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Original Article

ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL COMPOUND FROM FRUITS OF *ANTHOCEPHALUS INDICUS* A. RICH

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ABSTRACT

Objective: Aim of our study is to isolate and characterise antimicrobial compound from fruits of *Anthocephalus indicus* A. Rich.

Methods: *Anthocephalus indicus* A. Rich. crude ethanol fruit extract and different solvent fractions of fruit extract were screened for its antimicrobial activity against Gram negative and Gram positive bacterial cultures by MIC and agar well diffusion method. TLC bioautography method was used for isolation of compound from fraction of fruit extract showing maximum antimicrobial activity. Screening of isolated compound was carried out by preliminary phytochemical test and characterisation by FTIR, MS and ^{13}CNMR techniques.

Results: Crude ethanol fruit extract showed significant antimicrobial activity with MIC values of 12.5-6.25 mg/ml and zone of inhibition from 19.0-24.66 mm. Amongst the different solvent fractions used aqueous fraction showed maximum antimicrobial activity with MIC values of 0.78-3.12 mg/ml and zone of inhibition from 26.33-35.0mm. TLC bioautography method helps in the isolation of antimicrobial compound from aqueous fraction. Characterisation of isolated compound by FTIR, MS and NMR detects it as iridoid glucoside.

Conclusion: The results revealed that the fruit extract possess significant antimicrobial activity due to the presence of iridoid glucoside as a bioactive compound which can be used as a source of safe herbal antimicrobial agent.

Keywords: *Anthocephalus indicus*, Antimicrobial activity, Iridoid glucoside, TLC-Bioautography.

INTRODUCTION

Infectious diseases represent an important cause of morbidity and mortality amongst the general population, particularly in developing countries [1]. Infectious diseases are disorders caused by pathogenic microorganisms and even a healthy immune system sometimes fails to stop the bacteria from replicating and spreading the infection. As a result, bacteria thrive in the body and may emit toxins which damage cells and tissues leading to typical symptoms of bacterial disease [2]. 'The era of antibiotics' till the early 1970s led to optimism that infectious disease can be controlled and prevented by the modern medicines. However, infections are still the second-leading cause of death worldwide, causing over 13 million deaths each year. This fact is the result of the emergence of new diseases, the re-emergence of diseases once controlled and more specifically of the development of antimicrobial resistance [3]. This type of resistance may result from changes in the bacterial genome due to mutation [4]. It is essential to investigate newer drugs with lesser resistance. Systematic studies among various pharmacological compounds have revealed that any drug may have the possibility of possessing diverse functions and thus may have useful activity in completely different spheres of medicine [5].

Since ancient times, plants have been utilized as an important source of medicines as they are a reservoir of chemical agents with antimicrobial properties [6]. Medicinal plants are gaining popularity over antibiotics due to a rapid increase in the rate of infections, development of antibiotic resistance in microorganisms and side effects of synthetic antibiotics [7]. The therapeutic use of medicinal plants is becoming popular because of their minimum side effects and low resistance in microorganisms [8]. The different parts of the plant that can be used as antimicrobial agents include root, stem, leaf, flower, fruit, twig exudates, etc [9]. The antimicrobial activities of medicinal plants can be attributed to their secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, iridoid, etc [10].

Anthocephalus indicus A. Rich. is a large deciduous tree belonging to the family Rubiaceae commonly known as Kadamba and found all over India. Phytoconstituents in the plant consist of indole alkaloids, terpenoids, phenols, saponins, terpenes, steroids, fats and reducing sugars. It is used as herbal remedy that has been mentioned in ancient Indian medical literatures for the treatment of fever, anaemia, diabetes, uterine and liver complaints, menorrhagia, blood and skin diseases, diarrhoea, colitis, stomatitis, dysentery and in improvement of semen quality [11]. Literature survey reveals that ethanolic extract of *Anthocephalus indicus* A. Rich. showed significant antibacterial activity among the different solvent extracts used *viz*., petroleum ether, chloroform, acetone, water and hydroethanol [12]. Thus in the present study antibacterial activity of the ethanolic extract of fruit (unripe) of *Anthocephalus indicus* A. Rich. was carried out along with the isolation and characterisation of antimicrobial compound.

