# ISOLATION AND CHARACTERISATION OF ANTIMYCOBACTERIAL COMPOUNDS FROM *SCHKUHRIA PINNATA (Lam.) Kuntze ex Thell* AGAINST *MYCOBACTERIUM SMEGMATIS*

**BY**

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**A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY IN THE DEPARTMENT OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY, SCHOOL OF MOLECULAR AND LIFE SCIENCES, FACULTY OF SCIENCE AND AGRICULTURE, (UNIVERSITY OF LIMPOPO),**

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## Declaration

I declare that the dissertation titled: Isolation and characterisation of antimycobacterial compounds from *Schkuhria pinnata (Lam.) Kuntze ex Thell* against *Mycobacterium smegmatis*, hereby submitted to the University of Limpopo for the degree Master of Science in Microbiology has not been previously submitted by me to this or any other University and that the work contained herein is my own work in design and execution, and that all materials used are duly acknowledged.

______________________________ ______________________

Masiphephethu M.V. Date

## Dedication

This work is dedicated to my lovely parents (Mr. and Mrs. Masiphephethu), siblings (Fhumulani, Zwivhuya, Martin, Mitambo and Rikonise) and lastly to my daughter (Thalukanyo) and son (Rirhandzu).

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## List of abbreviations

| | |
|---|---|
| $^{13}C$ | Carbon-13 |
| $^{1}H$ | Hydrogen-1 |
| A | Acetone |
| AAE | Ascorbic acid equivalent |
| ABTS | 2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid |
| AIDS | Acquired immune deficiency syndrome |
| BAW | Butanol/Acetic acid/Water |
| BEA | Benzene/Ethanol/Ammonia hydroxide |
| C | Chloroform |
| CEF | Chloroform/Ethyl acetate/Formic acid |
| CE/MS | Capillary electrophoresis-Mass spectrometry |
| CM | Centimetre |
| COSY | Correlation spectroscopy |
| D | Dichloromethane |
| DEPT | Distortionless enhancement by polarization transfer |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPPH | 2, 2-diphenyl-1-picrylhydrazyl |
| E | Ethanol |
| EA | Ethyl acetate |
| EMW | Ethyl acetate/methanol/water |
| EMR | Electron magnetic resonance |

| | |
|---|---|
| FBS | Fetal bovine serum |
| FRAP | Ferric reducing antioxidant power |
| GAE | Gallic acid equivalence |
| H | Hexane |
| $H_2$DCF-DA | Dihydrodichlorofluorescein diacetate |
| HAT | Hydrogen atom transfer |
| HMBC | Heteronuclear multiple bond correlation |
| HMQC | Heteronuclear multiple quantum correlation |
| HPLC | High performance liquid chromatography |
| HSQC | Heteronuclear single quantum correlation |
| INT | ρ-iodonitrotetrazolium violet |
| $LC_{50}$ | Lethal concentration 50 |
| LPS | Lipopolysaccharides |
| M | Methanol |
| MEM | Minimal essential medium |
| MIC | Minimum inhibitory concentration |
| MS | Mass spectrometry |
| MTT | 3-(4, 5-dimethylthiazol-2- yl)-2, 4-diphenyltetrazolium bromide |
| NIC | National cancer institute |
| NMR | Nuclear magnetic resonance spectroscopy |
| PBS | Phosphate buffered saline |
| QE | Quercetin equivalence |
| ROS | Reactive oxygen species |

| | |
|---|---|
| rmp | Rotation per minute |
| SI | Selective index |
| SET | Single electron transfer |
| TLC | Thin layer chromatography |
| UV | Ultraviolet light |
| WHO | World health organisation |

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**Abstract**

*Schkuhria pinnata* was selected for this study based on its use in traditional medicine. This study was aimed at isolating and charactserising antimycobacterial compounds from *S. pinnata*. Different extraction procedures coupled with solvents of varying polarities were used in extraction of the plant materials. Solvents of intermediate polarity had the highest mass of the extracts and serial exhaustive extraction was the best extraction procedure which extracted high amounts of plant material obtained with dichloromethane solvent. The chromatograms were developed in three solvent systems (BEA, CEF and EMW) and sprayed with vanillin-sulphuric acid reagent for colour development. Different colours on the chromatograms indicated various phytochemical constituents. Standard chemical tests confirmed the presence of tannins, flavonoids, alkaloids, phlabotannins, terpenes, steroids, cardiac glycosides and saponins. It was discovered that *S. pinnata* possesses high phenolic and tannin content which could be behind the antioxidant and anti-inflammatory activities observed. Antioxidant activity was analysed using 2, 2–diphenyl-1-picrylhydrazyl (DPPH) qualitative and quantitative experiments. Chromatograms were sprayed with 0.2% DPPH solution, yellow bands or spot against the purple background indicated the presence of antioxidant compounds. On quantitative analysis methanol extracts had a good scavenging activity at various concentrations. Ferric ion reducing power of antioxidants from plant extracts was determined using FRAP assay. *S. pinnata* extracts had high ferric reducing power which was in a concentration-dependent manner. Antimycobacterial activity was evaluated using Bioautography and broth microdilution assays. Plant extracts indicated antimycobacterial activity observed on bioautograms with low MIC values ranging from 0.27 mg/ml to 2.5 mg/ml. African green monkey Vero kidney cells were used to evaluate the toxicity of crude extracts. The plant extract had cytotoxic value of 25 µg/ml with a selectivity index of 0.02 SI. It was observed that *S. pinnata* had anti-inflammatory activity on LPS-induced Raw 246.7 macrophage cells in a concentration-dependent manner. Bioassay guided fractionation on column chromatography managed to isolate two compounds which were characterised using nuclear magnetic resonance techniques. The compounds were elucidated to be helaingolide and eucannabinolide sesquiterpene lactones. Biological assays indicated that the compounds were active against *Mycobacterium smegmatis*. The compounds were toxic to Vero monkey kidney cells with less than 30

μg/ml $LC_{50}$ value and <1 selectivity index. These compounds had a good anti-inflammatory activity on LPS-induced Raw 246.7 macrophage cells which was in a concentration dependent manner. The compounds can be used as new leads in the development of anti-inflammatory and antimycobacterial drugs. The crude extracts and the isolated compounds from *S. pinnata* should be evaluated for their cytotoxicity and anti-inflammatory effects in *in vivo* experiments.

## Chapter 1

### 1. General introduction

Humans have been relying on nature to provide them with basic needs such as food, shelter, fragrances, fuel, clothing and medicines from their existence (Gurib-Fakim, 2006). According to Safowora (1982) medicinal plants are any plants which in one or more of its organs contain substances that can be used in the treatment of diseases. Plants produce compounds which are categorised as primary and secondary metabolites based on their role in growth and development (Dewick, 2009). Primary metabolites are responsible for normal growth, development and reproduction while secondary metabolites are for specific functions such as pollination attraction, production of colorants to attract or warn other species, defence against diseases and herbivores (Dewick, 2009). Secondary metabolites are effective against various diseases in human and they can be isolated from plants and serve as good agents that can be used in the development of new drugs (Soejarto, 1996; Hostettmann *et al.*, 2001).

Plants have gained their recognition in the development of new drugs due to challenges that arise when using chemically synthesised drugs. Some of the challenges include prolonged antibiotic therapy, inefficiency, high cost and increase in resistance of microorganism to antibiotics (Best and Best, 2009). As well as the demand of antibiotics due to increase in population growth (Joy *et al.*, 1998). In African countries, the knowledge of preparation and uses of medicinal plants was orally passed on from generation to generations (Masoko *et al.*, 2005; Gurib-Fakim, 2006; Reddy *et al.*, 2007). This knowledge is at risk of being lost, since in Africa we have the highest percentage of deforestation because most of these plants are trees. Therefore, formal documentation of each medicinal plant is significantly required for proper conservation and sustainable harvesting methods (Mander *et al.*, 1996; Gates, 2000; Betti *et al.*, 2013).

According to the world health organisation (WHO) (2002), about 80% of the population in developing countries are using medicinal plants for their primary health care needs. Medicinal plants are relied on by communities as they consider them to be effective, have less or no side effects and they are readily available (Philomena, 2011; Borokini

and Omotayo, 2012). Therefore, there is a need for clinical tests to validate their effectiveness which gives a clear understanding on their safety and efficacy.

Traditional healers use medicinal plants to treat diseases such as fever, wounds, sexually transmitted infections, inflammation, diarrhoea, cancer, malaria, tuberculosis and diabetes (Buwa *et al*., 2006; Bussmann *et al*., 2010; Anywar *et al*., 2016). Medicinal plants are administered through smoking, steaming, bathing, lotions, infusion as teas, as component mixtures in porridges and soups, and as concoctions (Céline *et al*., 2009). Plants secondary metabolites could results in severe side effects when consumed as a consequence of a diversity of phytochemicals (Talalay and Fahey, 2001; Gakuba, 2009). It is important to analyse medicinal plants toxicity for traditional healers and other consumers so as to have knowledge on their safety and dosage (Bagla, 2011).

Medicinal plants are of great importance in human's health due to the availability of secondary metabolites which have been reported to be therapeutic agents (Edeoga *et al*., 2005; Cos *et al*., 2006). Some of therapeutic activities which have been reported in literature are as follows: antibacterial, antifungal, antidiabetic, antioxidant, anticancer, antiviral and anti-inflammatory activities (Yue-Zhong Shu, 1998; van Wyk andWink, 2004; Mahesh and Satish, 2008). Oxidative stress and the use of synthetic antioxidants are the causing agents of degenerative and chronic conditions in human's health (Kuhn, 2003; Chen *et al*., 2008). Several research groups have indicated that dietary antioxidants offer an effective prevention of cell damage from oxidative stress. Therefore, screening for antioxidant compounds and new drugs from medicinal plants to find alternative ways of overcoming diseases caused by oxidative stress and synthetic drugs is important.

There are different processes followed in the analysis of natural products namely, plant identification and collection, extraction, biological assays, purification and characterisation (Sasidharan *et al*., 2011). Limitations associated with natural products include difficulties in slowness in working with natural products, access and supply (Doughari, 2012). The current advancement in science for some methods such as spectroscopic methods and good extraction techniques has helped significantly in the success of natural products analysis (Wang and Weller, 2006). These methods have helped with the speed in isolation of efficient bioactive compounds from plants that

can be used in drug discovery. The isolated bioactive compounds have to go through processes of bioassays, where different clinical and toxicological screening methods are applied before they are registered as medicines (Ulrich-Merzenich *et al*., 2009). Phillipson (2007) indicated that about 100 natural-products-derived compounds are currently in clinical trial process and at least 100 similar projects are in preclinical development and most of them are from plant origin. Isolation and characterisation of antimycobacterial compounds from *S. pinnata* against *Mycobacterium smegmatis* was the main focus in this study.

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## Chapter 2

## 2. Literature review

### 2.1. Medicinal plants

Throughout the history of human, plants have been used as medicinal remedies and as a source of food. Plants contain compounds which perform their biological functions in their metabolism. These compounds are of great importance in the human health system since they have therapeutic properties. A long history of trial and error (during the doctrine of signatures) by human gives significant evidences of plants being used in the treatment of various diseases (Halberstein, 2005). A plant such as liverwort (*Marchantiophyta or Hepatica*) is used in the treatment of liver and dandeions (*Taraxacum officinale*) is used in the treatment of urinary conditions and jaundice (Halberstein, 2005).

Different drugs originate from plants; drugs such as digoxin from *Digitalis* species, quinine and quinidine from *Cinchona* species, Atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum* (Rates, 2001). South Africa possess a unique and diverse botanical heritage with over 30.000 plant species of which around 3000 species are used therapeutically (van Wyk *et al.*, 1997). And they play an important role in the country's economic sector. Majority of people in South Africa still depend on traditional medicine and they still consult traditional healers (Elvin-Lewis, 2001).

Medicinal plants were considered to be an option for poor people in developing countries. However, literature has showed that the importance of natural products is clearly enormous (Wondimu *et al.*, 2007). In the past years, medicinal plants were used mostly in developing countries, but currently they are used worldwide (Hoareau and DaSilva, 1999; Rukangira, 2001). It has been reported that out of 250.000 plant species in all continents of the world, only 80.000 are used for treatment of diseases and they have the promising leads for development of new drugs (Joy *et al.*, 1998).

Pharmaceutical companies were increasingly growing economically because scientists were able to obtain pure compounds from plants and preform structural modifications to produce more active and safer drugs (Rates, 2001). However, due to increasing growth of human population, poverty, unpleasant side effects and

development of drug resistance by microorganisms, people turn to use medicinal plants as they consider them to be effective, affordable and safe (Grieson and Afolayan, 1999; Vermani and Grag, 2002). The importance of drugs from natural products have gained recognition since there have been problems associated with chemically synthesised drugs. The search for new drug development leads from medicinal plants continues to increase.

**2.2. Phytochemical constituents of medicinal plants**

Plants produce primary metabolites during primary metabolism that function to maintain their primary functions. Secondary metabolites are produced during secondary metabolism, where they are determined for specific functions such as defence against pathogens and for pollination (Dewick, 2009). The metabolites which are beneficial to human health are secondary metabolites. Medicinal plants contain a wide range of phytochemical constituents (secondary metabolites) that can be used in the treatment of diseases (Duraipandiyan *et al.*, 2006). Phytochemicals in plants are found in various plant organs, such as roots, stem, barks, fruits and leaves (Criagg and Newman, 2001). The composition and quantity of these metabolites differ and changes depending on the aging and habitat conditions of the plants, and they can be used in classification of some plant species (Kliebenstein, 2004).

Secondary metabolites have been reported to have therapeutic effects in human and animal's health system. These metabolites are classified into three main groups: first are phenolics which are phenolic acids, flavonoids, tannins and lignin compounds; the second ones are terpenes compounds which include sterols, carotenoids and cardiac glycosides and the third ones are nitrogen containing compounds which are alkaloids, terpenoids and glucosinates (Agostini-Costa *et al.*, 2012). The harvested medicinal plants can be used while they are still fresh or when they are dried. They are administered in deferent ways in order to obtain the bioactive compounds, depending on the preparation and the condition of the disease.

### **2.2.1.** Metabolites found in medicinal plants

#### 2.2.1.1. Flavonoids

Flavonoids are phenolic compounds abundantly distributed in plants. They are structurally built with two benzene rings joined by a linear carbon chain ($C_6C_3C_6$). There are over four thousand types of flavonoids that are derived from the main compounds called flavans, this include flavonoids such as anthocyanidins, flavones, flavonols, flavonones (Figure 2.1) and isoflavones. These compounds are found in fruits, tea, vegetables, seeds and wine (Grange and Davey, 1990; Doughari, 2012). In plants, they play an important role in pigments, defence against pathogens, predators and herbivores.

Available studies have reported that flavonoids possess biological activities such as antimicrobial, anticancer, anti-inflammatory, antiviral and antioxidant activities (Wink, 2015; Phanda and Kar, 2007; Doughari, 2012; Harborne, 1999). Researchers have gained the interest in flavonoids because of their antioxidant properties that have biological effects toward diseases such as cardiovascular diseases and cancer. Their antioxidant mechanism involves chelating metal ions and scavenging free radicals (van Acker *et al.*, 1996; Kumar and Pandey, 2013).

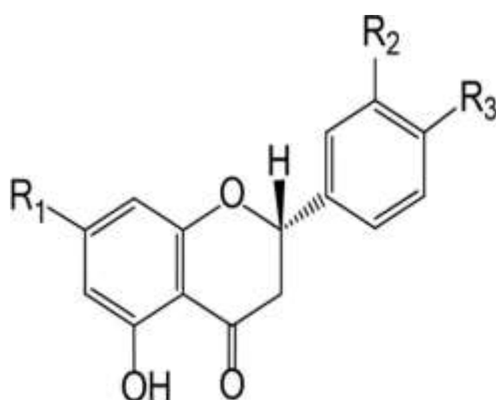


Figure 2.1: The chemical structure of flavanone (Kumar and Pandey, 2013).

#### 2.2.1.2. Terpernoids

Terpenoids (Figure 2.2) are a large family of volatile compounds such as carotenoids, tocopherol, phytol and sterols which are mostly distributed in plants. They can also be found in some marine organisms and insects. They have several biological activities

in both plants and human health. They protect plants from ultraviolet light, serve as the source of vitamin A that is important to human and they play a role in hormonal stimulation in animals (Wink, 2015). They are formed by hydrocarbons of general formula ($C_5H_8$) n.

Terpenoids are divided into eight classes which are classified based on the number of carbon atoms present in the structure. The classes include monoterpenoids, sesquiterpenoids, diterpenoids, sesterpenoids, troterpenoids, tetraterpenoids and polyterpenoids (Yadav *et al.*, 2014; Pattanaik and Lindberg, 2015). Literature has shown that terpenoids have different pharmaceutical activities such as antimalarial, anticancer, anti-inflammatory, antifungal and antiviral activity that benefits human health (Pattanaik and Lindberg, 2015). Tetraterpenoids are reported to be metabolites that have antioxidant activity and are used in medicinal drugs (Wink, 2015).

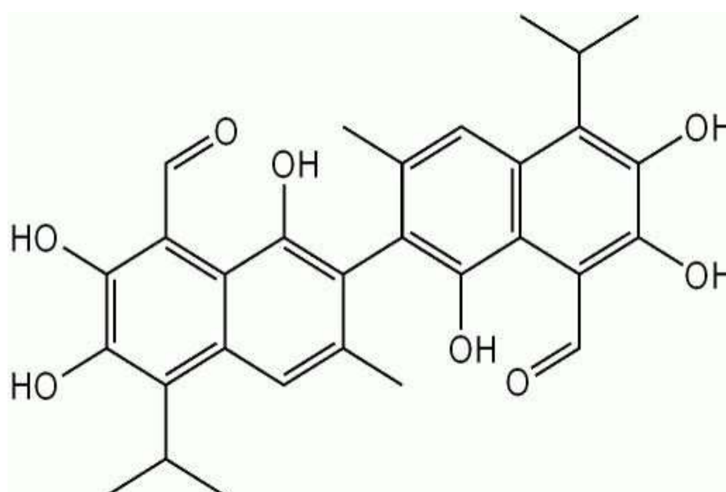


Figure 2.2: The structure of terpenoid compound called gossypol (Freeman and Beattie, 2008)

2.2.1.3. Alkaloids

Alkaloids are secondary metabolites that are widely distributed in plants and they contain one or more nitrogen atom on their structural formula which contributes to their basicity (Doughari, 2012). They are divided into few groups namely: non-heterocyclic alkaloids and heterocyclic alkaloids which are furtherly divided into 12 major groups (Robbers *et al.*, 1996). For example mescaline belongs to non-heterocyclic alkaloids and solasodine are triterpene alkaloids. These compounds are considered to be soluble in organic solvents. Alkaloid compounds have been reported to be growth

stimulants in plants and play a significant role in plant's germination and protection (Gurib-Fakim, 2006).

Plants use alkaloids as a defence chemical against herbivores. They are among the most active secondary metabolites with more than 12.000 isolated compounds. Some isolated compounds were discovered as insecticides for example nicotine and caffeine were incorporated in drugs to fight against pain, fever, and flu symptoms (Wink, 2015). Some are central nervous system stimulants and others are addictive stimulants, such as morphine (Figure 2.4), ergotamine, cocaine, nicotine, atropine, codeine and caffeine (Doughari, 2012). Alkaloids are subdivided into subgroups that are associated with diverse pharmacological activities such antimicrobial, anticancer, anti-hypersensitive, antiviral and antimalarial (Agostini-Costa *et al.*, 2012; Russo *et al.*, 2013; Cushnie *et al.*, 2014; Kittakoop *et al.*, 2014;Wink, 2015). Kinghorn *et al.* (2003) stated that alkaloid compounds isolated from *Erythroxylum pervillei* extracts has the ability to reverse multidrug resistance.

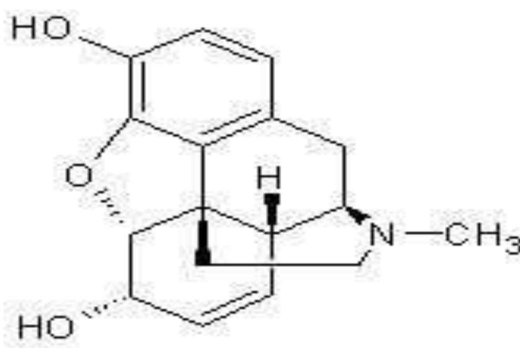


Figure 2.3: A morphine compound structure (Michael *et al.*, 2011)

2.2.1.4. Saponins

Saponins (Figure 2.5) are secondary metabolites that occur mostly in plants but can also be found in animals such as marine invertebrates (Francis *et al.*, 2002). They possess structural properties that are related to steroid or triterpenoid aglycone. Saponins have high molecular weight consisting of oligosaccharide units linked to steroids or triterpenoids by glycosidic linkage. These compounds are soluble in water and alcohol but insoluble in non-polar solvents. They possess foam properties that lead them to be used in cosmetics, some are added in shampoo, liquid detergents and toothpaste (Tanaka *et al.*, 1996).

Literature has showed that saponins are toxic to livestock and they have a bitter taste, Kar (2007) reported that they cause haemolysis of blood cells in cattle. Saponins have pharmacological effects such as antimicrobial, antitussives, anthelmintic, antidemertophytic and anti-inflammatory (Takagi *et al.*, 1980; Tamura *et al.*, 2001; Huang *et al.*, 2003; Sparg *et al.*, 2004). They also have anticancer and hypolopidemic activities. Some derivatives of saponins are used in the production of hormones (Doughari, 2007).

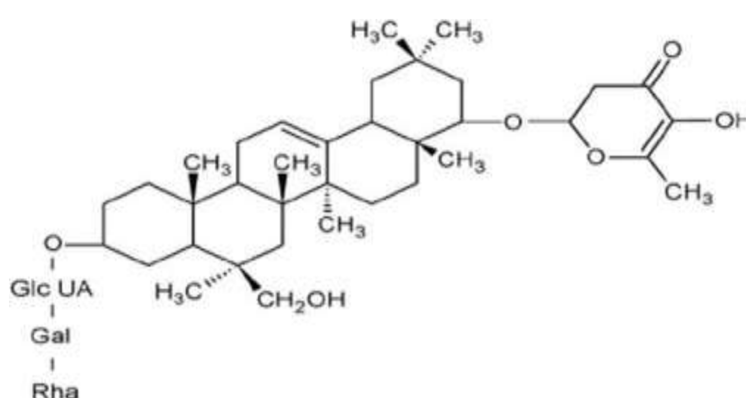


Figure 2.4: The structure of 2, 3-dihydro-2, 5-dihydroxy-4H-pyran-4-one saponin from peas (Reim and Rohn, 2014).

2.2.1.5. Tannins

Tannins are secondary metabolites referred to as plant polyphenols (Haslam 1989). They possess distinctive properties such as the ability to precipitate alkaloids, binding with protein, high molecular weight, soluble in water and alcohol, and they are acidic in reaction due to phenolic group on their structural formula; they give some red wine teas and unripe fruits bitter taste (Amarowicz, 2007).Tannins are detected in tea, coffee, bananas, chocolate, sorghum, grapes, spinach and red wine (Ghosh, 2015).

Tannins are divided in to two groups: the hydrolysed tannins produce gallic acid and allagic acid, and condensed tannins are flavonol-based compounds (Haslam, 1966; Amarowicz, 2007; Ashok and Updhyaya, 2012). Literature revealed that tannins possess therapeutic activities such as anti-inflammatory, anthelmintic, antimicrobial,

antiviral, anticancer and antioxidant activity (Chung *et al*,. 1998; Ketzis *et al*, 2006; Ferreira *et al*., 2008; Koleckar *et al*, 2008; Buzzini *et al*, 2008).

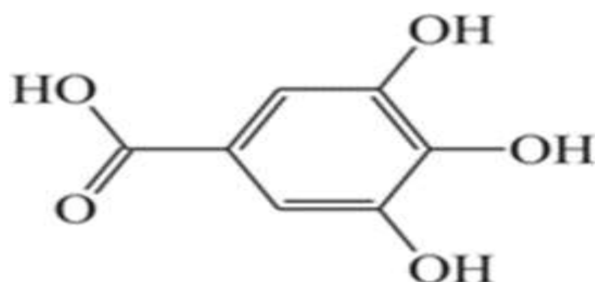


Figure 2.5: The structure of gallic acid compound of tannin (Lian *et al*., 2010).

**2.3. Interaction of plants compounds**

Plants produce compounds which plays a significant role in growth and production. Some of the compounds are useful in defence mechanisms. Microorganisms that attack plants over time turn to be resistant to defence by the plant, and plants that produce only a single type of defence compounds probably do not survive for a long period (Rasoanaivo *et al*., 2011). Secondary metabolites from plants may be present at all or certain seasons and in various plant organs. It has been discovered that phytochemicals from medicinal plants are of potential structural diverse bioactive compounds.

Pure drugs produced from plant compounds may be chosen for their high activity against human diseases. However, they have challenges such as not having an activity that is much better than of crude extracts at comparable concentrations (Wagner and Ulrich-Merzenich, 2008). This is due to an interaction of compounds within the crude extracts. Interaction of two compounds to accomplish an activity is referred to as synergistic interaction (synergy) and the phenomenon at which one compound inhibits the action of another is called antagonism reaction (Hemaiswarya *et al*., 2008; Rani *et al*., 2009). An example of antagonistic interaction, ginseng is a drug used for elevation of vital energy, when used in the presence of turnip its vital energy elevating activity would be reduced or eliminated (Che *et al*., 2013). Literature has shown the importance of combining antimicrobial agents from medicinal plants

and commercial antibiotics to enhance the antimicrobial activity against pathogens (Brinker, 2004; Betoni *et al*., 2006; Che *et al*., 2013).

### 2.4. Safety of medicinal plants

Medicinal plants produce secondary metabolites for specific functions to maintain their living environment. Some of metabolites are used in defence mechanism against pathogens, other plants and herbivores. The use of medicinal plants in developing countries is highly accepted because of better cultural acceptability and lesser side effects (Kamboj, 2000; Yadav and Dixit, 2008). Traditional healers and herbalists believe that medicinal plants have no side effects, whereas recent studies have shown that some plants are toxic. Some Medicinal plants are discovered to be poisonous, carcinogenic, and mutagenic, and some instead of combating the diseases they end up causing other diseases (Elgorashi et *al*., 2003; Haq, 2004).

The knowledge of identification, preparation and uses of medicinal plants is passed on orally from one generation to another. Medicinal plants and modern medicines often exist side by side and they do not often cooperate due to absence of scientific basis of medicinal plants (Addae-Mensah, 1992; Tylor *et al*., 2001). Therefore, scientific validation, standardisation and safety evaluation of traditional medicinal plants is of great importance (Tylor *et al*., 2001). Subratty *et al*. (2005) also mentioned that researchers are investigating scientific basis of traditional medicines to determine the potential bioactive compounds present in concoctions.

### 2.5. Bacterial resistance towards antibiotics

Diseases and other related ailments are unavoidable in human life and this has led humans to discover various ways of combating them. Antimycobacterial infections differ from others because mycobacteria tend to grow slowly in a condition that is nearly in a dormant state (Smith, 2004). Antimycobacterial infections are treated with several antimicrobial drugs that are losing their therapeutic power, drugs such as Isoniazid, Pyrazinamide, Ethambutol and Rifampicin (Smith, 2004). Morden medicines used in treatment of infections such as fever, gonorrhoea and tuberculosis are losing efficiency as microorganisms have developed resistance strains, high cost of prescribed drugs and increase in human population (levy, 1998; van de Bogaard *et al*., 2000; Lederberg *et al*., 2003).

Development of multidrug resistance by some bacterial strains and toxicity of synthetic drugs have revived the interest in the use of natural product such as medicinal plants. These challenges have placed more demand on using medicinal plants as an alternative way of overcoming the challenges of modern drugs. Medicinal plants have gained the attention of being used as an alternative due to less or no side effects, availability and low cost (Borokini and Omotayo, 2012). The importance of medicinal plants in therapeutic system is evident in the increasing presence of drugs derived from natural products. Cragg *et al*. (1997) have reported that about 75% of approved drugs have been derived from medicinal plants.

## 2.6. Therapeutic effects of medicinal plants

### **2.6.1.** Antioxidant activity

Oxygen is the most abundant element on earth. Dioxygen molecule that exists as air is of great significance to human and other living organisms excluding a small number of anaerobic bacteria. It is important in production of energy from food by the process called oxidation, and other mechanisms such as metabolic regulation, signal transduction, metabolic energy control and gene expression (Nunes *et al.,* 2012). Oxidative stress is a state in which the human body produces free radicals that exceed the amount of present antioxidants, resulting in oxidative stress. If those free radicals are not scavenged, they end up damaging bio-molecules such as lipids, proteins, mitochondria and DNA which induces chronic and degenerative diseases (Yoshikawa and Naito, 2002; Uddin *et al*., 2008). Significance of antioxidants is to protect cells against damage caused by reactive oxygen species such as super oxide, singlet oxygen, hydroxyl radical and peroxynitrite, and other free radicals (Kuhn, 2003; Mattson and Cheng, 2006). Free radicals are involved in chronic and degenerative diseases such as cardiovascular disorder, asthma, tumour inflammation, diabetes, neurodegenerative diseases and AIDS (Chen *et al*., 2006; Uddin *et al*., 2008).

Under normal conditions, the human body can eliminate or scavenge free radicals by the body's antioxidants defence mechanism such as catalyses (Sen, 1995). However, additional antioxidants from natural products such as plants are of great importance to protect against oxidative stress (Kasote *et al*., 2013). The increased interest in plants for their potential antioxidant activity was due to the discovery and subsequent isolation of ascorbic acid from plants (Szent-Giorgyi, 1963). Dietary antioxidants such

as vitamin C, vitamin E, flavones and β-carotene have been reported to have significant effects against free radicals (Madsen and Bertelsen, 1995; Reddy *et al*., 2007). Plants have a broad range of secondary metabolites that they use for their metabolisms. However, human and animals consume these metabolites to help their immune systems to fight against various diseases. Secondary metabolites such as phenolic acids, flavonoids, carotenoids, tannins and terpenoids have showed the antioxidant effects (Kasote *et al*., 2015).

Alam *et al*. (2013) indicated that there are about 19 *in vitro* and 10 *in vivo* methods which are used in accessing antioxidant activity of plant extracts. Some of the used methods are 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2, 2–diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power assay (FRAP). The presence of antioxidant compound is determined based on their mechanisms such as metal chelators, hydrogen donors or as reducing agents, this helps in categorising them as chain-breaking or preventive antioxidants (Miguel, 2010; Kasote, 2013).

### **2.6.2.** Antibacterial activity

Pharmaceutical companies have been manufacturing antibiotics which are the main treatment of various infections. In the past years, antibiotics started to lose their effectiveness due to microorganism that have the ability to acquire and transmit resistant genes, for them to survive in the presence of antibiotics (Nascimento *et al*., 2000; Sakagami and Kajimura, 2002). In some cases synthetic antibiotics are associated with the cause of adverse side effects such as allergic reactions, immune-suppression, hypersensitivity and toxicity (Ahmad *et al*., 1998). Most communities in developing countries are unable to access antibiotics due to poverty and unavailability of health centres near them. The search for new drugs from other sources such as medicinal plants, with less or no side effects, higher efficiency, affordable and readily available to the community was the way forward to overcome the challenge.

Many medicinal plants are used for treatment of diseases because of compounds they produce during secondary metabolism, of which about 12.000 have been isolated (Schultes, 1978). These compounds are the ones that inhibit or kill the microorganism's cells without harming the host cells in different mechanisms as compared to synthetic drugs (Fennell *et al*, 2004). Medicinal plants are used as

alternatives because communities believe that they are affordable to everyone, have less side effects, and are effective than synthetic drugs. Researchers have analysed various medicinal plants for their antimicrobial effects based on their traditional use confirmed by traditional healers and other herbalists. It was discovered that the compounds from plants have promising effects that can be used in the development of alternative antibiotics (Eloff 1998).

The importance of using the crude extracts was pointed out by Eloff and McGaw (2006) considering the compounds synergistic effects. Some of the secondary metabolites which are responsible for antimicrobial activity from plant extracts are flavonoids, Terpenoids, tannins and alkaloids (Silva and Femandes, 2010; Dai and Mumper, 2010). Researchers determine the presence of antibacterial activity from plant extracts using various methods such as dilution assays that include minimum inhibitory concentration method and bioautography.

#### **2.6.3.** Anti-inflammatory activity

When the body is exposed to injury, pathogens and other irritants, it triggers the release of hormones such as histamine, prostaglandin, serotonins and kinins resulting in inflammation. The current anti-inflammatory drugs are associated with gastric disorder, stomach pain, diarrhoea, nausea and other allergic reactions as side effects (Olsen *et al*., 2011). This imposes the need for development of new anti-inflammatory drugs with less or no side effects. Medicinal plants are a rich source of antioxidant compounds such as phenolic, alkaloids, flavonoids and vitamins which can be used in development of anti-inflammatory drugs. Phenolics and flavonoids compounds have been reported to inhibit the inflammatory process by regulating the production of pro-inflammatory molecule such as cytokine (TNF-α), nitric oxide and leukocyte adhesion (del Castillo *et al*., 2002; Cai *et al*., 2004; Yoon and Baek, 2005).

### **2.7. Methods of studying phytochemicals from medicinal plants**

Analysis of medicinal plants involves step-by-step processes including selection and collection of medicinal plants of interest, extraction, isolation of compounds, purification and characterisation of compounds. Medicinal plants are selected randomly or according to the knowledge of the traditional healer or indigenous people in the community (Martin, 1995). Through ethnobotany, researchers obtain information on the collection of the plant sample and their preparations. Medicinal plants can be

used either as whole extracts, fractions or pure compounds because they contain a wide range of phytochemical constituents which are deposited in various plant parts such as roots, stem, barks, leaves, flowers, seeds or fruits. Dried or fresh plant materials can be used for analysis of medicinal plants depending on the desire of the researcher.

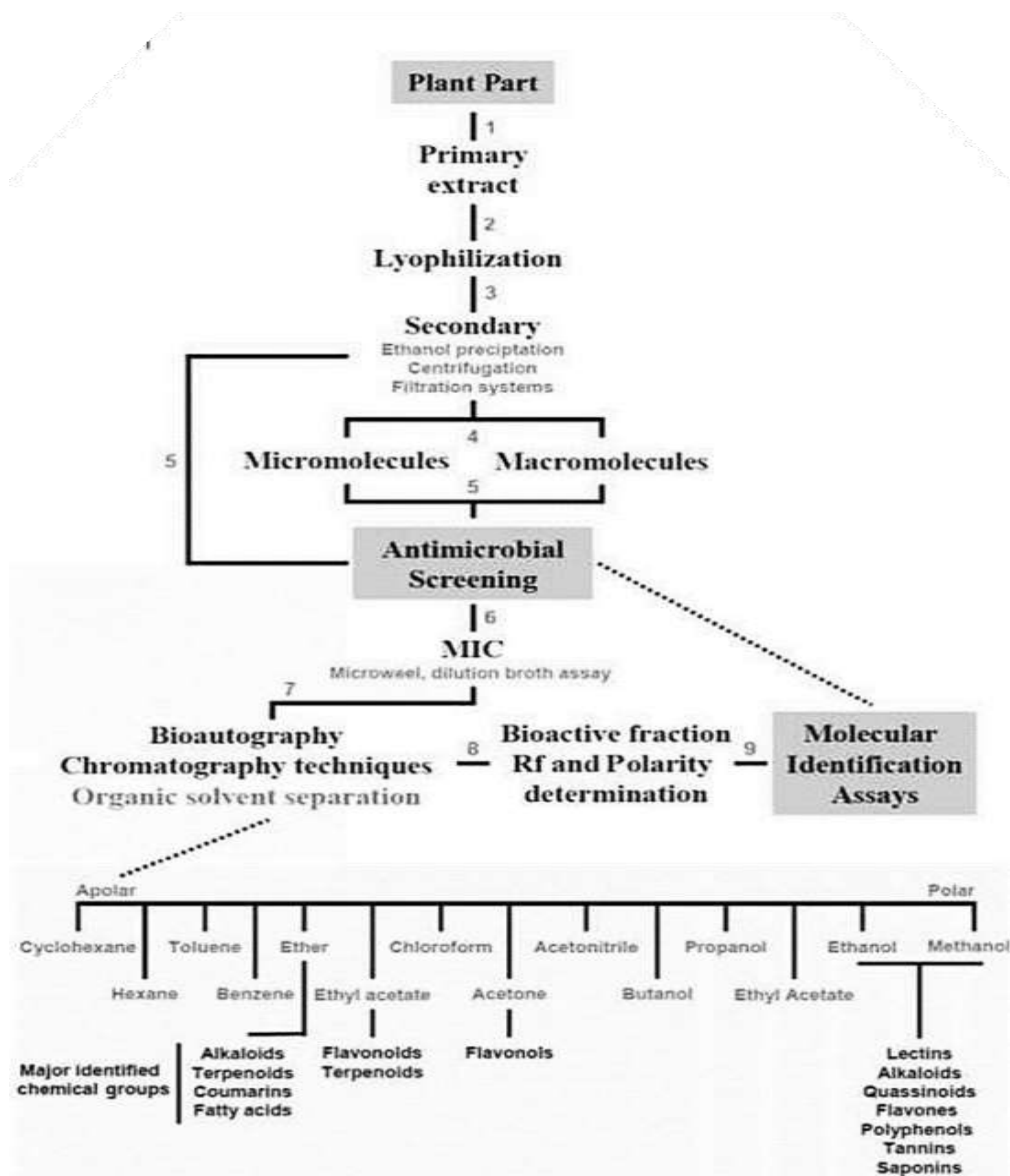


Figure 2.6: Flow chat of the processes which are followed when studying medicinal plants (Drugtimes, 2016).

### **2.7.1.** Extraction of phytochemicals

**2.7.2.** The important step in analysis of medicinal plants is extraction of the desired phytochemical constituents for identification and characterisation (Sasidharan *et al.*, 2011). Extraction is the separation of bioactive compounds from the plant materials with solvents of varying polarity (Handa *et al.*, 2008). There are different extraction procedures that researchers can choose to follow, namely decoction, digestion, infusion, maceration, percolation, hot continuous (Soxhlet), ultrasound extraction (sonication) and aqueous alcoholic extraction by fermentation (Handa *et al.*, 2008). In order to obtain high efficiency and efficacy, plant extracts depend on the type of extraction procedure, coupled with a good choice of extracting solvents and plant parts used (Gupta *et al.*, 2012).

The choice of solvent of varying polarities gives plant extracts a wide range of phytochemicals. Non-polar solvents extract non-polar compounds and polar compounds are extracted with polar solvents (Green, 2004). During extraction, it is important not to lose or destroy the potential bioactive compounds. This is important during the preparation of the sample, where a researcher might choose to wash the plant material, method of drying the sample which can be either freeze drying, oven drying or in the dark at room temperature, and the time exposure of sample to the solvents (Sasidharan *et al.*, 2011).

There are various methods that researchers use depending on the targeted compounds. The following are some of the commonly used extraction methods:

#### 2.7.2.1. Solvent extraction

Solvent extraction is a procedure in which solid plant materials come in contact with a solvent and soluble compounds move in to solvents, in a concentration gradient (Handa *et al.*, 2008). Thus the rate at which compounds are transferred in the solvent decreases as the concentration of compounds increase in the solvent until equilibrium is reached. Different extractants can be employed in this procedure to extract various phytochemical constituents (Doughari, 2012). This is based on the polarity of the solvent and compounds from plant material (Green, 2004). Heating the solvent can enhance the transfer of compounds to solvent and replacing the solvent with the fresh one change the concentration gradient to dissolve more compounds. This gives rise to more extraction procedures such as hot or cold percolation and concentration (Handa *et al.*, 2008).

#### 2.7.2.2. Soxhlet extraction (hot continuous)

In this procedure, a finely grounded sample is heated with solvent in a soxhlet apparatus that is connected to a condenser (Handa *et al.*, 2008). Soxhlet extraction cannot be used for thermolabile compounds as heating for some time may lead in degradation of the compounds (de Paira *et al.*, 2004). However, Gurib-Fakim (2006) mentioned that prolonged heating gives an advantage of extracting a number of compounds and it is suitable for insoluble materials such as waxes. The advantage of this procedure is that it requires a small amount of sample and solvent, time saving and inexpensive. It is considered a good extraction procedure even though some of the compounds are destroyed in the process (Gurib-Fakim, 2006; Handa *et al.*, 2008).

#### 2.7.2.3. Sonication extraction procedure

In sonication procedure, an ultrasound of wavelength ranging from 20-2000 kHz is used to facilitate the extraction of compounds from samples (Handa *et al.*, 2008). The applied ultrasound increases the surface contact of solvent and sample, where the plant cell walls are altered and disrupted leading to an increase release of compounds (Dhanani *et al.*, 2013). This method was considered an effective procedure when used in propolis extraction where high yield, less extraction time and high selectivity were observed (Trusheva *et al.*, 2007). Literature has showed the effectiveness of this procedure in extraction of thermolabile compounds from flowers (Ebrahim *et al.*, 2014).

