ASSESSMENT OF THE ANTIBACTERIAL ACTIVITY OF *ARTEMISIA AFRA*, *ERYTHRINA LYSISTEMON* AND *PSIDIUM GUAJAVA*

By

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Dissertation submitted in fulfilment for the requirements of the degree of Master of Technology in Biomedical Technology in the Faculty of Health Sciences at the Durban University of Technology

I Nhlanhla Wiseman Nsele do hereby declare that these investigations represent my original work, while registered for the M. Tech: Biomedical Technology degree in the Department of Biomedical and Clinical Technology at the Durban University of Technology, and have not been submitted in any form for any Diploma or Degree to another University. When use was made of the work of others it has been duly acknowledged in the text.

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ABSTRACT

Introduction

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics have led scientists to investigate the antimicrobial activity of medicinal plants

Aim

The aim of this study was to investigate the antimicrobial activity of extracts obtained from medicinal plants used in traditional medicine. A comparative study was carried out on the antimicrobial properties of extracts obtained by two different methods in order to choose that which extracts the most effective antimicrobial compounds.

Methodology

The plants used in this study *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* were harvested from the Silverglen Nature Reserve (Chatsworth) early in the morning (8 a.m.). The leaves of *A. afra* and *P. guajava* extracts and the bark of *E. Lysistemon* were used to prepare the extracts. All plant extracts were prepared according to modified method of the German Homeopathic Pharmacopoea. Two solvents, water and 60 percent ethanol were used to extract the antibacterial compounds from plant material.

The extracts were then assessed for their antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli.* The effect of the plant extracts on these bacteria was determined by the disk diffusion test, which was used as the screening test. Positive results were further subjected to the minimum inhibitory concentration and minimum bactericidal concentration assays. Tubes that showed no turbidity were then sub-cultured onto non-selective plates. Bacterial

sensitivity testing was carried out in accordance with modified Kirby-Bauer Antimicrobial Sensitivity Test.

An attempt was made to identify some antibacterial compounds using Thin Layer Chromatography and High Pressure Liquid Chromatography.

Results

None of the gram negative organisms were inhibited by *Artemisia afra, Erythrina cafra* and *Psidium guajava.* Only the ethanol extracts of all three plants were able to inhibit *Staphylococcus aureus* but not *Escherichia coli* and *Pseudomonas auruginosa*. None of the test organisms were inhibited by the aqueous extracts of all three plants used in this study. In the screening test, the zones of inhibition for ethanol extracts against *Staphylococcus aureus* ranged from 3mm – 7mm. The minimum inhibitory concentration ranged from 16.67 percent – 83.3 percent inhibition depending on the dilution of the extract.

Quercetin and *Catechin* were identified as some of the antibacterial compounds present in the leaves of *Psidium guajava.* These two compounds were not identified on *Erythrina lysistemon* and *Artemisia afra.*

Conclusion

The results obtained in this study have proven that *Artemisia afra, Erythrina cafra* and *Psidium guajava* ethanol extracts contain antibacterial substances. The ethanol extracts of all plants in this study inhibited the growth of *Staphylococcus aureus* but had no effect on the gram negative bacteria. Aqueous plant did not inhibit the growth of any bacteria in this study. This study has also shown that antibacterial effect of these extracts may be considerably enhanced in traditional treatment if traditional healers can include ethanol as one of the extraction solvents. The results obtained in this study might be considered sufficient for further studies aimed at isolating and identifying the active compounds and evaluating possible synergism of antimicrobial

activity among these extracts. Investigations on toxicity of these extracts should also be carried out.

DEDICATION

This work is dedicated with love and gratitude to my family for their support and for believing in me. I would also like to dedicate this work to the Department of Biomedical Science at Mangosuthu University of Technology.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Izel Botha and Mrs. J.N. Mtshali for their persistent guidance and encouragement throughout the duration of this study. Their valuable suggestions have been instrumental in shaping the project into its present form. I extend a word of thanks to the Biomedical Science staff that supported me throughout the duration of the project, especially the laboratory staff.

My sincere appreciation extends also to the Department of Homeopathy at the Durban University of Technology for allowing me to use their laboratory for the extraction of plants. Nevorn was also helpful in teaching me some of the extraction methods.

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I would like to thank all my friends for their support, encouragement and enthusiasm. The thought of knowing that you wanted the best for me kept me focused and going.

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CHAPTER ONE
INTRODUCTION

1.1 OVERVIEW

Infectious diseases are the leading cause of deaths worldwide. Antibiotic resistance has become a global concern (Westh *et al*., 2004). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens (Bandow *et al*., 2003). Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for discovering new drugs because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antibacterial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Rojas *et al*., 2003). Therefore, researchers are increasingly turning their attention to traditional medicine, looking for new ways to develop better drugs against microbial infections (Benkeblia, 2004). The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of certain medicinal plants for their potential antibacterial activity (Pakekh and Chanda, 2007).

Infectious diseases account for approximately one half of all deaths in most countries (Iwu, Duncan and Okunji, 1999). Infectious disease mortality rates are increasing in developing countries such as South Africa. The increase is attributed to the increase in Human Immunodeficiency Virus/Auto Immune Deficiency Syndrome infections (HIV/AIDS). Other contributing factors are the increase in the antibiotic resistance in the hospital acquired and community acquired infections (Iwu *et al*., 1999). The most dramatic increases occur among the 15 to 45 year old age group (Pinner *et al*., 1996). This could be due to the fact that young people are sexually hyperactive and this increases the risk of acquiring HIV infections.

Historically, plants have provided a good source of antimicrobial agents. Plants such as *Artemisia afra*, *Erythrina lysistemon* and *Psidium guajava* have been used to cure infections such as wounds, abscesses, arthritis, diarrhoea and influenza (Van Wyk *et al*., 2000). Traditional medicine has maintained a virile people in Africa, where a greater variety of herbal traditions exist than in any other continent (Luyt *et al*, 1999). Plants that are used in traditional medicine are likely to yield pharmacologically active compounds. It is important, however, that these plants, as well as traditional methods of treatments, undergo continuous scientific evaluation in order to facilitate the incorporation of traditional medicine into the health care system of South Africa (Luyt *et al*., 1999). Besides South Africa, plants have been, and are still, used worldwide in the treatment of various diseases (Meyer *et al*., 2002).The use of medicinal plants such as *Psidium guajava* by South Africans in curing diarrhoea, dysentery and wounds has been reported (Van Wyk *et al*., 2000). The leaves of *Psidium guajava* are being used as tea in cases of diarrhoea and topical in cases of certain wound infections. The guava plant is widely distributed in South Africa especially in KwaZulu Natal. Despite being widely utilised, not enough studies have been carried out to investigate the medicinal properties of the plants (Van Wyk *et al*., 2000).

In most developing countries, like South Africa, the chance of contracting diseases is high due to the poor hygienic and living conditions. Owing to inadequate accessibility to sufficient and inexpensive modern health care centres, especially in rural areas, people prefer to visit traditional healers. These traditional healers play a major role in the health sector. Worldwide, there has been a renewed interest in natural products (Iwu *et al*., 1999). Part of this interest is as a result of factors such as national concerns for health costs. Traditional healing is widely used in South Africa (Kelmanson *et al*., 2000). There are an estimated 200 000 indigenous traditional healers in South Africa, and up to 60 percent of South Africans consult these healers usually in addition to modern biomedical services (Van Wyk *et al*., 2000). Furthermore, up to 80 percent of developing country populations rely on traditional medicine as their primary health care (World Health Organisation, 2004). In South

Africa traditional healers are called '*Inyanga*'and '*Isangoma*' in Zulu. The terms *'Inyanga*' and *'Isangoma*' refer to a Herbalist and a Diviner, respectively.

Traditional healers in South Africa make use of medicinal plants, and some of these plants, including *Artemisia afra, Erythrina lysistemon,* and *Psidium guajava,* although not investigated scientifically, are used to cure certain infections (Van Wyk *et al*., 2000). With proper investigation these may serve as a source of modern drugs. South Africa has well over 30 000 species of these plants and approximately 3 000 of these are used as medicines. Some 350 species are commonly used and traded as medicinal plants (Van Wyk *et al*., 2000). Despite all these virtues, few studies have been conducted regarding indigenous medicines (Kaba, 1996). The trends in health call for a renewed interest in infectious diseases in the medical and health communities and in developing strategies for treatment and prevention. Development of new treatments is necessary if these challenges are to be dealt with. In this study the aim is to develop new treatment strategies that would encompass the development of new antimicrobials. The assessment of antimicrobial activity of *Artemisia afra*, *Erythrina lysistemon* and *Psidium guajava* on *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* could assist in the development of new antimicrobials. The species of plants used in this study were confirmed by the senior horticulturist, Mr. Barry Lang, at the Durban Botanical Gardens.

Samples of *Artemisia afra*, *Erythrina lysistemon* and *Psidium guajava* were collected from Silverglen Nature Reserve, Chatsworth. Extraction then took place using either water or 60 percent ethanol as extraction solvents. The two extracts were tested against the three different types of bacteria for their antibacterial activity. The method used was in accordance with the modification of the Kirby-Bauer antimicrobial test procedure (Thrupp, 1980). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by serially diluting the extracts using nutrient broth. Thin Layer Chromatography (TLC) was used to identify some active compounds. High Pressure Liquid Chromatography (HPLC) was utilised to confirm compounds identified by TLC method.

The aim of this investigation was to assess the antibacterial activity of various extracts of three traditional medicinal plants, i.e. *Artemisia afra*, *Erythrina lysistemon* and *Psidium guajava* on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* cultured *in vitro*.

The first objective was to determine zones of inhibition produced, MIC and MBC of two *Artemisia afra*, *Erythrina lysistemon* and *Psidium guajava* extracts on *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The second was to compare the antibacterial activity of water and ethanolic extracts for *Artemisia afra, Erythrina lysistemon* and *Psidium guajava*. The last was to identify some active antibacterial compounds.

It was hypothesised that *Artemisia afra, Erythrina lysistemon, and Psidium guajava* extracts will have antimicrobial effects on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.* It was further hypothesised that both water- based and ethanol extracts will exhibit antibacterial properties against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.* It was also thought that if *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* possess antibacterial effects on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, then antibacterial compounds are present in these plants.

It was concluded that the increasing failure of chemotherapeutics caused by antibiotic resistance exhibited by pathogenic microbial infectious agents is posing a threat to communities. Traditional medicine can play a major role in the treatment of these infections. Therefore, the screening of medicinal plants for their potential antibacterial activity may assist in the determination of new drugs from medicinal plants. There must be proper investigation of the traditional plants in order to discover new drugs that can complement the already existing drugs. The studies that have been done up to now suggest that there are antibacterial compounds in medicinal plants. If more funding can be made available, more scientists can engage in the extensive research on traditional plants.

CHAPTER TWO
LITERATURE REVIEW

2.1 INTRODUCTION

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents (Krishnaraju *et al*., 2005). Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy could be used for the treatment of bacterial infections (Pakekh and Chanda, 2007). Since antiquity, man has used various parts of plants in the treatment and prevention of various ailments (Tanaka *et al*., 2002).

2.2 MAJOR GROUPS OF ANTIMICROBIAL COMPOUNDS FROM PLANTS

2.2.1 *PHENOLIC* COMPOUNDS

The term *phenolic* compound embraces a wide range of plant substances which possess in common an aromatic ring bearing one or more hydroxyl substitutes (Harbone, 1984). They tend to be water-soluble, since they most frequently occur combined with sugar as glycosides and are usually located in the cell vacuole. Among the natural *phenolic* compounds, of which several structures are known, *flavonoids* form the largest group, but simple monocyclic *phenols, phenolpropanoids* and *phenolic quinines* all exist in considerable numbers (Harbone, 1984). In many cases, these substances serve as defence mechanisms of plants against predation by microorganisms, insects and herbivores (Marjorie, 1999). Some such as *terpenoids* give plants their odours; others such as *tannins* and *quinines* are responsible for plant pigment. Aromatic compounds are responsible for plant flavour. Useful antimicrobial phytochemicals can be divided into several categories, described below.

Simple *phenols* and *phenolic acids*

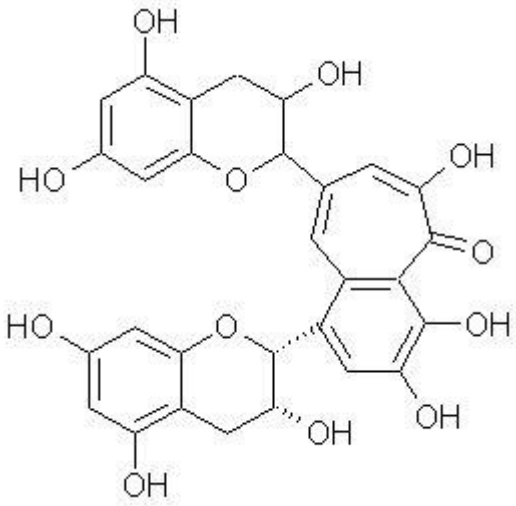

Figure 1: Structures of polyphenols found in green tea (Wikipedia, the free encyclopedia)

Phenols, sometimes called *phenolics*, are a class of chemical compounds consisting of a hydroxyl functional group (-OH) attached to an aromatic hydrocarbon group. The simplest of the class is *phenol* (C_6H_5OH). Some *phenols* are germicidal and are used in formulating disinfectants. *Phenol*, the parent compound, is used as a disinfectant and for chemical synthesis. *Propolis* is one of the few natural remedies that has maintained its popularity over a long period of time. The pharmacologically active molecules in the *propolis* are *flavonoids* and *phenolic acids* and their esters. These components have multiple effects on bacteria, fungi and viruses. In addition, *propolis* and its components have anti-inflammatory and immunomodulatory activities. Moreover, *propolis* has been shown to lower blood pressure and cholesterol levels. However, clinical studies to substantiate these claims are required. Coffee is particularly rich in bound *phenolic acids*, such as *caffeic acid, ferulic acid,* and *P-coumaric acid* (Nardini *et al*., 2002). *Quince* and *Aloe Ferox* both have *phenolic acids*

Spices are known to significantly contribute to the flavour, taste, and medicinal properties of food because of *phenolics*. Most spices contain *phenolic acids* such as *tannic, gallic, caffeic, cinnamic, chlorogenic, ferulic* and *vanillic acids*. A high amount of *tannic* and *gallic* acids are found in black mustard and clove. *Caffeic, chlorogenic* and *ferulic acids* are found in a high concentration in *cumin*. *Vanillic* and *cinnamic acids* are found in onion seeds (*Nigella sativa*).

