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ORIGINAL PAPER

Balkan endemic nephropathy and aristolochic acid I: an investigation into the role of soil and soil organic matter contamination, as a potential natural exposure pathway

Alexandra T. Gruia · Camelia Oprean · Alexandra Ivan · Ada Cean · Mirabela Cristea · Lavinia Draghia · Roxana Damiescu · Nikola M. Pavlovic · Virgil Paunescu · Calin A. Tatu

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Abstract Aristolochic acids (AAs) are carcinogenic and nephrotoxic plant alkaloids present in *Aristolochia* species, used in traditional medicine. Recent biomolecular and environmental studies have incriminated these toxins as an etiological agent in Balkan endemic nephropathy (BEN), a severe kidney disease occurring in the Balkan Peninsula. The questions on how the susceptible populations are exposed to these toxins have not yet been clearly answered. Exposure to AAs through the food chain, and environmental pollution (soil/dust), could provide an explanation for the presence of BEN in the countries where no folkloric use of the plant has been documented (Bulgaria, Croatia). Additional exposure pathways are likely to occur, and we have shown previously that AAs can contaminate crop plants through absorption from soil, under controlled laboratory environment. Here, we attempt to provide additional support to this potential exposure pathway, by revealing the presence of AAI in soil and soil organic matter samples collected from BEN and non-BEN areas. The samples were processed in order to be analyzed by high-pressure liquid chromatography, and ion trap mass spectrometry. Our results showed the presence of AAI in small concentrations, both in BEN and non-BEN soils, especially where *Aristolochia* plants and seeds were present.

A. T. Gruia · C. Oprean · A. Ivan · A. Cean · M. Cristea · L. Draghia · R. Damiescu · V. Paunescu · C. A. Tatu
OncoGen Centre, County Hospital "Pius Branzeu", Blvd. Liviu Rebreanu 156, 300736 Timisoara, Romania

C. Oprean (✉)
Department of Environmental and Food Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy "Victor Babes", Eftimie Murgu Sq. 2, 300041 Timisoara, Romania
e-mail: camelia.oprean@umft.ro

A. Ivan · C. A. Tatu
Department of Biology and Environmental Health, University of Medicine and Pharmacy "Victor Babes", Eftimie Murgu Sq. 2, 300041 Timisoara, Romania

L. Draghia · V. Paunescu
Department of Immunology, University of Medicine and Pharmacy "Victor Babes", Eftimie Murgu Sq. 2, 300041 Timisoara, Romania

N. M. Pavlovic
Serbian Medical Society, Branch Nis, Nis, Serbia

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Introduction

Balkan endemic nephropathy (BEN) is a dreadful kidney disease first described in the late 1950s and affecting discrete rural communities from the Balkan Peninsula (Serbia, Croatia, Bosnia, Romania, Bulgaria). Its etiology is still a matter of scientific debate (de Jonge and Vanrenterghem 2008; Mantle 2016); however, it is generally accepted that the disease is caused by certain environmental factors acting on a predisposing genetic background (Tatu et al. 1998; Stefanovic et al. 2006; Voice et al. 2006; O'Brien and Dietrich 2005; Clark and Snedker 2006; Kazantzis 1967; Ivić 1969; Dimitrov 2002; Trnacevic et al. 2002; Gluhovschi et al. 2002, 2011). During the past six decades the incidence and prevalence of the disease have varied from one endemic area to another but the historically described disease foci are still active, with new cases being diagnosed each year in the afflicted countries (Gluhovschi et al. 2011; Craciun and Rosculescu 1970; Tatu et al. 1998; Voice et al. 2006; Long and Voice 2007). Peculiar environmental factors (aristolochic acids, Pliocene lignite-derived compounds, mycotoxins) are likely candidates; however, although evidence has been gathered to make their implication in BEN more than likely, there are still major questions to be answered. For instance, a valid exposure pathway to aristolochic acids is still a matter of speculation and debate and there is a critical lack of a statistically justifiable evidence of exposure differences in BEN-endemic areas compared to nonendemic areas (Voice et al. 2006; Long and Voice 2007). For the Pliocene coal-derived compounds contaminating the drinking ground water supply sources, the toxicological data are still fragmentary and insufficient (Orem et al. 2007). Among the nephrotoxic mycotoxins, ochratoxin A (OTA) seemed like a suitable candidate for BEN causation; however, although it could be responsible to some extent for the BEN-associated urothelial carcinogenesis, it cannot really explain the mosaic distribution of the disease (Mantle et al. 2011). Moreover, BEN might not even be a unique clinical entity along the afflicted areas and consequently, its etiology could be heterogenous as well (Mantle et al. 2011).

