A COMPARATIVE *IN VITRO* STUDY OF THE ANTIMICROBIAL EFFECTIVENESS OF *BAPTISIA TINCTORIA* EXTRACT IN 62% ETHANOL AND *BAPTISIA TINCTORIA* EXTRACT IN DISTILLED WATER.

BY

Shamini Singh

Mini-dissertation submitted in partial compliance with the requirements of the Master's Degree in Technology: Homoeopathy in the Faculty of Health at the Durban Institute of Technology.

I, Shamini Singh do declare that this mini-dissertation represents my own work in both concept and execution.

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Signature of Student Date of Signature

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Signature of Supervisor Date of Signature

Dr Richard Steele B.A., H.D.E., M.tech (Hom)

FOREWORD

It gives me great pleasure to write this forward to the dissertation of Miss Shamini Singh.

I know her to be a very enthusiastic and enterprising student of homoeopathy.

Her dissertation is a study on the possible antimicrobial properties of *Baptisia tinctoria*.

As such, Homoeopathic remedies act on patients and are not generally meant to act on microbes. The action of the remedies is said to be on the vital force of the human, and by strengthening his own defenses, act curatively. However it is interesting to study if the herbal extract does have antimicrobial properties.

Shamini's dissertation and study represent her initial attempt to study this aspect.

Similar studies, which build upon this one, are needed to draw credible conclusions.

I wish her the very best in her endeavors.

Rajan Sankaran

DEDICATION

I would like to dedicate this work to my beloved parents, Baijanth and Priscilla Devi Singh, whose every waking moment was directed at my success and happiness. I love you both.

To my loving and gentle cousin, Rowark Singh, who was tragically taken from us on 22 August 2004. Your memory will always burn brightly in all our hearts. I love and miss you brother.

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A special thanks to a truly inspirational person, Dr Rajan Sankaran, for honouring me by kindly writing the foreword to my dissertation. I hope someday that I can emulate you in the field of Homoeopathy.

ABSTRACT

The purpose of this study was to determine the antimicrobial effect of *Baptisia tinctoria* extract (1:10) in 62% ethanol and *Baptisia tinctoria* extract (1:10) in distilled water on the *in vitro* growth inhibition of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* respectively as compared to ethanol and distilled water control.

Measurements were by means of the disc diffusion and well diffusion assays.

For this experiment, sixty Mueller-Hinton plates were prepared and inoculated with each test bacteria in turn. Thirty plates were used for the disc diffusion assay and the remaining thirty plates were used for the well diffusion assay. Filter discs were individually inoculated with the sample substances and the control using a micropipette, before being allowed to air dry. The three wells were made equidistantly apart on each plate by means of punching them with the base of a Pasteur pipette to the depth of 3 millimeters.

A Chloramphenicol antibiotic control was 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 inhibition measured with a pair of Vernier calipers at 24 hours.

Data entry was done using the SPSS statistical package. The Mann-Whitney U test was used for an inter-group comparison of the mean inhibition zones produced by the test and control substances after 24 hours of incubation. The test were performed at $\alpha = 0.05$ (5%) level of significance.

The results obtained were that the *Baptisia tinctoria* extract in 62% ethanol and the *Baptisia tinctoria* extract in distilled water did not produce a statistically significant inhibitive effect on any of the bacteria tested.

This study concluded that *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water, are ineffective in inhibiting the *in vitro* growth of any of the bacteria tested when evaluated by means of the disc diffusion and well diffusion assays.

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ABBREVIATIONS

ATCC	American Type Culture Collection
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
P. aeruginosa	*Pseudomonas aeruginosa*
Staph. aureus	*Staphylococcus aureus*
Staph. epidermidis	*Staphylococcus epidermidis*

DEFINITION OF TERMS

ANTIBIOTIC	A soluble substance derived from a mould or bacterium that inhibits the growth of other microbes (Dirckx, 1997:54). Destroys or inhibits microorganisms (Chevallier, 1996:321).
ANTIMICROBIAL	Tending to destroy microbes, to prevent their multiplication or growth, or to prevent their pathogenic action (Dirckx, 1997:57). Destroys or inhibits microorganisms (Chevallier, 1996:321).
ANTISEPTIC	An agent or substance capable of preventing infection by inhibiting the growth of infectious agents (Dirckx, 1997:58). Free from contamination by harmful bacteria, viruses and other microorganisms (Chevallier, 1996:321).
DECOCTION	Water-based preparation of bark, roots, berries, or seeds simmered in boiling water (Chevallier, 1996:321)
EXTRACT	To draw (as a juice or fraction) by physical or chemical process; *also*: to treat with a solvent as to remove a soluble substance (Merriam-Webster Online, 2004).

INFUSION A tea made by pouring water over a plant material (usually dried flowers, fruit, leaves, and other parts, though fresh plant material may also be used), and then allowed to steep. The water is usually boiling, but cold infusions are also an option (American Botanical Council, 2004).

POULTICE Herbal preparation usually applied hot to the affected area to alleviate pain and reduce swelling (Chevallier, 1996:321).

TINCTURE An extract of a plant made by soaking herbs in a dark place with a desired amount of either glycerine, alcohol, or vinegar for two weeks. The liquid is strained from the plant material and may then be used therapeutically (American Botanical Council, 2004).

- HERBAL TINCTURE A herbal tincture is usually prepared in a ratio 1:5 (1 part herb to 5 parts alcohol) (Chevallier, 1996:291).
- HOMOEOPATHIC TINCTURE A homoeopathic tincture is usually prepared in a ratio of 1:10 (1 part herb to 10 parts alcohol) (German Homoeopathic Pharmacopoeia Method HAB 4a, British Homoeopathic Association, 1991).

<u>NOTE</u>: The term "*herbal extract*" has been used in this mini-dissertation because the term "*extract*" as defined above can be applied to both tinctures and infusions, and so encompasses both the ethanolic (tincture) and the aqueous (infusion) solutions utilised in this study.

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

Due to the developing resistance of many bacteria to pharmaceutical antimicrobials, it has become necessary to find an alternative to current chemical antimicrobial substances (Carson, *et al*., 1995:421-424).

Herbs are the basis of many different medicinal systems around the world, being especially popular in the less developed countries where Western drugs prove to be too expensive to provide a basis of treatment for the majority of the population (Balik 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).

Studies so far have included *Baptisia tinctoria* as an ingredient (e.g. Wustenberg, *et al*., 1999), but no studies on *Baptisia* tinctoria alone were found in the literature search.

In this study, the extracts utilised, were manufactured by Parceval (Pty) Ltd., according to the German Homoeopathic Pharmacopoeia Method HAB 4a (British Homoeopathic Association, 1991), in the ratio 1:10 (1 part plant to 10 parts of solvent). Therefore these extracts were equivalent to homoeopathic mother tinctures.

In this study, the comparative effectiveness of *Baptisia tinctoria* in 62% ethanol and *Baptisia tinctoria* in distilled water, as an antimicrobial agent was tested against five different types of bacteria: *Staphylococcus aureus (Staph. aureus), Staphylococcus epidermidis (Staph. epiderimidis), Pseudomonas aeruginosa (P. aeruginosa), Escherichia coli (E. coli)* and *Enterococcus faecalis (E. faecalis)*.

This study was conducted *in vitro* using the disc diffusion and well diffusion assays. The ratio of the zone of inhibition of each sample compared to the zone of inhibition around the chloramphenicol was used to measure how effective an antimicrobial substance the test sample was. The expression of the test zones as a ratio to the zone around the chloramphenicol 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 the results of the experiment is the sample being tested (McGraw, Jager and van Staden, 2000:253).

1.2 PROBLEM STATEMENT

The purpose of this study was to investigate the effectiveness of *Baptisia tinctoria* in inhibiting the *in vitro* growth of *Staph. aureus*, *Staph. epidermidis, P. aeruginosa, E. coli,* and *E. faecalis*, in terms of the disc diffusion and well diffusion assays.

1.3 SUBPROBLEMS

1.3.1 Sub problem One

To compare the effectiveness of *Baptisia tinctoria* extract in 62% ethanol to a 62 % ethanol control, in inhibiting the *in vitro* growth of *Staph. aureus*, *Staph. epidermidis, P. aeruginosa, E. coli,* and *E. faecalis*, in terms of the size of the zones of inhibition.

