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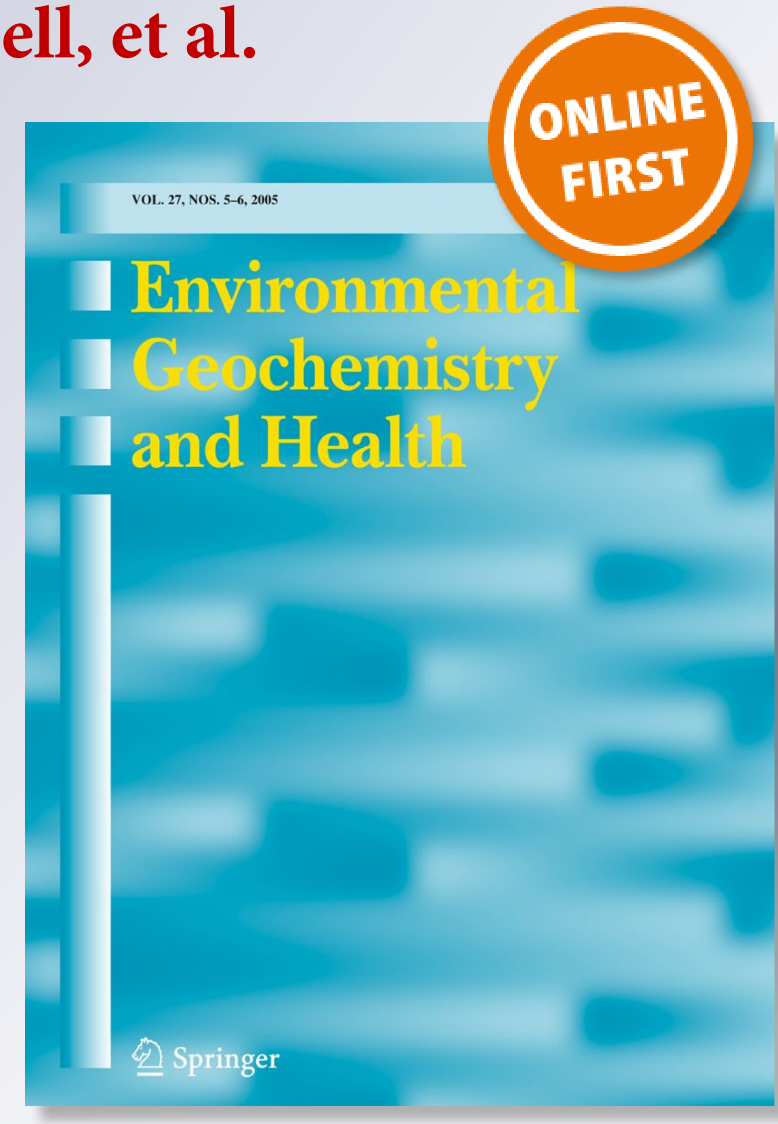
Environmental Geochemistry and Health
Official Journal of the Society for Environmental Geochemistry and Health

ISSN 0269-4042

Environ Geochem Health
DOI 10.1007/s10653-012-9477-8



Springer

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ORIGINAL PAPER

Possible health impacts of naturally occurring uptake of aristolochic acids by maize and cucumber roots: links to the etiology of endemic (Balkan) nephropathy

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Received: 21 March 2012 / Accepted: 20 July 2012

Abstract Aristolochic acids (AAs) are nephrotoxic and carcinogenic derivatives found in several *Aristolochia* species. To date, the toxicity of AAs has been inferred only from the effects observed in patients suffering from a kidney disease called "aristolochic acid nephropathy" (AAN, formerly known as "Chinese herbs nephropathy"). More recently, the chronic poisoning with *Aristolochia* seeds has been considered to be the main cause of Balkan endemic nephropathy, another form of chronic renal failure resembling AAN. So far, it was assumed that AAs can enter the human food chain only through ethnobotanical use (intentional or accidental) of herbs containing self-produced AAs. We hypothesized that the roots of some crops growing in fields where *Aristolochia* species grew over several seasons may take up certain amounts of AAs from the soil, and thus become a secondary source of food poisoning. To verify this possibility, maize plant (*Zea mays*) and cucumber (*Cucumis sativus*) were used as a model to substantiate the possible significance of naturally occurring AAs' root uptake in food chain contamination. This study

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showed that the roots of maize plant and cucumber are capable of absorbing AAs from nutrient solution, consequently producing strong peaks on ultraviolet HPLC chromatograms of plant extracts. This uptake resulted in even higher concentrations of AAs in the roots compared to the nutrient solutions. To further validate the measurement of AA content in the root material, we also measured their concentrations in nutrient solutions before and after the plant treatment. Decreased concentrations of both AAI and AAII were found in nutrient solutions after plant growth. During this short-term experiment, there were much lower concentrations of AAs in the leaves than in the roots. The question is whether these plants are capable of transferring significant amounts of AAs from the roots into edible parts of the plant during prolonged experiments.

