Journal of Ethnopharmacology 210 (2018) 88–94

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Toxic pyrrolizidine alkaloids provide a warning sign to overuse of the ethnomedicine *Arnebia benthamii*

Latif Ahmad[a], Yi He[a], Jia-Chen Hao[a], Andrew Semotiuk[b], Quan-Ru Liu[a,*], Paras Mazari[a]

[a] College of Life Science, Beijing Normal University, Beijing 100875, China
[b] Department of Botany and Plant Sciences, University of California, Riverside, CA 92507 USA

ARTICLE INFO

Keywords:
Herbal drug
Arnebia benthamii
Pyrrolizidine alkaloids
Toxicity
Neelum Valley
HPLC

ABSTRACT

Ethnopharmacological relevance: From early times man has used medicinal plants for the treatment of various ailments and basic health care needs. The use of herbal medicines has increased day by day and with this, so do reports of adverse events, poisoning, and suspected toxicity. Similarly, the indigenous communities of Neelum Valley in Azad Kashmir commonly use *Arnebia benthamii* (Wall. ex G.Don) I.M.Johnst. for medicinal purposes to treat various human aliments. Besides their medicinal uses, it also contains hepatotoxic pyrrolizidine alkaloids (PAs).

Aim of the study: This explorative study underscores two major aspects about this herbal medicine. Firstly we aimed to document the traditional therapeutic uses of *Arnebia benthamii* in Neelum Valley, Azad Kashmir. Secondly, to determine the presence or absence of hepatotoxic pyrrolizidine alkaloids and if they are within the suggested limit for the use of herbs in excess.

Materials and methods: Interviews, group discussions, and inquiries were carried out from July to September 2016 with local indigenous and elder people. In the laboratory, the plant was investigated for pyrrolizidine alkaloids by using high performance liquid chromatography (HPLC).

Results: A total of 30 respondents were interviewed. They explained the preferred preparation, parts used, and treatment indications. Treatment of fever along with kidney and liver problems are the three principle uses. Among the different parts of *Arnebia benthamii*, 43% respondents preferred aerial parts for the herbal formulation, followed by whole plants, and leaves. Decoction was the major mode of preparation and all herbal preparations were administrated orally. This study reports, for the first time according to our literature review, a study of *Arnebia benthamii* with regard to PA determination. By using column Zorbax SB-Aq and acetonitrile-water gradient as the mobile phase, HPLC results showed that the aerial parts of the plant were PA positive, and (1) Europine, Heliotrine (2), Lycopsamine (3), and Echimidine (4) were identified.

Conclusions: This study has revealed two new findings of significance to herbal medicine producers, practitioners, and consumers of *Arnebia benthamii* First, local knowledge regarding the medicinal uses of *Arnebia benthamii* were documented in five sites of Neelum Valley, Azad Kashmir. The use of this plant by a large part of the population in the study area shows the importance for their therapeutic benefits. Unfortunately, the second finding of this study shows that *Arnebia benthamii* contains hepatotoxic PAs. Hence, we advised to the government regulatory authorities and non-governmental organizations that use of this plant as herbal medicine should be excluded before more accurate quality control tests.

1. Introduction

The interaction of humans and plants form a long history that also extends into today. Humans depend on botanical resources to fulfill their needs such as food, clothing, shelter, transportation and medicine (Ahmad et al., 2006). Medicinal plants have played a critical role in human healthcare throughout history (Letsyo et al., 2017). While used historically, they also form a major part of present-day human healthcare (Sadat-Hosseini et al., 2017). The World Health Organization reported that approximately 65% of people throughout the world and 90% of people in developing countries depend on traditional or herbal medicine for primary healthcare (Vandebroek et al., 2008). Even if one does not rely on herbal medicine, their healthcare is still touched by plants. Medicinal plants are a major source of secondary metabolites used in drug discovery. Some estimates assert that plants and their derivatives are involved in 25% of

* Corresponding author.
E-mail address: liuquanru@bnu.edu.cn (Q.-R. Liu).

http://dx.doi.org/10.1016/j.jep.2017.08.009
Received 29 June 2017; Accepted 9 August 2017
Available online 12 August 2017
0378-8741/

commercial drugs (Bodeker and Burford, 2007). This shows their place in the lives of traditional and herbal medicine users, and even non-herbal medicine users.

Studies on the place of plants in the lives of humans lands in the field of ethnobotany. Ethnobotany includes studies on this interaction of humans and botanical resources (Rodrigues, 2007). Knowledge of these interactions is often encapsulated in traditional knowledge, but it can and is lost rapidly, especially in areas of social, cultural and environmental transformations (da Costa et al., 2017). While this knowledge is certainly valuable at a local scale, it is also important for scientific knowledge in general, and especially for conservation initiatives (Cussy-Poma et al., 2017; Muthu et al., 2006). To prevent losses, and provide benefit to all parties, ethnobotanists play a critical role in analyzing potential mechanisms of conservation, sustainable, and safe use of botanical resources (Gandolfo and Hanazaki, 2011).

Consumers of herbal medicine, often called ethnomedicine, provide alternatives for treatment. People often seek these ethnomedicines in hopes that they have less side effects and be safer than pharmaceutical medications (Rates, 2001). However, ethnomedicines can be considered to have harmful or toxic effects if taken in excess (Ahmad et al., 2015). Unfortunately, the testing and critical role of pharmacovigilance in local ethnomedicinal markets is often lacking to non-existent (Debbie et al., 2012). These dangers can be magnified with issues of product quality, processing methods, correct identification of species, and knowledge of potential physiological effects (Shaw, 2010).

