REV SEN
rev-sen.ec

Methods for extracting and isolating aristolochic acid from natural matrices.

María Cumandá Toral Tello [ID] [1], Lorena Viviana Mora Bravo [ID] [2]

1. Biochemistry and Pharmacy Degree, Faculty of Chemical Sciences, Universidad De Cuenca, Cuenca-Ecuador.

Received: December 30, 2023.
Accepted: January 23, 2024.
Published: February 13, 2024.
Editor: Dr. Salvador Magaña Mercado.

Cite:
Toral M, Mora L. Methods for extracting and isolating aristolochic acid from natural matrices. REV SEN 2024;12(1):32-40.

DOI: http://doi.org/10.56867/70
Sociedad Ecuatoriana de Nefrología, Diálisis y Trasplantes.

ISSN-L: 2953-6448

Abstract

Introduction: Medicinal plants from the *Aristolochia* genus have traditionally been employed to treat various pathologies. However, these plants are known to contain substantial quantities of aristolochic acids, both type I and II, which have been linked to the development of nephropathies and high-risk cancers due to their inherent toxicity. Thus, using these plants is considered a severe health risk for individuals.

Methods: This study aimed to identify effective extraction and isolation methods for aristolochic acid-desired natural matrices to obtain a standardized extract of the Aristolochia plant, Zaragoza liana (*Aristolochia*) from Puyo and Macas, Ecuador, was selected for analysis. The extraction methods employed included supercritical fluids and ultrasound-assisted extraction, with methanol as the solvent. Subsequently, the metabolites sing thin-layer chromatography (TLC) to confirm the presence of aristolochic acid.

Results: The results revealed that aristolochic acid exhibited a retention factor within Rf 0.61-0.77. These results were observable through yellow-colored bands under white light, which appeared dark or gray under UV light at 254 nm, and that the products displayed black coloration with a yellow halo under UV light at 366 nm. These distinctive characteristics provided conclusive evidence of the presence of aristolochic acid in the analyzed samples.

Conclusion: This study established effective methods for extracting and isolating aristolochic acid from *Aristolochia* plants. The results obtained through thin-layer chromatography confirmed the presence of this compound in the analyzed samples, underscoring the concern regarding the toxicity associated with medicinal plants. These findings emphasize the importance of standardizing *Aristolochia* extracts and highlight the potential risks linked to their consumption for human health.

Keywords:

Aristolochic Acid, Maceration, Aristolochia, Supercritical Fluids, Ultrasound, Thin Layer Chromatography.

* Corresponding author

Email: Lorena Viviana Mora Bravo < lorena.mora@ucuenca.edu.ec >/Address: Campus -UCUENCA, Biochemistry and Pharmacy Degree, University of Cuenca. Av. 12 de Abril and Av. Loja. Ecuador Basin. Tel. (593-7) 405 1000.

Kidney diseases have become a severe health problem, with nephrotoxicity being one of the most recurrent causes of hospital admissions. The kidney is the primary organ involved in detoxifying drugs and traditional herbal medicine [1].

Some species have been previously identified as medicinal agents but are unfamiliar with their metabolites, which can lead to possible tubulointerstitial kidney disease [2].

Aristolochic acid is a natural compound found in herbaceous plants of the genus *Aristolochia* that contains different toxic components, such as aristolochic acid type I (AA-I) and aristolochic acid type II (AA-II). This genus of bacteria is widely used in traditional medicine for treating different diseases due to its known anti-inflammatory, analgesic, expectorant, and antibacterial effects. However, in addition to its pharmacological properties, it can cause nephrotoxicity and carcinogenesis, mainly due to long-term administration [3, 4].

Several extraction and identification methods have been applied to natural sources of aristolochic acid in different countries, such as China, Sudan, Brazil, Chile, and Russia; however, the appropriate extraction method must be determined to obtain aristolochic acid effectively. This work compares two of the most commonly mentioned methods in the literature to determine which yields a better concentration of metabolites, including aristolochic acid.

Materials and methods

Study design

This project is based on a practical design that aims to extract and isolate aristolochic acid from the natural genus Aristolochia's natural matrices using standard maceration and more advanced and environmentally friendly extraction methods, such as maceration assisted by ultrasound and supercritical fluids. This study determined which method yielded the most aristolochic acid.

