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Determination of aristolochic acid I and II in North American species of *Asarum* and *Aristolochia*

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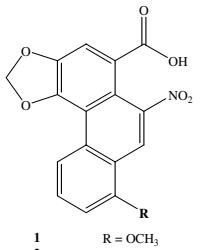
Wild ginger, *Asarum canadense*, which has folk uses as a medicinal and food plant, has been reported to contain aristolochic acid I. Rhizomes of North American species of Aristolochiaceae were surveyed for the presence of aristolochic acids by HPLC. Aristolochic acid I (**1**) and aristolochic acid II (**2**) were present in *Aristolochia* species and *Hexastylis*; **1** alone was detected in multiple accessions of *A. canadense* and *Asarum caudatum*, though not in *Asarum wagneri*. Concentrations in *A. canadense* were highly variable, reaching as much as 0.037 percent of dry weight.

1. Introduction

Asarum canadense L. (Aristolochiaceae), a rhizomatous perennial herb, is a significant member of the herbaceous understory in deciduous forests across much of the eastern half of the United States and Canada. Its aromatic rhizomes may be used as a flavoring or candied much like true ginger. At least a dozen Native American peoples used the rhizome and roots for medicinal purposes, especially for gastrointestinal discomfort or respiratory ailments and fever [1]; folk use still occurs, and the plant was until recently an article of commerce [2]. The common northwest American species, *Asarum caudatum* Lindl., which had similar traditional uses [1], is not found in commerce [2]. Many species of the related genus *Aristolochia* L., used medicinally worldwide, contain aristolochic acid [3–4]. Evidence from human case studies indicates that chronic medicinal use of *Aristolochia* species can result in a characteristic nephropathy and possibly renal cancer, attributed to aristolochic acids, i.e. aristolochic acid I (**1**) and aristolochic acid II (**2**) [5–9]. Though few reports exist of significant aristolochic acid content in *Asarum* species, small quantities of **1** were found in two of five species included in one study [4]. An early report that *Asarum canadense* contained **1** [10] induced the U.S. Food and Drug Administration (FDA) to place that species on the list of those banned from commerce due to safety issues involving aristolochic acids [11]. As the plant is commonly viewed as edible and medicinal [12, 13], it is important to determine whether chronic consumption of *Asarum canadense* may pose a health risk.



1 R = OCH_3
2 R = H

2. Investigations, results and discussion

2.1. Analysis of raw materials

Samples of American species of Aristolochiaceae were obtained from wild populations in late spring and early sum-

Table: Content of aristolochic acid I and II as % dry weight in North American species of Aristolochiaceae

Species	Voucher # *	Geographic origin	**1** as% dry weight**	**2** as% dry weight**
Aristolochia macrophylla	64	North Carolina, Macon Co.	0.39 (0.54)	0.66 (0.66)
Aristolochia serpentaria	58	Indiana, Perry Co.	0.13 (0.29)	0.0097 (0.45)
Hexastylis arifolia var. arifolia	69	North Carolina, Swain Co.	0.21 (1.44)	0.66 (0.37)
Asarum canadense	54	Wisconsin, Oconto Co.	0.0012 (1.39)	ND
	55	Wisconsin, Langlade Co.	0.0040 (2.53)	ND
	59	Indiana, Perry Co.	DUL	ND
	60	Indiana, Perry Co.	DUL	ND
	62	Indiana, Crawford Co.	DUL	ND
	66	North Carolina, Macon Co.	0.011 (2.01)	ND
	67	North Carolina, Macon Co.	0.021 (2.76)	ND
	68	North Carolina, Swain Co.	0.0092 (0.72)	ND
	77	South Carolina, Berkeley Co.	DUL	ND
	82	South Carolina, Berkeley Co.	DUL	ND
	84	Vermont, Addison Co.	0.0095 (1.31)	ND
	85	Vermont, Addison Co.	0.010 (0.60)	ND
	87	Vermont, Addison Co.	0.0099 (0.19)	ND
	88	New York, Cattaraugas Co.	0.037 (0.26)	ND
	89	Ohio, Morgan Co.	0.012 (0.85)	ND
	90	Missouri, St. Louis Co.	0.0090 (3.94)	ND
Asarum caudatum	104	Oregon, Lane Co.	DUL	ND
	106	Oregon, Linn Co.	DUL	ND
Asarum wagneri	110	Oregon, Jackson Co.	ND	ND

