Journal of Chromatography B, 917–918 (2013) 84–92

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of Eleutheroside B and Eleutheroside E in rat plasma by high performance liquid chromatography–electrospray ionization mass spectrometry and its application in a pharmacokinetic study

Bo Ma[a,1], Qi Zhang[a,1], Yinhui Liu[a], Jing Li[a], Qiuyu Xu[a], Xiaotian Li[b,*], Xiaojing Yang[a], Di Yao[a], Jingjing Sun[a], Guangbo Cui[a], Hanjie Ying[a]

[a] School of Pharmaceutical Sciences, Nanjing University of Technology, Nanjing 210009, People's Republic of China
[b] School of Pharmacy, Zhengzhou University, Zhengzhou, 450001, People's Republic of China

ARTICLE INFO

Article history:
Received 16 November 2012
Accepted 31 December 2012
Available online 14 January 2013

Keywords:
Eleutheroside B
Eleutheroside E
Eleutherococcus senticosus
LC–MS/MS
Pharmacokinetics

ABSTRACT

Eleutheroside B and Eleutheroside E, two kinds of the major bioactive saponins of *Eleutherococcus senticosus*, play a pivotal role in biologic activity. In this study, a specific and sensitive high performance liquid chromatography–electrospray ionization-tandem mass spectrometry method (HPLC–MS/MS) was developed and validated for simultaneous determination of Eleutheroside B and Eleutheroside E in rat plasma. The analytes were extracted from rat plasma via a simple protein precipitation procedure with methanol and polygonin was used as internal standard. Chromatographic separation was achieved on a C18 column using a gradient elution program with acetonitrile and water containing 0.1% ammonium hydroxide solution as the mobile phase, with a flow rate of 0.2 mL/min. The detection was performed on a triple–quadrupole tandem mass spectrometer by multiple reactions monitoring (MRM) mode in a negative ion mode via electrospray ionization (ESI). The transition monitored were m/z 371 $[M-H]^- \rightarrow$ 209 for Eleutheroside B, m/z 741$[M-H]^- \rightarrow$ 579 for Eleutheroside E and m/z 389$[M-H]^- \rightarrow$ 277 for internal standard. Linear calibration curves were obtained in the concentration range of 1–2000 ng/mL for both (Eleutheroside B and Eleutheroside E), with a lower limit of quantification of 1 ng/mL. Extraction recovery was over 80% in plasma. The intra- and inter-day precision (RSD) values were below 12% and accuracy (RE) was −2.80 to 5.70% at three QC levels for both. The assay was successfully applied to study pharmacokinetics behavior in rats after oral and intravenous administration of the single substances (Eleutheroside B and Eleutheroside E). And further research was performed by comparing the difference in pharmacokinetic behavior between the single substances and an aqueous extract of *E. senticosus* after oral administration. Significant difference in pharmacokinetic characteristics between the single substances and an aqueous extract was found in rat, which would be beneficial for the pre-clinical research and clinical use of *E. senticosus*.

1. Introduction

Acanthopanax senticosus (Rupr. et Maxim.) *Harms*, also called *Manyprickle Acathopanax Root*, *Eleutherococcus senticosus* or ciwujia, is a hardy shrub and widely distributed in the northeastern region of China, Japan, Korea and the fat-eastern region of Russia. As a well-known Traditional Chinese Medicine (TCM), the extracts from root and rhizome of *E. senticosus* have been used in traditional oriental medicine for the treatment of various ailments including rheumatism, arrhythmia, hypertension, cancers [1,2], fatigue [3,4], and asthenospermia [5]. In recent years, some studies have indicated that saponins are the main and effective components in the pharmacological efficacy of *E. senticosus*. Moreover, Eleutheroside B and Eleutheroside E, two kinds of the water extracts, are the major bioactive saponins of *E. senticosus* and play a pivotal role in biologic activity based on the material base and modern pharmacology research.

Eleutheroside B, also called syringin, is widely reported to be the key constituent of *E. senticosus* and has been used to immunomodulatory [6,7], anti-inflammatory, anti-nociceptive, and anti-hyperglycemic action [8]. Niu and his co-workers found that syringin could enhance glucose utilization and reduce plasma glucose level in streptozotocin-induced diabetic rats [9]. Further researches suggested that syringin would stimulate muscarinic M3 receptors in pancreatic cells and augment the insulin release to

* Corresponding author. Tel.: +86 371 67781889; fax: +86 371 67781908.
E-mail address: lixiaotian201211@163.com (X. Li).
[1] The first two authors contributed equally to this work.

1570-0232/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jchromb.2012.12.041

result in plasma glucose lowering action. And, this mechanism may be related to the release of acetylcholine (Ach) from nerve terminals [10,11]. Besides, Eleutheroside E, as another active ingredient from *E. senticosus*, was reported to have anti-inflammatory effect and protective effects in ischemia heat [12]. And recent studies shown Eleutheroside E could alleviate behavioral alterations in murine sleep deprivation stress model [3]. Huang et al. further investigate Eleutheroside E may be responsible for the pharmacological effect to anti-fatigue both in physical and mental fatigue by reducing the accumulation of blood urea nitrogen (BUN) [13]. In order to better use Eleutheroside B, Eleutheroside E and *E. senticosus*, it is necessary to study the pharmacokinetics of their major components in addition to the study of pharmacodynamics.

Recent publications have described several methods for the determination of Eleutheroside B and Eleutheroside E in biological samples or pharmaceutics using HPLC–UV [14–17] and LC–MS [18]. A few pharmacokinetics data of Eleutheroside B and Eleutheroside E after intravenous injection of *E. senticosus* extract were performed using HPLC-UV detection with the limit of detection (37.6 and 37 ng/mL) [19]. However, the pharmacokinetic characteristics of Eleutheroside B and Eleutheroside E are largely unknown so far, in particular absorption profile in the gastrointestinal tract due to lack of sensitive assays.