Keywords *Aristolochia* · Aristolochic acid · Balkan endemic nephropathy · Roots · Maize · Cucumber · Uptake · Soil · Contamination · Biogeochemistry

Introduction

Aristolochic acids (AAs) are nephrotoxic and carcinogenic nitrophenanthrene carboxylic acid derivatives that naturally occur only in several *Aristolochia* species (spp) (notably *A. clematitis, A. contorta, A. debilis, A. fangchi, and A. manschuriensis*), *Bragantia spp*. or *Asarum spp* (IARC Monographs 2002), and in butterflies that sequester AAs as a chemical defense to predation (von Euw et al. 1968; Fordyce 2000). Aristolactams (compounds involved in the natural plant synthesis of aristolochic acids but also in their metabolic detoxification in animal species) have a broader botanical range; most aristolactams occur in the *Aristolochiaceae* family, but there are authenticated reports of their presence in some members of the *Annonaceae*, *Menispermaceae*, and *Monimiaceae* (Mix et al. 1982). *Aristolochia spp* are commonly used as supplements in traditional Chinese medicines, as well as in botanicals and botanical-containing dietary supplements (IARC Monographs 2002). Herbal remedies containing species of the genus *Aristolochia* were recently classified as carcinogenic to humans (Group 1 compounds) by the International Agency for Research on Cancer (IARC 2002).

In 1970, a report proposed that Balkan endemic nephropathy (BEN) may be caused by chronic poisoning from seeds of *A. clematitis* (Ivić 1970). It was shown that wheat fields in endemic areas have an unusually high abundance of *A. clematitis*, and Ivić suggested that wheat grain, flour, and even bread could be contaminated with seeds of *A. clematitis*, which may lead to BEN and associated urothelial tumors (Ivić 1970; Ivić and Lović 1967; Hranjec et al. 2005). These original findings were later confirmed by several authors (Grollman et al. 2007; Arlt et al. 2007; Stiborová et al. 2007). An outbreak of rapidly progressive renal disease in Belgium in the early 1990s involved at least 100 patients, mostly middle-aged women undergoing a weight-loss regimen that included the use of a mixture of Chinese herbs containing *Aristolochia spp.* (Vanherweghem et al. 1993). This syndrome was originally termed ‘Chinese herbs nephropathy’ (CHN), and later on as “aristolochic acid nephropathy” (AAN) (Gillerot et al. 2001). There have been quite a few early cases of urothelial cancer among Belgian patients suffering from CHN (Depierreux et al. 1994; Cosyns et al. 1994). A subsequent investigation led to the identification of AAs in the herbal mixture consumed by these patients. Specific AA–DNA adducts were found in the urothelial tissue specimens from all the urothelial cancer patients, providing conclusive evidence of exposure to plants of the genus *Aristolochia* (Bieler et al. 1997; Schmeiser et al. 1996).

These and many later reports have led to a heightened concern about the presence of AAs in botanicals and raised awareness of a serious health risk associated with AAs originating from the *Aristolochia* species. The similarities between CHN and BEN soon developed into a hypothesis of a common etiological agent for both diseases (Cosyns et al. 1994; Arlt et al. 2007).

Ivić (1970) earlier observed the presence of *A. clematitis* in agricultural wheat fields in BEN areas. While conducting field research related to BEN etiology, we similarly observed the widespread presence of *A. clematitis* in cornfields as well as in fields cultivated with other crops (Fig. 1), in both BEN, as well as non-BEN areas.

It is generally assumed that AAs can enter the human food chain only through herbs containing self-produced AAs by the voluntary use of the plant as a natural remedy or by the accidental contamination of

Fig. 1 *Aristolochia clematitis* growing in a wheat field in Romania

food products. We hypothesize that some crops grown in fields where *A. clematitis* grows, senesces, and decomposes during successive years might accumulate certain amounts of AAs from the soil through root uptake, and subsequently transfer it to other plant structures (trunk, leaves, fruit, or seeds). In this way, agricultural plants could become a secondary source for AA poisoning. To test this hypothesis, we cultivated maize plant and cucumber in a culture media containing AAs and examined plant structures for the presence of AAs using a sensitive HPLC–MS method.

Materials and methods

Plant material and growth conditions

Maize plants (*Zea mays* L. Va$_{35}$, Zemun Polje) were grown from seeds that were surface sterilized in 10 % hydrogen peroxide, washed in distilled water, and germinated on double layer filter paper moistened with 0.5 mM $CaSO_4$ in darkness at 27 °C. Three days after sowing, the seedlings were transferred to pots for hydroponic cultivation using 25 % Knopp nutrient solution, pH 5.6 (Douglas 1975).

Cucumber (*Cucumis sativus* L. cv. Chinese long) seeds were germinated on filter paper moistened with 2.5 mM $CaSO_4$, and after 5 days, the seedlings were transferred to a standard full strength (100 %) nutrient solution for hydroponic cultivation.

The plants were grown under controlled environmental conditions in a growth chamber with a light/dark regime of 16/8 h, temperature regime of 24/20 °C, photon flux density of 300 µmol × m^{-2} s^{-1} at plant heights, and relative humidity of about 70 %. The nutrient solutions were continually aerated and replaced every second day to avoid nutrient deficiencies and pH changes. After 7 days of preculture at optimal conditions, plants were subjected to aristolochic acid (6.43 µM Form I and 11.72 µM Form II) in the nutrient solution for 48 h. The aristolochic acid standard (a mixture of Form I and II) used in the experiments was obtained from Sigma (St. Louis, MO, USA).

Additional treatment was conducted by mixing 0.2 g pulverized seeds of *Aristolochia clematitis* per 1 L of nutrient solution. Controls consisted of plants from the same seed batches that were germinated and cultivated in the same way as the treatment plants but without AAs in the nutrient solution. Aristolochic acids are known to be very stable in aqueous-based solutions, (Sun et al. 2001) so any naturally occurring degradation of the AAs during the 48 h experiment would be insignificant.

