ASSESSMENT OF ANTI-BACTERIAL POTENTIALS OF *GARCINIA KOLA* SEED EXTRACTS AND THEIR INTERACTIONS WITH ANTIBIOTICS

THULANI SIBANDA

Submitted in fulfillment of the requirements for the degree of MASTER OF SCIENCE IN MICROBIOLOGY

Department of Biochemistry and Microbiology,

Faculty of Science and Agriculture

UNIVERSITY OF FORT HARE, ALICE

SUPERVISOR: PROF. A.I. OKOH

DECEMBER 2007

DECLARATION

I, the undersigned, declare that this thesis and the work contained herein being submitted to the University of Fort Hare for the degree of Master of Science in Microbiology in the Faculty of Science and Agriculture, School of Science and Technology, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other university in partial or entirety for the award of any degree.

THULANI SIBANDA

_____

SIGNATURE

_____

DATE

ACKNOWLEDGEMENTS

My greatest thanks go to my supervisor, Professor A.I Okoh, for his support and guidance throughout my learning process. His knowledge and advice has been invaluable.

I would also like to thank the Zimbabwean Government which through the Presidential Scholarship Programme awarded me the scholarship that brought me to the University of Fort Hare. My profound gratitude also goes to the Department of Biochemistry and Microbiology at the University of Fort Hare for affording me the opportunity to undertake my studies, with special thanks to the Head of Department Professor G. Bradley, Mr L.V. Mabinya and Professor G. Pironcheva.

My thanks also go to the current and former technicians in the Department, for their kind assistance during my experimental work particularly Miss V. Malakate, Miss N. Mafu, Miss N. Giyose and Mr E. Green. I would also like to appreciate the support that I received from my colleagues in our research group, Miss N.S. Ncube, Miss T. Ndlovu, Mr O. Aiyegoro, Mr O. Igbinosa, Mr E. Odjadjare, Miss A. Osode, Miss C. Kunaka, Miss N. Mabentsela, Miss T.Y. Nquma and Mr M. Mubazangi.

My gratitude also goes to my family, my wife Sethi and son Nqobile who had to endure a long period of my absence, during my two years of study. Their wonderful support was important to get me to this point.

I would also like to pay tribute to the National Research Foundation (NRF) of South Africa for the financial support that made my experimental work possible.

TABLE OF CONTENTS

GENERAL ABSTRACT

The antibacterial potency of the extracts of the seed of *Garcinia kola* (bitter kola) was investigated in this study against a panel of referenced, environmental and clinical bacterial strains. The killing rates of the active extract as well as their potential for combination antibacterial therapy with standard antibiotics were also elucidated using standard procedures.

The aqueous and acetone extracts of the seed were screened for activity against 27 bacterial isolates. The aqueous extract exhibited activity mainly against Gram positive organisms with Minimum inhibitory concentration (MIC) values ranging from 5 $mgml^{-1}$ – 20 $mgml^{-1}$, while the acetone extract showed activity against both Gram negative and Gram positive organisms with MIC values ranging from 10 $mgml^{-1}$ - 0.156 $mgml^{-1}$. The acetone extract also showed rapid bactericidal activity against *Staphylococcus aureus* ATCC 6538 with a 3.097 Log_{10} reduction in counts within 4 hours at 0.3125 $mgml^{-1}$ and a 1.582 Log_{10} reduction against *Proteus vulgaris* CSIR 0030 at 5 $mgml^{-1}$ after 1 hour.

In addition, the aqueous, methanol and acetone extracts of the seeds also exhibited activity against four clinical strains of *Staphylococcus* isolated from wound sepsis specimens. The MIC values for the aqueous extract were 10 $mgml^{-1}$ for all the isolates while the acetone and methanol extracts had lower values ranging from 0.3125 - 0.625 $mgml^{-1}$. The acetone extract was strongly bactericidal against *Staphylococcus aureus* OKOH3 resulting in a 2.70 Log_{10} reduction in counts at 1.25 $mgml^{-1}$ within 4 hours of exposure and a complete elimination of the organism after 8 hours. The bactericidal

activity of the same extract against *Staphylococcus aureus* OKOH1 was weak, achieving only a 2.92 Log_{10} reduction in counts at 1.25 $mgml^{-1}$ (4× MIC) in 24 hours.

In the test for interactions between the acetone extract of the seeds and antibiotics, synergistic interactions were observed largely against Gram positive organisms using the FIC indices, (indices of 0.52 - 0.875) with combinations against Gram negatives yielding largely antagonistic interactions (indices of 2.0 to 5.0). Synergy (≥ 1000 times or ≥ 3 Log_{10} potentiation of the bactericidal activity) against both Gram negative and Gram positive organisms was detected by time kill assays mainly involving the antibiotics tetracycline, chloramphenicol, amoxycillin and penicillin G. Combinations involving erythromycin and ciprofloxacin consistently gave antagonistic or indifferent interactions.

We conclude that the acetone extract of *Garcinia kola* seeds possess strong bactericidal activities against both Gram positive and Gram negative organisms and can be therapeutically useful in the treatment of bacterial infections including the problematic staphylococcal wound infections. In addition, the acetone extract can be a potential source of broad spectrum resistance modifying compounds that can potentially improve the performance of antibiotics in the treatment of drug resistant infections.

CHAPTER 1

GENERAL INTRODUCTION

Throughout the history of mankind, infectious diseases have remained a major cause of death and disability accounting for about 22% of the global disease burden (Murray and Lopez, 1997). Over 50% of the deaths in children in Sub-Saharan Africa results from infectious causes (Lopez *et al.,* 2006). The discovery of penicillin in the 1940s and several other antibiotics in subsequent years led to great improvements in the management of infectious diseases particularly in developed countries. However, despite this success, the increased use of antibiotics led to the inevitable development of resistance, with the effect that diseases that were hitherto thought to have been controlled by antibiotics later re-emerged as resistant infections (Norrby *et al.,* 2005).

At present major pathogenic bacteria that contribute the most to the global infectious disease burden such as *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella* species *and Mycobacterium tuberculosis* are resistant to standard antibiotic therapies (Styers *et al.,* 2006; Fluit *et al.,* 2001; Gandhi *et al.,* 2006). This global emergence of multi-drug resistant bacterial strains has limited the effectiveness of current drugs, causing treatment failures (Hancock, 2005). The containment of this drug resistance requires that, new potent antimicrobial compounds be identified as alternatives to existing antibiotics (Overbye and Barrett, 2005). However, the current state of development of new antimicrobial drugs is not encouraging with only a few new ones being licenced in recent years (Levy and Marshall, 2004; Norrby *et al.,* 2005). This mismatch between the slow development of new drugs and the fast

emergence of resistant strains makes the future management of infectious diseases look bleak. As an alternative and perhaps a sustainable option, attempts to improve the efficacy of available antibiotics, particularly the older and cheaper ones have been suggested (Lomovskaya and Bostain, 2006).

Medicinal plants continue to play a central role in the healthcare systems of large proportions of the world's population, particularly in developing countries, where herbal medicine has a long and uninterrupted history of use (Koduru *et al.,* 2007). According to the World Health Organisation (WHO), up to 80% of the population in Africa depends on traditional herbal medicine for primary health care, accounting for around 20% of the overall drug market (WHO, 2004). The popularity of such plants in these communities owes largely to their local availability and price affordability (Voravuthikunchai and Kitpipit, 2005) and also confirms their effectiveness.

Plants produce a wide variety of secondary metabolites many of which have been reported to be of therapeutic value. Of the more than 250 000 species of higher plants in the world, only about 5 -10% have been chemically investigated (Tshibangu *et al.,* 2001). This raises the prospects of obtaining novel chemotherapeutic compounds if this vastly untapped resource could be adequately explored. The prospect of obtaining drugs from plants has been demonstrated by some notable examples of important pharmaceuticals derived from plant precursors. For instance, the antimalarial drug Quinine was derived from the quinoline alkaloid of *Cinchona* spp.; the topical analgesic Capsaicin was derived from a phenylalkyl-amine alkaloid of *Capsicum* spp; and the antineoplastic agent Camptothecina was derived from an indol alkaloid of *Camptotheca acuminate* (Raskin *et al.,* 2002). The rich chemical diversity in plants has also been reported to be a promising source of antibacterial

compounds (Bylka *et al.,* 2004; Smith *et al.,* 2007; Machado *et al.,* 2002), raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections.

In addition to plants being potential sources of direct antibacterial drugs, research has also shown that some secondary metabolites of plants with no intrinsic antimicrobial activity are useful in sensitizing bacterial cells to antimicrobial agents (Stermitz *et al.,* 2000; Tegos *et al.,* 2002). These compounds are believed to play a role in the plant's defence against infection by working in synergy with intrinsic antimicrobials. It has therefore been suggested recently, that such compounds can potentially be used to improve the efficacy of antibiotics against bacterial pathogens. The findings of Shibata *et al.* (2005), Stapleton *et al.* (2004), Marquez *et al.* (2005), Oluwatuyi *et al.* (2004) and Smith *et al.,* (2007) have confirmed that indeed plants can be sources of compounds that can potentiate the activity of antibiotics against resistant bacterial pathogens. These compounds have variably been termed resistance modifying, modulating or reversal agents.

While the routine practice has been to screen plant extracts for direct antimicrobial compounds, the second option of searching for resistance modifying compounds that can improve the efficacy of antibiotics when used in combination, appears more attractive as it allows for the recycling of old and relatively cheaper antibiotics that have been rendered ineffective due to resistance.

Description of the Study Plant: *Garcinia kola*

Garcinia kola is a tropical tree of the family Guttiferae commonly found in West and Central Africa with a geographical distribution extending from Angola, the Democratic Republic of Congo, Congo (Brazeville), Gabon, Cameroon, Central Africa Republic, Nigeria, Ghana, Benin, Togo, Ivory Coast, Liberia and Sierra Leone (Eyog-Matig *et al.,* 2007). The tree occurs in wet and moist semi deciduous

forests as well as in savannah regions where the average water availability is equivalent to 2000 - 2500 mm of rainfall per annum, temperature ranges of 21 ℃ to 32 ℃ and minimum relative humidity of 76.34% (Eyog-Matig *et al.,* 2007).

Figure 1: Part of the *Garcinia kola* plant showing the fruits.
Picture by P. Latham. Source: http://users.telenet.be/sf16063/pauwels/GarcKola.JPG

Garcinia kola plants grow to about 15 - 17 m tall with a fairly narrow crown. The plants bear fruits in about 10 - 12 years of their life cycle. The fruit is a drupe of about 5 - 10 cm in diameter (Figure 1) weighing approximately 30 - 50 g. It is usually smooth and contains a yellow-red pulp (Agyili *et al.,* 2006). Each fruit contains about 1 - 4 oval-shaped seeds of approximately 8 g. The seed coat is brown with branched lines (Figure 2), the kernels are pale and penetrated with pockets of resin. The

embryo is not well differentiated into cotyledons and embryonic axis, rather most of the seed is a mass of undifferentiated tissue (Agyili *et al.,* 2006).

Garcinia kola is one of the many non-timber forest plants that are of high socio-economic importance in West Africa. It is probably the most common source of chew sticks in West Africa. Split stems and twigs are used as chewing sticks in many parts of Africa, and have been commercialized in the major cities for years, offering natural dental care (Agyili *et al.,* 2006). The seed, commonly known as bitter kola, is a masticatory used in traditional hospitality, cultural and social ceremonies (Farombi *et al.,* 2000).

Figure 2: *Garcinia kola* seeds

The dried and ground seeds are mixed with honey to make a traditional cough mixture (Onunkwo *et al.,* 2004). Extracts of the seeds are also used by communities in the treatment of bronchitis and throat infections, for the relief of colic, curing of head or chest colds as well as in the treatment of liver disorders (Iwu *et al.,* 1999). In recent years, ground seeds have also been used as an industrial bittering agent in some Nigerian breweries (Okoro and Aina, 2007).

Garcinia kola is a plant that has shown tremendous potential as a source of therapeutic compounds. The seed has been reported to possess antibacterial, antiviral, anti-inflammatory, antioxidant, antidiabetic, as well as antihepatotoxicity potentials (Farombi *et al.,* 2002; Farombi, 2000; Akoachere *et al.,* 2002). The tree is therefore one of the prime medicinal plants of the African continent with a potential to provide relief to a continent with such a high infectious disease burden. A number of compounds with *in vitro* antibacterial activity have been isolated from the extracts of this plant. These include benzophenones such as kolanone and hydroxybiflavononols like GB1 (Madubunyi, 1995; Han *et al.,* 2005). The therapeutic activity of the plant has been attributed to flavonoids which are the dominant compounds of the plant. These vary from simple flavonoids such as apigenin and fisetin to biflavonoids such as amentoflavone and kolaflavonone (Iwu and Igboko, 1982). Some new flavonoids still continue to be identified from the plant (Han *et al.,* 2005).

While the antibacterial activities of the seeds of *Garcinia kola* have been investigated by other researchers, activity has largely been demonstrated for the aqueous, ethanol and petroleum ether extracts. This limited extractant variability is likely to underestimate the antibacterial potential of the plant. The quantity and diversity of extracted compounds has been shown to depend on the extracting solvent, with acetone and methanol (Eloff, 1998) having been observed to efficient

extractants. Often the minimum inhibitory concentrations (MICs) are used as the only tool for predicting the antimicrobial efficacy of the plant. There are limitations to the use of such data alone, mainly that it does not take into account the time related antimicrobial effects (Kiem and Schentag, 2006). It is also likely therefore that the antibacterial potentials of the plant could have been underestimated. In addition, the bactericidal efficacy of the extracts of this plant in terms of death kinetics has not been reported, yet this is a fundamental tool for determining the potency of any potential antimicrobial substance. Furthermore, recent research on the therapeutic potentials of medicinal plants has shown that in addition to them being sources of direct antimicrobial compounds, they can also be sources of compounds that can improve the performance of antibiotics when used in combination including in a number of cases against resistant pathogens (Gibbons, 2004). While the extracts of *Garcinia kola* seeds have been investigated for their antimicrobial activities, their interactions with antibiotics have also not been reported.

This research therefore aimed to contribute to the already existing knowledge about the therapeutic potentials of *Garcinia kola*, by investigating the antibacterial and resistance modifying activities of seed extracts of this plant. Specifically the research had the following objectives;

- to investigate the antibacterial activity of extracts of *Garcinia kola* seeds against a wide range of bacterial isolates, representing pathogenic microbes;
- to determine the Minimum Inhibitory Concentrations (MICs) of the extracts as a prediction index for the therapeutic potentials of the plant;
- to determine the bactericidal activity of the extracts by measurement of the rate of kill as an alternative prediction tool for the therapeutic potentials of the plant;

- to investigate the effect of combinations between extracts of the plant and antibiotics on the susceptibility of bacterial isolates; and
- to investigate the activity of the extracts of the plant against clinical strains of *Staphylococcus* isolated from cases of wound sepsis.

REFERENCES

Agyili J, Sacande M, and Kouame C (2006). *Garcinia kola* Heckel. Seed Leaflet. No. 113 of 2006.

Akoachere JF, Ndip RN, Chenwi EB, Ndip LM, Njock TE, and Anong DN (2002). Antibacterial effect of *Zingiber officinale* and *Garcinia kola* on respiratory tract pathogens. Eas. Afr. Med. J. 79 (11): 588-592.

Bylka W, Szaufer-Hajdrych M, Matlawska I, and Goslinska O (2004). Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris* L. Lett. Appl. Micro. 39(1): 93-97.

Eloff JN (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharm. 60(1): 1-8.

Eyog-Matig O, Aoudji AKN, and Linsoussi C (2007). *Garcinia Kola* Heckel seeds dormancy-breaking. App. Eco. Env. Res. 5(1): 63-71.

Farombi EO (2000). Mechanisms for the hepatoprotective action of kolaviron: Studies on hepatic enzymes, microsomal lipids and lipid peroxidation in carbontetrachloride-treated rats. Pharmacol. Res. 42(1): 75-80.

Farombi EO, Alabi MC, and Akuru TO (2002). Kolaviron modulates cellular redox status and impairment of membrane protein activities induced by potassium bromate ($KBrO_3$) in rats. Pharmacol. Res. 45(1): 63-68.

Farombi EO, Tahnteng JG, Agboola AO, Nwankwo JO, and Emerole GO (2000). Chemoprevention of 2-acetylaminofluorene- induced hepatotoxicity and lipid peroxidation in rats by kolaviron - A *Garcinia kola* seed extract. Food Chem. Tox. 38: 535-541.

Fluit AC, Schmitz FJ, Verhoef J, and European SENTRY Participants (2001). Multi-resistance to antimicrobial agents for the ten most frequently isolated bacterial pathogens. Int. J. Antimic. Agents. 18(2): 147-160.

Gandhi NR, Moll A, Sturm W, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, and Friedland G (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet. 368: 1575-1580.

Gibbons S (2004). Anti-staphylococcal plant natural products. Nat. Prod. Rep. 21: 263-277.

Han QB, Lee SF, Qiao CF, He ZD, Song JZ, Sun HD, and Xu HX (2005). Complete NMR assignments of the antibacterial biflavonoid GB1 from *Garcinia kola*. Chem. Pharm. Bull. 53(8): 1034-1036.

Hancock EW (2005). Mechanisms of action of newer antibiotics for Gram positive pathogens. Lancet Inf. Dis. 5(4): 209-218.

Iwu M, and Igboko O (1982). Flavonoids of *Garcinia kola* seeds. J. Nat. Prod. 650-650.

Iwu MW, Duncan AR, and Okunji CO (1999). New antimicrobials of plant origin. Janick J (ed.), Perspectives on new crops and new uses: 457-462.

Kiem S, and Schentag JJ (2006). Relationship of Minimal Inhibitory Concentration and Bactericidal Activity to Efficacy of Antibiotics for Treatment of Ventilator-Associated Pneumonia. Semin. Respir. Crit. Care. Med. 27: 51-67.

Koduru S, Grierson DS, and Afolayan AJ (2007). Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa. Curr. Sci. 92(7): 906-908.

Levy SB, and Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. Nat Med. 10: S122-S129.

Lomovskaya O, and Bostian KA (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic - A vision for applied use. Biochem Pharmacol. 7(1): 910-918.

Lopez AD, Mathers CD, Ezzati M, Jamison DT, and Murray CJL (2006). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 367: 1747-1757.

Machado TB, Leal ICR, Amaral ACF, Santos KRN, Silva MG, and Kuster RM (2002). Antimicrobial ellagitannin of *Punica granatum* fruits. J. Brazilian Chem. Soc. 13(5): 606-610.

Madubunyi II (1995). Antimicrobial activities of the constituents of *Garcinia Kola* seeds. Int. J. Pharmacog. 33(3): 232-237.

Marquez B, Neuville L, Moreau NJ, Genet JP, Santos AF, Andrade MCC, and Sant'Ana AEG (2005). Multidrug resistance reversal agent from *Jatropha elliptica*. Phytochem. 66: 1804-1811.

Murray CJL, and Lopez AD (1997). Mortality by cause for eight regions of the world: Global Burden of Disease Study. The Lancet. 349: 1269-1276.

Norrby RS, Nord CE, and Finch R (2005). Lack of development of new antimicrobial drugs: a potential serious threat to public health. Lancet Inf. Dis. 5(2): 115-119.

Okoro CC, and Aina JO (2007). Effect of storage on the brewing properties of tropical hop substitutes. Afr. J. Biotech. 6(12): 1479-1483.

Oluwatuyi MGW, Kaatz, and Gibbons S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochem. 65(24): 3249-3254.

Onunkwo GC, Egeonu HC, Adikwu MU, Ojile JE, and Olowosulu AK (2004). Some physical properties of tabletted seed of *Garcinia kola* (Heckel). Chem. Pharm. Bull. 52(6):649-653.

Overbye KM, and Barrett JF (2005). Antibiotics: Where did we go wrong? Drug Discov. Today. 10(1): 45-52.

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, and Fridlender B (2002). Plants and human health in the twenty-first century. Trends Biotech. 20(12): 522-531.

Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, and Higut T, (2005). Alkyl gallates, intensifiers of ß-lactam susceptibility in methicillin-resistant *Staphylococcus aureus* Antimic. Agents Chemo. 49(2): 549-555.

Smith ECJ, Williamson EM, Wareham N, Kaatz GW, and Gibbons S (2007). Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. Phytochem. 68(2): 210-217.

Stapleton PD, Shah S, Anderson JC, Hara Y, Hamilton-Miller JTM, and Taylor PW (2004). Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates. Int. J. Antimic. Agents. 23(5): 462-467.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, and Lewis K (2000). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. Appl. Biol. Sci. 97(4): 1433-1437

Styers D, Sheehan DJ, Hogan P, and Sahm DF (2006). Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. Ann. Clin. Micro. Antim. 5:2

Tegos G, Stermitz FR, Lomovskaya O, and Lewis K (2002). Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimic. Agents Chemo. 46(10): 3133-3141.

Tshibangu JN, Chifundera K, Kaminsky R, Wright AD, and Konig GM (2001). Screening of African medicinal plants for antimicrobial and enzyme inhibitory activity. J. Ethnopharm. 80: 25-35.

Voravuthikunchai SP, and Kitpipit L (2005). Activity of medicinal plant extracts against hospital isolates of methicillin-resistant *Staphylococcus aureus*. Clin. Micro. Inf. 11(6): 510-512.

WHO (2004). WHO issues guidelines for herbal medicines. Bull. World Health Organ. 82(3): 238-238.

CHAPTER 2

The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents

ABSTRACT

The problem of antibiotic resistance, which has limited the use of cheap and old antibiotics, has necessitated the need for a continued search for new antimicrobial compounds. Understanding the mechanisms of resistance is important in the development of strategies to solving the problem. Active efflux of drugs, alteration of target sites and enzymatic degradations are the strategies by which pathogenic bacteria acquire or develop intrinsic resistance to antibiotics. Multidrug resistance (MDR) pumps capable of recognizing and expelling a variety of structurally unrelated compounds from the bacterial cell conferring resistance to a wide range of antibiotics have since been chracterised in many Gram positive and Gram negative pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and, more recently, in mycobacteria. The ability of some chemical compounds (called MDR inhibitors or resistance modifying agents) to modify the resistance phenotype in bacteria by working synergistically with antibiotics *in vitro* has since been observed. The search for such compounds which can be combined with antibiotics in the treatment of drug resistant infections may be an alternative to overcoming the problem of resistance in bacteria. Crude extracts of medicinal plants stand out as veritable sources of potential resistance modifying agents and the African biosphere promises to be a potential source of such compounds owing to its rich plant species diversity.

Key words: Antibiotic resistance, resistance modifying agents, plant extracts.

INTRODUCTION

Since the discovery of antibiotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eradication of infectious diseases. However diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies (Levy and Marshall, 2004). Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concerns (Iwu *et al.,* 1999). The global emergence of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Hancock, 2005). Examples include methicillin-resistant staphylococci and pneumococci that are resistant to penicillin and macrolides; vancomycin-resistant enterococci as well as multidrug-resistant Gram negative organisms (Norrby *et al.,* 2005).

As resistance to old antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be curtailed. However, the past record of rapid, widespread and emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy (Coates *et al.,* 2002).

