

Phytochemical and Biological Investigation of *Aristolochia maurorum* L.

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Aristolochia maurorum L. of Jordanian origin has been investigated phytochemically, quantitatively, and biologically. Three atypical alkaloids, namely aristolochic acid I (**1**), aristolochic acid II (**2**) and aristolochic acid IIIa (**3**), have been isolated and identified. Of these known 1-phenanthrenecarboxylic acids, **2** and **3** are reported for the first time from this species. The identified compounds **1–3** were first evaluated biologically as cytotoxic agents against the brine shrimp lethality test (BST), in which compound **1** was found to be the most potent (LC₅₀, 4.9 µg/mL). The antiplatelet activity of the methanolic extracts, the acidic fractions of aerial and root parts, and the identified compounds **1–3** were evaluated using an automatic platelet aggregometer and coagulation tracer (APACT 2). Using external reference standards, and a reverse-phase isocratic method, the distribution of aristolochic acid I and aristolochic acid II in different plant parts of *Aristolochia maurorum* L. during flowering stage was analyzed by PDA-HPLC. A quantitative comparison between two previously reported extraction methods was also made. Roots were found to be the main storage of aristolochic acid I and aristolochic acid II during flowering stage with about 0.22 and 0.108% (w/w), respectively.

Key words: Aristolochic Acids, *Aristolochia maurorum*, Antiplatelet Activity

Introduction

Jordan population relies heavily on folk medicine. Contributed to the widespread use of medicinal plants in this area of the world is the richness in biodiversity of its flora which is attributed to Jordan's unique location between the three old continents' crossroads of botanic and climatic regions (Al-Khalil, 1995; Abu-Irmaileh and Afifi, 2000; Alkofahi *et al.*, 1990).

The genus *Aristolochia*, which belongs to the family Aristolochiaceae, comprises seven genera and 400 species, and flourishes mainly in tropical and warm temperate zones of both hemispheres (Watson and Dallwitz, 1992). In Jordan, four species of *Aristolochia* are reported to grow in the wild, namely: *A. maurorum* L., *A. parviflora* Sm., *A. bottae* Jaub. et Spach, and *A. billardieri* Jaub. & Spach (Al-Eisawi, 1998; Feinbrun-Dothan, 1986). Species of the genus *Aristolochia* are known commonly as Moorish Birthwort while locally as Khe-yar Al-Ghanam (Al-Eisawi, 1998).

Literature reviews report a variety of chemical compounds identified from the genus *Aristolochia* (Hong *et al.*, 1994; Achenbach and Fischer, 1997;

Wu *et al.*, 1994, 1999; Kery *et al.*, 1983) – aristolochic acids, which are a group of substituted 1-phenanthrenecarboxylic acids, in particular aristolochic acid I and II, are the major constituents (Budavri *et al.*, 1996).

Aristolochia species have well known beneficial effects. They were used traditionally as antidote in snakebites (Budavri *et al.*, 1996), an action which is confirmed by modern scientific investigation (Vishwanath *et al.*, 1987; Chandra *et al.*, 2002). Some *Aristolochia* species were found to possess anticancer activity in several bioscreening studies (Voloudakis-Baltatziz *et al.*, 1992; Mongelli *et al.*, 2000), moreover both aristolochic acids, I and II, possess good antithrombin activity (Wu *et al.*, 1994; Goun *et al.*, 2002). Among all of *Aristolochia* species' constituents, aristolochic acids I and II are usually the most potent (Goun *et al.*, 2002).

As part of our continuing demeanor to investigate and biologically evaluate Jordanian medicinal plants, phytochemical investigation, quantitative analysis and biological evaluation of *A. maurorum* L. are pursued. *Aristolochia maurorum* is found flowering from March to May in the middle and

northern parts of Jordan and characterized as perennial herb with many basal stems, spreading and forming hemispherical growth leaves with yellow spotted flowers (Al-Eisawi, 1998; Feinbrun-Dot-han, 1986).

