A CONTROLLED *IN VITRO* STUDY OF THE EFFECTIVENESS OF *TULBAGIA VIOLACEA* IN HERBAL TINCTURE AND HOMOEOPATHIC DILUTION (1X AND 6X) AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA.

BY

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I, Jonathan Reuben Rai Invernizzi do declare that this mini-dissertation represents my own work in both conception and execution.

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DEDICATION

I would like to dedicate this work to my parents, Rai and Avril Invernizzi, for all that they have done for me, and without whom I could never have achieved what I have.

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ABSTRACT

The purpose of this study was to determine the effect that *Tulbagia violacea* ethanolic herbal tincture, and *Tulbagia violacea* 1X and 6X homoeopathic potencies, had on the *in vitro* growth inhibition of *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus cereus* respectively, as compared to a ethanol negative control. The final results were expressed as a ratio to the values obtained from gentamycin and vancomycin. Measurement was by means of the disc-diffusion assay.

For this study fifteen Mueller-Hinton agar plates were prepared and inoculated with each test bacteria in turn. Filter paper discs were individually inoculated with the sample substances and the control using a micropipette, before being allowed to air dry. One disc each of the *Tulbagia violacea* herbal tincture, 1X potency, 6X potency, ethanol control, as well as a gentamycin and vancomycin disc were placed equidistantly apart on each plate. The gentamycin and vancomycin discs were included in the experiment with the sole purpose of accounting for plate-to-plate variations in the pharmacological sensitivity of the same species of bacteria.

The plates were incubated at 37°C, and the zones of inhibitions measured with a pair of Vernier callipers at 18 hour, 24 hour and 36 hour intervals.

Data entry and analysis was done using the SPSS® statistical package. The Friedman test was used for intra-group comparison of each test or control substance at 18 hours, 24 hours and 36 hours. The Mann-Whitney U test was used to compare the mean inhibition zones produced by the test and control substance after 18 hours, 24 hours and 36 hours of incubation. The tests were performed at α=0.05 (5%) level of significance.

The results obtained were that the *Tulbagia violacea* herbal tincture, and 1X and 6X homoeopathic potencies did not produce a statistically significant inhibitive effect on

any of the bacteria tested. No growth inhibition was evident around any of the *Tulbagia violacea* samples. This consequently resulted in all the values for the ratios of gentamycin and vancomycin to the *Tulbagia violacea* samples which was taken as the measure of the anti-microbial effectiveness of the samples, being reflected as zero, and the resultant p-values for all the Mann-Whitney U tests performed comparing the samples to the negative control being equal to 1.000.

The results concluded that the *Tulbagia violacea* ethanolic herbal tincture, and 1X and 6X homoeopathic potencies utilised, were ineffective in inhibiting the *in vitro* growth of any of the bacteria tested when evaluated in terms of the disc-diffusion assay.

The results were consistent with the findings of McGraw, Jager and van Staden (2000:254), but contradicted the writings of Watt and Breyer-Brandwijk (1962:717), who claimed a positive anti-microbial response against *Escherichia coli*.

Dilution tests on the efficacy of *Tulbagia violacea* essential oils and other constituents as anti-microbial agents are the next logical step in investigating the pharmacological properties of this indigenous plant. Following that, controlled *in vivo* clinical trials may contribute greatly to the relatively sparse knowledge we have about this herb.

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ABBREVIATIONS AND DEFINITION OF TERMS

HOMOEOPATHY The medical art and science developed by Samuel Hahnemann (Brewster O'Reilly, 1996:314).

POTENCY The especially produced capability in a medicine to effect a dynamic stimulus (Gaier, 1991:601).
Potency may also refer to the resultant degree of potentisation of a homoeopathic medicine (Brewster O'Reilly, 1996:339).

POTENTISATION The imparting of the pharmacological message of the sample substance by means of a process involving trituration and succussion (Gaier, 1991:441).

SUCCUSSION Vigorous shaking with impact. Part of the process of potentising substances to bring out their medicinal powers. (Brewster O'Reilly, 1996:353.)

TINCTURE An alcoholic or hydroalcoholic solution containing the active constituents of herbs (Murray, 1992:312).

TRITURATION A dry method of potentising substances, whereby the substance is finely ground in a mortar, with a certain proportion of milk sugar, thereby progressively attenuating it (Brewster O'Reilly, 1996:358).

X Represents a potency in the serial range of 1:10 dilutions, e.g. 6X is the sixth potency in the range, representing a dilution of 1:1000000 which has been succussed (Kayne, 1997:50).

ANOVA	- Analysis of variance
ATCC	- American Type Culture Collection
B. cereus	- *Bacillus cereus*
B. subtilis	- *Bacillus subtilis*
E. coli	- *Escherichia coli*
K. pneumoniae	- *Klebsiella pneumoniae*
M. tuberculosis	- *Mycobacterium tuberculosis*
Ps. aeruginosa	- *Pseudomonas aeruginosa*
Staph. aureus	- *Staphylococcus aureus*

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

Herbs are the basis of many different medicinal systems around the world, being especially popular in less developed countries where western drugs prove to be too expensive to provide a basis of treatment for the majority of the population (Balick and Cox, 1996:7). Herbal medicine can also provide clues to overcoming the increasing incidence of conventional drug-resistance in bacteria, and the associated escalation of nosocomial infections (De Smidt, 2001:7).

Tulbagia violacea [See figure 1.1] has long been recognised by South African ethnobotanists as an indigenous herb widely used in the traditional setting to treat many forms of sickness, from fever to respiratory disease, constipation and cancer (Van Wyk and Gericke, 2000:134). As with other many other indigenous African herbs, incomplete study has been made of the properties, characteristics and constituents of this plant (Eloff, 1997:2).

A member of the *Alliaceae* family, *Tulbagia violacea* shares the characteristic sulphurous odour of other *Alliums* such as *Allium cepa* and *Allium sativum* (Hutchings, *et al.* 1996:37). It is also suspected that *Tulbagia violacea* shares many of the physiochemical properties of *Allium sativum* (Van Wyk and Gericke, 2000:134), a renowned anti-microbial agent (Rode *et al.*, 1989:112).

Studies so far, to determine the anti-microbial properties of *Tulbagia violacea*, have had varying results.

Watt and Breyer-Brandwijk (1962:717) reported that *Tulbagia violacea* had a positive anti-microbial effect against *Escherichia coli* (*E. coli*) and *Mycobacterium tuberculosis* (*M. tuberculosis*), whilst having no action upon *Staphylococcus aureus* (*Staph. aureus*). Hutchings *et al.* (1996:38) noted a general bacteriostatic action of the

plant, whilst later studies by McGaw, Jager and van Staden (2000:254) reported negative results in anti-microbial tests against *E. coli*., *Klebsiella pneumoniae* (*K. pneumoniae)*, *Staph. aureus* and *Bacillus subtilis* (*B. subtilis)*.

In this study, the effectiveness of *Tulbagia violacea* as an anti-microbial agent was tested against five different bacteria: *K. pneumoniae, E. coli, Staph. aureus, Bacillus cereus (B. cereus)* and *Pseudomonas aeruginosa* (*Ps. aeruginosa)*. The *staphylococci, pseudomonads* and *E. coli* are recognised as being the three micro-organisms that historically have caused the most problems to health workers due to the acquiring of anti-microbial resistance (Weinstein, 1998:102).

Homoeopathic 1X and 6X potencies of *Tulbagia violacea* were included in the experiment to determine whether the homoeopathic potentisation of the herbal tincture brought out any latent properties, which were not exhibited in the crude form (Brewster O'Reilly, 1996:235).

Figure 1.1 *Tulbagia violacea* (van Wyk, 2000:263).

The study was conducted *in vitro* using the disc diffusion test (Capuccino and Sherman, 1992: 248). The ratio of the zone of inhibition of each sample compared to the combined zones of inhibition of the gentamycin and vancomycin discs was used as a measure of how effective an antibacterial agent each test substance was. The expression of the test zones as a ratio to the zones around the vancomycin and gentamycin was made to account for plate-plate variations in the overall sensitivity of the same species of bacteria. This ensures that the only variable affecting results is the sample being tested. (McGaw, Jager and van Staden, 2000:253.)

1.2 PROBLEM STATEMENT

The purpose of this study was to investigate the efficacy of a herbal tincture of *Tulbagia violacea* in inhibiting the *in vitro* growth of *E. coli, Staph. aureus, K. pneumoniae, B. cereus* and *Ps. aeruginosa*, in terms of the disc-diffusion test.

1.3 SUBPROBLEMS

1.3.1 Subproblem one

To compare the efficacy of *Tulbagia violacea* herbal tincture in 30% v/v ethanol to a 30% v/v ethanol control, in inhibiting the *in vitro* growth of *E. coli, Staph. aureus, K. pneumoniae, B. cereus* and *Ps. aeruginosa* in terms of the sizes of the zones of inhibition.

1.3.2 Subproblem two

To compare the efficacy of *Tulbagia violacea* 1X in 30% v/v ethanol to a 30% v/v ethanol control, in inhibiting the *in vitro* growth of *E. coli, Staph. aureus, K. pneumoniae, B. cereus* and *Ps. aeruginosa* in terms of the sizes of the zones of inhibition.

1.3.3 Subproblem three

To compare the efficacy of *Tulbagia violacea* 6X in 30% v/v ethanol to a 30% v/v ethanol control, in inhibiting the *in vitro* growth of *E. coli, Staph. aureus, K. pneumoniae, B. cereus* and *Ps. aeruginosa* in terms of the sizes of the zones of inhibition.

1.4 HYPOTHESIS

1.4.1 Hypothesis one

Tulbagia violacea herbal tincture in 30% v/v ethanol will have no significant antibacterial effects on *E. coli*.

1.4.2 Hypothesis two

Tulbagia violacea herbal tincture in 30% v/v ethanol will have no significant antibacterial effects on *Staph. aureus*.

1.4.3 Hypothesis three

Tulbagia violacea herbal tincture in 30% v/v ethanol will have no significant antibacterial effects on *K. pneumoniae*.

1.4.4 Hypothesis four

Tulbagia violacea herbal tincture in 30% v/v ethanol will have no significant antibacterial effects on *B. cereus*.

1.4.5 Hypothesis five

Tulbagia violacea herbal tincture in 30% v/v ethanol will have no significant antibacterial effects on *Ps. aeruginosa*.

1.4.6 Hypothesis six

Tulbagia violacea 1X in 30% v/v ethanol will have no significant antibacterial effects on *E. coli*.

1.4.7 Hypothesis seven

Tulbagia violacea 1X in 30% v/v ethanol will have no significant antibacterial effects on *Staph. aureus*.

1.4.8 Hypothesis eight

Tulbagia violacea 1X in 30% v/v ethanol will have no significant antibacterial effects on *K. pneumoniae*.

1.4.9 Hypothesis nine

Tulbagia violacea 1X in 30% v/v ethanol will have no significant antibacterial effects on *B. cereus*.

1.4.10 Hypothesis ten

Tulbagia violacea 1X in 30% v/v ethanol will have no significant antibacterial effects on *Ps. aeruginosa*.

1.4.11 Hypothesis eleven

Tulbagia violacea 6X in 30% v/v ethanol will have no significant antibacterial effects on *E. coli*.

1.4.12 Hypothesis twelve

Tulbagia violacea 6X in 30% v/v ethanol will have no significant antibacterial effects on *Staph. aureus*.

1.4.13 Hypothesis thirteen

Tulbagia violacea 6X in 30% v/v ethanol will have no significant antibacterial effects on *K. pneumoniae*.

1.4.14 Hypothesis fourteen

Tulbagia violacea 6X in 30% v/v ethanol will have no significant antibacterial effects on *B. cereus*.

1.4.15 Hypothesis fifteen

Tulbagia violacea 6X in 30% v/v ethanol will have no significant antibacterial effects on *Ps. aeruginosa*.

1.5 DELIMITATIONS

1.5.1 This study was limited to only five species of bacteria viz. *E. coli*, *Staph. aureus*, *K. pneumoniae*, *B. cereus* and *Ps. aeruginosa*.

