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Role of Terpenoids From *Elephantopus scaber* Against a Few Extended Spectrum β-Lactamase Producers

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Abstract: The extended spectrum β-lactamase producers are highly resistant to several conventional antibiotics. Hence efforts are now taken to screen few medicinal plants 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 ESBL producers. 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 extended spectrum β-lactamases (ESBL) producing multidrug-resistant enteric bacteria by the disc diffusion method. MICs were determined by micro broth dilution method. The crude plant extracts demonstrated zones of inhibition in the range of 5-16 mm against the chosen test bacteria. On the basis of promising activity, acetone extracts were selected to determine their efficacy in terms of Minimal Inhibitory Concentration (MIC), which ranged from 1.6-25 mg mL^{-1}. The acetone extract was subjected to activity-guided fractionation. The most effective fraction had a MIC of 62.5-250 μg mL^{-1}. 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 fraction was further tested for their *in vivo* cytotoxic activity to mammalian system using rats. No marked manifestations were observed. Normal liver and kidney functioning were also observed. The strong *in vitro* antibacterial activity of terpenoid derivatives against ESβL-producing Gram-negative bacteria suggests the compounds might find wide pharmaceutical use.

Key words: Minimum inhibitory concentration, Thin layer chromatography, Terpenoids, Extended spectrum β-lactamase

INTRODUCTION

Emerging resistant bacterial strains are a threat to the community. Drug resistance is one of the most serious global threats to the treatment of infectious diseases (Picazo *et al*., 2006; Kiffer *et al*., 2007). In addition to resulting in significant increases in costs and toxicity of newer drugs, antibiotic resistance is eroding our therapeutic armamentarium. Resistant strains of bacteria are continuing to increase, both in number and in variety, but not significantly different newer antibiotics are yet available. Treatment of infections caused by these resistant bacteria has become very difficult. Since they are resistant to many antibiotics, therapeutic options have become limited. Therefore, alternative methods of treatment are sought after. Despite the significant progress made in microbiology and the control of microorganisms, sporadic incidents of epidemics due to drug resistant microorganisms and hitherto unknown disease-causing microbes pose an enormous threat to public health. These negative health trends call for a global initiative for the development of new strategies for the prevention and

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treatment of infectious disease. For over several years medicinal plants have served as the models for many clinically proven drugs and are now being re-assessed as antimicrobial agents. The reasons for this renaissance include a reduction in the new antibacterial drugs in the pharmaceutical pipeline, an increase in antimicrobial resistance (Mohammed Akram, 2007) and the need of treatments for new emerging pathogens. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient. These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. Among the several drug resistant bacteria, β-lactamase production is the most important mechanism of resistance to penicillin and cephalosporins. The mechanism of this resistance was the production of extended spectrum β-lactamases (ESBLs) (Ayyagari and Bhargava, 2001). The most frequent co-resistances found among ESBL producing organisms are amino glycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethorprim (Nathisuwan *et al*., 2001). Major outbreaks involving ESBL strains have been reported from all over the worlds, thus making them emerging pathogens (Ananthakrishnan *et al*., 2000). Incidence of these organisms is being continuously increasing throughout the world with very limited treatment alternatives (Chaudhary and Aggarwal, 2004). Hence it becomes necessary to find alternative methods of treatment. Hence herbal drugs have gained significance now. Treatment of infections caused by these resistant bacteria has become very difficult, since they are resistant to many antibiotics. This limits therapeutic options (Supriya *et al*., 2004). Therefore, alternative methods of treatment are sought after. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections (Cos *et al*., 2006). Among the several plants screened, *Elephantopus scaber*, a member of the family Asteraceae known for its medicinal properties was also reported to possess antimicrobial activity (Avani and Neeta, 2005). Yet, there were no reports on the role of neither the plant nor its compound against the drug-resistant human pathogens. Hence this study was undertaken.

MATERIALS AND METHODS

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, stigmasterol, 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 (Avani and Neeta, 2005).

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 methanol in a Soxhlet apparatus for 18 h. Similar procedure was followed for extraction of acetone and hexane extracts. After filtration of the extract, it was evaporated at 30°C until dryness. The yields of the methanol, acetone and hexane extracts were 14.3, 12.1 and 10.9 g%, respectively.