Salicylic acid is a *phenolic* compound which is a precursor compound to aspirin (Montenegro *et al*., 2009). Aspirin (*acetylsalicylic acid*) is still the most commonly used *salicylate*. After oral administration as an aqueous solution, aspirin is rapidly absorbed at the low pH of the stomach. Less rapid absorption is observed with other formulations due to the rate limiting step of tablet disintegration - this latter factor being maximal in alkaline pH. The rate of aspirin absorption is dependent not only on the formulation but also on the rate of gastric emptying. Aspirin absorption follows first-order kinetics with an absorption half-life ranging from five to sixteen minutes. Hydrolysis of aspirin to *salicylic acid* by nonspecific esterases occurs in the liver and, to a lesser extent, the stomach so that only 68 percent of the dose reaches the systemic circulation as aspirin. Both aspirin and *salicylic acid* are bound to serum albumin (aspirin being capable of irreversibly acetylating many proteins), and both are distributed in the synovial cavity, central nervous system, and saliva. The serum half-life of aspirin is approximately 20 minutes. The fall in aspirin concentration is associated with a rapid rise in *salicylic acid* concentration. *Salicylic acid* is renally excreted in part unchanged and the rate of elimination is influenced by urinary pH, the presence of organic acids, and the urinary flow rate.

Benefit of *phenols*

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. *Phenolic* compounds are essential for the growth and reproduction of plants, and are produced as a response for defending injured plants against pathogens. Recent interest in *phenolic acids* stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart disease, stroke, and cancers).

The absorption and bioavailability of *phenolics* in humans are controversial. Data on these aspects of *phenolics* are scarce and merely highlight the need for extensive investigations of the handling of *phenolics* by the gastrointestinal tract and their subsequent absorption and metabolism. Plant *phenolic* compounds are diverse in structure but are characterised by hydroxylated aromatic rings (e.g. *flavan-3-ols*). They are categorised as secondary metabolites, and their function in plants is often poorly understood. Many plant *phenolic* compounds are polymerised into larger molecules such as the *proanthocyanidins* and *lignins*. Furthermore, *phenolic acids* may occur in food plants as esters or glycosides conjugated with other natural compounds such as *flavonoids, alcohols, hydroxyfatty acids, sterols,* and *glucosides*.

Some herbs such as *tarragon* and *thyme* both contain *caffeic acid,* which is effective against bacteria, viruses and fungi (Marjorie, 1999). *Catechol* and *pyrogallol* both are hydroxylated *phenols,* shown to be toxic against microorganisms (Marjorie, 1999). Some authors have found that more highly oxidized *phenols* are more inhibitory (Scalbert, 1991). The mechanisms thought to be responsible for *phenolic* toxicity to micro-organisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulhydryl groups or through more non-specific interactions with the proteins (Mason and Wasserman, 1987). *Phenolic* compounds possessing a C_3 side chain at a lower level of oxidation are classified as essential oils and often cited as antimicrobial as well.

2.2.2 *TERPENOIDS* AND ESSENTIAL OILS

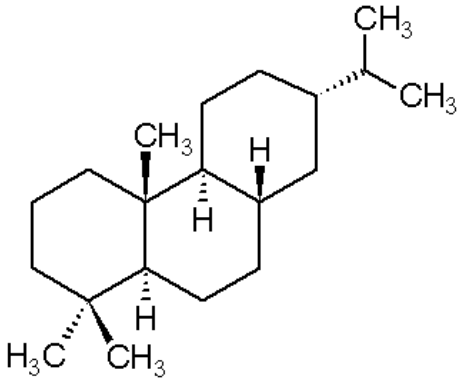

Figure 2: Basic structure of *terpenes* (Wikipedia, the free encyclopedia)

Plant fragrance is carried in the so-called essential oil component. These oils are secondary metabolites that are highly enriched in compounds based in isoprene structure (Marjorie, 1999). They are called *terpenes* and their general chemical structure is $C_{10}H_{16}$. If the compounds carry an additional element, usually oxygen, they are called *terpenoids*. *Terpenoids* are synthesised from acetate units and as such they share their origin with fatty acids (Marjorie, 1999). They differ from fatty acids in that they have extensive branching and they are cyclized. Some examples of common *terpenoids* include methanol and *Camphor* (*monoterpes*), *farnesol* and *Artemisin* (*sesquiterpenoids*) (Marjorie, 1999). *Terpenoids* are reported to be active against bacteria (Ahmed *et al*., 1993; Akpata and Akinrimisi, 1977). In 1977, it was reported that 60 percent of essential oil derivatives examined to date were inhibitory to fungi while 30 percent inhibited bacteria (Chaurasia and Vyas, 1977). It is speculated that the mode of action of the *terpenes* revolves around the membrane disruption by the lipophilic compounds (Marjorie, 1999). Food scientists have found the *terpenoids* present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Aureli and Zolea, 1992).

Terpenes are a class of hydrocarbons usually produced from plants. They are the building blocks of many essential oils used in perfumes and other fragrances.

2.2.3 *FLAVONES, FLAVONOIDS* AND *FLAVONOLS*

Flavones are *phenolic* structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a *flavonol*. *Flavonoids* are hydroxylated *phenolic* compounds but occur as a C_6-C_3 unit linked to an aromatic ring (Marjorie, 1999). They are known to be synthesised by plants in response to microbial infection (Dixon and Lamb, 1983). They have been found *in vitro* to be effective antimicrobial substances against a wide range of micro-organisms. Their activity is probably due to their ability to complex with bacterial cell walls, as described for *quinines*. More lipophilic *flavonoids* may also disrupt microbial cell membranes (Marjorie, 1999). *Catechins*, the most reduced form of the C_3 unit of flavonoid compound have been found to be present in oolong green tea (Marjorie, 1999).

It was found some time ago that teas exerted antimicrobial activity (Marjorie, 1999) and that they contain a mixture of *catechin* compounds. These compounds inhibited *in vitro Vibrio cholera 01* (Borris, 1996) and *Streptococcus mutans* (Batista *et al.*, 1994).There is a considerable range of *flavonol* glycosides in plants. More than 200 different glycosides of *quercetin* alone have been described, of which by far the most common is *quercetin* 3 rutinose, known as rutin (Harborne, 1984). *Flavones* occur as glycosides but the range of different glycosides is less than in the case of *flavonols*. More than one study has found that *flavone* derivatives are inhibitory to respiratory *syncytial virus* (RSV) (Barnard *et al*., 1993, Kaul *et al*., 1985).

2.2.3.1 *Quercetin*

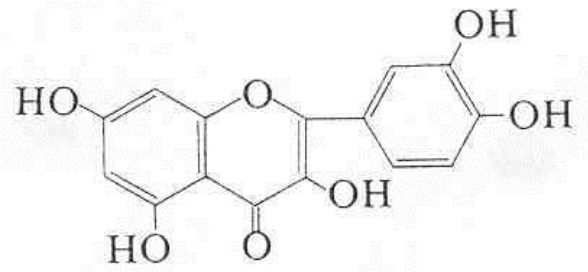

Figure 3: Structure of *Quercetin* (Wikipedia, the free encyclopedia)

Flavone is one of the main groups of *flavonoids*, and the most widespread *flavone* is *quercetin* serves as the backbone for other *flavonoids*, and is the most active of the *flavonoids* (Ikan, 1991). Many medicinal plants have significant *quercetin* content.

Quercetin behaves like auxins in stimulating the germination of wheat seeds. The possible function of this colouring matter in insect-pollinated flowers and edible fruits is to make these organs more conspicuous in order to aid seed dispersion by animals (Ikan, 1991).

Small quantities of *quercetin* may act as cardiac stimulants. It is used to strengthen weak capillary blood vessels (Ikan, 1991).

2.2.3.2 *Catechin*

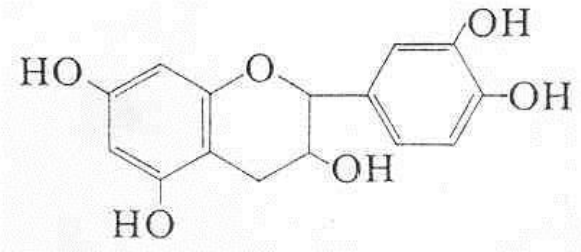

Figure 4: Structure of *Catechin* (Wikipedia, the free encyclopedia)

Flavonoids are able to join together to form other *flavonoids* e.g. *catechin*. *Catechin*s are monomeric *flavonoids* which, joined together, can form new compounds; it is a yellowish amorphous solid. There are different types of *catechin*s and they bond together in various ways to form different compounds with very different characteristics. Two *catechin*s joined together are called dimer and three *catechin*s bonded together a trimer and so on up to oligomers and polymers. These new compounds are called proanthocyanidis, or condensed *tannins*. *Oligomeric proanthocyanidins* are *oligomeric flavonoids*. These molecules are found in the bark of pine trees (Wijesekera, 1991).

Catechin easily binds to proteins, blocking bacteria from adhering to cell walls and thereby disrupting their ability to destroy them. It decreases cholesterol level in blood and prevents LDL cholesterol caused by oxidation, which prevents narrowing of blood vessels caused by the build-up of LDL cholesterol (Bishop *et al*., 2005)

Catechin is a useful component for the prevention and treatment of substances that suppresses the immune system (Wijesekera, 1991). It fights bacteria and viruses and prevents influenza. It is an antioxidant.

2.2.4 *TANNINS*

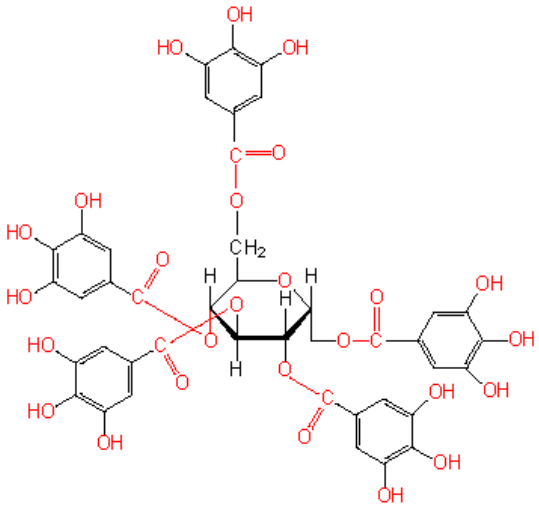

Figure 5: Basic Structure of a *Tannin* (Wikipedia, the free encyclopedia)

Tannins by definition have the ability to react with protein, forming stable water insoluble co-polymers (Harbone, 1998). They are of plant origin, which through their ability to cross-link with protein are capable of transforming raw animal skin into leather. Plant tissues high in tannin content impart an astringent taste, which acts as a deterrent to most feeders. *Tannins* are divided into two groups, hydrolysable and condensed *tannins*. Hydrolysable *tannins* are based on *gallic* acid, usually as multiple esters with D-glucose while the condensed *tannins* are derived from flavonoid monomers (Marjorie, 1999). *Tannins* may be formed by polymerisation of quinone units. Many human physiological activities, such as stimulation of phagocytic cells, host mediated tumour activity and a wide range of anti-infective actions have been assigned to *tannins* (Haslam, 1996). Their mode of antibacterial action is related to their ability to inactivate microbial adhesions, enzymes and cell envelope transport proteins. They also form complexes with polysaccharides. There is also evidence for direct inactivation of micro-organisms. Condensed *tannins* have been determined to bind cell wall of bacteria, preventing growth and protease activity (Jones *et al.*,

1994). *Tannins* consist mainly of *gallic* acid residues that are linked to glucose via glycosidic bonds.

2.2.5 *QUINONES*

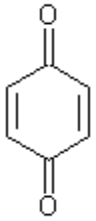

Figure 6: Basic structure of *p-benzophenone* (Wikipedia, the free encyclopedia)

Quinones contain aromatic rings (Marjorie, 1999). They are ubiquitous in nature and are characteristically highly reactive. Although they are widely distributed and exhibit great structural variation, they make relatively little contribution to colour in higher plants (Harbone, 1984). They are commonly present in bark, roots, or else in tissues (such as leaves) where their colours are masked by other pigments. These compounds, being coloured, are responsible for the browning reaction in cut or injured fruits and vegetables. They are also an intermediate in the melanin synthesis pathway of the human skin. Quinones provide a source of stable free radicals and are known to complex irreversibly with *nucleophilic amino acids* in proteins (Stern *et al.*, 1996), often leading to inactivation of protein and loss of function. For that reason, the potential range of *quinone* antimicrobial effect is great. Probable targets in the microbial cell are surface-exposed adhesions, cell wall polypeptides, and membrane-bound enzymes (Marjorie, 1999). *Quinones* may also render substrates unavailable to the microorganisms. One example of a *quinine* is *p-benzoquinone* and the basic chemical structure is given in Figure 6

2.3 TYPES OF HERBAL EXTRACTS

There are different types of herbal extracts that can be manufactured. These types are based on the method of extraction of the product. There are water-based, alcohol based, vinegar, *glycerine* and fat based extractions. The commonly used extraction solvents are water and alcohol, especially ethanol.

