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Pro-toxic dehydropyrrolizidine alkaloids in the traditional Andean herbal medicine “asmachilca”

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Abstract

Ethnopharmacological relevance—Asmachilca is a Peruvian medicinal herb preparation ostensibly derived from *Eupatorium gayanum* Wedd. = *Aristeguietia gayana* (Wedd.) R.M. King & H. Rob. (Asteraceae: Eupatorieae). Decoctions of the plant have a reported bronchodilation effect that is purported to be useful in the treatment of respiratory allergies, common cold and bronchial asthma. However, its attractiveness to pyrrolizidine alkaloid-pharmacophagous insects indicated a potential for toxicity for human consumers.

Aim of the study—To determine if commercial asmachilca samples, including fully processed herbal teas, contain potentially toxic 1,2-dehydropyrrolizidine alkaloids.

Materials and methods—Two brands of “Asmachilca” herbal tea bags and four other commercial samples of botanical materials for preparing asmachilca medicine were extracted and analyzed using HPLC-esi(+)MS and MS/MS for the characteristic retention times and mass spectra of known dehydropyrrolizidine alkaloids. Other suspected dehydropyrrolizidine alkaloids were tentatively identified based on MS/MS profiles and high resolution molecular weight determinations. Further structure elucidation of isolated alkaloids was based on 1D and 2D NMR spectroscopy.

Results—Asmachilca attracted many species of moths which are known to pharmacophagously gather dehydropyrrolizidine alkaloids. Analysis of 5 of the asmachilca samples revealed the major presence of the dehydropyrrolizidine alkaloid monoesters rinderine and supinine, and their *N*-oxides. The 6th sample was very similar but did not contain supinine or its *N*-oxide. Small quantities of other dehydropyrrolizidine alkaloid monoesters, including echinatine and intermedine, were also detected. In addition, two major metabolites, previously undescribed, were

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isolated and identified as dehydropyrrolizidine alkaloid monoesters with two “head-to-tail” linked viridifloric and/or trachelanthic acids. Estimates of total pyrrolizidine alkaloid and *N*-oxide content in the botanical components of asmachilca varied from 0.4 – 0.9% (w/dw, dry weight) based on equivalents of lycopsamine. The mean pyrrolizidine alkaloid content of a hot water infusion of a commercial asmachilca herbal tea bag was 2.2 ± 0.5 mg lycopsamine equivalents. Morphological and chemical evidence showed that asmachilca is prepared from different plant species.

Conclusions—All asmachilca samples and the herbal tea infusions contained toxicologically-relevant concentrations of pro-toxic 1,2-dehydropyrrolizidine alkaloid esters and, therefore, present a risk to the health of humans. This raises questions concerning the ongoing unrestricted availability of such products on the Peruvian and international market. In addition to medical surveys of consumers of asmachilca, in the context of chronic disease potentially associated with ingestion of the dehydropyrrolizidine alkaloids, the botanical origins of asmachilca preparations require detailed elucidation.

Abstract

Keywords

Asmachilca; Herbal tea; *Aristeguietia gayana*; *Eupatorium*; 1,2-Dehydropyrrolizidine alkaloids; Pyrrolizidine alkaloids; Hepatotoxicity; Rinderine; Supinine; Intermedine; Asmachilcadine; Asmachilcadinine

1. Introduction

“Asmachilca” is a traditional botanical medicine, ostensibly derived from *Eupatorium gayanum* Wedd. = *Aristeguietia gayana* (Wedd.) R.M. King & H. Rob. (Asteraceae: Eupatorieae), a bush up to 1 m in height found only in the Peruvian Andes at altitudes between 3,000 and 4,000 m. Originally used by Aymara people in the high Andes of Peru, it is used as an expectorant and for antitussive and antiasthma treatment (Madaleno, 2007; IICT, 2015). One mode of preparation involves boiling a handful of leaves and stalks in water for 10 min to provide an aqueous extract (IICT, 2015). Other documented modes of use are as a poultice (200 g of plant mixed with Balsamo de Buddha) and orally (5 g plant mixed with other herbal ingredients in 1 L water taken as 4 portions per day) (Bussmann and Glenn, 2010). There is a ready, internet-based availability of asmachilca raw materials as well as herbal tea blends that contain other plant species such as borage and eucalyptus. The health benefits of asmachilca have not been extensively investigated but a study of its phenolic and flavonoid components supported the hypothesized muscle-relaxing function in the control of respiratory ailments (Chico and Reyes, 2000; Bonilla et al., 2006).

Pyrrolizidine alkaloids (PAs) are comprised of two fused 5-membered rings with a nitrogen atom at one of the bridgeheads. The PAs include a number of sub-classes, including the 1,2-dehydropyrrolizidine alkaloid esters (dehydropAs) and their *N*-oxides. The dehydropAs, also referred more generally as “PAs” in many scientific as well as popular publications, are structurally diverse plant secondary metabolites biosynthesized by many taxa belonging to the Asteraceae, Boraginaceae, Fabaceae and Apocynaceae (Bull et al., 1968; Röder, 1995; Wiedenfeld et al., 2008). The dehydropAs are proven pro-toxins: They are metabolized mainly in the liver to form potent mono- and bifunctional alkylating agents that can form adducts with biomolecules including DNA and proteins. The toxicological effects include hepatotoxicity, pneumotoxicity, genotoxicity and carcinogenicity, thereby presenting health and welfare risks to livestock and humans (EFSA, 2011; Edgar et al., 2011; Molyneux et al., 2011; EMA, 2014). In addition, despite *N*-oxidation of the dehydropAs being an *in vivo* detoxifying metabolic process, the plant-derived dehydropA-*N*-oxides are reduced in the gastro-intestinal tract to their parent dehydropAs with subsequent absorption and toxin-forming metabolic activation (Mattocks, 1986).

Röder (1995, 2000), Röder and Wiedenfeld (2009, 2011, 2013) and Bolzan et al., (2007) have catalogued the occurrence of dehydropAs in a large number of plants, including species of *Eupatorium*, used as traditional medicines in a variety of traditional medicine systems. Accidental dehydropA exposures from contaminated food sources such as honey, grain and conventional tea tend to be sporadic and at low levels (Kakar et al., 2010; Bodi et al., 2014; Mathon et al., 2014). However, if traditional botanical medicines contain dehydropA-producing plants, the exposures to the dehydropAs are likely to be much higher and so the risk of adverse health effects is likely to be greater. Therefore, identifying and cataloguing the presence of dehydropAs in traditional medicines, in the form that they are recommended for use, is an important contribution to public health.

An ecological counterpoint to this toxicity are the diverse and peculiar relationships that many specialized insects exhibit towards withered or dry dehydropA-producing plants (Boppré, 2011). In particular, specialist insects that seek out dehydropA-producing plants in order to sequester dehydropAs and utilize them to increase their biological fitness, independent of nutritional requirements, are referred to as PA-pharmacophagous insects (Boppré, 1984). Asmachilca and dehydropAs were first associated in an ongoing entomological project in the Área de Conservación Privada “Panguana”, nr Yuyapitchis, Huánuco, Peru (9°73'S, 74°56'W). Among other aspects, this project uses pure dehydropAs and dehydropA-producing plants as baits to obtain PA-pharmacophagous butterflies and moths (Lepidoptera). In September 2014, recently cut asmachilca plant material purchased at a market in Lima, Peru, when placed in gauze bags, attracted 32 moths belonging to 15 known PA-pharmacophagous species (Fig. 1) over a 4-hour period (1800 – 2200 h). Subsequently many more specimens and species have been observed visiting asmachilca, strongly indicating the presence of dehydropAs. Similar behavioural observations of PA-pharmacophagous butterflies attracted to the neotropical, invasive aquatic plant *Gymnocoronis spilanthoides* (Asteraceae) (Senegal tea) also suggested the presence of dehydropAs which was subsequently confirmed by extraction and HPLC-esi(+)MS and MS/MS analyses (Boppré and Colegate, 2015).

Consequent to the entomological observations, it became an imperative to confirm whether the attraction to *asmachilca* plants was actually due to the presence of dehydroPAs. If so, then there is a potential for the dehydroPAs to contribute to the aetiology of chronically-developing disease in humans (Edgar et al., 2015), especially for young children, for whom *asmachilca* tisane is sometimes recommended in online advertisements, and for foetuses if pregnant women drink the tea. Therefore, commercially-obtained *asmachilca* samples were analyzed for the presence of potentially pro-toxic dehydroPAs.

2. Materials and methods

2.1. Plant material

Between September 2014 and February 2015, six *asmachilca* samples were purchased either at a market place in Lima, Peru or from internet-based vendors (Table 1). Leaves and seed heads from **II**, **III** and **V** were examined for morphological characteristics using a KEYENCE VHX-700FD digital microscope equipped with a VH-Z20R/VH-Z20W zoom lens 20–200x and a polarisation filter OP-87429.

