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The antimicrobial investigation of indigenous South African medicinal plants against oral pathogens

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Student number: 541949



A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the degree of Master of Pharmacy

Johannesburg, 2017

Declaration

I, Saajida Akhalwaya, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Pharmacy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

……

Saajida Akhalwaya

……day of……2017 in……

Dedication

I dedicate this dissertation to my family and friends,

for your unwavering love, support, encouragement and your tireless faith in me.

"Seek knowledge from the cradle to the grave"

Prophet Muhammed (PBUH)

Publications and presentations arising from this study

Publication

Research Publication: Akhalwaya, S., van Vuuren, S.F., Patel, M., 2017. An *in vitro* investigation of indigenous South African medicinal plants used to treat oral infections. Journal of Ethnopharmacology 210, (359-371). (Appendix A for abstract)

Conference presentations

Poster presentation: Akhalwaya, S., van Vuuren, S.F., Patel, M., 2015. The antimicrobial investigation of African plants against oral *Lactobacillus* and *Candida* spp. The Academy of Pharmaceutical Sciences of the Pharmaceutical Society of South Africa/ South African Association of Pharmacists in Industry (PSSA/SAAPI), 2015, 17th-19th September. (Appendix B for abstract and Appendix C for poster)

Oral presentation: Akhalwaya, S., van Vuuren, S.F., Patel, M., 2016. The antimicrobial efficacy of indigenous South African plants against oral pathogens and the effect of these plants on *Streptococcus mutans* biofilm. All Africa Congress on Pharmacy and Pharmacology, 2016, 5th-8th October. (Appendix D for abstract)

Abstract

Oral diseases in South Africa remain a huge public health problem due to the high cost, prevalence, severity and the influence on the patients well-being. Treatment for oral diseases requires the need for specialist dental health care workers and come at a high cost causing a great burden on the health system. The three most important oral diseases are dental caries, caused by *Streptoccocus mutans, Streptococcus sanguis, Lactobacillus acidophilus* and *Lactobacillus casei,* periodontal diseases caused by *Porphrymonas gingivalis* and *Fusobacterium nucleatum* and oral candidiasis caused by *Candida albicans, Candida glabrata* and *Candida krusei.* An ethnobotanical review has revealed that over a 120 South African medicinal plants are used for the treatment of oral diseases. This coupled with the lack of research on the subject, allowed for the investigation of the antimicrobial efficacy of some South African plants against oral pathogens.

A total of 140 aqueous and organic extracts and six essential oils were prepared from 31 different plant species. These plant samples were screened for antimicrobial efficacy against nine oral pathogens using the micro-titre plate dilution assay. Plant extracts that were found to have noteworthy antimicrobial activity against *Streptococcus mutans* were further evaluated on the effect on *S. mutans* biofilm formation using the glass slide method. The toxicity profiles of plant samples that were found to have noteworthy antimicrobial activity were evaluated using the brine shrimp lethality assay.

The plants did not exhibit antimicrobial efficacy against all nine pathogens in this study, instead, most were very specific to disease conditions. Some plants did show good antimicrobial activity against four of the nine pathogens tested (*A. afra* leaves, *C. torulosa* stems, *C, brachiata* leaves and *H. natalensis* leaves).The organic extract of *Cissampelos torulosa* stems displayed the lowest MIC value of 0.05 mg/ml against both *Lactobacillus* spp. This antimicrobial activity was also observed with the organic extract of *Spirostachys africana* leaves against *Candida albicans*. In some instances, a direct relationship was found between the traditional use of the plant and the antimicrobial activity observed. For example, noteworthy activity (MIC < 1.00 m/ml) was observed against all three *Candida* spp. for *Clematis brachiata* (leaves), a plant traditionally used to treat oral thrush. *Englerophytum*

magalismonatanum (stems) displayed notable activity against both *Streptococcus* spp. (MIC 0.83 mg/ml against *S. mutans* and MIC 0.67 mg/ml against *S. sanguis*).

Spirostachys africana leaves displayed the greatest anti-adherent properties against *S. mutans* biofilm formation at both 24 and 48 h, reducing the biofilm by 97.56% and 86.58% respectively. The majority of plant samples tested in the brine shrimp lethality assay (BSLA) were considered safe, however, 13 plant samples were considered toxic, at a concentration of 1 mg/ml, and their LC_{50} values were determined.

The findings from the results favour the potential use of these plants in treating oral diseases such as dental caries, periodontal diseases and oral thrush, and scientifically validates the traditional use of some of these plants.

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List of abbreviations

%	Percent
˚C	Degrees Celsius
µg	Microgram/s
µl	Microlitre/s
AIDS	Acquired immunodeficiency syndrome
Aq	Aqueous plant extract
ATCC	American type culture collection
CFU	Colony forming units
CHX	Chlorhexadine
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide gas
DCM: Meth	Dichloreomethane: methanol
EO	Essential oil
g	Gram/s
HIV	Human immunodeficiency virus
h	Hour/s
INT	*p*-Iodonitrotetrazolium violet
L	Litre/s
kg	Kilogram/s
LC_{50}	Concentration of test substance causing 50% brine-shrimp death
MIC	Minimum inhibitory concentration
mg	Milligram
ml	Millilitre
n	Number of experiments
NA	Not applicable
Org	Organic plant extract

PBS	Phosphate buffered saline
TSB	Tryptone Soya broth
UV	Ultra-violet
V	Volts
v/v	Volume per volume
w	watts
WHO	World Health Organisation

Chapter 1
Introduction

1.1. The oral cavity and the overview of oral diseases

The oral cavity is one of the most significant organs of the human body. It is the pioneer step in the digestive system and plays an important role as a sensory organ helping communication. The oral cavity is a unique environment that provides the perfect habitat for the proliferation of a diverse ecosystem of micro-organisms. It is the primary interface between the external environment and the body allowing for the entry of not only food and water, but micro-organisms (Marsh et al., 2009). There are over 500 micro-organisms that can be found colonizing the oral cavity (Huang et al., 2011). This is due to a number of features including the many distinctive surfaces that make up the oral cavity, the perfect temperature found in this environment and the constant supply of food and shredding of epithelial cells (Gupti, 2012; Henley-Smith et al., 2013).

The acidogenic and aciduric microflora that resides in the oral cavity can develop pathogenic properties when the pH balance is lowered. This occurs with the frequent consumption of sucrose and dietary carbohydrates that allow for the acidic environment to be maintained in the oral cavity (Loesche, 1996). Some of these micro-organisms contribute as causative agents of oral infections (Tanner and Stillman, 1993). Micro-organisms that can be implicated in oral diseases can be bacterial, fungal and viral. Pathogenic micro-organisms can cause common oral infections such as dental caries (tooth decay); periodontitis (gum disease) and endodontic infections (root canal) (Dewhirst, 2010). Common oral yeast infections (esophageal thrush) are caused by the opportunistic *Candida* spp.

Bacterial oral infections are the most common of all oral diseases and have the greatest prevalence which has increased drastically over the years (Petersen et al., 2005). The two most common bacterial infections are dental caries and periodontal diseases (Tanner and

Stillman, 1993). Dental caries most commonly occur in adolescence whilst periodontal diseases are more prevalent in adults and old age (Lemos and Burne, 2008). Dental plaque is a mass of variant bacteria that adhere to the surfaces of the oral cavity (Gupti, 2012). Dental caries and periodontal disease are caused by an accumulation of plaque (Tanner and Stillman, 1993). Dental plaque is a prime example of a biofilm. Biofilms are a mode of growth compromising layers of micro-organisms contained in an adhesive and protective matrix, thus allowing for easy communication between micro-organisms (Kolenbrander et al., 2002). Oral bacteria have an ability to produce biofilms on various surfaces of the oral cavity including the solid surface of teeth and the soft surfaces of epithelial tissues (Sandasi et al., 2011). When the biofilm is more cariogenic in nature, it consists of mainly Gram-positive acidogenic bacteria and when the biofilm is periopathogenic, it consists of mainly Gram-negative anaerobic periodontal bacteria (Chandki et al., 2011).

1.1.1. Dental caries

Dental caries can be defined as the destruction of the tooth and its surrounding tissue due to plaque forming bacteria and its acid by-products. These by-products attack the enamel and dentin of the teeth that leads to lesions and cavities (Henley-Smith el al., 2013). While dental caries is considered one of the most common diseases that manifest in children, all age groups can be vulnerable to the disease. There are a multitude of other factors that affect this chronic bacterial condition. These may include salivary production, dietary intake of sugars and exposure to fluoride (Selwitz et al., 2007). Cariogenic bacteria interact with the sugar, in particular sucrose and other complex carbohydrates, found in our diet and this aids in the formation of acids which cause the degradation of teeth (Featherstone, 2004). Some individuals who are at a higher risk of developing dental caries include those who suffer from hyposalivation or dry mouth. Hyposalivation (the production of little or no saliva) can be a side effect from the use of medications such as antihistamines and antidepressents, or from radiation therapy to the head and neck. There are also certain medical conditions that are known to cause hyposalivation including Sjogren's syndrome, diabetes and cystic fibrosis (Segura, 2014).

Caries affect various areas on the tooth surface and are classified by the site of these lesions, including the buccal and lingual smooth surfaces, approximal tooth surfaces, pit and fissure

surfaces and root surface caries (found in the dentine and cementum of the tooth) (Ismail et al., 2015). The tooth surfaces of incisors canines and premolars are popular areas where carious lesions can be found, however, the area with the highest rate of carious lesions are found on the occlusal fissure sites found in molars. Maxillary teeth, in general, are more susceptible to caries in comparison to mandibular teeth. Molars have the highest rate of caries, while central incisors found on the mandibular region are the least susceptible (Demirci et al., 2010).

1.1.1.1. Microbiology of dental caries

The primary causative micro-organisms implicated in dental caries are Gram-positive bacteria. Of the 200 to 300 species of bacteria that may be isolated from plaque, only *Streptococcus* and *Lactobacillus* spp. can be reliably associated with dental caries (Loesche, 1996). These species of bacteria are associated with dental caries due to their heightened cariogenic abilities of producing biofilms and lactic acid (Takarada et al., 2004; Gupti, 2012, Henley-Smith et al., 2013).

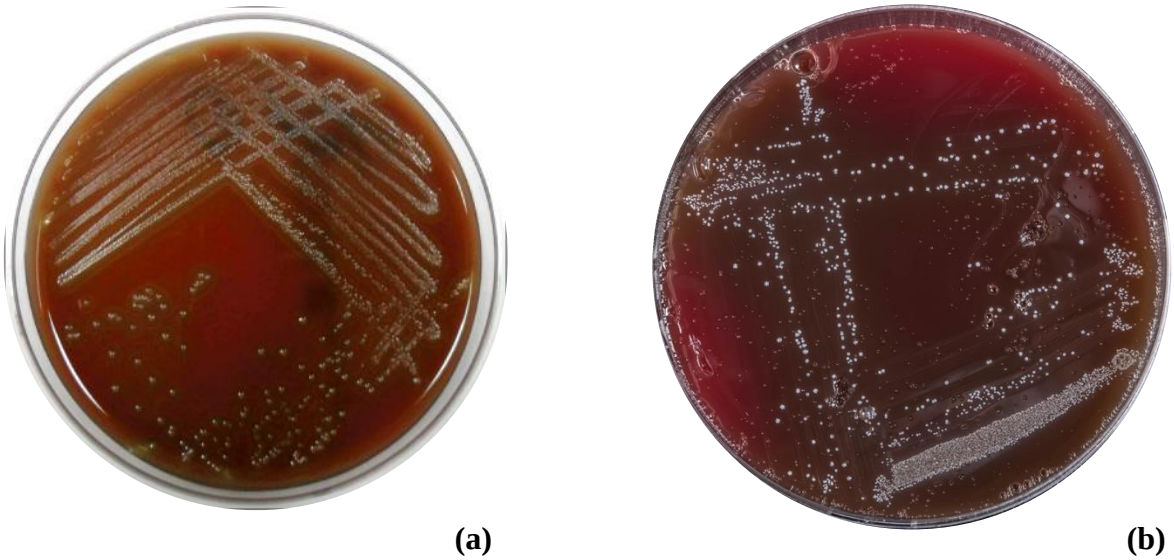
1.1.1.2. The *Streptococcus* spp.

There are around 25 different types of *Streptococcus* spp. that can be isolated from the oral cavity. Under certain conditions they can become pathogenic and aid in the development of carious lesions. Mutans Streptococci are considered the most important group of bacteria that are associated with dental caries (Nicolas and Lavoie, 2011). In particular, *Streptococcus mutans* has been discovered as the primary etiological bacteria that predominantly proliferate in the dental biofilm and aids in the development of carious lesions (Jakubovics and Kolenbrander 2010; Tahmourespour et al., 2010). *Streptococcus sanguis* is a Gram-positive heterogeneous group of bacteria that resides in the natural microflora of the oral cavity. Apart from its cariogenic ability, this bacterium is also known to be a causative agent in infective endocarditis (Caufield et al., 2000).

Streptococcus mutans is able to metabolize a number of sugars and plays an essential role in the adherence and development of biofilms (Karpinski and Szkaradkiewicz, 2013). It can grow in oxygen but optimum growth is achieved in the presence of CO_2 (Clarke, 1924). Both α or γ- haemolysis is displayed on sheep blood agar. They are acidogenic (produce acid) and

aciduric (withstand acid). In addition, they produce extra-cellular polysaccharides which facilitate the growth and adherence of bacteria (Hamada and Slade, 1980). Figure 1.1 (a) is an example of how *S. mutans* grows on a blood agar plate.

This cariogenic bacterium is Gram-positive, non-motile, non-spore forming and grows in short chains. *Streptococcus sanguis* is facultative anaerobe. It is a fermentative, catalase negative bacterium that displays α- haemolysis and slight greening due to partial haemolysis when cultured on blood agar plates (Kilian et al., 1989). Figure 1.2 (b) is an example of how *S. sanguis* grows on a blood agar plate.



(a) **(b)**

Figure 1.1: The spherical colonies of *Streptococcus mutans* ATCC 25175 (a) and *Streptococcus sanguis* ATCC 10566 (b)

1.1.1.3. The *Lactobacillus* spp.

The *Lactobacillus* spp. are rod shaped Gram-positive bacteria. *Lactobacillus acidophilus* and *Lactobacillus casei* fall into the homofermentive group of *Lactobacillus* found in the mouth, meaning that they mainly produce lactic acid during the breakdown of glucose. The lactobacilli are usually isolated from deep carious regions, including the cementum and root of the tooth, and are rarely found in the early onset of caries (Karpinski and Szkaradkiewicz, 2013).

Lactobacillus acidophilus is a Gram-positive, facultative anaerobe. It is a non spore-forming rod shaped bacterium, in pairs or short-chained that has the ability to produce lactic acid. (Fujisawa et al., 1992). Rough colonies are produced on acidic Rogosa medium (Figure 1.2 a)

Lactobacillus casei is a rod-shaped, Gram-positive bacterium that has the ability to produce lactic acid. *Lactobacillus casei* is also widely used in the production of dairy products. It occurs singularly and in short chains (Collins et al., 1989). They produce white colonies on an acidic Rogosa medium (Figure 1.2 b).

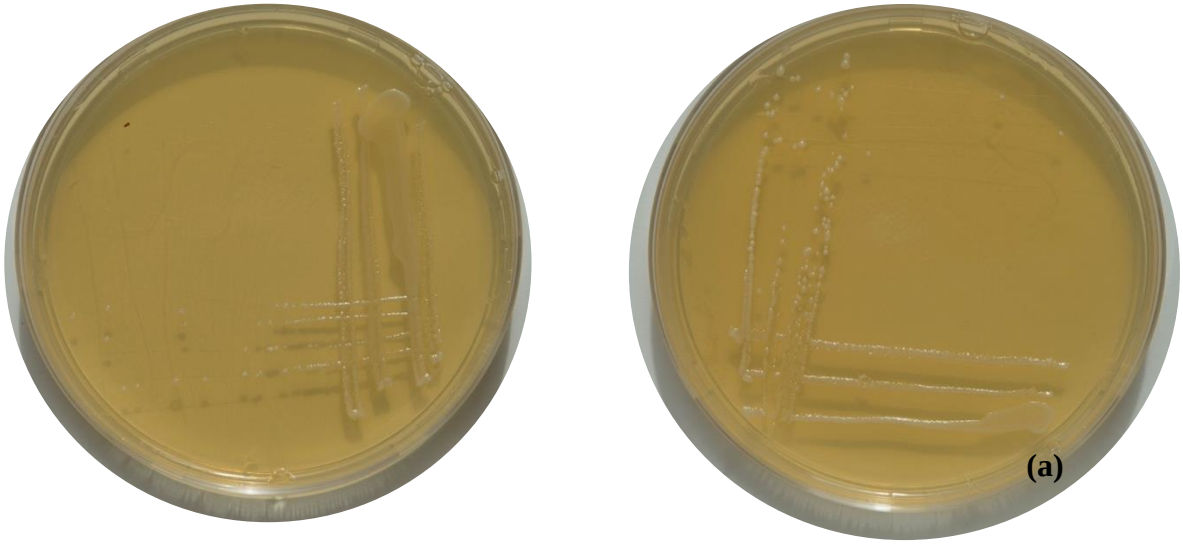


Figure 1.2: The white colonies of *Lactobacillus acidophilus* ATCC 4356 (a) and *Lactobacillus casei* ATCC 344 (b)

1.2.1. Periodontal diseases

Periodontitis is characterized by the 'degeneration and inflammation of gums, periodontal ligaments, alveolar bone and dental cementum' (Jain et al., 2008). The inflammation of the periodontium is caused by bacterial byproducts and host response which is triggered by the bacterial antigens. Periodontal diseases can be defined as a process that leads to the destruction of the supporting structures around the tooth which could lead to tooth loss (Guthmiller and Novak, 2002). Biofilms and dental caries usually precede periodontal infections. It is the leading cause of tooth loss in old age and is associated with more severe systemic conditions such as cancer, diabetes and human immunodeficiency virus (HIV) (Tanner and Stillman, 1993; Gupti, 2012). People suffering with periodontitis don't usually

experience pain until the disease has progressed to the later stages. Individuals usually seek help when the teeth have loosened or if they are suffering from bleeding gums and halitosis (Loesche, 1996).

As with dental caries, periodontitis is also a biofilm dependent disease. As the biofilm in the mouth continues to proliferate there is a shift in the makeup of micro-organisms found within the biofilm from a majority of Gram-positive acidic bacterium to strictly Gram-negative anaerobes. This occurs when the biofilm begins to enter the sub-gingival (below the gingiva) area. The maturity of the biofilm that surrounds the tissue in the sub-gingiva plays a vital role in the progression of periodontitis. This plaque has been found to contain a number of Gram-negative anaerobic rod species such as *Treponema, Bacteroides, Porphyromonas, Prevotella, Fusobacterium, Actinobacillus,* and *Eikenella* (Loesche, 1996; Guthmiller and Novak, 2002).

Periodontitis is usually graded by the depth of tissue loss (Loesche, 1996). This disease can be divided into two broad categories (the loss of teeth does occur in both categories): chronic periodontitis and aggressive periodontitis (Armitage, 1999). Chronic periodontitis is a localized progressive disease that usually affects people in their old age. Aggressive periodontitis is a more rapid, destructive disease usually associated with other systemic illnesses such as leukeamia, HIV and diabetes (Armitage, 2000).

The pathogenesis of periodontitis depends on certain characteristics that are owed to the periodontal pathogens. This firstly includes the pathogen's ability to adhere and proliferate in the biofilm, then the ability of the periodontal pathogens to interfere with hosts defence mechanism and lastly to penetrate and destroy tissue and bone surrounding teeth.

1.2.1.1. Adherence and proliferation in the biofilm

As the biofilm matures and Gram-negative bacteria begin to dominate, *F. nucleatum* increases in proportion to the biofilm formed. This bacterium acts as a link between the early and late colonizers during the progression and development of the biofilm and is a known causative agent in the proliferation of periodontal diseases as it has been found to be one of the most common bacteria that is consistently isolated from patients suffering from periodontitis (Bolstad et al., 1996). *Porphyromonas gingivalis* is one of the earlier colonizers found in the sub-gingival plaque. This micro-organism also interacts and co-aggregates with

other colonizers such as *F. nucleatum* and helps with the development of the biofilm found in the sub-gingival tissue (Lamont and Jenkinson, 2000).

1.2.1.2. Interferences with the host defence systems

Periodontal pathogens interfere with the host defence system by producing antigens that compromise this (Lamont and Jenkinson, 2000). *Porphyromonas gingivalis* interferes with this system through multiple mechanisms. This includes the secretion of proteinases that causes fragmentation of antibodies (IgG, IgA1, IgA2) (Frandsen et al., 1987). Some of these proteinases also cause the inactivation of leukocytes (Sundqvist et al., 1991). This bacterium can also produce a lipopolysaccharide that restricts diapedsis (the migration of leukocytes out of the circulatory system and to the place of infection) and causes a weak activation of the cytokine, tumor necrosis factor (TNF), which is responsible for the necrosis of cells (Darveau et al., 1995). Some strains of *P. gingivalis* can also form a capsule that bypasses the process of phagocytosis (Potempa and Banbula, 2000).

1.2.1.3. Invasion and destruction of host tissue

Invasion of the host tissue by periodontal pathogens is a fundamental step in the development of periodontitis (Guthmiller and Novak, 2002). *Fusobacterium nucleatum* helps invade and destroy the gingival tissue via multiple mechanisms requiring the aid of proteins and many other metabolites. It also causes the secretion of high levels of the cytokine interleukin- 8, IL-8 (Han et al., 2000). IL-8 is a pro-inflammatory cytokine that causes the activation of neutrophils (Bickel, 1993). In particular, a specific enzyme from the neutrophil known as neutrophil collagenase is activated by *F. nucleatum* proteinases and this causes severe and rapid destruction of the connective tissue (Bickel, 1993; Loeshe, 1996).

The invasion of *P. gingivalis* to the host tissue is a complex process involving both the bacterium and the host cells (Guthmiller and Novak, 2002). *Porphrymonas gingivalis* invades epithelial cells via endocytosis. Once invasion takes place, an immune response is triggered, with the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β). Once in the epithelial cells, *P. gingivalis* can proliferate. *Porphrymonas gingivalis* can also interfere with

the host defence mechanism by inhibiting the activation of neutrophils (Guthmiller and Novak, 2002). Neutrophils can be seen as the innate response and play a significant role in reducing the biofilm found in the sub-gingiva. Individuals who suffer from neutropenia, such as cancer patients, develop a very aggressive form of periodontitis with a rapid destruction of tissue (Loesche, 1996).

The rapid destruction of the supportive tissue surrounding the teeth is not only as result of the host defence mechanism being triggered, but also by the production of enzymes by periodontal pathogens. These enzymes are known to cause necrosis of the sub-gingival tissue (Guthmiller and Novak, 2002). *Porphrymonas gingivalis* is known to secrete a number of enzymes (Potempa and Banbula, 2000). One of the most commonly known enzymes secreted is the trypsin-like cysteine proteases known as gingipains. Gingipains are known to induce the pathways that aid in the progression of inflammation and helps with the degradation of alveolar bone. They are also responsible for the degradation of fibrin leading to increased bleeding at infected sites (Imamura, 2003).

1.2.1.4. Microbiology of periodontal diseases

Periodontal diseases are polymicrobial infections. The most common bacteria isolated from periodontal infections include Gram-negative anaerobic rod bacteria such as *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Trepenoma* spp. and *Eubacterium* spp. (Tanner and Stillman, 1993; Takarada et al., 2004; Gupti 2012).

Fusobacterium nucleatum is an anaerobic, spindle shaped rod Gram-negative bacterium. It forms white granular colonies on blood agar (Figure 1.3 a). They play a significant role in the development of periodontal diseases which is commonly and consistently isolated from deep periodontal pockets (Bolstad et al., 1996).

Porphyromonas gingivalis is a Gram-negative anaerobe that is part of the normal flora in the oral cavity, but has the ability to change into a severely destructive bacterium that flourishes in periodontal lesions (Mysak et al., 2014). It is an obligate anaerobe and therefore requires a strict anaerobic condition for its survival and growth. On blood agar it forms black colonies (Firgure 1.3 b). The dark pigment is due to the accumulation of hemin on the cell surface.

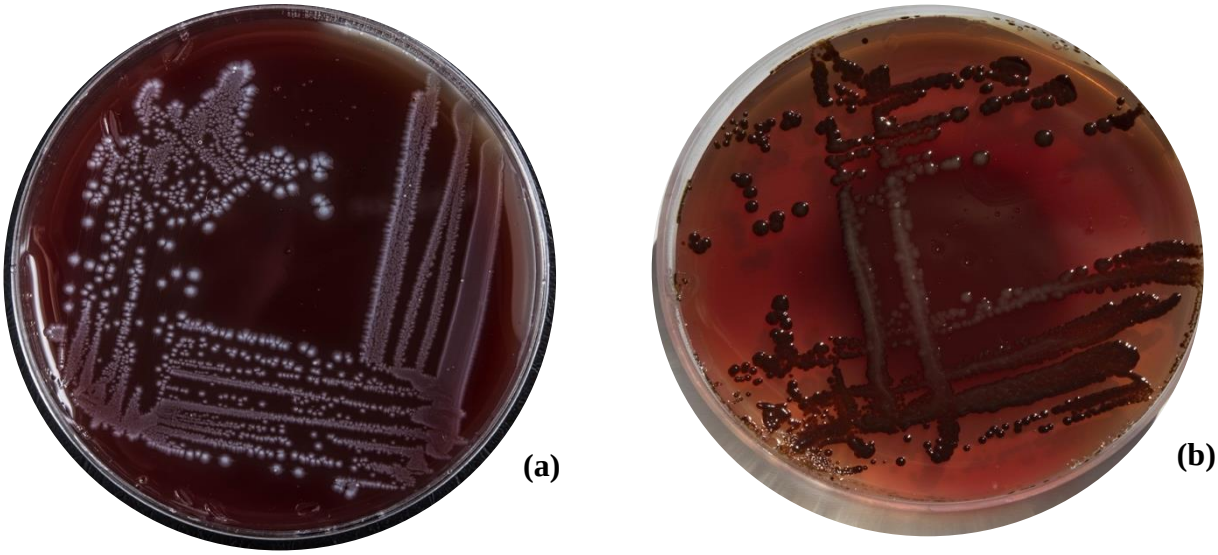


Figure 1.3: The granulated colonies of *Fusobacterium nucleatum* ATCC 25586 (a) and the black pigmented colonies of *Porphyromonas gingivalis* ATCC 33277 (b)

4.5.1. Oral candidiasis

Oral candidiasis is the most common mycotic infections that manifest in the oral cavity. The *Candida* spp. are opportunistic yeasts that reside as normal microflora and manifest in immunocompromised patients (Henley-Smith et al., 2013; Kaur et al., 2014). Oropharyngeal candidiasis has become an increasingly serious public health concern and is one of the most common and earliest fungal manifestations seen with infections involving the HIV and acquired immunodeficiency syndrome (AIDS). The worldwide prevalence of oral candidiasis in HIV and AIDS patients is 80-95% (Coogan et al., 2005; Kwamin et al., 2012). The occurrence of this fungal infection has also increased in individuals with certain predisposing factors including the use of dentures, dry mouth and hyposalivation, long term antibiotic use, smoking and people who have compromised immune systems (Garcia-Cuesta et al., 2014).

There are different types of oropharyngeal candidiasis. Some of the most common types include pseudomembranous (acute and chronic), atrophic (acute and chronic), hyperplastic (chronic) and *Candida*-associated lesions such as rhomboid glossitis and angular cheilitis (Akpan and Morgan 2002; Singh et al., 2014).

Pseudomembranous oral thrush is the most common type of thrush and is easily diagnosed. It can be described as white patches in the mouth that reveals a red inflamed mucosa when

removed. This type of candidiasis is commonly found in individuals who are immunocompromised. Acute atrophic candidiasis is mainly localized to the tongue. Patients who suffer from this type of oral candidiasis complain of a burning sensation on their tongue that usually is accompanied by a bright red colour. Chronic atrophic candidiasis is characterized by erythematous tissue that surrounds dentures. The use of dentures is a predisposing factor in this type of candidiasis. Chronic hyperplastic candidiasis is type of candidiasis that is largely associated with smoking. It is characterized by speckled white lesions. Rhomboid glossitis is characterized by lesions that are localized on the tongue. These lesions are associated with smokers and the use of aerosol inhalers. Angular cheilitis is fissuring on one or both corners of the mouth (Akpan and Morgan, 2002).

The pathogenesis of oral candidiasis depends on certain characteristics that are owed to the *Candida* spp. This include the polymorphism abilities of *Candida*, the adhesion of *Candida* to the host oral surfaces and epithelial tissues and the formation of a biofilm and contact sensing (Akpan and Morgan, 2002; Mayer et al., 2013). In addition, they produce hydrolytic enzymes which causes tissue damage and facilitates penetration of these organisms.

4.5.1.1. The *Candida spp.*

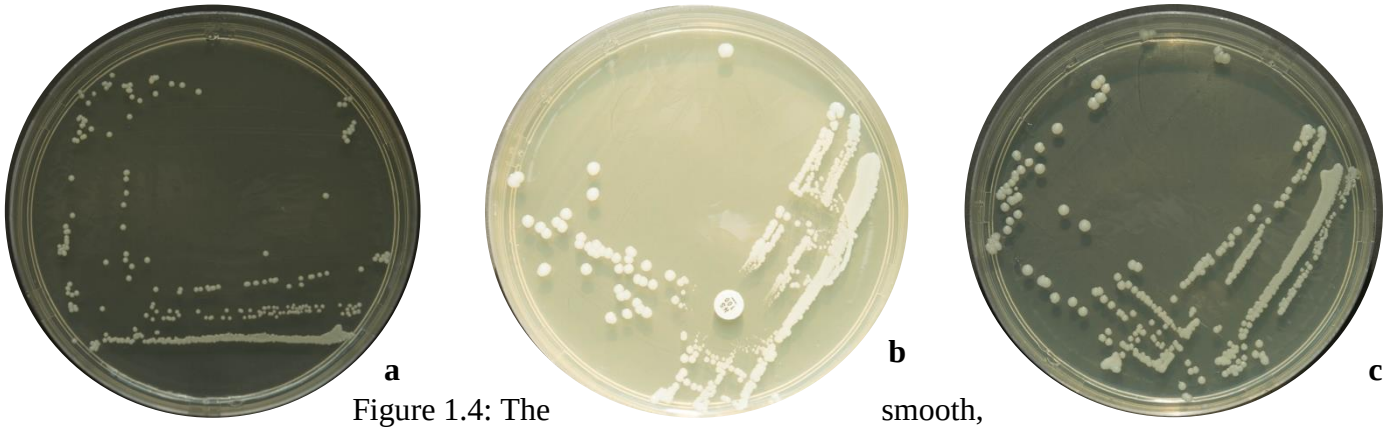
The *Candida* spp. are eukaryotic cells that have the ability to develop as both a unicellular oval cells or hyphae. The switching of this micro-organism depends on certain factors such as the pH, hyphae usually grow at pH >7 while oval cells grow at pH <6. Other factors include the presence of CO_2 which promotes hyphal growth and quorum sensing, high density of cells promotes yeast growth while low densities promote hyphal growth (Mayer et al., 2013). Both the hyphae and fungal forms of *Candida* spp. are important for pathogenesis. The hyphae form is important for the invasion into host cells and the yeast form (oval cells) is responsible for the dissemination of the disease (Jacobsen et al., 2012).