## 2.8. Separation and purification of bioactive compounds

Separation of plant extracts is a challenge since the extracts contain a mixture of various compounds. Medicinal plants possess hundreds of unknown mixture of compounds and many of them are in low amount (Doughari, 2012). Therefore, it is important to obtain the phytochemical fingerprints of bioactive phytochemicals present in medicinal plants. Compounds that have antibacterial and antioxidant activity or other therapeutic effects are separated and purified with chromatographic techniques.

Thin layer chromatography, paper chromatography, column chromatography, Sephadex chromatography and high pressure liquid chromatography can be used to obtain pure compounds depending on the property, solubility and volatility of the compounds to be separated (Harbone, 1998).

These methods have helped in the separation of compounds for the past five decades. Pure compounds help in the development of new drugs and their structural formula have to be known. This is determined with the use of spectroscopic techniques, where the interaction of electromagnetic radiation with matter is analysed. Nuclear Magnetic Resonance (NMR) spectroscopy and Mass spectrometry (MS) are the methods that chemist use in the study of chemical structure of molecular samples. NMR spectroscopy analyses the interaction of radio frequency of electron magnetic resonance (EMR) with unpaired nuclear spins to determine structural information of a given sample. In mass spectroscopy pure sample is analysed based on the mass-to-charge ratio of ionised atoms to separate them providing structural information (Kanu *et al*., 2008).

### 2.8.1. Thin layer chromatography

In the evaluation of phytochemicals from medicinal plants, TLC is the best technique that is used because of its advantages in simplifying research. It enables rapid analysis of plant extracts with less sample clean-up requirement and it gives qualitative, semi-quantitative information of separated compounds and it is inexpensive and save time (Liang *et al*., 2004; Doughari, 2012). In the analysis of medicinal plants, the TLC plates are either glass or aluminium coated with silica gel as a stationary phase. This technique is sensitive in such a way that it allows researchers to analyse the smallest amount of sample in a short time.

The process of analysing samples on TLC require a dilution of sample with an easy evaporating solvent, the sample will be spotted at around 1-2 cm from the bottom of the TLC plate. The spotted plate is placed in a chamber with mobile phase that assist in separation of compound present in the sample. Separated chromatograms are either visualised by spraying them with reagents or viewed under ultraviolet light. TLC analysis on silica gel has been used to compare phytochemicals of South African medicinal plants (Eloff, 2001; Kotze and Eloff, 2002).

### 2.8.2. High performance liquid chromatography

High performance liquid chromatography (HPLC) is a widely used method in isolation of natural products (Cannell, 1998). This method has gained the popularity in the analysis of components from natural products (Fan *et al.,* 2006). HPLC is commonly used in separation of volatile compounds such as higher terpenoids, phenolics,

alkaloids, lipids and sugar and for compounds that are easily detected using ultraviolet light (Harbone, 1998). It is a technique that involves the use of a column to hold the stationary phase (commonly silica gel), a pump that rotates liquid mobile phase to elute compounds and a detector that monitors the compounds as they elute off the column and also a fraction collector (Harbone,1998).

This method is considered the most significant and versatile method for qualitative analysis of natural products. Purification of compounds in HPLC is a process in which a targeted compound is separated or extracted from other compounds, this is based on the characteristic peak of compounds under certain chromatographic conditions. Identification of compounds with HPLC requires a detector that is properly set. The identified compound should show a peak that has a reasonable retention time. Apart from expensiveness of HPLC apparatus, this technique seems to be unable to detect non-chromophore compounds from medicinal plants (Liang *et al.*, 2004).

### 2.8.3. Column chromatography

Column chromatography is a method used in separation of individual compounds from a mixture of compounds. In this technique a glass with a diameter of about 50 mm and a height ranging between 5 cm to 1 m, with a tap at the bottom is a significant apparatus, since the mobile phase together with stationary phase are carefully loaded in to the glass and eluents are collected as fractions. Column chromatography is commonly used for preparative application because it can purify small amount of compounds, it is of low cost and the stationary phase is easily discarded after the process.

## 2.9. Bioassays for antimicrobial activity

Owing to the impact of drug resistance by microorganism, a better understanding of current methods used in evaluation of antimicrobial effects of plant extracts or pure compounds is significantly required for application in human health, agriculture and environment (Balouiri *et al*., 2016). Various methods can be employed in the evaluation of antimicrobial activity of plant extracts or pure compounds. The most commonly used methods are disk diffusion and broth or agar dilution methods. The following assays are commonly used in evaluation of antimicrobial effects of plant extracts:

### 2.9.1. Diffusion methods

#### 2.9.1.1. Agar disc diffusion assay

Agar disk diffusion method was invented in 1940 and it is still used in many clinical microbiology laboratories (Balouiri *et al*., 2016). Evaluation of bacterial resistance to antimicrobial compounds from plant extracts can be evaluated with agar diffusion assay. In this procedure, a disc of about 6 mm saturated with extracts of different concentrations is placed in an agar plate inoculated with the test microorganism. The antimicrobial compounds from plant extracts migrate or diffuse into the agar and inhibit the growth of tested microorganism. The effects of plant extracts is observed by the presence of clear zones called zones of inhibition (Figure 2.8). This procedure is similar to the E-test method were stripes are employed instead of discs (Kelly *et al*., 1999; Davies *et al*., 2000). The assay has a challenge of not testing for fastidious bacteria, but with standardisation, using specific culture media and different incubation conditions. This has made it easy to test for fastidious bacterial pathogens such as *Haemophilus influenza, streptococci, Neisseria gonorrhoeae and Neisseria meningitides* (Jacovides *et al*., 2012).

Figure 2.7: A Petri dish showing zones of inhibition formed by the antimicrobial compounds from plant extract (Sahu *et al*., 2013).

#### 2.9.1.2. Agar well diffusion assay

Agar well diffusion assay is a method that is commonly used to determine the antimicrobial activity of plant extracts or pure compounds (Magaldi *et al*., 2004). Agar well diffusion method is similar to disk diffusion method; the only difference is on the way extracts are loaded. The agar plate is seeded with the test strain over the entire

agar surface. Thereafter, holes with a diameter of about 6 to 8 mm are aseptically punched with a sterile cork-borer of a tip, and a volume of 20 to 200 microliter at desired concentrations is loaded in to the wells. Diffusion methods only provide a qualitative results by providing information on whether the bacteria is susceptible, intermediate or resistant to antimicrobial agents (Reller *et al.*, 2009). The observed inhibition zones of bacterial growth do not mean the death of bacteria. Therefore, this method cannot differentiate between bactericidal and bacteriostatic effects (Balouiri *et al*, 2016). Furthermore, for some microorganisms and antibiotics, a minimum inhibitory concentration (MIC) can be determined by comparing the inhibition zones with the known algorithms. This means that determination of MIC with diffusion methods is a challenge since it is difficult to determine the amount of antimicrobial compounds that diffused in to the agar (Balouiri *et al.*, 2016).

### 2.9.2. Dilution assays

Agar or broth dilution assays are the best method in determination of minimum inhibition concentration of antimicrobial agents from plant extracts.

#### 2.9.2.1. Broth dilution method

Broth dilution is one of the basic antimicrobial susceptibility evaluating method. Plants extracts obtained with various solvents are reconstituted to a certain concentration that is always known before serial dilution is performed. Researchers such as Eloff (1998) have been using microtiter plates in detection of antimicrobial activity from plant extracts. The use of microtiter plates involves serial dilution of plant extracts with water before the bacterial culture is added on each well. Positive and negative controls are also included. The plate is incubated at the required condition and there after a detecting reagent such asp-iodonitrotetrazolium violet (INT) is added to indicate the inhibition of bacterial growth, this is where the MIC value is determined. Bacteria convert the salt into formazan (pink colour); this shows the growth of bacteria in the wells, the inhibition of bacterial by plant extracts is represented by clear wells (Figure 2.8).

Figure 2.8: The broth microdilution method of plant extracts (Balouiri *et al.*, 2016).

2.9.2.2. Agar dilution method

Agar dilution method involves the incorporation of various concentrations of plant extracts into an agar medium using serial two-fold dilutions, followed by seeding the bacterial strain onto the agar plate surface (Grierson and Afolayan, 1999). The minimum inhibition concentration is determined by the lowest concentration of plant extract to inhibit the bacterial growth under suitable incubation condition. Agar dilution standardised method was considered a good method for fastidious pathogens.

### 2.9.3. Bioautography

Bioautography assay is a technique used to determine antimicrobial activity of bioactive compound from plant extracts. This technique can be combined with other chromatographic techniques such as planar electro-chromatography, high performance thin layer chromatography and thin layer chromatography (Choma and Grzelak, 2011). TLC-Bioautography assay combine chromatographic separation of plant compounds and localisation of antimicrobial compounds from the plant extracts (Shahverdi *et al.*, 2007). There are three ways in which bioautography can be approached: (a) Direct bioautography, where microorganisms are grown directly on TLC chromatograms in a humidified chamber, (b) Agar overlay bioautography, where an inoculated agar medium is applied directly on TLC plates, (c) Contact bioautography, involves the transfer of antimicrobial compounds from TLC plate to the inoculated agar plate (Hamburger and Cordell, 1987; Rahalison *et al.*, 1991).

### *2.10.* Selected plant for this study: *Schkuhria pinnata*

#### 2.10.1. Description

*Schkuhria pinnata* (*S. pinnata*) is a herbaceous, exotic plant that belong to the family *Asteraceae.* The family *Asteraceae* is a broad family of up to 25000 species and 14000 genera. The species within this family have distinctive phytochemicals that differentiates them. Perennial taxa contain polyfructanes, some contain sesquiterpene lactones that have been reported to be present in *Schkuhria pinnata*, and some taxa contain pyrrolizidine alkaloids while some have diterpenoids (Gurib-Fakin, 2006). It is commonly known as "*sebabane"* by Sepedi speaking people, and "*luswielo*" by Venda tribe and Dwarf marigold in English (Mupfure *et al.*, 2014; Masevhe *et al.*, 2012). It is a plant that originates from South America and it was first recorded in South Africa in 1898. *S. pinnata* was recorded to grow in mountainous and valley regions in South America and in African countries namely, Zimbabwe and South Africa, it grows in cultivated lands, along roadsides and disturbed ground like fields (Grabandt, 1985; Taylor, 2006; GRIN, 2012). Dwarf marigold is a plant that can grow to 60 cm by height with deep and finely separated leaves. It is an annual plant with flowers flat-topped inflorescences and is borne in a branched manner where the disc and ray florets are yellow (Deutschlander *et al.*, 2009).

#### 2.10.2. Medicinal uses

*S. pinnata* (Figure 2.9) has been used for medicinal purposes in the treatment of various diseases that affect human and animals. *S. pinnata* is used by traditional healers to treat tuberculosis related symptoms (Taylor, 2006). Traditional healers have long used this plant species as an effective blood cleanser. Usually the entire plant is uprooted and chopped up and brewed into an infusion (fresh plant) or a decoction (dried plant). Many types of skin problems, including eczema, dermatitis, and acne, black heads, are believed to be caused by toxins and partially digested bacteria circulating in the bloodstream, and this plant remedies (infusion or decantation) are used as a natural remedy for other types of skin conditions as well (Bussman *et al.*, 2008). It is also regarded as an anti-inflammatory, digestive, antitussive (stops coughing), capillary tonic, diuretic, and hypoglycemic (Taylor, 2006).

This plant has been employed as a herbal remedy for kidney, liver, and renal problems, malaria, diabetes, allergies, yeast infections, prostate inflammation, digestive

disorders and intestinal gas (Deutschlander *et al*., 2009; Njoroge *et al*., 2004). The plant has been reported to have therapeutic effects on livestock in treatment of eye infections, pneumonia, heart water, diarrhoea, wound infections and retained placenta (van der Merwe *et al*., 2001; Luseba *et al*., 2001). It was confirmed by Mupfure *et al*. (2014) that extracts of *S. pinnata* are effective against mastitis pathogens in dairy cattle.

Figure 2.9: The image of *S. pinnata* (www.zimbabweflora.co.zw).

**2.11. Microorganism used for the study (*Mycobacterium smegmatis*)**

*Mycobacterium smegmatis (M. smegmatis)* is a Gram positive and acid fast bacterium that falls under Mycobacteriaceae family, which includes *Mycobacterium tuberculosis*, *Mycobacterium fortuitum, Mycobacterium abscessus* and *Mycobacterium chelonae.* These bacteria are resistant against many antibiotics due to their protective outer layer that have specific resistant determinants (Nash, 2003). *M. smegmatis* and *M. fortuitum* are pathogens associated with wounds or lung infections (Pierre-Audigier *et al*., 1997; Brown-Elliot and Wallace, 2002; Wagner and young, 2004). *M. smegmatis* survives in aggregated layers of community called biofilm and they are commonly found on plants, soil and in water (Tsukamara, 1976). They are considered pathogenic and non-

pathogenic to human and animals under certain conditions (Shepard, 1957; Falcone *et al.*, 1994; Barker *et al.*, 1996; Ramakrishnan *et al.*, 1997).

Screening for antimycobacterial activity against *M. tuberculosis* is usually carried out using *M. smegmatis* because it is a fast growing and non-pathogenic strain (Newton *et al*, 2000). *M. smegmatis* causes diseases such as skin, soft tissue and bone infections (Best and Best, 2009). During vascular shunts, renal replacement therapy and cardiac therapy, contamination of materials and invasive procedures lead to transmission of *M. smegmatis* and results in infections (Brown-Elliot and Wallace, 2002; Wagner and Young, 2004). Literature has shown that this microorganism contribute in mastitis infection, wound infections, breast abscesses, endocarditis, lymphadenitis, cellulitis as well as aspiration pneumonia (Talaat *et al.*, 1998). Best and Best (2009) reported that *M. smegmatis* strain is resistant to isoniazid and rifampicin, two widely used antibiotics for treatment of tuberculosis.

Other treatments of infections resulting from *M. smegmatis* infection include prolonged antibiotics therapies which are toxic to the patients and surgical, debridement, which are expensive to poor rural people (Best and Best, 2009). Because the antibiotics and therapy are expensive and bacteria are resistant to the above mentioned drugs, alternative ways of developing drugs that everyone can afford, has led researchers to search for solutions using medicinal plants that are readily available to the community.

## 2.12. Aim and Objectives

### 2.12.1. Aim

The aim of the study was to isolate and characterise antimycobacterial compounds from *S. pinnata*

### 2.12.2. Objectives

The specific objectives of the study were to:

i. Evaluate the phytochemical constituents in *S. pinnata* using Thin Layer Chromatography (TLC).
ii. Determine the presence of antioxidant compounds in the *S. pinnata* using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and quantitative total antioxidant activity assay using ferric ion reducing antioxidant power (FRAP) procedure.

iii. Evaluate the antimycobacterial activity of crude extracts in *S. pinnata* using minimum inhibitory concentration assay and bioautographic assay.
iv. Isolate bioactive compounds with antimycobacterial activity using column chromatography assay and preparative TLC.
v. Analyse the effects of the crude extracts and isolated compounds for cytotoxicity on Vero monkey kidney cells using MTT assay and anti-inflammatory on Raw 264.7 macrophage cells.
vi. Characterise isolated compound from *S. pinnata* spectroscopy.

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# Chapter 3

## 3. Extraction and Phytochemical screening

### 3.1. Introduction

Medicinal plants contain bioactive compounds that are of importance for the treatment of various diseases. It is therefore important to extract these phytochemical constituents contained in them for evaluation in various tests to earnest their medicinal potential. Analysis of medicinal plants requires one to extract plant materials for further separation, purification and characterisation of the desired bioactive compounds (Evans 2008). The extraction process involves separation of bioactive compounds from plant materials by using selected solvents and extraction procedures (Gupta *et al*., 2012). It is an essential step in the analysis of plant phytochemicals (Jadhav *et al.,* 2009). This process include steps such as sample collection, pre-washing, drying and grinding of plant material, filtration, and reconstitution of plant extracts (Handa *et al.,* 2008).

Extraction of bioactive compounds from plant samples can be conducted with various procedures such as decoction, digestion, infusion, maceration, percolation, hot continuous (soxhlet), ultrasound extraction (sonication) and aqueous alcoholic extraction by fermentation (Handa *et al*., 2008; Das *et al*., 2010). There are a number of properties that determine a good solvent which include, the ease of evaporation at low temperature, have low toxicity and unable to cause plant extracts to complex or dissociate and those that promote rapid physiologic absorption of extracts, and do not interfere with ease of bioassays preparative actions (Hughes, 2002; Pandey and Tripathi, 2014). Various solvents with varying polarities are used in extraction, to obtain a wide range of phytochemical compounds from plants sample, thus non-polar solvent, extract non-polar compounds and polar compounds are extracted by polar solvent. Some of the solvents used in extraction procedure are hexane, water, ethanol, chloroform, ethyl acetate and methanol to obtain various bioactive compounds. To sustain the potential activity of the bioactive compounds extracted from plant material depends on the polarity of solvents, plant material size, temperature, pressure, time of plant material exposure to solvents, plant part used, solvent volume and extraction procedure (Sasidharan *et al.,* 2011; Gupta *et al.,* 2012).

Phytochemical screening of plant extracts and fraction can be conducted using Standard phytochemical methods. These are methods that validate the presence of phytochemical constituents from plant extracts and fractions. The methods described by Harborne (1973), Odebiyi and Sofowora (1978), Trease and Evans (1989), as well as Borokini and Omotayo (2012), can be used to determine the presence of compounds such as alkaloids, flavonoids, tannins, saponins and terpernoids. These methods determine the type of bioactive compounds targeted for isolation.

Thin layer chromatography (TLC) is a chromatographic technique that is considered to be affordable, simple and quick in giving researchers answers on how many components are presents in a mixture like plant extracts (Sasidharan *et al.,* 2011; Doughari, 2012 ). Thin layer chromatography is reported to be fast and effective in analysis of plant extract's phytochemical profiles, and it can separate small amount of plant extract (Harborne, 1998; McGaw *et al*., 2002). Known compounds and unknown compounds can also be compared through their retardation factor (Rf) values using this technique. Phytochemical constituents in plant extracts can be detected on a TLC plate where a reagent such as vanillin-sulphuric acid is sprayed on the chromatogram, which results in colour change of the compounds that reacted with the reagent to show their presence. The same TLC chromatograms can be viewed under different wavelength of ultraviolet light to detect the presence of fluorescing phytochemicals. The isolation of bioactive compounds is also validated by using this technique, where the compounds are identified and purified through TLC chromatograms. This chapter thus highlights the various extraction and phytochemical screening procedure employed in this study.

## 3.2. Methods and materials

### 3.2.1. Plant collection and storage

The S. *pinnata* plant was collected from the University of Limpopo, South Africa. A voucher specimen (UNIN 12298) of the plant material was authenticated by Dr B Egan a curator from the Larry Leach Herbarium (UNIN). The plant material was dried at room temperature for two weeks. The dried plant material was ground into fine powder using Sundy Hammer Crusher (SDHC150) and stored in a dark place in an air tight glass container.

### 3.2.2. Extraction procedures

In this study different extraction procedures were used.

#### 3.2.2.1. Preliminary extraction

The plant material was extracted by weighing 1 g of fine powder in different polyester centrifuge tubes. A volume of 10 ml of the following solvents namely: n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol and water were used to extract the plant materials. The tubes were shaken for ten minutes at 200 rpm in series 25 shaking machine (New Brunswick scientific. co., INC). The extracts were filtered in pre-weighed labelled bottles. This process was repeated three times to exhaustively extract the compounds and the extracts were combined. The extractants were removed under a stream of air at room temperature using a fan before dissolving extracts in acetone to a final concentration of 10 mg/ml.

#### 3.2.2.2. Serial exhaustive extraction

Finely ground plant material was exhaustively extracted by weighing 5 g of the powdered sample and extracted with 50 ml of n-hexane. The bottle was shaken for one hour at 200 rmp in series 25 shaking machine (New Brunswick scientific. co., INC). The supernatant was filtered in to pre-weighed bottles and the process was repeated three times. Thereafter, plant extracts were combined. The same procedure was followed on the same plant residues using 50 ml of different solvents namely: chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol, to exhaustively extract compound of varying polarities. The solvents were removed under a stream of air at room temperature and plant extracts were reconstituted with acetone to give a final concentration of 10 mg/ml.

#### 3.2.2.3. Extraction enrichment procedure

Two path ways were used in this procedure. The first involved the pretreatment of powdered plant materials with hexane (n-hexane wash), and extracted with n-hexane, acetone and ethanol. In the second method, the plant materials were extracted with different percentage (20%, 40%, 60%, and 80%) of acetone and ethanol in water. One gram of powdered plant material was weighed in polyester centrifuge tubes and extracted with 10 ml of the solvents. The tubes were vigorously shaken for 10 minutes. The supernatant were filtered in pre-weighed bottles and the extractants were

removed under the stream of air at room temperature. Plant extracts were reconstituted with acetone to a final concentration of 10 mg/ml.

n-Hexane wash

Finely ground plant material was pretreated by weighing 1 g of powdered sample and extracted with 10 ml of n-hexane; the same plant residues were dried and extracted with acetone and ethanol.

Acetone and ethanol in water.

Ten millilitres of each of the following solvents were used to extract 1 g of powdered plant materials: acetone, ethanol, water and 20%, 40%, 60%, 80% acetone and ethanol in water. Solvents were evaporated under a stream of air at room temperature. The final plant extracts were dissolved in acetone to give a concentration of 10 mg/ml.

#### 3.2.2.4. Optimal extraction

Extraction of plant material with acetone and ethanol was performed after three pre-treatment methods. One gram of powdered sample was extracted with 10 ml of each solvent (Table 1).

Table 3.1: The solvents used for pre-treatment and extraction methods.

| Pre-treatment | Extraction |
|---|---|
| n-Hexane "wash" | Acetone and ethanol extract. |
| 20% acetone and ethanol in water "wash" | 80% acetone and ethanol in water extract. |
| n-Hexane "wash" followed by 20% acetone and 20 % ethanol in water "wash" | Acetone and ethanol extract |

3.2.2.5. Preliminary Serial exhaustive extraction

3.2.2.5.1. Series 1, 2 and 3

Preliminary serial exhaustive extraction of 10 g plant materials was performed in three series with 100 ml of solvents of varying polarities. (a) Series 1: n-Hexane, dichloromethane, ethyl acetate, acetone and methanol. (b) Series 2: n-Hexane, ethyl acetate, acetone and methanol. (c) Series 3: n-Hexane, acetone and methanol. Extractants were removed under a stream of air at room temperature. Final extracts were reconstituted with acetone to give a concentration of 10 mg/ml.

### 3.2.3. Phytochemical constituent's profiles

Plant extracts of each solvent was reconstituted with acetone to a concentration of 10 mg/ml. An amount of 10 µl of each extracts was loaded at the bottom of the aluminium-baked TLC plates. The plates were placed in saturated solvent tanks containing three solvent systems namely: benzene/ethanol/ammonia hydroxide (BEA) (non-polar/basic) (18:2:0.2), chloroform/ethyl acetate/formic acid (CEF) (intermediate polarity/acidic) (10:8:2), ethyl acetate/methanol/water (EMW) (polar/neutral) (10:5.4:4) and Butanol/Acetic acid/Water [BAW] (polar) (4:1:5) (Kotze and Eloff, 2002). Thereafter, separated TLC plates were viewed under ultraviolet light. The plates were sprayed with vanillin sulphuric reagent (0.1 g vanillin (Sigma ®): 28 ml methanol: 1 ml concentrated sulphuric acid) and heated at 110°C for about a minute for colour development.

### 3.2.4. Phytochemical screening using chemical tests

3.2.4.1. Alkaloids

The presence of alkaloids was tested following a method described by Harborne (1973). An amount of 0.2 g of plant sample was weighed and extracted with 95% ethanol. Extracts were evaporated under a stream of air at room temperature. The plant extract was dissolved in 5 ml of 1% hydrochloric acid and 5 drops of drangendoff''s reagent was added. The formation of a reddish-brown inference indicated the presence of alkaloids in *S. pinnata* extracts.

3.2.4.2. Saponins

The Froth test was used whereby 1 g of plant sample was mixed with 30 ml of tap water in a test tube. The tube was vigorously shaken and heated at 100ºC. Formation of persistent froth indicated the presence of saponins in the plant sample (Odebiyi and Safowora, 1978).

3.2.4.3. Flavonoids

Aqueous extracts of *S. pinnata* was tested for the presence of flavonoids by adding 5 ml of diluted ammonia solution, followed by the addition of 1 ml of concentrated sulphuric acid. The sample was observed for a yellow colour that disappears on standing, indicating the presence of flavonoids (Borokini and Omotayo, 2012).

3.2.4.4. Tannins

The presence of tannins was determined by weighing 0.5 g of *S. pinnata* material in a test tube, and boiled with 5 ml of distilled water. The test tube was cooled and the mixture was filtered in to a clean test tube. A few drops of 0.1% ferric chloride was added to 1ml of the filtrate. Formation of brownish green or a blue-black colouration showed the presence of tannins (Trease and Evans, 1978).

3.2.4.5. Terpenoids

The ethanol extracts were obtained by extraction of *S. pinnata* material with ethanol. Thereafter, 0.5 g of extracts was dissolved in 2 ml of chloroform and 3 ml of concentrated sulphuric acid was cautiously added to form a layer. The sample was observed for a reddish brown colouration to draw interface which indicates the presence of terpenoids (Borokini and Omotayo, 2012).

3.2.4.6. Cardiac glycosides

Keller-Killiant test was used whereby 0.5 g of acetone extracts was dissolved in 5 ml of distilled water. A solution of glacial acetic acid 2 ml, with one drop of 0.1% ferric chloride was added to the dissolved extracts. Thereafter, 1 ml of concentrated sulphuric acid was carefully added and the formation of a brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides (Borokini and Omotayo, 2012).

#### 3.2.4.6.1. Steroids

Acetone extracts was weighed to an amount of 0.5 g into a test tube. Thereafter, 2 ml of acetic anhydride and 2 ml of sulphuric acid was added. The sample was observed for the blue or green colour change to draw inference, depicting the presence of steroids (Borokini and Omotayo, 2012)

## 3.2.5. Determination of total phenolic, flavonoids and tannin content

### 3.2.5.1. Total phenolic content

Total phenolic content from *S. pinnata* extracts was determined spectrophotometrically using a method described by Singleton *et al.,* (1999). Follin-ciocalteu method was used in determination of the total phenolic content from 70% aqueous acetone extracts. One millilitre of plant extracts was mixed with 9 ml of distilled water in a 25 ml volumetric flask. Thereafter, 1 ml of follin-ciocaltau phenol reagent was added to the mixture and it was well shaken. After 5 minutes, 1 ml of 7% sodium carbonate ($Na_2CO_3$) solution was added to the mixture and distilled water was added to make a final volume of 25 ml. Gallic acid (0.0625, 0.125, 0.25 and 1 mg/ml) was obtained using the same procedure used for plant mixture. The extracts and gallic acid mixture were allowed to stand for 90 minutes at room temperature in the dark. The absorbance of the mixtures was spectrophotometrically recorded at 550 nm. The total phenolic content was determined using linear regression from a gallic acid calibration standard curve.

### 3.2.5.2 Total flavonoid content

The aluminium chloride colorimetric method was used for determination of total flavonoid content from 70% aqueous acetone extracts (Tambe and Bhamber, 2014). One millilitre of plant extracts was added to 4 ml of distilled water in a 10 ml volumetric flask. Thereafter, 0.3 ml of 10% aluminium chloride was added to the mixture. After 5 minutes, 2 ml of 1 M sodium hydroxide was added and the flask was filled with distilled water to make a final volume of 10 ml. A set of reference standard mixtures of quercetin at various concentrations was prepared in the same manner as the extracts. The absorbance of the mixtures was recorded against the reagent blank at 510 nm using UV/visible spectrophotometer. The total flavonoid content was determined by linear regression from a quercetin calibration standard curve.

3.2.5.3. Total tannin content

Follin-ciocalteu method was used for determination of total tannin content in 70% aqueous acetone extracts. In a 10ml volumetric flask, 0.1 ml of plant extract was added to 7.5 ml of distilled water, 0.5 ml of follin-ciocalteu phenol reagent and 1 ml of 35 % $Na_2CO_3$ solution. Thereafter, the flask was filled with distilled water to make a final volume of 10 ml. The mixture was well shaken and allowed to stand for 30 minutes at room temperature. A set of reference standard solutions of gallic acid at various concentrations were prepared in the same manner as described above. The absorbance of the mixtures were recorded against the blank at 725 nm with UV/visible spectrophotometer. The tannin content was determined by linear regression from a gallic acid calibration standard curve (Tambe and Bhamber 2014).

## 3.3. Results

### 3.3.1. Preliminary extraction

The amount of plant extracts obtained in preliminary extraction was measured in milligrams (mg) as shown in figure 3.1. Chloroform (162 mg) extracted the largest amount of plant material followed by dichloromethane (153 mg), methanol and n-hexane (66 mg) the least. Phytochemical constituents from the crude extracts was analysed using aluminium-backed TLC plates which were developed in solvent systems of different polarity (BEA, CEF and EMW) and sprayed with vanillin-sulphuric acid reagent for colour development. Solvent system CEF, followed by BEA separated more bands of phytochemical constituents which reacted with vanillin-sulphuric reagent while EMW separated fewer bands (Figure 3.2).

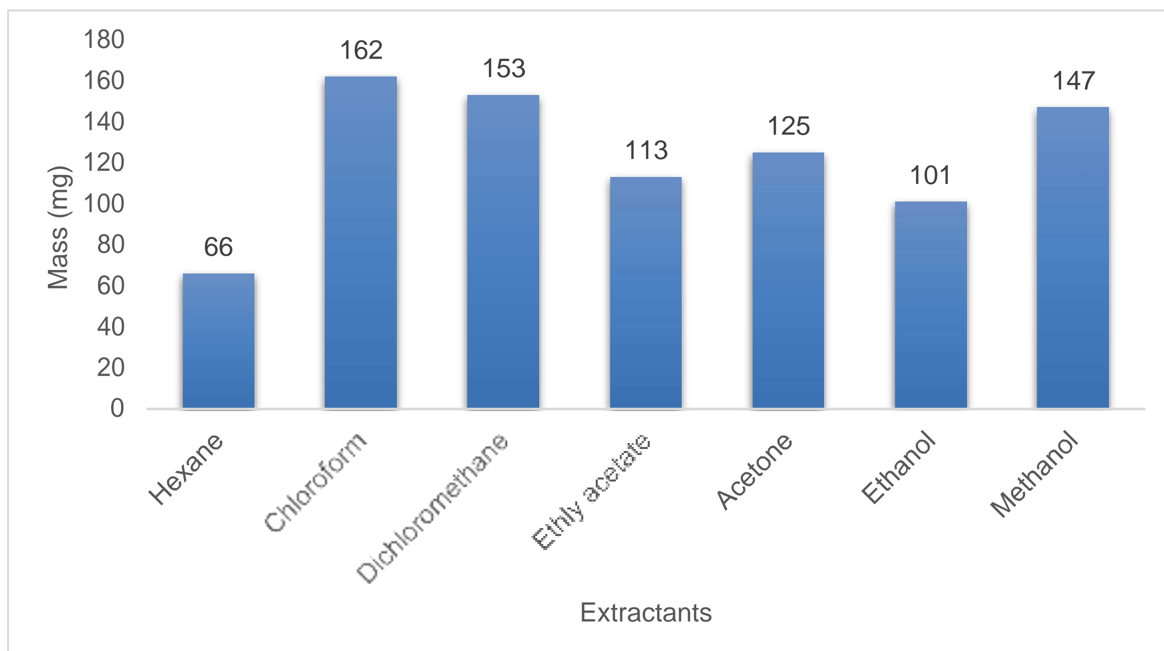


Figure 3.1: Mass of sample extracted with solvents of varying polarity, namely n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol.

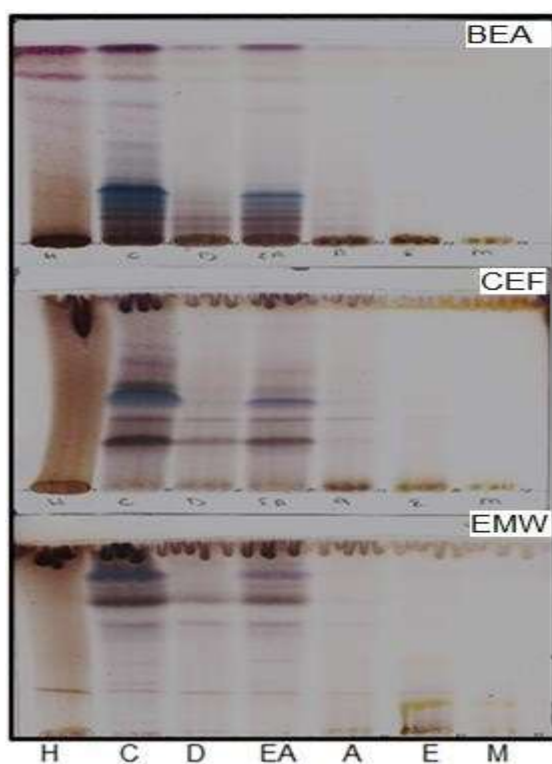


Figure 3.2: Phytochemical fingerprint of *S. pinnata* extracts separated in solvent system BEA, CEF and EMW, and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E) and methanol (M).

### 3.3.2. Serial exhaustive extraction

In order to obtain phytochemical constituents from *S. pinnata*, the sample was exhaustively extracted with solvents of varying polarity. Dichloromethane was the best solvent to extract the highest amount (1105 mg) of plant material, followed by chloroform (383 mg) and n-hexane (174 mg). The lowest amount was extracted with acetone (44 mg) (Figure 3.3). Phytochemical constituents from crude extracts were analysed using aluminium-baked TLC plates developed in three solvent systems and sprayed with vanillin-sulphuric acid reagent (Figure 3.4.). Solvent system CEF separated a number of phytochemicals followed by BEA and the least were separated in EMW.

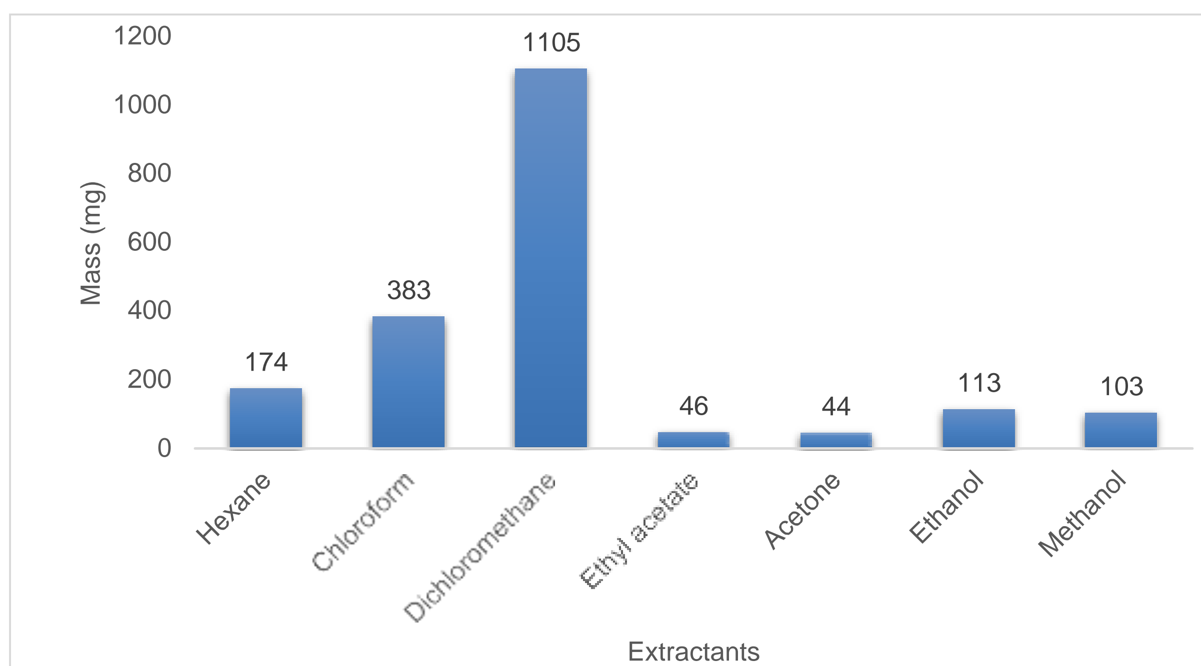


Figure 3.3: Extracted mass in mg of S. *pinnata* extracted by solvents of varying polarity: n-hexane chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol.

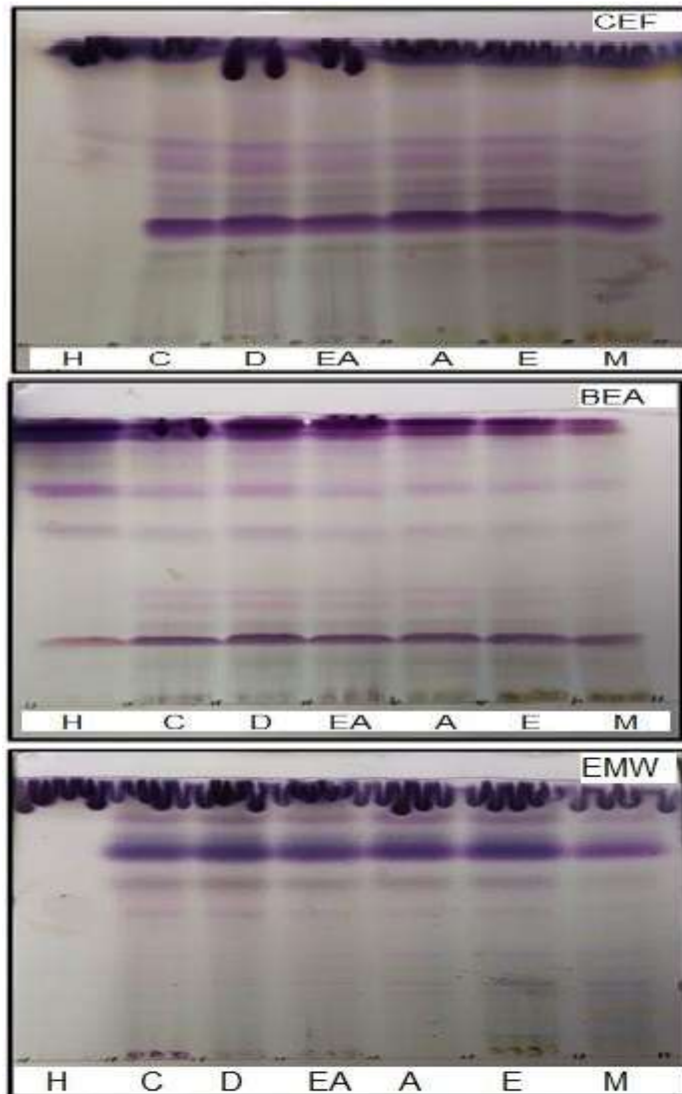


Figure 3.4: Chromatograms of *S. pinnata* extracts developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with vanillin sulphuric acid reagent. Lanes from left to right: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E) and methanol (M).

### 3.3.3. Extraction enrichment procedure

n-Hexane wash

The masses of plant extracts were increasing as the polarity of solvent increases, where ethanol obtained the highest extracts (68 mg) (Figure 3.5). Vanillin-sulphuric acid reagent was used to detect the profile of the phytochemical constituents in crude extracts on TLC plates. Solvent systems CEF and EMW were able to separate vanillin

reactive phytochemicals, while in BEA compounds couldn’t separate from the bottom of the plate (Figure 3.6).

