AN INVESTIGATION OF ANTIMICROBIAL COMPOUNDS FOR DENTAL HEALTH CARE FROM THE LEAF AND STEM EXTRACTS OF *CARISSA BISPINOSA* (L.) Desf. ex Brenan (APOCYNACEAE)

By

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[redacted]

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DECLARATION

I, **Wanda Shekwa**, declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Microbiology has not previously been submitted by me or anyone for a degree at this or any other university; that it is my work in design and in execution and that all the material contained herein has been duly acknowledged.

……..... ……..……

Signature Date

DEDICATION

I dedicate this work to:

My sister, Shekwa Thandeka, who supported me throughout the project, may this serve as motivation to her academic endeavours.

My late father who was always passionate and optimistic about the prospects of his children attaining good education. May his soul continue to rest in peace.

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LIST OF ABBREVIATIONS

^{13}C	Carbon-13
^{1}H	Hydrogen-1
A	Acetone
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
AMP	Amphotericin-B
AVG	Average
B	Butanol
BEA	Benzene ethanol ammonium hydroxide
C	Chloroform
CEF	Chloroform ethyl acetate
COSY	Correlation spectroscopy
D	Dichloromethane
DEPT	Distortionless enhancement through polarisation transfer
DMSO	Dimethyl sulfoxide

E	Ethanol
Ea	Ethyl acetate
EA	Ethyl acetate
EC_{50}	Half-maximal effective concentration
EMW	Ethyl acetate methanol water
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalence
Gen	Gentamicin
H	n-hexane
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPTLC	High-performance thin-layer chromatography
HSQC	Heteronuclear single quantum correlation
INT	ρ-iodonitrotetrazolium chloride
IR	Infra-red spectroscopy

M	Methanol
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHLS	National Health Laboratory Service
NMR	Nuclear magnetic resonance
OPLC	Over-pressured layer chromatography
PLC	Planar electrochromatography
PVM/MA	Polyvinyl methyl ether maleic acid
RF	Retention factor
ROS	Reactive Oxygen Species
SDA	Sabouraud’s dextrose agar
TLC	Thin layer chromatography
TLC-DPPH	Thin layer chromatography-2,2-diphenyl-1-picrylhydrazyl
UV	Ultraviolet
UV-vis	Ultraviolet-visible spectroscopy

W	Water

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ABSTRACT

Dental diseases are among the most prevalent diseases worldwide. A recent epidemiological survey has shown that about 49 to 83% people are affected by dental caries. *Carissa bispinosa (L.) Desf. ex Brenan* is one of the medicinal plants used traditionally to treat dental diseases. This study aims to investigate the effectiveness of antimicrobial compounds from the leaf and stem extracts of *C. bispinosa* on oral pathogens. Nine solvents (n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol and water) were used for the extraction of the leaf and stem powder of *C. bispinosa.* Biochemical tests were performed to determine the presence of phytochemical constituents and the phytochemical profile was generated through thin layer chromatography (TLC). TLC- 2, 2-diphenyl-1-picrylhydrazyl (TLC-DPPH) assay was used to determine the qualitative antioxidant activity while DPPH free radical scavenging and ferric reducing power assay were used for the quantification of antioxidant activity. The antimicrobial activity was tested on five pathogens (*Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes, Candida albicans* and *Candida glabrata*) qualitatively using the TLC-bioautography assay and quantitatively using the minimum inhibitory concentration (MIC) assay. Cytotoxicity was tested on the THP-1 cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The active compound was isolated through open column chromatography and preparative-TLC guided by bioassays. Structural elucidation was achieved from the analysis of spectral data generated by nuclear magnetic resonance (NMR) techniques. A variety of phytochemicals were detected on the leaf and stem extracts. The extracts had high antioxidant activity, especially those extracted with polar solvents. All the pathogens, except *C. glabrata*, demonstrated susceptibility to the extracts on the qualitative assay. For the leaf extracts, methanol extract had the lowest average MIC value (0.81 mg/ml) making it the most activity across the microorganisms, whereas hexane had the highest MIC value (1.63 mg/ml). *C. albicans* demonstrated higher susceptibility with an average of 0.73 mg/ml while *C. glabrata* displayed the least (2.8 mg/ml). Compared to the leaf extracts, lesser activity was observed for the stem extracts on bioautography and MIC. The percentage viability was decreasing as the concentration of the extract was increasing. However, the highest concentration resulted

in more than 50% cell viability. The compound was isolated as a white powder (10.5 mg). It was identified based on its physical properties, spectroscopic data and comparing the data with scientific literature and was found to be β-Sitosterol, a member of plant steroids (Phytosterols). The study is the first to detect the presence of the antimicrobial compound (β-Sitosterol) and isolate it from the leaves of *Carissa bispinosa.*

CHAPTER 1

1. GENERAL INTRODUCTION

Dental diseases are among the most prevalent diseases worldwide. A recent epidemiological survey has shown that about 49% to 83% people are affected by dental caries (Frencken *et al.,* 2017). Various microorganisms (normal flora) such as viruses, bacteria and fungi heavily inhabit the human oral cavity, which may pose a threat to dental or oral health (Alireza *et al*., 2014). Dental caries and periodontal diseases are the most common dental pathologies in humankind and may have serious implications, especially in immune-compromised patients. About 42.17% of South African children were reportedly having untreated dental caries in 2003 (Singh *et al*., 2010). The manifestation of oral or dental diseases can compromise the quality of life and psychology. An individual with an oral infection may have low self-esteem and discomfort when speaking, eating, and drinking (Needleman *et al.,* 2014). Modern technology-based dentistry treatment is not affordable in many developing countries (Yee and Sheiham, 2002). Even in countries with resources, dentistry is failing to meet the needs of the national population and is more focused on aesthetic treatments driven by profit interests (Holden, 2018). Most oral diseases are preventable and while they are not always life-threatening, they are a major public health problem in South Africa, posing an inordinate economic threat due to the growing demand for public health care. Despite the impact oral infections have on general health and quality of life, it is still one of the most neglected aspects of health in most developing countries, including South Africa (Ramphoma, 2016). Dental medication is usually expensive and not easily accessed, especially in developing countries such as South Africa (Akpata and Akinrimisi, 1977). Several disadvantages are linked to conventional medicine for oral infections, i.e. toxicity, low efficacy rate, and the limited number and high cost of medication, serious side effects and antibiotic resistance (McCullough and Farah, 2008; Mehta *et al*., 2002; Kathiravan *et al*., 2012; is one of the medicinal plants used traditionally to treat toothache (Muleya *et al*., 2014). This study aims to investigate the effectiveness of antimicrobial compounds from the leaf and stem extracts of *C. bispinosa* on oral pathogens.

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CHAPTER 2

2. LITERATURE REVIEW

2.1. Oral cavity and oral infections

The oral cavity is a vital organ in the human body, it is where mechanical digestion takes place and has a significant role in speech (Akhalwaya *et al.,* 2018). It links the external environment with the body, serving as an ingestion organ for food. The mouth is not only an entry point for water and food, but also for microorganisms (Marsh *et al.,* 2009). The structure of the oral cavity makes it a complex environment with distinct habitants such as the hard and soft palate, teeth, buccal mucosa, and tongue. The diversity in these micro-habitants creates a heterogeneous ecological system with a rich microbiota (Kilian, 2018). Like all the accessible parts of the human body such as the skin, upper respiratory tract, vagina and gastrointestinal tract, the oral cavity is colonised by microorganisms immediately after birth (Cephas *et al*., 2011). The oral cavity and its residents (normal flora) have a mutualistic or symbiotic relationship where the oral microbiota benefits from a warm environment and nutrients of the host, and in turn provides protection against pathogenic invaders. The number of oral microbial species is reported to be more than 700, including archaea bacteria, fungi, and viruses (Deo and Deshmukh, 2019; Hoare *et al.,* 2017). The major inhabitants of all the oral microbiota are bacteria (Segata *et al.,* 2012), primarily consisting of *Bacillus* spp, *Firmicutes* spp, *Proteobacteria* spp, and *Actinomycetes* spp (Mark, 2016). The earliest invaders are *Streptococcus* spp, *Eikenella* spp, *Prevotella* spp, *Actinomyces* spp, *Propionibacterium* spp, *Capnocytophaga* spp, *Haemophilus* spp, and *Veillonella* spp. Though these microorganisms thrive in communities of proximity, the distribution of each sub-habitant is distinct. This is due to several factors. Amongst these factors are: exposure to nutrients from the diet of the host, exposure to host products delivered by saliva and gingival crevicular fluid, oxygen availability and the characteristics of the surfaces available for attachment (Kolenbrander *et al.,* 2002; Palmer *et al.,* 2003). Some residents of the mouth outgrow other normal flora and lead to oral diseases in the area

where they are dominant. The three major infections of the oral cavity are dental caries, periodontal diseases, and oral candidiasis (Hoare *et al.,* 2017).

2.2. Normal oral flora

Various microorganisms (normal flora) such as viruses, bacteria, and fungi heavily inhabit the human oral cavity, which may pose a threat to the host. Of all the kingdoms found in the mouth, bacteria are the most prevalent with more than 500 species (Segata *et al*., 2012). The type of microorganisms residing in the mouth is influenced by several physiological factors such as temperature, pH, oxygen, and redox potential, which are responsible for the presence of different micro niches (Simon-Soro *et al*., 2013; Kleinberg and Jenkins, 1964). The oral microbiome changes with different stages of an individual's development. An infant is born with little or no microorganisms in the mouth. However, within a few h, vertical transmission takes place where microorganisms are transferred from the mother's or nurse's mouth to the infant. The streptococci genus is the primary coloniser, and it creates a favourable environment for other bacteria. The metabolites of the pioneer community may favour colonisation by bacteria such as *Actinomyces* spp. The frequently detected bacteria in infants are streptococci, staphylococci, Neisseria, and some Gram-negative anaerobes such as *Veillonella* spp while *Lactobacillus* spp*, Actinomyces* spp, *Prevotella* spp, and *Fusobacterium* spp are less frequent. A group of other microorganisms invade the mouth during and after the eruption of teeth and occupy the two newly formed micro-niches (the non-shedding hard-tissue surface of enamel and cementum, and the gingival crevice). During adolescence, the oral microbiome changes in response to hormonal changes as individuals progress to adulthood. *Spirochaetes* spp, *Veillonella* spp, *Prevotella* spp, and black-pigmented Bacteroides are some of the most common inhabitants at this stage. The diversity of the microbiome grows as more microorganisms invade the mouth until the equilibrium between the resident microflora and environmental conditions is established (Marsh, 2004; Kononen *et al.,* 1994).

2.3. Function of normal microflora

The presence of the normal microflora protects the host from being invaded by exogenous or transient microorganisms that can be pathogenic (Topoll *et al.*, 1990). A major decline in the oral microflora caused by different factors such as prolonged use of antibiotics may lead to the vulnerability of the host since the normal flora act as an innate defence (Marsh and Percival, 2006).

2.4. Dental Caries

Dental caries is among the most prevalent chronic diseases of the mouth (Bowen *et al*., 2018). It is characterised by the destruction of the hard tissue of the teeth (Valm *et al*., 2011). Caries is caused by the consumption of fermentable carbohydrates, including glucose, lactose, sucrose, fructose, maltose, and starch (Hujoel and Lingstrom, 2017). The oral biofilm ferments these carbohydrates and secretes acid by-products and during the process, the acid by-products demineralise the dental hard tissues and cause dental caries (Selwitz *et al.*, 2007). If the condition is not controlled, it worsens resulting in the formation of cavities on the affected tooth and ultimately allowing the invasion of the pulpal tissue (Fontana *et al.,* 2010). This disease is commonly known to manifest in children, but it can also affect all age groups (Selwitz *et al.,* 2007).

Before the onset of dental caries, the acidogenic bacteria residing on the biofilm break down dietary fermentable carbohydrates. The acids produced by these microorganisms result in the transformation of the microenvironment to a habitant which favours the growth of aciduric species. The pH of the mouth is lowered drastically by the aciduric species upon the continuous consumption of fermentable sugars (Marsh, 2003). The caries process involves a rapid alternating demineralisation and remineralisation of the tooth, where higher demineralisation results in a net loss of tooth enamel, hence the appearance of specific lesions at a certain site of the tooth (Pitts and Zero, 2016).

2.4.1. Microorganisms causing dental caries

Dental caries has been linked with a high proportion of *Streptococcus mutans*. *S. mutans'*s association with caries is due to its acid tolerance and rapid metabolism (Lee and Kim, 2014; Nakano *et al.*, 2005). Generally, the normal flora and pathogenic bacteria are in a balanced state. The consumption of sugars and a decrease in pH levels can

cause an environmental change and breakdown in the homeostasis of bacteria in the mouth (Marsh, 1994). An environmental change favours the growth of cariogenic bacteria, hence, the development of a cariogenic dental biofilm. A cariogenic biofilm includes, but is not limited to, *S. mutans, Streptococcus sobrinus*, and *Lactobacillus acidophilus* (Nakano *et al*., 2005).

2.4.1.1. *Streptococcus mutans*

Streptococcus mutans belongs to the streptococci genus. It is characterised by a spherical and Gram-positive cell and can thrive in both aerobic and anaerobic environments. This cariogenic species is non-motile and tests negative in catalase tests (Yoo *et al*., 2005; Hamada and Slade, 1980). *Streptococcus mutans* inhabits the oral cavity and is a major attributor to the formation of cariogenic biofilm and dental caries (Song *et al*., 2007). The pathogenicity of *S. mutans* is due to its ability to attach to the tooth and form a dental biofilm, produce acids by-products during the fermentation of carbohydrates, and produce extra- and intracellular polysaccharides (Lemos and Burne, 2008). *Streptococcus pyogenes* is another species from the streptococci genus with the potential to form biofilms in the oral cavity and nasopharynx (Doern *et al.,* 2009).

Figure 2.1: Spherical or oval, white colonies of *Streptococcus mutans* cultured on blood agar for 48 h at 37 °C under CO_2 (Naidoo, 2012).

2.4.1.2. *Lactobacillus* spp.

Lactobacillus spp. are Gram-positive, fastidious, mostly non-motile, non-spore-forming bacteria that use fermentative metabolism and produce lactic acid as a by-product (Duar *et al.,* 2017). This genus can adapt to different ecological niches since they are aero-tolerant or anaerobic. They are common inhabitants of environments that support fermentative metabolism, including soil, water, sewage, many animals, and humans (Makarova *et al.,* 2006). Lactobacilli have been studied over the years due to their economic importance in food, biotechnology, and therapeutic industries (Duar *et al*., 2017).

In humans, Lactobacillus reside in the gastrointestinal tract, oral cavity, and vagina (Walter, 2008). They are the first bacteria to be linked with dental caries development (Owen *et al*., 1949), and some of the species found in the oral cavity and gastrointestinal tract include *L. ruminis, L. reuteri, L. johnsonii, L. amylovorus*, and *L. salivarius* (Duar *et al*., 2017). The association of lactobacilli with dental caries is accounted for by their isolation from many caries lesions and their ability to create an environment with lower pH levels (Beighton, 2005).

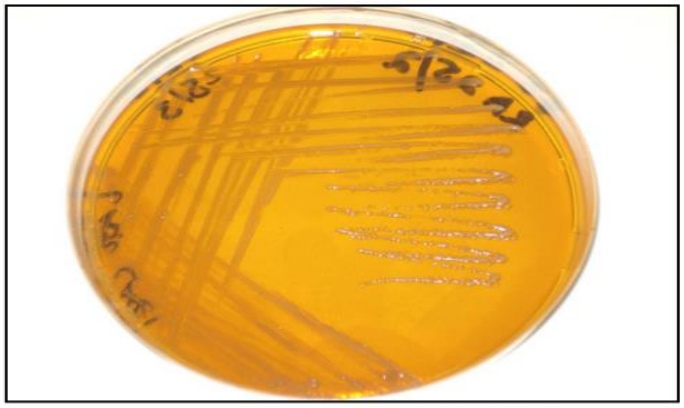

Figure 2.2: Round, white colonies of *Lactobacillus casei* cultured on Rogosa agar for 48 h at 37 °C under CO_2 (Naidoo, 2012).

2.4.1.3. *Enterococcus faecalis*

E. faecalis thrives in the root canal of teeth (Stuart *et al.,* 2006). It can cause teeth infection through its interconnections with *S. sanguinis*, and *S. mutans*. Before the onset of *E.*

faecalis infection in the root canal, dental caries resulting from untreated dental plaque formed by *S. sanguinis*, and *S. mutans* develop (Ge *et al*., 2008). Other systematic diseases may be implicated as well, if *E. faecalis* infection is not treated immediately (Li *et al.,* 2000). *E. faecalis* is believed to be attributable to 77% of failed endodontic cases and about 50% of cases of chronic apical periodontitis (Siqueira and Rocas, 2005). This infection entails the metabolism of all kinds of carbohydrates by *S. mutans*. Because glucose and sucrose are readily present in the extracellular space, S. mutans metabolise them to produce intracellular polysaccharide glycogen (IPSs) and bacteriocin, which is significant for the colonisation of teeth's biofilm (Karpinsky and Szkaradkiewicz, 2013). One way to maintain teeth affected by big dental caries on the surface of the pulp is performing endodontic treatment. However, about 24-77% of endodontic treatments fail due to the presence of *E. faecalis*. This microorganism can form a biofilm, penetrate the dentin tubule, thrive in low pH levels, and resist medications applied inside the canals (Singh and Kapoor, 2014).

Figure 2.3: *Enterococcus faecalis* on sheep blood agar (www.bacteriainphotos.com)

2.5. Periodontal diseases

Periodontal disease is any disorder affecting the tissues surrounding and supporting the teeth (periodontium). Such a disorder may be acquired or inherited (Pihlstrom, 2005). It is characterised by chronic inflammation and in its progression, it may result in the loss of periodontal ligament, destruction of surrounding alveolar bone, and ultimately, tooth

loss (de Pablo, 2009). The two diseases of the periodontium are gingivitis and periodontitis, the prior entails the inflammation of the gingiva (soft tissues) surrounding the teeth and the latter involves the destruction of the supporting structures of the teeth and periodontium (Kinane, 2001).

Periodontal diseases affect about 20-50% of the world population and their prevalence in young and old people make them a public health concern. Numerous risk factors are associated with periodontal diseases, including poor oral hygiene, smoking, diabetes, medication, stress, age, and heredity (Nazir, 2017).

2.5.1. Gingivitis

Gingivitis is a gingival inflammation characterised by reddened and swollen gum, which is susceptible to bleeding but without loss of connective tissue attachment (Beirne *et al*., 2007). Symptoms of gingivitis include bleeding and tenderness (Greene and Jackson, 2006), and if the symptoms are untreated, periodontitis may develop (Beirne *et al*., 2007). Gingivitis has a prevalence of 50–90% in adults worldwide, but it is reversible by simply adopting good oral hygiene and lifestyle changes. An adult with health gingiva may develop a dental biofilm 24 h after performing oral hygiene procedures such as tooth-brushing, and gingivitis in the range of 10 to 21 days after the oral hygiene procedures (Pihlstrom *et al*., 2005).

2.5.2. Periodontitis

Periodontitis can be defined as an infectious disease that leads to the inflammation of the tooth-supporting tissues (periodontium). Approximately 10-15% of adults suffer from progressive periodontitis worldwide. Furthermore, behavioural, environmental, and genetic factors are involved in the development of the disease, the exposure of susceptible individuals to its initiation, and the speed of progression (Eke *et al*., 2012; Kinane, 2001). Its development is influenced by the immune and inflammatory responses of the host. Periodontal diseases are initiated by dental plaque. Dental plaque leads to gingivitis, which precedes chronic periodontitis if untreated. The progression from plaque to periodontitis is associated with several factors such as medication, smoking, age, and systemic diseases (Beirne, 2007).

2.5.3. Microorganisms causing periodontal diseases

The development of dental plaque is the primary etiological factor in periodontal disease pathogenesis. Although the presence of plaque is a necessary and principal factor in periodontal disease, it cannot solely cause the disease (Xiong, 2016). Dental biofilm accumulates on gingival margins and triggers an inflammatory response, which in turn, alters the microbiota that may lead to serious consequences in susceptible individuals (Könönen, 2019). About 300 to 400 bacterial species colonise the sub-gingiva and form a plaque, however, only 10 to 20 species are associated with the pathogenesis of the destructive periodontal disease. It is only those with the capability to colonise by attaching themselves to the periodontium, multiplying and competing with other microorganisms and the immune system. The most common are Gram-negative anaerobic bacilli, some anaerobic cocci, and a large quantity of anaerobic spirochetes. The group associated with destructive capacity on the periodontium includes *Prevotella inter-media, Porphyromonas, gingivalis, Bacteroides, forsythus, Treponema denticola, Actinobacillus, and actinomycetum comitans* (Kinane, 2001).

2.5.3.1. *Porphyromonas gingivalis*

Porphyromonas gingivalis is a member of the oral microbiota but can be virulent in the presence of periodontal disease predisposing factors (Mysak *et al*., 2014). It is Gram-negative, non-motile, short and thrives in the absence of oxygen (obligate anaerobe) (Mysak *et al*., 2014; Samaranayake, 2002). *P. gingivalis* is prevalent in subgingival areas of patients suffering from severe periodontal disease (Samaranayake, 2002) and is considered as one of the main etiological factors of the disease in adults (Brochu *et al,* 2001).

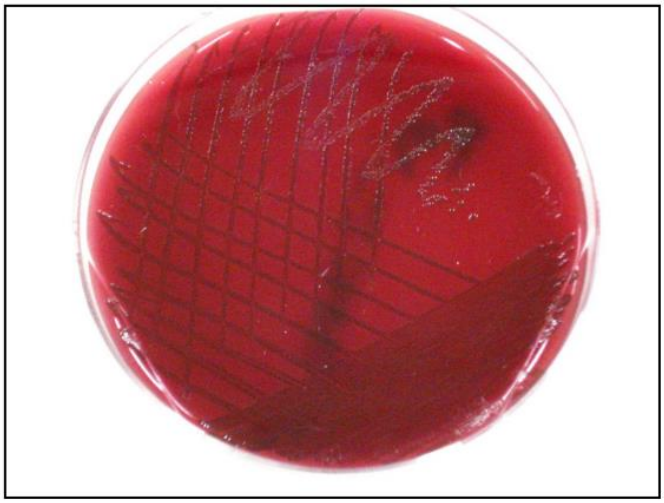

Figure 2.4: The black pigmented colonies of *Porphyromonas gingivalis* cultured on blood agar for 7 days at 37 °C under anaerobic condition (Naidoo, 2012).

2.5.3.2. *Prevotella intermedia*

The prevotella genus is characterised by obligate anaerobic, Gram-negative, and rod-shaped bacteria. They are identified by the shiny and smooth colonies with grey, light brown, or black colour on a blood agar plate (Shah and Collins, 1990). This genus is versatile and occupies different ecological niches such as the oral cavity, upper respiratory tract, urogenital tract, rumen, and human faeces (Eiring *et al*., 1998; Hayashi *et al.,* 2007). Most members of the the genus can invade host tissues and become pathogenic, this includes *Prevotella dentalis, Prevotella intermedia, Prevotella denticola*, and *Prevotella meleninogenica* (Nadkarni *et al*., 2012; Fujii *et al*., 2009; Field *et al*., 2010). *Prevotella intermedia* has been linked with periodontal diseases, periapical periodontitis, and gangrenous disease (Mombell *et al*., 2000; Gomes *et al*., 1994; Bolivar *et al*., 2012). The pathogenicity of *P. intermedia* is driven by its ability to degrade immunoglobulins, invade eukaryotic cells, and immune suppression of B and T cells (Silva *et al*., 2003). It also possesses tissue destructive and toxic materials, which makes it even more pathogenic (Nisengard and Newman, 1994).

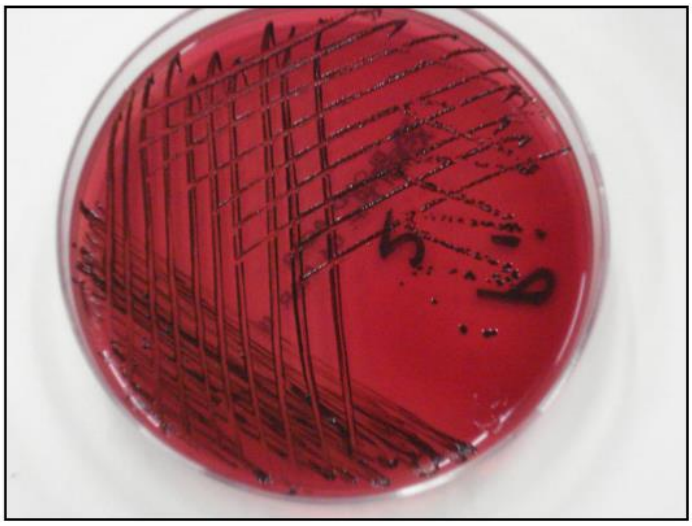

Figure 2 5: Black pigmented colonies of *Prevotella intermedia* cultured on blood agar for 7 days at 37 °C under anaerobic condition (Naidoo, 2012).

2.6. Oral candidiasis

Oral candidiasis is also known as thrush – an infection of the tongue and other areas of the oral mucosa and is characterised by an overgrowth of fungi and invasion of superficial tissues (Hellstein and Marek, 2019). Candidiasis is prevalent in the human population and readily affects numerous areas of the body. Candida species are found in the oral cavity of an estimated 30–60% of healthy adults, however, these microorganisms thrive as commensals rather than pathogens (Martin and Lamb, 1982).

Primarily, for candida to cause an infection, it must be retained inside the oral cavity. Therefore, detachment of loosely attached candida cells from the mucosal surfaces through the flow of saliva and swallowed is an important process that curbs the overgrowth of candida (Lewis and Williams, 2017). Various virulence factors, including biofilm formation, the morphological transition between yeast and hyphae, and secretion of hydrolytic enzymes, are associated with the pathogenesis of Candida species (Costa *et al.,* 2013; Shu *et al*., 2016).

2.6.1. *Candida spp*

Candida species are the most common fungal pathogens in human diseases despite being a commensal and member of the oral microbiome (Marsh and Martin, 2009; Neville, 2009). The term 'Candida' is derived from the Latin word "candid" meaning 'white'. Members of this genus are predominantly dimorphic and form hyphae or pseudohyphae along with standard blasto-spore (Odds, 1988). The genus is classified in the Saccharomycetaceae family which has more than 350 species (Marsh and Martin, 2009).

About 80% of the world population are asymptomatic carriers of *C. albicans* but do not necessarily cause diseases (Vila, 2020). Although *C. albicans* is by far the major cause of candidiasis, other etiological fungi from the candida genus also exist, including *C. glabrata, C. tropicali, C. kruesi, C. parapsilosis, C. lusitaniae, C. pseudotropicalis, C. stellatoidea,* and *C. guillermondii* (Singh *et al.,* 2014, Thompson *et al.,* 2010). The versatility of *C. albicans* enables it to adapt to its host and any change in its habitant favours its proliferation and potential to invade different sites (Vila, 2020).

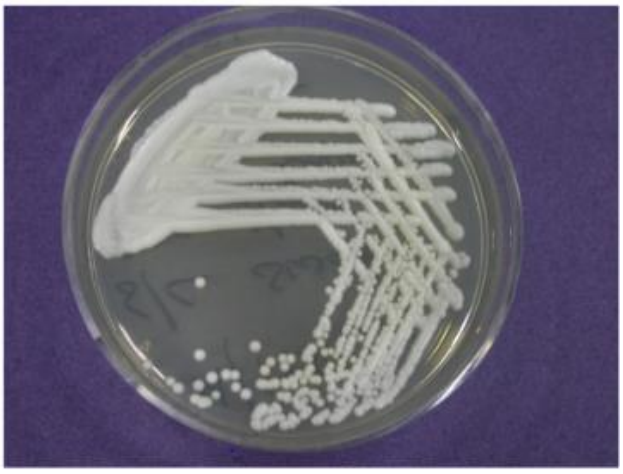

Figure 2.6: Sabouraud's dextrose agar (SDA) showing the isolates of *C. albicans* (Sharma *et al*., 2016).

2.6.2. *Candida albicans* association with cariogenic bacteria

The interactions amongst the oral microbiota are regarded as synergistic since the presence of one microorganism provides an ecological niche for the existence of another

(Avila *et al.,* 2009). An example of such interaction is when one organism excretes a metabolite that is useful to another (O'Donnell, 2015). Any change in the oral microenvironment may lead to the proliferation of one pathogenic species (dysbiosis) and ultimately the development of oral disease (Zaura, 2014).

The co-adhesion of *C. albicans* with bacteria is a significant interaction for the persistence of *C. albicans* thus enhancing colonisation in the host. The streptococci and *C. albicans* synergistic interactions are the most studied fungal-bacterial interaction in the oral cavity. Streptococci are the primary invaders of the mouth and their presence is essential for the establishment of a good habitant for *C. albicans* (Van Dijck, 2017). *Streptococcus oralis*, *Streptococcus gordonii, Streptococcus mitis*, and *Streptococcus mutans* are the specific primary species that readily adheres to the hyphae of *C. albicans* (Jakubovics, 2014). Moreover, streptococci provide *C. albicans* with a carbon source which is important for its proliferation (Jenkinson and Lamont, 2005). As a return, *C. albicans* creates a low oxygen tension environment by using lactic acid, which favours facultative streptococci (Jenkinson *et al*., 2008). Although the fungal-streptococci interaction is mutually beneficial, it may have detrimental effects on the oral cavity (Bertolini, 2019).

The interactions of the cariogenic bacteria *S. mutants* with *C. albicans* which cause dental caries (or tooth decay) have recently gained popularity (Zero *et al*., 2009). Dental caries is caused by the metabolic interactions of microorganisms found in the dental plaque, where the biofilm causes pH fluctuations, ultimately leading to an irreversible tooth decay (Metwalli *et al*., 2013; Krom *et al*., 2014). For years, *S. mutants* have been known for their cariogenic effects, however, recent studies have implicated *C. albicans* through its interactions with *S. mutants* (Falsetta *et al*., 2014; Klein *et al*., 2015). *S. mutants* are prevalent in dental biofilm where *C. albicans* thrives. Additionally, clinical studies have reported the increasing number of *C. albicans* isolation cases in dental caries patients (Pereira *et al.,* 2018). More importantly, the presence of a high level of sugar in the mouth, which is a predisposing factor for dental decay, is associated *with S. mutans–C. albican*s interactions (Koo and Bowen, 2014).

2.7. Oral health

2.7.1. Natural defence

Although the oral cavity contains saliva that has lysozyme and lactoperoxidase as antimicrobial agents, the availability of worn-out epithelial cells and food particles creates a good environment for microorganisms to thrive since the temperature (37ºC) and the pH (6) are favourable (Madigan, 2003). The presence of the normal flora also regulates and protects the host against pathogenic microorganisms (Dewhirst *et al*., 2010). The overgrowth of the normal flora along with other predisposing factors may lead to the development of oral or dental diseases (Hoare *et al.,* 2017). Therefore, it is important to take care of the mouth by washing regularly and avoid risky habits. Saliva plays an integral role in protecting the oral cavity from pathogens. There are four significant roles performed by saliva in maintaining oral health: (1) it acts as a lubricant and a medium that washes and carries bacteria from the oral cavity to the stomach where they are killed by gastric acid, (2) creates an alkaline environment of pH 6.7, which is not favourable to most pathogens and provides a buffering action, (3) maintains the integrity of the teeth, and (4) contains immunoglobulins that have to inhibit bacterial growth since they interact with surface receptors found on them (Humphrey and Williamson, 2001; Nisengard and Newman, 1994).