From extracts of the aerial and root parts, three compounds were isolated and characterized, namely: aristolochic acid I (**1**), aristolochic acid II (**2**) and aristolochic acid IIIa (**3**). Of these **2** and **3** were reported for the first time in this species. The chemical structures of all compounds were elucidated using a number of spectrometric and spectroscopic techniques, in principal NMR and MS. Compounds **1–3** were tested for their cytotoxicity using the brine shrimp lethality assay. The antiplatelet activity of the methanolic extracts and the acidic fractions of aerial and root parts, in addition to the identified compounds **1–3** were evaluated quantitatively according to Born (1962) using an automatic platelet aggregometer and coagulation tracer (APACT 2). This method is based on the turbidensitometric measurement of cell suspension. Since platelet-free plasma is more permeable for long wavelength light than platelet-rich plasma, the light transmitting capacity of plasma measured in percent can be used as a measure for the aggregation (Craig and Stitzel, 1994). Acetyl salicylic acid was used as standard platelet aggregation inhibitor and ADP (adenosine diphosphate) as standard platelet aggregation inducer.

Using PDA-HPLC, aristolochic acid I and II contents were found to vary considerably in different plant parts, where roots were found to be the main storage during flowering stage.

Results and Discussion

The dried aerial and root parts of *A. maurorum* L. were extracted and fractionated individually into four fractions (A–D). High concentrations of alkaloids were noted by TLC in the acidic fraction D. Thus, fraction D from each plant part was subjected to further purification using preparative TLC and semi-preparative HPLC. Three known alkaloids, namely aristolochic acid I (**1**) (171 mg, 0.007% w/w plant dry weight), aristolochic acid II (**2**) (27 mg, 0.001% w/w plant dry weight) and aristolochic acid IIIa (**3**) (22 mg, 0.0009% w/w plant dry weight), were isolated from the acidic fraction. Their chemical structures were identified using 1D-NMR and mass spectra analyses (Fig. 1). Iden-

tity of aristolochic acid I and II was further confirmed by matching their HPLC retention times of chromatographic peaks with those of the standards. The NMR and mass spectra obtained for the three compounds were in full agreement with those reported in the literature (Preistab, 1987).

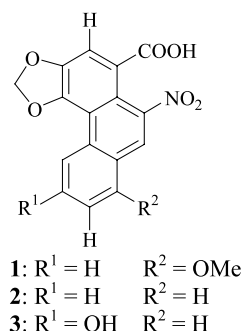


Fig. 1. Chemical structures of identified aristolochic acid I (**1**), II (**2**) and IIIa (**3**) in *A. maurorum*.

The identified compounds **1–3** and fractions A–D were biologically evaluated for cytotoxic activity using the brine shrimp lethality test (BST), in which compound **1** and the acidic fraction D of both the roots and the aerial parts were found the most potent with LC₅₀ of 4.9, 1.4, and 20.2 µg/mL, respectively (Table I).

Table I. Brine shrimp lethality test results for fractions and identified compounds of *Aristolochia maurorum*.

Compound	LC ₅₀ ^a	Confidence intervals 95%
Aristolochic acid I (1)	4.9	2.3–9.3
Aristolochic acid II (2)	10.8	4.4–27.2
Aristolochic acid IIIa (3)	168.9	40.0–718.2
Aristolochic acid standard ^b	3.3	1.2–6.6
(–)-Colchicine ^c	2.6	1.8–4.6

^a BST results are expressed as LC₅₀ values (µg/mL; concentration to kill 50% of the brine shrimp) using four data points, each run in triplicate.

^b Aristolochic acid standard is a mixture of aristolochic acid I (38%) and II (58%).

^c Positive control.

The antiplatelet activity of the methanolic extracts and the acidic fractions of aerial and root parts in addition to the identified compounds **1–3** were evaluated using an automatic platelet aggregometer and coagulation tracer (APACT 2). Pure compounds and aristolochic acid standard were

Table II. Platelets aggregation inhibition results.

Compound	0.40 mg/mL		0.20 mg/mL	
	Phase I ^a	Phase II ^a	Phase I ^a	Phase II ^a
Aristolochic acid I and II mixture (standard)	100%	100%	100%	100%
Aristolochic acid I (1)	100%	100%	39.5%	100%
Aristolochic acid II (2)	100%	100%	100%	75.8%
Aristolochic acid IIIa (3)	85.3%	100%	100%	100%

^a Inhibition of platelets aggregation induced by 10 μ M ADP; results were derived from two duplicate tests and calculated relative (normalized) to acetyl salicylic acid (Aspegic®), 5.5 mg/mL.

