Li Yun-Qing (Orcid ID: 0000-0003-3463-6560)

Li Cheng-Jian (Orcid ID: 0000-0003-1998-4778)

An UPLC-MS/MS method for simultaneous determination of five flavonoids from *Stellerachamaejasme* L. in rat plasma and its application to a pharmacokinetic study

Yun-Qing Li[a,b+], Cheng-Jian Li[a+], Lei Lv[a], Qing-qingCao[a], Xian Qian[a], Si wei Li[c], Hui Wang[a*], Liang Zhao[a,d *]

[a]Department of Pharmacy, Shanghai Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, China;

[b]Department of Pharmacy, The Children Hospital of Shanxi, Taiyuan 030000, China;

[c] Department of Pharmaceutical Science, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA;

[d] Center of Health Management, Zhejiang Changtai Hospital, Zhuji 311800, China

ABSTRACT

Stellerachamaejasme L. has been used as a Traditional Chinese Medicine for the treatment of scabies, tinea, stubborn skin ulcers, chronic tracheitis, cancer and

+ The first two authors contributed equally to this work.

*Corresponding authors: Department of Pharmacy, Eastern HepatobiliarySurgery Hospital, Second Military Medical University, Shanghai, 200438, China. E-mail addresses: wang_ehbh@126.com(H. Wang), zhaoliangphar@163.com(L. Zhao)

tuberculosis. A sensitive and selective ultra-high liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for the simultaneous determination of five flavonoids (stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasmin) of *Stellerachamaejasme* L. in rat plasma. Chromatographic separation was accomplished on an Agilent Poroshell 120 EC-C$_{18}$ column (2.1×100 mm, 2.7μm) with gradient elution at a flow rate of 0.4 mL/min and the total analysis time was 7 min. The analytes were detected using multiple reaction monitoring (MRM) in positive ionization mode. The samples were prepared by liquid–liquid extraction with ethyl acetate. The UPLC–MS/MS method was validated for specificity, linearity, sensitivity, accuracy and precision, recovery, matrix effect and stability. The validated method exhibited good linearity(r≥ 0.9956), and the lower limits of quantification ranged from 0.51 to 0.64 ng/mL for five flavonoids. The intra-day and inter-day precision were both less than 10.2%, and the accuracy ranged from -11.79-9.21%. This method was successfully applied to a pharmacokinetic study of five flavonoids in rats after oral administration of ethyl acetate extract of *Stellerachamaejasme* L.

KEYWORDS

UPLC-MS/MS; *Stellerachamaejasme* L.; Flavonoids;   Rat plasma; Pharmacokinetics

1. Introduction

Stellerachamaejasme L. (Thymelaeaceae), a perennial weed, distributes widely in the grasslands of Russia, Mongolia and China(Yan *et al.*, 2014; Shirai *et al.*, 2015;

Yan *et al.*, 2015). Its roots are commonly known as "Ruixianglang du" in Chinese, has been used in China as a Traditional Chinese Medicine for the treatment of scabies, tinea, stubborn skin ulcers, chronic tracheitis, cancer and tuberculosis(Xu *et al.*, 2001; Zhang *et al.*, 2013; Pan *et al.*, 2015). Previous phytochemical studies of *Stellerachamaejasme* L. has shown that it contains kinds of chemical components, including diterpene, lignans, coumarins, phenylpropanoid, flavonoids and volatile oil(Li *et al.*, 2014; Yan *et al.*, 2014; Zhang *et al.*, 2015). Modern studies have suggested that flavonoids from *Stellerachamaejasme* L. are the main components that mostly contribute to the pharmacological efficacy(Liu *et al.*, 2012; Liu and Zhu, 2012; Liu *et al.*, 2014b, a), such as anti-cancer activity(Liu *et al.*, 2012; Li *et al.*, 2014), anti-fatty liver(Wang *et al.*, 2015) and anti-HIV(Asada *et al.*, 2013).

In our earlier research, some flavonoids have been isolated and identified from ethyl acetate extract of *Stellerachamaejasme* L. (ESL), and five flavonoids including stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasmin have been detected in the rat plasma and tissues after oral administration of ESL. All of them are active compounds of the herbal preparation with pharmacological effects. Stelleranol was found to have potential antiviral activity against respiratory syncytial virus (Huang *et al.*, 2010). Yang et al demonstrated that chamaechromone have antiviral effect on hepatitis B virus(Yang and Chen, 2008). Neochamaejasmin A, meanwhile, was reported to induce apoptosis in human prostate cancer cells. According to previous research, chamaejasmine has a profound antiproliferative effects on human osteosarcoma cells, breast cancer cells, lung adenocarcinoma A549 cells and HEp-2 larynx carcinoma cells (Yu *et al.*, 2011; Zhang *et al.*, 2013; Yang,Wang and Ren, 2015). In addition, Zhang et al discovered that isochamaejasmin could induce apoptosis in leukemia cells through inhibiting the Bcl-2 family proteins(Zhang *et al.*, 2015).

However, there is almost no report for the pharmacokinetic study of simultaneous determination of those five flavonoids in rat plasma after administration of ESL. In this study, a rather selective and sensitive UPLC-MS/MS method was firstly developed and validated for simultaneously determining stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasmin in rat plasma. The results of this study would be helpful for improving clinical application and further pharmacological studies of *Stellerachamaejasme* L.