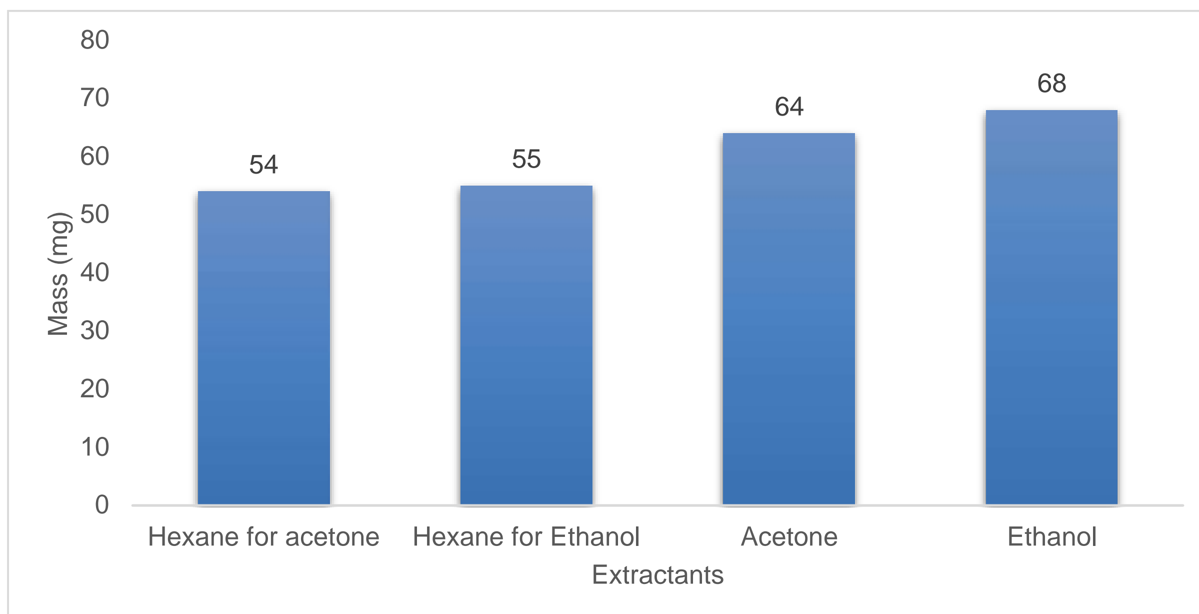


Figure 3.5: The mass in mg of *S. pinnata* extracts, extracted by solvents with varying polarity: n-hexane wash for acetone, n-hexane wash for ethanol, acetone and ethanol.

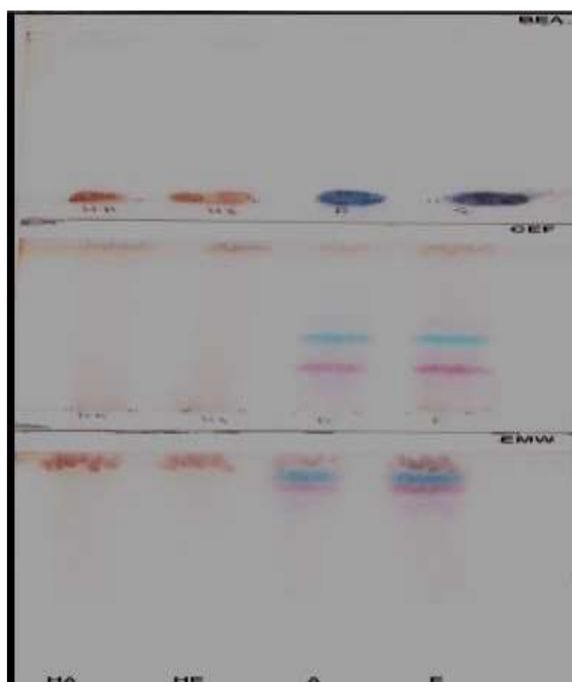

Figure 3.6: Chromatograms of *S. pinnata* extracts developed in BEA (top), CEF (middle) and EMW (bottom) solvent systems and sprayed with vanillin sulphuric acid

reagent. Lanes from left to right: n-hexane wash for acetone (HA), n-hexane wash for ethanol (HE), acetone (A) and ethanol (E).

Acetone and ethanol in water

Extraction of *S. pinnata* phytochemical constitutes was done by the use of acetone, ethanol, water and 20%, 40%, 60%, 80% of acetone and ethanol in water. The highest mass of extract was obtained with 40% acetone extract (128 mg), followed by the 20% acetone (106 mg). The lowest mass obtained was from the 80% ethanol extract (49 mg) (Figure 3.7). Phytochemical compounds which reacted with vanillin reagent were best separated by the solvent system CEF and EMW. Solvent system BEA showed the least bands of phytochemical compound which could not separate from the bottom of the TLC plate (Figure 3.8).

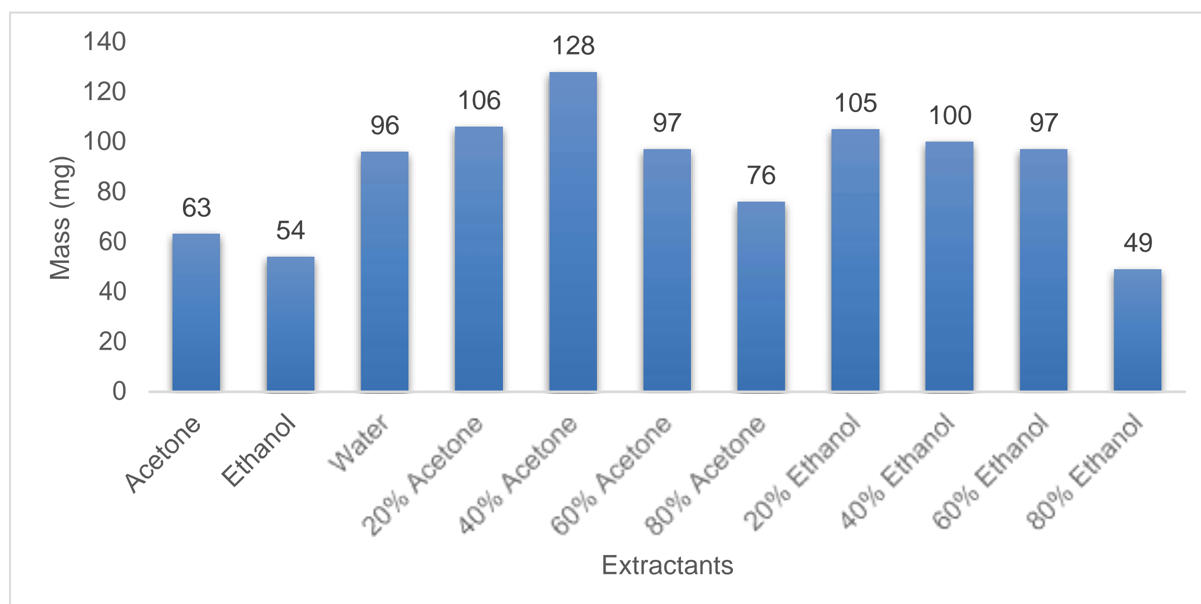


Figure 3.7: The mass in mg of plant extracts extracted using acetone, ethanol, water, and different percentages of acetone and ethanol in water.

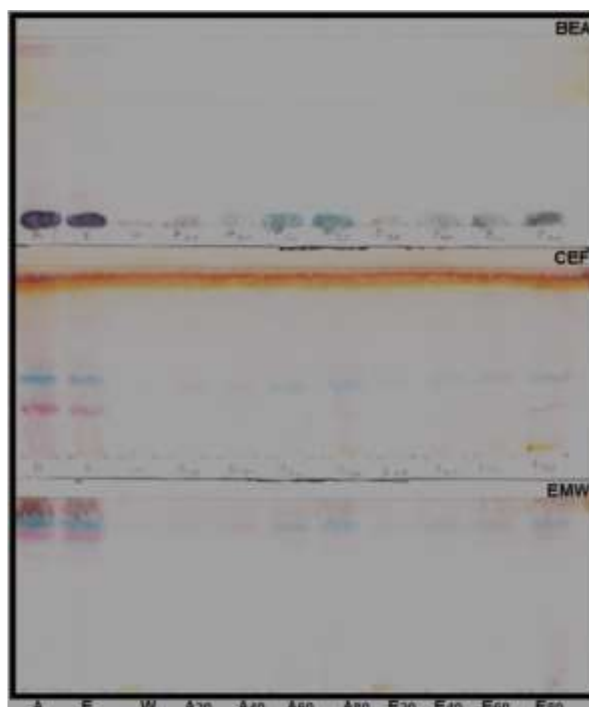


Figure 3.8: Chromatograms of crude extracts developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with vanillin sulphuric acid. Lanes from left to right: Acetone (A), ethanol (E), water (W), 20% acetone (A20), 40% acetone (A40), 60% acetone (A60), 80% acetone (A80), and 20% ethanol (E20), 40% ethanol (E40), 60% ethanol (E60), 80% ethanol (E80).

### 3.3.4. Optimal extraction methods

Extractants of varying polarities were used to extract the plant materials during pre-treatment and extraction method to obtain extract masses (Figure 3.9). In the pre-treatment method, n-hexane wash for 20% acetone extracted a higher amount (80 mg) as compared to other extractants. In extraction of pretreated sample procedure, the greater amount of extract was obtained for ethanol (177 mg) after pretreatment with 20% ethanol, followed by 80% acetone (136 mg), 20% acetone (119 mg) and 80% ethanol (104 mg) and acetone (56 mg) lowest amount. Phytochemical constituents from the extracts obtained were screened on aluminium-baked TCL plates (Figure 3.10 and 3.11). The plates were developed in different mobile phases and sprayed with vanillin-sulphuric acid reagent. Vanillin reactive phytochemical constituents were best separated in solvent system BEA followed by CEF, while EMW separate fewer bands (Figure 3.10 and 3.11).

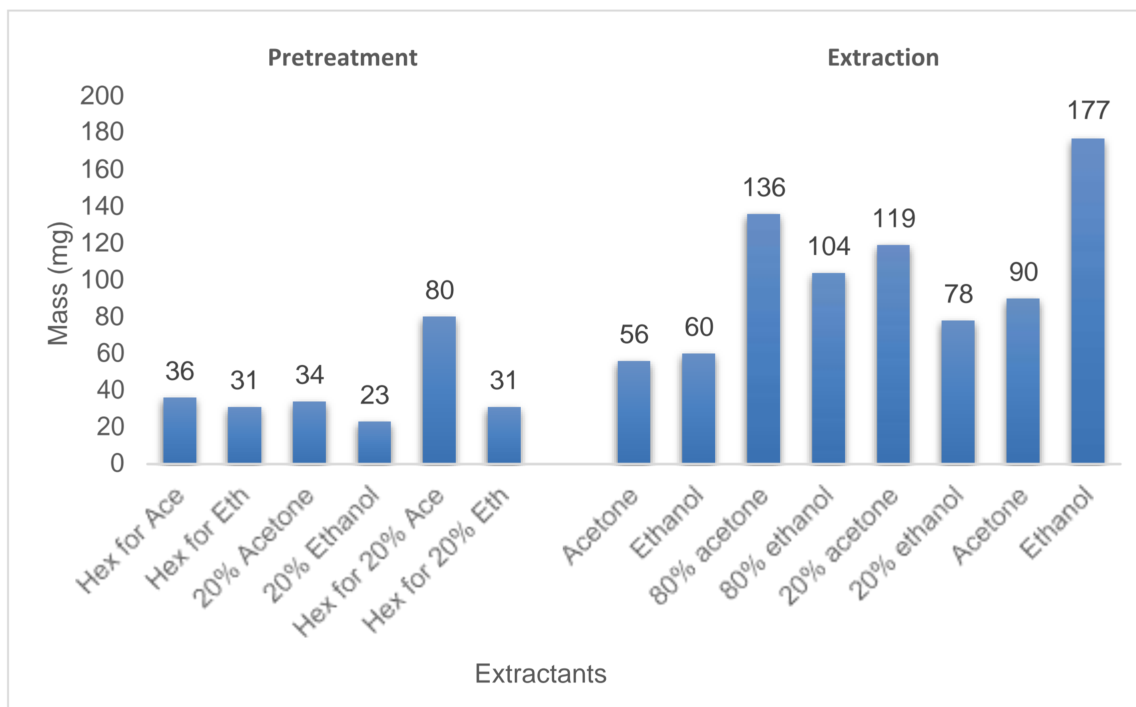


Figure 3.9: The mass of *S. pinnata* extracts in mg extracted by different extractants of varying polarities.

Pretreatment method

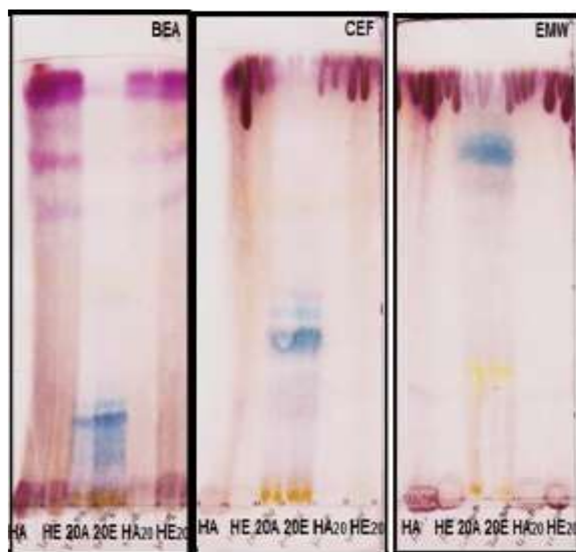


Figure 3.10: Chromatograms of *S. pinnata* extracts separated in three solvent systems BEA (left), CEF (middle), and EMW (right) and sprayed with vanillin sulphuric reagent. Lanes from left to right: n-hexane wash for acetone (HA), n-hexane wash for ethanol (HE), 20% acetone (A20), 20% ethanol (E20), n-hexane wash for 20% acetone (HA20), n-hexane wash for 20% ethanol (HE20).

Extraction of pretreated plant material

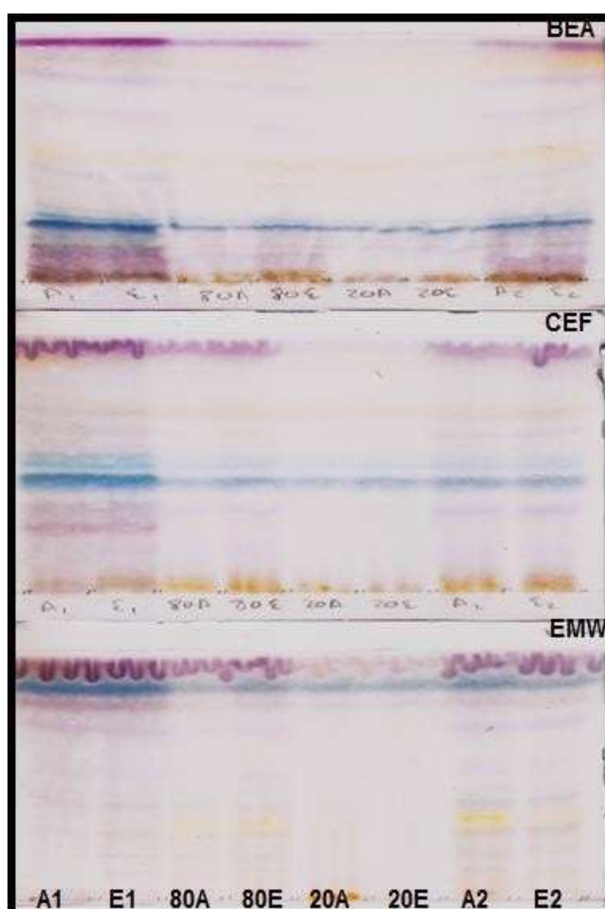


Figure 3.11: Chromatograms of *S. pinnata* extracts separated in BEA (top), CEF (middle) and EMW (bottom) solvent systems and sprayed with vanillin sulphuric acid reagent. Lanes from left to right: acetone after n-hexane wash (A1), ethanol after n-hexane wash (E1), 80% acetone (80A), 80% ethanol (80E), 20% acetone (20A), 20% ethanol (20E), acetone after 20% acetone wash (A2), and ethanol after 20% ethanol wash (E2).

### 3.3.5. Preliminary serial exhaustive extraction series (1, 2, 3)

Different solvents of varying polarities were used to exhaustively extract crude extracts of *S. pinnata* for preliminary isolation of compounds in large scale extraction. In series 1, dichloromethane (509 mg) extracted great amount of extract followed by methanol (262 mg), while the lowest amount was obtained with acetone (51mg). Ethyl acetate (525 mg) extracted the greatest amount of extracts and acetone extracted the least amount in series 2. In series 3, methanol (343 mg) also extracted high amount of extracts followed by acetone (210 mg) (Figure 3.12).

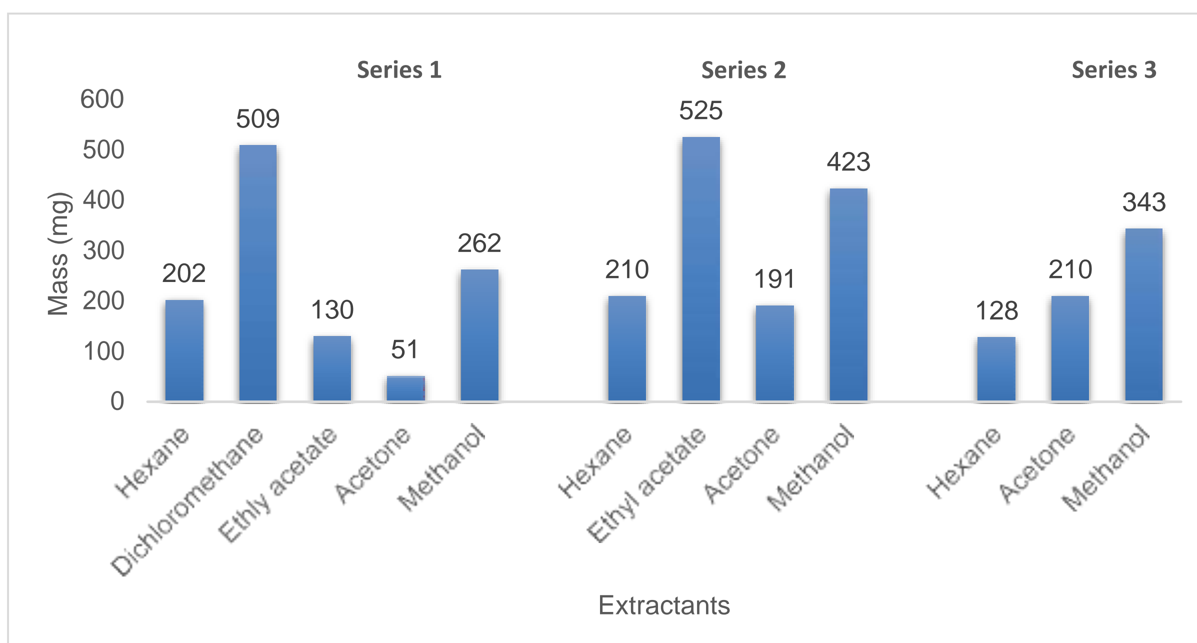


Figure 3.12: The mass of *S. pinnata* extracts in mg extracted with different solvents with varying polarities: n-hexane, dichloromethane, ethyl acetate, acetone and methanol, in three series.

(i) Series 1

*S. pinnata* crude extracts extracted in series 1 were analysed for phytochemical compound that were extracted with solvents of varying polarities. The phytochemical compounds on TLC plates that were separated by three solvent systems (BEA, CEF and EMW) were detected by spraying vanillin-sulphuric acid reagent. Solvent system BEA showed more bands followed by CEF and EMW (Figure 3.13).

(ii) Series 2

Aluminium baked-TLC plates were used to detect the phytochemical constituents of *S. pinnata* extracts. Vanillin-sulphuric acid reagent was sprayed on TLC plate for visualisation of bands that shows the phytochemical compounds. CEF solvent system showed more bands followed by BEA and EMW (Figure 3.14).

(iii) Series 3

Three different solvents of varying polarities: hexane, acetone and methanol were used in extraction of the plant material. TLC plates were sprayed with vanillin-sulphuric

acid reagent for visualisation of phytochemical compounds. More bands were present in BEA solvent system, followed by EMW and CEF (Figure 3.15).

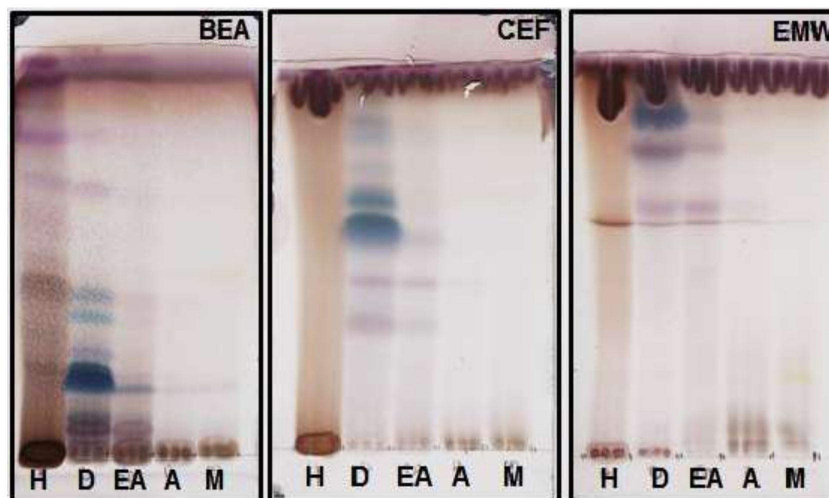


Figure 3.13: Chromatograms of *S. pinnata* extracts separated in BEA (left), CEF (middle) and EMW (right) solvent systems and sprayed with vanillin sulphuric acid reagent. Lanes from left to right: n-hexane (H), dichloromethane (D), ethyl acetate (EA), acetone (A) and M-methanol (M).

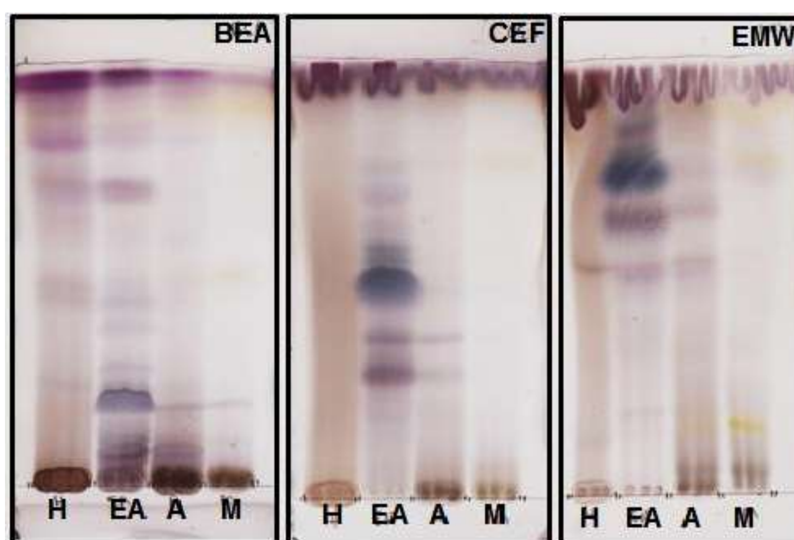


Figure 3.14: Chromatograms of phytochemical constituents from *S. pinnata* extracts separated in BEA (left), CEF (middle) and EMW (right) solvent systems and sprayed with vanillin sulphuric acid reagent. Lanes from left to right: n-hexane (H), ethyl acetate (EA), acetone (A) and methanol (M).

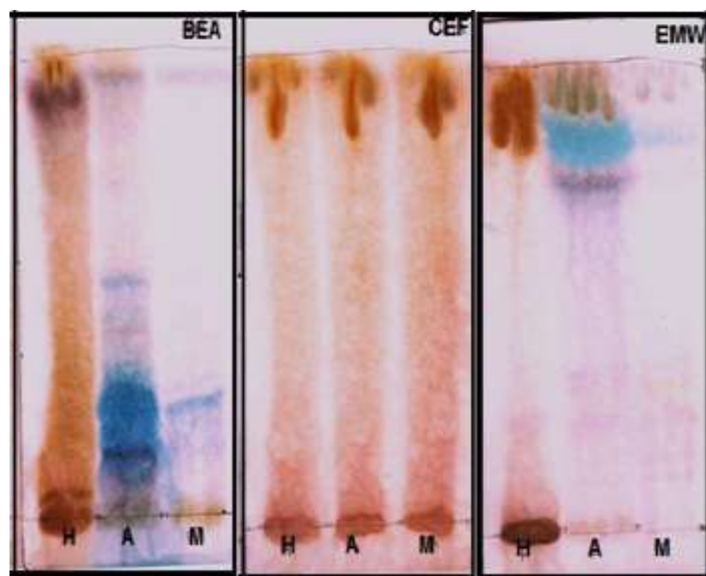


Figure 3.15: Chromatograms of *S. pinnata* extracts separated in BEA (left), CEF (middle) and EMW (right) solvent system and sprayed with vanillin sulphuric acid reagent. Lanes from left to right: n-hexane (H), acetone (A), methanol (M).

### 3.3.6. Phytochemical tests

Various standard phytochemical tests were conducted to test for the presence of different compounds. *S. pinnata* extracts tested positive for all tested compounds namely: tannins, saponins, phlabotannins, terpenoids, alkaloids, steroids and cardiac glycosides (Table 3.2).

Table 3.2: Phytochemical constituents of *S. pinnata* extracts

| Phytochemical constituents | Reaction |
|---|---|
| Tannins | + |
| Saponins | + |
| Phlabotannis | + |
| Flavonoids | + |
| Terpernoids | + |
| Alkaloids | + |
| Cardiac glycosides | + |
| Steroids | + |

Key: Present (+)

### 3.3.7. Determination of total phenolic, flavonoids and tannins content from *S. pinnata* aqueous acetone extracts.

Total phenolic and tannin content was determined from 70% aqueous acetone extracts using linear regression from gallic acid calibration standard curves (Figure 3.16 and 3.18). The total flavonoid content was also determined from 70% aqueous acetone extracts by extrapolating from quercetin calibration standard curve (Figure 3.17). The plant extracts indicated high amount of phenolic content compared to flavonoids and tannin content (Table 3.3).

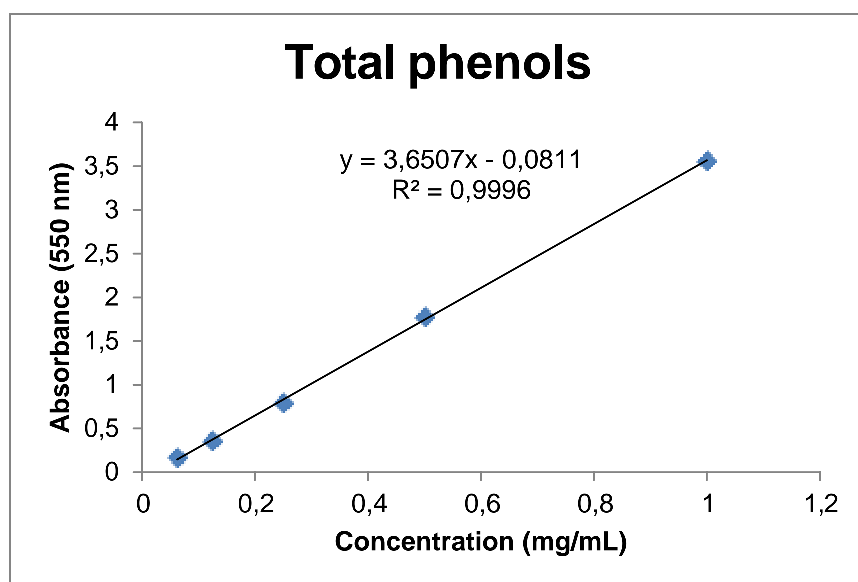


Figure 3.16: The gallic acid calibration standard curve for determination of total phenol content.

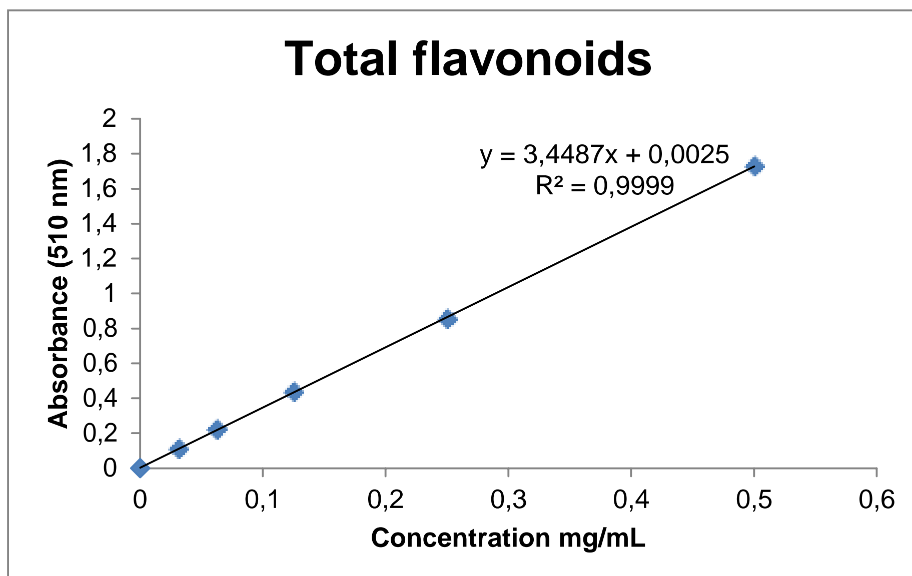


Figure 3.17: The quercetin calibration standard curve for total flavonoids content.

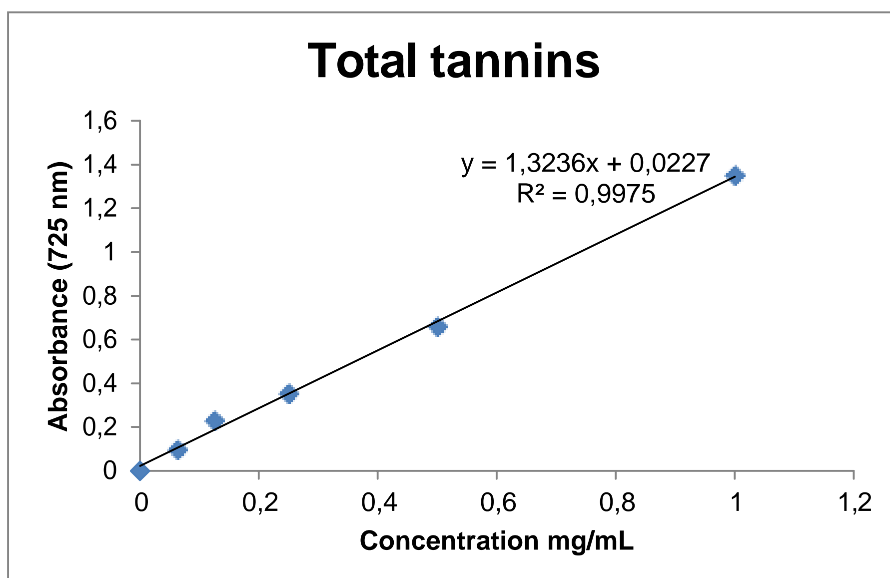


Figure 3.18: The total tannins content calibration standard curve.

Table 3.3: The values of total phenols, flavonoids and tannins content from *S. pinnata* extracts.

| Sample | Total phenols (mg of GAE/g) | Total tannins (mg of GAE/g) | Total flavonoids (mg of QE/mg) |
|---|---|---|---|
| *S. pinnata* | 55,33 ± 3,51 | 28,00 ± 1,73 | 4,00 ± 0,35 |

### 3.4. Discussion

The plant kingdom is significantly recognised in the production of new drugs due to the use of plants in medicinal remedies by humans. Drugs derived from medicinal plants are developed from plant phytochemical constituents such as alkaloids, flavonoids, tannins, terpenoids and saponins, which are of great importance in human health, veterinary and agriculture. Extraction is the first step in the analysis of plants to further identify and isolate the desired phytochemical compounds (Sasidharan *et al.,* 2011). The analysis of plant phytochemical constituents is necessary for synthesis of drugs and other therapeutic agents. Traditional healers have been using water to prepare their medicinal plant remedies, because of its availability and non-toxicity. The medical remedies prepared with water do not contain all active phytochemical constituents since it is polar (Gupta *et al.,* 2012). Hence, researchers have been using various extractants in order to obtain a wide range of bioactive compounds from medicinal plants.

This chapter involved the evaluation of different extraction procedures that can best extract a wide range of compounds from *S. pinnata* for possible biological activity testing and isolation. After extraction, the plant extracts were reconstituted to a concentration of 10 mg/ml with acetone based on reports by Eloff (1998) on its non-toxic effect on microorganism and ability to dissolve compounds of varying polarities. Harbone *et al.* (2013) reported that selection of extraction procedure is useful in order to study the stability of bioactive compounds from medicinal plants. Another report (Elkhair *et al.,* 2010) indicates that, maintenance of bioactive compounds stability is essential when choosing an extraction method.

Thin layer chromatography was used in the screening of the phytochemical profiles of *S. pinnata*, as it is considered to be highly efficient (McGaw *et al.,* 2002: Shahverdi, 2007). The vanillin-sulphuric acid reagent sprayed on chromatograms reacts with the compounds present in plant extracts. In preliminary extraction, BEA was the best eluent system to separate most of the compounds in plant extracts as also reported by Kotze and Eloff (2002). Few bands were separated in CEF while EMW separated the least compounds (Figure 3.4). Compounds in methanol extracts did not separate, this could be due to the polarity of the compounds relative to the solvent system.

In serial exhaustive extraction, solvents of varying polarities were employed to exhaustively extract compounds of wide polarity range. The highest mass of plant extract was obtained from dichloromethane, followed by chloroform and n-hexane (Figure 3.3). Dichloromethane is considered to be a good solvent in the extraction of high amount of lipophilic constituents and n-hexane in the removal of chlorophyll (Cos *et al.*, 2006; Sultana *et al.,* 2009; Sasidharan *et al.*, 2011). Most of the phytochemicals were best separated in CEF and BEA solvent system while EMW separated the least (Figure 3.4). Different colours observed on the chromatograms indicate that *S.pinnata* has different compounds of varying polarities. Since CEF mobile system separates compounds of intermediate polarity (Kotze and Eloff, 2002), this suggested that *S. pinnata* has high amount of intermediate polar compounds.

Sasidharan *et al.* (2011) reported that n-hexane is a non-polar solvent that best extract chlorophyll from plant material and the extracts mainly contains low or non-polar hydrophobic compounds. The removal of chlorophyll and lipids from the matrix of plant sample make it easy for other extractants to penetrate and extract compounds of similar polarities. From figure 3.5, ethanol extracted large amount of plant extracts followed by acetone, after n-hexane has removed chlorophyll and other compounds such as lipids. The mass obtained from n-hexane extracts were almost of the same amount. Chromatograms revealed the presence of separated compounds from acetone and ethanol extracts which were best separated in CEF and EMW (Figure 3.6). The extracts obtained from n-hexane wash did not react with the vanillin reagent. The observed results shows that *S. pinnata* extracts contain compounds of varying polarities.

Combination of solvents during extraction is also employed in order to get better extraction efficiency (Gupta *et al.,* 2012). Where extraction of plant material using different percentages of acetone and ethanol in water was employed, 40% acetone extracted the highest amount of plant extracts, followed by 20% acetone and 20% ethanol (Figure 3.7). The lowest amount of plant extracts was extracted with 80% ethanol. Acetone and water combination have been reported to be good solvent system in extraction of bioactive compounds from medicinal plants (Naczk and Shahidi, 2006; Alothman *et al.,* 2009; Michiels *et al.,* 2012; Zlotek *et al.,* 2016). Phytochemical profile for acetone and ethanol in water extracts showed that solvent system CEF separated most of vanillin reactive compounds while solvent system

EMW separated few compounds (Figure 3.8). In solvent system BEA, compounds couldn't separate from the bottom of TLC plate, but they reacted with the vanillin sulphuric acid reagent (Figure 3.8).

Azwanida (2015) stated that pre-extraction and extraction procedures are the initial steps in the study of medicinal plants. Among all the washes (Figure 3.9), n-hexane wash for 20% acetone yielded the highest amount of plant extracts followed by n-hexane wash for acetone and 20% acetone. The lowest amount of plant extracts was obtained with 20% ethanol wash. In the extraction of pretreated plant material, the highest amount of plant extracts was obtained with ethanol, 80% acetone and 20% acetone, while the least amount was obtained with acetone (Figure 3.9). Literature has indicated that ethanol is one of polar solvents that extract hydrophilic compounds from medicinal plants and it was reported to be the best solvent in extraction of flavonoids (Wang and Helliwell, 2001; Sasidharan *et al*., 2011). Phytochemical fingerprints (Figure 3.10) show that BEA solvent system separated a number of vanillin reactive compounds over the other solvent systems. CEF separated few bands of phytochemicals while EMW separated the least number of compounds. Extraction of pretreated plant material revealed more vanillin reactive compounds which were best separated with BEA, followed by CEF and EMW the least (Figure 3.11). From the extraction procedures, majority of the phytochemical constituents were observed to be well separated by solvent system BEA and CEF. This suggested that *S. pinnata* contains compounds with non-polar and intermediate polarity. Furthermore, serial exhaustive extraction was the best procedure which yielded high amount of plant extracts with a number of phytochemical constituents. This procedure was selected to be used during isolation.

Preliminary serial exhaustive extraction procedure was used in the screening for solvents to be used in isolation of antimycobacterial compounds. Serial exhaustive method is a successive procedure which involves the use of solvents with increasing polarity from n-hexane, a non-polar solvent, to methanol a more polar solvent in order to obtain compounds of wide polarity range. Solvents diffuse into the plant material and solubilise compounds of similar polarity (Green, 2004). Among the series, ethyl acetate was the best solvent that yielded the highest amount of plant extracts followed by dichloromethane and methanol (Figure 3.12) with acetone as the least. Methanol and ethyl acetate are one of the solvents which have been reported to extract most of

the hydrophilic compounds from medicinal plants (Sasidharan *et al.,* 2011). Fingerprint profile of the phytochemical constituents from *S. pinnata* shows that solvent system BEA separated a number of vanillin reactive compounds. Chromatograms show various compounds which reacted with vanillin-sulphuric acid reagent forming colourful bands (Figure3.13). In series 2 (Figure 3.14), phytochemical constituents contained in *S. pinnata* extracts were separated best with in solvent system BEA followed by CEF while in EMW, phytochemicals were not properly separated. This might be due to their polarity. In series 3, BEA solvent system separated a number of compounds while EMW separated few compounds (Figure 3.15). In all series, solvent system BEA separated most of the phytochemicals, and the blue compound was consistent.

In order to assess the general phytochemical composition in the plant, chemical tests were carried out on *S. pinnata*. Metabolites present in *S. pinnata* are known to have various pharmacological actions (Ndukwe *et al.,* 2007). The results on the phytochemical screening of the plant material (Table 3.1) indicated the presence of tannins, saponins, phlabotannins, flavonoids, terpernoids, alkaloids, cardiac glycoside and steroids. Oryema *et al.* (2011) also detected the presence of alkaloids, steroids and terpenoids in *S. pinnata* extracts. Ethanol extracts of *S. pinnata* have been reported to possess the sterol triterpenes and flavonoids compounds (Rodrigo *et al.,* 2010). The presence of terpenoids confirms the blue compounds which were observed from the phytochemical fingerprint profiles.

The amount of total phenolic content was determined from 70% aqueous acetone extract and expressed as GAE/g equivalent. The plant extract indicated the high amount of total phenolic content (55.33 GAE/g). Literature has reported that phenolic compounds play a role towards the unique taste, flavour, aroma and health-promoting properties found in vegetables and fruits (Tomas-Baberan and Espin, 2001). Total phenolic content have been reported from the family *Asteraceae* (Tawaha *et al*., 2007). The results showed that *S. pinnata* have an overall flavonoids amount of 4 QE/mg (Table 3.2). Flavonoids have been associated with anti-inflammatory, antibacterial and insecticide activities and have been used in food industries (Harbone, 2013).

Total tannin content was determined from 70% aqueous acetone extract. Tannin compounds are another group of secondary metabolites which are classified as ether

condensed or hydrolysed tannins. In this study gallic acid was used to derive a standard curve. The results showed that *S. pinnata* extracts contained an amount of 28 GAE/g. Tannins have been reported to have various biological activities such as antibacterial, antioxidant and anti-inflammatory (Mlombo *et al*., 2009). The presence of tannins in the plant extracts might be responsible for the bitterness taste of the plant extracts as it is called “sebabane” by Sepedi speaking people. This study is the first study that has determined the total phenolic, flavonoid and tannin content in *S. pinnata* extracts.

### 3.5. Conclusion

Different phytochemicals have various biological activities which can serve as potential leads that can help in protection against chronic diseases. The results obtained from *S. pinnata* extracts depend on the polarity of different compounds present in extracts. The results in this chapter suggests that *S. pinnata* possesses compounds of intermediate polarity due to the masses of the plant extracts obtained within the procedures, and contains a number of vanillin reactive phytochemical constituents. A serial exhaustive extraction procedure was the best extraction procedure due to high number of phytochemical constituents which reacted with vanillin sulphuric reagent. This supports the extraction condition mentioned by Eloff (1998) and Gupta *et al.* (2012). Furthermore the abundance of compounds such as phenolic, tannins and flavonoids could possibly provide potential leads for development of drugs against microbes.

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## Chapter 4

## 4. Antioxidant activity

### 4.1. Introduction

Oxidative stress can be defined as a state in which the balance between antioxidants and free radicals is lost (Pulido *et al*., 2000). Antioxidants defend against ageing and degenerative diseases caused by free radicals that accumulate in the body as by-products of metabolism (Cai *et al*., 2003). Oxidative stress results in cell damage (Krishnaih *et al*., 2011). Sánchez-González *et al*. (2005) stated that antioxidants can reduce or prevent imbalance of free radicals through three processes: metal chelating mechanism, repairing mechanism and scavenging mechanism. As oxidative stress is considered to be a major cause of numerous disorders and diseases in humans, natural antioxidant supplements have to be generated (Halliwell, 1994; Rackova *et al*., 2007).

