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WESTERN CAPE

**ANTIBACTERIAL ACTIVITY OF SOME SOUTH AFRICAN PLANT
EXTRACTS AGAINST *STREPTOCOCCUS MUTANS***

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DECLARATION

I, **Madeha Mohammed Kelani**, declare that “**Antibacterial Activity of Some South African Plant Extracts Against *Streptococcus mutans***” is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.

Madeha Mohamed Kelani
Student Number: 3481126



Date Signed

DEDICATION

*This study is dedicated to my Brother, Fouad, and my
Family.*

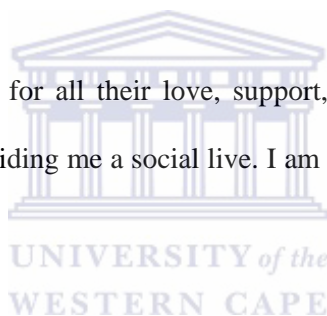


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ABSTRACT

Good oral health has a major influence on the general quality of life and well-being. Several chronic and systemic diseases have been attributed to poor oral health. With the increasing incidence of oral diseases, the global need for alternative prevention and treatment methods and safe, effective, and economical products has expanded. There are many studies showed that *Streptococcus mutans* has been implicated as major etiological agent of dental caries. South African has very rich natural flora especially Cape region. Several South African medicinal plants have been used since immemorial time for treatment of different human pathologies including dental problems. The antibacterial activities of plants extracts have been recognized and documented scientifically during the last few decades. In this project we aimed at the screening of 31 South African plants randomly collected from Cape region for their antibacterial activity against *Streptococcus mutans* initially at two concentrations (500 and 100 µg/ml). MIC's and MBC's were determined for the active plant extracts, as well as the qualitative studies of the effect of the active extracts on the biofilm formation. The best or the most active safe plant (*Psoralea fruticans*) was submitted to chromatographic separation to isolate the bioactive compounds which could be responsible for such activity. Identification of the pure active compounds was carried out using different spectroscopic techniques. Finally the biological evaluation of the isolated pure compounds was measured against *S. mutans* and also the cytotoxicity studies in-vitro against normal cells lines was carried out.

Keywords: Oral care, *Streptococcus mutans*, Cape flora, plant extracts, *Psoralea fruticans*, antibacterial activity, inhibition of biofilm formation, cytotoxicity, biological screening

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
DCM	Dichloromethane
DIW	Deionized water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EtOAc	Ethyl acetate
FBS	Foetal Bovine Serum
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectroscopy
IC₅₀	Half Maximal Inhibitory Concentration
INT	p-Iodonitrotetrazolium
IR	Infrared Spectroscopy
MBC	Minimal Bactericidal Concentration
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
min	Minute(s)
MMC	Minimum Microbicidal Concentration
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
<i>P. fruticans</i>	<i>Psoralea fruticans</i>
PBS	Phosphate Buffered Saline
<i>S. mutans</i>	<i>Streptococcus mutans</i>
TLC	Thin Layer Chromatography
UVS	Ultra Violet Spectroscopy
WST-1	Sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium salt

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Oral diseases are major health problems with tooth decay and gum disease representing a serious infectious disease that require global attention. Oral health affects the general quality of life and is associated with poor oral health, chronic diseases and systemic diseases. Many of the more than 750 species of bacteria that live in the mouth cavity can cause a number of oral diseases. The development of dental caries involve acidogenic bacteria and acid-tolerant Gram-positive bacteria (*Streptococcus mutans*, *Lactobacilli* and *Actinomyces*). Periodontal diseases have also been linked to anaerobic Gram-negative bacteria (*Porphyromonas gingivalis*, *Actinobacillus*, *Prevotella* and *Fusobacterium*). Due to the occurrence of oral disease, increasing resistance of bacteria to antibiotics, the negative or side effects of some antibacterial agents currently used in dental medicine and financial considerations in developing countries, there is a need for alternative prevention and treatment options that are safe, efficacious and cost-effective. Commercially available products contain different synthetic chemicals that alter the oral bacterial populations (microbiome or microbiota) and have undesirable side effects such as vomiting, diarrhoea and staining of teeth. Consequently, the search for alternative products continues and natural phytochemicals isolated from plants used as traditional medicines are considered good alternatives.¹

1.2 Oral Diseases

Periodontal diseases and tooth decay, as mentioned before, are the most prominent global oral health problems, although some conditions such as oral cancers and oral tissue lesions are also the cause of considerable health concerns.² Despite the popular progress in the overall health.

status, including oral and dental health, of populations living in industrialized countries, the spread of dental caries among children at schools is estimated at more than 90%, but also affects the majority of functions of craniofacial complex parts beyond adult life. There is considerable evidence linking poor oral health to chronic diseases, for instance, there is a significant association between rugged periodontal disease and diabetes.² Several lines of evidence suggest a link between poor oral health and systemic diseases, like rheumatoid arthritis, osteoporosis and cardiovascular diseases, whereas gum disease may also contribute to the risk of pregnancy complications such as preterm low-birth weight.³

Tooth loss caused by periodontal health (which affects up to 20% of the adult population in the world) could result in significant morbidity and premature death. The economic impact of oral disease is one of the important considerations with up to 10% of public health expenditure in developed countries allocated to dental care.² In most developing countries, spending in oral health care is much lower due to poor access to dental health care which is bounded and in general restricted to pain relief or contingency dental care.^{2,4}

The establishment of the relationships between oral disease and the activation of the microbial species that are part of the germs of the oral cavity is well defined.⁵ More than 750 species of bacteria live in the oral cavity (50% of which have not yet to be identified) and a number of those involved in oral disease.⁵ The development of tooth decay involves acidogenic bacteria and aciduric Gram-positive bacteria, primarily the *Streptococcus mutans* and *S. sobrinus*, *Lacobacilli* and *Actinomycetes*, which metabolize sucrose to organic acids (lactic acid in the first place) that dissolve the calcium phosphate in teeth, causing decalcification and eventual decay. Dental caries is thus supragingival condition.⁶ On the other hand, periodontal diseases are conditions under the gum that are linked to the Gram-negative bacteria such as *Actinobacillus sp*, *Prevoella sp*, *Porphyromonas gingival* and *Fusobacterium sp*.^{5,7} In periodontal disease, the areas at or below the gingival crevice become an infected incision which causes cellular inflammatory responses of the gums and

surrounding connective tissue. These inflammatory responses can manifest as gingivitis (very common and palpable as bleeding from the gums or gingival tissue) or periodontitis (the inflammatory response results in loss of collagen attachment of the tooth to the bone and loss of bone).⁶

1.3 Tooth Decay

Tooth decay occurs by the acid on the surface of the enamel. Acid is produced when sugars (mainly sucrose) in foods or beverages interact with bacteria in the dental biofilm (plaque) on the surface of the teeth. Acid production leads to loss of calcium and phosphate from the enamel. This process is called demineralization.⁸ Saliva works to weaken and neutralize the acid that causes demineralization. The enamel demineralizes and remineralizes many times during the course of a day. When the enamel ionic balance is constantly disrupted and demineralization exceeds remineralization tooth decay progresses. When this happens a lot and demineralization exceeds remineralization over several months, while purification occurs persistently and exceeds remineralization over time and over many months, there is a collapse of the enamel surface which leads to a cavity. Cavities, even in children who do not have their permanent teeth, can cause serious complications such as pain, tooth abscess, tooth loss, broken teeth, chewing problems and serious infections.⁹

1.4 Caries Process

The basic concept of the process of caries of the teeth is simple and was first described more than 100 years ago. The teeth are wrapped by bacteria which specify the dental plaque.^{8,10} Some of the bacteria in dental plaque have been identified, including such as *mutans streptococci* (e.g., *Streptococcus mutans* and *S. sobrinus*) and *lactobacilli*, which are acidogenic, i.e., they produce acids when the metabolism of carbohydrates enter a fermentation phase.⁸ These acids, such as lactic, acetic, propionic and formic acid can dissolve the mineral calcium phosphate from the tooth enamel or dentin.¹⁰ If this process is not halted or reversed, it progresses to carious lesions, eventually leading to a cavity. Many

carbohydrates such as glucose, sucrose, fructose or cooked starch can be metabolized by these bacteria with the evolution of organic acids (e.g., propionic, lactic, acetic) as byproducts.¹¹ The acids diffuse through the plaque and porous enamel (or dentin, if exposed), dissociating to produce hydrogen ions.¹² Hydrogen ions melt the metal easily, and release calcium and phosphate into solution, which can be spread from tooth. This is demineralization or, loss of mineral as illustrated in Figure 1.1.⁹

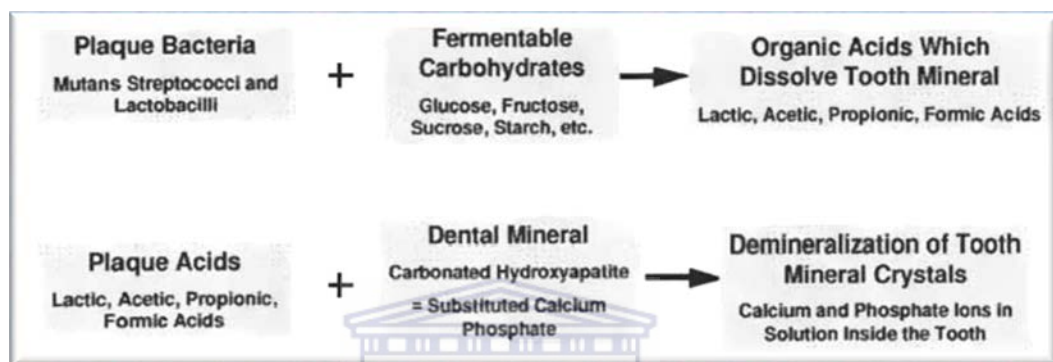


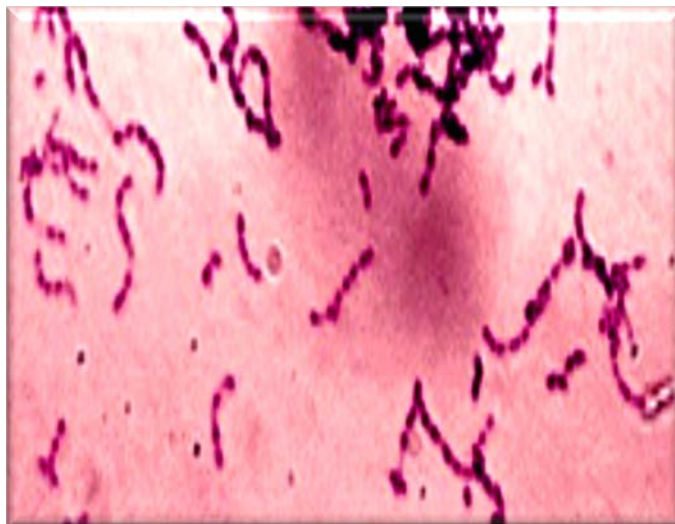
Figure 1.1: Schematic of acid production by plaque bacteria, followed by tooth demineralization

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1.5 Streptococcus mutans

Streptococcus mutans is a facultatively anaerobic, Gram-positive bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay. The microbe was first described by J Kilian Clarke in 1924.¹³ *Streptococcus* belongs to the firmicutes family and a group of lactic acid bacteria. *S. mutans*, a member of the human oral flora, is widely recognized as the main factor that causes tooth decay. Conditions in the oral cavity are influenced by a diversity of oral resident microbiota and commensal flora, and their usual balance with the host can change from one extreme to another, leading to the emergence of potentially pathogenic bacteria. *S. mutans* must tolerate the harsh environmental fluctuations quickly upon exposure to various antimicrobial agents to survive. *Streptococci* represent 20% of the oral bacteria and, along with sucrose, essentially determine the development of biofilms.¹⁴⁻¹⁶ Even though *Streptococcus mutans* could be antagonized by the leading oral

colonists, once they become dominant in mouth biofilms, tooth decay can develop and thrive.¹⁷ Figure 1.2 shows *S. mutans* stained in a thioglycollate broth culture.



Gram stain. Thioglycollate broth culture. Morphology is rod-like with chains when cultured on broth. Can cause subacute bacterial endocarditis and dental caries. Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #1043

Figure 1.2: *Streptococcus mutans* stained in a thioglycollate broth culture

While *S. mutans* grow in biofilm cells maintain a balance of metabolism involving the production and detoxification reactions. Biofilm is the sum of microorganisms and the cells that are committed to each other or the surface. Bacteria in the biofilm community can actually generate various toxic compounds that interfere with the growth of other competing bacteria. However, there have been very few studies on how to *S. mutans* can tolerate such an exposure to various toxic substances during their growth in the oral biofilm. Therefore, it is understandable that over time *S. mutans* developed strategies to successfully colonize and maintain a dominant position in the oral cavity.

1.6 Traditional Herbal Medicine and Oral Care

Medicinal plants have been used as traditional treatments for different diseases that affect humans for thousands of years in many parts of the world. In rural areas in developing countries, they continue to be used primarily as a source of medicine.¹⁸ Around 80% of

African populations use traditional medicines to take care of their health.¹⁹ Natural products derived from medicinal plants have proved to be a source of abundant bioactive compounds, many of which were the basis for the development of new lead chemicals to pharmaceuticals. The many diseases caused by microorganisms, and the increasing incidence of resistance among common pathogens to currently used therapeutic agents, like antiviral agents and antibiotics, has led to a renewed interest in the discovery of new anti-infective compounds. Of the nearly 500,000 species of plants that occur in all parts of world, only 1% has been investigated phytochemically, and there great potential for discovery of newer biologically active compounds.¹

Globally, the need for protection options and alternative products for oral diseases and treatments that are safe, effective and economical comes from the increase in the infection rate (especially in developing countries), the increase in the resistance of pathogenic bacteria to currently used antibiotics and chemotherapeutics, in addition to opportunistic infections in immunocompromized individuals.^{7,20} Although many agents are commercially available, these chemicals change the oral bacteria and have undesirable side effects such as vomiting, diarrhoea and staining of teeth.¹⁹

1.7 Traditional Plants Used to Treat Periodontal Diseases

There have been numerous reports of the use of traditional herbal medicine and pure natural compounds for the treatment of oral disease. Early studies have clearly confirmed that a number of substances had the potential to be used in the dental industry, due to their activity against cariogenic bacteria and the bacteria associated with periodontal disease. Several studies have investigated the activity of traditional plants against oral pathogens and the examination were mainly to validate the traditional use of the medicinal plants.

1.7.1 *Drosera peltata* (Droseraceae)

The use of *Drosera peltata* as a traditional treatment for tooth decay have been amply

validated. The chloroform extract of the aerial plant parts shows broad spectrum activity against many bacteria of the oral cavity, with greatest activity against *S. mutans* and *S. sobrium* (MIC=31.25 and 15.625 µg/ml, respectively).²¹ Plumbagin has been identified as the active ingredient of this extract which gave MIC values of 1 µg/ml against *S. mutans* and *S. sobrinus* and 2 µg/ml against *S. rattus* and *S. cricetus*.

1.7.2 Rhizoma coptidis (Ranunculacea)

The water extract of *R. coptidis*, a traditional Chinese medicinal plant, showed bactericidal activity against oral bacteria, especially against periodopathogenic bacteria (MIC=31-250 µg/ml).²²

1.7.3 Hamamelis virginiana (Hamamelidaceae)

Louk et al. assessed the ability of 10% decoctions and methanol extracts of a number of medicinal plants to inhibit bacterial isolates obtained from crevicular fluid of the periodontal pockets of periodontal patients. Generally, decoctions showed less activity than methanol extract. The extract from the leaves of *Hamamelis virginiana* had the greatest activity against all bacteria tested, with MIC values <512 µg/ml, particularly against *Porphyromonas spp.*, *Prevotella spp.* and *Actinomyces odontolyticus*.²³

1.7.4 Allium sativum (Liliaceae)

Allium sativum (garlic) has been used as a medicine since ancient time because of its antibacterial properties.²⁴ Whereas garlic has been shown to have such activity against a broad range of bacteria, the particular activity against Gram-negative oral pathogens, including *P. gingivalis* was only recently demonstrated. The garlic extract contains the major antimicrobial component, allicin, which is less active against Gram-positive bacteria (MIC range 35.7-142.7 mg/ml garlic; 13.7-55 µg/ml allicin), but more active toward a range of Gram-negative species (MIC range 1.1- 35.7 mg/ml garlic; 0.4-13.7 µg/ml allicin). The extract almost completely inhibited trypsin-like protease activity (implicated in the

pathogenesis of periodontitis) of *P. gingivalis*.²⁴

1.7.5 *Harungana madagascariensis* (Hypericaceae)

H. madagascariensis is a native African plant which contain numerous antimicrobial components.²⁵ Using successive soxhlet solvent extraction, an ethyl acetate extract of leaves was prepared and tested against many oral pathogens. While the extract was able to kill all the oral bacteria tested (including *Streptococcus species*, *Lactobacillus*, *Propionibacterium*, *Fusobacterium Actinomyces* and *Prevotella*), poly (D-L-lactide-co-glycolide) nanoparticles containing extract showed promising activity, with minimal bactericidal concentration (MBC) $\leq 5 \times 10^2$ mg/l, except for *Lactobacillus casei* with 7.5×10^2 mg/l. The authors suggested that this might be due to the bioadhesive polymer properties, which led to the extraction being in contact with the bacteria for prolonged periods.²⁵

1.7.6 *Pistacia lentiscus* (Anacardiaceae)

Resin exuded by *P. lentiscus* (*Anacardiaceae*) tree, known as the Mastic Gum, has been shown to have antimicrobial activity and is used in the preparation of food and as treatment for oral malodor, but the low solubility suggested that it might be useful for local application rather than a mouth rinse.²⁶ The *P. vera* extract has also been shown to possess antibacterial activity against oral *Streptococci* (MIC > 1 mg/ml), including the inhibition of adherence and glycolysis.²⁷

1.7.7 *Piper cubeba* (Piperaceae)

An ethanol extracts of *P. cubeba* showed good antibacterial properties against a range of cariogenic pathogens (MIC=90-200 μ g/ml). The ethanol extract was more active against *S. salivarius* (MIC value of 80 μ g/ml). (-)-Cubebin displayed MIC values ranging from 0.20 mM for *Streptococcus mitis* to 0.35 mM for *Enterococcus faecalis*.²⁸

1.7.8 *Helichrysum italicum* (Compositae)

H. italicum is widely found in the Mediterranean region and has been shown to have an assortment of biological properties. An ethanol extract of powdered flowering tops was demonstrated to have antimicrobial activity against *S. mutans*, *S. sanguis* and *S. sobrinus*, with MIC values of 31.25-62.5 µg/ml.²⁹

1.7.9 *Salvadora persica* (Salvadoraceae)

S. persica is a plant growing in the deserts from the region of western India to Africa. Other names for this plant are the Arak tree, chewing stick, natural toothbrush and Miswak or Siwaak. *S. persica* showed affectivity to prevent dental caries if it is used for a long time.³⁰ *S. persica* has been used for centuries as a traditional method for cleaning teeth. Roots of *S. persica* have been demonstrated to possess antimicrobial activity and produce a number of antimicrobial substances. The BITC (benzyle isothiocyanate) is the main antimicrobial component with high killing activity against Gram-negative periodontal pathogens.³¹ *S. persica* has antimicrobial effects against *S. mutans* and *S. faecalis*. Also, it has been showed that *S. faecalis* is the most sensitive microorganism affected by *S. persica*.³²

1.7.10 *Euclea natalensis* (Ebenaceae)

E. natalensis is an African traditional medicinal plant. The root is used for cleaning teeth and the gums with a chew, in the belief that it benefits the health of the mouth and teeth.³³ The root of *E. natalensis* contains naphthoquinones which are bactericidal.³³ Moreover, the root extract of the plant showed strong antibacterial activity against *S. mutans*. The authors suggested that the regular use to the *E. natalensis* might control the formation and activity of dental plaque and therefore reduce the incidence of gingivitis and possibly of dental caries.³⁴ Another study showed that the ethanol extract of *E. natalensis* has a positive inhibitory activity against five oral pathogens (*Actinomyces naeslundii*, *Actinomyces israelii*, *Porphyromonus gingivalis*, *Prevotella intermedia* and *S. mutans*).³⁵

1.7.11 *Psoralea corylifolia* (Fabaceae)

The antimicrobial activity of *P. corylifolia* leaves and its corresponding callus extract were tested against pathogenic bacteria causing periodontitis. The leaf callus methanol extract was found to be more effective against the pathogenic bacteria.^{36,37} Bakuchiol have been isolated from the seeds of *P. corylifolia*, and showed bactericidal effects against many oral bacteria, including *Streptococcus sanguines*, *S. salivarius*, *S. mutans*, *S. sobrinus*, *Enterococcus faecalis*, *E. faecium*, *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *Actinomyces viscosus* and *Prophyromonas gingivalis*. The bakuckiol might be a useful compound for the development of antibacterial agents against oral pathogens and also has a great potential to be used in food additives and mouth rinses to prevent and treat dental caries.^{36,38}

1.7.12 *Azadirachta indica* (Meliaceae)

A. indica (Neem) is commonly used as oral hygiene tools in different parts of the world. Neem has been considered to have various activities such as anti-ulcer and for cleaning the teeth in pyorrhea and other dental diseases. Leaves of the Neem have been used in the treatment of gingivitis and periodontitis. Neem has also showed enhanced efficacy in the treatment of oral infections and plaque growth inhibition in treating periodontal disorders. Neem has a good *in vitro* broad range antibacterial activity^{39,40}. Several studies have demonstrated the antibacterial activity and the anti-plaque anticariogenic properties of *A. indica*. Almas K reported that Neem chewing stick extract are effective at 50% concentration on *S. mutans* and *S. faecalis*^{32,41}.

1.7.13 Other Plants

Two recent studies examined the number of plants traditionally used in Brazil⁴² or South Africa,³⁵ respectively, for activity against oral pathogens *Prevotella intermedia*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *S. mutans* and *Lactobacillus casei*. Four Brazilian plant extracts, *Aristolochia cymbifera* (Aristolochiaceae), *Ziziphus joazeiro* (Rhamnaceae), *Caesalpinia pyramidalis* (Fabaceae) and *Cocos nucifera* (Palmae), were

shown to be active against the bacteria tested, with the ethanol extracts of *A. cymbifrica* being the most effective. Plants in South Africa (*Annona senegalensis*, *Englerophytum magalimontanum*, *Dicerocarym senecioides*, *Euclea divinorum*, *Euclea natalensis*, *Solanum panduriforme* and *Parinari curatellifolia*) were also tested, and all proved effective. In the case of *Breynia nivosus* (*Euphorbiaceae*) and *Ageratum conyzoides* (*Asteraceae*), cold and hot water and ethanolic extracts were tested for activity against *S. mutans*—the hot water and ethanol extracts showed weak activity (MIC>25 mg/ml).⁴³ Another study determined the ability of commercially available food that contain a high proportion of polyphenol to prevent the growth of *S. mutans* and other oral pathogens.⁴⁴ All extracts showed activity, with an extract of red grape seeds exhibiting the greatest activity against *S. mutans* (MIC=500 µg/ml). The unfermented cocoa extract and green tea were most active against other oral pathogen. The various extracts were also able to prevent the adhesion *S. mutans* on glass. These data indicate that extracts of polyphenol-containing foods might have a protective role against dental caries.⁴⁴

An investigation of a group of 27 medicinal plant extracts to identify the number that inhibits the growth of oral *Streptococci* showed that the most active extracts included those from *Abies canadensis* (*Pinaceae*), *Chelidonium majus* (*Papaveraceae*), *Albizia julibrissin* (*Fabaceae*), *Juniperus virginiana* (*Cupressaceae*), *Pinus virginiana* (*Pinaceae*), *Ginkgo biloba* (*Ginkgoaceae*), *Rosmarinus officinalis* (*Lamiaceae*), *Tanacetum vulgare* (*Asteraceae*), *Thuja plicata* (*Cupressaceae*) and *Sassafras albidum* (*Lauraceae*).⁷

1.8 Problem Statement

Dental caries is a major problem worldwide—*Streptococcus mutans* is a primary cause. The use of conventional preventive methods have proven effective in managing dental caries, however, excessive use of these synthetic products can change oral flora and cause other dental problems. For these reasons, there is a dire need to develop more efficacious anticariogenic compounds from alternative sources, including unexplored medicinal plants.

Many studies have investigated the antibacterial activity of different medicinal plant extracts against *S. mutans*, but there are few reports on the antibacterial potential of *Psoralea fruticans*, especially its chemical constituents which may be used effectively against *S. mutans*. The rationale of this study was to determine the antibacterial potential of the total extracts and chemical constituents of *P. fruticans* against *S. mutans*.

1.9 Aim of the Study

The initial aim of the study was to determine the antibacterial activity of extracts of 31 South African medicinal plants, collected randomly from the Cape Flats region against *S. mutans*. This led to the isolation and identification of bioactive compounds from *Psoralea fruticans* and, finally, biological evaluation of the pure isolated compounds for cytotoxicity against the normal human diploid fibroblast cell line (KMST-6).⁴⁵

1.10 Research questions of this study

1. What are the possible active extract(s) from the 31 plants collected from the Cape Flats region?
2. Does all the isolated compounds have antibacterial activity against *S. mutans*?
3. How we can isolate them?
4. What type of secondary metabolites exist within the active extract?
5. Can these compounds prevent *S. mutans* biofilm formation?
6. Are these compounds toxic to a normal human cell line?

1.11 Objectives of this study

The main objective of the study are:

1. Collection of sample from their natural habitats, documentation and identification.
2. Preparation of methanolic extracts from collected sample.
3. Antibacterial evaluation of the plant extracts.
4. Isolation of pure compounds from *Psoralea fruticans*.
5. Identification of the isolated compounds and biological evaluation of these compounds.

CHAPTER 2

SCREENING OF THE PLANT EXTRACTS AGAINST S.MUTANS AND BIOFILM FORMATION

2.1 Introduction

Throughout history, natural flora have served as a source of alternative medicines, new pharmaceuticals, health care products and medicinal plants and are important for drug development and pharmacological research, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active constituents.⁴⁶ The antibacterial activity of plant extracts has been recognized for many years.⁴⁷ Plant extracts have been used for a wide variety of purposes for many thousands of years.⁴⁸

In many parts of the world there is a long tradition of herbal medicine use for the treatment of many infectious diseases.⁴⁹ Because of the side effects of and resistance pathogenic microorganisms develop against antibiotics, a lot of attention has recently been focused on extracts and bioactive compounds isolated from plant species. Herbal medicinal plants may offer a new source of antibacterial agents for use.⁵⁰ Many drugs currently used to treat bacterial infections and other diseases have originally been isolated from natural sources, including medicinal plants.⁵¹

One of the major rationales leading to the development and design of new drugs from plants is to examine the uses claimed in traditional folklore medicine. Many reports on the pharmacological investigations of crude extracts have been published to validate the traditional uses.⁵² The aims of this study were to evaluate the antibacterial activity of 31 medicinal plant extracts on *Streptococcus mutans* (ATCC 25175), the bacteria that causes dental caries and to determine their minimum inhibitory concentration (MIC) as well as the

minimum microbicidal concentration (MMC) by the microdilution technique using 96-well plates.⁵³

2.2 Materials and Methods

2.2.1 General Reagents

The following general reagents were used in this study (Table 2.1).