Adhesion of *Candida* to the host oral surfaces and invasions of epithelial tissues is an important step in the development of infection. The adhesion of *C. albicans* is aided by proteins known as adhesins that are secreted by the micro-organism. Adhesins aid in the linkage process and allows for the *Candida* spp. to bond to the surfaces of the oral cavity (Mayer et al., 2013). Invasion and dissemination of the *C. albicans* into the epithelial tissues occurs through two pathways, endocytosis and active penetration (Dalle et al., 2010; Naglik

et al., 2011) Endocytosis, a process that does not require viable yeats cells, occurs via the expression of invasins and other proteins that permit the engulfment of the yeast cell into the host cell (Phan et al., 2005; Dalle et al., 2010). The other pathway of active penetration of the yeast cell into the host cell is mediated via unknown mechanisms that require active and viable yeast hyphae (Dalle et al., 2010). However, it is facilitated by the hydrolytic enzymes such as proteinase, phospholipase and lipases. In addition, the ability of *Candida* spp. to produce biofilm on viable (oral mucosa) and non-viable surfaces (dentures) contributes greatly to the pathogenicity of oral candidiasis and confers higher resistance to the disease (Fanning and Mitchell, 2012). Contact sensing is a unique characteristic that triggers the production of hyphae when the fungus is in contact with a surface. The hyphae then invade the host tissue (Kumamoto, 2008).

Candida albicans is the primary *Candida* spp. isolated from the oral cavity and was thought to be the predominant cause of infectious oropharyngeal candidiasis, however, *Candida* spp. such as *Candida glabrata* and *Candida krusei* have increasingly been isolated in patients suffering from oropharyngeal candidiasis (Soll, 2002; Meurman et al., 2007).

With a few exceptions, the microscopic and macroscopic characteristics of the different types of *Candida* spp. are similar in nature. *Candida* spp. are eukaryotic yeasts that have cell walls outside the plasma membrane. The plasma membrane contains sterols in particular, ergosterol (Akpan and Morgan, 2002; Singh et al., 2014). *Candida* spp. are versatile pathogens and have the ability to metabolize glucose in aerobic and anaerobic conditions. With the exception of *Candida glabrata* (which is not dimorphic and therefore does not produce pseudohyphae), Candida spp. can also survive at temperatures greater than 37˚C and produce pseudohyphae at these temperatures (Fidel et al., 1999). The term *Candida* is derived for the latin word candid meaning white. Spherical, white, smooth colonies are formed on agar plates (Figure 1.4). Different species of Candida are descriptively similar when cultured on agar plates (Fidel et al., 1999; Raju and Rajappa, 2011).



a **b** **c**

Figure 1.4: The smooth, spherical white colonies of *Candida albicans* (a) ATCC 10231, *Candida glabrata* (b) ATCC 90030 with nystatin disc to confirm purity of culture, and *Candida krusei* (c) ATCC 14243

1.4.1. Prevalence of oral diseases

It is important to note that one of the most common infectious diseases in humans today are oral infections. The 2010 Global Burden of Diseases, Injuries and Risk Factors Study estimated that oral conditions affect as many as 3.9 billion people worldwide. According to the same study, the burden of oral diseases has increased by almost 21% between 1990 and 2010. The diseases with the most significant increases included severe periodontitis and untreated caries. Untreated caries was also the most prevalent of all oral diseases with a global prevalence of 35% (WHO, 2016).

Oral diseases in South Africa remain a huge public health problem due to the high prevalence, severity and the cost of oral healthcare (Singh, 2011). In South Africa, a review of the oral health has revealed that over 50% of children under the age of 12 suffer from dental caries and 80% of these cases go untreated (van Wyk and van Wyk, 2010).

Oral diseases are considered noncommunicable diseases (NCDs) which are diseases that cannot be spread from one person to another. Apart from having a major impact on the overall health of an individual, oral diseases are linked to many other NCDs including cardiovascular disease and diabetes (Dewhirst, 2010). Like other NCDs, oral diseases have modifiable risk factors such as an unhealthy diet as well as alcohol and tobacco use (Varenne, 2015). However, in Africa there are some other unique risk factors that also affect oral health that were not highlighted in the Global Risk study. These include the low socioeconomic

status of the majority of the African population, the lack of specialist dental facilities as well as health care workers, and the high number of HIV infected individuals (WHO, 2016).

In developed countries that offer oral health care, dental infections are the fourth most expensive disease to treat. It is no surprise that the investment in oral health by developed countries is therefore low where the budget is assigned to the treatment of more severe diseases. When oral treatment is available it is usually only in limited oral health facilities. The number of specialist dental healthcare workers are also inadequate to meet the demand where in some parts of Africa there are less than one dentist for every 150 000 people compared to developed countries where the ratio is 1: 2000 (Petersen et al., 2005).

Oral lesions in the mouth are one of the first indications of an individual suffering from HIV or AIDS (Coogan et al., 2005). Approximately 50% of HIV infected humans suffer from some form of oral disease (bacterial, fungal or viral) in the early stages of the HIV. The swelling of the parotid glands and the reduction of salivary flow seen commonly in HIV patients could increase the incidence of dental caries (Petersen et al., 2005). The increasing incidence of periodontitis and oral candidiasis is inextricably linked to dysfunctional immunity experienced during HIV/AIDS when CD4 counts are low (Coogan et al., 2005). This is a serious problem especially in South Africa where recent statistics have shown that there are approximately 6.5 million South Africans living with HIV/AIDS (Statistics South Africa, 2014).

1.5.1. Conventional treatment of oral diseases

1.5.1.1. Treatment of dental caries

Treatment of oral infections are now focused on prevention approaches to help reduce the prevalence of these diseases (Loesche, 1996). Prevention approaches to both dental caries and periodontal diseases are fairly similar. These include the use of fluoride, increasing the strength and resistance of teeth by the use of pit and fissure sealants, effectively reducing the build-up of plaque and dietary modifications (National Institute of Health, 2001).

The eradication of plaque is done via mechanical and chemical means. Correct and regular brushing techniques (morning and at night) is vital for the reduction in plaque. Tongue cleaning and interdental cleaners such as floss are also highly recommended as well as

regular professional cleaning by a dentist. Chemical means of reducing plaque include the use of products such as mouthwashes that contain stannous fluoride, chlorhexidine and povidine-iodine (Loesche, 1996; Shah, 2005).

Diet largely affects the prevalence of dental caries and is a known risk factor (Petersen, 2005). Reducing the intake of highly refined sugary foods and complex carbohydrates that are known to be cariogenic will help reduce the disease. Substituting sucrose in our diet with other sugars such as xylitol will help reduce acid production by carious bacteria who find it difficult to breakdown xylitol (Loesche, 1996). Xylitol also has anti-cariogenic abilities and has been shown to reduce caries in people that chew gum containing this sucrose substitute (Tanzer, 1995).

1.5.1.2. Treatment of periodontal diseases

Oral hygiene is an integral part in the prevention of periodontal diseases (Loesche, 1996). Preventative measures are the same as that for dental caries (Shah, 2005). Mechanical treatments such as surgical procedures and debridements are the most common method of treatment. This, coupled with the empirical use of systemic antibiotics (such as tetracycline, metronidazole and doxycycline) is the mainstay in treatment of chronic periodontal diseases (Guthmiller and Novak, 2002). In some instances, the topical use of antibiotics and antimicrobial agents such as chlorhexidine in the form of a mouthrinse are used (Loesche, 1996).

1.5.1.3. Treatment of oral candidiasis

Antifungals agents are the main components used in the treatment of oral candidiasis. Topical antifungals are favoured because there is reduced toxicity and side effects. The most commonly used antifungal is nystatin. It is effective in the treatment of uncomplicated candidiasis, however, it does have some unfavourable side effects such as the unpleasant taste and the long period of therapy. Other topical antifungals such as fluconazole have also gained popularity and have effectively treated pseudomembranous candidiasis. Miconazole is available in a gel form but also must be taken with caution because miconazole is a potent P450 enzyme inhibitor (Akpan and Morgan, 2002; Garcia-Ceusta et al., 2014). Systemic antifungals are used concurrently with topical antifungals or if individuals are intolerant of

topical antifungals. Fluconazole is a popular systemic antifungal and has a good systemic dissemination. Itraconazole is a wide-spectrum systemic antifungal that is usually used when a patient develops resistance to fluconazole (Akpan and Morgan, 2002).

1.5.1. Limitation of conventional treatments

Surgeries, debridements and tooth extractions are common methods of treatment, it is costly and requires specialist dental health care workers, both of which are lacking in developing countries such as South Africa (Peterson, 2005). Modern day oral hygiene products such as mouthwashes contain chemical agents such as chlorhexidine and ethanol. Chlorhexidine is known to have cytotoxic effects and cause tooth staining (Palombo, 2011). A relationship has also been found between ethanol found in common mouthwashes and oral cancer (McCullough and Farah, 2008). The combination of chlorhexidine with the antifungal, nystatin, renders both antimicrobial agents inactive and should not be used together. Nystatin and other topical antifungals also contain high quantities of sucrose. This could negatively impact on patients suffering from dental caries or diabetes (Akpan and Morgan, 2002).

1.5.2. Natural products as dental treatments

There are a number of natural products on the market that are incorporated into commercial products that are used in the treatment and prevention of oral diseases. Products such as Aquafresh White® & Shine Herbal Fresh® (GlaxoSmithKline) toothpaste (with extracts of fennel, eucalyptus, thyme, lavender and spearmint) and Listerine Naturals Herbal Mint® (Pfizer) (contains eucalyptus and thyme oil). Eucalyptus and thyme oil has anti-cariogenic properties while spearmint oil inhibits the formation of fungal biofilms (Dagli et al., 2015). Parodantax® (GlaxoSmithKline), another herbal toothpaste used in the treatment of gingivitis also contains medicinal plants such as *Matricaria camomilla* (reduces gingival inflammation) and *Mentha piperita* (analgesic, antiseptic and anti-inflammatory) (Groppo et al., 2008). Mouthwashes that contain *Aloe vera* are shown to be more effective than Listerine® in the eradication of oral plaque (Kaim et al., 1998). *Aloe vera* also has also shown to reduce inflammation and bleeding of the gingiva (Scherer et al., 1998).

1.6.1. Medicinal plants used to treat oral diseases

1.6.1.1. Ethnobotanical review

Around 80% of the developing world population still use traditional medicine, including medicinal plants, as their first line of treatment (Kim, 2005). Dependence on medicinal plants for the treatment of oral diseases is observed worldwide (Tapsoba and Dechamps, 2006). Studies have documented the traditional use of plants in treating oral diseases on a global scale. This includes many African countries such as Burkino Faso (Tapsoba and Dechamps, 2006); Cameroon (Agbor and Naidoo, 2015); Equatorial Guinea (Akendengue, 1992); Kenya (Ngari et al., 2014), Madagascar (Novy, 1997), Uganda (Ocheng et al., 2014) and Tanzania (Ngilisho et al., 1994) amongst others. Apart from Africa, many other countries around the world have documented ethnobotanical plant use for the treatment of oral diseases including Brazil (Alviano et al., 2008), Gautemala (Hunter and Arbona, 1995), India (Almas et al., 1995), Nepal (Manandhar et al., 1998), Palestine (Ali-Shtayeh et al., 2000), Saudi Arabia (Al-Otaibi, 2004), and South Korea (Park et al., 2003). Studies have reported on plant extracts screened against oral pathogens (Lauk et al., 2003; Park et al., 2003; Badia and Zidan, 2004; Babpour et al., 2009; Ajaybhan et al., 2010; Abdollahzadeh et al., 2011; Yim et al ., 2013; Ocheng et al., 2014).

Some studies examined the efficacy of plant essential oils against oral pathogens (Takarada et al., 2004; Gursoy et al., 2009; Benbelaid et al., 2014). Some of the studies conducted focused specifically on the antimicrobial efficacy of plants against the *Streptococcus* spp., in particular, *Streptococcus mutans* (Tichy and Novak, 1998; Morgan et al., 2001; Almeida et al., 2008; Islam et al., 2012). A few studies concentrated specifically on plant sticks used as toothbrushes (AbdElRahman et al., 2002; van Vuuren and Viljoen, 2006; Aboul-Enein, 2014). Certain studies were dedicated to the efficacy of plant materials on biofilm formation (Brighenti et al., 2012; Zhao et al., 2014; Kouidhi et al., 2015). These, however, have been poorly explored as most plant-based antimicrobial studies have focused on planktonic micro-organisms, despite the fact that bacteria that form biofilms are known to be more resistant and more prevalent in the oral cavity (Sandasi et al., 2011).

1.6.1.2. The South African perspective

It is estimated that a major portion of the South African population (around 27 million South Africans) depends on traditional medicine as the first line treatment. This is due to convenience with regards to accessibility, general affordability and the high level of knowledge by local traditional healers (Street et al., 2008). A study by Lewis et al. (2004), studied the oral healthcare knowledge and practices of traditional healers in two regions in Gauteng and found that not only were traditional healers able to correctly diagnose and differentiate between oral diseases, but more than 50% of the patients who visited these healers sought treatment regarding oral manifestations such as toothache, swollen gums and oral candidiasis. A previous review of the South African medicinal plants and antimicrobial studies (van Vuuren, 2008, van Vuuren and Holl, 2017), has shown that despite the vast collection of antimicrobial studies done on South African plants that are used to treat a variety of ailments, there is very little research being done on the antimicrobial efficacy of South African plants used to treat oral infections. Some recent studies include two separate studies on *Dodonaea viscosa*, where plant extracts have been tested against *Candida albicans*. The antifungal properties were found to be more effective than conventional mouthwashes and high concentrations of the crude extracts were found to be bactericidal against *S. mutans* (Patel and Coogan, 2008; Naidoo et al., 2012).

A study involving the antimicrobial activity of eight different South African plant species used as chewing sticks was conducted by More et al. (2008). The results showed that six out of the eight plants tested exhibited minimum inhibitory concentration (MIC) values ranging from 25.0 to 0.8 mg/ml with the best activity observed for *Euclea natalensis* which inhibited the growth of both Gram-positive and Gram-negative oral pathogens. More recently, Henley-Smith et al. (2014), investigated the antimicrobial activity of a combination of South African plant species with green tea extracts against oral pathogens. These combinations were successful in inhibiting the growth of the oral pathogens tested. It is surprising that so few studies have been conducted, considering that when examining the ethnobotanical literature, there is 132 Southern African medicinal plants used for the treatment of oral diseases. Table 1.1 highlights these medicinal plants that are used traditionally to treat oral diseases.

Table 1.1: South African medicinal plants used traditionally to treat oral disease

Scientific and family name	Common name	Uses	References
Acacia karroo Hayne., Leguminosae[a]	Sweet thorn	The gum of the plant is used for food and is taken for oral thrush.	van Wyk et al., 2009; Nielsen et al., 2012
Acacia polyacantha Willd., Leguminosae[a]	White-stem Thorn	Used to heal tooth troubles.	Watt and Breyer-Brandwijk, 1962
Acokanthera oppositifolia L. Codd., Apocynaceae[a]	Bushman's poison	The leaves of the plant are used for toothache.	Philander, 2011; Nielsen, 2012
Afzelia quanzensis sensu Welw. Leguminosae-Caesalpiniaceae	Lucky bean tree	The bark is applied to aching teeth.	Watt and Breyer-Brandwijk, 1962
Agrimonia eupatoria Dumort. Rosaceae	Church Steeples	Unspecified parts of the plant are used as a mouth gargle.	Watt and Breyer-Brandwijk, 1962
Anemone caffra E. May. ex Prtiz. Renanculaceae	Wind flower	In Transkei the ground inner roots are used for toothache and inserted directly into the hole.	Hutchings, 1996
Anemone fanninii Harv. ex Mast Renanculaceae	Giant wild anemone	The ground roots are used for toothache and inserted directly into the hole.	Hutchings, 1996
Anemone tenuifolia var. tenuifolia Harv. Renanculaceae	Black widow	Leaves treat toothache.	Philander, 2011
***Annona chrysophylla* Boj. Anonnaceae[b]**	Wild custard apple	Used as a mouthwash to relieve toothache.	Watt and Breyer-Brandwijk, 1962; **More et al., 2008**
Asclepias crispa Berg. Asclepiadaceae	Bitterwortel	Used for toothache in the Karoo.	van Wyk, 2008
***Artemisia afra* Jacq. ex Willd., Asteraceae[abc]**	Wild African worm wood	Infusions of the leaves are used for toothache.	Watt and Breyer-Brandwijk, 1962; Rabe and van Staden, 1997; Liu et al., 2001; Moffett, 2010; **More et al., 2012**; Henley-Smith et al., 2013
Azima tetracantha Lam. Salvadoraceae	Bee sting bush	Sap is used for toothache, inserted into the wound after tooth removal.	Hutchings, 1996
Barleria prionitis L. Acanthaceae	Porcupine flower	Used as a mouthwash to relieve toothache.	Watt and Breyer-Brandwijk, 1962

Scientific and family name	Common name	Uses	References
Berula erecta Huds., Coville Apiaceae[a]	Water parsnip	Fresh rhizomes are chewed as a remedy for toothache.	van Wyk, 2008; van Wyk et al., 2009; Moffett, 2010; Watt and Breyer-Brandwijk, 1962
Blepharis capensis Pers. Acanthaceae	Creeping blepharis	The leaves are used as a toothache remedy by the Xhosa.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996)
Blepharis procumbens B. Heyne ex Roth Acanthaceae	Acanthus	A paste is made of the fresh leaves and is applied locally by the Southern Sotho to relieve toothache.	Watt and Breyer-Brandwijk, 1962
Brachylaena elliptica Less. Asteraceae	Bitter leaf or Silver oak	A decoction of the leaf is used as a gargle for mouth ulcers, thrush and quinsy.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996)
Carpobrotus acinaciformis L. Bolus Alzoaceae	Elands sour fig	The leaf juice is used as a gargle for mouth sores.	Watt and Breyer-Brandwijk, 1962
***Carpobrotus edulis* L N.E.Br., Aizoaceae[ab]**	Sour fig	The strained juice from the pounded leaf is used as a gargle to treat thrush and sore throats.	Watt and Breyer-Brandwijk, 1962; Thring and Weitz, 2005; van Wyk et al., 2008; van Wyk, 2008; **Maja, 2009**; Henley-Smith et al., 2013
Chaetacme aristata E. Mey. ex Planch Ulmaceae	Thorny elm	Powdered roots are used as dental anodynes.	Hutchings, 1996
Chironia baccifera L. Gentianaceae	Bitter bush	Leaves are rubbed onto aching teeth and gums.	Thring and Weitz, 2005
Chlorophora excelsa (Welw.) Benth & Hook f. Moraceae	African teak	The latex of the plant is used for dental caries.	Watt and Breyer-Brandwijk, 1962
Cissampelos capensis Thunb. Menispermaceae	Dawidjies	The root is chewed to relieve toothache.	van Wyk, 2008
Cissampelos torulosa E. Mey. Ex Harv., Menispermaceae[a]	Kidney- leaf	Roots are chewed for toothache.	Hutchings, 1996

Scientific and family name	Common name	Uses	References
Cissus lanigera Harv. Vitaceae	Cissus	The root of the wild vine is rubbed on to the gums to relieve toothache.	Watt and Breyer-Brandwijk, 1962
***Citrus aurantifolia* (Chrstim.) Swingle Rutaceae[b]**	Key lime	The leaf is used by the Cape Malay as a mouth wash and gargle for thrush and other conditions.	Watt and Breyer-Brandwijk, 1962; **Chaudhari et al., 2012**
Clausena anisata (Willd) Hook. f. ex., Rutaceae[ac]	Horse wood	Dried ground root bark is applied directly to aching tooth.	Hutchings, 1996; Philander, 2011, van Vuuren and Viljoen, 2006
Clematis brachiate Thunb., Ranunculaceae[a]	Traveller's joy	The root is cooked with salt and used as a remedy for thrush.	Watt and Breyer-Brandwijk, 1962
Clematopsis scabiosifolia Wele. ex Hiern f. obtusiloba Ranunculaceae	Feather duster	The stick of the plant is burnt and placed directly into the hollow tooth.	Watt and Breyer-Brandwijk, 1962
Coix lacryma Jobi L. Poaceae	Jobs tears	Placed on the infant to ward off teething.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Convolvulus bidentatus Bernh. Convolvulaceae	Bind weed	The root is chewed to relieve toothache.	Watt and Breyer-Brandwijk, 1962; Moffett, 2010)
***Cotyledon orbiculata* L., Crassulaceae[ab]**	Pigs ears	Warmed leaf juice is used as drops for toothache and earache.	Watt and Breyer-Brandwijk, 1962; Thring and Weitz, 2005; **Maja,** 2009; van Wyk et al., 2009; Moffett, 2010; Philander, 2011
Crabbea hirsuta Harv. Acanthaceae	Prickle head	Used for toothache.	Hutchings, 1996
Crabbea nana Nees subsp. Galpinii Acanthaceae	Sheep tree	The Zulu and Xhosa use the leaves as a toothache remedy and for painful esophageal cancer.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Croton gratissimus Burch., Euphorbiaceae[ac]	Lavender croton	The charred and powdered bark is used for bleeding gums.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Cyphostemma lanigerum Harv., Vitaceae[a]	Wild grape	The root is rubbed on the gums for toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Lin et al., 2001
Cyphostemma setosum Roxb., Vitaceae[a]	Cobas	Useful against mouth disease.	Watt and Breyer-Brandwijk, 1962

Scientific and family name	Common name	Uses	References
Dalbergia obovata E. Mey. Leguminosae[a]	Climbing flat bean	A paste of charred and powdered stems is mixed with water and used for mouth sores in infants.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Rabe and van Staden, 1997; Henley-Smith et al., 2013
***Datura stramonium* L., Solanaceae[ab]**	Jimson weed	The fresh green fruit is applied locally for toothache.	Watt and Breyer-Brandwijk, 1962; Thring and Weitz, 2005; **Maja, 2009**; van Wyk et al., 2009
***Dicerocaryum senecio* Koltzsch Abels Pedaliaceae[b]**	Boot protectors	Used as a chewing stick to relieve toothache.	**More et al., 2008**
***Dichrostachys cinerea* L., Fabaceae[ab]**	Sickle bush	Used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962; **Adejumo et al., 2008**
Dicoma anomala Sond. Asteraceae	Fever bush	Small amounts of the root are chewed for toothache.	Hutchings, 1996; Moffett, 2010
***Dodonaea viscosa* Jacq., Sapindaceae[ab]**	Hopbush	Plant is gargled for oral thrush or the twigs are chewed to clean teeth.	Watt and Breyer-Brandwijk, 1962; **Patel and Coogan, 2008**; van Wyk 2008, van Wyk et al., 2009; **Naidoo et al., 2012**; Henley-Smith et al., 2013
Elionurus muticus Nees. Poaceae	Wire grass	The root is chewed to relieve toothache.	Moffett, 2010
***Englerophytum magalismontanum* Sonder., Sapotaceae[ab]**	Stem fruit	Used as a chewing stick.	**More et al., 2008**
Entada phaseoloides G. Forst. Fabaceae	Matchbox bean	Given to infants during teething.	Watt and Breyer-Brandwijk, 1962
Equisetum ramosissimum Desf. Equisetaceae	Southern giant horsetail	Sap from plant is used to relieve toothache and applied to wounds after tooth extraction.	Kelmanson et al., 2000
***Erythrina lysistemon* Hutch., Fabaceae[ab]**	Coral tree	The Venda use the bark for toothache.	**More et al., 2008**

Scientific and family name	Common name	Uses	References
Dalbergia obovata E. Mey. Leguminosae[a]	Climbing flat bean	A paste of charred and powdered stems is mixed with water and used for mouth sores in infants.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Rabe and van Staden, 1997; Henley-Smith et al., 2013
***Datura stramonium* L., Solanaceae[ab]**	Jimson weed	The fresh green fruit is applied locally for toothache.	Watt and Breyer-Brandwijk, 1962; Thring and Weitz, 2005; **Maja, 2009**; van Wyk et al., 2009
***Dicerocaryum senecio* Koltzsch Abels Pedaliaceae[b]**	Boot protectors	Used as a chewing stick to relieve toothache.	**More et al., 2008**
***Dichrostachys cinerea* L., Fabaceae[ab]**	Sickle bush	Used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962; **Adejumo et al., 2008**
Dicoma anomala Sond. Asteraceae	Fever bush	Small amounts of the root are chewed for toothache.	Hutchings, 1996; Moffett, 2010
***Dodonaea viscosa* Jacq., Sapindaceae[ab]**	Hopbush	Plant is gargled for oral thrush or the twigs are chewed to clean teeth.	Watt and Breyer-Brandwijk, 1962; **Patel and Coogan, 2008**; van Wyk 2008, van Wyk et al., 2009; **Naidoo et al., 2012**; Henley-Smith et al., 2013
Elionurus muticus Nees. Poaceae	Wire grass	The root is chewed to relieve toothache.	Moffett, 2010
***Englerophytum magalismontanum* Sonder., Sapotaceae[ab]**	Stem fruit	Used as a chewing stick.	**More et al., 2008**
Entada phaseoloides G. Forst. Fabaceae	Matchbox bean	Given to infants during teething.	Watt and Breyer-Brandwijk, 1962
Equisetum ramosissimum Desf. Equisetaceae	Southern giant horsetail	Sap from plant is used to relieve toothache and applied to wounds after tooth extraction.	Kelmanson et al., 2000
***Erythrina lysistemon* Hutch., Fabaceae[ab]**	Coral tree	The Venda use the bark for toothache.	**More et al., 2008**

Scientific and family name	Common name	Uses	References
***Euclea divinorum* Hiern. Ebenaceae[b]**	Diamond shaped euclea	Root decoctions are used for toothache.	Hutchings, 1996; **More et al., 2008**
***Euclea natalensis* F. White. subsp. Ebenaceae[b]**	Natal Guari	This plant with *Glycyrrihiza glabra* roots are mixed and rubbed onto gums of teething children, also used for toothache and as a mouthwash.	Watt and Breyer-Brandwijk, 1962; **More et al., 2008**; van Wyk, 2011; Henley-Smith et al., 2013
Eucomis punctate L'Her. Hyacinthaceae[a]	Giant pineapple flower	Given to infants during teething.	Watt and Breyer-Brandwijk, 1962
Euphorbia gorgonis A. Berger. Euphorbiaceae	Golden lace cactus	The latex of the plant is used for toothache.	Watt and Breyer-Brandwijk, 1962
Euphorbia mauritanica L. var. Feuton Euphorbiaceae	Milkbush	A warm infusion of the root is used as a mouthwash for toothache.	van Wyk, 2008, Philander, 2011
Euphorbia systyloides Pax Euphorbiaceae	African milk plant	The Xhosa use the latex of the plant as a toothache remedy.	Watt and Breyer-Brandwijk, 1962
Gnidia cuneata Meisn. Thymelaeaceae	Fever bush	The Xhosa uses powdered roots for toothache.	Hutchings, 1996
Galenia Africana L. Aizoaceae	Yellow bush	Chewed by the Hottentot to relieve toothache.	Watt and Breyer-Brandwijk, 1962; van Wyk, 2008; Philander, 2011
Galium dregeanum Sond. Rubiaceae	Common marsh	Decoction of the root is used as a wash for teeth.	Watt and Breyer-Brandwijk, 1962
Galium rotundifolium L. Rubiaceae	Lanceleaf wild licorice	Decoction of the root is used as a wash for teeth.	Watt and Breyer-Brandwijk, 1962
Garulem bipinnatum Pax Rubiaceae	Snake wood	The root is used to prepare a mouthwash.	Watt and Breyer-Brandwijk, 1962
Gazania krebsiana Less. Asteraceae	Grassland gazania	A hot decoction of the root is held in the mouth to relieve toothache.	Watt and Breyer-Brandwijk, 1962; Moffett, 2010

Scientific and family name	Common name	Uses	References
Gethyllis afra Linn. Amaryllidaceae	Herb of milk	Flower decoction is used for toothache.	Louw et al., 2002
***Glycyrrhiza glabra* L. Amaryllidaceae**[b]	Licorice	Used for oral thrush.	Shai et al., 2008; **Sedighinia et al., 2012;** Henley-Smith et al., 2013
Gnidia capitata L. f. Thymelaeaceae	Little poison bush	Poultice applied to jaw to relieve toothache.	Watt and Breyer-Brandwijk, 1962; Moffett, 2010
Gnidia chrysantha (Saulms-Laub.) Gilg. Thymelaeaceae	Yellow heads	The root is applied locally to painful hollow teeth.	Watt and Breyer-Brandwijk, 1962
Gnidia cuneata Meisn. Thymelaeaceae	Little curry bush	Used for toothache where the powdered root is either inserted into the cavity or the root is chewed.	Watt and Breyer-Brandwijk, 1962
Heteropyxis natalensis Harv., Myrtaceae[a]	Lavender tree	Used by the Venda for bleeding gums.	Watt and Breyer-Brandwijk, 1962
Hydrocotyle bonariensis Lam. Apiaceae	Pennywort	The juice of the plant is used as a gargle for thrush and sore throats.	Watt and Breyer-Brandwijk, 1962
***Hymenocardia acida* Tul. Tiliaceae**[b]	Large red-heart	The ashed root is applied to mouth sores.	Watt and Breyer-Brandwijk, 1962; **Oshomoh and Idu, 2012**
Indigofera patens Eckl. and Zeyh. Fabaceae	Creeping indigo	The powdered root is applied locally to the hollowed tooth for the relief of toothache by the Xhosa.	Watt and Breyer-Brandwijk, 1962
Indigofera tinctoria Gouan. Leguminosae	True indigo	The juice of the leaf or a poultice of the leaf is applied to tooth wounds.	Watt and Breyer-Brandwijk, 1962
Kalanchoe thyrsiflora Harv. Crassulaceae	Paddle plant or Flapjacks	Poultice applied to jaw to relieve toothache.	Moffett, 2010
Knowltonia vesicatoria Hook. L. Alston. Ranunculaceae	Blister leaf	Aerial parts of the plant are used for tooth pain.	Nielsen et al., 2012

Scientific and family name	Common name	Uses	References
Piliostigma thonningii Hochst. Fabaceae	Camel's foot	The bark is used as a mouth wash.	Watt and Breyer-Brandwijk, 1962
Plantago major L. Plantaginaceae	Plantain	The leaf sap is applied directly for mouth.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Plumbago zeylanica L. Plumbaginaceae	Doctor bush	A root decoction mixed with milk is given to infants for oral inflammation.	Watt and Breyer-Brandwijk, 1962
Polygala myrtifolia L. Polygolaceae	Myrtle	The leaf is used for oral thrush.	Henley-Smith et al., 2013
Pycnostachys reticulate (E. Mey.) Benth. Labiatae	Blue soldier salvia	A root decoction is taken for aching teeth.	Hutchings, 1996
Ricinus communis L. Euphorbiaceae	Castor oil plant	Pounded root is applied to painful teeth.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Maroyi, 2013
***Rubia cordifolia* L. Rubiaceae**[b]	Common mader	A decoction of the plant is used as a mouthwash by the Southern Sotho.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; **Thombre et al., 2012**; Maroyi, 2013
Rubus pinnatus Willd. Rosaceae	Blackberry	Roots are used for toothache, either as warm water gargles or ground and inserted directly into the cavity.	Hutchings, 1996
Rubus rigidus Sm. Rosaceae	Wild bramble	Root decoction is used as a gargle for toothache.	Hutchings, 1996
Rumex acetosella subsp.	Sheep sorrel	Leaf decoction is used for toothache.	Hutchings, 1996
Rumex nepalensis L. Polygonaceae	Nepal dock	The roasted root is placed around a tooth abscess.	Watt and Breyer-Brandwijk, 1962
Rumex sagittatus Thbg. Polygonaceae	Climbing dock	Roots are used for toothache.	Hutchings, 1996
Ruta graveolens L. Rutaceae	Common rue	Bruised leaves are placed in hollow teeth to relieve toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Thring and Weitz, 2005
Sansevieria hyacinthoides Steud. Dracaenaceae[a]	Mother-in-laws tongue	Leaves and rhizomes are used for toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; van Wyk et al., 2009; Nielsen et al., 2012
Sapium ellipticum (Hochst.) Pax. Euphorbiaceae	Jumping seed tree	Root decoction is used as a mouthwash for toothache.	Hutchings, 1996