MATERIAL AND METHODS

Preparation of Plant Extract

Ethanol extract of dried powder (50 g) of fruit of *Anthocephalus indicus* A. Rich. was prepared using cold extraction method by continuous shaking in orbital shaker at 100 rpm (25°C). After 24 hours, the extract was filtered and residue of the sample was re-extracted by the same procedure using ethanol as the solvent for the period of one week [13]. After one week, the collected filtrates were concentrated by evaporating the ethanol at 50°C. Dried residue of the sample was re-dissolved in sterile Nutrient broth to make the particular concentration of fruit extract.

The concentrated crude ethanolic fruit extract of *Anthocephalus indicus* A. Rich. was subjected to fractionation by dissolving in distilled water (100 ml) followed by sequential partition with petroleum ether (4×50 ml), ethyl acetate (4×50 ml) and butanol (4×50 ml). The crude ethanolic fruit extract was separated into four different fractions *viz*. petroleum ether, ethyl acetate, butanol and aqueous [14]. Each fraction was evaporated to dryness and the dry mass from petroleum ether, ethyl acetate, butanol and aqueous fractions were re-dissolved in sterile Nutrient broth to make the particular concentration of each extract

Culture and Maintenance of Microorganisms

Pure cultures of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Corynebacterium diptheriae* and *Bacillus subtilis* were obtained from

the Department of Biotechnology, Birla College, Kalyan, Mumbai. The pure bacterial cultures were maintained on Nutrient agar slants.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of various concentration of ethanolic extract of fruit and different solvent fractions of fruit extract (petroleum ether, ethyl-acetate, butanol and aqueous fractions at the concentration of (250-0.781 mg/ml)of *Anthocephalus indicus* A. Rich. was carried out by tube dilution method for *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Corynebacterium diphtheriae* and *Bacillus subtilis*. Stock concentration of each sample was serially diluted (two fold) by adding 1.0 ml of Nutrient broth medium. The tubes were then inoculated with 0.1ml of bacterial suspension and incubated at 37ºC for 24 hours [15]. After incubation the tubes were examined for bacterial growth by adding 0.5 ml of triphenyl tetrazolium chloride (TTC-0.1%). The colour change of the triphenyl tetrazolium chloride was used as an indicator for the presence of viable cells. The colour change is due to the reduction of the colourless tetrazolium salt to the pink coloured formazan crystals by the action of NADH-generating dehydrogenase enzyme of bacterial cells [16].

Agar Well Diffusion Method

Antibacterial activity of ethanolic extract of fruit and different fractions (petroleum ether, ethyl-acetate, butanol and aqueous fractions) of ethanolic fruit extract of *Anthocephalus indicus* A. Rich. was further evaluated by agar well diffusion method against all the test bacterial cultures. Antibacterial activity was determined by measuring the diameter (mm) of zone of inhibition. The concentration of extracts to be used for the method was calculated on the basis of their respective MIC values and four times the MIC values were used to determine the zone of inhibition. The cultures were bulk seeded in sterile Nutrient agar medium and poured into sterile petri plates. The wells of 6mm diameter were made using sterile cork borer in solidified agar plates and then extracts were added into the wells. The diameter of zone of inhibition (ZOI) was measured in millimetres (mm). Ciprofloxacin (5 µg/ml) a standard antibiotic was used as a positive control. The plates were incubated at 37ºC for 24 hours and zones of inhibition were measured [17].

TLC-Bioautography

For the isolation of antibacterial compound from the fruit extract of *Anthocephalus indicus* A. Rich., the aqueous fraction of ethanolic fruit extract was analysed by agar overlay bioautography method. Bioautography belongs to microbiological screening method commonly used for the detection of antimicrobial activity of active compounds of plants [18]. The aqueous fraction of fruit extract was loaded on the TLC plates for the separation of compounds responsible for antibacterial activity. Preactivated TLC plate coated with silica gel G_{60} F254 (Merck) was used as stationary phase. Ethyl acetate: ethanol: water (8: 2: 0.2) was used as mobile phase [19]. In the agar-overlay bioautography method, the TLC plate with separated compounds is first placed on the surface of Nutrient agar medium in the petri plates and then covered with Nutrient agar medium seeded with the test organisms. The plates were then incubated at 37°C for 24 hours. The zone of inhibition formed due to antimicrobial agent on the TLC plates was visualized using triphenyl tetrazolium chloride reagent [20].