One common source of herbal product toxicity comes from pyrrolizidine alkaloids. Pyrrolizidine alkaloids (PAs) are secondary metabolites which are accumulated in plants that may cause hepatotoxicity in humans, and be hazardous to livestock and other wildlife (Avula et al., 2015). These accumulate in plants as PA-*N*-oxides that serve as defense compounds. They are produced by around 6000 plant species, which represents 3% of all angiosperms (Letsyo et al., 2017). Of these, the three families of Boraginaceae, Fabaceae, and Asteraceae contain largest number of PA producing species (Coulombe, 2003). Of these, the Boraginaceae has particular fame of having toxic PAs and their oxides (El-Shazly and Wink, 2014). According to previous studies, PAs are mutagenic, carcinogenic, and hepatoxic. This affects humans, wildlife, and livestock who ingest them (Mattocks, 1986; Prakash et al., 1999). The presence of PAs in herbal products and foodstuffs could lead to liver damage and then subsequently, hepatic veno-occlusive disease (Chen and Huo, 2010). If ingestion of these herbal products or foodstuffs with elevated concentrations of PAs is constant, death can result. Unfortunately, in animals and humans symptoms are not seen immediately. So the toxic effects are often diagnosed when it is too late. Exposure can come from direct consumption of foodstuffs, dietary supplements, herbal teas, or herbal products contaminated with PA containing plant material (Zhu et al., 2016). Sometimes, they can enter the food supply as a result of co-harvesting PA containing plant material with edible grains. (El-Shazly and Wink, 2014). Worldwide, around 10,000 PA poisoning cases have been reported (Dai et al., 2007; Fu et al., 2004).

Moreover, recently numerous cases of intoxications due to PAs are on the rise and researchers are pointing to PAs in herbal medicines (IRIN Asia, 2008a, 2008b; Molyneux et al., 2011; Edgar et al., 2011). The European Medicines Agency (EMA), implemented a limit of oral ingestion of herbal products containing PAs. This limit is 1 μg/day as a transitional measure for three years, then the threshold will be lowered to 0.35 μg/day for adults and 0.14 μg/day for children following a limit of 0.007 μg/kg body weight (BfR, 2011; EMA, 2016). For instance, the German Federal Health Administration limits internal exposure to no more than 1 μg/day (0.017 μg/kg/day for a 60 kg human) of PAs for no more than six weeks (German Federal Health Bureau, 1992). Furthermore, Belgium recommends a limit for PAs in herbs at 1 ppm (Dharmanada, 2013). Considering the risk of human exposure to these alkaloids, the World Health Organization (WHO) established the Health and Safety Guide (International Programme on Chemical Safety, 1989) on PAs and provided guidelines on the prevention of exposure with the dose limit of 15 μg/kg/day (IPCS, 1989).

One potential route of PA toxicity comes from *Arnebia* Forsskål (1775: 62) (Boraginaceae, *Lithospermeae*) with ca. 30 species distributed in the Himalayan region, Northeast Africa, Central and Southwestern Asia, and the Southeast Mediterranean region (Coppi et al., 2015). *Arnebia benthamii* (Wall. ex G.Don) I.M.Johnst. is a monocarpic perennial herb up to 2.7 feet tall with a swollen base. Its thick roots exude a red or purplish dye. It's simple, hollow, and solitary stem arises from a cluster of large basal leaves that are covered with long hairs that are 2–2.6 mm long. In Pakistan, it is distributed in the Pan-Himalayan region like in the Neelum Valley, Dir, Hazara (Flora of Pakistan, 1989). The present study has three aims: (1) Determine the presence of PAs in *Arnebia benthamii*. (2) Investigate and document the ethnobotany and use practices of *Arnebia benthamii* in the Neelum Valley, Pakistan (3) Assess if cultural practices fall within the EMA, IPCS, and German Federal Health Administration guidelines or if safety recommendations must be issued.

2. Materials and methods

2.1. Study area

This explorative study was carried out in Neelum Valley, Azad Kashmir, Pakistan. Neelum Valley is the largest district of Azad Kashmir having an area of 3621 square kilometers. It lies between 73–75°E longitude and 32–35°N latitude at an altitude of 900-6325 m above sea level (Ahmad et al., 2012; Mahmood et al., 2011; Dar, 2003).

The climate of Neelum Valley is temperate with cold winters and severe weather from mid-November until then end of April when temperatures average − 2.0 °C. While summers are moderate and very short lasting from June to August with an average temperature of 37.0 °C. The average rainfall is 165 cm annually (Ahmad et al., 2012; Qamar et al., 2010). The majority of the area is covered with thick vegetation, dominated by wild *Ficus palmata* Forssk. forests (Khan, 2008).

The main ethnic groups of the study area are the *Gujars, Syed, Kashmiris*, and *Awan*. Frequently spoken languages are *Kashmiri*, *Hindko*, and *Gujri* (Ishtiaq et al., 2012) (Fig. 1)

2.2. Ethno-pharmacological data collection and interviews with local people

Ethnobotanical data were collected from local respondents through open and semi-structured interviews (Martin, 1995; Cotton, 1996) between July and September of 2016. We conducted all interviews with prior informed consent regarding our academic research following the International Society of Ethnobiology Guidelines (2006). Due to the Line of Control (LOC), security risk, and the dangerous situation in this disputed area of Kashmir, we consulted with the security agency; Pakistan Army, local police and Forestry Department; and it was decided to conduct the research in five main localities (Athmuqam, Keren, Sharda, Kel, and Tao Butt). With the help of the local forestry department and inhabitants, 51 traditional healers and knowledgeable herbalists were identified in the five main localities. Of these, 30 agreed to voluntary interviews.

We interviewed the local people in public areas such as hotels and shops. Due to cultural and language differences, we selected the national language, Urdu, for interviews. For these ethnobotanical interviews, we conducted group discussions and asked questions regarding the local name of the plants, ethnomedicinal use, parts of the plants used, collection times, modes of preparation, route of administration, herbal preparation, and side effects if any.

