PHYTOCHEMICAL ANALYSIS AND BIOACTIVITY OF THE STEM BARK OF *COMBRETUM MOLLE* ON SOME SELECTED BACTERIAL PATHOGENS

By

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TABLE OF CONTENTS

Acknowledgements..…… v

List of figures...…… vi

List of tables...……. vii

Glossary of abbreviations...…… viii

Declaration…… x

Abstract……. xi

CHAPTER ONE: INTRODUCTION…….. 1

1.2 *Helicobacter pylori*…….. 2

1.3 *Pseudomonas aeruginosa*…… 2

1.4 *Plesiomonas shigelloides*……. 3

1.5 *Streptococcus pyogenes* …….. 4

1.6 Importance of traditional medicine in Africa……. 5

1.7 Statement of the problem…… 6

1.8 Hypothesis…… 7

1.9 Project objectives…….. 7

CHAPTER TWO: LITERATURE REVIEW…….…….. 8

2.1 ANTIBIOTIC RESISTANCE…….……. 8

2.2 *HELICOBACTER PYLORI*…….. 12

2.2.1Introduction…….. 12

2.2.2 Pathogenesis and clinical manifestation……. 12

2.2.3 Prevalance of *Helicobacter pylori* infection…… 13

2.2.4 Modes of transmission…….. 14

2.2.5 Treatment …….. 15

2.2.6 Laboratory diagnosis…….. 17

2.3 *PSEUDOMONAS AERUGINOSA*…… 20

2.3.1 Introduction…… 20

2.3.2 Pathogenesis and clinical manifestation…… 21

2.3.3 Modes of transmission…… 21

2.3.4 Treatment…… 22

2.3.5 Laboratory diagnosis……. 25

2.4 *PLESIOMONAS SHIGELLOIDES*……..... 28

2.4.1 Introduction…….. 28

2.4.2 Pathogenesis and clinical manifestation……. 29

2.4.3 Epidemiology …….. 39

2.4.4 Modes of transmission…….. 30

2.4.5 Treatment…….. 30

2.4.6 Laboratory diagnosis……..… 31

2.5 ST*REPTOCOCCUS PYOGENES*…..…….. 33

2.5.1 Introduction…….…… 33

2.5.2 Pathogenesis and clinical manifestation…… 33

2.5.3 Prevalance…… 35

2.5.4 Modes of transmission……. 36

2.5.5 Treatment……. 36

2.5.6 Laboratory diagnosis…… 37

2.6 PLANTS AS POTENTIAL SOURCES OF ANTIBIOTICS…..... 38

2.6.1 Introduction……. 38

2.6.2 Traditional medicinal use of *Combretum* species…… 39

2.6.3 *Combretum molle*……... 40

CHAPTER THREE: MATERIALS AND METHODS…….…… 42

3.1 Test bacterial strains…….. 42

3.2 Plant material……. 42

3.3 Plant extraction……. 42

3.4 Antimicrobial susceptibility tetsing……. 43

3.4.1 *Detremination of antimicrobial activity by agar well diffusion method*…….. 43

3.4.2 *Detremination of minimum inhibition concentration*……. 44

3.5 Phytochemical analysis……... 45

3.5.1*Thin layer chromatography*…… 45

3.5.2 *Bioautography*…… 46

3.5.3 *Column chromatography*……. 47

3.6 *Gas chromatography and mass spectrometry*… …….. 47

3.7 *High performance liquid chromatography*……..…….. 48

3.8 Statistical analysis……..... 48

CHAPTER FOUR: RESULTS……. 49

4.1 Extract yield…….. 49

4.2 Antimicrobial activity of the extracts against the test organisms…… 50

4.3 Fractionation of bioactive compounds by TLC..…… 55

4.4 Column chromatography analysis and MIC_{50} determination of the fractions…….. 57

4.5 GC-MS and HPLC analysis…….. 60

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS.. 64

5.1 Discussion……. 64

5.2 Conclusion……..…….. 71

5.3 Recommendations……... 71

REFERENCES……..….…… 72

APPENDICES……. 102

Appendix 1: Graphical representation of MIC..... …… 102

Appendix 2: TLC analysis……. 103

Appendix 3: Bioautography analysis…… 105

Appendix 4: Statistical analysis…….. 108

Appendix 5: *Combretum molle*…….. 113

Appendix 6: Manuscripts in preparation……. 114

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LIST OF FIGURES

Figure 1: Susceptibility of the extracts against the test organisms at 200mg/mL Concentration..... 53

Figure 2: GC-MS peak for EA 4..... 61

Figure 3: GC-MS peak for EMW1..... 62

Figure 4: HPLC peaks for EMW 1..... 63

Figure 5: HPLC peaks for EA 4..... 63

LIST OF TABLES

Table 1: Extract yield of *C. molle* stem bark..... 49

Table 2: Zone of inhibition of the organisms against extracts and antibiotic..... 51

Table 3: Mean zone diameter of inhibition range of *C. molle* extracts and antibiotic..... 52

Table 4: Antibacterial activity of *C. molle* and ciprofloxacin (MIC_{50}) against the test organisms..... 54

Table 5: Putative compounds fractionated on TLC plate..... 55

Table 6: Inhibition of bacterial growth by acetone extract by bioautography..... 56

Table 7: Antibacterial activity of the fractions (MIC_{50}) against the test organisms and their R_f values..... 58

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GLOSSARY OF ABBREVIATIONS

AFLP – amplified fragment length polymorphism

ARF – acute rheumatic fever

ATCC – American type culture collection

BEA- benzene/ ethanol/ ammonium hydroxide (36: 4: 0.4)

BHI – brain heart infusion

$C0_2$ – carbon dioxide

CEF – chloroform/ ethyl acetate/ formic acid (20: 16: 4)

CF – cystic fibrosis

CFU – colony forming unit

CLED – cystine lactose electrolyte deficient agar

DNA – deoxyribonucleic acid

ELISA- enzyme immunosorbent assay

EMW – ethyl acetate/ methanol/ water (40: 5.4: 4)

GAS – group A S*treptococcus*

GMOS – genetically modified organisms

H^+ - Hydrogen ion

HPLC- high performance liquid chromatography

IBB – inositol brilliant bile agar

IgG – immunoglobulin G

INT – *p*- idonitrotetrazolium

MIC – minimum inhibition concentration

MLSB – macrolide-lincosamide –streptogramin B

NADH – nucleotide adenine dehydrogenase

NF – necrotizing fasciitis

NMR- nuclear magnetic resonance

O_2 – oxygen

PBPs - penicillin binding proteins

PCR – polymerase chain reaction

PDA – *Plesiomonas* differential agar

PFGE – pulsed- field gel electrophoresis

RAPD – random amplified polymorphic DNA

R_f- retention factor

RHD – rheumatic heart disease

rRNA – ribosomal nucleic acid

SS- *Salmonella- Shigella* agar

STSS – streptococcal toxic shock syndrome

TLC- thin layer chromatography

UTI – urinary tract infections

UV light – ultra violet light

WHO – World Health Organization

XLD- xylose lysine desoxycholate

DECLARATION

I declare that this dissertation and the work contained herein is my original work and has not been presented to any other university for the award of any degree.

Name **MIRRIAM NYENJE**

Signature.....

Supervisor's signature.....

Date.....

ABSTRACT

Antimicrobial resistance is a worldwide problem that has deleterious long-term effects as the development of drug resistance outpaces the development of new drugs. Plants have been used for many generations for healing purposes, and screening of extracts of these plants has often yielded positive outcomes. This study was aimed at isolating and characterizing the major active antimicrobial compounds present in the stem bark of *C. molle,* in a bid to identify potential sources of cheap starting materials for the synthesis of new drugs. Various solvents (hexane, ethyl acetate, dichloromethane, acetone, ethanol and methanol) were used for extraction. The agar well diffusion technique was used to screen for antimicrobial activity of *C. molle* extracts against *Streptococcus pyogenes* ATCC 49399, *Plesiomonas shigelloides* ATCC 51903, *Pseudomonas aeruginosa* ATCC 15442, *Helicobacter pylori* ATCC 43526 and *Helicobacter pylori* 252C (clinical isolate); minimum inhibition concentration (MIC) of the most active extracts was determined by the broth dilution method. Fractionation of acetone extract was done by thin layer chromatography (TLC) and bioautography to determine the compounds present and their antimicrobial activity respectively. The acetone extract was purified by column chromatography and their MIC determined. The most potent fraction (EA4) was subjected to Gas chromatography- Mass spectrometry (GC-MS) and High performance liquid chromatography (HPLC) for identification of the active compounds. Results were analyzed by the Fisher's exact test. All the extracts tested demonstrated antimicrobial activity with zone diameters of inhibition ranging from 0–32 mm. Acetone was the most potent extract with its MIC ranging from 0.078–5.0 mg/mL. Seventeen fractions were collected from column chromatography and the most active fraction against all the organisms was EA 4 (eluted with 100% ethyl acetate), with its MIC ranging from 0.078 - 2.5mg/mL. There was no statistically significant difference ($P>0.05$) in the potency of the

four extracts (acetone, methanol, ethanol and ethyl acetate) and antibiotic (ciprofloxacin) on the different bacterial strains tested, likewise the crude extract and the fractions. No compound was detected by GC-MS whereas numerous peaks were identified by HPLC implying that the active compounds in this plant are non volatile. We could not identify the compounds thereby proposing further studies using Nuclear magnetic resonance to identify the compounds. The study revealed that the acetone extract of *C. molle* was the most active against all the test organisms and therefore justifies the use of this plant in traditional medicine.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Serious infections caused by resistant bacteria have become a major global healthcare problem in the 21st century. In developing countries, bacterial infections are still the main causes of death accounting for approximately half of all deaths (Fyhrquist *et al.*, 2006). Resistance to antimicrobials is a natural biological phenomenon that can be amplified or accelerated by a variety of factors such as: inadequate access to effective drugs, unregulated manufacture and dispensation of antimicrobials, poor adherence and lack of money to pay for appropriate and high-quality medications (WHO, 2000; Planta, 2007).

In the last sixty years, major improvements in the early recognition and treatment of infectious diseases resulted in an extraordinary reduction in the morbidity and mortality associated with these diseases. This has been due to the better understanding of the molecular mechanisms of these diseases and improved understanding of their pathophysiology and epidemiology; but most notably to the rapid development of safe and effective new antimicrobial agents. Unfortunately, bacteria and fungi have developed resistance to all classes of different antibiotics discovered to date (Alanis, 2005).

Streptococcus pyogenes (*S.pyogenes*) and *Pseudomonas aeruginosa* (*P.aeruginosa*), organisms that cause respiratory, cutaneous, and nosocomial infections in the immunocompromised as well as *Helicobacter pylori (H.pylori)* and *Plesiomonas shigelloides (P.shigelloides)* which cause gastrointestinal infections are now resistant to virtually all of the

older antibiotics (Eloff *et al*., 2005). The search for new antibacterial compounds with improved activity to replace those that have become inactive is therefore necessary.

1.2 *HELICOBACTER PYLORI*

H. pylori is a spiral-shaped bacterium that colonizes the stomach of half of the world's population. Once a person is infected, the organism can live in the stomach indefinitely and may not cause clinical illness (Stege *et al.*, 2006). *H. pylori* has been recognized as the etiological agent of chronic gastritis, gastric mucosa associated lymphoid tissue lymphoma, gastric and duodenal ulcer (Ndip. *et al*., 2003; Mégraud and Lehours, 2007; Ndip *et al*., 2008).

Therapy for *H. pylori* infection consists of one to two weeks of one or two effective antibiotics, such as amoxicillin, tetracycline, metronidazole, or clarithromycin, plus either ranitidine bismuth citrate, bismuth subsalicylate, or a proton pump inhibitor with success rate of 80-90% (Njume *et al.*, 2009). However, emerging resistance to antibiotics coupled with undesirable side effects such as nausea, vomiting, epigastric pains, abdominal discomfort and diarrhea as well as its significant cost of combination therapy poses enormous public health concerns (Iwu *et al.*, 1999; Njume *et al.*, 2009; Tanih *et al.*, 2010).

1.3 *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is a Gram-negative, aerobic rod that belongs to the family Pseudomonadaceae. It is found in soil, water, plants, animals (including human) and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also with little oxygen, and has thus colonized many natural and artificial environments (Prinsloo *et al*., 2008). Because it thrives on most surfaces, this bacterium is also found on and in medical equipment including catheters, causing cross infection in hospitals. The organism is a frequent cause of nosocomial infections such as pneumonia, urinary tract infections (UTIs),

bacteraemia and septicemia which can be life threatening. Infections due to this pathogen are especially prevalent among patients with cystic fibrosis (CF), acute leukemia, organ transplants and burns with 50% case fatality rate (Prinsloo *et al.,* 2008).

P. aeruginosa is notorious for its resistance to antibiotics and is therefore a dangerous and dreaded pathogen. The bacterium is naturally resistant to many antibiotics and the futility of treating *Pseudomonas* infections is most dramatically illustrated in CF patients, virtually all of whom eventually become infected with the strain that is so resistant that it cannot be treated, making the treatment of infections caused by this organism both difficult and expensive (Tanya, 2009).

1.4 *PLESIOMONAS SHIGELLOIDES*

P.shigelloides is the single homogenous species of *Plesiomona*s and belongs to the group of aquatic organisms recognized recently as a potential human enteropathogen (*Krovacek et al.,* 2000). The primary reservoirs of *P. shigelloides* are fresh and estuarine waters while fish and different kind of seafood act as secondary reservoirs in these environments.

P. shigelloides has been associated with extra and intra- intestinal infections in humans. Extra- intestinal infections such as bacteremia, cellulitis and meningitis are rarely reported in immunocompromised patients. Outbreaks are generally related to consumption of seafood or untreated water (Salerno, 2007).

Like other members of the family *enterobactericeae,* most of *P. shigelloides* strains are resistant to a broad spectrum of penicillin and macrolides (Avison *et al*., 2000; Stock and Weidman, 2001).

1.5 *STREPTOCOCCUS PYOGENES*

S. pyogenes is a major human pathogen causing both mild infections such as pharyngitis, impetigo and severe infections, including sepsis, necrotizing fasciitis (NF), and lethal streptococcal toxic shock syndrome (STSS). Nonsuppurative sequelae such as acute rheumatic fever (ARF), rheumatic heart disease (RHD) and glomerulonephritis are associated with high morbidity and mortality rates, especially in developing countries (Cunningham, 2000; Carapetis *et al.,* 2005).

In spite of more than half a century of extensive use of βeta- lactams, no resistance against these drugs among Group A Streptococcal (GAS) infections has been reported; however, resistance to macrolide antibiotics, recommended alternative treatment for patients allergic to penicillin is common in many countries (Morosin *et al*., 2003).

It has been realized that disease resistance is developing to almost all currently available drugs and this prevalence of drug resistant pathogens is occurring at a time when the discovery and development of new antibiotics is slowing down (Spellberg *et al*., 2008). Infections caused by resistant microbes are not only more severe and require longer and more complex treatments, but they are also significantly more expensive to diagnose and to treat. This is potentially disastrous in many low income countries because of lack of resources for purchasing expensive second or third– line drugs. This reiterates the need to revolutionize the search for alternative treatment regimens which seem to lie in medicinal plants (Njume *et al.,* 2009).

1.6 IMPORTANCE OF TRADITIONAL MEDICINE IN AFRICA

Traditional medicine is an important part of the health care system in most African countries. About 80-90% of the population in African countries depends on traditional medicine for their primary health care (Hostettman *et al.,* 2000). Plant materials provide the models for 50% of Western drugs and that many commercially proven drugs in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity (Trease and Evans, 2002). However, since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobial was to a lesser extent nonexistent. The interest in using plant extracts for treatment of microbial infections has increased in the late 1990s as conventional antibiotics became ineffective and also many of the antimicrobial drugs in use have undesirable toxic effects (Cowan, 1999).

1.6.1 *Traditional medicinal uses of Combretum species and their antimicrobial effects*

The family, Combretaceae consists of 20 genera and 300 species, the largest of which are *Combretum, Terminalia* and *Quisqualis* (Tan *et al.,* 2002). Species from the genus C*ombretum* and *Terminalia* are most widely used for medical purposes. *Combretum molle* (*C. molle*) is widely used in African traditional medicine for the treatment of various ailments and diseases. Various organs such as leaves, roots and stem bark of *C. molle* are predominantly used (Rodgers and Verotta, 1996). Traditional healers throughout Southern Africa employ species of Combretaceae for many medicinal purposes ranging from bacterial, fungal, viral and parasitic infections (Asres *et al.,* 2001; Fyhrquist *et al*., 2006; Ojewole, 2008).

1.7 STATEMENT OF THE PROBLEM

Conventional drugs usually provide effective antibiotic therapy for bacterial infections; but infections caused by resistant bacteria fail to respond to treatment, resulting in prolonged illness and greater risk of death. Treatment failures also lead to longer periods of infectivity, which increase the numbers of infected people moving in the community and thus expose the general population to the risk of contracting a resistant strain. It has been realized that disease resistance is developing to almost all currently available drugs because bacterial pathogens are emerging with new forms of virulence and new patterns of resistance to antimicrobial agents (WHO, 2002). Therefore novel classes of antibiotics are increasingly required due to the increasing population at risk and the growing prevalence of resistant pathogenic bacteria.

Plant based components are used as natural antimicrobials to treat bacterial pathogens and other gastrointestinal tract infections (Ndip *et al.,* 2008). These antimicrobial compounds from plants may inhibit bacteria by different mechanisms than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains (Eloff *et al.,* 1998).

Although the phytochemical constituents of some of the studied plant species are known, the exact bioactivity should be identified in order to standardize traditional medicine. This study evaluates the antimicrobial activity of extracts of the stem bark of *C. molle* in a bid to isolate and identify the main active constituents of this plant, and also to understand its biological activity against both Gram-negative and Gram-positive bacteria. This constitutes part of an effort to identify potential sources of cheap starting materials for the synthesis of new drugs to circumvent the problem of increasing drug resistance against these pathogens.