Scientific and family name	Common name	Uses	References
Sapium integerrimum (Hochst.) Leonard. Euphorbiaceae	Duiker berry	Root decoction is used as a mouthwash for toothache.	Hutchings, 1996
Securidaca longepedunculata Fresen. Polygalaceae	Violet tree	The Sotho chew the root of the plant for relieve of toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; van Wyk et al., 2009
Senecio coronatus (Thunb.) Harv. Compositae	Woolly grassland senecio	Infusion of roots is drunk for toothache.	Moffett, 2010
Senecio inornatus DC. Astereceae	Tall marsh senacio	Infusion of roots is drunk for toothache.	Moffett, 2010
Senecio serratuliodes L. Compositae	Two-day cure	The Zulu in small doses for swollen gums uses this plant with other Combretaceae plant species.	Watt and Breyer-Brandwijk, 1962
Siphonochilus aethiopicus Schweinf., Zingiberaceae[a]	Natal ginger	Used to treat oral thrush and other *Candida* infections.	Henley-Smith et al., 2013
Solanum aculeastrum Dunal Solanaceae	Soda apple	Root is powdered, boiled in water, cooled, strained and bottled. Gargle three times a day for toothache. The plant can also be placed into the wound after tooth extraction.	Hutchings, 1996; Felhaber, 1997; Philander, 2011
Solanum aculeatissimum Jacq. Solanaceae	Dutch eggplant	The smoke of the burning fruit is used for toothache.	Hutchings, 1996
Solanum capense L. Solanaceae	Nightshade	Powdered fruit is inserted into cavities and is also applied to wounds after tooth extraction.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Felhaber, 1997
Solanum hermannii Dunal Solanaceae	Devils apple	Steam from fruit decoction is used are used for toothache.	Hutchings, 1996
Solanum incanum L. Solanaceae	Bitter apple	The Southern Sotho use unspecified parts of the plant as a toothache remedy.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Solanum indicum L. Solanaceae	Poison berry	The vapour of the burning seed is used to relieve toothache.	Watt and Breyer-Brandwijk, 1962
Solanum lichtensteinii Willd. Solanaceae	Bitter apple	Used as a remedy for toothache and sore throats.	Moffett, 2010

Scientific and family name	Common name	Uses	References
Solanum melongena Mill. Solanaceae	Eggplant	The leaf has been used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962
Solanum merkeri Dammer var. ruandense Solanaceae	Healing-leaf tree	The root is used for swollen gums.	Watt and Breyer-Brandwijk, 1962
***Solanum panduriforme* E. Mey. Solanaceae**[b]	Bitter apple	The roots are used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; **More et al., 2008**; Moffett, 2010
Solanum sodomaeum L. Solanaceae	Devils apple	The crush leaf is held in the mouth.	Watt and Breyer-Brandwijk, 1962
Solanum tomentosum Sendtn. Solanaceae	Bitter apple	The roots are used as a toothache remedy.	Moffett, 2010
Spirostachys Africana Sond., Euphorbiaceae[a]	African mahogany tree	Used as a toothache remedy.	Philander, 2011
Spilanthes mauritiana DC. Asteraceae	Gourd	The leaves or flower of the plant is chewed to relieve toothache, pyorrhea and mouth sores.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Synadenium cupulare Boiss. Wheeler. Euphorbiaceae	Weeping tree	The latex is inserted into painful hollow teeth.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Tarchonanthus camphoratus L., Asteraceae[ac]	Camphor bush	Infusion of the leaves are used for toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Moffett, 2010
Tecoma capensis Lindl., Bignoniaceae[a]	Cape honey suckle	Powdered bark is rubbed into bleeding gums.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Tetradenia riparia Hochst., Lamiaceae[ac]	Ginger bush	Leaf infusions are used for mouth ulcers.	van Wyk et al., 2009
Teucrium africanum Thunb. Labiatae	Cancer bush	Leaf paste is used for toothache.	van Wyk, et al., 2008
Toddalia aculeata Pers. Rutaceae	Climbing orange	Used by Venda herbalist, the leaf is chewed or a thick poultice is placed on the tooth to relieve toothache.	Watt and Breyer-Brandwijk, 1962
***Vernonia mespilifolia* Less. Compositae**[b]	Black tea bush	Warm infusions are taken as a mouthwash for toothache.	Hutchings, 1996**; Anibijuwon et al., 2012**

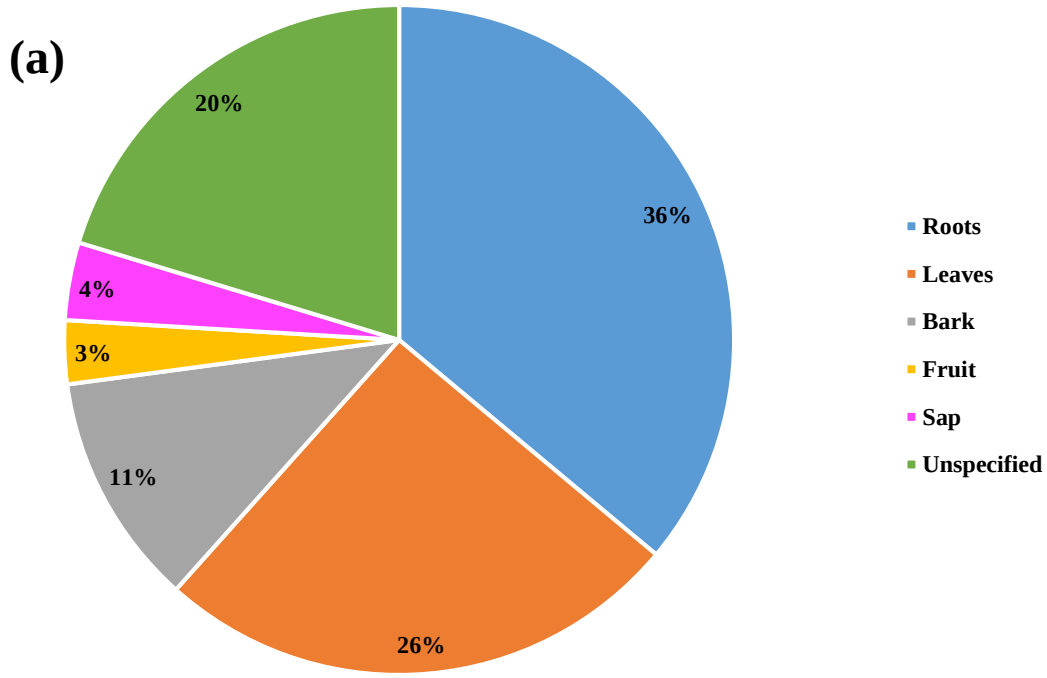
Scientific and family name	Common name	Uses	References
Warburgia salutaris G. Bertol., Canellaceae[a]	Pepper bark tree	The bark and leaf of the plant are used for oral and esophageal thrush.	Philander, 2011; van Wyk, 2011; Henley-Smith et al., 2013
Ximenia Americana L. Olacaceae	Yellow plum	Decoction of the leaf and twig are used as a mouthwash for toothache. The oil is also placed on a tooth before extraction.	Watt and Breyer-Brandwijk, 1962
Zanthoxylum capense Harv., Rutaceae[a]	Small knobwood	The powdered root, bark and leaf are used for toothache and dental plaque.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Adeniji et al., 1998; van Wyk et al., 2009; van Wyk, 2011
Zanthoxylum thunbergii DC. var. obtusifolia Harv. Rutacaea	Fever tree	Used as a toothache remedy.	Hutchings, 1996
Ziziphus mucronata Willd., Rhamnaceae[a]	Buffalo thorn	Roots are used for toothache.	Hutchings, 1996

a: Plants shaded in grey have been used in this study.
b: Plants and references in bold refer to antimicrobial efficacy studies that have been done on the plant against oral pathogens.
c: Selected aromatic plants have additionally undergone hydrodistillation to produce essential oils.

1.7. Summary of ethnobotanical review

As discussed previously, Table 1.1. is a breakdown of the 132 South African plants used traditionally to treat oral diseases. The table provides information on the plants used traditionally to treat oral diseases including the various parts of the plants used and how these plants were used. This table also highlights the plants that have been tested before against various oral pathogens (17.00%). These plants are marked in bold and emphasize the need for further research.

Figure 1.5. summarizes the information that is found in Table 1.1. Figure 1.5. (a) shows the most popular parts of plants used and Figure 1.5. (b) shows the mode of administration used traditionally. The most popular part of the plant used was the roots (36%) followed by leaves (26%). The most popular mode of administration of a plant was a plant decoction. A decoction is when the plant material is mixed with a certain amount of water and the mixture is brought to boiling point



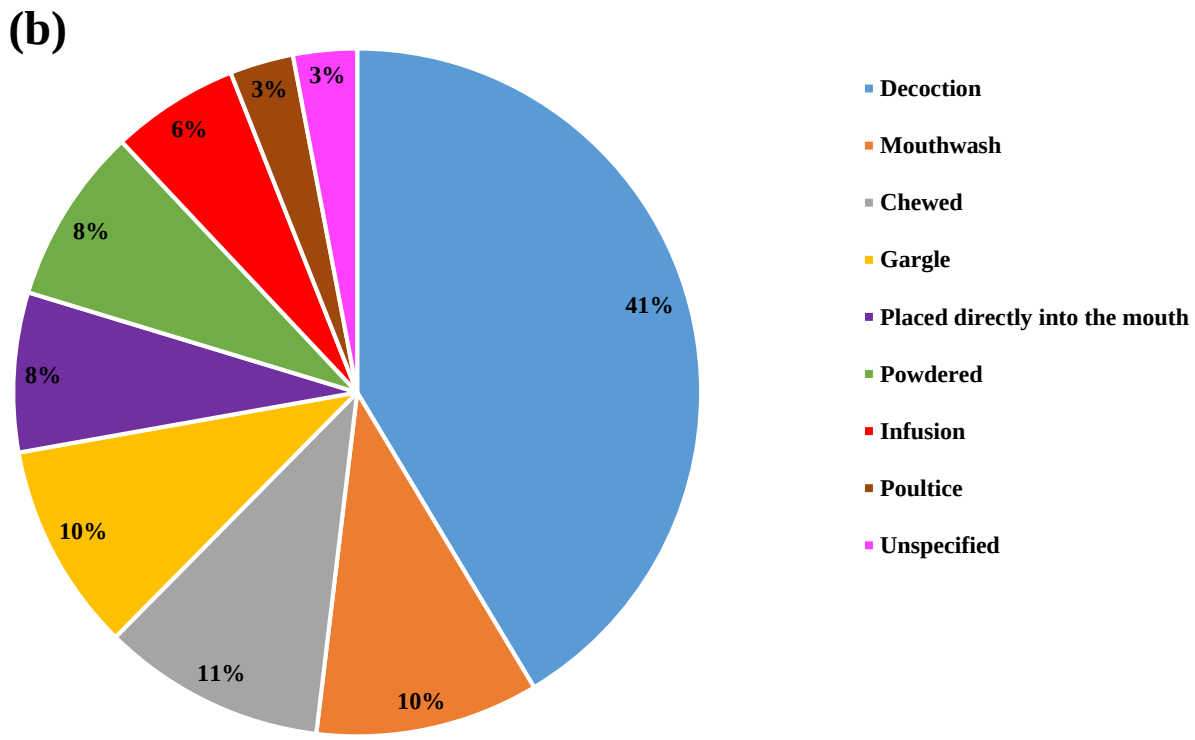


Figure 1.4: Summary of South African plants (Table 1.1) used traditionally to treat oral pathogens a. parts of the plants used b. modes of administration of plants

1.8. Aims and objectives of this study

This study aims to validate the use of South African medicinal plants used traditionally in the treatment of oral diseases. With consideration of the ethnobotanical text this study aims to provide a detailed account of the antimicrobial properties of selected South African plants, its effect on *Streptoccocus mutans* biofilm formation and the toxicity profiles of these plants. To achieve these aims the following objectives were set;

- To prepare aqueous and organic extracts, and essential oils of the relevant plant material collected from the Walter Sisulu Botanical Gardens or procured from Random Harvest Nursery.

- To evaluate the antimicrobial efficacy of plants used traditionally to treat oral infections using the minimum inhibitory concentration (MIC) assay.
- To evaluate the efficacy of selected plants on *Streptococcus mutans* biofilm formation.
- To evaluate the toxicity profiles of selected plants using the brine shrimp lethality assay (BSLA).

Chapter 2

Planktonic antimicrobial activity

2.1. Introduction

The ethnobotanical review revealed 132 plants that are traditionally used to treat oral ailments (Chapter 1 Table 1.1). A review of South African medicinal plants and antimicrobial studies has shown that despite the vast collection of antimicrobial studies being done on South African plants that are used to treat a variety of ailments (van Vuuren, 2008; van Vuuren and Holl, 2017), there is very little research undertaken on the antimicrobial efficacy of South African plants used to treat oral infections (More et al., 2008; Patel and Coogan, 2008; Naidoo et al., 2012, Henley-Smith et al., 2014). Consequently, the aim of this chapter is to investigate the antimicrobial efficacy of 31 plant species, traditionally used to treat oral infections, against nine pathogens representing the three most common oral infections: dental caries, periodontal diseases and oral candidiasis.

2.2. Material and methods

2.2.1. Collection and preparation of plant material

Collection of plant material was in accordance with current legislation of the Biodiversity and Bioprospecting act. The majority of the plant samples were identified and collected from the Walter Sisulu Botanical Gardens with permission and help from Mr. Andrew Hankey, specialist horticulturalist, who helped with plant identification and authentication. Some plant species were procured from Random Harvest Indigenous Nursery. Even though the ethnobotanical review revealed 132 plants used traditionally to treat oral infections, 31 plants were selected based on the availability. Where possible, the parts of the plant (roots, rhizomes, bark, leaves) were collected according to the part of the plant traditionally used. Voucher specimens were prepared and housed in the Department of Pharmacy and

Pharmacology. The bulk collected plant material was separated (roots, rhizomes, leaves, bark or stems) and left to dry at room temperature. Thereafter, these plants were ground to a fine powder using the Fritsch Pulverisette grinder (Labotec). Table 2.1 highlights the plant species used in this study with their voucher numbers.

Table 2.1: Voucher specimen numbers, plant parts used and extract yields of plants investigated

Plant species	**Part of plant collected**	**Collection sight and voucher number**	**Plant sample yields (%)**		
			Organic extract[a]	**Aqueous extract[a]**	**Essential oil**
Acacia karroo	Leaves	WSBG[b] 1.1	11.87	4.83	-
	Bark	WSBG[b] 1.2	12.71	8.02	-
Acacia polyacantha	Leaves	WSBG[b] 2.1	8.13	8.39	-
	Stems	WSBG[b] 2.2	4.12	10.04	-
Acokanthera oppositifolia	Leaves	WSBG[b] 3.1	19.21	4.13	-
	Stems	WSBG[b] 3.3	8.48	4.91	-
Artemisia afra	Leaves	WSBG[b] 4.1	16.39	7.22	-
	Stems	WSBG[b] 4.2	4.50	9.02	-
	Leaves	WSBG[b]EO4	-	-	0.78
Berula erecta	Leaves	WSBG[b] 5.1	4.25	16.61	-
	Stems	WSBG[b] 5.2	6.29	13.27	-
	Rhizomes	RHN[c,] 5.3	11.31	8.70	-
Carpobrotus edulis	Leaves	WSBG[b] 6.1	4.46	3.87	-
Cissampelos torulosa	Leaves	WSBG[b] 7.1	8.79	15.60	-
	Stems	WSBG[b] 7.2	16.68	14.32	-
Clausena anisata	Leaves	WSBG[b] 8.1	14.81	16.41	-
	Bark	WSBG[b] 8.2	8.43	4.99	-
	Twigs	WSBG[b] 8.3	3.72	8.36	-
	Leaves	WSBG[b]EO8	-	-	0.89
Clematis brachiate	Leaves	WSBG[b] 9.1	21.02	12.26	-
	Stems	WSBG[b] 9.2	5.23	0.76	-
	Flowers	WSBG[b] 9.3	13.63	22.40	-
	Leaves	RHN[c,] 9.4	7.61	20.40	-
	Roots	RHN[c,] 9.5	3.12	13.74	-
Cotyledon orbiculata	Leaves	WSBG[b] 10.1	9.49	15.89	-
Croton gratissimus	Leaves	WSBG[b] 11.1	12.01	4.07	-
	Bark	WSBG[b] 11.2	4.78	2.44	-
	Leaves	WSBG[b] EO11	-	-	1.42
Cyphostemma lanigerum	Leaves	WSBG[b] 12.1	10.23	13.43	-
	Stems	WSBG[b] 12.2	2.75	7.73	-
Cyphostemma setosum	Leaves	WSBG[b] 13.1	13.07	12.99	-
	Stems	WSBG[b] 13.2	4.57	11.30	-

Plant species	Part of plant used	Collection sight and voucher number	Plant sample yields (%)		
			Organic extract[a]	Aqueous extract[a]	Essential oil
Dalbergia obovata	Leaves	WSBG[b] 14.1	2.91	11.43	-
	Stems	WSBG[b] 14.2	2.94	14.32	-
Datura stramonium	Leaves	WSBG[b] 15.1	4.97	14.44	-
	Stems	WSBG[b] 15.2	3.42	9.82	-
	Fruit	WSBG[b] 15.3	8.57	6.80	-
Dichrostachys cinerea	Leaves	WSBG[b] 16.1	13.33	8.52	-
	Stems	WSBG[b] 16.2	3.04	6.35	-
Dodonaea viscosa	Leaves	WSBG[b] 17.1	11.35	7.77	-
	Stems	WSBG[b] 17.2	10.53	7.59	-
Englerophytum magalismontanum	Leaves	WSBG[b] 18.1	16.34	32.06	-
	Bark	RHN[c,] 18.2	5.95	10.71	-
Erythrina lysistemon	Leaves	WSBG[b] 19.1	15.89	7.70	-
	Bark	WSBG[b] 19.2	9.83	8.09	-
Eucomis punctata	Leaves	RHN[c,] 20.1	5.09	9.61	-
Heteropyxis natalenesis	Leaves	WSBG[b] 21.1	16.14	6.14	-
	Bark	WSBG[b] 21.2	6.13	7.77	-
Myrothamnus flabellifolius	Leaves	WSBG[b] 22.1	8.45	8.62	-
	Leaves	WSBG[b] EO22	-	-	0.73
Sansevieria hyacinthoides	Leaves	WSBG[b] 23.1	3.02	5.77	-
	Rhizomes	RHN[c,] 23.2	4.69	10.08	-
	Leaves	RHN[c,] 23.3	3.94	6.42	-
Siphonochilus aethiopicus	Leaves	WSBG[b] 24.1	13.17	12.32	-
	Stems	WSBG[b] 24.2	3.40	9.71	-
	Rhizomes	WSBG[b] 24.3	4.60	8.01	-
Spirostachys africana	Leaves	WSBG[b] 25.1	16.91	19.67	-
	Stems	WSBG[b] 25.2	7.31	14.56	-
Tarchonanthus camphoratus	Leaves	WSBG[b] 26.1	3.94	23.56	-
	Bark	WSBG[b] 26.2	4.17	31.62	-
	Leaves	WSBG[b] EO26	-	-	1.34
Tecoma capensis	Leaves	WSBG[b] 27.1	14.09	8.36	-
	Stems	WSBG[b] 27.2	3.43	4.02	-
Tetradenia riparia	Leaves	WSBG[b] 28.1	7.40	18.41	-
	Stems	WSBG[b] 28.2	1.51	12.30	-
	Leaves	WSBG[b] EO28	-	-	1.43
Warburgia salutaris	Leaves	WSBG[b] 29.1	13.91	14.21	-
	Bark	WSBG[b] 29.2	15.07	7.89	-
	Twigs	WSBG[b] 29.2	3.49	8.39	-

Plant species	Part of plant used	Collection sight and voucher number	Plant sample yields (%)		
			Organic extract[a]	Aqueous extract[a]	Essential oil
Zanthoxylum capense	Leaves	WSBG[b] 30.1	7.86	4.59	-
	Stems	WSBG[b] 30.2	8.15	8.01	-
Ziziphus mucronata	Leaves	WSBG[b] 31.1	11.43	7.45	-
	Stems	WSBG[b] 31.2	9.00	5.12	-

a: Percentage yield expressed for organic (dichloromethane: methanol, 1:1 v/v) aqueous extracts and essential oils per dry weight of grounded plant material weighed.
b: Walter Sisulu Botanical Gardens, Johannesburg South Africa.
c: Random Harvest Indigenous Nursery, Johannesburg South Africa.

2.2.2. Extraction of plant material

Two types of extracts (aqueous and organic), were prepared for this study. The method of obtaining these extracts is depicted in Figure 2.1. Dichloromethane and methanol (1:1) was the solvent chosen to extract both polar and non-polar compounds from the whole extracts. Organic plant extracts were prepared by immersing the fine powdered dry plant material in a 1:1 of dichloromethane and methanol (CH2 Cl: MeOH) and left in the shaker incubator at 37˚C for 24 h. Subsequently, the plant samples were filtered using sterile cotton wool this was repeated twice with the same plant material as more solvent was added after the first filtration and placed back in the shaker incubator for 24 h. The supernatant retrieved after both rounds of filtration was then left in the fume hood to allow for the solvent to evaporate and dry the organic plant sample. The traditional way of preparing plant samples usually includes the use of water (decoctions or infusions) therefore the need to include aqueous plant samples in this study was important. Aqueous plant extracts were prepared by immersing the macerated plant material in sterile distilled water (WFI). This was kept at ambient temperature overnight. The supernatant was retrieved and stored at -80˚C before being lyophilized. The lyophilized plant sample was exposed to UV light to ensure no microbial contamination of the samples. Percentage yield of extracts were calculated for both organic and aqueous extracts (Table 2.1).

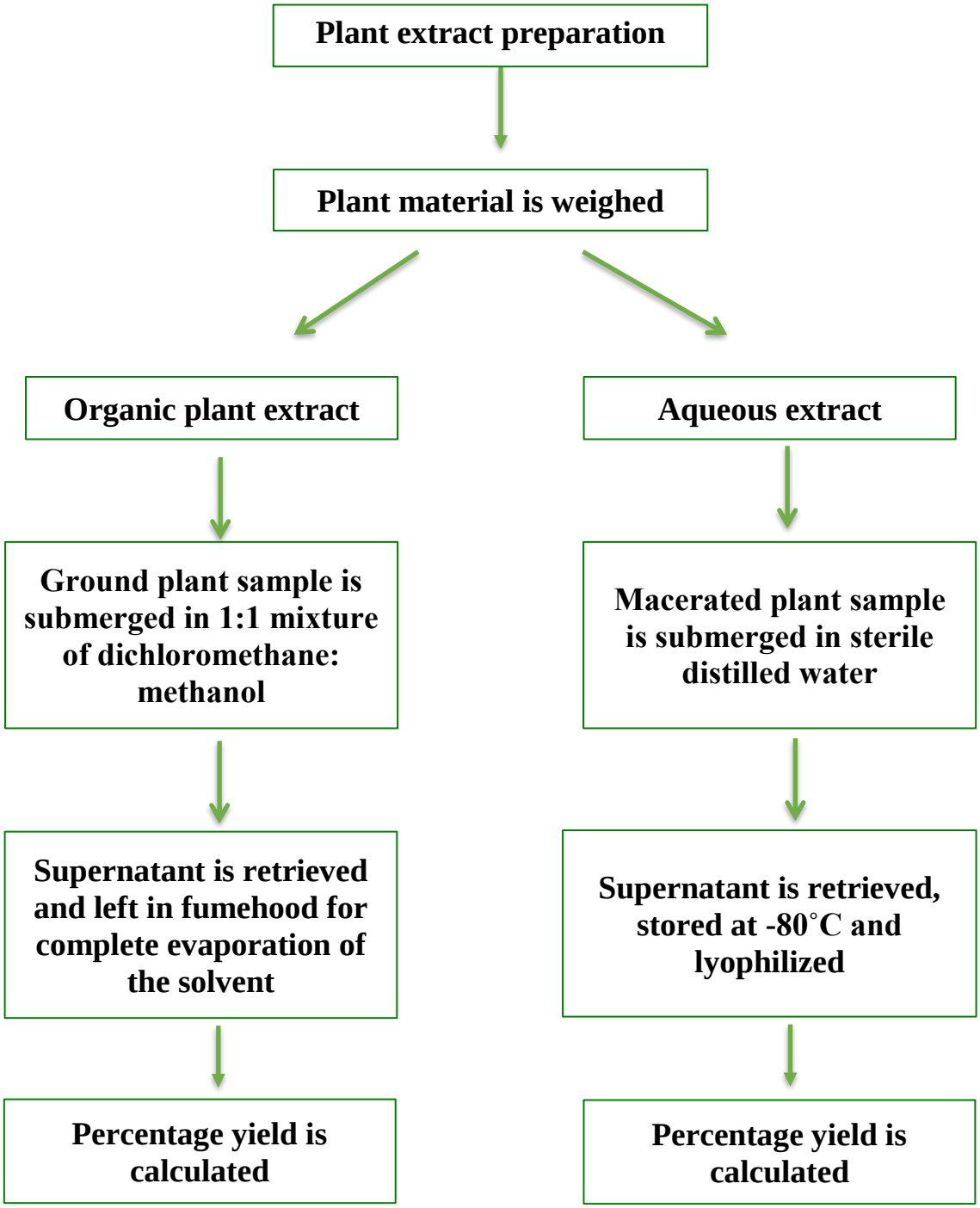


Figure 2.1: A schematic representation of the method used to prepare aqueous and organic extracts.

2.2.3. Preparation of essential oils (hydro-distillation)

Essential oils were obtained from the plant samples that were aromatic in nature. Six of the plant species in this study were selected, based on availability of plant material, for hydro-distillation namely, *Artemisia afra, Clausena anisata, Croton gratissimus, Myrothamnus flabellifolia, Tarconanthus camphoratus* and *Tetradenia riparia.* The leaves of all these plants were subject to hydro-distillation using a Clevenger-type apparatus (Figure 2.2). Fresh leaf material was weighed and packed into five litre round bottom flasks. Water was then added to the flask and the flask heated using a heating mantel (100˚C). The heating mantle allowed for the release of the essential oil. The collecting column was cooled down with the aid of a condenser with cool water running through it. After three hours the condensed essential oil was retrieved, weighed and stored in amber bottles at 4˚C. Percentage yields were calculated for the essential oils (Table 2.1).



Figure 2.2: Hydro-distillation of essential oil using a Clevenger-type apparatus.

2.2.4. Culturing of pathogens

Bacterial and yeast cultures were selected based on their ability to cause oral infections. Test micro-organisms used were American Type Culture Collection (ATCC) strains procured from Davies Diagnostics. Micro-organisms selected for the study included four Gram-positive cariogenic bacteria; *Streptococcus mutans* (ATCC 25175), *Streptococcus sangius (*ATCC 10556), *Lactobacillus acidophilus* (ATCC 4356) and *Lactobacillus casei* (ATCC 344), two Gram-negative periodontal bacteria;

Porphyromonas gingivalis (ATCC 33277) and *Fusobacterium nucleatum* (ATCC 25586) and lastly, three yeast strains; *Candida albicans* (ATCC 10231), *Candida glabrata* (ATCC 90030) and *Candida krusei* (ATCC 14243) which causes oral candidiasis. These micro-organisms were cultured in the respective media and incubation conditions as represented in Table 2.2. The media used in this study were designed specifically for the growth of these micro-organisms.

Table 2.2: Micro-organisms used in this study

Pathogen	**Macroscopic identification**	**Agar plate for purity assessment**	**Media for MIC analysis**	**Incubation conditions**	**Infection**
Streptococcus mutans (ATCC 25175)	Dome shaped colonies that are grey-purple in colour with hemolysis present	Mueller-Hinton agar with 5% blood	Mueller-Hinton broth with 5% blood	37˚C for 48 h under CO_2 conditions	Dental caries
Streptococcus sangius (ATCC 10556)	Dome shaped colonies that are grey-purple in colour with hemolysis present	Mueller-Hinton agar with 5% blood	Mueller-Hinton broth with 5% blood	37˚C for 48 h under CO_2 conditions	Dental caries
Lactobacillus acidophilus (ATCC 314)	Small, white, round, rough, raised and translucent colonies	Rogosa agar	Rogosa broth	37˚C for 48 h under CO_2 conditions	Dental caries
Lactobacillus casei (ATCC 393)	Circular, entire, white colonies	Rogosa agar	Rogosa broth	37˚C for 48 h under CO_2 conditions	Dental caries
Fusobacterium nucleatum (ATCC 25586)	Pinpoint, punctiform, translucent colourless, smooth and transparent	Blood agar	Todd Hewitt broth with 5% blood	37˚C for 4 days under strict anaerobic conditions	Periodontal diseases

Pathogen	Macroscopic identification	Agar plate for purity assessment	Media for MIC analysis	Incubation conditions	Infection
Porphyromonas gingivalis (ATCC 33277)	Forms black colonies	Blood agar: TSB with 5% blood, yeast extract hemin and menadione	Tryptone Soya broth with yeast extract, hemin and menadione	37˚C for 1 week under strict anaerobic conditions	Periodontal diseases
*Candida albicans (*ATCC 10231)	Colonies are small white smooth and circular.	Tryptone Soya agar	Tryptone soya broth	37˚C for 48 h under aerobic conditions	Oral candidiasis
Candida glabrata (ATCC 20030)	Colonies are smaller in comparison to other *Candida* species. Grows as glistening smooth cream coloured colonies.	Tryptone Soya agar	Tryptone Soya broth	37˚C for 48 h under aerobic conditions	Oral candidiasis
Candida krusei (ATCC 14243)	Dry, wrinkled white colonies. Spreading colonies with a white-yellowish surface.	Tryptone Soya agar	Tryptone Soya broth	37˚C for 48 h under aerobic conditions	Oral candidiasis

2.2.5. Minimum inhibitory concentration (MIC) assay

The aqueous and organic extracts were prepared in distilled water and acetone respectively, to a starting concentration of 32 mg/ml. The organic extracts that did not dissolve were placed in a Transsonic 540 sonicator (Labotec) for 3-5 minutes. If the plants were not dissolved even after sonification then the plant extracts were dissolved in Dimethyl sulfoxide (DMSO) where highest concentrations used in micro-titre plate were no higher than 12.5%. Essential oils samples were also prepared using acetone to a concentration of 32 mg/ ml.

The broth microdilution assay adapted from Eloff (1998), was used to quantify the antimicrobial activity of the selected South African plants. Using aseptic technique, 100 µl of broth (Table 2.2) was added to all the wells of the 96 well micro-titer plate. Thereafter 100 µl of respective diluted plant extract, at a starting concentration of 32

mg/ml, was added to the top row of the micro-titer plate along with the positive, negative and culture controls. The negative control (acetone, water or DMSO made up to 32 mg/ml) ensures that the solvent itself does not exert any antimicrobial effect. The positive control ciprofloxacin 0.01 mg/ml for bacteria and amphotericin B (0.1 mg/ml) for yeasts was used to confirm the microbial susceptibility. Serial dilutions were then performed by transferring 100 µl of the well content thereby diluting the extracts and controls by 50% each time. A 100 µl of a standardized culture suspension (1x 10^8 CFU/ml) prepared as a 0.5 McFarland's standard was added to all the wells of the micro-titre plate. The final micro-titre plate was then sealed with a sterile adhesive seal to ensure no evaporation of the plant extracts. Thereafter, the micro-titre plates were incubated at optimal conditions (Table 1). A CO_2 enriched environment for *Streptococcus* spp. and *Lactobacillus* spp. was produced using a candle in a sealed jar. A strict anaerobic environment was required for the growth of *P. gingivalis* and *F. nucleatum* which was produced using a gas pack system (Oxoid). To confirm purity of the cultures used, each diluted pathogen- broth mixture was streaked onto its respective media and incubated with the micro-titre plates.

