ANALYSIS OF ANTIBACTERIAL ACTIVITY OF *TERMINALIA SERICEA* AND *COMBRETUM IMBERBE* TWO COMBRETACEAE SPECIES FROM NAMIBIA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE IN MICROBIOLOGY

OF

THE UNIVERSITY OF NAMIBIA

BY

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APRIL 2019

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ABSTRACT

The study was conducted to investigate the antibacterial activities of stem and leaves from *Terminalia sericea* and the live and dead bark from *Combretum imberbe*. The aqueous and organic crude extracts of the two plants were tested on Gram-negative bacteria *Escherichia coli*, *Shigella sonnei*, *Serratia marcescens*, *Helicobacter pylori* and Gram-positive bacteria *Enterococcus faecalis*, *Staphylococcus aureus* and *Clostridium perfringens* using the disk diffusion method. All the crude extracts exhibited activity against the tested bacteria species except for extracts from *C. imberbe* (dead bark). Organic extracts had high activity with minimum inhibitory concentration (MIC) values of 0.1mg/ml for all plant parts against *S. aureus* , *S. marcescens*, and *H. pylori* whilst the aqueous extracts from *T. sericea* (stem and leaves) and *C. imberbe* (live bark) had MIC values of 1mg/ml all against *S. marcescens* and *H. pylori*. Fractions from the two plants were obtained by vacuum liquid chromatography (VLC) using different solvents with increasing polarity. The organic extract fractions from both plants were observed to have good activity against two of the seven bacterial species that were tested. The dead bark plant extracts from *C. imberbe* had MIC value of 10mg/ml with 100% Ethyl acetate extracts against the bacteria *H. pylori*. *T. sericea* leaf extracts had MIC value of 0.01mg/ml against *H. pylori* in 100% MeOH fractions whilst the stem extracts had activity at 0.01mg/ml against *E. faecalis* in 100% Acetone fractions. The results indicate that the two plants have good antibacterial activity hence further research is needed to isolate and identify active compounds which can be developed into effective drugs.

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LIST OF ABBREVIATIONS

AMR: Antimicrobial resistance

CDC: Centre for Disease Control and Prevention

CRE: Carbapenem-resistant Enterobacteriaceae

DCM: Dichloromethane

DMSO: Dimethyl sulfoxide

EARS-Net: European Antimicrobial Resistance Surveillance Network

ETEC: Enterotoxigenic *Escherichia coli*

FDA: Food and Drug Administration

MDR: Multiple Drugs Resistant

MeOH Methanol

MET: Ministry of Environment and Tourism

MIC: Minimum inhibitory concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

PPI: Protein pump inhibitor

VLC: Vacuum Liquid Chromatography

VRE: Vancomycin-resistant Enterobacteriaceae

WHO: World Health Organization

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisors Prof. Kazhila. C. Chinsembu and Prof. Marius Hedimbi for their patience, assistance, guidance and unwavering support throughout this study.

I would also like to thank my parents, Mr. and Mrs. Mapiye, for their faith in me and for their support both emotional and financially as no words can be enough for me to express the appreciation I have for their provision, encouragement and guidance from day one, I would not be where I am without them.

Above all else, I would like to thank my Almighty Heavenly Father who has held my hand from the beginning, for guiding and protecting me always and finishing what He started with me just as He promised; God deserves all the praise, Forever.

DEDICATION

I dedicate this research study to my late mother and sister who were my source of joy and inspiration and they shall forever be missed.

DECLARATIONS

I, Mapiye Samson hereby declare that this study is a true reflection of my own research and that this work or part thereof has not been submitted for a degree from any other institution of higher education.

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Mapiye Samson	Signature	Date

CHAPTER 1: INTRODUCTION

1.1 Background

The identification and use of medicinal plants for the maintenance of human health through the treatment of different infections due to their antimicrobial activity date back to prehistoric times (Tapsell et al. 2006). Plants have been identified that can synthesise hundreds of chemical compounds called phytochemicals with potential or established biological activity from the plant extracts displaying antimicrobial activity against all types of microorganisms including Gram positive and Gram negative bacteria (Hu et al. 2016). A single medicinal plant contains several different phytochemicals leading to the uncertainty of using a whole plant as medicine therefore rigorous scientific research to define efficacy and safety of phytochemical content and antimicrobial actions of many plants are required (Ahn 2017).

Globally, the absence of regulation on traditional medicine use caused the World Health Organization (WHO) to set up networks that monitor and encourage safe medicinal plants use (WHO 2014). In Namibia, medicinal plants are extensively used due to their availability, ease of access and are less expensive than modern medicines especially in rural communities which have made traditional medicinal practice as the only option in combating diseases (Iikasha 2016). Medicinal plants face both general threats, such as climate change and habitat destruction, and the specific threat of over collection to meet market demand. This is due to an increase in resistant microbial strains and a decline in the number of new effective antibiotic classes developed leading to people seeking alternative medicines (Marshall and Levy 2011).

1.1.1 Antimicrobial resistance

The discovery of antimicrobial drugs brought about a medical revolution were life threatening infections could be simply and effectively treated leading to the extension of the average human life span over the past decades (Van Boeckel et al. 2014). Without antimicrobials there will be no intensive care units were half of emergence cases of people treated suffer from microbial infections each year (Van Boeckel et al. 2014). The problem is the emergence of microbial infections that are rapidly becoming resistant to all antimicrobials due to overuse of these medical resources leading to loss in potency of the drugs (Laxminarayan et al. 2013).

The medical community and researchers are now locked in an arms race with microbes were on one hand antimicrobials in the health sector and agriculture industry have been spilled into the ecosystem for decades and on the other hand microbes have an infinite capacity to evolve and adapt in this environment (Penesyan et al. 2015). The development of each new antimicrobial drug has been followed by the detection of resistance to it (Laxminarayan et al. 2013). The development of resistance is a normal evolutionary process for microorganisms, but it is accelerated by the selective pressure exerted by widespread use of antimicrobial drugs. Resistant microbial strains are able to propagate and spread where there is non-compliance with infection, prevention and control measures (O'Neill 2014). In the last decades the number of new antimicrobial classes developed has declined whereas the amount of emerging and re-emerging resistant bacterial strains is increasing (Penesyan et al. 2015).

In the past, antibiotic resistance was associated with people with a weak immune system but now the bacterial infections are also occurring in healthy people (Laxminarayan et al. 2013). In a world where bacteria are increasing resistance to medicines there is an urgent need to turn to new and alternative medicines.

1.2 Statement of the problem

There is an increase in prevalence of resistant and multidrug-resistant bacterial strains to antimicrobials in Namibia due to the misuse of these drugs (Chinsembu 2016). This has been worsened by the decrease in potency of available antimicrobial drugs requiring the use of high doses and more toxic drug combinations which have adverse side effects and are also very expensive. This has further increased the burden of combating bacterial infections on the Namibian health system (Iikasha 2016).

1.3 Objectives

The objectives of the study were:

a) To evaluate the inhibitory activity of *T. sericea* and *C. imberbe* crude extracts on different bacterial strains.
b) To evaluate the antibacterial activity of fractionated *T. sericea* and *C. imberbe* extracts.

1.4 Significance of the study

This study serves to provide validity on the use of *T. sericea* and *C. imberbe* as alternative source of compounds to combat bacterial infections. In this study, the plant extracts of *T. sericea* and *C. imberbe* were evaluated for their antimicrobial activity against several bacterial strains. Findings of the study can be used to provide insight in future research toward the development of antimicrobial agents from selected plants. Medicinal plants unique to Namibia may be an important resource for drug discovery of new antibacterial compounds and offers potentially low-cost source of antimicrobials which can be used as complimentary or alternative medicine.

1.5 Limitations of the study

This study was limited to testing antibacterial activity of crude extracts.

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal plants

There is a long history on the use of medicinal plants as an abundant source of antimicrobial therapies for the treatment of various diseases and symptoms. Indigenous medicine remains the most important form of treatment which is culturally accepted and practiced by diverse traditional health systems (Itokawa et al. 2008).

The use of medicinal plants in third world countries such as Namibia contributes significantly to primary health care. According to the WHO (1993), 80 percent of the global population depends on traditional therapies which involve the use of active constituents from plant extracts (WHO 1993). Developing countries depend on plants as the primary source of medicine where they are easily accessible and affordable sources for traditional medication playing a major role in primary health care (Zakaria 1991). This is also due to the readily available plant-based products from different parts of the plant that have been known to have lesser or no side effects which makes them safer options as medicinal drugs (Itokawa et al. 2008; Kapewangolo 2013).

In recent years the use of herbs for treatment has increased noticeably as a result of continued funding for this kind of research and more recognition of traditional medicines in formal medicine (Kapewangolo 2013). This is due to the adverse side effects and undesirable health risks on hosts such as hypersensitivity, depletion of the beneficial gut and mucosal microorganisms, immunosuppression, allergic reactions and derangement of oral and intestinal bacterial flora that are caused by antimicrobial drugs (Thomas 2010). There is now a worldwide trend to go back to natural

resources, mainly traditional plants which are both culturally acceptable and economically viable (Thomas 2010). Medicinal plants extracts are used to treat several bacterial infections as they have very high potent antimicrobial activity which have lesser to no side effects and there is an urgent need to evaluate active molecules derived from medicinal plants which are identified as possible bioactive antimicrobial compounds. This is due to the rising threat of rapidly emerging MDR resistant bacterial strains which are heavily burdening the community and health system (Itokawa et al. 2008).

There is evidence that suggests that most traditional medicines are effective against pathogenic microorganisms but cannot be integrated into the current health system due to safety and efficacy concerns (Mohammed 2009). Plants generally have a wide range of phytochemicals and increase in dosages of the herbal remedies may harm the body, therefore, development of medicinal plants that are safe and efficient requires rigorous scientific critique and standardization (Street and Van Staden 2009).

2.2 Antimicrobial ethno medicinal plants used in Namibia

There is a long tradition of using plants in the treatment of infectious disease in Namibia where medicinal plants are used to relieve ailments caused by microbial infections (Chinsembu and Hedimbi 2010; Auala et al. 2012). Since 80 % of the world population relies on traditional medicine for therapy; Namibian medicinal plants may be an important resource for drug discovery of new antimicrobial compounds (Mahomoodally 2013; Pan et al. 2014). A study done by Chieckyouseff et al (2011), found that 36.4 % of 753 plants in Oshikoto region were used for medicinal purposes such as diarrhoea, skin infections, and oral hygiene mainly due to

their proximity, availability and reliability. Studies done by Chinsembu and Hedimbi (2010), revealed that several plant species in the northern region of Namibia were used to relieve symptoms of bacterial, fungal and viral infections as shown in the table 1 below.

Table 1: Ethnobotanical data of plants with antibacterial activity.

Plant species	**Part**	**Traditional use**	**References**
A. digitata	Seeds, pulp and leaves.	Dysentery, asthma and diarrhoea.	(Van Wyk and Gericke 2000).
A. indica	Leaves, stem, seeds and flower.	Malaria and respiratory diseases.	(Nadeem et al. 2013).
H. petersiana	Palm fruit, seeds, and sap	Malaria, cough, tuberculosis, sores related to STIs.	(Chinsembu 2016).
M. cerviana	Roots	STIs, fever, and burning urination.	(Malik et al. 2015).
P. africanum	Leaves, stem, roots	Antibacterial, anti-HIV, antioxidant, And anti-helminthic activities.	(Mazimba 2014).
D. lycioides	Twigs,	Antibacterial oral cariogenic	(Cai et al. 2000).

		microbes and periodontal pathogens.	

2.3 Medicinal use of Combretaceae species in Namibia

The Combretaceae family belongs to the order Myrtales and species from the genus *Combretum*, and to a lesser extent *Terminalia*, are most widely used for medicinal purposes (McGaw et al. 2001). Their fruits are avoided due to their toxicity whilst leaves, roots, stem and, barks are commonly used for medicine as shown in **Figure 1** (Rogers and Verotta 1996; Chinsembu et al. 2015). Traditional healers use plants in this family for various medicinal purposes that include treatment of abdominal disorders, diarrhoea, pneumonia, syphilis, toothache, gastric ulcers, venereal diseases, heart diseases, cleansing the urinary system, dysentery, gallstones, sore throats, and general weakness (Hutchings et al. 1996; van Wyk et al.1997; McGaw et al. 2001).

In Namibia, Combretaceae family particularly the two genera *Combretum* and *Terminalia* are used in the northern regions for treating viral, fungal and bacterial infections and offset side effects caused by antimicrobial medications (Chinsembu and Hedimbi 2010). *C. imberbe* shown in figure 2 below, is traditionally used for the treatment of diarrhoea and cough, symptoms which can be related to bacterial, fungal or viral infections. *T. sericea* has a wide range of uses in traditional medicine were decoctions of the roots of this plant are used to treat sexually transmitted infections such as gonorrhoea, syphilis and treatment of diarrhoea (Chinsembu et al. 2015).

Figure 1: The stem and leaves of *Terminalia sericea*.

T. sericea in figure 1 above, is used in traditional medicine where the infusion from boiled roots and leaves can be taken orally as a remedy for diarrhoea, coughs and stomach ache (Masupa 2012). Fyhrquist et al. (2014) described that hot decoctions of *T. sericea* are used in the management of gonorrhoea. *Terminalia* species possess ellagitannins, potent chemical ingredients with antimicrobial efficacy against multi-antibiotic-resistant *N. gonorrhoeae* (Semenya et al. 2013).

Figure 2: Bark of *Combretum imberbe*.

Combretum species are used in traditional medicine to treat infectious diseases including diarrhoea and many other ailments by rural people in Africa and most species are indigenous to Namibia as shown in **Table 2** (Chinsembu & Hedimbi 2010).

Table 2: Combretaceae family used for treating different bacterial infections (Chinsembu & Hedimbi 2010; Chinsembu et al. 2015).

Combretaceae family	**Local name**	**Plant part used**	**Bacterial infection treated**	**Preparation and administration**
Combretum apiculatum	Mukalanga	Shrub, leaves	General STI syndromes	Boil leaves in water, and drink warm solution
Combretum hereroense	Mububu	Shrub, leaves	Gonorrhoea, Chlamydia symptoms in men	Crush leaves, suspend in water, and drink cold infusion.
Combretum imberbe	Muzwili	Tree, leaves	General STI infections	Crush leaves, suspend in water, and drink infusion.
Combretum collinum		Leaves	Chronic diarrhoea	Drinking.
Combretum micranthum,		Leaves	Malaria, diarrhoea	Steaming, drinking
Combretum alaeagnoides	Pampa	Bark	Tuberculosis, Diarrhoea	Drinking
Combretum mossambicense.	Silutombolwa,	Climber, whole plant	Gonorrhoea, syphilis;	Cut into small pieces, pound, put in water, and drink cold infusion
Terminalia sericea	Mukenge	Tree, roots, leaves	Gonorrhoea, syphilis and meningitis.	Macerate materials together, boil in water, and drink decoction
Terminalia mollis	Muhonono	Bark, roots	Tuberculosis	Drinking

2.4 Secondary metabolites

There are two types of chemicals that are found in plants known as primary and secondary metabolites. Primary metabolites such as amino acids, nucleic acids, lipids, and simple sugars are compounds that are necessary for cellular processes (Kurmukov 2013). Secondary metabolites are referred to as natural products of the plant produced in response to abiotic and biotic factors such as heat, drought, herbivores, pathogens, and humans which induce stress on the plant (Zwenger and Basu 2008). These secondary metabolites are found in different plant parts such as leaves, roots, barks, and stems, and they are known to possess different properties that provide health benefits for humans they include alkaloids, terpenes, flavonoids, lignans, steroids, curcumins, saponins, phenolics and glycosides (Batista et al. 2009).