1.5.2 This study was limited to a specific species of *Tulbagia*, viz. *Tulbagia violacea*.

1.5.3 Only Mueller-Hinton agar was used as a growth media.

1.5.5 The non-polar (water insoluble) constituents of *Tulbagia violacea* were not effectively tested due to the hydrous nature of the agar diffusion medium in the disc-diffusion assay (Rios, Recio and Villar, 1988:142). This is further elaborated upon in chapter two.

1.5.6 Only *Tulbagia violacea* tincture produced in 30% ethanol was used.

1.5.7 This was an *in vitro* study.

CHAPTER TWO

LITERATURE REVIEW

2.1 ETHNOBOTANY

2.1.1 Definition

The term 'Ethnobotany' was coined in 1895 by the American botanist John W. Harshberger to describe the studies of plants used by "primitive and aboriginal people.". In a broader sense, it is a combination of the study of people ('ethno'), and the study of plants ('botany'). (Balick and Cox, 1996:3.)

2.1.2 Importance

Ethnobotany is important for a multitude of reasons. The link between people and plants in primitive societies is usually more clear and direct than in more industrialised ones. This is thought to be mostly because the mechanisms of production and marketing are so complex in developed society, that the botanical origins of a processed product are rarely known or understood by most individuals. In indigenous cultures however, people often have a greater degree of interaction with the plants they use, and consequently, possess knowledge about the properties of plants which western society has largely lost (Balick and Cox, 1996:6-7).

The World Health Organisation estimates that 80% of the people living in developing countries almost exclusively use traditional medicine as their primary form of medical care, with medicinal plants forming the principal constituent of these traditional therapeutics (Eloff, 1997:1). More primitive societies have through necessity maintained and passed on knowledge concerning plant medicine and cultivation (Balick and Cox, 1996:6-7). Most often, this knowledge is verbal and incorporated in local legend and folklore (Naranjo, 1995: 363).

Although it is often possible to scientifically validate certain medicinal properties or traditional uses of plants by western methods, science cannot explain how it is that

certain groups of people around the world look to these plants as sources of medicine in the first place (Wood, 1997:3).

Many modern western drugs also owe their development to the refinement of natural plant products. In 1984, one quarter of American prescription drugs were modelled on natural products. Of this, 74% of pharmacologically active plant derived components were discovered by following up on the ethnomedical use of the plant. Despite this, it has been estimated that to date, only around 15% of all angiosperms have been investigated chemically. Southern Africa contains almost 10% of the worlds plant diversity, however very little chemical work has been done on medicinal plants from this region. (Eloff, 1997:1-2.) The South African species remain an untapped reservoir of potentially important biologically-active compounds (Fennel and van Staden, 2001:23).

2.1.3 African ethnobotany

The ancient African healers have an extensive materia medica comprising various herbs, animal parts, minerals and clays. In traditional African medicine many food plants have medical usage, in contrast to orthodox western medicine where most drugs are viewed as 'poisons' which when administered in small doses, have the ability to heal. Apart from incorporating therapeutic plants into the daily diet, African medicine also contains many powerful drugs, as well as many African varieties of better-known western herbal-based drugs. (Iwu, 1993:1.)

Preliminary studies of the *in vitro* antibacterial effectiveness of some indigenous Southern African plants have been completed by people such as Rabe and van Staden (1997), Khan and Nkunya (1991), Lin *et al.*, (1999) and McGaw, Jager and van Staden (2000). However when compared to the extent of research of the indigenous herbs of countries such as America and Australia, there has been generally little attempt to evaluate African medicinal plants in general for biological activity and medicinal usefulness (Khan and Nkunya, 1991:48).

2.2 ETHNOBOTANICAL RESEARCH METHODOLOGY

2.2.1 Screening methods for natural products with anti-microbial properties

The three principal methods employed to evaluate the anti-microbial properties of natural products are diffusion assays, dilution tests and bioautography tests. Within these methods, standardisation of technique has been difficult, as many factors have been found to influence results. These factors include culture medium composition, micro-organisms tested, extractive method, pH, and solubility of the sample in the culture medium. (Rios, Recio and Villar, 1988:127.)

2.2.1.1 Diffusion assays

Diffusion methods of screening for anti-microbial properties of natural products classically employ either disc, hole or cylinder methods as reservoirs for the sample substance (Rios, Recio and Villar, 1988:128). This method is based on the principal that a reservoir containing an aqueous extract is brought into contact with an inoculated medium. The solute will diffuse through the interface into the agar gel until equilibrium is attained. After incubation the diameter of growth-free area around the reservoir can be measured and taken as the anti-microbial ability of that product. (Hewitt and Vincent, 1989:38.) [See figure 2.1.]

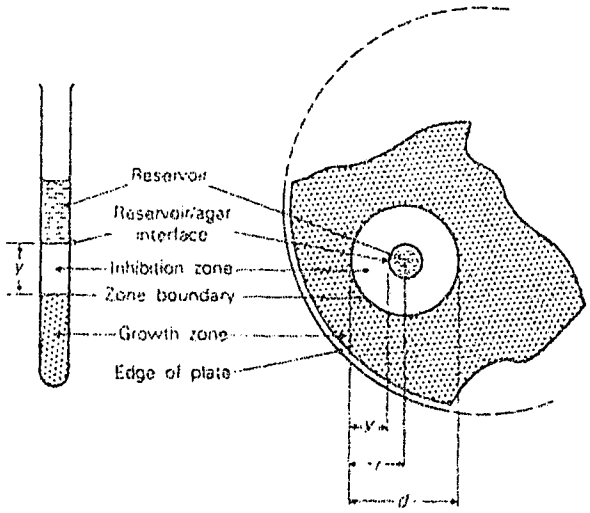

Figure 2.1 Model microbiological-assay. γ, distance from reservoir-agar interface to zone boundary. **r**, zone radius. **d**, zone diameter. (Hewitt and Vincent, 1989:39.)

Diffusion methods are well suited for the preliminary screening of pure substances, such as alkaloids, terpenoids and flavanoids. These methods however, cannot be used for samples that are difficult to diffuse in the media, as there is no direct correlation between diffusion power and anti-microbial activity. Therefore, when utilising agar plates (which are water-based) as a culture medium, this method is not acceptable for testing samples that are not highly soluble in water, regardless of whether the substance is originally applied in a non-polar solvent or not. Diffusion assays are therefore not suitable for testing the properties of essential oils or other non-polar substances. (Rios, Recio and Villar, 1988:127.)

Dry discs, as opposed to wet discs which contain a liquid sample, are used to test for anti-microbial properties of ethanol extracts (Rios, Recio and Villar, 1988:131), and have the advantage of nullifying the unknown effect that the rate of evaporation of the sample off the wet disc has on the results of a disc assay, as noted in the experiments by Reid (2002).

Comparisons of the zones of inhibition of natural products with those of synthetic antibiotics in disc-assay studies are only useful for establishing the sensitivity of test organisms. Comparisons of the anti-microbial potency of natural test substances and synthetic antibiotics cannot be made from these measurements. (Rios, Recio and Villar, 1988:142.) This is largely due to the fact that many other factors such as diffusion ability can influence the size of the zones of inhibition, resulting in misleading conclusions when comparing the two different types of substances (Hewitt and Vincent, 1989:40).

The optimum effectiveness of the disc diffusion method has been found to be obtained using Mueller–Hinton agar and standard micro-organisms (American Type Culture Collection or similar) (Rios, Recio and Villar, 1988:142).

2.2.1.2 Dilution tests

Dilution tests require a homogenous dispersion of the sample in water. Bacterial proliferation is measured by the turbidity of the solution, which is taken as a direct correlation to the amount of microbial growth (Rios, Recio and Villar, 1988:135).

These tests have the advantage of being able to test for pure substances as well as essential oils and other non-polar substances. They can also be used to yield a minimum inhibitory concentration value for anti-microbial samples. (Rios, Recio and Villar, 1988:128.)

Dilution methods are generally more complicated, time consuming and expensive to perform than disc-assay studies (Rios, Recio and Villar, 1988:142).

2.2.1.3 Bioautographic methods

The bioautographic method involves using paper chromatography, or thin layer chromatography, to isolate compounds which are then subsequently tested using the disc-assay method for anti-microbial activity. This technique is not as useful as diffusion and dilution methods for the preliminary screening of samples. (Rios, Recio and Villar, 1988:135.)

2.2.2 Choice of extractant

Various solvents have been employed to extract active compounds out of plant material in order to test for anti-microbial properties (Eloff, 1998:7).

The main considerations in choice of extractant is that it can extract the maximal amount of active ingredients from the sample, whilst not inhibiting the bioassay procedure (Eloff, 1998:7). In a study conducted by the Faculty of Medicine Research Committee, University of Pretoria, it was found that although no extractant was uniformly better than the others in all the parameters tested, ethanol proved to be a better extractant than water, while acetone was ultimately found to be overall the best extractant tested. Acetone was however found to have a possible inefficiency in extracting hydrophilic compounds. (Eloff, 1998:8.)

2.3 *TULBAGIA VIOLACEA*

2.3.1 Family

Tulbagia violacea forms part of the Alliaceae family (Van Wyk and Gericke, 2000:150).

Plants from this family are characteristically recognised as being perennial herbs with bulbs, bulblike corms, or a rhizome in the case of some South African species. Leaves form a sheath around a flowering stem, with membranous bracts at the bottom. Some species have a characteristic onion smell. The family consists of around 30 genera, four of which are native to Southern Africa. (Pooley, 1998:344.)

Within the Alliaceae, the genus *Allium* is distinguished from the genus *Tulbagia*, in that whereas the former has a bulb rootstock, campanulate flowers, connate tepals, and lacks a corona, the latter has a rhizome rootstock, more or less tubular flowers and tepals, and does contain a corona (Goldblatt and Manning, 2000:52).

2.3.2 Nomenclature

The *Tulbagia* species is named after Ryk Tulbagh, Governor of the Cape, 1751 – 1771 (Pooley, 1998:344). *Tulbagia violacea* is also sometimes known as *wild garlic*, or *wilde knoflok* (Roberts, 1990:233). In Zulu, it is known as either *isihaqa* (Hutchings, *et al.*, 1996:37), or *incinsini* (Roberts, 1990:233). In Sotho it is referred to as *mothebe* (Dyson, 1998:61). *Tulbagia violacea* may also be identified under the synonym of *Tulbagia cepacea* (Hutchings, *et al.*, 1996:37).

2.3.3 Description

Tulbagia violacea is a long-flowering perennial bearing round heads of small mauve flowers carried on long stalks (Joffe, 1993:300) [See figure 2.2]. The plants have tuberous rhizomes with many thick roots. Their leaves are long, narrow and hairless and have fleshy white bases. *Tulbagia violacea* are often found growing in clumps. (Van Wyk and Gericke, 2000:150.) The fully grown plants reach a height of about 20 – 35cm (Goldblatt and Manning, 2000:53).

Figure 2.2 *Tulbagia violacea* flowers (van Wyk, 2000:263).

2.3.4 Habitat and cultivation

Tulbagia violacea is indigenous to Southern Africa, occurring mainly in the South-east of South Africa, in the area between Knysna and KwaZulu-Natal (Goldblatt and Manning, 2000:53). It may however, be found as far north as Zimbabwe (Dyson, 1998:61).

It is an attractive garden plant, and as such is grown in many gardens across South Africa (Roberts, 1990:233). The plant is easy to grow, and may grow in poor soil. *Tulbagia violacea* may grow in sunny or partially shaded areas, and propagation may be from seed or by dividing larger clumps (Joffe, 1993:300).

The Zulu often encircle their huts with the growing plant in order to keep snakes away (Watt and Breyer-Brandwijk, 1962:717).

2.3.5 Parts used

The rhizomes and leaves are widely used in traditional South African medicine (Van Wyk and Gericke, 2000:150).

The Zulus often use the green parts and the flowers as a type of spinach, or as a condiment for meat (Watt and Breyer-Brandwijk, 1962:717).

2.3.6 Constituents

The presence of alkyl cysteine sulfoxide lyases in chemical studies of *Tulbagia violacea*, suggest that the odour production mechanisms may be similar to those produced by allinase-like enzymes in the genus *Allium* (Hutchings, *et al.*, 1996:37).