Fractionation of the Promising Extract

Based on the results obtained, the best extract among the three was chosen for fractionation. The acetone extract chosen by bioactivity was subject to fractionation on a silica gel column. Initial elution with discontinuous gradient of ethyl acetate and hexane, was followed then with acetone and ethyl acetate, with acetone and chloroform and finally with chloroform and hexane. This yielded 17 fractions (F1-17). The fractions F_{1-5}, F_{6-8}, F_{9-11}, F_{12-15} and F_{16-17} were combined according to their Rf values into five fractions finally.

Screening for ESBL Production

Bacterial isolates were obtained from patients infected with urinary tract infection attending CSI Mission General hospital, Tiruchirappalli for a period of 1 year (June 2004-June 2005). 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 μg), Cefotaxime /clavulanic acid (30/10 μg), ceftazimide (30 μg) and ceftazidime/clavulanic acid (30/10 μg). *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 μL 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.

Phytochemical Screening

The most bioactive fraction obtained from the acetone extract of *E. scaber* was selected for preliminary phytochemical screening. Test for alkaloids, steroids, flavonoids, terpenoids and proteins were carried out according to the standard methods (Harborne, 1973).

RESULTS

Antibiotic sensitivity of ESBL-producing strains of Gram-negative bacteria of laboratory and clinical origin showed resistance to multidrug β-lactam antibiotics. The MIC of penicillin, ampicillin, cefuroxime and cefotaxime ranged from 125-1024 μg mL^{-1}. Only few species like, *Aeromonas hydrophila* and *Citrobacter freundi* showed low resistance to ampicillin (MIC, 32 μg mL^{-1}), but produced β-lactamase, hydrolyzing ampicillin. All the test strains could hydrolyse the five common substrates, penicillin, ampicillin, cefotaxime, ceftazidime and cefuroxime, confirming their ESBL production as detected by double disc synergy test. The fractions of the acetone extract of *E. scaber* exhibited broad-spectrum activity against all the ESBL-producing multidrug-resistant strains tested. The antibiogram of the isolates selected for the study are shown in Table 1.

Measured zones of inhibition displayed in the Table 2 shows that the three extracts of ES demonstrate inhibitory activity against all the bacterial isolates under study. It is seen that the ES extract had no varied response to Gram reaction, hence could control both the Gram positive and negative almost equally. No inhibitory zones were produced by the suspending solvent, Dimethyl sulphoxide (DMSO) and the solvent only discs, indicating non-involvement of the suspending solvents in the inhibitory role. Among the activity of the three extracts, the acetone extract exhibited the maximum activity.

Effect of the Extracts on Esbl-Producing Bacteria

As shown in the data, the acetone extract was more effective against ESBL-producing *Acinetobacter baumannii*, producing a zone of 12 mm, than the other two extracts, methanol and hexane

Table 1: Antibiogram pattern of the screened EsβL-producers

ESBL Producing organisms (n = 236)	A	Ak	A-C	Cu	G	Nx	Ce	Ca	I*
Escherichia coli (N = 100)	(96)	(21)	(72)	(83)	(73)	(89)	(74)	(78)	S
	96%	21%	72%	83%	73%	89%	74%	78%	
Klebsiella pneumoniae (n = 36)	(34)	(23)	(28)	(26)	(22)	(28)	(29)	(30)	--
	93%	65%	78%	73%	62%	78%	80%	84%	S
Pseudomonas aeruginosa (n = 10)	(10)	(6)	(8)	(10)	(6)	(8)	(9)	(6)	--
	100%	62%	80%	100%	60%	80%	90%	60%	S
Citrobacter freundii (n = 8)	(8)	(4)	(5)	(8)	(4)	(6)	(5)	(6)	--
	100%	50%	60%	100%	50%	80%	60%	70%	S
Acinetobacter banumanii (n = 18)	(18)	(3)	(13)	(9)	(2)	(3)	(3)	(5)	--
	100%	20%	65%	50%	10%	20%	20%	30%	S
Aeromonas hydrophila (n = 20)	(20)	--	(20)	(12)	(20)	(20)	(20)	(20)	--
	100%	S	100%	60%	100%	100%	100%	100%	S
Enterobacter aerogenes (n = 14)	(13)	(4)	(6)	(7)	(4)	(6)	(3)	(5)	--
	90%	30%	40%	50%	30%	45%	20%	35%	S
Proteus mirabilis (n = 4)	(4)	(3)	(3)	(3)	(3)	(3)	(2)	(2)	--
	100%	80%	90%	80%	90%	80%	50%	50%	S
Morganella morganii (n = 10)	(9)	(1)	(2)	(6)	(1)	(2)	(6)	(7)	--
	90%	10%	20%	60%	10%	20%	60%	70%	S