2.4.1 Alkaloids

These are one of the largest single class of plant secondary metabolites found in almost 20% of all plant species which have pharmacological activity such as central nervous stimulant (e.g. brucine), central nervous depressant (e.g. morphine), antihypertensive (e.g. reserpine), anticholinergic (e.g. atropine), antiemetic (e.g. scopolamine), antitumor (e.g. vinblastine), antifungal (e.g. dicentrine) and antimalarial (e.g. quinine) activities (Evans 2009). Many alkaloids have been used as narcotics, stimulants, commercially as pharmaceuticals and in sufficient amounts, these compounds are poisonous to livestock, herbivores, and humans. In plants, alkaloids are used to inhibit the metabolism of glycosidase and trehalose to deter herbivores and protect plants against infection by acting as phytoanticipins and phytoalexins (Evans 2009). Many have found use in traditional medicine such as cinchona bark which is rich in quinine for malaria treatment or vinblastine in the treatment of cancer in modern medicine (Wangun et al. 2007; Robbers et al 1996).

Previous studies have revealed the antibacterial activity of the alkaloids chelerythrine, hapalindole and prosopilosidine which were nonhaemolytic or nontoxic to Vero cells and the effectiveness of squalamine against *Pseudomonas aeruginosa* pneumonia (Cushnie et al. 2014). The combination of alkyl methyl quinolone alkaloids 1-methyl-2[(Z)-7-tridecenyl]-4-(1H)-quinolone and 1-methyl-2[(Z)-8-tridecenyl]-4- (1H)-quinolone have demonstrated in vivo efficacy against *H. pylori* infection with MICs of 0.02 to 0.05 µg/ml (Chopra 2012). Different alkaloids with antimicrobial activity are depicted in **Figure 3** below (Vince and Zoltán 2011).

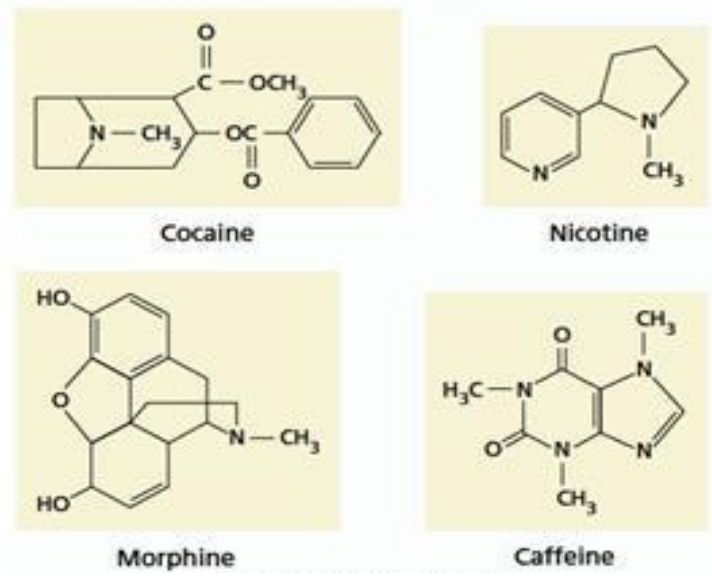


Figure 3: Examples of alkaloids isolated from plants.

2.4.2 Anthraquinones

These are derived from anthracene a compound found in the nucleus of anthraquinone compounds that have antimicrobial properties beneficial to human health (Kazmi et al. 1994). These have a great potential range on antimicrobial effects due to their ability to irreversibly bind to nucleophilic amino acids in proteins

by inactivating them leading to loss of function and in addition by providing a source of stable free radicals (Stern et al. 1996). *Cassia italica* tree which has anthraquinone is known to be bactericidal for *Pseudomonas pseudomalliae* and bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum* and *Pseudomonas aeruginosa* (Kazmi et al. 1994). Anthraquinones possess certain therapeutic properties including laxative, purgative and anti-inflammatory due to aldehyde group at C-2 as shown in **Figure 4** below (Kar 2008).

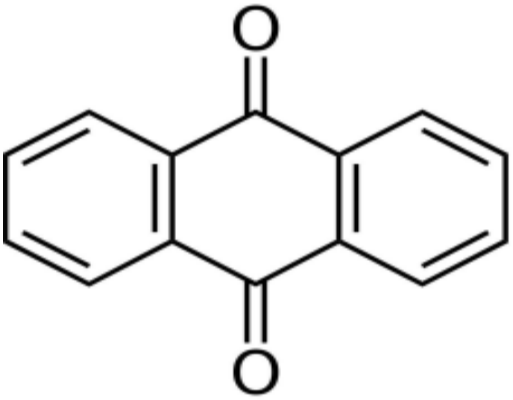


Figure 4: Structure of anthraquinone.

2.4.3 Flavonoids

These are phenolic structures containing one carbonyl group that are further divided into sub-groups such as flavones, flavonols, flavanones, chalcones, anthocyanins and isoflavones which are major classes shown in **Figure 5** below (Brielmann et al. 1999). They are synthesized by plants in response to microbial infection, therefore they have been found to be effective antimicrobial substances against a wide range of microorganisms (Borris 1996; Brielmann et al. 1999). The most reduced form of flavonoid compounds found in green teas are called catechins and they have

antimicrobial activity that includes inhibition of *Shigella spp.* and cholera toxin in *Vibrio cholerae* in vitro (Borris 1996). Catechins are also able to inactivate isolated bacterial glucosyltransferases in *Streptococcus mutans*, and where observed in reduction of fissure carries by 40% in rats which were fed a diet containing 0.1% tea catechins (Borris 1996).

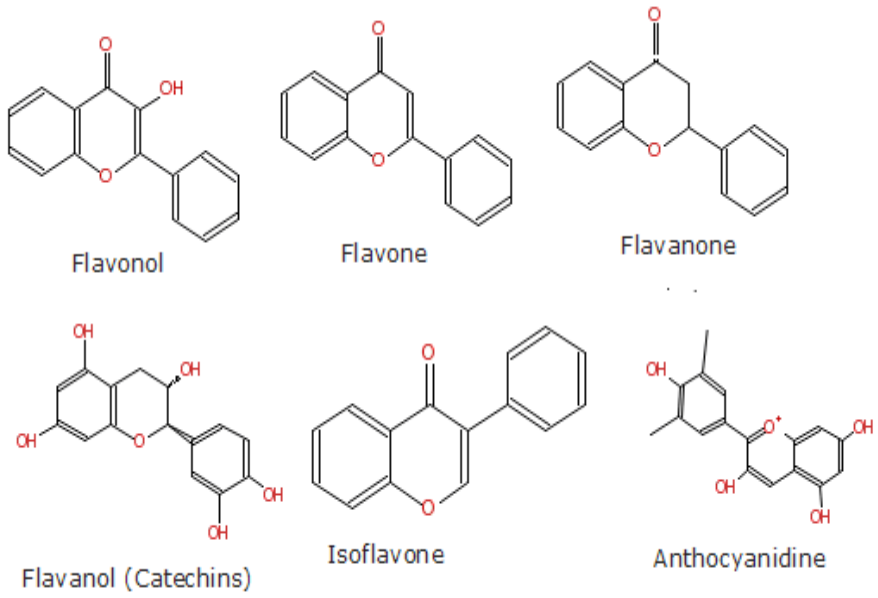


Figure 5: Major classes of flavonoids.

2.4.4 Terpenoids

These are oxygenated terpenes which are isoprene structures that carry the fragrance of plants and have antimicrobial properties due an internal peroxide link as shown in **Figure 6** below (Kar 2008). Terpenoids share their origins with fatty acids as they are synthesized from acetate units due to their extensive branching and cyclic structures. Terpenoids are active against bacteria e.g. capsaicin is bactericidal to *Helicobacter pylori* and ethanol-soluble fractions of a terpenoid called petalostemumol derived from the plant purple prairie clover showed excellent

activity against *B. subtilis* and *S. aureus* (Batista et al. 2009). Residents of Mali were observed to use the bark of a tree called *Ptelopsis suberosa* for the treatment of gastric ulcers and when terpenoid-containing fractions were tested on rats they were found to diminish and prevent severity of existing ulcers while a diterpene called trichorabdal A from a Japanese herb, directly inhibited *H. pylori* (Kadota et al.1997).

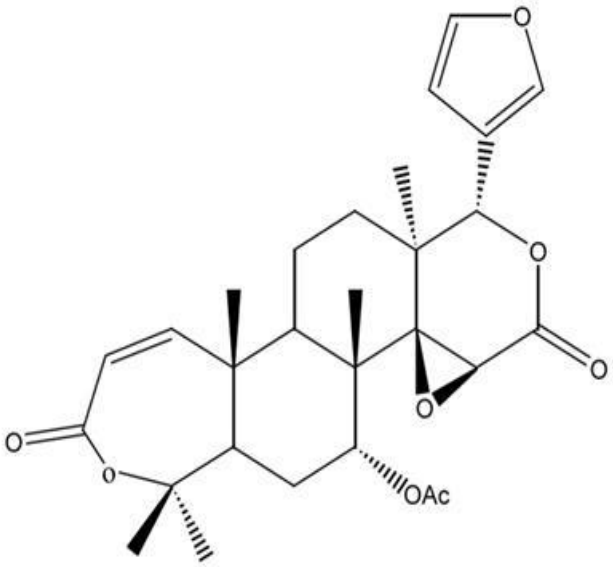


Figure 6: Terpenoid structure

2.4.5 Lectins

These are positively charged peptides that contain disulphide bonds. Their antimicrobial activity involves formation of ion channels in the microbial membrane and they competitively inhibit the adhesion of microbial proteins to host polysaccharide receptors (Zhang and Lewis 1997). Fabatin, a 47-residue peptide from fava beans, which is structurally related to thions from grains, inhibits *E. coli*, *P. aeruginosa*, and *Enterococcus hirae* (Zhang and Lewis 1997).

2.4.6 Tannins

They are found in roots, bark, wood, and fruits of many plants and they are commonly used in traditional medicine as anti-inflammatory, antioxidant and antimicrobial agents (Piwowarski et al. 2014). The antimicrobial activity of tannins involves mechanisms such as inhibition of microbial enzymes, and deprivation of substrates (Trentin et al. 2013). Extracts of some plants used in Brazilian traditional medicine, rich in proanthocyanidins and hydrolysable tannins induced morphological changes in *P. aeruginosa*, by preventing formation of biofilm through inhibition of bacterial adhesion (Trentin et al. 2013).

Hydrolysable tannins are secondary metabolites belonging to the family of vegetable tannins which are water soluble and have the ability to precipitate alkaloids and protein (Da Silva et al. 2017). The antibacterial activity of hydrolysable tannins have been shown to impede the growth of both MRSA and methicillin-sensitive (MSSA) strains of *S. aureus* (Da Silva et al. 2017). Aqueous extracts of hydrolysable tannins were shown to decrease the virulence of *H. pylori* by reducing cell surface hydrophobicity by provoking rapid cell aggregation (Liu et al. 2012). Hydrolysable tannins where observed to have antimicrobial activity against *S. sonnei*, *E.coli*, and *P. aeruginosa* by inhibiting bacteria cell adherence due to modification of cell surface receptors and suppression of glucan synthesis (Bennet and Bentley 2000).

2.5 Antimicrobial use

For more than 70 years, antimicrobial drugs have been regarded as the panacea to cure infections. Antimicrobial resistance is recognized as one of the greatest threats to human health worldwide that are rapidly outpacing available treatment options resulting in accelerated threats to global health security (WHO 2014). The difference in antimicrobial resistance problems in individual countries depends on how heavily they use antimicrobial drugs, with global consumption rising to 40% from 2000 to 2010 as shown in **Figure 7** below (Van Boeckel et al. 2014). This is usually worsened by large quantities of counterfeit and sub-standard antimicrobials permeating the pharmaceutical markets in some countries and regions especially in Africa and Asia.

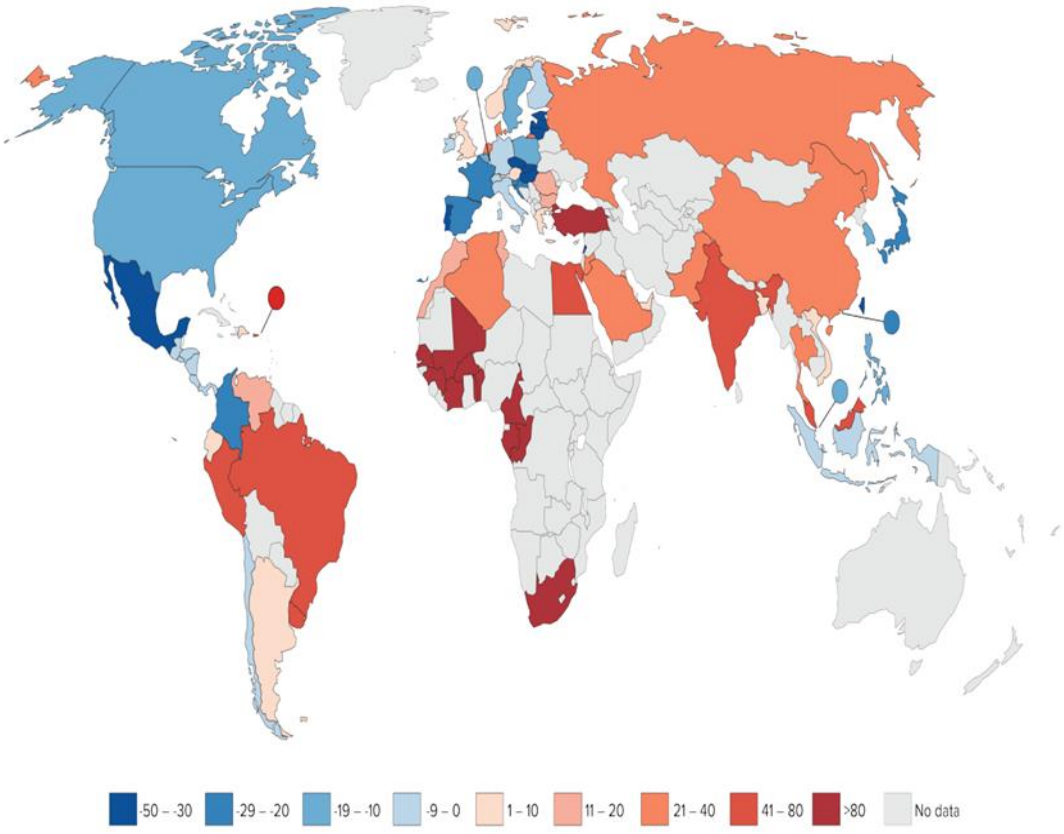


Figure 7: Global percentage change in antimicrobial consumption per capita 2000-2010 by country (Van Boeckel et al. 2014).

Between 2000 and 2010, total global antibiotic consumption grew by more than 30 percent, from approximately 50 billion to 70 billion standard units, based on data from 71 countries as shown in **Figure 7** above (Van Boeckel et al. 2014). Antimicrobial resistance is a global issue as no country can successfully tackle this problem alone due to the opportunities presented for the spread of antimicrobial pathogens by the speed and volume of intercontinental travel (Hayakawa et al. 2013).

2.5.1 Antimicrobial resistant bacteria

Evidence from around the world indicates an overall increase in resistance to all first-line and last-resort antimicrobials with a decline in the total stock of antibiotic effectiveness (WHO 2014). The Centers for Disease Control and Prevention (CDC) in the United States estimates that resistance to antimicrobials causes more than 2 million infections and 23,000 deaths each year with a direct cost of $20 billion (CDC 2013). In the developing world, it is estimated that 58,000 neonatal sepsis deaths are attributable to drug-resistant infections in India alone (Laxminarayan et al. 2013). Studies from Tanzania and Mozambique indicate that resistant bacterial infections result in increased mortality in neonates and children under five (Roca et al. 2008; Kayange et al. 2010).

2.5.2 Global burden

Antimicrobial resistance is recognised as one of the greatest threat to human health worldwide causing a low estimate of 700 000 deaths globally every year which is projected to reach 10 million deaths by 2050 (WHO 2014). Antimicrobial resistant bacteria infections claim at least 50 000 lives each year across Europe and the US alone with hundreds of thousands more dying in other areas of the world as reliable estimates of the true data are scarce (CDC 2013).

According to the WHO, global antimicrobial resistance estimates; *E. coli*, and *S. aureus* are listed as agents of greatest concern, associated with both hospital and community-acquired infections (WHO 2014). Some countries reported resistance of more than 50 percent to fluoroquinolones and third-generation cephalosporins from *E. coli* (WHO 2014).

