C-8), 73.4 (CH, C-4''), 71.3 (CH, C-2''), 70.6 (CH, C-3''), 70.1 (CH, C-5''), 17.5 (CH₃, C-6'').

Rutin (**3**), yellowish needles (MeOH), mp 178–180°C. UV $\lambda_{\text{max}}^{\text{MeOH}}$ (log ϵ): 360 (4.11), 265 sh, 257 (4.27) nm. IR $\nu_{\text{max}}^{\text{KBr}}$: 3421 (-OH), 1660 (-C=O), 1602, 1568, 1504 (-Ar), 1065 cm⁻¹. ESI-MS m/z : 609 [M-H]⁻, 463 [M-H-146]⁻, 301 [M-H-146-162]⁻. ¹H-NMR (DMSO-*d*₆, 400 Hz) δ_{H} : 12.61 (1H, s, HO-5), 10.82 (1H, s, HO-7), 9.65 (1H, s, HO-3'), 9.17 (1H, s, HO-4'), 7.62 (1H, d, J = 2.1 Hz, H-2'), 7.51 (1H, dd, J = 2.1, 8.1 Hz, H-6'), 6.80 (1H, d, J = 8.2 Hz, H-5'), 6.41 (1H, d, J = 1.8 Hz, H-8), 6.19 (1H, d, J = 2.0 Hz, H-6), 5.34 (1H, d, J = 7.0 Hz, H-1''), 4.38 (1H, brs, H-1'''), 0.98 (3H, d, J = 6.0 Hz, H-6''), 3.04–3.91 (10H, m, H-glucosyl). ¹³C-NMR (DMSO-*d*₆, 100 Hz) (DEPT) δ_{C} : 177.4 (C, C-4), 164.1 (C, C-7), 161.6 (C, C-5), 156.9 (C, C-9), 156.3 (C, C-2), 148.6 (C, C-4'), 144.9 (C, C-3'), 133.4 (C, C-3), 121.8 (CH, C-6), 121.2 (C, C-1'), 116.7 (CH, C-5'), 115.2 (CH, C-2'), 104.1 (C, C-10), 101.4 (CH, C-1''), 100.8 (CH, C-1'''), 98.7 (CH, C-6), 93.7 (CH, C-8), 76.6 (CH, C-3''), 75.9 (CH, C-5''), 74.1 (CH, C-2''), 73.2 (CH, C-4''), 71.9 (CH, C-3''), 70.6 (CH, C-4''), 70.1 (CH, C-2''), 68.9 (CH, C-5''), 66.9 (CH₂, C-6''), 18.4 (CH₃, C-6'').

Hyperin (**4**), yellowish needles (MeOH), mp 235–237°C. UV $\lambda_{\text{max}}^{\text{MeOH}}$ (log ϵ): 362 (3.76), 301 sh, 269 (2.10), 258 (4.05) nm. IR $\nu_{\text{max}}^{\text{KBr}}$: 3412 (-OH), 1652

(-C=O), 1608, 1508, 1462 (-Ar), 1038 cm⁻¹. ESI-MS m/z : 463 [M-H]⁻, 301 [M-H-162]⁻. ¹H-NMR (DMSO-*d*₆, 400MHz) δ_{H} : 12.63 (1H, s, HO-5), 10.85 (1H, s, HO-7), 9.68 (1H, s, HO-3'), 9.18 (1H, s, HO-4'), 7.68 (1H, d, J = 2.1 Hz, H-2'), 7.51 (1H, dd, J = 2.1, 8.0 Hz, H-6'), 6.78 (1H, d, J = 8.0 Hz, H-5'), 6.48 (1H, d, J = 1.8 Hz, H-8), 6.21 (1H, d, J = 1.8 Hz, H-6), 5.37 (1H, d, J = 7.5 Hz, H-1''), 3.34–3.89 (6H, m, H-2''–6''). ¹³C-NMR (DMSO-*d*₆, 100 MHz) (DEPT) δ_{C} : 176.9 (C, C-4), 164.8 (C, C-7), 160.8 (C, C-5), 156.7 (C, C-2), 156.1 (C, C-9), 149.1 (C, C-4'), 144.8 (C, C-3'), 133.6 (C, C-3), 122.4 (C, C-1'), 116.9 (CH, C-5'), 116.2 (CH, C-2'), 123.4 (CH, C-6'), 103.9 (C, C-10), 103.0 (CH, C-1''), 98.9 (CH, C-6), 93.6 (CH, C-8), 76.7 (CH, C-5''), 73.9 (CH, C-3''), 72.5 (CH, C-2''), 69.1 (CH, C-4''), 60.8 (CH₂, C-6'').