A: Ampicillin (10 mcg); Ak: Amikacin (30 mcg); A-C: Amoxy-clavulanicacid; Cu: Cefuroxime (30 mcg); G: Gentamycin (10 mcg); Nx: Norfloxacin (10 mcg); Ce: Cephephotaxime (30 mcg); Ca:Ceftazimide (30 mcg);*I: Imipenem (10 mcg)

Table 2: Inhibitory effects of the different extracts of *Elephantopus scaber* on ESBL-producing urinary pathogens

Bacteria	ESM[a]	ESA[b]	ESH[c]
Aeromonas hydrophila	12	11	9
Acinetobacter baumannii	6	12	10
Citrobacter freundii	5	11	6
Escherichia coli	6	8	6
Enterobacter aerogenes	7	11	7
Staphylococcus aureus	7	11	8
Klebsiella pneumoniae	6	11	6
Pseudomonas aeruginosa	9	6	9
Proteus mirabilis	9	13	9

(ZOI of 5 and 9 mm), respectively. β-lactamase producing *Citrobacter freundii* was moderately inhibited by the acetone extract, producing a zone of 11 mm as against the 5 and 6 mm by the methanol and hexane extracts respectively. Similar trend was seen in β-lactamase producing *Escherichia coli* and *Enterobacter aerogenes* and *Proteus mirabilis*, where the acetone extract displayed a better activity than the other two extracts. Unexpectedly, the acetone extract did not demonstrate significant activity against *Pseudomonas aeruginosa*. However the other two extracts exhibited moderate activity against this drug-resistant bacterium.

Effect of the Extracts on non Esbl-Producing Standard (ATCC) Stains

Table 3 depicts the continuing trend of the pronounced antibacterial activity of the acetone extract against the three standard strains as mentioned earlier. Though the methanol and hexane extracts also produced zones, they only had a moderate activity compared to the acetone extract.

Minimal Inhibitory Concentration (MIC) of the Extracts by Broth Dilution Method

However, in *Elephantopus scaber*, where the acetone extract as stated previously in the disc diffusion assay, demonstrated a significant result compared to the other extracts. The given data (Table 4 and 5) demonstrated the range of MIC of the acetone extract to be between 3.12 and 12.5 mg mL^{-1}, whereas the MIC of the other two extracts ranged between 3.12 and 50 mg mL^{-1}.

Antibacterial Activity by the Fractions

Based on the zones of inhibition and the MIC values, the acetone extract of *Elephantopus scaber* whole plant was chosen for fractionation in a silica gel column. The five fractions obtained in both

Table 3: Effect of the extracts of *Elephantopus scaber* on a few standard strains

Bacteria	ESM[a]	ESA[b]	ESH[c]
Escherichia coli ATCC	9	12	9
P. aeruginosa ATCC	12	6	8
Staphylococcus aureus ATCC	7	11	8

[a]: ESM-*Elephantopus scaber* methanol extract (100 mg mL^{-1}); [b]: ESA-*Elephantopus scaber* acetone extract (100 mg mL^{-1}); [c]: ESH-*Elephantopus scaber* hexane extract (100 mg mL^{-1})

Table 4: Minimal inhibitory concentrations of the different extracts of *Elephantopus scaber* on ESBL-producing urinary pathogens

	MIC (mg mL^{-1})		
Bacteria	ESM[a]	ESA[b]	ESH[c]
Aeromonas hydrophila	12.5	3.12	25.00
Acinetobacter baumannii	25.0	6.25	25.00
Citrobacter freundii	12.5	3.12	50.00
Escherichia coli	12.5	25.00	6.25
Enterobacter aerogenes	12.5	6.25	50.00
Staphylococcus aureus	12.5	12.50	50.00
Klebsiella pneumoniae	25.0	12.50	50.00
Pseudomonas aeruginosa	12.5	1.60	6.25
Proteus mirabilis	12.5	1.60	6.25