Other compounds isolated include sulphur compounds, 2,4,7-tetrathiaoctane-2.2-dioxide and 2,4,5,7-tetrathiaoctane, kaempferol, quercetin, thirteen more flavones, several sugars and steroidal saponins (Hutchings, *et al.*, 1996:37).

Sulphur compounds are often important in medicinal chemistry, as many drugs have a benzenesulfonamide nucleus (Lemke, 1983:80). The anti-microbial activity of *Allium sativum*, a plant with which *Tulbagia violacea* is thought to share common pharmacological mechanisms (Van Wyk and Gericke, 2000:150), is mainly due to its sulphur-containing compounds (Leung and Foster, 1996:261), and alliine, a methyl-containing amino acid (Evans, 1996:454).

2.3.6.1 Solubility of constituents

Water solubility of active-constituents is important when utilising an agar-based disc-diffusion type experimental model (Rios, Recio and Villar, 1988:127). Sulfonic acids and sulfonamides both contain the possibility of ion-dipole interaction with water which will favour water solubility. Amino and methyl groups, such as the type present in alliine, also greatly enhance water solubility. (Lemke, 1983:79.)

2.3.7 Actions

Tulbagia violacea is thought to be similar to *Allium sativum* in its medicinal activities (Van Wyk and Gericke, 2000:150).

2.3.8 Preparation

Traditionally *Tulbagia violacea* is administered in many varied ways. It may be prepared as an infusion from the tubers, or as a decoction to be used in the form of an enema. Tubers or leaves may be rubbed directly on the body, or rhizomes may be ground to form a powder. (Hutchings, *et al.*, 1996:37.) The leaves and flowers may be eaten whole (Van Wyk and Gericke, 2000:150).

2.3.9 Traditional and current uses

The Zulus and Sothos use *Tulbagia violacea* for treating respiratory ailments. The bulb has been recommended as a remedy for pulmonary tuberculosis and as an anthelminthic. Traditionally, infusions of the tuber have been used as love charm emetics, as well as administered as enemas for stomach ailments, constipation, rheumatism, paralysis and high fevers. The leaves may be used to treat sinus headaches, or colic, wind and restlessness in small children. The leaves may be used to treat esophageal cancer. (Hutchings, *et al.*, 1996:37; Neuwinger, 2000:536; Watt and Breyer-Brandwijk, 1962:717.)

The Early Cape Colonists used *Tulbagia violacea* as a remedy for pulmonary tuberculosis and as an anthelminthic (Hutchings, *et al.*, 1996:37).

Rastafarians have been known to drink a *Tulbagia violacea* decotion to treat coughs, colds and influenza (Dyson, 1998:61).

In the Transkei, the tubers are occasionally rubbed all over the body as a protection against evil spirits. (Hutchings, *et al.*, 1996:37.) The plants are also cultivated near the homestead to keep snakes away (Watt and Breyer-Brandwijk, 1962:717).

Young plants are sometimes eaten as vegetables, with the Zulus using the green parts and the flowers as a type of spinach, or as a condiment for meat. (Watt and Breyer-Brandwijk, 1962:717.)

2.3.10 Anti-microbial activity

A cold-water extract of an entire *Tulbagia violacea* plant tested positive in anti-microbacterial tests against *M. tuberculosis* and *E. coli*. In the same study, it was found that the extract had no effect against *Staph. aureus*. (Watt and Breyer-Brandwijk, 1962:717.) The results from this study are often cited as a reference in later publications (Hutchings, *et al.*, 1996:37; Neuwinger, 2000:536) to the anti-microbial properties of *Tulbagia violacea*. However the methodology followed by the researchers in obtaining these results is not clearly stated for review.

McGaw, Jager and van Staden (2000:254) reported negative results in a subsequent anti-microbial test against *E. coli*, *K. pneumoniae*, *Staph. aureus* and *B. subtilis*, using a crude hexanic, ethanolic and an aqueous extract of the entire plant, and measuring results by means of a disc-diffusion assay.

Bacteriostatic activity has been noted in water extracts from various parts of the plant, particularly when the extracts were fresh, from mature plants, and had not been heated (Hutchings, *et al.*, 1996:38). However, the methodology followed in obtaining these results is not stated.

Tulbagia violacea is thought to be similar to *Allium sativum* in its medicinal action (Van Wyk and Gericke, 2000:150). *Allium sativum* is a well known anti-microbial agent (Ross, 1999:33), with the antibacterial properties being much more pronounced in the hydroalcoholic extract than in the essential oil (Leung and Foster, 1996:261). This suggests that *Tulbagia violacea's* possible antibacterial constituents may also be more pronounced in a hydroalcholic extract, and as such diffuse well through the agar base of the disc-diffusion assay.

2.3.11 Homoeopathic dilutions of *Tulbagia violacea*

In this study, the homoeopathic dilutions of *Tulbagia violacea* in the 1X and 6X potencies will also be tested.

The Hahnemanian method of potentisation provides two separate scales, a centesimal one based on a dilution of one in a hundred, and a decimal one based on a dilution of one in ten.

The decimal potentisation process used in this study involves adding one drop of mother tincture to nine drops of diluents. This is followed by a burst of vigorous shaking or striking upon a hard surface, known as succussion, resulting in the first potency, which is named the 1X or D1 potency level. This process of dilution and succussion can be repeated to further raise the potency another level. Each potency level is designated the value of the number of times that it has been through the process of dilution and succussion. (Kayne, 1997:43.)

Potentisation is the imparting of the pharmacological message of the original substance by means of trituration or succussion, along with serial dilutions. It is the modification of medicines by means of a mechanical and mathematico-physical process. (Gaier, 1991: 441.)

In Aphorism 269 of his book the 'Organon of The Medicinal Art', the founder of homoeopathy, Samuel Hahnemann speaks of potentisation as follows:
"The homoeopathic medicinal art develops to a formerly unheard of degree the internal medicinal powers of crude substances. It does so by means of a procedure which belongs exclusively to it whereby these substances become altogether more than ever, indeed immeasurably, penetratingly effective and helpful, even those substances which, in their crude state do not manifest the least medicinal power in the human body." (Brewster O'Reilly, 1996:235).

2.4 BACTERIOLOGY

2.4.1 *E. COLI*

2.4.1.1 Classification

E. coli falls under the family Enterobacteriaceae, which form a large group of Gram-negative rods whose natural habitat is the intestinal tract of humans and animals (Jawetz, *et al.*, 1991:204). In the genus *Escherichia* to which it belongs, it is the only species of medical importance (Mims, *et al.*, 1998:523).

2.4.1.2 Morphology and identification

E. coli are short, motile, Gram-negative, non-spore-forming bacilli that can grow both aerobically and anaerobically on laboratory media (Mackie and McCartney, 1996:361). *E. coli* grows well on non-selective media, forming smooth, colourless, circular colonies 2-3mm in diameter after 18 hours incubation on nutrient agar, and larger red colonies when grown on MacConkey agar. They are able to grow over a large temperature range (15 – 45 °C), with some strains being able to survive temperatures of up to 60°C for 15 minutes, or 55°C for 60 minutes. (Greenwood, Slack and Peutherer, 1992:323.) Optimal growth temperature for *E. coli* is 36 – 37°C (Mackie and McCartney, 1996:361).

2.4.1.3 Epidemiology

E. coli is a member of the normal intestinal flora generally not causing disease, and often contributing to the normal function and nutrition of the intestine (Jawetz, *et al.*, 1991:215).

2.4.1.4 *E. coli* infections

E. coli usually only becomes pathogenic when it reaches areas outside of the intestines, such as the urinary tract, billiary tract, lungs, meninges, blood stream, bone or other anatomical sites. This bacteria is most often an opportunistic pathogen with infections most often arising in infancy, old age, during terminal stages of other disease, or during periods of immunosuppression. With severe infections, it may reach the blood stream causing sepsis.

E. coli may possess lipopolysaccharidal endotoxins in their cell walls. They may also sometimes produce exotoxins of clinical importance. (Jawetz, *et al.*, 1991:215-216.)

Four pathogenic groups of *E. coli* are known to be responsible for diarrhoeal disease:

- Enterotoxigenic *E. coli* (ETEC). Known to produce acute watery diarrhoea, which may lead to severe and possibly fatal dehydration, especially in infants and small children. This type also is responsible for what is known as travellers diarrhoea (turista).
- Enteroinvasive *E. coli* (EIEC). This type produces dysentery that is clinically indistinguishable from shigellosis. Blood, pus and mucous are often present in the faeces of infected individuals.
- Enteropathogenic *E. coli* (EPEC). This type is mainly associated with outbreaks of infantile diarrhoea.
- Vero cytotoxin-producing *E. coli* (VTEC). This type is also termed "enterohaemorrhagic" *E. coli*. This type is characterised by a mild to bloody diarrhoea that may precede a severe haemorrhagic colitis or haemolyticuraemic syndrome. Two different types of Vero cytotoxins produced by the bacteria are responsible for the condition.

(Mackie and McCartney, 1996:366.)

2.4.1.5 Anti-microbial sensitivity

Anti-microbials used to treat *E. coli* infections include all those that have action against Gram-negative organisms. These include pthalysulphathiazole, neomycin, doxycycline, trimethoprim, norfloxacin, and other fluoroquinolones. (Greenwood, Slack and Peutherer, 1992:333.) The two antibiotics most commonly used against *E. coli* infections are gentamycin and ciproflocin (Gladwin and Trattler, 2000:133).

2.4.2 *K. PNEUMONIAE*

2.4.2.1 Classification

K. pneumoniae falls under the genus *Klebsiella*, which are characteristically non-motile members of the Enterobacteriaceae family (Mackie and McCartney, 1996:366). Earlier in the nineteenth century, *K. pneumoniae* were included with other motile members of the *Enterobacter* species under the name, *Bacteria aerogenes* before subsequently being transferred to the genus *Klebsiella*. Later taxonomists gave the species name *pneumoniae* to the non-motile *aerogenes*-like organisms. (Greenwood, Slack and Peutherer, 1992:335.)

2.4.2.2 Morphology and identification

Members of the genus *Klebsiella* are straight rods about 1-2 micrometers long. They are generally shorter and thicker than the other Enterobacteria (Mims *et al.*, 1998:524). They are usually capsulate and form large, mucoid, greyish-white colonies when grown on laboratory media (Mackie and McCartney, 1996:366). *K. pneumoniae* stain Gram-negative (Mims, *et al.*, 1998:524), and grow between 12°C – 43°C, with optimum growth being at 37°C. Growth may occur under partially anaerobic conditions, but under strict anaerobic conditions, growth is poor. (Greenwood, Slack and Peutherer, 1992:335.)

2.4.2.3 Epidemiology

K. pneumoniae is present without pathology in about 5% of the population, residing in the respiratory tract and faeces.

2.4.2.4 *K. pneumoniae* infections

K. pneumoniae can cause bacterial pneumonias and may produce extensive hemorrhagic necrotising consolidation of the lung. (Jawetz, *et al.*, 1991:208.) It may also give rise to severe cases of bronchopneumonia, sometimes with chronic destructive lesions and multiple abscess formation in the lungs. Bacteraemia may result from this, with an associated high mortality rate. *K. pneumoniae* is most clinically significant in causing infections in hospital patients. Sepsis of surgical wounds and the urinary tract often lead to bacteraemia and possibly death. Colonisation of *K.*

pneumoniae in the respiratory tracts of patients receiving antibiotics has been frequently noted, although the clinical significance of this is not yet fully understood. (Greenwood, Slack and Peutherer, 1992:338.)

2.4.2.5 Anti-microbial sensitivity

Klebsiella developed resistance to antibiotics in their early days of usage, and have subsequently developed resistance to the newly developed antibiotics as well. The use of antibiotics is seen as undoubtedly the main contributive reason behind *Klebsiella* being a cause of infection in hospitals. Urinary tract infection by *Klebsiella* responds to treatment with trimethoprim, nitrofurantoin, co-amoxiclav and cephalosporins. Pneumonia and other more serious infections require treatment with cephalosporins or aminoglycosides. (Greenwood, Slack and Peutherer, 1992:339.) Gentamycin is the most commonly used of all the aminoglycosides to treat *Klebsiella* infections (Gladwin and Trattler, 2000:133).