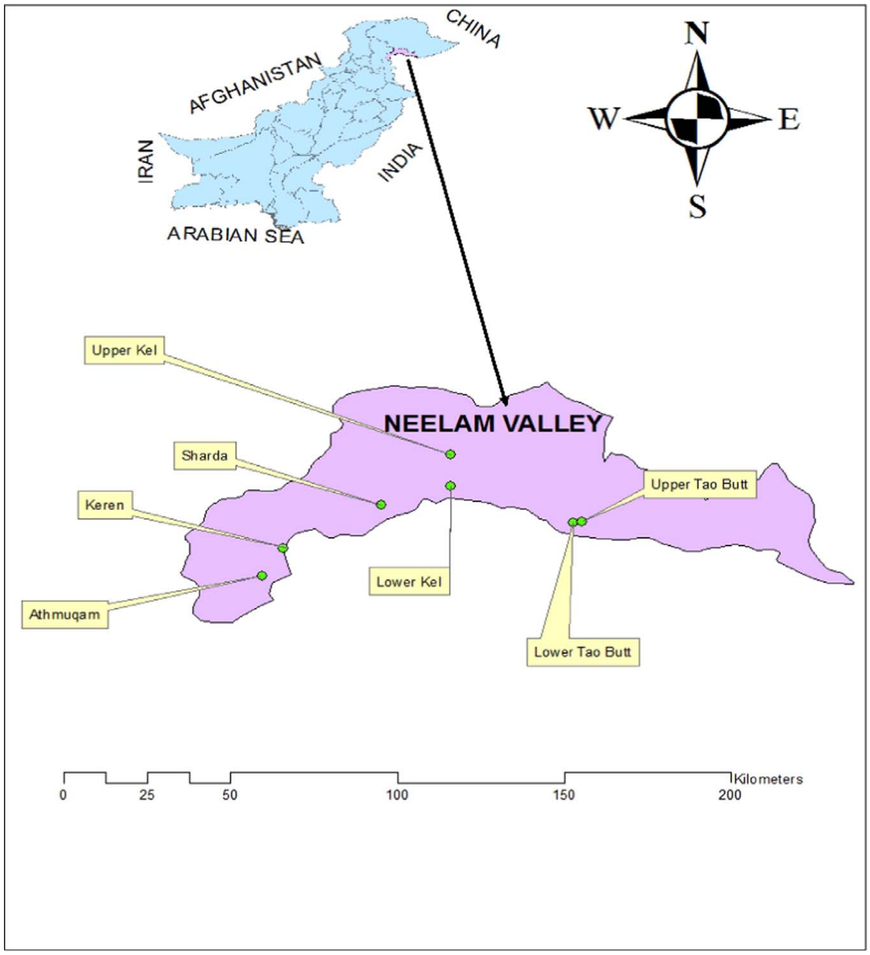


Fig. 1. Map of study area.

2.3. Botanical collection, identification and deposition in herbarium

From July to September 2016 we visited five sites three time each since this season is the flowering and fruiting season. We focused on Boraginaceae for these interviews in order to focus on our research question regarding PAs in this family. Whole plants were pressed for herbarium specimens and fresh plant material was collected in silica gel for phytochemical analysis. *Arnebia benthamii* (Wall. ex G.Don) I.M.Johnst. (voucher specimen number: BNU 0032542) along with other Boraginaceae members were brought to Beijing Normal University in Beijing, China where Prof. Quan-Ru Liu identified and compared the plants voucher specimens in the Chinese National Herbarium (PE) and Herbarium of Beijing Normal University. After complete identification, plants were assigned voucher specimen numbers and deposited as a ready reference for future studies.

2.4. Experimental

2.4.1. Chemicals and reagents

Reference standards of pyrrolizidine alkaloids, i.e. Europine (1) Heliotrine (2), Lycopsamine (3), and Echimidine (4) were purchased from ChemFaces (Hubei, China). All reagents were of HPLC grade. Methanol and acetonitrile was obtained from DikmaPure (USA). Formic acid was from Fluka (Sigma–Aldrich Co., Spruce St., Louis, USA). Ultra-pure water was used throughout the experimental process.

2.4.2. Medicinal plant material and sample preparation

The medicinal plant samples for HPLC were prepared according to the methods used by Avula et al. (2012, 2015). Briefly, dried aerial parts (500 mg) of mature flowering *Arnebia benthamii* was weighed and crushed in a mortar and pestle with liquid nitrogen. The sample was sonicated for 35 min with 3 mL of methanol immediately followed by centrifugation for 15 min at 4000*g*. After centrifugation the supernatant was transferred to another clean tube. The above procedure was repeated thrice and the respective supernatants combined. The final volume was adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection, 20 µL of sample was passed through a 0.45 µm FitMax Syringe Filter membrane (Dikma).

2.4.3. Preparation of standards

Stock solutions of Europine, heliotrine, lycopsamine, and echimidine in methanol were prepared separately at concentrations of 1.0 mg mL^{-1} (Avula et al., 2012, 2015; Griffin et al., 2013). The standards were characterized by NMR, and their purity was > 98%.

2.4.4. High performance liquid chromatography and chromatographic conditions

The liquid chromatographic system was the Waters Alliance 2695 Separation Module with water 2487 dual UV absorbance detector comprised of the following modular components: built-in quaternary pump, 4 channel degasser, auto-injector, and auto-sampler with 120 vials. The separation of the PAs from *Arnebia benthamii* was achieved on a Zorbax SB-Aq; 4.6 × 250 mm; 5 µm particle size column (Agilente, USA).

A gradient LC method was developed to separate the PAs from the aerial parts of *Arnebia benthamii*. The HPLC system was optimized with the mobile phase consisting of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.5 mL/min,

Table 1
Number of respondents of each area in ethnobotanical surveys.

Survey site	Aathmuqam	Keren	Sharda	Kel	Tao Butt
Total respondents	03	04	04	06	13
Age range	40–75	40–65	45–75	40–70	43–74
Education level					
Illiterate	0	0	1	3	6
Elementary school	0	2	1	1	3
Secondary school	1	1	1	2	3
High school	1	0	1	0	1
University	1	1	0	0	0

with the following gradient elution: 0–15 min: 87% B; 15–17 min: 50% B; 17–35 min: 0% B; 35–40 min: 0%; 40–53 min: 87% B. The total run time for analysis was 53 min. Before moving to the next sample, the system is washed by methanol for 25 min. Column temperature and injection volume were at room temperature and 20 µL, respectively. Column and chromatographic peaks were assigned with respect to comparison of the retention times to those of reference standards and the detection wavelength was set at 280 nm.