1.8 HYPOTHESIS

C. molle can provide cheap and potent bioactive compounds for the synthesis of new antimicrobials.

1.9 PROJECT OBJECTIVES

1.9.1 Overall objective

The overall objective of this study is to isolate and characterize the major active antimicrobial compounds present in the stem bark of *C. molle.*

1.9.2 Specific objectives

The specific objectives of this study are to:

1. Screen the plant extracts for antimicrobial activity.
2. Determine the minimum inhibition concentration of the extracts.
3. Fractionate the extracts to obtain the active fractions.
4. Isolate and identify the bioactive compounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANTIBIOTIC RESISTANCE

Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. In order to be fit to survive, all living organisms strive to adapt to their environment. Part of this adaptation process includes adjusting to weather conditions, food, water and in many cases oxygen availability and also the presence of potentially dangerous or even lethal external agents. The end result of this phenomenon is that many strains of bacteria have become resistant, and in many cases multi- resistant to these therapeutic agents thus rendering these drugs ineffective as treatments of choice for severe infections caused by these pathogens (Alanis, 2005).

Reports have shown a marked increase in antibiotic resistance of food-poisoning bacteria due to non-rational and excessive use of antibiotics as therapeutic agents or as growth promoters in livestock. Another factor of resistance potentially lies in the use of antibiotic resistance genes as selection markers in genetically modified organisms (GMOS). The main safety issues of concern are the release of these genes to sensitive organisms when these GMOS are introduced into the environment. The use of antibiotics to inhibit normal flora during cultivation of specimen from non sterile sites has also contributed to resistance as constant use of these antibiotics result in genetic modification (Angeh *et al.,* 2007). Due to emergence of drug resistant bacteria, the search for new antibacterial compounds with improved activity is necessary.

2.1.1 Mechanisms of antibiotic resistance

Bacterial resistance has its foundation at the genetic level; changes in the genetic makeup of the previously susceptible bacteria take place either via mutation or by the introduction of new genetic information. The expression of these genetic changes in the cell results in changes in one or more biological mechanisms of the affected bacteria and ultimately determines the specific type of resistance that the bacteria develop. Therefore, molecular (genetic) and biological mechanisms are the major mechanisms of resistance (Sefton, 2002; Levy and Marshall, 2004).

2.1.1.1 Genetic Mechanisms of resistance

The development of antibiotic resistance tends to be related to the degree of simplicity of the DNA present in the microorganism becoming resistant and to the ease with which it can acquire DNA from other microorganisms. For antibiotic resistance to develop, it is necessary that two key elements combine: the presence of an antibiotic capable of inhibiting the majority of bacteria present in a colony and a heterogeneous colony of bacteria where at least one of this bacterium carries the genetic determinant capable of expressing resistance to the antibiotic (Levy and Marshall, 2004). As susceptible bacteria in the colony dies, the resistant strains will survive possessing the genetic determinants that codify the type and intensity of resistance to be expressed by the bacterial cell. Selection of these bacteria results in the selection of these genes that can now spread and propagate to other bacteria (Alanis, 2005).

Once a genetic mutation occurs and causes a change in the bacterial DNA, genetic material can be transferred among bacteria by several means. The most common mechanisms of genetic transfer are conjugation which is normally mediated by plasmids (circular fragments of DNA) that are simpler than chromosomal DNA and can replicate independently of the

chromosome. The mechanism of transmission of plasmids among bacteria is via the formation of a ‘‘pilus’’ that forms between bacteria when they are next to each other, thus connecting them temporarily and allowing the passage of these DNA fragments.

Transformation is another form of transmission of bacterial resistance genes and takes place when there is direct passage of free DNA (also known as ‘‘naked DNA’’) from one cell to another. The ‘‘naked DNA’’ usually originates from other bacteria that have died and broken apart close to the receiving bacteria. Transduction is a third mechanism of genetic transfer and occurs via the use of a ‘‘vector’’, most often viruses capable of infecting bacteria also known as ‘‘bacteriophages’’. The virus containing the bacterial gene that codifies antibiotic resistance (the ‘‘resistant DNA’’) infects the new bacterial cell and introduces this genetic material into the receiving bacteria (Alanis, 2005).

2.1.1.2. Biological Mechanisms of Resistance

Whichever way a gene is transferred to a bacterium, the development of antibiotic resistance occurs when the gene is able to express itself and produce a tangible biological effect resulting in the loss of activity of the antibiotic. These biological mechanisms are many and varied but they can be summarized as follows:

2.1.1.2.1 *Antibiotic destruction or antibiotic transformation*

This destruction or transformation occurs when the bacteria produces one or more enzymes that chemically degrade or modify the antimicrobial, making them inactive against the bacteria. Gram-positive bacteria secretes enzymes that destroys the antibiotic before it reaches the organism, *e.g. Staphylococcus aureus* resistance to penicillin where as Gram-negative bacteria such as *Pseudomonas* and *Plesiomonas,* enzymes concentrated in the periplasmic space destroy the antibiotic when it enters the organism. Such enzymes are;

β-lactamase which hydrolyses the β-lactam ring before it can bind to penicillin binding proteins (PBPs), aminoglycoside modifying enzymes and chloramphenicol esterases. This is a common mechanism of resistance and probably one of the oldest one affecting several antibiotics but especially β-lactam antibiotics (Jacoby and Manoz-price, 2005).

2.1.1.2.2 Antibiotic active efflux.

The mechanism is relevant for antibiotics that act inside the bacteria and takes place when the microorganism is capable of developing an active transport mechanism that pumps the antibiotic molecules penetrated into the cell to the outside milieu until it reaches a concentration below antibacterial activity. This means that the efflux transport mechanism must be stronger than the influx mechanism in order to be effective (Hooper, 2005).

2.1.1.2.3 Receptor modification.

Receptor modification occurs when the intracellular target or receptor of the antibiotic drug is altered by the bacteria, resulting in the lack of binding and consequently the lack of antibacterial effect. Examples of this mechanism include modifications in the structural conformation of PBPs observed in certain types of penicillin resistance, ribosomal alterations that can render aminoglycosides, macrolides or tetracyclines inactive, and DNA-gyrase modifications resulting in resistance to fluoroquinolones (Sefton, 2002; Levy and Marshall, 2004).

Generally, Gram-negative bacteria are more resistant than Gram-positive bacteria to the action of antibiotics since they contain an outer membrane with a lipopolysacharide layer which renders them impermeable to certain antibiotics and bactericidal compounds (Nikaido, 1996). Some of the bacteria implicated in this problem of resistance are *H. pylori*, *P.*

aeruginosa, *P. shigelloides* and *S. pyogenes*, hence our choice to search for alternative means of treatment against them.

2.2. *HELICOBACTER PYLORI*

2.2.1 INTRODUCTION

Classified as a class 1 carcinogen, *H. pylori* is a microaerophilic Gram-negative coccobacillus (0.5 μm wide by 2 - 4 μm long), equipped with two to six flagella, that has chronically infected more than half of the world's population (Ndip *et al*., 2004; Ahmed *et al*., 2007; Tanih *et al*., 2008; Dube *et al*., 2009). *H. pylori* is responsible for a significant cause of morbidity and mortality imposing a major burden on health care systems worldwide. Eradication of the organism is the first therapeutic approach that constitutes a reliable long-term prophylaxis of peptic ulcer relapse, accelerating ulcer healing and reducing the rate of ulcer complications (Ndip *et al*., 2009).

2.2.2 PATHOGENESIS AND CLINICAL MANIFESTATION

To colonize the stomach, *H. pylori* must survive the acidic pH of the lumen and burrow into the mucus to reach its niche, close to the stomach's epithelial cell layer (Okeleye *et al*., 2010). *H. pylori* produce large amounts of the enzyme urease, which breaks down urea to carbon dioxide and ammonia. The ammonia is converted to ammonium by taking a proton (H^+) from water, which leaves only a hydroxyl ion. Hydroxyl ions then react with carbon dioxide, producing bicarbonate which neutralizes gastric acid. The ammonia produced is toxic to the epithelial cells, along with the other virulence products of *H. pylori* including proteases, vacuolating cytotoxin A (VacA), and certain phospholipases (Tanih *et al*., 2008).

The organism is considered as an important human pathogen causing gastritis, duodenal ulceration, proved to be associated with gastric lymphoma and seems to be a risk factor for adenocarcinoma of the stomach (Tanih *et al*., 2009). Acute infection with *H. pylori* may cause a transient clinical illness, characterized by nausea and abdominal pain that may last for several days. After these symptoms resolve, the majority of people progress to chronic infection. However there is no recognizable symptom complex or syndrome that can be ascribed to chronic gastritis, whether or not due to *H. pylori*. Peptic ulcers often present with dyspepsia. There may be pain at night; some patients report relief of pain with food or antacids and a recurrence of pain in two to four hours (Graham and Shiotani, 2005; Tanih *et al*., 2009).

2.2.3 PREVALENCE OF *H. PYLORI* INFECTION

The prevalence of *H. pylori* infection is approximately 50% worldwide, making it the most widespread infection in the world. The actual infection rates vary from nation to nation, about 40% in developed countries and 80-90% in the developing world (Lacy and Rosemore, 2001; Bakka and Salih, 2002; Asrat *et al*., 2004; Ndip *et al*., 2004; Delport *et al*., 2006; Fritz *et al*., 2006; Frenck *et al*., 2006; Mbulaiteye *et al*., 2006; Levin *et al*., 2007; Ndip *et al*., 2008; Dube *et al.*, 2009).

The onset of infection is from childhood, which once established may persist throughout life if not treated (Ndip *et al*., 2004; Aguemon *et al.*, 2005). Several studies demonstrated a high prevalence of the infection among children aged 3 – 5 years, suggesting that most acquisition occur before the age of 3 years (Nabwera *et al*., 2000; Frenck *et al*., 2006; Mohammed *et al*., 2007). However, Mbulaiteye *et al*. (2006) reported a rise in seropositivity with age from 76% in children aged 0-4years to 99% in adults, while another study by Kimanga *et al.* (2010) reported the prevalence to be 67.5% in all age groups (73.3% in children and 54.8% in

adults), similarly to the findings of Shmuely *et al*. (2003) who documented 60 - 73% in all age groups in dyspeptic patients.

In Democratic Republic of Congo, 62.4% of participants tested positive for *H. pylori* antibody (Longo-Mbenza *et al*., 2007) and 93% prevalance was reported among patients with peptic ulcer in Ethiopia (Henriksen *et al*., 1999). In South Africa, *H. pylori* prevalence among asymptomatic individuals was observed to be 84% (Fritz *et al*., 2006) while in Cameroon, high incidences of 52.7% and recently 92.2% *H. pylori* infections were reported in both asymptomatic and symptomatic individuals respectively (Ndip *et al*., 2004; 2008).

2.2.4 MODES OF TRANSMISSION

According to Dube *et al.* (2009), H. *pylori* prevalence and the rate of infection are inversely related to the standards of living and sanitary practice as revealed by a very high prevalence, especially in developing countries and in lower socio-economic groups in the developed world (Bardhan, 1997; Malaty *et al*., 1998; Ahmed *et al*., 2007). *H. pylori* transmission remains poorly understood, however it is believed to spread from person to person through oral-to-oral, feacal-to-oral or gastro-oral routes. Such observations suggest that these infections may be associated with low socioeconomic factors such as overcrowding, poor sanitation practices and contaminated environments (Delport *et al*., 2006; Tanih *et al.*, 2010).

Use of contaminated water including municipal tap water, groundwater, rivers and waste water systems has also been suspected to have a high impact in the transmission of *H. pylori* by the detection of *H. pylori* in water distribution systems (Bunn *et al*., 2002; Benson *et al*., 2004; Ahmed *et al*., 2006; Nayak and Rose, 2007; Dube *et al*., 2009).

Several studies have demonstrated an alternate life style of *H. pylori* as a biofilm, which is likely to be important to facilitate bacterial survival in hazardous environment (Donlan and Costerton, 2002; Cole *et al*., 2004). Biofilms are defined as “complex microbial ecosystems

adherent to each other and/or to surface or interface". Carron *et al*. (2006) demonstrated the existence of *H. pylori* biofilms on human gastric mucosa, which suggested involvement in the pathogenicity of gastric ulcers. The presence of *H. pylori* in subgingival biofilm and saliva samples of chronic periodontitis subjects has supported the possibility of the oral cavity being another possible reservoir of this bacterium (Souto and Colombo, 2008).

2.2.5 TREATMENT OF *H. PYLORI*

The standard first line therapy is one or two week(s) triple therapy consisting of a proton pump inhibitor such as omeprazole, lansoprazole, pantoprazole, rabeprazole, or esomeprazole and two of the following antibiotics; clarithromycin, metronidazole, tetracyline and amoxicillin (Malfertheiner *et al*., 2007; Eisig *et al*., 2009; Bakir *et al*., 2009; Njume *et al.*, 2009) with success rate of 80-90% (Peitz *et al.,* 1998). This problem is more in developing countries where resistance can be as high as 100% as reported in Nigeria for both metronidazole and clarithromycin (Aboderine *et al.*, 2007). Ndip *et al.* (2008) reported a 93.2% metronidazole and 44.7% clarithromycin resistance in Cameroon and in their study, more than 60% of the isolates exhibited multi- drug resistance to three or four antibiotics. Sherif *et al.* (2004) also recorded a high metronidazole resistance (60-80%) in Egyptian children and recommended that the use of metronidazole for the treatment of *H. pylori* in Egypt be avoided. A study by Tanih *et al*. (2010) indicated 95.5% metronidazole resistance and 20% clarithromycin resistance thus confirming high metronidazole resistance in developing world. However, emerging resistance to antibiotics, especially clarithromycin and metronidazole limits their use in the treatment of *H. pylori* infections. Furthermore, undesirable side effects of the drugs such as nausea, vomiting, epigastric pains, abdominal discomfort and diarrhoea as well as its significant cost of combination therapy poses enormous public health concerns (Njume *et al.*, 2009).

2.2.5.1 Mechanisms of resistance

H. pylori like a few other bacteria such as *Mycobacterium tuberculosis* acquires resistance by mutation to all the antibiotics used in the treatment regimens (Me´graud and Lehours, 2007). The mechanism does not involve plasmids which could be transmitted horizontally but point mutations which are transmitted vertically; however, transformation may be possible if two strains are present simultaneously in the stomach. The consequence is a progressive increase in the resistance rate due to the selection pressure.

2.2.5.1.1 ***Resistance to macrolides***

Macrolides act by binding to the machinery that builds proteins (ribosomes) at the level of the peptidyl transferase loop of the 23S rRNA gene. *H. pylori* resistance is the consequence of point mutations at two nucleotide positions, 2142 (A2142G and A2142C) and 2143 (A2143G), which lead to a conformational change and a decrease in macrolide binding. Resistance of *H. pylori* to macrolides is a major cause of failure of eradication therapies (Njume *et al*., 2009).

2.2.5.1.2 ***Resistance to amoxicillin***

Amoxicillin acts by interfering with peptidoglycan synthesis, especially by blocking transporters, PBPs. *H. pylori* strains harbour mutations on the *pbp-1a* gene and amino acid substitution *Ser-414-Arg* which appears to be responsible for the blockage of penicillin transportation (Mégraud and Lehours, 2007).

2.2.5.1.3. ***Resistance to tetracycline***

Tetracyclines interfere in protein synthesis at the ribosome level by binding to the 30S ribosomal subunit. The change in a nucleotide triplet (AGA-926 to 928-TTC), cognate of the positions 965 to 967 in *Escherichia coli*, has been associated with resistance to these

compounds probably because of lack of binding to the h1 loop, which is the binding site of tetracycline (Mégraud and Lehours, 2007). Tetracycline-resistant strains with no mutation in position 926 to 928 have also been described, and efflux is the mechanism most likely to be involved (Mégraud, 2004; Njume *et al*., 2009).

2.2.5.1.4 *Resistance to fluoroquinolones*

Fluoroquinolones block genetic replication by inhibiting the A subunit of the DNA *gyrase*, encoded by the *gyrA* gene. Mutations in the quinolone resistance-determining region of *gyrA* are found in *H. pylori* as well as in other bacteria. The amino acid positions concerned are mainly 87 and 91 (Tankovic *et al*., 2003).

2.2.6 LABORATORY DIAGNOSIS

There are various techniques of detecting *H. pylori* from specimens. These tests may be invasive such as endoscopy and gastric mucosal biopsy, microscopic examination of histological sections and rapid urease test or non-invasive tests such as Urea Breath Test (UBT), enzyme linked immunosorbent assay (ELISA), *H. pylori* stool antigen test (HpSTAR and HpSA), and latex agglutination tests (Shepherd *et al*., 2000; Krogfelt *et al*., 2005).

2.2.6.1 Microscopic examination of histological specimens

Histology can reveal the presence of bacteria as well as the type of inflammation. Many stains can be used to detect the organism, for example Warthine-Starry, Hp silver stain, Dieterle, Giemsa, Giminez, acridine orange, McMullen and immune-staining (Gatta *et al*., 2003; Ndip *et al*., 2003; Tanih *et al.,* 2008). Because of their weak contrast between the bacteria and mucus, haematoxylin and eosin stains are commonly used to evaluate the

inflammatory cells and Giemsa or Genta stain are used to detect the organism. Giemsa stain is most preferred because of its technical simplicity, high sensitivity and low cost (Gatta *et al*., 2003).

2.2.6.2 Culture of biopsy specimens

Specimen collection and transportation is very crucial in isolating *H. pylori*. Antibiotics and bismuth, when used in suboptimal therapy can suppress but not eliminate the organism. Therefore, the patient should not have used these agents for several weeks prior to culture. A single biopsy specimen gives good sensitivity but is not sufficient for a reliable diagnosis because *H. pylori* may have a patchy distribution and the more biopsy specimens analyzed, the higher the chance of detection (Ndip *et al*., 2007; Mégraud and Lehours, 2007; Ndip *et al*., 2008; Tanih *et al*., 2009). In most instances, the bacteria are not distributed homogeneously in the biopsy specimens; therefore grinding of the biopsy is mandatory (Ndip *et al*., 2008).

