doi.org/10.36721/PJPS.2020.33.2.REG.669-674.1

Analysis of chemical constituents in the extract and rat serum from the chloroform extract of *Oxytropis falcata* Bunge by HPLC-MS

Dejun Zhang[1,4], Wangtao Yuan[1,4], Benyin Zhang[1,4], Eun-Kyung Hong[3], Ping Shi[2], Zhanting Yang[1,4], Yuhao Zhang[1,4] and Honglun Wang[1]

[1]State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, China
[2]School of biotechnology of East China University of Science ＆ Technology, Shanghai, China
[3]Medvill Co., Ltd. Medvill Research Institute, Seoul, Korea
[4]College of Eco-Environmental Engineering, Qinghai University, Xining, China

Abstract: Analysis of the constituents of the chloroform extract of *Oxytropis falcata* Bunge (CEOF), a traditional Tibetan medicine, in rat's serum after oral administration, has been performed by HPLC-MS. We have identified 10 compounds in CEOF and 11 bioactive ingredients from rat's serum after given CEOF. Six bioactive ingredients from rat's serum are matched with original form of the compounds of CEOF. Other five bioactive ingredients were seemed to be respectively metabolites. HPLC-MS is rapid, sensitive method and suitable for identification of bioactive components absorbed into blood of CEOF providing information for further research of pharmacological mechanism.

Keywords: *Oxytropis falcata* Bunge, chloroform extract, rat serum, HPLC-MS.

INTRODUCTION

Oxytropis genus belonging to the family *Leguminosae* (subfamily *Papilionoideae*) comprises over 350 species which has been thought to be a locoweed, causing significant livestock poisoning and economic loss, especially in America, Russia and Canada (Zhu, 2004). In China, *Oxytropis falcata* Bunge, locally known as "E Da Xia", was first reported as an official herbal medicine in 1997 (Luo, 1997). As one of the "three anti-inflammatory drugs" and the "king of herbs", it has traditionally been used in many Tibetan and Mongolian medicinal formula for the treatment of diseases or symptoms of influenza, hyperpyrexia, pain, wounds, haemorrhage, inflammation, anthrax, *etc*. (Luo,1997). Recent studies on the biochemical activities of *O. falcata* have been focused on its hemostasis, antitumor, antioxidant, anti-inflammatory and analgesic activities (Dai *et al*., 2008).

For recent decades, more than 100 compounds including flavonoids, alkaloids, saponins, and polysaccharides have been isolated and identified in this plant (Li *et al*., 2012; Dong *et al*., 2010; Du *et al*., 2013, Li *et al*., 2014). Flavonoid aglycones such as chalcone and flavonone were considered to be responsible for the anti-inflammatory (Chen *et al*., 2010) and anti-myocardial ischemic activity (Chen *et al*., 2013). Our previous report (Zhang *et al*., 2013; Jiang *et al*., 2014) indicated that chloroform and ethyl acetate fractions could remarkably alleviate myocardial ischemia-reperfusion injury. Serum pharmaco-chemistry techniques *in vivo* are efficient methods for detecting bioactive ingredients from animal's serum (Wang *et al*., 2016). Zhong *et al.* established HPLC fingerprint of *Magnolia Officinal is*, and they used HPLC to analyse rats serum from given extracts of *Magnolia Officinalis* (Zhang *et al.,* 2013). Ma *et al.* have detected the constituents of the serum and the serum ingredients systematically for Huanglian Shangqing pill under the guide of theory of serum pharmacochemistry (Ma *et al.*, 2016).

In this study, we use HPLC-MS to investigate the constituents in rat's serum after giving chloroform extract of *Oxytropis falcata* Bunge (CEOF).

MATERIALS AND METHOD

Chemicals, reagents and other supplies

HPLC grade acetonitrile was purchased from Merck (Germany).Methanol was HPLC grade (Honeywell, USA). Anticoagulant tubes for temporary storage of blood samples were purchased from Tian Jin Chemical Reagents Development Center (China). *O. falcata* Bunge were collected from the third Tara, Gonghe County (Qinghai, China), July 28, 2014. All herbs were authenticated by professor Yong-Chang Yang (Northwest Institute of Plateau Biology, Chinese Academy of Science).