Most of the current research pertaining to BEN etiology is focused on the role aristolochic acids could play in causing the kidney damage, the chronic renal failure and the associated urinary tract tumors typically characterizing the disease. Aristolochic acids (AAs) are nitrophenanthrenic alkaloids found in *Aristolochia* species, and they are natively present in two different structural forms in various anatomical parts of the plant (the seeds having the highest concentration). The most abundant is AAI (Stiborová et al. 2016), a toxin found to have carcinogenic, nephrotoxic and mutagenic effects (Martincic 1957; Arlt et al. 2007; Vanherweghem et al. 1993; Depierreux et al. 1994; Cosyns et al. 1994; Gillerot et al. 2001; Mengs 1983). In the early 1990s, AAs have acquired a nefarious reputation by causing an outbreak of a few hundred cases of chronic renal failure and urothelial cancer in Europe. The cause of the outbreak was the consumption by young women of a herbal weight loss product sold in Belgium and accidentally contaminated with *Aristolochia fangchi* plant parts. The disease is now termed aristolochic acid nephropathy (AAN) and has been reported to occur in a few countries around the world (Voice et al. 2006). Grollman's molecular and epidemiological studies on endemic nephropathy patients from Croatia (Grollman et al. 2007) describe the similarity of pathophysiological features between BEN and AAN (Grollman et al. 2007; Grollman and Jelakovic 2007; Cosyns et al. 1994), thus confirming previous studies (Ivic and Lovic 1967; Ivić 1969) tentatively linking *Aristolochia clematitis* to BEN etiology.

Even though aristolochic acids were demonstrated to be toxic to the kidneys, *Aristolochia* is still widely used in tea infusions, cataplasms or enemas for its alleged therapeutical effects in various diseases (Rücker and Chung 1975; Priestap 1987; Gluhovschi et al. 2010). Dried *A. clematitis* leaves can be bought from farm markets around the year, or certain drugstores selling natural remedies, at least in Romania. However, in an attempt to quantify *Aristolochia* plant use in relation to BEN causation, Gluhovschi et al. (2010) could not find any significant epidemiological difference in terms of ethnobotanical exposure between the endemic and the nonendemic settlements, nor they could detect any AA in serum samples from BEN patients and exposed controls.

According to this study, the 'ethnobotanical route' most likely provides an exposure level to AAs too low to be of any toxicological relevance in terms of BEN etiology.

Although *A. clematitis* is widely present in the endemic, as well as nonendemic locations, growing as a weed plant, a definitive human exposure pathway to the aristolochic acid toxins, universal for all the BEN-affected areas, has not been proved yet (Long and Voice 2007; Mantle 2016).

Some early theories about AA exposure pathways were developed by Kazantzis (1967), who first suggested the possibility of flour contamination with AA from the plants that grow in wheat fields. This theory was later developed by Ivić (1969) who proposed that ingestion of flour contaminated with *A. clematitis* seeds may determine BEN. He noted that seeds from these plants commingled with wheat grain during the harvesting process, but his field surveys and data failed to provide convincing evidence (De Broe 2012).

In order to solve the aristolochic acids exposure pathway puzzle, some researchers have taken into consideration a possible AAs intrusion into the human food chain through contamination of soils and plant cultures (Pavlović et al. 2013, 2008; Li et al. 2016; Chan et al. 2016), *Aristolochia* plants being found especially in close proximity to or even growing among the plants from crop fields (e.g., corn, wheat) and farm gardens (e.g., cucumber). Whole plant or parts of it (e.g., seeds) could be collected simultaneously with the products resulted from cultures (e.g., wheat, corn, cucumbers.), or crop plants could uptake the AAs from soil (Pavlović et al. 2013).

The various exposure pathways discussed forward (i.e., natural remedy use, flour contamination, crop/-food chain contamination, dust) are not mutually exclusive. People could get exposed to the same one toxin (aristolochic acid) through multiple routes, depending on the geographical region, agricultural, social, geochemical and last but not least, cultural, conditions. The intensity of one versus another exposure pathway, profiled on a peculiar genetic susceptibility of the vulnerable population, could make the difference between becoming ill with the disease or not.

The present study covers one of the hypotheses that AA might contaminate garden and crop soils. Furthermore, we advance and bring some preliminary support to a new hypothesis: exposure to the surface layers of soil (having a high potential of dust aerosolization) contaminated with aristolochic acids of plant provenience, as an additional pathway contributing to the pathology of BEN. Accordingly, this study sought to answer the following questions: (1) Could the presence of A*ristolochia* plant influence the AA soil/soil organic matter composition? and (2) is there a difference between endemic and nonendemic soil composition in terms of AA contamination?

Materials and methods

Reagents

Aristolochic acid I (purity HPLC $\geq$ 90%) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Formic acid (for synthesis) and ortophosphoric acid (p.a. grade 85%) were purchased from Merck (Darmstadt, Germany). All solvents for liquid chromatography and mass spectrometry (MS) analysis (water, methanol, acetonitrile) were Chromasolv, HPLC grade and MS grade, respectively, and were purchased from Sigma-Aldrich.

