Revista Brasileira de Farmacognosia 28 (2018) 481–488

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Different processing methods change the oral toxicity induced by *Sophora alopecuroides* seeds and the contents of five main toxic alkaloids from the ethanol extracts determined by a validated UHPLC–MS/MS assay

Chenxu Zhang[a,1], Jie Chen[a,1], Juanhong Zhang[a], Shijie Wei[b], Hongyan Ji[b], Xiuli Wu[a,c,d,*], Wei Ma[e], Jing Chen[a,c,d,*]

[a] *School of Pharmacy, Ningxia Medical University, Yinchuan, China*
[b] *Institute of Clinical Pharmacology, General Hospital of Ningxia Medical University, Yinchuan, China*
[c] *Ningxia Engineering and Technology Research Center for Modernization of Hui Medicine, Yinchuan, China*
[d] *Key Laboratory of Hui Ethnic Medicine Modernization, Ministry of Education, Ningxia Medical University, Yinchuan, China*
[e] *Ningxia Ning-An Hospital, Yinchuan, China*

ARTICLE INFO

Article history:
Received 13 January 2018
Accepted 4 April 2018
Available online 14 May 2018

Keywords:
Vinegar-processing
Acute oral toxicity
Quinolizidine alkaloids
Cytisine
Histopathological analysis

ABSTRACT

This study investigated the influence of different processing methods on the oral toxicity of *Sophora alopecuroides* L., Fabaceae, seeds in mice and on the contents of five known toxic-effective quinolizidine alkaloids from the ethanol extracts quantified by ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometry. It provides an evidence to elucidate the possible reasons why vinegar-processing and parching methods significantly decrease the acute oral toxicity induced by *S. alopecuroides* and why wine-processing method increases it instead (demonstrated by measurement of LD_{50} and histopathological analysis). The analytical performance for the determination of the five analytes was evaluated by linearity, stability, repeatability, precision and accuracy, and recovery test. The lowest limit of quantification was determined to be 5 ng/ml for each substance and the precision and accuracy at lowest limit of quantification were below 20%. Cytisine, the most toxic alkaloid among the five alkaloids, declined 11.26, 3.98, and 2.73 folds after being vinegar-processed and fried in a ceramic or iron pan, respectively and had a very close correlation with the toxicity of *S. alopecuroides* seeds ($r = 0.8589$). Other matrine-type alkaloids with lower toxicity including matrine, sophcarpine, and sophoridine decreased after being wine-processed and fried in a ceramic pan, but increased 4.44, 7.20, and 7.23 folds when being processed by vinegar. Oxymatrine declined in all groups. It, therefore, reveals that vinegar-processing method reduces the oral toxicity of *S. alopecuroides* mainly due to a sharp decrease of cytisine, thus improves its clinical safety.

Introduction

Sophora alopecuroides L., Fabaceae, is a wild perennial herb of the xerophyte species and is widely distributed in the desert arid zone of northwestern China. It is an ethnomedicine that has been used by Uyghur nationality in Xinjiang Uyghur Autonomous Region of China for many years. Currently, it has also been listed in the six priority protective genuine medical herbs by Ningxia Hui Autonomous Region. The whole plant, roots and seeds of *S. alopecuroides* are bitter, cold, and venomous in nature, and have frequently been used to treat fever, inflammation, edema and pain (Xiao, 1993). Quinolizidine alkaloids, such as cytisine, matrine, oxymatrine, sophocarpine, sophoridine etc. in the seeds of *S. alopecuroides*, have been observed to exercise a similar active-toxic dual function as well as those in the root of *S. flavescens* Aiton named as 'Kushen' from family Fabaceae. These alkaloids possess extensive pharmacological activities of cardiac function improvement (Liu et al., 2015), antitumor or cancer suppression (Gao et al., 2009; Chang et al., 2014; Lu et al., 2014) as well as the inhibition and killing of various

* Corresponding authors.
E-mails: wu.xiuli2005@163.com (X. Wu), chenjing@nxmu.edu.cn (J. Chen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bjp.2018.04.007

microorganisms (Chang et al., 2014); however, their strong toxicity limits the popularization and wide application. The median lethal doses (LD_{50}) of matrine, oxymatrine, sophocarpine, sophoridine, and cytisine were determined to be 64.40 (Tian, 2016), 85.95 (Tian, 2016), 63.94 (Qian et al., 2012), 47.60 (Yu and Jiang, 2006), and 1.52 (Tian, 2016) mg/kg, respectively. Cytisine, therefore, has a highest toxicity dozens of times than other alkaloids according to the literatures.

To date, it was merely documented that *S. alopecuroides* should be parched to release smoke and turn surface black, and then grinded into powders before a clinical usage in order to decrease its toxicity (Ran, 1998). The influence of other conventional detoxifying methods on the toxicity of Traditional Chinese Medicines has not been studied. However, it has been demonstrated that vinegar- and rice wine-processing means decreased the toxicity of *S. flavescens* (Ye et al., 1999), which provides an important basis for our work. Furthermore, changes of these quinolizidine alkaloids in *S. alopecuroides* before and after being processed still remain unclear.

In the present study, therefore, the toxicity-attenuation effect of various processing methods was firstly evaluated by an acute oral toxicity test and pathologic analysis. The underlying reason was also elucidated by detecting the variation of five key toxic alkaloids including matrine, oxymatrine, sophocarpine, sophoridine, and cytisine in different processed *S. alopecuroides* samples. A rapid UHPLC–MS/MS assay was established and then successfully applied to quantify the contents of these alkaloids in this paper.

Materials and methods

Chemicals and reagents

Vinegar was purchased from Hengshun Vinegar Industry Co., Ltd (Jiangsu, China). Yellow rice wine was purchased from Guyue Longshan Shaoxing Wine Industry Co., Ltd (Zhejiang, China). Ethanol was purchased from Damao Chemical Reagent Factory (Tianjin, China). Methanol and acetic acid (HPLC-grade) was purchased from Fisher (Pittsburgh, USA). Ammonium acetate (HPLC-grade) was purchased from Dima (Richmondhill, USA). Ultra-pure water was obtained from a Milli-Q system (Bedford, USA) freshly.