Confronted with a possible shortage of new antimicrobials, there is need to ensure a careful use of our available drugs. This has led to calls for controlled use of antibiotics through the reduction of dosage used per regime of treatment or by regulating prescriptions in areas such as animal husbandry and aquaculture (Hernandez, 2005). While reduced use could lead to delayed resistance development, the emergence of resistant strains is from an evolutionary viewpoint inevitable. It becomes imperative therefore that alternative approaches are explored. Targeting and blocking

resistance processes could be an attractive approach. The presence of efflux pumps and multidrug resistance (MDR) proteins in antibiotic resistant organisms contribute significantly to the intrinsic and acquired resistance in these pathogens. The discovery and development of new compounds that either block or circumvent resistance mechanisms could improve the containment, treatment, and eradication of these strains (Oluwatuyi *et al.,* 2004). A few studies such as Gibbons *et al.* (2003), Dickson *et al.* (2006) and Braga *et al.* (2005) have reported that plant extracts can enhance the *in vitro* activity of certain antibiotics against strains of MDR *Staphylococcus aureus* and other pathogens. These studies have prompted the search for such MDR Pump or Efflux Pump inhibitors from medicinal plants. This paper reviews the mechanisms of resistance to antibiotic by pathogenic bacteria and how such processes can be curtailed by the use of plant extracts and plant derived compounds in a bid to highlight the importance of this untapped resource in the fight against the spread of antibiotic resistant pathogens.

THE CHALLENGE OF ANTIBIOTIC RESISTANCE

The development of resistance in bacteria is one of the mechanisms of natural adaptation to the presence of an antimicrobial agent that inhibits susceptible organisms and selects for the resistant ones. Under continued selection pressure, the selected resistant organisms multiply and spread to other geographic locations as well as to other microbes by transfer of resistance genes (Levy and Marshall, 2004). Selection of resistant strains occurs so rapid for some bacteria that clinical usefulness of the antibiotics is lost within a 5-year period (Bush, 2004).

The emergence and spread of microbes that are resistant to cheap and effective first-choice drugs has become a common occurrence. The problem is even more evident in bacterial infections which contribute most to the global infectious disease burden such as diarrheal, respiratory tract, meningitis, sexually transmitted infections, and tuberculosis (WHO, 2002). Resistance to penicillin in *Staphylococcus aureus* first appeared in 1942 immediately following its clinical use. By the late 1960s, more than 80% of both community- and hospital-acquired staphylococcal isolates were resistant to penicillin (Lowy, 2003). At present most clinical isolates of *Staphylococcus aureus* are multiple-drug resistant (resistant to three or more of agents such as ciprofloxacin, erythromycin, clindamycin, gentamycin, trimethoprim/sulphamethoxazole, linezolid, and vancomycin) (Styers *et al.,* 2006). Global resistance rates in *Streptococcus pyogenes* isolates are as high as 80% for erythromycin and 50% for penicillins (Low, 2005). Recently, strains of *Mycobacterium tuberculosis* that are resistant to virtually all classes of drugs currently available for the treatment of TB (isoniazid, rifampicin, fluoroquinolones, aminoglycosides (amikacin, kanamycin and capreomycin)) have been identified in the KwaZulu Natal Province of South Africa (Gandhi *et al.,* 2006) earning a new classification termed, Extremely Drug Resistant Tuberculosis (XDR TB).

When infections become resistant to first-choice or first-line antimicrobials, treatment has to be switched to second- or third-line drugs, which are nearly always expensive. In many poor countries, the high cost of such replacement drugs is prohibitive, with the result that some diseases can no longer be treated in areas where resistance to first-line drugs is widespread (WHO, 2002). Faced with such a challenge, there is need to develop alternative approaches in addition to the search for new antimicrobial compounds. Such approaches might include strategies that target resistance mechanisms coupled with antibiotics.

MECHANISMS OF ANTIBIOTIC RESISTANCE IN PATHOGENIC BACTERIA

Resistance to antimicrobials is as a result of three main strategies namely enzymatic inactivation of the drug (Davies, 1994), modification of target sites (Spratt, 1994) and extrusion by efflux (Nikaido, 1994). While chemical modifications could be significant in antibiotic resistance, exclusion from the cell of unaltered antibiotic represents the primary strategy in denying the antibiotic, access to its targets and this is believed to enhance resistance even in cases where modification is the main mechanism (Li *et al.,* 1994b).

Alteration of target sites

Chemical modifications in the antibiotic target may result in reduced affinity of the antibiotic to its binding site (Lambert, 2005). This is a mechanism employed by a number of pathogenic bacteria in evading the effect of antibiotics. Modifications are usually mediated by constitutive and inducible enzymes. Resistance to macrolides, lincosamide and streptogramin B antibiotics (MLS_B resistance) in pathogenic *Streptococcus* species is a result of methylation of the N^6 amino group of an adenine residue in 23S rRNA. This is presumed to cause conformational changes in the ribosome leading to reduced binding affinity of these antibiotics to their binding sites in the 50S ribosomal subunit (Seppala *et al.,* 1998; Kataja *et al.,* 1998). Beta-lactams antibiotics function by binding to and inhibiting the biosynthetic activity of Penicillin Binding Proteins (PBPs), thereby blocking cell wall synthesis. In *Staphylococcus aureus* and *Streptococcus pneumoniae,* resistance to beta-lactams can be a result of mutations leading to the production of PBP2a and PBP2b respectively. The two proteins have a reduced affinity for beta-lactams and yet they take over the functions of normal PBPs in the presence of inhibitory levels of beta-lactams (Golemi-Kotra *et al.,* 2003; Grebe and

Hakenbeck, 1996). This mechanism of resistance is also responsible for beta-lactam resistance in non- beta-lactamase producing *Haemophillus influenzae* (Matic *et al.,* 2003).

Enzymatic inactivation

The production of hydrolytic enzymes and group transferases is a strategy employed by a number of pathogens in evading the effect of antibiotics (Wright, 2005). Genes that code for antibiotic degrading enzymes are often carried on plasmids and other mobile genetic elements. The resistance to beta-lactam antibiotics by both Gram negative and Gram positive bacteria has long been attributed to beta-lactamases (Frere, 1995). These enzymes confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the four-membered beta-lactam ring (Wilke *et al.,* 2005). Resistance to aminoglycosides in Gram negative bacteria is most often mediated by a variety of enzymes that modify the antibiotic molecule by acetylation, adenylation or phosphorylation (Over *et al.,* 2001).

Antibiotic efflux

It is now widely recognized that constitutive expression of efflux pump proteins encoded by house-keeping genes that are widespread in bacterial genomes are largely responsible for the phenomenon of intrinsic antibiotic resistance (Lomovskaya and Bostian, 2006). Several studies have shown that active efflux can be a mechanism of resistance for almost all antibiotics (Li *et al.* 1994a; Gill *et al.* 1999 and Lin *et al.* 2002). The majority of the efflux systems in bacteria are non-drug-specific proteins that can recognize and pump out a broad range of chemically and structurally unrelated compounds from bacteria in an energy-dependent manner, without drug alteration or degradation (Kumar and Schweizer, 2005). The consequence of this drug extrusion is that, it leads to a reduced

intracellular concentration of the antimicrobial such that the bacterium can survive under conditions of elevated antimicrobial concentration (Marquez, 2005). The MIC of the drug against such organisms will be higher than predicted.

Multidrug resistance efflux pumps are ubiquitous proteins present in both Gram positive and Gram negative bacteria as either chromosomally encoded or plasmid encoded (Akama *et al.,* 2005). Although, such proteins are present constitutively in bacteria, the continued presence of the substrate induces over-expression (Teran *et al.,* 2003). This increased transcription is responsible for the acquired resistance. In Gram negative bacteria, the effect of the efflux pumps in combination with the reduced drug uptake due to the double membrane barrier is responsible for the high inherent and acquired antibiotic resistance often associated with this group of organisms (Lomovskaya and Bostian, 2006).

Efflux transporters constitute about 6% to 18% of all transporters found in any given bacterial cell (Paulsen *et al.,* 1998). Currently, much attention is being paid towards understanding the operating mechanisms of these pumps. This has potential applications in the design of transport inhibitors that could be used in combination with antibiotics in development of clinically useful drugs (McKeegan *et al.,* 2004).

The MDR pumps of pathogenic bacteria known so far, belong to five families of transporters namely; the major facilitator superfamily (MFS), the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family and the resistance-nodulation-cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) family (Kumar and Schweizer, 2005).

Some characterized Efflux proteins of pathogenic bacteria

The NorA protein of *Staphylococcus aureus* is the best studied chromosomally encoded pump in pathogenic Gram positive bacteria (Hooper, 2005). It is present in *Staphylococcus epidermidis* but appears to be absent in *Enterococcus faecalis* or in Gram negative organisms, such as *E. coli* and *K pneumoniae* (Kaatz *et al.,* 1993). Overexpression of the NorA gene in *Staphylococcus aureus* confers resistance to chloramphenicol and hydrophilic fluoroquinolone antimicrobials (Hooper, 2005; Kaatz and Seo, 1995).

QacA is a member of the major facilitator superfamily of transport proteins, which are involved in the uniport, symport, and antiport of a wide range of substances across the cell membrane (Mitchell *et al.,* 1998). The QacA multidrug exporter from *Staphylococcus aureus* mediates resistance to a wide array of monovalent or divalent cationic, lipophilic, antimicrobial compounds. QacA provides resistance to these various compounds via a proton motive force-dependent antiport mechanism (Brown and Skurray, 2001).

The mefA efflux protein of *Streptococcus pyogenes* is a hydrophobic 44.2-kDa transposon encoded protein, of the Major Facilitator superfamily that mediates efflux of macrolides (Kohler *et al.,* 1999) resulting in the M phenotype in *Streptococcus pyogenes* (Sutcliffe *et al.,* 1996). It shares a 90% amino acid homology with MefE (Roberts *et al.,* 1999) of *Streptococcus pneumoniae* that also mediates the efflux of macrolides.

PmrA (pneumococcal multidrug resistance protein) efflux of *Streptococcus pneumoniae* is a chromosomally encoded protein of the Major facilitator family that confers a resistance profile in *Streptococcus pneumoniae* similar to that of NorA in *Staphylococcus aureus* (Kohler *et al.,* 1999).

The efflux protein which is not expressed constitutively in pneumococcal strains is responsible for low-level fluoroquinolone resistance in pneumococci (Kohler *et al.,* 1999; Gill *et al.,* 1999).

AcrAB-TolC pump is a member of the Resistance-Nodulation-cell division (RND) family of tripartite multidrug efflux pumps ubiquitous throughout Gram negative bacteria. In *Escherichia coli*, the multidrug efflux pump has been shown to expel a wide range of antibacterial agents (Touze *et al.*, 2004). The resistance to fluoroquinolones, chloramphenicol-florfenicol and tetracycline in the food borne pathogen *Salmonella enterica* serovar Typhimurium definitive phage type 104 is highly dependent on the presence of AcrAB-TolC efflux pump (Baucheron *et al.,* 2004). The tripartite pump is also the major efflux mechanism of the nosocomial pathogen *Enterobacter aerogenes* (Masi *et al.,* 2003; Pradel and Pages, 2002). The pump has also been associated with baseline level resistance of *Haemophillus influenzae* Rd to erythromycin, rifampin, novobiocin, and dyes such as ethidium bromide and crystal violet (Sanchez *et al.,* 1997).

The RND family efflux pump, MexAB-OprM, of the opportunistic pathogen, *Pseudomonas aeruginosa* has been extensively characterized. Like other tripartite efflux proteins, it consists of three membrane bound subunits, MexA, MexB, and OprM, anchoring the inner and outer membranes. The MexB subunit is central to the pump function, which spans the cytoplasmic membrane 12 times, it selects antibiotics to be exported, and is assumed to transport the substrates expending the energy of the proton gradient across the cytoplasmic membrane (Akama *et al.,* 2004). Resistance to beta-lactams and non-beta-lactam antibiotics such as quinolones, tetracyclines, and trimethoprim has been attributed to efflux by the MexAB-OprM pump (Ziha-Zarifi *et al.,* 1999). Other Mex efflux proteins namely mexCD, mexEF MexXY mediating multidrug resistance have also been cloned from the chromosome of *Pseudomonas aeruginosa* (Mine *et al.,* 1999).

THE USE OF RESISTANCE MODIFYING AGENTS IN COMBINATION WITH ANTIBIOTICS TO OVERCOME RESISTANCE

The selection pressure exerted by the continued presence of bactericidal or bacteriostatic agents facilitates the emergence and dissemination of antibiotic resistance genes. Over generations, the genotypic makeup of bacterial populations is altered (Taylor *et al.,* 2002). The clinical implications of this are that many infections become untreatable resulting in serious morbidity and mortality. Although the introduction of new compounds into clinical use has helped to curtail the spread of resistant pathogens, resistance to such new drugs, has developed in some cases. For instance, resistance to the lipopeptide, daptomycin among clinical isolates of *Enterococcus faecium* has already been detected (Pankey *et al.* 2005). This is despite the fact that the drug was first licensed in 2003 (Norrby *et al.,* 2005).

It has been observed by several studies that antibiotic combinations can have synergistic benefits and interactions between existing antibiotics (Bayer *et al.,* 1980; Hooton *et al.,* 1984; Cottagnoud *et al.,* 2000; Hallander *et al.,* 1982). Several current therapeutic regimes are based on synergistic interactions between antibiotics with different target sites. As new antimicrobial compounds are discovered, there is need to assess their potentials in combination therapies with old antibiotics that have been rendered ineffective by the development of resistant strains, even when such compounds are not directly evidently inhibitory. Taylor *et al.* (2002) suggested that the use of agents that do not kill pathogenic bacteria but modify them to produce a phenotype that is susceptible to the antibiotic could be an alternative approach to the treatment of infectious disease. Such agents could render the pathogen susceptible to a previously ineffective antibiotic, and because the modifying agent applies little or no direct selective pressure, this concept could slow down or prevent the emergence

of resistant genotypes. The inhibition of resistance expression approach was successfully used in the production of Augmentin, a combination of amoxycillin and clavulanic acid (Reading and Cole, 1977). In this case, clavulanic acid is an inhibitor of class-A beta-lactamases which is co-administered with amoxicillin. The combination has been used clinically since the late 1970s (Neu *et al.,* 1993). A similar approach can be used for target-modifying enzymes and for efflux systems.

A number of *in vitro* studies have reported the use of plant extracts in combination with antibiotics, with significant reduction in the MICs of the antibiotics against some resistant strains (Al-hebshi *et al.,* 2006; Darwish *et al.,* 2002; Betoni *et al.,* 2006). The curative effect of plant extracts in this combination study has been variably referred to as resistance modifying/modulating activity (Gibbons, 2004). This ability of plant extracts to potentiate antibiotics has not been well explained. It is speculated that inhibition of drug efflux, and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics (Lewis and Ausubel, 2006; Zhao *et al.,* 2001).

Efflux pump inhibition in combination with antibiotics as a strategy for overcoming resistance

The discovery and development of clinically useful Efflux Pump Inhibitors (EPIs) that decrease the effectiveness of efflux pumps represents a significant advance in the development of therapeutic regimes for the treatment of MDR-related conditions. This approach termed the EPI strategy (Lomovskaya and Bostian, 2006), is based on blocking the activity of the pumps, resulting in the accumulation of the antibiotic inside the bacterial cell, consequently increasing access to its target sites. In addition, this will lead to increased susceptibility of the bacterium, thus implying that the therapeutic effect of the drug is achieved with low concentrations. Combining broad spectrum efflux

pump inhibitors with current drugs that are pump substrates can recover clinically relevant activity of those compounds and thus may provide new dimensions to the ever increasing need for development of new antimicrobial agents (Kaatz, 2002). This approach will in addition lead to the preservation and improvement of the usefulness of old and cheap antibacterial agents. Ultimately this could reduce the appearance and spread of resistant mutants (Kaatz, 2002).

Multiple targets and mutual interference strategies

A combination of antimicrobials with different target sites and mechanisms of action can be beneficial in reducing resistance development. The likelihood that a pathogen could simultaneously develop resistance against more than one drug is low (Dryselius *et al.,* 2005). Other combinations may involve antibiotics and other compounds that are not antimicrobial but can enhance the activity of the antibiotics. Combinations between antibiotics and known or new antimicrobial compounds might uncover some beneficial potential that might be useful in curbing resistance to antibiotics.

Some drug formulations in current use are already based on the concept of dual targets or mutual interference (Rossolini and Mantengoli, 2005). For instance, the combination of trimethoprim and sulphamethoxazole, (co-trimoxazole) involves a mutual interference of two sequential steps in the bacterial folate biosynthesis pathway. Sulphamethoxazole competitively inhibits bacterial dihydropteroate synthetase, an enzyme involved in the first step in the reaction leading to folic acid synthesis. Trimethoprim inhibits the enzyme dihydrofolate reductase, involved in the next step in the folic acid pathway (Jerry and Smilack, 1999). Beta-lactamase inhibitors, clavulanic acid and sulbactam have been used to enhance the activity of beta lactam antibiotics against beta lactamase producing organisms (Moosdeen *et al.,* 1988; Maddux, 1991).

The synergy between epigallocatechin gallate (EGCg) in tea catechins (the main compounds responsible for the antimicrobial activity of tea) and oxacillin observed by Zhao *et al.,* (2001) was attributed to the combined action of EGCg and Oxacillin on the biosynthesis of the cell wall thereby bypassing the resistance mechanism resulting from the reduced affinity of Penicillin Binding Proteins (PBP) to Oxacillin.

PLANTS AS SOURCES OF NEW ANTIMICROBIALS AND RESISTANCE MODIFYING AGENTS

Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being (Iwu *et al.,* 1999). Owing to their popular use as remedies for many infectious diseases, searches for substances with antimicrobial activity in plants are frequent (Betoni *et al.,* 2006; Shibata *et al.,* 2005). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Lewis and Ausubel, 2006; Cowan, 1999). Examples of some of these compounds are shown in Table 1. Literature is awash with compounds that have been isolated from a variety of medicinal plants. Despite this abundant literature on the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for clinical use as antibiotics (Gibbons, 2004).

A significant part of the chemical diversity produced by plants is thought to protect plants against microbial pathogens. Gibbons (2004), observes that a number of plant compounds often classified as antimicrobial produce MIC ranges greater than 1000 μgml^{-1} which are of no relevance from a clinical perspective. Tegos *et al.* (2002), suggests that a vast majority of plant compounds showing little *in*

vitro antibacterial activity are not antimicrobial but are regulatory compounds playing an indirect role in the plant defence against microbial infections.

The observation that plant derived compounds are generally weak compared to bacterial or fungal produced antibiotics and that these compounds often show considerable activity against Gram positive bacteria than Gram negative species has been made by many (Nostro *et al.,* 2000; Gibbons, 2004). This led to Tegos *et al.* (2002) hypothesizing that; Plants produce compounds that can be effective antimicrobials if they find their way into the cell of the pathogen especially across the double membrane barrier of Gram negative bacteria. Production of efflux pump inhibitors by the plant would be one way to ensure delivery of the antimicrobial compound. This hypothesis has been supported by the findings of Stermitz *et al.,* (2000a,b), who observed that *Berberis* plants which produce the antimicrobial compound, berberine, also make the MDR inhibitors 5-methoxyhydnocarpin D (5-MHC-D) and pheophorbide A. The MDR inhibitors facilitated the penetration of berberine into a model Gram positive bacterium, *Staphylococcus aureus*. In testing their hypothesis, Tegos *et al.* (2002), showed that two MDR inhibitors (INF_{271} and MC_{207110}) dramatically increased the effectiveness of thirteen putative plant antimicrobial compounds against Gram negative and Gram positive bacteria including isolates known to express efflux pumps.

Table 1: Examples of some plant derived compounds with antimicrobial properties

Class of Compound	Examples	Plant sources	Reference
Coumarins and their derivatives	asphodelin A 4'-*O*-β-D-glucoside	*Asphodelus microcarpus*	El-Seedi (2007)
	asphodelin A		
Simple phenols	Epicatechin	*Calophyllum brasiliense*	Pretto *et al.* (2004)
	Epigallocatechin Epigallocatechin gallate Epicatechin gallate	*Camellia sinensis*	Mabe *et al.* (1999) Hamilton-Miller (1995)
Flavonoids	Isocytisoside	*Aquilegia vulgaris* L.	Bylka *et al.* (2004)
	Eucalyptin	*Eucalyptus maculate*	Takahashi *et al.* (2004)
flavones	luteolin	*Senna petersiana*	Tshikalange *et al.* (2005)
	GB1(hydroxybiflavononol)	*Garcinia kola*	Madubunyi (1995), Han *et al.* (2005)
Tannins	Ellagitannin	*Punica granatum*	Machado *et al.* (2002)
Alkaloids	Berberine	*Mahonia aquifolium*	Cernakova and Kostalova (2002)
Terpenes	Ferruginol, (Diterpene)	*Chamaecyparis lawsoniana*	Smith *et al.* (2007)
	Epipisiferol (Diterpene)		
	1-Oxoferruginol	*Salvia viridis*	Ulubelen *et al.* (2000)

These studies have provided the basis for understanding the action of plant antimicrobials, namely that vast majority of such compounds are agents with weak or narrow-spectrum activities that act in synergy with intrinsically produced efflux pump inhibitors. There is reason therefore to believe that, plants could be a source of compounds that can increase the sensitivity of bacterial cells to antibiotics. Such compounds could be useful particularly against antibiotic resistant strains of pathogenic bacteria. The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying compounds and has yet to be adequately explored.