3. Results and discussion

3.1. Socio-demographic data

A total of 30 local inhabitants aged between 40 and 76 years old were interviewed in five different sites. We observed that most of the respondents belonged to an age between 51 and 80 years (Table 1). Most respondents were illiterate with education levels at illiteracy (33%), elementary school (23%), secondary school (26%), high school (10%), and university (6%) (Table 1). Ninety percent of respondents were married. Four of the interviewed people were government employees, 12 pastoralists, 4 herders, and 10 farmers. Most of them have monthly incomes of $150 USD or less.

When asked, 'In the last month, how many times did you use *Arnebia benthamii* as a medicine?' 48% of respondents answered one time, 20% not at all, 18% two times, 9% three times, and 5% five times. According to the questionnaire results the vast majority of respondents were involved in the collection of *Arnebia benthamii* with 43% collecting 0.5 kg per capita per month, 30% (1 kg per capita per month), 12% (1–2 kg per capita per month), and 10% (2–3 kg per capita per month).

3.2. Ethnopharmacological use of Arnebia benthamii

Arneb*ia benthamii* (Wall. ex G.Don) I.M.Johnst. Locally known as *Gao-zaban* is a perennial herb with a flowering period from July to September. The studied samples of *A. benthamii* were collected from upper Taio Butt, Neelum Valley with an altitude of 2313 m.

According to the questionnaire results, this species is used for three main ailments: kidney problems, liver problems, and fever (Fig. 2). In our literature search we found that *A. benthamii* has previous ethnopharmacological reports from Mahmood et al., (2011, 2012). They reported that the juice of leaves and flowers, and the decoction of the root are used for navel pain, fever, burning urine, and thirst. Interestingly, this species has the same local name in Indian Kashmir and is used for jaundice, cough, fever, blood purification, and chronic constipation (Lone et al., 2013).

3.3. Parts used, ethnomedicine preparation and intake of Arnebia benthamii

Different parts of *Arnebia benthamii* are used for three main ailments regarding kidneys, liver, and fever. During interviews, thirteen respondents mentioned the use of aerial parts as a remedy, seven

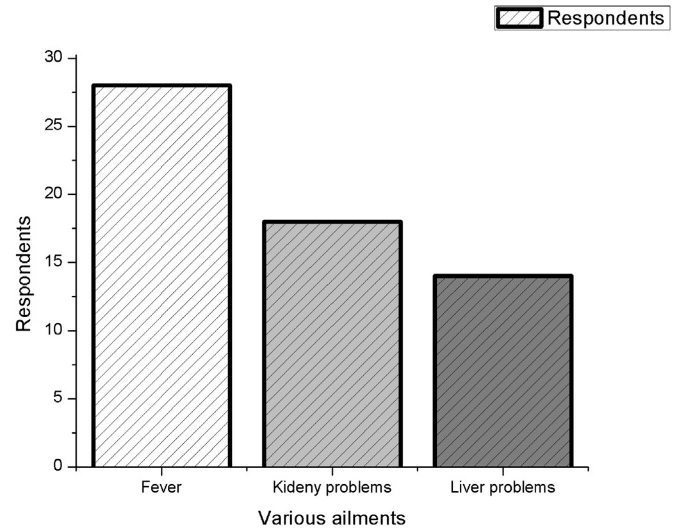


Fig. 2. Respondents mentioned the plant used for three main ailments.

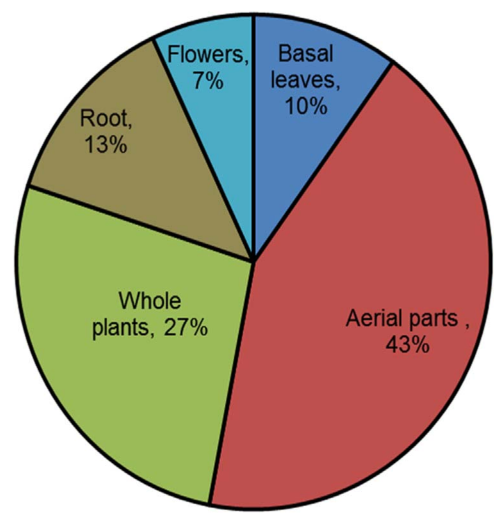


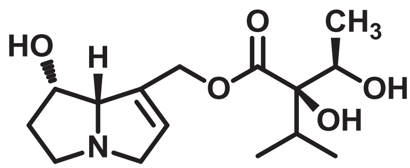
Fig. 3. Percentage of respondents who use various parts of *Arnebia benthamii.*

used whole plants, four used the roots, three used basal leaves, and two respondents used flowers (Fig. 3). The uses of these parts were also recorded in studies by Mahmood et al. (2011, 2012). The local people collect the plant and bring it to their homes. The whole plant or aerial parts of the plant are stored as dried powders in closed bottles or the whole plant is dried under shade and placed in a clean place.

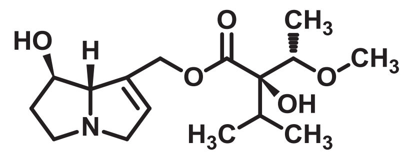
When asked, 'What is the best way of herbal medicine preparation with *Arnebia benthamii*?' twenty-six answered that decoction is the best way, and the remaining mentioned fresh juice of the flowers and leaves could be considered as the best preparation method. Almost all respondents mentioned that oral administration is the best way to intake this medicine. Two to three teaspoons of plant powder are added to 250–350 mL of boiling water, and sometimes local people mix in honey or sugar. In traditional herbal formulation, decoction is an easy way to prepare ethnomedicines, and it is one of the most common routes of internal administration in Pakistan (Nadembega et al., 2011; Mahmood et al., 2011).