The aim of the present study is to develop a selective and sensitive LC–MS/MS method for the simultaneous determination of Eleutheroside B and Eleutheroside E in rat plasma. The pharmacokinetic profiles of Eleutheroside B and Eleutheroside E were revealed after oral administration the single substances (Eleutheroside B and Eleutheroside E). On this basis, a comparative pharmacokinetic study with oral administration the single substances (Eleutheroside B and Eleutheroside E) and an aqueous extract of *E. senticosus* was further carried out. In additional, oral absolute bioavailability of Eleutheroside B and Eleutheroside E was also studied after oral and intravenous administration at a single substance of Eleutheroside B and Eleutheroside E. These researches would be helpful for reasonable usage of Eleutheroside B, Eleutheroside E and *E. senticosus*.

2. Experimental

2.1. Reagents and chemicals

Eleutheroside B (purity >98.1%), Eleutheroside E (purity >98.5%) and IS (polygonin, purity >98.3%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). An aqueous extract of *E. senticosus* (containing 1% Eleutheroside B and Eleutheroside E) was purchased from Xi'an Kai Lai Biological Engineering (Xi'an, China). HPLC grade reagents methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). HPLC grade 10% ammonium hydroxide solution was obtained from Sigma (St. Louis, MO). All the water used in this work was ultra-pure and produced by a Milli-Q system (Millipore, Bedford, MA, USA) (Fig. 1).

2.2. Apparatus and operation conditions

The HPLC system consisted of a Shimadzu HPLC-20A system consisting of two LC-20AD pumps, a DGU-20A3 degasser, a SIL-20AC auto sampler and a CTO-20AC column oven (Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was performed on a Hypersil GOLD AQ C18 column (150 mm × 2.1 mm, 5 μm, Thermo, USA) protected by a C18 Security guard (4 mm × 3.0 mm, ID 5 μm) and it was maintained at 35 °C with the flow rate was 0.2 mL/min. The mobile phase consisted of 0.1% ammonium hydroxide solution (10%, pH 10.5) (A) and acetonitrile (B) using a gradient elution of 15–15% B at 0–1.0 min;

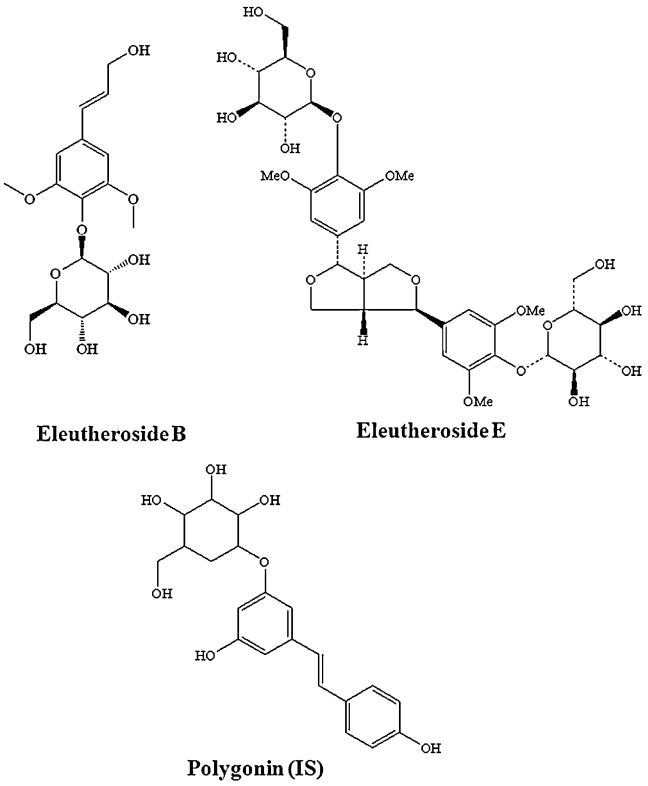

Fig. 1. The chemical structure of Eleutheroside B, Eleutheroside E and IS.

15–50% B at 1.0–1.8 min; 50–50% B at 1.8–5.0 min; 50–15% B at 5.0–5.1 min; 15–15% B at 5.1–10 min. For the first 5 min the eluent was diverted to LC–MS/MS analysis. The injection volume was 5 μL and the auto-sampler was conditioned at 4 °C.

Mass spectrometric analysis was achieved on an API 4000 Q-trap MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray inlet. All quantifications were performed in the negative ion electrospray ionization mode using multiple reaction monitoring (MRM) by recording ion currents for the following transitions: m/z 371.3 → 209.1 for Eleutheroside B, m/z 741.5 → 579.1 for Eleutheroside E and m/z 389.0 → 227.0 for internal standard. The optimized declustering potential (DP) for Eleutheroside B, Eleutheroside E and IS were −31 V, −70 V and −96 V, and collision energy (CE) for Eleutheroside B, Eleutheroside E and IS were −12, −15 and −23 eV, respectively. The product ions of these compounds are shown in Fig. 2. The optimized ion spray voltage and temperature were set at −4500 V and 450 °C, respectively. Gas 1 and gas 2 (nitrogen) were set at 50 and 50 psi, respectively. And, nitrogen was also used as the curtain gas controlled at 10 psi. Data acquisition was performed with Analyst 1.5.1 software (Applied Biosystems, Foster City, CA, USA).

2.3. Preparation of standards and quality control samples

Stock solutions were prepared by dissolving Eleutheroside B (8.5 mg), Eleutheroside E (11.0 mg) and internal standard (polygonin, 12.5 mg) into methanol to yield a concentration of 1.0 mg/mL, respectively. And then the stock solutions of Eleutheroside B and Eleutheroside E were diluted with methanol to get a series of working standard solutions at concentrations of 10, 20, 50, 200, 500, 2000, 5000, 10,000 and 20,000 ng/mL for calibration curves and 20, 1000 and 16,000 ng/mL concentrations for QC samples. In additional, working standard solution of IS was also at a concentration of 1 μg/ml. All stock solutions and working standard solutions were stored at −20 °C.