The standards of matrine (No. 519-02-8), oxymatrine (No. 16837-52-8), sophocarpine (No. 145572-44-7), sophoridine (No. 6882-68-4), and cytisine (No. 26904-64-3) with purity over 98% were purchased from Mansite Biological Technology Co., Ltd (Chengdu, China). Diazepam (No. 115-9302, IS) was purchased from National Institutes for Food and Drug Control (Beijing, China). All other chemicals and reagents were of analytical grade and commercially available.

Plant materials

The wild seeds of *Sophora alopecuroides* L., Fabaceae, were collected from Ningxia Hui Autonomous Region of China in July 2015 (coordinates 38°47′ N and 106°27′ E) and were authenticated Dr. Jing Chen (Ningxia Medical University). The voucher specimens were deposited in the College of Pharmacy, Ningxia Medical University, for further references (20150210).

Preparation of crude and processed materials

The crude seeds of *S. alopecuroides* were rapidly cleaned in running water and dried in the shade. Approximate 200 g in duplicate were separately parched in a ceramic (CSA – fried seeds in a ceramic pan) or iron pan (ISA – fried seeds in a iron pan) until the surface turned dark (200 °C). For wine-processed (WSA) and vinegar-processed (VSA) materials, 50 ml of yellow rice wine or 120 ml of vinegar diluted by a certain volume of purified water was individually added into a vitreous airtight container. Same amounts of *S. alopecuroides* seeds were put in rice wine or vinegar for 12 h until the vinegar or yellow rice wine had been completely absorbed and dried in a dry oven over a low temperature (50 °C).

Extract preparation

The seeds were grinded into powders and extracted for three times (30 min each time) with 65% ethanol (1:8, w/v) at ambient temperature in an ultrasonic water bath (40 kHz). The combined solution was filtered and evaporated to recover the solvents using a rotary-evaporator. Afterwards, the concentrated residues were dried to obtain the *S. alopecuroides*, ISA, CSA, WSA, and VSA extracts with a yield of 25.1%, 22.8%, 26.6%, 23.8% and 25.7% (w/w, dried extract/crude herb), respectively. All the extracts were stored at 4 °C before use.

Animal experiments and acute oral toxicity test in mice

Specific pathogen-free (SPF) ICR mice were purchased from the Laboratory Animal Center of Ningxia Medical University (Grade II, Certificate No. SYSK Ningxia 20050001). The weight difference within and between groups was less than ±20% of the sample population. The animals were allowed to acclimate to the housing condition under standard conditions (20 ± 3 °C, 40 ± 5% humidity, 12 h light/12 h dark cycle) and free access to standard pellets and water for one week prior to the experiment. The protocol was approved by the University Ethics Committee (Ningxia China, Ethic approval: 2015-013). All procedures involving animals were in accordance with the Regulations of the Experimental Animal Administration, State Committee of Science and Technology, People's Republic of China.

Total thirty healthy ICR mice (six weeks, 18–22 g) were randomly assigned to each of five groups containing six mice (three females and three males). The dried extracts of *S. alopecuroides*, ISA, VSA, WSA, and CSA were freshly dissolved in saline and five dose groups between the lowest (624 mg/kg) and highest (2200 mg/kg) dosages were set under a dose rate of 1:0.73. Mice were fasted overnight (12 h) with free access to water prior to oral administration of the ethanol extracts of *S. alopecuroides*, ISA, VSA, WSA, and CSA at single doses of 624, 855, 1172, 1606, and 2200 mg/kg. The volume administered by gavage in mice was approximately 0.4 ml per 10 g body weight for each animal (Xiao et al., 2007; Okoye et al., 2012; Ouyang et al., 2015). The acute oral toxicity test was carried out according to the Organization for Economic Cooperation and Development (OECD) Guideline 423 (OECD, 2001).

The behavior changes, toxic symptoms, and deaths were observed for 4 h after dosing, and then further observation was conducted for seven consecutive days. The status of skin and fur, eyes, mucous membranes, respiratory, autonomic effects (*e.g.* salivation), central nervous system effects (tremors and convulsions), changes in the level of motor activity, gait and posture, reactivity to handling and stereotypes or bizarre behavior (*e.g.* self-mutilation, walking backwards) were studied according to the literature (Wu et al., 2015). The time of death was recorded as precisely as possible. The LD_{50} was then calculated according to the formula below as previously described (Molle, 1986; Xu et al., 1992).

$$LD_{50} = lg^{-1}\left[X_m - i \times \left(\sum p - 0.5\right)\right]$$

X_m: the logarithm of maximum mortality dose; i: class interval (logarithmic difference between two adjacent doses); p: mice mortality under each dosage; $\sum p$: the sum of mortality.

The survivals were sequentially monitored for 7 days. At the end of the test, all animals were sacrificed for subsequent pathologic study.

Histopathological test

After an elaborate gross necropsy, the brain, heart, liver, lungs, spleen and kidneys of the mice were removed and the adherent tissues were carefully cropped. The dissected organs were fixed in 10% neutral formalin and processed adequately. The tissues were sliced to 5 μm thickness sections and placed onto glass slides, and then stained with hematoxylin and eosin (H.E.) before microscopic examination; the magnification power used was 400× (Tchamadeu et al., 2011).