RESISTANCE MODIFYING ACTIVITIES OF PLANT CRUDE EXTRACTS: THE BASIS FOR ISOLATION OF POTENTIALLY USEFUL COMPOUNDS

If the isolation of resistance modifying compounds from plants is to be realistic, screening for such activities in crude extracts is the first step in identifying leads for isolation of such compounds, and some plants have provided good indications of these potentials for use in combination with antimicrobial therapy. Typical examples are as follows:

Aqueous extracts of tea (*Camellia sinensis*) have been shown to reverse methicillin resistance in MRSA and also, to some extent, penicillin resistance in beta-lactamase-producing *Staphylococcus aureus* (Stapleton *et al.,* 2004). Forty to one hundred fold dilutions of tea extracts was able to reduce the MICs of high- level resistant MRSA (≥ 256 μgml^{-1}) to less than 0.12 μgml^{-1} for methicillin and penicillin (Yam *et al.,* 1998; Stapleton *et al.,* 2004). Aqueous crude khat (*Catha edulis*) extracts of Yemen showed varying antibacterial activities with a range of 5 - 20 $mgml^{-1}$ against periodontal bacteria when tested in isolation. Addition of the extracts at a sub- MIC (5 $mgml^{-1}$) resulted in a 2 to

4-fold potentiation of tetracycline against resistant strains *Streptococcus sanguis* TH-13, *Streptococcus oralis* SH-2, and *Fusobacterium nucleatum* (Al-hebshi *et al.,* 2006). Betoni *et al.* (2006), observed synergistic interactions between extracts of guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), lemongrass (*Cymbopogon citratus*), ginger (*Zingiber officinale*), carqueja (*Baccharis trimera*), and mint (*Mentha pieria*) from Brazil and some antibiotics which represented inhibitors of protein synthesis, cell wall synthesis, nucleic acid synthesis and folic acid synthesis against *Staphylococcus aureus*. Darwish *et al.* (2002), reported that sub-inhibitory levels (200 $\mu g ml^{-1}$) of methanolic extracts of some Jordanian plants showed synergistic interactions in combination with chloramphenicol, gentamycin, erythromycin and penicillin G against resistant and sensitive *Staphylococcus aureus*. The methanolic extract of *Punica granatum* (PGME) showed synergistic interactions with chloramphenicol, gentamycin, ampicillin, tetracycline, and oxacillin. The bactericidal activity of the combination of PGME (0.1 × MIC) with ampicillin (0.5 × MIC) by time-kill assays, reduced cell viability by 99.9% and 72.5% in MSSA and MRSA populations, respectively (Braga *et al.,* 2005). The ethanol extracts of the Chinese plants, *Isatis tinctoria* and *Scutellaria baicalensis* in combination with ciprofloxacin had synergistic activities against antibiotic resistant *Staphylococcus aureus* (Yang *et al.,* 2005). The combinations of penicillin with ethanolic extracts of *Paederia scandens* and *Taraxacun monlicum* showed a strong bactericidal activity on two strains of *Staphylococcus aureus* (Yang *et al.,* 2005). When ciprofloxacin was incorporated at sub-inhibitory concentrations (1/8 × MIC) to the crude chloroform extracts of *Jatropha elliptica* and the mixture assayed against NorA expressing *Staphylococcus aureus*, the activity of the extract was enhanced. This suggests the presence of an inhibitor of the pump which could restore the activity of ciprofloxacin (Marquez *et al.,* 2005). In another study, Ahmad and Aqil (2007) observed

that crude extracts of Indian medicinal plants, *Acorus calamus, Hemidesmus indicus, Holarrhena antidysenterica* and *Plumbago zeylanica* showed synergistic interactions with tetracycline and ciprofloxacin against Extended Spectrum beta-lactamase (ESβL), producing multidrug-resistant enteric bacteria with ciprofloxacin showing more synergy with the extracts than tetracycline.

Plant compounds with resistance modifying activities

Some isolated pure compounds of plant origin have been reported to have resistance modifying activities *in vitro*. Examples of some of the compounds are given in Table 2. This has prompted the search for such compounds from a variety of medicinal plants. Some of the compounds which have been observed to have direct antimicrobial activity have also been shown to potentiate the activity of antibiotics when used at below MIC levels.

Table 2: Some antibiotic resistance modifying compounds from plants.

Compound	Plant Source	Antibiotics Potentiated	Reference
Ferruginol	*Chamaecyparis lawsoniana*	Oxacillin	Smith *et al.* (2007)
		Tetracycline	
5-Epipisiferol		Norfloxacin	
		Tetracycline	
2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester	*Jatropha elliptica*	Ciprofloxacin	Marquez *et al.* (2005)
		Norfloxacin	
		Pefloxacin	
Carnosic acid carnosol	*Rosmarinus officinalis*	Erythromycin	Oluwatuyi *et al.* (2004)
Ethyl gallate	*Caesalpinia spinosa*	Beta-lactams	Shibata *et al.* (2005)
Methyl-1-α-acetoxy-7-α-14-α-dihydroxy-8,15-isopimaradien-18-oate	*Lycopus europaeus*	Tetracycline	Gibbons *et al.* (2003)
		Erythromycin	
Methyl-1-α-14-α-diacetoxy-7-α-hydroxy-8,15-isopimaradien-18-oate			
Epicatechin gallate	*Camellia sinensis*	Norfloxacin	Gibbons *et al.* (2004)
Epigallocatechin gallate		Imipenem	Hu *et al.* (2002)
		Panipenem	
		Beta-lactams	Zhao *et al.* (2001)

The antimicrobial properties of tea (*Camellia sinensis*) have been found to be a result of the presence of polyphenols (Yam *et al.,* 1998; Stapleton *et al.,* 2004; Si *et al.,* 2006). Bioassay directed fractionation of the extracts revealed that epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epicatechin (EC), and caffeine (CN) are the bioactive components. ECG and CG reduced MIC values for oxacillin from 256 and 512 to 1 and 4 mgl^{-1} against MRSA (Shibata *et al.,* 2005). Ethyl gallate, a conginer of alkyl gallates purified from a dried pod of Tara (*Caesalpinia spinosa*) native to South America, intensified beta-lactam susceptibility in MRSA and MSSA strains (Shibata *et al.,* 2005). The abietane diterpenes, (carnosic acid carnosol) isolated from the aerial parts of *Rosmarinus officinalis* by fractionation of the chloroform extract at 10 μgml^{-1}, potentiated the activity of erythromycin (16-32 fold) against strains of *Staphylococcus aureus* that express the two efflux proteins MsrA and TetK. Additionally, carnosic acid was shown to inhibit ethidium bromide efflux in a NorA expressing *Staphylococcus aureus* strain (Oluwatuyi *et al.,* 2004). A penta-substituted pyridine, 2, 6-dimethyl-4-phenylpyridine-3, 5-dicarboxylic acid diethyl ester and proparcine have been isolated from an ethanol extract of rhizome of *Jatropha elliptica* by bioassay guided fractionation. The pyridine at a concentration of 75 μgml^{-1} was shown to increase by 4-fold, the activity of ciprofloxacin and norfloxacin against NorA expressing *Staphylococcus aureus* when tested at sub-inhibitory concentrations (Marquez *et al.,* 2005). Smith *et al.* (2007), screened active compounds from the cones of *Chamaecyparis lawsoniana* for resistance modifying activities and observed that Ferruginol and 5-Epipisiferol were effective in increasing the efficacy of tetracycline, norfloxacin, erythromycin and oxacillin against resistant *Staphylococcus aureus*. The majority of researches on the combinations between plant extracts and antibiotics have been focused on the identification and isolation of potential resistance modifiers from such natural sources which are considered to be

positive results. However, it is likely that such combinations could produce antagonistic interactions that most studies have considered irrelevant and therefore ignored.

FUTURE DIRECTIONS

While there is an abundance of published data validating the antimicrobial activity of medicinal plants commonly used in folk medicine, this has not resulted in the identification of commercially exploitable plant derived antibacterial agents (Lewis and Ausubel, 2006). The majority of plant derived antimicrobial compounds generally have higher MICs than bacterial or fungal produced antibiotics, thus limiting their therapeutic potential (Gibbons, 2004). The findings of Tegos *et al.* (2002) have provided a foundation for a rationale on the potential actions of plant derived antimicrobial compounds and other compounds with no intrinsic antimicrobial value. It has already been established that crude extracts of some medicinal plants and some pure compounds from such plants can potentiate the activity of antibiotics *in vitro* (Marquez *et al.,* 2005; Smith *et al.,* 2007). This search for antibiotic resistance modulators in plants represents a new dimension to addressing the problem of antibiotic resistance. The chemical diversity available in plants still remains largely uninvestigated for potentials in improving the clinical efficacy of antibiotics. Most interestingly are medicinal plants and food plants which are inadvertently used with antibiotics in common community practices providing opportunities for interactions. As many medicinal plants still remain unexplored, there are enormous opportunities for the discovery of novel resistance modifying compounds of plant origins. Screening of antibiotic resistance modifying compounds from plants sources are expected to provide the basis for identifying leads for the isolation of therapeutically useful compounds. This

could in future be followed by *in vivo* assessments to determine the clinical relevance of such compounds. This represents a potential area of future investigation.

CONCLUSION

The quest for solutions to the global problem of antibiotic resistance in pathogenic bacteria has often focused on the isolation and characterization of new antimicrobial compounds from a variety of sources including medicinal plants. This has seen several medicinal plants being screened for antimicrobial activities. Investigations into the mechanisms of bacterial resistance have revealed that active efflux plays a significant role in the development of bacterial acquired and intrinsic resistance. Overcoming efflux has therefore been seen as an attractive alternative to circumventing the problem. Bacterial efflux pump inhibitors have since been isolated from some plants. The combination of such MDR inhibitors with antibiotics *in vitro* has shown that the activities of some antibiotics can be dramatically increased even against antibiotic resistant strains of bacteria. The large varieties of compounds produced by plants have proved to have therapeutic potentials as antimicrobials and as resistance modifiers. The African biosphere which is endowed with the highest plant species biodiversity promises to be a potential source of therapeutically useful compounds, especially from the perspective of their potentials in combination with antimicrobial chemotherapy which should form the subject of further extensive study.

ACKNOWLEDGEMENT

The authors thank the National Research Foundation (NRF) of the Republic of South Africa for financial support.

REFERENCES

Ahmad I, and Aqil F (2007). In vitro efficacy of bioactive extracts of 15 Medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. Microbio. Res: 1-12.

Akama H, Kanemaki M, Tsukihara T, Nakagawa A, and Nakae T (2005). Preliminary crystallographic analysis of the antibiotic discharge outer membrane lipoprotein OprM of *Pseudomonas aeruginosa* with an exceptionally long unit cell and complex lattice structure. Acta Cryst. F61: 131-133.

Akama H, Matsuura T, Kashiwagi S, Yoneyama H, Narita S, Tsukihara T, Nakagawa A, and Nakae T (2004). Crystal Structure of the Membrane Fusion Protein, MexA, of the Multidrug Transporter in *Pseudomonas aeruginosa*. J. Bio and Chem. 279 (25):25939-25942.

Al-hebshi N, Al-haroni M, and Skaug N (2006). *In vitro* antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms. Arch Oral Biol. 51:183-188.

Baucheron S, Tyler S, Boyd D, Mulvey MR, Chaslus-Dancla E, and Cloeckaert A (2004). AcrAB-TolC directs efflux-mediated multidrug resistance in *Salmonella enterica* Serovar Typhimurium DT104. Antimic. Agents Chemo. 48(10):3729-3735.

Bayer AS, Chow AW, Morrison JO, and Guze LB (1980). Bactericidal synergy between penicillin or ampicillin and aminoglycosides against antibiotic-tolerant lactobacilli. Antimic Agents Chemo. 17 (3):359-363.

Betoni JEC, Mantovani RP, Barbosa LN, Di-Stasi LC, and Fernandes A (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Mem. Inst. Oswaldo Cruz. 101 no.4.

Bylka W, Szaufer-Hajdrych M, Matlawska I, and Goslinska O (2004). Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris* L. Lett. Appl. Micro. 39(1):93-97.

Braga LC, Leite AAM, Xavier KGS, Takahashi JA, Bemquerer MP, Chartone-Souza E, and Nascimento AMA (2005). Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus.* Canad. J. Microbio. 51(7):541-547.

Brown MH, and Skurray RA (2001). Staphylococcal multidrug efflux protein QacA. J Mol. Micro. Biotech. 3(2):163-70.

Bush K (2004). Antibacterial drug discovery in the 21st century. Clin. Micro. Inf. 10(s4):10-17.

Cernakova M, and Kostalova D (2002). Antimicrobial activity of berberine, a constituent of *Mahonia aquifolium*. Folia Microbiol. (Praha). 47(4):375-378.

Coates A, Hu Y, Bax R, and Page C (2002). The future challenges facing the development of new antimicrobial drugs. Nat. Rev. Drug Discov. 1:895-910.

Cottagnoud P, Acosta F, Cottagnoud M, Neftel K, and Tauber MG (2000). Synergy between Trovafloxacin and Ceftriaxone against Penicillin-Resistant Pneumococci in the Rabbit Meningitis Model and *In Vitro*. Antimic. Agents Chemo. 44(8):2179-2181.

Cowan MM (1999). Plant Products as Antimicrobial Agents. Clin Micro. Rev. 12(4):564-582.

Darwish RM, Aburjai T, Al-Khalil S, and Mahafzah A (2002). Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Staphylococcus aureus*. J. Ethnopharm. 79:359-364.

Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. Science. 264:375-382.

Dickson RA, Houghton PJ, Hylands PJ, and Gibbons S (2006). Antimicrobial, resistance-modifying effects, antioxidant and free radical scavenging activities of *Mezoneuron benthamianum* Baill, *Securinega virosa* Roxb. Wlld. and *Microglossa pyrifolia* Lam. Phytother Res. 20(1):41-45.

Dryselius R, Nekhotiaeva N, and Good L (2005). Antimicrobial synergy between mRNA- and protein-level inhibitors. J. Antimic. Chemo. 56(1):97-103.

El-Seedi HR (2007). Antimicrobial Arylcoumarins from *Asphodelus microcarpus*. J. Nat. Prod. (1):118 -120.

Frere JM (1995). Beta-lactamases and bacterial resistance to antibiotics. Mol. Micro. 16 (3):385-395.

Gandhi NR, Moll A, Sturm W, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, and Friedland G (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 368:1575-1580.

Gibbons S, Oluwatuyi M, Veitch NC, and Gray AI, (2003). Bacterial resistance modifying agents from *Lycopus europaeus*. Phytochem. 62 (1): 83-87.

Gibbons S (2004). Anti-staphylococcal plant natural products. Nat. Prod. Rep. 21:263-277.

Gibbons S, Moser E, and Kaatz GW (2004). Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus*. Planta Med. 70(12): 1240-1242.

Gill MJ, Brenwald NP, and Wise R (1999). Identification of an efflux pump gene *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimic. Agents Chemo.43:187-189.

Golemi-Kotra D, Cha JY, Meroueh SO, Vakulenko SB, and Mobashery S (2003). Resistance to β-Lactam Antibiotics and Its Mediation by the Sensor Domain of the Transmembrane BlaR Signaling Pathway in *Staphylococcus aureus.* J. Biol. Chem. 278(20):18419-18425.

Grebe T, and Hakenbeck R (1996). Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. Antimic. Agents Chemo. 40(4): 829-834.

Hallander HO, Dornbusch K, Gezelius L, Jacobson K, and Karlsson I (1982). Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: interaction index and killing curve method. Antimic. Agents Chemo. 22(5):743-752.

Hamilton-Miller JM (1995). Antimicrobial properties of tea (*Camellia sinensis* L.). Antimic. Agents Chemo. 39(11):2375-2377.

Han QB, Lee SF, Qiao CF, He ZD, Song JZ, Sun HD, and Xu HX (2005). Complete NMR assignments of the antibacterial biflavonoid GB1 from *Garcinia kola*. Chem. Pharm. Bull. 53(8):1034-1036.

Hancock EW (2005). Mechanisms of action of newer antibiotics for Gram positive pathogens. Lancet Infect Dis. 5(4):209-218.

Hernandez SP (2005). Responsible use of antibiotics in aquaculture. FAO Fisheries Technical paper 469.

Hooper DC (2005). Efflux pumps and nosocomial antibiotic resistance: A primer for hospital epidemiologists. Healthcare Epidemiol. 40:1811-1817.

Hooton TM, Blair AD, Turck M, and Counts GW (1984). Synergism at clinically attainable concentrations of aminoglycoside and beta-lactam antibiotics. Antimic. Agents Chemo. 26(4):535-538.

Hu ZQ, Zhao WH, Asano N, Yoda Y, Hara Y, and Shimamura T (2002). Epigallocatechin gallate synergistically enhances the activity of carbapenems against methicillin-resistant *Staphylococcus aureus*. Antimic Agents Chemo. 46(2):558-560.

Iwu MW, Duncan AR, and Okunji CO (1999). New antimicrobials of plant origin. J. Janick (ed.), Perspectives on new crops and new uses: 457-462.

Jerry D, and Smilack MD (1999). Trimethoprim-Sulfamethoxazole. Mayo Clin. Proc. 74: 730-734.

Kaatz GW, Seo SM, and Ruble CA (1993). Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimic. Agents Chemo. 37(5):1086-1094.

Kaatz GW, and Seo SM (1995). Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. Antimic. Agents Chemo. 39(12):2650-5.

Kaatz GW (2002). Inhibition of bacterial efflux pumps: a new strategy to combat increasing antimicrobial agent resistance. Expert Opin Emerg Drugs. 7(2):223-33.

Kataja J, Seppala H, Skurnik M, Sarkkinen H, and Huovinen P (1998). Different Erythromycin Resistance Mechanisms in Group C and Group G Streptococci. Antimicrob Agents Chemother. 42(6):1493-1494.

Kohler T, Pechere JC, and Plesiat P (1999). Bacterial antibiotic efflux systems of medical importance. Cell. Mol. Life Sci. 56:771-778.

Kumar A, and Schweizer HP (2005). Bacterial resistance to antibiotics: Active efflux and reduced uptake. Adv. Drug Delivery Revs. 57:1486-1513.

Lambert PA (2005). Bacterial resistance to antibiotics: Modified target sites. Adv. Drug Delivery Revs. 57(10):1471-1485.

Levy SB, and Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. Nat Med. 10:S122 - S129.

Lewis K, and Ausubel FM (2006). Prospects for plant-derived antibacterials. Nat. Biotech. 24(12):1504- 1507.

Li XZ, Livermore DM, and Nikaido H (1994a). Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimic. Agents Chemo. 38(8):1732-1741.

Li XZ, Ma D, Livermore DM, and Nikaido H (1994b). Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to beta-lactam resistance. Antimic. Agents Chemo. 38(8):1742-1752.

Lin J, Michel LO, and Zhang Q (2002). Cme ABC functions as a multidrug efflux system in *Campylobacter jejuni*. Antimic. Agents Chemo. 46:2124-2131.

Lomovskaya O, and Bostian KA (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic - A vision for applied use. Biochem Pharmacol. 7(1):910-918.

Low DE (2005). Changing trends in antimicrobial-resistant Pneumococci: It's not all bad news. Clin. Inf. Dis. 41:S228-S233.

Lowy DF, (2003). Antimicrobial resistance: the example of *Staphylococcus aureus.* J. Clin. Invest. 111:1265-1273

Mabe K, Yamada M, Oguni I, and Takahashi, T. (1999). *In Vitro* and *In Vivo* Activities of Tea Catechins against *Helicobacter pylori*. Antimic. Agents Chemo. 43(7):1788-1791.

Machado TB, Leal ICR, Amaral ACF, Santos KRN, Silva MG, and Kuster RM (2002). Antimicrobial Ellagitannin of *Punica granatum* Fruits. J. Brazilian Chem. Soc. 13(5):606-610.

Maddux MS (1991). Effects of beta-lactamase-mediated antimicrobial resistance: the role of beta-lactamase inhibitors. Pharmacol. 11(2):40S-50S.

Madubunyi II (1995). Antimicrobial activities of the constituents of *Garcinia Kola* Seeds. Int. J. Pharmacog. 33(3):232-237.

Marquez B, Neuville L, Moreau NJ, Genet JP, Santos AF, Andrade MCC, and Sant'Ana AEG (2005). Multidrug resistance reversal agent from *Jatropha elliptica*. Phytochem. 66: 1804-1811.

Marquez B (2005). Bacterial efflux systems and efflux pumps inhibitors. Biochimie Vol. 87 12, 1137-1147.

Masi M, Pages JM, and Pradel E (2003). Overexpression and purification of the three components of the Enterobacter aerogenes AcrA-AcrB-TolC multidrug efflux pump. J Chromatogr B Analyt Technol Biomed Life Sci. 786(1-2):197-205.

Matic V, Bozdogan B, Jacobs MR, Ubukata K, and Appelbaum PC (2003). Contribution of beta-lactamase and PBP amino acid substitutions to amoxicillin/clavulanate resistance in beta-lactamase-positive, amoxicillin/clavulanate-resistant *Haemophilus influenzae*. J. Antimic. Chemo. 52(6): 1018-1021.

McKeegan KS, Borges-Walmsley MI, and Walmsley AR (2004). Structural understanding of efflux-mediated drug resistance: potential routes to efflux inhibition. Curr. Opinion pharmacol. 4(5): 479-486.

Mine T, Morita Y, Kataoka A, Mizushima T, and Tsuchiya T (1999). Expression in *Escherichia coli* of a New Multidrug Efflux Pump, MexXY from *Pseudomonas aeruginosa*. Antimic. Agents Chemo. 43(2): 415-417.

Mitchell BA, Brown MH, and Skurray RA (1998). QacA Multidrug Efflux Pump from *Staphylococcus aureus*: Comparative Analysis of Resistance to Diamidines, Biguanidines, and Guanylhydrazones Antimic. Agents Chemo. 42(2):475-477.

Moosdeen F, Williams JD, and Yamabe S (1988). Antibacterial characteristics of YTR 830, a sulfone beta-lactamase inhibitor, compared with those of clavulanic acid and sulbactam. Antimic. Agents Chemo. 32(6):925-927.

Nikaido H (1994). Prevention of drug access to bacterial targets: Permeability barriers and active efflux. Science. 264:382-388.

Neu HC, Wilson AP, and Gruneberg RN (1993). Amoxycillin/clavulanic acid: a review of its efficacy in over 38,500 patients from 1979 to 1992. J. Chemother. 5(2): 67-93.

Norrby RS, Nord CE, and Finch R (2005). Lack of development of new antimicrobial drugs: a potential serious threat to public health. Lancet Inf. Dis. 5(2):115-119.

Nostro A, Germarno MP, D'Angelo V, Marino A, and Canatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett in Appl Microbiol. 30: 379-384.

Oluwatuyi M, Kaatz GW, and Gibbons S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochem. 65(24): 3249-3254.

Over U, Gur D, Unal S, and Miller GH, Aminoglycoside Resistance Study Group (2001). The changing nature of aminoglycoside resistance mechanisms and prevalence of newly recognized resistance mechanisms in Turkey. Clin. Microbiol. Infection. 7(9): 470-478.

Pankey G, Ascraft D, and Patel N (2005). *In vitro* synergy of daptomycin plus rifampin against *Enterococcus faecium* resistant to both linezolid and vancomycin. Antimic. Agents Chemo. 49(12):5166-5168.

Paulsen IT, Sliwinski MK, and Saier Jr MH (1998). Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. J. Mol. Biol. 277: 573-592.

Pradel E, and Pages JM (2002). The AcrAB-TolC efflux pump contributes to multidrug resistance in the nosocomial pathogen *Enterobacter aerogenes*. Antimic. Agents Chemo. 46(8):2640-3.

Pretto JB, Cechinel-Filho V, Noldin VF, Sartori MRK, Isaias DEB, and Cruz AB (2004). Antimicrobial activity of fractions and compounds from *Calophyllum brasiliense* (Clusiaceae/Guttiferae). J. Biosciences. 59(9-10):657-662.

Reading C, and Cole M (1977). Clavulanic Acid: a Beta-Lactamase-Inhibiting Beta-Lactam from *Streptomyces clavuligerus*. Antimic. Agents Chemo. 11(5):852-857.

Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, and Seppala H (1999). Nomenclature for Macrolide and Macrolide-Lincosamide-Streptogramin B Resistance Determinants. Antimic Agents Chemo. 43(12): 2823-2830.

Rossolini GM and Mantengoli E (2005). Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. Clin. Micro. Inf. 11(s4):17-32.

Sanchez L, Pan W, Vinas M, and Nikaido H (1997). The acrAB homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. J. Bacteriol. 179(21):6855-6857.

