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Do South African medicinal plants used traditionally to treat infections respond differently to resistant microbial strains?

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ABSTRACT

Currently antimicrobial resistance is increasing at an alarming rate. Exposure to resistant strains hinders treatment outcomes both in rural and hospital settings. Thus, the aim of this study was to investigate five frequently used South African medicinal plants (*Artemisia afra, Lippia javanica, Osmitopsis asteriscoides, Croton gratissimus* and *Tetradenia riparia*) and test these against resistant bacterial strains (*Enterococcus faecalis, Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli* and *Serratia marcescens*) and comparatively evaluate efficacy with a reference strain. The conventional antibiotic ciprofloxacin was used as a positive control to also compare susceptibility of the various strains. Most plant samples demonstrated similar or better activity against the resistant strains. A general trend demonstrated that the organic extracts followed by the essential oils were able to withstand resistant strains better than the antibiotics which showed reduced susceptibility. This demonstrates great promise as natural products provide an alternative to fighting the onslaught of antibiotic resistance.

1. Introduction

Currently antimicrobial resistance is a global crisis, increasing at an alarming rate. The last few decades have shown an increase in clinical and community acquired multidrug resistant infections which have had an impact on treatment regimens (Canton and Morosini, 2011). It is estimated that by the year 2050, infections by antimicrobial resistant organisms will be the leading cause of death worldwide (O'Neill, 2014). Important bacterial strains conveying resistance include *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae* amongst others (Warnke et al., 2009; Khan and Zarrilli, 2012; Potron et al., 2015).

In South Africa, antimicrobial resistance has reached alarming proportions. Some statistical impact factors include studies that show more than 50% of all hospital-acquired *S. aureus* infections were of methicillin resistance origin (Bamford et al., 2011). In an editorial entitled "Wake up, South Africa! The antibiotic horse has bolted" (Mendelson et al., 2012), the very title leaves no subtlety as to the huge problem South Africa is facing with respect to antimicrobial resistance.

To address the crisis and challenges posed by the growing global resistance of micro-organisms to conventional antimicrobials, research has adapted a growing interest in examining alternatives such as natural products (Al-Mariri and Safi, 2014; Harvey et al., 2015; Moloney, 2016). Some recent studies to examine natural products and resistant strains include the investigation of *Uapaca togoensis* and related compounds when tested against Gram-negative multi-drug resistant phenotypes (Seukep et al., 2016). *Eucalyptus camaldulensis* has been studied against multi-drug resistant *Acinetobacter baumannii* (Knezevic et al., 2016). Antibacterial effects of cinnamon oil have been investigated against carbapenem resistant nosocomial *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates (Kaskatepe et al., 2016). The antimicrobial activity of *Artemisia judaica* (essential oil) against clinical multi-drug resistant bacteria (Benmansour et al., 2016) has been examined and studies on crude extracts against multiple resistant urinary tract infections (Mishra et al., 2017) have also had some attention. These are just a few studies that have focused on resistant strains.

South Africa is home to more than 3000 species of medicinal plants (Van Wyk et al., 1997), and many of these have shown great antimicrobial potential (Van Vuuren, 2008; Van Vuuren and Holl, submitted for publication). In spite of the number of publications dedicated to natural products and resistant strains, very little attention has been given to resistant strains and medicinal plants from a South African perspective. In a very recent review of antimicrobial studies and South African natural products (Van Vuuren and Holl, submitted for publication), only a few papers (Lall and Meyer, 1999; Heyman et al., 2009; Bisi-Johnson et al.,

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2011; Njume et al., 2011a, 2011b; Nielsen et al., 2012; Mabona et al., 2013; Khan et al., 2014; Nciki et al., 2016) included resistant strains and none of these studies comparatively evaluated the antimicrobial effects of natural products with conventional antibiotics. It is well known that medicinal plants are not as potent as single compound antibiotic drugs. Medicinal plants are considered active when efficacy (minimum inhibitory concentrations [MIC]) under 100 μg/mL are evident (Van Vuuren and Holl, submitted for publication). The effective MIC values for antibiotics varies between strains and have recommended acceptable breakpoint values (Andrews, 2004; CLSI guidelines, 2012), which are clearly much lower and in microgram quantities. Even though there is a large variation in potency, medicinal plants are more readily available, cheaper and cultural reasons validate selection and choice. Even though the efficacy of medicinal plants do not show the potency that single compound antibiotics offer they may be able to demonstrate more resilience against resistant microbial strains when compared to conventional antibiotics. This may in the future become a vital advantage in treating infections that harbour resistant strains.

With this in mind, the overall aim of this study was to compare the antimicrobial responses of resistant strains with that against a reference strain when exposed to indigenous South African medicinal plants. Furthermore the comparative response observed when exposed to conventional antibiotics was explored.

