The isolation and characterization of an antibacterial compound from *Terminalia sambesiaca* (Combretaceae)

By

Sehlapelo Irene Mokgoatsane

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North-West University (Potchefstroom campus)

Supervisor: Prof J.C Breytenbach

Co-Supervisor: Prof J.N Eloff

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DECLARATION

I, IRENE MOKGOATSANE, hereby declare that this thesis submitted for the award of the degree of Magister Scientiae (MSc) at North-West University, is my independent work and has not been previously submitted for a degree or any examination at any university.

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Mokgoatsane Irene

Date: November 2011

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Supervisor

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Co-supervisor

DEDICATION

This is dedicated to my mother and brothers, George, Isaiah and Solly; my wonderful husband, Simon, daughter, Lerato and son, Lethabo.

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First and foremost, I would like to thank God, Almighty for awarding me the opportunity, wisdom and ability to carry out the research. His mercies are new every morning, to Him be the glory.

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POSTER PRESENTATION

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90/10/1 v/v/v)
CEF	Chloroform/Ethyl acetate/Formic acid (5/4/1 v/v/v)
DCM	Dichloromethane
DPPH	2, 2- diphenyl-1-picrylhydrazyl
EMW	Ethyl acetate/Methanol/Water (40/5.4/4 v/v/v)
INT	Iodonitro-tetrazolium salts
LNBG	Lowveld National Botanical Garden
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
NMR	Nuclear Magnetic resonance (carbon 13 and proton)
R_F	Retardation factor
TLC	Thin Layer Chromatography
UV	Ultra violet radiation
V/v	volume per volume
VLC	Vacuum Liquid Chromatography

ABSTRACT

This was an investigation of the antimicrobial activity of the *Terminalia* species and isolation of the compound(s) responsible for such activity. *Terminalia* species are extensively used in the indigenous medicines in most parts of Africa. They are a source of many potent biologically active compounds. *Terminalia sericea* has been identified as one of the 51 most important African medicinal plants. There are several *Terminalia* species in South Africa and in this study the following species were investigated: *Terminalia sericea*, *Terminalia phanerophlebia*, *Terminalia mollis*, *Terminalia gazensis*, *Terminalia brachystemma*, and *Terminalia sambesiaca*.

The leaves of six *Terminalia* species were sequentially extracted with hexane, dichloromethane, ethyl acetate and methanol. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used to screen for the radical scavenging ability of the compounds present in the plant extracts. All plants had antioxidant activities present in different extracts. Most of the antioxidatively active compounds were present in the ethyl acetate and methanol extracts.

Minimum inhibitory concentration (MIC) was determined using a serial microdilution assay where tetrazolium violet reduction was used as an indicator of growth. This was done for both bacteria and fungi. Pathogens used included Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) as well as fungi; yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus* and *Microsporum canis*). All six *Terminalia* species were active against the selected pathogens. *E. faecalis* and *E. coli* were the most sensitive pathogens while *S. aureus* and *P. aeruginosa* were relatively resistant. Most extracts gave MIC values of 80 µg/ml while others (*T. sambesiaca*) gave values as low as 20 µg/ml after 24 hours of incubation. *Terminalia sambesiaca* gave the lowest average MIC value at 100 µg/ml, followed by *Terminalia mollis* with an MIC value of 118 µg/ml after 24 hours of incubation.

The total activity of the species was calculated by dividing the quantity extracted in milligrams (mg) from 1 gram leaves by the MIC value in mg/ml. This value indicates the volume to which the biologically active compound/s present in 1 gram of the dried plant material can be diluted

and still kill the bacteria. *T. sambesiaca* had the highest average total activity of 4312 ml/g while *T. gazensis* had the lowest average activity (1371 ml/g).

For antifungal activity, most extracts gave MIC values of 80 µg/ml. *T. sambesiaca* was the most active species showing values as low as 40 µg/ml after 24 hours of incubation. Amphotericin B was used as a positive control and there was no growth in any of the wells containing the antifungal agent, indicating a MIC of less than 20 µg/ml. *T. sambesiaca* and *T. brachystemma* had the lowest average MIC value of 0.20 mg/ml followed by *T. gazensis* with an average MIC value of 0.21 mg/ml after 24 hours of incubation. *T. sambesiaca* gave the lowest MIC values against the tested pathogens and was therefore selected for in depth investigation.

Isolation of compounds was undertaken on the crude extracts of *Terminalia sambesiaca* leaves. Open column chromatography was used to further separate compounds seen on TLC when phytochemical analysis was done. A compound was isolated from the ethyl acetate extract and its structure was elucidated. The isolated compound was identified as β-sitosterol and it was subjected to bioassays to ascertain activity reported in literature. It was active against all tested pathogens. The MIC values for the isolated compound against the tested pathogens were: 280 µg/ml for *E. faecalis,* 400 µg/ml for *E. coli* and 320 µg/ml for *S. aureus. E. faecalis* was more susceptible to the isolated compounds than the other pathogens used.

Although β-sitosterol was isolated from other plant species and from *Terminalia arjuna*, this is the first report of this compound from *Terminalia sambesiaca.* The fact that β-sitosterol was active against the selected pathogens confirms the findings from other studies. The excellent activity found in the *Terminalia sambesiaca* extracts could be a lead in the development of antimicrobial agents. It is a possibility that more compounds could still be isolated from *Terminalia* species that are responsible for antimicrobial activity of these plants. These findings justify the use of *Terminalia* species in ethnopharmacology.

UITTREKSEL

Hierdie studie was 'n ondersoek van die antimikrobiese aktiwiteit van *Terminalia*-spesies (vaalbos) en isolasie van die verbinding(s) wat verantwoordelik vir sodanige aktiwiteit is. *Terminalia*-spesies word op groot skaal in die inheemse medisyne van sentraal Afrika gebruik. Hulle is 'n bron van baie kragtige biologies aktiewe verbindings. *Terminalia sericea* is as een van die 51 belangrikste medisinale plante in Afrika geïdentifiseer. Daar is verskeie*Terminalia*-spesies in Suid-Afrika en in hierdie studie is die volgende spesies ondersoek: *Terminalia sericea, Terminalia phanerophlebia, Terminalia mollis, Terminalia gazensis, Terminalia brachystemma* en *Terminalia sambesiaca*.

Die blare van ses *Terminalia*-plante is met heksaan, dichloormetaan, etielasetaat en metanol geëkstraheer. Die 2,2-difeniel-1-pikrielhidrasiel (DPPH) -toets op DLC-plate is gebruik om die vermoë as radikaalvangers van die verbindings in die plantekstrakte te toets. Alle plante het anti-oksidantaktiwiteit in die verskillende ekstrakte getoon. Op grond van hierdie toetse was die mees aktiewe verbindings in die etielasetaat- en metanolekstrakte.

Die minimum inhibisiekonsentrasie (MIK) is bepaal deur gebruik te maak van 'n reeks verdunnings waar tetrasoliumviolet as 'n aanduiding van die groei van sowel bakterieë as fungi gebruik is. Patogene was onder meer Gram-negatiewe bakterieë *(Pseudomonas aeruginosa* en *Escherichia coli)* en Gram-positiewe bakterieë *(Enterococcus* faecalis en *Staphylococcus* aureus), swamme, giste *(Candida albicans* en *Cryptococcus neoformans*), termies dimorfiese fungi *(Sporothrix schenckii*) en swamme *(Aspergillus fumigates* en *Microsporum canis).* Al ses*Terminalia*-spesies was antibakterieel aktief teen die getoetsde patogene. *E. faecalis* en *E. coli* is die mees sensitiewe patogene terwyl *S. aureus* en *P. aeruginosa* relatief bestand is. Die meeste ekstrakte het MIK-waardes van 0,08 mg/ml, *terwyl* ander *(T.* sambesiaca) na inkubasie vir 24 uur waardes van so laag as 0,02 mg/ml vertoon het. *Terminalia sambesiaca* het die laagste gemiddelde MIK-waarde van 0,10 mg/ml gevolg deur *Terminalia mollis* met 'n MIK-waarde van 0,118 mg/ml na inkubasie vir 24 uur.

Die totale aktiwiteit van die spesies is bereken deur die hoeveelheid [in milligram (mg)] uit 1 gram blare geëkstraheer deur die MIK-waarde in mg/ml te deel. Hierdie waarde dui op die volume waartoe die biologies aktiewe verbinding teenwoordig in 1 gram van die gedroogde plantmateriaal verdun kan word en nog steeds die bakterieë doodmaak. *T. sambesiaca* het die hoogste gemiddelde totale aktiwiteit van 4312 ml/g terwyl *T. gazensis* die laagste gemiddelde aktiwiteit (1371 ml/g) het.

Die MIK-waardes van die meeste ekstrakte teen fungi was 0,08 mg/ml. *T sambesiaca* was die mees aktiewe spesie met waardes van so laag as 0,04 mg/ml na inkubasie vir 24 uur. Amfoterisien B is as 'n positiewe kontrole gebruik en daar was geen groei in enige van die putte wat die antifungusmiddel bevat het nie wat ‘n MIK van minder as 0,02 mg/ml aandui. *Terminalia sambesiaca* en *Terminalia brachystemma* het die laagste gemiddelde MIK-waarde van 0,20 mg/ml gevolg deur*Terminalia gazensis* met 'n gemiddelde MIK-waarde van 0,21 mg/ml na inkubasie vir 24 uur. *Terminalia sambesiaca* was die mees aktiewe spesie (lae MIK-waardes) teen getoetsde patogene en is dus vir ‘n diepteondersoek gekies.

Isolasie van verbindings is uit die ru-ekstrakte van die blare van van *Terminalia sambesiaca* gedoen. Oop kolomchromatografie is gebruik om verbindings soos op DLC gesien te skei. 'n Verbinding is uit die etielasetaatekstrak geïsoleer en die struktuur daarvan is bepaal. Die geïsoleerde verbinding is as β-sitosterol geïdentifiseer en bio-analises het die aktiwiteit soos in die literatuur gerapporteer bevestig. Dit is aktief teen alle getoetsde patogene. Die MIK-waardes van die geïsoleerde verbinding was 280 µg/ml vir *E. faecalis,* 400 µg/ml vir *E. coli* en 320 µg/ml vir *S. aureus. E. faecalis* is meer vatbaar vir die geïsoleerde verbindings as die ander patogene wat gebruik is.

Hoewel β-sitosterol uit ander plantspesies en uit *Terminalia arjuna* geïsoleer is, is dit die eerste verslag van hierdie verbinding uit *Terminalia sambesiaca*. Die feit dat β-sitosterol aktief teen die getoetsde patogene is, bevestig die bevindinge van ander studies. Die uitstekende aktiwiteit wat in die ekstrakte *Terminalia sambesiaca* gevind is, kan 'n leidraad in die ontwikkeling van antimikrobiese middels wees. Verdere verbindings wat verantwoordelik vir antimikrobiese aktiwiteit van *Terminalia*-spesies is kan nog steeds uit hierdie plante geïsoleer word. Die bevindinge van hierdie studie regverdig die gebruik van *Terminalia*-spesies in etnofarmakologie.

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CHAPTER 1: INTRODUCTION

Bacteria have developed numerous defences against antimicrobial agents with the number of drug-resistant pathogens being on the rise (Levy, 1998; Eloff *et al.,* 2005b). From the first case of antibiotic resistant *Staphylococcus aureus*, the problem of resistance has become a serious public health concern with economic, social and political implications, and continues to increase inexorably (Kunin, 1993; Weinstein, 2001; WHO, 2000). Penicillin-resistant pneumococci are also spreading rapidly and resistant malaria is rising, resulting in death of millions of adults and children each year (WHO, 2000).

This appearance of microbial resistance to antibiotics and the occurrence of fatal opportunistic infections associated with the Acquired Immunodeficiency Syndrome (AIDS) and cancer necessitates the search for new effective antimicrobial agents (Penna *et al.,* 2001). Plants have been used for many years to treat infectious diseases and they are being investigated as a source for new antimicrobial agents (Cowan, 1999). Most natural sources of compounds, such as higher plants, especially from the tropics have not been studied and they could be useful in the control of microbial infections (Fabry *et al*., 1998). Scientists are searching the earth for phytochemicals to be developed for treatment of infectious diseases especially given the emergence of drug-resistant microorganisms which increase the need for production of more effective antimicrobial agents (Tanaka *et al*., 2006).

Many people rely on herbal medicine as their primary source of health care. In Africa, millions of people depend on traditional medicine (Adewunmi *et al*., 2001; Van Wyk *et al*., 1997; Kelmanson *et al*., 2000). Almost 80% of the population in Africa still relies on traditional medicine to cure affections of early childhood, including malaria. Herbal medicines are believed to have stood the test of time because of their general safety, efficacy, cultural acceptability and lesser side effects. Being part of physiological functions in living flora, the chemical constituents present in herbal medicines are believed to have better compatibility with the human body (Kamboj, 2000).

Combretaceae is a large family of plants with the two most commonly occurring genera, *Combretum* and *Terminalia* being widely used in African traditional medicine (Katerere *et al*., 2003; Masoko and Eloff, 2005; Eloff *et al.,* 2008). Plants of the genus *Terminalia* are found in most parts of the world, with 250 species distributed in the tropics and subtropics (Leistner, 2000). Among species which have been identified, 14 are found in southern Africa while in

South Africa 4 species have been identified. These species are found in the Limpopo province (Leistner, 2000).

Many *Terminalia* species have uses in African traditional medicine. Some of these plants contain a large number of compounds. Kaur and colleagues (2002) identified polyphenols which include flavones, flavonols, phenylpropanoids and tannins from extracts of *Terminalia* plants. These compounds are claimed to treat different ailments including fractures, ulcers, blood diseases, anaemia and asthma (Kaur *et al*., 2002). Sabu and Kuttan (2002) have also identified gallic acid, which possesses antioxidant properties, and this may be useful in the management of diseases such as diabetes. *Terminalia arjuna* was found to contain cancer cell growth inhibitory constituents which are gallic acid, ethyl gallate and the flavone luteolin (Pettit *et al*., 1996). Eloff *et al* (2008) reported the isolation of arjunolone and arjunolic acid from *T. arjuna*.

Extracts of the *Terminalia* species are active against a variety of microorganisms (Silva *et al*., 1997; Iqbal *et al*., 1998). A study by Baba-Moussa and colleagues (1999) reveals that in addition to exhibiting antibacterial properties, some of these plants (*T. mollis* and *T.avicennioides*) possess antifungal properties. Extracts of *T. sambesiaca* roots possess antifungal activity (Fyhrquist *et* al., 2004). *Terminalia* plants are also sources for antiviral agents (Taylor *et al*., 1996). For example, Yukawa and colleagues (1996) have also shown that *T. chebula* is active against herpes simplex virus and cytomegalovirus. Other studies have proven that some *Terminalia* species contain active antiplasmodial agents, making them useful in the treatment of malaria (Valentin *et al*., 2000; Omulokoli *et al*., 1997). *T. catappa* has antioxidant properties (Chyau *et al*., 2002).

Bacterial infections such as those caused by *Neisseria gonorrhoeae,* are a major health problem especially in Africa (Silva *et al*., 2002). Due to the increasing prevalence of antimicrobial resistance (Silva *et al*., 2002), it is necessary to develop new drugs for the treatment of bacterial infections (Eloff *et al*., 2005b). Evidence exists that *Terminalia* species possess both antifungal and antibacterial activities (Pettit *et al*., 1996; Omulokoli *et al*., 1997; Silva *et al*., 2002; Fyhrquist *et al*., 2002; Fyhrquist *et al*., 2004; Masoko *et al*., 2005). Masoko (2006) isolated compounds with antifungal activity from leaf extracts of Combretaceae plants (*Combretum nelsonii*). Despite the depth of research already done on medicinal plants, the crisis of bacterial resistance to antibiotics, the AIDS virus, and other health related developments have increased the need for medicinal plant research which could be a solution to all the problems.

1.1 Aim and Objectives

1.1.1 Main objective

Though several investigations into the antimicrobial activity of members of the Combretaceae have been undertaken in recent years (Basséne *et al.*, 1995; Silva *et al.*, 1997; Baba-Moussa *et al.*, 1999; Kruger, 2004; Masoko *et al.*, 2005; Shai, 2008), the antibacterial and antifungal properties and compounds of various species of *Combretum* and *Terminalia* have not been exhaustively investigated. It was thus our aim to reinvestigate results previously obtained in our laboratories, to confirm the most active plant species and to isolate and characterise the active compound(s) from one of these species.

1.1.2 Specific objectives

(i) To screen selected plants for broad spectrum antimicrobial and antioxidant properties.

(ii) To select one species for further investigation based on antibacterial activity and availability.

(iii) To isolate the compound(s) that are responsible for the antibacterial properties

(iv) To determine the minimum inhibitory concentration of the isolated compound(s) against selected microorganisms.

(v) To characterise the molecular structure(s) of the active compound(s) isolated from the plant.

1.1.3 Hypothesis

The genus *Terminalia* contains antibacterial compounds that can be isolated by bioassay guided fractionation. The chemical structure of the isolated compound(s) can be determined and these compound(s) will have antibacterial activity that may be useful in human and animal medicine.

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal plants

Despite the availability of different approaches for the discovery of therapeuticals, natural products still remain one of the best reservoirs of structural types (Hostettmann, 1999). The use of medicinal plants as a source for relief from illness can be tracked back over five millennia to written documents of the early civilization in China, India and near east of Asia, and it is doubtless an art as old as mankind (Prozesky *et al*., 2001). Medicinal and poisonous plants have always played an important role in African society, with the first written records of Xhosa and Zulu medicinal plant usage in South Africa published as early as 1885 (Hutchings, 1989).

Many medicinal plants are used traditionally in most African countries, with the traditions of collecting, processing and applying plants and plant based medications having been handed down orally from generation to generation (Von Maydell, 1996). Research has been done on ethnobotanical use of plants in South Africa (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; Van Wyk *et al.,* 1997; Mander, 1998; Van Wyk and Gericke, 2000). It is reported that more than 27 million South Africans are users of indigenous medicine (Mander, 1998). Some of the medicinal plants have been studied scientifically and in most cases the results confirm the traditional therapeutic claims of these plants (Samy *et al*., 1998). For example, the leaves of *Entada abyssinica* have been found to contain flavonoids and triterpenoids which possess antiviral and anti-inflammatory properties; traditionally these leaves are powdered and applied as dressings to sores. Similarly, *Ximenia caffra* which is used for the treatment of dysentery and cholera has been found to contain tannins and coumarins and is also active against *Staphylococcus aureus* (Fabry *et al*., 1998).

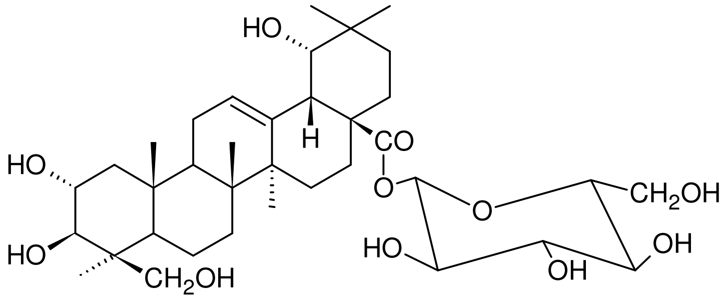
Most of the pharmacologically active, plant-derived components were discovered after the ethnomedicinal uses of the plants were investigated (Farnsworth and Soejarto, 1991). In traditional African medicine, the therapeutic activities of plant remedies used for minor ailments may be due to their chemical properties, for example, fresh leaves squeezed to stop bleeding contain tannins or other haemostatic components and plant remedies used for fever contain antipyretic principles (Williamson *et al*., 1996).

Interestingly, a plant may be used to treat different diseases, with different plant parts being used. Fabry and colleagues (1998) investigated the use of young branches and leaves of *T. spinosa* and found antibacterial properties. Omulokoli and colleagues (1997) mentioned that the stem bark of *T. spinosa* has antiplasmodial activity. The root extracts of *T. sericea* are used for the treatment of sexually transmitted diseases (STDs) and diarrhoea while the powdered bark is used by the Sotho for diabetes. Hot infusions of the outer layers of roots are used as fomentations for pneumonia and root decoctions are used as eye washes (Hutchings *et al.*, 1996).

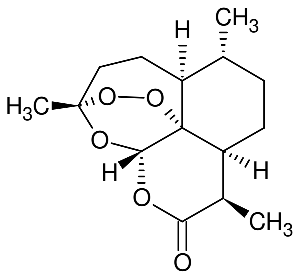
2.2. Plant derived antimicrobials

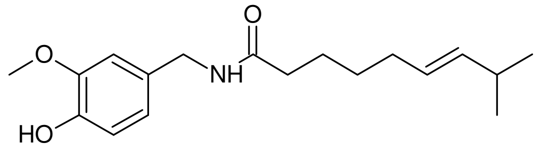
2.2.1. Terpenoids

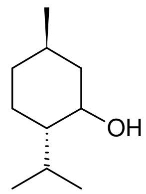
The essential oils of plants are secondary metabolites that are highly enriched with compounds known as terpenes. These compounds are based on an isoprene structure with the general formula of $C_{10}H_{16}$ (Cowan, 1999). Terpenes and terpenoids constitute a very large family of compounds. The structures of terpenoids are diverse and range from relatively simple linear hydrocarbon chains to highly complex ring structures (Back and Chappell, 1996). Terpene hydrocarbons may occur as monoterpenes (C_{10}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), hemiterpenes (C_5) and sesquiterpenes (C_{15}). Terpenes that contain an additional element (usually oxygen) are termed terpenoids (Cowan, 1999). Triterpenes have been found to be strong inhibitors of HIV-1 reverse transcriptase *in vitro* (Bessong *et al.*, 2004).