Chromatographic analysis system

The analytical conditions for HPLC were as following, C18 column (4.6×250 mm, Diamondsil) was packed with 5-µm particles (Dikma, China). Flow rate was 1 mL min^{-1}, of which 250µL min^{-1} was shunting for mass spectrometer. Mobile phase consisting of water(Solvent A) and ACN containing 0.1% formic acid(Solvent B) with gradient elution procedure as 0-30 min, 5% B to 95% B. Molecular mass analyses were conducted using an LCQ ion trap mass spectrometer (Thermo Fisher, USA) , electrospray voltage of the ion source is 3500 V, capillary temperature

**Corresponding author:* e-mail: djzhang@nwipb.ac.cn

is 120°C. The full scan of ions from m/z 100 to 1500 in the positive and negative ion mode was carried out. Analyses were operated Xcalibur software (Thermo Fisher) (Segall *et al.*, 2005).

Animal experiments

Male and female Sprague-Dawley rats (180-220g, body mass) were purchased from the laboratory animal center of Xn'an Jiaotong University (Shanxi Province, China). All rats were housed in an enironmentally controlled breeding with free access to standard animal chow and tap. The animals were allowed 12h acclimatization before experiment. All animal studies were approved by Institutional Animal Ethics Committee, Qinghai University.

Preparation of CEOF

CEOF was prepared by the protocol described previously (Jiang, 2014). The shade-dried whole herb of *O. falcata* (25 kg) was powdered completely, and wrapped with four layers of gauze, and then extracted three times with 95% ethanol (the ratio of *O. falcata* and ethanol is 1:10). The extraction temperature was 75°C and the duration time was 3 hours per extraction. Finally, the ethanol extract was concentrated in a rotary evaporator. Crude extract was dried in the oven, then it was fractionated by petroleum ether. The drug was extracted by chloroform and the chloroform fractions were combined, concentrated and freeze-dried. 500 mg of freeze-dried powder was dissolved in 50mL methanol. 1mL of this solution was filtered through 0.45 μm filter, the filtrate was applied to HPLC or HPLC-MS analysis. The filtrate was stored at 4°C.

Serum sample collection

The prepared CEOF was dissolved in distilled water as master slution, final concentration is 0.8 g L-1). The CEOF was administered orally to 6 rats (2.0mL/100 g body mass), male and female each half. After administration, the blood was collected from vena ophthalmic a of rat according to the following times: 0, 30, 60, 90, 120, 180, 240, 360, 480, 720, 1440 min. After anaesthetizing animals with inhaling ether, the blood samples was collected. Then, the serum was separated by centrifuging at 12000 rpm for 5 min at 4°C.

Then blood samples were centrifuged twice at 12 000 rpm for 5 min at 4°C, the supernatants were collected, dried under nitrogen gas at 50°C. The residues were re-constituted in 300μL methanol, then centrifuged 10 min at 12 000 rpm The sample was filtered through a 0.22 μm membrane filter before HPLC analysis. The serum samples which were collected at 0 min after oral administration were regarded as a vehicle. The other serum samples collected at different times after drug administration were all designated as CEOF-treated groups.

Collection and pretreatment of liver specimen

The rats were narcotized by injecting the urethane intraperitoneally and fixed after the last blood was taken from the vena ophthalmica. Then, making liver of the rat through the portal vein lavage with cold saline and cutting two certain sizes of the liver samples, wrapped with tin foil, placed in a sterile tube which was sterilized finally, the samples were marked well and saved in the liquid nitrogen tank.

Preparation of liver micro somes

Livers were removed from the liquid nitrogen and thawed in ice. Half a gram of livers was weighed accurately and put in a 4-mL centrifugal tube and 1.5mL Tris-HCl (pH=7.4) mol L-1 added per tube and 25% tissue homogenate prepared. Homogenates were centrifuged 30 min at 11 000 rpm at 4°C, the supernatant were moved to other ultracentrifuge tube, centrifuged 80 min at 100 000 rpm. Supernatant was discarded, micro somes were collected, added 600μL of 10mmol L-1 Hepes-HCl (pH 6.8) and homogenized, the resulting micro somes were stored at -80°C.