2.7.2. Measures for the prevention of oral infections

The act of brushing teeth is taught to children at a younger age in order for them to maintain pearly white teeth. However, oral health is much more than just white teeth. It encompasses the palate, gums and supporting tissues, tongue, lips, the lining of the mouth and throat, salivary glands, chewing muscles, and nerves. Over the past 50 years, fluoride toothpaste has played a remarkable role in oral hygiene. They are the sole attributor in the decline of dental diseases in developed countries (Longbottom *et al*., 2009; Wolff and Larson, 2009). The recommendation of brushing twice a day has been supported by strong scientific evidence (Twetman *et al*., 2003; Marinho *et al*., 2003) and is accepted globally for that reason (van der Weijden and Hioe, 2005). Today, brushing teeth and other means of oral hygiene to prevent dental diseases are a habit to many

people (Wolff and Larson, 2009). However, a mere mechanical removal of dental plaque is insufficient since a toothbrush cannot access some areas of the mouth, thus shoring up the need for a mouthwash (Sari and Birinci, 2007).

Mouthwashes have been proven to be effective in the reduction of bacterial dental biofilm (Yousefimanesh *et al.,* 2015), and their use after toothbrushing is increasingly becoming a habit among the masses (Sarner *et al*., 2012). Prevention of dental and periodontal diseases can be achieved by an abstinence of predisposing factors such as high sugar intake, alcohol, and smoking. Consumption of fermentable sugars may result in dental caries (Moynihan and Kelly, 2013), and has been associated with periodontal diseases (Lula *et al.,* 2014).

About 50% of periodontal diseases have been solely attributed to smoking, with numerous studies showing the link between smoking and loss of periodontal attachment (Haber, 1993; Haber *et al*., 1993). High alcohol consumption increases the risk of acquiring various conditions such as increased blood pressure, liver cirrhosis, cardiovascular disease, diabetes, and cancers of the mouth (Petersen and Ogawa, 2005). Recently, high alcohol consumption has been associated with severe periodontal disease (Tezal *et al.,* 2001; Pitiphat *et al.,* 2003). People who consume alcohol and tobacco products usually have an unbalanced diet, consuming mostly a lot of sugars and fats and food with low fibre content, which increases the risk of periodontal disease and oral cancer (Petersen and Ogawa, 2005).

2.7.3. Chemical treatment of oral infections

2.7.3.1. Chlorhexidine gluconate

Chlororhexidine has been proven to be effective against dental caries (Nisengard and Newman, 1994). It is a broad-spectrum antimicrobial agent that is active against Gram-positive bacteria, Gram-negative bacteria, and fungi. Chlororhexidine possesses both inhibition (bacteriostatic) and killing (bactericidal) mechanisms of action against bacteria, depending on its concentration. It kills bacteria by binding to the negatively charged sites of bacteria through electrostatic interactions leading to the spillage of intracellular components (Dogan *et al.*, 2003). A similar mechanism of action is observed on fungi,

where chlorohexidine compromises the integrity of the fungal cell wall causing leakage of cellular components. Chlorhexidine is incorporated in mouthwashes and can prevent the formation of plaque or remove already formed plaque (Dogan *et al.*, 2003).

2.7.3.2. Fluoride

Flourides are the most effective and widely used chemical agents against oral pathogens. They work as topical agents and exert a systemic effect if ingested (Marsh and Martin, 1999). As a topical agent, they work in three mechanisms of action, namely, the inhibition of bacterial metabolism when hydrogen fluoride diffuses into the bacterial cell if plaque is acidified; prevention of demineralisation and act a catalyst during remineralisation, driving the diffusion of phosphate and calcium into the tooth (Featherstone, 2000). Fluoride exerts a systemic effect when ingested as a supplement in tablets. The fluids on the oral cavity have low levels of fluoride which forms the thermodynamically stable and acid diffusion resistant fluorapatite when it interacts with the surface of the enamel or erupted teeth (Marsh and Martin, 1999).

2.7.3.3. Iodine

Iodine is an effective antiseptic used against oral pathogens (Shelaniki and Shelanaki, 1956). However, there is not much history in terms of the use of iodine in the oral cavity. Studies have indicated the effective use of iodine in the preparation of mucosal sites before surgery (Zinner *et al.,* 1961). The use of iodine compounds as topical agents has been shown to reduce the microbial population in the saliva and gingival crevice (Zinner *et al.,* 1961; Scopp and Orvieto, 1972). Numerous studies have found that iodine topicals can suppress *S. mutans* on affected teeth for several weeks in children suffering from dental caries (Gibbons *et al*., 1974). Moreover, povidone-iodine which is incorporated in some mouth rinses can effectively reduce microbial population in the gingival surface (Randall and Brenman, 1974; Scopp and Orvieto, 1972; Blake and Forman, 1967; Brenman and Randall, 1974).

2.7.3.4. Triclosan

Triclosan is active against a wide range of antimicrobial pathogens and has a low level of toxicity. It can be incorporated into kinds of toothpaste to reduce microbial population in the oral cavity. In addition to its compatibility with other toothpaste, triclosan does not have an unpleasant taste (Blinkhorn, 2009). However, no study has indicated the effectiveness of triclosan alone on gingivitis or caries, hence it is combined with a copolymer such as polyvinyl methyl ether maleic acid-PVM/MA (Gunsolley, 2006). PVM/MA facilitates the uptake and retention of the triclosan to enamel, oral epithelial cells, and plaque (Ciancio, 2007). It is believed that triclosan reduces plaque by inhibiting essential metabolic pathways in bacteria, it also reduces gingival inflammation which is a precursor for severe periodontal disease (Gunsolley, 2006).

2.7.3.5. Essential oils

Essential oils (also known as volatile oils) are aromatic oily liquids present in plant materials such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots. There are approximately 3000 essential oils that have been identified, of which 300 have been commercialised in the fragrance market (Van de Braak and Leijten, 1999). Some essential oils like cinnamon and tea tree oils have been traditionally used for oral healthcare (Edris, 2007). Essential oils contain small terpenoids and phenol compounds which are associated with antimicrobial activity and this has evoked interest from scientists to search for essential oils with antimicrobial activity (Prabuseenivasan, 2006). The pharmacological properties of essential oils are not limited only to antimicrobial activity, but also include anti-diabetic, antioxidants, treatment for cancer and cardiovascular diseases (Edris, 2007; Lahlou, 2004; Palombo, 2011).

2.8. Current therapy for dental diseases

The target for the treatment of oral infections is now based on preventative measures (Loesche, 1996). Prevention approaches include strengthening the teeth, dietary modifications, and removal of plaque through chemical and mechanical ways (National Institute of Health, 2001; Shah, 2005).

2.8.1. Treatment of dental caries

Water fluoridation has had a huge impact on oral health by reducing dental diseases at a low cost. The fluoride gets into the oral cavity and presents anti-caries activity in different mechanisms of action; promotion of enamel remineralisation and as an antimicrobial agent when rendering bacteria susceptible to an acidic environment (Featherstone, 2000; Courtel and Decroix, 2002).

Fluorides are also incorporated in dentifrices which enables efficient contact between fluorine and tooth enamel. Triclosan and chlorhexidine are also included in some kinds of toothpaste and mouthwashes, respectively. They are both antimicrobials with different mechanisms, the prior inhibits fatty acid synthesis, and the latter is involved in membrane disruption (Featherstone, 2000; Marsh, 1993).

The consumption of high carbohydrates (highly refined and complex sugary foods) is a predisposing factor in dental caries (Petersen *et al*., 2005). Thus, replacing the sugars with Xylitol reduces the chances of developing caries since Xylitol has an anti-cariogenic character (Loesche, 1996; Tanzer, 1995).

Researchers have also been trying other alternative ways such as immunisation to eliminate dental carries. However, the approach succeeded in animal models and has not been translated to a human vaccine (Russell *et al*., 2004).

2.8.2. Treatment of periodontal diseases

The most popular treatments for periodontal diseases are based on mechanical procedures such as surgery and debridements (Guthmiller and Novak, 2002). For a periodontal disease therapy to be considered effective, it should successfully establish the health of periodontium, prevent progression and recurrence of the disease, and preserve healthy teeth. Some of the antimicrobials used to achieve these are chlorhexidine, quaternary ammonium agents, phenolic compounds, stannous fluoride, and oxygenating compounds. Antibiotics such as penicillins, tetracycline, metronidazole, and macrolides are also used in periodontal therapy (Maja, 2009).

2.8.3. Treatment of oral candidiasis

There are several antifungal agents used in the treatment of oral candidiasis. They belong to the main groups of antifungals, which are polyenes, azoles, and Echinocandins. Polyenes consist of three antifungals, namely, amphotericin-B, nystatin and natamycin. The mechanism of action of polyenes entails the formation of a complex with ergosterol when a polyene binds to the lipid bilayer, which ultimately opens a pore and disrupts the cell membrane (Campoy and Adrio, 2016). Cellular constituents leak out of the cell through the pore resulting in cell death. Natamycin and nystatin are toxic to human cells and are applied as topical agents, and amphotericin-B is also applied topically since it is hydrophobic (Dowd, 2014).

Azoles are antifungal agents and are effective against a wide range of fungal pathogens. There are two subclasses of azoles, namely, imidazoles and triazoles. Imidazoles include ketoconazole, clotrimazole, itraconazole, and miconazole whereas triazoles have a broader spectrum compared to imidazoles and were created to replace the highly toxic imidazoles such as itraconazole and fluconazole (Campoy and Adrio, 2016).

Echinocandins are new antifungals that are effective against a wide spectrum of fungal pathogens, including Aspergillus species and candida. Echinocandins (caspofungin, anidulafungin, and micafungin) kill fungi by inhibition of β-glucan synthesis (Campoy and Adrio, 2016).

2.9. Shortcomings of current dental therapy

Modern technology-based dentistry treatment is not affordable in many developing countries (Yee and Sheiham, 2002). Even in countries with resources, dentistry is failing to meet the needs of the national population and is more focused on aesthetic treatments driven by profit interests (Holden, 2018). In South Africa, many people do not afford to pay specialist dental health care workers for Surgeries, debridements, and tooth extractions (Peterson *et al.,* 2005). A study has found a link between the alcohol constituted by mouthwashes and oral cancer (McCullough and Farah, 2008).

Though there are antifungals used to treat oral candidiasis, there are many challenges associated with them, including toxicity, low efficacy rate, and the limited number and high

cost of available antifungal agents (Mehta *et al.,* 2002; Kathiravan *et al*., 2012). Amphotericin-B is one of the widely used antifungals, is effective against a wide spectrum of fungal pathogens, and is mostly prescribed for more resistant cases of oral candidiasis (Dowd, 2014). However, it has its fair share of criticism as it is linked with severe side effects such as neurological toxicology, vomiting, and fever. Fluconazole is an azole without any toxic effect, but it has led to the emergence of resistance due to prolonged use (Marak and Dhanashree, 2018). Echinocandins inhibits the synthesis of b-(1,3) D-glucan which is present in the cell wall of all fungi and was believed to replace the azoles (Lee *et al*., 2018). However, recent clinical and *in-vitro* studies detected the emergence of resistant candida species (Medici and Poeta, 2015).

2.10. Medicinal plants used for dental diseases

2.10.1. Ethnobotanical review

About 70 to 80% of people depend on medicinal plants as the primary source of health care (Olsen, 1998). The enormous numbers are influenced by several factors, including inaccessibility, unavailability, and high cost of modern medicines (Shewamene, 2017). An increasing number of studies are reporting the use of medicinal plants in the treatment of oral infections.

Different parts of plants are used to treat oral infections, including roots (Muleya *et al*., 2014), rhizomes (van Wyk, 2008; van Wyk *et al.,* 2009; Moffett, 2010; Watt and Breyer-Brandwijk, 1962), bark (Watt and Breyer-Brandwijk, 1962), stem (Badgujar *et al.,* 2008), leaves (Achuta *et al.,* 2010), flowers (Bipul *et al*., 2010), fruits (Murthy *et al.*, 2008), seeds (Prusti and Behera., 2007) and whole plant (Achuta *et al.,* 2010). These different plants are used as a paste (Watt and Breyer-Brandwijk, 1962), mouth wash (More *et al.,* 2008), toothbrush (Chellaiah *et al*., 2006), chewing stick (More *et al.,* 2008), mouth freshener (Khan *et al*., 2004).

2.10.2. Dental care products with plant material

2.10.2.1. Toothpaste

Many kinds of toothpaste available over the counter constitute active plant-based ingredients within their formulation. Plant materials such as leaf extracts of olive, tea tree, cloves, mint, and aniseed, possess antimicrobial activity which upon incorporation in kinds of toothpaste, inhibit cariogenic bacteria (Wolinsky *et al.,* 1996).

2.10.2.2. Oral washes

Oral washes have been used since time immemorial as antiseptics, fresheners, and medicaments. However, today, they are produced and used with more credence as protection against oral and dental diseases (Mandel, 1988). An oral rinse has an advantage over a toothbrush since it can penetrate and reach areas where the toothbrush cannot. Using oral rinses helps to prevent the development of dental caries even in the parts that are difficult to access (Sari and Birinci, 2007).

Several plant-based products with antimicrobial activity have been incorporated in commercial mouthwashes, including green tea (Si *et al*., 2006), garlic (Houshmand *et al*., 2013), and *Aloe vera* (Allaker and Douglas, 2009). Mouthwashes are used all over the world by indigenous people, including in South Africa, where the Southern Sotho people use the *Rubia cordifolia* decoction as a mouthwash (Maroyi, 2013). Other plants such as *Euclea natalensis* and *Vernonia mespilifolia* are also used (Henley-Smith *et al*., 2013; Anibijuwon *et al*., 2012).

2.10.2.3. Chewing sticks and traditional toothbrushes

Teeth brushing is a mechanical means of removing the excess plaque from the oral cavity and an essential act in the prevention and treatment of dental caries (Park *et al*., 1985). Chewing sticks have been used as traditional toothbrushes for ages and are still used today in different parts of the world, including Africa, Asia, and the Middle East (Hilal, 2012; Wu *et al.,* 2001).

2.10.3. South African medicinal plants used against oral pathogens

In South Africa, there are more than 120 medicinal plants used in the treatment of oral infections. However, a few antimicrobial studies focused on oral pathogens (Akhalwayaa *et al.,* 2018). Akhalwayaa *et al*. (2018) prepared six essential oils from 31 different plant species used for the treatment of mouth infections in South Africa and found that most of these plant species have the potential to treat oral thrush, dental caries, and periodontal diseases. More *et al.* (2008) found that six of the eight plants tested on oral pathogens were effective with MIC values ranging from 0.8-25.0 mg/ml. Some of the plants used to treat oral diseases are listed in Table 2.1 below.

Table 2.1: Medicinal plants used to treat oral infections in South Africa

Scientific and family name	Common name	Uses	References
Acacia polyacantha Willd., Leguminosaea	White-stem Thorn	Used for healing of tooth problems.	Watt and Breyer-Brandwijk, 1962
Acokanthera oppositifolia L. Codd., Apocynaceae	Bushman's poison	The leaves are used for toothache.	Philander, 2011; Nielsen, 2012
Afzelia quanzensis sensuWelw. Leguminosae-Caesalpiniaceae	Lucky bean tree	The bark is applied to aching teeth.	Watt and Breyer-Brandwijk, 1962
Anemone caffra E. May. ex Prtiz. Ranunculaceae	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 Ranunculaceae	Giant wild anemone	The ground roots are used for toothache and inserted directly into the hole.	Hutchings, 1996
Anemone tenuifolia var. tenuifolia Harv. Ranunculaceae	Black widow	Leaves treat toothache.	Philander, 2011
Annona chrysophylla Boj. Annonaceaeb	Wild custard apple	Used as a mouthwash to relieve toothache.	Watt and Breyer-Brandwijk, 1962; More *et al*., 2008
Artemisia afra Jacq. ex Willd., Asteraceae	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

Asclepias crispa Berg. Asclepiadaceae	Bitterwortel	Used for toothache in the Karoo.	van Wyk, 2008
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
Berula erecta Huds., Coville Apiaceae	Water parsnip	Fresh rhizomes are chewed to relieve toothache.	van Wyk, 2008; van Wyk *et al.,* 2009; Moffett, 2010; Watt and Breyer-Brandwijk, 1962
Berula erecta Huds., Coville Apiaceae	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
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., Menispermaceaea	Kidney- leaf	Roots are chewed for toothache.	Hutchings, 1996
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
Clausena anisata (Willd) Hook. f. ex., Rutaceae	Horse wood	Dried ground root bark is applied directly to aching tooth.	Hutchings, 1996; Philander, 2011, van Vuuren and Viljoen, 2006
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	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	Lavender croton	The charred and powdered bark is used for bleeding gums.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996

Cyphostemma lanigerum Harv., Vitaceae	Wild grape	The root is rubbed on the gums for toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Datura stramonium L., Solanacea	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	Boot protectors	Used as a chewing stick to relieve toothache.	More *et al*., 2008
Dicerocaryum senecio Koltzsch Abels Pedaliaceae	Boot protectors	Used as a chewing stick to relieve toothache.	More *et al.,* 2008
Dichrostachys cinerea L., Fabaceaea	Sickle bush	Used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962; Adejumo *et al*., 2008
Dichrostachys cinerea L., Fabaceae	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
Dicoma anomala Sond. Asteraceae	Fever bush	Small amounts of the root are chewed for toothache.	Hutchings, 1996; Moffett, 2010
Dodonaea viscosa Jacq., Sapindaceae	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

Elionurus muticus Nees. Poaceae	Wire grass	The root is chewed to relieve toothache.	Moffett, 2010
Equisetum ramosissimum Desf. Equisetaceae	Southern giant horsetail	Sap from the plant is used to relieve toothache and applied to wounds after tooth extraction.	Kelmanson *et al*., 2000
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	Coral tree	The Vhavenḓa use the bark for toothache.	More *et al*., 2008
Euclea divinorum Hiern. Ebenaceae	Diamond shaped euclea	Root decoctions are used for toothache.	Hutchings, 1996; More *et al.*, 2008
Euclea natalensis F. White. subsp. Ebenaceae	Natal Guari	This plant with *Glycyrrihiza glabra* roots is 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
Euphorbia gorgonis A. Berger. Euphorbiacea	Golden lace cactus	The latex of the plant is used for toothache.	Watt and Breyer-Brandwijk, 1962
Euphorbia mauritanica L. var. Feuton Euphorbiaceae	Milk bush	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
Galenia Africana L. Aizoaceae	Yellow bush	Chewed by the Hottentots 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
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
Gethyllis afra Linn. Amaryllidaceae	Herb of milk	Flower decoction is used for toothache.	Louw *et al*., 2002
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	Fever bush	The Xhosa use powdered roots for toothache.	Hutchings, 1996
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
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

Pycnostachys reticulata (E. Mey.) Benth. Lamiaceae	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	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. Polygonacea	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	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

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 relief of toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; van Wyk *et al*., 2009
Senecio coronatus (Thunb.) Harv. Compositeae	Woolly grassland senecio	Infusion of roots is drunk for toothache.	Moffett, 2010
Senecio inornatus DC. Astereceae	Tall marsh senecio	Infusion of roots is drunk for toothache.	Moffett, 2010
Siphonochilus aethiopicus Schweinf., Zingiberaceae	Natal ginger	Used to treat oral thrush and other Candida infections.	Henley-Smith *et al.,* 2013
Solanum aculeastrum Dunal Solanaceae	Soda apple	Boiled root powder is cooled and gargled 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 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
Solanum melongena Mill. Solanaceae	Eggplant	The leaf has been used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962
Solanum panduriforme E. Mey. Solanaceae	Bitter apple	The roots are used as a toothache remedy.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; More *et al.,* 2008; Moffett, 2010
Solanum tomentosum Sendtn. Solanaceae	Bitter apple	The roots are used as a toothache remedy.	Moffett, 2010
Spilanthes mauritiana DC. Asteraceae	Gourd	The leaf or flower of the plant is chewed to relieve toothache, pyorrhea and mouth sores.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996
Spirostachys Africana Sond., Euphorbiaceaea	African mahogany tree	Used as a toothache remedy.	Philander, 2011
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., Asteraceaea	Camphor bush	Infusion of the leaves is used for toothache.	Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Moffett, 2010

Teucrium africanum Thunb. Labiateae	Cancer bush	Leaf paste is used for toothache.	van Wyk, *et al.,* 2008
Toddalia aculeata Pers. Rutaceae	Climbing orange	Used by Vhavenḓa herbalists: 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	Black tea bush	Warm infusions are taken as a mouthwash for toothache.	Hutchings, 1996; Anibijuwon *et al*., 2012
Zanthoxylum capense Harv., Rutaceae	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	Buffalo thorn	Roots are used for toothache.	Hutchings, 1996

2.11. Plant used in the study (*Carissa bispinosa)*

C. bispinosa belongs to the Apocynaceae family and is commonly known as forest num-num (English), *isibethankunzi* (isiZulu) and *bosnoemnoem* (Afrikaans). Plants that belong to the Carissa genus are evergreen shrubs and trees, with beautiful, glossy foliage and fragrance. The plant is distributed in South Africa's wooded areas in Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West, Western Cape and in some southern African countries (Stoffel, 2004). Stem and root extracts of *C. bispinosa* are used to stimulate male sex hormones and for treating toothache, respectively (Muleya *et al*., 2014).

Figure 2.7: Leaves and flowers of the plant used in this study (*C. bispinosa*) http://pza.sanbi.org/sites/default/files/styles/pow_content_image/public/images/plants/9888/carisbispin3.jpg?itok=gkEo-IXl).

2.12. Aim and objectives

2.12.1. Aim

To investigate the effectiveness of antimicrobial compounds from *C. bispinosa* leaf and stem extracts on oral pathogens.

2.12.2. Objectives

The objectives of this study are to:

I. Extract phytochemicals using various solvents (hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, butanol, and methanol) vs water extraction and determination of yield after extraction.
II. Determine the phytochemical constituents of the extracts.
III. Perform the ferric reducing power and DPPH radical scavenging activity and determine antioxidants potential.
IV. Carry out efficacy studies on the effectiveness of plant extracts against *Candida albicans, Candida glabrata, Streptococcus pyogenes, Staphylococcus aureus* and *Enterococcus faecalis*.
V. Evaluate the safety of the extracts by testing for toxicity against THP-1 cell line.
VI. Determine the structure of bioactive compounds using Mass spectroscopy and Nuclear Magnetic Resonance.

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CHAPTER 3

3. EXTRACTION AND PRELIMINARY PHYTOCHEMICAL ANALYSIS

3.1. INTRODUCTION

The compounds or standardised extracts from plants present an opportunity for novel drug leads due to their large and unmatched diversity of phytochemicals (Majekodunmi, 2015). Extraction is a fundamental process that can ultimately lead to the isolation and identification of compounds, analysis of bioactive compounds, and development of therapeutic products (Sasidharan *et al.,* 2011).

Various laboratory techniques are used for the extraction of plant secondary metabolites. The methods are categorised into two major methods: the conventional and non-conventional extraction methods. The prior method includes percolation, infusion, and maceration while the latter uses advanced extraction methods such as ultrasonic-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction (Sultana *et al*., 2009; Azmir *et al*., 2013; Belwal *et al.,* 2018). During an extraction process, proper actions must be taken to protect the compounds of interest from distortion or being destroyed. A basic extraction method entails the drying of the plant material and grinding it to a fine powder to increase the surface area of contact with the solvent (Fabricant and Farnsworth, 2001). A ratio of 10:1 (V/W) solvent to plant powder is ideal to achieve high quality and quantity of compounds (Das *et al*., 2010)

In addition to the type of extraction methods, the type of solvent used during extraction also influences the nature of secondary metabolites and the number of compounds extracted. This is because solvents tend to extract compounds that have the same polarity property as them. For example, polar solvents like water extract polar phenolic compounds, glycosides, and saponins, while hexane, a non-polar solvent, extracts non-polar steroids, and fatty acids.

The extracted sample is left at room temperature to evaporate the solvent, and the dried extract is reconstituted to the desired concentration on a solvent before specific chemical tests, biological activity, or other analysis (Majekodonmi, 2015). Screening for the

phytochemicals that are present in the extract is an inexpensive, quick, and simple procedure (Sasidharan *et al*., 2011). Different standard tests such as Keller killiani test, ferric chloride test, Wagner's and Hager's test, Borntrager's test, lead acetate, and sodium hydroxide test, and Salkowski's test are performed to screen for the presence of bioactive constituents (cardiac glycosides, tannins, alkaloids, anthraquinones, flavonoids, and steroids respectively) from the extract (Patil and Khan, 2016). Thin-layer chromatography is used to separate the compounds. The technique works on the principle that compounds have different solubilities on the eluent system and drawn up at different paces on an adsorbent material (Bele and Khale, 2011).

Since the secondary metabolites are usually extracted from the plant material as a mixture of compounds with different polarities, separating them to obtain a pure compound is very difficult. It requires the resolution power of chromatographic techniques i.e., TLC, Sephadex chromatography, flash chromatography, HPLC, and column chromatography. The structure of the isolated compound is then elucidated, and its biological activity is determined (Sasidharan *et al*., 2011). This chapter aimed to extract and determine the phytochemical constituents, TLC phytochemical profile, and quantify major phytoconstituents present in the extracts.

3.2. METHODOLOGY

3.2.1. Plant collection

The plant was collected from the Lowveld National Botanical Garden Nelspruit (Mpumalanga, South Africa). Voucher specimens of the plants were deposited at the University of Limpopo Larry Leach herbarium (UNIN) for future reference. The leaves and stem of *C. bispinosa* were air-dried in the absence of light and heat to protect the structures of heat-sensitive compounds. The dry plant materials were ground into powder with an electric grinder (Sundy hamercrusher SDHC 150) and kept in a polyethylene plastic bag away from light and heat.

3.2.2. Preliminary extraction procedure

The dried plant powder (1 g) was added into 50 ml centrifuge tubes and a volume of 10 ml solvent was added subsequently. Nine solvents of different polarities, namely: n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water were used for extraction. The plant material and solvent mixtures were shaken at a speed of 200 rpm for 20 min using a shaking incubator (New Brunswick Scientific Co., Inc). After extraction, the extracts were filtered with Whatman filter papers into empty vials of predetermined mass and the solvents were evaporated from the vial using a fan. The mass of phytochemicals extracted was calculated as the difference in mass of the vial with the dried plant extract and the empty vial. The crude extracts were kept at room temperature in the dark until use. The crude extracts were reconstituted in acetone to a final concentration of 10 mg/ml and preserved for phytochemical analysis.

3.2.3. Preliminary serial exhaustive extraction procedure

Five grams of leaf and stem powder were serially extracted with 50 ml of solvents of different polarities from non-polar to polar: Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water. The solvents were evaporated from the vial using a fan. The crude extracts were reconstituted in acetone to a final concentration of 10 mg/ml and preserved till further use.

3.2.4. Thin-layer chromatography analysis (TLC)

Aluminium-backed TLC plates (Merck, silica gel 60 F254) were used to generate the phytochemical profile of the extracts. The dried plant extracts were reconstituted to a final concentration of 10 mg/ml with acetone. A fraction of ten microliters plant extract was spotted one centimetre away from the edge of the TLC plate. The plates were run on three solvent systems of different polarities, which are, ethyl acetate/ methanol /water (40:5:4.5): [EMW] (polar/ neutral); chloroform/ ethyl acetate/ formic acid (5:4:1) [CEF] (intermediate polarity/acidic); benzene/ ethanol / ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). After developing the chromatograms, the plates were removed from the chromatographic tank and air-dried on a fume-hood cabinet, followed by the observation under an ultraviolet (UV) light at 365 nm for detecting

fluorescing compounds. To determine the presence of phytochemicals that are non-fluorescing under UV light, Vanillin-sulfuric acid reagent [0.1 g vanillin (sigma ®): 28 ml methanol: 1 ml concentrated sulphuric acid] was sprayed on the TLC plates. The plates were heated at 110 °C for 2 min to achieve optimal colour development. The plates were scanned and analysed.

3.2.5. Phytochemical analysis of serial exhaustive extracts

Phytochemical profile was determined using an aluminium-backed TLC (Merck, silica gel 60 F254) according to (Kotze and Eloff, 2002) as described in section 3.2.4.

3.2.6. Qualitative phytochemical analysis

3.2.6.1. Test for alkaloids

The presence of alkaloids was tested using Drangendoff's test, where 0.2 g of plant powder was extracted with 95% ethanol using a Soxhlet extractor. The ethanol was evaporated with a vacuum evaporator at 45 °C. The resulting crude extracts were dissolved in 5 ml hydrochloric acid (1% v/v) and about 5 drops of Drangendoff's reagent were added. The development of a reddish-brown colour indicated the presence of alkaloids (Harborne, 1973).

3.2.6.2. Test for flavonoids

Three drops of dilute ammonia solution were added to a 2 ml aqueous filtrate. A volume of 1 ml of concentrated sulphuric acid was added. A yellowish colour that disappears on standing indicates the presence of flavonoids (Borokini and Omotayo, 2012).

3.2.6.3. Test for steroids

The Liebermann-Burchard's test was used, where 0.5 g plant powder was extracted with ethanol. To the extract, two millilitres of acetic anhydride were added followed by the addition of 2 ml sulphuric acid. The formation of a blue or green indicates the presence of steroids (Borokini and Omotayo, 2012).

3.2.6.4. Test for terpenoids

To test for terpenoids, the Salkowski test was used. About 3 ml of concentrated sulphuric acid was added to the ethanol aqueous extract. An inference is drawn based on the appearance of a reddish-brown coloration (Borokini and Omotayo, 2012).

3.2.6.5. Test for tannins

Three drops of ferric chloride (0.1% v/v) were added to a 2 ml water aqueous filtrate. A formation of brownish-green or blue-black colour indicates the presence of tannins (Trease and Evans, 1989).

3.2.6.6. Test for saponins

About 30 ml tap water was added to a 1 g plant powder, the mixture was shaken vigorously and boiled at 100 °C. The presence of saponins is detected by the formation of froth (Odebiyi and Sowofora, 1977).

3.2.6.7. Test for phlobatannin

An aqueous filtrate of 2 ml was mixed with hydrochloric acid (2%) and boiled. Where the presence of phlobatannin is detected by the formation of a red colour (Borokini and Omotayo, 2012).

3.2.6.8. Test for cardiac glycosides

The Keller-Killiani test was used, where 5 ml of distilled water was added to the 0.5 g dry water extract. Two millilitres of glacial acetic acid with a drop of 0.1% ferric chloride solution was added. This was followed by the addition of 1 ml concentrated sulphuric acid. The presence of cardiac glycosides is detected by the appearance of a brown ring which is a characteristic of cardenolides (Borokini and Omotayo, 2012).

3.2.6.9. Test for anthraquinones

A volume of 10 ml sulphuric acid was added to a 0.5 g ethanol extract and the mixture was boiled and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The

Chloroform layer was transferred to another test tube and dilute ammonia (1 ml) was added. The development of the pink colour in the ammonium phase indicates the presence of anthraquinones (Ayoola *et al*., 2008).