Sericoside (1)







Artemisinin (2) **Capsaicin (3)** **Menthol (4)**

Figure 2-1. Examples of terpenoids with biological activities

2.2.2. Alkaloids

Alkaloids are organic bases containing nitrogen in a heterocyclic ring. Many have pronounced pharmacological activity (Williamson *et al*., 1996). The first medically useful example of an alkaloid is morphine which was isolated from opium poppy *Papaver somniferum* in 1805 (Fessenden and Fessenden, 1982). Some alkaloids have antimicrobial properties (Omulokoli *et al*., 1997; Karou *et al*., 2006), while others may be useful against HIV infection as well as intestinal infections associated with AIDS (McMahon *et al*., 1995). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine is attributed to their ability to intercalate with DNA (Cowan, 1999) while indoloquinoline alkaloids such as cryptolepine, cause cell lysis and morphological changes of *Staphylococcus aureus* (Sawer *et al*., 2005). Berberine **(Figure 2-2)** is found in roots, rhizomes and stem bark of plants. Extracts and decoctions of berberine have significant antimicrobial activity against organisms such as bacteria, viruses, fungi, protozoans, helminths and Chlamydia (Birdsall and Kelly, 1997). Clinically, berberine is used in the treatment of bacterial diarrhoea due to its ability to reduce intestinal secretion of water and electrolytes induced by cholera toxin, as well as inhibition of some *Vibrio cholerae* and *Escherichia coli* enterotoxins (Sack and Froelich, 1982)

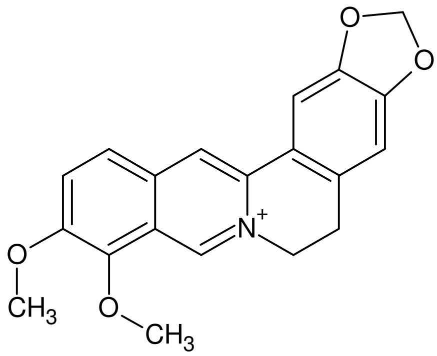


Figure 2-2. Berberine

2.2.3. Phenolics and polyphenols

Phenolic compounds include a wide range of secondary metabolites found in plants. They possess in common an aromatic ring substituted by one or more hydroxyl groups (Harborne, 1994). The main classes are simple phenols, hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones, hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (Chung *et al.*, 1998; Krueger *et al.*, 2003).

The common representatives of a wide group of phenylpropane-derived compounds that are in the highest oxidation state are cinnamic and caffeic acids. Caffeic acid, which is effective against viruses, bacteria, and fungi, is found in common herbs such as tarragon and thyme (Cowan, 1999). Catechin and pyrogallol are both hydroxylated phenols, shown to be toxic to microorganisms. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Mason and Wasserman, 1987). Eugenol is a well-characterised representative found in clove oil **(Figure 2-3)**. Phenolic constituents present in essential oils are generally recognized as active antimicrobial compounds. Eugenol, carvacrol, and thymol are phenolic compounds in cinnamon, cloves, sage, and oregano that possess antimicrobial activity. The exact cause-effect relation for the mode of action of phenolic compounds has not so far been determined; however, researches indicated that they may inactivate essential enzymes, reacting with the cell membrane or disturbing material functionality (Zaika, 1988).

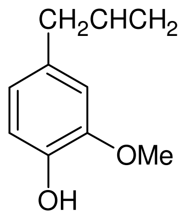
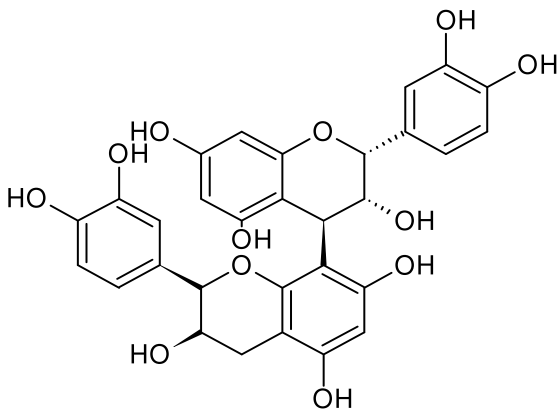


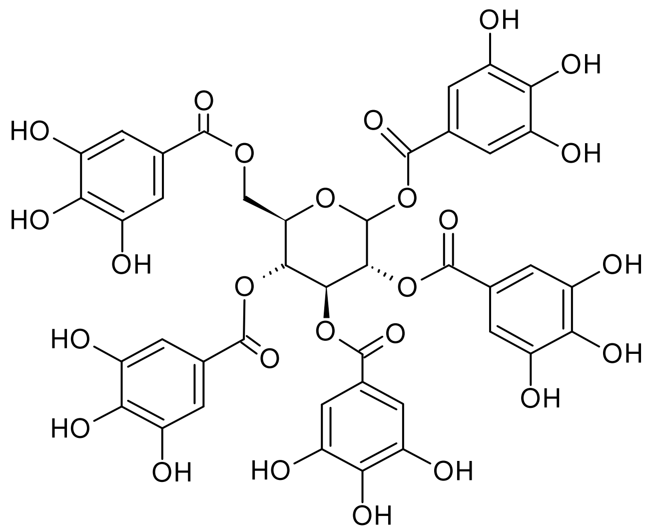
Figure 2-3. Eugenol

2.2.4. Tannins

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatine from solution, a property known as astringency (Haslam, 1996). Tannins are found in almost every plant part; bark, wood, leaves, fruits and roots. They are divided into two groups; hydrolysable tannins **(6)** which are based on gallic acid or ellagic acid, and usually occur as multiple esters with D-glucose; and condensed tannins **(5)** which are derived from flavonoid monomers. Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins etc. Both hydrolysable and condensed tannins have been found to be strong inhibitors of HIV-1 reverse transcriptase *in vitro* (Bessong *et al*., 2004).



Procyanidine B-2 (5)

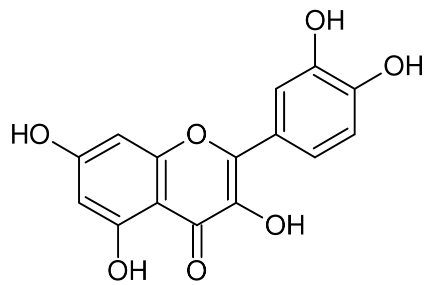


Pentagalloyl glucose (6)

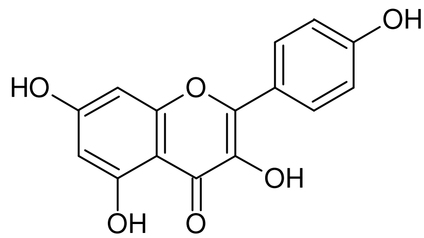
Figure 2-4. Examples of tannins

2.2.5. Flavonoids: Flavones and Flavonols

Flavonoids are an important group of polyphenols, widely distributed in plant flora. About 4000 flavonoids are known to exist and some of them are pigments in higher plants. Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). The common flavonoids found in plants are quercetin and kaempferol **(Figure 2-5)**. Flavonoids are derived from parent compounds known as flavans. Since they are known to be synthesised by plants in response to microbial infection, it should not be surprising that they have been found to be effective antimicrobial substances against a wide array of microorganisms. Their activity may be due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Tsuchiya *et al.*, 1996).



Quercetin (7)



Kaempferol (8)

Figure 2-5. Two biologically active flavonoids

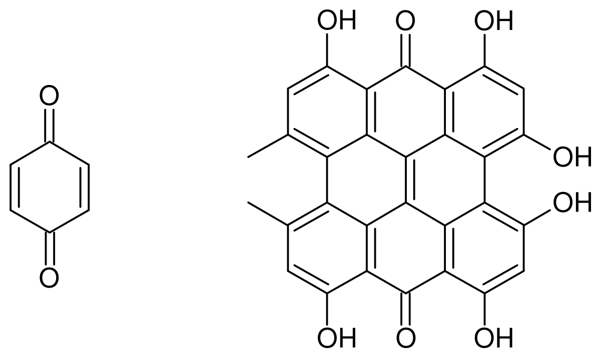
2.2.6. Saponins

Saponins are a vast group of glycosides, widely distributed in higher plants. They are abundant in many foods consumed by animals and man (Cheeke, 1971). Saponins are distinguished from other glycosides by their activity in decreasing surface tension. Many saponins have pharmacological properties and are used in phytotherapy and in the cosmetic industry (Sparg *et al.*, 2004). Saponins can be classified into two groups based on the nature of their aglycone skeleton. Steroidal saponins are almost exclusively present in the monocotyledonous angiosperms, while triterpenoid saponins occur mainly in the dicotyledonous angiosperms (Bruneton, 1995). Saponins are believed to form the main constituents of many plant drugs and folk medicine, and are considered responsible for numerous pharmacological properties (e.g. ginseng constituents). They have also been reported to possess antibacterial activity

(Sparg *et al.*, 2004). The two acylated bisglycoside saponins, Acaciaside A and B, isolated from *Acacia auriculiformis* have antifungal and antibacterial activity (Mandal *et al.,* 2005).

2.2.7. Quinones

Quinones may be defined as aromatic rings with two ketone substitutions **(Figure 2-6)**. Quinones are characteristically highly reactive. They are responsible for the browning reaction in cut or injured fruits and vegetables which happens because of polymerisation in the presence of oxygen and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). Oxidation and reduction reactions allow an easy switch between diphenol and diketone. Vitamin K which is a complex naphthoquinone possesses antihaemorrhagic activity which may be related to its ease of oxidation in body tissues (Harris, 1963). Quinones may complex irreversibly with nucleophilic amino acids in proteins (Stern *et al.*, 1996), which leads to inactivation of the protein and loss of function.



Benzoquinone (9) **Hypericin (10)**

Figure 2-6. Quinones

2.3. Combretaceae

2.3.1. Botanical overview

According to their characters, habitat and structural arrangement, plants are classified into different groups. The genus *Terminalia,* consisting of about 250 species, belongs to the family Combretaceae (Leistner, 2000).The Combretaceae is a large family with at least 600 species (Katerere *et al*., 2003).The Combretaceae family is placed in the myrtales order, whose members are characterised by simple estipulate leaves, flowers in racemose cluster, calyx fused with the ovary to form hypanthium and unilocular inferior ovary with 2 to 6 pendulous ovules (Bhattacharyya and Johri, 1998). Myrtales belongs to the Rosidae subclass which itself belongs to the class magnoliopsida (dicotyledons) and their division is magnoliophyta (angiosperms) as shown below.

Division	-	Magnoliophyta (angiosperms)
Class	-	Magnoliopsida (dicotyledons)
Subclass	-	Rosidae
Order	-	Myrtales
Family	-	Combretaceae
Genus	-	*Terminalia* L.

2.3.1.1. The genus *Terminalia* L.

The genus *Terminalia* is pantropical, including trees and shrubs. It is found in rain forests, woodlands, grasslands and shrublands. These plants are distributed throughout the tropics, extending to subtropics, with 14 species widespread in southern Africa except in the Free State, Lesotho, Western and Eastern Cape (Leistner, 2000). *Terminalia glaucescens* is found in Africa north of the equator on well-drained soils, in areas with an annual rainfall of 760-1500 mm and with 2 - 4 dry months (van der Maesen *et al*., 1994).*Terminalia sericea* is common on sandy soil, in the semi-arid to medium rainfall area of southern and south central Africa (Van der Maesen *et al*., 1994).

The plants of this genus are commonly called the myrobalans. Of the 20 genera belonging to the Combretaceae family, the myrobalans are the most important plants (Bhattacharyya and

Johri, 1998). **Table 2-1** shows a list of species found in southern Africa as well as their common names:

Table 2-1. List of *Terminalia* species and their common names (Carr, 1988)

SCIENTIFIC NAME	COMMON NAME
Terminalia brachystemma Welw. Ex Hiern	Kalahari cluster leaf/groenvaalboom
Terminalia mollis Laws	Large leaved terminalia
Terminalia phanerophlebia Engl. & Diels	Lebombo cluster leaf/Lebombo trosblaar
Terminalia prunioides M.A. Lawson	Purple-pod cluster leaf/Sterkbos
Terminalia randii Bak. f.	Spiny cluster leaf/Doring trosblaar
Terminalia sambesiaca Engl. & Diels	River terminalia, River cluster leaf
Terminalia sericea Burch. Ex DC	Silver cluster leaf/vaalboom/mogonono
Terminalia stenostachya Engl. & Diels	Rosette cluster leaf/Roset vaalboom
Terminalia stuhlmannii Engl.	Zigzag cluster leaf/Sigsag trosblaar
Terminalia trichopoda Diels	Tawny cluster leaf/bruinvaalboom
Terminalia erici-rosenii R. E. Fr.	N/A
Terminalia griffithsiana Liben	N/A
Terminalia kaiserana F. Hoffm.	N/A
Terminalia gazensis Bak. f.	Fringed leaf terminalia

The *Terminalia* genus is further divided into different sections based on plant characteristics, namely: Fatrea, Abbreviatae, Psidioides, Platycarpa, Pteleopsoides (Launert, 1978). Of the five sections, three include the species found in South Africa (Launert, 1978). **Table 2-2** shows all the species belonging to the three sections as well as their distribution.

Table 2-2. Distribution of *Terminalia* species (in southern and east Africa) (Launert, 1978)

SECTION	SPECIES	DISTRIBUTION
ABBREVIATAE	*Terminalia prunioides* *Terminalia randii* *Terminalia stuhlmannii*	Botswana, Zambia, Zimbabwe, South Africa Botswana, Zambia, Zimbabwe Botswana, Zambia, Zimbabwe, Mozambique
PSIDIOIDES	*Terminalia erici-*	Zambia

	risenii	
	Terminalia sericea	Botswana, Zambia, Zimbabwe, Malawi, Mozambique, South Africa, Tanzania
	Terminalia trichopoda	Botswana, Zambia, Zimbabwe, Malawi, Mozambique
	Terminalia brachystemma	Zambia, Zimbabwe, Mozambique, South Africa
	Terminalia kaiserana	Zambia, Malawi, Tanzania
PLATYCARPA	*Terminalia mollis*	Zambia
	Terminalia Stenostachya	Zambia, Zimbabwe, Mozambique, Malawi
	Terminalia gazensis	Zimbabwe, Malawi, Mozambique
	Terminalia Sambesiaca	Zambia, Zimbabwe, Mozambique, Tanzania
	Terminalia phanerophlebia	Mozambique, South Africa

Figure 2-7. ***Terminalia sambesiaca*** **tree**

Figure 2-8. ***Terminalia sambesiaca***

2.4. Ethnopharmacology of Combretaceae

The Combretaceae is an important resource in traditional medical practice. It is widely distributed in the tropical areas of Africa, South America and Asia (excluding Australia). Species belonging to the two main genera, *Combretum* and *Terminalia* have been used in the treatment of many ailments including syphilis, abdominal pains, conjunctivitis, diarrhoea and toothache (Hutchings *et al.,* 1996; Watt and Breyer-Brandwijk, 1962). Leaf extracts of several species of *Combretum*, *Terminalia*, *Pteleopsis* and *Quisqualis* all possess antibacterial activity (Eloff, 1999). *Combretum woodii* Duemmer and *Combretum microphyllum* Klotzsch are active against Gram-positive and Gram-negative bacteria (Eloff *et al*., 2005a; Kotzé and Eloff, 2002). *Combretum zeyheri* (Sond) also contains antimicrobial compounds (Breytenbach and Malan, 1989). According to Martini and Eloff (1998), *Combretum erythrophyllum* (Burch) contains at least 14 antibacterial compounds.

Baba-Moussa and colleagues (1999) tested seven Combretaceae species for antifungal activity and found that all the investigated species were active. *Terminalia avicennioides*, *Pteleopsis* species and *Combretum nigicans* contain large quantities of saponins and tannins which are believed to be responsible for their antifungal activity (Baba-Moussa *et al*., 1999).*Terminalia sericea* extracts are used to treat bacterial infections and diarrhoea (Fyhrquist *et al*., 2002). Intermediate and polar extracts of the roots of *T. sericea* exhibited antibacterial activity against *S. aureus, E. coli, P. aeruginosa* and antifungal activity against *C. albicans* (Moshi and Mbwambo, 2005).

2.4.1. Ethnopharmacology of *Terminalia*

The use of decoctions of several *Terminalia* species is widespread in Africa, and many species are known to contain antimicrobial constituents (Fyhrquist *et al*., 2002).

Terminalia macroptera

Ethanol extracts of *Terminalia macroptera* are active against *Shigella dysenteriae, Vibrio cholerae, Escherichia coli, Salmonella* spp. and *Campylobacter* spp. (Silva *et al*., 1997). Among the species investigated by Silva *et al.* (2002), *Terminalia macroptera* leaf extracts had the highest activity against *N. gonorrhoeae*.

Terminalia sericea

Powdered roots of *Terminalia sericea* are used with mylabris beetles for the treatment of schistosomiasis (Hutchings *et al*., 1996). The Vhavenda use the leaves of this plant for wounds and menorrhagia, the bark for wounds, while the roots are used for diarrhoea, infertility and STDs (Hutchings *et al*., 1996). The roots are also used in logical disorders, venereal diseases, general weakness, sore throats and nose bleeds (Hutchings *et al*., 1996). The powdered bark is taken with corn meal against diabetes (Watt & Breyer-Brandwijk, 1962). The dried fruits are used for the treatment of tuberculosis (Eldeen *et al.,* 2006). The root extracts of *T. sericea* have been found to be active against both Gram-positive and Gram-negative bacteria (Moshi and Mbwambo, 2005). Leaf extracts of *T. sericea* have also been found to possess antibacterial activity (Eloff, 1999). The beneficial effects of decoctions made from *Terminalia sericea* on HIV/AIDS patients may be linked to its inhibition of common opportunistic infections of bacterial or fungal aetiology, since *T. sericea* has been found to inhibit RNA-dependent DNA polymerase and ribonuclease H functions of HIV-1 reverse transcriptase (Bessong *et al*., 2004).

Terminalia sambesiaca

Terminalia sambesiaca is effective against a wide range of microorganisms. The methanolic root extracts of this species have been found to be nearly as effective as Amphotericin-B against *Candida albicans* (Fyhrquist *et al*., 2004). *Terminalia sambesiaca* is the most potent of 17 species of *Combretum* and *Terminalia* plants tested by Fyhrquist and colleagues (2002). Some biological tests of the antibacterial effects of this species have been carried out by Chhabra and colleagues (1981), who reported that the root extracts are active against *S. aureus*, *P. aeruginosa*, *S. typhi* and *S. boydii*. Extracts of the stem bark of *T. sambesiaca* showed antibacterial activity against *S. aureus*, *P. aeruginosa*, *S. typhi* and *S. boydii* (Chhabra *et al.,* 1989). Leaf extracts of *T. sambesiaca* also showed antifungal activity against *C. albicans* and *C. neoformans* (Masoko *et al.,* 2005; Masoko and Eloff, 2005). The powdered root bark of *T. sambesiaca* is mixed with porridge and used to treat bloody diarrhoea, while decoctions of the stem bark and leaves are used to treat cancer, stomach ulcers and appendicitis (Chhabra *et al.,* 1989).

Terminalia kaiserana

Methanolic root and leaf extracts of *Terminalia kaiserana* are bactericidal and this supports the use of this plant for the treatment of diarrhoea in traditional medicine (Fyhrquist *et al.*, 2002). The decoctions the roots of *T. kaiserana* are used for the treatment of backache and headache (Chhabra *et al.,* 1989).

Leaf, root and fruit extracts of *Terminalia brachystemma* are effective anthelmintics (Molgaard *et al.*, 2001). *Terminalia glaucescens* shows a wide spectrum of antibacterial activity against periodontic bacteria as well as antiplasmodial activity (Valentin *et al.*, 2000). *Terminalia chebula* and *Terminalia belerica* extracts are active against several pathogenic microorganisms (Ahmad *et al.*, 1998). When assayed for bacterial activity, *Terminalia arjuna* had significant activity against *Escherichia coli, K. aerogenes, P. vulgaris and P. aerogenes* which are Gram-negative organisms. This suggests the presence of an active antibacterial principle in the extract which supports the traditional use against infections by Gram-negative bacteria (Samy *et al.*, 1998).

2.4.2. Phytochemistry of *Terminalia* species

Among the Combretaceae genera, *Terminalia* is known as a rich source of pentacyclic triterpenes and their glycoside derivatives, flavonoids, tannins and other aromatic compounds (Garcez *et al.*, 2003).

2.4.2.1. *Terminalia sericea* and *T. superba*

Nerifolin, which is a glycoside isolated from *Terminalia sericea*, inhibits fibroblastic outgrowth in anural explanted heart tissue *in vitro* and inhibits the pulsation rate in a dilution of 1:700 or higher. Galls from trees contain 10,2% tanning matter. Triterpenoids, sericic acid and sericoside are the major constituents of roots of *T. sericea* trees from Mozambique. Sericic acid and sericoside possess anti-ulcer, anti-inflammatory and cicatrizing activity (Hutchings *et al.*, 1996; Rode *et al.*, 2003). The triterpene sericoside has been isolated from *Terminalia sericea* (Moshi and Mbwambo, 2005; Eldeen *et al.*, 2006).

The analysis of *Terminalia sericea* and *Terminalia superba* showed a complex sugar composition containing galacturonic, glucuronic, and 4-*O*-methylglucuronic acids as well as arabinose, xylose, galactose, mannose and rhamnose (Anderson and Bell, 1974).

Eldeen and colleagues (2006) isolated anolignan B **(Figure 2-9)** from *Terminalia sericea* which showed activity against Gram-positive and Gram-negative bacteria as well as anti-inflammatory activity (Eldeen *et al.*, 2006).

