An *in vitro* study of the antimicrobial effect of *Indigofera daleiodes* plant tinctures using Disc Diffusion and Well Diffusion Assay

BY

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Mini-dissertation submitted in partial compliance with the requirements of the Master's Degree in Technology: Homoeopathy

In the Faculty of Health Sciences

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DECLARATION

This is to certify that the work is entirely my own and not of any other person, unless explicitly acknowledged (including citation of published and unpublished sources). The work has not previously been submitted in any form to the Durban University of Technology or to any other institution for assessment or for any other purpose.

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DEDICATION

I would like to dedicate this study to my parents, Busisiwe and Siyabonga Mpangase. I love you greatly and honor you for raising me and always being there for me. I am proud to be your Son.

Acknowledgements

I would like to express my gratitude to everyone involved in making this dissertation possible.

In particular, I would like to thank:

My Lord and Saviour Jesus Christ for his guidance and overwhelming love, “in him I move, live and have my being”.

Dr D.F Naude- Thank you for your availability, dependability and professionality. I learnt a lot from observing you, beyond just academically.

DR. S.K.K Pillai- Thank you for the patience, support and professionality. You truly have a heart for people.

All of the lecturers who taught me throughout my academic years, I sincerely appreciate you. I am a product of your diligence.

Stanley Onwubu- Thank you for your time, kindness and humility. Your character speaks volumes of your faith in God.

Mrs G. Clark- Thank you for your warm welcome to the department and your willingness to help me every time I came to you.

Every nation Durban spiritual family- Thank you for all your kind words, prayers and so much more.I would not be where I am without you.

My friends- I can not mention you by name, but you know who you are. I appreciate and love you deeply. Thank you for being always there for me.

My family- I feel the best words to say is, thank you for being my ‘rock’.

ABSTRACT

Title: An *in vitro* study of the antimicrobial effect of *Indigofera daleiodes* using Disc Diffusion and Well Diffusion Assay

Background

With the steady and consistent rise of antibiotic resistance (WHO 2014) the health care sector around the world is currently under much stress because of bacterial infectious diseases. Many pharmaceutical companies across the world are trying to develop new types of antibiotics, which is a very difficult and expensive process (Gould and Bal 2013). Hence alternative therapies like Phytotherapy, Homeopathy are being looked into more as possible alternative areas of treatment (van Vuuren 2009: 462-472).

Aim of the study

The purpose of this study was to determine the effect of various ethanolic extracts of *Indigofera daleoides* against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using the Disc Diffusion and Well Diffusion Assay microbiological methodologies. The efficacy of the tinctures was compared against the ciprofloxacin antibiotic and ethanol as positive & negative controls, measuring the minimum inhibitory concentration of each tincture according to the antibacterial potency they possess.

Methodology

Disc Diffusion and Well Diffusion Assay were used to investigate the antimicrobial effects of the Indigofera daleoides tinctures (derived from roots, leaves and whole plants).

Ciprofloxacin was used as a positive control while 62% ethanol was used as the negative control. The tincture was prepared at a homeopathic dilution level (i.e. 1:10) according to the German Homeopathic Pharmacopoeia specifications (Bunyens 2005).

Disc Diffusion Assay:

Fifteen mueller-hinton agar plates were made. Each bacterium was then grown on three agar plates to make the experiments more valid i.e. 3 trials. Then six impregnated discs were placed at equidistance on the agar surface of each plate i.e. 3 discs were impregnated with the *Indigofera daleoides* tinctures (i.e. derived from the root, leaves and whole plant),1 disc was impregnated with 62% ethanol, another ciprofloxacin and the sixth one was plain. The 15 plates were then stacked and put in an incubator room at 37°C for 24hours. The results were then recorded by looking and measuring the zones of inhibition.

Mixed factorial ANOVA was used to test the difference across the treatment groups. One-way ANOVA was used to compare the differences between the whole plants, leaves and roots. Additionally, an independent t-test was further used to compare the differences between the two techniques employed. This was all done using SPSS version 25.

Results

The results of this study showed that all the *Indigofera daleoides* plant tinctures had no significant inhibitory effects on the selected panel of bacteria. Ciprofloxacin showed significant potency against all the bacteria, whilst ethanol was only slightly effective for some bacteria.

Conclusion

This study concluded that *Indigofera daleoides* plant tinctures in 62% ethanol are ineffective in inhibiting in vitro growth of the any of the selected panel of bacteria using Disc Diffusion and Well Diffusion Assay.

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LIST OF ACRONYMS AND ABBREVIATIONS

S. aureus- Staphylococcus aureus
P. aeriginosa- Pseudomonas Aeriginosa
E. faecalis- Enterococci faecalis
K. pneumonia- Klebsialla pneumonia
E. coli- Escherichia coli
WHO- Wealth Health Organisation
HIV- Human Immunodeficiency Virus
IV- Intravenous
ANOVA- Analysis of Varience
DMSO- Dimethyl sulfoxide
mm- millimetres
g/l- gram per litre
ml- millilitre

DEFINITION OF TERMS

Allopathic medicine- a system of medicine that aims to cure disease, using drugs and other therapies by a philosophy of opposition to disease (*contraria contraris*) (O' Reilly 1996: 8-9).

Antibiotic- a drug that is able to kill or inhibit bacteria (Prescott, Harley & Klein 2005: 780).

Antimicrobial- any substance that can inhibit or kill microorganisms Prescott, Harley & Klein 2005: 136).

Extract- a physical or chemical process of extrapolating the medicinal quality of a substance (Merriam-Webster online 2004)

German Homeopathic Pharmacopeoia- One of the main compendium's that gives specifications on how to make homeopathic remedies (Bunyens 2005).

Homeopathic- of or pertaining to homeopathy, which is a system of medicine that seeks to cure diseased patients throughough substances that when given to a healthy patient caused a set of symptoms and thus when given to a patient with a similar set of sypmtoms cures them i.e similaris simibilas curentur (O'Reilly 1996: 5).

Mother tincture- a preparation made in alcohol or other liquid media (i.e. glycerine, vinegar) that is used or has the potential to be used therapeutically (Blumenthal *et al.* 1998).

Phytotherapy- a system of medicine that uses herbs to treat patients. (Rotblatt and Ziment 2002).

Virulence- the degree of harmfulness a certain microorganism is able to cause when causing illness in an living entiry or being (Prescott, Harley & Klein 2005: 764).

CHAPTER ONE

1.1 OVERVIEW

In the history of medicine, antibiotics have been the most successful chemotherapeutic drugs in treating infectious diseases (Aminov 2010: 1-5). They literally shook the entire medical 'world' at their advent, and are presently the main drugs for the treatment. However in recent times many bacteria have developed strong resistance to antibiotics, which is a great challenge given the significant role antibiotics play in modern conventional medicine (Levy 2002: 25-30).In response to this continuing antibacterial resistance, pharmaceutical companies around the world have been consistently developing new antibiotics, but are unfortunately failing to keep up with the pace at which the bacteria are becoming resistant to new and more types of antibiotics (Aminov 2010: 1-5).

Antibiotics resistance has consequently opened up space for alternative systems of medicine to be used more again, as it was before the antibiotic 'revolution'Of all alternative systems of medicine, herbal medicine is the most common, especially in the undeveloped and developing world (Balix and Cox 1996: 219) because it costs less to use and it is more readily available. . In South Africa, herbal medicine has been used for centuries, most likely because of the rich plant biodiversity of the nation (van Vuuren 2008: 462-472).

Surprisingly, despite the history of herbal medicine in South Africa not enough information has been formally recorded on the therapeutic uses of the various traditional herbal remedies (Roblatt & Ziment 2002). This is disappointing because a significant amount of drugs including many antibiotics, have actually been developed from plant substances used originally in herbal medicine throughout the world (Zaidan et al 2005: 165-170).

It is with this insight that the researcher conducted this study in an attempt to test the antibacterial effect of the plant *Indigofera daleoides* which is used as to make a

traditional herbal remedy in the Limpopo province of South Africa (Ngobeli 2002); The current study was produced from whole plant, root and leaf extracts of *Indigofera daleiodes* according to method 4a (HAB 4a) of the German Homoeopathic Pharmacopoeia (Bunyens 2005) and tested its antibacterial properties against five common bacteria.

1.2 Problem Statement

The purpose of this study was to determine the effect of different ethanolic extracts of *Indigofera daleoides* against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using two microbiological methods, which are the disc diffusion and well diffusion. The efficacy of the tinctures was compared against the ciprofloxacin antibiotic and ethanol as positive & negative controls, measuring the minimum inhibitory concentration of each tincture according to the antibacterial potency they possess.

1.3 Hypothesis

1.3.1 Hypothesis one

Whole plant, root and leaf tinctures of *indigofera daleiodes* (62% v/v ethanol) display significantly different antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli.*

1.3.2 Hypothesis two

Whole plant, root and leaf tinctures of *indigofera daleiodes* (62% v/v ethanol) display significantly higher antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* than conventional antibiotics (Ciprofloxacin).

1.3.3 Hypothesis three

Whole plant, root and leaf tinctures of *indigofera daleiodes* (62% v/v ethanol) display significantly higher antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* than the 62% ethanol control (negative control)

1.3.4 Hypothesis four

The antibacterial activity of whole plant, root and leaf tinctures of *indigofera daleiodes* (62% v/v ethanol) on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* are different depending on whether disc diffusion or well diffusion methods are used.

1.3.5 Hypothesis five

The antibacterial activity *indigofera daleiodes* (62% v/v ethanol) depends on the interaction between type of tincture (whole plant, root and leaves), the type of bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli*) and the diffusion method used (disc or well diffusion).

1.4 Delimitations

The study was only limited to five species of bacteria namely; *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli*.

Only non-pathogenic strains of the bacteria were used for the study for safety reasons.

The study was limited to a specific species of *Indigofera* namely, *Indigofera daleoides*.

Only *Indigofera daleoides* in 62% ethanol was used.

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

This was an *in vitro* study

1.5 Assumptions

1.5.1 All cultures of *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* were grown under optimal conditions.

1.5.2 The tinctures were made according to strict German Homeopathic Pharmacopoeia method 4a standards.

Chapter 2

Literature Review

2.0 Utilisation of antimicrobial plant extracts

South Africa has a rich plant biodiversity (Van Vuuren 2008: 462-472)and consequently a very long history of plant medicinal usage (i.e. Traditional herbal medicine) (Gurib-Fakim 2006: 1-98). Despite the long history of Traditional herbal medicine, there is not much documentation and research that has been conducted and recorded on the effects of *Indigofera daleiodes* as a medical plant which is used by traditional medicinal healers in the province of Limpopo South Africa (Ngobeli 2002). Knowledge of therapeutic of different plants in Traditional herbal medicine is mainly disseminated orally across generations and borders (Van Vuuren 2008: 462-472). Written records and research is thus needed to more effectively record the precise medicinal plant actions and research with them.

For much of history, infectious diseases were the main cause of morbidity and mortality in the world, with treatment being by ineffective orthodox treatments (e.g. mercury treatment, bloodletting etc.) and indigenous medicinal systems (Davies & Davies 2010: 417-433). Then antibiotics were developed in the early 20th century which revolutionized medicine buy being very effective in treating bacterial infections (which were and are very common, and a great cause of morbidity and mortality) (Aminov 2010: 1-7). For many years' antibiotics were effective in treating various infectious conditions but this has now changed because many bacteria have become resistant to many antibiotics (Levy 2002: 25-30). Thus a search for new antibiotics and therapies has arisen to combat this dangerous phenomenon of antibiotic resistance (Van Vuuren 2008: 462-472).

2.1 Research methods in microbial studies

2.1.1 Screening methods for natural products with antimicrobial properties

There are three main common methods used to determine antimicrobial properties of natural products which are namely; Diffusion Assays, Dilution tests and Bioautographical tests (Rios, Recio & Villar 1988: 127-149). Each method has its own strengths and weaknesses in terms of affordability, easy utilization and in relation to the various factors that influence results which are pH, moisture, effects of the variation of divalent cations, solubility of the substance in sample, bacterial strains used (Rios, Recio & Villar 1988: 127-149). This study only used Diffusion Assays, specifically Disc and Well Diffusion Assays.

2.1.1.1 Diffusion Assays

This method was developed in the 1940's (Lalitha 2004). There have been subsequent modifications of this method with one of the most common being the Kirby-Bauer test.These methods are the most commonly used investigating the antimicrobial effects of natural products (Valgas *et al*. 2007: 369-380). Plant extracts tested for its antimicrobial activity is compared to an antibiotic known to have antimicrobial effects against the microorganism (s) being tested. The two common diffusion tests are Disc Diffusion & Well Diffusion assay (Valgas *et al.* 2007: 369-380). The Disc Diffusion Assays are lower in cost then other antimicrobial susceptibility tests & very easy to use.

2.1.1.2 Dilution tests

These are used to determine more specifically the minimum concentration of antimicrobial to inhibit or kill microorganisms (Lalitha 2004). They can be achieved by dilution of antimicrobial in either agar or broth media. Where broth media is used it is called the broth dilution method and mainly used when a small number of isolates are being tested whereas when the agar media is used it is called the agar dilution method with its main advantage being that you can test several microorganisms on one plate (Lalitha 2004). These tests specifically require a homogenous dispersion of a substance in water. The bacterial growth is measured by the turbidity of this solution, which is correlated to the bacterial growth (Rios, Recio & Villar 1988: 127-149). The tests are commonly conducted in measuring the antimicrobial effects of essential oils or non-polar substances and generally more time consuming, costly and complicated to perform than diffusion tests.

