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Is aristolochic acid nephropathy a widespread problem in developing countries?

**A case study of Aristolochia indica** L. in Bangladesh using an ethnobotanical–phytochemical approach

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**Abstract**

Ethnopharmacological relevance: Species of Aristolochia are associated with aristolochic acid nephropathy (AAN), a renal interstitial fibrosis and upper urinary tract cancer (UUC). Aristolochic acid nephropathy has been reported in ten countries but its true incidence is unknown and most likely underestimated. By combining an ethnobotanical and phytochemical approach we provide evidence for the risk of AAN occurring in Bangladesh. More specifically, we assess the intra-specific variation of aristolochic acid analogues in medicinally used *Aristolochia indica* samples from Bangladesh.

**Materials and methods:** Ethnobotanical information was collected from 16 kavirajes (traditional healers) in different study locations in Bangladesh. Plant samples were obtained from native habitats, botanical gardens, herbal markets and pharmaceutical companies. The samples were extracted using 70% methanol and were analysed using LC-DAD-MS and 1H-NMR.

**Results:** Roots as well as leaves are commonly used for symptoms such as snake bites and sexual problems. Among the informants knowledge about toxicity or side effects is very limited and *Aristolochia indica* is often administered in very high doses. Replacement of *Aristolochia indica* with other medicinal plants such as *Rauwolfia serpentina* (L.) Benth. ex Kurz was common. *Aristolochia indica* samples contained a variety of aristolochic acid analogues such as aristolochic acid I, aristolochic acid II, cepharadione A and related compounds.

**Conclusions:** AAN cases are likely to occur in Bangladesh and more awareness needs to be raised about the health risks associated with the use of *Aristolochia indica* and other species of *Aristolochia* as herbal medicines.

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**Abbreviations:** A, alstonine; AA, aristolochic acid; AAA, aristolochic acid analogue; AAN, aristolochic acid nephropathy; AL, aristolactam; BA, benzoic acid; BEN, Balkan endemic nephropathy; COSY, correlation spectroscopy; CP, commercial product; D2O, deuterated water; EBC, economic botany collection; ESI, electrospray ionisation; LC-DAD-MS, liquid chromatography-diode array detector-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; GNP, gross national product; HMBC, heteronuclear multiple-bond correlation spectroscopy; HMQC, heteronuclear multiple-quantum correlation spectroscopy; K2HPO4, di-potassium hydrogen orthophosphate; KH2PO4, potassium dihydrogen orthophosphate; J-RES, J-resolved; MeOD, deuterated methanol; MS, market sample; m/z, mass-to-charge-ratio; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PC, principle component; PCA, principle component analysis; PPM, parts per million; PTFE, polyfluoroethylene; RBG, Royal Botanic Gardens, Kew; RPM, revolutions per minute; S, serpentine; tR, retention time; TSP, 3-(tetramethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt; UUC, upper urinary tract cancer; UV, ultraviolet

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1. Introduction

Species of Aristolochia are known to cause aristolochic acid nephropathy (AAN), a renal interstitial fibrosis, which is associated with a high incidence of upper urinary tract cancer (UUC) (Nortier et al., 2000). It was initially reported in a Belgian cohort of over 100 patients after the intake of slimming pills containing a Chinese herb, Aristolochia fangchi Wu ex L.D. Chow & S.M. Hwang (Vanherweghem, 1998). Following this tragic incidence, species of Aristolochia were banned in many countries, including Germany, the UK, USA, Australia and Canada. However, drugs and medical preparations from species of Aristolochia are used widely (and often legally) in many countries and can be bought via the Internet (Gold and Slone, 2003; Schaneberg and Khan, 2004; Heinrich et al., 2009). So far AAN has only been reported in 15 countries but its true incidence is unknown and probably underestimated (National Toxicology Program, 2008).

Evidence for AAN being a major public health problem has only been provided for two small regions worldwide, specifically the Balkan region and Taiwan. In the Balkan region, the dietary intake of flour contaminated with Aristolochia clematitis L. seeds is proposed as the environmental causal factor for Balkan Endemic Nephropathy (BEN) (Grollman et al., 2007; Jelakovic et al., 2012). This disease has affected thousands of patients in the Danube basin and its clinical expressions and pathological lesions are strikingly similar to AAN (De Broe, 2012). Furthermore, recent research has shown that exposure to species of Aristolochia used in traditional Chinese medicines contributes significantly to the incidence of upper urothelial cancer (UUC) in Taiwan (Chen et al., 2012). However, it is likely that AAN and UUC are also prevalent in other countries where species of Aristolochia are used (Heinrich et al., 2009), creating a global public health problem of considerable but largely unknown magnitude (Debelle et al., 2008; Grollman et al., 2009; Grollman, 2013).