1 mg liver micro some and NADPH regeneration system were added to 0.05 mol L-1 Tris-HCl (pH 7.5) and incubated in 37°C water bath. After 2 min of pre-reaction, 10 L of 100 mg mL-1 aqueous distillate solution was added, continued with warm incubation; the total volume was 1mL. Oxygen was supplied to the surface for 0.5 min every 20 min. After 1.5 h, ice acetonitrile was added to terminate metabolic reaction, then raction solution was centrifuged 5 min at 12 000 rpm. Finally, the supernatant was filtered through a 0.22 μm membrane before HPLC-MS analysis (Wu *et al.*, 2014).

RESULTS

HPLC-MS analysis and identification of CEOF components

The positive ion mode and ESI source were used to analysis and identification bioactive ingredients in CEOF. The total chromatograms of ion current are shown in fig. 1. 10 compounds peaks in CEOF were detected and identified by mass spectrometry and confirmed by references (Que *et al.*, 2007). The identified compounds in CEOF were listed in table 1. They were identified as 7-dimethoxyflavonone, Ψ-baptigenin, rhamnetin, 7-methoxy-2,3-dihydro-2-flaconoid, 5-hydroxy-7-methoxy-2,3-dihydro -2-flaconoid, 2',4',4-trihydroxychalcone, 2'-hydroxy-4'-methoxy chalcone, 7-hydroxy-flavonone, oxytriphine, *m*-methoxyaniline. Which correspond to the peaks 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (Que, 2003; Yang *et al.*, 2008)

Compared with chromatogram of CEOF *between* control group and CEOF-treated group, the result were shown in fig. 2. The results shown 6 peaks which were identical in both groups. We think that six compounds (Peaks 1, 4,

Table 1: Structures of the constituents identified in CEOF

Number	Structure	Name	Formula	*M*r
1		7-Dimethoxyflavonone	$C_{16}H_{12}O_3$	252
2		Ψ-baptigenin	$C_{16}H_{10}O_5$	282
3		Rhamnetin	$C_{16}H_{12}O_7$	316
4		7-Methoxy-2,3-dihydro-2-flaconoid	$C_{16}H_{14}O_3$	254
5		5-Hydroxy-7-methoxy-2,3-dihydro-2-flaconoid	$C_{16}H_{14}O_4$	270
6		2’,4’,4-Trihydroxy -chalcone	$C_{15}H_{12}O_4$	256
7		2’-Hydroxy-4’ -methoxychalcone	$C_{15}H_{12}O_4$	256
8		7-Hydroxy -2,3-dihydro-2-flaconoid	$C_{15}H_{12}O_3$	240
9		Oxytriphine	$C_{15}H_{13}ON$	223
10		*m*-methoxyaniline	$C_{19}H_{21}O_4N$	327

Table 2: Structures of the constituents identified in the serum samples from CEOF-treated rats[a]

Number	Structure	Name	Formula	M_r
D1		Liquiritigenin	$C_{15}H_{10}O_4$	256
D2		Oxytropine C	$C_{19}H_{26}O_2N_2$	314
D3		Kaempfero(3-O-(6'-acetyl)-β-D -glucoside	$C_{27}H_{30}O_{11}$	530
D4		Pinocembrin	$C_{15}H_{12}O_4$	256
D5		2’, 4’ -Dihydroxychalcone	$C_{15}H_{12}O_3$	240

