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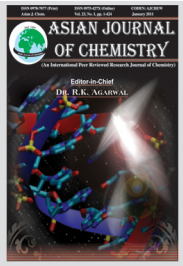

Separation and Quantification of a Natural Drug Component Against Human Pathogens from *Aristolochia bracteolata* Lam

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Aristolochia bracteolata Lam, whole plant was subjected to Soxhlet extraction with solvents like chloroform, acetone and methanol. Subsequently the extracted solvent phases were passed through thin layer chromatography and three distinct spots were separated as C_1, A_1 and M_2, respectively. Among all the three independent compounds C_1, A_1 compounds showed maximum level of antimicrobial activity against the bacterial pathogens like methicillin resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi* and *Aspergillus mucor*. Among all tested compounds C_1 and M_2 showed ten fold increased activity than A_1 compound. The ^{1}H NMR analysis of the purified active compounds C_1, A_1 and M_2, clearly shows that all the three compounds are independent. The HPLC and NMR results confirm that these three compounds are pure and entirely different from each other. ^{1}H NMR analysis individually confirmed that C_1, A_1 and M_2 compounds are pure and aliphatic in nature.

Key Words: *Aristolochia bracteolata*, Antimicrobial activity, Methicillin resistant *Staphylococcus aureus*.

INTRODUCTION

Many natural substances of plant origin may play a fundamental role in the host-pathogen relationship and products from different plant genera are reported biologically active molecules governed with antimicrobial, antifungal, allelopathic and antioxidant properties. *Aristolochia bracteolata*, is a polymorphous species responds to different geographic condition. The genus *Aristolochia* is in general composed of aromatic plants and many species are reported as to have medicinal properties. *Aristolochiaceae* plants have broad spectrum therapeutic uses in tumour growth inhibiting compound[1], having role as antiviral, antimiotic[2] involved in antivenom preparations and also used as an expectorant, analgestic[3]. *Aristolochia bracteolata* belongs to family *Aristolochiaceae*, distributed in the major part of India. This perennial prostrate herb used to grow 300-350 cm in height. This herb widespread and largely used in folk medicine to treat snake bites and used against malarial infection[4]. The leaves and roots are in bitter taste used to control intestinal worms (especially round worms), constipation, inflammations, amenorrhoea, dysmenorrhoea, foul ulcers, boils, syphilis, gonorrhoea, dyspepsia, colic, skin diseases, eczema, arthralgia and intermittent fevers[5]. The plant extract showed positive effect on wound healing with a significant increase in the level of two powerful antioxidant enzymes, superoxide dismutase and catalase in the granuloma tissue[6].

Roots of *Aristolochia bracteolata* were powdered and extracted in Soxhlet extractor with various solvent systems and subjected to antibacterial activity analysis. All crude extracts showed a broad spectrum on antibacterial activity. Ethyl acetate extract was more effective in the extraction of new compounds from the plant and again shows inhibiting of various microbial growths[7]. Another set of study was made by Elizabeth and Suryanarayana[8] revealed that *Aristolochia bracteolata* extract was tested against several human pathogens following the disc diffusion method and showed that *S. aureus* cultures are very sensitive towards the crude and solvent extract of the plant. They have also estimated the growth inhibition study of pathogens which was more than 78 %. In present study we have also tested the MIC values of different human pathogens and we have subsequently identified and isolated the novel plant compounds involved in the antimicrobial activity of human pathogens. Antimicrobial activity of *Aristolochia bracteolata* was tested against several human pathogenic micro-organisms by disc diffusion method and the amylase and protease production was assessed in few strains and it was found that there was 78.2 % reduction in the amylase activity of *Y. enterocolitica* and 100 and 60 % reduction in amylase and protease production of *P. aeruginosa* following the treatment with residual extract of this fruit extract. From these observations it can be attributed that *A. bracteolata* possesses strong antimicrobial activity[8]. Recently, Kavitha and Nirmaladevi[9]

proved that antimicrobial activity of the medicinal plant *Aristolochia bracteolata*. Aqueous, methanol and chloroform extracts of this plant were evaluated against the bacterial and fungal pathogens. Among the three extracts assessed, methanol extract was found to have the significant activity followed by the chloroform extract against certain bacteria. Water extract did not have any activity against bacteria. Antifungal activity assessment indicated that the tested fungal strains are more susceptible to aqueous extract followed by methanol extract and chloroform extract[9].

EXPERIMENTAL

Sample preparation: *Aristolochia bracteolata* Lam plants were obtained from the Tamilnadu Agricultural University nursery, Madurai. The whole plant was washed thoroughly in tap water followed by distilled water and allowed to dry in the shade at room temperature and subsequently the whole plant was powdered by using a laboratory chopper.

Active compound extraction: The dried and powdered, whole plant was subjected to various solvent extraction. About 10 g of dried powder was extracted with 200 mL of various organic solvent such as hexane, methanol, chloroform and acetone at 10 ºC above of their respective boiling point by hot percolation method using Soxhlet apparatus for *ca.* 8-10 h[10]. The respective organic phases of plant extract was passed through anhydrous sodium sulfate and subjected to rotary vacuum flash evaporator and concentrated.