The media components should include an agar base *e.g*. brain heart infusion agar, Columbia blood agar, and Wilkins Chalgren agar (Ndip *et. al*., 2003; 2008), growth supplements *e.g*. sheep and horse blood or serum as well as selective supplements containing antimicrobial compounds *e.g.* vancomycin or teicoplanin to inhibit Gram-positive cocci; polymyxin, nalidixic acid, colistin, trimethoprim, or cefsulodin to inhibit Gram-negative rods; and nystatin or amphotericin B to inhibit fungi (Ndip *et al.*, 2003; Mégraud and Lehours, 2007).

2.2.6.3. Biochemical characterization

Testing for the presence of certain enzymes such as cytochrome oxidase, catalase, urease, glutamyl transpeptidase, leucine aminopeptidase, and alkaline phosphatase is part of identification in cultured bacteria (Konturek, 2004; Samie *et al*., 2007). Cytochrome oxidase

is present in all members of the Helicobacteraceae family and it is usually detected with special reagents on a disk or a strip. Catalase is also present in all *Helicobacters* and most members of the *Campylobacteraceae* and is detected by introducing a loopful of bacteria into a drop of hydrogen peroxide and observing a very abundant production of bubbles. Nevertheless, catalase-negative mutants of *H. pylori* have been reported (Tanih *et al*., 2009).

To adapt to the acidic environments, *H. pylori* produces large amounts of urease enzyme to buffer the acidic medium and creates a micro-environment. Urease tests have been widely used because they are simple, cheap and easy to carry out; they can be performed readily in the endoscopy suite and give a rapid result. When a loopful of the organism is put in contact with a few drops of urease medium, a color change occurs instantaneously regardless of the formulation. Other diagnostic tests are indeed either strictly based on urease, like the rapid urease test and urea breath test, or partially based, like serology and PCR, which may target urease genes (Mégraud and Lehours, 2007).

2.2.6.4 Polymerase chain reaction (PCR)

PCR is the most commonly used nucleic acid amplification technique for the diagnosis of infectious disease, surpassing the probe and signal amplification methods (Mackay *et al*., 2003). PCR has been used extensively for the diagnosis of *H. pylori* from gastric biopsy specimens, saliva, faeces and archival specimens (Kidd *et al*., 2002; Smith *et al*., 2002; Samie *et al*., 2007).

2.2.6.5 Serological tests

The most common is enzyme-linked immunosorbent assay (ELISA), which detects the totality of the immunoglobulin in a patients' serum. The patient's body produces antibodies that are specific to antigen (*H. pylori*) after exposure. ELISA relies on the fact of the hydrogen bond formed between the antibodies and antigen which is monitored by a colour change when there is a reaction of antigen- antibody. Antibody tests play an important role in studies of pathogenesis and virulence. Antibodies against the important proteins of *H. pylori*, *cagA* and *vacA*, can be detected using different immunological techniques. After eradication of *H. pylori*, the antibodies disappear at different times and some, such as the anti-*cagA* antibodies, may persist for years (Oderda *et al*., 2001; Tanih *et al*., 2008).

An enzymatic immunoassay (EIA), detects the presence of *H. pylori* antigen in stool or faecal specimen. The test is used for diagnosis of antigen infection and for confirmation of eradication after treatment. The most widely used test in the assay uses polyclonal anti-*H. pylori* capture antibodies absorbed to microcells (Ndip *et al*., 2004; Ricci *et al*., 2007).

2.3 PSEUDOMONAS AERUGINOSA

2.3.1 INTRODUCTION

P. aeruginosa is an oxidase-positive, Gram-negative, aerobic rod that belongs to the family Pseudomondaceae. The genus *Pseudomonas* comprises of more than 140 species, only few of these are pathogenic to man and plants. The others are essentially saprophytic and occur widely in nature (Adedeji *et al*., 2007). Although classified as aerobic organisms, *P. aeruginosa* is considered by many as a facultative anaerobe as it thrives not only in normal atmosphere, but also with little oxygen; and has thus colonized many natural and artificial environments. Because it thrives on most surfaces, this bacterium is also found on and in

medical equipment including catheters, respirators, causing cross infection in hospitals (Prinsloo *et al.*, 2008). *P. aeruginosa*, a major nosocomial pathogen, is also responsible for community-acquired infections, generally associated with contaminated water and solutions (folliculitis, otitis and corneal ulcers) (Dubois *et al*., 2008).

2.3.2 PATHOGENESIS AND CLINICAL MANIFESTATION

The pathogenicity of *P. aeruginosa* is based on its ability to produce a variety of toxins and enzymes such as proteases and elastases and phenazine pigments, such as pyocyanin and rhamolipids; as well as its ability to resist phagocytosis (Kerr and Snelling, 2009). The organism is widely distributed and it is estimated that 5- 30% of normal individuals have *Pseudomonas* in their gastro intestinal tracts but the organisms rarely predominate (Abdulaali, 2009).

The organism is primarily an opportunistic pathogen that causes infections in hospitalized patients (nosocomial infections) particularly in burn patients where the skin host defenses is destroyed, orthopedic related infection, respiratory diseases, immunosuppressed and catheterized patients (Hawkey, 2008). Generally it contributes substantially to wound related morbidity and mortality as the organism enters into the blood, causing sepsis that may spread to the skin and lead to ecthyme gangrenosum, the black necrotic lesion. Several external otitis and skin lesion occurs in swimming pools and hot tubs users, particularly where chlorination is inadequate (Kim *et al*., 2001; Hawkey, 2008).

2.3.3 MODES OF TRANSMISSION

The mode of transmission of *P. aeruginosa* has not been fully elucidated, however it is clear that contact with contaminated surfaces, e.g. respiratory equipment, antiseptics, soap, sinks, mops, hot tubs, artificial fingernails, and physiotherapy and hydrotherapy pools and via indirect contact routes e.g. inadequate hand hygiene on the hands of healthcare workers are a

potential routes of transmission (Giamarellou, 2002). There is some evidence; however that transmission by large particle droplets may be implicated from a colonized or infected respiratory tract. This organism is capable of significant environmental survival on surfaces for period up to several days and this may contribute to cross transmission in a healthcare setting. Airborne transmission of this organism has been postulated; however scientific evidence on this subject is not robust (Jones *et al*., 2003; Panagea *et al*., 2005).

2.3.4 TREATMENT OF *PSEUDOMONAS* INFECTIONS

P. aeruginosa is notorious for its resistance to antibiotics and is therefore, a dangerous and dreaded pathogen. The bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its Gram-negative outer membrane and also its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics; also its natural habitat is the soil, living in association with the bacilli, actinomycetes and moulds (Falagas *et al.,* 2005).

P. aeruginosa is frequently isolated from non sterile sites such as mouth, swabs and sputum; under these circumstances, it often represents colonization and not infection. The isolation of the organism from non- sterile specimens should be interpreted cautiously. Aminoglycosides (gentamicin, amikacin, and tobramycin), quinolones (ciprofloxacin, levofloxacin), cephalosporins (ceftazidime, cefepime, and cefoperazone), carbapenems (imipenem, meropenem) and polmyxins (polymyxin B and colistin) have been traditionally used to treat *Pseudomonas* infections, but resistance to these agents is now common in many parts of the world. The rise in quinolone resistance has undermined the number of available agents to treat infections caused by these bacteria (Hachem *et al.,* 2007; Hawkey, 2008; Wright *et al.,* 2009).

A study in Pakistan among CF patients, found that among the three aminogylcosides, amikacin showed 21% resistance while gentamycin and tobramycin showed 66% and 27% resistance respectively. Cotrimoxazole and penicillin resistance was reported as high as 100% and 99% respectively. The third generation cephalosporin, ceftazidime showed 38% resistance and cefotaxime 76% while the first generation cephalosporines, cefuroxime showed 99% resistance (Anjum *et al*., 2010). Similar results were also found in another study in Jordan which reported resistance of 22% amikacin, 28% gentamicin, 31% tobramycin, 34% ciprofloxacin, 42% ceftazidime, 81% piperacillin, 74% meropenem, 91% cefepime and 94% tetracycline (Masaadeh *et al*., 2009). Lambert (2002) compared the resistance pattern of CF patients and non- CF and found that CF patients were more resistant than non-CF patients (36% CF vs. 3.9% non-CF for amikacin and 43% vs. 9.1% against gentamicin). Quinolones, cephalosporins, carbapenems and antipseudomonal penicillins demonstrated the same pattern (ciprofloxacin 24% vs. 7.3%, ceftazidime 14% vs. 1.7%, imepenem 31% vs. 6.7% and piperacillin 11% vs. 3.4%). The major mechanism of resistance to β-lactam and cephalosporin antibiotics is the production of the enzyme β-lactamase by the organism (Rupp and Fey, 2003).

2.3.4.1 Mechanisms of resistance

Basically, there are three mechanisms by which *Pseudomonas* resists the action of antimicrobial agents: restricted uptake and efflux; drug inactivation and changes in targets.

2.3.4.1.1 Penetration of antibiotics through the cell envelope of P. aeruginosa

All of the major classes of antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets. The aminoglycosides inhibit protein synthesis by binding to the 30S subunit of the ribosome. Quinolones bind to the A subunit of DNA *gyrase,* which

maintains the ordered structure of the chromosome inside the cells. The β-lactams (e.g. piperacillin, ceftazidime, imipenem, meropenem and aztreonam) inhibit the peptidoglycan-assembling transpeptidases located on the outer face of the cytoplasmic membrane. Finally the polymyxins bind to phospholipids in the cytoplasmic membrane, destroying its barrier function (Lambert, 2000).

2.3.4.1.2 Inactivation and modification of antibiotics

All *P. aeruginosa* strains possess the *ampC* gene for the inducible chromosomal β-lactamase. However, induction alone probably does not account for resistance unless the enzyme is over-expressed resulting in spontaneous mutation in the regulatory gene, *ampR*. β- lactamase produced by *P. aeruginosa* includes the extended spectrum β-lactamase (ESBLs) which are capable of hydrolyzing third and fourth generation cephalosporins such as ceftazidime, ceftoxime and cefepime as well as aztreonam (Lambert, 2002). Use of β- lactamase inhibitors (clavulanic acid with ticarcillin and tazobactam with piperacillin) provides protection of these antibiotics against some of the plasmid-mediated enzymes, but not the *ampC* enzyme.

Inactivation of aminoglycosides occurs through production of enzymes which transfer acetyl, phosphate or adenyl groups to amino and hydroxyl substituent on the antibiotics. Prior to the recognition that aminoglycosides are susceptible to efflux, inactivation was regarded as the major mechanism of resistance for this group of antibiotics (Maiti *et al*., 1998).

2.3.4.1.3 Changes in targets

This mechanism of resistance results from mutational changes in target enzymes. In *P. aeruginosa* it is most commonly encountered with the quinolones through mutation in the *gyrA* gene encoding the A subunit of the target enzyme, DNA *gyrase* (Akasaka *et al*., 2001).

2.3.4.1.4 Biofilm and resistance

In CF lung infections, *P. aeruginosa* grows as aggregates of cells (microcolonies) encased in a protective alginate polysaccharide. This mode of growth also occurs on surfaces, where it is referred to as a biofilm. The characteristic property of all biofilms is their remarkable resistance to eradication by physical and biochemical treatments, including antibiotics (Stewart and Costerton, 2001). Although this recalcitrance has been recognized for many years its biological basis has still not been thoroughly explained. Factors which might partly explain the resistance phenotype include the high bacterial cell density and physical exclusion of the antibiotic. Physiological changes might occur in cells within the biofilm involving a general stress response, in which key metabolic processes are shut down and protective mechanisms induced (Mah and OToole, 2001). It is clear that cells in the biofilm, like free-living 'planktonic' cells, can sense the presence of other cells (quorum sensing) and alter their properties accordingly (Riedel *et al.*, 2001). Finally, the population of cells within a biofilm is heterogeneous, containing fast- and slow-growing cells, some resist through expression of inactivating enzymes and efflux pumps, others conspicuously not expressing such systems. The overall resistance is therefore dependent upon an interaction between the entire population of cells and therapy needs to be directed against a multicellular community (Stewart and Costertan, 2001).

2.3.5 LABORATORY DIAGNOSIS

As a result of their clinical significance, many methods exist for the epidemiological investigation of infections caused by *P. aeruginosa*. In different parts of the world, biotyping, serotyping, antibiogram, phage typing, bacteriocin typing, plasmid profile, and more recent techniques like pulsed-field gel electrophoresis and random amplified polymorphic DNA analysis have been used in typing the organism (Ndip *et al*., 2005). However, most of these

methods are not widely available in routine diagnostic laboratories in developing countries because of the technical difficulties and expenses they involve.

2.3.5.1 Culturing

Pseudomonas can infect almost any site or organ, and therefore, can be isolated from various body fluids such as sputum, urine, wounds, and eye or ear swabs and from blood. The site of the specimen will determine the type of medium to use. Mac Conkey or CLEAD agars are used to culture specimens from sterile sites (pure specimens) and if the specimen is not pure, then the use of a selective medium is essential. Cetrimide agar has been traditionally used for this purpose. When grown on it, *P. aeruginosa* may express the exopigment pyocyanin which is blue-green in colour and the colonies will appear flat, large and oval. It also has a fruity smell characteristic (Hachem *et al*., 2007).

2.3.5.2 Biochemical tests

P. aeruginosa is catalase, oxidase, nitrate and lipase positive. When grown on triple sugar iron medium, it is basic and it does not produce gas or hydrogen sulfide. Oxidase test is used to determine if an organism posses the cytochrome oxidase enzyme or indophenols oxidase (an iron containing haemapoitein). These enzymes catalyze the transportation of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The cytochrome system is usually present in aerobic organisms. The test reagent N,N,N,N- tetra-methyl-P-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol (http://ww.hpa-standardmethods.org.uk/wg bacteriology).

2.3.5.3 Serological tests

Several studies have propagated serology and various antigens to diagnose initial *P. aeruginosa* infection. Antibodies against three important serum proteins alkaline protease, elastase, and exotoxin A have been detected using different immunological techniques [Exotoxin A and *Pseudomonas*- IgG ELISA, crossed immune electrophoresis (CIE), Western immunoblot] (Ratjen *et al.*, 2001). Precipitin measurement by CIE has been largely taken over by ELISA and Western blotting techniques. In CF, these tests are sensitive for diagnosing chronic *P. aeruginosa* colonization. However the failure of serological tests to detect early colonization in young patients and early infections emphasizes the need for continued reliance on cultures (Tramper-Stranders *et al*., 2006).

2.3.5.4 Molecular techniques

PCR is the most commonly used nucleic acid amplification technique for the diagnosis of infectious disease, surpassing the probe and signal amplification methods (Mackay *et al*., 2003; Bresson *et al*., 2010). Several PCR techniques are widely used e.g. Radom amplified polymorphic DNA PCR based on bacterial genotype and has been used as a typing technique. This technique is fast and reliable in discrimination among *P. aeruginosa* strains. However, Real time PCR (*gyr* B PCR) has been used extensively for the diagnosis of *P. aeruginosa* because of its specificity over other types [VIC, ETA and OPR real time PCR] (Qin *et al*., 2003).

2.4 PLESIOMONAS SHIGELLOIDES

2.4.1. INTRODUCTION

P. shigelloides was previously grouped in the family *Vibrionaceae* until molecular studies carried out by Martínez-Murcia *et al.* (1992) and Ruimy *et al.* (1994) indicated that *P. shigelloides* is phylogenetically related to the genus *Proteus*. Furthermore, Huys and Sings (1999) in an evaluation using the amplified fragment length polymorphism (AFLP) technique for genotyping *Aeromonas* species, found that *P. shigelloides* clearly falls out of the major *Aeromonas* cluster. In the light of these findings the genus *Plesiomonas* was moved to the family *Enterobacteriaceae*, and is the only oxidase-positive member of this family (Garrity *et al*., 2001).

The single specie in the genus *Plesiomonas*, *P. shigelloides* is a capsulated Gram-negative, flagellated and non-spore-forming facultative anaerobic rod (Garrity *et al*., 2001). This newcomer among the expanding group of known water and food-borne pathogens, *P. shigelloides* is increasingly regarded as an emerging and significant enteric pathogen (González-Rey *et al*., 2004).

2.4.2 PATHOGENESIS AND CLINICAL MANIFESTATIONS

P. shigelloides possesses several pathogenic properties such as production of heat-stable and heat-labile toxin, cytotoxin, hemolysin, haemagglutinin and other potential virulence factors. However, evidence so far has not been able to describe the direct association of these factors with clinical symptoms (Krovacek *et al*., 2000).

The bacterium causes mild, self-limiting diarrheal diseases in healthy adults and severe extra-intestinal complications such as septicemia and meningitis among children and immuno-compromised individuals with underlying maladies such as malignancy, splenectopy, alcoholic liver disease, cirrhosis, sickle-cell anaemia, and primary haemochromatosis (excess

deposition of iron throughout the body (Ampofo *et al*., 2001; Salerno *et al.,* 2007). *P. shigelloides* gastroenteritis is usually a mild self limiting disease with fever, chills, abdominal pain, nausea, diarrhea or vomiting. The onset of symptoms is 20 - 24 hours after consumption of contaminated food or water. Diarrhea is watery, non-mucoid and non-bloody, in severe cases, diarrhea may be greenish-yellow, foamy and blood tinged. Duration of illness in healthy people may be 1 to 7days (Wong *et al*., 2003).

2.4.3 EPIDEMIOLOGY OF *P. SHIGELLOIDES*

The primary reservoirs of *P. shigelloides* are fresh and estuarine waters throughout the world, while different kinds of seafood act as secondary reservoirs in these environments. This bacterium has also been isolated from humans as well as domestic animals such as dogs, cats, goats, sheep and cows (Krovacek *et al*., 2000; González-Rey *et al*., 2004).

Most of the reports on isolation of *Plesiomonas* are from countries situated in the tropical or subtropical areas. This bacterium has also been called the "Asian" bacteria because of the high incidence of isolations in countries such as Japan and Thailand (González-Rey *et al*., 2004). *P. shigelloides* is not limited to Asia, as recent studies in various African countries show that the organism plays an important role in gastro-enteritis and diarrheal cases (Oviasogie and Ekhaise**,** 2005; Obi *et al*., 2007; Ramalivhana and Obi, 2009).

