

**A controlled *in vitro* study of the effectiveness of
Alepidea amatymbica herbal tincture and homoeopathic
dilutions (D1 and D6) against Gram-positive and
Gram-negative bacteria**

by

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Degree in Technology: Homoeopathy in the Department of Health at the Durban Institute
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DEDICATION

I lovingly dedicate this work to my parents, Bert and Bev Williams, to whom I am eternally indebted.

I dare not try to express my gratitude, as any attempt will not convey the true extent of my feeling.

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ABSTRACT

The purpose of this study was to determine the efficacy of *Alepidea amatymbica* tincture and homoeopathic dilutions to the 1st and 6th decimal potency as compared to ethanol (negative control) in the *in vitro* inhibition of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus cereus* in terms of the disc diffusion test.

Vancomycin and gentamicin were included in the study as positive controls in order to account for plate-to-plate variations in the sensitivity of the bacterial strains to antimicrobial substances. Antimicrobial activity was expressed as the ratio of the inhibition zone (mm) produced by the test substance and the inhibition zone (mm) produced by the two combined antibiotic discs.

For this study 20 Mueller-Hinton agar plates were assigned to each bacterial species and were inoculated with their respective bacteria. Four dry filter paper discs and two antibiotic discs were placed equidistantly on each agar plate. The filter paper discs had been previously impregnated with one of the test substances or the negative control using a triple impregnation technique utilizing a micro-pipette. The plates were then incubated at 37° C. The diameters of the zones of inhibition were measured at 18 hours, 24 hours and 48 hours.

Data was analysed by means of the Statistical Package for Social Sciences (SPSS). Statistical methods utilized were Friedmans' test, Mann-Whitney U test, and Kruskal – Wallis Non-Parametric Analysis of Variance by Rank test.

The results showed that *Alepidea amatymbica* ethanol extract had a slight (non-significant) effect against the Gram-positive organisms, *Staphylococcus aureus* and *Bacillus cereus*, and no effect against the Gram-negative *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* or *Escherichia coli*. This was not surprising, as in general, Gram-negative bacteria are more resistant than Gram-positive bacteria (Rabe & van Staden, 1997). It was found that the homoeopathic dilutions of *Alepidea amatymbica* had no effect on any of the bacterial species.

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THE DEFINITION OF TERMS

◦ **Decimal Potency:**

Is based on the principle that the first potency should contain one tenth part of the homoeopathic base drug and each succeeding potency should contain one tenth part of the immediately preceding. The decimal potency is denoted by suffixing D (sometimes the Roman digit 'X' for 'decem' [ten] is used instead) to the numerals denoting the deconcentration stage of the drug. (Gaier, 1991:447.)

◦ **Decoction:**

The process of boiling down in order to concentrate a mixture. This is opposed to infusion, where the medicinal substance steeps in hot water or liquid. (Yasgur, 1998: 63.)

◦ **Infusion:**

A medicinal brew. Prepared by plant material being steeped in freshly boiled water (Gaier, 1991: 285).

◦ **Inoculum:**

A sample of a microorganism culture that is used to start a new culture (Ross, 1986:543).

◦ **In vitro:**

Refers to a biological reaction occurring in a laboratory apparatus. (Glanze *et al.*, 1990:645).

- ***In vivo:***

Refers to a biological reaction occurring in a living organism (Glanze *et al.*, 1990:645).

- **Potency:**

The stage of altered remedial activity to which a drug has been taken by means of a measured process of deconcentration, with succussion, or by trituration, of the medicinal substance, which is thus brought to a state of diminutive or infinitesimal subdivision (Gaier, 1991:432).

- **Potentisation:**

Is a physical process through which latent curative powers of medicines are aroused into activity, though these may have been inevident in their crude states. By this process quantitative deconcentration of drug substance occurs as qualitative increment takes place. (Gaier, 1991:444.)

- **Purgative:**

An agent which causes catharsis or movement of the bowels (Yasgur, 1992:117).

- **Succussion:**

To hit against a resilient object such as a leather bound book or the palm of your hand (Murphy, 1994:334).

- **Tincture:**

An alcoholic or water-alcohol solution, usually referring to a preparation from herbal materials (Blumenthal *et al.*, 1998:639).

- **Tonic:**

Invigorating, a remedy that enhances enfeebled functions and promotes well-being

(Gaier, 1991:556).

CHAPTER ONE

INTRODUCTION

Culturally, Africa would appear to be an extremely heterogenous continent with well over 2000 distinct tribes and as many languages. However, certain common threads run through most traditional medical systems in the continent, most particularly the near total reliance on plants for sources of ingredients for the formulation of remedies. (Iwu, 1994.)

One in four prescription medicines has an active ingredient originally derived from a plant source. Most of these plant-derived drugs were originally discovered through the study of traditional cures and folk knowledge of indigenous peoples - the ethnobotanical approach. (Balick and Cox, 1996:25.)

Many ethnobotanical surveys conducted in Africa do not record the general relationship between the local communities and plants, but are aimed at discovering whether any of the plants contain chemicals for development as drugs for European medicine. (Iwu, 1994.) This does at least bring the needed exposure that ethnobotanical research deserves.

Ethnobotany is becoming increasingly important as drug-resistant strains of bacteria are on the rise. There is thus an urgent need for new, inexpensive drugs that will be able to act for longer periods before resistance sets in. (McGaw, Jager & van Staden, 2000.) Antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains. (Eloff, 1998:2.)

The World Health Organization estimates that 80% of people living in developing countries almost exclusively use traditional medicine. This means that in the order of 3,3 billion people use medicinal plants on a regular basis. Medicinal plants used in traditional medicine should therefore be studied for safety and efficacy. (Eloff, 1998:2.)

In today's global economy, indigenous peoples are vulnerable to rapid economic and cultural change. Understanding of traditional ways, including uses of plants, can point to strategies for ameliorating negative consequences of that change. The study of ethnobotany and indigenous knowledge systems, developed over centuries, can teach us a lot about conservation of natural resources. (Balick & Cox, 1996:7.)

Highlighting the medicinal powers of plants will lead to greater awareness of their preservation. *Alepidea amatymbica* has a protected conservation status. The plant is presently common, but populations are dwindling as the habitat of the species is steadily declining (Mander *et al.*, 1995: 15). There is a lot to be learned regarding conservation of flora from ethnobotanists and traditional healers, who in order to sustain the use of their flora, follow certain rituals while collecting their medicines. An example of this would be that when collecting bark, they do not ring-bark the trees, but only take bark from one side of the tree. Failure to follow these rituals will render the medicines collected ineffective. (Mavi, 1994.)

With the increasing acceptance of traditional medicine as a valid form of health care, the screening of medicinal plants for active compounds is very important. Southern Africa contains roughly 10% of the world's plant diversity, but relatively little chemical work has been done on medicinal plants from this region. (Eloff, 1998:2.)

The purpose of this study was to investigate a South African plant, *Alepidea amatymbica* (*A. amatymbica*), for potential antibiotic activity in relation to *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*Ps. aeruginosa*), *Bacillus cereus* (*B. cereus*), *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). As far as can be determined, no laboratory research on the antimicrobial possibilities of this plant has been conducted before.

A. amatymbica is used in traditional medicine to treat colds, coughs (the root is sucked for relief), rheumatism, wounds, and to wash divining bones (Pooley, 1998:160). The plant is a Zulu remedy for colds and the Xhosa at one time used the plant in abdominal disorders. A root decoction is a widespread African remedy for stomach complaints and rheumatism. (Watt & Breyer-Brandwijk, 1962:1032.)

The study was conducted *in vitro* using the disc diffusion test (Capuccino & Sherman, 1992: 248). *A. amatymbica* as a herbal tincture and in the 1st and 6th decimal homoeopathic dilution was compared to ethanol (negative control) and vancomycin and gentamicin (positive controls). Antimicrobial activity was expressed as the ratio of the inhibition zone (mm) produced by the test substance to the inhibition zone (mm) produced by the two positive controls combined. The selection of the plant for evaluation was based on its traditional uses and literature surveys.

Staphylococci are widely distributed in the environment, being part of the normal microbial flora of the skin, upper respiratory tract, and intestinal tract. They are non-motile, non-capsulate, Gram-positive cocci of uniform size that occur characteristically in groups, but also singly and in pairs. (Cheesebrough, 1992:226.) They may cause

suppuration, abscess formation, a variety of pyogenic infections, and even fatal septicaemia (Jawetz *et al.*, 1991:194).

The genus *Bacillus* includes large aerobic, Gram-positive rods occurring in chains. Most members of this genus are saprophytic organisms prevalent in the soil, water, in air and on vegetation. (Jawetz *et al.*, 1991:180.) *B. cereus* has been found in a variety of infections, mostly in injured, debilitated or immunocompromised subjects, e.g. in abscess after injury (Mackie & McCartney, 1996:318).

E. coli is a Gram-negative, aerobic bacterium that occurs as a natural commensal in the intestinal tract of humans, especially in the ileum and the colon (Greenwood, Slack & Peutherer, 1992:323). From their normal site in the human body *E. coli* are able to cause frequent opportunistic infections, including urinary infections, wound infections, bacteraemias and diarrhoeal disease (Cheesebrough, 1992:254; Mackie & McCartney, 1996:364-365).

Klebsiellae are Gram-negative, non-motile, capsulate rods that usually produce large mucoid colonies when cultured on blood and Macconkey agar (Cheesebrough, 1992:263). *K. pneumoniae* is present in the respiratory tract and faeces of about 5% of normal individuals and infections occur most notably in the respiratory and urinary tracts (Jawetz *et al.*, 1991:217).