3.2.7. Quantification of major constituents

3.2.7.1. Determination of total phenolic content

The total phenolic content of the plant extracts (in 70% aqueous acetone) was determined using the spectrophotometric method described by Singleton *et al.* (1999) with minor modifications. The Folin-Ciocalteau method was used in the determination of total phenolic content, where a volume of 0.1 ml plant extract was added to 0.9 ml distilled water and mixed well in a 25 ml volumetric flask. A volume of 100 ul folin-ciocalteau phenol reagent was added to the mixture and the mixture was shaken well. After 5 min, about 1 ml of 7% Sodium carbonate (Na_2CO_3) solution was added to this mixture. Distilled water was added to a final volume of 2.5 ml. In the same manner, as described above, a set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/ml) were prepared. The mixtures were incubated for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV)/visible spectrophotometer. Total phenol content was expressed as mg of GAE/g of extract (Tambe and Bhambar, 2014).

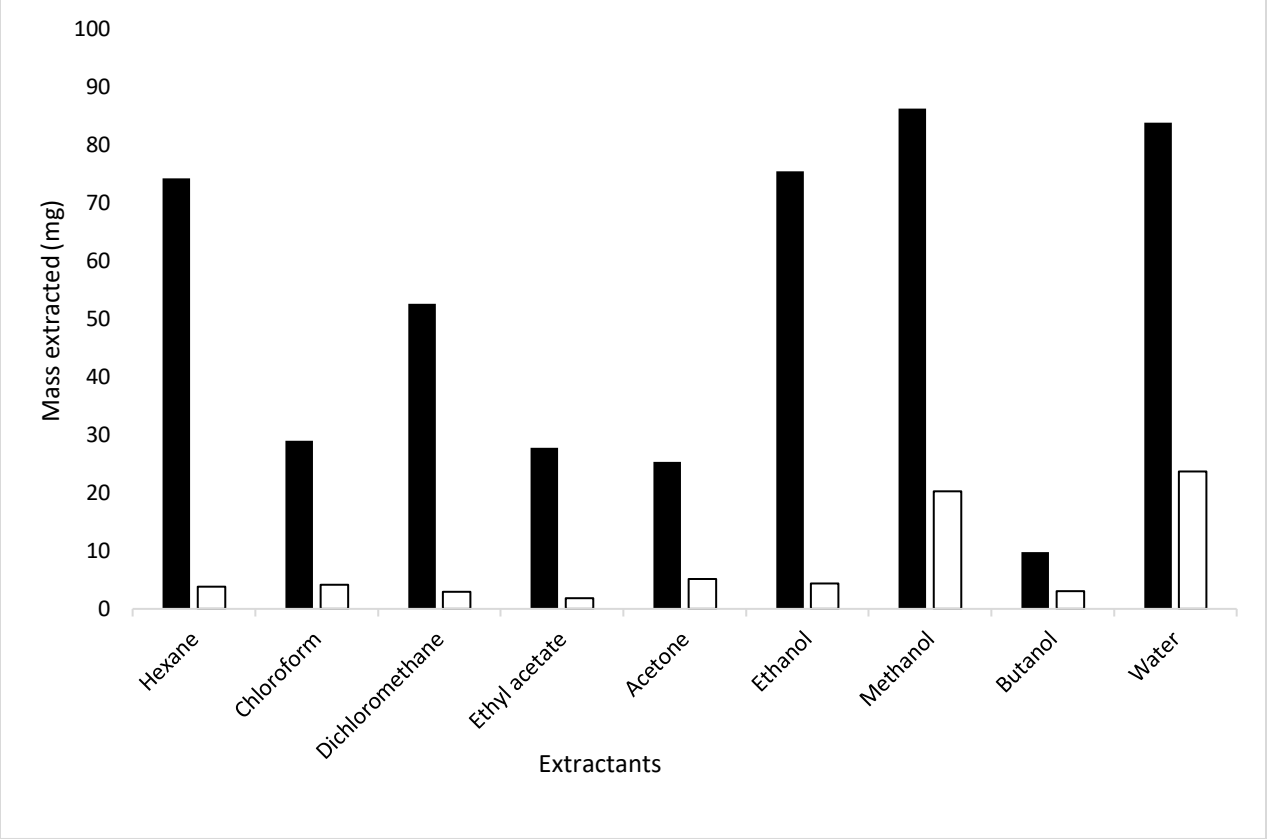
3.2.7.2. Determination of total tannin content

To determine the total tannin content, the Folin - Ciocalteau method was used, where 100 microlitres of 70% aqueous acetone extracts of the plant extracts and 5 ml distilled water were mixed in a 10 ml volumetric flask. About 0.2 ml of 2 M Folin- Ciocalteau phenol reagent and 1 ml of 35 % Na_2CO_3 solution were added to the mixture and this was made up to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/ml) were prepared in the same manner as described above. Absorbance for test samples and standard solutions were measured against the blank at 725 nm with a UV/Visible spectrophotometer. The tannin content was expressed as mg of GAE /g of extract (Tambe and Bhambar, 2014).

3.3. RESULTS

3.3.1. Preliminary extraction

C. bispinosa's leaf and stem powder (1 g) were extracted with solvents of different polarities: hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water. Figure 3.1 shows the mass in milligrams of leaf and stem crude extracts. The masses of the leaf extracts are relatively higher compared to the stem extracts. Methanol extract had the highest mass (86.2 mg) with the least being butanol (9 mg) of all the leaf extracts while water extract was the highest from the stem extracts with 23.7 mg and ethyl acetate (1.8 mg) being the least.

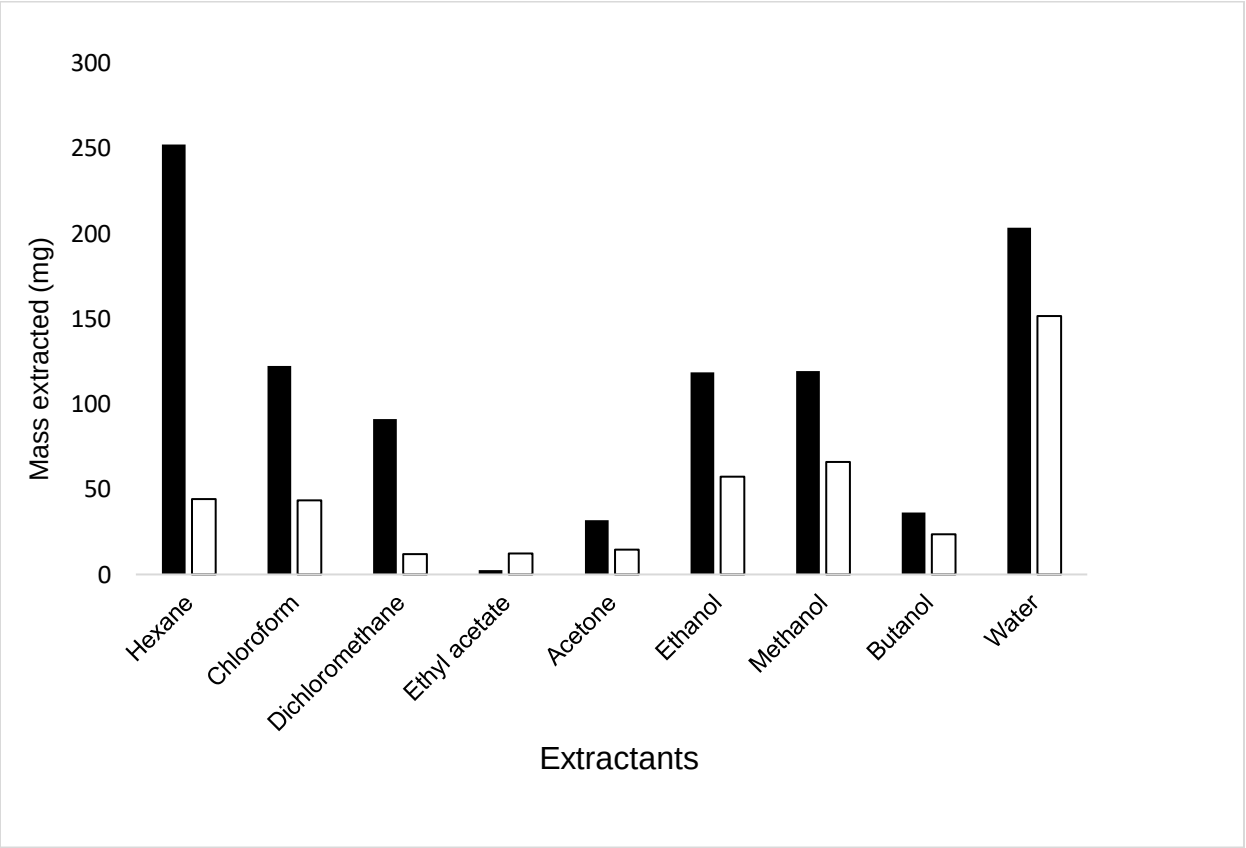


Key: Black= leaf, Blank= stem

Figure 3.1: The mass of leaf and stem crude extracts in milligrams extracted with different solvents.

3.3.2. Serial exhaustive extraction

A mass of 5 g dry plant powder was extracted with 50 ml of 9 solvents using the serial exhaustive extraction method. Hexane, a non-polar and the first solvent used, extracted the highest mass (252.1 mg) followed by water (203.5 mg) and the least extracted mass was observed on ethyl acetate's 2.7 mg from the leaf extracts. On the stem extracts, water had the highest mass extracted (121.7 mg) and dichloromethane the least (12 mg).

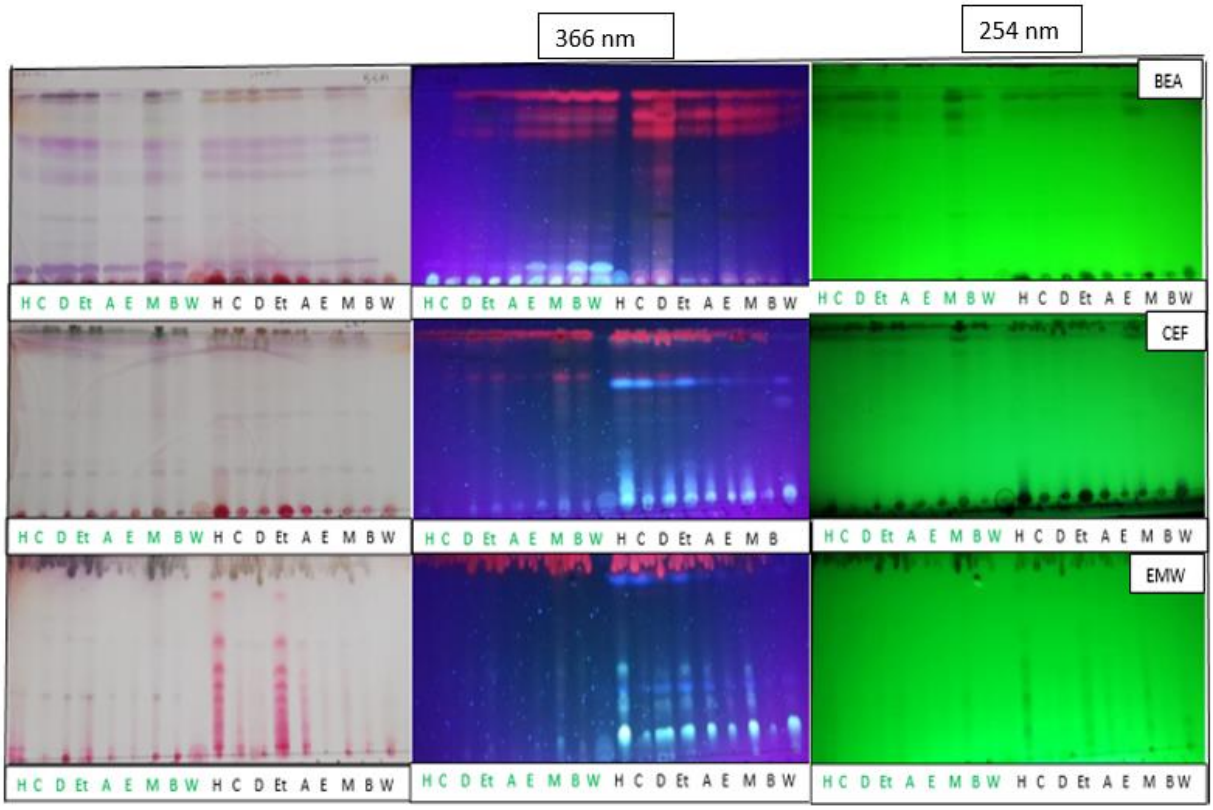


Key: Black= leaf, Blank= stem

Figure 3.2: The mass of leaf and stem crude extracts in milligrams extracted in serial exhaustive extraction with solvents of increasing polarity.

3.3.3. Thin layer chromatography analysis (TLC)

TLC was used in the analysis of *C. bispinosa*'s leaf and stem extracts. Figure 3.3 shows the phytochemical profile of the extracts observed under ultraviolet (UV) light at 254 nm and 366 nm. The plates were further sprayed with Vanillin-sulfuric acid reagent to detect non-flouriscing phytochemicals. Better resolution was observed on BEA mobile phase.

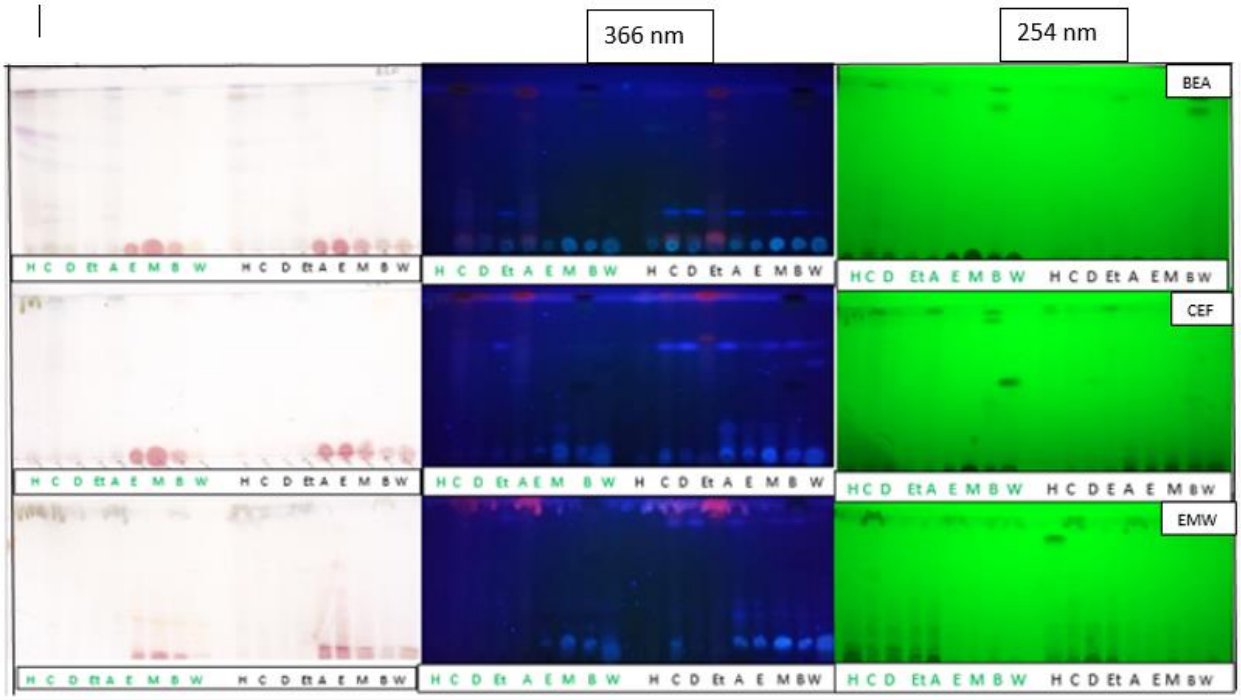


Key; green= leaf extracts; black= stem extracts

Figure 3.3: Chromatograms obtained after running TLC aluminium-backed plates loaded with the *C. bispinosa'*s leaf and stem extracts; (H) n-hexane, (D) dichloromethane, (Et) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (C) chloroform and (W) water in EMW, BEA and CEF mobile phases. The plates were observed under UV light at 366 nm and 254 nm and further sprayed with vanillin-sulphuric acid reagent and viewed.

3.3.4. Thin layer chromatography analysis (TLC) of serial exhaustive extracts

The phytochemicals of the leaf and stem powders of *C. bispinosa* were extracted through serial exhaustive extraction using nine solvents in a sequence of non-polar to polar: [(H) hexane, (C) chloroform, (D) dichloromethane, (Et) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol and (W) water]. CEF and EMW exhibited better resolution.



Key; green= leaf extracts, black= stem extracts

Figure 3.4: The chromatograms obtained after running the TLC aluminium-backed plates loaded with the *C. bispinosa*'s leaf and stem extracts; in EMW, BEA and CEF mobile phases. The plates were observed under UV light at 366 nm and 254 nm and further sprayed with vanillin-sulphuric acid reagent and scanned.

3.3.5. Phytochemical constituents

The leaf and stem of the plant were examined for the presence of different secondary metabolites with medicinal properties found in plants. Flavonoids were not found in both plant parts.

Table 3.1: Phytochemical constituents of *C. bispinosa*'s leaf and stem extracts.

Phytochemicals	Occurrence	
	Leaf	Stem
Alkaloids	+	+
Cardiac glycosides	+	+
Flavonoids	-	-
Phlobatannin	+	+
Saponins	+	+
Steroids	+	+
Tannins	+	+
Terpenoids	+	+

Keys: Present (+), Absent (-)

3.3.6. Determination of total phenolic and tannin content

The following calibration curve (Figure 3.5) was used to estimate total phenolic and tannin content. The curve indicated a positive linear correlation with the absorbance increasing in a dose dependant manner.

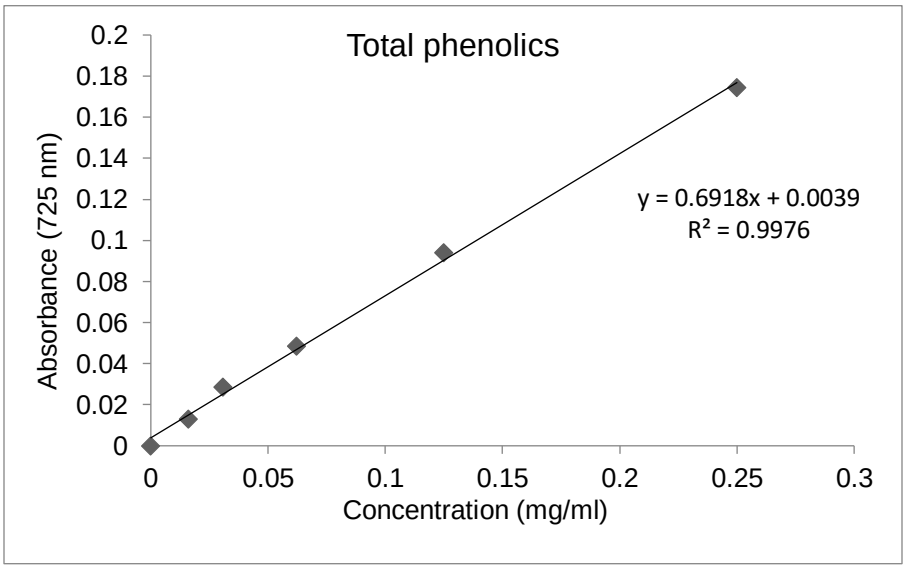


Figure 3.5: The gallic acid standard curve for the determination of total phenolic content.

The major phytochemical constituents (phenolics and tannins) of plants were quantified and their quantity was estimated using the calibration curves (figure 3.5 to 3.7). The stem extracts show higher total phenolic and tannin content than the leaf extracts. The leaf

water extracts had the highest total phenolic content (94.74 mg of GAE/g) while the acetone extract (338 mg of GAE/g) constituted the highest total phenolic content of all the stem extracts. The leaf extract's highest total tannin content was detected on hexane extract (22.98 mg of GAE/g) and ethanol extract contained the highest total tannin content (49.87 GAE/g) in the stem extracts.

Table 3.2: Total phenolic and tannin content of *C. bispinosa*'s leaf and stem extracts expressed as mg of gallic acid quivalent (mg of GAE/g of sample)

Solvent	Total phenolic content (mg of GAE/g of sample)		Total tannin content (mg of GAE/g of sample)	
	Leaf	Stem	Leaf	Stem
Hexane	29.98 ± 18.02	216 ± 30.55	22.98 ± 2.16	47.29 ± 2.83
Dichloromethane	70.41 ± 34.75	50 ± 18.16	8.36 ± 0.96	8.89 ± 0.68
Ethyl acetate	69.64 ± 25.46	36 ± 7.83	16.64 ± 2.57	6.79 ± 1.73
Acetone	29.21 ± 13.44	338 ± 14.94	15.04 ± 2.25	47.65 ± 2.51
Ethanol	33.79 ± 11.31	216 ± 59.39	13.46 ± 0.68	49.87 ± 5.02
Methanol	113.20 ± 10.41	163 ± 26.76	9.35 ± 5.04	28.07 ± 7.5
Butanol	40.01 ± 7.64	31 ± 10.73	10.65 ± 1.91	12.23 ± 3.27
Chloroform	30.23 ± 19.31	46 ± 17.57	15.37 ± 1.23	18.21 ± 4.35
Water	94.74 ± 12.64	244 ± 17.56	20.01 ± 1.43	38.68 ± 2.03

3.4 DISCUSSION

The plant's leaves and stem were dried, ground to fine powder and subjected to extraction using solvents of different polarities. Solvents mostly extract compounds of the same polarity as them, therefore, nine solvents of varying polarity were used to extract as many varieties of secondary metabolites as possible (Rebey *et al.,* 2012; Kchaou *et al*., 2013; Ngo *et al*., 2017).

In this study, extraction was achieved through shaking the mixture of solvent and leaf /stem powder, also with the use of the serial exhaustive extraction. The solvents, namely: Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, butanol, methanol, and water, ranging from non-polar to polar were used. The masses of the leaf extracts are relatively higher compared to the stem extracts. Methanol and water extracts had the highest number of secondary metabolites from both plant parts (86.2 mg and 83.7

mg on leaf and 20.2 mg and 23.7 mg, respectively). The ability of methanol to extract the highest mass of secondary metabolites correlates with the results observed by Masoko and Eloff (2007) and that of water, which was also shown by Bouhafsoun *et al.* (2019). From the serial exhaustive extracts, n-hexane and water extracted the highest mass.

The presence of phytochemicals was determined by TLC analysis where the development of various colours as bands on the TLC plates represents different compounds (Sasidharan *et al.,* 2011). The bands were observed under UV light (254 and 365 nm) and sprayed with Vanillin-sulphuric acid reagent and observed again to detect non-fluorescing compounds. Upon spraying with Vanillin-sulphuric acid reagent, it was observed that BEA solvent system separated the compounds better than CEF and EMW solvent system. For both the leaf and stem extracts, more bands were observed on EMW and BEA solvent systems suggesting the presence of polar and non-polar constituents.

The extracts were screened for secondary metabolites that are known to elicit therapeutic properties. The constituents tested were saponins, alkaloids, terpenes, cardiac glycosides, phlobatannin, tannins, steroids and flavonoids. All the secondary metabolites tested were present except for flavonoids. The major constituents were quantified and the leaf water extracts had the highest total phenolic content (94.74 mg of GAE/g) while the acetone extract (338 mg of GAE/g) constituted the highest total phenolic content of all the stem extracts. The leaf extract's highest total tannin content was detected on the hexane extract (22.98 mg of GAE/g) and the ethanol extract contained the highest total tannin content (49.87 GAE/g) in the stem extracts.

3.5 CONCLUSION

EMW solvent system demonstrated the better compound separation quality. The leaf and stem extracts of *C. bispinosa* constitute saponins, alkaloids, terpenes, cardiac glycosides, phlobatannin, tannins and steroids, which are phytochemical groups known to possess antimicrobial and antioxidant activity. The phenolic content is higher than the tannin content for both plant parts.

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CHAPTER 4

4. ANTIOXIDANTS

4.1. INTRODUCTION

Free radicals are defined as atoms or molecules that contain one or more unpaired electrons in the outer orbit and can exist on their own. Free radicals are short-lived, unstable, and highly reactive due to their odd number of electrons. Their high reactivity makes them abstract electrons from other compounds to gain stability. Thus, the compound whose electrons are abstracted becomes a free radical itself, triggering a chain reaction cascade that ultimately damages the cell (Mukherji and Singh, 1986). Normal cellular metabolism in humans produces free radicals. They are derived from both endogenous sources (phagocytic cells, mitochondria, endoplasmic reticulum, peroxisomes, etc.) and exogenous sources (alcohol, pollution, smoke, tobacco, heavy metals, industrial solvents, transition metals, pesticides, certain drugs like halothane, paracetamol, and radiation).

Free radicals have gained popularity in the biological world due to their central role in different physiological conditions and the influence they have on various diseases (Phaniendra *et al*., 2015). Excess free radicals in the body have been associated with damage to significant biomolecules, including DNA, lipids, and proteins, excruciating humans due to the resulting increase in oxidative stress in diseases like rheumatoid arthritis, neurodegenerative diseases, diabetes mellitus, cataracts, cardiovascular diseases, respiratory diseases and in the aging process (Yla-Herttuala, 1999; Stadtman and Levine, 2000; Marnett, 2000).

Plant secondary metabolites have gained popularity due to their health benefits and a possible source of antioxidants (Liu *et al*., 2017; Wang *et al*., 2018). People have been consuming synthetic antioxidants to scavenge free radicals; however, they are falling out of favour owing to their potential toxicity and other health risks. Thus, natural sources are being analysed and harnessed for natural antioxidants that can be incorporated in foods, pharmaceutical preparations, and cosmetics (Kicel *et al*., 2016; Liu and Yang, 2018).

The antioxidant activity of plant extracts has been shown to have a positive correlation with the extract's flavonoids, polyphenols, and proanthocyanidins content (Pellegrini *et al*., 2000; Pietta *et al*., 2000; Akyuz *et al*., 2014). The isolation and purification methods used to attain pure compounds are commonly costly, time-consuming, and require many resources. The crucial step to finding antioxidant compounds is the evaluation of extracts and fractions because of their potentials for high antioxidant potency (Amarowicz, 2004).

The frequently used methods in determining antioxidant activity qualitatively and quantitatively are the 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical's assays, ferric reducing antioxidant power (FRAP) assay, and ferrous-ion chelating assays (Molan *et al*., 2012). This chapter aimed to determine the antioxidant activity of the leaf and stem extracts of *C. bispinosa* using the DPPH free radical scavenging method on TLC and quantify the antioxidants present by determining the amount of free radical scavenged and assaying their antioxidant capacity using the reducing power method.

4.2. METHODOLOGY

4.2.1. DPPH free radical scavenging assay on TLC

The crude plant extracts were prepared and reconstituted, as mentioned in section 3.2.2, and used for qualitative antioxidant assay on TLC plates. The chromatograms were developed as in section 3.2.3 and dried in a fume-hood, sprayed with 0.2% (w/v) of DPPH (Sigma®) in methanol as an indicator. The presence of antioxidant compounds was detected by the development of yellow spots against a purple background (Deby and Margotteaux, 1970).

4.2.2. DPPH free radical scavenging assay

The free radical scavenging activity of the aqueous acetone plant extract was determined using the method described by Brand-Williams *et al.* (1995) with some modifications. Different concentrations of the range (0.031 to 1 mg/ml) were prepared through serial dilution with each test tube containing 1 ml of the plant extract, and about 1 ml 0.2 mmol/L DPPH was mixed with the plant extract of different concentrations. This mixture was then

diluted with 10 ml of methanol to a final volume of 12 ml. Different concentrations of L-ascorbic acid as a standard control were prepared in the same way as the plant extracts. DPPH solution was used as a standard control while methanol was used as a blank. The content was shaken well and incubated for 20 min in the dark. The absorbance was detected spectrophotometrically at 517 nm. The antioxidant activity was expressed as the half-maximal effective concentration (EC_{50}).

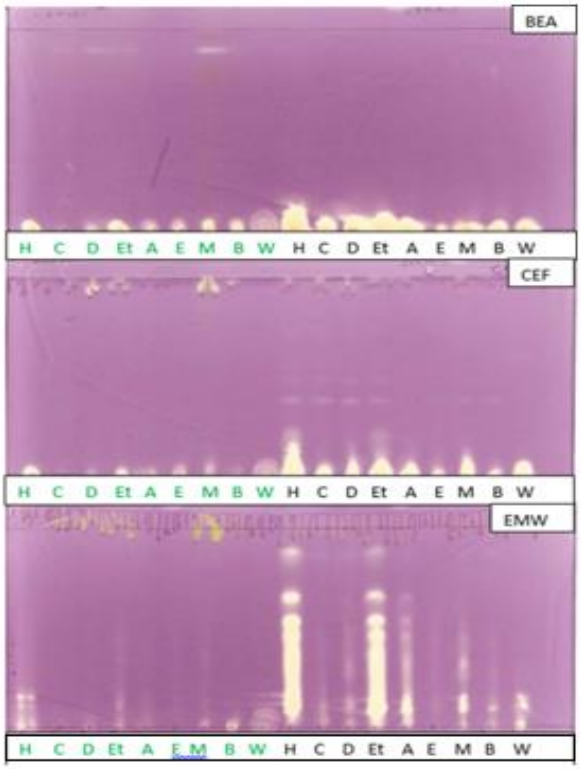
4.2.3. Ferric reducing power assay

The ability of the plant extract to reduce potassium ferricyanide (Fe3+) to potassium ferrocyanide (Fe2+), which reacts with ferric chloride to form a ferric-ferrous complex that is detected spectrophotometrically was determined using reducing power assay as described by Oyaizu (1986) with minor modifications. A set of concentrations ranging from 0.0625 mg/ml to 1 mg/ml of the 70% aqueous acetone plant extracts were prepared. Two millilitres of the plant extract were added to 2 ml sodium phosphate buffer (1 M, pH 6.6) and 2 ml potassium ferricyanide (1% w/v in distilled water), the mixture was mixed and incubated for 20 min in a water bath at 50°C. A volume of 2.5 ml trichloroacetic acid (10% w/v in distilled water) was added to the mixture to stop the reaction and then centrifuged for 10 min at 650 rpm. After centrifuging, 3 ml of the supernatant were added to a new test tube and 10 ml distilled water was added. To the new mixture, 1 ml ferric chloride (0.1% w/v in distilled water) solution. The content was mixed well, and the absorbance was measured at 700 nm. The blank was prepared in the same manner as the extracts but with acetone added instead of plant extracts.

4.3. RESULTS

4.3.1. TLC-DPPH assay

TLC DPPH assay was performed to qualitatively determine the antioxidant activity of *C. bispinosa*'s leaf and stem extracts. The assay is based on the ability of the extracts to scavenge free radicals of (0.2% DPPH in methanol) which was sprayed on the chromatograms. All the chromatograms had a yellow colouration upon spraying with DPPH indicating antioxidant activity. However, the EMW chromatogram showed a better separation of antioxidant compounds followed by CEF.