2.1.1.3 Bioautographical tests

The most classically used Bioautographical tests are paper chromatography and thin layer chromatography to isolate compounds in plants, these compounds are then subjected to diffusion tests against certain bacteria to test antimicrobial activity (Rios, Recio & Villar 1988: 127-149).

2.1.4 Plant Extracts

Most phytotherapuetic tinctures are made in alcohol especially between the range of 50% and 80% (Ketchum 1988). Ethanol enables the extraction of water insoluble substances from the plant substance which can have a significant effect in the plant tinctures action & potency. Ethanol itself between 50% & 80% has its own antimicrobial effects hence it is also a negative control in studies, where its used as an extractant to distinguish & compare its effect as a vehicle with that of the extract itself. In this study 62% ethanol was used.

2.2 *Indigofera daleoides*

2.2.1 Family

Indigofera daleoides forms a part of the leguminosa family (Figueiredo & Smith 2008).

2.2.2 Nomenclature

The plant has no known general common name and is found in the province of Limpopo, South Africa where its used in traditional medicine for the treatment of diarrhea (Mathabe *et al*. 2006: 286-293).

2.2.3 Description

Indigofera daleoides is a perennial semi erect plant with pinnate leaves which are pink in colour (Figueiredo & Smith 2008).

2.2.4 Habitat and cultivation

It is an afrotropical plant native to Angola, Botswana, Namibia and South Africa (Free State, Limpopo, Mpumalanga and North-West Province) (Figueiredo & Smith 2008). It is found in elevation levels between 1000-1500m above sea level and widely distributed in its environment which are mainly grasslands, open woodlands, on sand and even along roadsides (Figueiredo & Smith 2008).

2.2.5 Parts used

The roots and the barks are mainly used in making traditional medical preparations (Mathabe *et al*. 2006: 286-293).

2.2.6 Diseases used for

It is used by traditional medical healers of Limpopo Province South Africa in treating diarrhea of any type (Mathabe *et al*. 2006: 286-293). In other provinces and nations where it is endemic, there are no reports of it being used medicinally in any way.

2.2.7 Preparations

Traditional healers from Limpopo boil the roots and bark in water, and the solution made from that process is then used to treat patients with diarrheal complaints (Mathabe *et al.* 2006: 286-293).

2.3 Bacteriology

2.3.1 *Staphylococcus aureus*

2.3.1.1 Classification

It is a bacterium that belongs in the family *Micrococcaceae*. It is a commensal bacterium normally found in areas of the body like the skin and respiratory tract (Prescott, Harley and Klein 2005). It was 1st identified in the 19th century by Alexander Ogton and then later given the full name *staphylococcus* areas by Friedrich Rosenbach (Orent 2006). The other medically significant bacteria also found in the *staphylococcae* family are *S*. *epidermis* & *S. Saprophyticus* with 32 species well known in the genus *staphylococcus* many of which preferentially colonize the human body (Harris, Foster & Richards 2002: 39-60).

2.3.1.2 Morphology and identification

It is a gram positive coccal (round) non-motile, non-spore forming facultative anaerobe that grows by aerobic respiration or fermentation, with diameters from 0.5 to 1.5 micrometers (Harris, Foster & Richards 2002: 39-60). The cocci divide in various planes to form a grape-like structure hence the Latin name staphyl (Harris, Foster & Richards 2002: 39-60). The species name *aureus* is in reference to the fact that colonies often have a gold color when grown on solid media (Harris, Foster & Richards 2002: 39-60).

It is also tolerant to high concentrations of salt & highly resistant to heat because of its cell wall (Plata, Rosato & Wegrzyn 2009: 597-612).

2.3.1.3 Epidemiology

Humans are a natural reservoir and host of *Staphylococcus aureus*, mainly being a part of the skin flora and the anterior nares (Plata, Rosato & Wegrzyn 2009: 597-612). Between 30%-40% of the normal population carry the organism in the anterior nares at any given time (Plata, Rosato & Wegrzyn 2009: 597-612). Some individuals have extensive colonization of the perineum. Most *Staphylococcus aureus* infections acquired in the community are the result of autoinfection's from the anterior nares, the skin or both and in individuals with compromised immunities because of certain diseases e.g. Diabetes mellitus, HIV, etc. *staphylococci* are also among the most common hospital-based infections (Plata, Rosato & Wegrzyn 2009: 597-612). With certain strains being highly resistant to many types of antibiotics.

2.3.1.4 Staphylococcus aureus infections

Staphylococcus aureus infections usually occur as a result of previous skin injuries such as cuts, burns and surgical wounds (Prescott, Harley and Klein 2004: 898-899). Infections caused *Staphylococcus aureus* are commonly putrefactive. Common skin infections caused by *Staphylococcus aureus* are boils, carbuncles, furuncles, folliculitis and bullous impetigo.

The factors that contribute to the pathogenesis of *Staphylococcus areas* are mainly structural & the products it secrets i.e. exotoxins.

Enterotoxins produced by *Staphylococcus aureus* have been identified and associated with gastrointestinal conditions. Such food poisoning occurs when an individual ingests food contaminated with enterotoxin-producing strains (Plata, Rosato & Wegrzyn 2009: 597-612).

Other infections & conditions caused by Staphylococcus aureus include: -

* scalded skin syndrome);

* *staphylococcal* bacteremia leading to secondary pneumonia (ventilator-assisted) and endocarditis;

* *staphylococcal* osteomyelitis;

* septic arthritis (Prescott, Harley & Klein 2005)

2.3.1.5 Antimicrobial sensitivity

More than 80% of clinical isolates of *Staphylococcus aureus* in Britain are of the type that form penicillinase and are resistant to benzylpenicillin, phenoxymethylpenicillin, ampicillin and amoxicillin and partially resistant to cephaloridine (Plata, Rosato & Wegrzyn 2009: 597-612). A few of the penicillin-resistant strains are also resistant to methicillin. A suitable selection for sensitivity tests include; erythromycin, benzylpenicillin, methicillin, trimethoprim, Augmentin and fusidic acid. Sensitive strains may be treated with gentamicin, vancomycin, tetracycline, neomycin, etc.

2.3.2 *Pseudomonas aeruginosa*

2.3.2.1 Classification

P. aeruginosa, a gram-negative motile, bacillus, is an opportunistic pathogen that frequently causes hospital-acquired infections (Fazeli 2012: 332-337). *P. aeruginosa* infections can develop in many anatomic sites, including skin, subcutaneous tissue, bone, ears, eyes, urinary tract & heart valves.

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* & *P. pseudomallei* are recognized as the most important human pathogens in this genus (Prescott, Harley & Klein 2005).

2.3.2.2 Morphology and identification

P. aeruginosa is a motile, Gram-negative, rod-shaped bacillus, measuring about 0.2 to 5.0 micrometers in length (Prescott, Harley and Klein 2005). It may occur singularly, or in pairs, or occasionally in short chains. They are non-sporing, non-capsulate, and move via one or two polar flagella. They are usually aerobic, but are able to grow anaerobically in the presence of nitrates. *P. aeruginosa* can grow on a variety of media & over a wide temperature range.

Six types of *P. aeruginosa* may be observed:

- type 1 are large, low convex, oval & rough in appearance;

- type 2 are small, smooth & domed;

- type 3 are small & rough;

- type 4 are small

- type 5 are characterized by very mucoid growth, where colonial growth may merge & even drip onto the lid of the petri dish.

- type 6 are small dwarf colonies of the mucoid form

(Mackie & McCartney 1989)

The colonies may possess a sheen known as ‘iridescence’, as well as a characteristic sweet odour.

2.3.2.3 Epidemiology

P. aeruginosa is part of the normal human flora & it only becomes pathogenic when it is introduced into an area lacking in normal human defenses e.g. when a mucous membrane is disrupted by trauma (Fazeli 2012: 332-337).

2.3.2.4 *Pseudomonas* infections

Most pathology is mild & superficial. However, more severe infections may rise in hospitalized or immunocompromised patients. Although infection in these cases usually is still localized e.g. urinary tract infection or infected ulcers, more serious cases of septiceamia or necrotizing pneumonia do occur, & are associated with a high mortality rate (Fazeli 2012: 332-337). The lungs of children with cystic fibrosis are particularly susceptible to this bacterial infection.

2.3.2.5 Antimicrobial sensitivity

The virulence of *P. aeruginosa* is due to a number of factors (Prescott, Harley & Klein 2004). Exotoxin A in enzyme S have been identified to inhibit protein synthesis. Extracellular proteases & 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.

This bacterium has developed resistance to many antibiotics (Strateva & Daniel 2009: 1135-1148). Resistance to B-lactams (i.e. Penicillin, aminopenicillins, etc.), aminogylcosides & fluoroquinolones and the 1st two generations of cephalosporins (Strateva & Daniel 2009: 1135-1148). It easily acquires new additional resistance mechanisms constantly (Santana *et al*. 2016).

Many strains of *P. aeruginosa* however, do not respond well clinically to antibiotics that have appeared effective when treated *in vitro*.

2.3.3 *Enterococcus faecalis*

2.3.3.1 Classification

E. faecalis is an enterococcus belonging to the group *streptococci*, is one of the most pathogenic microorganism of the group *streptococci*. *E. faecalis* causes endocarditis, urinary tract infections, abdominal sepsis, cellulitis and wound infections as well as concurrent septiceamia (Fisher and Philips 2009: 1749-1757). *E. faecalis* is a member of the genus *Enterococcus* and the family *Streptococcaceae* (Kadhum *et al*. 2010).

2.3.3.2 Morphology & Identification

E. faecalis is a Gram-positive coccus that occurs in either pairs or chains (Kadhum *et al*. 2010). As with other *Streptococcus* Species *E. faecalis* thrives on complex media and is capable of growing in both aerobic and anaerobic conditions. They can be identified by rapid litmus milk reduction test. On MacConkey agar, *enterococci* produce distinctive small dark red colonies.

E. faecalis is a Gram-positive coccus that occurs in either pairs or chains (Kadhum *et al*. 2010)

2.3.3.3 Epidemiology

E. faecalis is normally resident in the intestinal tract of humans and most other animals (Kadhum *et al*. 2010). Some strains have been isolated from soil, food, water and plants. Their ability to grow and survive in extremes of temperature and salt concentrations, probably accounts for the ubiquitous distribution of the genus.

2.3.3.4 *Enterococcus faecalis* infections

As stated *E. faecalis* is commensal to the intestines of both humans and animals but in immunosuppressed individuals causes disease (Kadhum *et al*. 2010). It is thus an opportunistic pathogen, and can cause various conditions when found in an individual (even if healthy) outside of the intestines. It virulence is due its ability to produce various substances such as hemolysins, adhesions, aggregation substances & bacteriocins (Kadhum *et al*. 2010). Causing various conditions and states like blood poising, urinary tract infections, wound infections, etc (Levy 2002).

2.3.3.5 Antimicrobial sensitivity

Penicillin tolerance is common in *E. faecalis*, but a combination of penicillin

with an aminoglycoside is synergistic and clinically effective.

2.3.4 *Klebsiella pneumonia*

2.3.4.1 Classification

It is a Gram negative bacilli that is commensal to the digestive system and skin in a lot of individual normal humans (Somily *et al.* 2014: 1129-1136). Also occurs naturally in the soil even being involved in the nitrogen cycle which obviously is a very important cycle in agriculture.

2.3.4.2 Morphology and identification

Klebsiella spp. are Gram-negative, non-motile bacilli that belong to the family *Enterobacteriaceae* (Somily *et al.* 2014: 1129-1136). The genus *Klebsiella* consists of a number of species, including *K. pneumonia, K. oxytoca, K. planticola* and *K. terrigena*. The outermost layer of *Klebsiella spp*. consists of a large polysaccharide capsule that distinguishes the organisms from other members of the family.

2.3.4.3 Epidemiology

Klebsiella bacteria are normally found in the human intestines and feaces and are mostly spread through person-to-person contact. Less commonly, they are spread by contamination in the environment. As with other healthcare-associated infections, the bacteria are a common nosocomial infection (Aladag & Durak 2009: 630-639). The bacteria are not spread through the air.

2.3.4.4 Klebsiella pneumonia infections

Klebsiella infections can occur outside of the health care setting, but this is rare in healthy people. In hospitals and other health care locations, certain patients are at higher risk of developing *Klebsiella* infection. These include patients with devices such as ventilators (breathing machines) or intravenous (IV) catheters and patients who are taking certain antibiotics for a long time (Aladag & Durak 2009: 630-639).

Pneumonia can cause destructive changes to human lungs via inflammation and hemorrhage with cell death (necrosis) that sometimes produces a thick, bloody, mucoid sputum (currant jelly sputum) (Prescott, Harley & Klein 2005). These bacteria gain access typically after a person aspirates colonizing oropharyngeal microbes into the lower respiratory tract. As a general rule, *Klebselia* infections are seen mostly in people with a weakened immune system. Most often, illness affects middle-aged and

older men with debilitating diseases (Somily *et al*. 2014: 1129-1136). The opportunistic pathogen has been associated with various ailments such as urinary tract and respiratory tract infections (Aladag & Durak, 2009: 630-639). Many of these infections are obtained when a person is in the hospital for some other reason (a nosocomial infection). Feaces are the most significant source of patient infection, followed by contact with contaminated instruments.

Signs and Symptoms The signs and symptoms of *Klebsiella* infection depend on the location of infection. General signs of infection might include: fever; chills; erythema; oedema; pain; and drainage or pus from a wound or surgical site (Prescott, Harley & Klein 2005).

2.3.4.5 Antimicrobial sensitivity

Klebsiella infection can be treated with antibiotics. However, some *Klebsiella* bacteria have become resistant to antibiotics and can be very difficult to treat. In such cases, the antibiotic used to treat illness may need to be changed or a patient may need to take antibiotics for a longer period (Prescott, Harley and Klein 2005).