3.4. Separation and determination of PAs in Arnebia benthamii extracts

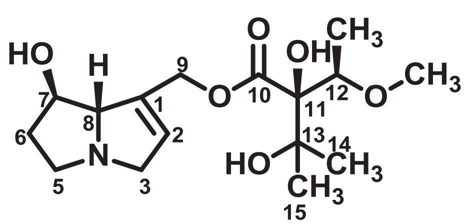
HPLC with UV detection is the most common method of analysis of pyrrolizidine alkaloids (Schaneberg et al., 2004). In the HPLC chromatogram, we used acetonitrile and water with 1% formic acid as a



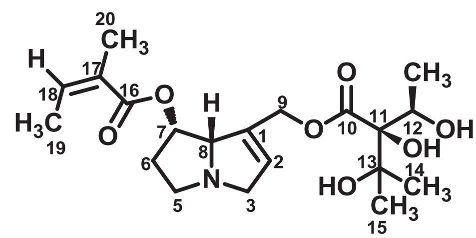
1



2



3



4

Fig. 4. Chemical Structure of 4 pyrrolizidine alkaloids.

Table 2
HPLC results of the pyrrolizidine alkaloids standards.

#	Compound name	Retention time (min)	Formula	Molecular weight
1	Europine	10.21	$C_{16}H_{27}NO_6$	329.1838
2	Heliotrine	10.77	$C_{16}H_{27}NO_5$	314.1962
3	Lycopsamine	37.16	$C_{15}H_{25}NO_5$	299.1733
4	Echimidine	46.64	$C_{20}H_{31}NO_7$	398.2173

mobile phase. The addition of 1% formic acid eliminates the tailing peak and shortens retention time (Zhang et al., 2007). The detector wavelength was set at 280 nm for maximum sensitivity. In this phytochemical investigation of *Arnebia benthamii* from Neelum Valley, the PAs present were determined and characterized by HPLC. Four PAs served as authentic reference samples, namely: Europine (1), Heliotrine (2), Lycopsamine (3), and Echimidine (4) and their chemical structures shown in the Fig. 4. These were used to identify the corresponding PAs in aerial parts of the plant. Using the HPLC

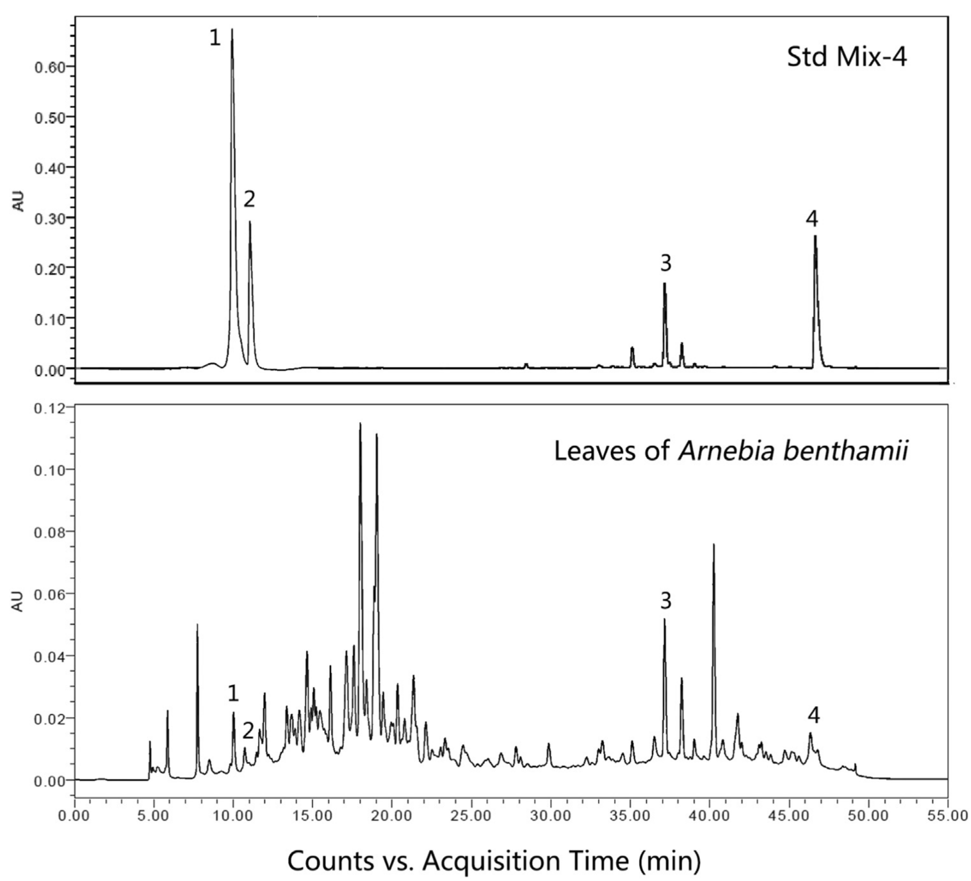


Fig. 5. Base peak chromatograms of standard mix and leave of *Arnebia benthamii*.

conditions stated above, the three standards of europine, heliotrine, lycopsamine, and echimidine were separated with retention times of 10.21, 10.77, 37.16, and 46.64 min respectively (Table 2). After identification of the retention time of standards, 20 µL of plant sample was injected and the retention times were compared to those of reference standards. Obtained data revealed that besides the four known peaks there were several unknown PAs or other compounds with different retention times shown in the HPLC chromatogram of *Arnebia benthamii* (Fig. 5).

This study shows, for the first time reported in the literature, the pyrrolizidine alkaloid profile of *Arnebia benthamii*. One of the difficulties in analysis of PAs is the unavailability of reference standards on the market (Wuilloud et al., 2004), and so the four available standards europine, heliotrine, lycopsamine, and echimidine have been used for this study. Pyrrolizidine alkaloids have been reported in some other *Arnebia* species such as *A. decumbens* (Vent.) Coss. & Kralik (El-Dahmy and Ghani, 1995), *A. euchroma* (Royle) I.M.Johnst. (Roeder and Rengel-Mayer, 1993), and *A. hispidissima* (Lehm.) A.DC. (Wassel et al., 1987). In this study we focused on the commonly harvested and consumed member, *Arnebia benthamii*.