Determination of AA content

After 7 days of preculture, and 48 h from the addition of aristolochic acid standards and/or pulverized *Aristolochia* seeds to the nutrient solution, the roots were washed, using a standard procedure, for 2 h in continuously aerated 2.5 mM $CaSO_4$ to remove adsorbed AAs from the root surface. Fresh plant materials (roots and leaves) were frozen in liquid nitrogen, homogenized using mortar and pestle, and extracted in methanol. Homogenates in methanol were centrifuged for 10 min at 10,000*g*, and supernatants were filtered (0.22-μm pore size, polyethersulfone filters) prior to analysis by high performance liquid chromatography (HPLC). Reverse-phase HPLC analysis was carried out on a Waters Breeze HPLC system (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector and with an EMD 1000 mass detector set in positive ESI mode. Signals were recorded in single ion mode for m/z 296 (NO_2+ H) for Form I and m/z 294 (H_2O+ H) for Form II. Separations were performed on Waters Xterra MS C-18 column, 2.1 × 50 mm with 3.5 μm particle size. Mobile phases were 0.1 % formic acid (Mobile Phase A) and 50 % acetonitrile (Mobile Phase B) with the following gradient profile: in first 20 min from 40 to 90 % B, followed by 20 min reverse to 40 % B with additional 5 min of equilibration time. Acetonitrile (J. T. Baker, Phillipsburg, NJ, USA), methanol (Carbo Reagenti, Milano, Italy), and p.a. grade formic acid were used. Data acquisition and evaluation were done using the Waters Empower 2 Software (Waters, Milford, MA, USA).

Results

To provide appropriate follow-up and comparison of AAI and AAII concentrations, we did control measurements of their spectrophotometric absorption

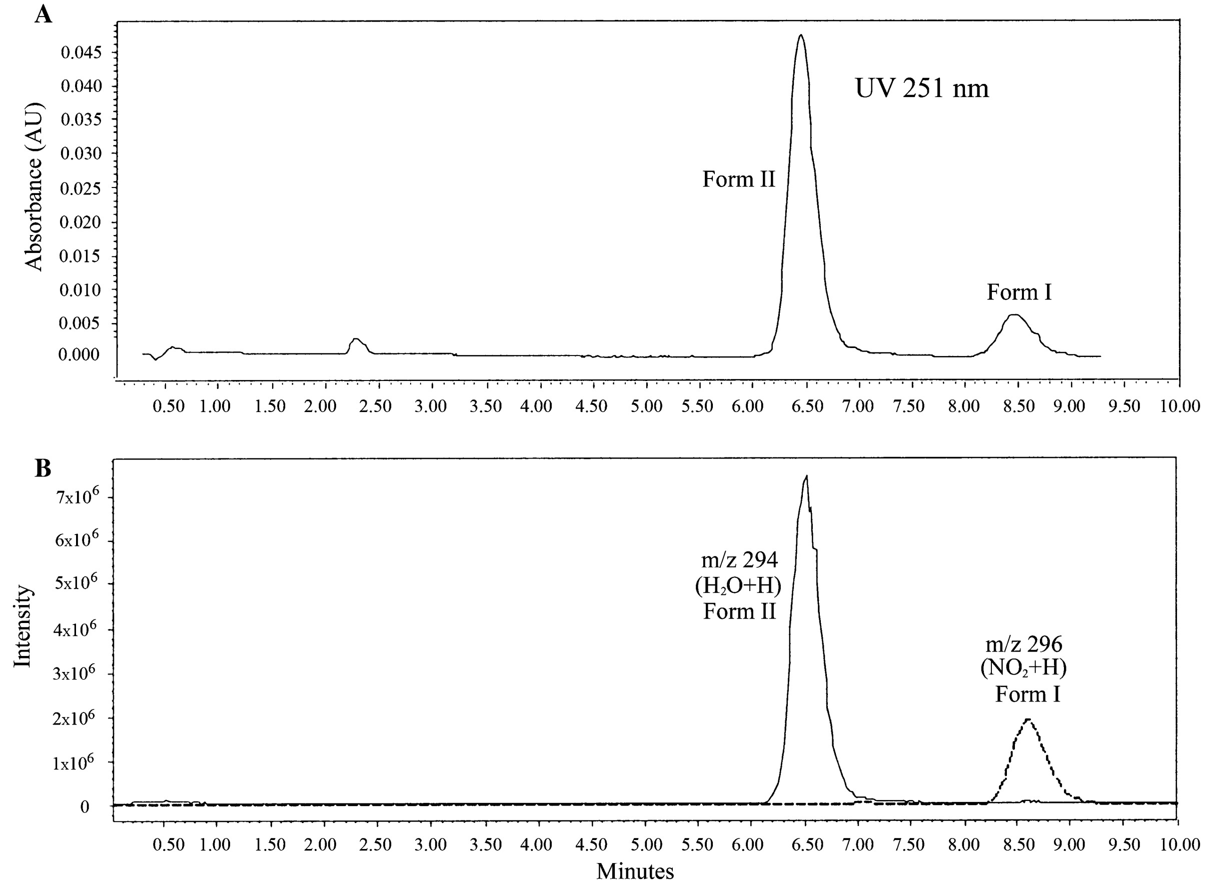

Fig. 2 Reverse phase HPLC UV (**A**) and LC MS ESI positive mode (**B**) chromatograms of commercial standard mixture of AAI and AAII

responses (Fig. 2) in the standard mixture (Sigma, St. Louis, MO, USA) containing 66 % AAII and 29 % AAI. Figure 2 shows the absorption peaks of AAI and AAII in standard solutions corresponding to their declared values.

