Original Article

Investigating the aristolochic acid content of different species of the genus Aristolochia available in the pharmaceutical market of Iran

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ABSTRACT

Various species of the genus *Aristolochia* contain a toxic compound called aristolochic acid (AA), which has been associated with reported side effects. Given that wounds and bites are among the most common conditions in martial medicine, and *Aristolochia* is one of the most widely used plants in the treatment of these conditions, there is a strong need to standardize the medicinal products obtained from *Aristolochia*. In this study, for the first time, the extract of *Aristolochia hyrcana* was standardized based on aristolochic acid, and it was then compared with *Aristolochia longa*. *Aristolochia hyrcana* and *Aristolochia longa* were obtained from the market, and aqueous and hydroalcoholic extracts were prepared from each. The level of aristolochic acid was measured by a HPLC method. In our study, the level of aristolochic acid in the extract of *Aristolochia hyrcana* and *Aristolochia longa* was found in the range of 0.17-0.20 mg/kg. These results indicate the safety of the recommended doses for *Aristolochia hyrcana* and *Aristolochia longa* in traditional Iranian medicine. However, it is important to note that natural compounds in plants can be significantly different even in different regions of Iran. Therefore, more studies are needed to evaluate the content of AA in *Aristolochia hyrcana* and *Aristolochia longa* in different geographical locations. Additionally, more research on different animal species, laboratory models, and analysis of specific compounds such as aristolochic acid is necessary to definitively prove the non-toxic nature of the doses used in traditional medicine.

Keywords: Aristolochia, Aristolochic acid, Laboratory models, HPLC method

Introduction

The herbal medicines in both developing and developed countries have been increasingly used [1]. Despite the growing popularity of traditional herbal medicines, there is often a lack of comprehensive information on their possible adverse effects [2]. The US Food and Drug Administration (FDA) is concerned about herbal ingredients and botanicals that may naturally contain aristolochic acid, or adulterated with aristolochic acid [9]. These acids are usually found in the roots of plants belonging to the *Aristolochiaceae* family [3]. The genus *Aristolochia* has more than 400 species that are both abundant and have therapeutic potential. Different species of *Aristolochia* are used in local and traditional medicine in different countries [4]. Studies have shown that rodents that received aristolochic acid developed lymphoma and cancer in the kidney, bladder, stomach, and lung, which indicates a potential increase in the risk of malignancy in humans [10,11].

In the traditional medicine of Iran, two widely used species of this genus are *Aristolochia longa* and *Aristolochia hyrcana*. Traditional medicine texts have mentioned several uses for these species separately or in combination [5]. Research has shown that the aqueous extract of *Aristolochia longa* root has toxic effects on liver and kidney function in Wistar rats [6]. In traditional medicine, *Aristolochia longa* has been used topically to treat gum infection and body lice [7]. *Aristolochia hyrcana* is mainly used as an antidote to eradicate infections and also to treat respiratory

diseases such as cough and dyspnea, nervous system disorders, and purulent wounds [8].

Given that the oral use of plants in the genus *Aristolochia* is limited due to having aristolochic acid, and wounds and bites are among the common conditions in martial medicine, and *Aristolochia* is one of the most widely used plants in the treatment of these conditions, there is a strong need to standardize medicinal products obtained from *Aristolochia*. In this study, the extracts of two species of *Aristolochia* (*Aristolochia longa* and *Aristolochia hyrcana*) was standardized based on aristolochic acid.

Materials and Methods

Preparation of materials

Two species, *Aristolochia hyrcana* and *Aristolochia longa*, were obtained from the market, and were marked with herbarium code 'pmp-1237' by the Faculty of Pharmacy of Tehran University of Medical Sciences. Standard aristolochic acid was prepared from Daya Exir Jam Company.