Table 2.1: General reagents and equipment used in this study

Chemicals	Supplier
<i>Streptococcus mutans</i> ATCC® 25175™	
Brain heart infusion	Quantum Biotech
Nutrient broth media	
Tryptic soy agar	
Glycerol	Merck
<i>p</i> -Iodonitrotetrazolium chloride (INT)	
Neomycin antibiotic	
Dimethyl sulfoxide (DMSO)	
Fluoroshield™ with DAPI (4',6-Diamidino-2-phenylindole)	Sigma Aldrich
Acetic acid	
Methanol	
Bisphenol-S (BPS)	
Instruments	
96-well culture plates	
6-well culture plates	Olympus
Olympus microscopes	
Vortex mixer	
Microscope slides	Lasec
Plastic loops	
Cover slides	
Incubator	Forma Scientific
Autoclave	
Benchtop centrifuge	Beckman-Coulter
Polystar omega microplate reader	BMG Labtech
Florescence microscope	Carl-Zeiss

2.2.2 Reagents and Solvents for Extraction

Methanol was bought from Crest Chemicals, South Africa. Ethyl acetate, and sulphuric acid from Kimix, South Africa. Vanillin was purchased from Merck Schuchardt, Germany.

2.2.3 Solvent Evaporation

The Rotavapor model Buchi Rotavapor RE 111 was used for solvent evaporation at 45°C.

2.2.4 Thin Layer Chromatography (TLC) Plates

Pre-coated plates of silica gel 60 F254 (Merck, Germany) was used for TLC analysis. Visualization of TLC plates was done by observing the bands or “spots” under UV at $\lambda 254$ nm and $\lambda 366$ nm using a UV lamp (CAMAG, Switzerland), and by spraying with the vanillin/sulphuric acid reagent.^{54,55}

2.2.5 Plant Collection

The plants were randomly collected from the Cape Flats Nature Reserve and surrounding areas, including the University of the Western Cape (UWC) campus in Cape Town, South Africa, in November 2012. except for *Aspalathus linearis* which was collected from local market (Afriflex, Cape town, South Africa) A herbarium was set up for each plant collection, and identified by Mr Frans Weitz (Department of Biodiversity and Conservation Biology, UWC) and deposited at the UWC herbarium (Table 2.2).

2.2.6 Plant Extraction

A known weight of each plant material (450g) was blended using a Waring blender with methanol (2.5 L) to increase the surface area and ease the extraction within little time. The blended plant material was soaked in a glass jar, warmed at 60°C for one hour and left for 24 hours at room temperature. The material was then filtered, and the residue extracted with fresh MeOH. The filtrate was concentrated *in vacuo* at reduced pressure using a Rotavapor. The dried crude extracts were transferred into labelled and washed vials and kept cooled until further use.

Table 2.2: List of plants collected for this study

No.	Plant Name	Family	TLC No.	Herbarium	Used Part
1.	<i>Brunsvigia orientalis</i>	Amaryllidaceae	13	Hu- 44	Leaves
2.	<i>Searisa lucida</i>	Anacardiaceae	3	Hu- 22	Leaves
3.	<i>Searsia laevigata</i>	Anacardiaceae	5	Hu- 46	Leaves
4.	<i>Metalisia muricata</i>	Asteraceae	7	Hu- 1b	Leaves
5.	<i>Athanasia trifurcate</i>	Asteraceae	25	Hu- 33	Leaves
6.	<i>Helichrysum cymosum</i>	Asteraceae	26	Hu- 47	Leaves
7.	<i>Helichrysm zeyheri</i>	Asteraceae	27	Hu- 18	Leaves
8.	<i>Helichrysm indicum</i>	Asteraceae	28	Hu- 43	Leaves
9.	<i>Athanasia crithmifolia</i>	Asteraceae	29	Hu- 22/1	Leaves
10.	<i>Arctotheca populifolia</i>	Asteraceae	31	Hu- 22/16	Leaves
11.	<i>Tarchonanthus camphoratus</i>	Asteraceae	32	Hu- 22/9	Leaves
12.	<i>Athanasia parvitflora</i>	Asteraceae	23	Hu- 20	Leaves
13.	<i>Senecio halimifolus</i>	Compositae	10	Hu- 8	Leaves
14.	<i>Scirpus antarcticus</i>	Cyperaceae	12	Hu- 59	Leaves
15.	<i>Psoralea fruticans</i>	Fabaceae	4-17	Hu-5	Leaves
16.	<i>Psoralea pinnata</i>	Fabaceae	18	Hu- 54	Seeds
17.	<i>Psoralea pinnata</i>	Fabaceae	19	Hu- 55	Leaves
18.	<i>Aspalathus linearis</i>	Fabaceae	21	-----	Leaves
19.	<i>Aspulathus hispida</i>	Faboideae	11	Hu- 54	Leaves
20.	<i>Castanea sativa</i>	Fagaceae	34	Hu- 17/3	Leaves
21.	<i>Salvia africana caerulea</i>	Lamiaceae	1	Hu- 47a	Leaves
22.	<i>Salvia africana lutea</i>	Lamiaceae	14	Hu- 50	Leaves
23.	<i>S. africana lutea</i>	Lamiaceae	15	Hu- 51	Root
24.	<i>Acacia karroo</i>	Leguminosae	16	Hu- 52	Leaves
25.	<i>Montania caryophyllacea</i>	Montiniaceae	33	Hu- 34	Leaves
26.	<i>Myrica quercifolia</i>	Myricaceae	8	Hu- 24	Leaves
27.	<i>Myrtus communis</i>	Myrtaceae	9	Hu- 42	Leaves
28.	<i>Phylica ericoides</i>	Rhamnaceae	6	Hu- 4	Leaves
29.	<i>Rosa canina</i>	Rosaceae	20	Hu- 57	Fruit
30.	<i>Halleria lucida</i>	Stilbaceae	24	Hu- 27	Leaves
31.	<i>Passerina rigida</i>	Thymelaeaceae	2	Hu-3	Leaves
32.	<i>Lichtensteinia lacera</i>	Apiaceae	36	Hu- 17/1	Leaves



Passerina rigida



Salvia african caerulea



Searisa lucida



Phyllica ericoides



Senecio halimifolius



Metalasia muricata



Aspalathus hispida



Psoralea pinnats seeds

All plant pictures were abstracted from Ispot in March 2015. www.ispotnature.org

Figure 2.1: Pictures of plants collected for this study



Rose canina (Chinese)



Aspalathus linearis



Acacia karroo



Salvia africana lutea



Psoralea fruticans



Myrica quercifolia



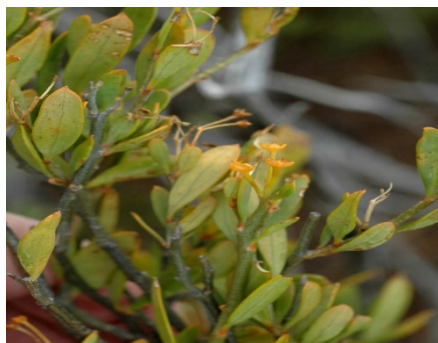
Myrtus communis



Athanasia trifurcata

All plant pictures were abstracted from Ispot in March 2015. www.ispotnature.org

Figure 2.1: Pictures of plants collected for this study (continued)



Montinia caryophyllacea



Tarchonanthus camphoratus



Athanasia crithmifolia



Arctotheca populifolia



Helichrysum zeyheri



Helichrysum indicum



Athanasia parviflora



Helichrysum cymosum

All plant pictures were abstracted from Ispot in March 2015. www.ispotnature.org

Figure 2.1: Pictures of plants collected for this study (continued)



Brunsvigia orientalis



Senecio halimifolius



Searsia laevigata



Psoralea pinnata



Halleria lucida



Castanea sativa



Scripus antarcticus



Lichtensteinia lacera

All plant pictures were abstracted from Ispot in March 2015. www.ispotnature.org

Figure 2.1: Pictures of plants collected for this study (continued)

2.2.7 Thin Layer Chromatography (TLC)

1 mg of crude extract (1-32) were dissolved in 100 µl of methanol to form a homogeneous solution in vials 1 to 35. The extract was then spotted onto TLC plates (10 X 20 cm), and developed in two different solvent systems (1% MeOH/DCM) and (8% MeOH/DCM).

2.2.8 Detection of Spots

The developed plates were viewed under an ultra violet (UV) lamp at wavelengths 254 and 366 nm. The TLC plates were sprayed with vanillin/sulphuric acid and gently heated according to the method described for plant drug analysis.⁵⁴ Figures 2.2 and 2.3 show the TLC plates of different plant extracts developed with 1% and 8% dichloromethane, respectively.

2.2.9 Preparation of Stock Solutions of the Extract

Approximately 10 mg of each extract was dissolved in 1 ml dimethyl sulphoxide (DMSO). Then 800 µl fresh nutrient broth medium was added to 200 µl of extract stock to a final concentration of 2 mg/ml.

2.2.10 Bacteria

Streptococcus mutans (ATCC 25175) was stored at -20°C. The bacteria were activated in brain heart infusion medium at room temperature and incubated for 24 hours at 37°C. After the incubation period, the Gram stain was carried out to check that the bacteria were clear and there was no contamination.

2.2.11 Qualitative Screening of Plant Extracts by Bioautography

The plant extracts were used to test for compounds with activity against the microorganisms using a simple and direct assay on TLC plates. Approximately 1 mg of each extract was dissolved in 100 µl methanol and spotted on TLC plates. The plates were developed in 3% MeOH/DCM and sprayed with *Streptococcus mutans* and incubated in a

humid atmosphere to allow for bacterial growth for 24 hours at 37°C. After incubation, the plates were sprayed with p-iodonitrotetrazolium violet salt (INT) and incubated for 2 hours at 37°C.^{56,57} The other plate was sprayed with vanillin/H₂SO₄ and heated.

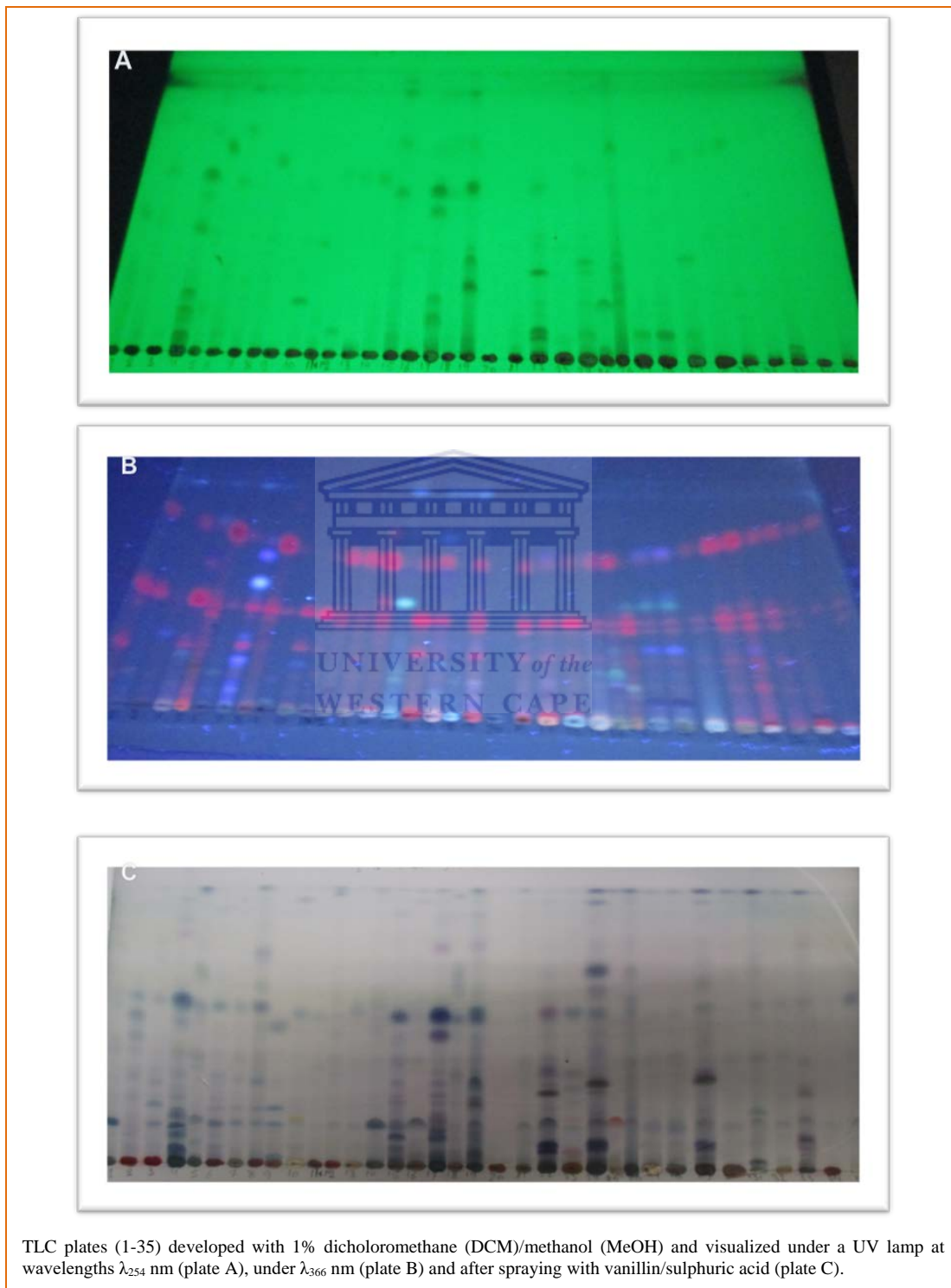
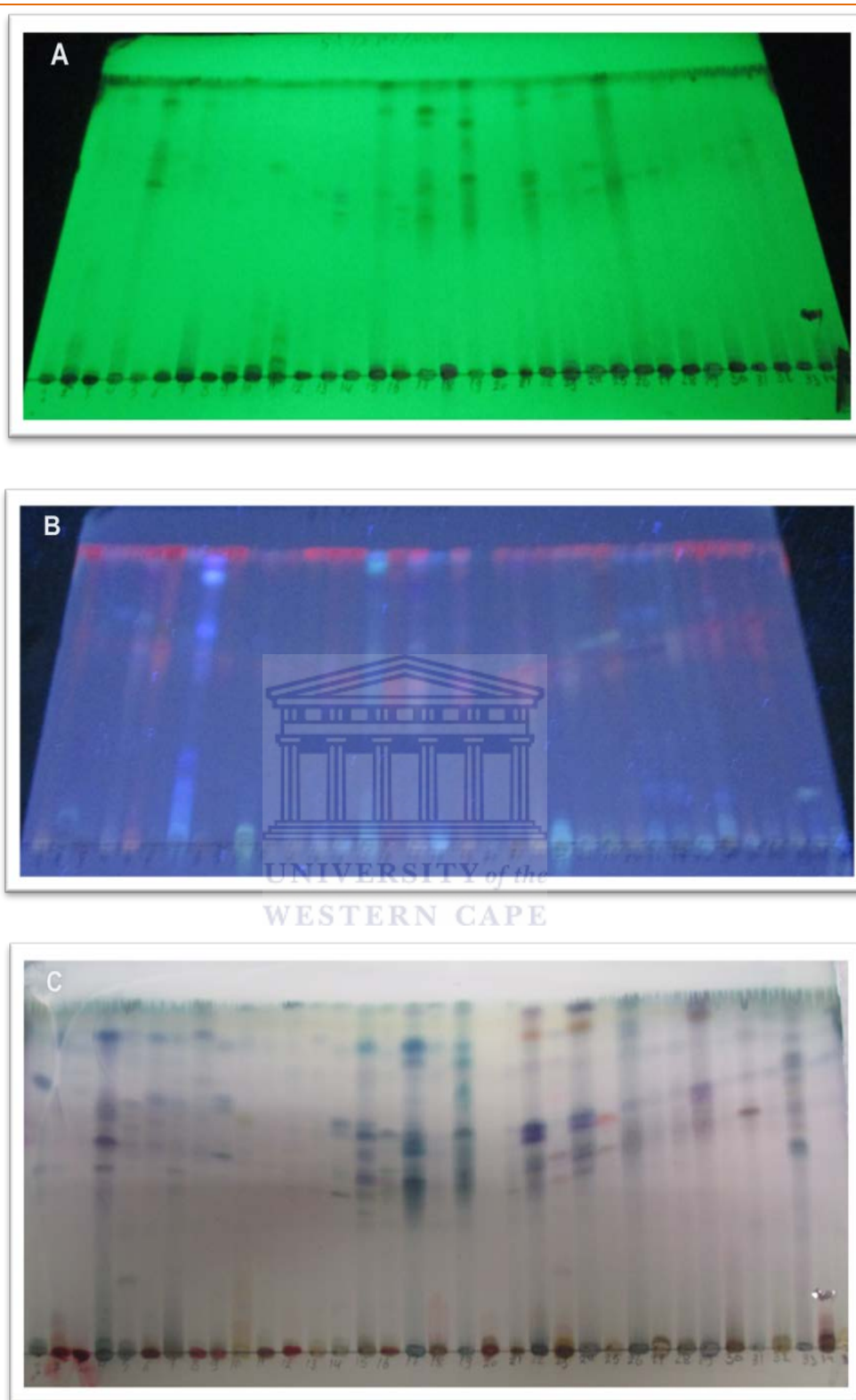


Figure 2.2: TLC plates of different plant extracts developed with 1% dichloromethane



TLC plates (1-35) developed with 8% dichloromethane (DCM)/methanol (MeOH) and visualized under a UV lamp at wavelengths λ_{254} nm (plate A), under λ_{366} nm (plate B) and after spraying with vanillin/sulphuric acid (plate C).

Figure 2.3: TLC plates of different plant extracts developed with 8% dichloromethane

2.2.12 Screening of Plant Extracts for Antibacterial Activity

The bacteria were transferred to nutrient broth media after centrifugation. The density was adjusted at 0.5 OD at 450 nm (McFarland standard) using a Polystar Omega microplate reader. All extracts were tested against *S. mutans* at two concentrations (500 and 100 µg/ml), then the plates were measured using a microplate reader. The first reading was just before incubation, then after 30 minutes, thereafter hourly over a period of 2 hours, then after 4 hours and the last reading was taken after 24 hours of incubation. After incubation for 24 hours at 37°C, microbial growth was indicated by adding 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet salt (INT) to microplate wells and incubated at 37°C for 1 hour. Each extract was analyzed in triplicate.

2.2.13 Determination of MIC and MMC

The microdilution technique using 96-well plates as described by Eloff⁵³ was used to obtain the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values of the crude extract against *S. mutans*. The active extracts were serially diluted in 96-well plates. The final concentration of extract and positive control (neomycin) ranged from 2 mg /ml to 0.005 mg/ml. Bacteria 24 hours old were added to the 96-well plate and incubated for 24 hours at 37°C, the density of bacteria was adjusted at 0.5 OD. Five concentrations were used to obtain the MIC (500, 250, 125, 62.5, and 31.25 mg/ml). The bacteria were treated with neomycin (positive control) and DMSO (negative control). Microbial growth inhibition was initiated by adding 40 µl of 0.2 mg/ml INT to microplate wells and incubated at 37°C for 1 hour. MIC was defined as the lowest concentration that inhibited INT colour change. The MMC was determined by adding 50 µl of the suspensions from the wells, which did not show any growth after incubation during the MIC assay, to 150 µl of fresh broth. These suspensions were re-incubated at 37°C for another 24 hours. The MMC was determined by adding 40 µl of 0.2 mg/ml INT to microplate wells and incubated at 37°C for 1 hour. The MMC was determined as the lowest concentration of extract that inhibited 100% growth of bacteria.^{58,59}

2.2.14 Biofilm Formation

The bacteria were activated in brain heart infusion medium and incubated for 24 hours at 37°C. After incubation, the culture was allowed to develop a biofilm on glass slides using 6-well plates. Approximately 50 µl bacteria and 50 µl fresh medium was cultured on a slide cover and incubated for 24 hours at 37°C. After incubation, the slide was either left untreated (negative control), treated with neomycin as a positive control or the MIC of the 8 active plants (*Salvia Africana caerulea*, *Psoralea fruticans*, *Acacia karroo*, *Salvia Africana lutea*, *Psoralea pinnata*, *Helicrsyum cymosum*, *Helichrysum zeyheri* and *Castania sativa*). After 24 hours incubation, the slides were removed from the plate and rinsed in sterile PBS. The fixation was done using 100% MeOH for 10 min, then 70:30 acetic acid:MeOH for 30 min followed with 100% for 2 min. Then the slide was rinsed again with PBS and dried. The slides were stained with Fluoroshield™ with DAPI and examined using a florescence microscope.⁶⁰

2.3 Results

2.3.1 Qualitative Screening Using TLC Bioautography

After the incubation period, the colour was clear the active extracts doing inhibition zones on the plate, as illustrated in Figures 2.4 and 2.5. Equal volumes of different plant extracts were spotted on silica gel plate. After development the plate was left to dry and either sprayed with bacteria or vanillin/H₂SO₄ for direct comparison. The purple colour indicates that the bacteria are still alive and the colourless to yellow zone denotes dead bacteria. The plates showed the extracts that have inhibitory activity of bacteria as well as the location of the active compounds.⁶¹

2.3.2 Microdilution Screening

From the 31 extracts tested, 8 plants were active against *Streptococcus mutans* at 500 µg/ml, and 3 plants were active at 100 µg/ml (Table 2.3). The active plants were *S. africana caerulea*, *Psoralea fruticans*, *S. africana lutea*, *Acacia karroo*, *Helichrysum cymosum*,

Psoralea pinnata, *Helichrysum zeyheri* and *Castanea sativa* 3 at 100 µg/ml. *Psoralea fruticans*, *Psoralea pinnata*, and *Helichrysum cymosum* were active at a concentration 100 µg/ml (Table 2.3, Figures 2.6 to 2.8).

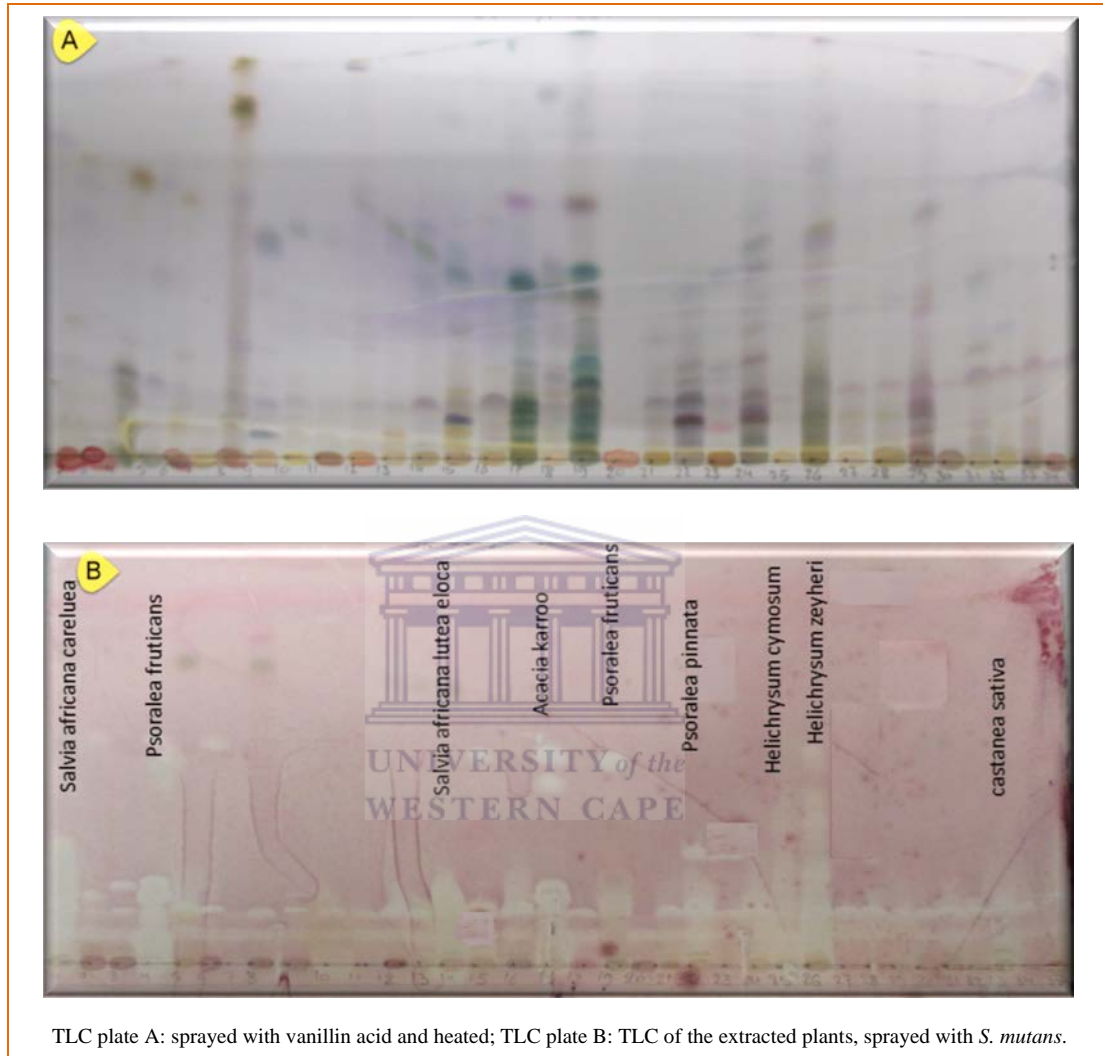


Figure 2.4: Qualitative screening of plant extracts using TLC bioautography—1

2.3.3 MIC and MMC

Preliminary screening for the plant extracts showed activities of eight extracts at 500 µg/ml and three of them showed potential activity at 100 µg/ml. The eight plant extracts were investigated for their MIC and MMC values. Table 2.4 shows the corresponding values of the extracts under study. The extracts of *P. fruticans* and *P. pinnata* were found to be the most effective against *S. mutans*, both exhibiting MICs of 31.25 µg/ml and MMCs of 62.5 µg/ml. *S. africana lutea*, *S. Africa caerulea*, *Acacia karro* inhibited bacterial growth at an MIC of

62.5 µg/ml with MMC of 125 µg/ml. *Helichrysum zeyheri*, *Helichrysum cymosum* and *Castanea sativa* inhibited the growth of bacteria at 125 µg/ml with an MMC of 250 µg/ml. The positive neomycine control inhibited the growth of *S. mutans* of 0.01 µg/ml with MMC of 0.31µg/ml (Table 2.4).