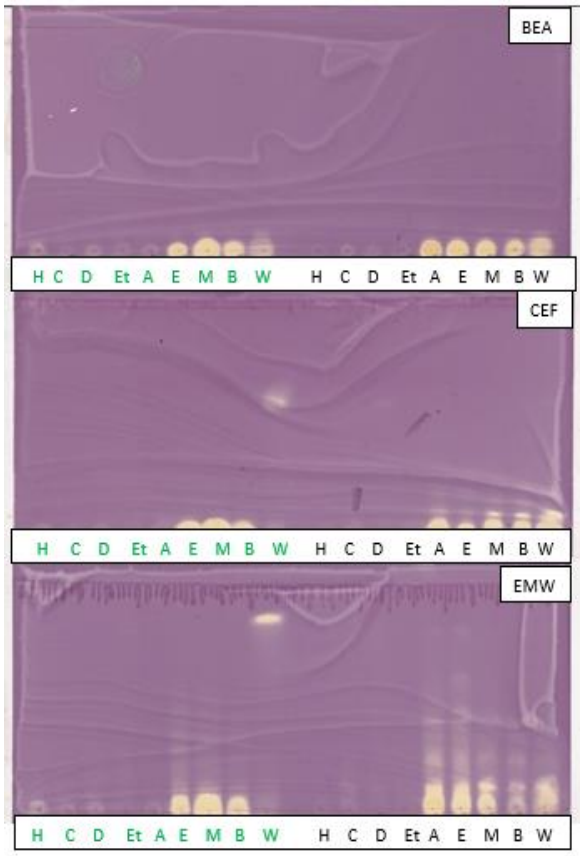


Key; green= leaf extracts; black= stem extracts

Figure 4.1: Chromatograms of the leaf and stem extracts: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol and (W) water of *C. bispinosa* developed in BEA, CEF and EMW mobile phases and sprayed with 0.2% DPPH.

4.3.2. TLC-DPPH assay serial exhaustive extracts

Figure 4.2 below shows the antioxidant potential of serial exhaustive extracts for the leaf and stem of *C. bispinosa*. The yellow colouration observed represents antioxidant activity. Only the polar extracts (ethanol, methanol, butanol, and water) indicated antioxidant activity for both stem and leaf extracts.



Key; green= leaf extracts; black= stem extracts

Figure 4.2: Chromatograms of *C. bispinosa*'s leaf and stem serial exhaustive extracts: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol and (W) water developed in BEA, CEF and EMW mobile phases and sprayed with 0.2% DPPH.

4.3.3. DPPH free radical scavenging assay

The leaf and stem extracts were analysed for antioxidant potential using quantitative DPPH free radical scavenging assay. The free radical scavenging activity of the extracts and L-ascorbic acid demonstrated a direct proportionality relationship with the concentration. From the leaf extracts ethanol, water, and methanol extracts exhibited the highest percentage of antiradical activity with 90.52%, 86.61%, and 78.52%, respectively, while ethyl acetate had the least percentage (13.33%). A slightly similar trend was observed on the stem extracts, where; methanol, ethanol and water extracts exhibited the highest activity.

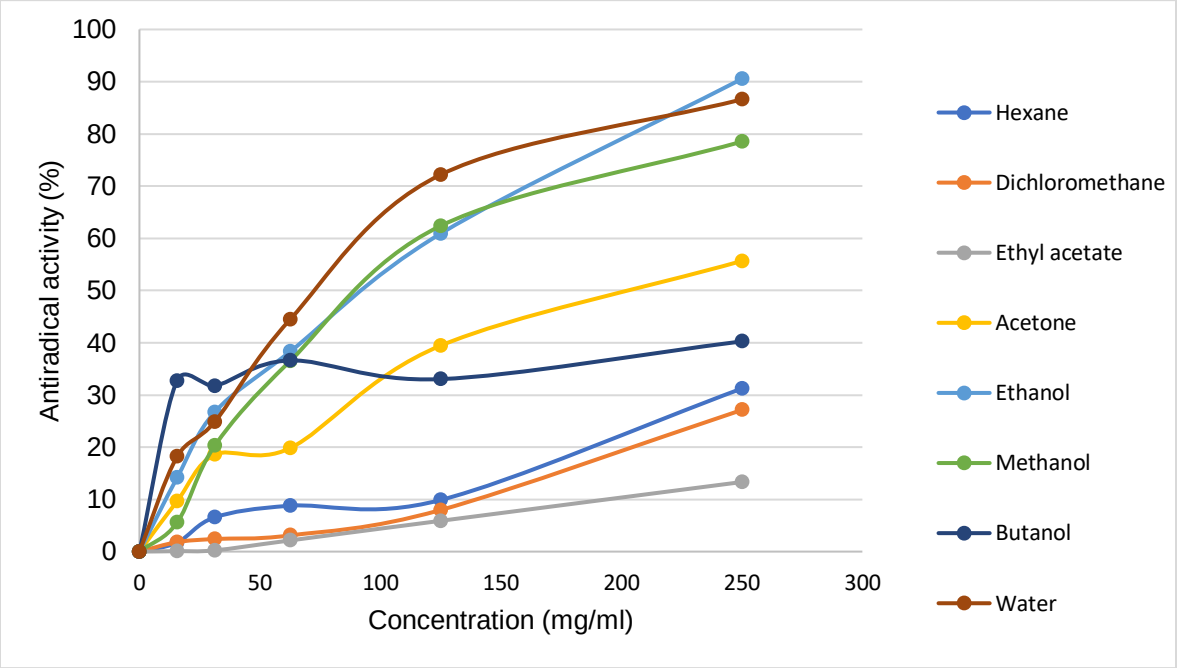


Figure 4.3: Antiradical activity percentage of *C. bispinosa*'s leaf extracts, extracted with different solvents.

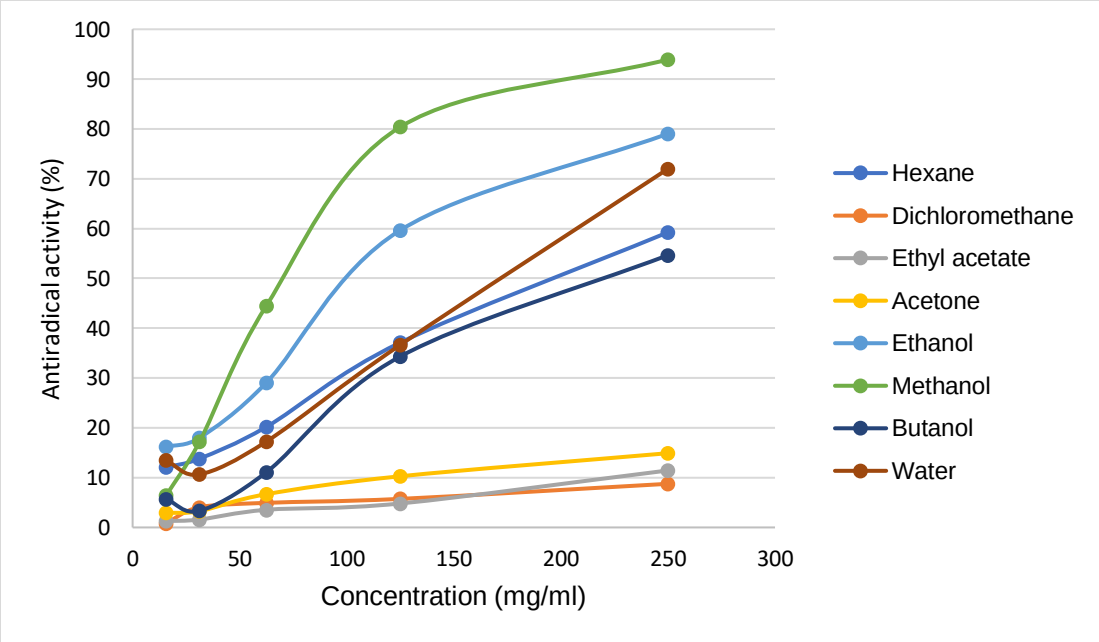


Figure 4.4: Antiradical activity percentage of *C. bispinosa*'s stem extracts, extracted using different solvents.

4.3.4. Ferric reducing power assay

The reducing power of the extracts was tested using the ferric reducing power assay. The assay is based on testing the reduction of potassium ferricyanide to potassium ferrocyanide by the antioxidant. This is followed by the reaction of potassium ferrocyanide with ferric chloride to form a ferric-ferrous complex that has a maximum absorption at 700 nm. Water and ethanol extracts were exhibited the highest reducing power respectively, while acetone and butanol displayed the least reducing ability for the leaf extracts. Ethanol and water extracts led the stem extracts with reducing power and chloroform extract exhibited the poorest reducing power.

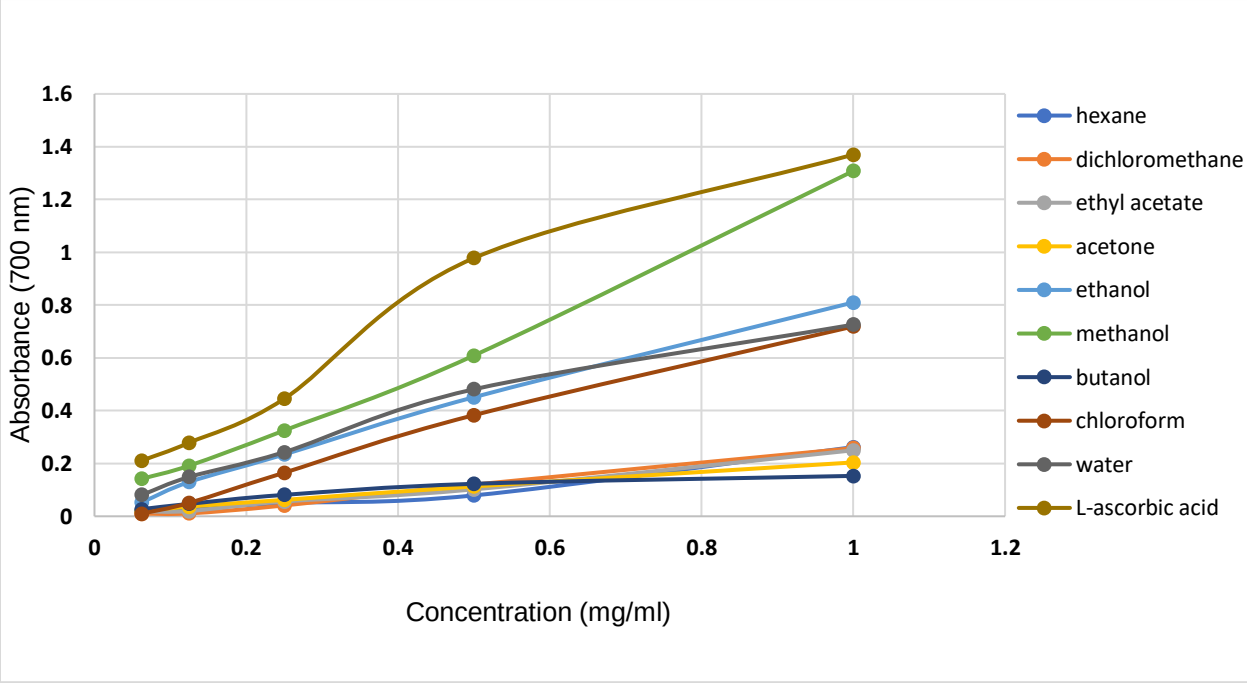


Figure 4.5: Reducing power of *C. bispinosa*'s leaf extracts.

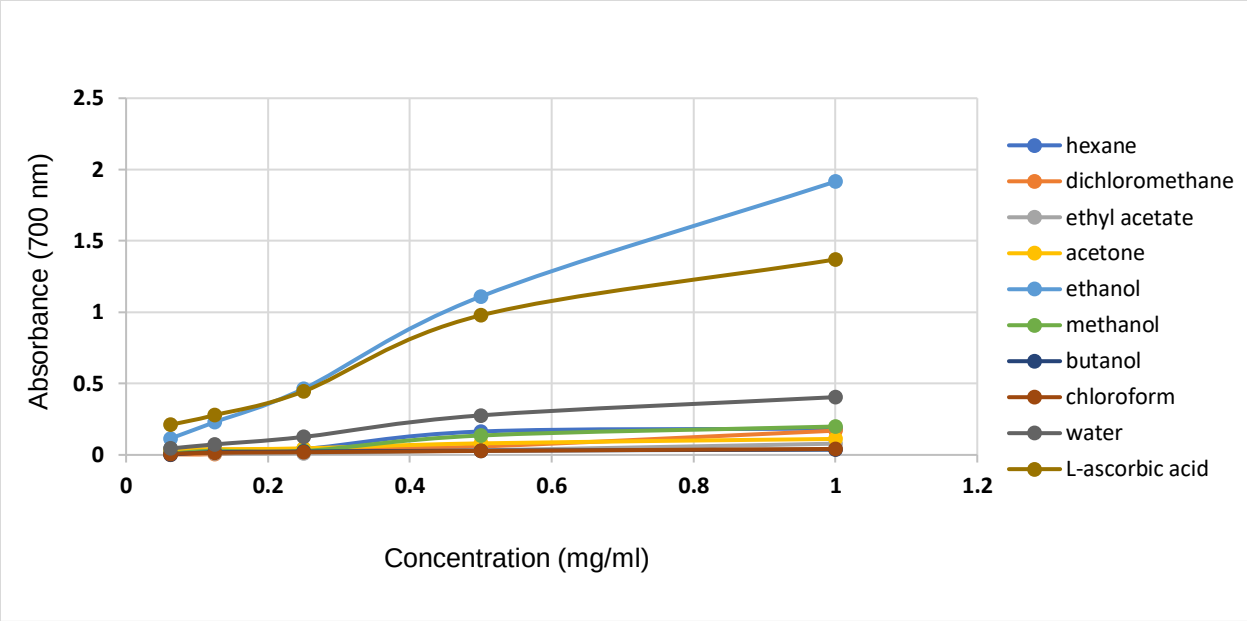


Figure 4.6: Reducing power of *C. bispinosa*'s stem extracts.

4.4 DISCUSSION

Free radicals have gained popularity in the biological world due to their central role in different physiological conditions and the influence they have on various diseases (Phaniendra *et al*., 2015). This has necessitated the search for antioxidant and antimicrobial activities from various plant extracts to explore alternative therapy against different oxidative reactions and microorganisms. In this study, the antioxidant activity of *C. bispinosa's* leaf and stem extracts was evaluated.

TLC was performed in different solvent systems (EMW, CEF, and BEA) to separate the phytoconstituents of the plants and ultimately test for their antioxidant activity. It can be seen in Figure 4.1 that the yellow spots were found predominantly on the EMW (polar) followed by the CEF (intermediary polar) solvent system, suggesting that most antioxidant molecules are polar. This is further supported by the high total phenolic content observed in Table 3.2. Numerous studies reported that a high phenolic content correlates with a high antioxidant activity (Moure *et al*., 2001; Tlili *et al.,* 2013; Tohma *et al*., 2017; Granato *et al*., 2018; Rahman *et al*., 2018).

According to El Jemli *et al*. (2016), it is important to perform more than one type of assay since antioxidant capacity may be affected by many factors. Thus, DPPH free radical

scavenging assay and ferric reducing power assay were also performed. Ethanol, water, and methanol extracts exhibited the highest free radical scavenging activity with 90.52%, 86.61%, and 78.52% antiradical activity, respectively, for leaf extracts. For stem extracts, methanol, ethanol, and water again led with the highest free radical scavenging activity with 93.93%, 79.03%, and 71.98%, respectively.

The reducing power of the extracts was tested using the ferric reducing power assay. The assay entails the reduction of potassium ferricyanide to potassium ferrocyanide by the antioxidant. This is followed by the reaction of potassium ferrocyanide with ferric chloride to form a ferric-ferrous complex that has a maximum absorption at 700 nm (Jayaprakasha *et al*, 2001). Methanol and ethanol extracts displayed high antioxidant potential for leaf (1.308) and stem (1.916) respectively, supporting the ability of methanol to extract antioxidants as observed by Ebrahimzadeh *et al*. (2008).

4.5 CONCLUSION

Both the leaf and stem extracts of *C. bispinosa* exhibited antioxidant activity in quantitative as well as qualitative assays. Polar solvent system chromatograms indicated the best antioxidant activity on the qualitative assay and the highest free radical scavenging activity as well as the reducing power on the polar solvents which demonstrated higher phenolic content.

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CHAPTER 5

5. ANTIMICROBIAL ACTIVITY

5.1. INTRODUCTION

Microbial diseases are the second and third leading killers in developing and developed countries, respectively (Luqman, 2005). Various antibiotics have been used over the years to treat different microbial infections. The misuse of antibiotics has led to a global health threat of antibiotic resistance (World Health Organization, 2012). Recently, a study has shown that about 66% of antibiotic prescribers in South African primary health care feel pressurised to prescribe antibiotics (Farley *et al*., 2018). The documented plethora of evidence that medicinal plants are the best source of a variety of antimicrobial agents has fuelled the harnessing of these natural resources (Selvamohan, 2012). Medicinal plants and products derived from them have been used as traditional medicine for centuries in the whole world. Today, about 80% of the world's population depends on ethnomedicine to treat different diseases (Joshi *et al.,* 2011; Pallant and Steenkamp, 2008).

Recently, a growing interest has aroused from researchers to develop new antimicrobial substances from different sources to eliminate microbial resistance, with much focus being directed to methods used in antimicrobial activity screening and evaluation. The commonly used bioassays include well diffusion, disk-diffusion, and broth or agar dilution, and other effective methods such as bioluminescent and flow cytofluorometric methods exist but are rarely used because they require specific equipment and evaluation for reproducibility and standardisation (Balouirin *et al.,* 2016).

Bioautography is a technique where planar chromatographic analysis is linked to biological detection methods (Muller *et al*., 2004). It is an inexpensive, yet effective technique used in phytochemical analysis of plant extracts to detect and identify bioactive compounds. Bioautography can thus be performed in both sophisticated laboratories and small research laboratories without access to expensive equipment (Marston *et al*., 1997). Planar chromatographic techniques such as TLC are generally used for bioautography. However, the detection method can be improved by the using advanced chromatographic tools like the high-performance thin-layer chromatography (HPTLC), planar

electrochromatography (PLC), and over-pressured layer chromatography (OPLC) (Heinemann *et al.,* 1967).

Antimicrobial activity is detected bioautographically using three methods, namely, direct TLC bioautographic detection, agar diffusion or contact bioautography, and agar diffusion or contact bioautography (Wagman and Bailey, 1969; Rios, 1988). It is applied in the screening of many samples for bioactivity such as antifungal, antibacterial, enzyme inhibition, antioxidant, etc., and the target-directed isolation of active compounds (Heinemann *et al*., 1967).

Dilution methods used for determining MIC values are the most appropriate since they make it possible to estimate the concentration of the antimicrobial agent in the broth medium (macro dilution or microdilution) or agar (agar dilution). Both the broth and agar dilution methods can be used to quantitatively test an antimicrobial agent on bacteria and fungi. The MIC values are defined as the lowest concentration of the antimicrobial agent tested that inhibits visible growth of the microorganism tested (Pfaller *et al*., 2004).

Broth microdilution is the most basic method for testing the susceptibility of microorganisms. This procedure entails the preparation of two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in a 96-well microtitre plate. Then, each well is inoculated with the test organism, and positive control is prepared similarly but a standard antimicrobial agent is used instead of the tested sample (CLSI, 2012). There are several calorimetric methods used to visualise inhibition that relies on the use of a dye reagent. The tetrazolium salt, 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is often used in MIC for antibacterial and antifungal microdilution assays (Liang *et al.,* 2012).

5.2. METHODS AND MATERIALS

5.2.1. Test microorganisms

Fungal and bacterial oral isolates were used as test organisms; *Candida albicans* and *Candida glabrata* (fungi), and three Gram-positive bacteria; *Streptococcus pyogenes, Staphylococcus aureus,* and *Enterococcus faecalis*. All the microorganisms were obtained from Polokwane Hospital, National Health Laboratory Service (NHLS). The

NHLS is a parastatal organization providing diagnostic laboratory services to public sector hospitals throughout South Africa.

5.2.2. Bioautography assay

The antimicrobial activity of the plant extracts was tested on the fungal and bacterial isolates using a bioautography assay described by Begue and Kline (1972). The crude extracts were reconstituted to 10 mg/ml on acetone, with 20 µl of the extract being loaded on the TLC plates, and chromatograms developed as in section 3.2.2. The plates were air-dried at room temperature for 3-5 days to completely evaporate the solvents. The test microorganisms were sprayed onto the chromatograms until they were completely wet and incubated for 24 h at 37 °C in 100 % relative humidity. After incubation, the chromatograms were sprayed with an aqueous solution of 2 mg/ml ρ-iodonitrotetrazolium chloride (INT) (Sigma) and incubated for about 2-3 h. The bioautograms were observed for fungal and bacterial growth, where clear zones against a red-pink background indicated growth inhibition by the compounds with antifungal antibacterial activity.

5.2.3. Serial broth microdilution assay

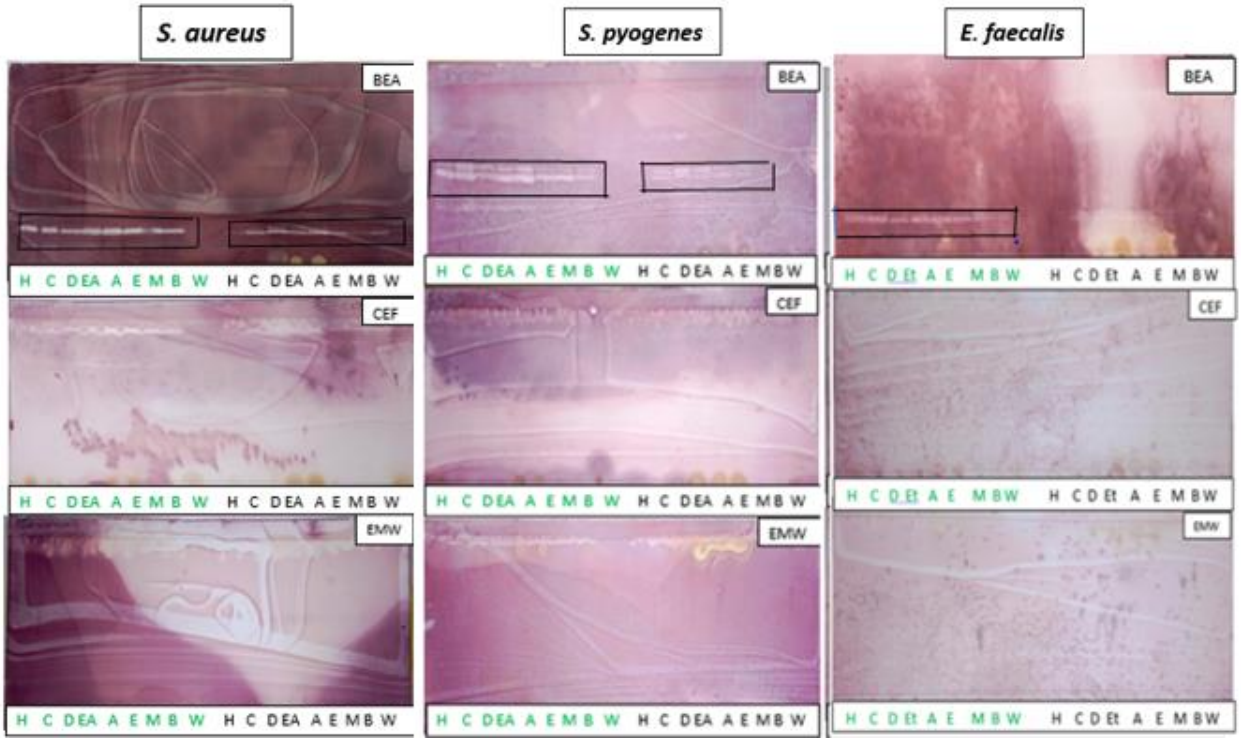
The minimum inhibitory concentration (MIC) values were determined using the serial microplate broth dilution methods developed by Eloff (1998). The crude plant extracts were reconstituted in acetone to a final concentration of 10 mg/ml. Microbial stock cultures were prepared by inoculating the fungal and bacterial culture into a 150 ml Sabouroud dextrose liquid media and nutrient broth and incubate at 30 °C and 37 °C, respectively. A hundred microlitres of the plant extracts were serially diluted to 50% with distilled water in 96 well microtitre plates. A volume of 100 µl of the plant extract was added into the first well and diluted serially with distilled water. Microbial cultures (100 µl) were added to each well. Acetone was used as a solvent control and gentamicin and (sigma®) in µg/ml was used as a positive control. For negative control, only the test organisms and water were added to the wells. The microtitre plates for the fungi and bacteria were covered with a plastic wrap (Glad) and incubated at 30 °C and 37 °C for 24 h, respectively, to allow for microbial growth. After incubation, 40 µl of 0.2 % w/v INT in water was added to each well as an indicator. All the tests were performed in triplicates, and the results are

represented as the mean of the three values. The plates were observed visually to determine the MIC, where clear wells represent positive results, and a purple-red colour indicates negative results. The total activity of the extracts was determined by dividing the MIC values by the mass extracted from 1 g of the plant material. The resultant values indicated the volume to which the amount obtained from 1 g of the plant material could be diluted and still inhibit the growth of the test microorganisms (Eloff, 1998).

5.3. RESULTS

5.3.1. Bioautography

To determine the antimicrobial activity of the extracts, bioautography was performed. The chromatograms were sprayed with *S. aureus, S. pyogenes* and *E. faecalis* and incubated at 37 °C. INT was sprayed on the chromatograms for visualisation following a 24-hour incubation. Figure 5.1 shows the antibacterial activity of the leaf and stem extracts and the marked areas represent the zones of bacterial inhibition. Antibacterial activity was observed on all bacteria with BEA chromatograms being the only one to demonstrate activity.



Key; green= leaf extracts; black= stem extracts

Figure 5.1: Bioautograms of leaf and stem extracts of *C. bispinosa's* extracted with different solvents; (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol and (W) water. The plates were sprayed with *S. aureus, S. pyogenes* and *E. faecalis* overnight cultures and sprayed with 2 mg/ml INT for visualisation.

Figure 5.2 shows the antimicrobial activity of *C. bispinosa's* leaf and stem extracts against *C. albicans* and *C. glabrata.* Faint inhibition zones were only observed on the BEA chromatogram sprayed with *C. albicans* culture indicating the susceptibility of the species to the extracts. No antifungal activity was observed on *C. glabrata.*

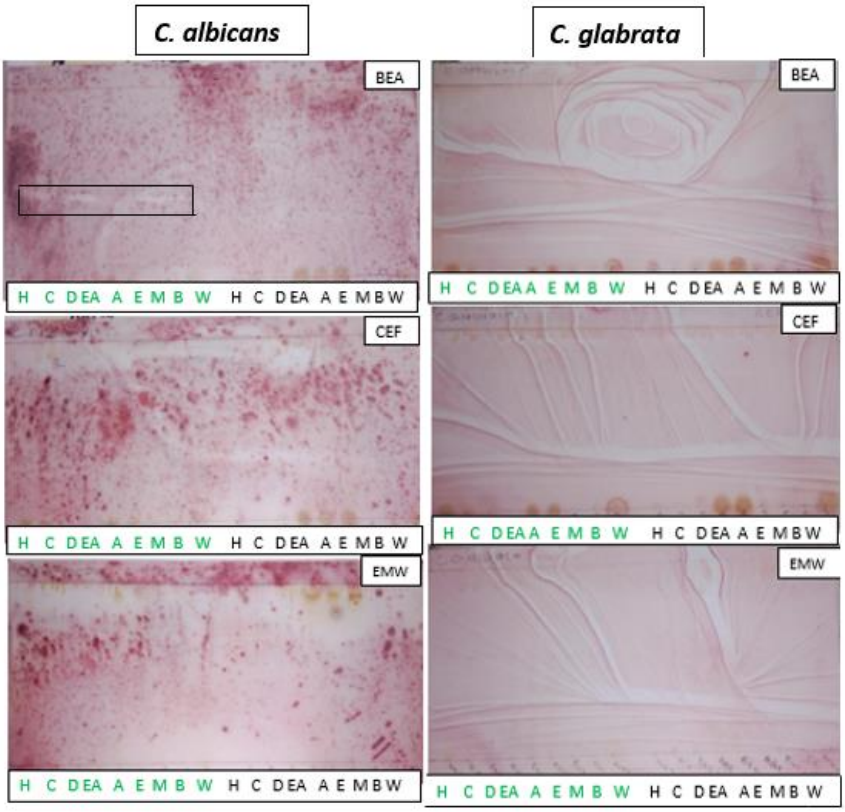
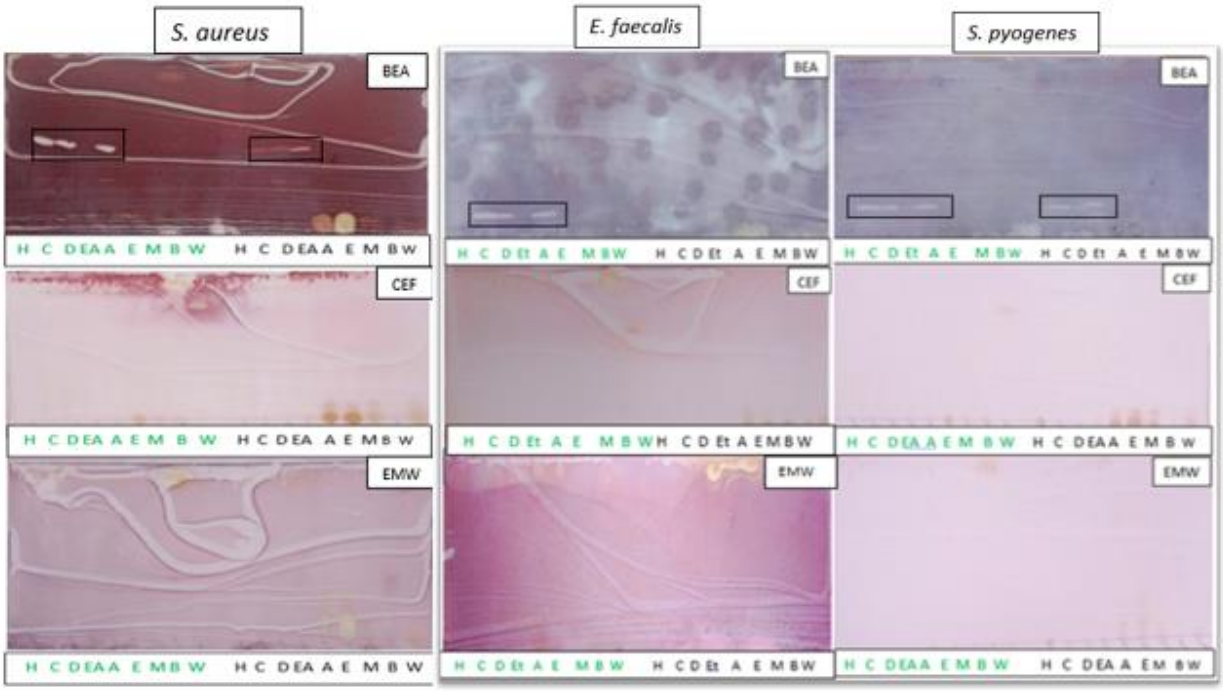


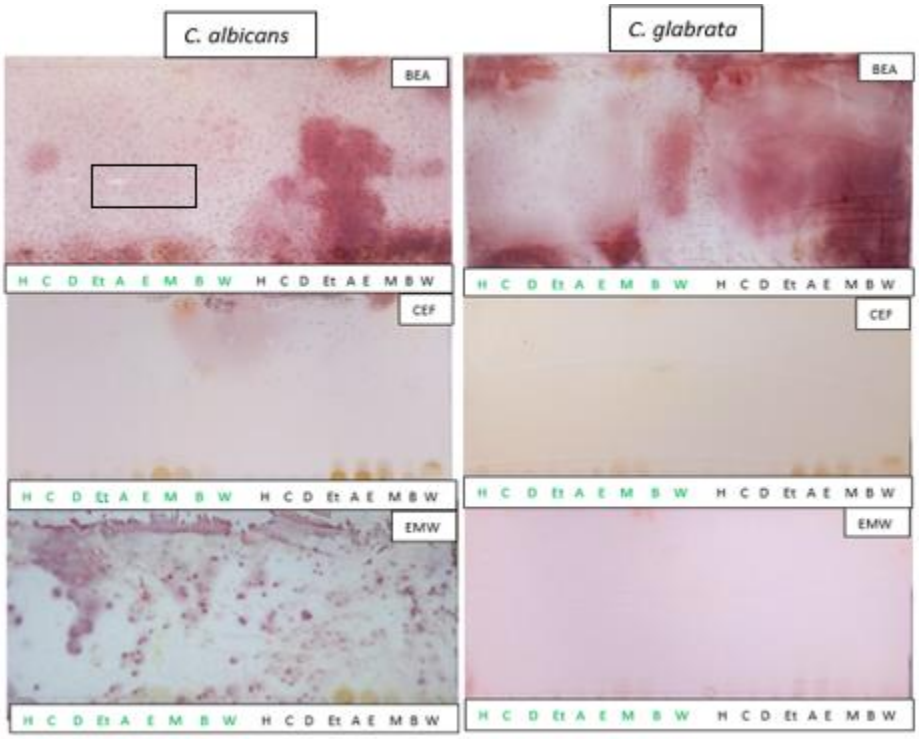
Figure 5.2: Bioautograms of leaf and stem extracts of *C. bispinosa's* extracted with different solvents; (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol and (W) water. The plates were sprayed with *C. albicans* and *C. glabrata* overnight culture and sprayed with 2 mg/ml INT for visualisation.