H. pylori are mainly responsible for chronic bowel diseases and affect more than half of the population worldwide which makes it one of the most common resistant chronic bacterial infections around the world (Boyanova et al. 2014). Increase in *H. pylori* resistance to clarithromycin and levofloxacin (mainly in developed countries) and to metronidazole (mostly in developing countries) has hindered successful eradication of the infection (Mentis et al. 2015).

2.5.3 Burden in Africa

In Africa, it is estimated that by 2050 antimicrobial resistance will cause 4.15 million deaths every year (WHO 2014). This is due to severe poverty which leads to

malnutrition, inadequate sanitation and lack of clean drinking water which exacerbates the burden of antimicrobial infections on the health systems. According to the WHO (2014), 66% of deaths in children under the age of five are caused by infectious diseases from drug-resistant microbial agents. In rural sub-Saharan Africa, community-acquired bacteraemia is a major cause of death for infants younger than 60 days where *E. coli*, *S. aureus* and streptococcus B are the predominant infectious agents (Berkley et al. 2005). Besides these gloomy statistics, evidence suggests that not much is being done to fight bacterial resistance due to limited resource settings and weak laboratory capacities coupled with little to no surveillance systems (WHO 2014)

2.5.4 Burden in Namibia

Morbidity and mortality that are caused by drug-resistant microbial infections are a major concern in developing countries such as Namibia. This is worsened by lack of surveillance data for antibiotic use and antimicrobial resistance due to poor laboratory infrastructure and a weak to no surveillance system. A study done by Pereko et al. (2015), revealed that the prescription of first-line antimicrobials by doctors was not in accordance with the Namibian treatment guidelines for local and regional antimicrobial sensitivity data due to lack of information.

2.6 Pathogenic bacteria

2.6.1 *Helicobacter pylori*

H. pylori are spiral-shaped bacteria that are present in 50% of the global population which grow in the digestive tract and have a tendency to attack the stomach lining causing peptic ulcers and gastritis in the stomach and the duodenum (Ghotaslou et al.

2015). The risk of *H. pylori* infection is high in children due to lack of proper hygiene and when sharing housing with others infected with the bacteria in overcrowded conditions (Ghotaslou et al. 2015). The most common route of transmission of *H. pylori* from one person to another is through oral-oral, faecal-oral or gastro-oral exposure and 85% of people infected with the bacteria never experience symptoms (Bytzer et al. 2011).

The colonisation of the stomach by *H. pylori* begins with the movement of the bacteria from the acidic layer in the lumen of the stomach using flagella to burrow into the mucus lining and adhere to the stomach epithelial cells using lipopolysaccharides as shown in **Figure 8** below (Amieva et al. 2008). *H. pylori* survive the acidic conditions in the stomach by using its enzyme urease to convert urea to carbon dioxide and ammonia which is alkaline (Ghotaslou et al. 2015). *H. pylori* will then secrete toxins such as vacuolating cytotoxin A (VacA) which induces cell apoptosis and cytotoxin-associated gene CagA which disrupts the tight junctions of the epithelial cells stimulating cytokines such as interleukin-8 which attracts neutrophils promoting inflammation of the stomach tissue (Amieva et al. 2008). This allows the hydrochloric acid to damage the stomach lumen layer causing the formation of ulcers. Inflammation causes excess production of hydrochloric acid due to histamine production and this leads to hydrochloric acid to enter the duodenum also causing ulcers (Ghotaslou et al. 2015).

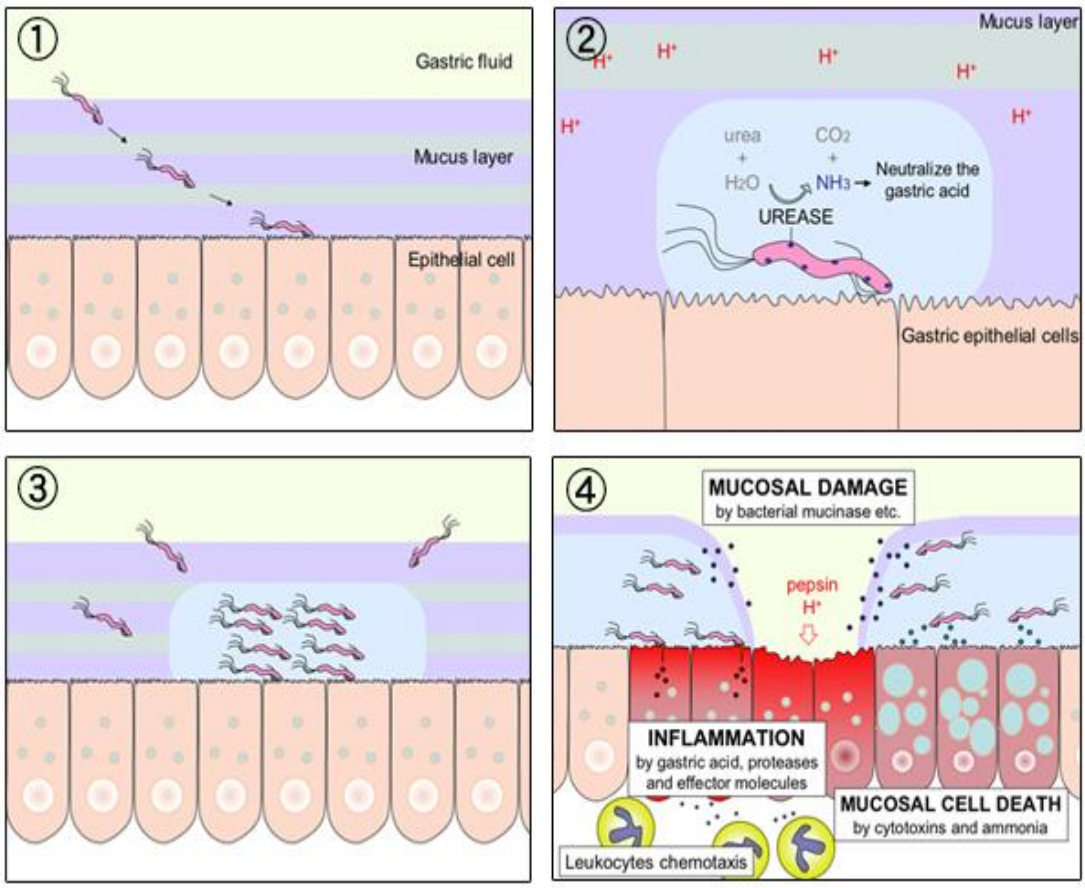


Figure 8*:* *Helicobacter pylori* infection and ulcer production begins by: (1) movement of bacteria from the acidic layer to the mucus lining, (2) adherence to the stomach epithelial cells, (3) enzyme urease neutralises the gastric acid, and (4) cell apoptosis and inflammation of stomach tissue leading to damage from hydrochloric acid causing ulcer formation (Ghotaslou et al. 2015).

Once *H. pylori* is detected in a person with a peptic ulcer, the standard first-line therapy is a one-week "triple therapy" which consists of proton pump inhibitors (PPI) such as omeprazole and the antimicrobials clarithromycin and amoxicillin (Malfertheiner et al. 2012). According to the Kyoto Global Consensus Meeting, only regimens with a 90% suppression rate of *H. pylori* should be used as an empiric therapy and focus should be on designing a regimen which results in a cure rate that at least approaches 100% (Sugano et al. 2015).

Therapy against *H. pylori* has turned out to be more difficult over the years, principally due to the great decrease of standard eradication therapies efficacy and an increase in antibiotic resistance by *H. pylori* due to increase in prescription drugs globally sown in **Table 3** below (Ghotaslou et al. 2015). Additionally, a study done in Japan by Kobayashi et al. (2007) revealed that there was an increase in clarithromycin resistance rates from 18% to 27% within three years. In Europe, countries demonstrated *H. pylori* resistance rates to clarithromycin, metronidazole, and levofloxacin at 17.5%, 34.9% and 14.1% respectively (Megraud et al. 2013). *H. pylori* resistance to antimicrobials is widely recognized as the main reason for treatment failure (Di Mario et al. 2006).

Table 3: Resistant antimicrobial agents in *H. pylori* (Ghotaslou et al. 2015).

Antibiotic	**Mode of action**	**Resistance mechanism**
Metronidazole	DNA damage by radicals	Increased drug efflux, enhanced activity of DNA repair enzyme.
Clarithromycin	Inhibit protein syntheses	rRNA point mutations
Amoxicillin	Inhibit cell wall synthesis	pbp gene mutations and efflux pumps.
Tetracycline	Inhibit protein synthesis	Nucleotides mutation in the 16S rRNA gene.
Fluoroquinolones	Inhibiting DNA gyrase and	point mutations resistance

	Topoisomerase II and IV	
Rifabutin	Inhibits the b-subunit of DNA-dependent RNA polymerase encoded by the rpoB gene	Mutation of the rpoB gene

In the developing world, *H. pylori* in infected individuals were found to be resistant to antimicrobials resulting in treatment failure and this is further exacerbated by the poor living conditions. A study done by Wang et al. (2004) revealed that ingesting lactic acid bacteria from sour milk products, such as koumiss, laban, yogurt, kefir, and some cottage cheeses has a suppressive effect on *H. pylori* infection. Broccoli and cauliflower have sulforaphane a compound which suppresses and may help to eradicate *H. pylori* infection (Moon et al. 2010).

2.6.2 *Staphylococcus aureus*

Globally, 2 billion people are estimated to carry some form of *S. aureus* and up to 53 million are thought to carry methicillin-resistant *S. aureus* (MRSA) (WHO 2014). Very high rates of MRSA are reported in East Asia and South America with prevalence rates above 50% whilst in Africa surveillance data is lacking for most countries with South Africa having high prevalence rates of 20-50% as shown in **Figure 9** (CDDEP 2015). The Namibian Institute of Pathology (NIP) isolated and performed susceptibility test on 600 *S. aureus* strains and they revealed that 92.4% were resistant to penicillin and 44.9% to cotrimoxazole and that the isolates were

found to be resistant to vancomycin 0%, and ciprofloxacin (4.4%) whilst resistance to methicillin was observed at 13.5% (Iileka et al. 2016).

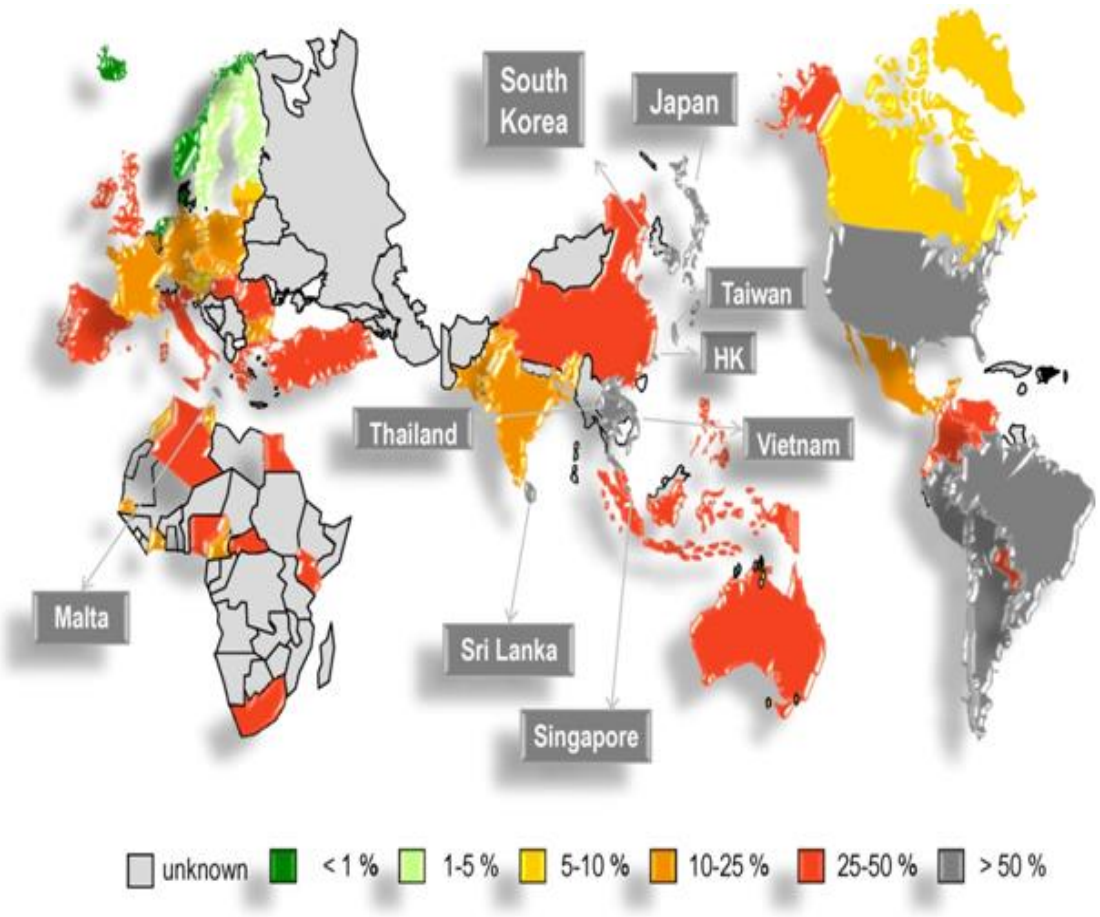


Figure 9: Worldwide prevalence of MRSA (CDDEP 2015).

Global surveillance has shown that MRSA represents a problem in all continents and countries where studies have been carried out (CDDEP 2015). *S. aureus* can easily become resistant to antimicrobials and produce many MDR strains which can acquire resistance to many structurally unrelated antimicrobials as shown in **Table 4** (Brown and Ngeno 2007). Many MRSA infections occur in hospitals and healthcare facilities and these are known as healthcare-acquired MRSA (HA-MRSA). The rates of

MRSA infection have been shown to increase in hospitalised patients who are treated with quinolones (CDC 2013). Healthcare provider-to-patient transmission is common, especially when healthcare providers move from patient to patient without performing necessary hygiene techniques such as hand-washing between patients (Tacconelli et al. 2008). Community-acquired MRSA (CA-MRSA) strains infect healthy people who had not been in contact with health care facilities and are more easily treated and less virulent than HA-MRSA (Calfee 2011). One possible contribution to the increased spread of MRSA infections comes from the non-medicinal use of antimicrobials such as in intensive farming for immunity and promotion of growth (Stefani et al. 2012).

Table 4: Mechanisms of resistance in *S. aureus* (Brown and Ngeno 2007).

Antibiotic	**Genetic Basis**	**Resistance mechanism**
Penicillin	blaZ gene	Hydrolyses the β-lactam ring
Methicillin	*mec*A gene	PBP2a active site blocks binding of all β-lactams.
Quinolones	NorA	Active efflux.
Vancomycin	*van*A operon	VRSA – modified target.
Linezolid	*cfr* gene	Methylation of ribosome
Fusidic acid	*fus*A gene	Horizontal acquisition of *fus*B gene inhibitory action of fusidic acid.
Daptomycin	*mprF* gene mutation	Decreased drug binding.

Methicillin resistance is conferred by the *mec*A gene, which encodes a penicillin-binding protein (PBP2A) with decreased affinity for *β*-lactam antimicrobials. PBP2a differs from other PBPs in that its active site blocks binding of all β-lactams but

allows the transpeptidation reaction to proceed (Lim and Strynadka 2002). MRSA has demonstrated a unique ability to rapidly respond to each new class of antimicrobials with the development of a resistance mechanism, starting with penicillin and methicillin, to vancomycin, fusidic acid, and teicoplanin, and until most recent to linezolid and daptomycin (Bayer et al. 2013). MRSA has a high capacity to adapt to different environmental conditions which make it the leading overall cause of nosocomial infections. MRSA has been able to acquire antimicrobial resistance by either drug-induced selection or drug-mediated mutagenesis at a fast pace resulting in the reduction of antibiotic shelf time and make essential the search of new active compounds that act against MRSA without any side effects to the host (Da Silva et al. 2017).

