ISOLATION, CHARACTERISATION AND CYTOTOXIC EFFECTS OF ANTIBACTERIAL AND ANTIOXIDANT COMPOUNDS FROM SELECTED MEDICINAL PLANTS

By

Refilwe Given Kudumela

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Declaration

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

…….. ……

Signature Date

Dedication

I dedicate this work to my mom, for her undying devotion and support and to everyone who made this work possible.

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Psalm 136 verse 1” Give thanks to the Lord, for he is good and his mercy endures forever”

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List of abbreviations

^{13}C	Carbon-13
^{1}H	Hydrogen-1
A	Acetone
ABTS	2, 2-azino-di-(3-ethylbenzothialozine-sulphonic acid
ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonia hydroxide
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CEF	Chloroform/Ethyl acetate/Formic acid
CLSI	Clinical and laboratory standard institute
COSY	Correlation spectroscopy
DCF	2, 7-dichlorofluorescein
D/DCM	Dichloromethane
DEPT	Distortionless enhancement through polarisation transfer
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EMW	Ethyl acetate/methanol/water
E	Ethanol
EC_{50}	Half maximal effective concentration
E. *coli*	*Escherichia coli*
EA	Ethyl acetate

E. faecalis	*Enterococcus faecalis*
FBS	Foetal bovine serum
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalence
H	n-hexane
H_2DCF-DA	dihydrodichlorofluorescein diacetate
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HSQC	Heteronuclear single quantum correlation
HPLC	High Performance Liquid Chromatography
INT	ρ-iodonitrotetrazolium violet
IR	Infra-red spectroscopy
LC_{50}	Lethal concentration 50
LPS	Lipopolysaccharide layer
M	Methanol
MEM	Minimal essential medium
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NCI	National cancer institute
NMR	Nuclear magnetic resonance
NSAID	Non- steroidal anti-inflammatory drugs
P. aeruginosa	*Pseudomonas aeruginosa*

PBS	Phosphate buffered saline
QE	Quercetin equivalence
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Rotation per minute
S. *aureus*	*Staphylococcus aureus*
SI	Selectivity index
TLC	Thin layer chromatography
USA	United States of America
UV	Ultraviolet
UV-vis	Ultraviolet-visible spectroscopy
W	Water
WHO	World Health Organization

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Abstract

This study was aimed at isolating and evaluating the antibacterial, antioxidant and cytotoxic effects of active compounds from selected medicinal plant species. The biological activities and cytotoxic effects of *Commelina africana*, *Dombeya rotundifolia*, *Elephantorrhiza elephantina* and *Schkuhria pinnata* were investigated based on their traditional uses. The plants were extracted with different solvents of varying polarities. Methanol was the best extractant with the highest yield. The presence of different phytoconstituents was screened using standard methods; this revealed the presence of terpenoids, flavonoids, tannins, and saponins. Meanwhile, the phytochemicals were screened on TLC, where different colours observed indicated the presence of different compounds. The total phenolic, tannin and flavonoid content were estimated using spectrophotometric methods. *D.* rotundifolia had the highest amounts of phenol, tannin and flavonoid content, while *S. pinnata* had the least. The antibacterial and antioxidant screening employed both qualitative and quantitative assays. Although not separated, this study demonstrated the antioxidant potential of *D*. *rotundifolia* extracts developed in BEA and CEF. *D. rotundifolia* also demonstrated the highest free radical scavenging and reducing power antioxidant potential. These activities could be attributed to the high phenolic, tannin and flavonoid content observed. All the selected plants had antibacterial potential against *E. coli* and *P. aeruginosa*. Only *S. pinnata* extracts were active against *E. faecalis* and *S. aureus*. The MIC values of the plants and ampicillin ranged from 0.02 to 2.50 mg/mL and 0.02 to 0.08 mg/mL, respectively. The plants were combined to determine any interaction effects in exhibiting antibacterial activity. Among all the possible combinations, combinations with *S. pinnata* had improved antibacterial activity. The effects of *D. rotundifolia* and *S. pinnata* extracts on the inhibition of the production of reactive oxygen species on LPS-induced RAW 264.7 macrophages was determined using the DCFHD-A assay. Both plants demonstrated anti-inflammatory potential by inhibiting the production of ROS in a dose-dependent manner. Cell viability assays revealed that *S. pinnata* had cytotoxic effects on African green monkey kidney (Vero) cells with the lowest LC_{50} (25 µg/mL). Bioassay guided fractionation and nuclear magnetic resonance spectroscopy of dichloromethane extracts of *S. pinnata* on column chromatography afforded isolation and characterisation of three sesquiterpene lactones. These were identified as 6-(2, 3-

dihydroangeloylory)-6α-hydroxyinuviscolide, heliangolide and eucannabiolide. All three compounds had antibacterial, anti-inflammatory and cytotoxic effects. The compounds may serve as leads for synthesis of less toxic therapeutic antibacterial and anti-inflammatory drugs in the pharmaceutical industry.

Chapter 1: Introduction

The healthcare system is currently facing many challenges, these include; the toxicity of the available antioxidant drugs, resistance of pathogenic bacteria to the available antibiotics and the increase in new and re-emerging infectious diseases. Although, effective some antibiotics have been associated with undesirable side effects, such as; nausea, depression of bone marrow, thrombocytopenic and agranulocytosis (Marchese and Shito, 2000). The currently used synthetic antioxidant drugs were proven to be toxic and carcinogenic to human beings (Lakshmi *et al*., 2014), as such, this necessitates the search for alternative safe and effective drugs, with different mechanisms of action so as to overcome these challenges (Palombo, 2011). These current drawbacks have led to the research on plants with medicinal effects (medicinal plants).

Medicinal plants are a potential source of phytochemicals that are of pharmaceutical interest such as flavonoids, sterols, alkaloids, phenolic compounds, terpenes etc. (Kapoor and Mishra, 2013). These chemical substances have therapeutic properties that can be used for treatment of human diseases. Some of these properties include; antioxidant, antimicrobial, anti-cancer, anti-diabetic, immune modulatory and reno-protection effects (Rafieian-Kopaei, 2011; Londonkar *et al*., 2013).

Scientists are searching the earth for phytochemicals to be developed into drugs for treatment of human and animal diseases. Isolation and structure elucidation of some of these compounds led to the synthesis and production of potent drugs such morphine and taxol. These compounds served as leads for synthesis of potent lower toxic compounds e.g. metformin and verapamil (Fabricant and Farnsworth, 2001; Bakht *et al*., 2013).

Most of these plants are used according to instructions and observations from traditional healers without any scientific validation, dosage and toxicity evaluation (Kumar *et al*., 2007). Therefore, medicinal plants with potent antioxidant and antibacterial activities should be evaluated for possible cytotoxic effects to validate their use in herbal medicine.

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Chapter 2: Literature review

2.1. Medicinal plants

People all over the world have been using medicinal plants for centuries as a form of treatment for human diseases and wounds. Some medicinal plants have been implicated as sources of antimicrobial agents. These are preferred over synthetic drugs because they have fewer side effects. The plant parts mostly used are flowers, roots, bulbs, twigs, leaves and stems (Mahesh and Shatish, 2008). Furthermore, herbal medicines may be in the form of powders, liquids, or mixtures which may be raw, boiled, ointments, liniments and incisions (Bakht *et al*., 2013).

Estimations have been made, that of the 14 to 28% of the higher plants that are used for medicinal purposes, about 74% of the pharmacologically active compounds derived from plants were discovered by following up on their use (Ncube *et al*., 2008; Das *et al*., 2010). South Africa has significant diversity of plants and over 3000 of these are used as traditional medicine, with 350 making the most commonly used and traded medicinal plants (van Wyk *et al*., 1997). World health organisation (WHO) (2002), reported that up to 80% of the population in Africa use traditional medicine to serve their health needs. Over 60% of the South African population, especially those in rural areas consult traditional healers, since they are numerous and easily accessible than western doctors. This was seen in Kwa-Zulu Natal Province, where a large number of people seem to seek medical advice from traditional healers in addition to Western medicine. This is either as a result of easy access, low costs or cultural and spiritual beliefs (Taylor *et al*., 2001).

2.2. Medicinal plants as a source of new drugs

Medicinal plants are now considered vital sources of new compounds with potential therapeutic effects because they contain a wide range of compounds that can be used for treatment of chronic and infectious diseases (Moussaoui and Alaoui, 2016). The components from medicinal plants play an important role in conventional Western medicine. Although a lot of species have been examined for antimicrobial and antioxidant properties, the vast majority of them have not yet been analysed (Viji

and Murugesan, 2010). Thus potent drugs may be discovered from plants which are recommended regularly and are observed to be effective (Borokini and Omotayo, 2012).

Farnsworth (1984) reported that at least 25% of the prescribed drugs that were issued in the United States and Canada were derived from natural plant products. Furthermore, Harvey (2001) reported that in the year 1993, in the USA only, 57% of all prescribed medicines comprised of at least one major active compound currently or once derived from plant sources. Many drugs sold world-wide are of plant origin, these include; Asatrophine, Ephedrine, Digoxin, Morphine, Quinine, Reserpine and Tubocurine (Gilani, 2005). About 119 secondary metabolites from plants were identified and are used globally as drugs. This is why medicinal plants used in traditional medicine should be studied for safety and efficiency (Eloff, 1998a).

2.3. Plant metabolites

Two classes of compounds that are found in plants include the primary and secondary metabolites. The primary metabolites are essential for growth and development while the secondary metabolites play a role in the defence against infections by microorganisms and even against pests. The secondary metabolites were proven to be effective in prevention and cure of diseases in humans and animals; hence they are called bioactive compounds (McChesney, 1993).

2.4. Secondary metabolites with medicinal effects

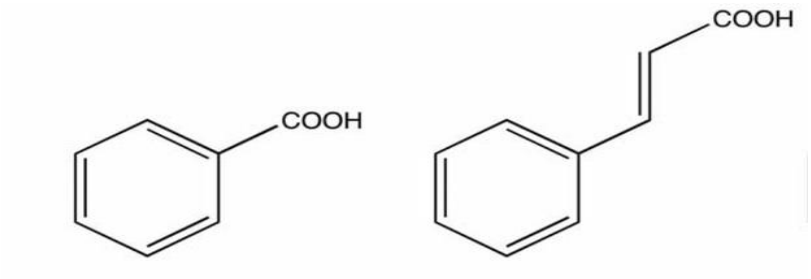
Almost all plants have the ability to synthesise aromatic compounds, most of which are phenols and at least 12000 secondary metabolites have been isolated (Hoult and Paya, 1996). These have been shown to play a role in the defence mechanism of the plant against predation by microorganisms and insects (Cowan, 1999). These include carotenoids, saponins, flavonoids, alkaloids, steroids, cardiac glycosides, and phlabotannins. Some are responsible for odours, which include; terpenoids, while others like quinones and tannins are responsible for the pigments (Karuppusamy, 2009). Phenolic compounds and flavonoids are largely distributed in higher plants and they are said to have multiple biological activities such as

anticarcinogenic, anti-inflammatory, antioxidant and antibacterial activities (Patel *et al*., 2010).

2.4.1. Phenolic compounds

Phenolic compounds are derivatives of pentose phosphate, shikimate, and phenylpropanoid pathway in plants. They possess an aromatic ring with one or more hydroxyl groups; their structure may range from simple phenolic molecules to complex polymers (Figure 2.1) (Balasundram *et al*., 2006; Ignat *et al*., 2011). The considerable physiological and morphological importance of these compounds in plants did not come as a surprise since they are widely distributed (Balasundram *et al*., 2006).

The main classes of simple phenols include; hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones, hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (Chung *et al*., 1998; Krueger *et al*., 2003; Mokgoatsane, 2011). These compounds possess a wide range of physiological properties which include; antioxidant, antimicrobial, anti-inflammatory, anti-artherogenic, anti-thrombotic, cardio protective and vasodilatory effects (Middleton *et al*., 2000; Manach *et al.,* 2005; Balasundram *et al*., 2006).



(a) **(b)**

Figure 2.1: General structures of phenolic acids (**a**) hydroxybenzoic acid and (**b**) hydroxycinnamic acid (Khoddami *et al.,* 2013).

2.4.2. Quninones

These are naturally ubiquitous and highly reactive aromatic rings with two ketones substitutions. These compounds are responsible for the browning reaction in injured or cut vegetables and are intermediates in the pathway of melanin synthesis in the human skin (Schmidt, 1988; Cowan, 1999; Mokgoatsane, 2011). An easy switch between di-phenol (hydroquinone) and di-ketone (quinone) is possible through oxidation and reduction reactions. Vitamin K is a naphthoquinone complex that exhibits anti-haemorrhagic activities (Cowan, 1999; Mokgoatsane, 2011).

Quinones (Figure 2.2) represent a source of stable free radicals. They form irreversible complexes with nucleophilic amino acids in proteins, thus rendering proteins inactive and dysfunctional. They also render substrates unavailable to microorganisms. This is why they are potential antimicrobial agents, targeting surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes on microbial cells (Cowan, 1999). Anthraquinones from *Cassia italica*, a Pakistani tree was reported to be bacteriostatic to *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, *Pseudomonas aeruginosa* and bactericidal to *Pseudomonas pseudomalliae* (Kazmi *et al*., 1994; Cowan, 1999).

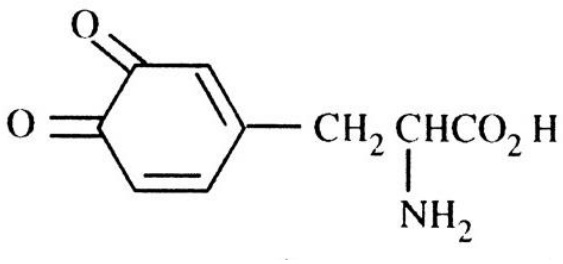


Figure 2.2: The structure of O-quinone (Cowan, 1999).

2.4.3. Flavonoids

Flavonoids are hydroxylated phenolic compounds with one carbonyl group, but occur as a unit linked to an aromatic ring (Figure 2.3) (Cowan, 1999). Most of them are known to be pigments in higher plants and derivatives of phenylalanine and tyrosine amino acids. Examples include catechins, iso-flavonols, flavones, anthocyanidins, anthocyanins, and proanthocyanidins (Khoddami *et al*., 2013). Quercetin and

kaempferol represent the common flavonoids found in plants. It is known that these compounds are synthesised by plants in response to microbial infections. Therefore, their effectiveness as antimicrobial substances against a wide range of microorganisms *in vitro* did not come as a surprise (Cowan, 1999).

Flavonoids may exhibit antimicrobial activity by forming complexes with bacterial cell walls, extracellular and soluble proteins. Some lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999). Oolong green teas have been reported to contain catechins compounds that exhibited *in vitro* antimicrobial activity against *Vibrio cholera* (Borris, 1996), *Streptococcus mutans* (Sakanaka *et al*., 1992), *Shigella* and other microorganisms (Tsuchiya *et al*., 1994; Vijaya *et al*., 1995). Nijveldt *et al*. (2001) has documented the antioxidant, antitumor, anti-inflammatory, anti-osteoporotic, antiviral and anti-thrombogenic effects of flavonoids.

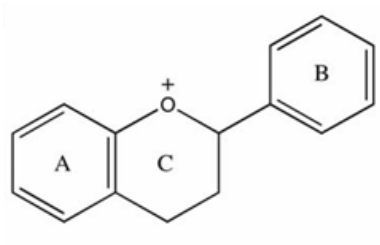


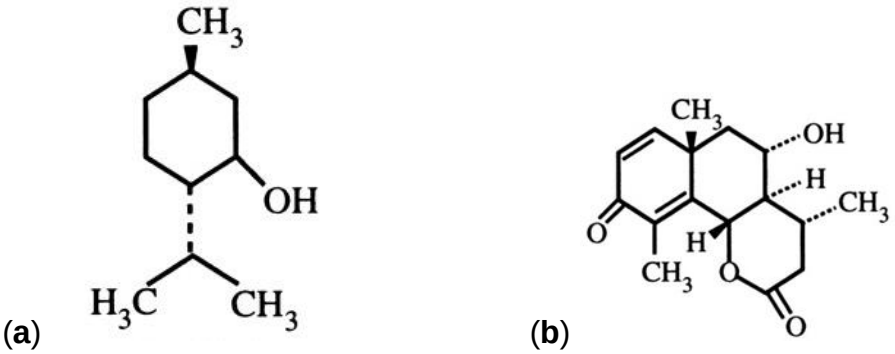
Figure 2.3: General structure of flavonoids (Khoddami *et al*., 2013).

2.4.4. Terpenoids

Terpenoids are a group of secondary metabolites containing carbon backbones made up of isoprene units which contain five carbon atoms, as a result, the number of carbon atoms in any terpenoids is a multiple of five (Figure 2.4) (Nassar *et al*., 2010). Terpenoids are divided into several groups based on the number of building blocks such as monoterpenes (e.g., carvone, geraniol, *d*-limonene, and perillyl alcohol), diterpenes (e.g. retinol and *trans*-retinoic acid), triterpenes (e.g., betulinic acid, lupeol, oleanic acid, and ursolic acid), and tetraterpenes (e.g., α-carotene, β-carotene, lutein, and lycopene) (Rabi and Bishayee, 2009; Thoppil and Bishayee, 2011).

Terpenes and their derivatives are widely recognized for their involvement in plant defence mechanism (Zwenger and Basu, 2007). The pharmacological activities

exhibited by this group of compounds include; anti-viral, antibacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities (Nassar *et al*., 2010; Mahato and Sen, 1997).



(**a**) (**b**)

Figure 2.4: The structures of common terpenoids (**a**) menthol and (**b**) artemisin (Abdallah and Quax, 2017).

2.4.5. Alkaloids

Alkaloids are heterocyclic nitrogen containing compounds classified in different categories based on their biosynthetic pathways. They are produced by plants in response to abiotic stress. Examples include; quinine, ephedrine, homoharringtonine and chelerythrine, which have a wide range of pharmacological activities such as antimalarial, antimicrobial, anticancer and antiasthma (Kittakoop *et al*., 2014). Morphine which was isolated from opium poppy *Papaver somniferum* in 1805 was the first medically useful example of an alkaloid (Fessenden and Fessenden, 1982; Mokgoatsane, 2011).

Berberine (Figure 2.5) is a natural alkaloid found in the roots, rhizomes, and stem bark of plants. The extracts and decoctions of berberine have significant antimicrobial activity against pathogens such as bacteria, viruses, fungi, protozoans, helminths, and Chlamydia (Birdsall and Kelly, 1997). Berberine has the ability to reduce intestinal secretion of water and electrolytes induced by cholera toxin, as well as inhibition of some *Vibrio cholerae* and *Escherichia coli* enterotoxins. This is why it is used in the treatment of bacterial diarrhoea (Sack and Froelich, 1982).

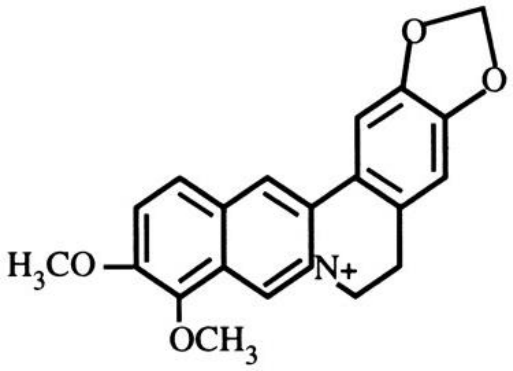
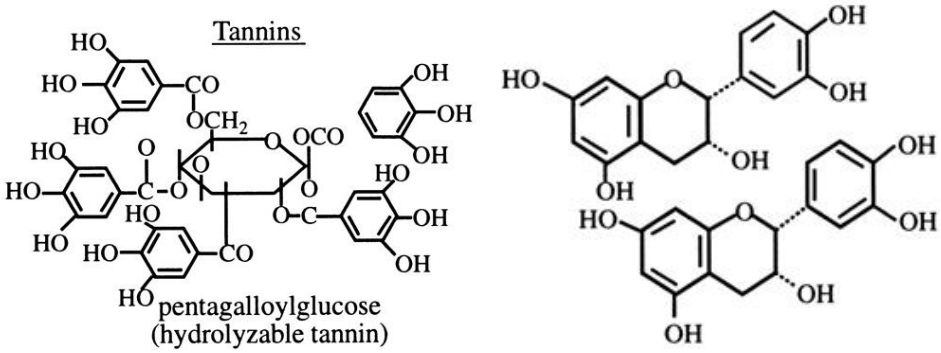


Figure 2.5: The structure of berberine a natural alkaloid (Cowan, 1999).

2.4.6. Tannins

Tannins are water soluble polyphenols commonly found in woody plants. They are divided into two groups; hydrolysable and non-hydrolysable (condensed) (Figure 2.6). The hydrolysable class include tannic acid while the non-hydrolysable include catechin (Akiyama *et al*., 2001). This group of compounds maybe formed either by condensations of flavan derivatives transported to woody plant tissues or by polymerisation of quinone units (Cowan, 1999). Many herbivores such as cattle, deer and apes do not eat plants with high tannins content because they cause a sharp sensation in the mouth of many mammalian herbivores due to their ability to bind to salivary proteins (Mazid *et al.,* 2011).

Tannins have the ability to inactivate microbial enzymes, adhesins and cell envelope transport proteins which could be their mode of antimicrobial action (Mokgoatsane, 2011). Chung *et al*. (1998) has reported on the bactericidal or bacteriostatic activity of tannins against *Staphylococcus aureus*. Medicinal plants containing tannins have been used for treatment of diarrhoea, as diuretics and anti-inflammatory remedy in India (Saxena *et al*., 2013). A review has been made on antimicrobial activities of tannins which listed 33 studies that documented inhibitory activities of tannins. These studies suggest that tannins are toxic to filamentous fungi, yeasts and bacteria (Scalbert, 1991).



(**a**) (**b**)

Figure 2.6: The structure of tannins (**a**) pentagalloylglucose (hydrolysable tannin) and (**b**) procyanidine B-2 (condensed tannin) (Cowan, 1999).

2.4.7. Saponins

Saponins are structurally diverse non-volatile surface active secondary metabolites widely distributed in higher plants. They consist of non-polar glycones coupled with one or more monosaccharide moieties (Figure 2.7) (Vincken *et al*., 2007). These compounds are divided into two groups based on their aglycone skeleton. The first group consists of the steroidal saponins, which are almost exclusively present in the monocotyledonous angiosperms. The second group consists of the triterpenoid saponins, which are most common in the dicotyledonous angiosperms (Bruneton, 1995; Sparg *et al*., 2004).

The pharmacological effects exerted by saponins include; expectorant, anti-inflammatory, vasoprotective, hypocholesterolemic, immunomodulatory, hypoglycaemic, molluscicidal, antimicrobial and anti-parasitic. (Sparg *et al*., 2004; Kräutler *et al*., 2008; Podolak *et al*., 2010). The antibacterial and antifungal activity of two acylated bisglycoside saponins, Acaciaside A and B, isolated from *Acacia auriculiformis* have been documented (Mandal *et al.,* 2005; Mokgoatsane, 2011).

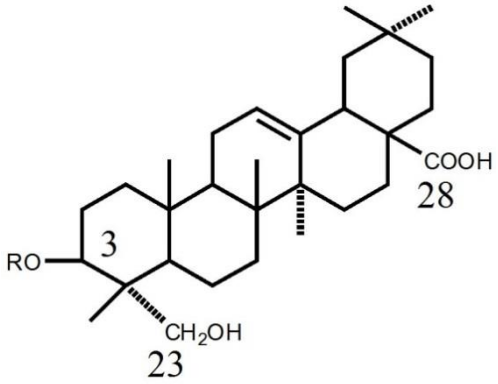


Figure 2.7: The structure of oleanane-type saponins analogues (Tamura *et al*., 2012).

2.5. Biological activities of medicinal plants

Medicinal plants used for treatment of illness by people are now receiving more attention in research because of their potential to yield benefiting health effects and to provide valuable therapeutic agents (Krishnaiah *et al*., 2009). This is due to the presence of bioactive compounds that produce definite physiological actions on the human body. Phytochemicals may possess one or more biological activities, with some acting in synergistic relationships in order to exhibit one biological activity, while others act antagonistically. These bioactive compounds have therapeutic potential and various biological activities; this is why most are characterised and separated so that each one can exhibit its own activity (Molan *et al*., 2012).

2.5.1. Antioxidant activity

Biochemical reactions are natural processes that take place in cells and organelles in our bodies in order to sustain life. However, these reactions contribute to formation of free radical molecules (Carocho and Ferreira, 2013). Free radicals are highly reactive and unstable molecules which may be of reactive oxygen species (ROS) and reactive nitrogen species (RNS) derivatives and non–derivatives. The highly reactive nature of free radicals leads to great damage of cells and other cell constituents including lipids, cell membrane, proteins and nucleic acids (DNA and RNA). The imbalance between the amount of oxidants and free radicals produced and their elimination by antioxidants lead to oxidative stress which cause diseases

such as; cancer, diabetes, cardiovascular diseases and inflammatory conditions (Lakshmi *et al*., 2014).

Antioxidants act by prolonging oxidation and reducing the amount of free radicals in the body, thus preventing cellular damage that results from oxidative stress (Mattson and Cheng, 2006). These antioxidants are classified into two groups based on solubility and line of defence. Hydrophilic antioxidants are soluble in water and they react with free radicals in the cytoplasm and blood plasma. Hydrophobic antioxidants are soluble in lipids and protect the cell membrane against lipid peroxidation. The defensive antioxidants include those that catalyses the reduction of oxidants, scavenge free radicals and repair damaged biomolecules (Panchawat *et al*., 2010).

Free radicals produced in the body are often removed by the body's naturally produced antioxidants such as glutathione or catalases (Sen, 1995). However, the amount produced by the human body alone is insufficient to prevent oxidative stress. Therefore, these have to be compensated with natural exogenous antioxidants such as vitamin C, vitamin E, flavones, and natural plant products (Madsen and Bertelsen, 1995; Rice–Evans *et al*., 1997; Diplock *et al*., 1998).

The currently available synthetic antioxidants include; butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), but these were proven to be toxic and carcinogenic to human beings (Lakshmi *et al*., 2014). As such, medicinal plants represent a potential source of new antioxidant drugs which could be used as exogenous antioxidants. Medicinal plants containing phenolic compounds such as phenols, tannins and flavonoids are rich sources of natural antioxidants which are non-toxic and easily accessible. Several studies have highlighted the strong antioxidant effects of flavonoids (Hollman, 2004).

There are a number of *in vitro* chemical-based assays that have been developed and are used to evaluate antioxidant activity in medicinal plants extract. These include the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and 2, 2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (ABTS) scavenging method for free radicals, ferric and cupric reducing capacity assays for metal ions reduction. The free radical scavenging assays are based on the rate of discoloration of colored radical compounds while the metal ions reduction assays are based on the reduction of metal complexes (López-Alarcón and Denicola, 2013; Marques *et al*., 2014).

2.5.2. Antimicrobial activity

Amongst other diseases, infectious diseases are a major cause of mortality worldwide and about 50 000 people are dying every day worldwide due to infectious diseases (Ashraf *et al*., 2015). Treatment of these diseases has become difficult due to the development of antibiotic resistance by the microbial strains and the extensive side effects brought by the current synthetic and microbial antibiotics. Most of *Escherichia coli* and *Staphylococcus aureus* isolates were reported to be resistant to ampicillin, amoxicillin, tetracycline, and trimethoprimsulfamethoxazole (Aibinu *et al*., 2004). This necessitates the need for discovery of antimicrobial compounds with different chemical structures and novel mechanism of action.

Medicinal plants represent a rich source of antimicrobial agents with better activity against multi-drug resistant bacteria and their antimicrobial potential had been accepted long before the mankind discovery of microorganisms (Anwar *et al*., 2009). Therefore, the screening of natural products for the discovery and development of novel antimicrobial agents is imperative (Yala *et al*., 2001; Edward-Jones, 2013; Aumeeruddy-Elalfi *et al*., 2016).

The bioactive compounds from plants may play a fundamental role in modern drug development of antimicrobials. Bioactive compounds such as phenolic compounds, alkaloids, flavones and tannins have shown antimicrobial potential. As such, plant-based drugs represent a promising lead in the development of drugs for treatment of infectious diseases (Ahmad and Beg, 2001; Ashraf *et al*., 2015). Plant extracts have exhibited strong inhibiting effects on a broad spectrum of microorganisms (Cowan, 1999; Nascimento *et al*., 2000). Plant-derived antimicrobials are based on structure, and their mechanism of action is related to disintegration of cytoplasmic membrane, destabilisation of the proton motive force, electron flow, active transport and coagulation of cell content (Silva and Fernandes, 2010).