2.3.1 ALCOHOL – BASED TINCTURES

Tinctures are traditionally made by placing the herb in an organic solvent and leaving it to soak for weeks. An alternative and faster method is for the solvent to be percolated through the herb (Werbach and Murray, 2002). For commercial preparations, an alcohol solvent is the most useful, usually ethyl alcohol (ethanol). In making an extract, the raw herb is submerged in an alcohol-water mixture for 2-4 weeks, depending on the pharmacopoeia and monograph followed. The liquid is then filtered to separate tincture from the exhausted herb. A water-alcohol mixture ensures the extraction of both the water soluble and alcohol soluble constituents. Alcohol–based extracts are usually stronger than infusions as alcohol can extract constituents that are water–insoluble (Rotblatt and Zimet, 2002). Alcohol is also a very effective natural preservative. Because a tincture is easily assimilated by the body, it is a very effective way to administer herbal compound. Tinctures are concentrated and cost-effective (Rotblatt and Zimet, 2002) and they have a longer shelf life.

2.3.2 WATER–BASED EXTRACTIONS

There are vast amounts of literature and research currently available on herbal tinctures in general. However, literature and research on water-based extractions are presently limited (Feiter, 2004). It is for this reason that water-based extractions are a very under-utilized resource.

Water-based extractions are manufactured as infusions or decoctions. An infusion is used for delicate plant parts such as leaves, flowers, soft stems and fruits (Singh, 2004). The herbal material is placed in a suitable vessel and boiling water is poured over. The infusion is steeped for 5 to 10 minutes. Decoctions are generally more concentrated than infusions, and the method is useful for fibrous plant material such as roots, stems and bark (Singh, 2004). To manufacture decoctins, the herbal material is placed in a pan, covered with cold water and brought to the boil. It is covered and allowed to simmer for 5 to 10 minutes (Rotblatt and Ziment, 2002).

To manufacture an infusion, the plant material and water is allowed to stand at room temperature overnight before being strained off. This is useful where there are many volatile oils that may be lost if heat is used. In both these cases, the water acts as a solvent to extract only those constituents that are soluble in water. It may be usefully applied to extract *tannins* and glycosides, but is not appropriate for the extraction of resins, volatile and non-volatile oils or alkaloids (Singh, 2002).

Water-based extracts are the safest type of extracts, however, as toxic alkaloids are usually insoluble in water. These extracts have a short shelf life due to bacterial contamination, and thus need to be refrigerated and discarded after few days. They are also difficult to standardise and are often bitter or unpleasant tasting unless flavour additives are incorporated (Rotblatt and Zimet, 2002). Situations exist where water-based extractions are more desirable than conventional alcohol tinctures. One such situation is where alcohol intake is prohibited by religion. The use of alcohol-containing medicines is also undesirable for use in babies and pregnant women (Beard, 2004), as it is not known what the safe level of alcohol intake is during pregnancy (ADF, 2004). Alcohol use during pregnancy may cause foetal alcohol syndrome (Singh, 2004b).

Water-based extractions have methodological advantages over alcohol-based tinctures in terms of anti-microbial studies. Research conducted at the Pretoria University by the Faculty of Medicine Research Committee states that if an extract is to be tested for anti-bacterial properties, the extractant should not inhibit the bioassay procedures. Alcohol itself possesses anti-microbial properties therefore antimicrobial studies using alcohol-based tinctures can be ineffective in determining the antimicrobial properties of the actual plant substance. The alcohol control must always be included when conducting such studies (Singh, 2004b).

2.3.3 OTHER METHODS OF HERBAL EXTRACTIONS

2.3.3.1 Vinegar

This extract is also called acetrata. Vinegar is a reasonably good solvent but the shelf life is only about 3 months (Singh, 2004b). Because of the unpleasant taste the medicine is frequently mixed with honey. It may be useful when administering herbs to a small child with compromised liver function because vinegar is very gentle on the body (Singh, 2004b).

2.3.3.2 Glycerine

This is also called *glycerata or glycerol*. *Glycerine* is a colourless, odourless and viscous fluid with solvent capacities somewhere between alcohol and water. A *glycerata* is commonly used to preserve fresh expressed plant juices and to make syrup (Singh, 2004b). The taste is sweet and the shelf life is 6 to 12 months.

2.3.3.3 Fat extractions

Using fat as a solvent will extract those constituents that are fat or alcohol soluble e.g. gums, resins, volatile oils, waxes and alkaloids. There are two methods used:

1. Enfleurage: Fresh plant material (usually flowers) is placed over a layer of fat with a low boiling point (e.g. cocoa butter) and allowed to stand for 3 days at room temperature. A mild organic solvent can then be used to extract the plant constituents from fat (Singh, 2004b).
2. Digestion: This is done in a similar way to effleurage but the fat is heated to about 35^{o}C and maintained at that level for several hours to a few days. The warm oil digests the plant material and draws out the fat-soluble constituents.

2.4 HERBS UTILIZED IN THIS STUDY

Antibacterial compounds have been isolated from a large number of plant species throughout the world (Hidetoshi and Gen-ichi, 2002). Many different types of antibacterial compounds play a role in plant defence. *Flavonoids* such as *naringenin, flavones* and *flavonols,* including *kaemforol, morin* and *quercetin,* constitute a large group of secondary plant metabolites that have been reported to have antibacterial activities (Hidetoshi and Gen-ichi, 2002).

2.4.1 *ARTEMISIA AFRA*

Figure 7: Fresh *Artemisia afra* (Van Wyk *et al*., 2000)

Artemisia afra is a highly aromatic plant named after the Greek goddess Artemis. *Artemisia afra* is one of the most popular plants in South Africa and its natural distribution extends northwards into tropical East Africa and North Africa in areas such as Ethiopia (Van Wyk *et al*., 2000). *Artemisia afra* is a common species in South Africa with a wide distribution from the Cedarberg Mountains in the Cape, northwards to tropical East Africa and stretching as far north as Ethiopia. In the wild it grows at altitudes between 2000 - 2440metres on damp slopes, along stream sides and forest margins (Van Wyk *et al*., 2000). It is an erect multi-stemmed perennial shrub of up to two metres in height. The feathery leaves are finely divided and usually have a greyish-green colour. The silver grey foliage is mainly used, but on occasions also the roots. This soft aromatic shrub is one of the most popular medicinal plants in South Africa. It is easy to grow and is an essential part of the herb garden (Van Wyk *et al*., 2000).

Artemisia afra grows in thick, bushy, slightly untidy clumps, usually with tall stems up to two metres high, but sometimes as low as 60 centimetres. The stems are thick and woody at the base, becoming thinner and softer towards the top. Many smaller side branches shoot from the main stems. The stems are ribbed with strong swollen lines that run all the way up. The soft leaves are finely divided, almost fern-like. The

upper surface of the leaves is dark green, whereas the undersides and the stems are covered with small white hairs, which give the shrub the characteristic overall grey colour. *A. afra* flowers in late summer, from March to May. The individual creamy yellow flowers are small (three to four millimetres in diameter), nodding and crowded at the tips of the branches. Very typical of *A. afra* is the strong, sticky sweet smell that it exudes when touched or cut (Germishuizen and Meyer, 2003).

A. afra needs full sun and heavy pruning in winter to encourage new lush growth in spring (Van Wyk *et al*., 2000). Actively growing in the summer months, it should be able to take quite low temperatures during the winter months. Fast-growing, established shrubs are very tough and will slowly spread to form thicker clumps. New plants can be propagated by division or from cuttings that root easily in spring and summer. Seeds can be sown in spring and summer.

A. afra is one of the oldest and best known medicinal plants, and is still used effectively today in South Africa by people of all cultures. The list of uses covers a wide range of ailments from coughs, colds, fever, loss of appetite, colic, headache, earache and intestinal worms to malaria. *A.afra* is used in many different ways and one of the most common practices is to insert fresh leaves into the nostrils to clear blocked nasal passages (Van Wyk *et al*., 2000). Another pedal hyperhydrosis practice is to place the leaves in socks for sweaty feet (Watt and Breyer-Brandwijk, 1962). Roberts (1990) lists many other interesting uses which include the use of *A. afra* in natural insecticidal sprays and as a moth repellent. The roots, stems and leaves are used in many different ways including enemas, poultices, infusions, body washes, lotions, smoked, snuffed or drunk as a tea. Wilde-als brandy is a very popular medicine still made and sold today. Roberts (1990) also mentions that wilde-als, with its painkilling and relaxing properties, combats the harmfull effects in society. *A. afra* has a very bitter taste and is usually sweetened with sugar or honey when drunk.

2.4.2 *ERYTHRINA LYSISTEMON*

Figure 8: *Erythrina lysistemon* (Van Wyk et al., 2000)

Figure 9: The bark of *Erythrina lysistemon* (Van Wyk *et al*., 2000)

The name *Erythrina* comes from the Greek *erythros* meaning red, both the flowers and the seeds are bright red. The species name *lysistemon* also comes from the Greek meaning 'with a loose or free stamen' and refers to the 'vexillary stamen' that is free

from the staminal tube (Pooley, 1993). The vexillary stamen is the stamen associated with the vexillum, which is another term for the standard petal, and in this species it is free, whereas in e.g. *Erythrina caffra* it is joined to the staminal tube below the middle.

E. lysistemon is a fast-growing, undemanding tree. It does best in fertile, well-aerated and well-drained soils (Van Wyk *et al*., 2000). It is fairly drought-tolerant, but performs better if given water during summer. It is sensitive to cold and grows best in frost free gardens, but will survive in regions with a winter minimum of -7° to -1°C (20°F to 30°F) provided it is planted in a sheltered position and protected from frost when young. This tree prefers dry winters, but it will thrive in the wet winters of the Western Cape as long as it is planted in well-drained soil and watered during the dry summers.

E. lysistemon is easily propagated from seed, cuttings and truncheons (Leistner, 2000). Seed is sown in spring and summer in a well-drained, general-purpose potting soil, placed in a warm but shaded spot and kept moist. Soaking the seed overnight in warm (not hot) water is not necessary for germination to occur, but should hurry things along. Dusting the seed prior to sowing or drenching after sowing with a fungicide that combats pre-emergence damping off, although not essential, will increase the percentage germination. Cuttings are best taken in spring to summer, and truncheons in late winter to spring. Truncheons are made from part of or even an entire branch which is left to dry and heal for a few days, then planted into a pot filled with sand or directly into the soil where the plant is to be grown, and kept damp but not wet. If a plant has to be transplanted, this is best done whilst it is dormant, during winter(Van Wyk *et al*., 2000).

The common coral tree is an excellent specimen tree for gardens and parks and is very effective planted in avenues or for street plantings. It is particularly recommended for any spot in the garden where sun in winter and shade in summer are needed.

There are ±100 species of *Erythrina* that occur in the warm regions of the world. Nine species occur in southern Africa: *E. acanthocarpa, E. baumii, E. caffra, E. decora, E. humeana, E. latissima, E. lysistemon, E. mendesii* and *E. zeyheri*. The Kirstenbosch National Botanical Garden has excellent specimens of *Erythrina lysistemon, E. caffra, E. humeana* and *E. latissima* that are attractive when in flower. *Erythrina lysistemon* is often confused with *Erythrina caffra*, the coast coral tree. *Erythrina caffra* grows in the coastal and riverine fringe forests from Port Shepstone in KwaZulu-Natal to the Humansdorp District in Eastern Cape and in a pocket further north on the KwaZulu-Natal coast (Van Wyk *et al*., 2000). It is generally taller than *Erythrina lysistemon*, the flowers are orange-scarlet, and a cream-flowered form is occasionally seen. The standard petal is shorter and broader so that the stamens stick out of the flower giving it a whiskered look. In most other respects they are very similar, and were in fact regarded as the same variable species for many years and, when not in flower, are difficult to tell apart.

Erythrina lysistemon is widely distributed in the Eastern and Northern parts of South Africa. It is commonly cultivated in gardens and parks. *Erythrina lysistemon* is a tree that reaches a height of about six to eight metres and can be easily recognised by the thick, thorny branches and bright red flowers. The bark is mainly used and sometimes the leaves and roots. The main use of the bark is to treat sores, wounds, abscesses and arthritis (Van Wyk *et al.,* 2000). The bark is also used for toothache by the Vhavenda people (Hutchings *et al*., 1996). To treat sores, wounds and swellings, the bark is applied as a poultice. Crushed leaves are reported to clear wounds caused by maggots and they are also used as eardrops to relieve earache. Active ingredients are a large number of *tetracyclic isoquinoline* alkaloids that include *erysovine* and *erythraline* (Van Wyk *et al*., 2000). Erythrina alkaloids are known to be highly toxic (Bruneton, 1995) but the traditional uses suggest antibacterial, anti-inflammatory and analgesic effects.

Coral trees are attacked by a boring insect that enters at the tip of a branch and causes die-back. As soon as this is noticed, the damaged branch should be cut back to unbored wood and the prunings should be burnt. It is difficult to control on big trees,

but it can be done with a systemic insecticide. Caterpillars can cause damage to the foliage, and an insect causes yellowish galls on the leaves, but these do not seem to affect the overall health or performance of the tree (Van Wyk *et al*., 2000).

