

Aristolochic acid nephropathy: A model of nephrotoxic lesion

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Summary. *Aristolochia clematitis*, or birthwort, is a very toxic cancerogenic plant, and is a representative of the genus *Aristolochia*, whose main toxins are aristolochic acids. The nephrotoxicity of this plant is especially potent. Two important human diseases likely caused by aristolochic acids are Chinese herbal nephropathy and Balkan endemic nephropathy (BEN). In the present study, we used laboratory NMRI mice as an experimental model of nephrotoxic lesions, in order to examine the relation between aristolochic acids and these diseases and to investigate the effect of chronic toxicity caused by *A. clematitis* infusion. The presence of aristolochic acids in *A. clematitis* infusions was confirmed by gas chromatography with mass spectrometric detection. Experimental animals were divided into three groups that received different concentration of infusion (10 g/1000 ml, 20 g/1000 ml and 40 g/1000 ml) per os. After 17 weeks of experiment, histological sections of kidneys for all experimental animals displayed characteristic infiltrates around glomeruli with clusters of lymphocytes and plasma cells. Also, changes in tubule parenchyma with visible damage of nephrocytes was observed. These pathological changes represent diffuse interstitial nephritis in the chronic phase of inflammation. The toxic effects of *A. clematitis* cause changes in kidneys that could manifest as initial stages of chronic diseases such as BEN and Chinese herb nephropathy.

Keywords: *Aristolochia clematitis*, gas chromatography, interstitial nephritis, mouse nephrotoxicity.

INTRODUCTION

For more than 2,500 years, plants of the genus *Aristolochia* and Aristolochiaceae family have been used in folk medicine. Extracts of these plants are traditionally used in the treatment of gout, rheumatism, arthritis, festering wounds, and asthma. All parts of the plant are used in extract preparation (root, leaf, stem and fruit). In earlier documents, there appears to be no available data about the possible toxic effects of these plants. However, today, it is known that almost all species of the genus *Aristolochia* contain toxins that cause diseases such as Chinese herbal nephropathy and Balkan endemic nephropathy (BEN) (Bukvić 2004).

Chinese herb nephropathy is a progressive interstitial fibrosis of the kidneys, which in most cases leads to urothelial cancer. Terminal stage interstitial renal disease and complete failure of renal function has been reported in patients with Chinese herbal nephropathy. There are several

risk factors related to this disease, but the most probable is the consumption of dried roots of *Aristolochia* species in herbal teas (Schmeiser et al. 1996; Lord et al. 2001). In order to model Chinese herb nephropathy, in the present study birthwort infusion was used to demonstrate the similarities between aristolochic acid nephropathy and Chinese herb nephropathy.

BEN is a chronic tubulointerstitial renal disease that affects residents of rural areas around the tributaries of the Danube throughout the Balkans. This disease leads to kidney dysfunction, primarily in the proximal tubules. The nephrotoxic and carcinogenic effects of birthwort have been confirmed in experiments on rabbits, which were fed with contaminated flour. Examination of renal tissue from these animals revealed histopathologic changes very similar to those associated with BEN in humans (Stefanović 1999). Today, after over 50 years of research on BEN and other nephrotoxic

disorders caused by *Aristolochia clematitis*, there are still a lot of unknowns, especially with respect to the etiology of these diseases (Batuman 2006).

The main toxins that have been detected in almost all *Aristolochia* species are aristolochic acids (AA). They can be found in *A. clematitis*, *A. contorta*, *A. debilis*, *A. fangchi*, *A. indica*, *A. manshuriensis*, and *A. serpentaria*, and also in some *Asarum* species (family Aristolochiaceae). In Europe, there are 18 different species of the genus *Aristolochia*, three of them are indigenous and grow in northwestern Europe. In Serbia, two species are present: *A. clematitis* and *A. palida* (Kojić and Janjić 1994). Aristolochic acids are a family of nitrophenanthrene carboxylic acids. Two most important derivatives of these acids are: aristolochic acid I (AA-I) and aristolochic acid II (AA-II) (Shibutani et al. 2007). Both of these aristolochic acids are genotoxic mutagens, because they can form covalent adducts with DNA. The most dominant adduct, dA-AA-I, is responsible for most of the mutagenic and carcinogenic properties of aristolochic acid (Arlt 2006). Aristolochic acid is absorbed through the gastrointestinal tract and distributed to the other parts of the body. According to previous data, AA-I is metabolized by both oxidative and reductive pathways, while AA-II is metabolized only by a reductive pathway. The metabolites of AA-I and AA-II are excreted in the urine and feces. It has been shown that metabolites of AA-I are excreted within 24 h, while metabolites of AA-II remain in the urine for 72 h (Arlt et al. 2007). Today, there are numerous methods for identifying the presence of aristolochic acid, such as thin-layer chromatography, gas chromatography, high-performance liquid chromatography (HPLC), nuclear magnetic resonance and capillary electrophoresis.