More *et al*. (2008) reported on the antimicrobial activities of ethanolic extracts of different plant species of South Africa used locally in traditional medicine against *Actinomyces israelii, Actinobacillus actinomycetemcomitans*, *Candida albicans*, *Actinomyces naeslundii*, *Porphyromonus gingivalis*, *Streptococcus mutans, and Privotella intermedia.* Liu *et al*. (2008) reported on essential oil of *Ganoderma*

japonicum (Fr.) Lloyd. exerting antimicrobial activity against eighteen microorganisms and concluded that it is a natural antibiotic.

In vitro antibacterial assays can be classified into three groups namely, diffusion, dilution and bioautographic methods. For Minimum Inhibitory Concentration determination of active plant extracts, a very sensitive 96-well microtitre plate method can be used (Eloff, 1998b). Bioautography combines TLC with a bioassay *in situ* and allows the localisation of active constituents in a complex sample. A variety of Southern African plants used in traditional medicine for treatment of infectious diseases, most likely caused by bacteria were screened for antibacterial activity using the disc-diffusion bioassay (Taylor *et al*., 20001).

2.5.3. Anti-inflammatory activity

Inflammation can be defined as a biological defence and repair mechanism of the innate immune system to protect against harmful stimuli, such as pathogens, damaged cells and tissues, toxic chemicals and irritants. This is a physiological process called acute inflammation and it is responsible for restoring normal tissue function (Jungbauer and Medjakovic, 2012). This type of response consist of three main vascular effects which include; vasodilatation and increased vascular flow, increased vascular permeability and leucocytes migration to the injured tissues (Rosa *et al*., 1971; Ching *et al*., 2009).

Symptoms of inflammation include the reversible features such as; pain, redness, heat and swelling which are often followed by loss of function of involved organs (Barbosa-Filho *et al*., 2006). Prolonged inflammation has been linked with various diseases such as; rheumatoid arthritis, atherosclerosis, asthma, cancer, neurological disease, metabolic disorders and cardiovascular diseases (Mueller *et al*., 2010; Pan *et al*., 2010).

Non-steroidal anti-inflammatory drugs (NSAID) are currently used for treatment of inflammatory related diseases. However, the use of NSAID is limited due to the adverse effects such as gastric lesions, stomach pain, allergic reaction and nausea (Adedapo *et al*., 2008). As such, new anti-inflammatory and analgesic drugs without

these side effects are therefore being researched as alternatives (Maphosa *et al*., 2009).

Preparations of natural products with anti-inflammatory properties have long been used as a folk remedy for inflammatory related conditions such as fevers, pain, migraine and arthritis (Huang *et al*., 2006). Several plant extracts and isolated compounds have been reported to have anti-inflammatory properties. The anti-inflammatory and anti-atherosclerotic activity of turmeric (*Curcuma longa*) has been documented. This validates the use of this plant for treatment of rheumatic disorders in Indian traditional medicine, (Mueller *et al*., 2010).

2.6. Separation and purification of compounds

Isolation and separation of bioactive compounds from medicinal plants is highly necessary for further analysis, since pure compounds isolated from these plants represent a major source of standards for screening and drug analysis (Patra *et al*., 2012). Biological assays and chemical screening allow localisation and targeted isolation of useful types of bioactive compounds with potential activities (Patra *et al*., 2012). As such, bioassay-guided isolation is employed to connect the chemical profiles of extracts or fractions with their biological activity in *in vitro* bioassays. These bioassays are performed at micro-scale; thereby significantly reducing the time of discovery since only those fractions with biological activities in *in vitro* will be re-fractionated until a pure compound is obtained (Bucar *et al*., 2013).

Separation and purification of compounds from medicinal plant materials usually begins with their extraction. But since plant extracts usually occur as mixtures of various bioactive compounds with different polarities, their separation remains a big challenge (Sasidharan *et al.,* 2011). Extraction is often followed by separation and purification of compounds, which involves the use of column chromatography and preparative thin layer chromatography. The components from crude extracts are separated into fractions based on the polarities using one or two solvents. The compound of interest is subjected to a purification process in order to exclude other accompanying compounds. This often employs techniques such as thin layer chromatography, column chromatography, preparative thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) (Bucar *et al*., 2013).

The purity of separated compounds is usually confirmed in the final step using TLC plates (Sasidharan *et al.,* 2011). Furthermore, the pure compounds are analysed for structure elucidation and characterisation using nuclear magnetic resonance (NMR) and Mass spectrometry (MS) (Patra *et al*., 2012).

2.7. Structure elucidation of compounds

Medicinal plants possess a mixture of compounds with complicated structures and potent biological activities (Matsumori *et al*., 1999; Harvey, 2007). The end point of isolation of compounds from medicinal plants is the structural elucidation of the identified compounds (Sarker *et al*., 2006). The advances in modern technology allow successful structural elucidation of isolated compounds from natural products by spectroscopic techniques such as NMR and Mass spectrometry (Matsumori *et al*., 1999; Harvey, 2007).

NMR spectroscopy is a useful technique in medicinal chemistry which allows complete analysis and interpretation of the structure of organic compounds. While mass spectrometry is a sensitive method for molecular analysis of compounds based on production of ions which are separated based on mass to charge ratio (Boloko, 2007). Isolation and separation of extracts from medicinal plants is a challenging and time consuming process, however, the combination of chromatographic separation techniques such as High Performance Liquid Chromatography (HPLC), hyphenated high performance liquid chromatography (LC)-NMR and LC-NMR-MS with NMR and MS allows separation and structural elucidation of unknown compounds and mixtures in less time (Levsen *et al*., 2003; Boloko, 2007).

2.8. Bacteria of clinical significance

There are a number of problematic drug-resistant pathogens that are encountered. These include the Gram-positive bacteria methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Streptococcus pneumoniae* and vancomycin- resistant *Enterococcus* spp. Meanwhile the Gram-negative bacteria include; multidrug-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* (Lister *et al*., 2009). For the purpose of this study only

Staphylococcus aureus, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* were selected and will be discussed below.

2.8.1. *Escherichia coli*

Escherichia coli is a facultative anaerobic, Gram-negative bacterium, which is a member of the Enterobacteriaceae family. This bacterium part of the gut normal flora and normally colonises the intestine of human and many animals (Pang *et al*., 2013). Most *E*. *coli* strains are harmless and they produce vitamin K2 to keep the human intestinal tract healthy and prevent the establishment of pathogenic bacteria. Nevertheless, some are virulent and can cause urinary tract infections and gastroenteritis (Sharma *et al*., 1992; Pang *et al*., 2013). The non-pathogenic ATCC 25922 strain of *E*. *coli* is Biosafety level 1 certified, as such, it is useful for various laboratory experiments (Lobry *et al*., 1992; Pang *et al*., 2013).

2.8.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunist aerobic Gram-negative bacterial pathogen which is found in many diverse environments (Lister *et al*., 2009). This pathogen is known to colonise immunocompromised individuals causing life threatening community and hospital-acquired infections. Community acquired infections include; otitis externa, skin and soft tissue infections in diabetes mellitus patients. While the hospital acquired infections include; pneumonias, urinary tract infections, blood stream and surgical site infections on burn wounds (Driscoll *et al*., 2007). *P. aeruginosa* ATCC 27853 is commonly used in Biotechnology and Microbiology laboratories for testing of the antibacterial activity of newly discovered drugs.

2.8.3. *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive streptococcus, which naturally colonise in the gastrointestinal tract of mammalians. It can also be found in soil, sewage, water and food frequently through faecal contamination. *E. faecalis* is an opportunistic pathogen which is a major cause of diseases such as urinary tract infections,

bacteremia, and infective endocarditis in humans (Rishi *et al*., 2009; Barge *et al*., 2013). Treatment of these infections is, however, often limited by antibiotic resistance of these isolates to conventional antibiotics. Furthermore, the acquired resistant genes confer resistance to antibiotics such as; chloramphenicol, clindamycin, erythromycin, tetracycline, high-level aminoglycosides, beta-lactamase and vancomycin (Murray, 1990; McBride, 2007).

2.8.4. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive facultative bacterium. This bacterium is known to be a common human and animal pathogen, which causes a wide range of clinical infections. The infections caused by this pathogen include; bacteremia, infective endocarditis, pneumonia, skin, endovascular and soft tissue infections (Tong *et al*., 2015; Giersing *et al*., 2016). People from all age groups and backgrounds are affected by *S*. *aureus* infections, but children, the elderly and immunocompromised individuals are the most susceptible (Giersing *et al*., 2016). There are strains that were reported to be resistant to antibiotics such as methicillin; these are called Methicillin-resistant *Staphylococcus aureus*. These strains are increasingly becoming prevalent among nosocomial and community acquired infections (Archer, 1998; François *et al*., 2010; Mokgoatsane, 2011; Giersing *et al*., 2016).

2.9. Plants selected in this study

2.9.1. *Commelina africana*

Commelina africana (Figure 2.8) is a perennial spreading herb, with hard and woody rootstock, small canary yellow flowers and flat or folded variously hairy leaves. This plant is widely distributed in forests, savannah, and grassland in Africa, Madagascar, and Arabian Peninsula. It spreads and flourishes rapidly in sandy soil in rocky areas during rain seasons and it is very common in Southern Africa.

The traditional uses of this plant include; using a decoction of the roots for treatment of venereal diseases and as a medicine for women suffering unduly during the

menstrual period by the Ndebele people. The ashes are applied to cuts for sterility and its infusion is drunk for the same purpose by the Sotho tribe (Leistner, 2000).

Figure 2.8: *Commelina africana* plant with flowers (adapted from http://www.plantzafrica.com/plantcd/commelafric.htm)

2.9.2. *Dombeya rotundifolia*

Dombeya rotundifolia (Figure 2.9) is a tree with rough, round, green leaves covered with star-like hairs, dark brown cocky bark and flurry white flowers, commonly known as wild pear. It grows in all soil types, allowing it to be planted in a wide range of garden conditions and in a diverse array of climatic zones. *D. rotundifolia* is distributed and mostly found in Mpumalanga, Kwazulu-Natal, Limpopo, Gauteng and North-West provinces of South Africa and northwards of Ethiopian woodland, wooded grassland and rocky mountain slopes.

Medicinal uses include making tea to relieve the discomfort of internal ulcers, haemorrhoids, or nausea in pregnancy, or to bring on or delay the onset of labour. The flowers are used to make a love portion. The hard bark is useful in making strong rope fibre and the woods are used to make fence posts, as it is termite resistant (Aubrey, 2001).

Figure 2.9: Leaves of *Dombeya rotundifolia.* (Adapted from http://www.africamuseum.be/collections)

2.9.3. *Elephantorrhiza elephantina*

Elephantorrhiza elephantina (Figure 2.10) is a low shrub from the Fabaceae family with unbranded and unarmed aerial stems, thickened rhizome, dark reddish brown bark, clustered raceme flowers that are golden yellow to pale yellowish white and dull green leaves which produces at ground level commonly known as elephant root. It often occurs in hot, dry areas with open shrub and grassland and widely distributed and found in Southern Africa, Namibia, Botswana, Zimbabwe, Mozambique and the Limpopo, Gauteng, North-West, Eastern Cape, Mpumalanga, Northern Cape, and Free State provinces of South Africa.

The traditional uses include; administration of roots infusion for dysentery and diarrhoea by the Xhosa and Zulu tribe (Hutchings *et al*., 1996) and these are also used for treatment of various diseases in cows including pneumonia, coughing and tick-borne diseases (Maphosa *et al*., 2009). It is also used externally for treatment of haemorrhoids and curing of skin diseases and acne by exposing the face to the vapour of the warm infusion.

Figure 2.10: *Elephantorrhiza elephantina* plant with flowers and fruits (Adapted from plantZAfrica.com)

2.9.4. *Schkuhria pinnata*

Schkuhria pinnata (Asteraceae), (Figure 2.11) is a herbaceous plant, usually 60 cm high commonly known as Dwarf Marigold. It has divided leaves and branched flower heads, with yellow disc and ray florets. It is native to South America and usually occurs in gardens and along roadsides, but it is widespread and distributed as a weed in South Africa (Deutschländer *et al*., 2009).

The traditional uses include; brewing the whole plant into infusion or decoction use for treatment of various disorders linked with infectious microorganisms including dermatitis, eczema and acne (McGaw and Eloff, 2008). It has been used for treatment of eye infections, pneumonia, and diarrhoea in cows and also retained placenta and wounds in livestock in South Africa (Mupfure *et al*., 2014). Several countries have been using it as a remedy for malaria and the ethanolic extract of the whole *S. pinnata* plant was reported to have antimalarial effects in animals (Muñoz *et al*., 2000).

Figure 2.11: *Schkuhria pinnata* plant (Adapted from http://www.sib.gov.ar/ficha/PLANTAE)

2.10. Aim and Objectives

2.10.1. Aim

To isolate compounds with antibacterial and antioxidant activity, from selected medicinal plant species and evaluate their cytotoxicity.

2.10.2. Objectives

The objectives of this study are to:

i. Perform phytochemical analysis and antibacterial assays with the test organisms on different plant extracts in order to determine the bioactive compounds against pathogens.

ii. Determine the chemical profile of the extracts using column chromatography.

iii. Perform bioassays to determine the antioxidant potential, anti-inflammatory activity and cytotoxicity of the plant extracts against mammalian non-cancerous cells.

iv. Perform synergistic/antagonistic study of the selected extracts or isolated and identified compound(s) from two plant species with significant antibacterial and antioxidant activities.

v. Determine the chemical structure of the identified compounds using NMR and Mass spectroscopy.

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Chapter 3: Extraction and preliminary phytochemical analyses

3.1. Introduction

Phytochemicals, also known as secondary metabolites are chemicals derived from plants. These form basic sources for the establishment of many pharmaceuticals and play a significant role in the identification and development of crude drugs. Phytochemical screening is a convenient and inexpensive procedure that gives an idea of the various types of phytochemicals present in crude extracts (Sasidharan *et al*., 2011). This is important in identifying new sources of therapeutically and industrially important compounds like tannins, alkaloids, phenolics, flavonoids, saponins, steroids, terpenoids etc. (Akindele and Adeyemi, 2007; Tadesse *et al*., 2012). This assay is also an important tool in the analysis of bioactive compounds (Sasidharan *et al*., 2011). It is therefore, necessary to separate the constituents from the plant material by extraction, because extraction is the crucial first step in the analysis of medicinal plants, which is necessary to extract the desired chemicals for further separation and characterisation (Sasidharan *et al*., 2011).

The different extraction procedures employed in the extraction of bioactive compounds from plants include; plant tissue homogenisation, serial exhaustive extraction, soxhlet (hot continuous extraction) extraction, maceration, infusion, distillation, decoction, and sonication (ultrasound extraction) (Tiwari *et al*., 2011). Different solvents are available for extraction of bioactive compounds from medicinal plants. However, the following should be considered when selecting a solvent for extraction; the intended use of the extract, the specific bioactive compounds being targeted, the toxicity and the influence on the bioassay (Sasidharan *et al*., 2011; Pandey and Tripathi, 2014).

Thin Layer Chromatography is a simple and inexpensive procedure which allows separation and identification of compounds in a mixture. For the detection of the compounds, different phytochemical screening reagents are sprayed on the TLC plates following separation. These reagents react with the phytochemicals and cause colour changes according to the phytochemicals present in plant extracts. For the same purpose, the plates are visualised under the Ultraviolet (UV) light at certain characteristic wavelengths (Sasidharan *et al*., 2011; Patil and Khan, 2016).

Several standard tests are performed to check for the presence of different active constituents in crude extracts. These include; the Wagner's and Hager's test for alkaloids, Borntrager's test for anthraquinones, Keller killiani test for cardiac glycosides, lead acetate and sodium hydroxide test for flavonoids, Fehling's test for reducing sugars, Ferric chloride test for tannins, Frothing and Haemolysis test for saponins and the Salkowski's test for steroids (Patil and Khan, 2016). The aim of this chapter was to determine the phytochemical constituents, TLC phytochemical fingerprint and quantification of phytoconstituents of the selected plants.

3.2. Methods and Materials

3.2.1. Plant collection

The selected plants which were: *Commelina africana* L. var. africana (UNIN 12295), *Dombeya rotundifolia* (Hochst.) Planch. var. *rotundifolia* (UNIN 12296), *Elephantorrhiza elephantina* (Burch.) Skeels (UNIN 12297) and *Schkuhria pinnata* (Lam.) Kuntze ex Thell (UNIN 12298) were collected in February 2016 at the University of Limpopo, in the Limpopo Province, South Africa. Plant collection was based on ethnopharmacological information provided by traditional healers in the Limpopo Province. Voucher specimen and tree labels were used to verify the identity of the plants. These were deposited at Larry Leach Herbarium (UNIN) for confirmation. The leaves of *Commelina africana, Dombeya rotundifolia,* and *Elephantorrhiza elephantina* were separated from the twigs, while only the roots of *Schkuhria pinnata* were removed. The plant materials were air-dried at ambient temperature. The dried plant materials were ground to fine powder using an electric grinder (Sundy hammer crusher SDHC150) and stored in the dark in airtight containers.

3.2.2. Extraction procedure

3.2.2.1. Preliminary extraction procedure

One gram each of the ground plant materials from the selected plants was extracted with 10 mL of n-hexane, dichloromethane, acetone, and methanol in different 50 mL

polyester centrifuge tubes. The tubes were shaken for 10 minutes in a series 25 shaking incubator (New Brunswick Scientific Co., Inc) at 200 rpm. The extracts were then filtered into pre-weighed labelled vials. The solvents were evaporated under a stream of cold air at room temperature, the mass obtained was determined and the extracts were reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.2.2. Serial exhaustive extraction

The leaves of *D. rotundifolia* were exhaustively extracted serially from 5 g of plant material with 50 mL of n-hexane. The bottles were vigorously shaken for an hour using a series 25 shaker incubator at 200 rpm and the supernatant was filtered into a pre-weighed bottle. The same process was repeated three times to exhaustively extract the compounds and the supernatants were combined. The same plant residue was extracted with dichloromethane, acetone and methanol. The solvents were evaporated under a stream of cold air at room temperature and the quantity of the plant material extracted was determined. The extracts were reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.2.3. Extraction enrichment procedures

The enrichment procedure employed two methods, the first pathway included defatting the finely ground plant material of the selected plants with n-hexane and subsequently extracting the pre-treated plant material with acetone and ethanol. The second pathway involved using different percentages (20%, 40%, 60% and 80%) of acetone and ethanol in water for extraction. The tubes were vigorously shaken for an hour using a series 25 shaker incubator at 200 rpm and the supernatant was filtered into a pre-weighed bottle. The solvents were evaporated under a stream of cold air at room temperature and the quantity of the plant material extracted was determined. The extracts were reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.2.3.1. n-Hexane wash

One gram of the ground *D. rotundifolia* leaves was extracted with 10 mL of n-hexane. The plant residues were dried between subsequent extractions, and later extracted with acetone and ethanol.

3.2.2.3.2. Acetone and ethanol in water mixtures

One gram of the *D. rotundifolia* leaves was extracted with 10 mL of each of the following solvents: acetone, ethanol, water, and 20%, 40%, 60% and 80% acetone and ethanol in water. The solvents were evaporated under a stream of cold air at room temperature and the quantity of the plant material extracted was determined. The extracts were reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.2.4. Optimal extraction method

This method involved three pre-treatment methods before subsequent extraction of the plant material with acetone and ethanol. One gram of *D. rotundifolia* leaves was extracted with 10 mL of each of the solvents as outlined in Table 3.1 below.

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

Pre-treatment	Extraction
n-Hexane “wash”	Acetone and ethanol
20% acetone and ethanol in water “wash”	80% acetone and ethanol in water
n-Hexane “wash” followed by 20% acetone and 20% ethanol in water “wash”	Acetone and ethanol

3.2.2.5. Preliminary serial exhaustive extraction

3.2.2.5.1. Series 1, 2 and 3

Ten gram of the *D. rotundifolia* leaves was serially extracted with 100 mL of solvents with varying polarities from non-polar to polar: (i) Series 1: n-hexane, dichloromethane, ethyl acetate, acetone and methanol. (ii) Series 2: n-hexane, ethyl acetate, acetone and methanol. (iii) Series 3: n-hexane, Acetone and methanol. The solvents were evaporated under a stream of cold air at room temperature and the quantity of the plant material extracted was determined. The extracts were reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.3. Preliminary screening for phytochemical compounds

The extracted chemical compounds (10 mg/mL) were analysed by separation with TLC using aluminium-backed TLC plates (Merck, silica gel 60 F_{254}) according to the method developed by Kotze and Eloff (2002). Ten microliters of the extracts was loaded on the TLC plates and developed in three mobile phases of different polarities i.e. benzene/ethanol/ammonium hydroxide (BEA) (non-polar/basic) (18:2:0.2), chloroform/ethyl acetate/formic acid (CEF) (intermediate polarity/acidic) (10:8:2) and ethyl acetate/methanol/water (EMW) (polar/neutral) (40:5.4:5). The separated compounds were examined under ultraviolet light (254 and 365 nm) for fluorescing and quenching compounds. To detect the separated compounds the chromatograms were sprayed with vanillin-sulphuric acid reagent [0.1 g of vanillin (Sigma®): 28 mL methanol: 1 mL concentrated sulphuric acid] and heated at 110 °C for 1-2 minutes for optimal colour development.

3.2.4. Phytochemical constituents screening

3.2.4.1. Terpenoids

The ethanol extracts of the selected plants were tested for terpenoids by weighing 0.5 g of the extract and dissolve into 2 mL of chloroform. Thereafter, 3 mL of concentrated sulphuric acid was carefully added to the mixture to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids (Borokini and Omotayo, 2012).

3.2.4.2. Flavonoids

The presence of flavonoids was tested on the aqueous extracts of the selected plants by adding 5 mL of diluted ammonia solution to a portion of the aqueous filtrate of plant extracts, followed by addition of 1 mL of concentrated sulphuric acid. A yellow colouration that disappears on standing indicates the presence of flavonoids (Borokini and Omotayo, 2012).

3.2.4.3. Cardiac glycosides

The Keller- Killiani test was employed to test for cardiac glycosides by weighing 0.5 g of plant extracts of the selected plants; the extracts were then diluted to 5 mL with water. The mixture of 2 mL of glacial acetic acid containing one drop of 0.1% ferric chloride solution was added to diluted plant extracts. This mixture was underplayed with 1 mL of concentrated sulphuric acid. A brown ring at the interface indicates the presence of a deoxysugar characteristic of cardenolides (Borokini and Omotayo, 2012).

3.2.4.4. Phlabotannins

The presence of phlabotannins was tested by weighing 0.2 g of powdered sample of the selected plants into 10 mL of distilled water and filtered. The filtrate was boiled with 2% hydrochloric acid solution. Sample was observed for the formation of red colour of precipitate to draw inference (Borokini and Omotayo, 2012).

3.2.4.5. Saponins

The persistent frothing test was used to test for saponins by weighing 1 g of the powdered plant material of the selected plants; this was mixed with 30 mL of tap water. The mixture was vigorously shaken and heated at 100 °C. The sample was observed for formation of persistent froth to draw inference (Odebiyi and Sofowora, 1977).

3.2.4.6. Tannins

The presence of tannins was tested by boiling 0.5 g of powdered plant material of the selected plants in 5 mL of distilled water in a test tube, then cooled and filtered. A few or three drops of 0.1% ferric chloride was added to 1 mL of the solution in a test tube and observed for brownish green or a blue-black colouration (Trease and Evans, 1989).

3.2.4.7. Alkaloids

Drangendoff's reagent was used to test for alkaloids by weighing 0.2 g of ground powdered plant material of the selected plants and extract with 95% ethanol using soxhlet extractor. The extracting solvent was evaporated to dryness using a vacuum evaporator at 45 °C. The plant residues were dissolved in 5 mL of 1% hydrochloric acid and 5 drops of drangendoff"s reagent was added. Reddish-brown colour change was observed to draw an inference (Harborne, 1973).

3.2.4.8. Steroids

Steroids were tested by adding 2 mL of acetic anhydride to 0.5 g of the selected plant extract, followed by addition of 2 mL of sulphuric acid to the mixture. Blue or green colour change was observed to draw inference (Borokini and Omotayo, 2012).

3.2.5. Total phenolic content determination

The concentration of phenolic content in 70% aqueous acetone extracts of the selected plants was determined using the spectrophotometric method described by Singleton e*t al*., 1999 with minor modifications. The determination of the total phenol content employed the Folin-Ciocalteau method, where 0.1 mL of extract and 0.9 mL of distilled water were mixed in a test tube. To this mixture 0.1 mL of Folin-Ciocalteau phenol reagent was added and the mixture shaken well. One millilitre of 7 % Sodium carbonate (Na_2CO_3) solution was added to the mixture after 5 minutes. The volume was made up to 2.5 mL with distilled water. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared as described above. The mixtures were incubated for 90 minutes at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV)/visible spectrophotometer. Total phenol content was expressed as mg of GAE/g of extract (Tambe and Bhambar, 2014).

3.2.6. Total tannin content determination

The tannin content was determined using Folin - Ciocalteau method. About 0.1 mL of the 70% aqueous acetone extracts of the selected plants was added to a 10 mL

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

3.2.7. Total flavonoid content determination

Total flavonoid content was determined by the aluminium chloride colorimetric assay. One millilitre of 70% aqueous acetone extracts of the selected plants was mixed with 4 mL of distilled water in a 10 mL volumetric flask. To the flask, 0.30 mL of 5 % sodium nitrite was added. About 0.3 mL of 10 % aluminium chloride was added to the mixture after 5 minutes, this was mixed. After 5 minutes, 2 mL of 1 M Sodium hydroxide was added and this was made up to 10 mL with distilled water. A set of reference standard solutions of quercetin (0.03125, 0.0625, 0.125, 0.25, 0.5 mg/mL) were prepared in the same manner as described above. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract (Tambe and Bhambar, 2014).

3.3. Results

3.3.1. Preliminary extraction procedure

The whole shrub (excluding the roots) of *S. pinnata* and the leaves of *E. elephantina, C. africana*, and *D. rotundifolia* were extracted using n-hexane, Dichloromethane, acetone and methanol. Figure 3.1 represents the quantity in milligrams of the plant material extracted from 1 g of the ground leaf powder using different solvents. The best extractant was methanol (178 mg) from *E. elephantina* and the least was n-hexane (4 mg) from *C. africana*. Overall, most of the plant material was extracted from *E. elephantina* followed by *S. pinnata* while the least was extracted from *C. africana*.

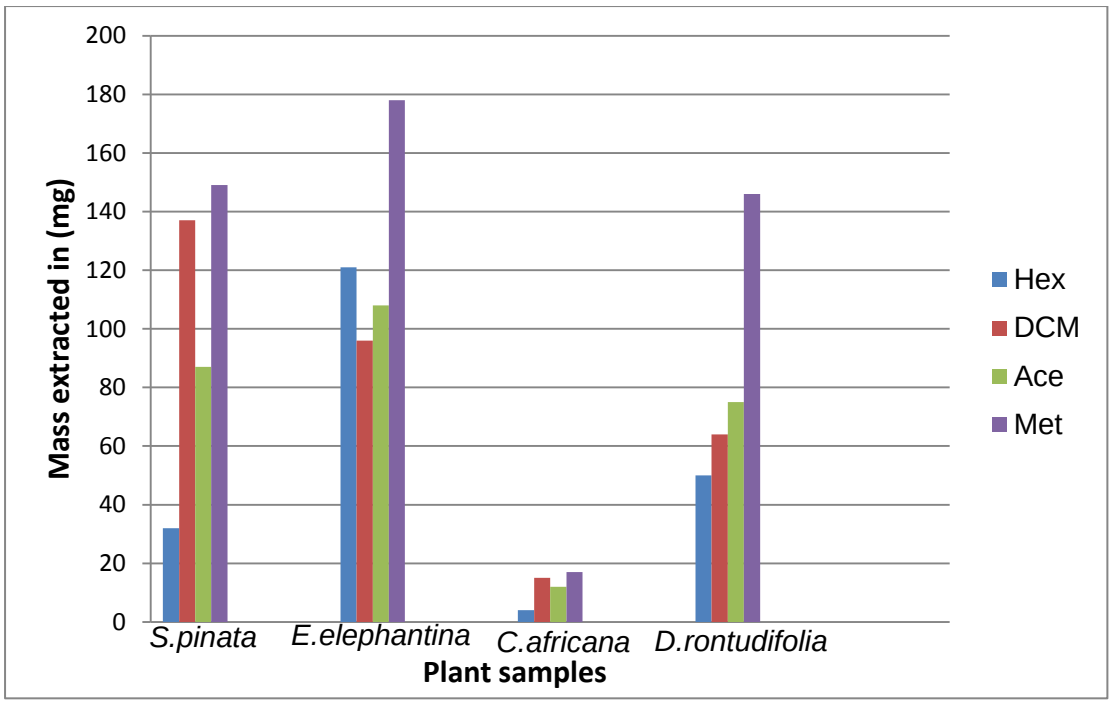

Figure 3.1: The mass extracted from 1 g of dried plants using 10 mL of different solvents i.e. n-hexane (**Hex**), Dichloromethane (**DCM**), Acetone (**Ace**) and methanol (**Met**).