The UV chromatograms of AAI and AAII in maize root extracts (Fig. 3, insets) indicate significant accumulation of both forms of AAs. High concentrations of absorped AAs enabled spectral confirmation of both AAI and AAII forms (Fig. 3), which correspond perfectly with the values obtained from commercial Sigma standard (Fig. 2). Furthermore, HPLC–MS analyses of the maize root extract showed significant amounts of both AA forms, following the same ratio of concentrations as that in the commercial AA standard added to the nutrient solution during treatment (Figs. 4, 6).

Chromatograms of the root content of AAI and AAII forms in cucumber (*Cucumis sativus* L. cv. Chinese long) grown on nutrient solutions containing 6.43 μM of AAI and 11.72 μM of AAII, respectively, show its capability for the uptake of AAs similar to that observed in maize plants (Fig. 5). However, much lower concentrations (more than tenfold difference) of both AA forms were calculated from the peak intensities (Fig. 6), compared to maize. A different pattern of AAI and AAII concentrations was observed in comparison with the chromatogram of commercial Sigma AAs standard (Fig. 2) added to nutrient solution during treatment (Figs. 5, 6). Moreover, the cucumber exhibited a different ratio of AAI to AAII (i.e., more AAI) as compared to the maize treatment and to Sigma standard AAs (Figs. 4, 5).

To further verify the measurement of AAI and AAII content in root material, we also measured AAI and AAII concentrations in nutrient solutions before (BT) and after (AT) plant treatment (Table 1). The measurements showed decreased concentrations of both AAI and AAII in the nutrient solution after plant growth. The results (Table 1) indicate that decreases in the nutrient concentrations of AAI and AAII generally correspond to increases in AAI and AAII observed in the roots of maize and cucumber after treatment (Figs. 5, 6, 7).

It is interesting to emphasize that, although the amounts of AAs decreased by about the same factor in both nutrient solutions (used for cucumber growth and for maize growth, respectively), the amounts of AAs found in the roots of cucumber were much smaller compared to those for the maize. This could be due to a much higher superficial retention/adsorption of the AAs by the cucumber roots (subsequently, the AAs being removed during the washing steps prior to analysis) and/or increased metabolic degradation of AAs by the cucumber plant.

In contrast to the high amounts of both AA forms in the roots of both plants (Fig. 6), we found much lower concentrations of AAI and AAII in the leaves (Fig. 7).

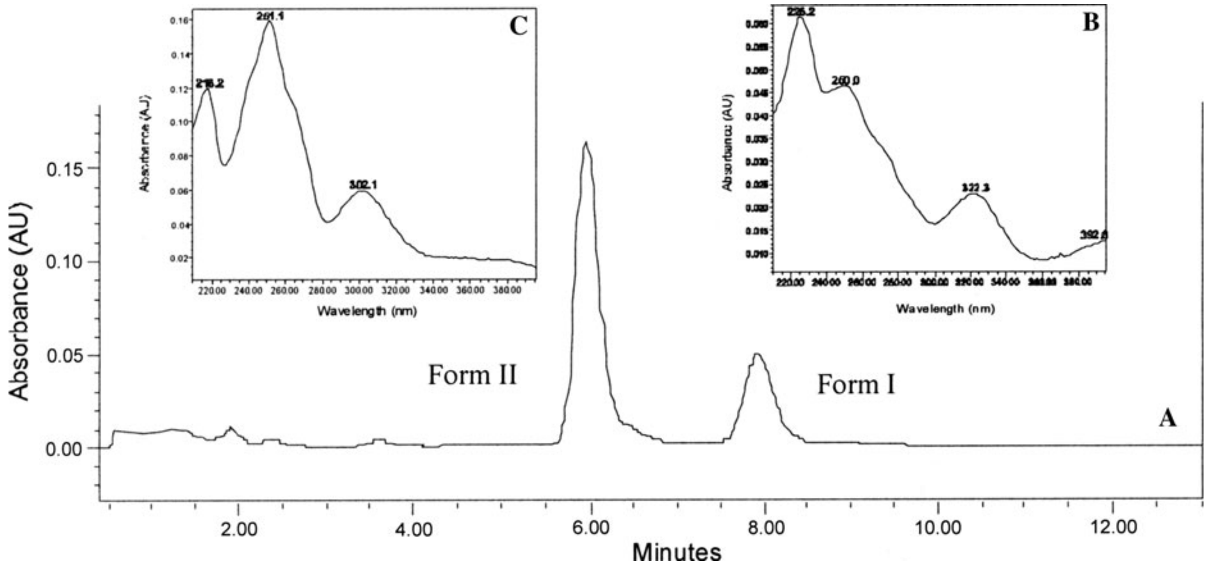

Fig. 3 Reverse phase HPLC UV chromatogram (*A*) of maize root extract from plants grown in nutrient solution containing 6.73 μM of AAI (*C*) and 11.72 μM of AAII (*B*)