UHPLC–MS/MS instrumentation and analytical conditions

The LC system for LC–MS/MS was Shimadzu Nexera. The instrument (UHPLC LC-30A) consisted of a binary LC-30AD pump, DGU-20A5 vacuum degasser, SIL-30AC autosampler and CTO-30A thermostat column compartment. An API4000 triple quadrupole mass spectrometer (AB SCIEX, Foster, USA) equipped with an electrospray ionization (ESI) source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode and set up in a multiple reaction monitoring (MRM) mode. Nitrogen was used as the nebulizer gas (50 psi) and the auxiliary gas (30 psi). The capillary temperature was 450 °C. The spray voltage was 4000 V. The collision gas was three units. The collision energy was 60 eV for each analyte and 57 eV for diazepam (IS). Base on the full-scan mass spectra of each analyte and the literature (Wang et al., 2012), the MRM transitions *m/z* 249.0 → 148.3, *m/z* 265.1 → 205.1, *m/z* 247.0 → 135.9, *m/z* 248.9 → 148.2, *m/z* 191.0 → 148.2, and *m/z* 285.2 → 193.2 were respectively selected for quantitative analysis of matrine, oxymatrine, sophocarpine, sophoridine, cytisine, and diazepam (IS). The resolution ratio of the mass spectrometer was set to be ±0.1.

Separation was performed on a Shim-pack XR-ODS column (2.0 mm × 100.0 mm, 2.2 μm) with a Shim-pack GVP-ODS column (2.0 mm × 5.0 mm, 2.2 μm). Isocratic elution was employed with mobile phase of acetonitrile-0.1% glacial acetic acid buffer solution containing 10 mM ammonium acetate (75:15, v/v). The flow rate was set at 0.3 ml/min. The column temperature and injection volume were set at 40 °C and 5 μl, respectively. The five quinolizidine alkaloids were identified by comparing the retention time (t_R) and measuring the contents with those of the standard substances within 2 min (1.19, 0.99, 1.15, 1.19, 0.97, 1.42 min for matrine, oxymatrine, sophocarpine, sophoridine, cytisine, and diazepam, respectively). The system was controlled by Analyst software version 1.5.1 (AB SCIEX, USA).

Preparation of calibration standards

Standard stock solutions from five separate weightings of matrine, oxymatrine, sophocarpine, sophoridine and cytisine and internal standard were prepared by dissolving the standards in methanol, giving a final concentration of 40 μg/ml for all the analytes and 50 μg/ml for IS. Standard working solutions were prepared by diluting the stock solution in methanol to obtain a serial of desired concentrations. All working solutions were stored at 4 °C and brought to room temperature before use. Calibration samples were freshly prepared before analysis.

Quality control (QC) samples at low, middle and high concentrations were prepared in methanol at strengths of 10, 100, and 4000 ng/ml. The QC samples were prepared independently using the same procedure as the calibration samples.

Assay validation

The UHPLC–MS/MS method was validated for linearity, stability, lowest limit of quantification (LLOQ), precision and accuracy, recovery, and repeatability (Wang et al., 2012; Gao et al., 2011).

Linearity and lowest limit of quantification

A series of standard stock solutions (concentration from 5 to 5000 ng/ml) containing 20 μl of IS were prepared for the establishment of calibration curves. The peak ratio between the analytes and the IS was plotted against nominal concentration to obtain calibration curves. Calibration curves with correlation coefficient (r^2) values of >0.99 and accuracy of ±15% (except for LLOQ where ±20% was applied) were considered to be acceptable.

Precision, repeatability and accuracy

The intra- and inter-day variations, which were chosen to determine the precision of the established method, were studied by determining the five analytes in five replicates during a single day and by duplicating the experiments on three consecutive days. Variations of the peak ratio were taken as the measures of precision and expressed as percentage relative standard deviations (RSD%). Repeatability was confirmed with six independent analytical sample solutions prepared from the same batch of sample and variations were expressed by RSD%. One of the sample solutions mentioned above was stored at room temperature and injected into the apparatus at 0, 2, 4, 8, 12, and 24 h, respectively, to evaluate the stability of the solution.

Extraction recovery

A recovery test was used to evaluate the accuracy of this assay. The test was performed by adding the five standards into three duplicates of known amounts of *S. alopecuroides* seeds. The spiked samples were extracted, processed, and quantified in accordance with the methods mentioned above. The average recovery percentage was calculated by the formula:

$$\text{Recovery}(\%) = \frac{(\text{observed amount} - \text{original amount})}{\text{spiked amount}} \times 100\%$$

Quantitative analysis of the extract samples

The dried powder of the extracts (0.2 g, $n = 3$) was weighed accurately into a 20 ml conical flask with a stopper, and 10 ml of methanol was added. After accurate weighing, ultrasonication (40 kHz) was performed at room temperature for 20 min. The same solvent was added to compensate for the weight lost during the extraction. The solution was adequately mixed centrifuged at 17,949 × *g* for 15 min. All the solutions were stored at 4 °C and filtered through a 0.22 μm membrane filter before injection into the UHPLC system for analysis.

Statistical analysis

Data were expressed as the mean ± SD. A paired-samples *t*-test was performed to determine whether the means of two groups were statistically different from each other. Analysis of variance (ANOVA) was a statistical measure used for determining whether differences existed among more than two groups, with the significance established at $p < 0.05$, 0.01, and 0.001. Statistical analysis was performed using SPSS software (version 16.0, SPSS Inc, USA). All the charts were plotted by Graph Pad Prism software system (version 3.0, San Diego, CA, USA).

Results and discussion

Toxic behavioral responses to the ethanol extracts of crude and processed Sophora alopecuroides *in single dose*

Behavioral responses to the acute toxicity of crude and processed *S. alopecuroides* at doses of 624–2200 mg/kg were elaborately summarized in Table 1. The severe poisoning symptoms including mouth scratching, gait ataxia, dull, arching, ptosis closing, convulsion and hyperspasmia were observed in crude *S. alopecuroides*- and WSA-treated mice at doses of 1606 and 2200 mg/kg and almost all the experimental animals died immediately. Abdominal breathing and strong twitching phenomenon were detected before the death of animals. These adverse effects completely disappeared after 4 days for surviving mice. CSA- and VSA-disposal obviously alleviated the poisoning symptoms. Meanwhile, *S. alopecuroides* and WSA at doses of 855–1172 mg/kg led to lower mice body weight gain than CSA and VSA (Fig. 1).