Ethnobotanical studies indicate that the Indian subcontinent is one of the hot spots for Aristolochia use (Heinrich et al., 2009). Aristolochia indica L. was found to be the most frequently cited species in the literature. A possible association between chronic interstitial fibrosis in Indians and the intake of Aristolochia has been proposed before (Vanherweghem, 1997; Debelle et al., 2008; Grollman et al., 2009). Therefore, it is likely that the medicinal use of Aristolochia indica results in a large number of undiagnosed AAN and UUC cases on the Indian subcontinent.

This work focuses specifically on the uses of Aristolochia indica in Bangladesh and their implications on public health. Bangladesh was chosen as a case study since Aristolochia indica, the most frequently cited Aristolochia species is native there. However, only fewer ethnobotanical reports on Aristolochia indica uses exist in comparison to India. While four species of Aristolochia are found in Bangladesh (Aristolochia elegans Mast., Aristolochia indica L., Aristolochia saccata Wall. and Aristolochia acuminata Lam., Ahmed et al., 2007), Aristolochia indica is the most widely distributed species and has the greatest importance as a medicinal plant (Heinrich et al., 2009; Mollik et al., 2010).

In Bangladesh only 1.5% of the gross national product (GNP) is spent on health care (Rahman, 2005). Non-communicable diseases including kidney disease are not priorities and patient care is deficient. The number of nephrologists in Bangladesh is limited and renal disease care is only available in six public hospitals and 10 private hospitals (Rashid, 2004). An overburdened health-care system with few renal facilities means that recording cases is not a priority. Since there are few statistics regarding kidney disease (Jha, 2009) it is not surprising that no records of AAN cases in Bangladesh exist. In addition to limited data recording there is a lack of knowledge regarding the condition and its causes. Furthermore, the medical care offered is rarely integrated with herbal medicine and it is unlikely that the connection between the use of Aristolochia as a medicine and kidney disease would to be made.

The use of herbal medicine in Bangladesh is widespread with estimates of up to 75% of the population using complementary and alternative medicines to manage their health needs (Ghani and Pasha, 2004). In addition to allopathic medicine a range of medical practices exist (and overlap) in Bangladesh including Ayurvedic, Unani, homeopathy, popular and spiritual medicines. Kavirajes are ‘folk’ or ‘traditional’ healers that use plant preparations to treat various ailments (Rahman et al., 2010). The practices of kavirajes are varied as they draw on Ayurvedic, Unami and spiritual models of healing (either in combination or exclusively). They are frequently found across Bangladesh catering to the primary health care needs of a large proportion of the population (Mollik et al., 2010).

The aim of this study was to assess the risk of AAN occurring in Bangladesh by combining an ethnobotanical and phytochemical approach. More specifically, we assessed the importance of Aristolochia indica, its medicinal uses and the knowledge about the health risks associated with its use by carrying out interviews with healers (kavirajes) in different study locations in Bangladesh. Furthermore, we studied the intraspecific phytochemical variation of Aristolochia indica samples obtained in Bangladesh using metabolomic methods based on LC-DAD-MS and 1H-NMR (Michl et al., 2011). We especially focused on the content of aristolochic acid analogues (AAAs), the group of compounds known to be responsible for the nephrotic and carcinogenic effects of Aristolochia species (National Toxicology Program, 2011; Kumar et al., 2003).

2. Materials and methods

2.1. Ethnobotanical survey

Prior to the start of the fieldwork ethical approval was obtained from the School of Pharmacy’s (University of London, UK) Ethics Committee (January 2012).

Ethnobotanical information was collected during two field studies in February 2012 and August 2012. Semi-structured interviews were carried out with 16 healers (kavirajes) in different study locations in Bangladesh (Rajshahi, Dhaka, Natore, Tangail, Bandarban and Sylhet, Fig. 1) in order to obtain data representative for the whole of Bangladesh. The interviews were carried out in Bengali with the help of a translator and generally took place in public spaces such as markets. Informed consent was obtained prior to the interviews. However, the aims of the study relating to the toxicity of Aristolochia indica could not be fully disclosed prior to the interview. Informants were asked to list medicinal uses of Aristolochia indica and were then asked to provide more detailed information about these uses. The questions covered in the interviews included vernacular names of the species, the parts used (leaves, roots, stems, fruits, seeds), the mode of preparation and administration, dose, duration of treatment and knowledge about toxicity or side effects. All informants were male, however they belonged to different ethnic groups: Bengali (10), Marma (1), Santal (2), Tripura (1), Mandai (1) and Bongshi (1). All informants were asked whether they would be willing to provide a sample of Aristolochia indica or to identify the plant in photographs if plant material was not available.