Ps. aeruginosa is a Gram-negative rod, non-sporing, non-capsulate, and usually motile by virtue of one or two polar flagella (Greenwood, Slack & Peutherer.1992: 345-349). It is widely distributed in nature and is commonly present in moist environments and

hospitals (Jawetz et al., 1991:224). *Ps. aeruginosa* has been implicated in skin, urinary and respiratory infections and septicaemic states (Cheesebrough, 1992:264).

Common treatment regimes for infection with any one of these bacterial species, usually involves the use of either gentamicin or vancomycin, both of which have serious side effects (Mims et al., 1998:419). This is just one of the many reasons that safer alternatives in the form of phytotherapy should be sought.

1.1 STATEMENT OF THE RESEARCH OBJECTIVES

The purpose of this study was to determine the efficacy of *A. amatymbica* in the inhibition of *in vitro* growth of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc diffusion test.

1.2 OBJECTIVES

1.2.1 The First Objective

To compare the efficacy of *A. amatymbica* tincture in 62% v/v ethanol against 62% v/v ethanol only (negative control) in the inhibition of *in vitro* growth of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc diffusion test.

1.2.2 The Second Objective

To compare the efficacy of *A. amatymbica* tincture in 62% v/v ethanol against *A. amatymbica* D1 in 62% v/v ethanol, in the inhibition of the *in vitro* growth of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc diffusion test.

1.2.3 The Third Objective

To compare the efficacy of *A. amatymbica* tincture in 62% v/v ethanol against *A. amatymbica* D6 in 62% v/v ethanol, in the inhibition of the *in vitro* growth of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc diffusion test.

1.2.4 The Fourth Objective

To compare the efficacy of *A. amatymbica* D1 in 62% v/v ethanol against 62% v/v ethanol only (negative control), in the inhibition of the *in vitro* growth of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc diffusion test.

1.2.5 The Fifth Objective

To compare the efficacy of *A. amatymbica* D6 in 62% v/v ethanol against 62% v/v ethanol only (negative control), in the inhibition of the *in vitro* growth of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc diffusion test.

1.3 GENERAL HYPOTHESIS

A. amatymbica herbal tincture as well as the 1st and 6th homoeopathic dilutions thereof, will have no antibacterial activity against *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* in terms of the disc-diffusion test.

1.4 THE DELIMITATIONS

1.4.1 This research project was limited to only five bacterial species, namely, *E. coli*, *Ps. aeruginosa*, *K. pneumoniae*, *B. cereus* and *S. aureus*.

1.4.2 This research project was limited to one species of the genus *Alepidea*, namely, *amatymbica*.

1.4.3 Only the following growth medium was used:

Mueller-Hinton Agar (Oxoid).

1.4.4 Only *A. amatymbica* tincture and homoeopathic dilutions (D1 and D6) were used as test substances.

1.4.5 This was an *in vitro* study.

1.5 THE ASSUMPTIONS

1.5.1 THE FIRST ASSUMPTION

All cultures of *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus* and *B. cereus* were grown under optimal conditions.

1.5.2 THE SECOND ASSUMPTION

The manufacture of *A. amatymbica* tincture and homoeopathic dilutions was in accordance with the German Homoeopathic Pharmacopoeia (GHP) method of preparation.

CHAPTER TWO

LITERATURE REVIEW

2.1 ETHNOBOTANY

Historically, the field of ethnobotany has belonged to the explorers and adventurers of Europe who observed and documented the uses of plants by the aboriginal peoples they encountered on their travels (Cotton, 1996), but the American Botanist John W. Harshberger coined the term 'ethnobotany' in 1895 to describe studies of "plants used by primitive and aboriginal people". This refers to people who follow traditional, nonindustrial lifestyles in areas that they have occupied for generations. (Balick & Cox, 1996.) Since then, the definition has been a topic of debate and is constantly being modified as it encompasses more and more disciplines within the field (Cotton, 1996).

In broad terms, 'ethnobotany' is the study of the relationship between plants and people. The study of the interactions of people and plants, including the influence of plants on human culture, is the focus of the interdisciplinary field of ethnobotany. The interests of ethnobotanists range from the functioning of indigenous healing systems to the imbibing of plants in rituals, from the cultural consequences of the extinction of a vine used to construct fish traps to the health consequences of a change in diet, from the class implications of forms of dress to the cultural role of body adornment. (Balick & Cox, 1996:3-7.)

In the African world, the natural environment is a living entity, whose components - the land, sea, atmosphere, and the faunas and floras - are intrinsically bound to humans. Plants therefore play a participatory role in healing (Iwu, 1994.) This scenario presents a wealth of opportunity for investigation.

Indigenous cultures retain much knowledge concerning plants that Western peoples have largely lost. They have of necessity maintained knowledge of plant medicines (Balick & Cox, 1996) and it is for this reason that ethnobotanical leads should not be ignored.

Paul Cox, a botanist who has done extensive research into the pharmacological activity of Samoan medicinal plants, lists 50 plant-derived drugs, which have been discovered on the basis of ethnobotanical leads. The list includes aspirin (from *Filipendula ulmaria*), digoxin (from *Digitalis purpurea*), morphine (from *Papaver somniferum*) and quinine (*Cinchona pubescens*). (Cotton, 1996.) These drugs are highlighted as examples of the medicinal potential that ethnobotanical studies have to offer.

Medicinal plants have become the focus of intense study recently in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore (Rabe & van Staden, 1997). The study being conducted here arises from the use of *A. amatymbica* within indigenous healing systems of Southern Africa, and is intended as a contribution to the development of a scientific knowledge base of indigenous medicinal plants.

2.1.1 ALEPIDEA AMATYMBICA

2.1.1.1 Family

A. amatymbica belongs to the Apiaceae (Umbelliferae) family (Van Wyk and Gericke, 2000:155), also known as the carrot family (Pooley, 1998:160).

2.1.1.2. Common names

It is known as *ikhathazo* by the Zulu (Van Wyk and Gericke, 2000:155). Other names include Giant Alepidea, Large Tinsel Flowers; Kalmoes, Slangwortel (Afrikaans); lesoko (South Sotho), inkatsankatsa (Swaziland) and iqwili (Xhosa) (Pooley, 1998:160).

2.1.1.3 Description

It is a robust, erect herb that grows up to 2 m in height. The stems are strongly grooved and leafy throughout. The flowering stalk is hollow with numerous small flowers arranged in dense, rounded heads. (Mander *et al.*, 1995:15.) The leaves are basal, approximately 300 x 100mm and not arranged in a rosette. This is important as it distinguishes it from *Alepidea natalensis* that does have its leaves in a basal rosette formation. The margins of the leaves are prominently toothed, each tooth ending in a bristle. (Pooley, 1998:160.)

The flowers are white and star-like (Mander *et al.*, 1995:15) and the flowerheads are approximately 20mm in diameter with 5 large, unequal bracts. The herb flowers from January to April. (Pooley, 1998:160).

There are two distinct forms of *A. amatymbica*: the typical form from the Eastern Cape, with leaves that gradually taper towards their bases and a northern form with distinctly heart-shaped leaves (Van Wyk, Van Oudtshoorn & Gericke, 1997:38). The rhizome is dark in colour and the crushed plant has a carrot-like smell and will stain hands a brown-orange colour (Mander *et al.*, 1995:15).

2.1.1.4 Habitat

This herb is found in grasslands and near streams, from the Cape to Zimbabwe, up to a height of 2100m above sea level (Pooley, 1998:160). It is found in the grasslands

throughout Kwazulu-Natal. This plant is presently common but populations are declining as the habitat of this species is being reduced. (Mander, *et al.* 1995:15.)

2.1.1.5 Parts used

The rhizome is used (Mander *et al.*, 1995:15).

2.1.1.6 Constituents

The rhizomes and roots contain very high concentrations (up to 27% dry weight) of several diterpenoids of the kaurene type. The major compounds are dehydrokaurenoic acids and kaurenoic acids, of which ent-16-kauren-19-oic acid is usually present in the greatest quantity. Screening tests indicate antimicrobial, antihypertensive and diuretic activity. (Van Wyk, Van Oudtshoorn & Gericke, 1997:38.)

2.1.1.7 Traditional uses and preparations

Van Wyk, Van Oudtshoorn and Gericke (1997:38) state that *A. amatymbica* is widely used for colds and chest complaints, including asthma, where an infusion is made together with *Cannabis sativa*. It is used as a drink or as an inhalant to treat chest ailments and is even used to prevent nervousness (Mander, *et al.*, 1995:15).

The plant is a Zulu remedy for colds (Watt & Breyer-Brandwijk, 1962:1032).

Van Wyk and Gericke (2000:155) state that the Zulu diviners smoke the dried rhizome or root, or take the powdered rhizome as snuff in order to communicate with the ancestors, or assist in divination. The rhizome is carried as a lucky charm, and is commonly used for colds, influenza and asthma (Van Wyk & Gericke, 2000:155).

The Zulu use an infusion of the root as an enema for children with coryza and cough. The Gauteng Zulus and the Swati drink a decoction of the root in influenza. The Xhosa use the plant in abdominal disorders, either powdered as an infusion, or as a tincture, for pains in the stomach. Large doses are purgative, small are tonic. A root decoction is a widespread African remedy for stomach complaints and rheumatism. (Watt & Breyer-Brandwijk, 1962:1032.)

In Zimbabwe, the tuberous root is used as a bee repellent, where a powder of the root is burnt and the smoke is blown into a beehive. The root is also used as a divination aid; to secure respect, prestige and security in one's job; as a lucky charm and to prevent witchcraft. (Gelfand *et al.*, 1993.)

2.2 ALEPIDEA AMATYMBICA D1 AND D6

In this study, *A. amatymbica* was diluted according to homoeopathic methodology in the ratio 1:10 to achieve the D1 and D6 dilution (see 3.3.4.2 and 3.3.4.3). This dilution process is known as potentisation, so called as it increases the therapeutic 'strength' of the substance, thus increasing its medicinal power. (Vithoulkis, 1986.)