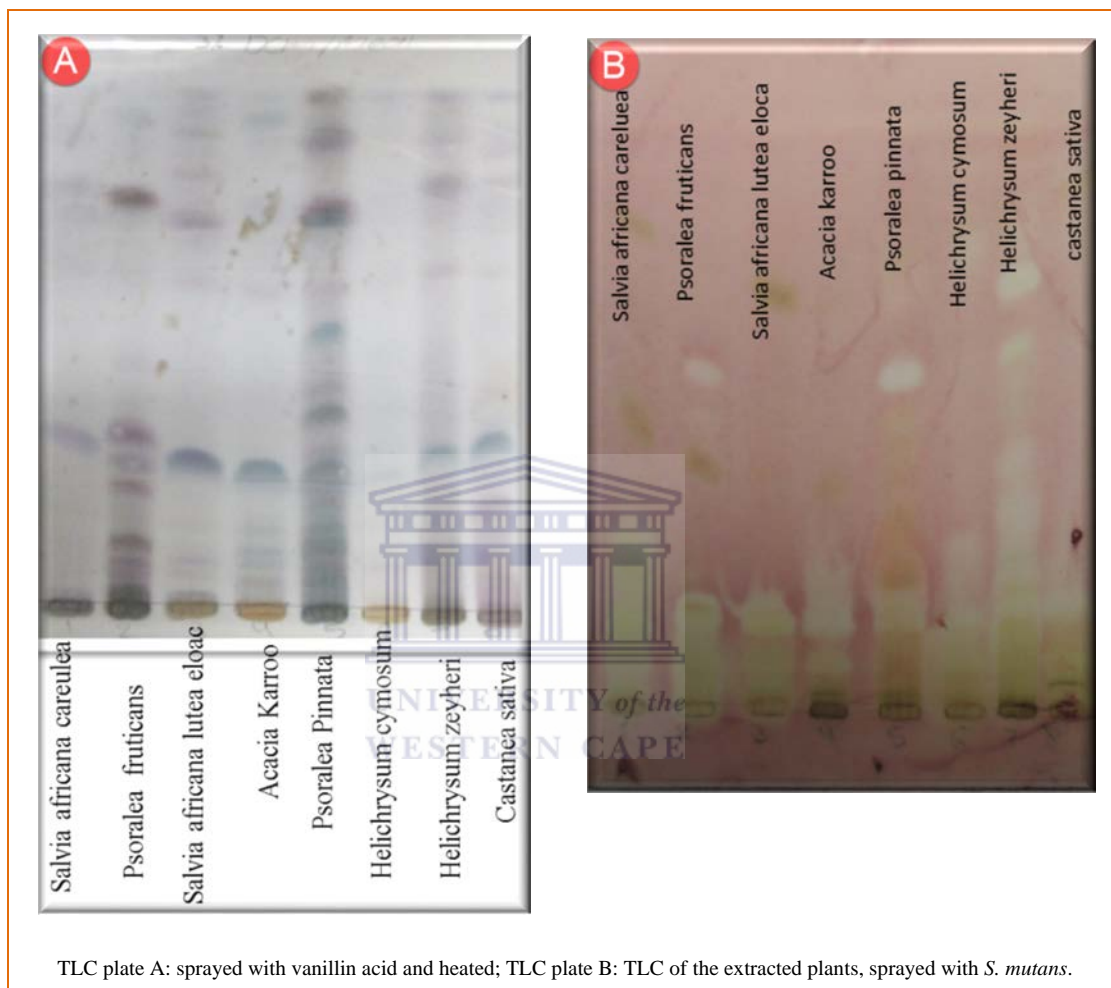


Figure 2.5: Qualitative screening of plant extracts using TLC bioautography—2

2.3.4 Streptococcus mutans Biofilm Formation

All the slides which were treated with extracts showed biofilm formation. The fluorescence microscope images show the treated slides decrease compared to the untreated slides or the control. The untreated cells formed a biofilm while the cells treated with extracts inhibited the formation of a *S. mutans* biofilm. Some extracts can reduce the biofilm development as *Psoralea fruticans* and *Salvia africana caerulea*, while the other extracts showed development in the biofilm (Figure 2.9).

Table 2.3: Activity of extracts prepared from plants collected for this study

Plant Extract	500 µg/ml	100 µg/ml
<i>Acacia Karroo</i>	Active	Not Active
<i>Arctotheca populifolia</i>	Not Active	Not Active
<i>Aspalathus linearis</i>	Not Active	Not Active
<i>Aspulathus hispida</i>	Not Active	Not Active
<i>Athanasia crithmifolia</i>	Not Active	Not Active
<i>Athanasia parvitflora</i>	Not Active	Not Active
<i>Athanasia trifurcata</i>	Not Active	Not Active
<i>Brunsvigia orientalis</i>	Not Active	Not Active
<i>Castanea sativa</i>	Active	Not Active
<i>Halleria lucida</i>	Not Active	Not Active
<i>Helichrysm indicum</i>	Not Active	Not Active
<i>Helichrysum zeyheri</i>	Active	Not Active
<i>Helichrysum cymosum</i>	Active	Active
<i>Lichtensteinia lacora</i>	Not Active	Not Active
<i>Metalisia muricata</i>	Not Active	Not Active
<i>Montinia caryophyllacea</i>	Not Active	Not Active
<i>Myrica quercifolia</i>	Not Active	Not Active
<i>Myrtus communis</i>	Not Active	Not Active
<i>Passerina rigida</i>	Not Active	Not Active
<i>Phylica ericoides</i>	Not Active	Not Active
<i>Psoralea pinnata</i> (seeds)	Not Active	Not Active
<i>Psoralea fruticans</i>	Active	Active
<i>Psoralea pinnata</i> (leaves)	Active	Active
<i>Rosa canina</i>	Not Active	Not Active
<i>S. africana lutea</i> (root bark)	Not Active	Not Active
<i>Salvia africana caerulea</i>	Active	Not Active
<i>Salvia africana lutea</i>	Active	Not Active
<i>Scirpus antarticus</i>	Not Active	Not Active
<i>Searisa lucida</i>	Not Active	Not Active
<i>Searsia laevigata</i>	Not Active	Not Active
<i>Senecio halimifolus</i>	Not Active	Not Active
<i>Tarchonanthus camphoratus</i>	Not Active	Not Active

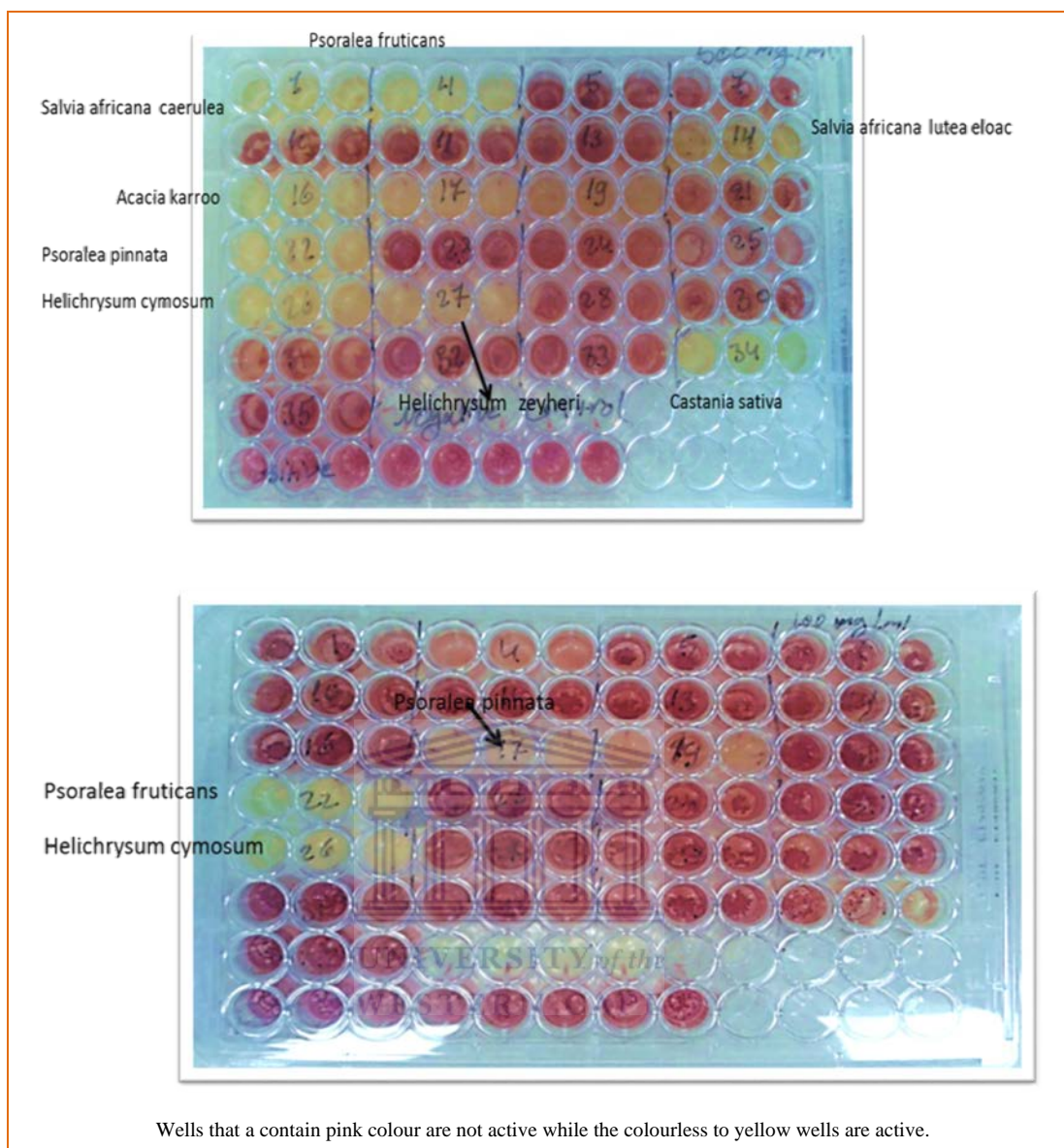


Figure 2.6: Microdilution screening colourimetric profiles of plant extracts active against *Streptococcus mutans*

Table 2.4: MIC and MMC values for eight plant active extracts

Plant Extract	MIC ($\mu\text{g/ml}$)	MMC ($\mu\text{g/ml}$)
<i>Salvia africana caerulea</i>	62.5	125
<i>Psoralea fruticans</i>	31.25	62.5
<i>S. africana lutea eloac</i>	62.5	125
<i>Acacia karroo</i>	62.5	125
<i>Psoralea pinnata</i>	31.25	62.5
<i>Helichrysum cymosum</i>	125	250
<i>Helichrysum zeyheri</i>	125	250
<i>Castanea sativa</i>	125	250
Positive Neomycin Control	0.01	0.31

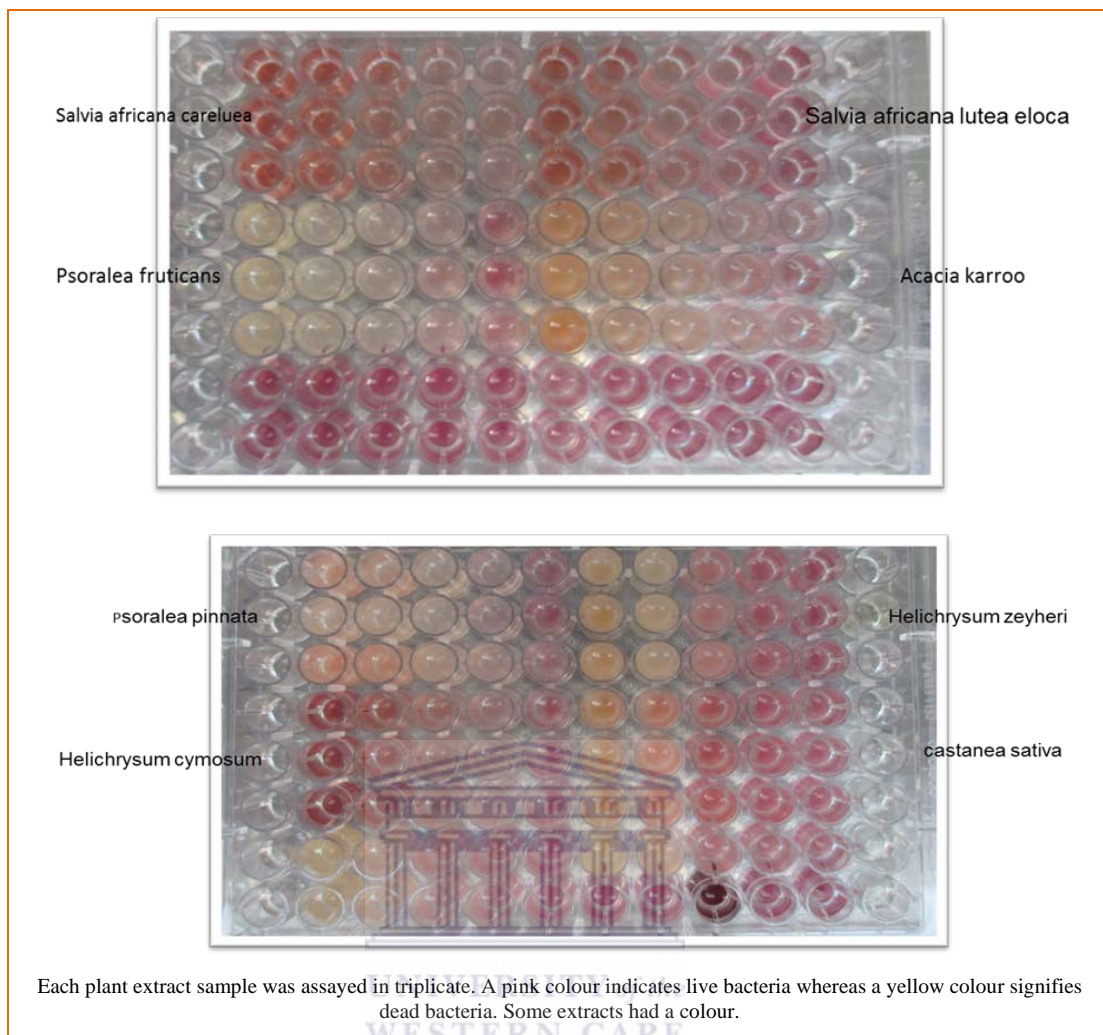


Figure 2.7: MIC colourimetric profiles of plant extracts active against *Streptococcus mutans*

2.4 Discussion

Of the 30 plants collected from the Cape Flats 32 extracts were generated and tested for their inhibitory activities against *S. mutans*. Initially, the plants extracts were screened at 500 and 100 $\mu\text{g/ml}$. The results showed that the extracts from eight plants (*Salvia africana caerulea*, *Psoralea fruticans*, *S. africana lutea*, *Acacia karroo*, *Helichrysum cymosum*, *Psoralea pinnata*, *Helichrysum zeyheri* and *Castanea sativa*) were active at 500 $\mu\text{g/ml}$, while extracts from only three plants (*P. fruticans*, *P. pinnata* and *H. cymosum*) demonstrated activity at 100 $\mu\text{g/ml}$.

MICs determined from the eight active plant extracts ranged from 250 to 31.25 $\mu\text{g/ml}$. *P. fruticans* and *P. pinnata* produced the most active extracts with MICs of 31.25 and MMCs of

62.5 $\mu\text{g/ml}$, respectively. The results showed that 42% of the plant extracts were active at 500 $\mu\text{g/ml}$ while 9% were active at 62.5 $\mu\text{g/ml}$. Furthermore, two plant extracts showed an MIC at 31.25 $\mu\text{g/ml}$. These plant extracts were selected for further phytochemical evaluation to isolate their bioactive compounds.



Figure 2.8: Absorbance values obtained for plant extracts evaluated by the microdilution technique

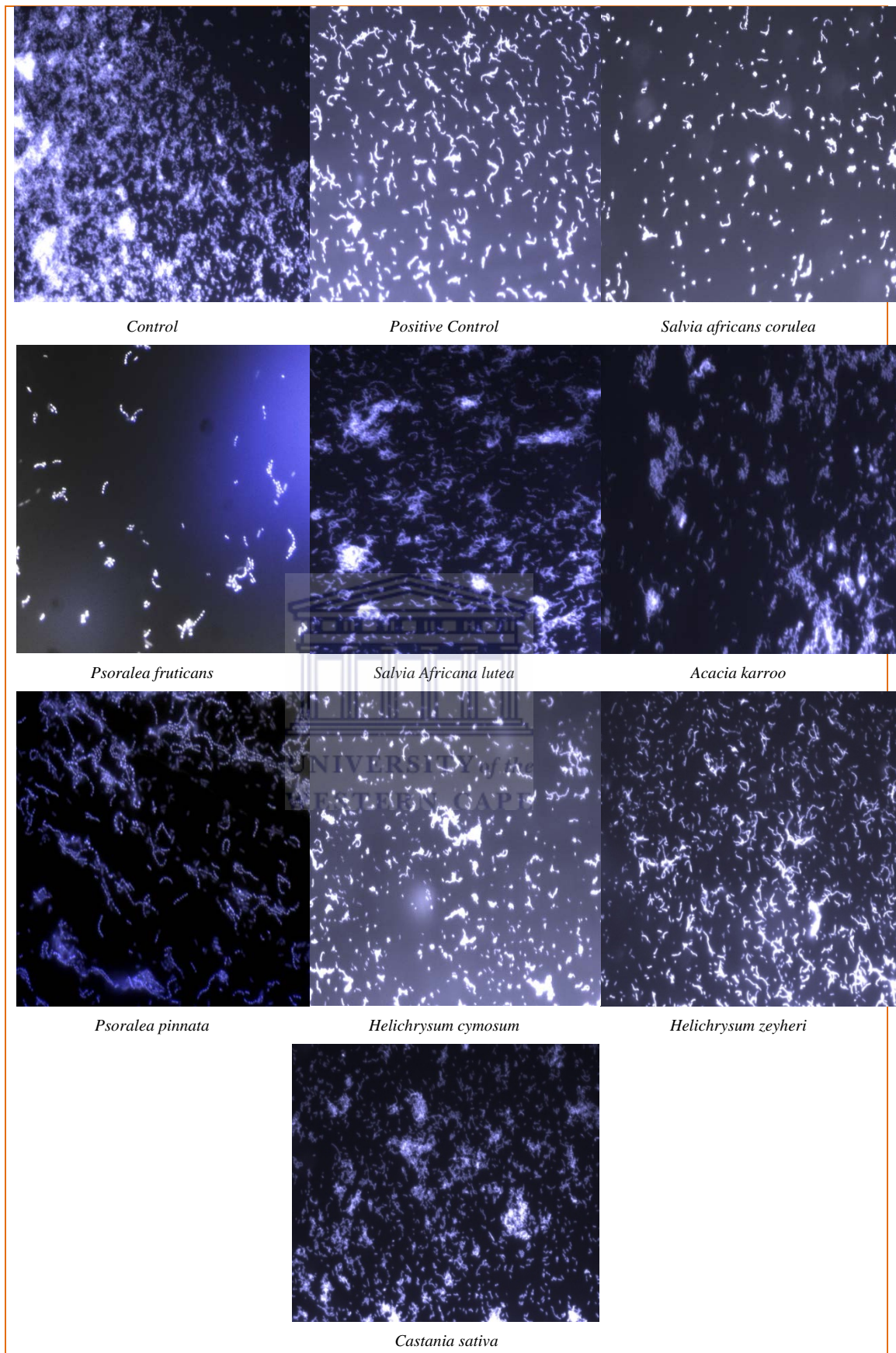


Figure 2.9: *Streptococcus mutans* biofilm formation after treatment with active plant extracts

The methanolic extract of *P. fruticans* was the most active against *S. mutans* with an MIC of 31.25 µg/ml and MMC 62.5 µg/ml. Thus, *P. fruticans* was indicated for detailed chemical characterization based on antibacterial activity because no such published data could be obtained in the literature and its TLC profiles suggested that it may be a rich source of compounds with inhibitory potential against *S. mutans*.

A study reported that the ethyl acetate extract of *Acacia karroo* showed antilisterial activity, exhibiting an MIC of 3.1 µg/ml against *L. monocytogenes*.⁶⁰ In another study, the methanol extract of *Acacia karroo* and *Salvia Africana* were able to prevent growth of *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans* and *Microsporum audouinii*. The *Acacia karroo* extract showed activity at MIC values below 100 µg/ml and *S. africana* showed the best activity with an MIC of 39.06 µg/ml against *E. coli*, *S. aureus* and *M. audainii*.⁶²

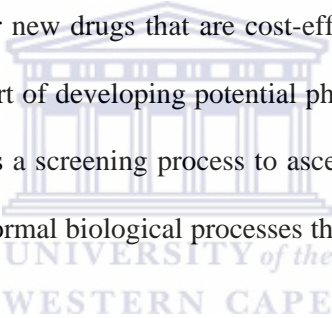
The aqueous extract of the dried leaves of *Salvia lutea* showed antimicrobial activity against *Staphylococcus aureus* at concentrations of 10 mg/ml and 5 mg/ml whereas the aqueous extract of dried leaves of *Salvia carulea* showed weak antimicrobial activity against the bacterium. No activity was demonstrated against *Pseudomonas aeruginosa*, *Candida albicans* or *Mycobacterium smegmatis*.⁶³ *Hilchrysum cymosum* showed bactericidal activity against *B. cereus* and it was also active against *S. aureus* and *S. pyogenes*.⁶⁴ *S. mutans* biofilms were developed using glass slides. Six active plants showed biofilms caused by cells as they adhered to each other. *Salvia africana caerulea* and *P. fruticans* showed a reduction in cell numbers and no development of a biofilm.

CHAPTER 3

CHEMICAL CONSTITUENTS OF *PSORALIA FRUTICANS* AND BIOLOGICAL EVALUATION OF THE ISOLATED COMPOUNDS

3.1 Introduction

In recent years, natural products have been shown to be more comprehensive and promising agents for the prevention of oral diseases, especially plaque-related diseases such as tooth decay. This, together with the increased antimicrobial resistance to available oral therapies attracted keen interest in the search for new drugs that are cost-effective, natural or of artificial origin.⁴⁰ Cytotoxic activity tests are part of developing potential pharmaceutical products into a clinically acceptable drugs and provides a screening process to ascertain that the compounds being tested are not more harmful to the normal biological processes than the effects for which they are being tested.⁶⁵



Various natural compounds have particular reactions against biological systems and cytotoxic evaluation of plants extracts is essential before they could be considered for new drug development.⁶⁶ Many plant extracts and isolated compounds have been used *in vitro* for cytotoxicity using different human and animal cell lines.⁶⁷ From the all tested extracts and according to the results obtained in the previous chapter, the methanolic extract of *P. fruticans* was the most active extract against *S. mutans* with an MIC of 32 µg/ml.

To date, no publication has reported on the chemical and/or biological aspects of *P. fruticans*. These two reasons provided an impetus and justification to start the chemical study of the methanolic extract of the plant to isolate and identify its main chemical constituents and evaluate the effects of isolated pure compounds against *S. mutans*.

Therefore, in this chapter the biological activity of the methanol extract of *P. fruticans* and isolated compounds against *S. mutans* are reported. The cytotoxicity activity of the crude extract of *P. fruticans* and isolated compounds is also reported for the normal KMST-6 fibroblasts cell line as measured colorimetrically by an *in vitro* assay using the water-soluble tetrazolium salt (WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, mono-sodium salt).^{68,69}

3.2 Literature Review on Genus Psoralea

3.2.1 Taxonomy, Morphology and Distribution of *Psoralea fruticans*

The taxonomy, morphology and distribution of *Psoralea fruticans* are summarized in Table 3.1 and Figure 3.1

Table 3.1: Taxonomy of *Psoralea fruticans*

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	Otholobium
Synonym(s)—Homotypic	Otholobium Fruticans (L.) Trifolium Fruticans (L.)
Synonym(s)—Heterotypic	Psoralea aculeate Thunb Psoralea cuneifolia Dum Psoralea bacteatal
Description	Straggling subshrub to 40 cm. Leaves 3-foliolate, leaflets obovate, glabrescent; stipules ciliate. Flowers purple to violet, calyx sparsely silky and glandular, lowest sepal much larger, very prominent, accrescent. Sep-Dec. Mountain fynbos, 160-400 m. SW (Cape Peninsula). ⁷⁰

The genus of *Psoralea* contains a wide range of shrubs and herbs subsidiary to the sub-papilionaceae family *Fabaceae*. The species, numbering about 120, are found in all parts of the tropical and subtropical regions of world. These plants are mainly found in South Africa (about 45 species), Australia (15 species) and North America (30 species). However *Psoralea* plants are also present in Asia, North Africa, South America and the Mediterranean region. They are annual or perennial, and they usually grow in arid or semi-arid zones.

