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Evaluating the Antibacterial Activity of *Elephantopus scaber* Extracts on Clinical Isolates of β -lactamase Producing Methicillin Resistant *Staphylococcus aureus* from UTI Patients

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Abstract: Methicillin Resistant *Staphylococcus Aureus* (MRSA) has gained much attention in the last decade, as the MRSA is a major cause of hospital acquired (nosocomial infections). β -lactam antibiotics are the preferred drugs against *S. aureus* infections, although *S. aureus* has developed resistance to the β -lactam antibiotics due to the production of chromosomal or plasmid mediated β -lactamases or by producing Penicillin Binding Proteins (PBPs). The Extended Spectrum β -Lactamase (ESBL) producers are highly resistant to several conventional antibiotics. This limits therapeutic options. Hence efforts are now taken to screen few medicinal plants, which are both economic and less toxic, against the ESBL producers. Among the several plants screened, we have chosen to screen the alcohol extracts of a traditional medicinal plant, *Elephantopus scaber* (Asteraceae) against several clinical strains of ESBL producing MRSA. ESBL producers were screened by double disc synergy test. Methanol, hexane and acetone extracts of *Elephantopus scaber* were investigated for their ability to inhibit the growth of the chosen ESBL producing multidrug-resistant bacteria by the disc diffusion method. Minimal Inhibitory Concentrations (MICs) were determined by micro broth dilution method. Synergistic interaction of plant extracts with certain antibiotics was also evaluated. On the basis of promising activity, acetone extracts were fractionated and their phytochemical analysis showed the presence of terpenoids, proteins and traces of steroids. TLC bioautography of the fraction showed the active compound to be terpenoids. The strong *in vitro* antibacterial activity of terpenoid derivatives against ES β L-producing MRSA bacteria suggests the compounds might find wide pharmaceutical use. Further investigations to elucidate the active compound are required.

Key words: *Elephantopus scaber*, ESBL, MRSA, MSSA

INTRODUCTION

Infectious diseases, the leading cause of premature deaths, in the world are killing most 50,000 people every day. An increase in antibiotic resistant bacteria is threatening world population with the recurrence of infectious diseases (e.g., Tuberculosis and cholera) that were once thought to be under control at least in developed countries. In the recent years incidence of multi-drug resistance in Gram positive (*S. aureus*, *S. pneumoniae*), Gram negative (*E. coli*, *Shigella*, *Haemophilus influenzae*) and other bacteria like *Mycobacterium tuberculosis* has been reported from all over the world (Mulligen *et al.*, 1993; Sajduda *et al.*, 1998; Sanches *et al.*, 1998). These multi-drug resistant bacteria have also created additional problems in cancer and AIDS patients. Methicillin Resistant *Staphylococcus aureus* (MRSA) has gained much attention in the last decade, as the MRSA is a major cause of hospital acquired

(nosocomial) infections. β -lactam antibiotics are the preferred drugs against *S. aureus* infections, although, *S. aureus* has developed resistance to the β -lactam antibiotics due to the production of chromosomal or plasmid mediated β -lactamases or by producing Penicillin Binding Proteins (PBPs). All the *S. aureus* strains have four PBPs (PBP1 to PBP4), but MRSA express a special PBP (PBP2 or PBP2a) from the *mecA* gene. PBP2a takes over the biosynthetic function of normal PBPs in the presence of inhibitory concentration of β -lactams because PBP2 has a decreased binding affinity to β -lactams (Bachi and Rohrer, 2002). This has resulted in the development of multidrug resistance against β -lactam and other antibiotics. Moreover, increased incidence of vancomycin-resistant MRSA has also been reported (Hiramatsu *et al.*, 1997). Thus, the number of effective exogenous antibiotics is decreasing, therefore concerted efforts are to be made to identify antimicrobial materials from natural products and traditional medicines.

