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A Novel Terpenoid from *Elephantopus scaber* with Antibacterial Activity Against Beta-lactamase-Producing Clinical Isolates

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Abstract: Bioactivity-guided fractionation of the acetone extract of *Elephantopus scaber* (ES) yielded a new terpenoid, 6-[1-(10,13-dimethyl-4,5,8,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[α]phenanthren-17-yl)ethyl]-3-methyl-3,6-dihydro-2 H-2-pyranone. The structure of the above mentioned compound was elucidated by interpretation of their spectroscopic data. Biological testing of the compound demonstrated significant antibacterial activity against a few multi drug-resistant ESBL-producing clinical isolates. 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.

Key words: *Elephantopus scaber*, bioactivity, fractionation, terpenoid

INTRODUCTION

Emerging resistant bacterial strains are a threat to the community. The emergence of antibiotic resistance (Kiffer *et al*., 2007) among both pathogenic and opportunistic microbes resident in hospitals represents a serious and recurrent problem for the treatment of infections (Mohammed Akram *et al*., 2007). Among the several drug resistant bacteria, β-lactamase production is the most important mechanism of resistance to penicillin and cephalosporins. The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against β-lactamase-mediated bacterial resistance to antibiotics. These cephalosporins had been developed in response to the increased prevalence of β-lactamases in certain organisms. These bacteria had the ability to confer resistance to the extended-spectrum cephalosporins (Jain and Mondal, 2007). Hence these new β-lactamases were coined Extended-Spectrum β-Lactamases (ESBLs).

However, the frequency of ESBL production in these organisms is low (Nathisuwan, 2001), but has now been on increase (Supriya Tankhiwale *et al*., 2004; Mohammed Akram *et al*., 2007). The mechanism of this resistance was the production of Extended Spectrum B-Lactamases (ESBLs) (Ayyagari and Bhargava, 2001). In addition, ESBL producing organisms exhibit coresistance to many other classes of antibiotics resulting in limitation of therapeutic option. The most frequent co-resistances found among ESBL producing organisms are amino glycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethorprim (Nathisuwan *et al*., 2001). The usual transmissibility of the responsible plasmids, however, allows resistance to spread readily to

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other pathogens (Medeiros, 1997), so that extended spectrum enzymes have been found in nearly all species of *Enterobacteriaceae*. 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. Though the biological activities of the compounds isolated from *Elephantopus scaber* have been studied (De-Silva *et al*., 1982; Paul Pui-Hay But *et al*., 1997), yet there are no reports on the effect on the active compound against multidrug-resistant bacteria. However, this study is the first of its kind.

MATERIALS AND METHODS

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).

Extract Preparation

Elephantopus scaber plants were collected from Kerala and authenticated at the Department of Botany of the College. The voucher specimen is available at the Department of Biotechnology, Holy Cross College, Trichy-2. The air-dried plants were powdered and 1 kg was extracted using methanol, acetone and hexane in a soxhlet apparatus and were evaporated to dryness under reduced pressure in rotary evaporator. The yields of the acetone, hexane and methanol extracts were 12.1, 10.9 and 14.3 g%, respectively. The dry residues of the crude extracts obtained was stored for further use. For convenience the methanol, acetone and hexane extracts of *E. scaber* were named ESM, ESA and ESH, respectively.

Fractionation of the Crude Extract with Promising Results

Fractionation of the crude extract was based on bioactivity. The most bioactive crude extract 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 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 and were named as F1, F2, F3, F4 and F5, respectively.

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 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

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were determined for the extracts by broth dilution method as described by Ayafor (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.

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); S: Succeptible

Table 2: Disc diffusion assay of the fractions of the acetone extract of *Elephantopus scaber*

	Zones of Inhibition (dia in mm)				
Organisms	F_1	F_2	F_3	F_4	F_5
Acinetobacter banumanii	9	4	14	11	-
Aeromonas hydrophila	11	7	11	8	-
Citrobacter freundii	11	9	13	10	4
Escherichia coli	11	7	11	8	-
Enterobacter aerogenes	9	9	11	7	6
Klebsiella pneumoniae	11	9	12	11	-
Pseudomonas aeruginosa	10	10	12	9	-
Proteus mirabilis	8	9	14	12	7
**Escherichia coli* ATCC 25922	12	13	17	13	-
***Escherichia coli* ATCC 35218	8	9	13	9	-

*ESBL negative control; **ESBL positive control

Table 3: MIC values of the fractions of ES on ESBL producers

	MIC (μg mL^{-1})				
Organisms	F_1	F_2	F_3	F_4	F_5
Acinetobacter banumanii	500	-	62.5	250	-
Aeromonas hydrophila	250	1000	125	500	-
Citrobacter freundii	250	500	62.5	62.5	-
Escherichia coli	250	1000	125	1000	-
Enterobacter aerogenes	500	500	125	500	-
Klebsiella pneumonia	250	500	125	500	-
Pseudomonas aeruginosa	250	500	125	1000	-
Proteus mirabilis	500	500	125	500	-
**Escherichia coli* ATCC 25922	31.75	62.5	15.87	62.5	-
***Escherichia coli* ATCC 35218	500	500	125	250	-

*ESBL negative control; **ESBL positive control

The results of the disc diffusion assay of the alcoholic crude extracts and the fractions of the methanol extracts are listed in Table 2 and 3, respectively. No zones were observed for solvent only discs and dimethyl sulphoxide. Varying levels of potency of these extracts were observed against different bacteria. MIC was found in the range of 62.5-1000 μg mL^{-1}. The finding clearly indicated overall high potency in terms of MIC values of the plant extract. Among the 5 fractions obtained, F3 exhibited good antibacterial activity against all the bacterial under study. No activity was found with F5 and moderate activity was seen with F1, F2 and F4. The MIC values of the 5 fractions of methanol extracts are given in Table 3. 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.

Report of the New Compound

TLC bioautography of the F3 fraction revealed terpenoids as the major bioactive phytoconstituent. It was further subjected to spectral studies and the compound was identified as a terpenoid, 6-[1-(10,13-dimethyl-4,5,8,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta [α]phenanthren-17-yl)ethyl]-3-methyl-3,6-dihydro-2 H-2-pyranone.

DISCUSSION

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, 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 present study, most ESBL positive strains which were resistant to several antibiotics (Table 1) were susceptible to our plant extract. Fraction 2 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 (Barre *et al*., 1997) 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 (1997) found that trichorabdal A, a diterpene from a Japanese herb, could inhibit *Helicobacter pylori*. Two diterpenes isolated by (Batista *et al*., 1994) were found to be effective against *S. aureus*, *V. cholera*, *P. aeruginosa* and *Candida* sp. 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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