Seppala H, Skurnik M, Soini H, Roberts MC, and Huovinen P (1998). A Novel Erythromycin Resistance Methylase Gene (*ermTR*) in *Streptococcus pyogenes*. Antimic. Agents Chemo. 42(2):257-262.

Sutcliffe J, Tait-Kamradt A, and Wondrack L (1996). *Streptococcus pneumoniae* and *Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimic. Agents Chemo. 40(8):1817-1824.

Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, and Higuti T (2005). Alkyl gallates, intensifiers of ß-lactam susceptibility in Methicillin-Resistant *Staphylococcus aureus* Antimic. Agents Chemo. 49 (2), 549-555.

Si W, Gong J, Tsao R, Kalab M, Yang R, and Yin Y (2006). Bioassay-guided purification and identification of antimicrobial components in Chinese green tea extract. J. Chromatography 1125(2):204-210.

Smith ECJ, Williamson EM, Wareham N, Kaatz GW, and Gibbons S (2007). Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. Phytochem. 68(2):210-217.

Spratt BG (1994). Resistance to antibiotics mediated by target alterations. Scince. 264:388-393.

Stapleton PD, Shah S, Anderson JC Hara Y, Hamilton-Miller JMT, and Taylor PW (2004). Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates. Int. J. Antimic. Agents. 23(5):462-467.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, and Lewis K (2000a). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. Appl. Bio. Sciences. 97(4):1433-1437.

Stermitz FR, Tawara-Matsuda J, Lorenz P, Mueller P, Zenewicz L, and Lewis K (2000b). 5'-Methoxyhydnocarpin-D and Pheophorbide A: *Berberis* Species Components that Potentiate Berberine Growth Inhibition of Resistant *Staphylococcus aureus.* J. Nat. Prod. 63(8): 1146 - 1149.

Styers D, Sheehan DJ, Hogan P, and Sahm DF (2006). Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. Ann. Clin. Micro. Antim. 5:2

Takahashi T, Kokubo R, and Sakaino M (2004). Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*. Lett. Appl. Micro. 39(1):60-64.

Taylor PW, Stapleton PD, and Luzio JP (2002). New ways to treat bacterial infections. DDT. 7(21): 1086-1091.

Tegos G, Stermitz FR, Lomovskaya O, and Lewis K (2002). Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials, Antimic. Agents Chemo. 46(10): 3133-3141.

Teran W, Antonia Felipe, Segura A, Rojas A, Ramos JL, and Gallegos MT (2003). Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the Drug Binding Repressor TtgR. Antimic Agents Chemo. 47(10): 3067-3072.

Touze T, Eswaran J, Bokma E, Koronakis E, Hughes C, and Koronakis V (2004). Interactions underlying assembly of the *Escherichia coli* AcrAB-TolC multidrug efflux system. Mol. Micro. 53 (2):697–706.

Tshikalange TE, Meyer JJM, and Hussein AA (2005). Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. J. Ethnopharmacology. 96(3): 515-519.

Ulubelen A, Oksuz S, Kolak U, Bozok-Johansson C, Celik C, and Voelter W (2000). Antibacterial diterpenes from the roots of *Salvia viridis*. Planta Med.Vol. 66(5): 458-462.

Wilke MS, Lovering AL, and Strynadka NCJ (2005). β-Lactam antibiotic resistance: a current structural perspective. Curr Opinion Microbiol. 8(5): 525-533.

Wright GD (2005). Bacterial resistance to antibiotics: Enzymatic degradation and modification. Adv. Drug Delivery Reviews. 57(10): 1451-1470.

World Health Organization (WHO) (2002). Antimicrobial resistance. Fact sheet No. 194.

Yam TS, Hamilton-Miller JM, and Shah S (1998). The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2' synthesis, and beta-lactamase production in *Staphylococcus aureus*. J. Antimic. Chemo. 42(2): 211-216.

Yang ZC, Wang BC, Yang XS, Wang Q, and Ran L (2005). The synergistic activity of antibiotics combined with eight traditional Chinese medicines against two different strains of *Staphylococcus aureus.* Colloids and surfaces B: Biointerfaces, 41(2-3): 79-81.

Zhao WH, Hu ZQ, Okubo S, Hara Y, and Shimamura T (2001). Mechanism of synergy between Epigallochatechin gallate and β-Lactams against methicillin resistant *Staphylococcus aureus*. Antimic. Agents Chemo. 45(6): 1737-1742.

Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, and Plesiat P (1999). *In vivo* emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimic. Agents Chemo. 43(2): 287-91.

CHAPTER 3

In vitro antibacterial regimes of crude aqueous and acetone extracts of *Garcinia kola* seeds

ABSTRACT

Aqueous and acetone extracts of *Garcinia kola* seeds were screened for activity against 27 bacterial isolates at 30 $mgml^{-1}$ and 10 $mgml^{-1}$ respectively. The aqueous extracts showed activity mainly against Gram positive organisms with MIC values ranging from 5 – 20 $mgml^{-1}$. The acetone extract showed activity against both Gram negative and Gram positive organisms with MIC values ranging from 0.156 - 10 $mgml^{-1}$. The bactericidal activity of the acetone extract was evaluated against *Staphylococcus aureus* ATCC 6538 and *Proteus vulgaris* CSIR 0030 by time-kill assay. The extract showed rapid bactericidal activity achieving a 3.097 Log_{10} reduction in counts within 4 hours at 0.3125 $mgml^{-1}$ against *S. aureus* ATCC 6538 and complete eradication of the organism in 8 hours at 2 × and 3 × MIC levels. A 1.582 Log_{10} reduction in counts was observed against *P. vulgaris* CSIR 0030 at 5 $mgml^{-1}$ after 1 hour of exposure and a complete eradication was observed in 2 hours. We propose that acetone extract of *Garcinia kola* seeds possess strong bactericidal activities and can be chemotherapeutically useful in the treatment of bacterial infections in humans.

Key words: *Garcinia kola*, acetone extract, bactericidal potency, killing rate, log reduction.

INTRODUCTION

The problem of bacterial resistance to commonly used antibiotics has necessitated the search for newer and alternative compounds for the treatment of drug resistant infections and the high cost of conventional drugs particularly in resource poor communities of the developing world has led to the increased use of plants as an alternative for the treatment of infectious diseases. Medicinal plants have for generations been used for the treatment of ailments including infectious diseases. Several findings on the chemotherapeutic potentials of some plants have shown that they can be sources of antimicrobial compounds of value (Rios and Recio, 2005).

Garcinia kola (Heckel), of the family of Guttiferae is a tropical tree of evergreen forests found in moist semi deciduous forest zones and savannah (Agyili *et al.,* 2006). It is cultivated and distributed throughout West and Central Africa where it is valued for its medicinal properties. The medicinal properties of the plant have been a subject of numerous investigations. The seeds commonly known as bitter kola are used by communities for the treatment of bronchitis and throat infections (Iwu *et al.,* 1999). It is also used to prevent and relieve colic, cure head or chest colds and relieve cough (Farombi, 2000). The stems and twigs of the plant are used as chewing sticks in maintenance of oral hygiene (Ndukwe *et al.,* 2005).

Aqueous, ethanolic and petroleum ether extracts of the seeds have been observed to possess antibacterial properties (Ezeifeka *et al.,* 2004), and Kolaviron, (a fraction of the defatted ethanol extract of the seed, containing *Garcinia* biflavonoid GB1, GB2 and kolaflavonone) has been reported to possess numerous therapeutic potentials (Farombi *et al.,* 2002; Uko *et al.,* 2001) along with such

components as mixtures of phenolic compounds, biflavonoids, xanthones, benzophenones and related triterpenes (Han *et al.,* 2005).

While numerous work have been done on the antimicrobial potentials of this plant, the overwhelming majority of the studies have concentrated on oral and respiratory tract pathogens (Akoachere *et al.,* 2002; Ndukwe *et al.,* 2005), thus underestimating the antimicrobial potentials of the plant. Also, reports so far available on the antibacterial potentials of the seed show that activity has been demonstrated for the aqueous, ethanolic and petroleum ether extracts of the seed (Ezeifeka *et al.,* 2004). There are no documented reports in literature on the antimicrobial potentials of the acetone extract of the seeds, despite the fact that acetone has been shown to be an efficient extractant of bioactive components (Eloff, 1998). In addition, while previous researchers have used MICs and MBCs as prediction tools for antimicrobial action of the plant extracts, there are limitations to the use of such data since it does not consider time-related antimicrobial effects (Kiem and Schentag, 2006), such as killing rate. The bactericidal potencies of the extracts of the plant in terms of the kinetics of bacterial death of the extracts of the seeds have not been reported. In this paper, we report the antibacterial potentials of the aqueous and acetone extracts of the seeds of *Garcinia kola* against a widened panel of bacterial pathogens especially those not normally related to respiratory tract infections, and using the killing rate of the extract as predicting tool of their bactericidal efficiency.

MATERIALS AND METHODS

Plant material

This study was conducted at the University of Fort Hare's Department of Biochemistry and Microbiology between the period April to July 2007. The plant material was prepared following the description of Farombi (2000). Peeled seeds of *Garcinia kola* were cut into pieces and dried in an oven at 40 ^{o}C for 48 hours. The material was then ground into a powder using a mechanical blender.

Preparation of extracts

The acetone and aqueous extracts of the plant were prepared in accordance to the description of Basri and Fan (2005). One hundred grams of seed powder was extracted in two steps with 500 ml and 300 ml of the respective solvent for 48 hour. Aqueous extracts were freeze dried at -50 ^{o}C under vacuum and acetone extracts were concentrated under reduced pressure using a rotary evaporator at 50 ^{o}C. The concentrated acetone extracts were then allowed to dry at room temperature to a constant weight. When not immediately used, the extracts were stored in air tight bottles at 4 ^{o}C.

Bacterial isolates used in the study

Bacterial strains used in this study consisted of reference strains obtained from the South African Bureau of Standards (SABS), namely, *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Pseudomonas aeruginosa* ATCC 7700, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 10031, *Klebsiella pneumoniae* ATCC 4352, *Proteus vulgaris* ATCC 6830, *Proteus vulgaris* CSIR 0030, *Serratia marcescens* ATCC 9986, *Acinetobacter*

calcoaceticus Aci1, *Acinetobacter calcoaceticus* Aci2. Also included in this study were environmental bacterial strains of *Klebsiella pneumoniae*, *Bacillus subtilis*, *Shigella flexineri*, *Salmonella* spp, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterococcus faecalis*, *E. coli*, *Staphylococcus aureus*, *Micrococcus luteus* and *Micrococcus kristinae*.

Assay for antibacterial activity

The antibacterial activities of the crude extracts were carried out using the agar dilution method (Afolayan and Meyer, 1997). Stock solutions of the extract were prepared by reconstituting the dried extract in the extracting solvents. This was used to prepare dilutions of the extract in molten Mueller Hinton agar maintained in a water bath at 50 ^{o}C to achieve concentrations of 30 mgml^{-1} for the aqueous extract and 10 mgml^{-1} for the acetone extract, while also achieving a final acetone concentration of 5% in the acetone extract media. An inoculum of each test organism prepared as described by Nostro *et al.* (2000) was used to seed the agar plates by streaking in duplicates. The inoculated plates were incubated under aerobic conditions at 37 ^{o}C for 24 hours. Positive controls consisted of extract free plates of Mueller Hinton agar inoculated with the test organism and negative control consisting of uninoculated plates. Control plates for the acetone extract consisted of Mueller Hinton agar plates with 5% acetone (which represented the final acetone concentration in the test plates). The absence of growth on the test plate compared with the positive control was used to indicate the inhibitory activity of the extracts (Afolayan and Meyer, 1997).

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the extracts was determined using the agar dilution method following the standard protocol of the European Committee for Antimicrobial Susceptibility Testing

(EUCAST, 2000). Acetone extracts were diluted in such a way that the highest concentration of the solvent in agar was 5% (at this concentration, the solvent was found to have no inhibitory effect on the test organisms). Control plates contained extract free Mueller Hinton agar plates for the aqueous extracts. Control plates for the acetone extract contained 5% acetone. Plates were inoculated with overnight broth cultures of the test organisms diluted 1:100 with fresh sterile nutrient broth and incubated for 18 hours at 37 ^{o}C. The MIC was defined as the lowest concentration of the extract that was able to inhibit the visible growth of the test organism (EUCAST, 2000).

Determination of the rate of kill of the crude extracts

The rate of kill determination was done by monitoring of bacterial cell death over time as in accordance with the description of Okoli and Iroegbu, (2005). The assay was done for the acetone extract on *Staphylococcus aureus* ATCC 6538 and *Proteus vulgaris* CSIR 0030 as representative Gram positive and Gram negative organisms respectively. The inoculum was prepared using the colony suspension method following the guidelines described in the EUCAST Discussion Document, (2003). The resultant suspension was diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volumes of Mueller Hinton broth incorporated with extract at multiples of the MIC to a final cell density of approximately 5×10^{5} cfuml^{-1}. The flasks were incubated with shaking at 37 ^{o}C. Samples (100 µl) were withdrawn at intervals and diluted appropriately and known volumes of diluted samples were plated out in triplicates on Mueller Hinton agar. Plates were incubated at 37 ^{o}C for 24 hours after which the numbers of survivors were enumerated. Controls consisted of extract free Mueller Hinton broth inoculated with the test organism.

RESULTS

Of the twenty seven bacterial isolates tested 17 were susceptible to the aqueous extract at a concentration of 30 $mgml^{-1}$, while 24 isolates were susceptible to the acetone extract at a concentration of 10 $mgml^{-1}$ (Table 1). *Enterobacter cloacae* ATCC13047, *Klebsiella pneumoniae* ATCC10031 and an environmental strain of *Klebsiella pneumoniae* were not susceptible to either the aqueous or acetone extract of the seeds. The minimum inhibitory concentrations (MIC) of the aqueous extract ranged between 5 and 20 $mgml^{-1}$, while that of the acetone extracts were generally lower and ranged between 0.156 and 10 $mgml^{-1}$ (Table 2). Differences were also observed in the susceptibilities of Gram positives (MIC values, 0.156 - 0.625 $mgml^{-1}$) and Gram negative test organisms (2.5 - 10 $mgml^{-1}$) to the acetone extract.

The killing rate studies revealed that the acetone extract exhibited bactericidal activities against *Staphylococcus aureus* ATCC 6538 and *Proteus vulgaris* CSIR 0030 resulting in the eradication of approximately 10^5 $cfuml^{-1}$ in 2 to 8 hours. Both the 2 × MIC and 3 × MIC of the acetone extract killed 100% of *Staphylococcus aureus* ATCC 6538 in 8 hours (Figure 1), while total eradication of *Proteus vulgaris* CSIR 0030 was achieved in 2 hours by the 2 × MIC of the extract against the organism (Figure 2).

Table 1: Antimicrobial activity of Aqueous and Acetone extracts of *Garcinia kola* seeds against bacterial isolates

Test organism	**Crude Extracts**	
	Aqueous **30 mgml^{-1}**	**Acetone** **10 mgml^{-1}**
Escherichia coli ATCC 8739	-	+
Escherichia coli ATCC 25922	-	+
Staphylococcus aureus ATCC 6538	+	+
Streptococcus faecalis ATCC 29212	+	+
Bacillus cereus ATCC 10702	+	+
Bacillus pumilus ATCC 14884	+	+
Pseudomonas aeruginosa ATCC 7700	+	+
Enterobacter cloacae ATCC 13047	-	-
Klebsiella pneumoniae ATCC 10031	-	-
Klebsiella pneumoniae ATCC 4352	-	+
Proteus vulgaris ATCC 6830	+	+
Proteus vulgaris CSIR 0030	+	+
Serratia marcescens ATCC 9986	-	+
Acinetobacter calcoaceticus Aci1	+	+
Acinetobacter calcoaceticus Aci2	+	+
Klebsiella pneumoniae	-	-
Bacillus subtilis	+	+
Shigella flexineri	-	+
Salmonella spp	-	+
Staphylococcus epidermidis	+	+
Pseudomonas aeruginosa	+	+
Proteus vulgaris	+	+
Enterococcus faecalis	+	+
Escherichia coli	-	+
Staphylococcus aureus	+	+
Micrococcus luteus	+	+
Micrococcus kristinae	+	+

Key: + susceptible to the extract, - not susceptible

Table 2: The minimum inhibitory concentrations (MIC) of the crude extracts of *Garcinia kola*.

Test organism	**MIC Values of Crude extracts ($mgml^{-1}$)**	
	Aqueous	**Acetone**
Staph. aureus ATCC 6538	10	0.156
Str. faecalis ATCC 29212	10	0.3125
B. cereus ATCC 10702	10	0.156
B. pumilus ATCC 14884	5	0.156
Ps. aeruginosa ATCC 7700	10	10
P. vulgaris ATCC 6830	20	5
P. vulgaris CSIR 0030	20	2.5
B. subtilis	10	0.156
Staph. epidermidis	10	0.3125
P. vulgaris	20	5
Ent. faecalis	20	0.3125
Staph. aureus	10	0.3125
Micro. luteus	10	0.625
Micro. kristinae	10	0.625
E. coli ATCC 8739	ND	10
E. coli ATCC *25922*	ND	10
Aci. calcoaceticus Aci1	ND	5
Aci. calcoaceticus Aci2	ND	5

Key: ND - not done.

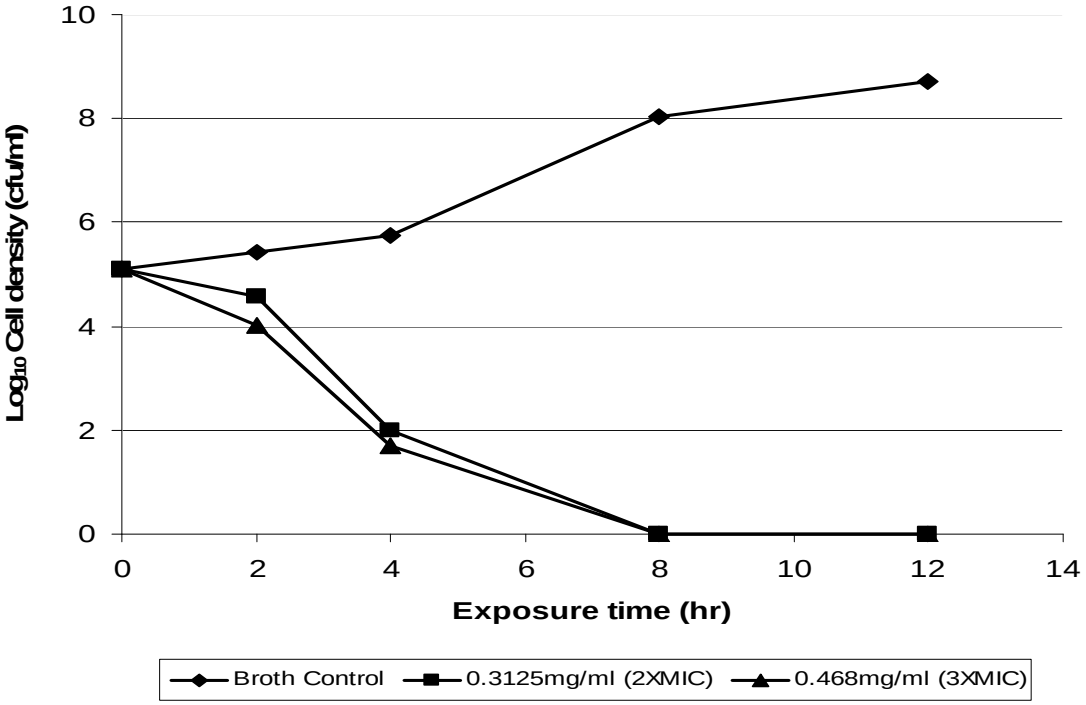

Figure 1: The killing rate regimes of acetone extract of *Garcinia kola* on *Staphylococcus aureus* ATCC 6538.

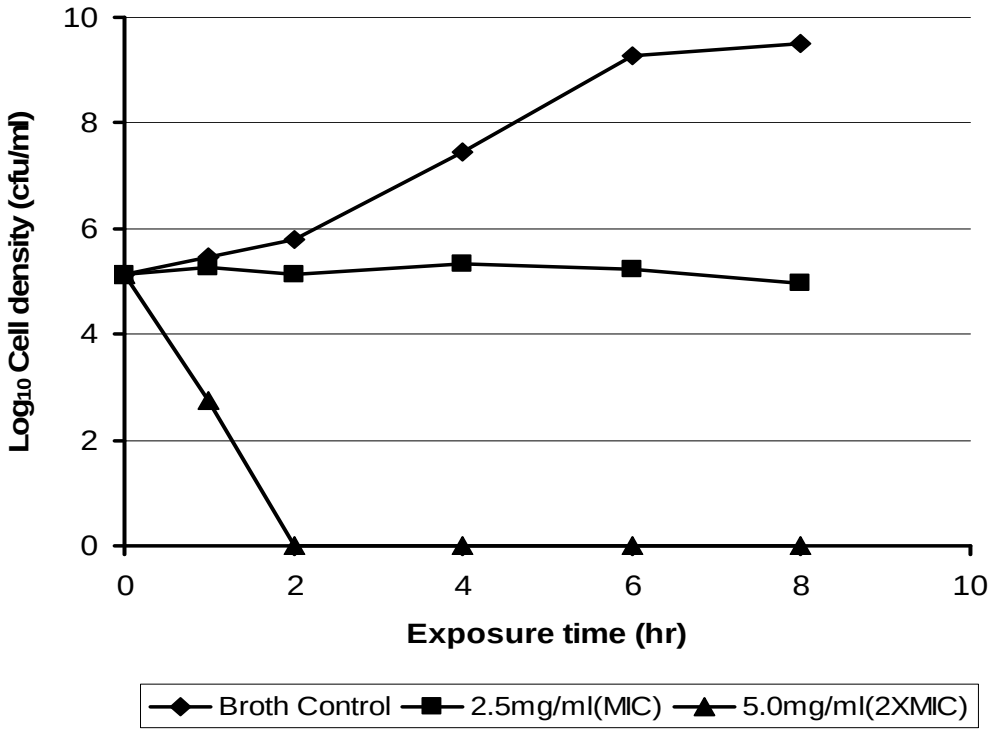

Figure 2: The killing rate regimes of acetone extract of *Garcinia kola* on *Proteus vulgaris* CSIR 0030

DISCUSSION

Garcinia kola is a plant that has shown tremendous potential as a source of novel chemotherapeutic agents. The plant is extensively utilized in traditional medicinal practices in West and Central Africa. It is therefore one of the prime medicinal plants of Africa that can provide relief to the millions of the poor people of the continent if its potentials are adequately explored.