Analysis and Characterisation of Antibacterial Compound

Antibacterial compound from the aqueous fraction of ethanolic fruit extract of *Anthocephalus indicus* A. Rich. was analysed by TLC-Bioautography method. The compound was scraped out from the TLC plate, dissolved in ethanol and analysed by standard methods of preliminary qualitative tests for the detection of phytochemical such as alkaloid, flavonoid, tannin and iridoid glucoside [21-24]. The characterisation of the compound was carried out by FTIR analysis from the Department of Chemistry, Birla College while MS (mass spectroscopy) and ^{13}C- NMR (nuclear magnetic resonance) analysis from SAIF-IIT, Bombay [19, 25].

Table 1: MIC

Extracts	E.c.	S.t.	K.p.	P.a.	S.a.	C.d.	B.s.
MIC values (mg/ml)							
ECF	6.25	6.25	12.5	12.5	6.25	6.25	6.25
PE	25	25	-	25	-	-	-
EtoAc	3.12	3.12	6.25	3.12	3.12	6.25	6.25
Bu	3.12	3.12	6.25	3.12	3.12	6.25	6.25
Aq	1.56	0.78	3.12	1.56	0.78	3.12	0.78

Values are mean ± SD of three replicates, -: no response E.c. = *Escherichia coli*, S.t. = *Salmonella typhi*, K.p. = *Klebsiella pneumoniae*, P.a. = *Pseudomonas aeruginosa*

S.a. = *Staphylococcus aureus*, C. d. = *Corynebacterium diphtheriae*, B.s. = *Bacillus subtilis*

ECF: Ethanol crude fruit extract, PE: Petroleum ether fraction of fruit extract

EtoAc: ethyl-acetate fraction of fruit extract, Bu: Butanol fraction of fruit extract, Aq: Aqueous fraction of fruit extract

Table 2: Agar Well Diffusion Method

	Zone of inhibition in mm (ZOI)						
Organism Extract	**E.c.**	**S.t.**	**K.p.**	**P.a.**	**S.a.**	**C.d.**	**B.s.**
Crude ethanolic Fruit extract	24.66 ±0.57	21.33 ±1.15	21.70 ±1.52	24.33 ±0.57	24.00 ±1.00	15.00 ±1.00	19.00 ±1.00
Ethyl-acetate fraction	23.00 ±1.00	19.00 ±1.00	19.70 ±0.57	24.00 ±1.00	21.70 ±1.52	14.33 ±0.577	18.33 ±0.57
Butanol fraction	23.33 ±1.15	18.70 ±1.15	21.00 ±0.57	24.00 ±1.00	22.0 ±1.00	14.70 ±0.57	19.33 ±0.57
Aqueous fraction	35.00 ±1.0	32.00 ±1.73	29.33 ±0.57	32.00 ±1.00	37.0 ±1.00	27.33 ±0.57	26.33 ±0.57
Ciprofloxacin (5µg/ml)	23.00 ±1.00	25.33 ±0.57	23.70 ±0.57	25.70 ±1.15	24.70 ±0.57	27.00 ±1.00	26.33 ±0.57

Values are mean ± SD of three replicates, Zone of inhibition (mm) includes the diameter of well

E.c. = *Escherichia coli*, S.t. = *Salmonella typhi*, K.p. = *Klebsiella pneumoniae*, P.a. = *Pseudomonas aeruginosa*

S.a. = *Staphylococcus aureus*, C. d. = *Corynebacterium diphtheriae*, B.s. = *Bacillus subtilis*

Table 3: Preliminary phytochemical analysis of isolated compound

Phytoconstituents	Observation
	Isolated compound
Alkaloids	-
Tannins	-
Flavonoids	-
Iridoid glucosides	+
Coumarins	-
Quinones	-

"+" represents presence of phytoconstituents, "- "represents absence of phytoconstituent

Table 4: FTIR data of isolated compound

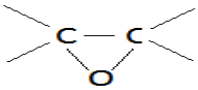
3340.1	OH stretching	Hydroxyl group of Glucopyranoside and Apiofuranosyl
2929.34	C-H stretching	Cyclopentane group of Aglycone (Iridoid group)
1721.16	C=O stretching	Ester group of Aglycone (Iridoid group)
1401.03	C-H stretching	CH_2 group of Apiofuranosyl
1209.15, 1066.44	C=C-O-C	Epoxide ring in Aglycone (Iridoid group)
915.058,812.849		C-H deformation in Epoxide ring
708.712		Cis isomers in Apiofuranosyl group
655.679, 668.214, 679.785	 CH=CH	Alkene in Alglycone group