The morphology of *Psoralea* genus is not very homogeneous. The habit of the species varies considerably; some attain a height of 15 feet, while others are low shrubs or diffuse herbs, while at least one species forms a dense cushion-like growth. A distinctive feature of the genus is the presence of resin glands on practically every part of the plant. Most species have leaves composed of three unequal leaflets, but some of them have one-foliated leaves, like *P. martinii* or the Indian *P. corylifolia*. The flowers have general shaped of the *Papilionoideae* family, usually are blue, purple, or yellow and the vast majority of species are sweetly scented flowers. The fruit is a small pod (3 to 8 mm long), indehiscent, one-seeded, and without albumen.^{71,72}

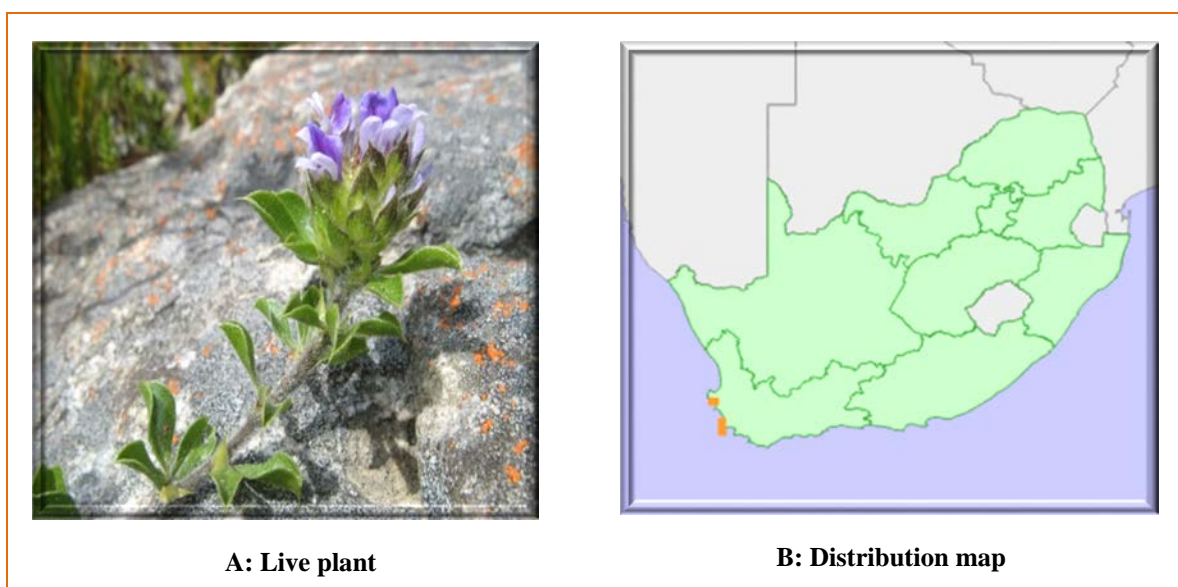


Figure 3.1: Morphology and distribution of *Psoralea fruticans*

3.2.2 Chemistry of *Psoralea* genus

Few studies on the *Psoralea* genus are found in the literature, however *P. corylifolia*, among other species, have been studied extensively and different secondary metabolites including coumarins, furanocoumarins, flavonoids, meroterpenoids and coumestanes have been reported.

3.2.2.1 Coumarins

The chemical studies of seeds of *P. corylifolia* revealed the presence of psoralen (1), psoralidin, psopsoralen or angelicin (2)⁷³⁻⁷⁵ coumarin (3)⁷⁶. isopsoralenoside (4) and psoralenoside (5)⁷⁷ and 8-methoxypsoralen (6),⁷⁵ as shown in Figure 3.2. Angelicin was also isolated from the dichloromethane extract of *Psoralea glandulosa*.⁷⁸ Psoralen was reported from the leafy branch, flowers, fruits and roots of *P. acaulis* and *P. bituminosa*.^{73,79,80}

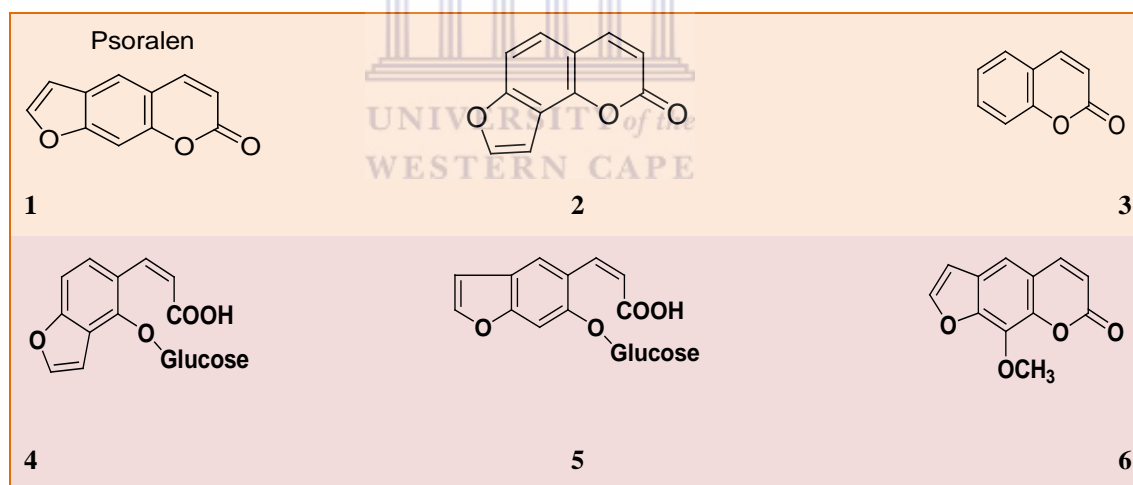


Figure 3.2: Coumarins and furanocoumarins from *Psoralea* species

3.2.2.2 Flavonoids

Bavachalcone (7), bavachinin (8), isobavachin (9) have been isolated from the seeds of *Psoralea corylifolia* Linn. and from *Psoralea fructus*,^{81,82} corylifols C (10),⁸³ 6-prenylnarigenin (11),^{73,84} coryfolia A (12),⁸⁵ corylifolin (13),⁸⁶ astragalin (14)⁷⁵ and bavachin (15)^{81,82} were isolated from

the seeds of *Psoralea corylifolia* (Figure 3.3).

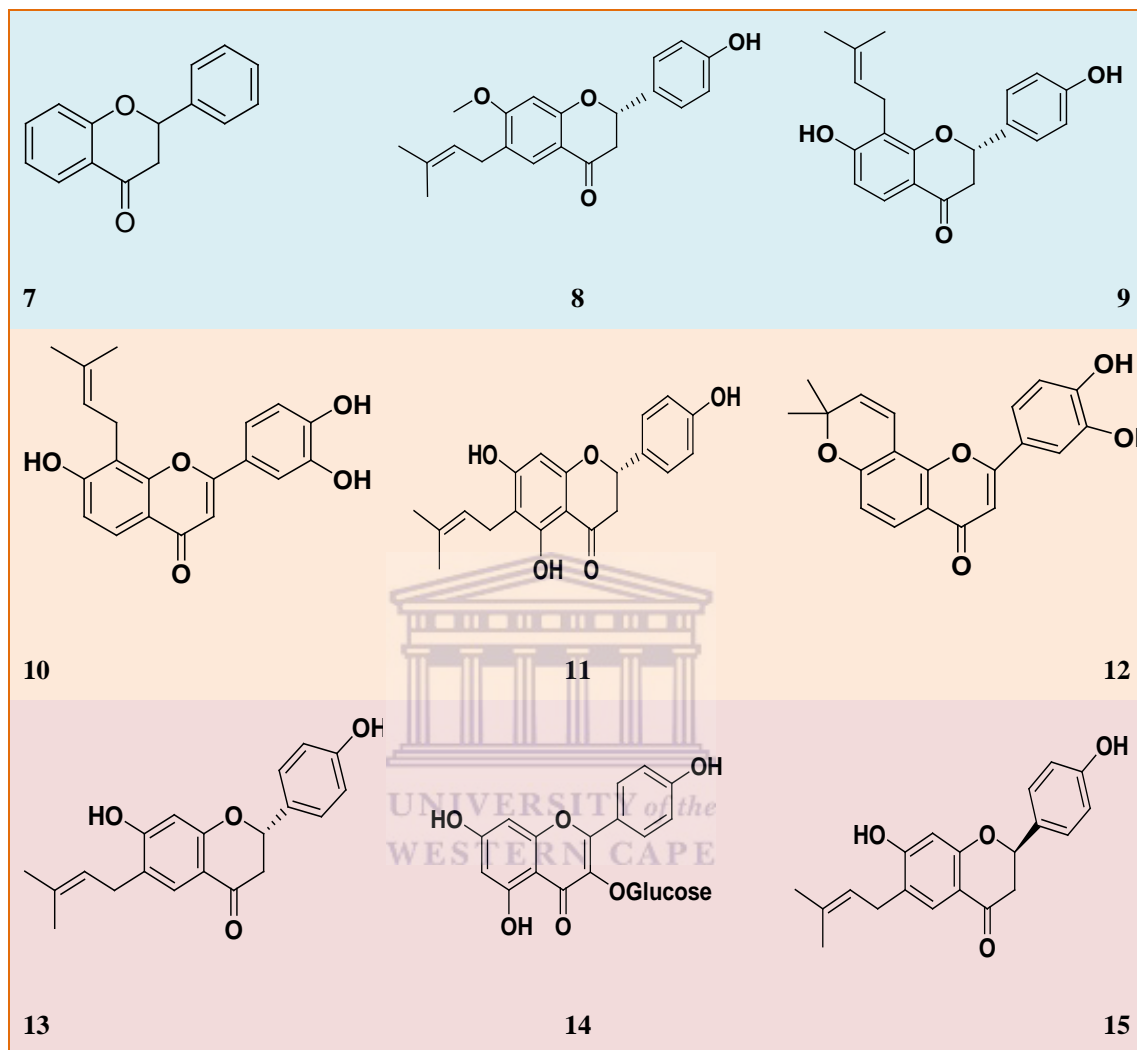


Figure 3.3: Flavonoid constituents isolated from *Psoralea* species

3.2.2.3 Isoflavonoids

Neobavaisoflavone (16), isobavachalcone,⁸⁷ corylinin (17),^{75,88} daidzein (18),⁸³ 8-prenyldaidzen (19), corylin (20), erythrinin A (21), isoneobavaisoflavone (22),⁸⁹ genstein (23),⁷⁶ bavadin (24),⁹⁰ bavarigenin (25)⁸⁵ and daidzin (26)⁹⁰ have been isolated from the seed of *Psoralea corylifolia* (Figure 3.4).

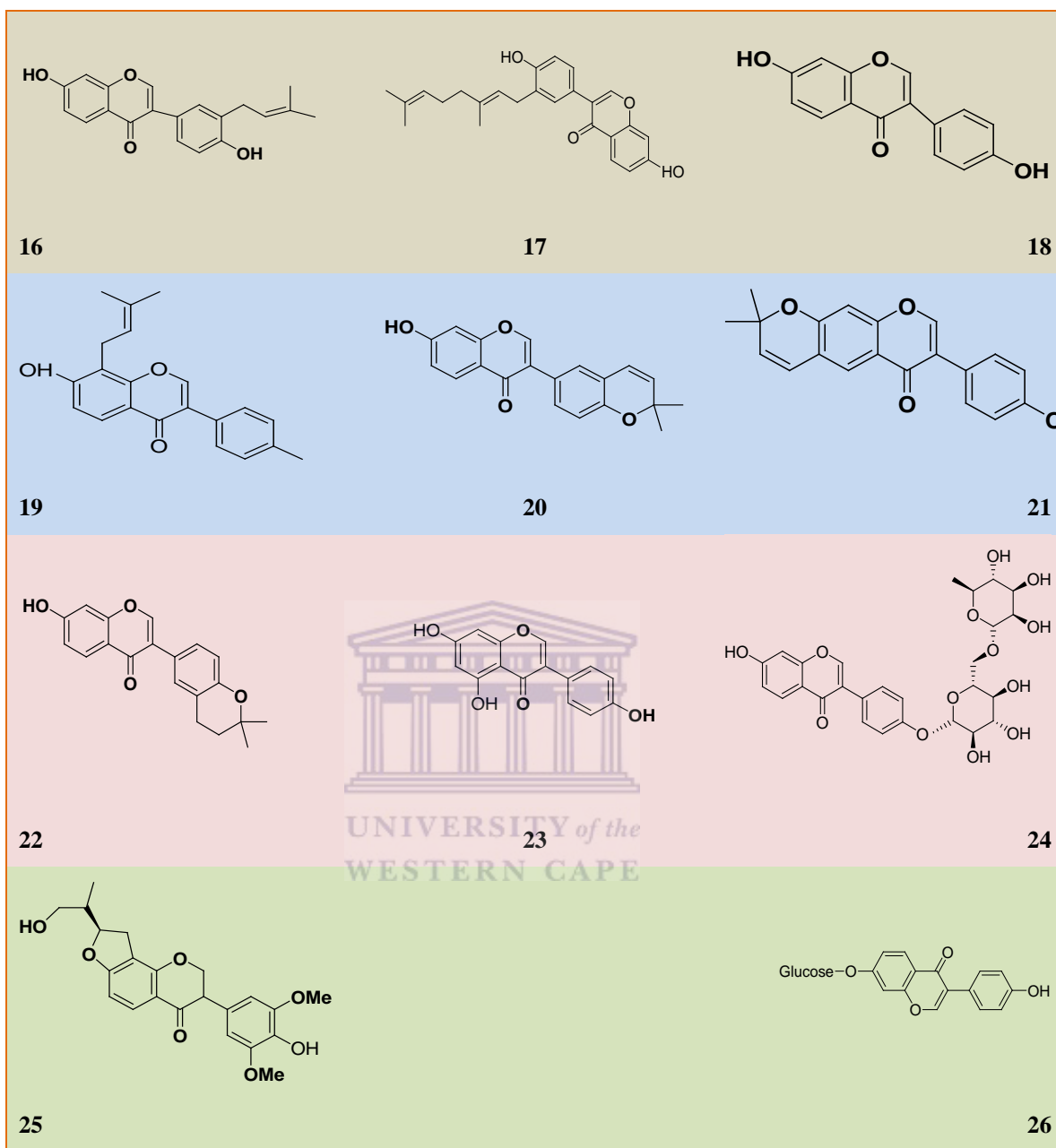


Figure 3.4: Isoflavonoid constituents isolated from *Psoralea* species

3.2.2.4 Comestance

The compounds psoralidin (27),⁸⁸ a new compound called psoraleflavanone (28),⁹¹ 4",5"-dehydroisopsoralidin (29)⁹² and sophoracoumestans-A (30) were isolated from the *Psoralea corylifolia*⁸⁸ as illustrated in Figure 3.5.

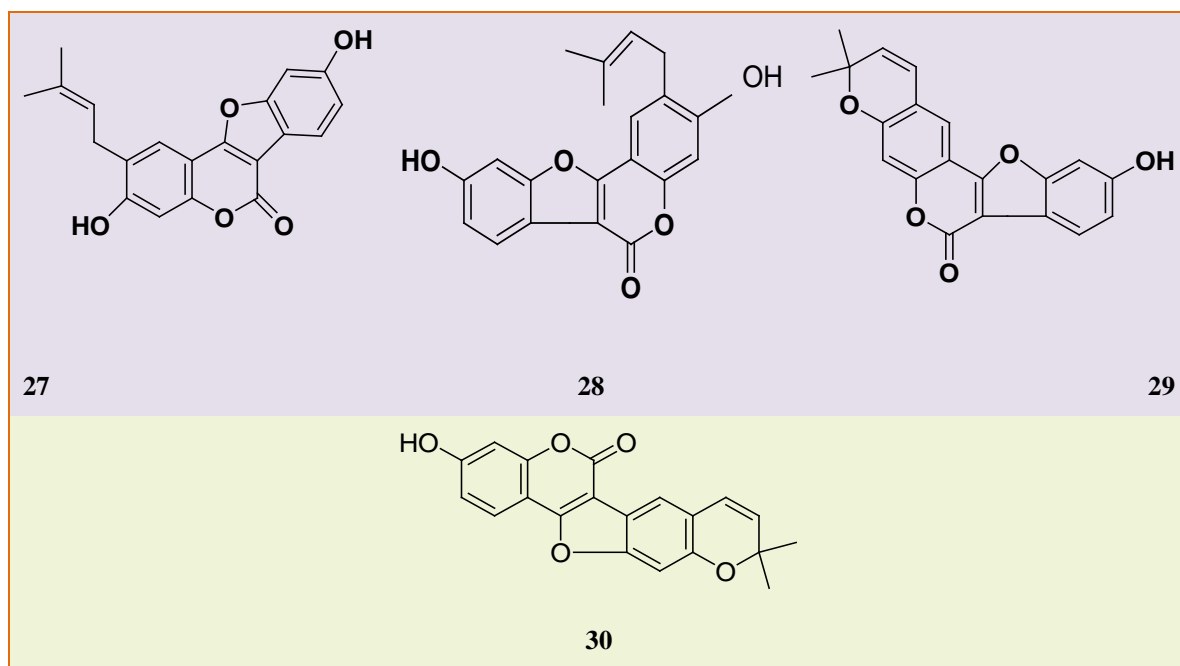


Figure 3.5: Comestance constituents isolated from *Psoralea* species

3.2.2.5 Chalcones

The compounds brosimacutin G (31), bakuchalcone (32),⁸³ a new compound, namely, 4,2'-dihydroxy-2''-(1'''-methylethyl)-2''-3''-dihydro-(4'',5'',3',4')-furano chalcone (33),⁹³ corylifol B (34),⁸³ obavachromene (35), 4-O-methylbavachalcone (36)⁸⁶ and corylifolinin (37) have been isolated from the seeds of *Psoralea corylifolia*⁸² as illustrated in Figure 3.6.

3.2.2.6 Meroterpenoids

The compound, bakuchiol (38), has been isolated from the seeds of the *Psoralea corylifolia* Linn. and from *Psoralea glandulosa*.⁹⁴⁻⁹⁶ Bisbakuchiols A (39), B (40),⁹⁷ five novel compounds—psoracorylifols A-E (41-45)—have been isolated from the seeds of *Psoralea corylifolia*.⁹⁸ The compound Δ 1,3-Hydroxybakuchiol (46) was isolated from the methanolic extract of *Psoralea corylifolia*.⁸⁴ The compounds cyclobakuchiol A (47), cyclobakuchiol B (48), 3-hydroxybakuchiol (49), 12-hydroxisobakuchiol (50) and the compound kuchiol (51) were

isolated from *Psoralea glandulosum*⁹⁶ as shown in Figure 3.7.

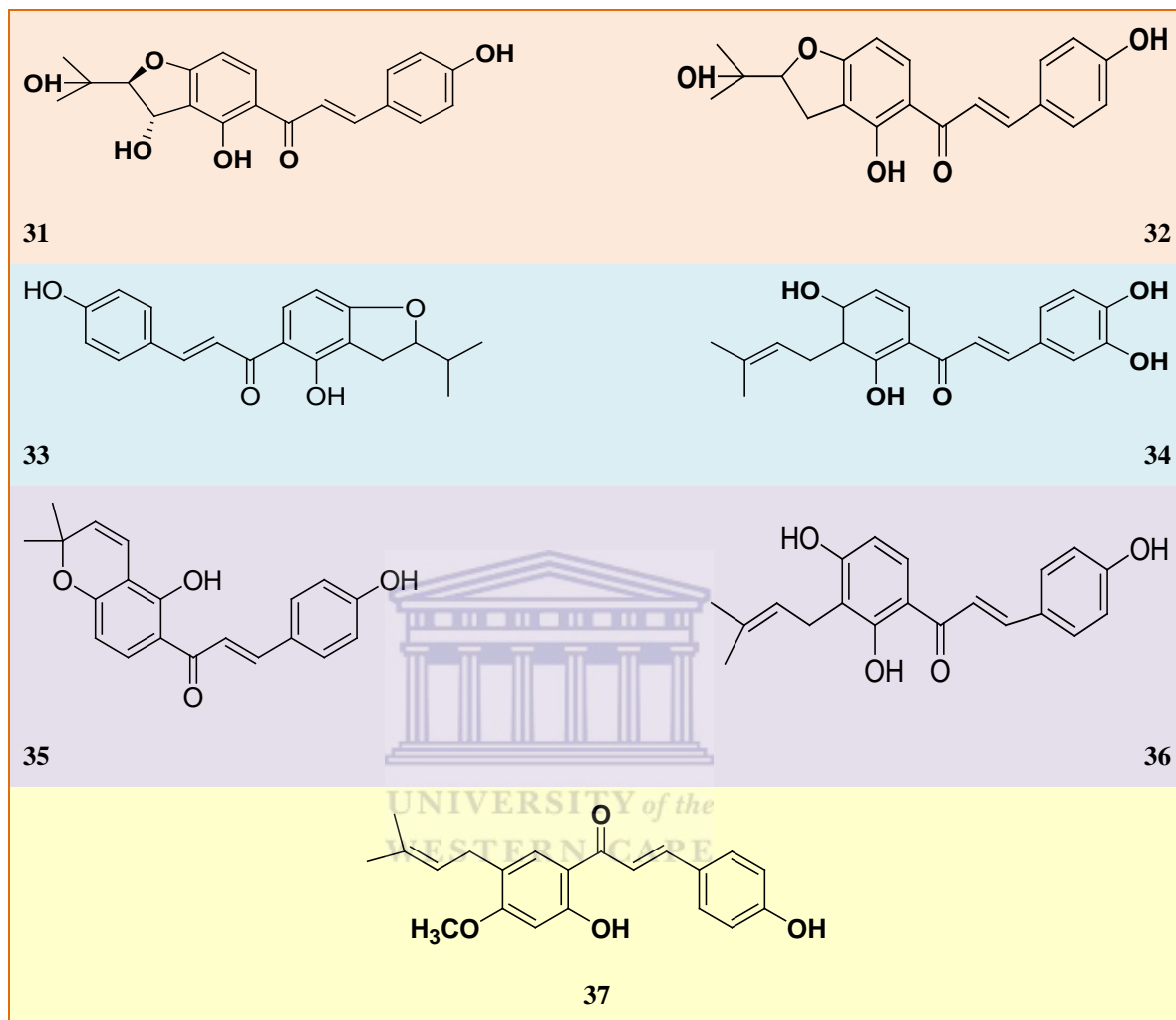


Figure 3.6: Chalcone constituents isolated from *Psoralea* species

3.2.2.7 Miscellaneous Compounds

The compounds uracil (52),⁸⁸ p-hydroxybenzaldehyde (53), 6-prenylnarigenin⁸⁴ and a new compound, namely methyl-4-hydroxybenzoat (54)⁹² and caryophyllene oxide (55) have been isolated from the dried fruits of *Psoralea fruticans*. The chemical compounds of the essential oils obtained by hydrodistillation from the aerial parts of *Psoralea mutisii* (Miq.), germacrene D (6.8%) (56) and β -caryophyllene (5.4%) (57). The major volatiles released by β -glucosidase

treatment of the aqueous plant residue were α -pinene (17.8%) (58) and tricyclene (5.6%) (59).⁹⁹ The same compounds were isolated independently with a new essential oil compound called y-cadinene (60) the first to be reported from this plant.¹⁰⁰ The compound anthraquinone (61) was representative of 3 species of *Psoralea* (*P. acaulis*, *P. bitumiosal* and *P. jaubertina fenzl*)⁷⁹ as depicted in Figure 3.8.

3.2.3 Pharmacology of *Psoralea* genus

Psoralea corylifolia Linn. is a plant of the family *Fabaceae* and distributed widely in China, India and Southeast Asia. Its fruit, known as fructose *Psoraleae* (Buguzhi in Chinese), is used traditionally to treat spermatorrhoea, pollakiuria, asthma, and nephritis.¹⁰¹ The seeds of *P. corylifolia* have been used as a tonic, to treat uterine bleeding, and as a coronary vasodilatory agent in traditional Chinese medicine. Seeds are effective against leucoderma, leprosy, psoriasis, asthma, as a stomachic, anthelmintic and diaphoretic.^{74,84}

3.2.3.1 Anticancer Activity

Psoralea corylifolia L., a traditional Chinese medicinal plant used to treat several diseases. The plant extracts have potent antibacterial, antitumour, antioxidant, anti-inflammatory, antifungal and immunomodulatory activity. A plethora of chemical substances such as psoralen, isopsoralen, bakuchiol, psoralidin, bakuchalcone, bavachinin, flavones, volatile oils and lipids are present in different parts of the plant. Extracts of *P. corylifolia* also show remarkable antitumour potential in preclinical studies.^{102,103}

3.2.3.2 Antibacterial Activity

The alcoholic extract produced death of microfilariae and showed antimycobacterial activity.³⁸ Bakuchiol, one of the major constituent of *P. corylifolia*, has shown activity against numerous Gram-positive and Gram-negative oral pathogens (MIC=1-4 μ g/ml).

