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Antibacterial Activity of *Cassia auriculata* Against ESBL Producing *E. coli* from UTI Patients

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Abstract: Urinary tract infection (UTI) is one of the most common diseases among all age groups encountered in medical practice today. The increasing drug resistance of bacteria has made the therapy of UTI difficult and has led to greater use of expensive broad spectrum drugs. The aim of the study was to evaluate the antibacterial efficacy of *Cassia auriculata* against Extended Spectrum Beta Lactamase (ESBL) producing *E. coli*. Methanol, ethanol, ethyl acetate, hexane and chloroform crude and Soxhlet extracts of ethyl acetate and hexane of leaves and flowers of *Cassia auriculata* were tested for antibacterial activity by well diffusion method against ESBL producing *E. coli*. It was concluded that *Cassia auriculata* has antibacterial activity against ESBL producing *E. coli* from UTI. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethyl acetate leaves and flowers extract were evaluated. The maximum activity was observed from a range between 50-125mg/ml.

Key words: ESBL · Urinary tract infection · *C. auriculata* · Minimum inhibitory Concentration · *E. coli*

INTRODUCTION

Microbes are remarkably adapted and amazingly versatile. Through the course of evolution, they have developed sophisticated mechanisms for preserving genetic information and disseminating it efficiently in the interest of their survival [1]. Urinary tract infection (UTI) posses a serious health threat in terms of antibiotic resistance and high recurrence rates [2]. It is estimated that about 35% of healthy individuals suffer from symptoms of UTI at some stages in their life. About 5% of women each year suffer with the problem of urinary pain (dysuria) and frequency [3]. The incidence of UTI is greater in women as compared to men either due to anatomical predisposition or urothelial mucosal adherence to the mucopolysaccharide lining or other host factors [4]. The Extended Spectrum Beta Lactamases (ESBL) producing bacteria identified in members of Enterobacteriaceae are increasingly causing urinary tract infection both in hospitalized and outpatients [5, 6]. The ESBL are plasmid mediated enzymes which are capable of hydrolyzing and inactivating a wide variety of β-lactams including third generation cephalosporins (3GC), penicillins and aztreonam [7].

These enzymes are the result of the mutations of the TEM-1, TEM-2 and SHV-1 enzymes. Normally the TEM-1, TEM-2 and SHV-1 enzymes confer a high resistance to the early penicillins and low level resistance to first generation cephalosporins [8]. In Enterobacteriaceae, classical ESBLs are evolved from the TEM and SHV families. In the recent years, new ESBLs of non TEM and non SHV types have emerged, such as the enzymes of CTX-M, PER, VEB and GES lineages [9]. Because of their extended substrate range, these enzymes were called as the Extended Spectrum Beta Lactamases (ESBL) [10]. The first ESBL isolates were discovered in Western Europe in the mid 1980s and subsequently in the US in late 1980s [11].

Plants produce a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry and it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens [12]. *Cassia auriculata* commonly known as tanners *Cassia*, also known as "Avaram" in Tamil is a shrub that belongs to the *Caesalpiniaceae* family [13]. The flower of the shrub is used in the treatment of skin disorders. Every part of this

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plant is valuable in medicine for ulcers, leprosy and liver disease [14]. The plant can also be used as an anti-diabetic, hypolipidemic and anti-oxidant [15]. The present study was conducted to evaluate the antibacterial activity of *C. auriculata* plant against ESBL producing *E. coli* isolated from UTI patients.

Since no previous attempts have been made to examine the anti bacterial effects of *C. auriculata*, against ESBL producing *E. coli*. The aim of this paper was to substantiate the antibacterial sensitivity of different extracts of *C. auriculata* leaves and flowers against ESBL producing *E. coli* to lengthen the queue of antibacterial herbs.

MATERIALS AND METHODS

Collection of Urine Samples: Fifty Urine samples were collected from a multi specialty hospital in Coimbatore (India) and transported to the laboratory in an ice cold condition after adding boric acid at a final bacteriostatic concentration of 1.8% without delay [16].

Isolation and Identification of Bacteria from Urine Samples: For the isolation of UTI causing organisms, a loopful of each urine sample was streaked on the blood and Mac Conkey agar medium and incubated at 37°C for 24 hrs [17]. After incubation, colonies were selected and characterized on the basis of morphological, cultural and biochemical [18] features and identified with the help of Bergey's Manual of Systemic Bacteriology [19].