2.6.3. Pathogenic *Escherichia coli*

A healthy human intestinal tract has harmless *E. coli* bacteria residing in the intestines. Some *E. coli* are pathogenic, meaning they can cause illness, such as diarrhoea, while others cause urinary tract infections, respiratory illness, pneumonia and neonatal meningitis that can be transmitted through contaminated water or food, or through contact with animals or persons (CDC 2013). Faecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Diarrhoea remains the second leading cause of death in children younger than 5 years globally, causing 500 000 deaths per year, and ranks sixth in global disability-adjusted life year burden for infants (Liu et al. 2012). There are different groups of pathogenic *E. coli* strains and these are Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC),

Enterohemorrhagic/Shiga toxin-producing *E. coli* (STEC) and Enteroaggregative *E. coli* (EaggEC) as shown in **Table 5** below (Pienaar et al. 2016). Each year, ETEC causes more than 200 million cases of diarrhoea and 380 000 deaths, mostly in children in developing countries (WHO 2010).

Table 5: Mechanism of virulence in pathogenic *E. coli* (Pienaar et al. 2016).

Pathogenic strain	**Virulence mechanism**
ETEC	Adhesion: produces heat liable (LTs) and heat stable (STs) enterotoxins. LTs activate the main chloride channels of epithelial cells, and STs activate adenylate cyclase (cAMP) activity both resulting in the influx of water into intestines and it is the most common cause of traveller's diarrhoea.
EPEC	Uses an adhesin called intimin which attaches to intestinal epithelium cells leading to loss of microvilli causing mucus diarrhoea.
EIEC	Invades and disseminates large intestine epithelial cells through endocytic vacuole lysis and spreading to nearby cells causing a syndrome similar to shigellosis, with profuse diarrhoea and high fever.
STEC	Adhesion and mucosal damage, produces shiga toxins (Stx1 and Stx2) that cleave and inactivate 60s ribosomes and causes bloody diarrhoea (strain O157: H7) and haemolytic-uremic syndrome (HUS) due to enhanced cytokine release causing kidney failure.

EaggEC	They have fimbriae which aggregate intestinal mucosa cells cause watery diarrhoea and produce ST enterotoxin.

Gram-negative *E. coli* strains have been observed to be resistant to many antimicrobials that are effective against Gram-positive organisms (Pienaar et al. 2016). When under stress, antimicrobial resistant *E. coli* has been shown to be able to pass on resistance genes to other bacterial species such as *S. aureus*, through horizontal gene transfer as they often carry multiple drug-resistance plasmids (Croxen et al. 2013). The prevalence of drug-resistant *E. coli* is of a great concern especially in the developing world where it is rampant due to a recent increase in access to antibiotics which are counterfeit and substandard causing the emergence of resistant strains. This has also been coupled with the hostile side effects associated with antimicrobials when used against STEC which increases the risk of haemolytic uremic syndrome (CDC 2014).

2.6.4 *Enterococcus faecalis (E. faecalis)*

It is a commensal bacteria inhabiting the gastrointestinal tracts of humans and other mammals which were formerly classified as part of the genus Streptococcus but now belongs to the genus Enterococcus. *E. faecalis* that are resistant to antimicrobials can cause life-threatening infections such as urinary tract infections, meningitis, endocarditis and septicaemia in a nosocomial environment. (Hidron et al. 2008). *E. faecalis* has been frequently found in root canal-treated teeth which harbour the

bacterium with a prevalence rate of 30% to 90% than cases of primary infections (Jaremko et al. 2013).

E. faecalis is resistance to many commonly used antimicrobial agents such as aminoglycosides, cephalosporins, clindamycin, the semisynthetic penicillins nafcillin and oxacillin, and vancomycin is becoming more common (Arias and Murray 2012). Multi-drug-resistant *E. faecalis* are well adapted to survive in the gastrointestinal tract and can become the dominant flora under antibiotic pressure, affecting the severely ill and immunocompromised patient thus becoming an invasive infection as shown in **Figure 10** below (Miller et al. 2014).

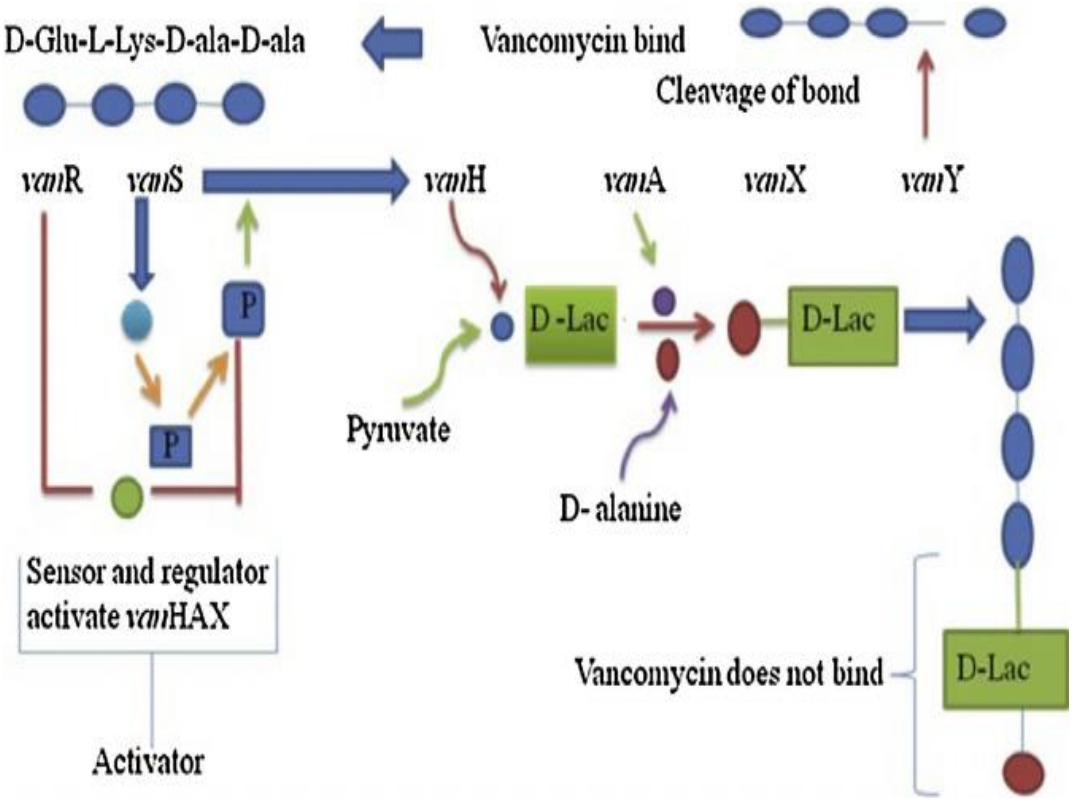


Figure 10: Resistant mechanism to vancomycin by *E. faecalis* (Miller et al. 2014).

The resistance of *E. faecalis* to vancomycin was a result of the use of the drug to treat MRSA which led to the selective pressure of the resistant *E. faecalis* strain (Hidron et al. 2008). Vancomycin blocks the construction of the cell wall of *E. faecalis* by preventing the formation of cross-linkages through binding with D-alanine groups at the end of the pentapeptide of N-acetylmuramyl pentapeptide, unlike the β-lactams which inhibit the activity of the enzyme peptidoglycane-transpeptidase (Arias and Murray 2012). This will cause the bacteria to synthesise a cell wall that is non-viable causing osmotic lysis and death of the bacteria. *E. faecalis* has a receptor, VanS to which vancomycin binds on its outer membrane causing phosphorylation and transfer of the phosphate group to VanR which will then bind to the cell nuclear membrane (Miller et al. 2014).

Phosphorylated VanR will then bind to the promoter region Van operon which will transcribe the four genes VanH, VanA, VanX and VanY producing a polycistronic mRNA which will be spliced and translated into four proteins VanH, VanA, VanX and VanY which confer resistance to vancomycin (Miller et al. 2014). VanH is a lactate dehydrogenase enzyme that catalyses the production of D-lactate form pyruvate where VanA which is a ligase will transfer the D-lactate and bind it in place of D-alanine. VanY, a carboxyl-peptidase cleaves D-alanine before D-lactate is added to the N-acetyl muramyl pentapeptide. Binding of D-lactate will prevent the ability of vancomycin to bind to the cell wall of *E. faecalis* thus conferring resistance to the drug (Miller et al. 2014).

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant material collection

The plant materials for *Terminalia sericea* and *Combretum imberbe* were collected from Ohangwena region in northern Namibia shown in **Figure 11** below. To avoid endangering *T. sericea* and *C. imberbe* plant population, cuttings of the stem, barks and leaves were collected and not the whole plants. The date and location was recorded and the collected materials were then stored and the part(s) used in the study were appropriately documented.

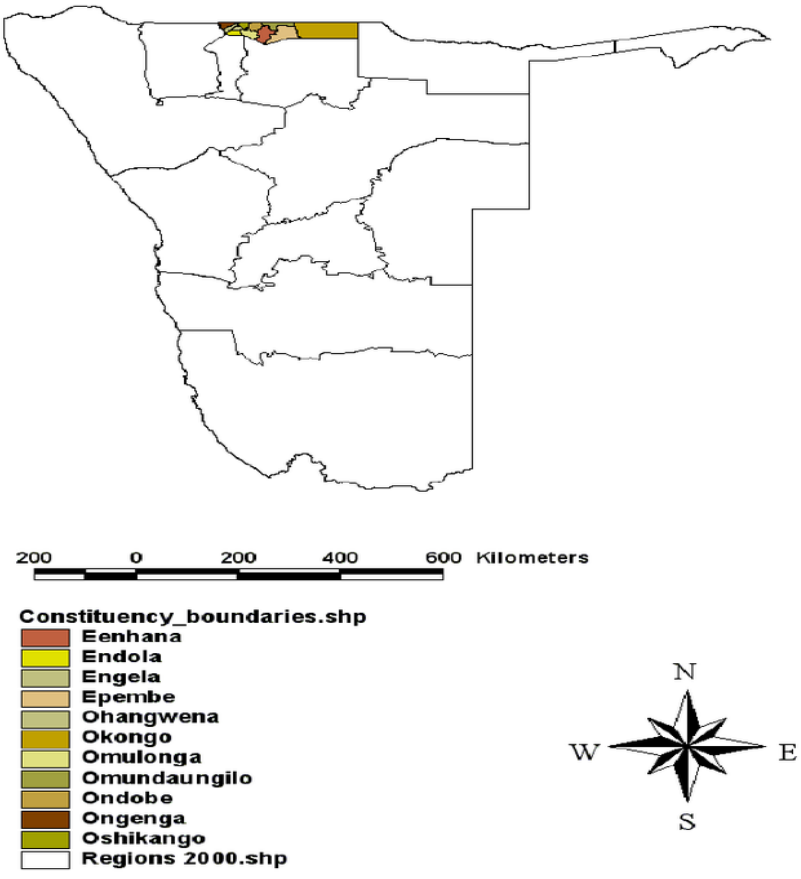


Figure 11: Map of Namibia showing Ohangwena region (Hedimbi and Chinsembu 2012).

Figure 12: The blended materials of *C. imberbe* and *T. sericea* plant parts.

3.1.1 Preparation of crude extracts

Stems and leaves of *T. sericea* and barks of *C. imberbe* were ground into smaller particles using a mortar and pestle and then blended into powder as shown in **Figure 12**. 100g of the plant material were then soaked in distilled water in conical flasks for aqueous extraction and for organic extraction, 250g of the plant material was soaked in a mixture of dichloromethane and methanol (DCM: MeOH) in 1:1 ratio in conical flasks for 12 hours. In the aqueous extraction, the samples were then boiled for 1 hour, filtered and re-washed by boiling again for another 1 hour in distilled water which were then removed by freeze-drying the crude extracts.

In organic extraction, the samples were filtered after soaking them for 12 hours and rewashed with the solvent DCM: MeOH by soaking them for another 12 hours. The organic samples were then concentrated by using a rotary evaporator at 55℃ and

completely dried in a fume hood. Both the aqueous and organic crude extracts were then stored at -76℃ until use.

3.1.2 Test Bacteria

The test organisms used in the study were Gram-negative bacteria *Escherichia coli, Shigella sonnei*, *Serratia marcescens*, *Helicobacter pylori a*nd Gram-positive bacteria *Enterococcus faecalis, Staphylococcus aureus* and *Clostridium perfringens* laboratory strains. The bacterial strain of *H. pylori* (ATCC4350) was thawed in a water bath at 37℃ and the entire suspension was transferred into 5ml Trypticase soy broth. This was then inoculated onto 5% blood agar plates and incubated at 37℃ for 3 days. The other bacteria were inoculated on nutrient agar at 37℃ for 24 hours.

3.1.3 Preparation of plant extracts solution

Antibacterial activity of the extracts was tested at various concentrations ranging from 100 to 0.01 mg/ml. The plant extracts for the selected plants were weighted and dissolved in DCM: MeOH and water for organic and aqueous extracts respectively; to prepare the stock solution of 100 mg/ml concentrations. The stock solution was prepared using the formula: $C = M/V$, where M= Mass of the plant extract and V= Volume of the solvent. The same stock solution was used to get the desired concentrations of 100, 1, 0.1 and 0.01 mg/ml by serial dilutions method using the equation: $C_1V_1 = C_2V_2$, where C= concentration and V= volume.

3.1.4. Susceptibility test

The seven bacterial strains were tested for susceptibility by the disk diffusion method at a concentration of 100mg/ml. Grade 1 Whatman filter paper was used for making discs. After sterilizing, the discs were then soaked in the different concentrations of plant extracts, dried and placed in bacteria agar plates. The plant extracts were each tested in triplicates with DCM: MeOH as the negative control for the organic extracts, distilled water for the aqueous extracts and gentamicin was used as the positive control. The plates were then incubated for 24 hours at 37℃ the results were recorded by measuring the zone of inhibition in millimetres (mm) for each microorganism by the different plant extracts. Serial dilutions were then done for each plant extract that inhibited the growth of bacteria from a stock solution of 100mg/ml to 0.01mg/ml so as to det*erm*ine the minimum inhibitory concentration.

3.1.5. Minimum inhibitory concentration (MIC)

This was performed to det*erm*ine the antimicrobial activity of selected plant extracts against specific bacteria by using the disk diffusion method. The bacteria were inoculated on nutrient agar and the plates were incubated for 24 hours at 37°C. The data was then recorded by measuring the zone of inhibition. Dilutions were done for plant extracts that had the highest inhibitory effect against each bacteria with concentrations ranging from 0.01 mg/mL to 100 mg/mL. The zones of inhibition were calculated in millimetres and recorded so as to determine the minimum inhibitory concentration (MIC).

3.1.6. Preparation of fractions

The crude extracts of *T. sericea* and *C. imberbe* were fractionated using vacuum liquid chromatography (VLC). Organic solvents; a) 10% Acetone (in DCM); b) 50% Acetone (in DCM); c) 20% Methanol (MeOH) (in DCM); d) 100% Acetone; e) 100% Ethyl Acetate and f) 100% MeOH were used on the different fractions for live bark, dead bark, leaves and stem extracts which were concentrated using a rotary evaporator at 55℃ and the samples were then completely dried in a fume hood as shown in **Figure 16** and **17** below.

(A) (B)

Figure 13: (A) Freeze dryer used to concentrate the water extracts. (B) Rotavapor used to concentrate the organic extracts.

Figure 14: Fractionated samples of crude extracts.

3.1.7. Data analysis

Analysis of variance was done to determine if there was a significant difference between the tested plants extract means and a Bonferroni post-hoc test was performed on ANOVA results which were significant i.e. p-value < 0.05 to reduce the chances of obtaining false-positive results (type I errors).

CHAPTER 4: RESULTS

4.1 Antimicrobial assay of crude extracts

All the plant parts had activity in aqueous extracts against the tested bacteria species with the exception of dead bark extracts from *C. imberbe* which showed no activity against any of the tested bacterial strains at 100mg/ml concentration as shown in **figure 17** below.