3.2 Repeatability, Precision and Recovery

The repeatability of the method was evaluated by analyzing five samples of the same batch *A. brachypus* leaves, and the precision was evaluated by the intra-day (three repetitions each sample, one day) and inter-day (three repetitions each sample, three days) analysis of the same sample of *A. brachypus*. The recoveries of quercetin, quercitrin, rutin and hyperin were determined by the injection of spiked samples, in triplicate, with standard solution. The mass of standards and the average recovery values, are shown in Table 2.

Table 2. The recovery of the method

Sample	No	Added (µg)	Found (µg)	Recovery (%)	Average recovery (%)	RSD (%)
Quercetin	1	5.00	4.98	99.6	99.5	0.91
	2	10.00	10.04	100.4		
	3	20.00	19.72	98.6		
Quercitrin	1	5.00	4.95	99.0	98.8	0.33
	2	10.00	9.89	98.9		
	3	20.00	19.68	98.4		
Rutin	1	5.00	5.08	101.6	101.1	0.55
	2	10.00	10.12	101.2		
	3	20.00	20.09	100.5		
Hyperin	1	5.00	5.12	102.4	102.4	0.68
	2	10.00	10.31	103.1		
	3	20.00	20.34	101.7		

RSDs data of the repeatability of quercetin, quercitrin, rutin and hyperin are 2.11, 1.93, 1.65 and 2.05%, respectively. RSDs were lower than 2.54% for the intra-day and lower than 3.41% for the inter-day assays. The recoveries of quercetin, quercitrin, rutin and hyperin are 99.5, 98.8, 101.1 and 102.4%, respectively. These results indicate that the present method provided acceptable repeatability, good recovery and precision.

3.3 Optimization of MAE

The influences of MAE conditions, such as solvent concentration, solution-solid ratio, microwave irradiation time and power on the extraction yield were determined by the L₉ (3⁴) orthogonal experiment. The factors and their levels, statistical analysis of the orthogonal experiment are shown in Table 3.

Table 3. Orthogonal experimental L₉ (3⁴) and analytical results.

Factor	Volume fraction of ethanol solution (%)				Microwave irradiation time/min				Microwave irradiation power/W				Solvent-solid ratio (mL·g ⁻¹)				Extraction yield/(mg·g ⁻¹)			
																	Q	Qr	R	H
1	25 (1)				6 (1)				200 (1)				20:1 (1)				0.44	1.06	1.51	1.75
2	25 (1)				8 (2)				300 (2)				30:1 (2)				0.73	1.49	1.98	2.48
3	25 (1)				10 (3)				400 (3)				40:1 (3)				0.64	1.36	1.82	2.28
4	50 (2)				6 (1)				300 (2)				40:1 (3)				0.81	1.58	2.11	2.57
5	50 (2)				8 (2)				400 (3)				20:1 (1)				0.70	1.46	1.93	2.42
6	50 (2)				10 (3)				200 (1)				30:1 (2)				0.76	1.52	2.01	2.52
7	75 (3)				6 (1)				400 (3)				30:1 (2)				0.63	1.39	1.84	2.29
8	75 (3)				8 (2)				200 (1)				40:1 (3)				0.68	1.41	1.90	2.36
9	75 (3)				10 (3)				300 (2)				20:1 (1)				0.67	1.48	1.94	2.45
Factor	Q	Qr	R	H	Q	Qr	R	H	Q	Qr	R	H	Q	Qr	R	H				
K ₁	1.81	3.91	5.31	6.51	1.88	4.03	5.46	6.61	1.88	3.99	5.42	6.63	1.81	4.00	5.38	6.62				
K ₂	2.27	4.56	6.05	7.51	2.11	4.36	5.81	7.26	2.21	4.55	6.03	7.50	2.12	4.40	5.83	7.29				
K ₃	1.98	4.28	5.68	7.10	2.07	4.36	5.77	7.25	1.97	4.21	5.59	6.99	2.13	4.35	5.83	7.21				
R	0.46	0.65	0.74	1.00	0.23	0.33	0.35	0.65	0.33	0.56	0.61	0.87	0.32	0.40	0.45	0.67				

Q: Quercetin Qr: Quercitrin R: Rutin H: Hyperin

As can be seen from Table 3, the influential extent of the affecting factors is different, which was: solvent concentration > microwave power > solution-solid ratio > microwave irradiation time. The optimized conditions for MAE are follows: solvent concentration (50% ethanol solution), solution-solid ratio (30 mL·g⁻¹), microwave irradiation time (8 min) and power (300 W).