Purification of bioactive compound through TLC: Thin layer chromatography technique was carried out using 0.25 mm thickness silica gel-G coated plates dried at room temperature (28 ± 1 ºC). 10 µL of acetone, chloroform, hexane and methanol extracts of the whole plant was loaded on the TLC plate individually. The solvent system used in the TLC analysis was benzene:methanol:acetic acid (7.5: 2.5: 1.0 v/v). The respective TLC resolved spots were identified by passing iodine vapour. The R_f values of the active compound in the crude plant extracts in the various solvent phase such as acetone, chloroform, hexane and methanol were determined.

HPLC analysis of bioactive compound: The resolved spots through TLC were scrapped aseptically and part of it was used for antimicrobial assay experiment. The remaining part was dissolved in methanol:water 60:40 v/v and a serial dilution were made. This filtrate was subjected to HPLC analysis using a Shimadzu HPLC system with Shim pack CLC ODS (4.6 mm × 15 cm) column, guard column, liquid pump LC-6 AD, system controller SCL-6B, detector (UV-Vis) SPD-6 AV, data processor CR-5A and the detection at 254 nm with mobile phase methanol:double distilled water 60:40, v/v at the flow rate of 1 mL/min. Identification and quantification of each peak was made by comparing with the retention time of the authentic standard peak and the related concentration in concern with the peak area[11].

Antifungal activity: The antifungal activity of the whole plant extract was done by the Kirbey-Bauer disc diffusion method. *Aspergillus mucor* cultivated on YPS broth, starting inoculam size 104 c.f.u./mL were prepared separately by the spectrophotometric method recommended by NCCLS[12]. These cultures were spread-plated on Mueller-Hinton agar separately. The TLC resolved spots from the extracts of various solvents such as acetone, chloroform, hexane and methanol were scraped of and 200 µg of this powder was dissolved in 1000 µL of sterile double distilled water. From the stock 50 µL was loaded onto sterile Hi-media discs and dried aseptically. These discs were placed onto the plates, inoculated with test organism, each disc was placed 5 cm apart from one disc and control discs with sterile double distilled water were also placed on the respective plates and were incubated at 37 ºC for 24-36 h.

Antibacterial activity: Antibacterial activity of the whole plant extract was also determined by the Kirbey-Bauer disc diffusion technique against *Salmonella typhi* and Methicillin resistant *Staphylococcus aureus* (MRSA). Log phase culture of above micro organisms were cultured on nutrient broth and spread-plated on Mueller-Hinton agar. The TLC passed through spots from various solvents like acetone, chloroform, hexane and methanol were scraped of and 200 µg of this powder was dissolved in 1000 µL of sterile double distilled water and 50 µL of aliquot was loaded onto sterile Hi-media discs and dried aseptically. These discs were placed within a distant of 5 cm in between onto the plates inoculated with bacterial cultures. A set of control disc with sterile double distilled water was also placed on the respective plates and incubated at 37 ºC for 24 h[13].

RESULTS AND DISCUSSION

Antimicrobial activity from the crude extract of *Aristolochia bracteolata* Lam: The antimicrobial activity pattern of various solvent extracts of *Aristolochia bracteolata* Lam against different microorganisms such as *Aspergillus mucor*, *Salmonella typhi* and MRSA are presented in Table-1. The antimicrobial activity results revealed that antimicrobial activity was found in chloroform, acetone and methanol extracts of the plant *Aristolochia bracteolata* Lam except the hexane extract. All the three crude extracts showed activity but chloroform extract showed maximum zone of inhibition against *Salmonella typhi*, this finding is well correlate with the findings of Rahman *et al.*[14]. Other sovents like acetone and methanol showed clearing zone of inhibition but the percentage of zone of inhibition is at higher side in the chloroform extract against *Salmonella typhi*.

TABLE-1
ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACT OF *Aristolochia bracteolata* Lam. AND ANTIMICROBIAL ACTIVITY PATTERN OF TLC RESOLVED SPOTS

Antimicrobial activity of crude extracts					Antimicrobial activity of TLC spots					
Micro-organism	Zone of inhibition (mm)				Spot	Solvent	R_f value	Zone of inhibition (mm)		
	Hexane	Chloroform	Acetone	Methanol				*S. aureus*	*S. typhi*	*A. mucor*
S. aureus	Nil	14	11	12	C_1	Chloroform	0.787	13	12	8
S. typhi	Nil	19	18	14	A_1	Acetone	0.545	12	13	8
A. mucor	Nil	16	12	12	M_1	Methanol	0.363	8	10	10

TLC identification of active compounds from crude extracts of *Aristolochia bracteolata* Lam: The R_f values of the TLC separated extracts from different solvents of *Aristolochia bracteolata* Lam are presented in Table-2. The separation of TLC pattern showed that hexane, chloroform and methanol extracts had two spots. But acetone extract has three spots. The individual compounds separated in each solvent were named as H_1 and H_2 in hexane extract, C_1 and C_2 in chloroform extract, A_1, A_2 and A_3 in acetone extract and M_1 and M_2 in methanol extracts. The present results are in agreement with the TLC pattern of Wagner and Bladt[15].