Reagents and Equipment

Table 1. Equipment and reagents used.

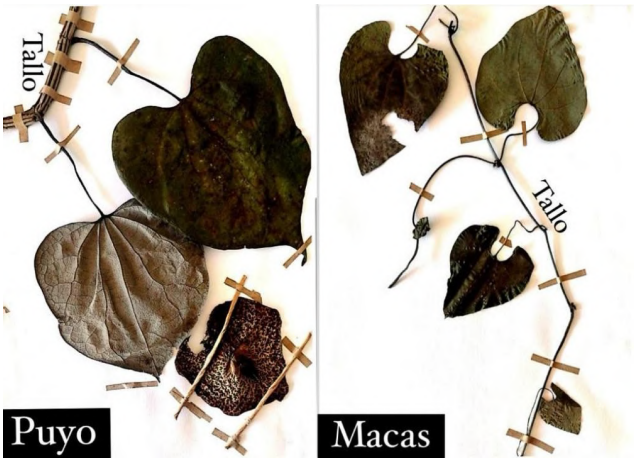
Reagents	Equipment
Methanol	Bathroom of ultrasound Branson 8893, state Joined
Chloroform	Rotavapor brand Laborota 4000 Efficient of Heidolph ® (Schwabach , Germany)
Acid acetic	Biofreezer Fisher Science ® R134A (Massachusetts, state Joined)
Acid hydrochloric	freeze dryer, FreeZone 2.5 of LABCONCO ®, (Kansas, state Joined)
Tin Chloride Dihydrate .	Equipment of fluent supercritical, brand Waters ®, model MV10 (Milford, state Joined)
Water type 2	Oven utility , model 61305 VWR,
Nitrogen gaseous degree 3	CAMAG ® FTA Visualizer.
Dioxide of carbon, Linde brand (Germany).	
Reagent of Dragendorff	

Collection of plant matter

The plant samples to be studied were *Aristolochia* sp., known as Zaragoza Lianae; they were collected in Pastaza (Puyo) and Morona Santiago (Macas) in the eastern zone of Ecuador.

The rattan samples were found to be domestically grown liana. The aerial parts (stems and leaves) of two samples of Zaragoza liana were collected during sampling. For the analysis, approximately 150 g of fresh samples were collected because plants between young and mature ages exhibit better characteristics, such as color and size, without signs of deterioration (leaf burning or leaf spots). The samples presented differences in the thickness of the stem and the size of the leaves, with the stem being thicker and the leaves of the plant collected in Puyo being larger (Figure 1).

Figure 1. Leaves and stems of the Puyo and Macas plants.



Preparation of the plant sample

The fresh sample was taken to the Laboratory of Medicinal Plants and Natural Products Department of Biosciences of the University of Cuenca to select and dry the analyzed parts. Each sample was weighed to choose the parts that did not deteriorate. Then, the plants were washed with drinking water, drained three times, placed in distilled water for 10 minutes, and emptied again. After this, the plants were placed on mesh covered with absorbent paper to remove excess water, and finally, on an electric dryer, meshes covered with newspaper were spread out and separated so that there was adequate airflow and drying was uniform throughout a temperature of 48 °C. The samples were reviewed after 48 hours to verify their drying status, and after 72 hours, the samples were removed from the electric dryer.

Sample crushing

Each dry sample was weighed before grinding, which was carried out in an Oster brand blender. The process consisted of placing a part of the dry matter in the blender for 10 seconds, turning it off, waiting a moment, and repeating the procedure three times. Subsequently, the plants were passed through a small mesh light sieve and stored in an airtight plastic bag. This process was carried out until the entire sample was crushed and sieved. In the case of the stems, they are chopped until they are approximately 1 to 3 mm in size. Once the two samples were utterly crushed and sieved, each was weighed again, and the final dry weights of the Puyo and Macas samples were obtained; these samples were referred to as T01 and T02, respectively. The samples were stored frozen at -80 °C.

Extraction process

This process consists of three phases to obtain the final concentration of metabolites: an extraction phase, a second concentration phase, and a lyophilization phase to help preserve the sample.