tested at two concentrations, 0.20 and 0.40 mg/mL on both phase I (adhesion of platelet) and phase II (platelet aggregation), while the methanolic extracts and the acidic fractions were tested at 4.4 mg/mL. Acetyl salicylic acid (5.5 mg/mL) has been used as a standard showing 100% platelet aggregation inhibition, *i.e.* 0% platelet aggregation was observed in both phase I and phase II, while 10 μ M ADP (adenosine diphosphate) was used as 100% platelet aggregator (Table II). Methanolic extracts of aerial and roots parts, in addition to acidic fractions, showed 100% activity at 4.4 mg/mL. Also, 100% inhibition of platelet aggregation has been noted with aristolochic acid standard, a mixture consisting of 38% aristolochic acid I and 58% aristolochic acid II. At 0.40 mg/mL, aristolochic acid I and II exhibited 100% inhibition of platelet aggregation. At 0.20 mg/mL aristolochic acid I selectively inhibited phase II with 100% activity and phase I with 39.5% inhibition while aristolochic acid II selectively inhibited phase I (adhesion) with 100% inhibition, and with less affinity

towards phase II, inducing 75.8% inhibition. At 0.20 mg/mL, aristolochic acid IIIa exhibited 100% inhibition of the two phases. At 0.40 mg/mL aristolochic acid IIIa showed 85.3% and 100% inhibition of both phase I and phase II, respectively. Noteworthy is the reproducible higher affinity of aristolochic acid IIIa towards phase I which is associated with the lower concentration level, a phenomenon which needs further investigation of the mechanism by which the compound inhibits platelet adhesion.

Applying PDA-HPLC, determination of aristolochic acid I and II content in the roots, stems and leaves of *A. maurorum* using two different, previously reported extraction methods was conducted. Aristolochic acid I and II contents in different plant parts, determined by method A (Rolf and Klaus, 1979) and method B (Rick *et al.*, 2001), are listed in Table III.

Aristolochic acid I and II contents in different plant parts vary considerably. During flowering stage, roots showed a higher content than the aboveground parts. Aristolochic acid I and II contents of roots were found to be 0.22 and 0.108% (w/w), respectively (method A), while that of stems and roots were only 0.03, 0.08% and 0.016 and 0.02% (w/w), respectively (method A) (Table III). Comparing the two extraction methods, method B (Rick *et al.*, 2001) gave lower yield of aristolochic acids than method A (Rolf and Klaus, 1979), an indication of less recovery of aristolochic acids. This may be attributed to the fact that these atypical alkaloids which contain an acidic carboxylic group in addition to a nitro group, reacted/un-ionized favorable under alcoholic-aqueous-/acidic-based conditions (method A) than just purely organic-based conditions (method B).

A reliable reversed-phase HPLC method has been used to analyze aristolochic acid I and II, in which satisfactory resolution, reproducibility, and accuracy have been obtained (Table III, Fig. 2).

Plant part	Aristolochic acid I ^a		Aristolochic acid II ^a	
	Method A	Method B	Method A	Method B
Roots	0.22 $\pm$ 0.01	0.09 $\pm$ 0.01	0.108 $\pm$ 0.003	0.038 $\pm$ 0.001
Stems	0.03 $\pm$ 0.01	0.014 $\pm$ 0.001	0.08 $\pm$ 0.001	0.003 $\pm$ 0.001
Leaves	0.016 $\pm$ 0.001	0.009 $\pm$ 0.001	0.02 $\pm$ 0.01	0.022 $\pm$ 0.003

^a Aristolochic acid I and aristolochic acid II content is expressed as w/w g% derived from the average of two extraction replicates, each run in duplicate.

Table III. Aristolochic acid I and II content of *Aristolochia maurorum*.

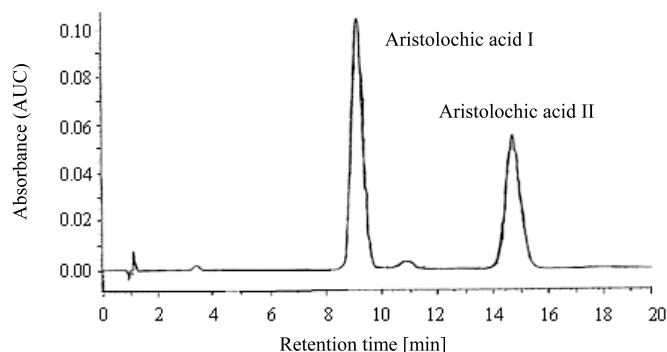


Fig. 2. HPLC chromatogram of 38 and 58 $\mu\text{g/mL}$ standards for aristolochic acids I and II, respectively.