Extraction

Aqueous and hydroalcoholic extracts were prepared from two species of *Aristolochia* by a soaking method. To prepare the aqueous extract, *Aristolochia hyrcana* and *Aristolochia longa* root powder was mixed separately with distilled water in a ratio of 1 to 5, and the mixture was soaked for 48 hours at room temperature. To prepare the alcoholic extract, 1000 ml of 70% alcohol was added to one kilogram of the powdered root of the plant, and kept for 3 days. The extract obtained from *Aristolochia hyrcana* then was separated using a filter paper and a funnel, and concentrated as much as possible by vacuum distillation, and the resulting extract was dried in an incubator at 37°C.

Aristolochic acid analysis by a HPLC method

AA I and AA II levels in the extract of *Aristolochia hyrcana* and *Aristolochia longa* were analysed using a High-Performance Liquid Chromatography (HPLC) [12]. The extraction process includes adding 100 mg of *Aristolochia* plant extract to 10 ml of acetonitrile; The solution is then placed in a Bain-Marie at 37°C, and passed through a 0.45 µm filter.

The HPLC instrument used in this study was an Agilent 1200. The HPLC conditions used are as follows: an Agilent column (C18, 4.6 x 150 mm, *5 µm)* of acetonitrile, and deionized double-distilled water, each with 100 µl of trichloroacetic acid as mobile phases at a flow rate of 0.5 ml/min was used. The observed retention time for AA I is 8.8 min. The analyte was detected by a HPLC UV detector at 390 nm [13].

Drawing the calibration curve

2 mg of standard aristolochic acid was added to 1 ml of acetonitrile-water solvent, each with a volume ratio of 50%. In order to draw a calibration curve and determine the relationship between the areas under the curve peaks and different concentrations of the standard aristolochic acid solution, the concentrations of 1 0.5, 0.25, and 2 mg/ml were made, and 20 microliters of each was injected into the HPLC device.

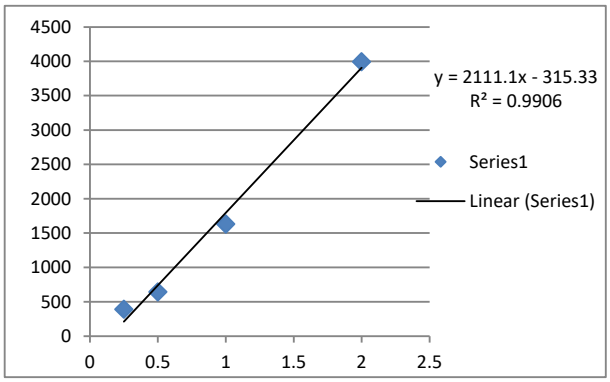

Figure 1. Calibration curve

Table 1. HPLC parameters

HPLC column	**Agilent, c18. (5 µm, 4.6 x 150 mm)**		
Mobile phase A	**0.1% trifluoroacetic acid-purified water**		
Mobile phase B	**0.1% trifluoroacetic acid-acetonitrile**		
Pump program			
Time,min	%A	%B	Gradient curve
0	80	20	NA[a]
25	30	70	1[b]
30	0	100	1
31	80	20	1
40	80	20	NA
Detection	Ultraviolet absorbance detector(390nm)		
Column temperature	40		
Flow rate	Approximately 0.5 mL/minute; flow rate should be adjusted so that desired retention time will be obtained		
Injected volume,µL	25		
Injection time,min	40		

Results and Discussion

Table 2. Aristolochic acid level of aqueous and alcoholic extracts of *Aristolochia hyrcana* and *Aristolochia longa*

Species	Extract	Aristolochic acid level (mg)
Aristolochia hyrcana	Alcoholic	0.18
	Aqueous	0.17
Aristolochia longa	Alcoholic	0.17
	Aqueous	0.20