In the first phase, two techniques were used: ultrasound-assisted maceration and prior moistening of the sample. Maceration is carried out with agitation to increase the extraction of metabolites using a polar solvent, an analytical type of methanol, in sufficient quantity to allow for an adequate contact surface. This process removes the metabolites present in the plants. Sonication increases the solvent contact with the components, increasing metabolite amounts.

The second phase concentrates the metabolites by evaporating the solvent through distillation and then condensing it. The third phase consists of a freeze-drying process in which the sample is dehydrated and preserved for later storage (Figure 2).

.

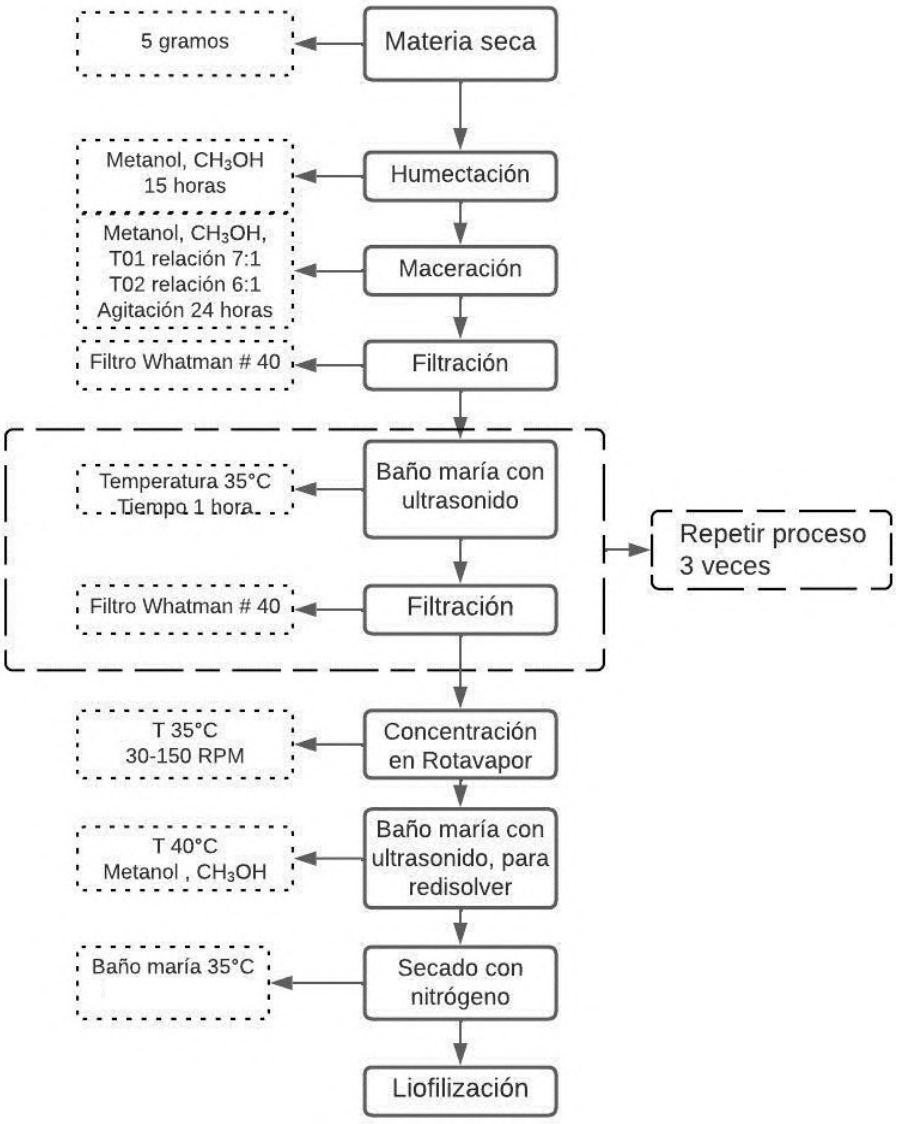


Figure 2. Metabolite extraction and concentration process diagram

Freeze-drying process

The freeze-dried extract tube was frozen in a freezer at -80°C. The tube was first placed horizontally for 5 minutes; after this time, the tube was rotated and left for 3 minutes, after which it was rotated again for 3 or 2 minutes; this process was repeated, but the time between turns was decreased until the tube wholly froze in a circular shape attached to the walls of the tube. The mixture was frozen for 2 hours, after which the tubes were subjected to lyophilization at a pressure of 0.090 mBar and a temperature of -52°C.

