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Anti-microbial Activity of Aristolochic Acid from Root of *Aristolochia bracteata* Retz

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***Abstract*—**The present research was designed to investigate the anti-microbial activity of aristolochic acid from the root of *Aristolochia bracteata*. From the methanolic & ethyl extract extracts of *Aristolochia bracteata* aristolochic acid I was isolated and conformed through IR, NMR & MS. The percentage purity of aristolochic acid I was determined by UV & HPLC method. Anti-bacterial activity of extracts of *Aristolochia bracteata* and the isolated compound was determined by disc diffusion method. The results reveled that the isolated aristolochic acid from methanolic extract was more pure than the compound from ethyl acetate extract. The various extracts (500µg/disc) of *Aristolochia bracteata* showed moderate antibacterial activity with the average zone of inhibition of 7-18 mm by disc diffusion method. Among the extracts, ethyl acetate & methanol extracts were shown good anti-microbial activity and the growth of *E.coli* (18 mm) was strongly inhibited. Microbial assay of isolated compound (Aristolochic acid I) from ethyl acetate & methanol extracts were shown good antimicrobial activity and the zone of inhibition of both at higher concentration 50 µg/ml was similar with the standard aristolochic acid. It may be concluded that the isolated compound of aristolochic acid I has good anti-bacterial activity.

***Keywords*—**Aristolochic acid I, Anti-microbial activity, *Aristolochia bracteata*, *Bacillus subtilis*, *E.coli*

I. Introduction

MEDICINAL plants have been known for their healing or disease-curing qualities for centuries [1]. Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. A rich heritage of knowledge to preventive and curative medicines was available in ancient scholastic works included in the Atharva veda, Charaka, Sushruta etc. Over 50% of all modern clinical drugs are of natural product origin and natural products play an important role in drug development programs in the pharmaceutical industry [2]-[4]. Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness. The characteristics of the plants that inhibit microorganisms and are important for human health have been researched in laboratories since 1926 [5].

Aristolochia bracteata Retz. (Aristolochiaea) commonly called as Worm killer in English and aadutheendaapaalai in Tamil. The whole plant was used as purgative, anthelmentic, antipyeretic & anti-inflammatory agents. The root parts was used to treat syphilis, gonorrhea and also used during labors to increase uterine contraction. The plant contain Aristolochic acid has many medicinal properties in various disease condition [6]. In the continuation of this strategy of new drug discovery we have studied the root parts of the plant *Aristolochia bracteata* and their isolated compound aristolochic acid I for their antibacterial activity.

II. Materials & Methods

A. Plant materials

The root of *Aristolochia bracteata* were collected from Tamilnadu in the month of May 2007 and identified by Department of Pharmacognosy, UCP, Tamilnadu, India. The fresh root were collected, shade dried for seven days and ground. All chemicals, reagents & aristolochic acid I was purchased from Sigma Chemicals, USA.

B. Extraction & isolation

The ground roots (500 g) were extracted in a round bottom flask with organic solvents (pet. ether, hexane, chloroform, ethyl acetate, methanol & water) at room temperature for 72 h with 1:2 sample & solvent ratio. After 72 h the extracts were filtered and dried by using rotary evaporator. The methanolic & ethyl acetate extracts were applied to a column of Silica gel (60-120 mesh) packed in benzene slurry and the column was developed with chloroform:ethyl acetate, from which were collected 16 fractions of 50 ml each. Fractions 6-16 were combined on the basis of similar TLC pattern (Si gel plates, chloroform-methanol & Iodine chamber). These fractions were further resolved by preparative TLC on silica gel G using chloroform-methanol as a mobile phase, resulting in isolation of one compounds (50 mg) from each extracts respectively, the percentage purity of isolated compound was determined by UV & HPLC method. IR, 1H NMR, & mass spectra of the compound were matched with standard aristolochic acid I.