2.2. Chemicals and reagents

Methanol for extractions was reagent ACS/USP/NF grade (Pharmaco Products, Brookfield, CT, USA). For HPLC, acetonitrile was HPLC-certified solvent (Honeywell Burdick and Jackson, Muskegon, MI, USA) and pure water (18.2 MΩ/cm) was prepared using a WaterPro PS Station (Labconco, Kansas City, MO, USA). For HPLC-esiMS analysis, the formic acid additive was “For Analysis” grade (>99%; Acros Organics/Thermo Fisher Scientific, NJ, USA) and the ammonium acetate additive was AR (ACS) grade (Mallinckrodt, Phillipsburg, NJ, USA). Ammonium hydroxide was certified ACS Plus (Fisher Scientific, Fair Lawn, NJ, USA). The free bases and *N*-oxides (Fig. 2) of lycopsamine (**1**), intermedine (**2**) echinatine (**3**) and supinine (**4**), and the free bases heliotrine (**5**), rinderine (**6**), amabiline (**7**) and lasiocarpine (**8**) were sourced from the stocks of extracted and purified or semi-purified pyrrolizidine alkaloids kept by the USDA/ARS Poisonous Plant Research Laboratory. The redox resin was prepared by stirring an aqueous solution of indigocarmine (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA) with a strong anion exchange resin (Amberlite IRA-410, chloride form) (Sigma-Aldrich, St. Louis, MO, USA) (Colegate et al., 2005). An HPLC-esi(+)MS analytical sample of rinderine-*N*-oxide (**6NO**) was prepared by adding hydrogen peroxide solution (30% aqueous solution, Mallinckrodt, KY, USA) to an analytical sample of rinderine in a 50:50 solution of methanol and 0.1% formic acid and warming at 37 °C for 16 hrs. Lithium aluminium hydride (LiAlH₄) was supplied as a 2 M solution in tetrahydrofuran (Sigma-Aldrich: Milwaukee, WI, USA). The trimethylsilylation reagent was *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich: Milwaukee, WI, USA).

2.3. Extraction and enrichment of alkaloids

For qualitative and quantitative purposes, **I** (4.8 g), **II** (3.1 g), **III** (2.5 g) and **IV** (3.1 g) were each inversion-mixed with methanol (15 mL) at room temperature (ca 22 °C) for 16 h. The extraction mixtures were centrifuged briefly and the supernatants decanted. This extraction procedure was repeated 5 times. The progress of each extraction was monitored using

HPLC-esi(+)MS, comparing the integrated area of extracted alkaloids with that of the lasiocarpine (**8**) internal standard. The 6 extracts were pooled for each sample, and concentrated *in vacuo* to about 10 mL. Samples **I**, **III** and **IV** were each accurately diluted to 25 mL with methanol while **II** was accurately diluted to 50 mL with methanol.

Sample **V** (ca. 5 g) was homogenised in methanol (100 mL) using a commercial blender and left to steep at room temperature (ca. 22 °C) for 24 h. Filtration afforded the crude methanol extract for subsequent qualitative HPLC-esi(+)MS and MS/MS analysis.

When required for analysis of dilute samples or for isolation purposes, concentration of the alkaloids from the crude methanol extracts was achieved using strong cation exchange (SCX), solid phase extraction (SPE) columns of an appropriate size (Strata, 55 µm 70 Å, Phenomenex, Torrence, CA, USA) as previously described (Colegate et al., 2005; Boppré and Colegate, 2015). Briefly, acidified samples, either in aqueous 0.05 M sulphuric acid or solutions of acetonitrile or methanol acidified with 0.1% formic acid, were applied to columns pre-conditioned by washing with methanol followed by 0.05 M sulphuric acid or 0.1% formic acid in water. A thus-loaded column was washed with methanol before the captured alkaloids were eluted with a 10% solution of saturated ammoniated methanol in methanol that was immediately evaporated to dryness at 35 °C in a flow of nitrogen. The applied sample and all column effluents (aqueous, methanolic and ammoniated methanolic) were monitored using HPLC-esi(+)MS.

2.4. HPLC and mass spectrometry

Two modes of HPLC separation i.e., C18 reversed phase (RP) under acidic conditions and porous graphitic carbon under basic conditions, followed by esi(+)MS and MS/MS analyses were conducted as previously reported (Boppré and Colegate, 2015). Basically, aliquots (2 µL) of analytical samples were injected (Agilent 1260 Infinity HPLC System, Agilent Technologies, CA, USA) onto a Synergi Hydro RP column (150 × 2 mm, 4µ) (Phenomenex, Torrence, CA, USA) fitted with a guard column of similar adsorbent (AC C18, 4 mm diameter × 2 mm) (Security Guard Cartridge system, Phenomenex, Torrence, CA, USA), or a Hypercarb porous graphitic carbon column (100 × 2.1 mm, 5 µ) (Thermo Scientific, USA) fitted with a Hypercarb guard filter (10 × 2.1 mm) and holder (Thermo Scientific, USA). Adsorbed analytes were eluted from the C18 column using a gradient flow (400 µL/min) of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Mobile phase B was held at 3% for 2 min before linearly increasing to 70% by 10 min. After holding at 70% for another 5 min, data acquisition was stopped and the column was re-equilibrated to 3% mobile phase B over 2 min and held for a further 7 min before the next injection. Adsorbed analytes were eluted from the porous graphitic carbon column using a gradient flow (400 µL/min) of 20 mM NH₄OAc and 0.02% NH₄OH in water (mobile phase A) and acetonitrile (mobile phase B). Mobile phase B was held at 3% for 2 min before linearly increasing to 70% by 15 min. After holding at 70% for another 10 min, data acquisition was stopped and the column was re-equilibrated to 3% mobile phase B over 5 min and held for a further 10 min before the next injection. The eluate from both HPLC columns was monitored using a Velos Pro LTQ mass spectrometer (Thermo Scientific, USA) in a 2 scan

(full MS scan followed by a data dependent MS/MS scan), positive ion mode and equipped with a heated electrospray ionization (HESI) source.

The high resolution mass measurements on HPLC peaks were acquired following their gradient elution from the C18 Hydro column with acidic mobile phase conditions. Thus, an aliquot (2 μ L) of a sample was injected onto the column using an Ultimate 3000 HPLC (Thermo Scientific). The column effluent was monitored using an Exactive Plus Orbitrap high resolution mass spectrometer (Thermo Scientific) calibrated as per the manufacturer's instructions and with a scan range 80 – 1000 Da; resolution 70000; microscans 1; sheath gas flow 35; auxiliary gas flow 10; spray voltage 4 kV; capillary temperature 320 $^{\circ}$ C; S lens RF field 55; auxiliary gas temperature 300 $^{\circ}$ C; and maximum inject time 200 ms.

2.5. Qualitative and quantitative analysis of pyrrolizidine alkaloids

Using both HPLC modes, the MS and MS/MS data for individual chromatographic peaks were examined for the protonated molecules (MH^{+}) and fragment ions characteristic of the various dehydroPAs or their *N*-oxides (Bull *et al.*, 1968; Colegate *et al.*, 2005; El-Shazly and Wink, 2014). The retention times and MS data of suspected dehydroPAs were compared with standards where available. Otherwise, tentative structures were initially based upon characteristic fragmentation patterns and high resolution mass measurements of protonated molecules and collision-induced dissociation fragments.

Using the C18 RP HPLC mode, quantitative estimates of dehydroPA content were determined using low-range (0.45 – 7 μ g/mL) and high-range (7 – 112.5 μ g/mL) calibration curves generated with lycopsamine (**1**), purified from *Symphytum officinale* (Colegate *et al.*, 2014), and normalized against the internal standard lasiocarpine (**8**). Therefore, quantitative estimates are reported as lycopsamine equivalents.

2.6. Isolation of selected alkaloids

For isolation purposes, an SCX SPE-derived alkaloid concentrate was dissolved in methanol (ca 50 mg/mL) and manually injected (500 μ L) onto a Synergi Hydro-RP semi-prep column (250 $\times$ 10 mm, 4 μ) (Phenomenex, Torrance, CA, USA) fitted with a guard column of similar adsorbent (AC C18, 10 mm $\times$ 10 mm) (Security Guard Cartridge system, Phenomenex, Torrance, CA, USA). The alkaloids were eluted from the column using an increasing linear gradient of acetonitrile into 0.1% formic acid in water at a flow rate of 4 mL/min generated using an Agilent 1260 Infinity semi-prep HPLC system comprising a quaternary pump, multi wavelength UV/vis detector and a fraction collector (Agilent Technologies, CA, USA). After holding at 3% for 2 min the acetonitrile component was increased to 70% at 15 min. The column was re-equilibrated after another 2 min back to 3% acetonitrile in 0.1% formic acid in water. Fractions (1 mL) were collected based on time from 7 min until 13 min. The collected fractions and the pre-collection (0 – 7 min) and post-collection (13 – 25 min) eluates were monitored using HPLC-esi(+)MS. Collected fractions from repeated injections were pooled accordingly.