2. Experimental

2.1 Chemicals and reagents

Stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine, isochamaejasmin and quercetin were purchased from Nature Standard (Shanghai, China) (purity >98.0%). Quercetin was used an an internal standard (IS). Chemical structures of those compounds are shown in Fig. 1. Acetonitrile, ethanol, ethyl acetate, petroleum ether and formic acid were of HPLC grades, and supplied by Merck (Germany). Ultra-pure water was prepared using a PW water system (Heal Force, Hong Kong).

2.2 Instruments and UPLC–MS/MS conditions

The UPLC system was Agilent technology 1290 series equipped with an online degasser, G7120A binary pump, G7167B auto-sampler and G7116B thermostatic-column. Chromatographic separation was achieved on an Agilent Poroshell 120 EC-C_{18} column (2.1×100 mm, 2.7μm) at 25 °C. The mobile phase consisted of methanol-water containing 0.1% formic acid with a gradient elution starting at 45% methanol and progressing linearly to 75% methanol over 6 min then

returning to 45% methanol. The flow rate was 0.4 mL/min and the total analysis time was 6 min. A 5 μL aliquot of sample solution was injected, with a needle wash process which is used to wash the outer wall of sample needle after each injection.

The mass spectrometric detection was performed on a 6470 triple quad mass spectrometer (Agilent, US) in positive ionization mode. An Agilent Mass Hunter workstation (Agilent, US) was used to control the equipment and for data acquisition and analysis. The quantification was obtained using multiple reaction monitoring (MRM) of precursor-product ion transition at m/z 559.2→153.0 for stelleranol, 543.3→198.9 for chamaechromone, 543.2→153.0 for neochamaejasmin A, 543.1→152.9 forchamaejasmin, 543.2→153.0 for isochamaejasmin and m/z 303.1→153.1 for quercetin (IS), respectively. Other parameters of the mass spectrometer were set as follows: a drying gas flow of 5 L/min; a drying gas temperature of 200 °C, a nebulizer pressure of 45 psig and a capillary voltage of 4000 V.

2.3 Preparation of ESL

For preparation of the extract, the dried powder of *Stellerachamaejasme* L. (1kg) was extracted under reflux with 4000 mL ethanol-water (80:20, v/v) for two times, 2 h each time, and then filtrated. Recovering ethanol under reduced pressure, and then extracted with petroleum ether and ethyl acetate successively for several times. Ethyl acetate extract was obtained by vacuum freeze drying. The extract was reconstituted in 15% ethanol-water to get a concentration equivalent to 0.25g/mL of ESL.

2.4 Preparation of calibration standards and quality control (QC) samples

The stock solutions of stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine, isochamaejasmin and the IS (quercetin) were individually prepared in

methanol. The stock solutions of the standards were further diluted in methanol to produce combined standard working solutions at a series of concentrations. The IS solution (94.2ng/mL) was obtained by diluting the stock solution in methanol. The samples for standard calibration curves were prepared by spiking appropriate amount of the standard solutions in 100 μL blank plasma to yield calibration concentrations of 1280, 640.0, 320.0, 128.0, 64.00, 32.00, 12.80, 6.400, 1.280 and 0.6400 ng/mL for stelleranol; 1060, 530.0, 265.0, 106.0, 53.00, 26.50, 10.60, 5.300, 1.060 and 0.5300 ng/mL for chamaechromone; 1055, 527.5, 263.8, 105.5, 52.75, 26.38, 10.55, 5.275, 1.055 and 0.5275 ng/mL for neochamaejasmin A; 1160, 580.0, 290.0, 116.0, 58.00, 29.00, 11.60, 5.800, 1.160 and 0.5800 for chamaejasmine; 1020, 510.0, 255.0, 102.0, 51.00, 25.50, 10.20, 5.100, 1.020 and 0.5100 ng/mL for isochamaejasmin, respectively. Quality control (QC) samples include low QC (LQC), middle QC (MQC), and high QC (HQC). The QC samples were prepared at 1.280, 128.0, and 1280 ng/mL for stelleranol;1.060, 106.0, and 1060 ng/mL for chamaechromone;1.055, 105.5, and 1055 ng/mL for neochamaejasmin A;1.160, 116.0, and 1160 ng/mL for chamaejasmine; 1.020, 102.0 and 1020 for isochamaejasmin. Calibration work solutions and QC samples were stored at 4 °C until UPLC–MS/MS analysis.

2.5 Preparation of plasma samples

The plasma samples were frozen and stored at -40°C in the refrigerator and were prepared to thaw at room temperature by vortex before use. The liquid–liquid extraction (LLE) method was applied to extract the analytes and IS from rat plasma. After 100 μL of plasma sample was transferred into a 1.5 mL centrifuge tube, 20 μL of IS work solution (94.2 ng/mL) was added. The mixture was vortex-mixed for 2 min and extracted with 900 μL of ethyl acetate by vortex-mixing for 5 min. Then the sample was centrifuged at 13,800 g for 10 min. The supernatant was transferred into

another test tube and evaporated to dryness with nitrogen gas at room temperature. The residue was reconstituted in 50 μL of the initial mobile phase by vortex-mixing for 2 min and centrifuged at 13,800 g for 10 min. Finally, 5 μL supernatant was injected for UPLC–MS/MS analysis.