3.3.2. Phytochemical analysis for preliminary extracts

Thin layer chromatography was employed for analysis of phytochemicals contained in the different extracts of the selected plants. Vanillin-sulphuric acid was used as a spraying reagent for visualisation of the different separated compounds. More bands were resolved in BEA than CEF in all the plants. CEF only resolved bands from *S. pinnata* and *D. rotundifolia*. More fluorescent compounds (circled with a pencil) were observed on chromatograms developed in BEA than in CEF (Figure 3.2).

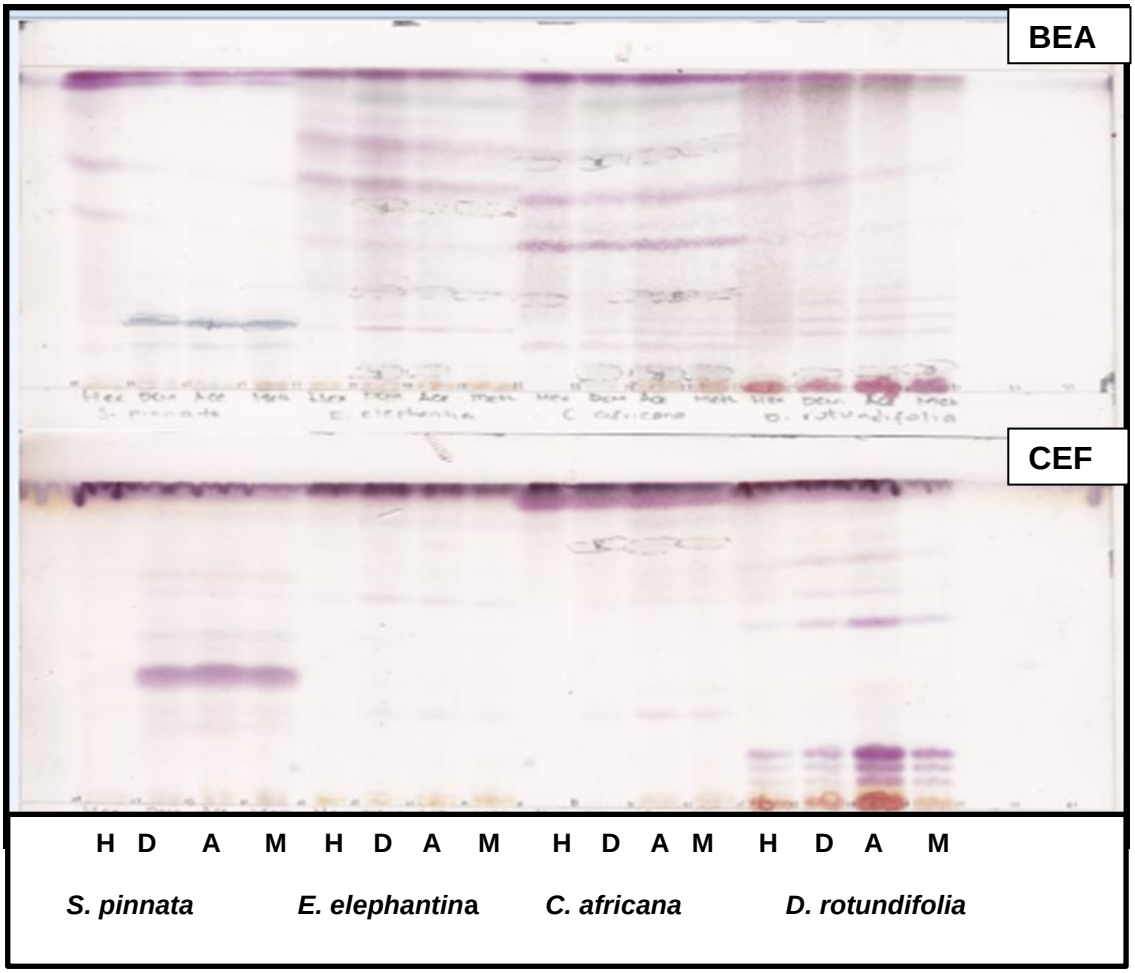

Figure 3.2: The chromatograms obtained after running the TLC aluminium-backed plates loaded with the plant extracts (*S. pinnata*, *E. elephantina*, *C. africana* and *D. rontudifolia*) in BEA and CEF mobile phases and sprayed with vanillin-sulphuric acid. The visualised compounds extracted with n-hexane (**H**), dichloromethane (**D**), acetone (**A**) and methanol (**M**).

3.3.3. Serial exhaustive extraction

In serial extraction procedure, methanol was the best extractant with the highest quantity of plant material extracted (886 mg) and the least was hexane with 183 mg (Figure 3.3). More polar compounds were extracted with a polar solvent (methanol).

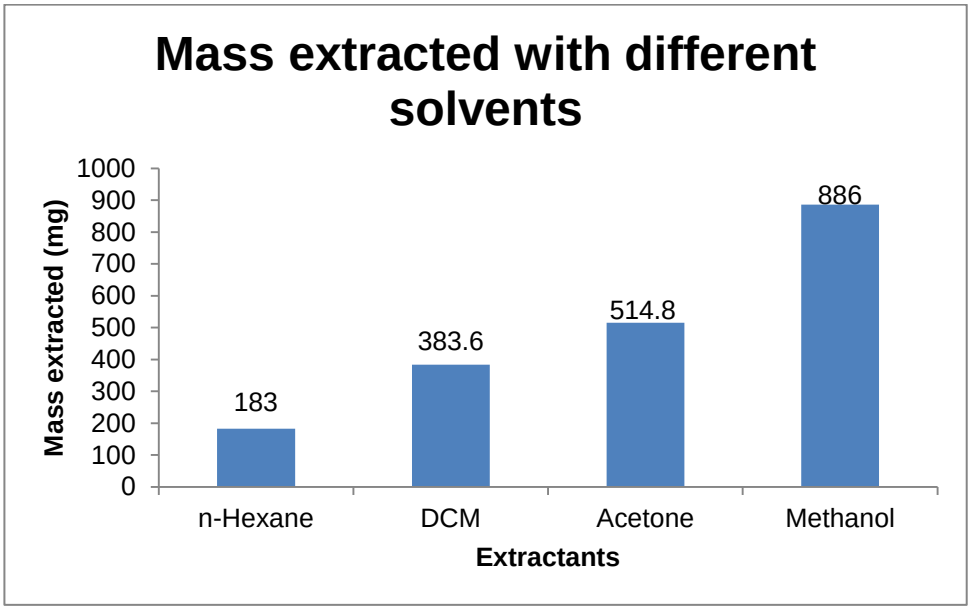

Figure 3.3: Mass exhaustively extracted serially from 5 g of the *D. rotundifolia* plant material with 50 mL of solvents of varying polarity (non-polar to polar).

3.3.4. Phytochemical analysis for serial exhaustive extracts

TLC analysis of phytochemicals exhaustively extracted serially with solvents of varying polarity from non-polar to polar. These were loaded on TLC plates and developed in different systems then sprayed with vanillin-sulphuric acid reagent. The different phytochemicals present are represented by the different colours observed (Figure 3.4). More bands were visible on the plates separated in EMW than the other separating systems.

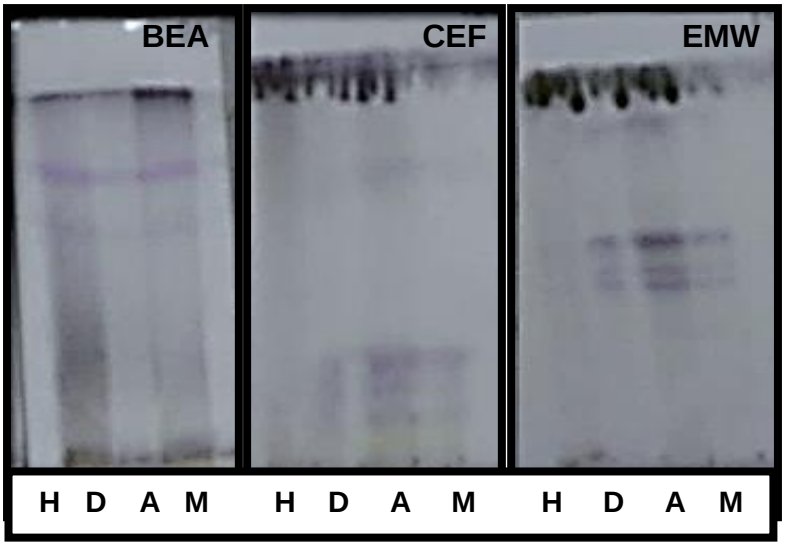

Figure 3.4: Chromatograms developed in BEA, CEF and EMW and sprayed with vanillin-sulphuric acid reagent to detect the phytochemicals present. The plant material was extracted with n-hexane (**H**), dichloromethane (**D**), acetone (**A**) and methanol (**M**).

3.3.5. Extraction enrichment procedures

3.3.5.1. n-Hexane wash

In n-hexane wash, the best extractant was ethanol (25.1 mg), followed by n-hexane for ethanol (20.5) and the least wash n-hexane for acetone (13.4) (Figure 3.5).

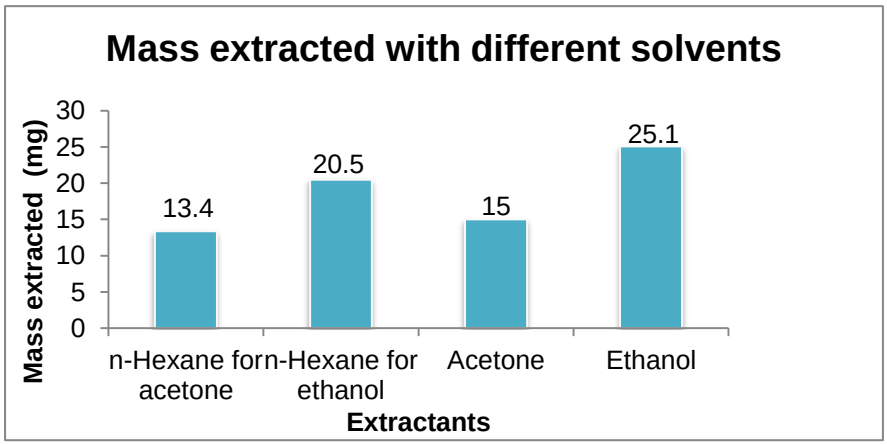

Figure 3.5: Mass extracted from 1 g of *D. rotundifolia* plant material with ethanol and acetone after washing with n-hexane

3.3.5.2. Phytochemical analysis for n-hexane wash

TLC was employed for separation of the plant extracts in three solvent systems, and the chromatograms sprayed with vanillin-sulphuric acid reagent are presented below (Figure 3.6). Different colours observed represent different phytochemicals. More bands were visible on the plates separated in CEF than BEA and EMW.

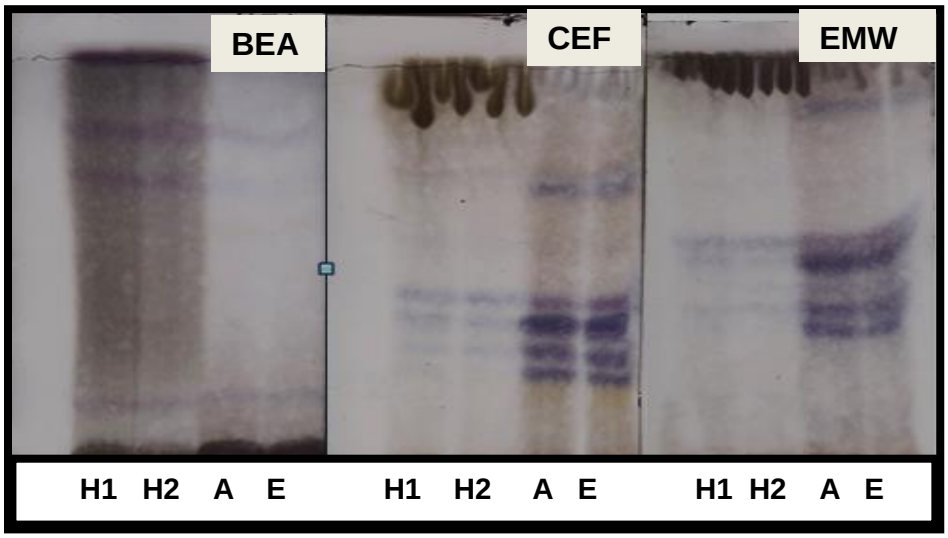

Figure 3.6: Chromatograms developed in BEA, CEF and EMW, then sprayed with vanillin-sulphuric acid reagent. Extracted with n-Hexane for acetone (**H1**)**,** n-hexane for ethanol (**H2**), acetone (**A**) and ethanol (**E**).

3.3.5.3. Acetone and ethanol in water mixtures

Acetone, ethanol, water, and different percentages of acetone and ethanol in water were employed for extraction of different phyto-constituents from *D. rotundifolia* leaves. The highest mass was extracted with 20% acetone (155.5 mg), followed by water (148.1 mg) while the lowest was extracted with ethanol (23.7 mg) (Figure 3.7).

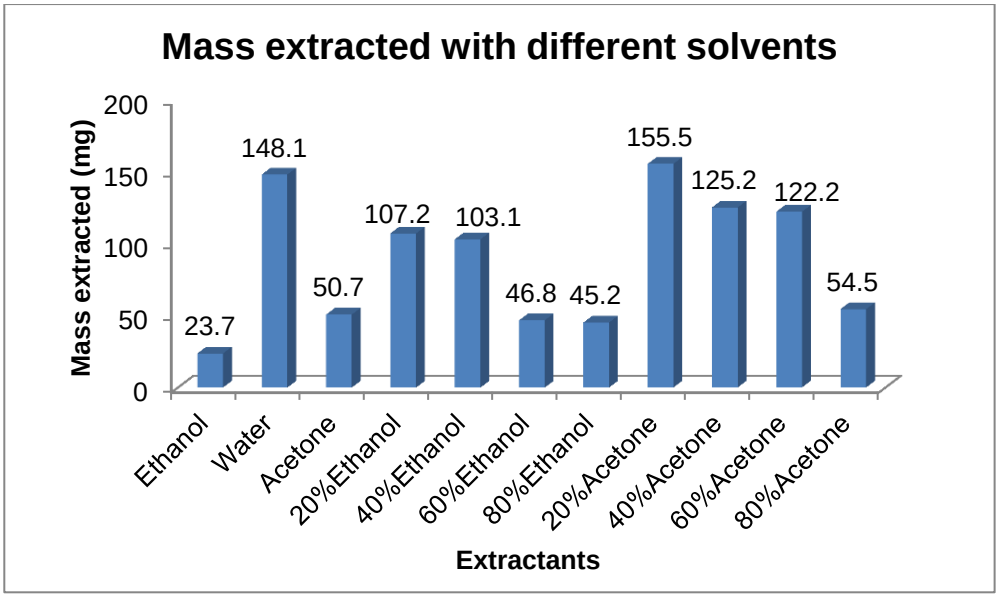

Figure 3.7: Mass extracted from 1 g of *Dombeya rotundifolia* plant material with acetone and ethanol.

3.3.5.4. Phytochemical analysis for acetone and ethanol in water extracts

Thin layer chromatography was used for analysis of the crude extracts. The chromatogram below (Figure 3.8) shows different phytochemicals which are represented by the different colours observed on TLC plates after spraying with vanillin-sulphuric acid reagent. Bands were only visible in extracts without water content and these were better resolved in CEF and EMW than BEA.

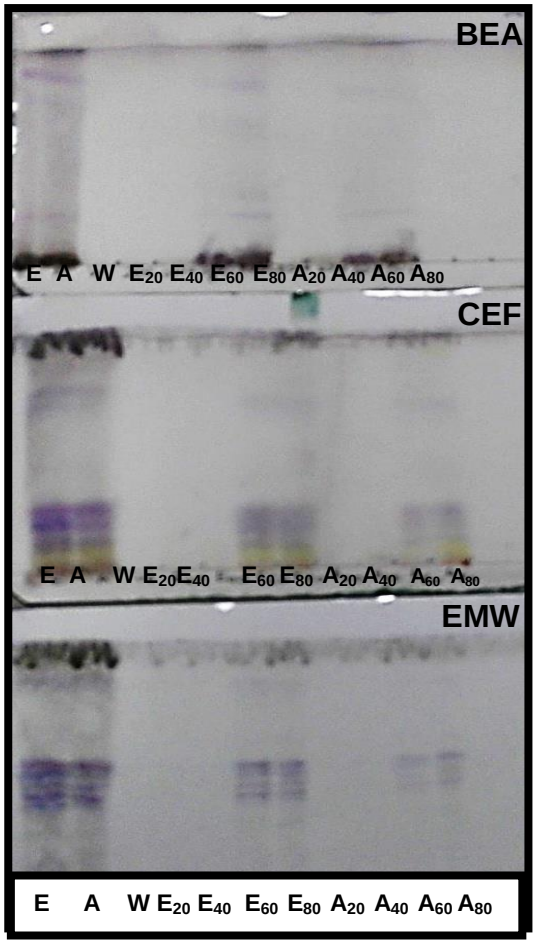

Figure 3.8: Thin layer chromatography fingerprint of *D. rotundifolia* leaf extracts developed in BEA, CEF, and EMW and sprayed with vanillin-sulphuric acid reagent. For compounds extracted with ethanol (**E**), acetone (**A**), water (**W)**, 20% ethanol ($\mathbf{E_{20}}$), 40% ethanol ($\mathbf{E_{40}}$), 60% ethanol ($\mathbf{E_{60}}$), 80% ethanol ($\mathbf{E_{80}}$), 20% acetone ($\mathbf{A_{20}}$), 40% acetone ($\mathbf{E_{40}}$), 60% acetone ($\mathbf{A_{60}}$) and 80% acetone ($\mathbf{A_{80}}$).

3.3.6. Optimal extraction method

Figure 3.9 represents different quantities of extracts obtained from optimal extraction method. Optimal extraction method consists of two steps; pre-treatment of the plant material with solvents of varying polarities followed by subsequent extraction of the pre-treated materials. In pre-treatment, the highest yield was with 20% ethanol (190.2 mg) followed by 20% acetone (125.6 mg) and the least was with n-hexane for 20% ethanol. In extraction, the highest mass was obtained with the plant material that was pre-treated with hexane and subsequently extracted with 20% acetone (96.6 mg), followed by the one pre-treated with 20% acetone and extracted with 80%

acetone (86.8 mg) and the least was obtained by the plant material pre-treated with n-hexane and subsequently extracted with 20% ethanol (25.3 mg).

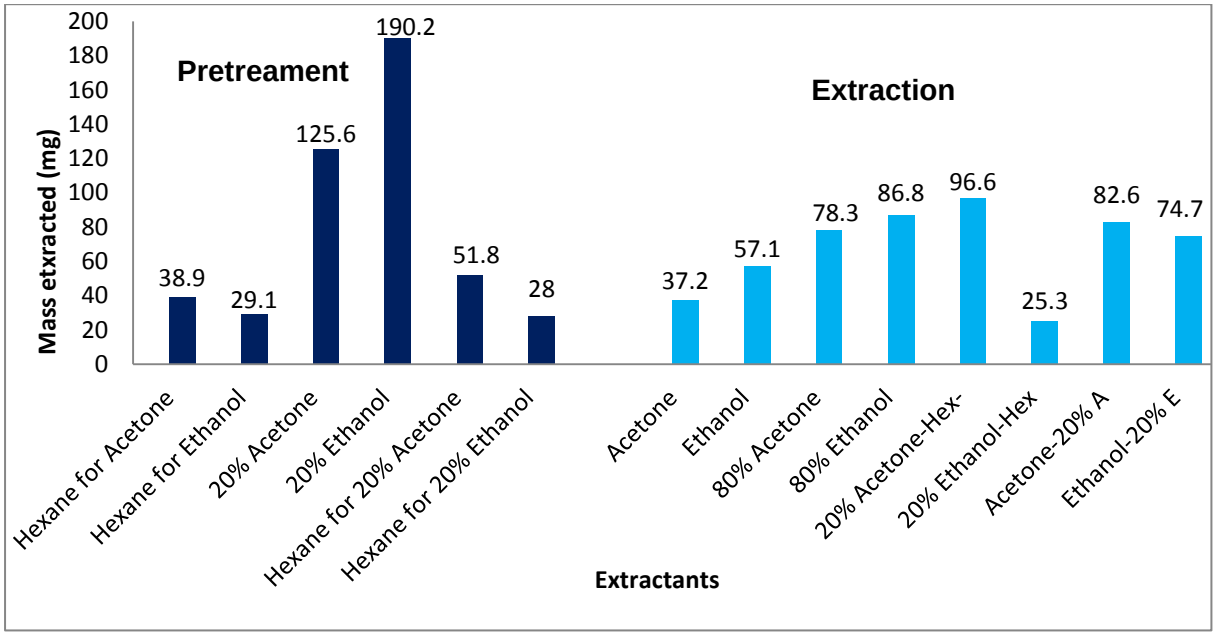

Figure 3.9: Mass optimally extracted from 1 g of *D. rotundifolia* plant material with acetone and ethanol after washing with n-hexane and acetone and ethanol in water.

3.**3.7. Phytochemical analysis for optimal extraction extracts**

TLC was used to analyse the phytochemicals of the pre-treated and subsequently extracted plant material. The plates were sprayed with vanillin-sulphuric acid reagent and the different colours observed represent different phytochemicals (Figure 3.10). The n-hexane extracts only had bands in BEA while the acetone and ethanol extracts had visible bands in plates separated in CEF and EMW.

Figure 3.10: Chromatograms developed in BEA, CEF and EMW, then sprayed with vanillin-sulphuric acid reagent. n-Hexane wash for acetone (**H_1**), n-hexane wash for ethanol (**H_2**), 20% acetone wash (**A_{20}**), 20% ethanol wash (**E_{20}**), n-hexane wash for 20% ethanol (**H_3**), n-hexane wash for 20% acetone (**H_4**), acetone extract pre-treated with n-hexane (**A**), ethanol extract pre-treated with n-hexane (**E**), 80% acetone extract pre-treated with 20% acetone (**A_{80}**), 80% ethanol extract pre-treated with 20% ethanol (**E_{80}**), 20% acetone wash for n-hexane pre-treated material (**A_2**), 20% ethanol wash for n-hexane pre-treated material (**E_2**), acetone extract for materials pre-treated with 20% acetone and n-hexane (**A_3**), ethanol extract for materials pre-treated with 20% ethanol and n-hexane(**E_3**).

3.3.8. Preliminary serial exhaustive extraction

3.3.9. Series I, II and III

A three series extraction was employed for preliminary isolation of compounds for large scale extraction (Figure 3.11). Methanol was the best extractant with the highest yield in all the series (1020.5 mg), (1004.7 mg) and (1516.6 mg) for series I, II and III, respectively, while DCM was the least in series I (121.6 mg).

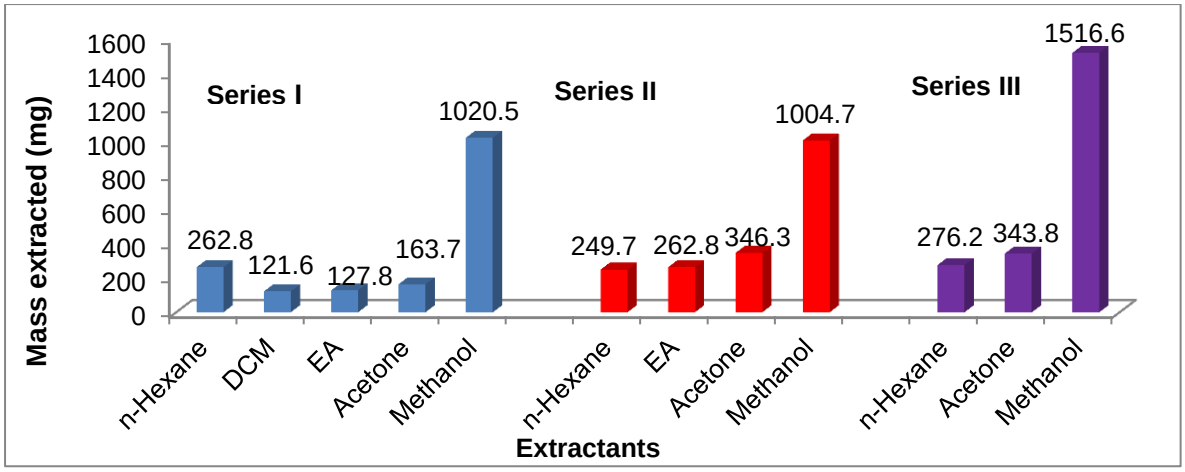

Figure 3.11: Mass serially extracted from 10 g of the *D. rotundifolia* leaf material with different solvents of varying polarities in three series from non-polar to polar; n-hexane, dichloromethane (**DCM**), ethyl acetate (**EA**), acetone and methanol.

(I). Series I

TLC fingerprint of phytochemicals developed in different solvent systems from the extracts serially extracted with different solvents of varying polarities and sprayed with vanillin-sulphuric acid reagent (Figure 3.12). Different phytochemicals are represented by different colours observed. Visible bands were only observed in ethyl acetate and acetone extracts on the plates separated in EMW.

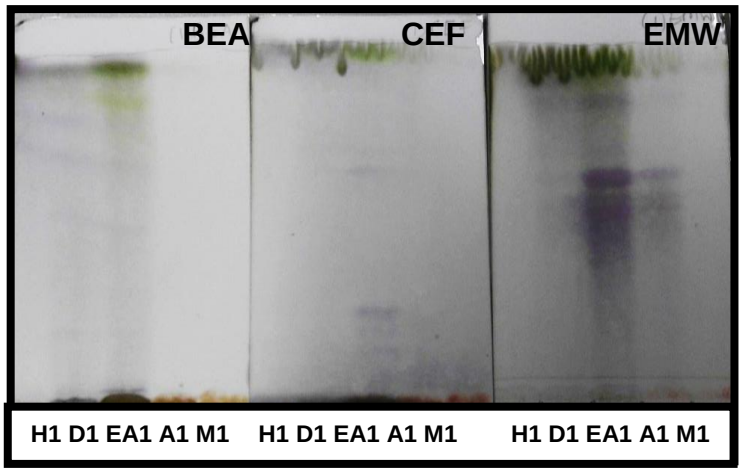

Figure 3.12: Chromatograms from *D. rotundifolia* leaf extracts developed in BEA, CEF, and EMW and sprayed with vanillin-sulphuric acid reagent for visualisation of the phytochemicals present. These represent compounds extracted serially with n-hexane (**H1**), dichloromethane (**D1**), ethyl acetate (**EA1**), acetone (**A1**), and methanol (**M1**).

(II). Series II

TLC analysis of phytochemicals developed in different solvent systems and sprayed with vanillin-sulphuric acid reagent for visualisation from the plant material serially extracted with different solvents of varying polarities (Figure 3.13). These phytochemicals are represented by the different colours observed on the plate. Only the n-hexane and methanol extracts separated in BEA and EMW had visible bands.