Instrumentation

High-pressure liquid chromatography (HPLC) analysis of samples was performed on an Agilent 1100 Series system coupled with an UV–VIS detector (Agilent Technologies, Palo Alto, CA, USA). Mass spectrometry analysis was performed on a Bruker mass spectrometer (MS) amaZon SL with ion trap (Bruker Daltonik GmbH, Bremen, Germany).

Fieldwork and sample collection

Sample collection was based on several selection criteria, which are presented and explained in detail below:

- Types of geographic areas: endemic and nonendemic;
- Dry periods, without rain for two consecutive weeks;
- Time frames related to plant growth and vegetation: autumn and spring;
- Samples location: gardens and farm crop fields;

- Types of samples: 1. soil organic matter (SOM) (top 5 cm); 2. soil samples from a depth of 5–20 cm.
- Presence and absence of *A. clematitis* plants;

For sampling during our field trip sessions, we selected BEN villages based on the number of patients diagnosed with the disease. Two endemic areas were historically described in Romania: one in Mehedinti County, which is still active and where new BEN patients are still reported each year; a second, smaller and non-active, endemic area, in Caras-Severin County, where no new BEN patients were identified during the last two decades or so. Irrespective of the endemic area, we noticed the presence of *A. clematitis* in similar abundances in all the sampled villages (Erghevita, Poroina and Bistrita from Mehedinti, and Secaseni from Caras-Severin). Moreover, *Aristolochia* plant is also widely spread around nonendemic areas such as Sasca Romana, Plugova and Ghioroc villages included in the present study and located entirely outside the BEN areas. All samples' collection was made in each sampling season from the same location of each garden or crop field, as specified in Table 1. The chosen sampling sites (private household gardens and/or crop fields located in the vicinity of the villages) are representative for all the endemic and nonendemic locations we have visited, and where *Aristolochia* contamination is usually present. People use such gardens/fields in the Balkans mostly for subsistence agriculture, growing vegetables for their own consumption, and to a lower extent, staple cereals for their farm animals.

Soil and soil organic matter sample collection was performed along three distinct field trips, during dry seasons (i.e., characterized by lack of rainfall for the previous 2 weeks) of 2015 and 2016, but also taking into account the life cycle and vegetation period of the *A. clematitis* plant: 1. autumn (November), when the plant starts to decay; 2. autumn (September), a season when the plant has ripe fruits, and some plants are also at the second or third vegetation cycle during the year (Fig. 1); spring (May) a season when *Aristolochia* plant is young and starts the flowering process.

Because *Aristolochia* plant grows as a weed especially on cultivable, disturbed, soils, we collected samples from gardens, as well as farm fields, characterized by the presence, or the absence, of the weed. Negative control soil samples were the ones collected from endemic and nonendemic areas, where *Aristolochia* plant was absent, at least at the time of collection. There were also negative control samples collected from nonendemic areas, where the plant is absent and there was no knowledge of its presence during the last 30 years.

We collected samples from the first two types of soil layers: the first layer, soil organic matter (SOM) is the surface layer of the first 5 cm (Fig. 2); the second layer, soil from a depth of 5–20 cm.

In order to prevent sample contamination and a possible cross-contamination, the collection tools were precleaned in the laboratory using high-grade methanol and ultrapure MilliQ water, and a decontamination was made on site as well, and thus, the scoop, with which samples were collected, was rinsed with methanol and washed three times with MilliQ water before and after samples' prelevation. Each soil sample was carefully double-bagged in ziplock bags (Fig. 2), closed with a minimum presence of air and coded, put it in a cooler box at 4 °C, transported to the laboratory, and preserved at – 20 °C. Voucher specimens of all analyzed samples were stored at – 80 °C for reference. All samples were collected on sunny, dry, days with relative atmospheric humidity of around 50%. We also mentioned in Tables 1 and 2 that some soil samples are collected from the close proximity of corn and grass. Data about the collected samples are presented in Table 1. All endemic and nonendemic locations are found in Romania.

Samples extraction method

Samples were extracted using a method adapted after Trujillo et al. (2006). All samples were dried at 60 °C before extraction. If *Aristolochia* seeds (intact or fragments) were visually identified in the collected soil samples, these were carefully removed before sample extraction. Five grams of soil were extracted with 3 mL of extraction solvent. Each sample was extracted in a number of n replicates, according to Table 1. The extraction solvent was 80% methanol and a 20% aqueous solution of 10% formic acid; all aqueous solutions were made in distilled water. The solid–liquid mixture was shaken at 1400 rpm for 24 h, and then the supernatant was separated from the residue by centrifugation at 4000 rpm for 10 min. A volume of 2 ± 0.2 mL of supernatant was collected with a syringe from each sample and then filtered