The antibacterial activity of the aqueous extract of the seed from this study was observed mainly against Gram positive organisms with limited activity against Gram negatives. Results from this study confirm that the aqueous extracts of the seeds of *Garcinia kola* seeds possess antibacterial properties thus validating the traditional use of the seeds in treatment of oral and respiratory tract infections. The limited activity against Gram negative bacterial isolates is in agreement with the findings of Ezeifeka *et al.* (2004), who reported that crude aqueous extracts of *Garcinia kola* seeds had activity against *S. aureus* and *Ps. aeruginosa* but lacked activity against *E. coli* by disc diffusion, although it is worth noting that the concentration of the extract used in that study was not reported. It is likely that the aqueous extracts can exhibit inhibitory activity against Gram negative organisms at higher concentrations than was used in this study. We considered 30 $mgml^{-1}$ comparable to the levels often reported in literature for the screening of crude extracts in the absence of a standard. Ndukwe *et al.* (2005) observed that aqueous extracts of *Garcinia kola* stems and twigs had no antimicrobial activity against reference strains of *E. coli* and *Pseudomonas aeruginosa* but had activity against *Staphylococcus aureus* and *Bacillus cereus*, although the same extracts exhibited activity against clinical isolates of Gram negative organisms from cases of oral infections. The differences in the susceptibilities of Gram positive and Gram negative bacteria to plant extracts have been observed by several researchers (Nostro *et al.,* 2000; Suffredini *et al.,* 2006; Parekh and

Chanda, 2006). Gram negative bacteria are inherently resistant to antimicrobials and this has been ascribed to the combined exclusion of the antimicrobial compounds by the double membrane barrier (present in this group) and transmembrane efflux (Zgurskaya and Nikaido, 2000).

In contrast with the aqueous extract, the acetone extract showed activity against both Gram positive and Gram negative organisms at 10 $mgml^{-1}$ (Table 1). Results from this study confirm that acetone is a potentially good solvent for the extraction of bioactive compounds of *Garcinia kola*. Acetone has been observed to be a relatively more efficient extractant of bioactive compounds. Eloff (1998), in a comparison of ethanol, acetone, methanol, methylenedichloride, methanol/chloroform/water and water) observed acetone to be the best in terms of the quantity and diversity of compounds extracted and water, the least. It is anticipated that the acetone extract of *Garcinia kola* seeds contains a higher quantity and concentration of active compounds than the aqueous extract since water is only able to extract hydrophilic compounds.

The MIC values for the aqueous extracts which ranged from 5 to 20 $mgml^{-1}$ were higher than the values for the acetone extract which ranged from 0.156 to 10 $mgml^{-1}$. Gram positive bacteria showed more susceptibility to the acetone extract than Gram negatives. It is likely that the extraction with acetone could be resulting in an increased diversity of compounds that interact with each other in a synergistic way resulting in an increased activity of the active principles (Tegos *et al.,* 2002). The interaction might include the increased permeation of the cell membrane of Gram negative bacteria which often presents an intrinsic resistance barrier.

Because the acetone extract showed more activity than the aqueous extract, its bactericidal efficacy was investigated against *Staphylococcus aureus* ATCC 6538 and *Proteus vulgaris* CSIR 0030 by

assay of bacterial death time. The extract showed good bactericidal activity at 0.3125 $mgml^{-1}$ (2 × MIC) and 0.468 $mgml^{-1}$ (3 × MIC) against *Staphylococcus aureus* (Figure 1). Reductions of 3.097 and 3.370 Log_{10} $cfuml^{-1}$ were achieved at 0.3125 and 0.468 $mgml^{-1}$ respectively against *Staphylococcus aureus* ATCC 6538 after 4 hours of exposure. At 8 hours, no survivors could be recovered in both the 2× and 3× MIC reaction cultures. The rate of killing of *Staphylococcus aureus* ATCC 6538 by the extract appeared to be both concentration and time dependent. Preliminary investigations revealed that the extract was rapidly bactericidal at 0.625 $mgml^{-1}$ (4 × MIC) achieving a complete elimination of the organism after 30 minutes of exposure (data not shown), while at 1 × MIC, it seemed to have a Bacteriostatic effect on the test organism with no major changes on the bacterial load with time (data not shown). The extract exhibited a strong bactericidal efficacy against *Proteus vulgaris* CSIR 0030 at 5 $mgml^{-1}$ (Figure 2). A 1.258 Log_{10} reduction in counts of the test organisms was achieved after 1 hour of exposure with a complete eradication after 2 hours.

A 3 Log_{10} or ≥ 99.9% reduction in viable bacterial density in an 18 - 24 hours period is the generally accepted definition of bactericidal activity in antibiotics (Pankey and Sabath, 2004). Although the MIC values of the crude extract were higher than often observed for antibiotics (Anadiotis *et al.,* 2002; Osburne *et al.,* 2006), the bactericidal potencies against the two test organisms showed a similar pattern to that often exhibited by antibiotics.

The bactericidal potentials of the extracts of *G. kola* from this study represent a significant finding on the therapeutic potentials of this plant. Since the acetone extracts showed such strong bactericidal activity against the test organisms used in this study, it is expected that if the compounds responsible for this activity could be isolated and crystalised, therapeutically useful drugs could be obtained.

CONCLUSION

This study has shown that the aqueous and acetone extracts of the seeds of *Garcinia kola* possess antibacterial activity with the acetone extract exhibiting activity against both Gram negative and Gram positive organisms. This confirms that acetone can be a good solvent for the extraction of biologically active components of the plant and also suggests that the acetone fraction could compare favorably well with standard antibiotics with regards to time of action, and this is the subject of an accompanying paper.

ACKNOWLEDGEMENT

We are grateful to the National Research Foundation (NRF) of South Africa for the grant (Ref: TTK2006061400023) which supported this study.

REFERENCES

Agyili J, Sacande M, and Kouame C (2006). *Garcinia kola* Heckel. Seed Leaflet. No. 113 of 2006.

Afolayan AJ, and Meyer JJM (1997). The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. J. Ethnopharm. 57 (3): 177-181.

Akoachere JF, Ndip RN, Chenwi EB, Ndip LM, Njock TE, and Anong DN (2002). Antibacterial effect of *Zingiber officinale* and *Garcinia kola* on respiratory tract pathogens. Eas. Afr. Med. J. 79 (11): 588-592.

Anadiotis L, Maskell JP, and Sefton AM (2002). Comparative *in-vitro* activity of penicillin alone and combined with gentamicin against clinical isolates of *Streptococcus pneumoniae* with decreased susceptibility to penicillin. Int. J. Antimicrob. Agents. 19(3): 173-181.

Basri DF, and Fan SH (2005). The potential of aqueous and acetone extracts of galls of *Queercus infectoria* as antibacterial agents. Ind. J. Pharm. 37: 26-29.

Eloff JN (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharm. 60(1): 1-8.

European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin. Micro. and Inf. 9(8): 1-7.

European Committtee for Antimicrobial Susceptibity Testing (EUCAST) (2000). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. Clin. Micro. and Inf. 6(9): 509-515.

Ezeifeka GO, Orji MU, Mbata TI and Patrick AO (2004). Antimicrobial activity of *Cajanas cajan, Garcinia kola* and *Xylopia aethiopica* on pathogenic microorganisms. Biotech. 3(1): 41-43.

Farombi EO (2000). Mechanisms for the hepatoprotective action of kolaviron: Studies on hepatic enzymes, microsomal lipids and lipid peroxidation in carbontetrachloride-treated rats. Pharmacol. Res. 42(1): 75-80.

Farombi EO, Alabi MC, and Akuru TO (2002). Kolaviron modulates cellular redox status and impairment of membrane protein activities induced by potassium bromate ($KBrO_3$) in rats. Pharmacol. Res. 45(1): 63-68.

Han QB, Lee SF, Qiao CF, He ZD, Song JZ, Sun HD, and Xu HX (2005). Complete NMR assignments of the antibacterial biflavonoid GB1 from *Garcinia kola*. Chem. Pharm. Bull. 53(8):1034-1036.

Iwu MW, Duncan AR, and Okunji CO (1999). New antimicrobials of plant origin. J. Janick (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA., 457-462.

Kiem S, and Schentag JJ (2006). Relationship of Minimal Inhibitory Concentration and Bactericidal Activity to Efficacy of Antibiotics for Treatment of Ventilator-Associated Pneumonia. Semin. Respir. Crit. Care. Med. 27: 51-67.

Ndukwe KC, Okeke IN, Lamikanra A, Adesina SK, and Aboderin O (2005). Antibacterial Activity of Aqueous Extracts of Selected Chewing Sticks. Journal of Contemp. Dent. Prac. 6(3): 086-094.

Nostro A, Germarno MP, D'Angelo V, Marino A, and Canatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett. App. Micro. 30: 379-384

Okoli S, and Iroegbu CU (2005). *In vitro* antibacterial activity of *Synclisa scabrida* whole root extracts. Afr. J. Biotech. 4(9): 946-952.

Osburne MS, Murphy CK, and Rothstein DM (2006). Enhanced Activity of Rifalazil in Combination with Levofloxacin, Linezolid, or Mupirocin against *Staphylococcus aureus in vitro*. J. Antib. 59(5): 303-308.

Pankey GA, and Sabath LD (2004). Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram positive bacterial infections. Clin. Inf. Dis. 38: 864-870.

Parekh J, and Chanda S (2006). Screening of aqueous and alcoholic extracts of some Indian medicinal plants for antibacterial activity. Ind. J. Pharm. Sci. 68: 835-838.

Rios JL, and Recio MC (2005). Medicinal plants and antimicrobial activity. J. Ethnopharm. 100:80-84.

Suffredini IB, Paciencia MLB, Varella AD, and Younes RN (2006). Antibacterial Activity of Brazilian Amazon Plant Extracts. Braz. J. Inf. Dis. 10(6): 400-402.

Tegos G, Stermitz FR, Lomovskaya O, and Lewis K (2002). Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials, Antimic. Agents and Chemo. 46(10): 3133-3141.

Uko OJ, Usman A, and Ataja AM (2001). Some biological activities of *Garcinia kola* in growing rats. Veterinarski ARHIV. 71(5): 287-297.

Zgurskaya HI, and Nikaido H (2000). Multidrug resistance mechanisms: drug efflux across two membranes. Mol. Micro. 37(2): 219-225.

CHAPTER 4

In vitro antibacterial activities of crude extracts of *Garcinia kola* seeds against wound sepsis associated *Staphylococcus* strains

ABSTRACT

Extracts of *Garcinia kola* seeds were evaluated for their activity against four *Staphylococcus* strains isolated from wound sepsis specimens. Three of the isolates were identified by 16S rDNA sequencing as *Staphylococcus aureus* with the GenBank Accession numbers, EU244633, EU244634, EU244636, and another was identified as *Staphylococcus sciuri* (Accession number EU244635). The aqueous, methanol and acetone extracts of *Garcinia kola* seeds showed activity against all four isolates at 30 $mgml^{-1}$ (aqueous extract) and 10 $mgml^{-1}$ (acetone and methanol extracts). The MIC values for the aqueous extract were the same (10 $mgml^{-1}$) for all the isolates. The acetone and methanol extracts had lower MIC values in the ranges of 0.3125 - 0.625 $mgml^{-1}$. The acetone extract showed strong bactericidal activity against *Staphylococcus aureus* strain OKOH3 resulting in a 2.70 Log_{10} reduction in counts at 1.25 $mgml^{-1}$ (2 × MIC) within 4 hours of exposure and a complete elimination of the organism after 8 hours. The same extract was weakly bactericidal against *Staphylococcus aureus* strain OKOH1, achieving only a 2.92 Log_{10} reduction in counts at 1.25 $mgml^{-1}$ (4 × MIC) in 24 hours. The interactions between the acetone extract and antibiotics were largely additive and indifferent with no combinations showing classical synergistic interactions. We conclude that extracts of *Garcinia kola* seeds can potentially be useful in the treatment of staphylococcal wound infections.

Key words: *Staphylococcus*; wound sepsis; *Garcinia kola*; antistaphylococcal activity; antibiotic potentiation.

INTRODUCTION

The genus *Staphylococcus* is widely distributed in nature being part of the indigenous microflora of the skin and nasal cavities of healthy persons. This association with the skin presents the organisms with an opportunity to cause local infection of wounds. Among members of this genus, *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus and Staphylococcus haemolyticus* are some of the species causing community and nosocomial human infections (Oliveira *et al.,* 2006). In particular, *Staphylococcus aureus* is the causative agent of a wide range of diseases, ranging from carbuncles and food poisoning, through more serious wound-related infections, to life threatening conditions, such as bacteremia, necrotizing pneumonia, and endocarditis (Holden *et al.,* 2004). This species is capable of expressing a variety of virulence factors that it is almost always considered medically relevant when encountered in clinical specimens.

In addition to strains of *Staphylococcus* being significant pathogens in terms of the variety of infections that they cause, the organisms have been recognized as having the ability to develop changes in their sensitivity to antimicrobials (Oliveira *et al.,* 2006). In the case of *Staphylococcus aureus*, strains resistant to methicillin (MRSA) were first identified following the introduction of the antibiotic in clinical use in the 1960s (Lowy, 2003). At present MRSA strains are a common occurrence and are now virtually resistant to all beta-lactam antibiotics (Cook, 1998; Archer, 1998). In addition to the problem of treatment failure in MRSA infections, the infections have often been associated with severe illnesses resulting in increased hospitalization periods and mortality (Rello *et al.,* 1994; Engemann *et al.,* 2003). Until recently, the glycopeptide antibiotic vancomycin has been used as the drug of last resort for many MRSA infections, but recent trends show that vancomycin resistant strains have also emerged (Tenover *et al.,* 2004). Reports of isolated cases of resistance to

the newest anti-staphylococcal agent linezolid (a member of the oxazolidinone group that has been heralded as a solution to MRSA infections) have also been noted (Wilson *et al.,* 2003).

Owing to their popular use in traditional medicine for the treatment of various ailments including infectious diseases, interest in medicinal plants as a source of novel antimicrobial compounds has been growing. A number of studies have validated the use of plants in the treatment of disease conditions (Samie *et al.,* 2005; Akinpelu and Onakoya, 2006). A typical example is *Garcinia kola*, a tropical plant of the African Continent which has been a subject of investigation as a potential source of antimicrobial compounds (Madubunyi, 1995; Han *et al.,* 2005).

While it is common practice that standard reference cultures of test isolates are used in evaluating the antimicrobial activity of plant extracts, the use of clinical isolates of pathogenic organisms may provide a more relevant and accurate prediction of the therapeutic potentials of plant extracts (Rios and Recio, 2005). While the antibacterial activity of *Garcinia kola* seed extracts has been demonstrated against reference strains of *Staphylococcus* (Ezeifeka *et al.,* 2003; Akoachere et al 2003), their efficacy against clinical strains of this organism particularly those associated with wound sepsis has not been documented. In this paper, we report the antibacterial activity of extracts of *Garcinia kola* seeds against clinical isolates of *Staphylococcus* obtained from cases of wound sepsis as well as the potentials of the plant extracts in combination with six selected antibiotics.

MATERIALS AND METHODS

Isolation and identification of *Staphylococci* from wound sepsis specimens

Pus swabs obtained from three patients presenting with septic abrasion of the hip, wrist and elbow respectively were suspended in sterile physiological saline. One milliliter of the suspension was inoculated into 50 ml of sterile nutrient broth and incubated aerobically at 37 ^{o}C for 24 hours. At the end of the incubation period, the broth cultures were streaked for isolation on mannitol salt agar. Presumptive *Staphylococcus* colonies were identified as golden yellow, circular, convex colonies that ferment mannitol. One colony from each specimen displaying the above morphology was purified by subculturing onto nutrient agar. Where more than one type of colonies displaying the cultural characteristics of *Staphylococcus* were observed on each plate, one colony from each colony type was selected and purified by subculturing onto nutrient agar. The presumptive *Staphylococcus* isolates were processed for molecular identification by 16S rRNA gene amplification and sequencing.

Amplification, sequencing and analysis of the 16S rRNA gene of the bacterial isolates

Total genomic DNA was isolated from LB-grown bacterial cultures using the QIAamp DNA miniprep kit, following the manufacturer's instructions and used directly as template for PCR amplification. The 16S rRNA genes of the bacterial isolates were amplified with the oligonucleotide primers: 63f (5'–CAGGCCTAACACATGCAAGTC-3') and 1387r (5'–GGGCGG(A/T)GTGTACAAGGC-3') described by Marchesi *et al.* (1998). The amplification reaction mixture contained standard *Taq* amplification buffer, 100 µM (each) deoxyribonucleotide triphosphate, 0.5 µM (each) primers, genomic DNA and 2.5 U of *Taq* DNA polymerase in a 50 µl reaction volume. The cycling parameters were 94 ^{o}C for 2 min followed by 30 cycles of 92 ^{o}C for 30 s, 55 ^{o}C for 30 s, and 75 ^{o}C for 45 s, with a final elongation

step of 75 ^{o}C for 5 min. Amplification products were directly cycle sequenced using the Spectrumedix SCE2410 genetic analysis system with 24 capillaries. Big Dye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems) was used for the sequencing reactions. The analysis of the 16S rRNA gene sequences of the bacterial isolates were carried out by comparing them with those in the GenBank database (http://www.ncbi.nlm.nih.gov) by using BLAST (Altschul *et al.,* 1997) to determine the most similar sequences.

Preparation of plant extracts

The extracts of the plant were prepared in accordance with the description of Basri and Fan (2005). One hundred grams of seed powder was steeped in 500 ml of the respective solvent (water, acetone and methanol) for 24 hours with shaking. The resultant extract was centrifuged at 3000 rpm for 5 minutes at 4 ^{o}C. The supernatant was then filtered through a Whatman No.1 filter paper while the residue was used for a second extraction with 300 ml of the respective solvents. After the second extraction process, the aqueous extract was freeze-dried at -50 ^{o}C under vacuum whereas the acetone and methanol extracts were concentrated under reduced pressure using a rotary evaporator at 50 and 65 ^{o}C respectively. The concentrated extracts were then allowed to dry at room temperature to a constant weight.

Preparation of bacterial inocula

The inocula of the test organisms were prepared using the colony suspension method (EUCAST, 2003). Colonies picked from 24 hour old cultures grown on nutrient agar were used to make suspensions of the test organisms in saline solution (0.85% NaCl) to give an optical density of

approximately 0.1 at 600 $_{nm}$. The suspension was then diluted 1:100 by transfer of 0.1 ml of the bacterial suspension to 9.9 ml of sterile nutrient broth before use.

Antibiotics used in this study

The following antibiotics which were available as powders were used in this study: Penicillin G sodium (Duchefa); Amoxycillin (Duchefa); Chloramphenicol (Duchefa); Tetracycline hydrochloride (Duchefa); Erythromycin (Duchefa) and Ciprofloxacin (Fluka).

Assay for antistaphylococcal activity

The antistaphylococcal activities of the crude extracts were carried out using the agar dilution method (Afolayan and Meyer, 1997). The extracts were incorporated directly into molten nutrient agar at 50 ^{o}C to achieve concentrations of 30 mgml^{-1} for the aqueous extract and 10 mgml^{-1} for the acetone and methanol extracts. Dilutions of the acetone and methanol extracts were done in such a way that the final solvent concentration was 5% in the media. Standardised bacterial suspensions were used to inoculate the agar plates by streaking in duplicates. The inoculated plates were incubated under aerobic conditions at 37 ^{o}C for 24 hours. Positive controls consisted of extract free plates of nutrient agar inoculated with the test organisms. For the acetone and methanol extracts, controls consisted of nutrient agar plates with 5% of the respective solvent (which represented the final solvent concentration in the test plates). The absence of growth on the test plates compared with the positive controls was used to indicate the inhibitory activity of the extracts (Afolayan and Meyer, 1997).

Determination of the Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations of the extracts and antibiotics were determined using the agar dilution method following the standard protocol of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2000a). The extracts and antibiotics were incorporated into molten nutrient agar at 50 ^{o}C and allowed to solidify at room temperature. The dilutions of the extract ranged from 0.039 – 10 mgml^{-1} (acetone and methanol) and 0.625 - 20 mgml^{-1} (aqueous). The antibiotic test plates had concentrations ranging from 0.004 – 512 mgl^{-1}. Standardised inocula of test strains were used to seed the test plates in duplicates by streaking. Plates were incubated at 37 ^{o}C for 24 hours. The controls consisted of extract- free and antibiotic- free nutrient agar plates. The MIC value was taken as the lowest concentration of the extract or antibiotic showing complete lack of growth after the incubation period (EUCAST, 2000a).

Rate of kill experiment of the crude acetone extract

The rate of kill determination for the acetone extract was done by assaying of bacterial cell death over time following the description of Okoli and Iroegbu (2005). The assay was carried out using two of the *Staphylococcus aureus* strains, OKOH1 and OKOH3. The assay method was a broth macrodilution based technique, with the extract incorporated into 50ml nutrient broth in flasks, at concentrations of 1, 2 and 4 times the MIC values. Control flasks consisted of extract-free nutrient broth. The flasks were inoculated with standardised bacterial suspensions to a final cell density of approximately 10^{5} cfuml^{-1}. The inoculated flasks were immediately incubated at 37 ^{o}C with shaking. At intervals of 1, 2, 4, 8, 12 and 24hours, samples (100 µl) were withdrawn from each flask, diluted in

tenfold series and plated out in duplicates on nutrient agar. Plates were incubated at 37^{o}C for 24hours after which the number of survivors were enumerated.

Extract - Antibiotic Combination Studies

The effect of combinations of the acetone extract of *Garcinia kola* seeds and antibiotics was evaluated by the use of the rate of kill assay following the descriptions of White *et al.* (1996) and Pankey *et al.* (2005) with modifications. The extract and antibiotics were incorporated into 50 ml of nutrient broth in flasks at concentrations equivalent to their respective MIC values for each test strain. Positive controls, consisting of the extract and antibiotic alone at the test concentrations were included in each experiment. The negative controls consisted of antibiotic- and extract-free broth.

The test and control flasks were inoculated with standardised suspensions of the test organisms to a final inoculum density of approximately 10^5 cfuml^{-1}. Immediately after inoculation, aliquots (100 µl) of the negative control flasks were taken, serially diluted in sterile saline and plated on nutrient agar in order to determine the zero hour counts. The test flasks were then incubated at 37 ^{o}C with shaking at 120 rpm. After 24 hours of incubation, aliquots (100 µl) were withdrawn from each test and control flask, serially diluted in sterile saline and plated (100 µl) on nutrient agar in duplicates. To improve the visual observation of colonies in the agar, 1 ml of a 0.5% aqueous solution of 2,3,5 triphenol tetrazolium chloride (Neugebauer and Gilliland, 2005) was added to 100 ml of molten agar at 50 ^{o}C before plating. The plates were then incubated at 37 ^{o}C for 24 hours under aerobic conditions after which, the number of colonies were enumerated.

The interactions between the extract and antibiotics were considered synergistic if there was a decrease of ≥ 2 Log_{10} cfuml^{-1} in colony counts at 24 hours by the combination compared to the most

active single agent (Pankey *et al.,* 2005). Additivity or indifference was defined as a < 2 Log_{10} $cfuml^{-1}$ change in the average of viable counts at 24 hours for the combination, in comparison with the most active single drug. Antagonism was defined as a ≥ 2 Log_{10} $cfuml^{-1}$ increase in colony counts at 24 hours by the combination compared with that by the most active single agent (Pankey *et al.,* 2005; Lee *et al.,* 2006).

