BIOLOGICAL ACTIVITIES AND MECHANISMS OF ACTION OF TWO ETHNOBOTANICALLY SELECTED SOUTH AFRICAN MEDICINAL PLANTS ON SOME BACTERIA ASSOCIATED WITH GASTROINTESTINAL INFECTIONS

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN ETHNOBOTANY (PHYTOMEDICINE)

IN THE DEPARTMENT OF BOTANY
FACULTY OF SCIENCE AND AGRICULTURE
UNIVERSITY OF FORT HARE

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AUGUST, 2012

DECLARATION

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Ethnobotany in the Faculty of Science and Agriculture is my original work with the exemption of the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

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Prof. Anthony J. Afolayan

ACKNOWLEDGEMENTS

I hereby acknowledge the grace of the Lord, Most High and Mighty, in my life. God, You have been so good to me in all the wildernesses of life I have gone. Thank you for being my best Friend, Guide and Mentor. By Your grace, this dream has come through to this end. I am also very grateful to my Supervisor, Prof Anthony Jide Afolayan. Thanks for your fatherly roles that go beyond research supervision during the cause of carrying out this study. Your constructive criticisms at every step of this study and for making me to be part of the Phytomedicine Research Group are inestimable. Your supervision and methods of dowsing tensions are impeccable. I really appreciate you and your family. I am also grateful to my HOD, Prof DS Grierson, for his contribution towards the success of this work.

To my loved ones - Elder and Dr (Mrs) Akinlolu Fakeye, Mrs, Mosun , Mr and Mrs Adefehinti, Mr, Okenyi and family, Mr. Adewale Fakoya and family, Elder and Mrs. Olaleye Maitanmi, Elder and Mrs. Tunde Afolayan and Pa and Mrs. Solabi - whose contributions to making this dream a reality, I cannot forget your love. Thanks for being there for me at the most critical time in this journey. May God reward your labour of love in multiple folds.

I am indebted and grateful to my father – Pa Joshua Adeoti Olajuyigbe (Late). You were my best friend in life. You were stronger than steel and truer than what needle is to a pole. Your words of instructions ring daily. Though this dream, your dream, is coming after your passage to the great beyond, I am grateful that your visions are actually materializing. To my mother, Mrs. Tolanikawo Z. Olajuyigbe, I am grateful for being the first Gold I have ever had in life. To my siblings, Olasunmibo, Mojisola, Olufemi and Ayodeji (aka Akobi), thanks for always putting your feet on ground at the appropriate time to me.

To the most important gift I have ever had in life, my wife - Aderonke A. Olajuyigbe, thanks for believing me. Without you, maybe I would still be the same old person. To my children, Oluwajomiloju and Oluwajeminipe, your presence rewrote the story of my life. With you, I am happy to having a happy and an ultimate home. Thanks for bearing this moment through with.

My sincere gratitude goes to my academic mentors, Dr (Mrs) Titilayo O Fakeye, Prof and Prof (Mrs) Deboye O Kolawole and Prof Otunola Adedayo. To my colleagues and research group members in Phytomedicine Research Centre, Drs Bola Okoh, Sunday Oyedemi, Sunday Arowosegbe, Abosede Wintola, Sinbad Olorunnisola, Callistus Bvenura, Wilfred Otang, Abimbola Badmus and others, thanks for your criticisms at one point or the other.

Without Govan Mbeki Research and Development Center of Fort Hare University and National Research Foundation of South Africa, this achievement would still be in the oblivion. I am very grateful for providing research grants to carry out my study in South Africa. I am also grateful to Babcock University Administration for their supports in realizing this goal. Lastly, I appreciate the Sangomas, rural dweller and herbalists who assisted me in providing ethnobotanical information that laid the foundation for the completion of this work.

DEDICATION

This research is dedicated to The Almighty God for being with me in the wilderness of life

and

To the loving memory of my father and mentor, Pa Joshua Adeoti Olajuyigbe

(14^{th} July, 1938 – 26^{th} June, 2011)

TABLE OF CONTENTS

CHAPTERS **PAGE**

General Abstracts

GENERAL ABSTRACTS

In this study, 36 plant species representing 24 families were found to be commonly used for the treatment of a variety of gastrointestinal disorders in Eastern Cape, South Africa. The family Fabaceae had the highest number of species. Out of these, 47.06% were used in the treatment of dysentery alone while 46.15% were used in the treatment of diarrhoea. *Acacia mearnsii* De Wild and *Ziziphus mucronata* subsp. *mucronata* Willd were selected for this research because they are extensively used in folkloric medicine in South Africa and there was lack of scientific reports that documented their biological activities. The phytochemical screening, antioxidant activities, *in vitro* antimicrobial activities, cytotoxicity, the synergistic potentials and mechanisms of actions of these plants were investigated.

The phytochemical screening and the antioxidant activities of the two species showed that the quantity of the phenolic compounds, flavonoids and proanthocyanidins detected differ significantly in the various extracts. Of the aqueous, acetone, ethanolic and methanolic extracts of *A. mearnsii*, the ethanolic extract had the highest flavonoids while the acetone extract had the highest phenolic contents. The proanthocyanidins were highest in the methanol extract while aqueous extracts had the least phytochemicals. Aqueous extract showed the least ferric reducing power but methanol extract indicated the highest reducing power. The reducing power of the extracts was lower than those obtained from the reference standard such as butylated hydroxytoluene (BHT), rutin and ascorbic acid. 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) diammonium salt showed that ethanol extract exhibited the highest antioxidant activity at the highest concentration tested. Also, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay indicated that ethanol extract had the highest radical scavenging activity at the lowest

concentration and the activities of all the extracts decreased with increase in their concentrations. In *Z. mucronata* subsp. *mucronata,* the phenolics were significantly higher than the flavonoids and proanthocyanidin contents in all the extracts investigated. The ethanol extract had the highest antioxidant activity, followed by the acetone extract while the aqueous extract was the least active. Reacting with ABTS, the 50% inhibitory concentrations (IC_{50}) were (0.0429 ± 0.04 mg/ml) for aqueous, (0.0317 ± 0.04 mg/ml) for acetone and (0.0306 ± 0.04 mg/ml) for ethanol extracts while they inhibited DPPH radical with 50% inhibitory concentration (IC_{50}) values of 0.0646 ± 0.02 mg/ml (aqueous), 0.0482 ± 0.02 mg/ml (acetone) and 0.0422 ± 0.03 mg/ml (ethanol). The investigation showed that a positive linear correlation existed between the total phenolic content and antioxidant activity of the extracts and that these plants have strong antioxidant property and free radical scavenging capability.

The *in vitro* antibacterial activities of *Acacia mearnsii* and *Z. mucronata* subsp. *mucronata* showed that their minimum inhibitory concentrations ranged between 0.039 mg/ml and 1.25 mg/ml. With the exception of acetone extract of *A. mearnsii* having MICs greater than 1.0 mg/ml for *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* KZN, all other isolates had MICs less than 0.7 mg/ml. In all the bacteria treated with *Z. mucronata* subsp. *mucronata* extracts, *Enterobacter cloacae* ATCC 13047 had MIC greater than 1 mg/ml in methanol extract, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 6538 had MICs greater than 1 mg/ml in acetone extract while all other isolates were highly susceptible to the different extracts of *Z. mucronata* subsp. *mucronata* and had MICs less than 0.7 mg/ml. While aqueous extract was as active as the alcoholic extracts in *A. mearnsii,* that of *Z. mucronata* had no effect. The ethanol extracts exhibited the highest degree of antibacterial activity in both plants. This study, also, showed that the antifungal activity of *A. mearnsii* ranging 0.3125 – 5.0 mg/ml was

higher than those of the different extracts of *Z. mucronata* subsp. *mucronata* ranging 1.25 – 10.0 mg/ml. It is evident from the results of the brine shrimp lethality assay that the crude extracts of *A. mearnsii* with the LC_{50} equaled 112.36 µg/ml and having the highest levels of toxicity (100%) death at 500 µg/ml was non toxic (LC_{50} > 100 µg/ml) while the LC_{50} for *Z. mucronata* subsp. *mucronata* equaled 90.27 µg/ml indicated a low level of toxicity.

The effects of combining the crude extracts of these plants with eight antibiotics were investigated by means of checkerboard and agar diffusion methods. On using the methanol extract of *A. mearnsii*, the agar diffusion assay showed that extract-kanamycin combination had zones of inhibition ≥ 20 ± 1.0 mm in all the bacteria tested (100%), followed by extract-chloramphenicol (90%) > extract-ciprofloxacin = extract-tetracycline (70%) > extract-amoxicillin (60%) > extract-nalidixic acid (50%) > extract-erythromycin (40%) > extract-metronidazole (20%). The checkerboard showed synergistic interaction (61.25%), additivity/indifference (23.75%) and antagonistic (15%) effects. I, therefore, concluded that the antibacterial potentials of the antibiotics were improved and combining natural products with antibiotic could be a potential source of resistance-modifying agents useful against multi-drug resistant bacteria.

The influences of these extracts on the ultrastructures, elemental components, protein and lipid leakages of five different bacteria were determined as the possible mechanisms of action of the extracts investigated. The scanning electron microscopy indicated varied ultrastructural changes in the morphology of bacterial cells treated with the extracts. The X-ray microanalysis showed significant differences between the elemental contents of extract-treated and untreated bacteria while lipids and proteins were leaked to a great extent from the extract-treated bacterial strains in comparison with the untreated ones. The possible mechanisms of action of the extracts

may include inhibition of a significant step in peptidoglycan assembly, inhibition of metabolic processes, disruption of cell wall and cell membranes resulting in the efflux of lipid and protein in all the bacteria tested. The possible mechanism of action involved in the lipid and protein leakages in the bacterial cells could be attributed to lipid peroxidation and protein oxidation owing to the antioxidant activities of the extracts that were active beyond the protective levels. I concluded that the morphological changes and the observed leakages showed rapid killing, significant membrane depolarization resulting in leakages and efflux of disintegrated cellular materials.

In general, this study has justified the ethnotherapeutic importance of *A. mearnsii* and *Z. mucronata* subsp. *mucronata* in the treatment of microbial infections by indicating the possible mechanisms of action of the crude extracts on the tested bacteria.

CHAPTER 1

General Introduction

General Introduction

1.1 Introduction

Infectious diseases are a significant cause of morbidity and mortality accounting for approximately 50% of all deaths in tropical countries (Khosravi and Behzadi, 2006). They are a leading cause of death worldwide (Ahmad and Aqil, 2009). Due to indiscriminate use of antibacterial agents in controlling infectious diseases, the frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide (Al-Bari et al., 2006). Multidrug resistance in bacteria has become a growing concern of the medical arena (Simões et al., 2007) and a great challenge to human health globally (Peters et al., 2008). As a result of the multidrug resistance, several mechanisms of resistance against antibiotics including antibiotic inactivation by enzymatic action, alteration of the efflux pump mechanisms, target mutation and decreased uptake of antibiotics were reported (Singh and Barrett, 2006).

With the prevalence of multi-drug resistant bacteria, appearances of strains with reduced susceptibility to antibiotics (Boucher et al., 2009) and their inexorable invasion of hospitals and communities (Ymele-Leki et al., 2012), there are increases in health care costs, appearance of many untreatable bacterial infections and the need to search for new infection-fighting strategies (Rojas et al., 2006). To effectively treat mixed and severe infections and prevent the emergence of resistant microorganisms (Levinson and Jawetz, 2002), attention has been focused on discovering new antimicrobial compounds of plant origin (Rossland et al., 2005). Natural products were, therefore, considered interesting alternatives for treatment of infections because they are rich in varieties of secondary metabolites with antimicrobial properties (Lewis and Ausubel, 2006; Lu et al., 2007).

1.2 Medicinal plants

Medicinal plants are important sources of therapeutic agents playing significant roles in the treatment of microbial infections (Selvamaleeswaran et al., 2010; Ichor and Ekoja, 2011). They synthesize a wide range of secondary metabolites that possess antimicrobial, antioxidant, cytotoxic and other pharmacologically valuable properties (Sasidharan et al., 2008; Olajuyigbe and Afolayan, 2011). Globally, they are viewed as unique sources of medicines and constitute the most common human use of biodiversity (Hiremath and Taranath, 2010). On all continents, natural products have been used for medicinal purposes for thousands of years (Levetin and McMahon, 2002; Patel and Coogan, 2008) before the introduction of antibiotics and other modern drugs (Kabir et al., 2005). In Africa, the use of remedies derived from plants in traditional healthcare practices is common and widespread (Rukangira, 2001). In South Africa, the rich plant biodiversity, with over 20000 different species, is a great source of interest to the scientific community (Borchers et al., 1997).

Traditionally, these plants are reliable sources for the treatment of diseases in different parts of the world (Hostettmann et al., 2000). The use of medicinal plants contributes significantly to primary health care delivery as they are regarded as an alternative to synthetic antimicrobial agents (Kumar et al., 2010). Since the development of new compounds and antimicrobial agents for the treatment of microbial infections is of increasing interest in the recent years (Trivedi and Hotchandani, 2004), the trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries (Ncube et al., 2008). They are less toxic and not very detrimental to human health (Satish et al., 2009). Since they are easily affordable, compatible with the human body and produce fewer side effects, there are increases in the consumption and demand for medicinal plants (Jayashree and Maneemegalai,

2008). In search for new sources of drugs and neutraceuticals (Ghosh; 2003; Sharma and Mujundar, 2003), several reports have indicated that antimicrobial activity of crude extracts and the bioassay-guided fractionation of those extracts yielded active principles (El-Seedi et al., 2002; Zgoda-Pola et al., 2002). Resulting from many indicated ethnotherapeutic effects of medicinal plants, several *in vitro* and *in vivo* studies on the efficacy of plant extracts have been investigated scientifically (Prusti et al., 2008; Montaz and Abdollahi, 2010). Several pharmacological importance have been accredited to different secondary metabolites including saponins, tannins, alkaloids, phenols, glycoalkaloids, flavonoids, sesquiterpenes, lactones and terpenoids in many medicinal plants (Nawrot et al., 2007).

Consequently, there is a growing interest in correlating the phytochemical constituents of a medicinal plant with their pharmacological activity (Al-Bayati and Al-Mola, 2008). Contemporary pharmacopoeias containing at least 25% of drugs derived from plants and many synthetic analogues built on prototype compounds isolated from plants have been reported (De Silva, 2005). Numerous drugs have entered the international pharmacopoeia through ethnobotany and traditional medicine (Ergene et al., 2006) because they contain active substances effective against human pathogens and relent or cure diseases (Hadi and Bremner, 2001). This is in response to the increasing trends in the emergence of resistance to antimicrobial agents resulting from poor quality drugs manufactured, patient non-compliance and spontaneous mutations within the microbial populations (Nester et al., 2002; Denyer et al., 2004) as well as under dose and their use as growth promoters in livestock. In ethnotherapy, there has been resurgence in the consumption and demand for medicinal plants (Jayashree and Maneemegalai, 2008) while effective treatment of bacteria resistant to currently used antibiotics (Monroe and

Polk, 2000) and the bioprospecting of new plant-derived drugs (Sharma et al., 2010) are on the increase.

Gastrointestinal infections refer to disorders that interfere with the workings of the intestine. They disrupt the body's ability to absorb nutrients and expel waste. Their symptoms may include abdominal cramping or pain, diarrhea, constipation, nausea, vomiting and signs of blood and mucus in stool (Spencer et al., 1983; Frank et al., 2000). They are caused by bacteria, viruses, protozoa, helminths and sometimes fungi. The most common bacteria in gastrointestinal infections are *Bacillus cereus*, Campylobacter, Salmonella, and *Escherichia coli* O157:H7 while rotavirus is the major cause of severe viral gastrointestinal infections (Gray et al., 2008). *Salmonella species* is a dangerous pathogen which can make its way into the bloodstreams to cause fatal infection. The verotoxin-producing *E, coli* O157:H7 may cause hemolytic uraemic syndrome, which is a serious life-threatening condition resulting in acute renal failure in children, if ingested (Tarr et al., 2005). While treatment of gastrointestinal infections is complicated by antibiotic resistance, the preventive and therapeutic effects of medicinal plants against gastrointestinal infections should not be underestimated.

Diarrhoea is an important clinical problem (Casbur-Jones and Farthing, 2004) and one of the leading causes of morbidity and mortality in developed and developing countries (Pruss et al., 2002; Kosek et al., 2003). It is an alteration in the normal bowel movement characterized by an increase in frequency and fluidity of stools (Baldi et al., 2009). It was considered the most frequent clinical manifestation of altered gastrointestinal ecology (Gordon et al., 1963; 1964) and enterohaemorrhagic (EHEC) and verotoxin producing *E. coli* (VTEC) such as O26, O103, O111 and O157 have been implicated in bloody diarrhoea (Law, 1997; Frank et al., 2011). While drug treatments, depending on the causal agents, is inevitable (Wynn and Fougere, 2007), its

management by the replacement of lost fluids and electrolytes has been indicated (Thapar and Sanderson, 2004). The use of antimicrobial agents for self-limiting diarrhoea is, however, discouraged to forestall the development of bacterial resistance. Consequently, a programme involving the use of herbal medicines for diarrhoeal control in the developing countries was introduced by WHO (2004). These medicinal plants are able to prevent, delay the onset or reduce the risk of this infection.

Shigellosis is a disease of public health importance causing self-limited diarrhea to severe dysentery (Gupta et al., 2010). Aside from clinical intestinal manifestations, it causes a wide variety of extra-intestinal signs such as bacteremia or neurologic manifestations (Ashkenazi, 2004). While enteropathogenic bacteria are mainly implicated (Tayung and Kar, 2005), Barman et al. (2011) and Vinh et al. (2011) indicated that shigellosis is caused by different species of Shigella. Although shigellosis is a major cause of gastroenteritis throughout the world with *Shigella spp.* being mainly implicated (Ram et al., 2008), other gastrointestinal pathogens such as Salmonella, Yersinia, enteropathogenic *Escherichia coli* (EPEC), *Entamoeba hystolytica, Bacillus subtilits, Bacillus cereus, Aeromonas hydrophyla* and *Campylobacter sp.* have been involved.

Although *Bacillus cereus*, *Bacillus subtilits*, Campylobacter, Salmonella, and *E. coli* O157:H7 have been implicated in gastrointestinal infections, the choice of most of the bacteria used in this study arose from their implications in different infections of clinical importance. *Staphylococcus aureus* causes a variety of suppurative, wound infections and food poisoning in human beings (Alvarez et al., 2006). *Pseudomonas aeruginosa* causes nosocomial respiratory tract infections, urinary tract and skin infections (Rossolini and Mantengoli, 2005; Gad et al., 2008) and intra-abdominal-sepsis and urogenital-sepsis (Page and Heim, 2009). *Bacillus subtilis*,

often used as probiotics, are nonpathogenic bacteria which may occasionally cause serious or even fatal disease in man (Weinstein and Colburn, 1950). While *Klebsiella pneumoniae* causes bloodstream infection (Pfaller et al., 1998) and nosocomially acquired pneumonia in immunocompromised patients (Bentzel et al., 2004), *Enterobacter cloacae* causes urinary tract infection (Kaminska et al., 2002). *Shigella sonnei* is a major cause of bacterial gastroenteritis and bacillary dysentery (Guerrant and Steiner, 2005; Dupont, 2005). These infections occur mostly in humans and often require proper attention because of the associated morbidity and mortality in some cases.

1.3 Mechanisms of action of antibacterial agents

In the last few decades, a number of studies on the *in vitro* and *in vivo* efficacy of plant extracts against pathogens causing microbial infections have been reported (Ahmed et al. 2002; Feresin et al., 2003; Natarajan et al., 2003; Subramoney et al., 2003; Amanloua et al. 2004; Kosalec et al., 2005; Sung et al., 2007). Although several studies elucidated the antimicrobial potentials vested in crude extracts of several higher plants, their mechanisms of actions, referring to specific biochemical interaction through which a pharmacologically active substance produces an effect on a living organism, remained poorly investigated.

However, the mechanisms of action of some isolated secondary metabolites of plants have been reported. Ahmed et al. (1993) showed terpenes to be involved in membrane disruption by lipophilic compounds. Ya et al. (1988), Chung et al. (1998) and Cowan (1999) suggested that terpenes promoted membrane disruption, coumarins caused reduction in cell respiration, and tannins acted on microorganism membranes and polysaccharides or enzymes promoting inactivation. Ulanowska et al. (2006) indicated that isoflavone genistein was able to change cell

morphology and inhibit DNA and RNA synthesis. Cushnie and Lamb (2005) and Zhang et al. (2008) indicated that flavonoids inhibited cytoplasmic membrane function and DNA gyrase and β-hydroxyacyl-acyl carrier protein dehydratase activities.

Although *A. mearnsii* and *Z. mucronata* have been associated with folkloric uses in Eastern Cape of South Africa, investigating their biological activities and mechanisms of action becomes essential to establish their effectiveness against bacterial pathogens. This may possibly assist in determining if a bioactive compound, when isolated, will produce the same effect in comparison to those of the established antibacterial agents, affect the same target sites in the same manner with those established for antibiotics or work in synergy with antibacterial agents to produce better pharmacological effects.

1.4 The choice of *Acacia mearnsii* De Wild. and *Ziziphus mucronata* subsp. *mucronata*

The folkloric use of *Acacia mearnsii* and *Ziziphus mucronata* subsp. *mucronata* for the treatment of gastrointestinal disorders, especially dysentery and diarrhoea, in Eastern Cape, South Africa, was discovered during the ethonobotanical survey for this research (Olajuyigbe and Afolayan, 2012). Although these plants are readily accessible to traditional healers in different locations, harvesting from trees around the University affords easy access to them as research materials for this study. The choice of alcoholics and water as extracting solvents was based on how they are being prepared by the traditional healers and varied degree of their ability to extract phytochemicals of medicinal importance. *Acacia mearnsii*, commonly known as Black Wattle tree, has globular inflorescence with 20-30 tiny pale yellow flowers and dark brown to black pods which are more or less straight, 5-10 cm long, 5-8 mm wide and strongly constricted

between seeds (Figure 1). It is basically known as one of the world's highest yielding sources of high quality condensed tannin (Young et al., 1986).

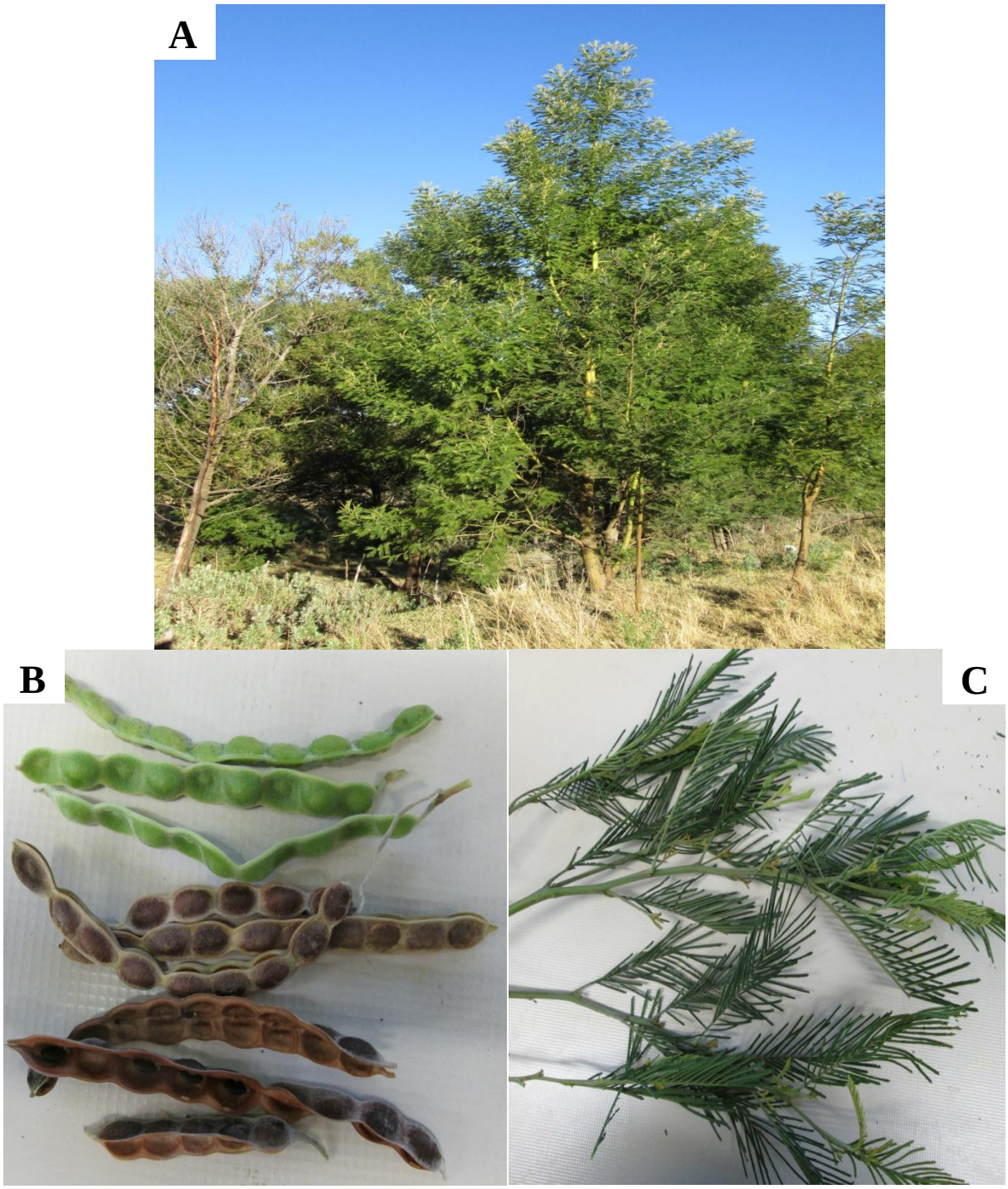

Figure 1: *Acacia mearnsii* de Wild, fruits and leaves. A = *Acacia mearnsii* in its natural habital, Hogsback road, Eastern Cape; B = *Acacia mearnsii* fruits; C = *Acacia mearnsii* leaves

Ziziphus mucronata. subsp. *mucronata,* also known as buffalo thorn in Figure 2, are used medicinally for the treatment of rheumatism, gastrointestinal complaints and snake bites (Tas et al., 1991). The infusions are used to relieve body pains, respiratory infections, chest problems, gonorrhea, diarrhoea and dysentery (Amusan et al., 2005).

Despite the acclaimed folkloric use of these two plant species, the two species were the most frequently mentioned for the treatment of gastrointestinal disorders during the survey and there is a lack of scientific reports on their pharmacological importance to substantiate the claims. Hence, these plant species were chosen and the stem barks frequently mentioned during the ethnobotanical survey were used for the investigation. This study was, therefore, aimed at investigating their biological activities, their potential effects in combination antimicrobial therapy and mechanisms of action with a view to validating their acclaimed use by the traditional healers of the Eastern Cape. A comparison of their biological activities may give credence to their use in ethnomedicine in this Province.

Figure 2: A = *Ziziphus mucronata subsp. mucronata* in its natural habitat. B = Leaves on spiny stem; C = Stem bark. The plant becomes deciduous with the onset of winter

1.5 Objectives of the study

1.5.1 Overall aim

The broad aim of this research is to investigate the biological activities and mechanisms of action of two ethnobotanically selected South African medicinal plants, *Acacia mearnsii* De Wild. and *Ziziphus mucronata* subsp. *mucronata*, on some bacteria associated with gastroenteritic infections.

1.5.2 Specific objectives

The specific objectives were:

1. To conduct an ethnobotanical survey of plants used in the treatment of gastrointestinal disorders in the study area and select the two most frequently used for further studies.
2. To investigate the phytochemicals and antioxidant activities of the two plants used in the treatment of dysentery and diarrhoea
3. To determine antimicrobial activities of the plant extracts against selected microorganisms associated with gastrogastroenteritic infections
4. To determine the possible cytotoxicity effects of the extracts using the brine shrimp lethality assay
5. To investigate synergistic and/or antagonistic effects of combining different extracts with some of the first line antibiotics including amoxicillin, erythromycin, ciprofloxacin, tetracycline, kanamycin, metronidazole, nalidixic acid and chloramphenicol
6. To determine the mechanisms of action of extracts of *A. mearnsii* and *Z. mucronata* as well as making comparative analytical studies

1.6 The structure of the thesis

This thesis consists of contributions in the form of reprints of published articles (Chapters 2, 3, 4, 5, 7, 8, 9, 10 and 11) and three manuscripts submitted for publication (Chapter 6, 12 and 13).

In Chapter 2, medicinal plants commonly used for the treatment of a variety of gastrointestinal disorders in Eastern Cape, South Africa are presented. Chapter 3 and 4 reported the quantitative phytochemical and antioxidant activities of the extracts of the two selected plants. The ethnotherapeutic potential of *A. mearnsii* and *Z. mucronata* were determined by investigating the antimicrobial potential and toxicity of their acetone extracts in Chapters 5 and 6. Since aqueous/water and ethanol are sometimes used traditionally in the preparation of herbal medicines, Chapters 7 and 8 report the results of the antibacterial activities of aqueous and ethanolic extract of *A. mearnsii* and *Z. mucronata*. Here, the antibacterial activity of aqueous extract of *Z. mucronata* was not reported because it had no activity against the selected bacteria at the test concentrations used. While Chapter 9 is a report of the antibacterial and time-kill assessment of methanolic extract of *A. mearnsii.* Chapter 10 is concerned with the results of the antibacterial activity of the methanol extract of *Z. mucronata*. A consideration for antimicrobial resistance to currently used antibiotic (D'Costa *et al.,* 2006; Endimiani and Paterson, 2007) led to investigating synergistic interactions between medicinal plant materials and some first line antibiotics used in chemotherapy. Chapter 11 showed that synergistic interactions resulted from a combination of *A. mearnsii* extracts and eight different antibiotics. Chapters 12 and 13 indicated that the mechanisms of action of crude extracts could be by inhibition of a significant step in peptidoglycan assembly, inhibition of metabolic processes or disruption of cell wall and cell membranes resulting in the efflux of lipid and protein in all the bacteria tested. The possible

mechanism of action involved in the lipid and protein leakages in the bacterial cells could be attributed to lipid peroxidation and protein oxidation owing to the antioxidant activities of the extracts being active beyond the protective levels.

In the General Discussion in Chapter 14, a clearer picture of the results of this study is presented. The medicinal potentials of *A. mearnsii* and *Z. mucronata* and their possible mechanisms of antibacterial activities are emphasized.

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CHAPTER 2

Ethnobotanical survey of medicinal plants used in the treatment of gastrointestinal disorders in the Eastern Cape Province, South Africa

(*Published in the Journal of Medicinal Plants Research*)

Journal of Medicinal Plants Research Vol. 6(18), pp. 3415-3424, 16 May, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1707
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Ethnobotanical survey of medicinal plants used in the treatment of gastrointestinal disorders in the Eastern Cape Province, South Africa

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Accepted 11 April, 2012

An ethnobotanical survey of plants used for the treatment of gastrointestinal disorders was carried out in the Eastern Cape Province, South Africa. Information on the names of plants, used parts and methods of preparation was obtained from traditional medical practitioners, herbalist, hawkers in traditional medicines and rural dwellers, using semi-structured questionnaire. 36 plant species representing 24 families were found to be commonly used in the treatment of a variety of gastrointestinal disorders in this study. The family Fabaceae had the highest number of species being used for treating gastrointestinal disorders. 47.06% of the plants used in treating dysentery and other gastrointestinal disorders were used in the treatment of dysentery alone while 46.15% of the plants used to treat diarrhoea and other gastrointestinal disorders, were used in the treatment of diarrhoea alone. 30.3% of the different plants were implicated in the treatment of various stomach problems. Rationales for the choice of these plants were also identified. The leaves were the most commonly used parts, followed by roots and bark while decoctions and infusions are the most frequent methods of preparation. The traditional healers in this Province possess rich ethno-pharmacological knowledge and depend largely on naturally growing plant species. The documented medicinal plants can serve as a basis for further and future phytochemical and pharmacological studies.

Key words: Medicinal plants, gastrointestinal disorders, dysentery, indigenous knowledge, over-exploitation.

INTRODUCTION

Traditionally, plants are reliable sources for the treatment of diseases in different parts of the world (Eisenberg et al., 1993; Cowan, 1999; Hostettmann et al., 2000). Their use contributes significantly to primary health care delivery (Holetz et al., 2002) as they are regarded as invaluable sources of pharmaceutical products (Olalde, 2005). Globally, medicinal plants have been unique sources of medicines and constituted the most common human use of biodiversity (Hamilton, 2004; Hiremath and Taranath, 2010). In African societies, the tradition of collecting, processing and applying plants and plant-based medications have been handed down from generation to generation. Traditional medicine, with medicinal plants as their most important component, are sold in market places or prescribed by traditional healers in their homes (Von Maydell, 1996). As a result of this strong dependence on plants as medicines, many ethnopharmacological studies have been conducted to determine their safety, efficiency and discovery of new active principles from them.

Ethnopharmacology, the science of application of indigenous or local medicinal remedies, including plants for treatment of diseases (Gurib-Fakim, 2006; Pande et al., 2008), is the investigation of biologically active agents traditionally used by humans (Bruhn and Holmested, 1981). These active agents included plant mixtures, whole plants and a portion of a plant as well as special preparations from plant materials. Being a multidisciplinary science, successful research in ethno-pharmacology requires the interaction of ethnobotanists, natural products chemists, pharmacologists, taxonomists, traditional healers and/or user communities. According to Vanden Berghe et al. (1986), Rojas et al. (1992) and Silva et al. (1996), the goal of ethnobotany or

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ethnopharmacology, therefore, is to utilize the impressive array of knowledge assembled by indigenous peoples about the plant and animal products they have used to maintain health.

Although the use of medicinal plants for subsistence, home remedies, trade and alleviating human suffering (Kunwar et al., 2006) plays important roles in the lives of rural people (Ahmad, 2003), the non-sustainable collection methods have caused threat from harvesting and many valuable medicinal herbs are becoming rare due to their continuous utilization (Swe and Win, 2005) and over-exploitation for commercial purposes (Tabuti et al., 2003; Kamatenesi-Mugisha and Oryem-Origa, 2005). In addition to these endangering effects of human-plant relationships, traditional folk knowledge and folklore information from many different cultures, which are the sum of attitudes, opinions, beliefs and customs handed down from generation to generation in a given society, (Anonymous, 1993) which are important tools in revealing plants with useful medicinal properties (Balandrin et al., 1993), are neither often found in written form, nor organized and structured in ways accessible to science. As a result, this knowledge changes because of indigenous creativity, innovativeness and contact with other knowledge systems. Along with the traditional lifestyles, traditional usage and folk knowledge of plants are disappearing due to copying of westernized lifestyles and economic systems.

Considering a sharp decrease in the biological species all across the globe and the increasing economic values placed on medicinal plants, documentation on ethno-botanical knowledge is a way to understand the use of different plant species to cure various ailments and means to conserve these natural resources. Globally, there is currently a renaissance of ethnobotanical surveys of medicinal plants and the need to screen specific parts of the plants (Paterson and Anderson, 2005; Igoli et al., 2005; Li and Vederas, 2009). Regardless of many ethnobotanical studies on medicinal plant resources in South Africa (Cunningham, 1988; Hutchings, 1989; Hutchings et al., 1996; Mander, 1998; Van Wyk et al., 1997; Van Wyk and Gericke, 2000; Appidi et al., 2008, Olorunnisola et al., 2011) and the world over, a large number of medicinal plants and associated indigenous uses still wait proper documentation (Tabuti et al., 2003). Although Appidi et al. (2008) had earlier reported some plants used in the treatment of diarrhoea, there is a lack of information on plants used in the treatment of gastrointestinal disorder such as dysentery while many plants relevant in treating diarrhoea and other gastrointestinal disorders are inexhaustible. This may be due to the thin line of distinction differentiating these infections especially diarrhea and dysentery. Hence, previous attentions may have been directed towards plants used in treating diarrhea while possibly and unknowingly addressing dysentery simultaneously. This ethnobotanical survey is, however, aimed at identifying plants and part(s) that are used in the treatment of some gastrointestinal disorders as well as indicating their methods of preparation and rationale for their trado-therapeutic effects in the Eastern Cape Province, South Africa.

MATERIALS AND METHODS

The study area falls within the latitude 30°00' to 34°15'S and longitudes 22°45' to 30°15'E. It is bounded by the sea on the East and the drier Karroo (Semi-desert vegetation) in the West. The elevation ranges from sea-level to approximately 2200 m in the North and the vegetation is veld type, known as the Eastern Cape thorn veld (Acocks, 1975; Masika and Afolayan, 2003). This area consists of many villages which are generally classified as rural and poor with difficulty in distinguishing between gastrointestinal disorders such as diarrhoea and dysentery infections.

Field visits for this study were carried out in May and July, 2011. Information was obtained from rural dwellers, traditional healers, hawkers of medicinal plant preparations and herbalists with the help of a semi-structured questionnaire and the guided field-walk method as described by Martin (1995) and Maundu (1995). The questionnaire was used to interview these individuals while the guided field-walk involved contacting and interviewing individuals recommended by other community members for their knowledge. The information collected included local names, the parts of the plant used and methods of preparation. The information was further validated by common response. The information from at least three or more respondents was considered as common response. Proper scientific identification of the plants and their uses in these communities were reaffirmed from the literature sources containing medicinal plants used in South Africa (Roberts, 1990; Hutchings et al., 1996; Van Wyk et al., 1997; Mander, 1998; Van Wyk and Gericke, 2000).

Intellectual property agreement statement

Prior to the interviews, the informants were duly informed about the objectives of the research. With verbal agreement that this research shall not be used for commercial purposes but to enlighten and document medicinal plants used for the treatment of gastrointestinal disorder, the interview was granted.

RESULTS

A total of 36 plant species distributed in 24 families were found to be used locally for treating various gastrointestinal disorders including diarrhoea, dysentery, abdominal cramps, gut disturbances, stomach disorders, upset and aches. The families are arranged in alphabetical order. Family names are followed by vernacular names or local names and plant part(s) used and their methods of preparations. The results are summarized in Table 1. The Fabaceae was represented by five plants, followed by Apiaceae, Asphodelaceae, Lamiaceae and Solanaceae (3 species each), Hyacinthaceae (2 species each) while other families (1 species each) were found to be used medicinally by the local communities. Of these plants, 19 (52.78%) different plants were indicated to be used for treating dysentery

Table 1. Ethnomedicinal plants used in Eastern Cape, South Africa, for the treatment of gastrointestinal disorders.

Plant	Family	Local name	Used part	Uses	Preparation
Acacia mearnsii	Fabaceae	Idywabasi	Bark and leaves	Dysentery	Infusions and concoctions of the bark; decoction of the bark
Acacia karroo Hayne	Fabaceae	Umngampunzi, intlaka, umnga	Leaves, bark and gum	Dysentery, diarrhoea and haemorrhage	Infusions and concoctions of the leaves, bark and gum
Alepidea amatymbica Eckl. and Zeyh.	Apiaceae	Iqwili	Root/rhizome	Abdominal cramps	Decoction of the roots
Bulbine abyssinica A.Rich., B.	Asphodelaceae	Iyeza lipulayiti, utswelana, Uyakayakana	Leaves and roots	Dysentery	Decoctions of the leaves and roots
Brachylaena ilicifolia (Lam.) E. Phillips and Scheweick.,	Asteraceae	Umgqeba	Leaves	Diarrhoea	Infusion and decoction of the leaves
Bulbine latifolia (L.f.) Roem. et Schult.	Asphodelaceae	Ibhucu, ingcelwane	Root	Diarrhoea	Decoctions of the root
Bulbine asphodeloides Roem. et Schult.	Asphodelaceae	Umthi kanoyayi, Uyakayakane	Tuber	Diarrhea, dysentery	Decoctions of the root/tuber
Cussonia spicata Thunb.,	Araliaceae	intsenge, umgezisa, umsenge,	Leaves	Stomach complaints	Infusion
Curtisia dentata (Burm.f.) C.A.Sm., Olea	Cornaceae	umLahleni, umGxina, Uzintlwa,	Bark	Stomach ailments	Decoctions of the bark
Centella asiatica (Linn.)	Apiaceae	Inyongwane, iphuzi	Roots and leaves	Stomach disorders, dysentery and diarrhoea	Infusion, decoction and concoction of the leaves and roots
Clausena anisata (Willd.) Hook.f. ex Benth.	Rutaceae	Iperepes	Root, bark, the fresh leaves	Stomach complaints	A decoction of the aromatic leaves or roots
Cissampelos capensis L.f.,	Menispermaceae	Umayisake	Roots, leaves	Stomach upset and diarrhoea	Infusion

Table 1. Contd

Eucomis autumnalis (Mill.)	Hyacinthaceae	Isithithibala Esimathunzi,	Bulbs	Stomach ache and colic	Decoctions of the bulb
Ekebergia capensis Sparrm.	Meliaceae	uManaye, umGwenya-wezinja	Bark	Dysentery	A decoction of the back
Foeniculum vulgare Mill.,	Apiaceae	Imbambosi	Leaves and stem	Stomach cramps	Infusion
Hydnora Africana (Thunb.)	Hydronaceae	Umavumbuka	Whole plant	Diarrhoea, dysentery	Infusions and decoctions
Ipomoea crassipes Hook.,	Convolvulaceae	Ubhoqo	Whole plant	Dysentery	Infusions and decoctions
Iconotis leonurus (L.) R. Br.	Lamiaceae	Imvovo	Whole plant	Dysentery	Infusion, decoction, rectum application
Mentha aquatica L., *Mentha longifolia* (L). L.	Lamiaceae	Inxina,	Leaves	Stomach aches	Infusion
Olea europaea L. subsp. africana (Mill.) P. S. Green	Oleaceae	Umnquma	Bark, leaves and roots	Diarrhoea	Infusions and decoctions of dried leaves, bark and roots.
Pelargonium reniforme Curtis	Geraniaceae	Umkumiso,	Root	Dysentery	Decoction
Persicaria lapathifolia (L.) Gray	Polygonaceae	Idolo lenkonyana	Roots and leaves or whole plant	Stomach complaints and diarrhoea	Infusion
Plantago lanceolata (L.)	Plantaginaceae	Ubendlela	Leaves	Diarrhoea, dysentery	Infusion
Rubia petiolaris DC.	Achanthaceae	Impendulo	Roots, leaves	Stomach problems, haemorrhagic diarrhoea and amoebic dysentery	Infusion, decoction and concoction
Sarcophyte sanguinea Sparrm.,	Balanophoraceae	umavumbuka	Whole plant	Diarrhea, dysentery	Infusion, Decoction
Schotia afra (L.) Thunb.	Fabaceae	Umgxam,	Bark and root,	Diarrheoa	Decoction
Schotia latifolia Jacq.	Fabaceae	Umgxam	Bark and root	Diarrheoa	Decoction
Schotia brachypetala Sond.	Fabaceae	Ishimnumyane,	Bark and roots	Dysentery, diarrhoea	Decoction
Strychnos henningsii Gilg,	Loganiaceae	umnonono, umnono,	Stem Bark	Stomach aches	Decoctions of the bark and infusions of the leaves

Table 1. Contd.

Syzygium cordatum Hochst.	Myrtaceae	Umsu	Bark, leaves and roots	Stomach complaints, diarrhoea	Decoction and concoction
Schizocarphus nervosus (Burch.) Van der Merwe	Hyacinthaceae	umagaqana, inkwitelu	Roots or rhizomes	Dysentery	Decoction of the roots
Solanum aculeastrum Dun., *Solanum tomentosum* L.	Solanaceae	Umthuma	Root, bark and berries	Dysentery	Infusion, decoction and concussion
Typha capensis (Rohrb.) N.E.Br.,	Typhaceae	inqoboka, ingcongolo	Rhizomes	Diarrhoea, dysentery	Decoction of the rhizomes
Ziziphus mucronata Willd. subsp. *mucronata* Willd	Rhamnaceae	Umphafa	Bark, leaves and roots	Diarrhoea, Dysentery	Decoction of the roots; concoction of bark and leaves

and other gastrointestinal disorders. Of these 19 plants used to treat dysentery and other gastrointestinal disorders, 8 (42.11%) plants were implicated in the treatment of dysentery alone. These plants include *Acacia mearnsii, Bulbine abyssinica, Ekebergia capensis*, *Ipomoea crassipes*, *Iconotis leonurus, Pelargonium reniforme, Schizocarphus nervosus* and *Solanum aculeastrum*. In addition, 14 (38.89%) plants were used to treat diarrhoea along with other gastrointestinal disorders. While six (42.86%) of these plants: *Brachylaena ilicifolia, Bulbine latifolia*, *Cissampelos capensis*, *Olea europaea*, *Persicaria lapathifolia* and *Schotia afra*, were implicated in the treatment of diarrhoea alone; eight plants (22.22%): *Acacia karroo*, *Hydnora Africana, Plantago lanceolata, Rubia petiolaris, Sarcophyte sanguine, Schotia brachypetala, Typha capensis* and *Ziziphus mucronata*, were implicated in treating dysentery and diarrhoea as shown in Figure 1. Though *Curtissia dentata* alone was mentioned as being used in the treatment of diarrhoea and stomach complaints, ten (27.78%) different plants were implicated in the treatment of various stomach problems. These plants include *Alepidea amatymbica, Cussonia spicata, Centella asiatica, Clausena anisata*, *C. capensis*, *Eucomis autumnalis, P. lapathifolia*, *R. petiolaris, Syzygium cordatum* and *Strychnos henningsii.*

In different communities, many plants are given different names. Many of these plants, eventually, have more than one local name. These plants are prepared and mostly administered orally in different ways, except *Iconotis leonurus* having rectum application in addition to its oral administration, in different ways to treat gastrointestinal and its associated disorders. In their preparations for therapeutic purposes, whole plants as well as various parts of each plant species were either used singly or in combined forms. Parts used also depend on the plant under consideration and severity of ailments. Leaves constituted the majority of uses (30.77%), followed by roots (25%) and bark (21.15%), whole plant (9.62%), rhizomes (5.77%), fruits (3.85%) as well as bulb and tuber (1.92%). The results are shown in Figure 2. Decoctions and infusions are the most frequently used methods of preparation as shown in Figure 3.

DISCUSSION

Over the last century, ethnobotany has evolved into a specific discipline that looks at the people–plant relationship in a multidisciplinary manner such as ecology, economic botany, pharmacology and public health (Balick, 1996). With extensive uses of medicinal plants, numerous drugs have been introduced into the international markets as a result of exploring ethnopharmacology and traditional medicines (Bussmann, 2002) which have expressed different pharmacological actions (Gregory, 2004). Hence, the traditional use of low profile and less known medicinal plants should

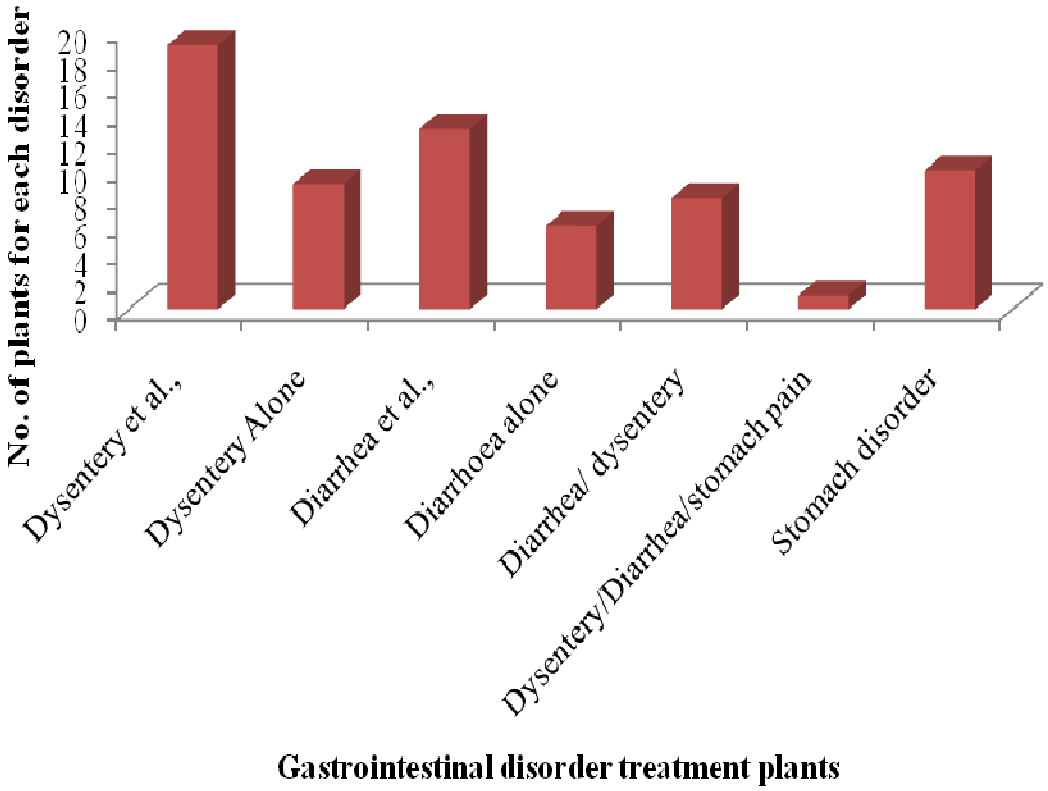

Figure 1. Number of plants relevant in treating each gastrointestinal disorder.

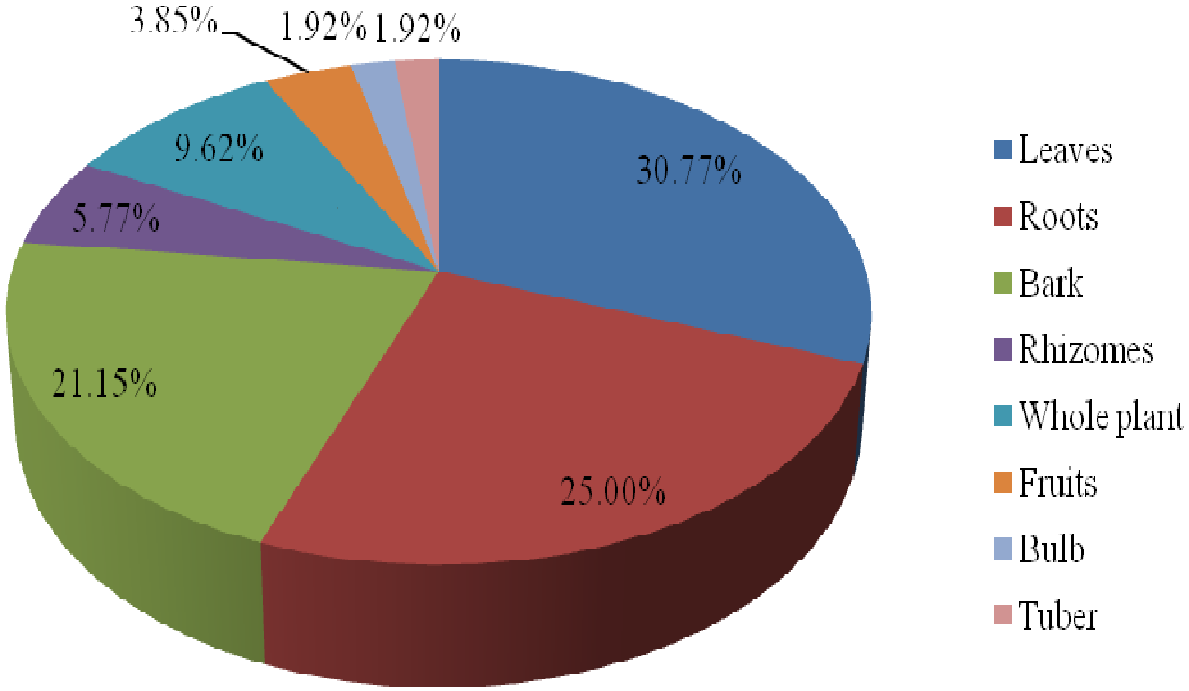

Figure 2. Frequency of plant parts used for treating gastrointestinal disorders.