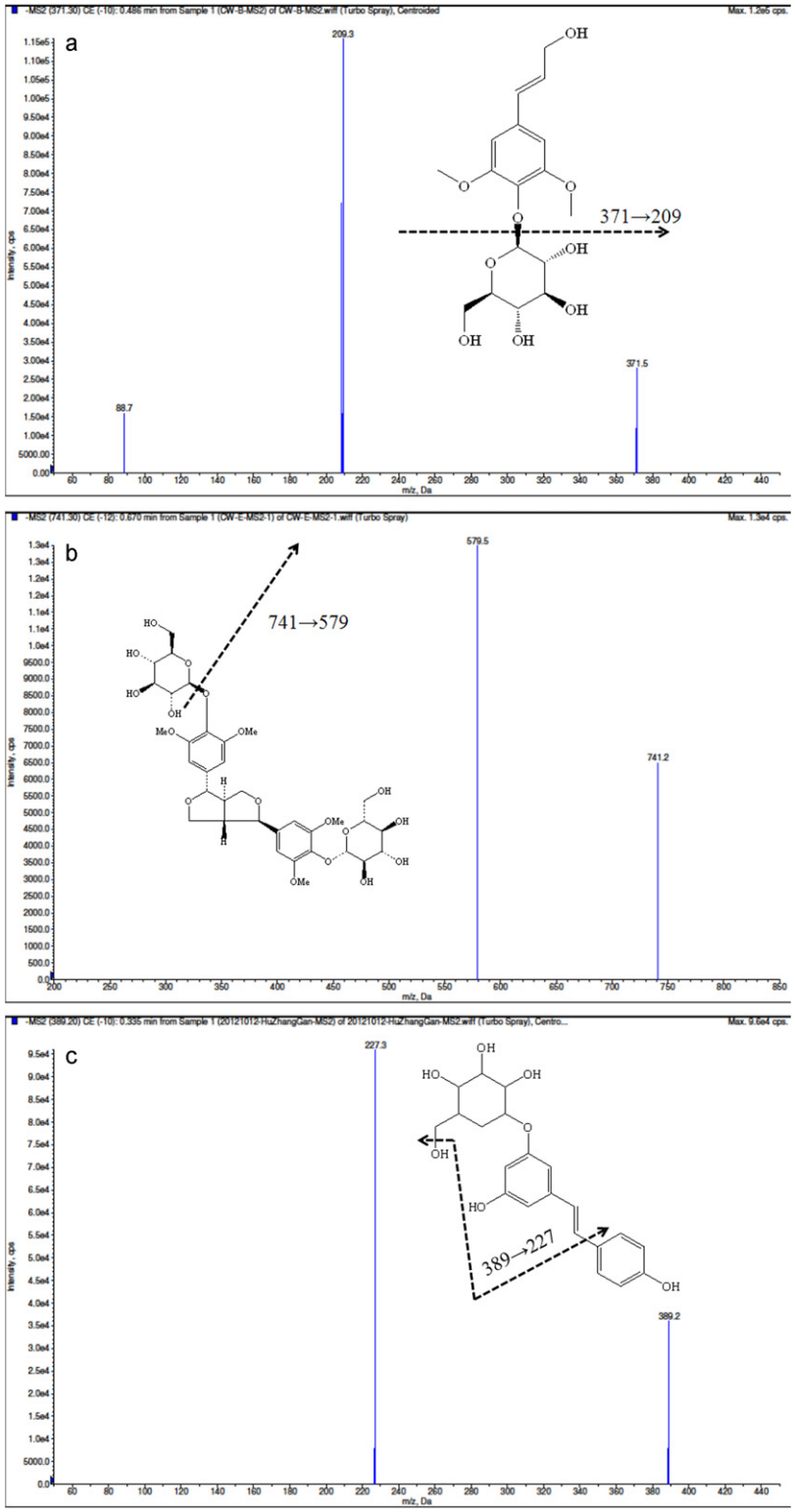

Fig. 2. Full-scan product ion spectra of $[M-H]^-$ for Eleutheroside B (A), Eleutheroside E (B) and IS (C).

The calibration curve samples were prepared by spiking 10 μL of one of the above-mentioned working solution into 90 μL blank rat plasma at concentrations of 1, 2, 5, 20, 50, 200, 500, 1000 and 2000 ng/mL for both. And quality control (QC) samples were also prepared with the same above process to acquire final concentrations of 2, 100 and 1600 ng/mL for Eleutheroside B and Eleutheroside E, respectively.

2.4. Plasma sample preparation

A 100 μL aliquot of each rat plasma sample was transferred into a 1.5-mL Eppendorf tube, and then precipitate with 400 μL methanol containing internal standard (polygonin 1 μg/mL) solution was added. The mixture was vortexed for 3 min and centrifuged at 20,000 × *g* for 10 min. Subsequently, 350 μL of