After incubation, 40 µl of 0.4% *p*-iodonitrotetrazolium violet solution (INT) solution was added to each well of the micro-titre plate. This was done when performing the MICs for *Candida* spp. and *Lactobacillus* spp. left at room temperature for 24 hours before reading. This indicator was also used for *P. gingivalis*, plates, the plates were placed back in an anaerobic gas chamber for three days before reading. The INT indicator will change colour from clear to pink/red in the presence of microbial growth. Once an observable colour change was noted in the culture control column, the plates were analysed. All other pathogens tested in this study had blood supplemented in the broth to support its growth. A row in the micro-titre plate was left with just the broth and no culture was added. This helps to decipher what no growth would look like in the plate; the results are compared to this control and confirmed with streaking out if necessary. The MIC was interpreted as the lowest concentration at which growth is inhibited (the first clear well), which would be considered the MIC of a particular plant extract or essential oil. Tests were done at least in triplicate for organic extracts and in duplicate or triplicate where necessary for aqueous extracts.

2.3. Results and discussion

The antimicrobial properties of 140 (70 organic and 70 aqueous) plant extracts (many plants had different plant parts studied) and six essential oils derived from 31 plants against nine pathogens associated with oral diseases (four Gram-positive bacteria, two Gram-negative and three yeasts) were determined. The antimicrobial results of the organic extracts, aqueous extracts and essential oils can be found in Table 2.4, 2.5 and 2.7 respectively. The antimicrobial activity, measured as MIC values, were classified according to Table 2.3. The values marked in bold in Table 2.4, 2.5 and 2.7 are MIC values that are considered noteworthy (< 1.00 mg/ml for plant extracts and ≤ 1.00 mg/ml for essential oils).

Table 2.3: A summary of the classifications of antimicrobial activity according to MIC values (mg/ml).

Plant sample	**MIC (mg/ml)**	**Classification of antimicrobial activity.**	**Reference**
Plant extract	< 1.00	Noteworthy activity	(Rios and Recio, 2005; van Vuuren, 2008)
	≥ 1.00 - < 4.00	Moderate activity	
	≥ 4.00 - ≤ 8.00	Weak activity	
	> 8.00	Very poor activity	
Essential oil	≤ 1.00	Noteworthy activity	(Orchard and van Vuuren, 2017)
	> 2.00 - ≤ 4.00	Moderate activity	
	> 4.00	Poor activity	

The MIC values of plant extracts and essential oils where no end point could be found were given a value of > 8.00 mg/ml as this was the highest concentration tested. At the test concentration, negative controls (acetone, water and DMSO) did not affect the growth of all test pathogens. For the positive controls (ciprofloxacin for bacteria and amphotericin B for yeasts) the activities varied between test pathogens with MIC values ranging from 0.04-0.78 µg/ml demonstrating that the conventional antimicrobials were responsive.

2.3.1. Antimicrobial effect of plant extracts against oral pathogens

The overall antimicrobial activity by both the aqueous and organic plant extracts have shown that the organic extracts have better antimicrobial activity against the oral pathogens. The results are shown in Figure 2.3, in comparison to the aqueous extracts. None of the aqueous extracts displayed noteworthy activity. The majority (50%) of

the aqueous extracts displayed weak antimicrobial activity (MIC values ≥ 4.00 - ≤ 8.00 mg/ml).

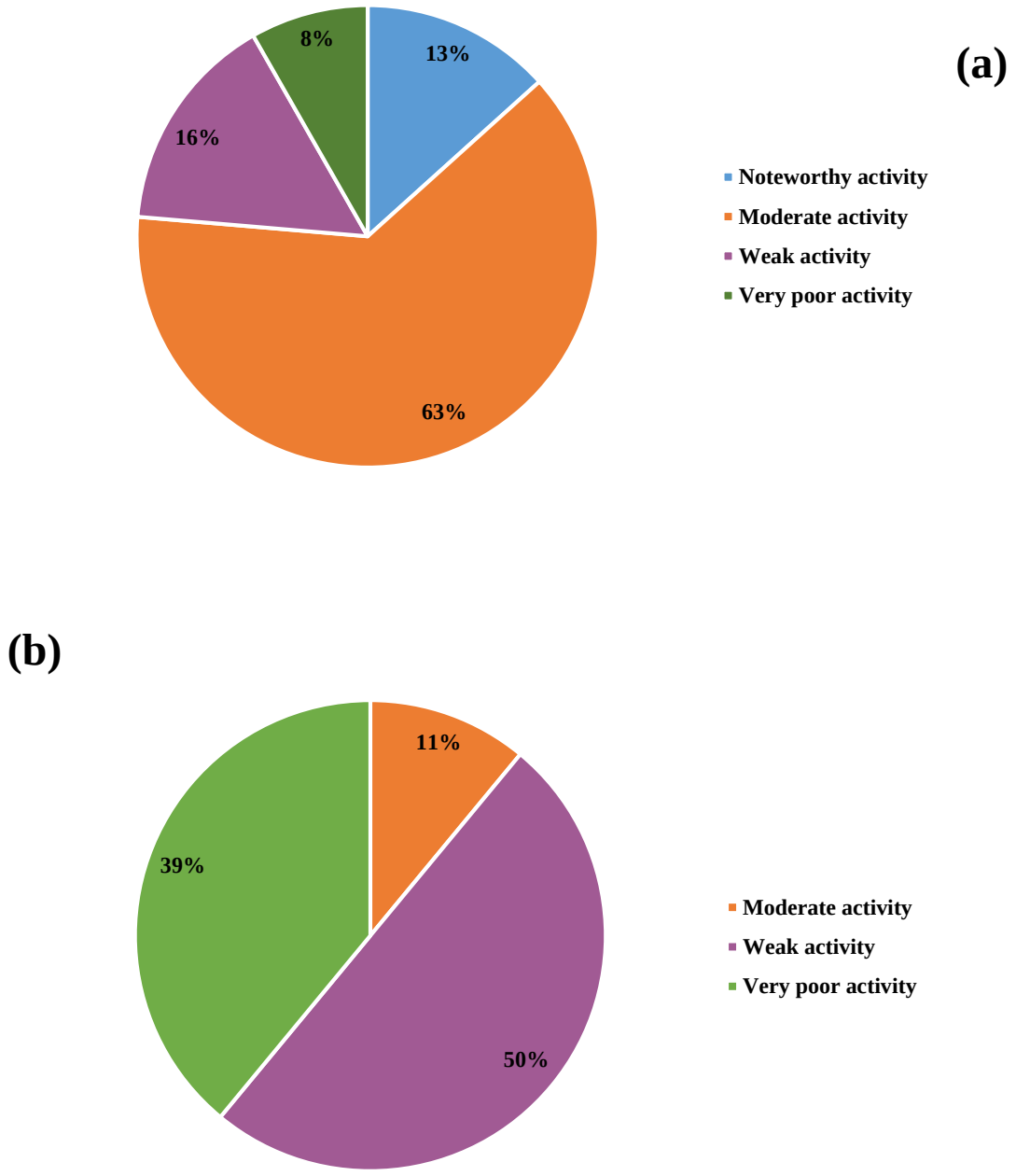


Figure 2.3: The overall antimicrobial activity of organic (a) and aqueous extracts (b)

A large portion (39%) of the aqueous extracts produced very poor activities (MIC > 8.00 mg/ml) and a small portion, 7.07%, of aqueous extracts MICs were classified with moderate activity (≥ 1.00 - < 4.00). The organic extracts displayed much greater

activity and 13% of extracts displayed noteworthy activity with MIC values > 1.00 mg/ml. The majority (63%) of the organic extracts produced moderate activity and only 8.00% of the organic extracts displayed very poor activity with MIC values > 8.00 mg/ml.

2.3.1.1. Antimicrobial activity of organic plant extracts

The activity of organic plant extracts can be found in Table 2.4. While many studies indicate that Gram-negative bacteria are more resistant to various antimicrobials, in this study the organic extracts displayed the most antimicrobial activity against *F. nucleatum* (18.84%). *Candida krusei* was the most susceptible yeast with 17.39% of all organic extracts displaying noteworthy activity against it (Figure 2.4).

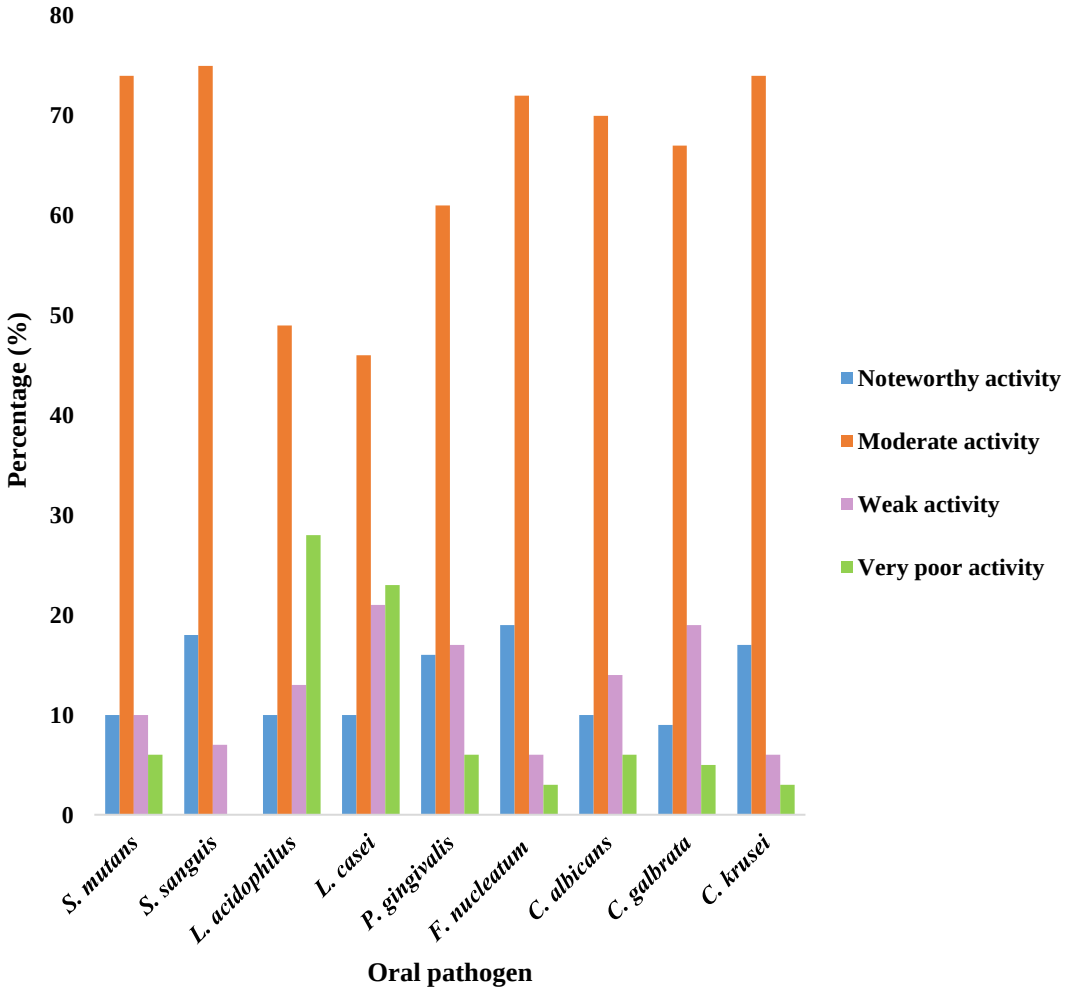


Figure 2.4: Overall activity of organic extracts against oral pathogens

Streptococcus mutans had the highest percentage of moderate activity (78.81%). No

weak activity (MIC values > 8.00 mg/ml) was seen with *S. sanguis*.

Cissampelos torulosa stems presented the lowest MIC values in this study of 0.05 mg/ml against both *Lactobacillus* spp. *Spirostachys africana* leaves also displayed this value against *C. albicans.* The plant extract with lowest mean MIC value (0.95 mg/ml) against all pathogens was *Heteropyxis natalensis* stems. Pathogen specific noteworthy activity was seen with *C. brachiata* leaves against the *Candida* spp. *Englerophytum magalismontanum* stems displayed pathogen specific noteworthy activity against *Streptococcus* spp. *Dodonaea viscosa* leaves, *T. riparia* leaves, *H. natalensis* leaves and *C. torulosa* stems all produced pathogen specific noteworthy activity against *Lactobacillus* spp.

2.3.2.1.1. Antibacterial properties of organic plant extracts against cariogenic pathogens

The *Streptococcu*s and *Lactobacillus* spp. are cariogenic Gram-positive bacteria that have the ability to cause tooth decay in the mouth (Takarada et al., 2004). Seven of the organic plant extracts displayed noteworthy activity against *S. mutans* and 12 displayed noteworthy activity against *S. sanguis*. Seven plant extracts were active against *L. acidophilus* as well as *L. casei*. *Cissempelos torulosa* stems, *D. viscosa* leaves, *H. natalensis* leaves and *T. riparia* leaves displayed noteworthy activity against both pathogens.

Englerophytum magalismonatanum stems displayed noteworthy activity against both *Streptococcus* spp. with MIC values of 0.83 mg/ml against *S. mutans* and 0.67 mg/ml against *S. sanguis*. The traditional use of *E. magalismonatanum* corroborates with its noteworthy antimicrobial activity against the cariogenic *Streptococcus* spp. as the sticks or bark are chewed to relieve toothache (More et al., 2008). In the study conducted by More et al., (2008) the MIC values against *S. mutans* were much higher displaying an MIC value of 12.50 mg/ml and an MBC concentration of 6.3 mg/ml. The disparity in antimicrobial activity in the two studies could be due to the different solvent used to extract the plant material. In this study a mixture of a non-polar and polar solvent, dichloromethane: methanol, was used. In comparison, More et al., (2008) employed the use of ethanol as the solvent. Hence, the active antimicrobial compounds from this plant could be non-polar and thus not able to dissolve in the polar solvent ethanol.

Table 2.4: The average MIC values (mg/ml) of organic (dichloromethane: methanol) extracts against nine oral pathogens

Plant name	**Mean MIC value (mg/ml) n=3**								
	S. mutans	***S. sanguis***	***L. acidophilus***	***L. casei***	***P. gingivalis***	***F. nucleatum***	***C. albicans***	***C. glabrata***	***C. krusei***
	ATCC 25175	**ATCC 10556**	**ATCC 314**	**ATCC 393**	**ATCC 33277**	**ATCC 25586**	**ATCC 10231**	**ATCC 20030**	**ATCC 14243**
Acacia karroo leaves	**0.50**	1.67	1.67	2.00	2.00	3.33	3.00	1.33	1.67
Acacia karroo bark	1.00	1.00	**0.50**	1.00	2.00	2.00	**0.50**	1.33	1.00
Acacia polyacantha leaves	2.67	1.00	> 8.00	>8.00	1.00	1.00	1.00	1.33	2.67
Acacia polyacantha stems	2.67	1.00	> 8.00	>8.00	**0.50**	2.67	8.00	4.00	1.33
Acokanthera oppositifolia leaves	8.00	1.00	> 8.00	5.33	4.00	3.33	2.00	1.67	2.00
Acokanthera oppositifolia stems	2.67	**0.50**	**0.50**	1.67	>8.00	1.33	1.67	1.67	1.33
Artemisia afra leaves	3.33	**0.50**	8.00	>8.00	**0.83**	**0.67**	**0.50**	1.00	1.67
Artemisia afra stems	2.00	1.33	4.00	2.67	1.00	**0.67**	5.00	4.00	**0.50**
Berula erecta leaves	1.67	**0.83**	> 8.00	8.00	**0.50**	1.00	3.00	2.67	**0.50**
Berula erecta stems	2.00	1.00	2.00	2.00	1.67	1.00	4.00	2.00	2.00
Berula erecta rhizomes (RHN)	**0.50**	1.67	2.00	2.67	3.00	2.00	> 8.00	> 8.00	> 8.00
Carpobrotus edulis leaves[a]	5.33	8.00	2.00	8.00	2.00	**0.50**	> 8.00	6.67	5.30
Cissampelos torulosa leaves[a]	4.00	2.00	1.00	2.00	2.00	2.00	2.00	2.00	2.00
Cissampelos torulosa stems	2.00	**0.50**	**0.05**	**0.05**	2.00	**0.83**	1.50	4.00	1.30
Clausena anisata leaves	1.67	2.00	2.00	2.00	1.33	3.33	3.50	4.00	2.00
Clausena anisata bark	2.00	1.33	2.00	1.33	4.00	**0.67**	5.33	5.33	1.67
Clausena anisata twigs	2.00	1.33	> 8.00	8.00	2.00	2.67	1.33	2.66	**0.33**
Clematis brachiata leaves	2.00	**0.67**	> 8.00	> 8.00	2.00	1.33	**0.33**	**0.33**	**0.42**
Clematis brachiata stems	2.00	1.67	3.33	8.00	2.00	2.67	2.00	1.33	2.00
Clematis brachiata flowers	2.00	**0.83**	> 8.00	8.00	8.00	2.00	**0.50**	**0.42**	2.00
Clematis brachiata leaves (RHN)	1.00	1.67	6.67	> 8.00	2.00	> 8.00	8.00	2.00	**0.67**
Clematis brachiate roots (RHN)	2.00	2.00	3.33	2.67	**0.50**	2.00	2.00	2.00	1.00
Cotyledon orbiculata leaves	4.00	**0.50**	2.00	2.00	8.00	**0.50**	1.33	1.00	1.00

Plant name	Mean MIC value (mg/ml) n=3								
	S. mutans **ATCC 25175**	***S. sanguis*** **ATCC 10556**	***L. acidophilus*** **ATCC 314**	***L. casei*** **ATCC 393**	***P. gingivalis*** **ATCC 33277**	***F. nucleatum*** **ATCC 25586**	***C. albicans*** **ATCC 10231**	***C. glabrata*** **ATCC 20030**	***C. krusei*** **ATCC 14243**
Croton gratissimus leaves	1.33	4.00	**0.67**	1.33	4.00	2.00	1.33	**0.83**	**0.83**
Croton gratissimus stems	3.33	**0.83**	2.00	1.67	1.67	**0.67**	4.00	1.33	2.00
Cyphostemma lanigerum leaves	2.00	2.00	> 8.00	> 8.00	2.00	1.00	1.00	1.33	2.00
Cyphostemma lanigerum stems	1.00	2.00	6.67	8.00	3.33	1.00	2.50	2.00	1.67
Cyphostemma setosum leaves	2.00	2.00	> 8.00	> 8.00	8.00	1.33	1.33	2.00	1.67
Cyphostemma setosum stems	2.00	2.00	2.67	2.00	8.00	2.00	2.67	2.00	2.00
Cyphostemma setosum fruit	2.00	1.33	8.00	4.00	4.00	2.00	2.00	8.00	4.00
Dalbergia obovata leaves	4.00	4.00	4.00	4.00	8.00	2.00	4.00	4.00	4.00
Dalbergia obovata stems	2.00	1.00	> 8.00	> 8.00	>8.00	**0.83**	2.67	2.00	2.00
Datura stramonium leaves	1.33	1.00	> 8.00	> 8.00	2.00	1.00	1.33	**0.25**	**0.42**
Datura stramonium stems	1.33	1.00	> 8.00	> 8.00	2.00	2.00	1.33	2.00	2.00
Datura stramonium fruit	1.33	3.33	> 8.00	> 8.00	1.33	**0.67**	1.67	1.67	> 8.00
Dichrostachys cinera leaves	1.33	4.00	6.67	5.33	2.67	2.00	1.33	3.33	1.00
Dichrostachys cinera stems	2.00	3.33	> 8.00	4.00	2.00	**0.67**	2.67	2.00	2.00
Dodonaea viscosa leaves	1.67	2.00	**0.25**	**0.50**	2.00	**0.33**	1.67	1.00	2.67
Dodonaea viscosa stems	1.00	2.00	2.00	4.00	2.00	2.00	2.67	2.00	2.00
Englerophytum magalismontanum leaves (RHN)	1.00	3.00	1.67	3.33	4.00	1.67	4.00	2.67	1.67
Englerophytum magalismontanum stems (RHN)	**0.83**	**0.67**	2.00	3.33	>8.00	2.67	1.67	3.33	2.67
Erythrina lysistemon leaves	1.00	1.00	> 8.00	> 8.00	>8.00	2.00	2.00	1.67	1.00
Erythrina lysistemon stems	**0.50**	2.00	1.00	**0.67**	1.67	> 8.00	1.00	1.00	1.67
Eucomis punctata leaves[a]	1.67	2.00	2.00	8.00	2.00	4.00	4.00	> 8.00	4.00
Heteropyxis natalensis leaves	1.33	2.00	**0.25**	**0.25**	1.00	2.00	1.00	1.00	3.33
Heteropyxis natalensis stems	2.00	**0.21**	1.33	1.00	**0.50**	1.33	**0.25**	**0.50**	1.00
Myrothamnus flabellifolia leaves	2.00	1.33	4.00	4.00	**0.50**	**0.33**	2.00	1.00	1.67
Sansevieria hyacinthiodes leaves	1.00	1.00	2.00	2.67	**0.25**	2.67	1.00	2.67	2.00

Plant name	Mean MIC value (mg/ml) n=3								
	S. mutans	***S. sanguis***	***L. acidophilus***	***L. casei***	***P. gingivalis***	***F. nucleatum***	***C. albicans***	***C. glabrata***	***C. krusei***
	ATCC 25175	**ATCC 10556**	**ATCC 314**	**ATCC 393**	**ATCC 33277**	**ATCC 25586**	**ATCC 10231**	**ATCC 20030**	**ATCC 14243**
Sansevieria hyacinthiodes rhizomes (RHN)	2 .00	2.00	2.00	2.00	2.00	1.67	2.00	**0.50**	2.00
Sansevieria hyacinthiodes leaves (RHN)	1.00	1.33	4.00	2.00	2.00	1.00	1.67	1.00	1.67
Siphonochilus aethiopicus leaves	> 8.00	1.00	1.33	2.00	1.00	**0.83**	1.00	1.00	**0.83**
Siphonochilus aethiopicus stems[a]	> 8.00	1.00	2.00	1.67	2.00	8.00	6.67	8.00	2.00
Siphonochilus aethiopicus roots	1.00	2.00	1.67	**0.25**	8.00	1.67	> 8.00	1.33	**0.83**
Spirostachys africana leaves	**0.50**	2.00	2.00	1.00	**0.67**	2.00	**0.05**	1.33	2.67
Spirostachys africana stems	1.33	**0.42**	1.67	2.00	2.00	2.67	0.67	1.33	2.00
Tarchonanthus camphoratus leaves	1.00	1.33	2.00	2.00	1.00	1.67	1.00	1.00	1.00
Tarchonanthus camphoratus bark	**0.67**	1.33	1.33	1.00	**0.13**	**0.67**	1.00	1.00	1.67
Tecomaria capensis leaves	**0.67**	2.00	2.67	3.33	1.00	1.30	1.33	1.00	3.33
Tecomaria capensis stems	2.00	4.00	> 8.00	> 8.00	1.00	2.67	1.33	> 8.00	**0.33**
Tetradenia riparia leaves	2.00	2.00	**0.42**	**0.25**	**0.50**	2.00	1.00	1.33	2.00
Tetradenia riparia stems	> 8.00	2.00	2.00	3.33	1.00	2.00	1.33	4.00	3.33
Warburgia salutaris leaves	1.00	1.67	1.00	2.00	8.00	3.33	1.67	4.00	2.00
Warburgia salutaris bark	2.00	2.00	1.33	**0.42**	**0.83**	4.00	2.00	1.67	2.00
Warburgia salutaris twigs	> 8.00	1.33	1.33	1.00	1.33	2.00	1.33	> 8.00	**0.25**
Zanthoxylum capense leaves	3.33	**0.83**	> 8.00	> 8.00	1.00	5.33	2.00	2.00	1.00
Zanthoxylum capense stems[a]	1.00	2.67	6.67	8.00	1.33	2.00	2.00	3.33	**0.50**
Ziziphus mucronata leaves	3.33	1.00	> 8.00	> 8.00	1.00	1.00	2.00	2.00	1.00
Ziziphus mucronata stems	2.00	1.33	4.00	4.00	**0.50**	1.00	2.67	2.00	2.00
Negative control (acetone or DMSO)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
Positive control (Ciprofloxacin or Amphotericin B) (in µl/ ml)	0.08	0.08	0.18	0.18	0.08	0.04	0.39	0.78	0.78
Culture control	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00

a: Plants were dissolved in 12.5% DMSO

MIC values marked in bold typeface are considered noteworthy antimicrobial activity

While *E. magalismonatanum* stem was the only extract that displayed noteworthy activity against both *Streptococcus* spp., different parts of *S. africana* plant species also displayed noteworthy activity against the *Streptococcus* spp. The leaf extract of *S. africana* showed notable activity against *S. mutans* (MIC 0.50 mg/ml) while the bark extract demonstrated good activity against *S. sanguis* (MIC 0.42 mg/ml). These activities may validate the traditional use of *S. africana* where unspecified parts of the plant were used as a remedy for toothache (Philander, 2011).

The organic extract of *C. torulosa* stems displayed the greatest antimicrobial activity with the lowest MIC value achieved in this study of 0.05 mg/ml against both *Lactobacillus* spp. tested. Noteworthy activity was also observed against other pathogens (*S. sanguis* with an MIC value of 0.50 mg/ml and *F. nucleatum* with an MIC value of 0.83 mg/ml). To the best of our knowledge no antimicrobial studies on this plant against oral pathogens have been documented, and yet the good antimicrobial activity particularly against the cariogenic bacteria correlates with the traditional use of *C. torulosa,* to relieve toothache (Hutchings, 1996). The parts of the plant tested in this study were the leaves and stems. In comparison to the stems, *C. toroulosa* leaves demonstrated mainly moderate antimicrobial activity.

Dodonaea viscosa or Hop bush as it is commonly known as, is one of the most popular South African plants used for oral infections, yet previous studies have not been conducted against *Lactobacillus* spp. The leaves and twigs are traditionally chewed to clean teeth. This plant was also found to be used as a gargle for oral thrush (Watt and Breyer-Brandwijk, 1962; van Wyk, 2008; van Wyk et al., 2008; Henley-Smith et al., 2013). *Dodonaea viscosa* leaves displayed noteworthy activity against both the *Lactobacillus* spp. with MIC values of 0.25 mg/ml against *L. acidophilus* and 0.50 against *L. casei*. The leaves also displayed good antimicrobial activity against *F. nucleatum* (MIC 0.33 mg/ml). Activity against other pathogens ranged from MIC of 1.00- 2.67 mg/ml. In comparison, the stems activity was weaker with activity mainly being moderate in nature. Previous studies that have focused on *D. viscosa* against oral pathogens include a study conducted by Naidoo et al., (2012). A time kill assay was employed and 71% of *S. mutans* was killed at the lowest concentration of *D. viscosa* tested (0.10 mg/ml) after exposure to the plant for 24 h. Patel and Coogan, (2008) tested the antifungal activity of *D. viscosa* leaves against *C. albicans* isolated

from HIV positive and negative patients where the average MIC found was 6.25 mg/ml. In this study the MIC values of *D. viscosa* leaves were lower against *C. albicans* (MIC 1.67 mg/ml). The difference in strains (clinical vs ATCC) and solvents could account for the discrepancies in MIC values between the two studies.

Heteropyxis natalensis leaves displayed noteworthy activity against both *Lactobacillus* spp. with MIC values of 0.50 mg/ml against *L. acidophilus* and *L. casei*. Unspecified parts of the plant are traditionally used to treat bleeding gums which is often related to a gingival infection that usually precedes dental caries (Gupti, 2012). No studies have been conducted before on *H. natalensis* leaves against *L. acidophilus* and *L. casei.* The stems displayed moderate activity against the rest of the pathogens with MIC values ranging from 1.00 mg/ml- 2.00 mg/ml. A recent study done by Henley-Smith, (2014) evaluated the antimicrobial efficacy of *H. natelensis* alone and in combination with green tea extracts against oral pathogens MIC values against *S. mutans* were documented as 2.66 mg/ml. The antimicrobial activity of this plant against *S. mutans* is comparable to this study where similar MIC values were produced (MIC 2.00 mg/ml).

The leaves of *T. riparia* also showed notable activity against the *Lactobacillus* spp. The plant also commonly known as ginger bush, showed MIC values of 0.42 mg/ml against *L. acidophilus* and 0.25 mg/ml against *L. casei.* In comparison, the activity of the stems of *T. riparia* were weaker. A leaf infusion of *T. riparia* is traditionally used to treat mouth ulcers and respiratory ailments (van Wyk et al., 2009).

2.3.2.1.2. Antibacterial properties of organic plant extracts against periodontal pathogens

The two Gram-negative bacteria (*F. nucleatum* and *P. gingivalis*) in this study are the causative agents in periodontal diseases (Gupti, 2012). While many studies indicate that Gram-negative bacteria are the most resistant pathogens to antimicrobials (de Angelis et al., 2014), in this study the extracts were generally the most susceptible to these bacteria with 15 plants displaying noteworthy activity against *F. nucleatum* and 12 plants exhibiting noteworthy activity against *P. gingivalis.* Three of these plant extracts displayed noteworthy activity against both periodontal pathogens; *A. afra* leaves, *M. flabellifolia* leaves and *T. camphoratus* bark (Table 2.4).

Notable antimicrobial activity was displayed against *P. gingivalis* (MIC 0.83 mg/ml) and *F. nucleatum* (MIC 0.67 mg/ml) by *A. afra* leaves. The stems of this plant also produced good antimicrobial activity against *F. nucleatum* (MIC 0.67 mg/ml). The antimicrobial efficacy of this plant has been well-studied (Liu et al., 2009). However, only one study by More et al. (2012) investigated the antimicrobial activity of *A. afra* plant extract and its constituents against periodontal pathogens. This study was congruent with previous results where the *A. afra* leaf extract displaying noteworthy activity *P. gingivalis* (MIC 0.06 mg/ml). The stems of this plant demonstrated weaker activity only displaying noteworthy activity against *F. nucleatum* (MIC 0.67 mg/ml). A study by More et al., (2012) investigated the antimicrobial activity of *A. afra* plant extract and its constituents against periodontal pathogens, *P. gingivalis* and *C. albicans* were tested and achieved similar results with the *A. afra* leaf extract displaying noteworthy activity against both pathogens.

The leaf of the *M. flabellifolia*, or resurrection plant as it is commonly known as, is traditionally chewed to treat Vincent's gingivitis (Watt and Breyer-Brandwijk, 1962; van Wyk, 2011). Vincent's gingivitis, also known as necrotizing ulcerative gingivitis is a periodontal disease caused by plaque forming bacteria in the oral cavity (Bascones- Martinez et al., 2011). The traditional use corroborates with the MIC values observed in this study as the organic leaf extract displayed activity worth noting against *P. gingivalis* and *F. nucleatum* with MIC values of 0.50 and 0.33 mg/ml.

Traditionally, the leaves of *T. camphoratus*, or Camphor bush, are used for the treatment of toothache (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Moffett, 2010). The bark and leaf had noteworthy activity against *S. mutans* (0.67 mg/ml). The bark also exhibited noteworthy activity against the periodontal pathogens, *P. gingivalis* (MIC 0.13 mg/ml) and *F. nucleatum* (MIC 0.67 mg/ml). To date, there are no studies that have reported on the antimicrobial efficacy of this plant against oral pathogens. However, the plant has been found to have antimicrobial activity against other Gram-positive and Gram-negative bacteria (Matasyoh et al., 2007). The plant has also been investigated against pathogens implicated in sexually transmitted infections where the organic extract was found to have broad-spectrum activity with

MIC values ranging from 0.50 mg/ml to 0.70 mg/ml against five of the six pathogens tested (Naidoo et al., 2013).