Klebsiella infections can be treated with antibiotics. However, some Klebsiella bacteria have become highly resistant to antibiotics, and some can be very difficult to treat (Santana *et al*. 2016). Persons diagnosed with a *Klebsiella*-related illness must follow the treatment as prescribed by the health care provider. If the health care provider prescribes an antibiotic, patients must take it exactly as instructed and complete the course of medication, even if symptoms are gone. This can help to prevent antibiotic resistance. the antibiotics it mainly is resistant too extended-spectrum cephalosporin's and penicillins due to the production of B-Lactamases (Aladag & Durak, 2009: 630-639).

2.3.5 *Escherichia coli*

2.3.5.1 Classification

E. coli is a gram-negative, enterobacter that normally inhabits the gastrointestinal tract. When *E. coli* organisms have colonizing, enterotoxic, cytotoxic, or invasive virulence traits, they become major causes of watery inflammatory, or bloody diarrhea, occasionally with heamolytic-ureamic syndrome (Sherris 1984). The extra intestinal site most often infected by *E. coli* is the urinary tract. This organism is also an

opportunistic pathogen, causing disease in patients who have received treatment with corticosteroids, radiation, antineoplastic drugs or antibiotics (Sherris 1984).

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 & animals (Prescott, Harley & Klein 2005). The genus *Escherichia* to which belongs the only species of medical importance.

2.3.5.2 Morphology and identification

E. coli are short, motile, gram-negative, non-spore forming bacilli that can grow both aerobically and anaerobically on laboratory media (Prescott, Harley & Klein 2004). 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 target red colonies when grown on macConkey agar (Jawetz et al. 1991). They are able to grow over large temperature range (15°C-45°C), with some strains being able to survive temperatures up to 60°C for 15 minutes, or 55°C for 60 minutes (Prescott, Harley & Klein 2005). Optimal growth temperature of E. coli is 37°C.

2.3.5.3 Epidemiology

E. coli is a member of normal intestinal flora generally not causing disease, and often contributing to the normal function & nutrition to the intestine (Prescott, Harley and Klein 2005).

2.3.5.4 Escherichia coli infections

E. coli may possess lopopolysaccaridal endotoxins in their cell walls. They may also sometimes produce exotoxins of clinical importance (Mahon & Lehman 2014). *E. coli* usually only becomes pathogenic when it reaches areas outside the intestines, such as the urinary tract, biliary tract, lungs, meninges, blood steam, bone or other anatomical sites (Mahon & Lehman 2014). 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. It causes urinary tract infections, blood poisoning, and wound infections (Levy 2002).

2.3.5.5 Antimicrobial sensitivity

Antimicrobials used to treat *E. coli* infections include all those that have action against gram negative organisms. it is resistant by producing B-lactamases (I.e. hydrolyze B-

lactam antibiotics such as penicillins, cephalosporins & monobactams) (Somily *et al.* 2014: 1129-1136).

2.4 Previous Research on *Indigofera daleiodes*

Ethnobotanical surveys by Ngobeli (2002) and Lin *et al*. (2002: 53-56) documented that *Indigofera daleoides* is used traditionally for treating diarrhea by traditional healers in the Province of Limpopo South Africa. In other regions and countries in which it is endemic there is was no recorded medical use. Other plants in the same family as *Indigofera daleoides* have demonstrated antimicrobial activity in various *in vitro* studies Pereira Leite *et al.* (2006: 261-265). Mathabe *et al*. (2006: 286-293) was the first to publish on *Indigofera daleoides'* antimicrobial activity. The Well Diffusion and Microplate Diffusion methods were used to test the antibacterial activity of extracts (methanol, acetone and ethanol) of *Indigofera daleoides,* of which antibacterial activity against both gram negative and positive bacteria was observed and measured, namely; *Shigella dysentery*, *Shigella sonnei*, *Shigella flexneri, Shigella boydie*, *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholera* and *Salmonella typhi* (Mathabe *et al*. 2006: 286-293). The study however only looked at bacteria causing diarrhoea and there is no evidence of a similar study on the antimicrobial effects of *Indigofera daleoides* being done. The parts used were the roots, leaves, bark and stem rhizome. No study of active compounds and metabolites has been currently done also, as suggested by Mathabe *et al*. (2006: 286-293). Hence there is not much information about the specific phytochemicals and active ingredients of the plant.

There has also been similar antimicrobial *in vitro* studies done at the Durban University of Technology by Motara (2007) on the plants *Withania somnifera* and *Xysmalobium undulatum* and Singh (2004) on *Baptisia tinctoria* extract to name just a few, which showed positive antibacterial activity.

Chapter 3

Methodology

3.1 The Data

The research constitutes two types of data which are; primary & secondary data. Their nature is as follows:

3.1.1 The Primary Data

3.1.1.1 Disc & Well Diffusion Method: Zones of inhibition

The antibacterial activity of three test substances manufactured from the whole plant, roots and leaves of *Indigofera daleoides* respectively as well as that of negative & positive controls were determined by measuring the zones of inhibition in mm using a metric ruler. A total of three trials were done on each of the five bacteria tested (i.e. *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia and Escherichia coli*) for both Disc and Well Diffusion methods. So a total of 30 plates were used.

The results of the experiment determining the antibacterial effect of *Indigofera daleoides* tincture (whole plant) in 62% ethanol was compared to the antibacterial effects of:

- 62% ethanol only
- Ciprofloxacin

The results of the experiment determining the antibacterial effect of *Indigofera daleoides* tincture (roots) in 62% ethanol was compared to the antibacterial effects of:

- 62% ethanol only
- Ciprofloxacin

The results of the experiment determining the antibacterial effect of *Indigofera daleoides* tincture (leaves) in 62 ethanol was compared to the antibacterial effects of:

- 62% ethanol only
- Ciprofloxacin

3.1.2 The Secondary Data

Methods for obtaining secondary data were from research articles derived from various international journal publications, books and manuals.

3.2 The criteria governing the admissibility of Data

Only data obtained from laboratory experiments carried out by the researcher at the Department of Biotechnology and Food Technology, microbiology laboratory (Steve Biko campus, level 0) was used.

3.3 Materials & Methods

3.3.1 Preparation of plant extracts

AfriNatural Phytomedicine cc sourced the plant and WLast incorporated made the respective three tinctures of *Indigofera daleoides*. Separate tinctures of the plant were made of the different parts; whole plant, roots and leaves using the German Pharmacopoeia Method HAB 4a specifications (Bunyens 2005). The precise method applied is as follows:

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

3.3.2 Preparation of medicated discs

3.3.2.1 Preparation of Indigofera daleoides in 62% ethanol base dry discs

- Sterile, 5mm, whatman filter paper number 4 discs were evenly placed upon the bottom of a sterile dish using a pair of sterile forceps, so that each petri dish contained 6 discs.
- Microlitres (10) of *Indigofera daleoides* extract was pipetted onto each disc using a calibrated micropipette.
- The petri dishes were then placed in an incubator room kept at 37ºC, and the discs allowed to dry over a period of 24hours. Until they were used the following day.

3.3.2.2 Preparation of the 62% ethanol only discs

- Ethanol (62%) was used as the medium in making the test substances (i.e. *Indigofera daleoides* mother tinctures) as stipulated by the German Homeopathic Pharmacopoeia method 4a specifications (Bunyens 2005).
- Sterile 5mm, whatman filter paper number 4, discs were evenly placed upon the bottom of a sterile petri dish using a sterile forceps, so that each petri dish contained 6discs.
- Microliters (10) of 62% ethanol were pipetted onto each disc using a calibrated micropipette.
- The petri dishes were placed in an incubator room kept at 37ºC, and discs allowed to dry.
- The dry discs were then stored in labelled sterile jars until used.

3.3.2.3 Preparation of the Ciprofloxacin positive control

- Sterile, 5mm, Whatman ® filter paper number 4 discs were placed on the bottom of a sterile dish using a pair of sterile forceps, so that each petri dish contained 6 discs.
- Ciprofloxacin (100mg) was diluted by 2ml of distilled water and stirred lightly to settle and mix.
- Microliters (10)of respective Ciprofloxacin solution were pipetted onto each disc using a calibrated micropipette.
- The petri dishes were then placed in an incubator room kept at 37^0C, and the discs allowed to dry.
- The dry discs were then labelled, placed in a sterile jar until used.

3.3.2.4 Preparation of Media

The medium of choice was Mueller-Hilton agar which is considered the best for routine antimicrobial susceptibility (Lalitha 2004). It was prepared according to the sigma Aldrich specifications (i.e. manufacturer), as follows:

- Mueller-Hinton agar powder (34g) was weighed out.
- The Mueller-Hinton agar powder was added to 1 liter of distilled water in a screw top flask.
- Then the mixture was shaken until well mixed.
- It was then autoclaved at 121ºC for 15 minutes.
- The flask was then allowed to cool to a relatively warm to touch temperature.
- When the flask was cool enough to hold comfortably it was poured in to empty agar plates. This was done in a sterilized laminar flow table space:
- The mouth of the flask was passed through a lit Bunsen burner before pouring to ensure proper sterility.
- agar (40ml) were poured into each plate.
- A total of 6 plates were prepared per bacterium.
- The plates were then stacked and allowed to solidify.
- After solidifying they were checked visually for any contamination and those contaminated were discarded.

3.3.2.5 Preparation of the inoculums

Single colonies were obtained from the Durban University of Technology Steve Biko Biotechnology Laboratory, and cultures of each bacteria were tested and used to inoculate separate Mueller-Hinton agar plates, and place in an incubator room which is kept at 37^0C.

The bacteria will be;

Staphylococcus aureus

Pseudomonas aeruginosa

Enterococci faecalis

Klebselia pneumonia

Escherichia coli

3.3.2.6 Controls

Positive control- Ciprofloxacin (purchased from Sigma Aldrich)

Negative control- Ethanol 62%

3.3.2.7 Preparation of saline test cultures

A couple of the colonies from the overnight Mueller-Hinton agar cultures of the selected bacteria were suspended in 10ml sterile solution (8.5 g/l) and the solution adjusted to 0.5 McFarland Equivalence Turbidity Standard, using saline and a vortex mixer (Lalitha 2004). See appendix F for make-up of McFarland standard.

3.3.2.8 Preparation of the plates for the Disc Diffusion Assay

- A black marker pen was used to label the base of the agar plate with abbreviations of the bacterium that was streaked on the plate to be tested.
- A black marker pen was used to partition the agar plate into 6 parts, and in each partition that the abbreviated name of the test substance was written.
- A sterile cotton swab was dipped into a well-mixed saline test culture and excess inoculum was removed by pressing slightly against the inner wall of the test tube.
- Using the swab, the entire agar surface of the plate was streaked 1st in a horizontal direction and then vertically. This was repeated 2 more times to ensure proper whole plate microbial growth.
- Sterile autoclaved forceps were used to place the impregnated discs on the agar surface.
- All the prepared plate cultures we placed in an inverted position in an incubator room that is set at 37°C.
- After a period of 24 hours the plates were examined for antimicrobial inhibitory activity, which is reflected by a clear zone of inhibition around the disc with the test substance in it.

3.3.2.9 Preparation of 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 were streaked on the plate.

- A black marker pen was used to partition the agar plate into 6 parts, and in each partition that the abbreviated name of the test substance was written

- A sterile cotton swab was dipped into a well-mixed saline test culture and excess inoculum was removed by pressing slightly against the inner wall of the test tube.
- Using the swab, the entire agar surface of the plate was streaked 1st in a horizontal direction and then vertically. This was repeated 2 more times to ensure proper whole plate microbial growth.
- A 3mm well was punched into the agar plate using a sterile pipette (kwon and Ricke, 1998).
- All the prepared plate cultures we placed in an inverted position in an incubator room that is set at 37°C.
- After a period of 24 hours the plates were examined for antimicrobial inhibitory activity, which is reflected by a clear zone of inhibition around the well with the test substance in it.

3.3.2.10 Measurements

The diameters of the zones of inhibition were measured in millimeters using a metric ruler held at the back of the of the inverted petri dish (Lalitha 2004). The results were recorded on a table for each relevant group (see appendix I).

3.3.2.11 Ethics and safety

The research was conducted at a microbiological lab under the supervision of Dr Santhosh Pillai (Department of Biotechnology and Food technology) at the Durban University of technology. All the bacteria used were non-pathogenic to ensure safety. The Faculty of Health sciences RHDC (Research and Higher Degrees' Committee) gave approval for the study since no human subjects were used in the study and thus the study was exempt from review by the DUT Institutional Research Ethics Committee.

3.4 Data Analysis and Statistical Procedures

3.4.1 Sample size of the study

The sample size throughout Part one (Disc Diffusion Assay) & Part two (Well Diffusion Assay) of the study was five i.e. each sensitivity test was done 3 times on each bacteria.

3.4.2 Statistical package

The Statistical Package for Social Sciences (SPSS) 25 was used in data capturing & analysis.

3.4.3 Analysis of Results

According to Crowder (2017), a mixed factorial ANOVA (repeated measures) is the most appropriate statistical technique when the same characteristics is measured on each case or subjects at several times or under several conditions. So after the data collection was done and results tabulated a Mixed factorial ANOVA test was used to test the difference across the microbial treatment groups in comparison with the negative (62% ethanol) and positive control (Ciprofloxacin). One-way ANOVA was used to compare the differences between tinctures of the whole plants, leaves and roots. Additionally, an independent t-test was further used to compare the differences between the two techniques employed.