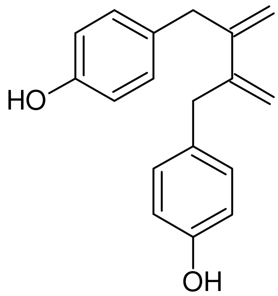


Figure 2-9. Anolignan B

2.4.2.2. *Terminalia mollis* and *T. brachystemma*

Leaves of *Terminalia mollis* contain tannins and saponins (Baba-Moussa *et al.*, 1999). The phytochemical investigation of *Terminalia mollis* and *Terminalia brachystemma* led to the isolation of six triterpenes , eight flavonoids , ellagitannins as well as gallic acid and 3-O-methylellagic acid 4'-O-α-rhamnopyranoside, some of which displayed good antifungal activity (Liu *et al.*, 2009).

2.4.2.3. *Terminalia stuhlmannii*

Katerere and colleagues (2003) have isolated triterpenoids from *Terminalia stuhlmannii* and *Combretum imberbe*. Pentacyclic triterpenes,1α,3β-hydroxyimberbic acid 23-*O*- α-L-4-acetylrhamnopyranoside **(11)** and 1α,3β,3,23-trihydroxy-olean-12-en-29-oate-23-*O*-α-[4-acetoxyrhamnopyranosyl]-29-α-rhamnopyranoside **(12)** have been isolated from the stem bark of *T. stuhlmanni* for the first time. These triterpenes are based on the 23-hydroxylated prototype aglycone of *Combretum imberbe*, thus, providing evidence of a chemotaxonomic link between *Combretum* and *Terminalia.* When subjected to microbial activity tests, both compounds were active against *Staphylococcus aureus* and the compound, **(12)** was also active against *Candida albicans*.

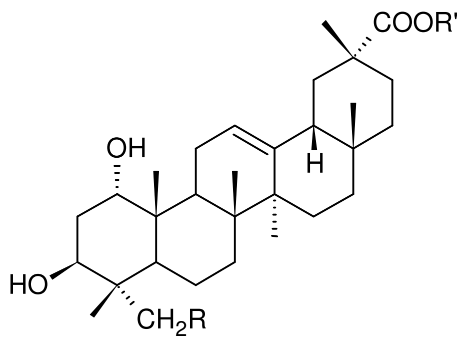


Figure 2-10. Pentacyclic triterpenes from *T. stuhlmanni*

11	**12**
R= 4-Ac-O-Rh	R= 4-Ac-O-Rh
R'= H	R'= Rh

2.4.2.4. *Terminalia glaucescens*

The phytochemical study on the stem bark extract of *Terminalia glaucescens* has led to the isolation of a novel triterpene compound, terminalin A (**Figure 2-11**) (Rahman *et al*., 2002). This plant is used extensively in African indigenous medicines, prescribed as an anti-dysenteric and antimicrobial agent (Rahman *et al*., 2002). Other compounds isolated from *T. glaucescens* are β-sitosterol, stigmasterol, lupeol, betulinic acid, β-amyrin and long chain fatty acids. Terminalin A showed inhibitory activity (IC_{50} = 73.23) against propyl endopeptidase (peptidase that hydrolyzes peptide bonds at the *L*- proline carboxyl terminal, also involved in learning and memory) (Rahman *et al*., 2002).

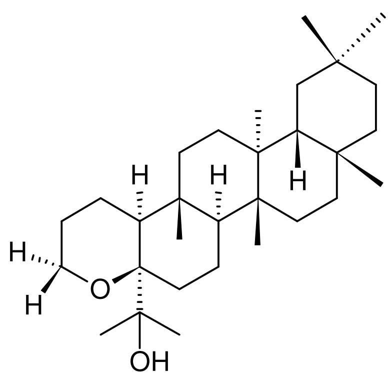


Figure 2-11. Terminalin A

2.4.2.5. *Terminalia macroptera*

The stem bark of *T. macroptera* contains antifungal and antibacterial triterpenes (Conrad *et al.,* 1998). Hydrolysable tannins isoterchebulin and 4,6-*O*isoterchebuloyl-D-glucose found in the stem bark of *T. macroptera* also possess bacteriostatic effects (Conrad *et al.,*2001). The leaves of *T. macroptera* contain chlorogenic acid, quercetin, isoorientin, ellagitannins and their monomers gallic acid and ellagic acid (Silva *et al.,* 2002). Terminolic acid **(Figure 2-12)** was isolated from *Terminalia macroptera* and was found to be active against bacterial pathogens including *Bacillus subtilis* and *Pseudomonas fluorescens* (Conrad *et al.*, 1998).

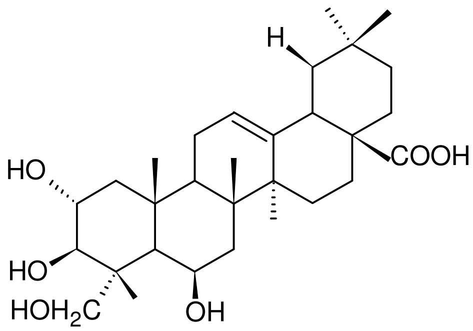
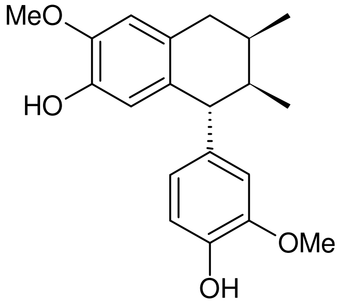


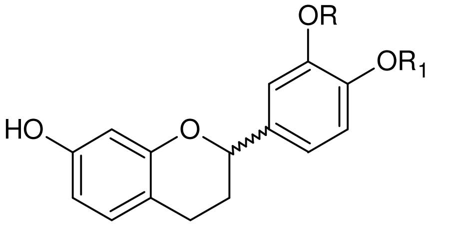
Figure 2-12. Terminolic acid

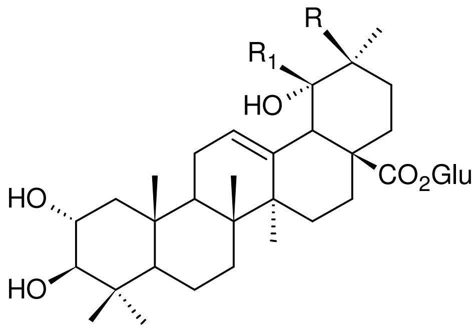
2.4.2.6. *Terminalia argentea*

Garcez *et al* isolated isoguaiacin **(13)**, 7,3'-dihydroxy-4'-methoxyflavan **(14)**, 7,4'-dihydroxy-3'-methoxyflavan **(15)**, tormentic acid **(16)** and arjunetin **(17)** from *T. argentea* as shown in **Figure 2-13** (Garcez *et al.*, 2003). Pentacyclic triterpenes have been reported in other *Terminalia* species, but the flavans which are not widespread in plants have only been reported in few families.



13





14: R = H, $R_1 = CH_3$
15: $R = CH_3$, R_1 = H

16: R = H, $R_1 = CH_3$
17: $R = CH_3$, R_1 = H

Figure 2-13. Compounds isolated from *T. argentea*

2.5. Conventional antimicrobial agents

Antimicrobial agents can be categorised according to whether they are antibiotics (derived from the growth of microorganisms), chemotherapeutic agents (synthetic compounds not found in nature) or derivatives from nonmicrobial natural sources (lichens, higher plants and animals) (Trease and Evans, 2002). According to Walkman's 1951 definition of antibiotics, the term is limited to substances produced by microorganisms. The term antibacterial is consequently used to include those active compounds prepared synthetically or isolated from higher plants. Most of the clinically used antibiotics are produced by soil microorganisms or fungi (Trease and Evans, 2002).

Antibacterial drugs are classified as either bacteriostatic or bactericidal. Bacteriostatic drugs arrest the growth and replication of bacteria at serum levels achievable in the patient, thus limiting the spread of infection while the body's immune system attacks, immobilises and eliminates the pathogens. If the drug is removed before the immune system has scavenged the organisms; enough viable organisms may remain to begin a second cycle of infection. Bactericidal drugs kill bacteria at drug serum levels achievable in the patient. Because of their more aggressive antimicrobial action, these agents are often the drugs of choice in seriously ill patients (Howland and Mycek, 2006).

The antibacterial drugs may be divided by the spectra of bacteria for which they are therapeutically effective i.e. narrow-spectrum, extended spectrum and broad spectrum:

(i) Narrow-spectrum antibacterial drugs are those that act only on a single or limited group of microorganisms. For example isoniazid which is active only against mycobacteria.
(ii) Extended spectrum antibacterial drugs are those that are effective against Gram-positive organisms and also against a significant number of Gram-negative bacteria, e.g. ampicillin which acts against Gram-positive and some Gram-negative bacteria.
(iii) Broad-spectrum antibacterial drugs are exemplified by tetracycline and chloramphenicol which affect a wide variety of microbial species (Howland and Mycek, 2006).

2.5.1. History of antimicrobial agents

Flemming noted the inhibition of bacteria by a colony of *Penicillum notatum* that had developed as a contaminant on a Petri dish (Howland and Mycek, 2006).

By 1940, significant quantities of the first penicillin from cultures of *Penicillum notatum* were produced. Penicillin G had clinical limitations which were its instability at acidic pH, susceptibility to destruction by beta-lactamase (penicillinase) and its relative inactivity against Gram-negative bacteria. These limitations were overcome by the production of semi-synthetic penicillins (Katzung, 1998).

Resources of industry and academic institutions were devoted to the study of penicillins and search for other antibiotics leading to the discovery of streptomycin, aureomycin, chloromycetin and other antibiotics involving various species of *Streptomyces, Cephalosporium* and *Penicillum* (Howland and Mycek, 2006; Kong and Liang, 2003).

Unfortunately, the widespread and indiscriminate use of antibiotics, together with poor hygiene has led to many pathogenic organisms acquiring resistance to specific antibiotic treatments. This problem is worsened by the fact that resistance to a particular antibiotic can be transferred from one organism to another. Thus, the clinician's antibiotic armamentarium is being steadily eroded, increasing the need for research in isolation or synthesis of new drugs (Trease and Evans, 2002).

2.5.2. Antibiotic resistance

The numbers of resistant strains of microbial pathogens are growing since penicillin resistant and multiresistant pneumococci caused a major problem in South African hospitals in 1977 (Eloff,1998b).This emergence of antibiotic resistance by bacteria has become a medical catastrophe and may lead to a post antibiotic era where antibiotics are no longer effective (Martini and Eloff, 1998). Currently, there are identified bacteria that are resistant to all available antibiotics. It is thus important to discover and develop new antimicrobial compounds that are effective against a wide spectrum of bacteria (Fyhrquist *et al.*, 2002).

Due to the increasing antibiotic resistance of bacterial isolates, efforts to find alternative antimicrobial components have been intensified. Scientists have consequently investigated plants for the presence of antimicrobial components (Eloff, 1998b). Traditional herbs have shown effectiveness against microorganisms making plants one of the bedrocks for modern medicine to attain new compounds. It is documented that plants have contributed about 7000 pharmaceutically important compounds and a number of top-selling compounds of modern time such as quinine, artemisinin, taxol and camptothecin (Bonjar *et al.*, 2003). The antimicrobial compounds from plants may inhibit bacteria through different mechanisms than conventional antibiotics and could therefore be of clinical value in the treatment of resistant microbes (Fyhrquist *et al.*, 2002).

2.5.2.1. Mechanism of resistance

Microbial species that are normally responsive to a particular drug may develop more virulent, resistant strains through spontaneous mutation or acquired resistance and selection. Resistance can be due to genetic alterations or altered expression of proteins. Bacterial resistance has been seen as the major obstacle since the beginning of antibiotic era. Nearly all groups of antibiotics have at least some bacterium that has developed resistance to them (Amyes, 2000). Methicillin-resistant *Staphylococcus aureus* is among the bacterial species that are capable of causing life-threatening illnesses (Bell *et al.,* 2002). Scientists have found strains of *S. aureus* to be resistant to vancomycin which is regarded as the antibiotic of last resort for the treatment of resistant infections.

2.5.2.1.1. Genetic alterations leading to drug resistance

Acquired antibiotic resistance requires the temporary or permanent gain or alteration of bacterial genetic information. Resistance develops due to the ability of DNA to undergo mutation or to move from one organism to another. There may be chromosomal alteration which occurs by insertion, deletion or substitution of one or more nucleotides within the genome. The cell thus replicates and transmits its mutated properties to progeny cells. This then results in organisms that proliferate under certain selective pressures, e.g. emergence of rifampicin-resistant *Mycobacterium tuberculosis* when rifampicin is used alone. Resistance can also be acquired due to DNA transfers from bacterium to another. Resistant properties are encoded in extra chromosomal R factors (resistant plasmids). Plasmids may enter cells by transduction, transformation or bacterial conjugation (Howland and Mycek, 2006).

2.5.2.1.2. Altered expressions of proteins

Resistance may be due to lack or alteration in an antibiotic site, lowered penetrability of the drug, increased efflux of the drug or presence of inactivating enzymes (Howland and Mycek, 2006).

2.5.2.1.3. Modification of the target site

Alteration of the antibiotic target site through mutation can confer organism resistance to one or more related antibiotics. E.g. *S. pneumoniae* resistance to β - lactam antibiotics involves alterations in one or more of the major bacterial penicillin binding proteins, resulting in decreased binding of the antibiotic to its target.

2.5.2.1.4. Decreased accumulation

Decreased uptake or increased efflux of a drug can lead to resistance, because the drug is unable to attain access to the site of its action in sufficient concentrations to kill the organism.

2.5.2.1.5. Enzymatic inactivation

The antimicrobial agent may be destroyed or inactivated by enzymes. These include β - lactamases that hydrolytically inactivate the β - lactam ring of penicillins, cephalosporins and related drugs, acetyltransferases that transfer an acetyl group to the antibacterial agent inactivating chloramphenicol or aminoglycosides, and esterases that hydrolyse the lactone ring of macrolides (Katzung, 1998; Howland and Mycek, 2006).

2.6. Bacteria of clinical significance as nosocomial agents

2.6.1. *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic pathogen that affects immunocompromised individuals and causes life-threatening infections in cystic fibrosis patients. Colonization by *P. aeruginosa* involves a biofilm mode of growth which is promoted by the production of exopolysaccharides (Sadovskaya *et al*., 2010). Biofilms are cellular aggregates encased in an extracellular matrix. Two loci, *pel* and *psi*, are involved in the production of carbohydrate-rich components of the biofilm matrix (Friedman and Kolter, 2004). In addition to biofilm formation, *P. aeruginosa* is capable of surface-associated motility which includes twitching and swarming (Kuchma *et al.,* 2010). *P. aeruginosa* is unable to cross the defence barriers of the healthy individual, but can cause life-threatening infections in immunocompromised hosts. Burn patients, patients with cystic fibrosis and patients with respiratory disease are the most likely to be threatened by *P. aeruginosa*. It may also cause meningitis and urinary tract infections (Bonjar *et al*., 2003).

2.6.2. *Staphylococcus aureus*

Infectious complications are a major source of post-operative morbidity and mortality (Martineau *et al*., 1998). *Staphylococcus aureus* causes a wide variety of infections ranging from benign skin infections to life threatening diseases (François *et al*., 2010). It is a major pathogen implicated in diabetic foot infections (Caputo *et al*., 2000). For adherence, bacterial cells form firm interactions with human cell surfaces and prevent their rapid elimination by physicochemical mechanisms. The surface components of the staphylococcal cell interact with complementary components on the eukaryotic host cell membranes. Methicillin-resistant *Staphylococcus aureus* strains are becoming increasingly prevalent among nosocomial and community acquired infections (Archer 1998; François *et al*., 2010).

2.6.3. *Escherichia coli*

Enterotoxigenic strains of *E. coli* have shown to be important aetiological agents among the spectrum of pathogens that cause acute diarrhoea. Colonization of the small intestine involves adherence to enterocytes (Nalin *et al.,* 1975; Guerrant *et al*., 1975). Diffusely adherent *Escherichia coli* pathogens have been found to be responsible for childhood diarrhoea (Scaletsky *et al*., 2002) Due to its tendency to cause outbreaks of severe and sometimes fatal diarrheal disease, *E. coli* has become a focus of public health attention. The uropathogenic *E. coli* is the main causative agent of urinary tract infections which cause more than 8 million clinic office visits annually (Litwin *et al*., 2005).

2.6.4. *Enterococcus faecalis*

E. faecalis, being among the most commonly isolated Gram-positive bacteria in the clinical microbiology laboratory, is capable of invading the urinary tract, causing wound infections such as surgical infections and intra-abdominal abscesses (Koenig and Kaye, 1961). It ranks third among the most common pathogens isolated from patients with urinary tract infections (Richards *et* a*l*., 2000). Infections due to multiple-drug-resistant strains are a serious medical problem.

CHAPTER 3: MATERIALS AND METHODS

3.1. Plant collection

Leaves of *Terminalia* species were collected in the Lowveld National Botanical Gardens (LNBG) in Nelspruit, South Africa in the summer of 2004. It has been found that summer is the best period to collect leaves, because new leaves are growing in numbers during this season. The origin of each tree is recorded in the database of the botanical garden. Voucher specimens of the trees are kept in the garden's herbarium. More information on the origin and references of these plants are presented elsewhere (Eloff, 1999). *Terminalia* species collected are listed in **Table 3-1.**

Table 3-1. *Terminalia s*pecies collected for antifungal and antibacterial screening

Terminalia **L.**	
Section	Species
Psidioides Exell	*T. brachystemma* Welw. ex Hiern
	T. sericea Burch ex DC
Platycarpa Eng. Diels emend Exell	*T. gazensis* Bak. f.
	T. mollis Laws
	T. sambesiaca Engl. & Diels
	T. phanerophlebia Engl. & Diels

3.2. Plant storage

Leaves were separated from stems, and dried at room temperature. Most scientists have tended to use dried material because there are fewer problems associated with large-scale extraction of dried plants rather than fresh plant material (Eloff, 1998a). The dried plants were milled to a fine powder in a Macsalab mill (Model 200 LAB, Eriez®, Bramley), and stored at room temperature in closed containers in the dark until used.

A reason for choosing dried leaves to work with is that the time delay between collecting plant material and processing it makes it difficult to work with fresh material because differences in water content may affect solubility or subsequent separation by liquid-liquid extraction. The secondary metabolic plant components should be relatively stable especially if it is to be used as an antimicrobial agent and many if not most plants are used in the dried form by traditional healers (Eloff, 1998a).

3.3. Extractants

When choosing the extractants the following parameters were considered: polarity (polar, intermediate or non-polar), the ease of subsequent handling of the extracts, the toxicity of the solvent in the bioassay process, and the potential health hazard of the extractants (Eloff, 1998b). The following extractants were chosen on the basis of the above parameters: methanol (polar); ethyl acetate (intermediate polarity); dichloromethane (intermediate polarity) and hexane (non-polar).

3.3.1. Extraction procedure

Plant samples from each species in **Table 3-1** were individually extracted by weighing four aliquots of 1 g of finely ground plant material and sequentially extracting with 10 ml of hexane, dichloromethane (DCM), ethyl acetate and methanol (all in technical grade - Merck) in polyester centrifuge tubes. Tubes were vigorously shaken for 3 - 5 minutes in a Labotec model 20.2 shaking machine at high speed. After centrifuging at 1.643 x g for 10 minutes the supernatant was decanted into pre-weighed labelled containers. The process was repeated 3 times to exhaustively extract the plant material and the extracts were combined. Extraction was done at room temperature. The solvent was removed under a stream of air in a fume cupboard at room temperature, to quantify the extraction. Methanol extracts were dried under high vacuum until they were completely dry.

3.4. Phytochemical analysis of compounds with different polarities and antioxidative activity

3.4.1. Thin Layer Chromatography (TLC)

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F_{254}). The TLC plates were developed under saturated conditions with one of the three eluent systems developed in the Phytomedicine Programme laboratory that separate components of Combretaceae extracts well, i.e.

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/ammonia hydroxide (90:10:1): [BEA] (non - polar / basic) (Kotzé and Eloff, 2002).

The dried extracts of the solvents were reconstituted to a concentration of 10 mg/ml in acetone. Acetone was the solvent of choice owing to its low toxicity towards the test organisms in the bioassay procedures (Eloff, 1998b).

Approximately 100 µg aliquots (10 µl of a 10 mg/ml solution) of each of the extracts were loaded in 1 cm bands on three 10 x 10 cm TLC plates (Merck, silica gel 60 F_{254}) and each of these was developed with EMW, CEF or BEA. The extracts were applied approximately 1 cm from the bottom of the plates with a micropipette and allowed to develop for 8 to 9 cm in a tank containing eluent. The atmosphere in the tank was saturated by placing filter paper wetted with the eluent against the walls of the tanks, which were then sealed with lids.

Once developed, the separated compounds were observed under Camac Universal TL-600 UV light at 360 nm and 254 nm and the fluorescing (360 nm) or quenching (254 nm) compounds marked. To detect the separated compounds, vanillin-sulphuric acid (0.1 g vanillin (Sigma®): 28 ml methanol: 1 ml sulphuric acid) was sprayed on the chromatograms and heated at 110 °C to optimal colour development.

3.4.2. TLC-DPPH screening for antioxidative activity

This method is generally used for the screening of potential antioxidant activity in crude plant extracts. It involves the chromatographic separation of the crude plant extract, after which the developed chromatogram is sprayed with a coloured radical solution and the presence of antioxidant compounds is indicated by the disappearance of the radical's colour. Ten microlitres (10 µl) of each extract was loaded as a 1 cm band on the origin of the TLC (Merck, silica gel 60 F_{254}) plates. Plates were developed using BEA, CEF and EMW (**Section 3.4**). Plates were viewed under UV (254 and 360 nm) light to locate the UV active compounds. To detect antioxidant activity, chromatograms were sprayed with a solution of 0.2 % 2, 2 – diphenyl -1-picrylhydrazyl (DPPH) (Sigma®) in methanol, as an indicator (Deby and Margotteaux, 1970) until just wet, and dried in the fume hood. The presence of antioxidant compounds was detected by yellow spots against a purple background on TLC plates sprayed with 0.2 % DPPH in methanol.