2.3.2.1.3. Anti-candidal properties of organic plant extracts

The plant samples were tested against three yeasts in this study; *C. albicans*, *C. glabrata* and *C. krusei*. The root of *C. brachiata* is traditionally used to treat oral thrush (Watt and Breyer-Brandwijk, 1962). The organic leaf extract of *C. brachiata* exhibited broad-spectrum noteworthy activity against all three *Candida* spp. (*C. albicans* 0.33 mg/ml, *C. glabrata* 0.33 mg/ml and *C. krusei* 0.42 mg/ml) and the organic flower extract showed noteworthy activity against *C. albicans* (MIC 0.50 mg/ml) and *C. glabrata* (MIC 0.42 mg/ml). Both the leaf and flower exhibited better activity in comparison to the root which is traditionally used. This could be because the root is traditionally cooked with salt and placed in the mouth. The salt could be enhancing the antifungal activity of the roots. A previous study (Naidoo et al., 2013), investigated the effect of *C. brachiata* organic leaf extract against *C. albicans* and reported an MIC value of 1.00 mg/ml, slightly lower activity than what was found in this study.

Other plants that have displayed activity worth noting include C*roton gratissimus* (leaf extract) against both *C. glabrata* and *C. krusei* with an MIC value of 0.83 mg/ml. The plant is noted in the literature as being used by traditional healers for the treatment of Candidiasis (Masevhe et al., 2015). *Datura stramonium* leaves also displayed noteworthy activity against *C. glabrata* (MIC 0.25 mg/ml) and *C. krusei* (MIC 0.42 mg/ml) while *H. natalensis* stems displayed noteworthy activity against *C. albicans* (MIC 0.25 mg/ml) and *C. glabrata* (MIC 0.50 mg/ml).

Lastly, it is surprising to note that the leaf and bark extract of *S. africana*, traditionally used for the treatment of toothache with no specific part of the plant being mentioned Philander, (2011), displayed noteworthy antimicrobial activity against *C. albicans* with MIC values of 0.05 mg/ml (the lowest MIC value in this study) and 0.67 mg/ml respectively. The leaves were also susceptible to *S. mutans* displaying noteworthy activity (MIC 0.50 mg/ml). In comparison to the leaves, *S. africana* stems displayed noteworthy activity against *C. albicans* (MIC 0.67 mg/ ml). Previous studies on *S.*

africana focused on the antimicrobial activity of this plant against bacteria that cause diarrhoea and food spoilage (Mathabe et al., 2008). A study on *Spirostachys africana* has found that the plant contains terpenoids (Mathabe et al., 2008 and Paiva et al., 2010).

2.3.1.2. Antimicrobial activity of aqueous plant extracts

The antimicrobial activity classifications of the aqueous plant extracts against each oral pathogen tested in this study can be seen in Figure 2.5 and the MIC values of the aqueous extracts against the oral pathogens can be found in Table 2.5. There was no noteworthy activity expressed by any of the aqueous plant extracts. The aqueous plant extracts were most active against the periodontal Gram-negative bacteria *P. gingivalis* showing the highest amount of moderate activity (24.29%). The aqueous plant extracts showed low percentages of moderate activity amongst the yeasts, with the highest percentage being 5.80% against *C. krusei.*

There was a lack of noteworthy activity (MIC <1.00 mg/ml) displayed by the aqueous extracts, however, the lowest MIC value of 1.00 mg/ml was displayed by many of the aqueous extracts including; *C. anisata* twigs, *Sansevieria hyacinthiodes* rhizomes and *T. camphoratus* leaves against *S. sanguis*. *Acacia karroo* bark, *H. natalensis* stems and *T. riparia* leaves against *P. gingivalis.* Lastly, *Warburgia. salutaris* bark also displayed the same activity against *F. nucleatum* and *C. krusei* (Table 2.5).

The common factor that is seen between the three aqueous plant extracts that produced the lowest MIC of 1.00 mg/ml against *S. sanguis* is that all three plants were recorded in ethnobotanical text as being used to treat toothache. The traditional use of *C. anisata* in the treatment of toothache is mentioned several times in literature (Hutchings, 1996; Philander, 2011; van Vuuren and Viljoen, 2006), the ground root bark is directly applied to the aching tooth. While we were unable to procure the root bark of this plant, an MIC value of 1.00 mg/ml was displayed by *C. anisata* twigs against. *S. sanguis.* Moderate activity was also displayed by by the bark of this plant (MIC 2.00 mg/ml) also against *S. sanguis*. The organic extract bark (MIC 1.33 mg/ml) and twigs (MIC 1.33 mg/ml) had similar antimicrobial activity against *S. sanguis.*

Table 2.5: The average MIC values (mg/ml) of aqueous extracts against nine oral pathogens

Plant name	**Mean MIC value (mg/ml) n=2**								
	S. mutans	***S. sanguis***	***L. acidophilus***	***L. casei***	***P. gingivalis***	***F. nucleatum***	***C. albicans***	***C. glabrata***	***C. krusei***
	ATCC 25175	**ATCC 10556**	**ATCC 314**	**ATCC 393**	**ATCC 33277**	**ATCC 25586**	**ATCC 10231**	**ATCC 20030**	**ATCC 14243**
Acacia karroo leaves	> 8.00	4.00	6.00	8.00	8.00	4.00	> 8.00	8.00	> 8.00
Acacia karroo bark	> 8.00	1.50	4.00	4.00	1.00	> 8.00	4.00	> 8.00	> 8.00
Acacia polyacantha leaves	> 8.00	3.00	> 8.00	> 8.00	8.00	8.00	8.00	2.00	8.00
Acacia polyacantha stems	2.00	2.00	> 8.00	> 8.00	8.00	4.00	6.00	4.00	8.00
Acokanthera oppositifolia leaves	> 8.00	8.00	> 8.00	> 8.00	>8.00	> 8.00	8.00	> 8.00	6.00
Acokanthera oppositifolia stems	> 8.00	4.00	> 8.00	> 8.00	>8.00	6.00	> 8.00	> 8.00	8.00
Artemisia afra leaves	3.00	8.00	> 8.00	> 8.00	8.00	3.00	8.00	8.00	4.00
Artemisia afra stems	4.00	> 8.00	> 8.00	4.00	>8.00	2.00	> 8.00	> 8.00	4.00
Berula erecta leaves	6.00	8.00	> 8.00	> 8.00	8.00	6.00	8.00	4.00	4.00
Berula erecta stems	8.00	8.00	8.00	> 8.00	4.00	8.00	8.00	> 8.00	4.00
Berula erecta rhizomes (RHN)	> 8.00	4.00	> 8.00	> 8.00	6.00	> 8.00	> 8.00	> 8.00	> 8.00
Carpobrotus edulis leaves	8.00	8.00	> 8.00	8.00	>8.00	6.00	> 8.00	> 8.00	8.00
Cissampelos torulosa leaves	> 8.00	3.00	4.00	4.00	4.00	1.50	> 8.00	8.00	> 8.00
Cissampelos torulosa stems	8.00	> 8.00	2.00	2.00	8.00	4.00	8.00	> 8.00	> 8.00
Clausena anisata leaves	> 8.00	2.00	> 8.00	> 8.00	>8.00	4.00	4.00	8.00	> 8.00
Clausena anisata bark	> 8.00	> 8.00	8.00	> 8.00	>8.00	8.00	6.00	4.00	> 8.00
Clausena anisata twigs	> 8.00	1.00	> 8.00	8.00	>8.00	> 8.00	4.00	4.00	8.00
Clematis brachiata leaves	2.00	6.00	> 8.00	> 8.00	4.00	> 8.00	> 8.00	8.00	8.00
Clematis brachiata stems	4.00	> 8.00	8.00	> 8.00	8.00	8.00	> 8.00	4.00	> 8.00
Clematis brachiata flowers	4.00	4.00	> 8.00	> 8.00	>8.00	8.00	8.00	8.00	8.00
Clematis brachiata leaves (RHN)	8.00	> 8.00	8.00	4.00	4.00	> 8.00	> 8.00	8.00	8.00
Clematis brachiate roots (RHN)	8.00	> 8.00	4.00	> 8.00	3.00	> 8.00	> 8.00	4.00	6.00
Cotyledon orbiculata leaves	6.00	8.00	8.00	> 8.00	8.00	> 8.00	8.00	8.00	> 8.00

Plant name	Mean MIC value (mg/ml) n=2								
	S. mutans **ATCC 25175**	***S. sanguis*** **ATCC 10556**	***L. acidophilus*** **ATCC 314**	***L. casei*** **ATCC 393**	***P. gingivalis*** **ATCC 33277**	***F. nucleatum*** **ATCC 25586**	***C. albicans*** **ATCC 10231**	***C. glabrata*** **ATCC 20030**	***C. krusei*** **ATCC 14243**
Croton gratissimus leaves	1.50	> 8.00	6.00	4.00	6.00	4.00	> 8.00	8.00	> 8.00
Croton gratissimus stems	6.00	8.00	8.00	> 8.00	4.00	4.00	> 8.00	8.00	> 8.00
Cyphostemma lanigerum leaves	2.00	4.00	> 8.00	6.00	4.00	4.00	> 8.00	> 8.00	> 8.00
Cyphostemma lanigerum stems	8.00	8.00	> 8.00	> 8.00	6.00	8.00	4.00	> 8.00	> 8.00
Cyphostemma setosum leaves	> 8.00	4.00	> 8.00	> 8.00	>8.00	8.00	8.00	> 8.00	8.00
Cyphostemma setosum stems	4.00	8.00	4.00	> 8.00	6.00	> 8.00	8.00	8.00	> 8.00
Cyphostemma setosum fruit	4.00	> 8.00	8.00	8.00	6.00	8.00	8.00	8.00	8.00
Dalbergia obovata leaves	1.50	4.00	8.00	8.00	8.00	2.00	8.00	8.00	> 8.00
Dalbergia obovata stems	2.00	8.00	> 8.00	> 8.00	>8.00	> 8.00	8.00	> 8.00	> 8.00
Datura stramonium leaves	8.00	8.00	> 8.00	> 8.00	4.00	6.00	> 8.00	8.00	> 8.00
Datura stramonium stems	> 8.00	> 8.00	> 8.00	> 8.00	4.00	4.00	8.00	4.00	6.00
Datura stramonium fruit	> 8.00	> 8.00	> 8.00	> 8.00	2.00	8.00	3.00	8.00	> 8.00
Dichrostachys cinera leaves	2.00	4.00	8.00	8.00	2.00	> 8.00	8.00	> 8.00	8.00
Dichrostachys cinera stems	> 8.00	> 8.00	> 8.00	> 8.00	2.00	8.00	> 8.00	> 8.00	8.00
Dodonaea viscosa leaves	4.00	8.00	8.00	8.00	3.00	3.00	> 8.00	6.00	> 8.00
Dodonaea viscosa stems	2.00	> 8.00	> 8.00	> 8.00	4.00	4.00	> 8.00	8.00	> 8.00
Englerophytum magalismontanum leaves (RHN)	> 8.00	8.00	4.00	6.00	4.00	> 8.00	8.00	8.00	8.00
Englerophytum magalismontanum stems (RHN)	2.00	8.00	4.00	4.00	>8.00	> 8.00	8.00	> 8.00	8.00
Erythrina lysistemon leaves	8.00	> 8.00	> 8.00	> 8.00	>8.00	8.00	8.00	> 8.00	8.00
Erythrina lysistemon stems	8.00	8.00	> 8.00	6.00	2.00	> 8.00	4.00	> 8.00	4.00
Eucomis punctata leaves	> 8.00	8.00	8.00	> 8.00	2.00	8.00	4.00	> 8.00	8.00
Heteropyxis natalensis leaves	> 8.00	8.00	> 8.00	8.00	2.00	> 8.00	4.00	> 8.00	> 8.00
Heteropyxis natalensis stems	4.00	6.00	4.00	> 8.00	1.00	> 8.00	4.00	8.00	> 8.00
Myrothamnus flabellifolia leaves	6.00	8.00	>8.00	>8.00	3.00	2.00	4.00	4.00	6.00
Sansevieria hyacinthiodes leaves	4.00	4.00	> 8.00	8.00	>8.00	6.00	> 8.00	4.00	8.00

Plant name	Mean MIC value (mg/ml) n=2								
	S. mutans **ATCC 25175**	***S. sanguis*** **ATCC 10556**	***L. acidophilus*** **ATCC 314**	***L. casei*** **ATCC 393**	***P. gingivalis*** **ATCC 33277**	***F. nucleatum*** **ATCC 25586**	***C. albicans*** **ATCC 10231**	***C. glabrata*** **ATCC 20030**	***C. krusei*** **ATCC 14243**
Sansevieria hyacinthiodes rhizomes (RHN)	8.00	1.00	> 8.00	> 8.00	2.00	> 8.00	> 8.00	> 8.00	> 8.00
Sansevieria hyacinthiodes leaves (RHN)	> 8.00	> 8.00	> 8.00	> 8.00	>8.00	8.00	> 8.00	> 8.00	8.00
Siphonochilus aethiopicus leaves	> 8.00	4.00	> 8.00	8.00	4.00	6.00	4.00	8.00	> 8.00
Siphonochilus aethiopicus stems	> 8.00	4.00	> 8.00	> 8.00	6.00	8.00	8.00	8.00	8.00
Siphonochilus aethiopicus roots	4.00	4.00	8.00	> 8.00	>8.00	8.00	> 8.00	8.00	8.00
Spirostachys africana leaves	8.00	8.00	> 8.00	8.00	>8.00	> 8.00	8.00	8.00	8.00
Spirostachys africana stems	8.00	8.00	2.00	> 8.00	4.00	4.00	> 8.00	4.00	4.00
Tarchonanthus camphoratus leaves	8.00	1.00	8.00	4.00	6.00	8.00	> 8.00	8.00	8.00
Tarchonanthus camphoratus bark	6.00	2.00	8.00	8.00	6.00	8.00	8.00	> 8.00	4.00
Tecomaria capensis leaves	2.00	8.00	> 8.00	> 8.00	8.00	8.00	4.00	8.00	8.00
Tecomaria capensis stems	2.00	> 8.00	> 8.00	> 8.00	>8.00	6.00	2.00	> 8.00	4.00
Tetradenia riparia leaves	> 8.00	4.00	> 8.00	> 8.00	1.00	8.00	4.00	4.00	8.00
Tetradenia riparia stems	4.00	8.00	4.00	8.00	4.00	3.00	4.00	8.00	4.00
Warburgia salutaris leaves	8.00	> 8.00	> 8.00	> 8.00	8.00	4.00	8.00	> 8.00	4.00
Warburgia salutaris bark	6.00	8.00	> 8.00	8.00	2.00	1.00	> 8.00	8.00	1.00
Warburgia salutaris twigs	> 8.00	8.00	8.00	> 8.00	4.00	8.00	4.00	> 8.00	> 8.00
Zanthoxylum capense leaves	4.00	> 8.00	> 8.00	> 8.00	6.00	2.00	> 8.00	> 8.00	8.00
Zanthoxylum capense stems	8.00	> 8.00	> 8.00	> 8.00	3.00	3.00	4.00	> 8.00	4.00
Ziziphus mucronata leaves	8.00	6.00	> 8.00	> 8.00	2.00	4.00	> 8.00	6.00	8.00
Ziziphus mucronata stems	4.00	8.00	8.00	8.00	4.00	8.00	8.00	4.00	>8.00
Negative control (WFI)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
Positive control (Ciprofloxacin or Amphotericin B) (in µl/ ml)	0.08	0.08	0.18	0.18	0.08	0.04	0.39	0.78	0.78
Culture control	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00

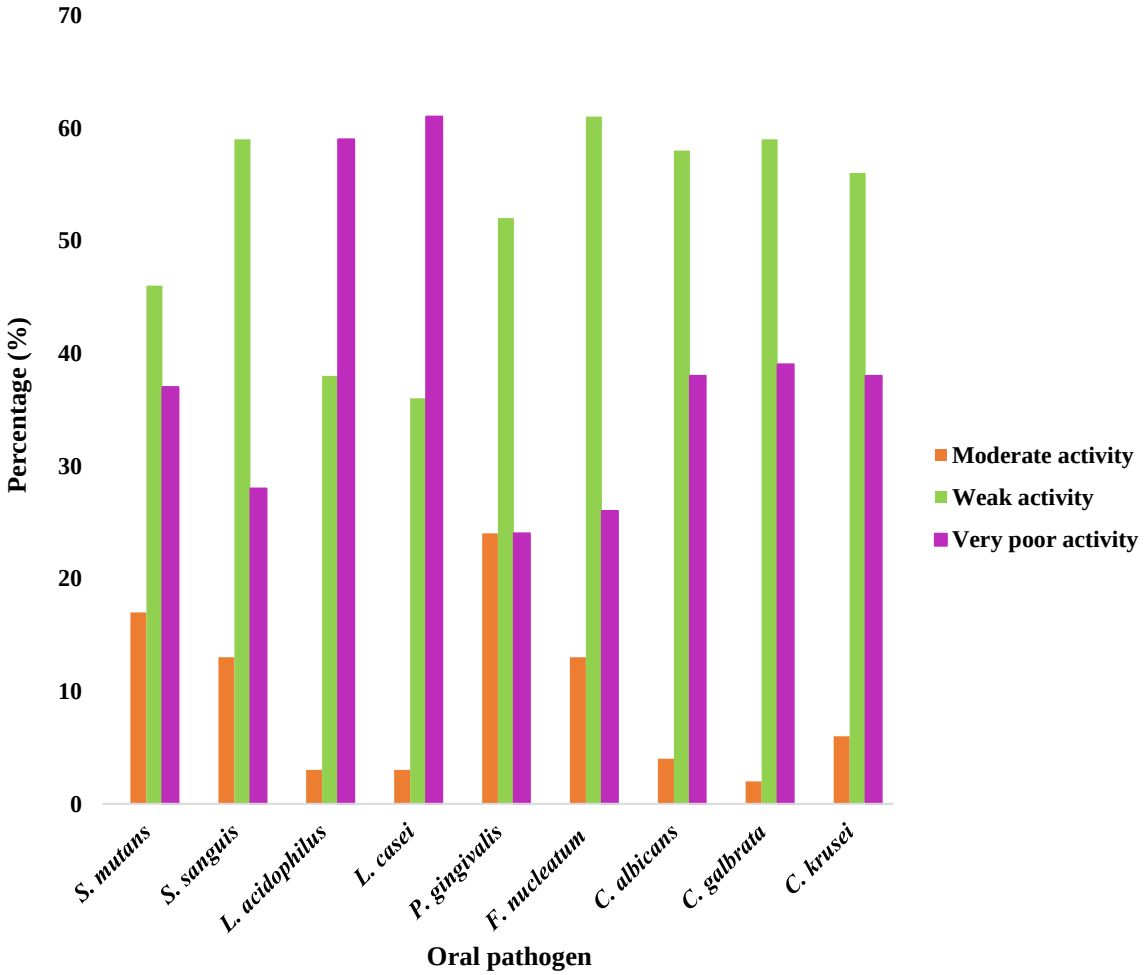


Figure 2.5: Overall activity of aqueous extracts against oral pathogens.

A study has been conducted by van Vuuren and Viljoen, (2006) and has found that the both the organic (MIC 8.00 mg/ml) and aqueous (MIC 4.00 mg/ml) bark extract of *C. anisata* had weak activity against *S. mutans.* In this study, the aqueous bark, leave and twig extract in this study did not perform well displaying very poor antimicrobial activity with MIC values > 8.00 mg/ml against S. *mutans*.

As discussed before, *T. camphoratus* has been used in the treatment of toothache (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Moffett, 2010). Both the leaves and stems of *T. camphoratus* displayed moderate activity against *S. sanguis* with MIC values of 1.00 mg/ml and 2.00 mg/ml respectively. Traditionally, an infusion of the leaves is used for toothache. This would explain the relatively low MIC value obtained by aqueous leaf extract against *S.*

sanguis.

Sansevieria hyacinthoides has also been found often in ethnobotanical literature, where the leaves and rhizomes have been used to treat toothache (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; van Wyk et al., 2009; Nielsen et al., 2012). The aqueous extract of the rhizomes, procured from Random Harvest Nursery, displayed the lowest activity against *S. sanguis* with an MIC value of 1.00 mg/ml moderate activity was also achieved against *P. gingivalis* (MIC 2.00 mg/ml). In comparison to the aqueous extract, *S. hyacinthoides* organic rhizome plant extract displayed similar moderate activity against *S. sanguis* (MIC 2.00 mg/ml) and the same activity against *P. gingivalis* (MIC 2.00 mg/ml).

Although the gum of *A. karroo* is traditionally used to for oral thrush van Wyk et al., (2009); Nielsen et al., (2012), it showed moderate acitivity against *P. gingivalis* and very poor activity against *Candida* spp. *Warburgia salutaris* is also used traditionally in the treatment of oral thrush where the bark is used. In this study the moderate activity by the aqueous twig plant extract was displayed by *C. krusei* as well as *F. nucleatum.*

Other aqueous plant extracts that have displayed the lowest activity at 1.00 mg/ml have been discuseed previously, *H. natelensis* stems and *T. riparia* leaves were the two aqueous plant extracts that showed a link between its antimicrobial efficacy (MIC 1.00 mg/ml) and its traditional use. Unspecified parts of *H. natelensis* have been documented in the treatment of bleeding gums (Watt and Breyer-Brandwijk, 1962), while an infusion of *T. riparia* is used traditionally to treat mouth ulcers (van Wyk et al., 2009).

2.3.2.4. Overview of plant extracts with noteworthy activity

Overall, the organic extracts performed well against the oral pathogens in this study. The plants did not exhibit antimicrobial efficacy against all nine pathogens in this study, instead, most were very specific to disease conditions (Appendix F). As the majority of plant samples showed pathogen specific noteworthy activity, it reiterates the importance of including a wide range of pathogens representing several infections when performing antimicrobial screening studies. Some plants did show good antimicrobial activity against four of the nine pathogens

tested (*A. afra* leaves, *C. torulosa* stems, *C, brachiata* leaves and *H. natalensis* leaves) while others showed activity against three of the nine pathogens (*C. brachiate* flowers, *C. gratissimus* leaves, *D. viscosa* leaves, *S. africana* leaves, *T. camphoratus* bark and *T. riparia* leaves). These plants should be investigated further as they have a wider range of activity against the pathogens tested in this study

2.3.2.5. Comparing the efficacy of South African plant extracts and plant extracts found in commercial products

As discussed previously in Chapter 1, Section 1.5.2., there are many commercial products that have incorporated plant extracts into their formulations. Commonly used ingredients such as *Mentha piperita, Matricaria camomilla* and *Aloe vera* have been investigated for their antimicrobial efficacy. *Mentha piperita* and *Matricaria camomilla* are ingredients found in the toothpaste Parodantax®. The antimicrobial activity of Parodantax® has been investigated before (de Rossi et al., 2014). The toothpaste was tested using the disc diffusion method and was highly effective against *S. mutans* and *C. albicans*, however, a study by Lee et al (2004), contradicted these results showing no effect on *S. mutans* or *C. albicans*. More studies have to be done on this toothpaste against a variety of oral pathogens to help fully undertsnad the antimicrobial efficacy of the toothpaste. *Aloe vera* has been used in gels and mouthwashes in the treatment of oral diseases. Fani and Kohanteb, (2012) investigated the antimicrobial efficacy of *A. vera* gel against *S. mutans* and *P. gingivalis.* The results showed good antimicrobial activity by the gel against both clinical and ATCC strains with MIC values as low as 0.025 mg/ml. In comparison to the present day study, where the lowest MIC value was 0.05 mg/ml, common plant extracts found in commercial products showed similar or lower MIC values, however, this study was the only study that used nine oral pathogens to include the most common dental infections.

2.3.3. Overall activity of selected essential oils against oral pathogens

The six essential oils tested against the nine pathogens associated with oral diseases yielded good results with the majority of the essential oils (52%) producing noteworthy activity against all oral pathogens (Figure 2.6). The MIC values can be found in Table 2.6. Of the six

essential oils tested *C. gratissimus* had the lowest (best activity) mean MIC value of 0.88 mg/ml. The lowest MIC value noted by *C. gratissimus* against oral pathogens was (MIC 0.25 mg/ml) against *L. casei*. Other plant species displaying the same efficacy include *T. riparia* against *F. nucleatum* and *M. flabellifolia* against *P. gingivalis* and *F. nucleatum*

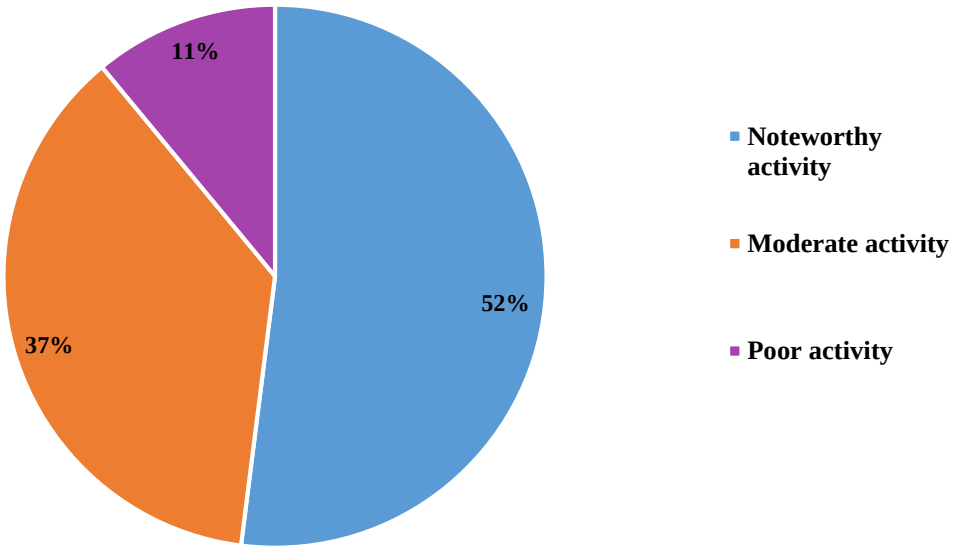


Figure 2.6: The overall activity of essential oils against oral pathogens

All six essential oils displayed good antimicrobial activity against *S. mutans*. The overall mean MIC value of all six essential oils against *S. mutans* was 0.79 mg/ml. *Candida albicans* was the most susceptible yeast to all six essential oils with a mean MIC value of 0.94 mg/ml.

C*roton gratissimus* was the essential oil with the best overall activity. The essential oil had the lowest MIC value noted (0.25 mg/ml) by all the essential oils in this study against *L. casei*. *Croton gratissimus* essential oil also displayed good activity against *S. mutans* with an MIC value of 0. 42 mg/ml. With the exception of *C. glabrata* (MIC 1.33 mg/ml), *C. gratissimus* displayed an MIC value of 1.00 mg/ml against the rest of the oral pathogens.The charred and powdered bark of *C. gratissimus* is traditionally used to treat bleeding gums (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996). Interestingly, although the traditional use does not pertain to an antimicrobial use, the essential oil displayed the best activity against the pathogens known to cause dental caries (i.e. *Streptococcus* and *Lactobacillus* spp.). Although no specific oral microbiology studies have been done on this species of *Croton*, this plant species has been investigated and found to have diterpenoids and in particular a casbane diterpene, a type of diterpene shown to have antibacterial and antibiofilm effect on bacteria (Sa´ et al., 2012).

As seen with the organic plant extracts, *T. riparia* has also rendered good results as an essential oil against the oral pathogens tested in this study. The essential oil displayed noteworthy activity (MIC 0.67 mg/ml) against *L. casei*, *F. nucleatum* (MIC 0.25 mg/ml) and *C. albicans* (MIC 0.50 mg/ml). All other MIC values ranged between 1.00 mg/ml and 2.00 mg/ml. The leaf infusions of *T riparia* are traditionally used to treat mouth ulcers (van Wyk *et al*., 2009). This gives credence to the good activity that *T. riparia* has against *F. nucleatum* which is a pathogen known to worsen and cause gingivitis and mouth ulcers (Takarada et al., 2004; Gupti 2012). A study was undertaken (de Melo et al., 2015), on the essential oil of *T. riparia* against cariogenic bacteria (*S. mutans, S. sanguis* and *L. casei*). The results correlated with results in this study as the essential oil displayed noteworthy activity against all three bacteria.

As seen with the organic extracts the *M. flabellifolia* essential oil also displayed noteworthy activity against the periodontal pathogens (*P. gingivalis* and *F. nucleatum*) with an MIC value of 0.25 mg/ml against both strains and an MIC value of 0.83 mg/ml against *C. albicans*. Although the essential oil and plant extract have not been investigated against periodontal pathogens, a study conducted by Viljoen et al. (2002), demonstrated that the essential oil has strong fungicidal activity against *C. albicans.*

The findings from the results of favour the potential use of these essential oils in treating oral diseases such as dental caries, periodontal diseases and oral thrush.

2.5. Summary

- The lowest MIC value (0.05 mg/ml) in this study was displayed by the organic extracts of *Cissampelos torulosa* stems against both *Lactobacillus* spp. and by *Spirostachys africana* against *C. albicans.*
- The lowest MIC value attained by aqueous extracts was 1.00 mg/ml. This was displayed by many of the aqueous extracts including; *Clausena anisata* twigs, *Sansevieria hyacinthiodes* rhizomes and *Tarchonanthus camphoratus* leaves against *S. sanguis*. *Acacia karroo* bark, *H. natalensis* stems and *T. riparia* leaves against *P. gingivalis.* Lastly, *W. salutaris* bark also displayed the same activity against *F. nucleatum* and *C. krusei.*
- Most of the plants exhibit antimicrobial activity specific to disease conditions.
- Organic plant extracts were most susceptible to Gram-negative bacteria with 15 plants displaying noteworthy activity against *F. nucleatum* and 12 plants exhibiting noteworthy activity against *P. gingivalis.*
- In comparison to the other yeasts in this study, the organic plant extracts were most active against *C. krusei* with 12 plants having good antimicrobial activity against it with MIC values ranging from 0.25 mg/ml- 0.83 mg/ml.
- Of the six essential oils tested *C. gratissimus* had the lowest (best activity) mean MIC value of 0.88 mg/ml.
- The lowest MIC value noted by *C. gratissimus* against *L. casei* (MIC 0.25 mg/ml). Other plant species displaying the same efficacy include *T. riparia* against *F. nucleatum* and *M. flabellifolia* against *P. gingivalis* and *F. nucleatum*.
- Some organic plant extracts showed noteworthy antimicrobial activity against four of the nine pathogens tested (*A. afra* leaves, *C. torulosa* stems, *C, brachiata* leaves and *H. natalensis* leaves).
- In some instances, there was a direct correlation between the antimicrobial activity and the traditional use displayed by the plants in this study.

