http://dx.doi.org/10.5012/bkcs.2013.34.6.1684

Liquid Chromatography Quadrupole Time-Of-Flight Tandem Mass Spectrometry for Selective Determination of Usnic Acid and Application in Pharmacokinetic Study

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Received January 28, 2013, Accepted March 13, 2013

A rapid and sensitive method for determining usnic acid of *Lethariella cladonioides* in rat was established using high performance liquid chromatography (HPLC) quadrupole time-of-flight (QTOF) tandem mass (MS/MS). Rat plasma was pretreated by mixture of acetonitrile and chloroform to precipitate plasma proteins. Chromatographic separation was achieved on a column (50 × 2.1 mm, 5 μm) with a mobile phase consisting of water (containing 5×10^{-3} M ammonium formate, pH was adjusted to 3.0 with formic acid) and acetonitrile (20:80, v/v) at a flow rate of 0.3 mL/min. A tandem mass spectrometric detection with an electrospray ionization (ESI) interface was conducted *via* collision induced dissociation (CID) under negative ionization mode. The MS/MS transitions monitored were *m/z* 343.0448 → *m/z* 313.2017 for usnic acid and *m/z* 153.1024 → *m/z* 136.2136 for protocatechuic acid (internal standard). The linear range was calculated to be 2.0-160.0 ng/mL with a detection limit of 3.0 pg/mL. The inter- and intra-day accuracy and precision were within ± 7.0%. Pharmacokinetic study showed that the apartment of usnic acid in vivo confirmed to be a two compartment open model. The method was fully valid and will probably be an alternative for pharmacokinetic study of usnic acid.

Key Words : Liquid chromatography, Time of flight, Mass spectrometry, Usnic acid

Introduction

Lichens are formed through symbiosis between fungi (mycobionts) and algae and/or cyanobacteria (photobionts).[1] They are well known for their production of organic compounds from several biosynthetic pathways. Many of these compounds, called lichen products, are only produced by lichens, or are very rarely detected in other organisms.[2] The occurrence of lichen substances is widely used as taxonomic markers, but lichenologists, ecologists, pharmaceutical chemists and others are becoming increasingly aware of the potential biological roles of these substances, and their functions have been well discussed during last decades.[3-6]

Of the hundreds of known secondary lichen metabolites, [2,6-diacetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3(2*H*,9b*H*)-dibenzo-furandione], known as usnic acid, is without a doubt the most extensively studied due to its physiological activities such as antimicrobial, antiviral, *anti*-inflammatory, antiproliferative, cardiovascular-protective activities as well as *anti*-tumor properties *via* different mechanisms.[7-11] High-performance liquid chromatography (HPLC) and thin layer chromatography have found to be widespreadly used in the analysis of secondary metabolites of lichens[12,13] and usnic acid has been analyzed routinely by HPLC.[14,15] Capillary electrophoresis has also been applied in the analysis of usnic acid using reverse polarity capillary zone electrophoresis.[16] In addition, HPLC tandem mass spectrometry method has also been developed to analyze salazinic, norstictic and usnic acid in Xanthoparmelia chlorochroa.[17,18] *Lethariella cladonioides* (Nyl.) Krog belongs to the family Parmeliaceae and grows on the cold hillside at an altitude of 4000-5000 m in the western part of China. It is used as remedies in folk medicine to prevent and cure sore throats, dizziness, neurasthenia, hyperlipidaemia, coronary heart disease and hypertension. The report by Wei *et al.* has identified usnic acid in *L. cladonioides* using HPLC with a diode array detector-mass spectrometry.[19]

In the literature, few methods were found to be established for determination of reference compounds including usnic acid of lichens in biological fluids, which limited the pharmacokinetic study of usnic acid and further use of lichens. This work is designed to develop a sensitive method for the determination of usnic acid in rat plasma. Moreover, the aims of this work also included the application of the proposed method in the pharmacokinetic study on usnic acid.