Experimental

General

NMR experiments were performed using a Bruker AC-200 MHz instrument (Fällanden, Switzerland). $\text{DMSO-}d_6$ (^1H , δ 7.26; ^{13}C , δ 77.0) with TMS as internal standard was used to obtain the ^1H and the ^{13}C NMR spectra. MS was performed at 70 eV on a VG-7070 E LREI instrument. HPLC was performed on a Lachrom[®] MERCK-HITACHI instrument (Tokyo, Japan), equipped with a quaternary gradient L-7150 pump, L-7455 diode-array detector, L-7200 autosampler, and D-7000 interface. The preparative HPLC column was a Hibar[®] MERCK pre-packed column RT 250–25, Lichrosorb[®] RP-18 (7 μm). The analytical HPLC column used was a LiChro-CART[®] 125–4 column, Purospher[®] STAR RP-18 endcapped (5 μm). Column chromatography was carried out using silica gel 60 (0.06–0.2 mm; 70–230 mesh) and TLC using silica gel 60 with gypsum and pigment addition for UV visualization (Scharlau Chemie S.A., Barcelona, Spain). TLC spots were visualized by a UV lamp (Desaga, Germany). The antiplatelet activity was carried out using an APACK 2 (automatic platelet aggregometer and coagulation tracer) dual channel aggregometer from Laborfibrintimer (Munich, Germany). Acidity in terms of pH was measured using a C.G. 840 pH meter from Schott (Mainz, Germany). Centrifugation was carried out using a centrifuge from Hermle (Gosheim, Germany). Aristolochic acid mixture standard was purchased from Sigma (USA), ADP from Helena Laboratories (Newcastle, UK), and acetyl salicylic acid (Aspegic[®]) from Synthelabo Laboratories (Gentilly, Cedex, France). *n*-Hexane for pesticide residue analysis was obtained from Lab Scan (Dublin, Ireland), methanol HPLC-grade, dichloromethane (analyti-

cal grade), hydrochloric acid (37%), acetic acid (glacial, analytical grade), and water HPLC-grade were all from Scharlau Chemie S.A., ammonia solution CP was from C.B.H Lab Chemicals (Nottingham, UK), citric acid and trisodium citrate were both from May and Baker Ltd (Dengham, Essex, England), and formic acid from G.C.C (Sandycroft, Deeside, UK). Brine shrimp eggs, 100% artemia cysts, were obtained from Ocean Star[®] International Inc. (Snowville, Utah, USA) and Instant Ocean[®], synthetic sea salt was from aquarium Systems (Ohio, USA).

Plant material

Aerial and root parts of *A. maurorum* L. were collected during the flowering stage in April 2002 in the northern part of Jordan from two areas in Irbid, namely Hoffa El-Mazar and El-Mugayer. The collected materials were identified by Prof. Jameel Al-Lahham, plant taxonomist, Department of Biology, Faculty of Science, Yarmouk University, Irbid, Jordan. A voucher specimen (PHC-103) was deposited in the herbarium of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan. Plant material was cleaned from the stocked mud and divided into two parts: aerial and roots, which were then shade-dried in warm well-ventilated area. Dried plant material was then ground using a laboratory mill and the exact weight was recorded.

Extraction and isolation

Dried plant material from both the aerial (2.3 kg) and root (208 g) parts was first defatted by soaking in *n*-hexane, a process that was repeated five times at room temperature, followed each time by filtration (fraction A). At room tempera-

ture dried plant material was then extracted several times with absolute methanol with intermittent shaking, followed each time by filtration. The filtrates were combined and dried under reduced pressure (fraction B) to yield 227 g and 84 g methanolic extract from aerial and root parts, respectively. The methanolic residue of both the aerial and root parts was then suspended in water, pH adjusted to 9.3 with 12.5% ammonia, and extracted three times with dichloromethane (fraction C). The basic aqueous residue was then made acidic (pH 3) using diluted HCl and extracted three times with dichloromethane (fraction D). All fractions were dried under vacuum and their exact weights recorded.

Pure compounds were isolated *via* prep-HPLC from acidic fraction D of the aerial and root parts using an isocratic solvent system of methanol and 1% acetic acid in water (70:30), with a 10 mL/min flow rate, monitoring at 310 nm, and a total run time of 30 min. The purities of the isolated compounds were checked by TLC. A further purification process using preparative TLC was carried out to improve the purity of the isolated compounds.