CHAPTER FOUR

Results

4.0 INTRODUCTION

This chapter presents the results of the data gathering process. In this study, the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plants, leaves, and roots (in 62% ethanol) respectively was assessed using two different techniques namely: (1) Disc Diffusion Assay and (2) Well Diffusion Assay. The antibacterial potency properties of *Indigofera daleoides* against *S. aureus*, *P. aeruginosa*, *E. faecalis*, *K. pneumonia*, and *E. coli* were compared against ethanol and Ciprofloxacin, respectively. Mixed factorial ANOVA was used test for the difference across the treatment groups. Furthermore, one-way ANOVA was used to compare the antibacterial differences between the whole plants, leaves and roots. In addition, an Independent t-test was further used to compare the differences between two techniques employed. This chapter concludes with a summary of the data that was analysed.

4.1. Assessing the antibacterial potency *Indigofera daleoides* mother tincture using Disc Diffusion Assay

This section assessed the antibacterial potency of *Indigofera daleoides* (in 62% ethanol) against five microorganisms (*S. aureus, P. aeruginosa, E. faecalis, K. pneumonia,* and *E. coli*). The results are summarised below.

4.1.1 Mauchly's Test of Sphericity for *Indigofera daleoides* mother tincture analyses using Disc Diffusion Assay

Table 4-1 showed the test for sphericity for data obtained from *Indigofera daleoides* mother tincture analyses using Disc Diffusion Assay techniques. As seen below, the Mauchly's test has a p-value of 0.009, which provide evidence against the sphericity of the data. Kinnear and Gray (2004) advised that when there is an absence of homogeneity of covariance in a data set, Greenhouse-Geisser is the most appropriate correction to interpret the set of data.

Table 4-1: Mauchly's Test of Sphericity for *Indigofera daleoides* mother tincture using Disc Diffusion Assay

Mauchly's Test of Sphericity[a]							
Measure:	MEASURE_1						
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Organism	0.009	20.682	9	0.020	0.505	1.000	0.250
Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.							
a. Design: Intercept + Group Within Subjects Design: Organism							
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.							

4.1.2 Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plants (62% ethanol)

Table 4-2 shows the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plants in 62% ethanol against *S. Aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* in comparison with ethanol and Ciprofloxacin, respectively. From the table below, it can be gathered that the antibacterial potency of Ciprofloxacin was consistently higher than other groups. Against *S. aureus* for example, the highest antibacterial potency (21.3±3.33) was measured for Ciprofloxacin while no antibacterial potency was observed for *Indigofera daleoides* mother tincture derived from whole plants in 62% ethanol. A similar pattern was noted for *P. aeruginosa. E. faecalis*. With regards to *E. coli*, although Ciprofloxacin (32.0±1.00) showed the highest antibacterial potency (32.0±1.00), the *Indigofera daleoides* mother tincture derived from whole plants in 62% ethanol displayed weak antibacterial potency (6.33.0±0.58) however this effect was comparable with 62% ethanol (3.67±6.35) thus arguably the effect achieved by *Indigofera daleoides* mother tincture derived from whole plants could be attributable to the 62% ethanol vehicle and not the plant.

Table 4.2: Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plants (62% ethanol)

Treatment group		Mean	Std. Deviation	N
S. aureus	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	5.3333	9.23760	3
	Ciprofloxacin (control)	21.3333	1.52753	3

P. aeruginosa	*Indigofera daleoides* in 62% ethanol	2.3333	4.04145	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	42.0000	2.64575	3
E. faecalis	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	22.6667	0.57735	3
K. pneumonia	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	14.0000	2.64575	3
	Ciprofloxacin (control)	39.6667	2.51661	3
E. coli	*Indigofera daleoides* in 62% ethanol	6.3333	0.57735	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	32.0000	1.00000	3

4.1.3 ANOVA tests of within-subjects and between effects for *Indigofera daleoides* mother tincture derived from whole plants (62% ethanol)

As shown in Table 4.3, the mean antibacterial potency of the prepared mother tincture derived from whole plants in comparison to 62% ethanol and Ciprofloxacin against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* showed significant differences (F (4.040, 12.120) = 5.848; *P*<0.001). Partial eta squared = 0.661 representing a large effect. This suggests that the antibacterial potency against the mentioned microorganisms were not the same across the treatment groups and the measured differences are large.

Table 4.3: Tests of within-subject's effects by antibacterial potency prepared from whole plants

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Organism * Treatment group	Greenhouse-Geisser	771.467	4.040	190.952	5.848	0.007	0.661
Error(Organism)	Greenhouse-Geisser	395.733	12.120	32.650			

The pairwise comparison test is given in Table 4.4. The antibacterial potency observed in the group treated with Ciprofloxacin was significantly higher than those

measured for 62% ethanol and mother tincture derived from whole plants (62% ethanol) (*P*<0.001), respectively. Figure 4.1 further demonstrates the differences in the antibacterial potency of the three treatment groups against the different bacteria. The antibacterial potency in the three group was clearly evident. Overall, Ciprofloxacin (31.53) had the highest mean antibacterial potency, followed by ethanol only (5.33) and lastly mother tincture derived from whole plants (62% ethanol) (1.73) for all five bacteria.

Table 4.4: Bonferroni test for antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plants

(I) Treatment group		Sig
Indigofera daleoides in 62% ethanol	62% ethanol only	0.004
	Ciprofloxacin (control)	0.000
62% ethanol only	*Indigofera daleoides* in 62% ethanol	0.004
	Ciprofloxacin (control)	0.000
Ciprofloxacin (control)	*Indigofera daleoides* in 62% ethanol	0.000
	62% ethanol only	0.000

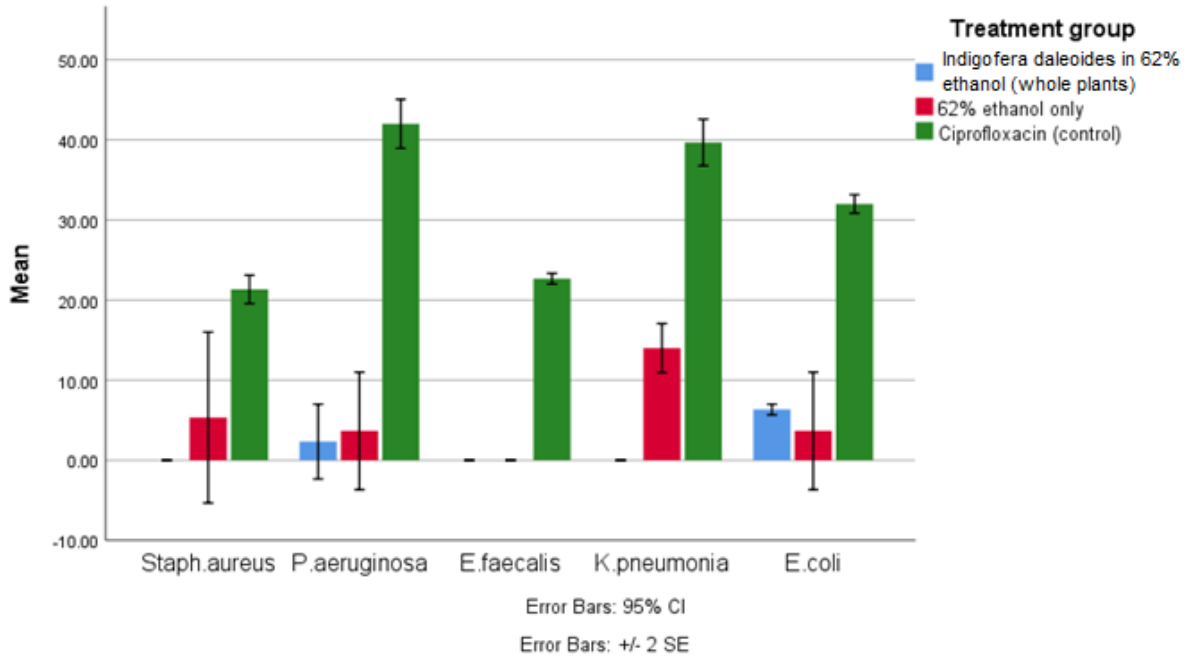

Figure 4.1: Differences in the mean antibacterial potency of the treatment groups

4.1.4 Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves

Table 4.5 shows the antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves in 62% ethanol against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* in comparison with ethanol and Ciprofloxacin. From the table below, it can be gathered that the antibacterial potency of Ciprofloxacin was consistently higher than other groups. Against *Staph. aureus* for example, the highest antimicrobial antibacterial potency (21.3±1.53) was measured for Ciprofloxacin, followed by *Indigofera daleoides* mother tincture derived from leaves (7.00±1.00). Similarly, and for *P. aeruginosa*, Ciprofloxacin measured the highest mean antibacterial potency (42.0±2.65), followed by *Indigofera daleoides* mother tincture derived from leaves (4.33±3.79). Interestingly, against *E. faecalis and K. pneumonia*, no inhibitory effect was measured for both *Indigofera daleoides* mother tincture derived from leaves in or 62% ethanol. Against, *E. coli*, Ciprofloxacin showed the highest antibacterial potency (32.0±1.00). It was also observed that the *Indigofera daleoides*

mother tincture derived from leaves had no antibacterial potency against *E. coli* while 62% ethanol only showed only slight antibacterial potency (3.67±6.35).

Table 4.5: Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves (62% ethanol)

Treatment group		Mean	Std. Deviation	N
S. aureus	*Indigofera daleoides* in 62% ethanol	7.0000	0.00000	3
	62% ethanol only	5.3333	9.23760	3
	Ciprofloxacin (control)	21.3333	1.52753	3
P. aeruginosa	*Indigofera daleoides* in 62% ethanol	4.3333	3.78594	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	42.0000	2.64575	3
E. faecalis	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	22.6667	0.57735	3
K. pneumonia	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	14.0000	2.64575	3
	Ciprofloxacin (control)	39.6667	2.51661	3
E. coli	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	32.0000	1.00000	3

4.1.5 ANOVA tests of within-subjects and between effects for *Indigofera daleoides* mother tincture derived from leaves (62% ethanol)

As shown in Table 4.6, the mean antibacterial potency of the prepared mother tincture derived from leaves in comparison to 62% ethanol only and Ciprofloxacin against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* showed significant differences (F (4.025, 12.076) = 6.804; *P*<0.001). Partial eta squared = 0.694 representing a large effect. This suggests that the antibacterial potency of the effect against the mentioned bacteria were not the same across the treatment groups and the measured differences are large.

Table 4.6: Tests of within-subject's effects by antibacterial potency prepared from leaves

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Organism * Treatment Group	Greenhouse-Geisser	886.978	4.025	220.347	6.804	0.004	0.694
Error(Organism)	Greenhouse-Geisser	391.067	12.076	32.384			

The pairwise comparison test is given in Table 4.7. The antibacterial potency observed in the group treated with Ciprofloxacin was significantly higher than those measured with 62% ethanol and mother tincture derived from leaves (*P*<0.001), respectively and the mother tincture with 62% ethanol derived from leaves (62% ethanol) (*P*<0.001). Figure 4.2 further demonstrates the differences in the antibacterial potency of the three treatment groups against the different bacteria. The antibacterial potency in the three group was clearly evident. Overall, Ciprofloxacin had the highest mean antibacterial potency (31.53mm), followed by ethanol only (5.33mm) and lastly mother tincture derived from leaves (62% ethanol) (2.27mm).

Table 4.7: Bonferroni test for antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves (62% ethanol)

(I) Treatment group		Sig.
Indigofera daleoides in 62% ethanol	62% ethanol only	0.008
	Ciprofloxacin (control)	0.000
62% ethanol only	*Indigofera daleoides* in 62% ethanol	0.008
	Ciprofloxacin (control)	0.000
Ciprofloxacin (control)	*Indigofera daleoides* in 62% ethanol	0.000
	62% ethanol only	0.000

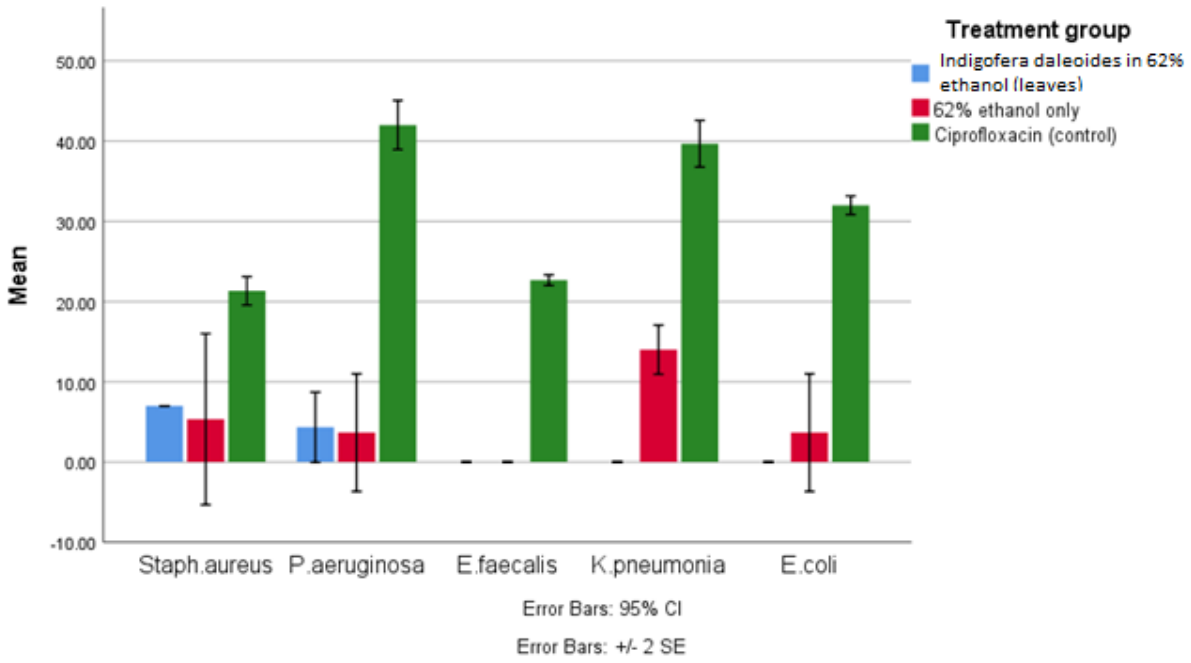

Figure 4.2: Differences in the mean antibacterial potency of the treatment groups

4.1.6 Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from roots

Table 4.8 shows the antibacterial potency of *Indigofera daleoides* mother tincture derived from roots in 62% ethanol against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* in comparison with 62% ethanol and Ciprofloxacin. From the table below, it was observed that the antibacterial potency of Ciprofloxacin was consistently higher than the other groups. Against *S. aureus* for example, the highest antibacterial potency (21.3±1.53) was measured for Ciprofloxacin, followed by *Indigofera daleoides* mother tincture derived from roots (7.33±0.58). Regarding the antibacterial potency against *P. aeruginosa and E. faecalis*, it can be gleaned that no antibacterial potency was measured using *Indigofera daleoides* mother tincture derived from roots in nor was any antibacterial potency was measured against *E. faecalis* using 62% ethanol.