Measurement of LD_{50}

As shown in Table 2, mortality rate (%) in ISA, CSA and VSA group were much lower than *S. alopecuroides* and WSA (VSA < CSA < ISA). Also, animals in ISA, CSA, and VSA achieved a higher LD_{50} than *S. alopecuroides* and WSA. It indicated that frying and vinegar-processing methods decreased the toxicity induced by *S. alopecuroides*. Meanwhile, gender difference on the acute toxicity was manifested. Female mice were more vulnerable than male with a higher death rate and a lower LD_{50}.

Histopathological analysis

Histologically, the distinct degenerative changes of the lung, liver, brain, kidney, and heart were described in Fig. 2 after oral treatment of *S. alopecuroides*, ISA, CSA, WSA, and VSA at lethal dose of 2200 mg/kg, implying that those organs were the main targets of *S. alopecuroides*-caused toxicity. The lesions observed in the lung tissue consisted of angiotelectasis, swelling and hyperemia, hemorrhage, and broadening pulmonary mesenchyme. In the liver, serious hepatic necrosis, remarkable central vein dilatation and congestion were observed. The changes in the brain including cellular swelling, crushing, and melting along with the formation of encephalomalacia foci, were indicative of nervous system lesions. Moreover, cell swelling in the kidney and heart, lightly stained cardiac myocyte nuclear, glomerulus pyknosis, and kidney tubules dilation and hyperemia were observable. No visible difference of the organ lesion degree was discovered among the dead animals when treated with unprocessed and processed *S. alopecuroides* under the lethal dosage (2200 mg/kg).

Analytical UHPLC–MS/MS method validation

Under the optimized analysis conditions, the representative MRM chromatograms for analytes and IS are presented in Fig. 3. No interference was observed under the optimized conditions. As shown in Table 3, calibration curves for the five analytes were well linear in the range 5–5000 ng/ml with LLOQ 5 ng/ml (RSD% was 8.61, 10.38, 1.71, 9.58, and 7.47, respectively for five analytes). Precision and accuracy, repeatability, stability, and extract recovery of the analytes meet the quantitative requirements (Tables 3 and 4).

Utility of the assay in determining the contents of the analytes in different processed Sophora alopecuroides *samples*

Contents of matrine, oxymatrine, sophocarpine, sophoridine, and cytisine in the extracts from different processed *S. alopecuroides* materials were observed to vary dramatically and had a correlation with the toxicity (LD_{50}) of different processed *S. alopecuroides* extracts (Fig. 4). Cytisine, the most toxic alkaloid among them, in the group CSA and VSA decreased 3.98 and 11.26 folds versus *S. alopecuroides*, respectively. Contents of matrine, sophcarpine, and sophoridine in ISA, CSA and WSA declined while those in VSA were

Table 1
Behavioral responses to the acute oral toxicity induced by the ethanol extracts of *Sophora alopecuroides*, ISA, CSA, WSA, and VSA in mice.

Group	Dosage (mg/kg)	Observed behavioral responses					
		Mouth scratching	Dystaxia	Dull	Arching	Ptosis closing	Convulsion and hyperspasmia
S. alopecuroides	2200	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)
	1606	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	6 min (5)
	1172	Immediately (6)	1 min (3)	1 min (5)	3 min (4)	4 min (4)	6 min (2)
	855	Immediately (6)	2 min (2)	2 min (4)	5 min (2)	6 min (2)	6 min (1)
	624	Immediately (4)	5 min (2)	5 min (2)	No	No	No
ISA	2200	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)
	1606	Immediately (6)	1 min (3)	1 min (5)	3 min (4)	4 min (4)	6 min (3)
	1172	Immediately (6)	1 min (3)	1 min (5)	3 min (4)	4 min (4)	4 min (1)
	855	Immediately (5)	2 min (2)	2 min (4)	5 min (2)	6 min (2)	No
	624	Immediately (4)	5 min (1)	5 min (2)	No	No	No
CSA	2200	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (5)	3 min (3)
	1606	Immediately (6)	2 min (2)	2 min (3)	3 min (2)	2 min (3)	5 min (1)
	1172	Immediately (5)	3 min (2)	1 min (3)	4 min (1)	3 min (3)	No
	855	Immediately (3)	3 min (1)	2 min (2)	No	4 min (1)	No
	624	Immediately (3)	No	No	No	No	No
WSA	2200	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)
	1606	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (6)	Immediately (5)
	1172	Immediately (6)	1 min (3)	1 min (5)	3 min (4)	3 min (4)	2 min (3)
	855	Immediately (6)	2 min (2)	2 min (4)	4 min (2)	4 min (2)	3 min (2)
	624	Immediately (3)	5 min (1)	5 min (2)	5 min (1)	No	No
VSA	2200	Immediately (6)	Immediately (4)	Immediately (5)	1 min (4)	Immediately (5)	3 min (3)
	1606	Immediately (3)	1 min (1)	Immediately (3)	3 min (2)	Immediately (3)	No
	1172	Immediately (2)	2 min (1)	1 min (2)	3 min (1)	4 min (1)	No
	855	No	No	4 min (1)	No	4 min (1)	No
	624	No	No	No	No	No	No

No, no toxic symptoms observed. The numbers in the parentheses indicate the amount of animals that emerged the signs of toxicity.