Experimental

Chemicals and Reagents. Standards of usnic acid and protocatechuic acid using as internal standard were acquired thanks to the help of the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ammonium formate and formic acid were from Sigma-Aldrich Company (St. Louis, MO). Acetonitrile was HPLC grade and purchased from Fisher Scientific (Springfield, NJ). *Lethariella cladonioides* was collected from Taibai mountain of Shaanxi province in China and was identified by Professor Mali Wang (Department of Pharmacognosy, Northwest University, China). Other reagents were analytical grade unless specified

Instruments and Conditions. Target compounds were determined using a LC–ESI–QTOF system acquired from

Agilent (Wilmington, DE, USA). The LC instrument was an Agilent 1200 Series, consisting of vacuum degasser unit, autosampler, an isocratic high pressure pump and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a dual-spray ESI source.

Chromatographic separation was performed on a Zorbax-C18 column (50 × 2.1 mm, 5 μm; Littleforts, Philadelphia, USA) at 25 °C. The mobile phase consisted of water (containing 5×10^{-3} M ammonium formate, pH was adjusted to 3.0 with formic acid) and acetonitrile (20: 80, v/v) at a flow rate of 0.3 mL/min.

Liquid nitrogen was used as nebulizing (35 psi) and drying gas (350 °C, 8.0 L/min) in the dual ESI source. The QTOF instrument was operated in the 4GHz high resolution mode and compounds were ionized in negative ESI, applying a capillary voltage of −3500 V. A reference calibration solution (Agilent calibration solution A) was continuously sprayed in the source of the QTOF system, employing the ions with *m/z* 112.9856 and *m/z* 1033.9881 for recalibrating the mass axis ensuring the accuracy of mass assignations throughout the chromatographic run. The Mass Hunter Workstation software was used to control all the acquisition parameters of the LC–ESI–QTOF system and also to process the obtained data.

The precursor ions for usnic and IS were obtained using a common fragmentor voltage of −160 V. Then, collision energy was optimized with the aim of obtaining a minimum of two product ions for each precursor. Mass patterns of usnic acid and IS were obtained with respect to maximum signal intensity of molecular ions and fragment ions, by consecutively infusing standard solutions of usnic acid (1.0 ng/mL), and IS (2.0 ng/mL), aided by a model 22 syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 500 μL/h. The optimal transitions were *m/z* 343.0448 $[M–H]^-$ of parent ion to *m/z* 313.2017 $[M–CH_3O]^-$ of daughter ion for usnic acid and *m/z* 153.1024 $[M–H]^-$ of parent ion to *m/z* 136.2136 $[M–H_2O]^-$ of daughter ion for IS.

Extraction of the Herb. The extract of *Lethariella cladonioides* was prepared by the following method. 40.0 g of *Lethariella cladonioides* was grinded to pieces of 40 bore size and refluxed two times with 8-fold 70% ethanol/water (v:v) for 1.0 h endurance each time. The suspension was filtered and the resulted solution was concentrated to 10.0 mL. The concentration of usnic acid in the extraction was determined as 0.16 mg/mL by HPLC method.

Preparation of the Plasma Samples. An aliquot of 0.5 mL rat plasma was transferred into a 5.0 mL eppendorf tube in presence of 2.0 uL of IS working solution (2.0 μg/mL). 1.0 mL acetonitrile and 0.6 mL chloroform were added to the sample for precipitating protein and extracting compound of interest by vortex-mixing 1.0 min. Then, the sample was centrifuged at 10000 rpm for 5.0 min. The supernatant was aspirated into a 5.0 mL tube and evaporated to dryness under an N_2 stream. Finally, the residue was reconstituted with 1.0 mL mobile phase and was filtered with Φ 0.22 μm polytetrafluoroethylene membrane prior to be analyzed by LC-MS/MS. The injection volume was 20.0 μL.