2.4.3 *PSIDIUM GUAJAVA*.

Figure 10: *Psidium guajava* (Van Wyk et al., 2000)

Figure 11: The fruits and leaves of *Psidium guajava* (Van Wyk *et al*., 2000).

In South Africa *Psidium guajava* is found in the warm subtropical areas of KwaZulu Natal, Mpumalanga and the Northern Province. *Psidium guajava* is a shrub or small tree, usually not more than four metres in height. The bark peels off in flakes revealing the characteristically smooth trunk. It has large leaves that are formed opposite each other in pairs with prominent veins, particularly on the lower side. It has small white flowers of about 25 millimetres in diameter with numerous stamens which are produced in the early summer, followed by rounded or pear-shaped yellow

fruits. Due to their delicious taste and high vitamin C content, guavas are an important commercial crop. *Psidium guajava* leaves are commonly used in South Africa as a remedy for diarrhoea (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996). The leaves are also used for several other ailments, including diabetes, fever, cough, ulcers, boils and wounds (Van Wyk *et al*., 2000). The leaves are crushed and boiled in water and the infusion is taken either orally as tea or as an enema.

For active ingredients, numerous *tannins* and other *phenolic* compounds have been identified, of which *amritoside* is of particular importance (Van Wyk *et al*., 2000). *Amritoside* is a glycoside of *ellagic* acid. Another biologically interesting compound in the plant is *guiajaverin*, a glycoside (*arabinopyroside*) of *quercetin* (Dictionary of Natural Products, 1996). The leaves also contain important oils and *triterpenoids*. Leaf extracts of *Psidium guajava* has been reported to be antibacterial (Pranee *et al*., 1999). *Ellagic* acid is an intestinal astringent and haemostatic (Merck, 1989; Bruneton, 1995). This explains the therapeutic value of the plant against diarrhoea and dysentery. The *tannins* are of value because of their vasoconstricting effect and the ability to form a protective layer on the skin and mucosa (Bruneton, 1995). *Quercetin* is an anti-oxidant with anticarcinogenic, anti-HIV and antibiotic effects (Dictionary of Natural Products, 1996). *Quercetin* has also been reported to be antibacterial (Jeffery *et al*., 1983) and has a hypoglycaemic effect (Ponglux, 1987). *Quercetin* is mostly found in the leaves and *ellagic acid* is mostly found in the bark.

The methanolic extract of *P. guajava* (leaves) was the only agent that showed significant inhibitory activities against the growths of some diarrhoeagenic pathogens in the study by Dr.J.Lin (Lin et al., 2002). Several studies (Lin et al., 2002) have also demonstrated that *P. guajava* possesses antidiarrhoeal and antimicrobial activities. In addition, leaf extract of *P. guajava* contains anticough (Jaiarj et al., 1999), antiamoebic and antispasmodic properties (Lin et al., 2002). Lutterodt, 1989 showed that quercetin from the leaf extract inhibits acetylcholine release in the gastrointestinal tract that might account for the antidiarrhoeal activity of the plant. Furthermore, several chemical compounds isolated from guava leaves possess antibacterial activities against different strains of gram negative bacteria (Caceres et

al., 1990) as well as gram positive bacteria (Jaiarj et al., 1999). All plant extracts significantly reduced castor-oil induced diarrhoea, slowed the propulsion of charcoal meal and significantly inhibited the PGE_2-induced enteropooling. It is well known that the administration of PGE_2 or PGE_1 induces the signs of inflammation, swelling and edema resulting from increased capillary permeability. Our results suggest that these plant extracts can reduce the capillary permeability and, therefore, decrease mucocal inflammation.

2.4.4 ANTIMICROBIAL SYNERGISMS IN PLANT PRODUCTS

Plant antimicrobials have been found to be synergistic enhancers in that, though they may not have any antimicrobial properties alone, when they are taken concurrently with standard drugs they enhance the effect of that drug (Kamatou *et al*., 2006). The synergistic effect from the association of antibiotic and plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment (Nascimento *et al*., 2000). The application of the synergistic principle is evident in commercial preparations for the treatment of various infections (e.g. the antibiotic augmentin). Traditional healers often use combinations of plants to treat or cure diseases (Kamatou *et al*., 2006). One notable example from the ethnobotanical literature is the concomitant administration of various *Salvia* species with *Leonotis leonurus* to treat various infections (Masika and Afolayan, 2003). Kamatou *et al*. (2006) confirmed the existence of synergism between *Salvia chamelaeagnea* and *Leonotis leonurus*, when these two plant extracts were combined together and tested against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*. They also reported synergism when the tincture of *L. leonurus* and various *Salvia* species were combined together against influenza. Boik (2001) conducted a large number of combination studies using various natural substances and their results strongly suggested that when used in combination, natural substances can produce synergistic effects. It is thought that *phenolic* compounds such as *flavonoids* may increase the biological activity of other compounds by synergistic or other mechanisms (Williamson, 2001). Experimental

evidence of synergistic actions between plants was also shown in a clinical study on the formulation of Chinese herbs used to treat eczema (Williamson, 2001).

2.4.5 COMBINATIONS OF BIOACTIVE PLANT PRODUCTS AND DIFFERENT CLASSES OF ANTIBIOTICS WITH SPECIFIC MECHANISM OF ACTION

In the treatment of drug resistant infections, combinations of antibiotics have often been used as this takes advantage of different mechanisms of action. The use of antimicrobial agents displaying synergy is one of the well established indications for combination antimicrobial therapy (Rybak and McGrath, 1996). Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting strains are more likely to result in successful therapeutic results. Thus, evidence of *in vitro* synergism could be useful in selecting most favourable combinations of antimicrobials for the practical therapy of serious bacterial infections (Hooton *et al*., 1984).

It has been proven that, in addition to the production of intrinsic antimicrobial compounds, plants also produce multi-drug resistant (MDR) inhibitors which enhance the activity of the antimicrobial compounds (Stermitz *et al*., 2000). Tegos *et al.* (2002) showed that the activity of presumed plant antimicrobials against Gram-positive and Gram-negative organisms was significantly enhanced by synthetic MDR inhibitors of associated efflux proteins. The findings provided a basis that plants can be prospective sources of natural MDR inhibitors that can modulate the performance of antibiotics against resistant strains. The screening of crude plant extracts for synergistic interaction with antibiotics can provide ways for the isolation of MDR inhibitors.

Darwish *et al*. (2002) carried out a study on some Jordanian plants and demonstrated that the efficacy of the antibiotics, *gentamycin* and *chloramphenicol* against *S. aureus* were reportedly improved by the use of plant materials. Ahmad and Aqil (2006), also reported that crude extracts of Indian medicinal plants demonstrated synergistic interaction with tetracycline and ciprofloxacin against extended spectrum

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

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

The synergies detected in the studies mentioned in this subsection were not specific to any group of organisms or class of antibiotics. This suggests that plant crude extracts are a blend of 30 compounds that can enhance the activity of different antibiotics. Plants have been known to contain myriads of antimicrobial compounds (Iwu *et al*., 1999) such as *polyphenols* and *flavonoids*. The antimicrobial and resistance modifying potentials of naturally occurring *flavonoids* and *polyphenolic* compounds have been reported in other studies such as Cushnie and Lamb (2005) and Sato *et al*. (2004).

Some of these compounds including *polyphenols* have been shown to exercise their antibacterial actions/activities through membrane perturbations. This disruption of the cell membrane coupled with the action of beta-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone *et al*., 2006). It has also been revealed that some plant-derived compounds can improve the *in vitro* activities of some peptidoglycan inhibiting antibiotics by

directly attacking the same site (i.e. peptidoglycan) in the cell wall (Zhao *et al*., 2001). While the above explanations may account for the synergy between the extracts and beta-lactam antibiotics that act on the cell wall, it might not apply in the case of the observed synergy with other classes of antibiotics with different targets such as *tetracyclines, erythromycin*, *ciprofloxacin* and *chloramphenicol*.

2.5 BACTERIA USED IN THIS STUDY

Pseudomonas aeruginosa is a highly prevalent opportunistic pathogen in hospitalized patients. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility.

Staphylococcus aureus is one of the major resistant pathogens that are found on the mucous membranes and the human skin of around a third of the population. MRSA (*methicillin*-resistant *Staphylococcus aureus*) is common in hospitals. Most *S. aureus* infections are resistant to *penicillin, methicillin, tetracycline* and *erythromycin*. This has left *vancomycin* as the only agent available. However, strains with intermediate (4-8 ug/ml) levels of resistance, termed VISA (*vancomycin* intermediate *Staphylococcus aureus*), began appearing in the late 1990s (Bozdogan *et al*., 2003). The first documented strain with complete (>16 ug/ml) resistance to *vancomycin*, appeared in the United States in 2002 (Bozdogan *et al*., 2003). It was termed VRSA (*Vancomycin*-resistant *Staphylococcus aureus*). A new class of antibiotics, *oxazolidinones*, are now available, and the first commercially available *oxazolidinone, linezolid*®, is comparable to *vancomycin* in effectiveness against MRSA (Bozdogan *et al*., 2003).

Leading etiological agents of urinary tract infections are *Escherichia coli, Candida albicans, Enterococcus faecalis, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus mirabilis* (Svanborg and Godaly, 1997), but *E. coli* is the most common. The incidence of acute uncomplicated urinary tract infection is estimated to exceed 0.5 episodes per annum among women between 18-30 years (Hooton *et al*., 1997). Despite the existence of potent antibiotics, resistant or multiresistant strains are

continuously appearing, imposing the need for a permanent search and development of new drugs. The test organisms in this study included the gram-positive *S. Aureus*, gram-negative *E. coli* and *P. aeruginosa*.

2.5.1 *STAPHYLOCOCCUS AUREUS*

Classification

S. aureus is a Gram-positive coccusr. The cocci are mainly arranged in grape-like clusters but some, especially when examined in pathological specimens, occur as single cells or pairs of cells (Greenwood *et al*., 1994). *S. aureus* are classified as a coagulase positive staphylococcus because it possesses the enzyme coagulase which has the ability to clot blood plasma (Howard *et al*., 1995).

Epidemiology

S. aureus, which is present in the nose and on the skin of a variable proportion of healthy people, is an opportunistic pathogen in that it causes infections most commonly at sites of lowered host resistance e.g. damaged skin or mucous membranes. The common infections caused by *S. aureus* are skin and wound infections, abscesses, osteomyelitis and food poisoning. *Staphylococcus aureus* is sensitive to many antimicrobial agents but some strains produce the enzyme β-lactamase which inactivates the action of β-lactam antibiotics. These are called multi-resistant strains of *S. aureus* (MRSA). *S. aureus* is the commonest cause of both social and community acquired infections. Patient to patient transmission via the hands of the personnel are the most common and prevalent means of spread (Berkow *et al*., 1992).

Growth Characteristics

S. aureus are facultative anaerobes that are able to grow on any nutrient media. Due to the fact that it does have some *haemolysins*, certain strains can cause β haemolysis on blood agar plates. It does not grow on MacConkey agar with crystal violet.

Toxins and enzymes

S. aureus is the primary toxin-producing type of *staphylococci* and produces a wide range of extracellular products viz. *haemolysins*, *leukocidin*, *enterotoxins*, *exfoliatin* and enzymes (Howard *et al*., 1995).

S. aureus produces four types of *haemolysins* viz. alpha, beta, gamma and delta. The exact role of each type of haemolysin is not known but it is believed that alpha *haemolysins* have dermonecrotic activity as well as haemolytic abilities (Howard *et al*., 1995).

Leukocidin toxin is capable of causing lysis of human leucocytes and macrophages. If these toxins are produced the patient will develop an impaired immune system. The leucocytes are a major part of the human immune system. This will expose the patient to infections(Iwase *et al*., 1990)

There are five types of *enterotoxins* (labelled as A through to E) produced by *S. aureus*. These toxins are responsible for *staphylococcal* food poisoning and conditions such as toxic shock syndrome which results when *staphylococci* grow and thrive within a cavity on the human body (Howard *et al*., 1995).

Exfoliatin is a toxin which has an epidermolytic or exfoliative effect. It causes layers within the epidermis of the skin to split thereby causing exfoliation of the skin (Ladhani *et al*., 1999).

There are numerous enzymes produced by *staphylococci* viz. lipase, fibrinolysins and proteases. However, *S. aureus* produces the enzyme coagulase which not only causes haemolysis but also inhibits the bactericidal activity of the normal serum. (Howard *et al*., 1995)

Staphylococcal Infections

S. aureus constitutes the largest amount of *Staphylococcal* infections due to coagulase enzyme production. Local infections are by far the most common of all

types of *staphylococcal* infection. More serious invasive *staphylococcal* infections are not very common in healthy individuals, but may occur in immunocompromised patients. Predisposing factors include injury to normal skin, prior viral infections, leucocyte defects, deficiencies in humoral immunity and alteration of normal flora due to use of antimicrobial agents to which *S. aureus* is not susceptible. This organism may also invade the blood stream and from there spread to numerous body sites (Howard *et al*., 1995).

2.5.2 *ESCHERICHIA COLI*

Classification

Escherichia coli is a member of the *Enterobacteriaceae*. It is a Gram-negative facultative anaerobe. It is found as a member of normal flora in the gut of humans and it is highly motile.