This process, which is poorly understood, has no satisfactory 'scientific' explanation. One possible explanation for this phenomenon might be that the process of potentisation changes the electrochemical structure of the solvent molecules, thus 'memorising' an imprint of the original remedy. Its mechanism of action seeks explanations beyond the scope of conventional biochemical interpretations of orthodox drug action. (Kayne, 1997.)

Research conducted previously on the effect of homoeopathic dilutions on the growth of wheat, has produced statistically significant evidence that potentised substances do

have an effect on plant growth. Plants negate any possible placebo effect (Kayne, 1997) as do bacterial cultures. Any antibacterial activity produced by the homoeopathic dilution can be directly attributed to the action of the homoeopathic dilution, and not to a placebo effect, which is impossible with prokaryotic organisms as subject matter.

2.3 BACTERIA

2.3.1 STAPHYLOCOCCUS AUREUS

2.3.1.1 Morphology and identification

The staphylococci are Gram-positive spherical cells, usually arranged in grape-like irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. (Jawetz *et al.*, 1991:194.)

The organisms are non-sporing, non-motile and usually non-capsulate (Greenwood, Slack & Peutherer, 1992:204). Their cell walls contain peptidoglycan (mucopeptide) and teichoic acids, important cell-adherence factors (Mackie & McCartney, 1996:245). When grown on nutrient agar, milk agar or blood agar for 24 hours at 37° Celsius individual colonies are circular, 2-3mm in diameter with a smooth shiny surface. Colonies appear opaque and are frequently pigmented, though a few strains are unpigmented (Greenwood, Slack & Peutherer, 1992:204).

2.3.1.2 Normal Habitat

Staphylococci are widely distributed in the environment. They form part of the normal microbial flora of the skin, upper respiratory tract, and intestinal tract. *S. aureus* is carried in the nose of 40% or more of healthy people. (Cheesebrough, 1992:225.)

2.3.1.3 Pathogenicity

Staphylococci are ubiquitous human parasites. The chief sources of infection are shedding human lesions, fomites contaminated from such lesions, and the human respiratory tract and skin. (Jawetz *et al.*, 1989:190.) According to Mackie & McCartney (1996:248), as well as Cheesebrough (1984:225-226), some of the commoner infections caused by *S. aureus* include the following:

1. *Pyogenic infections*: folliculitis; impetigo; furuncles, carbuncles, boils, styes, abscesses; breast abscesses; secondary wound infections of insect bites, ulcers, burns, wounds and skin disorders; cellulites; osteomyelitis; bronchopneumonia; lung abscesses and empyema.
2. *Disseminated infections*: septicaemia, often with consequent metastatic secondary foci.
3. *Toxin-mediated illness*: toxic shock syndrome; staphylococcal scalded skin syndrome in young children due to the toxin exfoliation; staphylococcal food poisoning from enterotoxin B produced by *S. aureus* in foods such as cooked meats and milk and milk-products.

2.3.1.4 Enzymes and toxins

- *Coagulase*, an enzyme that clots plasma and coats staphylococcal cells which probably prevents the bacteria from being destroyed by macrophages. Detection of free and bound coagulase forms the basis of the coagulase test used to identify *S. aureus*.
- *Hyaluronidase*, which helps *S. aureus* to spread in the tissues.
- *Nucleases* (Deoxyribonuclease).
- *Proteinase*.

- *Phosphatase*.
- Fibrinolysin or *Staphylokinase*, that causes fibrinolysis.
- *Lipase*, that breaks down fat.
- Leucocidin, that kills white blood cells.
- Enterotoxins A-E, that cause food poisoning.
- Toxic shock syndrome toxin (TSST-1).
- Epidermolytic toxins A and B.
- Haemolysins α , β , γ , δ . (Mackie & McCartney, 1996:246; Cheesebrough, 1992:226.)

2.3.1.5 Antimicrobial sensitivity

Antibiotics with activity against *S. aureus* include: penicillins, flucloxacillin, methicillin, cephalosporins, erythromycin, lincomycin, clindamycin, fucidin and vancomycin. Many strains of *S. aureus* are penicillin-resistant due to the production of penicillinase (a beta-lactamase). (Cheesebrough, 1992:226.)

2.3.2 BACILLUS CEREUS

2.3.2.1 Description and morphology

The genus *Bacillus* includes large aerobic, Gram-positive rods occurring in chains (Jawetz *et al.*, 1991:180). *B. cereus* is motile, non-capsulate, spore forming and respire aerobically (Mims *et al.*, 1998:518) and the spores are highly resistant to the lethal effects of heat, drying, chemical disinfectants and UV radiation (Mackie & McCartney, 1996:317).

2.3.2.2 Normal Habitat

Bacillus species are widely distributed in nature. Most live as saprophytes in the soil, dust, water, vegetation and in a variety of foods including cereals, spices, meats, and poultry. They are able to form resistant spores. (Cheesebrough, 1992:233.)

2.3.2.3 Toxins

Mackie & McCartney (1996:318) state that *B. cereus* produce several toxins: a lecithinase (phospholipase), two haemolysins, a diarrhoeal enterotoxin and an emetic toxin.

2.3.2.4 Pathogenicity

It has been found in a variety of infections, mostly in injured, debilitated or immunocompromised subjects, e.g. in abscess after injury (Mackie & McCartney, 1996:318). Local infections most commonly complicate post-surgical or traumatic wounds, burns, and affections of the eye. Cutaneous infections have also been reported, from orthopaedic departments, caused by the application of plaster of Paris casts or gauze rolls that were contaminated with *B. cereus* spores. (Koneman *et al.*, 1997:659.) *B. cereus* occasionally causes pneumonia and bronchopneumonia (Cheesebrough, 1992:234).

Most episodes of food poisoning caused by *B. cereus* are due to preformed toxin (Greenwood, Slack & Peutherer, 1992:276). Food poisoning caused by *B. cereus* has two distinct forms, the emetic type associated with rice dishes and the diarrheal type associated with meat dishes and sauces. The emetic form begins 1-6 hours after ingestion of contaminated food, whereas the diarrheal form has an incubation period of 1-24 hours. The presence of *B. cereus* in a patient's stool is not sufficient to make a

diagnosis of *B. cereus* disease, since the bacteria may be present in normal stool samples. (Jawetz *et al.*, 1991:182.)

2.3.2.5 Antimicrobial sensitivity

Bacillus species, with the exception of *B. anthracis*, are generally resistant to the older penicillins and cephalosporins. *B. cereus* is particularly β -lactamase resistant. Most strains are susceptible to the aminoglycosides, clindamycin, vancomycin, chloramphenicol, and erythromycin. (Koneman *et al.*, 1997:664.)

2.3.3 ESCHERICHEA COLI

2.3.3.1 Description and morphology

E. coli is a Gram-negative, aerobic bacterium that occurs as a natural commensal in the intestinal tract of humans, especially in the ileum and the colon. Most strains are motile, and produce a polysaccharide capsule. (Greenwood, Slack & Peutherer, 1992:323.)

E. coli and most of the other enteric bacteria form circular, convex, smooth colonies with distinct edges (Jawetz *et al.*, 1991:212).

2.3.3.2 Normal Habitat

E. coli organisms form part of the normal microbial flora of the intestinal tract of humans and animals and can also be found in soil, water, and vegetation (Cheesebrough, 1992:254).

2.3.3.3 Pathogenicity

As stated in Jawetz *et al.* (1991:216), the clinical manifestations of infection with *E. coli* depends on the site of the infection:

- Urinary tract infection: - *E. coli* is the most common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women.
- Traveller's diarrhoea: - Enterotoxigenic *E. coli* (ETEC) is a common cause of 'traveller's diarrhoea', which may occur by several mechanisms. These strains are important causes of enteritis in developing countries. They produce an acute, watery (secretory) diarrhoea that can lead to severe and often fatal dehydration in infants and young children (Cheesebrough, 1992:254).
- Sepsis: - When normal host defences are inadequate, *E. coli* may reach the bloodstream and cause sepsis.
- Meningitis: - *E. coli* and group *B streptococci* are the leading cause of meningitis in infants.

2.3.3.4 Antimicrobial sensitivity

Antibiotics that are used to treat *E. coli* infections include sulphonamides, trimethoprim, cotrimoxazole, nalidixic acid, nitrofurantoin, tetracycline, ampicillin, amoxycillin, cephalosporins, and aminoglycosides. In the treatment of *E. coli* diarrhoea, the antibiotic treatment is not as important a measure as is correct rehydration of the patient. (Cheesebrough, 1992:255.)

2.3.4 KLEBSIELLA PNEUMONIAE

2.3.4.1 Description and morphology

Klebsiellae are straight, Gram-negative rods about 1-2 micrometers long and 0.5-0.8 micrometers wide (Greenwood, Slack and Peutherer, 1992: 335). *Klebsiella* species

exhibit mucoid growth, large polysaccharide capsules, and a lack of motility. The colonies are large and very mucoid and tend to coalesce with prolonged incubation (Jawetz *et al.*, 1991:212,214).

2.3.4.2 Normal Habitat

K. pneumoniae is present in the respiratory tract and faeces of about 5% of normal individuals (Jawetz *et al.*, 1991:217) and can be found as a commensal in the mouth and upper respiratory tract, and also in moist environments in hospitals and elsewhere. (Cheesebrough, 1992:262.)

