Asian Journal of Chemistry; Vol. 25, No. 18 (2013), 10339-10343

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15428

Characteristics and Quality of Germplasm Resources in *P. niruri* from Different Source

YING F. NIU[1], DE Y. TANG[1,*], XUE L. LI[1] and WEI W. GAO[2]

[1]Yunnan District Inspectorate of Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 138 Xuanwei Road, Jinghong, Xishuangbanna 666100, Yunnan Province, P.R. China
[2]Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 151 Malianwa North Road, Haidian District, Beijing 100094, P.R. China

*Corresponding author: E-mail: niuyingfeng@163.com

(*Received*: 5 March 2013; *Accepted*: 14 November 2013) AJC-14398

For the quality assessment purposes, the paper reports a method for simultaneous determination of gallic acid and quercetin in *P. niruri* (Zhuzicao) by reversed phase high performance liquid chromatography (RP-HPLC) with ultraviolet light detection. Twenty-four samples from different source were planted in Xishuangbanna, China and then submitted to characters observation and quantitative determination. The average recoveries of gallic acid and quercetin were 96.66 % (RSD = 3.91 %) and 91.88 % (RSD = 2.69 %), respectively. The results indicated that there were distinct differences in the content of gallic acid and quercetin in *P. niruri* from different source and there were obvious correlation between botany characters and content. The method was simple and accurate with a good reproducibility and can be successfully applied to the quality assessment of *P. niruri*.

Key Words: *Phyllanthus niruri Linn*, Gallic acid, Quercetin, Character, RP-HPLC, Quality assessment.

INTRODUCTION

Phyllanthus niruri Linn, the dried herb of *Phyllanthus Linn* genera in the *Euphorbiaceae* family, is a traditional Dai national medicine. It distributed mainly in the Yunnan, Guangdong, Guangxi, Hainan and Taiwan province[1]. *P. niruri* was widely used as folk medicines for the treatment of various conditions, such as hepatitis, enteritis, dysentery, urinary tract infection and edema pain[2]. Previous studies showed it has many biological activities, including antihyperuricemic[3], antioxidant, hepatoprotective[4-7], antiplasmodial[8] and antinociceptive[9]. In addition, the preparations named "Zhu-zi-gan-tai capsule" used *P. niruri* as the main material[1].

Tannins, flavonoids and lignans have been reported to be constituents of *P. niruri*[10]. Among these compounds, gallic acid, a monomer of hydrolyzable tannins, has cancer prevention and anticancer activities[11] and the quercetin, one kind of the most important flavonoid, has been reported to have anti-cancer[12-14], antioxidation[15], antifree radical[16,17] and antivirus[18] activities. Therefore, gallic acid and quercetin can be used as indicative constituents for the quality assessment of the *P. niruri*. The chemical structures of gallic acid and quercetin are shown in Fig. 1.

The content of active components in *P. niruri* is rare report up to now. Thus, in the paper, a RP-HPLC method for the simultaneous quantitative determination of gallic acid and quercetin in *P. niruri* was established. In this study, 24 samples were collected from different source and then cultivated in the same place. The aim of the work was to determine if wild and cultivated plants showed differences in their chemical profiles and if the botany character can provide basis for *P. niruri* breeding.

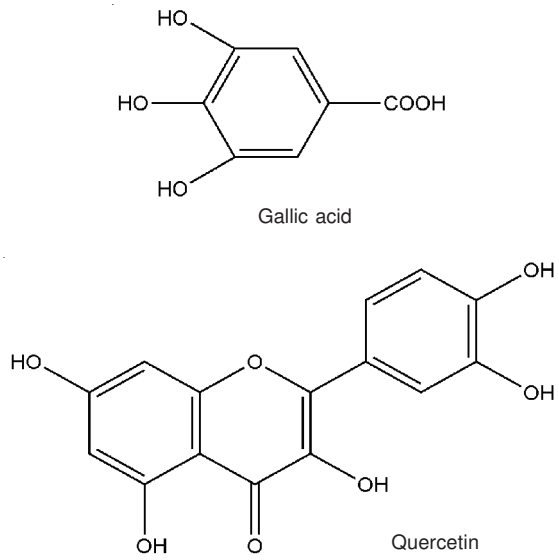

Fig. 1. Chemical structures of gallic acid and quercetin

EXPERIMENTAL

The standard sample of gallic acid was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Lot 110831-200803); its purity was above 90.1 %. The standard sample of quercetin was purchased from Tianjin Jince Analysis Co. Ltd; its purity was above 98 %.