The goal of the present study was to investigate the effects of chronic toxicity, caused by *A. clematitis* infusion, on the kidneys of laboratory NMRI mice. In addition, another goal was to confirm the presence of aristolochic acid derivatives in our *A. clematitis* infusion using gas chromatography with mass spectrometric detection.

MATERIALS AND METHODS

Aristolochia clematitis infusion

Samples of birthwort (*A. clematitis*) used in this experiment were collected during the month of May in 2010. Voucher specimens (*Aristolochia clematitis* L. 1753 N° 2-1793, Novi Sad, Ribarac, UTM 34TDR201, 07. 05. 2010. det.: Ružica Igić) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad (Holmgren PK and Holmgren NH 2003).

In 1000 mL of deionized boiling water, 40 g of dried leaves, roots and fruit were added and left to incubate for 3 hours. Afterward, the infusion was filtered and different solu-

tions were made (10 g/1000 mL, 20 g/1000 mL and 40 g/1000 mL), based on data from our previous pilot study, which considered the concentrations required to cause visible damage to kidneys (unpublished data).

GC-MS chromatography

Qualitative analysis of the *A. clematitis* infusion was conducted using gas chromatography with mass spectrometric detection (GC-MS). For the present study, only a 4% infusion was analysed, since this is the concentration most likely to cause renal damage. For this analysis, the infusion was prepared by adding 2 M H₂SO₄ in 2 mL of 4% infusion until the solution reached pH 2. Afterward, the infusion was applied to a Extrelut NT 3 column (Mereck, Germany). Twenty minutes later, elution was performed with a 15 mL mixture of solvent methylene chloride : methanol = 9:1. The eluate was evaporated in a nitrogen stream, then reconstituted in 0.4 mL of methylene chloride: 1 µL was injected into the GC-MS.

Gas chromatography analysis with mass spectrometric detection was performed using a gas chromatograph 6890 N (GC), equipped with a 5973 mass detector (MS) and automatic injector 7683; Agilent Technologies, in EI (Electron Impact) mode at 70 eV and in a capillary column DB-5MS (30 m x 0.25 mm, film thickness 0.25 µm, Agilent Technologies).

The temperature of the injector and detector were set at 250 °C and 280 °C, respectively. Chromatographic separation of compounds was performed by injecting 1 µL extract of methylene chloride extract in splitless mode (1 min), at a constant flow of helium as a carrier gas, 1 mL/min. The starting temperature of the column was 50 °C for 2 minutes, then 250 °C with a rising temperature rate of 20 °C/min, and finally 310 °C with a rising temperature rate of 30 °C/min, which was maintained for 10 minutes.

Qualitative GC-MS analysis of the 4% infusion was performed by scanning the masses in a wide range (50-550 m/z), and obtained mass spectra were compared with mass spectra from a commercial Wiley library.

Histological procedure

Experiments were performed at the Pasteur Institute in Novi Sad, on adult male laboratory NMRI mice of approximately the same weight (25 g) and age. Mice were harbored in the vivarium of the Pasteur Institute in Novi Sad, and acclimated to a temperature of (22 ± 2) °C with 12 hour light/dark cycles.

Experimental groups were divided into three groups of 20 mice, that were given different concentration of birthwort infusion per os every day: Experimental group 1 (10 g/1000 mL), Experimental group 2 (20 g/1000 mL), Experimental group 3 (40 g/1000 mL) and one control group of 20 mice

that was given only water. During this study only 6 experimental animals died, while others were sacrificed under ether anesthesia 17 weeks after the beginning of the study. Kidneys and whole urogenital system were taken and fixed in 10% formalin. After fixation, tissues were dehydrated in 96% ethanol and isopropyl alcohol, then embedded in paraffin and cut on a rotation microtome (Leica) in sections with a thickness of 3 to 5 μm , and then stained with hematoxylin and eosin (H&E).