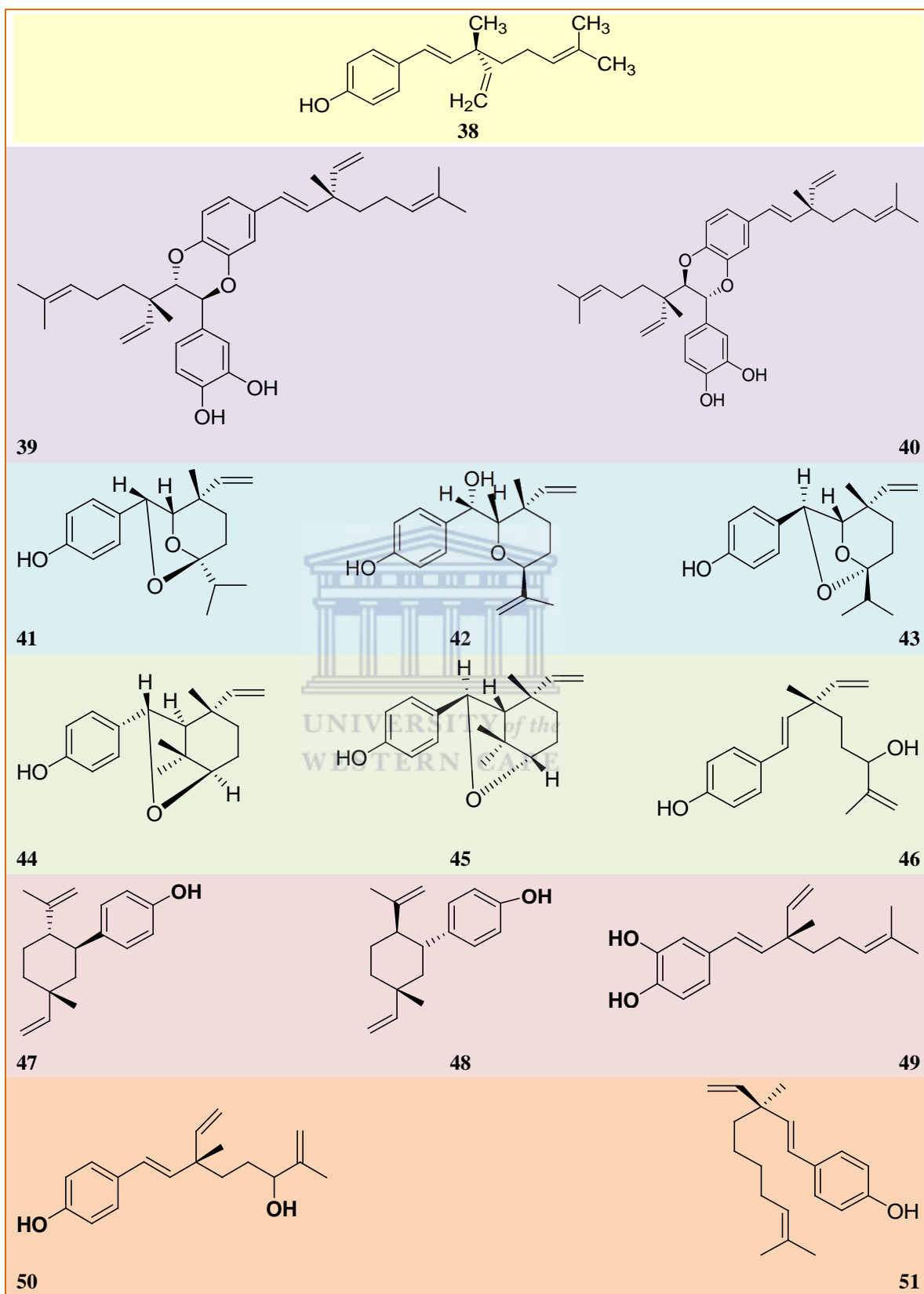


Figure 3.7: Meroterpenoid constituents isolated from *Psoralea* species

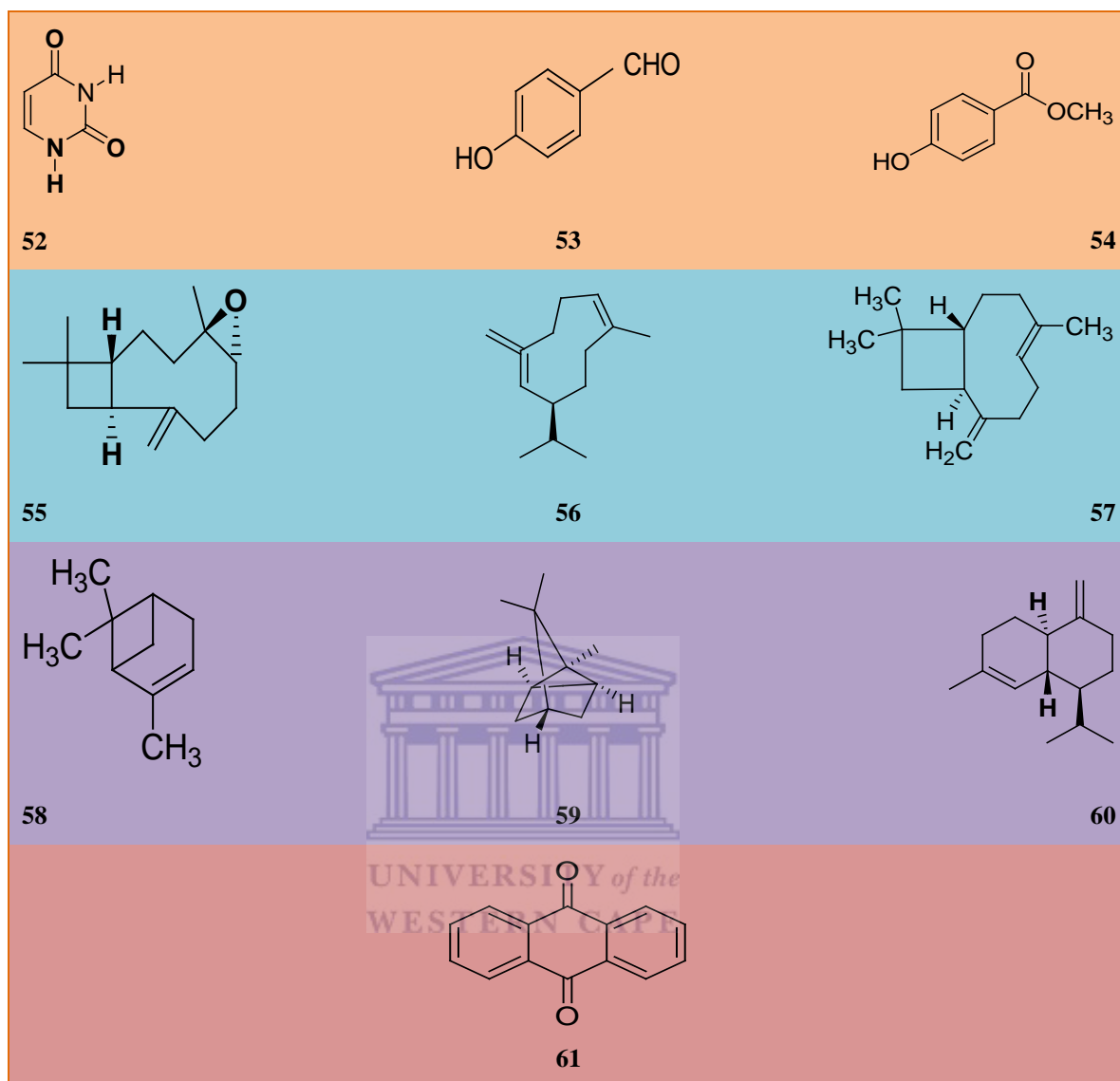


Figure 3.8: Miscellaneous constituents isolated from *Psoralea* species

It was able to inhibit the growth of *S. mutans* under a range of concentrations, pH values and in the presence of organic acids in a temperature-dependent manner also inhibited the growth of cells adhered to glass surface.^{37,74} The compounds corylifol A, corylifol B, corylidol C, neobavaisoflavone, isobavachalcone, isoneobavaisoflavone, bavachalcone, bavachin, bavachinin, corylin, 8-prenyldaidzein, bakuchalcone, brosimacutin G and erythrinin A were assayed for the antibacterial activities against two hospital pathogenic Gram-positive bacteria—*S. aureus* and *S.*

epidermidis *in vitro*. The compounds corylifol B, neobavaisoflavone, bavachinin and erythrinin A showed significant antimicrobial activity against *S. aureus* and *S. epidermidis* at the level of MICs 0.009-0.073 mM.

The compounds corylifol B, neobavaisoflavone, bavachalcone and bavachin showed comparable antibacterial activities as the positive controls (bakuchiol and magnolol). The compounds isobavachalcone, bavachinin and erythrinin A were even stronger than that two well-known natural antimicrobial agents, bakuchiol and magnolol.⁸³

The compounds, psoralidin, angelicin and psoralen that isolated from the seeds of *P. corylifolia* showed significant antibacterial activity against Gram-negative and Gram-positive bacteria. Psoralidin and angelicin showed efficacy against Gram-positive and gram-negative organisms, including *Bacillus subtilis*, *B. cereus*, *B. megatrium*, *Staphylococcus aureus*, *Sarcinalutea*, *Streptococcus*, *B. haemolyticus*, *Escherichiacoli*, *Shigelladysenteriae*, *S. shiga*, *S. boydii*, *S. flexneri*, *S. sonnei*, *S. sarcinaceae*, *Pseudomonasaeruginosa*. Psoralidin showed stronger activity against Gram-negative *Shigella sonnei* and *S. flexneri*. The compounds psoralen and angelicin exhibited the highest activity against Gram-positive *S. aureus*.⁷⁴

Coryifolinin isolated from the benzene extract of *P. corylifolia* produced coronary vasodilation and inhibitory action on Hela cells and an estrogenic effect.³⁸ The essential oil in a dilution of 1 in 50,000 and 1 in 10,000 has been found to kill *Paramecia* and *Streptococci* within 15 and 10 min, respectively.¹⁰⁴ The fruit extract inhibits the growth of *Staphylococcus citrates*, *Staphylococcus aureus*, *Staphylococcus albus*, including strains resistant to penicillin and other antibiotics. The compound psoralen shows strong inhibition of bacteria, including *Microsporium canis*, *Microsporium gypseum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *S. aureus*, *Candida albicans*, *Escherichia coli* and *P. aeruginosa*.³⁸

3.2.3.3 Anti-Inflammatory Activity

The *Psoralea fruticans* plant has been included to the formulations of traditional medicines in Southeast Asia as a prophylactic against osteoporosis caused by senescence, and for the treatment of impotence, cold, painful lower back, enuresis, alopecia, psoriasis, and vitiligo.¹⁰⁵

The methanolic extracts from the seeds of *P. corylifolia* was found to inhibit production of nitric oxide (NO) in lipopolyaccharide-activated mouse peritoneal macrophages. The isolated compounds, bavachinin (IC₅₀=26 μM), isobavachalcone (17 μM), neobavaisoflavone (ca. 29 μM), corylifol A (ca. 21 μM) and psoralidin (ca. 23 μM) significantly inhibited the accumulation of nitrite (NO₂) as a marker of production of NO.⁸⁴ However, the flavonone (bavachin and isobavachalcone) and isoflavones (neobavaisoflavone, corylifol A) and a coumarins (psoralidin) showed a moderate activity (IC₅₀=17- ca. 29 μM), but the active compounds (isobavachalcone and psoralidin) and other prenylated flavanones (bavachin and 6-prenylnaringein) showed cytotoxic effects at a high concentrations (100 μM).⁸⁴

Bavachinin A isolated from fruits revealed marked anti-inflammatory, antipyretic, and mild analgesic properties at a dose of 25-100 mg/kg. It has demonstrated better antipyretic activity than paracetamol and it showed no effect on the central nervous system, and the maximum lethal dose was greater than 1000 mg/kg in mice.¹⁰⁴ Bavachinin A also showed anti-inflammatory activity against carrageenan induced in rats.³⁸

3.2.3.4 Antioxidant Activity

The powder and extracts of *P. corylifolia* Linn. were investigated in lard at 100°C by using the oxidant stability instrum (OSI). It was found to have a strong antioxidant effect. The isolated compounds were investigated individually and compared with butylated hydroxytoluene (BHT) and α-tocopherol by the OSI at 100°C. The results showed that corylifolin, corylin, psoralidin and bakuchiol had strong antioxidant activity, especially psoralidin had stronger antioxidant

property than BHT, but isopsoralen and psoralen had no antioxidant activities at 0.02% and 0.04% levels.¹⁰⁶ Bakuchiol showed broad antioxidant activities in rat liver microsomes and mitochondria.¹⁰⁴ Bakuchiol showed weak inhibition at 10 μ M, and the cytotoxic effects were observed at 30 and 100 μ M using mouse peritoneal macrophages.⁸⁴

Bakuchiol oxidation was more potent in microsomes and inhibition of oxygen consumption induced lipid peroxidation dependent on time. Moreover, bakuchiol protects human red blood cells against oxidative haemolysis. These phenolic compounds in *P.corylifolia* are thus effective in protecting biological membranes against oxidative stress different.⁹⁵ The flavonoids isobavachin and isobavachalcone and bakuchiol were showed broad antioxidative activities in rat liver microsomes and mitochondria. They were found to inhibit NADPH-, ascorbate-, t-BuOOH- and CCl₄-induced lipid peroxidation in microsomes. They also prevented NADH-dependent and ascorbate-induced mitochondrial lipid peroxidation.⁹⁵

3.2.3.5 Hepatoprotective Activity

The water-soluble extracts containing bakuchiol has been found to possess hepatoprotective activity in tacrine-induced cytotoxicity in human liver-derived HepG2 cells. The IC₅₀ value of bakuchiol was 1 μ g/ml and of silymarin was 5 μ g/ml.¹⁰⁴

3.2.3.6 Cytotoxicity

Bakuchiol has been shown to possess a prominent cytotoxic effect on L929 cells in culture. It also showed cytotoxicity against cultured human cell lines, namely, A549, SKOV-3, SK-MEL-2, XF-498, and HCT-15.³⁸ It was reported to inhibit the expression of INOS(Inducible nitric oxide synthase) gene induced by interferon- γ and lipopolysaccharide in RAW macrophage-like cells at 1-40 μ M.¹⁰⁷ The coumestan derivative, psoralidin, isolated from the seeds of *P. corlifolia*, exhibited potent cytotoxic activity against HT-29 (colon) and MCF-7 (breast) cancer cell lines

with the IC_{50} values of 0.3 and 0.4 $\mu\text{g/ml}$, respectively. The coumarin, angelicin, showed a weak cytotoxic against the HT-29 (colon) and MCF-7 (breast) cancer cell lines with the IC_{50} values 17.7 and 11.9 $\mu\text{g/ml}$, respectively. Psoralen was considered to be inactive in the cytotoxic assay in this study.¹⁰⁵ It also showed a cytotoxic effect on stomach cancer cell lines with IC_{50} value of 53 $\mu\text{g/ml}$ in SNU-1 and 203 $\mu\text{g/ml}$ in SNU-16.¹⁰⁴

3.2.3.7 α -Glucosidase and α -Mannosidase Activity

The seeds of *P. corylifolia* were extracted using five different polar solvents: chloroform, methanol, 50% ethanol in water, water and ethanol. The chloroform extract showed the lowest IC_{50} value against α -glucosidase (82.9 $\mu\text{g/ml}$) and α -mannosidase (132 $\mu\text{g/ml}$). The isolated compounds possessed α -glucosidase inhibitory activities. Also, the compounds posralidin and corylifol A which exhibited potent inhibition with IC_{50} s of 13.7, 27.7 and 11.3 μM , respectively. Furthermore, compounds 4'-O-methylbavachalcone and posralidin showed α -mannosidase activity. Compounds psoralidin and neobavaisoflavone in the mechanistic analysis of inhibition mode against α -glucosidase showed noncompetitive inhibition, while that of corylifol was mixed. Interestingly, the most active glycosidase inhibitors were 4'-O-methylbavachalcone, posralidin and corylifol A.⁸⁶

3.2.3.8 Antidepressant Activity

A study of the antidepressant activity of total furanocoumarins present in *P. corylifolia* (TFPC) in the chronic mild stress model of depressed in mice revealed that TFPC possess potent and rapid antidepressant properties that are mediated via monoamine oxidase in the hypothalamic-pituitary-adrenal axis oxidative system. Thus, it makes *P. corylifolia* a potentially valuable drug to treat depression in the elderly.¹⁰⁸ Other researchers also proved psoralen's antidepressant effects, using forced swimming test model of depressed on male mice.¹⁰⁹

3.3 Materials and Methods

3.3.1 Materials

The specific reagents and equipment used in this study are outlined in Table 3.2.

3.3.2 Isolation and Characterization of Chemical Structures

3.3.2.1 Column Chromatography

Glass columns (different diameters) packed with silica gel 60 (0.063-0.0200 mm, Merck), were used for column chromatography.

3.3.2.2 High Performance Liquid Chromatography (HPLC)

Sample purification was carried out using Agilent Technologies 1200 series, equipped with UV detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), column compartment (G1316A) and reversed phase C18 column SUPELCO (25 X 1.0 cm). The flow rate was set at 1.5 ml/min and detection wavelength at λ_{254} nm.

3.3.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra were recorded at 25°C, using CDCl₃ as solvent, on a Bruker Avance 400 NMR spectrometer (1H 400 MHz, 13C 100 MHz). Chemical shifts of ¹H (δ H) and ¹³C (δ C) in ppm were determined relative to solvent signal.

3.3.2.4 Mass Spectroscopy (MS)

High resolution mass spectroscopy (HRMS) analysis was conducted using a Waters Synapt G2 Accurate Mass spectrometer using electrospray ionization (ESI) interface working in positive mode.

Table 3.2: Specific reagents and equipment used in this study

Chemicals	Supplier
Dulbecco's modified Eagle's medium (DMEM)	
Phosphate buffered saline (PBS)	Lonza
Penicillin-streptomycin	
Foetal bovine serum (FBS)	Thermo Scientific
Trypan blue stain (0.4%)	Invitrogen
Trypsin	Gibco
Dimethyl sulfoxide (DMSO), dichloromethane (DCM)	
Acetic acid, acetonitrile (ACN, HPLC grade), vanillin	
Methanol, ethyl acetate (EtOAc), Hexane (Hex)	Sigma-Aldrich or Merck
Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI)	
Bisphenol-S (BPS), sulphuric acid	
Commercial kits	
Cell proliferation reagent (WST-1)	Roche
Instruments	
Cell culture flasks (25 cm ²)	SPL Life Sciences
96-Multiwell culture plates	Sigma-Aldrich
Centrifuge 5417R	Eppendorf AG
CO ₂ incubator	Shellab
Countess II FI Automated Cell Counter	
Countess™ cell counting chamber slides	Invitrogen/Thermo Scientific
Tali® Image-Based Cytometer	
Tali® cellular analysis slides	
POLARstar Omega Microplate Reader	BMG Labtech
Sorvall TC-6 Centrifuge	Sorvall
Cover slides	Zeiss
Florescence microscope	

3.3.2.5 Ultra Violet (UV) Spectroscopy

A UV Nicolet Evolution (EV-100, Version 4.60) Spectrophotometer (Therma Electron Corporation, Madison, USA) was used for measurement of absorbance maxima between the wavelengths 200-450 nm.

3.3.2.6 Infrared (IR) Spectroscopy

Attenuated total internal reflectance Fourier transform infrared spectroscopy (FTIR) measurements were carried out using Spectrum 100 (PerkinElmer). Spectra recordings were accomplished using the Interface-Spectrum. Methanol was used to dissolve the samples.

3.3.3 Sample Collection and Identification

Psoralea fruticans was collected in August 2011 from the Cape Flats Nature Reserve, University of the Western Cape (UWC). The samples were collected by Dr Ahmed Mohammed (Chemistry Department, UWC) and identified by Mr Frans Weitz (Biodiversity & Conservation Department, UWC).

3.3.3.1 Preparation of Total Extract

The sample was collected and left to dry at room temperature (~25°C). Approximately 90.6 g of the dried plant was blended in MeOH. The extract was then warmed at the 60°C (in a water bath) for one hour and left overnight. The extract was filtered using Whatman filter paper and the plant residue was washed twice with fresh solvent. The combined total extracts were evaporated and the residue (19.03 g, 21.0 %) was kept at ~ 0°C, until used.

3.3.3.2 Fractionation of Total Extract

The extract (19.03 g) was applied to a silica gel column (7 X 27 cm, 500 g silica gel) (Figure 3.9)

and eluted using a mixture of Hex:EtOAc of increasing polarity, according to Table 3.3.

Table 3.3: Solvent system used for fractionation of the total extract of *Psoralea fruticans*

Solvent (%v/v)	Volume (Litre)	Fraction	Combined	Notes
Hex	1	1	I	
		2		
Hex:EtOAc 90:10	2	3	II	Purified
		4		
		5	III	
		6		
Hex: EtOAc 80:20	2	7	IV	Purified
		8		
		9		
		10		
Hex: EtOAc 70:30	2	11	V	Purified
		12		
		13		
		14		
Hex: EtOAc 60:40	2	15	VI	
		16		
		17		
		18		
Hex: EtOAc 50:50	1	19	VII	
		20		
Hex: EtOAc 40:60	1	21	VIII	
		22		
Hex: EtOAc 30:70	1	23	IX	
		24		
Hex: EtOAc 20:80	1	25	X	
EtOAc 100%	1	26	XI	
		27		
EtOAc: MeOH 98:2	1	28	XII	
		29		
EtOAc: MeOH 95:5	2	30	XIII	
		31		
		32		
		33		



Figure 3.9: Column chromatography setup

46 different fractions (volume=500 ml) were collected. The fractions were concentrated and chromatographed on silica gel TLC plates (Figure 3.10) using different solvent systems [Hex:EtOAc 4:6, DCM:MeOH (90:10; 95:5) and EtOAc:acetic acid:formic acid:H₂O (50:2:2:4)]. The fractions were pooled together according to their TLC profiles and were designated roman numerals (Table 3.3).

3.3.4 Purification of Chemical Constituents

3.3.4.1 Purification of Compounds 1-3

Fraction III (3.5 g) was subjected to column chromatography (3 x 33cm) on silica gel and eluted

with gradient mixture of increasing polarity from Hex and EtOAc 99:1 to 93:7 to yield 65 sub-fractions which were pooled together according to the TLC profile to six main fractions.

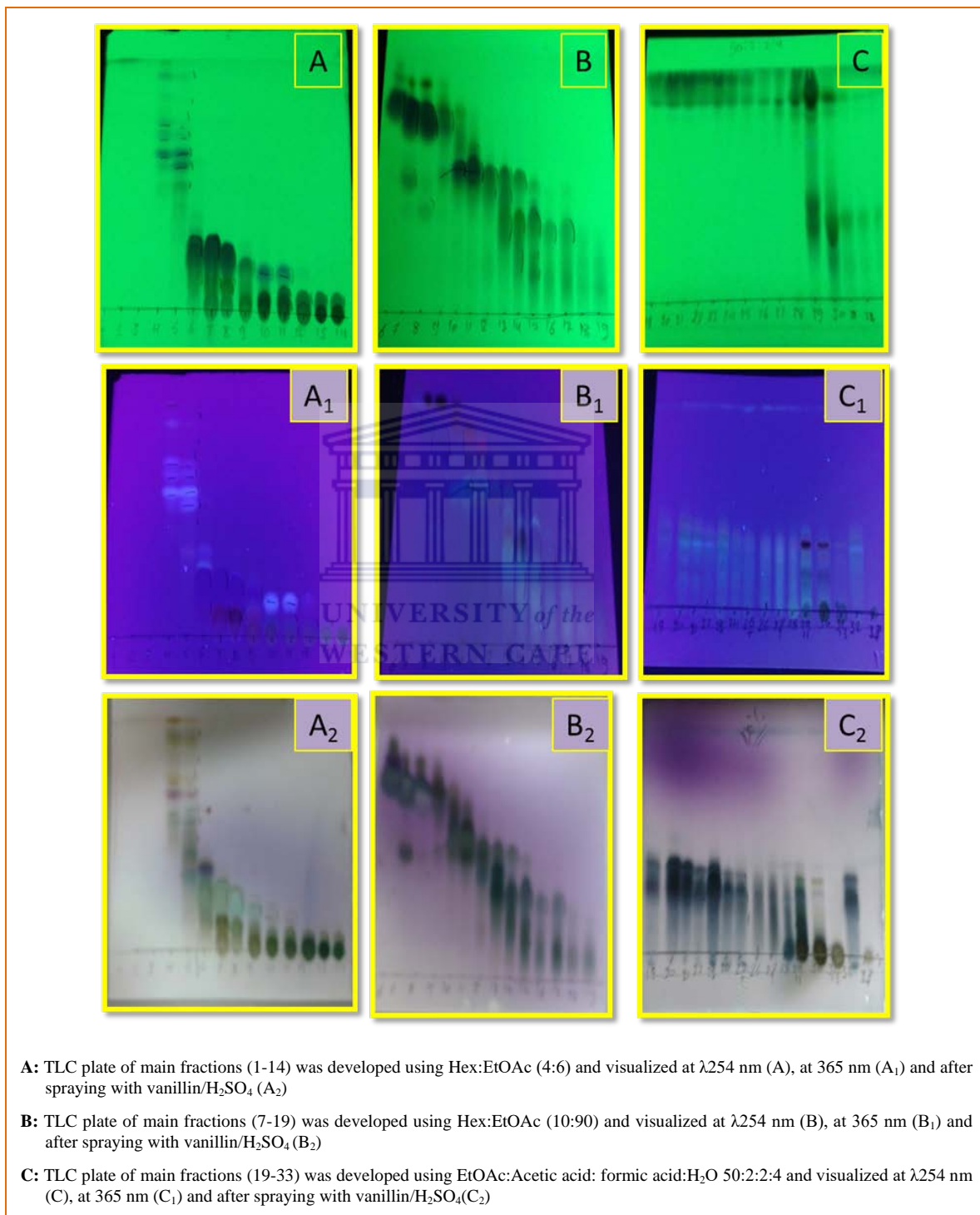


Figure 3.10: TLC chromatograms of fractions collected from the main column on silica gel 60 F254

The sub-fractions III_{2a} (120 mg) was divided into two equal parts; the first (60 mg) underwent HPLC fractionation. 30 µl of sub-fraction III₂ was injected into the HPLC column and eluted using a gradient system of MeOH and deionized water (DIW) (from 80:20 to 100 (MeOH) for 20 minutes, then continuing with 100% MeOH for 40 min to wash) the two peaks at 27.9 and 29.0 min were collected and examined by NMR. Peak 2 was pure compound (**1**) while peak 1 was a mixture of two compounds (**2** and **3**) (Figure 3.11). A trial to purify two compounds failed because of the small amount left after injection.

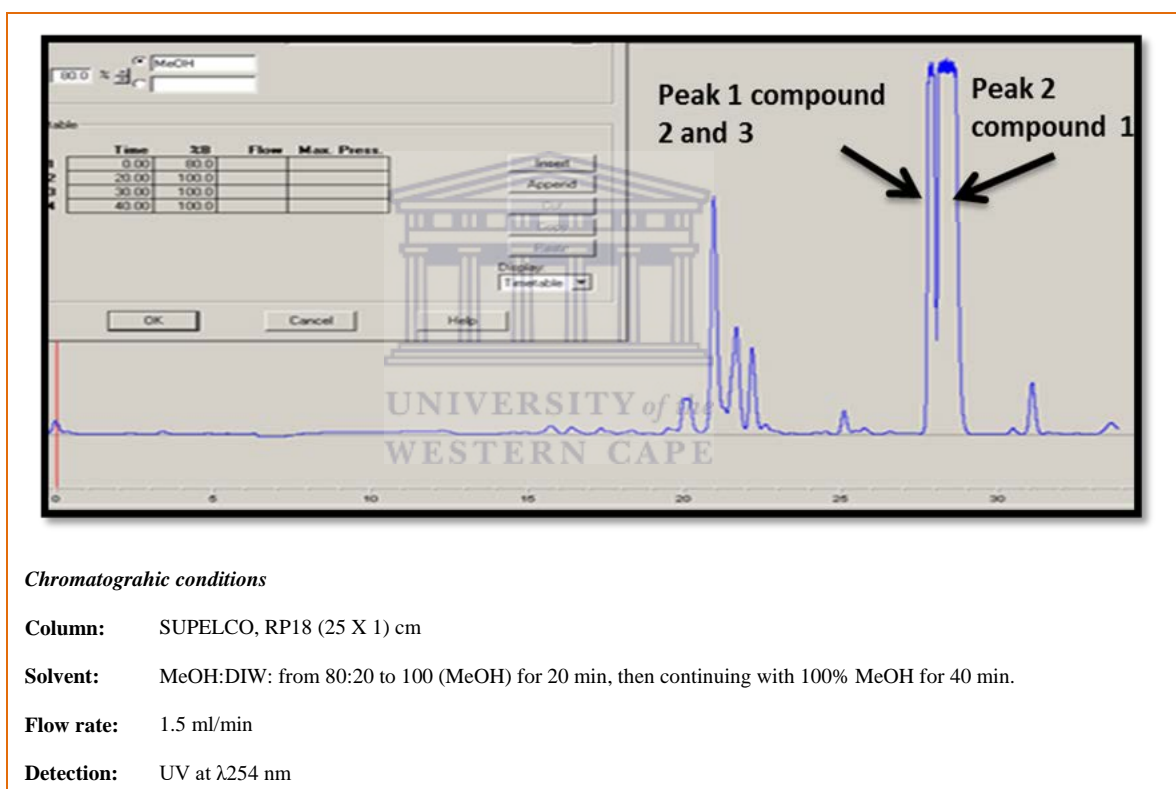


Figure 3.11: HPLC chromatogram of sub-fraction III₂

Fraction III_{2b} (60 mg) second part (60 mg) was applied to prep-TLC of silica gel (20 X 20 cm) and eluted with MeOH:DCM (99:1). Three bands were collected; the first one was identical with compound **1**, while bands 2 and 3 yielded pure compounds **2** and **3** (Figure 3.12).