Extraction with supercritical fluids

For the extraction, supercritical fluid equipment was used following the Standard Work Procedure established in the Phytochemistry Laboratory of the Medicinal Plants group of the University of Cuenca. This equipment uses carbon dioxide as a nonpolar solvent and has a purity of 99.99%. Initially, 2.3 g of each sample was weighed and placed in the cells; the cell was placed in the sample holder connected to the ports. For the extraction, an automatic method was used, where the polar cosolvent was HPLC grade methanol with a flow of 0.5 ml/min, which represents 10% of the flow, and CO2 had a flow of 4.5 ml/min at a temperature of 60°C and a pressure of 200 bar. The extraction took place in dynamic mode for 20 min, after which the mixture was static for 30 min and then dynamic again for 10 min for two cycles, for an extraction time of 120 min. The sample was collected in screw-cap tubes and subsequently concentrated with nitrogen until approximately 4 ml of the sample was left and stored refrigerated.

Identification via thin-layer chromatography of aristolochic acid

To identify the presence of aristolochic acid in the extracts obtained from Zaragoza vines, a process from the Phytochemistry Laboratory of the University of Cuenca was used based on the TLC methodology [5], which was modified to observe the presence of the aristolochic acid better. Silica gel 60 F254 plates 5 × 10 cm in length with glass and plastic backing were prepared and activated in a utility oil oven at a temperature of 100°C for 1 hour. Two mobile phases were prepared: the first was chloroform: methanol (6:1), and the second was chloroform:methanol: acetic acid (90:8:2). The mobile phases were conditioned for 30 min before they were added to the chamber; in addition, Dragendorff's reagent and tin chloride reagent (SnCl2*2H2O) were used as developers. After being developed with SnCl2*2H2O, the mixture was placed in an oven at 100°C for 10 to 15 minutes. White light was applied at 254 nm and 366 nm using CAMAG® TLC Visualizer equipment.

Analysis of the data

Notably, aristolochic acid was present under conditions of yellow coloration under white light, dark gray or black coloration under UV light at 254 nm, and black coloration with a yellow halo at 366 nm, which led to an Rf ranging between 0.53 and 0.80 [5-9].

Results

Collection of data

The Zaragoza vine species were collected from different places to compare the presence of aristolochic acid; thus, the Puyo sample was referred to as T01, and the Macas sample was referred to as T02 (Table 2).

Table 2. Geographic data of the collection location.

	T01	T02
Sector	Siguín, viaMacas	Neighborhood "La Florida"
Length	-77.907296	-78.122302
Latitude	-1.545143	-2.287472
Elevation	983.3 masl	1063.7 masl
Temperature	25°C	23°C
Date of harvest	02-03-2023	02-03- 2023

Drying of Zaragoza vine samples

The fresh samples initially weighed 176.34 g for T01 and 156.57 g for T02, which, after the drying process, resulted in dry weights of 72.25 and 39.26 g for T01 and T02, respectively. This could be because the percentage of humidity presented by each sample had a notable variation of 15%. The moisture content was more significant in the sample obtained in the city of Macas with 75% humidity than in the Puyo sample obtained with 60% humidity, which could be due to the maturity of the plants, which was defined by characteristics such as flowers and the thickness and size of the leaves. In general, the observed humidity range agrees with a study by [10], in which young leaves and stems taken at random had a humidity ranging from 60-80%, with young leaves and stems exhibiting greater humidity.

Extraction yield of Zaragoza rattan obtained by ultrasound-assisted maceration versus extraction yield by supercritical fluids

The extract yield obtained by ultrasound-assisted maceration of sample T02 was more significant than that obtained from sample T01 since 11.9% and 7.1% g of lyophilized extract were obtained from 10 g of dry sample, respectively. Similarly, after extraction by supercritical fluids (2.3 g), the yields were 10% and 15.2% for the extracts of T01 and T02, respectively, as appropriate, after reducing the sample with gaseous nitrogen. With these results, extraction with supercritical fluids performed better than ultrasound-assisted maceration. Therefore, supercritical fluid extraction is more efficient than ultrasound-assisted maceration.