Twenty four samples of *P. niruri* listed in Table-1 were collected from September to October in 2009 and then planted in Jinghong, China in 2010. The plant identified by Professor De-ying Tang.

Acetonitrile, HPLC grade, doubly distilled, water and phosphoric acid, A.R., both filtered through 0.45 μm organic Millipore membrane filters. Methanol, ethanol, hydrochloric acid, A.R. were used for extraction.

Chromatographic conditions: The analysis was carried out using a Waters600 System high performance liquid chromatograph equipped with an on-line degasser, a quadruplet pump and a Waters2489 UV detector. A Sunfire™ C_{18} (4.6 mm × 150 mm, 5 μm) column was used. All analyses were performed at the column temperature of 25 ºC. Gradient elution was used with a mobile phase consisted of (A) 0.2 % aqueous solution of phosphoric acid and (B) acetonitrile. The initial condition was set at 8 % of B and left for 6 min, then gradient up to 32 % of B from 6-9 min and kept consistent for next 13 min and finally, gradient down to 8 % of B from 22-25 min and kept consistent for last 13 min. The flow-rate was 1 mL min^{-1}, the UV detector was set at 254 nm and the injection volume was 10 μL. The retention times of reference gallic acid and quercetin are shown in Fig. 2.

Preparation of standard solutions: The standard samples of gallic acid and quercetin were separately weighed accurately and methanol was added to obtain respective stock solutions. The concentrations of gallic acid and quercetin in the stock solutions were 2.69 and 2.56 mg mL^{-1}, respectively.

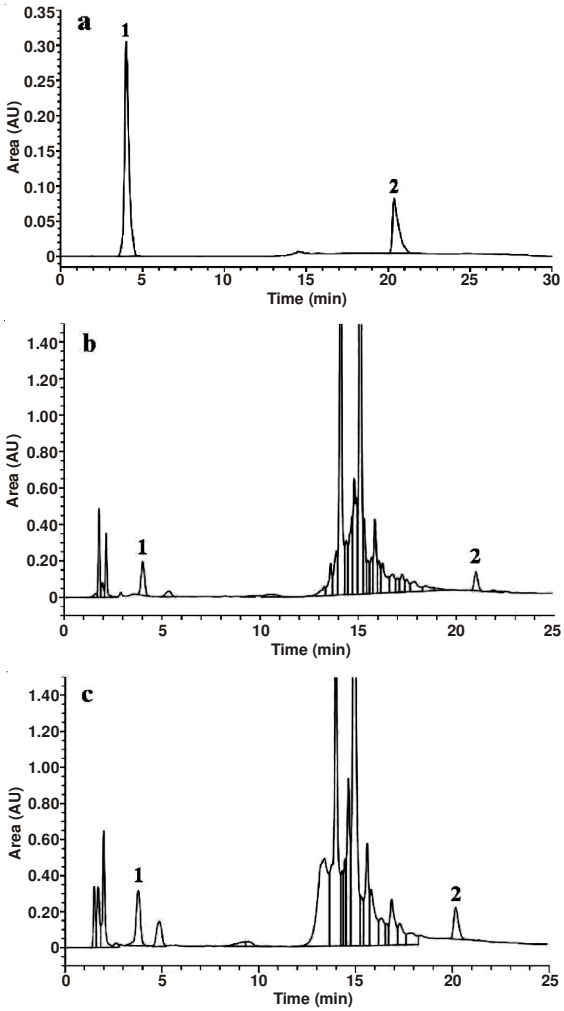

Fig. 2. HPLC chromatogram of reference substances(a); HPLC chromatogram of wild *P. niruri* (b); HPLC chromatogram of cultivated *P. niruri* (c). 1. Gallic acid; 2. Quercetin