3.5. Minimum Inhibitory Concentration (MIC)

3.5.1. Microdilution assay for bacteria

A serial microdilution assay (Eloff, 1998d) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts using tetrazolium violet reduction as an indicator of growth (Eloff, 1998d; McGaw, *et al*., 2001). For the preliminary tests, specific ATCC strains of two Gram-positive (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 21212) and two Gram-negative (*P. aeruginosa* ATCC 25922 and *Escherichia coli* ATCC 27853) bacteria were used. The selection of test organism strains was based on the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS). These species of bacteria are also the major cause of nosocomial infections in hospitals (Sacho and Schoub, 1993). The work was done in the laminar flow cabinet to limit contamination of the cultures.

3.5.1.1. The experimental design

Test group 1: Consisted of the pathogen plus different concentrations of the extracts. This group was used to determine activity in the extract (MIC value).

Test group 2: Positive control which contained the pathogen plus gentamycin.

This group was used to ensure that the pathogen was not a resistant strain and also to compare relative activities with the extracts.

Test group 3: A pure culture containing only the pathogen. This was necessary to distinguish poor growth from inhibition and to ensure that the laboratory conditions under which the pathogen had been placed did not affect its growth.

Test group 4: A negative control containing the pathogen together with the dissolution solvents. This ensured that the extraction solvents had no inhibitory effects on the pathogens.

3.5.1.2. Dilution of extracts

Two-fold serial dilutions were dispensed into 96 - well microplates as follows, distilled water (100 µl) was placed in each well using a Socorex multichannel micropipette and 100 µl of a 10 mg/ml extract concentration was placed in each first well of the relevant series of dilution, and thereby diluting the extracts in these wells by 50 %. Thereafter, 100 µl was removed from the wells and placed into the next wells. The concentration in these wells would then be 25 % of the original; the next would be 12.5 % and so on. The process was repeated all the way to the bottom of the plates. One hundred µl from the last row was discarded to ensure that all the wells contain 100 µl of the extract. Each column therefore had a series of two-fold dilutions of the extract concentration.

3.5.1.3. Addition of bacteria

Cultures of *S. aureus, E. faecalis, P. aeruginosa* and *E. coli*, grown overnight at 37 ^{o}C and stored in the refrigerator for up to 10 days before testing were used (Eloff, 1998b). The test organisms were prepared by diluting overnight broth culture with sterile diluent (0.1% Peptone water). The turbidity was adjusted to match 0.5 McFarland standards. The broth culture was then diluted 100-fold to give a concentration 10^{8} cfu/ml. One hundred µl of broth containing the relevant bacteria was placed in each of the wells and mixed by squirting the bacteria into wells. The microplates were incubated overnight at 37 ^{o}C. After incubation, 40 µl of 0.2 mg/ml *p* - iodonitrotetrazolium (INT) was added to each well. The microplates were visually examined for colour change after 24 hours of incubation. After addition of INT the microplates were left for 6 hours before being examined for bacterial growth. The lowest concentration that caused growth inhibition was noted and recorded, and the MIC values of the extract were calculated from the original concentration of the extracts. All the tests were conducted in triplicate.

3.5.2. Microdilution assay for fungi

A serial microdilution assay (Eloff, 1998d) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts against fungal species using tetrazolium violet reduction as an indicator of growth. This method had previously been used only for antibacterial activities (Eloff, 1998d; McGaw, *et al.*, 2001). To apply it for measuring antifungal activities, a slight modification was made to suit fungal growth conditions (Masoko *et al.*, 2005). Residues of the different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50 % with water in 96 well microtitre plates (Eloff, 1998d). Fungal cultures were transferred into fresh Sabouraud dextrose broth, and 100 µl of this was added to each well. Amphotericin B was used as the reference antifungal and positive control, and appropriate solvent blanks were included as negative control. As an indicator of growth, 40 µl of 0.2 mg/ml of *p* - iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the microplate wells. The covered microplates were incubated for two to three days at 35 °C and 100 % relative humidity. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 and 48 hours. All the tests were conducted in triplicate.

3.5.2.1. Addition of fungi

Five fungi were obtained from the Central Microbiology Laboratory Faculty of Veterinary Science, University of Pretoria and used as test organisms. These fungi represent the different morphological forms of fungi, namely yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus* and *Microsporum canis*) and are the most common and important disease-causing fungi of animals. All fungal strains were maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK).

3.5.2.2. The experimental design

Test group 1: Consisted of the pathogen plus different concentrations of the extracts. This group was used to determine activity in the extract (MIC value).

Test group 2: Positive control, which contained the pathogen plus Amphotericin B.

This group was used to ensure that the pathogen was not a resistant strain and also to compare relative activities with the extracts.

Test group 3: A pure culture containing only the pathogen. This was necessary to distinguish poor growth from inhibition and to ensure that the laboratory conditions under which the pathogen had been placed did not affect its growth.

Test group 4: A negative control containing the pathogen together with the dissolution solvents. This ensured that the extraction solvents had no inhibitory effects on the pathogens.

3.6. Bioautography

TLC plates (10 x 10 cm) were loaded with 100 µg (5 µl of 20 mg/ml) of each of the extracts. The prepared plates were developed in the three different mobile systems used: CEF, BEA and EMW **(Section 3.4)**. The chromatograms were dried at room temperature under a stream of air to remove the remaining solvent.

3.6.1. Antifungal tests

Fungal cultures were grown on Sabouraud agar for 3 – 5 days. Sabouraud broth was prepared in 250 ml bottles. Cultures were transferred into broth from agar with a sterile swab. The TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately 10^9 organisms per ml of actively growing fungi, e.g. conidia for filamentous fungi and yeast cells for the other fungi in a Biosafety Class II cabinet (Labotec, SA) cupboard. The plates were sprayed until they were just wet, and were then subsequently sprayed with a 2 mg/ml solution of *p* - iodonitrotetrazolium violet (INT) (Sigma®) and incubated overnight or longer in the case of *S. schenckii* and *M. canis* at 35 °C in a clean chamber at 100 % relative humidity in the dark. White areas on the TLC plate indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested fungi. A scanner was used to record results. To minimize fungal spreading and infections in our laboratory, the bioautograms were sealed in clear plastic envelopes before scanning for a permanent record.

3.6.2. Antibacterial tests

The bioautography procedure described by Begue and Kline (1972) was used. Dried TLC (Merck, silica gel 60 F_{254}) plates were sprayed with a concentrated suspension of actively growing cells of American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 21212), *Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853) obtained from the Microbiology Laboratory, Faculty of Veterinary Science, University of Pretoria. The plates were sprayed with bacteria until they were wet and opaque before incubation overnight at 38 ^{o}C in a clean chamber at 100 % relative humidity. Following incubation, the plates were sprayed with 2 mg/ml solution of *p* - iodonitrotetrazolium violet (INT). Clear zones against a red background on TLC plates indicated inhibition of growth after incubation for about an hour (Begue and Kline, 1972).

3.7. Isolation of an antimicrobially active compound from the leaves of *T. sambesiaca*

3.7.1. Bioassay guided extraction procedure

T. sambesiaca showed the presence of more active compounds than the other screened *Terminalia* species. This plant species is also readily available in South Africa and so far there is not much work done on it. *T. sambesiaca* was therefore chosen for the isolation of active compounds.

A number of factors were taken into consideration in choosing solvents that were to be used in the serial exhaustive extraction. The choice of solvent also depended on the kind of compounds to be extracted. Thus, the effect of solvent on subsequent bioassay was an important factor. From previously published work where authors screened plant material for anti-microbial properties, various extractants, from 80 % ethanol, methanol, petroleum ether, chloroform, ethanol and water (Salie and Eagles, 1996) were used. Eloff (1998b) found that acetone extracted a greater number of inhibitors of bacterial growth than other solvents used.

The leaves of *Terminalia sambesiaca* were carefully examined and old, insect damaged, fungus-infected leaves were removed. Healthy leaves were dried at room temperature. Once the leaves were dry, they were ground to a fine powder of *ca* 1.0 mm diameter. The powder was stored in a closed container at room temperature.

The defatting process by hexane is of importance in the isolation process since non-polar compounds will be extracted fast in this process and cellular membranes will be disrupted. Hence, serial exhaustive extraction was used with hexane as a starting solvent followed by dichloromethane (DCM), ethyl acetate and methanol as extractants. The polarity of solvents gradually increased and ranged from a non-polar solvent (hexane) to a more polar solvent (methanol). This was to ensure that a wide polarity range of compounds could be extracted in the process.

Extraction was initially performed on a Labotec Model 20.2 shaking apparatus with a 10 ml: 1 g solvent to dry weight ratio. With large quantities of plant material, the ratio was raised on a proportional scale. Dried leaves (500 g) of the *T. sambesiaca* were exhaustively extracted in a serial manner with solvents of increasing polarity. Finely ground plant material (500 g) was initially extracted with 5000 ml of hexane. The solvent was allowed to extract for 1 hour while shaking before being decanted. The same quantity of solvent was added to the marc and shaken once again for an hour. The process was repeated three times. The marc was allowed to dry and the process of extraction was repeated with dichloromethane, then ethyl acetate, and finally methanol.

The extracts were vacuum filtered through Whatman (no. 2) filter paper using a Buchner funnel, and solvent was removed by vacuum distillation in a Buchi rotary evaporator at 60 °C. Once concentrated to a small volume, the extracts were placed in pre-weighed beakers and allowed to dry completely in front of a cool stream of air. The mass extracted with each solvent was calculated.

3.7.2. Analysis of extract profiles by TLC

To determine the TLC chemical profile, 20 mg of each extract of *T. sambesiaca* was weighed into a pill vial and made up to a concentration of 10 mg/ml by re-dissolving in acetone. Solvent systems BEA, CEF and EMW were used (**Section 3.4**).

3.7.3. Microdilution assay

The serial microdilution method was used to determine MIC values for the four *T. sambesiaca* leaf extracts against bacteria and fungi (**Section 3.5**).

3.7.4. Bioautography

Bioautography (**Section 3.6**) was performed on all extracts using *S. aureus* and *E. faecalis* bacterial strains.

3.7.5. Open-ended column chromatography

The dichloromethane (DCM) and ethyl acetate extracts of *Terminalia sambesiaca* were subjected to column chromatography over silica gel. The dry packing method was used where silica gel 60 was poured slowly into the column (15.5 cm x 10 cm) on top of a small cotton wool. The dry sample of the extract was introduced into the column using a spatula. A filter paper cut to the internal diameter of the column and cotton wool were placed on top of the sample to prevent disturbance at the surface when the solvent is introduced. Eighteen elution systems were slowly added in the order as illustrated in **Table 3-2**. The vacuum was switched on after addition of solvent (about 1.5 L) into the column. The fractions were collected in pre-weighed beakers (250 ml) and allowed to evaporate overnight under a stream of cool air, whereafter analyses were carried out.

Table 3-2. Solvent systems used in column chromatography

Elution system	
Hexane:	100%
Hexane: Ethyl acetate	90%
Hexane: Ethyl acetate	80%
Hexane: Ethyl acetate	70%
Hexane: Ethyl acetate	60%
Hexane: Ethyl acetate	50%
Hexane: Ethyl acetate	40%
Hexane: Ethyl acetate	30%
Hexane: Ethyl acetate	20%
Hexane: Ethyl acetate	10%
Ethyl acetate:	100%
Ethyl acetate: Methanol	90%
Ethyl acetate: Methanol	80%
Ethyl acetate: Methanol	70%
Ethyl acetate: Methanol	50%
Ethyl acetate: Methanol	30%
Ethyl acetate: Methanol	10%
Methanol	100%

3.8. Analysis of fractions

After column chromatography, fractions were allowed to stand under a stream of cool air to facilitate concentration for TLC analysis and bioassays. When about half of the original volume of fractions had evaporated, fractions were analysed by TLC (**Section 3.4**).

3.8. 1. Combination of similar fractions using TLC

After TLC analysis, fractions were combined based on the similarity of their chemical profile. The combined fractions were placed under air to facilitate drying. The dry fractions were then weighed before being subjected to column chromatography (**Section 4.5.5**). Some fractions that crystallized were washed by dissolving in appropriate solvents and passing through a Pasteur pipette plugged with cotton wool to facilitate the removal of impurities. Various solvents were used, such as, hexane, ethyl acetate, chloroform, acetone, ethanol, and methanol. The fractions were investigated for their antimicrobial compounds using bioautography.

3.8.2. Melting point determination

The melting point of the isolated compound was determined using a melting point apparatus (Model FP 90, Mettler).

3.8.3. Nuclear Magnetic Resonance (NMR)

The pure samples were weighed and dissolved in either 2 ml chloroform or methanol depending on the solubility of the compounds, although other solvents were also attempted for compounds that could not dissolve completely. The samples were then pipetted into polytops (No.6) using a Pasteur pipette, dried, weighed and sent to the Chemistry Department, North-West University, Potchefstroom campus for analysis.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 600 spectrometer, using deuterated chloroform as solvent. The ^{1}H NMR and ^{13}C spectra were recorded at frequencies of 600.17 and 151.92 MHz respectively. All the chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (δ = 0). The splitting pattern abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet) and m (multiplet).

3.8.4. Mass spectrometry

MS spectra were recorded on an analytical VG 7070E mass spectrometer using fast electron impact (EI) as ionization technique.

3.8.5. MIC determination

A serial microdilution assay (Eloff, 1998d) was used to determine the minimum inhibitory concentration (MIC) values for the isolated compound using tetrazolium violet reduction as an indicator of growth (**Section 3.5**). For these tests, *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 21212) and *Escherichia coli* (ATCC 27853) bacteria were used.

CHAPTER 4: RESULTS

4.1. Plant extraction

The total mass extracted by each solvent from 1 g leaf material of *Terminalia* species was determined and calculated as a percentage extracted as shown in **Table 4-1**. The highest yield of extract was obtained with methanol with a percentage extract of 18.5, followed by ethyl acetate with a percentage of 1.31. Dichloromethane and hexane extracted the lowest percentage of 0.95 and 1.03 respectively. *Terminalia phanerophlebia* had the highest average percentage mass (7.40) followed by *Terminalia mollis* and *Terminalia sambesiaca* with percentage mass of 6.89 and 5.33 respectively. *Terminalia gazensis* had the lowest percentage mass of 3.95.

Table 4-1. The percentage mass of *Terminalia* species extracted with four extractants from dried powdered leaves

***Terminalia* species**	**Percentage mass residue extracted**				
	Hexane	**DCM**	**Ethyl acetate**	**Methanol**	**Average**
T. sericea	1.64	1.04	1.74	13.1	**4.38**
T. brachystemma	1.68	1.26	1.72	14.1	**4.70**
T. mollis	0.40	0.54	0.60	25.9	**6.89**
T. sambesiaca	1.08	0.90	1.36	18.0	**5.33**
T. phanerophlebia	1.12	1.42	1.88	25.2	**7.40**
T. gazensis	0.28	0.52	0.54	14.5	**3.95**
Average	1.03	0.95	1.31	18.5	**5.43**

The total percentages extracted from *Terminalia* species using different solvents (hexane, dichloromethane, ethyl acetate and methanol in a serial extraction) are shown in **Figure 4-1**.

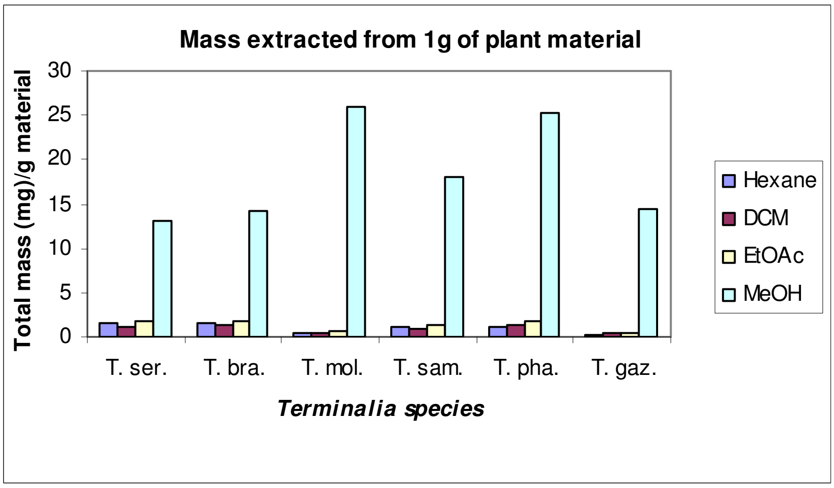


Figure 4-1. Percentage of powdered *Terminalia* leaf samples produced in a serial extraction with hexane, dichloromethane, ethyl acetate and methanol from six *Terminalia* species: T. ser. = *Terminalia sericea*, T. bra. = *Terminalia brachystemma*, T. mol. =*Terminalia mollis*, T. sam. = *Terminalia sambesiaca*, T. pha. = *Terminalia phanerophlebia*, T. gaz. = *Terminalia gazensis*.

4.2. Phytochemical analysis

The phytochemical analysis of the *Terminalia* species was carried out by thin layer chromatography using vanillin-sulphuric acid spray reagent for the visualisation of compounds. There was similarity in the chemical composition of the *Terminalia* extracts (**Figure 4-2**). The best solvent system was BEA because compounds in all extracts separated well in BEA, while in EMW (polar) most compounds moved with the solvent front indicating that most compounds present in the leaves of these plant species could be of non-polar or intermediate polarity and that the eluent polarity was too high.

4.3. Antioxidant screening

The *Terminalia* species under investigation showed antioxidant activity when different extracts where sprayed with 0.2 % DPPH in methanol. This method indicated the location of antioxidant compounds on the TLC plates making it easy to determine the R_f value of these compounds. The ethyl acetate and methanol extracts showed the most active compounds while dichloromethane and hexane had the lowest number or no compounds with antioxidant activity. *Terminalia gazensis* had the highest number of antioxidant compounds followed by *Terminalia sericea* and *Terminalia sambesiaca* while *Terminalia mollis*, *Terminalia phanerophlebia* and *Terminalia brachystemma* showed the lowest number of antioxidant compounds (**Figure 4-3**). Of the three elution systems used, EMW was the one able to elute compounds with antioxidant activity whereas in BEA and CEF compounds were stuck to the baseline, making it difficult to quantify them.

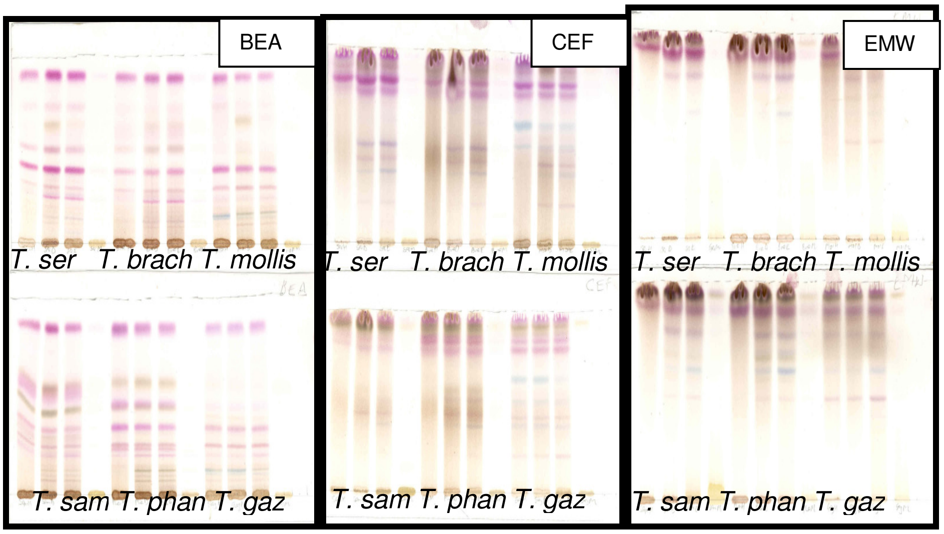
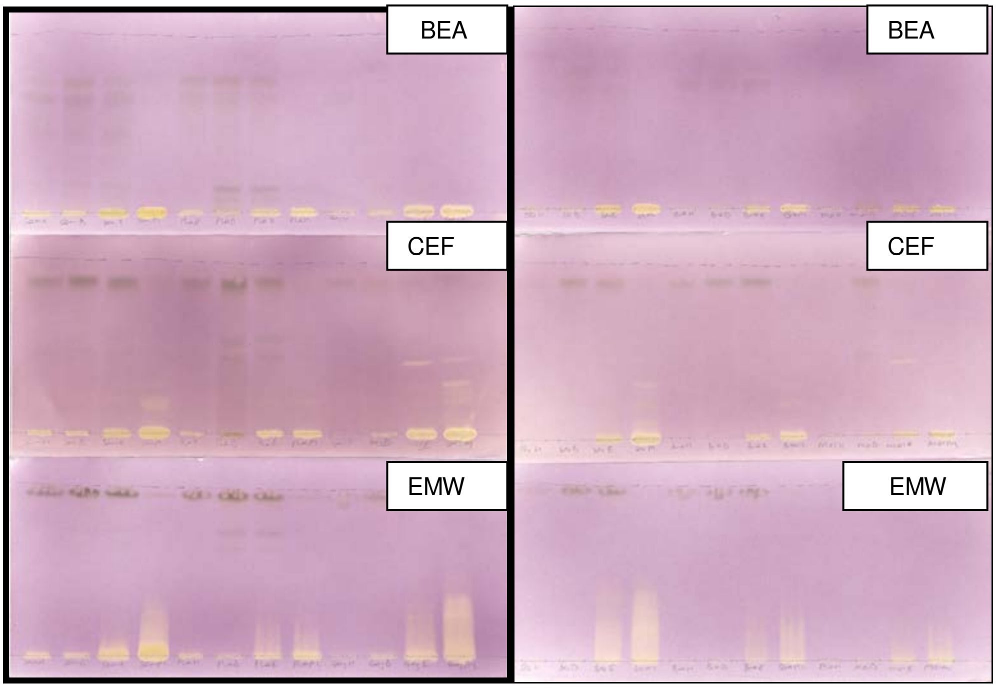


Figure 4-2. Chromatograms of six *Terminalia* species developed in BEA (left), CEF (middle) and EMW (right) solvent systems and sprayed with vanillin-sulphuric acid reagent to show compounds extracted with hexane, dichloromethane, ethyl acetate and methanol, in lanes from left to right in each group. T. ser. = *Terminalia sericea*, T. brach. = *Terminalia brachystemma*, T. sam. = *Terminalia sambesiaca*, T. phan. = *Terminalia phanerophlebia*, T. gaz. = *Terminalia gazensis*.