* All voucher numbers refer to W. L. Applequist's collection series.
** Standard deviation (%) given in parentheses. DUL = Detection Under the Limit of Quantitation. ND = Not Detected.

mer of 2001 (Table). Multiple accessions of *Asarum canadense* were screened to ensure that possible regional variation in aristolochic acid content would be detected. Rhizome material was dried, extracted in methanol by sonication, and analyzed by HPLC with a UV PDA detector.

2.2. Aristolochic acid I and II content as a percentage of dry weight

Aristolochic acid I (**1**) was detected in all samples with the exception of *Asarum wagneri* K. L. Lu & M. R. Mesler, an uncommon species endemic to the state of Oregon. Concentrations of **1** in two *Aristolochia* collections were 0.13% and 0.39% of dry weight; concentrations in several accessions of *Asarum canadense* were under the limit of quantitation, but in others were as high as 0.037%. *Hexastylis arifolia* Small, a rare North Carolina endemic related to *Asarum*, contained an unexpectedly high 0.21% of **1**. Amounts detected in each sample are provided in Table 1. By comparison, **2** was detected only in *Aristolochia* and *Hexastylis*, and in no samples of *Asarum*.

2.3. Evaluating the safety of Asarum canadense

All species of *Aristolochia* may be presumed to be potentially dangerous unless demonstrated to lack a significant concentration of aristolochic acids. The average minimum dose necessary to cause nephrotoxicity has not been established. The use of *Aristolochia* species in folk medicine worldwide provides strong evidence that infrequent or low-dose exposure does not cause terminal illness as a general rule. Case reports of aristolochic acid nephropathy describe long-term consumption [7, 14, 15], and in the unprecedented constellation of cases connected with a Belgian weight-loss clinic, most patients who were presumed to have had fairly high exposure remained healthy [15], although an unknown percentage developed progressive disease years after their last exposure [9]. It is also possible that other aspects of the Belgian weight-loss program exacerbated the natural toxicity of aristolochic acids, and no accurate determination of aristolochic acid dosages in those patients can be made [9], complicating efforts to estimate the toxic dose. We may assume that the content of a toxic compound in a product for human consumption should be at least two orders of magnitude below the amount expected to cause harm, to account for variations in individual tolerance. Notably, the accession of *Asarum canadense* with the most **1** contained about 10% of the amount found in a sample of *Aristolochia macrophylla* Lam., which might well be toxic if consumed chronically in quantity. Thus, *Asarum canadense* cannot be presumed to be completely safe.

Assessment of the safety of *Asarum canadense* is complicated by the fact that the content of **1** varied considerably among samples. Both environment and genetics may contribute to variation in aristolochic acid content. The highest concentration was found in a tiny New York population reportedly subject to great pressure from herbivory; the other particularly high values were found in small North Carolina populations in less than ideal habitat. Environmental pressure may induce extra production of **1** as a defensive compound. Also, collections from the northeastern United States (New York, Vermont, and Ohio) contained larger amounts of **1**, whereas collections from the Midwest and South Carolina had smaller amounts, regardless of population size or habitat quality. It is therefore possible that low-aristolochic acid chemical races could be identified for commercial use if a maximum safe dose were established.

2.4. Aristolochic acid content in other North American Aristolochiaceae

Aristolochic acid I (**1**) was detected in two samples of *Asarum caudatum* below the limit of quantitation. We observe that these samples, taken from a limited area, are insufficient to demonstrate that the species as a whole has low aristolochic acid content. Other chemical races could well exist, and a survey across the species' range would be needed in order to conclude that it was acceptably nontoxic. Aristolochic acid I was not detectable in one sample of *Asarum wagneri*, which is not common enough to be collected. The quantity of **1** and **2** in *Hexastylis arifolia* equaled that in American species of *Aristolochia*, a surprising result as *Hexastylis* is believed to be most closely related to *Asarum* [16]. The traditional use of this and other species of *Hexastylis* [17] probably did pose some health hazard. Fortunately, *Hexastylis* is scarce enough that it is unlikely to be collected today.

