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Research Article

PHYTOCHEMICAL ANALYSIS AND INVITRO ANTIBACTERIAL SCREENING OF SIDA ACUTA EXTRACTS AGAINST EXTENED SPECTRUM BETA-LACTAMASE PRODUCING PATHOGENS

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ABSTRACT

Urinary Tract Infection (UTI) is mainly a widespread disease among all age groups encountered in therapeutic practice today. Urinary tract pathogens have evolved frequent defence mechanisms against different antimicrobial agents. For this reason resistance to old and recently produced drugs is on the climb. The aim of the study is to evaluate the antibacterial efficacy of *Sida acuta* leaf extract against Extended Spectrum Beta Lactamase (ESBL) producing *E. coli*. Aqueous and acetone crude of *Sida acuta* was tested for antibacterial activity by disc diffusion method against ESBL producing *E. coli*. It was concluded that *Sida acuta* aqeous and acetone extracts has antibacterial activity against ESBL producing *E. coli* from UTI. Qualitative phytochemical analysis demonstrated the presence of alkaloids, flavonoids and other secondary metabolites which act as the enamours activity against the ESBL producing *E.coli.*

Keywords:

UTI, ESBL, Sida acuta, alkaloids.

1. INTRODUCTION

Now a day's we are major witnesses to an era where globalization and advancement in science is going on side by side with population increases. Contagious diseases are one of the most important causes of morbidity and mortality through worldwide, especially in developing countries (Zeigler, 2005; Yala *et al., 2001).* Urinary tract infection is defined as the large number of active microorganisms inside the urinary canal which are risky to their environment.

E. coli, Klebsiella, Streptococcus pyogenes, S. faecalis, Pseudomonas and *Proteus vulgaris* are the major causative agent for urinary tract infection (Singh *et al.,* 2012*).* Since the innovation of the different antibiotics and their uses as chemotherapeutic agents there was a confidence in the medical society that this would lead to the eventual eradication of infectious diseases. However, increase the usage of antibiotics has become the major issue for the emergence and spreading of multi-drug resistant strains of numerous groups of microorganisms. A microbe was extraordinarily adapted and miraculously versatile. Through the path of evolution, they have created the complicated mechanisms for preserving, hereditary information and disseminating it efficiently in the awareness of their survival (Greenwood, *1998).* Predominantly, β-lactam antibiotics were well-known to be equally active against most of the bacteria. But, the range of β-lactam antibiotics - the entire penicillin derivatives, and four generations of cephalosporins and monobactams, like azetreonam, have become notably hopeless when the resistance is afforded due to the creation of extended spectrum β-lactamase (ESBL) that attack the β-lactam ring with the ultimate antibiotic inactivation (Song *et al.,* 2009).

A strain of *E. coli is* the notable enteric pathogen producing CTX-M type cells (resistant to cefotaxime) with the ESBL action was first documented ever since 2 decades (Dropa *et al., 2009).* Consequently, *Klebsiella pneumoniae* also reported to produce β-lactamase. Thus, the interference with ESBL producing pathogen has become a common threat for today. For this reason, researchers are more and more turning their notice to herbal products for develop the better drugs against MDR strains *(*Braga *et al., 2005).*

Plants are rich in a extensive variety of secondary metabolic products such as tannins, alkaloids, phenol,terpenoids and flavonoids, which have been found in vitro to have antimicrobial properties *(* Lewis *et al., 2006).* Many plants metabolites have been found to cure urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections (Brantner *et al., 1994 and* Somchit *et al., 2003).* These evidences contribute to support and quantify the importance of screening natural products. *Sida acuta* (Malvaceae), is an rigid persistent plant found throughout the hotter parts of India. It is used for different therapeutic purposes such as liver disorders and diuretic in Ayurvedic preparations, asthma, fever, migraine, cough, cold, ulcer, antihelmintic and antifertility agents. The aim of the present study was to investigate the antibacterial and phytochemical activity of *Sida acuta* leaf extracts against ESBL producing strains isolated from urinary tract infections.

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL – SELECTION AND COLLECTION

A *Sida acuta* leaf was collected from Srirangam, Tiruchirapalli, and Tamilnadu. Collected leaves were dried in shade and ground into fine powder, stored in a closed container for further use.

2.2. PREPARATION OF AQUEOUS EXTRACT

The powdered plant material (150gm) was mixed with water and extracted completely. The leaves powder was mixed with sterile water and kept for 72 hours and filtered with a muslin cloth and it was condensed in hot air oven at 50˚C. The aqueous extracts were stored in a sterile container and refrigerated for future use (Jonathan, 2009).