Table 5: ^{13}C NMR data of isolated compound

Groups	Chemical shift (δc) in ppm
Glucopyranoside	
CH	71.473
CH	71.626
CH	71.946
CH	72.339
CH	74.345
CH	74.499
CH	76.652
Apiofuranosyl	
CH2	65.630
CH	73.864
CH2	74.449
CH	77.133
C	79.444
CH	82.717
Aglycone	
CH2	39.107
CH	42.558
C	45.558
OCH	58.945
CH2	64.768
CH2	68.721
$COOCH_3$	180.806

[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity
1	3340.1	70.1271	2	2929.34	80.5889
3	2364.3	85.5831	4	2341.16	86.7145
5	1721.16	73.2805	6	1401.03	75.2347
7	1209.15	70.4494	8	1066.44	58.3418
9	915.058	74.4118	10	888.059	74.7399
11	812.849	71.8648	12	708.712	66.2
13	679.785	65.3328	14	668.214	63.0752
15	655.679	62.6296			

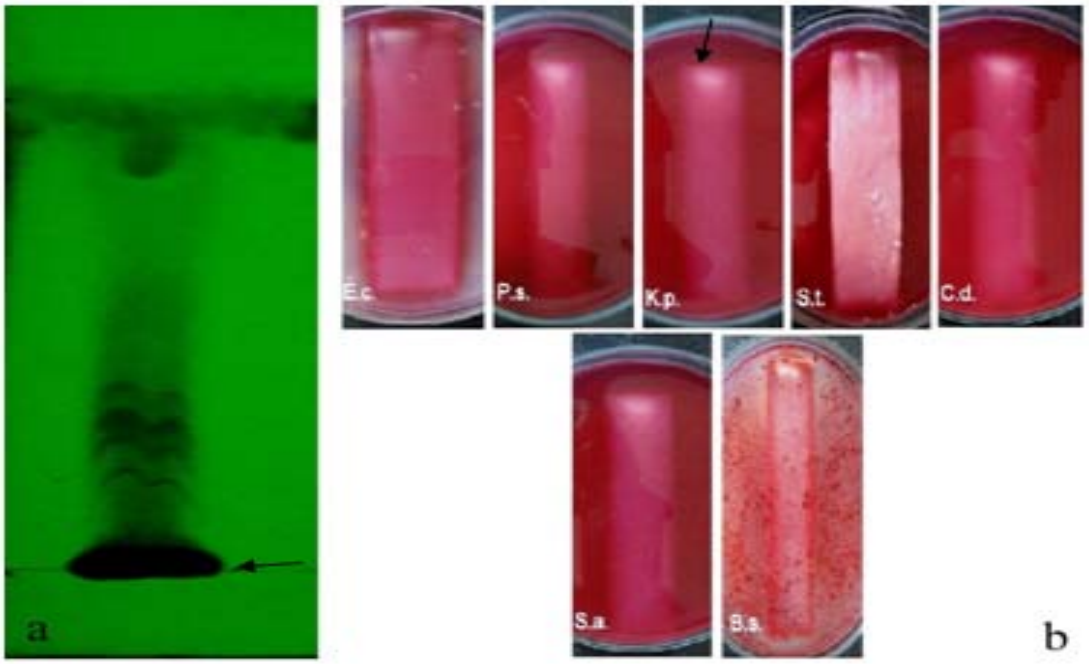


Fig. 1: TLC-Bioautography, a= Arrow indicates that the compound showed Zone of inhibition against the test organisms at the place where the sample (aqueous fraction) was loaded on the TLC plate. b = Arrow indicates the zone of inhibition of the antibacterial compound on the TLC plate.