In Conclusion, *Asarum canadense* usually contains only a tiny fraction of **1** found in *Aristolochia* species, which causes kidney damage only when consumed in some unknown quantity. It should be made clear that no evidence exists that any person has ever suffered nephrotoxicity attributable to consumption of *A. canadense*, and it is unlikely that significant numbers of illnesses among long-term consumers have gone undetected. Thus, to conclude that prior use of *A. canadense* should be considered a risk factor for nephropathy would be wholly inappropriate. Nonetheless, lacking an understanding of dosage effects, as well as the nephrotoxic activity of other aristolochic acids, we cannot rule out the possibility that long-term use of high-aristolochic acid varieties could pose a risk to susceptible persons. The FDA ban on commercial sale of the plant is therefore a wise precautionary measure, and we also suggest that educational efforts be made to reduce the promotion of the plant as edible in popular literature.

3. Experimental

3.1. Chemicals and reference compounds

HPLC grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Sigma (St. Louis, MO, USA). HPLC grade water was prepared by filtering nanopure water through a 45 μm membrane filter. A mixture of aristolochic acid I (**1**) and aristolochic acid II (**2**) was purchased from Sigma and separated into the individual components by RP-PTLC using a methanol : water (3 : 1) mobile phase.

3.2. Calibration of working standards

Approximately 5.0 mg of each standard compound (**1** and **2**) was placed in a 25 ml volumetric flask and diluted in methanol (stock solution). Further calibration levels were prepared by diluting the stock solution with methanol. Within the range of concentrations injected (200.0–8.0 μg/ml) the detector response was linear. Regression analysis at the various concentrations of standard solutions gave the correlation coefficient for each calibration curve (**1**, $R^2 = 0.9994$; **2**, $R^2 = 0.9998$). The limit of detection was 0.05 μg/ml for **1** and **2** and the limit of quantitation for **1** and **2** was 8.0 μg/ml. A recovery rate of 99% was recorded for both compounds. The Fig. represents the typical chromatogram of a standard solution containing **1** and **2** along with chromatograms for *A. macrophylla* and *A. canadense*.

3.3. Sample preparation

3.3.1. Plant collections

All American species of Aristolochiaceae that could be located in the field were collected. Multiple collections of *Asarum canadense* were made with