2.7. NMR analysis

One dimensional (1D) ^1H (300 MHz) and ^{13}C (75 MHz), 1D GOESY, and gradient-enhanced 2D (^1H - ^1H COSY, and ^{13}C - ^1H HSQC and HMBC) NMR data were acquired using a JEOL Eclipse NMR spectrometer using solutions in deuteriochloroform (Sigma-Aldrich, St Louis, MO, USA) and the residual proton in the CHCl_3 ($\delta = 7.2$) as the chemical shift reference.

2.8. Alkaloid hydrolysis and GCMS analysis

Solutions of heliotrine (**5**) (ca 300 μg), lycopsamine (**1**) (ca 280 μg), a mix of **5** (ca 300 μg) and **1** (ca 280 μg), and the new dehydroPA asmachilcadine (**10**) (ca 300 μg) (Section 3.4) in methanol were all evaporated to dryness under a flow of nitrogen at about 40 $^\circ\text{C}$.

Adapting a method described by Kempf *et al.* (2008) and applied to the necine base determination of the open chain diester dehydroPA cryptanthine (Colegate et al., 2013), LiAlH_4 solution (200 μL) was added to each sample vial and left to stand at room temperature for 2 h. Dichloromethane (1 mL) was added to each vial, causing very slight effervescence with the LiAlH_4 mixtures. Cautiously, 1 drop at a time, an aqueous solution of sodium hydroxide (10% w/v) was added resulting in vigorous effervescence and formation of a glutinous white solid. After addition of about 50 μL , the vigorous effervescence ceased and a further aliquot (50 μL) of 10% sodium hydroxide was added. Anhydrous sodium sulphate was added to each vial. After vigorous shaking, the contents of each vial were filtered into clean, dry vials and the residues washed with a further 2 aliquots (1 mL each) of dichloromethane that were also filtered into their respective vials. An aliquot (10 μL) of each dichloromethane solution was analyzed for any remaining presence of unreacted parent alkaloid using HPLC-esi(+)MS.

The dichloromethane solutions were evaporated to dryness and the residues dissolved in dry pyridine (50 μL) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (50 μL) and warmed at about 40 $^\circ\text{C}$ for 1 h. Using a Trace GC Ultra (Thermo: San Jose, CA, USA), an aliquot (2 μL) of each derivatized solution was injected (injector temperature 250 $^\circ\text{C}$) in a splitless mode onto a 30 m $\times$ 0.25 mm (0.25 μm) DB-5MS column (Agilent Technologies: Englewood, CO, USA) that was heated at 100 $^\circ\text{C}$ for 3 min then 5 $^\circ\text{C}/\text{min}$ to 180 $^\circ\text{C}$ held for 3 min then 22 $^\circ\text{C}/\text{min}$ to 300 $^\circ\text{C}$ held for 5 min with a flow of helium at 1.5 mL/min. The column effluent was transferred (transfer line temperature 275 $^\circ\text{C}$) to a Polaris Q ion trap mass spectrometer (Thermo: San Jose, CA, USA) and analysed in both the full scan and single ion monitoring (m/z 299, 183, 93) modes.

2.9. Pyrrolizidine alkaloid content of asmachilca-derived tisane

As a trial extraction, a single herbal tea bag (ca 1.2 g of herb mixture) from sample **VI**, a commercially-acquired “Asmachilca” herbal tea, containing “Eucalyptus, Asmachilca, Scorzonera, Borage, Cinnamon and Cloves” in undisclosed relative amounts, was immersed in methanol (60 mL) at about 35 $^\circ\text{C}$ for an hour. Subsequently, tisanes ($n = 5$) were prepared from individual herbal tea bags by adding an accurate volume of recently boiled hot water (ca 40 mL) and allowing to cool to room temperature with occasional gently mixing. All cooled extracts were monitored qualitatively using HPLC-esi(+)MS with both the Synergi

Hydro C18 RP column and the porous graphitic carbon Hypercarb column using an acidic or basic mobile phase respectively. An average ($n = 5$) semi-quantitative estimate of dehydroPA content (expressed as lycopsamine equivalents) extracted from a single tea bag with hot water was determined using the Synergi Hydro C18 RP column with the acidic mobile phase conditions (Section 2.4).

To establish a time course for extraction of the dehydroPAs, three herbal tea bags were individually immersed in recently boiled hot water (75 mL) and agitated by swirling. Aliquots of the aqueous extracts (100 μ L) were taken at 1, 2, 3, 4, 5, 10 and 60 min as the tisanes cooled. Individual and total dehydroPA contents were determined for each tisane and time point using HPLC-esi(+)MS with the Synergi Hydro C18 RP column and the acidic mobile phase conditions.

3. Results and discussion

3.1. Botanical considerations

Notwithstanding the recognized need for positive identification of plant sources in the production of herbal preparations (Hildreth et al., 2007; Applequist and Miller, 2013; Zöllner and Schwarz, 2013), the aim of this study was to assess *asmachilca per se* for the presence of dehydroPAs as indicated by the PA-pharmacophagous moths attracted to the plant. Therefore, the positive identification and source of all plants that are currently sold for the preparation of *asmachilca* were outside the scope of this current work. As demonstrated by others (BfR, 2013; Bosi et al., 2013; Bodi et al., 2014), the potential for adverse health consequences due to the presence of dehydroPAs in herbal teas, at concentrations that violate regulatory limits for these compounds in materials intended for human consumption, justifies this approach. Several available records (e.g., IICT, 2015) state that *asmachilca* is made of *Eupatorium gayanum* Wedd. = *Aristeguietia gayana* (Wedd.) R.M. King & H. Rob. (Asteraceae: Eupatorieae). However, traded *asmachilca* is not standardized and apparently is even prepared from different plant species. For example, *Aristiguieta [sic] persicifolia* (Chico and Reyes, 2000) and *Eupatorium triplinerve* (López and Vargas, 1992; Weight-Care, 2014) are also mentioned as sources of *asmachilca*. Additionally, there seems to be a mix-up with *ayapana*, another traditional medicine made of *Ayapana triplinervis* (M. Vahl) R.M. King & H. Rob. (*Eupatorium ayapana* Vent., *E. triplinerve*) which is not *Aristeguietia gayana* (Taylor, 2006). Nuñez (2009) and Nuñez et al. (2010) call *Baccharis vacciniifolia* Cuatrec. *asmachilca* while Bussmann et al. (2011) say *Asma Chilca [sic]* is derived from species of *Chromolaena* in addition to *E. gayanum*.

Species of *Eupatorium* are difficult to taxonomically separate from each other since there is neither a proper revision available nor a comprehensive key to identify the various species. As a prelude to a more thorough investigation of the species used for preparing *asmachilca*, since it is unknown where plants for sale are being harvested, 4 *asmachilca* samples (II - V, Table 1) were purchased from vendors at a marketplace in Lima, Peru. One sample of dry, powdered *asmachilca* material (I) and one of packaged *asmachilca* tea bags (VI) were acquired from internet sources. Sample I from Paraguay is somewhat enigmatic since *E. gayanum* is reported to be endemic in Peru (GBIF, 2015). Thus, it remains to be determined

whether the plant was actually harvested in Paraguay, and is, perhaps, a different species, or *E. gayanum*-derived material was exported from Peru to Paraguay.

Based on microscopic examination (Fig. 3), sample **II**, labelled as “Pulmonaria”, definitely contained plant material of an asteraceous plant and therefore was not a *Pulmonaria* sp. (Boraginaceae) *per se*. The latter, referred to as lungworts, are also marketed for the treatment of coughs and bronchitis. Furthermore, microscopically, while the surfaces of dry leaves of samples **II** and **III** appear very similar if not identical (Fig. 3A and B), that of sample **V** is completely different (Fig. 3C), as are the perianths and achenes. Also, the fresher, unpackaged plant sample **V**, in contrast to **II** and **III**, was sticky and had a strong α -ketobutyric acid-like smell. Thus, at least, two species of plants are marketed as *asmachilca*.

3.2. Qualitative HPLC-esi(+)MS and MS/MS analysis

The HPLC-esi(+)MS ion chromatograms of samples **I** – **V**, using the C18 reversed phase Hydro column with acidic mobile phase conditions (Fig. 4), and the MS/MS data for individual peaks (Table 2) revealed a qualitative similarity of all samples. Two distinct suites of PAs were observed. The earlier eluting group comprised monoester PAs and *N*-oxides with protonated molecules between m/z 284 and 342. The later eluting group comprised a suite of larger PAs and *N*-oxides with protonated molecules between m/z 428 and 460.

Seven of the alkaloids (numbers 5, 6, 8, 9, 11, 12 and 16, Fig. 5, Tables 2 and 3) in the analytical sample of the methanol extract of sample **I**, and 5 alkaloids (numbers 5, 6, 8, 11 and 16, Fig. 5, Tables 2 and 3) in **V**, were confirmed to be *N*-oxides after gently mixing with activated redox resin and observing their disappearance using HPLC-esi(+)MS (Colegate *et al.*, 2005).

Sample **I** was comprehensively representative of the PAs detected in the *asmachilca* samples (Fig. 4). Therefore, its suite of PAs was also investigated using HPLC-esi(+) high resolution CIDMS to provide molecular formulae for the parent ions as well as some significant fragment ions (Table 3). All calculated molecular formulae were good matches (< 5 ppm) for PA structures or their major fragment ions.