Synthetic antioxidants were categorised as phenolic structures that differs in the position of alkyl substitution (Velioglu *et al*., 1998). They are commonly used as additives in food industries. However, they were reported to cause illness in humans due to side effects such as liver damage and carcinogenicity (Dave, 2009). Other natural products such as medicinal plants are considered to be good alternatives in overcoming this challenge.

Plants have gained the attention of researchers due to the presence of various antioxidant compounds such as phenolic compounds (including phenolic acids flavonoids and tocopherols), nitrogen compounds (alkaloids, amino acids and amines) and terpenoids (Velioglu *et al*., 1998; Cai *et al*., 2003; Nunes *et al*., 2012; Bichra *et al*., 2013). Rice-Evans (2004) mentioned that medicinal plants are a great source of natural antioxidants. Furthermore, Kasote *et al*. (2015) supports the claim that plants serve as a good source of antioxidants which have the abilities of reducing or preventing oxidative damage.

Catalase, superoxide dismutase and glutathione peroxidase are antioxidant enzymes that play a vital role in scavenging free radicals (Yen *et al*., 2005). Antioxidants reduces the risk of ageing and degenerative diseases such as cancer, cardiovascular disease and stroke (Prior and Cao, 2000). Natural occurring antioxidants such as ascorbic

acid, carotenoids and phenolic compounds have been reported to be more effective against oxidative stress (Duh *et al*., 1999). They are highly recommended to be healthy than synthetic antioxidants (Abdalla and Roozen, 1999). High intake of antioxidants from natural products significantly helps in maintenance of the balance between free radicals and antioxidants (Pulido *et al*., 2000: Wang *et al.,* 2011).

Plants have been screened for the presence of potential antioxidants through several assays. Antioxidant activity screening methods are categorised on reaction-based mechanism, hydrogen atom transfer (HAT) and single electron transfer (SET) mechanism (Kasote *et al*., 2015). This study made use of 2, 2–diphenyl-1-picrylhydrazyl (DPPH) and ferric ion reducing antioxidant power (FRAP) assays of which both HAT and SET mechanisms are involved (Prior and Schaich, 2005). The presence of free radical reducing compounds from *S. pinnata* extracts was determined qualitatively and quantitatively using 1, 1 diphenyl-2-picrylhydrazyl (DPPH) and ferric ion reducing power (FRAP) assays. The reagent DPPH is a stable free radical which does not dimerise due to its ability to delocalise spare electron from a molecule (Eklund *et al*., 2005). It can be prepared in crystal form, through a quick procedure (qualitative) where DPPH is sprayed on TLC plate as a reagent to visualise the presence of antioxidant phytochemicals. The presence of antioxidant activity is detected by the change of initial purple background on TLC plate into yellow band or spot (Brand-Williams *et al*., 1995). Benzie and Strain (1999) developed an assay which determines ferric reducing capacity of plasma. However, the procedure was also used for other substances such as tea and wine (Pulido *et al.,* 2000). The ability of compounds to reduce ferric-trypyridyltriazine complex to ferrous state shows the presence of ferric ion reducing antioxidants. *S. pinnata* extracts were evaluated for the presence of ferric reducing antioxidants and free radical scavenging activities.

## 4.2. Methods and materials

### 4.2.1. Qualitative TLC- DPPH assay

All extracts obtained from the extraction procedures were screened for antioxidant activity. The TLC plates were prepared as in section 3.2.2, and they were dried under a stream of air at room temperature for 1 minute. Thereafter, they were sprayed with 0.2% 2, 2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma) in methanol. A positive result was

indicated by the presence of yellow bands against a purple background (Deby and Margotteaux., 1970).

### 4.2.2. Quantitative total antioxidant activity assay

The antioxidant potential of *S. pinnata* extracts was determined by using DPPH free radical scavenging activity assay (Brand –Williams *et al*., 1995). A working solution of 0.2% DPPH in methanol was prepared and 1 mg/ml of extract was serially diluted in test tubes. One millilitres of working solution was mixed with plant extracts of various concentrations. Methanol was used as blank and ascorbic acid was used as a positive control. DPPH solution was used as a standard control. The solutions were allowed to stand for 20 minutes in the dark at room temperature. The absorbance was recorded at 517 nm using UV/visible spectrophotometer. The experiment was repeated three times. The radical scavenging activity was determined using the following equation:

$$\%\ \text{scavenging activity} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{experiment}}}{\text{Absorbance}_{\text{control}}} \times 100$$

### 4.2.3. Ferric reducing power

Ferric ion reducing power of *S. pinnata* extracts was determined following a procedure described by Benzie and Strain (1999) with some modifications. Various concentrations of plant extracts (1 mg/ml to 0.0625 mg/ml) were prepared into test tubes. Ascorbic acid was used as a standard control and a blank solution was prepared without adding extracts. Two millilitres of 0.2 M sodium phosphate buffer and 2 ml of 1% potassium ferri-cyanide were added to the test tubes containing extracts of different concentrations. The solution was mixed well and incubated in a water bath at 50ºC for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid was added into the test tubes and centrifuged at 650 rpm for 10 minutes. The supernatant was mixed with 10 ml of distilled water and 1 ml of freshly prepared ferric chloride solution (0.1%). Thereafter, the solution was mixed. The absorbance of the solution was recorded at 700 nm against the blank solution. The experiment was repeated three times. The ferric reducing power of plant extracts was expressed as mg of ascorbic acid equivalents (AAE) per ml.

## 4.3. Results

### 4.3.1. Preliminary extraction

Antioxidant activity of plant extracts obtained in preliminary extraction procedure indicated the presents of radical scavenging compounds. The solvent system BEA separated a number of antioxidant compounds while in CEF and EMW, the antioxidant compounds moved with the solvent front (Figure 4.1).

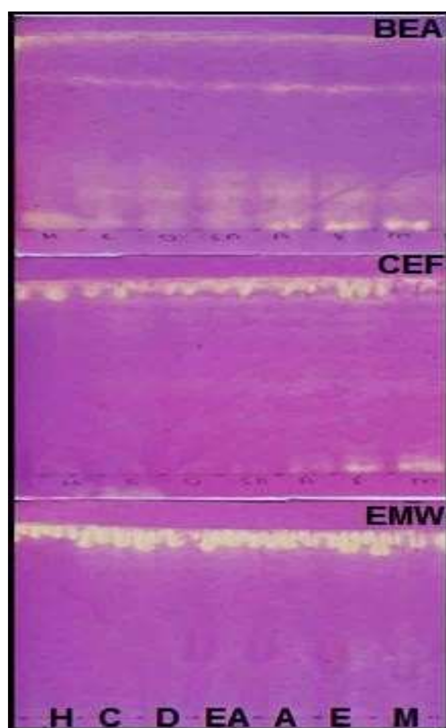


Figure 4.1: Chromatograms of *S. pinnata* extracts developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with 0.2% DPPH in methanol. Lanes from left to right: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E) and methanol (M).

### 4.3.2. Serial exhaustive extraction

*S. pinnata* sample was extracted with solvents of varying polarities, with n-hexane as a starting solvent and methanol was the last extractant (Figure 4.2). The plant extracts obtained with ethyl acetate and acetone indicated a good antioxidant activity which was best separated in EMW solvent system while in BEA and CEF, the compounds could not move from the bottom of the plate.

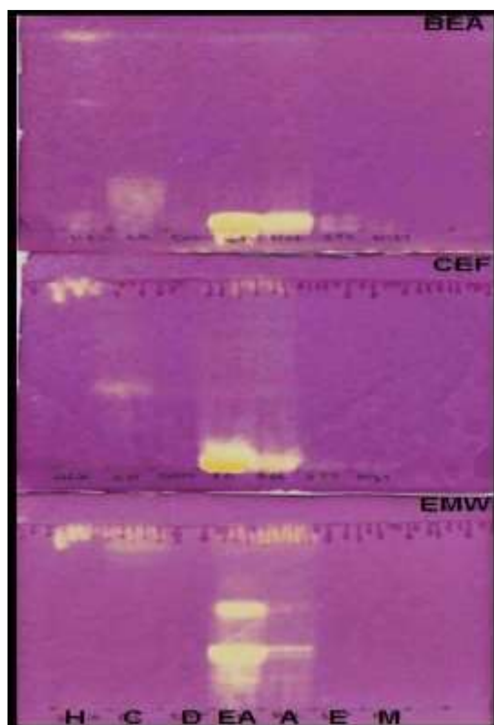


Figure 4.2: Chromatograms of *S. pinnata* extracts obtained after serially extraction, developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with 0.2% DPPH in methanol. Lanes from left to right: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E) and methanol (M).

#### 4.3.3. Extraction enrichment

n-Hexane wash

The plant material was washed with n-hexane and thereafter extracted with solvents of varying polarities. Acetone and ethanol extracts have showed the presence of antioxidant compounds. The antioxidant compounds were partially separated in solvent system BEA, while they were able to move with the solvent front in solvent system CEF and EMW (Figure 4.3).

Acetone and ethanol in water

The percentage fraction indicated antioxidant activity from 40% acetone, 60 % acetone, 80% acetone, 60% ethanol and 80% ethanol. Water extracts did not have antioxidant activity, while acetone and ethanol had the presence of antioxidant compounds separated in solvent system EMW (Figure 4.4).

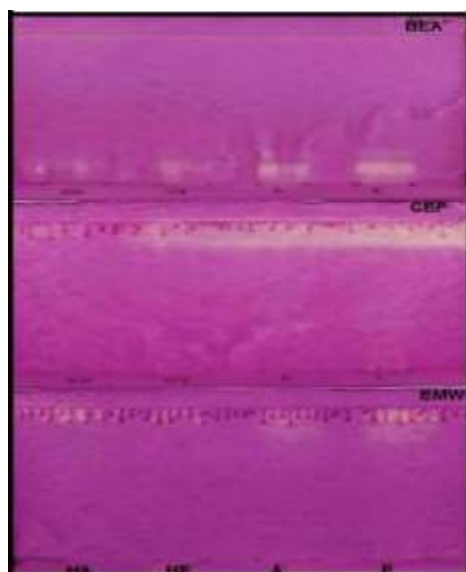


Figure 4.3: Chromatograms of *S. pinnata* extracts developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with 0.2% DPPH in methanol. Lanes from left to right: hexane for acetone (HA), hexane for ethanol (HE) acetone (A) and ethanol (E).

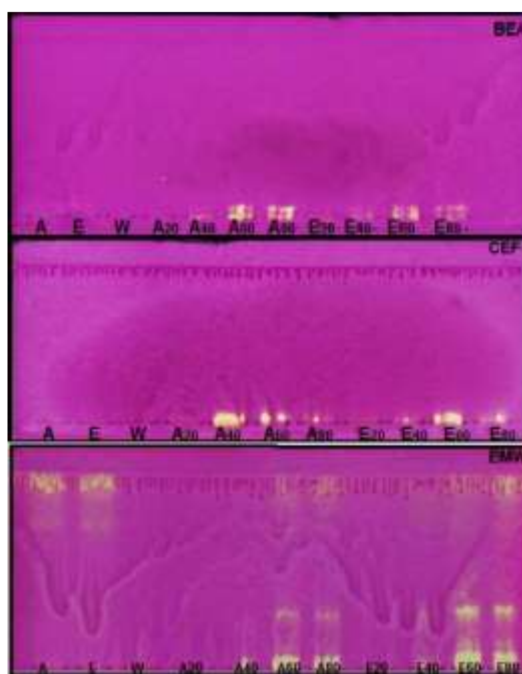


Figure 4.4: The chromatograms of *S. pinnata* extracts extracted with solvents of varying polarities and developed in three solvent systems: BEA (top), CEF (middle) and EMW (bottom) and sprayed with 0.2% DPPH in methanol. Lanes from left to right: acetone (A), ethanol (E), water (W), 20% acetone (A20), 40% acetone (A40), 60% acetone (A60), 80% acetone (A80), and 20% ethanol (E20), 40% ethanol (E40), 60% ethanol (E60), 80% ethanol (E80).

### 4.3.4. Optimal extraction

Yellow spots against the purple background indicated the presence of antioxidant compounds. The chromatograms of *S. pinnata* extracts were sprayed with 0.2% DPPH in methanol in order to determine the presence of antioxidant activity. In pre-treatment method, out of all extracts, only 20% acetone and 20% ethanol indicated the presents of antioxidant compounds. The antioxidant compounds could not separate from the bottom of the plates in all solvent systems (Figure 4.5). In extraction of pretreated sample, the compounds could not move from the bottom of the plates in BEA and CEF solvent systems (Figure 4.6).

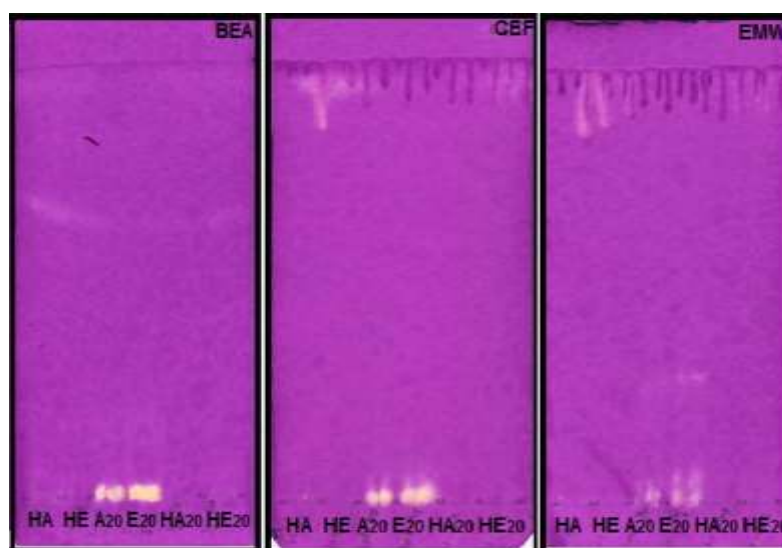


Figure 4.5: Chromatograms of *S. pinnata* extracts developed in BEA (left), CEF (middle) and EMW (right) solvent systems and sprayed with 0.2% DPPH in methanol. Lanes from left to right: hexane wash for acetone (HA), n-hexane wash for ethanol (HE), 20% acetone (A20), 20% ethanol (E20), n-hexane wash for 20% acetone (HA20) and, n-hexane wash for 20% ethanol (HE20).

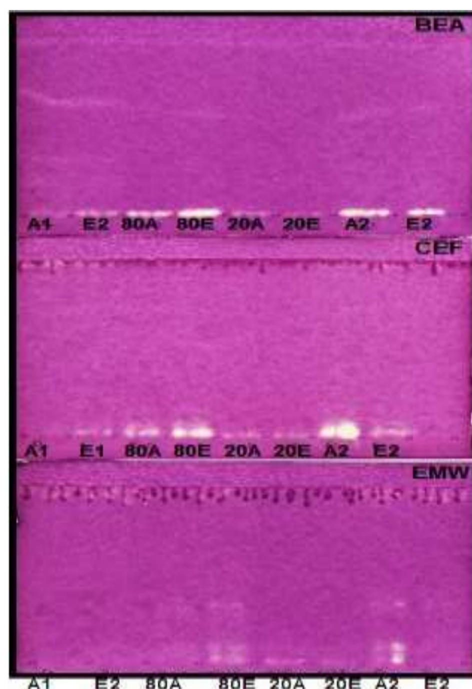


Figure 4.6: Chromatograms of *S. pinnata* extracts developed in BEA (top), CEF (middle) and EMW (bottom) solvent systems sprayed with 0.2% DPPH in methanol and separated with solvent systems of different polarity. Lanes from left to right: acetone after n-hexane wash (A1), ethanol after n-hexane wash (E1), 80% acetone (80A), 80% ethanol (80E), 20% acetone (20A), 20% ethanol (20E), acetone after 20% acetone wash (A2), and ethanol after 20% ethanol wash (E2).

#### 4.3.5. Preliminary serial exhaustive series (1, 2, 3)

Preliminary serial exhaustive extraction was done in order to screen for the isolation of compounds without changing the extracting solvents. The chromatograms of antioxidant activity are shown from figures 4.7 to 4.9 below:

Series 1

*S.pinnata* extracts were exhaustively extracted with solvents of varying polarity: n-hexane (H), dichloromethane (D), ethyl acetate (EA), acetone (A) and methanol (M). Antioxidant activity was detected from ethyl acetate, acetone and methanol extracts which were separated in BEA and CEF solvent system, while in EMW all the extracts did not show activity after spraying with DPPH (Figure 4.7).

Series 2

Antioxidant activity was screened from *S. pinnata* extracts extracted with: n-hexane (H), ethyl acetate (EA), acetone (A) and methanol (M). The screened extracts indicated the presence of antioxidant activity from extracts obtained with ethyl acetate, acetone and methanol under separation system BEA, while in CEF, acetone and methanol extracts showed more antioxidant activity than ethyl acetate extracts. Extracts which were separated in solvent system EMW could not react with DPPH sprayed on the TLC plates (Figure 4.8).

Series 3

*S. pinnata* extracts obtained from: n-hexane (H), acetone (A), methanol (M) solvents were screened for antioxidant activity using TLC plates. Acetone and methanol extracts indicated the presence of antioxidant compounds when separated in BEA solvent system. Methanol extracts indicated the presence of antioxidant compounds when separated in CEF while in EMW, no visible antioxidant activity was observed with all the extracts (Figure 4.9).

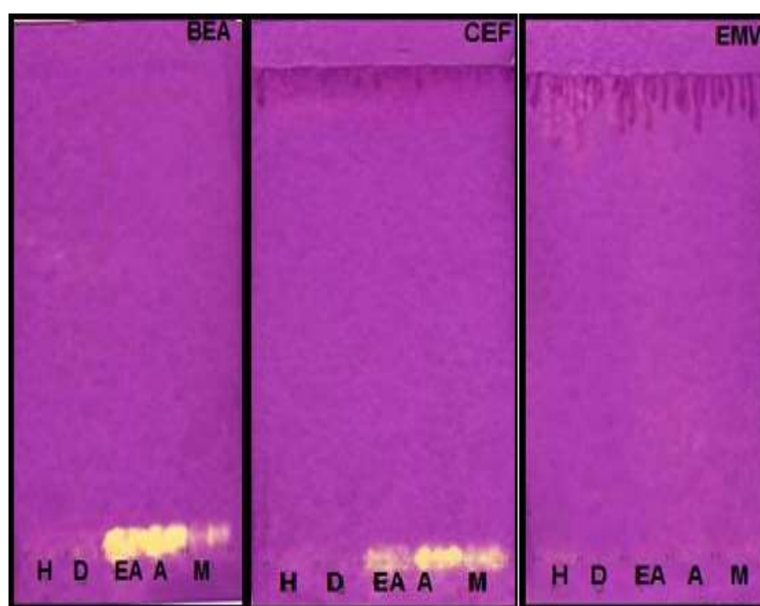


Figure 4.7: Chromatograms of *S. pinnata* extracts extracted with various solvents developed in BEA (left), CEF (middle) and EMW (right) solvent systems and sprayed with 0.2% DPPH in methanol. Lanes from left to right: n-hexane (H), dichloromethane (D), ethyl acetate (EA), acetone (A) and methanol (M).

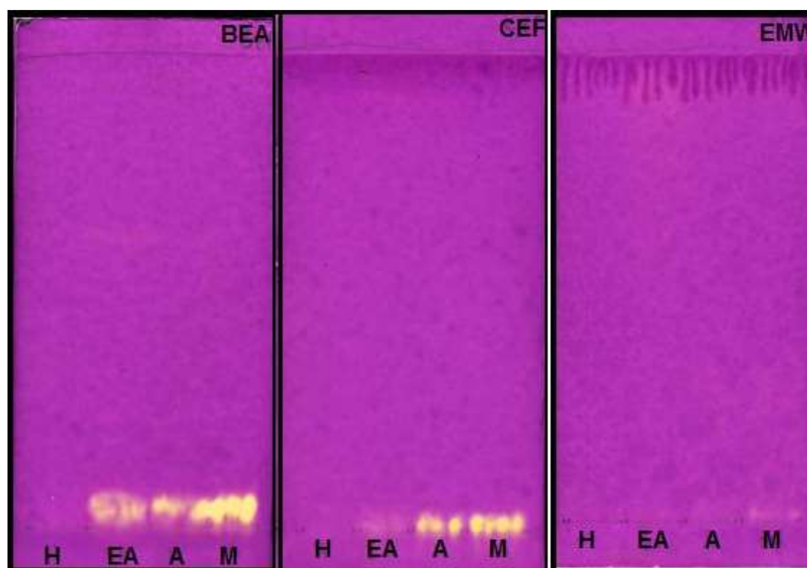


Figure 4.8: Chromatograms of *S. pinnata* extracts extracted with solvents of varying polarity developed in three solvent systems BEA (left), CEF (middle) and EMW (right) and sprayed with 0.2% DPPH in methanol. Lanes from left to right: n-hexane (H), ethyl acetate (EA), acetone (A) and methanol (M).

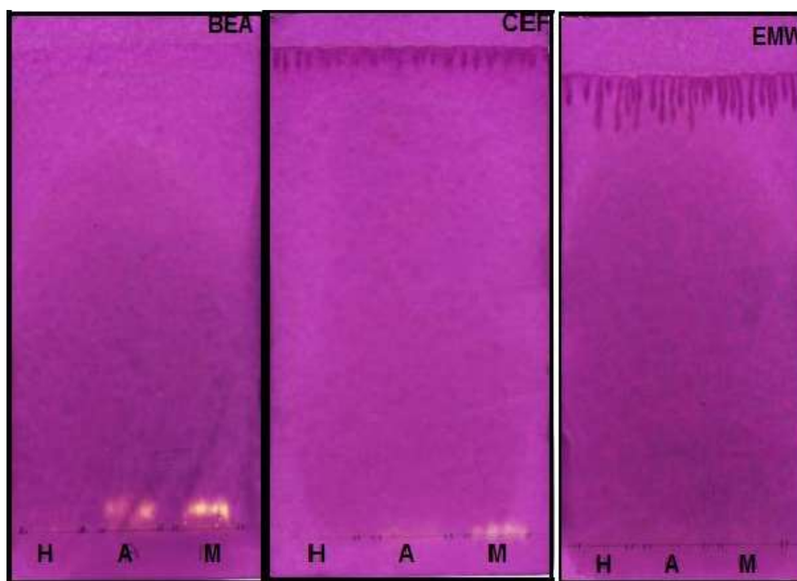


Figure 4.9: Chromatograms of *S. pinnata* extracts extracted with solvents of varying polarity developed in three solvent systems BEA (left), CEF (middle) and EMW (right) and sprayed with 0.2% DPPH in methanol. Lanes from left to right: n-hexane (H), acetone (A) and methanol (M).

### 4.3.6. Quantitative DPPH assay

The quantitative antioxidant activity of *S. pinnata* extracts was done using DPPH assay. The best antioxidant activity was observed from methanol extracts followed by ethyl acetate and acetone (Figure 4.10). All plant extracts at high concentrations had high percentage scavenging activity including the positive control (vitamin C).

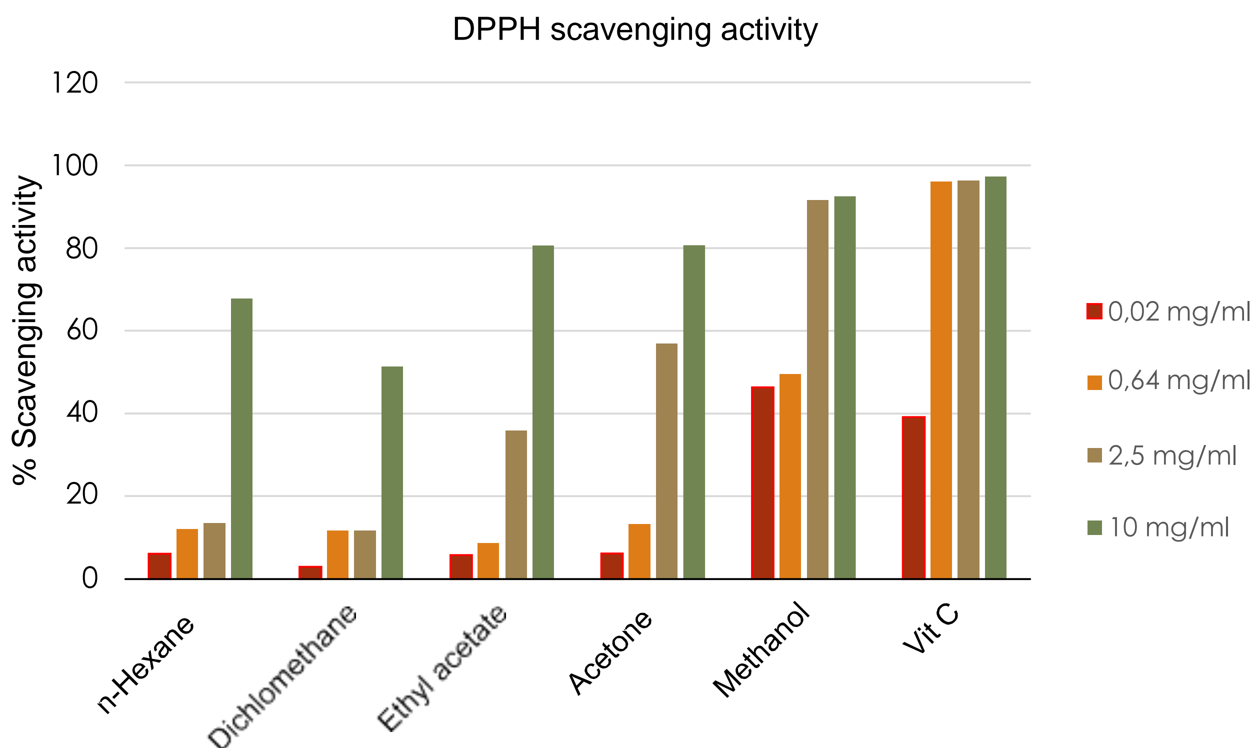


Figure 4.10: Quantitative percentage scavenging activity of *S. pinnata* extracts at different concentrations.

### 4.3.7. Ferric ion reducing power assay

Antioxidant activity of *S. pinnata* extracts at various concentration (0.0625 mg/ml – 1 mg/ml) was quantified using FRAP assay. This was based on the ability of antioxidant compounds in the plant extracts to reduce ferric ions into the ferrous complex. Ascorbic acid which was used as a standard control indicated high reducing power which was high at all concentrations when compared with the plant extracts (Figure 4.11).

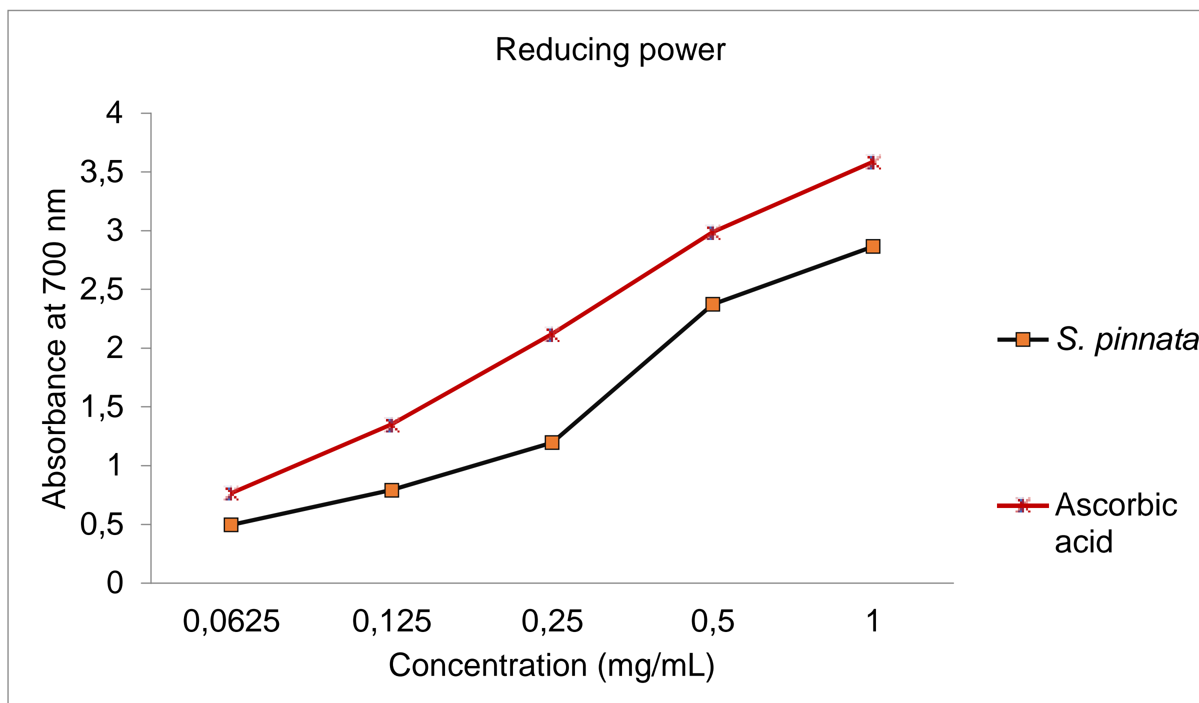


Figure 4.11: The reducing power of *S. pinnata* extracts at various concentration as compared to ascorbic acid.

**4.4. Discussion**

Plants have been used by human to relieve symptoms and cure various diseases (Ramawat and Merillon, 2008). Antioxidants from plants have drawn the attention of several research teams because of their ability in fighting against various diseases related to oxidative stress and free radical damage (Devasagayam *et al*., 2004). Researchers prefer the use of at least two methods when analysing antioxidant activity, in order to understand the mechanism and dynamics of the antioxidant action from the plant extracts.

The qualitative TLC-DPPH antioxidant procedure was used to screen extracts in order to detect potential antioxidant activity for further investigation such as identification and characterisation of the active compounds. The presence of antioxidant compounds was indicated by the yellow bands against the purple back ground after spraying the TLC plates with DPPH solution. The intensity of the yellow colour depends on the quantity and nature of antioxidant compounds present in extracts (Scio *et al*., 2012).

The obtained crude extracts from extraction procedures were screened qualitatively for the antioxidative properties. Antioxidant activity was observed from *S. pinnata*

extracts from extraction procedures (from Figure 4.1 to 4.9). The presence of antioxidant activity from plant sample might be due to flavonoid compounds which have been reported to possess antioxidant activity (Baharfar *et al*., 2015). In preliminary extraction, all extractants in figure 4.1 showed the presence of antioxidant activity with low intensity. A good separation of antioxidant compounds was observed in solvent system BEA, while in CEF and EMW, antioxidant compounds were present but they moved with the solvent front (Figure 4.1). This suggests that the plant extracts contain antioxidant compounds of various polarities, and indicates that the antioxidant compounds from plant extracts are polar compounds.

In the serial exhaustive extraction, ethyl acetate and acetone extracted strong antioxidant compounds which were observed in all solvent systems (Figure 4.2). In solvent system BEA and CEF compounds could not move from the bottom of the plate while in EMW the antioxidant compounds from ethyl acetate and acetone extracts were separated (Figure 4.2). This indicates that observed antioxidant compounds are of polar polarity as observed in the EMW. This could be due to the polarity of the mobile phase which has to be favourable with the phytochemicals within the plant extracts (Moteriya *et al*., 2014). Flavonoids and tannins present in *S. pinnata* extracts (Table 3.1) are likely to be responsible for the free radical scavenging activity observed. These compounds have been reported to act as primary antioxidants from plants (Zand *et al*., 2002; Ayoola *et al*., 2008). Researchers consider flavonoids compounds as “high level” natural antioxidants. This is due to their abilities to scavenge free radicals and reactive oxygen species (Fukumota and Mazza, 2000; Unno *et al*., 2000; Klahorst, 2002).

Waxes, fats and fixed oils from plant extracts were removed from the sample by the use of n-hexane, to unmask more antioxidant compounds. Chromatograms of *S. pinnata* extracts sprayed with DPPH reagent indicated the presence of antioxidant activity from acetone and ethanol extracts in BEA. However, the compounds moved with the solvent front in CEF and EMW (Figure 4.3). Mixtures of solvents help in extraction of compounds with a wide range of polarity (Gupta *et al.,* 2012). Extracts obtained after extraction with varying percentage of acetone and ethanol in water indicated the presence of antioxidant compounds. Antioxidant activity was observed from 60% acetone, 80% acetone, 60% ethanol and 80% ethanol extracts from all solvent systems, while in mobile phase CEF, antioxidant activity was observed from

40% acetone extracts (Figure 4.4). It was seen that few yellow bands against the purple background were separated in solvent system EMW while in solvent system BEA and CEF the compounds could not move from the bottom of the TLC plates (Figure 4.4), which indicates that the antioxidant compounds were more polar.

Pretreatment of plant sample revealed the presence of antioxidant compounds from 20% acetone and 20% ethanol extracts in solvent system BEA and CEF, while in solvent system EMW, the antioxidant activity was of low intensity (Figure 4.5). The antioxidant compounds extracted with solvents used in pretreatement procedure indicted that they are of polarity which could be separated with other solvent systems of varying ratios, because the compounds couldn't move with all solvent systems used (Figure 4.5). The presence of free radical scavenging activity was more after plant samples were pretreated. This suggests that more phytochemicals were unmasked after the wash of plant residues. Zlotek *et al.* (2016) reported that a mixture of acetone and water had good antioxidant activity. Plant extracts obtained with ethanol (E1), 80% acetone, 80% ethanol, acetone (A2) and ethanol (E2) extracts showed the presence of antioxidant compounds which could not move from the bottom of TLC plates (Figure 4.6). This suggests that mobile systems used were of low polarity for the compounds to separate.

Solvent system BEA and CEF from all series indicated the presence of antioxidant activity (Figure 4.7- 4.9). Antioxidant activity was seen from plant extracts obtained with ethyl acetate, acetone, and methanol in series 1 which were visible in mobile phase BEA followed by CEF (Figure 4.7). With EMW, antioxidant activity was not detected (Figure 4.7), which suggests that the antioxidant compounds present in *S. pinnata* extracts are of non-polar and intermediate polarities. In series 2, the presence of antioxidant compounds was observed with ethyl acetate, acetone, and methanol extracts from solvent system BEA, while in solvent system CEF, antioxidant activity was observed from acetone and methanol extracts (Figure 4.8). In series 3, the presence of antioxidant compounds was observed from methanol and acetone extracts separated in BEA, while in CEF only methanol extracts showed activity of low intensity with no activity observed in EMW (Figure 4.9). The antioxidant compounds could not move from the bottom of the plates in all the series, which is consistent with previous report (Katerere and Eloff, 2005). The recovery of antioxidant compounds from medicinal plants is said to be accomplished through different extraction

procedures and solvents (Sultana *et al*., 2009). This was also observed from the results of this study, where there was variation in polarity of antioxidant compounds amongst the extracts.

The free radical scavenging activity of *S. pinnata* extracts were evaluated quantitatively using DPPH solution. This provides a useful understanding of the reactivity of compounds with stable free radicals within the plant extracts. Vitamin C was used as a positive control for comparison with extracts. It had high scavenging activity at all concentrations as compared to the extracts (Figure 4.10). It was observed that the antioxidant activity of the plant extract increased in a concentration dependent manner. Methanol extracts showed the best scavenging activity at all concentrations, with concentration of 0.02 mg/ml higher than that of the control. Methanol extracts from *Asteraceae* family have been reported to have high radical scavenging activity (Candan *et al*., 2003; Stanojevic *et al*., 2009). This could be due to the presence of phenolic compounds which were detected in high amount (Table 3.2). Methanol is reported to be a good solvent to extract a number of phytochemicals including phenolic compounds which have good antioxidant activity (Rao *et al*., 2013). Acetone extracts indicated good scavenging activity at 10 mg/ml and 2.5 mg/ml concentrations and at the lower concentration (0.02 mg/ml) showed low scavenging activity. Dichloromethane showed the highest activity in extracts at high concentration and at lower concentration. n-Hexane showed scavenging activity at high concentration which was higher than that of dichloromethane extracts at higher concentration. Masevhe *et al*. 2012 found that *S. pinnata* was one of the plants which indicated weak antioxidant activity. This could be due to environmental condition where plant materials were collected, extraction procedure and experimental conditions.

Researchers have discovered that phenolic compounds are responsible for the antioxidant activities from plants (Fabri *et al*., 2009). Polyphenolic compounds such as saponins, tannins and flavonoids detected in *S. pinnata* extracts (Table 3.1) are considered to be the most effective contributors to the antioxidant activity of medicinal plants (Scio *et al*., 2012). There is a need for more research on *S. pinnata* since there is not much information on the study about its biological activities in qualitative assays. The qualitative and quantitative results indicate contradiction because the observed antioxidant activity from serial exhaustive extraction was from ethyl acetate and acetone extracts while methanol extracts did not show any activity. This could be due

to synergism of phytochemicals, since in the qualitative assay the antioxidant compounds are individually active whereas in the quantitative assay they could have synergistic mechanism. Another factor could be the separation of compounds on TLC plate that may disrupt the phytochemicals activity.

Ferric ion reducing power was used to analyse the ability of antioxidant compounds to reduce the ferric ion into ferrous complex. The increase in the absorbance of reaction mixture indicated a degree of reducing power of plant extracts (Vijayalakshmi and Ruckmani, 2016). The ferric reducing power of plant extracts is directly propotional to the positive control. Plant extracts exhibited a reducing power at low concentration of 0.0625 mg/ml at absorption of 0.495 nm which was close to that of ascorbic acid (0.765 nm). The plant extracts reducing power increased in a concentration depended manner. The results of radical scavenging and reducing power assays show a positive correlation with total phenol content which was found to be high (Table 3.2). These phytochemicals have been reported to possess antioxidant activity (Sulaiman and Balachandran, 2012). Phenolic acids are natural antioxidants found in fruits and vegetables which exhibit a number of therapeutic properties including antioxidation (Balasundram *et al*., 2006). Kaur *et al*. (2014) encouraged the evaluation of total phenol content in order to determine the relationship between antioxidant activity and the phytochemicals.

### 4.5. Conclusion

The observed antioxidant activity indicates that the polarity of the compounds depends on the extractants used in extraction procedure. Solvent system EMW indicates the polarity range of solvents that can be used in isolation of antioxidant compounds. Both qualitative and quantitative antioxidant analysis was found to positively correlate with the total phenol content of the plant extracts. The ferric reducing power indicates high reducing capacity of samples in a concentration dependent manner. Isolation of antioxidant compounds is required for development of natural antioxidant supplements which are regarded safe as compared to synthetic antioxidants.

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# Chapter 5

# 5. Antimycobacterial assays

## 5.1. Introduction

South Africa and other developing countries have a number of immune- compromised patients as a result of HIV/AIDS, tuberculosis, cancer, diabetes, organ transplantation and other opportunistic infections caused by *M. smegmatis* (Mphande, 2016). Patients with these infections face challenges such as prolonged antibiotic therapy, drug resistance to microorganisms, toxicity and high cost of drugs (World Health Organisation, 2002; Green *et al*., 2010). This therefore triggers the need to develop alternative drugs from different medicinal agents such as medicinal plants (Masoko *et al*., 2008). Medicinal plants have been used by human for centuries and the study of these plants in the fight against infections has a great impact towards the development of new drugs with less or no side effects, affordable and readily available to patients. The World health organisation reported that out of 121 drugs prescribed in the whole world and about 11% are developed from medicinal plants (Rates, 2001).

South Africa is considered as a rich source of medicinal plants and contains about 10% of the world's terrestrial plants, some of which possess medicinal properties (Arnold *et al*., 2002). Rosakutty and Roslin (2000) reported that medicinal plants are the greatest source of antimicrobial drugs. The challenge of passing on the information orally from one generation to another remains a big obstacle in retaining the information on medicinal plants in developing countries. It therefore imposes the importance of medicinal plants information recording, scientific validation, and their biological activities to be assessed (Houghton *et al*., 2005; Sasidharan *et al*., 2011). Reports indicate that several medicinal plants have been evaluated for their antibacterial properties (Lall and Meyer 1999; Cowan, 1999; Mativandlela *et al*., 2008). This study will add to the available reports of some of the recorded and screened medicinal plants with antibacterial effects.

Researchers prefer the use of antibacterial assay that save time, are simple, affordable, and effective in such a way that they can allow them to work with a number of extracts and fractions (Hostettman *et al*., 1997). Some of the assays commonly used by researchers are disk-diffusion and broth or agar dilution methods (Balouiri, 2016). In this study, TLC-bioautography and broth microdilution assays were used. In

TLC-bioautography, TLC plates were spotted with plant extracts, separated with three solvent systems (BEA, CEF and EMW) and sprayed with bacterial culture in broth. Thereafter, incubated and sprayed with a reagent ρ-iodinitrotetrazolium violet (INT) to detect the presence of antimycobacterial compounds from the plant extracts.