2.2. Plant material and herbal formulations

Aristolochia indica samples were collected either in their natural habitat or were obtained from botanical gardens. Voucher specimens of samples 3 (J. Michl BI-21582) and 4 (J.Michi
BI-21583) were identified by Dr. Martin Ingrouille and were deposited in the Herbarium, Royal Botanic Gardens, Kew (RBG Kew, UK). Root samples which were traded under the name ‘Ishwarmul’ (a common name for Aristolochia indica) were purchased from herbal markets. Further root samples were obtained from the Economic Botany Collection (RBG Kew, UK). The identity of these root samples was determined by their phytochemical profile. Commercial preparations obtained from phytopharmaceutical companies or from kavirajes were included as well. These preparations include two aqueous extracts, one semi-solid paste used to prepare pills containing Aristolochia indica as well as a variety of other plants such as Cinnamomum cassia L., Eclipta erecta L. and Curcuma phaeocaulis Valenton. Three samples (4, 10 and 11, Table 1) were extracted and analysed in triplicate in order to assess the reproducibility of the extraction and processing method. Information about the samples is shown in Table 1.

2.3. Chemicals

All chemical reagents used were of analytical grade. Deuterated water (D2O, 99.9%) and deuterated methanol (MeOD, 99.8%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). 3-(Tetramethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP, 98%) was obtained from Sigma–Aldrich (St. Louis, MO). Di-potassium hydrogen orthophosphate (K2HPO4) and potassium dihydrogen orthophosphate (KH2PO4) were obtained from BDH Laboratory Supplies (Poole, UK).

2.4. Preparation of extracts

The dried plant material was ground in a mortar and extracted with 70% methanol in a microtube for 16 h (50 mg/ml). The extracts were then centrifuged (10 min, 10 000 rpm) and filtered using a 0.45 μm PTFE filter. The liquid Ayurvedic formulations (Samples 16–17) were filtered using 0.45 μm PTFE filters and the filtrate was used for LC-MS analysis. An aliquot (700 μl) of each extract or formulation was transferred to LC-MS vials.

2.5. LC-DAD-MS analysis

The analysis was carried out using a Thermo Scientific LC-DAD-ESI-MS system (Thermo Scientific, MA, USA) consisting of an ‘Accela’ liquid chromatograph with diode array detector and an ‘LTQ-Orbitrap XL’ hybrid linear ion trap-orbitrap mass spectrometer. Chromatography was performed on a 150 mm × 3 mm, 3 μm Phenomenex Luna C18 column using a 400 μl/min flow rate. The separation was obtained using the following linear mobile phase gradient: 0 min: 90% A, 0% B, 10% C; 50 min: 0% A, 90% B, 10% C, 55 min: 0% A, 90% B, 10% C, 57 min 90% A, 0% B, 10% C, 60 min 90% A, 0% B, 10% C (A: water, B: methanol, C: acetonitrile containing 1% formic acid). The injection volume was 5 μl. Ammonia solution was added post column at a flow rate of 0.1 μl/min. The mass spectrometer was fitted with an electrospray source (Thermo ‘Ion Max’) operated in positive mode at a source voltage of 3.5 kV using sheath and auxiliary nitrogen flow rates of 60 and 20 units, respectively, and a capillary temperature of 300 °C. High-resolution MS1 scanning was performed in the orbitrap (m/z 250–2000; resolution 30 000). Low-resolution data dependent MS2 and MS3 scans were undertaken in the linear ion trap (isolation width 4 m/z units; collision energy 35%).

2.6. 1H-NMR analysis

After LC-DAD-MS analysis the samples were dried at room temperature and residual water was removed using lyophilisation. The plant extracts were redissolved in 490 μl MeOD containing 0.01% TSP and 210 μl KH2PO4 buffer (pH=6.0). The solutions were transferred into 5 mm NMR tubes. 1H-NMR spectra were recorded on a Bruker Avance 500 spectrometer operating at 500.13 MHz. For each sample 256 transients were recorded as 65 K data points with a spectral width of 10 330 Hz using 3.17 s acquisition time, 1.0 s relaxation delay, and a 30° pulse angle. FIDs were Fourier transformed with LB=0.3 Hz. The spectra were referenced to internal TSP. 1H-NMR spectra were manually corrected for phase and baseline distortions using Topspin (v 1.3, BrukerBiospin).