Identification by thin-layer chromatography

The coloration determined the presence of aristolochic acid in this metabolite: a yellow color in the visible light, a dark or gray color in the UV light at 254 nm, and a black color with a yellow halo in the UV light at 366 nm, the same as what was observed in the images. This coloration was compared with the results of published studies of different species of *Aristolochia*.

When using a mixture of chloroform:methanol: acetic acid as a mobile phase from the extracts obtained by supercritical fluids, the presence of a dark color with a yellow halo at a wavelength of 366 nm was observed, which may indicate the presence of aristolochic acid, with Rf values of 0.63 and 0.66 for extracts T02 and T01, respectively, which were revealed with tin chloride dihydrate (Figure 3). These values were similar for the plate developed with the dragendoff reagent; an Rf of 0.61 was obtained for T02, and an Rf of 0.63 was obtained for T01. However, it is worth mentioning that the color intensity increased more in T02 than in T01. On the other hand, for the extracts obtained by ultrasound-assisted maceration, bands corresponding to aristolochic acid were not observed (Figure 4).

A dark color with a yellow halo was also observed for the second mobile phase mixture of chloroform: methanol. However, unlike in the previous case, the Rf values were only 0.63 and 0.77 for the T02 extract obtained by the supercritical fluid and revealed with tin chloride dihydrate (Figure 5). These values and the coloration were similar in the plate developed with the Dragendorff reagent, with Rf values of 0.68 and 0.77 in the T02 extract (Figure 6). A more intense coloration was observed in both plates at an Rf of 0.77. Likewise, the absence of bands in the ultrasound-assisted maceration extraction indicated the presence of aristolochic acid.

Discussion

This study aimed to determine the appropriate extraction and identification methods for obtaining aristolochic acid from the Zaragoza bejuco species. Ultrasound-assisted maceration methods and supercritical fluids were evaluated, and the best method was selected by identifying aristolochic acid by thin-layer chromatography using two mobile phase mixtures.

In this project, two developers are used. The first is the Dragendorff reagent, which allows the presence of the metabolite to be visualized. However, the intensity of the coloration was lower than that of the SnCl2*2H2O reagent developer, which showed more intense coloration, allowing more precise identification. Thus, SnCl2*2H2O allows better visualization of the samples.

The retention factor ranged from 0.61-0.77, which, when compared with Dey, Blatter & Reich, Sudhakaran, Ioset, and Abdelgadir, was calculated based on similar methodologies, where a range of Rf between 0.53 and 0.80 was found.

In this study, it was observed that sample T02 obtained by supercritical fluid presented conditions that may indicate the presence of aristolochic acid in the two mobile phases. On the other hand, in sample T01, these conditions were observed only in the mobile phase chloroform:methanol: acetic acid.

Figure 3. TLC was performed with a mobile chloroform, methanol, and acetic acid phase at 254 and 366 nm. Rev. SnCl2*2H2O.