TABLE-1
COLLECTING SITES OF 24 SAMPLES OF *P. niruri*

No.	Source	Longitude	Latitude	Altitude (m)
1	Xiamen, Fujian, China	118º03.463'	24º26.703'	10
2	Maoming, Guangdong, China	111º00.033'	21º30.627'	30
3	Nanning, Guangxi, China	108º22'254"	22º51'600"	136
4	Wanning, Hainan, China	110º11.657'	18º44.024'	57
5	Changjiang, Hainan, China	109º07.674'	19º05.969'	378
6	Hekou, Honghe, Yunnan, China	103º57'46.7"	22º41'02.8"	225
7	Donge, Yuxi, Yunnan, China	101º51'09.9"	23º41'03.7"	770
8	Lijiang, Yuxi, Yunnan, China	102º00'21.5"	23º35'44.4"	384
9	Gasa, Yuxi, Yunnan, China	101º35'18.5"	24º00'38.5"	521
10	Mosha, Yuxi, Yunnan, China	101º45'21.8"	23º50'09.3"	508
11	Daluo, Xishuangbannan, Yunnan, China	100º08'07.2"	21º43'06.7"	702
12	Mengwang, Xishuangbannan, Yunnan, China	100º28'13.9"	22º23'12.4"	808
13	Mengla, Xishuangbannan, Yunnan, China	101º33'47.3"	21º28'43.2"	641
14	Mengman, Xishuangbannan, Yunnan, China	101º18'13.9"	21º16'53.7"	616
15	Menglun, Xishuangbannan, Yunnan, China	101º14'24."	21º56'08.6"	619
16	Mengyang, Xishuangbannan, Yunnan, China	100º53'28.3"	22º06'27.0"	748
17	Menglong, Xishuangbannan, Yunnan, China	100º40'57.2"	21º35'05.9"	624
18	Menghan, Xishuangbannan, Yunnan, China	101º58'56.05"	21º51'40.03"	534
19	Jinghong, Xishuangbannan, Yunnan, China	100º48'07.83"	22º00'47.83"	541
20	Yongde, Lincang, Yunnan, China	99º27'47.2"	24º11'46.9"	894
21	Gengma, Lincang, Yunnan, China	99º09'43.8"	23º36'44.5"	541
22	Myanmar	100º39'60.5"	21º23'32.8"	487
23	Nankan, Lao People's Democratic Republic	102º23'59.0"	20º34'33.9"	385
24	Lang Prabang, Lao People's Democratic Republic	102º08'12.1"	19º53'08.00"	277

Preparation of sample solutions: Samples pulverized to pass through 60 mesh sieve, 3 g of the powder was weighed accurately into a florence flask, followed by adding accurately 50 mL of ethanol-hydrochloric acid-water (16:1:3). The flask was weighed and the sample was subjected to reflux extraction for 1.5 h in a water bath at 85 ºC. Then the flask was cooled, weighed again and the weight loss was compensated for. The mixture was shaken up and filtered. Approximately 1 mL of filtrate was filtered through a 0.45 μm filter prior to obtain sample solution.

Characters observation: 24 samples from each portions of cultivated *P. niruri* were randomly selected, respectively and the characters include plant height, stem diameter, leaf length, leaves number, 1000-seed weight, ramification number, drying rate, weight per plant and germination rate were determined. Average values of each parameter of the 30 samples were calculated for analysis. The data was analyzed by SPSS statistics 17.0.

Validation of the method: The method was validated for linearity and range, precision, stability, reproducibility and accuracy.