Preparations of Standards Curves and Quality Control (QC) Samples. Stock solutions of usnic acid was prepared in 10% chloroform/methanol (v:v) at 0.16 mg/mL. The usnic stock solution was then serially diluted with methanol to produce a series of standard or QC working solutions at the desired concentrations. Stock solutions for IS were prepared at 0.2 mg/mL in methanol and diluted with methanol to yield an IS working solution at the concentration of 10.0 ng/mL.

The calibration standards were freshly prepared by adding 20 μL of the appropriate standard working solutions to 200 μL blank plasma and prepared using plasma treated method to provide the final concentrations of usnic acid at 2.0, 4.0, 8.0, 16.0, 25.0, 50.0, 80.0, 100.0 and 160.0 ng/mL. Low, medium and high levels of QC samples were prepared at the concentrations of 4.0, 25.0 and 100.0 ng/mL. All solutions described above were stored at 4.0 °C.

Matrix Effect and Extraction Recovery. Absolute matrix effect was used to evaluate the extent of MS signal suppression or enhancement. It was calculated by comparing the peak areas of analytes added in six different lots of plasma (A) with mean peak areas of the standards at the same concentrations in the reconstitution solvent (B), and expressed as (A/B × 100%). Relative matrix effect was used to evaluate the variations of different lots of plasma resulted from the matrix effect, and was calculated by the coefficients of variation [CV %] of peak area of analytes added post-extraction from six different lots of blank plasma.

Extraction recovery was calculated by comparing peak areas of QC samples (C) with the mean peak areas of analytes added post-extraction in six different lots of plasma (A), expressed as (C/A × 100%).

Method Validation. The validation of the current HPLC–QTOF–MS/MS assay was investigated including specificity, linearity, accuracy, intra-day and inter-day precision, recovery, and stability. The specificity was confirmed by analyze six different lots of blank rat plasma. Five validation batches were assayed to assess the linearity, accuracy and precision of the method. Each batch included a set of calibration standards and five replicates of QC samples at low, medium and high concentration levels, and was processed on five separate days. The linearity of each curve was assessed by plotting the peak area ratio of the analyte to IS versus the corresponding concentration of the analytes in the freshly prepared plasma calibrators. The accuracy of the assay was expressed by [(calculated concentration by the regression equations)/(spiked concentration)] × 100%, and the precision was evaluated by relative standard deviation (RSD). The stability of usnic acid in spiked samples was evaluated under possible conditions that should reflect situations likely to be encountered during actual sample handling and analysis, including thawed plasma at room temperature for 8.0 h, frozen plasma at −20 °C for 30 days, plasma samples after three cycles of freeze and thaw, and the processed samples kept at 4.0 °C for 48 h. The stability of the analyte in stock solution was also evaluated.

Pharmacokinetic Application. Sprague-Dawley rats, weighing 265 ± 15 g, were supplied by the Experimental

Animal Center of Xi'an Jiaotong University. The rats were kept in standard animal holding room at a temperature of 23 ± 2 °C and relative humidity of 60 ± 10%. The animals were acclimatized to the facilities for 7 days and then fasted with free access to water for 12 h prior to each experiment. The ethics of animal experiments were in accordance with the approval of the Department of Health Guidelines in Care and Use of Animals.

Eighteen rats were divided into three groups randomly (n = 6). The rats of one group were administered with 0.5% cardoxymethyl cellulose sodium salt (CMC-Na) with the dose of 5.0 mL/kg aqueous solution to form control group. For the rats of the other group, single oral dose of 5.0 mL/kg *Lethariella cladonioides* extract was administered. The extracts were suspended in 0.5% CMC-Na aqueous solution before each experiment. Blood samples were collected in heparinized eppendorf tube *via* the ear edge vein before dosing and subsequently at 5.0, 15.0, 30.0, 60.0, 90.0, 120.0, 180.0, 240.0, 300.0, 420.0, 540.0 and 720.0 min after oral administration. The blood samples were centrifuged at 10,000 rpm for 10 min and the plasma was separated and stored at −20 °C until assay.