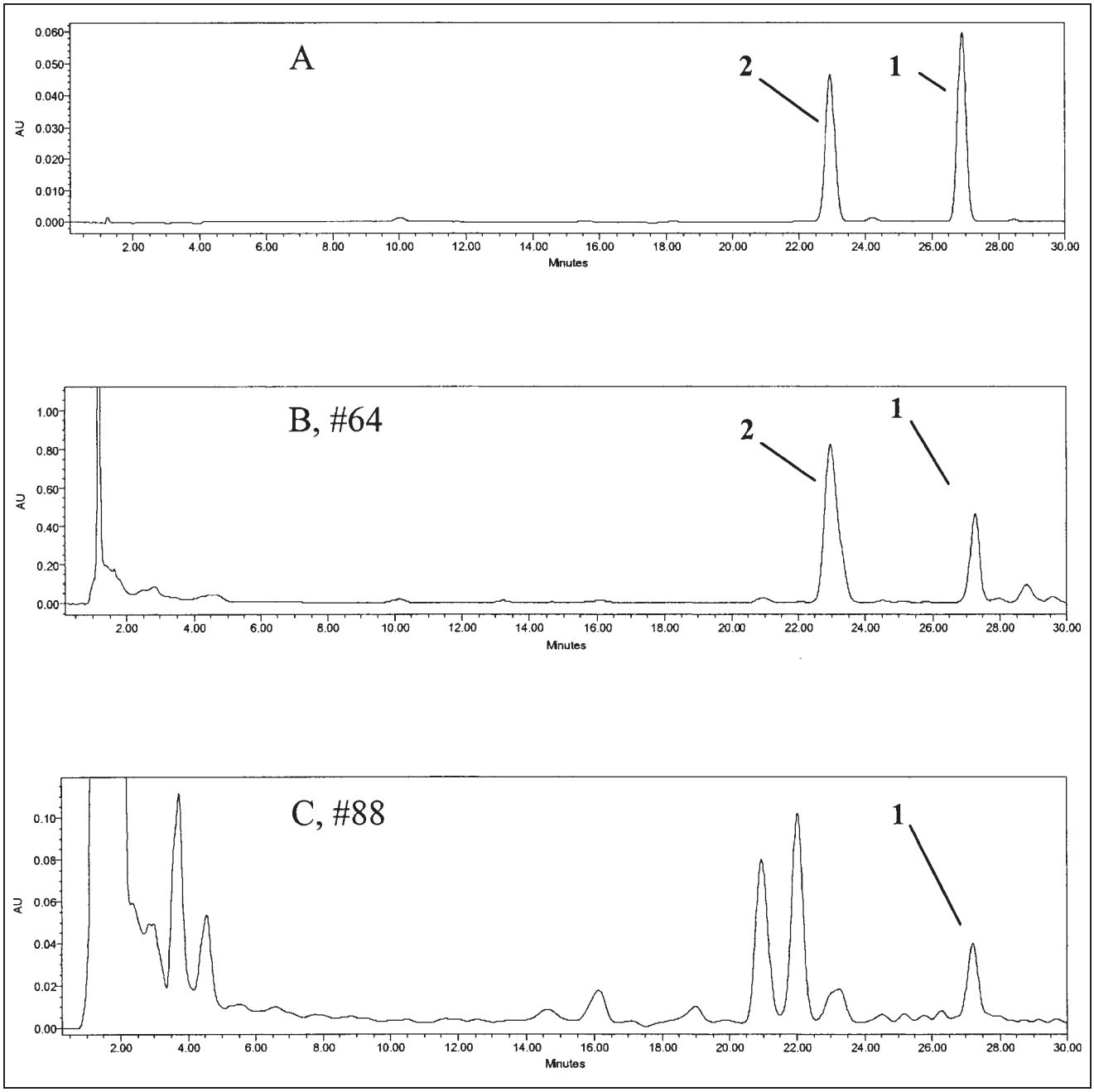


Fig.: Chromatograms of the standards **1** and **2** (A), *Arisolochia marcophylla* (B) and *Asarum canadense* (C)

the intention of covering as much of the United States range of the species as possible. Multiple rhizomes from each population were collected and dried in paper bags. Vouchers for each population were deposited in the Missouri Botanical Garden (MO) and University of Mississippi (HMISS) herbaria. Plant collection locations are listed in the Table.

3.3.2. Extraction procedure

Rhizomes were extracted in a similar fashion to the method recommended by the FDA [18]. Ground rhizome (0.5 to 1.0 g) was placed in a 15 ml screw capped polypropylene centrifuge tube (Falcon tubes from VWR Scientific Products, West Chester, PA, USA) and extracted three times in a FS20H Ultrasonic Cleaner (VWR Scientific Products, West Chester, PA, USA) with 3.0 ml of a mixture containing 80% methanol and 20% of 10% formic acid in water by sonication for 10 min. The emulsion was centrifuged (5.0 min at 3000 rpm) and the supernatants were combined in a 10 ml volumetric flask by pipette and diluted to the final volume with methanol and mixed thoroughly. All supernatants were filtered through a 0.45 μm PTFE syringe filter prior to injection.