4.1.1 Antibacterial activity of aqueous extracts at 100mg/ml

The leaf extracts had the highest mean zone of inhibition of 16.00mm against *E. coli* whilst antimicrobial activity of *T. sericea* stem extracts were found to have the lowest mean inhibition zone of 5.00mm against *H. pylori*. The extracts from *T. sericea* leaves had the highest mean zone of inhibition of 4.88mm compared to the other extracts from live bark and stem as shown in **Figure 17** below.

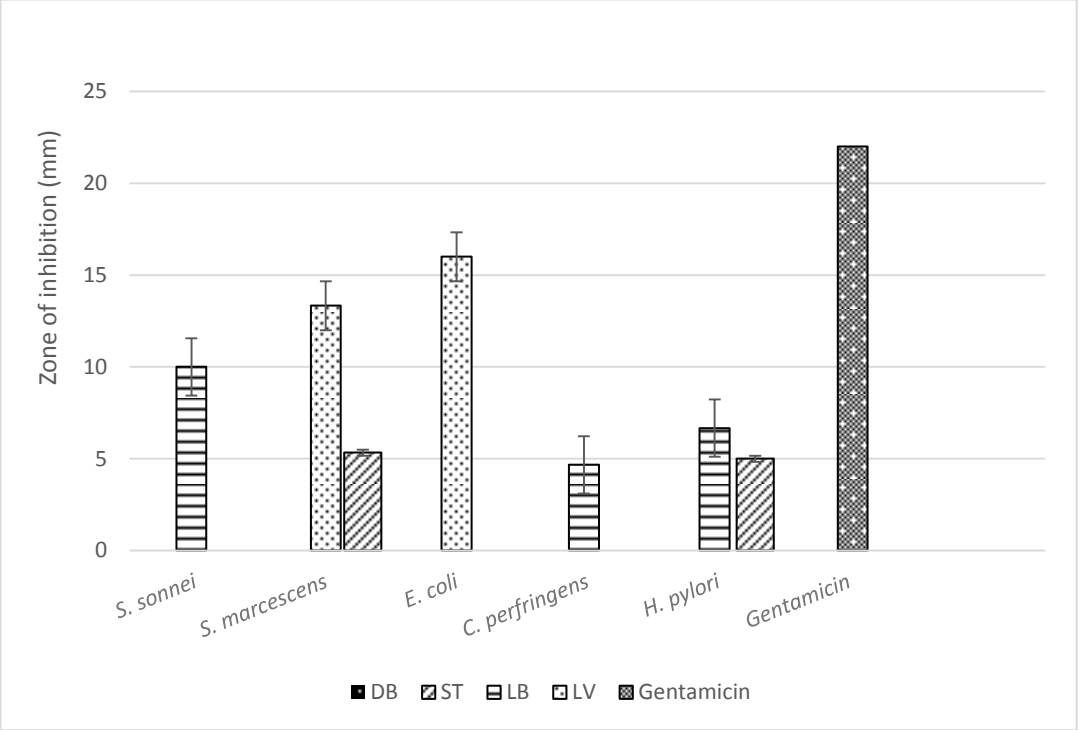


Figure 15: Zones of inhibition of aqueous crude extracts at 100mg/ml for both *T. sericea* and *C. imberbe* with mean zones of inhibition 3 replicates. No inhibition was observed with distilled water as a control.

4.1.2 Antibacterial activity of organic extracts at 100mg/ml

The stem extracts had the highest mean zone of inhibition of 13.62mm against all the susceptible bacteria that was tested whilst the extracts from the leaves had the lowest inhibition zone of 5.81mm. The live bark extracts had the highest antimicrobial activity against *H. pylori* with a mean inhibition zone of 18.67mm whilst organic extracts from stems had the lowest mean inhibition zone of 10mm against *S. sonnei*. The bacteria. *H. pylori* was the most susceptible to all three organic extracts of leaves, live bark, and stem with a mean inhibition zone of 17.56mm whilst *S. sonnei* was the least susceptible bacteria with a mean inhibition zone of 3.33mm.

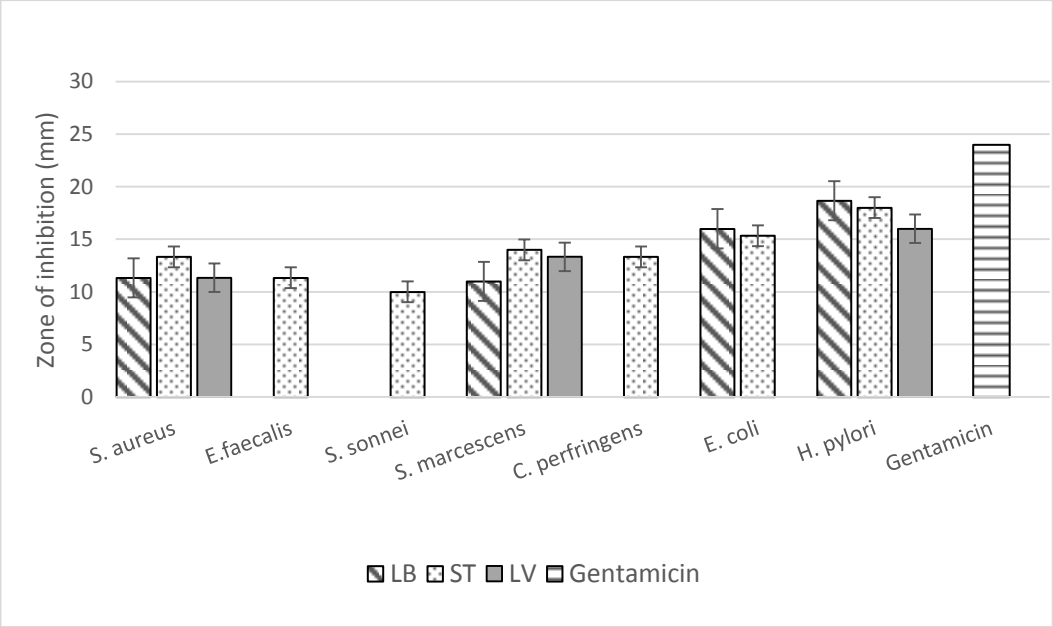


Figure 16: Zones of inhibition of organic crude extracts at 100mg/ml for both *T. sericea* and *C. imberbe* with mean inhibition zones of 3 replicates.

4.2. Minimum inhibitory concentration (MIC)

The MIC of live bark, stem and leaves for aqueous crude extracts was 1mg/ml whilst for the organic extracts it was 0.1mg/ml. The MIC for the aqueous extracts of *T. sericea* stem and leaves was 1mg/ml against *S. marcescens* and *H. pylori* whilst the MIC for *C. imberbe* live bark was also 1mg/ml against *S. marcescens* and *H. pylori*. The organic extracts of *T. sericea* stem and leaves all had MIC of 0.1mg/ml against *S. aureus, S. marcescens* and *H. pylori* whilst *C. imberbe* live bark had MIC of 0.1 mg/ml against *E. faecalis, S. aureus, S. marcescens* and *H. pylori* as shown in **Table 6** below.

Table 6: MIC of aqueous (aq) and organic (org) crude extracts.

Plant	Part	Bacteria	MIC (mg/ml)
T. sericea (aq)	ST	*S. marcescens*	1
	LV	*H. pylori*	1
C. imberbe (aq)	LB	*S. marcescens*	1
		H. pylori	1
T. sericea (org)	ST	*S. aureus*	0.1
	LV	*S. marcescens*	0.1
		H. pylori	0.1
C. imberbe (org)	LB	*S. aureus*	0.1
		S. marcescens	0.1
		E. faecalis	0.1
		H. pylori	0.1

Key: LV= Leaves; LB= Live bark; DB= Dead bark; ST =Stem

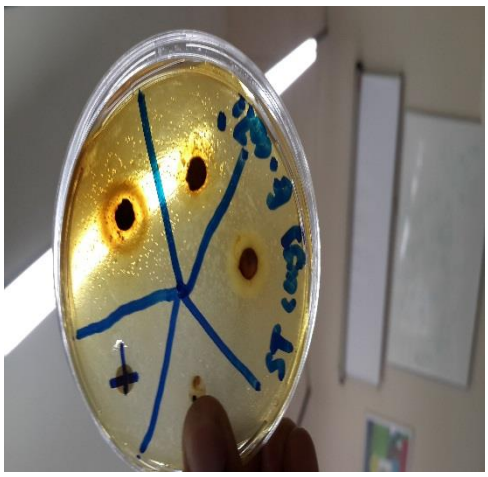
A  **B** 

Figure 17: Zones of inhibition **A**) ST organic extracts against *S. sonnei* at 0.1mg/ml and **B**) against *E.coli* at 0.01mg/ml.

A  B 

Figure 18: Zones of inhibition of **A**) *C. imberbe* DB organic extracts against *S. aureus at* 1mg/ml and **B**) *T. sericea* ST extracts against *H. pylori* at 0.01mg/ml.

4.2.1 Antibacterial activity of aqueous crude extracts

There was no activity observed against tested bacteria from all *C. imberbe* DB aqueous extracts at 10mg/ml. the aqueous extracts from *T. sericea* leaves had the highest mean inhibition zone of 13.67mm against *E. coli* and the *T. sericea* stem extracts had the lowest mean inhibition zone of 4.33mm against *H. pylori*. The bacteria *E. coli* was the most susceptible with an average inhibition zone of 8.11mm from all the aqueous extracts it had activity whilst

There was no activity observed from both *C. imberbe* dead bark aqueous and organic extracts. At a concentration of 10mg/ml, the organic extracts had the highest mean zone of inhibition ranging from 11.22 mm in *T. sericea* leaf extracts to 13.06 mm in stem extracts. The aqueous extracts had a much lower inhibition from both live bark and stem with zones of inhibition of 7.33 mm and 7.33 mm respectively. The highest mean from aqueous extracts was 12.50 mm from *T. sericea* leaf extracts. In the aqueous extracts group, the highest *S. aureus* had a mean inhibition zone of 3.78mm

from aqueous extracts at a concentration of 10mg/ml. the extracts from the *T. sericea* leaves had the highest average inhibition zone of 7.71mm whilst stem extracts had the lowest mean inhibition zone of 3.14mm.

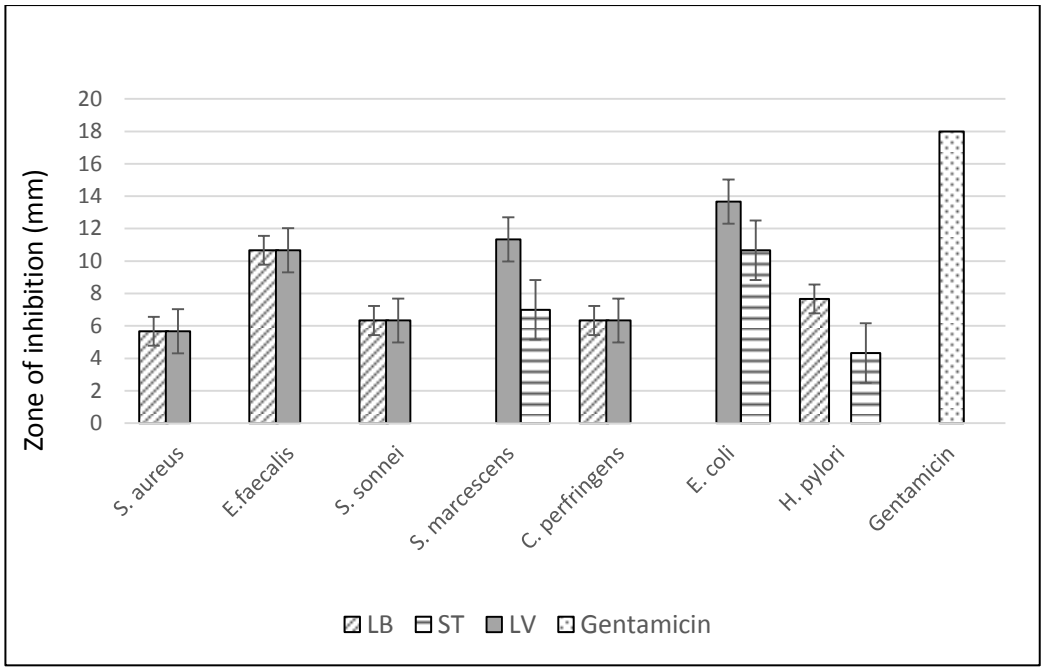


Figure 19: Zones of inhibition of *T. sericea* and *C. imberbe* aqueous extracts at 10 mg/ml with mean inhibition zones of 3 replicates.

4.2.2 Antibacterial activity of organic crude extracts

The organic crude extracts had higher mean inhibition zones when compared to the aqueous extracts with both the *T. sericea* stem and leaf extracts having the highest mean inhibition zone of 16.33mm against *H. pylori*. The lowest mean inhibition zone was 6.67mm from the *T. sericea* stem extracts against *S. marcescens*. The most susceptible bacteria was *H. pylori* with a mean inhibition zone of 16.22mm from the organic extracts of *T. sericea* leaves, stem, and *C. imberbe* live bark and *C. perfringens* was the least susceptible with a mean inhibition zone of 4.22mm. The

stem extracts had the highest antimicrobial activity with a mean inhibition zone of 13.00mm against all susceptible bacteria whilst the leaf extracts had the lowest activity with a mean inhibition zone of 6.73mm.

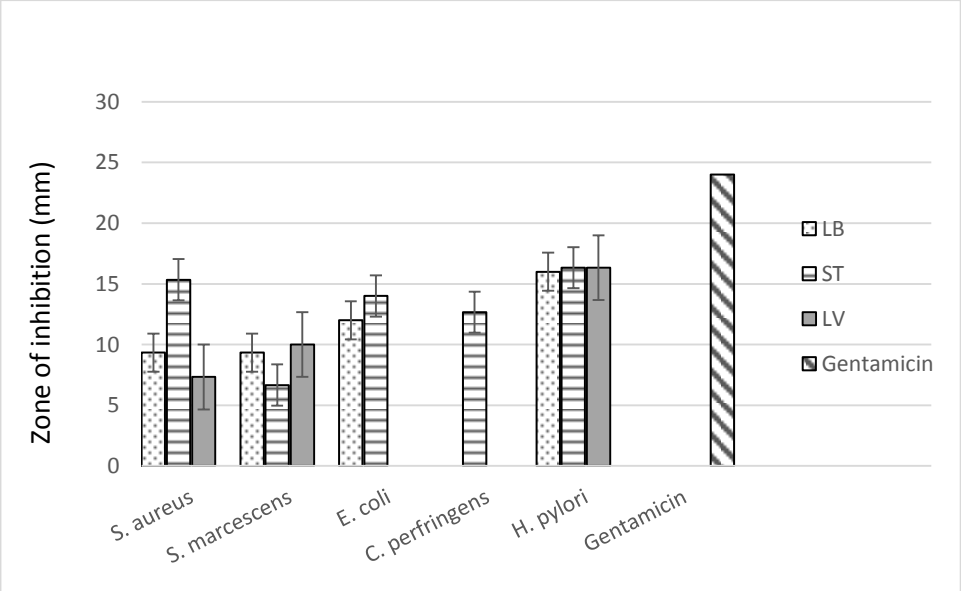


Figure 20: Zones of inhibition of *T. sericea* and *C. imberbe* organic extracts at 10mg/ml with mean inhibition zones of 3 replicates.

4.2.3 Antibacterial activity of aqueous extracts at 1mg/ml

Most of the bacteria were not susceptible to the plants aqueous extracts at concentration of 1mg/ml except for *S. marcescens* and *H. pylori*. The stem extracts had the highest mean inhibition zone of 6.00 mm whilst the other plant extracts had the lowest mean inhibition zone at 4.67mm. The ANOVA test p-value of 0.000000069 showed that there was a significant difference in the means of the plant extracts and the Bonferroni post-hoc test showed that there was no significant difference with a p-value of 0.42.