Results and Discussion

Optimization of Chromatographic Separation and MS/MS Conditions. The separation and ionization of usnic acid and IS were affected by the composition of mobile phase. Accordingly, different ratios (10:90, 20:80 and 30:70) of water/acetonitrile were used as mobile phase. The ratio of 20:80 of water/acetonitrile (v:v) was selected as the mobile phase in view of retention time and peak shape of usnic acid and IS. Ammonium formate was employed to supply the ionic strength. It was found that a mixture of 5.0×10^{-3} M ammonium formate buffer–water/acetonitrile could preferably improve peak shape and clear increase in mass spectral intensity. This solution was finally adopted as the mobile phase because the intensities of usnic acid and IS were deceased obviously when the concentrations of ammonium formate changed to 10-30 mM.

The selection of tandem mass transitions and associated acquisition parameters were evaluated for best response under negative and positive mode ESI conditions by infusing a standard solution, *via* a syringe pump. It was found that the analytes mainly generated negative product ions. The transitions for analysis of usnic acid and IS were selected at *m/z* 343.0448 $[M–H]^-$ → *m/z* 313.2017 $[M–CH_3O]^-$ for usnic acid and *m/z* 153.1024 $[M–H]^-$ → *m/z* 136.2136 $[M–H_2O]^-$ for IS because these two transitions were specific and the intensities of these ions were the strongest. Under the optimized conditions, good chromatographic separation and mass spectral signals were achieved in the assay of the plasma sample.

Matrix Effect and Extraction Recovery. The endogenous substances could interfere with the usnic acid determination due to the extreme low concentration of usnic acid in plasma samples. Choosing an appropriate internal standard is an important approach to reduce the matrix effects. In this study, protocatechuic acid was selected as internal standards. In Table 1, all the values (A/B × 100)% were between 96.0 and 105.0%, which means little matrix effect for usnic acid and IS in this method. The extraction recoveries of usnic acid from rabbit plasma were 90.6 ± 7.4, 95.8 ± 6.3, and 104.3 ± 4.5% at concentration levels of 4.0, 25.0.0 and 100.0 ng/mL, respectively.

Method Validation.

Specificity: The base peak of each mass spectrum for usnic acid and IS were observed from Q1 scans during the infusion of the neat solution in negative mode. Two [M–H] precursor ions, *m/z* 343.0448 $[M–H]^-$ for usnic acid and *m/z* 153.1024 $[M–H]^-$ for IS, were subjected to collision-induced dissociation (CID). The product ions were recorded as *m/z* 313.2017 $[M–CH_3O]^-$ and *m/z* 136.2136 $[M–H_2O]^-$, respectively. Mass transition patterns, *m/z* 343.0448 → 313.2017 and *m/z* 153.1024 → 136.2136 were selected to monitor usnic acid and IS. Representative MS/MS extracted chromatograms of blank sample, low, medium and high levels of QC samples plus plasma sample collected at 60.0 min after administration are shown in Figure 1. No endogenous peaks were found to be co-eluted with the analytes, indicating high specificity of the proposed method.

Linearity and Sensitivity. Nine-point calibration curves were prepared ranging from 2.0 to 160.0 ng/mL for usnic acid. The regression parameters of slope, intercept and correlation coefficient were calculated by 1/x-weighted linear regression. Excellent linearity was achieved with correlation coefficients greater than 0.9912 for all validation batches, shown in Table 2. The current method offered a limit of detection (LOD) of 3.0 ng/mL (S/N=3) and a limit of quantitation (LOQ) of 10.0 ng/mL (S/N=10), which is sensitive

Table 1. Matrix effects and extraction recovery of usnic acid and the internal standard in rat plasma

Analytes	Concentration (ng/mL)	Matrix effects (%, n = 6)	CV (%)	Extraction recovery (%, n = 5)	CV (%)
Usnic acid	4.0	96.3 ± 4.2	4.7	90.6 ± 7.4	8.0
	25.0	102.1 ± 5.4	6.1	95.8 ± 6.3	6.8
	100.0	98.7 ± 2.8	5.6	104.3 ± 4.5	5.9
I.S.	2.0	97.2 ± 3.6	3.8	96.5 ± 5.7	6.5
	2.0	104.9 ± 6.7	7.2	105.4 ± 3.9	4.6
	2.0	101.5 ± 4.3	5.6	99.7 ± 2.8	3.2