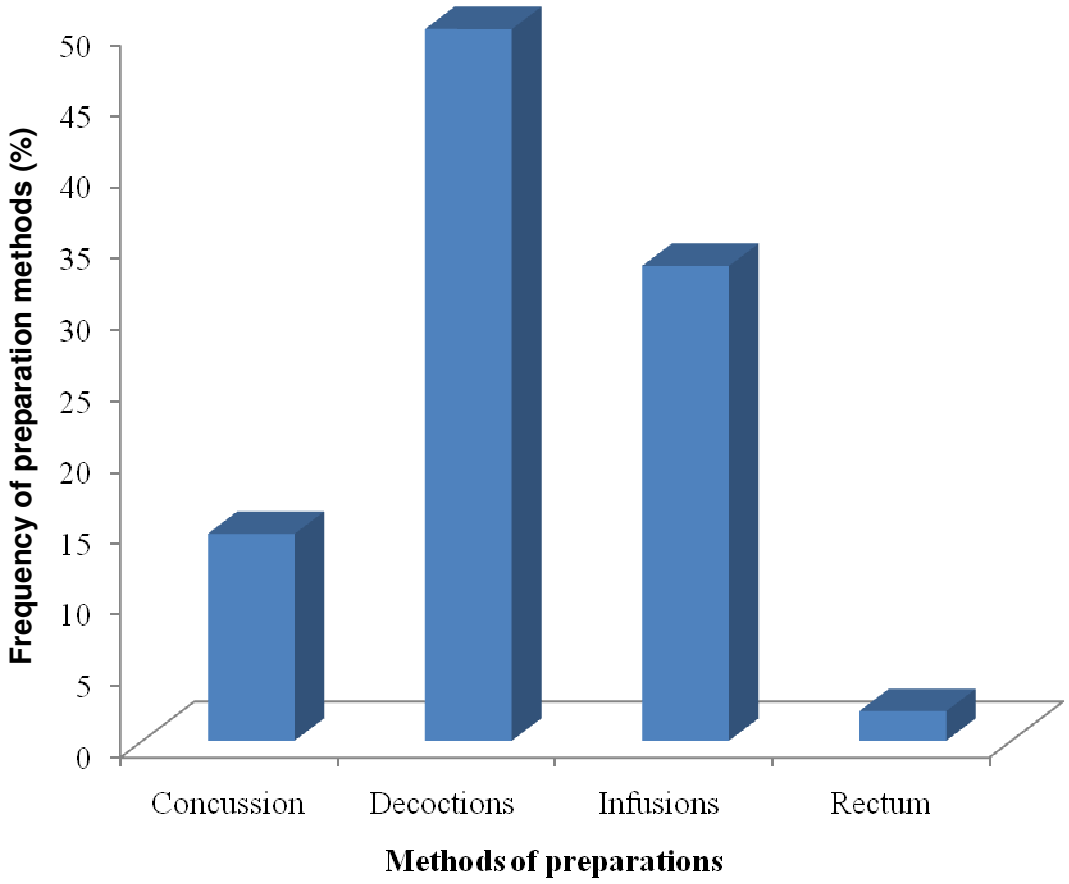

Figure 3. Frequency of different preparation methods for treating gastrointestinal disorders.

be documented to disseminate their therapeutic efficacy to pave the way for preparation of acceptable medicine and to reduce the pressure on overexploited species (Kala et al., 2006).

In South Africa, up to 60% of the population consults traditional healers (van Wyk et al., 1997), especially in rural areas where traditional healers are more numerous and accessible than Western health-care providers. The traditional healer are found within a short distance and are familiar with the patient's culture while the environment and the costs associated with treatments are negligible (Rinne, 2001). While the loss of valuable medicinal plants due to population pressure, agricultural expansion and deforestation have been widely reported (Abebe, 2001; Berhan and Dessie, 2002), documenting indigenous knowledge becomes essential to preserve the traditional knowledge and valuable information passed verbally from generation to generation and can be lost whenever a traditional medical practitioner passes without conveying his knowledge about traditional medicinal plants.

In this study, the number of indicated medicinal plants and their potential applications in the treatment of gastrointestinal disorders reflect the rich ethnomedicinal knowledge in the Eastern Cape. Here, traditional medicine remains the main resource of phytotherapy for a large majority of the people. The wide spread use of the various plants could be attributed to cultural acceptability, efficacy, physical accessibility and economic affordability as well as playing a major role in the treatment of gastrointestinal disorders in comparison to modern medicine. Based on the difficulty in distinguishing between diarrhoea and dysentery by the local people, many of the plants have been used in treating either of these infections unknowingly, thereby, indirectly showing their multipurpose efficacies. For instance, 38.89% of the plants mentioned are used for diarrhoea and other gastrointestinal disorders while 52.78% were indicated as being used against dysentery and other gastrointestinal disorders. While 22.22% of these plants are used in treating dysentery and diarrhoea, 27.78% are used in the treatment of various stomach problems. There are overlaps in plants used in treating both infections. Plants used in treating different stomach problems are not an exemption. In addition to earlier report of Appidi et al. (2008), *B. ilicifolia, C. capensis*, *P. lapathifolia* and *S. afra* have been used in the treatment of diarrhoea locally. The prevalence of the use of leaves for the preparation of

traditional herbal remedies as shown in this study corresponds with earlier reports in other studies (Brinkhaus et al., 2000; Yineger and Yewhalaw, 2007; Pradhan and Badola, 2008; Zainol et al., 2008). While the use of more than one plant or plants' parts in herbal preparations could be attributed to the additive or synergistic effect that extracts from the different plants are thought to have during treatment (Bussman and Sharon, 2006), Gessler et al. (1994) indicated that the use of concoctions suggests that the traditional medicines may only be active in combination due to the synergistic effects of several compounds that are acting singly. On the contrary, the use of bark, roots or uprooting the whole plant of a given species could be destructive means of obtaining the herbal remedies. These unfavorable extraction methods will eventually contribute to the loss of the forest trees.

Though the methods of preparing these medicinal plants vary, decoction and infusion methods are highly reputed and valued by traditional healers in Southern African native population for its curative and palliative effects in the treatments of diseases generally (Watt and Breyer-Brandwyk, 1962; Hutchings, 1996) while active compounds in preparations taken anally are more effectively re-absorbed by the mucus membranes of the rectum (Van Wyk and Wink, 2004). Decoction of a part or combination of different parts could be more effective as more active phytochemicals are likely to be extracted by boiling. In agreement with Nanyingi et al. (2008) and Bekalo et al. (2009), there is a lack of standardization and quality control in orally administered traditional medicine. Against these parameters, oral dosages are estimated using lids, spoons, cups, pinches and handfuls while most preparations are often prescribed through estimation in term of a full, half or one-fourth of a cup, depending on the age, physical condition of the patient being treated, severity and type of infection.

In addition, without scientific proofs from the traditional healers and local people, the rationales for the choice of some of these plants have been attributed to include some inherent properties of these plants. These attributes included being purgative, anti-dysenteric, anodyne, anti-inflammatory, carminative, demulcent, diaphoretic, emollient, styptic or astringent, refrigerant, stomachic, tonic and vasodilator. Usher (1984) and Koide et al. (1998) reported that the folk use of *A. mearnsii* (Fabaceae), *Mentha aquatic* (Lamiaceae), *P. lanceolata* (Plantaginaceae), *P. lapathifolia* (Polygonaceae), *R. petiolaris* (Achanthaceae), *S. sanguine* (Balanophoraceae), *S. afra* (Fabaceae) and *S. latifolia* (Fabaceae) as anti-dysenterics was due to their tannin content imparting astringent activity which helps to recuperate from diarrhoea and dysentery. Plants containing tannins are astringent, able to draw together or constrict body tissues and are effective in stopping the flow of blood or other secretions. Tannins strengthen veins by repairing the connective tissues surrounding veins and decrease capillary fragility. They are also known as antimicrobial (Cowan, 1999) and triterpenoids are beneficial for inflammation (Cipak et al., 2006). The anti-inflammatory activities may be due to the presence of alkaloids, flavonoids and saponins present in these plants like every other plants (Wong et al., 1992; Ono, 1994; Kerber, 1999; Fernanda et al., 2002; Fawole et al., 2009). The refrigerant, purgative and vasodilatory activities of these plants substantiate their ability to cause the blood to stop flowing and clog the arteries and veins as well as removing enough "heat" from the system (Littlewood, 1988; Lans, 2006).

Conclusion

Traditional knowledge of medicinal plants and their uses by indigenous cultures are not only useful for conservation of cultural traditions and biodiversity but also for community healthcare and drug development in the present and future. In this study, 36 plant species consisting of 24 families were used as ethnomedicines for gastrointestinal disorders in the Eastern Cape, South Africa. These plants treated diarrhoea, dysentery and various stomach problems. Reasons for the choice of these plants, plants' parts used and methods of preparations were indicated. Since traditional healers harvest roots and barks of some of these medicinal plants, there is need to educate them about the looming danger of wiping out some of the plant species if over-exploited. Further investigation of ethnopharmacology is worthwhile to affirm their antimicrobial activities against bacteria in diarrhoea and dysentery, isolate the plants' active chemical compounds, and decipher their modes of action.

ACKNOWLEDGEMENT

The authors are grateful to the National Research Foundation (NRF) of South Africa for supporting this research.

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CHAPTER 3

Phytochemical assessment and antioxidant activities of alcoholic and aqueous extracts of *Acacia mearnsii* De Wild

(*Published in the International Journal of Pharmacology*)

International Journal of Pharmacology 7 (8): 856-861, 2011
ISSN 1811-7775 / DOI: 10.3923/ijp.2011.856.861

Phytochemical Assessment and Antioxidant Activities of Alcoholic and Aqueous Extracts of *Acacia mearnsii* De Wild

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Abstract: Phenolic compounds are well known for their antioxidant activities. The objectives of this study were to determine the phenolic content of the crude extracts of *Acacia mearnsii* De Wild and to evaluate the antioxidant properties of these extracts. The Folin-ciocalteu procedure was used to assess the total phenolic compositions of the extracts as garlic acid equivalents. Antioxidant activity was evaluated using 2,2-Azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS) diammonium salt and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging methods. All the extracts showed antioxidant potential. Ethanolic extract had the highest total flavonoids. Acetone extract had the highest total phenolic contents. The total proanthocyanidins was highest in the methanol extract while aqueous extracts had the least of these phytochemicals. The reducing power of the extracts of *A. mearnsii* was dose dependent. Aqueous extract showed the least reducing power, methanol extract indicated the highest reducing power. The reducing power of the extracts is lower than those obtained from the reference standard such as Butylated Hydroxytoluene (BHT), Rutin and ascorbic acid. 2,2-Azinobis-3-ethyl Benzothiazoline-6-Sulfonic acid (ABTS) diammonium salt showed that ethanol extract exhibited the highest activity at the highest concentration tested. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay indicated that ethanol extract had the highest activity at the lowest concentration and the activities of all the extracts decreased with increase in their concentrations. This study revealed a positive linear correlation between the total phenolic content and antioxidant activity of the extracts of *A. mearnsii*.

Key words: Antioxidant activity, *Acacia mearnsii* extracts 2, 2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid, radical scavenging, reducing power

INTRODUCTION

Acacia is one of the important genera of the family Fabaceae. It is a cosmopolitan genus containing more than 1350 species (Seigler, 2003). In Australia, there are approximately 960 species, which makes Acacia the largest genus of vascular plants in that region. The *Acacia species* are of immense value for reforestation and reclamation of wastelands, for fuel wood, timber and shelter (Palmberg and Pasca, 1981). They are also used for conservation and improvement of soil fertility through nitrogen fixation. These species can provide the nutrients and therapeutic ingredients to prevent, alleviate or treat many diseases in humans. They contain a variety of bioactive components such as flavonoids, alkaloids, tannins and phenolic acids. The most prominent substances in many *Acacia* species, however, are complex phenolic compounds (condensed tannins) and polysaccharides or gums (Maslin and Stirton, 1997). These compounds are responsible for numerous biological and pharmacological properties of acacia due to their strong antioxidant and free radical scavenging activities (Chopra *et al*., 1999).

Antioxidants are vital substances with the ability to protect the body from damages caused by free radical-induced oxidative stress. While free radicals have been reported to cause cellular damages that result in chronic diseases, many studies have indicated that phenolic compounds play a crucial role in oxidative scavenging (Mateos *et al*., 2005). The antioxidative effect is mainly due to phenolic components such as phenolic acids, phenolic diterpenes, anthocyanins, caumarins and flavonoids (Cai *et al*., 2004; Chye and Sim, 2009). These phenolic compounds with antioxidant activity are believed to account mainly for the antioxidant capacity of many plants (Wu *et al*., 2004).

Acacia mearnsii de Wild (Fabaceae) is a fast-growing leguminous tree. It was introduced to South Africa about 150 years ago primarily for the tanning industry. The bark of *A. mearnsii* is known to contain about 20-40% tannins

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and 70% proanthocyanidins (Young *et al*., 1986). Although, the *A.* species is widespread, relatively little is known about its chemistry and antioxidant potentials. This may be due to the difficulty associated with the identification of *Acacia* species and the insufficient clarity about their taxonomic relationships (Seigler, 2003).

Prior to this study, there was a dearth of information on the phytochemical and antioxidant activity of *A. mearnsii* in the literatures. Hence, this study was designed to investigate the phytochemical composition and antioxidant potential of this plant.

MATERIALS AND METHODS

Collection of plant material: The bark materials of *A. mearnsii* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa in September, 2010 while the study was carried out immediately after the plant material was dried. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University.

The bark samples were air-dried at room temperature and pulverized using a milling machine. Portions of about 100 g each of the pulverized samples were extracted separately with acetone, methanol, ethanol and water for 48 h. The extracts were filtered through Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at 40°C using a rotary evaporator. The extracts were redissolved in their respective solvents to the required concentrations for the bioassay analysis.

Chemicals and reagents used: 2,2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS) diammonium salt, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), gallic acid, rutin, ascorbic acid (VC), quercetin and $FeCl_3$, were purchased from Sigma Chemical Co. (St. Louis, MO. USA) vanillin was from BDH Chemicals Ltd. (Poole, England) and Folin-Ciocalteu phenol reagent and sodium carbonate were from Merck Chemical Supplies (Darmstadt, Germany). All other chemicals used, including the solvents, were of analytical grade.

Determination of total flavonoids: Total flavonoids were estimated using the method of Ordonez *et al.* (2006).

Determination of ferric reducing power: The ferric reducing potential of the extract was determined according to the method of Kumar *et al.* (2005).

Determination of total phenol: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method (Wolfe *et al*., 2003).

Determination of total proanthocyanidins: The total proanthocyanidins were determined by using the procedure reported by Sun *et al.* (1998).

DPPH radical scavenging assay: For DPPH assay, the method of Liyana-Pathirana and Shahidi (2005) was adopted.

ABTS radical scavenging assay: The total antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method of Siddhuraju and Manian (2007).

Statistical analysis: The experimental results were expressed as the Mean±Standard Deviation. All assays were performed in triplicates. The data were subjected to one way analysis of variance using SPSS version 14.0. Differences between means at the 5% level were considered significant.

RESULTS AND DISCUSSION

Phytochemical compositions: The result showed that *A. mearnsii* has a considerable amount of total phenolic and flavonoids contents while the proanthocyanidins were very low in all the extracts. The quantities of the phenolic contents, total flavonoids and total proanthocyanidins were in descending order depending on the extract as shown in Fig. 1. The results indicated that acetone extract had the highest total phenolic content (47.88 mg g^{-1}) while ethanol extracts had the highest total flavonoid contents (7.98 mg g^{-1}). While total

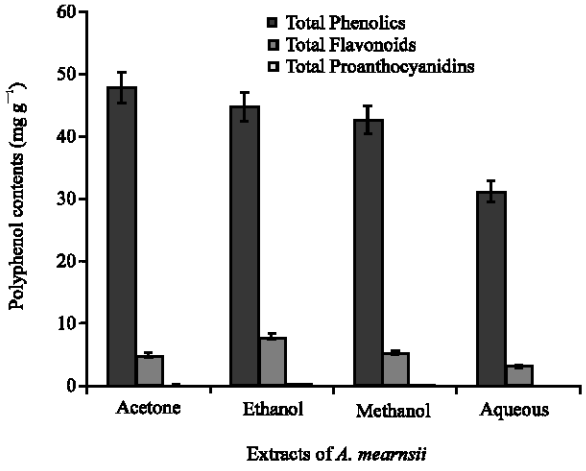

Fig. 1: Polyphenolic contents (mg g^{-1}) of *A. mearnsii*. Data are presented as Means±Standard Deviation of three replicate with significant increases from all samples tested

proanthocyanidin content (0.51 mg g^{-1}) was the highest in methanol extract, the polyphenols contents of this plant were least obtained in the aqueous extract. This result is similar to earlier report of Zongo *et al.* (2010) indicating that alcoholic extract exhibited higher level of total polyphenol contents than water extracts. Quantitatively, in *A. mearnsii*, total phenolic contents were more than the flavonoids while the proanthocyanidins were the least. Many related polyphenols, commonly found in plants, have been reported to have several different biological activities, including antioxidant property (Luo *et al.*, 2002; Afolayan *et al.*, 2008; Krishna *et al.*, 2010). According to Jayaprakasha and Patil (2007) and Hussein *et al.* (2010), there is a relationship between total phenolic content and antioxidant activity of plants. This is believed to be mainly due to redox properties of the phenolic compounds (Zheng and Wang, 2001) adsorbing and neutralizing free radicals, quenching active oxygen species as well as decomposing superoxide hydroxyl radicals. These phenolic compounds act as free radical terminators (Galvez *et al.*, 2003) while flavonoids show antioxidant activity through scavenging or chelating process (Torane *et al.*, 2011). El-Hela and Abdullah (2010) and Saikia and Adhyaya (2011) also noted a significant relationship between the free radical scavenging potency, the total phenolic and flavonoids contents of plant extracts. In this study, the extract having highest amount of flavonoids and phenolic compounds exhibited the highest antioxidant activity.

Total antioxidant power of extracts from *A. mearnsii* by the FRAP assay: The ferric reducing power of the different extracts of *A. mearnsii* was presented in Fig. 2. In both extracts and the standards studied, the reducing power or reductive capability of each of the extracts and the standards increased with increasing concentration. There are significant differences between the reductive capabilities of the extracts and those obtained for the standards such as Butylated Hydroxytoluene (BHT), Rutin and ascorbic acid. The reductive capabilities recorded was in the following order, Vitamin C>Rutin>BHT>Methanol>Acetone>Ethanol>Aqueous which showed that vitamin C exhibiting the highest reductive capability. At the highest concentration of 0.1 mg mL^{-1}, the reductive capability of each of the extract was 0.402 (aqueous), 0.421 (ethanol), 0.453 (acetone), 0.473 (methanol extracts), 0.633 (BHT), 0.706 (Rutin) and 1.218 (vitamin C) based on their spectrophotometric absorbance at 700 nm.

According to Chang *et al.* (2002) the observed reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant property of all the plant extracts. Furthermore, there is a linear relationship between concentrations and reducing power of the different extracts. This relationship is concentration dependent and may be associated with the presence of reductones (Duh, 1998) known to exert antioxidant activity by breaking the free radical chain via donating a hydrogen atom. These findings suggest that the *A. mearnsii* extracts are capable of donating electrons, and could, therefore, react with free radicals or terminate chain reactions.

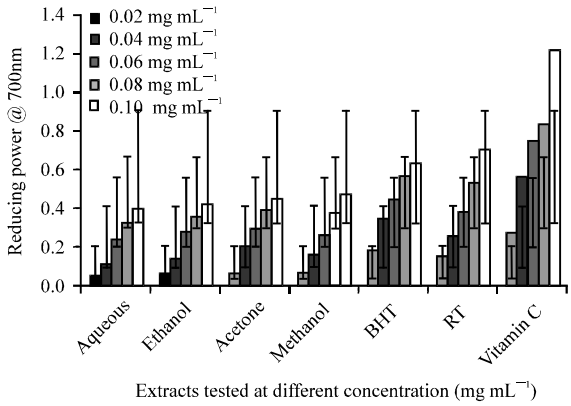

Fig. 2: Ferric reducing power determinations for the alcoholic and aqueous extracts of *A. mearnsii*. Data are presented as Means±Standard Deviation of three replicate with significant increases from all samples tested

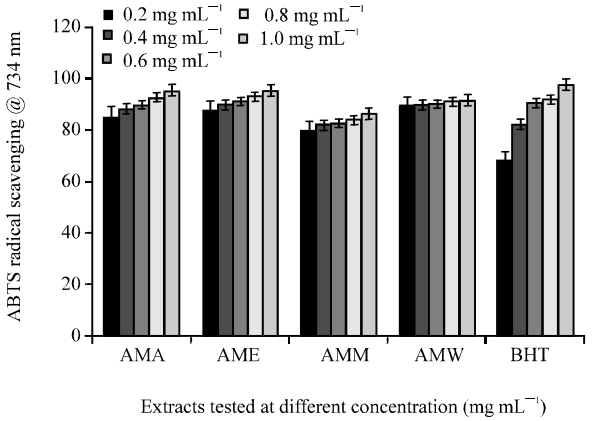

Fig. 3: ABTS radical scavenging activity of the alcoholic and aqueous extracts of *A. mearnsii*. Data are presented as Means±Standard Deviation of three replicate with significant increases from all samples tested

ABTS radical scavenging activity of the extract from *A. mearnsii*: The results of the free radical scavenging activity of the different extracts of *A. mearnsii* determined

by ABTS assay are shown in Fig. 3. Almost all the extracts had strong antioxidant abilities that exceeded that of BHT at varying concentrations. There was a steady increase in the ABTS radical scavenging capacity of all the extracts of *A. mearnsii* employed in present study. At the highest concentration of the extracts, the highest percentage inhibitions were recorded for the extracts and the standards.

The ABTS assay is based on the inhibition of the absorbance of the radical cation, ABTS, which has a characteristic long wavelength absorption spectrum (Sanchez-Moreno, 2002). While phenolic compounds scavenge radicals by forming a stable ABTS-H, the explicit method to measure the antioxidant activity of phenolic compounds is the decolorization of ABTS+radical. Decolorization of ABTS+,in present study, reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical cation in a concentration dependent manner. The scavenging activity of these extracts towards ABTS radicals is similar to those earlier reported by Miller and Rice-Evans (1997). These results showed that polyphenols from bark of *A. mearnsii* had high antioxidant activities and that the activity of $ABTS^{+}$ radical by the extracts was significant.

DPPH radical scavenging activity of extracts from *A. mearnsii*: In this study, the extracts of *A. mearnsii* exhibited varying degree of radical scavenging activity against the DPPH. At the lowest concentration of the extracts, high level of activity was observed in acetone, ethanol, methanol and aqueous extract. The activity of BHT was observed to be greater than activities of all the extracts at the lowest concentrations. This result showed that ethanol extract of the plant had the highest activity at the lowest concentration (Fig. 4). Their scavenging activities decreased with increase in the concentration of all the extracts as characterized by a rapid decline in the absorbance at 517 nm.

The ability of the extracts of *A. mearnsii* to act as a free radical scavenger or hydrogen donor was revealed by DPPH. This method is a rapid and sensitive way to survey the antioxidant activity of specific compounds or plant extracts. In this method, the antioxidant scavenges the DPPH radicals to form stable reduced DPPH molecules. The radicals formed are stabilized through the formation of non-radical products (Argolo *et al*., 2004). When the stable reduced DPPH molecules are formed in the presence of a free radical scavenger, the absorbance reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. The degree of reduction in absorbance value is indicative of

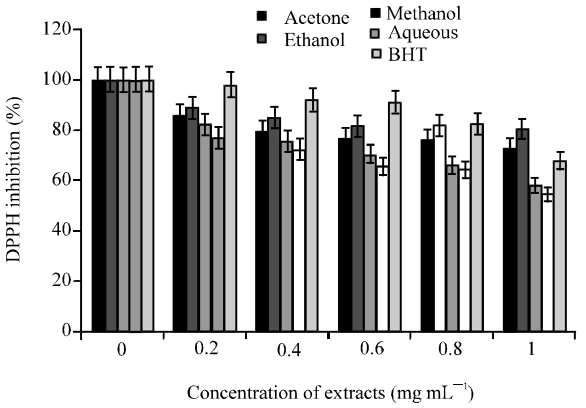

Fig. 4: DPPH radical scavenging activity of the alcoholic and aqueous extracts of *A. mearnsii*. Data are presented as Means±Standard Deviation of three replicate

the antioxidant power of the extract. More yellowish colour of DPPH shows more antioxidant activity of the compounds or extracts tested (Moein *et al*., 2008). The observed decreases in free-radical scavenging activities with increase in concentrations of extracts are statistically significant from one extract to another. While Yu *et al*. (2002) reported that higher concentrations of extracts are more effective in quenching free radicals in the systems, Goupy *et al*. (2003) observed that the rapid reaction between antioxidants and DPPH occurs with the transfer of the most labile hydrogen atoms to the radical, while the subsequent slow steps depends on the residual H-donating capacity of antioxidant degradation products.

CONCLUSIONS

In conclusion, it is well known that free radicals are one of the causes of several diseases. The present study demonstrated that *Acacia mearnsii* had significant antioxidant and radical scavenging activities. Irrespective of the method used for the analysis, all the extracts showed antioxidant activity and free radical scavenging capability. While their activities are less than those of the commercially available synthetic antioxidants, phenolic content of this plant could be a good source of natural antioxidant substances which could help to neutralize free radicals and play a beneficial role in oxidative stress prevention.

ACKNOWLEDGMENT

The authors are grateful to the National Research Foundation (NRF) of South Africa for supporting this research.

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CHAPTER 4

Phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

(Published in the BMC Complementary and Alternative Medicine)

BMC Complementary & Alternative Medicine

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Phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd

Olufunmiso O Olajuyigbe and Anthony J Afolayan*

Abstract

Background: Several plants traditionally used in treatment of a variety of infections in South Africa are reported in ethnobotanical surveys. Many of these plants including *Ziziphus mucronata* subsp. *mucronata* lack scientific reports to support their medicinal importance.

Methods: The antioxidant activities and phenolic contents of the acetone, ethanol and aqueous extracts of the stems of *Z. mucronata* subsp. *mucronata* were evaluated using *in vitro* standard methods. The total phenol, total flavonoids and proanthocyanidin content were determined spectrophotometrically. Quercetin, Tannic acid and catechin equivalents were used for these parameters. The antioxidant activities of the stem bark extracts of this plant were determined by ABTS, DPPH, and ferrous reducing antioxidant property (FRAP) methods.

Results: The quantity of the phenolic compounds, flavonoids and proanthocyanidins detected differ significantly in the various extracts. The phenolics were significantly higher than the flavonoids and proanthocyanidin contents in all the extracts investigated. The ferric reducing ability and the radical scavenging activities of the extracts were very high and dose-dependent. The ethanol extract had the highest antioxidant activity, followed by the acetone extract while the aqueous extract was the least active. Reacting with ABTS, the 50% inhibitory concentrations (IC_{50}) were (0.0429 ± 0.04 mg/ml) for aqueous, (0.0317 ± 0.04 mg/ml) for acetone and (0.0306 ± 0.04 mg/ml) for ethanol extracts while they inhibited DPPH radical with 50% inhibitory concentration (IC_{50}) values of 0.0646 ± 0.02 mg/ml (aqueous), 0.0482 ± 0.02 mg/ml (acetone) and 0.0422 ± 0.03 mg/ml (ethanol).

Conclusions: A correlation between the antioxidant activity and the total phenolic contents of the extracts indicated that phenolic compounds were the dominant contributors to the antioxidant activity of the plant. This study, therefore, demonstrated that *Z. mucronata* subsp. *mucronata* has strong antioxidant property and free radical scavenging capability.

Background

Free radicals and reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals are normal by-products of aerobic metabolism produced *in vivo* during oxidation [1]. These ROS are generated in the mitochondria and microsome organelles under normal physiological conditions. They can also be produced externally by exposure to radiation, toxic chemicals, cigarette smoking and alcohol consumption, and by eating oxidized polyunsaturated fats. Overproduction of ROS can result in oxidative damage to various biomolecules including lipids, proteins, DNA and cell membranes [2]. They also lead to the development of a variety of diseases such as coronary heart diseases, cancer, diabetes, hypertension and neurodegeneration [3,4]. While compounds capable of scavenging free radicals possess great potential in ameliorating these diseases [5,6], most of the ROS are scavenged by endogenous defense enzymes such as catalase, superoxide dismutase and peroxidase-glutathione system [7]. However, the activities of these endogenous defense systems may not be sufficient to mop up the free radicals.

Commonly used synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene

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(BHT), propylgallate (PG) and tertbutylhydroxyltoluene (TBHQ) [8]. Though important, they are known to constitute potential health risks and toxic effects [9]. Their applications are, therefore, strongly restricted. Hence, the need to search, develop and utilize more effective antioxidant from natural origin [10]. The medicinal properties of many plants have been attributed to the antioxidant properties of their constituents [11]. Some epidemiological studies also showed that the consumption of some plants can protect humans against oxidative damage by quenching free radicals and ROS [12,13]. Today, there are overwhelming interests in finding naturally occurring antioxidants for use in foods and in medicinal materials to replace synthetic antioxidants [14]. Interests in plant materials that are rich in phenols are increasing among scientists, food manufacturers and consumers because these materials retard oxidative degradation of lipids and improve the quality and nutritional value of food. People tend to eat functional foods with specific health benefits. The roles of natural antioxidants, mainly phenolic compounds, believed to have more antioxidant activity than vitamins C, E and β-carotene [15] is rapidly gaining attention.

Ziziphus mucronata Willd. subsp. *mucronata* Willd., also known as buffalo thorn, is a small to medium-sized tree, with a spreading canopy. It is distributed throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia. Its bark and roots are used medicinally for the treatment of various ailments, including rheumatism, gastrointestinal complaints and snake bites [16]. Warm bark infusions are used for body pains, expectorants in cough, respiratory infections and chest problems. The root infusions are used for treating gonorrhea, diarrhoea and dysentery. Decoctions of roots and leaves are applied externally to boils, sores and glandular swellings not to promote healing but for pain relief [17,18]. While several species of the genus *Ziziphus* have been investigated, there is a dearth of information on the phytochemical property and antioxidant activities of this plant. Hence, in line with the current trend of finding naturally occurring antioxidants, this study was designed to investigate the phenolic contents and antioxidant potential of different extracts of *Ziziphus mucronata* subsp. *mucronata* to justify its ethnomedicinal importance.

Methods

Collection of plant material

The bark materials of *Z. mucronata* subsp. *mucronata* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany by Prof. Grierson and a voucher specimen (OLAJ/2010/ZM/01) was prepared and deposited in the Griffin's Herbarium of the University.

The bark samples were air-dried at room temperature and pulverized using a milling machine. Portions of about 100 g each of the pulverized samples were extracted separately with 500 ml of each of the solvents, acetone, ethanol and water, for 48 h. The extracts were filtered through Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at 40°C using a rotary evaporator. The filtrate of aqueous extract obtained was quickly frozen at -40°C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA). The extraction yielded 5.3 g acetone extract, 14.2 g ethanol extract and 15.8 g water extract. The extracts were redissolved in their respective solvents to the required concentrations for the bioassay analysis.

Chemicals and reagents used

2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) diammonium salt, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), gallic acid, rutin, ascorbic acid (VC), quercetin and $FeCl_3$, were purchased from Sigma Chemical Co. (St. Louis, MO, USA); vanillin was from BDH Chemicals Ltd. (Poole, England) and Folin-Ciocalteu phenol reagent and sodium carbonate were from Merck Chemical Supplies (Darmstadt, Germany). All other chemicals used, including the solvents, were of analytical grade.

Determination of total flavonoids

Total flavonoids were estimated using the method of Ordonez et al. [19]. Here, 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of extract and allowed to stand for 60 min at room temperature before the absorbance was measured at 420 nm. The extract was evaluated at a final concentration of 1 mg/ml. Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve: $y = 0.025x$, $R^2 = 0.9812$, where × is the absorbance and y is the quercetin equivalent (QE).

Determination of total phenol

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method [20]. The extract (1 mg/ml) was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40°C for colour development. The absorbance was measured at 765 nm using a spectrophotometer. The content of total phenolic compounds was expressed as mg/g gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve:

$y = 0.1216x$, $R^2 = 0.9365$, where × is the absorbance and y is the tannic acid equivalent (GAE).

Determination of total proanthocyanidins

The procedure reported by Sun et al. [21] was used in the determination of the total proanthocyanidins. A volume of 0.5 ml of 0.1 mg/ml extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid. The mixture was allowed to stand for 15 min while the absorbance was measured at 500 nm. Total proanthocyanidin content was expressed as catechin equivalents (mg/g) using the equation based on the calibration curve: $y = 1.8223x + 0.0157$, $R^2 = 0.7246$, where × is the absorbance and y is the catechin equivalent (CE).

Determination of ferric reducing power

A spectrophotometric method [22] was used for the measurement of reducing power. The different concentrations of the extracts and the standards, rutin and BHT (0.02-0.10 mg/ml; 1 ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1% w/v). The mixture was incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid (TCA) (10% w/v) was added to the mixture which was centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml of (0.1% w/v) $FeCl_3$. While the absorbance was measured at 700 nm in a spectrophotometer, increasing absorbance of the reaction mixture indicated greater reducing power of the samples [23].

DPPH radical scavenging assay

For DPPH assay, the method of Liyana-Pathirana and Shadidi [24] was adopted. A solution of 0.135 mM DPPH in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. BHT was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Z. mucronata subsp. mucronata* extracts. The changes in colour from deep-violet to light-yellow were measured at 517 nm wavelength using 95% methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC_{50}) [25,26], IC_{50} was calculated based on the percentage of DPPH radicals scavenged. The lower the IC_{50} value, the higher is the antioxidant activity.

ABTS radical scavenging assay

For ABTS assay, the methods of Johnstone et al., [27] were modified and adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal proportions and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^+$ solution with 60 ml of methanol to obtain an absorbance of 0.708 ± 0.001 units at 734 nm using the spectrophotometer. $ABTS^+$ solution was freshly prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the $ABTS^+$ solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The $ABTS^+$ scavenging capacity of the extract was compared with that of butylated hydroxy toluene (BHT). The percentage inhibition was calculated as $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$ where A_{blank} is the absorbance of ABTS radical + methanol used as control; A_{sample} is the absorbance of ABTS radical + sample extract/standard. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC_{50}) [25,26], IC_{50} was calculated based on the percentage of ABTS radicals scavenged. The lower the IC_{50} value, the higher is the antioxidant activity.

Statistical Analysis

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). One way analysis of variance (ANOVA) and the Duncan's New Multiple-range test were used to determine the differences among the means. P values < 0.05 were regarded to be significant. The Pearson correlation analysis was performed between antioxidant activity and total phenolic content.

Results

Phytochemical compositions

In this study, the result showed that the amount of total phenolic content, flavonoids and proanthocyanidins differ significantly among the various extracts of the *Z. mucronata* subsp. *mucronata* (Figure 1). The values of total phenolic contents varied from 24.72 ± 0.01 to 31.96 ± 0.01 mg GAE/100 g dry weight of plant material. The flavonoid contents values ranged from 4.80 ± 0.01 to 9.02 ± 0.01 mg QE/100 g of dry plant material. The quantity of the proanthocyanidin contents ranged from 1.26 ± 0.01 to 2.08 ± 0.01 mg CE/100 g of dry plant material. The ethanol extract had the highest total phenolic content values, followed by the acetone extract while water extract contained the least. The highest value of flavonoid was recorded in the acetone extract, followed by the ethanol extract and the water extract. Though proanthocyanidin

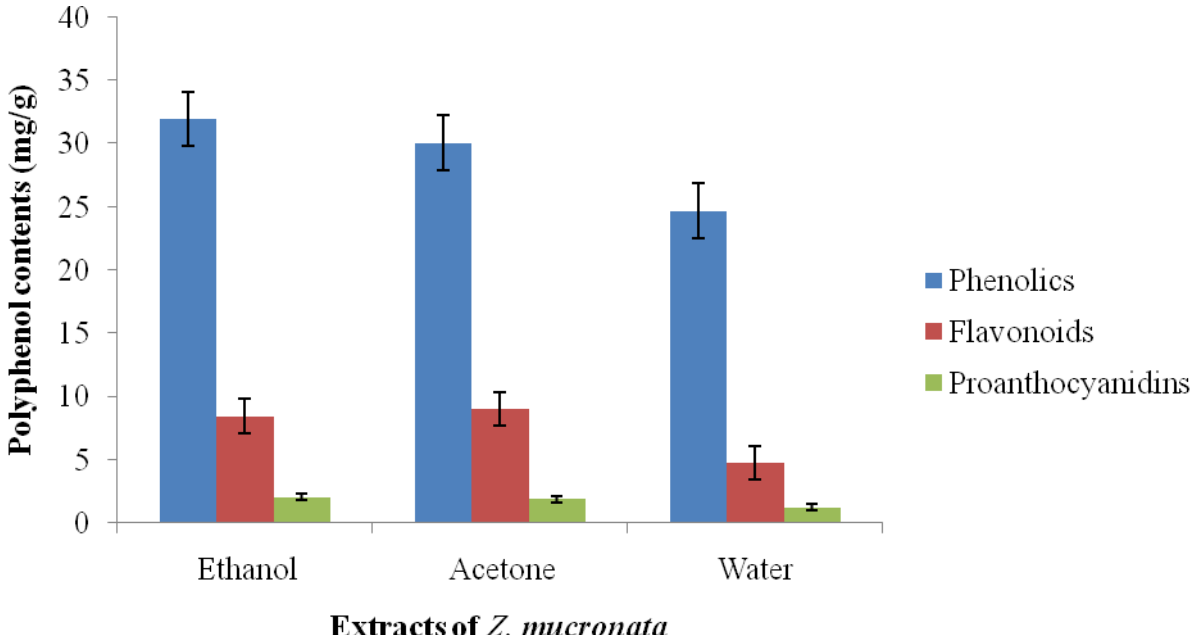

Figure 1 Polyphenolic contents (mg/g) of *Ziziphus mucronata* subsp. *mucronata*. Data are presented as means ± standard deviation of three replicate with significant increases from all samples tested.

was more in ethanol extract than other extracts, the proanthocyanidins content in this plant was relatively low and the differences in its quantity from one extract to another are significant. These results showed that, quantitatively, in *Z. mucronata*, total phenol contents were significantly higher than the flavonoid contents while the proanthocyanidins were the least.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP values of different extracts of *Z. mucronata* subsp. *mucronata* was determined in an attempt to compare their antioxidant activities The reducing abilities of the different extracts determined by FRAP method were measured spectrophotometrically by their absorbances at 700 nm and summarized in Figure 2. The reducing ability of the extracts showed a dose-dependent trend increasing with increases in the concentrations of the extracts. While highest reducing ability was observed at the highest concentration of each of the extracts, significant differences existed between the reducing ability of each all the extracts. Of the three extracts, acetone extract exhibited the highest reducing capability (0.454 ± 0.001) at the highest concentration. This was followed by (0.421 ± 0.002) ethanol extract while aqueous extract (0.14 ± 0.002) was the least. The reducing abilities recorded was in the following order, Rutin > BHT > Acetone > Ethanol > Aqueous which showed that rutin exhibited the highest reducing ability at 0.1 mg/ml. The significant differences for free radical scavenging activity among the different extracts may be attributed to the varied quantity of each of the phytochemical contents of the plant.

DPPH radical scavenging activity

The results of the DPPH scavenging activity of the extracts are as shown in Table 1. The alcoholic and aqueous extracts of *Z. mucronata* stem bark exhibited concentration dependent antiradical activity by inhibiting DPPH radical with inhibitory concentration 50% (IC_{50}) values of 0.0646 ± 0.02 mg/ml (aqueous), 0.0482 ± 0.02 mg/ml (acetone) and 0.0422 ± 0.03 mg/ml (ethanol) while those of the standards were 0.0406 ± 0.02 mg/ml (BHT) and 0.0411 ± 0.02 mg/ml (vitamin C). This result agreed with the earlier report of Motalleb et al. [28] which showed that the scavenging effects on the DPPH radical increases sharply with the increasing concentration of the samples and standards to a certain extent. In the order of activity, ethanol had the stronger antioxidant activity (0.0422 ± 0.03 mg/ml), followed by acetone extract while the least activity was obtained from aqueous extract. However, all the extracts exhibited significant DPPH free radical scavenging activity comparable to those obtained from the standards used as controls.

ABTS radical scavenging activity

Significant $ABTS^{+}$ free radical scavenging activity was evident in both alcoholic and aqueous extracts (Table 2). The rate of the decrease in absorbance at 734 nm depends on the type and amount of antioxidants and the

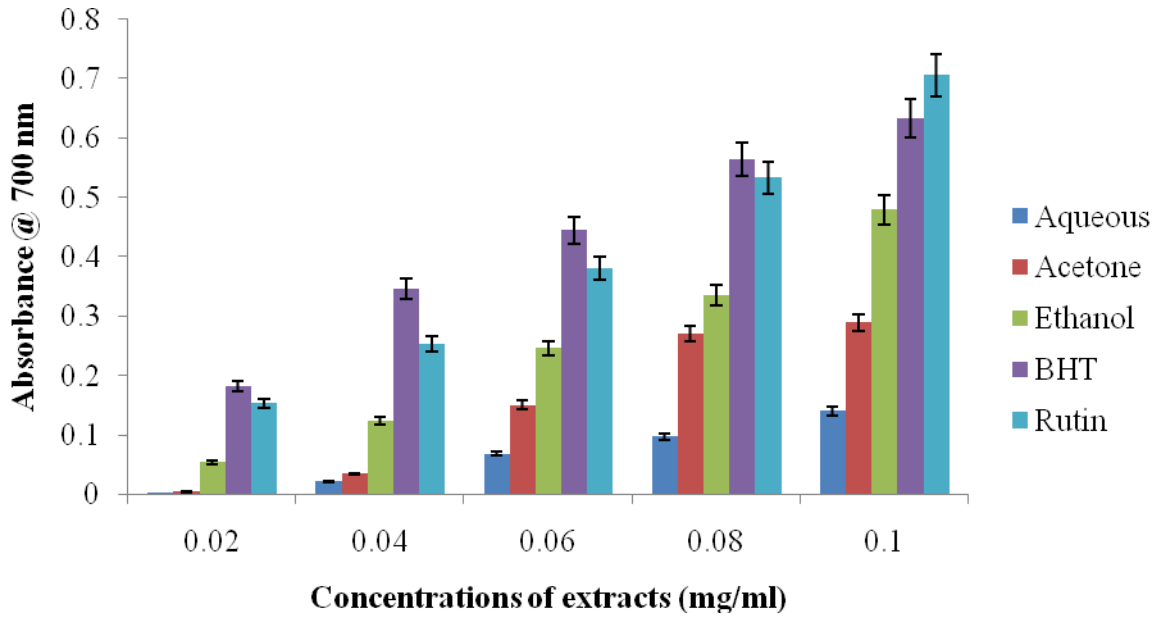

Figure 2 Ferric reducing power determinations for the alcoholic and aqueous extracts of *Z. mucronata* subsp. *mucronata*. Data are presented as means ± standard deviation of three replicate with significant increases from all samples tested.

results are thus expressed as antioxidant standard equivalents [29,30]. In this study, the suppression of the absorbance of $ABTS^+$ in a concentration-dependent manner is typically shown by all the extracts. Acetone (97.71 ± 0.03) and ethanol (95.73 ± 0.02) extracts showed $ABTS^+$ radical-scavenging activity higher than the reference standard BHT (93.47 ± 0.03) and aqueous extracts (88.96 ± 0.02) was lower than that of the BHT. Ethanol extract showed a 50% inhibitory concentration of IC_{50} = 0.0306 ± 0.04 mg/ml while acetone extract showed IC_{50} = 0.0317 ± 0.04 mg/ml which are higher than IC_{50} = 0.0429 ± 0.04 mg/ml of the aqueous extract. The differences in the antioxidant activities of the three extracts are statistically significant ($p < 0.05$).

Discussions

Phytochemical compositions

Phenolic compounds and flavonoids are the major constituents in most plants reported to possess antioxidant and free radical scavenging activity [31,32]. Phenolic compounds are efficient free radical scavengers [33] exhibiting antioxidant activity by inactivating lipid free radicals, or by preventing the decomposition of hydroperoxides into free radicals [34]. Medicinal plants containing flavonoids exhibit multiple pharmacological activities [35]. Flavonoids, probably the largest of the natural phenolics [36], possess antioxidant properties [37] acting as effective scavenger of deleterious free radicals and reactive oxygen species [38,39]. They have several biological activities including anti-inflammatory, antiallergic and immunomodulatory properties which could help to prevent age-related cardiovascular and neurodegenerative diseases as well as reducing the risk of cancer [40]. Their antioxidant activity depends on the number and location of hydroxyl groups of the flavonoid ring system and the relationship between peroxyl radical absorbing activity and the number of hydroxyl groups in flavonoids [41]. While they act through scavenging or

Table 1 DPPH radical scavenging activity of the alcoholic and aqueous extracts of *Z. mucronata* subsp. *mucronata*

	% inhibitions of $ABTS^+$ ± SD at different concentrations of the extracts					
Extracts	**0.02 (mg/ml)**	**0.04 (mg/ml)**	**0.06 (mg/ml)**	**0.08 (mg/ml)**	**0.1 (mg/ml)**	**IC_{50} (mg/ml)**
Aqueous	9.87 ± 0.02^a	21.86 ± 0.02^b	46.26 ± 0.02^c	62.18 ± 0.02^d	70.34 ± 0.02^e	0.0646 ± 0.02
Acetone	32.67 ± 0.02^a	39.35 ± 0.03^b	64.49 ± 0.02^c	82.53 ± 0.03^d	88.15 ± 0.02^e	0.0482 ± 0.02
Ethanol	34.35 ± 0.02^a	47.55 ± 0.03^b	71.04 ± 0.02^c	85.06 ± 0.01^d	94.47 ± 0.02^e	0.0422 ± 0.03
BHT	37.36 ± 0.02^a	48.50 ± 0.03^b	74.52 ± 0.01^c	92.56 ± 0.03^d	98.47 ± 0.02^e	0.0406 ± 0.02
Vitamin C	23.88 ± 0.01^a	47.65 ± 0.02^b	73.89 ± 0.03^c	90.94 ± 0.01^d	93.01 ± 0.03^e	0.0411 ± 0.02

Data are presented as means ± standard deviation of three replicate with significant increases from all samples tested.

Superscript a, b, c, d and e showed that means ± standard deviation in the same row with different superscript are significantly different ($p < 0.05$)

Table 2 ABTS radical scavenging activity of the alcoholic and aqueous extracts of *Z. mucronata* subsp. *mucronata*

	% inhibitions of $ABTS^+$ ± SD at different concentrations of the extracts					
Extracts	**0.02 (mg/ml)**	**0.04 (mg/ml)**	**0.06 (mg/ml)**	**0.08 (mg/ml)**	**0.1 (mg/ml)**	**IC_{50} (mg/ml)**
Aqueous	23.95 ± 0.02[a]	43.49 ± 0.06[b]	72.96 ± 0.02[c]	82.05 ± 0.03[d]	88.96 ± 0.02[e]	0.0429 ± 0.04
Acetone	42.13 ± 0.05[a]	52.47 ± 0.03[b]	75.64 ± 0.04[c]	92.48 ± 0.04[d]	97.71 ± 0.03[e]	0.0317 ± 0.04
Ethanol	43.10 ± 0.03[a]	54.85 ± 0.04[b]	82.76 ± 0.04[c]	93.76 ± 0.02[d]	95.73 ± 0.02[e]	0.0306 ± 0.04
BHT	33.31 ± 0.02[a]	54.27 ± 0.02[b]	79.06 ± 0.02[c]	91.05 ± 0.05[d]	93.47 ± 0.03[e]	0.0343 ± 0.02

Data are presented as means ± standard deviation of three replicate with significant increases from all samples tested. Superscript a, b, c, d and e showed that means ± standard deviation in the same row with different superscript are significantly different ($p < 0.05$)

chelating process [42], their ability to stabilize membranes by decreasing the fluidity of the membranes as well as partitioning flavonoids into the hydrophobic core of the membrane [43] have been considered a contributory mechanism to their antioxidant activities. Since antioxidants are used for the prevention and treatment of free radical-related disorders [44] as well as being essential in the prevention of diseases [5], the high quantity of phenolic and flavonoid contents of *Z. mucronata* subsp. *mucronata* may contribute to its potential antioxidant property and curative ability adsorbing and neutralizing free radicals.

Ferric Reducing Antioxidant Power (FRAP)

The best known antioxidants are phenolic compounds and flavonoids [45-47] exhibiting extensive free radical scavenging activities through their reactivity as hydrogen or electron-donating agents and metal ion chelating properties [7]. In this study, the reductive ability of the extracts reflected the reducing power of the *Z. mucronata* as a potential source of antioxidants. The FRAP assay showed that the antioxidants contained in the extracts acted as reductants in a redox-linked colorimetric reaction [32]. This report agreed with several reports that showed a close relationship between total phenolic content and high antioxidant activity [48-50]. This study, therefore, suggests that the recorded antioxidant capacity resulted from the contribution of different phytochemicals present in the plant and the reducing capacity of each extract may serve as a significant indicator of the potential activity of *Z. mucronata*.

DPPH radical scavenging activity

The reaction of DPPH with numerous antioxidants has earlier been published and the stoichiometry characterized [51]. The DPPH antioxidant assay is based on the principle that 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is able to decolourise in the presence of free radical scavengers (antioxidants). The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The odd electron in the DPPH radical is responsible for the absorbance at 517 nm and also for the visible deep purple colour [52]. Antioxidants in the different extracts of *Z. mucronata subsp. mucronata* reacted with DPPH which is reduced to the DPPH-H. Consequently, the absorbencies decreased from the DPPH radical to the DPPH-H formed. The degree of discoloration indicated the scavenging potential of the extracts in terms of hydrogen donating ability. The scavenging ability of this plant was significant and corresponded to the presence of high quantity of phenolic compounds. It is, therefore, reasonable to conclude that high antioxidants in the different extracts of *Z. mucronata* subsp. *mucronata* have resulted in the high level of DPPH radical scavenged in this study.

ABTS radical scavenging activity

The fact that phenolic antioxidants have been reported to scavenge $ABTS^+$ through hydrogen atom donation [53], electron transfer or even with a combination of the two mechanisms [54] may explain a current interest in the applicability of the $ABTS^+$ assay in determining the radical scavenging activities of plant extracts. Like the DPPH assay, ABTS assay measured the total antioxidant activity of the extracts. The different antioxidant activities of the different extracts and the ability of ethanolic extract to exhibit radical-scavenging greater than other extracts may indicate that active compounds of different polarity could be present in *Z. mucronata*. While the hydroxyl (OH) group in aromatic ring have been related to extracts' activity towards ABTS [54], the significant $ABTS^+$ scavenging activity of the different extracts may, however, depend on the presence of higher number of hydroxyl groups present in the plant while the high antioxidant activities may be due to their flavonoids and phenolic contents.

Conclusion

Today, antioxidative properties of extracts from plants have become a great interest due to their possible uses as natural additives to replace synthetic ones. This study was designed to investigate the phenolic contents and evaluate the *in vitro* antioxidant activities of *Z. mucronata* subsp.

mucronata. Phenolic compounds, flavonoids and proanthocyanidins were detected in the various extracts of the plant. The antioxidant potential of the extracts indicated that the alcoholic extracts exhibited higher antioxidant activities than the aqueous extracts. The results of FRAP, DPPH and ABTS assays showed that the extracts possess not only the antioxidant activities, but also potent free radical scavenger capability. The antioxidant activity of the extracts correlated well with the total phenolic contents and indicated that phenolic compounds are dominant contributors to the antioxidant activity of the extracts. The high polyphenolic contents, especially flavonoids, responsible for antioxidant activity may be the mechanism of action. *Z. mucronata* subsp. *mucronata* is a potential free radical scavenger and a useful source of natural antioxidants which may justify the therapeutic effectiveness of the plant.

Acknowledgements
The authors wish to acknowledge the financial support of the National Research Foundation and the University of Fort Hare. Thanks to Prof. Don Grierson for helping in the identification of the plant.

Authors' contributions
OOO participated in the design of the study, prepared the extracts, carried out the study and drafted the manuscript. AJA coordinated the study and revised the manuscript. Both authors approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 21 April 2011 Accepted: 16 December 2011
Published: 16 December 2011

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Pre-publication history

The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1472-6882/11/130/prepub

doi:10.1186/1472-6882-11-130
Cite this article as: Olajuyigbe and Afolayan: Phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. *BMC Complementary and Alternative Medicine* 2011 **11**:130.