2.6 Method validation

The method validation of stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasminin rat plasma was done following the FDA guidelines(FDA, 2013). The validation parameters included specificity, linearity, sensitivity, accuracy and precision, recovery, matrix effect and stability.

2.6.1 Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. In this paper, the specificity was ascertained by comparatively analyzing blank plasma samples from six individual rats, corresponding blank plasma spiked with the five analytes and IS and the plasma samples from the rats after oral administration of ESL.

2.6.2 Linearity, lower limits of quantification (LLOQ) and lower limit of detection(LLOD)

Calibration curves were prepared by assaying standard plasma samples at ten concentration levels. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to IS versus the nominal concentration (x) of analytes with a weighted ($1/x^2$) least square linear regression. Six replicate samples were used for evaluation. The LLOD and LLOQ were determined as the concentration of the analytes with a signal-to-noise ratio at 3 and 5, respectively.

2.6.3 Accuracy and precision

Three validation batches, each containing six replicates of LLOQ and QC samples at low, medium and high concentration levels were assayed to assess the precision and accuracy of the method at three different days. The intra-day and inter-day precisions were defined as relative standard deviation with criteria of less than 15% for QC samples and 20% for LLOQ; the accuracy was assessed by comparing the measured concentration with its nominal value with a criterion of within ± 15% for QC samples and 20% for LLOQ.

2.6.4 Recovery and matrix effect

The recovery of analytes and IS was determined by comparing the responses of the analytes from QC samples with the responses of analytes spiked in post-extracted blank rat plasma at equivalent concentrations. The matrix effects were evaluated quantitatively for six different lots of black rat plasma and evaluated at three QC concentrations in triplicate to know the individual distinctions of matrix effect. The matrix effects were calculated by determining the peak area ratios of the analytes in post-extracted spiked samples to those of pure standard solutions containing analytes at the equivalent concentration.

2.6.5 Stability

The stability of the five flavonoids in samples including room temperature stability (storage for 6 h at ambient temperature), long-term stability (storage for 30 days at -40 °C), freeze-thaw stability (three freeze at -40 °C and thaw cycles), post-preparation stability (storage for 12 h in the auto-sampler at 4 °C) was tested at LQC, MQC, HQC levels with six replicates at each level. All stability testing QC samples were determined by using the calibration curve of freshly prepared standard samples.

2.7 Pharmacokinetic study

Six healthy 6-8 weeks Sprague-Dawley rats were purchased from the Laboratory Animal Center of Second Military Medical University (Shanghai, China). The animals were kept under fasting for 12 h and fed with water prior to the oral administration of the ESL at a dose of 5 g/kg. Blood samples (approximately 400 μL) were collected from the fosse orbital vein into heparinized polythene tubes at 0 (before dosing), 0.17, 0.33, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after dosing, and then centrifuged immediately at 4600 g for 10 min. The obtained plasma was transferred into clean tubes and stored at -40 °C until analysis. In order to guarantee enough blood volume in rats, the rats were given free access to water after 2 h of administration. The animal handling procedures were approved by the Institutional ethical committee and conformed to the principles of the International Guide for the Care and Use of Laboratory Animals. Non-compartmentalpharmacokinetic parameters were calculated DAS 2.0 (China State Drug Administration).

3. Results and discussion

3.1 Optimization of LC conditions

Chromatographic conditions were optimized to improve peak shape, increase sensitivity and shorten run time for simultaneous analysis of five flavonoids. At the onset of the experiments, different mobile phase compositions were tried to achieve good resolution and symmetric peak shapes for each analyte and the IS. The mobile phase had been tested with acetonitrile-water and methanol-water binary solvent system using different buffers such as ammonium formate and formic acid. Finally, the mobile phase consisted of methanol-water containing 0.1% formic acid with a

gradient elution could improve symmetry of peak shape and enhance the signal response.

3.2 Optimization of MS conditions

In order to optimize AJS ESI determination conditions, we collected the mass spectra of five flavonoids and IS under both positive and negative ion detection mode. Results showed that the intensity of the most abundant molecule ion for each analyte produced in positive ion mode was much stronger than that in negative mode. Detection was finally operated in positive ion mode in this study. Precursor ions, product ions, collision energy and fragment voltage of each analyte were optimized by Optimizer software which comes with the tirple quad. The MS/MS transitions and energy parameters of the five compounds are shown in Table 1. Considering other MS parameters also play an important role on the ion response of analytes, we have done some fine-tuning on the basis of auto-tuning. Finally, other parameters were set as follows: a sheath gas temperature of 300 °C, a sheath gas flow of 12 L/min, a nozzle voltage of 2000 V, a nebulizer pressure of 45 psi, a capillary voltage 4000 V, a drying gas temperature of 200 °C, a drying gas flow of 5 L/min.

3.3 Optimization of sample preparation

We have compared protein precipitation with liquid–liquid extraction (LLE) during sample preparation. The former was discarded because of its high noise level and interference by endogenous substances. The latter offered a clean sample and high response. Ethyl acetate was used as the LLE solvent for sample preparation since these five flavonoids were extracted from the ethyl acetate part of *Stellerachamaejasme* L.