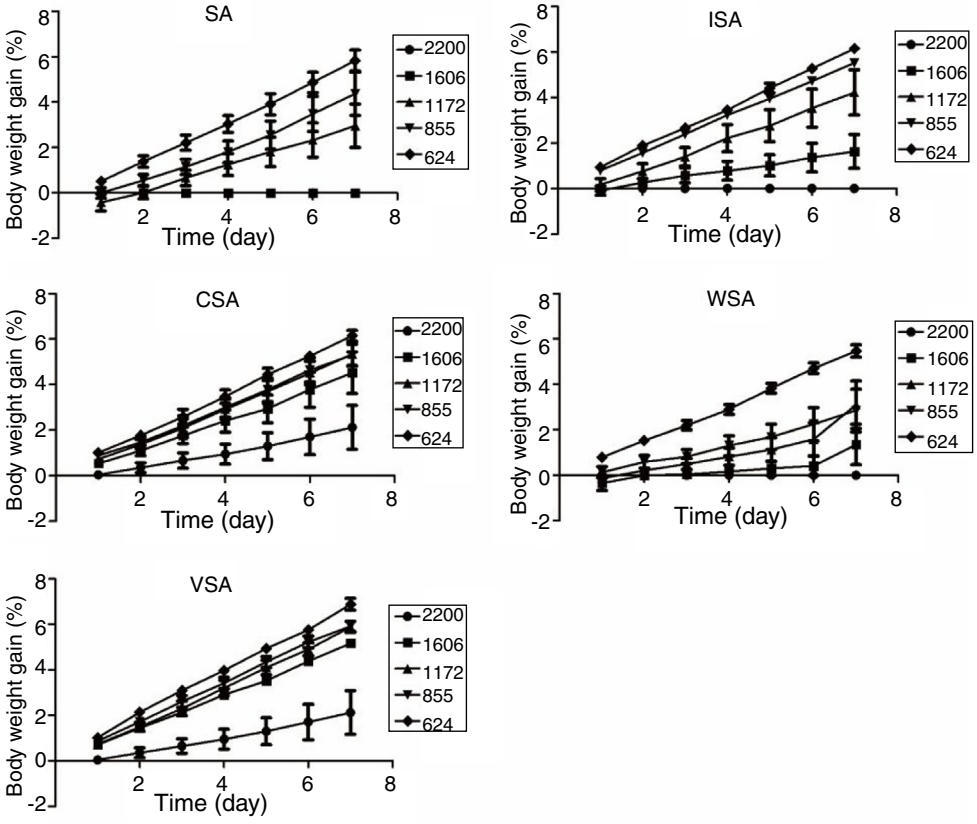

Fig. 1. Mice body weight gain after oral administration of the ethanol extracts of *Sophora alopecuroides*, ISA, CSA, WSA and VSA, respectively.

Table 2
The acute oral toxicity of ethanol extracts of unprocessed and processed *Sophora alopecuroides* in male and female mice.

Dosage (mg/kg)	Number of death/Mortality rate (%)									
	Sophora alopecuroides		ISA		CSA		WSA		VSA	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
2200	3/100.0	3/100.0	3/100.0	3/100.0	1/33.3	2/66.7	3/100.0	3/100.0	1/33.3	2/66.7
1606	2/66.7	3/100.0	1/16.7	2/66.7	0/0.0	1/16.7	2/66.7	3/100.0	0/0.0	0/0.0
1172	1/16.7	1/16.7	0/0.0	1/16.7	0/0.0	0/0.0	1/16.7	2/66.7	0/0.0	0/0.0
855	0/0.0	1/16.7	0/0.0	0/0.0	0/0.0	0/0.0	1/16.7	1/16.7	0/0.0	0/0.0
624	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
LD_{50}	1367.7	1109.2	1686.6	1367.7	2306.8	1870.7	1233.1	1000.0	2306.8	2074.9

enhanced 4.44, 7.20, and 7.23 folds, respectively. Oxymatrine content decreased in all the processed groups and was not detected in VSA.

Cytisine is a known partial agonist of α4β2 nicotinic acetylcholine receptor (Han et al., 2016), and has the teratogenicity (Barceloux, 2008) and high brain toxicity in spontaneously hypertensive rats (Simeonova et al., 2010). The negative side effects also include headache, nausea, vertigo, vomiting, diarrhea, chest pain, and in higher doses, convulsions and respiratory failure (Anderson et al., 2015). In this paper, we observed a very close correlation between the content of cytisine and the toxicity of different processing methods ($r = 0.8589$). It hinted that cytisine might be the most important toxicity-related chemical component in *S. alopecuroides* which deserves to further investigate its change mechanism after being processed. Unfortunately, we did not discover the alteration of cytisine when the powder of cytisine standard substance was heated in the waterbath overnight alone or with the vinegar. Some unknown chemicals in *S. alopecuroides* must participate in the complex reaction. More reasonable experiment should be designed later in order to search what chemicals involve in the reaction.

It was reported that oxidized-type alkaloids such as oxymatrine, oxysophcarpine, and oxysophoridine could be respectively transformed to the reduced-types (matrine, sophcarpine, and sophoridine) in a dynamic balance under heating and reductant existing conditions such as SO_2 according to the literature (Guo, 2006). In this study, we actually observed that the contents of matrine, sophocarpine, and sophoridine were elevated while oxymatrine was descended to below the detection limit in VSA. In order to ensure if the increase of reduced-types alkaloids in VSA was caused by conversing from their corresponding oxidized-types alkaloids, we heated oxymatrine, oxysophorcarpine, and oxysophoridine alone or with the vinegar in a waterbath overnight. However, no changes were found, too. The results closely conformed to the literature (Pan et al., 2008). Hence, as the authors speculate in that paper, some unpredictable reductants must be involved in the redox reaction. It, therefore, still needs a further investigation to elucidate the precise reasons in the further work.