Table 2.7: The average MIC values (mg/ml) of six essential oils against nine oral pathogens

Essential oil	**Mean MIC value (mg/ml) n=3**								
	***S. mutans* ATCC 25175**	***S. sanguis* ATCC 10556**	***L. acidophilus* ATCC 314**	***L. casei* ATCC 393**	***P. gingivalis* ATCC 33277**	***F. nucleatum* ATCC 25586**	***C. albicans* ATCC 10231**	***C. glabrata* ATCC 20030**	***C. krusei* ATCC 14243**
Artemisia afra	**0.67**	4.00	> 8.00	4.00	2.00	2.00	1.33	2.67	2.00
Clausena anista	**0.67**	**1.00**	> 8.00	2.00	1.33	2.67	**1.00**	2.00	2.00
Croton gratissimus	**0.42**	**1.00**	**1.00**	**0.25**	**1.00**	**1.00**	**1.00**	1.33	**1.00**
Tarchonanthus camphoratus	**1.00**	4.00	2.00	1.67	2.00	**1.00**	**1.00**	2.00	4.00
Tetradenia riparia	**1.00**	**1.00**	2.00	**0.67**	**0.50**	**0.25**	**0.50**	2.00	2.00
Myrothamnus flabellifolius	**1.00**	**1.00**	1.67	1.67	**0.25**	**0.25**	**0.83**	**1.00**	**1.00**
Negative control (acetone)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
Positive control (Ciprofloxacin or Amphotericin B) (in µl/ ml)	0.08	0.08	0.18	0.18	0.08	0.04	0.39	0.78	0.78
Culture control	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00

MIC values marked in bold typeface are considered noteworthy

Chapter 3

The effect of organic plant extracts on *Streptococcus mutans* biofilm formation

3.1.Introduction

3.1.1. The oral biofilm

The oral biofilm was discovered in the 17th century when Anton van Leeuwenhoek, the inventor of the microscope, studied the plaque formed on his own teeth and discovered the formation of microbial aggregates which were later termed as a biofilm (Chandki et al., 2011). An oral biofilm is compromised of a multitude of variant micro-organisms embedded in an extra-cellular matrix allowing them to adhere and communicate with each other (Tahmourespour et al., 2010; Jakubovics and Kolenbrander 2010; Huang et al., 2011). Oral bacteria have an ability to produce biofilms on various surfaces of the oral cavity including the solid surface of teeth and the soft surfaces of epithelial tissues (Kolenbrander et al., 2010; Sandasi et al., 2011). Biofilms have been implicated as the major factor in development of severe infections (Sandasi et al., 2011). The two most prevalent oral infections, dental caries and periodontal diseases, are found to be biofilm- dependent (da Silva et al., 2014; Kouidhi et al., 2015).

On the tooth surface, oral biofilm occurs supragingival or subgingival. It develops as either a cariogenic biofilm which consists mainly of Gram-positive bacteria or a periodontopathic biofilm which consists of mainly Gram-negative bacteria. Poor oral hygiene is a common factor in the development of both types of biofilms. However, a diet high in sucrose is responsible for the development of cariogenic biofilm (Chandki et al., 2011).

3.1.2. Microbiology of dental biofilm

The development of the biofilm is characterized by distinct phases including; an initial adherence and lag phase, a rapid growth phase and a steady state phase (Gurenlian, 2007). In the initial adherence phase the biofilm consists predominantly of *Streptococci* and *Actinomyce*s spp. (da Silva et al., 2014). In particular, *Streptoccous mutans* has been discovered as the primary etiological bacteria that predominantly proliferate in the dental biofilm (Jakubovics and Kolenbrander 2010; Kouidhi et al., 2015; Tahmourespour et al., 2010). As the biofilm enters the rapid growth phase there is an overpopulation of acidogenic bacteria (early colonizers) such as *Lactobacillus* spp. that become more dominant, thereafter, Gram-negative anaerobes such as *P. gingivalis* and *F. nucleatum* (late colonizers) begin to proliferate in the biofilm. The adherence of these pathogens; *Lactobacillus* spp. at an early stage and *P. gingivalis* and *F. nucleatum* at a later stage; respectively contribute to the proliferation of dental caries and periodontal diseases (Kolenbrander et al., 2002; da Silva et al., 2014).

Biofilms are known to confer higher amounts of resistance in comparison to planktonic cells (Huang et al., 2011). This can be caused by a variety of properties attributed to the biofilm including the biofilm mode of growth. Communication plays a vital role in the development of a biofilm, it allows for the expression of genes by micro-organisms found in the biofilm which regulate pathways that allow for binding and adhesion to surfaces (Kolenbander et al., 2002; Chandki et al., 2011). Furthermore, other properties of the biofilm mode of growth; its ability to grow on various surfaces and provide strong attachment to these surfaces contribute to its resistance abilities (Kolenbrander et al., 2010; Sandasi et al., 201q; Huang et al., 2011). The biofilm also serves as a defense against other competing micro-organisms. It does this by providing and regulating the physiochemical environment which in turn allows for the proliferation of the colonizers of the biofilm (Chandki et al., 2011). The physiological changes together with the production of enzymes are known to cause a breakdown of antimicrobial substances (Kumar and Anand., 1998). The adhesive nature of the oral biofilm coupled with resistance make biofilms very difficult to eradicate and contributes to the major oral health problem (Sandasi et al.,

2011).

3.1.3. The role of *Streptoccocus mutans* on the oral biofilm

The adhesion of *S. mutans* to the enamel surface is considered one of the primary steps in the biofilm formation (Ahn et al., 2008). Constituents within the saliva such as proteins, amylases and mucin form a salivary pellicle that coats the tooth surface. This aids in the formation of the biofim as *S. mutans* cells adhere to the salivary pellicle (Kryzysciak et al., 2014). This mechanism of adhesion is characterized by sucrose-dependent and sucrose-independent mechanisms (Cvitkovitch et al., 2003). Proteins such as glucan binding protein and glucotransferase protein, which are produced by *S. mutans*, aid sucrose-dependent pathways. Glucortansferance enzymes play a key role in aiding the aggregation of *S. mutans* not only to the tooth surface but to other micro-organisms forming micro-colonies that benefit and assist in the development of the biofilm. In addition, glucotransferase forms extra cellular polysaccharides that provide nutritive and adherent mechanisms for these bacteria (Krzysciak et al., 2014). Sucrose-independent mechanisms include the interaction of adhesions, such as salivary agglutinin and P1, which helps to facilitate the aggregation and adherence of *S. mutans* to the salivary pellicles found on the tooth surface (Ahn et al., 2008).

3.1.4. Current treatment plans and studies

There are many treatments plans available in treating oral biofilms this includes mechanical and chemical strategies (Sandasi et al., 2011). Mechanical means include physical removal of the dental plaque via brushing and scrubbing (Loesche, 1996; Kumar and Anand, 1998). Chemical means include the use of mouthwashes and other products that contain chlorhexidine and stannous fluoride as well as the use of conventional antimicrobials (Loesche, 1996). Most plant-based antimicrobials studied have focused on planktonic micro-organisms despite the fact that micro-organisms that form in biofilms are more resistant and therefore more problematic to control and should be investigated further (Sandasi et al., 2011). There have been a few studies that have been done on South African medicinal plants against biofilms this includes Naidoo et al., (2012) where the inhibitory effect of *Dodonea viscosa* leaves were

investigated against *S. mutans.* Two studies were also investigated on the effect of South African plants against *Listeria monocytogenes* biofilms (Nyila et al., 2012; Sandasi et al., 2011). With such few studies focusing on South African plants to eradicate biofilms there is an urgent need to investigate South African medicinal plants in eradicating biofilms.

3.2. Materials and methods

3.2.1. Plant material

The test plant organic extracts were selected based on their noteworthy antimicrobial activity against *S. mutans,* when average MIC values were < 1.00 mg/ml. Table one lists the seven plant extracts with their average MIC values and the part of the plant used. As discussed previously, plants were sourced from the Walter Sisulu Botanical Gardens or procured from random harvest nursery. Preparation of dichlormethane: methanol extracts can be found in Figure 2.2, Chapter 1, Page 51-52. Test plant extracts used in this study were prepared with acetone to three sub-inhibitory concentrations used in this study, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml.

3.2.2. Culture and inoculum preparation

Streptococcus mutans (ATCC strain 25175), sourced from Davies Diagnostics, was freshly grown on blood agar plates (TSA supplemented with 5% sheeps blood) at 37 ˚C for 48 h in a candle jar. Inoculum was then prepared by suspending the freshly grown *S. mutans* cells in phosphate buffered saline (PBS) with an optical density of 0.2 (405 nm) containing approximately 10^5-10^6 CFU/ml.

3.2.3. Biofilm assay

This assay was performed to determine the effect of crude plant extracts on the *S. mutans* biofilm formation. This was done using the glass slide technique adapted from Limsong et al., (2004) with a few modifications. Glass slides (25 mm x 12 mm in size) were first sterilized using Presept® disinfection tablets to get rid of the biofilm and then autoclaved. Prior to conducting the experiment, the slides were coated with filter sterilized saliva for 2 h. Tryptone Soya broth supplemented with 5% sucrose was

prepared as the media used in this assay, two ml of broth was added to four disposable bijou bottles. One bijou bottle was used as a control with no plant extract. The other three bijou bottles were used to test the three subinhibitory concentrations of the plant extracts. Two saliva coated slides were placed in each of the bijou bottles. (Figure 3.1). The plant extract was then added to each of the bottles depending on the subinhibitory concentration that was being tested. The control was made up to the same concentration as the plant extract using sterile water for injection. A positive control of chlorhexidine gluconate at a concentration of 0.2% to confirm biofilm susceptibility was added to the assay.

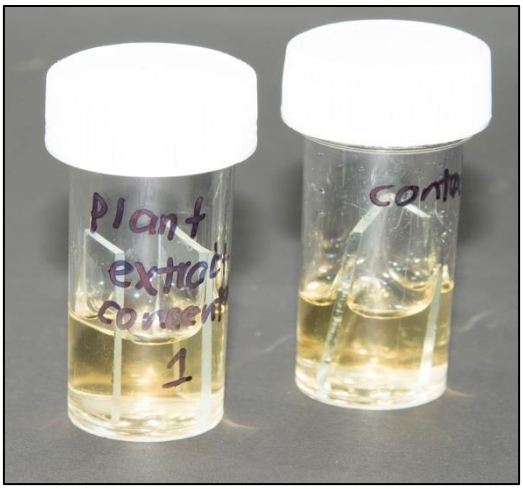


Figure 3.1: Disposable bijou bottles with sterile slides at 90˚

A 100 µl of inoculum was then added to each of the bottles. After 6 h, one glass slide is removed from each bottle and rinsed with PBS (Figure 3.2). The attached cells (biofilm) are then aseptically removed by scraping off the biofilm using a sterile slide and the cells were then resuspended and vortexed in one ml of PBS (Figure 3.3). Ten-fold serial dilutions from 1:10 to 1: 10000 were prepared in a micro-titer plate using PBS.



Figure 3.2: Rinsing slides with PBS



Figure 3.2: Scraping of the biofilm using a sterile slide

20 µl of each dilution was spread onto blood agar (TSA supplemented with 5% sheep's blood). Plates were incubated for 48 h under CO_2 conditions, the number of colonies on each plate was quantified and the counts multiplied by dilution factors and 50 to determine viable bacterial counts (CFU/ ml).

The resultant count, from the control bottle was taken as the 6 h control and the counts from the bottle with the plant extract were taken as the 6 h biofilm count in the presence of the organic dichloromethane: methanol plant extract. Bottles with the remaining slides were re-incubated. The above procedure was repeated after 24 hours. These experiments were repeated three times at three different subinhibitory concentrations. The bacterial counts of control and tests were compared and a percentage reduction of each test sample was calculated using the following equation:

$$\textbf{Percentage reduction} = \frac{\text{(CFU/ml) control count} - \text{(CFU/ml) test count}}{\text{(CFU/ml) control count}} \times 100$$

3.3. Results and discussion

The results of the effect of the seven organic plant extracts tested against the biofilm are represented in Table 3.1 and Table 3.2. The bacterial counts (CFU/ml) in the biofilm developed at 0, 6 and 24 h are represented in Table 3.1 and a summary table (Table 3.2) includes all the percentage reductions for each plant extract at the three subinhibitory test concentrations. The assays were done in triplicate for each of the plant extracts tested. All plant extracts reduced the attachment of *S. mutans* to the

glass slides, however, some plants performed better than others. The biofilm counts of *S. mutans* on control slides were always higher in comparison to the slides that were exposed to the plant extracts at their respective subinhibitory concentrations at both time intervals. CFU counts were highest at 0 h and similar between 6 and 24 h.

Spirostachys africana is the plant extract that displayed the highest reduction of adherent *S. mutans* cells at the highest subinhibitory concentrations tested, 0.25 mg/ml, at both 6 and 24 h with percentage reductions of 97.56% and 86.58% respectively. *Spirostachys africana* also displayed the highest reduction of the biofilm cells at the 24 h interval seen with all three subinhibitory concentrations. *Tarchonanthus camphoratus* displayed notable reduction of biofilm production at 6 h seen with all three subinhibitory concentrations. The effect of biofilm inhibition by the plant extracts were concentration dependent, meaning, plant extracts at higher concentrations have a greater inhibitory effect than those at lower subinhibitory concentrations, however, an exception was displayed with *Tarchonanthus camphoratus* at 6 h where the biofilm effect was similar at all three subinhibitory concentrations. *Tecoma capensis* tested at the lowest subinhibitory concentration of 0.0625 mg/ml produced the lowest percentage reductions at both 6 and 24 h.

3.3.1. Statistical analysis of data

To help confirm and compare the efficacy of test plant extracts in reducing the formation of the biofilm and to aid in data analyses the statistical program Stata ® was used. Comparison of the test plant extracts and their respective controls were first done using a T-test. Mean, standard deviations and *p* values were calculated from the T- test (Table 3.1). When *p* values were ≤ 0.05 the difference between the control and test plants was considered significant. As expected, all *p* values at 0 h comparing the control and test plants were considered insignificant. The 0 h count was done after exposure of the plant extract to the *S. mutans* inoculum and the colony forming units per ml were then similar to the control. With the exception of *E. magalismontanum* bark at the 6 h count, the rest of test plants significantly reduced formation of *S. mutans* biofilm in comparison to the control.

Table 3.1: The effect of the sub inhibitory concentrations of organic plant extracts on biofilm formation by *Streptococcus mutans*

Plant name	**Part used**	**Test concentration (mg/ml)**	**Repeats**	**Growth of *S. mutans* in biofilm (CFU/ ml)**					
				0 hours		**6 hours**		**24 hours**	
				Control	**Plant**	**Control**	**Plant**	**Control**	**Plant**
Acacia karroo	Leaves	0.25	1	$1.07x\ 10^8$	$9.85x\ 10^7$	$1.73x\ 10^7$	$5.88x\ 10^5$	$2.65\ x\ 10^6$	$1.15x\ 10^6$
			2	$1.20x\ 10^8$	$1.35x\ 10^8$	$6.20x\ 10^7$	$9.50x\ 10^6$	$1.55x\ 10^6$	$5.93x\ 10^5$
			3	$3.25x\ 10^7$	$2.70x\ 10^7$	$1.35x10^7$	$2.03x\ 10^6$	$2.58x\ 10^6$	$1.05x\ 10^6$
		0.125	1	$1.07x\ 10^8$	$1.44x\ 10^8$	$1.73x\ 10^7$	$9.00x\ 10^6$	$2.65\ x\ 10^6$	$1.63x\ 10^6$
			2	$1.20x\ 10^8$	$2.55x\ 10^8$	$6.20x\ 10^7$	$2.70x\ 10^7$	$1.55x\ 10^6$	$1.05x\ 10^6$
			3	$3.25x\ 10^7$	$2.63x\ 10^7$	$1.35x10^7$	$6.88x\ 10^6$	$2.58x\ 10^6$	$1.58x\ 10^6$
		0.0625	1	$1.07x\ 10^8$	$7.33x\ 10^7$	$1.73x\ 10^7$	$7.50x\ 10^6$	$2.65\ x\ 10^6$	$2.25x\ 10^6$
			2	$1.20x\ 10^8$	$1.80x\ 10^8$	$6.20x\ 10^7$	$2.90x\ 10^7$	$1.55x\ 10^6$	$1.35x\ 10^6$
			3	$3.25x\ 10^7$	$3.05x\ 10^7$	$1.35x10^7$	$6.60x\ 10^6$	$2.58x\ 10^6$	$2.25x\ 10^6$
		Results from t-test	**Mean**	$8.65x\ 10^7$	$1.08x10^8$	$3.09x10^7$	$1.09x10^7$	$2.26x\ 10^6$	$1.43x10^6$
			SD	$4.08x\ 10^7$	$7.85x10^7$	$2.34x10^7$	$1.01x10^7$	$5.33x10^5$	$5.57\ x10^5$
			***p* value**	0.2452		**0.0062**		**0.0010**	
Berula erecta	Rhizomes	0.25	1	$2.08x\ 10^7$	$2.60x\ 10^7$	$2.15x\ 10^6$	$1.55x\ 10^6$	$3.15x\ 10^6$	$1.95x\ 10^6$
			2	$2.90x\ 10^8$	$2.80x\ 10^7$	$5.80x\ 10^5$	$4.18x\ 10^5$	$2.30x\ 10^6$	$1.40x\ 10^6$
			3	$2.50x\ 10^7$	$2.30x\ 10^7$	$2.15x\ 10^6$	$1.50x\ 10^6$	$2.30x\ 10^6$	$1.48x\ 10^6$
		0.125	1	$2.08x\ 10^7$	$2.65x\ 10^7$	$2.15x\ 10^6$	$1.68x\ 10^6$	$3.15x\ 10^6$	$2.35x\ 10^6$
			2	$2.90x\ 10^7$	$2.50x\ 10^7$	$5.80x\ 10^5$	$4.60x\ 10^5$	$2.30x\ 10^6$	$1.70x\ 10^6$
			3	$2.50x\ 10^7$	$2.40x\ 10^7$	$2.15x\ 10^6$	$1.75x\ 10^6$	$2.30x\ 10^6$	$1.80x\ 10^6$
		0.0625	1	$2.08x\ 10^7$	$2.15x\ 10^7$	$2.15x\ 10^6$	$1.95x\ 10^6$	$3.15x\ 10^6$	$2.68x\ 10^6$
			2	$2.90x\ 10^7$	$2.90x\ 10^7$	$5.80x\ 10^5$	$5.20x\ 10^5$	$2.30x\ 10^6$	$1.95x\ 10^6$
			3	$2.50x\ 10^7$	$3.25x\ 10^7$	$2.15x\ 10^6$	$1.95x\ 10^6$	$2.30x\ 10^6$	$2.13x\ 10^6$
		Results from t-test	**Mean**	$4.49x10^7$	$2.62x10^7$	$1.63x10^6$	$1.31x\ 10^6$	$2.58x\ 10^6$	$1.94x\ 10^6$
			SD	$6.16x10^7$	$3.34x10^6$	$7.85x\ 10^5$	$6.51x\ 10^5$	$4.25x\ 10^5$	$4.08x\ 10^5$
			***p* value**	0.3827		**0.0023**		**0.0003**	

Plant name	Part used	Test concentration (mg/ml)	Repeats	Growth of *S. mutans* in biofilm (CFU/ ml)					
				0 hours		6 hours		24 hours	
				Control	Plant	Control	Plant	Control	Plant
Englerophytum magalismontanum	Bark	0.25	1	3.20×10^7	2.70×10^7	6.20×10^6	4.10×10^6	9.25×10^6	4.75×10^6
			2	2.56×10^7	2.25×10^7	6.00×10^6	4.05×10^6	4.50×10^6	2.20×10^6
			3	5.40×10^7	5.50×10^7	6.10×10^6	4.30×10^6	7.80×10^6	4.05×10^6
		0.125	1	3.20×10^7	2.85×10^7	6.20×10^6	4.65×10^6	9.25×10^6	5.15×10^6
			2	2.56×10^7	2.00×10^7	6.00×10^6	4.63×10^6	4.50×10^6	2.65×10^6
			3	5.40×10^7	4.40×10^7	6.10×10^6	4.68×10^6	7.80×10^6	4.55×10^6
		0.0625	1	3.20×10^7	3.10×10^7	6.20×10^6	5.20×10^6	9.25×10^6	6.10×10^6
			2	2.56×10^7	2.05×10^7	6.00×10^6	5.25×10^6	4.50×10^6	3.20×10^6
			3	5.40×10^7	5.10×10^7	6.10×10^6	5.15×10^6	7.80×10^6	5.45×10^6
		Results from t-test	**Mean**	3.72×10^7	3.33×10^7	1.22×10^7	4.67×10^6	7.18×10^6	4.23×10^6
			SD	1.29×10^7	1.33×10^7	1.83×10^6	4.60×10^5	2.24×10^6	1.32×10^6
			***p* value**	0.0055		0.2553		**0.0000**	
Erythrina lysistemon	Stems	0.25	1	3.80×10^7	4.00×10^7	1.55×10^7	4.25×10^6	7.00×10^6	3.00×10^6
			2	1.70×10^7	1.45×10^7	4.10×10^6	1.20×10^6	1.85×10^6	7.00×10^5
			3	3.10×10^7	3.00×10^7	3.70×10^6	9.50×10^5	3.25×10^6	1.40×10^6
		0.125	1	3.80×10^7	3.55×10^7	1.55×10^7	6.10×10^6	7.00×10^6	4.25×10^6
			2	1.70×10^7	1.30×10^7	4.10×10^6	1.55×10^6	1.85×10^6	1.15×10^6
			3	3.10×10^7	3.30×10^7	3.70×10^6	1.45×10^6	3.25×10^6	2.00×10^6
		0.0625	1	3.80×10^7	3.05×10^7	1.55×10^7	6.50×10^6	7.00×10^6	4.80×10^6
			2	1.70×10^7	1.25×10^7	4.10×10^6	1.75×10^6	1.85×10^6	1.35×10^6
			3	3.10×10^7	2.90×10^7	3.70×10^6	1.65×10^6	3.25×10^6	2.15×10^6
		Results from t-test	**Mean**	2.87×10^7	2.64×10^7	7.67×10^6	2.82×10^6	4.03×10^6	2.31×10^6
			SD	9.26×10^6	1.04×10^7	5.80×10^6	2.19×10^6	2.31×10^6	1.43×10^6
			***p* value**	0.0591		**0.0043**		**0.0017**	

Plant name	Part used	Test concentration (mg/ml)	Repeats	Growth of *S. mutans* in biofilm (CFU/ ml)					
				0 hours		6 hours		24 hours	
				Control	Plant	Control	Plant	Control	Plant
Spirostachys africana	Leaves	0.25	1	4.13×10^7	3.75×10^7	4.90×10^6	1.08×10^5	5.28×10^6	9.45×10^5
			2	2.53×10^7	2.23×10^7	1.85×10^6	1.58×10^4	2.08×10^6	2.13×10^5
			3	3.05×10^7	3.15×10^7	1.17×10^6	5.00×10^4	8.83×10^6	1.07×10^6
		0.125	1	4.13×10^7	2.90×10^7	4.90×10^6	1.35×10^6	5.28×10^6	1.29×10^6
			2	2.53×10^7	2.25×10^7	1.85×10^6	4.20×10^5	2.08×10^6	5.60×10^5
			3	3.05×10^7	2.98×10^7	1.17×10^6	3.18×10^5	8.83×10^6	2.20×10^6
		0.0625	1	4.13×10^7	2.75×10^7	4.90×10^6	1.90×10^6	5.28×10^6	2.60×10^6
			2	2.53×10^7	3.35×10^7	1.85×10^6	6.80×10^5	2.08×10^6	9.30×10^5
			3	3.05×10^7	3.40×10^7	1.17×10^6	4.18×10^5	8.83×10^6	4.15×10^6
		Results from t-test	**Mean**	3.24×10^7	2.97×10^7	2.64×10^6	5.84×10^5	5.40×10^6	1.55×10^6
			SD	7.07×10^6	5.11×10^6	1.72×10^6	6.41×10^5	2.92×10^6	1.23×10^6
			***p* value**	0.2915		**0.0024**		**0.0001**	
Tarchonanthus camphoratus	Bark	0.25	1	7.25×10^7	7.60×10^7	8.38×10^6	1.20×10^6	9.05×10^5	3.65×10^5
			2	7.35×10^7	7.60×10^7	1.25×10^6	2.10×10^5	9.00×10^5	4.40×10^5
			3	4.20×10^7	3.63×10^7	3.20×10^6	5.65×10^5	1.75×10^6	8.50×10^5
		0.125	1	7.25×10^7	5.40×10^7	8.38×10^6	1.15×10^6	9.05×10^5	4.58×10^5
			2	7.35×10^7	6.90×10^7	1.25×10^6	2.08×10^5	9.00×10^5	4.70×10^5
			3	4.20×10^7	4.25×10^7	3.20×10^6	5.45×10^5	1.75×10^6	9.33×10^5
		0.0625	1	7.25×10^7	5.93×10^7	8.38×10^6	9.30×10^5	9.05×10^5	6.15×10^5
			2	7.35×10^7	8.20×10^7	1.25×10^6	2.20×10^5	9.00×10^5	6.40×10^5
			3	4.20×10^7	4.15×10^7	3.20×10^6	3.90×10^5	1.75×10^6	1.20×10^6
		Results from t-test	**Mean**	6.27×10^7	5.96×10^7	4.28×10^6	6.02×10^5	1.19×10^6	6.63×10^5
			SD	1.55×10^7	1.70×10^7	3.19×10^6	3.98×10^5	4.24×10^5	2.78×10^5
			***p* value**	0.3136		**0.0044**		**0.0001**	

Plant name	Part used	Test concentration (mg/ml)	Repeats	Growth of *S. mutans* in biofilm (CFU/ ml)					
				0 hours		6 hours		24 hours	
				Control	Plant	Control	Plant	Control	Plant
Tecomaria capensis	Leaves	0.25	1	$2.60x10^7$	$2.55x\ 10^7$	$4.75x10^6$	$3.10x\ 10^6$	$2.20x\ 10^7$	$1.08x\ 10^7$
			2	$2.50x10^7$	$2.85x\ 10^7$	$9.23x10^6$	$4.50x\ 10^6$	$6.10x\ 10^6$	$3.20x\ 10^6$
			3	$4.30x10^7$	$4.10x\ 10^7$	$1.55x10^6$	$5.50x\ 10^5$	$1.15x\ 10^7$	$5.50x\ 10^6$
		0.125	1	$2.60x10^7$	$2.70x\ 10^7$	$4.75x10^6$	$3.15x\ 10^6$	$2.20x\ 10^7$	$1.35x\ 10^7$
			2	$2.50x10^7$	$2.50x\ 10^7$	$9.23x10^6$	$6.15x\ 10^6$	$6.10x\ 10^6$	$3.80x\ 10^6$
			3	$4.30x10^7$	$4.90x\ 10^7$	$1.55x10^6$	$7.00x\ 10^5$	$1.15x\ 10^7$	$7.00x\ 10^6$
		0.0625	1	$2.60x10^7$	$2.60x\ 10^7$	$4.75x10^6$	$4.40x\ 10^6$	$2.20x\ 10^7$	$1.85x\ 10^7$
			2	$2.50x10^7$	$2.90x\ 10^7$	$9.23x10^6$	$8.50x\ 10^6$	$6.10x\ 10^6$	$5.25x\ 10^6$
			3	$4.30x10^7$	$3.90x\ 10^7$	$1.55x10^6$	$1.00x\ 10^6$	$1.15x\ 10^7$	$1.00x\ 10^7$
			Mean	$3.13x10^7$	$3.22x\ 10^7$	$5.18x10^6$	$3.56x\ 10^6$	$2.33x\ 10^6$	$1.68x\ 10^6$
			SD	$8.76x10^6$	$8.60x\ 10^6$	$3.34x10^6$	$2.67x\ 10^6$	$7.00x\ 10^6$	$5.05x\ 10^6$
			***p* value**	0.4190		**0.0095**		**0.0039**	
Positive control: Chlorhexidine gluconate 0.2%				**-Control**	**+Control**	**-Control**	**+Control**	**- Control**	**+Control**
			1	$2.42x10^7$	$3.42x\ 10^7$	$4.24x10^6$	$4.30x\ 10^4$	$1.80x\ 10^7$	$7.20x\ 10^5$
			2	$3.78x10^7$	$3.10x\ 10^7$	$2.52x10^6$	$3.10x10^4$	$8.10x\ 10^6$	$1.20x\ 10^5$
			3	$1.48x10^7$	$9.80x\ 10^6$	$3.27x10^6$	$3.60x\ 10^4$	$6.15x10^6$	$2.20x\ 10^5$
			4	$2.42x10^7$	$3.42x\ 10^7$	$2.86x10^6$	$4.30x10^4$	$1.10x10^7$	$8.80x\ 10^5$
			5	$3.78x10^7$	$3.10x\ 10^7$	$1.47x10^6$	$7.50x10^4$	$2.42x10^7$	$2.40x\ 10^6$
			6	$1.48x10^7$	$9.80x\ 10^6$	$2.08x10^6$	$8.90x10^4$	$7.15x10^6$	$3.52x\ 10^5$
			7	$2.42x10^7$	$3.42x\ 10^7$	$1.68x10^6$	$9.20x10^4$	$6.84x10^6$	$4.58x10^5$
			8	$3.78x10^7$	$3.10x\ 10^7$	$2.64x10^6$	$3.10x10^4$	$1.55x10^7$	$3.34x10^5$
			9	$1.48x10^7$	$9.80x\ 10^6$	$1.45x10^6$	$2.18x\ 10^4$	$7.45x\ 10^6$	$2.25x10^5$
			Mean	$2.56x10^7$	$2.50x\ 10^7$	$2.47x10^6$	$5.13x10^4$	$1.16x\ 10^7$	$6.34x\ 10^5$
			SD	$1.00x10^7$	$1.15x\ 10^7$	$9.20x10^5$	$2.67x10^4$	$6.29x\ 10^6$	$7.06x\ 10^5$
			***p* value**	0.3815		**0.0001**		**0.0027**	

***p* values marked in bold are considered significant**

Table 3.2. Summary of percentages reduction in *S .mutans* biofilm formation by plant extracts

Plant name	Part used	Test concentration (mg/ml)	Repeats	Percentage reduction of *S. mutans* in biofilm			
				6 hour exposure		24 hour exposure	
				Reduction in biofilm (%)	Mean %	Reduction in biofilm (%)	Mean %
Acacia karroo	Leaves	0.25	1	96.59	88.76	56.60	59.20
			2	84.68		61.77	
			3	85.00		59.22	
		0.125	1	47.83	51.12	38.68	36.59
			2	56.45		32.26	
			3	49.07		38.83	
		0.0625	1	56.52	53.62	15.09	13.54
			2	53.23		12.90	
			3	51.11		12.62	
Berula erecta	Rhizomes	0.25	1	30.23	28.72	39.13	37.70
			2	27.91		38.10	
			3	28.02		35.87	
		0.125	1	18.60	20.46	21.74	24.41
			2	22.09		25.40	
			3	20.69		26.09	
		0.0625	1	9.30	11.96	7.61	12.64
			2	10.31		15.08	
			3	16.28		15.22	
Englerophytum magalismontanum	Bark	0.25	1	33.87	31.96	48.65	49.28
			2	32.50		51.11	
			3	29.51		48.08	
		0.125	1	25.00	23.76	44.32	42.37
			2	22.91		41.11	
			3	23.36		41.67	
		0.0625	1	16.13	14.73	34.05	31.02
			2	12.50		28.89	
			3	15.57		30.13	
Erythrina lysistemon	Bark	0.25	1	72.58	72.54	57.14	58.74
			2	70.73		62.16	
			3	74.32		56.92	
		0.125	1	60.65	61.22	39.29	38.49
			2	62.20		37.84	
			3	60.81		38.36	
		0.0625	1	58.06	56.93	31.43	30.77
			2	57.32		27.03	
			3	55.41		33.85	
Spirostachys africana	Leaves	0.25	1	97.81	97.56	82.09	86.58
			2	99.15		89.76	
			3	95.73		87.88	
		0.125	1	72.45	74.20	75.55	74.54
			2	77.30		73.01	
			3	72.86		75.07	
		0.0625	1	61.22	62.93	50.71	54.08
			2	63.24		55.18	
			3	64.32		52.97	

Plant name	Part used	Test concentration (mg/ml)	Repeats	Growth of *S. mutans* in biofilm			
				6 hour exposure		24 hour exposure	
				Reduction in biofilm (%)	Mean %	Reduction in biofilm (%)	Mean %
Tarchonanthus camphoratus	Bark	0.25	1	85.67	86.37	59.67	54.07
			2	83.20		51.11	
			3	82.34		51.43	
		0.125	1	86.27	84.21	49.45	47.98
			2	83.40		47.78	
			3	82.97		46.71	
		0.0625	1	88.90	83.74	32.04	31.74
			2	82.40		28.89	
			3	87.81		31.43	
Tecomaria capensis	Leaves	0.25	1	53.23	53.10	50.91	50.21
			2	51.22		47.54	
			3	54.84		52.17	
		0.125	1	33.68	34.16	38.64	38.49
			2	33.33		37.70	
			3	35.48		39.13	
		0.0625	1	7.27	7.19	15.91	14.29
			2	7.86		13.93	
			3	6.45		13.04	
Positive control: Chlorhexidine gluconate 0.2%			1	98.99		96.00	
			2	98.77		98.51	
			3	98.90		96.42	
			4	98.50		92.00	
			5	94.90		90.01	
			6	95.72		95.08	
			7	94.52		93.30	
			8	98.67		97.85	
			9	98.50		96.98	

The Kruskal-Wallis test was first employed to compare test plant extracts in reducing the formation of the biofilm. This test helped to determine if there was a statistically significant difference between the percentage inhibition of plant extracts at both 6 and 24 h. Once the statistically significant difference was confirmed using the Kruskal-Wallis test (p value =0.001 at both 6 and 24 h), a Dunns Pairwise comparison test was then undertaken between the plant extracts. This helps to pinpoint which percentage inhibition mean values of plant extracts are statistically significant from other plant extracts. Table 3.3 and Table 3.4 compares the p values of plant extracts and shows if there is a significant difference between percentage inhibitions. Values in bold typeface are those that have statistically significant difference ($p \leq 0.05$).