Broth microdilution assay involves the use of minimum inhibitory concentration (MIC) procedure developed by Eloff (1998) for quantitative analysis of antimicrobial activity of crude plant extracts and pure compounds. The procedure is carried out in a microtiter plate (96 well plates), where the plants extract is serially diluted, then treated with the bacterial culture in broth. Thereafter, a reagent ρ-iodinitrotetrazolium violet is administered to indicate the inhibition of the bacterial growth by plant extracts. This indicator is converted into a pink coloured formazan by the living bacteria in the culture and the positive result is indicated by clear wells. The recorded MIC value is the lowest concentration that inhibits the bacterial growth (Eloff, 1998). The total activity shows the highest volume at which the dried extracts (1 g) can be diluted to and still inhibit the growth of the bacteria (Eloff, 2000).

## 5.2. Methods and material

### 5.2.1. Bioautography assay

Qualitative antimycobacterial activity was evaluated following a procedure described by Begue and Kline (1972). The TLC plates were prepared as described in section 3.2.3, where 20 µl of plant extracts was loaded on the plates and they were developed in three solvent systems (BEA, CEF and EMW), and left in a stream of air at room temperature to dry the solvent system for 4-5 days. *M. smegmatis* was inoculated in 225 ml of middle-brook broth and incubated in a shaking incubator for 24 hours at 37ºC and used as a stock culture. About 10 ml of stock culture was further inoculated in 225 ml of freshly prepared broth and incubated for 24 hours at 37ºC. After the solvent systems had evaporated, the plates were sprayed with the bacterial culture and incubated at 37ºC for 24 hours. Thereafter, TLC plates were sprayed with 2 mg/ml INT in distilled water and incubated further for 2-3 hours. The plates were observed for inhibition of bacterial growth.

### 5.2.2. Broth microdilution assay

The lowest concentration of *S. pinnata* extracts to inhibit the growth of *M. smegmatis* was evaluated quantitatively using MIC procedure as described by Eloff (1998). Plant extracts were reconstituted in acetone to make a final concentration of 10 mg/ml in 96

well microtiter plates. Hundred µl of water was added in each well and 100 µl of plant extracts was added only in the first wells. The plant extracts from the first wells were serially diluted (50%). Thereafter, 100µl of bacterial culture was added to all wells and the plates were incubated at 37ºC for 24 hours. Acetone was used as a negative control and rifampicin as a positive control. After incubation, inhibition of *M. smegmatis* growth was indicated by clear wells and growth was indicated by formation of pink colour (Eloff, 1998).

## 5.3. Results

### 5.3.1. Preliminary extraction

Bioautography assay was used to evaluate the presence of antimycobacterial compounds in *S. pinnata* extracts using aluminium-baked TLC plates. The white bands against a pink background indicated the presence of antimycobacterial compounds which were able to inhibit the growth of *M. smegmatis* (Figure 5.1). A good antimycobacterial compound separation was observed in solvent system BEA followed by CEF and in EMW the compounds moved with the solvent front. the was antimycobacterial activity observed from n-hexane extracts.

Figure 5.1: Bioautograms of *S. pinnata* extracts separated in solvent systems BEA (top), CEF (middle) and EMW (bottom), sprayed with *M. smegmatis* culture in broth and INT reagent. Lanes from left to right: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E) and methanol (M).

### 5.3.2. Serial exhaustive extraction

The extracted antimycobacterial compounds were observed from chloroform, dichloromethane and ethyl acetate extracts (Figure 5.2). Antimycobacterial activity was not observed in ethyl acetate extracts separated in solvent system EMW.

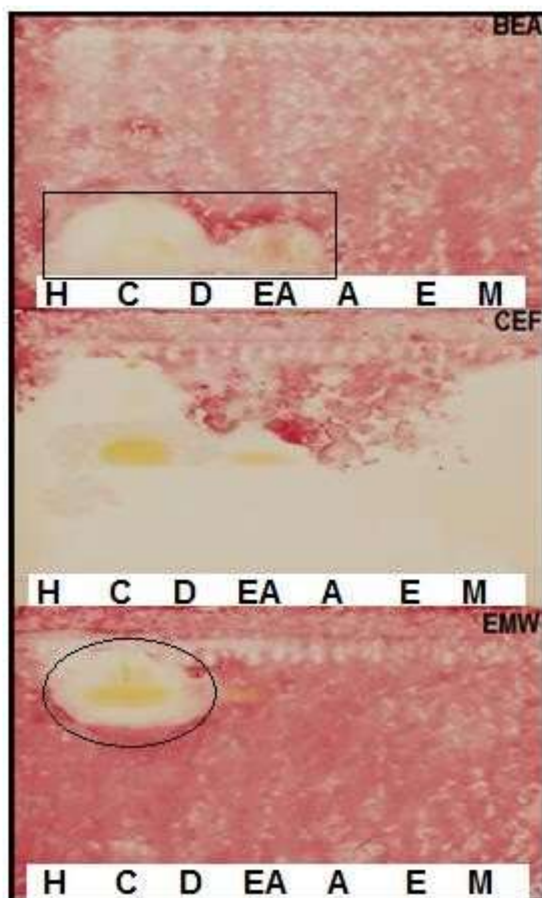


Figure 5.2: Bioautograms of *S. pinnata* extracts extracted with solvents of varying polarity developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with *M. smegmatis* culture in broth. Lanes from left to right: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E) and methanol (M).

### 5.3.3. Extraction enrichment procedure

n-Hexane wash

Antimycobacterial activity was observed with the ethanol extracts separated in CEF solvent system (Figure 5.3), while there was no activity observed with n-hexane and acetone extracts in all solvent system.

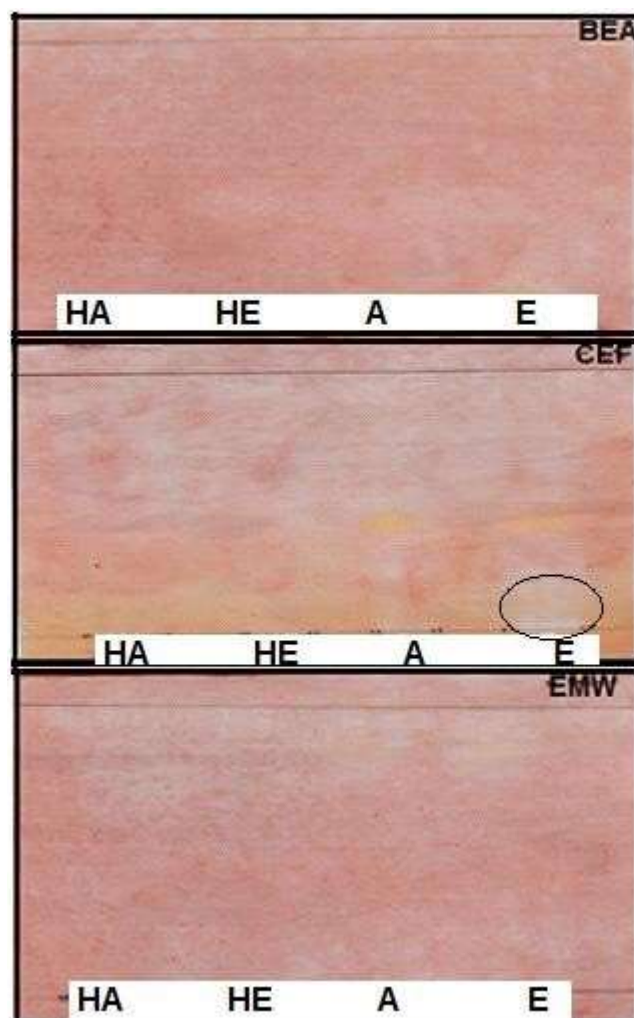


Figure 5.3: Bioautograms of *S. pinnata* extracts developed in BEA (top), CEF (middle) and EMW (bottom) and sprayed with *M.smegmatis* culture. Lane from left to right: n-hexane wash for acetone (HA), n-hexane wash for ethanol (HE), acetone (A) and ethanol (E).

Acetone and ethanol in water

*S. pinnata* extracts indicated antimycobacterial activity with all extracts excluding water extracts (Figure 5.4). A good separation of antimycobacterial compounds was observed in CEF solvent system while in EMW, the compounds were near the solvent front.

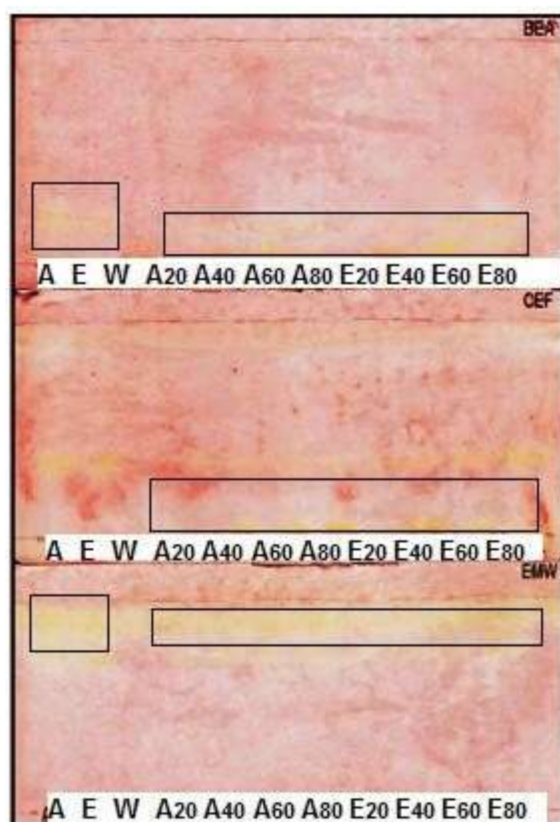


Figure 5.4: Bioautograms of *S. pinnata* extracts separated in solvent system BEA (top), CEF (middle), and EMW (bottom), sprayed with *M.smegmatis* culture and INT reagent. Lanes from left to right: Acetone (A), ethanol (E), water (W), 20% acetone (A20), 40% acetone (A40), 60% acetone (A60), 80% acetone (A80), and 20% ethanol (E20), 40% ethanol (E40), 60% ethanol (E60), 80% ethanol (E80).

### 5.3.4. Optimal extraction procedure

Pretreatment

Antimycobacterial activity was observed from 20% acetone and 20% ethanol extracts which were separated in CEF solvent system followed by BEA while in EMW the compounds moved with the solvent front (Figure 5.6).

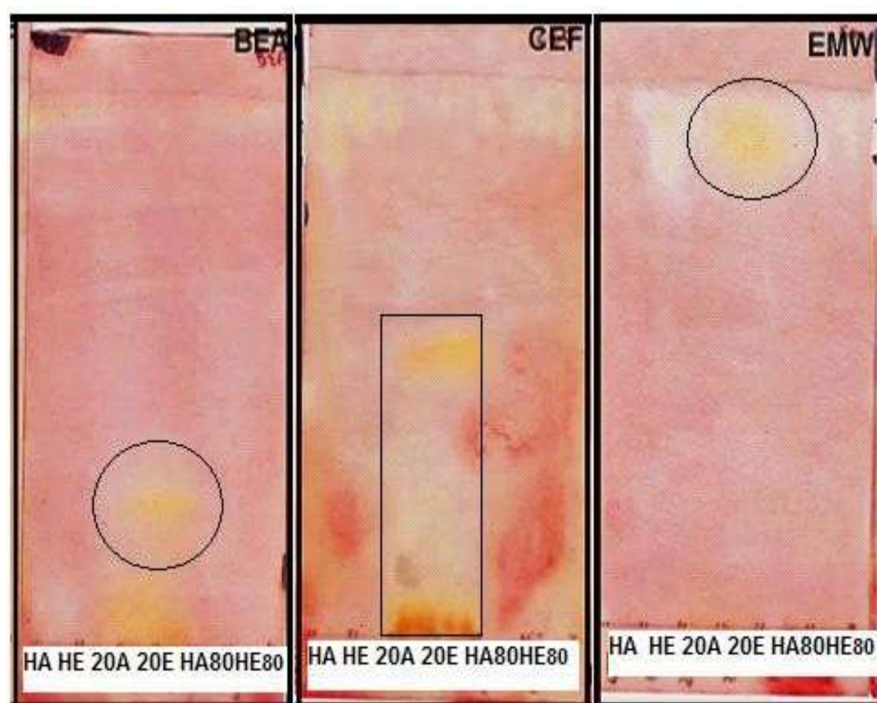


Figure 5.5: Bioautograms of the plant extracts extracted with various extractants, separated in solvent system BEA (left), CEF (middle), and EMW (right) and sprayed with *M. smegmatis* culture. Lanes from left to right: n-hexane wash for acetone (HA), n-hexane wash for ethanol (HE), 20% acetone (A20), 20% ethanol (E20), n-hexane wash for 80% acetone (HA80), n-hexane wash for 80% ethanol (HE80).

Extraction of pretreated sample

The plant extracts showed the presence of antimycobacterial compounds which were best separated in CEF and BEA solvent systems while in EMW the compounds moved with the solvent front (Figure 5.6).

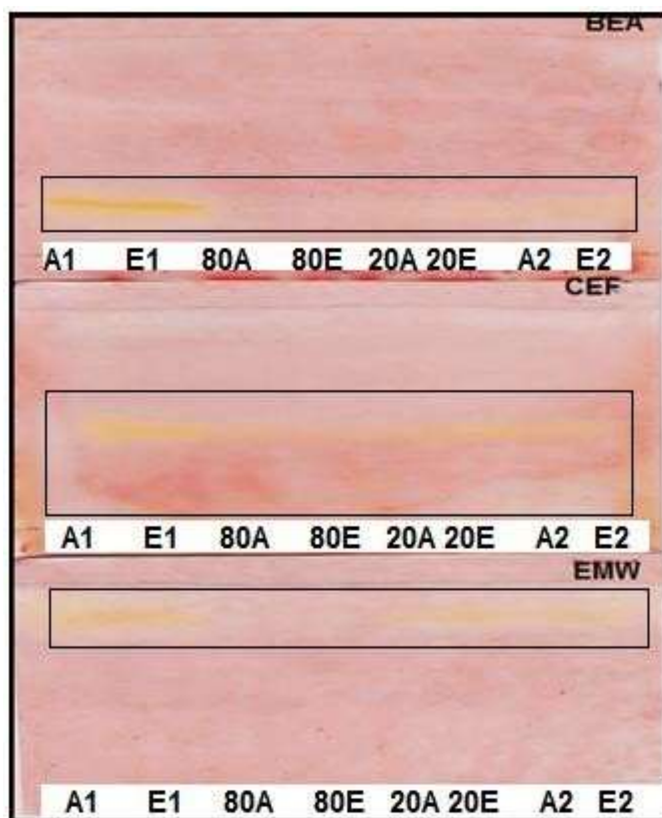


Figure: 5.6: Bioautograms of *S. pinnata* extracts separated in solvent system BEA (top), CEF (middle), and EMW (bottom) and sprayed with *M. smegmatis* culture in broth. Lanes from left to right: n-hexane wash (A1), ethanol after n-hexane wash (E1), 80% acetone (80A), 80% ethanol (80E), 20% acetone (20A), 20% ethanol (20E), acetone after 20% acetone wash (A2), and ethanol after 20% ethanol wash (E2).

#### 5.3.5. Preliminary serial exhaustive extraction series (1, 2, 3)

Antimycobacterial activity was evaluated on aluminium-baked TLC plates which were separated in three solvent systems (BEA, CEF, and EMW). The plant extracts indicated the presence of antimycobaterial compounds by the development of white area against the pink background (Figure 5.7 to 5.9). It was observed that from all chromatograms, dichloromethane, ethyl acetate, and acetone extracts indicated the presence of antimycobacterial compounds (Figure 5.7 to 5.9).

Series 1

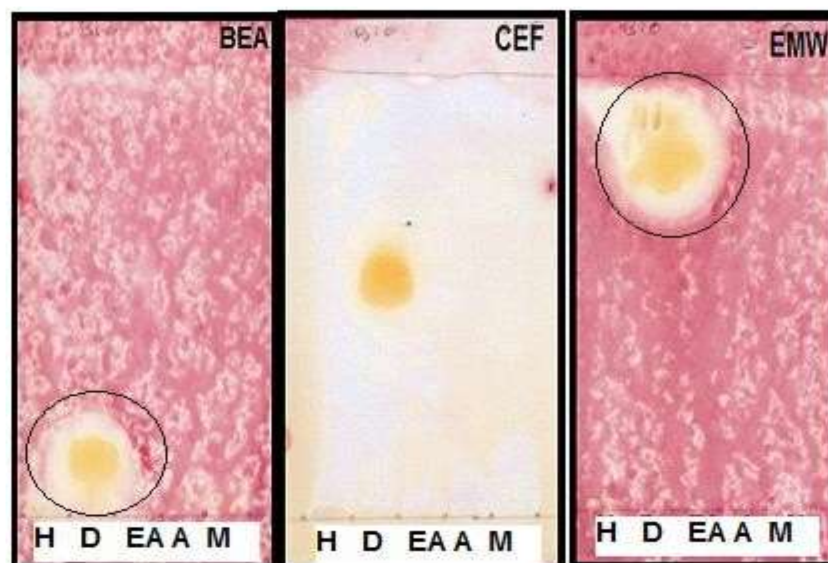


Figure 5.7: Bioautograms of *S. pinnata* extracts developed in three solvent systems BEA (left), CEF (middle) and EMW (right), sprayed with bacterial culture and 2 mg/ml INT reagent. Lanes from left to right: n-hexane (H), dichloromethane (D), ethyl acetate (EA), acetone (A) and methanol (M).

Series 2

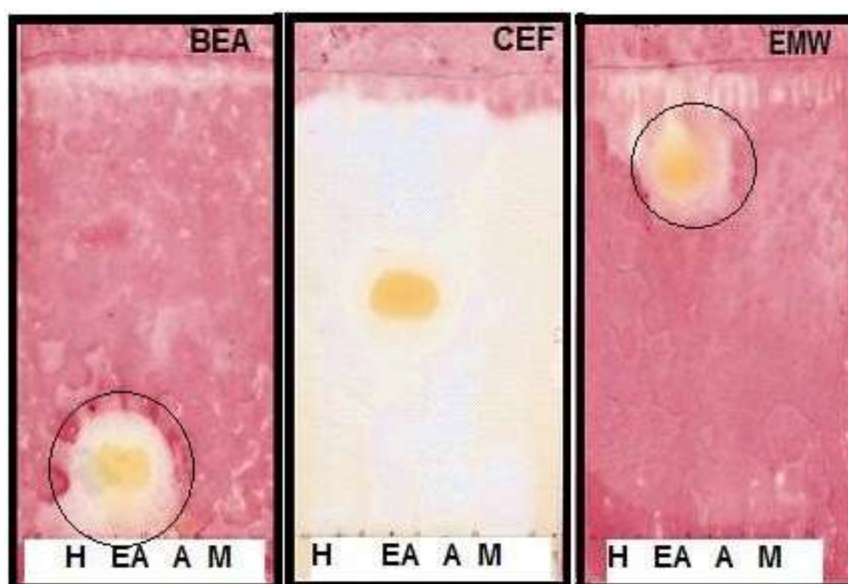


Figure 5.8: Bioautograms of *S. pinnata* extracts developed in three solvent systems BEA (left), CEF (middle) and EMW (right), sprayed with bacterial culture and 2 mg/ml INT reagent. Lanes from left to right: n-hexane (H), ethyl acetate (EA), acetone (A) and methanol (M).

Series 3

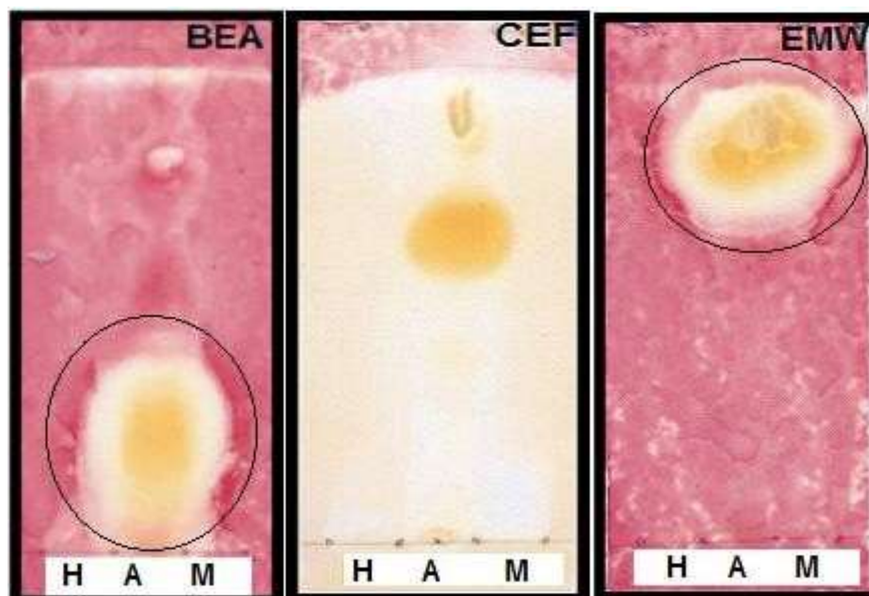


Figure 5.9: Bioautograms of *S. pinnata* extracts developed in three solvent systems BEA (left), CEF (middle) and EMW (right), sprayed with bacterial culture and INT reagent. Lanes from left to right: n-hexane (H), acetone (A) and methanol (M).

### 5.3.6. Determination of minimum inhibitory concentration values

*S. pinnata* extracts obtained through various extraction procedures coupled with solvents of varying polarities as indicated in table 5.1 to 5.5. The total activity values were determined by dividing the mass of the plant extracts by the MIC value (Eloff, 2000). It was observed from table 5.1-5.5, that the lower the MIC values the higher the total activity. Minimum inhibitory concentration values of various extracts were determined using broth microdilution assay where the extracts were serially diluted in 96 well microtiter plates:

5.3.6.1. Preliminary extraction

The lowest MIC value was observed with chloroform and dichloromethane extracts (0.43 mg/ml) which had the highest total activity. Rifampicin had the lowest MIC value (0.08 mg/ml) (Table 5.1).

Table 5.1: Minimum inhibitory concentration (MIC) (mg/ml) and total activity (ml/g) values of *S. pinnata* extracts obtained with preliminary extraction procedure.

| Plant extracts and MIC values (mg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|
| H | C | D | EA | A | E | M | Rifampicin |
| 1.46 | 0.43 | 0.43 | 0.64 | 1.25 | 2.5 | 2.5 | 0.08 |
| Total activity values (ml/g) | | | | | | | |
| H | C | D | EA | A | E | M | |
| 45.2 | 376.7 | 355.8 | 176.6 | 100 | 40.4 | 58.8 | |

5.3.6.2. Serial exhaustive extraction

MIC of plant extracts obtained with acetone indicated the lowest inhibitory concentration value (0.27 mg/ml) and total activity of 162.9 ml/g while n-hexane had the lowest MIC and total activity values (Table 5.2). The highest total activity value was obtained with dichloromethane extracts (D) (3453.1 mg/ml).

Table 5.2: The minimum inhibitory concentration in mg/ml and total activity ml/g values of *S. pinnata* extracts obtained with serial exhaustive extraction procedure.

| Plant extracts and MIC values (mg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|
| H | C | D | EA | A | E | M | Rifampicin |
| 2.5 | 0.43 | 0.32 | 0.32 | 0.27 | 0.37 | 0.53 | 0.08 |
| Total activity values (ml/g) | | | | | | | |
| H | C | D | EA | A | E | M | |
| 69.6 | 890.7 | 3453.1 | 143.8 | 162.9 | 305.4 | 194.3 | |

#### 5.3.6.3. Extraction enrichment procedure

*S. pinnata* extracts had the lowest MIC values with 80% ethanol (0.64 mg/ml), 60% acetone (0.64 mg/ml) and 80% acetone (0.64 mg/ml), while high total activity was obtained with 40% acetone (Table 5.3).

Table 5.3: Determination of minimum inhibitory concentration (mg/ml) and total activity (ml/g) of *S. pinnata* extracts obtained with extraction enrichment procedure.

| Plant extracts and MIC values (mg/ml) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HA | HE | A | E | Ace | Eth | 20A | 40A | 60A | 80A | 20E | 40E | 60E | 80E | Rifampicin |
| 2.5 | 2.5 | 0.74 | 1.03 | 1.25 | 2.5 | 1.67 | 0.84 | 0.64 | 0.64 | 2.5 | 1.67 | 1.25 | 0.64 | 0.08 |
| Total activity (ml/g) | | | | | | | | | | | | | | |
| HA | HE | A | E | Ace | Eth | 20A | 40A | 60A | 80A | 20E | 40E | 60E | 80E | |
| 21.6 | 22 | 86.5 | 66.02 | 51.2 | 21.6 | 63.5 | 152.4 | 151.7 | 118.6 | 42 | 59.9 | 77.6 | 76.6 | |

#### 5.3.6.4. Optimal extraction

Acetone extracts had the lowest MIC value of 0.27 mg/ml and high total activity value of 207.4 ml/g. n-Hexane extracts had high MIC (2.5 mg/ml) and lowest total activity (14.4) (Table 5.4). However, the highest total activity was observed with 80% acetone extracts (256.6 mg/ml).

Table 5.4: Determination of minimum inhibitory concentration (MIC) values (mg/ml) and total activity values (ml/g) of *S. pinnata* extracts obtained with optimal extraction procedure.

| Plant extracts and MIC values(mg/ml) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HA | HE | $A_{20}A_{80}$ | $E_{20}E_{80}$ | $HA_{20}$ | $HE_{20}$ | A1 | E1 | $A_{80}$ | $E_{80}$ | $A_{20}$ | $E_{20}$ | A2 | E2 | Rifampicin |
| 2.5 | 1.25 | 0.64 | 0.43 | 1.26 | 1.25 | 0.27 | 0.37 | 0.53 | 0.64 | 2.08 | 2.5 | 0.43 | 1.25 | 0.08 |
| Total activity (ml/g) | | | | | | | | | | | | | | |
| HA | HE | $A_{20}A_{80}$ | $E_{20}E_{80}$ | $HA_{20}$ | $HE_{20}$ | A1 | E1 | $A_{80}$ | $E_{80}$ | $A_{20}$ | $E_{20}$ | A2 | E2 | |
| 14.4 | 24.8 | 53.13 | 53.5 | 63.5 | 24.8 | 207.4 | 162.2 | 256.6 | 162.5 | 57.2 | 31.2 | 209.3 | 141.6 | |

5.3.6.5. Preliminary serial exhaustive extraction (series 1, 2, 3)

In series 1 and 2, ethyl acetate extracts had the lowest MIC values with the highest total activity while in series 3, acetone extracts had the lowest MIC value and high total activity (Table 5.5).

Table 5.5: Determination of minimum inhibitory concentration (MIC) values (mg/ml) and total activity values (ml/g) of *S. pinnata* extracts obtained with preliminary serial exhaustive extraction procedure

| Plant extracts and MIC values (mg/ml) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Series 1 | | | | | Series 2 | | | | Series 3 | | | Control |
| H | D | EA | A | M | H | EA | A | M | H | A | M | Rifampicin |
| 2.5 | 0.64 | 0.43 | 1.05 | 1.46 | 2.08 | 0.94 | 1.05 | 1.46 | 1.67 | 0.64 | 1.25 | 0.08 |
| Total activity (ml/g) | | | | | | | | | | | | |
| H | D | EA | A | M | H | EA | A | M | H | A | M | |
| 80.8 | 795.3 | 302.3 | 48.6 | 179.5 | 100.9 | 558.5 | 181.9 | 289.7 | 76.6 | 328.1 | 274.4 | |

## 5.4. Discussion

There is an increase in diseases caused by bacteria and other pathogenic microorganisms, and is thus pertinent to develop new drugs from medicinal plants as alternatives (Jones *et al*., 2008). Plants serve as a good source of phytochemicals which can be used as new lead in development of new alternative drugs (Ahmad *et al*., 2006). Eldeen and van Staden (2007) reported that over 350 plant species have been screened for antimycobacterial activities. *S. pinnata* was selected based on its uses in traditional medicine. The plant extracts were screened for antimycobacterial activity with the aim of isolation and characterisation of compounds which have desired biological activity.

The white spot on TLC plates against the pink background indicated bacterial inhibition zone. The pink colour represents the living bacterial cells that converted tetrazolium salt (INT) reagent, into a pink coloured formazan (Bugue and Kline, 1972). Antimycobacterial activity was observed from the bioautograms separated in solvent system BEA and CEF while in EMW the compounds moved with the solvent front (Figure 5.1). This could be due to the polarity of the antimycobacterial compounds. It has been indicated that *S. pinnata* extracts have positive antibacterial properties (Bussmann *et al*., 2008). In serial exhaustive extraction, phytochemicals which were serially extracted with chloroform, dichloromethane and ethyl acetate showed the antimycobacterial activity as observed in solvent system BEA, CEF and EMW (Figure 5.2). In solvent system CEF (Figure 5.2) the antimycobacterial compounds were separated and the observed reaction of white area at the bottom of the plate could be due to traces of formic acid that might have not evaporated and had a killing effect on microorganisms (Masoko and Eloff, 2006). Comparing compounds with antimicrobial activity with those that exhibited antioxidant activity, it shows that compounds with antioxidant activity were not responsible for the antibacterial activity observed (Figure 4.2 and 5.2). However, ethyl acetate extracts indicated good antioxidant and antimycobacterial compounds separated with deferent solvent systems (Figure 4.2 and 5.2). n-Hexane wash removes waxes and chlorophyll which expose more compounds that can be dissolved with other solvents (Sasidharan *et al*., 2011). Plants extracts obtained with ethanol showed the presence of antibacterial compounds from the bioautograms developed in the intermediate polar solvent system CEF (Figure 5.3) while there was no activity with hexane and acetone extracts. This could be due to

fatty acid and chlorophyll extracted which did not have antimycobacterial activity on hexane extracts and the compounds in acetone extracts could be active in a synergistic reaction. Acetone and ethanol in water extracts indicated the presence of antimycobacterial compounds in all solvent systems (Figure 5.4). However, it was observed that there was no activity with water extracts. Water has been reported to extract polar compounds (Gupta *et al.*, 2012). The antibacterial activity from *S. pinnata* extracts has been reported against Gram-positive microorganisms (Bussmann *et al.*, 2010; Suliman, 2010).

Pretreated plant extracts showed the presence of antibacterial compounds in 20% acetone and 20% ethanol extracts separated in solvent system BEA and CEF while in EMW solvent system the compounds moved with the solvent front (Figure 5.5). This indicates the polarity of antimycobacterial compounds from *S. pinnata* extracts. It was shown that antimycobacterial activity increased after the plant residues were pretreated (Figure 5.6) which is indicative that the antimycobacterial compounds in the plant extracts were unmasked after pretreatment. Kotze and Eloff, 2002 reported that combination of water and other solvents results in dissolving compounds which have saponin-like properties. Secondary metabolites such as tannins and terpenes are also reported to possess antibacterial activity which might be the compounds responsible for the observed S*. pinnata* antimycobacterial activity (Doughari, 2012).

Extraction of *S. pinnata* extracts using the series extraction procedures was done in order to select the best solvents to extract antimycobacterial compounds from large amount of plant material. Plant extracts extracted with dichloromethane, ethyl acetate and acetone from all bioautograms (Figure 5.7- 5.9) indicated the presence of antimycobacterial compounds. The bacterial culture could not grow properly in solvent system CEF (Figure 5.9), this happens when the formic acid couldn't evaporate completely from TLC plates, which has been reported to be toxic on microorganisms (Masoko and Eloff, 2006). The observed results suggest that antimycobacterial compounds from *S. pinnata* are of intermediate polarity since dichloromethane, ethyl acetate and acetone have been reported to extract compounds of intermediate polarities (Gupta *et al.*, 2012). This is supported by the masses of extracts obtained with all extraction procedures which were high for solvents of intermediate polarity (chapter 3).

Polyphenols are compounds which have been reported to possess antimicrobial properties in some medicinal plants (Cushnie and Lamb, 2005; Karou *et al.*, 2006; Amusan *et al.*, 2007). The total activity of plant extracts was considered in this study as suggested by Eloff (2000). Rifampicin is an antibiotic drug used in the treatment of infectious diseases such as cancer, leprosy and tuberculosis and in this study, it was used as appositive control (Kumar, 1998; Asif, 2013).

In preliminary extraction procedure the lowest MIC value (0.43 mg/ml) was obtained with dichloromethane and chloroform extracts, while methanol and ethanol extracts had high MIC value of 2.5 mg/ml (Table 5.1). Their total activity was found to be high indicating that the lower the MIC value the higher the total activity. In serial exhaustive extraction, acetone extracts (0.27 mg/ml), followed by chloroform (0.32 mg/ml), dichloromethane (0.32 mg/ml) and ethyl acetate (0.37 mg/ml) extracts had the low MIC values and high total activity values (Table 5.2). This suggests that *S. pinnata* bioactive compounds are of nonpolar and intermediate polarity. The total activity values observed depended on the mass of the extracts. This can be seen from the acetone extracts which had the lowest MIC value (0.27 mg/ml) and low total activity value (100 ml/g) as compared to chloroform extracts which had MIC value of 0.43 mg/ml and total activity of 890.7 ml/g (Table 5.2).

In n-hexane wash procedure, n-hexane extracts had high MIC value (2.5 mg/ml) while acetone had the lowest MIC value of 0.74 mg/ml. It was shown that after n-hexane wash, more antimycobacterial compounds were obtained with mixtures of acetone in water ranging from 0.64 mg/ml and 0.84 mg/ml, with high total activity values (Table 5.3). From optimal extraction procedure, high total activity values were obtained with acetone and ethanol extracts which had the lowest MIC values (Table 5.4) while the in series procedure, extracts obtained from solvents with intermediate polarity had high total activity and low MIC values from all series (Table 5.5).

From table 5.5, series 1 ethyl acetate extracts had the lowest inhibitory concentration (0.43 mg/ml) followed by dichloromethane extracts (0.64 mg/ml) with high total activity values as compared to other extractants. In series 2, ethyl acetate extract indicated a low MIC value (0.94 mg/ml) and high total activity value of 558.5 ml/g. In series 3, acetone extracts indicated the lowest inhibitory concentration of (0.64 mg/ml) and a total activity value of 328.1 ml/g. The MIC values obtained with the series greatly

correlates with the bioautography results. Mupfure *et al*. (2014) found that *S. pinnata* extracts had the lowest MIC values against mastitis pathogens. The antimycobacterial activity observed from *S. pinnata* extracts was not surprising as other species belonging to the family *Asteraceae* have been reported to have antibacterial properties (Boussaada *et al*., 2008); Borkataky *et al.,* 2013: Gakuubi *et al*., 2016) The detected phytochemicals in this study are associated with various biological activities including antimycobacterial activity observed with *S. pinnata* extracts.

The antimycobacterial results of both assays indicated that antimycobacterial compounds detected in *S. pinnata* are of non-polar and intermediate polarity. Masoko and Eloff (2005) also reported that the antimicrobial compounds behind the antibacterial activity are relatively non-polar in nature. Lack of correlation between some bioautography chromatograms and broth microdilution results could be due to possible loss of active compounds during evaporation of solvent systems from TLC plates, and antagonistic or synergistic effect of active compounds (Tylor *et al*., 2001; Masoko and Eloff, 2006; Mdee *et al*., 2009).

### 5.6. Conclusion

*S. pinnata* extract showed an effective antimycobacterial activity under serial exhaustive extraction procedure which makes it the extraction procedure of choice in isolation and characterisation of the active compounds. The observed results indicated that the plant extracts contain antimycobacterial phytochemicals of non-polar and intermediate polarity. Antimycobacterial properties observed indicated that *S. pinnata* has the potential to serve as a source of lead candidates for development of new antimycobacterial drugs.

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# Chapter 6

## 6. Isolation, purification and characterisation of bioactive compounds

### 6.1. Introduction

Plants offer variety of therapeutic agents used in treatment of several diseases and they play a significant role in development of new drugs (Newman and Cragg, 2012). Secondary metabolites such as alkaloids, terpenoids and phenolic compounds have therapeutic properties which can be used in development of new drugs (Brusotti *et al.*, 2014). Medicinal plants possess phytochemical compounds in combination, which poses a challenge to isolate, purify and characterise a single bioactive compound of interest. Various authors have highlighted an overview on sample preparation, isolation and characterization of the compounds to simplify the challenges faced during medicinal plants analysis (Chan *et al.*, 2011; Ciesla, 2012).

Analysis of medicinal plants requires an important step of extracting the bioactive compounds (Sasidharan *et al.*, 2011). Even though development of modern chromatographic and spectrometric techniques has simplified the analysis of bioactive compounds, however, extraction is still a significant aspect to be considered including the nature of plant parts used (Huie, 2002). Serial exhaustive extraction is an extraction procedure that fractionates crude extracts without introducing chemical change from non-polar to highly polar solvents (Tiwari *et al.,* 2011). Different solvents extract a wide range of various compounds with varying polarities (Zishiri, 2005).

Techniques used in isolation and purification of compounds include thin layer chromatography, column chromatography and high performance liquid chromatography (HPLC). Isolation of compounds using bioassay-guided fractionation method involves repetitive fractionation and assessment of biological activities up to the identification of the bioactive compound (Zishiri, 2005). In this study, thin layer chromatography and column chromatography techniques have been used to isolate antimycobacterial compounds. Antimycobacterial activity observed in preliminary work (Chapter 5) governs the isolation of the bioactive compounds. From the three series in preliminary serial exhaustive extraction (Chapter 3), series 1 was chosen as the first fractionation step in the isolation of antimycobacterial compounds from *S. pinnata* extracts. Different solvents with varying polarity have been used for better fractionation

and as eluents (Snyder and Kirkland, 1979). In this study, the following solvents were used in extraction and elution process: n-hexane, chloroform, dichloromethane, ethyl acetate, acetone and methanol.

## 6.2. Methods and materials

### 6.2.1. Serial exhaustive extraction

*Schkuhria pinnata* material was serially extracted by weighing 1.2 kg in a bottle and about 5 L of n-hexane solvent was transferred into the bottle. The bottle was vigorously shaken overnight at high speed (200 rpm) in a series 25 shaking machine (New Brunswick scientific. co., INC). The procedure was repeated two times for three hours with the same solvent and plant residues. The mixture was filtered and the solvent was concentrated using a rotary evaporator set at 50ºC. Thereafter, the same plant residues were exhaustively extracted with dichloromethane, ethyl acetate, acetone and methanol respectively. The plant extracts were transferred into preweighed beakers and placed under a stream of air at room temperature to dry up.

### 6.2.2. Phytochemical profiles

The phytochemical constituent's profiles were analysed on aluminium-backed TLC plates. The plates were prepared as described in section 3.2.3.

### 6.2.3. Antioxidant Assay

Evaluation of antioxidant compounds was done on aluminium-backed TLC plates following the procedure described in section 4.2.1.

### 6.2.4. Antimycobacterial assays

*M. smegmatis* culture used in bioautography and broth microdilution assay was prepared and maintained as mentioned in section 5.2.

#### 6.2.4.1. Bioautography

Bioautography assay was conducted as described in section 5.2.1.

#### 6.2.4.2. Broth microdilution assay

The lowest concentration to inhibit the growth of *M. smegmatis* was determined using broth dilution method as described in section 5.2.2.

### 6.2.5. Column chromatography

#### 6.2.5.1. Open column chromatography

Column chromatography was used in isolation, separation and purification of antimycobacterial compounds from *S. pinnata*. An open column (35 x $4^2$ cm) was packed with silica gel 60 (particle size 0.063-0.200 mm) (Fluka) mixed with 100% n-hexane. From the observed results antimycobacterial activity was from dichloromethane extracts (102.88 g) which was mixed with a small portion of silica gel and suspended into the column. The column was eluted with various solvent system listed in table 6.1 while collecting fractions. The fractions were concentrated with rotary evaporator set at 50ºC and the extracts were dried under a stream of air at room temperature. Collected plant extracts were reconstituted to a final concentration of 10 mg/ml. Thereafter, the plants extracts were screened for phytochemical constituents, antioxidant and antimycobacterial activity using aluminium-baked TLC plates as mentioned in section 6.2.3 and 6.2.4 respectively.

#### 6.2.5.2. Second open column chromatography

Plant extracts obtained with 70% n-hexane indicated the presence of antimycobacterial compounds. An amount of 4.6 g of the fraction was mixed with a small amount of silica gel and suspended into a column (33 x $3^2$ cm) packed with silica gel. The column was eluted with 90% chloroform in ethyl acetate, collecting fractions in to the test tubes. The test tubes were dried under a stream of air at room temperature and the plant extracts were evaluated for the presence of antimycobacterial compounds using TLC plates (described in section 6.2.4.1). Antimycobacterial activity was observed on the compounds which could not be evaluated further on column chromatography due to low mass (13.1 mg).