The serial exhaustive extracts were tested on *S. aureus, S. pyogenes* and *E. faecalis* using the bioautography assay. The marked white bands are zones of inhibition indicating the antimicrobial activity of the separated compounds. Only BEA mobile phase separated the active constituents which are present in chloroform, dichloromethane, and acetone. Antimicrobial activity was observed for *C. albicans* as indicated by the faint spots in figure 5.3 and 5.4 below. No activity detected against *C. glabrata.*



Key; green= leaf extracts; black= stem extracts

Figure 5. 3: Bioautograms of leaf and stem extracts of *C. bispinosa's* extracted serial exhaustively using different solvents; (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol and (W) water. The plates were sprayed with *S. aureus, S. pyogenes* and *E. faecalis* overnight cultures and sprayed with 2 mg/ml INT for visualisation.



Key; green= leaf extracts; black= stem extracts

Figure 5.4: Bioautograms of leaf and stem extracts of *C. bispinosa's* extracted serial exhaustively using different solvents. The plates were sprayed with *C.albicans* and *C. glabrata* overnight culture and sprayed with 2 mg/ml INT for visualisation.

5.3.2. Serial broth microdilution assay

The Serial broth microdilution assay was performed to quantitatively determine the antibacterial and antifungal activity of the extracts. Methanol extract had the lowest MIC value (0.81 mg/ml), making it the most active across the microorganisms and hexane had the highest MIC value (1.63 mg/ml). *C. albicans* demonstrated higher susceptibility with an average of 0.73 mg/ml while *C. glabrata* displayed the least susceptibility (2.8 mg/ml). The total antimicrobial activity was also determined and again, the methanol extract exhibited the highest susceptibility, where it can be diluted to 278.06 ml/g and still inhibit *S. aureus, S. pyogenes* and *C. albicans.* The overall antimicrobial activity was observed in methanol extract 201.10 ml/g.

Table 5.1: Minimum inhibitory concentration (MIC) values of leaf extracts against three bacterial species, two fungal species, gentamicin, and amphotericin.

	MIC (mg/ml)										Gen/Amp
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**	**mg/ml**
S. aureus	1.25	0.63	0.63	2.5	2.5	0.63	0.31	2.5	0.31	**1.25**	0.15
S. pyogenes	1.25	1.25	0.63	1.25	1.25	0.63	0.31	1.25	1.25	**1.01**	0.08
E. faecalis	2.5	2.5	2.5	2.5	0.63	1.25	0.63	1.25	1.25	**1.68**	0.15
C. albicans	0.63	0.63	1.25	1.25	0.63	0.31	0.31	1.25	0.31	**0.73**	0.08
C. glabrata	2.5	2.5	2.5	1.25	1.25	2.5	2.5	2.5	1.25	**2.08**	0.08
Average	**1.63**	**1.50**	**1.50**	**1.75**	**1.25**	**1.06**	**0.81**	**1.75**	**0.87**		

Key: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water, (AVG) average, (Gen) gentamicin and (Amp) amphotericin B.

Table 5.2: Total antibacterial and antifungal activity of the leaf extracts

	Total antimicrobial activity values (ml/g)									
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**
S. aureus	59,28	46,03	83,49	11,12	10,12	119,68	278,06	3,92	270	**97,97**
S. pyogenes	59,28	23,2	83,49	22,24	20,24	119,68	278,06	7,84	66,96	**75,67**
E. faecalis	29,64	11,6	21,04	11,12	26,35	60,32	136,83	7,84	66,96	**41,30**
C. albicans	117,62	46,03	42,08	22,24	40,16	243,23	278,06	7,84	270	**118,58**
C. glabrata	29,64	11,6	21,04	22,24	20,24	30,16	34,48	3,92	66,96	**26,70**
AVG	**59,09**	**27,69**	**50,23**	**88,96**	**23,42**	**114,61**	**201,10**	**6,272**	**148,18**	**79,95**

Keys: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water and (AVG) average.

The stem extracts were analysed to determine the minimum inhibitory concentration and total antimicrobial activity. The average minimum concentration that can inhibit microbial growth was 0.63 mg/ml of ethanol extract and the microorganism that is susceptible to the least concentration of stem extract was *S. pyogenes* inhibited on average by 1.29 mg/ml. Methanol and water extracts were observed to have the highest total antimicrobial activity with 16.11 and 13.27 ml/g, respectively.

Table 5.3: Minimum inhibitory concentration (MIC) values of stem extracts against three bacterial species, two fungal species, gentamicin, and amphotericin B.

	MIC (mg/ml)										Gen/Amp
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**	**Mg/ml**
S. aureus	1.25	5	1.25	2.5	1.25	0.31	2.5	2.5	2.5	**2.17**	0.31
S. pyogenes	5	1.25	0.63	0.63	0.31	0.63	1.25	0.63	1.25	**1.29**	0.15
E. faecalis	1.25	2.5	2.5	1.25	1.25	0.63	1.25	2.5	1.25	**1.60**	0.15
C. albicans	2.5	2.5	2.5	5	0.63	0.31	0.63	2.5	2.5	**2.12**	0.31
C. glabrata	5	5	2.5	2.5	1.25	1.25	2.5	2.5	2.5	**2.78**	0.15
Average	**3**	**3.25**	**1.88**	**2.38**	**0.94**	**0.63**	**1.63**	**2.13**	**2**		

Key: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water, (AVG) average, (Gen) gentamicin and (Amp) amphotericin B.

Table 5.4: Total antibacterial and antifungal activity of the stem extracts

	Total antimicrobial activity (ml/g)									
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**
S. aureus	3.04	0.84	2.32	0.72	4.16	14.19	0.08	1.2	9.48	**4.89**
S. pyogenes	0.76	3.36	4.60	2.86	16.77	6.98	16.16	4.76	18.96	**8.36**
E. faecalis	3.04	1.68	1.16	1.44	4.16	6.98	16.16	1.2	18.96	**6.09**
C. albicans	1.52	1.68	1.16	0.36	8.25	14.19	32.06	1.2	9.48	**7.77**
C. glabrata	0.76	0.84	1.16	0.72	4.16	3.52	8.08	1.2	9.48	**3.32**
Average	**1.82**	**1.68**	**2.08**	**1.23**	**7.50**	**9.18**	**16.11**	**1.91**	**13.27**	

Keys: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water and (AVG) average.

The serial exhaustive extracts of *C. bispinosa* were analysed to determine their antimicrobial activity against five microorganisms using the broth micro dilution assay. The activity is quantified by determining the minimum inhibitory concentration (MIC) values where the smaller the MIC value, the more effective the extract is. Table 5.5 shows the MIC values of leaf extracts, gentamicin, and amphotericin-B against the five pathogens. An average MIC value of 1.06 mg/ml was observed for acetone extract, making it the most effective organism. The most susceptible organism was *E. faecalis* which was inhibited on average by 0.97 mg/ml.

Table 5.5: Minimum inhibitory concentration (MIC) values of leaf serial exhaustive extracts against five bacterial species, two fungal species, gentamicin, and amphotericin-B.

	MIC values (mg/ml)										
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**	**GEN/AMP**
S. aureus	2,5	2,5	2,5	2,5	1,25	1,25	2,5	2,5	2,5	**2,22**	**0,15**
S. pyogenes	0,63	5	1,25	0,63	0,31	0,63	0,63	0,63	1,25	**1,22**	**0,08**
E. faecalis	1,25	0,63	1,25	1,25	1,25	0,63	1,25	0,63	0,63	**0,97**	**0,15**
C. albicans	2,5	2,5	1,25	2,5	1,25	1,25	2,5	2,5	2,5	**2,08**	**0,08**
C. glabrata	5	1,25	1,25	2,5	1,25	2,5	1,25	2,5	1,25	**2,08**	**0**
AVG	**2,38**	**2,38**	**1,5**	**1,88**	**1,06**	**1,25**	**1,63**	**1,75**	**1,63**		

Key: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water, (AVG) average, (Gen) gentamicin and (Amp) amphotericin B.

The MIC value is also used to determine total antimicrobial activity, which is an important measure. It measures the volume to which one gram of plant material can be diluted and still be effective against the tested microorganism. The highest total antimicrobial activity on average was recorded for hexane extract (170.79 ml/g) followed by water extracts (162.28 ml/g) and the least was ethyl acetate extract (1.94 ml/g).

Table 5.6: Total antibacterial and antifungal activity of the leaf serial exhaustive extracts.

Total antimicrobial activity (ml/g)										
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**
S. aureus	100,84	48,96	36,48	1,08	25,36	94,8	47,68	14,6	81,4	**50,13**
S. pyogenes	400,16	24,48	72,96	4,29	102,26	188,10	189,21	57,94	162,8	**133,58**
E. faecalis	201,68	194,29	72,96	2,16	25,36	188,10	95,36	57,94	323,02	**128,98**
C. albicans	100,8	48,96	72,96	1,08	25,36	94,8	47,68	14,6	81,4	**54,19**
C. glabrata	50,42	97,92	72,96	1,08	25,36	47,4	95,36	14,6	162,8	**63,1**
AVG	**170,79**	**82,92**	**65,664**	**1,94**	**40,74**	**122,64**	**95,06**	**31,93**	**162,28**	

Key: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water and (AVG) average.

Table 5.7 shows the MIC results obtained from broth micro dilution assay using the stem serial exhaustive extracts. The lowest minimum inhibitory concentration on average was recorded for methanol and acetone (1.63 mg/ml). The most susceptible pathogen was *C. albicans* (1.11mg/ml).

Table 5.7: Minimum inhibitory concentration (MIC) values of stem extracts against five bacterial species, two fungal species, gentamicin, and amphotericin B.

MIC values (mg/ml)											
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**	**GENT/AMP**
S. aureus	2,5	5	2,5	2,5	2,5	2,5	1,25	2,5	1,25	**2,5**	**0**
S. pyogenes	0,63	2,5	1,25	2,5	1,25	2,5	2,5	2,5	2,5	**2,01**	**0,08**
E. faecalis	2,5	2,5	2,5	5	1,25	1,25	0,63	1,25	0,63	**1,95**	**0,08**
C. albicans	0,63	1,25	0,63	0,63	0,63	1,25	1,25	2,5	1,25	**1,11**	**0,08**

C. glabrata	5	2,5	2,5	5	2,5	2,5	2,5	5	2,5	**3,33**	**0**
AVG	**2,25**	**2,75**	**1,88**	**3,13**	**1,63**	**2**	**1,63**	**2,75**	**1,63**		

Key: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water, (AVG) average, (Gen) gentamicin and (Amp) amphotericin B.

Table 5.8: Total antibacterial and antifungal activity of the stem extracts.

Total antimicrobial activity (ml/g)										
Microorganism	**H**	**C**	**D**	**EA**	**A**	**E**	**M**	**B**	**W**	**AVG**
S. aureus	17,76	8,66	4,8	4,88	5,88	22,96	52,8	9,48	121,36	**27,62**
S. pyogenes	70,48	17,32	9,6	4,88	11,76	22,96	26,4	9,48	60,68	**25,95**
E. faecalis	17,76	17,32	4,8	2,44	11,76	45,92	104,7619	18,96	240,79	**51,61**
C. albicans	70,48	34,64	19,05	19,37	23,33	45,92	52,8	9,48	121,36	**44,05**
C. glabrata	8,88	17,32	4,8	2,44	5,88	22,96	26,4	4,74	60,68	**17,12**
AVG	**37,07**	**19,052**	**8,61**	**6,80**	**11,72**	**32,144**	**52,63**	**10,428**	**120,97**	

Key: (H) hexane, (C) chloroform, (D) dichloromethane, (EA) ethyl acetate, (A) acetone, (E) ethanol, (M) methanol, (B) butanol, (W) water and (AVG) average

5.4. DISCUSSION

The antimicrobial activity of the leaf and stem extracts of *C. bispinosa* was tested in both qualitative and quantitative assays. TLC-bioautography was used for the prior and minimum inhibitory concentration (MIC) for the latter, all the assays iodonitrotetrazolium (INT) were used for indication of microbial growth. INT acts as an electron acceptor and is reduced to a purple-red colour in a reaction with a mitochondrial enzyme of active mitochondrial cells of bacteria (Berridge, 1996).

The qualitative antimicrobial activity is demonstrated in Figures 5.1 to 5.4, where the marked clear bands show zones of inhibitions. That is, there was no reduction of INT and therefore, there was the absence of microbial growth. BEA separated the antimicrobial compounds effectively and the least separation was observed on the polar mobile phase. Most of the inhibition zones were observed on the BEA chromatograms, suggesting that the antimicrobial compound(s) are possibly non-polar. This suggests that the most suitable solvent system for the isolation is a non-polar mobile phase.

All the leaf extracts, except for the water extract, demonstrated antimicrobial activity against bacterial pathogens; *S. aureus, E. faecalis,* and *S. pyogenes. C. albicans* was susceptible to all but hexane, acetone, and water extracts whilst its distant relative *C. glabrata* was not inhibited by any of the extracts, as seen in figure 5.1 to 5.3. The root of *C. bispinosa* is traditionally used to treat toothache and its effectiveness was recently proven by a study conducted by Muleya *et al.* (2014). The presence of antimicrobial compounds in the leaf may lead to the use of the plant leaves rather than the roots for conservation purposes (Jena *et al*., 2017).

Compared to the leaf extracts, lesser activity was observed in the stem extracts. Only *S. aureus* and *E. faecalis* were susceptible to the extracts. BEA chromatograms demonstrated a better separation of the active constituents and antimicrobial activity was observed from it alone. Faint bands of inhibition were observed for hexane and water extracts on the chromatograms sprayed with *S. aureus* while the rest of the extracts exhibited clear bands. Chloroform, dichloromethane, ethyl acetate, acetone, and ethanol extracts were the only ones to inhibit the growth of *E. faecalis*.

The antimicrobial activity was measured quantitatively using the broth microdilution assay to determine the minimum inhibitory concentration of each extract on the five pathogens. An MIC value equivalent to or above 2.5 mg/ml is considered nonsignificant (Ramadwa, 2011). For the leaf extracts, methanol extract had the lowest average MIC value (0.81 mg/ml), making it the most activity across the microorganisms, and hexane had the highest MIC value (1.63 mg/ml). *C. albicans* demonstrated higher susceptibility with an average of 0.73 mg/ml while *C. glabrata* displayed the least (2.8 mg/ml). With most of the extracts showing effectiveness in low concentration and the pathogens demonstrating susceptibility to the extracts, the *C. bispinosa's* leaf may be a suitable source of antimicrobial agent for the treatment of dental pathogens.

From the stem extracts, ethanol extract had an average MIC of 0.63 mg/ml and the microorganism that is susceptible to the least concentration was *S. pyogenes* inhibited on average by 1.29 mg/ml. The total antimicrobial activity was also determined, and methanol extract exhibited the highest, where it can be diluted to 278.06 ml/g and still inhibit *S. aureus, S. pyogenes*, and *C. albicans*. The overall antimicrobial activity was observed in methanol extract 201.10 ml/g. Total antimicrobial activity is a significant value as it measures the volume to which one gram of plant material can be diluted and still be effective against the tested microorganism (Eloff, 1998). Methanol and water extracts were observed to have the highest total antimicrobial activity with 16.11 and 13.27 ml/g, respectively

5.5. Conclusion

The leaf and stem extracts of *C. bispinosa* had antimicrobial activity against the four oral pathogens, namely: *S. aureus, S. pyogenes, E. faecalis* and *C. albicans*. However, leaf extracts exhibited higher activity than stem extracts. The antimicrobial activity was observed on both the bioautography and microdilution assays. It was also observed that the BEA mobile phase separated the compounds better than the other mobile systems.

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CHAPTER 6

6. CYTOTOXICITY TESTS

6.1. INTRODUCTION

Normal cellular metabolism generates Reactive Oxygen Species (ROS) during mitochondrial oxidative metabolism, which plays a significant role in cell signaling (Ray *et al*., 2012; Hemler and Lands, 1980). ROS are also produced in response to cytokines, bacterial invasion, and xenobiotics. Despite their role in cell signaling, ROS may damage the cell severely when there is an imbalance (oxidative stress) between them and antioxidants (Ray *et al*., 2012). Oxidative stress leads to direct or indirect ROS-mediated damage of lipids, nucleic acids, and proteins and has been implicated in diabetes, neurodegeneration, atherosclerosis, carcinogenesis, and aging (Paravicini and Touyz, 2006; Andersen, 2004; Shukla *et al.,* 2011; Trachootham *et al*., 2009; Haigis and Yankner, 2010).

The steroidal and non-steroidal drugs that are used as anti-inflammatory agents have adverse side effects, hence the exploration of medicinal plants as an alternative source is increasing significantly. Medicinal plants have been used for years and are trusted in treating different diseases. However, they have been associated with damage to red blood cells, irritation of the gastrointestinal tract, and damage of the kidney and heart (Nondo *et al.,* 2015). Hence, there is a need to test medicinal plants for cytotoxicity.

The methods for evaluation of anti-inflammatory and cytotoxic activities in plant extracts include enzymatic assays of enzymes involved in the biosynthesis of inflammatory mediators (Jadhav *et al.,* 2013), Nitric oxide (Li and Wang, 2011), and reactive oxygen species production determination (Su *et al*., 2010) and brine shrimp lethality tests (Ashraf *et al*., 2015) and cell line assays (McGaw *et al*., 2007). This chapter aims to evaluate the safety of the extracts by testing for toxicity against THP-1 cell line using MTT calorimetric assay.

6.2. Methods and materials

The MTT calorimetric assay described by Mosmann, (1983) was performed with modifications. The cell culture (THP-1 cell line) was maintained in a flask with RPMI 1640 medium (Whitehead scientific) supplemented with 10% foetal bovine serum (FBS) (Adcock-Ingram). Trypan blue was used to dye the cells and an automatic cell counter (model) was used to quantify viable cells. The cells were diluted with RPMI complete media to obtain 5×10^4 cells/ml cell suspension. Two hundred microliters of the cell suspension were added into each of the wells of the 96 well microtitre plate. The plates were incubated at 37 ºC in a 5% carbon dioxide (CO_2) incubator for 24 h

The stock solutions of the extracts were prepared to a concentration of 250 mg/ml dissolved in dimethyl sulfoxide (DMSO). The extracts were diluted to 1 mg/ml with complete media and a 0.25% of DMSO was maintained. One hundred microliters of the extracts (1000, 500, 250 µg/ml) prepared in a separate 95 well plate was transferred to the plate containing the cell cultures. The microtitre plates were incubated at 37 ºC in a 5% carbon dioxide incubator for 24 h. Following incubation, 20 µL of 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dissolved in 1X PBS was added to each well and the plates were further incubated for 4 h. After incubation, the media was removed from the plates and 100 µL of DMSO was added to each well. The plates were carefully swirled to dissolve the purple formazan crystals. Purple formazan crystals are formed when MTT is reduced by metabolically active cells. Thus, the amount of formed formazan products produced provides an indication of the number of viable cells. A microtitre plate reader (promega) was used to measure the absorbance of the purple colour at 540 nm. Cells treated with the extracts were compared with untreated cells.

6.3. Results

The cytotoxic effects of *C. bispinosa's* acetone extract was evaluated using MTT assay. The percentage viability was decreasing as the concentration of the extract was increasing, as shown in the figure below. However, the highest concentration resulted in more than 50% cell viability.

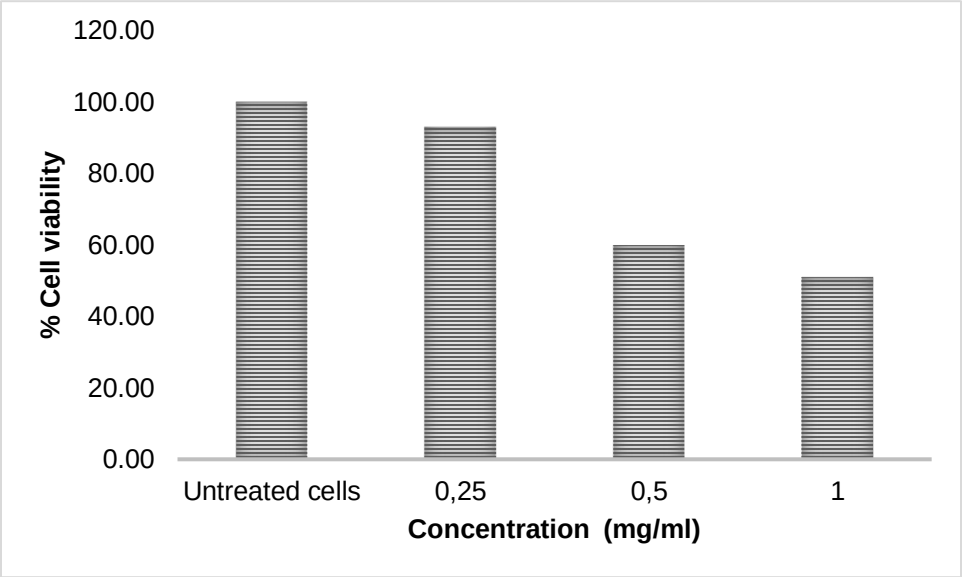

Figure 6.1: Percentage cell viability of *C. bispinosa*'s leaf acetone extract. Untreated cells were used as a positive control.

6.4. Discussion

A pharmaceutical drug should have minimal to no toxicity; thus, cytotoxicity studies should be the initial step in drug development. Cellular toxicity studies play a crucial role in determining the toxicity of plant extracts or biologically active compounds isolated from plant (McGaw, 2014). In this study, the cytotoxic effects of *C. bispinosa*'s acetone extract was evaluated using MTT assay. MTT assay is widely used to determine antimicrobial activity. This calorimetric assay is based on the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Maehara *et al.,* 1987), which results in a colour change from yellow to purple. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which carry the reduction reaction of MTT to formazan. A solubilisation solution is used to dissolve formazan crystals resulting in a coloured solution that can be quantified by measuring absorbance at 500-600 nanometers using a multi-well spectrophotometer (Berridge and Tan, 1993). The percentage cell viability was decreasing as the concentration of the extract was increasing, as shown in figure 6.1. However, the highest concentration resulted in more than 50% cell viability. The International Organization for Standardization established that cell viability above 80% is considered as non-cytotoxic

whereas within 80%–60% is considered weak; 60%–40% moderate and below 40% strong cytotoxicity, respectively (López-García *et al.,* 2014). The percentage cell viability ranged from 51% to 93% from the highest to the lowest concentration of the acetone extract. At a concentration of 0.25 mg/ml, the extract exhibited acceptable percentage cell viability levels and as the concentration was increasing, the toxicity rose to moderate levels.

6.5. Conclusion

The leaf extract of *C. bispinosa* exhibited a concentration dependant toxicity against the cell culture (THP-1 cell line). Despite the toxicity of the extract increasing with the increase in concentration, the highest concentration (1mg/ml) resulted in cell viability of more than 50%.

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CHAPTER 7

7. Bioactivity guided isolation of antimicrobial compounds

7.1. Introduction

Traditionally, herbal medicine is used as prescribed by a traditional healer or an herbalist and the knowledge of the specification of plants to different diseases is passed from generation to generation through folklore (Nondo *et al.,* 2015). Various forms of herbal medicine are prepared: liquids, liniments, ointments, powders, raw mixtures, boiled, and incisions (Bakht *et al*., 2013). In all the forms of remedy preparation, traditional healers prepare herbal medicine without any scientific testing or analysis (Zhou *et al*., 2009). Moreover, a phytochemical can demonstrate an antimicrobial activity on its own or when in combination with other phytochemicals (Richards *et al.,* 2016). Thus, it is necessary to isolate the active constituent(s) of a plant extract to formulate a novel drug.

The isolation step is guided by the bioactivity of the extract and is thus called bioassay-guided fractionation. Bioassays that are inexpensive, simple, and quick are used to screen for active constituents. After determining the activity, separation techniques such as column chromatography, thin-layer chromatography (TLC), flash chromatography, Sephadex chromatography, and high-performance liquid chromatography are used to isolate the compound. Thin-layer chromatography is used to determine if the compound is pure and can also be used to determine the identity of the compound in a mixture where the retention factor (Rf) of the test compound is compared to that of a known compound (Sasidharan *et al.,* 2011).

7.2. Methods and materials

7.2.1. Serial exhaustive extraction

The leaf extracts of *C. bispinosa* had a promising antimicrobial compared to the stem extracts, therefore, they were chosen for large-scale extraction and the subsequent isolation of bioactive compound(s). A mass of 0.6 Kg was extracted with 6 L of 4 different solvents ranging from non-polar to polar, namely, n-hexane, dichloromethane, acetone, and methanol. The serial exhaustive method was employed, where each solvent was left

to shake at 200 rpm overnight in the first batch followed by 2 h, and lastly, for 1 h. The extracts were filtered and concentrated using a rotary evaporator (Buchi B-490) and transferred to pre-weighed 250 ml beakers. The remaining solvent was evaporated using a stream of cold air at room temperature and the total mass extracted was determined as the difference in the mass of the beaker with the extract and the pre-weighed beaker.

7.2.2. Phytochemical analysis on TLC

The chemical profiles of *C. bispinosa* extracts were determined on aluminium-backed TLC plates (Merck, silica gel 60 F254) using a method developed by Kotze and Eloff, (2002), as described in section 3.2.3.

7.2.3. TLC-DPPH assay

Qualitative DPPH assay, using thin-layer chromatography was done according to the method described by Deby and Margotteaux (1970), as explained in section 4.2.1.

7.2.4. Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972), as described in section 5.2.2.1.

7.2.5. Serial broth microdilution assay

The broth micro-dilution method described by Eloff (1998) was used to determine the minimum inhibitory concentrations (MIC) values of crude extracts against four tested bacterial species, as explained in section 5.2.3.1.

7.2.6. Isolation of active compounds

7.2.6.1. First open column chromatography

The results of the bioassays performed on the serial exhaustive extracts were used to guide the chromatographic fractionation of the compounds using column chromatography. An open column (39 x 3,5 cm) was packed with silica gel 60 (particles

size 0.063-0.200 mm) (Fluka) using 100% n-hexane. Dichloromethane and acetone extracts demonstrated antimicrobial activity on bioautography and MIC assay and were thus ground into fine powder and mixed. The mixture was spread on top of the silica gel and a piece of cotton was placed on top to absorb the pressure when the solvents were poured. The compounds were eluted with solvent mixtures of different proportions, as shown on table 7.1. The collected fractions were concentrated using a rotary evaporator, which was dried and weighed. The fractions were tested for their chemical profile, antioxidant, and antimicrobial activity.

Table 7.1: Solvent systems used in the first open column chromatography

Solvent system	**Percentage (%)**
n-Hexane ,	100
n-Hexane: Ethyl acetate	90
	80
	70
	50
	30
	10
Ethyl acetate	100
Ethyl acetate: Methanol	90
	80
	70
	60
	50
	40
Methanol	100

7.2.6.2. Second open column chromatography

The active fractions collected from first column chromatography, which ranged from 50% n-hexane in ethyl acetate to 80% of ethyl acetate in methanol, were mixed to form a sub-fraction and finely ground and then subjected to open column chromatography for further

fractionation. An open column (38 x 3,5 cm) was packed with silica gel 60 (particles size 0.063-0.200 mm) (Fluka) using 50% n-hexane in ethyl acetate. The sample was spread on top of the silica gel, a piece of cotton was placed on top of the sample and the compounds were eluted with 50% n-hexane in ethyl acetate. The sub-fractions were collected with test tubes and concentrated through cold air and tested on TLC plates for bioactive compounds.

7.2.6.3. Third open column chromatography

The fractions that contained the active compound were combined into one target fraction and subsequently subjected to open column chromatography. The column (31 x 4 cm) was packed with silica gel 60. The active compound was eluted with n-hexane: ethyl acetate (70:30) and the eluents were collected in test tubes. The eluents were concentrated under a stream of air and subjected to TLC for further analysis.

7.2.6.4. Fourth open column chromatography

The fractions with a similar profile were combined and subjected to a small open column. The column was packed with silica gel 60 (particles size 0.063-0.200 mm) (Fluka) using n-hexane: ethyl acetate (70:30). The sub-fractions were collected in test tubes and concentrated under the stream of cold air and tested on TLC plates for bioactive compounds.

7.2.6.5. Preparative-TLC

The sub-fractions (test tube number 90-160) from the fourth open column chromatography were mixed and subjected to separation on TLC silica gel glass plates (Merck Silica gel 60 F254) using BEA. Since the compound was not UV active, a small portion of the TLC plate with the rest of the plate covered was sprayed with vanillin-sulphuric acid to visualise the target compound and determine its Rf value. The Rf value was used as a reference to trace the compound of interest and subsequently scraped off from the developed TLC plates. To separate the compound from the silica, it was immersed in ethyl acetate and filtered using cotton wool. The isolated compound was

tested on TLC for purity and bioautography for antimicrobial activity with *S. aureus* as test organism.