2.3.4.3 Pathogenicity

This species causes, according to Jawetz *et al.* (1991:217), Cheesebrough (1992:263), and Greenwood, Slack & Peutherer (1992:335-338), the following:

- Chest infections. Occasionally it causes a severe pneumonia, especially in patients being treated with ampicillin. It can produce extensive haemorrhagic necrotizing consolidation of the lung, sometimes with chronic destructive lesions and multiple abscess formation in the lungs.
- Urinary infections, particularly those that are hospital acquired.
- Septicaemia and meningitis.
- Wound infections and peritonitis.

2.3.4.4 Antimicrobial sensitivity

Cephalosporins and aminoglycosides are used in the treatment of *K. pneumoniae*. Most strains are resistant to ampicillin and show some multiple drug resistance. (Cheesebrough, 1992:2632.)

2.3.5 PSEUDOMONAS AERUGINOSA

2.3.5.1 Description and morphology

Ps. aeruginosa is a Gram-negative bacillus, non-sporing, non-capsulate, and usually motile by virtue of one or two polar flagella. It is an obligate aerobe but can grow anaerobically if nitrate is available.

The organism grows readily on a wide variety of culture media over a wide temperature range and emits a sweet grape-like odour that is easily recognised. (Greenwood, Slack & Peutherer, 1992:345-349.) It is usually recognised by the yellow-green pyocyanin pigment it produces (Cheesebrough, 1992:265).

Ps. aeruginosa cultured from the respiratory tracts of patients with cystic fibrosis produce large amounts of alginate, an exopolysaccharide which gives rise to strikingly mucoid colonies (Mackie & McCartney, 1996:414).

2.3.5.2 Normal Habitat

Pseudomonas species can be found in water, soil, sewage, and vegetation. They can also be found in the intestinal tract. *Ps. aeruginosa* is frequently present in hospital environments, especially in moist places such as sinks, bowls, drains, cleaning buckets, and humidifiers. It can also be found growing in eye drops, ointments, and weak antiseptic solutions. (Cheesebrough, 1992:264.)

2.3.5.3 Pathogenicity

Ps. aeruginosa is a classic opportunist pathogen with innate resistance to many antibiotics and disinfectants (Mackie & McCartney, 1996:413) and can infect almost any external site or organ (Greenwood, Slack and Peutherer, 1992:345).

Mims *et al.* (1998:395) state that this bacterium is the most devastating Gram-negative rod effecting burn wounds, and grows well on the moist environment of a burn wound. It is relatively rare, but carries a mortality rate exceeding 70% in patients compromised by severe burns, cancer or drug addiction (Mackie & McCartney, 1996:414).

Mims *et al.* (1998:215) also point out that *Ps. aeruginosa* is an important pathogen relating to cystic fibrosis as nearly all patients with this condition between the ages of 15-20 years will have colonies of this pathogen in their lungs that produce the characteristic thick mucoid secretions, thus exacerbating the present condition of the patient.

Cheesebrough (1992: 264) also holds *Ps. aeruginosa* responsible for some cases of otitis externa, eye infections (usually hospital-acquired), and septicaemia.

2.3.5.4 Antimicrobial sensitivity

Pseudomonas species are resistant to most of the commonly used antibiotics. Those antimicrobials that usually show activity against *Pseudomonas* species include aminoglycosides, polymyxins, and some penicillins and cephalosporins. (Cheesebrough, 1992:265.)

2.4 Screening Methods

Research on the antimicrobial activity of plants presents a few problems due to the diversity of criteria, techniques employed and the lipophilic properties of some samples.

It is due to these variables that different techniques and methods are utilized, and can be classified into three groups, namely: diffusion, dilution and bioautographic methods.

(Rios, Recio and Villar, 1988.)

Dilution techniques are those which require a homogenous dispersion of the sample in water and are used principally to determine the minimum inhibitory concentration (MIC) values of an extract, essential oil or pure substance. The grade of inhibition is related to the turbidity of the medium and is measured by spectrophotometry. The bioautographic method makes use of chromatography and is based on the agar-diffusion technique, whereby the antibacterial compound is transferred from the chromatographic layer to an inoculated agar plate. Inhibition zones are visualised by dehydrogenase-activity-detecting reagents. (Rios, Recio and Villar, 1988.)

As is explained, this study employs the use of one of the diffusion methods and the precise methodology is outlined in Chapter Three. A few possible modifications of this methodology are also outlined in Chapter Six. This method is not acceptable when the sample being tested is lipophilic, but the advantages of this diffusion method are the small size of the sample used in the screening and the possibility of testing 5 or 6 compounds against a single microorganism. (Rios, Recio and Villar, 1988.)

2.5 Other Related Studies

A number of studies testing South African plants for antibacterial activity using the air-dried disc-diffusion method have been conducted.

In 1997, Rabe and van Staden of the Department of Botany, University of Natal Pietermaritzburg, tested many South African plants for antibacterial activity by preliminary bioassay screening.

Dried plant material was used and the extraction process involved the use of either water, or 80% methanol. For their experiments, the disc-diffusion assay was used to determine the growth inhibition of bacteria by the plant extracts. The following bacteria were used: *S. aureus*, *Staphylococcus epidermis* (*S. epidermis*), *Bacillus subtilis* (*B. subtilis*), *E. coli* and *K. pneumoniae*. They inoculated molten MH agar, held at 48°C, with a broth culture of the test organism, then poured this mixture over the base plates to form a homogenous top layer. Air-dried filter paper discs containing 10µl of the extract being tested were placed on to the seeded top layer of the agar plates.

Antibacterial activity was expressed as the ratio of the inhibition zone (mm) produced by the plant extract and the inhibition zone (mm) caused by the negative control (methanol). The activity of neomycin (positive control) was included in this equation to adjust for plate-to-plate variations in the sensitivity of a particular bacterial strain. In their study, they found that any antibacterial activity expressed, was usually against the Gram-positive bacteria. A total of 54 extracts representing 21 plant species distributed among 15 families were investigated. Of the 21 plant species tested, 10, 8, and 12 of the species showed statistically viable activity against *S. aureus*, *S. epidermis* and *B. subtilis*, respectively.

In 1999, McGraw, Jager and van Staden tested a variety of South African medicinal plants for any antibacterial, anthelmintic and anti-amoebic activity. The bacteria selected were *B. subtilis*, *E. coli*, *K. pneumoniae* and *S. aureus*. They utilized the disc-

diffusion assay as an antibacterial screening method by seeding molten MH agar with a MH broth culture of the bacteria and then pouring this over the base plates to form a homogenous top layer. Their methodology is further similar to the current study in that they allowed the impregnated filter paper discs to air dry before placing them on the seeded MH agar plates.

A positive control (neomycin) was utilized in the study to adjust for plate-to-plate variation in the sensitivity of the bacterial strains. Hexane, ethanol and water were used as negative controls as these were the different solvents used as extractants during the tincture manufacture. The plates were incubated overnight at 37°C.

In total, 138 extracts belonging to 46 species were tested and any antibacterial activity exhibited by any of the extracts was expressed as a ratio to the activity exhibited by the positive control. Again, the Gram-positive bacteria (*B. subtilis* and *S. aureus*) were significantly more susceptible to the extracts tested than the Gram-negative ones (*E. coli* and *K. pneumoniae*). Only 4 extracts showed activity against *E. coli*, while 2 extracts were active against *K. pneumoniae*. It is interesting to note that the ethanolic extracts displayed the greatest antibacterial activity, while the water and hexane extracts showed the least activity (in that order).

In both of the above studies, p-values and statistical methods used are not given.

Similar studies conducted at the Department of Biotechnology at the Durban Institute of Technology have been conducted in recent years.

In 2002, Vosloo established the effects of *Thymus vulgaris* tincture in 43% and 70% ethanol upon *Streptococcus pyogenes* (*S. pyogenes*), *S. aureus*, *Ps. aeruginosa*, *S. epidermis*, *E. coli* and *Enterococcus faecalis* (*E. faecalis*).

She calculated the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of the tinctures in both ethanolic concentrations using the disc-diffusion method. 43% and 70% ethanol served as negative controls.

MH agar plates were inoculated with the bacterial cultures. Sterile filter paper discs were placed equidistantly on these plates, impregnated by a single procedure with 10µl of either test or control substance, and incubated at 37° C for 18 hours.

The diameters of the zones of inhibition of bacterial growth around the discs were measured and the data was analyzed statistically using the Mann-Whitney test to determine any inter-group change between the test and control sub-groups for both ethanolic concentrations.

All the strains of bacteria were sensitive to *Thymus vulgaris* tincture in both 43% and 70% ethanol and there was no significant difference between the effects of the two tinctures.

Langford (2001) performed a study to investigate the possibility of *Calendula officinalis* tincture, in both 30% and 60% ethanol, as an antibacterial agent against *E. coli*, *E. faecalis*, *Ps. aeruginosa*, *S. aureus*, *S. epidermis* and *S. pyogenes*.

She made use of the disc-diffusion method and 30% and 60% ethanol served as controls. Sterile filter paper discs were placed on inoculated agar plates. The discs had previously been impregnated with a known volume of either test or control substance. All the plates were incubated at 37°C and checked after 16, 24 and 48 hours for zones of inhibition of bacterial growth around the filter paper discs. The data was analyzed statistically using the Mann-Whitney test.

Calendula officinalis prepared in 30% ethanol and the 30% ethanol control yielded no results. The converse was true for *Calendula officinalis* prepared in 60% ethanol and the 60% ethanol control with respect to *E. faecalis*, *Ps. aeruginosa* and *S. pyogenes* where results were recorded. The results were negligible however when compared to the effects of antibiotics that were then utilized to combat infections with these three bacterial species. *Calendula officinalis* tincture therefore could not be recommended as an alternative to antibiotic use in cases of such infections.

Reid (2002) used the disc-diffusion method to determine any effects that *Rosmarinus officinalis*, *Salvia officinalis* and *Thymus vulgaris* as herbal extracts might display against *Candida albicans*. Ethanol and nystatin served as controls.