Table 6.1: Different combination of solvents used in elution of open column chromatography.

| Elution solvents | Percentages (%) |
|---|---|
| n-Hexane | 100 |
| n-Hexane: Ethyl acetate | 90:10 |
| | 80:20 |
| | 70:30 |
| | 50:50 |
| | 30:70 |
| | 10:90 |
| Ethyl acetate | 100 |
| Ethyl acetate: Methanol | 90:10 |
| | 80:20 |
| | 70:30 |
| | 60:40 |
| | 50:50 |
| | 40:60 |
| | 10:90 |
| Methanol | 100 |

6.2.5.3 Third open column chromatography

Plants extracts obtained with 10% n-hexane and 100% ethyl acetate from the first column (6.3.1) were combined (43.31 g) and mixed with a small amount of silica gel for it to be suspended into column (33 x $3^2$ cm) packed with silica gel. The column was eluted with 90% chloroform in ethyl acetate while collecting the fractions into the test tubes. Test tubes were dried under a stream of air at room temperature and pulled using TLC plates. Based on the similarity of phytochemical compounds, the combined test tubes made fraction 1 up to 4. The fractions were furtherly analysed for antimycobacterial activity (6.2.4.1) and fraction 4 indicated a good antimycobacterial activity which was taken to the next column.

6.2.5.4. Forth open column chromatography

From the pulled test tubes, fraction 4 (5.13 g) was mixed with a small amount of silica gel and suspended in an open column (32 x $2^2$ cm) packed with silica gel. The column was eluted with 70% ethyl acetate and 30% acetone solvents while collecting the fractions into test tubes. The test tubes were dried under a stream of air at room temperature and pulled to form 5 fractions. Thereafter, phytochemical analysis was done using TLC plates (6.2.2) and antimycobacterial activity was conducted as mentioned in section 6.2.4.

### 6.2.6. Preparative thin layer chromatography

The active fraction was further separated on TLC silica gel glass plates (Merck silica gel 60 F254) with 70% ethyl acetate in acetone. The plates were visualised under 365 nm UV light to trace the compounds on TLC plates. A pencil was used to mark the position of the compounds on the plates. Compounds in acetone were filtered into pre-weighed vials. Purity of isolated compounds was confirmed by the presence of a single band on TLC plate which was sprayed with vanillin sulphuric acid reagent for colour development.

## 6.3. Results

### 6.3.1. Serial exhaustive extraction

After serial exhaustive extraction of the plant material different amounts of plant extracts were obtained. From 1.2 kg of plant material, the obtained total mass of plant extract was 229.72 g (Table 6.2). Dichloromethane yielded the highest amount of the plant extracts (101.88 g) followed by methanol (78.52 g) and the least was from acetone which obtained 4.14 g.

Table 6.2: The masses of *S. pinnata* extracts obtained with different solvents of various polarities.

| **Extractants** | | **Mass of plant extracts (g)** | |
|---|---|---|---|
| | | **Mass** | **Total** |
| n-Hexane | A | 25.88 | |
| | B | 8.98 | 37.86 |
| | C | 3.0 | |
| Dichloromethane | A | 73.37 | |
| | B | 21.72 | 101.88 |
| | C | 7.79 | |
| Ethyl acetate | A | 3.49 | |
| | B | 1.73 | 6.32 |
| | C | 1.10 | |
| Acetone | A | 2.20 | |
| | B | 1.17 | 4.14 |
| | C | 0.77 | |
| Methanol | A | 45.25 | |
| | B | 20.19 | 78.52 |
| | C | 13.08 | |
| | **Total:** | | **229.72** |

Key: A= overnight, B= first three hours and C= last three hours.

### 6.3.2. Phytochemical constituents

The chromatograms revealed better separation of various phytochemicals compounds in all solvent systems; solvent system CEF separated most of the compounds as compared to BEA and EMW (Figure 6.1).

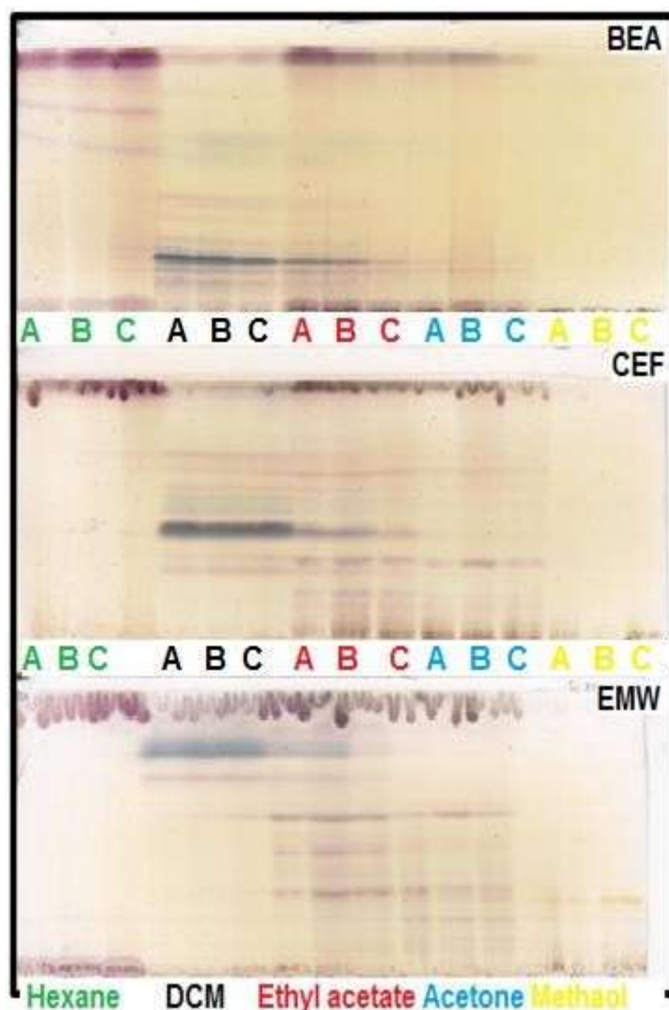


Figure 6.1: TLC profiles of *S. pinnata* extracts extracted with different solvents of varying polarities, separated with three mobile phase BEA (top), CEF (middle) and EMW (bottom) and sprayed with vanillin-sulphuric acid reagent. A= overnight, B= first three hours and C= last three hours.

### 6.3.3. Antioxidant activity

Antioxidant activity of *S. pinnata* plant was screened using TLC plates separated with three solvent systems (BEA, CEF and EMW) and sprayed with 0.2 % DPPH solution. The presence of antioxidant compounds was depicted by the yellow bands against the purple colour which was observed from ethyl acetate, acetone and methanol extracts which could not separate from the bottom of the plate in BEA solvent system (Figure 6.2). It was observed that in solvent system CEF there was antioxidant activity, while in EMW, only the methanol extract was showing the antioxidant activity (Figure 6.2).

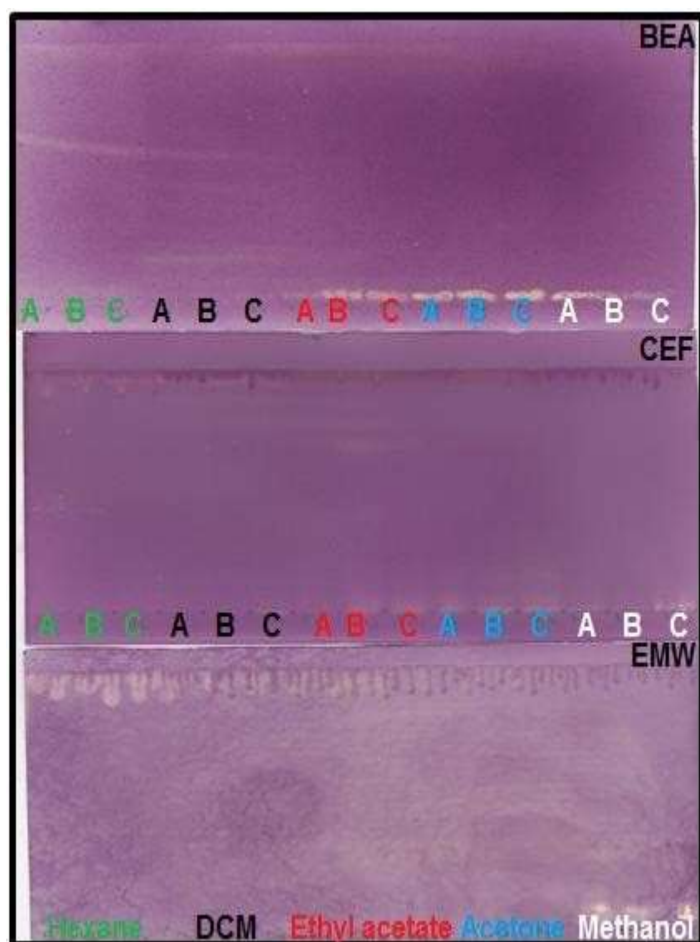


Figure 6.2: Chromatograms of *S. pinnata* extracts extracted with n-hexane, dichloromethane, ethyl acetate, acetone and methanol, separated in BEA (top), CEF (middle) and EMW (bottom) and sprayed with 0.2 % DPPH solution. A= overnight, B= first three hours and C= last three hours.

### 6.3.4. Antimycobacterial activity

6.3.4.1. Bioautography assay

White area against a pink background indicate the presence of antimycobacterial compounds which were observed with plant extracts obtained with n-hexane, dichloromethane, ethyl acetate and acetone (Figure 6.3). Antimycobacterial compounds were best separated in BEA solvent system.

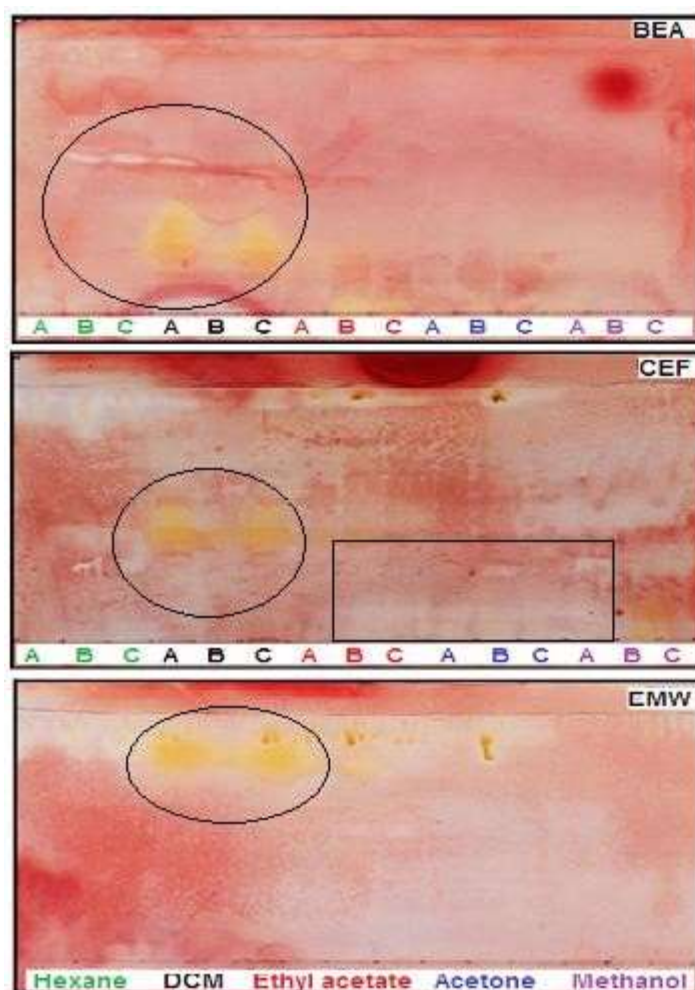


Figure 6.3: Bioautograms of *S. pinnata* extracts extracted with solvents of varying polarity, separated with three solvent systems BEA (top), CEF (middle) and EMW (bottom), and sprayed with *M. smegmatis* culture in broth. The antimycobacterial activity was detected by spraying 2 mg/ml INT in water. A= overnight, B= first three hours and C= last three hours.

6.3.4.2. Broth microdilution assay

Minimum inhibitory concentration of *S. pinnata* extracts was determined using broth microdilution procedure. The experiment was conducted in triplicate and the MIC values were expressed as mean values. The lowest inhibitory concentration was found to be from ethyl acetate (0.32 mg/ml) and acetone (0.39 mg/ml) extracts meanwhile methanol extracts had a high concentration (2.23 mg/ml) (Table 6.3).

Table: 6.3: Minimum inhibitory concentration (MIC) values (mg/ml) of *S. pinnata* extracts after 24 hours of incubation at 37°C.

| Extractants | | MIC values (mg/ml) | Average |
|---|---|---|---|
| n-Hexane | A | 0.64 | |
| | B | 1.25 | 1.46 |
| | C | 0.64 | |
| Dichloromethane | A | 0.32 | |
| | B | 0.64 | 0.43 |
| | C | 0.32 | |
| Ethyl acetate | A | 0.21 | |
| | B | 0.32 | 0.32 |
| | C | 0.43 | |
| Acetone | A | 0.32 | |
| | B | 0.43 | 0.39 |
| | C | 0.43 | |
| Methanol | A | 1.7 | |
| | B | 2.5 | 2.23 |
| | C | 2.5 | |
| Rifampicin | | 0.08 | |
| | | **Total Average** | **1.08** |

Key: A= overnight, B= first three hours and C= last three hours.

### 6.3.5. Isolation of antimycobacterial compounds

6.3.5.1. First open column chromatography

Dichloromethane extracts were subjected to open column chromatography, due to the mass of plant extract and eluted with combination of solvents listed in table 6.1. The masses of fractions collected were recorded and the total mass obtained was 72.52 g (Table 6.4). The highest amount of plant extract was obtained with 10% n-hexane

(30.42 g) followed by 100% ethyl acetate (12.89 g) while 100% methanol eluted the least (0.33 g) (Table 6.4).

Table 6.4: Masses of *S. pinnata* fractions collected from column chromatography eluted with different elution solvents.

| **Elution solvents** | **Percentage (%)** | | **Mass (g)** |
|---|---|---|---|
| n-Hexane | 100 | (**1**) | 0.46 |
| n-Hexane: Ethyl acetate | 90:10 | (**2**) | 1.45 |
| | 80:20 | (**3**) | 1.67 |
| | 70:30 | (**4**) | 1.35 |
| | 50:50 | (**5**) | 3.25 |
| | 30:70 | (**6**) | 7.30 |
| | 10:90 | (**7**) | 30.42 |
| Ethyl acetate | 100 | (**8**) | 12.89 |
| Ethyl acetate: Methanol | 90:10 | (**9**) | 8.39 |
| | 80:20 | (**10**) | 2.57 |
| | 70:30 | (**11**) | 1.17 |
| | 60:40 | (**12**) | 0.52 |
| | 50:50 | (**13**) | 0.30 |
| | 40:60 | (**14**) | 0.22 |
| | 10:90 | (**15**) | 0.23 |
| Methanol | 100 | (**16**) | 0.33 |
| | **Total** | | **72.52** |

6.3.5.1.1. Phytochemical profiles for the first column

The aluminium baked TLC plates were prepared as it was described in section 3.2.3. It was seen that solvent system BEA separated a number of vanillin reactive compounds followed by CEF and the least were separated in EMW under UV light (Figure 6.4).

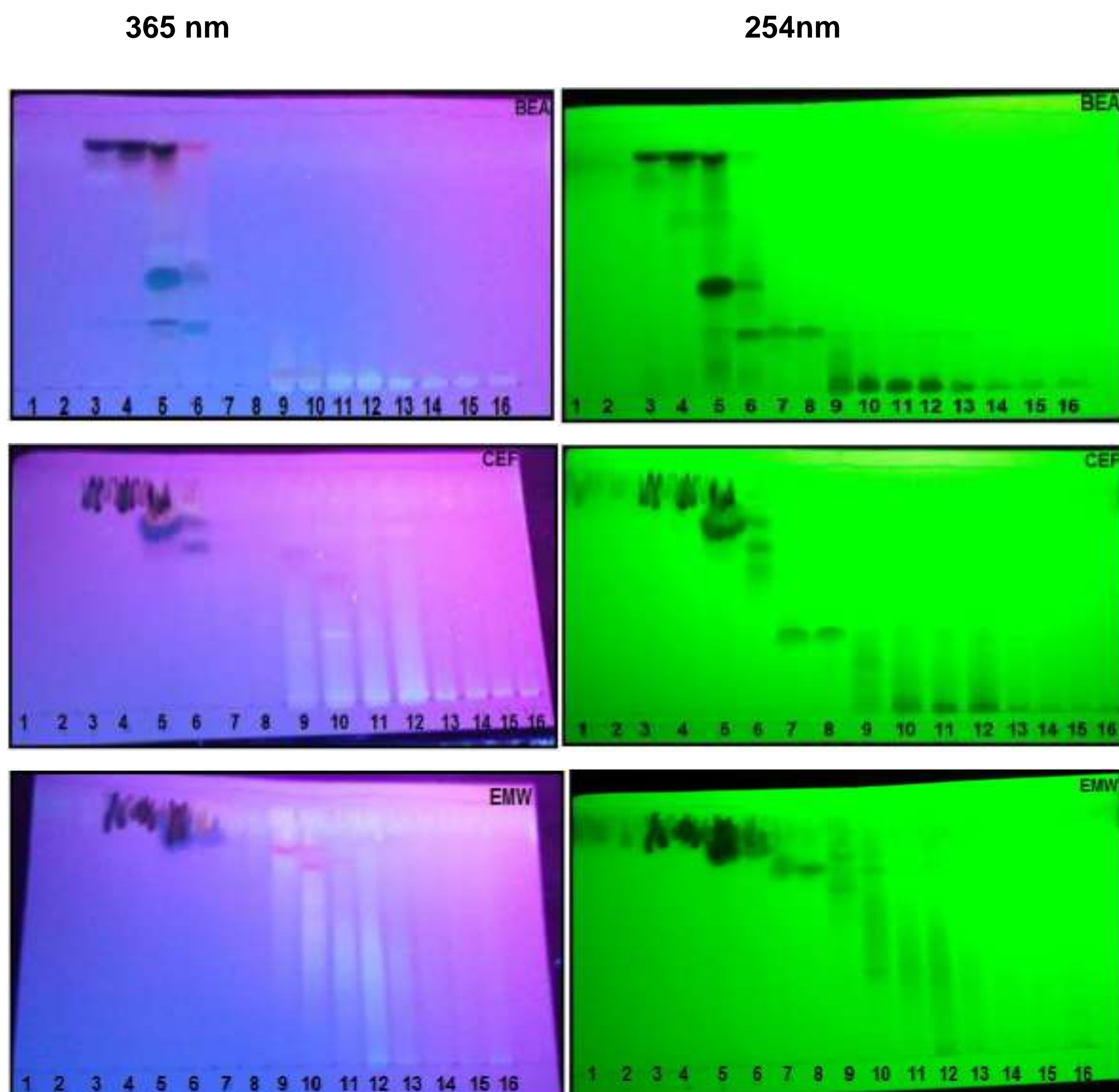


Figure 6.4: Thin layer chromatograms of *S. pinnata* extracts viewed under ultraviolet light (365 nm and 254 nm). Lanes from left to right: 100% n-hexane (1), 90% n-hexane (2), 80% n-hexane (3), 70% n-hexane(4), 50% n-hexane (5), 30% n-hexane (6), 10 % n-hexane (7); 100% Ethyl acetate (8), 90% ethyl acetate (9), 80% ethyl acetate (10), 70% ethyl acetate (11), 60% ethyl acetate (12), 50% ethyl acetate (13), 40% ethyl acetate (14), 10% ethyl acetate (15) and 100% methanol (16).

The best separation of vanillin reactive compounds was observed in BEA followed by CEF while few were observed in EMW (Figure 6.5).

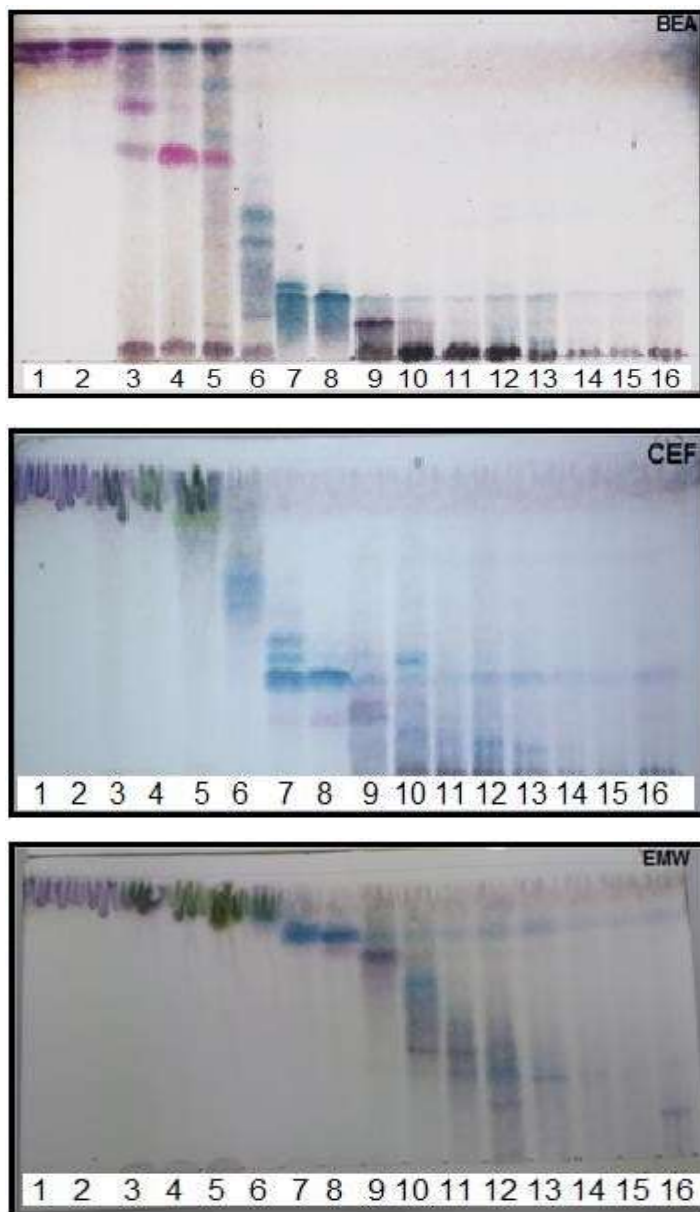


Figure 6.5: Chromatograms of *S. pinnata* fractions eluted with various solvent combinations, developed in three mobile phase: BEA (top), CEF (middle) and EMW (bottom) and sprayed with vanillin sulphuric acid reagent**.** Lanes from left to right: 100% n-hexane (1), 90% n-hexane (2), 80% n-hexane (3), 70% n-hexane(4), 50% n-hexane (5), 30% n-hexane (6), 10 % n-hexane (7); 100% Ethyl acetate (8), 90% ethyl acetate (9), 80% ethyl acetate (10), 70% ethyl acetate (11), 60% ethyl acetate (12), 50% ethyl acetate (13), 40% ethyl acetate (14), 10% ethyl acetate (15) and 100% methanol (16).

6.3.5.1.2. Antioxidant activity

Antioxidant activity of the fractions was seen by development of yellow colour against the purple background. A yellow band was observed from 70% n-hexane (4) and 50% n-hexane (5). Solvent system BEA indicated a better separation of antioxidant compounds from fraction 4 and 5, as compared to CEF and EMW where the compounds moved with the solvent front (Figure 6.5).

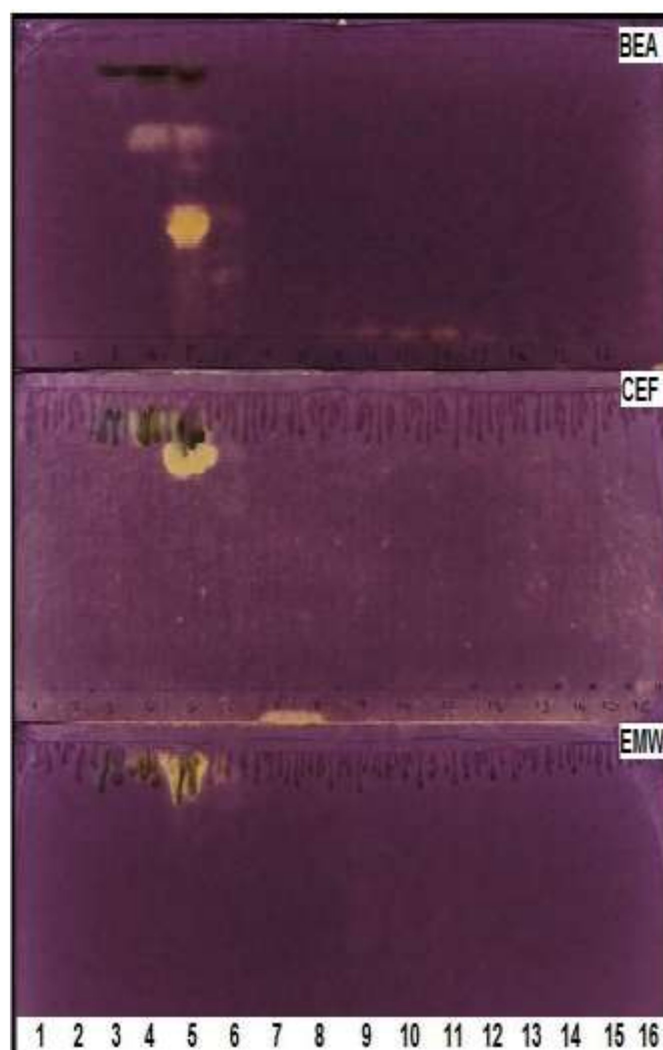


Figure 6.6: Chromatograms of *S. pinnata* extracts developed in BEA (top), CEF (middle) and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol. Lanes from left to right: 100% n-hexane (1), 90% n-hexane (2), 80% n-hexane (3), 70% n-hexane(4), 50% n-hexane (5), 30% n-hexane (6), 10 % n-hexane (7); 100% Ethyl acetate (8), 90% ethyl acetate (9), 80% ethyl acetate (10), 70% ethyl acetate (11), 60% ethyl acetate (12), 50% ethyl acetate (13), 40% ethyl acetate (14), 10% ethyl acetate (15) and 100% methanol (16).

6.3.5.1.3. Bioautography assay

It was observed that antimycobacterial compounds from fraction 7 and 8 were only separated in both BEA and CEF while in EMW they moved with the solvent front. Compounds from fractions 9, 10, 11 and 12 were well separated in CEF solvent system.

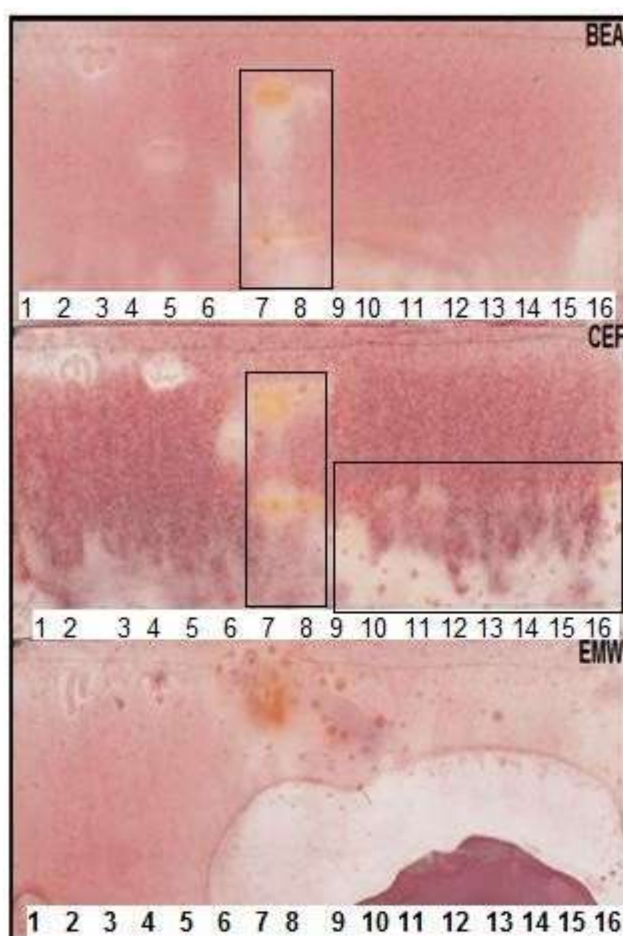


Figure 6.7: Bioautograms of *S. pinnata* extracts developed in three solvent systems BEA (top), CEF (middle) and EMW (bottom) and sprayed with *M. smegmatis* in broth and 2 mg/ml INT in water. Lanes from left to right: 100% hexane (1), 90% hexane (2), 80% hexane (3), 70% hexane(4), 50% hexane (5), 30% hexane (6), 10 % hexane (7); 100% Ethyl acetate (8), 90% ethyl acetate (9), 80% ethyl acetate (10), 70% ethyl acetate (11), 60% ethyl acetate (12), 50% ethyl acetate (13), 40% ethyl acetate (14), 10% ethyl acetate (15) and 100% methanol (16).

6.3.5.1.4. Broth microdilution assay

Lowest inhibitory concentration of the fractions was evaluated using the broth microdilution method. The fractions obtained with 50% hexane (5), 10% n-hexane (7) and 100% ethyl acetate (8) had the lowest MIC values of 0.11 mg/ml followed by 30% n-hexane (6) (0.19 mg/ml) and 70% n-hexane (4) (0.32 mg/ml) when compared to other fractions (Table 6.5).

Table 6.5: Minimum inhibition concentration (MIC) values (mg/ml) of *S. pinnata* fractions.

| **Fractions** | | | **MIC (mg/ml)** |
|---|---|---|---|
| Hexane | 100 | (**1**) | 0.63 |
| Hexane: Ethyl acetate | 90:10 | (**2**) | 1.04 |
| | 80:20 | (**3**) | 0.42 |
| | 70:30 | (**4**) | 0.32 |
| | 50:50 | (**5**) | 0.11 |
| | 30:70 | (**6**) | 0.19 |
| | 10:90 | (**7**) | 0.11 |
| Ethyl acetate | 100 | (**8**) | 0.11 |
| Ethyl acetate: Methanol | 90:10 | (**9**) | 0.36 |
| | 80:20 | (**10**) | 0.53 |
| | 70:30 | (**11**) | 0.84 |
| | 60:40 | (**12**) | 1.04 |
| | 50:50 | (**13**) | 1.67 |
| | 40:60 | (**14**) | 2.5 |
| | 10:90 | (**15**) | 2.08 |
| Methanol | 100 | (**16**) | 2.08 |
| **Total average:** | | | **0.88** |

### 6.3.6. Second column chromatography

Test tubes fractions collected were pulled together based on the similarity of phytochemical compounds and gave up to 24 fractions. It was observed that fraction 23 had a good separation of the compounds and high amount of fractions while in other fractions the compounds could not separate from the bottom of the plate (Figure 6.9). Antimycobacterial activity was observed from fraction 6, 10 and 17 (Figure 6.10). Isolated antimycobacterial compounds from the active fractions had masses which were less than 1 g which couldn't be subjected to another column for further isolation.

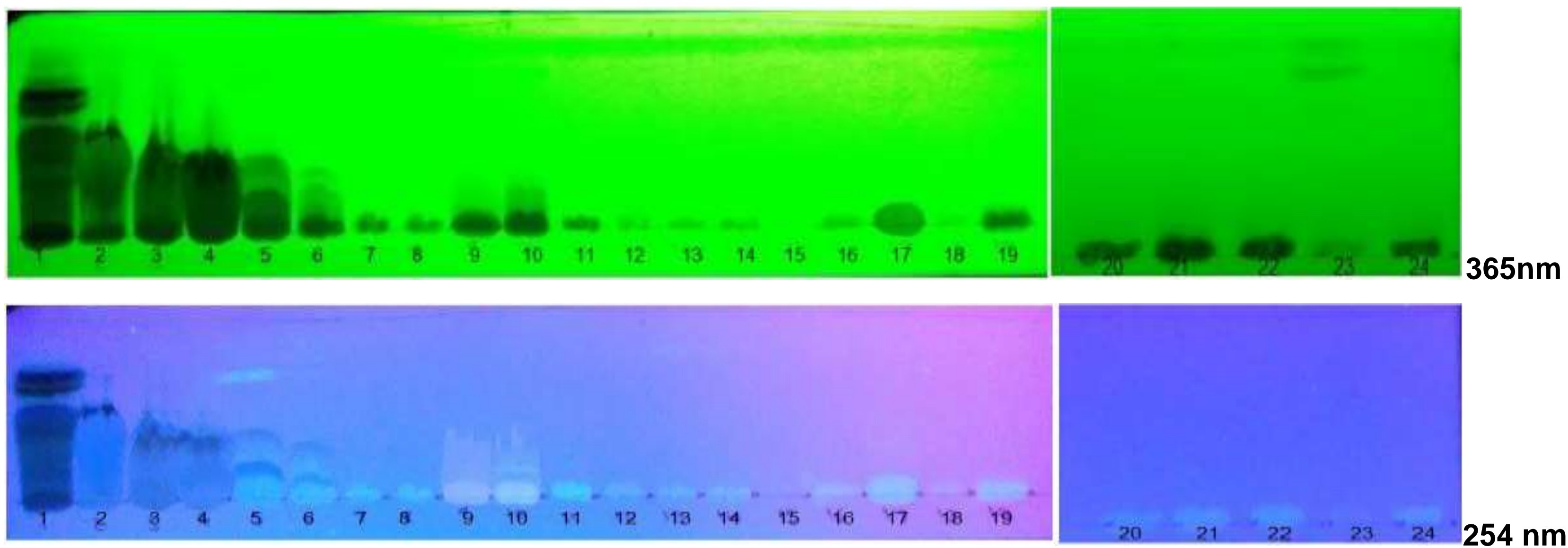


Figure 6.8: Chromatograms of *S. pinnata* fractions (1-24) developed with 90% chloroform in ethyl acetate and visualised under UV light (365 and 254 nm).

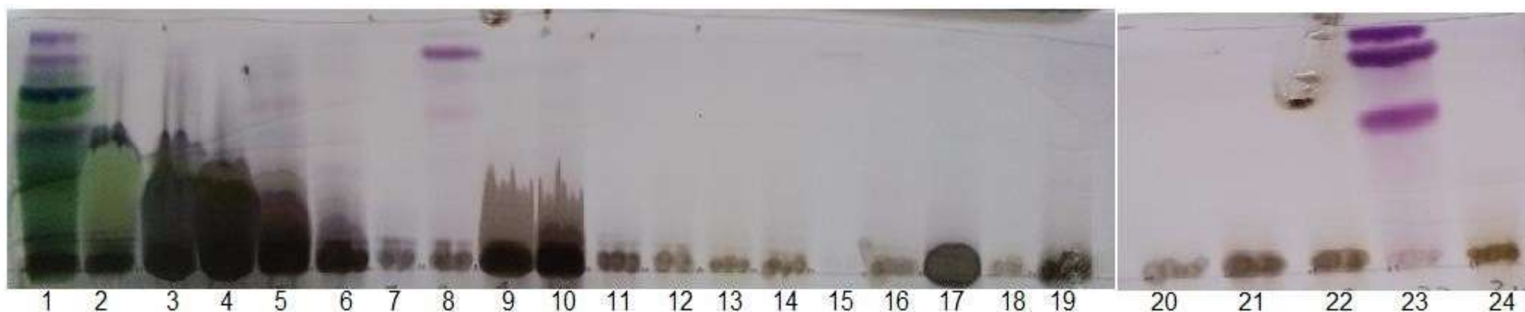


Figure 6.9: Chromatograms of *S.pinnata* fractions (1-24) developed in 90% chloroform in ethyl acetate and spayed with vanillin-sulphuric acid reagent for colour development.

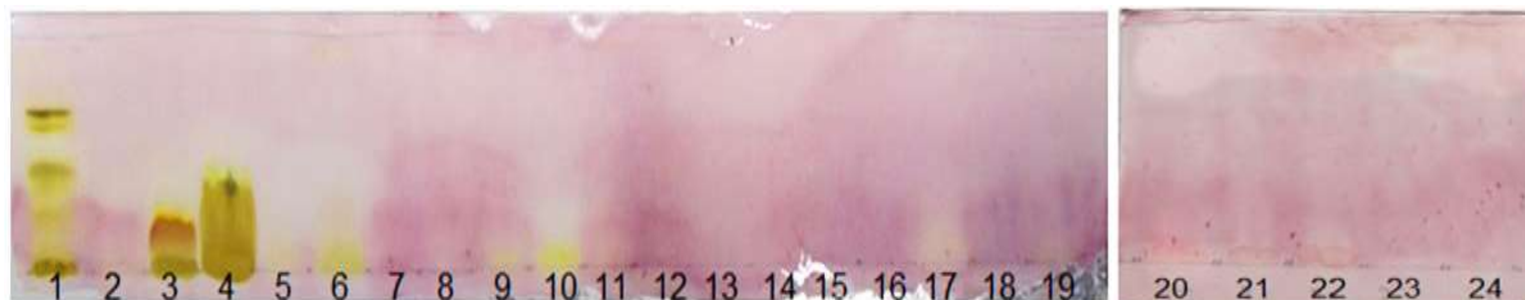


Figure 6.10: Bioautograms of *S. pinnata* fractions (1-24) developed in 90% chloroform in ethyl acetate, sprayed with *M .smegmatis* culture and 2 mg/ml INT in water. White area against the pink background indicates antimycobacterial activity.

### 6.3.7. Third column chromatography

Plant extracts obtained with 10% n-hexane and 100% ethyl acetate (fraction 7 and 8 from the first column section 6.3.1) was subjected to a column and which was eluted with 70% ethyl acetate in acetone. The fractions collected were pulled together based on the similarity of phytochemical compounds. The pulled fractions were evaluated for the phytochemical profiles using TLC plates which were sprayed with vanillin-sulphuric reagent for colour development (Figure 6.11A). Chromatograms indicated a number of vanillin reactive compounds. Antimycobacterial activity from the pulled fractions was observed from all fractions and the targeted compounds were from fraction 4 due to the number of compounds and high amount of the fraction 5.13 g (Figure 6.11B).

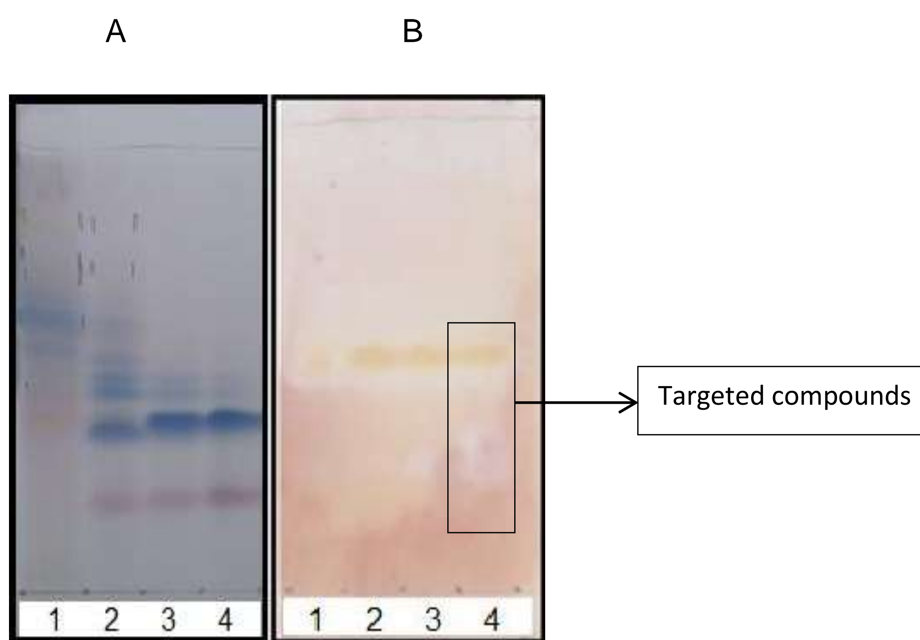


Figure 6.11: Chromatograms of the collected fractions (1 to 4), developed in 90% chloroform in ethyl acetate, and sprayed with vanillin sulphuric acid reagent **(A)** and *M. smegmatis* culture **(B)** to detect the targeted antimycobacterial compounds.

### 6.3.8. Fourth column chromatography

Fraction 4 (5.13 g) was chosen for further isolation using column chromatography which was eluted with 70% ethyl acetate in acetone. Aluminium-backed TLC plates were used to analyse the phytochemical compounds of the fractions (Figure 6.12 **A**). The chromatogram indicated that the fractions were still having a mixture of compounds. Antimycobacterial activity was observed from fraction 3, 4 and 5 (Figure 6.12 **B**).