Table 5: Minimal inhibitory concentrations of the different extracts of *Elephantopus scaber* on a few standard strains

	MIC (mg mL^{-1})		
Bacteria	ESM[a]	ESA[b]	ESH[c]
Escherichia coli ATCC	12.50	1.60	3.12
Pseudomonas aeruginosa ATCC	12.50	1.60	6.25
Staphylococcus aureus ATCC	3.12	6.25	25.00

[a]: ESM-*Elephantopus scaber* methanol extract (100 mg mL^{-1}); [b]: ESA-*Elephantopus scaber* acetone extract (100 mg mL^{-1}); [c]: ESH-*Elephantopus scaber* hexane extract (100 mg mL^{-1})

Table 6: Minimal inhibitory concentrations of the fractions of *Elephantopus scaber* acetone extract (μg mL^{-1}) on a few standard strains on ESBL-producing pathogens

Bacteria	F1	F2	F3	F4	F5
Aeromonas hydrophila	500	1000	125	250.0	0
Acinetobacter baumannii	250	1000	125	500.0	0
Citrobacter freundii	250	500	125	62.5	0
Escherichia coli	250	1000	250	1000.0	0
Enterobacter aerogenes	500	500	250	500.0	0
Staphylococcus aureus	500	1000	125	250.0	0
Klebsiella pneumoniae	250	500	250	500.0	0
Pseudomonas aeruginosa	250	500	250	1000.0	0
Proteus mirabilis	500	500	250	500.0	0

Table 7: Minimal inhibitory concentrations of the fractions of *Elephantopus scaber* acetone extract (μg mL^{-1}) on a few standard strains on a few standard strains

Bacteria	F1	F2	F3	F4	F5
Escherichia coli ATCC	500	500	250	250	0
Psedomonas aeruginosa ATCC	500	1000	250	500	0
Staphylococcus aureus ATCC	500	1000	125	250	0

*Concentration of fractions-5 mg mL^{-1}

extracts were designated as F1-F5. Due to their low quantities, the antibacterial activities of the fractions were determined only by MIC adopting microbroth dilution assay.

Antibacterial Activity of the Fractions of *E. scaber* Acetone Extract

Table 6 and 7 summarises the results of the antibacterial activity of the fractions against the ESBL and standard strains, respectively. Among the five fractions of the acetone extract of *E. scaber*, F5 exhibited no activity. F1, F2 and F4, though demonstrated inhibitory activity against all the bacterial pathogens, yet their activity was not significant, with the MIC values ranging between 250-1000 μg mL^{-1}. Among the fractions, F2 was more potent, whose MIC values oscillated between 125 and 250 μg mL^{-1}. Due to the consistently lower MIC values of F3 than the other fractions, F3 was considered as the best.

The MIC values of the most active fraction, F3 ranged between 62.5-250 μg mL^{-1}. Phytochemical analyses of the crude extracts demonstrated the presence of different phytocomponds, like terpenoids, proteins and steroids in the fractions. The result of the TLC bioautography of fraction, F3 was confirmed to be terpenoids.

DISCUSSION

Antibiogram Pattern of the Urinary Isolates

During the study period, among the 1872 urinary isolates, *E. coli* and *K. pneumoniae* have been reported as the most common organisms causing UTI of the 2046 clinical samples, 1689 samples showed the growth of bacteria and no growth were observed in 357 cultures. It was found that gram-negative organisms predominated in the etiology of the UTI infection. Although, *E. coli* was more frequently isolated than *K. pneumoniae*, ESBL production was more prevalent in *K. pneunoniae*, which is in accordance with findings reported in previous studies (Supriya *et al*., 2004). During the study period, few ESBL-positive isolates usually susceptible to carbapenems, developed resistance to carbapenems, the drug of choice for treating serious infections caused by ESBL-producing bacteria. This further limits therapeutic options, compelling alternative therapy. Hence plants possessing antibacterial activity against the multi drug-resistant bacteria were screened. Apart from the gram-negative bacterial pathogens, gram-positive *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA) also are often associated with UTI as opportunists (Gomez *et al*., 2007).