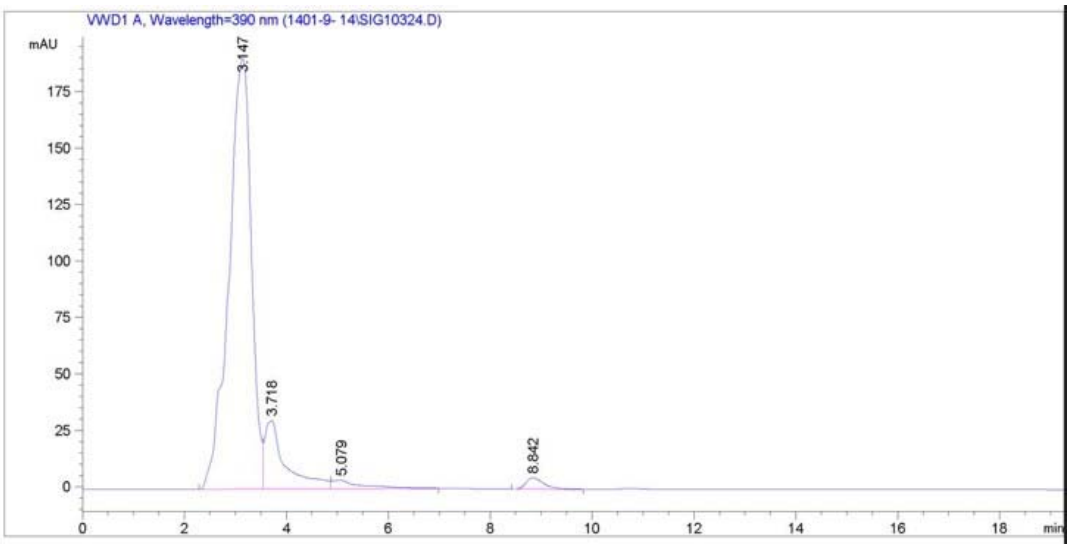

Figure 2. HPLC diagram of the aqueous extract of *Aristolochia longa*

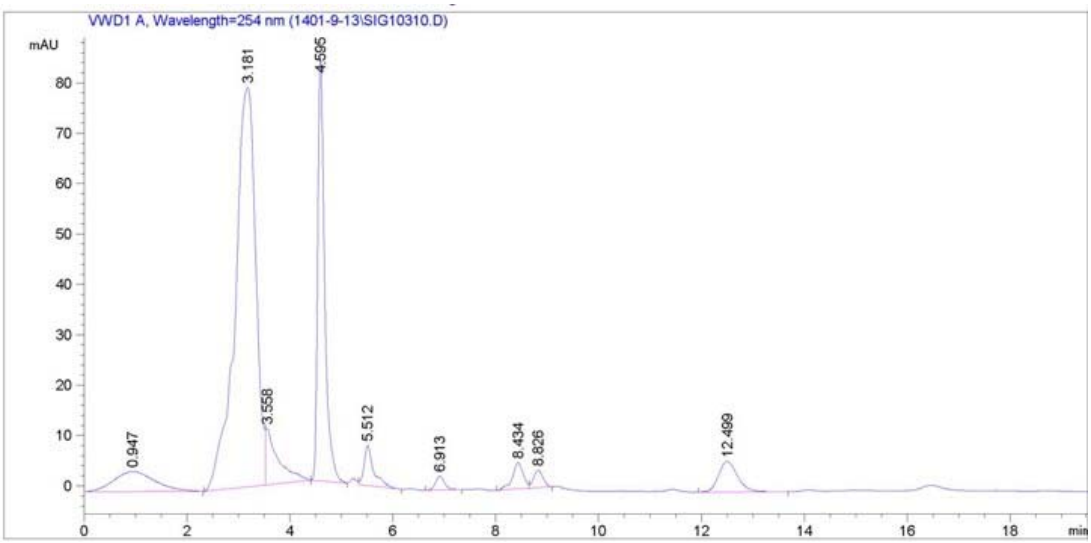

Figure 3. HPLC diagram of the alcoholic extract of *Aristolochia longa*

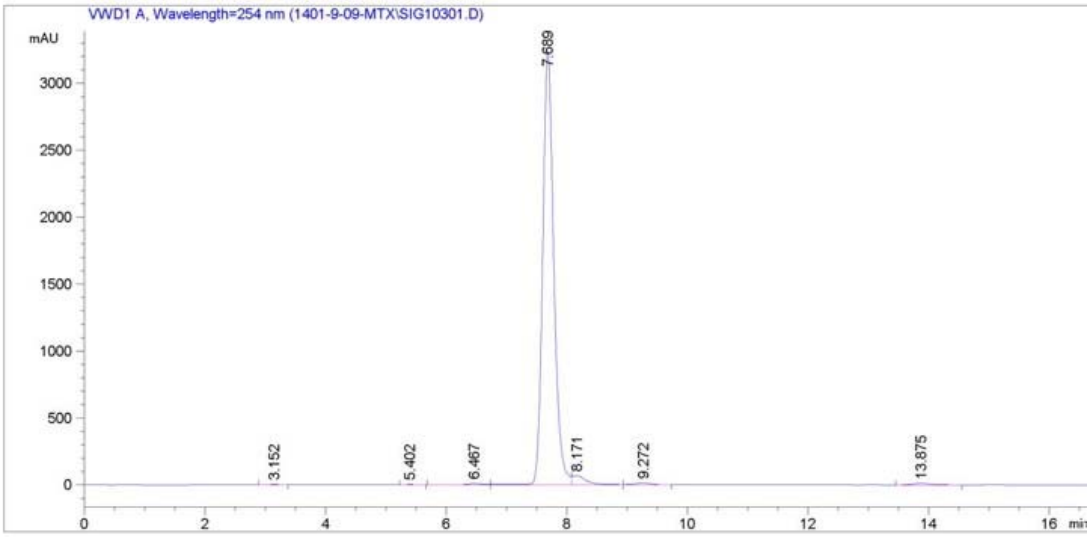

Figure 4. HPLC diagram of the aqueous extract of *Aristolochia hyrcana*

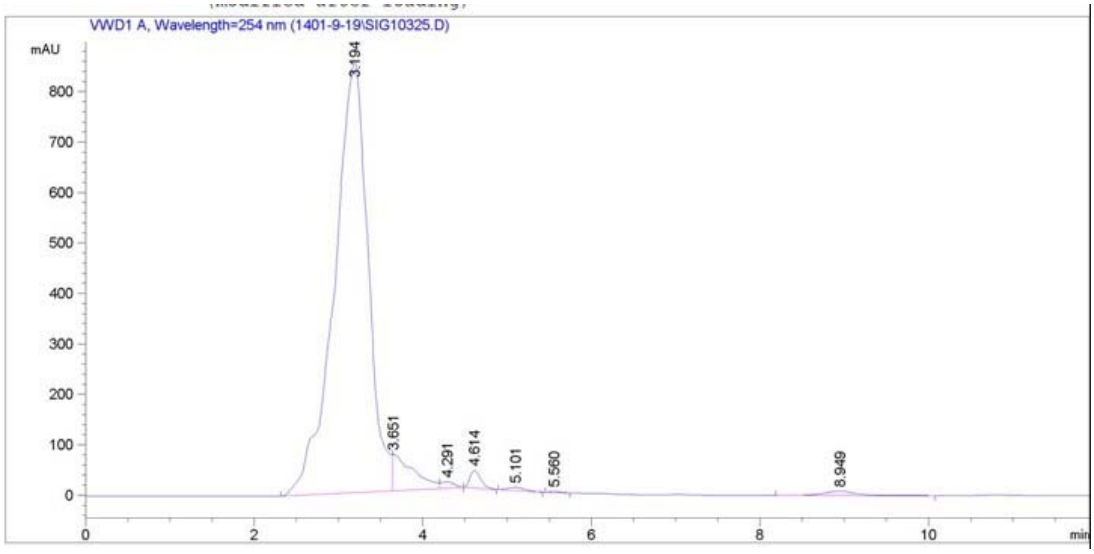

Figure 5. HPLC diagram of the alcoholic extract of *Aristolochia hyrcana*

Aristolochic acids found in *Aristolochia* species are a series of substituted nitrophenanthrene carboxylic acids whose main compounds are 3,4-methylenedioxy-8-methoxy-10-nitrophenanthrene-1-carboxylic acid (aristolochic acid I), and its demethoxylated derivative aristolochic acid II.