Linearity: The linearity of the response was verified at five concentration levels, ranging from 0.135-0.457 mg mL^{-1} for gallic acid and 0.0230-0.0783 mg mL^{-1} for quercetin, respectively. Linearity solutions were injected in triplicate. The calibration graph was obtained by plotting the peak area *versus* the concentration data and was treated by least-squares linear regression analysis. The slope and Y-intercept were calculated. The mean correlation coefficient (± RSD) for gallic acid and quercetin were 0.9992 (0.004) and 0.9991 (0.003), respectively (Fig. 3).

Precision: The precision of the assay method was evaluated by six injection of three different standard solutions of gallic acid (135, 215 and 457 μg mL^{-1}) and quercetin (230, 46.1 and 78.3 μg mL^{-1}) on the same day and the values of relative standard deviation were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision. The data obtained from precision experiments are given in Table-2 for intra- and inter-day precision studies. The RSD values for intra-day and inter-day precision study are < 2 %, which confirms that the method is sufficiently precise.

Stability: A sample solution was determined successively by HPLC at 0, 2, 4, 6, 8, 12 and 24 h after it was prepared. The results showed that the sample solution was stable at least in 24 h.

Reproducibility: Six portions of the same batch of sample were sampled and extracted for analysis. The contents of gallic acid and quercetin were determined and their RSD were 1.79 and 2.50 %, respectively.

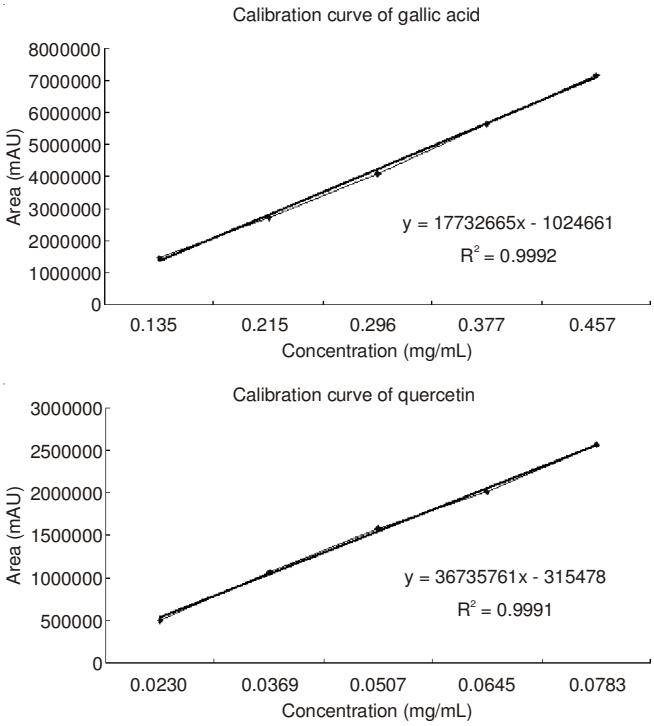

Fig. 3. Calibration curve of gallic acid and quercetin

Accuracy: The reference sample of gallic acid and quercetin were separately weighted accurately into a 100 mL volumetric flasks, ethanol was added to volume to obtain reference solution. The concentrations of gallic acid and quercetin were 0.3358 and 0.0651 mg·mL^{-1}, respectively. Six 1.5 g aliquots of a sample with known contents were weighed out accurately and 10 mL above reference solution (containing 3.358 mg gallic acid and 0.651 mg quercetin), 30 mL ethanol and 10 mL hydrochloric acid (25 %) were added into each aliquot. The samples were analyzed by the above method. The recovery rates and RSD were foud to be (96.7 ± 3.7) % for gallic acid and (91.9 ± 3.1) % for quercetin (n = 6) (Table-3).

RESULTS AND DISCUSSION

Optimization of extraction condition: Some vital factors, including extraction method, organic solvent and reflux time, will influence the degree of extraction of gallic acid and quercetin from the herbs. Therefore, the extraction conditions were optimized before HPLC analysis. The test compared the effects of different extraction methods such as ultrasonic extraction, reflux extraction, cold digestion to yield. The result revealed

TABLE-2
INTRA- AND INTER-DAY PRECISION STUDIES

Compound	Concentration (μg mL^{-1})	Intra-day		Inter-day	
		Mean*	RSD (%)	Mean*	RSD (%)
Gallic acid	135	135.24	1.41	134.86	1.62
	215	215.85	1.44	215.70	1.12
	457	457.63	1.04	457.12	1.47
Quercetin	23	23.78	1.22	22.45	1.90
	46.1	45.82	1.59	45.19	1.53
	78.3	77.96	1.34	77.35	1.65

*Mean of six determinations.