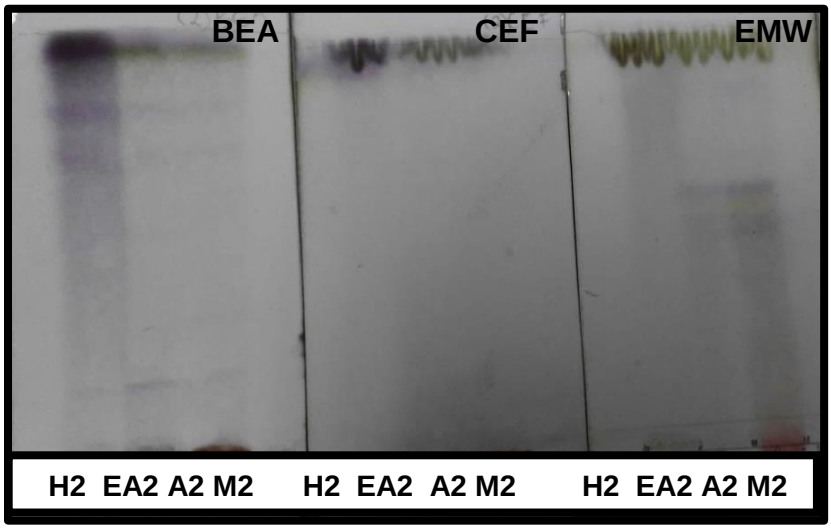

Figure 3.13: Chromatograms of *D. rotundifolia* leaf extracts developed in BEA, CEF, and EMW and sprayed with vanillin-sulphuric reagent to visualize the different phytochemicals present. These represent the compounds serially extracted with hexane (**H2**), ethyl acetate (**EA2**), acetone (**A2**) and methanol (**M2**).

(III). Series III

Extracts serially extracted with different solvents of varying polarities were analysed on TLC and sprayed with vanillin-sulphuric acid reagent for visualisation of the phytochemicals present (Figure 3.14). Different colours observed represent the different phytochemicals present. Better resolution of the bands was only observed in EMW than BEA and CEF.

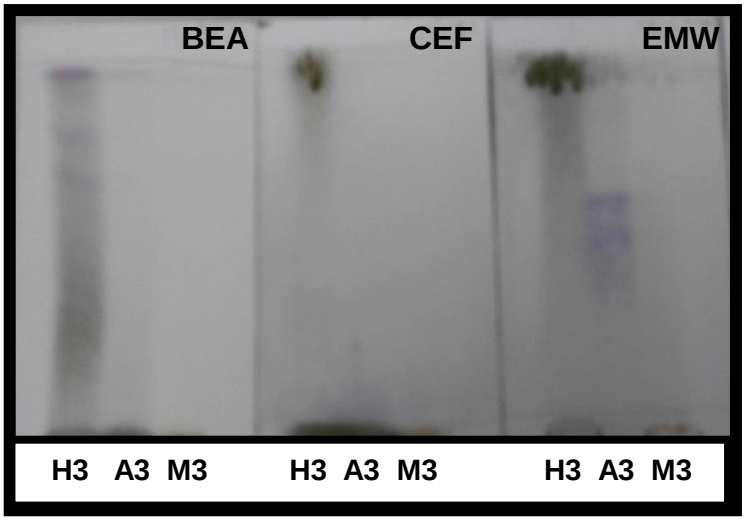

Figure 3.14: Chromatograms of *D. rotundifolia* leaf extracts obtained after development of the extracts in BEA, CEF and EMW and sprayed with vanillin-sulphuric acid reagent to visualise the different phytochemicals present. The compounds represented here were serially extracted with n-hexane (**H3**), acetone (**A3**) and methanol (**M3**).

3.3.10. Phytochemical constituents screening

Different secondary metabolites present in the extracts of the selected plants, which are responsible for the medicinal activity are presented in Table 3.2. All the plants showed the presence of cardiac glycosides, tannins and steroids and the absence of alkaloids. The rest of the constituents varied from plant to plant viz flavonoids, phlabotannins, terpenoids and saponins.

Table 3.2: Phytochemical constituents of the *S. pinnata*, *E. elephantina*, *C. africana,* and *D. rontudifolia* leaf extracts.

Phytochemical constituents	*C. africana*	*D. rontudifolia*	*E. elephantina*	*S. pinnata*
Terpenoids	+	+	-	+
Flavonoids	+	+	+	-
Cardiac glycosides	+	+	+	+
Phlabotannins	+	-	-	+
Saponins	+	+	-	+
Tannins	+	+	+	+
Alkaloids	-	-	-	-
Steroids	+	+	+	+

Key words: + = present; - = absent

3.3.11. Total phenolic, tannin and flavonoid content determination

The calibration curves used for estimation of the total phenolic, tannin and flavonoid content are represented in figures 3.15 to 3.17 below. These curves indicated a positive linear correlation with the absorbance increasing in a dose dependant manner.

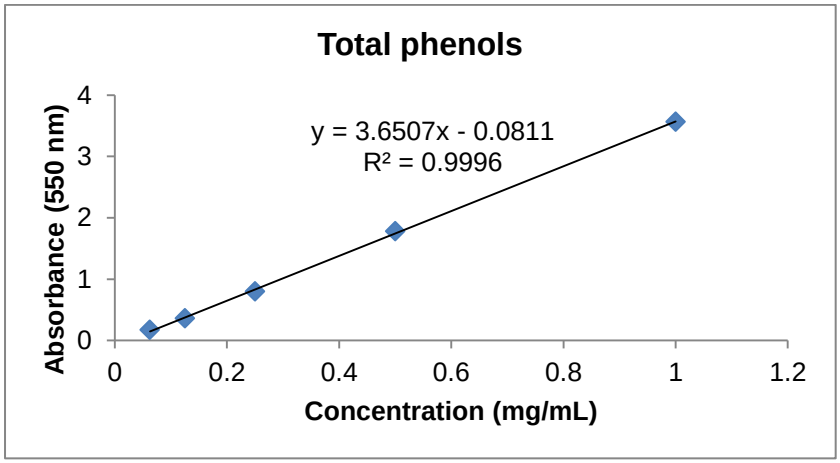

Figure 3.15: The garlic acid standard curve for total phenolic content determination.

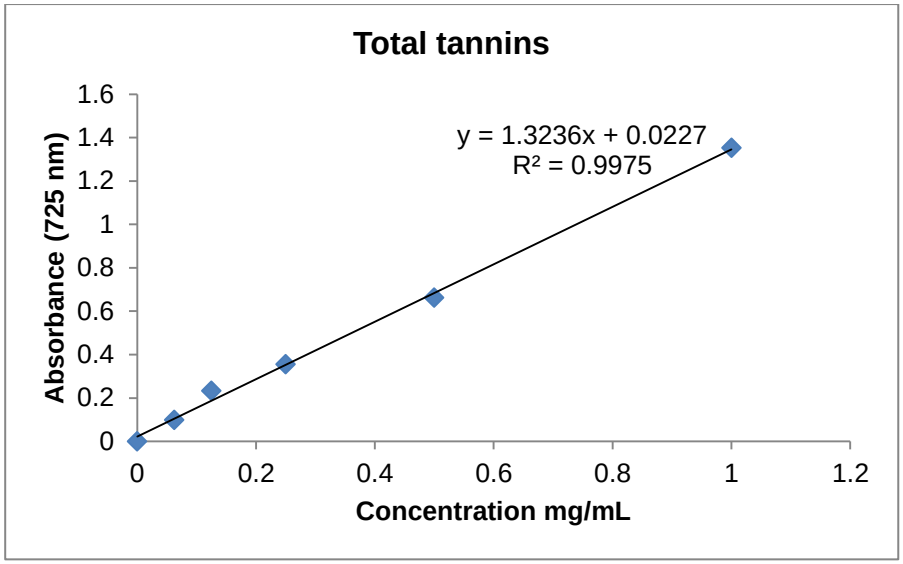

Figure 3.16: The garlic acid standard curve for total tannin content determination.

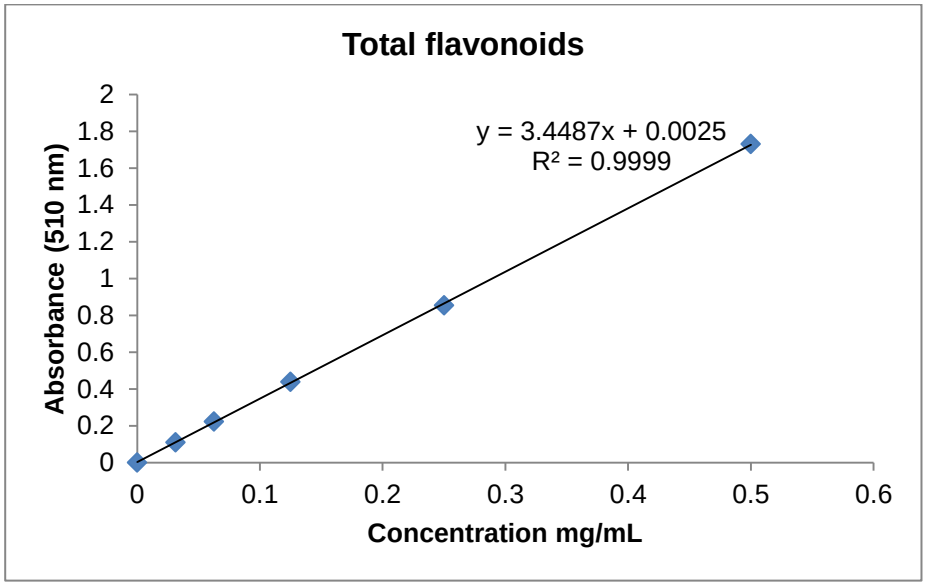

Figure 3.17: The quercetin standard curve for total flavonoid content determination.

The total phenolic content ranged from 259.00 to 55.33 mg of GAE/g of the sample, while the tannin and flavonoid content ranged from 330.33 to 28.00 mg of GAE/g of the sample and 19.90 to 4.00 mg of QE/g of the sample respectively (Table 3.3). *D. rotundifolia* had the highest concentration of phenols, tannins and flavonoids, while *S. pinnata* had the lowest.

Table 3.3: Total phenol, tannin, and flavonoid content of the 70% aqueous acetone extract of four selected plants.

Sample	Total phenols (mg of GAE/g of sample)	Tannins (mg of GAE/g of sample	Flavonoids (mg of QE/g of sample)
S. pinnata	55.33 ± 3.51	28.00 ± 1.73	4.00 ± 0.35
D. rotundifolia	259.00 ± 2.65	330.33 ± 15.63	19.90 ± 0.75
E. elephantina	58.67 ± 5.51	52,00 ± 2.65	5.10 ± 0.60
C. africana	113.67 ± 5.77	116.00 ± 3.46	14.10 ± 0.30

3.4. Discussion

Most researchers tend to use dry plant material because most traditional healers use medicinal plants in dried form and few problems were reported in connection with extractions of dry plant material than fresh ones (Eloff, 1998a). The plants used in this study were dried before extraction. Extraction is considered a very important step in the analysis of medicinal plants; as such, various solvents of varying polarities have been used for extraction of a variety of compounds from medicinal plants, thereby increasing the chances of extracting bioactive compounds (Eloff, 1998a).

For screening purposes the selected plants were extracted with four solvents of varying polarities, from non-polar to polar i.e. n-hexane, dichloromethane, acetone and methanol. The total masses of plant material extracted using different solvents were represented in figure 3.1. n-Hexane was a poor extractant since it had the least yield in all the plants, except for *E. elephantina* (121 mg). Meanwhile, methanol was the best extractant in all the plants since all the methanolic extracts had a great quantity of plant material compared to the other solvents. *S. pinnata* and *C. africana* appears to have intermediate to polar compounds since more plant material was extracted with dichloromethane and methanol. This suggests that the compounds present in *S. pinnata* and *C. africana* were soluble in intermediate to polar solvents.

Eloff (2004) reported on the importance of knowing the concentration of the extracts when analysing medicinal plants, as such, the extracts were dried and reconstituted in acetone to a final concentration of 10 mg/mL. This solvent was reported to be harmless against bacteria (Eloff, 1998b), therefore, it will not inhibit bacterial growth in the subsequent bioassays.

To evaluate and determine the number and type of compounds in each extract of the selected plants, thin layer chromatography was used. The chromatograms (Figure 3.2) were viewed under ultraviolet light to observe fluorescing compounds. More fluorescing compounds with different colours were observed in BEA and only one in CEF. This suggests that most of the compounds in the leaves of the selected plants are non-polar. Some compounds were not fluorescing, therefore, there was a need to detect them by spraying the plates with vanillin-sulphuric acid reagent. The observed different colours represent different phytochemical compounds, with more bands being observed in acetone extracts of *C. africana* and DCM extracts of *E. elephantina* in BEA than in CEF. The compounds in *C. africana* and *E. elephantina* were better resolved in BEA than in CEF, this suggests that they are non-polar.

Based on preliminary extraction and TLC analysis results of all the selected plants *D. rotundifolia* was chosen for further extraction with different extraction procedures. Enrichment, serial exhaustive and optimal extraction procedures were used to identify solvents that can be used individually, in mixtures, or serially as pre-treatment or extractant in extraction to optimise extraction of bioactive compounds (Zishiri, 2004) from this plant. The plant had a greater number of compounds on thin layer chromatography, as such, it had a potential of having bioactive compounds. Serial exhaustive extraction with solvent of varying polarities was the second best extraction method since it managed to increase the amount of plant material obtained after extraction. The amount of plant material extracted increased with increasing polarity (Figure 3.3). This is probably because the compounds were polar and were better dissolved in polar solvents. Moreover, the phytochemical fingerprint showed that the compounds were better resolved in EMW than CEF and BEA (Figure 3.4).

The *D. rotundifolia* plant material was washed with n-hexane to remove chlorophylls and fatty acids since this solvent is known to extract such constituents (Sasidharan

et al., 2011). The washed ethanol extract had a higher mass than the washed acetone extract (Figure 3.5). The phytochemical profiles (Figure 3.6) suggested that the compounds in these extracts were of intermediate polarity since they were resolved better in CEF than BEA and EMW.

The acetone and ethanol in water enrichment extraction procedure employed the use of different percentages of acetone and ethanol in water. This was to identify combinations that would extract active and/or inactive components since the different solvent percentages in water have different polarities (Zishiri, 2004). Increasing the water content in the mixture increased the amount of yield obtained in both acetone and ethanol in water extracts. The increase in the yield with increasing water content suggests that the compounds are soluble in polar solvents, because increasing the water content increased the polarity of the solvent. Water; followed by 20 and 40% acetone and ethanol in water extracts had the highest yield (Figure 3.7). The same extracts had no visible vanillin-sulphuric acid active compounds on the phytochemical fingerprint despite their high yields (Figure 3.8); Indicating that a greater yield does not always equals more bioactive compounds. The compounds were better resolved in CEF and EMW than BEA suggesting intermediate to polar polarity.

In the optimal pre-treatment method, 20% ethanol in water extract had the highest yield followed by 20% acetone in water extract. Meanwhile the least yield was obtained with the plant material pre-treated with n-hexane and subsequently extracted with 20% ethanol (Figure 3.9). This low yield could be as a result of the n-hexane pre-treatment which is known to extract chlorophylls and fatty acids (Sasidharan *et al*., 2011). No bands were visible on the extracts pre-treated with 20% ethanol and 20% acetone in water, as well as the 20% acetone and 20% ethanol washed material pre-treated with n-hexane (Figure 3.10). This is probably because the compounds were extracted in the second pre-treatment with 20% acetone and 20% ethanol.

Preliminary serial exhaustive extraction appears to be the best extraction method for compounds from *D*. *rotundifolia*. Methanol had the highest yield in all series while the least mass was obtained with DCM for series 1 and n-hexane for series 3. The greatest yield was obtained with series 3 since fewer solvents were used, as such,

the intermediate to polar compounds might have been extracted by methanol (Figure 3.11). The compounds extracted using the first series of solvents were better separated in EMW than the rest of the eluent systems (Figure 3.12). Meanwhile those in series 2 were better resolved in BEA and EMW, indicating the presence of non-polar and polar compounds (Figure 3.13). Only one band was observed on the phytochemical fingerprint of the acetone extract of the 3rd series (Figure 3.14). In all cases no bands were observed from the methanol extracts despite the greater yield obtained in all series.

Phytoconstituents screening of the selected plants revealed that *C. africana* contained most of the phytochemicals investigated while *E. elephantina* contained the least. Alkaloids were absent in all the plants while steroids, cardiac glycosides and tannins were present in all of them. Phlabotannins were present in *C*. *africana* and *S*. *pinnata* but absent in *E*. *elephantina* and *D*. *rotundifolia*. Flavonoids were present in all the selected plants except in *S. pinnata* meanwhile terpenoids and saponins were present in all the selected plants except in *E*. *elephantina* (Table 3.2). These bioactive compounds have therapeutic value and may possess one or more biological activity hence the importance of preliminary screening of phytochemicals when studying medicinal plants (Tadesse *et al*., 2012).

These observations are in line with those recorded by Agunbiade *et al*. (2012) on the presence of tannins, saponins, cardiac glycosides, and alkaloids in *C*. *africana*; however, no alkaloids were recorded in this plant in this study. This study is in agreement with the study conducted by Reid *et al*. (2001) on the presence of cardiac glycosides, saponins and tannins in the leaf, bark and shoot extracts of *D. rotundifolia*. The same study also reported the absence of alkaloids in the leaf, bark and shoot extracts of this plant. Mpofu e*t al*. (2014) reported the presence of saponins in *E. elephantina* ranging from 1.44 to 1.56 %; however, saponins were recorded to be absent in this study, probably because they were too low to be detected. There was no detection of alkaloids in all the plants but Mpofu *et al*. (2014) reported the presence of trace amounts of alkaloids in *E. elephantina*.

The Folin Ciocalteau and aluminium chloride methods were used to determine the total phenolic, tannin, and flavonoid contents respectively. The calibration curves (Figure 3. 15 to 3.17) were used to calculate the concentrations of the total phenolic,

tannin, and flavonoid content in the plant samples using gallic acid and quercetin as standards. The curves indicated a positive linear correlation with the absorbance increasing in a dose dependant manner. Among all the tested plant samples, the highest concentration of phenolic (259.00 mg GAE/g), tannin (330.33 mg GAE/g), and flavonoid (19.90 mg QE/g) content were observed on the *D. rotundifolia* extract followed by *C. africana*. The lowest concentration of phenolic (55.33 mg GAE/g), tannin (28.00 mg GAE/g), and flavonoid (4.00 mg QE/g of sample) content was recorded in *S. pinnata* (Table 3.3). These important phytochemicals have been reported to be responsible for a number of activities including antioxidant and antibacterial activities among other therapeutic effects (Rao *et al*., 2012). This is the first study reporting the phytochemical analysis and total phenolic, tannin and flavonoid content of *Schkuhria pinnata*.

3.5. Conclusion

The study demonstrated that the use of different solvents of varying polarities aids the extraction of different phytochemicals which were best resolved in CEF. This chapter recommends use of pre-treatment methods for the extraction of intermediate to polar compounds from *D*. *rotundifolia*. Water, which is an extractant used mostly by traditional healers had a high yield, however, no vanillin-sulphuric acid reactive compounds were present in all the water and high water content aqueous acetone and ethanol extracts. It is therefore not recommended for further bioassays. Phenolics, tannin, and flavonoid are known to possess antioxidant activity in *in vitro* assays. Therefore, all the plants should be further analysed for antioxidant activity since the presence of phenolic, tannin and flavonoid contents was demonstrated in all of them.

3.6. References

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Chapter 4: Antioxidant activity assays

4.1. Introduction

Oxidation is one of the most common contributing factors to producing free radicals. Accumulation of these free radicals in the body leads to oxidative stress and this causes human diseases such as cancer, diabetes, cardiovascular diseases and inflammatory conditions (Lakshmi *et al*., 2014). Therefore, antioxidants are needed to neutralise the action of free radicals in order to prevent such diseases because their treatment involves serious efficacy and safety issues (Ahmed *et al*., 2015).

Plants have been the basis of treatment of many diseases for centuries and they represent rich sources of therapeutics (Tambe and Bhambar, 2014; Ahmed *et al*., 2015). Phenolic compounds represent a major source of natural antioxidants capable of chelating metals, scavenging free radicals and preventing lipid peroxidation (Guleria *et al*., 2013). The antioxidative potential of phenolics may be attributed to their redox potential (Kaviarasan *et al*., 2007).

Plant-based antioxidant compounds were reported to be safe and beneficial, unlike synthetic ones, which were found to have adverse side effects including genotoxicity (Stanković, 2011). Therefore, search for new products with antioxidant properties is of utmost importance. As such, a number of methods were developed for screening and evaluation of antioxidant compounds from plant extracts. The most commonly used methods include; qualitative and quantitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals assays, ferric reducing antioxidant power (FRAP) assay and ferrous-ion chelating assays (Molan *et al*., 2012). The aim of this chapter was to determine the antioxidant activity of the selected plants using the DPPH radical scavenging method on TLC and quantify the antioxidants present by determining the amount of free radical scavenged and assaying their antioxidant capacity using the reducing power method.

4.2. Methods and materials

4.2.1. Qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC

The hydrogen donating potential of the extracts of the selected plants was screened using the method described by Deby and Margotteaux (1970). The phytochemicals were first separated using TLC (**section 3.2.3**). The chromatograms were dried in a fume-hood and then later sprayed with 0.2% (w/v) of DPPH (Sigma®) in methanol as an indicator. The antioxidant compounds present were detected by the yellow spots against a purple background on the TLC plates (Deby and Margotteaux, 1970).

4.2.2. Quantitative DPPH free radical scavenging activity assay

The free radical scavenging activity of the 70% aqueous acetone extracts of the selected plants was measure using the method described by Brand-Williams *et al*. (1995) with minor modifications. The stock solution of 0.2% DPPH in methanol was prepared and kept in a refrigerator until use. One millilitre of the DPPH solution was mixed with 1 mL of the different concentrations of the plant extracts (0.031 to 1 mg/mL), or the standard solution in a test tube. The absorbance was measured at 517 nm. The percentage of radical scavenging activity was calculated using the formula below, where, Ac and As are the absorbance of control and sample, respectively. The control contained 1 mL of methanol in place of the plant sample (Ahmed *et al*., 2015). The antioxidant activity was expressed as the half maximal effective concentration (EC_{50}).

$$\%\text{Scavenging activity } \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

4.2.3. Reducing power assay

The antioxidant capacity was evaluated using the reducing power assay as described by Oyaizu. (1986) with minor modifications. A set of concentrations ranging from 0.0625 mg/mL to 1 mg/mL of the 70% aqueous acetone extracts of the selected plants were prepared. Two millilitres of each of the prepared concentrations were added into a test tube, to this 2 mL of sodium phosphate buffer (1 M, pH 6.6) and 2 mL of potassium ferricyanide (1% w/v in distilled water) were added and mixed

well. This mixture was incubated in a water bath at 50 °C for 20 minutes. Following incubation, 2.5 mL of trichloroacetic acid (10% w/v in distilled water) was added and the mixture was centrifuged at 650 rpm for 10 minutes. About 3 mL of the supernatant was added into a test tube. To this, 10 mL of distilled water and 1 mL of ferric chloride (0.1% w/v in distilled water) solution was added and mixed well. The absorbances of the solutions were measured at 700 nm against a blank prepared as described above, but replacing the plant extract with an equal volume of a solvent (Ahmed *et al*., 2015).

4.3. Results

4.3.1. Qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC

4.3.1.1. Preliminary extraction procedure

The antioxidant activity of the extracts of the selected plants were screened using the qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates (Figure 4.1) developed in different separation systems. The presence of antioxidant compounds were indicated by the yellow spots against the purple background. All the extracts of *D. rotundifolia* demonstrated antioxidant potential in BEA and CEF mobile systems.

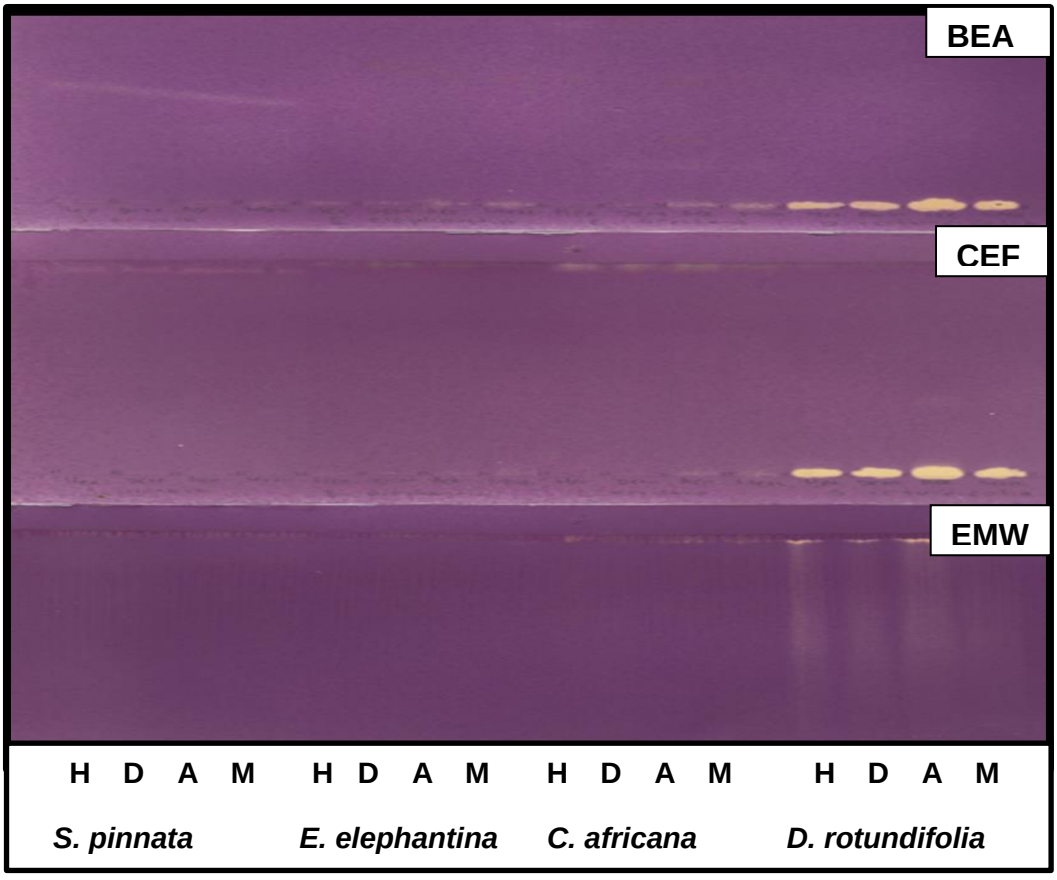

Figure 4.1: Chromatograms obtained after development of the plant extracts in BEA, CEF and EMW mobile phases and sprayed with 0.2% DPPH for compounds extracted with n-hexane (H), dichloromethane (D), acetone (A) and methanol (M).

4.3.1.2. Serial exhaustive extraction

To detect antioxidant-active compounds in *D. rotundifolia* plant material was extracted serially with n-hexane, dichloromethane; acetone and methanol were sprayed with 0.2% DPPH in methanol. All the extracts demonstrated antioxidant activity although no distinguished bands can be seen due to poor separation (Figure 4.2).

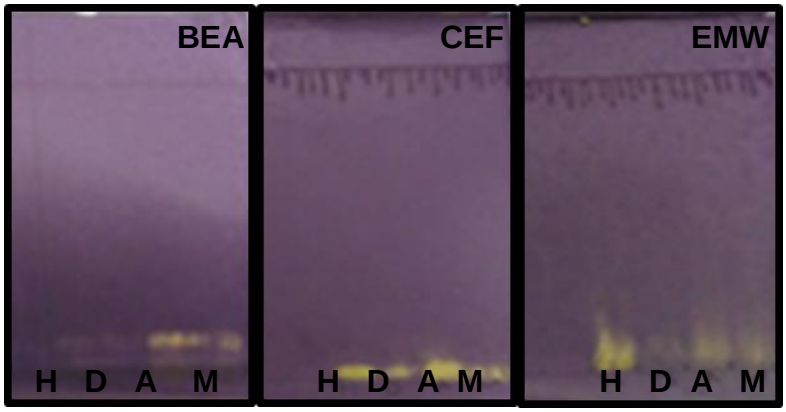

Figure 4.2: Chromatograms with *D. rotundifolia* leaf extracts developed in BEA, CEF, and EMW and sprayed with 0.2% DPPH for detection of antioxidant-active compounds serially extracted with n-hexane (**H**), dichloromethane (**D**), acetone (**A**) and methanol (**M**).

4.3.1.3. Extraction enrichment procedures

This procedure includes two extraction processes, n-hexane wash and acetone and ethanol in water extraction methods. The results of the antioxidant activity of both methods are shown in figures 4.3 and 4.4 below.

4.3.1.3.1. n-Hexane wash

The *D*. ro*tundifolia* plant material was pre-treated with n-hexane and subsequently extracted with acetone and ethanol. All the extracts including the pre-treated plant material had antioxidant activity in all the separation systems used. EMW managed to pull up the antioxidant compounds in the extracts, however, no distinguished separation was achieved (Figure 4.3).