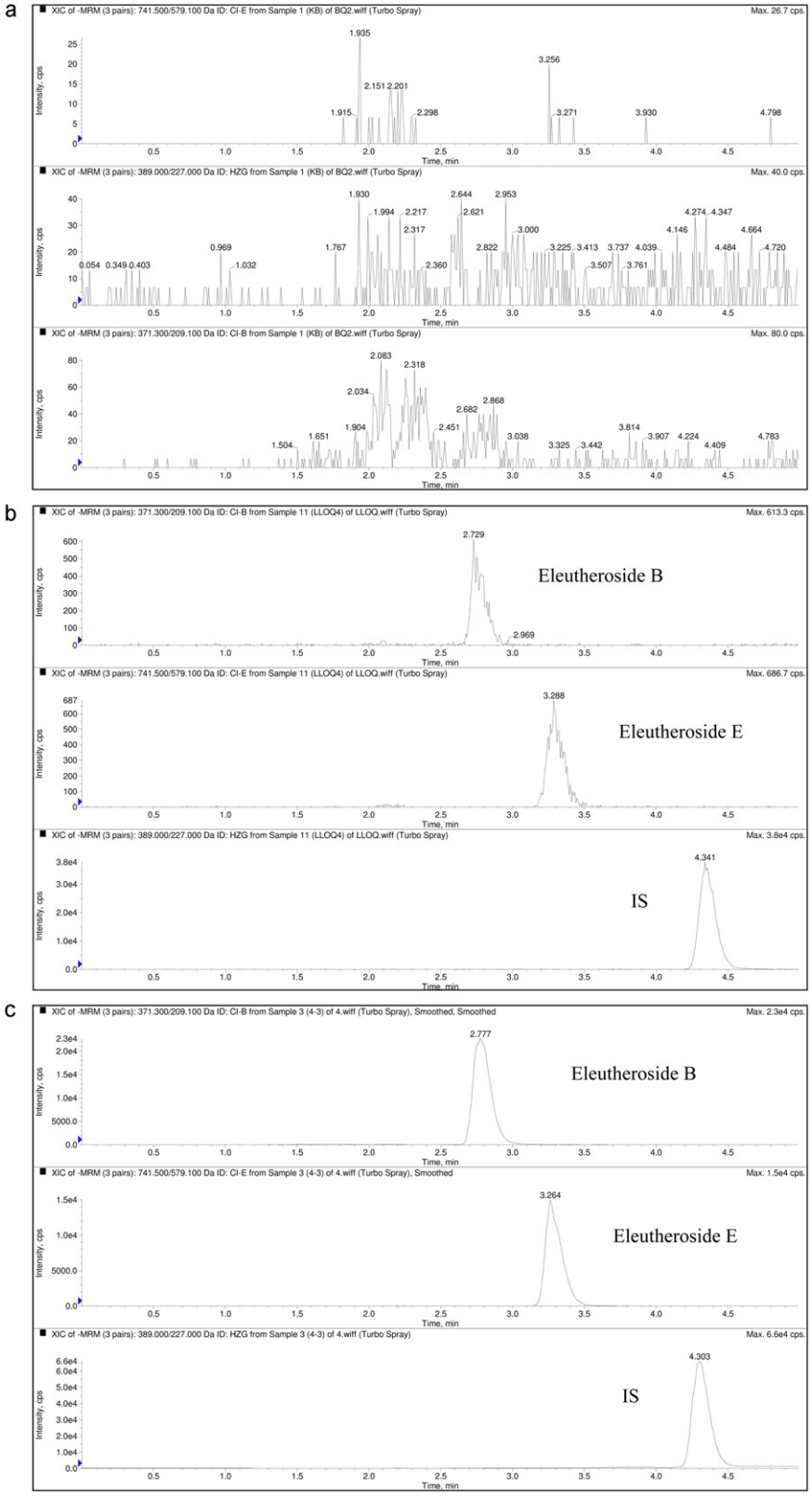

Fig. 3. Representative MRM chromatograms of (A) blank rat plasma, (B) rat plasma spiked with 1 ng/mL (LLOQ) raddeanin A, (C) a rat plasma sample obtained 1 h after an intravenous administration of Eleutheroside B and Eleutheroside E.

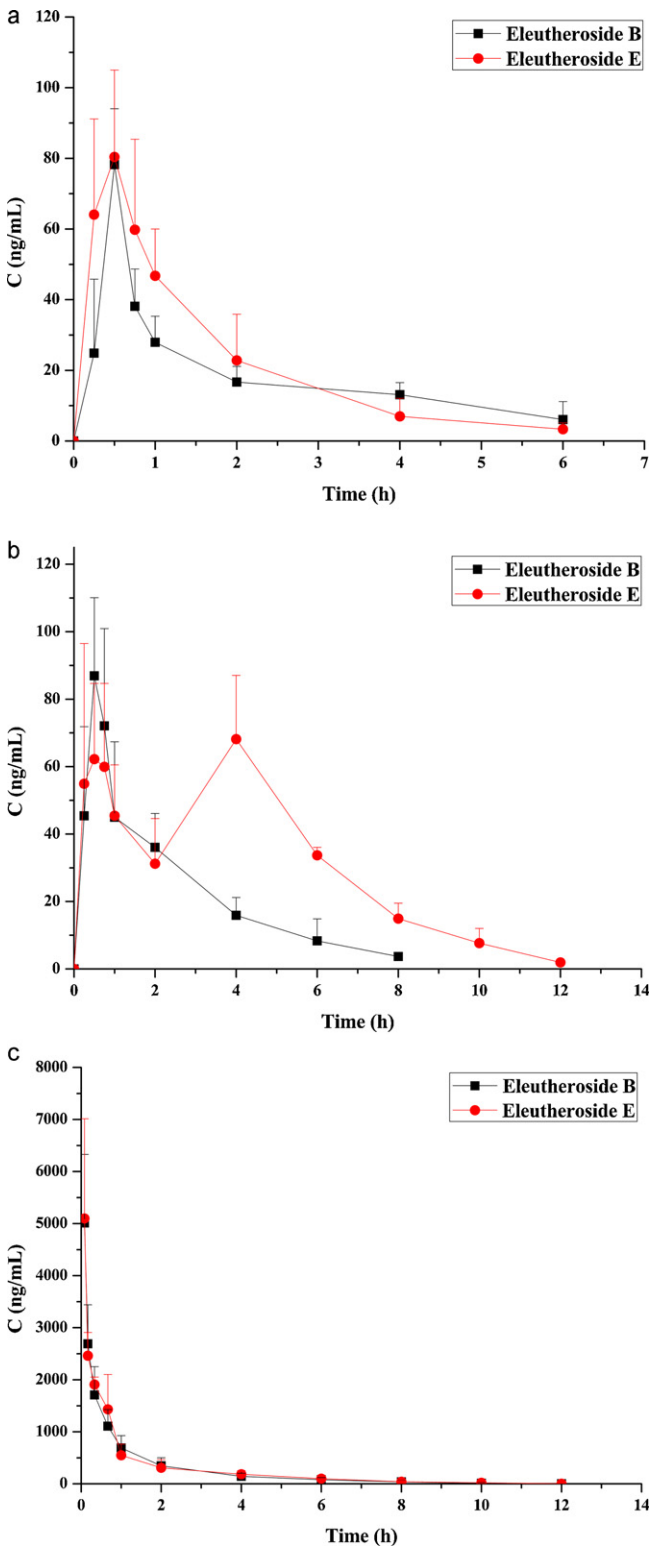

Fig. 4. Mean plasma concentration–time curve of Eleutheroside B and Eleutheroside E after a single oral administration the single substances (A), an aqueous extract of *Eleutherococcus senticosus* (B) and a single intravenous administration (C).

supernatant was transferred into a new 1.5-mL Eppendorf tube and evaporated to dryness under vacuum in speedvac concentrator. The residues were re-dissolved in 100 μL aliquots of re-dissolve solution of acetonitrile/methanol/water (10:20:70, v/v/v). And then the mixture was vortexed for 3 min and centrifuged at 20,000 × *g* for 10 min. Finally, 5 μL supernatant was injected for LC–MS/MS analysis.