RESULTS

The polymerase chain reaction amplification of the 16S rRNA gene of the bacterial isolates resulted in the expected 1.3 kb amplicons (Figure 1). Partial sequencing of the amplified DNA revealed that three of the isolates had high (≥ 98%) sequence homology to *Staphylococcus aureus* and have since been deposited in GenBank with Accession numbers EU244633, EU244634 and EU244636, while one of the isolates had a 100% sequence homology to *Staphylococcus sciuri* and has also been deposited in GenBank with Accession number EU244635 (Table 1).

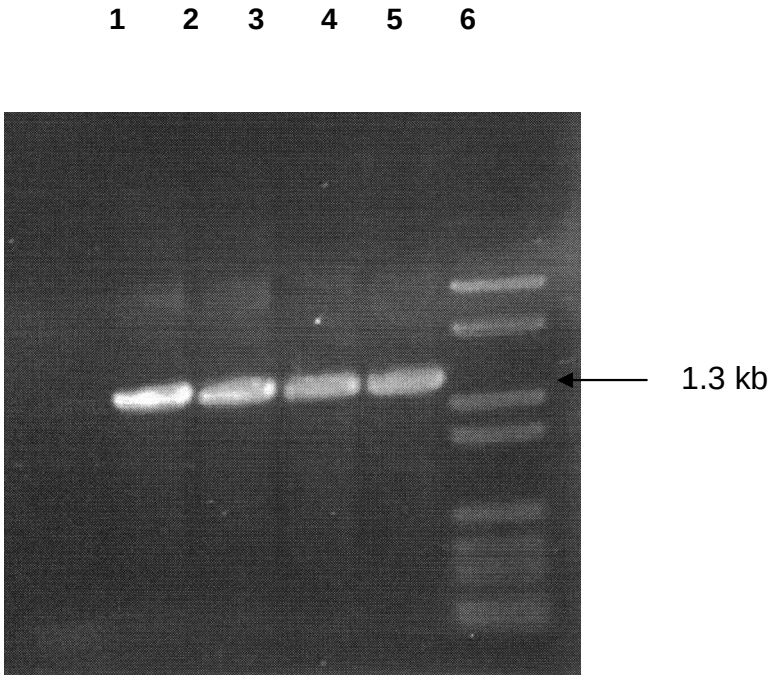

Figure 1: Gel picture of the 16S rRNA gene amplification for OKOH1 (Lane 2), OKOH2A (Lane 3), OKOH2B (Lane 4) and OKOH3 (Lane 5). Lane 1 represents the negative control and Lane 6 is a molecular weight ladder.

Table 1: Identity of bacterial isolates based on 16 rDNA sequencing.

Specimen Source	Isolate Identity	GenBank Accession Number
Septic abrasion (Hip)	*Staphylococcus aureus* OKOH1	EU244633
Septic abrasion (Wrist)	*Staphylococcus aureus* OKOH2A	EU244634
Septic abrasion (Wrist)	*Staphylococcus sciuri* OKOH2B	EU244635
Septic abrasion (Elbow)	*Staphylococcus aureus* OKOH3	EU244636

The susceptibility profiles of the *Staphylococcus* isolates to the extracts of *Garcinia kola* seeds revealed that all the strains showed susceptibility to the aqueous extract at 30 $mgml^{-1}$ as well as to the methanol and acetone extracts at 10 $mgml^{-1}$ (Table 2). All the isolates had identical MIC values for the aqueous extract (10 $mgml^{-1}$) and the methanol extract (0.3125 $mgml^{-}1$) (Table 3). Susceptibility to the acetone extract was slightly higher for the *Staphylococcus aureus* strains, OKOH1 and OKOH2A with MIC values of 0.3125 $mgml^{-1}$, while *Staphylococcus aureus* OKOH3 and *Staphylococcus sciuri* OKOH2B, had MIC values of 0.625 mg ml^{-1} (Table 3).

Table 2: Susceptibility of the staphylococcal isolates to extracts of *Garcinia kola* seeds.

Test Isolates	**Crude Extracts**		
	Aqueous	**Methanol**	**Acetone**
	30 mgml^{-1}	**10 mgml^{-1}**	**10 mgml^{-1}**
Staphylococcus aureus OKOH1	+	+	+
Staphylococcus aureus OKOH2A	+	+	+
Staphylococcus sciuri OKOH2B	+	+	+
Staphylococcus aureus OKOH3	+	+	+

Key: + denotes susceptibility

Table 3. Minimum inhibitory concentration (MIC) values for the crude extracts of *Garcinia kola* seeds and antibiotics against the *Staphylococcus* isolates.

Test Isolate	**Extract MIC values ($mgml^{-1}$)**			**Antibiotics MIC values (mgl^{-1})**					
	Aq	**Met**	**Ace**	**Amx**	**PenG**	**Chlo**	**Tet**	**Ery**	**Cip**
S. aureus OKOH1	10	0.312	0.312	1	0.25	4	0.25	0.25	0.5
S. aureus OKOH2A	10	0.312	0.312	1	0.5	4	0.25	0.5	0.5
S. sciuri OKOH2B	10	0.312	0.625	0.25	0.06	4	0.25	0.5	1
S. aureus OKOH3	10	0.312	0.625	1	1	4	0.25	0.5	0.5

Key: **Aq** - Aqueous; **Met** - Methanol; **Ace** - Acetone; **Amx**- Amoxycillin; **PenG**- Penicillin G; **Tet**-Tetracycline; **Chlo**- Chloramphenicol; **Ery**- Erythromycin; **Cip**- Ciprofloxacin

Two of the isolates, *Staphylococcus aureus* OKOH1 and OKOH3 were used to investigate the bactericidal activity of the acetone extract by time-kill assays. The extract was strongly bactericidal against isolate OKOH3 resulting in a 2.70 Log_{10} reduction in counts at 1.25 $mgml^{-1}$ (2× MIC) within 4 hours of exposure (Figure 2). A complete elimination of the test organism was achieved after 8 hours of exposure. In contrast, the extract was weakly bactericidal against isolate OKOH1 achieving only a 2.92 Log_{10} reduction in counts at 1.25 $mgml^{-1}$ (4× MIC) in 24 hours (Figure 3). At the MIC (0.3125 $mgml^{-1}$), the extract exhibited limited bactericidal activity during the first 8 hours but the organism showed evidence of regrowth as the exposure time was extended to 24 hours (Figure 3).

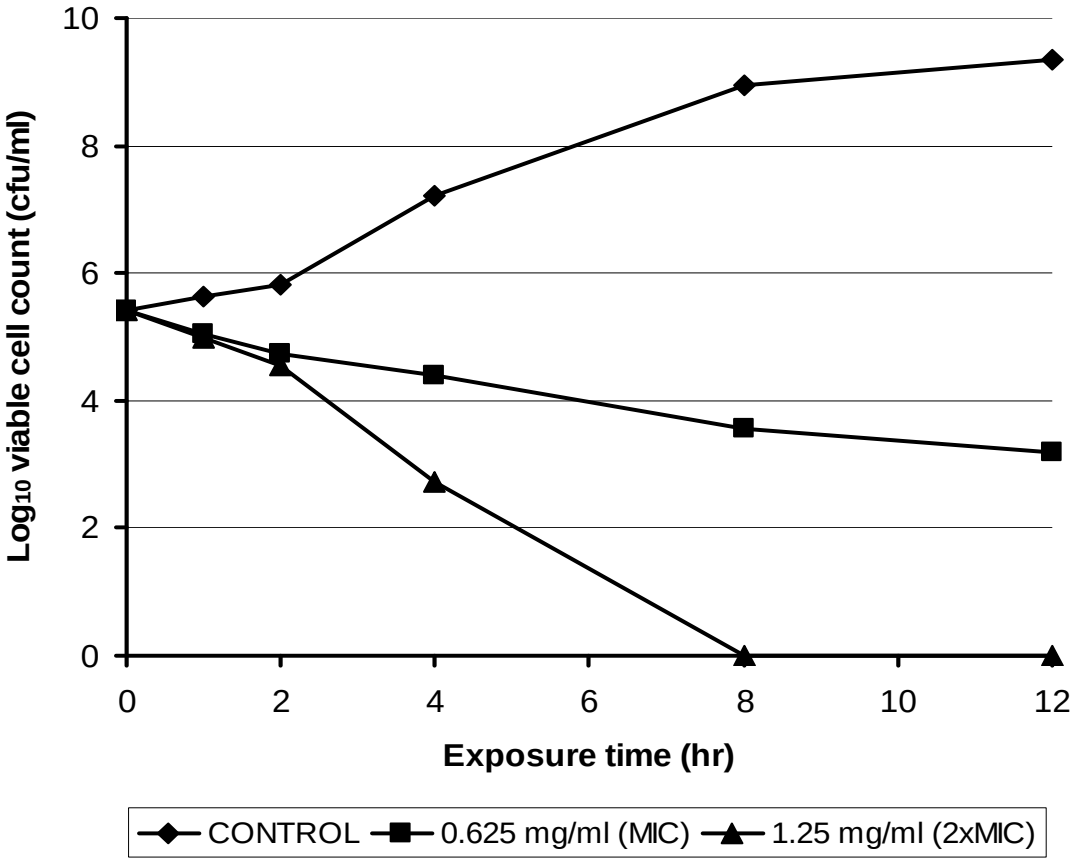

Figure 2. Effect of exposure to the acetone extract of *Garcinia kola* seeds on the viability of *Staphylococcus aureus* OKOH3.

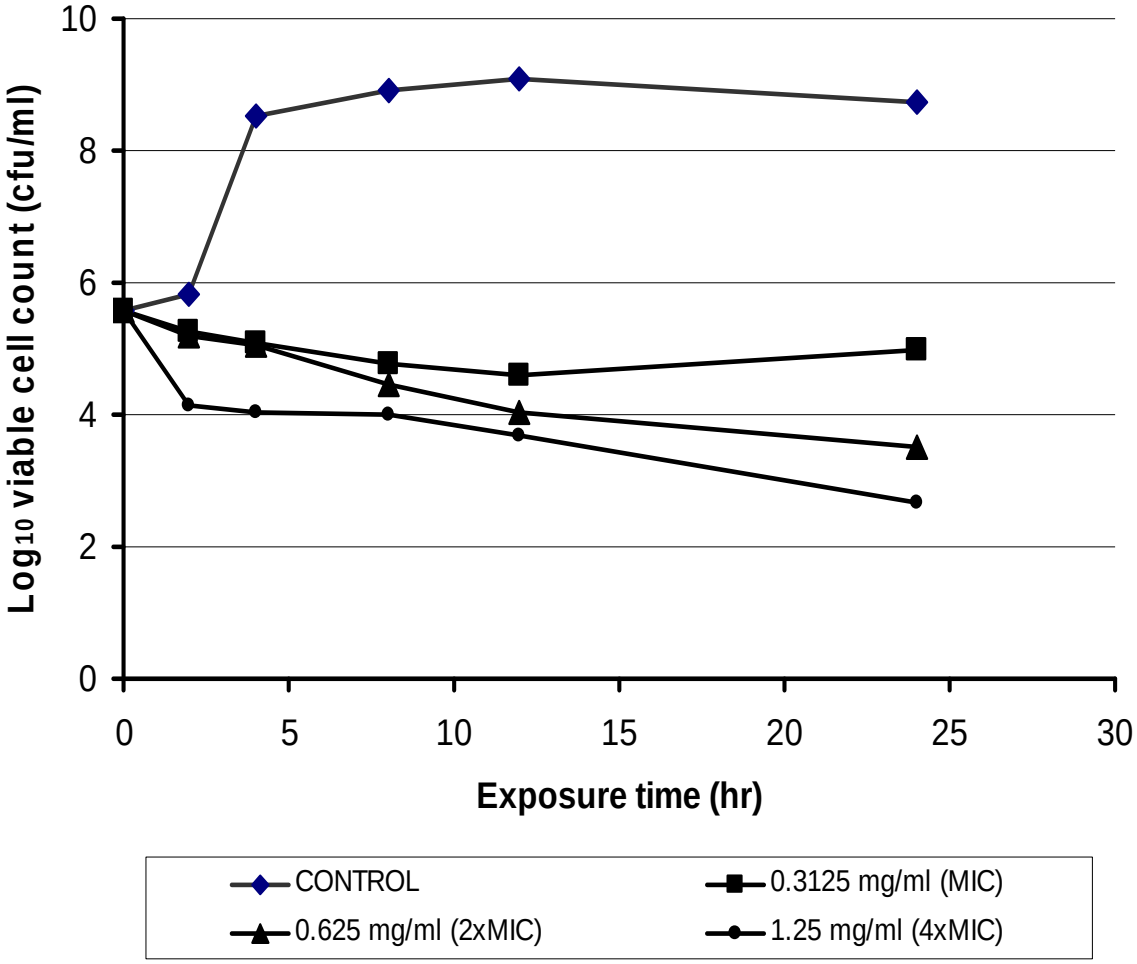

Figure 3. Effect of exposure to the acetone extract of *Garcinia kola* seeds on the viability of *Staphylococcus aureus* OKOH1.

The effect of combinations of the acetone extract and antibiotics on the susceptibility of the *Staphylococcus* isolates is shown in Table 4. The efficacy of all the antibiotics against isolates OKOH1 and OKOH3 was marginally improved in the presence of the extract. The extract-antibiotic combinations achieved decreases in bacterial counts ranging from 0.62 to 1.66 Log_{10} $cfuml^{-1}$ (Table 4). In contrast, extract-antibiotic combinations involving isolates OKOH2A and OKOH2B produced slight increases in bacterial counts for all but two antibiotics, with increases ranging from 0.04 to 1.27 Log_{10} $cfuml^{-1}$ (Table 4). Only the combinations involving amoxycillin (on isolate OKOH2A) and tetracycline (on isolate OKOH2B) showed marginal potentiation of antibiotic activity. The interaction between penicillin G and the extract on isolate OKOH3, was the only one to produce a synergistic effect with a 3.90 Log_{10} (> 1000 times) potentiation (Table 4).

Table 4. The effect of combinations of acetone extract of *G. kola* seeds and antibiotics on *Staphylococcus* isolates.

	Changes in bacterial counts (Log_{10} $cfuml^{-1}$) for the combination compared with the two agents used alone					
Test Organism	**Amx**	**PenG**	**Chlo**	**Tet**	**Ery**	**Cip**
S. aureus OKOH1	-1.35**(I)**	-1.38**(I)**	-0.64**(I)**	-1.66**(I)**	-0.78**(I)**	-0.62**(I)**
S. aureus OKOH2A	-0.19**(I)**	0.40**(I)**	0.44**(I)**	0.37**(I)**	0.51**(I)**	1.27**(I)**
S. sciuri OKOH2B	0.13**(I)**	0.37**(I)**	0.26**(I)**	-0.88**(I)**	0.13**(I)**	0.04**(I)**
S. aureus OKOH3	-1.00**(I)**	-3.90**(S)**	-0.62**(I)**	-0.73**(I)**	-1.30**(I)**	-0.82**(I)**

Key: **Amx** - Amoxycillin; **PenG** - Penicillin G; **Tet** - Tetracycline; **Chlo** - Chloramphenicol; **Ery** - Erythromycin; **Cip** - Ciprofloxacin. **(S)** - Synergy; **(I)** - Indifference/Additivity

DISCUSSION

Staphylococcus aureus is a prominent pathogen in hospital and community acquired infections as a major cause of wound suppuration (Archer, 1998). It is therefore always relevant when encountered in clinical specimens, particularly those of wound infections. The detection of *Staphylococcus aureus* in all the three specimens tested in this study underlines its importance as a cause of wound infections and is in agreement with the findings of other researchers such as Styers *et al.* (2006) and Moran *et al.* (2005) confirming that *Staphylococcus aureus* as a common pathogen frequently encountered in clinical specimens. In addition to *Staphylococcus aureus*, the coagulase negative *Staphylococcus sciuri* was also isolated from one of the specimens. *Staphylococcus sciuri* is a common inhabitant of the skin of rodents and other mammals such as dogs (Stepanovic *et al.,* 2001). The organism may also be found as a colonizing bacterium in humans, with low carrier rates in the nasopharynx, skin and urogenital tract (Stepanovic *et al.,* 2005). Occasionally, the organism has been isolated from patients with boils and wounds (Marsou *et al.,* 1999). While *Staphylococcus sciuri* is not so frequently encountered in clinical specimens, its presence is significant as the organism has been reported to carry a number of resistance plasmids (Schwarz *et al.,* 2002). Its co-infection with *Staphylococcus aureus* could therefore present problems of transmission of resistance genes to a true pathogen like *Staphylococcus aureus*.

The observation that all the strains of *Staphylococcus aureus* were susceptible to the crude extracts of *Garcinia kola* at concentrations as low as 0.3125 $mgml^{-1}$ supports the idea that extracts of this plant can be of value in the treatment of staphylococcal infections. The activity of the acetone and methanol extracts at concentrations of 0.3125 $mgml^{-1}$, were comparable to that of other plant extracts reported to possess antistaphylococcal activity (Palombo and Semple, 2002; Voravuthikunchai and

Kitpipit, 2005). *Garcinia kola* seeds have been known to possess compounds such as biflavonoids, xanthones and benzophenones (Iwu *et al.,* 1999). Some of these compounds such as Kolanone, hydroxybiflavononol (GB1) have been reported to have good antibacterial activity (Madubunyi, 1995: Han *et al.,* 2005). The antistaphylococcal activity particularly against clinical isolates is a very important finding as it demonstrates the potential of this plant in the treatment of problematic infections.

The killing rates experiments showed that the acetone extract possessed strong bactericidal activity against isolate OKOH3 achieving a > 99.9% (3 Log_{10}) reduction in counts after 8 hours. The same extracts exhibited a relatively weaker activity against isolate OKOH1 achieving a 2.92 Log_{10} reduction after 24 hours at 4× MIC. A greater than 99.9% killing activity in 24 hours is generally used as a standard of measurement of bactericidal efficacy (EUCAST, 2000b). The results also support the suggestion that extracts of this plant can be valuable in the treatment of some staphylococcal infections depending on the susceptibility of the infecting organism.

According to the MIC breakpoint values recommended by the British Society for Antimicrobial Chemotherapy (BSAC) and (EUCAST) (2005), two of the *Staphylococcus aureus* isolates (OKOH2A and OKOH3) had MIC values (Table 3) higher than the breakpoints for penicillin G (breakpoint MICs; 0.25 mgl^{-1}). This could be a reflection of the general trend in beta-lactam resistance among strains of *Staphylococcus aureus* in hospital and community settings. On the other hand, *Staphylococcus sciuri* exhibited high sensitivity to the beta-lactam antibiotics (MIC values; 0.06 and 0.25 mgl^{-1} for penicillin G and amoxycillin respectively). Susceptibility to tetracycline and erythromycin was high with MIC values for all the isolates, lower than the breakpoints (2 mgl^{-1} tetracycline and 1 mgl^{-1} erythromycin), thus suggesting that therapy by these drugs could be effective. However the closeness of the

erythromycin MIC values to the breakpoint for isolates OKOH2A, OKOH2B and OKOH3 (MIC values; 0.5 mgl^{-1}) could be a sign of emerging low level resistance to this drug. *Staphylococcus sciuri* was the only isolate to be classified as resistant to ciprofloxacin. This could be confirming the quinolone resistance gene carrying capacity that organisms of this group are known of (Schwarz *et al.,* 2002).

The level of interaction between the acetone extract of *Garcinia kola* seeds and antibiotics was investigated by the time-kill assay. In this method, concentrations of the extract and antibiotics equivalent to the MIC values were used in combination. Results from this study revealed a lack of synergistic interactions (i.e. > 100 times potentiation) between the extract and antibiotics on most of the isolates (Table 4). However, results still showed a positive picture of the interactions with marginal improvements (additive interactions) in the bactericidal activity of some antibiotics particularly against isolates OKOH1 and OKOH3. The varying levels of enhancement of antibiotic activity by the extracts is consistent with previous findings involving combinations of plant extracts and antibiotics on *Staphylococcus aureus* strains such as Darwish *et al.* (2002) and Yang *et al.* (2005). The likely reason for the observed lack of synergy could be the concentrations of the extract and antibiotics used in this study. Den-Hollander *et al.* (1997) emphasized that when determining synergy between two drugs, one of the drugs should have a concentration which does not affect the bacterial growth of the test organism. We also observed that some studies on the combinational effects of plant extracts and antibiotics such as Al-hebshi *et al.* (2006) and Braga *et al.* (2005) employed sub-inhibitory concentrations of the extracts and antibiotics. In this study, both the extract and the antibiotics at the MIC had some level bactericidal activity on the test isolates (data not shown) and this is likely to have interfered with synergy detection.

The enhancement in the killing effect (additivity) of the antibiotics suggests that extracts of *Garcinia kola* seeds could be containing compounds that can improve the efficacy of antibiotics. Such compounds are likely to be broad-spectrum resistance modifiers considering that this was observed across all classes of antibiotics tested.

CONCLUSION

Strains of *Staphylococcus* are important pathogens causing wound infections. Most clinical isolates of *Staphylococcus aureus* are at present resistant to a number of antibiotics necessitating the search for alternative treatment options using medicinal plants. Extracts of *Garcinia kola* seeds possessed good antibacterial activity against clinical isolates of *Staphylococcus*. The combinations of the acetone extract of this plant with six first-line antibiotics exhibited a marginal ability to improve the bactericidal potency of the antibiotics. The findings of this study are significant as they demonstrate the potential of obtaining some valuable compounds from this plant that can be combined with common antibiotics in the treatment of drug resistant staphylococcal infections. It is therefore necessary to carry out a bioassay directed fractionation of the acetone extract so as to isolate and identify the compounds responsible for the antistaphylococcal as well as the enhancement of antibiotic activity. Such compounds could be useful in the development of new antistaphylococcal drugs.

ACKNOWLEDGEMENT

The authors thank the National Research Foundation (NRF) of the Republic of South Africa for financial support.

REFERENCES

Afolayan AJ, and Meyer JJM (1997). The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. J. Ethnopharm. 57 (3): 177-181.

Akinpelu DA, and Onakoya TM (2006). Antimicrobial activities of medicinal plants used in folklore remedies in south-western Nigeria. Afr. J. Biotech. 5(11): 1078-1081.

Al-hebshi N, Al-haroni M, and Skaug N (2006). *In vitro* antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms. Arch Oral Biol. 51: 183-188.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Archer GL (1998). *Staphylococcus aureus*: A well-armed pathogen. Clin. Inf. Dis. 26: 1179-1180.

Basri DF, and Fan SH (2005). The potential of aqueous and acetone extracts of galls of *Queercus infectoria* as antibacterial agents. Ind. J. Pharm. 37: 26-29.

Braga LC, Leite AAM, Xavier KGS, Takahashi JA, Bemquerer MP, Chartone-Souza E, and Nascimento AMA (2005). Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus.* Canad. J. Microbio. 51(7): 541-547.

British Society for Antimicrobial Chemotherapy (BSAC) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2005). Establishing MIC breakpoints and the interpretation of *in vitro* susceptibility tests: 1-21.

Cook N (1998). Methicillin resistant *Staphylococcus aureus* versus the burn patient. Burns 24: 91-98.