Table 1

| Plant part | Source | Place of collection | Voucher number |
|------------|--------|---------------------|----------------|
| 1 Stem     | Wild   | Tangail             | BI-21568       |
| 2 Leaf     | Wild   | Tangail             | BI-21580       |
| 3 Stem     | Cultivated | Rajshahi             | BI-21582 (K)   |
| 4 Leaf     | Cultivated | Rajshahi             | BI-21583 (K)   |
| 5 Stem     | Cultivated | Natore              | BI-21571       |
| 6 Stem     | Cultivated | Natore              | BI-21572       |
| 7 Stem     | Cultivated | Natore              | BI-21573       |
| 8 Stem     | Cultivated | Rajshahi             | BI-21574       |
| 9 Fruit    | Cultivated | Rajshahi             | BI-21584       |
| 10 Root    | MS     | Dhaka               | BI-21570       |
| 11 Root    | MS     | Natore              | BI-21569       |
| 12 Root    | MS     | Dhaka               | BI-21577       |
| 13 Root    | MS     | EBC (Sri Lanka)     | BI-21555, EBC-45619 |
| 14 Root    | MS     | EBC (India)         | BI-21553, EBC-45617 |
| 15 Root    | MS     | EBC (India)         | BI-21551, EBC-45616 |
| 16 Liquid formulation | CP      | Rajshahi         | BI-21576       |
| 17 Liquid formulation | CP      | Rajshahi         | BI-21585       |
| 18 Paste   | CP     | Tangail             | BI-21581       |

Fig. 1. Map of different study locations in Bangladesh.
2.7. Data reduction and multivariate data analysis

The $^1$H-NMR spectra in the range of 0.1–10 ppm were divided into 990 regions (‘buckets’) of 0.01 ppm using AMIX software (v. 3.5.5., BrukerBiospin) and the signal intensity in each region was integrated. Water signals (4.65–5.15 ppm) and residual solvent signals (3.25–3.4 ppm) were excluded. The data could then be imported into Microsoft Excel for the addition of labels and normalisation. The spectral areas were normalised to the total sum of the spectral integral to avoid dilution effects.

Filtering, peak extraction, chromatogram de-convolution and peak alignment of the high-resolution LC-MS data were performed automatically using Mzmine 2 (http://mzmine.sourceforge.net). Peaks between 2 and 40 min retention time with a minimum peak intensity of 500 000 and $m/z$ between 250 and 650 were extracted. The resulting data set was normalised to the total raw signal.

Principal component analysis (PCA) was carried out on the normalised and pareto scaled dataset (for both, LC-MS and $^1$H-NMR data sets) using the software SIMCA P+ (v. 12, Umetrics, Umeå, Sweden).

For the analysis of aristolochic acid content peaks between 2 and 40 min retention time with a minimum peak intensity of 200 000 and $m/z$ between 250 and 650 were extracted. The dataset was not normalised.

2.8. Metabolite identification assignment

For the peaks extracted by Mzmine 2, the ion species (i.e. adduct) was determined so as to obtain an experimental accurate mass. This was then searched against the CRC Dictionary of Natural Products to identify candidate compounds having molecular masses within 5 ppm of the experimental value. The identity of characteristic biomarkers was verified using 2D NMR experiments ($^2$H–$^2$H J-RES, $^2$H–$^2$H COSY, $^2$H–$^2$H NOESY, $^2$H–$^{13}$C HMQC and $^2$H–$^{13}$C HMBC) on selected samples. AA I, AA II, AA III, AA Illa, AA IV, AA D and Al I were identified by comparison to reference standards. Further compounds were identified by comparing accurate mass data to an in-house database (UCL School of Pharmacy, London, UK) consisting of 193 aristolochic acid analogues that have previously been isolated from natural sources. Putative assignments for those AAAs were made based on their accurate mass, retention time, mass fragmentation and UV spectra.

3. Results and discussion

3.1. Importance of Aristolochia indica in Bangladesh

*Aristolochia indica* is widely available in herbal markets and the majority of healers were knowledgeable about its medicinal uses (11 out of 16 kavirajes). Local names include ishwarmul, ghorth, tang gway sobawai, and rudho jota (Table 2). While local names vary across ethnic groups and between different regions of Bangladesh ishwarmul was the most frequently reported local name. It is noteworthy that the name ishwarmul is also used for other plants besides *Aristolochia indica*, such as Pholodita pallida Lindl. (Orchidaceae).

*Aristolochia indica* is generally considered to be a very potent medicinal plant. This is also reflected in the meaning of the Bengali name ishwarmul, which can be translated as ‘god-given plant’. Numerous myths and beliefs about the plant exist, most of them in relation to snakes. One example is the belief that *Aristolochia indica* growing in the garden prevents snakes from entering a house.