Table 1 Sample type and location description. Both endemic and nonendemic sites were visited during the three field trips performed (November 2015, May 2016, September 2016), and multiplicates of samples were collected at the same site, in order to cover for the random environmental distribution of the aristolochic acid contamination

Time of collection	Endemic/ nonendemic location	Number of replicates	Presence (+)/ absence (−) of *Aristolochia* plants/seeds	Sample material collected	Sample location
November 2015	Endemic	$n = 11$	−	Soil	Garden 1, Erghevita, Mehedinti County
		$n = 3$	−	Soil, corn	Garden 1, Erghevita, Mehedinti County
		$n = 9$	+	Soil	Garden 2, Erghevita, Mehedinti County
		$n = 3$	+	Soil, grass	Garden 1, Erghevita, Mehedinti County
		$n = 9$	−	Soil	Garden, Secaseni, Mehedinti County
	Nonendemic	$n = 8$	+	Soil	Crop field, Plugova, Caras-Severin County
		$n = 9$	−	Soil	Garden, Sasca Romana, Caras-Severin County
		$n = 9$	+	Soil	Crop field, Sasca Romana, Caras-Severin County
		$n = 8$	+	Soil	Garden, Ghioroc, Arad County
		$n = 6$	−	Soil	Garden 1, Timisoara, Timis County
		$n = 4$	−	Soil	Garden 2, Timisoara, Timis County
May 2016	Endemic	$n = 4$	+	Soil	Garden 1, Erghevita, Mehedinti County
		$n = 4$	+	Soil, corn	Garden 1, Erghevita, Mehedinti County
		$n = 6$	−	Soil	Garden 1, Erghevita, Mehedinti County
		$n = 6$	+	SOM	Garden 1, Erghevita, Mehedinti County
		$n = 6$	−	SOM	Garden 1, Erghevita, Mehedinti County
		$n = 4$	+	Soil	Garden 2, Erghevita, Mehedinti County
		$n = 3$	−	Soil	Crop field, Poroina, Mehedinti County
		$n = 6$	−	SOM	Crop field, Poroina, Mehedinti County
	Nonendemic	$n = 3$	−	Soil, corn	Crop field, Plugova, Caras-Severin County
		$n = 4$	+	Soil, corn	Crop field, Plugova, Caras-Severin County
		$n = 4$	−	Soil	Garden 2, Timisoara, Timis County
September 2016	Endemic	$n = 12$	−	Soil	Garden 1, Erghevita, Mehedinti County
		$n = 12$	−	SOM	Garden 1, Erghevita, Mehedinti County
		$n = 3$	+	SOM	Garden 1, Erghevita, Mehedinti County
		$n = 12$	−	Soil	Garden 1, Erghevita, Mehedinti County
		$n = 6$	+	Soil	Garden 2, Erghevita, Mehedinti County
		$n = 6$	+	Soil	Garden 1, Bistrita, Mehedinti County
	Nonendemic	$n = 12$	+	Soil	Crop field, Plugova, Caras-Severin County
		$n = 4$	−	Soil	Garden 2, Timisoara, Timis County

through a 0.45 μm polyethersulfone (PES) filter prior to HPLC and MS injection (Trujillo et al. 2006; Liu et al. 2010).

HPLC analysis

All samples were analyzed by high-pressure liquid chromatography (Trujillo et al. 2006), on an ODS Ultrasphere Coulter C18 column (dp 5 μm, 4.6 mm, 250 mm length) at 40 °C temperature column, using a solvent gradient with a mobile phase of HPLC-grade water at a pH of 3 (acidified with 85% ortophosphoric acid) and acetonitrile. The mobile phase programme started with a ratio of 80% water and 20% acetonitrile, the ratio being subsequently changed at a constant rate to 30% water and 70% acetonitrile at 25 min and 100% acetonitrile at 30 min. The eluent flow rate was constant at 0.5 mL/min, the detection wavelength was

Fig. 1 Soil organic matter (SOM)/soil prelevation in the presence of *Aristolochia* plants and seeds, in an endemic village garden (Erghevita, Romania) in September 2016. A 3rd generation of *Aristolochia* plant can be observed, as well as ripe seeds from older plants

Fig. 2 Soil organic matter (SOM) prelevation from a garden in an endemic area (Erghevita village, SW Romania) in May 2016; *Aristolochia* plant growing near young corn plants

set up to 390 nm and the injected sample volume was 20 µL. Each sample was injected three times, and the results are presented as average ± standard deviation (SD).

In order to identify the presence of aristolochic acid I (AAI) in samples, AAI standard was injected under the same conditions as the samples. Standard dilutions from 7.14 µg/mL to 140 ng/mL were made from a stock solution of 71.43 µg/mL in MS-grade acetonitrile, and a calibration curve was generated in order to quantify AAI in samples. All dilutions were made in the same solvent mixture as the one used for sample extraction. The limit of quantification was 140 ng/mL, while the limit of detection was 100 ng/mL.