TABLE-3
RECOVERY RATES OF GALLIC ACID AND QUERCETIN (n = 6)

Control substance	m_{sample} (g)	$m_{control}$ (mg)	m_{added} (mg)	m_{found} (mg)	Recovery (%)	$Recovery_{average}$ (%)	RSD (%)
Gallic acid	1.501	6.47	3.358	9.80	99.2	96.7	3.7
	1.500	6.47	3.358	9.65	94.7		
	1.501	6.47	3.358	9.80	99.2		
	1.500	6.47	3.358	9.50	90.2		
	1.500	6.47	3.358	9.80	99.2		
	1.503	6.48	3.358	9.75	97.4		
Quercetin	1.501	1.85	0.651	2.44	90.6	91.9	3.1
	1.500	1.85	0.651	2.43	89.1		
	1.501	1.85	0.651	2.44	90.6		
	1.500	1.85	0.651	2.46	93.7		
	1.500	1.85	0.651	2.44	90.6		
	1.503	1.85	0.651	2.48	96.8		

that the reflux extraction is best. Because of that the quercetin almost exists as the quercitrin and gallic acid exists with tannin in most case, we chosen acid hydrolysis. The test also compared the effects of the methanol-HCl (25 %) and ethanol-HCl (25 %) as the extraction solvent. The result showed that the ethanol-HCl (25 %) had the better dissolving capacity for gallic acid and quercetin. The test also compared the effect of different extraction time including 0.5, 1, 1.5, 2, 2.5 and 3 h to yield. The result demonstrated that the yield increased with increasing time but increased slightly after 1.5 h. Based on energy and efficiency, 1.5 h was the optimal extraction time.

Optimization of chromatographic conditions: The maximum absorbance of gallic acid was between 260 to 270 nm and the maximum absorbance of quercetin is 254 nm. The gallic acid also has good absorbance at 254 nm, so the test chose 254 nm as the detection wavelength. The mobile phase was chosen after several trials with methanol, acetonitrile and phosphate buffer in various proportions and at different pH values. Because the polarity of gallic acid and quercetin are different, it is hardly to perform simultaneous quantitative determination of gallic acid and quercetin in short time under isocratic elution. Considering this, the test chose the linear gradient elution. Under the proposed chromatographic conditions, gallic acid and quercetin could be well separated in 0.5 h and the interference of low-polarity impurity was removed.

Samples content determination: The 24 portions of sample solution of *P. niruri* were obtained. The results of analysis shown in Table-4 were calculated on the basis of dried crude herbs. From Table-4, it is concluded that the contents of gallic acid and quercetin in the wild *P. niruri* are 0.273-0.516 and 0.0539-0.124 %, respectively and the contents of gallic acid and quercetin in the cultivated *P. niruri* are 0.372-0.543 and 0.0763-0.153 %, respectively.

Results of characters observation: The results of characters observation are shown in Table-5.

Result of correlation analysis: In the seven biological traits, drying rate is related to quality and others are related to yield. Correlation analysis among traits related to yield (Table-6) showed that there were certain correlation between weight per plant and plant height, stem diameter, leaf length, leaves number, ramification number. Then the correlation analysis between drying rate, weight per plant, gallic acid content and quercetin content in cultivated *P. niruri* (Table-7) were made to reveal the relationship between constituent content and quality and yield of cultivated *P. niruri.*

TABLE-4
CONTENTS OF GALLIC ACID AND QUERCETIN IN WILD AND CULTIVATED *P. niruri* (n = 3)