CHAPTER 5

Pharmacological Assessment of the Medicinal Potential of *Acacia mearnsii* De Wild.: Antimicrobial and Toxicity Activities

(Published in the International Journal of Molecular Science)

Int. J. Mol. Sci. **2012**, *13*, 4255-4267; doi:10.3390/ijms13044255

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Pharmacological Assessment of the Medicinal Potential of *Acacia mearnsii* De Wild.: Antimicrobial and Toxicity Activities

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Received: 13 February 2012; in revised form: 12 March 2012 / Accepted: 21 March 2012 / Published: 30 March 2012

Abstract: *Acacia mearnsii* De Wild. (Fabaceae) is a medicinal plant used in the treatment of microbial infections in South Africa without scientific validation of its bioactivity and toxicity. The antimicrobial activity of the crude acetone extract was evaluated by both agar diffusion and macrobroth dilution methods while its cytotoxicity effect was assessed with brine shrimp lethality assay. The study showed that both bacterial and fungal isolates were highly inhibited by the crude extract. The MIC values for the gram-positive bacteria (78.1–312.5) µg/mL, gram-negative bacteria (39.1–625) µg/mL and fungal isolates (625–5000) µg/mL differ significantly. The bacteria were more susceptible than the fungal strains tested. The antibiosis determination showed that the extract was more (75%) bactericidal than bacteriostatic (25%) and more fungicidal (66.67%) than fungistatic (33.33%). The cytotoxic activity of the extract was observed between 31.25 µg/mL and 500 µg/mL and the LC_{50} value (112.36 µg/mL) indicates that the extract was nontoxic in the brine shrimp lethality assay (LC_{50} > 100 µg/mL). These results support the use of *A. mearnsii* in traditional medicine for treatment of microbial infections. The extract exhibiting significant broad spectrum antimicrobial activity and nontoxic effects has potential to yield active antimicrobial compounds.

Keywords: *Acacia mearnsii*; antimicrobial activity; bactericidal; cytotoxic effects; extract

1. Introduction

In Africa, the use of remedies derived from plants in traditional health practices is common and widespread [1] even before the introduction of antibiotics and other modern drugs [2]. While more than 80% of the world's population still depends upon the traditional medicines for various diseases [3,4], Patel and Coogan [5] indicated that natural products have been used worldwide for medicinal purposes for thousands of years. Being nontoxic and easily affordable, today, there has been resurgence in the consumption and demand for medicinal plants [6] resulting from bacterial resistance to currently used antibiotics becoming a public health concern [7], rising costs of prescription drugs and the bioprospecting of new plant-derived drugs [8]. Though only 5–15% of the estimated 250,000 higher plants in the world [9] have been studied for a potential therapeutic value [10] and represent a potential source of new anti-infective agents [11,12], a large number remains to be investigated since the search for antimicrobial agents is largely concentrated on lower plants, fungi and bacteria.

Since the development of new compounds and antimicrobial agents for the treatment of microbial infections has recently become of increasing interest [13], the trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries [14]. The effects of plant extracts on microorganisms have been studied by a very large number of researchers in different parts of the world [15–17]. The presence of antibacterial, antifungal and other biological activities have also been demonstrated in extracts of different plant species used in traditional medicine practices [18–20]. On this basis, several reports have indicated that antimicrobial activity of crude plant extracts and the bioassay-guided fractionation of those extracts yielded active principles [21,22].

Although there is a growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity [23,24], the toxicological effects of most of these crude extracts are often overlooked based on the facts that plant medicines have better compatibility with the human body and produce fewer side effects [25]. However, to forestall adverse effects, sometimes life-threatening, allegedly arising consequential to taking herbal products or traditional medicines [26,27], cytotoxicity testing, an integral component of the biological evaluation of pharmacologically important materials and an essential part of standard screening procedures, becomes essential. Since toxicological evaluation of a plant extract seeks to determine its possible collateral effects to ensure the safety of its use, brine shrimp larvae, being sensitive to toxic substances, are commonly used for toxicity assays in pharmacology. The ratio between dead larvae (no motility) and living larvae (high motility) in comparison to a control without any toxic substance is used to estimate the toxicity of the test substances [28].

Acacia is a pantropical and subtropical genus with species abundant throughout Australia, Asia, Africa and America. The plant *Acacia mearnsii* De Wild. (Fabaceae), previously known as *Racosperma mearnsii* [29] and commonly known as Black Wattle tree, is a short medium lived woody perennial and spreading tree, about 15 m high with smooth and greenish-brown bark on young branches which are blackish and rough on trunk. The young branchlets are downy. While it is widespread and common in lowlands, open forest, heathy woodland and on cleared land, particularly on dry, shallow soils [30], it grows in open forest, woodland or tussock grassland, in gullies or on hillsides, in sandy or gravelly clay soils [31]. It has inflorescence globular with 20–30 tiny pale yellow flowers. Pods, dark brown to black in color, are more or less straight, 5–10 cm long, 5–8 mm wide and

strongly constricted between seeds [30]. Though little is known of the pharmacological importance of this plant, its phytochemical screening showed that the total phenolic content correlated well with the antioxidant activity of the extracts [32]. Since this plant is ethobotanically relevant in the treatment of microbial infections locally and scientific report on its pharmacological importance is limited, this study was aimed at assessing the antimicrobial and toxicity activities of the crude acetone stem bark extract of *A. mearnsii in vitro* to justify its ethnotherapeutic usage.

2. Results and Discussion

In this study, the antibacterial, antifungal and cytotoxicity activity of the crude acetone extract of the *A. mearnsii* was determined against twelve bacterial strains, twelve fungal isolates and brine shrimps respectively. The crude extract showed varied degrees of antibacterial activity against all bacteria tested (Table 1).

Table 1. Bacterial susceptibility to different concentrations of acetone stem bark extract of *A. mearnsii*.

Tested bacterial isolates	**Average inhibition zones produced by 100 µL of each concentration of antibacterial agents (±1.0 mm)**						
	Erythromycin	**Crude acetone extract of *A. mearnsii***					
	50	20,000	10,000	5000	2500	1250	625
			µg/mL				
Proteus vulgaris KZN	32	20	17	15	15	13	0
Staphylococcus aureus OK_1	31	19	14	0	0	0	0
Enterococcus faecalis KZN	0	20	16	14	0	0	0
Klebsiella pneumoniae KZN	14	22	20	18	16	15	14
Proteus vulgaris CSIR 0030	38	35	33	27	23	20	19
Bacillus cereus (ATCC 10702)	30	24	22	20	17	16	14
Escherichia coli (ATCC 25922)	13	22	20	18	16	15	15
Bacillus pumilus (ATCC 14884)	16	22	19	18	18	18	16
Salmonella typhi (ATCC 13311)	13	27	24	20	16	14	0
Serratia marcescens(ATCC 9986)	18	27	24	22	21	20	18
Klebsiella pneumoniae (ATCC 10031)	30	22	20	18	17	16	15
Pseudomonas aeruginosa (ATCC 19582)	37	20	18	16	14	13	0

Though both gram-positive and gram-negative were highly inhibited, the crude extract was most active against *Proteus vulgaris* CSIR 0030 inhibited at a minimum inhibitory concentration (MIC) of 39.1 µg/mL in comparison to other tested isolates. The MIC values for the gram-positive bacteria ranged 78.1–312.5 µg/mL while those of gram-negative bacteria ranged from 39.1–625 µg/mL (Table 2). The result of the agar diffusion and macrobroth dilution assay are complementary. Results showed susceptibility patterns of the bacteria dependent on extract concentration. At the highest concentration (2000 µg/mL) of extract, the inhibition zones for all the bacteria ranged between 19 and 35 ± 1.0 mm. While *Proteus vulgaris* CSIR 0030 with the least MIC value (39.1 µg/mL) had highest inhibition zones (35 ± 1.0 mm) in comparison to those of other isolates, *Staphylococcus aureus* OK_1 with the following lowest MIC value (78.1 µg/mL) had the least zone (19 ± 1.0 mm), and

Escherichia coli (ATCC 25922) and *Serratia marcescens* (ATCC 9986) with the highest MIC value (625 μg/mL) had intermediate zones of inhibition (22 and 27 ± 1.0 mm, respectively). With the exception of *Proteus vulgaris* KZN, *Staphylococcus aureus* OK_1, *Enterococcus faecalis* KZN, *Salmonella typhi* (ATCC 13311) and *Pseudomonas aeruginosa* (ATCC 19582), other bacteria had inhibition zones at the least concentration (625 μg/mL) tested.

Table 2. Degree of antibacterial activities of acetone stem bark extract of *A. mearnsii*.

Tested bacterial isolates	Erythromycin	*A. mearnsii*		
	MIC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)	MIC_{index}
Proteus vulgaris KZN	12.5	312.5	625	2
Staphylococcus aureus OK_1	0.1953	78.1	78.1	1
Enterococcus faecalis KZN	12.5	156.3	625	4
Klebsiella pneumoniae KZN	12.5	312.5	625	2
Proteus vulgaris CSIR 0030	0.048	39.1	156.3	4
Bacillus cereus (ATCC 10702)	0.0977	156.3	312.5	2
Escherichia coli (ATCC 25922)	0.3906	625	625	1
Bacillus pumilus (ATCC 14884)	12.5	312.5	312.5	1
Salmonella typhi (ATCC 13311)	6.25	156.3	156.3	1
Serratia marcescens (ATCC 9986)	3.125	625	625	1
Klebsiella pneumoniae (ATCC 10031)	0.09765	156.3	156.3	1
Pseudomonas aeruginosa (ATCC 19582)	0.1953	156.3	625	4

The antifungal activity of the extract showed that the fungal isolates were susceptible at varied concentrations. The MIC values ranged 625–5000 μg/mL. The asexually reproducing fungi exhibited higher MIC values than the non-spore forming fungi (Table 3). While the MBC values for the bacterial isolates ranged between 78.1 μg/mL and 625 μg/mL, the fungal MFC values ranged between 625 μg/mL and 20,000 μg/mL. Mechanism of antibiosis determination showed that the extract was more (75%) bactericidal than being bacteriostatic (25%) and more fungicidal (66.67%) than being fungistatic (33.33%). While the bacteria were more susceptible to the erythromycin, used as control, with zones of inhibition, except for *Enterococcus faecalis* KZN, ranging from 13 ± 1.0 mm to 38 ± 1.0 mm and MIC values ranging 0.048–12.5 μg/mL, the crude extract was not found to be ineffective against the microbial isolates test. Comparatively, the bacterial strains were more susceptible than the fungal strain.

Interacting the extract and brine shrimps at the concentrations used for the antimicrobial assay for the plant resulted in the death of the shrimps within 15 min. The assay was repeated at low concentrations ranging between 0.9765 μg/mL and 500 μg/mL. Mortality of the brine shrimps was noticed in the experimental group but the control group remained unchanged at the same time. The number of surviving brine shrimps in each vial was counted and the results were noted. The percent of mortality of the shrimp was calculated for every concentration of the test sample. The cytotoxic activity of the extract was observed between 31.25 μg/mL and 500 μg/mL. At 500 μg/mL, all the brine shrimps were killed and none was killed at 31.25 μg/mL after 24 h incubation period (Table 4). The mortality rate of the brine shrimps was found to increase with the increase in concentration of the sample. It is evident from the results of the brine shrimp lethality assay that the crude extract with the

LC_{50} being 112.36 µg/mL having the highest levels of toxicity (100%) death at 500 µg/mL was non toxic (LC_{50} > 100 µg/mL).

Table 3. Antifungal activities of acetone stem bark extract of *A. mearnsii*.

Tested fungal isolates	Antifungal activities of *A. mearnsii*		
	MIC (µg/mL)	MFC (µg/mL)	MIC_{index}
Candida krusei	1250	2500	2
Candida albicans	625	1250	2
Candida rugosa	625	625	1
Aspergillus niger	5000	20,000	4
Aspergillus terreus	5000	20,000	4
Aspergillus flavus	5000	20,000	4
Penicillium notatum	5000	20,000	4
Absidia corymbifera	625	625	1
Fusarium sporotrichioides	625	625	1
Trichophyton tonsurans	2500	5000	2
Candida glabrata (ATCC 2001)	625	625	1
Trichophyton mucoides (ATCC 201382)	2500	5000	2

Table 4. Cytotoxicity effects of acetone stem bark extract of *A. mearnsii* on brine shrimps.

Conc. of extract (µg/mL)	Test 1	Test 2	Test 3	Av. No. of shrimp alive (Sample)	Av. No. of shrimp alive (Control)	% Mortality	Log. of conc.	LC_{50} µg/mL
31.25	0	0	0	0	10	0	1.495	
62.5	5	4	4	4.33	10	43.3	1.796	
125	7	8	8	7.66	10	76.6	2.097	112.36
250	9	10	9	9.33	10	93.33	2.398	
500	10	10	10	10	10	100	2.699	

Resulting from the global increase in microbial resistance is a need to assess the antimicrobial activity of several medicinal plants with increased spectrum, potency and novel mechanisms of action which may be systemically active and offer new hope for improved therapeutic outcomes. Consequently, in addition to the significant antimicrobial activity exhibited by *A. mearnsii*, the varied degree of antimicrobial activity of the extract could be due to the nature and level of antimicrobial agents present in the plant, their mode of action and the typical differences in the microbial cell walls between the strains [33] as well as the synergistic effects of different phytochemicals present in the plant. The antimicrobial activity at relatively minimal concentrations of the extract could be attributed to the active phytochemical compounds present in the extract at different concentrations and potent enough to inhibit or kill microbial agents. Though there is a dearth of scientific reports on the pharmacological importance of this plant, Olajuyigbe and Afolayan [34] earlier indicated that its aqueous and ethanolic extracts exhibited significant antibacterial activities. The pronounced antibacterial effect of the extract in both gram-negative and gram-positive bacteria may be attributed to its ability to damage the different cell walls to allow the active compounds to adsorb, diffuse, penetrate and interact with the affected target sites as earlier indicated by Olajuyigbe and Afolayan [17]. Furthermore, the significant multifarious effects of the extract on the fungal isolates could be due to

the differences in their morphology, disruption of membrane [35] or cell wall integrity [36], inhibition of mycelia growth [37], high potential to block morphogenetic transformation [38], indirect inhibition of cell wall synthesis [39] and spore germination [40]. Hence, the degree of the fungicidal effects of the extract depends on its ability to significantly cause either, or all, of these processes.

The *in vivo* brine shrimp lethality test is a simple way of screening and fractionating physiologically active plant extract. It is based on whether the brine shrimp are dead or alive at the end of the test or on the ability to kill laboratory-cultured *Artemia nauplii* [41]. The LC_{50} (112.36 μg/mL) of this extract is in agreement with the earlier report of [42] which indicated that several extracts containing physiological active principles derived from natural products has $LC_{50} \leq 1000$ μg/mL using brine shrimp bioassay. However, in agreement with Moshi *et al.* [43], the crude acetone extract was nontoxic.

Despite a great lack of investigations linking phytochemical constituents, pharmacology and toxicological activity of many medicinal plants used in ethnomedicine, the pharmaceutical industry is moving away from drug discovery or screening towards medicinal plant materials which have become the subject of public attention. In view of the fact that there is a relationship between the pharmacological activity and toxicity of natural products from plants, the degree of antimicrobial activity and toxicity exhibited by *A. mearnsii* show a good relationship. While Schmitz *et al.* [44] indicated that the compounds that showed activity in the brine shrimp could be associated to some extent to the potency of the pharmacologically active principles in natural products, the varied antimicrobial activity exhibited by this extract may be attributed to its toxicity level resulting from the synergistic activity of the various phytochemicals present in the plant. An increase in the toxicity of the plant could possibly result in higher antimicrobial effects.

In addition, the significance of the inhibitory, bactericidal and fungicidal activities of this extract may not be underestimated. Since *in vitro* antimicrobial susceptibility testing assesses the relative susceptibility of microbial pathogens to selected therapeutic agents to optimize treatment of infections in clinical settings, determination of the microbicidal activity of these antimicrobial agents against an infecting organism may be useful in guiding therapy for serious infections especially when the host immune defense is compromised. The high percentages of the bactericidal and fungicidal activity activities of this extract at high concentrations showed the potentials of its active compounds to minimize the spread of an infecting organism from the site of infection and kill invading pathogens. In view of the fact that systemically active antimicrobial agents with increased spectrum and potency may offer new hope for improved therapeutic outcomes in both competent and immunocompromised individuals, a "cidal" regimen [45–48], for which *Acacia mearnsii* is an indicator, would be preferable. This, however, may justify the ethnotherapeutic relevance of this plant in the treatment of microbial infections in South Africa.

3. Experimental Section

3.1. Collection of Plant Material

The bark materials of *A. mearnsii* De Wild were collected in August 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the

Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University.

3.2. Extract Preparation

The bark sample was air-dried at room temperature, pulverized in a mill (Christy Lab Mill, Christy and Norris Ltd.; Process Engineers, Chelmsford, UK) and stored in a sterile air-tight container for further use. The extract of the bark was prepared in accordance to the description of Basri and Fan [49]. About 100 g of the pulverized sample was steeped in 500 mL of acetone for 72 h with shaking (Stuart Scientific Orbital Shaker, UK). The plant material was extracted for two other consecutive times. The extracts were combined, filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40 ℃ using a rotary evaporator (Laborarota 4000 efficient, Heldolph, Germany). The crude extract collected was allowed to dry at room temperature to a constant weight of 21.4 g. The extract was redissolved in acetone to the required concentrations for the bioassay analysis.

The reconstituted crude acetone extract solution was sterilized by filtering through 0.45 μm membrane filter. The extract was tested for sterility after membrane filtration by introducing 2 mL of the extract into 10 mL of sterile nutrient broth and incubated at 37 ℃ for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

3.3. Test Organisms

The bacterial isolates used in this study included *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 10702), *Proteus vulgaris* KZN, *Serratia marcescens* (ATCC 9986), *Pseudomonas aeruginosa* (ATCC 19582), *Enterococcus faecalis* KZN, *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* CSIR 0030, *Bacillus pumilus* (ATCC 14884), *Klebsiella* pneumoniae KZN, *Staphylococcus aureus* OK_1 and *Salmonella typhi* (ATCC 13311). The fungal isolates included *Candida krusei*, *Candida albicans*, *Candida rugosa*, *Candida glabrata* (ATCC 2001), *Absidia corymbifera*, *Fusarium sporotrichioides*, *Trichophyton tonsurans*, *Trichophyton mucoides* (ATCC 201382), *Penicillium notatum*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus*. These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organisms were maintained on nutrient broth, nutrient agar (Biolab), potato dextrose agar and sabouraud dextrose broth. The antibacterial assays were carried out using Mueller Hinton II agar and broth (Biolab). The antifungal assays were carried out using sabouraud dextrose agar and broth.

3.4. Preparation of Inocula

For the bacterial inoculums preparation, the inoculum of each test bacterial strains was prepared using the colony suspension method [50]. Colonies picked from 24 h old cultures grown on nutrient agar were used to make suspensions 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 transferring 0.1 mL of the bacterial suspension to 9.9 mL of sterile nutrient broth before use. The density of bacterial suspension

for susceptibility test was finally determined by comparison with 0.5 McFarland standard of Barium sulphate solution [51].

For the fungal inoculums preparation, spore suspension for fungal bioassay was prepared according to the procedure of Murugan *et al.* [52] as modified. Briefly, 1 cm^2 of seven day old spore producing cultures was dropped in sterile distilled water and vortexed for 30 s to release the fungal spores. The spore density of each fungus was adjusted with spectrophotometer (A_{595nm}) to obtain a final concentration of approximately 10^5 spores/mL. For the *Candida* spp., the inocula were prepared by adding 1 mL of overnight Candida cultures to 9 mL of sabouraud dextrose broth to yield 10^4 colony forming units (CFU) per microliter of the inoculum.

3.5. Antibiotic Susceptibility Testing—Agar Diffusion Method

The antibiotic susceptibility testing was determined using the modified Kirby-Bauer diffusion technique [51] by swabbing the Mueller-Hinton agar (MHA) (Oxoids, UK) plates with the resultant saline suspension of each strain. Wells were then bored into the agar medium with heat sterilized 6 mm cork borer. The wells were filled with 100 µL of different concentrations (625 µg/mL, 12,500 µg/mL, 2500 µg/mL, 5000 µg/mL, 10,000 µg/mL and 20,000 µg/mL) of the crude extract and 50 µg/mL of erythromycin antibiotic taking care not to allow spillage of the solutions onto the agar surface. The determinations were done in duplicates. After 24 h of incubation, the diameter of the inhibition zones of the extract and the antibiotic was measured and interpreted using the CLSI zone diameter interpretative standards [53].

3.6. Macrobroth Dilution for Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of the extract defined as the lowest concentration which resulted in maintenance or reduction of inoculums viability [54] was determined by serial tube dilution technique [55] against the bacterial and fungal isolates. For antibacterial assay, different concentrations of the extract ranging from 20 µg/mL to 10,000 µg/mL were prepared by serial dilutions in Mueller Hinton broth medium. Different concentrations of erythromycin (0.0122–50 µg/mL), used as positive control, were also prepared by serial dilution in Mueller Hinton broth. The tubes were inoculated with 100 µL of each of the bacterial strain. Blank Mueller Hinton broth was used as negative control.

For the antifungal assay, different concentrations of the extract ranging between 19.53 µg/mL and 40,000 µg/mL were prepared in sabouraud dextrose broth by serial dilutions. Each broth concentration was inoculated with 100 µL of the prepared fungal spores' solution. Two control tubes were included: one with spores and broth but no plant extract and one with broth and plant extract but no spores. The bacterial containing tubes were incubated aerobically at 37 °C for 24 h. The fungal containing tubes were incubated at 27 °C for 3–5 days. The first tube in the series with no visible growth after incubation period was taken as the MIC.

3.7. Determination of Minimum Bactericidal and Fungicidal Concentrations (MBC/MFC)

The MBC and MFC assays were carried out as described by Cheesbrough [56]. Here, fresh nutrient agar and sabouraud dextrose agar plates were inoculated with one loopful of culture taken from each of

the first five broth cultures that showed no growth in the MIC tubes. While MBC assay plates were incubated for at 37 °C for 24 h, MFC assay plates were incubated at 25 °C for 3–5 days. After the incubation periods, the lowest concentration of the extract that did not produce any bacterial or fungal growth on the solid medium was regarded as MBC and MFC values for this extract [57]. This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubating the bacteria or spore germination for the fungi after five days of incubation.

3.8. Determination of Mechanisms of Antibiosis (Bactericidal or Bacteriostatic)

The mechanism of antibiosis of the extracts was calculated using the ratio of MBC/MIC (MFC/MIC) or MIC_{index} as described by Shanmughapriya *et al.* [58] to elucidate whether the observed antibacterial effects were bactericidal, fungicidal, bacteriostatic or fungistatic. When the ratio of MBC/MIC or MFC/MIC was ≤2.0, the extract was considered bactericidal or otherwise bacteriostatic. If the ratio is ≥16.0, the extract was considered ineffective.

3.9. Brine Shrimp Lethality Test

The brine shrimp lethality test using the larvae of brine shrimp nauplii, *Artemia salina* L. was carried out using the standard procedure [42,59]. For the extract sample, 4000 µg of the crude acetone extract was initially dissolved in 1 mL of pure dimethyl sulfoxide (DMSO) to make the extract hydrophilic after which 3 mL of sterile distilled water was added to get a concentration of 1000 µg/mL of the extract used as a stock solution. Different concentrations (0.9765–500 µg/mL) of the extract were prepared from the stock solution by serial tube dilution technique in different vials. Ten nauplii were transferred into each vial using Pasteur pipettes and were not given food because hatched brine shrimp can survive for up to 48 h without food [60] as they still feed on their yolk-sac [28]. The control vials were prepared using DMSO only and the experiment was replicated three times. After 24 h of incubation, the vials were examined, the numbers of survivors in each vial were counted and percentages of deaths were calculated. Larvae were considered dead if they did not exhibit any observable movement during several seconds of observation. The extract is regarded as non-toxic if its LC_{50} is greater than 100 µg/mL in the brine shrimp lethality assay [43]. The mean mortality percentage and LC_{50} (lethal concentration for 50% of the population) were determined using statistical analysis and the graph of Logarithm of concentration against percent lethality [61].

4. Conclusions

The results show that the acetone extract of *A. mearnsii* had a significant antimicrobial activity and no toxic effects on the brine shrimps. This activity may indicate the medicinal potential, suggests the broad spectrum antimicrobial potential and validates the popular use of this plant in traditional medicine for the treatment of microbial infections by rural communities in South Africa since the extract was regarded as being nontoxic on brine shrimps.

Acknowledgments

The authors wish to acknowledge the financial support of the National Research Foundation and the University of Fort Hare. Thanks to Don Grierson for helping in the identification of the plant.

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CHAPTER 6

In vitro ethnotherapeutic potential of the acetone extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.*:* antimicrobial and toxicity evaluations

(*Published in the African Journal of Biotechnology*)

African Journal of Biotechnology Vol. 11(103), pp. 16783-16789, 25 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1553
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

In vitro ethnotherapeutic potential of the acetone extract of the bark of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.: Antimicrobial and toxicity evaluations

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Accepted 11 December, 2012

The antimicrobial and the cytotoxicity activities of the crude acetone bark extract of *Ziziphus mucronata* were determined by agar diffusion, macrobroth dilution assays and brine shrimp lethality assay, respectively, to ascertain its relevance in ethnomedicine. The results show that both Gram negative and Gram positive bacteria tested were equally inhibited. At the highest concentration (10000 µg/ml) of the extract, the bacterial inhibition zones ranged between 16 and 21 ± 1.0 mm, the fungal inhibition zones ranged between 13 and 19 ± 1.0 mm while *Fusarium sporotrichioides, Candida glabrata* and *Trichophyton mucoides* were not affected. The minimum inhibitory concentrations (MICs) values of the extract were between 78.1 and 312.5 µg/ml for the bacteria while the minimum bactericidal concentrations (MBCs) were between 156.3 and 2500 µg/ml. For the fungi, the MICs were between 1250 and 10000 µg/ml, the minimum fungicidal concentrations (MFCs) were between 5000 and 20000 µg/ml and the LC_{50} of the brine shrimp lethality assay was 90.27 µg/ml indicating a low level of toxicity. The degree of the pharmacological activity of *Z. mucronata* shows the significant medicinal potential of this plant in the treatment of microbial infections and justifies its use in complementary and alternative medicines in South Africa.

Key words: *Ziziphus mucronata*, antibacterial, cytotoxicity, fungicidal, ethanol extract, medicinal potential.

INTRODUCTION

Globally, pathogen resistance to antibiotics, an under-appreciated threat to public health in nations around the globe (Zhang et al., 2006), is a rapidly growing problem, leading to an urgent need for novel antimicrobial agents (Kumar and Schweizer 2005; Edgar et al., 2012). With this increased incidence of resistance to antibiotics and appearance of new infectious agents, many products have been evaluated directly for antimicrobial activity as well as their resistance modifying ability (Gibbons, 2004; Coutinho et al., 2009). These evaluations involved using new compounds, which are not based on the existing synthetic antimicrobial agents (Shah, 2005) but rather on natural products from plants considered interesting alternatives for treatment (Lu et al., 2007; Mbwambo et al., 2007). Plant-based traditional knowledge is a recognized tool playing a major role in the treatment of human traumas and diseases worldwide (Principe, 1991).

On all continents, people have used hundreds to thousands of indigenous plants for treatment of ailments since prehistoric times (Levetin and McMahon, 2002). In search for new sources of drugs and neutraceuticals (Ghosh, 2003; Sharma and Mujundar, 2003), there is a growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity (Al-Bayati and Al-Mola, 2008). While numerous

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drugs have entered the international pharmacopoeia through ethnobotany and traditional medicine (Ergene et al., 2006), contemporary pharmacopoeias contain at least 25% of drugs derived from plants and many synthetic analogues built on prototype compounds isolated from plants (De Silva, 2005) because they contain active substances effective against human pathogens and relent or cure diseases (Hadi and Bremner, 2001). However, to establish the potential therapeutic effects and ascertain the safety of the phytopharmaceuticals, many medicinal plants have been investigated *in vitro* for their antimicrobial and cytotoxicity activities pending their confirmation *in vivo* tests.

Ziziphus mucronata Willd. subsp. *mucronata* Willd. (Buffalo thorn) (Family: Rhamnaceae) widely distributed in South Africa (Foden and Potter, 2005; Raimondo et al., 2009), often found in thorny vegetation in both temperate and tropical climates, open scrubland, woodland, forest margins and riverine vegetation, is usually a shrub or small to medium sized tree up to 9 m tall. It has a trunk frequently crooked and branches spreading and drooping well above ground or near the base. While the thorns of the plant are in pairs, reddish brown, one straight and one bent, the flowers are small, yellow, inconspicuous, bisexual, in tight axillary clusters and often produce copious nectar (Bekele-Tesemma et al., 1993; Bein et al., 1996).

Traditionally, a poultice of the powdered and baked roots is used to relieve any pain. Boils, glandular swellings and other skin infections are treated with leaf paste. Leaf paste combined with an infusion of the roots is used to treat tubercular gland swellings, measles, dysentery, lumbago and chest complaints. Roots are used to treat snakebite. The bark containing 12 to 15% tannin is used as an emetic and steam bath to purify the complexion. Bark decoction is used for rheumatism and stomach troubles.

Bark infusion is used to treat coughs (Venter and Venter, 1996; van Wyk and Gericke, 2000). Though this plant has been implicated traditionally in the treatment of many infections, its pharmacological importance has not been scientifically documented. Therefore, this study was aimed at screening the acetone extract of the bark of *Z. mucronata* against bacteria and fungi of clinical importance and reporting the toxicity of the extract using brine shrimp lethality assay to ascertain its relevance in ethnomedicine.

MATERIALS AND METHODS

Collection of plant material

The stem bark materials of *Z. mucronata* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen (OLAJ/2010/ZM/01) was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark sample was air-dried at room temperature, pulverized in a mill (Christy Lab Mill, Christy and Norris Ltd; Process Engineers, Chelmsford, England) and stored in a sterile air-tight container for further use. The extract of the bark was prepared in accordance to the description of Basri and Fan (2005). About 100 g of the pulverized sample was seeped in 500 ml of acetone for 72 h with shaking (Stuart Scientific Orbital Shaker, Staffordshire, UK). The plant material was extracted for two other consecutive times. The extracts were combined, filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40°C using a rotary evaporator (Laborarota 4000 - efficient, Heldolph, Germany). The resulting crude extract was stored at -20°C until assayed. Stock solutions and serial dilutions of extracts and fractions were prepared in dimethylsulphoxide (DMSO) (Ambrozin et al., 2004).

Test organisms

The bacterial isolates used in this study included *Micrococcus luteus, Bacillus subtilis* KZN, *Enterococcus faecalis* KZN, *Staphylococcus aureus* OK_1, *S. aureus* OK_3, *Klebsiella pneumoniae* KZN, *Shigella sonnei* (ATCC 29930), *B. pumilus* (ATCC 14884), *Enterobacter cloacae* (ATCC 13047), *K. pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 19582) and *Acinetobacter calcaoceuticus anitratis* CSIR. The fungal isolates included Absidia *corymbifera, Candida krusei, Candida albicans, Cryptococcus neoformans, Candida rugosa, Candida glabrata* (ATCC 2001), *Aspergillus niger, Aspergillus terreus, Aspergillus flavus, Penicillium notatum* and *Trichophyton mucoides* (ATCC 201382). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The bacteria were maintained on nutrient agar while the fungal isolates were maintained on potato dextrose agar and broth which were used for the antifungal assays.

Preparation of inocula

For the bacterial inoculums preparation, the inoculum of each test bacterial strains was prepared using the colony suspension method (EUCAST, 2000). Colonies picked from 24 h old cultures grown on nutrient agar were used to make suspensions 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 transferring 0.1 ml of the bacterial suspension to 9.9 ml of sterile nutrient broth before use. The density of bacterial suspension for susceptibility test was finally determined by comparison with 0.5 McFarland standard of Barium sulphate solution (Cheesebrough, 2002).

The fungal inoculums were prepared by dropping 1 cm^2 of seven day old spore producing cultures in sterile distilled water and vortexed for 30 s to release the fungal spores. The spore density of each fungus was adjusted with spectrophotometer (A_{595} nm) to obtain a final concentration of approximately 10^5 spores/ml. For the *Candida spp.*, the inocula were prepared by adding 1 ml of overnight Candida cultures to 9 ml of potato dextrose broth to yield 10^5 colony forming units (CFU) per µL of the inoculum.

Antimicrobial assay by agar diffusion method (Inhibition zones)

The susceptibility screening of the test bacteria to the acetone extract of the bark of *Z. mucronata* and chloramphenicol, used as control, was done in accordance with the methods described by Irobi et al. (1996) and Akinpelu et al. (2008). For the antibacterial assay, the inoculum of each test bacterial strains was standardized

at 5 × 10^6 cfu/ml using McFarland Nephe-lometer standard (NCCLS, 1993). Sterile Mueller Hinton agar plates were seeded with each adjusted test bacterial strain and allowed to stand at 37°C for 30 min. Wells were then bored into the agar medium with heat sterilized 6 mm cork borer and were filled with 100 µL of different concentrations (2500 µg/ml, 5000 µg/ml and 10000 µg/ml) of the extract and chloramphenicol (7.8125 µg/ml, 15.625 µg/ml and 31.25 µg/ml).

For the antifungal assay, 100 µL of the fungal solutions was dispensed on potato dextrose agar plates and spread evenly with a sterile glass rod and allowed to stand for 1 h on the laboratory bench. Wells were then bored into the agar medium with a heat sterilized 6 mm cork borer. The wells were filled with 100 µL of different concentrations (5000, 10000 and 20000 µg/ml) of the extract. In both bacterial and fungal cultures, the antimicrobial solutions were not allowed to spill onto the surface of the agar while the culture plates were allowed to stand on the laboratory bench for 30 min to allow proper diffusion of the solutions before the bacterial culture plates were incubated aerobically at 37°C for 24 h and the fungal cultures incubated at 27°C for 3 to 7 days. Wells in blank Mueller Hinton and potato dextrose agar plates containing 5% DMSO representing the final DMSO concentration in the test plates without the extract served as positive controls. The tests were done in replicate for each of the bacterial and fungal isolates. After 24 h of incubation, the plates were examined if there is any zone of inhibition (Bauer et al., 1966). The diameter of the zones of inhibition of the extract and the antibiotic was measured and interpreted using the CLSI zone diameter interpretative standards (CLSI, 2008).

Macrobroth dilution for minimum inhibitory concentration (MIC)

MIC defined as the lowest concentration which resulted in maintenance or reduction of inoculums viability (Carson et al., 1995) was determined by serial tube dilution technique (Khan et al., 2007) for the microbial isolates. For antibacterial assay, different concentrations of the extract (9.766 to 5000 µg/ml) and chloramphenicol (0.9766 to 500 µg/ml), used as positive control, were separately prepared by serial dilutions in Mueller Hinton broth. The tubes were inoculated with 100 µL of each of the bacterial strains. Blank Mueller Hinton broth was used as negative control.

For the antifungal assay, macrobroth dilution assay was performed as described by Fromtling et al. (1993). Different concentrations (78.125 to 40000) µg/ml of the crude extract was prepared in potato dextrose broth by serial dilutions. Each broth concentration was inoculated with 100 µL inoculums of approximately 10^5 spores/ml of the prepared fungal spores' solution. Two control tubes were included: one with spores and broth but no plant extract and one with broth and plant extract but no spores. The fungal containing tubes were incubated at 27°C for 3 to 5 days. The MICs were determined as described by Dabur et al. (2004).

Determination of minimum bactericidal and fungicidal concentrations (MBC/MFC)

The MBC and MFC assays were carried out as described by Cheesbrough (2006). Here, extract-free nutrient and sabouraud dextrose agar plates were inoculated with one loopful of culture taken from each of the first five broth cultures that showed no growth and the first growth-containing tube in the MIC tubes. The MBC and MFC assay plates were respectively incubated at 37°C for 24 h and at 25°C for 3 to 5 days. After the incubation periods, the lowest concentrations of the extract that did not produce bacterial and fungal growth on the solid media were respectively regarded MBC and MFC values for this extract (Irkin and Korukluoglu, 2007). This observation was matched with the MIC test tube that did not show evidence of bacterial growth after 48 h or spore germination for the fungi after five days of incubation.

Brine shrimp (*Artemia salina* larvae) lethality test

The brine shrimp lethality test using the larvae of brine shrimp Fauplii, *Artemia salina* L, was carried out using the standard procedure (Meyer et al., 1982; McLaughlin and Rogers, 1998). Different concentrations (0.9766 to 500 µg/ml) of the extract were prepared from the stock solution by serial tube dilution technique in different vials. Ten nauplii were transferred into each vial using Pasteur pipettes and were not given food because hatched brine shrimp can survive for up to 48 h without food as they still feed on their yolk-sac. The control vials were prepared using DMSO combined with sea water only and the experiment was replicated three times. After 24 h of incubation, the vials were examined, the numbers of survivors in each vial were counted and percentages of deaths were calculated. Larvae were considered dead if they did not exhibit any observable movement during several seconds of observation. The extract is regarded as being mildly toxic if LC50 > 30<100 µg/ml and non-toxic if its LC_{50} is greater than 100 µg/ml in the brine shrimp lethality assay (Moshi et al., 2010). The mean mortality percentage and LC_{50} (lethal concentration for 50% of the population) were determined using statistical analysis and the graph of Logarithm of concentration against percent lethality (Gupta et al., 1996).

RESULTS

The preliminary antimicrobial screening of *Z. mucronata* shows that the crude acetone extracts of the stem bark exhibited a significant *in vitro* antibacterial activity against Gram negative and Gram positive bacteria and different fungal isolates tested. The zones of inhibition obtained from the crude extract and the chloramphenicol against the selected bacteria was indicated in Table 1. The extract exhibited a concentration dependent activity against the different bacterial strains. At the highest concentration (10000 µg/ml) of the extract, the zones of inhibition ranged between 16 and 21 ± 1.0 mm. At the lowest concentration (2500 µg/ml), the zones of inhibition ranged between 12 and 16 ± 1.0 mm. While *E. faecalis* KZN, *S. aureus* OK_1 and *S. aureus* OK_3 were not susceptible to the different concentrations of chloramphenicol, used as positive control, other bacterial strains had zones of inhibitions ranging between 20 and 27 ± 1.0 mm at the highest concentration (31.25 µg/ml) used. Table 2 summarizes the degree of the antibacterial activity of this crude extract as determined by the macrobroth dilution assay. The minimum inhibitory concentrations (MIC) values of the extract ranged between 78.1 and 312.5 µg/ml while the minimum bactericidal (MBC) values ranged between 156.3 and 2500 µg/ml. The MIC values of chloramphenicol ranged between 1.953 and 31.25 µg/ml for all the tested bacterial strains. While the activity of the extract was less than that of the chloramphenicol, the extract was not selective in its activity against the different tested groups of bacteria

Table 1. *In vitro* antibacterial activity of crude acetone extract of the bark of *Ziziphus mucronata.*

Tested bacterial isolate	Inhibition zones (± 1.0 mm) from chloramphenicol			Inhibition zones (± 1.0 mm) from *Z. mucronata*		
	31.25 (µg/ml)	15.63 (µg/ml)	7.81 (µg/ml)	10000 (µg/ml)	5000 (µg/ml)	2500 (µg/ml)
M. luteus	26	22	18	16	14	13
B. subtilis KZN	20	18	15	18	16	14
E. faecalis KZN	0	0	0	17	15	12
S. aureus OK_1	0	0	0	16	14	12
S. aureus OK_3	0	0	0	21	18	15
K. pneumoniae KZN	22	18	12	20	18	16
S. sonnei (ATCC 29930)	20	16	13	16	14	14
B. pumilus (ATCC 14884)	20	18	13	18	15	13
E. cloacae (ATCC 13047)	27	21	19	18	15	13
K. pneumoniae (ATCC 10031)	21	16	13	16	15	13
P. aeruginosa (ATCC 19582)	22	17	15	16	14	13
Acinetobacter calcaoceuticus anitratus CSIR	23	20	15	17	15	14

Table 2. Inhibitory and bactericidal activities of acetone extract of the bark of *Ziziphus mucronata.*

Tested bacterial isolate	Activity of chloramphenicol	Activity of *Z. mucronata*	
	MIC µg/ml	MIC µg/ml	MBC µg/ml
M. luteus	1.953	312.5	2500
B. subtilis KZN	3.906	312.5	625
E. faecalis KZN	31.25	312.5	2500
S. aureus OK_1	7.8125	78.1	156.3
S. aureus OK_3	7.8125	156.3	2500
K. pneumoniae KZN	7.8125	312.5	625
S. sonnei (ATCC 29930)	7.8125	78.1	156.3
B. pumilus (ATCC 14884)	7.8125	312.5	625
E. cloacae (ATCC 13047)	1.953	312.5	312.5
K. pneumoniae (ATCC 10031)	1.953	156.3	312.5
P. aeruginosa (ATCC 19582)	3.906	312.5	312.5
Acinetobacter calcaoceuticus anitratus CSIR	7.8125	312.5	625

having MIC values equally ranging between 78.1 and 312.5 µg/ml. On the contrary, the extract inhibited nine out of 12 fungal isolates tested. While *F. sporotrichioides, C. glabrata* and *T. mucoides* did not have zones of inhibition resulting from the different concentrations tested, other fungal isolates had zones of inhibitions that ranged between 13 and 19 ± 1.0 mm at the highest concentration (20 000 µg/ml). *Candida* species had the least zones of inhibition equaled to 13 ± 1.0 mm and *A. corymbifera* had the biggest zone of inhibition (19 ± 1.0 mm) being the

Table 3. *In vitro* antifungal activity of acetone extract of *Ziziphus mucronata*.

Tested fungal isolate	Inhibition zones (± 1.0 mm) from *Z. mucronata*				
	20000 (µg/ml)	10000 (µg/ml)	5000 (µg/ml)	MIC (µg/ml)	MFC
Absidia corymbifera	19	16	14	1250	5000
Aspergillus niger	15	14	13	10000	10000
Aspergillus terreus	15	15	14	5000	20000
Aspergillus flavus	14	14	13	10000	20000
Candida krusei	13	11	11	10000	10000
Candida albicans	13	11	11	5000	10000
Candida glabrata	0	0	0	5000	10000
Candida rugosa	13	12	10	5000	10000
Cryptococcus neoformans	17	14	12	10000	10000
Fusarium sporotrichioides	0	0	0	10000	20000
Penicillium notatum	14	13	12	5000	20000
Trichophyton mucoides	0	0	0	10000	20000

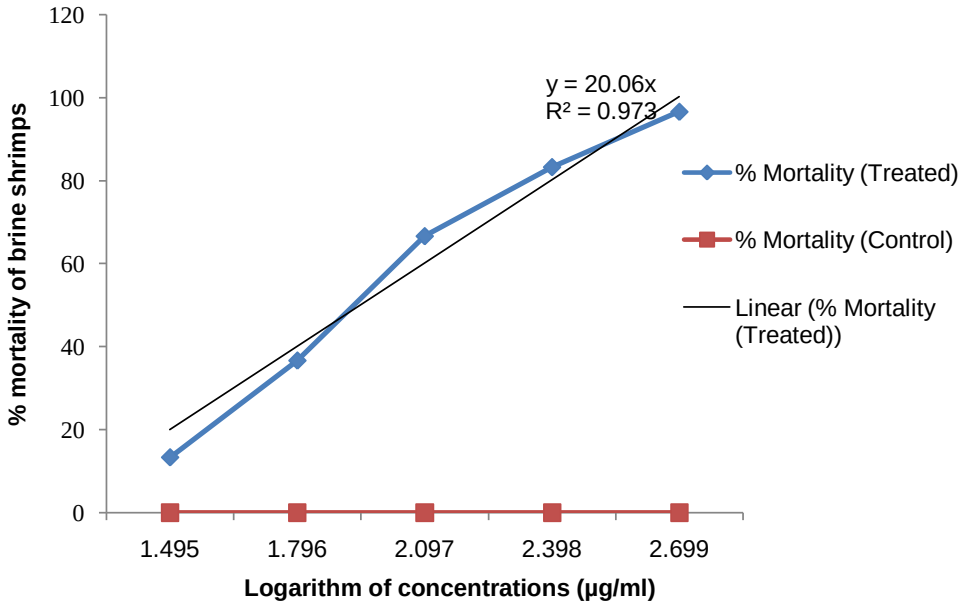

Figure 1. Graph of logarithm of concentrations versus percentage mortality of brine shrimps interacted with crude acetone extract of the bark of *Z. mucronata.*

most inhibited isolate by the various concentrations of the extract tested. Against these fungal isolates, the MICs of the extract were between 1250 and 10000 µg/ml while the minimum fungicidal concentrations (MFCs) were between 5000 and 20000 µg/ml (Table 3). The most inhibited fungal isolate, *A. corymbifera*, had the least MIC value of 1250 µg/ml. While *C. albicans, C. neoformans, C. rugosa, A. terreus* and *P. notatum* had MICs of 5000 µg/ml, the MICs of other fungal strains were 10000 µg/ml. The differences in the susceptibility of tested bacterial and fungal isolates as indicated by the antimicrobial assays could be due to the differences in their structural morphology and physiological characteristics. These characteristics could be attributed to the variations in the susceptibility of the fungal isolates to the extract as shown by the different assay methods.

From the *in vitro* cytotoxicity assay, treating brine shrimps (*Artemia salina* larvae) with acetone extract of the bark of *Z. mucronata* showed different mortality rate of brine shrimp which increased proportionately with the increasing concentration of the extract (Figure 1). The mean percentage mortality was plotted against the logarithm of concentrations and the concentration killing fifty percent of the larvae (LC_{50}) was determined. From the graph, the lethality concentration of the crude extract at which 50% (LC_{50}) mortality of brine shrimp nauplii

occurred was 90.27 µg/ml. This showed that the extract exhibited a mild cytotoxic activity against the brine shrimp.

DISCUSSION

In attempts to harness the antimicrobial substances in medicinal plants and to forestall the menace of microbial resistance and its associated complicacies, many alcoholic plant extracts have been investigated for antimicrobial activities and the effectiveness of several acetone extracts of many plants have been reported. Of these several studies, Mishra et al. (2009), Nkomo et al. (2011) and Ghamba et al. (2012) shows that acetone extracts of many plants have demonstrated significant antimicrobial activity against many tested organisms. In this study, the acetone extract of the bark of *Z. mucronata*, also, demonstrated remarkable activity against all the test organisms. The active components of this plant, having a good antimicrobial potential, may be highly soluble in acetone. The degree of antimicrobial activity may be accounted for by the flavonoids synthesized by plants in response to microbial infection (Hernandez et al., 2000; Schinor et al., 2007) and the exclusive ability of acetone to extract most flavonoids and steroids more than other solvents (Eloff et al., 2008; Afolayan and Lewu, 2009).

Considering the pathogenic potential of the tested organisms and upshots in microbial resistance, globally, the exhibited degree of antimicrobial activity of this crude extract indicated a positive step towards curtailing organisms with pathogenic potentials in both immunocompetent and immunocompromised individuals if, further, prosecuted clinically and the most potent bioactive compounds isolated. Since lower MIC and MBC values indicate higher efficacy (Cowan, 1999) and phytochemicals are routinely classified as antimicrobials when susceptibility tests had MICs in the range of 100 to 1000 µg/ml (Simões et al., 2009), the MIC/MBC and MIC/MFC ratios showed that the extract is bacteriostatic and fungistatic at lower concentrations while at higher concentrations, significant bactericidal and fungicidal activities were exhibited against these potential pathogens. Where MIC equals MBC/MFC, the bactericidal and fungicidal potency with a broad spectrum and great therapeutic potential of the plant is indicated.

Also, the brine shrimps, being highly sensitive to a variety of chemical substances, are considered a useful tool for preliminary toxicity assessment of plant extracts (Solis et al., 1993). The brine shrimp lethality is an indicative of many pharmacological actions (Klare et al., 2003) while toxicity study is considered essential in the identification and isolation of new compounds from crude extracts (Sasidharan et al., 2008). The degree of toxicity exhibited by the acetone extract could be attributed to the tannins, phenolic contents, flavonoids and proantho-cyanidins (Olajuyigbe and Afolayan, 2011) present in the stem bark. Tannins possess remarkable pharmacological activities including antibacterial (Jagetia and Baliga, 2002), and anti-infective (Baglin et al., 2003; Cichewicz and Kouzi, 2004) properties. The results could form a good basis for further phytochemical and pharmacological investigation.

In conclusion, though resistance is a global challenge and ethnotherapy or phytomedicine are often neglected because of civilization, western medicine, crude preparatory methods and lack of dosage regimen in traditional medicines, the observed antimicrobial and toxicity activities indicated that *Z. mucronata* could be a good source of essential and pharmacologically active compounds and justify the use of this plant in the treatment of microbial infections and its use in complementary and alternative medicines in South Africa.

ACKNOWLEDGEMENT

The authors wish to acknowledge the financial support of the National Research Foundation and the University of Fort Hare. Thanks to Prof Don Grierson for helping in the identification of the plant.

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CHAPTER 7

In vitro antibacterial activities of crude aqueous and ethanolic extracts of the stem bark of *Acacia mearnsii* De Wild

(*Published in the African Journal of Pharmacy and Pharmacology*)

African Journal of Pharmacy and Pharmacology Vol. 5(9), pp. 1234-1240, 8 September, 2011
Available online http://www.academicjournals.org/ajpp
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

In vitro antibacterial activities of crude aqueous and ethanolic extracts of the stem bark of *Acacia mearnsii* De Wild

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Accepted 11 July, 2011

The antibacterial activities of both water and ethanol extracts of *Acacia mearnsii* de Wild against Gram-positive and Gram-negative bacteria were investigated. The two extracts were very active against the two groups of bacteria. While the minimum inhibitory concentration (MIC) of the ethanol extract ranged between 78.1 and 625 µg/ml, the MIC for aqueous extract ranged between 156.3 and 625 µg/ml. The minimum bactericidal concentrations (MBC) of ethanol extract ranged between 156.3 and 625 µg/ml while that of aqueous extract was between 312.5 and 5000 µg/ml. Water appeared to be a good extraction medium and its extract could be as potent as alcoholic extracts.The MIC_{index} showed that the two extracts were bactericidal at high concentrations and bacteriostatic at low concentrations. The strong bactericidal activities of the two extracts may be attributed to the presence of some secondary metabolites which are soluble in both ethanol and water. The antibacterial activity of the extracts indicated the great therapeutic potential of this plant and has justified its ethnomedicinal use against infectious diseases.

Key words: *Acacia mearnsii*, extracts, antibacterial, bacteriostatic, bactericidal.

INTRODUCTION

Despite the huge advances in modern medicine, most people in the developing world still rely on traditional and effective knowledge to treat illness and disease. The valued trado-medical practices providing affordable healthcare have been recognized by the World Health Organization (WHO). While scientists anticipated that phytochemicals with adequate antibacterial efficacy will be useful for the treatment of bacterial infections (Balandrin et al., 1985), national and international policymakers are calling for partnerships between modern and traditional medicine to bridge the gap in global public health. Consequently, there are rising interests in the search for natural products from plants for the discovery of new antimicrobial agents in the recent times (Nascimento et al., 2000).

In view of the expensive treatment regimen by synthetic drugs already in practice, their gross side effects due to indiscriminate use (Sharif, 2001; Tomoko et al., 2002) as well as increasing trend in the emergence of resistance to antimicrobial agents resulting from poor quality drugs manufactured, patient non-compliance, and spontaneous mutations within the microbial populations (Nester et al., 2002; Denyer et al., 2004), there is a constant need to search for new and effective antimicrobial agents (Ahmad et al., 1998; Bhavnani and Ballow, 2000). In search of remedies for human ailments from the environment, particularly from natural resources (Habeeb et al., 2007), the plants turn out to be a significant source of therapeutics (Potier et al., 1990) and many potent and powerful drugs (Srivastava et al., 1996) used medicinally in different countries. In many developing countries, about 80% of available drugs come from medicinal plants. While progresses in antimicrobial drugs have introduced many antibiotics most of which are nontoxic but having side effects (Shafiei and Ghanbarpour, 1992), plants have provided a good source of anti-infective agents and many of them remain highly effective in the

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fight against microbial infections. Apart from being cost-effective, they have fewer side effects (Samsam and Moatar, 1991). Hence, ethnopharmacology and traditional knowledge-inspired approaches have become useful in drug discovery and development (Patwardhan, 2005; Cooper, 2008) while traditional medicine-inspired approaches remain important in the management of chronic diseases and discovery of drugs of natural origin (Patwardhan et al., 2004; Patwardhan and Mashelkar, 2009) as a result of their long history of use and better safety profiles than synthetic drugs.

Acacia mearnsii de Wild (Fabaceae) is a fast-growing leguminous tree. The genus *Acacia* is a cosmopolitan taxon containing more than 1350 species (Seigler, 2003). In Australia, there are approximately 960 species, which makes *Acacia* the largest genus of vascular plants in that region (Maslin, 2001). The species of Acacia was introduced to South Africa about 150 years ago primarily for the tanning industry (Sherry, 1971). They are of immense value for the reforestation and reclamation of wastelands (Skolmen, 1986). They are used for fuel wood, timber and shelter (Palmberg, 1981). While the bark of *A. mearnsii* is known to contain about 20 to 40% tannins and 70% proanthocyanidins (Young et al., 1986), ethanolic extract of *A. mearnsii* had the highest total flavonoid contents with aqueous extracts having the least of the phytochemicals while the total phenolic content correlated well with the antioxidant activity of the extracts (Olajuyigbe and Afolayan, 2011 accepted). Although *A. mearnsii* is widespread, relatively little is known about its pharmacological potentials. This may be due to the difficulty associated with the identification of *Acacia* species and the insufficient clarity about their taxonomic relationships (Seigler, 2003). There is lack of scientific studies on the pharmacological importance of this plant especially antimicrobial study. Though ethanol and water are the solvents traditionally used in herbal preparations by soaking or being allowed to simmer, this study is aimed at evaluating the antibacterial activities of both aqueous and ethanol extracts of this plant.