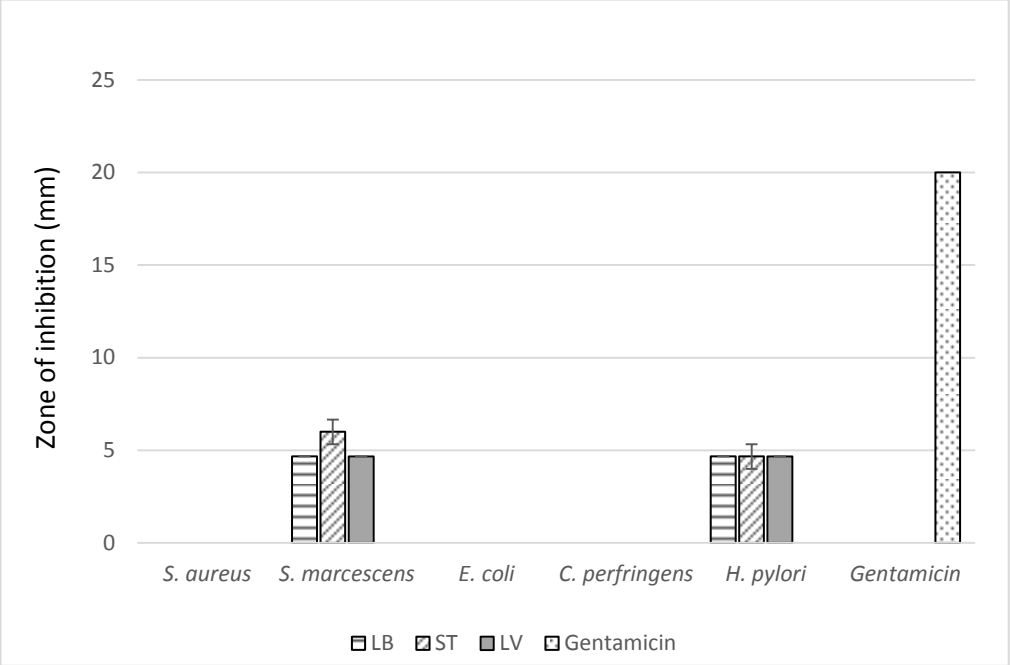


Figure 21: Zones of inhibition of aqueous *T. sericea* and *C. imberbe* extracts at 1mg/ml with mean inhibition zones of 3 replicates.

4.2.4 Antibacterial activity of organic extracts at 1mg/ml

The stem extracts had the highest mean inhibition zone of 16.67mm against *S. aureus whilst* the *C. imberbe* live bark extracts had the lowest mean inhibition zone of 5.33mm against *S. marcescens H. pylori* was the most susceptible bacteria from all four plant extracts with a mean inhibition zone of 12.32mm whilst *C. perfringens* was the least susceptible *with* a mean inhibition zone of 11.32mm and extracts from *C. imberbe* dead bark had the least average inhibition zone 4.23mm. ANOVA test p-value of 0.042 showed that there was a significant difference between plant extract means whilst the Bonferroni post-hoc test showed that there was no significant difference between the mean plant extracts.

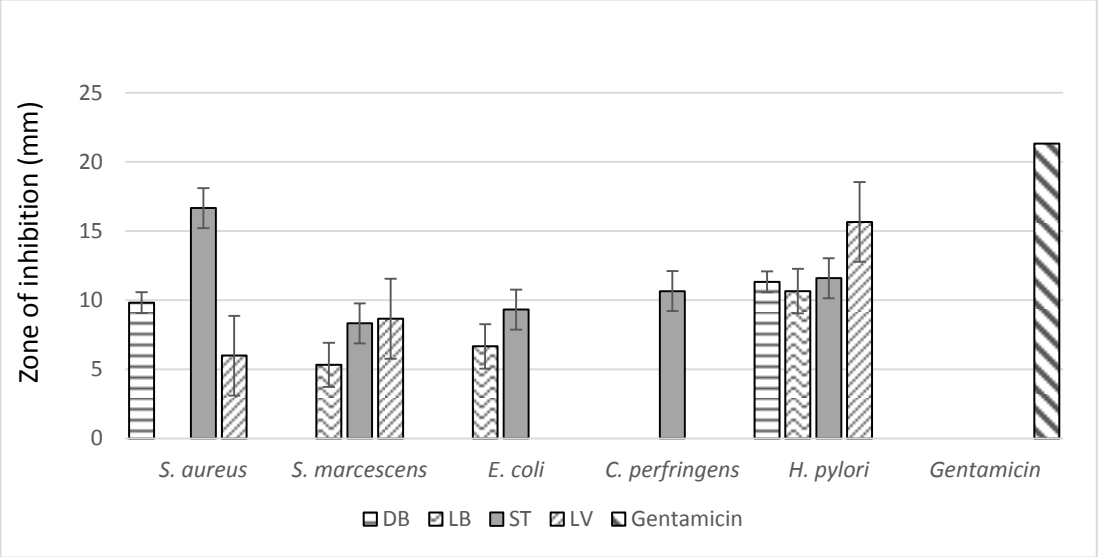


Figure 22: Organic crude of *C. imberbe* and *T. sericea* extracts at 1mg/ml with mean zones of inhibition of 3 replicates.

4.2.4 Antibacterial activity of organic extracts at 0.1mg/ml

The organic plant extracts of *C. imberbe* live bark and *T. sericea* leaves had the highest antimicrobial activity with mean inhibition zones of 11.33mm against the bacteria *S. marcescens* and the lowest mean inhibition zone was observed from *C. imberbe* live bark extracts against *S. aureus at* 6.00mm. *H. pylori* was observed as the most susceptible to the organic plant extracts with a mean inhibition zone of 10.22mm whilst *E. faecalis* was the least susceptible. The *C. imberbe* live bark organic extracts had the highest activity with a mean inhibition zone of 8.25mm whilst the *T. sericea* leaf extracts had the least activity with a mean inhibition zone of 6.66mm.

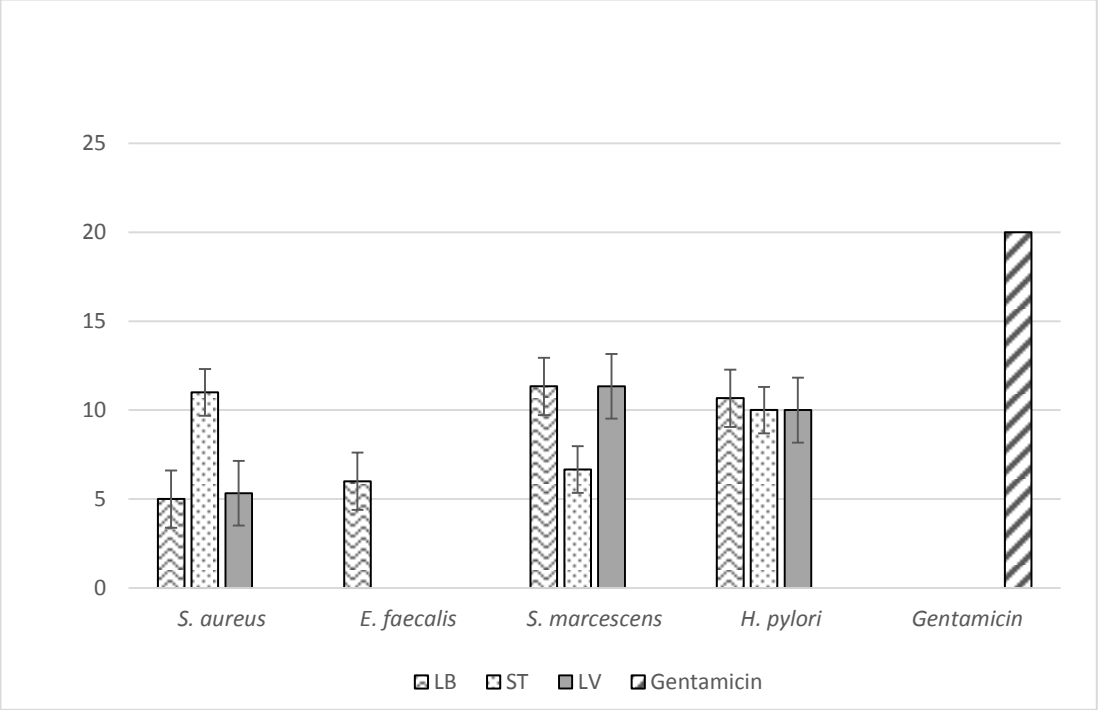


Figure 23: Organic extracts of *C. imberbe* and *T. sericea* with mean inhibition zones of 3 replicates at 0.1mg/ml.

4.3. Antibacterial activity of fractionated extracts

4.3.1 Dead bark fractions at 100mg/ml

The highest antibacterial activity was against *H. pylori* with mean inhibition zone of 12.67mm in 100% MeOH fractions which also had the highest mean zone of inhibition of 8.47mm from all the dead bark fractions it was susceptible to. The lowest observed mean zone of inhibition was 4.67mm against *S. sonnei* from 100% MeOH fractions. The fraction with the highest antimicrobial activity was 100% MeOH with a mean zone of inhibition of 8.46mm against all susceptible bacteria and the most susceptible bacteria was *H. pylori* with a mean inhibition zone of 10.89mm.

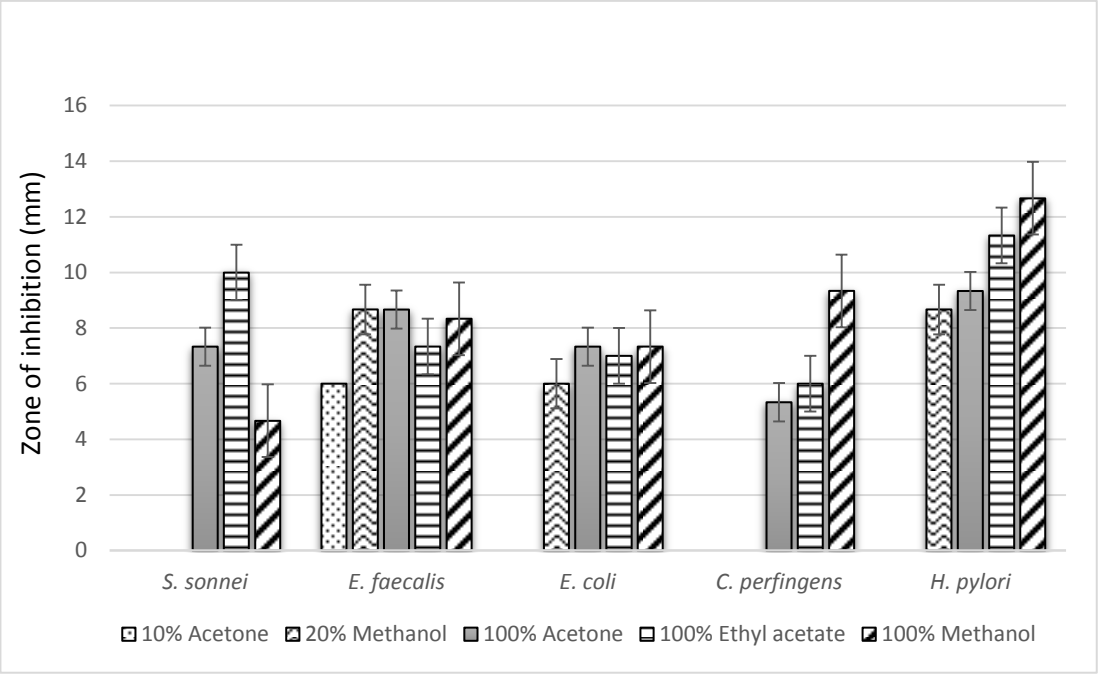


Figure 24: Zones of inhibition of different *C. imberbe* dead bark extract fractions at 100mg/ml with a mean zone of inhibition of 3 replicates.

4.3.2 Live bark fractions at 100mg/ml

There was no activity observed from 10% Acetone in DCM and 50% Acetone in DCM. The highest mean zone of inhibition was 13.33mm from 20% MeOH in DCM live bark fractions and 10% Acetone fractions had the highest antibacterial activity of 9.80mm against all susceptible bacteria. The lowest mean zone of inhibition was 4.33mm from Ethyl acetate fractions against *S. aureus* which also had the lowest mean zone of inhibition of 6.13mm at 100mg/ml.

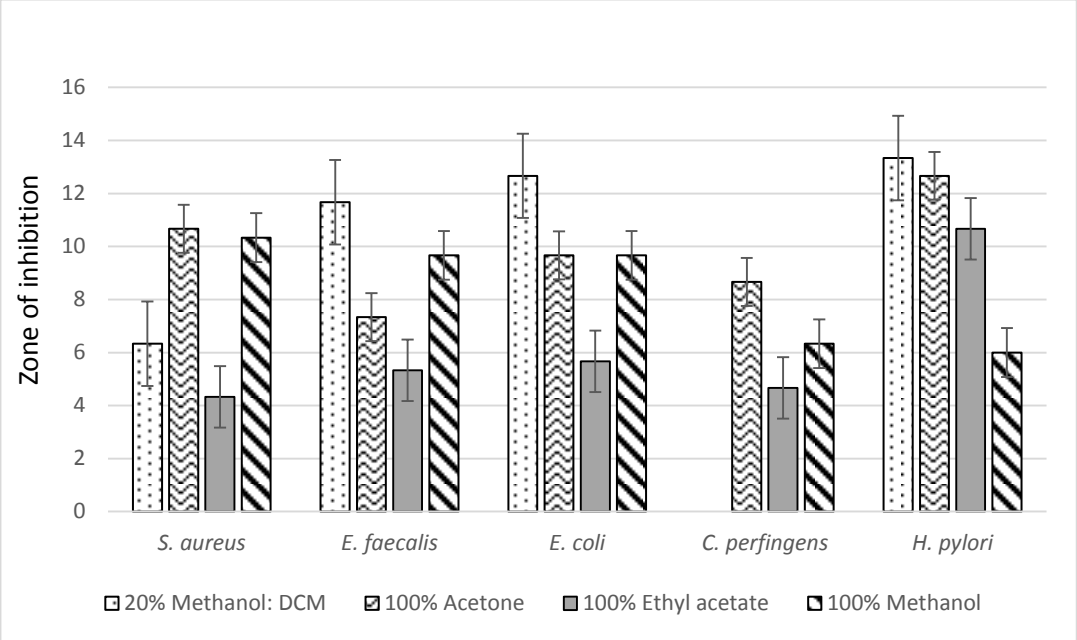


Figure 25: Zones of inhibition of different fractions from *T. sericea* live bark extracts with mean inhibition zones of 3 replicates at 100mg/ml.

4.3.3 Leaf fractions at 100mg/ml

There was no activity observed from 10% acetone in DCM with the lowest mean zone of inhibition from 100% Ethyl acetate of 3.33mm against *S. marcescens*. The highest mean zone of inhibition was 16.00mm from leaf fractions in 20% MeOH in DCM. The highest antibacterial activity mean zone of inhibition was from 20% MeOH in DCM fractions also against all susceptible bacteria and the most susceptible bacteria was *H. pylori* with a mean inhibition zone of 10.00mm against all leaf extract fractions to which it was tested.

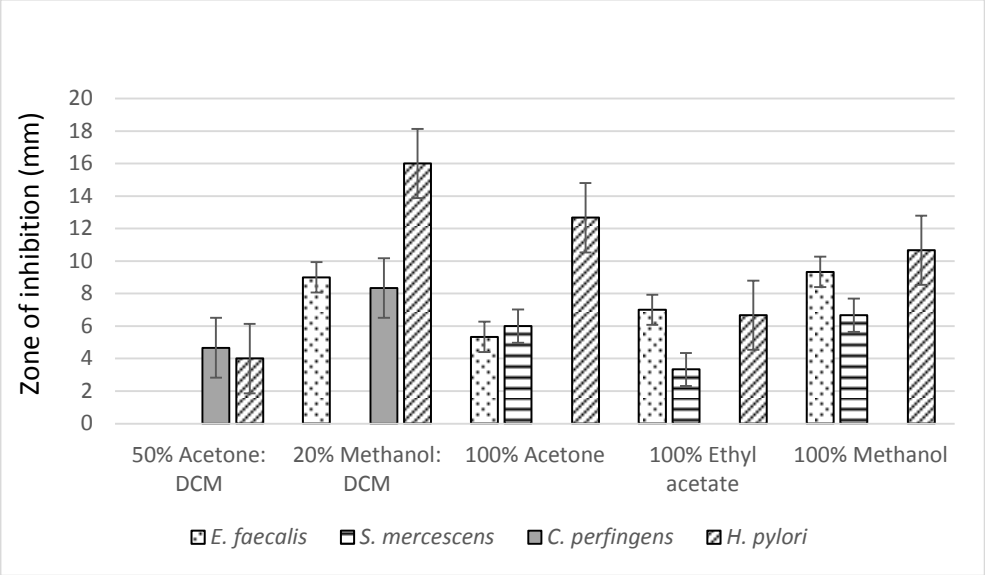


Figure 26: Zones of inhibition of *T. sericea* leaf extracts fractions with mean inhibition zone of 3 replicates at 100mg/ml.