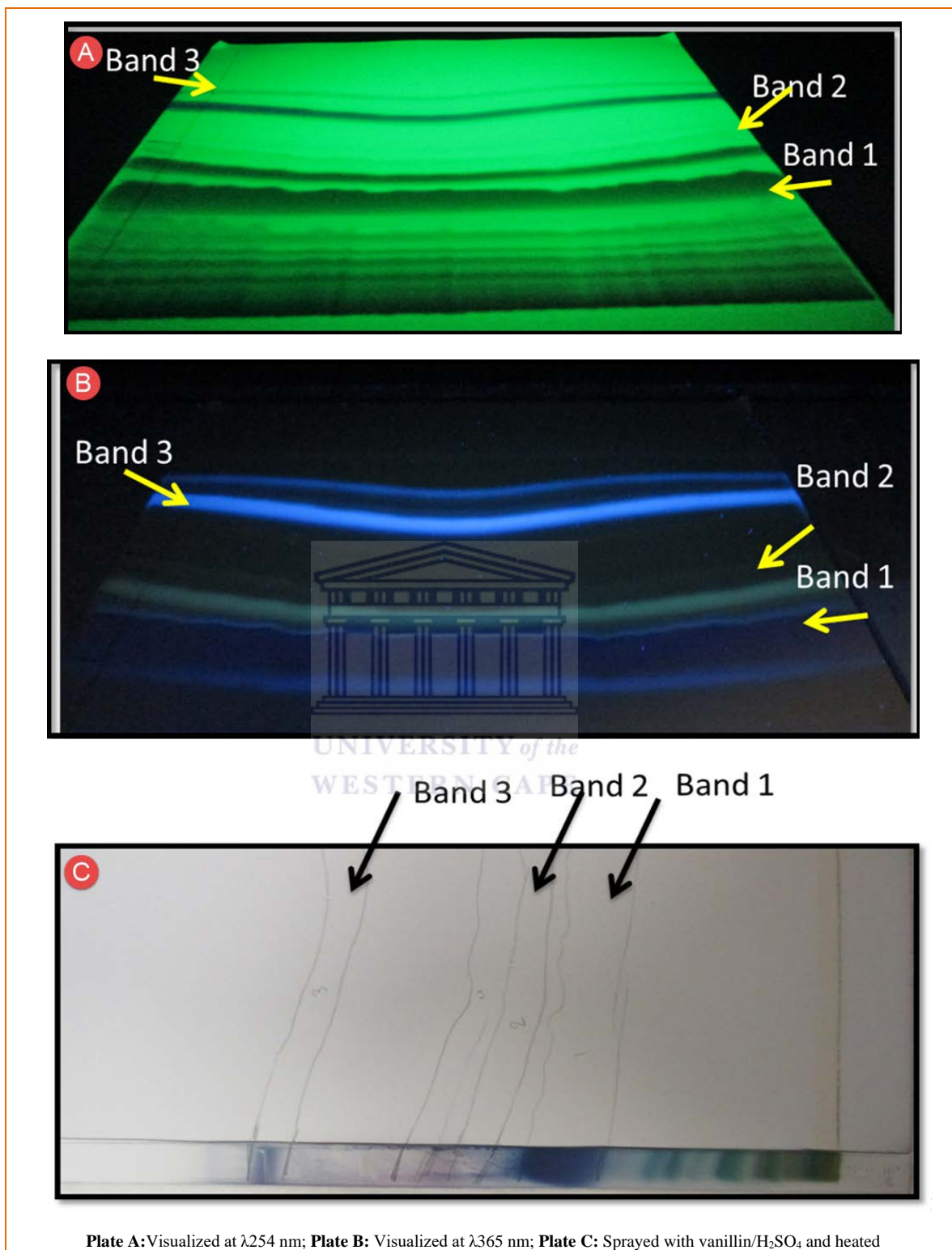


Figure 3.12: TLC plate for compounds 2 and 3 developed at 1% MeOH in DCM

3.3.4.2 Purification of Compounds 4 and 5

Fraction IV (1.1 g) was subjected to column chromatography (3 x 34 cm) on silica gel and eluted with mixture of increasing polarity from Hex and EtOAc 95:5 to 88:12 to yield 46 sub-fractions which were pooled together according to the TLC profile to eight main fractions. Fraction IV₂ upon standing at room temperature formed crystals, which were carefully washed with hexane to yield pure compound **4** (5 mg). The sub fraction IV₄ was injected into an HPLC chromatography column and eluted using MeOH:DIW (from 70:30, to 80:20 for 5 min, then continued to 100% for 35 min, washing through with 100% MeOH for 45 min); the peak at 29.0 min was collected to yield compound **5** (Figure 3.13).

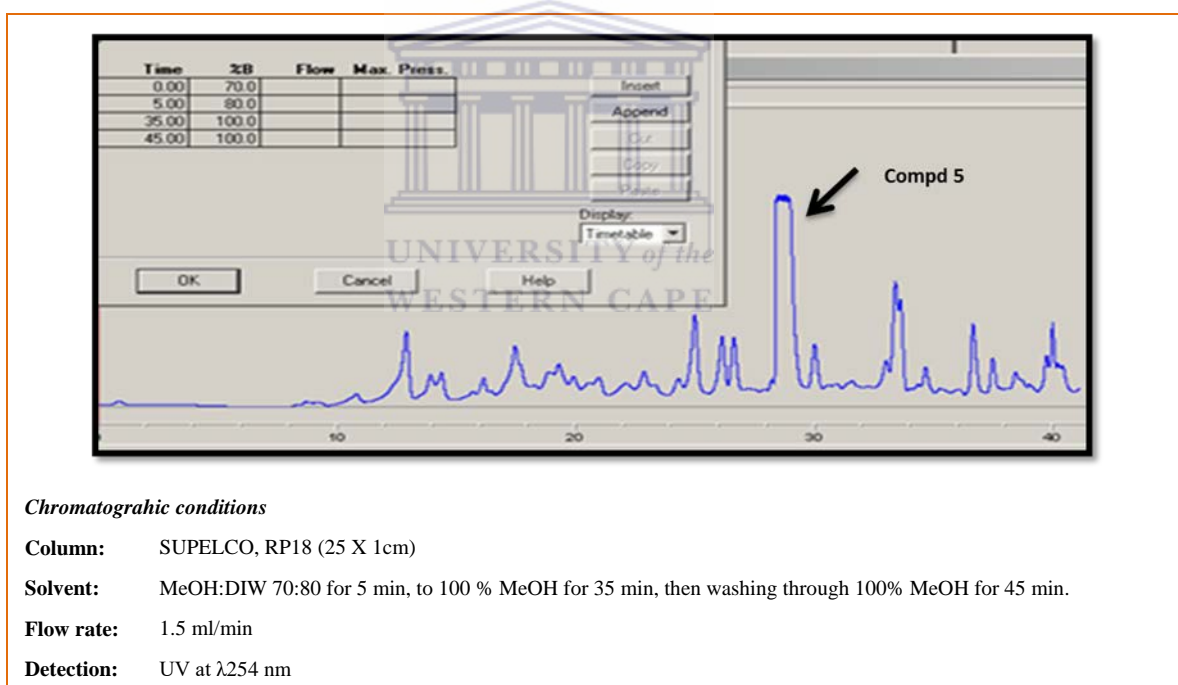


Figure 3.13: HPLC chromatogram of fraction IV that yielded compound 5

3.3.4.3 Purification of Compounds 6 and 7

Fraction V (0.96 g) was subjected to column chromatography (3 x 35 cm) on silica gel and eluted with mixture of increasing polarity from Hex and EtOAc 90:10 to 70:30 to yield 46 sub-fractions

which were pooled according to their TLC profile to 12 main fractions. Sub-fraction V₄ was injected into an HPLC chromatography column and eluted using MeOH:DIW (from 70% to 80% for 5 min, continued with 100% for 35 min, washing through 100% MeOH for 45 min). Two peaks at 13.5 and 33.0 min were collected to yield compounds **6** and **7** (Figure 3.14).

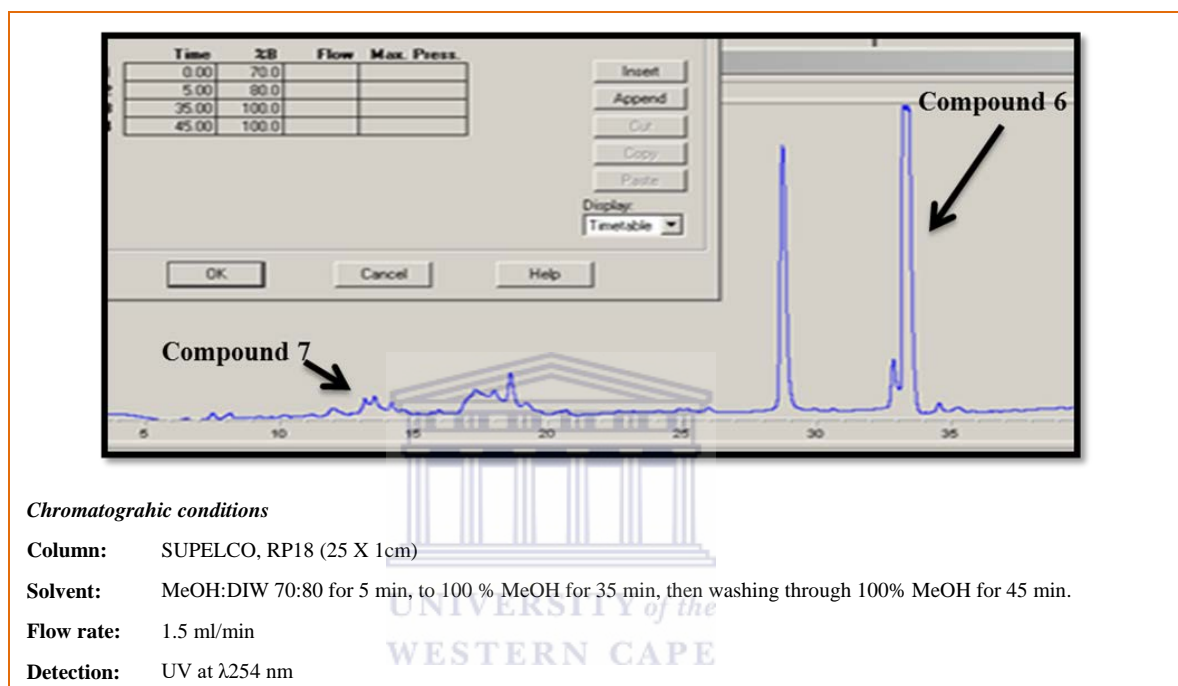


Figure 3.14: HPLC chromatogram of fraction V that yielded compounds **6** and **7**

3.3.5 Spectroscopic Details of Compounds

Spectroscopic data for the isolated compounds are summarized in Figures 3.15 to 3.19, and the isolation procedure followed is provided in the flow diagram in Figure 3.20.

3.3.5.1 Compound **1**

Yellowish oil; UV; λ_{max} 230, 314 nm. IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm⁻¹; ¹H and ¹³C NMR (CDCl₃): HRMS-ES negative mode m/z 313.1804[M-H]⁻ (calculated for C₂₀H₂₆O₃, 314.4186).

3.3.5.2 Compound 2

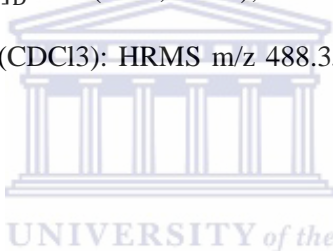
White, amorphous powder; UV: λ_{\max} 256, 298 nm. IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm^{-1} ; ^1H and ^{13}C NMR (CDCl_3): HRMS m/z 205.494 $[\text{M}-\text{H}]^+$ (calculated for $\text{C}_{11}\text{H}_{10}\text{O}_4$, 206.0579).

3.3.5.3 Compound 3

White, amorphous powder; $[\alpha]_{\text{D}}^{25}$ -1.5 (c 0.1, DCM); UV: λ_{\max} 260, 330 nm. IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm^{-1} ; ^1H and ^{13}C NMR (CDCl_3): HRMS m/z 311.1674 $[\text{M}-\text{H}]^+$ (calculated for $\text{C}_{20}\text{H}_{24}\text{O}_3$, 312.1725).

3.3.5.4 Compound 4

White, amorphous powder; $[\alpha]_{\text{D}}^{25}$ -1.5 (c 0.1, DCM); IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm^{-1} ; ^1H and ^{13}C NMR (CDCl_3): HRMS m/z 488.351 $[\text{M}]^+$ (calculated for $\text{C}_{30}\text{H}_{48}\text{O}_5$, 488.3502).



3.3.5.5 Compound 5

Yellowish crystals; Mp 103-106. UV: λ_{\max} 324 nm. IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm^{-1} ; ^1H and ^{13}C NMR (CDCl_3): HRMS m/z 313.1804 $[\text{M}-\text{H}]^+$ (calculated for $\text{C}_{20}\text{H}_{26}\text{O}_3$, 314.1882).

3.3.5.6 Compound 6

White crystals; Mp 90-92. UV: λ_{\max} 230, 300 nm. IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm^{-1} ; ^1H and ^{13}C NMR (CDCl_3): HRMS m/z 329.1748 $[\text{M}-\text{H}]^+$ (calculated for $\text{C}_{20}\text{H}_{26}\text{O}_4$, 330.21831).

3.3.5.7 Compound 7

White, amorphous powder; $[\alpha]_{\text{D}}^{25}$ -1.5 (c 0.1, DCM); IR: 3054, 2305, 1422, 1265, 1050, 895, 739, 705 cm^{-1} ; ^1H and ^{13}C NMR (CDCl_3): HRMS m/z 488.351 $[\text{M}]^+$ (calculated for $\text{C}_{30}\text{H}_{48}\text{O}_5$, 488.3502).

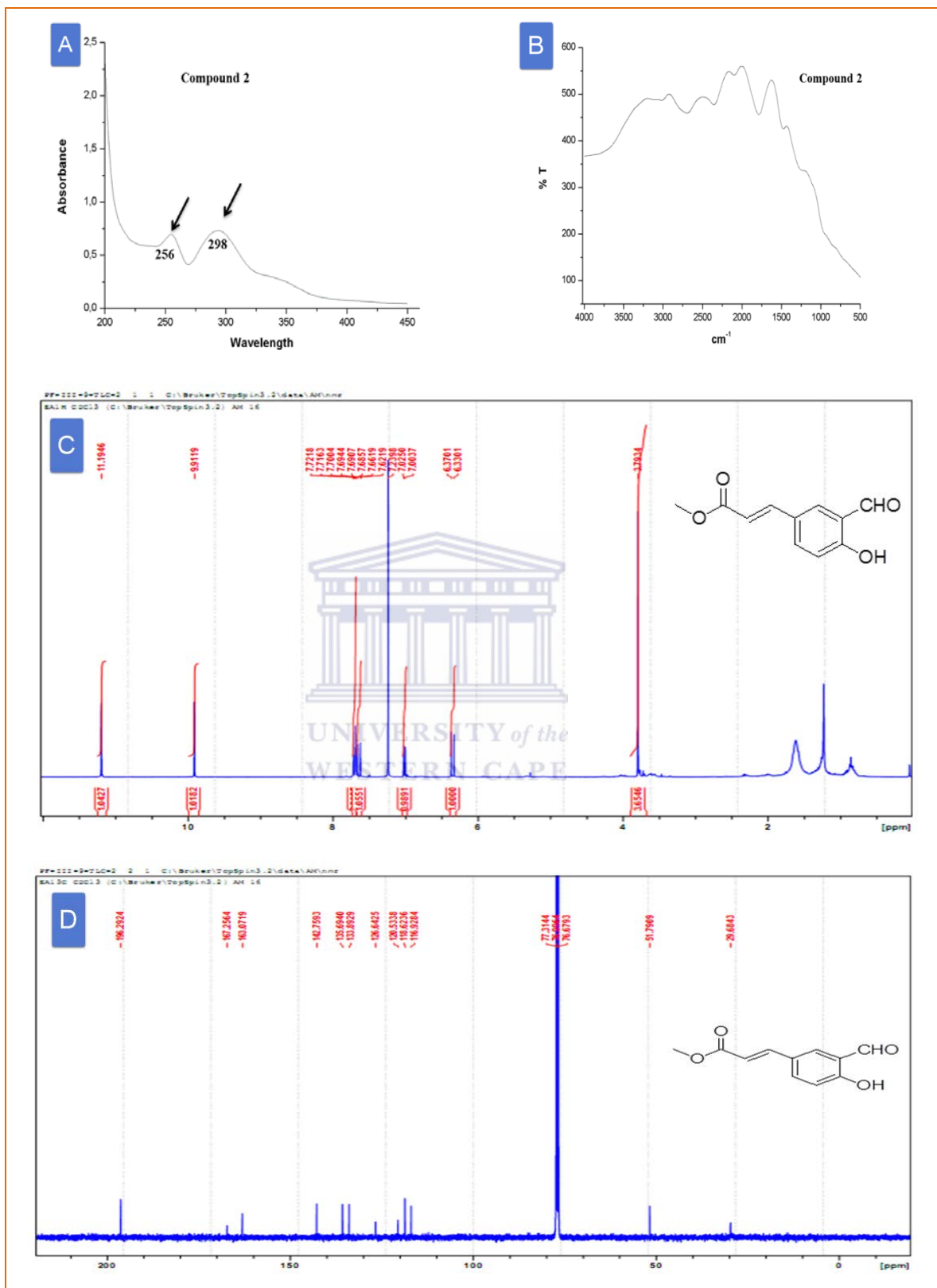


Figure 3.16: Spectroscopic data (UV (A), IR (B), ^1H (C) and ^{13}C (D) NMR) of compound 2

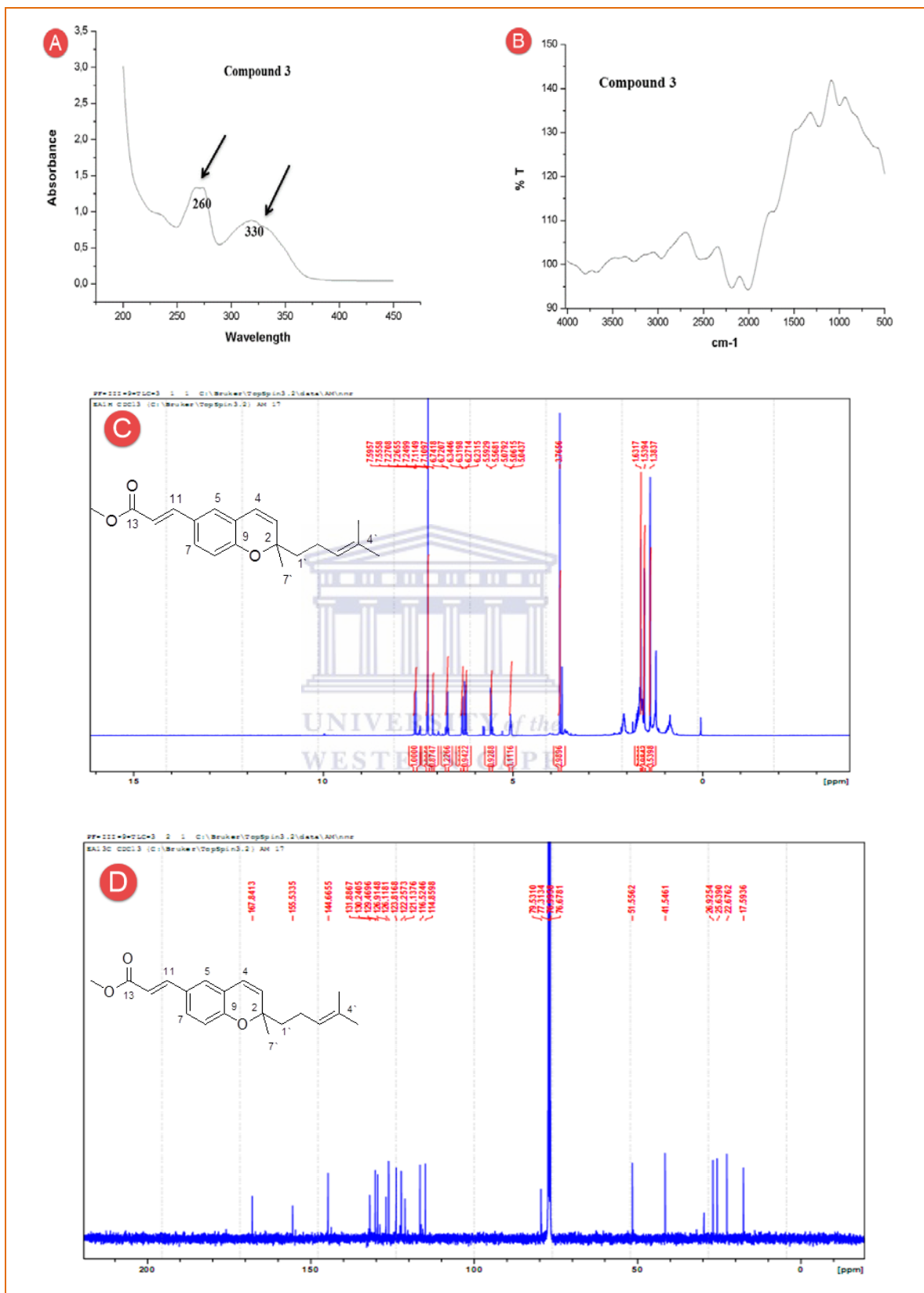


Figure 3.17: Spectroscopic data (UV (A), IR (B), ¹H (C) and ¹³C (D) NMR) of compound 3

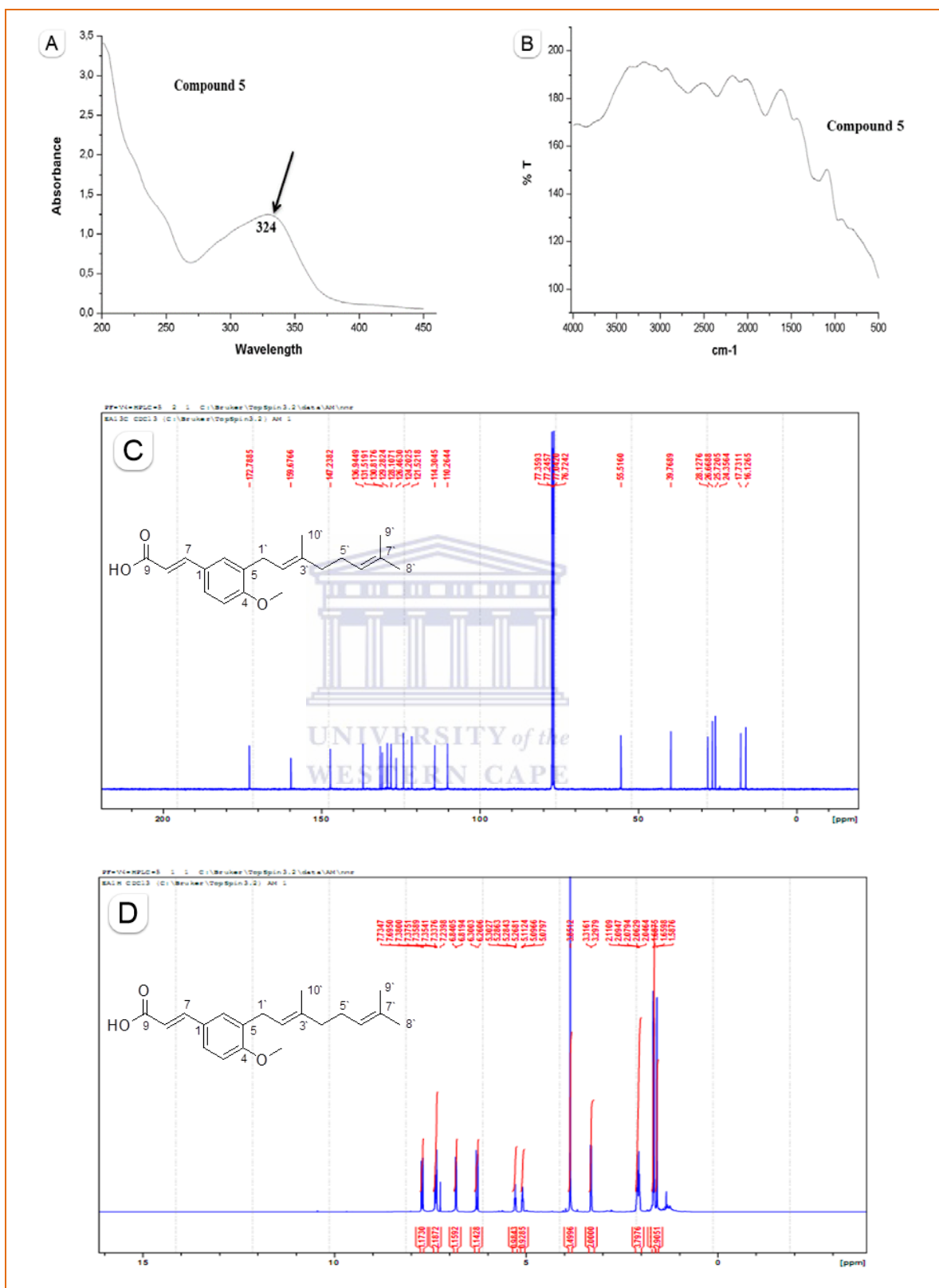


Figure 3.18: Spectroscopic data (UV (A), IR (B), ¹H (C) and ¹³C (D) NMR) of compound 5