In plants, PAs occur mainly as their N-oxides and these cannot be directly converted to the Hydroxy-PAs, but upon oral ingestion they are reduced in the liver microsomes by NADPH or by enzymes in the gut to free bases. Therefore they show equal toxicity to that of the free bases (Chou et al., 2003; Wang et al., 2005). Unfortunately in our study, all respondents reported oral administration in the form of decoction or juice. Heliotrine is classified as a heliotridine-type PA (Chen et al., 2010). These have been shown to induce chromosome damage and mutagenesis (Wiedenfeld and Edgar, 2011; Chen et al., 2010). In addition to this, it is especially carcinogenic to the liver (Chen et al., 2010). Additionally, lycopsamine is also shown to be hepatoxic and damage the liver (Liu et al., 2009).

Previously it was well established that PAs are hazardous for human health. According to Steyn (1933) nearly 100 years ago, liver disease was widespread in South Africa and this was shown to be caused by the consumption of bread contaminated with seeds from *Senecio* spp. which is high in PAs. In Russia, *Heliotropium lasiocarpum* Fisch. and C.A.Mey. contaminated foods like bread caused various liver diseases (Milenkov and Kizhaikin, 1952). In Afghanistan in 1976, due to contamination of cereal crops and food stuffs by *Heliotropium popovii* subsp. *Gillianum* Riedl 8000 people were affected and 3000 of them were severely poisoned with many fatalities (Mohabbat et al., 1976). There was a similar situation in Tajikistan in 1992, approximately 4000 people were hospitalized because of contamination of grain by *Heliotropium lasiocarpum* Fisch. and C.A.Mey. (Chauvin et al., 1994).

Based on documented historical cases and the known chemistry of PAs, we recommend that herbal products which contain high amounts of toxic PAs should be excluded from the market to safeguard the health of the numerous people who use these products. Moreover, the use of known PA containing plants in herbal preparation should be restricted and there is also a need for awareness among farmers, harvesters, transporters, and producers that the contamination of harvested herbs with PA weeds could result in higher PA levels (Letsyo et al., 2017).

4. Conclusion

This study has revealed two new findings of significance to herbal medicine producers, practitioners, and consumers of *Arnebia benthamii*. First, determination of toxic PAs lycopsamine, echimidine, and heliotrine in the *Arnebia benthamii* from Neelum Valley of Azad Kashmir has shown that besides their highly traditional therapeutic significances, the species is also a source of hepatoxic PAs. It is necessary that their potential health risks be recognized. Secondly, survey results revealed that nearly all-local people either use or collect this species for their traditional therapeutic value. Given this, we recommend that the use of *Arnebia benthamii* should be excluded from the local markets, and their herbal formulation should not be sold before PA levels are tested. We also suggest to the local forestry department, non-governmental organizations, and governmental organizations to motivate, encourage, and create awareness among the local people, harvesters, herbalists, farmers, herders, pastoralists, and transporters who use this herbal medicine should be monitored according to the European Medicines Agency implemented limits (i.e. 1 µg/day).

Acknowledgements

The authors thankfully acknowledge the plant taxonomy laboratory at the College of Life Sciences, Beijing Normal University for support of this research project and also, UC MEXUS for support of one author. The authors also thank the local people of the study area for providing expert information about wild food plant usage.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2017.08.009.

References

Ahmad, I., Aqil, F., Owais, M., 2006. Modern Phytomedicine: Turning Medicinal Plants into Drugs. John Wiley and Sons Ltd, New York.
Ahmad, K.S., Qureshi, R., Hameed, M., Ahmad, F., Nawaz, T., 2012. Conservation assessment and medicinal importance of some plants resources from Sharda, Neelum Valley, Azad Jammu and Kashmir, Pakistan. Int. J. Agric. Biol. 14, 997–1000.
Ahmad, L., Semotiuk, A., Zafar, M., Ahmad, M., Sultana, S., Liu, Q.R., Zada, M.P., Abidin, S.Z., Yaseen, G., 2015. Ethnopharmacological documentation of medicinal plants used for hypertension among the local communities of DIR Lower, Pakistan. J. Ethnopharmacol. 175, 138–146.
Avula, B., Wang, Y.H., Wang, M., Smillie, T.J., Khan, I.A., 2012. Simultaneous determination of sesquiterpenes and pyrrolizidine alkaloids from the rhizomes of Petasites hybridus (L.) GM et Sch. and dietary supplements using UPLC-UV and HPLC-TOF-MS methods. J. Pharm. Biomed. Anal. 70, 53–63.
Avula, B., Sagi, S., Wang, Y.H., Zweigenbaum, J., Wang, M., Khan, I.A., 2015. Characterization and screening of pyrrolizidine alkaloids and N-oxides from botanicals and dietary supplements using UHPLC-high resolution mass spectrometry. Food Chem. 178, 136–148.
BfR Bundesamt für Risikobewertung/Federal Institute of Risk Assessment, 2011. Chemical analysis and toxicity of pyrrolizidine alkaloids and assessment of the health risks posed by their occurrence in honey. BfR Opinion No. 038/2011. Berlin, Germany. ⟨http://www.bfr.bund.de/cm/349/chemical-analysis-and-toxicity-ofpyrrolizidine-alkaloids-and-assessment-of-the-health-risks-posed-by-theiroccurence-in-honey.pdf⟩. The full version of this BfR Opinion is available in German on ⟨http://www.bfr.bund.de/cm/343/analytik-und-toxizitaet-vonpyrrolizidinalkaloiden.pdf⟩ (Accessed 6 March 2017).
Bodeker, G., Burford, G., 2007. Traditional, Complementary and Alternative Medicine Policy and Public Health Perspectives. Imperial College Press, London, UK.
Chauvin, P., Dillon, J.C., Moren, A., 1994. E ´ pide´mie d' intoxication alimentaire a´ l' he´liotrope, Tadjikistan, Novembre 1992-Mars 1993. Cah. Sante´ 4, 263–268.
Chen, T., Mei, N., Fu, P.P., 2010. Genotoxicity of pyrrolizidine alkaloids. J. Appl. Toxicol. 30 (3), 183–196.
Chen, Z., Huo, J.R., 2010. Hepatic veno-occlusive disease associated with toxicity of pyrrolizidine alkaloids in herbal preparations. Neth. J. Med. 68, 252–260.
Chou, M.W., Wang, Y.P., Yan, J., Yang, Y.C., Berger, R.D., Williams, L.D., Doerge, D.R., Fu, P.P., 2003. Riddelliine N-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. Toxicol. Lett. 145, 239–247.
Coppi, A., Cecchi, L., Nocentini, D., Selvi, F., 2015. Arnebia purpurea: a new member of formerly monotypic genus Huynhia (Boraginaceae-Lithospermeae). Phytotaxa 204 (2), 123–136.
Cotton, C.M., 1996. Ethnobotany: Principles and Applications. JohnWiley and Sons Ltd., West Sussex, UK.
Coulombe, R.A., Jr, 2003. Pyrrolizidine alkaloids in foods. Adv. Food Nutr. Res. 45, 61–99.
Cussy-Poma, V., Fernández, E., Rondevaldova, J., Foffová, H., Russo, D., 2017. Ethnobotanical inventory of medicinal plants used in the Qampaya District, Bolivia. Bol. Latinoam. y del Caribe de Plantas Med. y Aromáticas 16 (1), 68–77.
da Costa, I.B., Bonfim, F.P., Pasa, M.C., Montero, D.A., 2017. Ethnobotanical survey of medicinal flora in the rural community Rio dos Couros, state of Mato Grosso, Brazil. Bol. Latinoam. Caribe Plant Med. Aromat. 16 (1), 53–67.
Dai, N., Yu, Y.C., Ren, T.H., Wu, J.G., Jiang, Y., Shen, L.G., et al., 2007. Gynura root induces hepatic veno-occlusive disease: a case report and review of the literature.