Antimicrobial activity of medicinal plants against drug-resistant bacteria, including MRSA and their interaction with β -lactam antibiotics/Beta-lactamase activity on mec gene product have been reported in one or the other plants, including *Camellia sinensis*, *Rosa canina*, *Scutellaria amoena* and *Arctostaphylos uvaursi*, from different parts of the world (Yam *et al.*, 1998; Shioto *et al.*, 2000; Liu *et al.*, 2000; Shimuzu *et al.*, 2001; Palombo and Semple, 2002). Such activities mainly in traditionally used Indian medicinal plants are less explored. India is fortunate in possessing the world's richest flora with about 120 families of plants, comprising of 1,30,000 species. The use of about 2400 of these plants have been mentioned in Ayurvedic and Unani texts and tribals use many others. Various preparations of these medicinal plants are used in treating ailments such as wounds skin diseases, dysentery, cough, cold, jaundice and leprosy etc (Chaudhary, 1996).

Screening of Indian medicinal plants revealed varying degree of antimicrobial activity against various pathogenic and opportunistic microorganisms (Ahmad *et al.*, 1998; Mehmood *et al.*, 1999; Ahmad and Beg, 2001). It is therefore, expected that such bioactive plant extracts may provide some alternative compounds to be used against MRSA with or without the combination of β -lactam antibiotics. In search of such an activity, crude alcoholic extracts and fractions of some Indian medicinal plants were evaluated for their antibacterial activity against β -lactamase producing-methicillin resistant *S. aureus* isolates and an isolate of methicillin sensitive *S. aureus* (MSSA). Preliminary data on the interaction between active plant extracts and certain antibiotics has also been generated.

MATERIALS AND METHODS

Antibiotic resistance profile of test strains: Urine samples of UTI infected patients from CSI Mission General hospital, Tiruchirappalli, India were processed. Ninety six strains of MRSA and a 127 strains of methicillin sensitive *S. aureus* (MSSA) were obtained during a period of 6 months (June 2005-December 2005). These strains were tested for their antibiotic sensitivity by disk diffusion method (Bauer *et al.*, 1966). Antibiotic discs/powders were purchased from Hi-Media Lab. Ltd, Mumbai (India).

Plant material: *Elephantopus scaber* Linn. is a small herb, which grows in the wild throughout the tropical regions of the world. The major phytochemical constituents of the plant are elephantopin, triterpenes, stigmaterol, epofriedelinol and lupeol (Rastogi and Mehrotra, 1990; Kritikar and Basu, 1991). The plant has

been used in the Indian system of medicine as analgesic, diuretic, astringent and antiemetic. The leaves of the plant were known to be used for bronchitis, small pox and diarrhea and as a brain tonic (Sankar *et al.*, 2001). Recently, it has been shown to possess anti-inflammatory and antitumour activity in animal models (Reico, 1989) and also found to have antibacterial activity against a few standard bacterial strains.

Plant extract preparation: The plant used in this study, *Elephantopus scaber* was obtained commercially and identified and voucher specimen was deposited at the Botany department of the College. The plants were shade dried and powdered. Air-dried powder (1 kg) was extracted with 2 L of acetone in a Soxhlet apparatus for 18 h. After filtration of the extract, it was evaporated at 30°C until dryness. The obtained crude extract (56 g L⁻¹) was chromatographed on a silica gel column. Initial elution with discontinuous gradient of ethyl acetate and hexane, then with acetone and ethyl acetate, with acetone and chloroform and finally with chloroform and hexane. This yielded 17 fractions (F1-17). The fractions F₁₋₅, F₆₋₈, F₉₋₁₁, F₁₂₋₁₅ and F₁₆₋₁₇ were combined according to their Rf values into five fractions finally.

Screening for ESBL production: The antibiogram obtained for the isolated bacteria revealed them to be multi-drug resistant clinical isolates. The tested isolates were screened for ESBL production following double disc synergy test (Miles and Amyes, 1996). ESBL presence was assayed using the following antibiotic discs: Cefotaxime (30 μ g), Cefotaxime/clavulanic acid (30/10 μ g), ceftazimide (30 μ g) and ceftazidime/clavulanic acid (30/10 μ g) *E. coli* ATCC 35218 and *E. coli* ATCC 25922 served as positive and negative controls, respectively.