2.4.3 *STAPH. AUREUS*

2.4.3.1 Classification

Staph. aureus falls under the family Staphylococcacea and genus *Staphylococcus*, which comprises of at least 20 different species of which *Staph. aureus* together with *Staphylococcus epidermidis*, and *Staphylococcus saprophyticus*, are recognised as being the most medically significant (Mims, *et al.*, 1998:513).

2.4.3.2 Morphology and identification

Staph. aureus are spherical organisms, usually about one micrometer in diameter, and occur in irregularly shaped clusters. They may also be seen singularly, in pairs, tetrads or chains. Younger cells stain Gram-positive, however older cells may stain Gram-negative. (Jawetz, *et al.*, 1991:194.) *Staph. aureus* are non-motile, non-sporing and generally non-capsulate. When grown on laboratory media, colonies are generally circular, 2-3 mm in diameter, and have a smooth, shiny surface. Colonies are opaque, and may have a golden-yellow, fawn or cream colour to them. (Greenwood, Slack and Peutherer, 1992:204.) When grown anaerobically, colonies are notably smaller, and more greyish in colour (Jawetz, *et al.*, 1991:194). The optimum growth temperature is

37°C, although pigment is formed best at 20 – 25°C (Mackie and McCartney, 1996:246).

2.4.3.3 Epidemiology

Staph. aureus occurs as part of the normal flora of the skin, and is present in the nasal cavities of 40 – 50% of healthy human beings (Jawetz, *et al.*, 1991:196).

2.4.3.4 *Staph. aureus* infections

Staph. aureus is by far the most clinically important *Staphylococcal* pathogen (Mackie and McCartney, 1996:248). Most strains are incapable of penetrating the normal barrier function of the skin, and tend only to cause infection when penetrating through breaks in the skin. Localised infections may progress to bacteraemia, whilst 'spontaneous' bacteraemias have been noted, where there is no origin of sepsis evident. This occurs more often in chronically debilitated patients. (Mackie and McCartney, 1996:248.)

Once past the barrier of the skin, *Staph. aureus* strains possess a large number of cell–associated and extracellular factors, which often enable the organism to survive the bodies defences and colonise the tissue. It is thought that these factors acting in unison make it possible for the organism to bind to connective tissue, resist the bactericidal response of the complement system, and prevent uptake by phagocytes.

Staph. aureus may cause:

- Pyogenic infections such as: carbuncles, boils, breast abscess, lung abscess, and empyema;
- Disseminated infections such as septicaemia;
- Toxin-mediated illness, such as toxic shock syndrome and staphylococcal food poisoning.

(Greenwood, Slack and Peutherer, 1992:204.)

Pathological changes caused by *Staph. aureus*, are usually due to enzymes and toxins produced by the bacteria. These include:

- Coagulase, an enzyme that both clots plasma and inhibits the uptake and phagocytosis of the bacteria by macrophages;
- Leucocidin, which kills white cells;
- Lytic exotoxins, which destroy red blood cells and platelets;
- Deoxyribonuclease, which destroys deoxyribonucleic acid;
- Lipase, which aids in the breakdown of fats;
- Staphylokinase, which causes fibrinolysis;
- Exfoliatin, which causes the peeling of the skin;
- Enterotoxin B, which causes food poisoning;
- Beta-lactamases that lead to penicillin-resistance.

(Greenwood, Slack and Peutherer, 1992:205.)

2.4.3.5 Anti-microbial sensitivity

Staph. aureus is resistant to most broad-spectrum penicillin such as ampycillin and amoxycillin due to the production of beta-lactamases by the bacterium. Clavulanic acid inactivates beta-lactamase, and sometimes is used in conjunction with amoxycillin (co-amoxiclav) as a therapy. Certain strains resistant to other antibiotics such as tetracycline or erythromycin, and even beta-lactamase-resistant penicillin, are not uncommon. All *Staph. aureus* strains remain susceptible to the glycopeptide antibiotics, vancomycin and teicoplanin. (Mackie and McCartney, 1996:247-248.)

2.4.4 *B. CEREUS*

2.4.4.1 Classification

The genus *Bacillus*, to which *B. cereus* belongs, contains nearly 50 different species. Of these, *B. cereus* and *Bacillus anthracis* are recognised as the only ones of major medical importance. (Mims, *et al.*, 1998:518.) The genus *Bacillus* used to include all rod-shaped bacteria, but now only includes large spore-forming Gram-positive bacilli. (Greenwood, Slack and Peutherer, 1992:271.)

2.4.4.2 Morphology and identification

B. cereus is a large Gram-positive bacilli which microscopically resemble *Bacillus anthracis* except for being motile and lacking a capsule (Greenwood, Slack and Peutherer, 1992:276). When grown on laboratory media, colonies formed are large (2-5 mm), greyish white in colour, and granular (Mackie and McCartney, 1996:323). *B. cereus* are non-fastidious in their growth, and respire aerobically. Spore-formation may occur after the organism is shed from the body. (Mims, *et al.*, 1998:518.)

2.4.4.3 Epidemiology

B. cereus is a common resident of our environment, often being present in soil and food (Mims, *et al.*, 1998:518).

2.4.4.4 *B. cereus* infections

B. cereus is the saprophytic species most closely related to *Bacillus anthracis*, and has a great potential to cause disease in humans, either as an opportunistic, or occasionally as a primary pathogen. Severe pathology may result, usually due to toxin production.

The toxins produced by *B. cereus* include:

- A lecithinase;
- Two haemolysins;
- A diarrhoeal enterotoxin;
- An emetic toxin.

These may be responsible for pathology including:

- Abscesses and cellulites;
- Endopthalmitis;
- Meningitis;
- Osteomyelitis;
- Ear and urinary tract infections;
- Food poisoning.

Most pathology only arises in debilitated or immune-suppressed people. (Mackie and McCartney, 1996:318,323.)

Vomiting is the main symptom of food poisoning caused by *B. cereus*, usually occurring within 6 hours of ingestion. Strains associated with this type of food poisoning are heat and acid-stable, surviving the cooking process.

A heat-labile toxin produced by the bacteria may cause a diarrhoeal form of food poisoning, similar to the enteritis caused by *E. coli*. (Greenwood, Slack and Peutherer, 1992:276.)

2.4.4.5 Anti-microbial sensitivity

B. cereus forms beta-lactamase, and as such is resistant to the penicillins and cephalosporins. The bacteria generally remain sensitive to gentamycin, clindamycin, erythromycin and vancomycin. (Mackie and McCartney, 1996:318,323.)

2.4.5 *PS. AERUGINOSA*

2.4.5.1 Classification

The *Pseudomonas* genus, of the family Pseudomonadaceae, contains more than 200 species, of which a few species are pathogenic to plants, insects or animals. *Ps. aeruginosa*, *Pseudomonas mallei* and *Pseudomonas pseudomallei* are recognised as the most important human pathogens in this genus. (Greenwood, Slack and Peutherer, 1992:345.)

2.4.5.2 Morphology and identification

Ps. aeruginosa is a motile, Gram-negative, rod-shaped bacillus, measuring about 0.6 x 2 micrometers. It may occur singularly, in pairs, or occasionally in short chains. (Jawetz, *et al.*, 1991:224.) They are non-sporing, non-capsulate, and move via one or two polar flagella. They are usually aerobic, but are able to grow anaerobically in the presence of nitrates. *Ps. aeruginosa* can grow on a wide variety of media, and over a wide temperature range. (Greenwood, Slack and Peutherer, 1992:345.)

Six different colonial types of *Ps. aeruginosa* may be observed:

- Type 1 are large, low convex, oval, and rough in appearance;
- Type 2 are small, smooth and domed;
- Type 3 are small and rough;
- Type 4 are small and 'rugose';
- Type 5 are characterised by very mucoid growth, where colonial growth may merge and even drip onto the lid of the Petri dish;
- Type 6 are small dwarf colonies of the mucoid form.

(Mackie and McCartney, 1996:415.)

The colonies produced may possess a sheen known as 'iridescence', as well as a characteristic sweet odour (Mims, *et al.*, 1998:526).

2.4.5.3 Epidemiology

Ps. aeruginosa is part of the common flora of the body, and becomes pathogenic only when it is introduced into areas lacking in normal human defences, e.g. when a mucous membrane is disrupted by trauma (Jawetz, *et al.*, 1991:225).

2.4.5.4 *Ps. aeruginosa* infections

Most pathology is mild and superficial. However, more severe infection may arise in hospitalised or immunocompromised patients. Although infection in these cases usually is still localised, e.g. urinary tract infection or infected ulcers, more serious cases of septicaemia or necrotising pneumonia do occur, and are associated with a high mortality rate. The lungs of children with cystic fibrosis are particularly susceptible to *Ps. aeruginosa* infection. (Greenwood, Slack and Peutherer, 1992:346.)

The virulence of *Ps. aeruginosa* is due to a number of factors. Exotoxin A and exo-enzyme S have been identified which helps to inhibit protein synthesis. (Greenwood, Slack and Peutherer, 1992:346.) Extracellular proteases and elastases destroy tissue at sites of infection, and extracellular slime production helps to prevent phagocytosis.

Pigments produced may also have a role in the pathogenicity of the bacteria. (Mims, *et al.*, 1998:526.)

2.4.5.5 Anti-microbial sensitivity

Ps. aeruginosa has developed resistance to many antibiotics. Presently, the best antibiotics to combat *Ps. aeruginosa* infections are the aminoglycosides tobramycin and gentamycin. These are often used in conjunction with anti-pseudomonal penicillin such as ticarcillin, azlocillin or piperacillin. Cephalosporins such as ceftazidime may also prove effective.

Many strains of *Ps. aeruginosa* however, do not respond well clinically to antibiotics that may have appeared effective when tested *in vitro*. (Mackie and McCartney, 1996:416-417.)

2.5 PREVIOUS RESEARCH

Previous research has been conducted utilising the biological-assay to evaluate the anti-microbial properties of natural products.

Reid (2002), utilised a disc-assay procedure to evaluate the effectiveness of *Rosmarinus officinalis*, *Salvia officinalis* and *Thymus vulgaris* herbal tinctures in 62% ethanol, in halting the *in vitro* growth of *Candida albicans*.

Reid concluded that the *Rosmarinus officinalis* and *Salvia officinalis* herbal tinctures tested in this study exhibited no statistically significant growth inhibiting effect on the *Candida albicans*. These results were inconsistent with the conclusions of previous research. *Thymus vulgaris* was however shown to have an anti-fungal effect, which is consistent with the results of previous studies. (Reid, 2002:144.)

Reid cited in her discussion her concern that the evaporation of the ethanol off the discs in the wet-disc method of the disc-assay may cause an unaccounted for effect on microbial growth and the subsequent production of the zones of inhibition in the experiment (Reid, 2002:121).

On further examination of the methodology utilised by Reid, various other potential flaws in her methodology became evident. These included:

- The use of filter paper discs punched from media which was not manufactured to be used in disc-diffusion studies;
- The application of wet ethanol samples to the paper discs after the discs had already been placed on the inoculated media. Prevention of the ethanol from spreading further than the edges of the disc is very difficult to limit or assess via this method, and can severely affect inhibition zone size.

Vosloo (2002), conducted disc-assay experiments utilising wet discs to determine the efficacy of *Thymus vulgaris* herbal tincture in 43% and 70% ethanol as an

antibacterial agent. Her research concluded that the herbal tinctures tested had a significant anti-microbial effect on the strains of bacteria tested.

Like Reid, Vosloo also expressed concern in her discussion regarding the evaporation of the ethanol and its possible effect on results.