[a] *Compounds not existing in CEOF.*

5, 7, 8 and 10 in fig. 2) from CEOF were absorbed into rat blood. They were identified as 7-dimethoxyflavonone, 7-methoxy-2, 3-dihydro-2-flaconoid, 5-hydroxy-7-methoxy-2,3-dihydro-2-flaconoid,2'-hydroxy-4'-methoxychalcone, 7-hydroxy-2,3-dihydro-2-flaconoid, *m*-methoxyaniline by references (Segall *et al.*, 2005; Jiang 2014; Que *et al.*, 2007). As shown in table 2, another five compounds (peaks D1, D2, D3, D4, D5 in fig. 2) were detected only in the serum of the treated animals. They were identified as liquiritigenin, oxytropine C, kaempfero(3-O-(6'-acetyl)-β-D-glucoside, pinocembrin, pinocembrin 2',4'-dihydroxychalcone, resp. These compounds were not found in the CEOF (Tian *et al.*, 2010; Wang *et al.*, 2008; Yao *et al.*, 2008; Lv *et al.*, 2006). The change in the composition of the constituents depending on time was observed. General changes were described as follows:7-dimethoxyflavonone, pinocembrin, 7-methoxy-2,3-dihydro-2-flaconoid,5-hydroxy-7methoxy-2,3-dihydro -2-flaconoid and m-methoxyaniline were detected after 1 to 24 hours after oral administration. However, 7-methoxy-2, 3 -dihydro-2-flaconoid and 5-hydroxy-7-methoxy-2,3-dihydro-2-flaconoid increased after oral administration and decreased after 6 h of oral administration. The serum level of *m*-methoxyaniline was maintained during the investigation time. Liquiritigenin, which was only detected in the treated serum, decreased 4 h after of oral administration. 2', 4'-dihydroxychalcone had appeared at 90 min after oral administration, and it disappeared in a short time. These compounds maybe metabolized into other chemical moieties after oral administration.

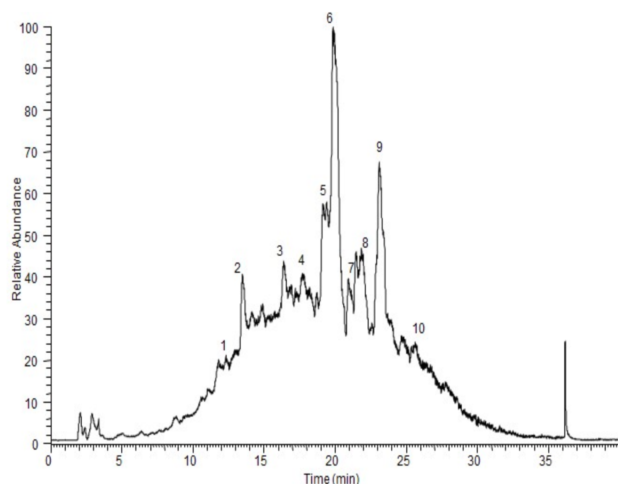

Fig. 1: HPLC-MS total ion current chromatograms of CEOF at the positive mode.

Analysis the proposed metabolic pathways of CEOF

The LC-MS total chromatograms of ion current at negative mode of Liver microsomes were shown in fig. 3. 2'-hydroxy-4'-methoxychalcone,Ψ-baptigenin,Kaempfero (3-O-(6'-acetyl)-β-D-glucoside, 7-hydroxy-2, 3-dihydro-2-flaconoid and 2-mono -linoleoyin were deceted in the liver microsomes of CEOF.