Table 3.3: Dunns Pairwise comparison test at 6 h (*p* value)

Plant samples	*Acacia karroo*	*Berula erecta*	*Englerophytum magalismontanum*	*Erythrina lysistemon*	*Spirostachys africana*	*Tecomaria capenis*
Berula erecta	**0.0033**	-	-	-	-	-
Englerophytum magalismontanum	**0.0065**	0.4065	-	-	-	-
Erythrina lysistemon	0.4619	**0.0024**	**0.0050**	-	-	-
Spirostachys africana	0.1026	**0.0000**	**0.0001**	0.1207	-	-
Tecomaria capensis	0.0197	0.2550	0.3364	0.0155	**0.0004**	-
Tarchonanthus camphoratus	0.0568	**0.0000**	**0.0000**	0.0686	0.3762	**0.0001**

***p*-values marked in bold are considered significant**

At 6 h the bark organic extract of *T. camphoratus* and the leaf extract of *S. africana* showed the most significant statistical difference to the other plant extracts. Both these extracts showed $p \leq 0.05$ against *B. erecta, E. magalismontanum* and *T. capensis* but did not show any statistical significant difference when compared to each other (p= 0.3762).

Table 3.4: Dunns Pairwise comparison test at 24 h (*p* value)

Plant samples	*Acacia karroo*	*Berula erecta*	*Englerophytum magalismontanum*	*Erythrina lysistemon*	*Spirostachys africana*	*Tecomaria capenis*
Berula erecta	0.0821	-	-	-	-	-
Englerophytum magalismontanum	0.3593	0.0399	-	-	-	-
Erythrina lysistemon	0.3002	0.0278	0.4351	-	-	-
Spirostachys africana	**0.0022**	**0.0000**	**0.0064**	**0.0100**	-	-
Tecomaria capensis	0.4087	0.1230	0.2772	0.2252	**0.0010**	-
Tarchonanthus camphoratus	0.2338	0.0171	0.3572	0.4197	0.0169	0.1692

***p*-values marked in bold are considered significant**

At 24 h there was only one plant extract, *S. africana* organic leaf extract, that showed the significant statistical difference to all other plant extracts in this assay with the exception of *T. camphoratus* bark where the *p* value was > 0.05 and therefore did not show any significant statistical difference between the two plant extracts.

Figure 3.4. shows the overall mean values of percentage inhibition n=9 (3 subinhibitory concentrations tested 3 times) at 6 and 24 h. *Tarconanthus camphoratus* organic bark extract had the highest overall percentage reduction at 6 h (88.80%) while *S. africana* leaf extract had the highest overall percentage reduction (78.20%) at 24 h. The majority of plant extracts had higher percentage reductions at 6 h in comparison to 24 h.

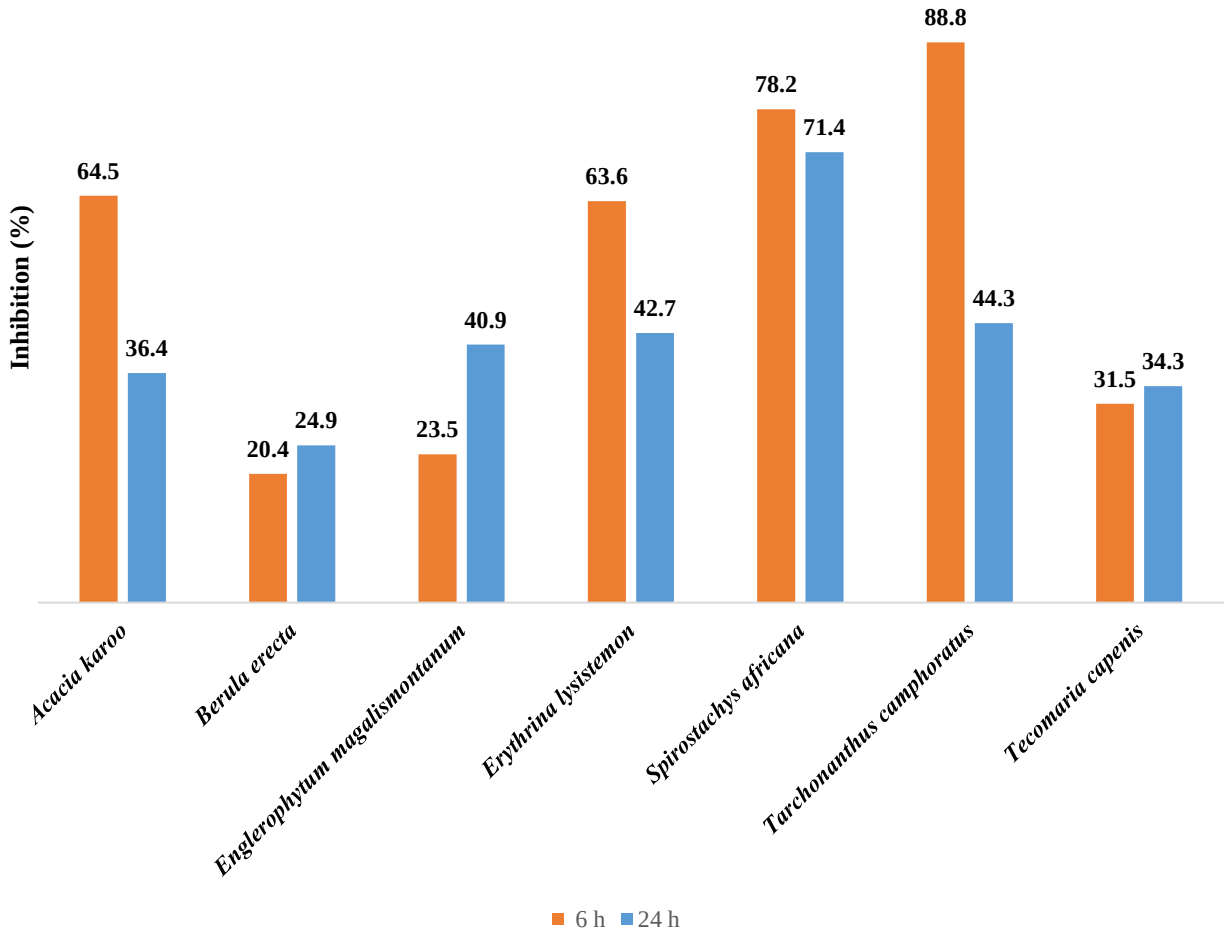


Figure 3.4. Overall mean percentage reduction of plant extracts inhibition of biofilm formation (n=9) at 6 and 24 h.

Streptococcus mutans is one of the initial bacteria that predominately proliferate in the oral dental biofilm (Jakubovics and Kolenbrander 2010; Kouidhi et al., 2015). The development of the biofilm is characterized by some distinct phases including; an initial adherence and lag phase, a rapid growth phase and a steady state phase (Gurenlian, 2007). This is the reasoning in testing the biofilm formation at 6 h (representing the adherence phase) and at 24 h (representing the growth phase). Apart from the 6 and 24 hour counts a 0 h count was also undertaken.

As the MIC concentration would not be consistent in the mouth and would be diluted by saliva and food as time goes by, this assay was therefore carried out at three subinhibitory concentrations to account for this. The properties that are required for these plant extracts to manage and reduce the biofilm that is formed not only necessitates the plant to inhibit the growth of the *S. mutans* bacterial cells but also needs anti-adhesive properties to further reduce the oral biofilm (Kouidhi et al., 2015). It is also for this reason that the concentration of plant extracts tested were subinhibitory, meaning the plant extract at this concentration could not cause death to the bacterial cells and the focus is therefore more on the effect of plant extracts on the virulence of *S. mutans.*

The results from this assay have shown that in most instances, percentage reduction for the 6 h biofilm counts were higher in comparison to the 24 h counts. This means that the plant extracts with higher percentage reductions at 6 h inhibited the adherence of *S. mutans* to the glass slides. The test plant extracts may have become too diluted over time or inactivated by the byproducts produced by *S. mutans.* Because of the presence of both sucrose and saliva in this assay, both sucrose-dependent and sucrose-independent pathways may have been inhibited by the plant extracts.

Spirostachys africana can be seen as the best test plant to use in inhibiting the formation of *S. mutans* biofilm. Compared to the control the plant significantly inhibited the biofilm formation at 6 and 24 h. The plant species also had the highest percentage inhibition at 0.25 mg/ml and had the most significant overall percentage inhibition at 24 h in comparison to the other test plants. No studies to date could be found that focuses on the biofilm formation of

this plant. Previous studies on *S. africana* have found that the plant contains terpenoids (Mathabe et al., 2008 and Paiva et al., 2010) as well as phytochemicals such as diosphenols and stachenone (Munkombwe et al., 1997). These constituents could account for the antibiofilm properties of the plant extract. *Camellia sinensis* a common shrub used to make green tea has been extensively studied against oral pathogens (Limsong et al., 2004; Hassani et al., 2008; Xu et al., 2011; Araghizadeh et al., 2013). The plant also contains phytochemicals which aids in inhibiting the attachment of cells to hard surfaces such as the enamel found in teeth (Xu et al., 2011) Another study by Limsong et al., (2004) determined the inhibitory effect of herbal extracts on the adherence of *S. mutans*, one of the herbal extracts included in the study was *Camellia sinensis*. The ethanol extracts of this plant inhibited *S. mutans* adhesion *in vitro*. A study done by Song et al., (2007) found that the plant *Polygonum cuspidatum* produced antibiofilm activity on both *S. mutans* and *Streptococcus sobrinus* at subinhibitory concentrations. The terpenoids and phenolic compounds found in the plant species were proposed in giving the plant these properties.

Tarchonanthus camphoratus bark overall produced the most significant percentage reduction at the 6 h count in comparison to the other plant species. The percentage reductions were not affected by the subinhibitory concentrations displaying the following results; at 0.25 mg/ml a mean percentage reduction of 83.74%, at 0.0125 mg/ml 84.21% and at 0.076 mg/ml 86.37%. This means that the reduction in biofilm was most likely due to the plants ability to stop *S. mutans* adhesion. A study on the phytochemistry and antioxidant properties of *T. camphoratus* by Nanyonga et al., (2013) revealed that the methanol bark extract was made up of flavonoids, saponins, phenols and tannins. These phytochemical constituents are known to have antibiofilm properties (Kouidhi et al., 2015). In particular, plants that contain flavonoids such as *Vaccinium macrocarpon* or cranberries have been studied extensively and its anti-adhesive properties well documented (Yamanaka et al., 2004; Koo et al., 2006). Another study by Hosseini et al. (2013), investigated the antibacterial activity of *Pistacia atlantica* extracts on *S. mutans* biofilm and found the anti-adherence properties of the plant to be credited to the flavonoid and tannin constituents found in the plant extract.

Acacia karroo and *E. lysistemon* were plant extracts that also displayed good anti-adherent properties in this assay. With both plant extracts, percentage reductions were higher at the 6 h

count in comparison to the 24 h count, however, the 24 h counts were still noteworthy with percentage reductions being >50.00%. As seen with *T. camphoratus,* previous studies have suggested that both these plants have been found to be rich in flavonoids (El Masry et al., 2002; Adedapo et al., 2008b). This could possibly account for these plants antibiofilm properties. Many plants that have been investigated for their anti-adhesive properties have been found to contain flavonoids (Yamanaka et al., 2004; Koo et al., 2006 ; Islam et al., 2008; Rukyadi et al., 2008; Lokegoankar et al., 2011; Feghali et al., 2012; Hosseini et al., 2013). Flavonoids not only seem to have anti-adherent properties but a study done by Hosseini et al. (2013), found that the *Morus alba* plant extract that is rich in flavonoids has anti-glucotransferase properties. This suggests that plant extracts that contain flavonoids were more likely to block sucrose-dependent pathways due to anti-glucotransferase inhibiting the formation of water-insoluble proteins that help with adherence of *S. mutans* to the tooth surface.

3.4. Summary

- All seven plant extracts that were tested had significant inhibitory effect on biofilm formation in comparison to the control.
- *Spirostachys africana* leaves had the highest inhibitory effect at a subinhibitory concentration of 0.25 mg/ml producing a percentage reduction of 97.56% at 6 h and 86.58% at 24 h. This plant extract also showed the highest overall mean percentage inhibitory effect (78.20%) at 24 h.
- *Tarchonanthus camphoratus* bark showed the highest overall mean percentage inhibitory effect (88.80%) at 6 h.
- Other plant extracts that had inhibition properties worth noting include *E. lysistemon* bark and *A. karroo* leaves
- In most instances, percentage reductions for the 6 h biofilm counts were higher in comparison to the 24 h counts.

Chapter 4

Toxicity of antimicrobially active plants

4.1. Introduction

Parcelsus, who was also known as the father of toxicology, documented the toxic effect of plants and animals as early as the 1500s (Parsuranam, 2011). Toxicology is a branch of science that deals with the adverse effects of chemical constituents on living organisms (Gowda et al., 2014). A major portion of the South African population still relies on medicinal plants as a first line treatment (Street et al., 2008). There is a misconception that natural remedies are safer in comparison to modern day conventional treatments, but, plants that display beneficial properties may also have cytotoxic properties (Hamidi et al., 2014). Compounds that are found within plants may interact with each other or with cells that can cause toxicity (Otang et al., 2013). Acute poisoning accounts for many deaths in South Africa (Malangu and Ogubanjo, 2009). Yet, there is a lack of epidemiological data available on biological toxins such as medicinal plants even though they are noted as one of the most frequent toxic exposures found in South Africa's major hospitals (Malangu and Ogubanjo, 2009; Veale et al., 2013). Therefore, it is of great importance to investigate the toxicity profiles of medicinal plants and establish the safety and efficacy of these plant extracts and essential oils.

Toxic exposure can cause damage to a number of organs in the body. Although the oral mucosa is made up of stratified epithelial tissue that acts as a protective barrier, it is still prone to toxic exposure (Gowda et al., 2014). Modern day oral hygiene products such as mouthwashes contain chemical agents such as chlorhexidine and ethanol. Chlorhexidine is known to have cytotoxic effects and cause tooth staining (Palambo, 2011). A relationship has also been found between ethanol found in common mouthwashes and oral cancer (McCullough and Farah, 2008). Therefore, there is a demand for safer, natural alternatives to these chemical agents that could be incorporated into oral hygiene products whilst producing minimal cytotoxic effects.

Toxicity studies are also imperative in the development of novel drugs and compounds (Parsuranam, 2011). When toxicity studies are run concurrently with antimicrobial studies, they help reduce the chance of false positive antimicrobial results and warrants the differentiation between toxic and antimicrobial properties of plants (Cos et al., 2006).

Toxicity studies have been employed to investigate the toxicity properties of individual plant samples that have shown noteworthy antimicrobial activity (MIC values <1.00 mg/ml for plant extracts and MIC values of ≤ 1.00 for essential oils Chapter 2, Table 3.1, 3.2 and 3.3). The brine shrimp lethality assay was used to carry out these investigations.

4.1.1. Brine shrimp lethality assay

The brine shrimp lethality assay has gained increased popularity over the years to investigate the toxicity of medicinal plants (Hamidi et al., 2014). The brine shrimp assay was developed by Micheal et al., (1956) and later modified by Vanhaeke et al., (1981), Meyer et al., (1982), and Sleet and Brendal, (1983). This crustacean model uses *Artemia franciscana* brine shrimp and is a rapid means of screening these plants (Sreeshma and Nair, 2014). Other advantages include its relatively low running cost and the small amount of plant material required. A good correlation has also been found between the LC_{50} values in this assay and the LC_{50} values obtained in animal studies (Hamidi et al., 2014).

4.2. Methods and materials

Artificial salt water was prepared by dissolving 16 g of Tropic Marine® in 500 ml of distilled water. Dried *Artemia franciscana* shrimp eggs, 0.5 g, (Ocean Nutrition) was added to the artificial seawater. To allow for maximum hatchability, the eggs were exposed to constant light (from a lamp 230 V) and the eggs were dispersed evenly with the help of a rotary pump (Kiho). The eggs were incubated at room temperature (25˚C) for 18-24 h. The brine shrimp are not fed during the assay as the nutrients found in the yolk sac of the shrimp egg is adequate for them to survive for the 48 h needed (Pelka et al., 2000).

4.2.1. Plant sample preparation

A total of 26 different plant species made up of 41 organic plant extracts and six essential oils, which were found to have antimicrobial activity (Chapter 2, Table 2.4 and 2.6), were further investigated for toxicity using the brine shrimp lethality assay. If a concentration of >1 mg/ml is required for the plant sample to exhibit toxicity, it is considered non-toxic (Bussmann et al., 2011). Therefore, samples were tested at a concentration of 1 mg/ml using DMSO (1% v/v).

4.2.2. Brine shrimp lethality assay

After incubation, the salt water containing the brine shrimp was placed in a shallow rectangle container, at an angle, and exposed to a light source (to attract the brine shrimp) thus allowing for a large amount of brine shrimp to be collected. Salt water (400 µl) containing on average 40-60 brine shrimp was added to each of the 48 wells in the micro-titre plate. Plant sample (400 µl) was then added to the wells (samples were tested in triplicate). A negative control (non-lethal) of salt water (32 mg/ml) and a positive control (lethal) 1.6 mg/ml concentration of potassium dichromate (Sigma) were added in triplicate. The viability of shrimp was confirmed prior to adding the test sample. When the brine shrimp is exposed to a substance that is toxic it will cause the shrimp to die, this will be observed using a light microscope (Olympus), if no movement is seen by the shrimp for a period of ten seconds the shrimp is considered dead (Carballo et al., 2002). Dead brine shrimp were then counted under the microscope after 24 and 48 h. After the 48 h count, 50 µl of glacial acetic acid was added to each well to kill all brine shrimps so that percentage mortality could be calculated. Percentage mortality was calculated by counting the number of dead shrimp at 24 and 48h and comparing it to the total number of dead brine shrimp.

Plant samples that displayed mortality rates > 50% were assumed to be toxic (Bussman et al., 2011). Plant samples considered toxic were then tested at six different concentrations (1, 0.5, 0.25, 0.125, 0.063 and 0.031 mg/ml). Percentage inhibitions at all six concentrations were calculated and helo to generate LC_{50} values that were determined using IBM® SPSS statistics and probit analysis. The LC_{50} value represents the concentration of a test substance necessary to have a lethal effect on 50% of the brine shrimp.

4.3. Results and discussion

The average percentage mortalities (n=3) of brine shrimp exposed to plant extracts can be found in Table 4.1. The majority of the 41 organic plant extracts investigated were considered non-toxic with percentage mortality rates below 50%.

Table 4.1: Average percentage mortality of brine shrimp exposed to South African plants extracts (1 mg/ml) used traditionally to treat oral diseases.

Plant extract	**% Mortality (n=3)**	
	24 h	**48 h**
Acacia karroo leaves	15.03	19.46
Acacia karroo bark	7.01	7.01
Acacia polyacantha stems	14.24	21.97
Acokanthera oppositifolia stems	26.89	47.31
Artemisia afra leaves	1.44	1.44
Artemisia afra stems	3.39	3.39
Berula erecta leaves	**100.00**	**100.00**
Berula erecta rhizomes	21.75	28.33
Carpobrotus edulis	47.43	48.06
Cissampelos torulosa stems	**100.00**	**100.00**
Clausena anisata leaves	**100.00**	**100.00**
Clausena anisata stems	**100.00**	**100.00**
Clematis brachiata leaves	23.42	31.68
Clematis brachiata flowers	33.27	46.12
Clematis brachiata leaves (RHN)	18.01	22.32
Clematis brachiata roots (RHN)	5.62	12.03
Cotyledon orbiculata leaves	35.28	35.28
Croton gratissimus leaves	**100.00**	**100.00**
Croton gratissimus stems	38.13	42.71
Dalbergia obovata stems	22.73	23.49
Dichrostachys cinera stems	10.21	15.05
Dodonaea viscosa leaves	34.69	39.03
Englerophytum magalismontanum stems	42.61	42.61
Erythrina lysistemon stems	12.02	16.31
Heteropyxis natalensis leaves	23.08	43.02
Heteropyxis natalensis stems	18.78	31.34
Myrothamnus flabellifolius leaves	3.16	3.16
Sansevieria hyacinthoides leaves	33.88	33.88
Sansevieria hyacinthoides stems	29.70	29.70
Siphonochilus aethiopicus leaves	33.71	36.77
Siphonochilus aethiopicus roots	30.48	39.66
Spirostachys africana leaves	**100.00**	**100.00**
Spirostachys africana stems	**99.38**	**100.00**
Tarchonanthus camphoratus stems	15.18	15.18
Tecomaria capensis leaves	25.48	25.48
Tecomaria capensis stems	27.80	44.10
Tetradenia riparia leaves	41.00	45.76

Plant extract	% Mortality (n=3)	
	24 h	48 h
Warburgia salutaris bark	**98.03**	**100.00**
Warburgia salutaris twigs	32.54	33.17
Zanthoxylum capense leaves	45.68	46.36
Zanthoxylum capense stems	9.07	9.07
Negative control: Tropic marine ® water (32 mg/ml)	0.00	0.00
Positive control: Potassium dichromate (1.6 mg/ml)	100.00	100.00

Values marked in bold typeface are considered toxic at a concentration of 1mg/ml (this is when percentage mortality was > 50%)

The average percentage mortalities of brine shrimp exposed to essential oils (n=3) can be found in Table 4.2. Five of the six essential oils tested were found to be toxic at a concentration of 1mg/ml.

Table 4.2: Average percentage mortality of brine shrimp exposed to South African plant essential oils (1 mg/ml).

Essential oil	% Mortality n=3	
	24 h	48 h
Artemisia afra	0.00	2.58
Clausena anisata	**100.00**	**100.00**
Croton gratissimus	**93.33**	**100.00**
Myrothamnus flabellifolius	**100.00**	**100.00**
Tarchonanthus camphoratus	**96.00**	**100.00**
Tetradenia riparia	**81.81**	**100.00**
Negative control: Tropic marine ® water (32 mg/ml)	0.00	0.00
Positive control: Potassium dichromate (1.6 mg/ml)	100.00	100.00

Values marked in bold typeface are considered toxic at a concentration of 1mg/ml (this is when percentage mortality was > 50%)

Using the brine-shrimp lethality assay, the plant samples were tested and many were deemed safe to use at a concentration of 1 mg/ml. In some instances brine shrimp when exposed to all parts of a particular plant tested showed low percentage mortalities (<50%). These plants included *A. karroo, A. afra, C. brachiata, S. hyacinthiodes, S. aethiopicus, T. capensis* and *Z. capensis.* In particular, the plant that had the least effect on the brine shrimp was *Artemisia afra* organic leaf extract with a percentage mortality of 1.44% at both 24 and 48 h. The stem and essential oil of *A. afra* were also considered safe against the brine shrimp killing <50% of the brine shrimp. *Acacia karroo* was another plant that showed minimum toxicity of both the bark and leaf at 24 and 48 h. The toxicity profiles of the bark and leaf extract were also considered safe against the brine shrimp with < 20% of the brine shrimp dying.

Acacia karroo is a plant commonly found in Southern Africa and has many medicinal uses (Mapiye et al., 2011). Both the bark and leaf organic extract showed minimal toxic effect to the brine shrimp with percentage mortality reductions of 15.03% at 24 h and 19.46% at 48 h for the leaf extract and 7.01% for the bark extract at both 24 and 48 h. A previous study by Adedapo et al. (2008a), tested acute toxicity of the aqueous bark extract of this plant and found it to cause death to the mice at an oral concentration of 1600 and 3200 mg/ml. At 800 mg/ml the plant was found to be hepatotoxic and decreased the platelet count of the mice significantly. The plants were tested at a much higher concentration in comparison to this study. One of the traditional uses that have been noted includes *A. karroo* being used to treat poisoning in cattle (Adedapo et al., 2008b). The plant has also been found to have high nutritional values and is used to supplement the feed of livestock (Adedapo et al., 2008b; Brown, 2016). From the widespread traditional use it is clear that this plant should be safe to use at the concentrations tested in this study.

The leaf and stem organic extract and the leaf essential oil of *A. afra* were all tested individually in the brine shrimp lethality assay with both parts being found to be non toxic. The plant had the least effect on the brine shrimp (percentage mortality rate 1.44%) seen in this assay at 24 and 48 h. The essential oil of *A. afra* also produced the least toxic effect on the brine shrimp in comparison to the other essential oils displaying no effect on the brine shrimp at 24 h and low toxic effect (2.58%) at 48 h. Similar percentage mortality values were reported by Hubsch, (2014). The aqueous and organic leaf extract and essential oil all showed minimal toxic effect to the brine shrimp. The general lack of toxicity of this plant could account for its widespread popularity. It has multiple antimicrobial uses against a variety of ailments and is one of the oldest plants documented for its use in traditional medicine in South Africa (Mangena and Muyima, 1999; Thring and Weitz, 2006).

All parts of *C. brachiata* were considered safe against the brine shrimp with all percentage mortalities being <50%. In particular, the roots had very little effect on the brine shrimp larvae with percentage mortality of 5.62% at 24 h and 12.03% at 48 h. This would explain why the roots are traditionally used (Hutchings, 1996). Toxicity tests have been undertaken against *C. brachiata* (Afolayan et al., 2009). This study investigated aqueous leaf plant extract on male

white rats and found that at 1 mg/ml the extract had no significant effect on the kidneys and only had effect on the liver bilirubin production after 21 days.

While the majority of the plant samples tested were considered safe, five out of six essential oils and eight plant extracts were considered toxic at a concentration of 1 mg/ml. This include the leaves and stems of *S. africana* and the essential oil, leaves and stems of *C. anisata*. Since toxicity of these plants have already been established at 1 mg/ml, the LC_{50} values were determined for these 13 plant samples to help establish the degree of toxicity. The LC_{50} values can be seen in Table 4.3. The LC_{50} value of a substance is the lethal concentration that will kill 50% of the test sample. Plant samples with a lower LC_{50} value are considered more toxic in nature. LC_{50} values below 249 µg/ml are considered highly toxic, 250 – 499 µg/ml are considered as moderately toxic and 500 – 999 µg/ml are considered weak or low in toxicity. LC_{50} values ≥ 1000 µg/ml are considered non-toxic (Bussmann et al., 2011).

Table 4.3: LC_{50} values of plant samples used traditionally to treat oral diseases.

Plant extract	**LC_{50} (µg/ml) n=3**	
	24 h	**48 h**
Berula erecta leaves	336	329
Cissampelos torulosa stems	**135**	**129**
Clausena anisata leaves	**132**	**130**
Clausena anisata stems	**126**	**120**
Croton gratissimus leaves	**103**	**103**
Spirostachys africana leaves	385	385
Spirostachys africana stems	297	277
Warburgia salutaris bark	**164**	**164**
Essential oils		
Clausena anisata	**194**	**194**
Croton gratissimus	323	323
Myrothamnus flabellifolius	**136**	**136**
Tarchonanthus camphoratus	**216**	**216**
Tetradenia riparia	**229**	**229**
Negative control: Tropic marine ® water (32 mg/ml)	0.00	0.00
Positve control: Potassium dichromate (1.6 mg/ml)	100.00	100.00

Values marked in bold typeface are considered highly toxic with values below 249 µg/ml

All parts of *C. anisata* plants were highly toxic. The leaves, stems and essential oil were tested and had LC_{50} values of 132 µg/ml, 126 µg/ml and 194 µg/ ml (all below 249 µg/ml).

Cissampelos torulosa stems, *C. gratissimus* leaves and *W. salutaris* bark were also considered highly toxic. Three other essential oils, *M. flabellifolius*, *T. camphoratus* and *T. riparia* were also found to be highly toxic with LC_{50} values < 24µg/ml.

The leaves of *B. erecta* have been found to be toxic in nature at a concentration of 1 mg/ml. Further investigation has revealed that the LC_{50} value of leaves were 336 µg/ml at 24 h and 329 µg/ml at 48 h, having a moderate degree of toxicity. In comparison, the rhizomes of this plant were found to be safe at a concentration of 1 mg/ml with a percentage mortality of 21.75% at 24 h and 28.33% at 48 h. This could be the reason the rhizomes and not the leaves are the part of the plant traditionally used to treat toothache (Van Wyk, 2008).

The *Cissempelos* spp. is noted in ethnobotanical literature for their toxic characteristics. The plant contains alkaloids such as bebeerine (Watt and Breyer-Brandwijk, 1962). Bebeerine is a bisbenzylisoquinoline alkaloid (Kocisko et al., 2003). Naturally derived bisbenzylisoquinoline alkaloids have been studied for their antiplasmodial and cytotoxic properties (Angerhofer et al., 1999; Steele et al., 1999). This constituent could therefore be responsible for the toxic properties of this plant on the brine shrimp. This species of plant has been documented not only for its beneficial antimicrobial use but for its traditional use as arrow poison and fumigant (Semwal et al., 2014). This plant has also been investigated and confirmed for its cytotoxic efficacy against a number of cancer cell lines including breast and renal (de Wet et al., 2009). This corroborated with results from this study where the plant caused 100% lethality of the brine shrimp at 1 mg/ml and was considered highly toxic when investigated further with LC_{50} values of 135 µg/ml at 24 h and 129 µg/ml at 48 h.

Toxic properties of *C. anisata* has been investigated before by Makirita et al, (2016) and Moshi et al, (2010). Both these studies were conducted in Tanzania where the plant is traditionally used. The brine shrimp lethality assay revealed similar results to this study. The dichloromethane extract of *C. anisata* root was toxic with an LC_{50} value of 71.9 µg/ ml (Moshi et al., 2010.) Makrita et al (2016), investigated the cytotoxic profiles of *C. anisata* leaves, fruit, twigs and bark. The results revealed LC_{50} values of less than 249 µg/ ml for all extracts tested with the exception of the methanol fruit extract which displayed LC_{50} values > 1000 µg/ml. The essential oil of *C. anisata* has been found to be rich in the compound

estragole, a compound that is proposed to cause the carcinogenic and toxic effects (Okunade and Olaifa, 1987). This compound could be the cause of death of the brine shrimp in this study.