On further examination of the methodology utilised by Vosloo, various other flaws also became apparent. These included:

- The use of filter paper discs not specifically manufactured for use in disc-assay studies;
- The application of 10 microlitres of sample onto the discs, once the discs had already been placed on the seeded agar. There is considerable doubt that a filter paper disc of the type used in the study can successfully hold that quantity of ethanol-based sample without the spreading of the sample beyond the edges of the disc (Rios, Recio and Villar, 1988:130);
- The agar plates were swabbed with a quantity of test culture to "ensure heavy growth" (Vosloo, 2002:39). In the disc-assay, it is correct procedure to discard any plates with heavy, rather than moderate growth (Cheesbrough, 1984:200), as too heavy an inoculum may cause disproportionately small zone diameters to be produced (Hewitt and Vincent, 1989:180);
- The use of wet ethanol discs, resulting in a concern about the effect of evaporation over time of the ethanol on micro-organism growth;
- Measurement of inhibition zone size was done with a plastic ruler. A technique which is strongly advised against, as it is seen to be very inaccurate (Hewitt and Vincent, 1989:176).

Langford (2001) tested the anti-microbial ability of *Calendula officinalis* herbal tincture in 30% and 60% ethanol by means of a disc-assay experiment utilising wet discs. Her results concluded that *Calendula officinalis* herbal tincture in 60% ethanol was an effective anti-microbial agent against selected bacteria, whilst the herbal tincture in 30% ethanol had no inhibitive effect on any of the bacteria tested.

Her conclusion based on these results was that the *Calendula officinalis* herbal tinctures could not provide an adequate *in vivo* anti-microbial alternative to synthetic anti-microbial drugs.

Possible flaws in this research evident upon further examination include:

- The use of filter paper discs not specifically manufactured for use in disc-assays;
- The agar plates were swabbed with a quantity of test culture to obtain "thick bacterial growth" (Langford, 2001:6). In the disc-assay, it is correct procedure to discard any plates with heavy, rather than moderate growth (Cheesbrough, 1984:200), as too heavy an inoculum may cause misleadingly small zone diameters to be produced (Hewitt and Vincent, 1989:180);
- The use of wet ethanol discs, resulting in a concern about the effect of evaporation over time of the ethanol on micro-organism growth;
- The *in vivo* antibacterial efficacy of a herbal tincture is compared to the *in vivo* efficacy of a synthetic antibiotic (Langford, 2001:114), based on direct comparison of the results of a disc-assay. The assay procedure cannot be used to directly compare results of a natural antibiotic to a synthetic one (Rios, Recio and Villar, 1988:142).

De Smidt (2001) tested the effectiveness of an *Allium sativum* aqueous extract, 3X and 8X potency in dilution, in inhibiting the *in vitro* growth of *Candida albicans*, and drug resistant strains of *E. coli, K. pneumoniae* and *Ps. aeruginosa*.

The aqueous dilutions were added to test tubes containing test organisms and nutrient broth. Tubes where activity was noted by spectrophotometry were subsequently evaluated by plating out and incubating the bacteria, and thereafter counting the Colony Forming Units (CFU's).

The experiments concluded that the *Allium sativum* extract exhibited a bacteriocidal effect against *E. coli* and *Candida albicans*. No bacteriocidal or bacteriostatic effect was exhibited against the drug-resistant strains of *K. pneumoniae* or *Ps. aeruginosa*. The homoeopathic potencies tested produced no bacteriocidal or bacteriostatic effect against any of the organisms tested.

This study utilised aqueous extracts as the samples. This had the advantage of nullifying the superfluous effect of ethanol on the results of the study as noted by Reid (2002:114), but had the disadvantage of not testing any of the lipid-soluble constituents of *Allium sativum* such as the essential oils.

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

3.1.1.2 Zones of Inhibition

The susceptibility of each bacteria to the test or negative control substance, was determined by the size of the zone of inhibition around each sample and negative control in turn, in relation to the combined sizes of the zones of inhibition (to enable statistical comparability of results), around the gentamycin and vancomycin discs. The expression of the test zones as a ratio to the zones around the vancomycin and gentamycin was made to account for plate-to-plate variations in the overall sensitivity of the same species of bacteria. This ensures that the only variable affecting results is the sample being tested. (McGaw, Jager and van Staden, 2000:253.)

3.1.1.2.1 Results of the experiment determining the effects of *Tulbagia violacea* herbal tincture in 30% ethanol, on *Staph. aureus, Ps. aeruginosa, E. coli, K. pneumoniae* and *B. cereus,* in comparison to 30% ethanol only by measurement of the zones of inhibition. The zones of inhibition of the *Tulbagia violacea* herbal tincture and the 30% ethanol are expressed as ratios to the combined inhibition zones of gentamycin 10μg, and vancomycin 30μg.

3.1.1.2.2 Results of the experiment determining the effects of *Tulbagia violacea* 1X in 30% ethanol, on *Staph. aureus, Ps. aeruginosa, E. coli, K. pneumoniae* and *B. cereus,* in comparison to 30% ethanol only by measurement of the zones of inhibition.

The zones of inhibition of the *Tulbagia violacea* 1X and the 30% ethanol, are expressed as ratios to the combined inhibition zones of gentamycin 10µg, and vancomycin 30µg.

3.1.1.2.3 Results of the experiment determining the effects of *Tulbagia violacea* 6X in 30% ethanol, on *Staph. aureus, Ps. aeruginosa, E. coli, K. pneumoniae* and *B. cereus*, in comparison to 30% ethanol only by measurement of the zones of inhibition. The zones of inhibition of the *Tulbagia violacea* 1X and the 30% ethanol, are expressed as ratios to the combined inhibition zones of gentamycin 10µg, and vancomycin 30µg.

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 METHODS

3.3.1 Preparation of media

Mueller-Hinton agar was the medium used in the experiments. It was prepared according to the Oxoid® manual (1979), as follows:

1) 38g of Mueller-Hinton agar powder was weighed out.
2) The Mueller-Hinton agar powder was added to 1 litre of distilled water in a screw top flask.
3) A magnetic stirrer was added to aid dissolution.
4) The mixture was shaken until well mixed.
5) The mixture was autoclaved at 121°C for 15 minutes.
6) The flask was allowed to cool whilst placed on a magnetic stirring machine. This ensured adequate mixing and prevented the mixture from solidifying.
7) Once the flask had cooled enough to hold, the agar was poured into agar plates as follows:

7.1 The top of the flask was flamed with a Bunsen burner before pouring each plate to prevent contamination;

7.2 Each plate was poured to a depth of approximately 4 millimetres;

7.3 A total of 20 plates were prepared per bacterium;

7.4 The plates were stacked and allowed to cool and solidify;

7.5 The plates were finally visually checked for contamination.

3.3.2 Preparation of the inoculums

Single colonies from the Durban Institute of Technology, Biotechnology Laboratory stock cultures of each of the standard bacteria to be tested were used to inoculate separate Mueller-Hinton agar plates, and allowed to incubate for 24 hours at 37°C. The only strain number available was for *Staph. aureus*, ATCC 25925.

3.3.3 Preparation of the saline test cultures

A few individual colonies from the overnight Mueller-Hinton agar cultures were suspended in 10ml of sterile saline solution (8.5g/l), and the solution adjusted to the 0.5 McFarland Equivalence Turbidity Standard (REMEL, nd).

3.3.4 Preparation of filter paper discs.

Whatman® filter paper number 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 (Hewitt and Vincent, 1989:57).

3.3.5 Preparation of 30% v/v ethanol.

30% v/v ethanol (negative control) was prepared according to the German Homoeopathic Pharmacopoeia standards (British Homoeopathic Association, 1985:11):

60,61 millilitres of 99% v/v ethanol (Batch no. 318/0/68) purchased from Illovo Sugar Ltd., was diluted with sufficient distilled water to produce 200 millilitres of 30% v/v ethanol. The concentration of the ethanol was verified using a hydrometer.

3.3.6 Preparation of *Tulbagia violacea*

3.3.6.1 Herbal tincture

A herbal tincture of *Tulbagia violacea* extract in 30% v/v ethanol (Batch no. 06130, expiry date: 05/2006) was obtained from Parceval (Pty) Ltd. Pharmaceuticals, and stored in a cool, dark cupboard until it was used. A tincture in 30% v/v ethanol was used, as this is the standard concentration of commercially available *Tulbagia violacea* herbal tincture.

The extract was prepared by Parceval (Pty) Ltd. as follows:

- The fresh herb, grown by Parceval (Pty) Ltd. Pharmaceuticals in Wellington, Eastern Cape, South Africa, was identified, harvested and cleaned.
- It was crushed into a pulp, before 30% v/v ethanol (purchased from Illovo Sugar Ltd.) and distilled water was added according to a formula that adjusts for the potentially variable moisture content of the fresh plant.
- The mixture was then macerated for a period of at least ten days during which it was stirred daily to ensure even extraction.
- Thereafter it was pressed and filtered, resulting in a herbal tincture with a final extract concentration of 0.4g/l. (Feiter, 2002.)

3.3.6.2 *Tulbagia violacea* 1X

A proportion of the *Tulbagia violacea* tincture was taken up to a 1X potency in 30% v/v ethanol, according to method 1 of the German Homoeopathic Pharmacopoeia (British Homoeopathic Association, 1985:16) as follows:

- 2ml of *Tulbagia violacea* herbal tincture was added to 18ml of 30% v/v ethanol contained in a 25ml amber-glass, screw-top bottle. This bottle was then succussed 100 times and labelled, *Tulbagia violacea* 1X.

The *Tulbagia violacea* 1X was stored in a cool, dark cupboard until it was used.

3.3.6.3 *Tulbagia violacea* 6X

A proportion of the *Tulbagia violacea* 1X was taken up to a 6X potency in 30% v/v ethanol, according to method 1 of the German Homoeopathic Pharmacopoeia (British Homoeopathic Association, 1985:16) as follows:

- 2ml of *Tulbagia violacea* 1X was added to 18ml of 30% v/v ethanol contained in a 25ml amber-glass, screw-top bottle. This bottle was then succussed 100 times and labelled, *Tulbagia violacea* 2X.
- The above procedure was repeated (each time using 2ml of the previous potency) another four times until 20ml of *Tulbagia violacea* 6X was produced.

The *Tulbagia violacea* 6X was stored in a cool, dark cupboard until it was used.

3.3.7 Preparation of medicated discs

3.3.7.1 Preparation of *Tulbagia violacea* herbal tincture dry-discs

The *Tulbagia violacea* herbal tincture dry-discs were prepared as follows:

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly spaced upon the bottom of two sterile Petri dishes using a pair of sterile forceps, so that each Petri dish contained 50 discs.
- 10 microlitres of *Tulbagia violacea* herbal tincture was pipetted onto each disc using a calibrated micropipette (Van Staden, Jager and McGaw, 2000:253).
- The Petri dishes were placed in a dark incubator at 37°C, and the discs allowed to dry (Rios, Recio and Villar, 1988; Wat *et al*, 1980:280).
- Once dry, a further 10 microlitres of herbal tincture was pipetted onto each disc, before being returned to the darkened incubator at 37°C to be allowed to dry once more.
- The dry discs were stored in labelled, sterile jars until they were used.

3.3.7.2 Preparation of *Tulbagia violacea* 1X dry-discs

The *Tulbagia violacea* 1X dry-discs were prepared as follows:

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly spaced on the bottom of two sterile Petri dishes using a pair of sterile forceps, so that each Petri dish contained 50 discs.
- 10 microlitres of *Tulbagia violacea* 1X was pipetted onto each disc using a calibrated micropipette (Van Staden, Jager and McGaw, 2000:253).
- The Petri dishes were then placed in a darkened incubator at 37°C to be allowed to dry (Rios, Recio and Villar, 1988; Wat *et al*, 1980:280).
- Once dry, a further 10 microlitres of *Tulbagia violacea* 1X was pipetted onto each disc, before being returned to the darkened incubator at 37°C to be allowed to dry once more.
- The dry discs were stored in labelled, sterile jars until they were used.