T. sambesiaca T. phanerophlebia T. gazensis *T. sericea T. brachystemma T. mollis*

Figure 4-3. Chromatograms of *Terminalia* species developed in BEA (top), CEF (centre) and EMW (bottom) solvent systems and sprayed with 0.2 % DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with hexane, dichloromethane, ethyl acetate and methanol, in lanes from left to right in each group.

4.4. Antimicrobial screening

4.4.1. Antibacterial screening

Minimum Inhibitory Concentration (MIC) was determined after 24 and 48 hours. Most extracts had MIC values of 80 µg/ml while others (*T. sambesiaca*) had values as low as 20 µg/ml after 24 hours of incubation (**Table 4-2**). Gentamycin was used as a positive control and there was no growth in any of the wells containing the antibiotic showing an MIC < 20 µg/ml. *Terminalia sambesiaca* had the lowest average MIC value at 0.097 mg/ml followed by *Terminalia mollis* with an MIC value of 0.118 mg/ml after 24 hours of incubation (**Table 4-3**).

The quantity of antibacterial compounds present in each extract was also determined for purposes of further isolation. The MIC is inversely related to the quantity of antibacterial compounds present. The arbitrary measure of the quantity of antibacterial compounds present was calculated by dividing the quantity extracted in milligrams (mg) from 1 gram leaves by the MIC value in mg/ml. This value indicates the volume to which the biologically active compound present in 1 gram of the dried plant can be diluted and still kill the bacteria (Eloff, 1999). **Table 4-4** shows the total activity of the *Terminalia* plant extracts. *T. sambesiaca* extracts had relatively higher average total activity values, while *T. gazensis* had the lowest average total activity values.

Table 4-2. Minimum inhibitory concentration (MIC) of the extracts of six *Terminalia* species after 24 and 48 h incubation at 37 ^{o}C

Organisms	Time (h)	MIC values (mg/ml)																								
		T. phanerophlebia				***T. brachystemma***				***T. sericea***				***T. gazensis***				***T. mollis***				***T. sambesiaca***				
		Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	**Average**
E. coli	24	0.16	0.16	0.08	0.16	0.16	0.16	0.08	0.08	0.16	0.16	0.08	0.16	0.16	0.16	0.16	0.08	0.16	0.16	0.08	0.08	0.32	0.08	0.08	0.08	**0.1**
	48	0.32	0.16	0.16	0.32	0.32	0.16	0.16	0.32	0.32	0.32	0.16	0.32	0.32	0.32	0.32	0.32	0.32	0.16	0.16	0.16	0.32	0.16	0.16	0.32	**0.3**
E faecalis	24	0.16	0.16	0.08	0.08	0.16	0.16	0.08	0.08	0.16	0.16	0.08	0.08	0.16	0.16	0.16	0.16	0.16	0.16	0.08	0.04	0.04	0.02	0.02	0.02	**0.1**
	48	0.32	0.16	0.16	0.16	0.32	0.32	0.16	0.16	0.64	0.32	0.16	0.16	0.16	0.16	0.16	0.32	0.32	0.16	0.16	0.16	0.16	0.16	0.04	0.04	**0.2**
P. aeruginosa	24	0.32	0.16	0.08	0.08	0.32	0.32	0.16	0.16	0.32	0.32	0.16	0.16	0.16	0.16	0.08	0.08	0.32	0.16	0.08	0.04	0.08	0.08	0.08	0.08	**0.2**
	48	0.32	0.16	0.16	0.32	0.64	0.64	0.64	0.64	0.64	0.64	0.32	0.64	0.32	0.32	0.32	0.32	0.64	0.64	0.32	0.32	0.32	0.32	0.08	0.08	**0.4**
S. aureus	24	0.16	0.16	0.08	0.08	0.16	0.16	0.04	0.04	0.32	0.16	0.08	0.04	0.16	0.16	0.08	0.08	0.16	0.04	0.08	0.08	0.32	0.08	0.08	0.08	**0.1**
	48	0.32	0.16	0.16	0.16	0.64	0.64	0.16	0.32	0.64	0.64	0.16	0.16	0.32	0.16	0.16	0.32	0.64	0.16	0.32	0.32	0.32	0.32	0.16	0.16	**0.3**
Average		**0.26**	**0.1**	**0.12**	**0.17**	**0.34**	**0.3**	**0.19**	**0.23**	**0.4**	**0.34**	**0.15**	**0.22**	**0.22**	**0.2**	**0.18**	**0.21**	**0.34**	**0.21**	**0.16**	**0.15**	**0.24**	**0.15**	**0.09**	**0.1**	**0.2**

Table 4-3. Average MIC values (antibacterial) of the extracts of different *Terminalia* species

Plant Species	**Average MIC values (mg/ml)**	
	24 h	**48 h**
T. phanerophlebia	0.13	0.22
T. brachystemma	0.14	0.39
T. sericea	0.16	0.39
T. gazensis	0.13	0.27
T. mollis	0.12	0.31
T. sambesiaca	0.10	0.19

Table 4-4. Total activity in ml/g of the extracts of six *Terminalia* species after 24 and 48 h incubation at 37 °C

Organisms	Time (h)	Total activity (ml/g)																							
		T. phanerophlebia				***T. brachystemma***				***T. sericea***				***T. gazensis***				***T. mollis***				***T. sambesiaca***			
		Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol
E. coli	24	70	89	235	1575	105	79	215	1767	102	65	217	817	17	26	34	1807	25	34	75	3235	34	112	170	2245
	48	35	89	117	787	53	79	107	442	51	32	109	406	9	17	17	451	12	34	37	1617	34	56	85	561.25
E. faecalis	24	70	177	235	3150	105	79	215	1767	102	65	217	1635	17	26	34	903	25	34	75	6470	270	450	680	8980
	48	35	89	117	1575	53	39	107	883	25	32	109	817	17	26	34	451	12	34	37	1617	67	56	340	4490
P. aeruginosa	24	35	89	235	3150	53	39	107	883	51	32	109	817	17	26	67	1807	12	34	75	6470	135	112	170	2245
	48	35	89	117	787	26	20	26	221	25	16	54	204	9	17	17	451	6	9	19	808	34	28	170	2245
S. aureus	24	70	177	235	3150	105	79	430	3535	51	65	217	3270	17	26	67	1807	25	135	75	3235	34	112	170	2245
	48	35	89	117	1575	26	20	107	442	25	16	109	817	9	26	34	451	6	34	19	808	34	28	85	1122
Average		**48**	**111**	**176**	**1968**	**66**	**54**	**164**	**1242**	**54**	**40**	**143**	**1098**	**14**	**24**	**38**	**1016**	**15**	**43**	**51**	**3032**	**59**	**119**	**233**	**3106**

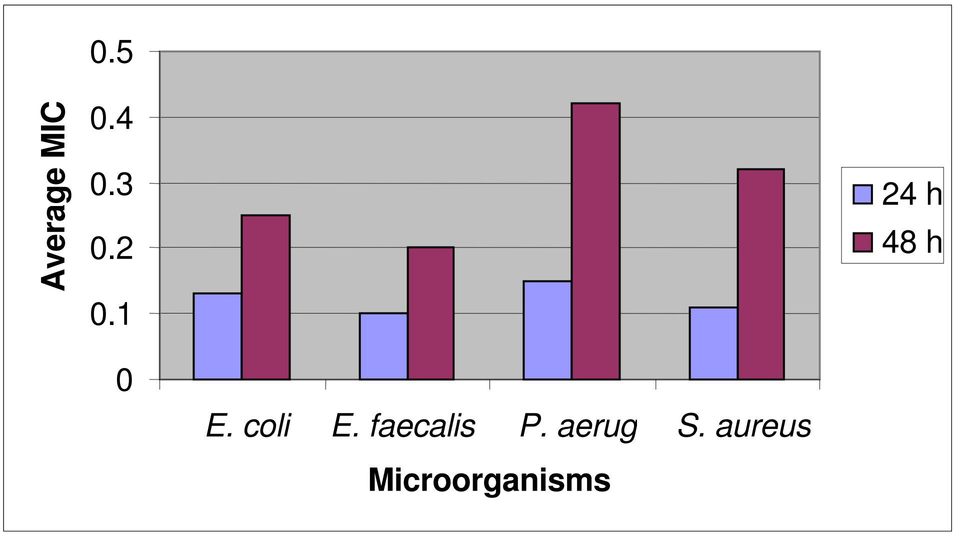


Figure 4-4. The sensitivity of different bacterial pathogens to the investigated species of *Terminalia* expressed as average MIC in mg/ml. *P. aerug* = *P. aeruginosa.*

The bacteria, *E. faecalis* and *S. aureus* showed equal sensitivity to the *Terminalia* species after 24 hours (MIC 0.1 mg/ml), but after 48 hours of incubation the average MIC values were 0.3 mg/ml for *S. aureus* and 0.2 mg/ml for *E. faecalis*, making *E. faecalis* the most susceptible microorganism (**Figure 4-4**). The difference in values between 24 and 48 hours indicates that a major part of the activity was bacteriostatic rather than bactericidal.

4.4.2. Antifungal screening

The growth of fungi was checked after 24 and 48 hours in order to determine the minimum inhibitory concentration (MIC). Most extracts had MIC values of 0.08 mg/ml. *T sambesiaca* was the most active species with values as low as 40 µg/ml after 24 hours of incubation. Amphotericin B was used as a positive control and there was no growth in any of the wells containing the antifungal agent, indicating a MIC of smaller than 20 µg/ml. *Terminalia sambesiaca* and *Terminalia brachystemma* had the lowest average MIC value of 0.20 mg/ml followed by *Terminalia gazensis* with an average MIC value of 0.21 mg/ml after 24 hours of incubation (**Table 4-6**). Masoko *et al.* (2005) found the lowest average MIC of 0.124 mg/ml among the *Terminalia* species they investigated.

Table 4-5. Minimum inhibitory concentration (MIC) of the extracts of six *Terminalia* species after 24 and 48 h incubation at 37 °C

Organisms	Time (h)	MIC values (mg/ml)																								
		T. phanerophlebia				***T. brachystemma***				***T. sericea***				***T. gazensis***				***T. mollis***				***T. sambesiaca***				
		Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	**Average**
Candida albicans	24	0.32	0.16	0.16	0.16	0.32	0.32	0.16	0.16	0.16	0.16	0.04	0.04	0.08	0.16	0.08	0.08	0.64	0.64	0.64	0.64	0.64	0.32	0.64	0.64	**0.37**
	48	1.25	1.25	1.25	1.25	2.5	1.25	1.25	1.25	1.25	2.5	1.25	1.25	2.5	1.25	2.5	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	**1.45**
Cryptococcus neoformans	24	0.16	0.16	0.08	0.08	0.16	0.16	0.16	0.16	0.32	0.32	0.08	0.08	0.16	0.08	0.08	0.08	0.64	0.16	0.08	0.08	0.04	0.04	0.04	0.04	**0.14**
	48	0.32	0.32	0.32	0.16	0.16	0.16	0.16	0.08	0.64	0.32	0.32	0.16	0.64	0.64	0.32	0.32	0.64	0.64	0.16	0.16	0.64	0.64	0.16	0.16	**0.34**
Aspergillus fumigatus	24	0.32	0.32	0.16	0.16	0.16	0.16	0.32	0.32	0.32	0.32	0.32	0.64	0.32	0.16	0.32	0.32	0.32	0.64	0.32	0.32	0.16	0.16	0.16	0.16	**0.28**
	48	0.64	0.64	0.32	0.64	1.25	1.25	1.25	1.25	0.32	0.64	0.64	0.64	1.25	1.25	1.25	0.64	0.64	0.32	0.32	0.64	0.64	0.64	0.64	0.64	**0.76**
Sporothrix schenckii	24	0.16	0.32	0.64	0.32	0.32	0.16	0.16	0.16	0.32	0.32	0.16	0.08	0.08	0.08	0.16	0.08	0.32	0.16	0.08	0.08	0.04	0.04	0.04	0.04	**0.18**
	48	0.64	0.64	0.32	0.32	0.16	0.16	0.16	0.32	0.32	0.16	0.16	0.32	0.32	0.16	0.16	0.32	0.64	0.64	0.32	0.32	0.64	0.64	0.16	0.16	**0.34**
Microsporum canis	24	0.64	0.64	0.32	0.32	0.32	0.16	0.16	0.16	0.32	0.16	0.16	0.16	0.64	0.32	0.32	0.64	0.32	0.32	0.16	0.16	0.32	0.32	0.16	0.16	**0.30**
	48	2.5	2.5	1.25	1.25	1.25	1.25	2.5	1.25	1.25	1.25	1.25	0.64	1.25	1.25	1.25	1.25	2.5	1.25	1.25	1.25	0.64	0.64	0.64	0.64	**1.33**
Average		**0.69**	**0.70**	**0.48**	**0.46**	**0.66**	**0.55**	**0.63**	**0.51**	**0.52**	**0.61**	**0.44**	**0.4**	**0.72**	**0.53**	**0.64**	**0.49**	**0.79**	**0.6**	**0.45**	**0.48**	**0.5**	**0.46**	**0.39**	**0.39**	**0.54**

Table 4-6. Average MIC values (against the investigated fungal species) of the extracts of different *Terminalia* species

Plant Species	**Average MIC values (mg/ml)**	
	24 h	**48 h**
T. phanerophlebia	0.28	0.88
T. brachystemma	0.20	0.94
T. sericea	0.22	0.76
T. gazensis	0.21	0.98
T. mollis	0.33	0.83
T. sambesiaca	0.20	0.66

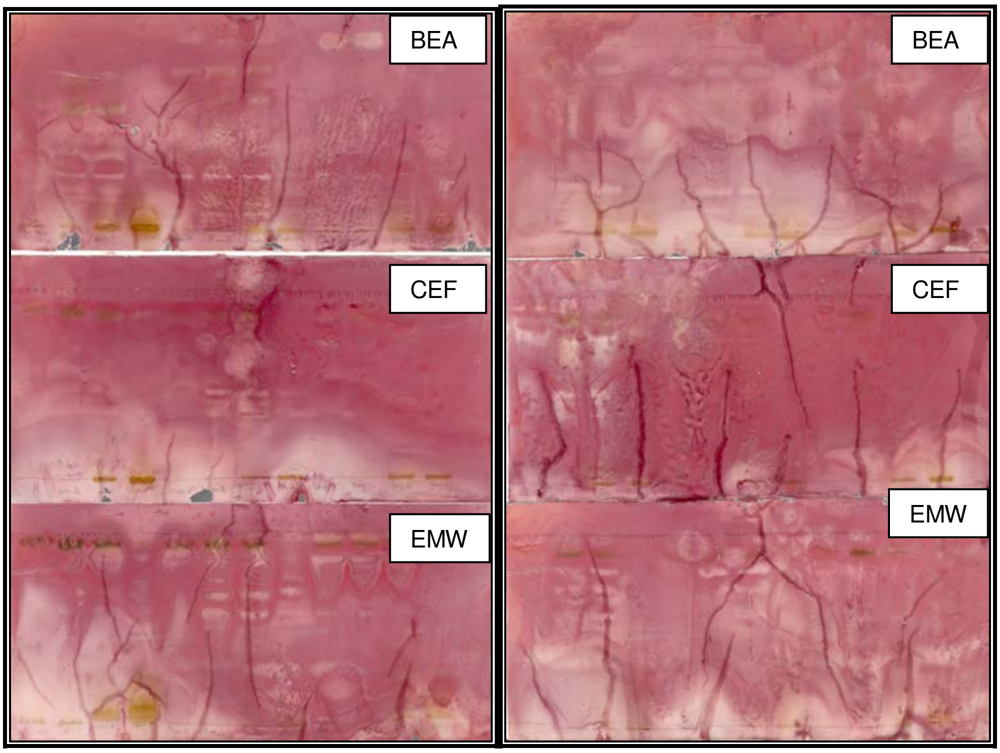
In order to determine the number of antifungal compounds present in each extract for purposes of further isolation, the inverse of MIC was used (**Section 4.4.1**). The species with the highest number of antifungal compounds was *T. sambesiaca* (**Table 4-7**). In all cases the major part of the antifungal activity was fungistatic rather than fungicidal.

Table 4-7. Total activity in ml/g of the extracts of six *Terminalia* species after 24 and 48 h incubation at 37 °C

Organisms	Time (h)	Total activity (ml/g)																							
		T. phanerophlebia				***T. brachystemma***				***T. sericea***				***T. gazensis***				***T. mollis***				***T. sambesiaca***			
		Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol	Hexane	DCM	Ethyl acetate	Methanol
Candida albicans	24	35	89	117	1575	52	39	107	884	102	65	435	3270	35	32	67	1807	6	8	9	404	17	28	21	281
	48	9	11	15	201	7	10	14	113	13	4	14	104	1	4	2	116	3	4	5	207	9	7	10	144
Cryptococcus neoformans	24	70	89	235	3150	105	79	107	884	51	32	217	1635	17	65	67	1807	6	34	75	3235	270	225	340	4490
	48	35	44	59	1575	105	79	107	1767	25	32	54	817	4	8	17	452	6	8	37	1617	17	14	85	1122
Aspergillus fumigatus	24	35	44	117	1575	105	79	54	442	51	32	54	204	9	32	17	452	12	8	19	808	67	56	85	1122
	48	17	22	59	394	13	10	14	113	51	1	27	204	2	4	4	226	6	17	19	404	17	14	21	280
Sporothrix schenckii	24	70	44	29	787	52	79	107	884	51	32	109	1635	35	65	34	1807	12	34	75	3235	270	225	340	4490
	48	17	22	59	787	105	79	107	442	51	65	109	409	9	32	34	452	6	8	19	809	17	14	85	1122
Microsporum canis	24	17	22	59	787	52	79	107	884	51	65	109	817	4	3	17	226	12	17	37	1617	34	28	85	1122
	48	4	6	15	201	13	10	7	113	13	8	14	204	2	4	4	116	2	4	5	207	17	14	21	280
Average		**31**	**39**	**76**	**1103**	**61**	**54**	**73**	**652**	**46**	**34**	**114**	**930**	**12**	**25**	**26**	**746**	**7**	**14**	**30**	**1254**	**74**	**63**	**109**	**1445**

4.4.3. Bioautography

This method was used to screen for antimicrobial (antibacterial and antifungal) compounds in the different extracts. The inhibition zones are observed as white spots on purple-red background. These white areas indicate where the reduction of INT to the coloured formazan did not take place due to the presence of antibacterial compounds that inhibited the bacterial growth (**Figure 4-5** to **Figure 4-8**).



