# The efficacy of *Calendula officinalis* tincture as an antibacterial on *in vitro* Pseudomonas aeruginosa

by

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Dissertation submitted in partial compliance with the requirements for the Master's Degree in Technology: Homoeopathy, in the Faculty of Health Sciences at the Durban Institute of Technology.

I, Mbuso Mabuza, declare that this dissertation represents my own work, both in conception and execution.

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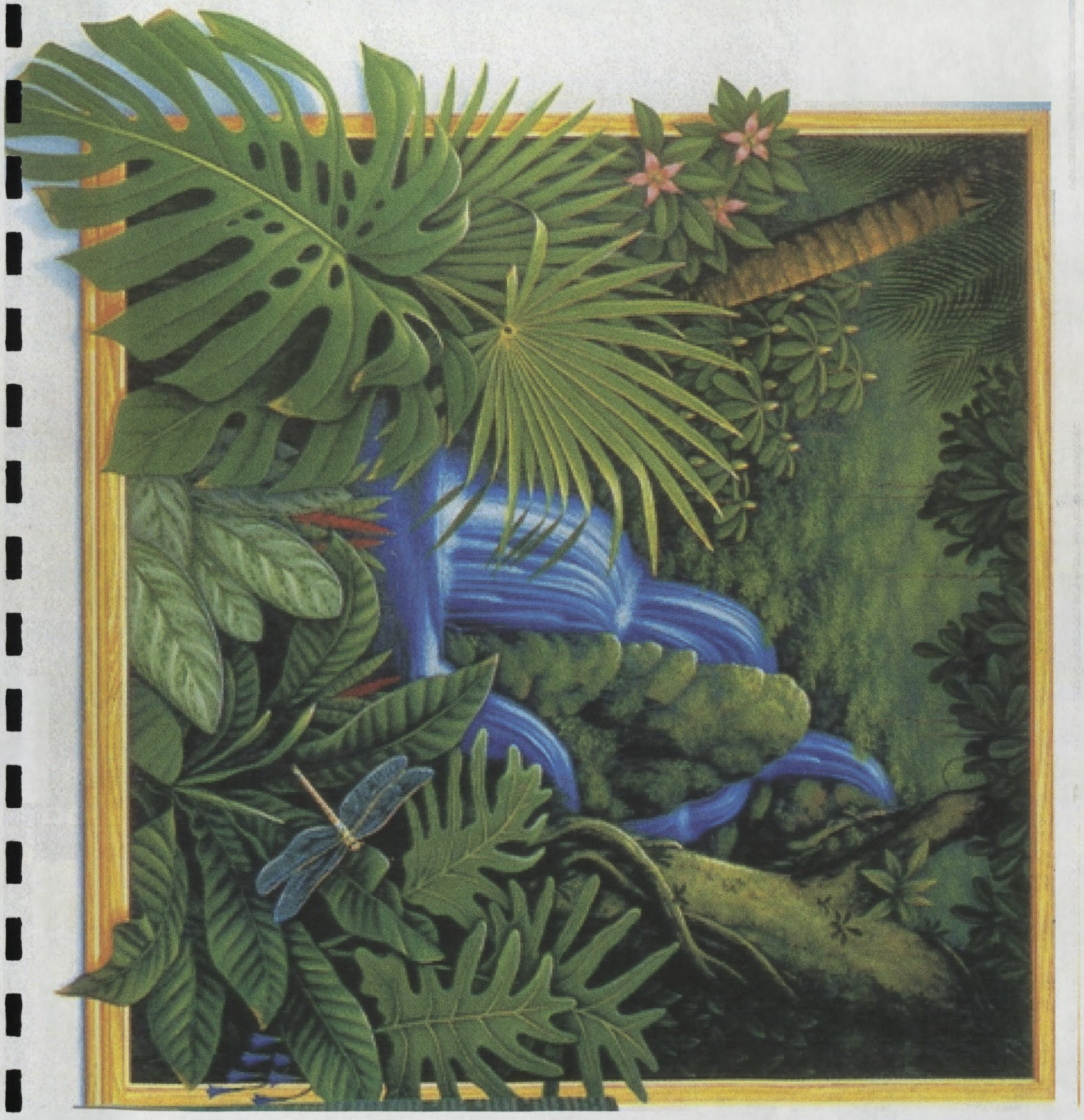


This one is for my parents, my siblings and our patients,

with much love.

When we reach beyond ourselves, and give effortlessly and wholeheartedly, we become different and fuller, and we find a deep happiness within ourselves.

Giving ourselves wholeheartedly opens up creativity and vision; through that we open doors and see, with levels of awareness that love conquers all pain and leads to true success in every area of our lives.

Living life with a sense of love, awareness, simplicity and meaning is the bridge home, the bridge to fulfilment; through it we truly know ourselves, others and spiritual peace.

The truth is that every day can be a great day in your life if you are prepared to appreciate and utilise the value of this present moment. Your attitudes today will influence your tomorrows so, really, today can be the greatest creative moment of life.

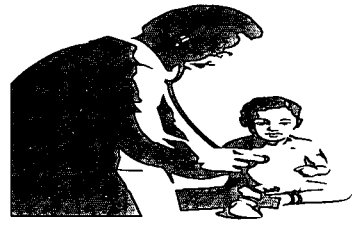
Let your heart – and – soul become a cool oasis of tranquillity.

Every experience is an arch where through gleams that untravelled world, whose margins fade forever and forever as we move.

Here's to immortality - cheers!

2002 *Mbuso Mabuza*

## EVERY PERSON MATTERS



*Since time began, clinicians have asked themselves two questions about their patients: "What is the diagnosis?" and "What is the treatment?". If they asked instead: "What is the problem?", "What is the cause?", "Is it preventable?", "Why was it not prevented in this case?", "What can I do to help*

*this patient, this family, this community?", "What can they do to help themselves?" and we try to find answers to all these questions and to act on them, great advances are possible in the health of communities, and also incidentally in the field of medicine in general.*

*At the same time, one needs to learn to respect the power of nature to heal and the relative importance of medicine to influence this process except, often, adversely.*

*Durban 2002* *Mbuso Mabuza*

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If, during the course of your life, you come into contact with a truly great human being, it can only enrich your soul, and possibly help you to become a better person.

Many people come and go. Some people stay for a while, and leave an experience of hope indelibly printed on our minds.

How vital it is that we keep a finger on the pulse of our own lives.

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It has been a long, worthwhile and exciting journey so far, one filled with hurt and joy. If I have triumphed, it is because of my faith and because of those who touched my life along the way, helped me through the worst of times, and left the lasting impression that they are, by any measure, the very best.

Finally, to the *Most High*, who gave me the courage to embark on my life as a doctor and who has stayed by my side through it all - all my love and heartfelt thanks.

*Durban 2002* *Mbuso Mabuza*

## ABSTRACT

The aim of this *in vitro* microbial study was to evaluate the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial on *in vitro* Pseudomonas aeruginosa. The standardised disc - diffusion method was employed. Seven pairs of Mueller - Hinton agar plates were used.

From each pair of the agar - containing plates, one was the experiment and the other was the control.

Pseudomonas aeruginosa broth cultures were grown for 24 hours. A sterile cotton swab was wetted in the Pseudomonas aeruginosa broth culture and then streaked evenly in three directions over the entire surface of the agar plates to obtain a uniform inoculum.

Sterile filter paper discs ( 0.45 micrometre pore size; 5 millimetre diameter) were impregnated with standardised amounts of *Calendula officinalis* tincture 60% (v/v) ethanol. The first set of discs was impregnated with a neat dilution of *Calendula officinalis* tincture 60% (v/v) ethanol. Thereafter, the subsequent sets of discs were impregnated with a series of twofold dilutions of *Calendula officinalis* tincture 60% (v/v) ethanol. That is, the second set of discs was impregnated with a ½ strength dilution; the third set with a 1/4

strength dilution; the fourth set with a 1/8 dilution; the fifth set with a 1/16 dilution; the sixth set with a 1/32 dilution; and the seventh set with a 1/64 dilution. One disc from the first set was aseptically placed on the inoculated agar surface of each plate of the first pair. Similarly, one disc from the second set was aseptically placed on the inoculated agar surface of each plate of the second pair. The same procedure was followed whereby one disc from the 1/4, 1/8, 1/16, 1/32, and 1/64 dilutions were placed on the inoculated agar surfaces of each plate of the third pair, fourth pair, fifth pair, sixth pair, and seventh pair, respectively.

The plates were inverted and incubated at 37 degrees Celsius in ambient air for 24 hours. After incubation, the presence of clear zones of inhibition, if any were observed against a light background and the diameters of such zones were measured.

The whole procedure was performed in triplicate. This implies that 42 Mueller - Hinton agar plates and 42 discs were used.

Therefore, the total sample comprised 21 *Calendula officinalis* and 21 ethanol impregnated discs, respectively.

After comparing the variances by *F – test*, a proper Student's paired *t – test* (with equal variance) was employed to compare the means between two samples (significance level: $P < 0.05$).

The mean activities of the two treatment groups were 6.88 and 6.69mm, respectively.

The antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol was assessed by regression analysis ( significance level: $P < 0.05$; ANOVA).

According to the results of this study, the antibacterial activities of *Calendula officinalis* tincture 60% (v/v) ethanol and the 60% ethanol control groups showed a similar pattern on *in vitro* Pseudomonas aeruginosa.

There was no statistically significant difference between the experiment and the control groups ($P > 0.05$; t-test).

This study has demonstrated that there was no evidence to prove the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial on *in vitro* Pseudomonas aeruginosa.

## Contents

Contents

Contents

LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

**ADP :** Adenosine diphosphate

**ANOVA:** Analysis of variance

**CF :** Cystic Fibrosis

**Ig A :** Immunoglobulin type A

**Ig G :** Immunoglobulin type G

**LPS :** Lipopolysaccharide

**NCCLS :** National Committee for Clinical Laboratory Standards

## DEFINITION OF TERMS

**Aerobe:** an organism that lives and grows only in the presence of oxygen (Freeland, 1999:146-149).

**Agar:** a gelatinous substance used as a culture medium for bacterial growth (Fong **et al.** 1995: 407-433).

**Aliphatic:** pertaining to a hydrocarbon that does not contain an aromatic ring (Warner and Warner, 1989:661-668).

**Anaerobe:** an organism that lives and grows in the absence of oxygen (Freeland, 1999:146-149).

**Antibiotics:** chemotherapeutic substances of microbial origin that are capable of inhibiting or killing bacteria and other microorganisms (Fong **et al.** 1995: 407-433).