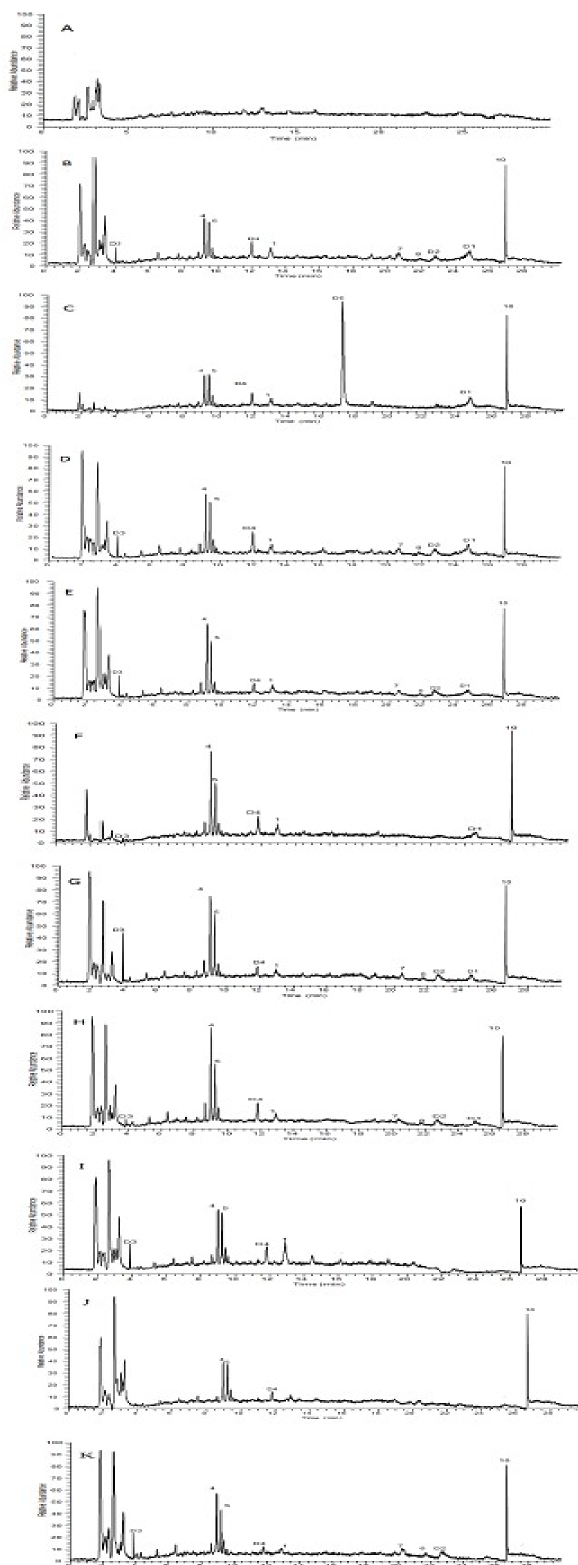

Fig. 2: HPLC-MS total ion current chromatograms at the positive mode of rat serum samples after oral administration (A: 0 min., B: 30 min., C: 60 min., D: 90 min., E: 120 min., F: 180 min., G: 240 min., H: 360 min., I: 480 min., J: 720 min., K: 1440 min).

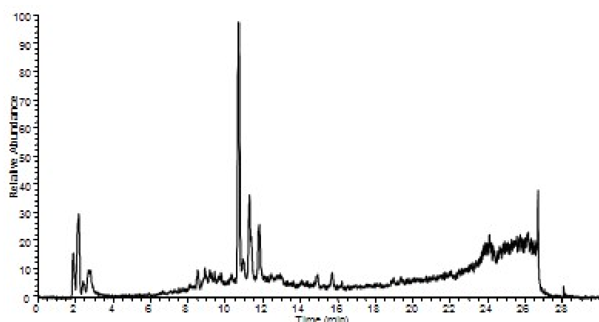

Fig. 3: The HPLC-MS total ion current chromatograms of liver microsomes at the negative mode

The proposed metabolic pathways of CEOF were as follows (fig. 4). It is possible that 7-dimethoxyflavonone which is prototype *in vitro* may occur after hydroxylation and demethylation to liquiritigenin *in vivo*. Rhamnetin may occur by demethylation and dehydroxylation to pinocembrin *in vivo*. Besides, 2'-hydroxy -4'-methoxychalcone may take part in demethylation to 2',4'-dihydroxy -chalcone *in vivo*. Meanwhile, it is possible that 2',4'-dihydroxychalcone may occur after methylation to 2'-hydroxy-4'-methoxychalcone in liver. The results showed that the metabolism of CEOF was -phase reaction, including hydroxylation and methylation mainly.

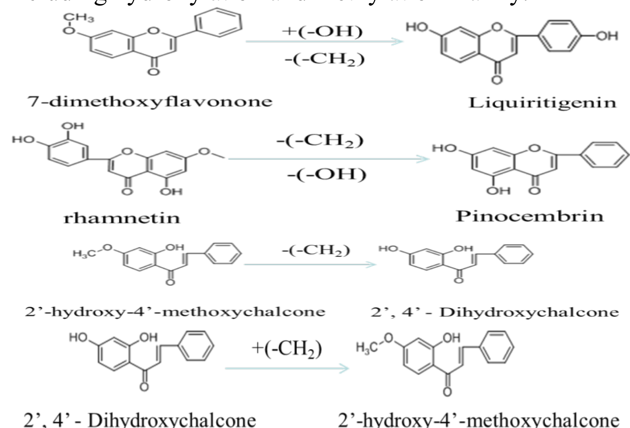

Fig. 4: The proposed metabolic pathways of CEOF.