2 .4 .4 MODES OF TRANSMISSION

Human infections due to this bacterium are mostly food and waterborne. The organism may be present in unsanitary (contaminated) water, which has been used as drinking water, recreational water, or water used to rinse foods that are consumed without cooking or heating; but also eating uncooked shellfish (contaminated with the organism). *P. shigelloides* infections occur in summer months and correlate with environmental contamination of freshwater [rivers, streams and ponds] (Krovacek *et al*., 2000).

2.4.5 TREATMENT OF *P. SHIGELLOIDES*

Treatment of *P. shigelloides* infections is based on the use of empiric antibiotic therapy especially in developing or rural communities without adequate facilities for antimicrobial susceptibility testing. This also contributes to the antibiotic resistance of the drug of choice.

Plesiomonas are generally sensitive to second- and third-generation cephalosporins (except cefoperazone, ceftazidime and cefepime), carbapenems, aztreonam, co-trimoxazole, tetracycline, aminoglycosides, aminopenicillins in combination with β- lactamase inhibitors (Stock & Wiedemann, 2001; Oviasogie and Ekhaise, 2005; Ramalivhana and Obi, 2009).

Like other members of the family *Enterobacteriaceae*, most of the *P. shigelloides* strains are resistant to a broad spectrum of penicillins, including ampicillin, piperacillin, ticarcillin, mezciocillin, carbenicillin, azlocillin and others (Avison *et al*., 2000; Stock and Wiedemann, 2001).

A study in Venda region of South Africa demonstrated that imipenem, meropenem, cephazoline, cefepime, ciprofloxacin, gentamicin and cefoxitin may be useful in the treatment of *P. shigelloides* infections. However, the organism showed resistance to multiple antibiotics such as penicillin G, ampicillin, vancomycin, amoxicillin, tetracycline and erythromycin (Ramalivhana and Obi, 2009). The results accord with the findings of Obi *et al*. (2004) in the

same region who reported resistance of 50% in erythromycin, 55% in chloramphenicol and 65% in ampicillin. The strains also demonstrated multiple antibiotic resistances (resistance to one or more classes of antibiotics) to ampicillin, tetracycline, erythromycin, chloramphenicol and co-trimoxazole which is in line with other findings (Asefa *et al.*, 1997; Obi *et al.*, 1997).

2.4.6 LABORATORY DIAGNOSIS

2.4.6.1 Culture.

Previously, enteric agars (MacConkey, Samonella Shigella (SS) and xylose lysine desoxycholate (XLD) agars) were used for isolation and identification of *P.shigelloides*. These media present diverse challenges for differentiation of *P. shigelloides* from other bacteria species. For instance, MacConkey agar supports the growth of *Plesiomonas* but because the genus contains both lactose positive and lactose negative strains it cannot be used for differential purposes (González-Rey *et al*., 2003). Schubert (1977) found SS agar as the most favourable medium for the growth of this bacillus but it could not differentiate *P. shigelloides* from other members of the family *Enterobacteriaceae* or *Aeromonas* spp. Therefore, other investigators have tried to formulate media that could overcome these problems. Von Gravenitz and Bucher (1983) recommended the use of inositol brilliant bile salt agar (IBB). This medium can differentiate between *Aeromonas* and *Plesiomonas*: *Aeromonas* appears colourless while *Plesiomonas* colonies are whitish to pinkish. Bismuth sulphite, deoxycholate- citrate-lactose- sucrose, Hektoen enteric, cefsulodin- Irgasan-novobiocin, sorbital- mackonkey, blood agar and campylobacter- selective agars can also be used for the detection of *P.shigelloides* (González-Rey *et al*., 2003).

2.4.6.2 Biochemical tests

P. shigelloides is oxidase, indole and lipase positive. When grown on triple sugar iron medium, it is basic and it does not produce gas or hydrogen sulfide. Being the only oxidase positive organism in the enterobacteriacae family, oxidase test is the most important diagnostic test of *P.shigelloides*. There are a variety of different commercial available kits, such as the TTE-AS and the API 20E, that give reliable results for identification of *P. shigelloides.* However, the value of biochemical typing is limited due to the phenotypic homogeneity of the species. Minor differences only are observed in carbohydrate fermentation (Krovacek *et al*., 2000).

2.4.6.3 Molecular techniques

PCR is a powerful tool that is used effectively in the identification and detection of food borne bacterial pathogens. PCR procedure is based on the 23S rRNA gene for identification of *P. shigelloides* strains from different environments. However, the use of DNA-based typing techniques, such as enterobacterial repetitive intergenic consensus (ERIC-PCR), repetitive extragenic palindromic-PCR (REP-PCR), random amplified polymorphic DNA (RAPD) and PFGE, improve the knowledge of *Plesiomonas* isolates (González-Rey *et al*., 2001).

2.5. STREPTOCOCCUS PYOGENES

2.5.1 INTRODUCTION

S. pyogenes are Gram-positive, non spore-forming cocci about 0.5-1.2 µm in size. They often grow in pairs or chains and are oxidase and catalase negative. *S. pyogenes* colonizes the upper respiratory tract of 5-15% of normal individuals (Lamagni *et al*., 2008). It is highly virulent as it overcomes the host defence system. The most common forms of *S. pyogenes* disease include respiratory and skin infections, with different strains usually responsible for each form. As normal flora, *S. pyogenes* can cause infection when immunity is compromised or when the organisms are able to penetrate the constitutive immune system (Todar, 2005).

2.5.2 PATHOGENESIS AND CLINICAL MANIFESTATION

S. pyogenes harbors a large number of virulence factors that contribute to its complex pathogenicity (Cunningham, 2000; Bisno *et al*., 2003). Some of the major virulence factors, the M protein, encoded by the *emm* gene, confers anti-phagocytic properties and induces a type specific host immune response, while the anti-T sera, are the pilin proteins, producing pilus-like structures (Mora *et al*., 2005) involved in adhesion and invasion of eukaryotic cells and in biofilm formation (Abbot *et al*., 2007; Mannetti *et al*., 2007). The streptococcal pyogenic exotoxins (Spe proteins), are potent immunostimulators associated with disease conditions such as acute rheumatic fever, scarlet fever, and STSS. These toxins trigger massive nonspecific activation of T cells and production of inflammatory interleukins and cytokines (Cunningham, 2000; Smoot *et al*., 2002).

Another important virulence factor found at the surface of GAS is the C5a peptidase, an endopeptidase that cleaves the complement-derived chemotaxin C5a, inhibiting the recruitment of phagocytic cells to the site of infection; serum opacity factor (SOF) is yet another virulence factor expressed at the surface of *S. pyogenes*. It binds apolipoprotein A1

and disrupts the structure of high density lipoproteins. It also binds fibronectin and fibrinogen (Gillen *et al*., 2008). Among other surface proteins are the fibronectin-binding proteins Sfb1, FBP54 and R28, which, together with the M protein and the hyaluronic acid capsule, allow the bacterium to adhere to, colonize and invade human skin and mucus membranes (Pahlman *et al*., 2006).

S. pyogenes is responsible for a diverse range of clinical manifestations, from mild skin/ soft tissue infections and pharyngitis to more serious diseases such as bacteraemia, cellulitis, peuperal sepsis, meningitis, erysipelas, pneumonia and necrotizing fasciitis. Development of streptococcal toxic shock syndrome in these patients increases risk of death substantially (Lamagni *et al*., 2008). The nonsuppurative complication, rheumatoid factor and glomerulonephritis continue to affect children in non- affluent countries, underscoring the medico-social impact of streptococcal disease. Rheumatic fever and glomerulonephritis are post streptococcal sequelae due to untreated streptococcal diseases. Rheumatic fever can result in permanent damage to the heart valves and recurrences are common, and life-long antibiotics prophylaxis is recommended following a single case (Cunningham, 2000). Therefore it is important to identify and treat GAS infections in order to prevent sequelae. Scarlet fever and puerperal sepsis have historically been the leading cause of morbidity and mortality with case fatality rates of 25-30% in developed countries (Lamagni *et al*., 2008).

2.5.3 PREVALENCE OF *S. PYOGENES* INFECTIONS

The burden of invasive *S. pyogenes* disease is high, with at least 663 000 new cases and 163 000 deaths worldwide each year. More than 111 million cases of S. *pyogenes* pyoderma and more than 616 million cases of pharyngitis have been reported annually (Carepitis *et al*., 2005). In the USA, approximately 10 000–15 000 cases of GAS occur annually, with a 10–13% mortality rate (Smith *et al*., 2005). Streptococcal pharyngitis continues to be one of the most common childhood illnesses throughout the world, with an estimated 7.3 million outpatient physician visits each year among children in the USA, 15% to 36% of which are due to GAS (Linder *et al*., 2005). The incidence of rheumatoid factor (RF) has declined in industrialized countries since the 1950s to reach today an annual figure of around 0.5 cases per 100 000 school age children. In contrast, it remains an endemic disease in developing countries with annual incidence rates ranging from 100 to 200 cases per 100 000 school-age children. It is also a major cause of cardiovascular mortality in these countries (Batzloff *et al*., 2004). The WHO has estimated that there are 30 million children with rheumatoid heart disease (RHD) in developing countries, compared with only 1.5 million in developed countries while Australia's aboriginal population suffers the highest incidence worldwide (O'Brien *et al*., 2002). In India isolation rates of GAS in children with pharyngitis ranged from 4.2% to 13.7%, which is comparable to the rates reported from developed countries. However, in closed and crowded communities the rates of isolation and spread of GAS infection is higher, 11.2–34% (Nandi *et al.*, 2002).

2.5.4 MODES OF TRANSMISSION

The major modes of transmission of GAS is by direct contact with secretions from infected persons and airborne. Patient to hospital personnel transmission is an example of nosocomial spread of GAS infection in hospital setting while personnel to patient transmission is common in sporadic outbreaks of surgical wound infections or postpartum infections of GAS (Factor, 2005). Among host factors, age, underlying diseases such as diabetes, cancer, HIV infection, chronic lung disease, varicella infection, or other acute or chronic skin lesions, or specific behaviors such as drug use, are known to influence risk of *S. pyogenes* infections. Environmental factors such as household size, and overcrowding, are also known to play an important role in influencing *S. pyogenes* transmission (Factor, 2005; Lamagni *et al*., 2008). As the majority of cases occur spontaneously in the community, possibilities for prevention remain limited.

2.5.5 TREATMENT OF *S. PYOGENES*

S. pyogenes is uniformly susceptible to penicillin, which remains the drug of choice for treating non serious infections by this organism. Erythromycin and other macrolides have been recommended as alternative treatment for patients allergic to penicillin; however, resistance to the macrolide–lincosamide–streptogramin B (MLSB) antibiotics has been reported in many countries (Fluit *et al*., 2001). Normally two mechanisms are involved in the resistance of *S. pyogenes* to macrolides: first, modification of the target by the *erm*-encoded gene products; and second, efflux provided by *mefA* products (Marosin *et al*., 2003; Silva-Costa *et al*., 2005). Treatment of toxic and severe invasive disease with antibiotics is not always effective, and mortality can exceed 50% (Cunningham, 2000).

2.5.6 LABORATORY DIAGNOSIS

2.5.6.1 Culture

Since *S. pyogenes* is responsible for a variety of diseases, various steps are taken to diagnose the bacteria. Streptococcus throat is commonly diagnosed through a throat culture which is recognized as the most reliable method for detecting the presence of GAS in the throat (Fox *et al*., 2006). Optimal recovery of GAS may be achieved by use of blood agar plates containing trimethoprim-sulfamethoxazole to inhibit some of the normal flora and growth under anaerobic conditions to enhance streptolysin O activity. In a positive throat culture, GAS appears as β-hemolytic, colonies on 5% sheep blood agar. Presumptive identification of the β-hemolytic GAS relies on susceptibility to bacitracin or a positive pyrolidonylarylamidase and catalase negative tests (Cunningham, 2000).

2.5.6.2 Serological tests

Serological diagnosis of GAS infections is based on immune responses against the extracellular products streptolysin O, DNase B, hyaluronidase and streptokinase, which induce strong immune responses in the infected host. Anti-streptolysin O (ASO) is the antibody response most often examined in serological tests to confirm antecedent streptococcal infection. The Lancefield serological grouping system, is another serological method for identification of Streptococci based on the immunological differences in their cell wall polysaccharides (groups A, B, C, F, and G) or lipoteichoic acids (group D) (Cunningham, 2000). While culture of GAS on sheep blood agar medium remains the gold standard for detection of GAS in throat swab specimen, newer and more rapid diagnostic tests, including direct carbohydrate antigen and nucleic acid probe detection, are now available (Fox et *al*., 2006).

2.6 PLANTS AS POTENTIAL SOURCES OF ANTIBIOTICS

2.6.1 INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active chemical compounds as antimicrobial agents. Medicinal plants are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, food supplements, and folk medicines, pharmaceuticals, intermediate and chemical entitled for synthetic drugs (Das *et al* ., 2009). It has been estimated that 14- 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno medicinal use of plants (Ncube *et al.,* 2008).

According to Ndip *et al*. (2008), medicinal plants constitute an effective source of both traditional and modern medicines. Herbal medicine has been shown to have profound utility with about 80% of rural population dependence on it for their primary health care; WHO has advocated the need for interaction between modern and traditional medicines with a view to exploiting and identifying compounds that could provide safe and effective remedies for ailments of both microbial and non-microbial origins.

Plants are continuously in contact with different microorganisms, including viruses, bacteria and fungi. In order to protect themselves, plants synthesize aromatic secondary metabolites which produce definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Edoega *et al*., 2005; Das *et al.,* 2009).

Extraction methods involve separation of medically active fractions of the plant tissue from inactive/inert components by using selective solvents and extraction technology. Solvents

diffuse into the solid plant tissues and solubilize compounds of similar polarity. Quality of plant extract depends on plant material, choice of solvents and extraction method. Properties of a good solvent in plant extractions include ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate. As the end product in extraction will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay (Ncube *et al.*, 2008; Das *et al*., 2009). Several studies have screened plant material for antimicrobial properties, various solvents e.g. ethanol, methanol, petroleum ether, chloroform, hexane, ethyl acetate, dichloromethane (DCM) and water has been used to extract active compounds (Eloff, 1998; Eloff, 2005; Masoko, 2005; Ndip *et al*., 2008; 2009; Ademola and Eloff, 2010). Eloff *et al* (1998) found that acetone extracted a greater number of inhibitors from Combretaceae species than other solvents used. Fresh or dried plant material can be used to extract secondary plant components. Most scientists have opted to use dry plant material because traditional healers frequently use dry plant material but also the delay between collection and processing makes it difficult to work with fresh material (Tiwari *et al.*, 2005).

2.6.2 TRADITIONAL MEDICINAL USES OF *COMBRETUM* SPECIES AND THEIR ANTIMICROBIAL EFFECTS

The family, Combretaceae consists of 20 genera and 300 species, the largest of which are *Combretum, Terminalia* and *Quisqualis*. Species from the genus C*ombretum* and *Terminalia* are most widely used for medical purposes. Traditional healers throughout Southern Africa employ species of Combretaceae for many medicinal purposes. These include the treatment of abdominal pain, abdominal disorders, gallstones, dysentery, gastric ulcers, headache, bilharzias, chest coughs, colds, nose bleeding, sore throats, pneumonia, conjunctivitis,

diarrhea, dysmenorrhea, earache, fever, hookworm, infertility in women, venereal diseases including syphilis, cleansing the urinary system, stomach and gastric problems, leprosy, scorpion and snake bite, swelling caused by mumps, fattening babies, jaundice, toothache and general weakness. Several of these uses point towards possible antimicrobial activity including antibacterial, antifungal and antiparasitic activity (Fyhrquist, 2006; Ojewole 2008).

2.6.3 *COMBRETUM MOLLE*

C. molle (R. Br. Ex G. Don) Velvet bushwillow in English (*Basterrooibos* in Afrikaans and *umBondwe* in Zulu), is one of the herbal plants belonging to the genus *Combretum*. *C. molle* is widely used in African traditional medicine for treatment of various ailments and diseases. Various organs such as leaves, roots and stem bark are predominantly used (Tan *et al.*, 2002; Bessong *et al*., 2004). Decoctions of the roots of *C. molle* seem to have a variety of uses against hookworm, stomach pains, snake bite, leprosy, fever, dysentery, general body swellings, and abortion as well as for swelling of the abdomen, sterility and constipation (Fyhrquist *et al*., 2002). Very often, *C. molle* is mixed with other species of *Combretum* as well as with other plant species since remedies made from mixtures of plant species are thought to be more powerful in their healing effects. The roots of *C. molle* are mixed with the roots of *Annona chrysophylla* or *Annona senegalensis*, and this remedy is used as an expectorant. There are few reports on the medicinal use of the stem bark of *C. molle*; an aqueous suspension of the stem bark is used for gargling and is drunk to treat angina and decoctions of the inner bark is used for the treatment of stomach problems. Even if the fruits of *Combretum* species in general are reported to be toxic, they are used as an aid in child birth in the same way as the leaves (Fyhrquist *et al*., 2002).

Some traditional health practitioners in KwaZulu- Natal Province of South Africa have also claimed that decoctions, infusions and other extracts of *C. molle* are effective remedies for the management and/or control of an array of human ailments, including arthritic and other inflammatory conditions (Ojewole, 2008). *C. molle* like other *Combretum* species has also been reported to posses antifungal, antimicrobial, anti-parasitic, antioxidant, and anti-inflammatory activity, as well as anti-HIV type 1 reverse transcriptase (Khan *et al*., 2000; Asres *et.al.* 2001; Eloff *et al*., 2005; Chaabi *et al*.,2006; Bessong *et al*., 2006; Angeh *et al*., 2007; Ojewole, 2008). However, we are not aware of studies which have tested the stem bark of the plant for its antimicrobial activity against the selected pathogens which constitute potential health problems in our environment.