Darwish RM, Aburjai T, Al-Khalil S, and Mahafzah A (2002). Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Staphylococcus aureus*. J. Ethnopharm. 79: 359-364.

Den-Hollander JG, Horrevorts AM, Van Goor MPJ, Verbrugh HA, and Mouton JW (1997). Synergism between tobramycin and ceftazidime against a resistant *pseudomonas aeruginosa* strain, tested in an *in vitro* Pharmacokinetic Model. Antimic. Agents Chemo. 41(1): 95-100.

Engemann JJ, Carmeli Y, Cosgrove SE, Fowler VG, Bronstein MZ, Trivette SL, Briggs JP, Sexton DJ, and Kaye KS (2003). Adverse clinical and economic outcomes attributable to methicillin resistance among patients with *Staphylococcus aureus* surgical site infection. Clin. Inf. Dis. 36: 592-598.

European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin. Micro. Inf. 9(8): 1-7.

European Committtee for Antimicrobial Susceptibity Testing (EUCAST), (2000a). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. Clin. Micro. Inf. 6(9): 509-515.

European Committee for Antimicrobial SusceptibilityTesting (EUCAST) (2000b). Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clin. Micro. Inf. 6(9): 503-508.

Han QB, Lee SF, Qiao CF, He ZD, Song JZ, Sun HD, and Xu HX (2005). Complete NMR assignments of the antibacterial biflavonoid GB1 from *Garcinia kola*. Chem. Pharm. Bull. 53(8): 1034-1036.

Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NPJ, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabbinowitsch E, Rutherford K, Mandy Sanders KM, Sharp S, Simmonds M, Kim Stevens K, Whitehead S, Bart G. Barrell BG, Brian G. Spratt BG, and Parkhill J (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the rapid evolution of virulence and drug resistance. PNAS, 101 (26): 9786-9791.

Iwu MW, Duncan AR, and Okunji CO (1999). New antimicrobials of plant origin. J. Janick (ed.), Perspectives on new crops and new uses : 457-462.

Lee JY, Oh WS, Ko KS, Heo ST, Moon CS, Ki HK, Kiem S, Peck KR, and Song JH (2006). Synergy of arbekacin-based combinations against vancomycin hetero-intermediate *Staphylococcus aureus*. J. Korean Med Sci. 21: 188-92.

Lowy DF (2003). Antimicrobial resistance: the example of *Staphylococcus aureus.* J. Clin. Invest. 111: 1265-1273.

Madubunyi II (1995). Antimicrobial activities of the constituents of *Garcinia kola* seeds. Int. J. Pharmacog. 33(3): 232-237.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, and Wade WG (1998). Design and evaluation of useful bacterium specific PCR primers that amplify genes coding for 16S rRNA. Appl. Environ. Microbiol. 64: 795-799.

Marsou R, Bes M, Boudouma M, Brun Y, Meugnier H, Freney J, Vandenesch F, and Etienne J (1999). Distribution of *Staphylococcus sciuri* subspecies among human clinical specimens, and profile of antibiotic resistance. Research Microbiol. 150(8): 531-541.

Moran GJ, Amii RN, Abrahamian FM, and Talan DA (2005). Methicillin-resistant *Staphylococcus aureus* in Community acquired Skin Infections. Emerg. Inf. Dis. 11(6): 928-930.

Neugebauer KA, and Gilliland SE (2005). Antagonistic action of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 toward spoilage Organisms in cottage cheese. J. Dairy Sci. 88: 1335-1341.

Okoli S, and Iroegbu CU (2005). *In vitro* antibacterial activity of *Synclisa scabrida* whole root extracts. Afr. J. Biotech. 4(9): 946-952.

Oliveira FP, Lima EO, Siqueira Junior JP, Souza EL, Santos BHC, and Barreto HM (2006). Effectiveness of *Lippia sidoides* Cham. (Verbenaceae) essential oil in inhibiting the growth of *Staphylococcus aureus* strains isolated from clinical material. Rev. Bras. Farmacogn. 16(4): 510-516.

Palombo EA, and Semple SJ (2002). Antibacterial activity of Australian plant extracts against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). J. Basic Micro. 42(6): 444-448.

Pankey G, Ashcraft D, and Patel N (2005). *In vitro* Synergy of daptomycin plus rifampin against *Enterococcus faecium* resistant to both linezolid and vancomycin. Antimic. Agents Chemo. 49(12): 5166-5168.

Rello J, Torres A, Ricart M, Valles J, Gonzalez J, Artigas A, and Rodriguez-Roisin R (1994). Ventilator-associated pneumonia by *Staphylococcus aureus*. Comparison of methicillin-resistant and methicillin-sensitive episodes. Am. J. Respir. Crit. Care Med. 150(6): 1545-1549.

Rios JL, and Recio MC (2005). Medicinal plants and antimicrobial activity. J. Ethnopharm. 100: 80-84.

Samie A, Obi CL, Bessong PO, and Namrita L (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. Afr. J. Biotech. 4(12): 1443-1451.

Schwarz S, Kehrenberg C, and Ojo KK (2002). *Staphylococcus sciuri* Gene *erm* (33), encoding inducible resistance to macrolides, lincosamides, and streptogramin B antibiotics, is a product of recombination between *erm*(C) and *erm*(A). Antimic. Agents Chemo. 46(11): 3621-3623.

Stepanovic S, Dakic I, Martel A, Vaneechoutte M, Morrison D, Shittu A, Jezek P, Decostere A, Devriese LA, and Haesebrouck F (2005). A comparative evaluation of phenotypic and molecular methods in the identification of members of the *Staphylococcus sciuri* group. Syst. Appl. Micro. 28(4): 353-357.

Stepanovic S, Dimitrijevic V, Vukovic D, Dakic I, Savic B, and Svabic-Vlahovic M (2001). *Staphylococcus sciuri* as a part of skin, nasal and oral flora in healthy dogs. Vet. Micro. 82(2): 177-185.

Styers D, Sheehan DJ, Hogan P, and Sahm DF (2006). Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. Ann. Clin. Micro. Antim. 5:2.

Tenover FC, Weigel LM, Appelbaum PC, McDougal LK, Chaitram J, McAllister S, Clark N, Killgore G, O'Hara CM, Jevitt L, Patel JB, and Bozdogan B (2004). Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimic. Agents Chemo. 48(1): 275-280.

Voravuthikunchai SP, and Kitpipit L (2005). Activity of medicinal plant extracts against hospital isolates of methicillin-resistant *Staphylococcus aureus*. Clin. Micro. Inf. 11(6): 510-512.

White RL, Burgess DS, Manduru M, and Bosso JA (1996). Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E test. Antimic. Agents Chemo. 40(8): 1914-1918.

Wilson P, Andrews JA, Charlesworth R, Walesby R, Singer M, Farrell DJ, and Robbins M (2003). Linezolid resistance in clinical isolates of *Staphylococcus aureus*. J. Antimic. Chemo. 51: 186-188.

Yang ZC, Wang BC, Yang XS, Wang Q, and Ran L (2005). The synergistic activity of antibiotics combined with eight traditional Chinese medicines against two different strains of *Staphylococcus aureus.* Colloids and surfaces B: Biointerfaces. 41(2-3): 79-81.

CHAPTER 5

In vitro evaluation of the interactions between the acetone extract of *Garcinia kola* seeds and some antibiotics

ABSTRACT

The effect of combinations of the acetone extract of *Garcinia kola* seeds and antibiotics was investigated by means of the fractional inhibitory concentration (FIC) indices as well as by the use of time-kill assays. Using the FIC indices, synergistic interactions were observed largely against Gram positive organisms (FIC indices of 0.52 - 0.875) with combinations against Gram negative bacteria mainly yielding antagonistic interactions (FIC indices of 2.0 to 5.0). The time-kill assay detected synergy against both Gram negative and Gram positive organisms with a ≥ 1000 times (≥ 3 Log_{10}) potentiation of the bactericidal activity of tetracycline and chloramphenicol (against *E. coli* ATCC 8739 and *K. pneumonia*e ATCC 10031) and amoxycillin and penicillin G against *Staphylococcus aureus* ATCC 6538. The observed synergy was not specific to a particular class of antibiotics although combinations involving erythromycin and ciprofloxacin consistently gave antagonistic or indifferent interactions. We conclude that the acetone extract of *Garcinia kola* seeds can be a potential source of broad spectrum resistance modifying compounds.

Key words: *Garcinia kola*; antibiotic resistance; interactions; resistance modifying compounds.

INTRODUCTION

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. Infections due to *Staphylococcus aureus* are presently resistant to beta-lactams (Cook, 1998), while *Enterococcus* strains are resistant to vancomycin, ampicillin, gentamycin and streptomycin (Montecalvo *et al.,* 1994). Gram negative pathogens such as *Salmonella* species, *Pseudomonas aeruginosa, Klebsiella pneumoniae* have become multi-drug resistant (Fluit *et al.,* 2001). With this emergence of resistance, most old and cheap antibiotics such as the penicillins, the tetracyclines and erythromycin have been rendered ineffective. The loss of clinical efficacy of such previously effective first-line drugs, means that treatment of infections, as a result has to be shifted to second-line or third-line antibiotics that are often more expensive with numerous side effects (Brook *et al.,* 2000). Notwithstanding the fact that new antimicrobial agents are being developed, the past record of resistance development shows that resistant strains often appear a few years after the first clinical use of any antibiotic (Perron *et al.,* 2005).

In the treatment of drug resistant infections, combinations of antibiotics have often been used as this takes advantage of different mechanisms of action. The use of antimicrobial agents displaying synergy is one of the well established indications for combination antimicrobial therapy (Rybak and McGrath, 1996). Antimicrobial synergism occurs when two or more antibiotics, in combination exert an inhibitory effect that is greater than the additive effects of the individual antibiotics. Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting strains are more likely to result in successful therapeutic outcome. Thus, evidence of *in vitro* synergism could be useful in selecting optimal combinations of antimicrobials for the empirical therapy of serious bacterial infections (Hooton *et al.,* 1984)

Plant extracts and plant derived compounds have long been established to possess antimicrobial activity. However, plant derived compounds have been seen to lack the broad spectrum and potent antimicrobial activity often displayed by bacterial or fungal produced antibiotics. Attempts therefore to find potent, nontoxic, broad-spectrum antibiotics from plants, have not yielded any good results even though large-scale screens have been undertaken by both pharmaceutical and biotech firms (Lewis and Ausubel, 2006).

It has been hypothesized that, in addition to the production of intrinsic antimicrobial compounds, plants also produce multi-drug resistance (MDR) inhibitors which enhance the activity of the antimicrobial compounds (Stermitz *et al.,* 2000). This hypothesis was tested by Tegos *et al.* (2002), who showed that the activity of putative plant antimicrobials against Gram positive and Gram negative organisms was significantly enhanced by synthetic MDR inhibitors of MDR efflux proteins. Those findings provided a basis to believe that plants can be potential sources of natural MDR inhibitors that can potentially improve the performance of antibiotics against resistant strains.

The screening of crude plant extracts for synergistic interactions with antibiotics is expected to provide leads for the isolation of MDR inhibitors. The ability of crude extracts of plants to potentiate the activity of antibiotics has been observed by some researchers and it is anticipated to form the basis for the bioassay directed isolation of potential resistance modulators from plants. In a study of some Jordanian plants by Darwish *et al.* (2002), the efficacy of the antibiotics, gentamycin and chloramphenicol against *Staphylococcus aureus* were reportedly improved by the use of plant materials. Ahmad and Aqil (2007), also reported that crude extracts of Indian medicinal plants demonstrated synergistic interactions with tetracycline and ciprofloxacin against extended spectrum beta-lactamases (ESβL)-producing multidrug resistant enteric bacteria. Betoni *et al.* (2006) also

observed synergistic interactions between extracts of Brazilian medicinal plants and eight antibiotics on *Staphylococcus aureus.* The use of *Catha edulis* extracts at sub-inhibitory levels, has been reported to reduce the minimum inhibitory concentration (MIC) values of tetracycline and penicillin G against resistant oral pathogens, *Streptococcus oralis*, *Streptococcus sanguis* and *Fusobacterium nucleatum* (Al-hebshi *et al.,* 2006).

A number of compounds with an *in vitro* activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Polyphenols (epicatechin gallate and catechin gallate) have been reported to reverse beta-lactam resistance in Methicillin Resistant *Staphylococcus aureus* (MRSA) (Stapleton *et al.,* 2004). Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to possess resistance modulating abilities in combination with various antibiotics against resistant strains of *Staphylococcus aureus* (Marquez *et al.,* 2005; Smith *et al.,* 2007; Shibata *et al.,* 2005 and Oluwatuyi *et al.,* 2004).

Garcinia kola is a plant that has shown immense potential as a source of chemotherapeutic compounds (Farombi *et al.,* 2002; Han *et al.,* 2005). The seeds of the plant, commonly known as bitter kola are used in West Africa for the treatment of liver disease, bronchitis, throat infections and in the relief of colic (Iwu *et al.,* 1999). Many phytochemical studies have revealed that the seed is rich in flavonoids and other water soluble polyphenolic compounds (Iwu and Igboko, 1982; Han *et al.,* 2005). While the antibacterial potentials of *Garcinia kola* seed extracts has previously been studied, the interactions between the extracts of this plant and antibiotics has not been documented, especially with regards to its potential as a source of resistance modifying compounds. In this paper, we report the effect of the acetone extract of *Garcinia kola* seeds on the antibacterial potencies of six first-line antibiotics.

MATERIALS AND METHODS

Plant extract preparation

The extracts of the seed were prepared in accordance to the description of Basri and Fan (2005). One hundred grams of seed powder was steeped in 500 ml of absolute acetone for 24 hours with shaking. The resultant extract was centrifuged at 3000 rpm for 5 minutes at 4 ^{o}C. The supernatant was then filtered through a Whatman No.1 filter paper while the residue was used for a second extraction with 300 ml of the solvent. After the second extraction, the filtrates were concentrated under reduced pressure using a rotary evaporator at 50 ^{o}C. The concentrated extract was then allowed to dry at room temperature to a constant weight.

Preparation of bacterial inocula

The inocula of the test organisms were prepared using the colony suspension method (EUCAST, 2003). Colonies picked from 24 hour old cultures grown on nutrient agar were used to make suspension of the test organisms in saline solution to give an optical density of approximately 0.1 at 600 $_{nm}$. The suspension was then diluted 1:100 by transfer of 0.1 ml of the bacterial suspension to 9.9 ml of sterile nutrient broth before use.

Antibiotics used in this study

The following antibiotics were used in this study: Penicillin G sodium (Duchefa); Amoxycillin (Duchefa); Chloramphenicol (Duchefa); Tetracycline hydrochloride (Duchefa); Erythromycin (Duchefa) and Ciprofloxacin (Fluka).

Determination of the Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations of the antibiotics and plant extracts were determined using the standard method of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2000). Dilutions of the antibiotics, ranging from 0.004 – 512 mgl^{-1} in nutrient agar were prepared by incorporating the antibiotic stock solution into molten agar at 50 ^{o}C. Dilutions of the extract ranging from 0.039 - 20 $mgml^{-1}$ were also prepared by incorporation of the extract in agar at 50 ^{o}C. After pouring onto plates and allowing the agar to set, the plates were inoculated with standardized inocula of the test bacteria by streaking in duplicates. Plates were incubated at 37 ^{o}C for 24 hours under aerobic conditions. The MIC was defined as the lowest concentration of the antibiotic that completely inhibited visible growth of the test organism as judged by the naked eye, disregarding a single colony or a thin haze within the area of inoculation (EUCAST, 2000).

Combination Studies

The checkerboard method

The study of the combined antimicrobial activity of the plant extracts and antibiotics was done using the agar dilution checkerboard method as described by Mandal *et al.* (2004). The extract and the antibiotics were combined by incorporation into molten nutrient agar at concentrations ranging from 1/8 to 2× MIC. After setting, the plates were inoculated with standardized cultures by streaking using a standard loop in duplicates. Plates were incubated for 24 hours at 37 ℃ after which the MIC values were estimated. The fractional inhibitory concentrations (FIC) were derived from the lowest concentration of antibiotic and extract combination permitting no visible growth of the test organisms (Mandal *et al.,* 2004). The FIC value for each agent was calculated using the formular;

$$\text{FIC (antibiotic)} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

$$\text{FIC (extract)} = \frac{\text{MIC of extract in combination}}{\text{MIC of extract alone}}$$

The interactions between the antibiotics and the extracts were evaluated by use of the FIC indices which were calculated using the formular;

$$\text{FIC Index} = \sum\text{FIC} = \text{FIC (antibiotic)} + \text{FIC (plant extract)}$$

Combinations were classified as synergistic, if the FIC indices were < 1, additive if the FIC indices were = 1, indifferent if the FIC indices were between 1 and 2 and antagonistic if the FIC indices were > 2 (Kamatou *et al.,* 2006). Where more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC value was expressed as the average of the individual FIC values as described by Pankey at al. (2005).

The time-kill method

The effects of combinations of the acetone extract of *Garcinia kola* seeds and antibiotics were also evaluated by use of the time-kill assay. This was performed by a broth macrodilution technique following the descriptions of White *et al.* (1996) and Pankey *et al.* (2005). The extract and antibiotics were incorporated into 50 ml of nutrient broth at 0.5× MIC and 1× MIC respectively. Controls consisting of nutrient broth incorporated with the extract and the respective antibiotic alone at the test concentrations were included in each experiment.

The test and control flasks were inoculated with each test organism to a final inoculum density of approximately 10^5 $cfuml^{-1}$. Immediately after inoculation, aliquots (100 µl) of the negative control flasks were taken, serially diluted in sterile saline and plated on nutrient agar in order to determine the zero hour counts. The test flasks were then incubated at 37 ^oC with shaking at 120 rpm. After 24 hours of incubation, samples were taken from each test and control flasks, serially diluted in sterile saline and plated (100 µl) on nutrient agar in duplicates. For a better visual observation of the colonies in the agar, 1 ml of 0.5% aqueous solution of 2,3,5 triphenol tetrazolium chloride (Neugebauer and Gilliland, 2005) was added to 100 ml of molten agar before plating.

The plates were incubated at 37 ^oC for 24 hours under aerobic conditions. After incubation, the number of colonies were enumerated and the mean counts ($cfuml^{-1}$) for each test and controls were determined and expressed as Log_{10} $cfuml^{-1}$.

The interactions were considered synergistic if there was a decrease of ≥ 2 Log_{10} $cfuml^{-1}$ after 24 hours by the combination compared to the most active single agent (Pankey *et al.,* 2005). Additivity or indifference was defined as a < 2 Log_{10} $cfuml^{-1}$ change in average viable counts after 24 hours for the combination, in comparison with the most active single agent. Antagonism was defined as a ≥ 2 Log_{10} $cfuml^{-1}$ increase in colony counts after 24 hours by the combination compared with that by the most active single agent (Pankey *et al.,* 2005; Lee *et al.,* 2006).

RESULTS

The MIC values of the antibiotics used in this study are shown in Table 1. Susceptibility to beta-lactam antibiotics, amoxycillin and penicillin G was higher against Gram positive organisms (MIC ranges of 0.015 – 0.25 mgl^{-1}) than against Gram negatives (MIC ranges of 2 – 32 mgl^{-1}). The macrolide, erythromycin had the highest MIC values of 128 mgl^{-1} against *E. coli* ATCC 8739 and 512 mgl^{-1} against *Proteus vulgaris* and *Enterococcus faecalis*. Gram negative organisms exhibited higher susceptibility to ciprofloxacin (MIC ranges of 0.015 – 0.25mgl^{-1}) than other antibiotics.

Table 1: MIC values for antibiotics.

Test Isolate	**MIC Values (mgl^{-1})**					
	Amx	**PenG**	**Tet**	**Chlo**	**Ery**	**Cip**
Staph. aureus ATCC 6538	0.015	0.008	0.25	2	0.25	0.5
Str. faecalis ATCC 29212	0.5	1	8	4	0.5	0.5
Ent. faecalis	0.25	8	32	64	512	0.5
E. coli ATCC 8739	4	32	1	4	128	0.312
K. pneumoniae ATCC 10031	32	64	0.5	1	4	0.015
P. vulgaris CSIR 0030	2	32	16	8	512	0.25

Key: **Amx** - Amoxycillin; **PenG** - Penicillin G; **Tet** - Tetracycline; **Chlo** - Chloramphenicol; **Ery** - Erythromycin; **Cip** - Ciprofloxacin

The FIC values of the acetone extract and the antibiotics, amoxycillin, ciprofloxacin, tetracycline and chloramphenicol are shown in Table 2. The activity of the antibiotics against Gram negative organisms was largely reduced by the presence of sub-inhibitory concentrations of the extract. The FIC indices for most of the combinations against Gram positive organisms ranged from 0.52 – 1.00 with only *Enterococcus faecalis* having an FIC index of 1.625 in the combination involving amoxycillin. The activity of all the antibiotics against *Klebsiella pneumoniae* ATCC 10031, was reduced by the presence of the extract with FIC indices for ciprofloxacin, chloramphenicol and tetracycline ranging from 2.00 – 5.00. The nucleic acid inhibitor, ciprofloxacin showed lack of synergy with the plant extract against all but one of the test organisms (*Streptococcus faecalis* ATCC 29212).

Table 2: Fractional inhibitory Concentration (FIC) values and indices for the combinations of the acetone extract of *G. kola* and antibiotics.

Antibiotic	Test isolate	Mean FIC (Antibiotic)	Mean FIC (Extract)	FIC Index	Interaction
Amoxycillin	*Staph. aureus* ATCC 6538	0.196	0.5	0.52	Synergy
	Str. faecalis ATCC 29212	0.5	0.5	1.00	Additivity
	Ent. faecalis	1.25	0.375	1.625	Indifference
Ciprofloxacin	*Str. faecalis* ATCC 29212	0.375	0.25	0.625	Synergy
	Ent. faecalis	0.375	0.5	0.875	Synergy
	E. coli ATCC 8739	2.00	0.06	2.06	Antagonism
	K. pneumoniae ATCC 10031	4.00	0.06	4.06	Antagonism
Chloramphenicol	*Str. faecalis* ATCC 29212	0.375	0.5	0.875	Synergy
	Ent. faecalis	0.234	0.5	0.734	Synergy
	E. coli ATCC 8739	0.5	0.25	0.75	Synergy
	K. pneumoniae ATCC 10031	1.00	1.00	2.00	Antagonism
Tetracycline	*Str. faecalis* ATCC 29212	0.375	0.5	0.875	Synergy
	Ent. faecalis	0.3125	0.374	0.686	Synergy
	K. pneumoniae ATCC 10031	4.00	1.00	5.00	Antagonism

The time-kill effect of combinations of the acetone extract of *Garcinia kola* and antibiotics is shown in Table 3. The extract showed ability to improve the bactericidal effect of beta-lactam antibiotics on Gram positive organisms. The bactericidal activity of amoxycillin and penicillin G was increased by 5.15 and 3.27 Log_{10} bases respectively against *Staphylococcus aureus* ATCC 6538. Marginal improvement (less than 2 Log_{10} bases potentiation) in the activity of amoxycillin against *Streptococcus faecalis* ATCC 29212 and *Enterococcus faecalis* was also observed. The bacterial killing activity of protein synthesis inhibitors, tetracycline and chloramphenicol was also increased against both Gram positive and Gram negative organisms with the bactericidal effect of tetracycline showing broad spectrum activity. Erythromycin was strongly potentiated against Gram positive organisms *Staphylococcus aureus* ATCC 6538 and *Streptococcus faecalis* ATCC 29212 but the activity of the same antibiotic was severely reduced against Gram negative bacteria, *E. coli* ATCC 8739 and *Klebsiella pneumoniae* ATCC 10031.