MATERIALS AND METHODS

Plant collection and identification

The bark materials of *A. mearnsii* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark samples were air-dried at room temperature and pulverized with a milling machine. About 100 g of the pulverized sample was extracted with 500 ml of ethanol for 48 h. The extract was filtered with Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at a maximum temperature of 40°C using a rotary evaporator. The water extract was prepared by soaking 100 g of the pulverized sample in 500 ml of sterile de-ionized distilled water. The mixture was brought to boil in a water bath for 30 min and allowed to stand for 24 h on rotary shaker. The mixture was filtered through Whatman No. 1 filter paper. The filtrate of water extract obtained was quickly frozen at -40°C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA).The dried crude aqueous and ethanol extracts were redissolved in their respective solvents to the required concentrations for the bioassay analyses.

The reconstituted extract solutions were sterilized by filtering through 0.45 µm membrane filter. They were tested for sterility after filtration by introducing 2 ml of each extract into 10 ml of sterile nutrient broth and incubated at 37°C for 24 h. A sterile extract was indicated by the absence of turbidity of the broth after the incubation period (Ronald, 1995).

Test organisms

Organisms used in this study included seven Gram positive and eight Gram negative bacteria. The Gram positive strains included three American Type Culture Collection [*Staphylococcus aureus* (ATCC 6538), *Streptococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 10702)], two environmental isolates [*Bacillus subtilits* (KZN) and *Micrococcus luteus*] as well as two clinical isolates [*S. aureus* (OK1) and *S. aureus* (OK2a)]. The Gram negative strains were five American Type Culture Collection [*Pseudomonas aeruginosa* (ATCC 19582), *Shigigella sonnei* (ATCC 29930), *Salmonella typhi* (ATCC 13311), *Escherichia coli* (ATCC 25922) and *Enterobacter cloacae* (ATCC 13047)] and three environmental isolates [*Klebsiella pneumonia* (KZN), *Proteus vulgaris* (KZN), *Shigella flexneri* (KZN)]. All the strains were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organisms were maintained in nutrient broth and nutrient agar (Biolab) slant incubated at 37°C for 24 h.

Determination of the minimum inhibitory concentration (MIC)

The activities of the extracts and their minimum inhibitory concentrations were determined by the macrobroth dilution methods (Jones et al., 1985; NCCLS, 1993). The inoculum of each test strain was standardized at 5×10^6 cfu/ml using McFarland Nephe-lometer standard. The extracts were serially diluted with nutrient broth to give final concentrations of 19.53, 39.06, 78.13, 156.25, 312.50, 625.00, 1250.00, 2500.00, 5000.00 and 10,000 µg/ml. The tubes were inoculated with 100 µl of each bacterial suspension. Blank nutrient broth was used as negative control while different concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 and 5.2 µg/ml ciprofloxacin prepared by serial dilution was used as positive controls. The tubes were incubated aerobically at 37°C for 24 h. The MIC was the lowest concentration of the extract that yielded no visible growth after the incubation (Sung et al., 2006).

Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined according to the method of Doughari (2006). Sterile nutrient agar plates were inoculated with a loopful of culture taken from each of the first three test tubes that showed no growth in the MIC tubes. After incubation for 24 h at 37°C, the least concentration of the extracts that showed no colony formation on the agar was taken as the MBC.

Table 1. Antibacterial activities of ethanol and aqueous extracts of *A. mearnsii.*

Listofbacteria used	Ciprofloxacin			Ethanol extract			Water extract		
	MIC (µg/ml)	MBC (µg/ml)	MIC index	MIC (µg/ml)	MBC (µg/ml)	MIC index	MIC (µg/ml)	MBC (µg/ml)	MIC index
Pseudomonasaeruginosa(ATCC 19582)	0.08	0.16	2	156.25	312.50	2	312.50	312.50	1
Shigellasonnei(ATCC 29930)	0.16	0.32	2	156.25	312.50	2	312.50	625.00	2
Salmonellatyphi(ATCC 13311)	0.02	0.08	2	156.25	156.25	1	312.50	312.50	1
Escherichia coli(ATCC 25922)	0.02	0.02	1	312.50	312.50	1	625.00	2500.00	4
Enterobactercloacae(ATCC 13047)	0.08	0.32	4	156.25	156.25	1	625.00	1250.00	2
Klebsiellapneumoniae(KZN)	0.02	0.04	2	156.25	312.50	2	312.50	625.00	2
Proteusvulgaris(KZN)	0.32	0.64	2	312.50	625.00	2	625.00	2500.00	4
Shigellaflexneri(KZN)	0.16	0.32	2	78.13	312.50	4	156.25	1250.00	8
Serratiamercescens(ATCC 9986)	0.04	0.08	2	625.00	625.00	1	625.00	5000.00	8
Klebsiellapneumonia(ATCC 4352)	0.16	0.32	2	156.25	312.50	2	625.00	2500.00	4
Staphylococcus aureus(ATCC 6538)	0.32	0.64	2	312.50	312.50	1	625.00	2500.00	4
Streptococcusfaecalis(ATCC 29212)	0.64	2.56	4	156.25	312.50	2	625.00	2500.00	4
Bacilluscereus(ATCC 10702)	0.08	0.08	1	156.25	312.50	2	312.50	312.50	1
Bacillussubtilits(KZN)	0.08	0.08	1	312.50	312.50	1	625.00	2500.00	4
Staphylococcus aureus(OK2a)	0.64	0.64	1	156.25	312.50	2	312.50	312.50	1
Micrococcusluteus	0.16	0.32	2	78.13	156.25	2	625.00	1250.00	2
Staphylococcus aureus(OK1)	0.04	0.04	1	78.13	156.25	2	312.50	1250.00	1

Determination of mechanisms of antibiosis (bactericidal or bacteriostatic)

The mechanism of antibiosis of the extracts was calculated using the ratio of MBC/MIC as described by Shanmughapriya et al. (2008) to elucidate whether the observed antibacterial effects were bactericidal or bacteriostatic. When the ratio of MBC/MIC was less than or equal to 1.0, the extract was considered bactericidal. However, when the ratio of MBC/MIC was equal to or greater than 2.0, the extract was considered bacteriostatic. If the ratio was equal to or greater than 16.0, the extract was considered ineffective.

RESULTS AND DISCUSSION

The two extracts of *A. mearnsii* were active against both Gram-negative and Gram-positive bacteria (Table 1). The antibacterial activities of ethnol extract against the different bacterial strains are presented in Figure 1 while those of water extract are as indicated in Figure 2. From Figure 1, *S. flexneri* (KZN), *M. luteus* and *S. aureus* (OK1) had the least minimum inhibitory concentrations (78.13 µg/ml) and *S. mercescens* (ATCC 9986) had the highest minimum inhibitory concentration (625 µg/ml). With the exception of *S. flexneri* (KZN) for which the minimum bactericial concentration (MBC) was four times higher than the MIC values, the MBC values of other bacteria were either similar or two folds higher than the MIC values. From Figure 2, *S. flexneri* (KZN) was the most susceptible of all the bacteria to water extract of *A. mearnsii*. The organism had the least MIC value (156.25 µg/ml) while other bacteria had MIC values ranging between two and four folds higher. With the exception of *P. vulgaris* (KZN) and *S. mercescens* (ATCC, 9986), the MBC values of all the bacteria were similar or two to four folds higher than the MIC values. Comparatively, Figure 3 showed that the two extracts were not as effective as ciprofloxacin used as control. The MIC and MBC values of the ethanol extract were lower than those of water extract. While the two extracts were active at very low concentrations and exerted a high degree of inhibitory effect on the organisms; ethanol extract had better antibacterial effects against all the organisms than the water

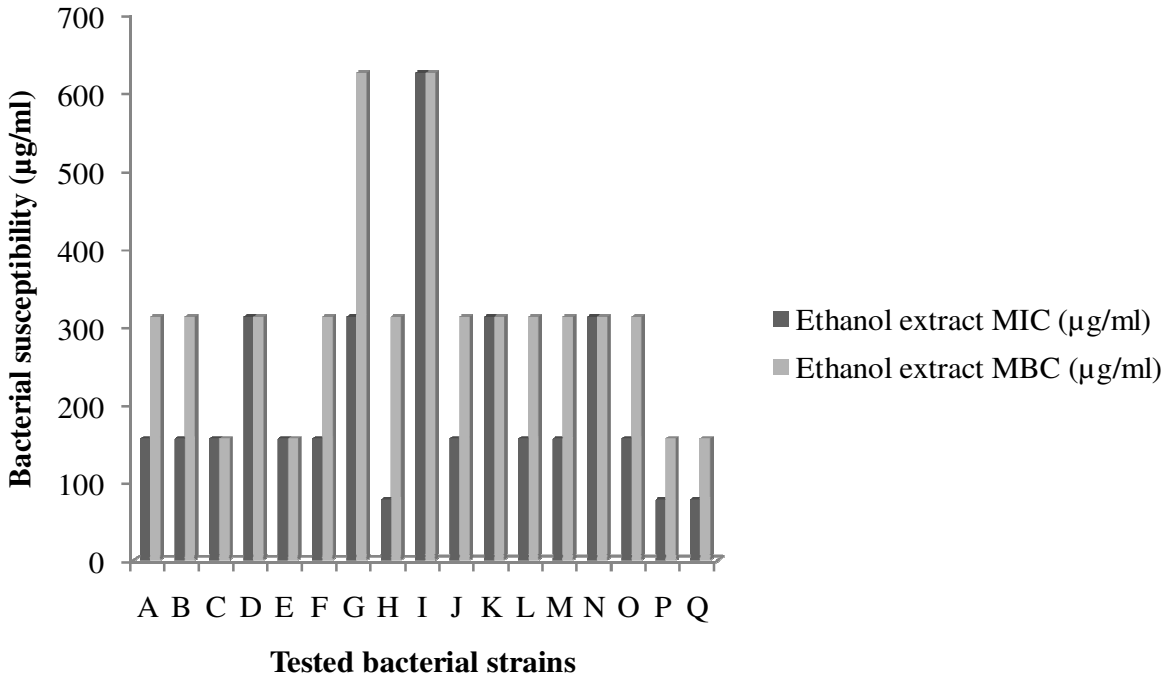

Figure 1. Antibacterial activity of ethanol extract of *A. mearnsii* against tested bacterial strains. A, *P. aeruginosa* (ATCC 19582); B, *S. sonnei* (ATCC 29930); C = *S. typhi* (ATCC 13311); D, *Escherichia coli* (ATCC 25922); E, *Enterobacter cloacae* (ATCC 13047); F, *K. pneumoniae* (KZN); G, *P. vulgaris* (KZN); H, *S. flexneri* (KZN); I, *S. mercescens* (ATCC 9986); J, *K. pneumonia* (ATCC 4352); K, *S. aureus* (ATCC 6538); L, *S. faecalis* (ATCC 29212); M, *B. cereus* (ATCC 10702); N, *B. subtilits* (KZN); O, *S. aureus* (OK2a); P = *M. luteus;* Q = *S. aureus*(OK1).

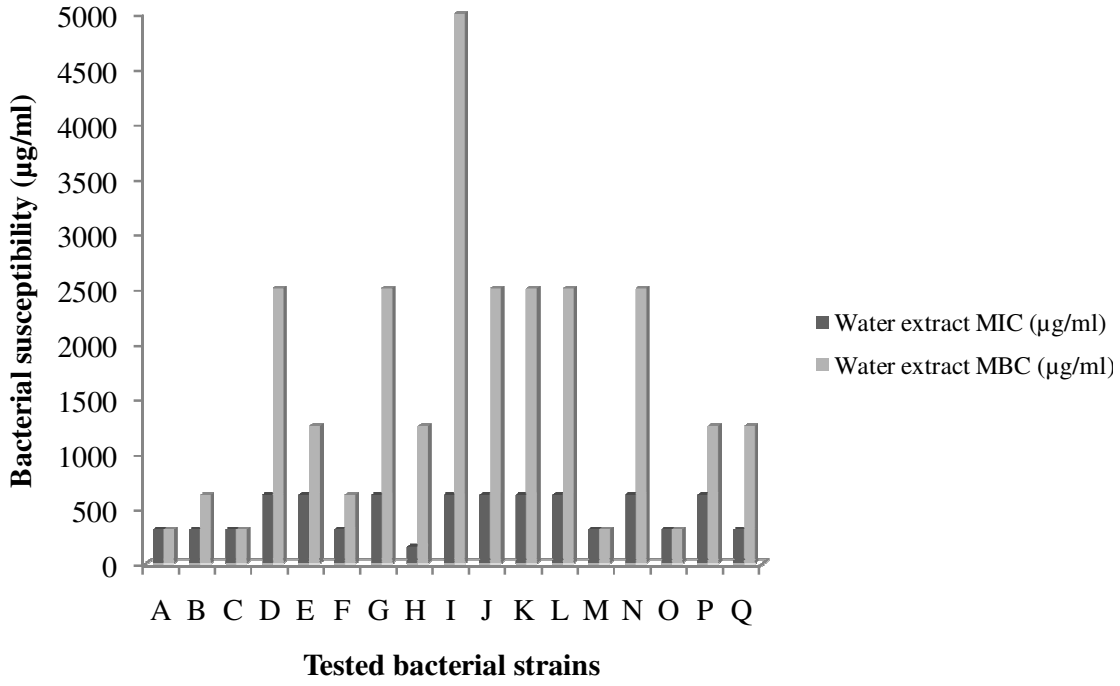

Figure 2. Antibacterial activity of water extract of *A. mearnsii* against tested bacterial strains. A, *P. aeruginosa* (ATCC 19582); B, *S. sonnei* (ATCC 29930); C = *S. typhi* (ATCC 13311); D, *Escherichia coli* (ATCC 25922); E, *Enterobacter cloacae* (ATCC 13047); F, *K. pneumoniae* (KZN); G, *P. vulgaris* (KZN); H, *S. flexneri* (KZN); I, *S. mercescens* (ATCC 9986); J, *K. pneumonia* (ATCC 4352); K, *S. aureus* (ATCC 6538); L, *S. faecalis* (ATCC 29212); M, *B. cereus* (ATCC 10702); N, *B. subtilits* (KZN); O, *S. aureus* (OK2a); P = *M. luteus;* Q = *S. aureus*(OK1).

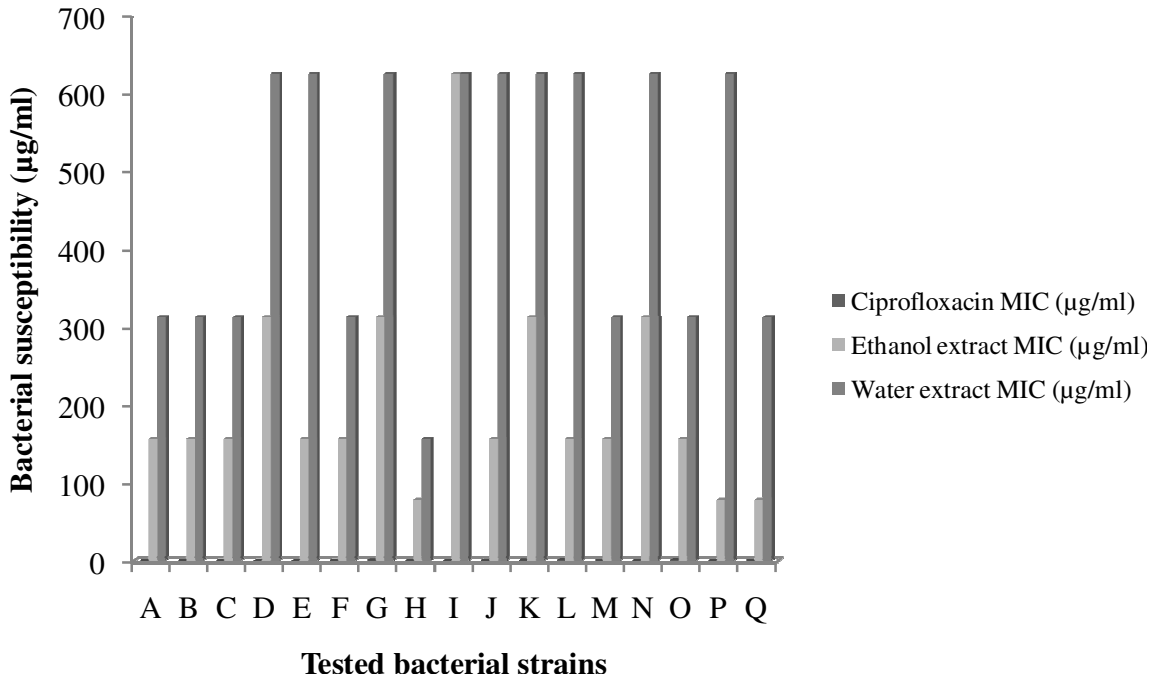

Figure 3. Comparative antibacterial effects of ciprofloxacin, ethanol and water extracts of *A. mearnsii* on tested bacterial strains. A, *P. aeruginosa* (ATCC 19582); B, *S. sonnei* (ATCC 29930); C = *S. typhi* (ATCC 13311); D, *Escherichia coli* (ATCC 25922); E, *Enterobacter cloacae* (ATCC 13047); F, *K. pneumoniae* (KZN); G, *P. vulgaris* (KZN); H, *S. flexneri* (KZN); I, *S. mercescens* (ATCC 9986); J, *K. pneumonia* (ATCC 4352); K, *S. aureus* (ATCC 6538); L, *S. faecalis* (ATCC 29212); M, *B. cereus* (ATCC 10702); N, *B. subtilits* (KZN); O, *S. aureus* (OK2a); P = *M. luteus;* Q = *S. aureus*(OK1).

extract mainly due to the better solubility of the active compounds in the organic solvents. Though MIC_{index} greater than 1 was considered as bacteriostatic, none of the MIC_{index} was equal to or greater than16.0. Hence, the extracts were considered effective against the organisms.

Many pharmacologically bioactive compounds such as alkaloids, flavanoids, tannins, anthraquinones and phenolic compounds have been implicated in the antibacterial activities of many plants (Hostettman et al., 1995; Edeoga et al., 2005; Nawrot et al., 2007). The antibacterial activities of both extracts of *A. mearnsii* may be attributed to the presence of the pharmacologically important compounds such as flavonoids which are soluble in ethanol and water. It may also be due to the presence of broad spectrum antibacterial compounds or metabolic toxins in addition to other pharmacologically active compounds in the plant. While the antibacterial activity of the extracts depended on the plant chemotype, extract preparation, solvent used and the sensitivity of the bacteria (Loziene et al., 2007), the variation of the MIC values among the isolates depended on the presence of intrinsic levels of tolerance to antimicrobials in the tested microorganisms (Ahmad and Aqil, 2007).

The antibacterial activities of ethanol and water extracts had earlier been reported (Farrukh et al., 2008; Sharma et al., 2010). In this study, ethanol extract showed greater antibacterial activity as compared to water extract. The stronger extraction capacity of ethanol could have produced greater active constituents responsible for the antimicrobial activity. This is similar to the general reports of many workers (Aliero and Afolayan, 2006; Annamalai et al., 2007; Parekh and Chanda, 2007; Khond et al., 2009). However, contrary to previous reports indicating that water extract could be ineffective or have low antibacterial activity against microbial agents (Paz *et al.,* 1995; Vlietinck et al., 1995; Jayaraman et al., 2008), water extract of *A. mearnsii* exhibited a significant antibacterial activity against all bacteria tested.

Although many workers have reported that water is a poor extractor of antibacterial compounds from plant (Ibekwe et al., 2001; Karaman et al., 2003), this study has indicated that water may be a good extraction medium and its extract may be as potent as alcoholic extract. This potency may, however, be due to the presence of anionic components such as thiocyanate, nitrate, chloride and sulphates along with other water soluble antibacterial compounds present in the plant material (Darout et al., 2000). With the exception of *S. mercescens* and *S. flexneri* where the MIC_{index} equaled 8, the inhibitory effect of the water extract was significant. The ability of the water and ethanol extracts to inhibit the organisms at relatively low concentrations has justified the use of *A. mearnsii* by the rural communities in the treatment of some infections.

ACKNOWLEDGEMENT

The authors wish to acknowledge the financial support of the National Research Foundation of South Africa and the University of Fort Hare.

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CHAPTER 8

Antimicrobial potency of the ethanolic crude bark extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

(*Published in the African Journal of Pharmacy and Pharmacology*)

African Journal of Pharmacy and Pharmacology Vol. 6(10), pp. 724-730, 15 March, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.749
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Antimicrobial potency of the ethanolic crude bark extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

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Accepted 15 February, 2012

***Ziziphus mucronata* Willd. subsp. *mucronata* Willd. is a medicinal plant widely used in South Africa for the treatment of various diseases. Like many medicinal plants to which traditional claims including the treatment of ailments of infectious origin but without scientific reports have been associated, *Z. mucronata* is not an exemption. The ethanolic stems bark extract of *Z. mucronata* against medically important pathogens was investigated for the antimicrobial activity by the agar dilution method while the degree of its antimicrobial activity was determined by the macrobroth dilution method. The extract showed good antibacterial and antifungal activities. Both Gram-negative and Gram-positive bacteria were highly inhibited by the extract at very low concentrations. All the bacteria had inhibition zones greater than 20 mm at a concentration of 5 mg/ml with the exception of *Pseudomonas aeruginosa* (ATCC 19582) and *Bacillus pumilus* (ATCC 14884). For the bacteria, the minimum inhibitory concentration (MIC) values ranged between 0.078 and 0.625 mg/ml. For the fungi, the MIC values were between 2.5 to 10 mg/ml. The bacteria were more susceptible to the extract than the fungi. The antimicrobial activity of the extract indicated a broad spectrum, great therapeutic potential and justified the use of this plant by the rural communities in the treatment of microbial infections.**

Key words: Agar dilution, antimicrobial, broad spectrum, ethanol extract, fungicidal concentration.

INTRODUCTION

Medicinal plants constitute an effective source of effective therapy for both traditional and modern medicine. They are important for pharmacological research and drug development. Their constituents are used directly as therapeutic agents as well as starting materials for the syntheses or as models for pharmacologically active compounds. Due to a rapid increase in the rate of infections, antibiotic resistances in microorganisms and side effects of synthetic antibiotics, uses of medicinal plants in the treatment of microbial infections are gaining fast recognitions over antibiotics (Babu and Subhasree, 2009). Hence, plants have turned out to be an important source of new potent antibiotics, new drug leads and new chemical entities (Saklani and Kutty, 2008). They are considered cheapest and safer alternative sources of antimicrobials (Sharif and Banik, 2006; Doughari et al., 2007) to which pathogens are not resistant (Fabricant and Farnsworth, 2001).

Consequently, phytomedical investigations have revealed several plants with anti-infective properties (Stafford et al., 2005; Kambizi et al., 2007; Anwar et al., 2011). The effects of many different plant extracts on pathogenic microorganism have been indicated by numerous studies (Vundac et al., 2007; Háznagy-Radnai et al., 2008). Pharmacologically bioactive compounds such as alkaloids, flavanoids, tannins and phenolic compounds (Nawrot et al., 2007; Beltrame et al., 2011; Ullah et al., 2011; Samali et al., 2012) producing definite physiological actions on the humans have been

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identified. Antimicrobials of plant origin effective in the treatment of infectious diseases and simultaneously mitigating many of the side effects often associated with synthetic antimicrobial agents have been discovered (Iwu et al., 1999; Rajeshwar et al., 2005).

Ziziphus mucronata Willd. subsp. *mucronata* Willd, a small to medium-sized tree with a spreading canopy, is distributed throughout summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia. In ethnomedicine, pastes of the roots and leaves are applied to treat boils, swollen glands, wounds and sores while steam baths from the bark are used to purify and improve complexion (Palmer and Pitman, 1972). In East Africa, the roots are used for treating snake bites, gonorrhea, diarrhoea and dysentery (Hutchings et al., 1996). In South Africa, ethnobotanical survey indicated that it is used for gastrointestinal disorder, dysentery and diarrhoea. Unlike some members of the *Ziziphus* genus, there is a lack of scientific reports to indicate the pharmacological importance and antimicrobial activities of this plant. Hence, this study was aimed at investigating the antimicrobial activities of ethanolic bark extract of *Z. mucronata* and justifies its ethnomedicinal importance in complementary and alternative medicine.

MATERIALS AND METHODS

Collection of plant material

The bark materials of *Ziziphus mucronata* subsp. *mucronata* were collected in August, 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark sample was air-dried at room temperature and pulverized using a milling machine. About 100 g of the pulverized sample was extracted with 500 ml of ethanol for 72 h. The extract was filtered through Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at 40°C using a rotary evaporator. The extract was redissolved in ethanol to the required concentrations for the bioassay analysis.

The reconstituted ethanolic extract solution was sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract into 10 ml of sterile nutrient broth and incubated at 37°C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period (Ronald, 1995).

Test organisms

The microorganisms used in this study included *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Proteus vulgaris* (KZN), *Serratia marcescens* (ATCC 9986), *Enterococcus faecalis* (KZN), *Staphylococcus aureus* (OK3), *Pseudomonas aeruginosa* (ATCC 19582), *Klebsiella pneumoniae* (KZN), *Staphylococcus aureus* (OK1), *Salmonella typhi* (ATCC 13311), *Acinetobacter calcaoceuticus* (UP), *Candida krusei, Candida albicans, Cryptococccus neoformans, Cryptococccus rugosa, Penicillium notatum, Aspergillus niger, Aspergillus flavus and Aspergillus terreus*. These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organisms were maintained on nutrient broth, nutrient agar (Biolab), potato dextrose agar and sabouraud dextrose broth. The antibacterial assays were carried out using Mueller Hinton II Agar (Biolab) and broth. The antifungal assays were carried out using sabouraud dextrose broth and potato dextrose agar.

Antimicrobial assay by agar diffusion method (Inhibition zones)

The susceptibility screening of the test bacteria to the ethanolic bark extract of *Z. mucronata* and ciprofloxacin, used as control, was done in accordance with the methods described previously (Irobi et al., 1994; Akinpelu et al., 2008). For the antibacterial assay, the inoculum of each test bacterial strains was standardized at 5×10^6 cfu/ml using McFarland Nephe-lometer standard (NCCLS, 1993). Sterile Mueller Hinton agar plates were seeded with each adjusted test bacterial strains and allowed to stand at 37°C for 1 h. For the antifungal assay, 1 cm^2 of seven day old fungal cultures was dropped in sterile distilled water and vortexed for 2 min to release the fungal spores. Potato dextrose agar plates were seeded with 200 µl of the fungal spore solutions, allowed to stand for 1 h on the laboratory bench. Wells were then bored into the agar media using a sterile 6 mm cork borer. The wells were filled with 100 µl of different concentrations of the extract and ciprofloxacin taking care not to allow spillage of the solutions onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 30 min to allow proper diffusion of the extract and antibiotics before being incubated at 37°C for 24 h for bacterial cultures and 27°C for 72 h for fungal cultures. Wells in blank Mueller Hinton agar and potato dextrose agar plates containing 10% ethanol representing the final ethanol concentration in the test plates without the extract served as positive controls. After 24 h incubation period, antimicrobial activities were determined by measuring the inhibition zones against the test organisms using a calibrated transparent meter rule. Both antibacterial and antifungal assays were carried out in triplicates.

Macrobroth dilution for minimum inhibitory concentration (MIC)

The activity of the extract and minimum inhibitory concentration were determined by the macrobroth dilution methods (NCCLS, 1993). For antibacterial assay, different concentrations of the extract ranging from 0.02 to 10 mg/ml were prepared by serial dilutions in Mueller Hinton broth medium. Different concentrations of ciprofloxacin ranging from 0.01 and 5 µg/ml used as positive control were also prepared by serial dilution in Mueller Hinton agar. The tubes were inoculated with 100 µl of each of the bacterial strain. Blank Mueller Hinton broth was used as negative control.

For the antifungal assay, different concentrations of the extract ranging between 0.2 and 20 mg/ml were prepared in sabouraud dextrose broth by serial dilutions. Each broth concentration was inoculated with 100 µl of the prepared fungal spores' solution. Two control tubes were included: One with spores and broth but no plant extract, and one with broth and plant extract but no spores. The bacterial containing tubes were incubated aerobically at 37°C for 24 h. The fungal containing tubes were incubated at 27°C for 3 to 5 days. The first tube in the series with no visible growth after incubation period was taken as the MIC.

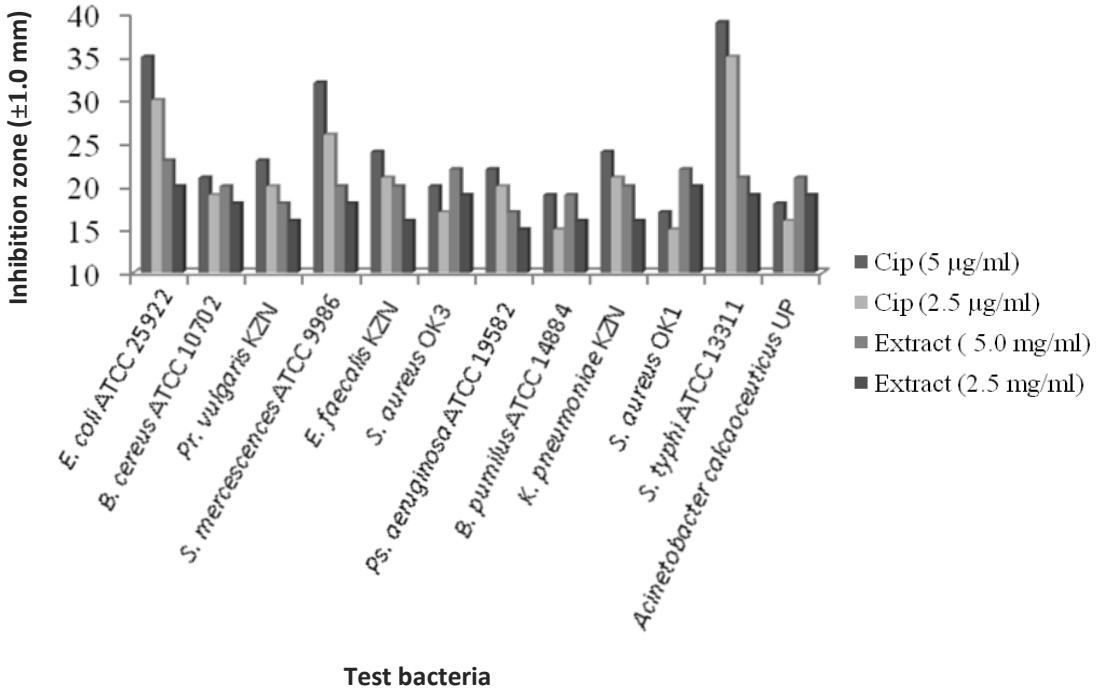

Figure 1. Antibacterial activity of ethanolic extract of *Z. mucronata* and ciprofloxacin against tested bacteria.

Determination of minimum bactericidal and fungicidal concentrations (MBC/MFC)

For the determination of the MBC and MFC, fresh nutrient agar and potato dextrose agar plates were inoculated with one loopful of culture taken from each of the first three broth cultures that showed no growth in the MIC tubes. While MBC assay plates were incubated for 24 h, MFC assay plates were incubated for 5 days. After the incubation periods, the lowest concentration of the extract that did not produce any bacterial or fungal growth on the solid medium was regarded as MBC and MFC values for this extract (Irkin and Korukluoglu, 2007). This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubating the bacteria or spore germination for the fungi after five days of incubation.

Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA) and the mean values were separated at ($p<0.05$) using Duncan's multiple range test. The one way ANOVA test was used to determine if there was any statistically significant difference in the diameter of the zones of inhibition obtained from the different concentrations of the extract tested against the microorganisms. All statistical analyses were done using SAS software (1999) model.

RESULTS

The antibacterial activities of the stem bark extracts of *Z. mucronata* against medically important pathogens were assessed by the presence or absence of inhibition zones. To determine the degree of antibacterial activity, the extract was subjected to minimum inhibitory concentration (MIC) assay by serial two fold dilution method (Florey et al., 1989). The result revealed that the ethanolic extract of *Z. mucronata* possess good antibacterial activity against both Gram-positive and Gram-negative bacteria (Figure 1) and antifungal activity against all the tested fungal isolates (Figure 2). The antibacterial activity assessed in terms of inhibition zone indicated that *E. coli* ATCC 25922 had the highest inhibition zone and *P. aeruginosa* ATCC 19582 had the least inhibition zone from 5 mg/ml concentration of the extract (Figure 1). The minimum inhibitory concentrations assay indicated that the two groups of bacteria were susceptible to the extract at very high concentrations with *S. aureus* OK_1 having the highest minimum inhibitory concentration of 0.0781 µg/ml (Figure 3). With the exception of *P. aeruginosa* ATCC 19582 and *B. pumilus* ATCC 14884, all the bacteria had inhibition zones greater than 20 mm at a concentration of 5 mg/ml. The extract was less potent than the standard antibiotic, ciprofloxacin, to which the bacteria were highly susceptible at 5 µg/ml.

The antifungal assay indicated that all the fungal isolates were highly susceptible to different concentrations of the extract and their susceptibilities were concentration dependent. With an increase in the concentration of the extract, there are increases in the inhibition zones of each of the microorganisms. Minimum fungicidal concentrations of the plant extracts were expectedly higher than the minimum inhibitory concentration while the MIC values were found ranging from 2.5 to 10 mg/ml for fungi. The MIC values of *P. notatum* and *A. flavus* was 2.5 mg/ml, *A. niger*, *A. terreus*

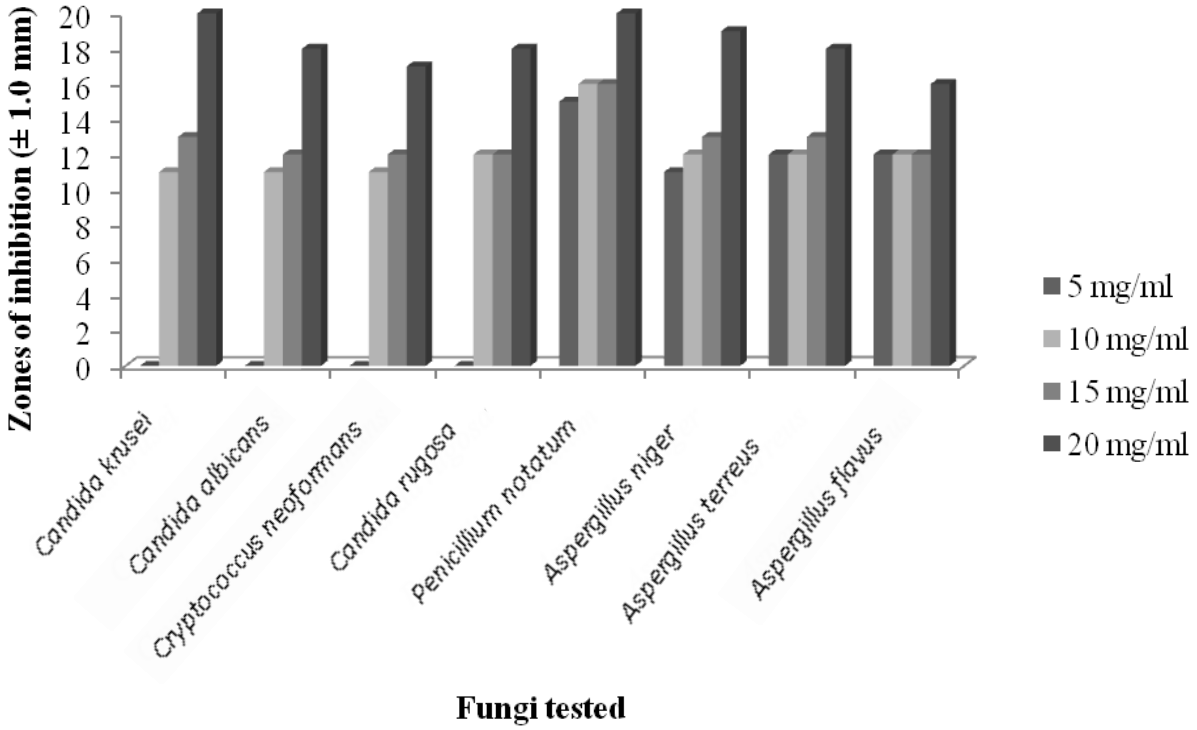

Figure 2. Antifungal activities of different concentration of ethanolic extract of *Z. mucronata* subsp. *mucronata*.

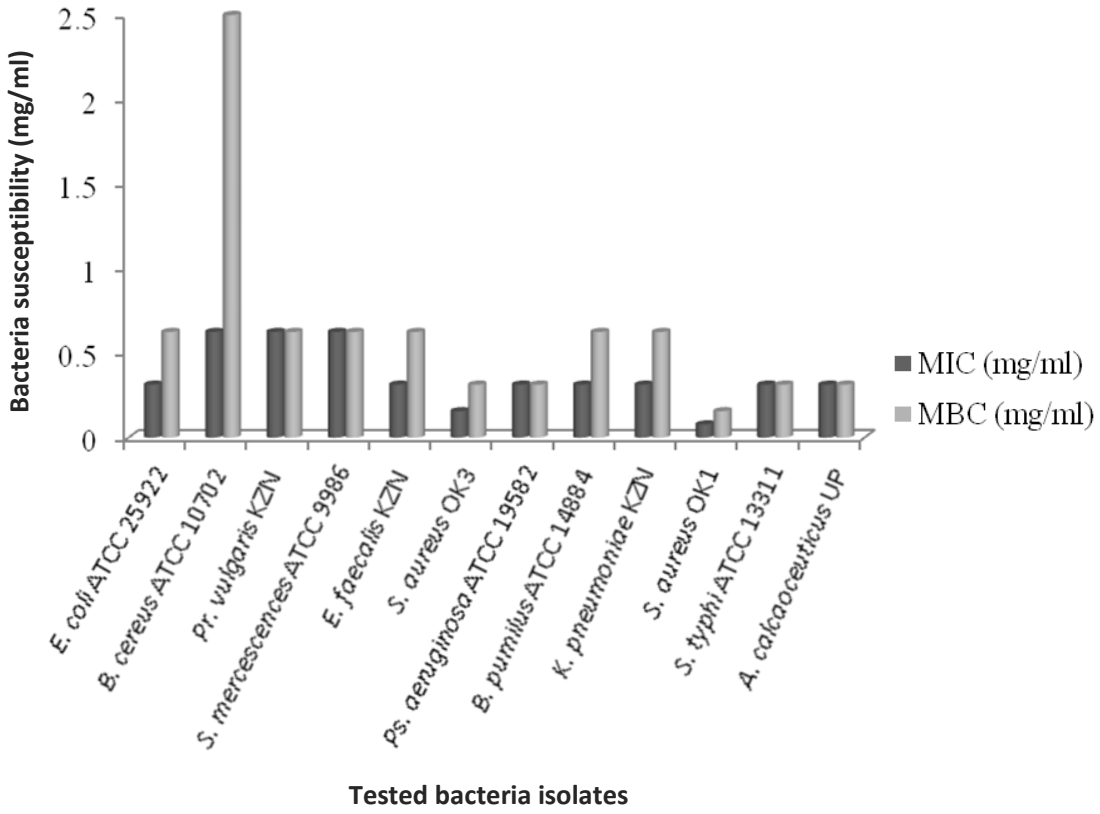

Figure 3. MIC and MBC of ethanolic extract of *Z. mucronata* subsp. *mucronata* against tested bacteria.

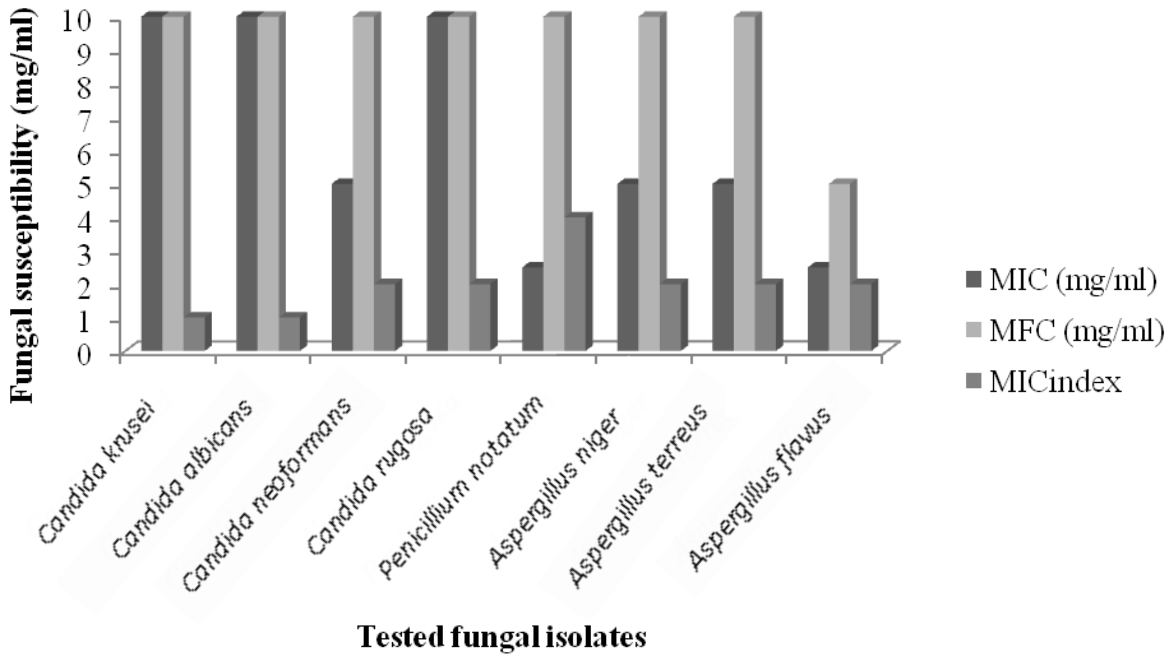

Figure 4. MIC and MFC of ethanolic extract of *Z. mucronata* subsp. mucronata against tested fungi.

and *Cryptococccus neoformans* was 5 mg/ml, *C. krusei, C. albicans* and *C. rugosa* was 10 mg/ml (Figure 4). Although the MIC values of the extract against bacteria were considerably low, they were higher than those obtained for the fungi indicating that the bacteria were more susceptible to the extract than the fungi. The MIC/MBC and MIC/MFC ratios indicated that the extract is bacteriostatic and fungistatic at lower concentrations while at higher concentrations, it is bactericidal and fungicidal. The activity of the extract on the fungal species is significant as these fungi are usually implicated in atrophic candidiasis, vaginal thrush and aflatoxin production. Where MIC equals MBC/MFC, the similarity revealed that the plant bark possesses potent antibacterial and fungicidal components against the test isolates. The low MIC and MBC values showed that the extract has strong antimicrobial activity. The antimicrobial activity of the extract, however, indicated a broad spectrum and great therapeutic potential of the plant.

DISCUSSION

The antibacterial and antifungal activities of the ethanol extract of *Z. mucronata* were investigated in this study. The results showed that the extract had significant antimicrobial potency against both bacteria and fungi tested. While the active components in the crude extract may be acting synergistically to produce good antimicrobial effects (Eloff, 1998), the disparity between the activities of the extract and the standard antimicrobial drug may be due to the mixtures of bioactive compounds present in the extract compared to the pure compound contained in the standard antibiotics (Gatsing et al., 2010).

Screening of plants for their pharmacological activity has indeed been the vast source of innumerable therapeutic effects representing molecular diversity engineered by nature. Plant extracts and their purified compounds have shown antibacterial and fungicidal effects both *in vitro* and *in vivo* (Saglam and Arar, 2003). Extracts with known antimicrobial properties can be of great importance in therapeutic treatments (Erdogrul, 2002; Rojas et al., 2006) and their active principles have been considered alternative cheap and effective herbal drugs against common microbial infections (Kareru et al., 2008). In agreement with several reports on antimicrobial activities of many medicinal plants (Afolayan et al., 2002; Mathabe et al., 2006; Lategan et al., 2009; Gavanji et al., 2011; Govindappa et al., 2011), the significant antimicrobial activities of *Z. mucronata* indicated its medicinal potential that could be used against infectious pathogens. Its ethanolic extract, at different concentrations, possesses inhibitory effects indicating a broad spectrum of antimicrobial activity.

Although the mechanism of action of this extract is not understood, its action against the bacteria and fungi may be due to the inhibition of cell wall through pore formation in the cell and leakage of cytoplasmic constituents by the bioactive components of the extract (Bais et al., 2002; Hassan et al., 2007). While phytochemical compounds such as tannin coagulate the wall proteins, saponins

facilitated the entry of toxic material or leakage of vital constituents from the cell (Onwuliri and Wonang, 2005). Flavonoids inhibit the activity of enzymes (Dathak and Iwu, 1991) by forming complexes with bacterial cell walls, extracellular and soluble proteins, more lipophilic flavonoids disrupt cell wall integrity (Kurtz et al., 1994) or microbial membranes (Tsuchiya et al., 1996) at low concentrations.

Since the MIC values indicated the definite nature of the antimicrobial activities of this plant, the inhibition zones values, only, indicated extent of effectiveness of the extract with increasing concentration. The difference in the antibacterial activities between the macrobroth and agar diffusion assays may be attributed to the degree of exposure of each of the tested organisms and the rate of diffusion of the extract into the surrounding agar from the wells. In broth assay, the organisms have direct contacts with the extract. The diffusion in agar assay, in effect, created an extract gradient around the wells with microbial inhibition being achieved only when the extract concentration in the agar approximately reached that of the tube MIC. Progressively, higher extract concentrations yielded progressively greater degrees of inhibition (larger inhibition zones). The tube assay provided a more accurate determination of the MIC and antibacterial activity of the extract than the well diffusion assay. Under liquid conditions, the microbial cells are in direct contact with the extract which was more homogenously present throughout the culture liquid and no differential gradient was established. The obtained result is similar to the findings of Banso et al. (1999) and Rasooli et al. (2006) that showed that higher concentrations of antimicrobial substance yielded greater degrees of growth inhibition.

In conclusion, this study demonstrated that crude extract of *Z. mucronata* exhibited good antimicrobial activity against different infectious agents but not as effective as the standard antibiotic used for comparison. The activity of the extract against both bacteria and fungi has confirmed its broad spectrum antimicrobial activity. The present study, therefore, justifies the rationale for the traditional use of the ethanolic stem bark extract of *Z. mucronata* in the treatment of microbial infections by rural communities in South Africa.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support of the National Research Foundation and the University of Fort Hare.

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Chapter 9

In Vitro Antibacterial and Time-Kill Assessment of Crude Methanolic Stem Bark Extract of *Acacia mearnsii* De Wild against Bacteria in Shigellosis

(*Published in the Molecules*)

Molecules **2012**, *17*, 2103-2118; doi:10.3390/molecules17022103

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Article

In Vitro Antibacterial and Time-Kill Assessment of Crude Methanolic Stem Bark Extract of *Acacia mearnsii* De Wild against Bacteria in Shigellosis

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Received: 13 January 2012; in revised form: 13 February 2012 / Accepted: 15 February 2012 / Published: 21 February 2012

Abstract: Shigellosis is an important cause of worldwide morbidity and mortality among young children and old people for which treatment with antimicrobial agents is limited. Hence, the need for curative potentials obtainable from medicinal plants becomes inevitable. This study was carried out to assess the antibacterial potentials of crude methanolic extract of the stem bark of *Acacia mearnsii* against some selected bacteria of clinical importance in shigellosis. The bacteria were inhibited by the extract to produce concentration dependent inhibition zones. The extract exhibited a varied degree of antibacterial activity against all the tested isolates. The MIC values for Gram negative (0.0391–0.3125) mg/mL and those of Gram positive bacteria (0.0781–0.625) mg/mL indicated that the Gram negative bacteria were more inhibited by the extract than the Gram positive bacteria. Average log reduction in viable cell count in time-kill assay ranged between −2.456 Log_{10} to 2.230 Log_{10} cfu/mL after 4 h of interaction, and between −2.921 Log_{10} and 1.447 Log_{10} cfu/mL after 8 h interaction in 1× MIC and 2× MIC of the extract. The study provided scientific justification for the use of the crude methanolic extract from the stem bark of *A. mearnsii* in shigellosis. The degree of the antibacterial activity indicated that the crude extract is a potential source of bioactive compounds that could be useful for the development of new antimicrobial agents capable of decreasing the burden of drug resistance and cost of management of diseases of clinical and public health importance in South Africa.

Keywords: *A. mearnsii*; antibacterial; shigellosis; time-kill; methanolic extract

1. Introduction

Shigellosis, an important cause of worldwide morbidity and mortality [1], is a disease of public health importance in developing countries causing self-limited diarrhea to severe dysentery [2]. It is primarily a disease of poor, crowded communities that do not have adequate sanitation or clean water [3]. Aside from clinical intestinal manifestations, it causes a wide variety of extra-intestinal signs such as bacteremia or neurologic manifestations [4]. Though Enteroinvasive *Escherichia coli* (EIEC) and Enterohemorrhagic *E. coli* (EHEC) are less frequently implicated in bacillary dysentery [5], Enteropathogenic bacteria are mainly implicated [6]. Barman *et al.* [7] and Vinh *et al.* [8] indicated, also, that shigellosis is caused by different species of Shigella (*Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii* and *Shigella sonnei*) belonging to the family Enterobacteriaceae. *Shigella sonnei* is the serogroup of Shigella most frequently responsible for sporadic and epidemic enteritis in developed countries while *S. flexneri* is the most dominant strain in developing countries [9]. EHEC known as verocytotoxin producing *E. coli* (VTEC) [10] produce a Shiga-like toxin (SLT) and the enteroinvasive *E. coli* (EIEC) possess genetic and biochemical characteristics similar to *Shigella* [11]. Although shigellosis is a major source of gastroenteritis throughout the world with *Shigella spp.* being majorly implicated [12], other gastrointestinal pathogens such as Salmonella, Yersinia, enteropathogenic *Escherichia coli* (EPEC), *Entamoeba hystolytica*, *Bacillus subtilits*, *Bacillus cereus*, *Aeromonas hydrophyla* and *Campylobacter sp.* have been involved.

Epidemiologically, 165 million children and young adults suffer worldwide annually with 1 million associated deaths from shigellosis. 99% of these cases occur in developing countries [13,14] with 69% occurring in children aged less than five years [15,16]. While Lee *et al.*, [17], Niyogi, [14] and Wang *et al.*, [18,19] reported that there is still a relatively high incidence of bacillary dysentery among children and old people, Alam and Bhatnagar, [20], Seol *et al.*, [21] and Von Seidlein *et al.*, [22] indicated that easy variability of the pathogen, lack of cross-immunity among different types of Shigella bacteria, increase of drug-resistant strains among the carriers and the patients' immunity status that cannot be sustained after the infection are responsible for the observed incidence rate among the young children and old people.

Shigella spp. and most of the associated gastrointestinal pathogens are usually spread directly from person to person by the fecal–oral route or indirectly by ingestion of fecal contaminated food or water [23,24]. The pathogenesis showed that an inoculum of 10^2 *Shigella* cells and as many as 10^6 EIEC cells are sufficient for the onset of the infection [25]. Though both EIEC and *Shigella* cause bacillary dysentery in humans by invading and multiplying within epithelial cells of the colonic mucosa to cause an intense inflammatory response characterized by abscesses and ulcerations [26,27], early detection and intervention in disease outbreaks has enabled timely public health measures to limit illness and death [28]. This could be in addition to improved nutrition in many countries, improved healthcare delivery in some areas and more widespread use of measles vaccine [29], vitamin A supplementation [30] and proper case management [31] to reduce the severity of intestinal infections.

Though shigellosis is a global human health problem and a public health challenge, especially in endemic areas, emergence of multi-drug resistant (MDR) strains involving all microbial pathogens and antimicrobial drugs is a simultaneous growing concern globally [2] and multidrug resistance in shigellosis is not exempted [14]. Due to the global emergence of drug resistance, the choice of antimicrobial agents for treating shigellosis is limited. Hence, the curative potentials of medicinal plants locked-up and embedded in some chemical components that effect physiological responses are needed for the treatment of the infection.