CHAPTER THREE

MATERIALS AND METHODS

3.1 BACTERIAL STRAINS

Bacterial strains used in the study consists of reference strains*; Streptococcus pyogenes* ATCC 49399, *Plesiomonas shigelloides* ATCC 51903, *Pseudomonas aeruginosa* ATCC 15442, *Helicobacter pylori* ATCC 43526 and *Helicobacter pylori* clinical isolate which was isolated from gastric biopsies, lyophilized and kept at – 80^{o}C in our laboratory (Tanih *et al*., 2010). These organisms were selected based on their disease burden and increasing trend of antibiotic resistance in the developing world (Eloff *et al*., 2005).

3.2 PLANT MATERIAL

The stem bark of *C. molle* was selected based on ethno-botanical information. The plant was harvested in the vicinity of Venda, Limpopo Province. Identification was done by botanists at the University of Venda where voucher specimen (CNU FHO 5) has been deposited. The bark was washed and dried at room temperature for 2 weeks. It was then ground to fine powder using a mechanical blender (ATO MSE mix, 702732, England).

3.3. PLANT EXTRACTION

Plant extraction was done in accordance with the method of Ndip *et al.* (2009) slightly modified. Technical grade solvents (hexane, dichloromethane (DCM), ethyl acetate, acetone, ethanol and methanol) were employed for extraction. Ground plant material (300g) was macerated in three folds excess of the solvent in extraction bottles such that the solvent was above the plant material. The slurry was placed in a shaker (Edison, N.J., USA) for 48hours then centrifuged at 3000rpm for 5 minutes and filtered using filter paper of pore size 60A. The process was repeated twice for a total of three extractions (exhaustive extraction) for each solvent. The collected extracts were concentrated under reduced pressure in a rotavapor

(Strike 202, Steroglass) to recover the solvents. The yielded extracts were weighed and stored in a labeled tight lid container for further bioassay.

3.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING

3.4.1 Determination of antimicrobial activity by agar well diffusion method

The antimicrobial susceptibility was determined in accordance with method previously described by Boyonova *et al*. (2005). Inocula were prepared from subcultures of bacteria as follows: four to five colonies of the isolate were emulsified in sterile normal saline and turbidity adjusted to 1x 10^9CFU/mL (corresponding to 0.5 McFarland standards). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates (Oxoid, Basingstoke, England). The plates were allowed to dry for 3- 5 minutes. Wells about 6mm in diameter were aseptically punched with a sterile cork borer (5 holes per plate) and the wells were filled with 50 µl extracts of different concentrations (200mg/mL, 100mg/mL and 50 mg/mL). The plates were left for 30 minutes before incubation in order for the extracts to diffuse into the agar. The plates were incubated at 37^0C for 24 hours and the zone of inhibition measured to the nearest millimetre. The plates were in duplicate and the mean zone diameter of inhibition was calculated for each solvent; 0.025mg/mL of ciprofloxacin was used as a positive control and 80% acetone as a negative control. The break point of ≥ 11mm was considered susceptible (CLSI, 2008).

H. pylori was inoculated on Columbia blood agar base (Oxoid, Basingstoke, England) supplemented with 7% sterile defibrinated sheep blood and skirrows {amphotericinB (2.5 mg) and *Campylobacter* supplements; trimethoprim (2.5 mg/mL), vancomycin (5.0 mg/mL) and cefsulodin (2.5mg/mL)}. Plates were incubated at 37ºC for 3 - 5 days under micro-aerophilic conditions (6% O_2 and 10% CO_2) [Anaerocult, Basingstoke, England] (Tanih *et al*., 2010).

3.4.2 *Determination of minimum inhibitory concentration (MIC)*

MIC was determined using broth microdilution method previously described by Njume *et al*. (2010). Briefly, the extracts were dissolved in 80% acetone giving a stock concentration of 10 mg/mL. BHI broth (100µl) was dispensed into the wells except the last row of wells which contained distilled water. A 100µl of the stock solution was dispensed into the first well; a two-fold serial dilution was carried out up to well number 12 from which 100µl was discarded. 20µl of standardized bacterial suspension (1 x 10^9 CFU/ mL) was added to the wells except the control wells (control wells contained broth only, broth+ extract, broth + isolate and distilled water only). The plates were read using micro plate reader (Bio-Rad 680, Japan) adjusted to 620 nm before and after incubation.

For *H. pylori* isolates, BHI broth was supplemented with 7% sterile horse serum and skirrows [amphotericin B (2.5 mg) and *Campylobacter* supplements; trimethoprim (2.5 mg/ml), vancomycin (5.0 mg/mL) and cefsulodin (2.5mg/mL)], plates read at the same wavelength. Plates were incubated at 37ºC for 48 hours under micro-aerophilic conditions (6% O_2 and 10% CO_2) [Anaerocult, Basingstoke, England]. The two absorbencies were compared, the rise in absorbance reflected bacterial growth. MIC was determined as the lowest concentration (highest dilution) that inhibited bacterial growth.

3.5 PHYTOCHEMICAL ANAYLSIS

3.5.1 Thin layer chromatography (TLC)

Fractionation of the most active extract, acetone was done using Silica gel TLC plates (Merck, Kieselgel 60 F_{254}) according to the method of Masoko *et al*. (2006). Acetone extract (5µl) of different concentrations (25, 50 and 100mg) was spotted on TLC plates, and eluted using the three different mobile solvent systems, ethyl acetate/ methanol/ water (EMW) in the ratio of 40: 5.4 :4 to separate the polar compounds; chloroform/ethyl acetate/fomic acid (CEF) in the ratio 5:4:1 to separate intermediate compounds and benzene/ethanol/ammonium hydroxide (BEA) in the ratio 90:10:1 to separate non polar compounds. These solvent systems have been optimized to separate components of the family Combretaceae (Eloff, 1998). Samples were applied rapidly and developed without delay to minimize oxidation or photo-oxidation of constituents. The plates were done in duplictes (plate A for TLC and plate B for bioautography screening). The developed plates (plate A) were visuallized under ultraviolet light (254 and 360 nm), which was subsequently sprayed with vanillin sulphuric acid spray reagent (2mg of vanillin in 28 mL of methanol plus 1mL of concentrated sulphuric acid) and carefully heated for 5 minutes at 100^{o}C to allow colour development. The retention factor value (R_f) of the visible bands were marked under daylight.

$$R_f = \frac{\text{distance moved by analyte (compound)}}{\text{distance moved by solvent}}$$

3.5.2 Bioautography

Bioautography was carried out as described by Masoko *et al*. (2006). Developed TLC plates (plate B) were dried under a stream of fast moving air for 3 days to remove traces of solvent on the plates. *P. shigelloides, P. aeruginosa* and *S. pyogenes* were subcultured in 20 mL of freshly prepared BHI broth and incubated overnight, to ensure that actively growing bacteria is sprayed. The plates were sprayed with the bacterial suspension until wet, incubated overnight at 37°C and 100% relative humidity. The plates were sprayed with 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) [0.2mg of INT + 99mL of water + 1mL of methanol] and further incubated for 1hour. White bands indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of organisms. The R_f of the inhibition zones on the plate (plate B) was compared with the R_f of the reference chromatogram (plate A) to determine the R_f of the active compound.

H. pylori is a fastidious organism which requires nutritional supplements as well as special growth conditions, as such, the organism did not grow on the TLC plate and indirect bioautography (agar overlay) method was used prevously described by Valgas *et al*. (2007). Developed, dried TLC plates were placed in sterile petri dish. A thin layer of BHI agar containing 10^5CFU/mL of *H.pylori* isolates , skirrows and 7% horse serum was poured over the plates. After solidification of the medium, the plates were incubated for 3-5 days under micro-aerophilic conditions (5 - 6% O_2 and 10% CO_2) [Anaerocult, Basingstoke, England]. Inhibition of growth was indicated by zone of inhibition around the active compounds on the chromatogram. The R_f of the zones on the plate was compared with that on the reference plate to find the R_f of the active compound.

3.5.3 Column Chromatography

Column chromatography was used to purify the individual chemical compounds from the extract, as previously described by Ndip *et al*. (2009). Briefly, 50g of silca gel (MN Kieselgel 60, 0.063- 0.2mm/ 70 – 230 mesh, ASTM) was weighed, packed in the column and equilibrated with ethyl acetate for two days before loading the extract. Six grams of acetone extract plus 12g of silica gel powder was mixed and ground to very fine particles. The mixture was then loaded onto a silica gel column equilibrated with ethyl acetate. The combination which gave good activity, EMW (40:5.4: 4) was used to elute the column; fractions (200 mL) were collected and concentrated on a rotary evaporator (Steroglass 202 Rotavapa). Fractions were weighed and stored in air tight containers for further bioassay. MIC of the fractions was determined and the active fractions were further analyzed on TLC to determine the purity.

3.6 Gas chromatography and mass spectrometry (GC-MS)

GC-MS anaylsis was carried out using Helett- Packard HP 5973 mass spectrometer interfaced with an HP- 6890 gas chromatograph with an HP5 column (30mx 0.25mm 0.25 μm film thicknesses) and an FID detector. The oven temperature was programmed from 70^{o}C (after 2 minutes) to 325^{o}C at 4 ^{o}C / minute, final temperature was held for 10 minutes at 240 ^{o}C. The ion source was set at 240^{o}C and electron ionization at 70 Ev. Helium was used as the carrier gas (1mL / min). The split ratio was 1:25 with the scan range of 35 to 425amu (Senatoe *et al.*, 2004).

3.7 *High performance liquid chromatography (HPLC)*

The detection and identification of non-volatile compounds were performed using Thermo Fisher Scientific series model surveyor HPLC System (Hypersial Gold C18 column of 5µm particle size). Solvents were HPLC- grade methanol and ethyl acetate. A gradient involving two mobile phases was used with mobile A consisting of methanol: water (75:25 v/v) and mobile B ethyl acetate. The running time was 15mins, pressure 1177 Psi, and flow rate 1ml/min. We did not have commercial standards of the known compounds to co-inject with samples in order to identify and confirm the presence of the compounds in the extracts. A graphical print out of the retention time of the compounds was obtained (Wei *et al.,* 2006).

3.8 STATISTICAL ANALYSIS

Analysis was performed using the SPSS version 17.0 (Illinois USA, 2009). The One way ANOVA test was used to determine if there was any statistically significant difference in the zone diameter of inhibition of the plant extract and antibiotic; the MIC of the extracts and the antibiotics and the MIC of the extracts and the fractions. P-values <0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 Extract yield

Dried and ground *C.molle* stem bark (300g) was serially extracted with hexane, ethyl acetate, DCM, acetone, ethanol and methanol. Methanol was the best solvent with a yield of 6.77g followed by acetone (3.97g), ethyl acetate (1.25g), DCM (0.93g) while hexane was the least with 0.002g as shown in Table 1.

Table 1: Extract yield of *C. molle* stem bark powder (300g) serially extracted with six solvents.

Solvents	**Mass residue extracted (g)**				
	1st extraction	2nd extraction	3rd extraction	Total mass	Yield %
Hexane	0.001	0.001	-	0.002	0
Ethyl acetate	0.5	0.4	0.35	1.25	0.4
DCM	0.5	0.3	0.13	0.93	0.3
Acetone	2.5	0.99	0.48	3.97	1.3
Ethanol	0.23	0.19	0.22	0.64	0.2
Methanol	3.32	1.95	1.5	6.77	2.3

4.2 Antimicrobial activity of the extracts of *C. molle* against the test organisms.

Results obtained revealed that the acetone extract was active against all the organisms; *P. shigelloides* was the most susceptible organism to all extracts while *H. pylori* (clinical isolate 252C) was the most resistant with partial zones of inhibition. Generally the zone of inhibition ranged from 0 - 32 mm, with the best activity (11 - 32 mm) demonstrated by the acetone, and least, 0 - 17 mm by DCM (Table 2). An inhibition zone diameter of ≥ 11mm was chosen as a break-point of bacterial susceptibility of the extracts and the antibiotic. Ciprofloxacin (0.025mg/ml) which was used as a positive control had a zone diameter of inhibition of 16 - 36mm; acetone (80%) used as the negative control, showed no activity. Since the yield of hexane was small, no further tests were carried out with this extract. The zones of inhibition of the extracts and antibiotic were compared; no statistical significant difference was observed for four extracts (acetone, methanol, ethanol and ethyl acetate).

Table 2: Zone of inhibition (mm) of the organisms against extracts and ciprofloxacin.

Organism	Solvent extracts of the stem bark of *C. molle* / antibiotic										
	Acetone		Ethanol		Methanol		Ethyl acetate		Dichloromethane		Ciprofloxacin
	(mg/ mL)										
	200	100	200	100	200	100	200	100	200	100	0.025
S.pyogenes	24±2.1	19±0.7	21±0.7	15±0.7	20±4.2	17±5.6	16	14±0.7	17±2.1	15±3.5	30±0.7
P. aeruginosa	25±1.4	18±1.4	17	15±1.4	8±2.8	3±3.5	7± 0.7	0	11±0.7	8±1.4	33±1.4
P.shigelloides	32±4.9	29±2.1	32±2.8	28±0.7	25±1.4	21±3.5	17±1.4	12	21±0.7	20	36±2.8
H. pylori 252C	15±1.4	11±2.1	11±2.8	7±0.7	13±0.7	0	7±2.1	0	0	0	16±2.8
H.pylori 43526	16±2.1	11±2.1	11±1.4	9±2.1	15±4.24	0	12±1.4	7±3.5	0	0	35

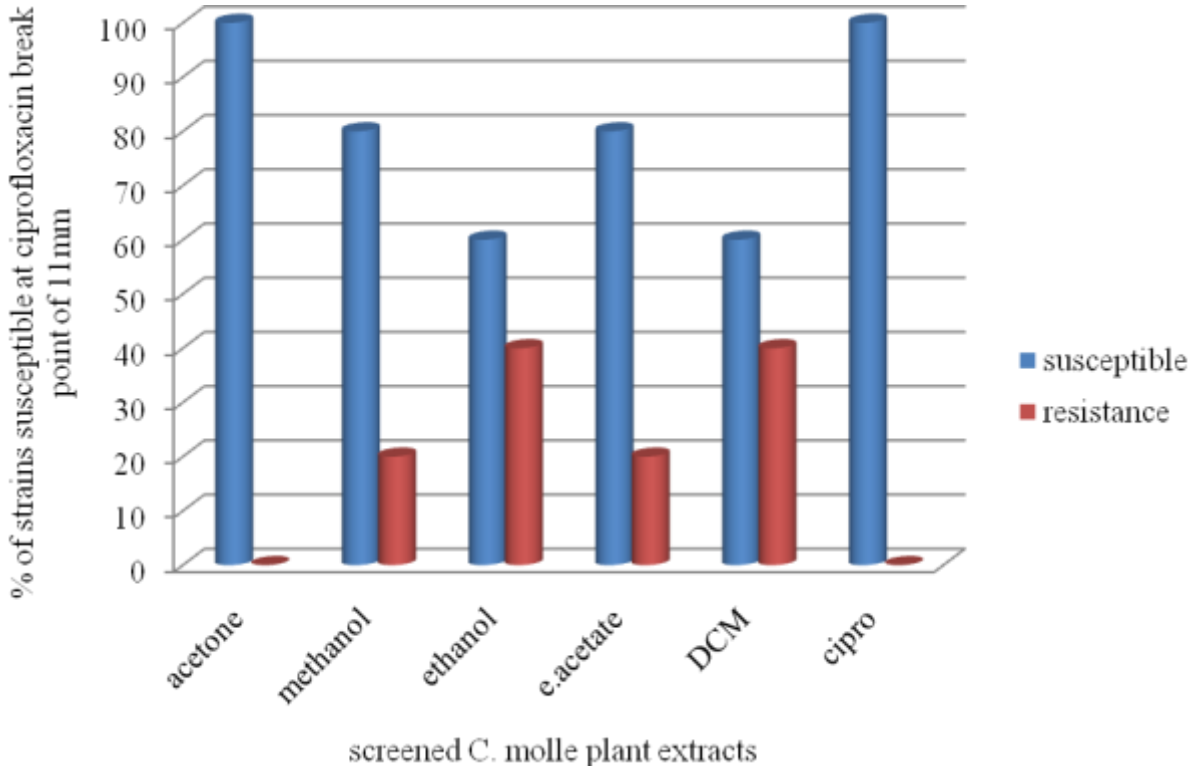
Experiments were repeated twice and mean ± standard deviation recorded

The mean zone diameter of the individual extracts against the organisms was calculated and the results showed that acetone had potent activity (22.5 ±7.0) followed by ethanol (18.8 ± 8.3); the least was the DCM extract (12.8 ± 9.6) {Table 3}; hence acetone extract was chosen for subsequent bioassay.

Table 3: Zone of inhibition of the organisms against extracts and antibiotic

Extract/ control antibiotic	Mean zone diameter (mm)	Inhibition range (mm)
Acetone	22.5 ± 7.0	8-32
Ethanol	18.8 ± 8.3	0-32
Methanol	17.8 ± 5.9	0-29
Ethyl Acetate	16.2 ± 6.5	0-25
Dichloromethane	13 ± 3.9	0-17
Ciprofloxacin	33.2± 2.3	16-36

Of the five organisms subjected to the plant extracts and antibiotic, 100% susceptibility was recorded for the acetone extract and antibiotic. Susceptibility of methanol and ethyl acetate was 80% while ethanol and DCM was 60% respectively (figure1).



e. acetate, ethyl aceate; DCM., Dichloromethane

Fig 1: Susceptibility of the extracts against the test organisms at 200mg/mL.

4.2.1 MIC_{50} Determination

MIC_{50} of the extracts was determined against the organisms alongside ciprofloxacin. Acetone extract was the most active with a MIC_{50} ranging from 0.078 - 2.5mg/mL followed by ethanol, 0.625 - 5mg/mL while ethyl acetate was the least (2.5mg/mL). On the other hand, the MIC_{50} of ciprofloxacin ranged from 0.00312- 0.0156 mg/mL (Table 4). There was no statistical significance difference between the MIC of the extracts and control antibiotic ($P > 0.05$).