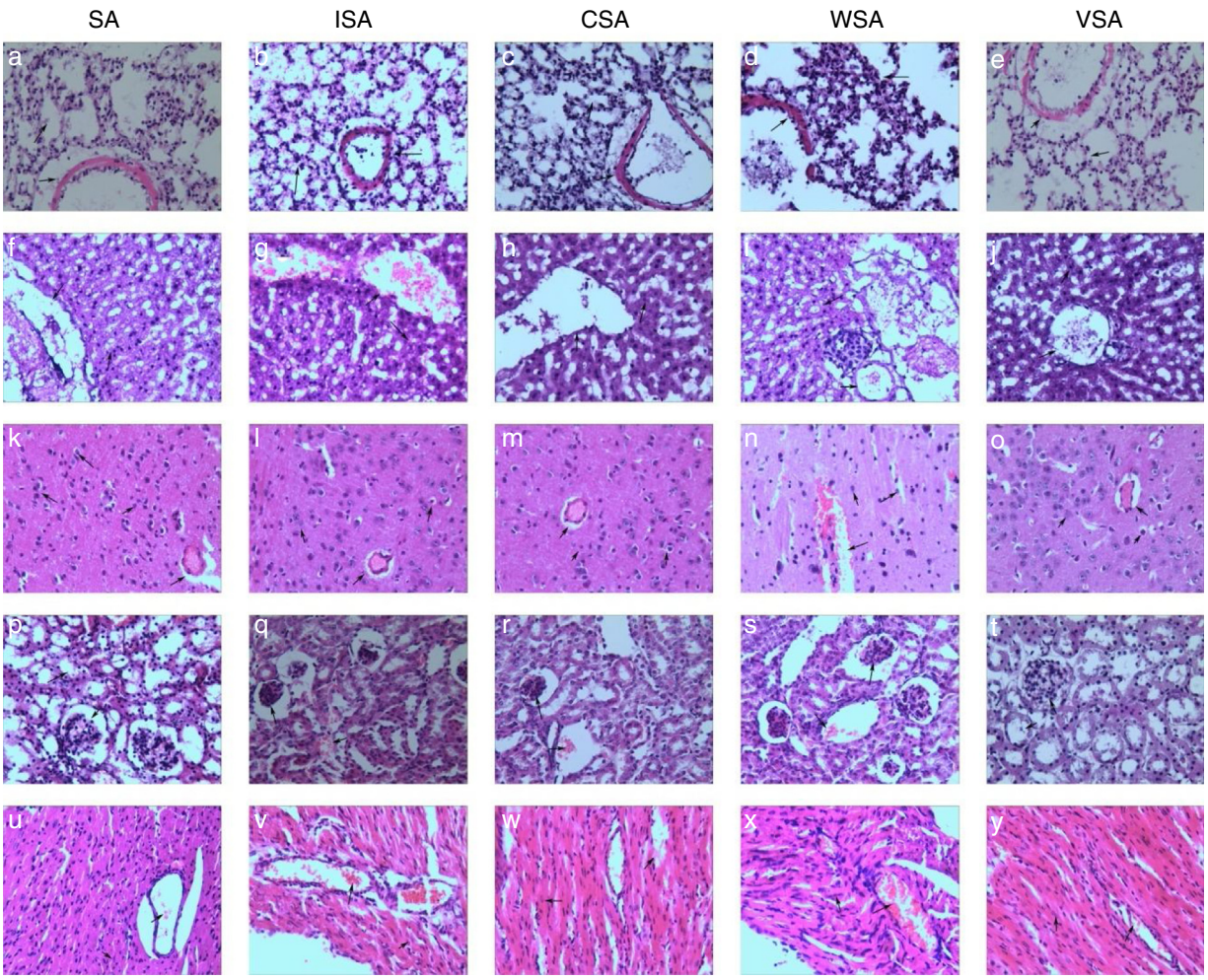

Fig. 2. Effects of *Sophora alopecuroides*, ISA, CSA, WSA, and VSA ethanol extracts on the microstructures of various mice organs after 7 days (HE 400×). (a), (b), (c), (d) and (e): Lung; (f), (g), (h), (i) and (j): Liver; (k), (l), (m), (n) and (o): Brain; (p), (q), (r), (s) and (t): Kidney; (u), (v), (w), (x) and (y): Heart.

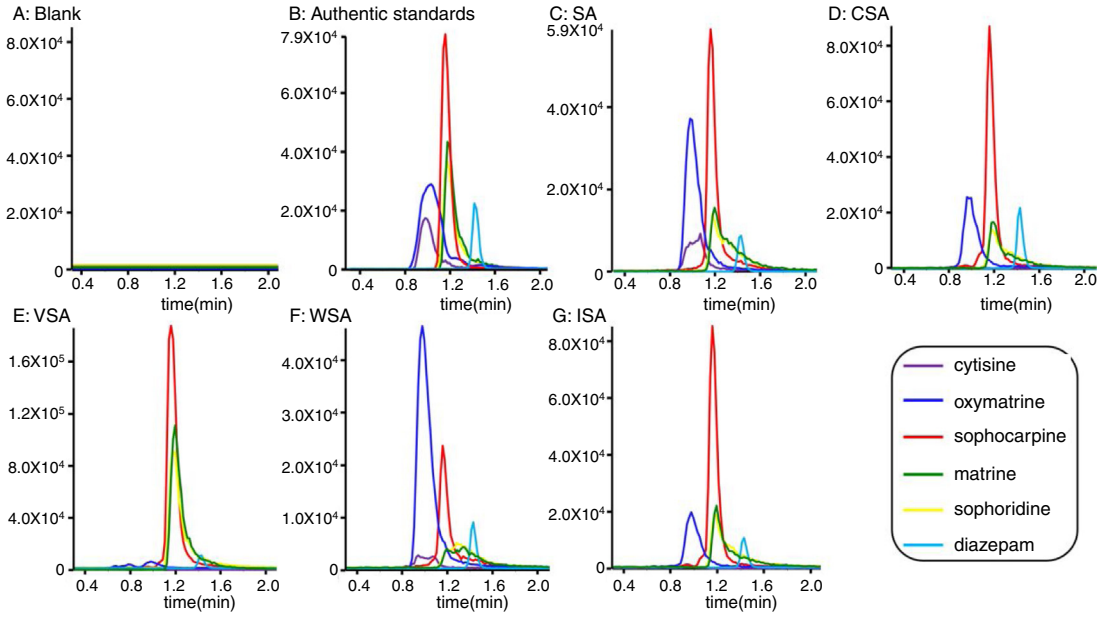

Fig. 3. Representative multiple reaction monitoring (MRM) chromatograms of analytes: (A) Blank; (B) Authentic standards; (C) *Sophora alopecuroides*; (D) CSA; (E) VSA; (F) WSA; (G) ISA.

Table 3
Calibration curves of 5 analytes determined by a rapid UHPLC–MS/MS method.