**Antibiotic Sensitivity Test:** a test in which chemical substances capable of killing or inhibiting the growth of microorganisms are added to suspensions of the intended microorganism and allowed to grow overnight in order to determine the bactericidal or bacteriostatic effect of the chemical substance (Fong **et al.** 1995: 407-433).

**Antiseptic:** a substance that inhibits bacterial growth both *in vitro* and *in vivo:* a chemical compound that can inhibit or stop the growth of bacteria without necessarily killing them (Freeland, 1999: 146-149).

**Bacteremia:** presence of living bacteria in the blood, with or without significant response on the part of the host (Fong **et al.** 1995: 407-433).

**Broad-spectrum antibiotics:** chemotherapeutic substances of microbial origin that are capable of inhibiting or killing both gram-negative and gram-positive bacteria (Fong **et al.** 1995: 407-433).

**Buffer:** a substance that, by its presence in a solution increases the amount of acid or alkali necessary to restore the hydrogen ion concentration to a neutral pH (Fong **et al.** 1995: 407-433).

**Diffusion:** process of spreading out or movement of molecules through a substance. The larger the molecules the slower the movement. Molecules of a solution of a high concentration move more rapidly toward molecules of a lesser concentration. Temperature and the size of the material holding the substances also influence the rate of movement (Fong **et al.** 1995: 407-433).

**Disinfectant:** any agent or chemical compound that kills pathogens in the inanimate environment or prevents microorganisms from causing a disease (Fong **et al.** 1995: 407-433).

***Enterobacteriaceae*:** family of gram-negative facultative anaerobic rod-shaped bacilli that inhabit the large intestine as well as soil, water, and plants. Several species can be opportunistic or nosocomial pathogens and some cause urinary and respiratory infections (Fong **et al.** 1995: 407-433).

**Facultative anaerobes:** organisms that can live and grow with or without the presence of molecular oxygen (Fong **et al.** 1995: 407-433).

**Fastidious:** organisms that are difficult to grow, requiring specialised or enhanced culture media for growth (Fong **et al.** 1995: 407-433).

**Flagella:** flexible whip-like structure found on cells and microorganisms, used as an organ of locomotion (Fong **et al.** 1995: 407-433).

**Gram stain:** standardised procedure for staining bacteria according to their ability to accept and retain dyes based on certain characteristics in the composition of their cell walls. The gram characteristics are used as the starting point in the identification process for all bacteria. Gram-positive bacteria retain the initial crystal violet stain due to the peptidoglycan and

teichoic acid in the cell wall that resist dissolution by the acid-alcohol decolorizer. Gram-negative bacteria lose their lipoprotein and lipopolysaccharide layer in the decolorizing process, leaving the now naked peptidoglycan layer to accept the safranin counter-stain (Fong **et al.** 1995: 407- 433).

**Immuno-compromised:** having the immune system weakened by the administration of immuno-suppressive drugs, irradiation, malnutrition, and by certain disease processes, such as AIDS (Fong **et al.** 1995: 407-433).

**Immuno-suppression:** inhibition of the formation of antibodies to antigens that may be present. It may be induced as part of therapy (as after organ transplantation), or it may be a result of clinical symptoms (Fong **et al.** 1995: 407-433).

***In vitro*:** refers to biological experiments done in Petri plates (Petri dishes) or test tubes (Fong **et al.** 1995: 407-433).

**In vivo:** "in living"; refers to laboratory experiments testing chemical agents in a living organism (Fong **et al.** 1995: 407-433).

**Morphology:** Form, structure and shape of organism (Fong **et al.** 1995: 407-433).

**Mutate:** to change the genetic material, which can eventually lead to a change in the characteristics of an organism (Fong **et al.** 1995: 407-433).

**Narrow-spectrum antibiotics:** chemotherapeutic substances of microbial origin that are capable of inhibiting or killing gram-negative or gram-positive bacteria, but not both types (Fong **et al.** 1995: 407-433).

**Nosocomial infection:** infection or disease not carried by the patient but that originated from within a hospital environment (Fong **et al.** 1995: 407-433).

**Obligate anaerobe:** microorganism that can live and grow only in the absence of molecular oxygen (Fong **et al.** 1995: 407-433).

**Opportunistic infection:** infection that does not pose a threat to a person with a normal healthy immune system but seizes the opportunity to infect those with a weakened immune system (Fong **et al.** 1995: 407-433).

**Pathogenic bacteria:** bacteria that are capable of producing disease (Fong **et al.** 1995:407-433).

**Pili:** fine, slender, hair-like structures arising from the basal bodies in the cytoplasmic membrane of many bacterial cells (Fong **et al.** 1995: 407-433).

**Pseudomonas aeruginosa:** gram-negative obligate aerobic rod-shaped bacillus that produces a greenish-blue pigment (cyanin) and fluorescein. It is a major cause of severe, often fatal, nosocomial infections as well as being associated as a secondary infection in patients with severe burns (Fong **et al.** 1995: 407-433).

**Tincture:** alcoholic or hydroalcoholic solution prepared from a chemical substance or an animal or vegetable drug (Fong **et al.** 1995: 407-433).

**Turbid:** cloudy state of a solution or substance (Fong **et al.** 1995: 407-433).

**Turbidimetric counting of microbes:** Within limits, the turbidity of a microbial suspension is proportional to the number of cells present. The size and shape of cells, as well as their refractive properties, all influence the relationship between cell numbers and turbidity. A rough indication of the number of microbes present in a suspension may be obtained by comparing the turbidity of the suspension with that of McFarland's standard tubes (Heritage **et al.** 1996:103).

**Virulence:** degree of pathogenicity or disease-producing ability of an organism (Fong **et al.** 1995: 407-433).

## CHAPTER ONE

### 1.0 Introduction

With the widespread emergence of antibiotic resistance, especially among potential nosocomial pathogens such as **Pseudomonas aeruginosa**, evaluation of alternative antimicrobial products such as *Calendula officinalis* is important (Cox **et al.** 2000:170-175). Such increases in antibiotic resistance among Pseudomonas species are in line with a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria.

Pseudomonas aeruginosa continues to be a major cause of infections even in Western society, in part because of its high intrinsic resistance to antibiotics. Hancock (2000: 247-255) has demonstrated that this intrinsic resistance arises from the combination of unusually restricted outer membrane permeability and secondary resistance mechanisms such as energy-dependent multi-drug efflux and chromosomally encoded periplasmic beta-lactamase.

Given this high level of natural resistance, mutational resistance to most classes of antibiotics can readily arise.

It has been established that Pseudomonas aeruginosa infections are the most troublesome in terms of nosocomial infections (Gordon **et al.** 2001:725-730).

For example, contaminated non-touch fittings in hospitals are a great hazard, especially when they are installed in risk areas such as in intensive-care units (Halabi **et al.** 2001:117-121). Moreover, Pseudomonas aeruginosa infections may accelerate lung disease in children, and this may result in a very high rate of mortality in paediatric wards (Kosorok **et al.** 2000:277-287).

Many pathogens are developing resistance to most currently used antibiotics, and there are increasingly frequent reports of pathogens which are resistant to almost all available antibiotics (Pennington, 2000:93-95). Antibiotic resistance in bacteria has been linked to over-use of antibiotics in animals and humans (Davies, 1998: 2-3).

***Calendula officinalis*** tincture is made from the leaves and flowers of the common marigold plant ( Pot Marigold ). Plant extracts such as *Calendula officinalis* tincture typically contain a number of active components. As a result, some provide multiple benefits and some provide a mixture of benefits and adverse effects (Petry and Hadley, 2001:55-59).

*Calendula officinalis* has antimicrobial properties, insignificant toxicity both with its one-time and chronic administration. It is also devoid of local irritation properties. However, inappropriate and extravagant use of *Calendula officinalis* can cause neurological toxicity (Iatsyno **et al.** 1979:77-81).

In essence, not much research has been done on *Calendula officinalis* as an antibacterial agent against the most troublesome antibiotic resistant bacterial species such as Pseudomonas aeruginosa (Petry and Hadley, 2001:57-60).

Quantitative assay of antibacterial agents by microbiological methods represents a special application of microbial inhibition. The tests have been so designed that a relationship exists between the degree of antimicrobial activity and the quantity of the antibacterial agent; that is, within certain limits of antibacterial concentration, proportionality exists between the amount of antibacterial and the degree of inhibition (Nicholls **et al.** 1989:1291-1303).

Therefore, the purpose of this study was to evaluate the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial agent on *in vitro* Pseudomonas aeruginosa. The results from this study may be of clinical significance in terms of contributing towards the development of an alternative to antibiotics.

## 1.1 Objectives

**1.1.1** To investigate the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial agent on *in vitro* Pseudomonas aeruginosa.

**1.1.2** To employ the NCCLS - approved standardised simple disc - diffusion method to determine the susceptibility of *in vitro* Pseudomonas aeruginosa to *Calendula officinalis* tincture 60% (v/v) ethanol.

**1.1.3** To compare the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol on *in vitro* Pseudomonas aeruginosa with that of 60% ethanol, by using a series of twofold dilutions of *Calendula officinalis* tincture 60% (v/v) ethanol as well as twofold serial dilutions of 60% ethanol.

# CHAPTER TWO

## 2.0 REVIEW OF THE RELATED LITERATURE

### 2.1 Pseudomonas aeruginosa

#### 2.1.1 Bacterioloy

Pseudomonas aeruginosa is an aerobic, motile, Gram - negative rod; its outstanding bacteriologic feature is the production of colourful water-soluble pigments. It is commonly found free living in moist environments, but is also a pathogen of plants, animals, and humans (Dart, 1996:113).

As a cause of infection, it is particularly important in patients with severe burns, cystic fibrosis (CF), haematologic malignancies, and other immuno-compromised states. Pseudomonas aeruginosa also demonstrates the most consistent resistance to all the medically important bacteria (Hancock, 2000:247-255).

#### 2.1.2 Morphology and Structure

Pseudomonas aeruginosa is generally slimmer and more pale staining than members of the *Enterobacteriaceae* family, but its length is comparable (0.5 x 2.5 micrometre). Its flagella are polar, but other morphologic differences from other Gram - negative bacteria are not sufficiently consistent to be diagnostically useful. Ultrastructural features are similar to those of other Gram - negative bacteria.