2.3. PREPARATION OF ACETONE EXTRACT

Acetone extract was collected by making use of soxhlet extraction. It was performed by placing 50gm plant material with 1:1 ethanol and methanol. Extraction was performed at 90˚C for 12 hours. The extracts were filtered under the vacuum through Whatman filter paper (No. 1) under gravity. Extract was dried under vaccum evaporator for removing the solvent. The remaining residues were stored in refrigerator till further use (Shi *et al.,* 2005).

2.4. SAMPLE COLLECTION

Urine sample were collected in different patients from Government Hospital**,** Srirangam. About 50 samples were collected for a period of one month and were subjected to microbiological investigation. All samples were smeared on clean microscopic slide and were stained by using Gram's staining technique. Stained smears were observed under oil immersion microscope and also all the sample were subjected to isolation and identification of the ESBL producing *E.coli,Pseudomonas,Staphylococuus aureus* and *Streptococcus* spp (Koneman *et al.,* 1994).

2.5. CONFIRMATION OF CLINICAL ISOLATES (KONEMAN *ET AL.,* 1994)

Selected colonies from selective and differential media were subjected to macros copy, microscopy and biochemical tests for identification.

2.6. ASSESSMENT OF ANTIBIOTIC SENSITIVITY PATTERN

All isolates were subjected into antibiotic sensitivity test according to Bauer *et al.,* 1966. The susceptibility of isolates was examined by a disc diffusion assay.

2.7. DETERMINATION OF ANTIBACTERIAL ACTIVITY

Disc diffusion method was followed (Bauer *et al.,* 1966) to determine the anti bacterial activity. Petri plates containing 20 ml of Mueller Hinton agar were seeded with 4 hours old fresh culture of clinical isolates and referral strains. By making use of template drawn discs were

dispensed on the solidified Mueller Hinton agar with test organisms. This was incubated at 37°C for 24 hours in an incubator (Rands SBC). The test was performed in triplicates. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - Media). The resistance patterns were interpretated as per CDC recommendations.

2.8 ESBL DETECTION

2.8.1. SCREENING FOR ESBL PRODUCERS - DOUBLE DISC SYNERGY ASSAY (CLSI, 2006)

The DDST was performed as a standard disc diffusion assay on Muller Hinton Agar (MHA). Discs containing 30µg aztreonam and 30µg of ceftazidime, ceftriaxone and cefotaxime each were placed 30mm apart (centre to centre) around a disc containing amoxicillin plus clavulanic acid (augmentin 20µg + 10µg). The MHA plate was incubated at 37ºC for 24 hrs. Enhancement of inhibition zone of any one of the test antibiotics towards augmentin disc was regarded as presumptive ESBL production and subjected to phenotypic confirmatory test. If the screening test was negative it was repeated placing the discs 20mm apart.

2.9. ANTIBACTERIAL STUDY OF PLANT EXTRACT

2.9.1. PREPARATION OF DISC WITH PLANT EXTRACTS

Known quantity of extracts of both aqueous and acetones were dissolved in DMSO: Methanol of 1:1 ratio. This in turn was diluted with equal volume of phosphate buffered saline (PBS pH 7). It was then filter sterilized by making use of sortorious syringe filter of pore size 0.22µm. Sterile discs of 6 mm diameter (Hi-Media) were loaded with 50µg - 250 µg / disc concentration of the extract and were dried. Dried discs were stored in sterile containers till use. Solvent loaded discs were also prepared and used as negative control. Oxytetracycline loaded Hi-Media discs were used as positive control.

2.9.2.DETERMINATION OF ANTIBACTERIAL ACTIVITY

Disc diffusion method was followed (Bauer *et al.,* 1966) to determine the antibacterial activity of the leaves extract of *Sida acuta*. Petriplates containing 20 ml of Mueller-Hinton agar were seeded with 4 hours old fresh culture of clinical isolates and referral strains. By making use of template drawn extracts and fractions loaded discs were dispensed on the solidified Mueller Hinton agar with test organisms. Oxytetracycline antibiotic disc (30µg/disc) obtained from M/s Hi-Media laboratories Ltd, Mumbai was used as positive control for bacteria and solvent loaded discs were used as negative control. The plates were incubated at 37°C for 24 hours in an incubator (Rands SBC). The test was performed in triplicates. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - Media).

2.10. PHYTOCHEMICAL SCREENING

Phytochemical analysis of secondary metabolites Powder material was extract with various solvent in the order of increasing polarity and the extract were subjected to phytochemical screening of secondary metabolites like alkaloids, flavonoids and steroids by standard methods.

3. RESULTS

UTI are most common in developing countries like India, due to the poor hygienic condition. Now day's hospitals are highly accommodated with accidental cases and was over crowded which will lead to unhygienic condition.