3.3.7.3 Preparation of *Tulbagia violacea* 6X dry-discs

The *Tulbagia violacea* 6X dry-discs were prepared as follows:

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly spaced on the bottom of two sterile Petri dishes using a pair of sterile forceps, so that each Petri dish contained 50 discs.
- 10 microlitres of *Tulbagia violacea* 6X was pipetted onto each disc using a calibrated micropipette (Van Staden, Jager and McGaw, 2000:253).
- The Petri dishes were then placed in a dark incubator at 37°C to be allowed to dry (Rios, Recio and Villar, 1988).
- Once dry, a further 10 microlitres of *Tulbagia violacea* 6X was pipetted onto each disc, before being returned to the darkened incubator at 37°C to be allowed to dry once more.
- The dry discs were stored in labelled, sterile jars until they were used.

3.3.7.4 Preparation of 30% v/v ethanol dry-discs

The 30% v/v ethanol dry-discs were prepared as follows:

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly spaced on the bottom of two sterile Petri dishes using a pair of sterile forceps, so that each Petri dish contained 50 discs.
- 10 microlitres of 30% v/v ethanol was pipetted onto each disc using a calibrated micropipette (Van Staden, Jager and McGaw, 2000:253).
- The Petri dishes were then placed in a dark incubator at 37°C to be allowed to dry (Rios, Recio and Villar, 1988; Wat *et al*, 1980:280).
- Once dry, a further 10 microlitres of ethanol was pipetted onto each disc, before being returned to the darkened incubator at 37°C to be allowed to dry once more.
- The dry discs were stored in labelled, sterile jars until they were used.

3.3.7.5 Preparation of vancomycin 30µg discs

Five tubes each containing 50 vancomycin 30µg discs were purchased from Continental Laboratory and Medical Supplies, Durban, and stored at 5°C until they were used.

3.3.7.6 Preparation of gentamycin 10µg discs

Five tubes each containing 50 gentamycin 10µg discs were purchased from Continental Laboratory and Medical Supplies, Durban, and stored at 5°C until they were used.

3.3.8 Preparation of the plates

3.3.8.1 Innoculation of the plates

Using sterile techniques, the plates were inoculated as follows:

- Using a sterile 4mm wire loop, two loopfuls of a saline test culture were applied to a previously prepared Mueller-Hinton agar plate (Rabie and van Staden, 1997:85).
- The bacteria were then spread evenly across the plates using a sterile glass spreader (Mackie and McCartney, 1996:115).
- The inoculants were allowed to dry for a few minutes with the Petri dish lids in place.

- This process was separately repeated 20 times for each of the five bacteria to be tested. One streaked plate for each test and control group was produced per culture.
- A marker pen was used to label the under surface of each agar plate with a letter to denote which bacteria was streaked on the plate.

The plates were labelled as follows:

A) E. coli

B) Staph. aureus

C) Ps. aeruginosa

D) B. cereus

E) K. pneumoniae

3.3.8.2 Placement of discs on the plates

The under surface of each agar plate was marked with the numbers one to six evenly around its edge using a marker pen, with each number referring to a particular disc, namely:

1) *Tulbagia violacea* herbal tincture
2) *Tulbagia violacea* 1X
3) *Tulbagia violacea* 6X
4) Ethanol 30% v/v
5) Vancomycin 30 µg
6) Gentamycin 10 µg

One of each previously prepared dry disc was placed by means of a sterile needle onto each plate. The discs were placed equidistant from each other in their corresponding pre-marked places.

Innoculation and then placement of the discs was done in batches of five plates. This was to minimise the time lapse between the inoculation of each plate, and the placing of the discs. The minimal time lapse ensures that zone production is not adversely affected (Hewitt and Vincent, 1989:180).

3.3.9 Incubation of the plates

The plates were incubated in an inverted position at 37°C.

3.3.10 Recording of results

The plates were inspected at 18, 24 and 36 hours for the presence of growth inhibition, which was indicated by a clear zone surrounding the corresponding disc.

The zone diameters of the samples, control and antibiotic discs were measured in millimetres using a pair of vernier callipers to ensure accuracy (Hewitt and Vincent, 1989:65). The measurement was performed in triplicate. The average inhibition zone diameter was calculated and recorded on a table (See Appendix A through E).

The susceptibility of each bacteria to the test or negative control substance, was determined by the size of the zone of inhibition around each sample and negative control in turn, in relation to the combined sizes of the zones of inhibition (to enable statistical comparability of results), around the gentamycin and vancomycin discs.

3.4 DATA ANALYSIS

3.4.1 Sample size of 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 bacteria (five in total) 15 times to make the study statistically viable. This resulted in five groups each containing 15 sets of results.

3.4.2 Statistical methods

3.4.2.1 Intra-group comparison of *Tulbagia violacea* herbal tincture in 30% v/v ethanol with regard to the observations at 18 hours, 24 hours and 36 hours

The Friedman's test was used to compare results from related samples (The significance of the differences in response for K treatments applied to n subjects).

(i) Hypothesis testing

The null hypothesis H_0, states that there was no change in diameter of the zone of inhibition with regard to observations at 18 hours, 24 hours and 36 hours, at the α=0.05 (5%) level of significance.

The alternative hypothesis H_1, states that there was a change in the diameter of the zone of inhibition with regard to observations at 18 hours, 24 hours and 36 hours, at the α=0.05 (5%) level of significance.

H_0: There was no change in the diameter of the zone of inhibition

H_1: There was a change in the diameter of the zone of inhibition

(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 ? α.

P is the observed significance level or probability value.

If the null hypothesis H_0 is rejected for Friedman's T test, then a multiple applied, comparison procedure will have to be using the Dunn Procedure to determine which of the times are significantly different. Let R_j and $R_j^{/}$ be the j^{th} and $j^{i\,th}$ time rank totals. Let α be the experiment wise error rate. Usually α= 0.10

If $|R_j - R_j^{/}| = z \vee {}^{bk(k+1)}/_{6}$, 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, α=0.10, z=2.12

This procedure is repeated for each bacteria in turn.

(Kanji, 1999:113; Fisher and van Belle, 1993:430; Reid, 2002:36).

3.4.2.2 Intra-group comparison of *Tulbagia violacea* 1X in 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 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 *Tulbagia violacea* 6X in 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 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 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 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 *Tulbagia violacea* herbal tincture in 30% v/v ethanol and 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 hours

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

(i) Hypothesis testing

The null hypothesis H_0, states that there was 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 was a difference at the $\alpha = 0.05$ 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? 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 ? α.

P is the observed significance level or probability value

This procedure is repeated for each bacteria in turn.

(Kanji, 1999:82; Fisher and van Belle, 1993:315; Reid, 2002:36).

3.4.2.6 Inter-group comparison between *Tulbagia violacea* 1X in 30% v/v ethanol and 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 hours

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

(i) Hypothesis testing

As per 3.4.2.5 (i).

(ii) Decision rule

As per 3.4.2.5 (ii).

3.4.2.7 Inter-group comparison between *Tulbagia violacea* 6X in 30% v/v ethanol and 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 hours

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

(i) Hypothesis testing

As per 3.4.2.5 (i).

(ii) Decision rule

As per 3.4.2.5 (ii).

3.4.2.8 Inter-group comparison between *Tulbagia violacea* herbal tincture in 30% v/v ethanol, *Tulbagia violacea* 1X in 30% v/v ethanol and *Tulbagia violacea* 6X in 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 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 herbal tincture, 1X potency and 6X potency to each other, with regard to observations at 18 hours, 24 hours and 36 hours.

(i) Hypothesis testing

In each test, the null hypothesis states that there was no difference in diameter among the means of the samples being compared to each other. The alternative hypothesis states that there was a difference among the means.

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

$H_1 : \mu_1 ? \mu_2 ? \mu_3$. (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 0,05 level of significance.

Reject H_0 if $P < \alpha$.

Accept H_0 if P ? α.

P is the observed significance level or probability value.

This procedure is repeated for each bacteria in turn.

(Kanji, 1999:89; Fisher and van Belle, 1993:430; Reid, 2002:36).

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. See Appendix A-E for raw data.

4.2 STATISTICAL ANALYSIS OF DATA

4.2.1 Intra-group comparison of *Tulbagia violacea* herbal tincture in 30% v/v ethanol with regards to observations at 18, 24, and 36 hours

4.2.1.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* herbal tincture in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours. (See Table 4.1 and 4.2.)

Table 4.1 *E.coli*. Descriptive Statistics for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

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

Table 4.2 *E. coli*. Friedmans' test for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameter of the zones of inhibition for *E. coli* at 18, 24 or 36 hours.

4.2.1.2 *Staph. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* herbal tincture in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.3 *Staph. aureus*. Descriptive Statistics for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

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

Table 4.4 *Staph. aureus*. Friedmans' test for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameter of the zones of inhibition for *Staph. aureus* at 18, 24 or 36 hours.

4.2.1.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* herbal tincture in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.5 *K. pneumoniae*. Descriptive Statistics for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

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

Table 4.6 *K. pneumoniae*. Friedmans' test for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *K. pneumoniae* at 18, 24 or 36 hours.

4.2.1.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* herbal tincture in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.7 *B. cereus*. Descriptive Statistics for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

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

Table 4.8 *B. cereus*. Friedmans' test for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *B. cereus* at 18, 24 or 36 hours.

4.2.1.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* herbal tincture in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.9 *Ps. aeruginosa*. Descriptive Statistics for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

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

Table 4.10 *Ps. aeruginosa*. Friedmans' test for *Tulbagia violacea* herbal tincture in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.2 Intra-group comparison of *Tulbagia violacea* 1X in 30% v/v ethanol with regards to observations at 18, 24, and 36 hours

4.2.2.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 1X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.11 *E. coli*. Descriptive Statistics for *Tulbagia violacea* 1X in 30% v/v ethanol.

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

Table 4.12 *E. coli*. Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *E. coli* at 18, 24 or 36 hours.

4.2.2.2 *Staph. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 1X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.13 *Staph. aureus*. Descriptive Statistics for *Tulbagia violacea* 1X in 30% v/v ethanol.

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

Table 4.14 *Staph. aureus*. Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Staph. aureus* at 18, 24 or 36 hours.

4.2.2.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 1X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.15 *K. pneumoniae*. Descriptive Statistics for *Tulbagia violacea* 1X in 30% v/v ethanol.

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

Table 4.16 *K. pneumoniae*. Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *K. pneumoniae* at 18, 24 or 36 hours.

4.2.2.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 1X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.17 *B. cereus*. Descriptive Statistics for *Tulbagia violacea* 1X in 30% v/v ethanol.

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

Table 4.18 *B. cereus*. Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *B. cereus* at 18, 24 or 36 hours.

4.2.2.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 1X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.19 *Ps. aeruginosa*. Descriptive Statistics for *Tulbagia violacea* 1X in 30% v/v ethanol.

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

Table 4.20 *Ps. aeruginosa*. Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.3 Intra-group comparison of *Tulbagia violacea* 6X in 30% v/v ethanol with regards to observations at 18, 24, and 36 hours

4.2.3.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 6X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.21 *E. coli*. Descriptive Statistics for *Tulbagia violacea* 6X in 30% v/v ethanol.

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

Table 4.22 *E. coli*. Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *E. coli*. at 18, 24 or 36 hours.

4.2.3.2 *Staph. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 6X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.23 *Staph. aureus.* Descriptive Statistics for *Tulbagia violacea* 6X in 30% v/v ethanol.

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

Table 4.24 *Staph. aureus.* Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Staph. aureus* at 18, 24 or 36 hours.

4.2.3.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 6X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.25 *K. pneumoniae.* Descriptive Statistics for *Tulbagia violacea* 6X in 30% v/v ethanol.

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

Table 4.26 *K. pneumoniae.* Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of K. pneumoniae at 18, 24 or 36 hours.

4.2.3.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 6X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.27 *B. cereus.* Descriptive Statistics for *Tulbagia violacea* 6X in 30% v/v ethanol.

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

Table 4.28 *B. cereus.* Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *B. cereus* at 18, 24 or 36 hours.

4.2.3.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the *Tulbagia violacea* 6X in 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.29 *Ps. aeruginosa.* Descriptive Statistics for *Tulbagia violacea* 6X in 30% v/v ethanol.

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

Table 4.30 *Ps. aeruginosa.* Friedmans' test for *Tulbagia violacea* 1X in 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.4 Intra-group comparison of 30% v/v ethanol with regards to observations at 18, 24, and 36 hours

4.2.4.1 *E. coli*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.31 *E. coli.* Descriptive statistics for 30% v/v ethanol.