Role of the Solvents Used

The absence of the zones of inhibition by the methanol, acetone and hexane solvent only discs, demonstrated the non-involvement of the solvents used for extracting the plant materials in the production of inhibitory zones. Also the dimethyl sulphoxide (DMSO) showed no inhibitory effect, as already reported (Srinivasan *et al*., 2001; Dhanabal *et al*., 2006).

The *E. scaber* results reveal no notable differences in the activity of the ES plant extracts between the gram positive and gram-negative bacteria. The zones of inhibition by the acetone extract of *E. scaber* ranged between 6 and 12 mm. This fact may be due to the plant being independent to gram reaction, suggesting a different mode of action by *E. scaber*. Though the acetone extract of *E. scaber* exhibited a higher inhibitory activity, yet no inhibitory zones were observed against *Pseudomonas* spp., while the methanol and hexane extracts of *E. scaber* was effective against *Pseudomonas* spp. The similar activity of the plant, *E. scaber* against both gram-positive and gram-negative bacteria may be indicative of the presence of broad-spectrum antibiotic compounds in *E. scaber*. Gram-positive and Gram-negative MDR bacteria are almost equally sensitive to these extracts, indicating their broad-spectrum nature. However, strain-and plant extract-dependent variations in the antibacterial activity were also evident (Aqil and Ahmad, 2007).

Though our results of the antibacterial potential by the two chosen plants are in accordance with earlier reports (Rahman *et al*., 2001; Avani and Neeta, 2005), yet there are no reports on the potential of the two plants against multi drug-resistant bacterial isolates. We have reported that EJS extracts were effective against many ESBL-producing drug-resistant bacterial isolates, so also both the plants active against MRSA (Jasmine *et al*., 2007).

Among the fractions of *E. scaber* acetone extract, F5 lacked activity, which may be due to the absence of compounds or due to very low concentration of the compounds. Often times, there is loss of compounds in the fractions that were present in the crude. This loss occurs during extraction (Shariff, 2001). The MIC values of the other four fractions ranged between 125 and 1000 μg mL^{-1}, where the consistency was observed in F3 (125-250 μg mL^{-1}).

β-lactam resistance among clinical isolates is growing problem (Livermore *et al*., 2001). Many Gram-negative bacilli produce ESBL, which are enzymes that mediate resistance to all β-lactams except cephamycins and carbapenems (Philippon *et al*., 1989). Organisms producing ESBL are typically multi-drug resistant (Supriya *et al*., 2004). Compared with ESBL-negative isolates, ESBL-positive isolates are more often resistant to amino glycosides, ciprofloxacin and cotrimoxazole. In this study, most ESBL positive strains which were resistant to several antibiotics (Table 1) were susceptible to our plant extract. Fraction F3 was highly bactericidal against all the ESBL isolates under study. Since the MIC and the Minimum Bactericidal Concentration (MBC) values were the same, the activity of F3 was concluded as bactericidal. Terpenoids, the compound detected in F3 might be responsible for the antibacterial effect. Terpenoids have been reported to be active against several bacteria (Kubo *et al*., 1992) and also against viruses, fungi and protozoans (Ayafor *et al*., 1994; Ghoshal *et al*., 1996). Food scientists have found that the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Aureli *et al*., 1992). Kadota *et al*. (1997) found that trichorabdal A, a diterpene from a Japanese herb, could inhibit *Helicobacter pylori*. Two diterpenes isolated by (Batista, 1994) were found to be effective against *S. aureus*, *V. cholera*, *P. aeruginosa* and *Candida* spp. The mechanism of action of terpenes is not fully understood, but is speculated to involve in membrane disruption by the lipophilic compounds (Cowan, 1999). Though few reports are available on the role of terpenoids against several bacteria, there is not much literature available for the inhibitory action of terpenoids on multidrug resistant ESBL producing human pathogens. The exact mechanism is yet to be investigated. Attention to this issue could usher in a badly needed new era of chemotherapeutic treatment of infection by using plant-derived principles.

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