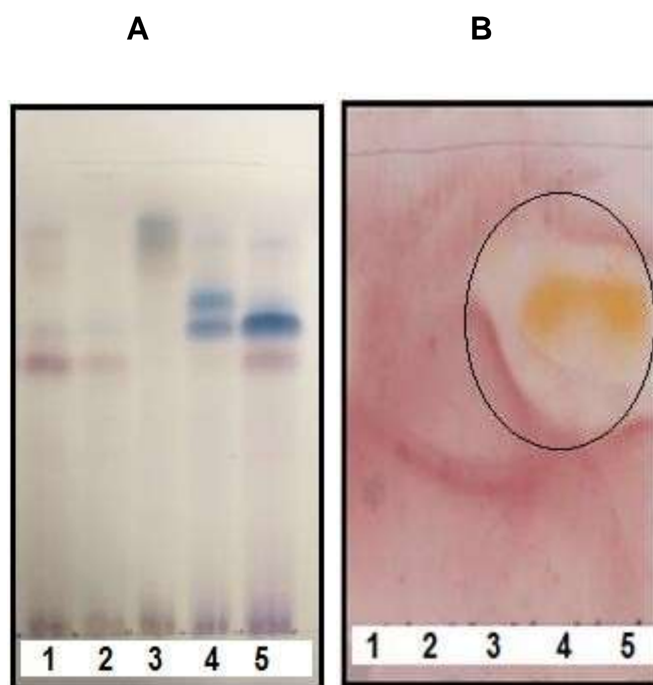


Figure 6.12: Chromatograms of the pulled fractions (1-5), developed in 70% ethyl acetate in acetone, sprayed with vanillin sulphuric acid for colour development **(A)** and *M. smegmatis* culture **(B)**.

### 6.3.9. Preparative TLC plates

Fraction 5 was chosen to be subjected on preparative TLC procedure due to good separation of two compounds which makes it easy to scrap and had high amount of the extract. Preparative TLC plates were used to scrap the antimycobacterial compounds from fraction 5 which resulted into compound I and II (Figure 6.13).

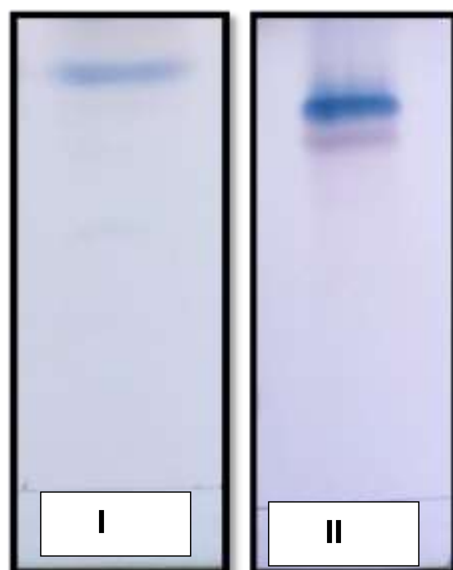


Figure 6.13: Chromatograms of the scrubbed compound I and II developed with 70% ethyl acetate in acetone and sprayed with vanillin-sulphuric acid regent for colour development.

**6.4. Discussion**

Serial exhaustive extraction was the first step conducted in order to exhaustively extract large amounts of antimycobacterial compounds using solvents of varying polarity for subsequent fractionation with column chromatography. A total mass of *S. pinnata* extracts obtained was 229.72 g (Table 6.2). A wide range of phytochemical constituents were obtained (Table 6.2), this could be seen by the amount of plant extracts obtained with different solvents. Dichloromethane extracted the largest amount of plant material (101.88 g) followed by methanol (78,52g) and the least was from acetone with a mass of 4.14 g. The phytochemical profiles of plant extracts was evaluated using TLC, where the best separation of more compounds was observed under solvent system CEF followed by BEA and the least were separated in EMW (Figure 6.1). It was observed that the chlorophylls or fatty acids extracted by n-hexane (Sasidharan *et al.*, 2011), were only separated in BEA solvent system. The presence of antioxidant compounds after serial exhaustive extraction was observed at low intensity in extracts of ethyl acetate, acetone and methanol which were separated in solvent system BEA and EMW (Figure 6.2). The best separation of white bands against the pink background indicating the presence of antimycobacterial compounds was observed from n-hexane and dichloromethane extracts developed in BEA solvent system (Figure 6.3). The MIC values were expressed as averages. The lowest

average MIC value was obtained from ethyl acetate (0.32 mg/ml), acetone (0.39 mg/ml) and dichloromethane extracts (0.43 mg/ml), while methanol extracts had high average MIC value of 2.23 mg/ml. From the antimycobacterial activity observed and high mass of plant extracts, dichloromethane extracts were chosen to be subjected to column chromatography to isolate the active compounds.

An open column chromatography was eluted with different solvent combination (Table 6.1) and the obtained masses of the fractions were recorded (Table 6.4). it was seen that the highest mass of collected fractions was obtained with 10% n-hexane (7) (30.42 g) followed by 100% ethyl acetate (8) (12.89 g) and the least amount was from 40% ethyl acetate (14) (0.22 g) (Table 6.4). The targeted antimycobacterial compounds were traced by performing TLC phytochemical profiles and antimycobacterial assays for the fractions. TLC Phytochemical profiles were visualised under UV light (254 and 365 nm) to mark the compounds which might not be visible after vanillin-sulphuric reagent treatment (Figure 6.4). Both UV wavelengths indicated a number of compounds which were visible after spaying with vanillin-sulphuric acid reagent (Figure 6.5). Separation of phytochemical was observed to be increasing as the polarity of the mobile systems increased, nonpolar phytochemicals extracted with nonpolar solvents best separated under BEA mobile phase, this was also observed with solvents of intermediate polarity and CEF mobile phase and in EMW (Figure 6.5). Antioxidant activity was detected from 70% n-hexane (4) and 50% n-hexane (6) fractions which were best separated in solvent system BEA (Figure 6.6).

The fractions were evaluated for their antimycobacterial activity using TLC-bioautography (Figure 6.7) and broth microdilution assays (Table 6.5). From Figure 6.8, antimycobacterial compounds were observed from 70% n-hexane (4), 30% n-hexane (6), 10% n-hexane (7) and 100% ethyl acetate (8) fractions which were best separated in solvent system BEA, In CEF, antimycobacterial compounds from 90% ethyl acetate (9), 80% ethyl acetate (10), 70% ethyl acetate (11) and 60% ethyl acetate (12) factions were observed at the bottom of the TLC plate. In EMW, the white area which doesn't resemble the activity on the plate could be due to the presence of solvents traces which didn't evaporate well, and have been reported to be toxic to the microorganisms (Eloff, 1998; Masoko and Eloff, 2006). The lowest MIC value was determined from 50% hexane, 10% hexane (7) and 100 % ethyl acetate (8) fractions

at 0.11 mg/ml, followed by 30% hexane (6) at 0.19 mg/ml while 40% ethyl acetate (14) had the highest (2.5 mg/ml) (Table 6.5).

Isolation of antimycobacterial compounds was done on 10% hexane (7) and 100% ethyl acetate (8) fractions which had high amount of plant extracts (43.31 g combined) (Table 6.4), similar phytochemical compounds (Figure 6.5) and good separation of antimycobacterial activity (Figure 6.8) with lowest MIC values of 0.11 mg/ml (Table 6.5). The combined fractions were suspended into an open column chromatography which was eluted with 90% chloroform in ethyl acetate. Phytochemical and antimycobacterial analysis were conducted to trace the targeted compounds using TLC profiles (Figure 6.11). The chromatograms indicated the presence of antimycobacterial compounds which were still combined with other compounds (Figure 6.12). Fraction 4 from the pulled test tubes was subjected to another column which was eluted with 70% ethyl acetate in acetone. Phytochemical analysis of the pulled test tubes indicated impurity of the active compounds which lead to purification of the compounds using preparative thin layer chromatography (prep-TLC) procedure. The plates were separated in 70% ethyl acetate in acetone to scrap off a single compound depicted in figure 6.13. The antimycobacterial activity observed with *S. pinnata* extracts could be due to the presence of tannin compounds which have the ability to in activate microbial adhesions, enzymes and ability to inhibit the growth of bacteria (Scio *et al*., 2012). Antimycobacterial activity of terpenoid compounds which are able to inactivate or destroy the genetic material of the bacteria (Kim *et al*., 1995; Bagamboula *et al*., 2004). Other phytochemical detected from *S. pinnata* extracts in this study have been reported for a number of biological activities including antibacterial activity.

### 6.5. Conclusion

The plant extracts of *S. pinnata* indicated the presence of compounds which can be used in the development of new antimycobacterial drugs. The observed antimycobacterial activity validates the use of *S. pinnata* in traditional medicines for treatment of fever, cough, wounds and other bacterial infections in animals and human. Structural elucidation of the isolated compounds will be analysed in chapter 9.

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## Chapter 7

## 7. Cytotoxicity and anti-inflammation activity assays

### 7.1. Introduction

Natural products are considered to be the healthiest products. However, researchers have discovered that some plants possess toxic compounds that when administered they may result in server side effects overtime. The use of wrongly identified plants can result in intoxication of patients and can lead to death. This has been reported in a Brazil community where other plants were incorrectly used for preparation of coffee and tea (Rates, 2001). Some of the medicinal plants contain poisonous metabolites which have good therapeutic effects at certain concentrations and cannot be administered, prepared or prescribed to patient by unqualified persons (Nasri and Shirzad, 2013). The use of medicinal plant is based on the knowledge which was passed on from generation to generation. However, their effectiveness, safety and drug intentions require careful clinical trials (Sasidharan *et al.*, 2011).

Inflammation is a process which the body undergo in response to cell damage, pathogens and other irritants. It is categorised under primary immune responsive mechanism in human and animals (Charles *et al.*, 2001). This reaction is helpful in prevention of blood loss during injury and elimination of irritants from the damaged tissue (Chowdhury *et al.*, 2009). Inflammation is diagnosed through symptoms such as pain, swollen, heat and loss of function from the affected tissue (Ferrero-Miliani *et al.*, 2007). Current anti-inflammatory drugs are associated with severe side effects such as stomach ulcers and heart frailer (Harirforoosh *et al.*, 2014). Therefore, this necessitates evaluation of medicinal plants for development of anti-inflammatory drugs with less or no side effects.

Toxicological studies play an important role in evaluation of medicinal plants safety (Chanda *et al.*, 2015). There are different methods used in the analysis of toxicity and inflammatory effects of medicinal plants which include the use of animal models that are treated with potential medicinal plants and *In vitro* assays where cell lines are used (McGaw *et al.*, 2007; Sara *et al.*, 2017). In this study, Vero monkey kidney cells and lipopolysaccharide (LPS) induced macrophages Raw 264.7 cell lines were used to evaluate the toxicity and anti-inflammatory effects of *S. pinnata* crude extracts using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay and anti-

inflammatory assay, respectively.

## 7.2. Methods and materials

### 7.2.1. MTT assay

Toxicity of *S. pinnata* extracts was evaluated on African green monkey kidney (Vero) cells using tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983). Cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). An amount of 200 µl cell suspension (5 x $10^4$ cells/ml) was seeded in each well of sterile 96-well microtiter plates. The plates were incubated at 37ºC, 5% $CO_2$ for 24 hours until the growth reached the exponential phase. After incubation, the cells were washed with150 µl phosphate buffered saline (PBS, White Scientific). Thereafter, cells were treated with 200 µl of the acetone plant extracts at different concentrations (0.025 -1 mg/ml). Untreated cells were used as a negative control and doxorubicin chloride (Pfizer Laboratories) as positive control. The plates were further incubated at 37ºC, in 5% $CO_2$ for 48 hours. Thereafter, 30 µl of MTT (Sigma) solution (5mg/ml) in PBS was added to each well and the plates were further incubated at 37ºC, in 5% $CO_2$ for 4 hours. After incubation the MEM medium was removed leaving MTT crystals. Fifty microliter of dimethyl sulfoxide (DMSO) was added to the plates and the plates were gently shaken to dissolve MTT crystals. The experiment was performed in quadruplicates. The absorbance was measured at 570 nm using a microtiter plate reader (Bio Tek Synergy). The wells containing medium and MTT without cells were used as blank. The $LC_{50}$ values were calculated as the concentration of test sample resulting in a 50% reduction of absorbance compared to untreated cells.

### 7.2.2. Anti-inflammatory assay

An anti-inflammatory effect of *S. pinnata* extracts was evaluated on Raw 264.7 macrophage cells following a procedure described by Karthikeyan and Chunduru (2016). A total volume of 200 µl of cells was plated in a sterile 96- well plate and incubated at 37ºC, in 5% $CO_2$ overnight to allow cells to attach. After incubation, cells were treated with 100 µl of acetone extracts at different concentrations (0.32 mg/ml, 0.64 mg/ml, and 8 mg/ml) and 10 mg/ml of lipopolysaccharide (LPS) for 24 hours. Curcumin (µM) was included as a positive control. After incubation, the cells were washed with phosphate buffered saline (PBS). Thereafter, 100 µl of (20 µM)

hydrophilic alcohol dihydro dichlorofluorescein ($H_2DCF$-DA) dye was added in each well and incubated for 30 minutes in the dark. The fluorescence was measured at 480 nm.

## 7.3. Results

### 7.3.1. MTT assay

*S. pinnata* was evaluated for its cytotoxicity effects using MTT assay on African green monkey kidney (Vero) cells. During MTT analysis, the viable cells converted MTT by mitochondrial dehydrogenases into formazan crystals. The selectivity index (SI) is determined by dividing the $LC_{50}$ value in mg/ml by the MIC value in mg/ml (Elisha *et al*., 2017). The cytotoxicity value of *S. pinnata* extracts was determined to be less than 25 µg/ml with selective index of 0.02 SI (Table 7.1).

Table 7.1: Determination of cytotoxicity value (µg/ml), minimum inhibitory concentration (µg/ml) and selective index (SI) of acetone extracts.

| **Plant extracts** | **$LC_{50}$ (µg/ml)** | **MIC (µg/ml)** | **Selective index (SI)** |
|---|---|---|---|
| Acetone | <25.0 | 1250 | 0.02 |

### 7.3.2. Anti-inflammatory assay

Anti-inflammatory activity of *S. pinnata* extracts was determined by measuring the inhibition percentage of reactive oxygen species in Raw 264.7 macrophage cells induced by LPS stimulant. It was observed that the inhibition of ROS production by the plant extracts was in a concentration dependant manner (Figure 7.1). Lowest ROS inhibition was obtained in with the lowest concentration (0.32 mg/ml). Curcumin (50µM), as a positive control, indicated a significance inhibition of ROS production when compared to the extracts.

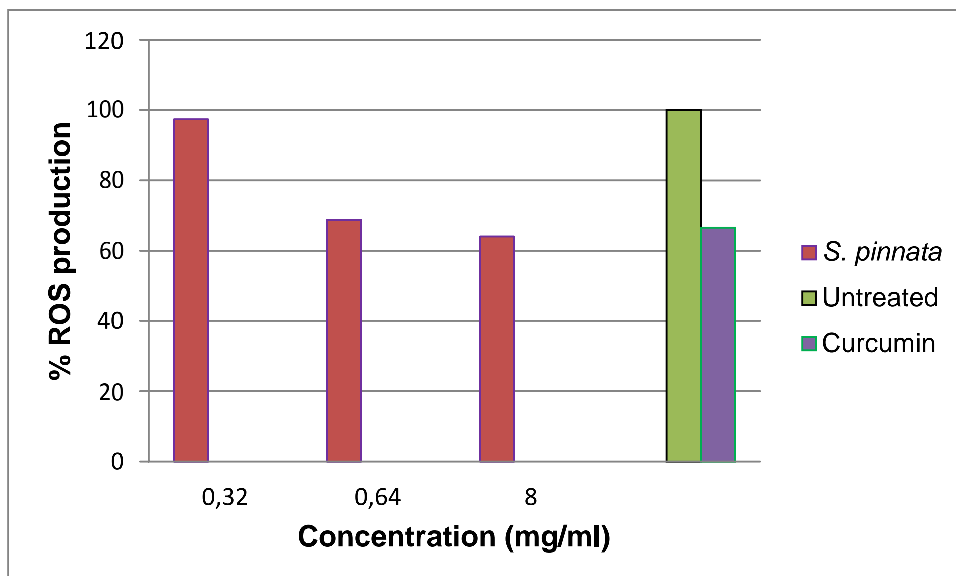


Figure 7.1: The effects of isolated compounds I and II at different concentrations on Raw 264.7 macrophage cells in response to oxidative stress and inflammation determined by measuring the production of ROS in percentages.

### 7.4. Discussion

The search for new leads in drug development is continuous since plants have been proven to have unlimited biological activities. However, the safeness of medicinal plants has to be evaluated. The use of medicinal plants without the knowledge of their toxicity is life threatening (Makhafola *et al*., 2012). Plants phytochemicals could be toxic to microorganism and not to cells. Therefore, selective index has to be evaluated before concluding about their effectiveness (Elisha *et al*., 2017).

Evaluation of the safety of medicinal plants is important even for the traditional healers and herbalist in order for them to use medicinal plants without the fear of toxicity. Literature has indicated the importance of determining the toxicity of plant extracts on host cells and pathogens through selectivity index (dividing the $LC_{50}$ in mg/ml with MIC in mg/ml). Cytotoxic effects of *S. pinnata* extracts were determined on Vero monkey kidney cells and the selectivity index of the extracts against *M. smegmatis* was calculated. Inhibition of reactive oxygen species production was determined on LPS-induced Raw 264.7 macrophage cells. From Table 7.1, the concentration in which the plant extracts was able to inhibit 50% of the cells was determined to be less than 25 µg/ml which was out of the required $LC_{50}$ value determined by America National Cancer Institute (NCI) ($LC_{50}$ < 30 µg/ml) (Steenkamp and Gouws, 2006) . From the

$LC_{50}$ value obtained (Table 7.1), it shows that the crude extracts of *S. pinnata* were toxic to Vero cells and it suggests that the antimycobacterial activity could be due to the toxicity of the compounds within the plant. Makhafola *et al.* (2012) indicated that if the selectivity index is greater than one, the plant extract is less toxic to the host cells than bacteria. It was found that *S. pinnata* has selectivity index which was lower than one (0.02 SI). Therefore, antiproliferative activity found by Rodrigo *et al.* (2010) might be due to the cytotoxicity of *S. pinnata* extracts killing the cells. Toxicity of *S. pinnata* could be due to the presence of saponin compounds which have been reported to be toxic to livestock (Kar, 2007) and was detected in this study. Medicinal plants containing pyrrolizidine-alkaloid compounds are also reported to have hepatotoxicity effects.

Anti-inflammatory activity of *S. pinnata* was evaluated on Raw 264.7 microphage cells. Reactive oxygen species were induced by introduction of LPS in to the cells. It was observed that inhibition percentage of ROS production was in concentration dependent manner (Figure 7.1). *S. pinnata* extracts at lower concentration (0.32 mg/ml) inhibited 97 % of the ROS when compared to the untreated cells. At a concentration of 0.64 mg/ml the plant extracts managed to inhibit 68% of the ROS which was closer to that of the positive control (66 %). A good ROS inhibition was observed at higher concentration which indicated 64% inhibition lower than that of a positive control (66%). It was found that methanol and dichloromethane extracts of *S. pinnata* have positive anti-inflammatory effects (Luseba *et al.*, 2007). Medicinal plants have played a vital role in minimising the inflammation in patients (Akhtar and Haqqi, 2012). Anti-inflammatory activity observed with *S. pinnata* extracts corresponded with the antioxidant activity observed in chapter 5. This could be due to the presence of flavonoids, tannins, terpenes and phenolic compounds in the plant extracts. Flavonoids have been reported to have a good anti-inflammatory activity (De Kok *et al.*, 2008).

### 7.5. Conclusion

Toxicity of *S. pinnata* extracts found in this study indicates that the antibacterial activity observed could be from the toxic effects. The study indicated a good anti-inflammatory activity of the plant extract, where responsible compounds can be isolated and used in development of anti-inflammatory drugs. This study provides scientific validation for the use of *S. pinnata* extracts in treatment of inflammatory diseases by traditional

healers. Nonetheless, it is however recommended that the use of this plant should be taken with precautionary measures as it may course detrimental effects overtime.

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## Chapter 8

## 8. Structure elucidation

### 8.1 Introduction

Isolation of bioactive compounds from *S. pinnata* extracts involved step by step processes including extraction, biological activity screening, fractionation and characterisation of the targeted compounds. Medicinal plants contain a number of phytochemicals in a mixture which make it a challenge during isolation of the bioactive compound due to the amount, solubility and volatility of the compound (Sasidharan *et al*., 2011; Gupta *et al*., 2012, Doughari, 2012). Structural elucidation of the isolated compounds is done through spectroscopic techniques such as nuclear magnetic resonance (NMR), Mass spectroscopy (MS), ultraviolet spectroscopy (UV) and infrared spectroscopy (IR).

Nuclear magnetic resonance is a definite technique used in the structural analysis of any sample possessing nuclei. Scientists analyse the structural components of molecules with the use of simple one dimensional and two dimensional techniques. The following are the techniques used to elucidate structures of compounds: proton NMR, carbon NMR, correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation HSQC and distortionless enhancement through polarisation transfer DEPT (Buevich *et al*., 2014). One dimension proton NMR ($^{1}$H-NMR) gives information about the number of protons in the molecule and 2D-carborn NMR ($^{13}$C-NMR) determines the type of carbon in the molecule. Correlated spectroscopy (COSY) gives information on the coupling between protons. Heteronuclear multiple quantum correlation (HMQC) provide information on the attachment of proton to different carbons based on their signalling while heteronuclear multiple bond correlation (HMBC) gives information on the coupling of proton and carbon based on their bonds. These techniques give a good characterisation of proton-rich natural products and advancement of the NMR thereby making it easy for researchers to analyse a number of molecules (Breton and Reynolds, 2013; Derome, 2013).

Mass spectroscopy is a technique which analyses molecules based on the charged particles (ions) and provides information of the molecular weight of the compounds. It

is also sensitive to the size of the sample. There are a number of techniques combined with MS including gas chromatogram (GC/MS), liquid chromatography (LP/MS), and capillary electrophoresis (CE/MS) which makes it easier to elucidate structures of molecules.

## 8.2. Methods and materials

The purified compounds were dissolved in deuterated acetone and sent to the Chemistry Department at the University of Limpopo for structural analysis using both one and two dimensional NMR techniques ($^{1}H$, $^{13}C$, COSY, DEPT and HMBC,). The prepared samples were ran using 400MHz NMR spectrometer (Bruker) at 400MHz, a number of scans were 10240 and at a temperature of 295.5 K. Prof O. Mazimba at Botswana International University of Science and Technology assisted with the analysis of NMR spectrum and structure elucidation of the compounds.

## 8.3. Results

### *8.3.1.* Structural analysis of an isolated compound I from *S. pinnata*

The NMR techniques ($^{1}H$, $^{13}C$, DEPT, COSY and HMBC) were used to analyse the molecules from the isolated compound I (Figure 8.1 to 8.5). Table 8.1 gives a summary of $^{13}C$ shift values as compared to the obtained values from literature. The structure of isolated compound I was elucidated as heliangolide sesquiterpene lactone (Figure 8.6).

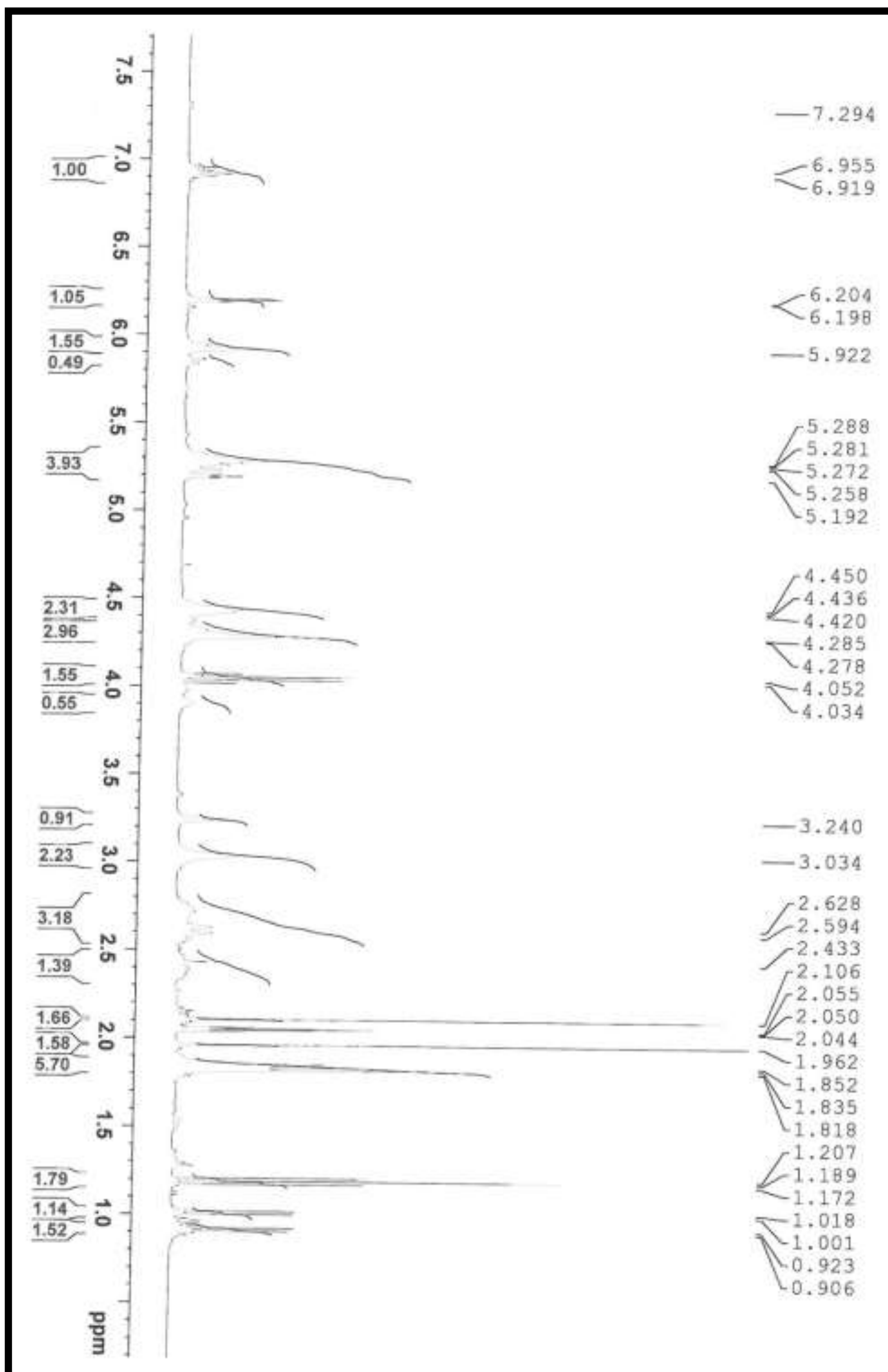


Figure 8.1: The $^{1}$H-NMR spectrum for compound I isolated from dichloromethane extracts of *S. pinnata*.

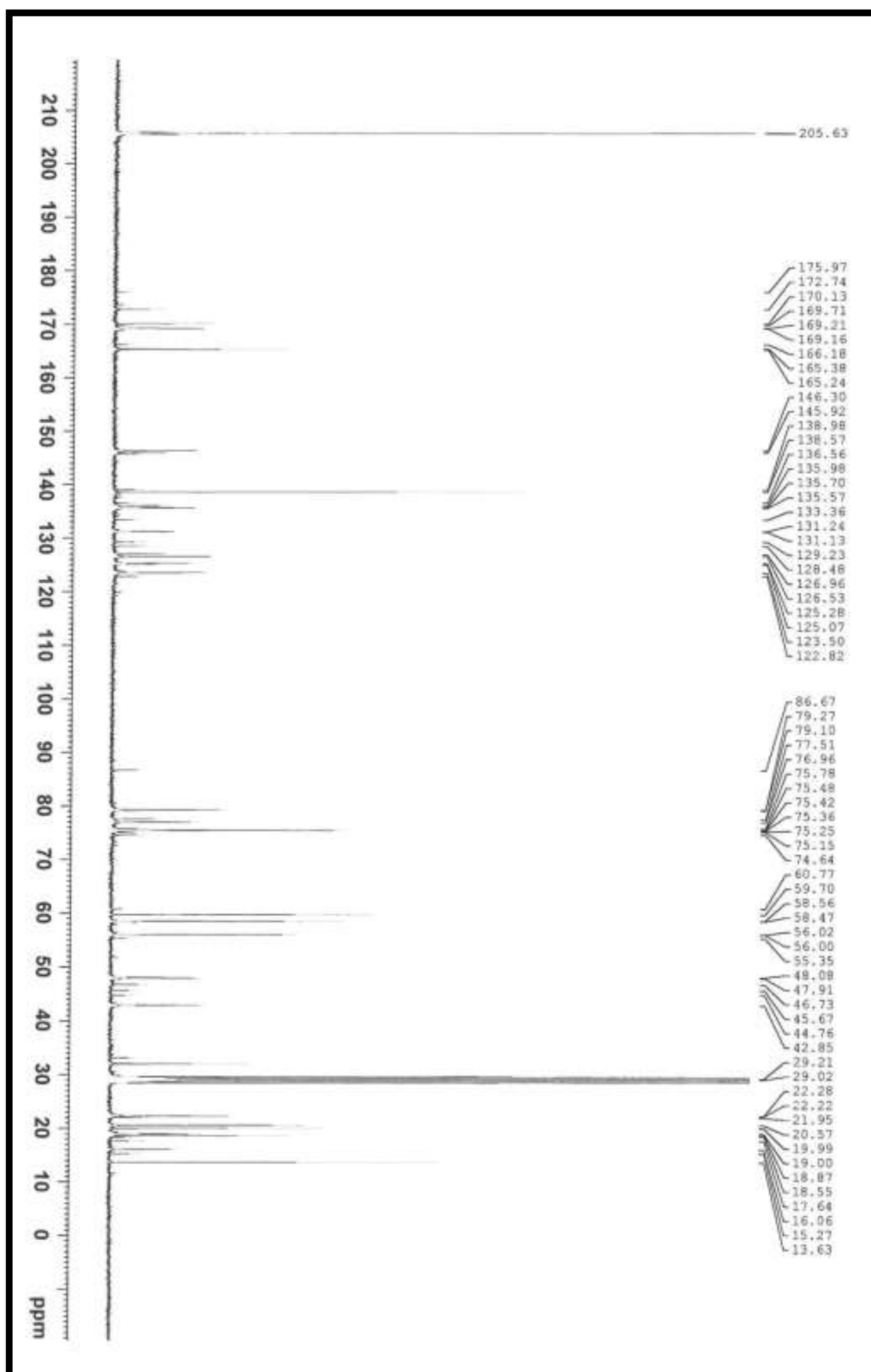


Figure 8.2: The $^{13}$C-NMR spectrum for compound I isolated from dichloromethane extracts of *S. pinnata*.

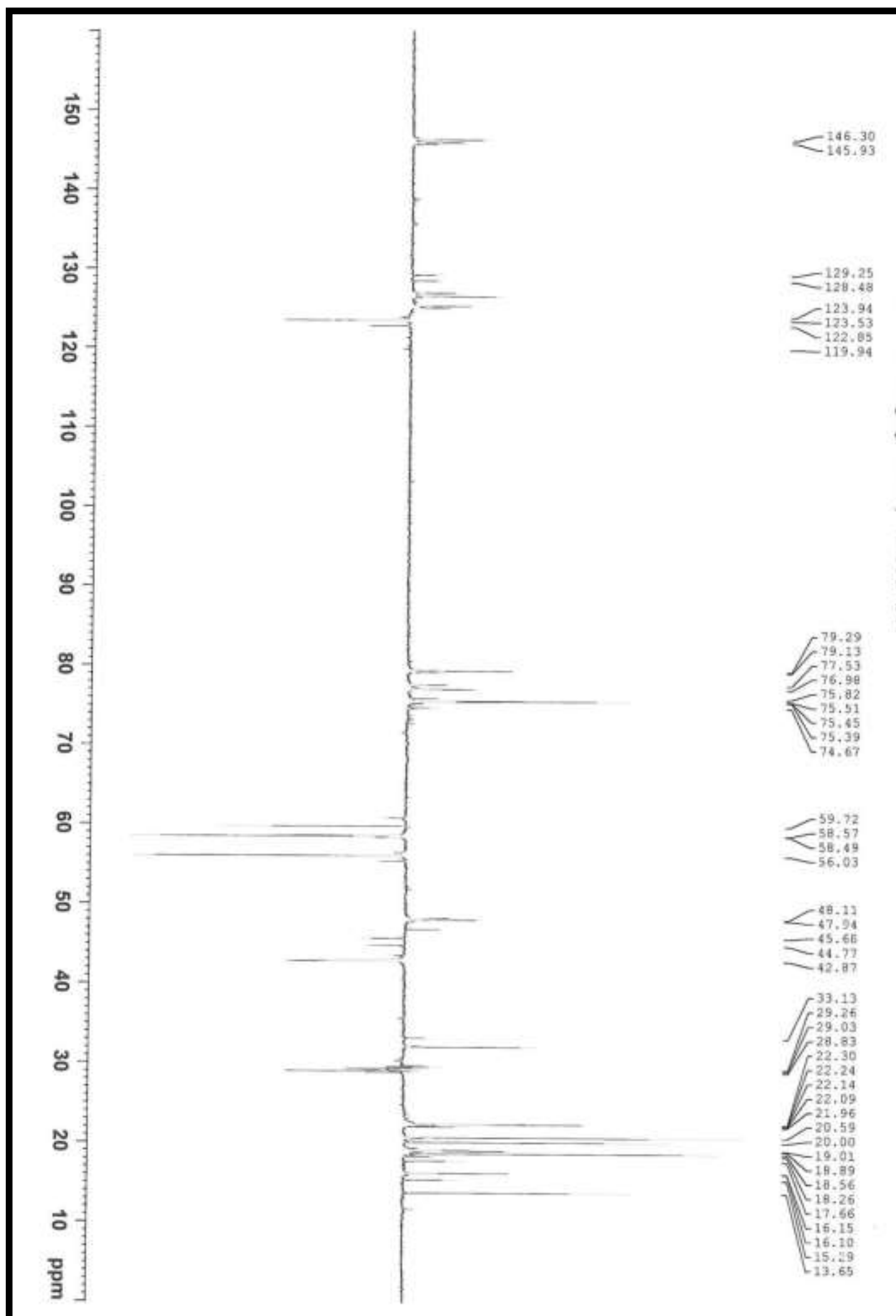


Figure 8.3: The DEPT-NMR spectrum for compound I isolated from dichloromethane extracts of *S. pinnata*.

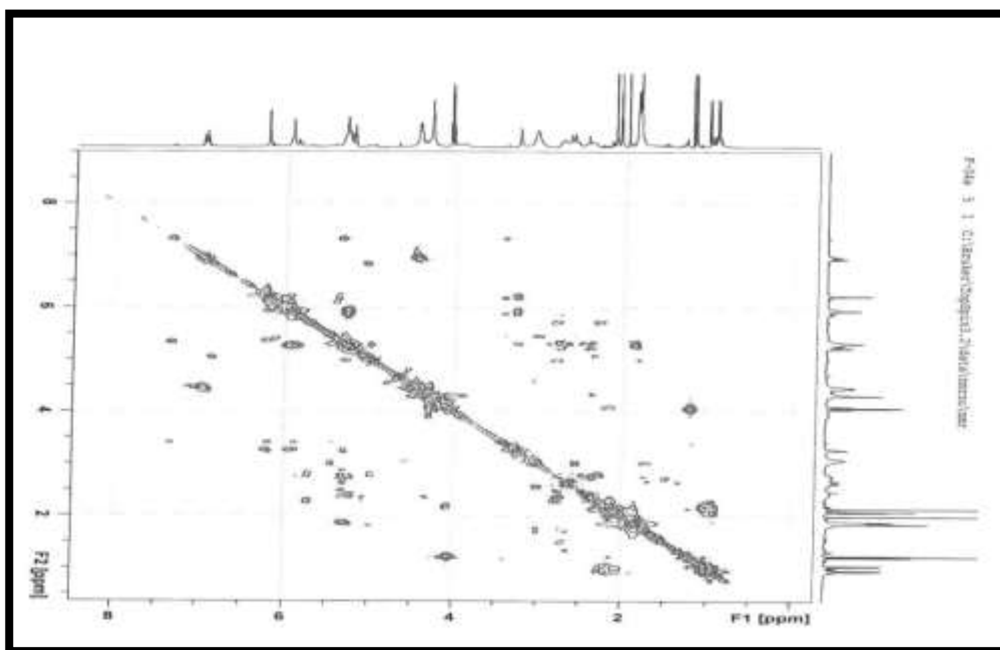


Figure 8.4: The COSY-NMR spectrum for compound I isolated from dichloromethane extracts of *S. pinnata*.

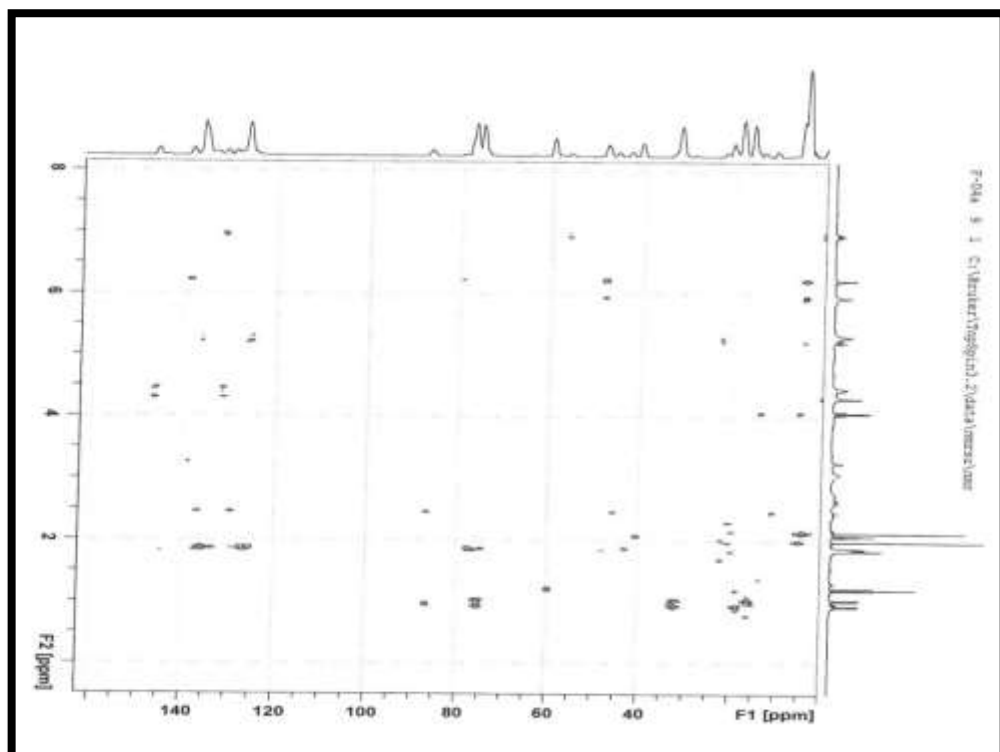


Figure 8.5: The HMBC-NMR spectrum for compound I isolated from dichloromethane extracts of *S. pinnata.*

Table 8.1: $^{13}$C-NMR shift values for isolated compound I compared to the compound from literature.

| Positions | Pacciaroni *et al.*, 1995 | | Compound I |
|---|---|---|---|
| 1 | 126.4 | CH | 125.3 |
| 2 | 29.5 | $CH_2$ | 29.0 |
| 3 | 77.6 | CH | 76.9 |
| 4 | 137.1 | | 138.5 |
| 5 | 126.4 | CH | 126.9 |
| 6 | 78.9 | CH | 79.1 |
| 7 | 48.5 | CH | 48.1 |
| 8 | 75.9 | CH | 75.54 |
| 9 | 43.4 | $CH_2$ | 42.8 |
| 10 | 136.1 | | 135.7 |
| 11 | 135.1 | | 131.2 |
| 12 | 165.6 | | 165.4 |
| 13 | 125.3 | $CH_2$ | 123.5 |
| 14 | 19.5 | $CH_3$ | 20.5 |
| 15 | 23.0 | $CH_3$ | 22.2 |
| 1’ | 170.1 | | 169.1 |
| 2’ | 135.9 | | 135.5 |
| 3’ | 145.5 | CH | 146.2 |
| 4’ | 56.6 | $CH_2$ | 56.0 |
| 5 | 59.0 | $CH_2$ | 58.5 |
| 1’’ | 172.5 | | 170.1 |
| 2’’ | | $CH_2$ | 45.6 |
| 3’’ | 32.3 | CH | 31.9 |
| 4’’ | 18.8 | $CH_3$ | 19.1 |
| 5’’ | 16.5 | $CH_3$ | 18.5 |

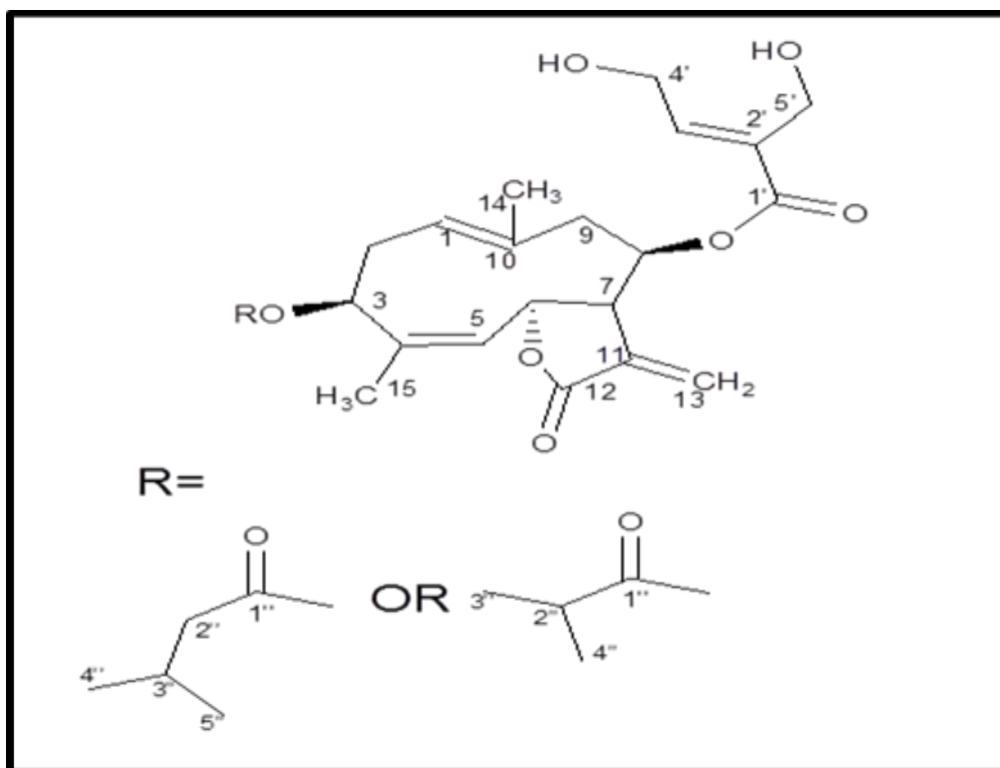


Figure 8.6: The Structure of compound I (Heliangolide) isolated from dichloromethane extracts of *S. pinnata*.

