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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

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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

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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.

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