Antibacterial activity: The antibacterial activity of the extract was evaluated by the disc diffusion method (Bauer *et al.*, 1966). Mueller Hinton agar plates were prepared and inoculated on the surface with the test organism whose concentration was adjusted using 0.5 std. McFarland's opacity tube (McFarland, 1907). About 10 μ L of the test extracts (1 g in 10 mL DMSO) were impregnated on sterile discs (Himedia, Mumbai, India) and on drying; the discs were placed on Mueller Hinton plates. After incubation for 24 h at 37°C, positive results were established by the presence of clear zones of inhibition around the active extracts. Also DMSO and solvent only discs were used as controls. The assessment of the antibacterial activity was based on the measurement of diameter of the zone of inhibition formed around the standard antibiotic discs (NCCLS, 2002).

Determination of minimal inhibitory concentration and Minimal bactericidal concentration: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were determined for the extracts by broth dilution method as described by Ayafor *et al.* (1994). The concentration at which there was no visually detectable bacterial growth was taken as the MIC and the concentration at which there was no bacterial growth after inoculation in Mueller Hinton agar was taken as MBC.

Synergistic interaction of plant extracts with antibiotics: Synergistic interaction between antibiotics like ampicillin, tetracycline and chloramphenicol with crude plant extracts was studied by agar well diffusion method. For determining the synergistic effects of plant extracts with antibiotic, the wells were punched at a predetermined distances so that their inhibitory circles touch each other only tangentially without influencing each other as recommended by Chattopadhyay *et al.* (1988). The wells were inoculated with plant extract and antibiotic separately. Plates were then incubated at 37°C, for 18 h. Enlargement of inhibition zones indicates a positive interaction (synergism).

Phytochemical analysis of plant extracts: Major phytochemicals, in the crude extracts of plants, were detected by standard colour tests and thin layer chromatography, as described elsewhere (Wagner and Bladt, 1995; Ahmad and Beg, 2001).

TLC bioautography: For direct bioautographic assay, agar overlay assay (Slusarenko *et al.*, 1989) was used, with little modification, to detect antimicrobial compounds in plant extracts active against MRSA. Five to ten microliter of each plant extract, was spotted on preparative E-MERCK chromatographic Silica gel f 254 thickness 0.25 mm plates of 3×8 cm. Only one solvent system (acetone: ethanol: 1:1) was used. One milliliter of 10⁶ CFU mL⁻¹ broth culture was used for every 10 mL of nutrient agar. Developed chromatograms were placed in autoclave petri plates. Culture was added to the melted nutrient agar medium and a thin layer was poured over the chromatograms. Plates were incubated at 37°C, for 24 h. Zones of inhibition of bacterial growth could be seen around the active chromatogram spot. The spot was also confirmed by flooding the plates with 0.02 mg mL⁻¹ solution of p-iodonitro-tetrazolium violet.

RESULTS

Antibiotic sensitivity of Methicillin Resistant *Staphylococcus aureus* (MRSA) strains showed resistance to multi-drugs, while methicillin sensitive

Table 1: Minimal inhibitory concentrations of the different extracts of *E. scaber* against MRSA and MSSA

Strain	MIC (mg mL ⁻¹)		
	Methanol extract	Acetone extract	Hexane extract
MRSA	50	6.25	25
MSSA	25	3.12	12.5

Table 2: Minimal inhibitory concentrations of the different fractions of the acetone extract of *E. scaber* against MRSA and MSSA

Strain	MIC (µg mL ⁻¹)				
	F1	F2	F3	F4	F5
MRSA	500	1000	250	250	NA
MSSA	250	500	125	250	NA

