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Isolation and identification of antibacterial compound from the leaves of *Cassia auriculata*

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Abstract. – *Objectives:* **Antimicrobial properties of medicinal plants and plant parts such as flowers, roots, fruits, seeds and oils are being used to cure some chronic and acute diseases throughout the world. In the present study, an attempt has been made to isolate and identify the antibacterial compound present in the leaves of the *Cassia auriculata.***

Materials and Methods: **A preliminary screening of antibacterial activity was carried out with fine different plant extracts viz., *Aegle marmelos, Chloris Virgata, Clausena anisata, Feronia limonia* and *Cassia auriculata* against different human pathogenic bacteriae such as *Escherichia coli*, *Salmonella typhi, Proteus mirabilis* and *Klebsiella pneumoniae* at different concentrations.**

Results and Conclusions: **Based on the results, the plant *Cassia auriculata* was selected as the efficient plant, which shows antibacterial activity against the tested organisms. Further compound responsible for its antibacterial activity was isolated and identified by IR spectrum, ^{1}HNMR, ^{13}CNMR and Mass spectrum studies, as oleanolic acid, which has the molecular formula of $C_{30}H_{48}O_3$.**

Key Words:

Antibacterial activity, *Cassia auriculata,* Oleanolic acid.

Introduction

Herbal medicines have been known to man for centuries.Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine[1]. Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world.

India has about 2000 species of medicinal plants and a vast geographical area with high production potential and varied agro-climatic conditions[2]. For a long period of time, plants have been a valuable source of natural products for maintaining human health, last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purpose has gradually increased[3]. According to the World Health Organization (WHO), medicinal plants would be the source to obtain a variety of drugs. Several synthetic antibiotics are employed in the treatment of infections and communicable diseases[1]. The harmful microorganisms can be controlled with drugs and the presence of multiple drug resistant bacteria has created alarming clinical situations in the treatment of infectious diseases. The pharmacological industries have produced a number of new antibiotics. However, resistance to these drugs by microorganisms has increased[4]. In general, bacteria have the genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents[5]. Therefore, research is being carried out in order to minimize the use of antibiotics, and to develop new drugs, either synthetic or natural to control pathogenic antibiotic-resistant microorganisms[6]. Certain natural products such as plants and different parts of plant-leaves, roots, flowers, fruits, barks, seeds and oils are used to cure chronic and acute diseases.

Materials and Methods

Collection of Plant Materials

The fresh and healthy leaves of *Cassia auriculata* were collected from Cuddalore district, Tamil Nadu, India. The leaves were washed thoroughly with tap water and then with sterilized distilled water for the removal of dust and sand

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particles. The leaves were shade dried for few days and then powdered. This was used for the extraction of antimicrobial compounds against the microbes.

Culture Used

Microorganisms chosen for the present study were isolated from the clinical specimens that came for testing in the Department of Microbiology RMMCH Annamalai University. The cultures identified and used in the study were *Escherichia (E) coli, Salmonella (S) typhi, Proteus (P) mirabilis and Klebsiella (K) pneumoniae*.

Preparation of Plant Extracts

The preparations of different plant extracts were done through a modified method[7].

Solvent Extraction Methods

The shade dried leaf materials were used for the solvent methanol extraction. About 5 g of leaf powder were weighed and mixed with methanol (1:3 w/v), which was incubated for two days. After the incubation period the slurry was filtered through Whatman no. 1 filter paper in a beaker and allowed it for evaporation. The residue was dissolved with dimethyl sulfoxide (DMSO) with different concentrations and checked it for antimicrobial activity[8].

Antimicrobial Susceptibility Test

Disc diffusion method was adopted for evaluation of antimicrobial activity of *Cassia auriculata*. Muller Hinton agar was prepared and autoclaved at 15lbs pressure for 20 minutes and cooled to 45°C. The cooled media was poured on to sterile Petri plates and allowed for solidification. The plates with media were seeded with the respective microbial suspension using sterile swab. The disc impregnated with respective leaf extracts at different concentrations (100, 200 and 300 mg/ml) individually were placed on the four corners of each Petri dishes. Control disc was also placed. After incubation period, the diameter of the zone formed around the paper disc were measured and expressed in mm[4].

Cassia Auriculata

Preparation of Methylene Chloride (Dichloromethane) Extracts

The collected healthy and well grown leaves of *Cassia auriculata* were immediately brought to the laboratory. The leaves were first washed with tap water, then surface sterilized in 10 per cent sodium hypochlorite to prevent the contamination of any microbes. They were thoroughly rinsed with sterile distilled water. The leaf samples were shade dried followed by oven drying (60°C) and milled in an electrical blender[9].