No.	Gallic acid (%)		Quercetin (%)	
	Wild samples	Cultivated samples	Wild samples	Cultivated samples
1	0.372	0.514	0.0657	0.107
2	0.273	0.425	0.0635	0.105
3	0.417	0.377	0.0863	0.112
4	0.355	0.543	0.0748	0.153
5	0.378	0.454	0.0626	0.130
6	0.516	0.436	0.112	0.140
7	0.319	0.490	0.0607	0.114
8	0.391	0.408	0.0684	0.0763
9	0.3	0.524	0.059	0.125
10	0.319	0.471	0.0539	0.111
11	0.361	0.504	0.0911	0.119
12	0.345	0.461	0.102	0.121
13	0.299	0.408	0.0843	0.131
14	0.397	0.454	0.113	0.103
15	0.431	0.440	0.123	0.113
16	0.324	0.447	0.102	0.106
17	0.347	0.460	0.0993	0.0972
18	0.387	0.487	0.094	0.125
19	0.401	0.501	0.124	0.110
20	0.374	0.482	0.0622	0.106
21	0.354	0.434	0.0863	0.111
22	0.349	0.426	0.106	0.120
23	0.408	0.438	0.115	0.117
24	0.36	0.372	0.104	0.110

Conclusion

In this work, a RP-HPLC method for simultaneous determination of gallic acid and quercetin in *P. niruri* was established. The method fulfilled all the requirements to be identified as a simple and accurate method. Therefore the method is useful for the quality assessment of *P. niruri.*

There are distinct difference in the contents of gallic acid and quercetin in both wild and cultivated *P. niruri* from different source. It might be related to genetic factors and environmental factors such as climate, soil and rainfall. The concrete reasons remain need further investigation. There is no obvious correlation between wild and cultivated *P. niruri*. This result showed that the environmental factors have greatly influence on the quality of *P. niruri.*

Correlation analysis of traits and contents of cultivated *P. niruri* shows that there were significant negative correlation ($P < 0.01$) between weight per plant and gallic acid content

TABLE-5
RESULTS OF CHARACTERS OBSERVATION OF CULTIVATED SAMPLES (n = 30)

No.	Plant height (cm)	Stem diameter (mm)	Leaf length (cm)	Leaves number	Ramification number	Weight per plant (g)	Drying rate (%)
1	31.95	3.11	6.28	29	5	8.45	0.27
2	51.30	3.22	7.49	23	6	12.18	0.33
3	67.83	4.03	11.57	31	7	18.20	0.31
4	32.80	3.12	6.30	25	6	6.77	0.17
5	50.27	3.20	7.61	29	4	10.03	0.27
6	47.00	3.63	7.47	23	4	9.08	0.35
7	57.47	5.17	7.74	25	5	13.83	0.31
8	86.90	3.88	9.48	28	8	20.23	0.21
9	47.07	5.28	8.18	32	7	13.42	0.32
10	56.87	4.55	8.84	33	10	25.64	0.31
11	49.67	4.24	7.39	23	4	10.87	0.33
12	66.13	3.93	13.10	35	6	13.85	0.32
13	73.23	3.99	8.98	30	7	18.25	0.33
14	59.47	3.88	8.81	31	7	16.63	0.29
15	68.46	3.69	8.11	29	5	16.64	0.33
16	54.73	3.39	8.37	28	5	14.31	0.30
17	54.57	4.23	8.49	31	8	19.22	0.29
18	65.63	3.86	9.60	32	6	15.75	0.22
19	51.20	3.35	8.99	35	7	14.12	0.38
20	40.63	3.36	8.13	32	5	13.60	0.28
21	69.53	3.96	8.10	24	6	17.72	0.29
22	58.60	3.94	8.91	31	8	20.30	0.28
23	54.57	4.23	8.49	31	8	19.22	0.31
24	74.53	4.08	9.29	32	7	19.51	0.28

TABLE-6
RESULTS OF CORRELATION ANALYSIS AMONG TRAITS RELATED TO YIELD (n = 24)

Trait	Plant height	Stem diameter	Leaf length	Leaves number	Ramification number
Stem diameter	0.290	–	–	–	–
Leaf length	0.623**	0.221	–	–	–
Leaves number	0.206	0.137	0.614**	–	–
Ramification number	0.359	0.366	0.359	0.516**	–
Weight per plant	0.678**	0.447*	0.476*	0.467*	0.811**

*,**mean significant differences at 0.05 and 0.01 levels, respectively.