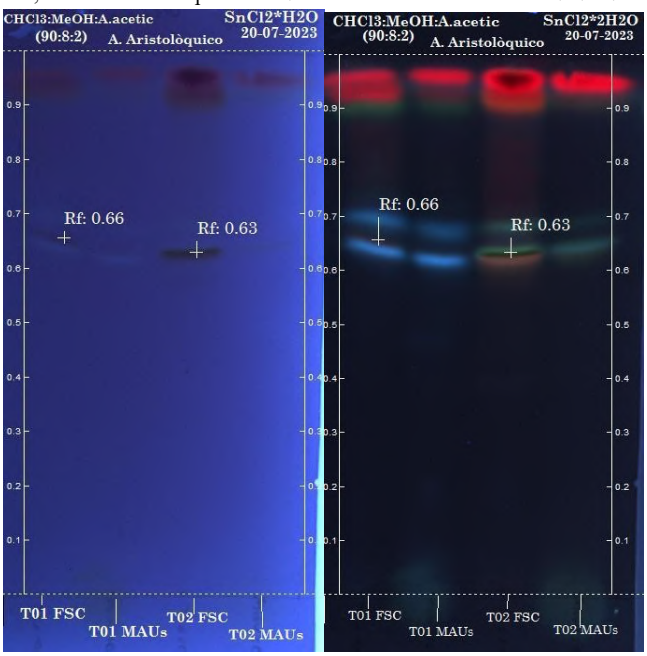
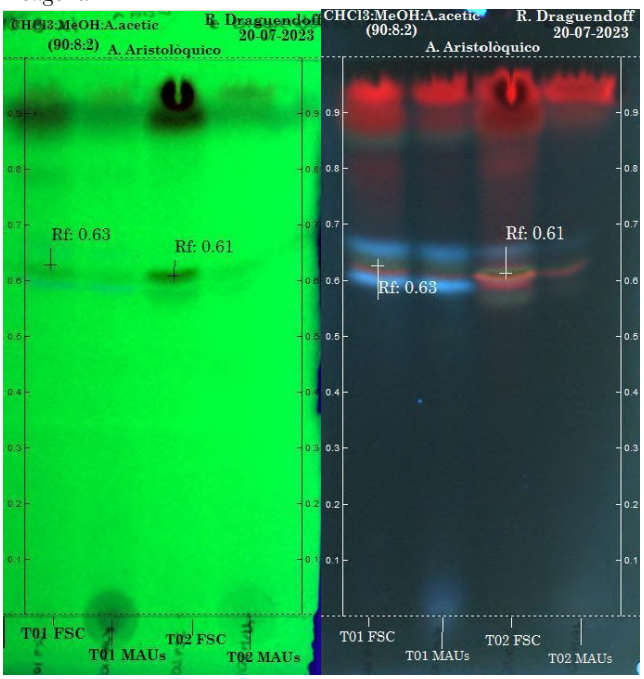


Figure 4. TLC was performed with a mobile phase of chloroform:methanol: acetic acid at 254 and 366 nm with Rev. Dragendorff Reagent.



This means that the presence of bands that indicate the presence of a metabolite depends on several factors, such as the extraction method, the nature of the compound, the concentration of the compound, and its affinity for the stationary and mobile phases [11].The concentration of metabolites varies depending on the efficiency of the extraction method. An efficient extraction method allows the recovery of the most significant number of metabolites possible from the sample. If extraction is complete, metabolites may be included in the sample matrix and will not be detected at substantial concentrations [12].

When performing the extraction, only with supercritical fluids could the presence of the metabolite be observed on the thin layer chromatography plates. This may be due to the parameters used in this method since a nonpolar solvent, carbon dioxide (CO_2), which has a critical temperature of 31°C and a pressure of 74 bar, is used for the extraction. By exceeding the essential points of pressure and temperature, a supercritical fluid is obtained; this fluid has a density and viscosity that allows greater diffusion of the solvent through the membrane, increasing penetration into the pores, which, in this way, results in an excellent yield of the final extract. In addition, a cosolvent that confers a particular polarity to the supercritical fluid increases its capacity as a solvent [13].

On the other hand, ultrasound-assisted maceration extraction involves using high-frequency and powerful sounds ranging up to 100 kHz to release the compounds of interest [12]. For this purpose, the temperature, extraction time, and sonication duration must be considered. This approach is essential for obtaining a higher concentration of metabolites, considering the substance to be extracted. This study considered these factors using three one-hour cycles at 40°C. However, the presence of the metabolite of interest could not be confirmed.

According to the results, the absence of metabolites in the thin-layer chromatography plates may be mainly due to the extraction method since an adequate extraction method can purify the sample and eliminate unwanted compounds. Concentrating the sample by removing unwanted solvents or diluents is also possible. A more concentrated sample can produce more intense bands via chromatography [11].

Furthermore, the mobile phase used in chromatography significantly influences the presence of bands in thin-layer chromatography [11]. Since the expected substances must be able to dissolve, if a substance is poorly soluble in the mobile phase, it may not move adequately across the plate, resulting in a weak or absent band. Compared with the results obtained in the chloroform: methanol mobile phase, the absence of a band on the plate may be due to the concentration of the metabolite and the affinity of this sample for this phase.

Conclusion

Based on these tests, the optimal extraction method is the supercritical fluid method due to the conditions presented in the chromatography plates, raising suspicion of the presence of aristolochic acid. Furthermore, the mobile phase that showed the best affinity was a mixture of chloroform:methanol: acetic acid, together with the development of tin chloride dihydrate, which allowed better visualization of the bands.