S. aureus (MSSA) strains were sensitive to all antibiotics tested. The Minimum Inhibitory Concentration (MIC) of β-lactam antibiotics (benzyl penicillin, ampicillin, cloxacillin, cefixime) ranged from 250 to >2000 µg mL⁻¹ against the strains of methicillin resistant *Staphylococcus aureus* while the MIC values of these antibiotics against the MSSA strain ranged from 6.25 to 25 µg mL⁻¹. Production of β-lactamases was a common characteristic for these MRSA and MSSA strains. The extract of the traditionally used Indian medicinal plant was tested against the above mentioned clinical strains of β-lactamase producing MRSA and MSSA. The extract of *Elephantopus scaber* showed little activity against MRSA strains, compared to the MSSA strains. The antibacterial potency of the crude plant extracts was determined in term of Minimum Inhibitory Concentration (MIC) against *S. aureus* strains. MIC of the plant extracts ranged from 3.12 to 50 mg mL⁻¹ against the MRSA strains (Table 1). On the basis of the promising activity, the acetone extract of *Elephantopus scaber* were fractioned in a silica gel column. Antimicrobial activity was located in 4 acetone fractions among the nine obtained. Phytochemical analysis showed the presence of alkaloids, phenols, glycosides, tannins, saponins, steroids and terpenoids (Table 2). TLC-bioautography of the plant extracts revealed terpenoids as the major bioactive phytoconstituents.

DISCUSSION

The choice of drugs, to be used against MRSA, is shrinking day by day as susceptibility of MRSA to drugs is decreasing by target site alteration, enzyme modification and permeability changes (Brumfitt and Hamilton-Miller, 1989). Different substrate specificity/spectrum has been reported in various type of MRSA strains, like β-lactamase positive and PBP2, inducible β-lactamase negative and PBP2 constitutive, chromosomal and plasmid-encoded β-lactamases, production of β-lactamases may also directly influence the resistance level in these strains since these strains are resistant to methicillin where PBP2 is expected to play

major role in the development of resistance against not only with β -lactam antibiotics but also to unrelated antibiotics. The β -lactamase produced by MRSA also hydrolyzed methicillin. These strains were resistant to β -lactam antibiotics with high MIC values, including methicillin ($>100 \mu\text{g mL}^{-1}$). Therefore, it is expected that this clinical strain might belong to MRSA group and not to BORSA/MODSA group which confers low level of resistance against methicillin. To confirm the presence of *mecA* gene in this strain, further characterization is needed. In the present study, the zones of inhibition was recorded in the range of 11 mm to 27 mm at $100 \mu\text{g mL}^{-1}$ concentration. The antibacterial potency of the fractions of the acetone extract was determined in terms of MIC, which ranged from $125\text{-}1000 \mu\text{g mL}^{-1}$. The behavior of *S. aureus* strains, against individual plant extract differed significantly, which requires further investigations in terms of their active constituents and its effect on the MRSA strains. Fractionations of the plant extracts revealed that the acetone fractions showed good activity while less activity was found in the methanol and hexane fractions. Our findings are in agreement with the reports on other plants (Yam *et al.*, 1998; Palombo and Semple, 2002). However, this is probably the first report on the chosen Indian medicinal plants against β -lactamase producing MRSA and MSSA strains from Tamil Nadu, South India. The interaction of the plant extracts with tetracycline showed synergistic interaction, while the extract exhibited less synergism with Tc as well as with β -lactam antibiotic (ampicillin). The possible mechanism for such an interaction might be exploited in combination chemotherapy but requires in-depth understanding. Present study is in agreement with few others (Iinuma *et al.*, 1996; Lee *et al.*, 1998) who have also demonstrated positive interaction of certain plant extracts with antibiotics.

Phytochemical analysis by color test and TLC bioautography of certain extracts revealed the presence of terpenoids, phenols, steroids, glycosides, saponins as major phytoconstituents. To identify the exact chemical nature of active compounds in these plant extracts detailed phytochemical investigations are needed. Therefore, activity based fractionations of these extracts and determination of active principle and its mechanism of interaction with antibiotics have to be explored in order to uncover the real therapeutic potential of these extracts in the management of infectious diseases caused by MRSA strains and probably by other multidrug resistant bacteria.

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