While the collection of *Aristolochia indica* in the wild seems to be common, three informants reported that the plant is now very rare. The fruits of *Aristolochia indica* were reported to be used as food in the past, when the plant was commonly found in its natural habitat. This reported use is particularly concerning, given that it is likely that high doses were consumed and that fruits often accumulate high amounts of aristolochic acid analogues.

3.2. Medicinal uses

Ethnobotanical data were collected from 11 kavirajes. The healers interviewed during this study came from a variety of ethnic backgrounds and practiced different medical systems, such as Unani or Ayurveda. All parts of the plant are used medicinally. A variety of medicinal uses were reported for *Aristolochia indica* (Table 2). However, snake bites (5 use reports) was the most frequently reported use. Other reported uses include sexual problems (3), gastric problems (2) and the use as a tonic (2). *Aristolochia indica* species are used in childbirth or as an abortifacient in many parts of the world, including Europe (Heinrich et al., 2009). Interestingly, these uses were not reported by any of the informants in this study.

3.3. Doses and formulations

Formulations and doses used are rarely reported in the ethnobotanical literature. However, due to the species’ toxic potential, detailed records on the commonly used dose ranges were recorded. A variety of doses and formulations were reported by the kavirajes (Table 2). In terms of formulations, in most cases *Aristolochia indica* plant material (roots, leaves or stems) was used directly rather than as an extract. The most frequently reported formulation was a pill made of different parts of *Aristolochia indica*, often in combination with other plants such as Asparagus racemosus Willd., Withania somnifera (L.) Dunal or Piper nigrum L. and honey or sugar. Furthermore the use of pills containing *Aristolochia indica* in combination with chemicals obtained from local markets was reported. The identity of these chemicals is unknown but might be of toxicological concern, especially since this formulation was used for pneumonia in children. Other common formulations include the use of juice obtained from *Aristolochia indica* roots or leaves or the intake of the plant powder with small amounts of water. Interestingly, extraction of the plant material using infusion with water was only reported by one kaviraj. However, phytopharmaceutical companies frequently manufacture water extracts containing *Aristolochia indica* and a variety of other medicinal plants according to Ayurvedic recipes.

The reported doses varied greatly. Extremely high doses were reported, such as the use of 25–50 g of root powder against snakebites or the ingestion of 3 g of root powder per day for one month as a tonic. Furthermore, *Aristolochia indica* is sometimes used for a long duration. One example is the use of 3 pills made of *Aristolochia indica* leaves per day for 4 month against parasitic worms. Assuming that one pill contains around 0.5 g of plant material this results in an estimated total dose of 180 g. However, in other cases the reported doses were extremely small or the plant material was applied topically. One informant reported that small pieces of roots are often worn around the neck in an amulet.

3.4. Reports of toxicity and side effects

In general knowledge about toxicity and side effects was very limited (Table 2) and the majority of informants reported no side effects or toxic effects (8 out of 11 kavirajes). Sleepiness, gastric problems and rashes were reported as rare side effects occurring in a few of the patients. Interestingly, one informant was aware of toxic effects of *Aristolochia indica* leaves, but reported that the roots of the plant are not toxic. However, none of the informants reported nephrotoxicity as a risk resulting from the use of *Aristolochia indica* as a herbal medicine.

3.5. Phytochemical variation in ‘Ishwarmul’ (*Aristolochia indica* and other taxa) samples

We carried out a metabolomic study of *Aristolochia indica* samples collected from their natural habitat or from botanical
The phytochemical variation of these samples was studied using LC-MS and $^1$H-NMR. A three component PCA model explained 39.8% of the variation in the LC-MS data set (Fig. 2a) and 64.4% of the variation in the $^1$H-NMR data set (Fig. 2b).

gardens. Root samples, which were traded under the name *Ishwarmul*, and herbal formulations reported to contain *Aristolochia indica* plant material were also included in the analysis.
The PCA scores plot for the LC-MS data shows that the samples are clustered into various groups. According to the loadings plot only samples with negative PC1 scores (1, 2, 3, 4, 5, 6, 7, 9, 10, 13 and 14) were characterised by the presence of aristolochic acid I (m/z 359.0870, tR = 35.93) and other aristolochic acid analogues. This result suggests that not all analysed samples contained Aristolochia indica plant material, even though they were traded under the plants common name ‘Ishwarmul.’