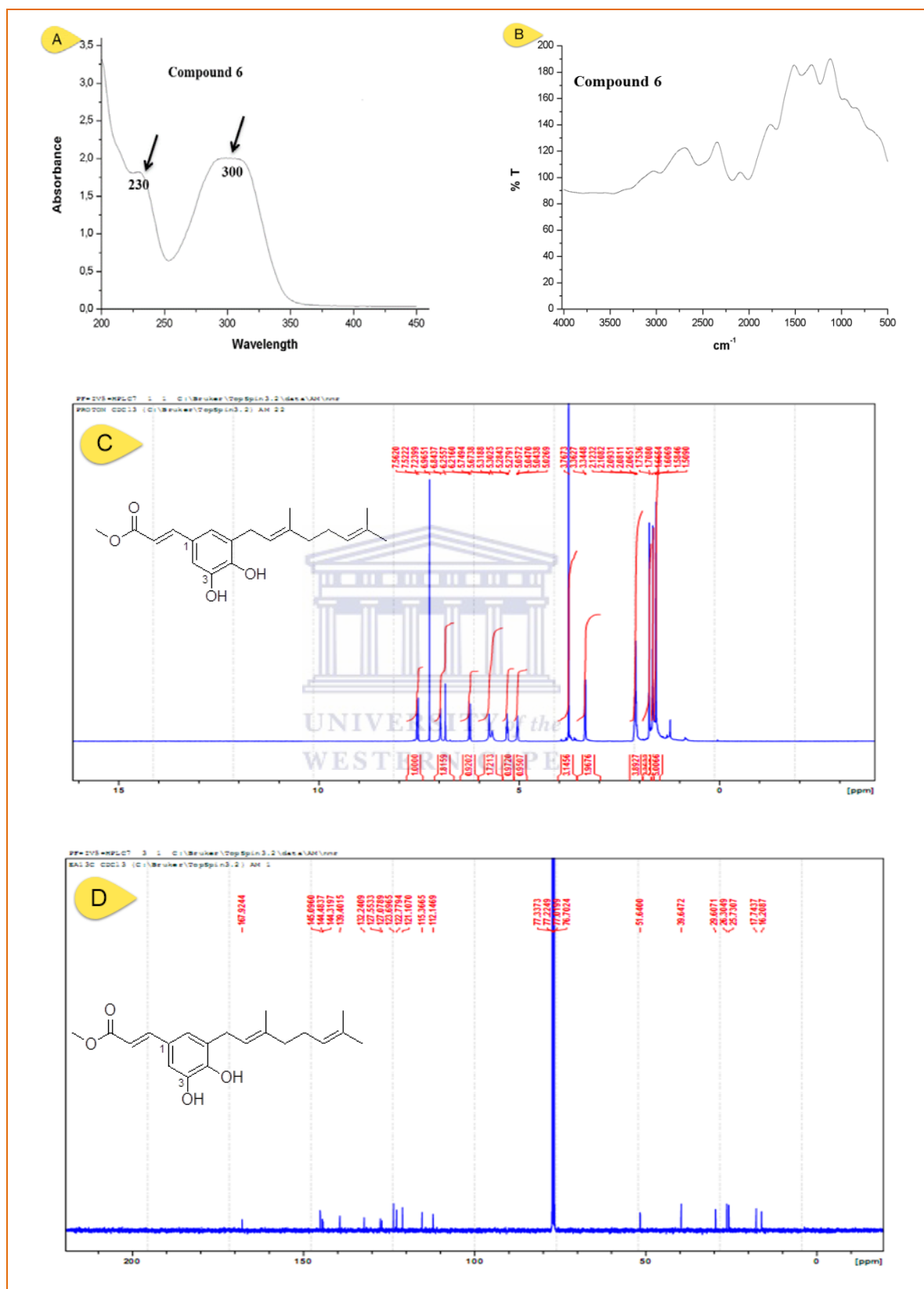


Figure 3.19: Spectroscopic data (UV (A), IR (B), ¹H (C) and ¹³C (D) NMR) of compound 6

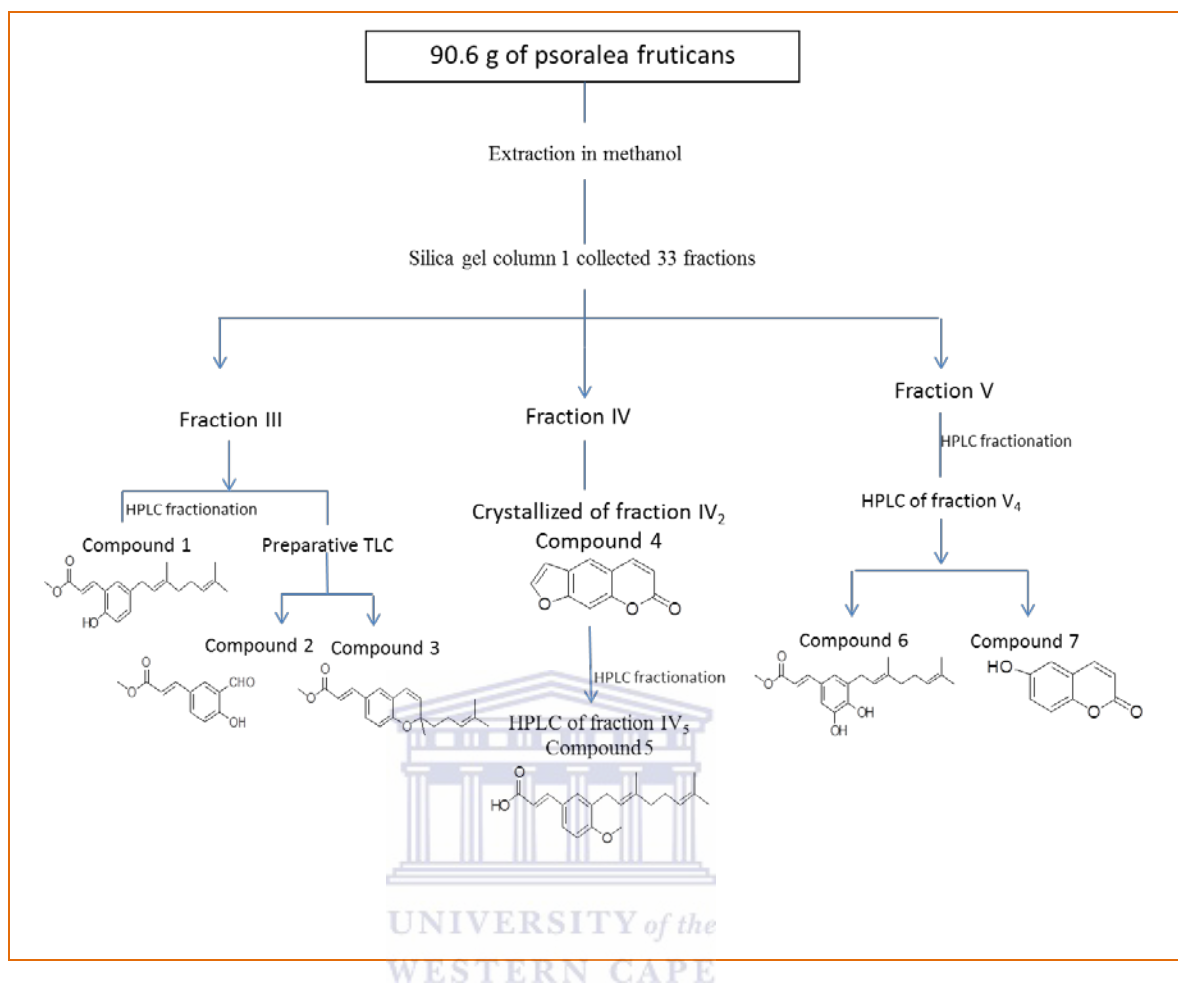


Figure 3.20: Schematic of isolation steps followed

3.4 Results

Screening of 32 plant extracts against *S. mutans* showed potent activity of the *P. fruticans* methanolic extract. The extract was subjected to chromatographic purification to yield seven pure compounds. Five of the isolated compounds are new metabolites isolated for the first time from a natural source, and belong to the geranyl derivative of coumaric acids (Figure 3-21).

3.4.1 Psortican A (1)

The molecular formula of compound **1** (Figure 3-21) was determined by HRMS as $C_{20}H_{26}O_3$. The NMR spectra (Figure 3.15) exhibited signals for four methyls, three of them are olefinic at

δ_{H} 1.66 (*s*, Me-8'), 1.58 (*s*, Me-9'), 1.74 (*s*, Me-10'), and methyl ester at 3.77, two olefinic proton at 5.30 (1H, *td*, $J=7.2$, 1.2 Hz, H-2'), and 5.06 (1H, *br t*, $J=7.0$ Hz, H-6''); three methylene groups two of them directly coupled to the above double bonds (as indicated from COSY and HMBC correlations) at 3.34 (CH₂, *d*, $J=7.2$ Hz, H-1'), 2.08 (2 CH₂, *m*, H-4', -5'), the above data with carbon-13 signals [δ_{C} at 29.3 (C-1'), 121.1 (C-2'), 138.8 (C-3'), 39.6 (C-4'), 26.4 (C-5'), 123.8 (C-6'), 131.9 (C-7'), 25.6 (C-8'), 17.7 (C-9'), and 16.2 (C-10')] indicated the presence of geranyl group.¹¹⁰

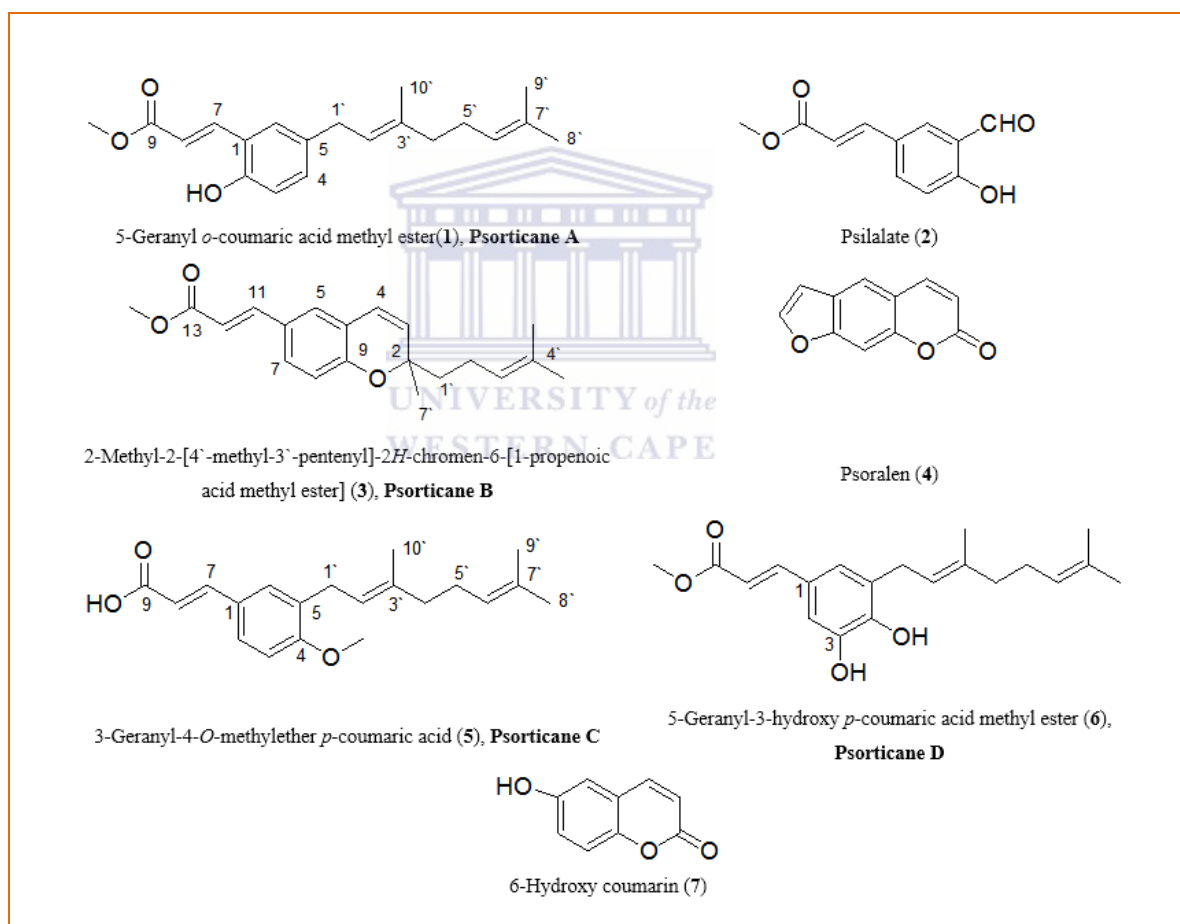


Figure 3.21: Chemical structures of compounds 1-7 isolated from *P. fruiticans*

Additionally, the compound showed a typical signals of *o*-coumaric acid methyl ester [δ_{H} 6.8 (1H, *d*, 8.8 Hz, H-3); 7.26 (2H, *m*, H-4, -6); 6.26 (1H, *d*, 16.0 Hz, H-8); 7.60 (1H, *d*, 16 Hz, H-

7)/ δ_C 127.6 (C-1), 156.8 (C-2), 116.1 (C-3), 127.7 (C-4), 126.9 (C-5), 130.1 (C-6), 145.2 (C-7), 114.6 (C-8), 168.2 (C-9)]. The coupling pattern of the benzene ring required 1,2,5-trisubstituted system which indicates the location of the geranyl group at C-5, this fact was supported by the HMBC spectra which showed cross peaks (among others) between H-1'/C-4, C-6; H-6/C-1, C-5 and H-7/C-6, C-2 Other 2D spectra HMBC, HSQC, and COSY confirmed the structure of **1** as 5-geranyl *o*-coumaric acid methyl ester and given the trivial name psorticane A (Table 3.4).

3.4.2 Compound 3 (Psorticane B)

The molecular formula of compound **3** (Figure 3-16) was determined by HRMS as C₂₀H₂₄O₃, two units less than compound **1**. The ¹H NMR spectrum showed signals of *p*-coumaric acid methyl ester similar to compound **1**, the HMBC correlations were in agreement with the 1,3,4-trisubstituted benzene ring as shown in Figure 3.22. The presence of *cis* coupled olefinic protons at 5.57 (*d*, 9.8 Hz, δ_C 130.2) and 6.32 (*d*, 9.8, δ_C 122.3), in addition to the absence of the methylene group signal of compound **1**, indicated the formation of a pyran ring between C4-C3' of compound **1**.

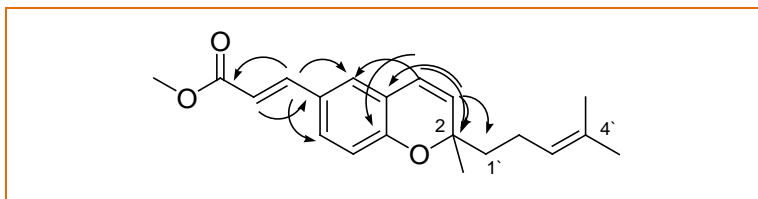


Figure 3.22: Some important HMBC correlations of compound 3 (from H to C)

The structural modification was supported by the chemical shift of the first double bond in the geranyl group, and the HMBC correlation of H-3/C-2, C-4, C-10, C-1' and H-4/C-5, C-9, C-2 (among others, Figure 3.21). The above data confirmed the structure of compound **3** as 2-methyl-2-[4'-methyl-3'-pentenyl]-2*H*-chromen-6-[1-propenoic acid methyl ester], and given the trivial name psorticane B (Table 3.4).

3.4.2 Compound 5

Compound **5** has molecular formula $C_{20}H_{26}O_3$, and showed very similar NMR spectra of compound **1** (Figure 3.18)—the only difference is the chemical shift C9 to 172.8 (168.2 compound **1**) which indicate free carboxylic group. The hydroxylated carbon was located at C4 as deduced from the HMBC correlation of H1'/C4, C5, C6; and H7/C6, C2, C1, additionally, the methoxyl proton showed HMBC with C4. Other 2D NMR spectra confirmed the structure of compound **5** as (Figure 3.18), and given the trivial name psorticane C (Table 3.4).

3.4.6 Compound 6

Compound **6** has molecular formula of $C_{20}H_{26}O_4$ from HRMS. The NMR spectra was very similar to compound **1** (Figure 3.19), except for the existence of extra hydroxyl group attached to the benzene ring which confirmed from the presence of only *m*-coupled two aromatic proton signals at δ_H 6.84 (br. s), and 6.97 (br. s) both protons located at C2, C6 positions of the coumaric side chain, which confirmed by the HMBC correlations between H7/C1, C2, C6 and H1'/C5, C4, C6. Other 2D NMR spectra confirmed the structure of **6** as (Figure 3.19) and given the trivial name psorticane D (Table 3.4).

3.4.7 Psilalste, Psoralen and 6-Hydroxy Coumarin

Additionally, three known compounds were isolated from the same extract and identified as psilalate (**2**)¹¹¹, psoralen (**4**) and 6-hydroxy coumarin (**7**).¹¹² To the best of our knowledge, compound **2** is reported for the second time from a natural source and both compounds **2** and **7** were isolated for the first time from *Psoralea* genus.

Table 3.4: ¹H (400 MHz) and ¹³C (100 MHz) of compounds 1, 2, 3, 5 and 6

No	Psorticane A (1)		Psilalate (2)		Psorticane B (3)		Psorticane C (5)		Psorticane D (6)	
1	127.6 s		126.6 s				127.1 s		126.5 s	
2	156.8 s		135.7 d	7.69*	79.5 s		112.2 d	6.97 br s	129.3 d	7.34 br s
3	116.1 d	6.8 d	120.5 s		130.2 d	5.57 d, 9.8	144.4 s		130.8 s	
4	127.7 d	7.26*	163.1 s		122.3 d	6.32 d, 9.8	144.5 s		159.7 s	
5	126.9 s		118.6 d	7.02 d, 8.8	126.1 d	7.11 d, 2.0	127.6 s		110.3 d	6.82 d, 8.4
6	130.1 d	7.26*	133.9 d	7.71 dd, 2.0, 8.8	126.9 s		122.8 d	6.84 br s	128.1 d	7.36 dd, 8.4, 1.6
7	145.2 d	7.60 d, 16.0	142.6 d	7.64 d, 16.0	129.5 d	7.26 dd, 8.5, 2.0	145.1 d	7.54 d	147.2 d	7.71 d 15.6
8	114.6 d	6.26 d 16.0	116.9 d	6.35 d, 16.0	116.5 d	6.73 d, 8.5	115.4 d	6.23 d	114.3 d	6.28 d 15.6
9	168.2 s		167.3 s		155.5 s		167.9 s		172.8 s	
10					121.1 s					
11					144.5 d	7.57 d, 16.0				
12					114.9 d	6.25 d, 16.0				
13					167.8 s					
1'	29.3 d	3.34 d	196.3 d	9.91 s	41.5 t	1.65 m	29.6 t	3.35 d	28.1 t	3.30 d, 7.0
2'	121.1 d	5.30 br t			22.7 t	2.11 m	121.1 d	5.28 t	121.5 d	5.28 br t, 7.0
3'	138.8 s				123.8 d	5.06 t, 6.4	139.4 d		136.9 s	
4'	39.6 t	2.08 m			131.9 s		39.7 t	2.08*	39.8 t	2.06, m
5'	26.4 t	2.08 m			25.6 q	1.63 s	26.3 t	2.09*	26.7 t	3.09 m
6'	123.8 t	5.06 br t			17.6 q	1.54 s	123.7 d	5.04 t	124.2 d	5.10 t 6.0
7'	131.9 s				26.9 q	1.38 s	132.3 s		131.5 s	
8'	25.6 q	1.66 s					25.7 q	1.67 s	25.7 q	1.66 s
9'	17.7 q	1.58 s					17.7 q	1.61 s	17.7 q	17.7 s
10'	16.2 q	1.74 s					16.2 q	1.75 s	16.1 q	1.69 s
OMe	51.6 q	3.77 s	51.8 q	3.79 s	51.6 q	3.77 s	51.6 q	3.77 s	55.5 q	3.85 s
2-OH										
3-OH										
4-OH										

3.5 Discussion

Chromatographic separation of the active extract from *P. fruticans* using different techniques, including semi-prep HPLC yielded seven pure compounds, viz. psortican A (**1**), psilalate (**2**), psortican B (**3**), psoralen (**4**), psortican C (**5**), psortican D (**6**) and 6-hydroxycoumarine (**7**). Psorticanes A-D are new compounds reported for the first time from a natural source. The identification of the isolated compounds was effectively completed using extensive analysis of the NMR spectroscopic data. Different metabolites have been reported from *Psoralea* genus, including coumarins, furanocoumarins, flavonoids, meroterpenoids and comestanes (see section 3.2.2 of this chapter).

The isolation of coumaric acid derivatives have not been reported so far from this genus. It is of interest to indicate that *C*-geranyl derivatives of phenolic acids and especially coumaric acids are rare metabolites and have been described from *Chrysothamnus pulchellus*.¹¹³ The isolation of the *C*-geranyl derivatives of coumaric acid adds a new dimension to the secondary metabolites profile of *Psoralea* genus. However, prenylated derivatives have been isolated from the genus, but the geranyl derivatives are reported for the first time in this study.

3.3 Biological Evaluation of the Isolated Compounds

3.3.1 Determination of Antibacterial Activity by TLC Bioautography

The pure compounds were diluted in methanol and spotted onto TLC plates. The plates were thoroughly sprayed with a dense culture of *S. mutans*, incubated for 24 hours in a humid atmosphere to allow growth of bacteria. After the incubation period, one of the plates was sprayed with INT and incubated for 2 hours.^{56,57} The other plate was developed with 3% MeOH:DCM and sprayed with vanillin acid and heated.

3.3.2 Determination of MIC and MMC of the Pure Compounds

The methodology is essentially as described before in Chapter 2. Different concentrations of the compound samples were tested to obtain the MIC values using serial dilutions (100, 50, 25, 12.5 and 6.25 µg/ml).^{58,59}

3.3.3 Determination of Biofilm Formation

The same methodology used for extracts was applied for the pure compounds, except that the slides were covered with compounds and left to dry completely. Then 50µl of fresh medium and 50 µl of bacteria were add and incubated at 37°C for 24 hours in a humid atmosphere to allow growth of bacteria.⁶⁰ Neomycin was used as a positive control and untreated cells as a control. After 24 hours, the slides were rinsed with sterile PBS and fixation performed as described in Chapter 2.

3.3.4 Cell Culture

KMST-6 fibroblasts, a normal human cell line, were cultured in DMEM (Dulbecco's Modified Eagles medium), supplemented with (10% v/v) foetal bovine serum and antibiotic (1% v/v). Cryogenic vials containing the cells were taken from the -150°C freezer and slowly thawed to 37°C. The cells were plated into 25-cm² flasks and cultured in a humidified CO₂ incubator (5% CO₂:air) at 37°C until confluency was reached. The culture medium was replaced every 48 hours.

3.3.5 Subculturing of Cells

After the cells were grown to 90% confluency, the culture medium was discarded and cells washed with 5 ml phosphate buffered saline solution and 3 ml of pre-warmed trypsin was added to detach the cells. The flasks were incubated for 3 minutes at 37°C until cells detached from the bottom of flasks. About 2 ml of growth medium was added to deactivate the action of trypsin and the mixtures were transferred into sterile 50-ml conical tubes and centrifuged at 2500 rpm for 3

minutes using a Sorvall TC6 centrifuge. After centrifugation, fresh growth medium was added to the cell pellets after discarding the supernatant. The cell pellets were either re-suspended in growth medium or growth medium containing 10% DMSO depending on whether the cells were going to be seeded into new flasks or whether the cells were going to be stored at -150°C .

3.3.6 Storage of Cells

After trypsinization, the cell pellet was re-suspended in growth medium containing 10% DMSO. The suspension was transferred to labelled cryogenic vials and stored at -150°C .

3.3.7 Cell Counting

Cell counts were performed as per the manufacturer's instructions using a CountessTM automated cell counter (Invitrogen).

3.3.8 Preparation of Stock Solutions of the Extract and Compounds

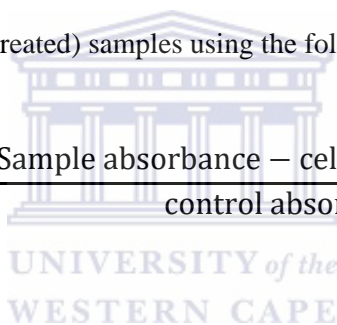
The extract and compounds were weighed first and dissolved in DMSO. The final concentration of DMSO in this step did not exceed 5%. After that the extract and compounds were diluted in growth medium to reach a concentration of $1000\ \mu\text{g}/\text{ml}$. The DMSO final concentration after dilution was 1%.

3.3.9 WST-1 Cell Proliferation Assay

The growth inhibitory effects of the methanolic extract of *P. fruticans* and some of the isolated compounds were evaluated using the tetrazolium salt, i.e., the WST-1 colourimetric assay, as recommended by the manufacture (Roche Diagnostics GmbH, Mannheim, Germany).¹¹⁴ The cells were cultured to 90% confluence and trypsinized as described before. The cells were seeded in a 96-well culture plates at a density of 5×10^4 cells/100 μl per well. The plates were incubated at 37°C in a humidified CO_2 incubator for 24 hours to allow the cells to attach to the bottom of the plate. A dilution series was made of the extract with increasing concentrations (62.5, 125,

250, 500 $\mu\text{g/ml}$) and the concentrations of isolated compounds were (25, 50, 75 and 100 $\mu\text{g/ml}$). Then the culture medium was removed and the cells were treated. As a positive control, the cells were treated with 6% DMSO, a known inducer of apoptotic cell death and since DMSO is known to be toxic to cells;¹¹⁵ an additional control was set up to evaluate its toxicity. The cells were therefore also treated with 1% DMSO. Untreated cells were used as a negative control. All treatments were done in triplicate. After 24 hours, 10 μl WST-1 reagent was added to the wells, and the plates were incubated at 37°C for another four hours. The optical density/absorbance of the wells was determined using a BMG Labtech POLARstar Omega microplate reader and the plates were read at 430 nm, and 630 nm was used as a reference wavelength. The percentage of cell viability was calculated by comparing the absorbance of the test samples with the absorbance of the control (untreated) samples using the following calculation:

$$\% \text{ cell viability} = \frac{\text{Sample absorbance} - \text{cell free sample blank}}{\text{control absorbance}} \times 100$$



3.3.10 Results

3.3.10.1 Qualitative Screening Using TLC Bioautography

The active compounds showed inhibition zones on the plate. The place of growth inhibition on the plate are shown via clear or creamy-white spots against the intensely coloured background indicative of bacterial growth (Figures 3.23 and 3.24). Zones of inhibition by compounds on the plate were visualized using the P-iodonitrotetrazolium violet (INT) detection system.^{56,61}

3.3.10.2 Minimum Inhibitory (MIC) and Microbicidal Concentration (MMC)

Of the six tested compounds six were active and one compound showed no activity. Compound 3 was not active as confirmed by the bacterial growth in the five tested concentrations with MIC

and MMC values in excess of 100µg/ml. Compounds **1** and **5** were the most active as they inhibited the growth at 6.25 µg/ml with MMC 12.5 µg/ml. Compound **4** was the second most active exhibiting an MIC of 12.5 µg/ml and MMC of 25 µg/ml. Compound **6** was inhibited bacterial growth at 50 µg/ml with an MMC of 100 µg/ml whereas compound **2** exhibit growth inhibition at 100 µg/ml as illustrated in Table 3.5.