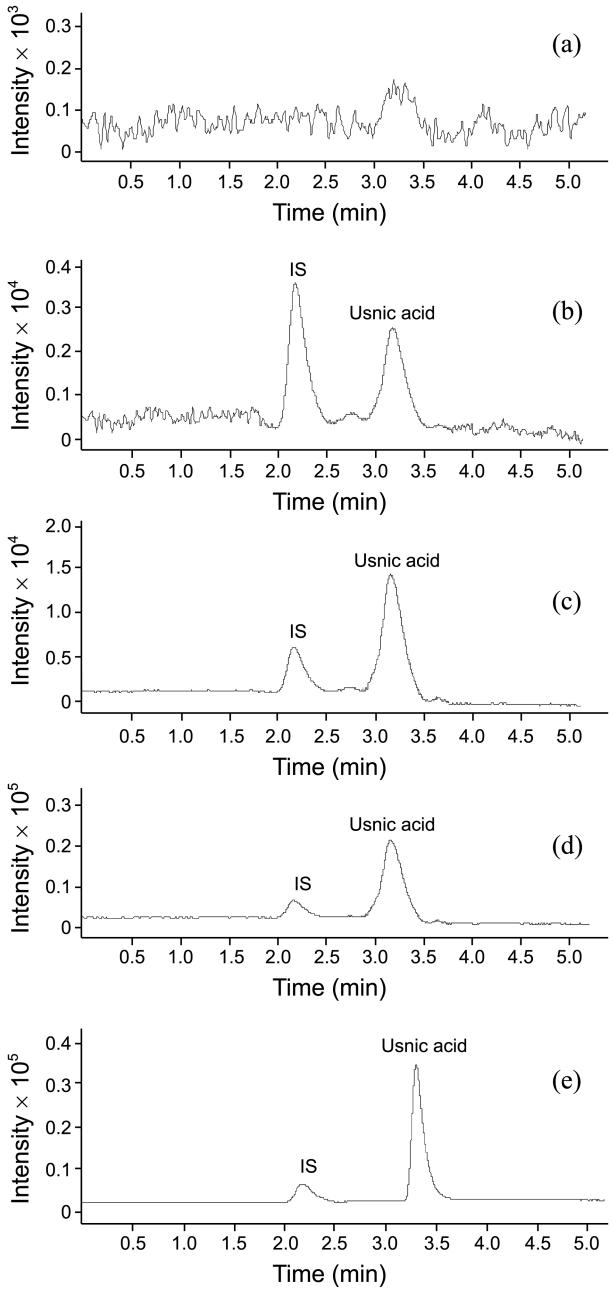

Figure 1. Representative extracted chromatograms for (a) blank plasma, (b) blank plasma containing 4.0 ng/mL usnic acid and 4.0 ng/mL I.S, (c) blank plasma containing 25.0 ng/mL usnic acid and 4.0 ng/mL I.S, (d) blank plasma containing 100.0 ng/mL usnic acid and 4.0 ng/mL I.S, (e) plasma sample collected at 60.0 min after single oral dose of *Lethariella cladonioides* extract (5.0 mL/kg).

enough to investigate our pharmacokinetic behaviors of the compound.

Accuracy and Precision. The accuracy and the precision were analyzed by the QC samples at three concentrations. The assay accuracy and precision results are summarized in Table 3. The data obtained was within the acceptable limits to meet the guideline for bioanalytical methods (www.fda.gov).

Stability. The stability of usnic acid in plasma tested in this work implied that no significant degradation occurred at room temperature for 6.0 h and at −20 °C for 25 days. The plasma samples after three freeze and thaw cycles and the processed samples kept in the autosampler (4.0 °C) for 48 h were stable. The stock solutions of usnic acid and IS in methanol gave a good stability at 4 °C for 21 days.