Fifteen Sabouraud's dextrose agar plates were inoculated with *Candida albicans*. Filter paper discs, previously impregnated with test or control substance were placed equidistantly on the plates which were incubated at 37°C. The diameters of the zones of inhibition were measured at 18, 24 and 36 hours.

Data was analyzed by the Mann-Whitney, the Friedman and the Kruskal-Wallis tests. The results showed that *Rosmarinus officinalis* and *Salvia officinalis* were ineffective, whilst *Thymus vulgaris* was effective in the inhibition of *in vitro* growth of *Candida albicans* at 18 and 24 hours. Nystatin however proved to be more effective than any of these herbs.

CHAPTER THREE

METHODOLOGY

3.1 THE DATA

The research involves two types of data: primary and secondary. The nature of the data is as follows:

3.1.1 The Primary Data

- 1.a. Results of the experiment determining the antimicrobial effect of
A. amatymbica tincture prepared in 62% v/v ethanol on *E. coli*.
- b. Results of the experiment determining the antimicrobial effect of
A. amatymbica D1 prepared in 62% v/v ethanol on *E. coli*.
- c. Results of the experiment determining the antimicrobial effects of
A. amatymbica D6 prepared in 62% v/v ethanol on *E. coli*.
- d. Results of the experiment determining the antimicrobial effect of 62% v/v ethanol
only (negative control) on *E. coli*.
- 2.a. Results of the experiment determining the antimicrobial effect of *A. amatymbica*
tincture prepared in 62% v/v ethanol on *Ps. aeruginosa*.
- b. Results of the experiment determining the antimicrobial effect of *A. amatymbica* D1
prepared in 62% v/v ethanol on *Ps. aeruginosa*.
- c. Results of the experiment determining the antimicrobial effects of *A. amatymbica* D6
prepared in 62% v/v ethanol on *Ps. aeruginosa*.
- d. Results of the experiment determining the antimicrobial effect of 62% v/v ethanol
only (negative control) on *Ps. aeruginosa*.

- 3.a. Results of the experiment determining the antimicrobial effect of *A. amatymbica* tincture prepared in 62% v/v ethanol on *S. aureus*.
- b. Results of the experiment determining the antimicrobial effect of *A. amatymbica* D1 prepared in 62% v/v ethanol on *S. aureus*.
- c. Results of the experiment determining the antimicrobial effects of *A. amatymbica* D6 prepared in 62% v/v ethanol on *S. aureus*.
- d. Results of the experiment determining the antimicrobial effect of 62% v/v ethanol only (negative control) on *S. aureus*.

- 4.a. Results of the experiment determining the antimicrobial effect of *A. amatymbica* tincture prepared in 62% v/v ethanol on *B. cereus*.
- b. Results of the experiment determining the antimicrobial effect of *A. amatymbica* D1 prepared in 62% v/v ethanol on *B. cereus*.
- c. Results of the experiment determining the antimicrobial effects of *A. amatymbica* D6 prepared in 62% v/v ethanol on *B. cereus*.
- d. Results of the experiment determining the antimicrobial effect of 62% v/v ethanol only (negative control) on *B. cereus*.

- 5.a. Results of the experiment determining the antimicrobial effect of *A. amatymbica* tincture prepared in 62% v/v ethanol on *K. pneumoniae*.
- b. Results of the experiment determining the antimicrobial effect of *A. amatymbica* D1 prepared in 62% v/v ethanol on *K. pneumoniae*.
- c. Results of the experiment determining the antimicrobial effects of *A. amatymbica* D6 prepared in 62% v/v ethanol on *K. pneumoniae*.
- d. Results of the experiment determining the antimicrobial effect of 62% v/v ethanol only (negative control) on *K. pneumoniae*.

3.1.2 The Secondary Data.

Research articles from journal publications, books and manuals.

3.2 CRITERIA GOVERNING THE ADMISSIBILITY OF DATA

Only data obtained from laboratory experiments carried out by the researcher at the Department of Biotechnology, Durban Institute of Technology was used.

3.3 MATERIALS AND METHOD

The basic methodology followed, unless otherwise stated, was as per Cappucino and Sherman (1992).

3.3.1 Preparation Of Media

Mueller-Hinton Agar was prepared according to the directions in the Oxoid Manual (1979) as follows:

- 35 grams Mueller-Hinton agar powder was weighed out.
- This was added to 1 litre distilled water in a screw top flask.
- A magnetic stirrer was added to ensure adequate mixing.
- The mixture was sterilized by autoclaving at 121°C for 15 minutes.
- The flask was allowed to cool in a beaker of cold water, which had been placed on a magnetic stirring machine. This ensured adequate mixing and prevented the mixture from solidifying.

3.3.2 Preparation Of The Plates

Once the flask had cooled enough to hold, the agar was poured into sterile 90 mm agar plates as follows:

- The top of the flask was flamed with a bunsen burner before pouring each plate to prevent contamination.
- Each plate was poured to a depth of approximately 5 mm.
- A total of 110 plates were prepared.
- The plates were stacked and allowed to cool and solidify.
- They were then visually checked for contamination.

Once the agar had solidified, the plates were divided into 5 groups of 20 each and each group was assigned a letter from A–E, which denoted the bacterium that that group was to be inoculated with. The bacteria were grouped as follows; group A (*S. aureus*), group B (*K. pneumoniae*), group C (*Ps. aeruginosa*), group D (*E. coli*) and group E (*B. cereus*).

The numbers 1 to 6 along with the assigned group letter, were written on the under side of each petri dish using a black marker pen, in a manner that was small enough not to obscure any results obtained but large enough to remain legible. The numbers were written along the circumference of the plates, equidistant from each other. Each number was allocated a test or control substance as follows:

1. *A. amatymbica* tincture,
2. *A. amatymbica* D1,
3. *A. amatymbica* D6,
4. Ethanol 62% v/v,
5. Vancomycin,
6. Gentamicin.

3.3.3 Preparation Of Filter Paper Discs

Whatman[®] filter paper No. 4 was used. The filter paper was punched into discs 5 millimetres in diameter. These discs were placed in a jar and autoclaved at 121°C for 15 minutes to ensure sterilization. Using sterile forceps, the discs were placed in sterile petri dishes, and each disc was impregnated with an appropriate test substance or negative control using a triple-impregnation technique. The first impregnation stage consisted of 15 µl of the appropriate substance, the second consisted of 10 µl and the third of 5 µl. Between each impregnation stage, the discs were dried at 37°C for one hour with the lids of each petri dish off.

3.3.4 Preparation Of Test Substances

3.3.4.1 Preparation Of *A. Amatymbica* Tincture

A. amatymbica tincture in 62% ethanol (batch no. 052301, expiry date 04/2007) was prepared by Parceval (Pty) Ltd. Pharmaceuticals and made up according to the HAB 4a method of the German Homoeopathic Pharmacopoeia (British Homoeopathic Association, 1985:22). According to Parceval, this pharmacopoeia is recognised as an international standard in the manufacture of plant extracts and dilutions, and they manufacture all their tinctures according to these standards. A 62% ethanolic concentration was used as at this concentration a very broad spectrum of both lipophilic and hydrophilic plant constituents are extracted during the tincture manufacturing process. (Gorlich, 2003.)

3.3.4.2 Preparation Of *A. Amatymbica* D1

A. amatymbica D1 in 62% v/v ethanol was prepared according to the German Homoeopathic Pharmacopoeia Method 3a (British Homoeopathic Association 1985:18).

A standard of 62% v/v ethanol was utilised through all dilutions to ensure a standard in the ethanol percentage for the experimental requirements.

3.3.4.3 Preparation Of *A. Amatymbica* D6

A. amatymbica D6 in 62% v/v ethanol was prepared according to the German Homoeopathic Pharmacopoeia Method 3a (British Homoeopathic Association, 1985:18).

A standard of 62% v/v ethanol was utilised through all dilutions to ensure a standard in the ethanol percentage for the experimental requirements.

3.3.5 Preparation Of Controls

A negative control of 62 % v/v ethanol was included in the experiment so as to validate any results obtained by the test substances and to ensure that any zones of inhibition obtained were not due to the ethanol solvent.

Gentamicin and vancomycin served as positive controls and references, to account for plate-to-plate variations in the sensitivity of the bacteria to antimicrobial agents. Zones of inhibition produced by a test substance were expressed as a ratio to the combined diameters of the two positive controls.

3.3.5.1 Preparation Of 62% V/V Ethanol

62% v/v ethanol (negative control) was made up according to the German Homoeopathic Pharmacopoeia standards (British Homoeopathic Association, 1985:11): 129.2 millilitres of 96% v/v ethanol was diluted with sufficient water to produce 200 millilitres.

3.3.5.2 Preparation Of Gentamicin

Gentamicin (batch 261401, expiry date 2005/01) was prepared by Oxoid Limited.

3.3.5.3 Preparation Of Vancomycin

Vancomycin (batch no. 263551, expiry date 2005/02) was prepared by Oxoid Limited.

3.3.6 Preparation Of The Inoculum

Each bacterial species was obtained from a single colony of the culture of the organism from the Biotechnology department at the Durban Institute of Technology. Each bacterial species was then inoculated onto a petri dish containing Mueller–Hinton agar. Each of these was then incubated at 37°C for 48 hours. Using a sterile wire loop, each bacterial species was inoculated into their respective nutrient broth. The solution was adjusted to Mcfarland's Equivalence Turbidity Standards 0,5 to ensure a standard concentration of bacteria in each broth culture.