The major alkaloid, based upon peak areas, showed a protonated molecule and retention time indicative of a lycopsamine diastereoisomer i.e., lycopsamine (**1**) or its C13 epimer intermedine (**2**), or their respective C7 epimers echinatine (**3**) and rinderine (**6**) (Fig. 2) (Colegate *et al.*, 2014). The MS/MS data (Table 2) indicated it was one of the heliotridine-based epimers echinatine or rinderine (Boppré and Colegate, 2015). It has previously been shown that HPLC using a porous graphitic carbon column with basic mobile phase conditions can significantly alter the relative retention times of the dehydroPAs and their *N*-oxides and, in some cases, improve resolution of some alkaloids when compared to HPLC using the C18 RP column with an acidic mobile phase (Boppré and Colegate, 2015). Similar chromatographic changes were observed when the *asmachilca* extracts were re-analyzed using the porous graphitic carbon column with basic mobile phase conditions. More significantly for this study, these changes included the baseline resolution of the lycopsamine diastereoisomers (**1**, **2**, **3** and **6**, Fig. 2) and, thereby, confirmed the major

presence of rinderine (**6**) in all samples. For example, HPLC of sample **I** using both columns and sets of mobile phase conditions shows a reversal of elution order of *N*-oxides and their respective free base dehydroPAs, resolution of the lycopsamine diastereoisomers, and resolution of a minor isomeric form of **10** (Fig. 6). In addition to sample **I** (Fig. 6), sample **IV** also showed a minor concentration of rinderine's C13 epimer echinatine (**3**), and the retronecine-based analogue of rinderine, intermedine (**2**). It is unusual for a plant to biosynthesize both heliotridine and retronecine-based PAs but has been proven for the invasive aquatic plant *Gymnocoronis spilanthoides* in which the major presence of lycopsamine and intermedine is accompanied by a minor presence of their heliotridine-based analogues echinatine and rinderine respectively (Boppré and Colegate, 2015). The co-occurrence of rinderine (**6**) and its retronecine epimer intermedine (**2**) has also been reported in *Chromolaena odorata* which has a similar profile to the *asmachilca* samples analyzed herein (Biller et al., 1994).

The major presence of rinderine (**6**) over echinatine (**3**) and intermedine (**2**) was reflected in the relative concentrations of their respective *N*-oxides that were also well resolved from each other using the porous graphitic carbon column with basic mobile phase conditions. Similar to the subtle differences observed in the MS/MS profiles of the retronecine-based alkaloids **1** and **2** compared to the heliotridine-based **3** and **6**, their *N*-oxides also display subtle differences that can be used to confirm the tentative identification based on retention time (Fig. 7, Table 2).

Reconstructed ion chromatograms displaying *m/z* 316 for the protonated *N*-oxide molecules showed that rinderine-*N*-oxide (**6NO**) was the major *N*-oxide in all samples. Intermedine-*N*-oxide (**2NO**) was also detected in all samples whereas echinatine-*N*-oxide (**3NO**) was only detected in samples **I**, **IV** and **V**. The relative % abundances of **2NO**, **6NO** and **3NO** were 31, 65, 4; and 8, 92, 0; and 7, 93, 0; and 24, 63, 13; and 10, 75, 15 for samples **I** – **V** respectively.

Using the C18 RP column with acidic mobile phase conditions, the other major *N*-oxide observed (alkaloid 9, Fig. 5, Tables 2 and 3), in all samples except for **V**, was identical (retention time and MS/MS) to standard supinine-*N*-oxide (**4NO**). Reduction in the presence of the redox resin caused an increase in the supinine (**4**) peak (alkaloid 7, Fig. 5, Tables 2 and 3) that was readily differentiated from amabiline (**7**) based on HPLC retention times. The differentiating absence of supinine and its *N*-oxide in Sample **V** compared to Samples **II** and **III** (Fig. 4) is consistent with the morphological examination of the leaves that also clearly differentiated **V** from **II** and **III**.

All the peaks in the second group of alkaloids (Fig. 4) had analogous MS/MS fragmentations (Table 2). They all showed an initial loss of 44 Da which is indicative of a terminal hydroxy(methyl)methine group (CH₃-CH-OH) (Colegate et al., 2005). Furthermore, they all showed major fragments resulting from losses of 144 Da and 162 Da from the protonated molecule, together indicative of a viridifloric-like acid (Fig. 2) entity. For alkaloids 10, 13 and 15, the base ion peak corresponded to loss of an intact viridifloric-like acid entity (MH⁺ - 162 Da) whereas for alkaloids 12 and 16, which were both identified as *N*-oxides, it was the loss of a fragmented viridifloric-like acid entity (MH⁺ - 144 Da) that

gave the base ion peak. High resolution mass measurements of the major peaks (alkaloids 10 and 13, Fig. 5) indicated a protonated molecular formula of $C_{22}H_{38}NO_8$ and $C_{22}H_{38}NO_7$ for alkaloid 10 and 13 respectively (Table 3). The loss of an oxygen atom and the observation of a fragment ion at m/z 122 for alkaloid 13, compared to alkaloid 10, are analogous to the comparison between alkaloids 4 and 7 (Tables 2 and 3), identified as rinderine (**6**) and supinine (**4**) respectively, and indicates that alkaloid 13 may be the C7 deoxy analogue of alkaloid 10 thereby implying that alkaloid 10 may be a monoester with a larger esterifying acid rather than an open chain diester of two smaller acids.

Potentially useful from a biosynthesis aspect are the trace levels of saturated PA analogues of the major dehydroPAs. Detected by observing MS/MS profiles with fragment ions 2 Da higher than the corresponding fragments from the dehydroPA, these included dihydro-rinderine (MH^+ m/z 302), and its *N*-oxide (MH^+ m/z 318); and the dihydro-derivative of alkaloid 10 (MH^+ m/z 446).

A minor dehydroPA (MH^+ m/z 342) was observed eluting at about 6.8 min in Samples **IV** and **V**. With a base ion peak at m/z 138, the MS/MS showed a fragment at m/z 282 (60% relative abundance) indicating a C13 acetylated lycopsamine-like derivative (**12**) (Colegate et al., 2005), probably 13-acetylrinderine since rinderine is the major dehydroPA present. Reconstructed ion chromatograms displaying m/z 342 only demonstrated trace levels of this acetylated derivative in the other samples. Along with the absence of supinine (**4**) and its *N*-oxide in **V**, this greatly increased presence of the acetylated derivative in samples **IV** and **V** may be a subtle indicator of source plant differences, either taxonomically or chemotypical.

3.4. Structure elucidation

The two major free base alkaloids from the later-eluting “Group 2” suite of dehydroPAs, i.e., alkaloid 10 (MH^+ m/z 444) and alkaloid 13 (MH^+ m/z 428) (Figs. 4 and 5) were isolated, to a purity of >90%, as yellow/orange gums using semi-preparative HPLC.

The 1H and ^{13}C NMR data (Tables 4 and 5) confirmed the similarity in structure that was apparent from the MS/MS data. Two 2,3-dihydroxy-2-*iso*-propylbutanoic acids units were identified for each alkaloid, similar to viridinatine (**9**) isomers isolated from *Cynoglossum furcatum* (Ravi et al., 2008) and *Onosma erecta* (Damianakos et al., 2013). However, instead of the C7 and C9 hydroxyls of the necine base each being esterified with a single butanoic acid unit, the two butanoic acid units were linked “head-to-tail” and esterified the C9 hydroxyl of the necine base. In support of this, the terminal hydroxy(methyl)methine ($CH_3-CH-OH$) proton (H13, Fig. 2) of the acid unit esterifying the necine base C9 hydroxyl is considerably deshielded by the extended esterification with the second butanoic acid unit relative to the chemical shift of its analogous proton (H17) in the second acid unit. Furthermore, the H13 quartet shows long-range correlations to both carbonyl carbons (C11 and C15) (Table 4). The C9 methylene protons show a long-range correlation to carbonyl C11 thus confirming the site of esterification.

Since it has been previously shown that 2D NOESY interactions between H7 and H8 are an unreliable indicator of the relative configuration of these protons in some dehydroPAs (Colegate et al., 2013), an attempt was made to demonstrate the heliotridine or retronecine

configuration of alkaloid 10 using a 1D GOESY NMR experiment. However, using heliotrine and lycopsamine as model heliotridine and retronecine monoesters respectively, a GOESY interaction was observed for both. Therefore, the heliotridine configuration of the necine base for alkaloid 10 was determined by reductive hydrolysis of the ester and subsequent GCMS and MS/MS analysis of the trimethylsilylated necine base. Therefore alkaloid 10 is described as a heliotridine base derivatized at C9 with two “head-to-tail” linked 2,3-dihydroxy-2-*isopropyl*butanoic acid units and is given the trivial name *asmachilcadine* (**10**).