DISCUSSION

Our present investigation is a systematical study on identifying the transitional components *in vivo* after rat's oral administration by CEOF by using LC-MS technology. Comparing with the literatures sources CEOF (Tian *et al.*, 2010; Wang *et al.*, 2008; Yao *et al.*, 2008; Lv *et al.*, 2006), we discovered that the transitional ingredients were flavonoids mostly, and some of its have been changing to a certain extent over time. Moreover, it is consistent with the previous preliminary experimental results that the CEOF is likely to be the effective part in myocardial ischemia-reperfusion injury. This study will lay the foundation to the scientific clinical treatment of disease in myocardial ischemia-reperfusion injury, as well as provide a way of developing new drugs, which can treat the related diseases. At the same time, we will further examine each of the relevant single compounds in for their pharmacological effect on myocardial ischemia and reperfusion injury model.

CONCLUSION

In this study, we investigated the bioactive constituents of rat's serum after oral administrating CEOF by HPLC-MS. We discovered that the ingredients were mostly flavonoids and some of them have been changed depending on time. Moreover, they were consistent with the previous results that CEOF is likely to be the effective part in myocardial ischemia-reperfusion injury. The information from the analysis by HPLC-MS will be helpful to define mode of action of CEOF or pharmacokinetic study.

HPLC-MS analytical system for the serum pharmacochemistry will provide validated and rapid methodology for the identification of bioactive constituents of traditional Tibetan herbal medicine.

ACKNOWLEDGEMENTS

This research was supported by grant from the Opening Project of State Key Laboratory of Bioreactor Engineering, Qinghai Province Science and Technology Office (No.2017-SF-A8), Qinghai Provincial Science Foundation (2016-ZJ-01), the Open Project of State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University (2016-KF-05).

REFERENCES

Chen QH, Li Q, Wang WJ, Zhao Y and Zhang XY (2013). Related mechanism's research progress of flavonoids against myocardial ischemia - reperfusion injury. *Chin. J. Clin. Pharmacol.*, **29**: 958-960.

Chen WH, Wang R and Shi YP (2010). Flavonoids in the poisonous plant *Oxytropis falcate*. *J. Nat. Prod.*, **73**(8): 1398-1403.

Dai YP, Yang H and Tong L (2008). Haemostatic effect of falcate crazyweed herb. *J. Nanjing Univ. Chin. Med.*, **24**(2): 107-108.

Dong W, Wei T, Guang-Ming Y and Bao-Chang C (2010). Anti-inflammatory, antioxidant and cytotoxic activities of flavonoids from *Oxytropis falcata* Bunge. *Chin. J. Nat. Med.*, **8**(6): 461-465.

Du H, Liu MC, Li FA, Ren SC and Ma JB (2013). Influence of *Oxytropis Falcata* Bunge on expression of Bcl-2 And Bax of myocardial ischemia and reperfusion injury in rats. *J. Qinghai Med. Coll.,* **34**: 203-206.

Jiang H, Zhan WQ, Liu X and Jiang SX (2008). Antioxidant activities of extracts and flavonoid compounds from *Oxytropis falcate* Bunge. *Nat. Prod.*