TABLE-2
TLC PATTERN OF SEPARATED SPOTS OF *Aristolochia bracteolata* Lam. SOLVENT EXTRACTS

TLC resolved spots	Solvent	R_f value
H_1	Hexane	0.857
H_2	Hexane	0.943
C_1	Chloroform	0.868
C_2	Chloroform	0.937
A_1	Acetone	0.857
A_2	Acetone	0.920
A_3	Acetone	0.787
M_1	Methanol	0.400
M_2	Methanol	0.874

Antimicrobial activity of TLC resolved spots: The antimicrobial activity pattern of the TLC resolved spots were shown against the tested organisms as the zone of inhibition (Table-1). Except hexane extract, other solvent extracts showed remarkable antimicrobial activity and it is very clear that hexane as crude extract and the TLC passed extract do not show any activity. It is further confirmed that the compounds which are extracted by hexane have no antimicrobial property. The tested spots which showed activity for C_1 of chloroform and A_1 of acetone showed maximum activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Salmonella typhi*. But the M_2 of methanol extract showed maximum activity against *Aspergillus mucor*. Eventhough all the three tested spots showed antimicrobial activity, the C_1 of chloroform and A_1 of acetone have more antibacterial activity, but the M_2 of methanol has more antifungal activity and present findings are well correlate with the results of Deborah *et al.*[16].

Spectrophotometric and HPLC analysis: The UV absorption pattern of the TLC resolved spots undergone for antimicrobial activity were subjected to UV-Vis spectrophotometer analysis. Maximum absorbance was noted between 250 and 260 nm. The TLC resolved spots of chloroform, acetone and methanol extract of *Aristolochia bracteolata* Lam were analyzed (Table-2) along with the antibiotic standards ciprofloxacin and oflaxacin and showed a close relative compounds. HPLC retention time, area of the spots and the respective standards are presented in Table-3. The result revealed that ciprofloxacin and ofloxacin have the retention time of 3.219 and 3.781 min, respectively. The retention time of antibiotic standards and the TLC separated active compounds of *Aristolochia bracteolata* Lam are different because they are natural compounds and a correlation was made with synthetically available antibiotics such as ciprofloxacin and ofloxacin[10] but having antibiotic role needs deeper study.

^{1}H NMR analysis of the active compounds: Several presenters from the United Kingdom described the use of proton NMR of crude plant extracts, followed by multivariate analysis, to cluster data sets to highlight differences. This type of analysis gives a comprehensive summation fingerprint of all (hydrogen-containing) metabolites extracted and can provide direct structural information regarding individual metabolites in the mixture. Therefore, it is suitable for high-throughput, rapid, first-pass screening. Subtraction of data sets generates virtual NMR spectra and hence important structural data on compound(s) contributing to differences between samples[17]. The ^{1}H NMR spectral analysis showed that the TLC separated active compounds of C_1, A_1 and M_2 are independent compounds with aliphatic in nature. The analyzed compounds C_1, A_1 and M_2 are having some antibiotic moiety and it will initiate antimicrobial and antifungal activity.

Conclusion

The two compounds C_1 and A_1 isolated from *Aristolochia bracteolata* Lam are effective antibacterial bioactive compounds, which can be used against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Salmonella typhi* whereas the compound M_2 can be used as effective antifungal agent especially *Aspergillus mucor*. An attempt was made to exploit the plant's antimicrobial property and the successfully isolated bioactive compounds of C_1, A_1 and M_2 can be used for the effective treatment of methicillin-resistant *Staphylococcus aureus*, *Salmonella typhi* and *Aspergillus mucor* infections. All the extracts showed a broad range of activity against tested bacteria. Further studies on large scale purification study will provide more information on drug and pathogen relationship. Therefore if a systematic investigation is initiated the traditional medicinal systems practiced in India can offer promising leads for the discovery of different potent antibiotics that can have therapeutic and dietary use globally.

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TABLE-3
ABSORPTION MAXIMA FOR THE TLC SEPARATED SPOTS OF DIFFERENT SOLVENT EXTRACTS AND HPLC ANALYSIS OF TLC RESOLVED ACTIVE COMPOUNDS AND ANTIBIOTIC STANDARDS

TLC Separated spots of different solvents extracts				HPLC analysis of TLC compounds				
Solvents	Spots	Wavelength (nm)	OD	Sample	Dilution	Volume	Retention time (min)	Area
Acetone	A_1	290	3.000	A_1	10^{-2}	50	2.142	15518
Chloroform	C_1	290	3.000	C_1	10^{-1}	50	2.719	15291
Methanol	M_1	287	1.704	M_2	10^{-1}	50	3.421	15083

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