Morphology and Identification

E. coli are facultative anaerobic, short, straight, gram-negative bacilli that are non-sporing, usually motile with flagella and occur singly or in pairs in rapidly growing liquid cultures (Williams *et al*., 2004). *E. coli* grows well on non-selective media forming circular, convex, smooth colonies with distinct edges (Jawetz *et al*., 1991). *E. coli* readily ferments glucose, lactose and certain other sugars producing both acid and gas, a property used in preliminary identification (Pattison *et al*., 1995)

E. coli can be easily recovered from samples such as urine, stools, blood and pus on lactose containing media e.g. MacKonkey agar plate. Colonies become visible within a day. Lactose fermentation together with other biochemical tests helps to differentiate *E. coli* from other highly gram-negative rods (Howes, 2002).

Clinical Manifestations

Despite the fact that *E. coli* is a member of the normal flora and is not highly pathogenic, it is of great medical importance due to the frequency and sacrity of infections it causes. The organism is a commensal in the small intestines, however, because of its presence in stools, it often reaches and incites disease in other parts of the body, especially the urinary tract and peritoneum (Myrvik and Weiser, 1989) *E.*

coli produce two types of toxins, viz. heat-labile enterotoxin and heat-stable enterotoxin (Howard *et al*., 1995), thus causing different types of clinical symptoms.

Urinary Tract Infection

The normal urinary tract is sterile and resistant to bacterial colonisation. However, urinary tract infections are the most common bacterial infection in all age groups (Simon, 2002). *E. coli* is the most common cause of urinary tract infections and accounts for 90 percent of first urinary tract infections in young women. In hospitalised patients, *E. coli* accounts for 50 percent of cases (Howes, 2004).

E. coli enters the urinary tract by natural means or may be introduced via catheters or other instruments. Their persistence in the urinary tract is favoured by anesthesia, paralysis by any other agent that interrupts the normal voiding reflex, or by anatomical abnormalities that permit retention of urine. Although obstruction alone does not cause urinary tract infection, its presence does predispose to infection and makes infection more difficult to eradicate with medical therapy. Urine is an excellent culture medium and readily supports the growth of *E. coli* (Berkow and Beers, 1999).

Diarrhoeal Diseases

Diarrhoea-causing *E. coli* are claified into different categories based on their virulence properties. The major categories are *enteropathogenic* (EPEC), *enterotoxigenic* (ETEC), *enteroinvasive* (EIEC), *enteroaggeregative* (EaggEC) (Parry *et al*., 2002) and *enterohaemorragic* (EHEC) (Mackie and McCartney, 1996).

Enteropathogenic (EPEC) is associated with acute outbreaks of diarrhoea in young children (Parry *et al*., 2002). Illness results from the bacteria adhering to the intestinal wall and subsequent destruction of the microvilli (Centre for Food Safety and Applied Nutrition, 2003).

Enterotoxigenic (ETEC) is associated with infant diarrhoea in developing countries and traveller's diarrhoea. Acute watery diarrhoea, similar to cholera, is produced by

heat stable or heat labile toxins. It is acquired by consuming contaminated water or food (Howard *et al*., 1993).

Enteroinvasive (EIEC) is associated with dysentery-type illness caused by the invasion of the gut epithelial cells (Parry *et al*., 2002). Clinically the illness is characterised by fever, abdominal cramps, malaise, toxaemia and watery diarrhoea consisting of blood and mucous (Centre for Food Safety and Applied Nutrition, 2003).

Enteroaggregative (EaggEC) is associated with persistent diarrhoea in people in developing countries (Parry *et al*., 2002). These bacteria are emerging as a potential cause of diarrhoea in patients with AIDS (Berkow and Beers, 1999).

Enterohaemorrhagic (EHEC) is associated with haemorrhagic colitis and haemolytic uraemic syndrome. It affects the cells lining the gastrointestinal tract and kidneys (Parry *et al*., 2002)

Neonatal Sepsis

Neonatal sepsis can be described as an invasive bacterial infection of the bloodstream occurring in the first four weeks of life (Berkow and Beers, 2000). Newborns may be highly susceptible to *E. coli* sepsis because they lack IgM antibodies (Jawetz *et al*., 1989). A newborn may be predisposed to neonatal sepsis by obstetric complications, maternal bleeding, toxaemia, precipitous delivery or maternal infection particularly of the urinary tract or endometrium (Myrvik and Weiser, 1989). The symptoms of sepsis are not specific and they vary from one child to another.

Neonatal Meningitis

Neonatal meningitis is defined as the inflammation of the meninges due to bacterial invasion in the first four weeks of life (Berkow and Beers, 2000). Neonatal meningitis most frequently results from pre-existing bacteraemia associated with neonatal sepsis. Meningitis may also result from skin lesions of the scalp that, along with developmental defects, lead to communication of the skin surface with the

subarachnoid space. Direct extension to the central nervous system from the ear rarely occurs (Berkow and Beers, 2002). Symptoms of the infection in the newborns are not very specific and may include crying, irritability, sleeping more than usual, lethargy, refusing to take breast milk or bottle milk, low or unstable body temperature, jaundice, pallor, breathing problems, rash, vomiting and diarrhoea. There may also be bulging of the fontanelle. Cranial nerve abnormalities may also be present (Berkow and Beers, 2000).

Antimicrobial sensitivity

Antimicrobials used to treat *E. coli* infections include all those that have action against gram negative organisms. These include *pthalysulphathiazole, neomycin, doxycycline, trimethoprim, norfloxacin, chloramphenicol* and other fluoroquinolones (Singh, 2004b). Some strains of *E. coli* are developed resistance to these antibiotic agents (Singh, 2004b).

2.5.3 *PSEUDOMONAS AERUGINOSA*

Classification

Pseudomonas aeruginosa belongs to the genus *Pseudomonas* of the family *Pseudomonadaceae*. (Wilson *et al*., 1979). *Pseudomonas maltophilia*, the only consistently oxidase-negative pseudomonad is an occasional opportunistic pathogen, associated with a variety of serious infections (Davies *et al*., 1980). It is resistant to most antimicrobials. Other occasional opportunistic pathogens are *P. alcaligens, P. stutzeri, P. mendocina* and *P. putrefaciens.*

Morphology and Identification

Pseudomonas aeruginosa is a Gram-negative, non-capsulate bacterium, which is usually motile by virtue of one or two polar flagella. It is a strict aerobe but can grow anaerobically if nitrate is available. It differs from members of *Enterobacteriaceae* by deriving energy from carbohydrates by an oxidative rather than a fermentative metabolism. *P. aeruginosa* grows readily on standard laboratory media. Strains from clinical material are usually β-haemolytic. Most strains produce a bluish green *phenazine* pigment, *pyocyanin* as well as *fluorescein* a greenish yellow *pteridine* that fluoresces (Davis *et al*., 1980). These pigments colour the medium surrounding the

colonies. About 10 percent of strains do not form the pigment (Davis *et al*., 1980). The biochemical reactions are also useful in the identification of *P. aeruginosa.*

Clinical Manifestations

The community infections caused by *P. aeruginosa* are mostly mild and superficial. In hospitalised patients, *Pseudomonas* infections are more common, more severe and more varied. Infection is usually localised, as in catheter-related urinary tract infections, infected ulcers, bed sores or burns, and eye infections.

P. aeruginosa can be found occasionally in the axilla and anogenital areas of normal skin but rarely in stools of adults unless antibiotics are administered. The organism is commonly a contaminent of lesions populated with more virulent organisms, but occasionally it causes infection in tissues that are exposed to the external environment (Berkow *et al*., 1992).

P.aeruginosa produces three potent, serologically distinct exotoxins, which are more important in its pathogeniocity than the endotoxins (Davis *et al*., 1980). The organism also produces collagenase, lipase and haemolysins which all contribute to its pathogenicity (Davies *et al*., 1980).

Antimicrobial Sensitivity

This bacterium has developed resistance to many antibiotics. Currently the best antibiotics to inhibit growth of *P. aeruginosa* are the aminoglycosides such as *amikacin, tobramycin* and *gentamicin*. These are often used in-conjunction with antibiotics such as *piperacillin*. *Cephalosporins* such as *ceftazidine* and *cefotaxime* may also prove effective. Many strains of *P. aeruginosa* however, do not respond well clinically to antibiotics that have appeared effective when treated *in vitro* (Singh, 2004b). There are many herbs that yield useful medicinal compounds including those having antibacterial activity to *Pseudomonas* and other bacteria (Singh *et al*., 2002).

2.6 RESEARCH METHODS

2.6.1 SCREENING METHODS FOR NATURAL PRODUCTS WITH ANTIMICROBIAL PROPERTIES

The three most common methods employed to evaluate the antibacterial properties of natural products are diffusion assay, dilution tests and bioautography tests. This study has used the disc diffusion assay which was followed by dilution assay for minimum inhibitory concentration

Diffusion assays

Diffusion methods of screening for antibacterial properties of natural products employ either a disc or reservoirs for the sample substance (Singh, 2004a). This method is based on the principle that the reservoir containing an extract is brought into contact with an inoculated medium. The solute will diffuse into the agar. After incubation the diameter of the growth-free area around the reservoir is measured and taken as the antibacterial activity of that product.

Diffusion assays are well suited for the preliminary screening of pure substances, such as *alkaloids*, *terpenoids* and *flavonoids* (Singh, 2004a). These methods cannot be used for samples that are difficult to diffuse in the media, because the correlation between diffusion power and antimicrobial activity has not been established.

Comparison of the zones of inhibition of natural products with those of synthetic antibiotics in disc assay is useful for establishing the sensitivity of the test organism. Comparison of the antibacterial potency of the natural test substances and the synthetic antibiotic cannot be made from these measurements (Singh, 2004a). This is due to the fact that many other factors such as diffusion ability which can affect the zone size of inhibition can be influential, resulting in misleading conclusions. The optimum effectiveness of the disc diffusion method is obtained by using Mueller-Hinton agar and standardised microorganisms (American Type Culture Collection) (Singh, 2004a)

Dilution tests

Dilution tests require a homogeneous dispersion of the sample. Bacterial multiplication is measured by the turbidity of the solution, which is taken as a direct correlation to the amount of bacterial growth (Singh, 2004a). These tests can be used to produce the minimum inhibitory concentration for the antibacterial sample. The dilution tests are usually more complicated, time consuming and expensive to perform than the disc diffusion methods.

Bioautographic methods

This method involves using paper chromatography or thin layer chromatography to isolate compounds which are then subsequently tested using the disc assay method for anti-bacterial activity. This technique is not as feasible as the disc assay and dilution methods for preliminary screening of sample due to the associated costs (Singh, 2004a).

2.7 CHOICE OF EXTRACTANT

Ethanol is usually used in the manufacture of plant extracts to enable the extraction of water-insoluble constituents from the source material as well as preservative for the extract (Singh, 2004a). However ethanol itself has antibacterial effects. This is why a 60 percent ethanol control was used in this study

In order to neutralise the variable effect of ethanol altogether, a water-based extract is also assessed. Invernizzi (2002) recommended that trials should be done using different types of extractants to see which is the most effective in extracting the active compounds from the plants. He also makes mention of the use of acetone, but this is not a viable option as a therapeutic agent owing to its toxic nature (Invernizzi, 2002).

2.8 CONCLUSION

Medicinal plants have been considered a healthy source of life for people. Therapeutic properties of medicinal plants are very useful in healing various diseases. They contain a number of antibacterial compounds. Bacterial resistance to

known antibiotics is increasing especially against *P.aeruginosa*, *E.coli* and *S.aureus.* That is why the use of medicinal plants can represent the best solution.

CHAPTER THREE
METHODOLOGY

3.1 STUDY DESIGN

This is an experimental study that involves laboratory analysis of the activity of *Artemisia afra*, *Erythrina lysistemon* and *Psidium guajava* on *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

3.2 THE DATA

This research involved two types of data: primary and secondary. Primary data were collected through experiments conducted during the course of this research study, whereas the secondary data were obtained through research articles published in journals, books and manuals

3.3 CRITERIA GOVERNING THE ADMISSIBILITY OF DATA

Only data gathered from experiments conducted by the researcher at the Mangosuthu University of Technology, Department of Biomedical Science Microbiology and Chemistry laboratory were included in the data analysis

3.4 MATERIALS AND METHODS

3.4.1 SAMPLE COLLECTION

Samples of *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* were obtained from Silverglen Nature Reserve (Chatsworth). The plants were harvested early in the morning (8a.m.) when the cells are more active (Lang, 2010). For *Artemisia afra* and *Psidium guajava* extracts, leaves were used and the bark for *Erythrina lysistemon*.
These plants were identified by Barry Lang at Durban Botanic Gardens on 03 May 2009.