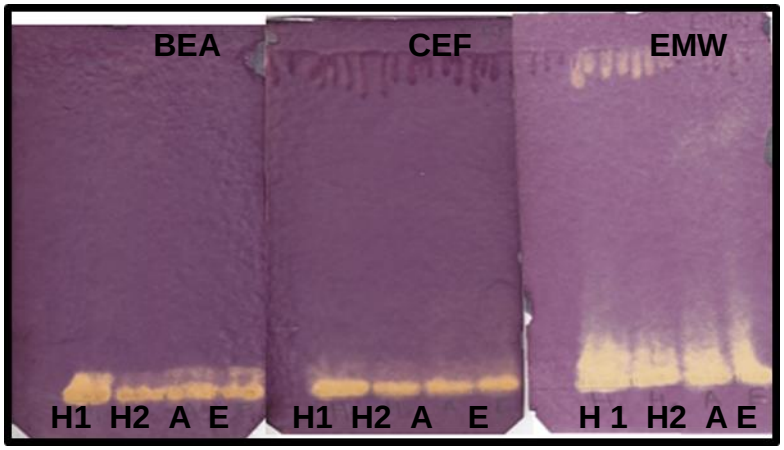

Figure 4.3: Chromatograms obtained after separation of *D. rotundifolia* leaf extracts in BEA, CEF and EMW and spraying with 0.2% DPPH to visualise the antioxidant-active compounds from plant material washed with n-hexane (**H1**), (**H2**) and subsequently extracted with acetone (**A**) and ethanol (**E**) respectively.

4.3.1.3.2. Acetone and ethanol in water treatment

Different percentages of acetone and ethanol in water were used to optimise the extraction of antioxidant-active compounds in *D. rotundifolia* leaves. To detect these compounds, the chromatograms were sprayed with 0.2% DPPH. In all the separation systems, water and 20% aqueous acetone had no detectable antioxidant activity, while ethanol and most of the aqueous ethanol extracts had antioxidant activity (Figure 4.4).

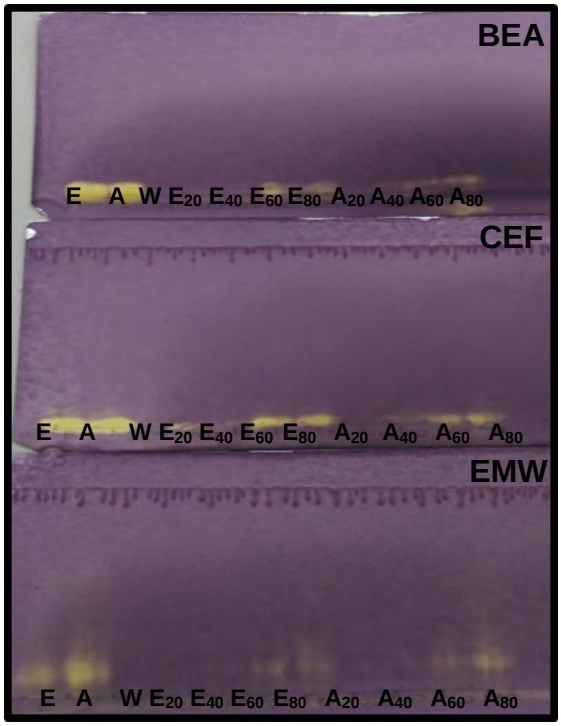

Figure 4.4: Chromatograms obtained after development of the *D. rotundifolia* leaf extracts in BEA, CEF and EMW and sprayed with 0.2% DPPH to detect for antioxidant-active compounds extracted with ethanol (**E**), acetone (**A**), water (**W)**, 20% ethanol ($\mathbf{E_{20}}$), 40% ethanol ($\mathbf{E_{40}}$), 60% ethanol ($\mathbf{E_{60}}$), 80% ethanol ($\mathbf{E_{80}}$), 20% acetone ($\mathbf{A_{20}}$), 40% acetone ($\mathbf{E_{40}}$), 60% acetone ($\mathbf{A_{60}}$) and 80% acetone ($\mathbf{A_{80}}$).

4.3.1.4. Optimal extraction procedure

This method involved pre-treatment of the *D. rotundifolia* leaves with n-hexane and 20% aqueous acetone and ethanol before subsequent extraction with acetone and ethanol. No significant antioxidant activity was detected for the pre-treatment while all the extracts obtained after the pre-treatment had antioxidant activity. This antioxidant compounds were only separated in EMW (Figure 4.5).

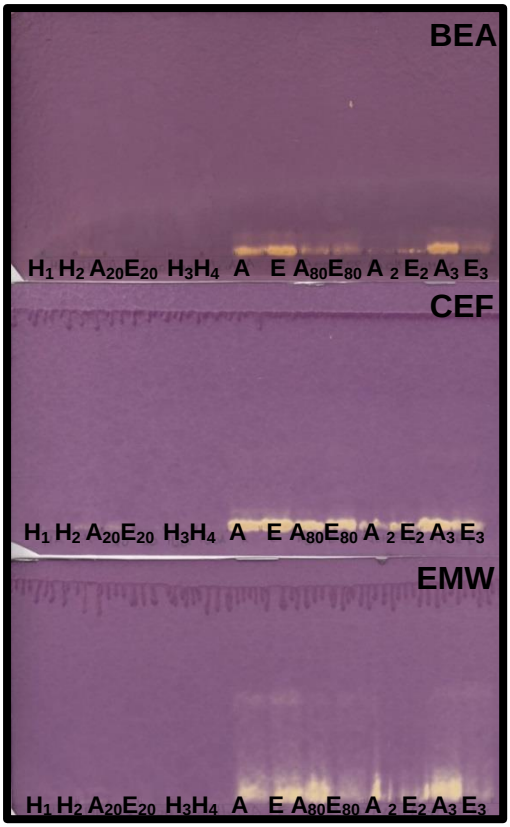

Figure 4.5: Chromatograms obtained following development of the *D. rotundifolia* leaf extracts in BEA, CEF and EMW and spraying with 0.2% DPPH for detection antioxidant-active compounds from n-hexane wash for acetone (**H_1**), n-hexane wash for ethanol (**H_2**), 20% acetone wash (**A_{20}**), 20% ethanol wash (**E_{20}**), n-hexane wash for 20% ethanol (**H_3**), n-hexane wash for 20% acetone (**H_4**), acetone extract pre-treated with n-hexane (**A**), ethanol extract pre-treated with n-hexane (**E**), 80% acetone extract (**A_{80}**), 80% ethanol extract (**E_{80}**), 20% acetone wash for n-hexane pre-treated material (**A_2**), 20% ethanol wash for n-hexane pre-treated material (**E_2**), acetone extract for materials pre-treated with 20% acetone and n-hexane (**A_3**), ethanol extract for materials pre-treated with 20% ethanol and n-hexane(**E_3**).

4.3.1.5. Serial exhaustive extraction

Three series of serial exhaustive extraction were employed to optimise the antioxidant compounds extracted from *D. rotundifolia* leaves. To detect these compounds the chromatograms were sprayed with 0.2% of DPPH in methanol (Figures 4.6 to 4.8).

4.3.1.5.1. Series 1

In series 1, antioxidant compounds were present in ethyl acetate, acetone and methanol in chromatograms developed in BEA, acetone and methanol in chromatograms developed in CEF and Methanol only for chromatograms developed in EMW (Figure 4.6).

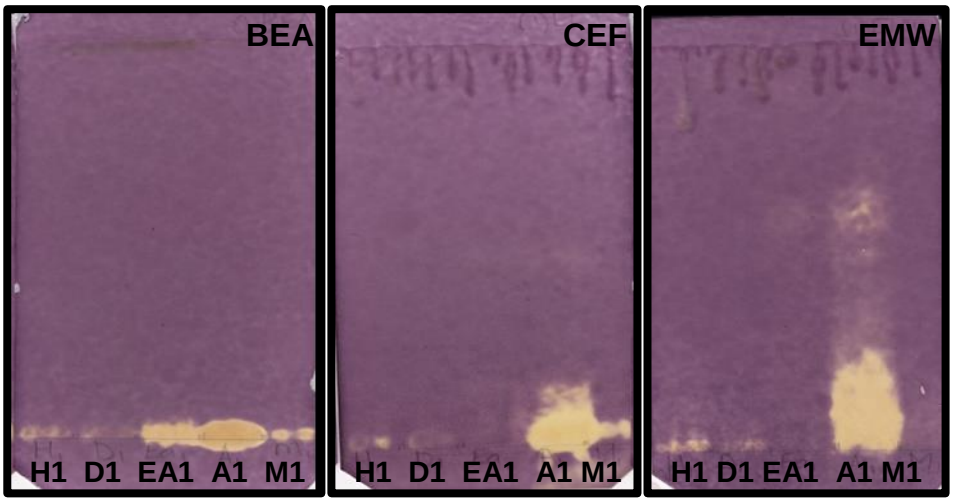

Figure 4.6: Chromatograms with *D. rotundifolia* leaf extracts developed in BEA, CEF and EMW and sprayed with 0.2% DPPH for detection of antioxidant-active compounds extracted with n-hexane (**H1**), dichloromethane (**D1**), ethyl acetate (**EA1**), acetone (**A1**) and methanol (**M1**).

4.3.1.5.2. Series 2

All the n-hexane extracts in this series had no detectable antioxidant activity on all the chromatograms. BEA separation system revealed antioxidant activity on the acetone and methanol extracts, while for CEF it was ethyl acetate, acetone and methanol and EMW only acetone and ethyl acetate extracts had antioxidant activity. CEF and EMW managed to separate the antioxidant compounds in this series (Figure 4.7).

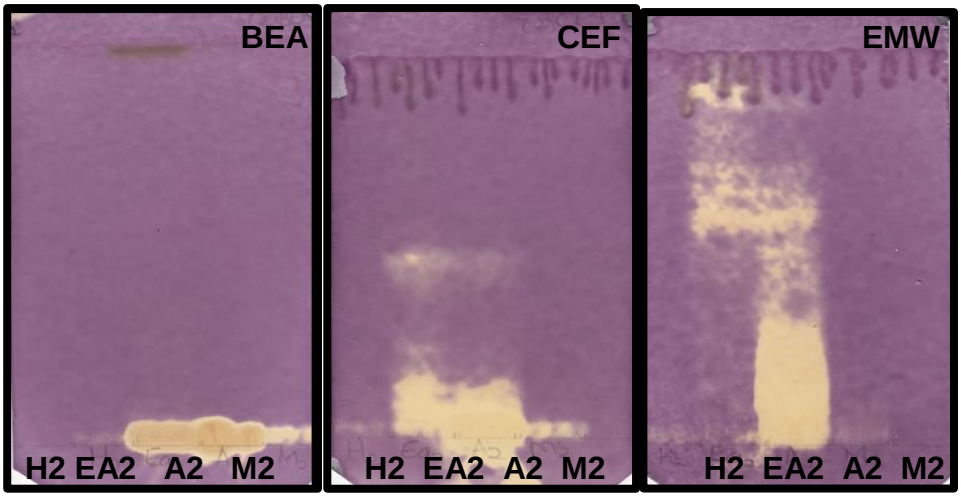

Figure 4.7: Chromatograms with *D. rotundifolia* leaf extracts developed in BEA, CEF, and EMW and sprayed with 0.2% DPPH for detection of antioxidant. Active compounds extracted with hexane (**H2**), ethyl acetate (**EA2**), acetone (**A2**) and methanol (**M2**).

4.3.1.5.3. Series 3

This series used three solvents of increasing polarity to extract compounds with antioxidant activity from the *D. rotundifolia* leaves. In all the separation systems n-hexane had no detectable antioxidant activity, while acetone and methanol extracted antioxidant-active compounds which were separated in EMW (Figure 4.8).

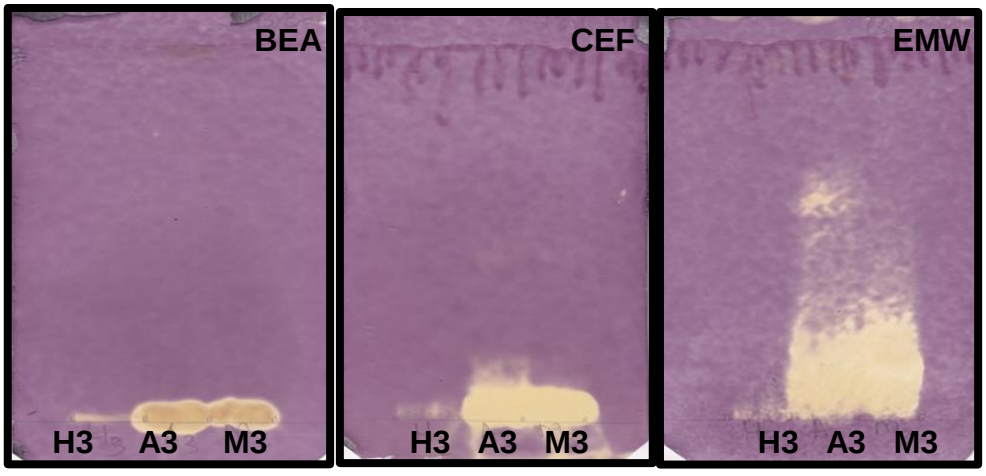

Figure 4.8: Chromatograms with *D. rotundifolia* leaf extracts developed in BEA, CEF, and EMW and sprayed with 0.2% DPPH for detection of antioxidant-active compounds extracted with n-hexane (**H3**), acetone (**A3**) and methanol (**M3**).

4.3.2. Quantitative antioxidant activity assay

To quantitatively analyse the antioxidant activity in the selected plants, two methods were used, these were; the quantitative DPPH scavenging assay and the reducing power method for antioxidant capacity assay.

4.3.2.1. DPPH free radical scavenging activity assay

The free radical scavenging ability of the selected plants was quantified using the DPPH radical scavenging method. Table 4.1 below represents the concentrations of the extracts of the selected plants which scavenged half of the DPPH free radical. It is worth noting that the lower the half maximal effective concentration (EC_{50}) the higher the antioxidant activity. The highest antioxidant activity was demonstrated by *D. rotundifolia* with the lowest EC_{50} (0.27 ± 0.0007) while the lowest was recorded with *S. pinnata* with the highest EC_{50} (2.98 ± 0.0071).

Table 4.1: The free radical scavenging potential (EC_{50} values) of the 70% aqueous acetone extracts of the selected plants against ascorbic acid as a positive control.

Sample	DPPH scavenging potential EC_{50}
S. pinnata	2.98 ± 0.0071
D. rotundifolia	0.27 ± 0.0007
E. elephantina	0.94 ± 0.0014
C. africana	0.95 ± 0.0021
Ascorbic acid	0.10 ± 0.0014

4.3.2.2. Reducing power method

The antioxidant capacity of the selected plants was evaluated using the reducing power method. *D. rotundifolia* displayed the highest reducing power while the rest of the plants showed a similar trend. In any case, increasing the concentration of the extracts increased the reducing power (Figure 4.9).

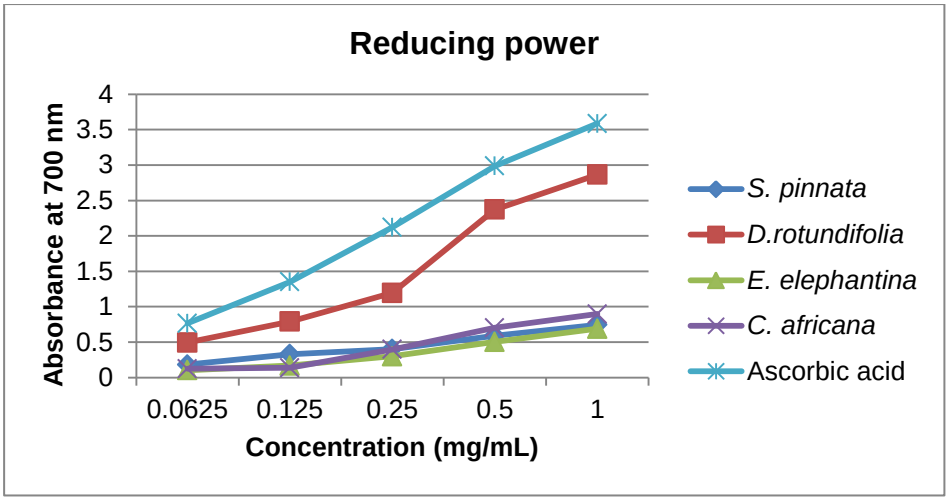

Figure 4.9: The reducing power potential of the selected plants and ascorbic acid, with absorbance increasing with increasing concentration.

4.4. Discussion

There is an increasing interest of plants as potential sources of new drugs with antioxidant activity to replace the synthetic ones (Chanwitheesuk *et al*., 2005). The selected plants were evaluated for antioxidant potential. Figure 4.1 show the presence of antioxidant constituents in the extracts of *D. rotundifolia* that are polar as yellow spots against the purple background, even though they were not well separated when developed in BEA and CEF solvent systems. This suggests that the extracts contain hydrogen-donating antioxidant compounds which were able to reduce DPPH to a yellow DPPH-H. The observed antioxidant compounds were not separated, probably due their polarity relative to the solvent systems.

Different extracts of *D. rotundifolia* from the different extraction procedures used in chapter 3 were evaluated for hydrogen donating antioxidant potential. This was to find an extraction procedure that will extract significant amounts of antioxidant compounds which will be separated in BEA, CEF or EMW. Figures 4.2 to 4.8 shows the antioxidant potential of the *D. rotundifolia* extracts from all the extraction procedures used in this study. All the extracts from the serial exhaustive extraction had antioxidant activity on the chromatograms separated in BEA, CEF, and EMW although no distinguished separation of the antioxidant compounds was obtained (Figure 4.2). All the n-hexane wash extracts also had antioxidant compounds which

were not separated probably, because of their polarity relative to the solvent systems used (Figure 4.3). The acetone and ethanol in water enrichment procedure managed to extract high concentrations of antioxidant-active compounds with acetone and ethanol; this is evidenced by the intensive zones of inhibition. Meanwhile, water and 20 and 40 % aqueous acetone and ethanol extracts from the former extraction procedure had no antioxidant-active compounds despite their high yields in extraction (Figure 4.4).

All the pre-treatment extracts in the optimal extraction method had no detectable antioxidant compounds in BEA and EMW; however, 20% aqueous acetone and ethanol extracts had small quantities of antioxidant compounds in CEF separation systems. Meanwhile the subsequent extracts all had antioxidant-active compounds in all the separation systems, although the extracts from material pre-treated with n-hexane and 20% acetone and 20% ethanol had small quantities of antioxidant compounds indicated by faint zones of inhibition (Figure 4.5). This is probably due to the subsequent pre-treatment with 20% acetone and ethanol which extracted most of the antioxidant compounds present. Three series of serial exhaustive extractions were developed in order find the best series of solvents for extraction of antioxidant-active compounds. The first series (Figure 4.6) contained less antioxidant compounds compared to the second (Figure 4.7) and third series (Figure 4.8). This is probably because the first series contained more solvents than the 2^{nd} and 3^{rd} ones. Despite all the different extraction procedures used for extraction of antioxidant-active compounds (in this study), no optimal separation with distinguished bands of the *D. rotundifolia* extracts was achieved.

The quantitative DPPH free radical scavenging method and reducing power assay were used as a measure of antioxidant activities, to determine the mode of antioxidant-active compounds in the selected plants. The DPPH radical scavenging method is based on the decrease in absorbance upon donation of protons by potential antioxidants which reduce the radical DPPH to a non-radical DPPH-H (Türkoğlu *et al*., 2007; Lee *et al*., 2012). This decrease in absorbance was taken as a measure of the extent of radical scavenging activity by the extracts, i.e. the lower the absorbance the higher the antioxidant activity (Türkoğlu *et al*., 2007). The free radical scavenging activities of the extracts of the selected plants were expressed as

the effective concentration for scavenging 50% of the free radicals (EC_{50}) and these are presented in Table 4.1. The highest radical scavenging activity was observed in *D. rotundifolia* with the lowest EC_{50} (0.27 ± 0.0007), this was followed by *E. elephantina* (0.94 ± 0.0014) and *C. africana* (0.95 ± 0.0021). The lowest was recorded in *S. pinnata* with the highest EC_{50} (2.98 ± 0.0071). The EC_{50} of the positive control ascorbic acid was 0.10 ± 0.0014.

The reducing power assay measures the antioxidant capacity of the plant extracts. This method is based on the ability of antioxidants within the extracts to reduce the Fe^{3+}/ferricyanide complex to its ferrous (fe^{2+}) form by donating electrons. The concentration of Fe^{2+} ions were taken as a measure of the Perl's Prussian complex. Therefore an increase in absorbance signalled an increase in reducing power capacity of the plant extracts (Lee *et al*., 2012). From figure 4.9 it was revealed that *D. rotundifolia* had the highest reducing power while the other plants showed almost equal efficacy that is lower. The reducing power capacity of all the plants increased in a dose dependant manner. *D. rotundifolia* plant which had the highest total phenolic, tannin, and flavonoid content in chapter 3 also displayed the highest free radical scavenging and ferric reducing capacity. This correspondence may be attributed to the well-known fact that phenolics and flavonoids possess high antioxidant potential (Ahmed *et al*., 2015) as free radical scavengers and metal ion reducers (Guleria *et al*., 2013).

4.5. Conclusion

This study has tested four plants for antioxidant activity using three methods which gave an indication on the presence and mode of antioxidant compounds in the extracts. The presence of potent antioxidant compounds with free radical scavenging and reducing power abilities were demonstrated in *D. rotundifolia* leaf extracts. This observed antioxidant efficacy could be due to the presence of phenolics, tannins and flavonoids which were present in this plant in high amounts. These phytochemicals were reported to have a number of activities including antioxidant activity.

4.6. References

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Chapter 5: Antibacterial activity assays

5.1. Introduction

The constant emergence of multi-drug resistant pathogens is threatening the efficacy of the currently used antibiotics (Bandow *et al*., 2003; Parekh and Chanda, 2007a). As a result medicinal plants are researched as possible new sources of antimicrobial agents with possibly novel mechanisms of action and fewer side effects since they have therapeutic relevance in folklore (Farnsworth and Moris, 1975; Machado *et al*., 2003; Motsei *et al*., 2003; Barbour *et al*., 2004; Parekh and Chanda, 2007b). These medicinal plants are used in the form of herbal remedies which are prepared from one plant or a combination of different plant species (Ncube *et al*., 2012). Some researchers focus on combinations of plants with conventional antibiotics, while others focus on plant-plant combinations to improve efficacy (Chung *et al* 2011; Ncube *et al*., 2012; Moussaoui and Alaoui, 2016; Komape *et al*., 2017).

Herbal mixtures containing a combination of different plant species were reported to have better biological activities than isolated active compounds and herbal mixtures prepared from one plant specie (Williamson, 2001; Nahrstedt and Butterweck, 2010; Ncube *et al*., 2012). Combinational therapy is not only limited to herbal mixtures, but have also been used in the treatment of diseases such as cancer, infectious diseases and HIV/AIDS because these were reported to have not only one but several targets (Williamson, 2001; Wagner, 2011). Although there is significant information on the screening of medicinal plants used in folklore medicine for antimicrobial activity, most of these only focus separate groups of compounds (Ncube *et al*., 2012).

This has led to an increase in the interest in screening of medicinal plants for antimicrobial activities and synergistic studies (Eloff, 1998a; Parekh and Chanda, 2007a). Medicinal plants may be screened through random sampling (Mojab *et al*., 2010) or following up on their traditional uses as leads (Fabricant and Farnsworth, 2001).

Screening is an imperative process for the discovery of novel compounds with potent biological activities. Therefore, *in vitro* biological assays were developed for

screening of antimicrobial activities in traditional medicinal plants (da Costa *et al*., 2010). These assays are dived into diffusion and dilution methods for antimicrobial susceptibility testing and minimum inhibitory concentration (MIC) determinations (Ncube *et al*., 2008). The diffusion methods include; agar well diffusion, agar disk diffusion and bioautography, while the dilution tests include agar and broth micro/macro dilution methods (Balouiri *et al*., 2016).

Bioautography is a variation of the diffusion methods, where the antimicrobial compounds are adsorbed on a thin layer chromatography (TLC) plate (Ncube *et al*., 2008). This method uses less samples and the polarity of the active compounds can be determined. Furthermore, it offers reproducible results, simplicity and overcomes the challenges of isolating and identifying antimicrobial compounds (Silva *et al*., 2005; Patil *et al*., 2013). The three variations of bioautography include; (i) contact bioautography, where antimicrobial compounds are transferred from a TLC plate onto an inoculated agar medium, (ii) agar overlay bioautography, where the chromatograms are covered with a seeded agar medium and (iii) direct bioautography where chromatograms are sprayed with a suspension of test microorganisms (Hamburger and Cordell, 1987; Rahalison *et al*., 1991; Sasidharan *et al*., 2011). Direct bioautography is the mostly used method among all the other bioautographic methods and it was chosen for qualitative analysis of antibacterial activity in this study. Antimicrobial activity is represented by zones of inhibition of microbial growth which are visualised with dehydrogenase activity-detecting reagents such as tetrazolium salts (Choma and Grzelak, 2011).

Broth micro-dilution is amongst the basic techniques of quantitative antimicrobial susceptibility testing. This method is useful for the determination the minimum inhibitory concentration of the observed antimicrobial activity (Balouiri *et al*., 2016). A concentration range (5 to 8) is made using a two-fold serial dilution of the antimicrobial agent from the first well (Das *et al*., 2010). Unlike the agar dilution method, this method uses broth and there is a direct contact between the test sample and the test microorganisms (Eloff, 1998b; Shahid, 2012). Visualisation employs the colorimetric indicators (Ncube *et al*., 2008). This method offers advantages over diffusion methods in that it has increased sensitivity even on smaller sample quantities, reproducibility, convenience and the ability to distinguish between bacteriostatic and bactericidal effects (Reller *et al*., 2009). However the

limiting factor is the solubility and miscibility of non-polar bioactive compounds like terpenes and alkaloids (Shahid, 2012). The broth micro-dilution method is considered the most sensitive method for screening antimicrobial activity in plant extracts (Eloff, 1998b; Shahid, 2012).

MIC values of observed antimicrobial activity in plants does signify the total quantity present in plant part, since the extracts are dried and made to a known concentration prior to the bioassay (Eloff, 2001). The activity of the quantity present is known as total activity and it refers to the amount to which the quantity extracted from one gram of the plant material could be diluted to and still inhibit the growth of the test microorganism. The total activity can be calculated using the equation below (Sakong, 2012). The aim of this chapter was to qualitatively and quantitatively evaluate the antibacterial properties of the selected plants against bacteria with pathogenic effects against human and animals as well as implications in multi-drug resistance and infectious diseases.

$$\text{Total antibacterial activity} = \frac{\text{Quantity extracted (mg/g)}}{\text{MIC value (mg/mL)}}$$

5.2. Methods and Materials

5.2.1. Test microorganisms

Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) and two Gram-negative bacteria (*Escherichia coli* ATCC 28922 and *Pseudomonas aeruginosa* ATCC 27853) are the strains primarily recommended for use by the Clinical and Laboratory Standards Institute (CLSI) (NCCLS, 1992). These bacterial species were maintained on nutrient agar at 4 °C. Prior to bioautography and broth micro-dilution assays the cultures were inoculated in nutrient broth and incubated at 37 °C for 12 hours.

5.2.2. Qualitative antibacterial assay

5.2.2.1. Bioautography assay

The Bioautography procedure was done according to the method described by Begue and Kline (1972). The plates were loaded with 20 µL of the plant extracts and separated as described in **section 3.2.3** and placed under a stream of air for a period of five days to allow the solvents used for development to evaporate. The actively growing cultures of the test microorganisms mentioned above were sprayed on the plates until they were completely wet then incubated at 37 °C for 24 hours under 100% relative humidity. Following incubation, the plates were sprayed with an aqueous solution of 2 mg/mL ρ-iodonitrotetrazolium chloride (INT) (Sigma) and further incubated for 2-3 hours. The bioautograms were observed for bacterial growth, clear zones against the red-pink background indicated growth inhibition by the compounds with antibacterial activity.