3.3.7 Inoculation Of The Plates

From each nutrient broth with its respective bacterial species, a sterile 4mm wire loop was used to inoculate each Mueller-Hinton agar plate, and a glass spreader was used to ensure uniform seeding of each plate. Between each inoculation stage, the glass spreader was stored in 70% ethanol and flamed to ensure sterile practice and minimize cross contamination of the agar plates. For each bacterial species, 20 agar plates were inoculated in this manner. From the 20 inoculated plates for each bacterial species, 15 plates that yielded the best results were used to record any results obtained. This allowed for a small margin of error as was bound to occur during the inoculation stage.

3.3.8 Placement Of The Discs

Once the plates had been inoculated, each test substance was placed onto each agar plate in a manner that corresponded with their allocated number on the underside of each petri dish. This was done using a sterile needle.

3.3.9 Incubation

Once this part of the procedure had been completed, all of the agar plates were incubated at 37°C.

3.3.10 Recording Of Results

The plates were examined at 18, 24 and 48 hours for the presence of any growth inhibition, as indicated by a clear zone around each disc. The susceptibility of each bacterial species to any of the test substances was determined by the size of this zone. The zone diameter was measured in millimetres (mm) using a ruler and recorded. Photographs were taken at 18 hours as a visual record.

3.4 DATA ANALYSIS

3.4.1 Sample Size Of The Study

The sample size of the study was 15, which means each test yielded 15 data sets. The efficacy of each test and control substance was tested against each of the bacterial species 15 times to make the study statistically viable.

3.4.2 Statistical Methods

3.4.2.1 Intra-Group Comparison Of *A. amatymbica* In 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Friedman's test was used to compare results from related samples.

(i) Hypothesis testing

The null hypothesis H_0 , states that there is no change in diameter of the zone of inhibition with regard to observations at 18 hours, 24 hours and 48 hours, at the $\alpha=0.05$ level of significance. The alternative hypothesis H_1 , states that there is a change in the

diameter of the zone of inhibition with regard to observations at 18 hours, 24 hours and 48 hours, at the same level of significance.

Reject H_0 if $P < \alpha$.

Accept H_0 if $P \geq \alpha$.

P is the observed significance level or probability value. If the null hypothesis H_0 is rejected for Friedman's T test, then multiple applied, comparison procedures will have to be carried out using the Dunn Procedure to determine which of the times are significantly different. Let R_j and R_j' be the j^{th} and j'^{th} time rank totals.

Let α be the experiment wise error rate. Usually $\alpha = 0.10$

If $|R_j - R_j'| \geq z \sqrt{bk(k+1)/6}$, then R_j and R_j' are declared significant.

In the above formula:

b = the number of blocks

k = the number of times

z = value in the inverse normal distribution corresponding to

$(1 - [\alpha/k(k-1)])$

To compute the treatment rank totals, rank values in each block and then compute the sum of the ranks for each time.

When $k=3$, $\alpha=0.10$, $z=2.12$

(Fisher and van Belle, 1993:430).

3.4.2.2 Intra-Group Comparison Of *A. amatymbica* D1 With Regard To Observations At 18, 24 And 48 Hours.

The Friedman's test was used to compare results from related samples.

i) Hypothesis testing

As per 3.4.2.1 (i).

(ii) Decision rule

As per 3.4.2.1 (ii).

3.4.2.3 Intra-Group Comparison Of *A. amatymbica* D6 With Regard To Observations At 18, 24 And 48 Hours.

The Friedman's test was used to compare results from related samples.

i) Hypothesis testing

As per 3.4.2.1 (i).

(ii) Decision rule

As per 3.4.2.1 (ii).

3.4.2.4 Intra-Group Comparison Of 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Friedman's test was used to compare results from related samples.

(i) Hypothesis testing

As per 3.4.2.1 (i).

(ii) Decision rule

As per 3.4.2.1 (ii).

3.4.2.5 Inter-Group Comparison Between *A. amatymbica* Tincture In 62% V/V Ethanol And 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Mann-Whitney U test was used to compare the samples.

(i) Hypothesis testing

The null hypothesis H_0 , states that there is no difference in diameter of the zone of inhibition between the substances, with respect to the variable comparison at the $\alpha =$

0.05 level of significance. The alternative hypothesis H_1 , states that there is a difference at the same level of significance.

H_0 : there was no difference between the 2 groups, $M_1=M_2$.

H_1 : there was a difference between the 2 groups, $M_1 \neq M_2$.

(ii) Decision rule

At $\alpha = 0,05$ level of significance, the null hypothesis is rejected if $P < \alpha$ where P is the observed significance level or probability value. Otherwise the null hypothesis is accepted at the same level of significance.

Reject H_0 if $P < \alpha$.

Accept H_0 if $P \geq \alpha$.

P is the observed significance level or probability value

(Fisher and van Belle, 1993:315).

3.4.2.6 Inter-Group Comparison Between *A. amatymbica* D1 In 62% V/V Ethanol And 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Mann-Whitney U test was used to compare the samples.

(i) Hypothesis testing

As per 3.4.2.7 (i).

(ii) Decision rule

As per 3.4.2.7 (ii).

3.4.2.7 Inter-Group Comparison Between *A. amatymbica* D6 In 62% V/V Ethanol And 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Mann-Whitney U test was used to compare the samples.

(i) Hypothesis testing

As per 3.4.2.7 (i).

(ii) Decision rule

As per 3.4.2.7 (ii).

3.4.2.8 Inter-Group Comparison Between *A. amatymbica* In 62% V/V Ethanol And *A. amatymbica* D1 In 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Mann-Whitney U test was used to compare the samples.

(i) Hypothesis testing

As per 3.4.2.7 (i).

(ii) Decision rule

As per 3.4.2.7 (ii).

3.4.2.9 Inter-Group Comparison Between *A. amatymbica* In 62% V/V Ethanol And *A. amatymbica* D6 In 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Mann-Whitney U test was used to compare the samples.

(i) Hypothesis testing

As per 3.4.2.7 (i).

(ii) Decision rule

As per 3.4.2.7 (ii).

3.4.2.10 Inter-Group Comparison Between *A. amatymbica* D1 In 62% V/V Ethanol And *A. amatymbica* D6 In 62% V/V Ethanol With Regard To Observations At 18, 24 And 48 Hours.

The Mann-Whitney U test was used to compare the samples.

(i) Hypothesis testing

As per 3.4.2.7 (i).

(ii) Decision rule

As per 3.4.2.7 (ii).

3.4.2.11 Inter-Group Comparison Between *A. Amatymbica* In 62% V/V,

A. amatymbica D1 in 62% V/V Ethanol And *A. amatymbica* D6 In 62% V/V Ethanol

With Regard To Observations At 18, 24 And 48 Hours.

The Kruskal-Wallis non-parametric Analysis of Variance by Ranks method was used to compare the diameters of the zones of inhibition of the tincture and homoeopathic potencies, to each other, with regard to observations at 18 hours, 24 hours and 48 hours.

(i) Hypothesis testing

In each test, the null hypothesis states that there is no difference in diameter among the means of the tincture and homoeopathic potencies being compared to each other. The alternative hypothesis states that there is a difference among the means.

$$H_0 : \mu_1 = \mu_2 = \mu_3.$$

$$H_1 : \mu_1 \neq \mu_2 \neq \mu_3. \text{ (All 3 means are not equal, at least one mean differs from the rest)}$$

(ii) Decision rule

At $\alpha = 0,05$ level of significance, the null hypothesis is rejected if $P < \alpha$ where P is the observed significance level or probability value. Otherwise the null hypothesis is accepted at the same level of significance.

Reject H_0 if $P < \alpha$.

Accept H_0 if $P \geq \alpha$.

P is the observed significance level or probability value.

The Dunn Procedure for use with the Kruskal-Wallis test:

If the null hypothesis H_0 is rejected for Kruskal-Wallis test, then multiple comparison procedure will have to be used to determine which of the medians (test substances) are significantly different.

Let R_i and R_j be the means of the ranks of the i^{th} and j^{th} samples respectively. Let α be the experiment wise error rate. The values of α are usually 0.15, 0.20, 0.25 depending on the value of k . (as k increases, α increases)

If $|R_i - R_j| > Z_{(1-\alpha/k(k-1))} \sqrt{N(N+1)/12 (1/n_i + 1/n_j)}$, then the difference $|R_i - R_j|$ is declared significant at the α level.

In the above formula:

k =the number of samples

N =the number of observations in all samples combined

z =the value in the inverse normal distribution corresponding to

$(1-[\alpha/k(k-1)])$

If $k=3$, $\alpha=0.15$; $z=1.96$

If $k=4$, $\alpha=0.20$; $z=2.12$

If $k=5$, $\alpha=0.25$; $z=2.326$ etc..

(Fisher and van Belle, 1993: 430).

If there are extensive ties in the data, the inequalities will be adjusted to ensure a conservative result. The appropriate inequality for equal sample sizes is:

$$|R_i - R_j| \leq z \sqrt{k[N(N^2-1) - (\sum t^3 - \sum t)] / 6N(N-1)}$$

where t is the number of values in the combined sample that are tied at a given rank

(Daniel, 1978: 213).

3.4.3 Statistical Package

The statistical package for Social Sciences (SPSS) was used for data entry and analysis.

CHAPTER FOUR

RESULTS

4.1 INTRODUCTION

This chapter covers the results obtained from statistical analysis of the data obtained.

4.2 STATISTICAL ANALYSIS OF DATA

4.2.1 Intra-group comparison of *A. amatymbica* herbal tincture in 62% v/v ethanol with regards to observations at 18, 24, and 48 hours.

4.2.1.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* herbal tincture in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.1 and 4.2.

Table 4.1 Descriptive Statistics for *A. amatymbica* herbal tincture in 62% v/v ethanol against *E. coli*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.2 Friedmans' test for *A. amatymbica* herbal tincture in 62% v/v ethanol against *E. coli*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$, therefore the null hypothesis was accepted.