Antibiotic Susceptibility Testing: Antibiogram of the UTI isolates was determined using Muller Hinton agar (MHA) and disc diffusion method [20]. Antibiotics commonly used for the treatment of UTI i.e. Ampicillin 10µg, amikacin 30µg, ciprofloxacin 10µg, cefotoxime 30µg, cefazolin 30µg, ceftazidime 30µg, ceftriaxone 30µg, gentamycin 10µg, imipenem 10µg, ofloxacin 5µg, penicillin 2µg, piperacillin 100µg, sulphamethazole 10µg, trimethoprim 10µg and vancomycin 30µg concentration were used. The diameter of the zone of inhibition was measured and the isolates were classified as "resistant", "intermediate" and "sensitive" based on standard chart.

Screening for ESBL Producers by Disk Diffusion Method: The screening was done by disk diffusion test as recommended by the CLSI [21, 22]. ceftazidime 30mcg was used as the indicator drug. Zone diameter = 22mm was suspected to be due to possible ESBL producers.

Detection of ESBL by Confirmatory Tests

Double Disk Synergy Test (DDST): A disk of augmentin (20µg amoxicillin+10µg clavulanate) was placed on MHA containing test inoculum. Then the discs of cefotoxime (30µg) and ceftazidime (30µg) were placed 16 to 20 mm apart from the augmentin disc (centre to centre). After incubation (37°C for 24 hrs) the zone of cephalosporin disc towards the clavulanic acid disc was considered as ESBL producers [23].

Phenotypic Disc Confirmatory Test (PDCT): The test was performed as recommended by CLSI. Disks of ceftazidime (CA) 30µg and ceftazidime-clavulanic acid (CAC) 20+10µg or cefotoxime (CE) 30 µg and cefotoxime-clavulanic acid (CEC) 20+10 µg were placed on MHA at a distance of 30mm between each other. Increase in zone diameter (=5mm) for CAC versus CA or CEC versus CE is confirmed as ESBL producing organisms [23].

Collection of Plant Material: Leaves and flowers of *C. auriculata* were collected from villages in and around Coimbatore District, South India. Plant materials were dried under room temperature for about 10 days and ground into fine powder using a blender.

Crude Extraction: The shade dried coarsely powdered leaves were subjected to cold extraction using hexane, chloroform, ethyl acetate, acetone and methanol. After one week, the frequently shaken mixture was filtered through Whatmann No. 1 filter paper. The extract was used for antibacterial activity testing [24].

Soxhlet Extraction: To obtain the ethyl acetate extracts, dried and finely powdered leaves and flowers of *C. auriculata* (about 10gms each) homogenized using 100ml ethyl acetate were added to Soxhlet apparatus. The boiling point was set at 40°C. The solvent was recycled. The compounds present in the sample were extracted continuously until the solution lost the color. The extract was then transferred to a sterile Petri dish and kept for evaporation of ethyl acetate at room temperature. The residue was collected and stored in the refrigerator [25].

Antibacterial Activity of Extracts: Antibacterial activity of crude and Soxhlet extracts of *C. auriculata* (leaves and flowers) was tested using the agar well diffusion method (24, 25). The test inoculum (0.5 McFarland's turbidity) was spread onto MHA by using a sterile cotton swab. Then the wells were made by a sterile well puncture.

Twenty µl of crude and Soxhlet extracts were added to each well and incubated at 37°C for 24 hrs and the diameter of zone of inhibition was measured in mm.

Minimum Inhibitory Concentration: Nutrient broth (0.5ml) was added in a series of sterile tubes and a same amount of adjusted isolated organisms (0.5% Mc Farland) was inoculated into the tubes. Different concentrations (25µl, 50µl, 75 µl, 100 µl and 125 µl) of the extracts were added and the tubes were incubated at 37°C for 24 hrs and examined for turbidity [26].

Minimum Bactericidal Concentration: After 24 hrs of incubation, samples from the tubes showing turbidity were streaked on Muller Hinton agar plates and the plates were incubated at 37°C for 24 hrs.

RESULTS AND DISCUSSION

Forty one out of the 50 examined urine samples showed prominent bacterial count. In the present study the most commonly isolated pathogen was *E. coli* (23 and 46%) and the percent of ESBL producing *E. coli* was 78.2% (Table 1).

Jesus *et al*. [27], reported 5% susceptibility of ESBL producing *E.coli* to ceftriaxone and 63% to ceftazidime. Also in his study, 21% susceptibility of *E. coli* was found for ceftriaxone and ceftazidime and 73.9% susceptibility to imipenem. The degree of resistance against third generation cephalosporins can be highly variable among different ESBL enzymes and the sensitivity of screening for ESBL can vary depending on the type of antimicrobial agent tested. While some ESBL enzymes confer frank resistance to Extended Spectrum Cephalosporins, many isolates show only intermediate resistance or even susceptibility to one or more of these antimicrobial agents despite carriage of an ESBL [28].