Table 4: Antibacterial activity of *C. molle* and ciprofloxacin (MIC_{50}) against the test organisms

Organism	MIC_{50} of the extracts/antibiotic (mg/mL)					
	Acetone	**Ethanol**	**Methanol**	**DCM**	**E.A**	**Cipro**
S.pyog.	0.156	0.625	2.5	0.625	2.5	0.00312
P. shig.	0.078	0.625	0.156	0.312	2.5	0.00312
P.aerug	0.625	1.25	2.5	2.5	2.5	0.0156
*H.p*252C	2.5	1.25	ND	ND	ND	0.0125
H.p 43526	1.25	5	ND	ND	ND	0.0125

DCM, Dichloromethane; E.A., Ethyl acetate; Cipro, Ciprofloxcin; *P.shig*, *Plesiomonas shigelloides* ATCC 51903; *P.aerg.*, *P aeruginosa* ATCC 15442; *H.p.*, *Helicobacter pylori* ATCC 43526; *Helicobacter pylori* 252C (clinical isolate); *S. pyog.*, *Streptococcus pyogenes* ATCC 49399; ND, not determined (no activity at 10mg/mL).

4.3 Fractionation of bioactive compounds by thin layer chromatography

Since acetone extract was the most active, it was fractionated on silica gel TLC plate. Intermediate polarity solvent combination, CEF, separated 10 compounds, while the more polar combination, EMW, separated 9 compounds. The least polar combination BEA had the least number of compounds (3). Twenty- two compounds were observed with Vanillin spray while 9 were visualized under UV; under UV, 5 compounds fluoresced on CEF, 3 on BEA and only 1 on EMW (Table 5).

Table 5: Putative compounds fractionated on TLC plate.

	EMW	CEF	BEA	Total no. of compounds
Vanillin	9	10	3	22
UV	1	5	3	9

UV, ultra violet; EMW, ethyl acetate/methanol/water; CEF, Chloroform/ethyl acetate/formic acid; BEA, Benzene/ ethanol/ammonium hydroxide.

4.3.1 Bioautography assay

Although more compounds were observed on CEF plate, EMW separated more active compounds. Since the R_f value is constant for the same compound under defined conditions, the presence of clear bands with the same R_f value may imply that the same compounds are probably responsible for the antimicrobial activity in the extract. Therefore, to determine the R_f values of the active compounds against the test organisms, the plates were compared with that of the reference plate.

Table 6: Inhibition of bacterial growth by acetone extract by bioautography

Organism	EMW		CEF	
	a	B	a	b
H. pylori	0.181	+	0.031	+
	0.361	+	-	-
	0.515	+	-	-
P.shigelloides	0.181	+	0.031	+
	0.361	+	-	-
	0.515	+	-	-
S.pyogenes	0.818	+	0.437	+
P.aeruginosa	0.818	+	0.562	++
	0.878	+	0.437	++

a, R_f of the active compound; b, degree of inhibition; +, active; ++, more active; -, no activity

CEF, chloroform/ ethyl acetate/fomic acid; EMW, ethyl acetate/ methanol/water; R_f , retention factor

4.4 Column chromatography analysis and MIC_{50} determination of the fractions

Seventeen fractions (200mL each) were collected from the column chromatography assay. Fractions E.A 1- 4 were eluted with 100% ethyl acetate and fractions EMW 1-13 eluted with EMW (40:5.4: 4). The fractions were concentrated on a rotavapor, weighed and stored in air tight containers. EMW 1 had the highest yield (1.6 g) while EA1 had the least (0.001g). To determine the purity, fractions were further analyzed on TLC plate. Fractions, EMW 8 and EMW 13 were identified as pure because one band was observed on TLC plate and fractions EMW 6 and EMW 9 had compounds with similar R_f value and different MIC values. The same was true for EMW 11 and 12 (Table7).

Although fractions EMW 8 and EMW 13 were pure, they demonstrated activity against *S. pyogenes* only; whereas fractions E.A 3, EMW 1 and EMW 3 were active against all the test organisms except *H. pylori* with a MIC_{50} ranging from 0.0097 – 2.5mg/mL. E.A 4 (eluted with 100% ethyl acetate) was the only fraction which had broad activity (Table 7).

Table 7: Mass of the eluted fractions and their MIC_{50} against the test organisms

Fraction/ antibiotic	Rf value of the compounds in fractions	MIC_{50} against test organisms			
		S. pyogenes	***P. shigelloides***	***P. aeruginosa***	***H. pylori* 252C**
E.A 1	0.810, 0.878	ND	ND	ND	ND
E.A 2	0.135, 0.168, 0.439, 0.608	ND	ND	ND	ND
E.A 3	0.186, 0.202, 0.472, 0.506	0.195	0.078	1.25	ND
E.A 4	0.641, 0.810	0.195	0.078	0.156	2.5
EMW 1	0.472, 0.608, 0.709	0.0097	0.0097	0.625	ND
EMW 2	0.067, 0.202, 0.506	0.0195	0.156	ND	ND
EMW 3	0.168, 0.202, 0.405, 0.472	0.078	0.156	2.5	ND
EMW 4	0.033, 0.067, 0.202, 0.4391	0.078	0.312	ND	ND
EMW 5	0.033, 0.439, 0. 810, 0.844	0.312	0.625	ND	ND
EMW 6	0.013, 0.439, 0.810	0.312	0.625	ND	ND

EMW 7	0.031, 0.824	0.625	1.25	ND	ND
EMW 8	0.202	1.25	ND	ND	ND
EMW 9	0.013, 0.439, 0.810	1.25	1.25	ND	ND
EMW 10	0.439, 0.810	0.625	ND	ND	ND
EMW 11	0.013, 0.810	ND	0.156	ND	ND
EMW 12	0.013, 0.810	ND	ND	ND	ND
EMW 13	0.878	1.25	ND	ND	ND
Ciprofloxacin		0.024	0.048	0.024	0.075

E.A, ethyl acetate; EMW, ethyl acetate/ methanol/ water; ND, not determined (no activity at 10mg/mL), *P.shigelloides*, *Plesiomonas shigelloides* ATCC 51903; *P. aerginosa*, *P. aeruginosa* ATCC 15442; *H.pylori* 252C, *Helicobacter pylori* 252C (clinical isolate); *S. pyogenes*, *Streptococcus pyogenes* ATCC 49399.

4.5 GC-MS and HPLC Analysis

The two most potent fractions with fewer compounds on TLC plate (E.A 4 and EMW1) were further analyzed by GC-MS to identify the bioactive compounds. The results demonstrated a single peak for both fractions without a clear identity (Figures 2&3). Since GC-MS detects volatile compounds only; it was therefore likely that the active compounds in the fractions were non volatile. The fractions were then further analyzed by HPLC to detect the non volatile compounds obtaining numerous peaks (Figures 4&5) indicating the presence of compounds. The highest peak for each fraction was thought to possibly, represent a compound present in high propotion.

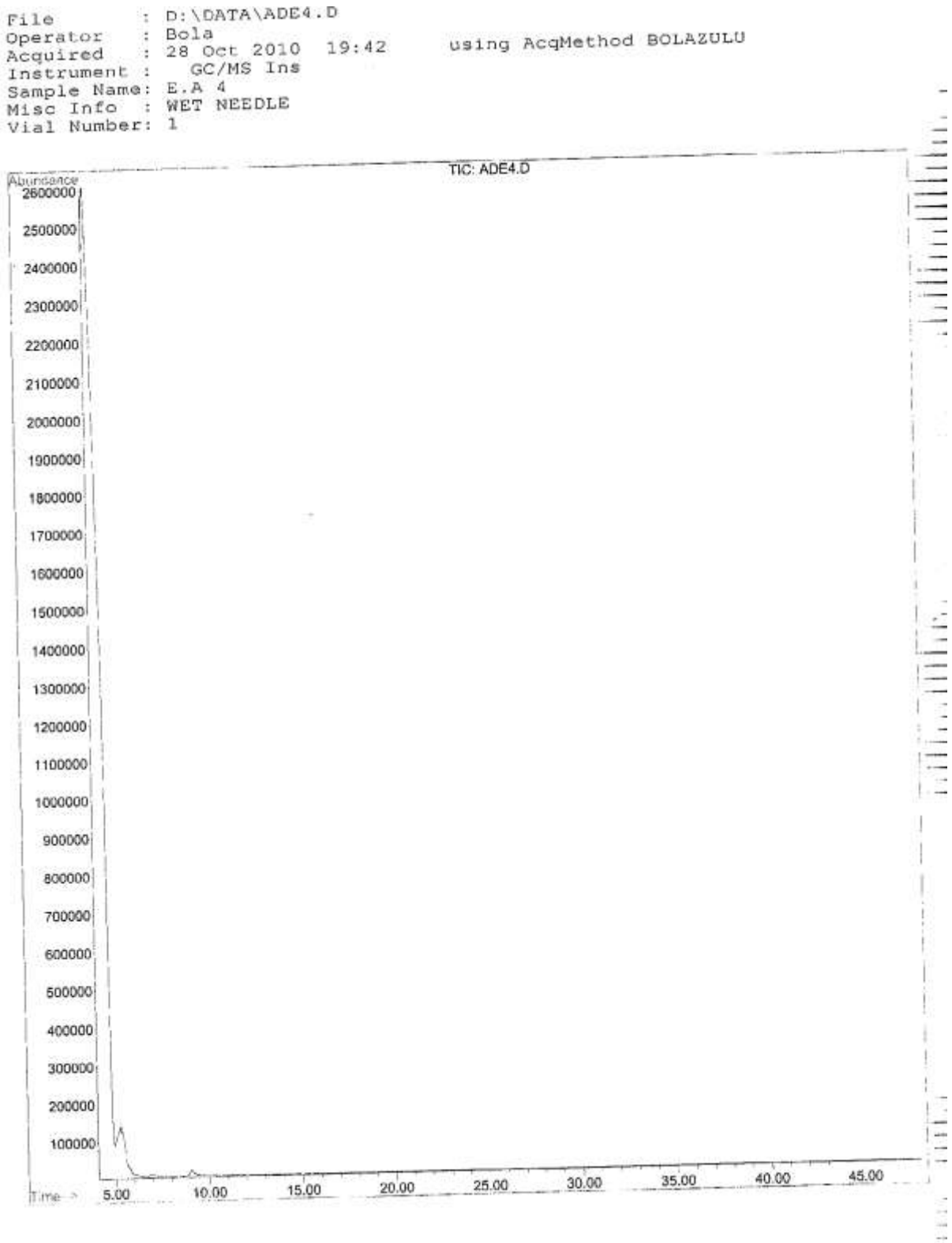


Figure 2: GC-MS peak of EA4 fraction

```
File         : D:\DATA\ADE1.D
Operator     : Bola
Acquired     : 28 Oct 2010   16:01      using AcqMethod BOLAZULU
Instrument   :   GC/MS Ins
Sample Name: EMW1
Misc Info    : WET NEEDLE
Vial Number: 1
```

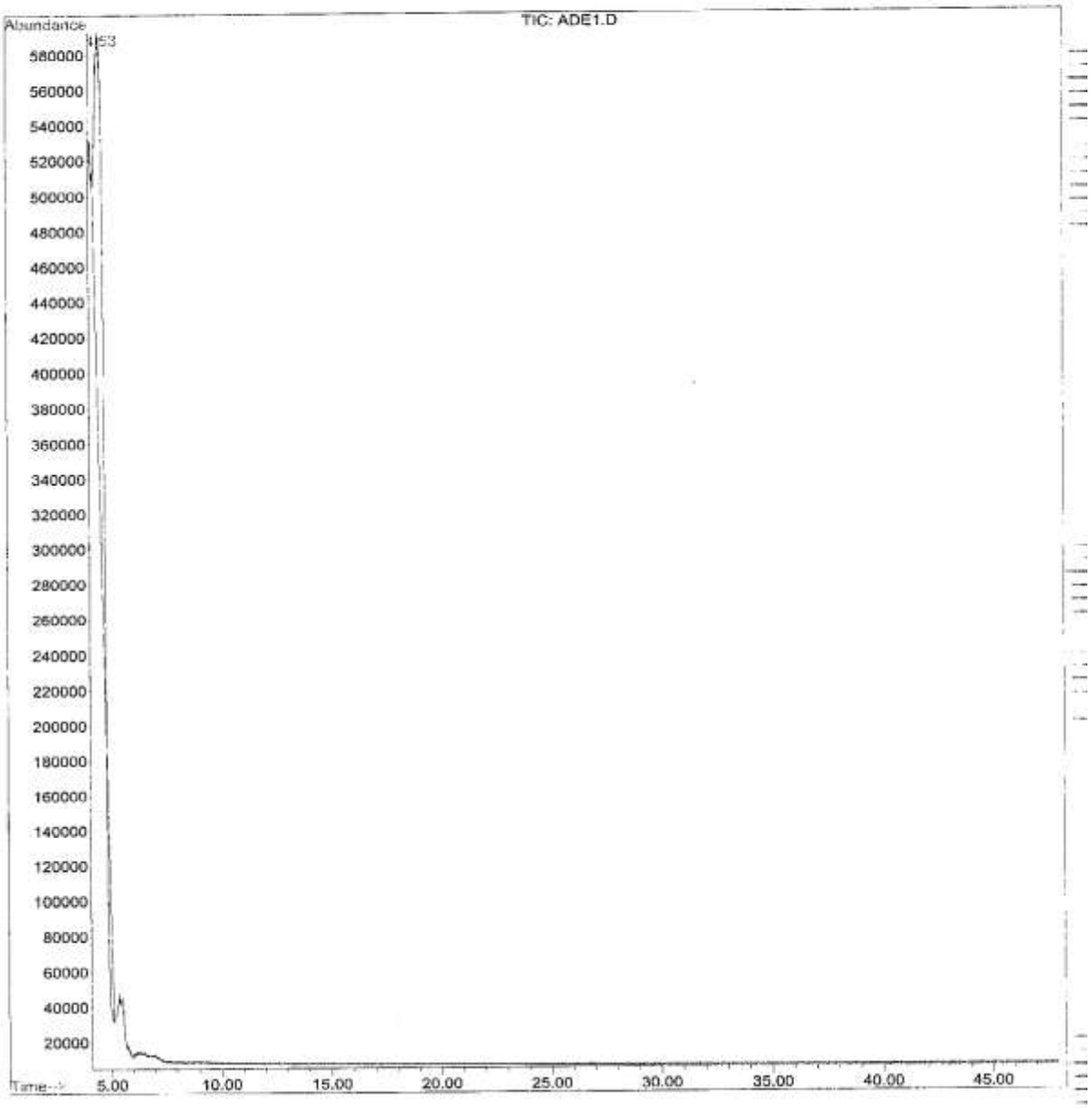


Figure 3: GC-MS peak for EMW1

Area % Report

Data File: C:\ChromQuest\EMW 1 3.5 min.dat
Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Bola1.met
Acquired: 11/1/2010 4:57:54 PM
Printed: 11/1/2010 5:07:52 PM

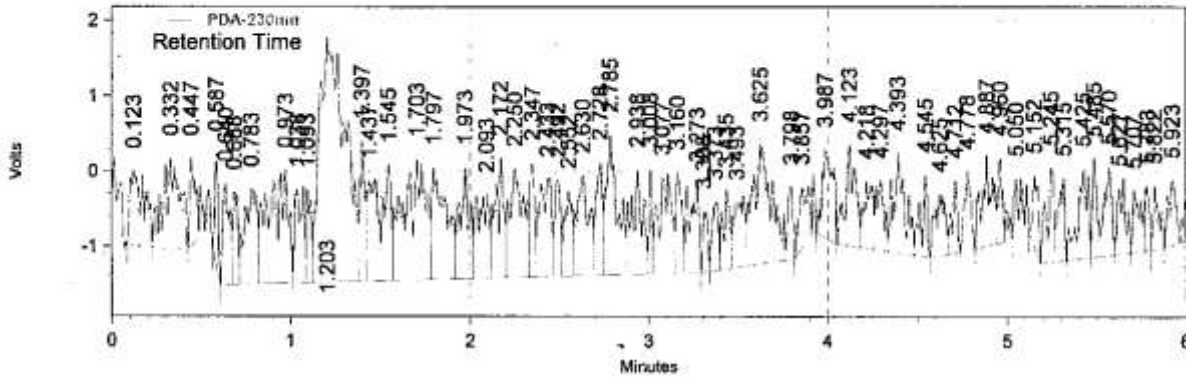


Figure 4: HPLC peaks for EMW 1

Area % Report

Data File: C:\ChromQuest\EA4- 3.5 min.dat
Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Bola1.met
Acquired: 11/1/2010 4:12:47 PM
Printed: 11/1/2010 4:19:46 PM

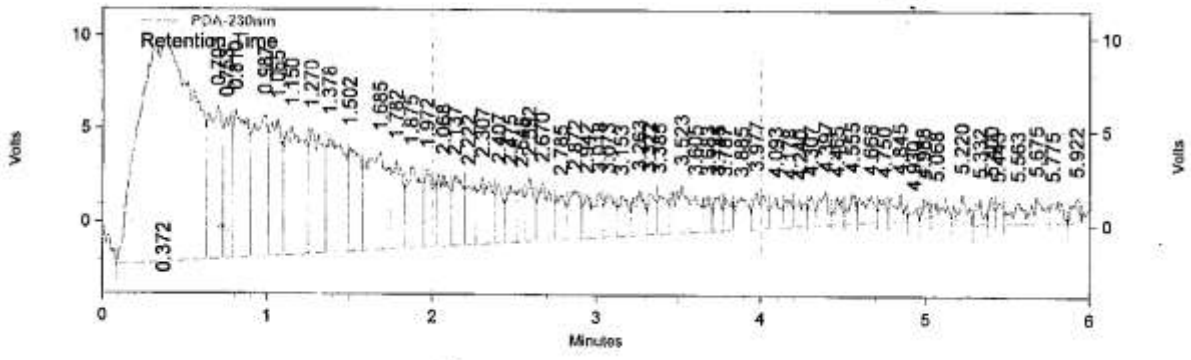


Figure 5: HPLC peaks for EA 4

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

The increasing resistance to conventional antibiotics by microorganisms has necessitated the search for new, efficient and cost effective ways for the control of infectious diseases (Samie *et al*., 2005; Ndip *et al*., 2007). Several studies have reported the antimicrobial activity of *C. molle* against bacteria, fungi and helminths (Asres *et.al.* 2001; Eloff *et al.*, 2005; Ojewole, 2008). Although the phytochemical constituents of the stem bark of *C. molle* are known, to our knowledge, the exact bioactivity against these test organisms has not been established. Therefore our study evaluated the antimicrobial activity of extracts and fractions of the stem bark of *C. molle* in a bid to isolate and identify the main active constituents of this plant against the test organisms.