Unsurprisingly, the three herbal formulations 16, 17 (two liquid water extracts obtained from phytoceutical companies) and 18 (a semi-solid paste for making pills obtained from a kaviraj) form a cluster with positive PC1 scores in the LC-MS data set. The formulations contain a variety of different herbs and are therefore not likely to contain high amounts of aristolochic acids. Examination of the PCA loadings plots showed that the differentiation is caused mainly by a variety of compounds with short retention times (of which sucrose is likely to be a component of the unretained peak at tR = 2.00 min giving [M+NH4]+ at m/z 360.1511). The 1H-NMR data loadings plot is characterised by a variety of overlapping peaks in the carbohydrate region as well as aromatic peaks corresponding to benzoic acid (δ 7.95 (dd, J = 6.5 Hz), δ 8.40 (d, J = 8.5 Hz), δ 8.37 (d, J = 6.6 Hz), δ 7.81 (d, J = 8.1), δ 7.76 (m) δ 7.75 (d, J = 1.4 Hz), δ 7.49 (m), δ 4.95 (m), δ 4.79 (m), δ 4.76 (dd, J = 12.5, 5.5 Hz), δ 4.66 (m), δ 3.82 (s), δ 3.22 (d, J = 5.5 Hz), δ 3.15 (d, J = 6.4 Hz), δ 2.82/δ 2.79 (m) and δ 1.38 (s)) apart from a small difference in chemical shifts for H-20 (δ 2.81 vs. δ 2.79). A plant commonly used for snakebites, insomnia and insanity in Ayurvedic medicine is Rauvolfia serpentina (L.) Benth. ex Kurz (Keshavan, 2011). Local names in Bangladesh include chamdrumul and sarpa-gandha. The presence of alstonine and serpentine in samples 11 and 12 would be consistent with roots of this species.

Samples 8 and 15 have very similar phytochemical profiles but do not contain Rauvolfia alkaloids or aristolochic acid analogues. This indicates that these samples belong to a, third unidentified species.

LC-MS total ion chromatograms and 1H-NMR spectra representative for the different groups of samples (Aristolochia indica Fig. 2.
samples, *Rauvolfia serpentina* samples and herbal formulations) and are shown in Fig. 3.

The result of the metabolomic analysis indicates that replacements or misidentifications of plant species occur frequently in Bangladesh. It also highlights that metabolomic analysis is a suitable tool for the authentication of herbal medicines, allowing the identification of replacements or contaminated plant material.

### 3.6. Intraspecific variation in *Aristolochia indica* samples

Samples 8, 11, 12, 15, 16, 17 and 18 were removed from the analysis and a PCA model of the remaining samples was calculated based on their LC-MS and 1H-NMR data (Fig. 2c and d). A 3-component PCA model explained 58.2% of the variation in the LC-MS dataset and 59.8% of the variation in the 1H-NMR dataset. Samples 1 (stem), 2 (leaf), 2 (stem), 3 (leaf) and 4 (leaf) are shown as outliers in the PCA scores plot of the LC-MS data set. While the cluster consisting of samples 1 (stem), 3 (leaf) and 4 (leaf) was characterised by high amounts of aristolochic acid I ([M+NH₄]⁺, \( m/z \) 359.0870, \( t_R \) = 35.93 min), aristolactam I N-β-D-glucoside ([M+H]⁺, \( m/z \) 456.1285, \( t_R \) = 26.53 min) and aristolactam I ([M+H]⁺, \( m/z \) 294.0759, \( t_R \) = 36.18 min), samples 2 (stem) and 2 (leaf) had high amounts of aristolochic acid II (\( t_R \) = 34.09 min, \( m/z \) 329.0766). It is noteworthy that these outliers consisted of fresh, green plant material. In contrast, samples, which were obtained from older, woody stems or roots (2, 3, 5, 6, 7, 10, 13 and 14) were located in the centre of the scores plot. These samples were characterised by smaller amounts of aristolochic acid analogues and a major metabolite giving \( m/z \) 342.1706 (\( t_R \) = 7.38 min). Among the possible candidate
compounds suggested by the formula of this ion (C_{20}H_{24}NO_{4}^{+}; 1.8 ppm) is the aporphine derivatives magnoflorine. The compound showed the same MS/MS spectrum as the ion assigned to M^+ of magnoflorine in an LC-MS analysis of a leaf extract of Magnolia grandiflora L. held in the analytical archives at RBG Kew. These results suggest that the intraspecific variation in the phytochemical profile Aristolochia indica samples is influenced by factors such as the age of the plant and the plant part used.