The lipopolysaccharide (LPS) present in the cell wall has a core-lipid A structure with both common and variable components. The polysaccharide side chains extending from the outer membrane LPS are believed to determine serologic specificity of the variable component. A mucoid exopolysaccharide slime layer may be present in some strains. Pili composed of repeating monomers of the pilin structural subunit extend from the cell surface (Dart, 1996:113).

### 2.1.3 Growth and Metabolism

Pseudomonas aeruginosa is an aerobe sufficiently versatile in its growth and energy requirements to use simple molecules such as ammonia and carbon dioxide as sole nitrogen and carbon sources. Thus, it does not require enriched media for growth, and it can survive and multiply over a wide temperature range (20 to 42 degrees Celsius) in almost any environment, including those with a high salt content. The organism uses oxidative energy producing mechanisms and has high level of cytochrome oxidase (oxidase positive). Although an aerobic atmosphere is necessary for optimal growth and metabolism, most strains will multiply slowly in an anaerobic environment if nitrate is present as an electron acceptor (Calfee, 2001: 11633-11637).

Growth on all common isolation media is luxurious, although not as rapid as that of the *Enterobacteriaceae*. Colonies are well developed after

overnight incubation, usually show blue / green pigmentation, and have a delicate, fringed edge. Confluent growth often has a characteristic metallic sheen and intense "fruity" odour. Haemolysis is usually produced on blood agar. In broth, a surface pellicle is formed, reflecting the organism's preference for aerobic conditions and chemotaxis toward oxygen (Calfee, 2001: 11633-11637).

### 2.1.4 Toxins and Extracellular Products

Most strains of Pseudomonas aeruginosa produce extracellular products, including a toxin termed exotoxin A, proteolytic enzymes destructive to tissues, lecithinase, collagenase, and an elastase that could account for the destruction seen in arterial walls (Passador **et al.** 1993: 1127-1130). Exotoxin A is 10,000 times more toxic to experimental animals than Pseudomonas endotoxin, and is found in more than 90% of clinical isolates. Mutants that lack exotoxin A have much decreased virulence for experimental animals, and antitoxin protects animals against otherwise fatal challenge with exotoxin - producing strains. The exotoxin A molecule possesses separate domains for cell membrane binding, translocation, and catalytic activity, all of which are distinct from diphtheria toxin. Exotoxin A enters cells via receptor - mediated endocytosis and is internalised into a low pH vesicle from which it translocates and reaches its target molecule. The toxin acts to inhibit protein synthesis by a mechanism identical to that of diphtheria toxin. It catalyses the ADP-ribosylation and thus the

inactivation of elongation factor 2, leading to shutdown of protein synthesis and cell death (Passador **et al.** 1993: 1127-1130).

Exotoxin S has been implicated as a virulence factor required by Pseudomonas aeruginosa for dissemination from burn wounds and for tissue destruction in patients with chronic lung infection. *In vitro* it ADP-ribosylates several proteins including vimentin and a *ras* protein, but the physiologically important target protein(s) is not yet identified. Pseudomonas aeruginosa also expresses an elastase that acts to inactivate a variety of biologically important proteins and processes. Its role as a virulence factor is supported by the list of substrates that it attacks, including elastin, human IgA and IgG, complement components, and some collagens. Pseudomonas aeruginosa elastase shows homology with other proteases including those produced by *Legionella pneumophila* and *Vibrio cholerae* (Passador **et al.** 1993:1127-1130).

### 2.1.5 Pseudomonas aeruginosa Disease

#### 2.1.5.1 Epidemiology

The primary habitat of Pseudomonas aeruginosa and other pseudomonads is environmental. They are found in water, soil, and various types of vegetation throughout the world (Dart, 1996:113).

Pseudomonas aeruginosa has been isolated from the throat and stool of 2 to 10% of healthy persons. Colonisation rates may be higher in hospitalised patients. Infection with Pseudomonas aeruginosa, rare in previously healthy persons, is one of the most important causes of invasive infection in compromised patients with serious underlying disease, such as leukemia, CF, and extensive burns. The organism's ability to survive and proliferate in water with minimal nutrients can lead to heavy contamination of any unsterile water, such as that in the humidifiers of respirators. Inhalation of aerosols from such sources can bypass the normal respiratory defence mechanisms and initiate pulmonary infection (Halabi, 2001:117-121).

Infections have resulted from the growth of Pseudomonas in medications, contact lens solutions, and even in some disinfectants. Sinks and faucet aerators may be heavily contaminated and serve as the environmental source for contamination of other items. It is important to recognise, however, that the simple finding of a few Pseudomonas aeruginosa in solutions or sites not normally sterile ( for example, drinking water or food)

is not in itself abnormal or a cause for alarm. The risk lies in the proximity between items susceptible to contamination and patients uniquely predisposed to infection (Wyatt, 1993:227-245).

### 2.1.5.2 Pathogenesis and Immunity

Although Pseudomonas aeruginosa is an opportunistic pathogen, it is one of particular virulence. The organism usually requires a significant break in first - line defences (such as a wound) or a route past them (such as a contaminated solution or intractable tube) to initiate infection (Dart,1996:113).

Attachment to epithelial cells is the first step in infection and is likely mediated by pili that belong to the family N-methylphenylalanine type pili similar to those of gonococcus, and *Vibrio cholerae*. Several other adhesins have been described that could be involved in the pathogenesis of respiratory infection. As yet, it is unclear which of these adhesins are operative during the infectious process (Prince, 1992: 251-260).

The virulence of Pseudomonas aeruginosa is therefore multifactorial and is under control of several regulatory pathways (Prince, 1992: 251-260).

Human immunity to Pseudomonas infection is not well understood, although some inferences can be drawn from animal studies and clinical observations. The strong propensity of Pseudomonas aeruginosa to infect

the immune - compromised host, particularly those with defective cell - mediated immunity, indicates that these responses are also important (Passador **et al.** 1993: 1127-1130).

### 2.1.5.3 Clinical Manifestations

Pseudomonas aeruginosa can produce any of the opportunistic extraintestinal infections caused by members of the *Enterobacteriaceae* family. Burn, wound, urinary tract, skin, eye, ear, and respiratory infections all occur and may progress to bacteremia. Pseudomonas aeruginosa is also one of the most common causes of infection in environmentally contaminated wounds (for example, osteomyelitis after compound fractures) (Howard, 1995:116).

Pseudomonas aeruginosa pneumonia is a severe infection particularly in patients with granulocytopenia (Howard, 1995:116). It is also a common cause of otitis externa, including "swimmer's ear" and a rare but life-threatening "malignant" otitis externa seen in diabetics. Folliculitis of the skin may follow soaking in inadequately decontaminated hot tubs that can become heavily contaminated with the organism (Wyatt, 1993:227-245).

The organism can cause conjunctivitis, keratitis, or endophthalmitis when introduced into the eye by trauma or contaminated medication or contact lens solution. Keratitis can progress rapidly and destroy the cornea within 24 to 48 hours.

In some cases of Pseudomonas aeruginosa bacteremia, cutaneous papules develop that progress to black, necrotic ulcers. It is called **ecthyma gangrenosum** and is the result of direct invasion and destruction of blood vessel walls by the organism (Howard, 1995:116).

### 2.1.5.4 Pseudomonas aeruginosa and Cystic Fibrosis

Pseudomonas aeruginosa is now the most common bacterial pathogen to complicate the management of patients with CF, an inherited disease of exocrine glands associated with excessive viscid mucus in the smaller respiratory passages. In a high proportion of cases the respiratory tract becomes colonised with Pseudomonas aeruginosa, which once established, becomes almost impossible to eradicate. This infection is a leading cause of morbidity and eventual death of these patients (May, 1991:191-206).
In CF patients the organisms do not invade the lung tissue, but remain in the bronchi, forming a kind of biofilm with associated microcolonies. A striking feature of this association is the presence of alginate-producing mucoid strains rarely seen in other circumstances. Several enzymes are involved in alginate biosynthesis, including a GDP mannose dehydrogenase that effectively channels carbohydrate intermediates into alginate. No less than four gene loci are involved in the transcriptional regulation of alginate biosynthesis. The environmental stimuli regulating the mucoid phenotype is unknown, although some investigators propose osmolarity and desiccation

signals that may exist in the lungs of CF patients. The end result is enhanced survival of Pseudomonas aeruginosa through adherence and avoidance of phagocytosis. Some studies indicate that the alginate also interferes with the access and /or action of antimicrobials (May, 1991:191-206).

## *2.2 Calendula officinalis*

### *2.2.1* Common Name

*Calendula officinalis* is commonly called Marigold or Pot Marigold (Roberts, 2000:12)

### 2.2.2 Scientific Classification

The marigold belongs to the composite family, *Compositae (Asteraceae)* (The World Book Encyclopedia, 1993:206).

### 2.2.3 Description

**Marigold** is a hardy flowering plant commonly grown in gardens. Marigolds are named for Mary, the mother of Jesus (Hammond, 1999:56). Marigolds range from 15 to 90 centimetres in height. They generally have feathery, fernlike leaves and yellow, orange, or reddish - brown flowers. The dwarf varieties are suitable for edging and for containers. In its wild state the flowers are smaller, single and bright orange. Most marigolds have a strong odour. All cultivated marigolds are *annuals* - that is, they live for only one year (The World Book Encyclopedia, 1993:206).

The name marigold also refers to the pot marigold (genus *Calendula*) and unrelated plants of several families. There are about 50 species of marigolds.

Six of these species are commonly cultivated: (1) African, also called Aztec or big, (2) French, (3) sweet - scented, (4) signet, (5) Irish lace and (6) Muster - John - Henry.

African marigold ( *Tagetes erecta* ), French marigold ( *Tagetes patula* ), and several other species are grown as garden ornamentals, although most species have strong - scented leaves. Members of the genus *Tagetes* have attractive yellow, orange, or red flowers that are solitary or clustered; leaves opposite each other on the stem that usually are finely cut; and bracts ( leaf-like structures ) that form a cup - shaped base below each flower head (The New Encyclopaedia Britannica, 1990:846-847).

### 2.2.4 Habitat and cultivation

All marigolds are native to an area that extends from the South western United States to Argentina. Spanish explorers took marigolds to Europe in the early 1500's. *Calendula officinalis* has thrived in Southern Europe (The World Book Encyclopedia, 1993:206).