In the present study, it was observed that methanol and acetone were good extractants with yield of 6.77g (2.3%) and 3.97g (1.3%) respectively while hexane was the least 0.002g (0%). The findings concurs with the work of others (Asres *et al.,* 2001; Eloff *et al.,* 2005; Masoko *et al.,* 2006) who also found that acetone and methanol extract more compounds of *Combretum* species. Angeh *et al*. (2007) worked on different species of *Combretum* and found that the yield differed with species but methanol yield was high in all species. Saponins and Tannins which are reported to be in abundance could be extracted by methanol and acetone (Masoko *et al*., 2006). This might explain why methanol and acetone yielded more than other solvents. Non- polar solvents yield more lipophilic components, while alcoholic solvents give a larger spectrum of polar material. Acetone is usually preferred because it extracts polar and non polar components (Masoko *et al*., 2007).

All the five extracts tested showed varying degree of antibacterial activity against the test bacterial species (Table 3). The antibacterial activity of acetone, ethanol, methanol and ethyl acetate extracts compared favourably with that of the standard antibiotic (ciprofloxacin) ($p > 0.05$) and appeared to be broad spectrum as its activity was independent of Gram reaction. The results imply that these extracts contain compounds with therapeutic potential comparable to the antibiotic.

Previous works have demonstrated potent antimicrobial activity against Gram-positive bacteria (Kelmanson *et al*., 2000; Kumar *et al*., 2010). However, in this study it was realized that *P. shigelloides,* a Gram-negative bacterium was the most susceptible organism with a MIC ranging from 0.078 - 0.625 mg/mL. Most plant extracts have been reported to be more active against Gram-positive than Gram-negative bacteria and has been attributed to the fact that Gram-negative bacteria contains an outer membrane with lipopolysaccharide layer which render them impermeable to certain antibiotics and bactericidal compounds (Nikaido, 1996; Fennell *et al*., 2004).

The weak activities demonstrated by some of these extracts *in vitro*, does not neccessarily imply that they would demonstrate weak activities *in vivo* because of immuno-modulation of chemical compounds from medicinal plants which has been proven to be inactive or weakly active *in vitro*. Also, as with some drugs, some of these plant extracts may be more potent *in vivo* due to metabolic transformation of their components into highly active intermediates (Ngemenya *et al*., 2006; Ndip *et al*., 2009).

The acetone extract was the most potent among the five extracts with a zone diameter of inhibition ranging from 11-32 mm. The findings corroborate previous works (Asres *et al*., 2001; Krugger, 2004; Masoko *et al*., 2006; Mishra *et al*., 2009). In their study, Asres *et al*. (2001) demonstrated good activity of the acetone extract against *Mycobacterium tuberculosis, Trypanosoma brucei rhodesiense* STIB900 and *Plasmodium falciparum* 3D7. The activity was attributed to high amount of hydrolysable tannins present in the stem bark of *C. molle*. It is generally believed that tannins are non-selective enzyme inhibitors due to their polyphenolic groups. However, it has been shown that some hydrolysable tannins display selective cytotoxicity. Therefore, the potential of this group of compounds for drug development should not be undermined, especially when they are proved to be the active ingredients of plants used in traditional medical practices (Asres *et al*., 2001; Funtogawa *et al*., 2004).

Krugger (2004) worked on *Terminalia sericea* and also reported marked activity with the acetone extract against *Staphylococcus aureus* compared to other solvents. Similarly, the acetone extracts of *C. nelsonii, C. albopuctactum, and C. imberbe* and *T.sericea* possessed growth inhibitory activities against fungal pathogens *in vivo* (Masoko *et al*., 2006).

Mishra *et al*. (2009) screened the antifungal activity of *Cinnamomun zeylanicum* (CZ) against the growth of two moulds (*Alternana solani* and *Curvulana lunta*) and found that acetone extract was the most potent exhibiting 100% inhibition of spore germination.

These findings therefore suggest that an organic solvent in particular, acetone is a good solvent as it extracts more active compounds from plant material. Flavonoids and steroids have been reported to be extracted using acetone (Eloff, 1998); plants produce flavonoids in response to microbial infection (Schinor *et al*., 2007) which may account for *in vitro* activity of these compounds against a wide array of microorganisms.

Crude extracts are used for initial screening of plants for antimicrobial activities followed by fractionation of the active extract on TLC plate with several solvent systems to elute soluble antimicrobial compounds. The quantity and types of bio-molecules eluted from extracts will depend on the polarity of the solvents (Eloff *et al*., 1998; Masoko *et al*., 2006; Ndip *et al*., 2009).

Phytochemical anaylsis of the acetone extract revealed the presence of varied chemical components of the plant. This was notable from the different colour changes depicted by individual compounds due to their reaction with the spray reagent used (vanillin/sulphuric acid). Vanillin spray revealed twenty-two putative compounds compared to nine from UV light. Saponins which are believed to be extracted by acetone and react well with vanillin sulphuric acid reagent were hardly extracted due to the fact that few compounds seemed to be present at the origin of most polar solvent system used {EMW} (Masoko *et al.,* 2006).
The intermediate polarity solvent combination CEF gave good separation followed by EMW while BEA was the least with three compounds (Table 5). The results are contrary to the findings of Eloff *et al*. (2005) who investigated the efficacy of the leaf of *Combretum woodii* and reported good separation with BEA followed by CEF. Species diversity and difference in climatic conditions might explain this discrepancy.

Prior studies have demonstrated that more polar solvents generally elute more active molecules (Eloff *et al*., 1998; Masoko *et al*., 2006; Ndip *et al*., 2009) which is in agreement with our findings where more active compounds were separated with the polar solvent combination E/M/W (Table 6). There was a variation in the R_f values of the active compounds on direct and overlay methods used in the study. There was one major antibacterial compound (R_f 0.818 from EMW) against *S. pyogenes* and *P. shigelloides.* The

findings suggest that some of the antimicrobial compounds of this plant are of intermediate polarity; while the indirect method (overlay) showed antibacterial compounds (R_f 0.181, 0.361 and 0.515 on EMW) against *H. pylori* isolates implying that the anti-*H.pylori* compounds of this plant are of high polarity. Worthy to note is that bioautography is not a quantitative measure of antimicrobial activity. It only indicates separated compounds with antimicrobial activity. The fact that of the bacteria tested, *H.pylori* had the highest number of inhibition bands does not imply it was the most susceptible organism. It is also important to note that the absence of activity could be due to evaporation of the active compounds, photo-oxidation or very little amount of the active compound (Masoko and Eloff, 2005). On the other hand it is also possible that synergism plays a major role in extracts that were active when the minimum inhibitory concentration of the mixture was determined, but no activity when the compounds are separated on bioautography.

The biological activity of a given plant extract reflects contributions from a number of constituents. Consequently, the initial observation of biological activity in a plant extract is typically followed by bioassay-guided fractionation which is designed to isolate and purify the bioactive constituents. The most active crude extract (acetone) was fractionated using silica gel column chromatography. The polar solvent combination (EMW) that separated more active compounds on bioautography was used to elute seventeen fractions. Fourteen of the seventeen fractions screened exhibited potent antibacterial activity against *S.pyogenes* while fraction E. A 4 showed broad spectrum activity (Table 7). The MIC_{50} of the fractions ranged from 0.0079 to 2.5 mg/mL with *S. pyogenes* and *P. shigelloides* being more susceptible. These MIC values were lower than those of the crude extract which ranged from 0.078 - 5 mg/ml. However, statistically there was no significant difference ($p > 0.05$). The low MIC values observed for these fractions are a good indication of high efficacy

against the organism at low concentrations. Ndip *et al*. (2009) had earlier indicated that the amount of active components in crude extracts from medicinal plants maybe small or diluted and when fractionated, these components become concentrated and therefore exhibit activity. Thus, fractions from crude medicinal plant extracts have great potential as antimicrobial compounds against microorganisms and can be used as potential sources for antibacterial agents in the treatment of infectious diseases caused by microbes.

The TLC profile of fractions EMW 6 and 9 were similar likewise EMW 11 and 12; surprisingly, they had different MIC values. It is therefore likely that these fractions were different but contained compounds with similar R_f values. Since EA 4 showed broad-spectrum activity, it could be speculated that some of the principal antimicrobial components of this plant had intermediary polarity. Fractions with the least number of compounds on the TLC plate but which also had broad-spectrum activity (E.A 4 and EMW 1), were further analysed to identify the bioactive compounds present. No compounds were detected on GC-MS; however, HPLC results showed numerous peaks suggesting that the fractions contained some compounds. GC-MS detects only volatile compounds which are mostly lipids (essential oils). The identification was based on the comparison of their retention indices and mass spectra with those in Wisely 275 Library database, implying that the fractions did not contain volatile compounds within the spectrum.

Numerous peaks were identified on HPLC for both fractions, with the highest peak representing a compound in high proportion. Their retention time was not similar indicating that they were different compounds. The compound EMW 1 with highest peak had a retention time of 1.203 and area percentage of 11.07 while that of EA 4 had a retention time of 0.372 and area percentage of 24.62. The compounds responsible for antimicrobial activity

in these fractions were however not identified. Notwithstanding it can be assumed that the compounds in high proportion were the major ones responsible for their activity. Further investigations are required to further purify and identify these compounds.

Previous phytochemical studies of this plant led to the isolation of triterpenoid glycosides, tannins, alkaloids, saponins, stilbenes, triterpene saponin- oleanone trypetitepene, arjunolic acid and mollic acid glucosides which demonstrated cytotoxic, antifungal, antimicrobial and anti-inflammatory activity, as well as anti-HIV type 1 reverse transcriptase (Asres *et al*., 2001; Ojewole, 2008; Ponoue *et al*., 2008; Bessong *et al*., 2010). Asres *et al*. (2001) isolated triterpenoid glycosides and tannins from acetone extract of the stem bark of *C. molle*. Punicalagin, arjunglucoside and sericoside were isolated after further purification. These compounds were evaluated for antimycobacterial activity and only punicalagin inhibited the growth of *M. tuberculosis* typus humanus ATCC 27294. Burapadaja and Bunchoo (1995) reported antimicrobial activity of the same compound against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*.

Previous studies into the effects of the terpenoids on isolated bacterial membranes revealed their site of action to be at the phospholipid bilayer. They affect bacterial processes that include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Seenivasan *et al*., 2006). Hydrolysable tannins have been reported to have anti-*H. pylori* (Funtogawa *et al.,* 2004) and antiplasmodial activity (Asres *et al*., 2004). Tannins are found in large quantities in the bark of trees where they act as a barrier for micro-organisms like bacteria and fungi. They have been found to form irreversible complexes with proline rich protein (Shimada, 2006) resulting in the inhibition of cell protein synthesis. Herbs that have tannins as their main components are astringent in

nature and are used for treating inflamed or ulcerated tissues, intestinal disorders such as diarrhoea and dysentery (Parekh and Chanda, 2007). Perhaps similar mechanisms of action were responsible for the antimicrobial actions of the plant extracts under study.

5.2 CONCLUSION

From the results obtained in this study, the following conclusions can be drawn:

- The acetone extract of the stem bark of *C.molle* demonstrated good antimicrobial activity against *P. aeruginosa, S. pyogenes, P. shigelloides* and *H. pylori* supporting the use of this plant in traditional medicine.
- EMW solvent combination separated more active compounds from the acetone extract implying that most active compounds from this plant are of high polarity.
- The active compounds of *C. molle* are non volatile.

5.3 RECOMMENDATIONS

We recommend further studies on;

- Further purification and elucidation of the active compounds in order to provide novel or lead compounds for the synthesis of new drugs.
- Antimicrobial activities against other organisms including bacteria and fungi in order to identify the broad spectrum activity of the plant extract and fractions.
- Toxicity and mechanism of action of the plant extract and fractions.

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APPENDICES

Appendix 1

Graphical representation of MIC

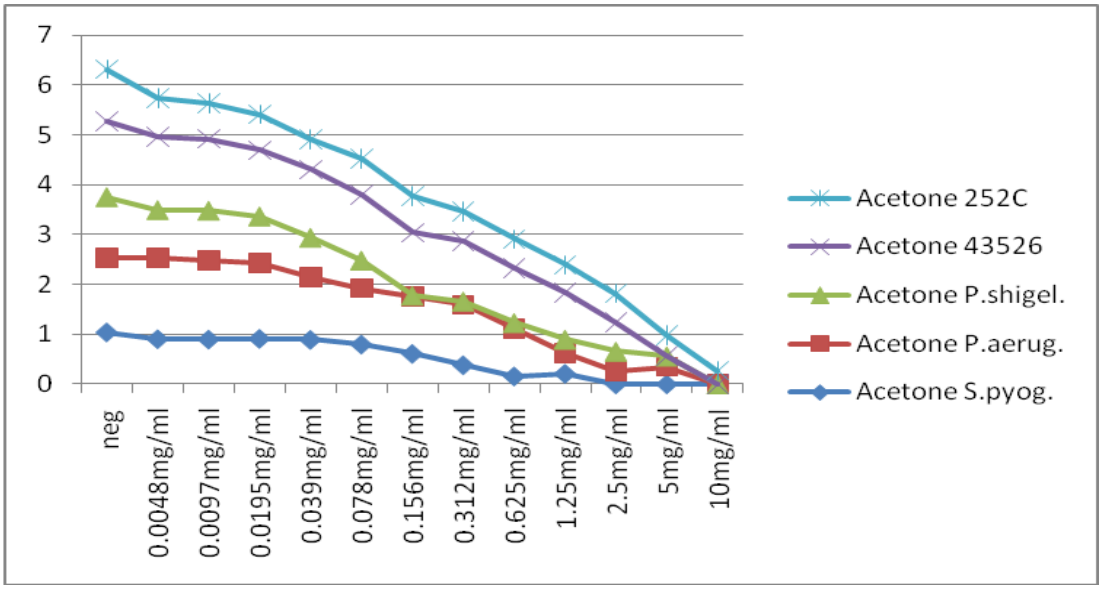


Figure 1: A graphic presentation of MIC of acetone extract against the test organisms

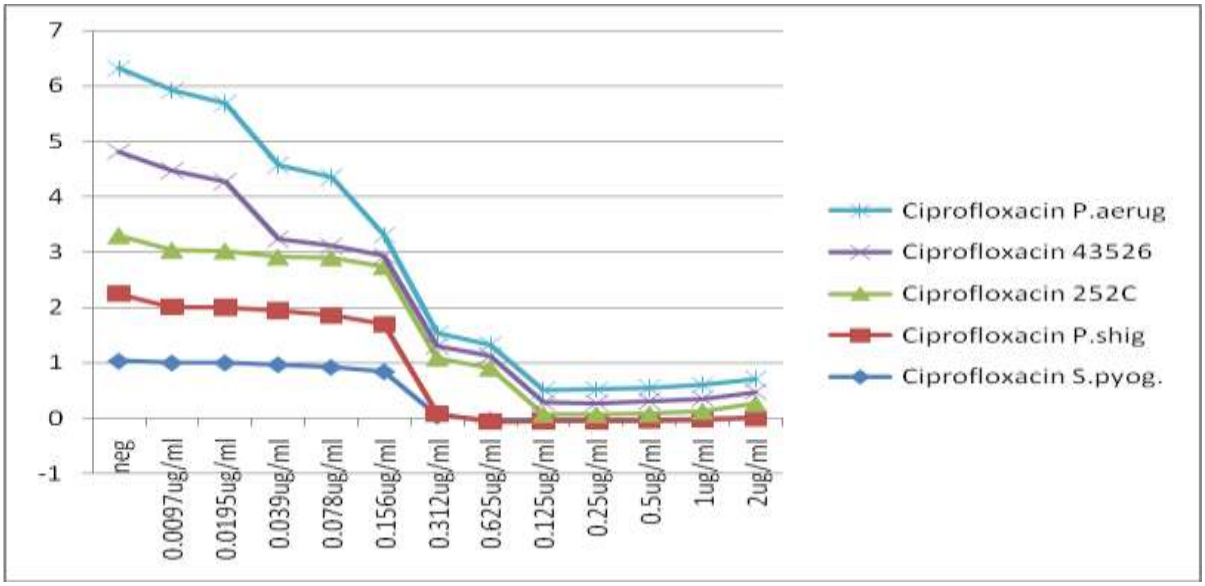
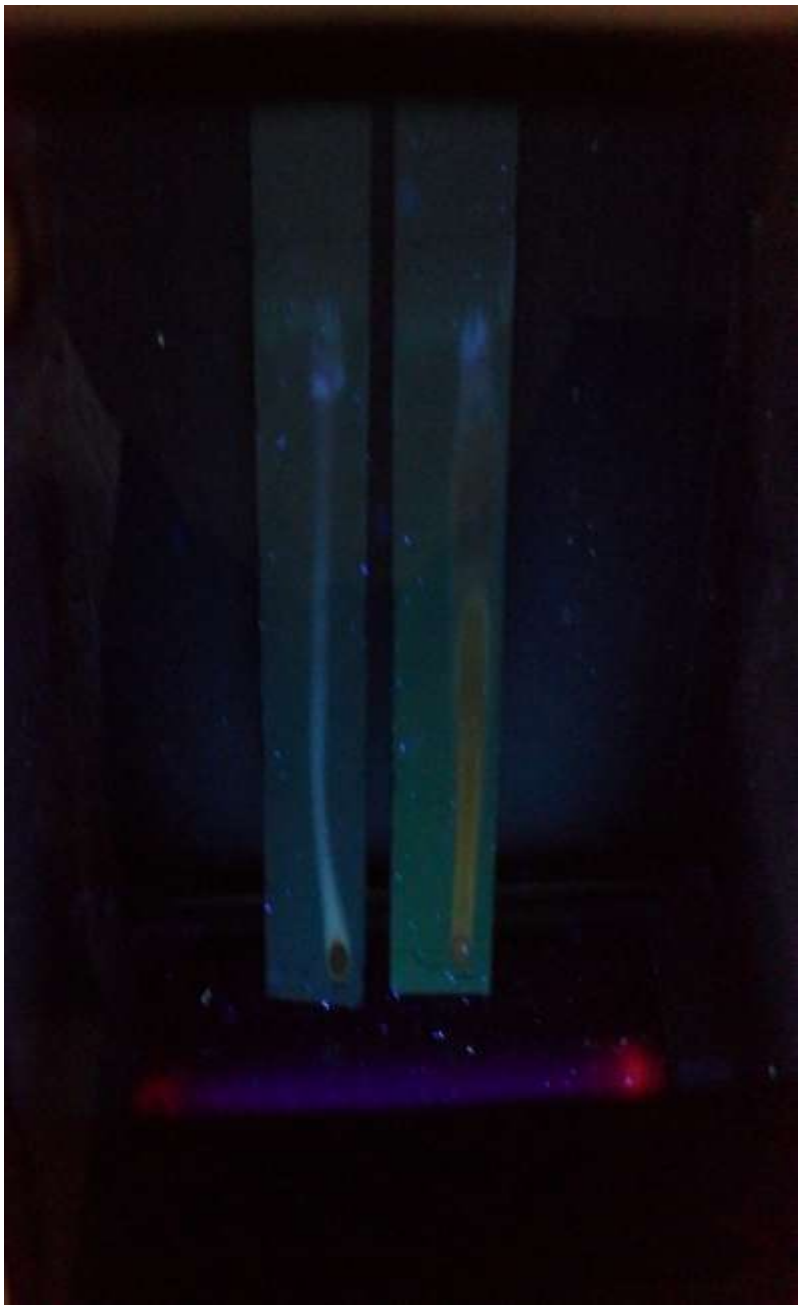