Table 3: The effect of combinations of the acetone extract of *Garcinia kola* seeds and antibiotics determined by the time-kill assay

	Changes in bacterial counts (Log_{10} $cfuml^{-1}$) for the combination compared with the two agents used alone					
Test Organism	**Amx**	**PenG**	**Chlo**	**Tet**	**Ery**	**Cip**
Staph. aureus ATCC 6538	-5.15 **(S)**	-3.27 **(S)**	-1.04 **(I)**	-3.24 **(S)**	-2.44 **(S)**	0.00 **(I)**
Str. faecalis ATCC 29212	-0.88 **(I)**	0.69 **(I)**	-1.15 **(I)**	-1.46 **(I)**	-2.02 **(S)**	-2.96 **(S)**
Ent. faecalis	-1.79 **(I)**	0.63 **(I)**	0.003 **(I)**	-0.37 **(I)**	-0.21 **(I)**	0.33 **(I)**
E. coli ATCC 8739	0.59 **(I)**	-2.78 **(S)**	-3.28 **(S)**	-5.94 **(S)**	2.73 **(A)**	4.18 **(A)**
K. pneumoniae ATCC 10031	1.03 **(I)**	-0.47 **(I)**	-3.21**(S)**	-3.34 **(S)**	4.78 **(A)**	5.06 **(A)**
P. vulgaris CSIR 0030	3.72 **(A)**	3.54 **(A)**	2.56 **(A)**	-0.73 **(I)**	-0.02 **(I)**	0.10 **(I)**

Key: **Amx**- Amoxycillin; **PenG**- Penicillin G; **Tet**-Tetracycline; **Chlo**- Chloramphenicol; **Ery**- Erythromycin; **Cip**-Ciprofloxacin **(S)** - Synergy; **(I)** - Indifference/Additivity; **(A)** - Antagonism

DISCUSSION

The organisms used in this study were reference as well as environmental strains of pathogenic organisms often posing problems of drug resistance in clinical settings. In order to assess the effects of combinations of the extracts of the plant and antibiotics, the MIC values of the antibiotics had to be determined as these provided the reference point for defining the interactions. The objective of testing plant extracts for potentials of synergy with antibiotics is to assess if combinations of such extracts with antibiotics can bring about positive changes in the susceptibility of the test strains, thus necessitating the use of strains resistant to the test antibiotics. For that reason therefore, the British Society for Antimicrobial Chemotherapy (BSAC) and EUCAST (2005), recommended MIC breakpoints were used as a way of determining the presence or lack of resistance in the test isolates. Although this data is often used in surveillance studies to monitor trends in resistance development, we saw it convenient to apply it in our studies in the absence of a standard.

According to the MIC breakpoints, strains of *Staphylococcus and Streptococcus* with MIC values of ≥ 0.25 mgl^{-1} (for penicillin G), ≥ 2 mgl^{-1} (for amoxycillin), ≥ 2 mgl^{-1} (for tetracycline), ≥ 1 mgl^{-1} (for erythromycin), ≥ 4 mgl^{-1} (for chloramphenicol) and ≥ 1 mgl^{-1} (for ciprofloxacin) are classified as resistant. From our results, *Streptococcus faecalis* ATCC 29212 and *Enterococcus faecalis* were resistant to penicillin G, tetracycline, chloramphenicol, and erythromycin. The MIC values for these organisms ranged from 4 to 512 times higher than the predicted breakpoint values. The breakpoint values for enteric bacteria are; 16 mgl^{-1} (penicillins), 2 mgl^{-1} (tetracycline), 16 mgl^{-1} (chloramphenicol) and 1 mgl^{-1} (ciprofloxacin) (BSAC and EUCAST, 2005). The enteric bacteria used in this study showed varying levels of susceptibility to the test antibiotics. *Klebsiella pneumoniae* ATCC 10031 exhibited low susceptibility to both penicillin G and amoxycillin while *E. coli* ATCC 8739 and *Proteus*

vulgaris CSIR 0030 were more susceptible to amoxycillin. The enteric organisms were generally susceptible to chloramphenicol and ciprofloxacin but had high MIC values against erythromycin. The presence of such elevated MIC values for some of the organisms used in this study against such common front-line antibiotics may reflect a common presence of resistance mechanisms universally present in bacteria, and may justify the need to seek strategies to inhibit such mechanisms.

Combinations of antibiotics and the acetone extract of *Garcinia kola* seeds were investigated for possible synergistic interactions. In the checkerboard method, synergy is based on the increased susceptibility of the test organism to the presence of both antimicrobial agents which is reflected by changes in the MIC values (Odds, 2003). Using the FIC indices, synergy was detected mainly against Gram positive organisms. The synergy against *Streptococcus faecalis* ATCC 29212 and *Enterococcus faecalis* is an important observation as these organisms were resistant to penicillin G, tetracycline, chloramphenicol, and erythromycin with MIC values much higher than their predicted breakpoints. Although the level of antibiotic potentiation was low (FIC Indices of 0.52 - 1.00) as not to lead to a restoration of susceptibility (lowering the MIC values to below the breakpoint values) the results seem promising considering that crude extracts were used. The potentiation is likely to have been much more pronounced had purified compounds been used. Since the synergy was not specific to any class of antibiotics, it is likely that the target for this interaction could be cell membrane since it is the fundamental difference between Gram negative and Gram positive bacteria.

As an alternative method, the time-kill assay was also employed to assess the effect of the combinations. This method is based on a comparison of the killing rate of the combination to that of the individual agents. In the experiment, the extract was incorporated at sub-inhibitory concentrations (1/2× MIC) with the antibiotic used at the minimum inhibitory concentration.

In contrast to the checkerboard method, the time-kill assay detected synergy against both Gram positive and Gram negative organisms. Strong synergistic interactions on *Staphylococcus aureus* ATCC 6538 were observed in combinations involving beta-lactams (amoxycillin and penicillin G) as well as the protein synthesis inhibitors, tetracycline and erythromycin. Combinations involving tetracycline and chloramphenicol were highly bactericidal against *E. coli* ATCC8 7339 and *K. pneumoniae* ATCC 10031 with a more than 1000 fold (> 3 Log_{10}) potentiation of the antibiotic (Table 3). Combinations involving erythromycin and ciprofloxacin against the same Gram negative organisms were largely antagonistic.

The synergy detected by the time-kill assay was not specific to any group of organisms or class of antibiotics. This suggests that the crude extracts of this plant could be containing a mixture of compounds that can enhance the activity of different antibiotics. The seeds of *Garcinia kola* have been known to contain a number of antimicrobial compounds (Iwu *et al.,* 1999) such as polyphenols and flavonoids. The antimicrobial and resistance modifying potentials of some naturally occurring flavonoids and polyphenolic compounds have been reported by other studies (Cushnie and Lamb, 2005; Sato *et al.,* 2004). This would suggest that, the synergy with antibiotics observed in this study could be attributable to such compounds. Some of these compounds like polyphenols have been shown to exert their antibacterial action through membrane perturbations. This perturbation of the cell membrane coupled with the action of beta-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone *et al.,* 2006). It has also been shown that some plant derived compounds can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e. peptidoglycan) in the cell wall (Zhao *et al.,* 2001).

While the above explanations may account for the synergy between the extracts and beta-lactam antibiotics that act on the cell wall, it might not apply in the case of the observed synergy with other classes of antibiotics with different targets such as tetracycline, erythromycin, ciprofloxacin and chloramphenicol. Bacterial efflux pumps are responsible for a significant level of resistance to antibiotics in pathogenic bacteria (Kumar and Schweizer, 2005). Some plant derived compounds have been observed to enhance the activity of antimicrobial compounds by inhibiting MDR efflux systems in bacteria (Tegos *et al.,* 2002). 5'-methoxyhydnocarpin is an example of an inhibitor of the NorA efflux pump of *Staphylococcus aureus* isolated from *Berberis fremontii* (Stermitz *et al.,* 2000). It is likely that the acetone extract of *Garcinia kola* seeds could be containing potential MDR efflux pump inhibitors. Such compounds are likely to be broad spectrum efflux inhibitors considering that the synergistic effect of the extract was observed on both Gram positive and Gram negative organisms as well as in combination with, cell wall inhibiting and protein synthesis inhibiting antibiotics. In fact, some broad spectrum efflux pump inhibitors have been isolated from some plants. Smith *et al.* (2007) reported one efflux pump inhibitor (ferruginol) from the cones of *Chamaecyparis lawsoniana* that inhibited the activity of the quinolone resistance pump (NorA), the tetracycline resistance pump, (TetK) and the erythromycin resistance pump, (MsrA) in *Staphylococcus aureus*.

The strong synergy observed between the extracts of *Garcinia kola* and common first-line antibiotics is a significant finding demonstrating the therapeutic potentials of this plant. However, there is a need to establish the molecular basis of these interactions.

CONCLUSION

The extracts of *Garcinia kola* seeds showed potentials of synergy in combination with some antibiotics against reference strains of pathogenic organisms often posing problems of drug resistance. The detection of synergy between crude extract of *Garcinia kola* and antibiotics demonstrates the potential of this plant as a source of antibiotic resistance modifying compounds. It is necessary to carry out a bioassay guided fractionation of the acetone extract of this plant in a bid to isolate and identify the compounds responsible for the enhancement of antibiotic activity. An elucidation of the mechanisms of action of these compounds must be followed by toxicity and *in vivo* tests to determine the therapeutic application of such compounds in combination therapy. These are subjects of on-going investigation in our research group.

ACKNOWLEDGEMENT

The authors are grateful to the National Research Foundation (NRF) of the Republic of South Africa for financial support.

REFERENCES

Ahmad I, and Aqil F (2007). *In vitro* efficacy of bioactive extracts of 15 medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. Micro. Res.162: 264-275.

Al-hebshi N, Al-haroni M, and Skaug N (2006). *In vitro* antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms. Arch Oral Biol. 51: 183-188.

Basri DF, and Fan SH (2005). The potential of aqueous and acetone extracts of galls of *Queercus infectoria* as antibacterial agents. Ind. J. Pharm. 37: 26-29.

Betoni JEC, Mantovani RP, Barbosa LN, Di-Stasi LC, and Fernandes A (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Mem. Inst. Oswaldo Cruz. 101 no.4.

Brook I, Gooch WM, Jenkins SG, Pichichero ME, Reiner SA, Sher L, and Yamauchi T (2000). Medical management of acute bacterial sinusitis: Recommendations of a clinical advisory committee on pediatric and adult sinusitis. Ann. Otol. Rhinol. Laryngol. 109: 1-19.

British Society for antimicrobial Chemotherapy (BSAC) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2005). Establishing MIC breakpoints and the interpretation of *in vitro* susceptibility tests: 1-21.

Cook N (1998). Methicillin resistant *Staphylococcus aureus* versus the burn patient. Burns 24: 91-98.

Cushnie TPT, and Lamb AJ (2005). Antimicrobial activity of flavonoids. Int. J. Antimic. Agents. 26(5): 343-356.

Darwish RM, Aburjai T, Al-Khalil S, and Mahafzah A (2002). Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Staphylococcus aureus*. J. Ethnopharm. 79: 359-364.

Esimone CO, Iroha IR, Ibezim EC, Okeh CO, and Okpana EM (2006). *In vitro* evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus.* Afr. J. Biotech. 5(11): 1082-1086.

European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin. Micro. Inf. 9(8): 1-7.

European Committtee for Antimicrobial Susceptibity Testing (EUCAST) (2000). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. Clin. Micro. Inf. 6(9): 509-515.

Farombi EO, Alabi MC, and Akuru TO (2002). Kolaviron modulates cellular redox status and impairment of membrane protein activities induced by potassium bromate ($KBrO_3$) in rats. Pharmacol. Res. 45(1): 63-68.

Fluit AC, Schmitz FJ, Verhoef J, and European SENTRY Participants (2001). Multi-resistance to antimicrobial agents for the ten most frequently isolated bacterial pathogens. Int. J. Antimic. Agents 18: 147-160.

Han QB, Lee SF, Qiao CF, He ZD, Song JZ, Sun HD, and Xu HX (2005). Complete NMR assignments of the antibacterial biflavonoid GB1 from *Garcinia kola*. Chem. Pharm. Bull. 53(8): 1034-1036.

Hooton TM, Blair AD, Turck M, and Counts GW (1984). Synergism at clinically attainable concentrations of aminoglycoside and beta-lactam antibiotics. Antimic. Agents Chemo. 26(4): 535-538.

Iwu M, and Igboko O (1982). Flavonoids of *Garcinia kola* seeds. J. Nat. Prod. 650-650.

Iwu MW, Duncan AR, and Okunji CO (1999). New antimicrobials of plant origin. J. Janick (ed.), Perspectives on new crops and new uses: 457-462.

Kamatou GPP, Viljoen AM, van Vuuren SF, and van Zyl RL (2006). *In vitro* evidence of antimicrobial synergy between *Salvia chamelaeagnea* and *Leonotis leonurus*. SA J. Bot. 72: 634-636.

Kumar A, and Schweizer HP (2005). Bacterial resistance to antibiotics: Active efflux and reduced uptake. Adv. Drug Deliv. Revs. 57: 1486-1513.

Lee JY, Oh WS, Ko KS, Heo ST, Moon CS, Ki HK, Kiem S, Peck KR, and Song JH (2006). Synergy of arbekacin-based combinations against vancomycin hetero-intermediate *Staphylococcus aureus*. J. Korean Med Sci. 21: 188-92.

Lewis K, and Ausubel FM (2006). Prospects for plant-derived antibacterials. Nature Biotech. 24(12): 1504-1507.

Mandal S, Mandal MD, and Pal NK (2004). Evaluation of combination effect of ciprofloxacin and cefazolin against *Salmonella enterica* serovar *typhi* isolates by *in vitro* methods. Calicut Med. J. 2(2): e2.

Marquez B, Neuville L, Moreau NJ, Genet JP, Santos AF, Andrade MCC, and Sant'Ana AEG (2005). Multidrug resistance reversal agent from *Jatropha elliptica*. Phytochem. 66: 1804-1811.

Montecalvo MA, Horowitz H, Gedris C, Carbonaro C, Tenover FC, Issah A, Cook P, and Wormser GP (1994). Outbreak of vancomycin-, ampicillin-, and aminoglycoside-resistant *Enterococcus faecium* bacteremia in an adult oncology unit. Antimic. Agents Chemo. 38(6): 1363-1367.

Neugebauer KA, and Gilliland SE (2005). Antagonistic action of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 toward spoilage organisms in cottage cheese. J. Dairy Sci. 88: 1335-1341.

Odds FC (2003). Synergy, antagonism, and what the chequerboard puts between them. J. Antimic. Chemo. 52(1): 1-1.

Oluwatuyi M, Kaatz GW, and Gibbons S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochem. 65(24): 3249-3254.

Pankey G, Ashcraft D, and Patel N (2005). *In vitro* synergy of daptomycin plus rifampin against *Enterococcus faecium* resistant to both linezolid and vancomycin. Antimic. Agents Chemo. 49(12): 5166-5168.

Perron GG, Zasloff M, and Bell G (2005). Experimental evolution of resistance to an antimicrobial peptide. Proc. Biol. Sci. 273(1583): 251-256.

Rybak MJ, and McGrath BJ (1996). Combination antimicrobial therapy for bacterial infections. Guidelines for the clinician. Drugs. 52(3): 390-405.

Sato Y, Shibata H, Arai T, Yamamoto A, Okimura Y, Arakaki N, and Higuti T (2004). Variation in synergistic activity by flavone and its related compounds on the increased susceptibility of various strains of methicillin-resistant *Staphylococcus aureus* to β-lactam antibiotics. Int. J. Antimic. Agents. 24(3): 226-233.

Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, and Higuti T (2005). Alkyl gallates, intensifiers of β-lactam susceptibility in methicillin-resistant *Staphylococcus aureus* Antimic. Agents Chemo. 49(2): 549-555.

Smith ECJ, Williamson EM, Wareham N, Kaatz GW, and Gibbons S (2007). Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. Phytochem. 68(2): 210-217.

Stapleton PD, Shah S, Anderson JC, Hara Y, Hamilton-Miller JMT, and Taylor PW (2004). Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates. Int. J. Antimic. Agents. 23(5): 462-467.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, and Lewis K (2000). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. Appl. Biol. Sci. 97(4): 1433-1437.

Tegos G, Stermitz FR, Lomovskaya O, and Lewis K (2002). Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimic. Agents Chemo. 46(10): 3133-3141.

White RL, Burgess DS, Manduru M, and Bosso JA (1996). Comparison of three different *in vitro* methods of detecting synergy: Time-Kill, Checkerboard, and E test. Antimic. Agents Chemo. 40(8): 1914-1918.

Zhao WH, Hu ZQ, Okubo S, Hara Y, and Shimamura T (2001). Mechanism of synergy between epigallochatechin gallate and β-lactams against methicillin resistant *Staphylococcus aureus*. Antimic. Agents Chemo. 45(6): 1737-1742.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Resistance to antibiotics by major bacterial pathogens has limited the efficacy of current antibacterial drugs. This has necessitated the search for new alternative compounds. In addition, the combined use of antibiotics and compounds that inhibit resistance mechanisms allowing the antibiotic to regain its potency against resistant pathogens has also been seen as an alternative approach to mitigate the effect of resistance among bacterial pathogens.

Medicinal plants, owing to their rich diversity of secondary metabolites, promise to provide a potential source of these two types of compounds, if the rich chemical diversity could be adequately tapped. A number of plant derived compounds have been reported to exhibit *in vitro* synergy with antibiotics against resistant strains of pathogenic bacteria. The synergy has been reported in a few cases, to involve dual targeting or mutual interference between the plant compounds and antibiotics (Zhao *et al.,* 2001; Yam *et al.,* 1998). To a greater extent, however, the synergy has been attributed to inhibition of antibiotic MDR efflux proteins (Smith *et al.,* 2007; Marquez *et al.,* 2005; Oluwatuyi *et al.,* 2004). It is the prospects of obtaining potent broad spectrum MDR efflux pump inhibitors from plants that has been considered attractive (Lomovskaya and Bostain, 2006; Lewis and Ausubel, 2006). Obtaining drug preparations based on combinations between efflux pump inhibitors and antibiotics can help to recover the clinical efficacy of old antibiotics that have been rendered ineffective due to resistance.

In this report, seed extracts of *Garcinia kola*, a tropical plant of the family Guttiferae which is widely distributed in West and Central Africa where it is valued for its medicinal properties, were assessed for their antimicrobial properties as well as the potentials of their combinations with antibiotics.

In the antibacterial activity assays, the aqueous and acetone extracts of the seed were evaluated for their activity against 27 bacterial strains. The aqueous extract showed activity mainly against Gram positive organisms with MIC values ranging from 5 – 20 $mgml^{-1}$ while the acetone extract exhibited activity against both Gram negative and Gram positive organisms with MIC values ranging from 0.156 - 10 $mgml^{-1}$. The bactericidal activity of the acetone extract revealed that the extract was strongly bactericidal against *Staphylococcus aureus* and *Proteus vulgaris* at concentrations of 0.3125 and 5 $mgml^{-1}$ respectively.

In the antistaphylococcal activity assay, the aqueous, methanol and acetone extracts of the seeds were investigated for their activity against four clinical strains of *Staphylococcus* obtained from cases of wound sepsis. All the strains were susceptible to the aqueous extract at 30 $mgml^{-1}$ with MIC values of 10 $mgml^{-1}$. The methanol and acetone extracts exhibited more potency with the strains susceptible at 10$mgml^{-1}$ and MIC values ranging from 0.312 to 0.625 $mgml^{-1}$. The bactericidal activity of the acetone extract revealed that the extract was strongly bactericidal against one of the isolates (OKOH3) but weakly bactericidal against another isolate (OKOH1).

In the study of the effect of combinations between the extracts of the plant and antibiotics, combinations of the acetone extract and the antibiotics penicillin G, amoxycillin, tetracycline, chloramphenicol, erythromycin and ciprofloxacin were investigated by means of the fractional inhibitory concentration (FIC) indices as well as by the use of time-kill assays. Synergistic

interactions with some antibiotics were observed largely against Gram positive organisms using the FIC indices, with combinations against Gram negative organisms yielding largely antagonistic interactions. The time-kill assay detected synergy involving all antibiotics against both Gram negative and Gram positive organisms with a ≥ 1000 times (≥ 3 Log_{10}) potentiation of the bactericidal activity for some antibiotics.

In total, the results of this research unveiled some significant findings on the therapeutic potentials of the seeds of *Garcinia kola* laying a foundation for possible future studies that may lead to the production of some chemotherapeutically useful compounds. Considering these significant findings, this research therefore concludes with the following recommendations:

- There is need to widen the extractant variability in the antibacterial activity of the plant since the activity of the plant will depend on the properties of the extracting solvent used. Notably solvents such as chloroform, dichloromethane, hexane among others have not been previously investigated.

- There is need to investigate the bactericidal potency of the plant against a wider range of clinical isolates of pathogenic organisms in order to obtain a more accurate evaluation of the plant's therapeutic potential.

- There is also need to carry out a bioassay guided fractionation of the acetone extract of the plant in a bid to isolate the compounds responsible for the antibacterial as well as the potentiation of antibiotic activity. Further, it will be necessary to elucidate the mechanism of action of such compounds as well as their levels of toxicity to assess their clinical applicability.

REFERENCES

Lewis K, and Ausubel FM (2006). Prospects for plant-derived antibacterials. Nat. Biotech. 24(12): 1504-1507.

Lomovskaya O, and Bostian KA (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic - A vision for applied use. Biochem Pharmacol. 7(1): 910-918.

Marquez B, Neuville L, Moreau NJ, Genet JP, Santos AF, Andrade MCC, and Sant'Ana AEG (2005). Multidrug resistance reversal agent from *Jatropha elliptica*. Phytochem. 66: 1804-1811.

Oluwatuyi M, Kaatz GW, and Gibbons S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochem. 65(24): 3249-3254.

Smith ECJ, Williamson EM, Wareham N, Kaatz GW, and Gibbons S (2007). Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. Phytochem. 68(2): 210-217.

Yam TS, Hamilton-Miller JM, and Shah S (1998). The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2' synthesis, and beta-lactamase production in *Staphylococcus aureus*. J. Antimic. Chemo. 42(2): 211-216.

Zhao WH, Hu ZQ, Okubo S, Hara Y, and Shimamura T (2001). Mechanism of synergy between epigallochatechin gallate and β-lactams against methicillin resistant *Staphylococcus aureus*. Antimic. Agents Chemo. 45(6): 1737-1742.