By contrast, the supinidine character of the necine base for alkaloid 13, tentatively indicated by the MS and MS/MS data, was confirmed by the observation of the upfield shift of C7 relative to alkaloid 10 (δ 73.38 $\rightarrow$ 29.13). Additionally, two C7 protons were observed, coupled to each other and with COSY interactions with H8 and the C6 methylene protons (Table 5). Therefore, alkaloid 13 is the supinidine analogue of **10** and is given the trivial name *asmachilcadinine* (**11**).

While there is ample precedent for “head-to-tail” linked acid units forming monoester dehydroPAs, that include various acid units (eg. acetic, angelic, *isovaleric* and *p*-coumaric acids) terminally linked to a viridifloric or trachelanthic acid esterifying C9 of the necine base, a SciFinder search indicated no previous reports of viridifloric and/or trachelanthic acid units thus linked to form a monoester dehydroPA. Differences in some ^1H and ^{13}C NMR chemical shifts have been associated with the stereochemistry at C13 in dehydroPAs with a single viridifloric or trachelanthic acid entity (Colegate et al., 2014). Assessment of similar resonances for compounds **10** and **11**, each with the “head-to-tail”-coupled diacid unit, provided conflicting deductions and is therefore unreliable. The lower field chemical shift of C13 (δ 74.42 and 74.2 for **10** and **11** respectively) and the methyl protons on C22 (δ 1.32 and 1.31 for **10** and **11** respectively) together imply an S configuration at C13 similar to echinatine (**3**) and lycopsamine (**1**) but contrary to the major alkaloid present, rinderine (**6**). Furthermore, the chemical shift of C17 (δ 69.59 and 69.4 for **10** and **11** respectively) and the methyl protons on C18 (δ 1.18 and 1.16 for **10** and **11** respectively) together imply an R configuration at C18 similar to rinderine (**6**). Together these observations indicate a C9 viridiflorate further linked to a trachelanthic acid. However, by contrast and based again on observations with the lycopsamine isomers **1**, **2**, **3** and **6**, the relatively small differences in chemical shift of the C9 methylene protons, and between the two *isopropyl* methyl groups (H20 and H21) for **10** and **11** imply an R configuration at C13. Similarly, the larger difference in chemical shift for the two *isopropyl* methyl groups (H24 and H25) for **10** and **11** imply an S configuration at C17. Therefore, because of this conflicting ambiguity, the relative stereochemistries at carbons 12, 13, 16 and 17 in the two acid units remain undefined.

Similar to observations when acquiring NMR data for echiuplatine, an open chain diester from *Cryptantha* and *Echium* spp. (Colegate et al., 2013), and for reasons that are not unequivocally defined, some chemical shifts for the necine base protons and carbons, especially H8 that can shift downfield by about 0.2 and 0.8 ppm for **10** and **11** respectively, were variable depending upon the age of the NMR solutions of **10** or **11**. Similar variation in chemical shifts were observed if a CDCl_3 solution of **11** was concentrated by being

evaporated to dryness under nitrogen and reconstituted in a smaller volume of CDCl_3 to improve the signal to noise ratio. The chemical shifts of the necic acid nuclei remained largely unaffected and the observations therefore indicated a facile protonation of the pyrrolizidine nucleus as previously demonstrated (Colegate et al., 2013).

Due to the similarity of MS and MS/MS data it is tentatively proposed that alkaloids 12 and 11, both of which are reduced on treatment with the redox resin, are isomers of asmachilcadine-*N*-oxide (**10NO**), and that alkaloid 16 is the *N*-oxide of asmachilcadinine (**11NO**). This was confirmed by treating analytical samples of **10** and **11** with hydrogen peroxide *in situ* and observing the development of the expected *N*-oxide peaks using HPLC-esi(+)MS. Similarly it can be tentatively proposed that the MS and MS/MS data for alkaloids 14 and 15 are isomers of 1,2-dihydroasmachilcadinine. This latter suggestion is in accord with the observation of trace amounts of other putative dihydro derivatives of the more major alkaloids present in the asmachilca samples.

3.5. Quantitative HPLC-esi(+)MS analysis

Because of the lack of individual standards for the PAs detected in the asmachilca samples, a semi-quantitative assessment of total and individual PA content was achieved using lycopsamine (**1**) as the calibration standard. Two 5-point calibration curves were used to cover the lower and upper concentrations ranges of the detected alkaloids in the samples i.e., $y = 10.2(x^2) + 11.2x$, $R^2 = 0.999$ and $y = 27.7x - 7.4$, $R^2 = 0.998$ for the ranges 0.45 – 7 $\mu\text{g/mL}$ and 7 – 112.5 $\mu\text{g/mL}$ respectively, where “y” is the concentration and “x” the analyte peak area divided by the area of the internal standard (**8**). It can be confidently inferred that the rinderine (**6**), echinatine (**3**) and intermedine (**2**) concentrations reported are acceptably accurate since the response factor for each of these diastereoisomers was very similar to that for lycopsamine (Colegate et al., unpublished). However, the response factors for the *N*-oxides and the other reported PAs can be quite different to each other (Betteridge et al., 2005) and therefore the quantitation of individual alkaloids and total PA content are tentatively expressed as lycopsamine (**1**) equivalents.

Sample **III**, the dried and packaged “Asmachilca”, and **IV**, the “Infusion Asmachilca” herbal tea bags, were each estimated to contain 0.4% (w/dw plant material) total PAs and their *N*-oxides. Sample **I**, the asmachilca powder from Paraguay, analyzed for 0.7% (w/dw plant material) total PAs and their *N*-oxides while **II**, the dried and packaged “Pulmonaria”, was estimated to contain 0.9% (w/dw plant) total PAs and their *N*-oxides.

Notwithstanding the small sample size for a statistically confident conclusion, the relative concentrations of the various alkaloids detected (Fig. 8) show a consistency across **I** – **IV** in that alkaloids 3/4, 9, 5/6, 10 and 13 are the major alkaloids in all samples. There are some apparently anomalous observations such as the relatively higher concentrations of alkaloid 10 (asmachilcadine, **10**) in the commercial tisane (**IV**) and the powder sourced from Paraguay (**I**). The relatively minor differences in dehydroPA and *N*-oxide content that were observed between the samples could be due to an intrinsic variability in chemotypes of the same species; the use of different plant species *per se*; or, in the case of the herbal teas and

powdered sample, the contamination of the major plant species with another dehydroPA-producing, or non dehydroPA-producing plant.

3.6. Pyrrolizidine alkaloid content of tisane prepared from a single herbal tea bag

In addition to sample **IV**, the “Infusion Asmachilca” commercial herbal tea bags, that was quantitatively analyzed for methanol-extracted PAs, a different tisane product (**VI**) was sourced from a USA-based internet supplier to investigate the extractability of the potentially toxic dehydroPAs into hot water.

A pilot extraction of a single herbal tea bag from **VI** with methanol afforded a pale green solution that, when analyzed using HPLC-esi(+)MS and MS/MS, readily showed the presence of the asmachilca alkaloids, including supinine (**4**), and its *N*-oxide, and the acetylated derivative (**12**). The quantity of the main alkaloids extracted from a single tea bag was estimated at 1.9 mg lycopsamine equivalents or about 0.16% of the herbal tea mixture. Also observed, based on HPLC retention time and the MS/MS profile, was a trace of lycopsamine-*N*-oxide (**1NO**) presumably from the Borage (*Borago officinalis*) component of the herbal mixture (Dodson and Stermitz, 1986).

Addition of hot, recently boiled water to the herbal tea bags (**VI**) resulted in orange/brown infusions that were allowed to cool to room temperature before analysis. The HPLC-esi(+)MS ion chromatograms of the 5 separate herbal tea infusions were very similar qualitatively and quantitatively. The relative amounts of major alkaloids, including free base PAs as well as their more water-soluble *N*-oxides, (Fig. 9) were very similar to the profile for sample **IV**, the “Infusion Asmachilca” herbal tea product (Fig. 8). The mean content of major alkaloids of the 5 infusions was estimated at 1.7 ± 0.1 mg lycopsamine equivalents per infusion. A comparison of the methanol extract with the hot water extract revealed similar concentrations of the individual PAs, and their *N*-oxides, in both solvents.

Another three herbal tea bags from Sample **VI**, but sourced from a different box of 12 sachets, were used to determine a time course of extraction of the dehydroPAs and their *N*-oxides into hot water. It was evident that after steeping times of 3 – 5 min, recommended on the packaging, the extraction of dehydroPAs was effectively maximized yielding about 2.6 ± 0.1 mg lycopsamine equivalents per infusion (Fig. 10A). The individual alkaloids, including *N*-oxides, were extracted at similar rates (Fig. 10B). Some minor differences in relative concentrations of the alkaloids were observed between this time course extraction of 3 herbal tea bags and the mean estimate of dehydroPAs determined using 5 herbal tea bags. Thus, asmachilcadine (**10**, alkaloid 10) was the major alkaloid in the former extracts (Fig. 10) while the rinderine (**6**)/intermediate (**2**) (alkaloids 3/4) pair was predominant in the latter (Fig. 9). This, and the difference in mean alkaloid estimates (1.7 ± 0.1 mg and 2.6 ± 0.1 mg), indicates a slight variability in production or source materials.