3.4 Flavonoid Content

Under the optimized MAE conditions, flavonoids were extracted from the leaves of *A. brachypus* Harms. According to the above HPLC conditions, the contents of quercetin, quercitrin, rutin and hyperin were determined to be 0.86, 1.65, 2.19 and 2.68 mg·g⁻¹, respectively.

4. CONCLUSION

The leaves of *A. brachypus* are abundant in flavonoids. Under optimal MAE extraction conditions, *i.e.* 50% aqueous solution of ethanol as extractant, solution-solid ratio 30 mL·g⁻¹, microwave irradiation power 300 W and time 8 min, the highest extraction yields of flavonoids from the leaves of *A. brachypus* Harms are obtained. The HPLC analysis method for flavonoids is accurate with good reproducibility. The results indicate that the employment of MAE provides an efficient, precise, as well as labor- and time-saving procedure.

ACKNOWLEDGEMENTS

This work was financially supported by the Science and Technology Support Program of Gansu Province (Grant No. 1104FKCM133), P.R. China.

REFERENCES

1. N. Ni, X. Liu. *Chin. Tradit. Herb. Drugs*, **37**, 1895, (2006).
2. T. Fujikawa, A. Yamaguchi, I. Morita, H. Takeda, S. Nishibe. *Biol. Pharm. Bull.* **19**, 1227, (1996).
3. S.-Y. Park, C.-S. Yook, T. Nohara. *Tetrahedron Lett.* **42**, 2825, (2001).
4. S.-Y. Park, C.-S. Chang, C.-S. Yook, T. Nohara. *J. Nat. Prod.* **63**, 1630, (2000).
5. Z. Wang, L. Liu, G. Tian, J. Shen. *Chin. Biodiver.* **5**, 251, (1997).
6. J.-M. Yi, M.-S. Kim, S.-W. Seo, K.-N. Lee, C.-S. Yook, H.-M. Kim. *Clin. Chim. Acta*, **312**, 163, (2001).
7. C. Lin, P. Huang. *Phytother. Res.* **14**, 489, (2000).
8. P.C.H. Hollman, M.G.L. Hertog, M.B. Katan. *Food Chem.* **57**, 43, (1996).
9. G. Cao, E. Sofic, R.L. Prior. *Free Radic. Biol. Med.* **5**, 749, (1997).
10. L.W. Wattenburg. *Cancer Res.* **45**, 1, (1985).
11. H. Wei, L. Tye, E. Bresnick, D.F. Birt. *Cancer Res.* **50**, 499, (1990).
12. H. Li, B. Chen, Z. Zhang, S. Yao. *Talanta*, **63**, 659, (2004).
13. C.S. Eskilsson, E. Bjorklund. *J. Chromatogr. A*, **902**, 227, (2000).
14. S. Chen, M. Spiro. *Flavour Frag. J.* **10**, 101, (1995).
15. J. Dai, V.A. Yaylayan, G.S.V. Raghavan. *J. Agric. Food Chem.* **49**, 4584, (2001).
16. Y. Shu, M. Ko, Y. Chang. *Microchem. J.* **74**, 131, (2003).
17. M. Li, J. You, Z. Liu, H. Zhang. *Chem. J. Chin. Univ.* **25**, 850, (2004).
18. L. Chen, H. Jin, L. Ding, H. Zhang, J. Li, C. Qu, H. Zhang. *Sep. Purif. Technol.* **59**, 50, (2008).
19. M. Yu, Z. Li, N. Li, X. Li. *J. Shenyang Pharm. Univ.* **25**, 633, (2008).
20. T. Hatano, T. Yasuhara, R. Yoshihara. *Planta Med.* **57**, 83, (1991).
21. J. Wang, H. Yang, Z. Lin, H. Sun. *Phytochemistry*, **46**, 1275, (1997).
22. Y. Feng, X. Meng, J. Wang. *Food Sci.* **28**, 298, (2007).