Structural elucidation of active components

The structures of the purified compounds were elucidated using a series of spectroscopic and spectrometric techniques, principally: 1D-NMR and low resolution EI-MS, HPLC retention time matching with standards and by comparison with the reported spectral data in the literature (Preistab, 1987). An authentic standard material of aristolochic acid I and II was available.

Brine shrimp lethality test (BST)

The BST was performed as described previously (Meyer *et al.*, 1982; McLaughlin and Rogers, 1998).

Antiplatelet activity of aristolochic acids

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained from healthy male donors according to the procedure reported by Williamson *et al.* (1981). Acetyl salicylic acid (Aspegic®) solution of 5.5 mg/mL was used as platelet aggregation inhibitor standard. ADP, 10 μ M final concentration in test, was used as a standard platelet aggregator over 8 min using a dual channel aggregometer APACT 2. Solutions of plant extracts

and pure compounds were prepared in HPLC-grade methanol to give a final concentration of 80 mg/mL and 8 mg/mL, respectively. The test samples were further diluted with saline to adjust to the isotonic pH value to provide a final concentration of 4.4 mg/mL for extracts and 0.4 mg/mL for pure compounds.

Determination of aristolochic acid I, II content in A. maurorum

A comparison between two previously reported extraction methods of *A. maurorum* L. has been made. In method A (Rolf and Klaus, 1979), a portion of 2 g of each ground plant part (roots, leaves, and stems) was accurately weighed and dissolved in 50 mL of a mixture of 80% HPLC-grade methanol and 20% formic acid in water, and then shaken for 1 h at 250 rpm, followed by centrifugation for 4 min at 4000 rpm. The supernatant was decanted, adjusted to 50 mL with methanol, and finally filtered with a 0.45 μ L pore size Teflon filter (Rolf and Klaus, 1979). In method B (Rick *et al.*, 2001), 1 g of each ground plant material was treated with *n*-hexane (30 mL) by frequent shaking followed by filtration, a step that was carried out to remove non-polar components. Solid residues were air-dried at room temperature and then extracted with HPLC-grade methanol (2 $\times$ 30 mL) with frequent shaking for 30 min, followed each time by filtration. The collected filtrates were combined and centrifuged at 3000 rpm for 5 min. The supernatant volume was adjusted to 50 mL with methanol and then filtered with a 0.45 μ L pore size filter to offer an analytical sample for HPLC (Rick *et al.*, 2001). Standard aristolochic acid (Sigma) is composed of two main derivatives of aristolochic acids: aristolochic acid I (38%) and aristolochic acid II (58%). The stock solution was prepared by dissolving 50 mg of the standard in 50 mL of HPLC-grade methanol. The stock solution was then diluted using HPLC-grade methanol to construct two calibration curves of seven points [(0.38, 3.8, 9.5, 19, 28.5, 38, and 76 μ g/mL) and (0.58, 5.8, 14.5, 29, 43.5, 58, and 116 μ g/mL)] for aristolochic acid I and aristolochic acid II, respectively. To check for accuracy, two quality control (QC) points [(7.6 and 30.4 μ g/mL) and (11.6 and 46.4 μ g/mL)] for aristolochic acid I and aristolochic acid II, respectively, were also prepared. PDA-HPLC was used to assay aristolochic acids. Mobile phase was an isocratic blend of 60% methanol and 40%

of 1% acetic acid with water. Flow rate was 1 mL/min, the detector was set at 310 nm and the injection volume was 25 μ L. The two QC samples at 7.6 and 30.4 and 11.6 and 46.4 μ g/mL of aristolochic acid I and II, respectively, were accurate within 5.7 and 10.1% and 6.9 and 8.9% (RSD%), respectively, from actual concentration. All plant samples, calibration points and QC samples were injected twice. Aristolochic acid I and aristolochic acid II were eluted at 9.33 and 14.35 min, respectively. Measuring peaks heights, two linear calibration curves, $r^2 > 0.9999$, were constructed.

Plant samples were analyzed by the above described method. All plant samples were injected twice, and the results obtained are given in Table III. The percentage of aristolochic acid I and II in the samples was calculated against external standards using the following equation:

$$\% W/W = C \cdot FV \cdot 100\% / W,$$

where C is the sample's aristolochic acid I/aristolochic acid II concentration (g/mL), extrapolated from the calibration curves' linear regression, FV is the final volume of the sample (mL), and W is the sample weight (g).

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