2.5. Method validation

The specificity of the method was assessed by comparing lowest concentration in the calibration curves with five different batches of blank rat plasma that had undergone the same pretreatment and analysis.

Calibration curves were individually carried out using least-squares linear regression analysis of a 9-point calibration curve prepared in five replicates by plotting the peak area of the analytes versus the peak area of the internal standard and using $1/X^2$ as a weighting factor. Calibration curves had to have a correlation coefficient (r) of 0.995 or better. The lower limit of quantification (LLOQ) was determined as the lowest concentration with a signal-to-noise (S/N) ratio of 10. The acceptance criteria for each point calculated standard concentration were less than or equal to ±15% deviation from the nominal value except at LLOQ, which was set at ±20%.

The extraction recovery and absolute matrix effect were evaluated for Eleutheroside B and Eleutheroside E samples prepared at three QC concentrations (2, 100, and 1600 ng/mL) and for the internal standard polygonin at a concentration of 100 ng/mL. Each set of samples was analyzed in five replicates. The extraction recoveries were evaluated by comparing the peak areas of extracted standards to that post-extraction plasma spiked with analytes at corresponding concentrations. The absolute matrix effect was calculated by comparing the peak areas of post-extraction plasma spiked with analytes to those prepared in re-dissolve solution of acetonitrile/methanol/water (10:20:70, v/v/v).

The intra-day and inter-day precision and accuracy were investigated by analyzing five sets of QC samples at three concentration levels (2, 100 and 1600 ng/mL for both Eleutheroside B and Eleutheroside E) on three different days. The accuracy was expressed by the relative percentage error, (R.E., %), and the precision by relative standard deviation (R.S.D., %). The RE of the mean value should be within ±15% at each concentration except for the LLOQ, where the RE should be within ±20%. The precision was required to be less than 20% at the LLOQ level and less than 15% at other concentrations.

The stability of Eleutheroside B and Eleutheroside E in rat plasma was carefully investigated by analyzing the QC samples at 3 concentration levels (2, 100, and 1600 ng/mL) under the following conditions: (1) the short-term stability was studied after exposure of the plasma samples to room temperature (20 °C) for 12 h; (2) the long-term stability was tested after keeping the samples at low temperature (−70 °C) for 14 days; (3) freeze–thaw stability was investigated by freezing the QC samples at −70 °C overnight, and thawing at 25 °C, for a total of three freeze–thaw cycles; (4) post-preparative stability was analyzed following storage in the auto-sampler at 4 °C for 24 h. The results were evaluated by the values of R.E.% and R.S.D.%.

The dilution integrity experiment was researched because some of the studied sample concentrations were expected to be higher than the upper limit of quantification (above ULOQ). Dilution integrity experiment was carried out by a 5-fold dilution of the ULOQ concentration (2000 ng/mL × 5) with blank plasma for five replicates. The acceptable precision and accuracy were required to be within ±15%. And carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the ULOQ and the response was checked, as previously reported [20].

2.6. Application to pharmacokinetic study

Eighteen Sprague-Dawley rats (both sexes) weighing 200–220 g were obtained from the Animal Center of Nanjing Medical

University (Nanjing, China) and were kept in an environmentally controlled room (temperature: 25 ± 2 °C, humidity: 50 ± 20%, 12 h dark–light cycle) for at least 5 days before the experiment. The rats were fasted overnight before drug administration. All rats were randomly assigned to three groups ($n = 6$). Blood samples (0.2 mL) were collected from the ocular vein into heparinized tubes at 0 (pro-drug), 5 min, 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h after intravenous administration of single substances (containing Eleutheroside B 5 mg/kg and Eleutheroside E 4.5 mg/kg), and at 0 (pro-drug), 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h after oral administration of single substances and an aqueous extract from *E. senticosus* (at a dose containing Eleutheroside B 5 mg/kg and Eleutheroside E 4.5 mg/kg), respectively. Blood samples were placed in heparinized Eppendorf tubes and plasma was immediately separated by centrifugation at 3000 × *g* for 10 min and the samples were stored at −70 °C until analysis. All animal experiments were carried out according to the Guidelines for the Animal Ethics Committee of Nanjing University of Technology (Nanjing, China).

Pharmacokinetic parameters were calculated by non-compartmental methods using the DAS (Drug and Statistics) 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China) from the plasma concentration–time data. The maximum plasma drug concentration (C_{max}) and the time to reach the maximum plasma drug concentration (T_{max}) values were obtained directly from the experimental data. The elimination half-life ($T_{1/2}$) was determined by linear regression of the terminal portion of the plasma concentration–time data. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration point (AUC_{0-t}) was calculated by the linear trapezoidal method. Extrapolation to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + Ct/ke$, where Ct is the last measurable plasma concentration and ke is the terminal elimination rate constant, as previously reported [21]. The absolute bioavailability is the dose-corrected area under curve (AUC) oral divided by AUC intravenous. All results were expressed as arithmetic mean ± standard deviation (S.D.).