The genus *Tagetes* consists of about 30 species native to South western North America, tropical America, and South America (The World Book Encyclopedia, 1993:206).

Marigolds are easy to grow. They can survive periods of dry weather better than most other garden flowers can. Many gardeners plant marigold seeds indoors in late Winter or early Spring. The seedlings are transplanted outdoors in a warm, sunny place in late Spring. Some marigolds produce an oil that repels *nematodes,* small worms that live as parasites on plant roots. Gardeners sometimes grow such marigolds with other plants to protect those plants from nematodes (The World Book Encyclopedia, 1993:206)

The *Tagetes* species has a strong, pungent, insect - repelling odour. It is often planted amongst vegetables to keep them insect free. It is of paramount importance to remember that none of the *Tagetes* species should in any way be used for medicine or cooking (Roberts, 1999:12).

*Calendula officinalis* has no insect - repelling properties, but this old fashioned Winter flowering herb is an amazing medicinal plant, its therapeutic properties having been well documented since the earliest times. In a 12$^{th}$ century Herbal it was suggested that merely looking into the brilliant bright orange calendula flowers would clear up eye ailments, improve the eyesight and clear the head! (Roberts, 1999:12).

*Calendula officinalis* is widely grown in southern Europe but grown as a garden plant in temperate regions around the world. It is easily grown from seeds, and it flourishes in almost all soils. It prefers a sunny site (Chevallier, 1998:72-74).

### 2.2.5 Harvesting

The flowers are cut as soon as they open from June onwards and dried in the shade (up to 55 degrees Celsius). For medicinal use, the whole plant is cut at ground level and the root is discarded (Hey, 1994:134).

### 2.2.6 Medicinal Uses

*Calendula* flowers have been used for healing since ancient times, but these healing properties were only recently substantiated by medical science.

Most of the key medicinal properties are resident in the *resins*, mainly concentrated at the base of the flowers.

*Calendula* heals wounds, as well as internal and external ulcers. It is an antibacterial (bacteriostatic), improves blood flow to the affected area and encourages formation of granulation tissue. It is antifungal and can be used to treat athlete's foot, ringworm and candida. The tincture applied neat to cold sores (herpes simplex) hastens healing and it can also improve acne. *Calendula* has a mild bitter tonic action which improves general health via the liver. It soothes the digestive tract, and can be used for colitis and any ailment infecting the mucous membrane of the intestinal tract. An infusion of calendula can be taken to regulate menstrual and menopausal problems (Hey, 1994:134).

### 2.2.7 Culinary Uses

*Calendula* is mainly used for eye appeal, as the spicy, tangy flavour is mild. The petals are carefully separated from the flower heads (they bruise easily) and used as a substitute for saffron. The petals can be used in rice dishes, stews, soups, custards, salads and cheese dips (Hey **et al**. 1996:28).

### 2.2.8 Other Uses

*Calendula* flowers steeped in boiling water make a brightening rinse for fair hair. The flowers are also decorative, both in the garden and as a cut flower in floral arrangements. To brighten potpourri, the petals are carefully removed from the flower heads, spread out to dry and then added to potpourri when ready (Hey **et al.** 1996:28).

### 2.2.9 Administration

TINCTURE (as prescribed); INFUSION (eyewash for inflamed eyes, mouthwash for ulcers and bleeding gums); CREAM (burns, sunburn, cracked nipples, nappy rash, eczema, ringworm); DOUCHE (vaginal thrush); COMPRESS (Hey, 1994:134).

### 2.2.10 Constituents and Pharmacology

The mother tincture is prepared from the upper part of the aerial portion, which consists of the flowering top and about 15cm of stalk. The flowers contain most of the active components (Demarque **et al.** 1997:86).

- *flavonoids*, *saponins* derived from *oleanolic acid* and *tannins* with a hypotensive activity;

***Saponins***: saponins are naturally occurring plant compounds that are

* similar in chemistry to the glycosides
* lather-producing when irritated under water.

Many saponins are extremely poisonous and will rapidly cause haemolysis (breaking down of red blood corpuscles) in the circulatory system. However, many common vegetables contain small amounts of saponins, but as they are poorly absorbed by the human digestive system they can be eaten with impunity. Others produce rapid symptoms of toxicity. Plants known to contain saponins should NEVER be eaten until their safety has been fully established (Hallowell, 1996:20-47).

***Tannins:*** tannins are organic by - products of plant growth. They are both astringent and irritant. Tannins can be found in all the plant families, but mostly the *Geraniaceae*, *Papilionaceae*, *Rosaceae.*

Tannins have a variety of medicinal properties. They are used because of their astringent action in the treatment of burns and varicose ulcers, thus giving some credibility to the old-folk remedy that suggests dipping burnt fingers in cold tea which is rich in tannins (Hallowell, 1996:20-47).

- organic acid, in particular salicylic acid with an antiplatelet activity;
- carotene, in large amounts, and manganese; these have the following effects when used externally:
    * anti-inflammatory, especially against mosquito bites,
    * antiseptic
    * antibiotic
- alcohols and *triterpene lactones* with the following actions:
    * antifungal (the **resins** especially),
    * antiviral
    * antibiotic
    * antitumour
- *essential oils* and organic acids with a choleretic activity.

***Essential oils:*** essential or volatile oils are actually waste products found within the glandular cells of certain plants.

The use of volatile oils in the treatment of disease is a highly specialised medical science. Although some essential oils can be taken internally, the usual mode of application is to apply the oil to the skin in a massage rub.

The oil is quickly absorbed and acts on the nervous system. However, essential oils should never be applied directly to an open wound as they may cause severe irritation (Hallowell, 1996:20-47).

- ***Resins***, *Bitter glycosides*, *Sterols* and *Mucilagens*.

***Glycosides:*** glycosides are a complex group of chemicals which contain in their chemical bonding:

* one non-sugar part (Aglycin)
* one or more sugar parts.

There are several types of glycosides, the most common of which are

Digitalis glycosides

Anthraquinone glycosides

Salicylic glycosides

Flavonoid glycosides

Phenolic glycosides

Most glycosides have a powerful action on the heart. It is NOT recommended that heart disease be subjected to self medication, and plants rich in glycosides must be used with great discretion (Hallowell, 1996:20-47).

***Mucilagens:*** mucilagens are by-products of metabolic processes that occur within the plant. They:

* swell when in contact with water
* take on a viscous or plastic constituency.

*Calendula officinalis* appears to astringe (tighten up) capillaries, explaining its therapeutic effectiveness in cuts, wounds and inflammatory conditions. It is one of the few herbs which is astringent but has a low tannin content, the astringency being due to the ***resins*** ( Chevallier, 1998:72-74).

### 2.2.11 Key actions

Anti-inflammatory, wound healer, antispasmodic, anti-haemorrhagic, bacteriostatic, stimulates sweating, stimulates onset of menstruation (Chevalier, 1998:72-74).

### 2.2.12 Key indications

**Internally:** Inflammatory disorders of the digestive system; infection or congestion of the lymphatic system; painful and irregular periods.

**Externally:** Healing inflamed and sore rashes / wounds / cuts / grazes etc; fungal skin infections; vaginal infections (Chevallier, 1998:72-74).

### 2.2.13 Combinations

With *Echinacea* and *Nettle* for chronic skin conditions; urticaria; chronic infections and chronic inflammatory states.

With *Wild Yam*, *Agnus Castus* and *Cramp Bark* for irregular and painful periods (Chevallier, 1998:72-74).

## 2.3 Ethanol

### 2.3.1 General Characteristics

Ethanol or ethyl alcohol is a small water soluble molecule (Warner and Warner, 1989:661-668).

Many compounds decrease the bacterial count when applied directly to a surface, but ethanol often has a very low therapeutic index, which makes it unsuitable for systemic administration (Warner and Warner, 1989:661-668).

Depending on use, ethanol can be classified as an antiseptic, an agent that destroys or inhibits microorganisms when applied to living tissue; and a disinfectant, an agent that performs the same function but is intended for use on inanimate objects.

The alcohols have been studied more thoroughly than any other class of disinfectants or antiseptics. Currently, only ethyl and isopropyl alcohols are used for their germicidal action (Warner and Warner, 1989:661-668).

### 2.3.2 Structure - Activity Relationships

The effectiveness of aliphatic alcohols is greater with increasing molecular weight and chain length. Bactericidal effectiveness in order of decreasing activity is primary normal > primary iso > secondary normal > tertiary (Warner and Warner, 1989:661-668)

### 2.3.3 Mechanism of Action

The antibacterial action of alcohols is thought to be due to a denaturing effect on proteins, a process that requires the presence of water. The fact that absolute ethyl alcohol is a less effective agent than aqueous alcohol helps confirm this mechanism of action.

Alcohols also inhibit phosphorylation systems. The greatest effect is seen in mitochondria with nicotinamide adenine dinucleotide (NAD) - linked substrates, probably because of the depletion of NAD (Warner and Warner, 1989:661-668).

### 2.3.4 Uses

A 70% aqueous solution of ethyl alcohol is considered optimal for activity. Ethyl alcohol in 70% concentration is bactericidal in 1 - 2 minutes at 30 degrees Celsius but less effective at lower and higher concentration. Experimentally, it is extremely difficult to differentiate degrees of activity for concentrations of 60% to 95%. In place of the 70% aqueous ethanolic solution, a mixture of 50% ethanol, 10% acetone, and 40% water is also used in the preoperative preparation of skin. This treatment does not sterilise the skin, but reduces the number of viable organisms. Ethyl alcohol is widely used for sterilisation of clinical thermometers (Warner and Warner, 1989:661-668).

Because of the many uses of and restrictions on ethyl alcohol, isopropyl alcohol is now widely used in its place. It is the largest alcohol by molecular

weight that is miscible in water, a property that facilitates the preparation of solutions. At higher concentrations, isopropyl and ethyl alcohol show equal antiseptic activities; at lower concentrations, isopropyl alcohol is more effective. It shows nearly equal activity at concentrations between 50% and 95% (Warner and Warner, 1989:661-668).

Other alcohols used as antibacterial agents are benzyl alcohol, phenyl alcohol and chlorobutanol. They are used as preservatives.

Glycols such as ethylene, propylene, and trimethylene are used in aerosols for air sterilisation (Warner and Warner, 1989:661-668).