Consequently, in the last few decades, the study of medicinal plants and their traditional use in different parts of the World has increased [32]. Hundreds of plants have been used as herbal remedies in indigenous medicine systems [33]. While herbal medicines are assumed to be of great importance in the primary healthcare of individuals in rural communities [34,35], plant-based traditional knowledge coupled with the high cost involved in the development of patentable chemicals and drugs [36] are recognized as essential tools in search for new sources of drugs and neutraceuticals [37]. Thus, antimicrobial activity of crude and semi-purified extracts of many plants has been widely reported [38–41]. The increasing use of traditional therapies which the laypeople considered as a part of their heritage [42] now requires more scientifically sound evidence for the principles behind plants' therapeutic effectiveness in complementary and alternative medicines [43].

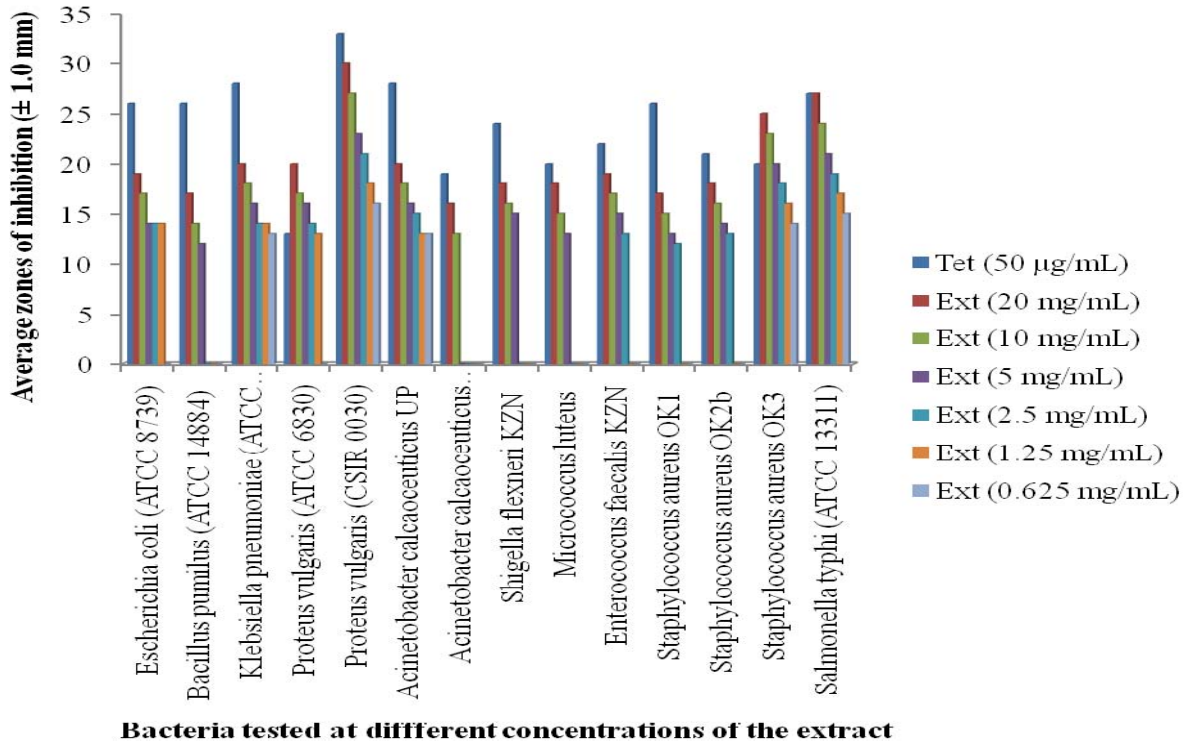
The genus *Acacia* (Wattle) is a member of the pea family (Leguminosae). While it is the largest group of vascular plants in Australia with about 1,000 species currently recognized, it is a group of 1,404 species distributed throughout tropical and warm temperate areas of the World. Leaves, twigs and bark are used for a range of medicinal purposes, including decoctions for the treatment of flu, colds, skin ailments and smoke from certain species has been used to promote health. Seeds of about 30 species are used as food [44], and many acacias are used as stock fodder in the arid zone rangelands [45,46]. Though *Acacia* with diverse morphological, biological and ecological attributes offers great scope for economic, environmental and social utilization, *Acacia mearnsii* is basically known as one of the World's highest yielding sources of high quality condensed tannin. Its pharmacological importance is less explored despite its being grown in large-scale plantations in South Africa and other parts of the world. This study was designed to assess its antibacterial activity and justify its relevance in the treatment of dysentery for which it has been implicated in the rural areas in South Africa.

2. Results and Discussion

The antibacterial activity of the stem bark methanol extract of *A. mearnsii* was evaluated by measuring the inhibition zone against medically important pathogens. The degree of the antibacterial activity was assayed by the serial two fold dilution method to determine the minimum inhibitory concentration (MIC) of the extract while the bactericidal activity was assessed by time kill assay *in vitro*. The result showed that the extract had good antibacterial activity against both Gram positive and Gram negative bacteria at different concentrations (Figure 1). The agar diffusion assay indicated that the bacteria were inhibited by the extract to produce concentration dependent inhibition zones. At the least used concentration, five of the bacteria were inhibited. At the highest concentration, all the bacteria were inhibited. The inhibition zones produced by 100 µL of 20 mg/mL of the extract ranged between

16 mm and 30 mm for all the bacteria. Of the Gram negative bacteria, *Salmonella typhi* (ATCC13311) and *Proteus vulgaris* (CSIR 0030) had the highest inhibition zones of 27 mm and 30 mm, respectively, while those of other Gram negative bacteria ranged between 16 mm and 20 mm. Of the Gram positive bacteria, *Staphylococcus aureus* OK_3 had the highest inhibition zone (25 mm) while those of others ranged between 17 mm and 18 mm. The extract exhibited a varied degree of antibacterial activity against all the tested isolates for which the MIC values ranged between 0.0391 mg/mL and 0.625 mg/mL. While the MIC values for Gram negative ranged between 0.0391 mg/mL and 0.3125 mg/mL, those of Gram positive bacteria ranged between 0.0781 mg/mL and 0.625 mg/mL. The MBC values were similar or 2–4 folds higher than MIC values for the extract (Table 1). While a good agreement exists between the agar diffusion and macrobroth dilution assays, the antibacterial effects showed that Gram negative bacteria were more inhibited by the extract than the Gram positive bacteria. The extract was less potent than the standard antibiotic, tetracycline, of which 100 µL of 50 µg/mL had produced inhibition zones ranging between 13 mm and 33 mm in diameter. The variation between the activities of the extract and the standard antimicrobial drug may be due to the mixtures of bioactive compounds present in the extract compared to the pure compound contained in the standard antibiotics that rarely have the same degree of activity as the unrefined extract at comparable concentrations or dose of the active component.

Figure 1. Bacterial susceptibility to different concentrations of crude methanolic extract of *A. mearnsii*.



Key: Tet represents Tetracycline; Ext represents Extract.

The results of time-kill studies are presented in Table 2. Data are presented in terms of the $\log_{10}$ cfu/mL change and are based on the conventional bactericidal activity standard, that is, a 3 Log_{10} cfu/mL or greater reduction in the viable colony count [47]. Average log reduction in viable cell count in time-kill assay ranged between −2.456 Log_{10} to 2.230 Log_{10} cfu/mL after 4 h of interaction, and

between −2.921 Log_{10} and 1.447 Log_{10} cfu/mL after 8 h interaction in 1× MIC and 2× MIC of the extract. The greatest reduction in cell density with the extract was observed with *Klebsiella pneumoniae* (10031) (−2.921 log_{10}), *Acinetobacter calcaoceuticus* UP (−2.638 Log_{10}), *Micrococcus luteus* (−2.444 Log_{10}), *Acinetobacter calcaoceuticus* CSIR (2.387Log_{10}) and *Enterococcus faecalis* KZN (−2.246 Log_{10}). At these concentrations, the significant reduction in the bacterial population suggests that the extract was highly bactericidal after a 4 h incubation period, while the bacterial colonies were almost wiped out after incubating for 8 h. On the contrary, there was a net growth of all the test isolates when subjected to ½ MIC concentration of the extract. Growth inhibition and efficacy of the crude methanolic stem bark extract were observed to be dose and time dependent producing distinct time-kill profiles for the tested bacteria.

In the last few decades, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the classical antibiotics have led researchers to investigate the antimicrobial activity of several medicinal plants utilized as popular folk medicines or finished products collectively known as phytomedicines. While the continuous spread of multidrug-resistant pathogens has become a serious threat to public health and a major concern for infection control practitioners worldwide [48], re-emergence of previously controlled diseases contributes substantially to the high frequency of opportunistic and chronic infection cases in developing countries [49,50]. Hence, since resistance to currently available antimicrobial agents used to treat infections requires new appraisals [51,52], *in vitro* susceptibility testing and determination of the bactericidal activity with the aim of indicating the efficacy of an antimicrobial agent against infecting organisms to provide informed decision regarding treatment of serious infections become necessary. The reason being that, in certain unique clinical settings involving sites not easily accessed by host defenses [53,54] and neutropenic immunosuppressed hosts [55], the ability of the agent administered to kill the pathogen outright may be quite important [56,57].

Table 1. Antibacterial activity of the methanolic extract of *Acacia mearnsii* De Wild.

	Tetracycline (µg/mL)	**Methanolic extract (mg/mL)**		
	MIC	**MIC**	**MBC**	**MIC/MBC**
Escherichia coli (ATCC 8739)	0.977	0.3125	0.625	2
Bacillus pumilus (ATCC 14884)	3.125	0.3125	0.625	2
Klebsiella pneumoniae (ATCC 10031)	0.488	0.1563	0.1563	1
Proteus vulgaris (ATCC 6830)	7.8125	0.1563	0.625	4
Proteus vulgaris (CSIR 0030)	0.488	0.0391	0.1563	2
Acinetobacter calcaoceuticus UP	125	0.1562	0.3125	2
Acinetobacter calcaoceuticus anitratis CSIR	0.0305	0.3125	0.625	2
Shigella flexneri KZN	0.1966	0.0391	0.0781	2
Micrococcus luteus	31.25	0.078	0.1563	2
Enterococcus faecalis KZN	15.625	0.156	0.3125	2
Staphylococcus aureus OK_1	0.976	0.1563	0.625	4
Staphylococcus aureus $OK2_b$	0.1966	0.625	1.25	2
Staphylococcus aureus OK_3	0.1953	0.3125	0.625	2
Salmonella typhi (ATCC 13311)	0.0305	0.3125	0.3125	1

Table 2. *In vitro* time kill assessment of the rude methanolic stem bark of *A. mearnsii*.

	Log_{10}Kill ½ X MIC			**Log_{10}Kill MIC**			**Log_{10}Kill 2 X MIC**		
	0 h	**4 h**	**8 h**	**0 h**	**4 h**	**8 h**	**0 h**	**4 h**	**8 h**
Escherichia coli (ATCC 8739)	2.083	3.131	4.248	2.199	1.294	1.125	2.340	0.121	−1.542
Bacillus pumilus (ATCC 14884)	2.212	3.422	4.449	2.392	2.167	1.033	2.422	0.111	−1.815
Klebsiella pneumoniae (ATCC 10031)	2.170	3.386	4.292	2.207	1.121	0.427	2.185	−1.456	−2.921
Proteus vulgaris (ATCC 6830)	2.164	3.401	4.468	2.217	1.187	0.274	2.200	1.132	−1.951
Proteus vulgaris (CSIR 0030)	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acinetobacter calcaoceuticus UP	2.091	2.237	3.427	2.375	1.185	0.233	2.345	−1.0915	−2.638
Acinetobacter calcaoceuticus anitratis CSIR	2.276	3.324	4.519	2.354	1.199	0.122	2.375	−0.951	−2.387
Shigella flexneri KZN	ND	ND	ND	ND	ND	ND	ND	ND	ND
Micrococcus luteus	2.193	2.093	3.137	2.241	1.420	0.121	2.210	0.292	−2.444
Enterococcus faecalis KZN	2.272	2.121	3.179	2.272	1.358	0.246	2.265	0.130	−2.246
Staphylococcus aureus OK_1	2.386	2.203	3.322	2.268	2.140	1.032	2.321	1.083	−1.614
Staphylococcus aureus $OK2_b$	2.234	2.025	3.340	2.336	1.137	0.053	2.303	1.358	−1.287
Staphylococcus aureus OK_3	3.342	4.690	6.633	3.167	2.230	1.447	3.248	1.916	1.062
Salmonella typhi (ATCC 13311)	2.292	2.328	3.155	2.270	2.201	1.274	2.288	2.185	−0.727

Key: ND represents Not Determined.

In this study, our data showed that the response of the bacteria to the tested extract varied among the strains and are concentration and time dependent. The differences in susceptibility may be due to the differences in cell wall composition and/or genetic content of their plasmids [58]. While the active components in the crude extract may be acting synergistically to produce good antimicrobial effects [59], the disparity between the activities of the extract and the standard antimicrobial drug may be due to the mixtures of bioactive compounds present in the extract compared to the pure compound contained in the standard antibiotics [60]. The MBC values obtained suggested that a biocidal effect of the crude extract could be expected on most of the tested organisms [61,62]. This effect which could necessitate isolation and development of new antimicrobial agents was exhibited by the time-kill assessment of the extract. The observed degree of antibacterial activity may be attributed to the inherent astringent property associated with the high percentage of tannin sometimes called polyphenols [63,64] in the plant while its phytochemical assessment showed that both total phenolic content and the antioxidant activity correlated well [65]. Thus, in agreement with other reports, the antibacterial activity of the extract may be due to the presence of phytoconstituents such as tannin [66], alkaloids [67], terpenoids [68], flavonoids [69,70], and saponins [71,72].

In addition, the differences between the susceptibility of the Gram positive and Gram negative bacteria may be attributed to the differences in their cell wall components and thicknesses [73]. However, the fact that Gram negative bacteria were more susceptible to the extract is significant as Gram positive bacteria are usually reported as being more affected by plant extracts [74–76]. In the Gram negative bacteria, the extract was able to overcome the permeability barrier provided by the cell wall and the membrane accumulated resistance mechanisms [77]. Though several mechanisms of action in the growth inhibition of bacteria including destabilization of cytoplasmic and plasma membranes, inhibition of extracellular microbial enzymes and metabolisms, and deprivation of the substrate required for microbial growth [78] have been reported, the mode of antimicrobial action of plant tannin may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, and mineral uptake [79] or polymerization through oxidation reactions [80]. Hence, the disintegrative ability of tannin compounds on the bacterial colonies probably resulted from their interference with the bacterial cell wall to inhibit their growth [81,82] while the degree of toxicity resulting from their polymerization may be responsible for the degree of the bactericidal activity of the extract over a short period of time in time-kill assay.

3. Experimental

3.1. Collection of Plant Material

The bark materials of *Acacia mearnsii* De Wild were collected in August, 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen (OLAJ Med 2010/01) was prepared and deposited in the Griffen Herbarium of the University.

3.2. Extract Preparation

The bark sample was air-dried at room temperature and pulverized using a milling machine. The extract of the bark material was prepared in accordance to the description of Basri and Fan [83]. About 100 g of the pulverized sample was extracted with 500 mL of methanol for 48 h with shaking (Stuart Scientific Orbital Shaker, Staffordshire, UK). The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40 °C using a rotary evaporator (Laborota 4000–efficient, Heldolph, Germany). The crude extract collected was allowed to dry at room temperature to a constant weight of 18.7 g. The extract was redissolved in dimethylsulfoxide (DMSO) to the required concentrations for bioassay analysis. The reconstituted extract solution was sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 mL of the extract into 10 mL of sterile nutrient broth before being incubated at 37 °C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

3.3. Test Organisms and Bacterial Inocula Preparation

The bacteria used in this study included *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 10031), *Bacillus pumilus* (ATCC 14884), *Proteus vulgaris* (ATCC 6830), *Proteus vulgaris* (CSIR 0030), *Acinetobacter calcaoceuticus* UP, *Acinetobacter calcaoceuticus anitratis* CSIR, *Shigella flexneri* KZN, *Salmonella typhi* (ATCC 13311), *Micrococcus luteus*, *Enterobacter faecalis* KZN, *Staphylococcus aureus* OK_{2b}, *Staphylococcus aureus* OK_1 and *Staphylococcus aureus* OK_3. These strains were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The inocula of the test bacteria were prepared using the colony suspension method [84]. Colonies picked from 24 hold cultures grown on nutrient agar were used to make suspensions 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 transferring 0.1 mL of the bacterial suspension to 9.9 mL of sterile nutrient broth before being used.

3.4. Antimicrobial Assay by Agar Diffusion Method (Inhibition Zones)

For the initial determination of the antibacterial activity of the crude methanolic extract of *A. mearnsii*, the susceptibility screening of the test bacteria to the extract and tetracycline, used as control, was determined using the modified Kirby-Bauer diffusion technique [85] by swabbing Mueller-Hinton agar (MHA) (Oxoids Ltd, Basingstoke, Hampshire, UK) plates with the resultant saline suspension of each adjusted bacterial strain. Wells were then bored into the agar media using a sterile 6 mm cork borer. The wells were filled with 100 µL of different concentrations of the extract and tetracycline taking care not to allow spillage of the solutions onto the surface of the agar. The culture plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of these solutions before being incubated at 37 °C for 24 h. Wells in blank Mueller Hinton agar containing 10% DMSO representing the final concentration of the DMSO in the test plates without the extract served as positive controls. The determinations were done in duplicates. After 24 h of incubation, the plates were examined if there is any inhibition zone [86]. The organisms were not susceptible to 10% DMSO used in the control assay. The diameters of the inhibition zones produced by each of the concentrations of

the solutions were measured in millimeters [87] and interpreted using the CLSI zone diameter interpretative standards [88].

3.5. Macrobroth Dilution for Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) defined as the lowest concentration which resulted in maintenance or reduction of inoculums viability [89] was determined by serial tube dilution technique [90,91] for the bacterial isolates. Different concentrations (0.02–10.0) mg/mL of the extract and (0.03–250) µg/mL of tetracycline were differently prepared by serial dilutions in Mueller Hinton broth medium. Each tube was then inoculated with 100 µL of each of the adjusted bacterial strain. Two blank Mueller Hinton broth tubes, with and without bacterial inoculation, were used as the growth and sterility controls. The bacterial containing tubes were incubated aerobically at 37 °C for 24 h. After the incubation period, the tubes were observed for the MICs by checking the concentration of the first tube in the series (ascending extract and antibiotic concentrations) that showed no visible trace of growth. The first tube in the series with no visible growth after the incubation period was taken as the MIC.

3.6. Determination of Minimum Bactericidal Concentrations (MBC)

Since the clinical occurrences of tolerance usually necessitate bactericidal testing, the MBC was determined by sampling all the macroscopically clear tubes and the first turbid tube in the series. Before being sampled, the tubes were gently mixed by flushing them with a sterile pipette, and a 100 µL aliquot was removed. Each aliquot was placed on a single antibiotic-free nutrient agar plate in a single streak down the center of the plate in accordance with the method of Shanholtzer *et al.*, [92]. The samples were allowed to be absorbed into the agar until the plate surface appeared dry (about 30 min). The aliquot was then spread over the plate by lawning technique. In many studies on microbial susceptibility, this subculturing method has been found satisfactory in eliminating the problem of antimicrobial agent carryover from the 100 µL subculture volume [93–96]. The growth and sterility controls were sampled in the same manner. The MBC lawned plates were incubated for 24 h at 35 °C. After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as MBC values for this extract [97]. This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation.

3.7. Determination of Rate of Kill

Assays for the rate of killing bacteria by the crude methanolic extract were carried out using a modified plating technique of Eliopoulos amd Eliopoulos [98] and Eliopoulos and Moellering [99]. The extract was incorporated into 10 mL Mueller Hinton broth in McCartney bottles at ½ MIC, MIC and 2× MIC. Two controls, one Mueller Hinton broth without extract inoculated with test organisms and Mueller Hinton broth incorporated with the extract at the test concentrations without the test organisms, were included. Inoculums density, approximately 10^5 cfu/mL further verified by total viable count, was used to inoculate 10 mL volumes of both test and control bottles. The bottles were incubated at 37 °C on an orbital shaker at 120 rpm. A 100 µL aliquot was removed from the culture medium at 0, 4 and 8 h for the determination of cfu/mL by the plate count technique [100] by plating

out 25 µL of each of the dilutions. The problem of extract carryover was addressed by dilution as described previously by Pankuch *et al.*, [101]. After incubating at 37 °C for 24 h, emergent bacterial colonies were counted, cfu/mL calculated and compared with the count of the culture control without extract.

4. Conclusions

The results of the present study provided scientific justification for the use of the methanol extract from the stem bark of *Acacia mearnsii* in shigellosis. The degree of the antibacterial activity indicated that the crude extract is a potential source of bioactive compounds that could be useful for the development of new antimicrobial agents capable of decreasing the burden of drug resistance and cost of management of diseases of clinical and public health importance in this South Africa. Since plants produce a diverse range of bioactive molecules making them rich sources of different types of medicines, further pharmacological and toxicity will be necessary to establish their safety as antimicrobial agents.

Acknowledgement

The authors wish to acknowledge the financial support of the National Research Foundation and the University of Fort Hare. Thanks to Don Grierson for helping in the identification of the plant.

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Sample Availability: Contact the authors.

CHAPTER 10

In vitro antibacterial activities of the methanol extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

(*Published in the Journal of Medicinal Plants Research*)

Journal of Medicinal Plants Research Vol. 5(16), pp. 3791-3795, 18 August, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

In vitro antibacterial activities of the methanol extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

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Accepted 23 June, 2011

The antibacterial activity of crude methanolic extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. against both gram-positive and gram-negative bacteria were investigated using agar and macrobroth dilution methods. The extract had effective activities against both Gram-positive and Gram-negative bacteria. The agar dilution method indicated that the organisms were susceptible at concentrations ranging between 0.5 and 2.5 mg/ml. While the minimum inhibitory concentration (MIC) values ranged between 0.0391 and 0.625 mg/ml, the minimum bactericidal concentration (MBC) values range between 0.3125 and 1.25 mg/ml. Generally, the Gram-negative bacteria were more susceptible to the extract than the Gram-positive bacteria. The MIC_{index} indicated that the extract was bactericidal at higher concentrations and bacteriostatic at lower concentrations. Our study revealed the broad-spectrum potential of the plant as well as established its ethnobotanical relevance in the traditional treatment of diarrhoeae and dysentery.

Key words: *Ziziphus mucronata*, methanol extract, antibacterial assay, macrobroth dilution.

INTRODUCTION

Herbal medicine, being used by all cultures throughout history, is the use of plants for their therapeutic value (Duke, 2002). According to Farnsworth (1984), a number of herbal plants and their compounds have been used, and have served as models for modern medicine. Apart from 30 to 40% of plants used in today's conventional drugs, other plants are used as herbal supplements, botanicals and teas (Kirby, 1996; Hostetmann and Marston, 2002). World Health Organization (WHO) recognized that 74% of the 119 plant-derived pharmaceutical medicines used in modern medicine correlated directly with their traditional uses as plant medicines by native cultures (Schulz et al., 2001). According to Lopez et al. (2001) and Karaman et al. (2001), these plants contain numerous biological active compounds. Many of these compounds have been shown to have antimicrobial activity acting individually or in combination on the human body to prevent health disorders (Palombo and Semple, 2001; van Wyk and Wink, 2004). Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic values (Nostro et al., 2000). They have been shown to have genuine utility and long been utilized as source of therapeutic agents worldwide. About 80% of the world population depend on it as sources of their primary health care (WHO, 1993) and have increasingly been used to treat many diseases (Basile et al., 1999). The scientific studies available on a good number of medicinal plants indicate that promising phytochemicals can be developed for many human health problems (Gupta, 1994; Dahiru et al., 2005). They are also a possible source for new potent antibiotics to which pathogenic strains are not resistant (Mohanasundari et al., 2007).

The genus *Ziziphus* found in desert areas (Jawanda and Bal, 1978) belongs to the Rhamnaceae family. The members of the taxon are known to be drought tolerant and very resistant to heat (Paroda and Mal, 1989). *Ziziphus* species are important versatile fruit trees in many arid countries and are planted as hedges to protect livestocks from predators (Cherry, 1985). *Ziziphus mucronata* Willd. subsp. *mucronata* Willd., also known as buffalo thorn, is a small to medium-sized tree, with a spreading canopy. It is distributed throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia. Its bark and roots are used medicinally for the treatment of various

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ailments, including rheumatism, gastrointestinal complaints, and snake bites (Tas et al., 1991). Warm bark infusions are used to relieve body pains, as expectorants for cough, against respiratory infections and for chest problems. The root infusions are used for treating gonorrhea, diarrhoea and dysentery. Decoctions of roots and leaves are applied externally to ooze boils, treat sores and glandular swellings (Venter and Venter, 2002; Amusan et al., 2005). While the fruits are sometimes sucked by small children, some rural dwellers believe that the tree serves as a protection against lightning and others cultivate this species to mark burial sites.

Although, the antimicrobial activity of some members of the *Ziziphus* genus has been reported in literatures (Sarfaraz et al., 2002; Adamu et al., 2006; Abalaka et al., 2010), there is a dearth of such information on *Z. mucronata* since the pharmacological importance of the plant has not been scientifically documented. For example, the root extract of *Z. mauritiana* was reported to be antimycobacterial (Taylor et al., 1995) while *Z. spina-christi* has been reported to have activity against bacteria and fungi (Shahat et al., 2001) including some other resistant pathogens (Nazif et al., 2002). Although, *Z. mucronata* is a South African plant used in treating infections, there is lack of scientific studies on the pharmacological importance of this plant especially antimicrobial study. The aim of this study was to investigate the antibacterial potential of the methanolic extract of the bark of *Z. mucronata* subsp. *mucronata* in order to validate its ethnomedical use in the treatment of infectious diarrhoea and dysentery.

MATERIALS AND METHODS

Plant collection and identification

The bark materials of *Z. mucronata subsp. mucronata* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University. The bark samples were air-dried at room temperature and pulverized using a milling machine before extraction. Portions of about 100 g each of the pulverized samples were extracted with methanol for 48 h. The extraction was repeated for another two consecutive periods. The extracts were filtered with Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at a maximum temperature of 40°C using a rotary evaporator. The extract was redissolved in methanol to the required concentrations for the bioassay analysis. The corresponding concentration was expressed in term of mg of extract per ml of solvent (mg/ml).

Test organisms

Ten bacteria used in this study included five Gram-positive (*Staphylococcus aureus* (ATCC 6538), *Streptococcus faecalis* (ATCC 29212), *Micrococcus luteus, Bacillus subtilits* KZN, *Staphylococcus aureus* (OK2a)) and five Gram-negative bacteria (*Escherichia coli* (ATCC 8739), *Klebsiella pneumonia* (ATCC 4352), *Proteus vulgaris* (CSIR 0030), *Shigella flexneri* KZN, *Shigella sonnei* (ATCC 29930). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organisms were maintained in nutrient broth and nutrient agar (Biolab) while Mueller Hinton II Agar (Biolab) was used for susceptibility, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay.

Antibacterial assay

The antibacterial screening was essentially by the agar dilution method described by Afolayan and Meyer (1997). The dried plant extracts were dissolved in methanol to final concentration of 50 mg/ml and sterilized by filtration through 0.45 µm millipore filters (Schleicher and Schuell, Microscience, Dassel, Germany). This was used to prepare dilutions of the extract in molten Mueller Hinton agar maintained in a water bath at 50°C to concentrations ranging between 0.1 and 10 mg/ml. The inoculum of each test strain was standardized at 5×10^6 cfu/ml using McFarland Nephe-lometer standard (NCCLS, 1993). The organisms were streaked in radial patterns on the agar plates. Two nutrient agar plates containing 5% methanol representing the final methanol concentration in the test plates without the extract served as positive controls. Another two blank plates containing only nutrient agar served as negative controls (Afolayan and Meyer, 1997). Plates were incubated under aerobic conditions at 37°C for 24 h. Each test was done in triplicate and lack of visible growth on the test plates was used to indicate the inhibitory activity of the extracts.

Determination of minimum inhibitory concentration (MIC)

The *in vitro* antibacterial activity of the extract and minimum inhibitory concentration was determined by the macrobroth dilution methods (NCCLS, 1993). Nutrient broth medium was used to prepare different concentrations ranging from 0.0195 to 10 mg/ml by serial dilutions. Each prepared concentration in tubes was inoculated with 100 µl of each of the 10^6 cfu/ml bacterial strain. Blank nutrient broth was used as negative control. The tubes were incubated aerobically at 37°C for 18 to 24 h. The first tube in the series with no sign of visible growth was taken as the MIC.

Determination of minimum bactericidal concentration (MBC)

For the determination of the MBC, one standard loopful of culture was taken from each of the first three broth tubes that showed no growth in the MIC tubes and inoculated on fresh nutrient agar plates. After incubation for 24 h, the least concentration of the extracts that showed no colony formation on the agar was taken as the MBC. In order to elucidate whether the observed antibacterial effects were bactericidal or bacteriostatic, MIC_{index} (MBC/MIC) ratios were calculated against each test strain. MBC/MIC ratios greater than 1 was considered bacteriostatic while other MBC/MIC ratios were considered bactericidal (Shanmughapriya et al., 2008).

RESULTS AND DISCUSSION

The result of the antibacterial assay indicated that methanol extract of *Z. mucronata* subsp. *mucronata* showed good antibacterial activity against both Gram-positive and Gram-negative bacteria by agar dilution method (Table 1). The result obtained from macrobroth

Table 1. Antibacterial activity of methanolic extract of the bark of *Ziziphus mucronata* subsp. *mucronata* by agar dilution method.

Organisms	Gram +/- ve	MIC (mg/ml)
Escherichia coli (ATTC 8739)	-	1
Proteus vulgaris (CSIR 0030)	-	1
Klebsiella pneumoniae (ATCC 4352)	-	0.5
Shigella flexneri (KZN)	-	2.5
Shigella sonnei (ATCC 29930)	-	1
Staphylococcus aureus (ATCC 6538)	+	2.5
Streptococcus faecalis (ATCC 29212)	+	0.5
Bacillus subtilits (KZN)	+	2.5
Micrococcus luteus	+	2.5
Staphylococcus aureus (OK2a)	+	2.5

Table 2. Antibacterial activity of methanolic extract of the bark of *Ziziphus mucronata* subsp. *mucronata* by macrobroth dilution method.

Organisms	MIC (mg/ml)	MBC (mg/ml)	MIC_{index}
Escherichia coli (ATTC 8739)	0.1562	0.6250	4
Proteus vulgaris (CSIR 0030)	0.0391	0.6250	16
Klebsiella pneumoniae (ATCC 4352)	0.3125	0.625	2
Shigella flexneri (KZN)	0.3125	0.3125	1
Shigella sonnei (ATCC 29930)	0.1562	0.6250	4
Staphylococcus aureus (ATCC 6538)	0.6250	1.2500	2
Streptococcus faecalis (ATCC 29212)	0.6250	1.2500	2
Bacillus subtilits (KZN)	0.6250	1.2500	2
Micrococcus luteus	0.1563	0.1563	1
Staphylococcus aureus (OK2a)	0.3125	0.3125	1

assay indicated that this extract exerted a high degree of inhibitory effect on these test organisms (Table 2).

However, the MIC and MBC values varied from one organism to the other. Generally, the MBC values were higher than the MIC values. The differences in the values of MICs and MBCs suggested a selective antibacterial activity of the extract. The susceptibility of all these bacteria to the methanol extract of *Z. mucronata* subsp. *mucronata* in both agar and macrobroth dilution methods were complementary. The extract exhibited the highest MIC against *Proteus vulgaris* and highest MBC on *Micrococcus luteus*. Comparatively, the Gram-negative bacteria were more susceptible to the extract than the Gram-positive strains. This is contrary to the general reports of many workers who have reported that plant extracts are more active against Gram-positive than Gram-negative bacteria (Jigna and Sumitra, 2006; Sofidiya et al., 2009; Afolayan and Ashafa, 2009). The difference in sensitivity between Gram-positive and Gram-negative bacteria to the plant extract could be ascribed to the anatomical or structural differences between these microorganisms. Gram-positive bacteria lack the normal outer membrane and the cell wall is usually much thicker than that of Gram-negative species (Archibald et al., 1993; Ghuysen and Hakenbeck, 1994).

The Gram-positive cell wall is made up of multiple layers of peptidoglycan, a polymer comprising repeating subunits of N-acetylglucosamine (NAG) and N-acetylmuranic acid (NAM) which are attached by a β(1-4) linkage, and a small string of four amino acids (L-alanine, D-glutamic acid, diaminopimelic or lysine, and D-alanine) that extend from NAM. The subunits are covalently attached by peptide glycine interbridges formed between the amino acid chains. Physiologically, the peptidoglycan in the cell wall gives shape, rigidity and protects the cell, but remains porous allowing certain biomolecules to translocate. The Gram-negative cell wall is structurally more complex than the Gram-positive cell wall. It comprises the peptidoglycan biopolymers which are only a few layers when compared to several hundreds present in Gram-positive bacteria. Gram-negative bacteria possess inner and outer cell membranes unlike the Gram-positive bacteria. The amino acid side chains of the peptidoglycan subunits are not connected by peptide interbridges but are directly covalently bonded to each other and are suspended in the periplasmic space

(Nelson et al., 2009). The cell wall in Gram-positive bacteria does not restrict the penetration of antimicrobials (Lambert, 2002). In Gram-negative bacteria, the outer membrane together with a set of multi-drug resistance pumps are effective barriers to antimicrobial compounds (Tegos et al., 2002). The extract of *Z. mucronata* could have promoted a local disturbance and the alteration of the physicochemical properties of the outer membrane, the membrane proteins and porin pathways to cause an increase in membrane permeability and the inflow of the extract (Chapple et al., 2004; Lohner and Blondelle, 2005). These events probably allowed sufficient amount of the extract to adsorb, diffuse, penetrate and interact with the target sites thereby preventing the active mechanism of resistance in the Gram-negative bacteria. This property of the extract is particularly noteworthy as it might be an indication of its broad spectrum antibacterial capability.

While Reuben et al. (2008) once stated that the MIC and MBC are often close or hooked values, El-Mahmood (2009), however, reported that the MBC values can either be the same or higher than the corresponding MIC values. Using turbidity as a measure of growth in this study, the MBC values appeared to be more reliable than the MIC values because it indicated that the extract is bactericidal. The MIC_{index} however, and expectedly, suggested that the plant extract was bacteriostatic at lower concentration and bactericidal at higher concentration. This observation is of potential importance in the health care delivery system. It showed that the plant could be used as an alternative to orthodox antibiotics in the treatment of infectious diseases caused by these microbes.

CONCLUSION

In conclusion, the activity of the extract against both Gram-negative and Gram-positive bacteria has testified to its broad spectrum antimicrobial activity. The results of this study have further revealed the great therapeutic potentials of *Z. mucronata* subsp*. mucronata* and justified its ethnobotanical use in the treatment of diarrhoeae and dysentery in this part of the world.

ACKNOWLEDGEMENT

The authors acknowledge the financial support of the National Research Foundation of South Africa and the GMRDC of the University of Fort Hare.

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CHAPTER 11

Synergistic interactions of methanolic extract of *Acacia mearnsii* De Wild with antibiotics against bacteria of clinical relevance

(*Published in the International Journal of Molecular Science*)

Int. J. Mol. Sci. **2012**, *13*, 8915-8932; doi:10.3390/ijms13078915

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Synergistic Interactions of Methanolic Extract of *Acacia mearnsii* De Wild. with Antibiotics against Bacteria of Clinical Relevance

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Received: 27 March 2012; in revised form: 27 June 2012 / Accepted: 10 July 2012 / Published: 17 July 2012

Abstract: With the emergence of multidrug-resistant organisms, combining medicinal plants with synthetic or orthodox medicines against resistant bacteria becomes necessary. In this study, interactions between methanolic extract of *Acacia mearnsii* and eight antibiotics were investigated by agar diffusion and checkerboard assays. The minimum inhibitory concentrations (MICs) of all the antibiotics ranged between 0.020 and 500 µg/mL while that of the crude extract varied between 0.156 and 1.25 mg/mL. The agar diffusion assay showed that extract-kanamycin combination had zones of inhibition ≥20 ± 1.0 mm in all the bacteria tested (100%), followed by extract-chloramphenicol (90%) > extract-ciprofloxacin = extract-tetracycline (70%) > extract-amoxicillin (60%) > extract-nalidixic acid (50%) > extract-erythromycin (40%) > extract-metronidazole (20%). The checkerboard showed synergistic interaction (61.25%), additivity/indifference (23.75%) and antagonistic (15%) effects. The synergistic interaction was most expressed by combining the extract with tetracycline, metronidazole, amoxicillin, ciprofloxacin, chloramphenicol and nalidixic acid against *E. coli* (ATCC 25922), erythromycin, metronidazole, amoxicillin, chloramphenicol and kanamycin against *S. aureus* (ATCC 6538), erythromycin, tetracycline, amoxicillin, nalidixic acid and chloramphenicol against *B. subtilis* KZN, erythromycin, metronidazole and amoxicillin against *E. faecalis* KZN, erythromycin, tetracycline, nalidixic acid and chloramphenicol against *K. pneumoniae* (ATCC 10031), erythromycin, tetracycline, metronidazole and chloramphenicol against *P. vulgaris* (ATCC 6830), erythromycin, tetracycline, amoxicillin and chloramphenicol against *S. sonnei* (ATCC 29930), metronidazole, amoxicillin and chloramphenicol against *E. faecalis* (ATCC 29212) and ciprofloxacin and chloramphenicol against *Proteus vulgaris* KZN. The synergistic interactions indicated that

the bactericidal potentials of the antibacterial agents were improved and combining natural products with antibiotic could be potential sources for resistance-modifying agents useful against infectious multi-drug resistant bacteria.

Keywords: *Acacia mearnsii*; checkerboard assay; drug-herbal interaction; multi-drug resistance; synergistic effects

1. Introduction

Infectious diseases are important causes of morbidity and mortality [1–3]. They remain the biggest killer of children and young adults as well as the second-leading cause of death worldwide [4,5] despite remarkable successes of antimicrobial drug generations of the third part of the last century. While host and pathogen interactions are complex and requires several effort to limit the spread of causal organisms [6], uncontained infections result in systemic inflammatory response syndrome that may progress into severe sepsis, septic shock, or mortality [7]. In recent years, bacterial infections caused by resistant strains have been increased dramatically [8]. Multiple drug resistance, an intrinsic and inevitable aspect of microbial survival that continually challenges human health [9], have resulted from the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases [10,11]. With the emergence of multidrug-resistant organisms, there are few or no treatment options for infections with certain microorganisms [12,13] as bacteria are resistant to 21 different antibiotics and each isolate is on average resistant to 7–8 antibiotics [14]. Under these circumstances where treatment becomes challenging, physicians have requested clinical laboratory to assess the adequacy of therapy [15–17], especially, when bactericidal antimicrobial agent therapy is considered necessary [17].

As a result, the need for new antimicrobial agents becomes greater than ever [18] and development of such agents faces significant challenges causing some pharmaceutical companies to curtail or abandon researches on anti-infective agents [19–21] while the cost of pharmaceutical research and development becomes high [22] and a number of factors make antimicrobial agents less economically attractive targets for development of other drug classes [23]. To this end, plants with related bioactive compounds varying in potency [24,25] and antimicrobial activity have attracted great attention [26–30], concurrent uses of pharmaceuticals with herbal remedies by laymen in self-medications are reported [31–33] and potential interactions of antibiotics with allopathic treatment and herbal remedies of various infections are inevitable. While the beneficial effects are expected as synergistic amplification or diminishment of possible adverse side effects, these combined bioactive compounds in plants prevented the gradual decline in efficacy frequently observed when single drugs are used over a long period of time [34]. Although South Africa's rich plant biodiversity, with over 20,000 different species, is a great source of interest to the scientific community [35] and one way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents [36] in a global village with ineffective synthetic antibiotics, investigating medicinal plants combined with synthetic or orthodox medicines against bacteria of clinical relevance might not be an invaluable effort. Medicinal plants, an indispensable part of the traditional medicine

practiced all over the world because of low costs, easy access and ancestral experience [37], have many traditional claims which included the treatment of ailments of infectious origin [38]. They are an important source of new drugs, new drug leads and new chemical entities [39] to which pathogens are not resistant [40].

The pantropical genus *Acacia* (Fabaceae) includes over 1200 species [41,42] of which *Acacia mearnsii* De Wild is a member. *A. mearnsii* is a fast growing leguminous tree indigenous to Argentina but was introduced to South Africa over 150 years ago primarily for tanning industry [43]. The ethnobotanical studies showed it is a medicinal plant essential in the treatment of microbial infections in South Africa. While there is a dearth of information on its pharmacological importance, Olajuyigbe and Afolayan [44] indicated that the ethanolic and aqueous extract of *A. mearnsii* had a significant antibacterial activity. However, as a further study in cognizance of challenges of multidrug resistant bacteria, its antibacterial effect in combination with different first-line antibiotics commonly used against infectious agents needs to be investigated. Hence, to forestall drug-herbal interaction of clinical importance and indicate the degree of efficacy of these potential combinations to avert unexpected side effects, prolonged use of antibacterial agents and development of microbial resistance, this study was aimed at investigating the influence of combining methanolic extract of *A. mearnsii* with some of the commonly used antibiotics on the susceptibility of resistant bacteria of clinical importance.

2. Results and Discussion

From this study, the extract, antibiotics and their combinations exhibited significant antibacterial activities as shown in Table 1. With the exception of metronidazole (Met) not inhibiting any of the bacteria and erythromycin (Ery) that inhibited *E. faecalis* (ATCC 29212), *K. pneumoniae* (ATCC 10031) and *S. sonnei* (ATCC 29930) only, the bacterial inhibition zones produced by the methanolic extract ranged between 15 and 19 ± 1.0 mm, those of antibiotics ranged from 13 to 31 ± 1.0 mm while those of their combinations ranged between 13 and 29 ± 1.0 mm. While amoxicillin (Amx) inhibited all the bacteria to produce zones of inhibition ranging between 13 and 30 ± 1.0 mm, tetracycline (Tet) inhibited eight of the ten bacterial isolates, except *S. aureus* (ATCC 6538) and *P. vulgaris* KZN, with zones of inhibition ranging from 19 to 22 ± 1.0 mm. The zones of inhibition from chloramphenicol (Chl), nalidixic acid (Nal), and kanamycin (Kan) ranged between 17 and 25 ± 1.0 mm and ciprofloxacin (Cip) had zones of inhibition ranging between 19 and 31 ± 1.0 mm.

Table 1. Average zones of inhibition (±1.0 mm) from AMM alone, antibiotics alone and their combinations concentrations.

	Average Zones of Inhibition (±1.0 mm) Produced by Methanolic Extract (AmM) Alone, Antibiotics Alone and Their Combinations																	
Bacteria Used	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	Q	R	S
S. aureus (ATCC 6538)	17 ± 0.58	0	18 ± 0.58	0	17 ± 0.58	0	15 ± 0.58	13 ± 0.58	18 ± 0.58	17 ± 0.00	22 ± 1.00	23 ± 0.58	17 ± 0.58	19 ± 0.58	21 ± 1.00	18 ± 0.00	25 ± 1.00	24 ± 0.58
E. faecalis (ATCC 29212)	15 ± 0.58	22 ± 0.58	23 ± 0.00	19 ± 0.00	20 ± 0.58	0	16 ± 0.58	20 ± 0.58	18 ± 1.00	15 ± 0.58	20 ± 1.00	19 ± 0.58	17 ± 1.00	18 ± 0.58	23 ± 1.00	20 ± 1.00	21 ± 0.58	22 ± 0.58
E. coli (ATCC 25922)	16 ± 0.00	0	18 ± 0.58	20 ± 0.58	22 ± 0.58	0	15 ± 0.58	22 ± 1.00	20 ± 0.58	19 ± 0.58	30 ± 1.00	27 ± 0.58	25 ± 0.00	29 ± 0.58	22 ± 0.00	21 ± 0.58	20 ± 1.00	21 ± 0.58
B. subtilis KZN	18 ± 0.00	0	20 ± 0.58	19 ± 0.58	21 ± 0.58	0	19 ± 0.58	24 ± 1.53	16 ± 0.00	18 ± 0.58	26 ± 1.00	29 ± 0.58	20 ± 0.58	22 ± 0.58	17 ± 0.58	20 ± 0.58	22 ± 0.58	21 ± 0.58
P. vulgaris KZN	16 ± 1.00	0	18 ± 0.58	0	19 ± 0.00	0	15 ± 1.00	30 ± 1.53	25 ± 1.00	17 ± 0.58	27 ± 0.58	25 ± 0.00	21 ± 0.58	19 ± 0.00	23 ± 0.58	24 ± 0.58	25 ± 0.58	26 ± 0.00
E. faecalis KZN	18 ± 0.58	0	18 ± 0.00	21 ± 0.58	24 ± 0.58	0	20 ± 0.58	23 ± 0.58	22 ± 0.00	18 ± 0.00	30 ± 1.00	29 ± 0.58	24 ± 0.58	25 ± 0.58	23 ± 0.58	21 ± 0.58	22 ± 0.58	25 ± 0.58
E. cloacae (ATCC 13047)	17 ± 0.00	0	17 ± 1.15	17 ± 1.52	19 ± 0.58	0	13 ± 0.58	19 ± 1.15	22 ± 1.00	16 ± 1.00	27 ± 1.53	24 ± 0.58	22 ± 0.58	20 ± 0.58	22 ± 0.58	20 ± 0.58	22 ± 0.58	21 ± 1.00
K. pneumoniae (ATCC 10031)	17 ± 0.58	23 ± 0.58	23 ± 1.53	20 ± 1.00	22 ± 0.58	0	17 ± 0.58	18 ± 0.58	18 ± 1.15	17 ± 0.00	19 ± 1.53	22 ± 0.58	18 ± 0.00	19 ± 0.58	21 ± 0.58	22 ± 0.58	21 ± 0.58	24 ± 1.00
P. vulgaris (ATCC 6830)	18 ± 0.00	0	19 ± 0.00	21 ± 1.53	23 ± 1.15	0	15 ± 0.58	17 ± 0.58	21 ± 0.58	18 ± 0.00	22 ± 1.00	17 ± 0.58	19 ± 0.58	19 ± 0.58	23 ± 0.58	20 ± 0.58	21 ± 0.00	21 ± 1.15
S. sonnei (ATCC 29930)	19 ± 0.58	22 ± 1.00	23 ± 0.58	22 ± 1.53	23 ± 0.58	0	21 ± 0.58	21 ± 0.58	22 ± 0.58	19 ± 0.58	20 ± 0.58	18 ± 0.00	19 ± 0.58	21 ± 0.58	24 ± 1.00	21 ± 0.58	22 ± 0.00	23 ± 0.00

A = Methanol extract (AmM) (10 mg/mL); B = Erythromycin (Ery) (25 µg/mL); C = AmM (10 mg/mL) + Ery (25 µg/mL); D = Tetracycline (Tet) (62.5 µg/mL); E = AmM (10 mg/mL) + Tet (62.5 µg/mL); F = Metronidazole (Met) (125 µg/mL); G = AmM (10 mg/mL) + Met (125 µg/mL); H = Amoxicillin (Amx) (62.5 µg/mL); I = AmM (10 mg/mL) + Amx (62.5 µg/mL); J = Methanol extract (AmM) (10 mg/mL); K = Ciprofloxacin (Cip) (2.5 µg/mL); L = AmM (10 mg/mL) + Cip (2.5 µg/mL); M = Nalidixic acid (Nal) (125 µg/mL); N = AmM (10 mg/mL) + Nal (125 µg/mL); P = Chloramphenicol (Chl) (25 µg/mL); Q = AmM (10 mg/mL) + Chl (25 µg/mL); R = Kanamycin (Kan) (125 µg/mL); S = AmM (10 mg/mL) + Kan (125 µg/mL).

All the antibiotics and their combinations with the extract were more active than the extract. Apart from *S. aureus* (ATCC 6538), *P. vulgaris* (ATCC 6830) and bacteria not inhibited by metronidazole (Met) and erythromycin (Ery), the susceptibility of other bacteria indicated that zones of inhibition from antibiotics were wider in size than those of methanolic extract. The zones of inhibition produced by the antibacterial combinations, however, varied in size and were mostly wider than those obtained from either the extract or the antibiotics. In all the bacteria tested, extract combined with erythromycin (AE) had average zones of inhibition of 18 to 23 ± 1.0 mm. Extract combined with tetracycline (AT) had zones of inhibition ranging between 17 and 24 ± 1.0 mm. While that of extract combined with metronidazole (AM) ranged 15 to 21 ± 1.0 mm, those of extract combined with the amoxicillin (AA) were from 16 to 25 ± 1.0 mm. In the combined extract and erythromycin (AE), *B. subtilis* KZN, *E. faecalis* (ATCC 29212), *K. pneumoniae* (ATCC 10031) and *S. sonnei* (ATCC 29930) had the widest zones of inhibition equal to or greater than 20 ± 1.0 mm, others were less than 20 ± 1.0 mm. In extract combined with tetracycline (AT), *S. aureus* (ATCC 6538), *P. vulgaris* KZN and *E. cloacae* (ATCC 13047) had zones of inhibition less than 20 ± 1.0 mm, others had theirs being equal to or greater than 20 ± 1.0 mm. While *E. faecalis* KZN and *S. sonnei* (ATCC 29930) had zones of inhibition higher than 20 ± 1.0 mm in extract combined with metronidazole (AM), *E. coli* (ATCC 25922), *P. vulgaris* KZN, *E. faecalis* KZN, *E. cloacae* (ATCC 13047), *P. vulgaris* (ATCC 6830) and *S. sonnei* (ATCC 29930) had their zones of inhibition higher than 20 ± 1.0 mm in the combination of extract and amoxicillin (AA). AC exhibited highest antibacterial activity with the biggest zones of inhibition sizes, followed by AK, while other combinations exhibited varied degree of antibiotics activity. Based on the percentage of the number of bacteria with zones of inhibition equal to or greater than 20 ± 1.0 mm, the effect of combining the extract with the different antibiotics showed the following order: AK (100%) > ACh (90)% > AC = AT (70%) > AA (60%) > AN (50%)> AE (40%) > AM (20%).

In comparison to other combinations against a particular bacterium, AK was the most active against *S. aureus* (ATCC 6538), *P. vulgaris* KZN, *K. pneumoniae* (ATCC 10031), *P. vulgaris* (ATCC 6830) and *S. sonnei* (ATCC 29930). AC was the most active against *E. coli* (ATCC 25922), *B. subtilis* KZN, *E. cloacae* (ATCC 13047) and *E. faecalis* KZN. While the activity of AC was greater than those of AK, AE produced the highest inhibitory effect against *E. faecalis* (ATCC 29212) and AM produced the least inhibitory effect against the highest number of the test isolates. Of all the bacteria tested, *B. subtilis* KZN and *E. faecalis* KZN were the most inhibited bacteria by the AC. *E. faecalis* KZN was the most inhibited bacteria by the AN, AT and AM combinations. While *P. vulgaris* KZN was the most inhibited by AA, ACh and AK combinations, *E. faecalis* (ATCC 29212), and *K. pneumoniae* (ATCC 10031) and *S. sonnei* (ATCC29930) were the most inhibited bacteria by the AE combination.

Distinguishing synergistic from antagonistic interactions is of major importance for the development of improved strategies for the management of microbial infections. The *in vitro* antibacterial activity of the extract and the different antibiotics combinations was further assessed on the basis of the fractional inhibitory concentration (FIC) index representing the sum of the FICs (∑FICs) of each drug tested, where the FIC is determined for each drug by dividing the MIC of each drug when used in combination by the MIC of each drug when used alone. According to these MIC breakpoints of CLSI [45], the bacteria were classified as being susceptible, intermediate and resistant based on their susceptibility to each test antibiotic. The results showed that the MICs of the crude extract varied between 0.156 and 1.25 mg/mL and those of the antibiotics varied between 0.020 and 500 µg/mL

(Table 2). When the evaluation criteria of Eliopoulos and Eliopoulos [46], Giertsen *et al.*, [47], Grytten *et al.* [48], Isenberg [49], Bhusal *et al.* [50], Petersen *et al.* [51] and Kamatou *et al.* [52] were applied to the results obtained and antibacterial combination resulting in $\sum FIC \leq 1.0$ as synergism, $1.0 < \sum FIC \leq 4$ as indifference and $\sum FIC > 4$ as antagonism were considered, the antibacterial combinations showed synergistic interactions (61.25%), additivity/indifference (23.75%) and antagonism (15.0%) against the test organism. Considering synergism as $\sum FIC \leq 0.5$, additivity/indifference as $5 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$, the extract-antibiotics combinations indicated synergism (20%), additivity/indifference (71.25%) and antagonism (8.75%) (Table 3). The number of synergistic interaction between the AmM and the antibiotics against different bacteria in each treatment is in the following order: ACh > AA > AM ≥ AE > AT > AC = AN > AK. That is, ACh was synergistic against more bacteria, followed by AA, while AK was synergistic against the least number of the bacterial isolates. While AK had additive/indifferent effect against eight of the ten organisms, followed by AN and AT, others showed varied degree of additive/indifferent effects against different numbers of test bacteria. On the other hand, synergistic interaction was most expressed against *E. coli* (ATCC 25922) by AT, AM, AA, AC, ACh and AN, *S. aureus* (ATCC 6538) by AE, AM, AA, ACh and AK, *B. subtilis* KZN by AE, AT, AA, AN and ACh, *E. faecalis* KZN by AE, AM and AA, *K. pneumoniae* (ATCC 10031) by AE, AT, AN and ACh, *P. vulgaris* (ATCC 6830) by AE, AT, AM and ACh, *S. sonnei* (ATCC 29930) by AE, AT, AA and ACh, *E. faecalis* (ATCC 29212) by AM, AA and ACh, *Proteus vulgaris* KZN by AC and ACh. The differences in the resultant synergistic, indifferent and antagonistic interactions were due to the elevated MIC values obtained from the resistance of these bacteria to some of the antibiotics.