TABLE I : Prevalence of UTI isolates

S.No	Number of samples	Number and percentage of isolates			
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***E. coli***	***Streptococcus spp***
1	50(100%)	13(26%)	10(20%)	20(40%)	6(12%)

About 50 Urine samples was collected from Government Hospital, Srirangam and samples were categorized based on the presence of microbes and was presented in Table. No.I. The microorganisms were isolated from the urine samples as per the methodology and isolated *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus spp. E.coli* (40%) is the predominant pathogen is present in compare to the others pathogen.(Table.No.I.). Table.No.II. represents various biochemical and microscopic features of UTI isolates.

Antibiotic sensitivity pattern of the isolates are performed by using disc diffusion method. Maximum number of microorganisms showed resistance against multiple numbers of antibiotics which was presented in Table.No.III. Table No.IV showed the double disc diffusion synergy test (DDST) is the most widely used test due to its simplicity and ease with which the results can be interpreted. It is reliable method of ESBL detection. The results shows the *E.coli* is the major pathogen to express the high ESBL activity when compare to the others.

TABLE .II. Isolation and identification of pathogens

S.No	Test	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Esherichia coli*	*Streptococcus pyogens*
1	Gram staining	Gram positive	Gram negative	Gram negative	Gram positive
2	Motility	Non motile	Motile	Motile	Non motile
3	Indole	Negative	Negative	Positive	Negative
4	Methyl red	Positive	Negative	Positive	Negative
5	Voges proskauer	Negative	Negative	Negative	Negative
6	Citrate utilization	Positive	Positive	Negative	Negative
7	Triple Sugar Iron agar test	Not performed	K/K	A/A	Not performed
	Gas production	Not performed	Negative	Positive	Not performed
	H_2S production	Not performed	Negative	Negative	Not performed
8	Catalase	Positive	Negative	Positive	Positive
9	Oxidase	Positive	Positive	Negative	Negative
10	Phenyl alanine deaminase	Not performed	Negative	Not performed	Not performed
11	Esculin hydrolysis	Positive	Negative	Negative	negative
12	Growth on				
	Blood agar	Beta hemolysis	Not performed	Not performed	Beta hemolysis
	MacConkey agar	NLF colonies	NLF colonies	LF colonies	Not performed
	Baired parker agar	Black colour colonies	Not performed	Not performed	Not performed
	Cetrimide agar	Not performed	Greenish colour colonies	Not performed	Not performed
	Mannitol salt agar	Yellow colour colonies	Not performed	Not performed	Not performed
	EMB agar	Not performed	Not performed	Metallic sheath colonies	Not performed

TABLE III: ANTIBIOTIC SENSITIVITY ASSAY

PERCENTAGE OF RESISTANCE					
S.NO	**ANTIBIOTICS**	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***E.coli***	***Streptococcus pyogens***
1	AZITHROMYCIN	S	S	S	S
2	AZTREONAM	R	R	R	R
3	CHLORAMPHENICOL	R	R	S	R
4	CEFUROXIME	R	R	R	R
5	DOXYCYCLIN	R	R	R	R
6	FUCONAZOLE	R	R	R	R
7	CLOTRIMAZOLE	R	R	R	R
8	COTRIMAXAZOLE	R	R	R	R
9	CLINDAMYCIN	R	R	R	R
10	MIMOCYCLIN	R	R	R	R
11	VANCOMYCIN	S	S	R	S
12	KANAMYCIN	R	S	R	S
13	CLARITHROMYCIN	R	R	R	R
14	TRIMETHOPRIM	R	R	R	R
15	GENTAMYCIN	R	R	R	R
16	SPECTINOMYCIN	R	S	R	R
17	AMOXYCILLIN	S	R	R	R
18	ERYTHROMYCIN	R	R	R	R
19	AUGMENTIN	R	R	R	R

TABLE IV: ESBL ACTIVITY ASSAY

S.NO	**ANTIBIOTIC**	**ZONE in mm**			
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***E.coli***	***Streptococcus pyogens***
1	Aztreonam	-	-	-	-
2	ceftriaxone	7	8	-	9
3	cefotaxime	-	-	-	-
4	amoxicillin + clavulanic acid	-	18	25	12

TABLE: V. ANTIBACTRIAL ACTIVITY ON SIDA ACUTA WATER EXTRACT

S.NO	Pathogens	Concentration in µg				
		50	100	200	400	600
1	*E.coli*	10 mm	11 mm	13 mm	14 mm	15 mm
2	*Streptococcus pyogens*	9mm	11 mm	12 mm	13 mm	14 mm
3	*Staphylococcus aureus*	8 mm	10 mm	11 mm	14 mm	14 mm
4	*Pseudomonas aeruginosa*	10 mm	12 mm	13 mm	14 mm	15 mm