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C. HPTLC fingerprinting of extracts

Standard stock solution (1 mg/ml) of aristolochic acid was dissolved in methanol and extracts of plant were prepared by using respective solvents. CAMAG HPTLC system (Switzerland) with a Linomate 5 sample applicator was used for the analysis. The analysis was performed in air-conditioned room maintained at 22° C and 55% humidity. TLC was performed on precoated siliga gel HPTLC aluminium plates 60F254 (20 cm x 10 cm, 0.2 mm thickness, 5-6 µm particle size, E-Merck, Germany). Five microliters of the standard solutions were spotted as bands of 6 mm width by using the auto sampler fitted with a 100 µl Hamilton syringe. The plates were developed using chloroform-methanol (10:1 v/v) in a CAMAG twin-trough plate development chamber which was lined with filter paper and presaturated with 30 ml mobile phase. The developed plates were air dried and scanned. A spectrodensitometer (Scanner 3, CAMAG) equipped with 'win CATS' planar chromatography manager (version 1.3.0) software was used for the densitometry measurements, spectra recording and data processing. Absorption/remission were the measurement mode at a scan speed of 20 mm/s. spots of aristolochic acid was scanned from 200 to 600 nm so as to record their UV-vis spectrum and to obtain their wavelength of maximum absorption. Densitograms were recorded at the wavelength of maximum absorption (254 nm) of aristolochic acid. Each concentration of reference compound was spotted two times on the plates and analyzed. The concentration of reference compound was spotted against peak area to obtain calibration curves.

D. HPLC analysis of isolated compounds

HPLC analysis was performed in a Shimadzu HPLC system (Shimadzu, Japan) with LC-10AD model pump, a Rheodyne injector fitted with 20 µl sample loop and a SPD-10A UV-vis detector. A reverse phase column (Phenomenex, C-18, ODS-2, 5 µm, 250 x 4-6 mm) with an extended guard column was used as stationary phase and an isocratic elution of methanol-water-acetic acid (67:32:1) at 1 ml/min as mobile phase. Chromatograms were recorded at 390 nm. Isolated compounds from methanol & ethyl acetate extracts and standard aristolochic acids were appropriately diluted with mobile phase, filtered through 0.45 µm PTFE microfilters and injected in to HPLC. The percentage purity of isolated compounds was determined by the following formula:

$$\frac{\text{Sample Area}}{\text{Standard Area}} x \frac{\text{Standart Dilution}}{\text{Sample Dilution}} x \ 100$$

E. Antimicrobial activity

The antibacterial assay was performed by disc diffusion technique [7]. The sample solution of the material (various extracts of the plant) to be tested were prepared by dissolving a definite amount of material in appropriate solvent to attain a concentration of 50mg/ml. 10 µl of such solution was applied on sterile disc (5mm diameter, filter paper) and allowed to dry off the solvent in an aseptic hood. Thus, such discs contain 500 µg of crude extracts. To compare the activity with standard antibiotics, Kanamycin (30 µg/disc) was used. The extracts of Aristolochia bracteata were tested against Gram-positive and Gram-negative (Bacillus subtilis & Escherichia coli) bacteria. The antibacterial activities of the extracts were then determined by measuring the respective zone of inhibition in mm. Antimicrobial assay of isolated compound were determined by same method described above. The activity was compared with standard aristolochic acid (50 µg/ml) and two different concentrations (25 µg/ml & 50 µg/ml) were used for this study.

III. Results AND Discussion

The freshly prepared extracts were subjected to preliminary phytochemical screening test for various constituents. This revealed the presence of alkaloids, tannins, saponins, flavonoids, glycosides, and terpenoids. In the present study, the identification of aristolochic acid from various extracts of plant was verified by profiling reference sample of aristolochic acid I under the proposed HPTLC conditions. HPTLC profile of methanol & ethyl acetate extracts spiked with these reference compounds is given in Fig 1 (A,B,C,D). In order to further establish the presence of aristolochic acid I in the extracts, the compounds were isolated from both extracts and subjected to IR, NMR & MS spectra obtained are present in Table 1-3. The percentage purity of isolated aristolochic acid I was compared with standard aristolochic acid I by UV & HPLC method. In UV the % purity of isolated aristolochic acid I from ethyl acetate and methanolic extracts were 90.29 % w/v & 95.75 % w/v respectively. In HPLC the % purity of isolated aristolochic acid I from ethyl acetate and methanolic extracts were 89.91 % w/v & 94.01 % w/v respectively and the peak area of isolated aristolochic acid I from ethyl acetate and methanolic extracts were 7752 & 8105 mV respectively (Fig 2). The obtained peak area was closely related with peak area of standard aristolochic acid I (8621 mV). The results reveled that the isolated aristolochic acid I from methanolic extract was more pure than the compound from ethyl acetate extract.