3. Results and discussion

3.1. Optimization of mass spectrometric and chromatographic conditions

Different ionization methods (including positive and negative modes) were tested and compared to obtain good specificity and sensitivity for Eleutheroside B and Eleutheroside E determination. Negative ESI was found to be more sensitive than positive ESI by infusing an approximately 200 ng/mL solution of Eleutheroside B and Eleutheroside E in acetonitrile/water (50/50, v/v) using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA). During a direct infusion experiment, the mass spectra for Eleutheroside B and Eleutheroside E revealed peaks at m/z 371 and 741, respectively as protonated molecular ions $[M-H]^-$. The product ion mass spectrum for Eleutheroside B shows the formation of characteristic product ions at m/z 209 and 88. For Eleutheroside B and Eleutheroside E, the m/z 209 and 579 fragment was dominant and was therefore used for quantification.To achieve the efficient separation of Eleutheroside B, Eleutheroside E and IS, different mobile phases (methanol and acetonitrile) and additives (ammonium acetate, formic acid and ammonia water) were tested. The retention time was lengthened and the peak shape was widened using methanol as organic phase, compared with using acetonitrile as organic phase. Therefore, acetonitrile was chosen as the organic phase because its ability on the peak shape and ionization in negative modes is superior to that of methanol. The addition of 0.1% ammonium hydroxide in water could enhance the PH value of the mobile phase, which could improve the ionization efficiency and intensity of the signal response in negative modes during the ESI process. Gradient elution is usually used in order to improve the peak shape and eliminate higher matrix effect due to co-elute the analyte and the endogenous plasma components. Polygonin selected as internal standard has similar chromatographic and mass spectrometric behaviors to the analyte, and affinis sample preparation in plasma with the analyte.

3.2. Selection of extraction method

To obtain better extraction efficacy and less endogenous interference, various sample extraction approaches including protein precipitation and liquid–liquid extraction with different solvents were investigated. Above all, several solvent combinations were tested for liquid–liquid extraction of the analyte and IS. However, liquid–liquid extraction is not a viable option that could not provided satisfactory extraction efficiency due to the analytes highly hydrophilic nature. Subsequently, we used the protein precipitation including acetonitrile and methanol, which provided satisfactory extraction efficiency. In the end, methanol was chosen as the extraction solvent because of its higher extraction efficiency than that of acetonitrile.

3.3. Method validation

3.3.1. Specificity

Selectivity was investigated by comparing the chromatograms of five different of blank rat plasma with the corresponding spiked plasma. The retention times of Eleutheroside B, Eleutheroside E and IS were about 2.7, 3.2 and 4.3 min, respectively. A representative chromatogram of blank plasma, spiked plasma sample with analytes in LLOQ level and IS, and plasma sample from rat 1 h after an intravenous injection administration is illustrated in Fig. 3. No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma.

3.3.2. Linearity and LLOQ

Five calibration analyses were carried out on five consecutive days. A typical equation for calibration curve at the range from 1 to 2000 ng/mL was $y = 0.00267x - 0.000422$ ($R = 0.9992$) for Eleutheroside B and $y = 0.00154x - 0.000595$ ($R = 0.9995$) for Eleutheroside E, indicating a good linearity. The LLOQ was 1 ng/mL (S/N > 10), with %RSD = 9.66 and 11.07, %RE = −7.10 and −5.96 for Eleutheroside B and Eleutheroside E.

3.3.3. Precision and accuracy

As shown in Table 1, the method gave good precision and accuracy with the intra- and inter-day precision. The inter-day accuracy ranged 1.17–1.83% for Eleutheroside B and −1.70 to 3.68% for Eleutheroside E as well as the intra-day accuracy ranged −2.80 to 3.30% for Eleutheroside B and 0.60–5.70% for Eleutheroside E. The intra- and inter-day precisions were within 11.46% for Eleutheroside B and 10.68% for Eleutheroside E, respectively. The values for both intra- and inter-day accuracy and precision were found to be within the acceptable criteria.

3.3.4. Extraction recovery and matrix effect

The extraction recoveries of Eleutheroside B from rat plasma were 84.83 ± 2.41%, 83.95 ± 5.01% and 82.63 ± 8.85% at concentration levels of 2.00, 100 and 1600 ng/mL, respectively ($n = 5$), and the average extraction recoveries of Eleutheroside E was 86.45 ± 6.54%, 87.29 ± 7.65% and 87.45 ± 9.70% at three QC samples ($n = 5$). The extraction recovery of the internal standard (100 ng/mL) was 88.27 ± 10.40% ($n = 5$).

Table 1
Summary of the inter-day as well as inter-day accuracy and precision of Eleutheroside B and Eleutheroside E in rat plasma ($n = 15$).

Compound	Nominal concentration (ng/mL)	Intra-day			Inter-day		
		Measured concentration (ng/mL)	Precision (%)	Accuracy (%)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Eleutheroside B	2	1.94	11.46	−2.80	2.04	3.45	1.83
	100	103.30	9.28	3.30	101.26	10.66	1.26
	1600	1646.20	5.50	2.88	1618.66	4.53	1.17
Eleutheroside E	2	2.01	8.55	0.60	1.97	4.94	−1.70
	100	105.70	5.46	5.70	103.68	10.68	3.68
	1600	1656.40	6.18	3.53	1618.94	3.30	1.18

The matrix effects (enhancement/suppression of ionization) were evaluated comparing the absolute peak area of plasma samples at three QC concentrations after extraction with the mean peak area of standard solutions at the same concentration. The matrix effects of Eleutheroside B at concentrations of 2.00, 100 and 1600 ng/mL were 102.83 ± 4.58, 97.86 ± 2.73 and 94.41 ± 4.46%, respectively. And the average matrix effects of Eleutheroside E were 96.02 ± 6.97, 90.90 ± 7.51 and 90.11 ± 5.88% at three QC samples ($n = 5$), respectively. Additional, the matrix effect value was 104.32 ± 5.24% for IS. No significant matrix effect for Eleutheroside B, Eleutheroside E and IS was observed, indicating that no co-eluting substance influenced the ionization of the analyte and IS significantly. The extraction recoveries and matrix effects of Eleutheroside B, Eleutheroside E and IS from rat plasma samples are shown in Table 2.

3.3.5. Stability

The stability of the analytes in rat plasma under different temperature and timing conditions was evaluated and the data of the stability studies are listed in Table 3. Autosampler stability test suggested that Eleutheroside B and Eleutheroside E on three QC concentrations were stable in the mobile phase at 4 °C for at least 24 h (RE: −4.22 to 2.67% for Eleutheroside B and −7.10 to 8.00% for Eleutheroside E, RSD < 13% for both). Three freeze–thaw cycles of the QC samples, appeared to have little effect on the stability, with accuracy ranging from −7.90 to −1.16% for Eleutheroside B and from −8.20 to 3.30% for Eleutheroside E (RSD < 12% for both). The long-term stability tests indicated that Eleutheroside B and Eleutheroside E were stable in rat plasma on three QC samples at −70 °C for at least 14 days (RE: −3.70 to 9.82% for Eleutheroside B and −4.90 to −2.91% for Eleutheroside E, RSD < 10% for both). In the short-term stability (12 h), the two compounds were stable in plasma at three QC concentrations, with accuracy ranging from −4.20 to 1.60% for Eleutheroside B and from −8.10 to 2.96% for Eleutheroside E (RSD < 10% for both).