7.3. Results

7.3.1. Serial exhaustive extraction

A mass of 0.6 Kg *C. bispinosa* leaf powder was subjected to serial exhaustive extraction using n-hexane, dichloromethane, acetone, and methanol, respectively, as solvents. A total of 143.3345 g was obtained from the four solvents with methanol extracting the highest mass (88.7508 g) and acetone extracting the lowest mass (14.524 g).

Table 7.2: The mass of extracts obtained after performing serial exhaustive extraction with solvents of increasing polarity.

Extracts		**Mass residue (g)**	
		Mass	**Total**
n-Hexane	I	16.2684	21.79
	II	3.9548	
	III	1.5665	
Dichloromethane	I	10.6174	18.27
	II	4.7566	
	III	2.896	
Acetone	I	9.2569	14.52
	II	3.4341	
	III	1.833	
Methanol	I	67.5733	88.75
	II	15.833	
	III	5.3445	
	Total		**143.33**

7.3.2. Phytochemical analysis on TLC

Serial exhaustive extraction was done on *C. bispinosa's* leaf powder using four solvents of increasing polarity (n-hexane, dichloromethane, acetone, and methanol). The compounds were separated on TLC with BEA, CEF and EMW mobile systems and the Chromatograms were sprayed with vanillin sulphuric acid for visualisation. Different compounds are indicated by the bands and the different colours, better separation was observed CEF and EMW mobile phases.

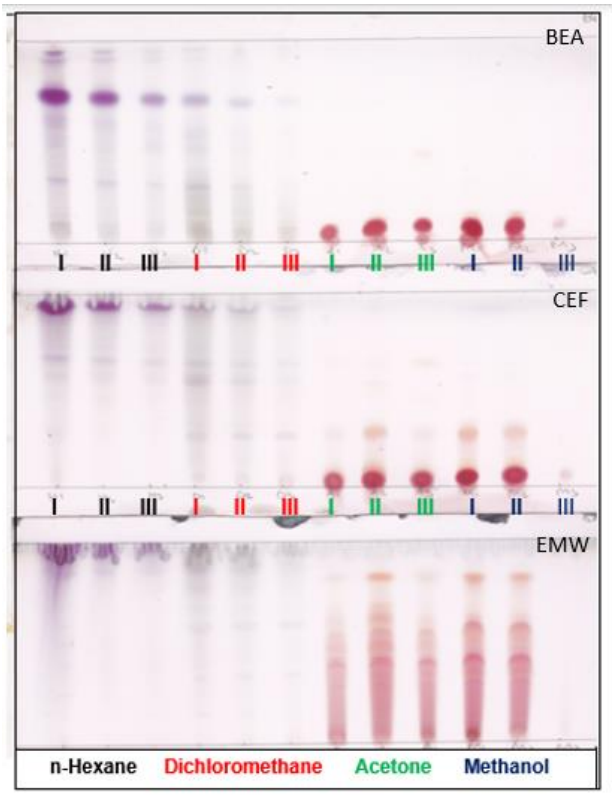

Figure 7.1: The phytochemical profile of *C. bispinosa*'s leaf extracts extracted with n-hexane, dichloromethane, acetone, and methanol. The chromatograms were sprayed with vanillin-sulphuric acid and observed.

7.3.3. TLC-DPPH assay

Chromatograms of n-hexane, dichloromethane, acetone, and methanol extracts were developed on BEA, CEF and EMW mobile phases. A solution of 0.2% DPPH in methanol was sprayed on the chromatograms for visualisation of antioxidant compounds where

yellow spots against a purple background represent antioxidant activity. Antioxidant compounds were detected on the acetone and methanol extracts and they were best separated on the EMW mobile system.

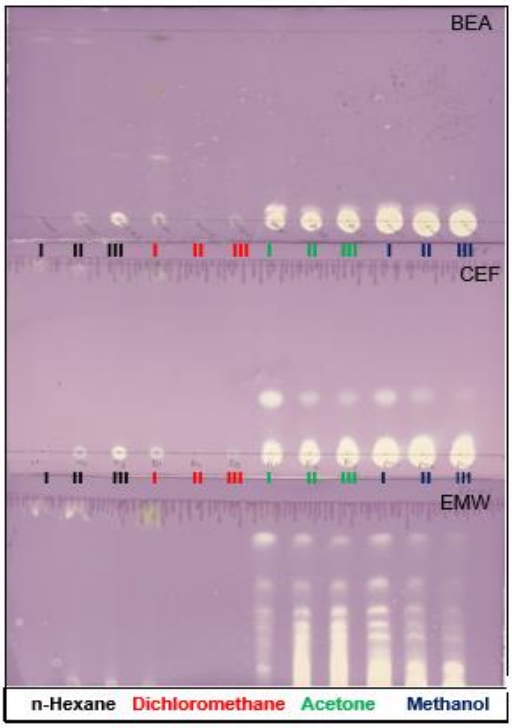

Figure 7.2: The antioxidant activity of the serial exhaustive extracts, extracted with solvents of increasing polarities. The chromatograms were sprayed with DPPH dissolved in methanol and observed.

7.3.4. Bioautography assay

Bioautography assay was performed to determine the antimicrobial activity of the n-hexane, dichloromethane, acetone, and methanol extracts. The chromatograms were developed in BEA, CEF and EMW mobile phases. Microbial cultures of *S. aureus, E. faecalis, S. pyogenes, C. albicans,* and *C. glabrata* were sprayed on the chromatograms and incubated overnight. INT was sprayed on the chromatograms for visualisation where a white band against a pink background indicates a zone of inhibition. Dichloromethane extracts had antimicrobial activity against all the five pathogens. Acetone extracts were effective against all pathogens except *C. albicans.*

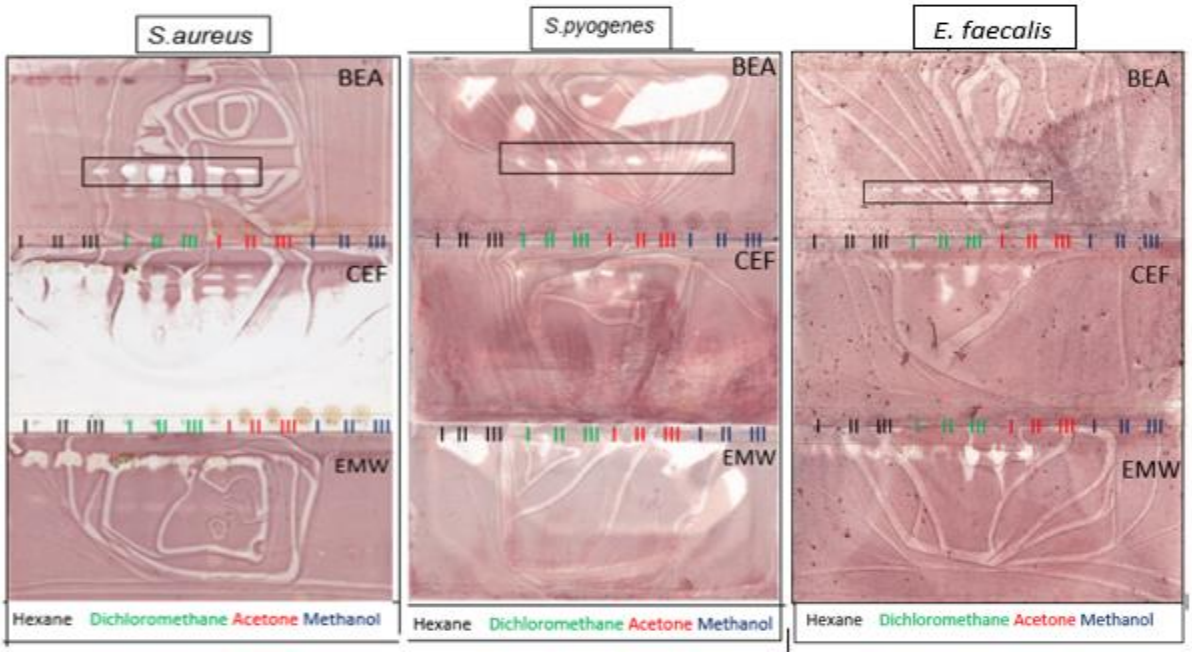

Figure 7.3: Bioautograms of *C. bispinosa*'s leaf serial exhaustive extracts developed in BEA, CEF and EMW mobile system. The chromatograms were sprayed with 24 h culture of S*. aureus, S. pyogenes* and *E. faecalis*, then incubated overnight, sprayed with INT, and observed.

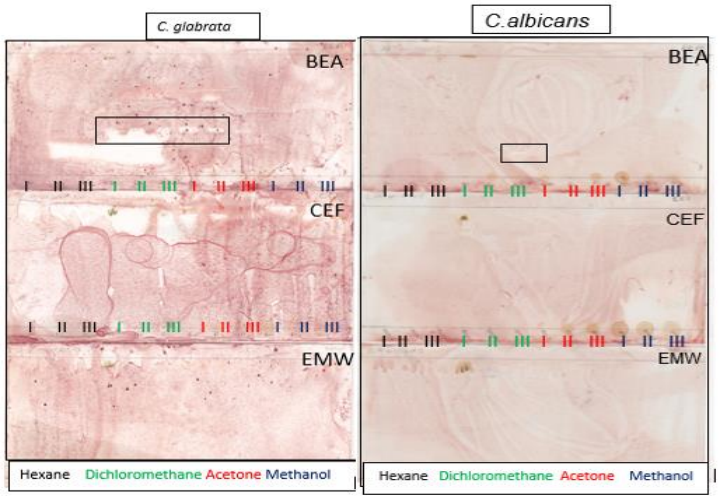

Figure 7.4: Bioautograms of *C. bispinosa's* leaf extracts developed in BEA, CEF and EMW mobile phases. The chromatograms with *C. glabrata* and *C. albicans* were sprayed with 2 mg/ml for visualisation.

7.3.5. Serial broth microdilution assay

Serial broth microdilution assay was used to determine the minimum inhibitory concentration of the n-hexane, dichloromethane, acetone, and methanol extracts. Acetone extracts exhibited the lowest MIC value on average (0.46 mg/ml) and the most susceptible pathogens were *S. aureus* and *E. faecalis* inhibited on average by (1.28 mg/ml).

Table 7.3: The MIC values of *C. bispinosa*'s leaf extracts, extracted with four solvents of increasing polarity and tested on *S. aureus, E. faecalis, S. pyogenes, C. albicans,* and *C. glabrata*.

Microorganisms	Extractants													AMP
	n-Hexane			**Dichloromethane**			**Acetone**			**Methanol**			**AVG**	(mg/ml)
	I	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**		
S. aureus	1.25	0.63	0.63	0.31	1.25	1.88	0.63	1.25	1.25	1.25	2.5	2.5	**1.28**	0.15
E. faecalis	1.25	0.63	0.94	0.94	1.25	1.25	0.63	0.94	1.25	1.88	2.5	1.88	**1.28**	0.08
S. pyogenes	1.25	0.63	0.94	0.31	1.25	1.25	0.63	1.25	1.88	2.5	1.88	1.88	**1.30**	0.15
C. albicans	1.25	1.25	0.94	0.47	0.94	1.25	0.94	1.88	0.94	1.88	2.5	2.5	**1.40**	0.15
C. glabrata	1.88	1.25	1.25	0.94	1.25	1.25	0.94	1.25	1.25	2.5	2.5	2.5	**1.56**	0.15
Average	1.38	0.88	0.94	0.59	1.19	1.38	0.75	1.31	1.31	2.00	2.38	2.25		
Total average	**1.07**			**1.05**			**0.46**			**2.21**				

The dichloromethane and acetone extracts had a promising antimicrobial activity and were chosen for first column chromatography as thus. An open column (35 × 4 cm) was packed with silica gel 60 (particles size 0.063 - 0.200 mm) (Fluka) using 100% n-hexane. Various eluent systems were used and 70% ethyl acetate in methanol eluted the highest mass (4.094 g) followed by 30% n-hexane in ethyl acetate (2.071 g). The lowest mass (0.034 g) was eluted by 100% n-hexane.

Table 7.4: The masses of fractions from fractionation of the dichloromethane and acetone extracts.

Numbering	**Solvent system**	**Percentage (%)**	**Mass (g)**
1	n-Hexane (1)	100	0.050
2	n-Hexane (2)	100	0.034
3	n-Hexane: Ethyl acetate	90	0.037
4		80	0.383
5		70	0.501
6		50	1.550
7		30	2.071
8		10	1.663
9	Ethyl acetate	100	1.143
10	Ethyl acetate: Methanol	90	1.529
11		80	1.882
12		70	4.094
13		60	0.980
14		50	1.431
15		40	0.795
16	Methanol	100	0.461
	Total		**18.604**

The phytochemical profile of the first column chromatography fractions was determined using TLC. The chromatograms were developed on BEA, CEF and EMW mobile phases and observed under UV light at 254 and 366 nm. The plates were further sprayed with vanillin sulphuric acid to visualise non-fluorescing compounds. At 254 nm, better separation was observed on the EMW mobile system while at 366 nm BEA had better resolution.

Figure 7. 5: Chemical profile of fractions collected from the first open column chromatography and separated on BEA, CEF and EMW mobile phases. The chromatograms were observed under UV light at 254 and 366 nm, respectively. The plates were further sprayed with vanillin sulphuric acid to visualise non-fluorescing compounds.

The TLC-DPPH assay was employed to analyse the antioxidant activity of the first column chromatography fractions. The chromatograms were sprayed with 0.2% DPPH in

methanol and the yellow bands on a purple background represent antioxidant activity. Antioxidant activity was observed from the fractions eluted with 90% ethyl acetate in methanol to 100% methanol, however, the antioxidant compounds were only separated on the EMW mobile phases.

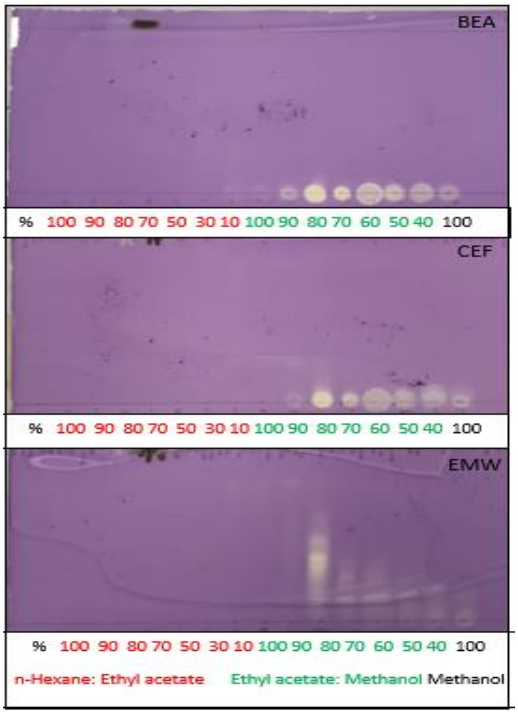

Figure 7. 6: Antioxidant activity of first column chromatography fractions isolated with various eluent systems obtained after performing TLC-DPPH assay. The chromatograms were developed on BEA, CEF and EMW mobile phases and sprayed with 0.2% (w/v) of DPPH.

The antimicrobial activity of the first column chromatography fractions was determined using bioautography assay. Figure 7.7 below shows the bioautograms of the fractions obtained from the first column chromatography and tested against *E. faecalis*, *S. aureus* and *S. pyogenes.* The white bands on a pink background observed on the BEA mobile phase represent antimicrobial activity. The active fractions ranged from 50% n-hexane in ethyl acetate to 80% of ethyl acetate in methanol on the *E. faecalis* and *S. aureus* bioautograms**.**

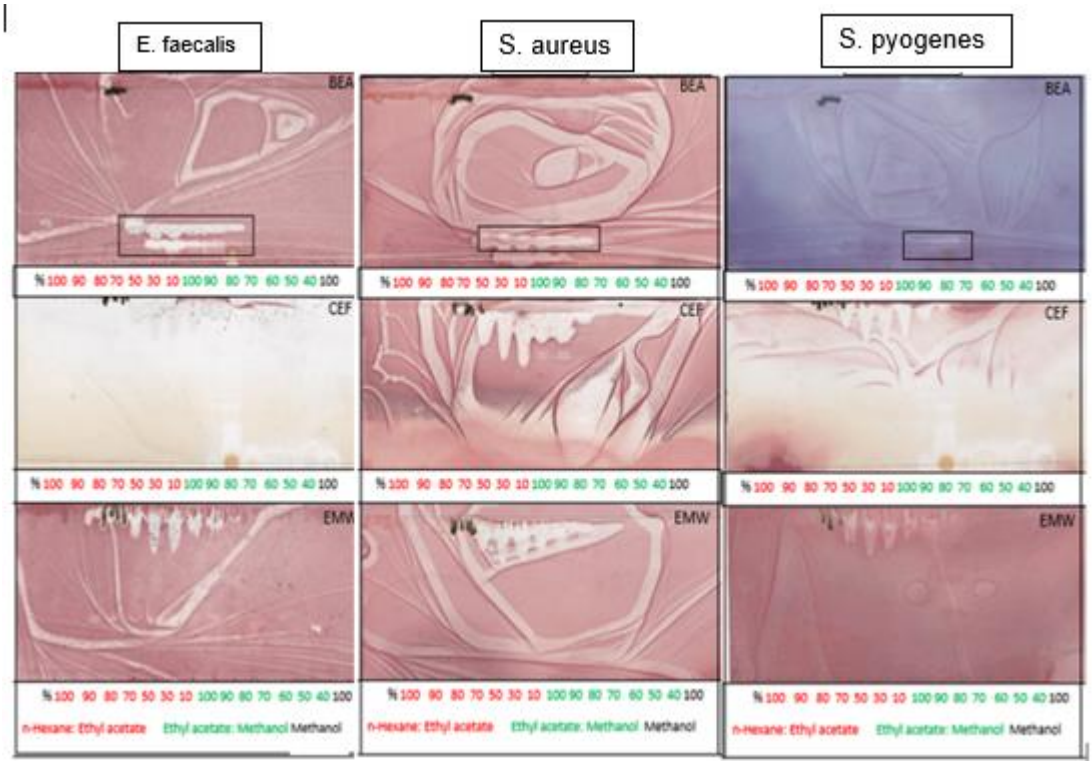

Figure 7.7: Antimicrobial activity of fractions obtained from the first column chromatography. The chromatograms were developed on BEA, CEF and EMW mobile systems and sprayed with *E. faecalis, S. aureus* and *S. pyogenes* overnight cultures.

Figure 7.8 below shows the bioautograms of the first column chromatography fractions. The chromatograms were developed on the BEA, CEF and EMW mobile phases and tested against *C. albicans* and *C. glabrata.* The active fractions ranged from 50% n-hexane in ethyl acetate to 80% of ethyl acetate in methanol on the *C. albicans.*

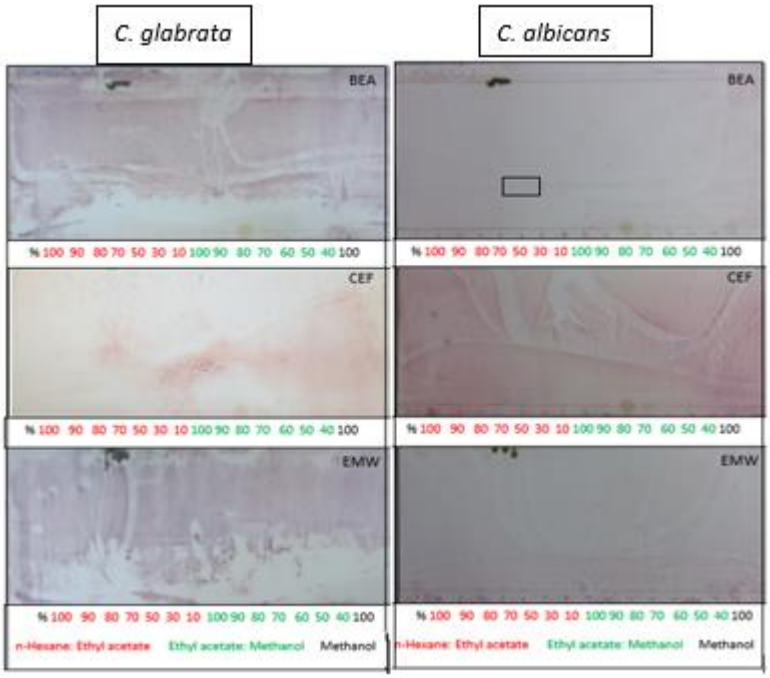

Figure 7.8: Bioautograms of the first column chromatography fractions. The chromatograms were developed on the BEA, CEF and EMW mobile phases and tested against *C. albicans* and *C. glabrata*.

Table 7.5: MIC values of the fractions obtained from first column chromatography and tested on the five pathogens.

Microorganisms	**MIC values (mg/ml)**																**Amp/ Gen (mg/ml)**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Avg**	
S. aureus	>2.5	>2.5	>2.5	2.5	2.5	0.84	1.25	0.84	0.52	1.04	0.84	2.5	1.46	>2.5	2.5	2.08	**1.57**	0..08
E. faecalis	>2.5	>2.5	>2.5	1.88	2.5	0.63	0.52	0.84	1.04	0.63	0.73	1.46	1.88	1.46	2.5	2.5	**1.43**	0.08
S. pyogenes	>2.5	>2.5	>2.5	>2.5	2.08	1.67	1.46	1.88	1.04	1.46	1.67	2.08	2.5	2.08	>2.5	2.5	**1.70**	0.08
C. albicans	>2.5	>2.5	>2.5	2.08	1.46	1.04	2.08	1.46	1.25	1.67	1.04	2.08	2.08	2.5	2.08	>2.5	**1.74**	0.31
C. glabrata	>2.5	>2.5	>2.5	2.5	>2.5	1.46	1.88	1.67	1.04	2.08	1.04	2.08	2.08	>2.5	2.08	2.5	**1.86**	0.16
Average	**>2.5**	**>2.5**	**>3.5**	**2.24**	**2.14**	**1.88**	**1.47**	**1.34**	**0.98**	**1.41**	**1.06**	**2.04**	**2.0**	**2.01**	**2.29**	**2.40**		

7.3.6. Second column chromatography

The antimicrobial active fractions obtained from first column chromatography were mixed and subjected to further purification in second column chromatography. Various combinations of n-hexane and ethyl acetate were tested using TLC and bioautography to determine the best solvent system for second column chromatography. The 50:50 combination was chosen as the most suitable.

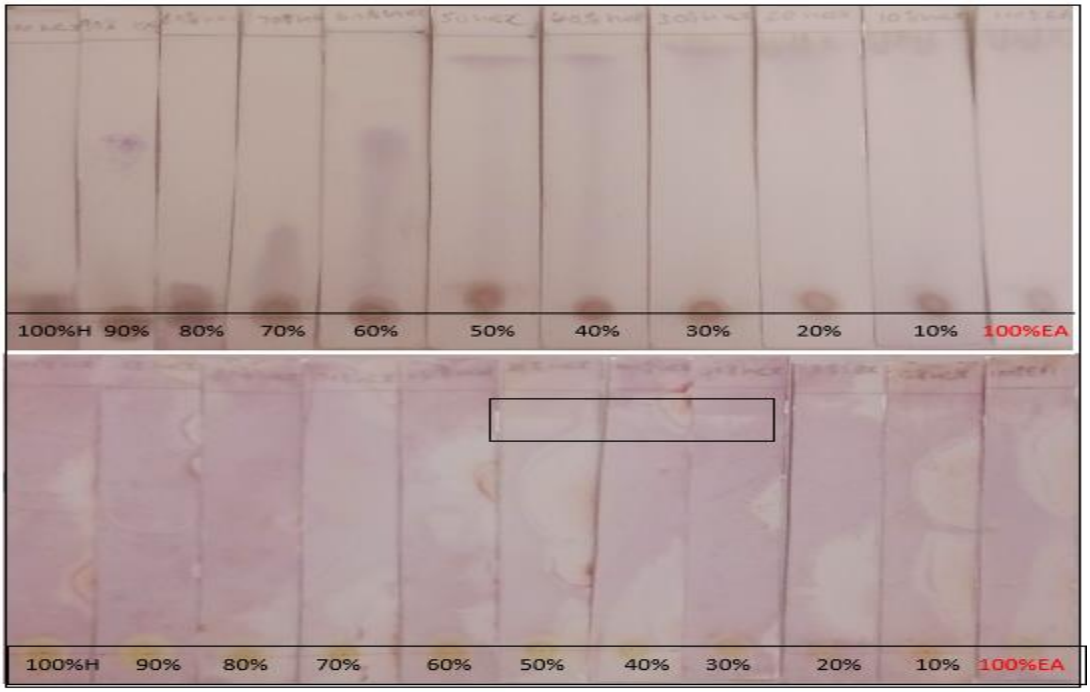

Figure 7.9: Strips of chromatograms developed in different solvent combinations of n-hexane and ethyl acetate. The chromatograms were sprayed with vanillin-sulphuric acid. The bioautomatograms were sprayed with *S.aureus* overnight culture.

The active sub-fractions ranging from 50% n-hexane in ethyl acetate to 80% of ethyl acetate in methanol in the first column were mixed and subjected to second column chromatography where they were eluted with 50% n-hexane in ethyl acetate. The sub-fractions were collected with test tubes and tested on TLC to trace bioactive compound(s).

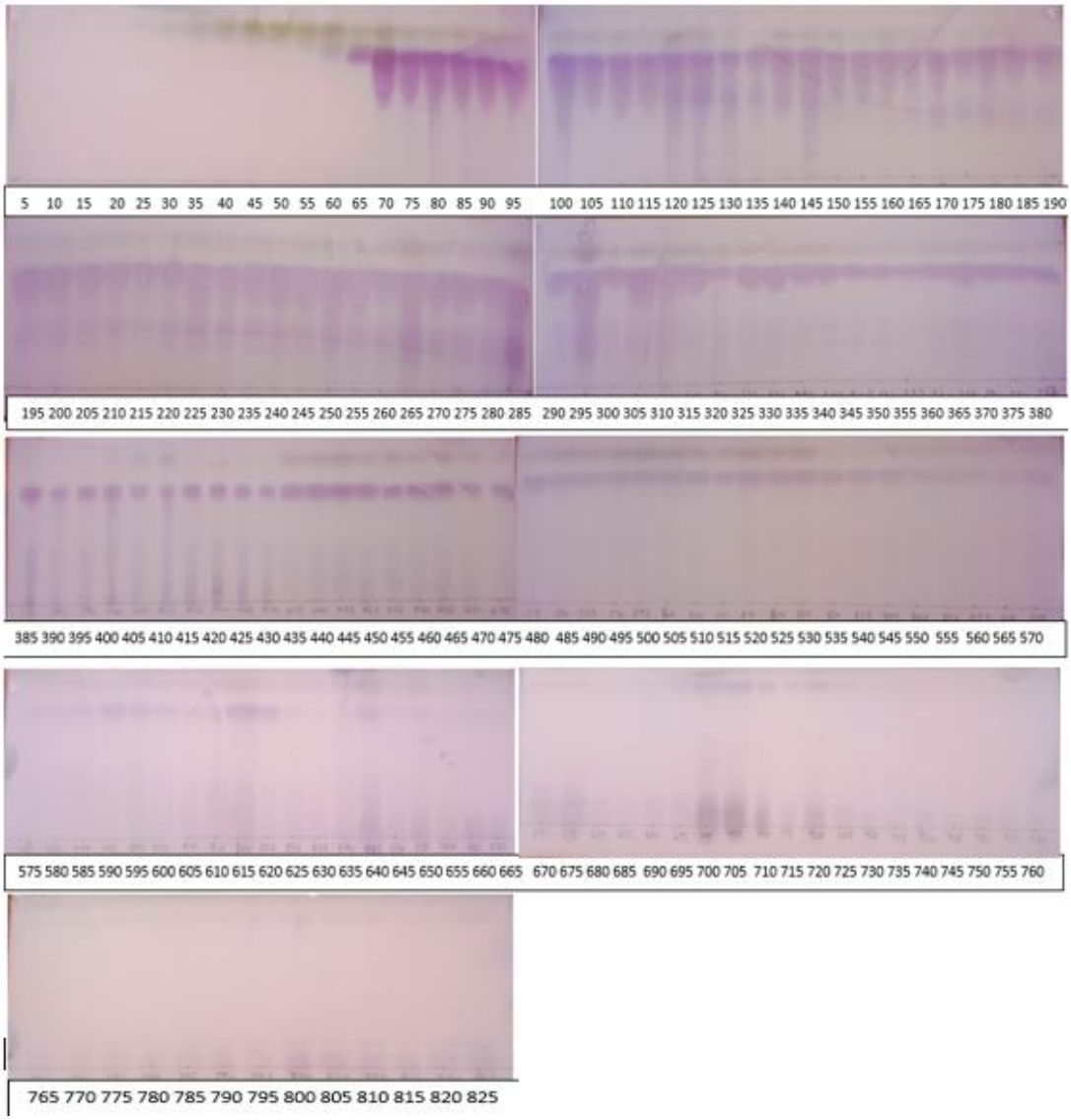

Figure 7.10: The chromatograms of the second column chromatography fractions ran with n-hexane and ethyl acetate in a 50:50 ratio mobile phase.

The second column chromatography sub-fractions with similar chemical profile were mixed into ten fractions. The factions were subjected to TLC where BEA, CEF and EMW were used as mobile phases. Bioautography assay was also performed to determine which fractions had antimicrobial activity and trace the band with bioactive compound(s). The compounds were better separated by the BEA mobile system, as shown in figure 7.11 below, where two prominent bands are observed. Antimicrobial active compounds

were detected on fractions 1-7 and the most prominent band observed in figure 7.15 contained the bioactive compound(s).

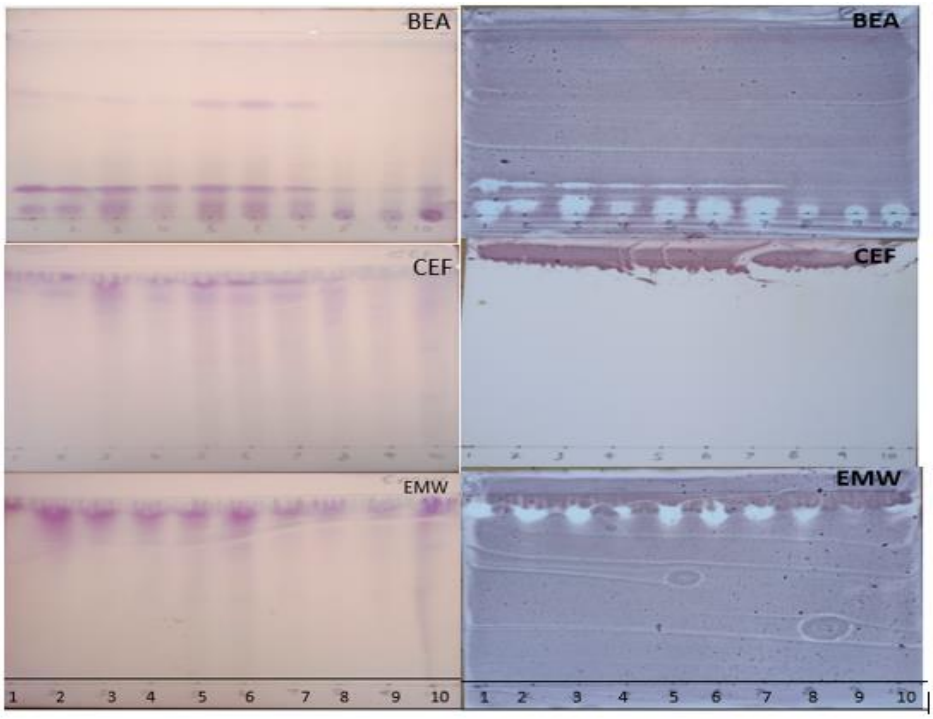

Figure 7.11: The chemical profile and bioautograms of the 10 sub-fractions obtained after performing second column chromatography developed in BEA, CEF and EMW mobile phases. The plates were sprayed with vanillin-sulphuric acid for visualisation while the bioautograms were sprayed with *S.aureus* overnight culture.