3.4 Method validation

3.4.1 Specificity

Typical chromatograms obtained from blank plasma, blank plasma spiked with six analytes and plasma samples from the rats after oral administration of ESL are shown in Fig.2. Retention time of stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine, isochamaejasmin and IS were 2.4 min, 3.5 min, 3.9 min, 4.3 min, 5.1 min and 2.9 min, respectively. All the peaks of the analytes and I.S. were detected with excellent peak resolution (R>2.0) as well as peak shapes, and no interference from the endogenous substances was observed at the retention time of the analytes and IS.

3.4.2 Linearity, LLOQ and LLOD

The equation of linear regression and linearity range for the six analytes were shown in Table 2. The results showed good linearity and all correlation coefficients were higher than 0.9950. The LLOD with a signal-to-noise (S/N) ratio >3 ranged from 0.1700 to 0.2133 ng/mL, whereas the LLOQ with an S/N ratio >5 ranged from 0.5100 to 0.6400 ng/mL, which was sensitive enough for our pharmacokinetic studies using rat plasma.

3.4.3 Accuracy and precision

The intra-day and inter-day accuracy and precision data of five flavonoids in rat plasma are listed in Table 3. At each QC and LLOQ level, the inter-day and intra-day precisions (RSD) offive flavonoids were 0.3−10.2%, the accuracy was -11.79−9.21%.The results on both intra-day and inter-day were found to be within the acceptance criteria.

3.4.4 Recovery and matrix effect

The data of recovery and matrix effect of the five flavonoids are summarized in Table 3. Mean absolute recoveries of stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasmin were 83.84-97.32%, 87.41-98.93%, 81.26-97.14%, 88.44-93.77% and 80.16-94.15% at three QC levels. Results suggested the sample preparation method the extraction method was effective and stabile. The matrix effects derived from QC samples were between 80.48% and 99.83%. These results confirmed that there were no significant matrix effects.

3.4.5 Stability

The stability data of the five flavonoids in rat plasma including room temperature stability (storage for 6 h at ambient temperature), long-term stability (storage for 30 days at -40 °C), freeze–thaw stability (three freeze at -40 °C and thaw cycles), post-preparation stability (storage for 12 h in the auto-sampler at 4 °C) were shown in Table 4. The results indicates that all analytes were stable in rat plasma and processed samples under different storage conditions.

3.5 Pharmacokinetic study

The newly developed method was sufficiently sensitive to determine the plasma concentration of stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasmin following a single oral administration of ESL. The concentration-time curves of five flavonoids are shown in Fig.3 and its estimated pharmacokinetic parameters calculated by DAS 2.0 software are presented in Table 5.As shown in Table 5, the range of C_{max} of these five flavonoids was from 64.21 to 152.07 μg/L because the content of five flavonoids varied greatly in the ESL. All the five flavonoids achieved the maximum plasma concentration within 0.55 h after oral administration, meanwhile, their elimination half-life time ($t_{1/2}$) were hovering around

5 h, which suggests that these five flavonoids had similar elimination rates in rat plasma after oral administration of ESL.

In previous study, only chamaechromone has been investigated for *vivo* analysis method(Lou *et al.*, 2011), but its plasma concentration of LLOD was not low enough, which was not satisfied the requirements of our study. In that literature, a double-peak phenomenon has been found in the plasma concentration-time curve after oral administration of purified chamechromone, which probably was caused by enterohepatic circulation. However, no double-peak phenomenon found in our study. Their T_{max} is about 11 h and $t_{1/2}$ is about 30 h, which are much later than reported in our results. The reason for those may be that we administered orally to rat with ESL rather than purified chamaechromone and the drug-drug interaction would influence the absorption and elimination of flavonoids, which also need further experiments to prove. These results should be useful for further studies on the pharmacokinetics, pharmacies and clinical practice of *Stellerachamaejasme* L.

4. Conclusion

An UPLC–MS/MS method was developed and validated for the simultaneous determination of stelleranol, chamaechromone, neochamaejasmin A, chamaejasmine and isochamaejasmin in rat plasma. This is the first paper to simultaneously determinate these five flavonoids in rat plasma after oral administration of ESL. The advantages of this method include simple sample preparation, high sensitivity, and short run time (7.0 min per sample). Moreover,this method was successfully applied to pharmacokinetic study of ESL in rats. The results might be helpful for investigating the bioactivity mechanism and clinical application of *Stellerachamaejasme* L.

Acknowledgements

References

Asada Y, Sukemori A, Watanabe T, Malla KJ, Yoshikawa T, Li W, Kuang X, Koike K, Chen CH, Akiyama T, Qian K, Nakagawa-Goto K, Morris-Natschke SL, Lu Y and Lee KH. Isolation, structure determination, and anti-HIV evaluation of tigliane-type diterpenes and biflavonoid from Stellera chamaejasme. *Journal of natural products* 2013; **76**: 852-857. DOI:10.1021/np300815t.

Huang W, Zhang X, Wang Y, Ye W, Ooi VE, Chung HY and Li Y. Antiviral biflavonoids from Radix Wikstroemiae (Liaogewanggen). *Chinese medicine* 2010; **5**: 23. DOI:10.1186/1749-8546-5-23.

Li J, Shen Q, Bao CH, Chen LT and Li XR. A new dicoumarinyl ether from the roots of Stellera chamaejasme L. *Molecules : a journal of synthetic chemistry and natural product chemistry* 2014; **19**: 1603-1607. DOI:10.3390/molecules19021603.