On the other hand, and against *K. pneumonia*, Ciprofloxacin measured the highest antibacterial potency (39.67±2.52) followed by 62% ethanol (14.0±2.65) whilst *Indigofera daleoides* mother tincture derived from roots showed the least antibacterial potency (7.67±6.66). In terms of the antibacterial potency against *E. coli*, while

Ciprofloxacin measured the highest antibacterial potency (32.0±1.00), there was comparable antibacterial potency was observed for both *Indigofera daleoides* mother tincture derived from roots and 62% ethanol (3.67±6.35).

Table 4.8: Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from roots

Treatment group		Mean	Std. Deviation	N
S. aureus	*Indigofera daleoides* in 62% ethanol	7.3333	0.57735	3
	62% ethanol only	5.3333	9.23760	3
	Ciprofloxacin (control)	21.3333	1.52753	3
P. aeruginosa	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	42.0000	2.64575	3
E. faecalis	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	22.6667	0.57735	3
K. pneumonia	*Indigofera daleoides* in 62% ethanol	7.6667	6.65833	3
	62% ethanol only	14.0000	2.64575	3
	Ciprofloxacin (control)	39.6667	2.51661	3
E. coli	*Indigofera daleoides* in 62% ethanol	3.6667	6.35085	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	32.0000	1.00000	3

4.1.7 ANOVA tests of within-subjects and between effects for *Indigofera daleoides* mother tincture derived from roots

As shown in Table 4.9, the mean antibacterial potency of the prepared mother tincture derived from roots in comparison to 62% ethanol and Ciprofloxacin against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* showed significant differences (F (4.979, 14.937) = 4.597; *P*<0.01). Partial eta squared = 0.605 representing a large effect. This suggests that the antibacterial potency of the effect against the mentioned microorganisms were not the same across the treatment groups and the measured differences are large.

Table 4.9: Tests of within-subject's effects by antibacterial potency prepared from roots

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Organism * Group	Greenhouse-Geisser	749.689	4.979	150.573	4.597	0.010	0.605
Error(Organism)	Greenhouse-Geisser	489.200	14.937	32.751			

The pairwise comparison test is given in Table 4.10. The antibacterial potency observed in the group treated with Ciprofloxacin was significantly higher than those measured with 62% ethanol and mother tincture derived from roots (P<0.001), respectively. There were no statistical differences observed in the antibacterial potency measured for 62% ethanol and mother tincture derived from roots (62% ethanol) (P>0.05). Figure 4.3 further demonstrates the differences in the antibacterial potency of the three treatment groups against the different bacteria. The antibacterial potency in the three group was clearly evident. Overall, Ciprofloxacin had the highest mean antibacterial potency (31.53mm), followed by 62% ethanol (5.33mm) and lastly mother tincture derived from roots (3.73mm).

Table 4.10: Bonferroni test for antibacterial potency of *Indigofera daleoides* mother tincture derived from roots

(I) Treatment group		Sig.
Indigofera daleoides in 62% ethanol	62% ethanol only	0.653
	Ciprofloxacin (control)	0.000
62% ethanol only	*Indigofera daleoides* in 62% ethanol	0.653
	Ciprofloxacin (control)	0.000
Ciprofloxacin (control)	*Indigofera daleoides* in 62% ethanol	0.000
	62% ethanol only	0.000

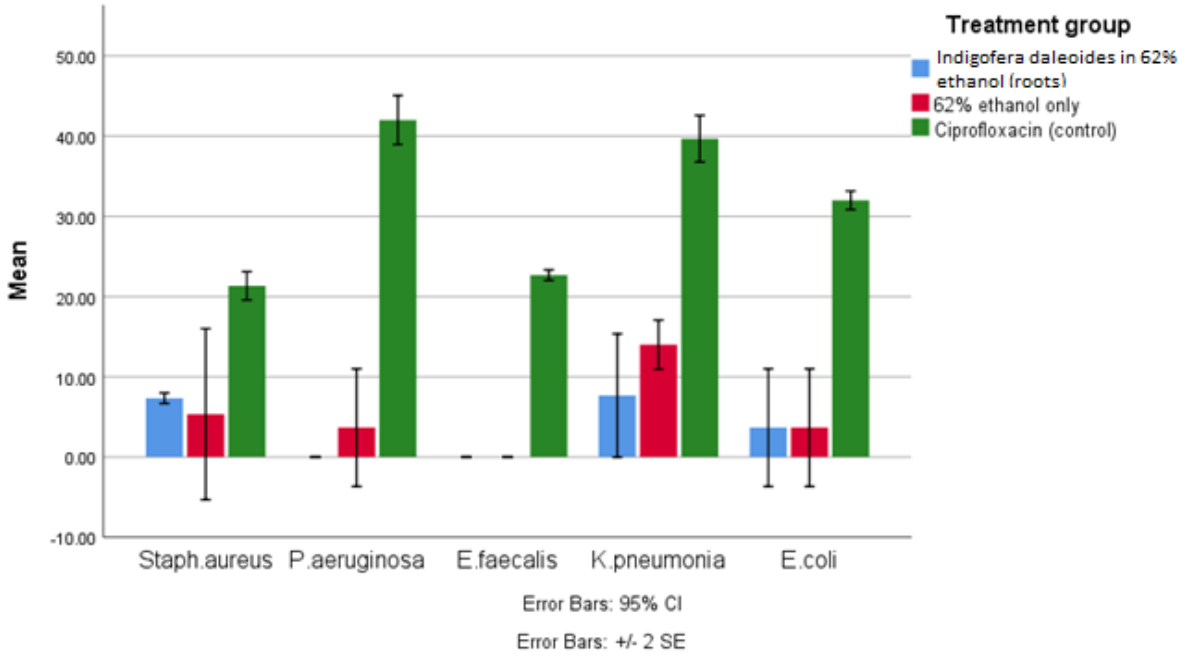

Figure 4.3: Differences in the mean antibacterial potency of the treatment groups derived from roots

4.1.8: Comparison the antibacterial potency of respective *Indigofera daleoides* mother tinctures derived from various parts of the plant using the Disc Diffusion Assay

The one-way ANOVA test, mean, standard deviation of the antibacterial potency of *Indigofera daleoides* mother tincture derived from the respective parts of the plant is shown in Table 4.11. Although the mean antibacterial potency of the roots was higher than tinctures manufactured from other parts of the plant, the one-way ANOVA test, however failed to show any significant differences beyond the 0.05 interval level (P>0.05). This suggests that statistically the antibacterial potency of *Indigofera daleoides* mother tincture is similar irrespective from which part of the plant it is derived.

Table 4.11: ANOVA test for *Indigofera daleoides* mother tincture

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		F	Sig.
					Lower Bound	Upper Bound		
Whole plants	15	1.7333	2.98727	0.77131	0.0790	3.3876	1.359	0.268
Leaves	15	1.8000	3.09839	0.80000	0.0842	3.5158		
Roots	15	3.7333	4.92032	1.27042	1.0086	6.4581		
Total	45	2.4222	3.80483	0.56719	1.2791	3.5653		

4.2. Assessing the antibacterial potency *Indigofera daleoides* mother tincture using Well Diffusion Assay

This section reports on the statistical analysis of the antibacterial potency of *Indigofera daleoides* (in 62% ethanol) against five microorganisms (*S. aureus, P. aeruginosa, E. faecalis, K. pneumonia,* and *E. coli*) using data derived from the well diffusion assay method. The results are summarised below.

4.2.1 Mauchly's Test of Sphericity for *Indigofera daleoides* mother tincture analyse using Well Diffusion Assay

Table 4.12 shows the test for sphericity for data obtained from *Indigofera daleoides* mother tincture analyses using Well Diffusion Assay techniques. As seen below, the Mauchly's test has a p-value of 0.102, which provide no evidence against the sphericity of the data.

Table 4.12: Mauchly's Test of Sphericity for *Indigofera daleoides* mother tincture using Well Diffusion Assay

Mauchly's Test of Sphericity[a]							
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Organism	0.102	10.090	9	0.376	0.554	1.000	0.250
Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.							
a. Design: Intercept + Group Within Subjects Design: Organism							
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.							

4.2.2 Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plant

Table 4.13 shows the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plant against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli* in comparison with 62% ethanol and Ciprofloxacin. From the table below, it can be seen that the antibacterial potency of Ciprofloxacin was consistently higher than the two other groups. Against *S. aureus* for example, the highest antibacterial potency was measured for Ciprofloxacin (29.3±1.15), followed by *Indigofera daleoides* mother tincture derived from whole plants (11.67±1.53). Similarly, and against *P. aeruginosa*, the highest antibacterial potency (47.7±2.52) was measured for Ciprofloxacin, followed by *Indigofera daleoides* mother tincture derived from whole plant (10.33±1.54). Of interest, no antibacterial potency was measured against *E. faecalis, K. pneumonia, and E. coli* using *Indigofera daleoides* mother tincture derived from whole plants and 62% ethanol respectively.

Table 4.13: Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plant

Treatment group		Mean	Std. Deviation	N
S. aureus	*Indigofera daleoides* in 62% ethanol	11.6667	1.52753	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	29.3333	1.1547	3
P. aeruginosa	*Indigofera daleoides* in 62% ethanol	10.3333	1.1547	3
	62% ethanol only	4	6.9282	3
	Ciprofloxacin (control)	47.6667	2.51661	3
E. faecalis	*Indigofera daleoides* in 62% ethanol	0	0	3
	62% ethanol only	0	0	3
	Ciprofloxacin (control)	34	4.3589	3
K. pneumonia	*Indigofera daleoides* in 62% ethanol	0	0	3
	62% ethanol only	0	0	3
	Ciprofloxacin (control)	36.3333	4.04145	3
E. coli	*Indigofera daleoides* in 62% ethanol	0	0	3
	62% ethanol only	0	0	3
	Ciprofloxacin (control)	35.6667	0.57735	3

4.2.3 ANOVA tests of within-subjects and between effects for *Indigofera daleoides* mother tincture derived from whole plant

As shown in Table 4.14, the mean antibacterial potency of the prepared mother tincture derived from whole plant in comparison to 62% ethanol only and Ciprofloxacin against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia, and E. coli* showed significant differences (F (8, 24) = 6.094; *P*<0.001). Partial eta squared = 0.670 representing a large effect. This suggests that the antibacterial potency of the effect against the mentioned bacteria were not the same across the treatment groups and the measured differences are large.

Table 4.14: Tests of within-subjects' effects by antibacterial potency prepared from whole plants

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Organism * Group	Sphericity Assumed	498.356	8	62.294	6.094	0.000	0.670
Error(Organism)	Sphericity Assumed	245.333	24	10.222			

The pairwise comparison test is given in Table 4.15. The antibacterial potency observed in the group treated with Ciprofloxacin was significantly higher than those measured with 62% ethanol and mother tincture derived from whole plant (*P*<0.001), respectively. Figure 4.4 further demonstrates the differences in the antibacterial potency of the three treatment groups against the different microorganisms. The antibacterial potency in the three group was clearly evident. Overall, Ciprofloxacin had the highest mean antibacterial potency (36.6mm), followed by mother tincture derived from whole plant (4.4mm), and lastly 62% ethanol (1.53mm).