RESULTS

Results of qualitative analysis of *Aristolochia clematitis* infusion

The presence of aristolochic acid in the 4% infusion was confirmed by identification of their three products: M-294 at a retention time of 16.95 min, M-293 at a retention time of 19.16 min and M-355 at a retention time of 19.71 min. A total ion current (TIC) chromatogram for a 4% infusion of *A. clematitis* is shown in Figure 1.

The results are consistent with published data (Podea et al. 2001), indicating that the identified derivatives M-294, M-293 and M-355 have the same fragment ions as aristolochic acids. Mass spectra of appropriate derivatives M-294, M-293 and M-355 are shown in Figures 2-4.

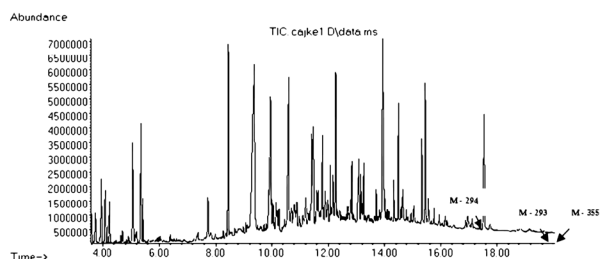


Figure 1. Total ion current (TIC) chromatogram for 4% infusion of *Aristolochia clematitis*.

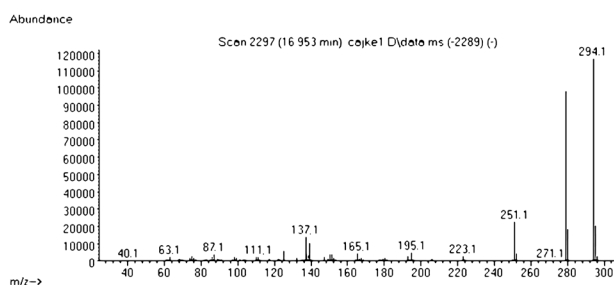


Figure 2. Mass spectrometric detection of derivative M-294 in 4% infusion of *Aristolochia clematitis*.

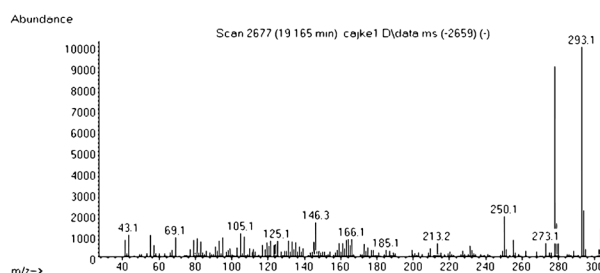


Figure 3. Mass spectrometric detection of derivative M-293 in 4% infusion of *Aristolochia clematitis*.

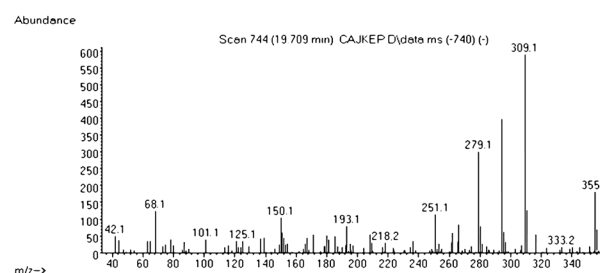


Figure 4. Mass spectrometric detection of derivative M-355 in 4% infusion of *Aristolochia clematitis*.

Histological analysis of kidneys

Based on histological sections, all experimental groups had different levels of damage in the kidney parenchyma, depending on the concentration of infusion solution. Tubule parenchyma in Experimental group 3 was parenchymatously degenerated with a widely extended lumen of the tubules (Fig. 5). In the cortex of the kidney, almost all experimental animals had diffusely collapsed glomeruli with characteristic infiltrates around them made of clusters of lymphocytes and plasma cells (Fig. 6). All of these pathological changes

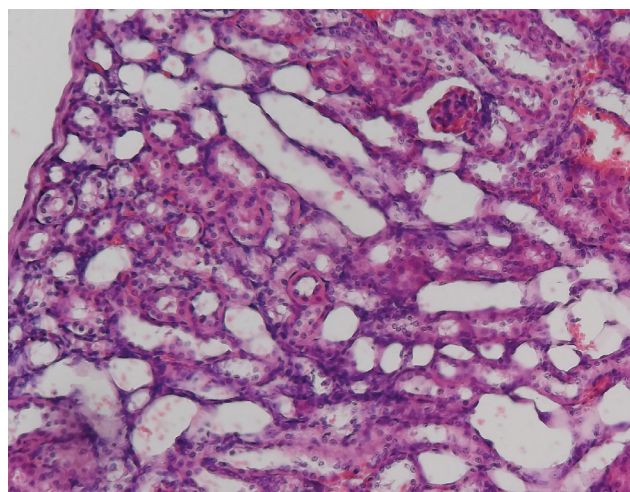


Figure 5. Histological section of NMRI mouse kidney treated with 40 g/1000 mL *Aristolochia clematitis* infusion for 17 weeks. Widely extended lumen of tubules in renal cortex. Hematoxylin and eosin stain (x200).