TABLE: VI. ANTIBACTRIAL ACTIVITY ON SIDA ACUTA ACETONE EXTRACT

S.NO	Pathogens	Concentration in µg				
		50	100	200	400	600
1	*E.coli*	10 mm	12mm	13 mm	15 mm	16 mm
2	*Streptococcus pyogens*	11 mm	12 mm	14 mm	15 mm	16 mm
3	*Staphylococcus aureus*	11 mm	12 mm	13 mm	14 mm	15 mm
4	*Pseudomonas aeruginosa*	9 mm	11 mm	13mm	15 mm	16 mm

TABLE .VII. PHYTOCHEMICAL ANALYSIS SIDA ACUTA LEAF EXTRACTS

S.NO	Test	Water extract	Acetone extract
1	Alkaloids	Positive	Positive
2	Steroids	Positive	Positive
3	Terpenoids	Positive	Positive
4	Flavonoids	Negative	Negative
5	Tannins	Positive	Positive
6	Flavones	Positive	Positive
7	Cardiac glycoside	Positive	Positive
8	Proteins	Positive	Positive
9	Carbohydrate	Negative	Negative
10	Ninhydrin	Positive	Positive
11	Lignin	Positive	Positive
12	Inulin	Negative	Negative
13	Phenol	Positive	Positive

Active principles of medicinal plants were extracted by aqueous, acetone extraction. Both the extracts confer the antibacterial activity against all the ESBL producing *E.coli, Staphylococcus aureus, Streptococcous pyogens, and Pseudomonas aeruginosa*. The results were tabulated in Table. V and VI. Table VII showed the phytochemical analysis of acetone and water extracts the presence of phenols, alkaloids, flavanoids, steroids, terpanoids ,tannins,and amino acids.

4. DISCUSSION

Urinary tract infection (UTI) is one of the major widespread infections standing next to upper respiratory infection with an rising conflict to antimicrobial agents. These sicknesses affect patients in all age of groups and sexes. Majority of UTIs are not life aggressive and do not cause any permanent damage. Multiple antimicrobial resistances among gram negative and gram positive organism have been a long term and well documented trouble with urinary tract infection. The possible pathogens to cause the UTI has been observed in table I & II. It shows the *E.coli, Staphylococcus aureus*, *Streptococcus* and *Pseudomonas spp* are the major pathogen to cause the UT infection. *Escherichia coli* have been documented as the most important pathogen related with urinary tract infection in a lot of countries. *E.coli* in generally be alive in gastrointestinal tract, but they enter into the urinary tract in unfavorable condition. The frequency of UTI is larger in women than men who may be either due to anatomical inclination or urothelial mucosal adherence to the muco polysaccharide coating or other factor.

The ESBL producing geneus has been detected in members of Enterobacteriaceae, are increasingly causing UTI (Virender Singh *et al.*, 2012). Our results also similar to the above statements (Table.4). β-lactamase enzymes was frequently divided into four groups like A, B, C and D based on sequence homology. A, C and D group enzymes was using at the active site of serine for hydrolyzation of β-lactam antibiotics (Matagne *et al.*,1998), while B group enzymes utilizes zinc ions to catalyze the hydrolysis of the β-lactam elements (Livermore *et al.,* 2000). Indeed, β-lactam coding genes are transferred by extra chromosomal DNA like plasmids and transposons to same or unrelated species of bacteria. Although the extent to which *E.coli* developed the resistance to antimicrobial drugs and the movement with which they do so change with different types of drugs, so far drug resistance has developed to all synthetic antimicrobial drugs. Herbal medicines are in great claim in the urbanized world for prime health care. *Sida acuta* is an herb available throughout the India as weed lacking any proper cultivation. Traditional people uses the plants for leaving supprative ulcer, the leaves and roots are used in indigenous medicines as mustard. The juice of the leaves is boiled in oil and applied to testicular swelling and in elephantiasis. A decoction of leaves

and roots is credited with emollient and tonic properties and is used in the cure of haemorrhoids and impotence. Leaf juice is given for release in chest pain. In the present study *Sida acuta* water and acetone extract active against ESBL producing microorganism. Both the extracts was gave the high antimicrobial activity. ESBL producing pathogens was the serious problem in developed countries but it will arrested by the *Sida acuta* leaf extracts. *Sida acuta* has presence of phenols, alkaloids, flavonoids, steroids, terpanoids, tannins and amino acids in both the extracts. The secondary metabolites of the plants have given the major activity against the ESBL producing UTI pathogens.

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