INVESTIGATING THE MECHANISM OF ACTION OF TERPENOIDS AND THE EFFECT OF INTERFERING SUBSTANCES ON AN INDIAN MEDICINAL PLANT EXTRACT DEMONSTRATING ANTIBACTERIAL ACTIVITY

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ABSTRACT

Elephantopus scaber, an Indian medicinal plant, reported to possess potent bactericidal activity against several drug-resistant bacteria was investigated for their mode of action. By performing salt tolerance assays, it was found that the extract of the whole plant of Elephantopus scaber, compromised the integrity of the cytoplasmic membrane of Staphylococcus aureus, leading to a decrease in ability to exclude NaCl. The bactericidal action of the Elephantopus scaber extract was concluded to be due to its membrane-active properties. The effect of contaminants on the efficacy of this extract was also investigated. Organic contaminants (bakers' yeast and skim milk powder) decreased the efficacy of all extracts investigated, while hard water had no effect. Greater understanding of the biocidal properties of the plant extracts investigated may determine if they have medical, industrial or environmental applications. Since Elephantopus scaber possessed antibacterial activity, the experiment was further carried out to isolate and identify the putative antibacterial compounds based on bioassay-guided fractionation. Bioactivity-guided fractionation of the acetone extract of Elephantopus scaber (ES) yielded a new terpenoid compound (already reported) as, 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-2H-2-pyranone. The active compound was purified by repeat column and structure was determined on the basis of chemical and physiochemical evidence.

KEYWORDS: Elephantopus scaber; terpenoid; salt tolerance; antibacterial.

INTRODUCTION

Antibiotic resistance has increased rapidly during the last decade, creating a serious threat to the treatment of infectious diseases. Drug resistance is one of the most serious global threats to the treatment of infectious diseases (1,2). 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. For over several years medicinal plants have served as the models for many clinically proven drugs, and are now being reassessed as antimicrobial agents. Literally thousands of plant species have been tested against hundreds of bacterial strains in vitro and many medicinal plants are active against a wide range of gram-positive and gram-negative bacteria. However very few of these medicinal plant extracts have been tested against resistant bacteria. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Plants produce a wide array of organic compounds, usually secondary metabolites, which in addition to imparting characteristic odour, pigment and flavour properties, sometimes exhibit antimicrobial action [3]. The extraction, and possible subsequent therapeutic application, of these biologically active phytochemicals is not a recent development, with many plant-derived antimicrobials undergoing clinical trials for human use [3]. Studies conducted worldwide, for example, the investigation of traditional African [4,5,6], Caribbean [7] and Indian [8,9,10] medicinal plants, function not only to scientifically validate purported properties of traditional medicinal plants, but also in the identification of possible sources of effective drugs. It follows, therefore,
that as plant products become more widely exploited as a source of antimicrobial agents, those products in-turn require rigorous characterization, both in identification of active constituents, and in the study of mechanisms by which they exert their antimicrobial activity. However, few studies have included further investigations of the biological action of antimicrobial plant extracts. The current study was designed to characterise selected Indian medicinal plant extracts, previously demonstrated to exhibit antibacterial activity against drug-resistant Gram positive and negative bacteria [10]. In addition, we recently identified the active component of the *Elephantopus scaber* extract as a terpenoid, a class of antimicrobial phytochemicals whose mechanism of action is membrane disruption. The experiments described here were specifically undertaken to investigate (i) the mode of antibacterial action of the *Elephantopus scaber* extract and (ii) the effect of contaminants on the activity of this medicinal plant extracts.

**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 [11,12]. 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 [13]. Recently, it has been shown to possess anti-inflammatory and antitumour activity in animal models [14], and also found to have antibacterial activity against a few standard bacterial strains.

**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 1kg 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 gm %, 10.9 gm % and 14.3 gm% 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 F1_5, F6_8, F9_11, F12_15 and F16_17 were combined according to their Rf values into five fractions finally and were named as F1, F2, F3, F4 and F5 respectively.

**Test Organisms**

Urinary isolates from symptomatic Urinary tract infected patients attending or admitted to CSI Mission General Hospital in Tiruchirappalli, South India, from October 2005-March 2006, were identified by conventional methods. Clinical isolates were included for the study. The bacterial strains were grown and maintained on Nutrient Agar slants.

**Salt tolerance assay**

The salt tolerance assay [15] involved investigating the ability of *S. aureus* cells treated with the *E. scaber* extract to grow on NA supplemented with NaCl. Triplicate suspensions of bacteria were prepared as described above and treated with extract at 0.5 × minimum inhibitory concentration (MIC) (1: 4000 dilution of extract in sterilised water), 1 × MIC (1: 2000), and 2 × MIC (1: 1000). MIC was determined as outlined below.

After 30 min, samples were removed, serially diluted, plated onto NA or NA supplemented with 50 mg/ml and 70 mg/ml NaCl and incubated at 37 °C for 24 h. The mean number of colony-forming units/ml on the NA–NaCl plates were reported as a percentage of those on the NA-only plate. Controls consisted of untreated cells incubated under the same conditions.

**Effect of interfering substances on biocide activity**
To assess the effect of contaminants and cations (hard water) on the efficacy of the studied extracts [16], skim milk powder (Bonlac Foods, Melbourne, Australia) and bakers’ yeast (Defiance Milling Co., UK) were added to NB to a final concentration of 10% (w/v) and 5% (w/v), respectively. The effect of hard water was investigated using NB prepared in distilled water to which CaCl$_2$ (0.304 mg/ml) and MgCl$_2$ (0.065 mg/ml) [17] had been added. Plant extract was added to sterile tubes containing NB (or NB in hard water) and serial 2-fold dilutions were carried out. The interfering substances were added to the tubes along with bacterial culture (10 µl of an overnight NB culture) and these were incubated at 37 °C for 24 h. The minimum bactericidal concentration was then determined. NB-only cultures served as controls. The assay was carried out in triplicate and any effect of the contaminants was observed as an increase in MBC compared to the control.

### Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts

MICs were determined using a broth dilution method where doubling dilutions of extract in NB (in a final volume of 2 ml) were inoculated with 10 µl of an overnight NB culture. Following incubation at 37 °C for 24 h, the MIC was recorded as the lowest dilution that inhibited bacterial growth. To determine MBCs, the tubes were sampled, inoculated onto NA, and incubated for a further 24 h. The MBC was recorded as the lowest concentration that prevented the recovery of viable bacteria.

### RESULTS

#### Determination of the mechanism of antibacterial action

Investigations of the mechanism of action were carried out on the acetone extract of _E. scaber_ as this was the most potent of the extracts determined from preliminary screening of Indian medicinal plants. The activity of the extract and the chemical nature of its active component also suggested that it might be membrane-active.

Sub-lethal injury of bacterial cell membranes can alter their permeability and affect the ability to adequately osmoregulate or exclude toxic materials. The loss of salt tolerance has been used in previous studies to reveal membrane damage in sub-lethally injured bacteria [15]. The salt tolerance assay was used to investigate the membrane-active nature of the _E. scaber_ extract. Fig.1 illustrates the relationship between the salt tolerance of _S. aureus_ and the concentration of _E. scaber_ extract to which the bacteria were exposed. The results are reported as the percentage decrease in CFU/ml on the NA–NaCl plates compared to the NA-only control plates. Treatment of _S. aureus_ with the _E. scaber_ extract at MIC level or greater reduced the ability of the survivors to form colonies on the NaCl containing media. Exposure to sub-MIC levels did not affect survival as there was no significant difference in survival on NA–NaCl plates between bacteria not exposed to extract and those exposed to 0.5 × MIC levels of extract. This decreased ability to exclude salt (at or above the MIC) indicated a loss of membrane fidelity in the presence of the _E. scaber_ extract.

#### Table 1: Effect of a few interfering substances on the efficiency of _Elephantopus scaber_ extracts on the growth of _S. aureus_

<table>
<thead>
<tr>
<th>Interfering substances</th>
<th>Minimal Bactericidal concentration (mg/ml)</th>
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<td>Hard water (0.304 g/l CaCl$_2$)</td>
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*ESM- Elephantopus scaber methanol extract, ESA- Elephantopus scaber acetone extract, ESH- Elephantopus scaber hexane extract

#### Table 2: Effect of a few interfering substances on the efficiency of _Elephantopus scaber_ extracts on the growth of _E. coli_

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*ESM- Elephantopus scaber methanol extract, ESA- Elephantopus scaber acetone extract, ESH- Elephantopus scaber hexane extract
Effect of interfering substances
The bactericidal efficacy of all four plant extracts in the presence of various interfering substances was investigated. The MBC rather than MIC was determined for several reasons. In terms of practicality, the turbidity of the bakers’ yeast and skim milk powder added to NB meant that it was not possible to visually distinguish the concentration at which growth was inhibited. Secondly, it has been suggested that the determination of the MBC of a biocidal compound is more valuable to its characterisation than its MIC \[18\]. The MBC of all extracts increased in the presence of skim milk powder and bakers’ yeast but was unaltered in NB prepared in hard water (Table 1&2). Specifically, the addition of skim milk powder and bakers’ yeast typically caused a four- and two-fold increase in MBC, respectively.

DISCUSSION
Understanding the mechanisms of biocidal action and the factors influencing their activity, such as the effect of interfering substances, have become key issues for the better use of biocides \[18\]. The membrane disruption seen could still be a function of the biocide-induced autolytic events described above \[19, 20, 18\]. Regardless of the sequence of events, however, the results indicate that membrane damage is occurring in the presence of the E.scaber extract. Demonstrating loss of salt tolerance has been utilised in other studies to investigate the mode of action of antibacterial agents against S. aureus \[15\]. Overall, the results obtained from these investigations suggested membrane-activity as a plausible mode of action for the E.scaber extract, supporting the finding that the active component of this extract is a terpenoid.
conjunction with other products. For example, incorporation of biocides into pharmaceutical or general household formulations would necessitate a study of how this would affect their antimicrobial activity [15]. Loss of efficacy not only impacts on the immediate potency of a biocide but also has implications for increased resistance. The use of biocides at sub-effective concentrations can confer bacterial resistance to third-party therapeutic agents, such as antibiotics [21]. Therefore, while the loss of efficacy due to the presence of interfering substances may be the primary result, future problems of resistance to the biocide itself as well as to other antimicrobial agents may arise. The current study indicated efficacy of extracts was not affected by the presence of hard water, but was compromised by the presence of organic matter. As recognized by Hammer et al. (1999), this data may be useful in assessing the potential commercial applications of the extracts. Organic matter is able to compromise the activity of antimicrobial agents by several modes [15]. Adherence of organic matter to the bacterial cell may act to prevent exposure to the antibacterial substance, thereby decreasing the level of activity [22, 23]. A similar effect is also proposed for the interaction between organic mater and the antimicrobial compound. This results in the usually active substance being less readily absorbed by the bacteria, and therefore less effective [16]. The data presented in this paper provide important information about the biocide properties of the Indian medicinal plants extracts. In addition to further studies, such as the identification of the active components of all extracts, these data will help to determine if these extracts will have useful medical, industrial or environmental applications. Further investigation of other factors or conditions that may affect efficacy of the extracts, such as pH and temperature, is also warranted.

REFERENCES