3.4.2 PREPARATION OF THE WATER–BASED EXTRACTION OF *ARTEMISIA AFRA*

Artemisia afra was prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia (Benyunes, 2005):

- *Artemisia afra* (fresh plant part above ground) were harvested early in the morning (8a.m.)
- Plant material was immediately minced in an electrical mincer and weighed into a glass jar
- One part of minced plant material was added to three parts of distilled water (1:3) according to calculation 1(Appendix A)
- The mixture was shaken for five minutes and then left in a glass jar for 10 days at a temperature not exceeding 20^{o}C, agitating the mixture once a day
- Thereafter it was pressed through 100 percent cotton muslin cloth and filtered through a No 1 Whatman filter paper (Singh, 2004b)
- Distilled water was added to make up the volume

3.4.3 PREPARATION OF THE WATER–BASED EXTRACTION OF *ERYTHRINA LYSISTEMON*

Erythina lysistemon was prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia (Benyunes, 2005):

- *Erythrina lysistemon* (fresh bark) was harvested early in the morning (8a.m.)
- Plant material was immediately minced in an electrical mincer and weighed into a glass jar
- One part of minced plant material was added to three parts of distilled water (1:3) according to calculation 2 (Appendix A)
- The mixture was shaken for five minutes and then left in a glass jar for 10 days, agitating the mixture once a day. This was refrigerated
- Thereafter it was pressed through 100 percent cotton muslin cloth and filtered through a No 1 Whatman filter paper (Singh, 2004b)
- Distilled water was added to make up volume

3.4.4 PREPARATION OF THE WATER–BASED EXTRACTION OF *PSIDIUM GUAJAVA*

Psidium guajava was prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia (Benyunes, 2005):

- *Psidium guajava* (fresh plant part above ground) was harvested early in the morning (8a.m.)
- Plant material was immediately minced in an electrical mincer and weighed into a glass jar
- One part of minced plant material was added to three parts of distilled water (1:3) according to calculation 3 (Appendix A)
- Three parts of distilled water were added to one part of minced plant material
- The mixture was shaken for five minutes and then left in a glass jar for 10 days at temperature not exceeding 20^{o}C, agitating the mixture once a day.
- Thereafter it was pressed through 100 percent cotton muslin cloth and filtered through a No 1 Whatman filter paper (Singh, 2004b)
- Distilled water was added to make up volume

3.4.5 PREPARATION OF THE ETHANOL TINCTURE OF *ARTEMISIA AFRA*.

Artemisia afra was prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia (Benyunes, 2005):

- *Artemisia afra* (fresh plant part above ground) were harvested early in the morning (8a.m.)
- Plant material was immediately minced in an electrical mincer and weighed into a glass jar
- One part of minced plant material was added to three parts of 86% ethanol (1:3) according to calculation 4 (Appendix A)
- The mixture was shaken for five minutes and then left in a glass jar for 10 days at temperature not exceeding 20^{o}C, agitating the mixture once a day
- Thereafter it was pressed through 100 percent cotton muslin cloth and filtered through a No 1 Whatman filter paper (Singh, 2004b)
- After the calculation and preparation, the final ethanol tincture was at 60 percent
- 60 percent ethanol was added to make up volume

3.4.6 PREPARATION OF THE ETHANOL TINCTURE OF *ERYTHRINA LYSISTEMON*

Erythrina lysistemon, was prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia (Benyunes, 2005):

- *Erythrina lysistemon* (fresh plant part above ground) were harvested early in the morning (8a.m.)
- Plant material was immediately minced in an electrical mincer and weighed into a glass jar
- One part of minced plant material was added to three parts of 86 percent ethanol (1:3) according to calculation 5 (Appendix A)
- The mixture was shaken for five minutes and then left in a glass jar for 10 days at temperature not exceeding 20^{o}C, agitating the mixture once a day
- Thereafter it was pressed through 100 percent cotton muslin cloth and filtered through a No 1 Whatman filter paper (Singh, 2004b)
- After the calculation and preparation, the final ethanol tincture was at 60 percent
- 60 percent ethanol was added to make up volume

3.4.7 PREPARATION OF THE ETHANOL TINCTURE OF *PSIDIUM GUAJAVA*.

Psidium guajava was prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia (Benyunes, 2005):

- *Psidium guajava* (fresh plant part above ground) were harvested early in the morning (8 a.m.)
- Plant material was immediately minced in an electrical mincer and weighed into a glass jar
- One part of minced plant material was added to three parts of 86 percent ethanol (1:3) according to calculation 6 (Appendix A)
- The mixture was shaken for five minutes and then left in a glass jar for 10 days at temperature not exceeding 20^{o}C, agitation the mixture once a day
- Thereafter it was pressed through 100 percent cotton muslin cloth and filtered through a No 1 Whatman filter paper (Singh, 2004b)
- After the calculation and preparation, the final ethanol tincture was at 60 percent
- 60 percent ethanol was added to make up volume

3.4.8 PREPARATION OF DISTILLED WATER CONTROL

Distilled water was obtained from the Department of Homeopathy at the Durban University of Technology.

3.4.9 PREPARATION OF 60 PERCENT (V/V) ETHANOL CONTROL

The 60 percent (v/v) ethanol was prepared according to the German Homeopathic Phamacopoea standard: 65.90ml of 96 percent (v/v) ethanol was diluted with sufficient distilled water to produce 100ml of 60 percent ethanol (Singh, 2004b).

3.4.10 ANTIBIOTIC ASSAY (AA) DISCS

Antibiotic Assay discs were used as recommended by Invernizzi (2002).
They were purchased from Davies Diagnostics (Batch number 277653)

3.4.11 THE PREPARATION OF MUELLER HINTON AGAR

Fresh Mueller Hinton (MH) was prepared according to the manufacture's instruction (Oxoid). Thirty eight grams of powder was weighed out into three separate one-litre glass bottles. Distilled water was added until the one litre mark of each bottle was reached using a measuring cylinder. This was mixed until the powder had completely dissolved. Bottles were sterilised by autoclaving for 15 minutes at 121^{o}C. The agar was poured into plates to solidify.

3.4.12 THE PREPARATION OF THE NUTRIENT BROTH

Fresh Nutrient Broth was made up according to the manufacturer's instructions (Oxoid). Forty grams of Nutrient Broth powder was weighed into a one litre glass bottle. Distilled water was added until the one litre mark was reached. This was mixed until the powder had completely dissolved. Five millilitres was dispensed into bijou bottles before autoclaving. Bijou bottles were sterilised by autoclaving for 15 minutes at 121^{o}C.

3.4.13 PREPARATION OF NUTRIENT AGAR SLOPES

Fresh Nutrient Broth was made up according to the manufacturer's instructions (Oxoid). Twenty eight grams of Nutrient agar powder was weighed into a one litre glass bottle. Distilled water was added until the one litre mark was reached. This was mixed until the powder had completely dissolved. Ten millilitres was dispensed into MacCathy bottles before autoclaving. These were sterilised by autoclaving for 15 minutes at 121^{o}C. They were then allowed to slope before setting.

3.4.14 PREPARATION OF BLOOD AGAR PLATES

Blood Agar Base (BA) was prepared according to the manufacturer's instructions (Oxoid). Thirty eight grams of blood agar base powder was weighed out into 3 separate one litre glass bottles. Distilled water was added until the one litre mark of each bottle was reached using a measuring cylinder. This was mixed until the powder had completely dissolved. Bottles were sterilised by autoclaving for 15 minutes at 121^{o}C. It was allowed to cool to 50^{o}C. Fifty millilitres of fresh horse blood obtained from the National Health Laboratory Service media department was added and it was mixed. The agar was poured into plates to solidify.

3.4.15 MICROBIAL CULTURES

The cultures of *Staphylococcus aureus* (ATCC29213), *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853) were maintained on nutrient agar slopes at 4^{o}C and subcultured on to blood agar plates for 24 hours before use. These are known American Type Culture Collection strains obtainable from Davies Diagnostics.

3.4.16 PREPARATION OF INOCULUMS

A few colonies from the overnight cultures of *E. coli*, *S. aureus* and *P. aeruginosa* were suspended in 5ml of nutrient in bijou bottles. This was swirled to allow even distribution of the culture. The suspension was then vortexed in a vortex mixer to enable adequate mixing. The suspension was made up to the equivalent of 0.5 McFarland turbidity standard as described by Thrupp (1980)

3.4.17 BACTERIAL SENSITIVITY TESTING (SCREENING)

The method that was used was in accordance with a modification of the Kirby-Bauer Antimicrobial Sensitivity Test Procedure (Cappucino and Sherman, 1992). Inoculum containing $1x10^6$ colony forming units (CFU) per millilitre (ml) was introduced on to the surface of Mueller Hinton Agar plates.

Inoculation of plates

The inoculation of six agar plates with *E. coli*, six agar plates with *S. aureus* and six agar plates with *P. aeruginosa* was done using a sterile swab which was dipped into the well mixed overnight nutrient broth culture prepared above. The agar surface of each plate was inoculated such that there was uniform growth.

Placement of Disc on the agar plate

The plates were divided into 5 quadrants and labelled as water control, 60 percent ethanol control, *vanillic acids* control for gram negative bacteria, *vancomycin* control for *S. aureus*, water-based extract of each plant and ethanol tincture of each plant. The antibiotic assay discs were soaked for 10 seconds into each solution except for the *amikacin* and *vancomycin* discs which were bought ready for use from Davies Diagnostics. Each disc was then gently placed on the surface of the Mueller Hinton agar plate such that they firmly adhered to the surface of the agar plates.

Incubation of plates

Inoculated plates were incubated at 37^oC for 24 hours. After incubation, the plates were examined for the inhibition of growth around the discs. This was indicated by the clear zone of inhibition surrounding the discs. The zone of inhibition was measured using a ruler and they were compared to the controls. Each extract testing was repeated six times to ensure consistency and also to allow for statistical analysis.

3.4.18 DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION (MOILORI *et al.*, 1996).

Preparation of serial dilutions for MIC's

Nutrient broth (2ml) was pipetted using a sterile micropipette into 5ml test tubes. The number of test tubes was eight. For each plant, 2ml of ethanolic extract was added to the first tube in the series. It was then mixed and 2ml of that mixture was pipetted to

the second tube. This procedure was repeated up to tube eight. After mixing in tube number eight, 2ml was discarded. This process thus resulted in 2mls volume present in all the test tubes. The dilutions were as follows: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.

Minimum inhibitory concentration and minimum bactericidal

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined by serially diluting the extracts using nutrient broth in test tubes as mentioned above. Each test tube was inoculated with 100ul of each bacterial suspension from three different bacteria. The suspensions were the same as those used for the screening test. They were made up to the equivalent of 0.5 McFarland turbidity standard. They were incubated at 37^{o}C for 24 hours. There were three rows of tubes for each extract, since each plant was tested against three bacteria. The water control and ethanol control was included with each extract tested. Therefore there were five rows of 8 tubes per row for each extract that yielded zones of inhibition on the screening test. The MIC was regarded as the lowest concentration of the extract that did not permit any visible growth. The results were compared with the 0.5 McFarland standard as follows:

- No turbidity no growth
- 1+ turbidity scanty
- 2+ turbidity moderate
- 3+ turbidity dense

Minimum bactericidal concentration

The MBC was determined by taking tubes with no visible growth after 24 hours and streaking them onto fresh blood agar plates, incubating them at 37^{o}C for twenty four hours and examining for growth. The MBC was regarded as the lowest concentration of the extracts that prevented the growth of any bacterial colony on a solid medium. Each extract was tested six times with each bacterium.

3.4.19 PHYTOCHEMICAL CHARACTERISATION METHOD OF THE ACTIVE PLANTS.

Extracts prepared previously were used to detect the presence of *flavonoids* and *flavonols* in the leaves or bark of the active plants. Ethanol extracts and water extracts of the active plants were prepared using sufficient amounts of plant material. These extracts were filtered using a membrane filter. The resultant filtrate was then used to perform the Thin Layer Chromatography and High Pressure Liquid Chromatography.

Thin Layer Chromatography (TLC) (Harbone, 1984)

Thin Layer Chromatography plates were run using BAW: n-Butanol (80 ml); Acetic acid (20 ml); Water (50 ml) as the mobile phase. Aliquots of prepared extracts of the active plant were spotted onto 20x20 cm silica gel plates. The plates were allowed to run for ±4 hours in BAW and allowed to dry. Spots that were visible under normal light and ultraviolet light (254 and 366 nm) were demarcated. *Quercetin* was used as the standard. The other aliquots of plant extracts were also run on the TLC plate using methanol (98 ml): Water (2 ml) as the mobile phase. The standard used on this plate was *catechin*. The bands that are formed were compared to the *catechin* standard. The spray used for *quercetin* identification was 0.5ml anisaldehyde, 10ml glacial acetic acid and 5ml concentrated sulphuric acid in 85ml methanol and the spray used for *catechin* identification was 1ml concentrated sulphuric acid, 1ml acetic anhydride and 50ml chloroform. The Rf value was regarded as the distance leading edge of component moves divided by the total distance the solvent front moves.

High Pressure Liquid Chromatography (HPLC)

Harbone (1984) stated that HPLC is mainly used for those classes of compounds that are non-volatile, e.g. higher *terpenoids*, *phenolics* of all types, alkaloids, lipids and sugars. The chromatographic system used was the Beckman HPLC system consisting of a double pump. Programmable solvent Module 126, Diode Array Detector module 168, with 32 Karat Gold software supplied with Beckman, Column C18 Bondapak 5 μm dimension (250 x 4.6mm). The chromatographic conditions were as follows:

Mobile phase was Acetonitrile (80): water (20) for *quercetin* and methanol (4): 1 percent Acetic acid in water (1) for *catechin.* Flow rate: 0.9 ml/min; Reference standard: *quercetin* and *catechin* 2.0 g dissolved in 10 ml methanol; Sample size: initial extract in the assay was 25g/100 ml. Injected volume was 5 µl. The run time was 30 min. The retention time for each standard was then compared to the retention time for the ethanolic extracts of plants.

3.4.20 STATISTICAL PROCEDURES

Water and ethanolic extracts from each plant were tested six times with each bacterium. In total, the experiment was repeated 36 times for each plant. For each replication the zone of inhibition produced, MIC and MBC were recorded. The response of each bacterium was also coded: growth or no growth. The number of replicates was determined in consultation with the statistician.

A Fisher's exact test was used to compare the number of replications that respond (grow) among the three bacterium in each extract (ethanol and water) separately. Where a bacterium responded, the response was compared between the two extracts using Fisher's exact test to determine which extract was the more effective. If there was no difference between the extracts, the data was pooled. The analysis was repeated for each plant.