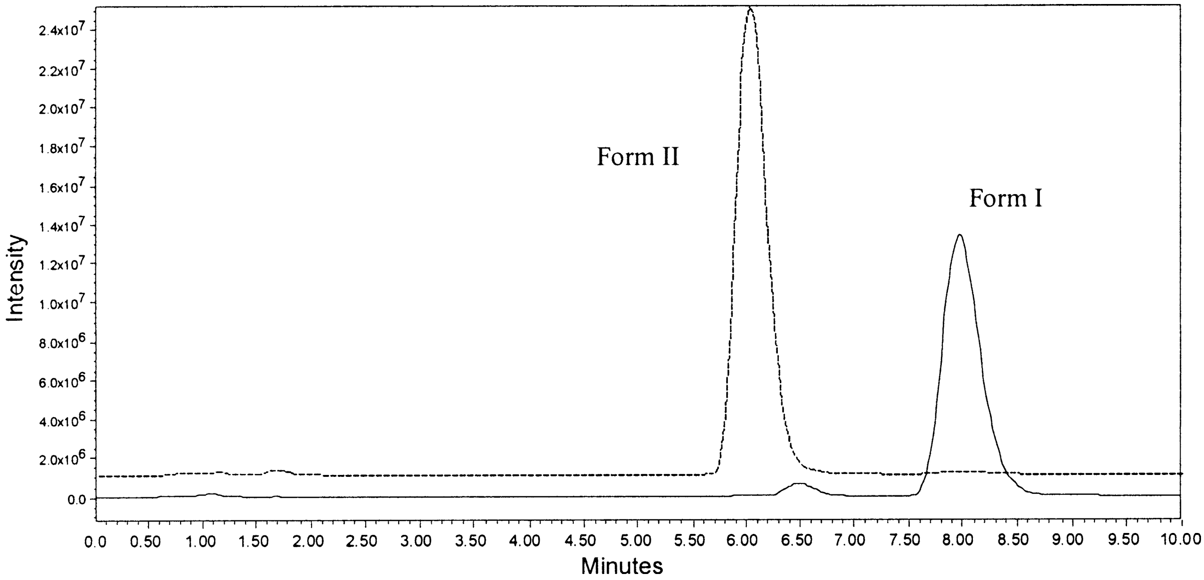

Fig. 4 HPLC-MS chromatogram of AAs in maize plants (*Zea mays* L. Va_{35}, Zemun Polje) grown on nutrient solution containing 6.73 μM of AAI and 11.72 μM of AAII

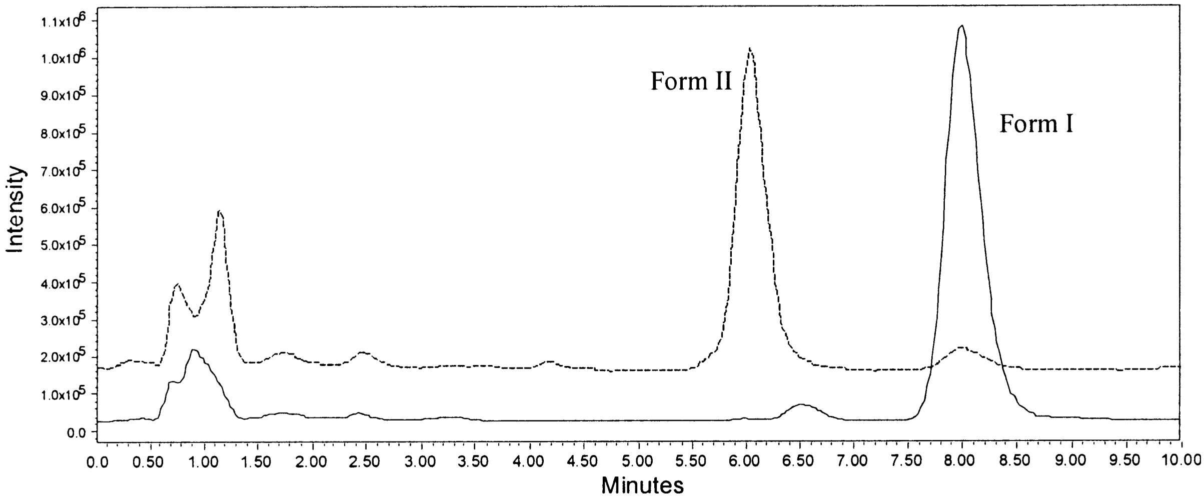

Fig. 5 HPLC-MS chromatogram of AAs in cucumber (*Cucumis sativus* L. cv. Chinese long) grown on nutrient solution containing AAI (6.73 μM) and AAII (11.72 μM)

Although the concentrations of AAs in leaves were very low, the same pattern of concentration ratios was present in leaves as compared to values measured in roots (Figs. 6, 7). The results suggest similar ways of root-to-shoot translocation for AAI and AAII, both in cucumber and in maize. With respect to the lower amounts of AAs taken by cucumber roots in comparison with maize, it is clear that cucumber more readily translocates AAs (at least the AAI form) to the leaves than maize throughout the same treatment duration.