E.c. = *Escherichia coli*, S.t. = *Salmonella typhi*

K.p. = *Klebsiella pneumoniae,* P.a. = *Pseudomonas aeruginosa*

S.a. = *Staphylococcus aureus,* C. d. = *Corynebacterium diphtheriae*

B.s. = *Bacillus subtilis*

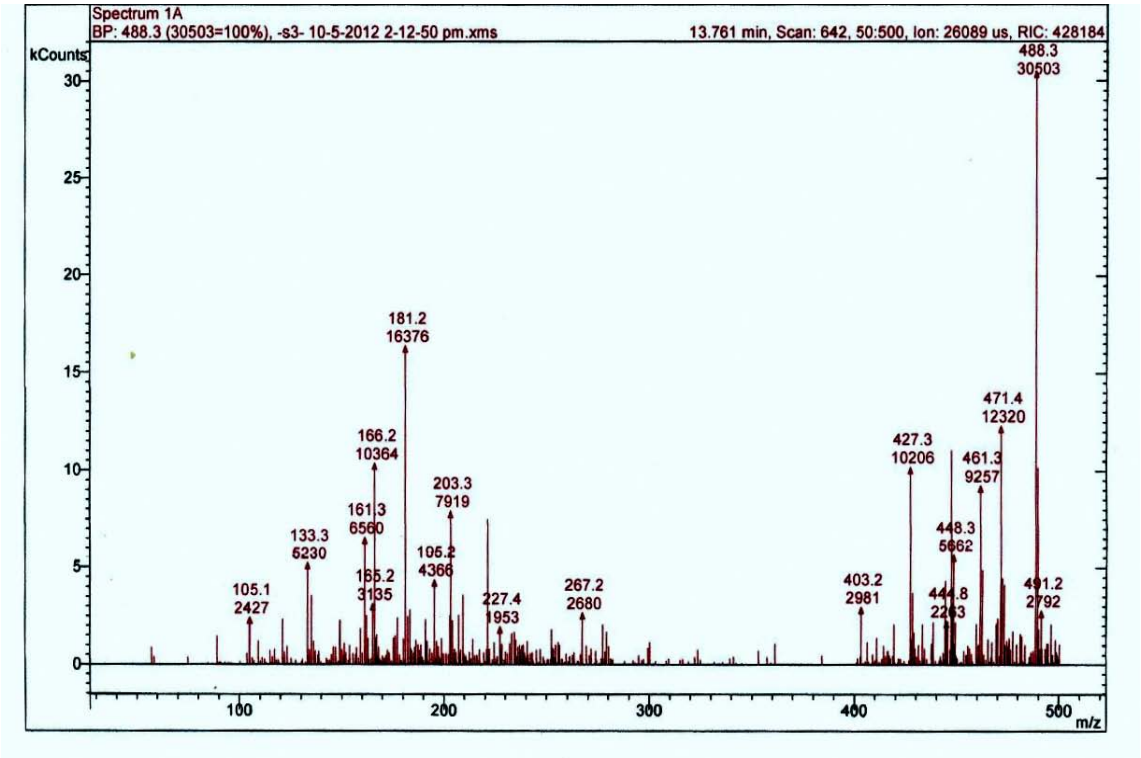


Fig. 2: MS (Mass Spectroscopy) data of isolated compound

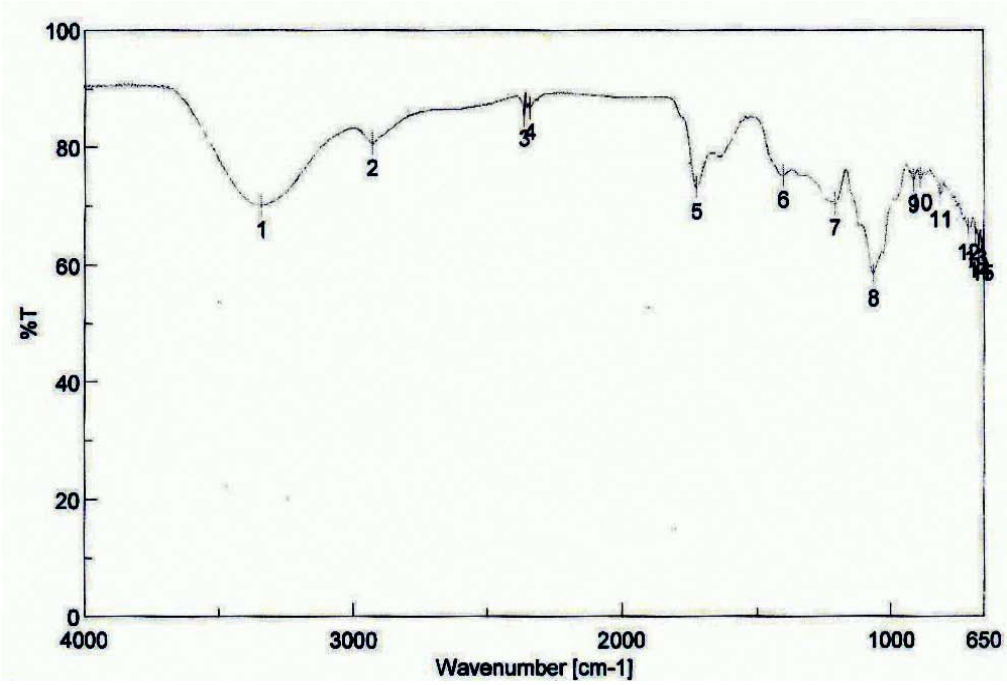


Fig. 3: FTIR data of isolated compound

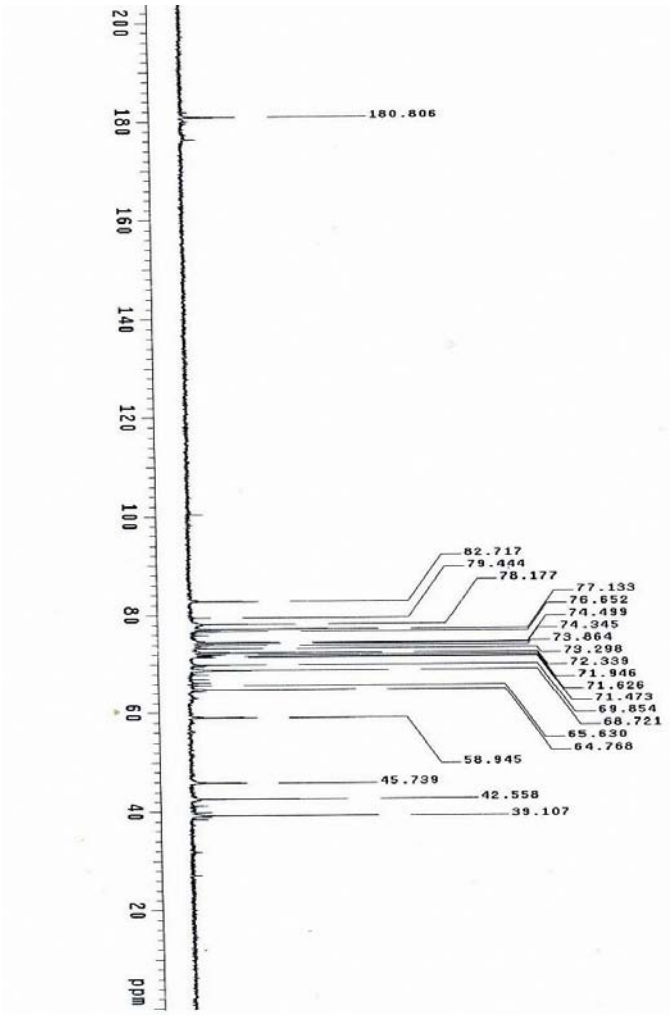


Fig. 4: ^{13}CNMR data of isolated compound

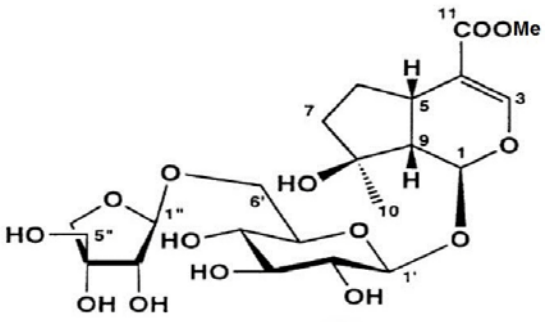


Fig. 5: Structure of isolated compound (Iridoid glucoside)

RESULT AND DISCUSSION

Minimum Inhibitory Concentration

The most commonly used method to determine the antibacterial activity is tube dilution assay [26]. In the present study, the low MIC value of ethanolic fruit extract indicates that *Anthocephalus indicus* A. Rich. fruit extract may act as an effective antibacterial agent. The aqueous fraction of ethanolic fruit extract showed lower MIC value as compared to petroleum ether, ethyl-acetate and butanol fractions which clearly indicate that active compound fractionated in aqueous solvent is more active as an antibacterial agent as compared to compounds fractionated in petroleum ether, ethyl-acetate and butanol solvents (Table 1).