Analytes	Calibration curves	r^2	Linear range (ng/ml)	LLOQ (ng/ml)	Repeatability (RSD%, $n = 5$)	Stability (RSD%, $n = 5$)	Recovery (%)	
							Mean	RSD%
Matrine	$Y = 0.0272X + 3.43$	0.9944	5–5000	5 (8.61[a], 9.32[b])	7.95	6.34	89.51	2.34
Oxymatrine	$Y = 0.0513X + 4.24$	0.9996	5–5000	5 (10.38,-15.20)	4.85	4.83	75.69	1.34
Sophocarpine	$Y = 0.558X + 0.309$	0.9712	5–5000	5 (1.71, -2.40)	4.82	10.07	77.37	3.25
Sophoridine	$Y = 0.0203X + 2.94$	0.9950	5–5000	5 (9.58, 1.40)	3.21	5.56	89.81	1.52
Cytisine	$Y = 0.272X + 0.257$	0.9916	5–5000	5 (7.47, 5.00)	6.06	8.49	71.18	2.41

[a] Represents RSD% (% relative standard deviation) = (SD/mean) × 100.
[b] Represents CE% (% relative error) = [(measured concentration – theoretical concentration)/theoretical concentration] × 100).

Table 4
Precision and accuracy (intra- and inter-day) of the UHPLC–MS/MS method for the analysis of five alkaloids.

Analytes	Theoretical concentration[a] (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 3$)		
		Measured conc. (ng/ml)	Precision (RSD%)	Accuracy (RE%)	Measured conc. (ng/ml)	Precision (RSD%)	Accuracy (RE%)
Matrine	10	10.66 ± 0.61	5.73	6.60	10.71 ± 1.17	10.90	7.10
	100	96.04 ± 3.91	4.07	−3.96	99.93 ± 6.59	6.60	−0.07
	4000	4134.00 ± 216.98	5.25	3.35	3772.00 ± 304.49	8.07	−5.70
Oxymatrine	10	9.20 ± 0.27	2.96	−8.0	9.66 ± 0.96	9.98	−3.40
	100	88.64 ± 3.33	3.76	−11.36	92.66 ± 7.52	8.12	−7.34
	4000	4020.00 ± 195.58	4.87	0.50	3885.33 ± 173.53	4.47	−2.87
Sophocarpine	10	9.00 ± 0.37	4.11	−10.00	10.04 ± 1.39	13.80	0.40
	100	86.7 ± 1.16	1.34	−13.30	96.73 ± 10.56	10.92	−3.27
	4000	4240.00 ± 204.45	4.82	6.00	4096.67 ± 332.88	8.13	2.42
Sophoridine	10	8.60 ± 0.45	5.26	−14.00	9.62 ± 1.12	11.67	−3.80
	100	90.86 ± 1.09	1.20	−9.14	100.19 ± 8.84	8.83	0.19
	4000	4210.00 ± 144.05	3.42	5.25	3876.00 ± 287.99	7.43	−3.10
Cytisine	10	8.92 ± 0.73	8.16	−10.80	9.52 ± 1.25	13.16	−4.80
	100	93.24 ± 1.62	1.73	−6.76	99.42 ± 8.07	8.11	−0.58
	4000	3848.00 ± 201.42	5.23	−3.80	3898.00 ± 232.54	5.96	−2.55

Intra-day and inter-day precision and accuracy were determined with $n = 5$ or $n = 3$ for each concentration, respectively.
RE % (% relative error) = [(measured concentration – theoretical concentration)/theoretical concentration] × 100.
RSD (% relative standard deviation) = (SD/mean) × 100.
[a] Selected concentrations represent the low, medium and high (LQC, MQC, and HQC) concentrations.

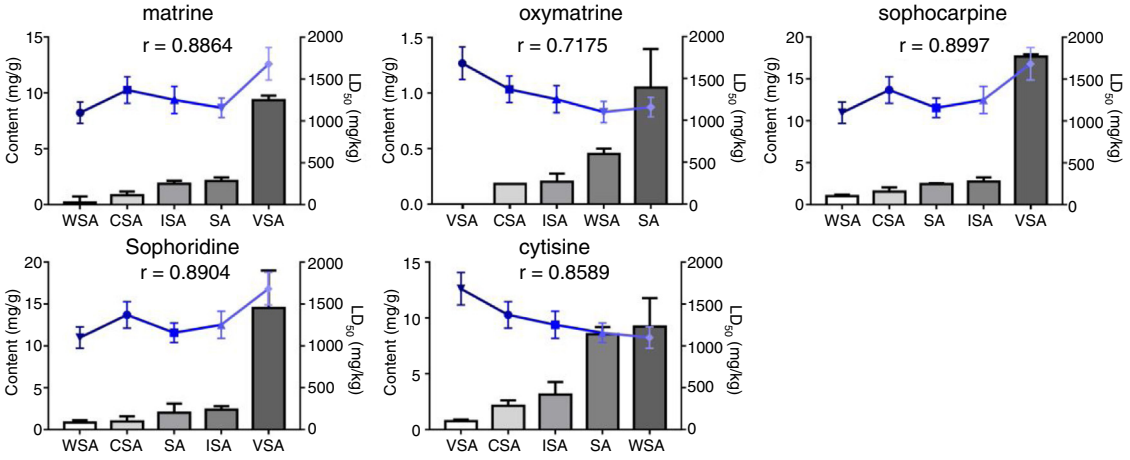

Fig. 4. Correlation between content variation of five alkaloids and LD_{50} of different processed *Sophora alopecuroides*. Data were expressed as mean with SD ($n = 3$).

Conclusions

In conclusion, the present work firstly investigated the acute toxicity profiles of oral use of ethanol extract of *S. alopecuroides*, ISA, CSA, WSA, and VSA. Contents of the quintessential quinolizidine alkaloids in *S. alopecuroides*, especially cytisine, varied prominently before and after being processed. It might be the key mechanism of CSA and VSA on the attenuation of *S. alopecuroides*-induced toxicity. The data will be beneficial for future clinical application of the medical safety of SA.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with

those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Acknowledgements

This work was supported by grants from Ningxia Natural Science Foundation (NZ17056).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.04.007.