5.2.3. Quantitative antibacterial assay

5.2.3.1. Broth micro-dilution assay

The minimum inhibitory concentration (MIC) values were determined using the serial microplate broth dilution methods developed by Eloff (1998b). The plant extracts were dissolved in acetone to give a final concentration of 10 mg/mL. The bacterial species were inoculated into 150 mL nutrient broth and incubated at 37 °C for 24 hours, this served as the stock culture. From the stock culture 10 mL was removed and inoculated in 150 mL nutrient broth and incubated at 37 °C for 24 hours. Hundred microlitres of the plant extract was serially diluted (50%) with sterile distilled water in 96-well microtitre plates, and 100 µL of the bacterial culture was added into each well. Acetone was used as a negative control; the microtitre plates were covered and incubated at 37 °C for 24 hours. Following incubation, 40 µL of 0.2% ρ-iodonitrotetrazolium chloride (INT) (sigma) dissolved in water was added to each well as an indicator. The covered plates were further incubated for 30 minutes at 37 °C at relative humidity. The plates were observed for clear wells (activity) which resulted from reduction of the purple colour and the MIC values were recorded as the lowest

concentration that inhibited bacterial growth. The tests were done in triplicates. Total activity of the extracts was calculated by dividing the MIC values with the mass extracted from 1 g of the plant material. The resultant values indicated the volume to which the amount obtained from 1 g of the plant material could be diluted to and still inhibit growth of the test microorganisms (Eloff, 2001).

5.2.3.2. Antibacterial interaction effects of the selected plants

Stock solutions (10 mg/mL) of acetone extracts of each plant were prepared by re-dissolving the extracts in acetone. For 1:1 test combinations, 50 µL of each of the two extracts were mixed to make up a volume of 100 µL in the first wells of a 96-well microtitre plate. Each extract contributed 33.3 µL and 25 µL for the 1:1:1 and 1:1:1:1 combinations respectively, to make up 100 µL in the first wells of a 96-well microtitre plate (Ncube *et al*., 2012). MIC values were determined for each of these combinations to establish any interaction effect following the antibacterial assays described in **section 5.2.3.1**. Following investigations of the independent MIC of the selected plants, the synergistic or antagonistic interactions between the plants were investigated. This was achieved by determining the MIC of the combinations exhibiting antibacterial activity to establish any interaction effect. The fractional inhibitory concentration (FIC) was calculated for the 1:1 combinations of the plants. This was determined with the equation below, where (i) and (ii) represented the different 1:1 plant combinations (Mabona *et al*., 2013). The FIC index was expressed as the sum of $FIC_{(i)}$ and $FIC_{(ii)}$ and this was used to classify the interaction as either synergistic (≤0.50), additive (0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00) (van Vuuren and Viljoen, 2008).

$$\mathrm{FIC(i)}\,\frac{\text{MIC of (a) in combination with (b)}}{\text{MIC of (a) independently}}$$

$$\mathrm{FIC(ii)}\,\frac{\text{MIC of (b) in combination with (a)}}{\text{MIC of (b) independently}}$$

5.3. Results

5.3.1. Bioautography assay

Figure 5.1 represents potent antibacterial activities of the extracts of the selected plants against *E. coli* and *P. aeruginosa*. *S. pinnata* was the most active plant with all the extracts active against two bacteria on the chromatograms developed in BEA, CEF and EMW. This was followed by *E. elephantina* on chromatograms developed in BEA. Meanwhile, only n-hexane, DCM and acetone extracts of *C. africana* and *D. rotundifolia* were active against these bacteria on chromatograms developed in BEA and EMW.

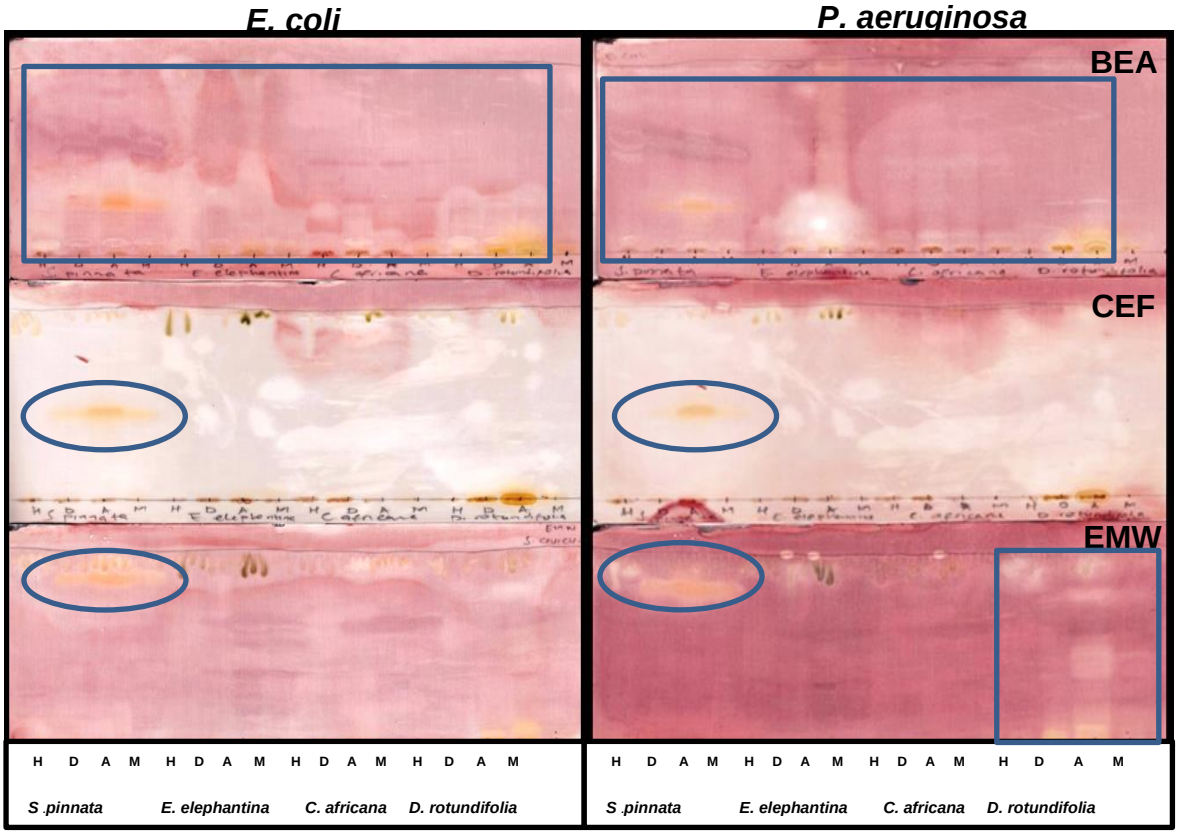

Figure 5.1: The bioautograms of different extracts of the selected plants separated in BEA, CEF and EMW. These were sprayed with overnight *E. coli* and *P. aeruginosa* cultures and visualised by spraying with 2 mg/mL INT. These represents compounds extracted with n-hexane (H), dichloromethane (D), acetone (A) and methanol (M).

The chromatograms below show antibacterial potential against *E. faecalis* and *S. aureus*. The activity against *E. faecalis* and *S. aureus* was only observed with the DCM, acetone, and methanol extracts of *S. pinnata* on chromatograms developed in BEA and CEF. Meanwhile, the activity against *S. aureus* was only demonstrated on acetone extract of *D. rotundifolia* on chromatograms developed in EMW (Figure 5.2).

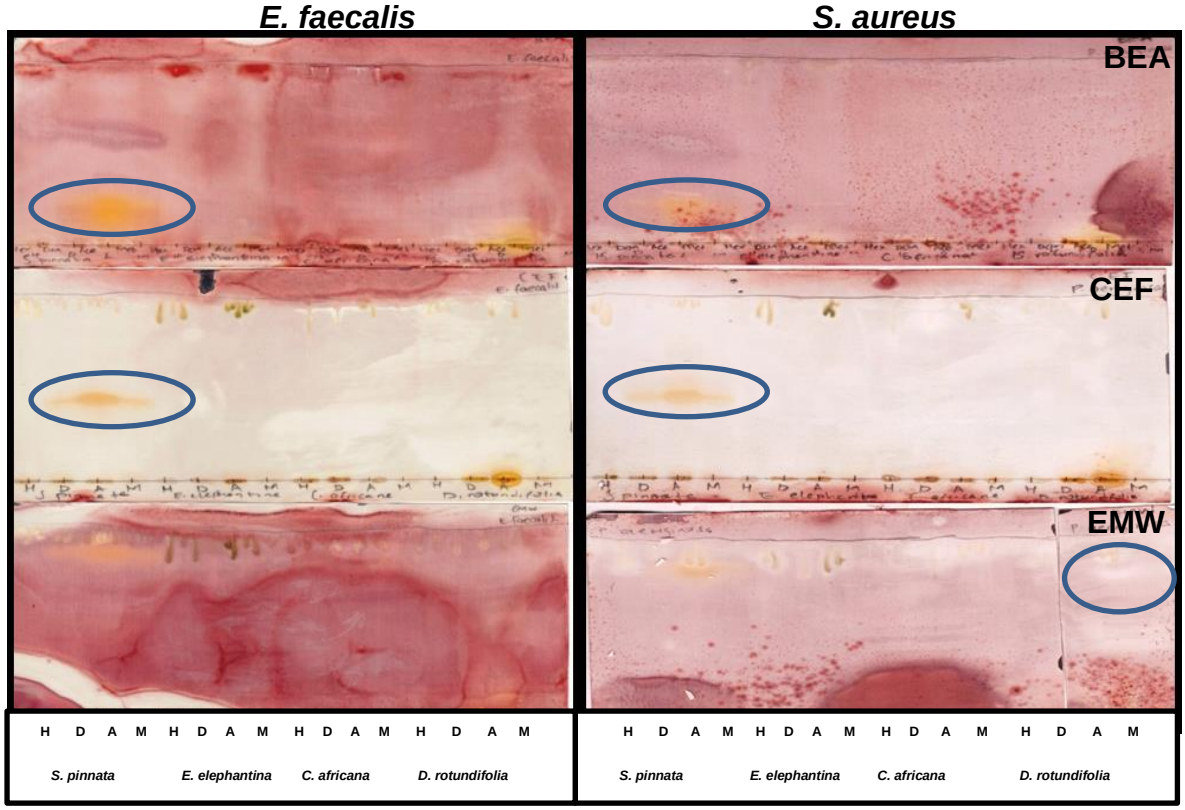

Figure 5.2: Bioautograms of different extracts of the selected plants separated in BEA, CEF and EMW. These were sprayed with overnight *E. faecalis* and *S. aureus* cultures and visualised by spraying with 2 mg/mL INT. These represents compounds extracted with n-hexane (H), dichloromethane (D), acetone (A) and methanol (M).

5.3.2. Broth Micro-dilution assay

The minimum inhibitory concentrations (MIC) and the total activities of the selected plant extracts against the tested bacterial species are presented in Table 5.1 and 5.2, respectively. Acetone extract of *C. africana* had the lowest MIC value (0.02 mg/mL) against *E. coli*. *E. coli* was the most susceptible bacteria with the lowest average MIC value of 0.87 mg/mL, while *E. faecalis* was the least with the highest average MIC value of 2.07 mg/mL (Table 5.1). The total activity was obtained by

dividing the mass extracted from one gram of the plant material with the MIC values. This measures the amount to which the quantity extracted from one gram of the plant material could be diluted to and still inhibit the growth of the test microorganism. The total activity values revealed that acetone extract of *C. africana* was the most active against *E. coli* as its antibacterial contents could be diluted to 600 mL/g and still inhibit the growth of *E. coli* (Table 5.2). The best overall total activity was observed with the DCM extract of *S. pinnata* against all the tested bacteria with an overall average of 242 mL/g.

Table 5.1: The MIC values of the selected plant extracts (mg/mL) against the test organisms.

Microorganisms	*C. africana*				*D. rotundifolia*				*E. elephantina*				*S. pinnata*				Avg	Amp (mg/mL)
	H	D	A	M	H	D	A	M	H	D	A	M	H	D	A	M		
E. coli	2.5	1.25	0.02	2.5	1.25	0.32	0.32	0.32	1.25	0.64	0.64	0.64	1.25	0.32	0.32	0.32	**0.87**	0.03
P. aeruginosa	2.5	2.5	2.5	2.5	1.25	1.25	1.25	2.5	2.5	2.5	2.5	2.5	2.5	0.64	0.64	0.64	**1.92**	0.02
E. faecalis	2.5	2.5	2.5	2.5	2.5	1.25	1.25	2.5	2.5	2.5	2.5	2.5	2.5	0.64	1.25	1.25	**2.07**	0.03
S. aureus	2.5	2.5	2.5	2.5	1.25	1.25	1.25	1.25	0.64	2.5	2.5	2.5	2.5	1.25	1.25	2.5	**1.92**	0.08
Average	**2.5**	**2.2**	**1.9**	**2.5**	**1.56**	**1.02**	**1.02**	**1.64**	**1.72**	**2.0**	**2.0**	**2.0**	**2.2**	**0.71**	**0.87**	**1.18**		

Key words: H= n-hexane; D= dichloromethane; A= acetone; M= methanol; Avg= average; Amp= ampicillin.

Table 5.2: The total activities of the selected plant extracts (mL/g) after 24 hours incubation.

Microorganisms	*C. africana*				*D. rotundifolia*				*E. elephantina*				*S. pinnata*				Average
	H	D	A	M	H	D	A	M	H	D	A	M	H	D	A	M	
E. coli	1.6	12	600	6.8	40	200	234	456	97	150	169	278	26	428	272	466	**215**
P. aeruginosa	1.6	6	4.8	6.8	40	51	60	58	48	38	43	71	13	214	136	233	**64**
E. faecalis	1.6	6	4.8	6.8	20	51	60	58	48	38	43	71	13	214	70	119	**52**
S. aureus	1.6	6	4.8	6.8	40	51	60	117	189	38	43	71	13	110	70	119	**59**
Average	**1.6**	**8**	**154**	**6.8**	**35**	**88**	**104**	**172**	**96**	**66**	**75**	**123**	**16**	**242**	**137**	**234**	

Key words: H= n-hexane; D= dichloromethane; A= acetone; M= methanol.

5.3.3. Antibacterial interaction effect studies of the selected plants

The acetone extracts of the selected plants had potent antibacterial activities against the tested bacteria (**Section 5.3.2**); as such, they were further studied in combination for any antibacterial interaction effects using the broth micro-dilution assay. The Minimum inhibitory concentration (MIC) values of acetone extracts of the selected plants individually and in combination are presented in Table 5.3, while the fractional inhibitory concentration (FIC) values are presented in Table 5.4. *C. africana* had lowest MIC value of 0.03 mg/mL against *E. coli*. Out of all the combinations, the A+B+C and A+B+C+D combination had potent antibacterial activity against all the tested microorganisms with equal average MIC values of 0.28 mg/mL. Overall the Gram-negative bacteria were more sensitive to the *S. pinnata*, *C. africana*, and *D. rotundifolia* (A+B+C) plant combination (Table 5.3). The FIC values were calculated as outlined in **section 5.2.3.2** for the 1:1 combinations to establish any synergistic or antagonistic interactions between the plants using the MIC values of the plants independently and in combinations. The sum of two FIC values for each combination was used to determine the fractional inhibitory index which aided in determining the interaction as either synergistic (≤0.50), additive (0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00) effect of the combinations. Synergistic effects were only exhibited against *P. aeruginosa* with 0.22, 0.24, and 0.19 FIC index values for the A+B, C+A and C+D combinations, respectively. Meanwhile, the antagonistic effects were only observed against *E. coli* with 5.52 and 4.58 FIC index values for combinations A+B and B+C respectively (Table 5.4).

Table 5.3: MIC (mg/mL) values of the acetone extracts of the selected plants independently and in combination.

Microorganisms	Plant combinations													Avg	Amp (mg/mL)
	A	A+B	A+B+C	A+B+C+D	B	B+C	B+C+D	C	C+A	C+A+D	D	D+C	D+A+B		
E. coli	0.84	0.16	0.09	0.13	0.03	0.13	0.13	0.52	0.73	0.52	1.04	0.63	0.16	**0.42**	0.03
P. aeruginosa	0.27	0.04	0.06	0.07	0.53	0.67	0.67	0.42	0.04	0.07	0.41	0.04	0.67	**0.27**	0.02
E. faecalis	1.67	0.63	0.63	0.63	1.67	0.63	0.63	1.25	1.25	1.04	1.67	1.67	0.63	**1.08**	0.03
S. aureus	1.67	0.53	0.32	0.27	0.84	0.32	0.21	0.63	0.84	0.84	0.84	0.84	0.27	**0.65**	0.08
Average	**1.11**	**0.34**	**0.28**	**0.28**	**0.87**	**0.44**	**0.41**	**0.61**	**0.72**	**0.62**	**0.99**	**0.80**	**0.43**		

Key words: A= *Schkuhria pinnata*; B= *Commelina africana*; C= *Dombeya rotundifolia*; D= *Elephantorrhiza elephantina;* Amp= ampicillin; Avg= Average.

Table 5.4: Fractional inhibitory concentration indexes of the 1:1 combinations of the selected plants

Microorganisms	Plant combinations				Average
	A+B	B+C	C+A	C+D	
E. coli	**5.52**	**4.58**	2.27	1.82	**3.55**
P. aeruginosa	**0.22**	2.86	**0.24**	**0.19**	**0.88**
E. faecalis	0.75	0.88	1.75	2.34	**1.43**
S. aureus	0.95	0.89	1.84	2.33	**1.50**
Average	**1.86**	**2.30**	**1.53**	**1.67**	

Key words: A= *Schkuhria pinnata*; B= *Commelina africana*; C= *Dombeya rotundifolia*; D= *Elephantorrhiza elephantina*.

5.4. Discussion

Screening of antibacterial potential of the extracts employed both qualitative and quantitative methods, using both Gram-negative and Gram-positive bacteria as test microorganisms, so as to check the spectrum of activity of the plant extracts. Direct bioautography helped in predicting the number of active components in the extracts as well as their polarity (Silva *et al*., 2005; Ncube *et al*., 2008). Figure 5.1 to 5.2 shows the bioautograms obtained after spraying the chromatograms with *S. aureus*, *E. coli*, *E. faecalis*, and *P. aeruginosa*. The white areas against the purple-pink background indicate antimicrobial activity of the extracts, where the tetrazolium salts were not reduced to formazan product because the plants contain compounds that inhibited microbial growth (Choma and Grzelak, 2011).

The results demonstrated that all the selected plants exhibited antibacterial activity against *P. aeruginosa* and *E. coli* on chromatograms eluted in BEA and EMW (Figure 5.1). Although not as significant as the other tested bacteria, only the DCM, acetone, and methanolic extracts of *S. pinnata* had activity against *E. faecalis* and *S. aureus* (Figure 5.2). The presence and separation of the antibacterial components in BEA (non-polar system) suggests that they could be non-polar. No significant activity was observed on the chromatograms developed in CEF, this is probably due to the toxicity of the traces of formic acid included in the CEF (Masoko and Eloff, 2005; Masoko *et al*., 2008).

The broth micro-dilution method was used as a quantitative measure to determine the lowest concentration of the plant extracts which lead to growth inhibition of the tested microorganisms, expressed as the MIC values (Table 5.1). The highest tested concentration was 2.5 mg/mL and this was considered as not significant (Ramadwa, 2011). Findings revealed the plants to have antibacterial activity against the tested bacteria with the most susceptible being *E. coli*, with an average MIC value of 0.87 mg/mL, followed by *P. aeruginosa* (1.92 mg/mL). *E. faecalis* (2.07 mg/mL*)* and *S. aureus* (1.92 mg/mL*)* were the least susceptible to the effect of the extracts.

The acetone extract of *C. africana* was the most effective with MIC value of 0.02 mg/mL, followed by the DCM against *E. coli*. The acetone and methanol extract of *D.*

rotundifolia and those of *S. pinnata* had MIC values of 0.32 mg/mL, as well as the DCM, acetone, and methanol extract of *E. elephantina* against *E*. *coli*. The MIC values for the positive control (ampicillin) were ranging from 0.02 to 0.08 mg/mL. The results suggest that the extracts of the selected plants have antibacterial compounds with broad a spectrum of activity, but are more effective in inhibiting growth of Gram-negative bacteria than Gram-positive bacteria. This is in agreement with the proposal made by Zaika. (1988) that the Gram-positive bacteria are more resistant than Gram-negative bacteria.

Eloff (2000) reported on the importance of considering the total activity of the extracts altogether with the MIC when assessing the activity of the extracts. The total activities of the selected plant extracts against the tested microorganisms are presented in Table 5.2. The highest activity was observed with the acetone extract of *C. africana* (600 mL/g) followed by the methanolic extract of *S. pinnata* (466 mL/g) both against *E. coli* and the lowest was observed in the n-hexane extract of *C. africana* (1.6 mL/g) against all the test organisms. This means that the quantity of the acetone extract of *C. africana* can be diluted to 600 mL/g and still inhibit the growth of *E. coli*. The antibacterial potential of the selected plants observed may be due to the presence of tannins, flavonoids, and terpenoids which were reported to have various pharmacological activities including antibacterial activity (Dilika *et al*., 1997).

Based on screening, acetone extracts of the selected plants were studied in combinations in order to assay their interactive effects against the test bacteria. The MIC values of the plants independently and in combination are presented in Table 5.3. When the plants were combined in a 1:1:1 and 1:1:1:1 ratios, the antibacterial efficacy were either enhanced (lower MIC), had no effect (equal MIC) or reduced (higher MIC) than when the plants were studied independently (van Vuuren and Viljoen, 2008). When *S. pinnata* was combined with *C. africana* and *D. rotundifolia* (combination A+B+C) the efficacy was enhanced against all the tested microorganisms with MIC values lower than the plants independently except for *C. africana* against *E. coli*. Combination with *C. africana*, *D. rotundifolia* and *E. elephantina* (B+C+D) enhanced the efficacy against all the tested microorganisms except *P. aeruginosa*, whose efficacy was reduced (0.67 mg/mL). No potent activity was observed against *S. aureus* (0.84 mg/mL) with the *D. rotundifolia*, *S. pinnata*,

and *E. elephantina* combination (C+A+D); however, the same combination enhanced the efficacy against *E. coli*, *P. aeruginosa* and *E. faecalis*. On the other hand the combination with *E. elephantina*, *S. pinnata* and *C. africana* (D+A+B) enhanced the efficacy against *E. coli*, *E. faecalis*, and *S. aureus* but reduced the one against *P. aeruginosa*. When all the selected plants were combined (combination A+B+C+D) the efficacy against all the tested bacteria was enhanced with average MIC value (0.28 mg/mL) which is lower than the average MIC values of the plants independently. Overall potent antibacterial activity was observed against *P. aeruginosa* with combination A+B+C (0.06 mg/mL) and C+A+D (0.07 mg/mL).

Fractional inhibitory concentration values aided the classification of the 1:1 combinations as synergistic (≤0.50), additive (0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00). Synergistic effects were only demonstrated against *P. aeruginosa* by the A+B (0.22), C+A (0.24) and C+D (0.19) combinations. Antagonistic effects were only demonstrated against *E. coli* with combination A+B and B+C. The same combinations had additive effects against *E. faecalis* and *S. aureus*, while A+B had indifferent effect against *P. aeruginosa*. On the other hand, combination C+A and C+D had indifferent effect against *E. coli*, *E. faecalis*, and *S. aureus*.

The results obtained in this study are consistent with previous studies on the antibacterial potential of the selected plants against the test microorganisms. (Hedberg and Staugård, 1989; Aaku *et al*., 1998; Reid *et al*., 2001; Pretorius *et al*., 2003; Mathabe *et al*., 2006; Mupfure *et al*., 2014). The MIC for *E. elephantina* methanolic and acetone extracts against *S. aureus* were 0.16 mg/mL and 0.32 mg/mL, respectively. However, *E. coli* was reported to be resistant to both extracts (Mathabe *et al*., 2006). To the best of my knowledge nothing has been reported on the antibacterial potential of both *C. africana* and *S. pinnata* against the tested microorganisms used in this study using the bioautography and broth-micro-dilution method.

5.5. Conclusion

Bioautography demonstrated that antibacterial compounds in *S. pinnata* extracts are non-polar. This study reports for the first time the antibacterial activity of *C. africana*

and *S. pinnata* against the tested organisms using the bioautography and broth micro-dilution methods. The present study demonstrated that the extracts of the selected plants have antibacterial compounds with broad spectrum activity and are more effective in inhibiting growth of Gram-negative bacteria than Gram-positive bacteria.

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Chapter 6: Bioactivity guided isolation of antibacterial compounds

6.1. Introduction

Herbal mixtures consist of a wide range of bioactive compounds with a wide range of biological activities and these mixtures are used for treatment of many diseases (Hostettman *et al*., 1997). Nevertheless, most of the plants are used based on instructions and observations from traditional doctors without any scientific validation and dosage evaluation (Kumar *et al*., 2007). Therefore, these medicinal plants should be explored as sources of new lead with compounds with potential incorporation in therapeutic development (Hostettman *et al*., 1997). In order to understand the efficacy of plant constituents in treatment and prevention of diseases as well as the safe dosages, the bioactive compounds needs to be extracted and isolated (Hostettman *et al*., 1997).

Isolation of bioactive compounds employs a process that integrates separation of compounds in a mixture with their *in vitro* biological activity; this is known as bioassay guided fractionation. This process begins with the screening of different crude extracts for any biological activity; followed by separation of the active compounds within the crude extracts and testing the fractions for biological activity. The fractions with activity are further separated and tested for biological activity until a pure compound (s) is obtained (Jamil *et al*., 2012). The separation usually involves the use of column chromatography, where the components from crude extracts are separated into fractions based on the polarities, using one or two solvents (Bucar *et al*., 2013). Thin layer chromatography is often used to confirm the purity of the isolated compounds (Sasidharan *et al.,* 2011). The aim of this chapter was to isolate bioactive compounds using bioassay guided fractionations.

6.2. Methods and materials

6.2.1. Serial exhaustive extraction

Serial exhaustive extraction was used to extract bioactive compounds from the *Schkuhria pinnata* plant material since it had promising antibacterial activity. About 1.2 kg of the plant material was weighed and dissolved into 6 litres of n-hexane in a

bottle. The bottle was vigorously shaken for a day at 200 rpm. The supernatant was filtered, concentrated using rotary evaporator at 50 °C, and transferred into pre-weighed labelled beakers. The same process was repeated three times. The same plant residues were extracted as mentioned above in the following order with 6 litres of dichloromethane, ethyl acetate, acetone, and methanol. Solvents were removed under a stream of cold air at room temperature and the masses of the crude extracts were determined.

6.2.2. Phytochemical analysis

The chemical profiles of the *S. pinnata* extracts were analysed on aluminium backed TLC plates (Merck, silica gel 60 F_{254}) using a method developed by Kotze and Eloff, (2002) as described in **section 3.2.3**.

6.2.3. TLC-DPPH assay

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

6.2.4. Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972) as described in **section 5.2.2.1** and the bacterial species were maintained as described in **section 5.2.1**.

6.2.5. Broth micro-dilution assay

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

6.2.6. Isolation of antibacterial and antioxidant compounds

6.2.6.1. Open column chromatography

Column chromatography was used for isolation, separation, and purification of the active compounds with antioxidant and antibacterial activities from the dichloromethane crude extracts. An open column (35 × 4 cm) was packed with silica gel 60 (particles size 0.063 - 0.200 mm) (Fluka) using 100% n-hexane. The samples were mixed with small amounts of silica gel and subjected to column chromatography. The constituents of the extracts were eluted through an open column using solvents in Table 6.1 with increasing polarity from non-polar (n-hexane) to polar (methanol). The fractions were collected and the solvents were removed under a stream of cold air at room temperature and the masses of the crude extracts were determined. Furthermore, the fractions were tested for antioxidant and antibacterial activity using TLC-DPPH (**Section 6.2.3**) and bioautography (**Section 6.2.4**) and broth micro-dilution (**Section 6.2.5**).

Table 6.1: Different percentages of the different solvents used for elution of compounds from DCM extract of *S. pinnata* from an open column chromatography.

Elution solvent	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

6.2.6.2. Second open column chromatography

Biological activities of the fractions obtained from the first column (**Section 6.3.1**) revealed that the n-hexane: ethyl acetate (10:90) fraction had potent antibacterial activities. Therefore, it was chosen for further separation and purification of the bioactive compounds. The target fraction was subjected to an open column chromatography (39 x 4 cm) packed with silica gel 60 for further fractionation. The column was eluted with 100% ethyl acetate. The eluents were collected in small test

tubes and placed under a stream of air to concentrate. The fractions were analysed on the thin layer chromatography plates and those with similar profiles were combined. The concentrated fractions were further tested for antioxidant activity using TLC-DPPH (**Section 6.2.3**) and bioautography (**Section 6.2.4**) and broth micro-dilution (**Section 6.2.5**).