The leaf extract and essential oil of *C. gratissimus* has been documented in the ethnobotanical literature as being toxic, however, is commonly used traditionally as a purgative (Watt and Breyer-Brandwijk, 1962). The cytotoxic and antiproliferative properties have been demonstrated in several studies (Mulholland et al., 2010; Okokan and Nwafor, 2009, Mukanganyama et al., 2012). Subchronic toxic studies in a rat model have found that the root of *C. gratissimus* is safe and had no significant effect on the liver (Okokan et al., 2010). Another study that investigated the toxic effects of *C. gratissimus* leaves in a rat model found the methanol extract to be safe up to concentrations of 5000 mg/ml (Onwusonye et al., 2016). Although the leaf extract and essential oil were found to be toxic in this study the part used traditionally to treat bleeding gums is the bark and stem of the plant which was found to be safe at 1 mg/ml with percentage reductions <50%.

Both the leaf and stem organic extract of *S. africana* were toxic at a concentration of 1 mg/ml. The LC_{50} values of the leaves and stem extracts were found to be moderate in toxicity. This plant has been documented in the ethnobotanic literature for causing diarrhoea, headache and nausea (Kuete, 2014). The wood is burnt to release toxins in the preparation of poison used on arrows (Bradfield et al., 2015). Previous cytotoxic studies have isolated active compounds from this plant and found that two of these compounds, a known triterpenoid isolated as Lupeol and a diterpene, were toxic when tested using the XTT assay using the vero cell-line (Mathabe et al., 2008).

The essential oil of *T. camphoratus* was found to have a high degree of toxicity with an LC_{50} value of 216 µg/ml in this study. A study investigating the cytotoxic properties of the bark, fresh leaf and dry leaf essential oils was undertaken by Nanyonga et al, (2013). The brine shrimp lethality assay revealed that the essential oils were considered toxic at a concentration of 1 mg/ml with all percentage reductions of the brine shrimp being >50%, however, LC_{50} values were determined using the human embryonic kidney cells and the LC_{50} values obtained were greater than 249 µg/ml and were considered less toxic in comparison to this

study. One of the reasons for the difference in LC_{50} values could be due to the difference in assays. A crustacean model (brine shrimp lethality assay) can yield different results in comparison to results from a human cell line (MTT assay) (Steenkamp and Gouws, 2006).

Tetradenia riparia is one of the most aromatic medicinal plants found in South Africa (Gazim et al., 2014). The essential oil was found to be toxic in this study. An MTT assay was conducted previously on the essential oil against two tumor cell lines with high percentage inhibitions of both cell lines (Gazim et al., 2014).

The use of *W. salutaris* plant is widespread where it is used both traditionally and commercially in products (Kotina et al., 2014). The bark was found to be toxic in this study. The bark constituents have been studied before and have been found to have powerful antifeedant activity that could possibly cause toxicity (Nakanishi, 1980; Drewes et al., 2001). A study by Nibret et al (2010), studied the trypanocidal and cytotoxic activity of the bark of *W. salutaris.* The methanol and dichloromethane extracts produced high degrees of toxicity with IC_{50} values of 114.91 and 40.62 µg/ml respectively against HL-60 cells.

While the essential oils of *T. riparia*, *T. camphoratus* and *M. flabellifoilus* were all found to be highly toxic to the brine shrimp, the plant extracts made of the same leaf material were considered safe at a concentration of 1.00 mg/ml with killing <50% of the brine shrimp. Essential oils are considered more volatile compounds that are highly concentrated when compared to actual plant extracts. Essential oils are also lipophilic in nature allowing for easy access into membranes in the body, this could account for the variations in toxic effects of the essentials oils and plant extracts (Williamson, 2006).

The toxicity of plant samples depends on the composition of the plant samples that can be influenced by a number of factors including the geographical source of the plants, seasonal and ecological variations, type and time of extraction process used, and part of the plant used (Rani, 2013). This coupled with the variations in assays used in these studies could be proposed as the reason there are sometimes discrepancies between results in this study and other literature. Another factor that needs to be considered is that even though a plant can be found to have a high degree of toxicity, the MIC value could be lower than the LC_{50} value.

This was seen with *C. torulosa* stems where the MIC value against both *Lactobacillus* spp. was 0.06 mg/ml or 60 µg/ml and the LC_{50} values from the brine shrimp lethality assay were 135 µg/ml and 129 µg/ml at 24 and 48 h.

4.4. Summary

- The majority of plant extracts tested in the brine shrimp lethality assay were considered safe to use.
- *Artemisia afra* was found to be the safest plant in this study with percentage mortalities of 1.44% at 24 and 48 h for the leaves and 3.39% at 24 h and 48 h for the stem plant extract. The essential oil of *A. afra* was the only safe oil in this study with percentage mortalities of 0.00% and 2.58% at 24 and 48 h respectively.
- Eight plants samples, five plant extracts and three essential oils, were considered highly toxic with LC_{50} values below 249 µg/ml. *Cissampelos torulosa* stems, *C. anisata* leaves and stems, *C. gratissimus* leaves and *W. salutaris* bark were the organic extracts and *C. anisata, T. camphoratus* and *T. riparia* were the essential oils with LC_{50} values below 249 µg/ ml.

Chapter 5
Conclusions and future recommendations

5.1. Summary

The use of traditional medicine and in particular medicinal plants is a very common and abundant practice in South Africa. This study focused particularly on the traditional use of these medicinal plants for the treatment of oral infections. The ethnobotanical literature review revealed that there are 132 plants that are used for the treatment of various types of oral diseases. Based on availability and sustainable harvesting protocols, 31 plants were collected and investigated further for antimicrobial efficacy. Aqueous and organic (dichloromethane: methanol) plant extracts were prepared from the 31 plants. Six of the 31 plants selected in this study also underwent distillation and the essential oils obtained. The plants samples were first investigated for their antimicrobial activity against nine pathogens associated with the most common dental diseases; dental caries, periodontal diseases and oral candidiasis. Toxicity profiles were then undertaken for plant samples that were found to have noteworthy antimicrobial activity (MIC <1.00 mg/ml) against any of the pathogens in this study. Seven plant extracts showed active antimicrobial activity against *S. mutans* and were further investigated for its efficacy against *S. mutans* biofilm production.

5.1.1. Antimicrobial activity of plants

The findings from the results of the MIC assay favour the potential use of many of these essential oils and plant extracts in treating dental caries, periodontal diseases and oral thrush. When testing the plant samples extracts the oral pathogens in this study it was found that the organic (dichloromethane: methanol) extracts achieved a greater inhibitory antimicrobial activity in comparison to the aqueous extracts. The lowest MIC value (0.05 mg/ml) in this study was displayed by the organic extract of *C. torulosa* stems against both *Lactobacillus* spp. and by *S. africana* leaves against *C. albicans.* The lowest MIC value of 1.00 mg/ml displayed by the aqueous extracts was *C. anisata* twigs, *S. hyacinthiodes* rhizomes and *T.*

camphoratus leaves against *S. sanguis*. *W. salutaris* bark also displayed the same activity (1.00 mg/ml) against *F. nucleatum* and *C. krusei.* Of the six essential oils tested, *C. gratissimus* had the lowest (best activity) mean MIC value of 0.88 mg/ml. The lowest MIC value noted by *C. gratissimus* against oral pathogens was (MIC 0.25 mg/ml) against *L. casei*. Other plant species displaying the same efficacy include *T. riparia* against *F. nucleatum* and *M. flabellifolia* against *P. gingivalis* and *F. nucleatum*.

5.1.2. The effect of crude plant extracts on *S. mutans* biofilm formation

All seven of the plant extracts that were tested had an inhibitory effect on biofilm formation in comparison to the negative control. *Spirostachys africana* leaves had the greatest inhibitory effect at a subinhibitory concentration of 0.25 mg/ml producing a percentage reduction of 97.56% at 6 h and 86.58% at 24 h. Overall, this plant had the most significant reduction of biofilm formation at 24 h. Another plant extract that had inhibition properties worth noting is *T. camphoratus* bark, at a subinhibitory concentration of 0.25 mg/ml, *T. camphoratus* displayed percentage reductions of 83.74% at 6 h and 54.07% at 24 h. In comparison to other test plant, *T. camphoratus* bark had the most significant reduction in *S. mutans* biofilm production at 6 h. In most instances, percentage reductions for the 6 h biofilm counts were higher in comparison to the 24 h counts. Due to the presence of both sucrose and saliva in this assay both sucrose-dependent and sucrose-independent pathways could have been inhibited by the plant extracts.

The results from this assay have shown that some plant extracts tested, in particular, *S. africana* and *T. camphoratus,* have not only the ability to inhibit the growth of *S. mutans* but also display good anti-adherent properties making them prime candidates as active ingredients in oral hygiene products such as toothpastes and mouthwashes.

5.1.3. Toxicity of plant samples

A total of 26 plant species made up of 41 organic plant extracts and six essential oils were investigated using the brine-shrimp lethality assay.The majority of the plant extracts tested in the brine shrimp lethality assay were considered non-toxic with the exception of *B. erecta*

leaves, *C. torulosa* steams, *C. anista* leaves and stems, *C. gratissimus* leaves *S. africana* leaves and stems and *W. salutaris* bark. Five (*C. anista, C. gratissimus, M. flabellifolius, T. camphoratus* and *T. riparia*) of the six essential oils tested were considered toxic. All plant samples (eight plant extracts and five essential oils) that were considered toxic at a concentration of 1 mg/ml were further tested at six different concentrations and their LC_{50} values were determined. In some instances, plants were found to be toxic, but still traditionally used and dose is always a deciding factor in traditional use.

There is a lack of in-depth scientific research on the toxicity profiles of all the plants that have been investigated. The brine shrimp lethality assay is also considered more of a preliminary test and further emphasizes the need for more *in vivo* studies that focus on toxic exposure in the oral cavity. Better reporting of symptoms due to toxic exposure of plants by both traditional healers and hospitals are also needed to help determine and fully understand the safety and efficacy of all these plants.

5.1.4. Traditional use of the plant

In some instances, there was a direct correlation between the antimicrobial activity and the traditional use displayed by the plants in this study. Pathogen specific noteworthy activity was also more common. This validates the traditional use of these plants in the treatment of oral diseases. *Cissempelos torulosa* stems are traditionally used in the treatment of toothache. *Streptococcus* spp. and *Lactobacillus* spp. are the causative organisms implicated in toothache and this plant displayed antimicrobial efficacy against both *Lactobacillus* spp. with the lowest MIC value in this study of 0.05 mg/ml and also showed good antimicrobial efficacy against *S. sanguis* (MIC 0.50 mg/ml). A similar relation has been found by *S. africana* where unspecified parts of the plant are used traditionally to treat toothache. *Englerophytum magalismonatanum* bark was another plant where the MIC values corroborates with the traditional use of the plant, displaying notable activity against *S. mutans* (MIC 0.50 mg/ml) and *S. sanguis* (MIC 0.42 mg/ml), the bark is traditionally chewed to relieve toothache.

Some plants that were traditionally used to treat periodontal diseases were also found to have a direct relationship to the MIC values obtained in this study. This was seen with *M. flabellifolia*, the leaves are traditionally documented as being used to treat Vincents gingivitis, a type of periodontal gum disease, the leaf extract displayed good antimicrobial efficacy against both the periodontal pathogens in this study; *P. gingivalis* (MIC 0.50 mg/ml) and *F. nucleatum* (MIC 0.33 mg/ml) the leaf essential oil also displayed noteworthy activity against both *P. gingivalis* and *F. nucleatum* (MIC 0.25 mg/ml).

A leaf infusion of *T. riparia* is traditionally used in the treatment of gum disease, the organic extract produced antimicrobial efficacy against *P. gingivalis* (MIC 0.50 mg/ml) while the leaf essential oil had good activity against *F. nucleatum* (MIC 0.25 mg/ml) and *P. gingivalis* (MIC 0.50 mg/ml) thus giving credibility to the plants traditional use. The last plant worth noting is *C. brachiata*. This plant has been used traditionally to treat oral thrush.

5.2. Further recommendations

This study can be considered as the first in depth study to provide *in vitro* scientific information on a range of medicinal plants used traditionally in the treatment of oral diseases. One needs to take cognisance, however, only 31 out of 132 plants documented have been investigated and this leaves a large scope still to be investigated. Only 17% of the plants found in the ethnobotanical review (Chapter 1, Table 1.1) have been previously investigated before against oral pathogens.

Now that the antimicrobial efficacy of these plants have been confirmed the next step would be to identify and isolate the active compounds found in the plants that showed the most antimicrobial activity. Isolation of active compounds can be the first step in the development of novel drug compounds that could be used for the treatment of oral infections. Compounds that are derived from plants are also considered safer and more effective than the actual plant extract (Rates, 2001). Isolation of these compounds can also help identify its structural activity. This allows us to understand and determine the correct pathways and mechanism of action that these compounds exert on oral pathogens at a cellular and biochemical level.

Investigating the pathways that the plant extracts use to inhibit the growth of *S. mutans* biofilm production and affect the virulence of the bacterium can be analysed. Specifically, investigations can be conducted that determine the effect of the plant extract on *S. mutans* extra polysaccharide production. Other studies can also be done to see if the plant extracts interfere with adhesins and proteins that are needed for adherence of the biofilm to the tooth surface. Lastly, studies on the effect of the plant extracts on lactic acid production by *S. mutans* can be investigated. There are also other in-depth studies that can investigate the mechanistic antimicrobial efficacy of plant extracts against periodontal pathogens. These studies include analysing the effect of periodontal active plants on collagenase and gingipains production. Periodontal diseases are also associated with pain and inflammation and therefore analysing active plants for anti-inflammatory properties and cytokine inhibition would be beneficial.

As mentioned frequently in this study, the oral biofilm or dental plaque consists of a multitude of different micro-organisms that interact and adhere to each other. This study has particularly focused on the effect of active plants on *S. mutans* biofilm production. Further studies could investigate the use of a polymicrobial biofilms that mimic the plaque that is found in the mouth. Other studies should also analyse not only the effect of the plants on biofilm formation but also if the plant species are able to eradicate preformed biofilms.

As this study focused on *in vitro* studies, further research should conduct *in vivo* studies that would help confirm the *in vitro* antimicrobial and toxic findings from this study. This would allow for a better understanding of the biological pathways that the plant samples could affect the human body and in particular the oral cavity. Special consideration for studies that focus on the effect and interaction of these plants on the oral cavity, for example, *in vivo* studies that focus on the toxic exposures in the oral cavity. This would help in the development of safe and effective novel drugs and delivery systems that can be used to treat oral infections.

Lastly, *C. brachiata* has been used traditionally to treat oral thrush, as discussed previously, the roots are traditionally cooked with salt to treat thrush, however, in this study the stems displayed the best overall activity against all three *Candida* spp. This invites the question;

does the traditional use of salt to enhance the anti-candidal effect of this plant and further studies should look into this?

From the number of natural products already incorporated into commercial products it is clear that there is a need for further investigation into the possibility of new treatments that incorporate South African natural resources for antimicrobial use to treat and prevent periodontal diseases, dental caries and oral candidiasis. Formulation studies should look into the development of mouthwashes, toothpastes and chewing gum that include natural South African products. This product should have good absorption and permeability into the oral mucosa and have inhibitory antimicrobial efficacy at low concentrations with limited side effects.

5.3. Final conclusions

Overall, the majority of the plants traditionally used to treat oral diseases have been found to have some antimicrobial activity against the tested oral pathogens. The antimicrobial findings from this study validate the traditional use of these plants for the treatment of dental caries, periodontal diseases and oral candidiasis. These promising results could be used as the first step in future endeavours that produces novel drugs that help in combating the increasing problem of oral diseases where previously oral pathogens have been neglected. The toxicity profile findings in this study could aid as a warning for the over use of plants that have been found to be toxic in nature.

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Appendix A:

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An *in vitro* investigation of indigenous South African medicinal plants used to treat oral infections

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Toxicity

ABSTRACT

Ethnopharmacological relevance: Over a 120 South African medicinal plants are used for the treatment of oral diseases. Despite the vast collection of antimicrobial studies being done on South African plants, there is still limited research on pathogens associated with oral infections. In consultation with the available ethnobotanical literature, this study investigates the antimicrobial efficacy of some South African medicinal plants against oral pathogens.

Aim of the study: To provide a detailed account of the antimicrobial properties of selected South African medicinal plants used traditionally to treat oral infections. The effect on *Streptococcus mutans* biofilm formation and the toxicity profiles of these plants are also investigated.

Materials and methods: A total of 136 aqueous and organic extracts and six essential oils were prepared from 31 different plant species. These plant samples were screened for antimicrobial efficacy against nine oral pathogens using the micro-titre plate dilution assay. Plant extracts that were found to have noteworthy antimicrobial activity against *S. mutans* were further evaluated on the effect on *S. mutans* biofilm formation using the glass slide technique. The toxicity profiles of plant samples that were found to have noteworthy antimicrobial activity were evaluated using the brine shrimp lethality assay.

Results: The organic extract of *Clausmpolos tomentosa* stems displayed the lowest MIC value of 0.05 mg/ml. against both *Lactobacillus* spp. This high antimicrobial activity was also observed with the organic extract of *Spirostachys africana* leaves against *Candida albicans*. In some instances, a direct relationship was found between the traditional use of the plant and the antimicrobial activity observed. For example, noteworthy activity (MIC < 1.00 mg/ml.) was observed against all three *Candida* spp. when tested against *Clematis brachiata* (leaves), a plant traditionally used to treat oral thrush. *Englerophytum magalismontanum* stems displayed notable activity against both *Streptococcus* spp. (MIC 0.83 mg/ml. against *S. mutans* and MIC 0.67 mg/ml. against *S. sanguis*). *Spirostachys africana* leaves displayed the greatest anti-adherent properties against *S. mutans* biofilm formation at both 24 and 48 h, reducing the biofilm by 97.56% and 86.58% respectively. The majority of plant samples tested in the brine shrimp lethality assay (BSLA) were considered safe, however, 13 plant samples were considered toxic, at a concentration of 1 mg/ml..

Conclusion: Noteworthy antimicrobial activity for plants species such as *C. brachiata* and *E. magalismontanum* provides validation for the traditional use of these plants. *Spirostachys africana* displayed the greatest reduction of adherent *S. mutans* cells. The BSLA results revealed that the majority of the plant samples were not toxic in nature. The findings from the results favour the potential use of these plants in treating oral diseases such as dental caries, periodontal diseases and oral thrush.

1. Introduction

Oral diseases in South Africa remain a huge public health problem due to the high prevalence, severity and the cost of oral healthcare (Singh, 2011). Conventional treatment of periodontal infections includes surgery, debridements and tooth extractions making this a great

Abbreviations: Aq, aqueous extract; AIDS, acquired immune deficiency syndrome; ATCC, american type culture collection; BSLA, brine shrimp lethality assay; CFU/ml., colony forming units per millilitre; D:M, 1:1 mixture of dichloromethane: methanol; DMSO, dimethyl sulfoxide; HIV, human Immunodeficiency Virus; INT, p-iodonitrotetrazolium chloride; MIC, minimum inhibitory concentration; PBS, phosphate buffered saline; TSA, tryptone Soya agar; TSB, tryptone soya broth

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Appendix B:

The antimicrobial efficacy of African medicinal plants against oral pathogens.

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[2]Department of Clinical Microbiology and Infectious Diseases
[1,2]Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

Purpose: Oral diseases in South Africa remain a huge public health problem due to the high prevalence, severity and influence on the patients well-being. A major portion of the South African population relies on medicinal plants as the first line treatment. The aim of this study was to investigate the antimicrobial efficacy of selected African plants against pathogens known to cause oral diseases.

Methods: Organic (dichloromethane: methanol, 1:1) extracts were prepared from the African medicinal plants used traditionally in treating oral infections. The antimicrobial activity was assessed using the minimum inhibitory concentration (MIC) assay against the micro-organisms associated dental caries and periodontal diseases; *Streptococcus mutans* (ATCC 25175), *Lactobacillus acidophilus* (ATCC 4356) and *L. casei* (ATCC 344). The MIC assay was also tested against micro-organisms associated with oral candidiasis; *Candida albicans* (ATCC 10231), *C. glabrata* (ATCC 90030) and *C. krusei* (ATCC 14243).

Results: From the antimicrobial studies *Datura stramonium* displayed noteworthy activity (0.25 mg/mL) against *C. glabrata* and *C. krusei*. *Clematis brachiata* and *Heteropyxis natalensis* also displayed good activity against *C. glabrata* with MIC values of 0.25 mg/ mL and 0.50 mg/ mL respectively. *Artemisia afra* (0.50 mg/ mL), *Berula erecta* (0.50 mg/ mL), *Tecomaria capensis* (0.38 mg/ mL) and *Warburgia salutaris* (0. 25 mg/ ml) all showed good activity against *C. krusei. Tarchonanthus camphoratus* displayed one of the best activities against *S. mutans* with mean MIC values as low as 0.50 mg/ mL. *Cissampelos torulosa* displayed one of the lowest MIC values against *L. acidophilus* and *L. casei* with an MIC value of 0.06 mg/ mL. *Heteropyxis natalensis, Dodonaea viscosa*, and *Tetradenia riparia* all displayed noteworthy activity against both *Lactobacillus* spp.

Conclusion: This study validates the traditional use of some African medicinal plants for the treatment of oral infections

Appendix C:

The antimicrobial investigation of African plants against oral *Lactobacillus* and *Candida* spp.

Saajida Akhalwaya[1], Sandy van Vuuren[1] and Mrudula Patel[2]

[1] Dept. of Pharmacy and Pharmacology, Faculty of Health Sciences, University of Witwatersrand

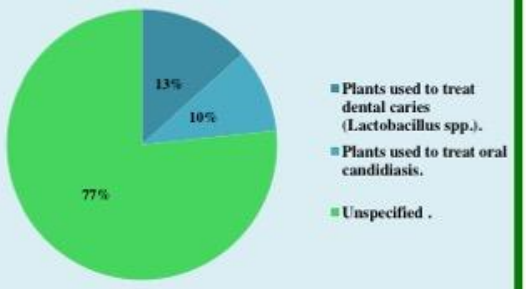
[2] Dept. of Clinical Microbiology and Infectious Diseases, Faculty of Health Sciences, University of the Witwatersrand



INTRODUCTION

The *Lactobacillus* spp. are one of the most common bacterial species with cariogenic ability i.e. the ability to form plaque and produce lactic acid. A strong correlation has been found between *Lactobacillus* counts in the mouth and dental caries[1]. The *Candida* species are opportunistic micro-organisms that reside as normal microflora and manifest as an infection in the mouth in immunocompromised conditions[2,3].A review of the ethnobotanical literature (Figure 1), is an overview of over 100 African medicinal plants used to treat oral diseases. A major portion of the South African population relies on medicinal plants as the first line treatment for oral diseases[4]. It is therefore of great importance to scientifically validate the antimicrobial efficacy of these indigenous African plants for oral infections.

Figure 1: Traditional use of African plants to treat oral infections.



AIM

The aim of this study is to investigate the antimicrobial efficacy of African plants used for oral infections against *Lactobacillus* and *Candida* spp.

METHODS

Organic (dichloromethane: methanol, 1:1) extracts were prepared from a selection of African medicinal plants used traditionally in treating oral infections. The antimicrobial activity was assessed using the micro-titre plate dilution assay to determine the minimum inhibitory concentration (MIC) against the micro-organisms associated with dental caries; *Lactobacillus acidophilus* (ATCC 4356) and *L. casei* (ATCC 344). The MIC assay was also tested against micro-organisms associated with oral candidiasis; *Candida albicans* (ATCC 10231), *C. glabrata* (ATCC 90030) and *C. krusei* (ATCC 14243). Ciprofloxacin was used as a positive control at starting concentrations of 0.01 mg/ml and included in each bacterial assay to confirm antimicrobial susceptibility. Amphotericin B was used at 0.1 mg/ml against *Candida* spp. The solvent (acetone) was included as a negative control. All assays were undertaken in triplicate[5].

RESULTS AND DISCUSSION

Of the 39 plant samples tested 15 displayed good activity and 2 displayed significant activity with MIC values as low as 0.05 mg/ ml. Table 1 displays the results obtained for the most active plant samples.

CONCLUSION

Cissampelos torulosa was found to have the best activity against both *Lactobacillus* spp. with MIC values as low as 0.05 mg/ml. *Spirostachys africana* displayed the most significant activity against *C. albicans* with MIC values as low as 0.05 mg/ ml. *Heteropyxis natalensis* showcased broad-spectrum activity against all micro-organisms tested with the exception of *C. krusei*. From the MIC values obtained, this *in vitro* investigation validates the traditional use of some African plants against oral diseases caused by *Lactobacillus* and *Candida* spp.

Table 1: Plant extracts with the lowest MIC values against the respective pathogens.

Pathogen		Plant	MIC value (mg/ ml)
L. acidophilus		*Acacia karoo* (stems) *Acacia polycantha* (stems) *Tetradenia riparia* (leaves)	0.50
		Heteropyxis natalensis (leaves) *Dodonaea viscosa* (leaves)	0.25
		Cissamepelos torolusa (stems)	0.05
L. casei		*Dodonaea viscosa* (leaves)	0.50
		Heteropyxis natalensis (leaves) *Tetradenia riparia* (leaves)	0.25
		Cissamepelos torolusa (stems)	0.05
C. albicans		*Acacia karoo* (stems) *Artemisia afra* (leaves)	0.63
		Heteropyxis natalensis (stems) *Clematis brachiata* (leaves)	0.25
		Spirostachys africana (leaves)	0.05
C. glabrata		*Heteropyxis natalensis* (stems)	0.50
		Clematis brachiata (flower)	0.42
		Datura stramonium (leaves) *Clematis brachiata* (leaves)	0.25
C. krusei		*Zanthoxylum capense* (stems) *Artemisia afra* (stems) *Berula erecta* (leaves)	0.50
		Tecomaria capensis (leaves)	0.38
		Datura stramonium (leaves) *Warbugia salutaris* (twigs)	0.25

. MIC values equivalent or less then 0.50 mg/mL have good activity and anything less then 0.10 mg/mL are considered to have significant activity.[6]

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Appendix D:

Society: APSSA
Theme: Natural medicine
Presentation preference: Oral
Taking part in the young scientist competition: Yes

The antimicrobial efficacy of indigenous South African plants against oral pathogens and the effect of these plants on *Streptococcus mutans* biofilm formation.

Saajida Akhalwaya[1], Sandy F. van Vuuren[1*], Mrudula Patel[2]

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[2]Department of Clinical Microbiology and Infectious Diseases
[1,2]Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, 2193, Johannesburg, South Africa.
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Purpose: A review of the ethnobotanical literature has revealed over a 100 South African plants that have traditionally been used to treat and manage oral diseases. Oral diseases are one of the most common infectious diseases in humans today In South Africa they remain a huge public health problem due to the high prevalence, severity and influence on the patients well-being. The aim of this study was to investigate the antimicrobial efficacy of selected South African plants against pathogens known to cause oral diseases and to investigate the effect of these plants on the formation of *Streptococcus mutans* biofilm.

Methods: Aqueous and organic (dichlormethane: methanol, 1:1) extracts and essential oils were prepared from the South African medicinal plants. The antimicrobial activity was assessed using the minimum inhibitory concentration (MIC) assay against micro-organisms associated with the two most common oral infections; dental caries and periodontal diseases. *Streptococcus mutans* (ATCC 25175), *S. sanguis* (ATCC 10556), *Lactobacillus acidophilus* (ATCC 4356) *L. casei* (ATCC 344), *Fusobacterium nucleatum* (ATCC 25586) and *Porphyromonas ginigvalis* (ATCC 33277) were investigated. The MIC assay was also tested against micro-organisms associated with oral candidiasis; *Candida albicans* (ATCC 10231), *C. glabrata* (ATCC 90030) and *C. krusei* (ATCC 14243). Plants that displayed noteworthy antimicrobial activity (MIC values of < 1.00 mg/ml) against *S. mutans* was further investigated for its effect on *S. mutans* biofilm formation using the glass slide technique.

Results: From the antimicrobial studies *Cissampelos torulosa* displayed one of the lowest MIC values against *L. acidophilus* and *L. casei* with an MIC value of 0.06 mg/ mL. All plant extracts reduced the attachment of *S. mutans* to the glass slides. *Spirostachys africana* leaves is the plant extract that displayed the most significant reduction of adherent *S. mutans* cells at both 6 and 24 hours with percentage reductions of 97.56% and 86.58% respectively.

Conclusion: This study validates the traditional use of some South African medicinal plants for the treatment and management of oral infections.

Appendix E:

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2rd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-150504-1 10/04/2015

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof S van Vuuren, Saaida Akhalwaya. (Student number 5491949)**

Project title: **Antimicrobial investigation of indigenous South African medicinal plants against oral pathogens.**

Reason: This is a laboratory study using established microbial cultures *S mutans, S sanguis, L acidophilus, L casei, P gingivalis, F nucleatum, C albicans, C glabrata, C krusei.* There are no human participants

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HREC (MEDICAL)
2015 -05- 04
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Langutani Masingi.

Appendix F:

Organic plant extracts with noteworthy antimicrobial activity (MIC < 1.00 mg/ml)	***S. mutans***	***S. sanguis***	***L. acidophilus***	***L. casei***	***P. gingivalis***	***F. nucleatum***	***C. albicans***	***C. glabrata***	***C. krusei***
Acacia karroo leaves	**X**								
Acacia karroo bark		**X**					**X**		
Acacia polyacantha stems					**X**				
Acokanthera oppositifolia stems		**X**	**X**						
Artemisia afra leaves		**X**			**X**	**X**	**X**		
Artemisia afra stems					**X**				**X**
Berula erecta leaves		**X**			**X**				
Berula erecta rhizomes (RHN)	**X**								
Carpobrotus edulis leaves						**X**			
Cissampelos torulosa stems		**X**	**X**	**X**		**X**			
Clausena anisata bark						**X**			
Clausena anisata twigs									**X**
Clematis brachiata leaves		**X**					**X**	**X**	**X**
Clematis brachiata flowers		**X**					**X**	**X**	
Clematis brachiata leaves (RHN)									**X**
Clematis brachiate roots (RHN)					**X**				
Cotyledon orbiculata leaves		**X**				**X**			
Croton gratissimus leaves			**X**					**X**	**X**
Croton gratissimus stems		**X**					**X**		
Dalbergia obovata stems							**X**		
Dichrostachys cinera stems							**X**		
Dodonaea viscosa leaves			**X**	**X**			**X**		
Englerophytum magalismontanum stems (RHN)	**X**	**X**							
Erythrina lysistemon stems	**X**			**X**					
Heteropyxis natalensis leaves			**X**						
Heteropyxis natalensis stems		**X**			**X**		**X**	**X**	
Myrothamnus flabellifolia leaves					**X**	**X**			
Sansevieria hyacinthiodes leaves					**X**				
Sansevieria hyacinthiodes rhizomes (RHN)								**X**	
Siphonochilus aethiopicus leaves						**X**			**X**
Siphonochilus aethiopicus roots				**X**					

Organic plant extracts with noteworthy antimicrobial activity (MIC < 1.00 mg/ml)	***S. mutans***	***S. sanguis***	***L. acidophilus***	***L. casei***	***P. gingivalis***	***F. nucleatum***	***C. albicans***	***C. glabrata***	***C. krusei***
Spirostachys africana leaves	**X**				**X**		**X**		
Spirostachys africana stems		**X**							
Tarchonanthus camphoratus bark	**X**				**X**	**X**			
Tecomaria capensis leaves	**X**								
Tecomaria capensis stems									**X**
Tetradenia riparia leaves			**X**	**X**	**X**				
Warburgia salutaris bark				**X**	**X**				
Warburgia salutaris twigs									**X**
Zanthoxylum capense leaves		**X**							
Zanthoxylum capense stems*					**X**				**X**