When we measured the concentrations of AAI and AAII in the nutrient solution with 0.2 g pulverized seeds of *Aristolochia clematitis* per 1 L of nutrient solution (Fig. 8), we found that the peak representing AAI was much higher than that of AAII (Fig. 8a). It seems that a much higher abundance of AAI in *Aristolochia clematitis* pulverized seeds may account for higher uptake rates of AAI detected in the maize root grown in the described solution (Fig. 8b). The UV spectra

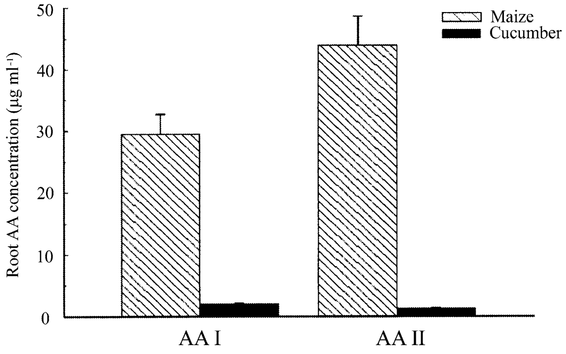

Fig. 6 Root content of AA in maize (*Zea mays* L. Va$_{35}$, Zemun Polje) and cucumber (*Cucumis sativus* L. cv. Chinese long) plants grown on nutrient solution containing AAI (6.73 µM) and AAII (11.72 µM)

Table 1 Concentration of AAI and AAII forms in nutrient solution before (BT) and after (AT) treatment in which maize and cucumber were grown

Knopp concentration		AA I (µg/ml)	AA II (µg/ml)
Maize	BT	200.00	400.00
	AT	0.12	0.99
Cucumber	BT	200.00	400.00
	AT	0.10	0.86

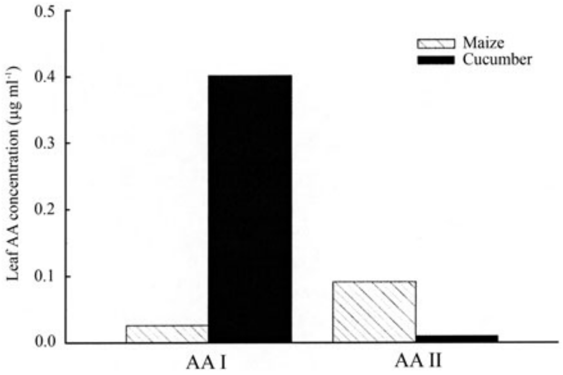

Fig. 7 Leaf content of AA in maize (*Zea mays* L. Va$_{35}$, Zemun Polje) and cucumber (*Cucumis sativus* L. cv. Chinese long) plants grown on nutrient solution containing 6.73 µM of AAI and 11.72 µM of AAII

corresponding to AAI peaks from both analyses are shown in Fig. 8c.

Maize plants (*Zea mays* L. Va$_{35}$, Zemun Polje) grown on nutrient solutions containing 6.43 µM of AAI and 11.72 µM of AAII (Fig. 9b) appeared pale and smaller, with poorly developed roots as compared to maize plants grown in the nutrient solution without added AAs (Fig. 9a). The toxicity symptoms of cucumber plants (*Cucumis sativus* L. cv. Chinese long) grown on identical nutrient solutions were also observed and were primarily expressed in older leaves (Fig. 10).

Discussion

The maize plant (*Zea mays* L. Va$_{35}$, Zemun Polje) and cucumber (*Cucumis sativus* L. cv. Chinese long) were used as a model to substantiate the possible involvement of naturally occurring AAI and AAII root uptake in food chain contamination.

The toxic effects of AAI and AAII have been inferred so far only from the effects seen in patients suffering from acute renal failure as a result of consuming herbal mixtures containing *Aristolochia* species. Exposure leads to rapidly progressive fibrosing interstitial nephropathy (Vanherweghem et al. 1993; Depierreux et al. 1994; IARC Monographs 2002), which is a consistent feature of AAN. Contamination of wheat fields with *A. clematitis* suggests that wheat grain, flour, and even bread could also be contaminated with AAs. The consumption of AAs from contaminated bread has been hypothesized as a factor in the onset of BEN and associated urothelial tumors (Ivić 1970; Hranjec et al. 2005; Ivić and Lović 1967; IARC Monographs 2002), and there is some recent evidence supporting this assumption (Jelaković et al. 2012). However, although these most recent studies bring proof to the AA-induced urothelial carcinogenesis and possibly kidney damage, the proposed exposure pathway, that is, intoxication through *Aristolochia* seeds-tainted wheat flour, still remains conjectural and hypothetical. Moreover, in the BEN areas in Romania, wheat has only been grown sporadically and if the wheat contamination hypothesis is true, then one would expect a significant decrease in the BEN incidence during the last couple of decades or so, since modern harvesting and herbicide application methods have been extensively employed. This does not seem to be the case, at least for the Romanian BEN area, where BEN prevalence has remained constant. Alternative exposure pathways to AAs derived from *Aristolochia* must be in play, and also additional nephrotoxic cofactors. The fact that *A.*