The powdered leaves (400 g) were extracted in a Soxhlet apparatus for 72 h with methylene chloride (dichloromethane) (300 ml × 3). The extracts were pooled and the solvent was evaporated using a rotary evaporator under reduced pressure at 40°C.

Isolation of Antimicrobial Compound

The diethyl ether extract (50 g) was submitted to silica gel column chromatography (Air-Blow Equipments, Chennai, India) (size 5 × 60 cm) using with hexane: $CHCl_3$ (8:2, 0:1) and MeOH as eluent, to give eight fractions (A-H). Fraction D (600 mg) with highest antimicrobial activity was separated by silica gel column chromatography with hexane: EtOAc (7:3) to obtain six fractions (C1-C6). Fraction C5 was submitted to silica gel column chromatography with hexane: EtOAc (3:1) and then $CHCl_3$: EtOAc (19:1) to give compound 1 (29 mg, Rf = 0.19 in $CHCl_3$: hexane, 4:1).

Identification of Antimicrobial Compound

The compound was identified by spectral studies like, IR, ^{1}H NMR, ^{13}C NMR and mass spectrum. IR spectra were recorded in AVATAR-330 FT-IR spectrophotometer (Thermo Nicolet) (Thermo Fisher Scientific, MA, USA) and only noteworthy absorption levels (reciprocal centimeters) are listed. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker AMX 400 MHz Spectrometer (Bruker BioSpin GmbH, Ettlingen, Germany) using deuterated chloroform ($CDCl_3$) as solvent and TMS (tetramethylsilane) as internal standard. ^{11}C NMR spectra were recorded at 400 MHz on a Bruker AMX 400 MHz (Bruker BioSpin GmbH, Germany) using standard parameters using $CDCl_3$ as solvent. All the NMR measurements were made on 5 mm NMR tubes. For recording ^{1}H NMR spectrum, solutions were prepared by dissolving 10 mg of the active principle in 0.5 ml of $CDCl_3$ while for ^{13}C NMR spectra about 20 mg of the compound was dissolved in the same volume of the solvent. Here, TMS was used as an internal standard. Mass spectrum was recorded using Varian 1200L Mass Spectrometer (Varian India Pvt. Ltd., Powai, Mumbai).

Results

The antimicrobial activity of crude leaf extracts of *Cassia auriculata* were studied in different concentrations (100 mg/ml, 200 mg/ml, 300 mg/ml)[10]. Antibacterial potential of leaf extract was assessed in terms of zone of inhibition of bacteria growth. The results of the antibacterial activities carried out in 100, 200, and 300 mg/ml of each leaves was used for antimicrobial screening. The antibacterial activity of the extract increased linearly with increase in volume of extract (mg/ml). The methanol extract have shown more sensitive to *Proteus mirabilis* and *Klebsiella pneumoniae, Escherichia coli* and *Salmonella typhi*, the growth inhibition zone measured ranged from 5-19 mm for all the sensitive bacteria. *Cassia auriculata* measured a zone size ranging from (15, 16 and 17 mm) for *Proteus mirabilis* (18, 19 and 19 mm) for *Klebsiella pneumoniae* (10, 11 and 13 mm) for *Escherichia coli* and (9, 10 and 11 mm) for *Salmonella typhi*. The results showed that leaves of *Cassia auriculata* was found to be more effective against the microbes used and hence the plant was selected for compound isolation.

IR Spectrum Studies

The IR spectrum of oleanolic acid (**1**) is presented. The IR spectrum of oleanolic acid (**1**) exhibited peaks at 3466-3534 and 1694 cm^{-1} are due to hydroxyl and carboxylic acid carbonyl carbon moiety.

Nuclear Magnetic Resonance (NMR) Studies 1H NMR

The ^{1}H NMR spectrum of oleanolic acid is presented in Figure 1. The ^{1}H-NMR spectrum of compound **1** showed seven tertiary methyl groups at δ 0.75, 0.77, 0.90, 0.91, 0.93, 0.98 (each 3H,s,CH_3 × 6) and 1.13 (3H,s, H-27) on an oleanane skeleton. A doublet doublet of one proton at δ 1.96 (1H, dd, *J*= 13.39, 4.03Hz, H-9) and another doublet doublet of one proton at δ 2.82 (1H, dd, *J*= 13.75, 4.18Hz, H-18) and a triplet of one vinyl proton at δ 5.28 (1H, t, *J*= 3.50 H-12) were assigned to H-9, H-18 and H-12, respectively, suggesting an olea-12-ene skeleton. One methine proton at δ 3.22 (1H, dd, *J*= 11.05, 4.68 Hz, H-3) showed that **1** has at least one hydroxyl group. The other 21 protons (both CH and CH_2 protons) give unresolved absorption at δ 1.06-1.09 (1H, m) and δ 1.16-1.91 (20H, m)[11].