4.2.1.2 *S. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* herbal tincture in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.3 and 4.4.

Table 4.3 Descriptive Statistics for *A. amatymbica* herbal tincture in 62% v/v ethanol against *S. aureus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	6.4667	.5614	6.00	7.00
24 hours	15	6.4667	.5614	6.00	7.00
48 hours	15	6.4667	.5614	6.00	7.00

Table 4.4 Friedmans' test for *A. amatymbica* herbal tincture in 62% v/v ethanol against *S. aureus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.1.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* herbal tincture in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.5 and 4.6.

Table 4.5 Descriptive Statistics for *A. amatymbica* herbal tincture in 62% v/v ethanol against *K. pneumoniae*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.6 Friedmans' test for *A. amatymbica* herbal tincture in 62% v/v ethanol against *K. pneumoniae*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.1.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* herbal tincture in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.7 and 4.8.

Table 4.7 Descriptive Statistics for *A. amatymbica* herbal tincture in 62% v/v ethanol against *B. cereus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	7.0000	.0000	7.00	8.00
24 hours	15	7.0667	.2585	7.00	8.00
48 hours	15	7.0667	.2585	7.00	8.00

Table 4.8 Friedmans' test for *A. amatymbica* herbal tincture in 62% v/v ethanol against *B. cereus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.1.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* herbal tincture in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.9 and 4.10.

Table 4.9 Descriptive Statistics for *A. amatymbica* herbal tincture in 62% v/v ethanol against *Ps. aeruginosa*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.10 Friedmans' test for *A. amatymbica* herbal tincture in 62% v/v ethanol against *Ps. aeruginosa*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.2 Intra-group comparison of *A. amatymbica* D1 in 62% v/v ethanol with regards to observations at 18, 24, and 48 hours.

4.2.2.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D1 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.11 and 4.12.

Table 4.11 Descriptive Statistics for *A. amatymbica* D1 in 62% v/v ethanol against *E. coli*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.12 Friedmans' test for *A. amatymbica* D1 in 62% v/v ethanol against *E. coli*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.2.2 *S. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D1 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.13 and 4.14.

Table 4.13 Descriptive Statistics for *A. amatymbica* D1 in 62% v/v ethanol against *S. aureus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.14 Friedmans' test for *A. amatymbica* D1 in 62% v/v ethanol against *S. aureus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.2.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D1 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.15 and 4.16.

Table 4.15 Descriptive Statistics for *A. amatymbica* D1 in 62% v/v ethanol against *K. pneumoniae*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.16 Friedmans' test for *A. amatymbica* D1 in 62% v/v ethanol against *K. pneumoniae*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.2.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D1 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.17 and 4.18.

Table 4.17 Descriptive Statistics for *A. amatymbica* D1 in 62% v/v ethanol against *B. cereus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.18 Friedmans' test for *A. amatymbica* D1 in 62% v/v ethanol against *B. cereus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.2.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D1 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.19 and 4.20.

Table 4.19 Descriptive Statistics for *A. amatymbica* D1 in 62% v/v ethanol against *Ps. aeruginosa*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.20 Friedmans' test for *A. amatymbica* D1 in 62% v/v ethanol against *Ps. aeruginosa*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.3 Intra-group comparison of *A. amatymbica* D6 in 62% v/v ethanol with regards to observations at 18, 24, and 48 hours.

4.2.3.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D6 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.21 and 4.22.

Table 4.21 Descriptive Statistics for *A. amatymbica* D6 in 62% v/v ethanol against *E. coli*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.22 Friedmans' test for *A. amatymbica* D6 in 62% v/v ethanol against *E. coli*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.3.2 *S. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D6 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.23 and 4.24.

Table 4.23 Descriptive Statistics for *A. amatymbica* D6 in 62% v/v ethanol against *S. aureus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.24 Friedmans' test for *A. amatymbica* D6 in 62% v/v ethanol against *S. aureus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.3.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D6 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.25 and 4.26.

Table 4.25 Descriptive Statistics for *A. amatymbica* D6 in 62% v/v ethanol against *K. pneumoniae*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.26 Friedmans' test for *A. amatymbica* D6 in 62% v/v ethanol against *K. pneumoniae*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $P>\alpha$. Therefore the null hypothesis was accepted.

4.2.3.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D6 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.27 and 4.28.

Table 4.27 Descriptive Statistics for *A. amatymbica* D6 in 62% v/v ethanol against *B. cereus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.28 Friedmans' test for *A. amatymbica* D6 in 62% v/v ethanol against *B. cereus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.3.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *A. amatymbica* D6 in 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.29 and 4.30.

Table 4.29 Descriptive Statistics for *A. amatymbica* D6 in 62% v/v ethanol against *Ps. aeruginosa*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.30 Friedmans' test for *A. amatymbica* D6 in 62% v/v ethanol against *Ps. aeruginosa*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.4 Intra-group comparison of 62% v/v ethanol with regards to observations at 18, 24, and 48 hours.

4.3.4.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.31 and 4.32.

Table 4.31 Descriptive statistics for 62% v/v ethanol against *E. coli*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.32 Friedmans' test for 62% v/v ethanol against *E. coli*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.4.2 *S. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.33 and 4.34.

Table 4.33 Descriptive statistics for 62% v/v ethanol against *S. aureus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.34 Friedmans' test for 62% v/v ethanol against *S. aureus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.4.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.34 and 4.35:

Table 4.35 Descriptive statistics for 62% v/v ethanol against *K. pneumoniae*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.36 Friedmans' test for 62% v/v ethanol against *K. pneumoniae*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.4.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.37 and 4.38.

Table 4.37 Descriptive statistics for 62% v/v ethanol against *B. cereus*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.38 Friedmans' test for 62% v/v ethanol against *B. cereus*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.4.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 62% v/v ethanol to the combined diameters of inhibition of the gentamicin and vancomycin, against each other at 18, 24, and 48 hours. See Tables 4.39 and 4.40.

Table 4.39 Descriptive statistics for 62% v/v ethanol against *Ps. aeruginosa*.

	N	Mean	Std. Deviation	Minimum	Maximum
18 hours	15	.0000	.0000	.00	.00
24 hours	15	.0000	.0000	.00	.00
48 hours	15	.0000	.0000	.00	.00

Table 4.40 Friedmans' test for 62% v/v ethanol against *Ps. aeruginosa*.

N	15
Chi-Square	.000
df	2
Asymp. Sig.	1.000
Exact Sig.	1.000
Point Probability	1.000

CONCLUSION: $P=1.000$. Thus $p>\alpha$. Therefore the null hypothesis was accepted.

4.2.5 Inter-group comparison between *A. amatymbica* herbal tincture in 62% v/v ethanol and 62% v/v ethanol with regard to observations at 18 hours, 24 hours and 48 hours.

4.2.5.1 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.41.

Table 4.41 Results of Mann-Whitney test for *A. amatymbica* herbal tincture against *E. coli*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P>\alpha$. Therefore the null hypothesis was accepted.

4.2.5.2 S. aureus

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.42.

Table 4.42 Results of Mann-Whitney test for *A. amatymbica* herbal tincture against *S. aureus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.5.3 K. pneumoniae

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.43.

Table 4.43 Results of Mann-Whitney test for *A. amatymbica* herbal tincture against *K. pneumoniae*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.5.4 *B. cereus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.44.

Table 4.44 Results of Mann-Whitney test for *A. amatymbica* herbal tincture against *B. cereus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.5.5 *Ps. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.45.

Table 4.45 Results of Mann-Whitney test for *A. amatymbica* herbal tincture against *Ps. aeruginosa*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.6 Inter-group comparison between *A. amatymbica* D1 in 62% v/v ethanol and 62% v/v ethanol with regard to observations at 18 hours, 24 hours and 48 hours.

4.2.6.1 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.46.

Table 4.46 Results of Mann-Whitney test for *A. amatymbica* D1 against *E. coli*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.6.2 *S. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.47.

Table 4.47 Results of Mann-Whitney test for *A. amatymbica* D1 against *S. aureus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.6.3 K. pneumoniae

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.48.

Table 4.48 Results of Mann-Whitney test for *A. amatymbica* D1 against *K. pneumoniae*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.6.4 B. cereus

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.49.

Table 4.49 Results of Mann-Whitney test for *A. amatymbica* D1 against *B. cereus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.6.5 *Ps. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.50.

Table 4.50 Results of Mann-Whitney test for *A. amatymbica* D1 against *Ps. aeruginosa*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.7 Inter-group comparison between *A. amatymbica* D6 in 62% v/v ethanol and 62% v/v ethanol with regard to observations at 18 hours, 24 hours and 48 hours.

4.2.7.1 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.51.

Table 4.51 Results of Mann-Whitney test for *A. amatymbica* D6 against *E. coli*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.7.2 *S. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.52.

Table 4.52 Results of Mann-Whitney test for *A. amatymbica* D6 against *S. aureus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.7.3 *K. pneumoniae*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.53.

Table 4.53 Results of Mann-Whitney test for *A. amatymbica* D6 against *K. pneumoniae*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was

accepted.

4.2.7.4 *B. cereus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.54.

Table 4.54 Results of Mann-Whitney test for *A. amatymbica* D6 against *B. cereus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.7.5 *Ps. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the samples. See Table 4.55.

Table 4.55 Results of Mann-Whitney test for *A. amatymbica* D6 against *Ps. aeruginosa*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.8 Inter-group comparison between *A. amatymbica* herbal tincture in 62% v/v ethanol, *A. amatymbica* D1 in 62% v/v ethanol and *A. amatymbica* D6 in 62% v/v ethanol with regard to observations at 18 hours, 24 hours and 48 hours.