Table 4.15: Bonferroni test for antibacterial potency of *Indigofera daleoides* mother tincture derived from whole plant

(I) Treatment group		Sig.
Indigofera daleoides in 62% ethanol	62% ethanol only	0.026
	Ciprofloxacin (control)	0.000
62% ethanol only	*Indigofera daleoides* in 62% ethanol	0.026
	Ciprofloxacin (control)	0.000
Ciprofloxacin (control)	*Indigofera daleoides* in 62% ethanol	0.000
	62% ethanol only	0.000

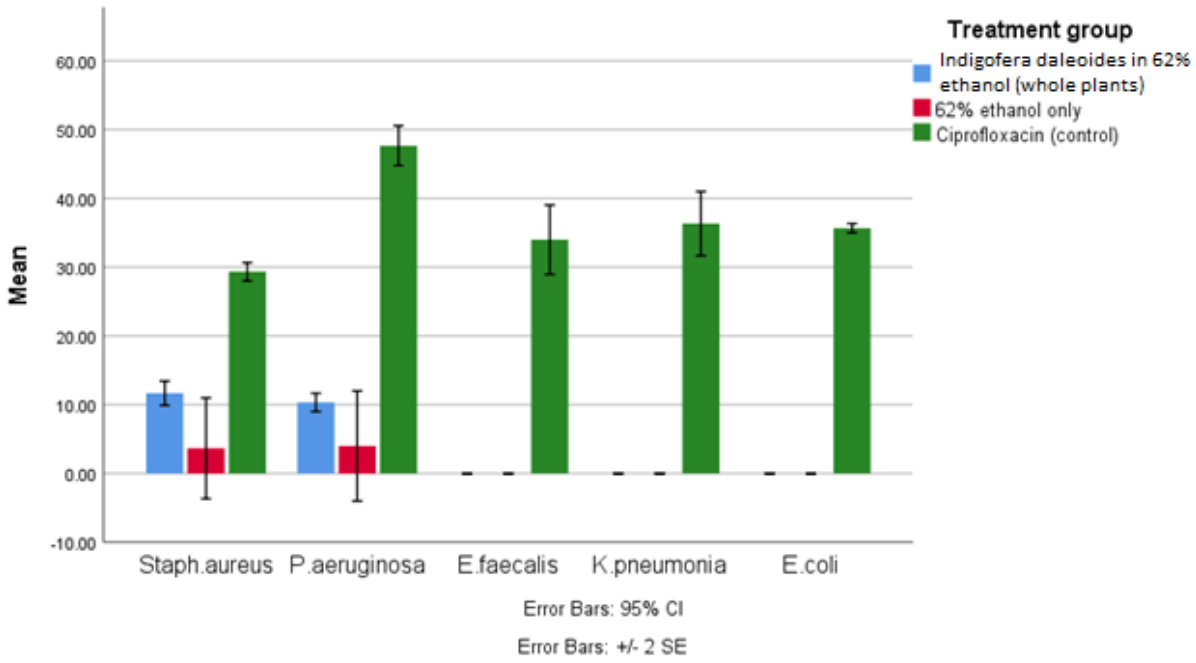

Figure 4.4: Differences in the mean antibacterial potency of the various treatment groups

4.2.4 Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves

Table 4.16 showed the antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and

E. coli in comparison with 62% ethanol and Ciprofloxacin. From the table below, it can be seen that the antibacterial potency of Ciprofloxacin was consistently higher than other two groups. Against *S. aureus* for example, the highest antibacterial potency (29.3±1.15) was measured for Ciprofloxacin, followed by *Indigofera daleoides* mother tincture derived from leaves (12.33±0.58). Similarly, and against *P. aeruginosa*, the highest antibacterial potency (47.7±2.52) was measured for Ciprofloxacin, followed by *Indigofera daleoides* mother tincture derived from leaves (11.33±1.55). Of interest, no antibacterial potency was measured against E. *faecalis, K. pneumonia, and E. coli* using *Indigofera daleoides* mother tincture derived from leaves and 62% ethanol only, respectively.

Table 4.16: Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves

Treatment group		Mean	Std. Deviation	N
S. aureus	*Indigofera daleoides* in 62% ethanol	12.3333	0.57735	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	29.3333	1.15470	3
P. aeruginosa	*Indigofera daleoides* in 62% ethanol	11.3333	1.15470	3
	62% ethanol only	4.0000	6.92820	3
	Ciprofloxacin (control)	47.6667	2.51661	3
E. faecalis	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	34.0000	4.35890	3
K. pneumonia	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	36.3333	4.04145	3
E. coli	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	35.6667	0.57735	3

4.2.5 ANOVA tests of within-subjects and between effects for *Indigofera daleoides* mother tincture derived from leaves.

As shown in Table 4.17, the mean antibacterial potency of the prepared mother tincture derived from leaves in comparison to 62% ethanol and Ciprofloxacin against *S. aureus, P. aeruginosa, E. faecalis, K. pneumonia, and E. coli* showed significant differences ($F(8, 24) = 6.494$; $P<0.001$). Partial eta squared = 0.684 representing a large effect. This suggests that the antibacterial potency against the mentioned

bacteria were not the same across the treatment groups and the measured differences are large.

Table 4.17: Tests of within-subjects' effects by antibacterial potency prepared from leaves

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Organism * Group	Sphericity Assumed	523.022	8	65.378	6.494	0.000	0.684
Error(Organism)	Sphericity Assumed	241.600	24	10.067			

The pairwise comparison test is given in Table 4.18. The antibacterial potency observed in the group treated with Ciprofloxacin was significantly higher than those measured with 62% ethanol only and mother tincture derived from leaves (P<0.001), respectively. Figure 4.5 further demonstrates the differences in the antibacterial potency of the three treatment groups against the different bacteria. The antibacterial potency in the three group was clearly evident. Overall, Ciprofloxacin had the highest mean antibacterial potency (36.6mm), followed by mother tincture derived from leaves in (4.73mm), and lastly 62% ethanol (1.53mm).

Table 4.18: Bonferroni test for antibacterial potency of *Indigofera daleoides* mother tincture derived from leaves

(I) Treatment group		Sig.
Indigofera daleoides in 62% ethanol	62% ethanol only	0.015
	Ciprofloxacin (control)	0.000
62% ethanol only	*Indigofera daleoides* in 62% ethanol	0.015
	Ciprofloxacin (control)	0.000
Ciprofloxacin (control)	*Indigofera daleoides* in 62% ethanol	0.000
	62% ethanol only	0.000

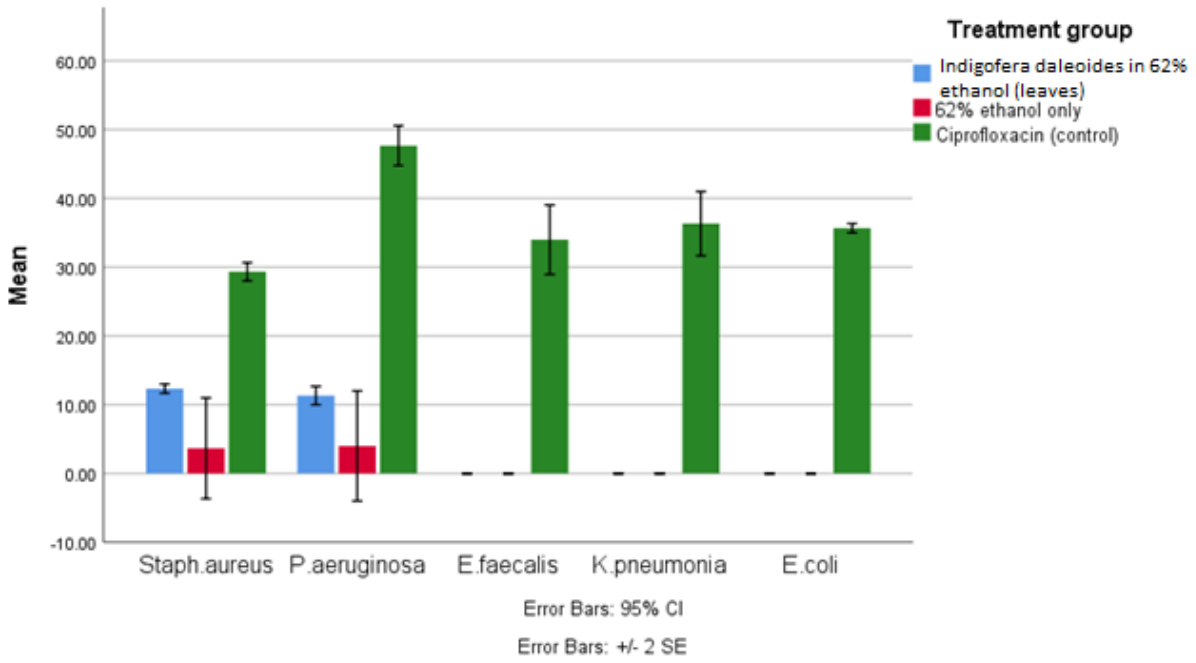

Figure 4.5: Differences in the mean antibacterial potency of the treatment groups

4.2.6 Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from roots

Table 4.19 showed the antibacterial potency of *Indigofera daleoides* mother tincture derived from roots in 62% ethanol against *S. Aureus, P. aeruginosa, E. faecalis, K. pneumonia, and E. coli* in comparison with 62% ethanol and Ciprofloxacin. From the table below, it can be seen that the antibacterial potency of Ciprofloxacin was consistently higher than other two groups. Against *S. aureus* for example, the highest antibacterial potency (29.3±1.15) was measured for Ciprofloxacin, followed by *Indigofera daleoides* mother tincture derived from roots (6.67±5.86). There was, however, no antibacterial potency against *P. aeruginosa* using *Indigofera daleoides* mother tincture derived from roots. In contrast, Ciprofloxacin measured highest antibacterial potency (47.7±2.52), followed by 62% ethanol (4.0±6.92) against *P. aeruginosa*.

On the other hand, no antibacterial potency was measured for *Indigofera daleoides* mother tincture derived from roots and 62% ethanol against *E. faecalis and K. pneumonia*, respectively. Interestingly, and against *E. coli*, *Indigofera daleoides* mother tincture derived from roots had antibacterial potency (6.67±5.78) whilst no antibacterial potency was observed for 62% ethanol, this suggests an antibacterial effect beyond that of the 62% ethanol vehicle.

Table 4.19: Assessing the antibacterial potency of *Indigofera daleoides* mother tincture derived from roots

Treatment group		Mean	Std. Deviation	N
S. aureus	*Indigofera daleoides* in 62% ethanol	6.6667	5.85947	3
	62% ethanol only	3.6667	6.35085	3
	Ciprofloxacin (control)	29.3333	1.15470	3
P. aeruginosa	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	4.0000	6.92820	3
	Ciprofloxacin (control)	47.6667	2.51661	3
E. faecalis	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	34.0000	4.35890	3
K. pneumonia	*Indigofera daleoides* in 62% ethanol	0.0000	0.00000	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	36.3333	4.04145	3
E. coli	*Indigofera daleoides* in 62% ethanol	6.6667	5.77350	3
	62% ethanol only	0.0000	0.00000	3
	Ciprofloxacin (control)	35.6667	0.57735	3

4.2.7 ANOVA tests of within-subjects and between effects for *Indigofera daleoides* mother tincture derived from roots

As shown in Table 4.20, the mean antibacterial potency of the prepared mother tincture derived from roots in comparison to 62% ethanol and Ciprofloxacin against S. *aureus, P. aeruginosa, E. faecalis, K. pneumonia, and E. coli* showed significant differences ($F(8, 24) = 4.827$; $P<0.001$). Partial eta squared = 0.617 representing a large effect. This suggests that the antibacterial potency of the effect against the mentioned bacteria were not the same across the treatment groups and the measured differences are large.

Table 4.20: Tests of within-subjects' effects by antibacterial potency prepared from roots

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Organism * Group	Sphericity Assumed	574.089	8	71.761	4.827	0.001	0.617
Error(Organism)	Sphericity Assumed	356.800	24	14.867			

The pairwise comparison test is given in Table 4.21. The antibacterial potency observed in the group treated with Ciprofloxacin was significantly higher than those measured with 62% ethanol and mother tincture derived from roots in (P<0.001), respectively. No statistical differences were observed in the antibacterial potency measured for mother tincture derived from roots n and 62% ethanol (P>0.05). Figure 4.6 further demonstrates the differences in the antibacterial potency of the three treatment groups against the different bacteria. The antibacterial potency in the three group was clearly evident. Overall, Ciprofloxacin (36.6mm) had the highest mean antibacterial potency, followed by mother tincture derived from roots in (2.67mm), and lastly 62% ethanol only (1.53mm).

Table 4.21: Bonferroni test for antibacterial potency of *Indigofera daleoides* mother tincture derived from roots

(I) Treatment group		Sig.
Indigofera daleoides in 62% ethanol	62% ethanol only	0.854
	Ciprofloxacin (control)	0.000
62% ethanol only	*Indigofera daleoides* in 62% ethanol	0.854
	Ciprofloxacin (control)	0.000
Ciprofloxacin (control)	*Indigofera daleoides* in 62% ethanol	0.000
	62% ethanol only	0.000

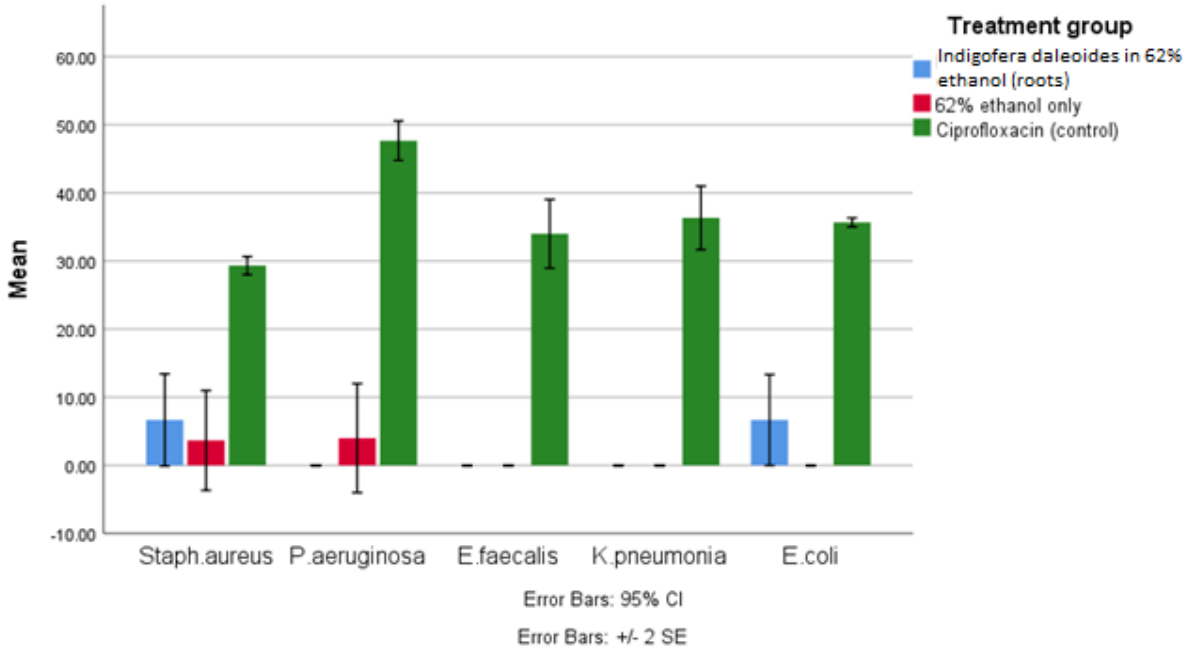

Figure 4.6: Differences in the mean antibacterial potency of the treatment groups

4.2.8: Comparison the antibacterial potency of respective *Indigofera daleoides* mother tinctures derived from various parts of the plant using the Well Diffusion technique.