2. Methodology

2.1. Plant selection and preparation

A selection of five common South African medicinal plants [*Artemisia afra* Jacq. ex Willd. (Asteraceae), *Osmitopsis asteriscoides* (L.) Less. (Asteraceae), *Lippia javanica* Spreng. (Verbenaceae), *Croton gratissimus* Burch. (Euphorbiaceae) and *Tetradenia riparia* (Hochst.) Codd (Lamiaceae)] was chosen based on popularity, traditional use to treat infections and the fact that they are aromatic which allowed for the inclusion of the essential oils in the study. These plants are all antimicrobially well studied (Mangena and Muyima, 1999; Viljoen et al., 2003; Viljoen et al., 2005; Van Vuuren and Viljoen, 2008; Shikanga et al., 2010; Suliman et al., 2010; York et al., 2012), yet little attention has been given to how these medicinal plants respond to resistant bacterial strains. Plant species (with the exception of *O. asteriscoides* which was collected from a population near Betty's Bay in the South Western Cape region of South Africa) were collected from the Walter Sisulu botanical gardens with permission and assistance from Mr. Andrew Hankey, Specialist horticulturist and plant conservationist. Identification was confirmed and voucher specimens prepared and stored in the Department of Pharmacy and Pharmacology, University of the Witwatersrand. Dried plant samples were ground and immersed in a 1:1 mixture of dichloromethane and methanol. This organic extract was selected for the ability to extract a combination of both polar and nonpolar compounds. The plant samples in solvent was retained in the shaker incubator (Labcon) at 37 °C for 24 h. Aqueous extracts were prepared by submerging the macerated plant material in sterile distilled water which was kept at ambient temperature overnight. Thereafter the extracts were filtered, stored at −80 °C and lyophilized (VirtTis) (Van Vuuren and Viljoen, 2006). For the essential oils, a known quantity of weighed fresh leaf material was subjected to hydrodistillation using a Clevenger-type apparatus. After 3 h, the essential oil was collected, weighed and stored in amber bottles at 4 °C.

2.2. Culture strains

Bacterial strains (Table 1) included in the study were selected on the basis of emerging antimicrobial resistance patterns. These included the "ESKAPE" pathogens which have come to encompass *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus* spp. and *Acinetobacter* spp. The selected strains represent pathogens with growing multidrug resistant virulence on a global scale. The number of test strains included was based on availability. Where clinical resistance is common, more strains were included. These included *Enterococcus faecalis* (five resistant strains), *Staphylococcus aureus* (eight resistant strains) and *Klebsiella pneumoniae* (three resistant strains).

A non-resistant reference strain was included for each strain type to compare the antimicrobial response. Resistant strains were obtained from various sources as indicated in b–d (Table 1). All cultures were confirmed pure before commencement of the study and resistance patterns were predetermined by the relevant suppliers. Eight different bacterial species (three Gram-positive and five Gram-negative strains) comprising of a total of 32 different strains were included in the study (Table 1).

Culture purity was monitored throughout the study by using the streak plate method. Sterility of media was confirmed by incubating un-inoculated broth with the tests.

2.3. The minimum inhibitory concentration (MIC) method

The MIC method was modified from Eloff (1998) and is in accordance with the CLSI guidelines (2012). All plant samples (essential oils, organic and aqueous extracts) were prepared to a starting concentration of 32 mg/mL. The organic extracts and essential oils were diluted in acetone. The aqueous plant samples were diluted in sterile water. The positive control was prepared by diluting ciprofloxacin to a concentration of 0.01 mg/mL in sterile water. This was an important comparator to include as a response of inhibitor (either tests or antibiotic) was crucial to determine if resistant strains respond differently. A negative control was included to determine if solvent (acetone) exhibited any antimicrobial effects. The method, in brief, involved incorporating 100 μL of test sample and or control together with 100 μL of sterile Tryptone Soya broth (TSB) into the top row of a micro-titre plate. The test samples were then serially diluted in TSB to give concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.075 mg/mL. A McFarland standard (approximately 1×10^6 colony forming units [CFU]/mL) was used as a guide to prepare cultures and 100 μL added into all the wells of each plate. Each micro-titre plate was sealed with a sterile adhesive film to prevent evaporative loss of the plant extracts, especially the essential oils during the incubation period. All the micro-titre plates were then incubated at 37°C for 24 h. After incubation 40 μL (0.04%) of *p*-iodonitrotetrazolium violet solution (INT) was added to each well of the plate. The plates were allowed to stand for anything between 3 to 6 h depending on the micro-organism to allow for a colour change to occur. The MIC was determined by the lowest dilution which presented with no colour change. The study was done in triplicate on alternate days. Comparative evaluation between the reference and resistance strains were examined and only differences of more than one dilution factor were considered worthy of noting.

3. Results

The MIC results for the essential oils as well as the organic and aqueous extracts of the five selected plant species are given in Table 2 (Gram-positive test micro-organisms) and Table 3 (Gram-negative test micro-organisms). Efficacies ranged between noteworthy (where MIC values were below 1.00 mg/mL) to poor activity (>8.00 mg/mL). The inhibitory concentration of the negative control (acetone solvent) for all strains was >8.00 mg/mL indicating that the solvent had no influence on the activity. The reference strain (shaded area in Tables 2 and 3) showed in most cases higher susceptibility (0.08–0.63 μg/mL) to the positive control ciprofloxacin than the resistant strains which demonstrated various degrees of resistance (0.08–>2.50 μg/mL). There were some cases (Sa7, Sa8 and Sa9; Ef5 and Ef6; Bc1and Bc2) where the Gram-positive resistant strains demonstrated the same susceptibility pattern to the reference strains. One needs to take into cognisance in