Aristolochic acids appear to be ubiquitous in plants, and are found in roots, stems, leaves, and fruits. *Aristolochia* species also contain related aristolactams, which are cyclic phenanthrene amides. It has been reported that aristolochic acids do not exist outside the *Aristolochiaceae* family.

Aristolochic acids have been reported to stimulate defense mechanisms against infections and inflammation in various mammalian species, including humans, and this acid stimulate the phagocytic activity of peripheral granulocytes in healthy volunteers at oral daily doses of 3 times 0.3 mg per person for 10 days.

This daily dose is approximately 0.015 mg/kg for a 60 kg person [14]. However, the recommended dose of *Aristolochia hyrcana* is approximately 5-7 grams for a person weighing 70 kg in the field of Iranian traditional medicine [15].

The pharmacokinetics of aristolochic acid I and II has been studied in mice, rats, guinea pigs, dogs and humans after oral treatment. The studied doses ranged from 0.6 to 85 mg/kg. Most of the results are related to mice. After oral administration, aristolochic acid I was readily absorbed from the gastrointestinal tract. After oral administration of aristolochic acid I to mice, about 91% of the dose was equally distributed in urine and feces. It has been reported that three lactating mothers treated with oral doses of 1.35 mg of aristolochic acid for three days excreted 2.5-5% of the dose of aristolochic acid in their milk [14].

In rats, oral LD50 of aqueous solutions of the active ingredient in aristolochic acids is 203.4 mg/kg in males, and 183.9 mg/kg in females. LD50 has been reported as 82.5 mg/kg in males, and 74.0 mg/kg in females after intravenous injection. In mice, oral LD50 of aristolochic acids is 55.9 mg/kg in males, and 106.1 mg/kg in females. LD50 is reported as 38.4 mg/kg in males, and 70.1 mg/kg in females after intravenous injection [14].

El Omari et al. (2020) showed that *A. longa* aqueous extract (ALAE) has a low toxicity potential in long-term oral administration. However, in continuous and higher oral doses, *A. longa* has significant toxicity on liver, kidney, and intestine. The molecular mechanism of AA toxicity in the studied organs is related to mutagenesis, and capable of forming covalent DNA adducts during metabolic activation in various organs through cyclic nitrenium ion, which leads to DNA damage and cell cycle arrest [16].

A 4-week study on AA toxicity in mice showed that an oral dose of 25.0 mg/kg AA resulted in degenerative lesions mainly in the kidneys, bladder, and testes. The level of non-toxic effect was detected at 0.2 mg/kg AA [17].

Xu et al. (2021) reported that aristolochic acid (AA) is widely used for anti-infective, anti-viral and anti-bacterial treatment since antibiotics were banned in the poultry industry. But long-term use of this drug in high doses can cause harmful damage to all animals. In this study, the LD50 of AA in Tianfu male broilers was 14.52 mg/kg. Exposure to high doses of AA in broilers can cause nephrotoxicity by breaking the redox balance and causing oxidative stress, along with promoting apoptosis of kidney cells. As a result, it has been found that AA in high doses damages the kidneys of broiler chickens [18].

Intraperitoneal injection of aristolochic acid at a dose of 15 mg/kg for 3 days resulted in a significant increase in inflammatory cell filtration and tubular atrophy in C57BL/6 mice [19]. Another study showed that the administration of 10, 50 and 100 mg/kg of aristolochic acid for 3 days causes necrosis of renal tubular epithelium cells and a significant increase in glucose and protein levels in urine; They found that administration of an oral dose of 100 mg/kg AA resulted in extensive necrosis affecting almost all nephrons in the kidneys of

mice. However, when a lower dose of 10 mg/kg was administered, no necrotic lesions were observed [20].