The one-way ANOVA test, mean, standard deviation of the antibacterial potency of *Indigofera daleoides* mother tincture derived from the different parts of the plants is shown in Table 4.22. Although the mean antibacterial potency of the leaves was higher than any of the other parts of the plants, the one-way ANOVA test, however failed to show any significant differences beyond the 0.05 interval level (P>0.05). This suggests that statistically the antibacterial potency of *Indigofera daleoides* is similar irrespective from which part of the plant it is derived

Table 4.22: ANOVA test for *Indigofera daleoides* mother tincture

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		F	Sig
					Lower Bound	Upper Bound		

Whole plants	15	4.4000	5.64168	1.45668	1.2757	7.5243	0.621	0.543
Leaves	15	4.7333	6.02929	1.55676	1.3944	8.0722		
Roots	15	2.6667	4.59296	1.18590	0.1232	5.2102		
Total	45	3.9333	5.40791	0.80616	2.3086	5.5580		

4.3 Comparison between Disc and Well Diffusion techniques

Table 4.23-Table 4.25 summarises the Independent t-test results obtained from the two different techniques used. It can be observed that there were no significant differences in the antibacterial potency results obtained based on the measurement techniques used (P>0.05). This notwithstanding, it can be gathered from Table 4.23 that the results obtained from the well diffusion (3.47±5.23) assay was more favourable when compared against Disc Diffusion Assay (2.88 ±3.34) for *Indigofera daleoides* mother tincture. Similarly, Well Diffusion techniques showed higher antibacterial potency when used for Ciprofloxacin. In contrast, disc diffusion results were more favourable for 62% ethanol.

Table 4.23: Differences between Disc and Well Diffusion Assay in assessing *Indigofera daleoides* mother tincture antibacterial potency

Technique		N	Mean	Std. Deviation	Std. Error Mean	Sig
Antibacterial potency	Disc Diffusion	15	2.8760	3.34437	0.86351	0.710
	Well Diffusion	15	3.4780	5.22816	1.34991	

Table 4.24: Differences between Disc and Well Diffusion Assay in assessing ethanol only antibacterial potency

Technique		N	Mean	Std. Deviation	Std. Error Mean	Sig
Antibacterial potency	Disc Diffusion	5	10.4000	6.18870	2.76767	0.180
	Well Diffusion	5	4.6000	6.30872	2.82135	

Table 4.25: Differences between Disc and Well Diffusion Assay in assessing Ciprofloxacin antibacterial potency

Technique		N	Mean	Std. Deviation	Std. Error Mean	Sig
Antibacterial potency	Disc Diffusion	5	31.5400	9.47644	4.23799	0.369
	Well Diffusion	5	36.4000	6.37887	2.85272	

4.4 Conclusion

In summary, the mixed factorial ANOVA suggests Ciprofloxacin exhibits the highest antibacterial potency against *S. Aureus, P. aeruginosa, E. faecalis, K. pneumonia*, and *E. coli*. Although *Indigofera daleoides* mother tincture showed some antibacterial potency, it was however, limited in most instances against E*. faecalis and K. pneumonia* only. Equally important, the chapter revealed that the parts of the *Indigofera daleoides* mother tincture plants used made no significant contribution to its antibacterial potency. In terms of measurement technique applied the data suggests that the Well Diffusion is a more sensitive technique as data derived from this method was more favourable for Ciprofloxacin and *Indigofera daleoides.*

Table 4.26: Mean antibacterial potency per organism

		Mean antibacterial potency (mm) per organism				
Method	**Treatment**	***S aureus***	***P. aeruginosa***	***E. faecalis***	***K. pneumonia***	***E. coli***
DD	Ciprofloxacin	21	42	23	40	32
	62% ROH	4	4	0	14	4
	Whole plant	0	4	0	0	6
WD	Ciprofloxacin	29	48	34	36	36
	62% ROH	4	4	0	0	0
	Whole plant	12	4	0	0	0
DD	Ciprofloxacin	21	42	23	40	32
	62% ROH	4	4	0	14	4
	Leaves	7	4	0	0	0
WD	Ciprofloxacin	29	48	34	36	36
	62% ROH	4	4	0	0	0
	Leaves	12	4	0	0	0
DD	Ciprofloxacin	21	42	23	40	32
	62% ROH	4	4	0	14	4
	Roots	7	4	0	8	4
WD	Ciprofloxacin	29	48	34	36	36
	62% ROH	4	4	0	0	0
	Roots	7	4	0	0	7

Chapter 5

Discussion

5.1 Disc Diffusion Method

The results of this study showed that the respective mother tinctures made from varying parts of the plant *Indigofera daleoides* had no statistically significant inhibitory effect on the selected panel of bacteria (i.e. *S. Aureus, P. aeruginosa, E. Faecalis, K. pneumonia and E. coli*). Ethanol (62%) had little antibacterial potency whilst ciprofloxacin showed the greatest antibacterial potency against all respective bacteria, as reflected statistically by the Mauchly's sphericity tests.

The one-way ANOVA test was employed to compare the differences amongst the tinctures. The mean size of the zone of inhibition of the tinctures made of *Indigofera daleoides* roots was (3.7333mm), leaves (1.8000) and whole plants was (1.7333). The tincture made from the roots of *Indigofera daleoides* thus showed the most antibacterial potency compared to the tinctures made from leaves and whole plant against the various bacteria, but such effects were not statistically significant reflected by an interval level which is greater than 0.05.

Some bacteria were specifically completely insensitive to the tinctures of *Indigofera daleoides*. The possible reasons why the tinctures had so little antimicrobial effects are, namely:

- The dilution ratio (i.e. the homeopathic mother tincture dilution ratio is 1:10) was too high as opposed to the normal herbal ratio which is 1:5. Hence active ingredients of the plants are less.
- The drying process of the discs might have nullified the potency of the tinctures.
- The bacteria may be resistant to *Indigofera daleoides*
- Ethanol as an extractant was possibly not effective in extracting the antimicrobial properties of the *Indigofera daleoides* plant. Perhaps another extractant would be more suitable i.e. methanol, acetone etc.

5.2 Well Diffusion Method

The Mauchly's test for sphericity was employed when analyzing results obtained from the well diffusion assay experiments. Ciprofloxacin showed the greatest inhibitory activity followed by *Indigofera daleoides* mother tinctures and 62% ethanol showing the least activity. When the comparisons were made between the inhibitory effects of the tinctures there were no significant differences beyond the 0.05 interval level ($p>0.05$). This reflects that the antibacterial potency of the different mother tinctures was relatively the same even though they are made of different parts of the same plant. According to Mathabe *et al.* (2006) *Indigofera daleoides* showed significant antimicrobial activity *S. aureus* and *E. coli* using the Well Diffusion Assay. This activity was not confirmed in the current study which can be due to a number of factors, namely;

- The homeopathic dilution ratio (1:10) as according to the German Homeopathic Pharmacopeia (Bunyens 2005) is too dilute of a concentration with not enough active ingredients of the plant in it to exhibit proper antimicrobial effect.
- The choice of solvents and plant extract formation process applied by Mathabe et al. (2006) differed from the present study i.e. dried powdered plant materials (10 grams) were used and dissolved in 150 ml's of different solvents (i.e. methanol, acetone and ethanol), they were then dissolved in dimethyl sulphoxide (DMSO i.e. the negative control) to a final concentration of 100mg/m.

Of the three tinctures produced, the leaves tincture showed the greatest inhibitory effect with a mean zone of inhibition of 4.7333mm, whole plant (4.400mm), and roots (2.6667). Overall the were no significant effects or differences in antibacterial potency of the tinctures made from different parts of *Indigofera daleiodes*, and tested in the study using Well Diffusion Assay.

5.3 General Discussion

When Disc Diffusion and Well Diffusion tests were compared using independent t-tests there were no significant differences between the 2 techniques used to conduct the research ($P>0.05$). The Well Diffusion method did however produce data indicating greater effectiveness with tinctures (3.47±5.23) in comparison to Disc Diffusion (2.88±3.34) and also showed higher antibacterial potency for ciprofloxacin, while the Disc Diffusion was more effective with the ethanol control only discs.

The study shows that *Indigofera daleoides* has limited antibacterial effect against this panel study of bacteria. A change of methodology and tincture manufacture method make up could potentially yield different results. The different parts used or the different measurement techniques applied in this study made no significant contribution to *Indigofera daleoides* mother tincture's antibacterial potency.

Chapter 6

Conclusion and Recommendations

6.1 Conclusion

The purpose of this study was to determine the effect of various ethanolic extracts of *Indigofera daleoides* made from whole plant, leaves and roots against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using the Disc Diffusion and Well Diffusion Assay microbiological methodologies. The efficacy of the tinctures was compared against the ciprofloxacin antibiotic and ethanol as positive & negative controls respectively, measuring the minimum inhibitory concentration of each tincture according to the antibacterial potency they possess.

- *Indigofera daleoides* 62% mother tinctures did not show significant antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.
- *Indigofera daleoides* 62% mother tinctures did not show significant antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.
- 62% ethanol did not have significant antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.
- 62% ethanol did not have significant antibacterial effects on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.
- Ciprofloxacin had significant antibacterial effects on *Staphylococcus aureus*, *Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.
- Ciprofloxacin had significant antibacterial effects on *Staphylococcus aureus*, *Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.
- The antibacterial effect of *Indigofera daleoides* 62% v/v tinctures was not significantly superior to that of 62% ethanol on *Staphylococcus aureus,*

Pseudomonas aeruginosa, *Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.

- The antibacterial effect of *Indigofera daleoides* 62% v/v tinctures was not significantly superior to that of 62% ethanol on *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.
- Ciprofloxacin was significantly superior in antibacterial effects compared to *Indigofera daleoides* 62% v/v on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.
- Ciprofloxacin was significantly superior in antibacterial effects compared to *Indigofera daleoides* 62% v/v on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.

There were also little differences between the potency results of the two methodologies. With Well Diffusion Assay showing slightly greater sensitivity of the selected of panel to the *Indigofera daleoides* tinctures and ciprofloxacin, while ethanol showed slightly greater antibacterial in effect on the Disc Diffusion method.

6.2 Recommendations

In light of the results obtained from this study the researcher makes the following recommendations:

- The standard herbal extract be used (1:2 & 1:5) in future studies.
- Antimicrobial effect of *Indigofera daleoides* should be tested against other strains of bacteria.
- *In vivo* testing of the effects of *Indigofera daleoides.*
- Future studies should consider other variations of the types of tests applied e.g. Agar Dilution tests.
- Active ingredients in *Indigofera daleoides* be isolated and tested against a selected panel of bacteria.

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Appendices

Appendix A:

Preparation of plant extracts by German Pharmacopeia Homoeopathic Method 4a

- Plant material is milled using a hammer mill with 1mm sieve size.
- The milled material is added to the solvent (62% v/v ethanol) in a ratio of 1 part dried materials to 10 parts solvent.
- The mix will then be 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 (amber glass bottles or stainless steel containers are used)
- The mixture is stirred twice daily (by hand using a paddle, morning and evening).
- Once maceration is complete, the mixture is then poured through a 100% cotton cloth (loomstate) and pressed using a hydraulic press. Pressing is continued until no more liquid comes out.
- The liquid will be obtained and 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 is then discarded.

Appendix B:

Preparation of inoculums and saline cultures

Preparation of the inoculums

- Single colonies obtained from the Durban University of Technology, Steve Biko S10 level 0 Laboratory cultures of each bacteria will be tested and used to inoculate separate Mueller-Hinton agar plates, and allowed to incubate for 24 hours at 37ºC.
- The bacteria will be;
  - *Staphylococcus aureus*
  - *Pseudomonas aeruginosa*
  - *Enterococci faecalis*
  - *Klebselia pneumonia*
  - *Escherichia coli*

Controls
Positive control- Ciprofloxacin (purchased from sigma Aldrich)
Negative control- Ethanol 62% (purchased from Parceval manufacturers)

. Preparation of saline test cultures

- A few colonies from the overnight Mueller- Hinton agar cultures of the selected bacteria will be suspended in 10ml sterile solution (8.5 g/l) and the solution adjusted to 0.5 McFarland Equivalence Turbidity Standard

Appendix C: Well Diffusion Method

- A marker pen will be used to label the side of the agar plate with a number to denote which bacteria were streaked on the plate.
- the marker pen will then be used on the under surface of the agar plates, referring to a particular disc, namely:
  Group A
  1) *Indigofera daleoides* root tincture in 62% ethanol based extract
  2) 62% ethanol
  3) Ciprofloxacin

  Group B

  1) *Indigofera daleoides* stem tincture in 62% ethanol based extract
  2) 62% ethanol
  3) Ciprofloxacin

  Group C

  1) *Indigofera daleoides* leaves tincture in 62% ethanol based extract
  2) 62% ethanol
  3) Ciprofloxacin
- A sterile cotton swab is then 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.
- With a sterile Pasteur pipette a well 3mm in diameter will be punched into the agar surface.
- With sterile forceps filtered paper discs will be distributed over the agar surface.
- All the plate cultures will be inoculated in an inverted position for 24 hours at 37®C.
- Following incubation, the plates will be examined for the presence of growth inhibition, indicated by a clear zone surrounding each disc. The susceptibility of an organism will be determined by the size of this zone.
- The zone diameters will be measured in millimeters using a pair of Vernier calipers to ensure accuracy and recorded in a table for each relevant Group.