World J. Gastroenterol. 13, 1628–1631.
Dar, M.E.U., 2003. Ethnobotanical use of Lawat district Muzaffarabad, Azad Jamu and Kashmir. Asian J. Plant. Sci. 2, 680–682.
Debbie, S., Graeme, L., Pierre, D., Elizabeth, W., Kelvin, C., 2012. Pharmacovigilance of herbal medicine. J. Ethnopharmacol. 140 (3), 513–518.
Dharmanada, S. , 2013. Safety issue affecting herbs: Pyrrolizidine alkaloids. Monograph on the internet. Available at ⟨http://www.itmonline.org/arts/pas.htm⟩ (Accessed 5 May 2014).
Edgar, J.A., Colegate, S.M., Boppré, M., Molyneux, R.J., 2011. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food Addit. Contam. 28, 308–324.
El-Dahmy, S., Adel Ghani, A., 1995. Alkaloids of *Arnebia decumbens* Vent. Az. J. Pharm. Sci. 15, 24–34.
El-Shazly, A., Wink, M., 2014. Diversity of pyrrolizidine alkaloids in the boraginaceae structures, distribution, and biological properties. Diversity 6 (2), 188–282.
EMA, 2016. European Medicines Agency. Committee on Herbal Medicinal Products (HMPC) Public statement on contamination of herbal medicinal products/traditional herbal medicinal products with pyrrolizidine alkaloids. Transit. Recomm. Risk Manag. Qual. Control. ⟨http://www.ema.europa.eu/docs/en_GB/document_library/Agenda/2014/11/WC500177850.pdf⟩ (Accessed 19 April 2017).
Flora of Pakistan, 1989. ⟨http://www.tropicos.org/Name/40001957?Projectid=32⟩.
Fu, P.P., Xia, Q., Lin, G., Chou, M.W., 2004. Pyrrolizidine alkaloids–genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab. Rev. 36, 1–55.
Gandolfo, E.S., Hanazaki, N., 2011. Etnobotânica e Urbanizaçã o- Conhecimento e utilização de plantas de restinga pela comunidade nativa do distrito do Campeche (Florianópolis, SC). Acta Bot. Bras. 25, 168–177.
German Federal Health Bureau, Bundesanzeiger,1992. June 17, 4805, Dtsch. Apoth. Ztg. 132, pp. 1406–1408.
Griffin, C.T., Danaher, M., Elliott, C.T., Kennedy, D.G., Furey, A., 2013. Detection of pyrrolizidine alkaloids in commercial honey using liquid chromatography–ion trap mass spectrometry. Food Chem. 136 (3), 1577–1583.
International Programme on Chemical Safety, 1989. Pyrrolizidine Alkaloids Health and Safety Guide No. 26. World Health Organisation, Geneva.
International Society of Ethnobiology, 2006. International Society of Ethnobiology Code of Ethics (with 2008 additions). ⟨http://ethnobiology.net/code-of-ethics/⟩.
IRIN Asia, 2008a. Afghanistan: WHO Confirms 'Charmak' Disease in Herat Province Kabul, 15 May 2008. Available from: ⟨http://www.irinnews.org/report.aspx?Reportid=78218⟩.
IRIN Asia, 2008b. Afghanistan: 'Charmak' disease still killing people, livestock in West Herat, 16 December 2008. Available from: ⟨http://www.irinnews.org/report.aspx?Reportid=81971⟩.
Ishtiaq, M., Mumtaz, A.S., Hussain, T., Ghani, A., 2012. Medicinal plant diversity in the flora of Leepa Valley, Muzaffarabad (AJK), Pakistan. Afr. J. Biotechnol. 11 (13), 3087–3098.
Khan, M.A., 2008. Biodiversity and Ethnobotany of Himalayan Region Poonch Valley Azad Kashmir Pakistan (Ph. D. Thesis). Department of Botany, Quaid-i-Azam University, Islamabad, 1–292.
Letsyo, E., Jerz, G., Winterhalter, P., Beuerle, T., 2017. Toxic pyrrolizidine alkaloids in herbal medicines commonly used in Ghana. J. Ethnopharmacol. 202, 154–161.
Liu, F., Wan, S.Y., Jiang, Z., Li, S.F.Y., Ong, E.S., Osorio, J.C.C., 2009. Determination of pyrrolizidine alkaloids in comfrey by liquid chromatography–electrospray ionization mass spectrometry. Talanta 80 (2), 916–923.
Lone, P.A., Bhardwaj, A.K., Bahar, F.A., 2013. A study of some locally available herbal medicines for the treatment of various ailments in Bandipora district of J & K, India. Int. J. Trad. Nat. Med. 2 (3), 164–178.
Mahmood, A., Malik, R.N., Zabta, K.S., Aqeel, M., 2011. Ethnobotanical survey of plants from Neelum, Azad Jammu and Kashmir, Pakistan. Pak. J. Bot. 43, 105–110.
Mahmood, A., Mahmood, A., Malik, R.N., 2012. Indigenous knowledge of medicinal plants from Leepa valley, Azad Jammu and Kashmir, Pakistan. J. Ethnopharmacol. 143 (1), 338–346.