4.3.4 Stem fractions at 100mg/ml

In the stem fractions the highest activity was from *H. pylori* with a mean zone of inhibition of 14.00mm in 100% MeOH fractions and the lowest mean zone of inhibition was 4.33mm from 100% Acetone fractions against *S. aureus* . The fractions with the highest antimicrobial activity were from 100% MeOH with a mean inhibition zone of 6.95mm against all susceptible bacteria and the most susceptible bacteria was *H. pylori* with a mean inhibition zone of 9.39mm against all fractions from which it was tested.

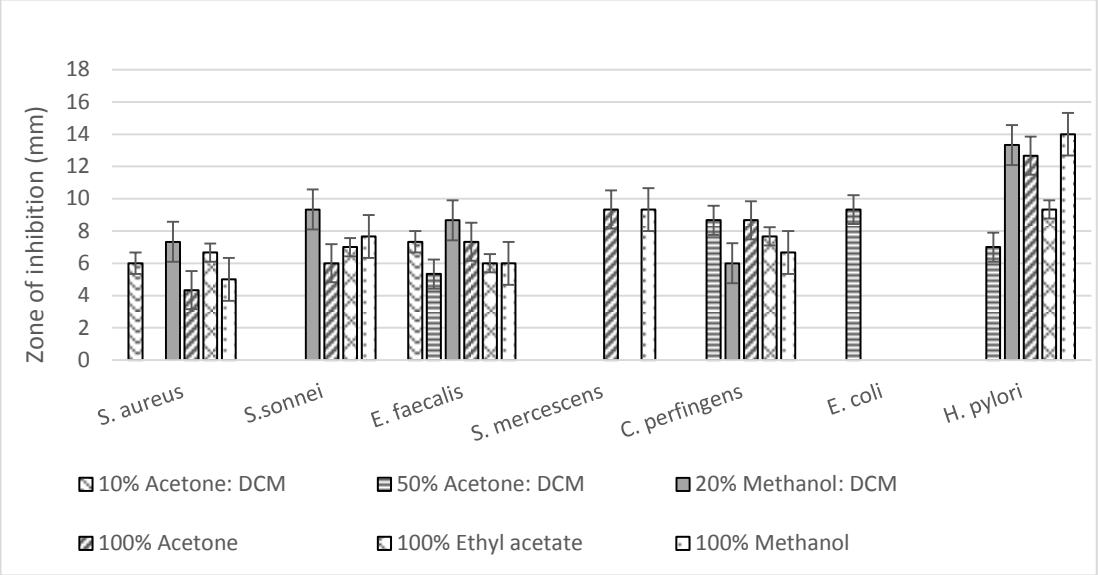


Figure 27: Zones of inhibition of *T. sericea* stem extract fractions with mean inhibition zones of 3 replicates at 100mg/ml.

4.4 Minimum inhibitory concentration (MIC) of fractions

The fractions from all the plant extracts had antibacterial activity at 10mg/ml except for live bark fractions and the MIC for dead bark fractions was 10mg/ml in which the highest mean inhibition zone was 8.33mm against *C. perfringens* in 20% MeOH: DCM. Both the stem and leaf fractions had MIC at 0.01mg/ml where they had activity against *E. faecalis* and *H. pylori* and the highest activity was 11.33mm for the leaf fractions and 7.33mm for the stem fractions all against *H. pylori* as shown in **Table 7** below.

Table 8: MIC of active extract fractions.

Plant extracts	Part	Fraction	Bacteria	MIC (mg/ml)
C. imberbe	DB	20% MeOH: DCM	*H. pylori*	10
		100% Acetone	*C. perfringens*	10
		100% Ethyl acetate	*S. sonnei*	10
		100% MeOH		10
T. sericea	ST	100% MeOH	*H. pylori*	0.01
		100% Acetone	*E. faecalis*	0.01
	LV	100% MeOH	*H. pylori*	0.01

Key: LV= Leaves; LB= Live bark; DB= Dead bark; ST =Stem

Figure 28: Zone of inhibition of *C. imberbe* LB and DB fractions against *H. pylori* at 0.1mg/ml. and 10mg/ml respectively.

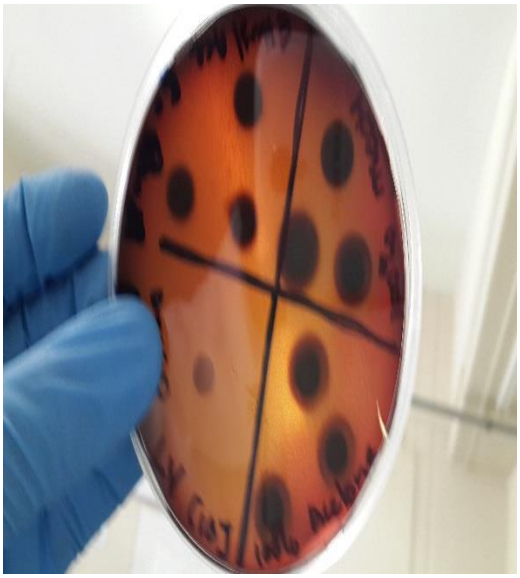
A  **B** 

Figure 29: Zone of inhibition of *T. sericea*) LV fractions against *H. pylori* and **B**) ST fractions against *E. faecalis* at 0.01mg/ml.

4.41 Dead bark fractions at 10mg/ml

The only bacteria that were susceptible to dead bark fractions at 10mg/ml were *S. sonnei*, *C. perfringens* and *H. pylori*. The highest mean inhibition zone of 8.33mm in 20% MeOH in DCM was observed from *C. perfringens* whilst *S. sonnei* had the least mean inhibition zone of 4.67mm in 100% Acetone. The fraction with the highest antimicrobial activity was 100% Acetone with a mean inhibition zone of 6.67mm against all susceptible bacteria that was tested.

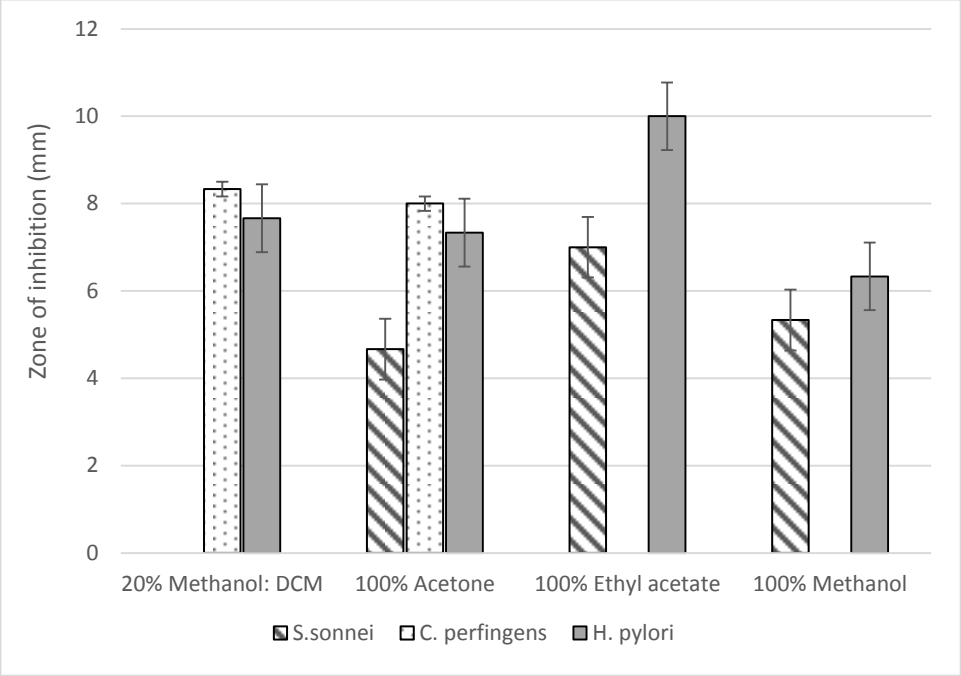


Figure 30: Zones of inhibition of *C. imberbe* dead bark extracts with mean inhibition zones of 3 replicates at 10mg/ml.

4.4.2 Leaf and stem fractions at 10mg/ml

At 10mg/ml fractions from leaves and stems were only active against *E. faecalis* and *H. pylori* bacterial strains. The highest mean zone of inhibition was 14.33mm from100% MeOH leaf fractions against *H. pylori* whilst in stem extracts the highest mean zone of inhibition was 10.33mm from 100% Ethyl acetate fractions also against *H. pylori*. The fractions with the highest antimicrobial activity were from 100% MeOH with a mean inhibition zone of 9.67mm against all susceptible bacteria tested whilst the most susceptible bacteria between the two was *H. pylori* with a mean inhibition zone of 8.50mm.

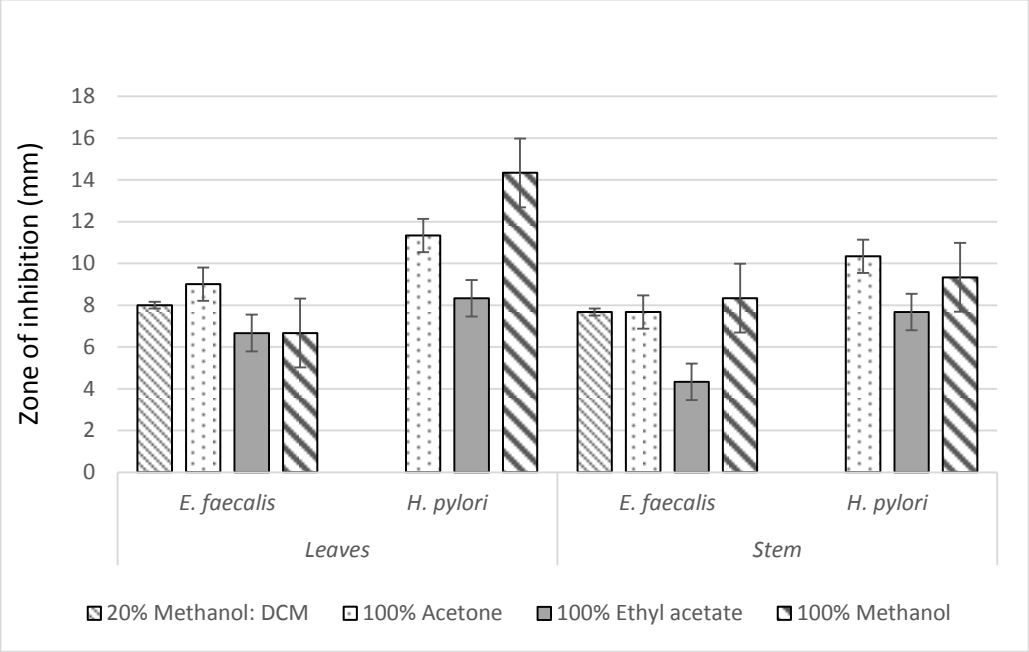


Figure 31: Comparison of antibacterial activity of different fractions from *T. sericea* leaf and stem extracts with mean of zones of inhibition of 3 replicates at 10mg/ml.

4.4.3 Leaf and stem extract fractions in 1mg/ml

The highest antimicrobial activity in leaf extracts was observed in 100% acetone fractions against *H. pylori* with a mean inhibition zone of 12.00mm whilst in the stem extracts, the highest mean inhibition zone was 9.67mm also against *H. pylori* in 20% MeOH in DCM stem fractions. The fractions from 100% Ethyl acetate also against *H. pylori* had the highest antimicrobial activity with a mean inhibition zone of 9.25mm against all susceptible bacteria tested whilst the most susceptible bacteria was *H. pylori* with a mean inhibition zone of 9.92mm against all fractions that had activity.

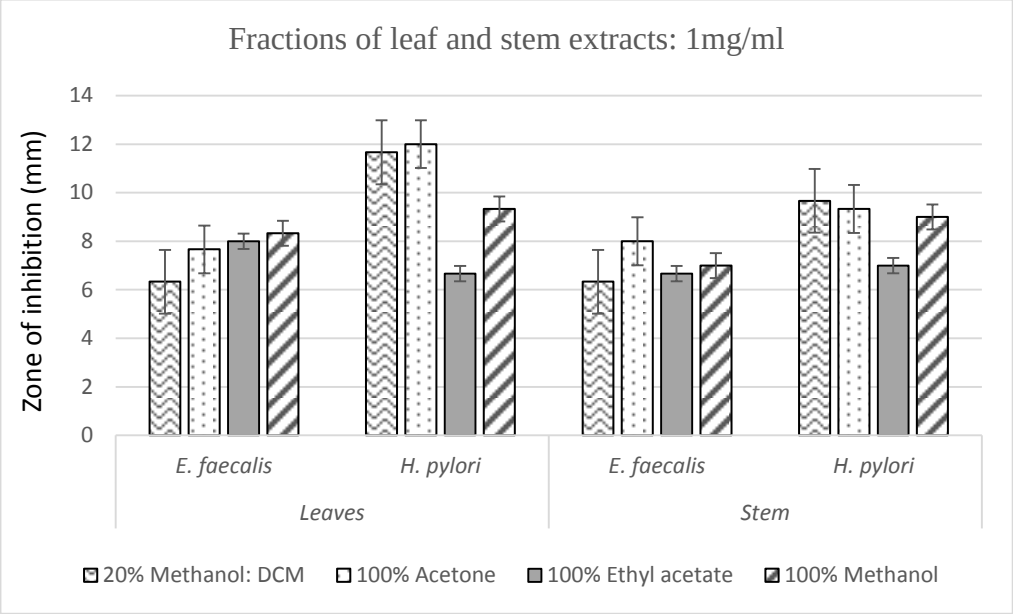


Figure 32: Comparison antibacterial activity of *T. sericea* leaf and stem fractions with a mean inhibition zone of 3 replicates at 1mg/ml.

4.4.4 Leaf and stem extract fractions at 0.1mg/ml

The highest mean zone of inhibition between the two extracts was observed from 100% MeOH fractions of leaves against *H. pylori* with a mean zone of inhibition of 12.33mm. The bacteria *H. pylori* was the most susceptible bacteria with a mean zone of inhibition of 9.78mm against all fractions used whilst 100% MeOH fractions had the highest antimicrobial activity with a mean zone of inhibition of 11.44mm against all susceptible bacteria tested.