It was found that 78.2% isolates of *E. coli* were positive for ESBL enzymes. World wide data shows that there is an increasing resistance among UTI pathogens to conventional antibiotics. Resistance has emerged even to newer, more potent antibiotic agents. Antibiotic resistance surveillance is necessary to determine the size of the problem and to guide the empirical selection of antibiotic agents for treating infected patients. Use of appropriate antimicrobial and the early removal of unnecessary interventional apparatus are of importance for the control and decreasing the prevalence of ESBL-producing *Escherichia coli* [30].

Table 1: Prevalence of microbes in collected sample

Total No of Samples	Types of Organisms Isolated	No. Of Samples Showing Positive	%
41	*Escherichia Coli*	23	46
	Klebsiella sp	18	3 6
23	ESBL *E.coli*	9	39
18	ESBL *Klebsiella* sp	6	33

Table 2: Antibiotic sensitivity of bacterial isolates

Serial No.	Antibiotics	Zone of inhibition diameter (mm)
1	Amp	6.3 ± 1.5
2	Ak	15.3 ± 2.4
3	CIP	23.5 ± 3.3
4	CTX	-
5	CZ	-
6	CAZ	-
7	CTR	21.3 ± 5.1
8	GEN	18 ± 5.5
9	IMP	26.3 ± 3.7
10	OF	17.5 ± 4.2
11	P	-
12	Pi	21.3 ± 2
13	S	-
14	TR	14.8 ± 3
15	VA	-

Zone of inhibition was calculated by using four replicas

Table 3: Sensitivity pattern of the tested bacterial isolates against crude extracts of *Cassia auriculata*

	Zone of Inhibition (mm)						
Bacteria	Methanol (Leaves)	Ethanol (Leaves)	Ethyl Acetate (Leaves)	Chloroform (Leaves)	Aqueous (Leaves)	Hexane (Leaves)	Methanol (Flower)
E. coli	18	-	24	-	-	-	27
E. coli ESBL	28	-	27	-	-	-	30

Table 4: Sensitivity pattern of the tested bacterial strains against soxhlet extract of *Cassia auriculata*

	Zone of Inhibition (mm)		
Bacteria	Ethyl Acetate (Leaf)	N-hexane (Flower)	Ethyl Acetate (Flower)
E. coli	28	-	26
E. coli ESBL	30	-	31

Table 5: Minimum inhibitory concentration of ethyl acetate of fresh flower and dry leaves of *C.auriculata* against microorganisms

Bacteria	Fresh Flower(mg/mL) EA	Dry Leaves (mg/mL) EA
E.coli	75-125	25-125
ESBL *E.coli*	25-125	25-125

EA- Ethyl acetate

Crude methanol, ethanol, ethyl acetate, n-hexane and chloroform extracts of the dried leaves and flowers of *C. auriculata* were tested for antibacterial activity against the isolated organisms. Crude leaf extracts of methanol and ethyl acetate showed high activity against ESBL producing *Escherichia coli*. The methanol extract of flowers showed high effectiveness against ESBL producing *Escherichia coli* when compared to non-ESBL producing *Escherichia coli*. Methanol and ethyl acetate extracts (Leaves) exhibit higher degree of anti bacterial activity against (ESBL) *E. coli* than non ESBL *E. coli* (Table 2). The medicinal properties and pharmacological actions of *C. auriculata* are well known to Indian traditional medicine. These plants are known to contain various active principle of therapeutic value and possess biological activity against a number of diseases [31].

The soxhlet n-hexane and ethyl acetate extracts of *C. auriculata* (leaves and flower) were tested for antibacterial activity against the isolated organisms. The extract of ethyl acetate showed higher activity against ESBL producing *Escherichia coli* than non-ESBL producing *Escherichia coli* (Table 3). The antibacterial activity of *C. auriculata* extract may be due to the presence of phenolic constituents [32]. Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic anti microbials [33].

The MIC and MBC of the extracts against the isolated bacteria were determined by the tube-dilution method. Ethyl acetate extract of *C. auriculata* (leaves and flowers) was highly effective against *E. coli* (MIC value of 25 -125µg/ml) (Table 5). The result of MIC suggested that ethyl acetate flower and leaf extracts of *C. auriculata* could possibly act as a bactericidal agent against ESBL *E.coli* and non ESBL *E.coli.*

In can be concluded that the inhibitory effects of the extracts justify the medicinal use of *C. auriculata* against uropathogens. Further study is required to find out the active components of medicinal value.

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