1.3.2 Sub problem Two

To compare the effectiveness of *Baptisia tinctoria* extract in distilled water to a distilled water control, in inhibiting the *in vitro* growth of *Staph. aureus*, *Staph. epidermidis, P. aeruginosa, E. coli,* and *E. faecalis*, in terms of the size of the zones of inhibition.

1.3.3 Sub problem Three

To compare the effectiveness of *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water, in inhibiting the *in vitro* growth of *Staph. aureus*, *Staph. epidermidis, P. aeruginosa, E. coli,* and *E. faecalis*, in terms of the size of the zones of inhibition.

1.4 HYPOTHESIS

1.4.1 Hypothesis One

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *Staph. aureus* using the disc diffusion method.

1.4.2 Hypothesis Two

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *Staph. epidermidis* using the disc diffusion method.

1.4.3 Hypothesis Three

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *P. aeruginosa* using the disc diffusion method.

1.4.4 Hypothesis Four

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *E. coli* using the disc diffusion method.

1.4.5 Hypothesis Five

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *E. faecalis* using the disc diffusion method.

1.4.6 Hypothesis Six

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *Staph. aureus* using the disc diffusion method .

1.4.7 Hypothesis Seven

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *Staph. epidermidis* using the disc diffusion method.

1.4.8 Hypothesis Eight

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *P. aeruginosa* using the disc diffusion method.

1.4.9 Hypothesis Nine

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *E. coli* using the disc diffusion method.

1.4.10 Hypothesis Ten

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *E. faecalis* using the disc diffusion method.

1.4.11 Hypothesis Eleven

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *Staph. aureus* using the disc diffusion method.

1.4.12 Hypothesis Twelve

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *Staph. epidermidis* using the disc diffusion method.

1.4.13 Hypothesis Thirteen

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *P. aeruginosa* using the disc diffusion method.

1.4.14 Hypothesis Fourteen

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *E. coli* using the disc diffusion method.

1.4.15 Hypothesis Fifteen

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *E. faecalis* using the disc diffusion method.

1.4.16 Hypothesis Sixteen

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *Staph. aureus* using the well diffusion method.

1.4.17 Hypothesis Seventeen

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *Staph. epidermidis* using the well diffusion method.

1.4.18 Hypothesis Eighteen

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *P. aeruginosa* using the well diffusion method.

1.4.19 Hypothesis Nineteen

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *E. coli* using the well diffusion method.

1.4.20 Hypothesis Twenty

Baptisia tinctoria extract in 62% ethanol will have no significant antimicrobial effects on *E. faecalis* using the well diffusion method.

1.4.21 Hypothesis Twenty One

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *Staph. aureus* using the well diffusion method.

1.4.22 Hypothesis Twenty Two

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *Staph. epidermidis* using the well diffusion method.

1.4.23 Hypothesis Twenty Three

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *P. aeruginosa* using the well diffusion method.

1.4.24 Hypothesis Twenty Four

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *E. coli* using the well diffusion method.

1.4.25 Hypothesis Twenty Five

Baptisia tinctoria extract in distilled water will have no significant antimicrobial effects on *E. faecalis* using the well diffusion method.

1.4.26 Hypothesis Twenty Six

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *Staph. aureus* using the well diffusion method.

1.4.27 Hypothesis Twenty Seven

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *Staph. epidermidis* using the well diffusion method.

1.4.28 Hypothesis Twenty Eight

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *P. aeruginosa* using the well diffusion method.

1.4.29 Hypothesis Twenty Nine

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *E. coli* using the well diffusion method.

1.4.30 Hypothesis Thirty

There will be no significant difference between the antimicrobial effects of *Baptisia tinctoria* extract in 62% ethanol compared with *Baptisia tinctoria* extract in distilled water on *E. faecalis* using the well diffusion method.

1.5 DELIMITATIONS

- This study was limited to only five species of bacteria namely, *Staph. aureus, Staph. epidermidis, P. aeruginosa, E. coli, E. faecalis.*

- This study was limited to *Baptisia tinctoria* only.

- Only Mueller-Hinton agar was used as a growth medium.

- Only *Baptisia tinctoria* extract produced in 62% ethanol and distilled water was used.

- This was an *in vitro* study.

CHAPTER TWO

LITERATURE REVIEW

2.1 RESEARCH METHODS

2.1.1 Screening methods for natural products with antimicrobial properties

The three most common methods employed to evaluate the antimicrobial properties of natural products are diffusion assay, 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, microorganism being tested, extractive method, pH and solubility of the sample in the culture medium (Rios, Recio and Villar, 1988:127). This study used two versions of the diffusion assay, namely, disc diffusion and well diffusion assays.

2.1.1.1 Diffusion assays

Diffusion methods of screening for antimicrobial properties of natural products classically employ either a disc, well or cylinder methods as reservoirs for the sample substance (Rios, Recio and Villar, 1988:128). This method is based on the principal that the 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 the growth-free area around the reservoir can be measured and taken as the antimicrobial ability of that product.

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 antimicrobial 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 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, 1988:131).

Dry discs, as opposed to wet discs, which contain a liquid sample, are used to test for antimicrobial properties of ethanol extracts (Rios, Recio and Villar, 1998:131), and have the advantage of nullifying the unknown effects that the rate of evaporation of the sample off the wet disc has on the results of the disc assay, as noted in experiments by Reid (2002) and Invernizzi (2002). This study made use of dry discs.

Comparisons of the zones of inhibition of natural products with those of synthetic antibiotics in disc assay or well assay are useful for establishing the sensitivity of test organisms. Comparisons of the antimicrobial potency of the natural test substances and the synthetic antibiotic cannot be made from these measurements (Rios, Recio and Villar, 1988:142). This is due to the fact that many other factors can be influential, such as diffusion ability, which can affect the size of the zones of inhibition, resulting in misleading

conclusions when comparing two different types of substances (Hewitt and Vincent, 1989:40).

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

2.1.1.2 Dilution tests

Dilution test require a homogenous dispersion of a sample in water. Bacterial proliferation is measured by the turbidity of the solution, which is taken as a direct correlation to the amount of bacterial 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)

The dilution tests are generally more complicated, time consuming and expensive to perform than the disc assay or well assay studies (Rios, Recio and Villar, 1988:142).

2.1.1.3 Bioautographic methods

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

2.1.2 Choice of extractant

Ethanol is usually used in the manufacture of plant extracts to enable the extraction of water-insoluble constituents from the source material as well as serving as a preservative for the extract. However ethanol itself has antimicrobial effects between the 50% and 80% concentrations (Ketchum, 1988:131). This is why 62% ethanol only control will be used.

In order to try and neutralise the intervening variable effect of ethanol altogether, a water-based extract will also be assessed. Invernizzi (2003), cited in his recommendations that trials should be done using different types of extractants to see which is the most effective in extracting the active ingredients from plant materials. He makes mention of the use of acetone, but this is not viable as a therapeutic agent due to its toxic nature (Invernizzi, 2003:84)

2.2 *BAPTISIA TINCTORIA*

2.2.1 Family

Baptisia tinctoria forms part of the Leguminosae family (Chevallier, 1996: 175).

2.2.2 Nomenclature

Baptisia tinctoria is commonly known as wild indigo, indigo weed, horsefly weed, yellow broom, clover broom, rattle bush and yellow indigo. The name *Baptisia* is derived from the Greek *bapto* or *baptizo* (to dye, to colour), the plant having been formerly used as a colouring agent (Foster and Duke, 1990).

2.2.3 Description

Baptisia tinctoria is characteristically recognised as being a perennial plant having a stem from 2 to 3 feet high, blabrous and branching, yellowish-green in colour, and studded with small black dots. The leaves are sub sessile, 3-foliate-palmate; the leaflets are small, roundish, or obovate, acute at the base, very acute at the apex, bluish-green in colour, and turning black on drying. The stipules are setaceous and caducous. The flowers are bright yellow, few, and borne in small, loose, terminal racemes. The fruit is subglobose, bluish-black pod the size of a pea, on a stalk longer than the calyx, and contains several seeds (Foster and Duke, 1990).

2.2.4 Habitat and cultivation

Baptisia tinctoria (Wild indigo) is indigenous to the eastern parts of North America and grows from North Carolina to Southern Canada in dry, hilly woods (Chevallier, 1996: 175).