Li J, Zhang JJ, Pang XX, ZhengChen XL and Gan LS. Biflavanones with anti-proliferative activity against eight human solid tumor cell lines from Stellera chamaejasme. *Fitoterapia* 2014; **93**: 163-167. DOI:10.1016/j.fitote.2014.01.002.

Liu LP, Han K, Chen W, Zhang YY, Tong LJ, Peng T, Xie H, Ding J and Wang HB. Topoisomerase II inhibitors from the roots of Stellera chamaejasme L. *Bioorganic & medicinal chemistry* 2014b; **22**: 4198-4203. DOI:10.1016/j.bmc.2014.05.042.

Liu X, Li Y, Yang Q, Chen Y, Weng X, Wang Y, Li N and Zhu X. In vitro inhibitory and pro-apoptotic effect of Stellera chamaejasme L extract on human lung cancer cell line NCI-H157. *Journal of traditional Chinese medicine = Chung i tsa chih ying wen pan* 2012; **32**: 404-410 .

Liu X, Yang Q, Zhang G, Li Y, Chen Y, Weng X, Wang Y, Wang Y and Zhu X. Anti-tumor pharmacological evaluation of extracts from stellera chamaejasme L based on hollow fiber assay. *BMC complementary and alternative medicine* 2014a; **14**: 116. DOI:10.1186/1472-6882-14-116.

Liu X and Zhu X. Stellera chamaejasme L. extract induces apoptosis of human lung cancer cells via activation of the death receptor-dependent pathway. *Experimental and therapeutic medicine* 2012; **4**: 605-610. DOI:10.3892/etm.2012.643.

Lou Y, Hu H, Liu Y, Yu Q, Li L, Ping L, Yu L, Jiang H and Zeng S. Determination of chamaechromone in rat plasma by liquid chromatography-tandem mass spectrometry: application to pharmacokinetic study. *Journal of pharmaceutical and biomedical analysis* 2011; **55**: 1163-1169. DOI:10.1016/j.jpba.2011.04.009.

Pan L, Hu H, Wang X, Yu L, Jiang H, Chen J, Lou Y and Zeng S. Inhibitory effects of neochamaejasmin B on P-glycoprotein in MDCK-hMDR1 cells and molecular docking of NCB binding in P-glycoprotein. *Molecules : a journal of synthetic chemistry and natural product chemistry* 2015; **20**: 2931-2948. DOI:10.3390/molecules20022931.

Shirai K, Okamoto Y, Tori M, Kawahara T, Gong X, Noyama T, Watanabe E and Kuroda C. Diversity in the flavonoid composition of Stellera chamaejasme in the Hengduan Mountains. *Natural product communications* 2015; **10**: 53-56 .

US Food and Drug Administration, Guidance for Industry: Bioanalytical Method Validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070107.pdf

Wang Y, Li JY, Han M, Wang WL and Li YZ. Prevention and treatment effect of total flavonoids in Stellera chamaejasme L. on nonalcoholic fatty liver in rats. *Lipids in health and disease* 2015; **14**: 85. DOI:10.1186/s12944-015-0082-6.

Xu ZH, Qin GW, Li XY and Xu RS. New biflavanones and bioactive compounds from Stellera chamaejasme L. *Yao xue xue bao = Acta pharmaceutica Sinica* 2001; **36**: 669-671 .

Yan Z, Guo H, Yang J, Liu Q, Jin H, Xu R, Cui H and Qin B. Phytotoxic flavonoids from roots of Stellera chamaejasme L. (Thymelaeaceae). *Phytochemistry* 2014; **106**: 61-68. DOI:10.1016/j.phytochem.2014.07.013.

Yan Z, Zeng L, Jin H and Qin B. Potential ecological roles of flavonoids from Stellera chamaejasme. *Plant signaling & behavior* 2015; **10**: e1001225. DOI:10.1080/15592324.2014.1001225.

Yang D, Wang P and Ren X. Apoptosis induced by chamaejasmine in human osteosarcoma cells through p53 pathway. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2015; **36**: 5433-5439. DOI:10.1007/s13277-015-3209-5.

Yang G and Chen D. Biflavanones, flavonoids, and coumarins from the roots of Stellera chamaejasme and their antiviral effect on hepatitis B virus. *Chemistry & biodiversity* 2008; **5**: 1419-1424. DOI:10.1002/cbdv.200890130.

Yu H, Zhang T, Cai L, Qu Y, Hu S, Dong G, Guan R, Xu X and Xing L. Chamaejasmine induces apoptosis in human lung adenocarcinoma A549 cells through a Ros-mediated mitochondrial pathway. *Molecules : a journal of synthetic chemistry and natural product chemistry* 2011; **16**: 8165-8180. DOI:10.3390/molecules16108165.

Zhang C, Zhou SS, Feng LY, Zhang DY, Lin NM, Zhang LH, Pan JP, Wang JB and Li J. In vitro anti-cancer activity of chamaejasmenin B and neochamaejasmin C isolated from the root of Stellera chamaejasme L. *Acta pharmacologica Sinica* 2013; **34**: 262-270. DOI:10.1038/aps.2012.158.

Zhang SD, Shan L, Li W, Li HL and Zhang WD. Isochamaejasmin induces apoptosis in leukemia cells through inhibiting Bcl-2 family proteins. *Chinese journal of natural medicines* 2015; **13**: 660-666. DOI:10.1016/S1875-5364(15)30063-7.