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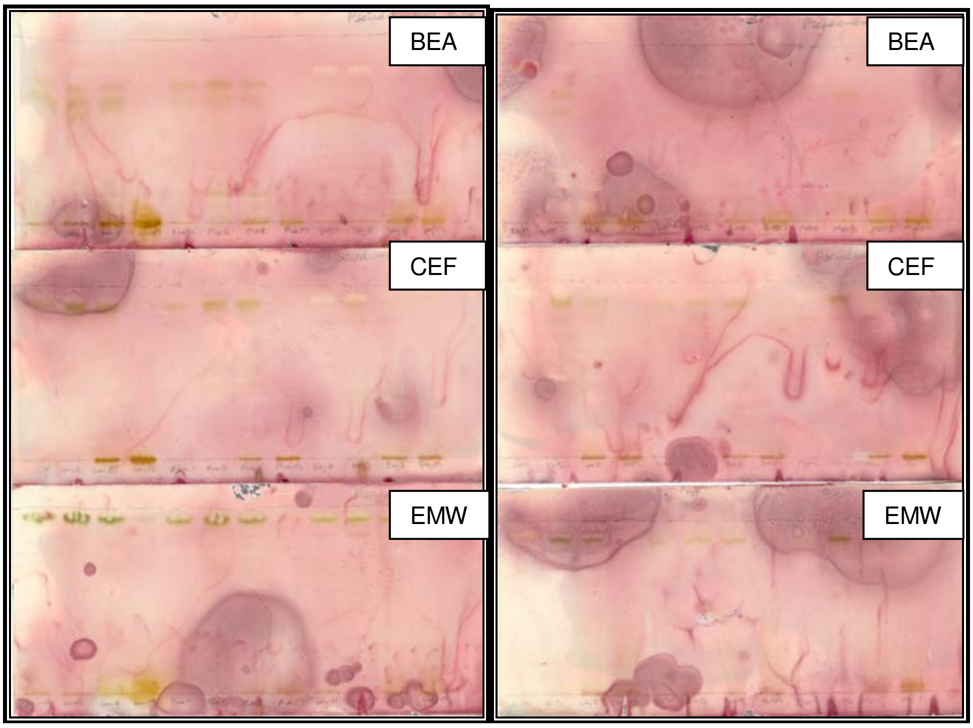
T. phanero T. sambesiaca T. gazensis T. sericea T. brachystemma T. mollis

Figure 4-5. Bioautography of *Terminalia* species extracted with hexane (Hex), dichloromethane (D), ethyl acetate (Et), methanol (Me) in lanes from left to right for each group, separated by BEA, CEF, EMW and sprayed with *Enterococcus faecalis*. White areas indicate where reduction of INT to the coloured formazan did not take place because of the presence of compounds that inhibited the growth of *E. faecalis*



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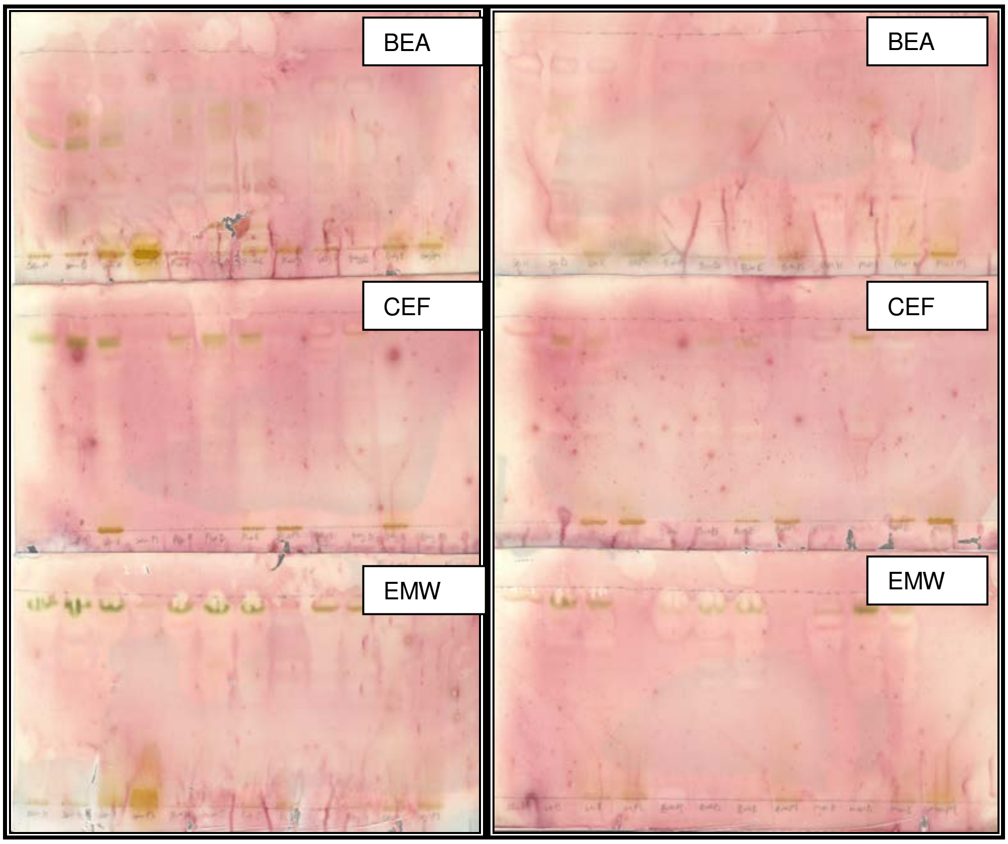
T. phanero T. sambesiaca T. gazensis T. sericea T. brachystemma T. mollis

Figure 4-6. Bioautography of *Terminalia* species extracted with hexane (Hex), dichloromethane (D), ethyl acetate (Et), methanol (Me) in lanes from left to right for each group, separated by BEA, CEF, EMW and sprayed with *Pseudomonas aeruginosa*. White areas indicate where reduction of INT to the coloured formazan did not take place because of the presence of compounds that inhibited the growth of *P. aeruginosa*



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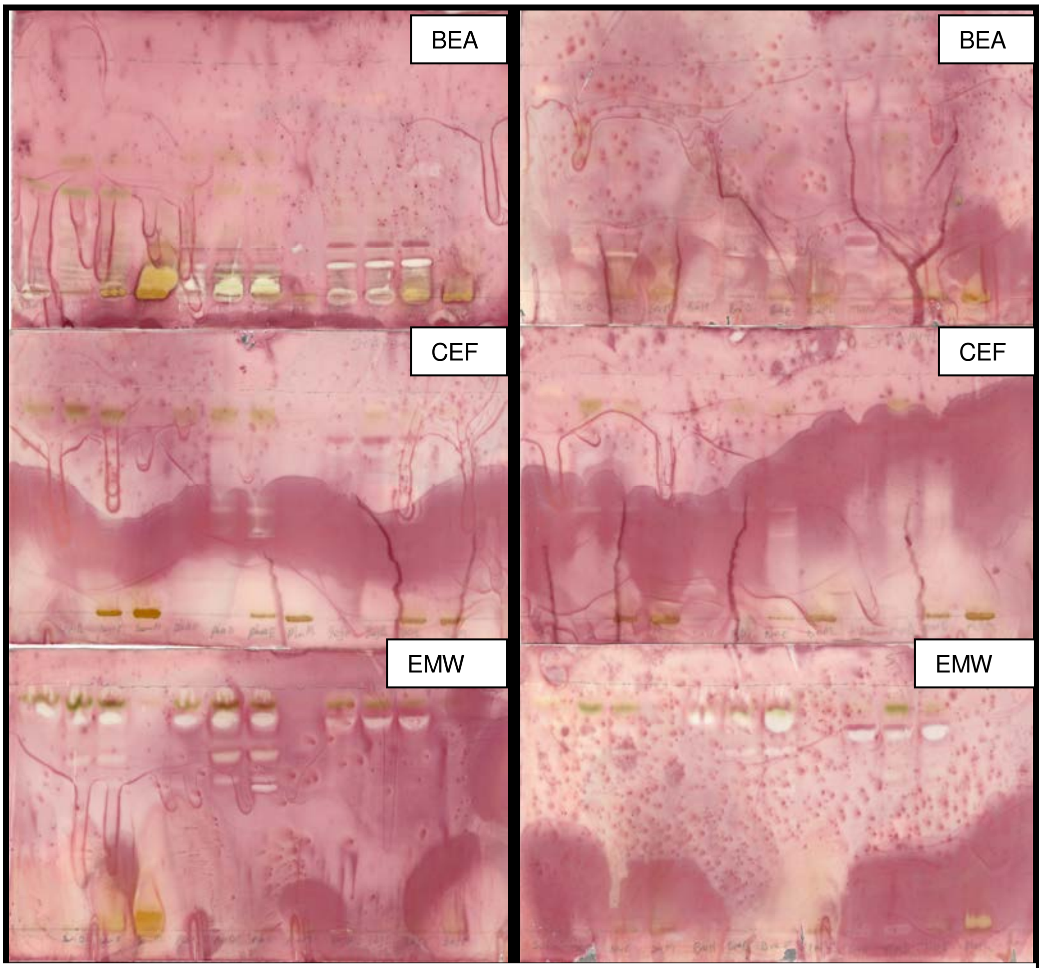
T. phanero T. sambesiaca T. gazensis T. sericea T. brachystemma T. mollis

Figure 4-7. Bioautography of *Terminalia* species extracted with hexane (Hex), dichloromethane (D), ethyl acetate (Et), methanol (Me) in lanes from left to right for each group, separated by BEA, CEF, EMW and sprayed with *Escherichia coli*. White areas indicate where reduction of INT to the coloured formazan did not take place because of the presence of compounds that inhibited the growth of *E. coli*



Hex D Et Me Hex D Et Me Hex D Et Me Hex D Et Me Hex D Et Me Hex D Et Me

T. phanero T. sambesiaca T. gazensis T. sericea T. brachystemma T. mollis

Figure 4-8. Bioautography of *Terminalia* species extracted with hexane (Hex), dichloromethane (D), ethyl acetate (Et), methanol (Me) in lanes from left to right for each group, separated by BEA, CEF, EMW and sprayed with *Staphylococcus aureus*. White areas indicate where reduction of INT to the coloured formazan did not take place because of the presence of compounds that inhibited the growth of *S. aureus*

Table 4-8. The R_f values and relative inhibition of compounds present in *Terminalia* species as determined by bioautography

	BEA/Hexane				BEA/DCM				BEA/Ethyl acetate				BEA/Methanol			
R_f	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a
Terminalia sambesiaca																
0.28	XX				XX	XX			XXX	XX				X		
0.31	XX					XX	XX			XX				X		
0.33	XX			X	X	XX	X		XXX	XX	X			X	XX	
0.38		X	X		X	X			XX					X		
0.46	XX			X		X	X		XX							
0.66	XX	X	X			XX			XX	XX						
0.84	XX	X	X			XX			XXX	XX						
0.75		XX	XX	XX						XX						
Terminalia phanerophlebia																
0.20	XX	XX	X		XX	XX		X		XX		X				
0.27	X	XX		X		XX	X			XX						
Terminalia gazensis																
0.36	X	X														
0.75				XX				XX		X						
0.84		XX				XX				XX						
Terminalia mollis																
0.50	XX				XX				X							
0.60	XX				XX											
Terminalia sericea																
0.65	XX															
0.46								XX								
0.28		X				X										
0.75		X				X			XX							
Terminalia brachystemma																
0.12				XX												
0.46							XXX									
0.75		XX				XX			XX	XX						

E.c = *Escherichia coli,* E.f = *Enterococcus faecalis*, S.a = *Staphylococcus aureus*, P.a *Pseudomonas aeruginosa*. The relative degree of inhibition: X = moderate inhibition, XX = high inhibition, XXX = very high inhibition. BEA = Benzene: Ethyl acetate: Ammonia

Table 4-9. The R_f values and relative inhibition of compounds present in *Terminalia* species as determined by bioautography

	CEF/Hexane				**CEF/DCM**				**CEF/Ethyl acetate**				**CEF/Methanol**			
R_f	**E.c**	**E.f**	**S.a**	**P.a**	**E.c**	**E.f**	**S.a**	**P.a**	**E.c**	**E.f**	**S.a**	**P.a**	**E.c**	**E.f**	**S.a**	**P.a**
Terminalia sambesiaca																
0.36	X					XX			XX							
0.51					XX		XX									
0.48																
0.75	XX															
0.66		X			XX	XX									XX	
0.74					XX		XX				X					
0.79			X				XX									
0.92	XX				XX		X									
Terminalia phanerophlebia																
0.45		X				X										
0.66		X			X											
0.73											X					
Terminalia gazensis																
0.84	X			X				X								
0.75		X				X	X			X			X			
0.60		X		X		X				X						
Terminalia mollis																
0.50	XX												XX		XX	
0.60							XX									
0.73			X		XX											
0.84					X										XX	
Terminalia sericea																
0.65	XX	X				X		XX		X	XX			XX		
0.46		X				XX		X		X		X				
0.28												XX				
0.66															XX	
Terminalia brachystemma																
0.12	X			X						X					X	
0.46		XX											X			

E.c = *Escherichia coli,* E.f = *Enterococcus faecalis*, S.a = *Staphylococcus aureus*, P.a *Pseudomonas aeruginosa*. The relative degree of inhibition: X = moderate inhibition, XX = high inhibition. CEF = Chloroform: Ethanol: Formic Acid

Table 4-10. The R_f values and relative inhibition of compounds present in *Terminalia* species as determined by bioautography

	EMW/Hexane				**EMW/DCM**				**EMW/Ethyl acetate**				**EMW/Methanol**			
R_f	**E.c**	**E.f**	**S.a**	**P.a**	**E.c**	**E.f**	**S.a**	**P.a**	**E.c**	**E.f**	**S.a**	**P.a**	**E.c**	**E.f**	**S.a**	**P.a**
Terminalia sambesiaca																
0.92	XX				XX	XX	X		XXX	XX	X					
0.73	XX				XX	XX	X		XXX	XX					XX	
0.98	XX	X			X				XX							
0.75	XX						X		XX		X			X		
0.66	XX	X				XX	X		XX	XX	X			X		
0.84	XX	X	X			XX	X		XXX	XX	X			X		
Terminalia phanerophlebia																
0.75	XX		X		XX			X				X				
0.84	X			X			X			XX						
Terminalia gazensis																
0.92	X		XX				XX				XX					
0.75		X				X				X		X				
0.84		X				X				X						
Terminalia mollis																
0.66	XX				XX				X	X				X		
0.92	XX		XX		XX		XX				XX					
Terminalia sericea																
0.65	XX					XX				XX						
0.61								XX								
0.95																
0.66						XX			XX	XX						
Terminalia brachystemma																
0.92			XX	XX			XX				XX					
0.56		X				X	XXX			X						
0.73						X			XX	X						

E.c = *Escherichia coli,* E.f = *Enterococcus faecalis*, S.a = *Staphylococcus aureus*, P.a *Pseudomonas aeruginosa*. The relative degree of inhibition: X = moderate inhibition, XX = very high inhibition, XXX = very high inhibition. EMW = Ethyl acetate: Methanol: Water

4.5. Isolation of antibacterial compounds from the leaves of *T. sambesiaca*

T. sambesiaca, being readily available in South Africa has shown the presence of more active compounds than the other screened *Terminalia* species. It was thus chosen for the isolation of active compounds.

4.5.1. Extraction

Extraction was carried out as explained in **Section 3.7.1**. Powdered leaves (500 g) of *Terminalia sambesiaca* were serially extracted with hexane, dichloromethane, ethyl acetate and methanol. The mass extracted per solvent is presented in **Table 4-11**.

Table 4-11. Total mass extracted from *T. sambesiaca* species using solvents of varying polarities

Extract	**Mass**
Hexane	50.20 g
Dichloromethane	48.76 g
Ethyl acetate	47.68 g
Methanol	60.80 g
Total mass	**207.44 g**

Methanol extracted the highest mass (60.80 g), ethyl acetate and DCM extracted almost the same quantity of 47.68 g and 48.76 g respectively. The total mass extracted from the *T. sambesiaca* plant leaves was 207.44 g (41.48%).

4.5.2. Phytochemical analysis

TLC analysis was done as explained in **section 3.4**, using aluminium backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F_{254}). The TLC plates were developed in BEA, CEF and EMW as eluting systems. The TLC profile for the four extracts is shown in **Figure 4-9**. After spraying with vanillin-sulphuric acid reagent and heating at 105 ^{o}C, hexane and DCM extracts had similar components in BEA while ethyl acetate and methanol did not separate well due to their relatively high polarity. In CEF most of the compounds visible in the ethyl acetate extract were present in the DCM extract as well. Methanol did not show a clear separation of components with all the eluting systems.

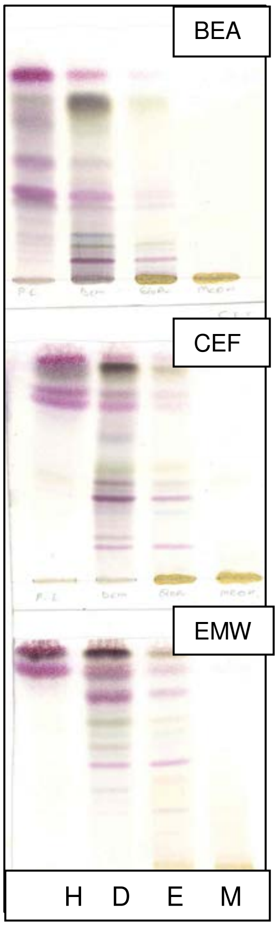


Figure 4-9. Chromatograms of *T. sambesiaca* extracts developed in BEA (top), CEF (middle) and EMW (bottom) solvent systems and sprayed with vanillin-sulphuric acid reagent to show compounds extracted with hexane (H), dichloromethane (D), ethyl acetate (E) and methanol (M)

4.5.3. Bioautography

For quantitative analysis, bioautography was carried out and results are shown in **Figure 4-10**. The figure shows active compounds with different R_f values. When BEA was used as a separating system, the hexane extract showed the presence of two compounds with R_f values of 0.33 and 0.75 respectively, the DCM extract showed two compounds with R_f values of 0.38 and 0.33 respectively while the ethyl acetate and the methanol extracts did not show any active compounds. In CEF, the hexane extract showed two compounds (R_f 0.79 and 0.92), DCM five compounds with R_f values of 0.51, 0.66, 0.74, 0.79 and 0.92.The ethyl acetate and methanol extracts did not show separation of the active compounds. In EMW, hexane showed one compound (R_f 0.84), DCM two compounds (R_f 0.66 and 0.78) and ethyl acetate three compounds (R_f 0.66, 0.78 and 0.93). Methanol did not show any activity in EMW.

Figure 4-10. Bioautography of *T. sambesiaca* extracts developed in BEA (top), CEF (middle) and EMW (bottom) solvent systems and sprayed with *Staphylococcus aureus* to show active compounds extracted with hexane (H), dichloromethane (D), ethyl acetate (E) and methanol (M). White areas indicate where reduction of INT to the coloured formazan did not take place because of the presence of compounds that inhibited the growth of *S. aureus*

4.5.4. Minimum Inhibitory Concentration of extracts of *T. Sambesiaca* leaves

Antifungal testing

All four extracts of *T. sambesiaca* leaves had activity against the fungal strains used (**Table 4-12**). On average, *C. neoformans* and *S. schenckii* were the most sensitive fungi tested; with an average MIC of 0.04 mg/ml. *C. albicans* was the least sensitive of the fungal species used in this investigation giving an average MIC of 0.56 mg/ml. The DCM extract was the most active extract giving an average MIC of 0.17 mg/ml against all pathogens tested.

Table 4-12. Minimum Inhibitory Concentration (MIC) of *T. sambesiaca* extracts using different solvents after 24 h incubation at 37 °C.

Microorganisms	**MIC Values mg/ml**				
	Hexane	DCM	Ethyl acetate	Methanol	**Average**
C. albicans	0.64	0.32	0.64	0.64	**0.56**
C. neoformans	0.04	0.04	0.04	0.04	**0.04**
A. fumigatus	0.16	0.16	0.16	0.16	**0.16**
M. canis	0.32	0.32	0.16	016	**0.24**
S. schenckii	0.04	0.04	0.04	0.04	**0.04**
Average	**0.24**	**0.17**	**0.20**	**0.20**	**0.20**

Results from antibacterial screening

The bacterial strains used were all sensitive to the *T. sambesiaca* extracts. Methanol, ethyl acetate and DCM extracts had an average MIC of 0.07 mg/ml while hexane had an average MIC of 0.19 mg/ml. The most sensitive microorganism was *E. faecalis* with an average MIC of 0.04 mg/ml followed by *P. aeruginosa* (MIC of 0.08 mg/ml). *S. aureus* and *E. coli* showed the same sensitivity at an average MIC of 0.14 mg/ml (**Table 4-13**).

Table 4-13. Minimum Inhibitory Concentration (MIC) of *T. sambesiaca* extracts using different solvents after 24 h incubation at 37 °C

Microorganisms	**MIC Values mg/ml**				
	Hexane	DCM	Ethyl acetate	Methanol	**Average**
S. aureus	0.32	0.08	0.08	0.08	**0.14**
E. coli	0.32	0.08	0.08	0.08	**0.14**
E. faecalis	0.04	0.04	0.04	0.04	**0.04**
P. aeruginosa	0.08	0.08	0.08	0.08	**0.08**
Average	**0.19**	**0.07**	**0.07**	**0.07**	**0.10**

4.5.5. Bio-assay guided fractionation

The dichloromethane (DCM) extract

The DCM (48.76 g) and ethyl acetate (47.68 g) extracts were chosen for further analysis since they gave promising MIC values and were less complex to separate, and then subjected to column chromatography (23 cm X 3 cm). Different eluting systems were used as outlined in **Chapter 3** and all fractions obtained were weighed **(Table 4-14)**

Table 4-14. The mass (g) of fractions of *T. sambesiaca* leaf DCM and ethyl acetate extracts as recovered from the column using eluents of varying polarity

Eluent	Percentage	Mass (g)	
		DCM	Ethyl acetate
Hexane:	100%	0.328	0.088
Hexane: Ethyl acetate	90%	0.275	0.202
Hexane: Ethyl acetate	80%	0.380	0.194
Hexane: Ethyl acetate	70%	0.690	0.318
Hexane: Ethyl acetate	60%	1.250	0.195
Hexane: Ethyl acetate	50%	0.857	0.259
Hexane: Ethyl acetate	40%	0.590	0.219
Hexane: Ethyl acetate	30%	1.567	0.319
Hexane: Ethyl acetate	20%	0.279	0.217
Hexane: Ethyl acetate	10%	0.368	0.320
Ethyl acetate:	100%	0.230	0.187
Ethyl acetate: Methanol	90%	0.190	2.580
Ethyl acetate: Methanol	80%	0.156	3.254
Ethyl acetate: Methanol	70%	0.125	3.435
Ethyl acetate: Methanol	50%	0.200	3.070
Ethyl acetate: Methanol	30%	0.230	2.562
Ethyl acetate: Methanol	10%	0.120	2.050
Ethyl acetate: Methanol	100%	0.115	1.980
Total		**7.950**	**21.448**

The starting mass for the DCM and the ethylacetate extracts was 48.76g and 46.68 respectively. The total mass recovered from DCM and ethyl acetate extracts was 7.950 g (16.30%) and 21.448 g (44.98%) respectively. The fractions from DCM column were separated using the three eluting systems (BEA, CEF and EMW) to locate the different compounds (**Figure 4-11**).