Mass spectrometry analysis

In order to confirm the mass of AAI, all samples were analyzed by direct injection into the mass spectrometer. All mass spectra data were analyzed with Bruker Compass 1.5 DataAnalysis software version 4.1 (Bruker Daltonik GmbH, Bremen, Germany). Mass spectrometer settings were established after a continuous direct infusion of the AAI standard, with a flow rate of 5 µL/min, and a manual scan in positive electrospray ionization mode with enhanced resolution was found to be a sensitive method. Ion source conditions were set up as following: capillary exit 140 V, dry temperature 180 °C, nebulizer 7.30 psi, dry gas 4 L/min, capillary voltage 4500 V, high voltage (HV) end plate offset 500 V. The range for the full ESI scan was set between 200 and 400 m/z. After an injection of AAI, a signal of 364 m/z was observed, and thus all samples were analyzed by single ion monitoring (SIM) mode; 364 m/z represents the molecular weight of AAI (341 g/mol) conjugated with one sodium ion (23 g/mol) $[M + Na]^+$.

Results

In order to detect and quantify the presence of AAI, all extracted samples were analyzed by high-pressure liquid chromatography coupled with UV detection. The quantification of AAI was based on the calibration curve, which was made starting from a stock solution of AAI standard in acetonitrile and some serial dilutions in extraction solvent (Fig. 3A). The calibration curve was linear in the range of 140 ng/mL–7.14 µg/mL with a precision and accuracy less than 15% both for the limit of quantification and for the other concentrations. Regression coefficient was higher than 0.99, and the recovery rate was 84.95% for a concentration of 357 ng/mL. The specificity of the method was confirmed by mass spectrometry detection (Fig. 3C).

We were able to detect and quantify the aristolochic acid I at nanogram (part per trillion) levels, with a