7.3.7. Third column chromatography

The seven active fractions obtained from the second column chromatography were mixed and subjected to the third column chromatography for further purification. Various combinations of n-hexane and ethyl acetate were investigated to determine the most suitable mobile phase for the column chromatography. The 70% n-hexane in ethyl acetate combination was selected.

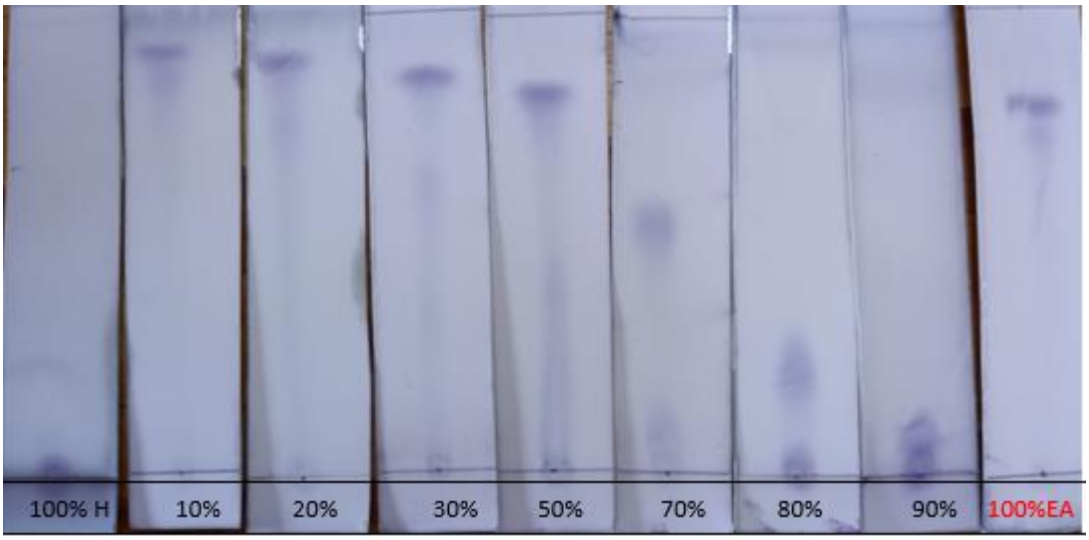

Figure 7.12: Strips of chromatograms developed in different solvent combinations of n-hexane and ethyl acetate, the chromatograms were sprayed with vanillin-sulphuric acid.

The seven active fractions obtained from the second column chromatography were mixed to and subjected to the third column chromatography for further purification. A mobile phase consisting of 70% n-hexane in ethyl acetate was used and the fractions were collected using 400 test tubes. Figure 7.13 below shows the chemical profile of the fractions after running TLC and using BEA as a mobile system. The plates were sprayed with vanillin-sulphuric acid in methanol to visualise the compounds. The compound of interest is not pure as there are three distinct profiles where more than one band can be observed on the chromatograms, as seen from test tube 70 to 120, 125 to 300 and 305 to 400.

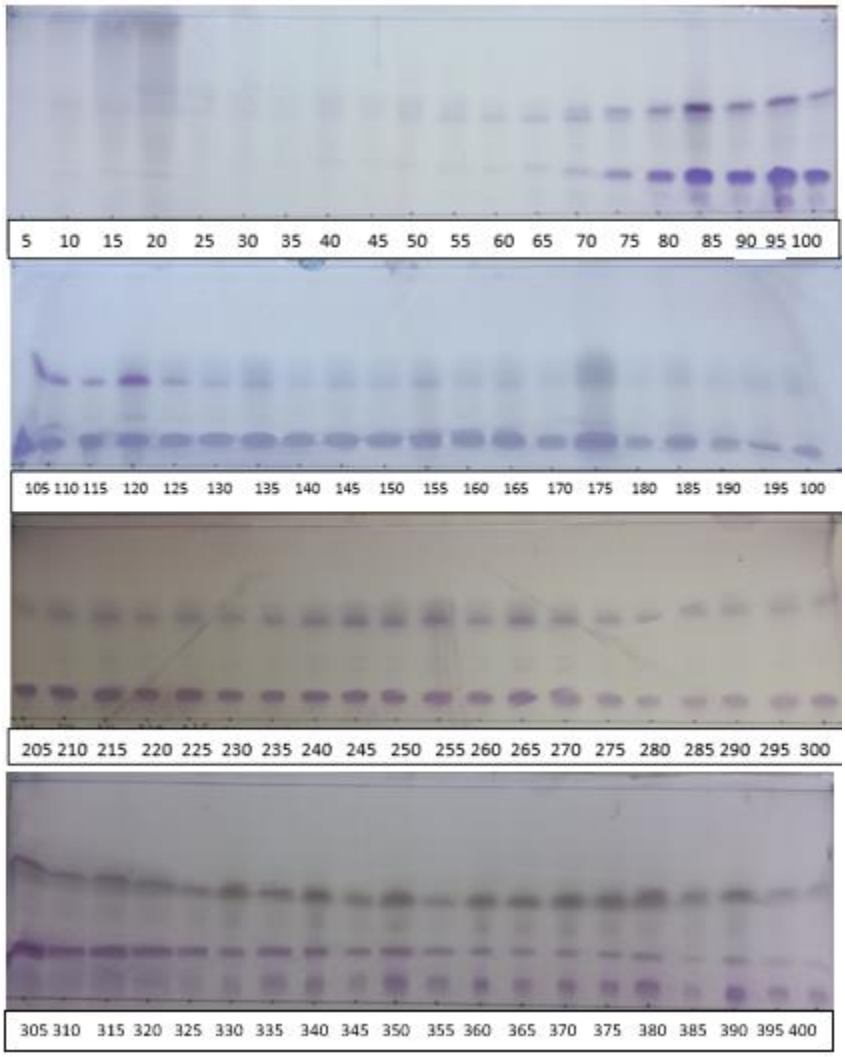

Figure 7.13: Phytochemical analysis of third column chromatography fractions. The chromatograms were developed on the BEA mobile system and sprayed with vanillin-sulphuric acid for visualisation.

Similar fractions from the third column chromatography were pooled together to form three fractions, namely, A, B and C. TLC was run to compare the profiles of the three fractions, fraction A and B were similar as observed in figure 7.14.

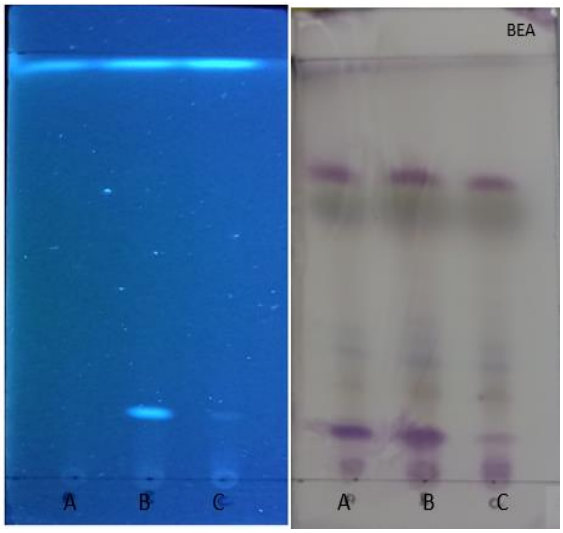

Figure 7.14: The chromatograms of the third column chromatograms pooled together, the plate was developed in BEA mobile system and sprayed with vanillin-sulphuric acid for visualisation.

7.3.8. Fourth column chromatography

To determine the most suitable solvent for elution of the target compound in the fourth open column chromatography, different ratios of n-hexane and ethyl acetate solvent systems were prepared and used to run strips of TLC plates spotted with the fractionated compound. The 70% n-hexane in ethyl acetate solvent system was selected.

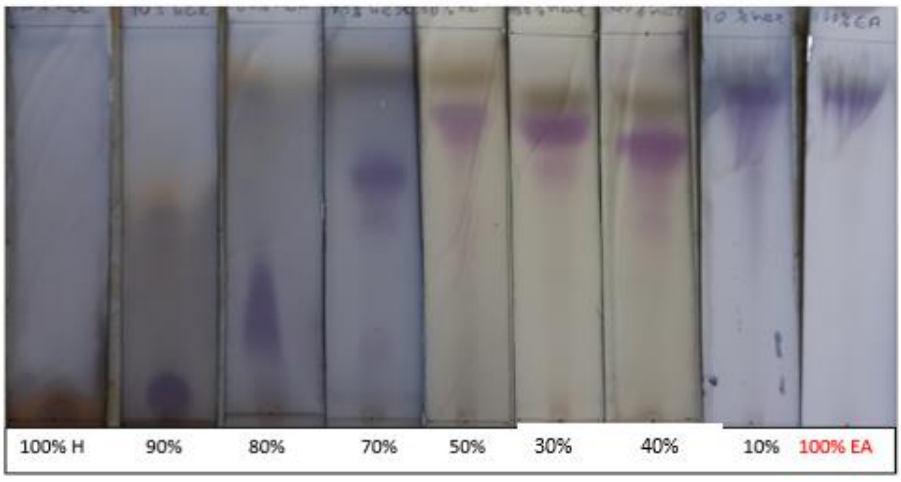

Figure 7.15: Determination of suitable mobile system for elution of target compound in open column chromatography.

For further fractionation of the target compound, Fraction A and B from the third open column chromatography were mixed and subjected to the fourth open column chromatography using 70% n-hexane in ethyl acetate as a solvent system. The fractions were collected in 200 test tubes and the target (purple) compound was detected from test tube number 90 to 160 as seen on figure 7.16 below. However, the compound was not pure and needed further fractionation.

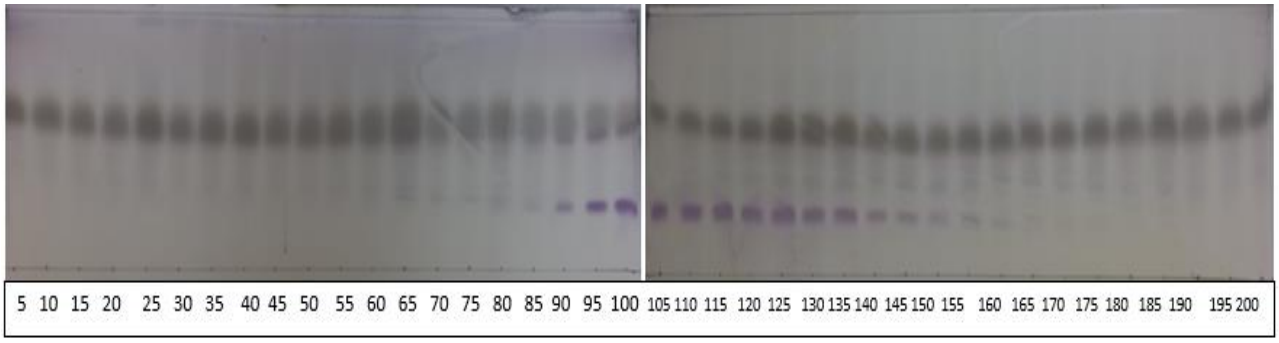

Figure 7. 16: Phytochemical profile of the fourth open column chromatography fractions. The plates were developed on BEA mobile phase and sprayed with vanillin-sulphuric acid for visualisation.

The plate with the purple compound was analysed under UV at 254 and 365 nm to determine if it is UV active. The compound of interest could only be traced after spraying the plates with vanillin-sulphuric acid.

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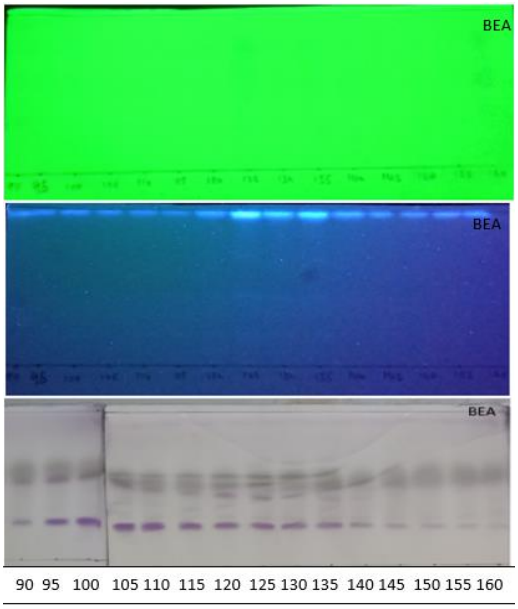

Figure 7.17: Phytochemical profile of the fourth open column chromatography fractions. The plate were developed on BEA mobile phase, observed under UV light at 254 and 365 nm. The plates were further sprayed with vanillin-sulphuric acid and observed.

TLC-bioautography assay was performed to analyse the antimicrobial activity of the purple compound against the test organism (*S. aureus)*. The phytochemical profile was used as a reference. Zone of inhibition was observed, hence the antimicrobial activity of the compound.

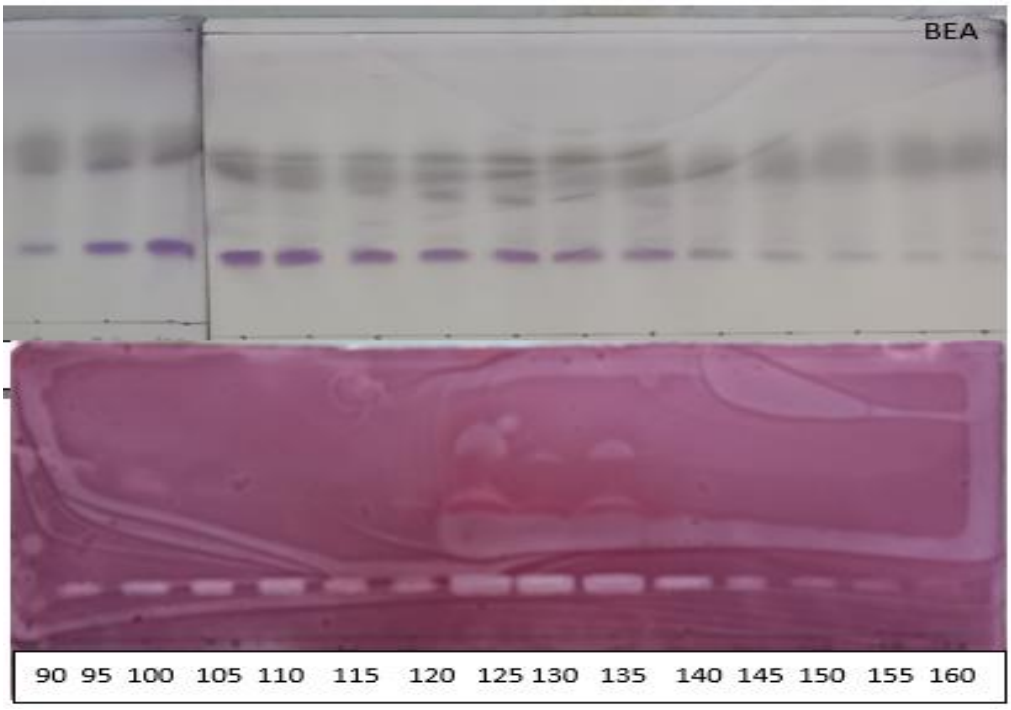

Figure 7.18: Phytochemical profile and antimicrobial activity of the purple target compound, where both plates were developed on BEA and sprayed with vanillin-sulphuric acid and *S.aureus* overnight culture, respectively.

7.3.9. Preparative TLC

Fractions 90 to 160 were pooled together and subjected to TLC silica gel glass plates (Merck Silica gel 60 F254) using BEA as a mobile phase. The purple compound of interest was traced using the plate sprayed with vanillin-sulphuric acid since the compound was not UV active. The scraped off compound was immersed in ethyl acetate, filtered and analysed on TLC. Figure 7.24 below shows the isolated and purified compound.

Figure 7.19: Fingerprint of the isolated and purified compound. The chromatogram was developed on BEA mobile system.

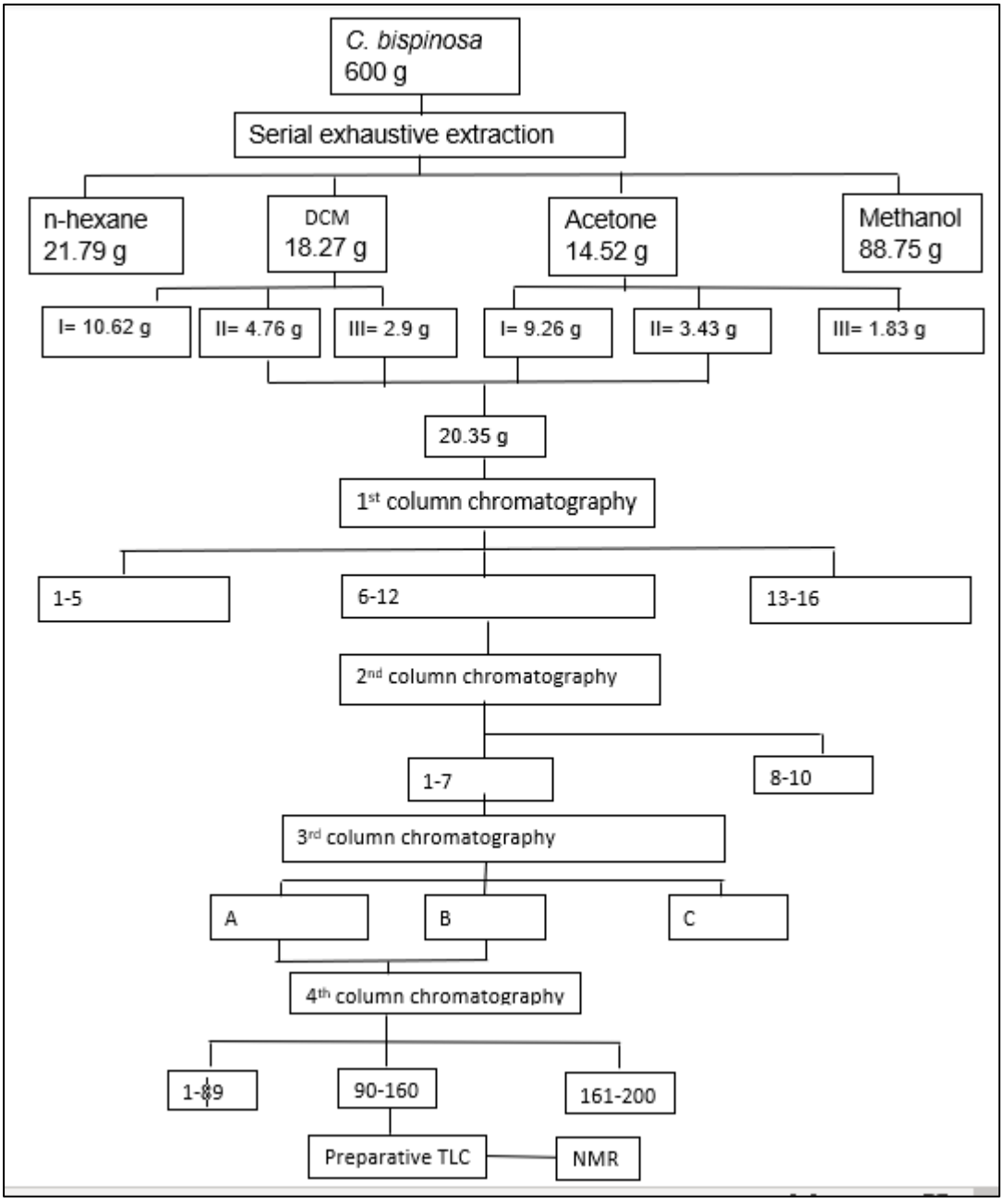

Figure 7.20: Overview of the steps followed in the bioactivity guided fractionation of the target compound.

7.4. Discussion

Biological active compounds derived from plants and microbes have recently given humankind a potential lead to discover potent drugs that can combat different human

diseases (Haruna and Yahaya, 2021). Therefore, ground-breaking research should continue in the field of phytochemistry. In the current study, the leaves of *C. bispinosa* exhibited promising antimicrobial activity against oral pathogens and were thus chosen for further analysis and isolation of the bioactive compound.

The dried *C. bispinosa* leaf powder (0.6 kg) was subjected to serial exhaustive extraction using n-hexane, dichloromethane, acetone and methanol. The total mass extracted was 143.33 g with methanol extracting the highest mass (88.75 g) followed by n-hexane (21.79 g), while acetone extracted the least (14.52 g). The plant contains mostly polar compounds, as seen on table 7.2, where methanol accounts for most of the extracted material.

The extracts were analysed for their phytochemical profile using TLC, where the chromatograms were developed on a non-polar (BEA), intermediately polar (CEF) and polar (EMW) mobile phases. The non-polar extracts were separated well on the BEA mobile system and for the polar extracts better separation was observed on EMW. The TLC-DPPH assay was employed to determine the qualitative antioxidant activity where the chromatograms of n-hexane, dichloromethane, acetone, and methanol extracts were developed on BEA, CEF and EMW mobile phases. The plates were sprayed with 0.2% DPPH in methanol for visualisation of antioxidant compounds where yellow spots against a purple background represent antioxidant activity. Antioxidant compounds were detected on the acetone and methanol extracts and they were best separated on the EMW mobile system.

Bioautography was employed to determine the antimicrobial activity of the *C. bispinosa* leaf extracts that were obtained through serial exhaustive extraction. The Dichloromethane and acetone extracts were effective against all the bacteria (*S. aureus, E. faecalis* and *S. pyogenes*) and fungi (*C. albicans* and *C. glabrata*) test organisms. The n-hexane and methanol extract only inhibited one test organism which are *S. aureus* and *S. pyogenes*, respectively. The inhibition was only observed on the plates resolved with BEA mobile phase. Serial broth microdilution assay was used to quantify the antimicrobial activity of the n-hexane, dichloromethane, acetone, and methanol extracts. Acetone extracts exhibited the lowest MIC value on average (0.46 mg/ml) and the most susceptible

pathogens were *S. aureus* and *E. faecalis* inhibited on average by (1.28 mg/ml). The dichloromethane (II and III) and Acetone (I and II) extracts had a promising qualitative antimicrobial activity and were selected for further fractionation using open column chromatography.

The dichloromethane and acetone extracts were subjected to open column chromatography and eluted with the mobile phases in table 7.1. The fractions were collected in beakers and analysed for antimicrobial activity using bioautography assay. Active fractions ranged from 50% n-hexane in ethyl acetate to 80% of ethyl acetate in methanol on the *E. faecalis* and *S. aureus* bioautograms and were narrowed in between the range against the other pathogens. Since the compound was not isolated in the first column chromatography as indicated by the presence of many bands on figure 7.5, the antimicrobial active fractions were mixed and further fractionated on the second column chromatography using n-hexane: ethyl acetate (50:50) as a mobile phase. The compound was collected in 825 test tubes, concentrated and analysed using TLC. The chemical profile was used to mix the samples into 10 fractions with similar bands. TLC and Bioautography were used to analyse the phytochemical profile and antimicrobial activity of the resulting ten fractions. Only fractions 1 to 7 demonstrated antibacterial activity against *S. aureus.* The antimicrobial activity is due to the purple band, as observed in figure 7.11. However, the compound was not pure since other bands were present on the same plate. The active fractions (1-7) were mixed and separated through the third column chromatography with n-hexane: ethyl acetate (70:30) as a mobile phase. The fractions were collected in 400 test tubes, analysed and grouped into three fractions (A, B and C), according to their profile. Fractions A and B had a similar profile and had the band of interest; however, impurities were still present and required further fractionation.

The fourth open column chromatography did not separate the target compound as well, therefore, preparative TLC was required as a resolution. In preparative TLC, TLC silica gel glass plates (Merck Silica gel 60 F254) were spotted with the sample and developed in BEA mobile phase. Since the compound was not UV active, a small portion of the TLC plate with the rest of the plate covered with aluminium foil, was sprayed with vanillin-sulphuric acid to visualise the target compound and determine its Rf value. The Rf (0.2)

value was used as a reference to trace the compound of interest and subsequently scraped off from the developed TLC plates. To separate the compound from the silica, the scraped material was immersed in ethyl acetate and filtered using cotton wool. The filtered compound was concentrated under a stream of cold air and found to be a pure purple compound following analysis in TLC. The pure compound was sent to the chemistry department (University of Limpopo) for NMR analysis.

7.5. Conclusion

The antimicrobial active compound present in dichloromethane and acetone extracts of *C. bispinosa's* leaves were isolated using open column chromatography and preparative TLC. The isolated compound has a potential in the formulation of novel drugs for dental ailments and was as thus analysed using NMR for structural elucidation, as presented in the next chapter.

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CHAPTER 8

8. Structural elucidation

8.1. Introduction

Identification and characterisation of a bioactive compound is a big challenge due to the combination of different types of bioactive constituents which exist in different polarities (Sasidharan *et al*., 2011). Numerous techniques have been developed over the years to simplify the process, including Nuclear Magnetic Resonance (NMR), Mass spectroscopy (MS), infra-red spectroscopy (IR), and ultraviolet-visible spectroscopy (UV-vis) (Sakong, 2012). Over the years, the isolation and characterisation of antimicrobial compounds based on their physio-chemical properties have made it possible for scientists to classify them into different categories. The classes are categorised in terms of the constituents and chemical properties possessed by the antimicrobial compound, such as polarity, whether the compound has proteins, lipids, or carbohydrates, the relative mass of the compound, and the UV spectrum of the compound (Fedenuik and Shand, 1998). Thus, an unknown compound can be identified through the characterisation of molecules.

Modern thin layer chromatography (TLC) has been used since the late 1970s to characterise compounds based on their polarity (Poole and Poole, 1989). However, the technique has its drawback owing to its inability to retain molecules adequately and inaccuracy when the sample is loaded on the plate abundantly. Despite the drawback, TLC is still employed when preparing small quantities of crude extracts to be further purified through high-performance liquid chromatography (HPLC) (Boyer, 2006).

Recently, TLC is coupled with HPLC in the characterisation of molecules; the prior determines the polarity of the compound and helps in choosing the HPLC system (Poole and Poole, 1989). HPLC works on a similar principle as TLC, however, HPLC also has other techniques separating with different dimensions such as reverse phase-HPLC, gel exclusion chromatography, and ion-exchange chromatography (Boyer, 2006). The structural characterisation is also required after mass and polarity characterisation and is achieved by mass spectrometry (MS). In this technique, the sample is bombarded with

electrons to reduce the analytes into ions. Analytes are then identified based on their mass-to-charge ratios (m/z) over time (Siuzdak, 1994).

To determine the secondary and tertiary structure of the unknown compound, magnetic resonance (NMR) can be used (Boyer, 2006). The technique has gained popularity and has become the most significant technique in the determination of chemical structures since the birth of Fourier transform spectrometer, which was a great development of radionuclide research such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, and the advancement of two-dimensional and three-dimensional nuclear magnetic technology. The principle of NMR is that molecules are irradiated by electromagnetic waves in a magnetic field, energy level transitions occur after the atomic nuclei with magnetic distance absorb a certain amount of energy, and then, NMR spectrum is obtained by mapping the absorption strength with the frequencies of the absorption (Monakhova *et al*., 2013).

8.2. Materials and methods

The isolated compound was subjected to NMR techniques for the determination of its chemical structure. About 10.5 mg of the compound was dissolved in chloroform and sent to the Chemistry Department at the University of Limpopo for analysis. The NMR analysis was performed through 1D (1H, 13C and DEPT 135) and 2D (HMBC, HSQC, and COSY) techniques using 400 MHz NMR Spectrometer (Bruker) at 400 MHz, and the number of scans were 10240 at a temperature of 295.5 K. Following NMR analysis, the results were sent to Prof Ofentse Mazimba, of the Chemical & Forensic Sciences Department at Botswana International University of Science and Technology, who analysed the NMR results and elucidated the structure of the compound.

8.3. Results

The isolated compound was analysed using NMR to determine spectra of the compound under different pulse programs (1H Proton, 13C, DEPT 135, COSY, HSQC, and HMBC). The spectra of the compound, as shown on Figure 8.1 to 8.6, aided in the characterisation of the compound.