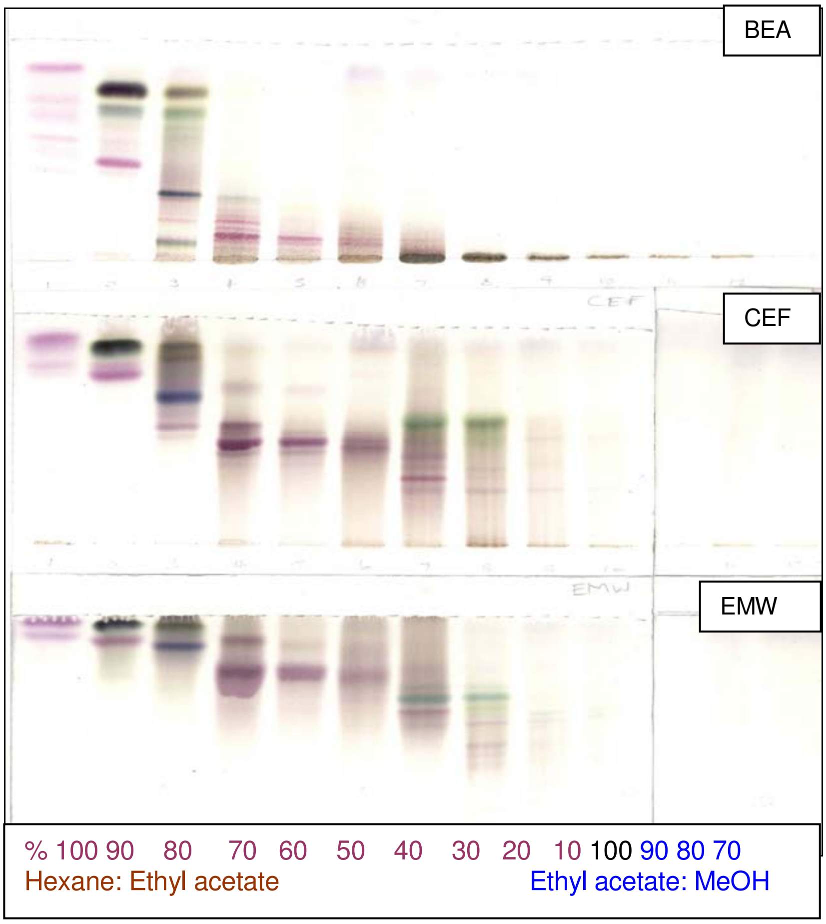


Figure 4-11. Chromatograms of *T. sambesiaca* DCM extracts developed in BEA, CEF and EMW solvent systems and sprayed with vanillin-sulphuric acid to reveal the different compounds

Bioautography was performed on all fractions to locate active compounds. Results are shown in **Figures 4-12** and **4-13**. Fractions 90% hexane in ethyl acetate, 80% hexane in ethyl acetate, 70% hexane in ethyl acetate, 60% hexane in ethyl acetate as well as 40% and 30% showed active compounds.

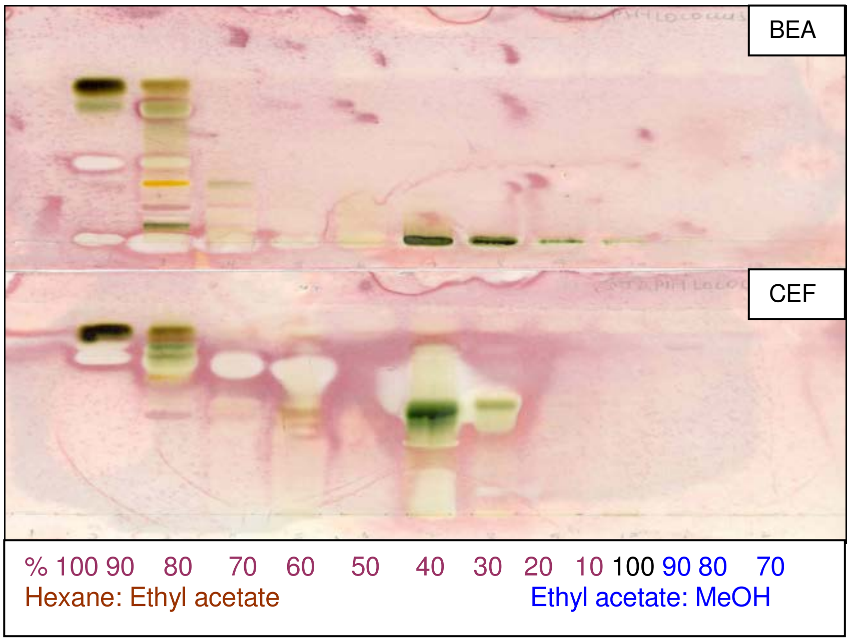


Figure 4-12. Bioautography of *T. sambesiaca* DCM extract separated by BEA and CEF and sprayed with *S. aureus*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *S. aureus.*

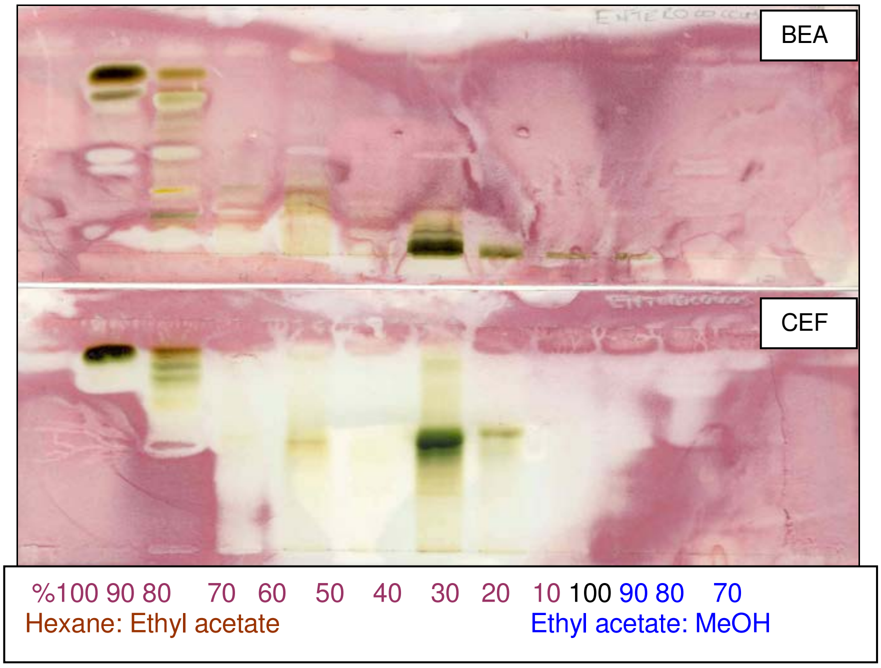


Figure 4-13. Bioautography of *T. sambesiaca* DCM extract separated by BEA and CEF and sprayed with *E. faecalis*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *E. faecalis.*

An overview of the isolation process is shown in a flow chart (**Figure 4–14**). The compounds isolated were sent for analysis by NMR (shown as NMR).

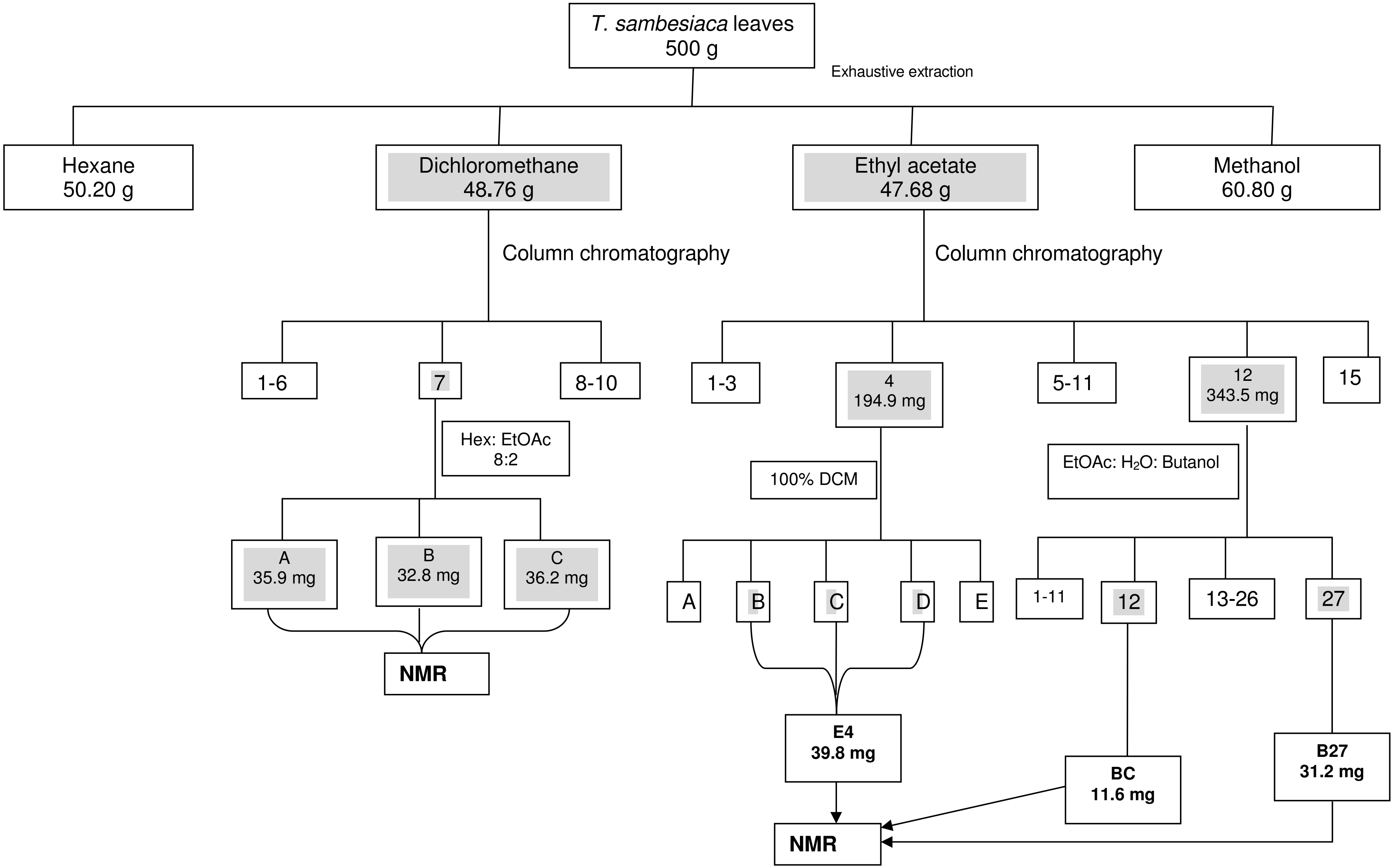


Figure 4-14. Fractionation of *T. sambesiaca* leaves

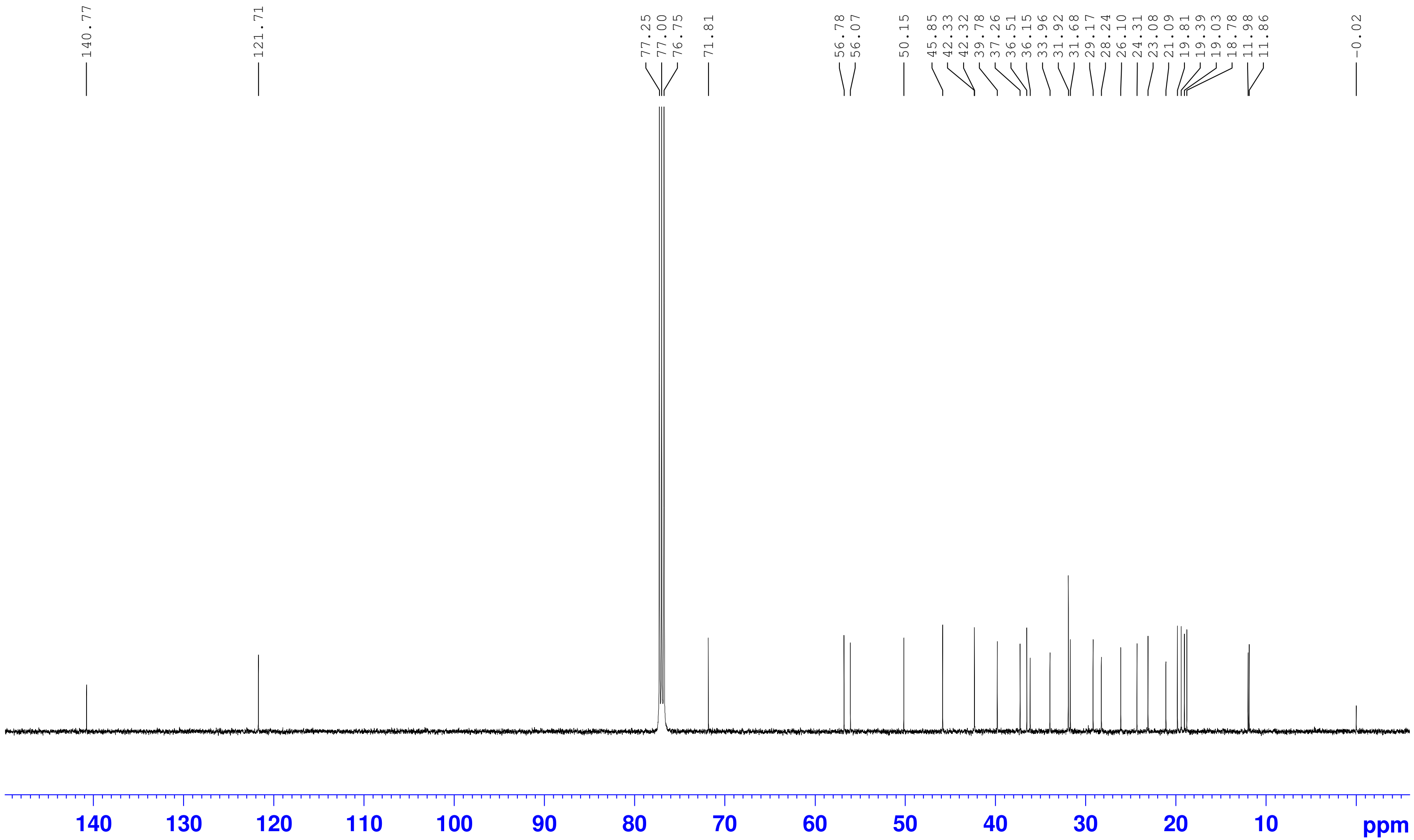


Figure 4-15. ^{13}Carbon NMR spectrum of compound **E4**

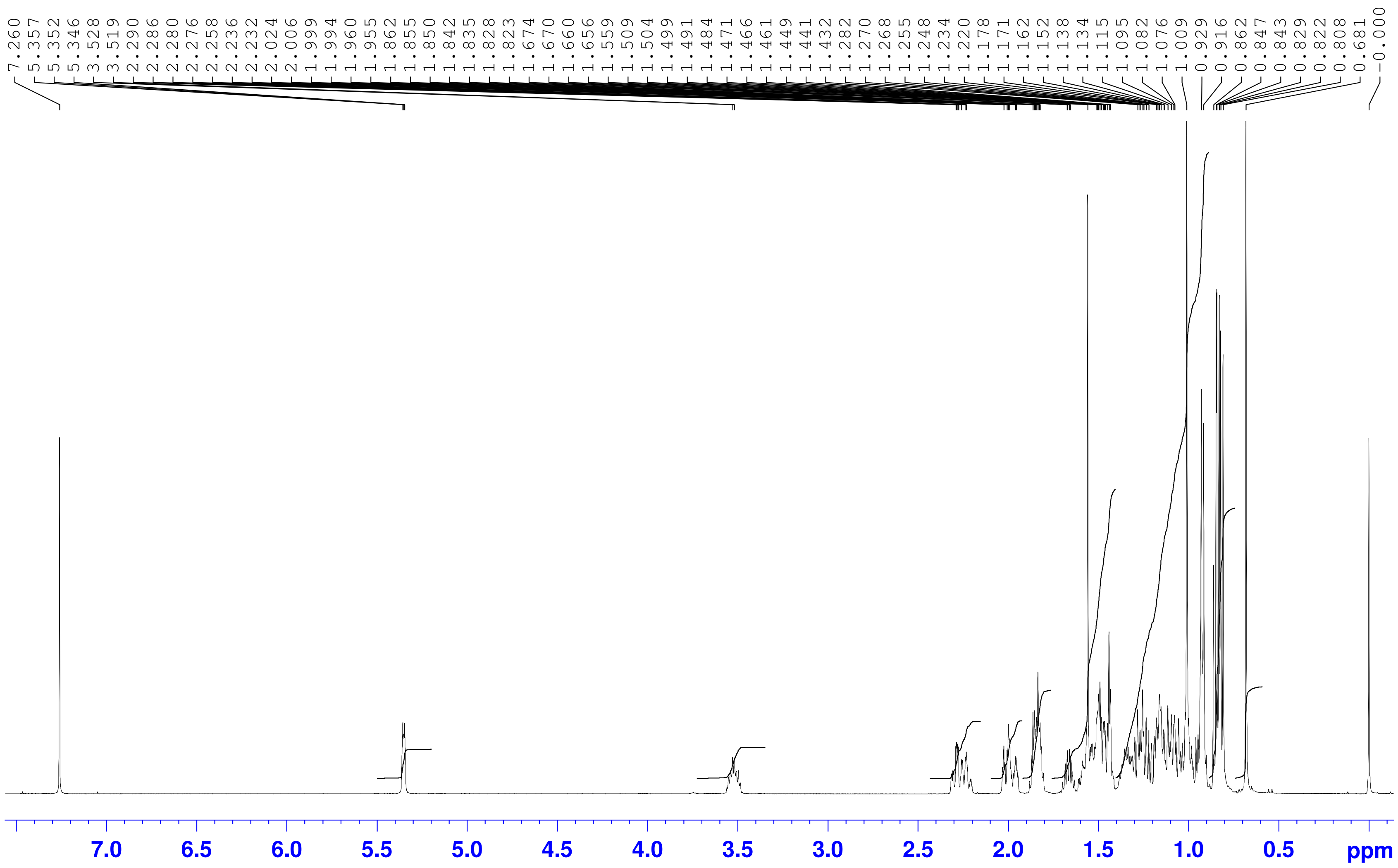


Figure 4-16. Proton NMR spectrum of compound **E4**

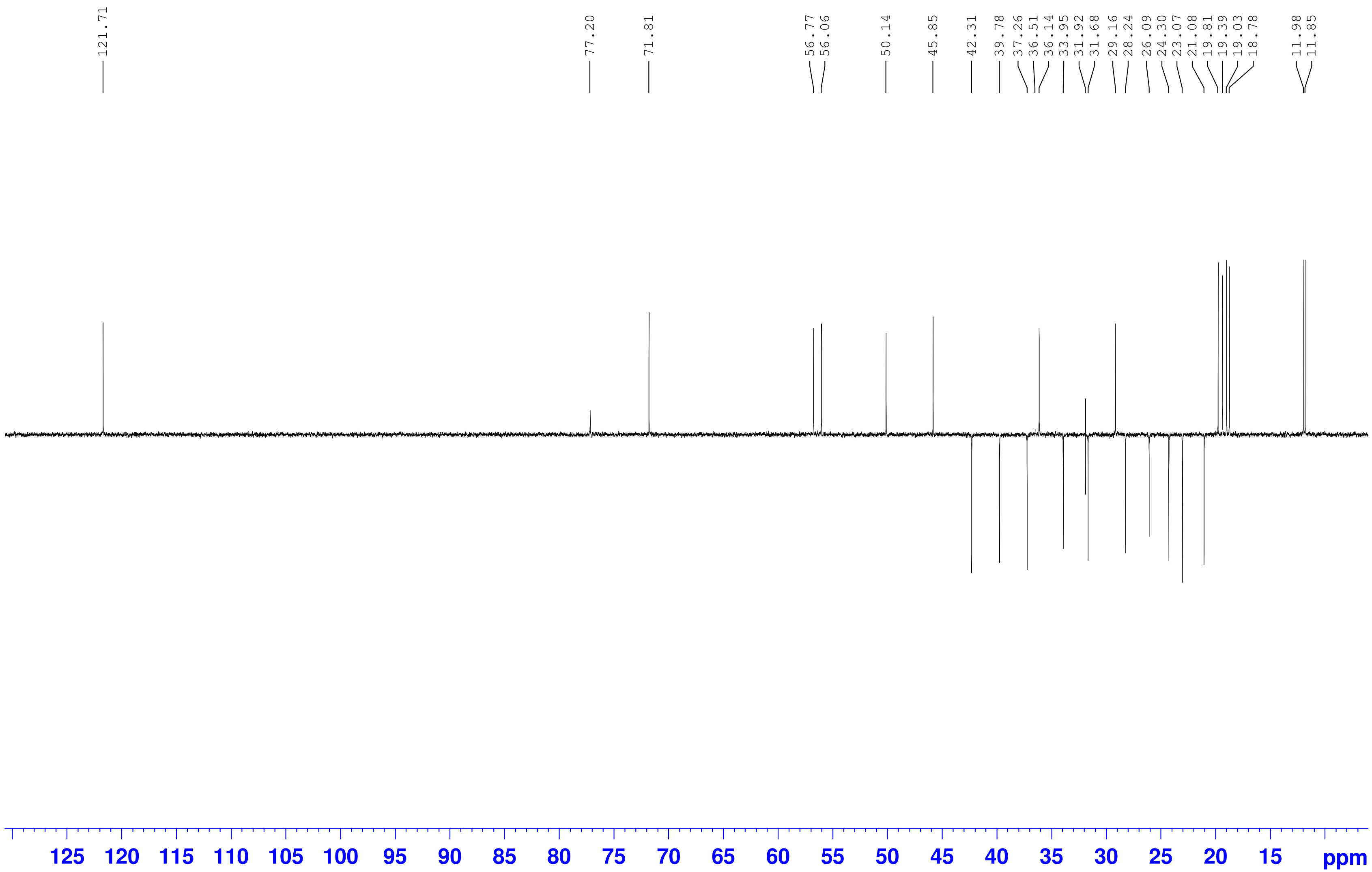


Figure 4-17. DEPT spectrum of compound **E4**

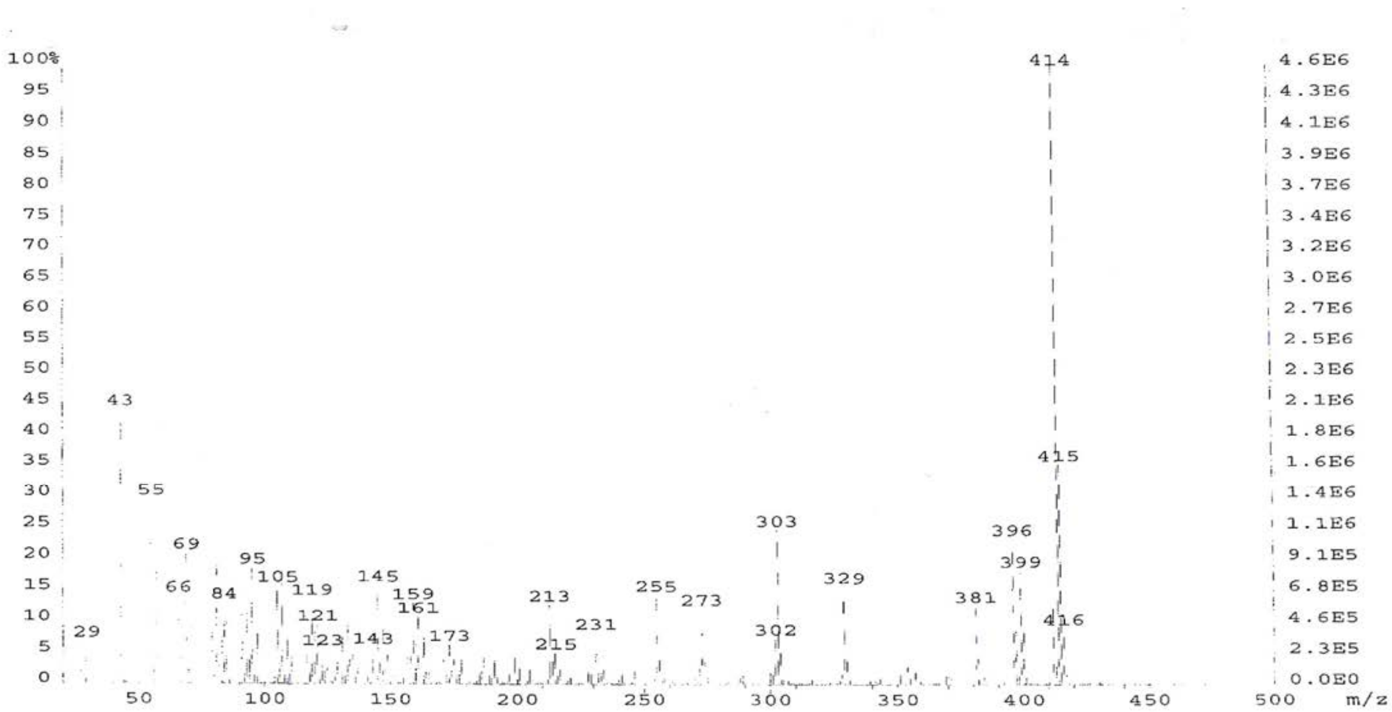


Figure 4-18. MS spectrum of compound **E4**

4.5.6. Nuclear Magnetic Resonance

4.5.6.1. Compounds in DCM extract

Compounds **A**, **B** and **C** were isolated from column chromatography fraction 4 of DCM extract and submitted for NMR analysis. The spectra showed the presence of impurities in all compounds and the purification of the compounds was impossible due to their presence in very small quantities. A compound **(E4)** similar to Compound **A** was however present in the ethyl acetate extract and was isolated.