### Table 3
Retention times, UV maxima and fragmentation ions of identified aristolochic acid analogues. The compounds are sorted by descending average peak areas across all samples.

| No. | Compound | Retention time (min) | [M+H]^+ (m/z) | [M+NH4]^+ (m/z) | Fragmentation ions (m/z) | UV maxima (nm) |
|-----|----------|----------------------|----------------|----------------|-------------------------|----------------|
| 1   | Aristolochic acid Ia | 30.76 | 345.0717 | 327, 310, 284, 254 | 235, 320, 392 |
| 2   | Aristolochic acid IIa | 21.12 | 344.0768 | 327, 309 | 235, 280, 426 |
| 3   | Cepharadione A\(^b\) | 16.35 | 442.1130 | 424, 322, 280, 222 | 234, 266, 283, 336, 403 |
| 4   | Aristolactam IIIa; N-\(\beta\)-D-glucopyranoside\(^b\) | 33.60 | 296.0757 | 279, 251 | 235, 278, 391 |
| 5   | Aristolactam Ia; N-\(\beta\)-D-glucopyranoside\(^b\) | 22.71 | 359.0870 | 324, 296, 294, 268 | 252, 320, 392 |
| 6   | Aristolactam IVa | 30.73 | 345.0713 | 327, 310, 284, 254 | 235, 320, 392 |
| 7   | Aristolactam IIIb | 34.09 | 329.0766 | 324, 296, 250, 238 | 250, 300, 356 |
| 8   | Aristolactam Ib | 32.61 | 306.0759 | 288, 278, 248, 220 | 238, 301, 314, 343 |
| 9   | Aristolactam IVb | 34.09 | 329.0766 | 324, 296, 250, 238 | 250, 300, 356 |
| 10  | Aristolactam IIIb | 33.60 | 296.0757 | 279, 251 | 235, 278, 391 |
| 11  | Aristolactam IIa | 30.09 | 294.0758 | 279, 251 | 234, 316, 450 |
| 12  | Aristolactam IIb | 29.06 | 294.0758 | 279, 251 | 234, 316, 450 |
| 13  | Aristolactam IIIb | 28.40 | 294.0758 | 279, 251 | 234, 316, 450 |
| 14  | Aristolactam Ia; N-\(\beta\)-D-glucopyranoside\(^b\) | 22.71 | 359.0870 | 324, 296, 294, 268 | 252, 320, 392 |
| 15  | Aristolactam Ib | 30.09 | 294.0758 | 279, 251 | 234, 316, 450 |
| 16  | Aristolactam IIb | 29.06 | 294.0758 | 279, 251 | 234, 316, 450 |
| 17  | Aristolactam IIIb | 28.40 | 294.0758 | 279, 251 | 234, 316, 450 |
| 18  | Aristolactam IIb | 29.06 | 294.0758 | 279, 251 | 234, 316, 450 |
| 19  | Aristolactam Ia; N-\(\beta\)-D-glucopyranoside\(^b\) | 22.71 | 359.0870 | 324, 296, 294, 268 | 252, 320, 392 |
| 20  | Aristolactam IIb | 29.06 | 294.0758 | 279, 251 | 234, 316, 450 |
| 21  | Aristolactam Ia | 30.76 | 345.0717 | 327, 310, 284, 254 | 235, 320, 392 |

\(^a\) Identified by comparison with reference standard.

\(^b\) Tentative assignment based on accurate mass, UV spectra and mass fragmentation.

\(^c\) Fragmentation ions were extracted from MS2 and MS3 scans.

3.7. Variation in aristolochic acid analogue content

Several aristolochic acid analogues were revealed by the LC-DAD-MS analyses and these were either identified by comparison with standards or given tentative assignments from their accurate mass, fragmentation ions and UV spectra (Table 3 and Fig. 4). Aristolochic acid I had the highest average peak area across all samples, followed by aristolochic acid II and cepharadione A.
Other common aristolochic acid analogues include aristolactam I N-p-o-glucopyranoside, aristolochic acid V, aristolactam I, aristolochic acid IIa and aristolochic acid D.

Based on the sum of their peak areas sample 13 (root) has the highest total amount of aristolochic acid analogues. While this sample contains low amounts of AA I and AA II it contains a large variety of aristolochic acid analogues, which are not present in any other sample. Possible candidates for these compounds include aristolactam AII, aristolactam III, aristolactam IVa and aristolactam AIII. In general, root samples contained a wider variety of different aristolochic acid analogues compared to other plant parts.

Sample 9 (fruit) had the second highest overall aristolochic acid content. However, this is due to its extremely high contents of aristolochic acid I and II. This may indicate that Aristolochia indica fruits accumulate high amounts of aristolochic acid analogues.