### 2.3.5 Making Tinctures

Sometimes called macerations. Alcohol is much more effective than water for drawing out the medicinal properties of plants. The resultant mixture after soaking fresh or dried plants in alcohol is an extremely potent medicine that need only be administered in extremely small amounts. There are various methods used in the manufacture of tinctures, but the simplest way is to soak one ounce of the plant in one pint of alcohol for a period of eight weeks. The container should be shaken daily for the first four weeks.

CAUTION: NEVER use wood alcohol or methanol as this can be deadly poison (Hallowell, 1996:20-47).

## CHAPTER THREE

## 3.0 Materials and Methods

### 3.1 Materials

#### 3.1.1 Media and reagents (storage conditions)

**a.** Mueller- Hinton agar in 100 mm petri plates (2-8 degrees Celsius) - agar depth 5mm.

**b.** *Calendula officinalis* tincture 60% (v/v) ethanol stock solution 100 ml; was purchased from Floro Force, Cape Town, SOUTH AFRICA.

#### 3.1.2 Supplies

**a.** Sterile cotton - tipped swabs

**b.** Sterile Pasteur pipettes

**c.** McFarland turbidity standard

**d.** Sterile 5mm filter paper discs

**e.** Clean dry surface

## 3.2 Methodology

The standardised simple disc - diffusion method was employed in order to evaluate the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial agent on *in vitro* Pseudomonas aeruginosa. *Calendula officinalis* tincture 60% (v/v) ethanol was compared to 60% ethanol as a control. The whole procedure was performed in triplicate in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) (Jones, 2001:1285-1289).

### 3.2.1 Agar preparation

Seven pairs of 100mm petri plates were used. From each pair one was used as the experiment and the other as a control. Therefore, 14 plates of Mueller - Hinton agar were poured and allowed to solidify. The agar depth was set at 5mm per plate.

### 3.2.2 Preparation of serial dilutions of treatment

A series of twofold dilutions of *Calendula officinalis* tincture 60% (v/v) ethanol were made. That is, a series of seven sets of dilutions were made ( i.e. neat, ½, 1/4, 1/8, 1/16, 1/32, and 1/64). An analogous series of twofold tube dilutions of the 60% ethanol control (final volume 1.0 ml) were also made.

### 3.2.3 Impregnation of discs

Seven pairs of filter paper discs were set aside. One set of each pair comprised seven discs.

One set of each pair was impregnated with the corresponding dilution of *Calendula officinalis* tincture, and the other set was impregnated with the corresponding dilution of 60% ethanol. For example, one set of the first pair of discs was impregnated with the neat dilution of *Calendula officinalis* tincture 60% (v/v) ethanol, while the other set was impregnated with the neat dilution of 60% ethanol. Similarly, one set of the second pair of discs was impregnated with the ½ dilution of *Calendula officinalis* tincture 60% (v/v) ethanol, while the other set was impregnated with the ½ strength dilution of 60% ethanol. The same procedure was followed until the first and second set of discs of the seventh pair were appropriately impregnated with 1/64 dilution of *Calendula officinalis* tincture 60% (v/v) ethanol and 1/64 dilution of 60% ethanol, respectively.

### 3.2.4 Inoculum preparation

Using a sterile loop, 5 Pseudomonas aeruginosa colonies of similar colony morphology ( from overnight agar plate) were picked and placed in 3.0 ml phosphate - buffered saline as a diluent. This was shaken until an organism suspension equivalent to McFarland 0.5 turbidity standard was

obtained.

### 3.2.5 Inoculation and incubation

A sterile cotton swab was dipped into the Pseudomonas aeruginosa suspension, and excess fluid was expressed against the side of the tube. The dry surface of each Mueller - Hinton agar plate was inoculated by streaking the swab in three directions over the entire surface of the plate, in order to obtain a uniform inoculum. After each plate the swab was discarded in a battery jar containing a disinfectant solution (i.e, methylated spirit).

One of the discs that were impregnated with the neat dilution of *Calendula officinalis* tincture 60% (v/v) ethanol was aseptically and carefully placed on the surface of one of the Mueller - Hinton agar plates of the first pair and it was ensured that contact was made with the agar. One of the discs that were impregnated with the neat dilution of 60% ethanol was aseptically placed on the surface of the second Mueller - Hinton agar plate of the first pair.

Similarly, one of the discs that were impregnated with the ½ - strength dilution of *Calendula officinalis* tincture 60% (v/v) ethanol was aseptically placed on the surface of the first Mueller - Hinton agar plate of the second pair. One disc impregnated with the ½ -strength 60% ethanol was aseptically placed on the surface of the second Mueller - Hinton agar plate

of the second pair. The same procedure was followed until the seventh pair of Mueller - Hinton agar plates was reached. Therefore, the 1/4, 1/8, 1/16, 1/32, and 1/64 dilutions corresponded with the third, fourth, fifth, sixth, and seventh pairs of Mueller - Hinton agar plates, respectively.

The plates were inverted, and incubated at 37 degrees Celsius in ambient air for 24 hours. After incubation, the presence or absence of clear zones of inhibition was observed by examining each plate against a light background.

With the aid of a millimetre ruler, the diameter of such zones was measured. If zones of inhibition were absent or indistinct, the plates were incubated for an additional 24 hours before determining the final result. Any indistinct growth was considered as absence of antibacterial activity. Antibacterial activity was considered to be absent if the zone size was <7mm, and present if the zone size was >11mm. Antibacterial activity was considered to be intermediate if the zone size was between 7 and 11mm. Intermediate antibacterial activity would warrant further investigation by the agar dilution method. The diameter of each disc was included in the measurement of the zone of inhibition.

Once again, it is important to note that the foregoing procedure was performed in triplicate. This implies that a total of 42 Mueller - Hinton agar plates were used ( i.e. 21 experiments and 21 controls ).

Therefore, the total sample comprised 42 discs.

One disc per agar plate was preferred over five discs per plate in order to avoid any possibility of overlap of zones of inhibition.

### 3.3 Statistical Analyses

After comparing the variances by *F- test,* a proper Student's two-tailed *t – test* (with equal variance) was employed to compare means between two samples (significance level: $P < 0.05$). The antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol was assessed by regression analysis (significance level: $P< 0.05$; ANOVA). All statistical analyses were conducted with Stata (Stata Corporation, 4905 Lakeway Drive, Texas 77845, USA).

#### 3.3.1 Assumption:

The null hypothesis (Ho) states that there is no significant difference between the two treatment groups.

The alternative hypothesis (Ha) states that there is a significant difference between the two treatment groups.

## CHAPTER FOUR

### 4.0 Results

### 4.1 Antibacterial activity of *Calendula officinalis* tincture

Table 1 – Table 4 show that under the test conditions, *in vitro* Pseudomonas aeruginosa showed some susceptibility to a neat dilution of *Calendula officinalis* tincture 60% (v/v) ethanol. The twofold serial dilutions of *Calendula officinalis* tincture 60% (v/v) ethanol showed no antibacterial activity on *in vitro* Pseudomonas aeruginosa.

*In vitro* Pseudomonas aeruginosa was also susceptible to a neat dilution of 60% ethanol. The series of twofold dilutions of 60% ethanol diminished its antibacterial activity on *in vitro* Pseudomonas aeruginosa.

**Table 1:** Antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa.

| TEST | TREATMENT<br>1-Calendula<br>2-Ethanol | DILUTION<br>[Concentration] | ACTIVITY<br>(mm) |
|---|---|---|---|
| 1 | 1 | 1 | 15.5 |
| 1 | 1 | 0.5 | 6 |
| 1 | 1 | 0.25 | 6 |
| 1 | 1 | 0.125 | 6 |
| 1 | 1 | 0.0625 | 5.5 |
| 1 | 1 | 0.03125 | 5 |
| 1 | 1 | 0.015625 | 5 |
| 1 | 2 | 1 | 16 |
| 1 | 2 | 0.5 | 6 |
| 1 | 2 | 0.25 | 5 |
| 1 | 2 | 0.125 | 5 |
| 1 | 2 | 0.0625 | 5 |
| 1 | 2 | 0.03125 | 5 |
| 1 | 2 | 0.015625 | 5 |

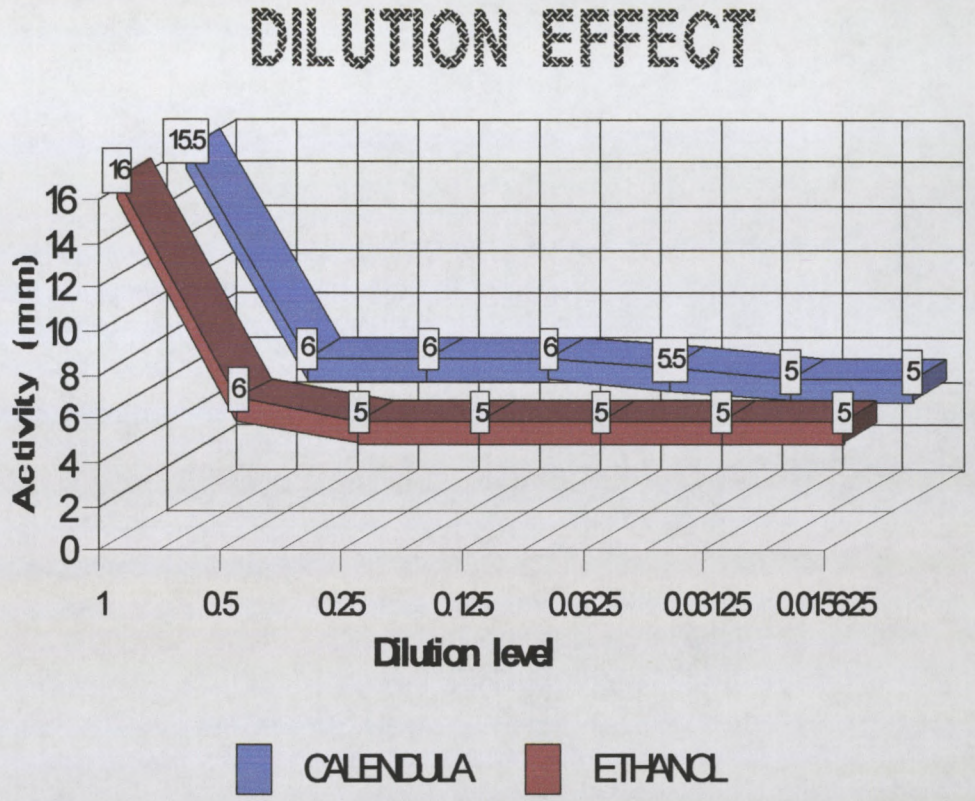


**Figure 1.** Effect of dilution on the antibacterial activity of *Calendula officinalis* 60% (v/v) ethanol versus 60% ethanol on *in vitro* *Pseudomonas aeruginosa*