2.2.5 Parts used

The roots and leaves are the most commonly used part of the plant (Chevallier, 1996: 175). This study utilised root material.

2.2.6 Constituents

The constituents of *Baptisia tinctoria* are alkaloids, glycosides and oleoresin (Hoffmann 1984: 229). Chevallier (1996) reports that *Baptisia tinctoria* also contains isoflavones which are oestrogenic, flavonoids and polysaccharides.

2.2.7 Actions

Baptisia tinctoria was commonly used as a poultice by Native Americans and New World settlers to treat snakebite. The Mohicans used a decoction of the root to bathe cuts and wounds (Chevallier, 1996: 175).

In present day, the key uses for *Baptisia tinctoria* are as an immunostimulant, antiseptic (Chevallier, 1996: 175), antimicrobial, anticatarrhal and febrifuge (Hoffmann, 1984: 229). It is considered particularly effective for upper respiratory tract infections such as tonsillitis and pharyngitis and is also valuable in treating infections of the chest, gastro-intestinal tract and skin (Chevallier, 1996: 175, Hoffmann, 1984: 229). Systemically it may help in the

treatment of enlarged and inflamed lymph glands (lymphadenitis) and to reduce fevers (Chevallier, 1996: 175, Hoffmann, 1984: 229).

Baptisia tinctoria is also used in creams topically in the treatment of leucorrhoea, infected ulcers and to ease sore nipples (Chevallier, 1996:175). If taken both internally and as a mouthwash it is reputed to heal mouth ulcers, gingivitis and help in the control of pyorrhoea (Hoffmann, 1984: 229).

2.2.8 Preparations

It may be prepared as an alcoholic extract or as a decoction from the roots (Hoffmann, 1984: 229).

2.3 BACTERIOLOGY

2.3.1 *STAPH. AUREUS*

2.3.1.1 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 *Staph. epidermidis* and *Staph*. *saprophyticus* are recognised as being the most medically significant (Mims, *et al*., 1998:513). *Staph. aureus*, a coagulase-positive staphylococci, can be the cause of pustular skin diseases, sycosis barbae, wound infection, scalded skin syndrome, toxic shock syndrome, diarrhoea and vomiting, pneumonia, acute osteomyelitis, septic arthritis, intravenous catheter infection and endocarditis (Pattison, *et al*., 1995:51).

2.3.1.2 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 the older cells may stain Gram-negative (Jawetz, *et al*., 1991:187). *Staph. aureus* are non-motile, non-sporing and generally non-capsulate. When grown on laboratory media, colonies are generally circular, 2-3mm in diameter, and have a smooth, shiny surface. Colonies are opaque, have a golden-yellow, fawn or cream colour to them (Greenwood, Slack and Peutherer, 1992:204). When grown anaerobically, colonies are notably smaller and greyish in colour (Jawetz, *et al*., 1991:187). The optimal growth

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

2.3.1.3 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*., 1991:189).

2.3.1.4 *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 bacteraemia have been noted where there is no sign of sepsis evident. This occurs in more chronically ill 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 body's defence mechanism and colonise the tissues. 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 conditions caused by *Staph. aureus*, are usually due to enzymes and toxins produced by the bacteria. These enzymes and toxins include:

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

(Greenwood, Slack and Peutherer, 1992:205)

2.3.1.5 Anti-microbial sensitivity

Staph. aureus is resistant to most broad-spectrum penicillins such as ampycillin and amoxycillin due to the production of beta-lactamases by the

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

2.3.2 *STAPH. EPIDERMIDIS*

2.3.2.1 Classification

Staph. epidermidis belongs to the family Staphylococcacea and the genus *Staphylococcus*.

2.3.2.2 Morphology and identification

Staph. epidermidis is a Gram-positive coccus. Colonies are usually non-haemolytic and white (Cheesbrough, 1984:227). *Staph. epidermidis* cultures can be distinguished from *Staph. aureus* by their lack of clumping factor and their failure to coagulate plasma (Greenwood, Slacker and Peutherer, 1992:210).

2.3.2.3 Epidemiology

Staphylococci, particularly *Staph. epidermidis*, are members of the normal flora of the human skin and respiratory and gastrointestinal tract (Jawetz, *et al*., 1991:189). The chief source of infection are shedding human lesions, fomites contaminated from such lesions, and the human respiratory tract and skin. Contact spread of infection has assumed added importance in hospitals, where a large number of the staff and patients carry antibiotic-resistant staphylococci in their nasal cavities and skin (Jawetz, *et al*., 1991:191).

2.3.2.4 *Staph. epidermidis* infections

Staph. epidermidis, is increasingly a cause of nosocomial bacteraemia associated with catheters and other foreign bodies, and is an important cause of morbidity and mortality in debilitated patients (Beers *et al*., 1999:1148).

2.3.2.5 Anti-microbial sensitivity

Staph. epidermidis is resistant to most broad-spectrum penicillin and methicillin (Mims, *et al*., 1998:526).

2.3.3 *P. AERUGINOSA*

2.3.3.1 Classification

P. aeruginosa, a Gram-negative motile bacillus, is an opportunistic pathogen that frequently causes hospital-acquired infections. *P. aeruginosa* infections can develop in many anatomic sites, including skin, subcutaneous tissue, bone, ears, eyes, urinary tract and heart valves (Beers, *et al*.,1999:1173).

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

2.3.3.2 Morphology and identification

P. aeruginosa is a motile, Gram-negative, rod-shaped bacillus, measuring about 0.6 x 2 micrometers. It may occur singularly, or 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 presences of nitrates. *P. aeruginosa* can grow on a wide variety of media, and over a wide temperature range (Greenwood, Slacker and Peutherer, 1992:345).

Six different types of *P. 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, 1995:415).

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

2.3.3.3 Epidemiology

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

2.3.3.4 *P. aeruginosa* infections

Most pathology is mild and superficial. However, more severe infections 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 morality rate. The lungs of children with cystic fibrosis are particularly susceptible to this bacterial infection (Greenwood, Slacker and Peutherer, 1992:346).

2.3.3.5 Anti-microbial sensitivity

The virulence of *P. aeruginosa* is due to a number of factors. Exotoxin A and exoenzyme S have been identified to inhibit protein synthesis (Greenwood, Slacker and Peutherer, 1992:346). Extracellular proteases and elastases destroy tissues 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).

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

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

2.3.4 *E. COLI*

2.3.4.1 Classification

E. coli is a Gram-negative, enterobacter that normally inhabits the gastrointestinal tract. When *E. coli* organisms have colonising, enterotoxic, cytotoxic, or invasive virulence traits, they become major causes of watery, inflammatory, or bloody diarrhoea, occasionally with haemolytic-uremic syndrome. The extraintestinal site most often infected by *E. coli* is the urinary tract. This organism is also an opportunistic pathogen, causing disease in patients who have defects in host resistance as a result of other disease or who have received treatment with corticosteroids, radiation, antineoplastic drugs or antibiotics (Beers, *et al*., 1999:1158).

E. coli falls under the family Enterobacteriaceae, which forms the largest group of Gram-negative rods whose natural habitat is the intestinal tract of humans and animals (Jawetz, *et al*., 1881:204). The genus Escherichia to which it belongs is the only species of medical importance (Mims, *et al.*, 1998:523).

2.3.4.2 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

(15ºC - 45ºC), with some strains being able to survive temperatures of up to 60ºC for 15 minutes, or 55ºC for 60 minutes (Greenwood, Slacker and Peutherer, 1992:323). Optimal growth temperature for *E. coli* is 37ºC (Mackie and McCartney, 1996:361).

2.3.4.3 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).

2.3.4.4 *E. coli* infections

E. coli may possess lipopolysaccaridal endotoxins in their cell walls. They may also sometimes produce exotoxins of clinical importance. *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 bacterium is most often an opportunistic pathogen with infections most often arising in infancy, old age, during terminal stages of other diseases, or during periods of immunosuppression. With severe infections, it may reach the blood stream, causing sepsis (Jawetz, *et al.*, 1991:216).