3.4. Chromatography

Sample analysis by HPLC was done on a Waters 2690 Separations Module with an in-line mobile phase degasser at 0.6 psia and a Waters 996 PDA detector (Waters, Millford, MA, USA). The analysis software was Millennium[32] by Waters. The operating conditions: column, Symmetry C_{18} 3.5 μm 4.6 × 75 mm by Waters (Millford, MA, USA: Part No. WAT066224); Guard column, Security Guard C18 cartridge system (Phenomenex, Torrance, CA, USA); Mobile phase, water (0.1% TFA) (solvent A), acetonitrile (solvent B), and methanol (solvent C); Gradient, start at 60 : 0:40 (A:B:C) with a change to 55 : 0:45 over 15 min, then end at 40 : 55 : 5 after another 15 min. Wash with B for 8 min, and re-equilibrate for 10 minutes. Total run time was 45 min with a retention time (rt) of 27.2 min for **1** and 23.3 min for **2**; Flow rate, 0.70 ml/min; Detection wavelength, 254 nm; Injection volume, 20 μl; Temperature, 35 °C. The HPLC method was validated by the standard deviation in percent (SD%) of each sample (n = 3) reported in the Table. All SDs were less than 4.0%.

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References

1 Moerman, D. E.: Native American Ethnobotany. p. 105, Timber Press, Portland OR 1998
2 McGuffin, M.; Kartesz, J. T.; Leung, A. Y.; Tucker, A. O.: Herbs of Commerce. p. 20, American Herbal Products Association, Silver Spring MD 2000
3 Mix, D. B.; Guinaudeau, H.; Shamma, M.: J. Nat. Prod. **45**, 657 (1982)
4 Hashimoto, K.; Higuchi, M.; Makino, B.; Sakakibara, I.; Kubo, M.; Komatsu, Y.; Maruno, M.; Okada, M.: J. Ethnopharmacol. **64**, 185 (1999)
5 Depierreux, M.; Van Damme, B.; Vanden Houte, K.; Vanherweghem, J.-L.: Am. J. Kidney Dis. **24**, 172 (1994)
6 Vanhaelen, M.; Vanhaelen-Fastre, R.; But, P.; Vanherweghem, J.-L.: Lancet **343**, 174 (1994)
7 Pena, J. M.; Borras, M.; Ramos, J.; Montoliu, J.: Nephrol. Dial. Transplant. **11**, 1359 (1996)
8 Tanaka, A.; Nishida, R.; Maeda, K.; Sugawara, A.; Kuwahara, T.: Clin. Nephrol. **53**, 301 (2000)
9 Nortier, J. L.; Martinez, M.-C. M.; Schmeiser, H. H.; Arlt, V. M.; Bieler, C. A.; Petein, M.; Depierreux, M. F.; De Pauw, L.; Abramowicz, D.; Vereerstraeten, P.; Vanherweghem, J.-L.: New Engl. J. Med. **342**, 1686 (2000)
10 Doskotch, R. W.; Vanevenhoven, P. W.: Lloydia **30**, 141 (1967)
11 http://www.fda.gov/ora/fiars/ora_import_ia5410.html
12 Elliott, D.: Wild Roots. p. 54, Healing Arts Press, Rochester VT, 1995
13 Foster, S.; Duke, J. A.: A Field Guide to Medicinal Plants. p. 138, Houghton Mifflin Co., Boston/New York, 1990
14 Lord, G. M.; Tagore, R.; Cook, T.; Gower, P.; Pusey, P. D.: Lancet **354**, 481 (1999)
15 Vanherweghem, J.-L.; Depierreux, M.; Tielemans, C.; Abramowicz, D.; Dratwa, M.; Jadoul, M.; Richard, C.; Vandervelde, D.; Verbeelen, D.; Vanhaelen-Fastre, R.; Vanhaelen, M.: Lancet **341**, 387 (1993)
16 Kelly, L. M.: Am. J. Bot. **85**, 1454 (1998)
17 Moerman, D. E.: Native American Ethnobotany. p. 265, Timber Press, Portland OR, 1998
18 Flurer, R. A.; Jones, M. B.; Vela, N.; Ciolino, L. A.; Wolnik, K. A.: The FDA Laboratory Information Bulletin No. 4212, Dietary Supplements. http://vm.cfsan.fda.gov/ãcrobat/lib4212.pdf

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