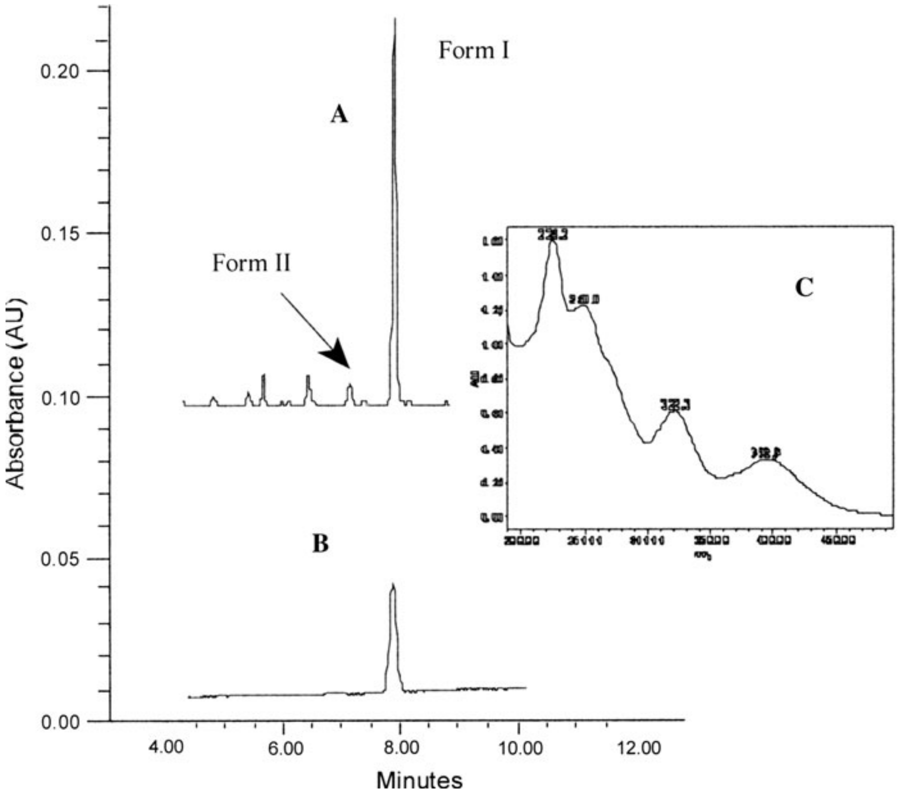

Fig. 8 Reverse phase HPLC–UV chromatogram of *Aristolochia clematitis* pulverized seed extract (*A*), HPLC–MS chromatogram of maize root extract (*B*), and UV spectrum of AAI from both seed and maize extracts (*C*)

clematitis may grow with similar intensity and frequency in other areas remote from the BEN region (Fig. 11), but with similar rural population and ethnic groups and no identified cases of nephropathy, raises the question whether *Aristolochia* by itself is sufficient to explain the causation of BEN and its associated urothelial tumors. Cofactors such as toxic organic compounds leached into the drinking groundwater from low-rank (Pliocene) lignite deposits found to underline the endemic (but not the nonendemic) BEN areas, along with a particular genetic susceptibility of the exposed population, might contribute along with AAs to a total toxic burden effect leading to the peculiar kidney disease and tumors (Tatu et al. 1998; Orem et al. 2007; Pavlovic et al. 2008).

While there is sufficient evidence in humans for the nephrotoxicity and carcinogenicity of herbal remedies containing plant species of the genus *Aristolochia* (IARC Monographs 2002), no data are available in the literature on whether some plants other than *Aristolochia* species can effectively absorb sufficient amounts of AAI and AAII to produce nephrotoxic and carcinogenic effects in humans after prolonged exposure. The feasibility of similar uptake of ochratoxin A, another nephrotoxic agent and possible carcinogenic to humans (IARC Monographs 2002), into coffee plants has been tested experimentally using ochratoxin A radiolabeled with ^{3}H and 14C (Mantle 2000).

Since the absorption of AAs by plants other than *Aristolochia* species may vary, we should point out that differences in soil qualities that may control differential absorption of AAs by crops could have an impact on the AA contamination of plants through the soil. However, there is very limited information on the biogeochemical stability of AAs or about their natural cycles in general. Our recent HPLC–MS studies on soil samples (from *A. clematitis* infested vegetable gardens and crop fields) collected from BEN, as well as non-BEN areas indicate the presence of both AAI and AAII in toxicologically significant amounts (μg/kg of dried soil). Similar amounts were detected in soils collected in the fall (i.e., containing "fresh" *A. clematitis* plant parts) as well as in spring, suggesting a high biogeochemical stability and insignificant seasonal decay of AAs released by the various

Fig. 9 Appearance of maize plants (*Zea mays* L. Va$_{35}$, Zemun Polje) grown without (**a**) or on nutrient solutions containing AAI and AAII (**b**)

plant parts (seeds, leaves, roots) [manuscript in preparation]. AAs seem to have a significant thermal and geomicrobiological stability; thus, accumulation in soils could occur; subsequently, the AAs may become available for absorption by crop/staple plants and enter the human food chain. BEN requires several decades to develop, so even at low levels such an absorption process of AAs could become a significant exposure pathway for the population at risk.

Although the idea that AAs from soil could be uptaken as contaminants by various crop plants into the human food chain ecosystem is novel, the concept has been previously proven at least for similar organic structures. For instance, it has been demonstrated that phenanthrene and pyrene can be taken up and accumulated by a diversity of leguminous plants in amounts proportional to their soil concentration (Gao and Zhu 2003). AAs being phenanthrenic structures themselves, a similar mechanism is not unlikely to occur. Our idea should apply not only in the context of Balkan endemic nephropathy etiology, but also in the potential causation of other AAN cases occurring worldwide.

It has been shown that *A. clematitis* seeds contain AAI and AAII at a ratio of ∼10:1 and that the determined total AA content of 1 gram of *A. clematitis* seeds is significantly higher than that reported for ethanol extracts of the roots (Hranjec et al. 2005). In our study of maize and cucumber roots and leaves, a proportionally much higher increase in the concentration of AAI than that of AAII (Figs. 6, 7) was found, although the standard mixture used for plant treatment contained a much higher concentration of AAII (67 %) than that of AAI (33 %), respectively (Fig. 2). This could be a result of the more effective uptake of AAI (the more nephrotoxic/carcinogenic form) or the metabolic transformation of one form of AA into the other, following uptake by the plant. These results may support the findings of Shibutani et al. (2007), who concluded that AAI and AAII have similar genotoxic and carcinogenic potential, and that although both compounds are cytotoxic, AAI is solely responsible for the nephrotoxicity associated with AAN.