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 is also known to be responsible for what is known as traveller's diarrhoea (turista).
- Enteroinvasive *E. coli* (EIEC). This type produces dysentery that is clinically indistinguishable from shigellosis. Blood, pus and mucous are often found in the faeces of infected individuals.
- Enteropathogenic *E. coli* (EPEC). This type is mainly associated with infantile diarrhoea.
- Vero Cytotoxin-producing *E. coli* (VTEC). This type is also called 'enterohaemorrhagic' *E. coli*. It is characterised by mild to moderate 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).

2.3.4.5 Anti-microbial sensitivity

Antimicrobials used to treat *E. coli* infections include all those that have action against Gram-negative organisms. These include pthalysulphathiazole, neomycin, doxycycline, trimethoprim, norfloxacin, chloramphenicol and other fluoroquinolones (Greenwood, Slacker and Peutherer, 1992:233).

2.3.5 *E. FAECALIS*

2.3.5.1 Classification

E. faecalis is an enterococcus belonging to the group D streptococci, is one of the most pathogenic microorganism of the Group D streptococci. *E. faecalis* causes endocarditis, urinary tract infections, abdominal sepsis, cellulitis and wound infections as well as concurrent septicaemia (Beers, *et al*., 1999:1151). *E. faecalis* is a member of the genus Enterococcus and the family Streptococcaceae (Hardie and Whiley, 1997:75).

2.3.5.2 Morphology and identification

E. faecalis is a Gram-positive coccus that occurs in either pairs or chains (Howard, *et al*., 1994:267).

As with other *Streptococcus* species *E. faecalis* thrives on complex media and is capable of growing in both aerobic and anaerobic conditions (Howard, *et al*., 1994:260). They can be identified by rapid litmus milk reduction test. On MacConkey agar, enterococci produce distinctive small dark red colonies (Cheesborough, 1984:230).

2.3.5.3 Epidemiology

E. faecalis is normally resident in the intestinal tract of humans and most other animals (Prescott, Harley and Klein, 1999:503). Some strains have been isolated from soil, food, water and plants. Their ability to grow and survive under a wide range of environmental conditions, including extremes of

temperature and salt concentrations, probably accounts for the almost ubiquitous distribution of the genus (Hardie and Whiley, 1997:85).

2.3.5.4 *E. faecalis* infections

E. faecalis is an opportunistic pathogen that can cause urinary tract infection and endocarditis (Prescott, Harley and Klein, 1999:502). Of particular concern is the increasing prevalence of enterococci in hospital-acquired infections and their increasing levels of resistance to antimicrobial agents (Hardie and Whiley, 1997:85).

Enterococci are often found in intra-abdominal and pelvic wound infections, but since these are normally polymicrobial, it is difficult to assess their role in such conditions (Hardie and Whiley, 1997:85). Bacteraemias due to enterococci are common, often occurring in elderly patients with serious underlying medical conditions or in immunocompromised individuals who have undergone anti-microbial therapy (Hardie and Whiley, 1997:85).

Enterococcus is also associated with septicaemia, cholecystitis and peritonitis (Greenwood, Slacker and Peutherer, 1992:221).

2.3.5.5 Anti-microbial sensitivity

Penicillin tolerance is common in *E. faecalis*, but a combination of penicillin with an aminoglycoside is synergistic and clinically effective (Mackie and McCartney, 1996:269).

2.4 PREVIOUS RESEARCH

Invernizzi (2002), conducted a study to evaluate the antimicrobial effectiveness of *Tulbagia violacea* herbal extract in 30% ethanol and homoeopathic dilution (1x and 6x) against the *in vitro* growth of *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus, Klebsiella pneumonia* and *Bacillus cereus*. Invernizzi makes reference to three principal methods used to evaluate the antimicrobial properties of natural products i.e.. diffusion assays, dilution tests and bioautography tests. In his study, Invernizzi performed the disc diffusion assay. Following the critique made by Reid (2002), Vosloo (2002) and Langford (2001) that the evaporation of the ethanol of the wet disc may cause an unaccounted for effect on the microbial growth and the subsequent production of zones of inhibition in the experiment, Invernizzi made use of the dry discs in preference to wet discs, so as to nullify the superfluous antimicrobial effect of ethanol on the bacteria. Invernizzi's study revealed that *Tulbagia violacea* herbal extract and homoeopathic dilutions were ineffective in inhibiting the *in vitro* growth of the bacteria tested. In consequence, Invernizzi searched for possible flaws in his experimental design. As a result of his findings, Invernizzi then went on to make several recommendations so as to eliminate these flaws. This study is largely based on the recommendations of Invernizzi.

As with Invernizzi, Dummer (2003) conducted a study on the antimicrobial effectiveness of *Lithuania somniferous* extract in 62% ethanol against the *in vitro* growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia* and *Bacillus cereus* by means of the disc

diffusion assay and the agar dilutions sensitivity test. This study revealed that the herbal extract was ineffective against all the bacteria tested except for the *Staphylococcus aureus*. Dummer cited possible flaws in her methodology that may have resulted in her negative results. She recommended that an alternative diffusion method be performed. In her recommendation, she mentioned the hole or reservoir method specifically. The new method introduced in this study was the well diffusion assay following the recommendation from Dummer. The well diffusion assay is a standardised method for the screening of properties of natural products (Kwon and Ricke, 1998).

There has been some research conducted on the activity of *Baptisia tinctoria* combined with other natural products.

Freudenstein and Bodinet (1999), performed an *in vivo* study to test the effects of an orally applied aqueous-ethanolic herbal extract of a mixture of *Thuja occidentalis herba, Baptisia tinctoria radix, Echinacea purpurea radix* and *Echinacea pallidae radix* on antibody response against sheep red blood cells in mice. Their study investigated the immunomodulatory effect of the long-term use of this mixture. In this study, the mice were given repeated doses of the mixture at a comparative human therapeutic level. Application was through the drinking water and food to the subjects which mirrors also the human method of administration. The study demonstrated a potent antibody enhancing ability of *Thuja, Echinacea* and *Baptisia* whilst indicating no immunosuppression with long-term use. However, as the researchers

provided no statistical analysis of their results, there is no way of evaluating the significance of their results.

Wustenberg, Henneicke-von Zepelin, *et al*. (1999), conducted a double-blind, placebo-controlled study involving 263 people. The study showed that a herbal formula, Esberitox® N, was significantly more effective than placebo in relieving cold symptoms. Treatment response was seen in as early as three days. Esberitox® N is an alcohol extract containing *Thuja occidentalis herba, Baptisia tinctoria radix, Echinacea purpurea radix* and *Echinacea pallidae radix.*

These studies supported the fact that *Baptisia tinctoria* has a role in immunomodulatory agents, but little research has been conducted on *Baptisia tinctoria* in isolation. It was with this in mind that this study was conducted.

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

Zones of Inhibition

The susceptibility of each bacterium 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 Chloramphenicol antibiotic discs. The expression of the test zones as ratios around the Chloramphenicol 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 the results is the sample being tested. (McGraw, Jager and van Staden, 2000:253.)

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 Microbiology, M. L. Sultan Campus, Durban Institute of Technology was used.

3.3 MATERIALS AND METHODS

3.3.1 Methodology

The herbal extracts were prepared by Parceval (Pty) Ltd. in a 1:10 ratio according to the German Homoeopathic Pharmacopoeia Method HAB 4a (British Homoeopathic Association, 1991) and the Kinetic Extraction method (Lilje, 2003).

The disc diffusion assay was prepared by the researcher according to the methodology of Cheesbrough (1984:198). However, Cheesbrough outlines the methodology in broad terms only. Therefore the researcher, in consultation with Dr. K. Perumal (2003), expanded these in order to be able to have a detailed step-by-step methodology. For instance, Cheesbrough has the following sentence only: “A disc… is impregnated with a known volume and appropriate concentration of an antimicrobial, and this is placed on a plate of sensitivity testing agar inoculated with the test organism.” (1984:198). This was then expanded as per 3.3.1.5 and 3.3.1.9.

The well diffusion assays were prepared by the researcher, in consultation with Dr. K. Perumal (2003), according to the methodology of Kwon and Ricke (1998).