Appendix D: Disc Diffusion Method

- A marker pen will be used to label the side of the agar plate with a number to denote which bacteria were streaked on the plate.
- the marker pen will then be used on the under surface of the agar plates, referring to a particular disc, namely:
  Group A
  4) *Indigofera daleoides* root tincture in 62% ethanol based extract
  5) 62% ethanol
  6) Ciprofloxacin

  Group B

  4) *Indigofera daleoides* stem tincture in 62% ethanol based extract
  5) 62% ethanol
  6) Ciprofloxacin

  Group C

  4) *Indigofera daleoides* leaves tincture in 62% ethanol based extract
  5) 62% ethanol
  6) Ciprofloxacin
- A sterile cotton swab is then 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 will be streaked 1st in a horizontal direction and then vertically to ensure a heavy growth over the entire surface.
- With sterile forceps filtered paper discs will be distributed over the agar surface.
- All the plate cultures will be inoculated in an inverted position for 24 hours at 37®C.
- Following incubation, the plates will be examined for the presence of growth inhibition, indicated by a clear zone surrounding each disc. The susceptibility of an organism will be determined by the size of this zone.

Appendix E: Preparation of medicated discs and ethanol only dry discs

Preparation of medicated discs

Preparation of Indigofera daleoides in 62% ethanol base dry discs

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

Preparation of the 62% ethanol only discs

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

Appendix F: Preparation of media and Macfarland standard

The medium of choice will be the Mueller-Hilton agar due to its pH of 7.2 to 7.4. It will be prepared as follows:

- 38g of Mueller-Hinton agar powder will be weighed out.
- The Mueller-Hinton agar powder will be added to 1 liter of distilled water in a screw top flask.
- A magnetic stirrer will be added to aid dissolution.
- Then the mixture will be shaken until well mixed.
- Then the autoclaved at 121ºC for 15 minutes.
- The flask will then be allowed to cool whilst placed on a magnetic stirrer machine. This ensures adequate mixing and prevents the mixture from solidifying.
- Once the flask has been cooled enough to hold, the agar will be poured into the agar plates as follows:
  - The top of the flask will be flamed with a burner before pouring each plate to prevent contamination.
  - Each plate will then be poured to a depth of approximately 4 millimeters.
  - A total of 16 plates will be prepared per bacterium.
  - The plates will then be stacked and allowed to solidify.
- They will then be checked for contamination.

Mcfarland Standard

- A $BaSO_4$ 0.5 McFarland standard may be prepared as follows:
1. A 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.

2. The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standard.

3. The Barium Sulfate suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing and diluting the bacterial inoculum.
These tubes should be tightly sealed and stored in the dark at room temperature.

5. The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer (Lalitha 2004).

Appendix G: Quotation for statistics

Deepak Singh (DUT senior lecture

Faculty of Applied Sciences

Department of Mathematics, Physics & Statistics

Cell: 031 373 5266

Email: singhd@dut.ac.za

May 2016

The cost of your research would be R2500. It will be a complete chapter with tables and graphs, with all the necessary stats.

Deepak Singh

Appendix H (a): Permission Application Letter to use Microbiological Laboratory
HOD: Food and Technology Department

E-1123 Linda Mnomiya Road
Umlazi township
4066

Faculty of Applied Sciences
Department of Biotechnology and Food technology
Head of Department
P.O. BOX 1334
Durban
4000

Dear Dr Swalaha

Permission Application Letter to use the Homoeopathic Day Clinic (HDC)

Thank you for reading this letter. My name is Mr Senzo Mpangase (21008596). I am currently registered for M. Tech. Homoeopathy and I am requesting to use the laboratory at Steve Biko, S10 level 0. My Co-supervisor is Dr S.K.K Pillai, a senior lecturer in your department. The tittle of my study is: An *in-vitro* study of the antimicrobial effect of *Indigofera daleoides* plant tinctures using disc diffusion and well diffusion assay.

Outline of the Procedures: The experimental study will take approximately a week to be conducted of which only a few hours will be used each day. Namely for make pure cultures of bacteria; medicated discs and to perform diffusion tests (well & disc). Everything will be done under the supervision of the lab technician or Co-supervisor.

Yours faithfully.

Mr S.H. Mpangase (21008596)-Researcher 081 8414 731

Dr. D. Naude (Supervisor) – 031 373 2514 (David@dut.ac.za)

Dr. S. K.K Pillai (Co-Supervisor)- 031 373 5329 (Santhoshk@dut.ac.za)

Appendix H (b): Permission Application Letter to use Steve biko microbiological laboratory
Director: Research and Postgraduate Support

E-1123 Linda
Mnomiya road
Umlazi township
4066

Director: Research and Postgraduate Support
Tromso Annex, 1st Floor
Gate 1, Steve Biko Campus
P.O. BOX 1334
Durban
4000

Dear Professor Moyo

Permission Application Letter to use the DUT facility and staff

Thank you for reading this letter. My name is Mr Senzo Mpangase (21008596). I am currently registered for M. Tech. Homoeopathy and I am requesting to use the microbiological laboratory at Steve Biko S10 level 0. The tittle of my study is: An *in-vitro* study of the antimicrobial effect of *Indigofera daleoides* plant tinctures using disc diffusion and well diffusion assay

Outline of the Procedures: The experiment will take approximately a week to be conducted of which only a few hours will be used each day. Namely for make pure cultures of bacteria; medicated discs and to perform diffusion tests (well & disc). Everything will be done under the supervision of the lab technician or Co-supervisor.

Yours faithfully.

Mr S.H. Mpangase (21008596)-Researcher 081 8414 731

Dr. D. Naude (Supervisor) – 031 373 2514 (David@dut.ac.za)

Dr. S.K.K. Pillai (Co-Supervisor) – 031 373 5329 (SanthoshK@dut.ac.za)

APPENDIX I

Table 1: Data collection tables of zones of inhibition for *Indigofera daleoides* mother tincture (derived from whole plants) in 62% ethanol on the growth of *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococci faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion assay.

Organisms		*Indigofera daleoides* in 62% ethanol	62% ethanol only	*Ciprofloxacin (+ control)
S. aureus	Trial 1	0	0	23
	Trial 2	0	16	21
	Trial 3	0	0	20
	Average	0	4	21
P. aeruginosa	Trial 1	0	0	40
	Trial 2	0	0	41
	Trial 3	7	11	45
	Average	2	4	42
E. faecalis	Trial 1	0	0	22
	Trial 2	0	0	23
	Trial 3	0	0	23
	Average	0	0	23
K. pneumonia	Trial 1	0	15	42
	Trial 2	0	16	40
	Trial 3	0	11	37
	Average	o	14	40
E. coli	Trial 1	7	11	33
	Trial 2	6	0	31
	Trial 3	6	0	32
	Average	6	4	32

Table 2: Data collection tables of zones of inhibition for *Indigofera daleoides* mother tincture (derived from leaves) in 62% ethanol on the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococci faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.

Organisms		*Indigofera daleoides* in 62% ethanol	62% ethanol only	*Ciprofloxacin (+ control)
S. aureus	Trial 1	7	0	23
	Trial 2	7	16	21
	Trial 3	6	0	20
	Average	7	5	21
P. aeruginosa	Trial 1	7	0	40
	Trial 2	6	0	41
	Trial 3	0	11	45
	Average	4	4	42
E. faecalis	Trial 1	0	0	22
	Trial 2	0	0	23
	Trial 3	0	0	23
	Average	0	0	23
K. pneumonia	Trial 1	0	15	42
	Trial 2	0	16	40
	Trial 3	0	11	37
	Average	0	14	40
E. coli	Trial 1	0	11	33
	Trial 2	0	0	31
	Trial 3	0	0	32
	Average	0	4	32

Table 3: Data collection tables of zones of inhibition for *Indigofera daleoides* mother tincture (derived from roots) in 62% ethanol on the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococci faecalis, Klebsiella pneumonia* and *Escherichia coli* using Disc Diffusion Assay.

Organisms		*Indigofera daleoides* in 62% ethanol	62% ethanol only	*Ciprofloxacin (+ control)
S. aureus	Trial 1	8	0	23
	Trial 2	7	16	21
	Trial 3	7	0	20
	Average	7	5	21
P. aeruginosa	Trial 1	0	0	40
	Trial 2	0	0	41
	Trial 3	0	11	45
	Average	0	4	42
E. faecalis	Trial 1	0	0	22
	Trial 2	0	0	23
	Trial 3	0	0	23
	Average	0	0	23
K. pneumonia	Trial 1	12	15	42
	Trial 2	11	16	40
	Trial 3	0	11	37
	Average	8	14	40
E.coli	Trial 1	0	11	33
	Trial 2	11	0	31
	Trial 3	0	0	32
	Average	4	4	32

Table 4: Data collection tables of zones of inhibition for *Indigofera daleoides* mother tincture (derived from whole plant) in 62% ethanol on the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococci faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.

Organisms		*Indigofera daleoides* in 62% ethanol	62% ethanol only	*Ciprofloxacin (+ control)
S. aureus	Trial 1	13	11	30
	Trial 2	10	0	30
	Trial 3	12	0	28
	Average	12	4	29
P. aeruginosa	Trial 1	11	0	45
	Trial 2	11	12	48
	Trial 3	9	0	50
	Average	10	4	48
E. faecalis	Trial 1	0	0	36
	Trial 2	0	0	29
	Trial 3	0	0	37
	Average	0	0	34
K. pneumonia	Trial 1	0	0	32
	Trial 2	0	0	40
	Trial 3	0	0	37
	Average	0	0	36
E. coli	Trial 1	0	0	36
	Trial 2	0	0	36
	Trial 3	0	0	35
	Average	0	0	36

Table 5: Data collection tables of zones of inhibition for *Indigofera daleoides* mother tincture (derived from leaves) in 62% ethanol on the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococci faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.

Organisms		*Indigofera daleoides* in 62% ethanol	62% ethanol only	*Ciprofloxacin (+ control)
S. aureus	Trial 1	12	11	30
	Trial 2	12	0	30
	Trial 3	13	0	28
	Average	12	4	29
P. aeruginosa	Trial 1	10	0	45
	Trial 2	12	12	48
	Trial 3	12	0	50
	Average	11	4	48
E. faecalis	Trial 1	0	0	36
	Trial 2	0	0	29
	Trial 3	0	0	37
	Average	0	0	34
K. pneumonia	Trial 1	0	0	32
	Trial 2	0	0	40
	Trial 3	0	0	37
	Average	0	0	36
E. coli	Trial 1	0	0	36
	Trial 2	0	0	36
	Trial 3	0	0	35
	Average	0	0	36

Table 6: Data collection tables of zones of inhibition for *Indigofera daleoides* mother tincture (derived from roots) in 62% ethanol on the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococci faecalis, Klebsiella pneumonia* and *Escherichia coli* using Well Diffusion Assay.

Organisms		*Indigofera daleoides* in 62% ethanol	62% ethanol only	*Ciprofloxacin (+ control)
S. aureus	Trial 1	11	11	30
	Trial 2	9	0	30
	Trial 3	0	0	28
	Average	7	4	29
P. aeruginosa	Trial 1	0	0	45
	Trial 2	0	12	48
	Trial 3	0	0	50
	Average	0	4	48
E. faecalis	Trial 1	0	0	36
	Trial 2	0	0	29
	Trial 3	0	0	37
	Average	0	0	34
K. pneumonia	Trial 1	0	0	32
	Trial 2	0	0	40
	Trial 3	0	0	37
	Average	0	0	36
E. coli	Trial 1	10	0	36
	Trial 2	0	0	36
	Trial 3	10	0	35
	Average	7	0	36

Appendix J: Application Letter for increase of research budget

E- 1123 Linda Mnomiya Road
Umlazi Township
4066

Director: Research and Postgraduate Support
Tromso Annex, 1st Floor
Gate 1, Steve Biko Campus
P.O. BOX 1334
Durban

Dear Professor Moyo

Application Letter for increase of research budget

Thank you for reading this letter. My name is Mr Senzo Mpangase (21008596). I am currently registered for M. Tech. Homoeopathy and I am requesting an increase of budget for my research study with an additional amount of **R5000**. Most of my research budget is for statistical analysis and consumables. The tittle of my study is: *An in-vitro* study of the antimicrobial effect of *Indigofera daleoides* plant tinctures using disc diffusion and well diffusion assay

Outline of the Procedures: The experimental study will take place at the Durban University of Technology (DUT), Steve Biko S10 level 0. It will take approximately a week to be conducted of which only a few hours will be used each day. Namely for make pure cultures of bacteria; medicated discs and to perform diffusion tests (well & disc). Everything will be done under the supervision of the lab technician or Co-supervisor.

Yours faithfully.

Mr S.H. Mpangase (21008596)-Researcher 081 8414 731

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