Zhang T, Yu H, Dong G, Cai L and Bai Y. Chamaejasmine arrests cell cycle, induces apoptosis and inhibits nuclear NF-κB translocation in the human breast cancer cell line MDA-MB-231. *Molecules : a journal of synthetic chemistry and natural product chemistry* 2013; **18**: 845-858.

Zhang Y, Liu C, Qi Y, Li Y, Li S, Wang Y, Ren J and Tang Y. Efficient combination of circulating ultrasound-assisted extraction and centrifugal partition chromatography for extraction and on-line separation of chemical constituents from Stellera chamaejasme L. *Phytochemical analysis : PCA* 2015; **26**: 301-309. DOI:10.1002/pca.2564.

.

Figure legends

Fig.1 The chemical structures of five flavonoids and the internal standard.

Fig.2 MRM chromatograms of five flavonoids and internal standard in (A) blank
plasma, (B) blank plasma spiked with LLOQ samples, (C) plasma obtained
from a rat 2 h after oral administration. 1,stellerano; 2,quercetin(IS);
3,chamaechromone; 4,neochamaejasmin A; 5,chamaejasmine;
6,isochamaejasmin.

Fig.3 Mean (±SD) plasma concentration-time curves of five flavonoids in rats after
oral administration of ESL (n = 6).

Table 1. Tandem mass spectrometric parameters of five flavionds and IS

Analytes	Precursor ion	Produce ion	Fragmentor(V)	CE (eV)
Stelleranol	559.2	153.0	125	24
Chamaechromone	543.3	198.9	125	4
Neochamaejasmin A	543.2	153.0	125	16
Chamaejasmine	543.1	152.9	125	16
Isochamaejasmin	543.2	153.0	125	16
Quercetin	303.1	153.1	125	35

Table 2 Regression equation, LOD and LOQ of five flavonoids in rat plasma.

Analytes	Regression equation	Linear rang (ng/mL)	r	LOQ (ng/mL)	LOD (ng/mL)
Stelleranol	Y=0.0472X+0.5680	0.6400-1280	0.9967	0.6400	0.2133
Chamaechromone	Y=0.0180X+0.0789	0.5300-1060	0.9990	0.5300	0.1767
Neochamaejasmin A	Y=0.0581X+0.2892	0.5275-1055	0.9991	0.5275	0.1758
Chamaejasmine	Y=0.0182X+0.2660	0.5800-1160	0.9956	0.5800	0.1933
Isochamaejasmin	Y=0.0308X+0.1657	0.5100-1020	0.9994	0.5100	0.1700

Table 3 Summary of precision, accuracy, recovery and matrix effect in rat plasma (n=5-6).

Analytes	Concentration (ng/mL)	Intra-day precision RSD (%)	Inter-day precision RSD (%)	Accuracy RE(%)	Absolute Recovery (%, mean ± SD)	Matrix effect (%, mean ± SD)
Stelleranol	0.640	6.4	8.2	6.21	-	-
	1.280	5.6	6.1	-2.31	84.65 ± 5.91	93.21 ± 7.79
	64.00	2.1	4.9	-9.44	97.32 ± 4.26	89.89 ± 6.25
	640.0	2.6	4.4	5.19	83.84 ± 3.56	80.48 ± 7.15
Chamaechromone	0.530	5.2	7.2	9.21	-	-
	1.060	0.3	5.5	-10.21	87.41 ± 3.17	95.35 ± 7.99
	53.00	3.9	4.8	-9.06	96.82 ± 7.6	83.09 ± 6.25
	530.0	4.9	4.6	3.28	98.93 ± 5.63	99.83 ± 3.09
Neochamaejasmin A	0.528	4.5	10.2	1.23	-	-
	1.055	3.1	9.3	0.29	97.14 ± 7.47	85.34 ± 6.13
	52.75	2.2	3.5	5.31	94.12 ± 3.32	97.66 ± 4.47
	527.5	7.4	4.8	-7.31	81.26 ± 5.68	85.94 ± 5.80
Chamaejasmine	0.580	6.4	3.4	4.13	-	-
	1.160	6.3	2.3	-11.79	88.44 ± 6.44	86.73 ± 5.21
	58.00	4.1	7.4	-9.02	92.74 ± 5.71	87.44 ± 5.98
	580.0	4.3	2.9	2.76	93.77 ± 3.44	84.65 ± 4.72

Isochamaejasmin	0.510	4.2	2.3	4.22	-	-
	1.020	3.2	3.0	3.99	80.16 ± 7.00	96.82 ± 5.40
	51.00	1.9	4.5	-9.39	94.15 ± 5.43	87.73 ± 3.24
	510.0	6.4	4.9	-9.14	83.74 ± 6.71	85.24 ± 7.08
Quercetin (IS)	94.20	-	-	-	95.34 ± 8.14	90.67 ± 4.65

Table 4 Stability data of five flavonoids in rat plasma (n=3).