Bourhia et al. (2019) showed that rhizome decoction of *A. Paucinervis* (RDA) causes toxicity in mice at a dose of 1 g/kg/day under subacute toxicity conditions. RDA is safe at a dose of less than 4 g/kg. The plant extract prepared by decoction showed more toxic effects than the extract prepared by soaking at room temperature [21].

In Mengs' (1987) study, the acute effects of a mixture of aristolochic acids I (77.2%) and II (21.2%), intragastrical or intravenous administration at high doses in male and female rats (NMRI) and rats (Wistar) resulted in death caused by acute kidney failure within 15 days. Depending on the species and genus, the oral LD50 ranged from 56 to 203 mg/kg and the intravenous LD50 ranged from 38 to 83 mg/kg. The predominant histological features were severe necrosis of the renal tubules, atrophy of the spleen and thymus, superficial lesions of the pre-gastric from both routes, followed by hyperplasia and hyperkeratosis of the squamous epithelium [22].

Guinnin et al. also reported short-term clinical signs after administration of ethanol extract of *A. albida* to animals [23]. Yuan et al also found similar results on mice treated with *A. fructus* for 28 days [24]; Additionally, according to Liu et al., administration of 4 grams of *A. manshuriensis* and 4 mg of aristolochic acid per day using the same animal model caused acute kidney failure [25].

However, in a study that examined the ethanolic extract of *A. manshuriensis* for 8 weeks, it was found that the kidney function of the tested mice was not affected [26].

According to the studies conducted, the amount of aristolochic acid in the extract of *Aristolochia hyrcana* and *Aristolochia longa* was found in the range of 0.17-0.20 mg/kg extract in our study. According to these results, the amount of aristolochic acid in *Aristolochia longa* and *Aristolochia hyrcana* was 0.038 and 0.030 mg/kg respectively, which does not reach the toxicity level according to previous studies.

These results show that although in our study, the toxicity dose of *Aristolochia longa* and *Aristolochia hyrcana* was not in the danger zone according to other studies; However, various studies showed that frequent oral administration of these plants causes damage to various organs of the body, including the kidney. Therefore, for the ultimate control of AA toxicity, we need to further study the content of AA in related medicinal materials and preparations to determine the minimum effective and toxic dose. We also need to study methods for effective detoxification of drugs that can reduce AA toxicity and control the incidence and development of AA-induced side effects. Some factors such as pharmaceutical ingredients, dosage form, extraction process, composition, dose, and treatment duration can affect AA exposure. This scientific approach helps provide a clearer understanding of research findings on AA toxicity in laboratory animals and humans and its implications for traditional medicine. However, it is important to acknowledge that our conclusions should be limited to the data provided within the scope of this study.

Conclusion

These results indicate the safety of the recommended doses for *Aristolochia longa* and *Aristolochia hyrcana* in traditional Iranian medicine. However, it is important to note that natural compounds in plants can be significantly different even in different regions of Iran. Therefore, more studies are needed to evaluate the content of AA in *Aristolochia longa* and *Aristolochia hyrcana* in different geographical locations. Additionally, more research on different animal species, laboratory models, and analysis of specific compounds such as aristolochic acid is necessary to definitively prove the non-toxic nature of the doses used in traditional medicine.

Availability of data and materials

The data that support the findings of this study are available from AJA University of Medical Sciences, but restrictions apply to the availability of this data, which were used under license for the current study, and are not publicly available. Data are however available from the authors upon reasonable request and with permission AJA University of Medical Sciences.

Authors' contributions

All authors contributed to the study conception and design. The manuscript has been read and approved by all named authors and there are no others persons who satisfed the criteria for authorship but are not listed. The order of authors listed in the manuscript has been approved by all of us. All authors read and approved the fnal manuscript.

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Conflict of interest: None

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Ethics statement: None

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