4. Conclusions

The unequivocal determination of potentially toxic dehydroPAs in dried and fresh plant material sold as asmachilca, and in “Asmachilca” herbal teas indicates a potential health risk to consumers. Even a mild preparation of tisane, in comparison to some herbal recipes, using

a single “Asmachilca” herbal tea bag (sample **VI**) examined in this study would expose the consumer to about 2.2 ± 0.5 mg of dehydroPAs which is far in excess of existing regulations and/or recommendations in various countries relating to exposures to dehydroPAs, e.g., 0.1 µg/day (Germany); 0.007 µg/kg BW/day (UK); 0.1 µg/kg BW/day (Netherlands); and 1 µg/kg BW/day (Australia/New Zealand) (EMA, 2014). This concern is exacerbated for foetuses, nursing neonates and young children because of their particular sensitivity to the toxic effects of dehydroPAs. However, toxicological studies would be required to better define this potential risk specifically for asmachilca since not all dehydroPA esters exhibit the same degree of hepatotoxicity, pneumotoxicity and/or genotoxic carcinogenicity. While larger exposures to the dehydroPAs can lead to instances of hepatic sinusoidal obstruction syndrome (also known as hepatic veno-occlusive disease), relatively easy to associate with exposure to the dehydroPAs, an epidemiological study of the indigenous population of users of asmachilca may identify more chronically-developing associated diseases such as cirrhosis, pulmonary arterial hypertension and various cancers (Edgar et al., 2015).

The asmachilca preparations are apparently lacking in standardization and the recipes for utilization of the plant vary. With respect to the latter, and similar to observations by Betz et al. (1994) who showed that, contrary to untested beliefs, the dehydroPAs in comfrey were soluble in hot water, it is significant that the asmachilca-derived dehydroPAs are efficiently extracted in to the tisane along with their more water-soluble *N*-oxides. Therefore, not only do the plant species actually used to prepare asmachilca need to be unambiguously identified (including their phytochemical characterization) (see Applequist and Miller, 2013), many further questions need to be addressed including: i). what are the beneficial bioactives? ii). is asmachilca harvested throughout the year with consequent variations in the composition of the secondary metabolites over the vegetation period? iii). how is the plant material treated and processed (methods of harvesting, drying and storing)? iv). how frequently and in which amounts is asmachilca consumed? v). with respect to sustainability, do traded plant materials originate from cultivated plants or from those collected in the wild? When these, and other questions can be answered and correlated to phytochemical analyses, perhaps recommendations for collection and processing can be developed to help reduce the exposure to the toxic dehydroPAs while simultaneously enhancing exposure to the beneficial metabolites. Otherwise, if trading of crude drugs is abandoned and laborious methods are applied for producing a standardized medicine, then pre-extraction and elimination of dehydroPAs, e.g., by supercritical fluid extraction with carbon dioxide (EPO, 1992), might be a way to take advantage of the pharmacologically beneficial metabolites in asmachilca. For example, such an approach has been developed with Common Butterbur (*Petasites hybridus*), used to treat migraine symptoms, to develop medicinal products containing the presumed bioactive sesquiterpene petasins, but without the potentially toxic dehydroPAs (Pothmann and Danesch, 2005).

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Glossary

CIDMS	collision-induced dissociation mass spectrometry
COSY	two dimensional proton-proton correlation spectroscopy
dehydoPA	1,2-dehydropyrrolizidine alkaloid
ESI(+)-MS	electrospray ionization positive ion mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GOESY	gradient enhanced nuclear Overhauser effect NMR spectroscopy
HMBC	two dimensional carbon-proton heteronuclear multiple bond correlation NMR spectroscopy
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
HSQC	two dimensional carbon-proton heteronuclear single quantum coherence NMR spectroscopy
LiAlH₄	lithium aluminium hydride
NMR	nuclear magnetic resonance spectroscopy
NOESY	nuclear Overhauser effect NMR spectroscopy
PA	pyrrolizidine alkaloid
RP	reversed phase
SCX SPE	strong cation exchange solid phase extraction

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Fig. 1. Arctiine moths attracted to withered *asmachilca* plant material presumably collecting dehydropyrrolizidine alkaloids. Note the extended proboscides (arrows).

Milwaukee, WI, USA).

Fig. 2.

The structures of dehydropyrrolizidine alkaloids and their necine bases and necic acids discussed in the text.

Fig. 3. Macrophotographs of leaf surfaces (**a** upper, **b** lower), perianths and achenes (**c**) of: **A** sample **II** (dried plant material labelled “Pulmonaria”); **B** sample **III** (dried plant material labelled “Asmachilca”); and **C** sample **V** (recently harvested, unprocessed plant material), showing that sample **II** is clearly an Asteraceae similar to sample **III**, and not, as the label might suggest, a Boraginaceae. Furthermore, sample **V** is significantly different indicating a separate species. Scale bars: **a,b** 1 mm, **c** 2 mm.

Fig. 4.

HPLC-ESI(+)MS base ion (m/z 80-1000) chromatograms of methanol extracts of asmachilca samples **I** (dried powdered plant sourced from Paraguay), **II** (dried plant material labelled "Pulmonaria"), **III** (dried plant material labelled "Asmachilca"), **IV** (packaged herbal tea bags, labelled "Infusion Asmachilca") and **V** (recently harvested, unprocessed plant material). Also shown are reconstructed ion chromatograms displaying m/z 300, 316, 428, 430, 444 and 460 for significant dehydropyrrolizidine alkaloids in each of the samples.

Fig. 5. HPLC-esi(+)MS base ion (m/z 80-1000) chromatograms of sample **I**, the dried asmachilca powder sourced from Paraguay, and **V**, unprocessed asmachilca herbal tea plant material, before (**A**) and after (**B**) treatment with indigocarmine-based redox resin to reduce *N*-oxides. MS/MS data for the numbered peaks are shown in Table 2. “IS” is the internal standard, lasiocarpine (**8**). The peak labelled with “*” is an acetylated monoester described in the text.

Fig. 6.

A comparison of the HPLC-esi(+)-MS ion chromatograms of an extract of sample **I** acquired using: **A.** the C18 Reversed Phase column with acidic mobile phase conditions, and **B.** the porous graphitic carbon column with basic mobile phase conditions. **A1** and **B1** show the reversal of elution times of *N*-oxides and their respective free base dehydropyrrolizidine alkaloids. **A2** and **B2** also show this reversal of elution times in addition to showing resolution of **10** and an isomer displaying the same MS/MS profile. Peak annotations correspond to structure numbers shown in figure 2.

Fig. 7.

HPLC-esi(+)MS reconstructed ion chromatograms displaying: **A.** m/z 300 for the diastereoisomers lycopsamine (**1**), intermediine (**2**), rinderine (**6**) and echinatine (**3**), and **B.** m/z 316 for their respective *N*-oxides. Shown also, to exemplify the subtle but significant differences in fragment abundances, are the MS/MS profiles for: **C.** intermediine (**2**), **D.** rinderine (**6**), **E.** intermediine-*N*-oxide (**2NO**), and **F.** rinderine-*N*-oxide (**6NO**). The peak annotations in **A** and **B** refer to structure numbers (Fig. 2).

Fig. 8.

Estimated concentrations of various pyrrolizidine alkaloids in asmachilca samples **I** (dried powdered plant from Paraguay), **II** (dried plant material labelled “Pulmonaria”), **III** (dried plant material labelled “Asmachilca”) and **IV** (packaged herbal tea bags, labelled “Infusion Asmachilca”). Concentrations are given as “µg lycopsamine equivalents/g plant”. The alkaloid numbers refer to Tables 2 and 3 and Fig. 5. For ease of display, some alkaloids have been grouped for quantitative estimation. The approximate relative concentrations of the grouped alkaloids are similar to those exemplified for **I** in Table 2.

Fig. 9.

Estimated content, expressed as lycopsamine equivalents, of various pyrrolizidine alkaloids in tisane prepared using a commercial “Asmachilca” herbal tea bag (sample **VI**). The error bars represent the range of measurements. The average total content of pyrrolizidine alkaloids in a hot water infusion of a tea bag ($n = 5$) from **VI** was determined to be 1.7 ± 0.1 mg equivalents of lycopsamine. The alkaloid numbers refer to Tables 2 and 3 and Fig. 5. For ease of display, some alkaloids have been grouped for quantitative estimation. The approximate relative concentrations of the grouped alkaloids are similar to those exemplified for **I** in Table 2.

Fig. 10.

A time course for the extraction of pyrrolizidine alkaloids (expressed as lycopsamine equivalents following HPLC-esi(+)MS analysis) into water during the preparation of tisanes from commercial “Asmachilca” herbal tea bags (sample **VI**). **A.** The average total content of pyrrolizidine alkaloids in a hot water infusion of a herbal tea bag ($n = 3$) from **VI** determined at 1, 2, 3, 4, 5, 10 and 60 minutes following addition of hot water. The error bars represent the range. **B.** Individual or closely eluting pairs of alkaloids determined for an infusion of one herbal tea bag at the various times. The alkaloid numbers refer to Tables 2 and 3 and Fig. 5. For ease of display, some alkaloids have been grouped for quantitative estimation. The approximate relative concentrations of the grouped alkaloids are similar to those exemplified for **I** in Table 2.