A second analysis was comparing the zones of inhibitions measured in millimetres using a Kruskal Wallis test because of the small numbers of samples. The comparisons followed the same analysis plan as the bacterial response. The size of the zone of the zone of inhibition between the three bacteria was compared first. Where possible, the zones for each bacterium were compared between the two extracts. If there was no difference the extracts were pooled and the plants were compared.

3.5 CONCLUSION

The methods used in this study were able to provide answers to the questions about the antibacterial nature of the plants. On the screening tests the zones of inhibition were observed and the minimum inhibitory concentrations for each plant against each bacteria were performed. TLC and HPLC techniques were used for the identification of some active antibacterial compounds.

CHAPTER FOUR

RESULTS

The tables of the data for the antibacterial screening test are displayed in Appendix A and the statistical tables for the minimum inhibitory concentrations are also displayed in Appendix A. Other tables appearing in Appendix A are the tables for the identification of two antibacterial compounds using TLC and HPLC.

4.1 CRITERIA GOVERNING THE ADMISSIBILITY OF DATA

Only data obtained from the experiments carried out by the researcher at the Mangosuthu University of Technology in the department of Biomedical Technology were included in the statistical analysis. It was hypothesised that *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* extracts will have antimicrobial effects on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.* It was further hypothesised that both water-based and ethanol extracts will exhibit antibacterial properties against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.* It was also thought that if *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* possess antibacterial effects on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, then antibacterial compounds are present in these plants.

4.2 EFFECTS OF *A. AFRA* WATER BASED EXTRACTS VERSUS WATER CONTROL ON *E. COLI, P. AURUGINOSA* AND *S. AUREUS*

The *A. afra* water–based extracts and the water control did not have any antimicrobial effect on *E. coli*, *P. aeruginosa* and *S. aureus*. This would be evident by the zone of inhibition (Table 1) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Table 1: Zones of inhibition for *A. afra* water-based extract against bacteria

	E. coli	***P. aeruginosa***	***S. aureus***
Water control	0mm	0mm	0mm
A. afra water extract	0mm	0mm	0mm

4.3 EFFECTS OF *E. LYSISTEMON* WATER BASED EXTRACTS VERSUS WATER CONTROL ON *E. COLI, P. AERUGINOSA* AND *S. AUREUS*

The *E. lysistemon* water based extract and the water control did not have any antimicrobial effects on *E. coli, P. aeruginosa* and *S. aureus* as this would be shown by the zones of inhibition (Table 2) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Table 2: Zones of inhibition for *E. lysistemon* water-based extract against bacteria

	E. coli	***P. aeruginosa***	***S. aureus***
Water control	0mm	0mm	0mm
E. lysistemon water extract	0mm	0mm	0mm

4.4 EFFECTS OF *P. GUAJAVA* WATER–BASED EXTRACT VERSUS WATER CONTROL ON *E. COLI, P. AERUGINOSA* AND *S. AUREUS*

The *Psidium guajava* water based extract versus water control on *E. coli, P. aeruginosa* and *S. aureus* did not have any antimicrobial activity as this would be shown by the zones of inhibition (Table 3) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Table 3: Zones of inhibition for *P. guajava* water-based extract against bacteria

	E. coli	***P. aeruginosa***	***S .aureus***
Water control	0mm	0mm	0mm
P. guajava water extract	0mm	0mm	0mm

4.5 EFFECTS OF *A. AFRA* TINCTURE IN 60 PERCENT ETHANOL VERSUS 60 PERCENT ETHANOL CONTROL ON *E. COLI, P. AERUGINOSA* AND *S. AUREUS*

4.5.1 STATISTICAL ANALYSIS OF RESULTS

	E. coli	*P. aeruginosa*	*S. aureus*
P.value	0.8	0.09	0.003

Interpretation of results for *E. coli*

$\alpha = 0.05$

$P = 0.8$

The null hypothesis (H_O) is accepted since $P \geq \alpha$. Thus there is no significant difference in diameter of the zones of inhibition between *A. afra* ethanol tincture and 60 percent ethanol control on *E. coli*. This is also shown by the zones of inhibition (Table 4) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Table 4: Zones of inhibition for *A. afra* (60%) ethanol tincture against bacteria

	E. coli	***P. aeruginosa***	***S. aureus***
Ethanol control	2mm	2mm	1mm
A. afra (60%) ethanol tincture	2mm	2mm	6mm

Interpretation of results for *P. aeruginosa*

$\alpha = 0.05$

$P = 0.09$

The null hypothesis (H_O) is accepted since $P \geq \alpha$. Thus there is no significant difference in diameter of zones of inhibition between *A. afra* ethanol tincture and 60 percent ethanol control on *P. aeruginosa.* This is also shown by the zones of inhibition (Table 4) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Interpretation of results for *S. aureus*

$\alpha = 0.05$

$P = 0.003$

The alternative hypothesis (H_1) is accepted since $P \leq \alpha$. Thus there is significant difference in diameter of zones of inhibition between *A. afra* ethanol tincture and 60 percent ethanol control on *S. aureus.* This is also shown by the zones of inhibition (Table 4) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

4.6 EFFECTS OF *E. LYSISTEMON* TINCTURE IN 60 PERCENT ETHANOL VERSUS 60 PERCENT ETHANOL CONTROL ON *E. COLI, P. AERUGINOSA* AND *S. AUREUS*

4.6.1 STATISTICAL ANALYSIS OF RESULTS

	E. coli	*P. aeruginosa*	*S. aureus*
P.value	0.11	0.007	0.005

Interpretation of results for *E. coli*

$\alpha = 0.05$

$P = 0.11$

The null hypothesis (H_O) is accepted since $P \geq \alpha$. Thus there is no significant difference in diameter of the zones of inhibition between *E. lysistemon* ethanol tincture and 60 percent ethanol control on *E. coli*. This is also shown by the zones of inhibition (Table 5) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Table 5: Zones of inhibition for *E. lysistemon* (60%) ethanol tincture against bacteria

	E. coli	***P. aeruginosa***	***S. aureus***
Ethanol control	2mm	2mm	1mm
E. lysistemon (60%) ethanol tincture	2mm	1mm	4mm

Interpretation of results for *P. aeruginosa*

$\alpha = 0.05$

$P = 0.007$

The alternative hypothesis (H_1) is accepted since $P \leq \alpha$. Thus there is significant difference in diameter of zones of inhibition between *E. lysistemon* ethanol tincture and 60 percent ethanol control on *P. aeruginosa*. This is also shown by the zones of inhibition (Table 5) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Interpretation of results for *S. aureus*

$\alpha = 0.05$

$P = 0.005$

The alternative hypothesis (H_1) is accepted since $P \leq \alpha$. Thus there is significant difference in diameter of zones of inhibition between *E. lysistemon* ethanol tincture and 60 percent ethanol control on *S. aureus.* This is also shown by the zones of inhibition (Table 5) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

4.7 EFFECTS OF *P. GUAJAVA* TINCTURE IN 60 PERCENT ETHANOL VERSUS 60 PERCENT ETHANOL CONTROL ON *E. COLI, P. AERUGINOSA* AND *S. AUREUS*

4.7.1 STATISTICAL ANALYSIS OF RESULTS

	E. coli	*P. aeruginosa*	*S. aureus*
P.value	0.002	0.001	0.003

Interpretation of results for *E. coli*

$\alpha = 0.05$

$P = 0.002$

The alternate hypothesis (H_1) is accepted since $P \leq \alpha$. Thus there is significant difference in diameter of the zones of inhibition between *P. guajava* ethanol tincture and 60 percent ethanol control on *E. coli*. This is also shown by the zones of inhibition (Table 6) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Table 6: Zones of inhibition for *P. guajava* (60%) ethanol tincture against bacteria

	E. coli	***P.aeruginosa***	***S.aureus***
Ethanol control	2mm	2mm	1mm
P. guajava (60%) ethanol tincture	0mm	0mm	5mm

Interpretation of results for *P. aeruginosa*

$\alpha = 0.05$

$P = 0.001$

The alternative hypothesis (H_1) is accepted since $P \leq \alpha$. Thus there is significant difference in diameter of zones of inhibition between *P. guajava* ethanol tincture and 60 percent ethanol control on *P. aeruginosa*. This is also shown by the zones of inhibition (Table 6) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

Interpretation of results for *S. aureus*

$\alpha = 0.05$

$P = 0.003$

The alternative hypothesis (H_1) is accepted since $P \leq \alpha$. Thus there is significant difference in diameter of zones of inhibition between *P. guajava* ethanol tincture and

60 percent ethanol control on *S. aureus*. This is also shown by the zones of inhibition (Table 6) produced in the Kirby Bauer Antimicrobial Sensitivity Test.

4.8 MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC)

The MICs and MBCs were performed on ethanolic extracts of *P. guajava, E. lysistemon* and *A. afra* since they all gave zones of inhibition against *S. aureus.*

4.8.1 STATISTICAL ANALYSIS OF RESULTS FOR *A. AFRA* (1 IN 2) DILUTION

The MIC and MBC for a 1 in 2 dilution *A. afra* tincture against *S. aureus* was 83.33 percent no growth and 16.67 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is presented in Table 7.

Table 7: Statistical analysis of results for *A. afra* (1 in 2) dilution

	Growth (G)	**No Growth (NG)**	**Total**
60% ethanol control	100%	0%	100%
A.afra (60%) ethanol	16.67%	83.33%	100%

4.8.2 STATISTICAL ANALYSIS OF RESULTS FOR *A. AFRA* (1 IN 4) DILUTION

The MIC and MBC for a 1 in 4 dilution *A. afra* tincture against *S. aureus* was 67.67 percent no growth and 33.33 percent growth when compared to the 100 percent growth for the 60 percent ethanol control as it is shown in Table 8.

Table 8: Statistical analysis of results for *A. afra* (1 in 4) dilution

	Growth (G)	**No Growth (NG)**	**Total**
60% ethanol control	100%	0%	100%
A.afra (60%) ethanol	33.33%	66.67%	100%

4.8.3 STATISTICAL ANALYSIS OF RESULTS FOR *A. AFRA* (1 IN 8) DILUTION

The MIC and MBC for a 1 in 8 dilution *A. afra* tincture against *S. aureus* was 33.33 percent no growth and 66.67 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 9.

Table 9: Statistical analysis of results for *A. afra* (1 in 8) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
A. afra (60%) ethanol	66.67%	33.33%	100%

4.8.4 STATISTICAL ANALYSIS OF RESULTS FOR *A. AFRA* (1 IN 16) DILUTION

The MIC and MBC from a 1 in 16 dilution to 1 in 258 dilution of *A. afra* tincture against *S. aureus* was 0 percent no growth and 100% percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 10.

Table 10: Statistical analysis of results for *A. afra* (1 in 16) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
A.afra (60%) ethanol	100%	0%	100%

4.8.5 STATISTICAL ANALYSIS OF RESULTS FOR *P. GUAJAVA* (1 IN 2) TO 1 IN 32 DILUTION

The MIC and MBC for a 1 in 2 dilution to 1 in 32 dilution of *P. guajava* tincture against *S. aureus* was 100 percent no growth and 0 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 11.

Table 11: Statistical analysis of results for *P. guajava* (1 in 2) to (1 in 32) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
P. guajava (60%) ethanol	0%	100%	100%

4.8.6 STATISTICAL ANALYSIS OF RESULTS FOR *P. GUAJAVA* (1 IN 64) TO 1 IN 128 DILUTION

The MIC and MBC for a 1 in 64 dilution to 1 in 128 dilution of *P. guajava* tincture against *S. aureus* was 66.67 percent no growth and 33.33 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 12.

Table 12: Statistical analysis of results for *P. guajava* (1 in 64) to (1 in 128) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
P.guajava (60%) ethanol	33.33%	66.67%	100%

4.8.7 STATISTICAL ANALYSIS OF RESULTS FOR *P. GUAJAVA* (1 IN 256) DILUTION

The MIC and MBC for a 1 in 256 dilution *P. guajava* tincture against *S. aureus* was 0 percent no growth and 100 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 13.

Table 13: Statistical analysis of results for *P. guajava* (1 in 256) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
P. guajava (60%) ethanol	100%	0%	100%

4.8.8 STATISTICAL ANALYSIS OF RESULTS FOR *E. LYSISTEMON* (1 IN 2) DILUTION TO (1 IN 8) DILUTION

The MIC and MBC for a 1 in 2 dilution to 1 in 8 dilution of *E. lysistemon* tincture against *S. aureus* was 100 percent no growth and 0.00 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 14.

Table 14: Statistical analysis of results for *E. lysistemon* (1 in 2) to (1 in 8) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
E .lysistemon (60%) ethanol	0%	100%	100%

4.8.9 STATISTICAL ANALYSIS OF RESULTS FOR *E. LYSISTEMON* (1 IN 16) DILUTION TO (1 IN 32) DILUTION

The MIC and MBC for a 1 in 16 dilution to 1 in 32 dilution of *E. lysistemon* tincture against *S. aureus* was 83.33 percent no growth and 16.67 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 15.

Table 15: Statistical analysis of results for *E. lysistemon* (1 in 16) to (1 in 32) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
E. lysistemon (60%) ethanol	16.67%	83.33%	100%

4.8.10 STATISTICAL ANALYSIS OF RESULTS FOR *E. LYSISTEMON* (1 IN 64) DILUTION

The MIC and MBC for a 1 in 64 dilution of *E. lysistemon* tincture against *S. aureus* was 16.67 percent no growth and 83.33 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 16.