4.5.6.2. Compounds in ethyl acetate extract

Compound **BC** was found to be impure, while compound **B27** could not dissolve in any of the solvents attempted. NMR spectra for compound **E4** are presented in **Figure 4-15, Figure 4-16** and **Figure 4-17**.

4.5.7. Structure elucidation

Compound **E4**: White needle-shaped crystalline substance; mp. = 136-137 ^{o}C; $C_{29}H_{50}O$; FAB MS *m/z* (%), (spectrum); 414, 396, 381, 329, 303, 273, 255, 231, 213, 173, 159, 145, 119, 95, 69, 55. ^{1}H NMR has given signals at δ 0.68, 0.81, 0.82, 0.84, 0.92, 1.00, 3.52, 5.35. The ^{13}C - NMR spectrum: 11.86 (C-29), 11.98 (C-18),18.78(C-21), 19.03(C-19), 19.39 (C-27), 19.81 (C-26), 21.09 (C-11), 23.08 (C-28), 24.31 (C-15), 28.24 (C-16), 26.10 (C-23), 29.17 (C-25), 31.68 (C-2), 31.92 (C-8), 33.96 (C-7), 36.15 (C-22), 36.51 (C-20), 37.26 (C-10), 39.78 (C-1), 39.78 (C-12), 42.33 (C-4), 42.32 (C-13), 45.85 (C-24), 50.15 (C-9), 56.07 (C-17), 56.78 (C-14), 71.81 (C-3), 121.71 (C-6), 140.77 (C-5).**Table 4-15** shows the comparison of spectral data for compound **E4** and that reported for β- sitosterol.

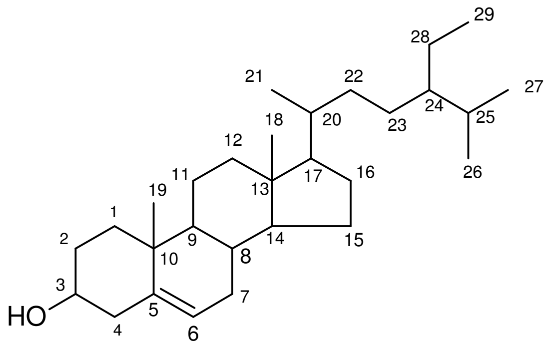


Figure 4-19. β – sitosterol structure

The physical properties and spectral data of compound **E4** were identical with those of β -sitosterol and therefore compound **E4** was identified to be β - sitosterol. The structure is shown in **Figure 4-19**.

Table 4-15. A comparison of ^{13}C - NMR spectral data of **E4** and that reported for β - sitosterol

Position	Moghaddam *et al.*, (2007)	Patra *et al.*, (2010)	Pateh *et al.*, (2009)	Jamal *et al.*, (2009)	Habib *et al.*, (2007)	Burdi *et al.*, (1991)	Haque *et al.*, (2008)	Zhang *et al.*, (2005)	Compound E4
	δ	δ	δ	δ	δ	δ	δ	δ	δ
1	37.7	37.28	37.3	37.9	32.4	37.30	37.41	37.25	39.78
2	32.3	31.69	31.6	33.3	36.1	31.73	31.82	31.65	31.68
3	72.2	71.82	71.8	79.3	71.8	71.64	71.96	71.79	71.81
4	42.8	42.33	42.2	39.0	42.3	42.36	42.25	-	42.33
5	141.2	140.7	140.8	158.3	140.7	140.77	140.91	140.75	140.77
6	122.1	121.72	121.7	117.1	121.7	121.70	121.87	121.71	121.71
7	32.1	31.69	31.9	33.6	31.6	31.73	31.94	31.90	33.96
8	32.3	31.93	31.9	36.0	28.2	31.96	31.94	-	31.92
9	50.6	50.17	51.2	48.9	42.3	50.20	50.15	50.13	50.15
10	36.9	36.52	36.5	36.9	39.7	36.55	36.30	36.50	37.26
11	21.5	21.10	21.1	26.1	20.9	21.13	21.10	21.08	21.09
12	40.2	39.80	39.8	38.2	31.8	39.83	39.93	39.77	39.78
13	42.8	42.33	42.3	39.2	40.4	42.26	42.33	42.30	42.32
14	57.2	56.79	56.8	55.7	45.8	56.82	56.78	56.76	56.78
15	24.7	24.37	24.3	28.2	21.2	24.33	24.33	24.30	24.31
16	28.7	28.25	28.3	30.0	21.0	28.27	28.27	28.24	28.24
17	56.5	56.09	56	49.5	50.1	56.12	56.22	56.05	56.07
18	12.4	11.86	11.9	15.6	20.5	11.90	12.01	11.98	11.98
19	19.8	18.40	19.4	19.0	20.2	19.42	19.54	19.39	19.03
20	36.6	36.52	36.2	35.3	29.6	36.16	36.17	36.14	36.51
21	19.2	18.79	18.8	17.7	18.7	19.08	18.93	18.77	18.78
22	34.4	33.98	33.9	33.9	33.7	34.01	33.96	33.94	36.15

23	26.5	26.14	26.1	29.0	29.1	26.19	26.08	26.07	26.10
24	46.2	45.88	45.9	41.5	42.3	45.91	45.85	45.83	45.85
25	29.6	28.91	29.2	30.1	30.5	29.24	29.16	29.14	29.17
26	20.2	19.80	19.8	26.1	19.8	18.82	19.84	19.82	19.81
27	19.5	18.79	19.3	21.5	19.3	19.84	19.05	19.03	19.39
28	23.5	23.10	23.1	27.4	24.2	23.13	23.08	23.06	23.08
29	12.3	11.99	12.2	15.6	11.8	12.01	12.28	11.85	11.86

4.5.8. MIC determination

The compound **E4** was found to be active against bacterial pathogens (**Table 4-16**). The average MIC value for compound E4 was 330 µg/ml while gentamycin had an average MIC of 0.31 µg/ml. The isolated compound was more active against *E. faecalis* compared to the other pathogens used. This was also the case when the crude extract was tested; *E. faecalis* was the most susceptible microorganism.

Table 4-16. Minimum Inhibitory Concentration (MIC) of compound **E4** after 24 h incubation at 37 ^{o}C

	MIC (µg/ml) after 24h			
	E. faecalis	*E. coli*	*S. aureus*	**Average**
Compound **E4**	280	400	320	330
Gentamycin	0.63	0.16	0.16	0.31

CHAPTER 5: DISCUSSION

5.1. Plant extraction

The six *Terminalia* species were selected for antimicrobial (antifungal and antibacterial) and antioxidant screening based on their wide traditional use and availability in South Africa. The total percentage of mass extracted was calculated and presented in **Table 4-1**. After using a serial extraction procedure, methanol was the best extracting solvent and extracted 18.5 % of the dry mass. This extract contains high molecular weight, polar compounds like tannins and carbohydrates in late summer. The species that yielded the highest mass was *T. phanerophlebia*. Among the species screened by Eloff (1999), *Terminalia sericea* showed the highest mass percentage of 22.6 when acetone was used as a single extractant. Extraction was followed by evaporation of the extracting solvent and all the extracts were redissolved in acetone because it was not found harmful towards bacteria (Eloff, 1998b) and fungi (Masoko *et al.*, 2005) and it also dissolves compounds with a wide range of polarities.

5.2. Phytochemical analysis

Thin Layer Chromatography (TLC) was used to analyse the components of *Terminalia* species using vanillin-sulphuric acid reagent to reveal compounds that were separated. According to TLC, the different *Terminalia* species had similar components when eluted with different solvent systems of varying polarity **(Figure 4-2)**. This was not unexpected as the plants belong to the same genus. The BEA mobile phase, which separates non - polar compounds, separated the *Terminalia* components best on normal phase silica. This may indicate the nature of compounds that are in abundance in the *Terminalia* species. CEF mobile phase also revealed a good number of compounds that were of medium polarity and acidic nature.

5.3. Antioxidant screening

When the TLC plates were sprayed with 0.2 % DPPH in methanol to screen for antioxidant activity of different extracts, activity was found in the ethyl acetate and methanol extracts. EMW separated compounds with antioxidant activity, though the separation was not good, since only normal phase silica was used. This indicates that *T. sambesiaca* leaves contain polar compounds with antioxidant activity. Many *Terminalia* species have been reported to possess antioxidant activity especially in roots and bark. Among the plants screened by Naik *et al.* (2003), *Terminalia chebula* gave the best antioxidant activity. A water extract of the leaves of *Terminalia catappa* are reported to give strong antioxidant activity (Kinoshita *et al*., 2007). Chyau *et al.* (2002) reported excellent scavenging effects of methanolic extracts of *Terminalia catappa* on DPPH radicals.

5.4. Antimicrobial screening

5.4.1. Minimum inhibitory concentration

To determine antibacterial activity, growth of microorganisms was checked after 24 and 48 hours to determine the minimum inhibitory concentration (MIC). *Terminalia sambesiaca* and *Terminalia mollis* gave low average MIC values (0.097 mg/ml and 0.118 mg/ml respectively) compared to other plants. Masoko *et al*. (2005) found similar results, with the lowest average MIC for *Terminalia* species (0.124 mg/ml after 24 hours) when tested against fungi. We found that *Terminalia sambesiaca* leaf extracts gave the highest activities and this is confirmed by the findings of Kruger (2004) who reported *Terminalia sambesiaca* as the most active of the tested *Terminalia species*. These findings indicate that *Terminalia* species possess substantial antibacterial and antifungal activities, which may be explored in the development of new antimicrobial drugs. The results also explain the use of these species by traditional healers in treating diseases caused by microbial infections.

The total activity of the plants was also determined in order to select the plant species to be investigated further. This gives an indication of the quantity of antibacterial or antifungal compounds present in a particular extract. MIC is inversely related to the quantity of antimicrobial compounds present. The total activity was calculated by dividing the quantity extracted in milligrams from 1 g leaves by the MIC value in mg/ml.

This value indicates the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the microorganism (Eloff, 1999). *Terminalia sambesiaca* extracts gave higher values for total activity and were considered worthy of further investigation.

5.4.2. Bioautography

The bioautography method was used to fingerprint the plant components responsible for antimicrobial activity as observed in MIC determination. The white spots against a purple-red background indicate the R_f value of antibacterial compounds. This means that reduction of INT to the coloured formazan did not take place due to the presence of an antimicrobial agent. Chromatograms developed in solvent systems BEA, CEF and EMW were used. EMW did not separate the components well, possibly because the neutral pH did not inhibit the ionizations of acidic and basic moieties in the extracts. Several antimicrobial compounds with different R_f values were found when BEA and CEF were used as eluting systems indicating that they are of non-polar or intermediate polar nature. Though the methanol extract had the highest percentage mass extracted, the active compounds could not separate well on TLC even when a polar eluent (EMW, normal phase silica) was used.

When the methanol extract was screened for antimicrobial activity using the serial-dilution method, activity was observed, but in bioautography individual compounds could not show activity. This could be because the TLC caused disruption of synergism between active constituents, thereby reducing the activity of the compounds (Schmourlo *et al.*, 2004). Synergism plays an important role in antimicrobial activity of plant extracts and this makes it difficult to isolate a single active compound to address antibiotic resistance. However, simple manipulation such as selective extraction could be used as a way of increasing activity of crude extracts (Eloff *et al.*, 2008).

All six *Terminalia* species were active against the tested pathogens. *E. faecalis* and *E. coli* were the most sensitive pathogens while *S. aureus* and *P. aeruginosa* were relatively resistant with fewer white spots on the TLC plates. This could just mean that the species screened were more active against *E. faecalis* and *E. coli* as activity of different antibacterial agents against bacteria varies.

Terminalia sambesiaca showed more active compounds than the other *Terminalia* species **(Table 4-8)**, even though *T. sambesiaca* extracts gave no activity against *P.*

aeruginosa. The fact that the serial dilution method showed that *T. sambesiaca* is active against *P. aeruginosa* could be due to a synergistic effect among compounds which may be lost when the compounds are separated on the chromatogram.

5.5. Isolation of antimicrobial compounds from a leaf extract of *T. sambesiaca*

T. sambesiaca was chosen for further analysis since it shows presence of more active compounds than the other species tested. It is readily available in South Africa has not been investigated in depth before. To isolate active compounds, the starting mass of plant material should be reasonable. Extraction was done serially in order to reduce the complexity of the crude extract as it has proven successful in other studies. This method removes unwanted waxy material from the extract to make it easy for the compounds to be extracted.

The DCM extract was first selected as it contained most of the active compounds and had less of the polar matter which is difficult to work with. The ethyl acetate extract showed partly the same compounds present in DCM extract **(Figure 4-2)**; hence it was also subjected to fractionation. The methanol extract was attempted, but was found to contain mostly very polar matter that tends to stick to the normal phase TLC plate when analysed, so it was disregarded. After the extraction using four solvents, antimicrobial (antibacterial and antifungal) tests were performed again to ascertain activity. During fractionation, bioautography was also carried out in order to trace the active compounds being isolated since this was a bio-assay guided fractionation. This method ensures that the isolated compound is active against the tested pathogens.

5.6. Characterization of active compound

Compound **E4** was obtained as white needle-shaped crystalline substance, with a melting point of 136-137 ^{o}C. The MS spectra of compound **E4** showed the major molecular ion peak, at m/z 414, which was compatible to the molecular formula $C_{29}H_{50}O$. The observed peak at m/z 396 was due to loss of H_2O which indicated that the compound contain an OH group. This compound was soluble in chloroform, acetone and ethyl acetate. ^{1}H NMR spectrum of compound **E4** in $CDCl_3$ showed a multiplet at δ 3.52 (1H; m; H-3) and a doublet at δ 5.35 (1H; d; J = 3; H-6). The signals seen at δ 0.68, 0.81, 0.82, 0.84, 0.92, and 1.00 correspond to the six methyl protons. The ^{13}C NMR spectrum of the compound **E4** in $CDCl_3$ showed the signals at 140.77 (C-5) and 121.71 (C-6) ppm which were due to olefinic carbons in the ring system.

^{13}C NMR and DEPT showed twenty nine carbon signals which include six methyl, nine methylene, eleven methane and three quartenary carbons. The signal at δ 71.81 was due to oxymethine carbon (C-3). Signals at δ 11.98 (C-18), 19.03 (C-19), 18.78 (C-21), 19.81 (C-26), 19.39 (C-27), 11.86 (C-29) ppm were due to six methyl groups of the compound. Compound **E4** was identified as β-sitosterol by MS and NMR experiments and by comparison with spectral data from literature (Djemgou *et al*., 2009; Moghaddam *et al*., 2007; Patra *et al.,* 2010; Pateh *et al.,* 2009; Jamal *et al.,* 2009; Burdi *et al.,* 1991; Haque *et al.,* 2008; Zhang *et al.,* 2005).

Row and Rao (1962) have isolated β-sitosterol from the heartwood of *Terminalia paniculata*. β-sitosterol was found to be one of the two compounds present in the most active fraction when Mokbel and Hashinaga (2005) evaluated antimicrobial activity of Buntan (*Citrus grandis* Osbeck) Fruit peel extract. Although this compound is reported in other *Terminalia* species, we are not aware of any report of the presence of this compound in *Terminalia sambesiaca*.

β-sitosterol is a well known compound widely used in medicine. It has anthelminthic and antimutagenic activities (Villaseňor *et al*., 2002). β-sitosterol isolated from Aloe Vera has angiogenic activities. It enhances new vessel formation in gerbil brains damaged by ischaemia (Choi *et al*., 2002). β-sitosterol also shows activity against *Trypanosoma brucei brucei* in vitro with a MIC value of 12.5 µg/ml (Nweze *et al*., 2011). Saw palmetto which is mainly used to treat BPH, has β-sitosterol as one of its components responsible for the pharmacological effects (Heinrich *et al*., 2004). The seeds of the soya plant which are rich in oil and protein contain appreciable quantities of the phytosterols stigmasterol and sitosterol. These are used extensively for steroid synthesis (Trease and Evans, 2002). The active fraction (containing the major compound β-sitosterol and minor compound stigmasterol) from *Pluchea indica* Less. (*Asteraceae)* neutralises viper and cobra venom-induced actions (Gomes *et al*., 2007).

CHAPTER 6: CONCLUSION

Six *Terminalia* species were assayed for antimicrobial activity and some of the compounds responsible for activity isolated from the leaves of the antimicrobially most promising species, *T. sambesiaca*. In this study, β - sitosterol was isolated from *Terminalia sambesiaca* and tested active against the bacterial strains used. It has been suggested elsewhere that this compound may be used as a preservative if its toxicity profile is known since it is found in certain fruits of plants. *Terminalia* species are known as rich sources of terpenoids. A triterpenoid glycoside was isolated from *Terminalia arjuna* and the wound healing activity of this plant was attributed to the isolated triterpenoid glycoside (Patnaik *et al*., 2007).

All the screened *Terminalia* plant extracts showed activity against the selected microorganisms. This supports the use of these plants in the treatment of diseases which are caused by microbial infection (Fyhrquist *et al.,* 2002; Masoko *et al.,* 2005). β-sitosterol isolated from the leaves of *T. sambesiaca* was active against bacterial and fungal strains though the activity was smaller than that shown by the crude extract. This could be attributed to possible synergism among compounds found in the extracts or that the most active compound was not isolated. Terpenoids are active against bacteria and fungi (Taylor *et al*., 1996). Although after isolation, the MIC for the compounds is in some cases lower than that of the crude extract; these compounds may still be useful in the treatment of many ailments.

Terminalia species is also being used in combination with other species. Triphala, a dry powder of equal proportion of *T. chebula*, *T. belerica* and *Emblica officinalis* has antidiabetic and antioxidant activity and is used extensively in Indian system of medicine (Sabu and Kuttan, 2002). Some *Terminalia* species may be used for infections caused by viruses; for example, *T. chebula* showed activity against the cytomegalovirus (Yukawa *et al*., 1996).

The microdilution assay is a reliable method to use in the determination of antimicrobial activity because it gives an idea of the concentration of plant extract able to inhibit or stop bacterial growth. Bioautography is helpful in identifying the compounds to be isolated as it enables location of compounds on TLC relative to other compounds which may or may not be active. Bio-assay guided isolation of compounds aims to the isolation of active compounds.

This study shows that the *Terminalia* species assayed exhibited both antibacterial and antifungal properties and this supports the use of these plants in African traditional medicine in the treatment of ailments caused by these bacteria and fungi. More investigation is necessary to test these plants for other activities and for toxicity tests in order to make them more useful and accessible to the African society.

The bioautography results showed that there is more than one compound responsible for the biological activity of these plant extracts. In an effort to increase accessibility and affordability of medicine, it is recommended that more active compounds would be isolated from *Terminalia* species in order to develop active antimicrobial agents.

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