4.2.8.1 *E. coli*

The Kruskal-Wallis non-parametric Analysis of Variance by Ranks method was used to compare the diameters of the zones of inhibition of the herbal tincture, D1 potency and D6 potency to each other, with regard to observations at 18 hours, 24 hours and 48 hours. See Table 4.56.

Table 4.56 Results of Kruskal-Wallis test for *A. amatymbica* herbal tincture and D1 and D6 homoeopathic potencies against *E. coli*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.8.2 *S. aureus*

The Kruskal-Wallis non-parametric Analysis of Variance by Ranks method was used to compare the diameters of the zones of inhibition of the herbal tincture, D1 potency and D6 potency to each other, with regard to observations at 18 hours, 24 hours and 48 hours. See Table 4.57.

Table 4.57 Results of Kruskal-Wallis test for *A. amatymbica* herbal tincture and D1 and D6 homoeopathic potencies against *S. aureus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.8.3 *K. pneumoniae*

The Kruskal-Wallis non-parametric Analysis of Variance by Ranks method was used to compare the diameters of the zones of inhibition of the herbal tincture, D1 potency and D6 potency to each other, with regard to observations at 18 hours, 24 hours and 48 hours. See Table 4.58.

Table 4.58 Results of Kruskal-Wallis test for *A. amatymbica* herbal tincture and D1 and D6 homoeopathic potencies against *K. pneumoniae*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.8.4 *B. cereus*

The Kruskal-Wallis non-parametric Analysis of Variance by Ranks method was used to compare the diameters of the zones of inhibition of the herbal tincture, D1 potency and D6 potency to each other, with regard to observations at 18 hours, 24 hours and 48 hours. See Table 4.59.

Table 4.59 Results of Kruskal-Wallis test for *A. amatymbica* herbal tincture and D1 and D6 homoeopathic potencies against *B. cereus*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

4.2.8.5 *Ps. aeruginosa*

The Kruskal-Wallis non-parametric Analysis of Variance by Ranks method was used to compare the diameters of the zones of inhibition of the herbal tincture, D1 potency and D6 potency to each other, with regard to observations at 18 hours, 24 hours and 48 hours. See Table 4.60.

Table 4.60 Results of Kruskal-Wallis test for *A. amatymbica* herbal tincture and D1 and D6 homoeopathic potencies against *Ps. aeruginosa*.

Time	P-value
18 hours	1.000
24 hours	1.000
48 hours	1.000

CONCLUSION: At 18, 24 and 48 hours, $P > \alpha$. Therefore the null hypothesis was accepted.

CHAPTER FIVE

DISCUSSION

A. amatymbica herbal tincture did not exhibit any significant antimicrobial properties against any of the bacteria in the study. The only results that were obtained at all, were very minor results produced by the tincture against *B. cereus* ($P=1.000$) and *S. aureus* ($P=1.000$). Very small zones of inhibition were recorded for these two bacteria. However, these results were not statistically significant. The remaining three bacteria were completely resistant to the herbal tincture. The same is true for the D1 and D6 homoeopathic dilutions, where no results were obtained at all.

The disc diffusion test for antimicrobial activity is not a definitive one. Although the results obtained were disappointing, it is important to remember that this method is actually more of a screening method for any antimicrobial properties. (Rios, Recio & Villar, 1988.) Very minor results were obtained against *S. aureus* and *B. cereus* using the disc diffusion test, but there are more definitive methods that are discussed in the following chapter that may yield more encouraging results for these two bacterial species.

Research on the antimicrobial activity of medicinal plants has encountered some problems because of the diversity of criteria and techniques employed and the lipophilic properties of some samples. Diffusion and dilution methods have been employed to study the antimicrobial activity of medicinal plants. Since some factors (culture medium composition, microorganisms tested, inocula volume, extractive method, pH, solubility of the sample in the culture medium, incubation temperature) can change results, it is

difficult using these methods to standardize a procedure for the study of antimicrobial plants. (Rios, Recio & Villar, 1988.)

Diffusion methods are models with a low credibility for samples that are difficult to diffuse in the media because there is no relation between diffusion power and antimicrobial activity. They are well suited however for the preliminary screening of pure substances. (Rios, Recio & Villar, 1988.)

Apart from varying the methodology as an *in vitro* study, *A. amatymbica* could also be tested *in vivo* by means of a controlled clinical trial. This plant may not have been effective *in vitro*, but perhaps when used *in vivo*, in clinical trials, it may have more of an inhibitory effect on the growth of these bacteria. It is possible that a substance showing no inhibition *in vitro* could have a significant effect *in vivo*, due to an indirect pathway, e.g. via the immune system.

The results of this experiment do not correlate well with a review of the related literature, concerning the bacteria and herbal tincture chosen. *A. amatymbica*, briefly, is used as a remedial agent mainly for abdominal and chest complaints. The bacteria included in the study have a predilection for these sights, yet were insensitive to any antibacterial properties that the tincture may have had. It is possible that further experimentation with other bacteria that are commonly found in the same sites might yield more encouraging results.

CHAPTER SIX

6.1 CONCLUSIONS

The purpose of this study was to investigate the efficacy of *A. amatymbica* tincture, D1 and D6 dilutions as antibacterial agents against *S. aureus*, *Ps. aeruginosa*, *K. pneumoniae*, *B. cereus* and *E. coli* in terms of the disc diffusion test.

The selection of bacteria was based on the ethnobotanical use of *A. amatymbica* in Southern Africa as a remedial agent, and this study sought to determine whether there was any scientific validation for its use in disorders that this plant is commonly used for.

The results of the experiment did not reveal any significant antimicrobial activity for *A. amatymbica* tincture and homeopathic dilutions thereof against any of the bacterial species.

6.2 RECOMMENDATIONS REGARDING MATERIALS AND METHODOLOGY

- Use of the agar-overlay method using a hole or reservoir in the agar medium is recommended. A reservoir that has been drilled into inoculated agar and that contains the sample to be tested, is brought into contact with the inoculated medium. After incubation, the diameter of the clear zone around the reservoir (inhibition diameter) is measured. In order to lower the detection limit, the inoculated system can be kept at a low temperature before incubation, which favours diffusion through the culture medium and this increases the inhibition diameter. (Rios, Recio & Villar, 1988.)

- Dilution methods are another alternative and are those that require a homogenous dispersion of the sample in water. They can be used in the preliminary screening for antimicrobial activity, but are principally used to determine the minimum concentration values of an extract, essential oil or pure substance, which this study does not concern itself with. (Rios, Recio & Villar, 1988.)
- Bioautography is an important detection method for new or unidentified antimicrobial compounds and could be another consideration. The test substance initially undergoes Thin Layer Chromatography. The typical procedure is based on the agar-diffusion technique, whereby the antibacterial compound is transferred from the chromatographic layer to an inoculated agar plate. Inhibition zones are visualised by dehydrogenase-activity-detecting reagents. (Rios, Recio & Villar, 1988.)
- An alternative method for inoculating the agar plates is to suspend molten Mueller-Hinton agar at 48°C and then inoculate this with a predetermined concentration of bacteria. This mixture is then poured over pre-prepared base plates forming a homogenous layer, and allowed to set. Filter paper discs are placed accordingly and the plates are incubated as per normal. (Rabe & van Staden, 1997.)
- An improvement on the dry disc technique utilized in this study might be to use wet discs with re-pipetting of a certain amount of the test substance onto the discs a few times daily over the 48 hour period.

- It is important that prior to tincture manufacture, a voucher specimen of the plant must be deposited at a herbarium for accurate identification. This is to ensure that the correct species of plant is used in the tincture manufacture. (McGaw, Jager & van Staden, 2000). Cotton (1996) states that the voucher specimen must exhibit the main features required for its identification and the range in variation in these features (such as leaf shape or size). A good collection should ideally include samples of all the plant organs, and at all stages of development. It is particularly important that intact examples of reproductive structures are represented wherever possible, for quick identification.
- Thin Layer Chromatography (T.L.C) of the tincture to be used in the study is recommended in order to identify and quantify all its chemical constituents. Even though of the same species, the plant being tested might have differing quantities of constituents depending on various factors, e.g the location where the plant grew.
- The *A. amatymbica* tincture was manufactured from dry material by Parceval (pty) Ltd. Preparation from fresh material might have yielded different results.
- The choice of extractant may also yield different results. Various solvents have been used to extract plant metabolites. Solvents with increasing polarity (ether, petroleum ether, chloroform, ethyl acetate and ethanol) are employed in the extraction from dried plant material. If extraction is to screen plants for antimicrobial components, the extractant must not exert too toxic an effect on the

test organisms. For bioassay screening methods, acetone instead of ethanol, methanol and water as an extractant might be better as acetone is able to dissolve both hydrophilic and lipophilic components of the plant, is miscible with water, is volatile and has a low toxicity to the test organisms. It is interesting that traditionally plant extracts are prepared with water (for example, infusions, decoctions, and poultices). (Eloff, 1998.)

- As in this study, if the method of air-drying the filter paper discs prior to placing them on the inoculated agar plates is chosen, a multi-impregnation technique for the filter paper discs is advised. Use as many stages to impregnate the discs with the desired amount of substance as time factors will allow for. This will ensure maximal absorption of the test/control substance by the filter paper discs.
- Ensure that standard microorganisms (ATCC or similar) are being used. Geographically isolated pathogenic microorganisms should never be used. (Rios, Recio & Villar, 1988).

6.3 OTHER RECOMMENDATIONS

- Apart from recommending alternatives to the methodology of an *in vitro* study, it is important that this plant be subject to controlled clinical trials and *in vivo* studies. It is possible that a remedy showing no inhibition *in vitro* could have a significant effect *in vivo*.
- During impregnation of filter paper discs with test/control substance, leave a large enough space around each disc so that no coalescence occurs between

neighbouring discs. This ensures that each disc absorbs the correct amount of test/control substance.

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