References

Anderson, M.J., Kurtycz, D.F.I., Cline, J.R., 2015. Baptisia poisoning: a new and toxic look-alike in the neighborhood. J. Emerg. Med. 48, 39–42.

Barceloux, D.G., 2008. Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs. Plants and Venomous Animals. John Wiley and Sons, Inc., New Jersey.

Chang, A.H., Cai, Z., Wang, Z.H., Sun, S.K., 2014. Extraction and isolation of alkaloids of *Sophora alopecuroides* and their anti-tumor effects in H22 tumor-bearing mice. Afr. J. Tradit. Complement. 11, 245–248.

Gao, Y., Li, G., Li, C., Zhu, X., Li, M., Fu, C., Li, B., 2009. Anti-nociceptive and anti-inflammatory activity of sophocarpine. J. Ethnopharmacol. 125, 324–329.

Gao, H.Y., Li, G.Y., Wang, J.H., 2011. Studies on the dynamic accumulations of *Sophora alopecuroides* L. alkaloids in different harvest times and the appropriate harvest time. J. Chromatogr. B 879, 1121–1125.

Guo, A., 2006. Studies on technology for extracting and purifying matrine and oxymatrine from *Sophora flavescens*. J. Southwest Forestry Coll. 26, 37–39.

Han, J., Wang, D.S., Liu, S.B., Zhao, M.G., 2016. Cytisine, a partial agonist of α4β2 nicotinic acetylcholine receptors, reduced unpredictable chronic mild Stress-Induced depression-like behaviors. Biomol. Ther. 24, 291–297.

Liu, Z., Wang, J., Qiu, C., Guan, G., Liu, X., Li, S., Deng, Z., 2015. Matrine pretreatment improves cardiac function in rats with diabetic cardiomyopathy via suppressing ROS/TLR-4 signaling pathway. Acta Pharmacol. Sin. 36, 323–333.

Lu, X., Lin, B., Tang, J.G., Cao, Z., Hu, Y., 2014. Study on the inhibitory effect of total alkaloids of *Sophora alopecuroides* on osteosarcoma cell growth. Afr. J. Tradit. Complement. 11, 172–175.

Molle, J., 1986. Limites de tolérance et toxicités de quelques amino acides (Formes L et D), Amino-peptides-protéines. In: Paris, Cahier N°4. Ed AEC, pp. 207–232.

OECD, 2001. Procedures for Toxicological Assessment on Food Safety.

Okoye, T.C., Akah, P.A., Ezike, A.C., Okoye, M.O., Onyeto, C.A., Ndukwu, F., Ohaegbulam, E., Ikele, L., 2012. Evaluation of the acute and sub acute toxicity of *Annona senegalensis* root bark extracts. Asian Pac. J. Trop. Med. 5, 277–282.

Ouyang, C.B., Liu, X.M., Liu, Q., Bai, J., Li, H.Y., Li, Y., Wang, Q.X., Yan, D.D., Mao, L.G., Cao, A., 2015. Toxicity assessment of cadinene sesquiterpenes from *Eupatorium adenophorum* in mice. Nat. Prod. Bioprospect. 15, 2319–2325.

Pan, G.Z., Li, F., Li, J., 2008. Effect of converter of matrine and oxymatrine on extraction method of *Radix Sophorace Flavescentis*. Res. Pract. Chin. Med. 22, 52–54.

Qian, L.W., Dai, W.H., Wang, L.L., Zhou, G.Q., Zhang, Y., Wang, H.S., 2012. Toxicity study on sophocarpine and oxysophocarpine in mice. Chin. J. Experim. Trad. Med. Formulae 13, 266–268.

Ran, X., 1998. Zhong Hua Yao Hai (Book Two). Harbin Publishing House, Harbin.

Simeonova, R., Vitcheva, V., Mitcheva, M., 2010. Effect of cytisine on some brain and hepatic biochemical parameters in spontaneously hypertensive rats. Toxicol. Lett. 229, 21–25.

Tchamadeu, M.C., Dzeufiet, P.D., Nana, P., Kouambou Nouga, C.C., Ngueguim, T.F., Allard, J., Blaes, N., Siagat, R., Zapfack, L., Girolami, J.P., 2011. Acute and sub-chronic oral toxicity studies of an aqueous stem bark extract of *Pterocarpus soyauxii* Taub (Papilionaceae) in rodents. J. Ethnopharmacol. 133, 329.

Tian, X.S., 2016. Research progress on toxicity of alkaloids in *Sophorae Tonkinensis Radix et Rhizoma*. Chin. J. Exp. Tradit. Med. Formulae 22, 230–234.

Wang, H.Q., Guo, S., Qian, D., Qian, Y., Duan, J.A., 2012. Comparative analysis of quinolizidine alkaloids from different parts of *Sophora alopecuroides* seeds by UPLC–MS/MS. J. Pharm. Biomed. 67–68, 16–21.

Wu, X., Wan, D., Xie, C., Li, T., Huang, R., Shu, X., Ruan, Z., Deng, Z., Yin, Y., 2015. Acute and sub-acute oral toxicological evaluations and mutagenicity of N-carbamylglutamate (NCG). Regul. Toxicol. Pharm. 73, 296–302.

Xiao, P., 1993. A Pictorial Encyclopaedia of Chinese Medical herbs, Japanese Edition. Chuokoron- Shinsha, Inc., Tokyo.

Xiao, J., Li, B., Zhang, X., 2007. The research progress of acute oral toxicity evaluation test method. Foreign Med. Sci. (Section Hygiene) 34, 84–88.

Xu, S.Y., Bian, R.L., Chen, X., 1992. Methodology of pharmacological experiment, publication and distribution of the second edition. Chin. Pharmacol. Bull. 1, 19.

Ye, D., Zhang, S., Pan, S., 1999. Processing Science of Chinese Materia Medica. People's Medical Publishing House, Beijing.

Yu, J.Q., Jiang, Y.X., 2006. Effects of sophoridine on central nervous system in mice. Chin. Tradit. Herb. Drugs 37, 1688–1689.