This study showed that the roots of the maize plant and cucumber are not only capable of active uptake of both AAI and AAII from nutrient solution, but this uptake is also capable of yielding comparatively higher concentrations of AAs in the roots than in the nutrient solutions (Table 1). During this short-term experiment (48-h exposure to AAs), we found much lower concentrations of AAI and AAII in the leaves than in the roots (Figs. 6, 7), which was to be expected.

An important question to consider in the context of our results is whether the plants are capable of transferring significant amounts of AAI and AAII into other anatomical parts during long-term experiments. Some studies have shown that *A. clematitis* itself contains a much higher concentration of AAI and AAII in seeds compared to roots (Hranjec et al. 2005) (up to ten times higher). However, there are no data available on whether *A. clematitis* synthesizes AAs in seeds and/or in the roots only or on whether AAs, after being synthesized, are translocated to other parts of the plant.

The present study not only confirms the uptake of AAI and AAII by the roots of maize and cucumber (Figs. 4, 5, 6), but also the appearance of a significant

Fig. 10 Toxicity symptoms of cucumber plants (*Cucumis sativus* L. cv. Chinese long) grown on nutrient solutions containing AAI and AAII

Fig. 11 A cornfield in Romania (from a non-BEN area) heavily contaminated with *Aristolochia clematitis.* Soil contamination with aristolochic acids from *Aristolochia* seeds (*inset*) could be responsible for the impaired growth of corn plants

phenotypic toxic effect in exposed plants (Figs. 9, 10). An important question to address is whether such crop plants from endemic regions have varying AA adsorbing capability as compared to similar plants from nonendemic regions owing to certain soil peculiarities. However, earlier studies have failed to detect any significant geochemical differences in the soil from endemic and nonendemic households, making this possibility unlikely (Long et al. 2001). If there is indeed differential AA uptake by crop plants in endemic versus nonendemic regions, other mechanisms might be in play to explain this variability.

Watanabe et al. (1988) showed the phytotoxic activities of AAI and AAII in cucumber through the inhibition of seed germination and shoot and root elongation of the seedlings, as well as chlorosis. The current investigation, with ten times lower concentration of AA treatment, did not affect the physiological and morphological developing parameters of plants but verified the same trend of AA root accumulation. On the other hand, maize appeared to show a higher sensitivity to AA exposure, primarily manifested in the reduced growth of the overall plant. In an uncontrolled natural experiment, we could observe that crops of maize growing in areas heavily contaminated with *A. clematitis* plants have impaired growth and a reduced number of kernels (Fig. 11). As the soil from these areas has been shown to contain AAs, this could provide an explanation for the altered growth, but other factors may also contribute to this situation: allelopathic interactions between the maize and *Aristolochia* plants, the presence of other weeds, variations in the amount of rainfall, slightly different agricultural practices, and the use of herbicides, among others.

If further studies confirm that AAI and AAII taken up by plant roots can be transferred during the process of maturation to other plant parts and concentrated there, then there may be a number of pathways for AAs to enter the food chain of animals and humans. Consequently, the chronic exposure of both animals and humans to varying doses of AAs in food, without the exposure being recognized, may be highly significant, particularly when it is assumed that the renal damage caused by AA appears cumulative and irreversible regardless of the dose and exposure period (Cosyns 2003).

Since AAs were proposed by Ivić as an etiologic agent for BEN (Ivić 1970), differential exposure to this nephrotoxin among endemic and nonendemic villages could not be documented, bringing again the issue of other (co)factors involved. Ivić originally proposed that AAs enter the human food chain through the contamination of wheat/flour with *Aristolochia* seeds growing in the wheat fields. One problem with this hypothesis is that there is only very limited wheat cultivation in the Romanian endemic areas, mainly due to the hilly terrain. Instead, corn is much more frequently grown. Our current research does not provide any new insights into the differential exposure mechanisms, but might explain an alternative and more unifying pattern of exposure to AAs (i.e., bioaccumulation in crop plants and subsequent entry into the human food chain) for all BEN afflicted countries.

AAN is currently described as a worldwide problem (Debelle et al. 2008), and while most of the AAN cases have been caused by misuse of botanical remedies, involuntary exposure to AAs via tainted foods (e.g., contaminated wheat flour or corn, etc.) may play a role in at least some geographical areas (for instance, in the Balkans).

Moreover, our findings raise the question of whether people can effectively be protected only by issuing warnings to the public, industry, and health professionals on the adverse health risks associated with the consumption of products potentially containing AAs, or whether regular analysis of plants or products containing plants proven capable of the uptake of AAs should be undertaken. The same could be true for meat and dairy products obtained from animals fed with plants that are possibly contaminated with AAs.

Finally, the presence of AAs in soil raises another potential exposure pathway: dust loaded with particles carrying AAs. In many Balkan villages, dust is a major problem, especially during the dry summer days, when the people work their gardens and farm land. Additional research will be needed to establish whether this is a valid exposure pathway.

Acknowledgments This work was supported by the Serbian Ministry of Science (grant 143020B). All the experiments were performed on equipment provided by the Institute for Multidisciplinary Research and Serbian Ministry of Science. Part of this work was supported by grants from the US Geological Survey (Reston, VA, USA), University of Medicine and Pharmacy, Timisoara, Romania, and NATO (CLG grant # 980104).

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