^{13}C NMR

The ^{13}C NMR spectrum of the compound **1** is produced . It indicates the presence of 30 carbon atoms (7 methyls, 10 methylenes, 5 methines, 1 acid carbonyl and 7 quaternary carbons). The presence of oxygenated carbon at C-3 resonates at δ 79.04. The signal around δ 183.45 is due to acid carbonyl carbon at C-28. The three methyl groups at C-25, C-26 and C-27 appear at δ 15.31, 17.14 and 25.93. The gem-dimethyl carbons at δ C-23, C-24, C-29 and C-30 appear at 28.09, 15.93, 32.44 and

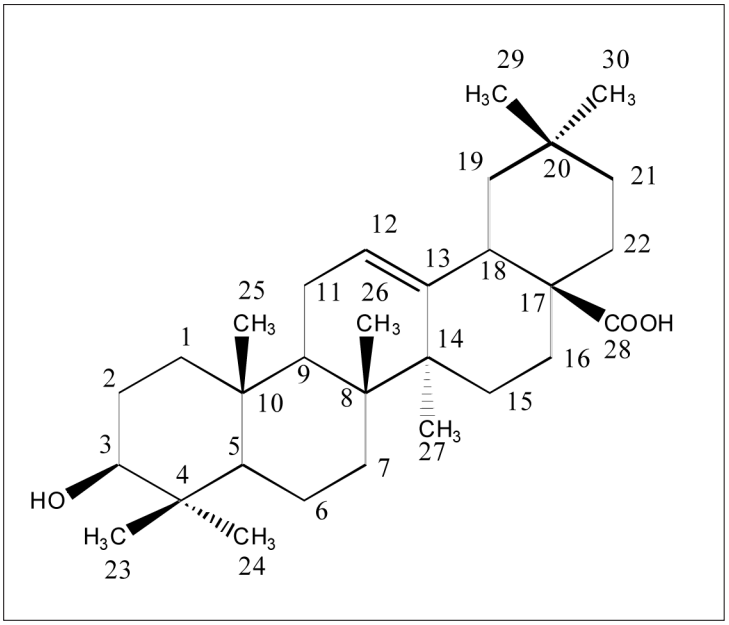

Figure 1. Oleanolic acid.

Table I. Antimicrobial activity of crude leaf extract of Cassia auriculata in comparision to methanol extract.

Serial No	Plants	Microorganism	Zone of inhibition in millimeter Concentration in mg/ml			
			Control	100	200	300
1	*Cassia auriculata*	*Escherichia coli*	–	10	11	13
		Salmonella typhi	–	9	10	11
		Proteus mirabilis	–	15	16	17
		Klebsiella pneumoniae	–	18	19	19

23.39 respectively. The quaternary carbons at δ C-4, C-8, C-10, C-13, C-14, C-17 and C-20 resonate at 38.75, 39.28, 37.09, 143.60, 41.59, 46.53 and 30.66. Obviously the remaining signals at δ 38.40, 27.16. 18.29, 33.05, 22.91, 27.69, 23.57, 45.88, 33.80 and 32.62 are due to methylene carbons at C-1, C-2, C-6, C-7, C-11, C-15, C-16, C-19, C-21 and C-22 carbons respectively. The signals at δ 55.23, 47.64, 122.62 and 40.98 are due to C-5, C-9, C-12 and C-18 carbons. The spectral data were similar to the ones reported for oleanolic acid (Figure 1)[12].

Mass Spectrum

The mass spectrum of the compound **1** is produced. The mass spectrum of it showed a molecular ion at *m/z* 456 corresponding to $C_{30}H_{48}O_3$. From the above results, it is concluded that the compound is oleanolic acid.

Discussion

Recently much attention has been directed towards plant extracts and biologically active compounds isolated from popular plant species[13]. The use of medicinal plants play a vital role in covering the basic health needs in developing countries and the plants may offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms[14].

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world populations[15].

In the present work methanolic extract of *Cassia auriculata* shows higher activity against the test bacteriae such as *Klebsiella pneumoniae*, *Proteus mirabilis, Escherichia coli* and *Salmonella typhi*. The present study suggests the presence of oleanolic acid (triterpenoids) in the leaves of *Cassia auriculata* showing the antimicrobial activity, and also that the organic solvent extraction is suitable to verify the antimicrobial properties of medicinal plants[2]. The present study justifies the claimed uses of leaves in the traditional system of medicine to treat various infectious disease caused by the microbes.

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