Pharmacokinetics. Following validation, the method was successfully applied to the pharmacokinetic study of usnic acid in rats. The mean plasma concentration-time profile of usnic acid after administration is shown in Figure 2. The main pharmacokinetic parameters of usnic acid were calculated by second version of an excel software named Drug and Statistics (Shanghai, China). The pharmacokinetic process of usnic acid confirmed to be a two-compartment open model. The maximum plasma concentration (C_{max}) was calculated to be 15.0 ± 2.3 ng/mL with a value of 60.0 ± 5.6 min for the time to reach maximum plasma concentration (t_{max}). The half-life for distribution ($t_{1/2\alpha}$) and elimination ($t_{1/2\beta}$) were 11.19 ± 1.64 and 69.32 ± 8.57 min, respectively, providing the evidence that usnic acid can exert its therapeutic effect quickly but only last around one hour. The total exposure measured as $AUC_{(0-\infty)}$ (area under concentration-time curve) and $AUC_{(0-t)}$ were calculated to be 2.76 ± 0.40 and 2.60 ± 0.35 μg/mL min. The time-points for plasma collection are accordingly believed to be acceptable due to the fact that ratio of $AUC_{(0-\infty)}$ versus $AUC_{(0-t)}$ is lower than 120%. In summary, the proposed method proves to be suitable to the determination of usnic acid in biological fluids and will probably be an alternative for pharmacokinetic study on usnic acid.

Conclusion

A sensitive, selective and rapid HPLC–QTOF–MS/MS method for the determination of usnic acid in rat plasma was described. The method had the properties of reducing ion suppression and offering superior sensitivity with an LOQ of 10.0 ng/mL, satisfactory selectivity and short run time of 5.0 min. The method has been successfully applied to a pharma-

Table 2. Linearity for assay of usnic acid in rat plasma

Analytical batch	Slope	Intercept	Regression equation	Correlation coefficient
1	0.0362	0.0486	$y = 0.0486x + 0.0362$	r = 0.9912
2	0.0435	0.0484	$y = 0.0484x + 0.0435$	r = 0.9945
3	0.0374	0.0490	$y = 0.0490x + 0.0374$	r = 0.9936
4	0.0359	0.0480	$y = 0.0480x + 0.0359$	r = 0.9972
5	0.0511	0.0482	$y = 0.0482x + 0.0511$	r = 0.9989

Table 3. Intra-day (n = 5) and inter-day (n = 5) precision and accuracy for assay of usnic acid in rat plasma

Concentration (ng/mL)	Intra-day (n=5)			Inter-day (n=5)		
	Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)	Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)
4.0	4.24 ± 0.12	2.8	106.0	3.72 ± 0.26	7.0	93.0
25.0	23.2 ± 1.56	6.7	92.8	26.8 ± 1.82	6.8	107.2
100.0	97.6 ± 3.45	3.5	97.6	103.4 ± 5.23	5.1	103.4

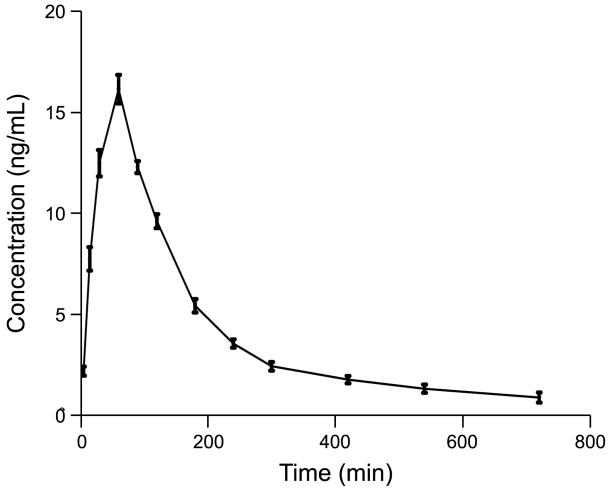

Figure 2. Mean plasma concentration-time profile of usnic acid after administration of *Lethariella cladonioides* extract in rats (n = 6).

cokinetic study of usnic acid in rat, providing an alternative for clinical determination of usnic acid.