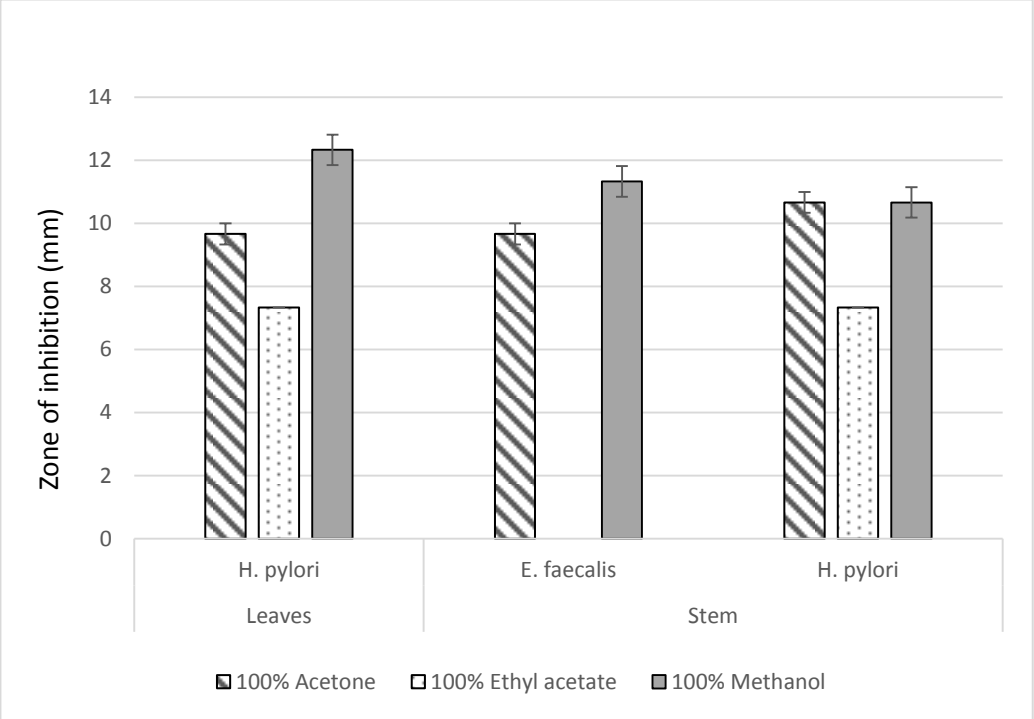


Figure 33: Comparison of different fractions of *T. sericea* stem and leaf extracts with a mean zone of inhibition of 3 replicates at 0.1mg/ml.

4.4.5 Leaf and stem extract fractions at 0.01mg/ml

There was low activity observed from both stem and leaf fractions where the leaf fractions had the highest mean zone of inhibition of 11.33mm against *H. pylori* in 100% MeOH whilst the stem extracts were only active against *E. faecalis* with the highest zone of inhibition of 7.67mm from 100% Acetone fractions.

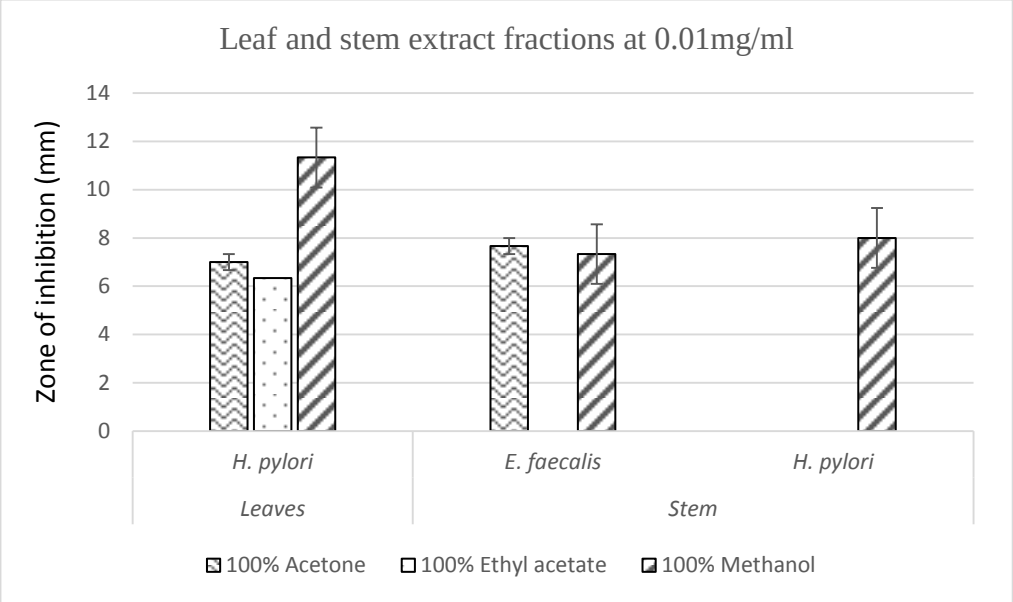


Figure 34: Comparison of antibacterial activity between leaf and stem extracts of *T. sericea* with a mean zone of inhibition of 3 replicates at 0.01mg/ml.

CHAPTER 5: DISCUSSION

5.1. Antibacterial activity of crude extracts

The diminishing efficacy and increasing toxicity of synthetic drugs due to problems associated with antimicrobial resistant bacteria present a serious global medical crisis, requiring constant surveillance, which continuously challenges the scientific community (Lai et al. 2014). Traditional medicine has been practiced in Africa and other regions for centuries through the application of herbal plants for therapeutic purposes. The studied plants, *T. sericea*, and *C. imberbe* are used locally for their traditional medicinal properties, however; their efficacies against resistant bacteria have not been determined. Namibia is a developing country and it is important that effective but less expensive antimicrobial drugs should be developed to accommodate all patients, regardless of financial status, in order to eliminate the health burdens caused by resistant bacteria.

There has been an increase in published reports in recent years showing successful antimicrobial activities of various traditional medicinal plants against protozoa, fungal, viral and bacterial infections which indicate some efficacy in the ethno medicinal plants used by traditional healers in Namibia (Cheikhyoussef et al. 2011). In the present study, favourable antagonistic activities against tested bacteria were exhibited by the crude extracts and fractions of *T. sericea* and *C. imberbe*.

5.1.1 *T. sericea*

In this study, the MIC values of aqueous extracts showed that *T. sericea* stem and leaves had antimicrobial activity against *S. marcescens* and *H. pylori* at 1mg/ml with the stem extracts exhibiting high activity against both bacteria. The organic extracts had activity at a low MIC of 0.1mg/ml with the extracts from the stem and leaves

showing antimicrobial activity against *S. aureus*, S. *marcescens* and *H. pylori*. The mean growth inhibition zones of the concentrations were slightly higher than that of the positive control.

Terminalia species have been reported to possess active compounds that have antiviral, antifungal and antibacterial activity (Singh et al. 2005). Antimicrobial activity against *S. aureus* with MIC of 1.60 mg/ml from *T. sericea* have been shown (Mabona et al., 2013). Masoko and Eloff (2008) also revealed that acetone and methanol fractions of *T. sericea* had health benefits that were linked with strong anti-oxidant and oxygen scavenging activities. *T. sericea* has been shown to be involved in the wound healing process due to its antimicrobial and antioxidant activity (Parkar et al. 2017). *T. sericea* leaves were found to possess higher antibacterial activity against clinical and laboratory diarrheal bacteria pathogens such *Salmonella*, *E. coli* and *Shigella* species in children under the age of five in Namibia with low MICs against *Salmonella* and *E. coli* 250 µg/ml as well as laboratory *E. coli* of 62.5µg/ml (Iikasha 2016).

The leaves, roots and bark of *T. sericea* were used in the Northern regions of Namibia as antimicrobial agents against most sexually transmitted infections by drinking decoctions from the plant to manage opportunistic infections (Chinsembu and Hedimbi 2010). *T. sericea* roots are used to treat diarrhoea, pneumonia, colic and bilharzia, while the leaves are used for stomach disorders (Bessong et al. 2005; Amri 2011). In India, *Terminalia bellerica* is traditionally used as an antibacterial remedy and studies done by Singh et al. (2011) revealed that it contained lignans that had antimicrobial activity *in vitro* (Singh et al. 2011). *Terminalia arjuna* fruit paste is

applied topically on wounds and its bark powder is boiled with water and inhaled to cure a headache and to kill worms in teeth (Ignacimuthu et al. 2006).

5.2.2 *C. imberbe*

The MICs of *C. imberbe* live bark aqueous extracts was 1mg/ml against the bacteria *S. marcescens* and *H. pylori* whilst the organic extracts had a lower MIC of 0.1mg/ml against *S. aureus*, *E. faecalis* and *H. pylori*. This shows that the live bark extracts of *C. imberbe* is an effective inhibitor of the growth of the bacteria *S. aureus* , *E. faecalis* and *H. pylori* which are known to be increasingly less susceptible to antimicrobial drugs (Ghotaslou et al. 2015; Da Silva et al. 2017). The mean growth inhibition zones of all the concentrations of the plant extract were slightly less than that of the positive control

The significance of *C. imberbe* as a traditional medicinal plant is its use throughout Southern Africa as shown by the different local names of the plant (Madikizela et al. 2014). *C. imberbe* disc diffusion assay of the ethanolic extracts were found to be active at a low MIC of 125 μg/ml against *Mycobacterium smegmatis* and had a potential as an efflux pump inhibitor in mycobacteria (Magwenzi et al. 2014)

The MICs of *Combretum microphyllum* from methanol, dichloromethane, and tetrahydrofuran varied from 0.01 to 1.25 mg/ml and were found to have antibacterial activity against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* (Kotze et al. 2002). The leaves of *Combretum erythrophyllum* were found to have antibacterial compounds which had activity against *S. sonnei* at 25 μg/ml (Martini et al. 2004). The Ethyl acetate fractions of *Combretum woodiii* were found to have MIC values as low as 0.08 mg/ml and the active compound Combretastin B5 was identified to have activity against *S. aureus*.

Combretaceae species have been reported to be used by the majority of traditional healers in Eastern, Southern and Western Africa for the treatment of various diseases (Eloff et al. 2008). The roots from *C. imberbe* are used to make a decoction that is taken orally to treat diarrhoea, while an infusion made from the root bark is used for the treatment of schistosomiasis (Venter and Venter 1996). Green leaves from the plant are placed on hot coals producing a smoke that can be inhaled to relieve coughs, colds (Venter and Venter 1996). In Venda, it has been reported that the roots of *C. imberbe* that grow horizontally and cross the foot path can be mixed with other plant species such as *Sclerocarya birrea subsp. caffra*, *Diospyros lycioides*, *Combretum erythrophyllum* and other species to restore or revive fertility in Vhavenda women (Mabogo 1990).

In Namibia, boiled *C. imberbe* bark filtrates are drunk to treat sexually transmitted infections (Chinsembu and Hedimbi 2010). In Zambia, Mozambique, Malawi and Zimbabwe, the dried leaves of *Combretum paniculatum* are used to treat diarrhoea, malaria, and fevers and the hot water root extracts are used as an anthelmintic whilst decoctions are used to treat pulsating anterior fontanelle in infants, venereal disease, and menorrhagia, and twigs are used as an appetizer (Masoko and Eloff 2008). The findings demonstrate the high activity of plants against bacteria but also validates their use in traditional medicine as a treatment against bacterial infections.

5.2 Antibacterial activity of fractions

The fractionated organic extracts of *C. imberbe* live and dead bark and of *T. sericea* leaves and stems all had antimicrobial activity against the bacterial strains that were tested. There was a low antibacterial activity from the solvents; 10% acetone: DCM

and 50% Acetone: DCM with an increase in activity from 20% MeOH: DCM to 100% MeOH. The MIC of active extract fractions exhibited antimicrobial activity against *H. pylori* and *C. perfringens* and *S. sonnei* with *C. imberbe* dead bark having activity in 100% Ethyl acetate, 100% Acetone, 20% MeOH in DCM and 100% MeOH fractions at 10mg/ml.

The fractions from *T. sericea* stem had antimicrobial activity at 0.01mg/ml against *H. pylori* and *E. faecalis* in 100% MeOH and 100% Ethyl acetate whilst the leaf fractions had activity against *H. pylori* with MIC of 0.01mg/ml in 100% MeOH. The results obtained here are in line with the low MIC values of 0.08 mg/ml to 0.02 mg/ml obtained in different extracts of *Combretum* species which were found to have antimicrobial activity with *C. imberbe* MIC values ranging from 0.7 mg/ml to 0.28mg/ml (Masoko et al. 2006). In comparison with the previous reported studies on antimicrobial activity of both *C imberbe* and *T. sericea*, the results are in good agreement in terms of antibacterial activities showing that the plants can serve as possible alternative medicines to combat bacterial infections

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

This study was conducted to demonstrate the antibacterial activity of *C. imberbe* and *T. sericea* which could potentially serve to treat bacterial strains that have become less susceptible to most of the common antimicrobials used for bacterial infection treatments. The results of the study have revealed the antibacterial potential of both *C imberbe* and *T. sericea* with the methanol fractions of stem and leaf extracts from *T. sericea* exhibiting low MIC values of 0.01mg/ml hence significantly supporting their use in resource poor settings for treatment of bacterial infections.

The overall effectiveness of the fraction extracts were shown to have greater antibacterial activity than the crude extracts and both the crude and the fractionated extracts were observed to have high activity with MICs as low as 0.1mg/ml and 0.01mg/ml against the bacteria *H. pylori.*.

Further research should be conducted to evaluate the effectiveness of extracts that were found to have MIC value as low as 0.01mg/ml, such as those from the live bark of *C. imberbe* and the stem and leaves of *T. sericea* on *S. aureus, E. faecalis* and *H. pylori.* The bacteria are known to be increasingly resistant to some of the available antimicrobial drugs. This includes determining the chemical structure of the active compound in the crude extracts and the active principal from the active fractions with low MICs.

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APPENDICES

APPENDIX A

MINISTRY OF ENVIRONMENT AND TOURISM
Private Bag 13306, Windhoek, Namibia

APPLICATION TO CONDUCT RESEARCH AND/OR COLLECT BIOLOGICAL SPECIMENS

To be submitted to the Permit Office, Ministry of Environment and Tourism at least 3 months before date of commencement of project. Late submissions will not be accepted.

1. **PRINCIPAL INVESTIGATOR:** (name, address, contact numbers):
MAPIYE SAMSON
25 MARSHALL ROCK
ROCKY CREST
WINDHOEK
Cell number: 0814134479 / 0811515706 ; EMAIL: samsonmapiye@gmail.com

2. **TITLE OF PROPOSED PROJECT:**
ISOLATION AND CHARACTERISATION OF ANTI-HIV COMPOUNDS FROM TWO COMBRETACEAE SPECIES.

☑ **FULL PROPOSAL** detailed protocol submitted at this time.
☐ **PILOT PROPOSAL** detailed protocol to be submitted after fieldwork begins.

3. **SPECIFIC DATES OF INVESTIGATION:** ~~30~~ OCTOBER - ~~30~~ NOVEMBER 2016

4. **LOCALITY OF PROPOSED RESEARCH:** OHANGWENA REGION NOTHERN NAMIBIA.

5. **PARTICULARS OF ALL CO-WORKERS WHO WILL ASSIST YOU IN NAMIBIA**

Name	Nationality
PROF. K. C. CHINSEMBU	ZAMBIAN
DR. MARIUS HEDIMBI	NAMIBIAN
Pamela Claassen	Namibian

Attach list if necessary

1

I hereby certify that the information included in this application is correct. Moreover, I have read and understand the conditions and I agree to abide by them.

Signature of the Principal Investigator: _____

Place: Windhoek Date: 07/09/2016

Recommendation by research committee, head of institution, or employer of principal investigator:

I _____

Recommend the project proposed by the principal investigator in my

Capacity as _____

_____

Signature: _____ Date: _____

PLEASE APPEND TO THE APPLICATION:

1) Curriculum vitae of principal investigator, including a list of publications for the past five years.

2) A detailed description of the proposed research. Please pay special attention to:
   a) Objectives, motivation, key questions and hypotheses to be tested. Differentiate between short and long-term studies.
   b) Describe and discuss previous related research by yourself.
   c) Discuss your approach to the study, and especially the methodology.
   d) Motivate species and numbers of biological specimens to be collected.
   e) Provide detail of assistance required from MET (keep this to a minimum).
   f) State clearly when collected specimens will be deposited in which collection (see below).

GENERAL CONDITIONS:

1. Please liaise with local institutions/authorities in your same field of interest.

2. When appropriate, please supply a copy of your field catalog of specimens collected to the Ministry of Environment and Tourism's Permit Office before leaving Namibia.

3. Plant specimens should be deposited with the National Botanical Research Institute, palaeontological specimens with Geological Survey, and animal, archaeological and anthropological specimens with the State Museum of Namibia. Alternative arrangements must be fully motivated for in advance. Type specimens of new species and at least 30% of all secondary types must be deposited with either the National Museum of Namibia or the National Herbarium irrespective of