Antibacterial Activity using Agar Well Diffusion Method

Amongst the crude fruit extract and different solvent fractions (petroleum ether, ethyl-acetate, butanol and aqueous) of fruit extract of *Anthocephalus indicus* A. Rich., aqueous fraction of showed maximum zone of inhibition against all the test organisms as compared to other extracts (Table 2)**.**

TLC-Bioautography

Antibacterial compound responsible for the antibacterial activity of aqueous fraction of ethanolic fruit extract was analysed by agar overlay bioautography method. The separated compounds, when analysed for antibacterial activity by agar overlay bioautography method, showed zone of inhibition against the test organisms at the place where the sample (aqueous fraction) was loaded on the TLC plate. This might have helped in the purification of the compound which was responsible for the maximum antibacterial activity of aqueous fraction of fruit extract (Figure 1).

Bioautography procedure enables recognition of known antimicrobial compounds in extracts at the early stages of separation and is thus economically very important [27]. It is a simplest and cheapest method for detecting plant constituents because the method is easy to run, sensitive, reproducible and requires little equipment. This detection method can be successfully combined with MS, FTIR, NMR, HPLC, HPTLC and UV for the characterisation of bioactive compounds [18].

Analysis and Characterisation of Antibacterial Compound

The compound that showed antibacterial activity by bioautography method was scrapped off from the TLC plate, dissolved in ethanol and analysed by standard methods of preliminary qualitative tests for the detection of phytochemical such as alkaloid, flavonoid, tannin, coumarin, quinone and iridoid glucoside. The result of qualitative test was found to be positive for the iridoid glucoside and negative for the alkaloid, flavonoid and tannin (Table 3).

The characterisation of the compound was carried out by FTIR and ^{13}C- NMR (nuclear magnetic resonance). The mass spectroscopy showed a molecular peak at 491.2 (Figure 2) with the molecular formula $C_{21}H_{33}O_4$. The FTIR spectrum indicated the presence of organic groups in the isolated compound (Table 4 and Figure 3). The ^{13}C NMR data of compound confirmed the presence of apiofuranosyl-glucopyranoside in addition to cyclopentane ring of iridoid group. The chemical shifts were similar to those obtained for iridoid glucoside (Table 5 and Figure 4). The antibacterial compound was characterised as iridoid glucoside (Figure 5).

The compound responsible for antibacterial activity of aqueous fraction of ethanolic extract of fruit of *Anthocephalus indicus* A. Rich. was identified as an iridoid glucoside by qualitative analysis, MS, FTIR and ^{13}C NMR techniques. The iridoid glucoside showed significant antibacterial activity against Gram negative bacterial cultures such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and Gram positive bacterial cultures like *Corynebacterium diphtheriae*, *Staphylococcus aureus* and *Bacillus subtilis.* Hence Iridoid glucoside present in fruit of *Anthocephalus indicus* A. Rich. may be used as a potent broad spectrum herbal antibacterial agent.

CONCLUSION

The results for antibacterial activity of *Anthocephalus indicus* A. Rich. were found to be similar as obtained by other authors. They reported that ethanolic extract of *Anthocephalus cadamba* showed significant antibacterial against Gram positive and Gram negative organsims [18]. Alcoholic extract of fruits of *Anthocephalus indicus* A. Rich. showed significant antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* with the zone of inhibiton of 22-24 cm and low MIC value upto 1.00 mg/ml [28]. Review for the natural antimicrobial compounds, showed that iridoid glycosides present in the plant belonging to Apocynaceae, Scrophulariaceae, Diervillaceae, Lamiaceae, Loganiaceae and Rubiaceae possess significant antimicrobial activity with the lower MIC values and can be isolated from alcoholic extract of the plants [29]. Phytoconstituents present in plants are used for the treatment of microbial infection, as an alternative to chemically prepared synthetic drugs because of safer therapeutic effect [30]. Initial screening for the potential antibacterial compounds from plants may be performed by crude extracts. Crude plant extract is the mixture of various compounds. Bioassay-guided isolation integrates the processes of separation of compounds in a mixture, using various analytical methods. The process involves solvent partition of crude extract using solvents from low polarity to high polarity solvents by solvent partition method and checking the activity of each fraction. This method separates the active compounds from less non-active or less-active compounds [14].

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