Due to the emergence of multidrug-resistant pathogens, treatment with antibacterial combinations, using two or more antibacterials has become commonplace [53]. In search of more effective chemotherapeutic agents for treating microbial infections, combination therapy becomes an important strategy as synergistic interactions can potentially increase efficacy, reduce toxicity, cure faster, prevent the emergence of resistance, and provide broader-spectrum of activity than monotherapy regimens [54]. It has been shown to be beneficial for several difficult-to-treat infections such as human immunodeficiency virus and mycobacterial infections, which do not respond well to single-drug therapy due to either lack of efficacy or rapid emergence of resistance [55]. Since drug-drug combinations are convenient models of additivity, their experimental properties can, then, give valuable insights into the significance of synergistic and antagonistic interactions of dissimilar drugs [56,57]. Hence, it is important to interact the first line antibiotics, used in this study, with medicinal plant materials in order to identify synergistic and/or antagonistic effects able to provide guidance for empirical use, reduce cost and duration of antimicrobial therapy.

Table 2. The minimum inhibitory concentrations (MICs) of the extract and the antibiotics used.

	Minimum Inhibitory Concentrations								
	AmM	**Ery**	**Tet**	**Met**	**Amx**	**Cip**	**Nal**	**Chl**	**Kan**
	(mg/mL)	**(μg/mL)**							
S. aureus (ATCC 6538)	0.313	6.250 (R)	7.813 (R)	15.625 (R)	31.250 (R)	0.020 (S)	31.250 (R)	1.953 (S)	1.953 (S)
E. faecalis (ATCC 29212)	1.250	0.195 (S)	3.906 (S)	31.250 (R)	3.906 (S)	0.313 (S)	31.250 (R)	1.953 (S)	125.000 (R)
E. coli (ATCC 25922)	0.313	1.563 (R)	1.953 (S)	31.250 (R)	7.813 (I)	0.039 (S)	1.953 (S)	3.906 (S)	125.000 (R)
B. subtilis KZN	1.250	6.250 (R)	0.977 (S)	31.250 (R)	62.500 (R)	0.020 (S)	7.813 (S)	3.906 (S)	3.906 (S)
P. vulgaris KZN	0.313	12.500 (R)	7.813 (R)	62.500 (R)	0.488 (S)	0.313 (S)	62.500 (R)	0.977 (S)	7.8125 (S)
E. faecalis KZN	0.156	12.500 (R)	31.250 (R)	15.625 (R)	0.976 (S)	0.313 (S)	62.500 (R)	31.250 (R)	250.000 (R)
E. cloacae (ATCC 13047)	0.625	6.250 (R)	15.625 (R)	31.250 (R)	500.000 (R)	0.156 (S)	62.500 (R)	1.953 (S)	62.500 (R)
K. pneumoniae (ATCC 10031)	0.313	0.195 (S)	0.488 (S)	31.250 (R)	0.977 (S)	0.039 (S)	3.906 (S)	1.953 (S)	15.625 (S)
P. vulgaris (ATCC 6830)	0.313	25.000 (R)	15.625 (R)	62.250 (R)	250.000 (R)	0.156 (S)	1.953 (S)	7.813 (I)	31.250 (R)
S. sonnei (ATCC 29930)	0.156	0.391 (S)	1.953 (S)	62.250 (R)	500.000 (R)	0.020 (S)	15.563 (I)	1.953 (I)	31.250 (R)

Table 3. Fractional inhibitory concentrations of different combination of the extract and the antibiotics.

	Fractional Inhibitory Concentration			**Remarks**	**Fractional Inhibitory Concentration**			**Remarks**
	FICI (AmM)	**FICI (Ery)**	**FICI index**		**FICI (AmM)**	**FICI (Tet)**	**FICI index**	
S. aureus (ATCC 6538)	0.5	0.25	0.75	Synergy	0.5	0.5	1	Indifference
E. faecalis(ATCC 29212)	0.0156	1	1.0156	Indifference	0.125	1	1.125	Indifference
E. coli (ATCC 25922)	0.25	0.5	0.75	Synergy	0.0624	0.25	0.3125	Synergy
B. subtilis KZN	0.25	0.5	0.75	Synergy	0.0156	0.5	0.5156	Synergy
P. vulgaris KZN	1	0.25	1.25	Indifference	0.5	0.5	1	Indifference
E. faecalis KZN	0.5	0.0625	0.5625	Synergy	2	0.5	2.5	Indifference
E. cloacae (ATCC 13047)	0.5	0.5	1	Indifference	0.5	0.5	1	Indifference
K. pneumoniae (ATCC 10031)	0.0312	0.5	0.5312	Synergy	0.0156	0.25	0.2656	Synergy
P. vulgaris (ATCC 6830)	0.5	0.0625	0.5625	Synergy	0.5	0.25	0.75	Synergy
S. sonnei (ATCC 29930)	0.015625	0.0625	0.0781	Synergy	0.0312	0.0625	0.0937	Synergy

Table 3. *Cont.*

	Fractional Inhibitory Concentration			Remarks	Fractional Inhibitory Concentration			Remarks
	FICI (AmM)	FICI (Met)	FICI index		FICI (AmM)	FICI (Amx)	FICI index	
S. aureus (ATCC 6538)	0.5	0.25	0.75	Synergy	0.5	0.125	0.625	Synergy
E. faecalis (ATCC 29212)	0.125	0.125	0.25	Synergy	0.0078	0.0625	0.0703	Synergy
E. coli (ATCC 25922)	0.25	0.0625	0.3125	Synergy	0.25	0.25	0.5	Synergy
B. subtilis KZN	0.5	0. 5	1.0	Indifference	0.5	0.25	0.75	Synergy
P. vulgaris KZN	1	0.25	1.25	Indifference	0.125	2	2.125	Indifference
E. faecalis KZN	0.5	0.125	0.625	Synergy	0.0625	0.25	0.3125	Synergy
E. cloacae (ATCC 13047)	0.5	0.25	0.75	Synergy	0.5	0.015625	0.5156	Synergy
K. pneumoniae (ATCC 10031)	1	0.25	1.25	Indifference	0.25	2	2.25	Indifference
P. vulgaris (ATCC 6830)	0.5	0.0625	0.5625	Synergy	1	0.03125	1.03125	Indifference
S. sonnei (ATCC 29930)	2	0.25	2.25	Indifference	0.015625	0.000122	0.0157	Synergy
	Fractional Inhibitory Concentration			**Remarks**	**Fractional Inhibitory Concentration**			**Remarks**
	FICI (AmM)	**FICI (Cip)**	**FICI index**		**FICI (AmM)**	**FICI (Nal)**	**FICI index**	
S. aureus (ATCC 6538)	0.25	4	4.25	Antagonistic	1	0.25	1.25	Indifference
E. faecalis (ATCC 29212)	0.25	1	1.25	Indifference	0.5	0.5	1.0	Indifference
E. coli (ATCC 25922)	0.0625	0.5	0.5625	Synergy	0.125	0.5	0.625	Synergy
B. subtilis KZN	0.0625	4	4.0625	Antagonistic	0.3125	0.125	0.4375	Synergy
P. vulgaris KZN	0.25	0.25	0.5	Synergy	2	0.25	2.25	Indifference
E. faecalis KZN	1	0.5	1.5	Indifference	4	0.25	4.25	Antagonistic
E. cloacae (ATCC 13047)	0.125	0.5	0.625	Synergy	1	0.25	1.25	Indifference
K. pneumoniae (ATCC 10031)	0.25	2	2.25	Indifference	0.25	0.5	0.75	Synergy
P. vulgaris (ATCC 6830)	0.5	1	1.5	Indifference	1	4	5	Antagonistic
S. sonnei (ATCC 29930)	2	16	18	Antagonistic	2	0.5	2.5	Indifference
	Fractional Inhibitory Concentration			**Remarks**	**Fractional Inhibitory Concentration**			**Remarks**
	FICI (AmM)	**FICI (Chl)**	**FICI index**		**FICI (AmM)**	**FICI (Kan)**	**FICI index**	
S. aureus (ATCC 6538)	0.125	0.5	0.625	Synergy	0.125	0.5	0.625	Synergy
E. faecalis (ATCC 29212)	0.03125	0.5	0.53125	Synergy	1	0.25	1.25	Indifference
E. coli (ATCC 25922)	0.125	0.25	0.375	Synergy	1	0.0625	1.0625	Indifference
B. subtilis KZN	0.015625	0.125	0.140625	Synergy	0.125	1	1.125	Indifference
P. vulgaris KZN	0.0625	0.5	0.5625	Synergy	0.5	0.5	1.0	Indifference
E. faecalis KZN	2	0.25	2.25	Indifference	8	0.125	8.125	Antagonistic
E. cloacae (ATCC 13047)	0.5	4	4.5	Antagonistic	2	0.5	2.5	Indifference
K. pneumoniae (ATCC 10031)	0.125	0.5	0.625	Synergy	1	0.5	1.5	Indifference
P. vulgaris (ATCC 6830)	0.25	0.25	0.5	Synergy	1	0.25	1.25	Indifference
S. sonnei (ATCC 29930)	0.125	0.25	0.375	Synergy	2	0.25	2.25	Indifference

Consequently, to identify synergistic or antagonistic antibacterial combinations capable of providing empirical use and treatment of poly-microbial infections where combination therapy is required [53], *in vitro* combination of plant materials with different antibiotics had been investigated against potential pathogens. As a result, several studies [58–62] showed that there are varied interactions between plant extracts and antibiotics. In agreement with these studies, our study demonstrated synergism, additivity/indifference and antagonism between methanolic extract of *Acacia mearnsii* and different classes of first line antibiotics. The synergistic effects indicated that the antibacterial combinations were more effective than the activity of the individual agents. The increase in the sizes of the zones of inhibition and the decreased MICs resulting from the extract and antibiotic combinations indicated the improved bactericidal potentials of the extract and the antibiotics as combined antibacterial agents. These variations were adjudged by Pei *et al.* [63] to have resulted from differences in mechanisms of action and variation of combined antibacterial action in the tested bacteria.

Although the extract combined with antibiotics, having different target sites, exhibit varied degree of antibacterial activity, the ability of these antibacterial combinations to inhibit resistant bacteria showed that they have either attacked different target sites singly or combined to overcome inherent resistant mechanisms in the isolates. One of the phytoconstituents, such as flavonoids or the polyphenols, may have interacted with the antibiotics to enhance its mechanisms of action at the target sites for which the antibiotic was designed. Since Cushnie *et al.* [64] indicated synergy between flavonoids and chemotherapeutics and Sato *et al.* [65] and Cushnie *et al.* [64] reported antimicrobial and resistance modulating potentials of flavonoids and polyphenols, combining the methanolic extract with the antibiotics could have altered the inherent resistant properties in the bacteria to be more effective [65–68]. In addition, the initial role of each phytoconstituent in antimicrobial activity and in their combination with antibiotics may not be underestimated. Aromatic planar quaternary alkaloids in extracts interchelate with DNA [69]. Lipophilic flavonoids disrupt microbial membranes [70]. Tannins precipitate microbial protein [71]. Saponins having detergent properties serve as lytic agents [72]. These phytochemicals in the extract could have acted singly or synergistically with other compounds for effective antibacterial activities. While the double attack of extracts with antibiotics on different target sites was noted [73], the phytoconstituents coupled with the antibiotics targeting specific sites could have formed a complex with enhanced antibacterial properties. On the other hand, the cell membrane, vital for any microorganism, is the primary target for most antibacterial agents [74]. Against resistant bacteria, a component of the phytoconstituents could have acted singly to cause membrane disruption or translocate through the membrane to a target receptor inside the cell to pave ways for each of these antibiotics to reach their target sites of action to upshot their concerted activities [75] while blocking the inhibitory effects of protective enzymes [76–78]. While Zhao *et al.* [79] reported that some phytochemicals can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by attacking the same site in the cell wall, Esinome *et al.* [73] showed that polyphenols couple with β-lactams could enhance antibacterial activity to disrupt transpeptidation of the cell membrane. While these may account for the varied degree of antibacterial activities of the different combinations, it could account for the differences in the rate at which the antibiotics get to their target sites.

Though efflux pumps have been associated with resistance mechanisms in bacteria, resistance modifying agents [80] and the ability of combinations between extracts and antibiotics [78,81] to

inhibit these resistance mechanisms [82] have been reported. In agreement with these earlier reports, the ability of methanolic extract combined with each antibiotic to inhibit bacteria that exhibited low susceptibility and high MICs showed that these combinations could mediate in bacterial resistance and serve as a source of plant derived natural products with antibiotic resistance-modifying activity to be used against multi-drug resistant bacteria in hospital and community acquired infections.

3. Experimental Section

3.1. Collection of Plant Material

The bark materials of *Acacia mearnsii* De Wild were collected in August 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen (OLAJ Med 2010/01) was prepared and deposited in the Griffen Herbarium of the University.

3.2. Extract Preparation

The bark sample was air-dried at room temperature and pulverized using a milling machine. The extract of the bark material was prepared in accordance to the description of Basri and Fan [83]. About 100 g of the pulverized sample was extracted with 500 mL of methanol for 48 h with shaking (Stuart Scientific Orbital Shaker, UK). The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40 °C using a rotary evaporator (Laborota 4000 efficient, Heldolph, Germany). The crude extract collected was allowed to dry at room temperature to a constant weight of 18.7 g. The extract was redissolved in dimethylsulfoxide (DMSO) to the required concentrations for bioassay analysis.

The reconstituted extract solution was sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 mL of the extract into 10 mL of sterile nutrient broth before being incubated at 37 °C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

3.3. Bacterial Strain

The bacteria used in this study included *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Baciilus subtilis* KZN, *Proteus vulgaris* KZN, *Enterococcus faecalis* KZN, *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (ATCC 6830) and *Shigella sonnei* (ATCC 29930). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The antibacterial assays were carried out using Mueller-Hinton II Agar (Biolab) and broth. The inocula of the test bacteria were prepared using the colony suspension method [84]. Colonies picked from 24 h old cultures grown on nutrient agar were used to make suspensions 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 transferring 0.1 mL of the bacterial suspension to 9.9 mL of sterile nutrient broth before being used.

3.4. Antibiotics Used in This Study

Antibiotic powders of amoxicillin, chloramphenicol, ciprofloxacin, erythromycin, tetracycline hydrochloride, metronidazole, kanamycin and nalidixic acid were used. Stock antibiotic solutions were prepared and dilutions made according to the CLSI (Clinical Laboratory Standardization Institute) method or manufacturer's recommendations [85,86].

3.5. Antibiotic Susceptibility Testing—Agar Diffusion Method

Each of the isolates was standardized using colony suspension method. Each strain's suspension was matched with 0.5 McFarland standards to give a resultant concentration of 1.5×10^8 cfu/mL. The antibiotic susceptibility testing was determined using the modified Kirby–Bauer diffusion technique [87] by swabbing the Mueller-Hinton agar (MHA) (Oxoids UK) plates with the resultant saline suspension of each strain. Wells were then bored into the agar medium with heat sterilized 6 mm cork borer. The wells were filled with 100 μL of different concentrations prepared for the methanolic extract alone, antibiotics alone and their combinations taking care not to allow spillage of the solutions onto the surface of the agar. The plates were allowed to stand for at least 30 min before being incubated at 37 °C for 24 h [88]. The determinations were done in duplicate. After 24 h of incubation, the plates were examined for zones of inhibition [89]. The diameter of the zones of inhibition produced by the extract alone, antibiotic alone and their combinations were measured and interpreted using the CLSI zone diameter interpretative standards [45].

3.6. Determination of Minimal Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) for the extract and the antibiotics under study were determined in duplicate by the macrobroth dilution method in Mueller-Hinton broth according to CLSI (Clinical Laboratory Standardization Institute) [86]. To determine the MICs of each antibiotic, the concentrations used for each of the antibiotics (0.0019–500) μg/mL and those of extract (0.078125–5) mg/mL were prepared by serial dilution in Mueller-Hinton broth. To determine their combinatorial effects, combinations of different concentrations ranging from $^1/_2\times$ MIC to 8× MIC of each of the antibiotics and the extract were used. The tubes were inoculated with 100 μL of each of the bacterial strains. Blank Mueller-Hinton broth was used as negative control. The bacterial containing tubes were incubated aerobically at 37 °C for 24 h. Each combination assay was performed two times. The MIC was defined as the lowest dilution that showed no growth in the Mueller-Hinton broth.

3.7. Checkerboard Assay

The interactions between the extract and the antibiotics were determined using the checkerboard as previously described [51]. The range of drug concentration used in the checkerboard assay was such that the dilution range encompassed the MIC for each drug used in the analysis. The fractional inhibitory concentration (FIC) was derived from the lowest concentrations of the extract and the antibiotics in combination permitting no visible growth of the test organisms in the Mueller-Hinton broth after incubation for 24 h at 37 °C [90]. FIC indices were calculated using the formula: FIC index = (MIC of extract in combination/MIC of extract alone) + (MIC of antibiotics in combination/MIC of

antibiotics alone). In antimicrobial combination, Eliopoulos and Eliopoulos [46], Isenberg [47] and Petersen *et al.* [51] defined synergy as $\sum FIC \leq 0.5$, additivity as $5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$. Giertsen *et al.* [47], Grytten *et al*. [48], and Kamatou *et al.* [52] defined synergy to occur when $\sum FIC < 1.0$, additivity when $\sum FIC = 1.0$ and antagonism when $\sum FIC > 1.0$. Bhusal *et al.* [50] also showed synergism as $\sum FIC \leq 0.75$, indifference as $0.75 < \sum FIC \leq 4$, and antagonism as $\sum FIC > 4$. This implies that synergy by the checkerboard method may be defined as $FIC \leq 0.5$ or $FIC \leq 1$.

4. Conclusions

The combinations of antibacterial agents demonstrating *in vitro* synergism against infectious agents are most likely to be a means of achieving pragmatic and effective treatment for bacterial infection especially in patients with infections difficult to treat. Since the development of new classes of antibacterial agents is of paramount importance, the crude methanolic extract of *A. mearnsii* showed potential synergy on being combined with some antibiotics against resistant bacteria of clinical importance and suggested that varied degree of effective therapy will be more attained with these antibacterial combinations. Hence, assessing the therapeutic potentials of this plant allows us to know how best it can be used in the treatment of diseases, especially, when the synergistic competency between plants and standard antibiotics is highly required to complement effective therapy. This work has indicated the potential of this plant as a source of resistance modulating and novel chemotherapeutic agents for the production of synthetically improved therapeutic agents that could be combined with antibiotics to enhance their antimicrobial activities. Further works including the isolation of the bioactive phytochemical compounds, investigation of the mechanisms of actions and *in vivo* studies to validate the therapeutic potentials of these combinations are ongoing in our Research Centre.

Acknowledgments

The authors wish to acknowledge the financial support of the National Research Foundation of South Africa and the University of Fort Hare, South Africa.

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CHAPTER 12

Mechanisms of Action, Ultrastructure and X-ray microanalysis of the stem bark extract of *Acacia mearnsii* against some selected bacteria

(*Submitted to the BMC Complementary and Alternative Medicine in this format*)

Mechanisms of Action, Ultrastructure and X-ray microanalysis of the effects of stem bark extract of *Acacia mearnsii* De Wild against some selected bacteria

Abstract

The significance of medicinal plants as unique sources of antimicrobial agents has been established through many *in vitro* studies. However, determining their mechanisms of action becomes relevant to establish their pharmacological roles in antimicrobial therapy. In this study the possible mechanisms of action of ethanol extract of *A. mearnsii* were investigated. The scanning electron microscopy indicated varied ultrastructural changes in the morphology of bacterial cells treated with the ethanol extract. The X-ray microanalysis showed significant differences between the elemental contents of extract-treated and untreated bacteria while lipids and proteins were leaked to a great extent from extract-treated bacterial strains in comparison with the untreated ones. The study suggests that the possible mechanisms of action of the ethanol extract of *A. mearnsii* may include inhibition of a significant step in peptidoglycan assembly, inhibition of metabolic processes, as well as the cell wall and cell membrane disruptions resulting in the efflux of lipid and protein in all the bacteria tested. The physiological damages to the cell wall, however, justify the bactericidal ability of the extract.

Keywords: Scanning electron microscopy, microanalysis, elemental contents, mechanism of action

Introduction

Medicinal plants are of significant importance because of their roles in the treatment of microbial infections. They have provided a good source of anti-infective agents and many of them remain highly effective in the fight against microbial infections. Their antibacterial activities as indicated by their minimum inhibitory concentration (MIC_{index}) index against any give pathogen are important in defining the nature of their antibacterial effects. The antibacterial effects, however, depend on the nature and types of phytochemicals present in the plant materials, extract preparation, solvent used and the susceptibility of test bacteria (Loziene et al., 2007) while the variation in different bacterial susceptibility depended on the presence of intrinsic levels of tolerance to antimicrobials in the tested microorganisms (Ahmad and Aqil, 2007) and the structural physiology of the bacterial cell wall.

Acacia mearnsii De Wild. (Fabaceae) is a member of the genus Acacia. It is considered a wild and a notorious plant because of its ability to compete with indigenous plants and populate a large expanse of land sporadically. While it was introduced into South Africa over 150 years ago basically for its high tannin contents (Young et al., 1986), there have been a dearth of scientific reports indicating its pharmacological importance. However, recently, Olajuyigbe and Afolayan, (2011, 2012a, b) reported that *A. mearnsii* is a medicinal plant of ethnobotanical and pharmacological importance. Sequel to these earlier reports indicating significant antibacterial activities, investigating the mechanisms of action of *A. mearnsii* becomes imperative to establish the means through which it produces an effect on different bacterial strains. Hence, this study was designed to determine the possible mechanisms of action and investigate the ultrastructure and X-ray microanalysis of the effects of its ethanol extract on some selected bacteria.

Materials and methods

The bacteria used for the study

The bacteria used in this study included *Escherichia coli* (ATCC 8739), *Shigella flexneri* (KZN), *Proteus vulgaris* (ATCC 6830), *Staphylococcus aureus* (ATCC 6538) and *Bacillus pumilis* (ATCC 14884). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa.

Collection of plant material

The bark materials of *the Acacia mearnsii De Wild* were collected in August, 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen (OLAJ Med 2010/01) was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark sample was air-dried at room temperature and pulverized using a milling machine. The extract of the bark material was prepared in accordance with the description of Basri and Fan (2005). About 100 g of the pulverized sample was extracted with 500 ml of ethanol for 48 h with shaking (Stuart Scientific Orbital Shaker, UK). The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40^{o}C using a rotary evaporator (Laborota 4000 – efficient, Heldolph, Germany). The crude extract collected was allowed to dry at room temperature to a constant weight. The extract was redissolved in dimethylsulfoxide (DMSO) before being diluted with sterile distilled water to the required concentrations for bioassay analysis.

The reconstituted extract solution was sterilized by filtering through 0.45 μm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract into 10 ml of sterile nutrient broth before being incubated at 37^{o}C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

Determination of the effect of ethanolic extract on cell morphology

Overnight broth cultures of the different bacteria strains were standardized at 10^{6} cfu/ml using McFarland Nephe-lometer standard (NCCLS, 1993). One milliliter of each of the adjusted bacterial culture was added to 9 ml of the plant extract at 4 x MIC and kept at laboratory temperature (28^{o}C) for 2 h. Untreated controls were prepared in Mueller Hinton broth medium and incubated for the same period of time. The bacterial cells were harvested by centrifuging at 10000 rpm for 10 mins and subsequently washed with phosphate buffer (pH 7.2). The bacterial pellets were re-suspended in 2.5% glutaraldehyde and kept for 2 h to fix the bacterial cell. The fixed bacterial cell suspensions were centrifuged at 10000 rpm for 10 min, washed and re-suspended in buffer (pH 7.2) from where the cells were then deposited on a 0.45-μm-pore-size membrane filter (Schleicher & Schuell, Dassel, Germany). The bacterial cells on the membrane filters were dehydrated with a graded series of acetone/water washes (20%, 50%, 70%, 90% and 100% acetone) to gradually remove water from the cells without physical damage. The dehydrated bacterial cells were mounted on a stub, allowed to dry before being sputtered with a small amount of gold/palladium (Ione coater: EIKO IB.3) to avoid charging in the microscope. Microscopy was performed with a JEOL JSM-6390LV Scanning Electron Microscope (Japan). Secondary electron images were taken with an accelerating voltage of 15 kV.

Energy Dispersive spectroscopy (EDS) of the bacterial cells

To determine the influence of the extract on the elemental components of the bacteria cells, a beam of electrons was focused on both extract-treated and extract-untreated bacteria cells to scan the cells at the point where examination of its chemical composition was desired. The detection and determination of elements with the EDS was based on the emission of characteristic X-rays by the bacteria cells under bombardment with electrons using JEOL JSM-6390LV Scanning Electron Microscope equipped with Electron Dispersive Spectroscope. The dispersed spectra produced a pattern of X-rays characteristic of the element excited. Only the most intense emissions, the so-called K′ and Kα lines, were analyzed with the spectrometers (Figueras and Guarro, 1997).

Determination of Lipid leakage from bacteria cells

Phospho-vanillin was prepared as previously described by Frings and Dunn (1970). 0.6 g of Vanillin (Sigma Chemical Co., St. Louis, Mo. 63118) was dissolved in 10 ml of absolute ethanol before diluting to 100 ml with distilled water. This solution was mixed with 400 ml of concentrated phosphoric acid with constant stirring before being stored at room temperature in a brown bottle. The lipid leakage assay was carried out using the method described by Van Handel and Day (1988) and Kaufmann and Brown (2008). Briefly, bacterial cells were harvested from an overnight broth culture by centrifuging at 10000 rpm for 10 min and used to prepare 10^8 cfu/ml. The standardized cell suspension was treated with 1 x MIC and 2 x MIC of the extract and the samples were incubated at 37^oC for 120 min. After incubating at 37^oC for 30 min, each cell suspension was sampled at 30 min interval and centrifuged at 10000 rpm for 10 min. 0.2 ml of sulfuric acid was added to a replicated small portion of the supernatant (10 µl) in tubes and

heated in water bath for 10 min at 100^{o}C. Vanillin-phosphoric acid reagent was added to the mixture before being removed from water bath, vortexed and allowed to cool while the optical density was measured at 525 nm. The concentration of lipid leakage was estimated from linoleic acid standard curve used as standard.

Determination of Protein leakage from bacteria cells

To determine the leakage of intracellular materials from the cells, the bacteria cells were treated with the 1 x MIC and 2 x MIC of the extract and the samples were incubated at 37^{o}C for 120 min. Immediately the bacteria was introduced into the extract solutions and at an interval of 1 h incubation period, 1 ml of the bacteria-extract mixture was centrifuged at 12000 rpm for 10 min. Ten microlitre (10 µl) of the supernatant was dispensed into 96 - well microtitre plate and 250 µl of Bradford's reagent was added. The mixtures were incubated at room temperature for 15 min with intermittent shaking. The amount of protein released from the cells was determined spectrophotmetrically at 525 nm by the method of Bradford (1976). The concentrations of protein leaked were extrapolated from Bovine serum albumin (BSA) which was used as a standard.

Results

In this study, the influence of ethanol extracts of *Acacia mearnsii* on the ultrastructures of five different bacterial strains was investigated to determine the possible mechanisms through which crude extracts of medicinal plants exhibit their antibacterial activities. The results showed that the extract had varied effects on the ultrastructures of the individual cells and bacterial populations. In Figures 1 - 5, all the bacterial strains not treated with the extract of *A. mearnsii*

had their morphological features intact. Untreated *Escherichia coli* had short rods, smooth surface and round ends with no cellular materials attached to their surfaces. *Shigella flexneri* had regular rod-shaped cells. *Proteus vulgaris* remained intact within matrices. *Bacillus pumilis* cells appeared as intact, smooth and shorts rods while *Staphylococcus aureus* cells were intact and were typically spherical in shape. However, on treating them with the extract, there were significant changes in their morphology. In *E. coli* treated with the extract (Fig. 1B), no cellular materials were found on the surfaces of most of the cell. Many cells were totally collapsed with depressions in the middle of the cells. Few dead cells were elongated with rough surfaces while some were bulged. In Figure 2B, treating *S. flexneri* with the extract resulted in changing the rod-shaped cells to polygon-shaped, collapsed or squeezed cells that looked coccoid in feature suggesting inflammation and bursting of cells. When *P. vulgaris* was treated with the extract (Fig. 3B), the cells were totally damaged. High percentage of the cells collapsed and many cells were shred into pieces. The cell collapsing was observed at both end of each bacterial cell while most treated cells appear longer than the untreated cells. Treating *B. pumilis* with the extract caused a massive death of cells (Fig. 4B). In affected but not shredded cells, there were collapsed twisting cells while few cells were swollen. In Fig. 5B, *S. aureus* treated with the extract appeared rough on the surface and many cells were destroyed. While many cells appeared unaffected, some cells appeared swollen or bigger in size than the untreated cells. The surfaces of the unaffected but inflamed cells were smooth and no pores were observed.

In the determination of the elemental contents of these bacterial isolates, the presence of Carbon (C), Nitrogen (N), Oxygen (O), Sodium (Na), Magnesium (Mg), Aluminum (Al), Chlorine (Cl), Silicon (Si), Phosphoruc (P), Sulphur (S), Potassium (K), Calcium (Ca) and Copper (Cu) were determined. The electron dispersive X-ray spectroscopy of these bacterial strains revealed the

elemental components of both treated and untreated bacterial strains as shown in Figure 6 – 10 and Table 1. The results showed that there were significant differences in the weight (%) of the element observed in the extract-treated and untreated bacterial strains. From the micrographs in Fig. 6 – 10, the peak of the elements present in the extract-untreated bacterial strain were higher than those obtained from the untreated bacterial strains. These differences were, however, indicated by the data in Table 1. In comparison to the extract-untreated *B. pumilis*, extract-treated *B. pumilis* had increases in the weight (%) of C, N, O, Na, Mg and Cl and decreases in the weight (%) of Si, P, S, K, Ca and Cu. The increases in the C, N and O which are major elements essential to life suggested rapid metabolic and growth processes in this bacterial isolates, possibly, in response to the environmental perturbation due to the presence of the extract. With the exception of *P. vulgaris*, this observation may be appropriated to other bacterial strains treated with this extract since one of the major elements essential to life was higher in the treated bacteria than the untreated specimen. In comparison to the untreated bacterial cells, Na and Cl were found to increase in weight (%) in all the test isolates. While N, O, Na, Mg, Al, Si, Cl, K and Cu were found to increase in weight (%) in extract-treated *E. coli*, O, Na, Al, S, Cl and K were found to increase in weight (%) in *S. flexneri* and Na, Mg, Cl, K, Ca and Cu had increased weight (%) in *P. vulgaris*.

A consideration for the carbon contents of these cells showed that while the carbon (C) content of untreated *S. flexneri* was the highest, that of the treated *E. coli* was the highest and that of the *S. flexneri* was the least in the extract-untreated controls. For the nitrogen content, there is an increase in the nitrogen content of treated *E. coli* and *B. pumilis* while there is a loss in those of *S. flexneri, P. vulgaris* and *S. aureus*. Considering other micro and major elements in these bacterial strains, Mg, Al and K were found to increase in all the bacteria except in *S. flexneri, P.*

vulgaris and *B. pumilis* respectively. Except in *P. vulgaris*, the weight of Ca was decreased in all the extract treated bacteria while that of Cu was decreased in *S. flexneri*, *P. vulgaris* and *B. pumilis*. It may, therefore, be appropriate to infer from the differences between the elemental components of extract-treated and untreated bacteria that the increments observed was due to the rapid replication, synthesis of micromolecules and rapid growth of these isolates in order to survive in the challenged environment. The varied decreases in the elemental components of the treated isolates, compared to the untreated ones, could be attributed to the ability of the extract to breakdown the synthesized micromolecules and attack the basic mechanisms underlining replication, growth and development in bacteria.

The determination of the lipid and protein leakage ability of this extract showed time and concentration dependent lipid and protein leakage ability of the extract. The results are as shown in Figures 11 and 12. The lipid and protein leakages that resulted from 2xMIC were higher than those obtained from 1 x MIC of the extract. In both assays, leakages obtained from *S. aureus* were the highest compared to other tested isolates. While the protein leakage was very high in *S. flexneri*, the bacterial had the least lipid leakage resulting from its treatment with the different concentrations of the extract in comparison with those obtained from the other strains. On the other hand, protein released from *S. aureus* at MIC and 2 x MIC were generally higher than those obtained from other bacterial strains. The concentration of the lipid leakages obtained from the bacteria treated with the MIC ranged from 68 μg/ml to 325 μg/ml while those of the 2 x MIC ranged between 83.07 μg/ml and 285.97 μg/ml. While the protein released at the MIC ranged between 333.3 μg/ml and 427.3 μg/ml, those obtained at the 2 x MIC ranged between 354.0 μg/ml and 542.7 μg/ml. The results showed that there are differences between the concentrations of the leaked lipids and proteins.

Discussion

Many studies have investigated the effect of crude plants extracts on different bacterial strains to determine one of the possible mechanisms of action through which they exert their antibacterial activity *in vitro*. In this study, the scanning electron microscopy (SEM) demonstrated clearly the antibacterial effect of the ethanol extract of *A. mearnsii*. There were distinct morphological changes of varied degrees as indicated by the differences between the treated bacteria and their untreated controls. The bulging, swelling and elongation of intact but affected cells indicated the inflow of solutes containing phytochemicals into the cells, followed by bursting as indicated by the shredded bacterial cells. The observed shrunken, collapsed and depressed cells could be attributed to ex-osmosis that resulted in plasmolysis of bacterial cells or imperfect synthesis of the cell wall after treatment. Of significance is the ability of the extract to change the morphology of *S. flexneri* from rods to polygon shaped cells while most *S. aureus* cells appeared unaffected. In *S. aureus*, thickening of the cell wall was a form of defense against antibiotics and compounds that inhibit the cell wall synthesis (Gemmell and Lorian, 1996; Giesbrecht et al., 1998). The thickened cell walls were more rigid and affected the membrane permeability so that the cells become more easily broken. Katzung (2007) indicated that cell membranes are also damaged through this process. Hence, the morphological changes observed in these bacterial strains meant that the extract inhibited cell wall synthesis, caused membrane damages and possessed different antibacterial components exerting different mechanisms of action at different target sites.

In respect to rod-shaped *Shigella flexneri* that was changed to squeezed, collapsed, polygon-shaped cocci and varied elongation observed in other bacterial strains, the ability of the ethanol extract to inhibit a significant step in peptidoglycan assembly is suggested. Schwarz et al. (1969)

reported that lateral wall elongation and septum formation are biochemical processes involved during the growth of rod-shaped bacterial cells. Higgins and Shockman (1971) indicated that cocci expansion occurred through cross wall formation and that the presence of additional genetic information is required for the lateral wall formation. Satta et al. (1979; 1983) suggested that shape determination and maintenance of bacterial rods depend on the activity of the two biochemical reactions. Satta et al. (1980) showed that the actual shape of rod-shaped bacteria is determined by the balance between these two competing biochemical reactions occurring in the terminal stages of peptidoglycan synthesis. Canepari et al., (1984) and Satta et al. (1983; 1985) reported that correct balance between the two biochemical reactions would result in normal shaped rods, anomalous prevalence of the reactions for lateral wall elongation would result in long rods and prevalence of the reactions for septum formation would lead to formation of cocci or coccobacilli. In view of these earlier reports, the distorted shapes of *S. flexneri* and the elongations of some cells in other bacterial strains, used in this study, could be attributed to the ability of the phytochemicals in the ethanol extract to inhibit the reactions leading to septum formation in *S. flexneri* to produce elongated rods and inhibition of reactions leading to lateral wall elongation in other isolates to produce cocci.

In nature, there is a great breadth of microbial metabolism. Cultivated bacteria convert the chemical energy of a growth substrate into the work and biosynthetic processes underlining both survival and replication. Consequently, major elements such as H, C, O, N, P, S, Cl, K, Na, Ca, Mg, Se, Zn, Fe, Mn, Cu, Co, Ni and Mo synthesized have been identified in bacterial cells (Steinberg, 1938; Sokoloff, 1954; Rouf, 1964). These elements have been related to the microorganisms' genes that enable them to persist under selective pressure in a complex and often changing chemical environment (Wackett et al., 2004). According to Rouf 1964, the

inorganic elements present in bacterial samples can be classed into three groups: major, minor and trace elements. Mg, P, K and S may be considered major elements being present in relatively high concentrations. Ca, Fe, Zn, and perhaps, Cu and Mn may be considered minor and the rest trace elements. In biological system, C, N, O, P and S are commonly bonded with H. Since 60% of the cell mass is H_2O, C, N, O, S and P, clustered together with hydrogen formed approximately 97% of an *E. coli* cell. While Troussellier et al. (1997) indicated that the elemental content can vary in taxa while the growth condition can also affect the elemental composition (Goldman et al., 1987; Nakano, 1994; Fagerbakke et al., 1996; Vrede 1998). The macromolecule composition of a bacterial cell is affected by both growth rate and nutrient availability (Vrede et al., 2002). In agreement with these earlier reports, this study showed variables in the weight (%) of the elemental contents of the different bacteria strains used as controls while the differences between the extract-treated and untreated bacteria could be attributed to the influence of the growth conditions they were subjected to.

Considering that metabolic processes result in cell growth, a lack, inhibition or breakdown of metabolic processes will result in inadequacy of the bacterial elemental contents. Thus, in agreement with Mårdén et al., (1985), Holmquist and Kjelleberg (1993) and Troussellier et al., (1997) who indicated that growth limitation of C, N, and P decreases cell volumes and ultramicrocells of bacteria, the reduction in the weight (%) of some elemental contents of the extract-treated bacteria in comparison with the control showed that the extract was able to inhibit metabolic processes essential to bacterial existence and cause a breakdown of some elemental components. On the contrary, the observed increase in the elemental components of extract-treated bacteria as compared to their controls indicated that the bacteria metabolized and replicated actively in attempts to survive the perturbed environment while their synthesized

elemental contents were not completely degraded, thereafter, by the phytochemicals in the growth medium.

Bacterial cell walls are made of peptidoglycan which is made from polysaccharide chains cross-linked by unusual peptides containing D-amino acids (van Heijenoort, 2001). The cell membrane, built from lipids and proteins and lying just beneath the cell wall, is a biological membrane that separates the interior of all cells from the outside environment (Kimball's Biology Pages, Cell Membranes - website). It is selectively permeable to ions and organic molecules, controls the movement of substances in and out of cells (Alberts et al., 2002) and consists of the lipid layer which embedded proteins. In prokaryotic cells, proteins comprise about one-half of the dry weight (Ingraham et al., 1983). In most bacterial cell membranes, the major phospholipids are neutral phosphoethanolamine (PE), anionic phosphoglycerol (PG) and cardiolipin (CL) (Ratledge and Wilkinson, 1998; Lohner and Prenner, 1999). In *E. coli* cell membrane, PE, PG and CL contents are approximately 80, 6 and 12% (Morein *et al.*, 1996) while *S. aureus* cell membrane PE, PG and Cl contents are approximately 0, 58 and 42% (Beining et al., 1975). The lipid and protein leakages obtained over a period of time, in this study, signify the disintegration of the cell membrane and bacterial cellular compounds, the kinetic effects of the extract and correlates with the antibacterial activity of the extract. This correlation indicates that the disruption of the cell membranes has played a vital role in the observed morphological changes in the different bacterial strains. Therefore, coupling the morphological changes, breakdown of bacterial elemental contents with the lipid and protein leaked, it may be hypothesized that the mechanism of the antibacterial activity of the extract against these isolates seems to be through inhibition of metabolic processes, cell wall and cell membrane disruption and interference with the cell membrane resulting in the efflux of lipid and

protein in all bacteria tested. The physiological damages to the cell wall may have started within the first few minutes of treating the bacteria with the extract as indicated by the amount of leakages and the increasing intensity of the lipid and protein in the medium over the sampling periods.

Conclusion

In conclusion, this is the first report associating disintegration of elemental contents of bacterial isolates with the mechanisms of action of a medicinal plant. This study showed that morphological damages, differences in the elemental contents of treated and untreated bacteria and the lipid and protein leakages, could be attributed to the binding of the antimicrobial agents to the membrane surfaces and subsequent disintegration of the cell membranes resulting in various morphological disorders. These pharmacological effects could be linked together to justify the bactericidal ability of the extract and indicate its possible mechanisms of action against the test isolates.

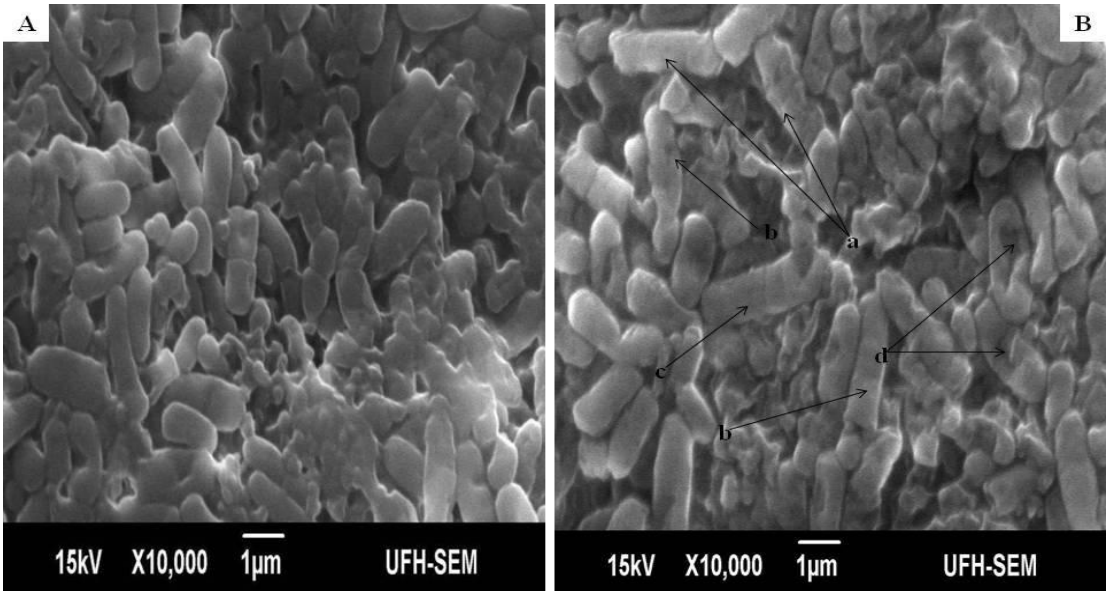

Figure 1: Influence of ethanol extract on the morphology of *E. coli*. A = Extract untreated *E. coli*; B = Extract treated *E. coli*. (a=dead cells with rough surfaces; b=collapsed and elongated dead cells; c=bulged or swollen dead cells; d=depressions in dead cells).

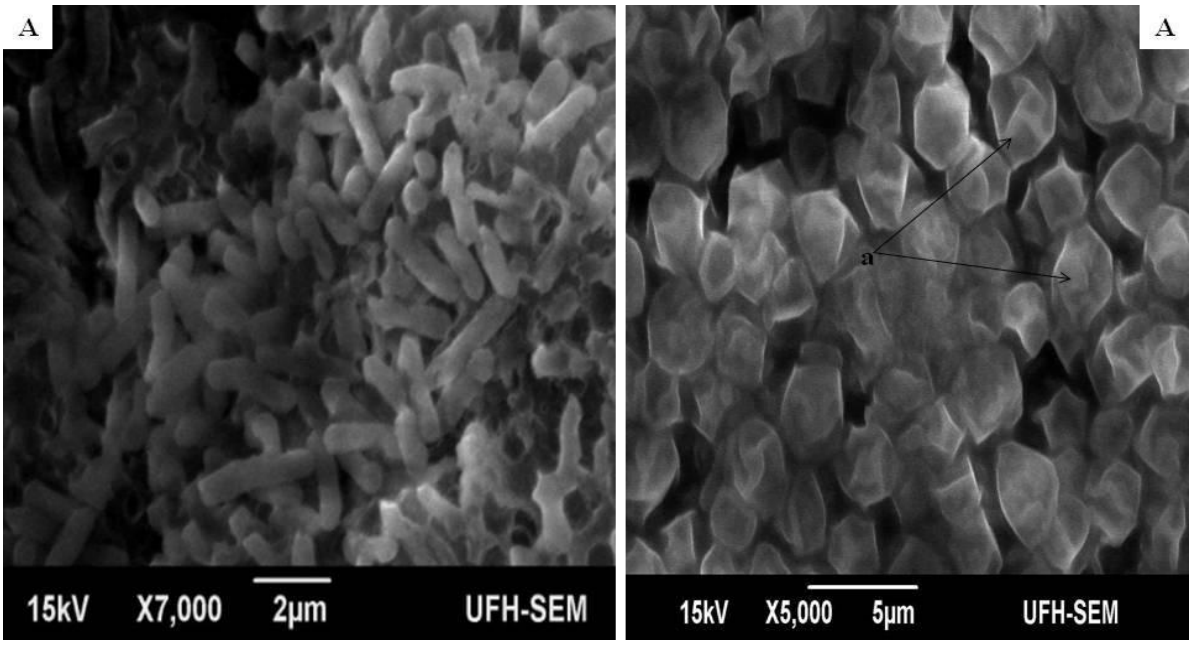

Figure 2: Influence of ethanol extract on the morphology of *S. flexneri*. A = Extract untreated *S. flexneri*; B = Extract treated *S. flexneri*. (a=polygon shaped, collapsed or squeezed cells).

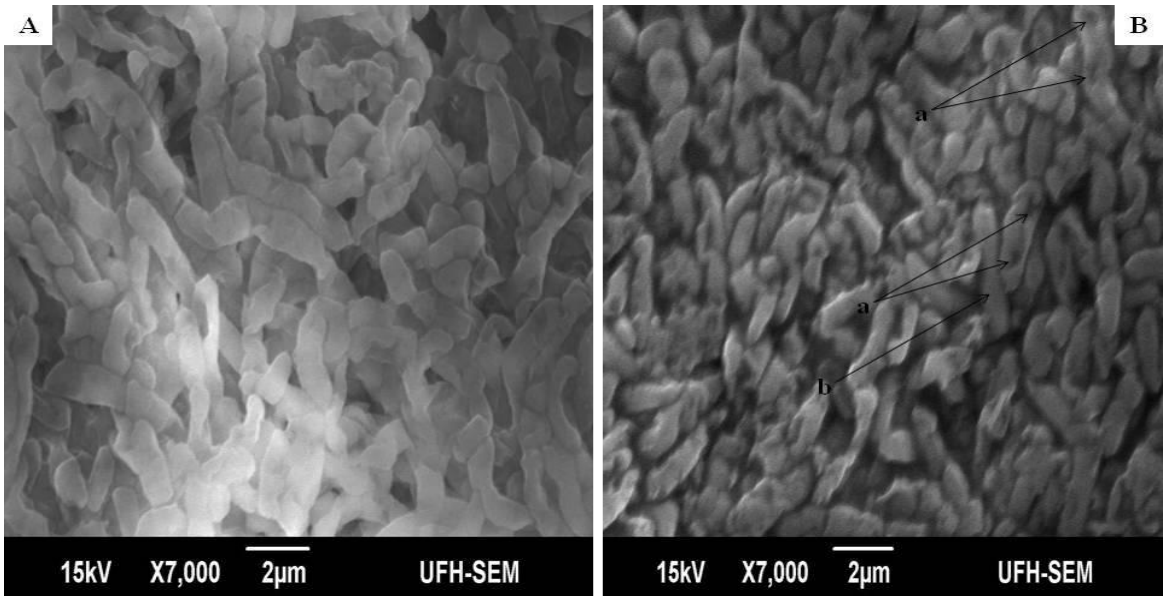

Figure 3: Influence of ethanol extract on the morphology of *P. vulgaris*. A = Extract untreated *P. vulgaris;* B = Extract treated *P. vulgaris*. (a=dead cell with depression at the polar ends; b=elongated cell)

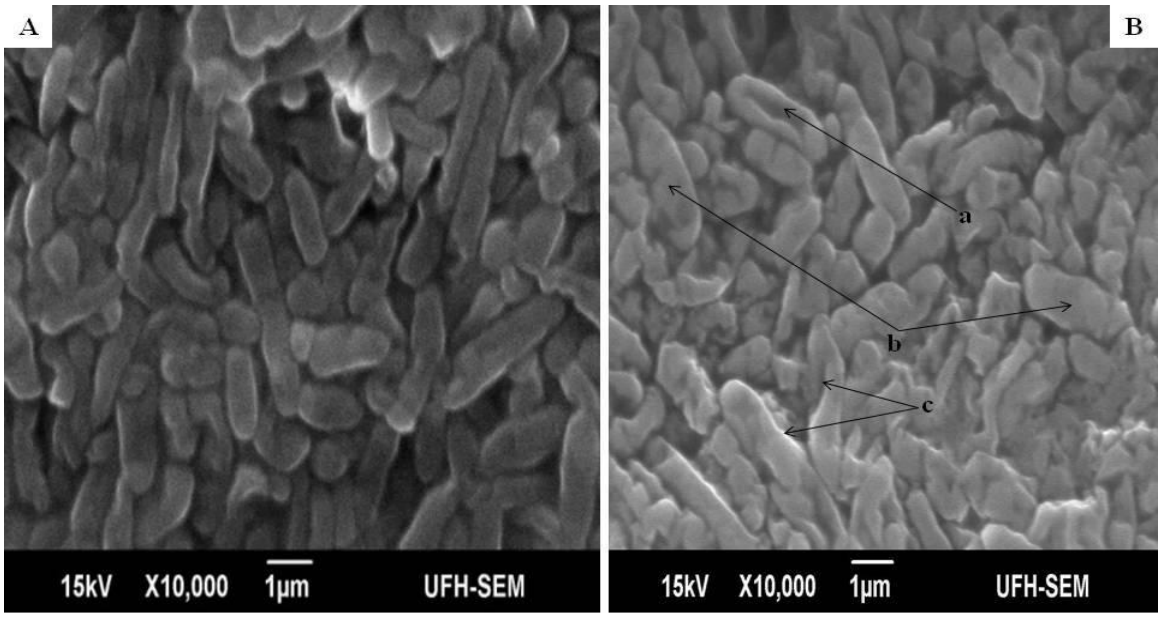

Figure 4: Influence of ethanol extract on the morphology of *B. pumilis*. A = Extract untreated *B. pumilis;* B = Extract treated *B. pumilis*. (a=depressed or collapsed cell; b=swollen cells; c=collapsed cells with twisting).