Table 16: Statistical analysis of results for *E. lysistemon* (1 in 64) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
E. lysistemon (60%) ethanol	83.33%	16.67%	100%

4.8.11 STATISTICAL ANALYSIS OF RESULTS FOR *E. LYSISTEMON* (1 IN 128) DILUTION TO (1 IN 256) DILUTION

The MIC and MBC for a 1 in 128 dilution to 1 in 256 dilution of *E. lysistemon* tincture against *S. aureus* was 0.00 percent no growth and 100 percent growth when compared to the 100 percent growth for the 60 percent ethanol control. This is shown in Table 17.

Table 17: Statistical analysis of results for *E. lysistemon* (1 in 128) to (1 in 256) dilution

	Growth (G)	No Growth (NG)	Total
60% ethanol control	100%	0%	100%
E. lysistemon (60%) ethanol	100%	0.00%	100%

4.9 IDENTIFICATION OF THE ACTIVE COMPOUND

An attempt was made to isolate two active compounds that are responsible for the inhibition of bacterial growth. Developed TLC plates were first viewed under UV 254 nm light, sprayed with anisaldehye reagent and sulphuric acid: acetic anhydride: chloroform reagent and again viewed under the same wavelength. The presence of

flavonoids was detected in the form of visible spots on TLC plates. Spots similar to *quercetin* standard on one plate and *catechin* standard on another plate were observed. The R_F value of the *quercetin* standard was 0.91 and that of the sample was also 0.91. After spraying the plate the band of *quercetin* standard was yellow and it was similar to one of the bands of *P. guajava* tincture test sample. On another plate, the R_F value of *catechin* standard was 0.86 and that of the sample was also 0.86 (Table 18). After spraying this plate, the band of *catechin* standard was brown-yellow and it was similar to one of the bands of *P. guajava* tincture test sample. These spots were not observed for both *A.afra* extracts and *E.lysistemon* extracts.

Table 18: Results of Thin Layer Chromatography

Compound	Standard	*A. afra*	*E. lysistemon*	*P. guajava*
Quercetin	Rf value = 0.91	-	-	Rf value = 0.91
Catechin	Rf value = 0.86	-	-	Rf value = 0.86

The bands that were produced from the TLC plate were scraped off from the silica gel plates. They were then resuspended on 60% ethanol. There were tested against *S. aureus*, *P. aeruginosa* and *E. coli* for their antibacterial activity. The zones of inhibition were produced against *S. aureus*. Zones were 6mm for *quercetin* and 5mm for *catechin*. To verify the TLC results, the extracts were examined by HPLC. The active peak (Rt 2.42min and Rt 2.47min) were identified as *catechin* and *quercetin*, by comparison with the retention time for *catechin* standard and *quercetin* standard (Table 19).

Table 19: Retention times of the compounds separated by HPLC

Compound	Standard	*A. afra*	*E. lysistemon*	*P. guajava*
Quercetin	Retention time = 2.33	-	-	Retention time = 2.47
Catechin	Retention time = 2.43	-	-	Retention time = 2.42

4.10 CONCLUSION

The results in this study indicated that the water extracts of *A. afra, E. lysistemon* and *P. guajava* plants were not antibacterial active against *E. coli, P. aeruginosa* and *S. aureus*. The ethanolic extracts do not possess any antibacterial activity against *E. coli*

and *P. aeruginosa*. The antibacterial activity demonstrated by *A. afra*, *E. lysistemon* and *P. guajava* was against *S. aureus*. The activity of each plant against *S. aureus* was not the same. *P. guajava* was more active with the larger zone of inhibition. This was confirmed by the minimum inhibitory concentration which was higher for *P. guajava* than the other two plants. The two compounds that were identified were *catechin* and *quercetin* from *P. gaujava* ethanolic extracts. These two compounds are antibacterial.

CHAPTER FIVE
DISCUSSION

A total of two extracts for each of the plants were tested against each of the three bacterial strains. Two of the bacterial strains were Gram-negative and one was Gram-positive. Extracts were obtained from the leaves of *A. Lysistemon* and *P. Guajava* and from the bark of *E. Lysistemon*. The water extracts of *A. afra, E. lysistemon* and *P. guajava* did not exhibit any antibacterial activity against any of the bacteria tested. According to Fischer's exact test there was no P-value because, for both water control and water-based extracts there were no zones of inhibition produced.

Ethanol extracts of the three plants did not exhibit any antimicrobial activity against *E. coli* and *P. aeruginosa* in this study. The p-value for *A. afra* ethanol tincture against *E. coli* was 0.8 and 0.09 against *P. aeruginosa*. For *E. lysistemon* the P-value was 0.11 against *E. coli* and was 0.007 on *P. aeruginosa*. This p-value against *P. aeruginosa* did not indicate that this plant is effective, but that it was due to the fact that the ethanol control produced bigger zones of inhibition than *E. lysistemon* ethanol tincture. For *P. guajava* the p-values were 0.002 and 0.001 on *E. coli* and *P. aeruginosa* respectively. This was also due to the fact that the ethanol control produced bigger zones of inhibition than *P. guajava* ethanol tincture.

The method of extraction and the solvent used did influence the resultant antimicrobial activity of *A. afra*, *E. lysistemon* and *P.guajava* in this study. All three plants utilised for this study demonstrated the presence of antimicrobial activity. This experiment was able to produce zones of inhibition against *S. aureus,* which is a Gram-positive bacterium. It did not inhibit the growth of *E. coli* and *P. aeruginosa*. The negative results obtained against *E. coli* and *P. aeruginosa* support the fact that Gram-negative bacteria are more resistant, probably because of their thick murein layer which prevents the entry of inhibitors (Martin, 1995). Traditionally, most plant extracts are prepared with water. In this study the ethanol extract produced zones of inhibition, but water extracts did not produce any inhibition zones. Since the

antibacterial activity was shown to be high in ethanol extracts, it means that it is likely that most traditional healers do not sufficiently extract the compounds, which are responsible for antibacterial activity on these bacteria. This could mean that water failed to extract sufficient concentration of antibacterial compounds to produce any effect on bacteria used in this study. The metabolic effects occuring *in vivo* could also activate certain compounds in the body. These metabolic processes depend on temperature, pH and other factors present *in vivo* but absent in vitro.

The zones of inhibition were different for each plant, indicating that the activity of the plants against bacteria, especially *S.aureu* is not the same. This was supported by the difference in the MICs and MBCs for *A. afra, E. lysitemon* and *P. guajava* on *S. aureus*. The minimum inhibitory concentration and minimum bactericidal concentration for *A. afra* indicated that the most effective dilution against *S. aureus* is 1 in 4 with 83.33 percent effectivity and the least effective dilution is 1 in 8 with 33.33 percent effectivity. For *E. lysistemon* the most effective dilution is 1 in 32 with 83.33 percent inhibition of growth and the least effective dilution being 1 in 64 with 16.67 percent inhibition of growth. Lastly the minimum inhibitory concentration and minimum bactericidal concentration for *P. guajava* demonstrated that the most effective dilution that is against *S. aureus* is 1 in 32 with 100 percent no growth and the least effective dilution was 1 in 128 with 66.67 percent no growth.

Using TLC and HPLC techniques, two active compounds were identified as *quercetin* and *catechin*. It was discovered that these two compounds are not dissolved completely in water and as a result, the controls were prepared using 60 percent ethanol. These two compounds were isolated from the ethanolic tincture of *Psidium guajava* and they were not present on *E. lysistemon* and *A. afra*. This demonstrates that there are other compounds responsible for antibacterial activity in *E. lysistemon* and *A. afra*. The other observation is that the water-based extract of these plants did not possess the two compounds. It is not surprising that there are differences in the antimicrobial effects of plant species, due to the phytochemical properties and differences among species. It is quite possible that some of the plants that were ineffective in this study do not possess antibiotic properties, or the plant

extracts may have contained antibacterial constituents, but not in sufficient concentrations so as to be effective. The fact that contamination of *E. lysistemon* aqueous extract by *E. coli* and *Klebsiela* species which are both gram-negative bacteria, demonstrate that this extract does not posess or posesses poor antibacterial activity against gram-negative organisms. It is also possible that the active chemical constituents were not soluble in ethanol or water (Pakekh and Chanda, 2007). However, even though the activity against *S. aureus* is providing evidence of biological activity, the plant extracts still warrant further investigations.

Prior to the study, the pilot study comparing the dried and fresh plants was performed. The association between the state (dried or fresh) and the zone of inhibition was significant. According to the Kruskal Wallis test the Chi-square was 0.001for *P. guajava*, and 0.035 for both *E. lysistemon* and *A. afra* against *S. aureus*. This is because the zones of inhibition were reduced in the dried plants for the ethanol extracts. This is a clear indication that dried are less active than fresh plants. In his study, Dr Lin has demonstrated that *P. guajava* can treat diarrhoea (Lin et al, 2002) . *S. aureus* is one of the organisms that cause food poisoning and this study has shown that *P. guajava* ethanolic extract can inhibit *S. aureus*. This could mean that if diarrhoea is due to *S. aureus*, it can be treated because of this antibacterial activity of *P. guajava* against *S. aureus*. It must be noted that diarrhoea can be caused by a wide range of bacteria.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The objectives of this study were to determine zones of inhibition produced, MICs and MBCs of two *A. afra* extracts on three bacteria, two *E. lysistemon* extracts on three bacteria and two *P. guajava* extracts on three bacteria. The other objective was to analyse different responses of bacteria tested to different extracts and also to compare the antibacterial activity of different extracts. It is evident from the results that the traditional plants used in this study possess antibacterial activity. It is also evident that different bacteria will not respond the same to the plant extract, although the plant has antibacterial activity. This means that some bacteria are resistant to the antibacterial activity of plant extracts while others are sensitive. The extraction technique is also vital for the maximum effect of the plant on bacteria.

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Iwu *et al*., 1999). Continued further exploration of plant-derived antimicrobials is needed today. Further research is necessary to determine the identity of the antibacterial compounds from these plants and also to determine their full spectrum of efficacy. However, the present *in vitro* antimicrobial evaluation of some plants forms a primary platform for further phytochemical and pharmacological studies.

6.2 RECOMMENDATIONS

This work can serve as the basis for future developments of antibiotics from the traditional plants. The herbs should be tested *in vivo* by means of clinical trials and they should also be tested for their toxicity to cells. Different parts of the plants should also be tested for antibacterial activity to a wide range of bacteria. Trials should be run with different types of extractants such as glycerine, vinegar and

acetone to see which is the most effective in extracting the active compounds of the three plants used in this study. With the use of column chromatography the compounds in each plant can be separated and made into a powder form. The concentration can be then determined using HPLC. This can then be tested against bacteria. Lodging of a voucher specimen has an advantage of verifying the plant material used in the experiment at a later stage, should the subsequent review of the experiment by other researchers take place.

The literature has indicated that there is synergism between plants but for the plants used in this study, it has not been investigated. This is another area that warrants further research as these plants are all antibacterial active against *S. aureus*.

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APPENDIX A

Calculation 1: Water-based extraction of *A. afra*

The weight of water from the plant was eliminated using the following calculation:

E_3 = 2MD ÷ 100 where M = plant weight (50g) and D = % of drying (74%)

Start weight = 5g

End weight = 1.3g

Water lost weight = 3.7g

Therefore % weight loss = 74%

Substitution in the formula = 2x50x74÷100

= 74ml of distilled water was added to 50g of minced plant

Calculation 2: Water-based extraction of *E . lysistemon*

The weight of water from the plant was eliminated using the following calculation:

E_3 = 2MD ÷ 100 where M = plant weight (70g) and D = % of drying (74.6%)

Start weight = 5g

End weight = 1.27g

Water lost weight = 3.73g

Therefore % weight loss = 74.6%

Substitution in the formula = 2x70x74.6÷100

= 104.4ml of distilled water was added to 70g of minced plant

Calculation 3: Water-based extraction of *P. guajava*

The weight of water from the plant was eliminated using the following calculation:

E_3 = 2MD ÷ 100 where M = plant weight (45g) and D = % of drying (67.5%)

Start weight = 4g

End weight = 1.35g

Water lost weight = 2.7g

Therefore % weight loss = 67.5%

Substitution in the formula = 2x45x67.5÷100

= 60.75ml of distilled water was added to 45g of minced plant

Calculation 4: Ethanol tincture preparation of *A. afra*

The weight of water from the plant was eliminated using the following calculation:

$E_3 = 2MD \div 100$where M = plant weight (50g) and D = % of drying (74%)

Start weight = 5g

End weight = 1.3g

Water lost weight = 3.7g

Therefore % weight loss = 74%

Substitution in the formula = 2x50x74÷100

= 74ml of 86% ethanol was added to 50g of minced plant

Calculation 5: Ethanol tincture preparation of *E. lysistemon*

The weight of water from the plant was eliminated using the following calculation:

$E_3 = 2MD \div 100$ where M = plant weight (50g) and D = % of drying (74%)

Start weight = 5g

End weight = 1.3g

Water lost weight = 3.7g

Therefore % weight loss = 74%

Substitution in the formula = 2x50x74÷100

= 104.4ml of 86% ethanol was added to 70g of minced plant

Calculation 6: Ethanol tincture preparation of *P. guajava*

The weight of water from the plant was eliminated using the following calculation:

$E_3 = 2MD \div 100$ where M = plant weight (50g) and D = % of drying (74%)

Start weight = 5g

End weight = 1.3g

Water lost weight = 3.7g

Therefore % weight loss = 74%

Substitution in the formula = 2x50x74÷100

= 60.75ml of 86% ethanol was added to 45g of minced plant