The various extracts (500µg/disc) of Aristolochia bracteata showed moderate antibacterial activity with the average zone of inhibition of 7-18 mm by disc diffusion method. Among the extracts, ethyl acetate & methanol extracts were shown good anti-microbial activity and the growth of E.coli (18 mm) was strongly inhibited. Microbial assay of isolated compounds from ethyl acetate & methanol extracts were shown good antimicrobial activity and the zone of inhibition of both at higher concentration was similar with the standard aristolochic acid I. The results were presented in Table 4 & Fig 3.

The results obtained from the structural elucidation of isolated compound revealed that the isolated compound was aristolochic acid I and the data also compared with previous reports for further clarification [8]-[10]. In previous studies, Negi et al., reported the antibacterial activity of dried extracts of *Aristolochia bracteata* against a few Gram-positive and Gram-negative bacteria. All the crude extracts showed a broad

spectrum of antibacterial activity [11]. The ethyl acetate extract was found to be the most effective. In present investigation also ethyl acetate extract shows effective against Gram-positive and Gram-negative bacteria. In another study, the species of aristolochia like *Aristolochia paucinervis*, *Aristolochia longa* were reported as good antimicrobial drugs [8]-[12]. In another study, methanolic extract of *Aristolochia bracteata* was found to be good antimicrobial agent [13]-[14]. When compared with previous reports our research also showed good anti-bacterial activity of ethyl acetate & methanlic extracts. But, no one reported which active principle is responsible for antibacterial activity of *Aristolochia bracteata*. In this present study, we have isolated aristolochic acid I from both ethyl acetate & methanolic extract and the isolated aristolochic acid I showed good antimicrobial activity against both gram positive & gram negative bacteria.

TABLE I
IR SPECTRUM FOR ISOLATED AND STANDARD ARISTOLOCHIC ACID I

Groups	Wave Numbers in cm-1		
	AA I-S	AA I-EAE	AA I-ME
OH stretching in carboxylic acid group	3395.76	3380.72	3390.16
CH stretching in Aromatic hydrocarbons	3080.42	3082.12	3081.33
CH stretching in methoxy group	2852.81	2902.42	2867.91
CH stretching in O-CH2-O group	2922.25	2851.91	2946.28
C=O Stretching in carboxylic acid group	1729.24	1724.44	1720.20
C=C stretching in ring structure	1591.99	1589.13	1594.96
N=O stretching in Nitro group	1460.16	1462.32	1468.24
C-O stretching in lactam ring	1268.24	1267.13	1274.61
C-N stretching in aromatic rings	896.93	897.34	898.38
CH bending in aromatic hydrocarbons	939.36	938.24	888.44
N=O bending in nitro group	749.37	748.38	739.12

TABLE II
NMR SPECTRUM FOR ISOLATED AND STANDARD ARISTOLOCHIC ACID I

Groups	δ Values in ppm		
	AA I-S	AA I-EAE	AA I-ME
Aromatic proton	8.851	8.721	8.743
Aromatic proton	8.602	8.584	8.491
Aromatic proton	7.970	7.989	7.894
Aromatic proton	7.733	7.661	7.652
Proton in carboxylic acid group	7.256	7.241	7.238
Aromatic proton	7.125	7.147	7.123
Proton in methoxy group	6.371	6.293	6.210
Proton in methyl group	5.830	5.825	5.797

AA I-S: Standard aristolochic acid I, AA I-EAE: Aristolochic acid I isolated from ethyl acetate extract, AA I-ME: Aristolochic acid I isolated from methanolic extract.

TABLE III
MASS SPECTRUM FOR ISOLATED AND STANDARD ARISTOLOCHIC ACID I

Fragmentation pattern	m/e values in amu		
	AA I-S	AA I-EAE	AA I-ME
Due to parent ion	341	341.3	341.4
Due to loss of (M-H2O+H)+	323	323.1	323.3
Due to loss of (M-NO2+H)+	295	295.2	295.4
Due to loss of (M-CO2+H)+	252	252.2	253.20
Due to loss of (M-CH3+H)+ and breakage of lactam ring	239	239.2	239.3
Due to loss of (M-CH3+H)+ after the breakage of lactam ring	224	224.4	224

AA I-S: Standard aristolochic acid I, AA I-EAE: Aristolochic acid I isolated from ethyl acetate extract, AA I-ME: Aristolochic acid I isolated from methanolic extract.