3. 3. 1.1 Preparation of *Baptisia tinctoria* extract

3.3.1.1. Preparation of *Baptisia tinctoria* made up as a 62% ethanol based extract

At 62% v/v ethanol a broad spectrum of ingredients are extracted, both lipophilic and hydrophilic. The *Baptisia tinctoria* extract in 62% ethanol (Batch No 11026 expiry date 10/2005) was prepared by Parceval (Pty) Ltd. according to the German Homoeopathic Pharmacopoeia Method HAB 4a (British Homoeopathic Association, 1991), using the roots of *Baptisia tinctoria*. Parceval manufactures all of their tinctures according to this methodology, and calls then herbal tinctures. The precise method followed by Parceval was as follows (Lilje, 2003):

- The plant material was milled using a hammer mill with 1mm sieve size.
- The milled material was added to the solvent (62% v/v ethanol) in a ratio 1 part dried part material to 10 parts solvent.
- The mix was stirred gently and left to macerate (left to stand) for a minimum of 10 days at a set temperature not exceeding 25ºC, away from direct light (use amber glass bottles or stainless steel containers).
- The mixture was stirred twice daily (by hand using a paddle, morning and evening).
- Once maceration was complete, the mixture was then poured through 100% cotton cloth (loomstate) and pressed using a hydraulic press. Pressing continued until no more liquid came out.

- The liquid obtained was filtered through filter paper (Whatman® filter paper number 1) and stored in glass, away from direct light at temperatures not exceeding 25ºC.
- The press cake was discarded.

Once obtained, the extract was stored in a refrigerator at the M. L. Sultan Biotechnology Laboratory, M. L Sultan Campus, Durban Institute of Technology, Durban.

3.3.1.1.2 Preparation of *Baptisia tinctoria* made up as water based extract

The *Baptisia tinctoria* extract in distilled water was prepared by Parceval (Pty) Ltd. Pharmaceuticals according to the Kinetic Extraction Method. The precise method followed by Parceval was as follows (Lilje, 2003):

- The plant material was milled using a hammer mill with 1mm sieve size.
- The milled material was added to the solvent (distilled water in this instance) in a ratio 1 part dried part material to 10 parts solvent.
- The mix was stirred mechanically using a paddle mixer set at 50 rpm for 24 hours.
- This mixture was protected from direct light (use amber glass bottles or stainless steel containers) and kept at a temperature of 25ºC.
- The mixture was stirred twice daily (by hand using a paddle, morning and evening).

- Once stirring was complete, the mixture was then poured through 100% cotton cloth (loomstate) and pressed using a hydraulic press. Pressing continued until no more liquid came out.
- The liquid obtained was filtered through filter paper (Whatman® filter paper number 1) and stored in glass, away from direct light at temperatures not exceeding 25ºC.
- The press cake was discarded.

Once obtained, the extract was stored in a refrigerator at the M. L. Sultan Biotechnology Laboratory, M. L Sultan Campus, Durban Institute of Technology, Durban.

3.3.1.2 Preparation of Negative Controls

3.3.1.2.1 Preparation of 62% ethanol control

62% ethanol was obtained from the M. L. Sultan Biotechnology Laboratory.

3.3.1.2.2 Preparation of distilled water control

Distilled water was obtained from the M. L. Sultan Biotechnology Laboratory.

3.3.1.3 Preparation of Positive Control

One tube containing 50 Chloramphenicol 10μg antibiotic discs (Batch No. 44599) manufactured by Oxoid was obtained from the M. L. Sultan laboratory stock and stored at 5°C until used.

3.3.1.4 Preparation of the 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 Schott bottle and autoclaved at 121°C for 15 minutes to ensure sterility.

3.3.1.5 Preparation of the Medicated Discs

3.3.1.5.1 Preparation of the *Baptisia tinctoria* extract in 62% ethanol base dry-discs

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly placed upon the bottom of a sterile petri dish using a pair of sterile forceps, so that each petri dish contained 18 discs.
- 10 microlitres of *Baptisia tinctoria* extract was pipetted onto each disc using a calibrated micropipette.
- The petri dishes were then placed in a dark incubator at 37°C, and the discs allowed to dry.
- The dry discs were then stored in labelled sterile jars until used.

3.3.1.5.2 Preparation of the *Baptisia tinctoria* extract in water base dry-discs

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly placed upon the bottom of a sterile petri dish using a pair of sterile forceps, so that each petri dish contained 18 discs.
- 10 microlitres of *Baptisia tinctoria* extract was pipetted onto each disc using a calibrated micropipette.

- The petri dishes were then placed in a dark incubator at 37°C, and the discs allowed to dry.
- The dry discs were then stored in labelled sterile jars until used.

3.3.1.5.3 Preparation of the 62% ethanol only dry-discs

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly placed upon the bottom of a sterile petri dish using a pair of sterile forceps, so that each petri dish contained 18 discs.
- 10 microlitres of 62% ethanol was pipetted onto each disc using a calibrated micropipette.
- The petri dishes were then placed in a dark incubator at 37°C, and the discs allowed to dry.
- The dry discs were then stored in labelled sterile jars until used.

3.3.1.5.4 Preparation of the water only dry-discs

- Sterile, 5mm, Whatman® filter paper number 4, discs were evenly placed upon the bottom of a sterile petri dish using a pair of sterile forceps, so that each petri dish contained 18 discs.
- 10 microlitres of distilled water was pipetted onto each disc using a calibrated micropipette.
- The petri dishes were then placed in a dark incubator at 37°C, and the discs allowed to dry.
- The dry discs were then stored in labelled sterile jars until used.

3.3.1.6 Preparation of the Media

The medium of choice is the Mueller-Hilton agar due to its pH of 7.2 to 7.4. It was prepared according to the Oxoid® manual (1979), as follows:

- 38g of Mueller-Hinton agar powder was weighed out.
- The Mueller-Hinton agar powder was added to 1 litre of distilled water in a screw top flask.
- A magnetic stirrer was added to aid dissolution.
- The mixture was shaken until well mixed.
- The mixture was autoclaved at 121°C for 15 minutes.
- The flask was then allowed to cool whilst placed on a magnetic stirrer machine. This ensured adequate mixing and prevented the mixture from solidifying.
- Once the flask had cooled enough to hold, the agar was poured into agar plates as follows:
    - The top of the flask was flamed with a Bunsen burner before pouring each plate to prevent contamination;
    - Each plate was poured to a depth of approximately 4 millimetres;
    - A total of 12 plates was prepared per bacterium;
    - The plates were stacked and allowed to solidify;
- The plates were finally checked for contamination.

3.3.1.7 Preparation of the Inoculums

Single colonies obtained from the M. L. Sultan Biotechnology Laboratory stock cultures of each of the bacteria to be tested were used to inoculate

separate Mueller-Hinton agar plates, and allowed to incubate for 24 hours at 37°C. The stock numbers for the cultures were:

- *Staph. aureus,* ATCC 27821;
- *Staph. epidermis,* ATCC 700563;
- *P. aeruginosa,* ATCC 27107;
- *Esch. coli,* ATCC 39403*;*
- *E. faecalis,* ATCC 27792.

3.3.1.8 Preparation of the Saline Test Cultures

A few colonies from the overnight Mueller-Hinton agar cultures of *S. pyogenes, S. aureus, S. epidermidis, P.aeruginosa, E. coli* and *E. faecalis* were suspended in 10 ml sterile saline solution (8.5g/L) and the solution adjusted to the 0.5 McFarland Equivalence Turbidity Standard (REMEL, nd).

3.3.1.9 Preparation of the Plates for the Disc Diffusion Assay

- A marker pen was used to label the side of the agar plate with a number to denote which bacteria was streaked on the plate.
- The plates were labelled as follows:

    1) *Staph. aureus*
    2) *Staph. epidermidis*
    3) *P. aeruginosa*
    4) *Esch. Coli*
    5) *E. faecalis.*
- A marker pen was used to label the under surface of the agar plate with a letter evenly around its edge, with each letter referring to a particular disc, namely:

GROUP A

A1) *Baptisia tinctoria* made up as 62% ethanol based extract
A2) 62% ethanol
A3) Chloramphenicol.

GROUP B

B1) *Baptisia tinctoria* made up as a water based extract
B2) Water only
B3) Chloramphenicol.

- A sterile cotton swab was dipped into a well-mixed saline test culture and excess inoculum removed by pressing the saturated swab against the inner wall of the culture tube.
- Using the swab, the entire agar surface of the plate was streaked first in a horizontal direction and then vertically to ensure a heavy growth over the entire surface.
- With sterile forceps filter paper discs were distributed over the agar surface.
- All the plate cultures were incubated in an inverted position for 24 hours at 37°C.
- Following incubation, the plates were examined for the presence of growth inhibition, indicated by a clear zone surrounding each disc. The susceptibility of an organism to a substance was determined by the size of this zone.