**Table 2:** Antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa

| TEST | TREATMENT<br>1-Calendula<br>2-Ethanol | DILUTION<br>[Concentration] | ACTIVITY<br>(mm) |
|---|---|---|---|
| 2 | 1 | 1 | 17 |
| 2 | 1 | 0.5 | 5.5 |
| 2 | 1 | 0.25 | 5.5 |
| 2 | 1 | 0.125 | 5 |
| 2 | 1 | 0.0625 | 5 |
| 2 | 1 | 0.03125 | 5 |
| 2 | 1 | 0.015625 | 5 |
| 2 | 2 | 1 | 17 |
| 2 | 2 | 0.5 | 6 |
| 2 | 2 | 0.25 | 5.5 |
| 2 | 2 | 0.125 | 5 |
| 2 | 2 | 0.0625 | 5 |
| 2 | 2 | 0.03125 | 5 |
| 2 | 2 | 0.015625 | 5 |

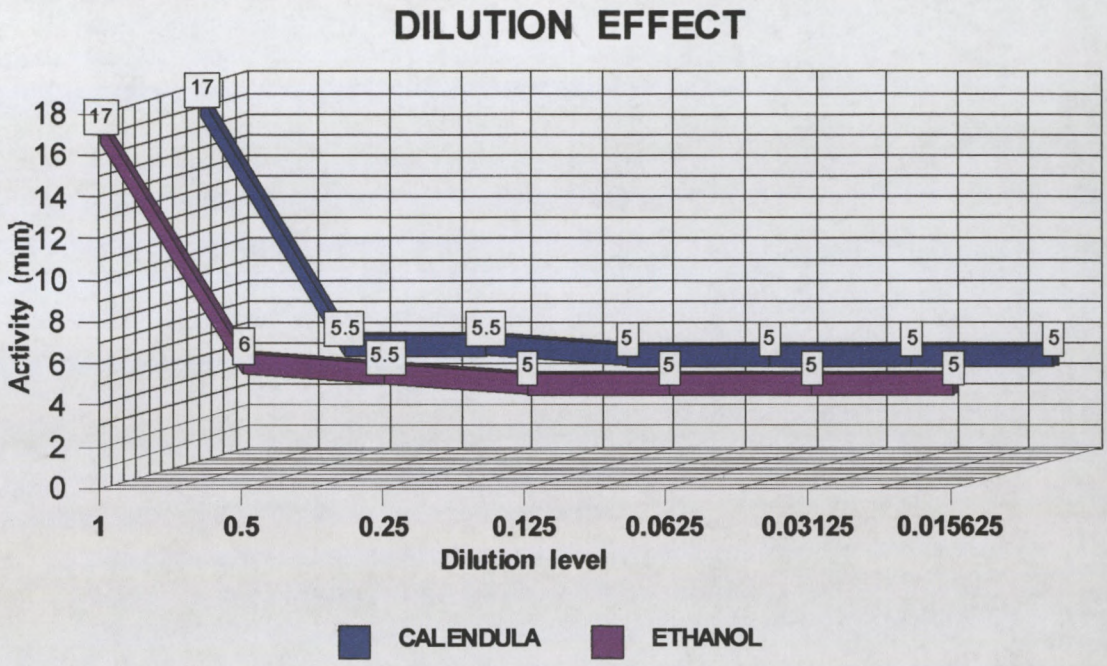


**Figure 2:** Effect of dilution on the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa.

**Table 3:** Antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa.

| TEST | TREATMENT<br>1-Calendula<br>2-Ethanol | DILUTION<br>[Concentration] | ACTIVITY<br>(mm) |
|---|---|---|---|
| 3 | 1 | 1 | 16 |
| 3 | 1 | 0.5 | 6 |
| 3 | 1 | 0.25 | 5 |
| 3 | 1 | 0.125 | 5 |
| 3 | 1 | 0.0625 | 5 |
| 3 | 1 | 0.03125 | 5 |
| 3 | 1 | 0.015625 | 5 |
| 3 | 2 | 1 | 15 |
| 3 | 2 | 0.5 | 5 |
| 3 | 2 | 0.25 | 5 |
| 3 | 2 | 0.125 | 5 |
| 3 | 2 | 0.0625 | 5 |
| 3 | 2 | 0.03125 | 5 |
| 3 | 2 | 0.015625 | 5 |

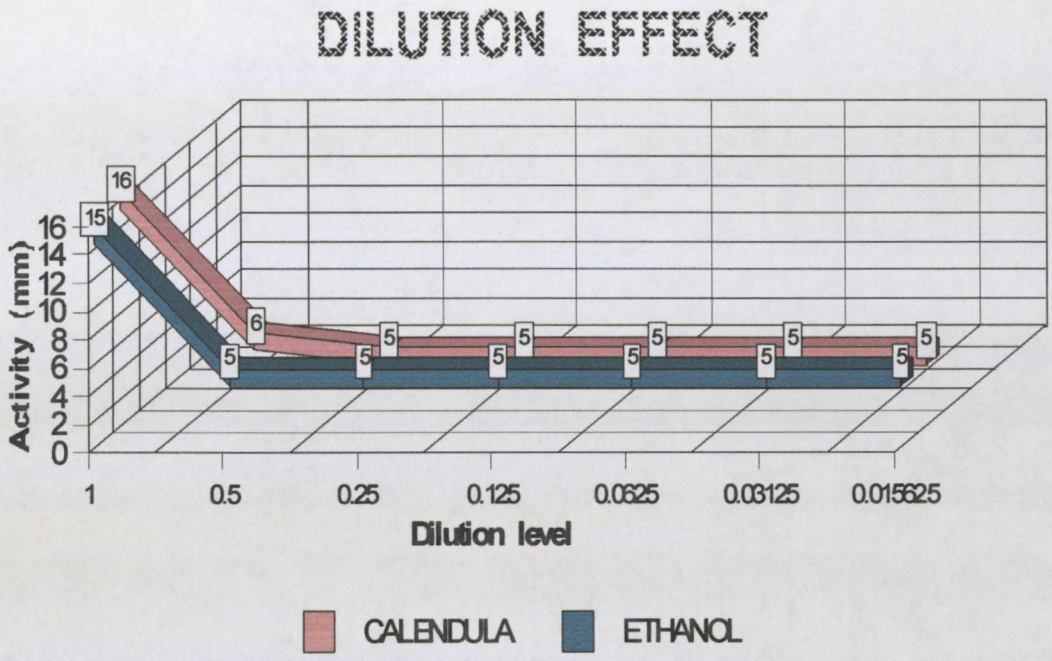


**Figure 3.** Effect of dilution on the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa.

**Table 4:** Mean effect of dilution on the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa.

| **TREATMENT**<br>1-Calendula<br>2-Ethanol | **DILUTION**<br>[Concentration] | **ACTIVITY**<br>(mm) |
|---|---|---|
| 1 | 1 | 16.2 |
| 1 | 0.5 | 5.8 |
| 1 | 0.25 | 5.5 |
| 1 | 0.125 | 5.3 |
| 1 | 0.0625 | 5.2 |
| 1 | 0.03125 | 5.0 |
| 1 | 0.015625 | 5.0 |
| 2 | 1 | 16.0 |
| 2 | 0.5 | 5.8 |
| 2 | 0.25 | 5.2 |
| 2 | 0.125 | 5.0 |
| 2 | 0.0625 | 5.0 |
| 2 | 0.03125 | 5.0 |
| 2 | 0.015625 | 5.0 |

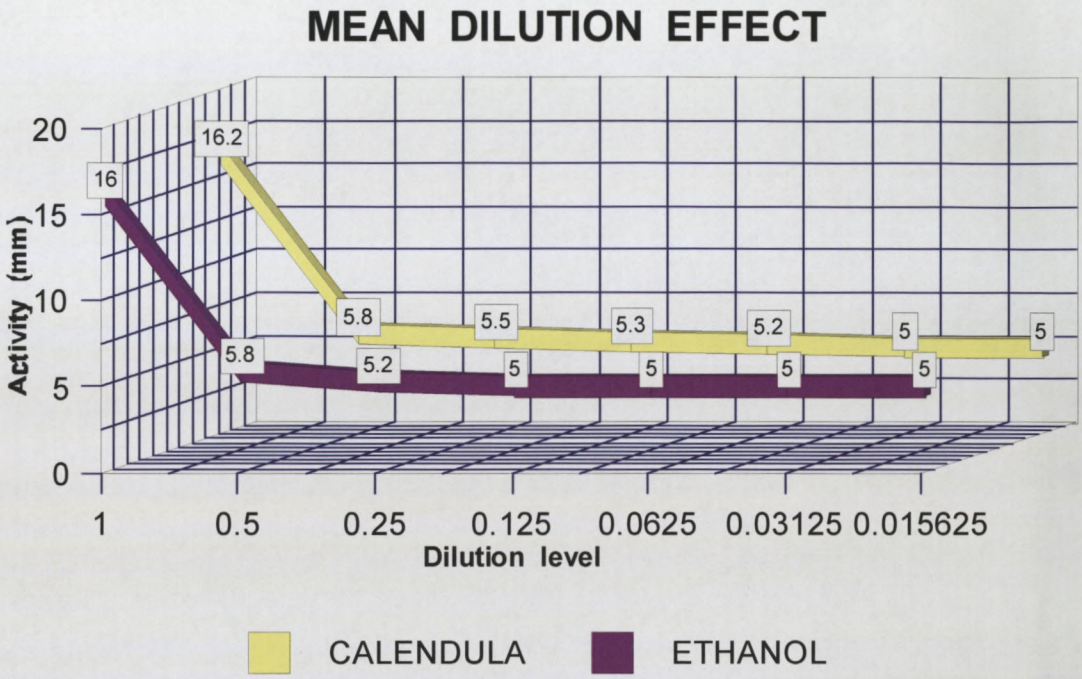


**Figure 4:** Mean effect of dilution on the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* Pseudomonas aeruginosa.