### *8.3.2.* Structural analysis of isolated compound II from *S. pinnata*

The structure of isolated compound II was elucidated using the information depicted by NMR spectra from figure 8.7 to 8.11. This technique characterises a compound due to chemical shift on resonant frequencies of the nuclei within the molecule of the sample. Table 8.2 indicate a summary of carbon shift values compared to the ones from literature and the structure of isolated compound II was determined as a derivative of eucannabinolide sesquiterpene lactone (Figure 8.12).

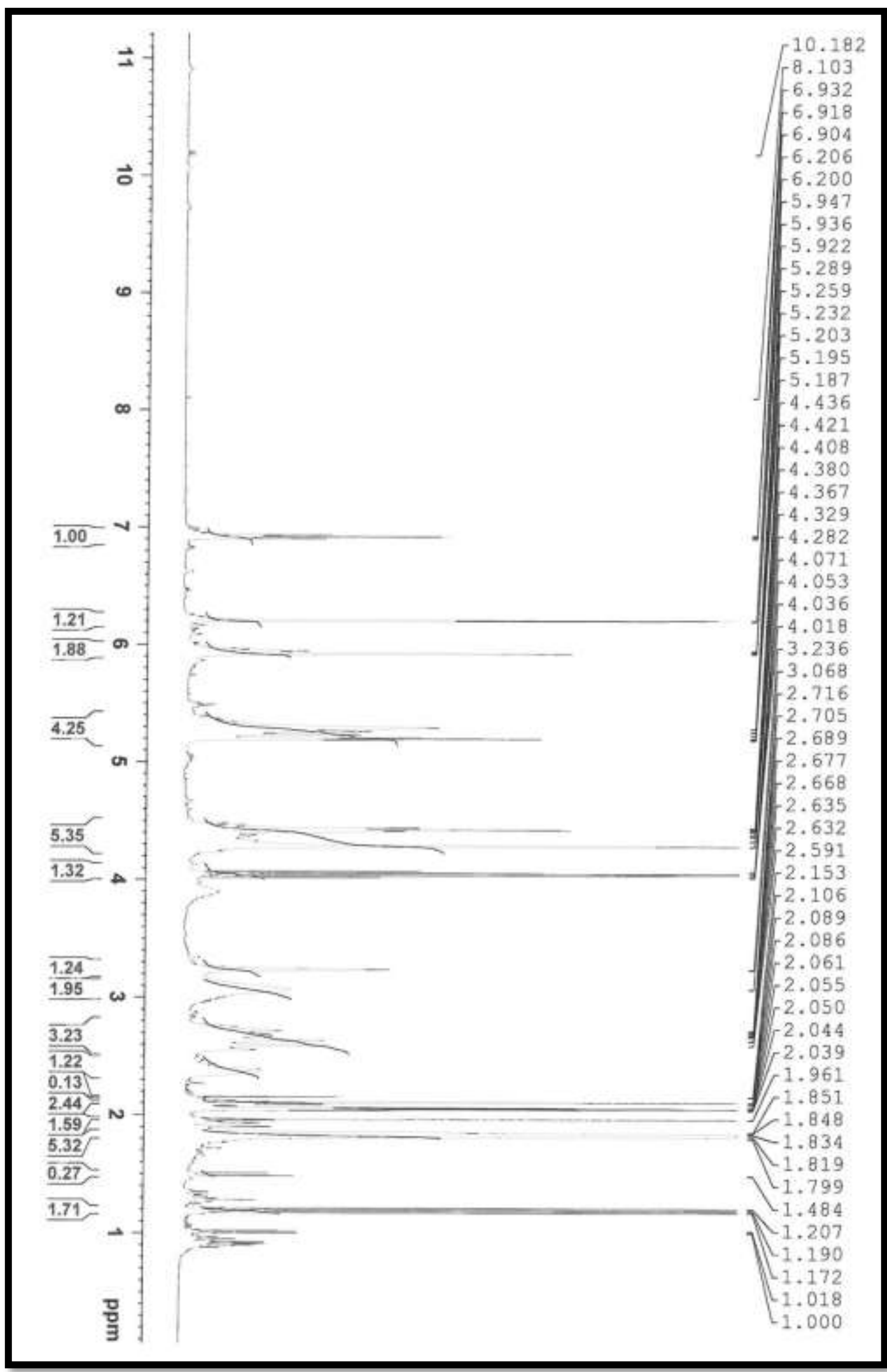


Figure 8.7: The $^{1}H$-NMR spectrum for compound II isolated from dichloromethane extracts of *S. pinnata*.

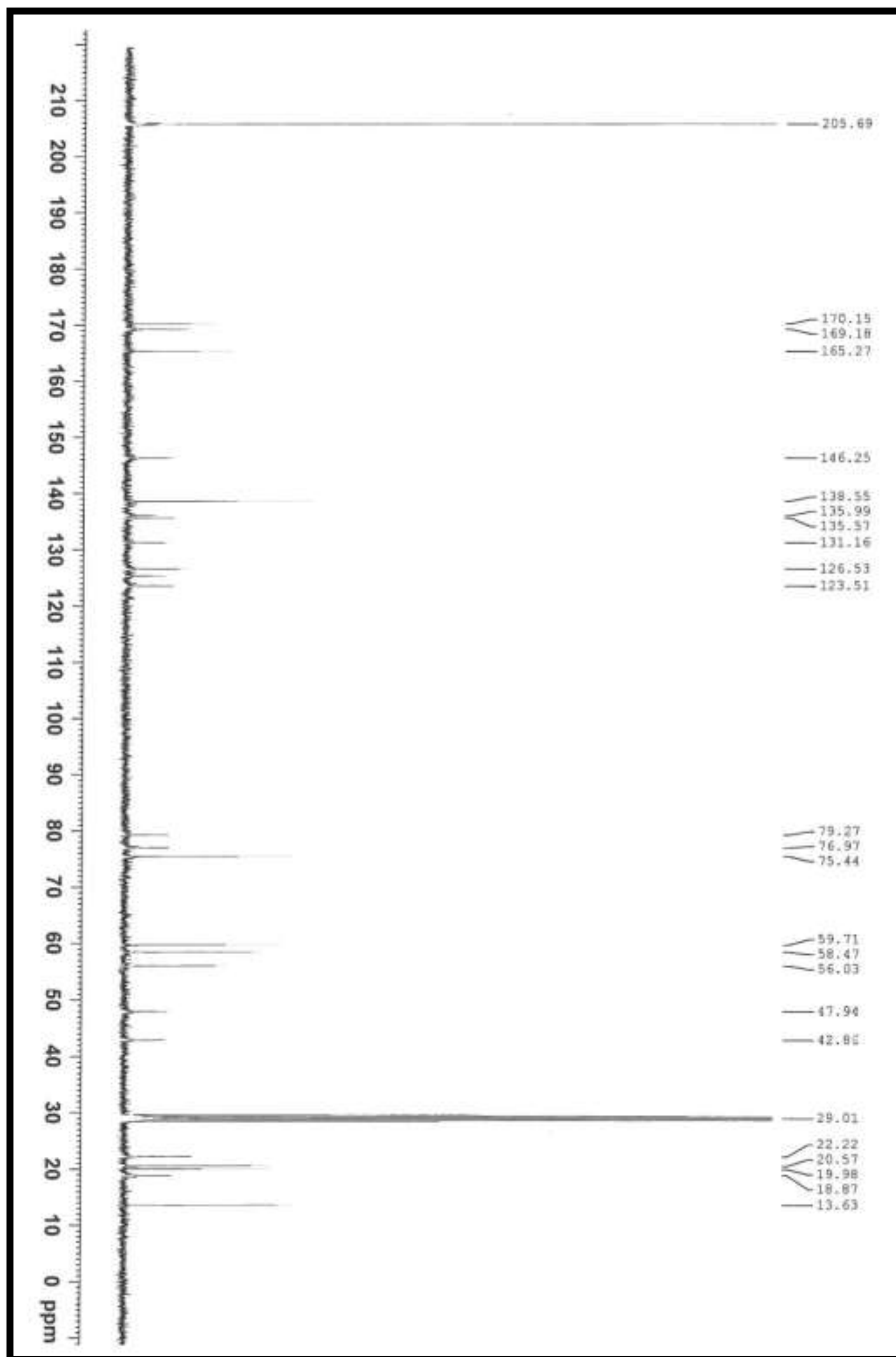


Figure 8.8: The $^{13}$C-NMR spectrum for compound II isolated from dichloromethane extracts of *S. pinnata*.

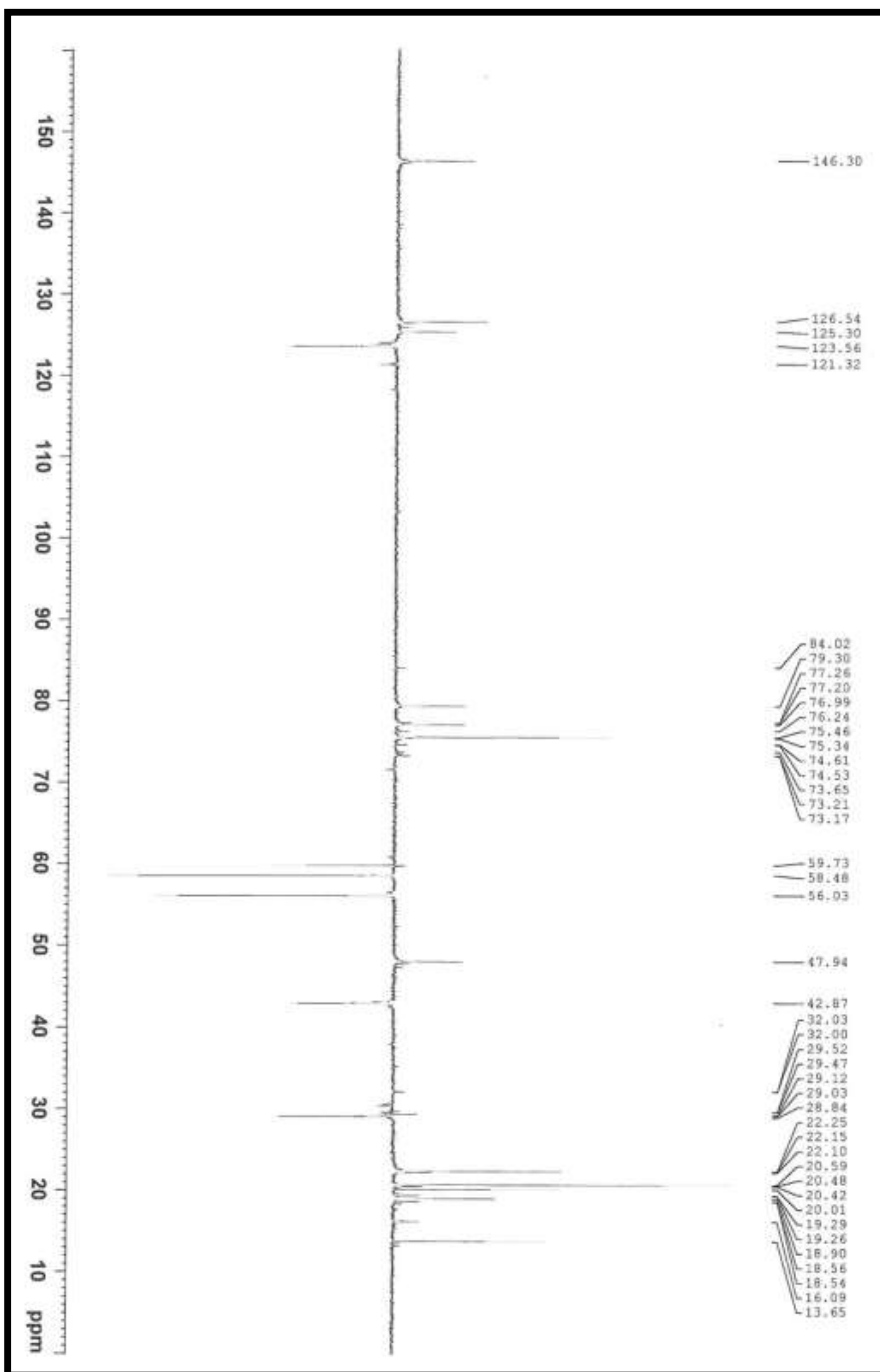


Figure 8.9: The DEPT-NMR spectrum for compound II isolated from dichloromethane extracts of *S. pinnata*.

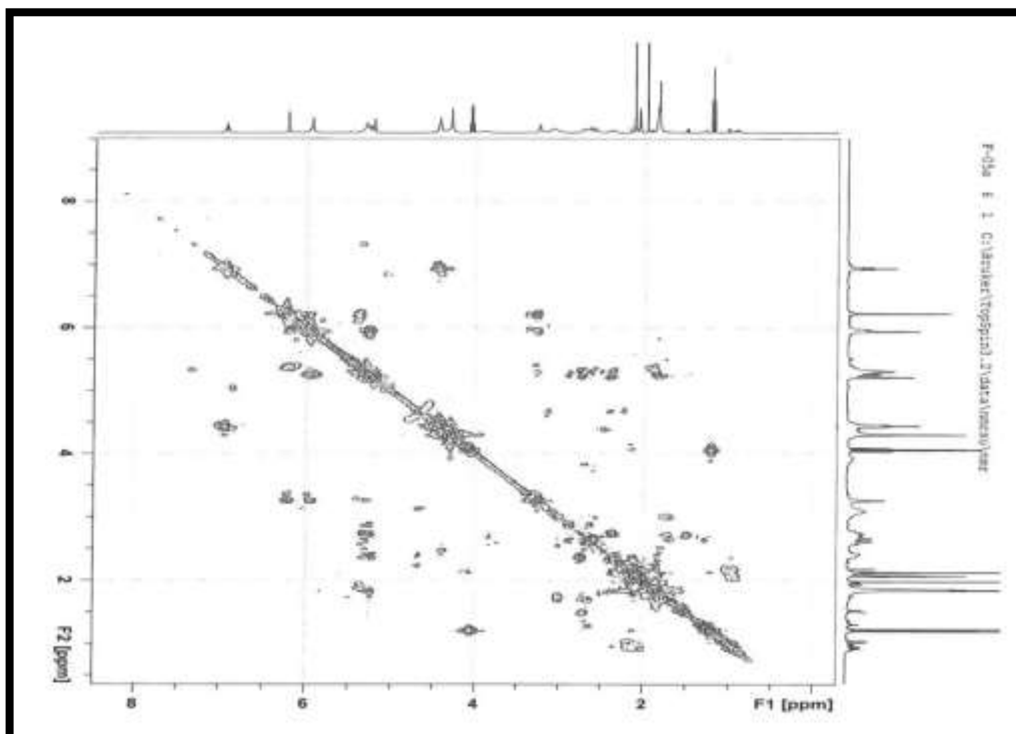


Figure 8.10: The COSY-NMR spectrum for compound II isolated from dichloromethane extracts of *S. pinnata*.

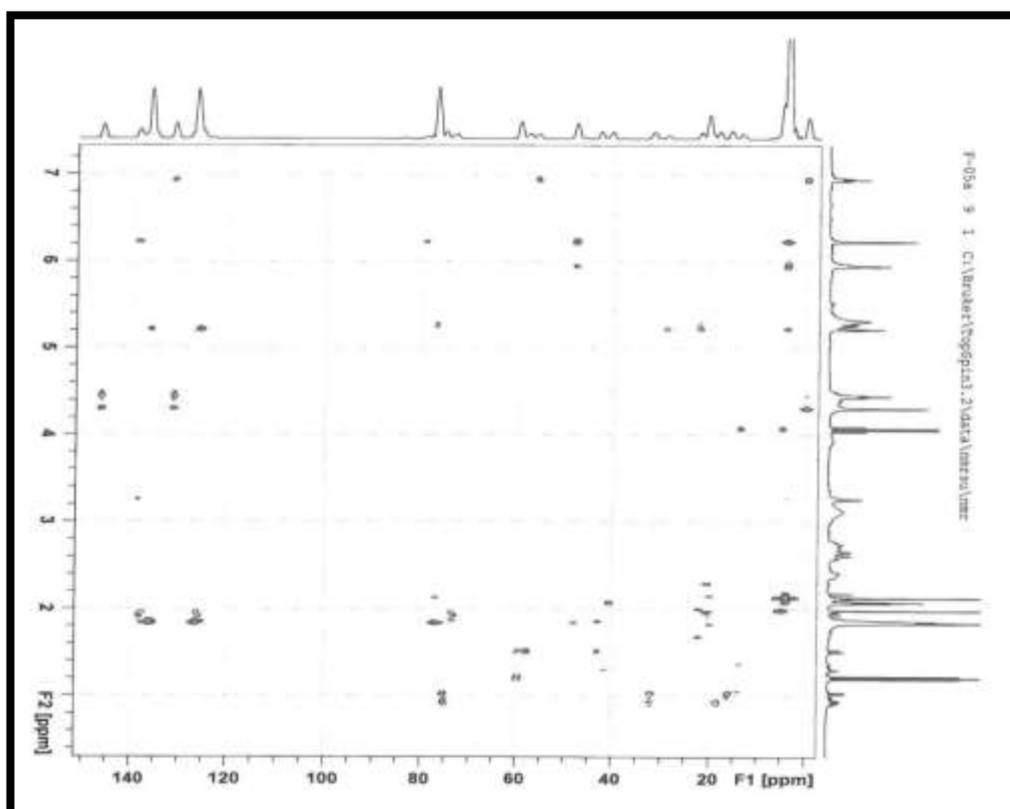


Figure 8.11: The HMBC-NMR spectrum for compound II isolated from dichloromethane extracts of *S. pinnata.*

Table 8.2: $^{13}$C-NMR shift values for isolated compound II compared to the compound from literature.

| Positions | Herz and Govindan, 1980 | | Compound II |
|---|---|---|---|
| 1 | 125.07 | CH | 125.30 |
| 2 | 29.38 | $CH_2$ | 29.01 |
| 3 | 76.83 | CH | 76.97 |
| 4 | 137.27 | | 138.55 |
| 5 | 126.03 | CH | 126.53 |
| 6 | 79.07 | CH | 79.27 |
| 7 | 48.32 | CH | 47.94 |
| 8 | 76.05 | CH | 75.44 |
| 9 | 43.25 | $CH_2$ | 42.86 |
| 10 | 136.56 | | 138.55 |
| 11 | 135.44 | | 135.99 |
| 12 | 170.25 | | 170.15 |
| 13 | 125.07 | $CH_2$ | 125.30 |
| 14 | 19.39 | $CH_3$ | 18.89 |
| 15 | 22.99 | $CH_3$ | 22.22 |
| 1’ | 165.49 | | 165.27 |
| 2’ | 131.09 | | 131.16 |
| 3’ | 145.45 | CH | 146.25 |
| 4’ | 58.78 | $CH_2$ | 58.47 |
| 5 | 56.52 | $CH_2$ | 56.03 |
| 1’’ | 169.90 | | 169.18 |
| 2’’ | 21.13 | $CH_3$ | 20.57 |

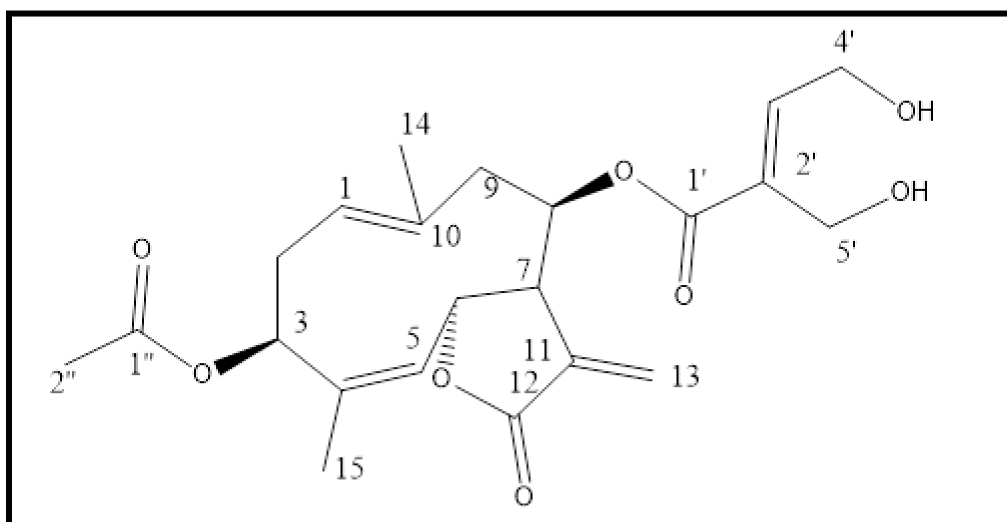


Figure 8.12: The structure of compound II (Derivative of eucannabinolide) isolated from dichloromethane extracts of *S. pinnata*.

## 8.4. Discussion

Fractionation of dichloromethane extracts from *S. pinnata* in column chromatography resulted in isolation of two antimycobacterial compounds. Nuclear magnetic resonance spectroscopy was used in the elucidation of heliangolide and eucannabinolide compounds isolated from *S. pinnata*. Different techniques from NMR spectroscopy (proton, carbon, COSY, HQSC, and DEPT) assisted in elucidation of the compounds which belong to sesquiterpene lactones. These techniques provided the information on the number of proton, types of carbon and coupling system between carbon and hydrogen atoms of the molecule. Table 8.1 shows the carbon NMR shift values of compounds I in comparision with those obtained by Pacciaroni *et al*. (1995). The structure of the isolated compound I was elucidated as heliangolide sesquiterpene lactone compound (Figure 8.6). Table 8.2 summarises carbon shift values from Herz and Govindan (1980) and of the isolated compound II. The isolated compound II was elucidated as structure of a derivative of eucannabinolide sesquiterpene lactone (Figure 8.12). Plants belonging to family *Asteraceae* have been reported to be the source of sesquiterpene lactones compounds (Shen *et al*., 2005). Hengaliangolide sesquiterpene lactone has been isolated from *Eupatorium kiirunense* (Yang *et al*., 2005).

## 8.5. Conclusion

Nuclear magnetic resonance successfully elucidated the structure of isolated compounds from dichloromethane extracts of *S. pinnata* which were previously isolated from the literature. Isolated compounds have to be tested for the biological activities to evaluate if the antimycobacterial activity, cytotoxicity and anti-inflammatory effects observed with the crude extracts were due to synergism or antagonistic effect of the compounds.

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# Chapter 9

## 9. Biological activities of isolated compounds

### 9.1. Introduction

Bioactive compounds might have the effective activity in a synergistic reaction or single compound (Balunas and Kinghorn, 2005). For that reason isolated compounds have to be evaluated to check if they still have the biological activities. *S. pinnata* belongs to the family *Asteraceae* which has been reported to possess most of the sesquiterpene lactone compounds. A number of these compounds have been isolated from different plants belonging to this family. Sesquiterpenes have been reported to be effective against bacteria, fungi, malaria and inflammatory diseases (Matejić *et al.*, 2014). The following compounds are some of the compounds isolated from plants belonging to genus *Schkuhria*: germacranolides, elemanolides and labdanes (Ganzer and Jakupovic, 1990), heliangolides (Bohlman and Zdero, 1981) and Schkuhripinnatolide-C and pectolaringenin (Pacciaroni *et al.*, 1995). The most reported biological activity of these compounds is the anticancer activity (Zhang *et al.*, 2005; Ghantous *et al.*, 2010). In this study the antimycobacterial, cytotoxicity and anti-inflammatory activities of the isolated compounds were evaluated using bioautography, broth microdilution, MTT and anti-inflammatory activity assays.

### 9.2. Methods and materials

#### 9.2.1. Phytochemical analysis

Phytochemical analysis of the isolated compounds was done using thin layer chromatography plates as mentioned in section 3.2.3.

#### 9.2.2. Antimycobacterial activity

9.2.2.1. Bioautography assay

Thin layer chromatography was used to screen antimycobacterial activity of the compounds following a procedure described in section 5.2.1.

9.2.2.2. Broth microdilution assay

Minimum inhibitory concentration of the compounds was evaluated as mention in section 5.2.2.

### 9.2.3. MTT assay

Cytotoxic effects of the compounds were evaluated following a procedure described in section 7.2.1.

### 9.2.4. Anti-inflammatory assay

Anti-inflammatory activity of the compounds was determined following a procedure mentioned in section 7.2.2.

## 9.3. Results

### 9.3.1. Phytochemical analysis

The chromatograms indicated a purified single compound I and compound II which has another phytochemical compound (Figure 9.1).

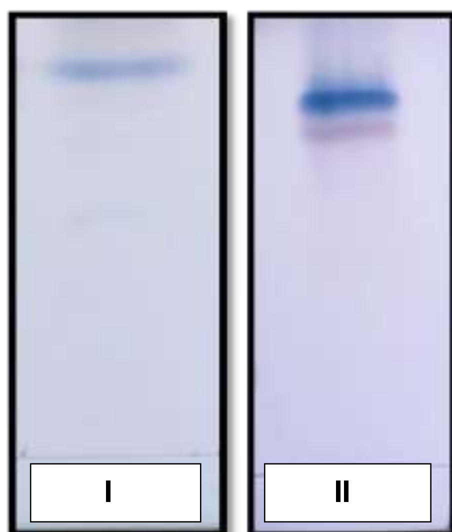


Figure 9.1: Chromatograms of compound I and II, developed with 70% ethyl acetate in acetone and sprayed with vanillin sulphuric acid regent for colour development.

### 9.3.2. Antimycobacterial activity

9.3.2.1. Bioautography assay

Antimycobacterial activity was evaluated using TLC plates, sprayed with the bacterial culture in broth and INT (2 mg/ml) in distilled water. The white area against the pink background indicates antibacterial activity which was observed on both compounds (Figure 9.2)

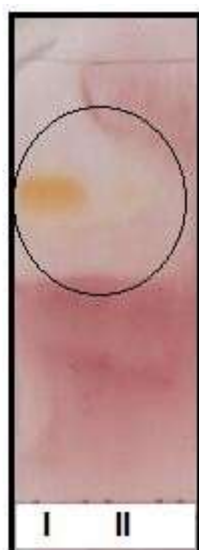


Figure 9.2: Bioautograms of heliangolide **(I)** and a eucannabinolide derivative **(II)** developed in 70% ethyl acetate in acetone, sprayed with *M. smegmatis* culture and INT (2 mg/ml) reagent. The white area against a pink background indicated growth inhibition of the microorganism.

9.3.2.2. Broth microdilution assay

The lowest MIC value of 0.53 mg/ml was obtained with eucannabinolide compound when compared to compound I which had the MIC value of 0.63 mg/ml and the positive control (0.08 mg/ml) (Table 9.1).

Table 9.1: Minimum inhibitory concentration (MIC) values (mg/ml) of Compound I and II.

| **Compounds** | **MIC (mg/ml)** |
|---|---|
| Compound I | 0.63 |
| Compound II | 0.53 |
| Rifampicin | 0.08 |
| Average | 0.58 |

### 9.3.3. MTT assay

Cytotoxicity of the compounds was evaluated on Vero monkey kidney cells following an MTT procedure described by Mosmann (1983). Cytotoxic value of heliangolide

compound was found to be 13.54 µg/ml and for eucannabinolide to be 14.15 µg/ml (Table 9.2). The selectivity index (SI) for both compounds was below 1.

Table 9.2: Determination of cytotoxicity value (µg/ml), minimum inhibitory concentration (µg/ml) and selectivity index (SI) of isolated compounds.

| Compounds | $LC_{50}$ (µg/ml) | MIC (µg/ml) | Selective index (SI) |
|---|---|---|---|
| Heliangolide | 13.54 | 630 | 0.02 |
| Eucannabinolide | 14.15 | 530 | 0.03 |

### 9.3.4. Anti-inflammatory assay

Anti-inflammatory effects of the compounds at different concentrations were determined on LPS induced Raw 246.7 cells (Figure 9.3). It was observed that the activity of the compounds was in a concentration dependent manner as compared to the untreated cells. The ability of curcumin (positive control) to reduce the production of ROS was almost of the same percentage of compound II at the lowest concentration.

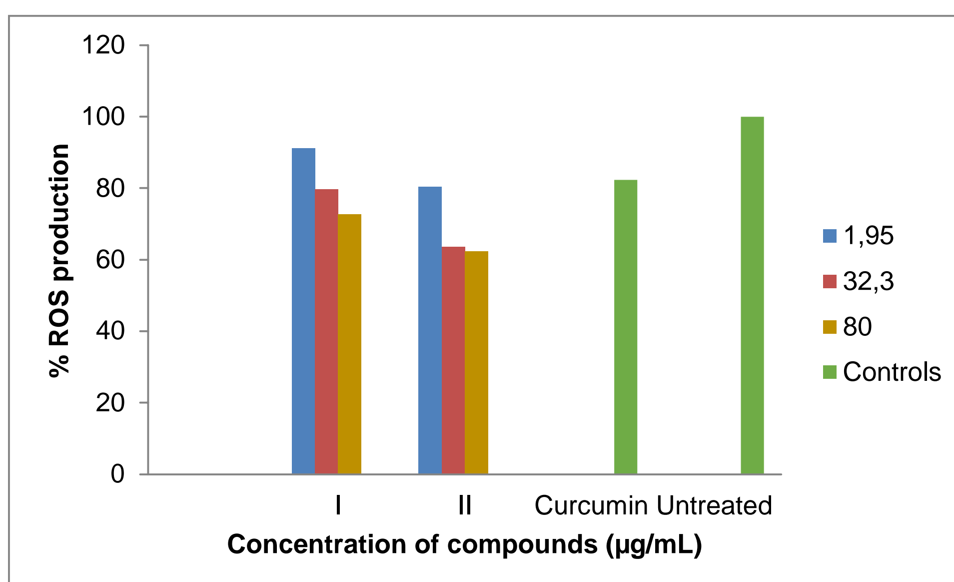


Figure 9.3: The effects of isolated compounds I and II at different concentrations on Raw 264.7 macrophage cells in response to oxidative stress and inflammation determined by measuring the production of ROS in percentages.

## 9.4. Discussion

Analysis of phytochemical profiles from crude extracts assisted in determining the possible bioactive compounds as it was observed that there were blue bioactive compounds in the preliminary extractions procedures (chapter 3). When it comes to isolation, the blue compounds were isolated as heliangolide and eucannabinolide sesquiterpene lactone compounds (Figure 9.1) which were having antimycobacterial activity against *M. smegmatis* (Figure 9.2). Suliman (2010) detected the blue and purple compounds from *S. pinnata* leaves. Sesquiterpene lactones from *S. pinnata* have been reported to possess antiprotozoal activity (Kimani *et al.*, 2018). Biological activities from sesquiterpenes are connected to the presence of α, β-unsaturated-γ-lacton ring (Palvlovic *et al.*, 2006). Biological activities such as antifungal, antimalarial and antibacterial activities have been reported in a number of studies (Matejić *et al.*, 2014).

Antimycobacterial activity of the isolated compounds was evaluated using broth microdilution method described by Eloff, 1998. Eucannabinolide had the lowest MIC values of 0.53 mg/ml as compared to heliangolide which had 0.63 mg/ml (Table 9.1). Rifampicin was used as a positive control and had the lowest MIC value of 0.08 mg/ml. Sesquiterpene lactones compounds from *Laurus spp* had an effective activity against *Mycobacterium tuberculosis* (H37Rv) with the lowest MIC values ranging from 6.25 to 12.5 mg/L (Luna-Herrera *et al.*, 2007). Isolated compounds retained their antimycobacterial effects, indicating that the antimycobacterial activity observed with the crude extracts was not in antagonistic or synergism mechanism.

Cytotoxic effects of the compounds have been evaluated on Vero monkey kidney cells using MTT assay. The isolated compounds heliangolide and eucannabinolide compounds indicated $LC_{50}$ values of 13.54 µg/ml and 14.15 µg/ml respectively (Table 9.2). The compounds as well as extracts were toxic to the Vero monkey kidney cells with $LC_{50}$ < 30 µg/ml which are regarded toxic (Elisha *et al.*, 2017). The compounds had toxic effects towards the host cells than the microorganism as the selectivity indices for both compounds were less than one (Table 9.2) (Makhafola *et al.*, 2012). The observed antimycobacterial activity of the compounds indicated that the compounds are individually active.

The anti-inflammatory activity of the isolated compounds was evaluated on Raw 264.7 macrophage cells. Inhibition of reactive oxygen species production was observed to be in concentration dependent manner (Figure 9.3). The highest concentration of heliangolide inhibited 72% while the lowest inhibition was obtained with lowest concentration. The same trend was observed with eucannabinolide compound as compared to untreated cells. Abe *et al.* (2015) reported the anti-inflammatory activity of sesquiterpene lactones from *Tithonia diverifolia* on human neutrophils. Antiproliferative activity of compounds from plants belonging to family *Asteraceae* have been reported against Caco-2 cells (Rodrigo *et al.*, 2010). The biological activities such as anti-inflammatory, antibacterial, antimalarial and antifungal have been reported from plant belonging to *Asteraceae* family (Lang *et al.*, 2002; Garcia *et al.*, 2003; Habtemariam, 2001).

### 9.5. Conclusion

The compounds can be used as new leads in the development of antimycobacterial drugs. However, they have to be tested against *Mycobacterium tuberculosis.* The compounds can be used in the development anti-inflammatory drugs as well. Nonetheless, the compounds have to be analysed further in *in vivo* assays to justify their biological activities found in this study.

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## Chapter 10

## 10. General discussion and conclusion

*S. pinnata* was evaluated for the presence of potential bioactive compounds against *Mycobacterium smegmatis* aiming to isolate and characterise the active antimycobacterial compounds. Medicinal plants have been used for thousands of years and their dominancy in modern medicine validates their use in traditional medicines. Drug resistance, side effects and high cost of antibiotics have a negative impact on the health care system. Health care systems have some challenges in treatment of infectious and degenerative diseases as the drugs used to combat diseases course server side effects, are unaffordable and also the issue of drug resistance to microorganisms. These challenges resulted in the search for new leads in development of new drugs. Medicinal plants possess phytochemicals which have therapeutic properties. Evaluation of these medicinal plants for their biological activities such as antioxidant, antibacterial and anti-inflammatory activities, gives better understanding on the efficacy, safety and synergistic interactions.

As extraction is regarded as the first essential step in the analysis of natural products, solvent polarity plays a vital role in the type of phytochemicals to be obtained. *S.pinnata* material was extracted with solvents of varying polarity using different extraction procedures. High amount of plants extracts were obtained with solvents of intermediate polarity. The mass transfer between the sample and the solvent is highly dependent to the solubility of the extractants. Phytochemical profiles of the plant extracts were analysed using thin layer chromatography plates developed in three solvent systems (BEA, CEF, EMW) at different polarities. Different colours on the chromatograms indicated the compounds which are present from the plants extracts. Chemical test also confirmed the presence of tannins, saponins, flavonoids, alkaloids, terpenoids and steroids. These phytochemicals have been reported to have therapeutic potential. Serial exhaustive extraction procedure was the best extraction procedure to extract high amounts of bioactive compounds.

The plant extracts were evaluated for the biological activities such as antioxidant, antibacterial, anti-inflammatory and cytotoxic effects. In this study, plant extracts were reconstituted in acetone to conduct bioassays. Qualitative and quantitative analysis of

antioxidant activity was evaluated using 1, 1 diphenyl-2-picrylhydrazyl (DPPH) and ferric ion reducing power (FRAP) assays. The presence of antioxidant compounds was depicted by yellow spots or bands against the purple background on TLC plates. Most of the detected antioxidant compounds were not separating from the bottom of the plates. However, some were separated in EMW solvent system. Methanol had the best scavenging activity during quantitative analysis but could not show much activity on the TLC, this indicates that the antioxidant active compounds were working in a synergistic mechanism rather than as single compounds on TLC plates. *S. pinnata* extracts had high reducing power when compared to ascorbic acid. Antioxidant activity could be due to high content of phenolic and tannins compounds detected from the plant extracts. The observed result gives a potential lead for further isolation of the antioxidant compounds which could be used in development of antioxidant supplements.

Solvents with intermediate polarity showed the presence of antimycobacterial compounds on TLC-bioautograms and lowest MIC values. The antimycobacterial activity against *M. smegmatis* might be due to the detected phytochemicals which have been reported to possess a number of biological activities including antibacterial. *S. pinnata* has antibacterial potential against Gram negative and Gram positive microorganisms. It was found that the crude extracts with $LC_{50}$ value of 25 µg/ml had cytotoxic effects on Vero monkey kidney cells and the selective index was less than one. Oxygen reactive species production was inhibited in a concentration dependent manner on LPS- induced Raw 264.7 macrophage cells when compared to the control. However, they require further analysis for them to be used in the development of anti-inflammatory drugs.

Serial exhaustive extraction was chosen for extraction of *S. pinnata* extracts as it had indicated better results in the preliminary work. After serial exhaustive extraction, dichloromethane extracts had high amount of extracts and good antimycobacterial activity. Column chromatography aided in further fractionation of the extracts with solvents of varying polarity. Preparative TLC developed with 70% ethyl acetate in acetone was used to purify the isolated compounds I and II. Characterisation of the compounds structures was analysed with NMR techniques which determined the chemical shifts of the nuclei available in the compounds. The chemical shifts were compared with the ones from literature and it was suggested that compound I is

heliangolide sesquiterpene lactone and compound **II** was a derivative of eucannabinolide sesquiterpene lactone. These compounds have been isolated before from the plants belonging to family the *Asteraceae.* Sequiterpene lactones compounds are responsible for a number of biological activities including antibacterial and anti-inflammatory activity. The compounds were discovered to be toxic to Vero monkey kidney cells with the less than 30 µg/ml $LC_{50}$ values and they are regarded to be more toxic to the host cells because their selectivity indices was less than one. These compounds had a good anti-inflammatory activity which was in a concentration dependent manner. The observed anti-inflammatory activity could be due to the ability of the compounds to scavenge free radicals which was observed in the qualitative assay. Therefore, these compounds can be used as new leads in development of anti-inflammatory drugs. The effective use of this plant in treatment of wounds and other infections could be due to the antioxidant compounds from the plant extracts. Therefore, *in vivo* assays have to be performed to determine the compounds toxicity and anti-inflammatory effects for development of new drugs with less toxicity. The objectives of the study were successfully achieved. This study recommends that the evaluation of the mechanisms behind the toxic and anti-inflammatory effects of the compounds and crude extracts to be carried out.