3.3.1.10 Preparation of the Plates for the Well Diffusion Assay

- A marker pen was used to label the side of the agar plate with a number to denote which bacteria was streaked on the plate.
- The plates were labelled as follows:

1) *Staph. aureus*
2) *Staph. epidermidis*
3) *P. aeruginosa*
4) *Esch. Coli*
5) *E. faecalis.*

- A marker pen was used to label the under surface of the agar plate with a letter evenly around its edge, with each letter referring to a particular disc, namely:

GROUP A

A1) *Baptisia tinctoria* made up as 62% ethanol based extract
A2) 62% ethanol
A3) Chloramphenicol.

GROUP B

B1) *Baptisia tinctoria* made up as a water based extract
B2) Water only
B3) Chloramphenicol.

- A sterile cotton swab was dipped into a well-mixed saline test culture and excess inoculum removed by pressing the saturated swab against the inner wall of the culture tube.
- Using the swab, the entire agar surface of the plate was streaked first in a horizontal direction and then vertically to ensure a heavy growth over the entire surface.
- With a sterile Pasteur pipette a well 3mm in diameter was punched into the agar surface (Kwon and Ricke, 1998).
- All the plate cultures were incubated in an inverted position for 24 hours at 37°C.
- Following incubation, the plates were examined for the presence of growth inhibition, indicated by a clear zone surrounding each disc. The susceptibility of an organism to a substance was determined by the size of this zone.

3.3.1.11 Measurement of the Results

- The plates were then photographed for record purposes.
- The zone diameters were measured in millimetres using a pair of vernier callipers to ensure accuracy and were recorded on a table (Appendix A) for each relevant group.

3.3.1.12 Analysis of Results

The data collected from the test and control plates in each group, i.e. the measurement of the diameter of the zone of inhibition, was used to look for inter-group change by use of the Mann-Whitney U-test using the SPSS package with confidence levels set at 95%, between the test and control subgroups in both group A and B. The mean and standard deviation values were compared in order to look for possible trends.

3.4 DATA ANALYSIS

3.4.1 Sample size of study

The sample size was 15, which means that each test yielded 15 sets of data. The efficacy of each test and control substance was tested against each bacterium (five in total) 3 times to make the study statistically viable.

3.4.2 Statistical methods

3.4.2.1 Inter-group comparison between Baptisia tinctoria extract in 62% ethanol and 62% ethanol only using the disc diffusion method

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

(i) Hypothesis testing

The null hypothesis Ho, states that there is no significant difference in the diameter of the zone of inhibition between the samples, with respect to the variable comparison at the $\alpha = 0.05$ level of significance.

The alternative hypothesis H1, states that there is a significant difference at the $\alpha = 0.05$ level of significance.

H_0: there is no significant difference between the 2 groups, $M_1=M_2$

H_1: there is a significant difference between the 2 groups, $M_1 \neq M_2$

(ii) Decision rule

At $\alpha = 0.05$ level of significance, the null hypothesis is rejected if $P < \alpha$ where P is the observed significance level or probability value. Otherwise the null hypothesis is accepted at the same level of significance.

Reject H_0 if $P < \alpha$.

Accept H_0 if $P \geq \alpha$.

P is the observed significance level or probability value.

This procedure is repeated for each bacterium in turn.

(Kanji, 1999:82; Fisher van Belle, 1993:315; Reid, 2002:36; Invernizzi, 2002:43).

3.4.2.2 Inter-group comparison between Baptisia tinctoria extract in distilled water and distilled water only using the disc diffusion method

(i) Hypothesis testing

As per 3.4.2.1(i)

(ii) Decision rule

As per 3.4.2.1 (ii)

3.4.2.3 Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion method

(i) Hypothesis testing

As per 3.4.2.1(i)

(ii) Decision rule

As per 3.4.2.1 (ii)

3.4.2.4 Inter-group comparison between Baptisia tinctoria extract in 62% ethanol and 62% ethanol only using the well diffusion method

(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 Baptisia tinctoria extract in distilled water and distilled water only using the well diffusion method

(i) Hypothesis testing

As per 3.4.2.1(i)

(ii) Decision rule

As per 3.4.2.1 (ii)

3.4.2.6 Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion method

(i) Hypothesis testing

As per 3.4.2.1(i)

(ii) Decision rule

As per 3.4.2.1 (ii)

3.4.3 Statistical package

The Statistical Package for Social Sciences (SPSS®) was used for data entry and analysis.

CHAPTER FOUR

RESULTS

4.1 INTRODUCTION

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

4.2 STATISTICAL ANALYSIS OF DATA

Refer to Appendix A for Raw Data

Refer to Appendix B for Graphs

4.2.1 Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and 62% ethanol only using the disc diffusion assay

4.2.1.1 *Staph. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.1).

Table 4.1 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Staphylococcus aureus* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the disc diffusion assay.

4.2.1.2 *Staph. epidermidis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.2).

Table 4.2 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Staphylococcus epidermidis* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the disc diffusion assay.

4.2.1.3 *P. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.3).

Table 4.3 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Pseudomonas aeruginosa* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted. Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the disc diffusion assay.

4.2.1.4 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.4).

Table 4.4 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Escherichia coli* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted. Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the disc diffusion assay.

4.2.1.5 *E. faecalis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.5).

Table 4.5 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Enterococcus faecalis* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the disc diffusion assay.

4.2.2 Inter-group comparison between *Baptisia tinctoria* extract in distilled water and distilled water only using the disc diffusion assay

4.2.2.1 *Staph. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.6).

Table 4.6 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Staphylococcus aureus* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the disc diffusion assay.

4.2.2.2 *Staph. epidermidis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.7).

Table 4.7 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Staphylococcus epidermidis* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.500
Wilcoxon W	1.500
Z	0.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the disc diffusion assay.

4.2.2.3 *P. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.8).

Table 4.8 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Pseudomonas aeruginosa* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.500
Wilcoxon W	1.500
Z	0.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the disc diffusion assay.

4.2.2.4 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.9).

Table 4.9 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Escherichia coli* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.500
Wilcoxon W	1.500
Z	0.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the disc diffusion assay.

4.2.2.5 *E. faecalis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.10).

Table 4.10 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Enterococcus faecalis* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.500
Wilcoxon W	1.500
Z	0.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the disc diffusion assay.

4.2.3 Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay

See Figure 1, Appendix B, for a graphical representation of the results.

4.2.3.1 *Staph. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.11).

Table 4.11 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Staphylococcus aureus* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay.

4.2.3.2 *Staph. epidermidis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.12).

Table 4.12 Statistical analysis of *Baptisia tinctoria* tincture in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Staphylococcus epidermidis* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay.

4.2.3.3 *P. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.13).

Table 4.13 Statistical analysis of *Baptisia tinctoria* tincture in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Pseudomonas aeruginosa* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.500
Z	0.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay.

4.2.3.4 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.14).

Table 4.14 Statistical analysis of *Baptisia tinctoria* tincture in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Escherichia coli* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay.

4.2.3.5 *E. faecalis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.15).

Table 4.15 Statistical analysis of *Baptisia tinctoria* tincture in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Enterococcus faecalis* according to the Mann-Whitney U Test using the disc diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay.

4.2.4 Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and 62% ethanol only using the well diffusion method

4.2.4.1 *Staph. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.16).

Table 4.16 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Staphylococcus aureus* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the well diffusion assay.

4.2.4.2 *Staph. epidermidis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.17).

Table 4.17 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Staphylococcus epidermidis* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the well diffusion assay.

4.2.4.3 *P. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.18)

Table 4.18 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Pseudomonas aeruginosa* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the well diffusion assay.

4.2.4.4 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.19).

Table 4.19 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Escherichia coli* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the well diffusion assay.

4.2.4.5 *E. faecalis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.20).

Table 4.20 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to 62% ethanol control against *Enterococcus faecalis* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% and the 62% ethanol only using the well diffusion assay.

4.2.5 Inter-group comparison between *Baptisia tinctoria* extract in distilled water and distilled water only using the well diffusion method.