Martin, G.J., 1995. Ethnobotany: a Methods Manual. Chapman and Hall, London.
Mattocks, A.R., 1986. Chemistry and Toxicity of Pyrrolizidine Alkaloid. Academic Press, New York.
Milenkov, S.M., Kizhaikin, Y., 1952. Toxic hepatitis with ascites. In: Proceedings of Symposium on V. M. Molotov Medical Institute, Tashkent.
Mohabbat, O., Srivastava, R.N., Younos, M.S., Sediq, G.G., Menzad, A.A., Aram, G.N., 1976. An outbreak of hepatic venoocclusive disease in north-western Afghanistan. Lancet 308, 269–271.
Molyneux, R.J., Gardner, D.L., Colegate, S.M., Edgar, J.A., 2011. Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? Food Addit. Contam. 28, 293–307.
Muthu, C., Ayyanar, M., Raja, N., Ignacimuthu, S., 2006. Medicinal plants used by traditional healers in Kancheepuram district of Tamil Nadu, India. J. Ethnobiol. Ethnomed. 2, 43. http://dx.doi.org/10.1186/1746-4269-2-43.
Nadembega, P., Boussim, J.I., Nikiema, J.B., Poli, F., Antognoni, F., 2011. Medicinal plants inbaskoure, kourittenga province, Burkina Faso: an ethnobotanical study. J. Ethnopharmacol. 133, 378–395.
Prakash, A.S., Pereira, T.N., Reilly, P.E., Seawright, A.A., 1999. Pyrrolizidine alkaloids in human diet. Mutat. Res. 443, 53–67.
Qamar, Z.Q., Anwar, M., Dar, N.I., Ali, U., 2010. Ethno-botanical study of wild medicinal plants of Neelum Valley, Azad Jammu and Kashmir, Pakistan. Pak. J. Wildl. 1 (1), 25–30.
Rates, S.M.K., 2001. Plants as a source of drugs. Toxicon 39, 603–613.
Rodrigues, J.S.C., 2007. Estudo etnobotânico das plantas aromáticas e medicinais. In: Figueiredo, A.C., Barroso, J.G., Pedro, L.G. (Eds.), Potencialidades e aplicações das plantas aromáticas e medicinais. Curso Teórico-Prático. Faculdade de Ciências da Universidade de Lisboa -Centro de Biotecnologia Vegetal, Lisboa, Portugal.
Roeder, E., Rengel-Mayer, B., 1993. Pyrrolizidine alkaloids from *Arnebia euchroma*. Planta Med. 59, 192.
Sadat-Hosseini, M., Farajpour, M., Boroomand, N., Solaimani-Sardou, F., 2017. Ethnopharmacological studies of indigenous medicinal plants in the south of Kerman, Iran. J. Ethnopharmacol. 199, 194–204.
Schaneberg, B.T., Molyneux, R.J., Khan, I.A., 2004. Evaporative light scattering detection of pyrrolizidine alkaloids. Phytochem. Anal. 15 (1), 36–39.
Shaw, D., 2010. Toxicological risks of Chinese herbs. Planta Med. 76, 2012–2018.
Steyn, D.G., 1933. Poisoning of human beings by weeds contained in cereals (bread poisoning). Onderstepoort J. Vet. Sci. Anim. Ind. 1, 219–266.
Vandebroek, I., Thomas, E., Sanca, S., Van Damme, P., Van Puyvelde, L., De Kimpe, N., 2008. Comparison of health conditions treated with traditional and biomedical health care in a Quechua community in rural Bolivia. J. Ethnobiol. Ethnomed. 4 (1), 1.
Wang, X., Yan, J., Fu, P.P., Chou, M.W., 2005. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. Int. J. Environ. Res. Public. Health 2, 74–79.
Wassel, G., El-Menshawi, B., Saeed, A., Mahran, G., 1987. Toxic pyrrolizidine alkaloids of certain Boraginaceae. Acta Pharm. Suec. 24, 199–204.
Wiedenfeld, H., Edgar, J., 2011. Toxicity of pyrrolizidine alkaloids to humans and ruminants. Phytochem. Rev. 10 (1), 137–151.
Wuilloud, J.C., Gratz, S.R., Gamble, B.M., Wolnik, K.A., 2004. Simultaneous analysis of hepatotoxic pyrrolizidine alkaloids and N-oxides in comfrey root by LC-ion trap mass spectrometry. Analyst 129 (2), 150–156.
Zhang, F., Wang, C.H., Xiong, A.Z., Wang, W., Yang, L., Branford-White, C.J., Bligh, S.A., 2007. Quantitative analysis of total retronecine esters-type pyrrolizidine alkaloids in plant by high performance liquid chromatography. Anal. Chim. Acta 605 (1), 94–101.
Zhu, L., Ruan, J.Q., Li, N., Fu, P.P., Ye, Y., Lin, G., 2016. A novel ultra-performance liquid chromatography hyphenated with quadrupole time of flight mass spectrometry method for rapid estimation of total toxic retronecine-type of pyrrolizidine alkaloids in herbs without requiring corresponding standards. Food Chem. 194, 1320–1328.