Figure 5. TLC was performed with a chloroform: methanol mobile phase at 254 and 366 nm. Rev. SnCl2*2H2O.

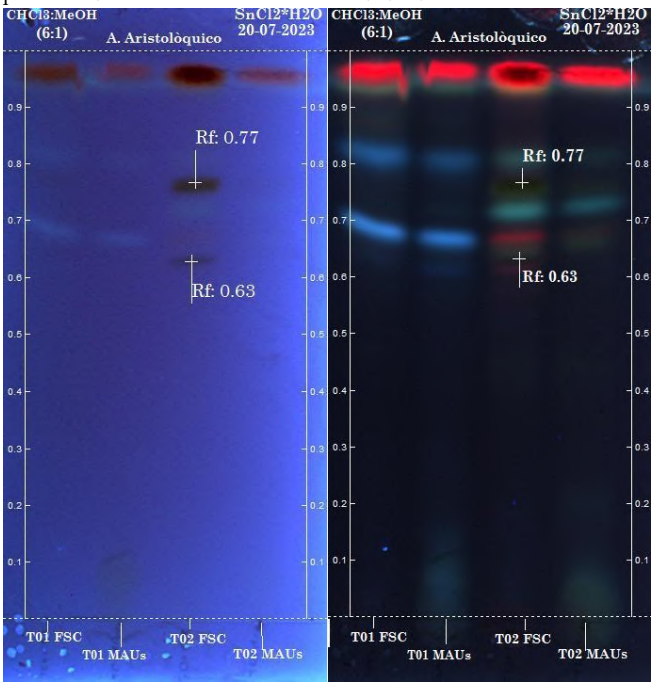
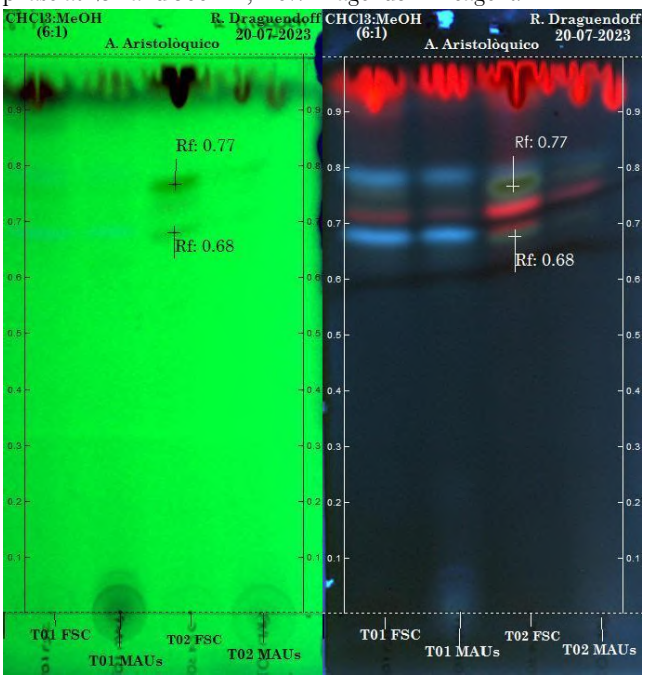


Figure 6. TLC was performed with chloroform: methanol mobile phase at 254 and 366 nm, Rev. Dragendorff Reagent.



However, the ideal identification method cannot be determined because a reference standard is required to verify the presence of aristolochic acid and the conditions applied.

Abbreviations
Does not apply

Supplementary information
The supplementary materials have not been provided.

Acknowledgments
Does not apply.

Author contributions
María Cumandá Toral Tello: Data curation, Formal analysis, Funding acquisition, Research, Methodology, Project administration, Resources, Software, Writing – original draft.
Lorena Viviana Mora Bravo: Conceptualization, supervision, validation, visualization, writing: review and editing.
All the authors read and approved the final version of the manuscript.

Financing
The Faculty of Chemical Sciences of the University of Cuenca funded the study.

Availability of data or materials
The data sets generated and analyzed during the current study are not publicly available but can be shared upon academic request.

Statements

Ethics committee approval and consent to participate
Does not apply.

Consent for publication
Patient photographs, tomography scans, or X-ray studies were not needed.