3.3.6. Dilution and carry-over effect

Dilution integrity experiments were preformed in five replicates by a 5-fold dilution with blank plasma, and assay precision and accuracy were tested using the same sample pretreatment method. The result indicated that the precision was less than 9.5%, and the accuracy was within ±10.6%. A potential carry-over effect was carried out by analyzing extracted blank rat samples after the highest calibrators (2000 ng/mL) and no carry-over was observed.

3.4. Pharmacokinetic application

The above method was successfully applied to the pharmacokinetic study of Eleutheroside B and Eleutheroside E after oral administration at single dose of the single substances and an aqueous extract of *E. senticosus* as well as intravenous injection the single substances in healthy rats. Mean plasma concentration–time curve of Eleutheroside B and Eleutheroside E in single dose study is shown in Fig. 4. The main pharmacokinetic parameters of Eleutheroside B and Eleutheroside E are calculated and listed in Table 4. A fast absorption process was found after oral administration of an aqueous extract from *E. senticosus* and single substances, respectively. The mean values of T_{max} were 0.45 ± 0.112 h for single substances and 0.583 ± 0.144 h for an aqueous extract, respectively. The elimination half-life of Eleutheroside B and Eleutheroside E was less than 2.5 h for both. Oral absolute bioavailability of Eleutheroside B and Eleutheroside E was 3.30 ± 0.63% and 3.82 ± 0.86%, respectively, after oral and intravenous administration at a single of Eleutheroside B and Eleutheroside E.

Pharmacokinetic parameters revealed interesting differences in the plasma between oral administration of an aqueous extract from *E. senticosus* and single substances. The AUC_{0-t} of Eleutheroside B after oral administration of an aqueous extract of *E. senticosus* was found significantly elevated ($P = 0.034$) compared with oral administration of single substances. In additional, AUC_{0-t} and $AUC_{0-\infty}$ of Eleutheroside E were significantly increased ($P = 0.009$ for AUC_{0-t} and $P = 0.011$ for $AUC_{0-\infty}$) by oral administration of an aqueous extract of *E. senticosus*, compared with oral administration of single substances. No significant difference was observed in C_{max} of Eleutheroside B and Eleutheroside E in rat plasma for an aqueous extract of *E. senticosus and* single substances. Moreover, enterohepatic circulation was found in Eleutheroside E after oral administration an aqueous extract of *E. senticosus.* Further researches should be carried out to discover the reasons of pharmacokinetic behavior difference. The knowledge obtained could be used to evaluate the effect of these differences on the efficacy and safety of the *E. senticosus* in clinical applications.

Table 2
Extraction recovery and matrix effect of Eleutheroside B and Eleutheroside E in rat plasma ($n = 5$).

Compound	Nominal concentrations (ng/mL)	Extraction recovery (%)	CV (%)	Matrix effect (%)	CV (%)
Eleutheroside B	2	84.83 ± 2.41	2.85	102.83 ± 4.58	4.46
	100	83.95 ± 5.01	5.96	97.86 ± 2.73	2.79
	1600	82.63 ± 8.85	10.71	94.41 ± 4.46	4.73
Eleutheroside E	2	86.45 ± 6.54	7.57	96.02 ± 6.97	7.26
	100	87.29 ± 7.65	8.76	90.90 ± 7.51	8.26
	1600	87.45 ± 9.70	11.09	90.11 ± 5.88	6.53
IS	100	88.27 ± 10.40	11.78	104.32 ± 5.24	5.02

Table 3
Stability of Eleutheroside B and Eleutheroside E in rat plasma ($n = 5$).

Sample condition	Analyte	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Accuracy (%)	Precision (%)
The short-term stability (12 h)	Eleutheroside B	2	1.92	−4.20	5.64
		100	101.60	1.60	8.51
		1600	1563.50	−2.31	6.51
	Eleutheroside E	2	1.84	−8.10	8.31
		100	94.48	−5.52	8.39
		1600	1647.40	2.96	7.64
The long-term stability (14 days, −70 °C)	Eleutheroside B	2	1.93	−3.70	7.22
		100	109.80	9.82	6.91
		1600	1569.60	−1.90	9.22
	Eleutheroside E	2	1.90	−4.90	9.60
		100	95.66	−4.34	6.39
		1600	1553.40	−2.91	7.43
Three freeze/thaw cycles	Eleutheroside B	2	1.84	−7.90	7.36
		100	98.84	−1.16	11.46
		1600	1565.00	−2.19	3.85
	Eleutheroside E	2	1.84	−8.20	7.78
		100	103.30	3.30	9.06
		1600	1520.30	−4.98	5.99
The post-preparative stability (24 h, 4 °C)	Eleutheroside B	2	1.94	−3.20	8.21
		100	95.78	−4.22	7.62
		1600	1642.80	2.67	8.24
	Eleutheroside E	2	1.86	−7.10	12.25
		100	108.00	8.00	11.55
		1600	1620.45	1.27	7.39

Table 4
Non-compartmental pharmacokinetic parameters for (1) Eleutheroside B and (2) Eleutheroside E in rat plasma after a single oral administration of single substances and an aqueous extract from *Eleutherococcus senticosus* as well as intravenous administration of single substances ($n = 6$).