4.2.5.1 *Staph. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.21).

Table 4.21 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Staphylococcus aureus* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the well diffusion assay.

4.2.5.2 *Staph. epidermidis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.22).

Table 4.22 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Staphylococcus epidermidis* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the well diffusion assay.

4.2.5.3 *P. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.23).

Table 4.23 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Pseudomonas aeruginosa* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the well diffusion assay.

4.2.5.4 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.24).

Table 4.24 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Escherichia coli* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the well diffusion assay.

4.2.5.5 *E. faecalis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.25).

Table 4.25 Statistical analysis of *Baptisia tinctoria* extract in distilled water compared to distilled water control against *Enterococcus faecalis* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in distilled water and the distilled water only using the well diffusion assay.

4.2.6 Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion method

See Figure 2, Appendix B, for a graphical representation of the results.

4.2.6.1 *Staph. aureus*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.26).

Table 4.26 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Staphylococcus aureus* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion assay.

4.2.6.2 *Staph. epidermidis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.27).

Table 4.27 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Staphylococcus epidermidis* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion assay.

4.2.6.3 *P. aeruginosa*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.28).

Table 4.28 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Pseudomonas aeruginosa* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion assay.

4.2.6.4 *E. coli*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.29).

Table 4.29 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Escherichia coli* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion assay.

4.2.6.5 *E. faecalis*

The Mann-Whitney U test (Wilcoxon inversion test) was used to compare the two samples (see Table 4.30).

Table 4.30 Statistical analysis of *Baptisia tinctoria* extract in 62% ethanol compared to *Baptisia tinctoria* extract in distilled water against *Enterococcus faecalis* according to the Mann-Whitney U Test using the well diffusion assay.

Mann - Whitney U test	0.000
Wilcoxon W	1.000
Z	-1.000
Asymp. Sig. (2-tailed)	0.317
Exact Sig. [2*(1-tailed Sig.)]	1.000

- Not corrected for ties
- Grouping variable: Substance used

CONCLUSION: P>α. Therefore the null hypothesis was accepted.

Therefore there was no significant difference in the zones of inhibition produced by the *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion assay.

CHAPTER FIVE

DISCUSSION

The results of this study demonstrated that the *Baptisia tinctoria* extract in 62% ethanol had no antimicrobial effect on any of the bacteria tested, namely, *Staph. aureus*, *Staph. epidermidis, P. aeruginosa, E. coli,* and *E. faecalis*, when evaluated using the disc diffusion or the well diffusion assays. No growth inhibition was exhibited with any of the samples when compared to the negative control.

Furthermore, the results of this study demonstrated that the *Baptisia tinctoria* extract in distilled water had no antimicrobial effect on any of the bacteria tested, namely, *Staph. aureus*, *Staph. epidermidis, P. aeruginosa, E. coli,* and *E. faecalis*, when evaluated using the disc diffusion or the well diffusion assays. No growth inhibition was exhibited with any of the samples when compared to the negative control.

The absence of any zone of inhibition of the sample being tested and the 62% ethanol negative control, was due to the use of dry discs in preference to wet discs. This technique nullified the antimicrobial effect of the ethanol on the bacteria, as all the ethanol present had evaporated off the disc before the placement of the disc onto the media. This finding is the same as Invernizzi (2002) and similar to Dummer (2003). These researchers also utilised dry discs in preference to wet discs.

None of the samples yielded any antimicrobial results.

Possible reasons for the lack of results are:

- The use of a too low concentration of the herbal extract;
- The antimicrobial properties of *Baptisia tinctoria*, which resides in its essential oils could not diffuse though the agar medium;
- Contamination of the *Baptisia tinctoria* extract by unknown microbes during the manufacture process, as there was no preservative added to the herbal extract. This was especially evident in the distilled water based extract when colonies of brown coloured microbes began growing on the research plates after 36 hours. These were not scientifically analysed, but the laboratory supervisor, Dr Perumal, was of the opinion that they were fungal growth (Perumal, K. 2003).
- The use of dry discs is questionable as a method of disc diffusion;
- The extractant used was not effective in extracting the antimicrobial properties of *Baptisia tinctoria*.

The dry disc methodology was recommended by Invernizzi (2003). From the results of this study, as well as Invernizzi and Dummer, it would follow that the dry disc methodology is unsatisfactory. It would appear that the wet disc method is more feasible for research purposes considering the positive results obtained utilising this method by Reid (2002), Vosloo (2002) and Langford (2001).

Freudenstein and Bodinet (1999) found that the herbal extract was more effective when tested *in vivo* (see page 30). Thus, it would be beneficial to conduct an *in vivo* study utilising *Baptisia tinctoria* extracts as manufactured in this study.

The results obtained in this study question the validity of assertations made by phytotherapists such as Hoffman (1984) and Chevalier (1996) who claim that *Baptisia tinctoria* has antimicrobial properties. It leads one to question how their results were obtained and the methodologies utilised by them in order to obtain these results.

Knowledge of the ratio of a plant to solvent is important in assessing the results of any study. Unfortunately, most studies do not state the exact ratio of ingredients which makes it difficult to evaluate the validity of any results obtained.

On reflection, the use of a homoeopathic dilution ratio (1:10) in this study may be a factor underlying the negative results obtained, because this ratio contains 50% less of the active ingredients than a herbal dilution ratio (1:5).

In this regard, the fact that the major herbal manufacturer in South Africa, Parceval (Pty) Ltd., produces and sells its mother tinctures in a 1:10 ratio rather than the standard 1:5 ratio is a cause for concern, because of the possible clinical implications.

Further studies of the antimicrobial effects of herbal extracts should be undertaken using standard herbal dilution ratios.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The purpose of this study was to investigate the effectiveness of an ethanolic tincture and a distilled water tincture of *Baptisia tinctoria* in inhibiting the *in vitro* growth of *Staph. aureus, Staph. epidermidis, P. aeruginosa, E. coli* and *E. faecalis* in terms of the discs diffusion assay and well diffusion assay.

Baptisia tinctoria extract in 62% ethanol proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the disc diffusion assay.

Baptisia tinctoria extract in 62% ethanol proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the well diffusion assay.

Baptisia tinctoria extract in distilled water proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the disc diffusion assay.

Baptisia tinctoria extract in distilled water proved to be ineffective in inhibiting the *in vitro* growth of the bacteria tested, in terms of the well diffusion assay.

Thus, the results of the study showed that *Baptisia tinctoria* was not effective as an *in vitro* antimicrobial substance.

6.2 RECOMMENDATIONS

6.2.1 Identification of source material

The plants used to manufacture the *Baptisia tinctoria* herbal tincture should be expertly identified and voucher specimens deposited in 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.

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 such as glycerine, vinegar, acetone and fat, to see which is most effective in extracting the active ingredients of *Baptisia tinctoria* plants.

6.2.3 Concentration of the herbal extract

The exact concentration of the herbal extract should be determined at the onset of the experiment. The herbal extract should be dried into a powder form. This powder can be added to a solution and the exact concentration can thus be determined.

6.2.4 Manufacture of the herbal extract

Manufacture of the herbal extract by the experimenter, rather than purchasing a pre-manufactured herbal extract, 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 the final tincture concentration can be specifically chosen to suit the precise methodology of the experiment. This will also help to prevent contamination of the final product.

6.2.5. Standard herbal dilution

Repeat the study using a standard herbal dilution such as 1:2 and 1:5 in tincture, infusion and decoction, as per for instance, Chevallier (1996:290) and Hoffman (1984:141-166).

6.2.6 *In vivo* testing

Baptisia tinctoria should be tested *in vivo* for antimicrobial effectiveness, by means of a controlled clinical trial.

6.2.7 A homoeopathic clinical trial

While this homoeopathic mother tincture antimicrobial study produced negative results, it is possible that homoeopathically potentised *Baptisia tinctoria* may have clinical antimicrobial properties, due to a possible energetic stimulation of the vital force (Organon Aphorism 16, Brewster O'Reilly, W. 1996). Thus it is recommended that a clinical trial of *Baptisia tinctoria* in potency be undertaken.

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APPENDIX A

Table 1: Data regarding the inhibitory effect of *Baptisia tinctoria* tincture in 62% ethanol compared to 62% ethanol control on the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* using the disc diffusion assay.