The content of aristolochic acids analogues also varied greatly between different parts of a single plant. For samples 2 and 3, leaf, stem and root material from the same plant were analysed. Interestingly sample 2 (leaf) did not contain aristolochic acid I, whereas the root and stem of the same plant did. While in sample 3 all parts of the plant contained high amounts of aristolochic acid I, the highest quantity was found in the leaf sample. Leaves, fruits and young stems contain significantly higher amounts of aristolochic acid I and II than roots and woody stems (average sum of peak areas $2.78 \times 10^6$ vs. $4.86 \times 10^5$, $p < 0.05$).

3.8. Toxicological risk assessment based on ethnobotanical and phytochemical evidence

The toxicological risk that is posed by the consumption of Aristolochia indica as a herbal medicine is dependent on a variety of factors. In relation to drug preparation, which used these factors include the dose, the mode of administration and the processing method of the plant material. Other factors such as the time of plant collection or interactions with other drugs that are taken simultaneously might have minor influences on the toxicological risk. Additionally the phytochemistry of the plant material is an important factor that determines the toxicological risk associated with the intake of Aristolochia indica. Besides the amount, the type of aristolochic acids that are present also is important, given that the toxicity of aristolochic acid analogues varies greatly (Balachandran et al., 2005; Li et al., 2010).

In this study, the use report with the highest reported dose was 200 g of Aristolochia indica leaves soaked in 1 l of water, taken once a day for 3 days, resulting in a total estimated dose of 600 g of Aristolochia indica leaves. However, the fact that aristolochic acids are very apolar compounds has to be considered. Kite et al. (2002) showed that the relative amount of AA I in a water extract is less than 6% compared to a 70% methanol extract. Therefore the amount of aristolochic acid analogues present in a water extract is comparatively low, and as a result the toxicological risk of this formulation is reduced. On the other hand the use of Aristolochia indica powder was reported in estimated total doses as high as 90 g of powder. In these cases the toxicity of the plant is not reduced due to its processing method. In addition, the age of the plant material influences the toxicological risk. Aristolochic acid (I and II) contents in fruits, leaves and young stems are higher and young plants can therefore be associated with higher toxicological risk. It is noteworthy that some of the doses reported in this study are comparable to the cumulative doses, which were reported for patients of the Belgian cohort who developed urethelial carcinoma after taking Chinese slimming pills (around 200 g of Aristolochia fangchi Wu ex L.D. Chow & S.M. Hwang) (Nortier et al., 2000). A cumulative dose of 200 g of A. fangchi contains approximately 130 mg aristolochic acid. Furthermore this study showed that replacements of Aristolochia indica with other medicinal plants such as Rauvolfia serpentina (L.) Benth. ex Kurz are common. Therefore it is likely that the problem exists vice versa. I.e. Aristolochia indica plant material is used as a replacement for other herbal medicines.

Taking this into consideration it is extremely likely that aristolochic acid nephropathy cases have occurred in Bangladesh and that AAN is an overlooked public health problem.

4. Conclusions

AAN and aristolochic acid associated UUC has been identified as an important health risk in some countries, but the wide distribution of the genus globally requires a systematic assessment of regions where AAN or UUC may be of concern. Here we demonstrate that Aristolochia indica is widely available on herbal markets and through healers’ networks in Bangladesh, a country from which so far no cases of AAN have been reported. Our results indicate that knowledge among kavirajes about the health risks associated with its use is extremely limited.

Using a novel approach of directly combining an ethnobotanical study with a metabolomic analysis we were able to provide an assessment of potential risks associated with the use of Aristolochia indica in Bangladesh. Based on the data obtained using this ethnobotanical–phytochemical approach it is likely that aristolochic acid nephropathy cases occur in Bangladesh creating an important public health problem. Consequently future research needs to focus on an epidemiological assessment of AAN and UUC in populations potentially at risk in Bangladesh.

This work also highlights that awareness needs to be raised about the health risks associated with the use of Aristolochia indica and that there is a need for intervention by public health authorities. Since Aristolochia species are commonly used in many parts of the world including India, Africa and Central America, it is likely that so far their effects on public health in these regions have been overlooked. Therefore, this case study has wider implications and should be expanded to other countries.

There is a need to carefully study supply and value chains in herbal medicines and the impact this has on the products composition, quality and safety (Booker et al., 2012). Overall, this study demonstrates the value of using an ethnopharmacological approach combining ethnobotanical fieldwork with a metabolomic analysis in addressing one of the key concerns in the area of medicinal plant research – the exposure of endogenous toxins found in a few taxa commonly used local and traditional medical systems.

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