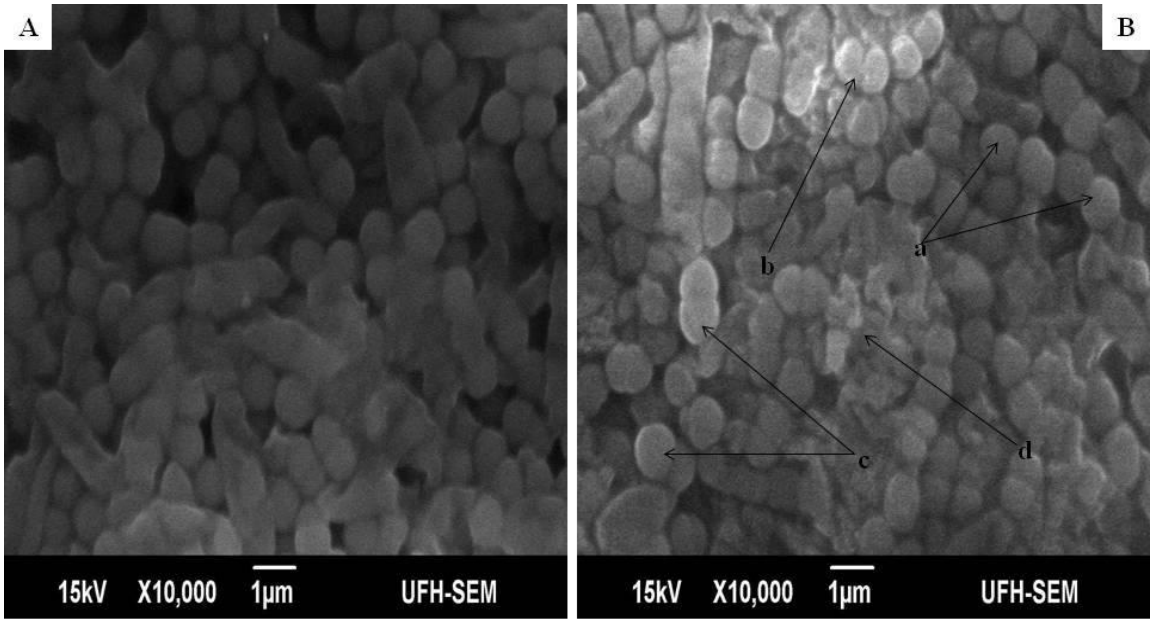

Figure 5: Influence of ethanol extract on the morphology of *S. aureus*. A = Extract untreated *S. aureus*; B = Extract treated *S. aureus*. (a= intact but affected cells; b=dead cells with destroyed cells; c=swollen cells; d=shredded cells)

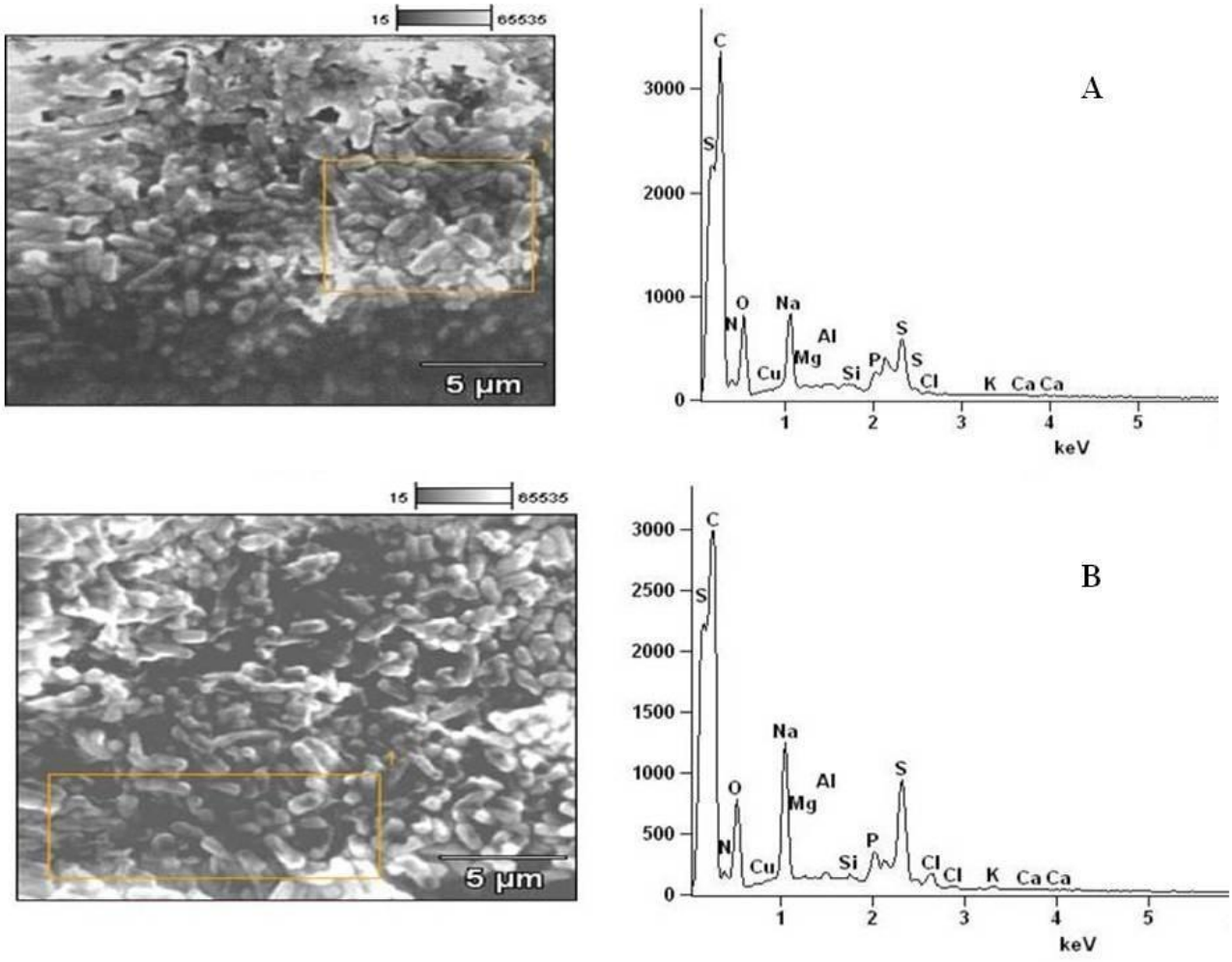

Figure 12.6: Elemental components of (A) extract-untreated *E. coli* and (B) extract-treated *E. coli*.

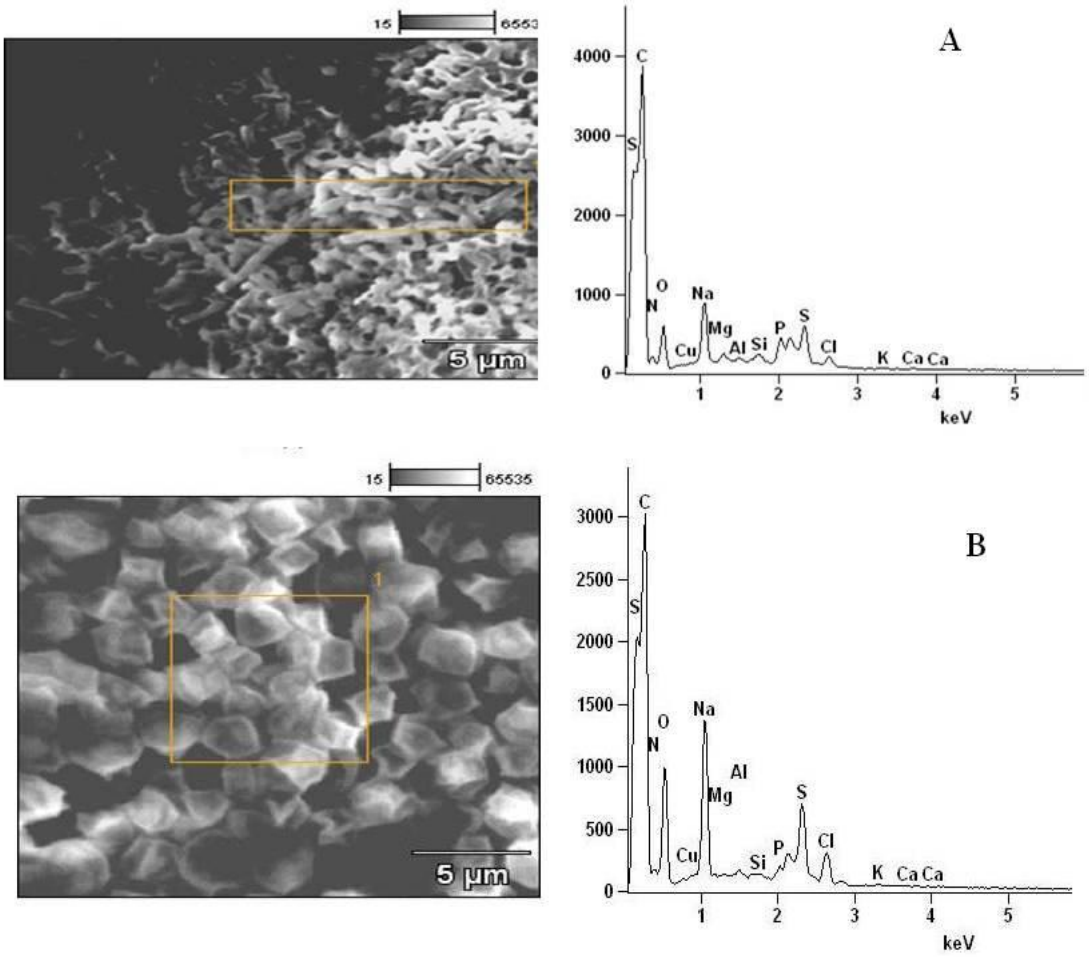

Figure 12.7: Elemental components of (A) extract-untreated *S. flexneri* and (B) extract-treated *S. flexneri*.

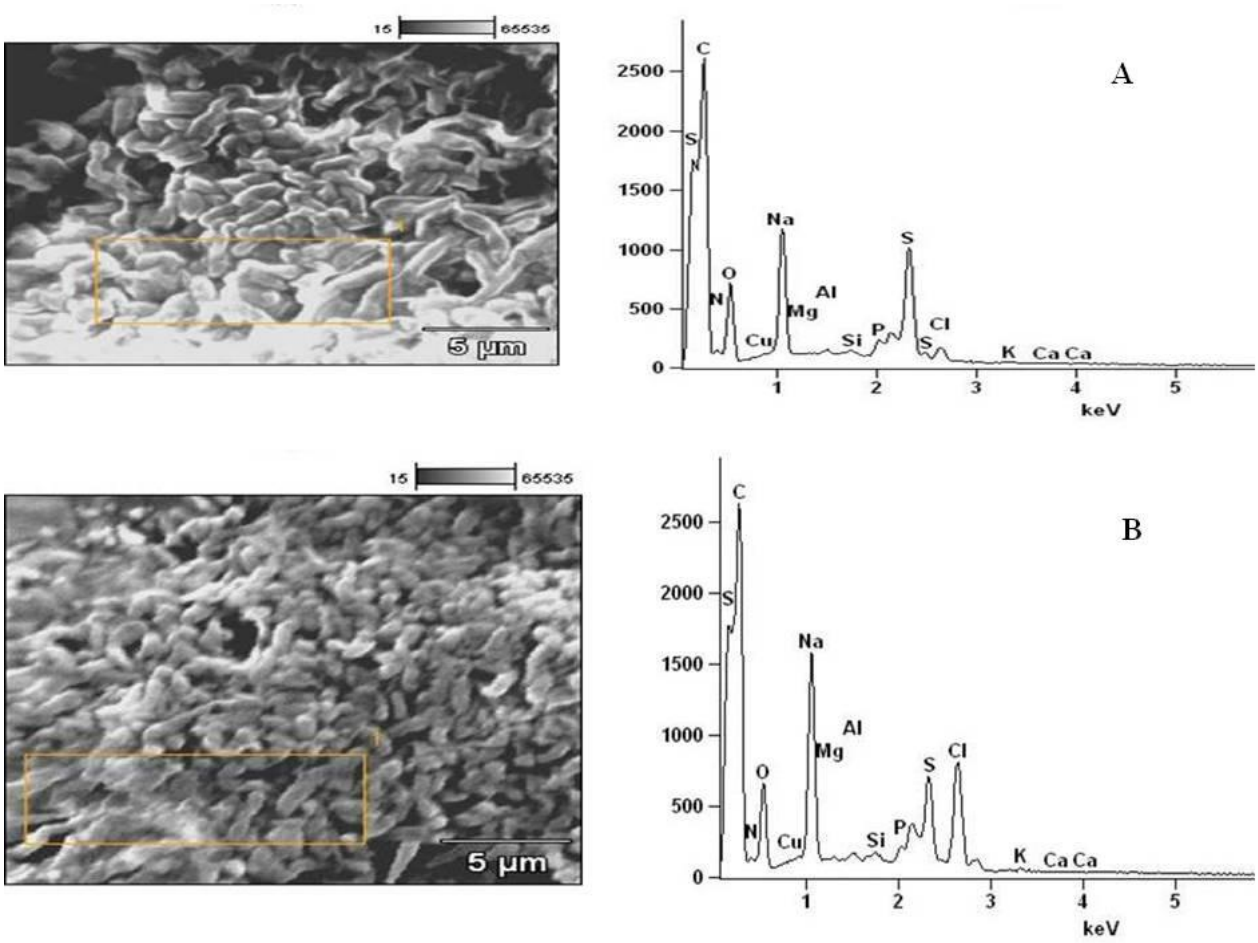

Figure 12.8: Elemental components of (A) extract-untreated *P. vulgaris* and (B) extract-treated *P. vulgaris*.

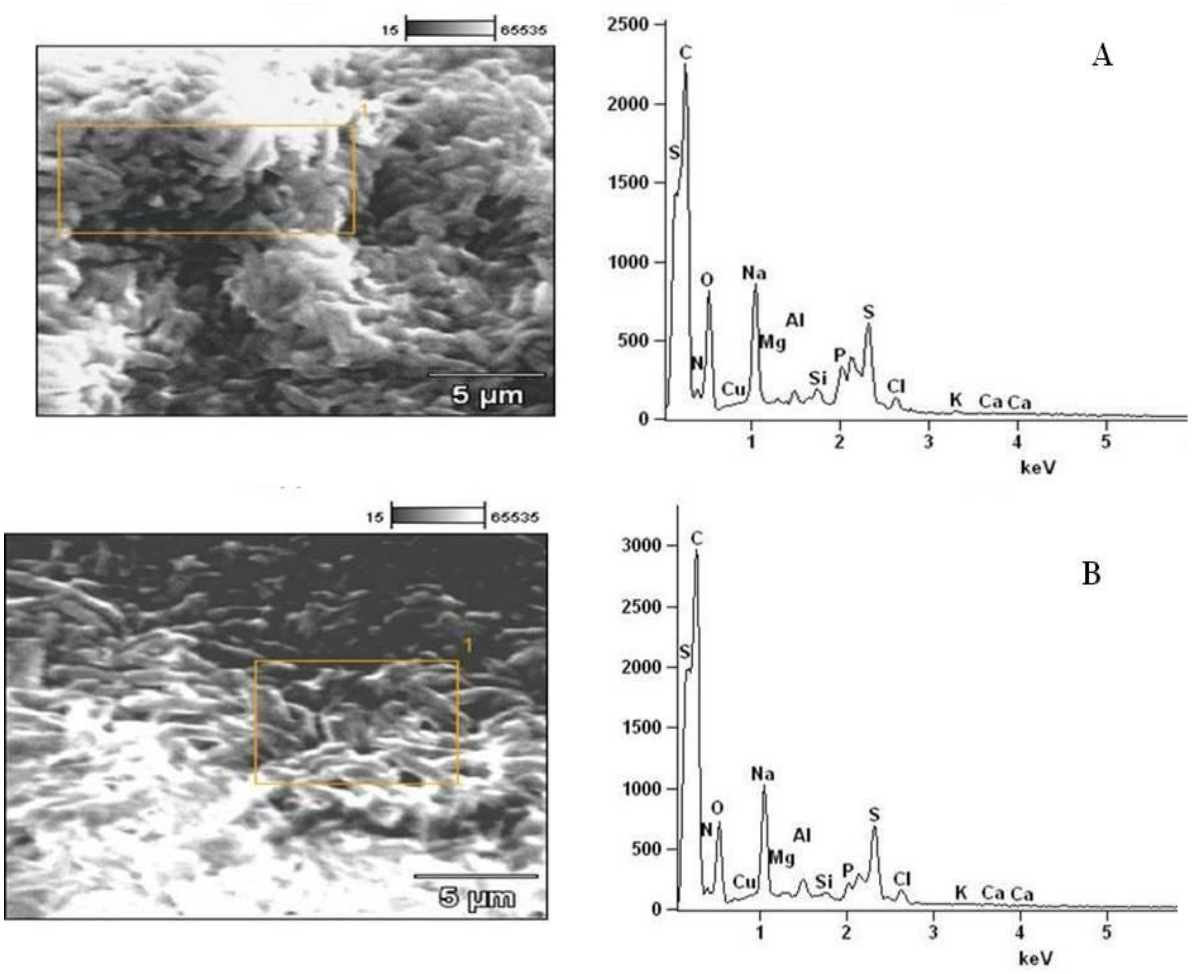

Figure 12.9: Elemental components of (A) extract-untreated *B. pumilis* and (B) extract-treated *B. pumilis*.

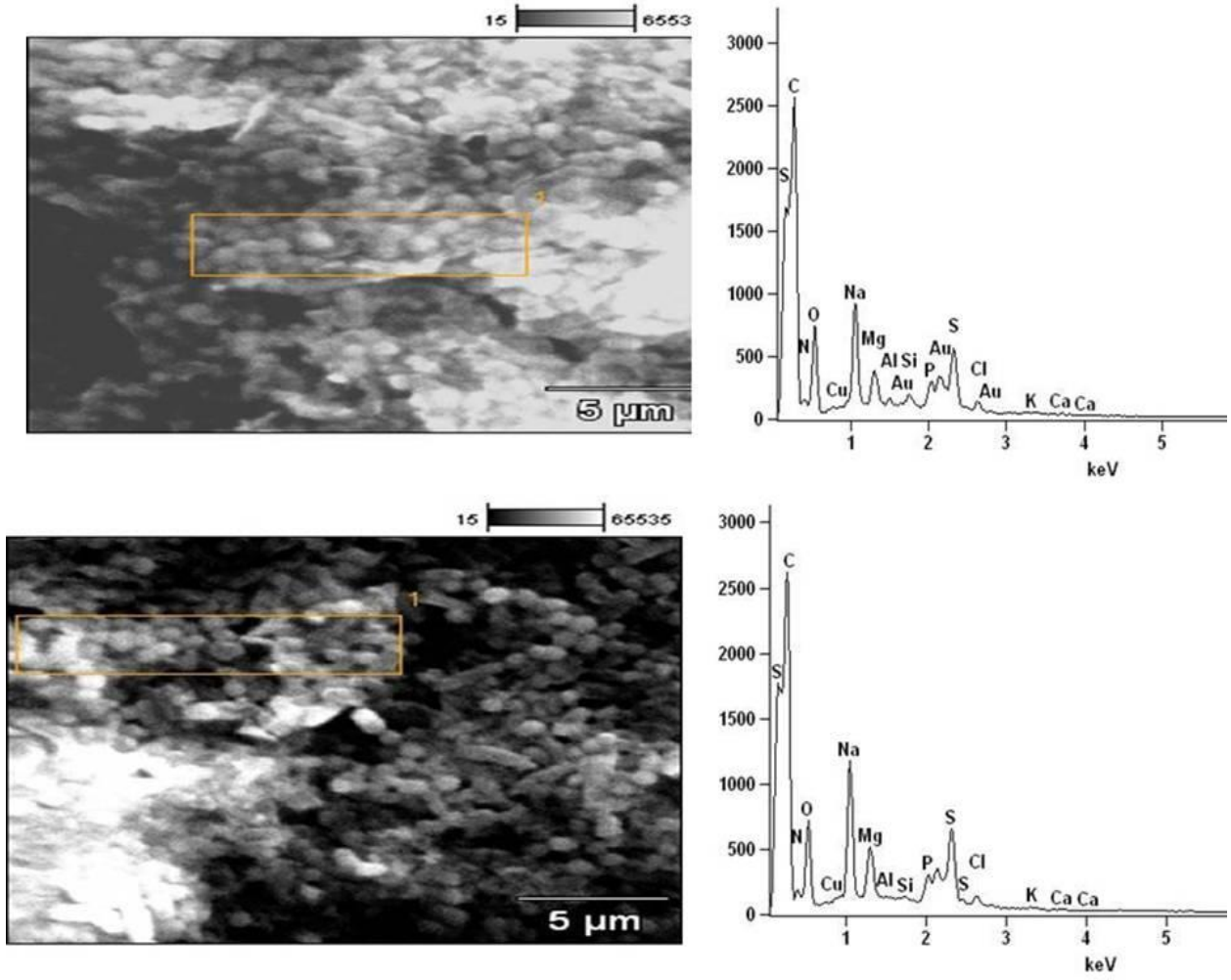

Figure 12.10: Elemental components of (A) extract-untreated *S. aureus* and (B) extract-treated *S. aureus*.

Table 12.1: Elemental components of extract-treated and untreated bacterial strains detected by Electron Dispersive Spectroscopy

	Average Weight (%) of the elemental components of the extract-treated and untreated bacteria strains												
AME - EDS Result	**C**	**N**	**O**	**Na**	**Mg**	**Al**	**Si**	**P**	**S**	**Cl**	**K**	**Ca**	**Cu**
Untreated *E. coli*	55.14±0.51	0.18±0.45	23.23±0.47	8.76±0.13	0.04±0.02	0.20±0.04	0.39±0.05	1.98±0.09	8.23±0.13	0.28±0.06	0.13±0.05	0.24±0.06	1.79±0.33
Treated *E. coli*	53.25±0.55	0.47±0.42	21.64±0.48	9.55±0.15	0.13±0.07	0.74±0.05	0.43±0.06	1.37±0.12	7.88±0.20	1.66±0.12	0.45±0.06	0.10±0.06	2.34±0.38
Untreated *S. flexneri*	62.79±0.49	0.64±0.48	16.52±0.40	7.04±0.12	0.55±0.07	0.05±0.02	0.61±0.05	2.26±0.12	5.21±0.16	1.51±0.10	0.13±0.06	0.32±0.06	2.34±0.34
Treated *S. flexneri*	50.50±0.53	0.18±0.48	2.60±0.50	10.50±0.15	0.02±0.07	0.66±0.05	0.24±0.06	0.75±0.12	6.20±0.20	2.74±0.12	0.26±0.06	0.04±0.06	1.84±0.39
Untreated *P. vulgaris*	52.87±0.50	0.49±0.45	21.85±0.41	9.39±0.12	0.06±0.07	0.36±0.02	0.54±0.05	1.14±0.13	10.11±0.16	1.62±0.11	0.27±0.06	0.05±0.06	1.25±0.35
Treated *P. vulgaris*	52.29±0.49	0.67±0.42	18.51±0.38	10.81±0.13	0.08±0.05	0.20±0.04	0.33±0.04	0.96±0.08	6.28±0.16	7.35±0.05	0.28±0.05	0.07±0.05	2.11±0.32
Untreated *B. pumilis*	50.02±0.55	0.45±0.42	22.49±0.48	9.44±0.15	0.25±0.07	0.35±0.05	0.94±0.06	2.50±0.12	8.77±0.20	1.51±0.12	0.28±0.06	0.10±0.06	2.87±0.38
Treated *B. pumilis*	52.47±0.70	0.99±0.46	25.07±0.74	9.72±0.19	0.27±0.09	1.08±0.08	0.61±0.06	0.29±0.08	5.42±0.18	1.74±0.16	0.18±0.07	0.06±0.07	2.09±0.48
Untreated *S. aureus*	53.24±0.55	1.21±0.13	18.76±0.38	9.42±0.15	0.17±0.08	0.43±0.02	1.23±0.07	3.80±0.23	6.56±0.20	1.92±0.16	0.20±0.02	1.17±0.06	1.88±0.38
Treated *S. aureus*	52.61±0.51	0.95±0.00	20.33±0.39	9.93±0.14	0.26±0.05	0.84±0.04	0.96±0.04	2.23±0.08	5.62±0.17	2.27±0.16	0.32±0.05	0.89±0.05	2.69±0.33

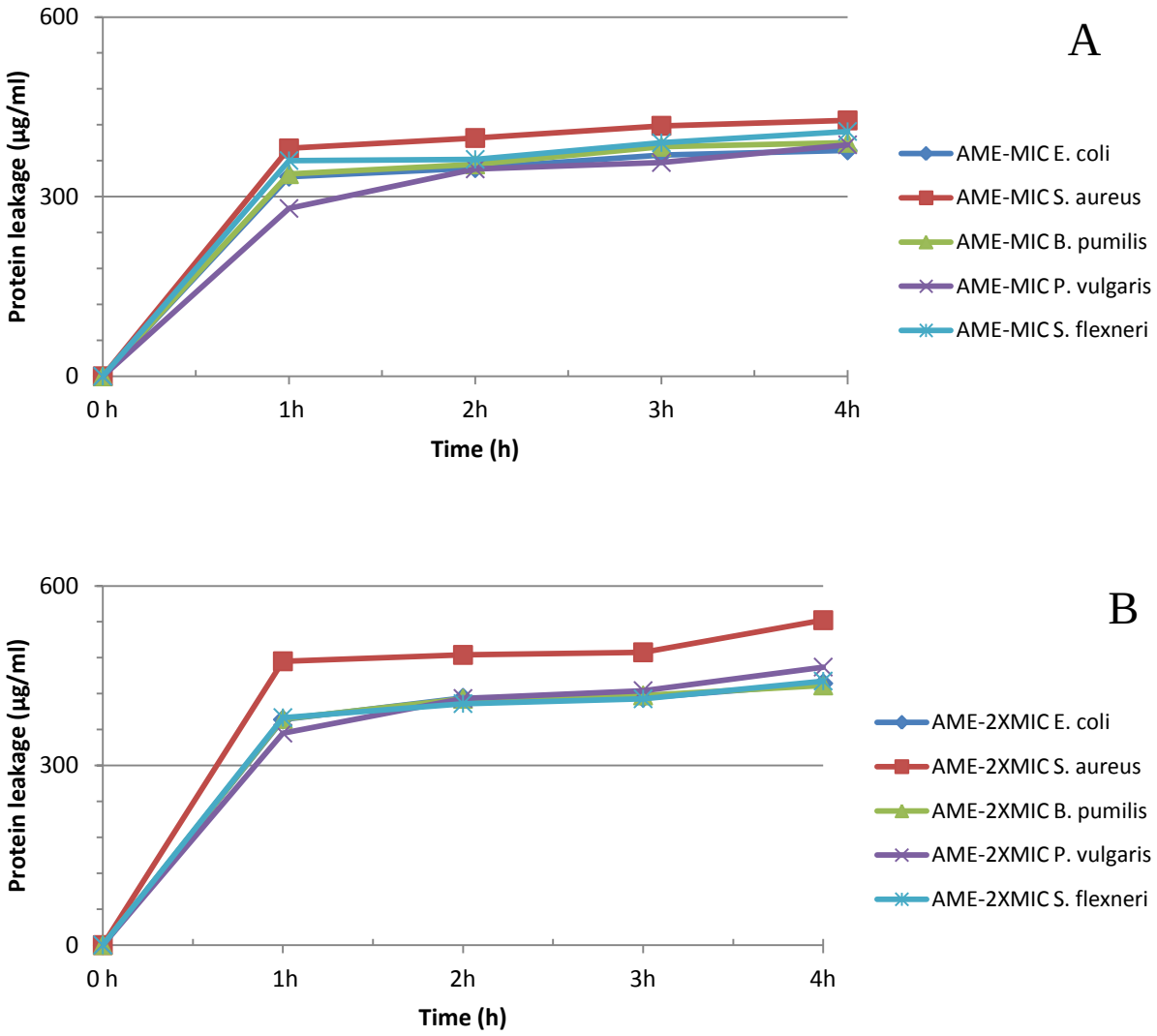

Figure 11: Leakage of protein from different bacteria after treatment at their MIC and 2xMIC.

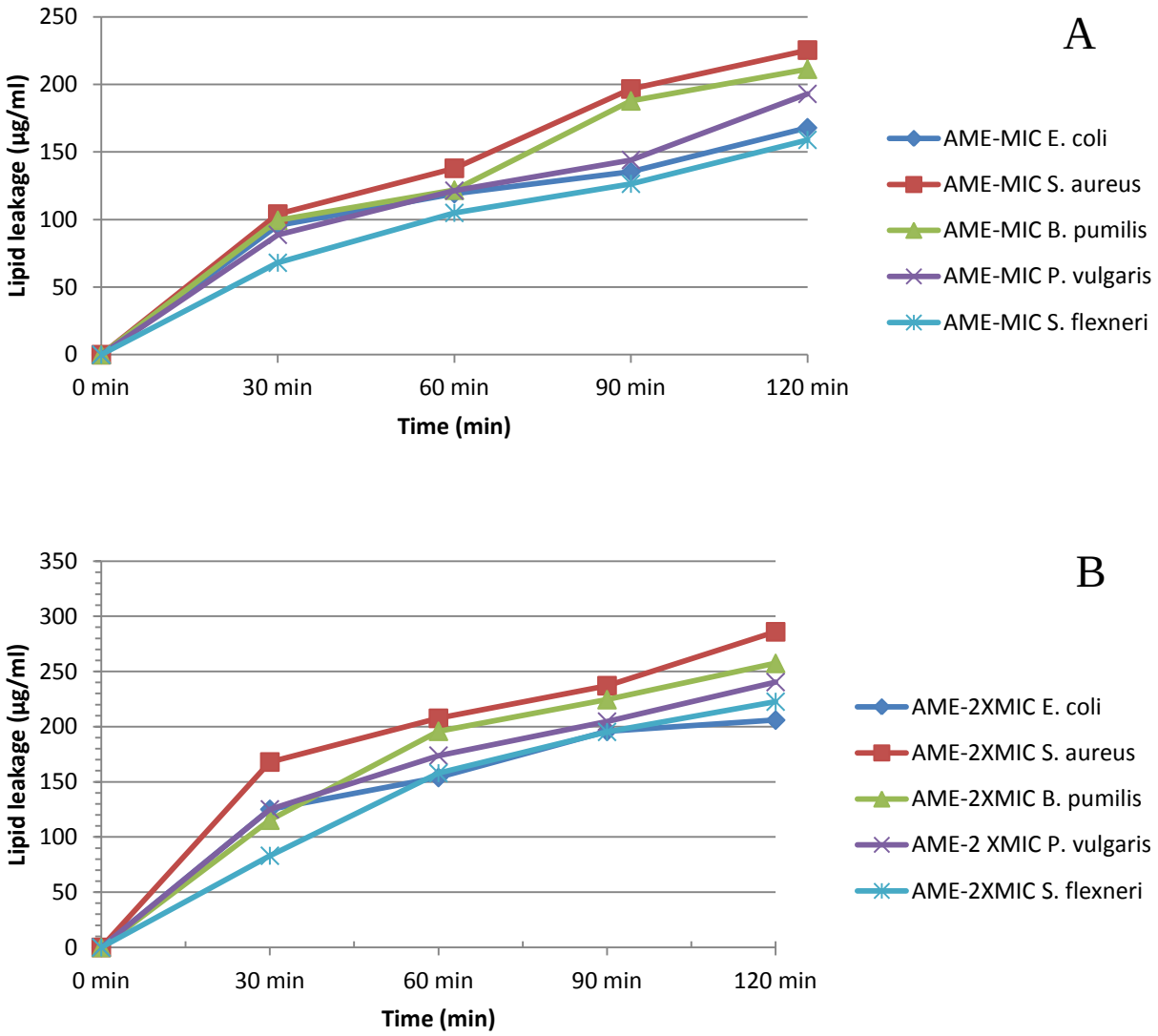

Figure 12: Leakage of lipid from different bacteria after treatment at their MIC and 2xMIC.

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CHAPTER 13

A comparative effect of the alcoholic and aqueous extracts of *Acacia mearnsii* De Wild on protein leakage, lipid leakage and ultrastructural changes in some selected bacterial strains as possible mechanisms of antibacterial action

(*Accepted in the Journal of Pure and Applied Microbiology in this format*)

A comparative effect of the alcoholic and aqueous extracts of *Acacia mearnsii* De Wild on protein leakage, lipid leakage and ultrastructural changes in some selected bacterial strains as possible mechanisms of antibacterial action

Abstract

In this study, the possible mechanisms of action of acetone, methanol and aqueous extracts of *Acacia mearnsii* was investigated. The influences of these extracts on the ultrastructures, protein and lipid leakages of five different bacteria were determined. The results showed that the different extracts had varied effects on the different extract-treated isolates while the untreated isolates remained intact. On the extract-treated isolates, the extracts caused significant ultrastructural changes, protein and lipid leakages. The morphological changes and the observed leakages showed rapid killing, significant membrane depolarization resulting in leakages and efflux of disintegrated cellular materials. Disruptions in the outer wall and cytoplasmic membranes, especially, at the polar regions of the cells, whole cell collapse and presence of extruded cellular materials situated close to the collapsed end of the cytoplasm, and eventual cell death were observed. The observed distinct morphological changes such as cell elongation and roughening of the surfaces suggested that the extract may have interfered with bacterial cell wall synthesis leading to cell deformations. While aqueous extract was the most active in causing protein leakages, methanol extract was the leading cause of lipid leakages. The leakages were time and concentration dependent and, in some instances, significantly different from extract to extract. The possible mechanism of action involved in the lipid and protein leakages in the bacterial cells could be attributed to lipid peroxidation and protein oxidation owing to the antioxidants activities of the extracts being beyond protective levels. The study showed that the different extracts of *A. mearnsii* had bactericidal effects against the test isolates,

caused ultrastructural changes and leakages leading to disruption of the cytoplasmic membranes of the bacterial cells and populations.

Keywords: Scanning electron microscopy, ultrastructure, leakages, lipid peroxidation, mechanism of action

13.1 Introduction

Medicinal plants have been the traditional source of raw materials for medicine readily available for use by the local populace. In many developing countries, using medicinal plants as an alternative medicine is well known (Sandhu and Heinrich, 2005; Gupta et al., 2005). They have been used to treat infectious diseases in most parts of the world for thousands of years (Chariandy et al., 1999) because of their antiseptic and bactericidal properties (Lassak and McCarthy, 2001) and their eventual important roles in drug development in pharmaceutical industry (Jeyachandran and Mahesh, 2007). In many parts of the world, the extracts of medicinal plants are used as antibacterial, antifungal and antiviral agents (Hassawi and Khama, 2006). The successful prediction of their antimicrobial activities has been largely dependent on the type of solvent used in the extraction procedure. While alcohol and aqueous extraction methods are often adopted by traditional medical practitioners, the degree of antibacterial activities of medicinal plants have been attributed to the solubility of their phytochemicals in the solvents used in the extraction procedures (De Boer et al.. 2005). Since medicinal plants could be a rich source of antimicrobial agents (Voravuthikunchai and Kitpipit, 2005) and the systematic screening of these plants may result in the discovery of novel effective compounds (Newman et al., 2000; Tomoko

et al., 2002), determining their mechanisms of action becomes necessary for the proper elucidation of the degree of their bactericidal activities.

Acacia mearnsii de Wild, Fabaceae, indigenous to South Eastern Australia, was introduced to South Africa over 150 years ago for the tanning industry (Sherry, 1971). Being an invasive plant known as a threat in South Africa, it is considered an economic plant requiring scientific intervention for control (Sherry, 1971; Richardson, 1998) because it affects hydrological balances in areas where found (Dye and Jarmain, 2004). Although it is a plant of industrial value because of its high tannin content, there is a dearth of information on its pharmacological importance. However, its ethnopharmacological importance as a medicinal plant with astringent property and significant antimicrobial activities had been reported by Olajuyigbe and Afolayan, (2012a,b). Resulting from the reported antimicrobial activities, we have, in this study, made a comparative analysis of the possible mechanisms of the antibacterial activities of this plant. Hence, this study was designed to investigate the comparative effects of the alcoholic and aqueous extracts of *A. mearnsii* on protein leakage, lipid leakage and ultrastructural changes in some selected bacterial strains. Since knowledge on the mechanisms of action may allow the rational development of antimicrobial agents that target cell functions, visual information may be useful in providing insight on the ultrastructural changes in the cell, assist in characterizing the type and magnitude of changes occurring in cell composition in response to treatments with the extracts and help to understand how and why a treatment is bactericidal or bacteriostatic against a particular organism.

13.2 Materials and Methods

13.2.1 *Bacteria used for the study*

The bacteria used in this study included *Escherichia coli* (ATCC 8739), *Shigella flexneri* (KZN), *Proteus vulgaris* (ATCC 6830), *Staphylococcus aureus* (ATCC 6538) and *Bacillus pumilis* (ATCC 14884). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa.

13.2.2 *Collection of plant material*

The bark materials of *Acacia mearnsii* De Wild were collected in August, 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen (OLAJ Med 2010/01) was prepared and deposited in the Griffen Herbarium of the University.

13.2.3 *Extract preparation*

The bark sample was air-dried at room temperature and pulverized using a milling machine. The extract of the bark material was prepared in accordance to the description of Basri and Fan (2005). About 100 g of the pulverized sample was extracted with 500 ml of acetone, methanol and water for 48 h with shaking (Stuart Scientific Orbital Shaker, UK). The extracts were filtered through Whatman No. 1 filter paper. While the alcoholic extracts were concentrated under reduced pressure at 40^{o}C using a rotary evaporator (Laborota 4000 – efficient, Heldolph, Germany), the aqueous extract was obtained by freeze drying. The crude alcoholic extracts collected were allowed to dry at room temperature to a constant weight. The extract was

redissolved in dimethylsulfoxide (DMSO) before being diluted with sterile distilled water to the required concentrations for bioassay analysis.

The reconstituted extract solution was sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract into 10 ml of sterile nutrient broth before being incubated at 37^{o}C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

13.2.4 *Determination of the effect of alcoholic and aqueous extracts on cell morphology*

Overnight broth cultures of the different bacteria strains were standardized at 10^6 cfu/ml using McFarland Nephe-lometer standard (NCCLS, 1993). One milliliter of the adjusted bacterial culture was added to 9 ml of each of the plant extracts at 4 x MIC and kept at laboratory temperature (28^{o}C) for 2 h. Untreated controls were prepared in Mueller Hinton broth medium and incubated for the same period of time. The bacterial cells were harvested by centrifuging at 10000 rpm for 10 mins and subsequently washed with phosphate buffer (pH 7.2). The bacterial pellets were re-suspended in 2.5% glutaraldehyde and kept for 2 h to fix the bacterial cells. The fixed bacterial cell suspensions were centrifuged at 10000 rpm for 10 min, washed and re-suspended in buffer (pH 7.2) from where the cells were then deposited on a 0.45-µm-pore-size membrane filter (Schleicher & Schuell, Dassel, Germany). The bacterial cells on membrane filters were dehydrated with a graded series of acetone/water washes (20%, 50%, 70%, 90% and 100% acetone) to gradually remove water from the cells without physical damage. The dehydrated bacterial cells were mounted on a stub, allowed to dry before being sputtered with a small amount of gold/palladium using Ion coater: EIKO IB.3 to avoid charging in the microscope. Microscopy was performed with a JEOL JSM-6390LV Scanning Electron

Microscope (Japan). Secondary electron images were taken with an accelerating voltage of 15 kV.

13.2.5 *Determination of Protein leakage from bacteria cells*

To determine the leakage of intracellular materials from the cells, the bacteria cells were treated with the 1 x MIC and 2 x MIC of the extract and the samples were incubated at 37^{o}C for 120 min. Immediately the bacteria was introduced into the extract solutions and at an interval of 1 h incubation period, 1 ml of the bacteria-extract mixture was centrifuged at 12000 rpm for 10 min. Ten microlitre (10 µl) of the supernatant was dispensed into 96 - well microtitre plate and 250 µl of Bradford's reagent was added. The mixtures were incubated at room temperature for 15 min with intermittent shaking. The amount of protein released from the cells was determined spectrophotmetrically at 525 nm by the method of Bradford (1976). The concentrations of protein leaked were extrapolated from Bovine serum albumin (BSA) which was used as a standard.

13.2.6 *Determination of Lipid leakage from bacteria cells*

Phospho-vanillin was prepared as previously described by Frings and Dunn (1970). 0.6 g of Vanillin (Sigma Chemical Co., St. Louis, Mo. 63118) was dissolved in 10 ml of absolute ethanol before diluting to 100 ml with distilled water. This solution was mixed with 400 ml of concentrated phosphoric acid with constant stirring before being stored at room temperature in a brown bottle. The lipid leakage assay was carried out using the method described by van Handel and Day (1988) and Kaufmann and Brown (2008). Briefly, bacterial cells were harvested from an overnight broth culture by centrifuging at 10000 rpm for 10 min and used to prepare 10^{8} cfu/ml. The standardized cell suspension was treated with 1 x MIC and 2 x MIC of the extract

and the samples were incubated at 37^{o}C for 120 min. After incubating at 37^{o}C for 30 min, each cell suspension was sampled at 30 min interval and centrifuged at 10000 rpm for 10 min. 0.2 ml of sulfuric acid was added to replicated small portions of the supernatant (10 µl) in tubes and heated in water bath for 10 min at 100^{o}C. Vanillin-phosphoric acid reagent was added to the mixture before being removed from water bath, vortexed and allowed to cool while the optical density was measured at 525 nm. The concentration of lipid leakage was estimated from linoleic acid standard curve used as standard.

13.2.7 *Statistical analysis*

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). One way analysis of variance (ANOVA) and the Duncan's New Multiple-range test were used to determine the differences among the means. P values < 0.05 were regarded to be significant.

13.3 Results

In this study, the possible mechanism of action of acetone, methanol and aqueous extracts of *Acacia mearnsii* was investigated. The influences of these extracts on the ultrastructures, protein and lipid leakages of five different bacterial isolates including *Escherichia coli* (ATCC 8738), *Shigella flexneri* (KZN), *Proteus vulgaris* (ATCC 6830), *Staphylococcus aureus* (ATCC 6538) and *Bacillus pumilis* (ATCC 14884) were determined. The results showed that the different extracts had varied effects on the extract-treated isolates. The effects of the different extracts on the surface morphology of the different bacteria during its logarithmic growth phase

were shown in Figures 1 – 5 where the degree of antimicrobial activities on the surface morphology of individual cells and bacterial populations were indicated. In Figures 1 - 5, the untreated bacteria retain the morphological features. Untreated *E. coli*, *S. flexneri*, *P. vulgaris* and *B. pumilis* cells were typically rod-shaped and the cells surfaces were relatively smooth while the untreated *S. aureus* were typically spherical in shape. In *E. coli* treated with acetone extract (Fig. 1B), all cells showed significant effect of the extract. Most cells had very mild depressions on their surfaces. Few cells were collapsed in the middle. Some cells were wrinkled with rough surfaces. Others had pores on their surfaces which were not significantly distinct but were depicted by scattered dots of extruded intracellular materials while some intact but affected cells bulged with round tapering ends. As a result of the depressions, almost all the bacterial cells had extruded cellular materials which either clustered at one of the polar end of the cell or sparsely scattered on the cell surfaces. In *E. coli* treated with methanol extract (Fig. 1C), higher numbers or percentages of cells were structurally and badly damaged. Many cells had holes or pores on their surfaces but with a distinct pore at a polar end of the cell or just a little away from the polar end. Some cells were collapsed in the middle but did not extend to the polar ends. Some cells were elongated with wrinkled surfaces. Many cells had the extruded cellular materials scattered on cell surfaces or cluster to a spot in some cells. In cells where the extruded materials accumulated on a location, they were rarely found on other parts of the cell surfaces and vice versa. Few cells, also, remained intact with rough surfaces. In *E. coli* treated with aqueous extract (Fig. 1D), the pores were more prominent on cell surfaces with a larger hole at the opposite polar ends. Both scattered and accumulated extruded cellular materials were found on most cells contrary to what were observed with methanol extract-treated *E. coli*. The clustered or accumulated cellular materials were not restricted to the polar end of the cells where found and

were more whitish on the cell surfaces than was observed in acetone extract-treated *E. coli*. In Figure 2B, *S. flexneri* treated with acetone extract, the cells were constricted, shorter and superficially depressed at the centre. Pores and extrusion of cellular materials were not found on the surfaces of the bacterial cells. In Figure 2C, *S. flexneri* treated with methanol extract were mostly shorter, accumulated extruded cellular materials were found mostly at the middle part of the cells or at the polar ends in few cases while cell collapse or depressions are found at the tip or crown of a polar end of the bacterial cells. In *S. flexneri* treated with aqueous extract (Fig. 2D), there were constrictions only. No collapse or pores were observed. When *Proteus vulgaris* treated with acetone extract (Figure 3B), the extracellular matrices were destroyed. The exposed treated cells were intact, thinner and longer than the untreated cells. When treated with methanol extract (Fig. 3C), the cells were badly damaged. The cells that were not totally damaged were shorter and collapsed while some were twisted. No extrusion and attachment of extruded cellular materials to the cell surfaces were observed but there were pores on each affected cells. Affected but intact cells were swollen at one end and tapered towards the other end. Contrary to what were obtained with methanol extract-treated *P. vulgaris*, its treatment with aqueous extract (Fig. 3D) resulted in many cells having significant puffy-like depressions with few cells having a pore at the polar end while many cells were unaffected and intact.

On treating the *B. pumilis* cells with acetone extract (Fig. 4B), a great elongation of the cells occurred. Each cell was collapsed to become flat-shaped dead cells with irregular edges. In methanol treated cells (Fig. 4C), the cell elongation effects were greater than was observed with acetone extract-treated isolates and no pores or the presence of aggregated cellular material was recorded. In aqueous extract treated cells (Fig. 4D), cell elongations and death almost equal to that of methanol was observed in the affected cells. The effects of methanol and aqueous extracts

on the cells were similar but seemed greater than those observed with acetone treated isolates. In Fig. 5B, C and D, *S. aureus* treated with both aqueous and alcoholic extracts were not lysed or remained intact but had rough surfaces and increases in sizes.

These results showed that the different extracts had different effects on the different extract-treated isolates, while varied degree of morphological changes were observed on the affected cells and bacterial populations. Of notes are the shapes, colours, distribution and locations of the extruded cellular materials on the bacterial cell surfaces. The diversity in the shape, size, distribution and location of the extruded cellular materials showed that the extracts contain different phytochemicals which are target site specific. The points at which the cellular materials were located on the cells surfaces signify the points at which there are leakages resulting from the pore forming activities of the extracts. Having extruded cellular materials scattered on some cells' surfaces and/or located and congealed at either or both polar ends of the cells showed that the antibacterial agents in the extracts were able to attack a particular component of the bacterial cells located at the points of extrusions. The differences in the colour and appearances of the extruded cellular materials at different locations on the cell surfaces may imply that different components of the extracts was able to attack different cellular and cytoplasmic materials in a particular bacterial strain resulting in the colour variations. Comparative analysis of the differently affected cell morphology showed that, while some cells had extruded cellular materials being attached to cell surfaces, other cells had their cellular materials totally washed into the medium environments. The congealed cellular materials may be attributed to the astringent property of the tannin contents of the plant.

A comparative analysis of the protein leakage ability of acetone, methanol and aqueous extracts of *A. mearnsii* was considered in this study. Each data in Table 1 represented combined

Mean ± Standard deviation of triplicate samples of protein leakages from each bacteria treated with each extract and leakages was analyzed with consideration for the most effective extract. The protein leakage ability of the extracts was concentration dependent as the effects of 2 x MICs was significantly higher than those obtained from the MICs of the extracts. On treating the bacterial strains with the MICs and 2 x MICs of each extract, the ability of the extracts to release protein leakages from *S. aureus* (471.17 ± 41.24 µg/ml) ($p = 0.003$ @ MICs and $p = 0.045$ @ 2 x MICs), *P. vulgaris* (419.17 ± 19.18 µg/ml) ($p = 0.001$ @ MICs) and *B. pumilis* (481.00 ± 12.06 µg/ml) ($p = 0.001$ @ MICs and $p = 0.014$ @ 2 x MICs) were significantly different from each other while those obtained from other extract-treated organisms were not significantly different from each others. Of the three extracts at 2 x MIC, aqueous extract released the highest quantity of protein from *S. aureus* (577.17 ± 35.32 µg/ml), followed by acetone extract (*S. aureus* – 492.75 ± 66.71 µg/ml) and the least protein leakage, at this concentrations, was from the methanol extract (476.83 ± 45.93 µg/ml). Of the MIC-treated strains, aqueous extract released more protein from *P. vulgaris* (419.17±19.18 µg/ml) and *B. pumilis* (481.00 ± 12.06 µg/ml) at 2 x MIC in comparison to those of other organisms apart from *S. aureus*. For the methanol extract and apart from the *S. aureus*, highest protein was released from *S. flexneri* (364.59 ± 15.46 µg/ml) and *E. coli* (443.02 ± 39.74 µg/ml) respectively with the MIC and 2 x MIC treatments in comparison with other isolates. The acetone extract, however, showed that its protein leakage ability, at both MIC and 2 x MIC, was the highest in *S. aureus*, followed by that of *B. pumilis* > *E. coli* > *S. flexneri* > *P. vulgaris* (Table 1).

In Table 2, each data represented combined Mean ± Standard deviation of triplicate samples of protein leakages from each organism treated with each extract with a consideration for the sampling time interval. The protein leakage ability of the different extract at different

sampling time interval showed that the activities of the extracts to effect protein leakages were time dependent and significantly different on hourly basis (Table 2). However, with the exception of the protein leakages from *S. flexneri*, at both MIC and 2 x MIC, there were no significant differences in the protein leakage ability of the different extracts from the other bacterial strains by considering the different sampling time intervals. By pairing the bacteria and making a multiple comparison of each paired treated bacteria, Bonferoni's multiple comparison test comparing protein leakages between two paired bacteria over the sampling periods showed that protein leakages from *E. coli* compared with *S. aureus*, *E. coli* and *B. pumilis*, *S. aureus* and *P. vulgaris*, *S. aureus* and *B. pumilis*, *S. aureus* and *S. flexneri* as well as *B. pumilis* and *S. flexneris* were significantly different over the sampling periods while those of *E. coli* compared with *P. vulgaris*, *E. coli* and *S. flexneri*, *B. pumilis* and *P. vulgaris* as well as *P. vulgaris* and *S. flexneri* were not significantly different (Table not shown). From Table 3 showing each data representing combined Mean ± Standard deviation of triplicate samples of protein leakages from each organism treated with each extract to indicate the susceptibility of each bacteria to each extract in the treatment periods, *S. aureus* was the most inhibited by all the extracts by having the highest quantity of protein leaked by all the extracts. However, while *B. pumilis* (319.00 ± 21.62 µg/ml) was the least affected as indicated by the quantity of protein leaked by MIC of methanol extract, other organisms exhibited varied degrees of susceptibility to the different extracts. The statistical analysis showed that the susceptibility of each bacterial strain to aqueous and methanol extracts was significantly different while their susceptibility to acetone extract was not significantly different from each other. On the basis of the amount of protein leaked, the bacteria were more susceptible to aqueous extract, followed by methanol extract while the acetone extract was the least.

The lipid leakage ability of the different extracts was also investigated and the results were as presented in Tables 4 – 6. In Table 4, each data represented combined Mean ± Standard deviation of triplicate samples of lipid leakages from each organism treated with each extract and leakages was analyzed with consideration for the most effective extract. The ability of the extract to cause lipid leakages was concentration dependent and significantly different at MICs for extract treated *S. aureus* ($p = 0.038$) and *P. vulgaris* ($p = 0.000$ @ MICs and 2 x MICs) while the effects of the extracts were not significantly different from each other for the rest of the bacterial bacteria treated with the different MICs and 2 x MICs. Generally from the different extracts at MICs, the Mean ± Standard deviation of lipid leakages from the organism ranged between 81.85 ± 40.12 µg/ml and 224.65 ± 31.25 µg/ml while at 2 x MICs, the lipid leakages ranged between 109.63 ± 47.47 µg/ml and 309.40 ± 40.42 µg/ml. For methanol, lipid leakages range from 109.06 ± 32.63 µg/ml to 224.65 ± 31.25 µg/ml at the MIC and 140.49 ± 45.22 µg/ml to 309.40 ± 40.42 µg/ml at the 2 x MICs, followed by aqueous extract with lipid leakages ranging from 81.85 ± 40.12 µg/ml to 169.32 ± 64.30 µg/ml at the MIC and 109.63 ± 47.27 µg/ml to 207.78 ± 69.06 µg/ml at the 2 x MICs and least with acetone extract with lipid leakages ranging from 88.61 ± 48.43 µg/ml to 152.71 ± 27.82 µg/ml at the MIC and 128.12 ± 52.81 µg/ml to 229.46 ± 31.07 µg/ml at the 2 x MICs. With consideration for their lipid leakage ability at the MICs, methanol extract may have indicated higher antibacterial activity, followed by aqueous extract and the least activity from the acetone extract.