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

Table 4.32 *E. coli.* Friedmans' test for 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *E. coli* at 18, 24 or 36 hours.

4.2.4.2 *Staph. aureus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.33 *Staph. aureus.* Descriptive statistics for 30% v/v ethanol.

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

Table 4.34 *Staph. aureus.* Friedmans' test for 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Staph. aureus* at 18, 24 or 36 hours.

4.2.4.3 *K. pneumoniae*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.35 *K. pneumoniae*. Descriptive statistics for 30% v/v ethanol.

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

Table 4.36 *K. pneumoniae*. Friedmans' test for 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *K. pneumoniae* at 18, 24 or 36 hours.

4.2.4.4 *B. cereus*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.37 *B. cereus.* Descriptive statistics for 30% v/v ethanol.

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

Table 4.38 *B. cereus.* Friedmans' test for 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *B. cereus* at 18, 24 or 36 hours.

4.2.4.5 *Ps. aeruginosa*

The Friedmans' test was used to compare the ratios of the diameters of inhibition of the 30% v/v ethanol to the combined diameters of inhibition of the gentamycin and vancomycin, against each other at 18, 24, and 36 hours.

Table 4.39 *Ps. aeruginosa.* Descriptive statistics for 30% v/v ethanol.

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

Table 4.40 *Ps. aeruginosa.* Friedmans' test for 30% v/v ethanol.

Ranks

	Mean Rank
18 hours	2.00
24 hours	2.00
36 hours	2.00

Test Statistics[a]

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

a. Friedman Test

CONCLUSION: P=1.000, therefore P>α. Therefore the null hypothesis was accepted. There was no change in the diameters of the zones of inhibition of *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.5 Inter-group comparison between *Tulbagia violacea* herbal tincture in 30% v/v ethanol and 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 hours

4.2.5.1 *E. coli*

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

Table 4.41 *E. coli*. Results of Mann-Whitney test for *Tulbagia violacea* herbal tincture.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *E. coli* at 18, 24 or 36 hours.

4.2.5.2 *Staph. aureus*

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

Table 4.42 *Staph. aureus*. Results of Mann-Whitney test for *Tulbagia violacea* herbal tincture.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Staph. aureus.* at 18, 24 or 36 hours.

4.2.5.3 *K. pneumoniae*

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

Table 4.43 *K. pneumoniae*. Results of Mann-Whitney test for *Tulbagia violacea* herbal tincture.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *K. pneumoniae* at 18, 24 or 36 hours.

4.2.5.4 *B. cereus*

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

Table 4.44 *B. cereus*. Results of Mann-Whitney test for *Tulbagia violacea* herbal tincture.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *B. cereus* at 18, 24 or 36 hours.

4.2.5.5 *Ps. aeruginosa*

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

Table 4.45 *Ps. aeruginosa.* Results of Mann-Whitney test for *Tulbagia violacea* herbal tincture.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.6 Inter-group comparison between *Tulbagia violacea* 1X in 30% v/v ethanol and 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 hours

4.2.6.1 *E. coli*

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

Table 4.46 *E. coli.* Results of Mann-Whitney test for *Tulbagia violacea* 1X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *E. coli* at 18, 24 or 36 hours.

4.2.6.2 *Staph. aureus*

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

Table 4.47 *Staph. aureus.* Results of Mann-Whitney test for *Tulbagia violacea* 1X.

Time	**P-value**
18 hours	1.000
24 hours	1.000
36 hours	1.000

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Staph. aureus* at 18, 24 or 36 hours.

4.2.6.3 *K. pneumoniae*

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

Table 4.48 *K. pneumoniae.* Results of Mann-Whitney test for *Tulbagia violacea* 1X.

Time	**P-value**
18 hours	1.000
24 hours	1.000
36 hours	1.000

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *K. pneumoniae* at 18, 24 or 36 hours.

4.2.6.4 *B. cereus*

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

Table 4.49 *B. cereus*. Results of Mann-Whitney test for *Tulbagia violacea* 1X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *B. cereus* at 18, 24 or 36 hours.

4.2.6.5 *Ps. aeruginosa*

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

Table 4.50 *Ps. aeruginosa*. Results of Mann-Whitney test for *Tulbagia violacea* 1X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.7 Inter-group comparison between *Tulbagia violacea* 6X in 30% v/v ethanol and 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 hours

4.2.7.1 *E. coli*

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

Table 4.51 *E. coli*. Results of Mann-Whitney test for *Tulbagia violacea* 6X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *E. coli* at 18, 24 or 36 hours.

4.2.7.2 *Staph. aureus*

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

Table 4.52 *Staph. aureus*. Results of Mann-Whitney test for *Tulbagia violacea* 6X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Staph. aureus* at 18, 24 or 36 hours.

4.2.7.3 *K. pneumoniae*

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

Table 4.53 *K. pneumoniae*. Results of Mann-Whitney test for *Tulbagia violacea* 6X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *K. pneumoniae* at 18, 24 or 36 hours.

4.2.7.4 *B. cereus*

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

Table 4.54 *B. cereus*. Results of Mann-Whitney test for *Tulbagia violacea* 6X.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *B. cereus* at 18, 24 or 36 hours.

4.2.7.5 *Ps. aeruginosa*

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

Table 4.55 *Ps. aeruginosa.* Results of Mann-Whitney test for *Tulbagia violacea* 6X.

Time	**P-value**
18 hours	1.000
24 hours	1.000
36 hours	1.000

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Ps. aeruginosa* at 18, 24 or 36 hours.

4.2.8 Inter-group comparison between *Tulbagia violacea* herbal tincture in 30% v/v ethanol, *Tulbagia violacea* 1X in 30% v/v ethanol and *Tulbagia violacea* 6X in 30% v/v ethanol with regard to observations at 18 hours, 24 hours and 36 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, 1X potency and 6X potency to each other, with regard to observations at 18 hours, 24 hours and 36 hours.

Table 4.56 *E. coli*. Results of Kruskal-Wallis test for *Tulbagia violacea* herbal tincture and 1X and 6X homoeopathic potencies.

Time	**P-value**
18 hours	1.000
24 hours	1.000
36 hours	1.000

CONCLUSION: At 18, 24 and 36 hours, $P > \alpha$. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *E. coli* at 18, 24 or 36 hours.

4.2.8.2 *Staph. 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, 1X potency and 6X potency to each other, with regard to observations at 18 hours, 24 hours and 36 hours.

Table 4.57 *Staph. aureus.* Results of Kruskal-Wallis test for *Tulbagia violacea* herbal tincture and 1X and 6X homoeopathic potencies.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Staph. aureus* at 18, 24 or 36 hours.

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, 1X potency and 6X potency to each other, with regard to observations at 18 hours, 24 hours and 36 hours.

Table 4.58 *K. pneumoniae.* Results of Kruskal-Wallis test for *Tulbagia violacea* herbal tincture and 1X and 6X homoeopathic potencies.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *K. pneumoniae* at 18, 24 or 36 hours.

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, 1X potency and 6X potency to each other, with regard to observations at 18 hours, 24 hours and 36 hours.

Table 4.59 *B. cereus*. Results of Kruskal-Wallis test for *Tulbagia violacea* herbal tincture and 1X and 6X homoeopathic potencies.

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

CONCLUSION: At 18, 24 and 36 hours, $P > \alpha$. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *B. cereus* at 18, 24 or 36 hours.

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, 1X potency and 6X potency to each other, with regard to observations at 18 hours, 24 hours and 36 hours.

Table 4.60 *Ps. aeruginosa*. Results of Kruskal-Wallis test for *Tulbagia violacea* herbal tincture and 1X and 6X homoeopathic potencies.

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

CONCLUSION: At 18, 24 and 36 hours, P> α. Therefore the null hypothesis was accepted. There was no difference between the zones of inhibition for *Ps. aeruginosa* at 18, 24 or 36 hours.

CHAPTER FIVE

B) DISCUSSION

5.1 *TULBAGIA VIOLACEA* HERBAL TINCTURE

The results of this study demonstrate that the *Tulbagia violacea* herbal tincture in 30% v/v ethanol had no antibacterial effect on any of the bacteria tested, *i.e.*, *E. coli, K. pneumoniae, B. cereus, Staph. aureus* or *Ps. aeruginosa*, when evaluated using the disc-diffusion assay. No growth inhibition was exhibited with any of the samples, resulting in a p-value of 1.000 for all the samples tested when compared to the negative control using the Mann-Whitney U test.

The absence of any significant statistical difference between the antibacterial effect of the sample tested, and the 30% v/v ethanol negative control, can be seen to be due to the use of dry discs in preference to wet discs. This technique effectively nullified the superfluous anti-microbial effect of the ethanol on the bacteria, as all the ethanol present had been evaporated off before the placement of the discs on the inoculated media. This resulted in no zones of inhibition being formed around the ethanol discs.

There was no difference in the inhibition zone sizes at 18, 24 and 36 hours.

5.2 *TULBAGIA VIOLACEA* 1X

The results of this study demonstrate that the *Tulbagia violacea* 1X in 30% v/v ethanol had no antibacterial effect on any of the bacteria tested, *i.e.*, *E. coli, K. pneumoniae, B. cereus, Staph. aureus* or *Ps. aeruginosa*, when evaluated using the disc-diffusion assay. No growth inhibition was exhibited with any of the samples, resulting in a p-value of 1.000 for all the samples tested when compared to the negative control using the Mann-Whitney U test.

The absence of any significant statistical difference between the antibacterial effect of the sample tested, and the 30% v/v ethanol negative control, can be seen to be due to the use of dry discs in preference to wet discs. This technique effectively nullified the

superfluous anti-microbial effect of the ethanol on the bacteria, as all the ethanol present had been evaporated off before the placement of the discs on the inoculated media. This resulted in no zones of inhibition being formed around the ethanol discs.

There was no difference in the inhibition zone sizes at 18, 24 and 36 hours.

5.3 *TULBAGIA VIOLACEA* 6X

The results of this study have shown that the *Tulbagia violacea* 6X in 30% v/v ethanol had no antibacterial effect on any of the bacteria tested, *i.e.*, *E. coli*, *K. pneumoniae*, *B. cereus*, *Staph. aureus* or *Ps. aeruginosa*, when evaluated using the disc-diffusion assay. No growth inhibition was exhibited with any of the samples, resulting in a p-value of 1.000 for all the samples tested when compared to the negative control using the Mann-Whitney U test.

The absence of any significant statistical difference between the antibacterial effect of the sample tested, and the 30% v/v ethanol negative control, can be seen to be due to the use of dry discs in preference to wet discs. This technique effectively nullified the superfluous anti-microbial effect of the ethanol on the bacteria, as all the ethanol present had been evaporated off before the placement of the discs on the inoculated media. This resulted in no zones of inhibition being formed around the ethanol discs.

There was no difference in the inhibition zone sizes at 18, 24 and 36 hours.

5.4 GENERAL DISCUSSION

None of the samples tested yielded any positive anti-microbial results.

There is a possibility that the failure of the experiment to produce any statistically significant results, may have been due to one of the following possible flaws in experimental procedure:

- The incorrect identification of the original source material;
- The use of too low a concentration of herbal tincture;
- The anti-microbial properties of *Tulbagia violacea* residing mainly in its essential oil, which in turn could not diffuse through the agar medium.

These possible flaws are discussed further in Chapter Six.

The failure of the *Tulbagia violacea* herbal tincture in 30% v/v ethanol to yield any positive results is consistent with the findings of McGaw, Jager and van Staden (2000:254).

The absence of a positive anti-microbial response against *E. coli* contradicts the findings of Watt and Beyer-Brandwijk regarding that microbe, but this study agrees with them regarding their findings that *Tulbagia violacea* has no effect on *Staph. aureus* (1962:717). However, the experimental procedure followed by Watt and Breyer-Brandwijk was not stated, so it may not be appropriate to make a direct comparison between the findings in this study and theirs.