Pharmacokinetic parameters	Unit	I.V.	Oral.	Oral.
		The single substances	The single substances	An aqueous extract of *Eleutherococcus senticosus*
(1)				
AUC_{0-t}	μg/Lh	3340.98 ± 761.70	110.11 ± 20.93*	187.04 ± 53.23
$AUC_{0-\infty}$	μg/Lh	3353.61 ± 768.50	130.19 ± 34.54	193.89 ± 55.46
MRT	H	1.42 ± 0.45	2.05 ± 0.27	2.17 ± 0.51
$T_{1/2}$	H	1.43 ± 0.37	2.03 ± 1.05	1.47 ± 0.41
T_{max}	H	–	0.412 ± 0.14	0.58 ± 0.14
CL	L/h/kg	0.94 ± 0.28	24.25 ± 6.16	16.73 ± 4.80
V	L/kg	1.91 ± 0.51	65.02 ± 24.21	33.95 ± 8.18
C_{max}	μg/L		78.25 ± 15.80	97.75 ± 12.64
Absolute bioavailability	%	–	3.30 ± 0.63	–
(2)				
AUC_{0-t}	μg/Lh	3404.38 ± 656.58	129.95 ± 29.12**	367.60 ± 83.10
$AUC_{0-\infty}$	μg/Lh	3434.06 ± 655.32	135.19 ± 32.27**	375.14 ± 87.90
MRT	H	1.60 ± 0.18	1.42 ± 0.42	4.07 ± 0.45
$T_{1/2}$	H	1.77 ± 0.70	1.13 ± 0.43	1.49 ± 0.18
T_{max}	H	–	0.42 ± 0.14	2.75 ± 2.17
CL	L/h/kg	0.90 ± 0.19	23.03 ± 5.33	8.35 ± 2.24
V	L/kg	2.36 ± 1.22	37.01 ± 15.49	17.61 ± 2.46
C_{max}	μg/L		91.33 ± 12.53	75.50 ± 26.62
Absolute bioavailability	%	–	3.82 ± 0.86	–

Data are means ± S.D. Significantly different from an aqueous extract of *Eleutherococcus senticosus* (*$P < 0.05$, **$P < 0.01$).

4. Conclusion

In this study, a sensitive and reliable LC–MS/MS method was developed and validated to simultaneous determination of Eleutheroside B and Eleutheroside E in rat plasma using a simple protein precipitation procedure. Linear calibration curves were established over the concentration range of 1–2000 ng/mL in plasma for both. The LC–MS/MS assay was successfully applied to a comparative pharmacokinetic study of Eleutheroside B and Eleutheroside E after oral administration of the single substances and an aqueous extract of *E. senticosus*. The data shown that notable differences in the pharmacokinetic behavior and enterohepatic circulation were found after oral administration of an aqueous extract of *E. senticosus* compared with the single substances. Therefore, pharmacokinetic studies play a pivotal role in improving therapeutic effect and avoiding toxicity when plant extract used in the treatment of disease.

Acknowledgment

The study was supported by grant from the "333 High Level Talent Cultivation Project" of Jiangsu Province (2012) as well as Program for Changjiang Scholars and Innovative Research Team in University (Grant No. IRT1066).

References

[1] H. Wagner, E. Jordan, B. Feil, Oncology 43 (Suppl. 1) (1986) 16–22.

[2] D.H. Kim, K.T. Lee, E.A. Bae, M.J. Han, H.J. Park, Arch. Pharm. Res. 22 (1999) 30–34.
[3] L.Z. Huang, B.K. Huang, Q. Ye, L.P. Qin, J. Ethnopharmacol. 133 (2011) 213–219.
[4] C. Tohda, M. Ichimura, Y. Bai, K. Tanaka, S. Zhu, K. Komatsu, J. Pharmacol. Sci. 107 (2008) 329–339.
[5] L. Huang, H. Zhao, B. Huang, C. Zheng, W. Peng, L. Qin, Pharmazie 66 (2011) 83–97.
[6] J.Y. Cho, K.H. Nam, A.R. Kim, et al., J. Pharm. Pharmacol. 53 (2001) 1287–1294.
[7] Y.Y. Song, Y. Li, H.Q. Zhang, Yao Xue Xue Bao 45 (2010) 1006–1011.
[8] H.S. Niu, F.L. Hsu, I.M. Liu, J.T. Cheng, Horm. Metab. Res. 39 (2007) 894–898.
[9] H.S. Niu, I.M. Liu, J.T. Cheng, C.L. Lin, F.L. Hsu, Planta Med. 74 (2008) 109–113.
[10] H.S. Niu, F.L. Hsu, I.M. Liu, Neurosci. Lett. 445 (2008) 113–116.
[11] K.Y. Liu, Y.C. Wu, I.M. Liu, W.C. Yu, J.T. Cheng, Neurosci. Lett. 434 (2008) 195–199.
[12] S. Nakamura, Y. Zhang, H. Matsuda, K. Ninomiya, O. Muraoka, M. Yoshikawa, Chem. Pharm. Bull. 59 (2011) 1020–1028.
[13] L.Z. Huang, L. Wei, H.F. Zhao, B.K. Huang, K. Rahman, L.P. Qin, Eur. J. Pharmacol. 658 (2011) 150–155.
[14] Y.C. Ma, X.Q. Wang, F. Hou, et al., J. Pharm. Biomed. Anal. 55 (2011) 908–915.
[15] B.T. Gaffney, H.M. Hugell, P.A. Rich, Phytochem. Anal. 15 (2004) 231–234.
[16] Q. Li, L.X. Sun, L. Xu, et al., Biomed. Chromatogr. 20 (2006) 1315–1320.
[17] J.S. Kang, P.T. Linh, X.F. Cai, H.S. Kim, J.J. Lee, Y.H. Kim, Arch. Pharm. Res. 24 (2001) 407–411.
[18] S.P. Liu, J.T. An, R. Wang, Q. Li, Molecules 17 (2012) 7903–7913.
[19] S. Feng, F. Hu, J.X. Zhao, X. Liu, Y. Li, Eur. J. Pharm. Biopharm. 62 (2006) 315–320.
[20] S. Ghassabian, S.M. Moosavi, Y.G. Valero, K. Shekar, J.F. Fraser, M.T. Smith, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 903 (2012) 126–133.
[21] X. Zhao, X. Liu, Y. Wang, J. Zhong, Y. Chen, G. Wang, J. Pharm. Biomed. Anal. 41 (2006) 906–911.