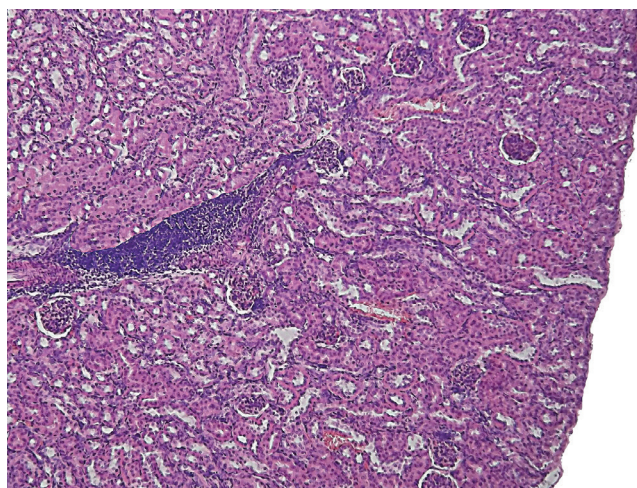


Figure 6. Histological section of NMRI mouse kidney treated with 10 g/1000 mL *Aristolochia clematitis* infusion for 17 weeks. Characteristic infiltrate around glomeruli in renal cortex made of clusters of lymphocytes and plasma cells. Hematoxylin and eosin stain (x100).

represent diffuse interstitial nephritis in the chronic phase of inflammation.

DISCUSSION

The nephrotoxic effects of birthwort (*A. clematitis*) have been shown, as has already been pointed out, and represents the initial stage of severe pathological changes in the renal interstitium. Later, these pathological changes could result in the development of chronic diseases that are similar to Chinese herbal nephropathy and BEN. The morphological and clinical picture of these diseases are nearly identical; suggesting that one of the possible causes of the occurrence of both diseases is aristolochic acid, which Ivić pointed out in 1969.

In addition, BEN causes glomerular and chronic interstitial nephritis. In patients, there is a slow progression of this disease, probably because of the low long-term intake of toxins from this plant (Long and Voice 2007). Considering this, in the present study a long-term intake of birthwort infusion was used as a model of chronic toxicity, which resulted in damage to the renal parenchyma and especially the renal tubules. It is believed that aristolochic acid causes this disease, because AA-DNA adducts have been found in renal tissues and urothelial cancers of affected patients. Urine analysis of these patients showed tubular proteinuria, glycosuria and enzymuria. Tubular proteinuria, detected in asymptomatic patients, shows reduced function of proximal renal tubules, most probably as a result of the toxic effects of aristolochic acid in the body (Cronin et al. 2002).

Previous experimental studies on animals have also shown the chronic toxicity of aristolochic acid, which was applied orally or peritoneally (Debelle et al. 2002). These

results showed that animals exposed to higher doses of this acid developed renal failure (Nortier and Vanherweghem 2007). Today, all medicines and teas that contain any extracts of plants of the genus *Aristolochia* are classified as carcinogenic to humans (Group 1 carcinogen) by the International Agency for Research on Cancer (IARC).

The results of the present study show that not all concentrations of *A. clematitis* infusion resulted in severe chronic toxicity for shorter periods of time, which may be the explanation for why this plant was previously used in ethnomedicine, probably in smaller concentrations, and therefore was not recognized as toxic. Severe toxicity of *A. clematitis* can be observed after longer exposure of experimental animals to toxins e.g. chronic toxicity (6–12 months), similar to BEN in humans, due to low level intake of this plant's toxin for over 20 years (Grollman and Jelaković 2007).

The chronic nephrotoxic effect of *Aristolochia clematitis* infusion is evident in NMRI mice, due to the presence of diffuse infiltrates of lymphocytes and plasma cells in the renal cortex of most of the mice. Also, the presence of derivatives of aristolochic acids, which caused the mentioned nephrotoxicity, was demonstrated in the *A. clematitis* infusion.

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