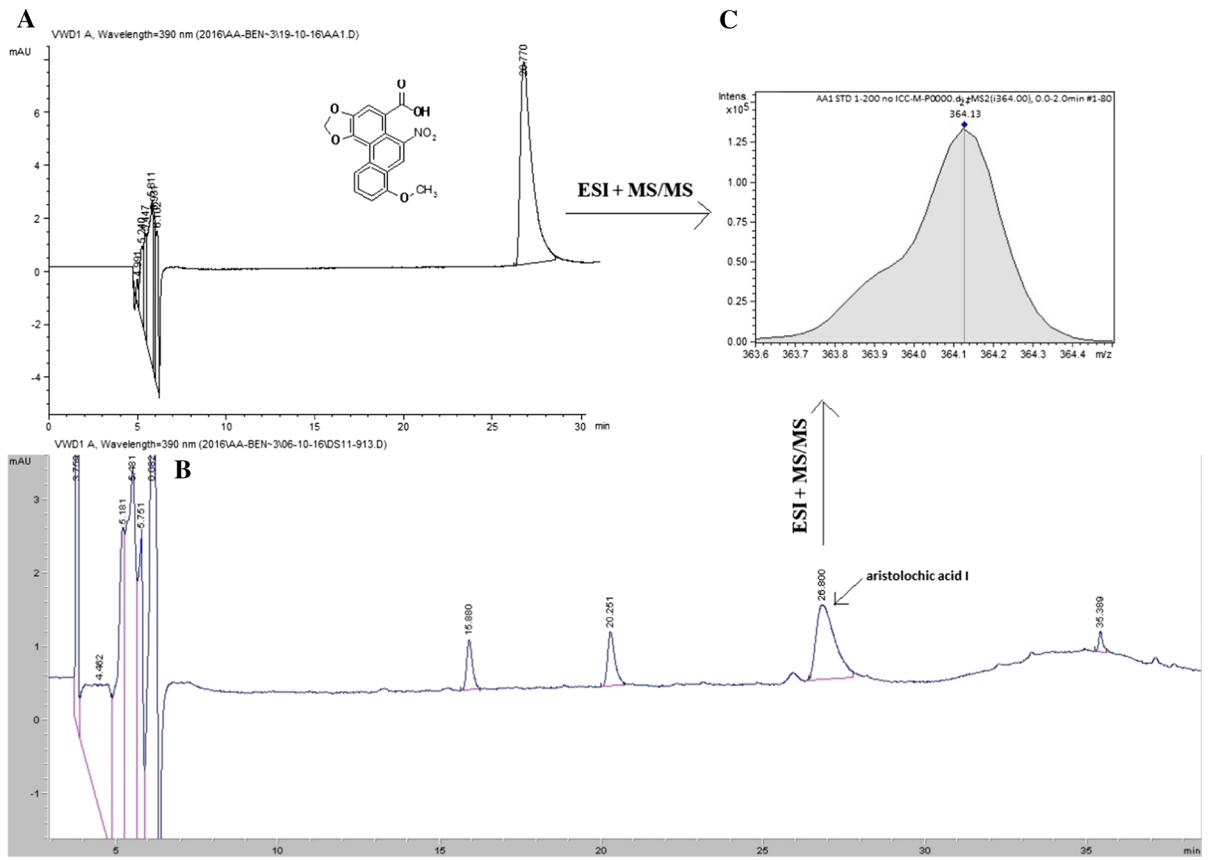

Fig. 3 AAI chromatography analysis: **A** HPLC–UV AAI standard dilution analysis; **B** chromatogram of soil organic matter sample from a garden in Erghevita, an endemic village in Romania, sample collected with seeds in September 2016. The presence of AAI could be detected at a RT of 26.800 min as indicated by the arrow. **C** ESI + MS/MS spectra of AAI extracted ion chromatogram of the $[M + Na]^+$ ion of AAI (m/z 364)

range of concentrations from 140 to 606 ng/mL of soil/SOM extract, in endemic, but also in nonendemic, soils. The presence of AAI was detected in most of the samples where *A. clematitis* plant and seeds could be visually identified (Fig. 3B). However, certain soil samples collected in the close proximity of decayed plant parts or seeds did not show any detectable AAI. On the other hand, we were able to detect AAI in soil samples without *Aristolochia* plant growing or decaying in the proximity.

After AAI UV detection, a mass confirmation was necessary; thus, a sensitive method was employed, using an ion trap mass spectrometer. When a dilution of 1:200 AAI in extraction solvent (concentration of 357 ng/mL) was injected directly into the mass spectrometer in the positive ionization mode, the analytes yielded predominantly $[M + Na]^+$ ions at m/z 364 (Fig. 3C). For a much better sensitivity, the ion trap was closed for a couple of seconds at a time, in order to accumulate a significant number of AAI ions, thus leading to a higher intensity signal. MS/MS spectra of samples were compared to those obtained for AAI and were identical.

A description of the analyzed samples is detailed in Table 2.

During the three field trips performed in 2015–2016, we collected 125 samples from endemic areas and 71 samples from nonendemic areas; 35 samples from endemic areas and 4 from nonendemic areas were found positive for AAI.

Most of the samples where AAI was found were SOM/soil samples from endemic areas; also in these samples, we found the highest concentration levels of AAI (up to 606 ng/mL SOM extract), some of the samples being collected in the presence of *Aristolochia* plant, but without any apparent

Table 2 Types of samples collected and analyzed for the presence of aristolochic acid I

Time of collection	Endemic/ nonendemic location	Presence (+)/absence (−) of *Aristolochia* plants/seeds	Material collected	Number of replicates	Number of samples with AAI	AAI (ng/ml)
November 2015	Endemic	−	Soil	$n = 11$	$n = 0$	–
		−	Soil, corn	$n = 3$	$n = 0$	–
		+	Soil	$n = 9$	$n = 1$	172.815
		+	Soil, grass	$n = 3$	$n = 3$	BDL
						BDL
						BDL
		−	Soil	$n = 9$	$n = 1$	BDL
	Nonendemic	+	Soil	$n = 8$	$n = 1$	BDL
		−	Soil	$n = 9$	$n = 0$	–
		+	Soil	$n = 9$	$n = 0$	–
		+	Soil	$n = 8$	$n = 1$	BQL
		−	Soil	$n = 6$	$n = 0$	–
		−	Soil	$n = 4$	$n = 0$	–
May 2016	Endemic	+	Soil	$n = 4$	$n = 1$	BDL
		+	Soil, corn	$n = 4$	$n = 0$	–
		−	Soil	$n = 6$	$n = 0$	–
		+	SOM	$n = 6$	$n = 0$	–
		−	SOM	$n = 6$	$n = 2$	BDL
						BDL
		+	Soil	$n = 4$	$n = 2$	BDL
						BDL
		−	Soil	$n = 3$	$n = 1$	BDL
		−	SOM	$n = 6$	$n = 0$	–
	Nonendemic	−	Soil, corn	$n = 3$	$n = 1$	BDL
		+	Soil, corn	$n = 4$	$n = 1$	BDL
		−	Soil	$n = 4$	$n = 0$	–
September 2016	Endemic	−	Soil	$n = 12$	$n = 5$	BDL
						BDL
						BDL
						170.158
						BDL
		−	SOM	$n = 12$	$n = 8$	BDL
						BDL
						326.755
						303.076
						351.054
						BDL
						BQL
						BDL
		+	SOM	$n = 3$	$n = 3$	605.829
						BQL
						BQL
		−	Soil	$n = 12$	$n = 2$	BDL
						BDL
		+	Soil	$n = 6$	$n = 5$	BDL
						BDL
						BDL