Organisms		Baptisia tinctoria in 62% ethanol	62% Ethanol only	*Chloramphenicol
Staph. aureus	Trial 1	3	5	10
	Trial 2	4	5	10
	Trial 3	3	5	11
	AVE	3.33	5	10.33
Staph. epidermidis	Trial 1	4	5	14
	Trial 2	3	4	13
	Trial 3	3	6	13
	AVE	3.33	5	13.67
P. aeruginosa	Trial 1	3	5	4
	Trial 2	3	4	6
	Trial 3	3	5	5
	AVE	3	4.67	5
E. coli	Trial 1	4	4	10
	Trial 2	4	4	10
	Trial 3	3	4	9
	AVE	3.67	4	9.67
E. faecalis	Trial 1	4	3	6
	Trial 2	4	4	7
	Trial 3	3	3	7
	AVE	3.67	3.33	9.67

* The zones of inhibition produced by the positive control Chloramphenicol.

Table 2: Data regarding the inhibitory effect of *Baptisia tinctoria* tincture in distilled water compared to distilled water control on the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* using the disc diffusion assay.

Organisms		Baptisia tinctoria in 62% ethanol	62% Ethanol only	*Chloramphenicol
Staph. aureus	Trial 1	3	4	10
	Trial 2	3	4	10
	Trial 3	3	4	10
	AVE	3	4	10
Staph. epidermidis	Trial 1	3	3	9
	Trial 2	3	3	11
	Trial 3	3	3	10
	AVE	3	3	10
P. aeruginosa	Trial 1	3	3	4
	Trial 2	3	3	6
	Trial 3	3	3	6
	AVE	3	3	5.33
E. coli	Trial 1	3	3	8
	Trial 2	3	3	12
	Trial 3	3	3	10
	AVE	3	3	10
E. faecalis	Trial 1	3	3	6
	Trial 2	3	3	6
	Trial 3	3	3	6
	AVE	3	3	6

* The zones of inhibition produced by the positive control Chloramphenicol.

Table 3: Data regarding the inhibitory effect of *Baptisia tinctoria* tincture in 62% ethanol compared to 62% ethanol control on the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* using the well diffusion assay.

Organisms		Baptisia tinctoria in 62% ethanol	62% Ethanol only	*Chloramphenicol
Staph. aureus	Trial 1	5	4	11
	Trial 2	6	5	12
	Trial 3	5	5	11
	AVE	5.33	4.67	11.33
Staph. epidermidis	Trial 1	5	4	11
	Trial 2	6	3	11
	Trial 3	6	5	13
	AVE	5.67	3.33	11.67
P. aeruginosa	Trial 1	5	3	7
	Trial 2	7	3	8
	Trial 3	5	4	7
	AVE	5.67	3.33	7.33
E. coli	Trial 1	6	5	11
	Trial 2	6	5	12
	Trial 3	5	4	11
	AVE	5.67	4.67	11.33
E. faecalis	Trial 1	8	5	8
	Trial 2	7	5	11
	Trial 3	8	5	8
	AVE	7.67	5	9

* The zones of inhibition produced by the positive control Chloramphenicol.

Table 4: Data regarding the inhibitory effect of *Baptisia tinctoria* tincture in distilled water compared to distilled water control on the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* using the well diffusion assay.

Organisms		Baptisia tinctoria in 62% ethanol	62% Ethanol only	*Chloramphenicol
Staph. aureus	Trial 1	4	3	11
	Trial 2	3	3	10
	Trial 3	3	3	13
	AVE	3.33	3	11.33
Staph. epidermidis	Trial 1	4	3	13
	Trial 2	4	3	12
	Trial 3	4	3	12
	AVE	4	3	12.33
P. aeruginosa	Trial 1	5	4	7
	Trial 2	4	3	8
	Trial 3	3	3	8
	AVE	4	3.33	7.67
E. coli	Trial 1	5	3	14
	Trial 2	4	3	11
	Trial 3	4	3	14
	AVE	3.67	3	13
E. faecalis	Trial 1	5	3	7
	Trial 2	5	3	8
	Trial 3	4	3	8
	AVE	4.67	3	7.67

* The zones of inhibition produced by the positive control Chloramphenicol.

APPENDIX B

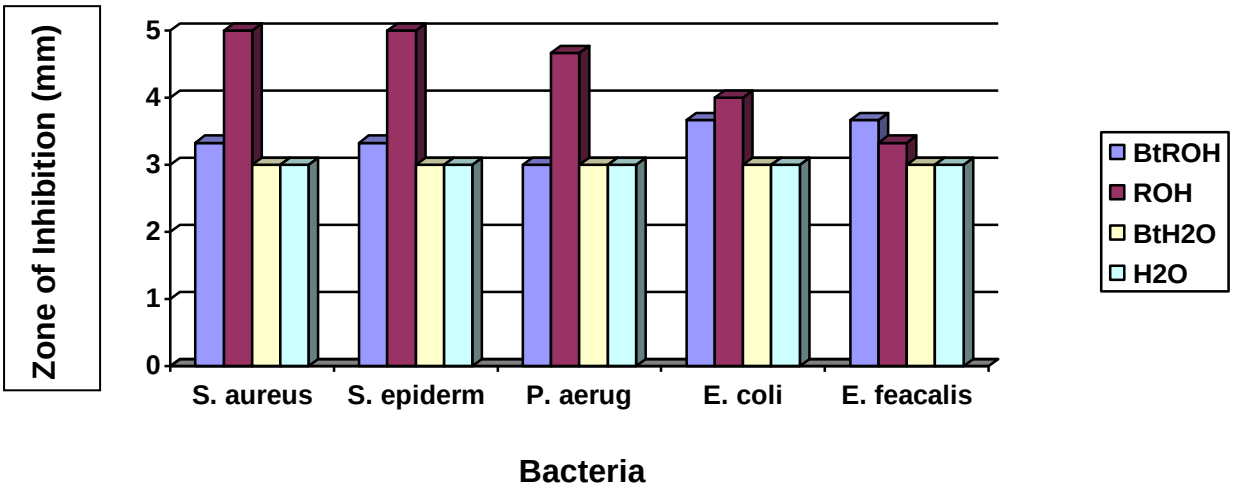

Figure 1. Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the disc diffusion assay

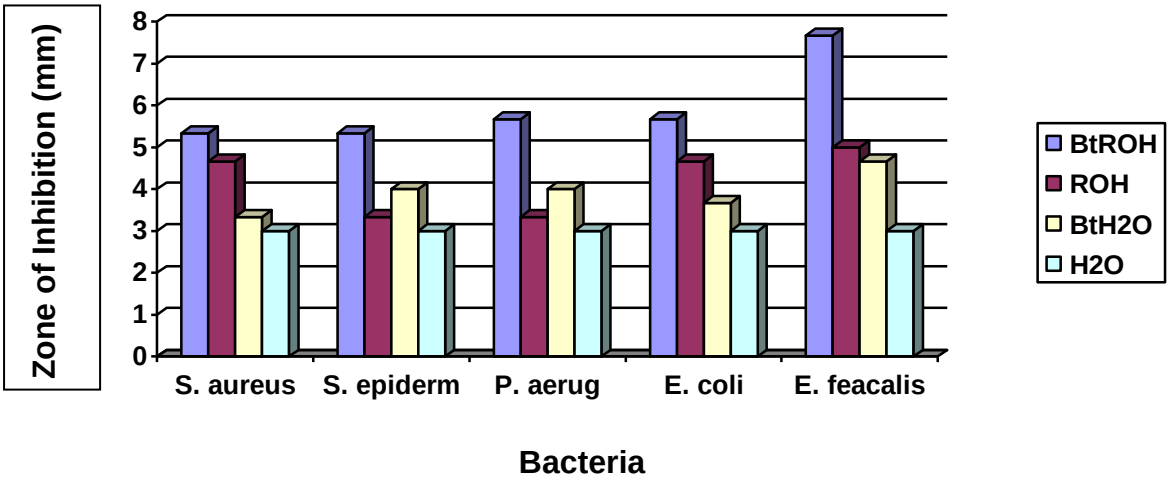

Figure 2. Inter-group comparison between *Baptisia tinctoria* extract in 62% ethanol and *Baptisia tinctoria* extract in distilled water only using the well diffusion method