Res., **22**(18): 1650-1656.
Jiang RF (2014). The effective parts of *Oxytropis falcata* Bunge protects against myocardial ischemic-reperfusion injury in rats, Qinghai University Thesis for M.S. pp.10-20.
Jiang RF, Li FA, Li YP and Zhang DJ (2014). The effective parts of *Oxytropis falcata* Bunge protects against myocardial ischemic-reperfusion injury in rats. *J. Qinghai Med. Coll.*, **35**: 183-187.
Li MX, Lan ZH, Wei LL, Zhang WJ, Zhang RX and Jia ZP (2012). Phytochemical and Biological Studies of Plants from the GenusOxytropis. *Rec. Nat. Prod*., **6**(1): 1.
Li Z, Liu MC, Li FA, Ren SC, Ma JB and Zhang DJ (2014). The antioxidative effect of *Oxytropis falcata* Bunge ethanol extract on rats' hearts against myocardial ischemia and reperfusion injury. *Nat. Prod. Res. Dev.*, **26**: 423-426.
Luo DS (1997). China Traditional Tibetan Herbal. Nation Press Publishing Co., Beijing.
Lv F and Xu YJ (2006). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge. *J. Chin. Med. Mat.*, **29**: 1304.
Ma H, Liu Y, Mai X, Liao Y, Zhang K, Liu B, Xie X and Du Q (2016). Identification of the constituents and metabolites in rat serum after oral administration of Huanglian Shangqing pills by ultra high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. *J. Pharm. Biomed. Anal.*, **125**: 194-204.
Que S (2003). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge, Northwest Normal University Thesis for M.S. pp.22-49.
Que S, Zhang YS and Zhao YY (2007). Chemical constituents of Tibetan medicine *Oxytropis falcate*. *Chin. Trad. Herbal Drugs*, **38**(10): 1458-1460.
Segall SD, Artz WE, Raslan DS, Ferraz VP and Takahashi JA (2005). Analysis of triacylglycerol isomers in Malaysian cocoa butter using HPLC–mass spectrometry. *Food Res. Int.*, **38**(2): 167-174.
Tian YQ, Zhao HB, Liu H, Tian M and Hou XM (2010). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge. *J. Anhui. Agric. Sci.*, **38**: 14340-14341.
Wang D, Yang H, Dai YP, Tong L and Cai BC (2008). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge (II). *Chin. Pharm. J.*, **43**: 1292-1294.
Wang Y, Li G, Zhou Y, Yin D, Tao C,Han L, Yue X, Pan Y, Yao Y, Peng D and Xu F (2016). The difference between blood-associated and water-associated herbs of Danggui-Shaoyao San in theory of TCM, based on serum pharmacochemistry. *Biomed. Chromatogr*., **30**(4): 579-587.
Wei Q, He YP and Li JC (1979). The pharmacological effects of *Oxytropis falcate* Bunge on hypothalamo-pituitary-adrenocortical system. *Nat. Med. J. China*, **59**: 677-679.
Wu N, Xu W, Zhang YB and Yang XW (2014). Biotransformation of malabaricone C by rat hepatic microsomes and cytotoxic activities against gastric cancer cells *in vitro*. *Chin. Pharm. J.*, **4**: 241-245.
Yang H, Wang D, Tong L and Cai BC (2008). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge (III). *J. Nanjing Univ. Chin. Med.*, **24**: 43-44.
Yang H, Wang D, Tong L and Cai BC (2008). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge (I). *Chin. Pharm. J.*, **43**: 338-340.
Yang H, Wang D, Tong L and Cai BC (2008). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge (IV). *Chin. Pharm. J.*, **43**: 1538-1540.
Yao SY, Ma YB, Tang Y, Chen JJ and Zhang XM (2008). Studying on chemical constituents of Tibetan medicine *Oxytropis falcata* Bunge. *Chin. Pharm. J.*, **33**: 1418-1441.
Zhang DJ, Du H, Liu MC, Li FA, Li DX and Ma JB (2013). Influence of *Oxytropis falcata* Bunge on NO and NOS of myocardial ischemia-reperfusion injury in rats. *Chin. J. Modern Appl. Pharm.*, **30**: 1054-1058.
Zhang H, Geng Y, Wang Y, Tang JW, Ma CY, Kong L, Wang X, Yan ZY, Wei Y, Wu XQ and Li N (2013). Preliminary study on serum pharmacochemistry of Magnolia officinalis. *J. Chem. Pharm. Res.*, **6**: 1337-1341.
Zhu XY (2004). Outline of the classified system of the Chinese Leguminosae. *Bull. Bot. Res*., **24**(1): 20-27.