Analytes	Concentration (ng/mL)	Accuracy (%) (mean ± SD)			
		Autoinjector	Short-term	Freez-thaw	Long-term
Stelleranol	1.280	97.78 ±6.06	95.05 ±8.32	83.98 ±7.68	90.27 ± 10.4
	64.00	91.55 ± 9.80	91.56 ± 7.16	93.90 ± 6.96	96.85 ± 9.92
	640.0	96.06 ± 4.33	95.33 ± 9.21	92.47 ± 7.95	86.01 ± 9.49
Chamaechromone	1.060	92.40 ±8.47	98.74 ± 4.80	96.81 ± 4.03	87.16 ± 7.74
	53.00	90.28 ± 8.54	90.12 ± 3.31	98.18 ± 4.79	94.58 ± 9.73
	530.0	96.74 ± 5.84	93.66 ± 5.88	94.20 ± 8.40	91.67 ± 6.30
Neochamaejasmin A	1.055	90.33 ± 10.33	97.44 ± 7.24	85.70 ± 6.05	91.73 ± 10.26
	52.75	101.98 ± 3.25	91.31 ± 5.68	96.10 ± 8.73	85.35 ± 7.16
	527.5	94.25 ± 5.90	90.93 ± 7.06	98.68 ± 4.58	87.05 ± 8.22
Chamaejasmine	1.160	92.06 ±7.99	90.71 ± 5.99	93.48 ± 7.44	89.07 ± 5.13
	58.00	98.08 ± 8.17	99.50 ± 8.73	103.87 ±8.91	92.83 ± 7.64
	580.0	97.91± 6.01	92.14 ± 4.42	98.36 ± 6.02	91.74 ± 9.25
Isochamaejasmin	1.020	94.53 ±7.38	95.10 ± 7.69	97.54 ± 8.21	88.70 ± 5.71
	51.00	94.21 ± 6.46	92.33 ± 6.80	102.44 ± 6.40	96.50 ± 10.47
	510.0	90.15 ± 10.00	93.45± 8.51	94.89 ± 3.49	89.74 ± 4.18

Table 5 Pharmacokinetic parameters of five flavonoids in rats after oral administration.

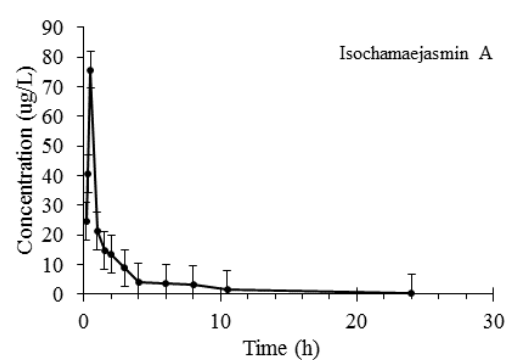| Parameters	Stelleranol	Chamaechromone	Neochamaejasmin A	Chamaejasmine	Isochamaejasmin
C_{max} (µg/L)	73.47±1.19	152.07±3.62	104.56±9.28	64.21±7.92	75.73±12.31
T_{max}(h)	0.51±0.10	0.33±0.18	0.55±0.12	0.33±0.18	0.50±0.05
$t_{1/2}$ (h)	5.28±0.34	5.28±0.24	5.32±0.91	5.42±1.27	5.12±1.38
AUC_{0-t} (h·µg/L)	67.32±11.03	525.51±14.29	99.47±2.33	81.56±14.32	108.97±21.49
$AUC_{0-\infty}$ (h·µg/L)	68.11±9.94	544.01±11.35	101.29±3.84	84.84±16.27	111.49±19.39
$AUMC_{0-\infty}$(h·h·µg/L)	176.41±10.22	4062.04±23.08	351.46±26.75	483.11±20.90	482.37±30.24
$MRT_{0-\infty}$ (h)	2.24±1.47	7.47±1.02	3.47±0.99	5.70±1.62	4.33±2.02
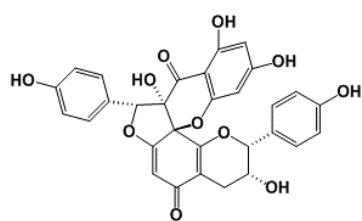
Stelleranol
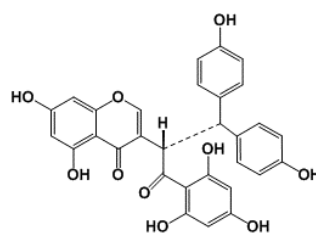
Chamaechromone
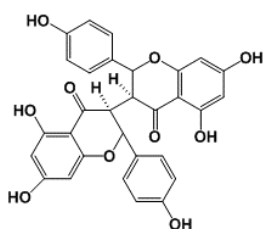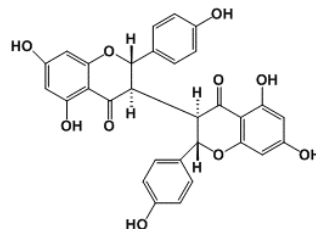
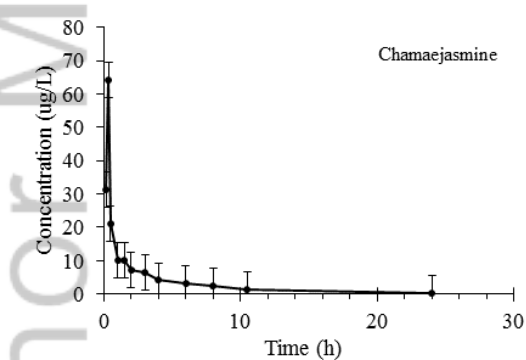**Neochamaejasmin A**
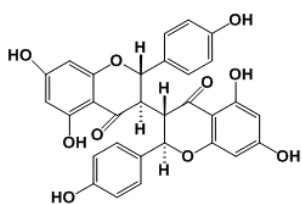
Chamaejasmine
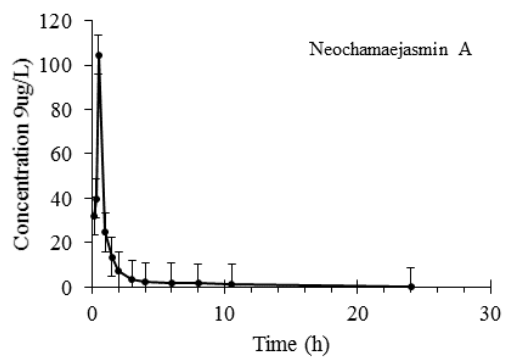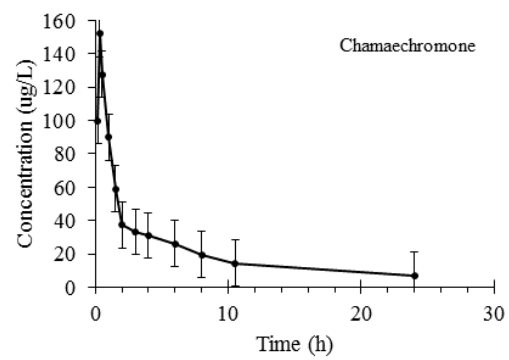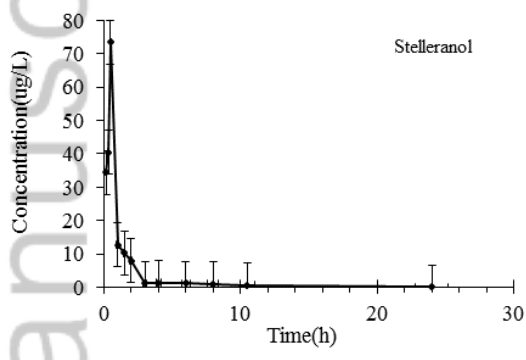
Isochamaejasmin
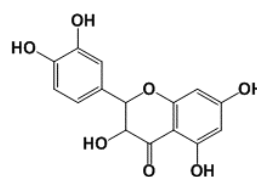
Quercetin