6.2.6.3. Third column chromatography

The fractions from above **section 6.3.2** with active compounds were combined to make one target fraction. The target fraction was subjected in a small open column (38.5 x 5 cm) packed with silica gel 60 for further fractionation. The antibacterial compound was eluted from the column with ethyl acetate: acetone (70:30). The eluents were collected in small test tubes and placed under a stream of air to concentrate the fractions for further analysis on the thin layer chromatography plates.

6.3. Results

6.3.1. Serial exhaustive extraction

Finely ground *S. pinnata* plant material was serially extracted three times with each of the following solvents from non-polar to polar (n-hexane, dichloromethane, ethyl acetate, acetone, and methanol). The total mass of 229.72 g was obtained from 1.2 kg of the plant material (Table 6.2). Most of the plant material was extracted with dichloromethane (102.88 g) while the least was extracted with acetone (4.14 g).

Table 6.2: The masses obtained after serial exhaustive extraction of 1.2 kg of *S. pinnata* with different solvents of varying polarity.

Extractants		Mass residue extracted (g)	
		Mass	Total
n-Hexane	I	25.88	37.86
	II	8.98	
	III	3.0	
Dichloromethane	I	73.37	102.88
	II	21.72	
	III	7.79	
Ethyl acetate	I	3.49	6.32
	II	1.73	
	III	1.10	
Acetone	I	2.20	4.14
	II	1.17	
	III	0.77	
Methanol	I	45.25	78.52
	II	20.19	
	III	13.08	
Total			**229.72**

6.3.2. Phytochemical analysis

The phytochemical profile of the n-hexane, dichloromethane, ethyl acetate, acetone, and methanol extracts of *S. pinnata* was analysed using thin layer chromatography. The extracts were separated in BEA, CEF, and EMW solvent systems then sprayed with vanillin-sulphuric acid reagent for visualisation of the compounds (Figure 6.1). Different colours on the TLC plate represent different compounds. The compounds were better resolved in CEF and EMW mobile phase.

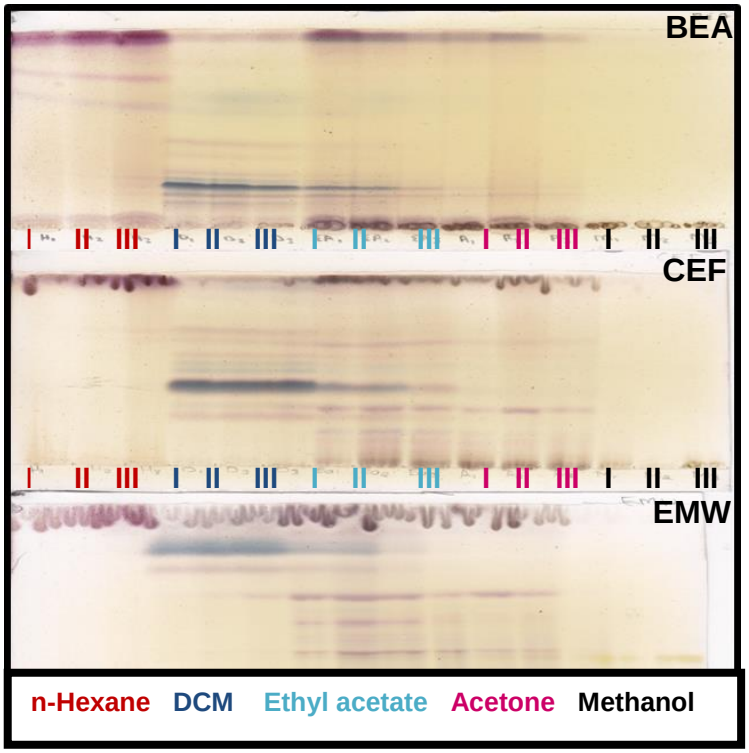

Figure 6.1: Chromatograms obtained following development of the *S. pinnata* extracts in BEA, CEF, and EMW then spraying with vanillin-sulphuric acid reagent for visualisation.

6.3.3. Antioxidant activity

The n-hexane, dichloromethane, ethyl acetate, acetone, and methanol extracts of *S. pinnata* separated in three solvent systems and were sprayed with 0.2% of DPPH in methanol as an indicator to detect free radical scavenging activities. The observed yellow spots against the purple background indicated antioxidant activity. Although not separated, ethyl acetate, acetone, and methanol extracts showed free radical scavenging activity in BEA (Figure 6.2).

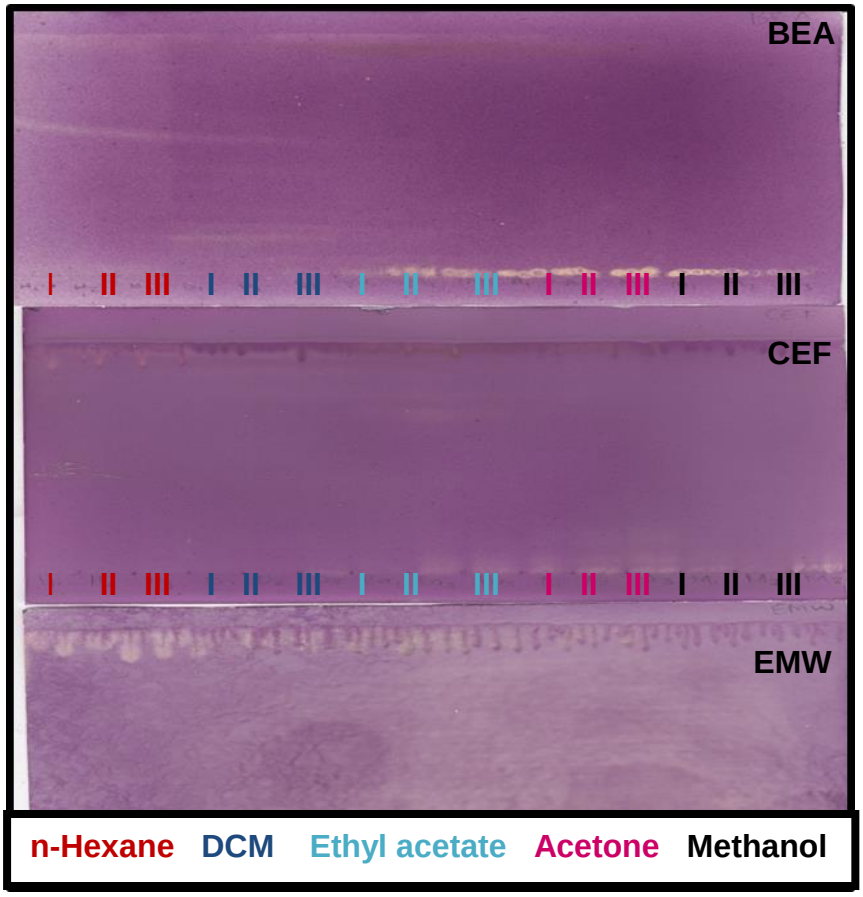

Figure 6.2: Chromatograms of *S. pinnata* n-hexane, DCM, ethyl acetate, acetone, and methanol extracts developed in BEA, CEF, and EMW. These were sprayed with 0.2% DPPH for visualisation of the antioxidant-active compounds.

6.3.4. Bioautography

Bioautography was used to evaluate the antibacterial activities of the *S. pinnata* extracts. The crude extracts were separated in BEA, CEF, and EMW and sprayed with *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. The white zones against pink background on the chromatograms below indicate antibacterial activity (Figure 6.3 and 6.4). The n-hexane, dichloromethane and ethyl acetate extracts had potent activity against all the tested bacteria on bioautograms developed in BEA and CEF.

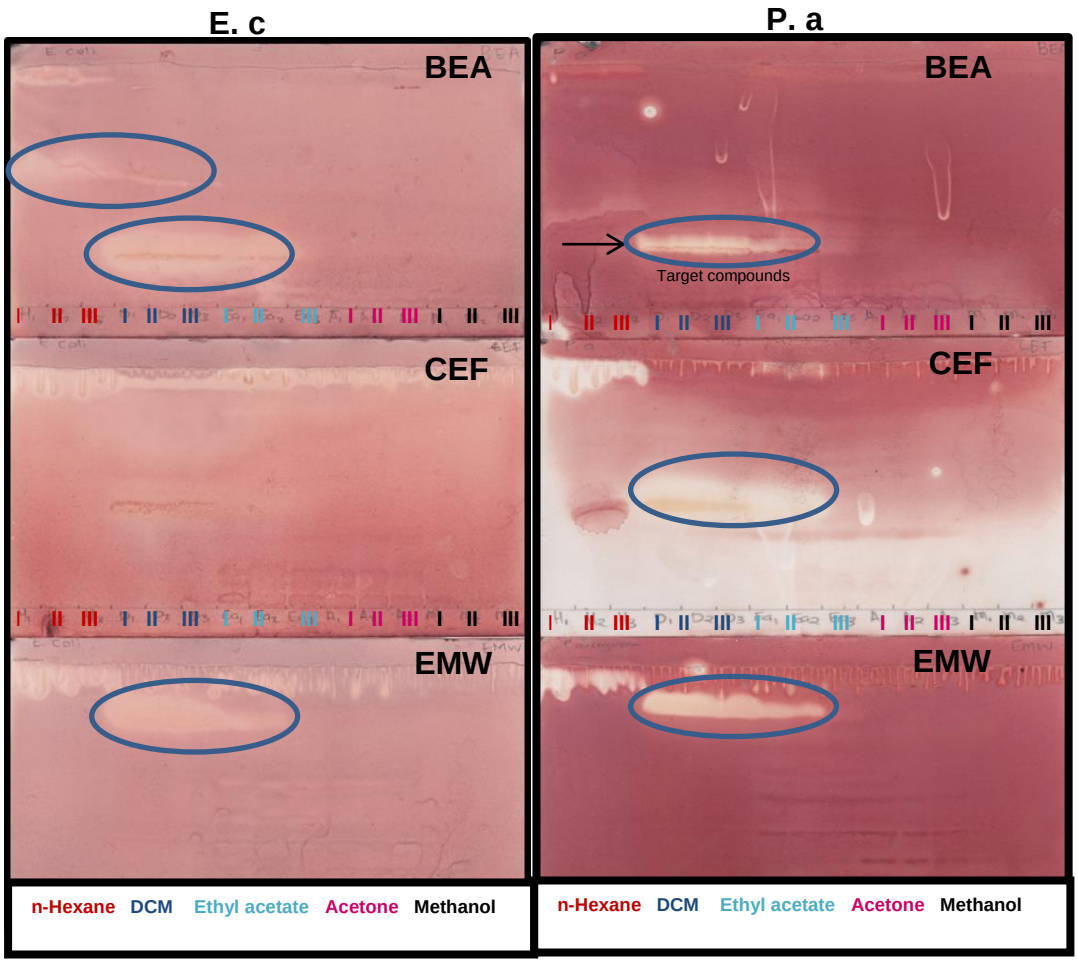

Figure 6.3: The bioautograms of different *S. pinnata* extracts obtained after separation BEA, CEF and EMW and sprayed with overnight *E. coli* (E. c) and *P. aeruginosa* (P. a) cultures and visualised by spraying with 2 mg/mL INT.

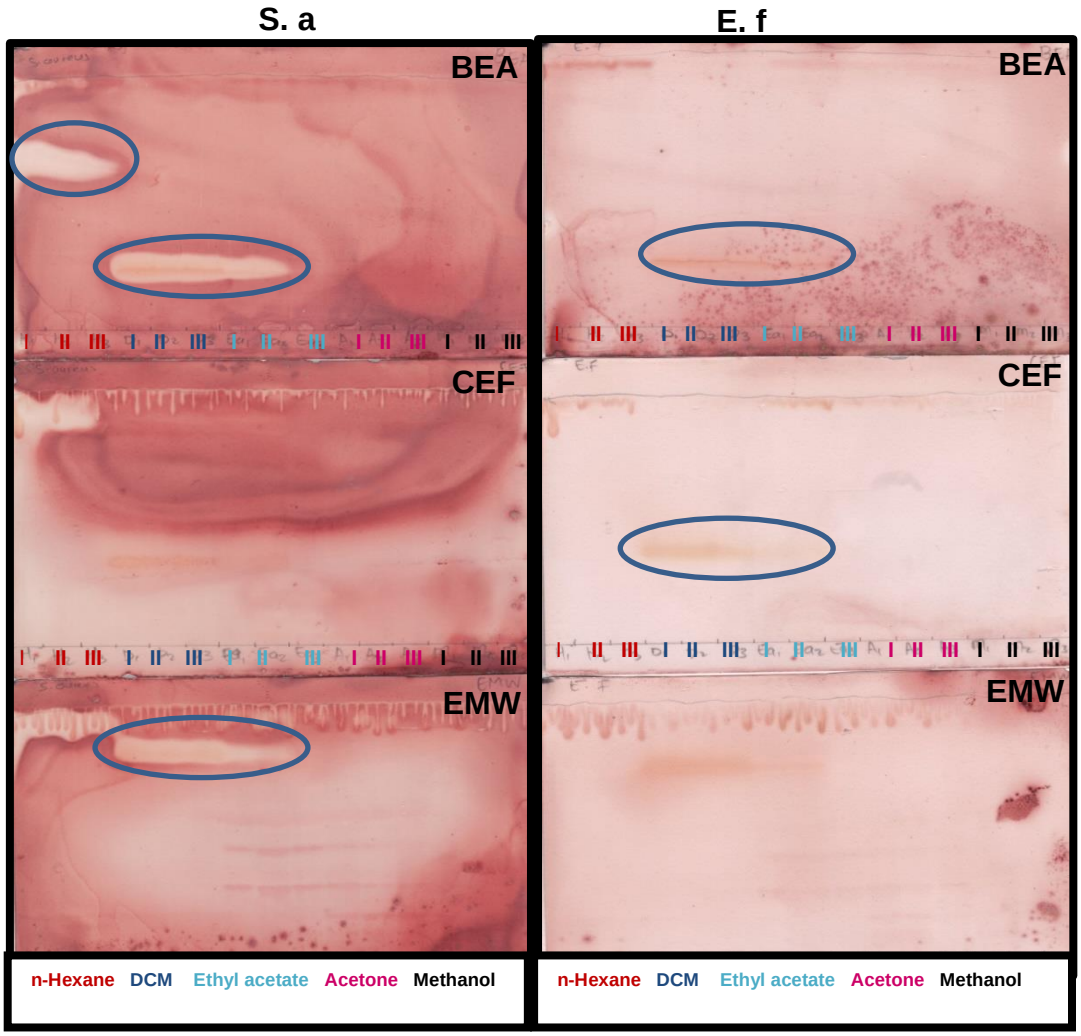

Figure 6.4: The bioautograms of different *S. pinnata* extracts obtained after separation in BEA, CEF and EMW and sprayed with overnight *E. faecalis* (E. f) and *S. aureus* (S. a) cultures and visualised by spraying with 2 mg/mL INT.

6.3.5. Broth micro-dilution assay

The quantitative antibacterial activity was evaluated using the broth micro-dilution assay to determine the minimum inhibitory concentrations of the *S. pinnata* extracts against the four tested bacteria. Ampicillin was used as a positive control and the MIC values ranged from 0.02 to 0.08 mg/mL. *E. faecalis* was the most susceptible bacteria with an average MIC value of 0.27 mg/mL, followed by *P. aeruginosa* (0.54 mg/mL), *E. coli* (0.70 mg/mL), and *S. aureus* (0.84 mg/mL). Dichloromethane extracts had best overall activity with the lowest average MIC value of 0.24 mg/mL against all the tested bacteria, followed by ethyl acetate (0.42 mg/mL), acetone (0.49 mg/mL), n-hexane (0.54 mg/mL), and methanol (1.25 mg/mL) (Table 6.3).

Table 6.3: MIC (mg/mL) values of the serial exhaustive extraction extracts of *S. pinnata*.

Microorganisms	Extractants															Avg	Amp (mg/mL)
	n-Hexane			Dichloromethane			Ethyl acetate			Acetone			Methanol				
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III		
E. coli	1.05	0.84	0.74	0.19	0.35	0.19	0.37	0.43	1.67	0.43	0.43	0.84	0.53	1.25	1.25	**0.70**	0.03
P. aeruginosa	0.32	0.32	0.08	0.16	0.08	0.08	0.32	0.32	0.64	0.16	0.16	0.16	0.32	2.5	2.5	**0.54**	0.02
E. faecalis	0.08	0.16	0.16	0.04	0.16	0.04	0.08	0.08	0.16	0.08	0.32	0.16	0.64	0.64	1.25	**0.27**	0.03
S. aureus	1.05	0.94	0.74	0.64	0.53	0.43	0.32	0.37	0.32	1.25	1.25	0.64	1.57	1.3	1.25	**0.84**	0.08
Average	**0.63**	**0.57**	**0.43**	**0.26**	**0.28**	**0.19**	**0.27**	**0.30**	**0.70**	**0.48**	**0.54**	**0.45**	**0.77**	**1.42**	**1.56**		
Total average		**0.54**			**0.24**			**0.42**			**0.49**			**1.25**			

Key; Amp= ampicillin; Avg= average

6.3.6. Isolation of antibacterial compounds from dichloromethane extracts

6.3.6.1. First open column chromatography

Dichloromethane extracts of *S. pinnata* exhibited overall potent antibacterial activity against all the tested bacteria, as such, it was chosen for isolation of antibacterial compounds. The three extracts were combined to give a total mass of 102.88 g which was subjected to column chromatography. Column chromatographic separation of the dichloromethane extracts used the different percentages of the solvents listed in Table 6.4 as eluents and the masses of the fractions collected are also listed. A total of 100.41 g was collected. The highest mass was eluted with 10% n-hexane in ethyl acetate (60.75 g), while the least was eluted with 40% ethyl acetate in methanol (0.25 g) (Table 6.4).

Table 6.4: The mass (g) of fractions collected from column chromatographic separation of *S. pinnata* dichloromethane extracts using different solvents.

Elution solvent	Percentages (%)	Mass (g)
n-Hexane	100%	0.60
n-Hexane: Ethyl acetate	90:10	1.16
	80:20	2.66
	70:30	3.05
	50:50	3.99
	30:70	9.27
	10:90	60.75
Ethyl acetate	100	6.93
Ethyl acetate: Methanol	90:10	8.78
	80:20	1.36
	70:30	0.67
	60:40	0.33
	50:50	0.31
	40:60	0.25
Methanol	100	0.30
Total		**100.41**

6.3.6.1.1. Phytochemical analysis of 1st column chromatography fractions

Following the column chromatographic separation of the DCM extracts; the fractions collected were analysed on TLC for the phytochemicals. This involved separation and development of the plates in BEA, CEF and EMW and visualisation of the compounds under ultraviolet light (Figure 6.5). The number of compounds observed decreased with increasing polarities of the eluent system.

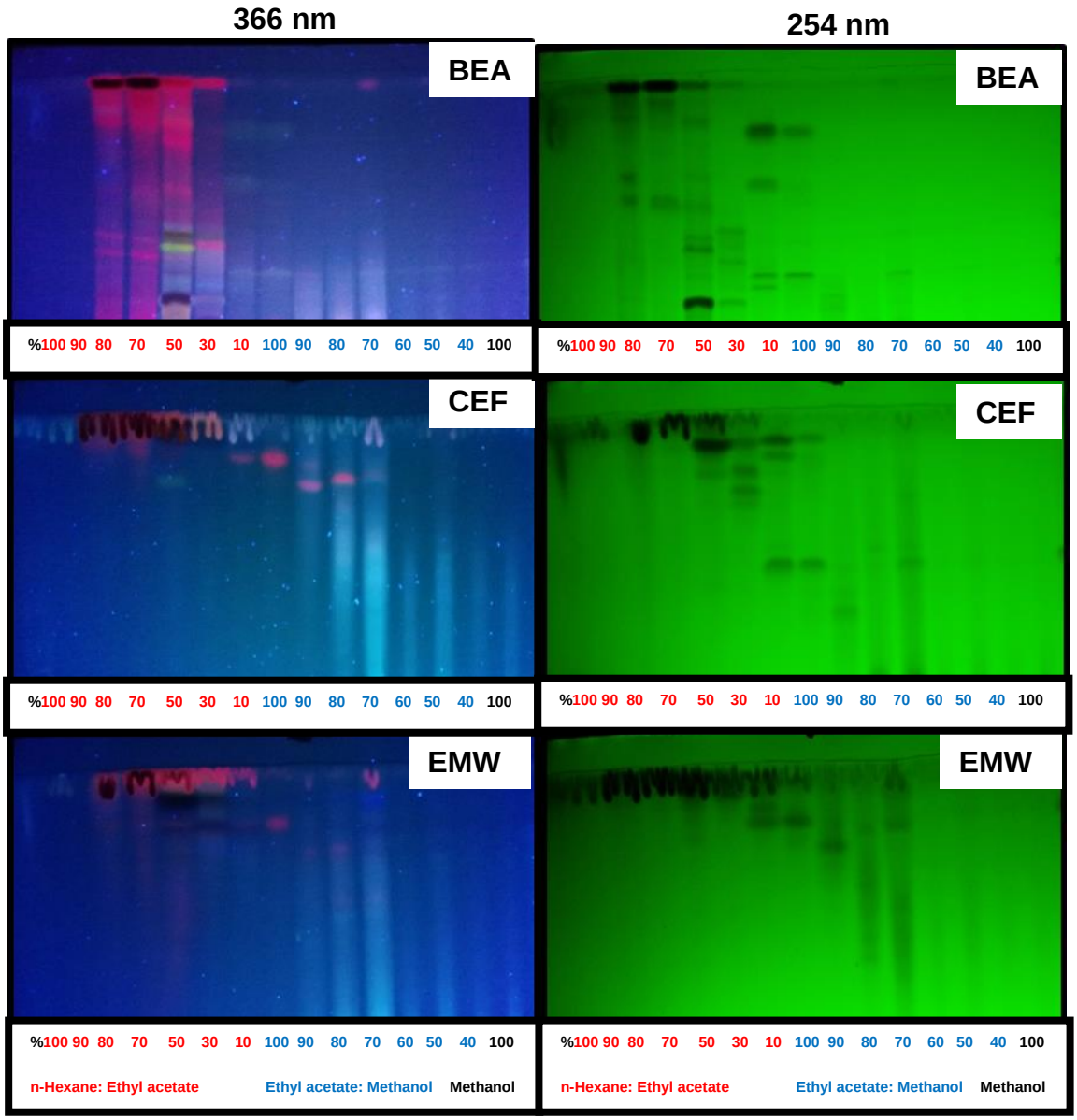

Figure 6.5: The phytochemical fingerprint of the dichloromethane fractions separated in BEA, CEF, and EMW and viewed under ultraviolet light at 366 and 254 nm wavelengths.

The plates developed in BEA, CEF and EMW were sprayed with vanillin-sulphuric acid for visualisation of the non-fluorescing compounds within the fractions. More compounds were observed in the plates developed in BEA than CEF and EMW (Figure 6.6).

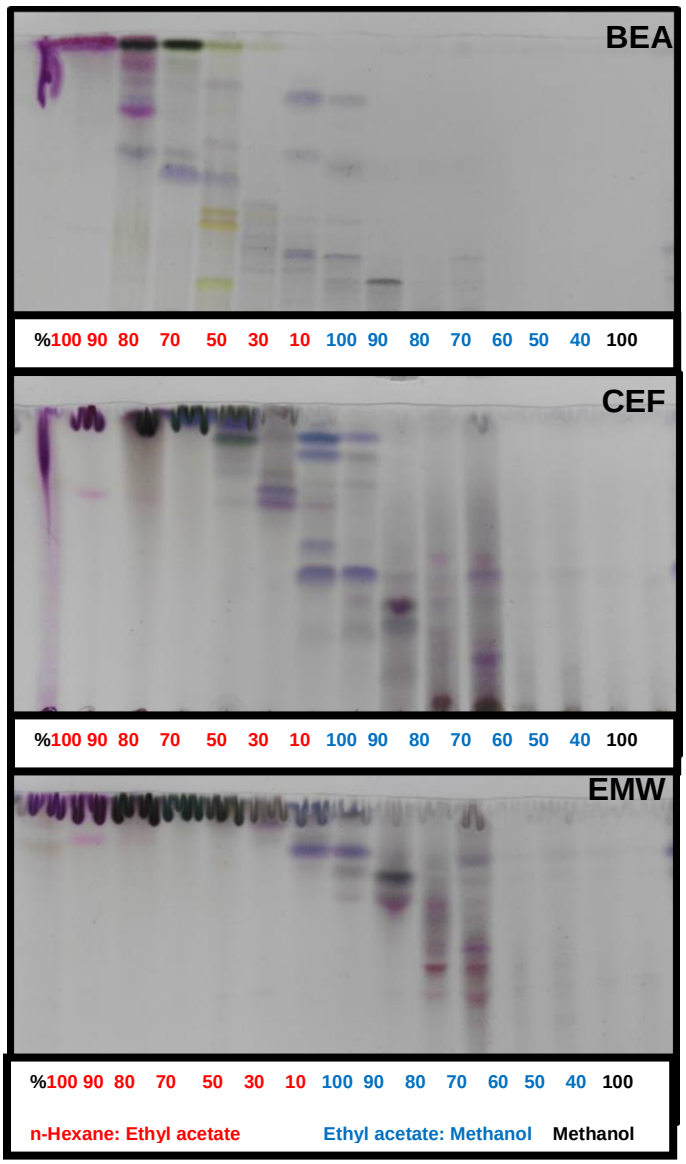

Figure 6.6: The phytochemical fingerprint of the dichloromethane fractions developed in BEA, CEF, and EMW and sprayed with vanillin-sulphuric acid reagent.

6.3.6.1.2. Bioautography for the 1st column fractions

To test for the antibacterial potential of the fractions, chromatograms developed in BEA, CEF and EMW were sprayed with Gram-negative bacteria *E. coli* and *P. aeruginosa* (Figure 6.7) and Gram-positive bacteria *E. faecalis* and *S. aureus* (Figure

6.8). The following fractions had antibacterial activity against the tested bacteria; 70% n-hexane: ethyl acetate, 50% n-hexane: ethyl acetate, 30% n-hexane: ethyl acetate, 10% n-hexane: ethyl acetate, 100% ethyl acetate, and 90% ethyl acetate: methanol.

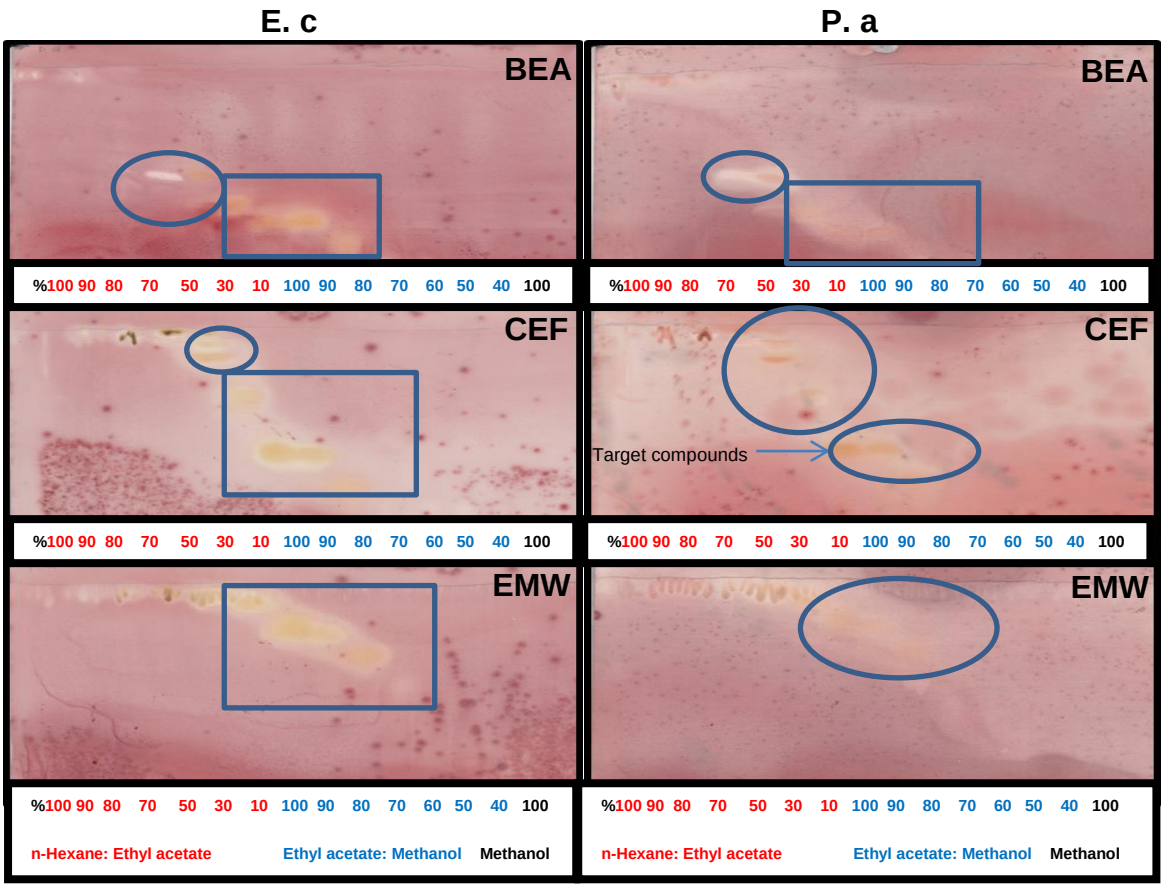

Figure 6.7: The bioautograms of different fractions from DCM extracts of *S. pinnata* obtained after separation in BEA, CEF and EMW and sprayed with overnight *E. coli* (E. c) and *P. aeruginosa* (P. a) cultures and visualised by spraying with 2 mg/mL INT.