## 4.2 Statistical analyses

Neat dilution alone had a significant antibacterial effect on in vitro Pseudomonas aeruginosa ($P < 0.001$; ANOVA) (Table 8).

The mean activities of the two treatment groups were 6.88 and 6.69mm, respectively (Table 10).

The results demonstrate that there was no statistically significant difference between the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol and that of the 60% ethanol control ($P > 0.05$; t-test) (Table 10).

**Table 5: xi: regression log activity i. test i. treatment i. dilution**

i.test _Itest_1-3 (naturally coded; _Itest_1 omitted)

i.treatment _Itreatment_1-2 (naturally coded; _treatment_1 omitted)

Number of observations = **42**
F( 4, 37) = **57.50**
Prob > F = **0.0000**
R-squared = **0.8614**
Adj R-squared = **0.8464**
Root MSE = **.15842**

| Source | SS | df | MS |
|---|---|---|---|
| Model | **5.77235798** | **4** | **1. 4430895** |
| Residual | **.928617929** | **37** | **.02509778** |
| Total | **6.7 0097591** | **41** | **0.163438437** |

| Log act. | Coef. | Std.Err. | t | P> I t I | [95% Conf. Interval] | |
|---|---|---|---|---|---|---|
| _Itest_2 | **-.0083096** | **.0598782** | **-0.14** | **0.890** | **-.1296344** | **.1130151** |
| _Itest _3 | **-.0482189** | **.598782** | **-0.81** | **0.426** | **-.1695436** | **.0731058** |
| _Itreatment~2 | **-.0321459** | **.488903** | **-0.66** | **0.515** | **-.1312072** | **.0669153** |
| dilution | **1.114412** | **.0736712** | **15.13** | **0.000** | **.9651403** | **1.263684** |
| _cons | **1.532387** | **.0531642** | **28.82** | **0.000** | **1.424666** | **1.640108** |

**Table 6: xi: regression act i. test i. treatment dilution**

i.test _Itest_1-3 (naturally coded; _ Itest _1 omitted)

i.treatment _Itreatment_1-2 (naturally coded; _Itreatment_1 omitted)

Number of observations = **42**
F( 4, 37) = **44.23**
Prob > F = **0.0000**
R-squared = **0.8270**
Adj R-squared = **0.8083**
Root MSE = **1.6943**

| Source | SS | df | MS |
|---|---|---|---|
| Model | **507.8619** | **4** | **126.964047** |
| Residual | **106.215239** | **37** | **2.8068214** |
| Total | **614.071429** | **41** | **14.9773519** |

| Activity | Coef. | Std. Err. | t | P>I t I | [95% Conf. | Interval ] |
|---|---|---|---|---|---|---|
| _Itest _2 | .0714286 | .6403885 | 0.11 | 0.9212* | -1.226122 | 1.368979 |
| _Itest _3 | -.2857143 | .6403855 | -0.45 | 0.658* | -1.583265 | 1.011836 |
| _Itreatment~2 | -.1904762 | .522875 | -0.36 | 0.718 | -1.249922 | .8689692 |
| dil | 10.46548 | .787903 | 13.28 | 0.000 | 8.869041 | 12.06193 |
| _cons | 3.985566 | .5685839 | 7.01 | 0.000 | 2.833505 | 5.137626 |

**Table 7:** xi: regress act i.treatment dil

i. treatment _Itreatment_1-2 (naturally coded; Itreatment_1 omitted)

Number of obs = **42**
F(2, 39) = **92.19**
Prob > F = **0.0000**
R-squared = **0.8254**
Adj R-squared = **0.8164**
Root MSE = **1.658**

| Source | SS | df | MS |
|---|---|---|---|
| Model | **506.85619** | **2** | **253.428095** |
| Residual | **107.215239** | **39** | **2.74910869** |
| Total | **614.071429** | **41** | **14.9773519** |

| Activity | Coef. | Std. Err. | t | P>\|t\| | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| _Itreatment~2 | -.1904762 | .5116834 | -0.37 | 0.712 | -1.225453 | .8445011 |
| dil | 10.46548 | .7710387 | 13.57 | 0.000 | 8.905911 | 12.02506 |
| _cons | 3.914137 | .4227132 | 9.26 | 0.000 | 3.059119 | 4.769155 |

**Table 8:** anova act treatment dil

Number of obs = **42** R-squared = **0.9910**

Root MSE = **.403504** Adj R-sqaured = **0.9891**

| Source | Partial SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Model | **608.535714** | **7** | **86.93336735** | **533.94** | **0.0000** |
| | | | | | |
| Treatment | **.380952381** | **1** | **.380952381** | **2.34** | **0.1354** |
| Dil | **608.154762** | **6** | **101.359127** | **622.54** | **0.0000 *** |
| | | | | | |
| Residual | **5.53571429** | **34** | **.162815126** | | |
| Total | **614.071429** | **41** | **14.9773519** | | |

**Table 9:** tab dil

| dil. | Freq. | Percent | Cum. |
|---|---|---|---|
| .015625 | **6** | **14.29** | **14.29** |
| .03125 | **6** | **14.29** | **28.57** |
| .0625 | **6** | **14.29** | **42.86** |
| .125 | **6** | **14.29** | **57.14** |
| .25 | **6** | **14.29** | **71.43** |
| .5 | **6** | **14.29** | **85.71** |
| 1 | **6** | **14.29** | **100.00** |
| Total | **42** | **100.00** | |

**Table 10:** t test activity, by (treatment)

Two - sample *t test* with equal variances

| Group | Obs. | Mean | Std. Err. | Std. Dev | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | **21** | **6.880952** | **.8541947** | **3.914412** | **5.099134** | **8.662771** |
| 2 | **21** | **6.690476** | **.855289** | **3.919427** | **4.906375** | **8.474578** |
| Combined | **42** | **6.785714** | **.597163** | **3.870058** | **5.579719** | **7.991709** |
| diff. | | **.1904762** | **1.208788** | | **-2.252575** | **2.633527** |

Degrees of freedom: **40**

Ho: mean **(1)** - mean **(2)** = diff = 0

| Ha: diff < 0 | Ha: diff ~ = 0 | Ha: diff > 0 |
|---|---|---|
| t = 0.1576 | t = **0.1576** | t = 0.1576 |
| P < t = 0.5622 | P > \| t \| = **0.8756** * | P > t = 0.4378 |

## CHAPTER FIVE

### 5.0 Discussion

The emergence of antibiotic resistance is a major problem confronting clinicians and new approaches must be explored. Antibacterial activity of *Calendula officinalis* tincture and other plant extracts has received limited study (Abu - Ghazaleh, 2000: 433-440).

Therefore, in this study we have examined the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol on *in vitro* Pseudomonas aeruginosa.

The results of this study show that there was no significant difference between the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol and the 60% ethanol control on *in vitro* Pseudomonas aeruginosa ($P > 0.05$; t test) (Table 10).

The mean activities were 6.88 and 6.69 mm in groups 1 and 2, respectively, but there is no evidence that this is more than can be expected from sampling variation ($p=0.88$).

Even after adjusting for dilution and test in a regression model, the difference in activity is still not inconsistent with the null hypothesis of differences being due to chance ($p=0.72$). Converting the dilution and / or the activity to logarithms does not alter the result materially.

This lack of evidence against the null hypothesis of the treatments being equivalent, does not imply any evidence that they are the same. That is, it would not be proper to claim that we have proved them to be equivalent, rather we have merely failed to prove that they are different (which could be due to the sample being small).

Given the constituents of *Calendula officinalis* one would expect *Calendula officinalis* tincture to have a significantly pronounced antibacterial activity than ethanol. In fact, a synergistic antibacterial activity would have been expected between the constituents of *Calendula officinalis* and the 60% ethanol present in the tincture.

It is possible to postulate the reasons for the limited antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol. Firstly, the concentration of the components of *Calendula officinalis* in the tincture was probably far too low or absolutely nil. Secondly, the stability of the components of *Calendula officinalis* was possibly hampered by the storage conditions of the tincture and by the environmental conditions under which the study was carried out.

Furthermore, it must be borne in mind that *in vitro* environments are markedly different from *in vivo* environments. Therefore, results from *in vitro* studies are not always compatible with results from *in vivo* studies.

For example, a major difference between the bacterial colonies and biofilms in patients (such as those on vascular, urinary or peritoneal catheters; or on orthopaedic prostheses) or in aqueous environmental locations, is that the antibacterial agent - containing medium to which the colonies are exposed is static. Because of this lack of mixing, the antibacterial agent in the immediate vicinity of the colonies will rapidly be depleted, and this can be expected on apparent reduction in antibacterial susceptibility. However, even in the case of biofilms bathed in a well mixed liquid medium, there is still a diffusive mass - transfer region close to the film surface (Palenik **et al.** 1989:351-366).

The standardised simple disc - diffusion method is normally used to examine the efficacy of antibiotics in accordance with NCCLS guidelines. However, there are no guidelines for testing the antibacterial efficacy of plant extracts such as *Calendula officinalis* tincture. For instance, some of the guidelines that were employed in this study are those that are normally used for examining the susceptibility of *enterococci* to aminoglycosides.

By employing the standardised disc - diffusion method, the antibacterial efficacy of *Calendula officinalis* tincture is influenced by a complex of factors, such as the rate of diffusion of the tincture through the agar, the size of the inoculum, the rate of growth of the bacterial colonies, the

viability of the bacteria, and its susceptibility to the tincture. Pseudomonas aeruginosa is actually a non - fastidious and rapid growing bacterial species (Jones,2001:1285-1289).

Certainly, the stability of *Calendula officinalis* tincture at different temperatures must be taken into consideration. To illustrate this point, the stability of ozonised sunflower oil (oleozon) is documented. Oleozon is stable for up to 1 year in the temperature range -10 to +8 degrees Celsius. Moreover, it is stable for up to 6 months at room temperature (27-30 degrees Celsius); after this period, the antimicrobial properties diminish. The pH is also stable for up to 1 year in the temperature range -10 to +8 degrees Celsius. At 30 degrees Celsius, the pH is stable for up to 6 months (Sechi **et al.** 2001:279-284).

Therefore, there is a possibility that the efficacy of the constituents of *Calendula officinalis* is also influenced by factors such as temperature, pH and concentration.