Table 1

Asmachilca-related samples investigated in this study. Commercial names of suppliers are withheld intentionally.

Sample Number	Description	Source
I	powdered plant material, not labelled	Paraguay, via German internet vendor
II	ca. 60 g crushed plant material in a cellophane bag, labelled “Pulmonaria”	Peru, Lima market
III	ca. 60 g crushed plant material in a cellophane bag, labelled “Asmachilca (Eupatorium gayanum wedd.)”[sic]	Peru, Lima market
IV	herbal tea bags, 1.5 g labelled “Infusion Asmachilca”	Peru, Lima market
V	bundle of freshly cut plant material (stems with leaves), called asmachilca by the vendors	Peru, Lima market
VI	herbal tea bags, 1.2 g labelled “Asmachilca”	Peru, via USA internet vendor

Table 2

HPLC-esi(+)MS and MS/MS of the pyrrolizidine alkaloids in Sample **I** (Fig. 5), the dried *asmachilca* powder sourced from Paraguay. The sample was injected onto the Synergi Hydro RP column and components eluted with the acetonitrile – 0.1% formic acid gradient system. Mass spectra data were acquired using the Velos Pro Linear Trap mass spectrometer.

Alkaloid ^a (Structure Number) ^b	Retention Time (min)	Relative Alkaloid Abundance ^c (%)	MH ⁺ (m/z)	MS/MS ^d (m/z)
1	4.97	0.6	318	300(65) ^e , 282(19), 274(17), 256(32), 238(9), 174(100), 156(20), 138(30), 120(6), 96(4)
2	5.1	0.8	302	284(9), 266(2), 258(21), 256(2), 212(4), 202(10), 158(100), 140(29), 122(32), 98(2), 94(2)
3 (2)	5.15	2.6	300	256(3), 210(2), 156(6), 138(100), 120(19), 94(49)
4 (6)	5.31	22.8	300 (599, 10) ^f	282(7), 256(2), 156(6), 138(100), 120(5), 96(6), 94(3)
5 (6NO)	5.57	12.5	316 (631, 18)	298(6), 272(18), 254(1), 226(25), 210(3), 172(100), 156(2), 155(11), 154(4), 138(9), 137(3), 136(5), 124(1), 120(1), 112(2), 111(2), 108(1), 94(3)
6 (2NO)	5.68	6.8	316 (631, 16)	298(7), 272(18), 2554(2), 226(29), 210(4), 210(4), 172(100), 156(1), 155(8), 138(21), 137(4), 136(9), 120(2), 112(2), 111(2), 108(1), 106(1), 94(6)
7 (4)	5.98	4.2	284 (567, 3)	240(4), 194(2), 140(6), 122(100), 120(1), 94(4)
8	6.12	4.6	302 (603, 15)	284(12), 258(21), 252(33), 240(4), 225(3), 212(5), 158(100), 142(4), 141(5), 140(6), 124(32), 122(6), 100(4)
9 (4NO)	6.20	14.4	300 (599, 15)	282(8), 256(18), 238(2), 210(35), 194(4), 184(1), 156(100), 140(2), 139(14), 138(4), 124(1), 122(6), 121(5), 120(10), 110(3), 108(2), 96(1), 97(1)
10 (10)	7.33	10.4	444	426(6), 400(3), 300(37), 282(100), 254(1), 238(1), 156(1), 138(28)
11	7.57	2.6	460 (919, 4)	442(8), 416(6), 316(100), 298(44), 282(1), 172(4)
12 (10NO)	7.66	1.6	460 (919, 4)	442(7), 416(5), 316(100), 298(43), 282(2), 172(4)
13 (11)	7.74	9.3	428	410(3), 384(3), 284(27), 266(100), 140(1), 122(13)
14	7.78	0.9	430	412(3), 386(3), 286(14), 285(1), 268(100), 267(5), 266(3), 240(1), 142(4), 124(3), 122(1)
15	7.97	3.9	430	412(3), 386(5), 286(26), 268(100), 252(2), 250(2), 142(5), 124(1)
16 (11NO)	8.00	2.1	444 (887, 5)	426(7), 400(5), 300(100), 282(41), 266(2), 156(4)

^a peak numbers refer to Figure 5

^b structure numbers refer to Figure 2

^c based upon individual peak areas as percentage of total peak area

^d 35% collision-induced dissociation energy

^e % relative abundance

^f m/z and relative abundance of dimer ion

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Table 3

HPLC-esi(+)collision-induced dissociation high resolution MS of the pyrrolizidine alkaloids in Sample **I** (Fig. 5), the dried asmachilca powder sourced from Paraguay. The sample was injected onto the Synergi Hydro RP column and components eluted with the acetonitrile – 0.1% formic acid gradient system.

Alkaloid Number ^a (Structure Number) ^b	Observed MH ⁺ (<i>m/z</i>)	Observed Fragment Ion (<i>m/z</i>)	Relative Ion Abundance (%) ^c	Calculated Molecular Formula (MH ⁺)	<i>d</i> (ppm)	Identity
1	318.19123		60	C ₁₅ H ₂₈ NO ₆	0.4	unknown, possibly a 2-hydroxy analogue of dihydrorinderine
		174.11221	100	C ₈ H ₁₆ NO ₃	−1.5	
		156.10177	30	C ₈ H ₁₄ NO ₂	−0.9	
		138.0912	20	C ₈ H ₁₂ NO	−1	
2	302.19625		60	C ₁₅ H ₂₈ NO ₅	0.2	1,2-dihydrorinderine (or isomer)
		158.11724	100	C ₈ H ₁₆ NO ₂	−2	
		140.10681	22	C ₈ H ₁₄ NO	−1.3	
		122.09636	15	C ₈ H ₁₂ N	−0.6	
3 (2)	300.18060		42	C ₁₅ H ₂₆ NO ₅	0.2	intermediate
		156.10159	100	C ₈ H ₁₄ NO ₂	−2	
		138.09108	62	C ₈ H ₁₂ NO	−1.9	
		120.08069	10	C ₈ H ₁₀ N	−0.7	
		94.06503	35	C ₆ H ₈ N	−1	
4 (6)	300.18051		30	C ₁₅ H ₂₆ NO ₅	−0.1	rinderine
		156.10148	80	C ₈ H ₁₄ NO ₂	−2.7	
		138.09098	100	C ₈ H ₁₂ NO	−2.6	
		120.08064	15	C ₈ H ₁₀ N	−1.1	
		94.06502	4	C ₆ H ₈ N	−1.1	
5 (6NO)	316.17468		100	C ₁₅ H ₂₆ NO ₆	−2.5	rinderine- <i>N</i> -oxide
		172.09628	70	C ₈ H ₁₄ NO ₃	−3.1	
		155.09377	14	C ₈ H ₁₃ NO ₂	−2	
		138.09102	20	C ₈ H ₁₂ NO	−2.3	
		136.07549	5	C ₈ H ₁₀ NO	−1.5	
6 (2NO)	316.17471		100	C ₁₅ H ₂₆ NO ₆	−2.4	intermediate- <i>N</i> -oxide
		172.09636	52	C ₈ H ₁₄ NO ₃	−2.7	
		155.09381	12	C ₈ H ₁₃ NO ₂	−1.7	
		138.09100	32	C ₈ H ₁₂ NO	−2.5	
		136.07559	10	C ₈ H ₁₀ NO	−0.7	
		93.05720	10	C ₆ H ₇ N	−1.1	
7 (4)	284.18556		30	C ₁₅ H ₂₆ NO ₄	−0.3	supinine