TABLE IV
ZONE OF INHIBITION OF EXTRACTS & ISOLATED COMPOUNDS FROM ARISTOLOCHIA BRACTEATA

Drugs	Zone of Inhibition (mm)	
	Bacillus subtilis	E.coli
Pet. Ether Extract 500 µg/ml	0	8
Hexane Extract 500 µg/ml	0	7
Chloroform Extract 500 µg/ml	9	10
Ethyl Acetate Extract 500 µg/ml	11	18
Methanol Extract 500 µg/ml	10	12
Aqueous Extract 500 µg/ml	0	8
AA I-EAE 50 µg/ml	0	15
AA I-ME 50 µg/ml	17	16
AA I-S 50 µg/ml	19	16
Ciprofloxacin 50 µg/ml	16	18

AA I-S: Standard aristolochic acid I, AA I-EAE: Aristolochic acid I isolated from ethyl acetate extract, AA I-ME: Aristolochic acid I isolated from methanolic extract.

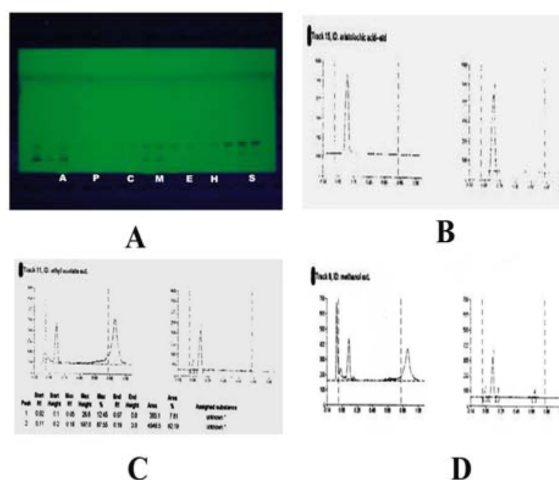
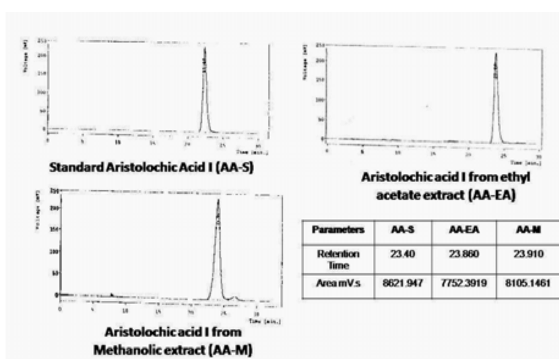


Fig. 1 (A,B,C,D) represents the HPTLC data of standard aristolochic acid and various extracts of *Aristolochia bracteata*



Parameters	AA-S	AA-EA	AA-M
Retention Time	23.40	23.860	23.910
Area mV.s	8621.947	7752.3919	8105.1461

Fig. 2 HPLC data for standard aristolochic acid and isolated compound from *Aristolochia bracteata*

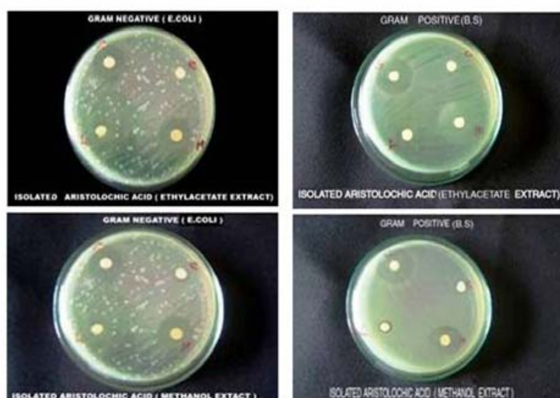


Fig. 3 Antimicrobial activity of aristolochic acid

IV. Conclusion

From these findings it may be concluded that the isolated compound of aristolochic acid I has good anti-bacterial activity.

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