From Table 5, each data represented combined Mean ± Standard deviation of triplicate samples of lipid leakages from each extract-treated organism. The lipid leakage ability of the extracts was analyzed with consideration for their activity over the sampling periods to determine their effectiveness. Here, the lipid leakage ability of all the extracts was time

dependent and the ability of the extracts to cause leakages in the sampling period was significant different with time in *E. coli* and *S. flexneri* treated at the different concentrations as well as *S. aureus* and *B. pumilis* treated with the 2 x MICs of the extracts. The lipid leakage ability of the extracts were not significantly different with time in *B. pumilis* and *S. aureus* treated at MICs and *P. vulgaris* treated at both concentrations. Generally, the Mean ± Standard deviation of the lipid leakages ranged between 58.57 ± 14.86 µg/ml and 214.22 ± 42.73 µg/ml in the MICs treated bacteria while at 2 x MICs, the lipid leakages ranged between 84.44 ± 1.36 and 270.81 ± 15.31 µg/ml over the sampling period. At 30 min sampling period, the lipid leakages ranged between 58.57 ± 14.86 and 178.30 ± 33.70 µg/ml while at 120 min, the lipid leakage ranged between 145.49 ± 9.35 and 270.81 ± 15.31 µg/ml. Determining the susceptibility of the different bacterial strains with their lipid leakage profiles as indicated by the combined Mean ± Standard deviation of triplicate samples of lipid leakages from each extract-treated organism in Table 6, the varied lipid leaked at the different concentrations showed the degree of susceptibility of each bacterial strain to the different extracts. With the exception of bacterial susceptibility to the aqueous extract which was not significantly different based on the quantity of protein leaked, the bacterial susceptibility to methanol and acetone extracts was significantly different. However, the susceptibility of these bacteria to the different extracts based on the quantity of lipid leaked was in the following order: *P. vulgaris* > *S. aureus* > *E. coli* > *B. pumilis* > *S. flexneri*. With different p = values, the bacteria may be said to said to more susceptible to methanol extract, followed by acetone extract and least susceptible to aqueous extract.

Determining the protein and lipid leakages showed a possible mechanism of the antibacterial activity of the different extracts. The results showed that the ability of the extracts to cause leakages may or may not be significantly different from each other depending on the types

of bacteria being treated. However, while the leakages are time and concentration dependents, the degree of each leakage from each bacterial strain indicated the degree of antibacterial activity exerted by the extracts. Consequently, it was observed that while aqueous extract was the most active in causing protein leakages, methanol extract was the leading cause of lipid leakages. The ultrastructural changes and the leakages resulting from treating the bacteria strains with the different extracts showed the dynamic natures of the different extracts as antibacterial agents.

13.4 Discussion

In eliminating the side effects of antibacterial agents, studying their modes of action becomes essential to foresee the damages done to the microorganisms while the knowledge of the mechanisms of action of these agents could help to define or understand and predict the effects of the antibacterial agents in some metabolic pathways. In this study, the morphological changes and the observed leakages showed rapid killing, significant membrane depolarization that resulted in leakages and efflux of disintegrated cellular materials. The disruption in the outer wall and cytoplasmic membranes especially at the polar regions of the cells, whole cell collapse and presence of extruded cellular materials situated close to the collapsed end of the cytoplasm, and eventual cell death were observed. The observed distinct morphological changes such as cell elongation and roughening of the surfaces suggested that the extract may have interfered with bacterial cell synthesis leading to cell deformations. These findings agree with a previous report on food-borne pathogens treated with epigallocatechin gallate (Si et al., 2006). These responses in agreement with other researches on the antimicrobial activity of phenolic compounds affecting cellular membranes (Davidson 1997; Ultee et al., 1999) could be attributed to the membrane

permeating agents present in the different extracts as observed in spheroplasts of Gram-negative and intact Gram-positive bacteria by Papo et al. 2002.

With respect to the responses obtained as a result of treating bacteria with plant materials having antibacterial properties, several mechanisms of action including destabilization of cytoplasmic and plasma membranes, inhibition of extracellular microbial enzymes and metabolisms, and deprivation of the substrate required for microbial growth (Puupponen-Pimia et al., 2004) have been proposed. Generally, the antimicrobial properties of plants are due to many active phytochemicals including quinine, berberine, tannins, alkaloids, flavanoids, terpenoids, carotenoids, coumarins, curcumines and saponins (Gurdip et al., 2008; Handa et al., 2008). Vaara (1992) and Tsuchiya and Iinuma (2000) reported the ability of their phytochemicals to disrupt the plasma membrane by localized hyper-acidification and disruption of membrane transport and/or electron transport. Sikkema et al. (1995) implicated their ability in causing structural and functional damages to plasma membrane. Damage to the membrane functions, also, has been proposed as a mechanism of action for phenols and phenolic compounds (Aizenman, 1978; Mitscher, 1978; Keweloh et al., 1990; Sikkema et al., 1995; Sashidhar NS, 2002). Tannins have been found to form irreversible complexes with proline-rich proteins (Hagerman and Butler, 1981), cause polymerization through oxidation reactions (Field and Lettinga, 1992) and inactivate microbial adhesions, enzymes, cell envelope transport proteins, and mineral uptake (Min et al., 2003). While saponins are implicated in interference with membrane integrity (Bangham and Horne, 1962), thymol inhibits ATP-generating pathways and causes membrane perforation as its principal mode of action (Shapiro and Guggenheim, 1995). In acting on the permeability barriers in cytoplasmic membrane, an increase in cytoplasmic membrane permeability is shown by leakages of the important intracellular material (Denyer and

Hugo, 1991) and many of these phytochemicals have been reported to cause leakages of ions, adenosine triphosphate (ATP), nucleic acids and amino acids (Tranter et al., 1993; Gonzalez et al., 1996; Tahara et al., 1996; Tassou et al., 2000). In agreement with these earlier reports, the observed morphological changes and leakages, in this study, may therefore, be attributed to the effect(s) of one or many phytochemicals acting singly or in synergy with others to effect different mechanisms of actions since a mixture of phenolic compounds acts synergistically better than its individual components separately (Havsteen, 2002).

Considering the leakages recorded in this study, lipid peroxidation, a continuous process in bacterial cells maintained at low level by antioxidants (Munkres, 1976), may be implicated. Although phytochemicals with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems (Cao et al., 1996) and *Acacia mearnsii* possessed antioxidant properties (Olajuyigbe and Afolayan, 2011), the antioxidant activities could have enhanced membrane free radical reactions beyond the protective level and cause lipid peroxidation (Cerutti, 1985). Consequentially, from the lipid and protein leakages in the bacterial cells, it may be hypothesized in agreement with Cerutti (1985), as a possible mechanism of action, that the extracts of *A. mearnsii*, attacked the DNA, proteins, lipids, polyunsaturated fatty acids of cells and initiate lipid peroxidation in the cytoplasmic membranes. As a result of these possible lipid peroxidation whose products are toxic to cells (Ashkar et al., 1981; Ueda et al., 1985), polysaccharide fatty acids may have been biodegraded to a variety of reactive products like (C_3 - C_9) aldehydes which may include 2-propenal, 4-hydroxynonenal and malondialdehyde which are able to damage proteins (Humpries and Sweda, 1998). These aldehydes could have attacked the base and sugar moieties of DNA to produce single and double strand breaks in the backbone, allowing joining of base and sugar groups and cross-links to other molecules to

obstruct replication (Cabiscol et al., 1994; Grant et al., 1998). The oxidation of protein could have resulted in damages which may include oxidation of sulfhydryl groups, reduction of disulphides, oxidative adduction of amino acids residues close to metal-binding sites through metal catalyzed oxidation, reactions with aldehydes, modification of prosthetic groups of metal groups or metal clusters, protein-protein cross-linking and peptide fragmentation. While some amino acids are oxidized to carbonyl derivatives (Berlett and Stadtman, 1997), 4-hydroxynonenal produced during the peroxidation of polyunsaturated fatty acids reacts with the sulfhydryl groups of protein to form stable covalent thiolether adducts carrying a carbonyl function (Humpries and Sweda, 1998; Szweda et al., 1993), highly oxidized proteins inhibit preoteases to degrade other oxidized proteins (Dean et al., 1997; Grune et al., 1997) while *in vivo* protein carbonylation is generally used as a marker of irreversible and unrepairable oxidative protein damage (Nystrom, 2005). As a result of these effects, the lipid peroxidation and protein oxidation proposed as the possible mechanisms of action of these extracts could have caused degradation of the cell wall, damage to cytoplasmic membrane proteins and lipids as well as leakages of cell contents resulting in morphological changes observed and deaths of the bacterial cells.

13.5 Conclusion

In conclusion, this study showed that different extracts of *A. mearnsii*, used in this study, had bactericidal effects against the test isolates as indicated by the ultrastructural changes and leakages leading to the disruption of the cytoplasmic membranes of the bacterial cells and populations. While the possibility of using different mechanisms of action, due to the presence of

different phytochemicals, may not be underestimated, indicating lipid peroxidation and protein oxidation as possible mechanisms of antibacterial action may not be an overstatement. Resulting from being able to disrupt the outer membranes, the extracts were considered being hydrophobic as they were able to partition the lipids of bacterial cell membranes to effect ultrastructural changes and rendered the cells more permeable to the influx of the phytochemicals in the extracts. The differences in the ultrastructural damages and the leakages effected by the different extracts could be due to the differences in the type, quantity and degree of bioactivity of each of the phytochemical components extracted by each solvent. Although this study implicated lipid peroxidation and protein oxidation as the possible mechanisms of action resulting from the possibility that the antioxidant properties of this plant could have gone beyond protective levels due to high concentrations, further quantitative and determinative studies on the proteins and lipid components released from cells would be able to elucidate the sources and types of released cellular materials.

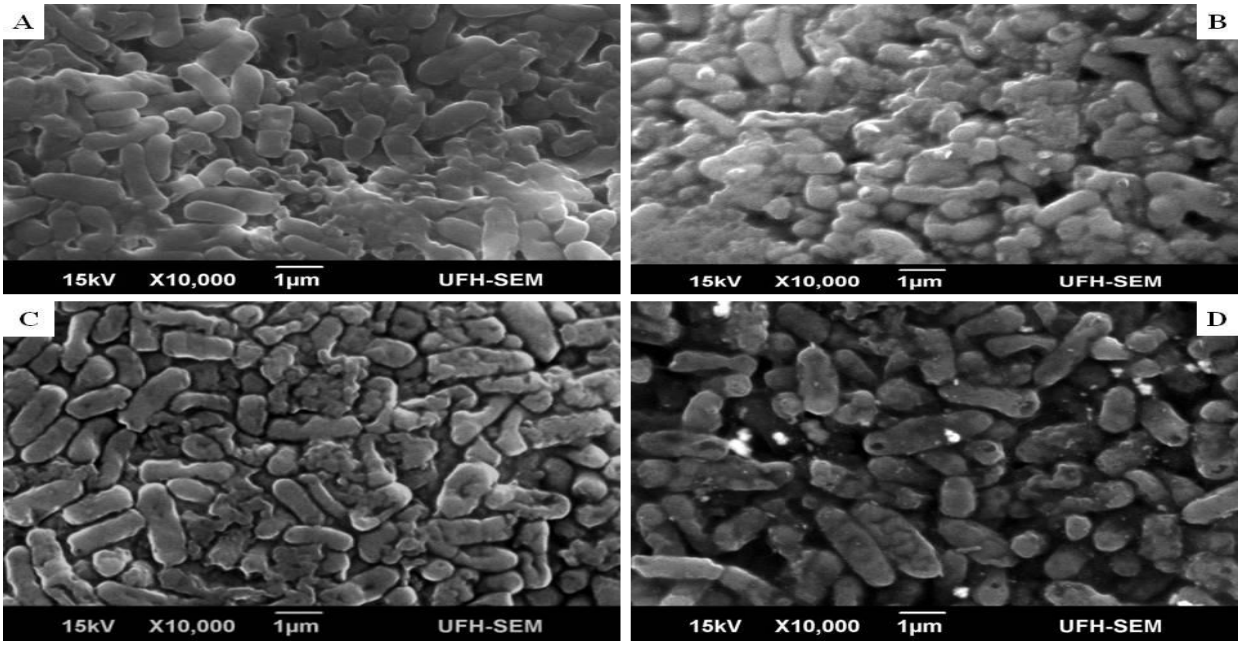

Figure 13.1: Influence of alcoholic and aqueous extracts of *A. mearnsii* on *E. coli*. A = Untreated *E. coli*; B = *E. coli* treated with acetone extract; C = *E. coli* treated with methanol extract; D = *E. coli* treated with aqueous extract

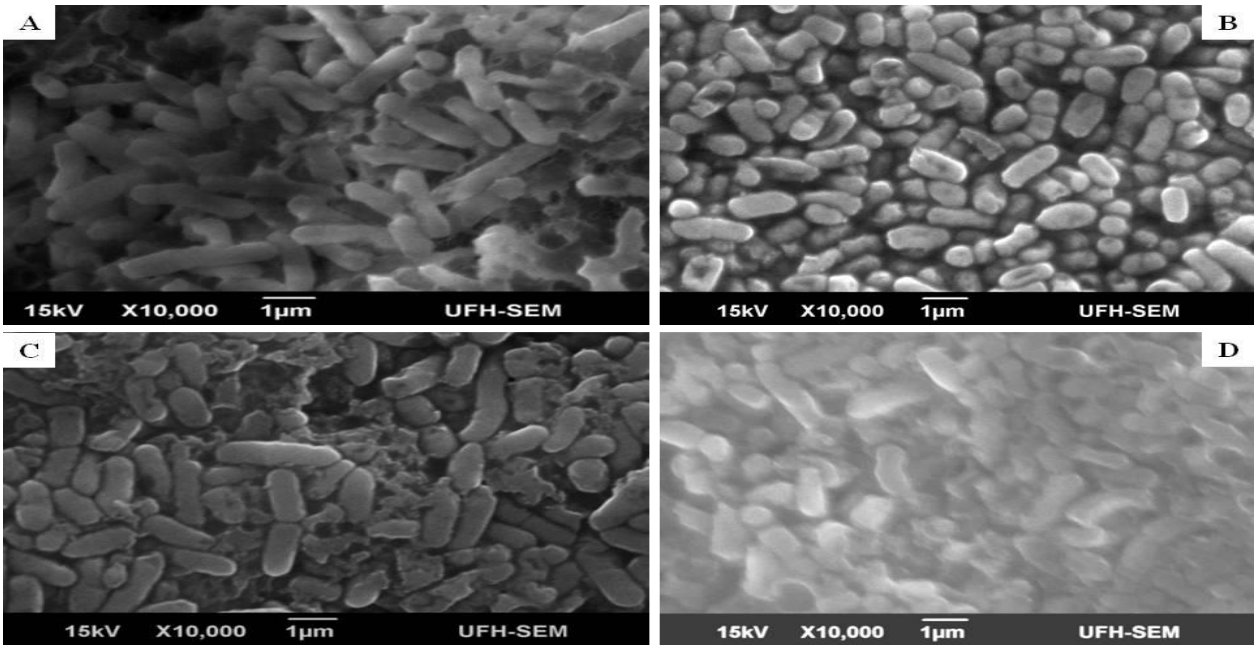

Figure 13.2: Influence of alcoholic and aqueous extracts of *A. mearnsii* on *S. flexneri*. A = Untreated *S. flexneri*; B = *S. flexneri* treated with acetone extract; C = *S. flexneri* treated with methanol extract; D = *S. flexneri* treated with aqueous extract.

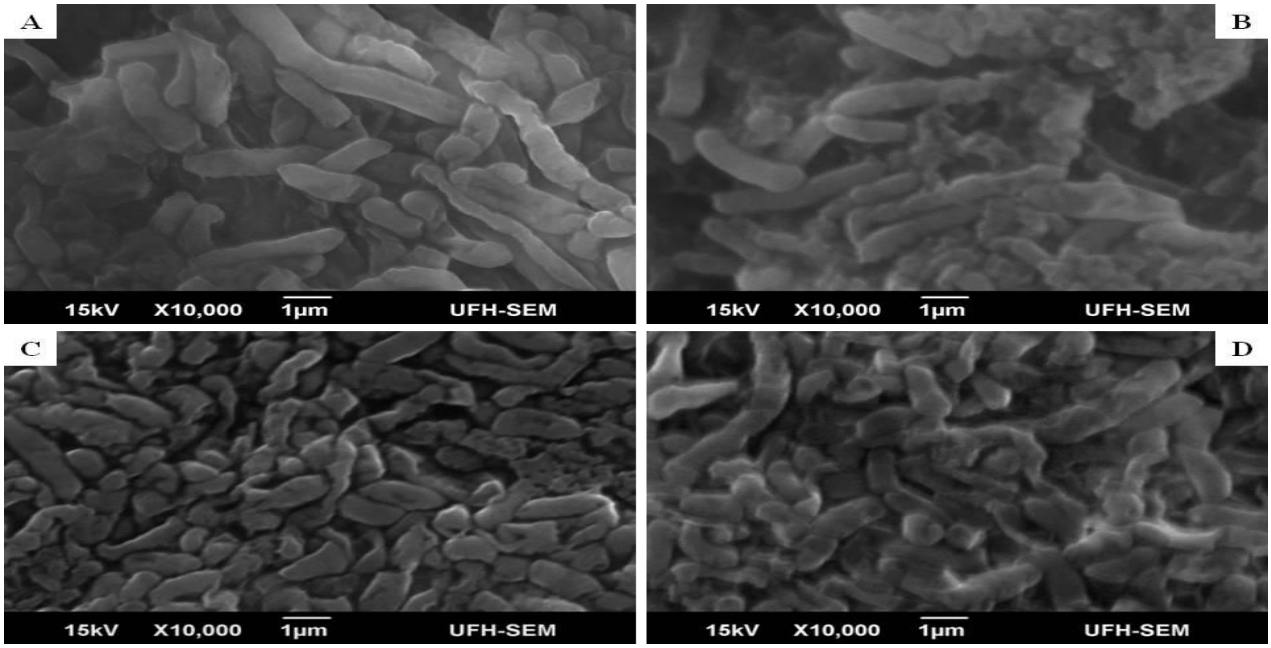

Figure 13.3: Influence of alcoholic and aqueous extract of *A. mearnsii* on *P. vulgaris*. A = Untreated *P. vulgaris*; B = *P. vulgaris* treated with acetone extract; C = *S P. vulgaris* treated with methanol extract; D = *P. vulgaris* treated with aqueous extract.

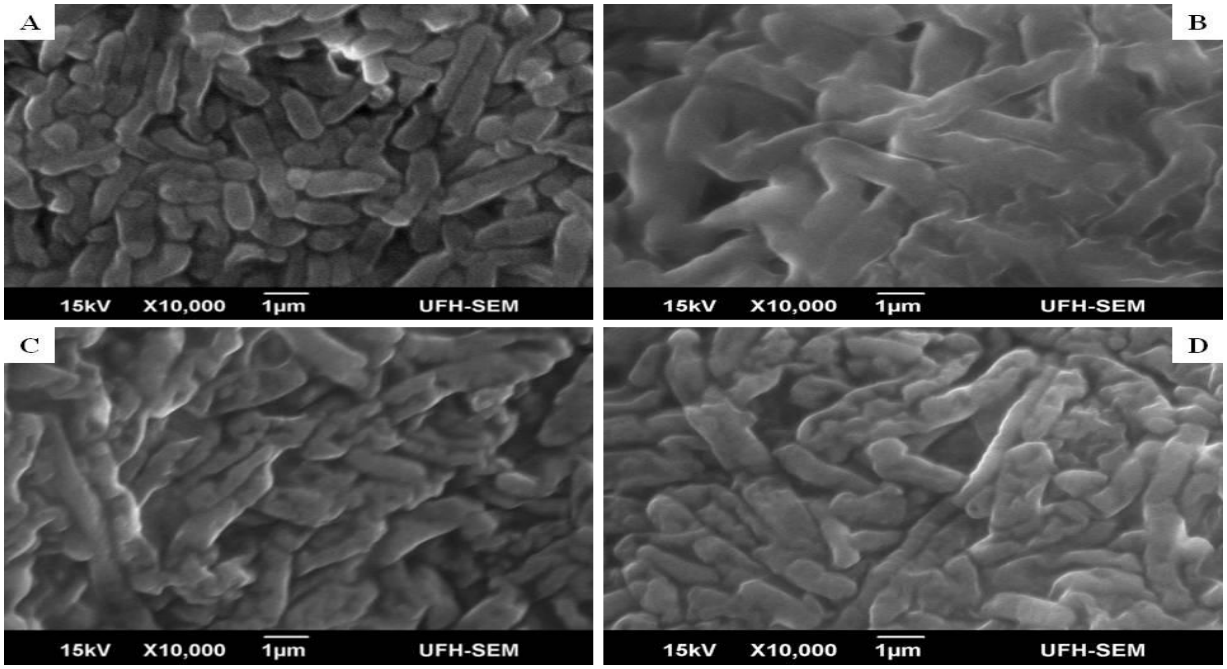

Figure 13.4: Influence of alcoholic and aqueous extracts of *A. mearnsii* on *B. pumilis*. A = Untreated *B. pumilis*; B = *B. pumilis* treated with acetone extract; C = *B. pumilis* treated with methanol extract; D = *B. pumilis* treated with aqueous extract.

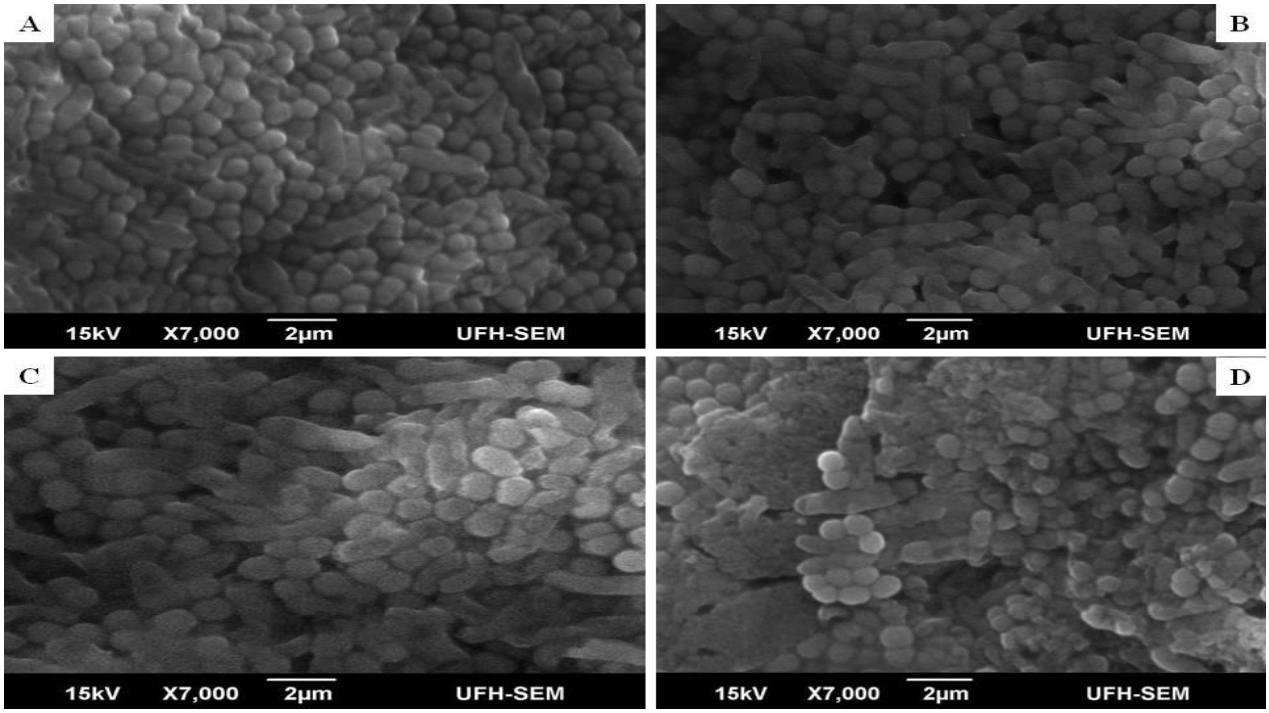

Figure 13.5: Influence of alcoholic and aqueous extracts of *A. mearnsii* on *S. aureus*. A = Untreated *S. aureus*; B = *S. aureus* treated with acetone extract; C = *S. aureus* treated with methanol extract; D = *S. aureus* treated with aqueous extract.

Table 13.1: Comparative analysis of the protein leakage ability of each extract from each bacterial strain

	Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain									
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Extract	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
Methanol	357.08 ± 29.75	443.02 ± 39.74	394.58 ± 15.58	476.83 ± 45.93	319.00 ± 21.62	397.66 ± 32.18	364.50 ± 20.68	410.91 ± 27.93	364.59 ± 15.46	404.75 ± 20.07
Aqueous	349.42 ± 41.03	393.00 ± 37.21	471.17 ± 41.24	577.17 ± 35.23	413.09 ± 13.22	481.00 ± 12.06	419.17 ± 19.18	460.83 ± 31.34	372.16 ± 26.8	408.58 ± 31.21
Acetone	359.08 ± 40.08	453.83 ± 44.72	386.83 ± 14.17	492.75 ± 66.71	385.83 ± 33.78	489.42 ± 55.73	351.34 ± 13.10	426.83 ± 44.83	359.02 ± 24.79	434.00 ± 36.89
	p value = 0.928	P value = 0.133	P value = 0.003	p value = 0.045	p value = 0.001	p value = 0.014	p value = 0.001	p value = 0.182	p value = 0.726	p value = 0.371

Table 13.2: Comparative analysis of the protein leakage ability of the extracts from each bacterial strain at different time interval

Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain at different time interval										
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Time	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
1 h	308.89 ± 16.01	371.91 ± 29.95	390.33 ± 34.53	459.33 ± 68.44	341.89 ± 55.17	412.44 ± 60.65	355.67 ± 35.51	391.22 ± 24.51	337.67 ± 5.21	381.00 ± 18.50
2 h	344.44 ± 48.81	437.00 ± 26.31	412.67 ± 41.64	500.78 ± 56.50	372.56 ± 40.91	457.11 ± 44.22	375.89 ± 30.72	425.44 ± 27.81	360.89 ± 17.99	414.44 ± 10.56
3 h	376.67 ± 20.83	449.33 ± 35.10	422.67 ± 37.60	525.22 ± 50.85	384.33 ± 45.62	464.33 ± 46.94	385.22 ± 40.80	441.22 ± 32.07	374.48 ± 3.21	418.67 ± 12.34
4 h	371.33 ± 29.08	461.56 ± 40.61	444.44 ± 72.79	577.00 ± 44.70	391.78 ± 55.49	490.22 ± 64.69	396.56 ± 37.52	473.56 ± 24.14	388 ± 9.85	449.00 ± 32.04
	p value = 0.098	p value = 0.044	p value = 0.617	p value = 0.151	p value = 0.641	p value = 0.424	p value = 0.588	p value = 0.035	p value = 0.002	p value = 0.022

Table 13.3: Comparative analysis of the bacterial susceptibility to each extract based on quantity of protein leaked

	Mean ± Standard Deviation of the bacterial susceptibility in response to extract treatments					
Organisms	AMM-MIC	AMM-2xMIC	AMW-MIC	AMW-2xMIC	AMA-MIC	AMA-2xMIC
E. coli	357.08 ± 29.75	443.02 ± 39.74	349.42 ± 41.03	393.00 ± 37.21	359.08 ± 40.08	453.83 ± 44.72
S. aureus	394.58 ± 15.58	476.83 ± 45.93	471.17 ± 41.24	577.17 ± 35.23	386.83 ± 14.17	492.75 ± 66.71
B. pumilis	319.00 ± 21.62	397.66 ± 32.18	413.09 ± 13.22	481.00 ± 12.06	385.83 ± 33.78	489.42 ± 55.73
P. vulgaris	364.50 ± 20.68	410.91 ± 27.93	419.17 ± 19.19	460.83 ± 31.34	351.34 ± 13.10	426.83 ± 44.83
S. flexneri	364.59±15.46	404.75 ± 20.07	372.16 ± 26.80	408.58 ± 31.21	359.02 ± 24.79	434.00 ± 36.89
	p value = 0.003	p value = 0.028	p value = 0.000	p value = 0.000	p value = 0.256	p value = 0.266

Key: AMM = Methanol extract; AMW = Aqueous extract; AMA = Acetone extract

Table 13.4: Comparative analysis of the lipid leakage ability of each extract from each bacterial Strain

	Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain									
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Extract	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
Methanol	169.75 ± 40.68	210.15 ± 50.65	216.13 ± 28.70	252.32 ± 35.56	149.00 ± 28.14	172.75 ± 25.31	224.65 ± 21.35	309.40 ± 40.42	109.06 ± 32.63	140.49 ± 45.22
Aqueous	169.32 ± 64.30	207.78 ± 69.06	159.44 ± 47.42	201.16 ± 39.11	124.40 ± 27.70	194.16 ± 51.75	81.85 ± 40.12	109.63 ± 47.47	107.31 ± 32.50	134.33 ± 34.64
Acetone	152.71 ± 27.82	229.46 ± 31.07	148.10 ± 15.29	208.57 ± 39.66	88.61 ± 48.43	133.71 ± 48.43	90.68 ± 41.45	128.12 ± 52.81	98.00 ± 52.60	148.32 ± 61.19
	p value = 0.844	P value = 0.819	P value = 0.038	p value = 0.178	p value = 0.111	p value = 0.192	p value = 0.000	p value = 0.000	p value = 0.918	p value = 0.920

Table 13.5: Comparative analysis of the lipid leakage ability of the extracts from each bacterial strain at different time interval

Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain at different time interval										
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Time	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
30 min	113.03 ± 7.46	154.04 ± 29.62	140.97 ± 33.89	178.30 ± 33.74	79.02 ± 43.66	114.73 ± 35.64	87.71 ± 92.18	123.1 ± 119.7	58.57 ± 14.86	84.44 ± 1.36
60 min	152.77 ± 12.39	203.11 ± 27.72	166.57 ± 35.55	205.80 ± 24.46	109.48 ± 33.22	157.90 ± 34.25	128.71 ± 86.69	172.7 ± 111.1	88.10 ± 17.73	126.04 ± 9.01
90 min	175.67 ± 10.71	235.22 ± 8.27	180.46 ± 46.37	232.08 ± 28.72	134.77 ± 29.92	180.72 ± 29.50	144.35 ± 76.46	200.9 ± 101.1	127.00 ± 2.07	162.71 ± 8.65
120 min	214.22 ± 42.73	270.81 ± 15.31	210.23 ± 44.27	266.58 ± 25.78	159.41 ± 17.37	214.14 ± 33.20	168.81 ± 64.88	232.9 ± 110.0	145.49 ± 9.35	191.00 ± 30.50
	p value = 0.004	p value = 0.001	p value = 0.279	p value = 0.027.	p value = 0.075	p value = 0.035	p value = 0.672	p value = 0.673	p value = 0.000	p value = 0.000

Table 13.6: Comparative analysis of the bacterial susceptibility to each extract based on quantity of lipid leaked

	Mean ± Standard Deviation of the bacterial susceptibility in response to extract treatments					
Organisms	AMM-MIC	AMM-2xMIC	AMW-MIC	AMW-2xMIC	AMA-MIC	AMA-2xMIC
E. coli	169.73 ± 40.68	210.15 ± 50.65	169.32 ± 64.30	207.78 ± 69.06	152.71 ± 27.82	229.46 ± 31.07
S. aureus	216.13 ± 28.70	252.32 ± 35.56	159.44 ± 47.42	201.16 ± 39.11	148.10 ± 15.29	208.57 ± 39.66
B. pumilis	149.00 ± 28.14	172.75 ± 25.31	124.40 ± 27.70	194.16 ± 51.75	88.61 ± 48.43	133.71 ± 48.43
P. vulgaris	224.65 ± 21.35	309.40 ± 40.42	81.85 ± 40.12	109.63 ± 47.47	90.68 ± 41.45	128.12 ± 52.81
S. flexneri	109.06 ± 32.63	140.49 ± 45.22	98.00 ± 52.60	148.32 ± 61.19	107.31 ± 32.50	134.33 ± 34.64
	p value = 0.000	p value = 0.000	p value = 0.088	p value = 0.100	p value = 0.047	p value = 0.008

Key: AMM = Methanol extract; AMW = Aqueous extract; AMA = Acetone extract

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CHAPTER 14

General Discussions, Recommendations and Conclusions

General Discussions, Recommendations and Conclusions

14.1 General Discussions

Since a large number of medicinal plants and associated indigenous uses still await proper documentation (Tabuti et al., 2003), this study investigated medicinal plants used in the treatment of gastrointestinal disorders in South Africa. This investigation documented the traditional use of medicinal plants used in the treatment of these disorders. It was also meant to disseminate the therapeutic efficacy of the identified plants to pave the way for preparation of acceptable medicine and to reduce the pressures on over exploited species such as *Gunnera perpensa* L. (Xuma and Naidoo, 2007) and *Curtissia dentata* (Shai et al., 2009). Of the identified medicinal plants, *Acacia mearnsii* De Wild and *Ziziphus mucronata* subsp. *mucronata* Wild were chosen for this research.

The main objective of this study was to validate the ethnomedicinal importance of *A. mearnsii* and *Z. mucronata* subsp. *mucronata* in the treatment of gastrointestinal disorders such as diarrhoea and dysentery. This objective would be achieved by investigating their phytochemicals and antioxidant activities, determining their *in vitro* antimicrobial activities, determining their cytotoxicity effects using brine shrimps lethality assay, investigating interactions between their crude extracts and some of the first line antibiotics as well as determining the mechanisms of action(s) of their extracts and making comparative analysis.

Although many members of the Acacia *genus* and Ziziphus *genus* have been implicated in the treatment of various infections, there is a dearth of information on *Acacia mearnsii* and *Ziziphus mucronata* subsp. *mucronata* belonging to the same family, *Fabaceae*, investigated in this study. This study is just scientifically documenting and publishing information on their ethnotherapeutic importance. To validate their ethnomedicinal importance, twenty seven bacteria

strains, including 13 species, made up of 16 Gram-negative and 11 Gram-positive bacteria were screened for antibacterial susceptibility to aqueous, ethanol, methanol and acetone extracts of these two plant species. In this study, the plants exhibited varied degrees of broad spectrum of antibacterial and antifungal activities at the concentrations tested. Generally, for bacteria treated with *A. mearnsii* extracts, the minimum inhibitory concentrations (MICs) of methanol extracts ranged between 0.039 mg/ml and 1.25 mg/ml; that of acetone and ethanol extracts ranged between 0.039 mg/ml and 0.625 mg/ml while those of aqueous extract ranged between 0.156 mg/ml and 0.625 mg/ml. With the exception of acetone extract having MICs greater than 1.0 mg/ml for *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* KZN, all the isolates had MICs less than 0.7 mg/ml. Ethanol extract exhibited the greatest degree of antibacterial activity against all the test isolates. For all the bacteria treated with *Z. mucronata* subsp. *mucronata* extracts, the MICs of methanol extract ranged between 0.039 mg/ml and 1.25 mg/ml, acetone extract ranged between 0.078 mg/ml and 1.25 mg/ml, ethanol extract ranged between 0.078 mg/ml and 0.625 mg/ml while the aqueous extract of *Z. mucronata* was not effective at the highest concentration of 20 mg/ml used against all the bacteria. While *Enterobacter cloacae* ATCC 13047 had MIC greater than 1 mg/ml in methanol extract, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 6538 had MICs greater than 1 mg/ml in acetone extract but more susceptible to ethanol extract. While all other isolates were highly susceptible to the different extracts of *Z. mucronata* subsp. *mucronata* and had MICs less than 0.7 mg/ml, its ethanol extract was also found to exhibit the highest degree of antibacterial activity.

Despite the chemical structure of the outer membrane in Gram-negative bacteria and the presence of teichoic acids in the cell wall of Gram-positive bacteria (Mackey, 2000), the extracts from the two plant species were able to achieve significant bactericidal effects on all the tested

bacterial strains. In most cases, the minimum bactericidal concentrations (MBCs) of the different extracts indicated high percentages of bactericidal than bacteriostatic. Lower MIC and MBC values indicate higher efficacy (Cowan, 1999). The phytochemicals were classified as effective antimicrobial agents when susceptibility tests had MICs in the range of 100 – 1000 µg/ml (Simões et al., 2009). The MIC/MBC ratios or $MIC_{indices}$ showed that these extracts were bacteriostatic at lower concentrations and bactericidal at higher concentrations against the bacterial isolates. Where MIC equals MBC, the bactericidal potency with a broad spectrum and great therapeutic potential of the plant is indicated. The MBC values suggested that a biocidal effect of the extracts could be expected on most of the tested organisms (Carbonnelle et al., 1987; Mims et al., 1993).

A comparison of the antibacterial activities of the two plants showed that *A. mearnsii* was more effective than *Z. mucronata* subsp. *mucronata*. Of significance are the antibacterial activities exhibited by the ethanol and aqueous extract of *A. mearnsii*. These are the basic solvents commonly used by the rural dwellers in the preparation of traditional medicines. To forestall fermentation, ethanol extract is mostly adopted locally while plant extracts from organic solvents have been found to produce more consistent antimicrobial activity compared to those of water extracts (Parekh et al., 2005). The antibacterial activities of the ethanol and aqueous extracts of *A. mearnsii* showed their potential therapeutic efficacies in the treatment of gastrointestinal infections, especially dysentery and diarrhoea, for which the plants have been implicated in ethnobotanical survey. Although *Z. mucronata* subsp. *mucronata* was recorded more in the treatment of gastrointestinal disorders than the *A. mearnsii*, this study showed that alcohol extracts of *A. mearnsii* would be more effective in the treatment of these infections. This is in agreement with Parekh et al. (2006) who indicated that organic solvent extract were more

active than aqueous extracts. While the antibacterial activity of aqueous extract of *Z. mucronata* subsp. *mucronata* agreed with the earlier reports (Nang et al., 2007; Rangama et al., 2009) indicating that aqueous extracts of some plants were mostly not effective as antibacterial agents, this study showed that aqueous extract of *A. mearnsii* was almost as effective as its alcoholic extracts. It had MICs ranging between 0.156 mg/ ml and 0.625 mg/ml of which 0.625 mg/ml was the maximum MICs for a high percentage of the bacteria tested with *A. mearnsii* extracts.

Also, the bacteria in this study exhibited varied degree of susceptibility to the plants' extracts, their susceptibility may be attributed to their intrinsic morphological and physiological features. The variation may be due to the differences in their cell wall composition and/or genetic contents of their plasmids (Yao and Moellering, 1995; Karaman et al., 2003). That many Gram-negative bacteria were highly susceptible to the extracts of both plants may suggest that the phytochemicals in the plants were able to overcome the permeability barrier provided by the cell wall and the membrane combined resistance mechanisms including efflux of antibacterial agents (Adwan and Abu-Hasan, 1998).

To investigate the antifungal activities, the two plants were tested against *Candida krusei*, *Candida albicans*, *Candida rugosa*, *Candida glabrata* (ATCC 2001), *Absidia corymbifera*, *Fusarium sporotrichioides*, *Trichophyton tonsurans*, *Trichophyton mucoides* (ATCC 201382), *Penicillium notatum*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus.* These fungi were included in the investigation to verify the broad spectrum antimicrobial potentials of the two plants. The plants showed that the antifungal activity of *A. mearnsii* ranging from 0.3125 to 5.0 mg/ml was higher than those of the different extracts of *Z. mucronata* subsp. *mucronata* ranging from 1.25 to 10.0 mg/ml. The asexually reproducing fungi exhibited higher MIC values

than the non-spore forming fungi. Mechanism of antibiosis determination showed that they were more fungicidal than being fungistatic.

Despite the anatomical variations, the effective inhibition of both Gram-negative and Gram-positive bacteria as well as fungal isolates by the extracts of both plants indicated that they were not selective in their antimicrobial activities and possess broad spectrum of antimicrobial activities. This is in agreement with earlier reported antibacterial activities of some plants (Siddique et al., 2004; Ramzi et al., 2005; Suresh et al., 2008). Since the antibacterial activity of an extract depends on its phytochemical contents, there have been varied reports on the effectiveness of plant extracts. Ethanol, methanol and water are commonly used solvents for antimicrobial activity (Parekh et al., 2005; Rojas et al., 2005) and the antimicrobial activities of extracts of these solvents have been widely reported. However, while Masoko and Eloff, (2006) discovered that acetone and methanol extracted more phytochemicals from leaves, Basri and Fan, (2005), Lourens et al., (2005) and Olajuyigbe and Afolayan, (2012a,b) used acetone to extract phytochemicals from the plants. In agreement with these reports from various studies, this investigation showed that acetone, ethanol and methanol extracts of these two plant species had varied antibacterial activities with ethanol extracts showing more antimicrobial activities than others.

On testing for the cytotoxicity of the plant materials, brine shrimps were treated with extracts at concentrations ranging between 0.9765 µg/ml and 500 µg/ml. Mortality of the brine shrimps was noticed in the experimental group but the control group remained unchanged. Since plant extracts were regarded as mildly toxic if LC_{50} > 30<100 µg/ml and non-toxic if LC_{50} is greater than 100 µg/ml in the brine shrimp lethality assay (Moshi et al., 2010), it is evident from this study that the crude extracts of *A. mearnsii* with the LC_{50} equaled 112.36 µg/ml and having

the highest levels of toxicity (100% death) at 500 µg/ml was non toxic (LC_{50} > 100 µg/ml) while the LC_{50} for *Z. mucronata* subsp. *mucronata* equaled 90.27 µg/ml indicated a low level of toxicity.

Since there is growing interest in identifying the source of the therapeutic effects (Awadh et al., 2001) as well as correlating the phytochemical constituents of medicinal plant with its pharmacological activity (Al-Bayati and Al-Mola, 2008; Costa et al., 2008), the phytochemical analyses of *A. mearnsii* and *Z. mucronata* subsp. *mucronata* showed the presence of varied degree of phytochemicals with antioxidant properties. Quantitatively, their phenolic, flavonoid and proanthocyanidin contents differ significantly in the various extracts. The phenolic contents were significantly higher than the flavonoids and proanthocyanidin contents in all the extracts and all the extracts showed significant antioxidant potentials. In *A. mearnsii*, ethanolic extract had the highest total flavonoids. Acetone extract had the highest total phenolic contents. The total proanthocyanidins was highest in the methanolic extract while aqueous extracts had the least of these phytochemicals. In *Z. mucronata* subsp. *mucronata*, the phenolics were significantly higher than the flavonoids and proanthocyanidin contents in all the extracts investigated.

The two plants, also, exhibited significant ability to donate electrons in the ferric reducing power assays and had very high radical scavenging effects which were dose dependent. In *A. mearnsii*, aqueous extract showed the least reducing power, methanolic extract had the highest reducing power. The reducing power of the extracts was lower than those obtained from the reference standard such as butylated hydroxytoluene (BHT), Rutin and ascorbic acid. 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) diammonium salt showed that ethanolic extract exhibited the highest radical scavenging activity at the highest concentration tested. 1,1-

diphenyl-2-picrylhydrazyl (DPPH) assay indicated that ethanolic extract had the highest scavenging activity at the lowest concentration and the activities of all the extracts decreased with increase in their concentrations. In *Z. mucronata* subsp. *mucronata,* the ethanolic extract had the highest antioxidant activity, followed by the acetone extract while the aqueous extract was the least active. Reacting with ABTS, the 50% inhibitory concentrations (IC_{50}) were (0.0429 ± 0.04 mg/ml) for aqueous, (0.0317 ± 0.04 mg/ml) for acetone and (0.0306 ± 0.04 mg/ml) for ethanolic extracts while they inhibited DPPH radical with 50% inhibitory concentration (IC_{50}) values of 0.0646 ± 0.02 mg/ml (aqueous), 0.0482 ± 0.02 mg/ml (acetone) and 0.0422 ± 0.03 mg/ml (ethanol).

In previous studies, the pharmacological importances of these phytochemicals have been documented. Pharmacologically bioactive compounds such as alkaloids, flavanoids, tannins and phenolic compounds (Nawrot et al., 2007; Beltrame et al., 2011; Ullah et al., 2011; Samali et al., 2012) have been identified. Phenolic compounds and flavonoids act as strong antioxidants and free radical scavengers, chelate metals and interact with enzymes, adenosine receptors and biomembranes (Middleton and Kandaswami, 1993). Antimicrobial activity of the saponins and phenolics had been reported (Karamanoli, 2002; Benhammou et al., 2008). This study showed that *A. mearnsii* and *Z. mucronata* subsp. *mucronata* had strong antioxidant activities and free radical scavenging capability which are less than those of the commercially available synthetic antioxidants. Hence, their phenolic contents could be a good source of natural antioxidant substances useful in neutralizing free radicals and play beneficial roles in oxidative stress prevention in disease states. The observed antimicrobial activities could be attributed to the presence of these phytochemicals in the various extracts while their antioxidant ability justifies

their healing potentials when used in the treatment of various infections for which they were ethnobotanically implicated.

In view of the fact that concurrent uses of pharmaceutical with herbal remedies are, often, not declared (Kassler et al., 1991; Buchness, 1998; Donaldson, 1998) in self-medications by the rural dwellers and synergistic antimicrobial agents are commercially used in the treatment of various infections (Chatterjee et al., 2009), the potential interactions of antibiotics with these plants was investigated by means of agar dilution and checkerboard methods. This study showed that the various extracts of the two plants exhibited varied degree of interactions with the antibiotics to which they were combined against all the test bacterial isolates. The checkerboard showed synergistic interaction (61.25%), additivity/indifference (23.75%) and antagonistic (15%) effects for methanolic extract of *A. mearnsii*. From literature, different mechanisms of action(s) have been indicated to explain the synergistic effects of combining extracts with different antibiotics. These mechanisms included simultaneous double attack of extracts with antibiotics on different target sites (Esimone et al., 2006), phytochemicals disrupting membranes or translocating through the membrane to a target receptor inside the cell to pave ways for each of these antibiotics to reach their target sites of action to upshot their concerted activities (Haukland et al., 2001), blocking of the inhibitory effects of protective enzymes (Aburjai et al., 2001; Darwish et al., 2002; Hemaiswarya et al., 2008), improvement of the *in vitro* activity of some peptidoglycan inhibiting antibiotics by attacking the same site in the cell wall (Zhao et al., 2001) and possession of enhanced antibacterial activity to disrupt transpeptidation of the cell membrane (Esimone et al., 2006). These were proposed to substantiate the effectiveness of the antimicrobial activities of combined herbal-drugs in therapy. This study showed that the synergistic interactions indicated that the bactericidal potentials of the antibacterial agents were

improved and combining natural products with antibiotic could be potential sources for resistance-modifying agents useful against infectious multi-drug resistant bacteria.

Although the combination of the extract and different antibiotics showed evidence of *in vitro* synergism, antagonisms and indifferences were also recorded in the combinations of these antibacterial agents to a lesser extent. According to Rowe et al. 2009, chemical interactions between antibacterial agents can affect the chemical nature, the stability and bioavailability of drug products, and consequently, their therapeutic efficacy and safety. The indifferent and antagonistic interactions could be evidence that complexes which may have been formed when the antibacterial agents were combined were not able to diffuse through the agar medium. While the additive interactions showed each of the antibacterial agents were still able to act independently when combined against the bacterial isolates to a lesser extent, the antagonism showed that there are phytoconstituents which are inhibitory or exist at micron levels without sufficient antibacterial capabilities to be in synergy with the antibiotics used.

The possible mechanisms of action of these plants' extracts was investigated by determining the influence of the different extracts of each plant on the ultrastructure, protein and lipid leakages, elemental components and extracellular release of some metal cations in some treated and untreated selected bacterial strains. The ultrastructural changes and elemental components of treated and untreated isolates were determined by using Scanning Electron Microscope (SEM) coupled with an Electron Dispersive Spectroscope (EDS). The selected bacteria included *Escherichia coli* (ATCC 8739), *Shigella flexneri* (KZN), *Proteus vulgaris* (ATCC 6830), *Staphylococcus aureus* (ATCC 6538) and *Bacillus pumilis* (ATCC 14884). The study showed that the extracts had varied effects on the ultrastructures of the individual cells and bacterial populations, caused lipid and protein leakages as well as significant differences

between elemental components of extract-treated and untreated bacterial isolates. The morphological changes and the observed leakages showed rapid killing, significant membrane depolarization resulting in leakages and efflux of disintegrated cellular materials. Disruptions in the outer wall and cytoplasmic membranes, especially, at the polar regions of the cells, whole cell collapse and presence of extruded cellular materials situated close to the collapsed end of the cytoplasm and eventual cell death were observed. The observed distinct morphological changes such as cell elongation and mummification of the surfaces suggested that the extracts could have interfered with bacterial cell wall synthesis leading to cell deformations. The differences between the elemental components of extract-treated and untreated bacteria suggest that the increments observed may be due to the rapid replication, synthesis of micromolecules and rapid growth of these isolates in order to survive in the challenge environment. The varied decreases in the elemental components of the treated isolates, compared with the untreated ones, could be attributed to the ability of the extract to breakdown the synthesized micromolecules and the basic mechanisms underlining replication, growth and development in bacteria.

Although the influence of several medicinal plants on the morphology of microbial cells had been reported with little or no attention to the surface textures of the treated bacterial cells, this study observed differences in the surface textures and features of the treated isolates. While this study may be the first to observe differences in the elemental components of bacterial cells as a possible mechanism of action, reported information on leakages have not linked leakages in extracted-treated bacterial cells to any possible mechanism of action.

Schwarz et al. (1969) reported that lateral wall elongation and septum formation are processes involved during the growth of rod-shaped bacterial cells. Satta et al. (1980) showed that the actual shape of rod-shaped bacteria is determined by the balance between these two

competing reactions occurring in the terminal stages of peptidoglycan synthesis. In agreement with these early reports, the ultrastructrual changes in *S. flexneri* and elongations in other bacterial cells could be attributed to the imbalances between lateral wall elongation and septum formation caused by the extracts. Hence, this study may suggest that the possible mechanisms of action of the ethanolic extract of *A. mearnsii* changing rod-shaped *S. flexneri* to squeezed, collapsed and polygon-shaped coccoid and elongations of some of the other isolates could be an inhibition of a significant step in peptidoglycan assembly.

The mechanisms of action underlying the lipid and protein leakages have also been related to the lipid peroxidation and protein oxidation. This was due to the fact that the antioxidant activities of these plants could have enhanced membrane free radical reactions beyond the protective level and cause lipid peroxidation (Cerutti, 1985). The extracts from these plants could have attacked the DNA, proteins, lipids, polyunsaturated fatty acids of cells and initiate lipid peroxidation in the cytoplasmic membranes. Lastly, the ability of the extracts to cause variations between the elemental components of treated and untreated strains could be attributed to the inhibition of metabolic processes involved in the biosynthesis of bacterial cellular materials and the breakdown of some of these elemental components or element containing cellular materials that were rapidly synthesized in response to environmental perturbation due to the presence of the phytochemicals in the growth medium. However, the divergent antibacterial effects of these plants showed that there may be several mechanisms of action involved in their antimicrobial activities.

14.2 Conclusions

Acacia mearnsii and *Ziziphus mucronata* subsp. *mucronata* are used in South Africa for ethnotherapeutic purposes. Many of the uses are associated with treatment of infections including gastrointestinal disorders such as dysentery and diarrhoea. Subject to *in vivo* experimentation, this study showed that the two plants may have great capabilities in the treatment of bacterial infections because of the degree of their antibacterial activities at high concentrations as indicated by the obtained minimum inhibitory concentrations (MICs). Since medicinal plants are an inexhaustible source of natural drugs that may be employed in combating microbial infections and a source of resistance modulating agents, these plants are reservoirs of significantly active phytochemicals that should be isolated for pharmacological purposes or used in curtailing the development of resistance to existing antibiotics. This could complement the efforts of synthesizing new generation of antibiotics in response to the global bacterial resistance development. Although there have been dearth of scientific reports on their medicinal importance, this study showed their medicinal values, justified their ethnomedicinal uses in the local settings and proposed some mechanisms of actions to indicate some possible biochemical interactions through which their pharmacologically active substance produces an effect(s) on a living organism. The study, also, showed that *A. mearnsii* would be more effective in treating gastrointestinal infections than *Z. mucronata*.

From this study, I concluded:

- That *A. mearnsii* and *Z. mucronata* subsp. *mucronata* are medicinal plants implicated in the treatment of gastrointestinal disorders especially dysentery and diarrhoea in South Africa.

- That these two plants possess free radical scavenging and antioxidant properties lower than those of standard antioxidant agents and could be used effectively in ethnomedicine with little or no toxicity
- That the degree of *in vitro* antimicrobial activities exhibited by *A. mearnsii* and *Z. mucronata* subsp. *mucronata* against a wide range of bacterial and fungal isolates associated with diverse kinds of infections suggested that these plants are potential sources of antibiotics.
- That the crude extract of these plants exhibited significant antibacterial activities at concentrations less than 1 mg/ml suggested that they possess a broad spectrum antibacterial activity.
- That their broad spectrum antibacterial activity is against a wide range of Gram-negative and Gram-positive bacteria supported their ethnomedicinal importance and uses in folkloric medicines for the treatment of various gastrointestinal disorders and other microbial infections.
- That their herbal-drug interactions could result in synergistic interaction suggested that they could be a source of resistance modulating agents
- That *A. mearnsii* and *Z. mucronata* subsp. *mucronata* possess diverse mechanisms of action by which they express their antibacterial activities in bacterial cells.

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