Conflicts of interest
The authors declare no conflicts of interest.

Author information
Does not apply.

References

1. Yang B, Xie Y, Guo M, Rosner MH, Yang H, Ronco C. Nephrotoxicity and Chinese Herbal Medicine. Clin J Am Soc Nephrol. 2018 Oct 8;13(10):1605-1611. doi: 10.2215/CJN.11571017. Epub 2018 Apr 3. PMID: 29615394; PMCID: PMC6218812.

2. Zhang Q, Luo P, Chen J, Yang C, Xia F, Zhang J, Tang H, Liu D, Gu L, Shi Q, He X, Yang T, Wang J. Dissection of Targeting Molecular Mechanisms of Aristolochic Acid-induced Nephrotoxicity via a Combined Deconvolution Strategy of Chemoproteomics and Metabolomics. Int J Biol Sci. 2022 Feb 21;18(5):2003-2017. doi: 10.7150/ijbs.69618. PMID: 35342337; PMCID: PMC8935225.

3. Fang L, Tian M, Row KH, Yan X, Xiao W. Isolation of aristolochic acid I from herbal plant using molecular imprinted polymer composited ionic liquid-based zeolitic imidazolate framework-67. J Sep Sci. 2019 Oct;42(19):3047-3053. doi: 10.1002/jssc.201900554. Epub 2019 Jul 31. PMID: 31313494.

4. Zhang M, Liu H, Han Y, Bai L, Yan H. A review of the pharmacological properties, toxicological characteristics, pathogenic mechanism, and analytical methods of aristolochic acids. Toxin Reviews 2021;40(4):606-615. doi: 10.1080/15569543.2020.1811344.

5. Abdelgadir AA, Ahmed EM, Eltohami MS. Isolation, Characterization and Quantity Determination of Aristolochic Acids, Toxic Compounds in Aristolochia bracteolata L. Environ Health Insights. 2011 Feb 27;5:1-8. doi: 10.4137/EHI.S6292. PMID: 21487531; PMCID: PMC3072213.

6. Dey A, Nongdam P, Nandy S, Mukherjee S, Mukherjee A, Tikendra L, Hazra K, Pandey D. Polyamine elicited aristolochic acid production in vitro clonally fidel Aristolochia indica L.: An ISSR and RAPD markers and HPTLC based study. South African Journal of Botany 2021;140:326-335. doi: 10.1016/j.sajb.2020.06.018.

7. Blatter A, Reich E. High Performance Thin-Layer Chromatographic Analysis of Aristolochic Acids in Chinese Drugs. JPC – Journal of Planar Chromatography – Modern TLC 2004;17(5):355-359. doi: 10.1556/JPC.17.2004.5.6

8. Sudhakaran M. Pharmacognostic and HPTLC Fingerprint Profile of the Root of Aristolochia indica Linn. In addition, Quantification of the Marker Compound. European Journal of Medicinal Plants. 2014;4(5):1113-1124. doi: 10.9734/EJMP/2014/9570.

9. Ioset J, Raoelison G, Hostettmann K. Detection of aristolochic acid in Chinese phytomedicines and dietary

supplements used as slimming regimens. Food and Chemical Toxicology, 2003;41(1):29-36. doi: 10.1016/S0278-6915(02)00219-3.

10. Claro R, Ruiz N. Aceptación de una dieta artificial por larvas de la mariposa Battus polydamas polydamas (LEPIDOPTERA: PAPILIONIDAE). Acta Biol. Colomb 2010;15(1):47-62. Scielo: v15n1a4

11. Miller J. Chromatography: Concepts and Contrasts, 2nd Edition 2009, ISBN: 978-0-470-53025-2.

12. Azuola R, Vargas P. Extracción de sustancias asistida por ultrasonido (EUA). Tecnología en marcha. 2007;20(4):30-40. dialnet.unirioja.es/4835781

13. Páez M, Narváez C, Bermúdez L, Muñoz L, Gómez D, Carvalho C, Rico J. (2016). Guía de extracción por fluidos supercríticos: fundamentos y aplicaciones. ISBN: 978-958-15-0229-5.

DOI: Digital Object Identifier. **PMID**: PubMed Identifier.

Editor's Note

REV SEN remains neutral about jurisdictional claims over published maps and institutional affiliations.