## CHAPTER SIX

### 6.0 Conclusion and Recommendations

This study has demonstrated that there was no evidence to prove the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial on *in vitro* Pseudomonas aeruginosa.

There was no statistically significant difference between the antibacterial activity of the experiment and the control groups.

Based on the results of this study, it would be easy to conclude that the antibacterial properties of *Calendula officinalis* tincture 60% (v/v) ethanol can be attributed to the 60% ethanol present in the tincture.

In retrospect, it is essential to consider the fact that the concentration of the constituents of *Calendula officinalis* tincture was not determined. It would be essential to determine the stability of *Calendula officinalis* tincture 60% (v/v) ethanol, under the storage and experimental conditions.

Therefore, a quality control programme must be adopted in order to accurately examine the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial on *in vitro* Pseudomonas aeruginosa.

Further study is needed to determine the effects of additional factors such as inoculum size, inoculum pre-compared to post-addition of *Calendula officinalis* tincture 60% (v/v) ethanol, short term kill - curves, long term survival and possible selection of resistant variants.

Thus, since Pseudomonas aeruginosa is a common contaminant which causes infections with various vascular catheters, medications, contact lens solutions, non - touch fittings in hospitals, and even some disinfectants, *Calendula officinalis* may warrant further study as an additive to topical preparations or in other areas such as harvesting of blood or blood products, or even to peritoneal dialysis solutions, in order to reduce Pseudomonas aeruginosa contaminants.

This study should lead to the setting up of some clinical trials in order to compare the efficacy of *Calendula officinalis* tincture with other antibacterial agents. The wide availability of *Calendula officinalis* could make it a competitive antibacterial agent.

It will require the combined effort of both researchers in the clinical and laboratory fields to verify the usefulness of *Calendula officinalis* and other potential antibacterial agents and their clinical importance. Hopefully, in the near future, continued research and cooperation between clinical and laboratory researchers will bring Pseudomonas infection treatment protocols closer to the needs of the individual patient.

**Comment**

Important advances have been made in recent years to enable practitioners to gain a greater understanding of the medicines that they use. This has to be commended, for obvious reasons.

However, the methodological correlate of the mechanistic and deterministic materialist ontology which assumes that what is scientifically real (i.e., a necessary quality of scientific knowledge) is *what is physically measurable*, can be somewhat confusing in most instances.

We seem to be more concerned about disease than about health and we presume that attending to the health of individuals is sufficient to improve the health of society. We claim to be scientific and yet many new forms of therapy are introduced into practice before they have been scientifically validated, (for example, coronary artery bypass surgery). We claim that we have preserved the art of medicine, of the care for the whole individual, but daily we witness examples of failure in this vital aspect of medical practice.

Expectations are not being met, and as we claim that medicine is becoming more and more scientific, so patients begin to declare that this is just what they fear and dislike.

The growing demand on doctors to weigh the individual good against the common good creates further distrust at the individual level.

What we should contribute to the greater glory of medicine is not (only) the innovation of tools and their efficiency. We should strive to perfect the doctor, his or her insights and the depth of feelings. We must be concerned with the quality of the doctor's relationships and the mirror they provide.

If we consider ourselves healers, with power in our hands, it gets a bit metaphysical. The hardest part is to reduce oneself by humility, as there is so much that is not known. The path of healing is a process. It is not a matter of clearing up one issue or prescribing medicine and fixing people for life.

None of this suggests that we should retreat into therapeutic nihilism, or that patients should despair over medicine's uncertainties. There can only be benefits if we manifest more humility and honesty. Patients, too, might well become somewhat more realistic in their expectations.

The essence of good medical care must be to provide a good service to our patients. This must incorporate reviewing and assessing of what we do and how we do it. Today all of us must account for what we do. One of the major criticisms against practitioners is that we do not account for our actions. Accountability must incorporate our responsibility to ourselves, our patients, our colleagues and our profession to maintain appropriate standards.

It is a truism that we should learn from our mistakes, but how can we do it with the minimum time and effort? While statistics may suggest that all is not well, they do not tell us what to do. By keeping records of medical problems and asking why did it happen and then carrying out an intensive review, performance of clinical care can often be dramatically improved. It is our responsibility to restore and sustain trust and confidence in the medical profession.

Let research make its progress. It increases us all. But for the physician, it is the years scuffed softly underfoot - the odd moments of noting and welcoming what ripples from a single, momentary act - like serving a patient in love - that remains our saving grace. Our true business is serving the sick as a moral responsibility. That is all. Our achievement is more like a snapshot - not often clear or beautiful or well composed, but revealing in the virtues we strive for, the relationships we form, and all the chaotic elements that clutter the clinical frame. We are compensated for a lack of technical expertise by the variety of methods at our disposal - the test of time, the use of our senses, the auger of compassion propelling us into the good work and real lives of our patients.

All of us need to search deeply within ourselves for the strength and the courage to embark on new ways of thinking and working which will bring scholarship to bear on the realities of progress in our society and which

will enable us to be proud of the work we do as humane physicians concerned with the advancement of knowledge, the maintenance of professional ideals, high standards in the care of people who seek our services, and for the future health of our communities. We all have doubts about our ability to adapt, but adapt we must, with imagination and optimism, if we are to contribute to the survival of our future generations.

I believe we are on the way to a paradigm revolution in the ontology of science which will enable a new understanding of life (a new biology) and a new understanding of conscious life which will not negate what can be said from a materialist viewpoint, but will transcend the limitations of that point of view.

A more comprehensive approach is inevitable. A unified health service is required to achieve similar goals in health and the encouragement of centres of excellence. The current imbalance between private and public health services needs to be redressed.

Our health services are not isolated structures but are important components of whole systems. The demands for immediate piece-meal changes without planning for the health care system as a whole are unacceptable.

Given the enormous disparities and selfishness which have characterised our profession these challenges can only be met if we can successfully

bring together and create harmony between the needs of individuals and the collective needs of our society.

Our efforts in planning for the future will be severely hampered if we do not face the enormous challenges squarely.

The more fragmented and stereotyped we are, the more complex the tapestry of the healing system becomes, interweaving the intrinsic and the extrinsic, the self and others. The threads extend in every direction – but perhaps the strongest and most sustaining are the ties that bind.

May we have the courage and the wisdom to see things as they are, and not as we are.

May we continue to grow in understanding, and the ability to act on that understanding.

In the meantime, the air is filling with the perfume of promise.

Thanks for reading along with me. I hope our paths will cross someday.

Mbuso Mabuza

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## APPENDIX 1

*[RAW DATA]*

### TEST 1

***Table A.1*** **The antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus that of 60% ethanol on *in vitro* Pseudomonas aeruginosa**

| **Sample No. &[ dilution]** | **EXPERIMENT** [Calendula officinalis tincture 60%(v/v)ethanol] | | **CONTROL** [60% Ethanol] | |
|---|---|---|---|---|
| | Zone of inhibition | Activity | Zone of inhibition | Activity |
| 1. [neat] | 15.5mm | + | 16.0mm | + |
| 2. [1/2] | 6.0mm | _ | 6.0mm | _ |
| 3. [1/4] | 6.0mm | _ | 5.0mm | _ |
| 4. [1/8] | 6.0mm | _ | 5.0mm | _ |
| 5. [1/16] | 5.5mm | _ | 5.0mm | _ |
| 6. [1/32] | 5.0mm | _ | 5.0mm | _ |
| 7. [1/64] | 5.0mm | _ | 5.0mm | _ |

## APPENDIX 2

## [RAW DATA]

***TEST 2***

***Table A.2*** **The antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus that of 60% ethanol on *in vitro* Pseudomonas aeruginosa.**

| **Sample No. & [dilution]** | **EXPERIMENT** [Calendula officinalis tincture 60%(v/v)ethanol] | | **CONTROL** [60% Ethanol] | |
|---|---|---|---|---|
| | Zone of inhibition | Activity | Zone of inhibition | Activity |
| 1. [neat] | 17.0mm | + | 17.0mm | + |
| 2. [½] | 5.5mm | _ | 6.0mm | _ |
| 3. [1/4] | 5.5mm | _ | 5.5mm | _ |
| 4. [1/8] | 5.0mm | _ | 5.0mm | _ |
| 5. [1/16] | 5.0mm | _ | 5.0mm | _ |
| 6. [1/32] | 5.0mm | _ | 5.0mm | _ |
| 7. [1/64] | 5.0mm | _ | 5.0mm | _ |

## APPENDIX 3

## [RAW DATA]

***TEST 3***

***Table A.3*** **The antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus that of 60% ethanol on *in vitro* Pseudomonas aeruginosa.**

| **Sample No. & [dilution]** | **EXPERIMENT** [Calendula officinalis tincture 60%(v/v)ethanol] | | **CONTROL** [60% Ethanol] | |
|---|---|---|---|---|
| | Zone of inhibition | Activity | Zone of inhibition | Activity |
| 1. [neat] | 16.0mm | + | 15.0mm | + |
| 2. [½] | 6.0mm | _ | 5.0mm | _ |
| 3. [1/4] | 5.0mm | _ | 5.0mm | _ |
| 4. [1/8] | 5.0mm | _ | 5.0mm | _ |
| 5. [1/16] | 5.0mm | _ | 5.0mm | _ |
| 6. [1/32] | 5.0mm | _ | 5.0mm | _ |
| 7. [1/64] | 5.0mm | _ | 5.0mm | _ |

## APPENDIX 4

**FINAL RESULT [ i.e. AVERAGE OF THE THREE TESTS]**

Table A.4 The antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus the antibacterial activity of the 60% ethanol control on *in vitro* Pseudomonas aeruginosa.

| **Sample No. & [dilution]** | **EXPERIMENT** [Calendula officinalis tincture 60%(v/v)ethanol] | | **CONTROL** [60% Ethanol] | |
|---|---|---|---|---|
| | Zone of inhibition | Activity | Zone of inhibition | Activity |
| 1. [neat] | 16.2mm | + | 16.0mm | + |
| 2. [½] | 5.8mm | _ | 5.8mm | _ |
| 3. [1/4] | 5.5mm | _ | 5.2mm | _ |
| 4. [1/8] | 5.3mm | _ | 5.0mm | _ |
| 5. [1/16] | 5.2mm | _ | 5.0mm | _ |
| 6. [1/32] | 5.0mm | _ | 5.0mm | _ |
| 7. [1/64] | 5.0mm | _ | 5.0mm | _ |