Alkaloid Number ^a (Structure Number) ^b	Observed MH ⁺ (<i>m/z</i>)	Observed Fragment Ion (<i>m/z</i>)	Relative Ion Abundance (%) ^c	Calculated Molecular Formula (MH ⁺)	<i>d</i> (ppm)	Identity
		140.10662	100	C ₈ H ₁₄ NO	−2.7	
		122.09625	80	C ₈ H ₁₂ N	−1.4	
8	302.19552		100	C ₁₅ H ₂₈ NO ₅	−2.3	dihydrosupinine- <i>N</i> -oxide
		158.11715	88	C ₈ H ₁₆ NO ₂	−2.6	
		140.10676	10	C ₈ H ₁₄ NO	−1.7	
		124.11183	60	C ₈ H ₁₄ N	−2	
9 (4NO)	300.17978		100	C ₁₅ H ₂₆ NO ₅	−2.6	supinine- <i>N</i> -oxide
		156.10148	88	C ₈ H ₁₄ NO ₂	−2.7	
		139.09884	26	C ₈ H ₁₃ NO	−2.3	
		138.09113	6	C ₈ H ₁₂ NO	−1.5	
		122.09624	16	C ₈ H ₁₂ N	−1.5	
		120.08071	10	C ₈ H ₁₀ N	−0.6	
10 (10)	444.25976		60	C ₂₂ H ₃₈ NO ₈	1.3	asmachilcadine
		300.18009	10	C ₁₅ H ₂₆ NO ₅	−1.5	
		282.16981	38	C ₁₅ H ₂₄ NO ₄	−0.6	
		138.09100	100	C ₈ H ₁₂ NO	−2.5	
		120.08065	5	C ₈ H ₁₀ N	−1.1	
11	460.25359		100	C ₂₂ H ₃₈ NO ₉	−1.1	asmachilcadine- <i>N</i> -oxide isomer
		316.17532	20	C ₁₅ H ₂₆ NO ₆	−0.5	
		298.16470	16	C ₁₅ H ₂₄ NO ₅	−0.7	
		172.09639	5	C ₈ H ₁₄ NO ₃	−2.5	
		138.09107	8	C ₈ H ₁₂ NO	−2	
12 (10NO)	460.25412		100	C ₂₂ H ₃₈ NO ₉	0.03	asmachilcadine- <i>N</i> -oxide
		316.17534	25	C ₁₅ H ₂₆ NO ₆	−0.4	
		298.16470	22	C ₁₅ H ₂₄ NO ₅	−0.7	
		172.09636	4	C ₈ H ₁₄ NO ₃	−2.7	
		138.09107	6	C ₈ H ₁₂ NO	−2	
13 (11)	428.26445		65	C ₂₂ H ₃₈ NO ₇	0.4	asmachilcadinine
		284.18507	10	C ₁₅ H ₂₆ NO ₄	−2	
		266.17475	44	C ₁₅ H ₂₄ NO ₃	−1.2	
		122.09616	100	C ₈ H ₁₂ N	−2.2	
14	430.28040		100	C ₂₂ H ₄₀ NO ₇	1.1	dihydroasmachilcadinine isomer
		268.19043	45	C ₁₅ H ₂₆ NO ₃	−1.1	
15	430.28041		100	C ₂₂ H ₄₀ NO ₇	1.1	dihydroasmachilcadinine

Alkaloid Number ^a (Structure Number) ^b	Observed MH ⁺ (<i>m/z</i>)	Observed Fragment Ion (<i>m/z</i>)	Relative Ion Abundance (%) ^c	Calculated Molecular Formula (MH ⁺)	<i>d</i> (ppm)	Identity
		286.20093	14	C ₁₅ H ₂₈ NO ₄	-1.2	
		268.19041	65	C ₁₅ H ₂₆ NO ₃	-1.2	
		124.11194	14	C ₈ H ₁₄ N	-1.1	
16 (11NO)	444.25950		100	C ₂₂ H ₃₈ NO ₈	0.7	asmachilcadinine- <i>N</i> -oxide
		300.18018	28	C ₁₅ H ₂₆ NO ₅	-1.2	
		282.16965	28	C ₁₅ H ₂₄ NO ₄	-1.2	

^a peak numbers refer to Figure 5

^b structure numbers refer to Figure 2

^c based upon individual peak areas as percentage of total peak area

^d (Observed MW - Expected MW) * 10⁶ / Expected MW

Table 4¹H and ¹³C NMR data for asmachilcadine (**10**) (alkaloid 10, Tables 2 and 3).

Carbon	¹³ C (ppm) ^a	¹ H (ppm)	COSY	HMBC ¹ H → ¹³ C
1	134.88	na	na	na
2	126.48	5.79, bs	3 _u , ^b 3 _d , 8(w)	3, 8, 9
3	61.63	4.29, H3 _d , bd J _{3d,3u} =15.8	3 _u	NC
		3.52, H3 _u , bd, J _{3u,3d} =15.6	3 _d , 8	
5	54.78	3.65, H5 _d , m	5 _u , 6 _{d,u}	NC
		2.9, H5 _u , m	5 _d , 6 _{d,u}	3, 7
6	33.11	ca 2.02, 2H, m	5 _d , 5 _u , 7	NC
7	73.38	4.3, m	6 _{d,u} , 8(w)	NC
8	80.90	4.6, bs	3 _u , 7 (w), 2 (w)	NC
9	61.79	4.88, H9 _d , bd, J _{9d,9u} =13.6	9 _u	1, 2, 8
		4.78, H9 _u , bd, J _{9u,9d} =13.3	9 _d	1, 2, 8, 11
11	173.71	na	na	na
12	81.86	na	na	na
13	74.42	5.31, q, J _{13,22} =6.3	22	11, 12, 15, 22
15	174.23	na	na	na
16	82.71	na	na	na
17	69.59	4.01, q, J _{17,18} =6.4	18	15, 16, 18
18	17.44	1.18, d, J _{18,17} =6.4	17	16, 17
19	32.74 or 33.05	2.04, m	20, 21	11, 12, 20, 21
20 [*]	17.14	0.96, sd, J _{20,19} =6.9; J _{24,23} =6.9	19	12, 19
21 [*]	17.03	0.92, d, 3H, J _{21,19} =6.9	19	12, 19
22	14.02	1.32, d, J _{22,13} =6.3	13	12, 13
23	32.74 or 33.05	2.11, m	24, 25	15, 16, 24, 25
24 ^{**}	16.84	0.96, sd, J _{20,19} =6.9; J _{24,23} =6.9	23	16, 23
25 ^{**}	16.58	0.89, d, 3H, J _{25,16} =7.0	23	16, 23

NC: no ¹H → ¹³C correlation observed

na: not applicable, quaternary carbons

d: doublet; m: multiplet; bd: broad doublet; sm: superimposed multiplet; sd: superimposed doublet; w and vw: weak and very weak correlation

^a all ¹³C assignments based on gradient enhanced HSQC^b d and u subscripts denote the low field and high field partners in a geminal proton pair^{*} the isopropyl methyl group chemical shifts could be interchanged^{**} the isopropyl methyl group chemical shifts could be interchanged

Table 5¹H and ¹³C NMR data for asmachilcadinine (**11**) (alkaloid 13, Tables 2 and 3).

Carbon	¹³ C (ppm) ^a	¹ H (ppm)	COSY	HMBC (¹ H → ¹³ C)
1	137.23	na	na	na
2	126.18	5.72, s	3 _d , ^b 3 _u , 8, 9 _d	1, 3, 8
3	61.63	3.99, H3 _d , bd, J _{3d,3u} =16.1	2 (vw), 3 _u	1, 2, 5
		3.40, H3 _u , bd, J _{3u,3d} =ca 15	2 (vw), 3 _d , 8	
5	56.84	3.22, H5 _d , ddd, J _{5d,5u} =10, J _{d2} =J _{d3} =5.5	5 _u , 6	3, 6 (w), 7, 8
		2.55, H5 _u , m	5 _d , 6	3, 6, 7, 8 (w)
6	25.87	1.81, sm, H6 _d , H6 _u	5 _d , 5 _u , 7 _d , 7 _u	5, 7, 8
7	30.10	2.08, H7 _d , sm	6, 7 _u , 8	1, 5, 6, 8
		1.54, H7 _u , m	6, 7 _d , 8	1, 5, 6, 8
8	71.71	4.3, bs	2 (vw), 3 _u , 7 _d , 7 _u	NC
9	62.46	4.79, H9 _d , AB d, J _{9d,9u} =13.2	9 _u	1, 2, 8, 11
		4.71, H9 _u , AB d, J _{9d,9u} =13	9 _d	1, 2, 8, 11
11	174.09	na	na	na
12	81.39	na	na	na
13	74.21	5.26, q, J _{13,22} =6.3	22	11, 12, 15, 22
15	174.44	na	na	na
16	82.58	na	na	na
17	69.4	3.94, q, J _{17,18} =6.4	18	15, 18
18	17.66	1.16, d, J _{18,17} =6.3	17	16, 17
19	32.83	2.08, sm	20, 21, 24, 25	11, 12, 20, 21
20 [*]	17.2	0.92, sd, J _{20,19} =6 - 7	19, 23	12, 16, 19, 23
21 [*]	17.66	0.92, sd, J _{21,19} =6 - 7	19, 23	12, 16, 19, 23
22	14.05	1.31, d, J _{22,13} =6.3	13	12, 13
23	32.47	2.08, sm	20, 21, 24, 25	15, 16, 24, 25
24 ^{**}	16.52 [*]	0.86, d, J _{24,23} =6.8	19, 23	12, 16, 19, 23
25 ^{**}	17.05 [*]	0.94, J _{25,23} =6 - 7	19, 23	12, 16, 19, 23

NC: no ¹H → ¹³C correlation observed

na: not applicable, quaternary carbons

d: doublet; m: multiplet; bd: broad doublet; sm: superimposed multiplet; sd: superimposed doublet; w and vw: weak and very weak correlation

^a all ¹³C assignments based on gradient enhanced HSQC^b d and u subscripts denote the low field and high field partners in a geminal proton pair^{*} the isopropyl methyl group chemical shifts could be interchanged^{**} the isopropyl methyl group chemical shifts could be interchanged