Table 2 continued

Time of collection	Endemic/ nonendemic location	Presence (+)/absence (−) of *Aristolochia* plants/seeds	Material collected	Number of replicates	Number of samples with AAI	AAI (ng/ml)
						BDL
						BDL
		+	Soil	$n = 6$	$n = 1$	BDL
	Nonendemic	+	Soil	$n = 12$	$n = 0$	–
		−	Soil	$n = 4$	$n = 0$	–

SD $\leq \pm$ 35 ng/mL; *BDL*-below detection limit; *BQL*-below quantification limit. Presented results are the average value of three separate injections of the same sample (replicates)

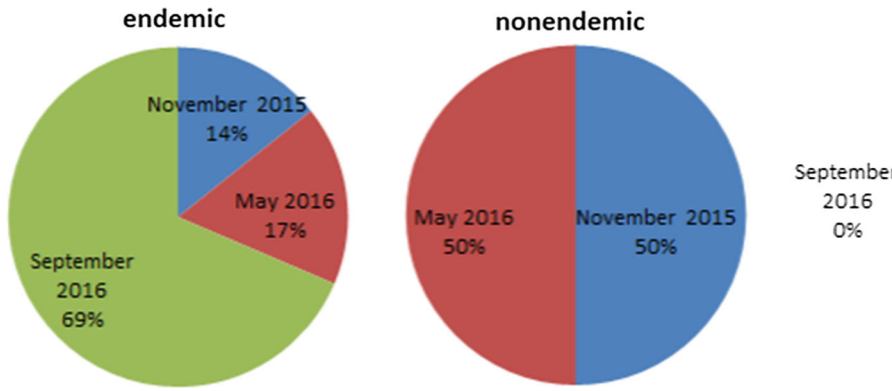

Fig. 4 Percentage of AAI-positive samples from endemic (**a**) and nonendemic (**b**) areas collected during the three field trips

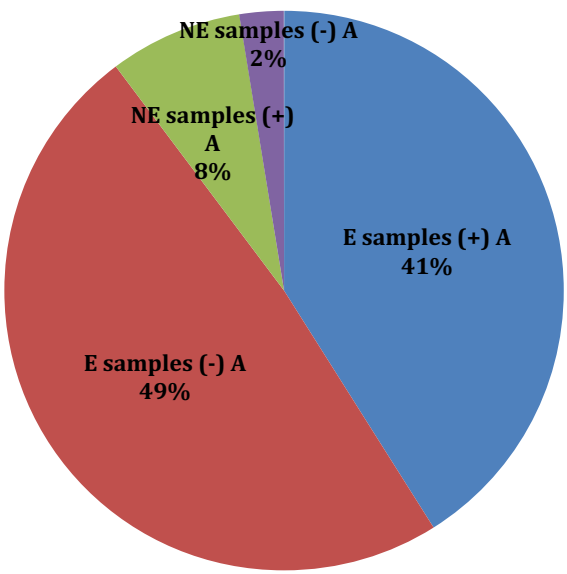

Fig. 5 Percentage of endemic (E) and nonendemic (NE) samples with or without *Aristolochia*

proportionality connection between the presence of the plant/seeds and the AAI concentration level.

Probably due to certain seasonal variation factors, no AAI-positive samples from nonendemic areas were found in September 2016, while for the endemic areas, 69% of all AAI-positive samples were collected during the September field trip (Fig. 4). Overall, there is a higher number of AAI-positive samples from endemic areas, samples collected from places with *A. clematitis* plant being visually spotted, or being absent (Fig. 5).

Discussion

Our current study has addressed a possible exposure pathway to AA, that is the natural contamination of crop soils and gardens. The fact that AAI can be detected in soil/soil organic matter samples from both endemic and nonendemic areas, sometimes in similar concentrations, raises again the question if aristolochic acid is the sole factor responsible for BEN. Although *A. clematitis* grows all across the Balkan Peninsula, and other species (e.g., *Aristolochia indica*) from the same genus (*Aristolochia* sp.) grow in many other

parts of the world, (e.g., India), which all contain the same aristolochic acids (Michl et al. 2013), it is possible that certain local environmental factors specific only for the BEN areas could be controlling the levels of exposure for the affected population. Of course, other cofactors are likely to play a crucial role: a certain genetic susceptibility in the xenobiotic metabolising genes, possible epigenetic factors, coal-derived contaminants of the drinking water supplies (Orem et al. 2007), mycotoxins and others.

Taking into account the fact that organic compounds can actively and passively circulate from plants to soil and viceversa (Dettenmaier 2008), the AA could also enter the soil through several pathways: pass from the plant root cell; from a plant during the decaying process; or from the seeds which fall on the ground surface when they mature. And furthermore, this process of circulating organic compounds from plants to soil could be bidirectional, and these compounds could be picked up by the plants of provenience or by other plants from the vicinity. Soil as a structure has multiple layers, but the first two are playing an important role in ecosystem's dynamics (Mehrabanian 2013). Also the agricultural processes, like digging and plowing, contribute to the AA spreading, both in soil and air (with the potential respiratory airway exposure), this compound having a high biogeochemical stability (Tangtong 2014).