Acknowledgments. Financial support is kindly from the Program for Changjiang Scholars and Innovative Research Team in University (no. IRT1174), Ministry of Science and Technology of Shaanxi Province (no. 2012JQ4022) and the Department of Education of Shaanxi Province (no. 11JK0661). And the publication cost of this paper was supported by the Korean Chemical Society.

References

1. Ingolfsdottir, K. *Phytochemistry* **2002**, *61*, 729.
2. Bjerke, J. W.; Elvebakk, A.; Domínguez, E.; Dahlback, A. *Phytochemistry* **2005**, *66*, 337.
3. Huneck, S. *Naturwissensch.* **1999**, *86*, 559.
4. Molnár, K.; Farkas, E. *Z. Naturforschung C* **2010**, *65*, 157.
5. Mitrović, T.; Stamenković, S.; Cvetković, V.; Tošić, S.; Stanković, M.; Radojević, I.; Stefanović, O.; Comić, L.; Dačić, D.; Curčić, M.; Marković, S. *Int. J. Mol. Sci.* **2011**, *12*, 5428.
6. Kosanić, M. M.; Ranković, B. R.; Stanojković, T. P. *J. Sci. Food. Agr.* **2012**, *92*, 1909.
7. Guo, L.; Shi, Q.; Fang, J. L.; Mei, N.; Ali, A. A.; Lewis, S. M.; Leakey, J. E.; Frankos, V. H. *J. Environ. Sci. Health. C* **2008**, *26*, 317.
8. Behera, B. C.; Mahadik, N.; Morey, M. *Pharm. Biol.* **2012**, *50*, 968.
9. O'Neill, M. A.; Mayer, M.; Murray, K. E.; Rolim-Santos, H. M.; Santos-Magalhaes, N. S.; Thompson, A. M.; Appleyard, V. C. *Braz. J. Biol.* **2010**, *70*, 659.
10. Einarsdottir, E.; Groeneweg, J.; Bjornsdottir, G. G.; Harethardottir, G.; Omarsdottir, S.; Ingolfsdottir, K.; Ogmundsdottir, H. M. *Planta. Medica.* **2010**, *76*, 969.
11. Song, Y. J.; Dai, F. J.; Zhai, D.; Dong, Y. M.; Zhang, J.; Lu, B. B.; Luo, J.; Liu, M. Y.; Yi, Z. F. *Angiogenesis* **2012**, *15*, 421.
12. Bhattarai, H. D.; Paudel, B.; Hong, S. G.; Lee, H. K.; Yim, J. H. *J. Nat. Med.* **2008**, *62*, 481.
13. Gupta, V. K.; Darokar, M. P.; Shanker, K.; Negi, A. S.; Srivastava, S. K.; Gupta, M. M.; Khanuja, S. P. S. *J. Liq. Chromatogr. R. T.* **2007**, *30*, 97.
14. Cansaran, D.; Atakol, O.; Halici, M. G.; Aksoy, A. *Pharm. Biol.* **2007**, *45*, 77.
15. Ji, X.; Khan, I. A. *J. AOAC. Int.* **2005**, *88*, 1265.
16. Falk, A.; Green, T. K.; Barboza, P. *J. Chromatogr. A* **2008**, *1182*, 141.
17. Dailey, R.; Siemion, R.; Raisbeck, M.; Jesse, C. *J. AOAC. Int.* **2010**, *93*, 1137.
18. Roach, J. A. G.; Musser, S. M.; Morehouse, K.; Woo J. Y. J. *J. Agr. Food. Chem.* **2006**, *54*, 2484.
19. Wei, A. H.; Zhou D. N.; Ruan, J. L.; Cai, Y. L.; Xiong, C. M.; Li, M. X. *J. Sci. Food. Agr.* **2012**, *92*, 373.