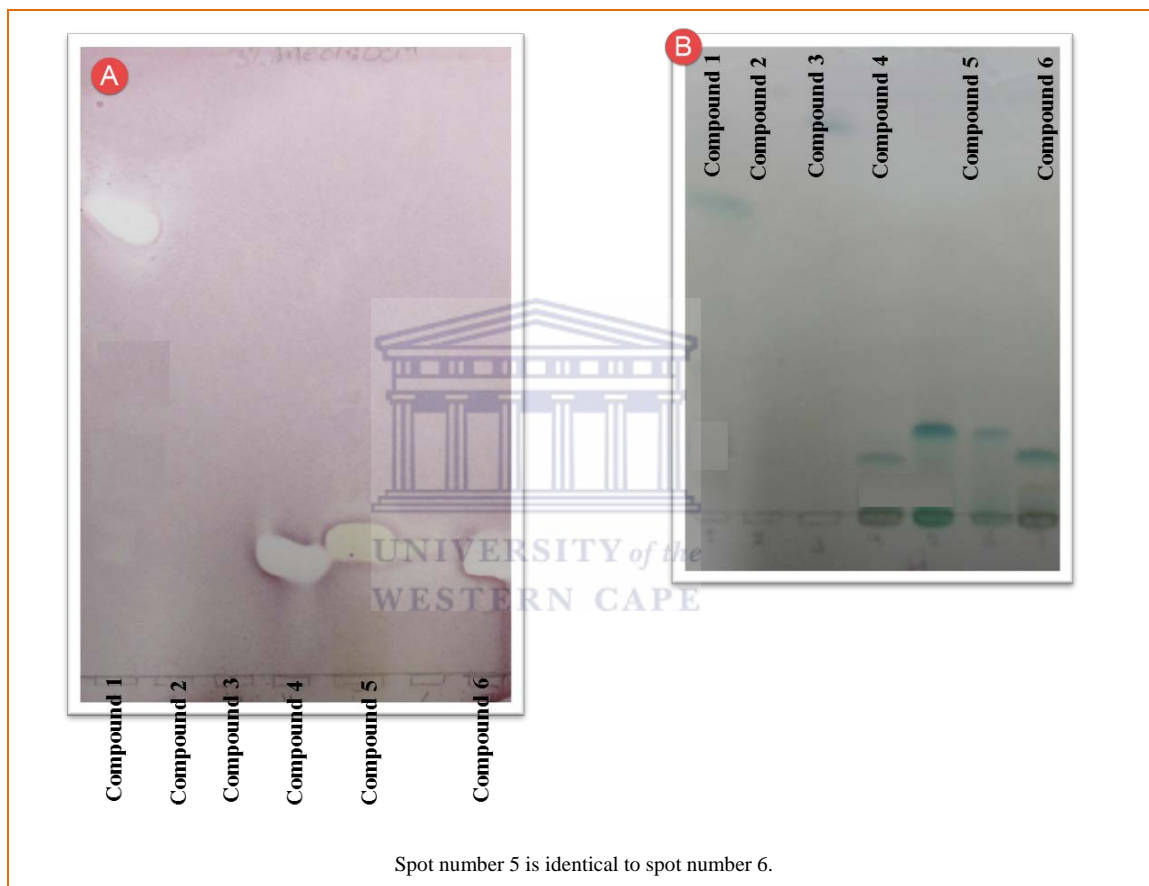


Figure 3.23: TLC plates of the pure compounds sprayed with bacteria (A) and vanillin acid (B)

3.3.10.3 Streptococcus Mutans Biofilm Formation

The cells treated with the compounds showed a reduction in cell numbers and no development of a biofilm. Compound **1** showed no development of *S. mutans* on the glass slide and few bacteria that attached to the slide. Compounds **3** and **5** showed that cells adhered to each other. The other compounds showed no biofilm development (Figure 3.25).

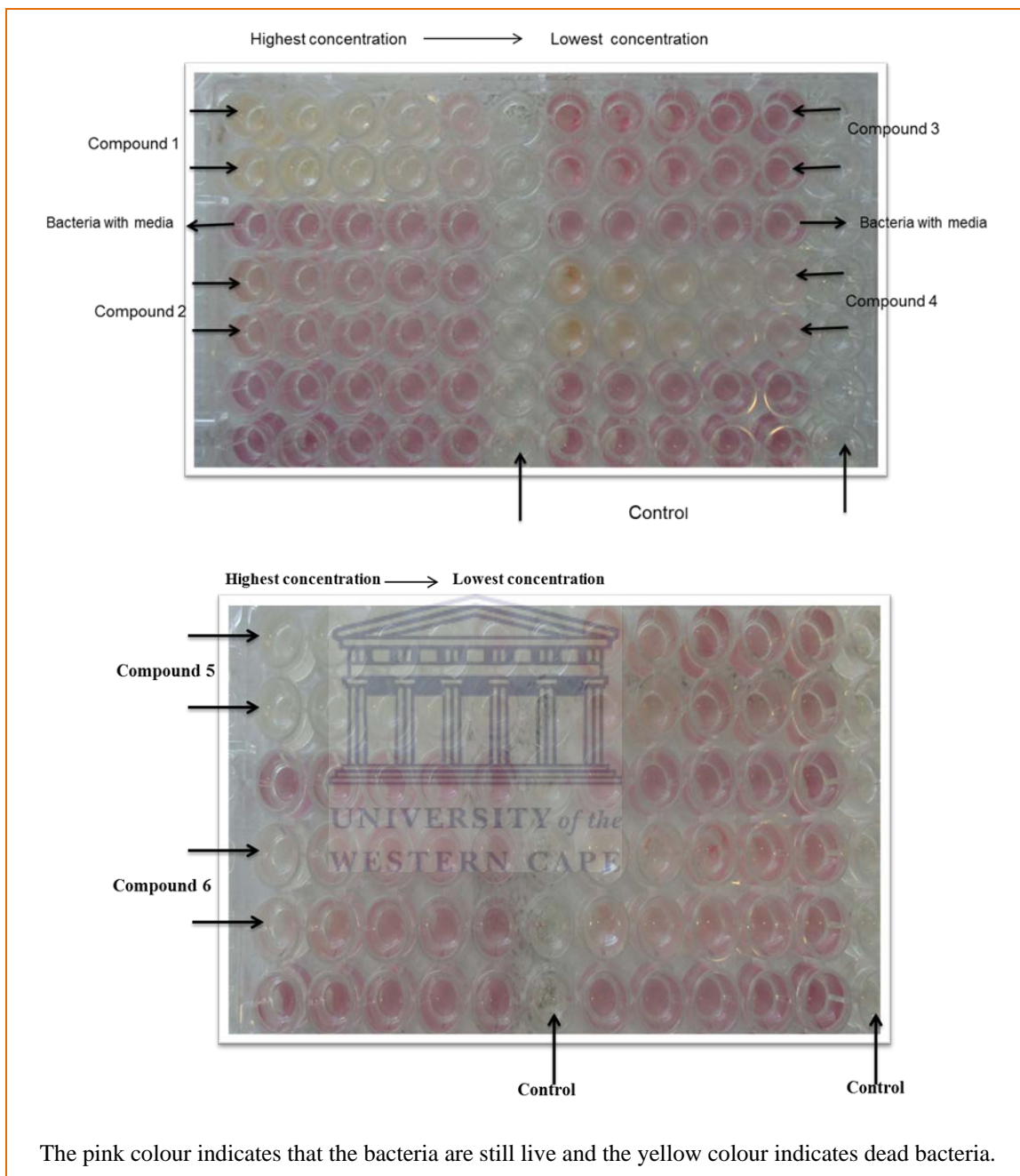


Figure 3.24: The minimum inhibitory concentration 96-well plates for the compounds

3.3.10.4 Cytotoxic Effects of the Total Extract of *Psoralea fruticans*

Non-tumorigenic immortalized normal human human diploid fibroblasts cells (KMST-6)⁴⁵ were treated for 24 hours with the total methanolic extract of *P. fruticans* as described before.

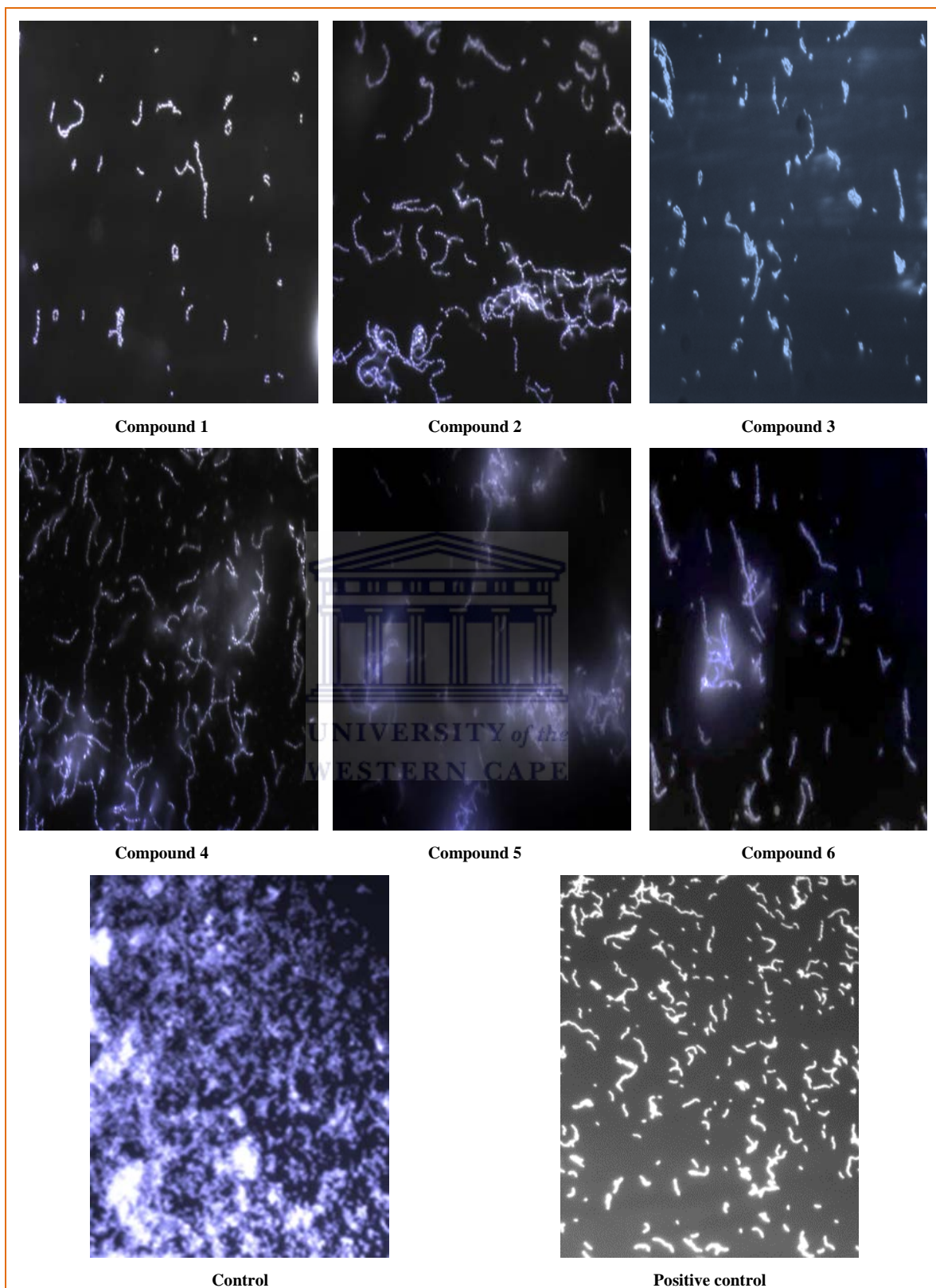


Figure 3.25: *Streptococcus mutans* biofilm developed on glass slides and treated with the compounds

Table 3.5: MIC, MMC and IC₅₀ values of the crude extract and isolated compounds of *Psoralea fruticans*

Sample	MIC (µg/ml)	MMC (µg/ml)	IC ₅₀ (µg/ml)
Total extract	31.25	62.25	71
Compound 1	6.25	12.5	<25
Compound 2	50	100	>100
Compound 3	>100	>100	>100
Compound 4	12.5	25	>100
Compound 5	6.25	12.5	44
Compound 6	50	100	>100

The cell viability following treatment was determined using the WST-1 assay.⁶⁸ The viability of KMST-6 was unaffected at 62.5 µg/ml, while no viability was observed at the highest dose of 125 µg/ml. with an IC₅₀ value of 71 µg/ml (Table 3.5).

3.3.10.5 Cytotoxic Effects of the Isolated Compounds of *Psoralea fruticans*

Six compounds were selected to assess their cytotoxic effects of KMST-6 normal human fibroblasts. The cells were treated for 24 hours with the pure compounds as described before and cell viability were assessed using WST-1 assay.¹¹⁴ The IC₅₀ values for these compounds were determined (Table 3.5). Compounds **2**, **3**, **4**, and **6** were inactive in the cytotoxic assay with IC₅₀ valued >100µg/ml. Compound **5** was active with IC₅₀ value of 44 µg/ml and compound **1** was the most active compound in cytotoxic assay with IC₅₀ <25 µg/ml (Table 3.5, Figure 3.26). Figure 3.27 shows the effect of the total methanolic extract of *P. fruticans* on KMST-6 cell viability.

3.3.11 Conclusion

TLC bioautography plate with isolation compounds showed a clear inhibition growth spots. Compounds **1**, **4**, **5**, and **6** showed creamy-white inhibition zones. Quantification of the antibacterial activity of isolated compounds was carried out using serial plate microdilution.⁵³

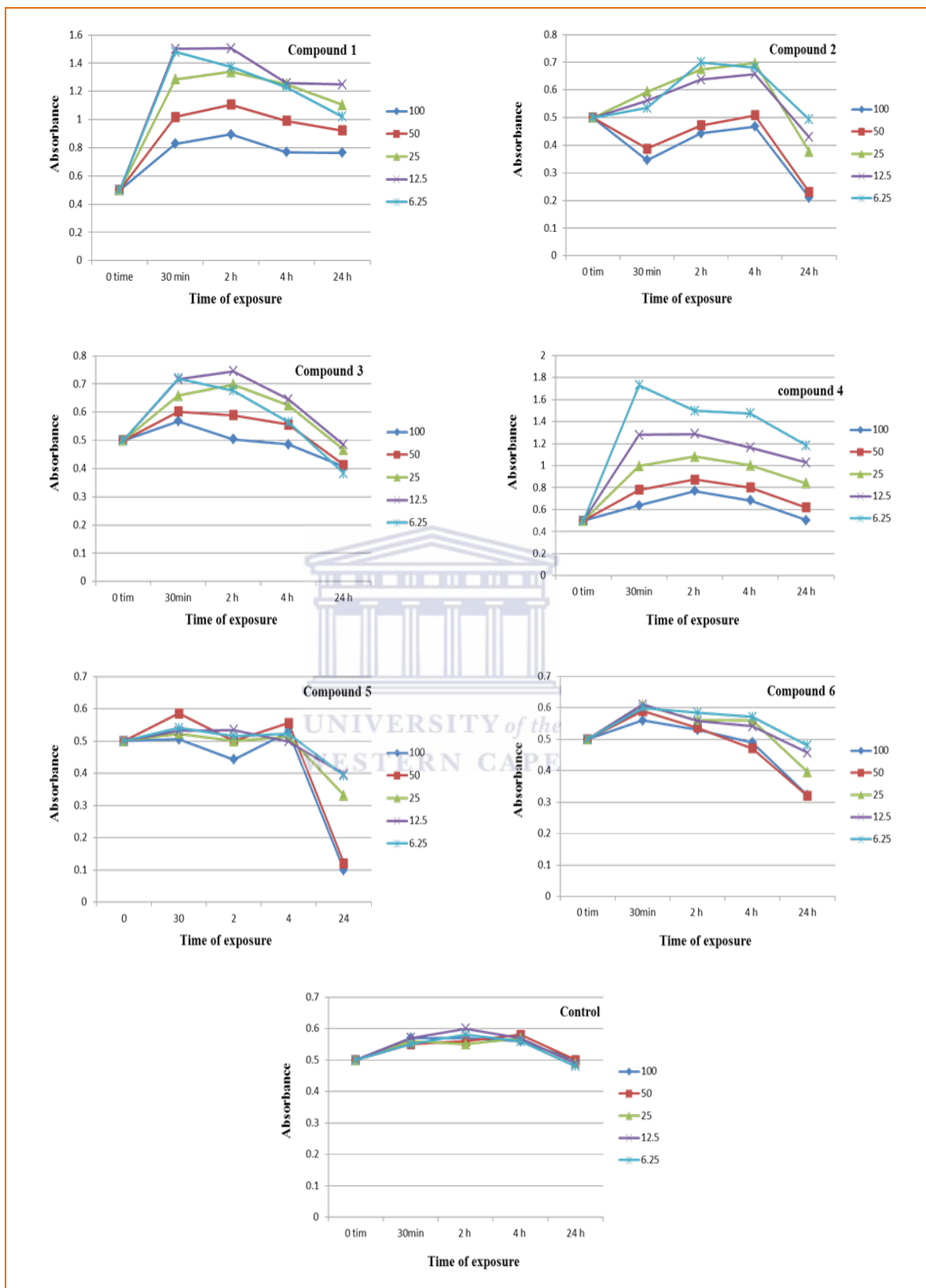


Figure 3.26: Absorbance profiles for the MIC of the compounds

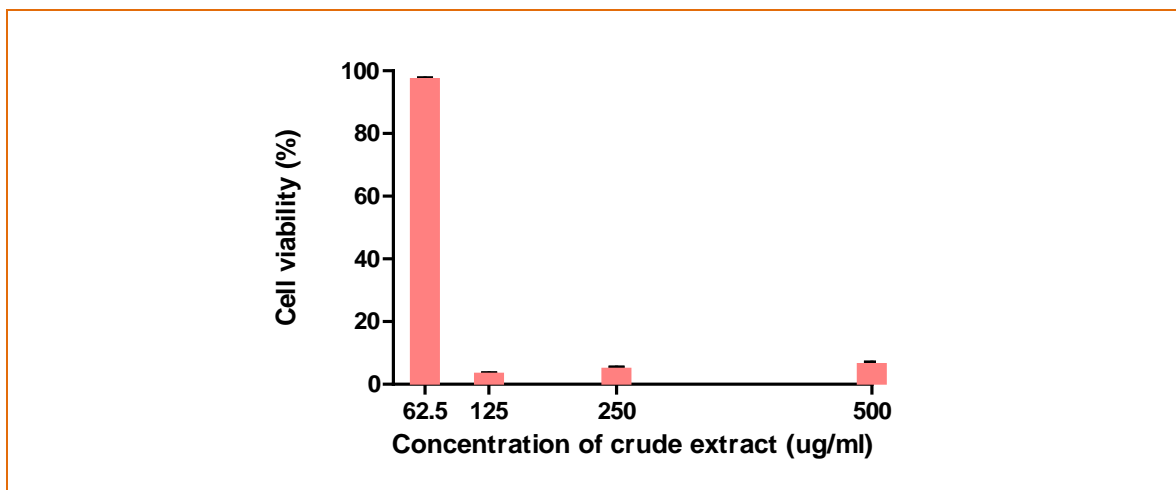


Figure 3.27: Effect of the total methanolic extract of *P. fruticans* on KMST-6 cell viability

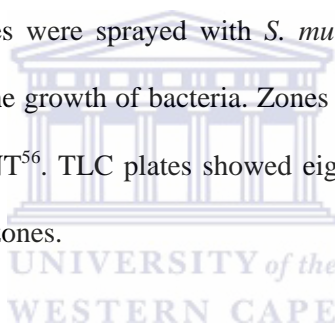
Compounds **1** and **5** were the most active in inhibiting the growth of *S. mutans* at 6.25 $\mu\text{g/ml}$ with MMC of 12.5 $\mu\text{g/ml}$. Compound **4** also inhibited bacterial growth at 12.5 $\mu\text{g/ml}$ with MMC of 25 $\mu\text{g/ml}$ while compound **6** did so at 50 $\mu\text{g/ml}$ with MMC of 100 $\mu\text{g/ml}$. *S. mutans* biofilm development determined using glass slides treated with the isolate compounds showed a decrease in cell number. The cytotoxic assay was carried out for the crude methanol extract and six isolated compounds to assess if the extract and compounds exerted toxic effects on normal KMST-6 human fibroblasts. The cells viability was assessed using the WST-1 assay.

The total crude extract were not toxic which showed IC_{50} value of 71 $\mu\text{g/ml}$. Compounds **1** and **5** were found to be the most active with IC_{50} values <25 and 44 $\mu\text{g/ml}$, respectively. Compound **1** had toxic effect on the human cell line and compound **5** showed some cytotoxic effect, while the all other compounds were regarded as safe in human cell line, i.e., inactive in cytotoxic assay with $\text{IC}_{50}>100\mu\text{g/ml}$. Compound **4** which is psoralen was considered to be inactive as measured by the cytotoxic assay as previously reported.³⁶ According to another study,¹⁰⁴ psoralen showed to have cytotoxic effect on stomach cancer cell line with IC_{50} value of 53 $\mu\text{g/ml}$ in SNU-1 and 203 $\mu\text{g/ml}$ in SNU-16.

CHAPTER 4

CONCLUSION

Many South African plants are now being investigated for potential antimicrobial activities. In this study, about 32 South African medicinal plants were collected from the Cape Flats region and tested against *S. mutans*. The plants were extracted with methanol and thin layer chromatography (TLC) used as a screening method for the detection of antimicrobial activity of the compounds.^{57,116} The 32 plant extracts were spotted on TLC plates and developed in 3% MeOH:DCM. Then the plates were sprayed with *S. mutans* and incubated for 24 hours in a humid atmosphere to allow the growth of bacteria. Zones of inhibition by the compounds on the plate was envisaged using INT⁵⁶. TLC plates showed eight extracts that exhibited antibacterial activity with clear inhibition zones.



The microdilution assay was used to investigate the inhibition of bacterial growth.⁵³ Eight plants were active at 500 µg/ml concentration, namely, *Salvia africana caerulea*, *Psoralea fruticans*, *S. africana lutea eloca*, *Acacia karro*, *Helichrysum cymosum*, *Psoralea pinnata*, *Helichrysum zeyheri* and *Castanea sativa*) and three were active at 100 µg/ml, namely, *Psoralea fruticans*, *Psoralea pinnata* and *Helichrysum cymosum*). The minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) were determined for the eight active extracts: *Psoralea fruticans* and *Psoralea pinnata* yielded the most active extracts which inhibit the growth of bacteria at MIC 31.25 µg/ml with MMC of 62.5 µg/ml. *S. africana lutea eloca*, *S. Africa acerulea* and *Acacia karroo* inhibited bacterial growth at MIC 62.5 µg/ml and MMC of 125 µg/ml. *Helichrysum zeyheri*, *Helichrysum cymosum* and *Castanea sativa* inhibited the growth of bacteria at MIC 125 µg/ml and MMC 250 µg/ml.

Psoralea fruticans was selected for phytochemical studies to isolate the active constituents because of its antibacterial activity at lower concentrations and according to TLC plate which showed that *Psoralea fruticans* have potential bioactive compounds. This finding is noteworthy because there are few chemical or biological studies published on *Psoralea fruticans*.

Chromatographic separation of the active extract from *P. fruticans* using different techniques, including semi-prep HPLC yielded seven pure compounds, viz., psorticane A (**1**), psilalate (**2**), psorticane B (**3**), psoralen (**4**), psorticane C (**5**), psorticane D (**6**) and 6-hydroxycoumarine (**7**); psorticanes A-D are new compounds reported for the first time from this natural source. The identification of the isolated compounds was effectively completed using extensive NMR spectroscopy.

Different metabolites have been reported from *Psoralea* genus, including coumarins, furanocoumarins, flavonoids, meroterpenoids and comestanes. The isolation of coumaric acid derivatives have not hitherto been reported for this genus. It is of thus of interest to indicate that C-geranyl derivatives of phenolic acids and especially coumaric acids are rare metabolites and have been described from *Chrysothamnus pulchellus*.¹¹³ The isolation of the C-geranyl derivatives of coumaric acid add a new dimension to the secondary metabolites profile of *Psoralea* genus. However, prenylated derivatives have been isolated from the genus, but the geranyl derivatives are reported for the first time in this study.

Seven compounds were selected to antibacterial and cytotoxic evaluation. The MIC of pure compounds were determined using the serial plate microdilution assay.^{53,59} The MIC is demarcated as the lowest concentration that inhibited the growth of the bacteria. Six compounds inhibited bacterial growth. One compound was not active at the tested concentration. Compounds **1** and **5** were the most active compounds which inhibited the growth of bacteria at MIC 6.25

$\mu\text{g/ml}$ with MMC of $12.5 \mu\text{g/ml}$ and compound **4** next at MIC $12.5 \mu\text{g/ml}$ with MMC of $25 \mu\text{g/ml}$. Compounds **2** and **6** inhibited bacterial growth at MIC $50 \mu\text{g/ml}$ with MMC of $100 \mu\text{g/ml}$.

An improved method was used for investigating the effect of the active extracts and the purified compounds from *Psoralea fruticans* on *S. mutans* biofilm formation.¹¹⁷ *S. mutans* biofilm development was measured on glass slides for 24 hours in a humid atmosphere. After incubation, glass slides were treated with the crude extracts of the eight active plants and isolated compounds.⁶⁰ Some extracts showed biofilm development, including *Acacia green*, *Salvia Africana lutea*, *Psoralea pinnata*, *Helichrysum cymosum*, *Helichrysum zeyheri* and *Castania sativa*. Two plants showed no development on biofilm (*Salvia africana careulea* and *Psoralea fruticans*). The compounds were not preventing *S. mutans* to attach to the glass, but showed reduction in cell numbers and no biofilm development.

Psoralea fruticans extract and seven compounds were selected for cytotoxic evaluation on KMST-6 human fibroblasts. The cell viability was determined using WST-1 assay.¹¹⁴ The results showed that the methanol extract of *Psoralea fruticans* is safe on KMST-6 cells with an IC_{50} value of $71 \mu\text{g/ml}$. Five of the test compounds were found to be non-toxic to KMST-6 cells (IC_{50} value $>100 \mu\text{g/ml}$), while compounds **1** and **5** showed some toxic effect on KMST-6 cells (IC_{50} value $<25 \mu\text{g/ml}$ and $44 \mu\text{g/ml}$, respectively). These results may have significant implications in the treatment of oral disease caused by cariogenic bacteria, including *S. mutans*.

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