However, finding AAI contamination in soil and SOM samples does not provide a quantitative measure for the respiratory exposure route per se. These first two layers of soil, which encompasses AA, could be a source of dust or air particulate generation, leading to respiratory exposure in the local inhabitants. In this regard, we have performed an additional, air sampling study, using advanced high volume total particulate aerosol sampling, a method that will provide new, quantitative insights into the potential respiratory exposure route (manuscript in preparation).

Most publications related to the topic describe the high frequency and abundance of *Aristolochia* in endemic areas, and thus the hypothesis that AAs can lead to crops contamination and enter the food chain inadvertently (Ivic 1969; Grollman et al. 2007; Arlt et al. 2007; Stiborová et al. 2016; Pavlović et al. 2008, 2013; Li et al. 2016; Chan et al. 2016). However, based on our field observations, the weed can be present with similar frequency and density in many BEN-free areas as well, or not found at all in some BEN areas, raising the legitimate question why the disease is geographically limited only to the historically described areas of the afflicted Balkan countries, or if it really has the claimed impact in inducing BEN. Other, even more puzzling, field observations show that in non-BEN areas, where *Aristolochia* plant is widely spread, there are no reported BEN patients, even if the population uses the plant for decoction preparation, in a similar manner to the people from endemic villages. Furthermore, some non-BEN locations are in very close proximity to BEN locations but have never had BEN patients.

Considering the omnipresence of *A. clematitis* in many fields and gardens in both the endemic and nonendemic areas, and taking into account the similar social and cultural habits in regard to the medicinal use of *Aristolochia* and agricultural practices, a differential exposure could be explained by certain local environmental factors, related to soil biogeochemistry, dust generation potential, local hydrogeochemistry, etc. Such factors could also control the selective bioavailability of aristolochic acid to absorption by crop plants and influence the level of contamination of the human food chain. We have demonstrated a selective uptake of AAs by maize and cucumber under controlled laboratory conditions (Pavlović et al. 2013), while Long and Voice (2007), discussing about the environmental mobility of aristolochic acids, mentioned that AAs are slightly soluble at a pH of 6 or above, values that are specific to agricultural soil (Chemical Abstracts Registry); this fact suggests that, under the right pedological conditions, a variable AA release from the seeds and uptake by other plants could occur, making differential exposure possible and confining BEN geographically.

Conclusions

Using sensitive liquid chromatography and ion trap mass spectrometry methods, we were able to demonstrate in our study soil and soil organic matter contamination with aristolochic acid I, a potent nephrotoxin and carcinogenic substance claimed to be the culprit for Balkan endemic nephropathy and other kidney diseases. Both endemic and nonendemic areas were investigated for the presence of AAI, and it has become apparent that the environmental contamination levels in endemic versus nonendemic areas are

to some extent similar. A key factor for the presence of AA in soils is the local presence of the *Aristolochia* plants or parts of it. Another observation, based on the experimental studies performed in the current work, is that a high degree of AAI soil contamination was found in September, probably because of the higher abundance of the plants in multiple stages of vegetation, and/or due to more seeds being present, but all these claims necessitate further confirmation.

Finding AAI in SOM samples could provide support for an additional potential exposure pathway to AAs, through the respiratory route, via dust generated from SOM; such an exposure could act in addition to other voluntary (dietary or medicinal use) or involuntary (food chain contamination) exposure pathways to aristolochic acids. The potential for dust formation in the endemic (but also nonendemic) regions is high, and it is likely that certain agricultural activities, like manually plowing the gardens (still common practice in many villages in Romania and Serbia), could facilitate exposure to air particulates carrying adsorbed aristolochic acids. Each of the above-mentioned pathways may have a partial contribution to the critical concentration threshold necessary for the AAs to provoke kidney damage and other health effects, through a cummulative action, but more extensive research will be needed in order to quantify the significance of each exposure route. Aristolochic acid-induced kidney disease might have transcended the boundaries of the Balkan Peninsula, turning into a global medical threat (Grollman 2013). Chronic renal failure is a serious disease and, in the case of AAs at least, a disease that can be prevented or averted. Wherever kidney disease due to aristolochic acid is suspected, exposure of any kind—dietary, iatrogenic, environmental—should be evaluated by establishing the abundance and prevalence of *Aristolochia* plants in gardens and crop fields, correlated with the presence of aristolochic acids in soil, air particulates, crop plants (e.g., vegetables, wheat, corn, flour) and food items (e.g., flour, bread) and specific measures should be taken to minimize the risks of exposure.

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