These results suggest that *Tulbagia violacea* does not share the antibacterial actions of *Allium sativum* (Ross, 1999:33), when tested *in vitro*. However, a clinically controlled *in vivo* study may prove that *Tulbagia violacea* does share some of the other actions of *Allium sativum*, as suggested by Van Wyk and Gericke (2000:134).

Tulbagia violacea may prove to be an effective antibacterial agent when tested *in vivo*, as the herb may have an indirect mechanism of action e.g. enhancing the bodies natural defence mechanisms against bacteria.

The 1X and 6X potencies of *Tulbagia violacea* may in the future prove to be effective in limiting the spread of infection *in vivo*, if prescribed according to classical homoeopathic rather than phytotherapeutic principles. A complete homoeopathic proving of *Tulbagia violacea* may contribute greatly to the homoeopathic *materia medica*.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The purpose of this study was to evaluate the efficacy of an ethanolic extract, and a homoeopathic 1X and 6X potency of *Tulbagia violacea* in inhibiting the *in vitro* growth *of E. coli, K. pneumonia, Staph. aureus, B. cereus* and *Ps. aeriginosa*, in terms of the disc-diffusion test.

Tulbagia violacea herbal tincture in 30% v/v ethanol proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the disc-diffusion test.

Tulbagia violacea 1X in 30% v/v ethanol proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the disc-diffusion test.

Tulbagia violacea 6X in 30% v/v ethanol proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the disc-diffusion test.

6.2 RECOMMENDATIONS

Due to the absence of any statistically significant positive results arising from this experiment, the methodology followed was carefully reviewed in the light of further research of related literature, with the intent of identifying possible flaws.

Arising from the review, the following points are areas which should be given careful consideration by researchers embarking upon similar experiments.

6.2.1 Identification of source material

The plants used to make the *Tulbagia violacea* herbal tincture should be expertly identified and voucher specimens deposited at a reputable herbarium, to ensure that similar sub-species of the same family are not mistakenly identified and used as the original sample plants for the herbal tincture. According to Dr. Neil Crouch (2002), Head Ethnobotanist at the Natal Herbarium, Durban, the lodging of voucher specimens is particularly important when utilising indigenous plants, especially those that are not in flower, as these run the greatest risk of being mistakenly identified.

Lodging of a voucher specimen has the added advantage of verifying the plant material used in the experiment at a later date, should subsequent review of the experiment by other researchers call this point into question.

6.2.2 Extractant

Trials should be run with different types of extractants to see which is most effective in extracting the active ingredients from the fresh *Tulbagia violacea* plants.
Eloff (1997), for instance, found that acetone was a far better extractant than ethanol or water when extracting active ingredients from *Anthocleista grandiflora* and *Combretum erythrophyllum.*

Different concentrations of ethanol used for extraction should also be tested to discover which has the most advantageous extractant ability for this herb.

6.2.3 Concentration of the herbal tincture

The exact concentration of the herbal tincture used should be determined at the outset of the experiment.

It is recommended that the original *Tulbagia violacea* herbal tincture in 30% v/v ethanol, should be dried and the residue stored at –15°C. The residue should then be resuspended in ethanol of the same strength, at a predetermined concentration, e.g. 100mg of residue per millilitre of ethanol. When a known amount of this solution is then pipetted onto a filter paper disc, the exact amount of residue on the disc can be calculated. (McGaw, Jager and van Staden, 2000:252.)

Example: 10 microlitres of 100 mg/ml solution pipetted onto a filter paper disc will result in 1mg of residue being present on each disc.

6.2.4 Manufacture of herbal tincture

Manufacture of the herbal tincture by the experimenter, rather than purchasing of a pre-manufactured herbal tincture, will ensure greater control over the various factors influencing the final sample. The correct identification of sample material can be ensured, and the type of extractant used and final tincture concentration can be specifically chosen to suit the precise methodology of the experiment.

6.2.5 Identification of stock culture

Each culture used should be clearly identified with an ATCC or other type-culture collection number. This will verify that a standard strain, essential for a microbiological assay test, is used, as well as ensuring that the experiment can be accurately reproduced. (Rios, Recio and Villar, 1988:142.)

6.2.6 Concentration of innoculant

Serial dilution methods or spectrophotomic techniques should be used to gain a precise estimate of the number of bacterial cells present in the innoculant before it is seeded onto the agar (McGaw, Jager and van Staden, 2000:252).

6.2.7 Paper discs

Specifically manufactured assay-discs of uniform thickness and diameter should be used in preference to discs cut from filter paper. The size of the inhibition zone may be greatly influenced by the thickness and composition of the filter paper used to make the discs. (Hewitt and Vincent, 1989:57.)

6.2.8 Inoculation of media

The molten agar should be inoculated with the cultures before it is poured, to ensure that even concentrations of inoculate and ultimately uniform perimeters of the zones of inhibition are produced. When the microbes are only swabbed on the top of the plates, only surface growth occurs on the medium. It has been found that measurement of surface growth only may give inaccurate representations of inhibition zone sizes. (Hewitt and Vincent, 1989:67.)

Discs must be placed on the plates as soon as possible after inoculation of the media. Even one or two hours of standing time at room temperature before placement of discs may result in doubling or trebling of the bacterial cell numbers, and consequent diminishment of the zones of inhibition. (Hewitt and Vincent, 1989:180.) [See figure 6.1.]

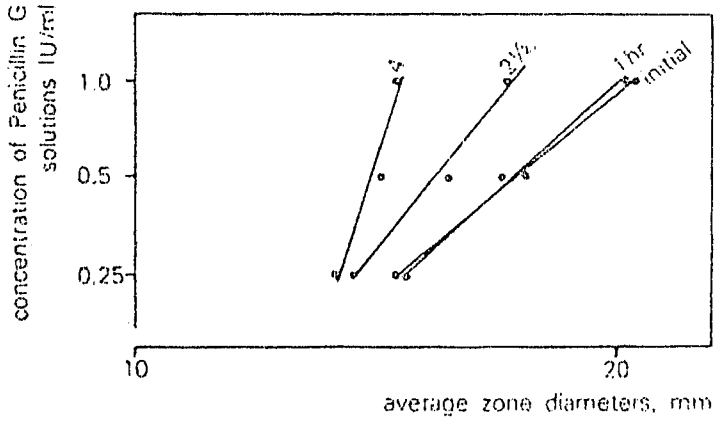

Figure 6.1 Graph illustrating the effect of leaving seeded assay medium at room temperature before applying test solutions. Not only are zone diameters getting smaller after longer standing, but the slope of the assay is also adversely affected resulting in loss of sensitivity. (Hewitt and Vincent, 1989:180.)

6.2.9 Refrigeration of plates

The inoculated plates should be refrigerated overnight at 5°C as soon as the discs have been placed. This will allow for diffusion of the sample into the medium before bacterial proliferation occurs. This has the effect of lowering the detection limit, and increasing the inhibition diameter. (Rios, Recio and Villar, 1988:128.)

6.2.10 Dilution tests

Dilution tests (See 2.2.1.2) should be performed in conjunction with disc-diffusion tests in order to test for the antibacterial effectiveness of any non-polar extracts (e.g. essential oils) which do not diffuse easily through the hydrous agar medium.

Dilution methods are also more sensitive than diffusion methods, and can yield a minimum inhibitory concentration in the event of positive anti-microbial results. (Rios, Recio and Villar, 1988:142.)

6.2.11 *In vivo* testing

Tulbagia violacea should be tested *in vivo* for antibacterial effectiveness, by means of a controlled clinical trial.

6.2.12 A homoeopathic proving

A complete homoeopathic proving of *Tulbagia violacea* should be undertaken. This will enable prescription of this herb in homoeopathic potencies, according to homoeopathic principles.

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List of Appendices

Appendix A – *E. coli*									
					diameter (mm)				
TEST AND CONTROL		Test 1			Test 2			Test 3	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	20	20	20	25	25	25	25	25	25
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 4			Test 5			Test 6	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	23	23	23	25	25	25	25	25	25
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 7			Test 8			Test 9	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	24	24	24	24	24	24	24	24	24
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 10			Test 11			Test 12	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	24	24	24	24	24	24	20	20	20
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 13			Test 14			Test 15	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	22	22	22	22	22	22	25	25	25
6)Vancomycin	0	0	0	0	0	0	0	0	0

Appendix B – *K. pneumoniae*									
					diameter (mm)				
TEST AND CONTROL		**Test 1**			**Test 2**			**Test 3**	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% m/m	0	0	0	0	0	0	0	0	0
5)Gentamycin	24	24	24	24	24	24	24	24	24
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 4			**Test 5**			**Test 6**	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% m/m	0	0	0	0	0	0	0	0	0
5)Gentamycin	26	26	26	25	25	25	24	24	24
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 7			**Test 8**			**Test 9**	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% m/m	0	0	0	0	0	0	0	0	0
5)Gentamycin	24	24	24	24	24	24	25	25	25
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 10			**Test 11**			**Test 12**	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% m/m	0	0	0	0	0	0	0	0	0
5)Gentamycin	25	25	25	23	23	23	24	24	24
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 13			**Test 14**			**Test 15**	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% m/m	0	0	0	0	0	0	0	0	0
5)Gentamycin	24	24	24	25	25	25	26	26	26
6)Vancomycin	0	0	0	0	0	0	0	0	0

Appendix C – *Staph. aureus*									
				diameter (mm)					
TEST AND CONTROL		Test 1			Test 2			Test 3	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	30	30	30	31	31	31	31	31	31
6)Vancomycin	21	21	21	20	20	20	22	22	22
		Test 4			Test 5			Test 6	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	33	33	33	34	34	34	30	30	30
6)Vancomycin	22	22	22	23	23	23	21	21	21
		Test 7			Test 8			Test 9	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	34	34	34	30	30	30	32	32	32
6)Vancomycin	23	23	23	21	21	21	21	21	21
		Test 10			Test 11			Test 12	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	33	33	33	31	31	31	31	31	31
6)Vancomycin	22	22	22	21	21	21	22	22	22
		Test 13			Test 14			Test 15	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	32	32	32	32	32	32	31	31	31
6)Vancomycin	22	22	22	22	22	22	22	22	22

Appendix D – *B. cereus*									
	diameter (mm)								
TEST AND CONTROL		Test 1			Test 2			Test 3	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	25	25	25	28	28	28	23	23	23
6)Vancomycin	14	14	14	15	15	15	14	14	14
		Test 4			Test 5			Test 6	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30 %m/m	0	0	0	0	0	0	0	0	0
5)Gentamycin	27	27	27	24	24	24	26	26	26
6)Vancomycin	17	17	17	15	15	15	14	14	14
		Test 7			Test 8			Test 9	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	28	28	28	25	25	25	25	25	25
6)Vancomycin	14	14	14	15	15	15	14	14	14
		Test 10			Test 11			Test 12	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	26	26	26	26	26	26	25	25	25
6)Vancomycin	15	15	15	14	14	14	16	16	16
		Test 13		Test 14				Test 15	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	27	27	27	26	26	26	26	26	26
6)Vancomycin	16	16	16	15	15	15	14	14	14

Appendix E – *Ps. aeruginosa*									
					diameter (mm)				
TEST AND CONTROL		Test 1			Test 2			Test 3	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	16	16	16	17	17	17	17	17	17
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 4			Test 5			Test 6	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	18	18	18	19	19	19	19	19	19
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 7			Test 8			Test 9	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	18	18	18	22	22	22	19	19	19
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 10			Test 11			Test 12	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	18	18	18	18	18	18	18	18	18
6)Vancomycin	0	0	0	0	0	0	0	0	0
		Test 13			Test 14			Test 15	
	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs	18hrs	24hrs	36hrs
1) *Tulbagia violacea* tincture	0	0	0	0	0	0	0	0	0
2) *Tulbagia violacea* 1X	0	0	0	0	0	0	0	0	0
3) *Tulbagia violacea* 6X	0	0	0	0	0	0	0	0	0
4)Ethanol 30% v/v	0	0	0	0	0	0	0	0	0
5)Gentamycin	16	16	16	17	17	17	17	17	17
6)Vancomycin	0	0	0	0	0	0	0	0	0