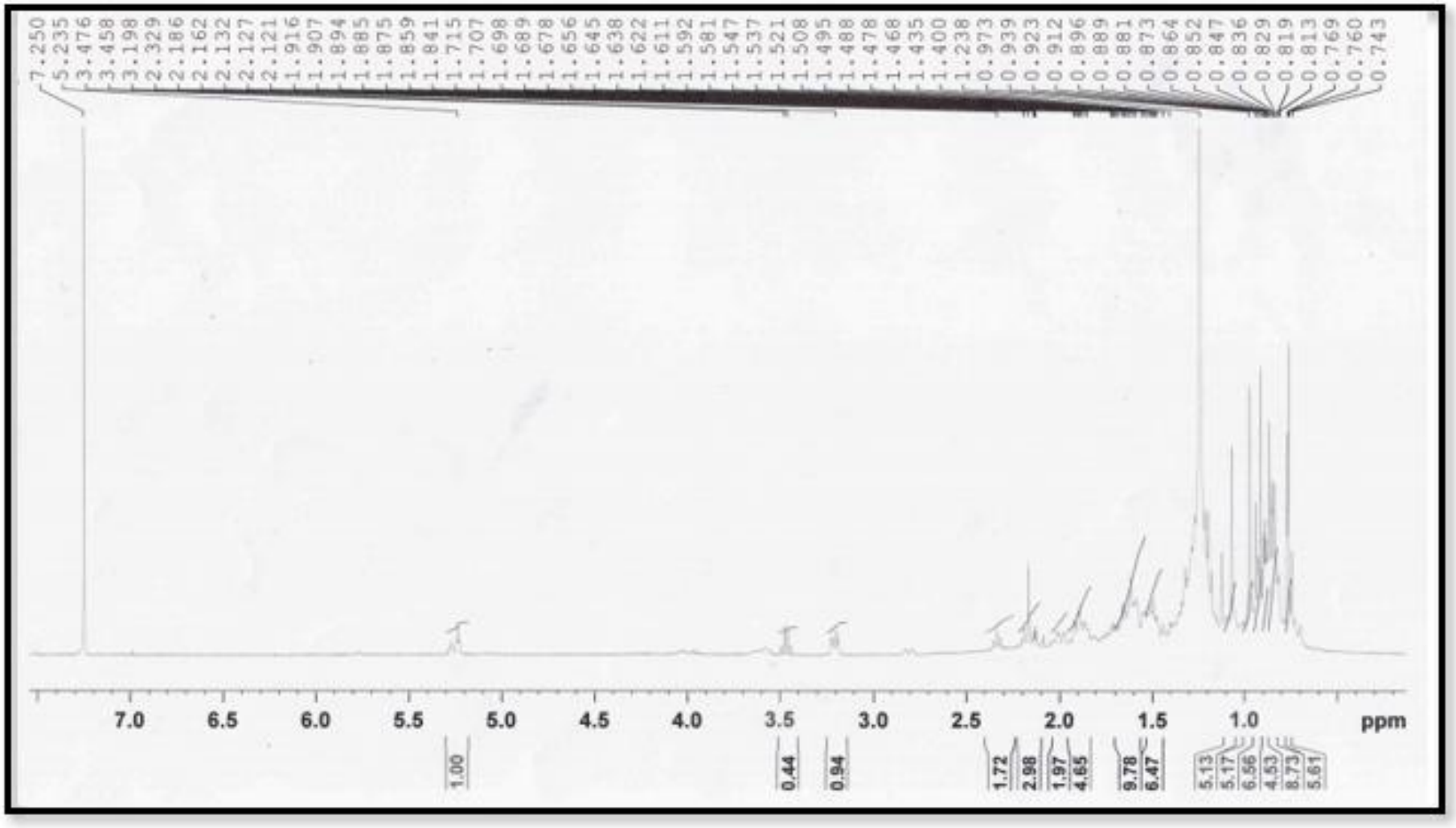

Figure 8.1: ^{1}H NMR spectrum of isolated compound

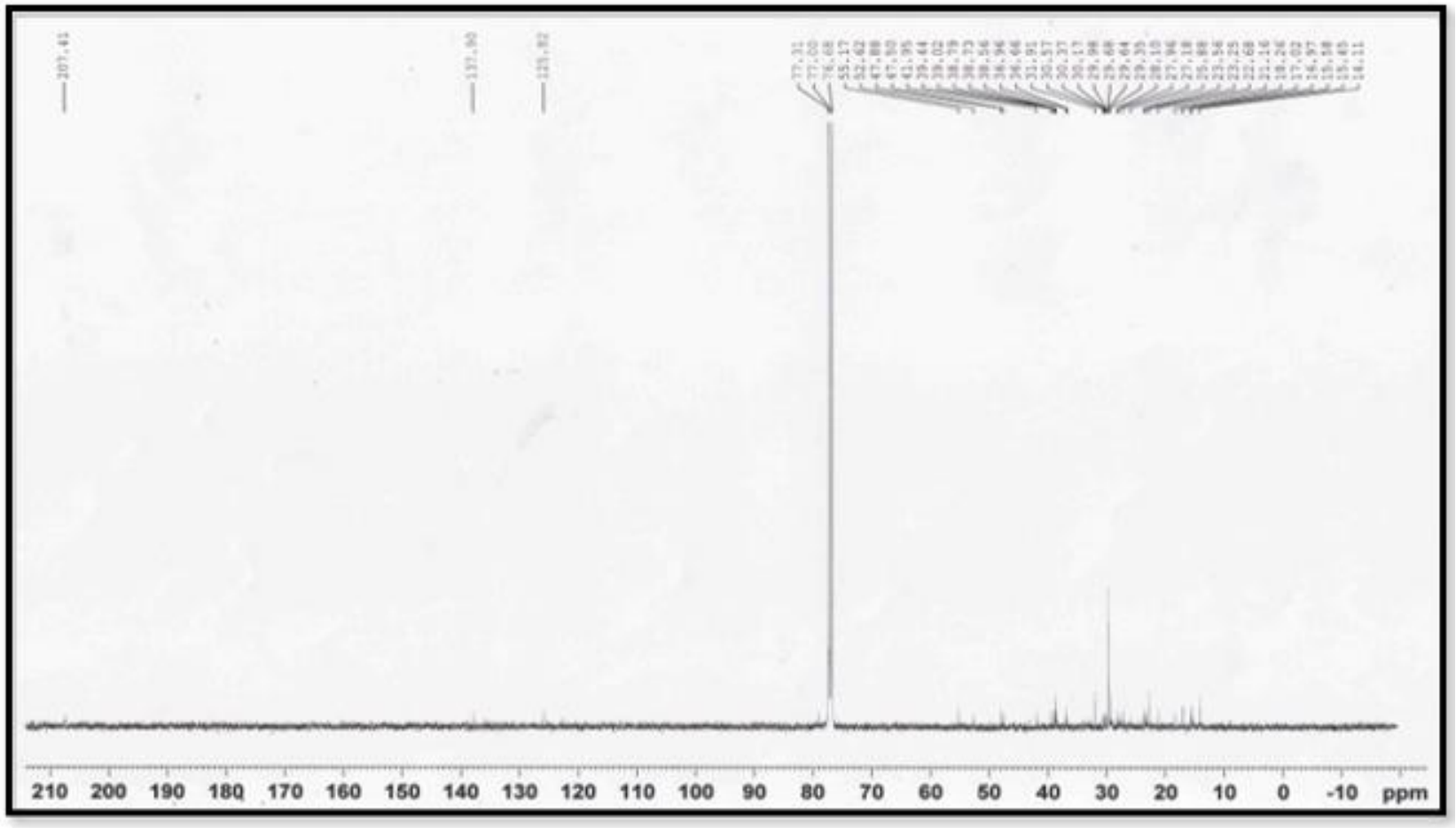

Figure 8. 2: ^{13}C CPD NMR spectrum of the isolated compound

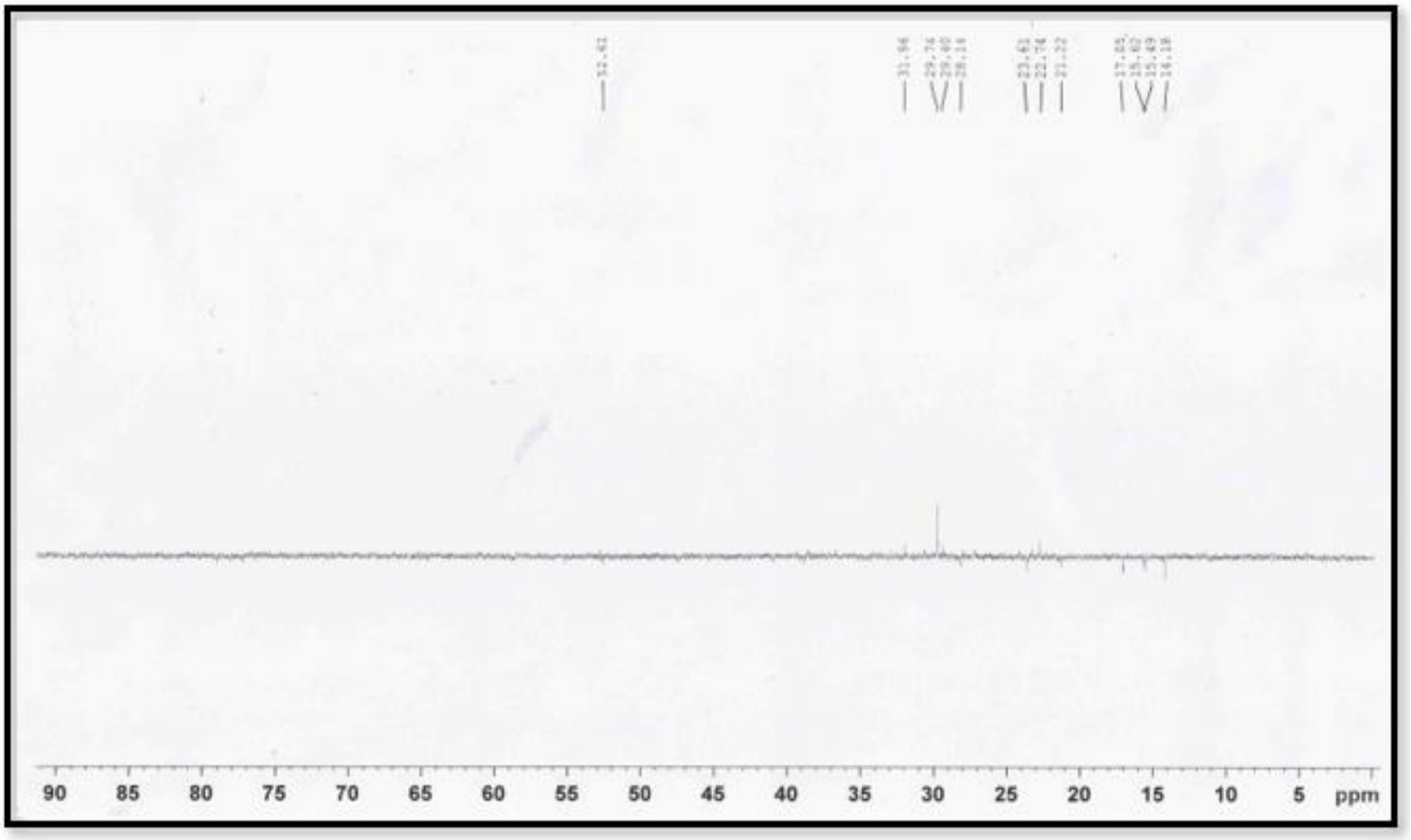

Figure 8.3: DEPT 135 NMR spectrum of the isolated compound

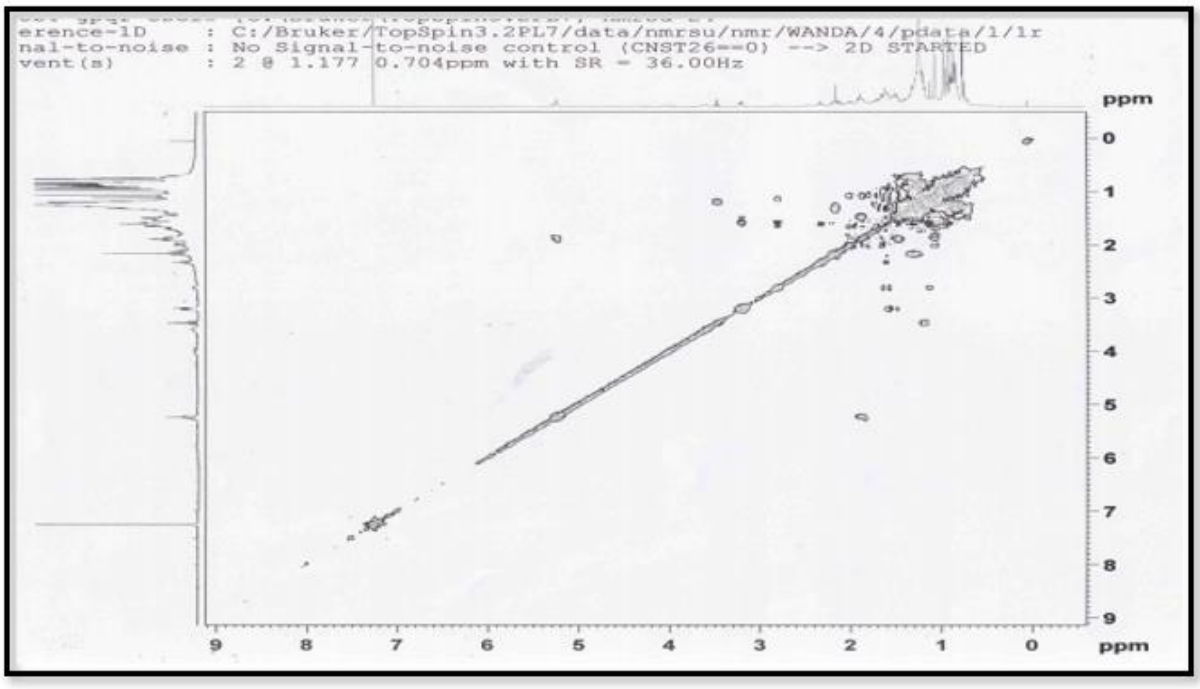

Figure 8.4: COSY NMR spectrum of the isoleted compound

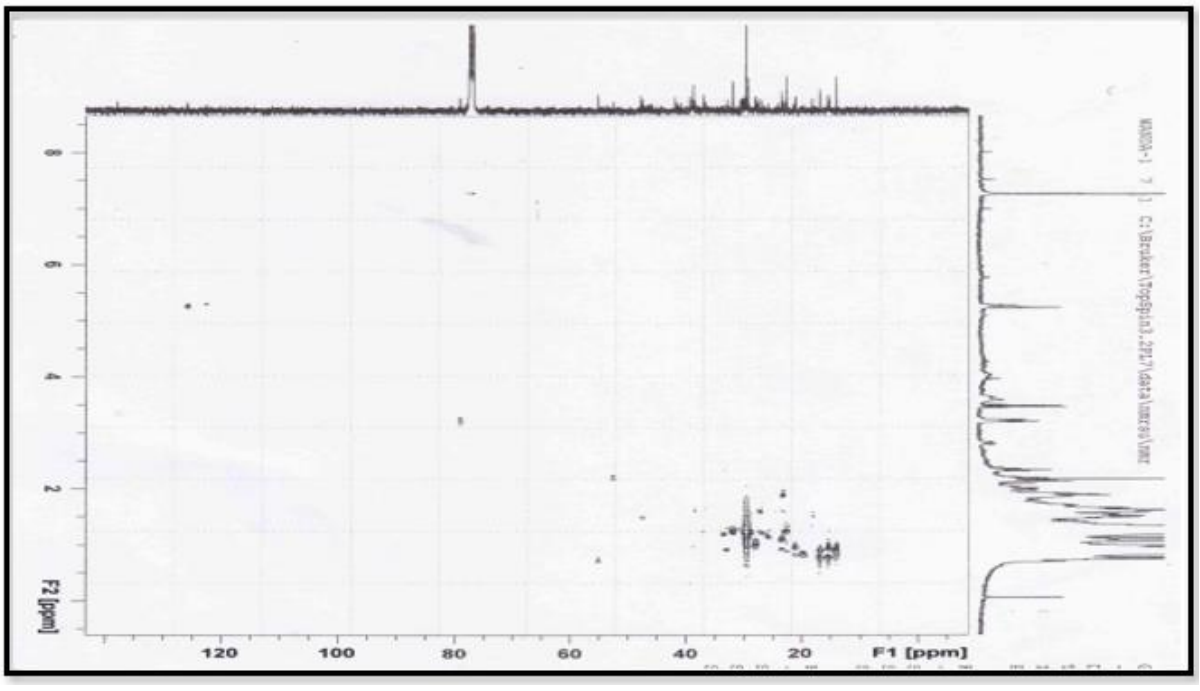

Figure 8.5: HSQC spectrum of isolated the isolated compound

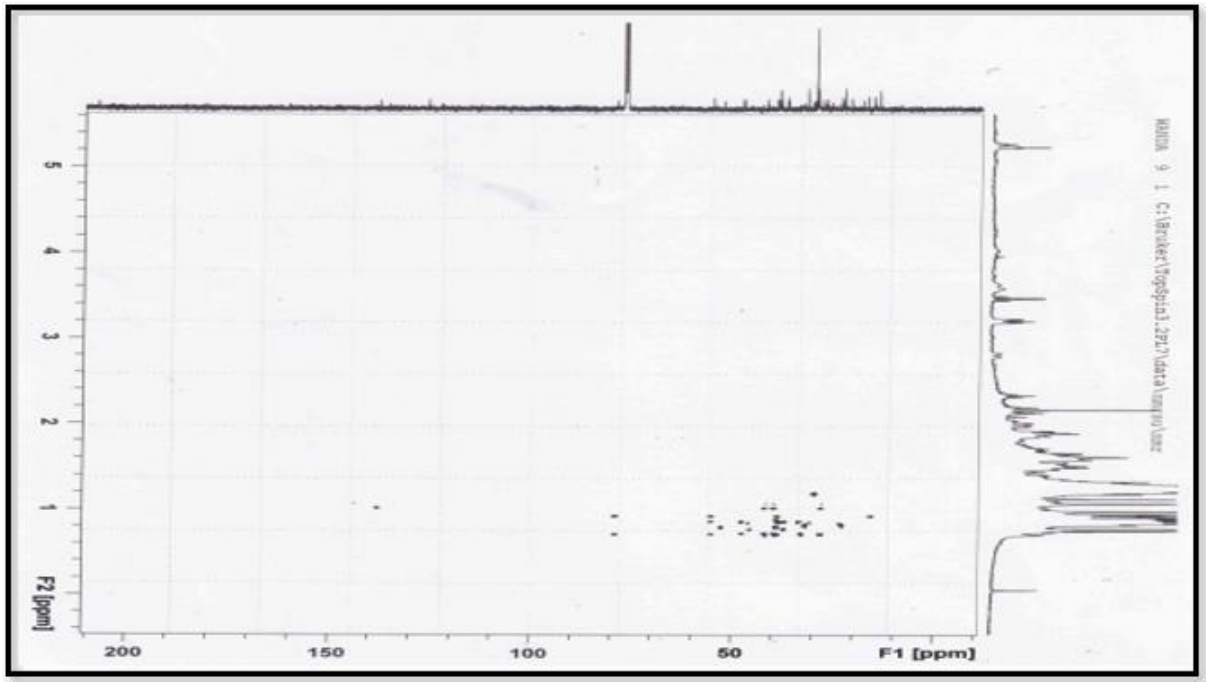

Figure 8.6: HMBC spectrum of the isolated compound

8.3.2. Spectroscopic data

Table 8.1: Summarised spectroscopic data of ^{13}C shifts of the isolated compound and β-Sitosterol from literature.

Position	**Type**	**β-Sitosterol (Ododo *et al*., 2016)**		**Isolated compound**	
		Chemical Shift, δ (ppm) value			
		13C NMR	**1 H NMR (multiplicity**	**13C NMR**	**1 H NMR (multiplicity**
1	CH_2	37.28	1.46 (m)	38.77	1.65-1.60 3H
2	CH_2	31.69	1.56 (m)	30.5	191-1.85 3H
3	CH(OH)	71.82	3.54 (m)	79.01	3.21 (m)

4	CH_2	42.33	2.32 (m)	41.92	2.32 (m)
5	QC(=)	140.77	-	137.87	-
6	CH(=)	121.73	5.37 (overlapping, t)	125.79	5.23 t (J= 3.5 Hz)
7	CH_2	31.93	2.04 (m)	31.91	2.15 (m)
8	CH	31.93	1.69 (m)	31.91	2.12 (m)
9	CH	50.16	1.55 (m)	47.86	1.91-1.85
10	QC	36.51	-	36.66	-
11	CH_2	21.11	1.52 (m)	21.16	1.65-1.60
12	CH_2	39.80	1.51 (m)	39.41	1.65-1.60 8H
13	QC	42.34	-	41.57	-
14	CH	56.79	1.50 (m)	55.13	1.65-1.60 8H
15	CH_2	24.33	1.58 (m)	23.55	1.74-1.67
16	CH_2	28.27	1.85 (m)	27.94	1.99 (m) 2H
17	CH	56.08	1.45 (m)	52.57	1.65-1.60 8H
18	CH_3	11.89	0.70 (s)	15.29	0.91 (s)
19	CH_3	19.42	1.03 (s)	15.44	1.12 (s)
20	CH	36.17	1.60 (m)	36.94	1.74-1.67 3H

21	CH_3	18.84	0.94 (overlapping, d)	15.57	1.06
22	CH_2	33.98	0.93 (m)	32.88	0.89 (m) 2H
23	CH_2	26.11	1.15 (m)	27.15	1.52-1.46 (m) 4H
24	CH	45.86	1.38 (m)	47.4	1.58 (m) 1H
25	CH	29.19	1.57 (m)	24.35	1.74-1.67
26	CH_3	19.84	0.84 (overlapping, d)	18.24	0.91 (s)
27	CH_3	19.06	0.86 (d)	17.00	0.97 (s)
28	CH_2	23.10	1.10 (m)	22.68	1.52-1.46 4H
29	CH_3	12.01	0.82 (overlapping, t)	14.12	0.86 (s)
-	OH	-	1.98 (s)		

8.3.3. Structure of the compound

The figure below shows the structure of the isolated compound, which was determined with the aid of NMR analysis.

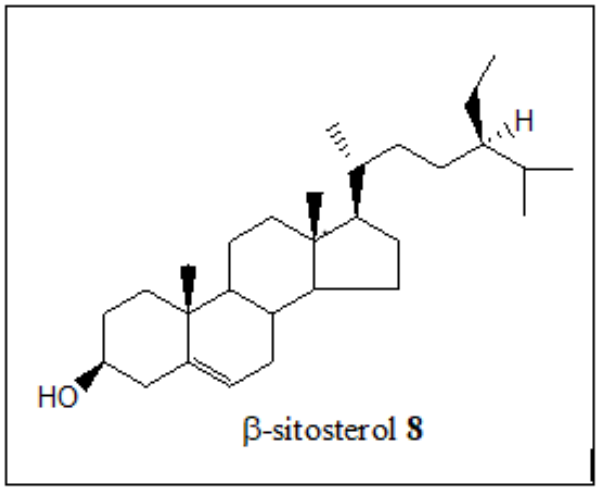

Figure 8.7: Structure of the isolated compound

8.4. Discussion

The isolation of the compound was accomplished using a series of open column chromatography and preparative-TLC guided by bioassays. The resulting pure compound was analysed using NMR techniques (1H, 13C, DEPT 135, HMBC, HSQC, and COSY). Analysis of the NMR spectra aided the structural elucidation of the compound. The compound was found to be a member of plant steroids (Phytosterols). The current study has isolated an antimicrobial compound, which was identified as β-Sitosterol. The compound was identified based on its physical properties (crystal with white colour), spectroscopic data (NMR), as shown in figure 8.1 to 8.6 and table 8.1 and comparing the data in the scientific literature (Ododo *et al*., 2016). β-Sitosterol has also been isolated from three carissa species; *C. edulis C. opaca* and *C. caranda* (Al-Youssef and Hassan, 2010; Saeed and Ahmed, 2015; Pakrashi and Datta, 2015; Hegde and Joshi, 2010). It is mixed with other phytosterols such as stigmasterol and campesterol in preparative amounts and commercialised. Studies have proven the safety, non-toxicity, effective nutritional supplementation and health benefits in different applications, including antibacterial activity (Sen *et al*., 2012).

8.5. Conclusion

This study has demonstrated the isolation and identification of β-Sitosterol, a member of plant steroids (Phytosterols) from the leaves of *C. bispinosa*. The compound has also been isolated from three Carissa species, namely, *C. edulis C. opaca* and *C. caranda*. Nuclear overhauser effect spectroscopy (NOESY) and Mass spectroscopy (MS) of this compound should be performed to determine its 3-D structures and confirm molecular weights, respectively.

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CHAPTER 9

9. Biological activities of isolated compounds

9.1. Introduction

C. bispinosa belongs to the Carissa L. genus under the family of Apocynaceae comprising about 250 genera (Lindsay *et al.,* 2000; Stoffel, 2004). Numerous species under this genus have been used in traditional medicines (Lindsay *et al.,* 2000) and studies have found many biological activities, including antibacterial (Ahmed and Fatima, 2016) and antioxidant (Itankar and Lokhande, 2011) activities.

The current study has isolated an antimicrobial compound, which was identified as β-Sitosterol. This compound is a natural micronutrient present in the cells and membranes of all oil producing plants, fruit, vegetables, grains, seeds and trees. It is mixed with other phytosterols such as stigmasterol and campesterol in preparative amounts and commercialised. Studies have proven the safety, non-toxicity, effective nutritional supplementation and health benefits in different applications, including antibacterial activity (Sen *et al*., 2012). β-Sitosterol has been isolated from three carissa species; *C. edulis C. opaca* and *C. caranda* (Al-Youssef and Hassan, 2010; Saeed and Ahmed, 2015; . Pakrashi and Datta, 2015; Hegde and Joshi, 2010). Sen *et al*. (2012) and Joy *et al*. (2012) have reported the antimicrobial activity of this compound against *S. aureus* and *E. coli*.

9.2. Methods and materials

9.2.1. Phytochemical analysis on TLC

The chemical profile of the isolated compound was determined on aluminium-backed TLC plates (Merck, silica gel 60 F254) using a method developed by Kotze and Eloff, (2002), as described in **section 3.2.3.**

9.2.2. TLC-DPPH assay

Qualitative DPPH assay, using thin-layer chromatography was done according to the method described by Deby and Margotteaux (1970), as explained in **section 4.2.1.**

9.2.3. Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972), as described in section **5.2.2.1.**

9.2.4. Serial broth microdilution assay

The broth micro-dilution method described by Eloff (1998) was used to determine the minimum inhibitory concentrations (MIC) values of crude extracts against four tested bacterial species, as explained in **section 5.2.3.1.**

9.3. Results

9.3.1. Phytochemical analysis

The figure below shows the phytochemical profile of the isolated compound. The single purple band observed indicates the presence of one compound after running TLC with BEA as the mobile phase.

Figure 9.1: Phytochemical profile of the compound isolated from *C. bispinosa's* leaves. The chromatogram was run on BEA mobile phase.

9.3.3. TLC-DPPH

TLC-DPPH assay was performed to determine the antioxidant activity of the isolated compound. The compound did not demonstrate antioxidant activity.

Figure 9.2: Antioxidant activity of the compound isolated from *C. bispinosa's* leaves. The chromatogram was run on BEA mobile phase and sprayed with 0.2% DPPH in methanol.

9.3.3. Bioautography

The isolated compound was subjected to bioautography assay to determine the antimicrobial activity. The compound was active against the test organisms (*E. faecalis* and *S. aureus*), as shown on the figure below.

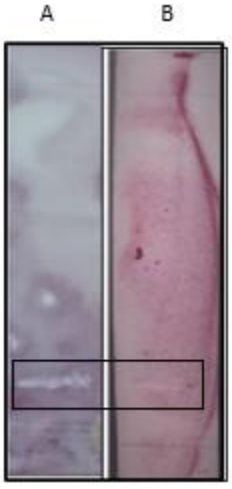

Figure 9.3: Antimicrobial activity of the compound isolated from *C. bispinosa*'s leaves. The compound was tested against *E. faecalis* (A) and *S. aureus* (B).

9.3.4. Minimum inhibitory concentration

The antimicrobial activity of the isolated compound was quantified using the minimum inhibitory concentration assay. Gentamicin was used as a positive control and *E. faecalis* and *S. aureus* were used as test organisms. *S. aureus* was the most susceptible, as it was inhibited by 125 µg/ml and the average inhibitory concentration was 187.5 µg/ml.

Table 9.1: MIC values of the isolated compound against *E. faecalis* and *S. aureus*.

Microorganisms	MIC values (µg/ml)	
	Isolated compound	Gentamicin
E. faecalis	250	125
S. aureus	125	62.5
Average	**187.5**	**93.75**

9.4. Discussion

The isolated compound (β-Sitosterol) was analysed for antioxidant activity using the TLC-DPPH assay. No antioxidant activity was observed on the TLC plate, as shown of figure 9.2. However, Ayaz *et al.* (2017) found that β-Sitosterol has a concentration dependant antioxidant activity. The absence of activity may be due to lower concentration used on the assay. Bioautography assay was used to determine qualitatively the antimicrobial activity of the compound against *E. faecalis* and *S.aureus.* The compound inhibited both organisms. These results support the findings of Sen *et al*. (2012) and Joy *et al*. (2012) who detected the antimicrobial activity of the compound against *S. aureus* and *E. coli*. The compound was also analysed using the minimum inhibitory concentration assay to quantify its activity. Gentamicin was used as a positive control and *E. faecalis* and *S. aureus* were used as test organisms. *S. aureus* was the most susceptible as it was inhibited by 125 µg/ml and the average inhibitory concentration was 187.5 µg/ml.

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CHAPTER 10

10. General discussion, conclusions and recommendations

10.1. General discussion

C. bispinosa has been traditionally used in the treatment of dental diseases. Phytochemical, antimicromicrobial, antioxidant and cytotoxicity studies were conducted to evaluate the effectiveness of the plant species as a possible source of a novel drug for dental diseases. Phytochemicals play a significant role in human health and people are increasing becoming aware of them. Numerous benefits are associated with them, including antibacterial, antifungal, anti-inflammatory and antioxidant activity (Prakash and Kumar, 2011; Packer and Weber, 2001). The current study has determined the phytochemical constituents and chemical profile of *C. bispinosa*'s leaf and stem extracts. Saponins, alkaloids, terpenes, cardiac glycosides, phlobatannin, tannins and steroids were detected on both plant materials. The major constituents were quantified and the leaf water extracts had the highest total phenolic content (94.74 mg of GAE/g) while the acetone extract (338 mg of GAE/g) constituted the highest total phenolic content of all stem extracts. The leaf extract's highest total tannin content was detected on the hexane extract (22.98 mg of GAE/g) and the ethanol extract contained the highest total tannin content (49.87 GAE/g) in the stem extracts.

Antioxidant activity was detected using TLC-DPPH assay and quantified through DPPH free radical scavenging assay and ferric reducing power assay. Ethanol, water, and methanol extracts exhibited the highest free radical scavenging activity with 90.52%, 86.61%, and 78.52% antiradical activity, respectively, for leaf extracts. For stem extracts, methanol, ethanol, and water again led with the highest free radical scavenging activity with 93.93%, 79.03% and 71.98%, respectively. On the ferric reducing power assay, methanol and ethanol extracts displayed high antioxidant potential for leaf (1.308) and stem (1.916), respectively. The cytotoxic effects of *C. bispinosa*'s acetone extract was evaluated using MTT assay. The percentage viability was decreasing as the concentration of the extract was increasing. However, the highest concentration resulted in more than 50% cell viability.

Antimicrobial activity was detected using TLC-bioautography assay and quantified through broth microdilution assay. All the leaf extracts except for the water extract demonstrated antimicrobial activity against bacterial pathogens; *S. aureus, E. faecalis,* and *S. pyogenes. C. albicans* was susceptible to all but hexane, acetone, and water extracts whilst its distant relative *C. glabrata* was not inhibited by any of the extracts on bioautography. For the leaf extracts, methanol extract had the lowest average MIC value (0.81 mg/ml), making it the most activity across the microorganisms, and hexane had the highest MIC value (1.63 mg/ml). *C. albicans* demonstrated higher susceptibility with an average of 0.73 mg/ml while *C. glabrata* displayed the least (2.8 mg/ml). Compared to the leaf extracts, lesser activity was observed for the stem extracts on bioautography and MIC.

The antimicrobial active compound present in *C. bispinosa*'s leaves was isolated using open column chromatography and preparative TLC guided by bioassays. The compound was isolated as a white powder (10.5 mg) and analysed using NMR (^{1}H, ^{13}C, DEPT 135, HMBC, HSQC, and COSY). NMR spectra aided the structural elucidation of the compound. It was identified based on its physical properties, spectroscopic data and comparing the data with scientific literature (Ododo *et al*., 2016) and was found to be β-Sitosterol, a member of plant steroids (Phytosterols).

10.2. General conclusions and recommendations

The study indicated the leaves of antimicrobial activity of *C. bispinosa* against dental pathogens. Furthermore, the extracts of this plant indicated potent antioxidant activity and low toxicity levels. Upon isolation and structural elucidation, the active compound was identified as β-Sitosterol. The study is the first to isolate β-Sitosterol as an antimicrobial compound for dental health from the leaf extract of *C. bispinosa*

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