Figure 2: A graphic presentation of MIC of ciprofloxacin against the test organisms

Appendix 2

TLC analysis

Figure 1: TLC plate after vanillin spray

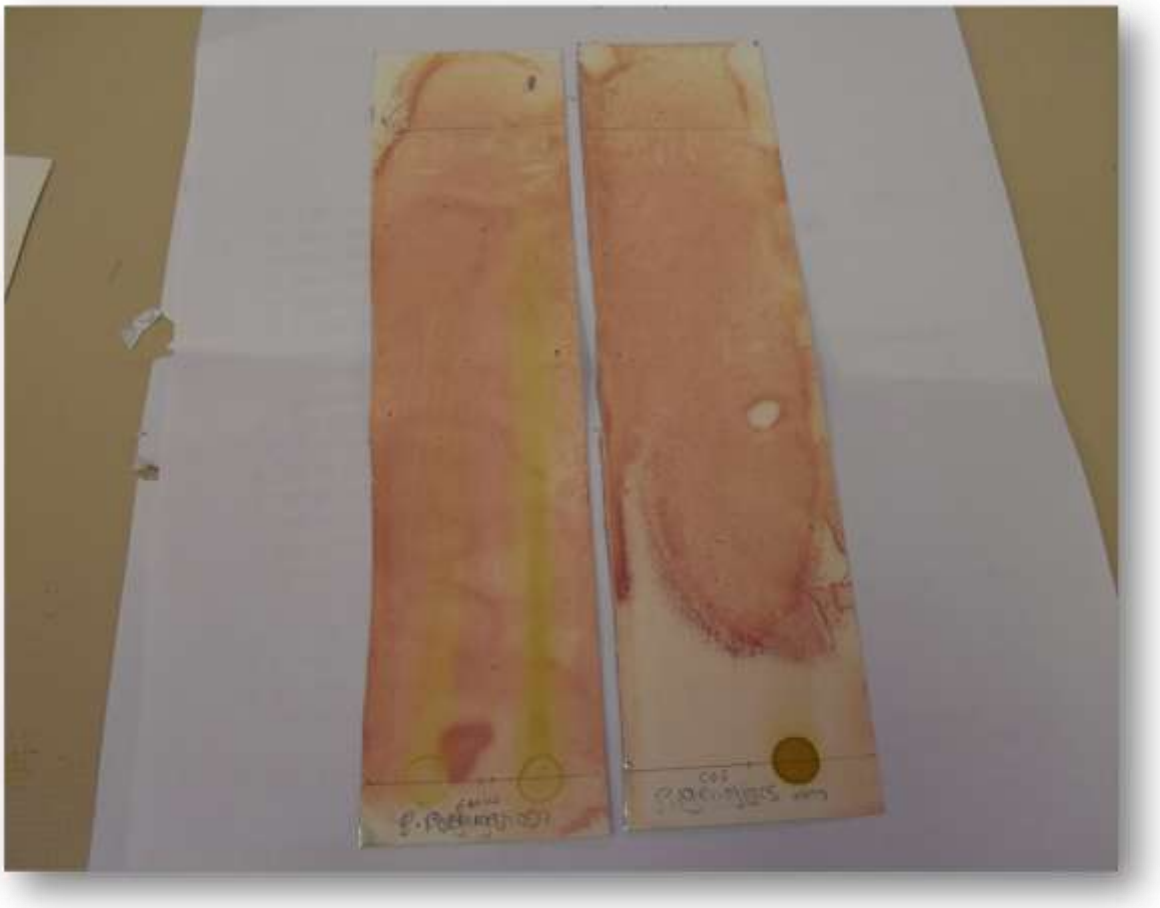


CEF EMW

Figure 2: TLC plate under UV

Appendix 3

Bioautographic analysis



EMW CEF

Figure1: Bioautography of *P.aeruginosa*

CEF EMW

Figure 2: Bioautography of *S.pyogenes*

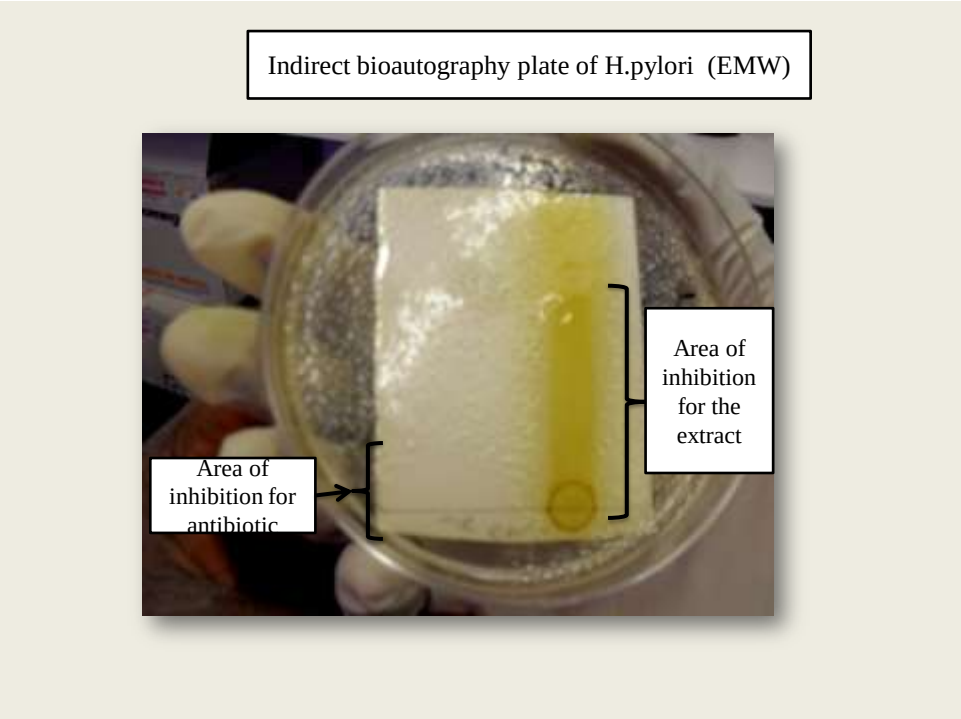


Figure3: Indirect bioautography plate of *H.pylori* 43526 for EMW

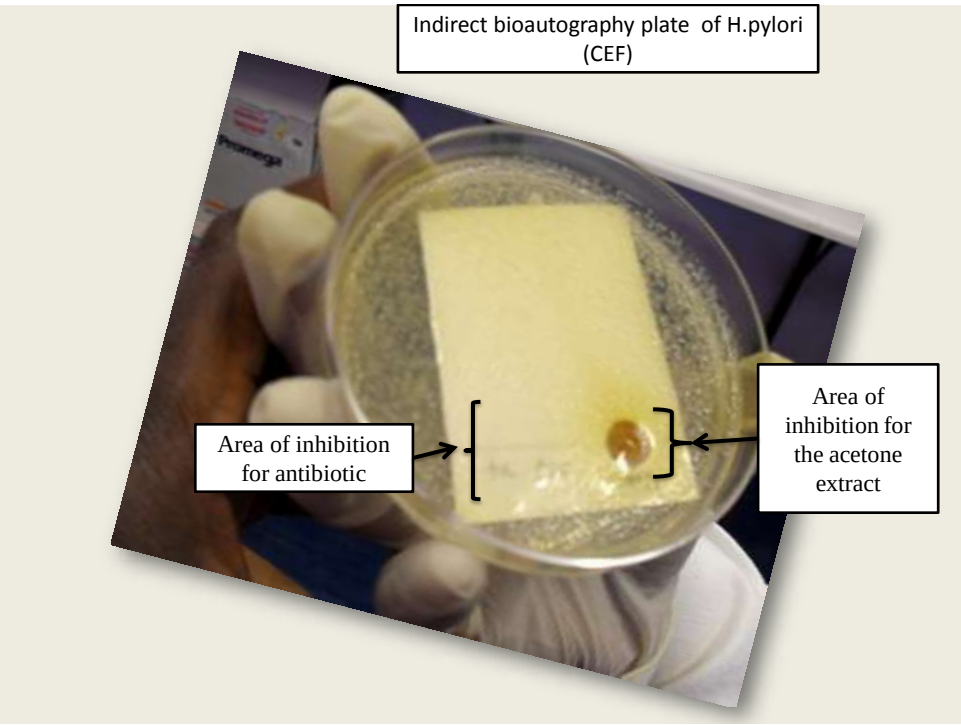


Figure4: Indirect bioautography plate of *H.pylori* 43526 for CEF

Appendix 4

Statistical anaylsis

Multiple Comparisons of the extracts and antibiotic

Tukey HSD

(I) ext	(J) ext	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	4.000000	4.753345	.978	-11.07823	19.07823
	3	4.600000	4.753345	.957	-10.47823	19.67823
	4	6.200000	4.753345	.844	-8.87823	21.27823
	5	10.600000	4.753345	.312	-4.47823	25.67823
	6	12.600000	4.753345	.149	-2.47823	27.67823
	7	-7.600000	4.753345	.684	-22.67823	7.47823
2	1	-4.000000	4.753345	.978	-19.07823	11.07823
	3	.600000	4.753345	1.000	-14.47823	15.67823
	4	2.200000	4.753345	.999	-12.87823	17.27823
	5	6.600000	4.753345	.803	-8.47823	21.67823
	6	8.600000	4.753345	.553	-6.47823	23.67823
	7	-11.600000	4.753345	.220	-26.67823	3.47823
3	1	-4.600000	4.753345	.957	-19.67823	10.47823
	2	-.600000	4.753345	1.000	-15.67823	14.47823
	4	1.600000	4.753345	1.000	-13.47823	16.67823
	5	6.000000	4.753345	.863	-9.07823	21.07823
	6	8.000000	4.753345	.632	-7.07823	23.07823
	7	-12.200000	4.753345	.175	-27.27823	2.87823
4	1	-6.200000	4.753345	.844	-21.27823	8.87823
	2	-2.200000	4.753345	.999	-17.27823	12.87823
	3	-1.600000	4.753345	1.000	-16.67823	13.47823
	5	4.400000	4.753345	.965	-10.67823	19.47823
	6	6.400000	4.753345	.824	-8.67823	21.47823
	7	-13.800000	4.753345	.090	-28.87823	1.27823
5	1	-10.600000	4.753345	.312	-25.67823	4.47823
	2	-6.600000	4.753345	.803	-21.67823	8.47823
	3	-6.000000	4.753345	.863	-21.07823	9.07823
	4	-4.400000	4.753345	.965	-19.47823	10.67823
	6	2.000000	4.753345	.999	-13.07823	17.07823
	7	-18.200000*	4.753345	.010	-33.27823	-3.12177
6	1	-12.600000	4.753345	.149	-27.67823	2.47823
	2	-8.600000	4.753345	.553	-23.67823	6.47823
	3	-8.000000	4.753345	.632	-23.07823	7.07823

	4	-6.400000	4.753345	.824	-21.47823	8.67823
	5	-2.000000	4.753345	.999	-17.07823	13.07823
	7	-20.200000*	4.753345	.004	-35.27823	-5.12177
7	1	7.600000	4.753345	.684	-7.47823	22.67823
	2	11.600000	4.753345	.220	-3.47823	26.67823
	3	12.200000	4.753345	.175	-2.87823	27.27823
	4	13.800000	4.753345	.090	-1.27823	28.87823
	5	18.200000*	4.753345	.010	3.12177	33.27823
	6	20.200000*	4.753345	.004	5.12177	35.27823

*. The mean difference is significant at the 0.05 level.

1, Acetone; 2 Ethanol; 3, Methanol; 4, Ethyl Acetate; 5, DCM; 6, Water; 7, Ciprofloxacin

Multiple Comparisons; MIC of extracts and antibiotics

Tukey HSD

(I) E	(J) E	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.7842800	.7486952	.895	-3.163659	1.595099
	3	-.8249467	.8645187	.926	-3.572417	1.922524
	4	-1.6062800	.8645187	.457	-4.353751	1.141191
	5	-.2519467	.8645187	1.000	-2.999417	2.495524
	6	.6877200	.7486952	.937	-1.691659	3.067099
2	1	.7842800	.7486952	.895	-1.595099	3.163659
	3	-.0406667	.8645187	1.000	-2.788137	2.706804
	4	-.8220000	.8645187	.927	-3.569471	1.925471
	5	.5323333	.8645187	.988	-2.215137	3.279804
	6	1.4720000	.7486952	.398	-.907379	3.851379
3	1	.8249467	.8645187	.926	-1.922524	3.572417
	2	.0406667	.8645187	1.000	-2.706804	2.788137
	4	-.7813333	.9665613	.962	-3.853099	2.290432
	5	.5730000	.9665613	.990	-2.498766	3.644766
	6	1.5126667	.8645187	.519	-1.234804	4.260137
4	1	1.6062800	.8645187	.457	-1.141191	4.353751

	2	.8220000	.8645187	.927	-1.925471	3.569471
	3	.7813333	.9665613	.962	-2.290432	3.853099
	5	1.3543333	.9665613	.726	-1.717432	4.426099
	6	2.2940000	.8645187	.135	-.453471	5.041471
5	1	.2519467	.8645187	1.000	-2.495524	2.999417
	2	-.5323333	.8645187	.988	-3.279804	2.215137
	3	-.5730000	.9665613	.990	-3.644766	2.498766
	4	-1.3543333	.9665613	.726	-4.426099	1.717432
	6	.9396667	.8645187	.880	-1.807804	3.687137
6	1	-.6877200	.7486952	.937	-3.067099	1.691659
	2	-1.4720000	.7486952	.398	-3.851379	.907379
	3	-1.5126667	.8645187	.519	-4.260137	1.234804
	4	-2.2940000	.8645187	.135	-5.041471	.453471
	5	-.9396667	.8645187	.880	-3.687137	1.807804

1, Acetone; 2 Ethanol; 3, Methanol; 4, Ethyl Acetate; 5, DCM; 6, Ciprofloxacin

Multiple Comparisons; Active fractions and antibiotic

Tukey HSD

(I) F	(J) F	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.2245833	.5540035	.976	-1.919477	1.470310
	3	.2928667	.5922547	.959	-1.519051	2.104784
	4	.4641667	.5540035	.835	-1.230727	2.159060
2	1	.2245833	.5540035	.976	-1.470310	1.919477
	3	.5174500	.5540035	.788	-1.177444	2.212344
	4	.6887500	.5129076	.559	-.880417	2.257917
3	1	-.2928667	.5922547	.959	-2.104784	1.519051
	2	-.5174500	.5540035	.788	-2.212344	1.177444
	4	.1713000	.5540035	.989	-1.523594	1.866194
4	1	-.4641667	.5540035	.835	-2.159060	1.230727
	2	-.6887500	.5129076	.559	-2.257917	.880417
	3	-.1713000	.5540035	.989	-1.866194	1.523594

1, Ethyl acetate 3; 2, Ethyl acetate 4; 3, EMW1; 4, Ciprofloxacin

Multiple Comparisons; Active fractions and crude

Tukey HSD

(I) F	(J) F	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.2245833	.7289768	.989	-2.454783	2.005616
	3	.2928667	.7793090	.981	-2.091317	2.677050
	4	-.3320833	.7289768	.967	-2.562283	1.898116
2	1	.2245833	.7289768	.989	-2.005616	2.454783
	3	.5174500	.7289768	.891	-1.712749	2.747649
	4	-.1075000	.6749014	.998	-2.172263	1.957263
3	1	-.2928667	.7793090	.981	-2.677050	2.091317
	2	-.5174500	.7289768	.891	-2.747649	1.712749
	4	-.6249500	.7289768	.826	-2.855149	1.605249
4	1	.3320833	.7289768	.967	-1.898116	2.562283
	2	.1075000	.6749014	.998	-1.957263	2.172263
	3	.6249500	.7289768	.826	-1.605249	2.855149

1, Ethyl Acetate 3; 2, Ethyl Acetate4; 3, EMW1; 4, Crude

Appendix 5

Combretum molle

Appendix 6

Manuscript in preparation

1. *In-vitro* antimicrobial activity of the stem bark of *Combretum molle* against selected bacterial pathogens of medical importance.
2. Phytochemical analysis and bioactivity of the acetone extract of the stem bark of C*ombretum molle* on selected bacterial pathogens.