TABLE-7
RESULTS OF CORRELATION ANALYSIS AMONG CONTENT AND TRAITS (n = 24)

	Gallic acid	Quercetin	Drying rate
Quercetin	0.329	–	–
Drying rate	-0.161	-0.044	–
Weight per plant	-0.547**	-0.492*	0.077

*,**Mean significant differences at 0.05 and 0.01 levels, respectively.

and distinct negative correlation ($P < 0.05$) between weight per plant and quercetin content. There was no correlation between drying rate and contents.

ACKNOWLEDGEMENTS

This research was supported by a grant 2008IF018 from The Magnitude Innovation Project funded by the Yunnan Provincial Science and Technology Department, China.

REFERENCES

1. F.D.A. Yunnan, Standard of Chinese Medicinal Materials in Yunnan, Yunnan Art Press, Yunnan, p. 158 (2005).
2. S. Chin, Bot. Inst., Chinese Academy of Sciences: Flora of Hainan, Sci. Press, Beijing, p. 131 (1965).
3. V. Murugaiyah and K.L. Chan, *J. Ethnopharmacol.*, **124**, 233 (2009).
4. R. Harish and T. Shivanandappa, *Food Chem*., **95**, 180 (2006).
5. R. Bhattacharjee and P.C. Sil, *Food Chem. Toxicol*., **45**, 817 (2007).
6. M. Chatterjee, K. Sarkar and P.C. Sil, *Pathophysiology*, **13**, 95 (2006).
7. M.K. Sarkar and P.C. Sil, *Pathophysiology*, **14**, 113 (2007).
8. R.K. Cimanga, L. Tona, N. Luyindula, K. Mesia, M. Lusakibanza, C.T. Musuamba, S. Apers, T.D. Bruyne, S.V. Miert, N. Hermans, J. Totté, L. Pieters and A.J. Vlietinck, *J. Ethnopharmacol.*, **95**, 399 (2004).
9. A.R.S. Santos, V.C. Filho, R.A. Yunes and J.B. Calixto, *Gener. Pharmacol.: Vas. Sys*., **26**, 1499 (1995).
10. X.P. Cheng, X.F. Niu, Z.C. Zhu and W.F. Li, *J. Xi'an Med. Univ*., **22**, 162 (2001).
11. H.X. Li, Z. Wang and Y.Z. Liu, *Chin. Med. Mater*., **26**, 444 (2003).
12. C. Wang, S.N. Tan, C.L. Lu, N. Yao and X. Ma, *J. Shanxi Med. Univ*., **40**, 504 (2009).
13. H.Q. Fu, G.S. Tao, J. Zhang and Z. Li, *J. Cent. South Univ. (Med. Sci.)*, **32**, 890 (2007).
14. K.P. Zhang, Q. Zhang, D.H. Yu, C.J. Chen, Z.N. Cheng and B.Q. Li, *J. Bengbu Med. Coll*., **34**, 379 (2009).
15. C.C. Gong, N.G. Zheng, J.L. Wu, P.X. He and Y.L. Wang, *Basic Clin. Med*., **29**, 52 (2009).
16. Y. Jin, Y. Lu, G.Z. Han, H.J. Sun, H.S. Yu and F.X. Jin, *Chin. Tradit. Herbal Drugs*, **38**, 408 (2007).
17. J.J. Wang and C.X. He, *J. Sci. Teachers' Coll. Univ*., **30**, 71 (2010).
18. W.J. Zhang, B.T. Chen, Q.H. Zhu, G.B. Chen, L.B. Wei and B. Xiong, *Chin. Tradit. Herbal Drugs*, **36**, 76 (2005).