BMC_4189_F1.png
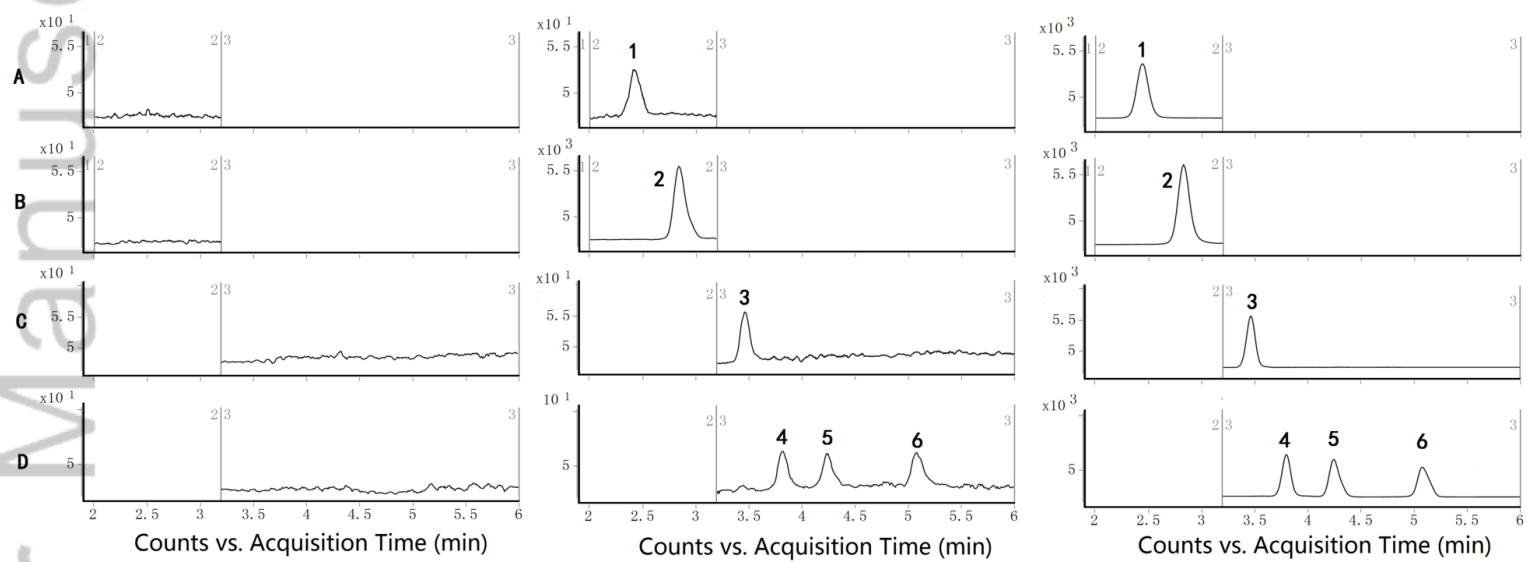
Counts vs. Acquisition Time (min)

BMC_4189_F2.tif

BMC_4189_F3.png