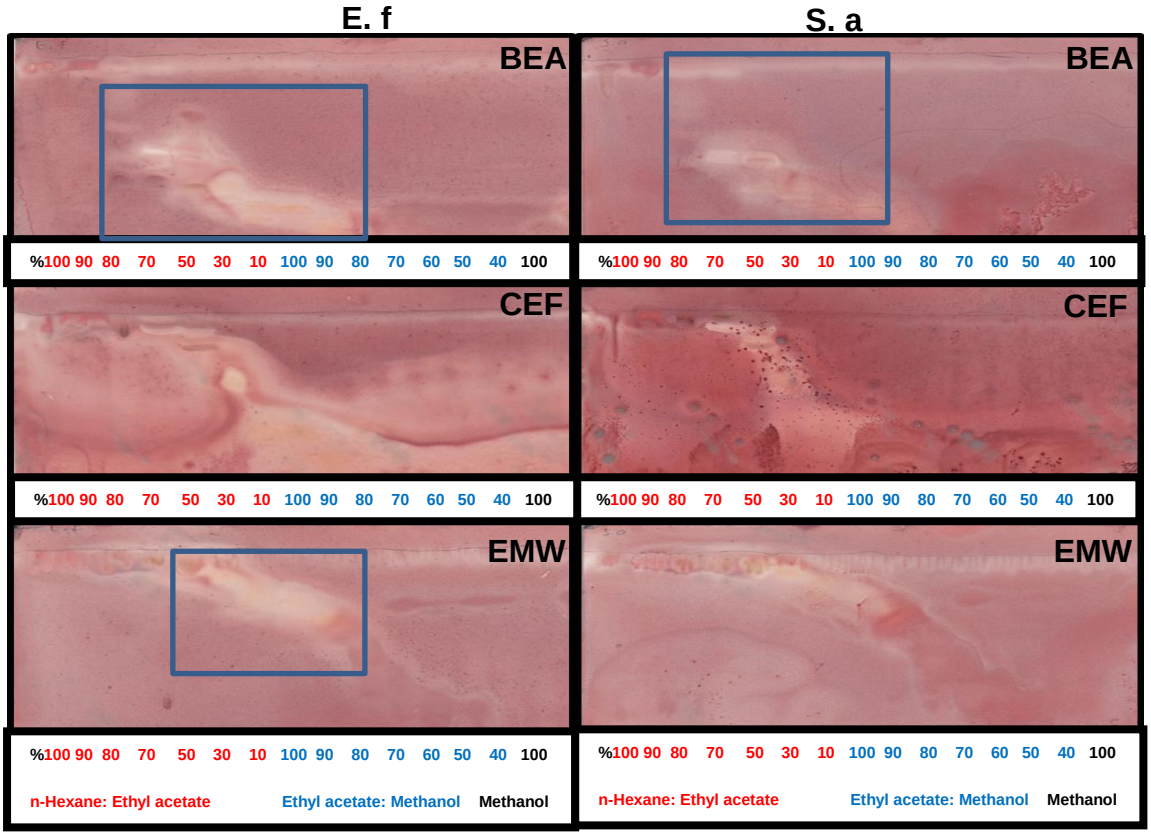

Figure 6.8: The bioautograms of different fractions from DCM extracts of *S. pinnata* obtained after separation BEA, CEF and EMW and sprayed with overnight *E. faecalis* (E. f) and *S. aureus* (S. a) cultures and visualised by spraying with 2 mg/mL INT.

6.3.6.1.3. Broth micro-dilution assay for 1st column fractions

The broth micro-dilution results for determination of minimum inhibitory concentrations of the fractions are shown in Table 6.5. Fraction 6 had the highest antibacterial activity against all the tested bacteria with the lowest average MIC (0.06 mg/mL), while fraction 14 had the lowest antibacterial activity with the highest average MIC (1.16 mg/mL).

Table 6.5: MIC values (mg/mL) of fractions from column chromatography against four tested bacteria.

Microorganisms	MIC values (mg/mL)																Amp
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Avg	(mg/mL)
E. coli	2.5	2.5	0.32	0.16	0.16	0.08	0.04	0.16	0.32	1.25	1.25	0.64	0.32	0.64	0.64	**0.73**	0.03
P. aeruginosa	0.53	1.25	1.42	0.53	0.27	0.13	0.27	0.68	0.86	0.12	0.25	0.50	0.33	1.36	1.46	**0.67**	0.02
E. faecalis	0.08	0.13	0.13	0.09	0.09	0.02	0.08	0.08	0.08	0.29	0.16	0.09	0.03	0.12	0.25	**0.11**	0.03
S. aureus	0.08	0.16	0.08	0.04	0.08	0.02	0.08	0.04	0.04	2.5	1.25	0.64	0.32	2.5	1.25	**0.61**	0.08
Average	**0.80**	**1.01**	**0.50**	**0.21**	**0.15**	**0.06**	**0.12**	**0.24**	**0.33**	**1.04**	**0.73**	**0.47**	**0.25**	**1.16**	**0.90**		

Key: Avg= average; Amp= ampicillin; 1= 100% n-hexane; 2= 90% n-hexane: ethyl acetate; 3= 80% n-hexane: ethyl acetate; 4= 70% n-hexane: ethyl acetate; 5= 50**%** n-hexane: ethyl acetate; 6= 30% n-hexane: ethyl acetate; 7= 10% n-hexane: ethyl acetate; 8= 100% ethyl acetate; 9= 90% ethyl acetate: methanol; 10= 80% ethyl acetate: methanol; 11= 70% ethyl acetate: methanol; 12= 60% ethyl acetate: methanol; 13= 50% ethyl acetate: methanol; 14= 40% ethyl acetate: methanol; 15= 100% methanol.

6.3.6.2. Second open column chromatography

Fraction 7 (10% n-hexane: ethyl acetate) had potent antibacterial activities in both the bioautography and broth micro-dilution dilution assays; as such, it was chosen for further separation of the antibacterial compounds. The sub-fractions from the second column chromatography were eluted with 100% ethyl acetate and collected in tests tubes. Following evaporation of solvents, the sub-fractions were spotted on TLC and sprayed with vanillin sulphuric acid to check their profiles. The purplish color represents the compounds eluted from column chromatographic separation of fraction 7 (10% n-hexane: ethyl acetate) (Figure 6.9).

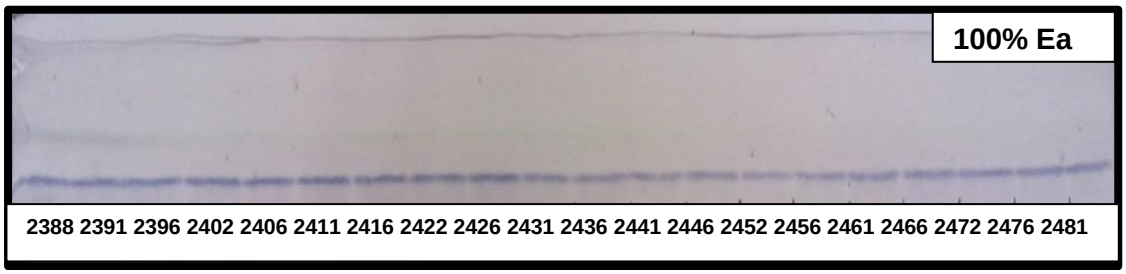

Figure 6.9: Fractions from open column chromatography for separation of antibacterial compounds. The compounds were developed in BEA and prayed with vanillin-sulphuric acid reagent. Ea (ethyl acetate).

The sub-fractions with similar profiles from the second column chromatography (loaded with 10% n-hexane: 90% ethyl acetate) were combined to give ten fractions. These were labelled 1-10 and spotted on TLC and sprayed with vanillin-sulphuric acid reagent. Fraction 2 and 6 had similar profiles (purple colour), while fraction 3, 4, 5, 7, 8 and 10 had a bluish compound (Figure 6. 10).

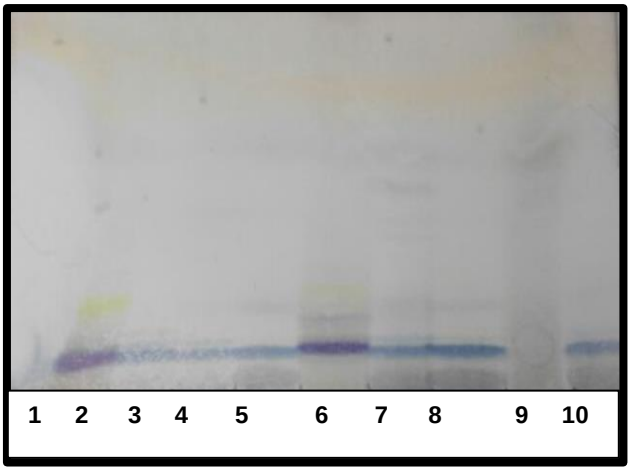

Figure 6.10: The combined fractions from second chromatography developed in BEA and sprayed with vanillin-sulphuric acid reagent.

6.3.6.2.1. Bioautography of the 2nd column combined fractions

Bioautography was performed to evaluate the antibacterial potential of the combined fractions from second column chromatography against *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*. Fraction 1, 3, 4, 7, 8 and 9 were active against all the tested bacteria, while 2, 5, 6 and 10 were not active against all of them (Figure 6.11 and 6.12).

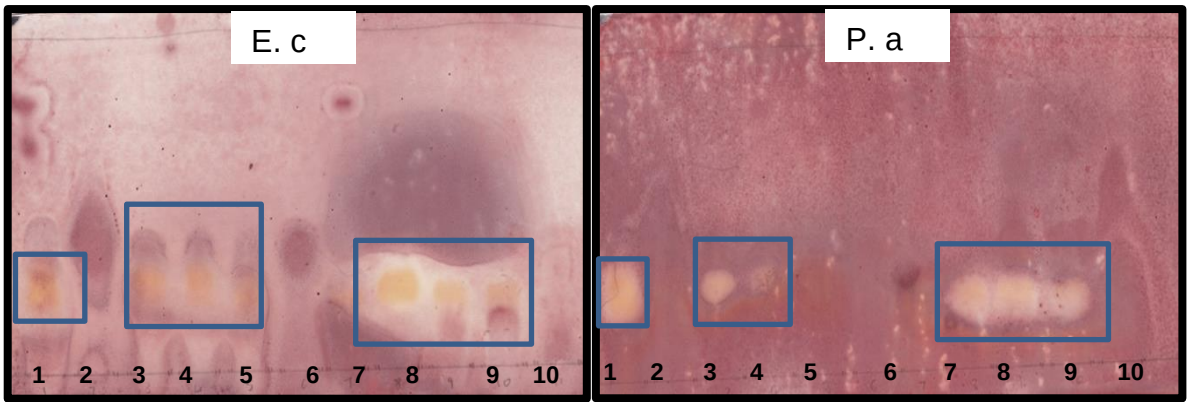

Figure 11: The bioautograms of the combined fractions from the second column chromatography obtained after separation in BEA separation system, spraying with overnight *E. coli* (E. c) and *P. aeruginosa* (P. a) cultures and visualised by spraying with 2 mg/mL INT.

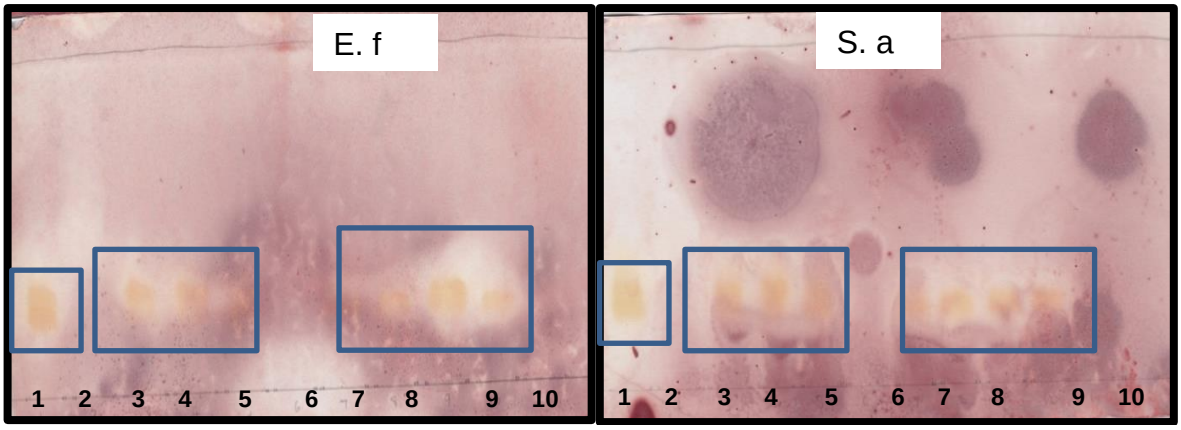

Figure 6.12: The bioautograms of the combined fractions from the second column chromatography obtained after separation in BEA separation system, spraying with overnight *E. faecalis* (E. f) and *S. aureus* (S. a) cultures and visualised by spraying with 2 mg/mL INT.

6.3.6.3. Third column chromatography

The fractions with similar profiles i.e. fraction 3, 4, 7 8 and 10 were combined to give a mass of 16.65 g which was combined with small amounts of acetone and separated on column chromatography eluted with 70% ethyl acetate in acetone. The blue compounds were obtained after spraying the chromatograms with vanillin-sulphuric acid (Figure 6.13).

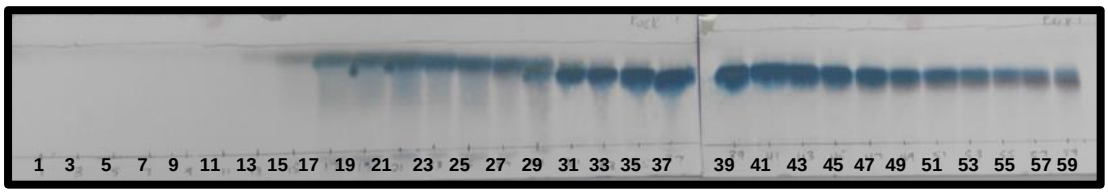

Figure 6.13: Chromatograms of fractions from the third column chromatography developed in ethyl acetate: acetone (70:30) and sprayed with vanillin-sulphuric acid.

The chart below (Figure 6.14) represents a summary of the process used for isolation of antibacterial compounds. This involved the bioassay guided fractionation of DCM extracts through a series of three open column chromatography, where some fractions with similar profiles were combined. Pure compounds were further characterised using nuclear magnetic resonance.

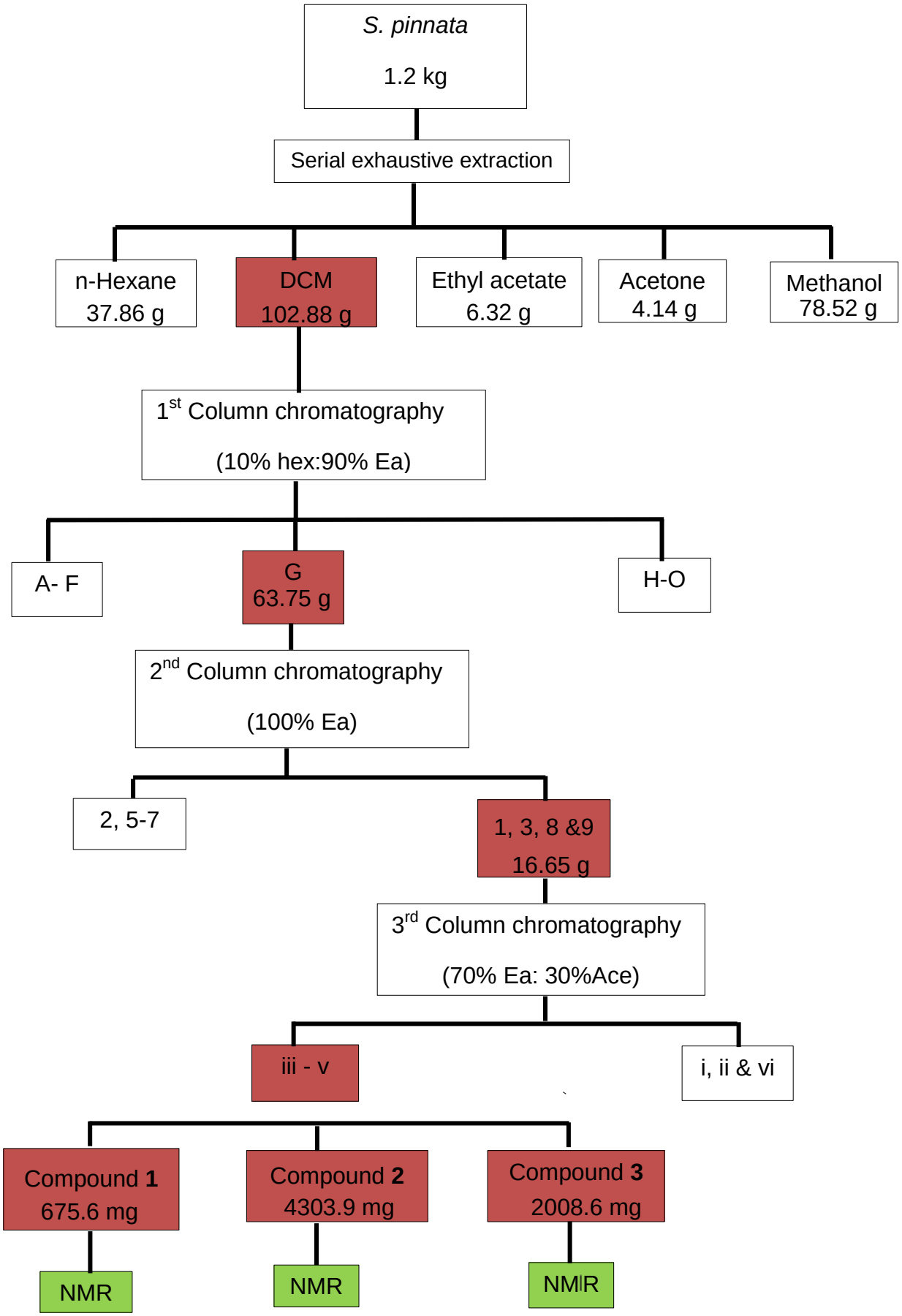

Figure 6.14: A schematic diagram of the summary of the isolation process.

6.4. Discussion

Schkuhria pinnata was chosen for further analysis and isolation of the antibacterial compounds since it exhibited promising antibacterial potential, and is readily available in South Africa and not much has been documented about its phytochemical analysis and biological activities. Bioactivity guided fractionation aided the isolation of the antibacterial compounds from this plant and the process began with extraction, because this is the first step of isolation and purification of compounds from medicinal plants (Sasidharan *et al.,* 2011).

Serial exhaustive extraction employed the use of five solvents with increasing polarity i.e. n-hexane, dichloromethane, ethyl acetate, acetone and methanol. A total mass of 229.72 g was extracted from 1.2 kg of *S. pinnata*. The highest mass extracted with dichloromethane (102.88 g), followed by methanol (78.52 g) and n-hexane (37.86 g). The lowest was extracted with acetone (4.14 g) and ethyl acetate (6.32 g) (Table 6.2). These findings suggest that most of the compounds present in the *S. pinnata* plant material are intermediate to polar since most plant material was extracted with dichloromethane (DCM) and methanol.

The phytochemical profiles of the extracts from serial exhaustive extraction were analysed on thin layer chromatography (TLC). The compounds from *S. pinnata* extracts were better resolved in CEF and EMW than BEA (Figure 6.1); this also suggests the presence of intermediate to polar compounds. The antioxidant potential of the extracts was evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC. Although in low concentrations, antioxidant activities were demonstrated in the ethyl acetate, acetone and methanol extracts of *S. pinnata*. These were represented by the less intense yellow spots on the chromatogram developed in BEA only (Figure 6.2).

Bioautography was used to evaluate the antibacterial potential of the extracts against four test bacteria. All the DCM and some ethyl acetate extracts (I and II) inhibited the growth of all the test bacteria (Figure 6.3 and 6.4). Meanwhile, n-hexane extracts inhibited the growth of *E. coli* and *S. aureus*. The minimum concentration of the observed inhibition was determined using the broth micro-dilution assay. DCM extracts had overall potent antibacterial activity against the tested bacteria with the

lowest average MIC value (0.24 mg/mL), followed by ethyl acetate (0.42 mg/mL) and acetone (0.49 mg/mL) extracts. The lowest activity was observed with methanol (1.25 mg/mL). The order of susceptibility of the microorganisms is as follows; *E. faecalis* (0.27 mg/mL) > *P. aeruginosa* (0.54 mg/mL) > *E. coli* (0.70 mg/mL) > *S. aureus* (0.84 mg/mL). It is worth pointing out that some antibacterial activities with pharmacological relevance (MIC value less than 0.1 mg/mL) were demonstrated against *P. aeruginosa* and *E. faecalis*.

Masoko and Eloff (2007) reported on the usefulness of bioassays in localisation and identification of potential active compounds. Based on activity, the DCM extracts were chosen for isolation of the antibacterial compounds using column chromatography. The column was eluted with different combinations of n-hexane, ethyl acetate, and methanol to elute a wide range of compounds with different polarities. Table 6.4 represents the masses of the collected fractions. The highest mass was eluted with 10% n-hexane in ethyl acetate (60.75 g), followed by 30% n-hexane: ethyl acetate (9.27 g) and 90% ethyl acetate: methanol (8.78 g). The least was eluted with 40% ethyl acetate in methanol (0.25 g).

The phytochemicals (Figure 6.5 and 6.6) and antibacterial potential (Figure 6.7 and 6.8) of the fractions were analysed in order to trace the compounds of interest. The fractions had compounds that were visible at both fluorescing (366 nm) and quenching (254 nm) wavelengths (Figure 6.5). The number of compounds observed was decreasing with increasing polarity. The same trend was observed when the chromatograms were sprayed with vanillin-sulphuric acid reagent (Figure 6.6). The fractions had potent antibacterial activities against the tested bacteria in both the bioautography (Figure 6.7 and 6.8) and broth micro-dilution assay (Table 6.5). The following fractions had antibacterial activity on bioautography against the tested bacteria; 70% n-hexane: ethyl acetate, 50% n-hexane: ethyl acetate, 30% n-hexane: ethyl acetate, 10% n-hexane: ethyl acetate, 100% ethyl acetate, and 90% ethyl acetate: methanol. The MIC values ranged from 0.02 to 2.5 mg/mL, while those of positive control (ampicillin) ranged from 0.02 to 0.08 mg/mL. Fraction 6 (30% n-hexane: ethyl acetate) had the highest antibacterial activity against all the tested bacteria with the lowest average MIC (0.06 mg/mL), followed by fraction 7 (10% n-

hexane: ethyl acetate) (0.12 mg/mL). Fraction 14 (40% ethyl acetate: methanol) had the lowest antibacterial activity with the highest average MIC (1.16 mg/mL).

Based on activity and good separation on TLC, fraction 7 was chosen for further fractionation of the antibacterial compounds on column chromatography and was eluted with 100% ethyl acetate. Phytochemical analysis (Figure 6.9 and 6.10) and bioautography (Figure 6.11 and 6.12) were performed to trace and ascertain activity of the compounds. The compounds were still not fully resolved hence it was necessary to separate them further. Fraction 3, 4, 7 8 and 10 had similar profiles and were active against all the tested bacteria; therefore, they were combined to make one big fraction. This was separated on a column chromatography eluted with 70% ethyl acetate in acetone to give single bands represented in figure 6.13. This study reports for the first time on the isolation of antibacterial compounds from DCM extracts of *S. pinnata*.

6.5. Conclusion

This study has demonstrated that the DCM extracts of *S. pinnata* had promising antibacterial compounds against the tested bacteria. Hence the extracts were chosen for further analysis and separation of the active compounds. Bioactivity guided isolation remains a promising procedure for isolation of active constituents from plants. This study serves as validation for the use of this plant for treatment of eye infections and diarrhoea in traditional medicine. Biological activities and structural elucidations of the isolated compounds will be documented in the next chapters.

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Chapter 7: Cytotoxicity and anti-inflammatory activity assays

7.1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are amongst the free radicals that are produced by the body's metabolic system under normal circumstances. Despite their importance in gene expression and activation of receptors, when present in excess amounts, free radicals can cause oxidative stress which may lead to cancer and inflammatory related diseases (Diaz *et al*., 2012). Several plants are used successfully for treatment and management of different types of inflammation and related disorders (Shaikh *et al*., 2016). Given the adverse side effects of the steroidal and non-steroidal anti-inflammatory drugs that are used currently, there is a need to explore such plants as an alternative (de Oliveira *et al*., 2014).

The use of traditional medicine systems to treat various aliments has been in existence for years and continues to provide the human population with new medicines (Serpeloni *et al*., 2011). However, most of the plants in traditional medicine are used based on folklore knowledge transmitted through generations with no information about their efficacy, safety and clinical evaluation (Nondo *et al*., 2015). Furthermore, the plants are assumed to be safe based on their long history of use in the treatment and management of diseases (Fennell *et al*., 2004).

The toxicity, mutagenicity, and carcinogenicity potential of many plants used as food and in traditional medicine is well documented (Fennell *et al*., 2004). The use of medicinal plants is associated with irritation of the gastrointestinal tract, destruction of red blood cells, and damage of the heart and kidney (Nondo *et al*., 2015). Therefore, this necessitates the need for toxicity evaluation of medicinal plants used in ethnopharmacology. The methods for evaluation of anti-inflammatory and cytotoxic activities in plant extracts include; enzymatic assays of enzymes involved in the biosynthesis of inflammatory mediators (Jadhav *et al*., 2013), Nitric oxide (Li and Wang, 2011) and reactive oxygen species production determination (Su *et al*., 2010) and brine shrimp lethality tests (Ashraf e*t al*., 2015) and cell line assays (McGaw e*t al*., 2007). Thus the aim of this chapter was to evaluate the anti-inflammatory activity and cytotoxic effect of plant extracts on LPS-induced macrophages Raw 264.7 cell

line and African green monkey kidney (Vero) cell line using DCFHD-A assay and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assays, respectively.

7.2. Methods and materials

7.2.1. Cell viability assay

The effects of acetone extracts of the selected plants on cell viability were assessed on African green monkey kidney (Vero) cells using the tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983). The cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Two hundred microlitre of the cell suspension (5 x 10^4 cells/mL) was added into each well of a sterile 96-well microtitre plate. The plates were incubated for 24 hours at 37 °C in a 5% CO_2 incubator for the cells to attach. The MEM was aspirated and the cells were washed with 150 µL phosphate buffered saline (PBS, Whitehead Scientific). The cells were treated with 200 µL of different concentrations of the extracts (1-0.025 mg/mL) prepared in MEM. The microtitre plates were incubated for 48 hours with the extracts in the same conditions as described earlier. Untreated cells were included as negative control. Following incubation, 30 µL of MTT (5 mg/mL) in PBS (Sigma) was added to each well and the plates were incubated further for 4 hours at 37 °C. Following incubation, the medium was removed and replaced with 50 µL of DMSO; the 96-well plates were swirled gently to dissolve the MTT formazan crystals. The amount of MTT reduction was measured by detecting absorbance in a microplate reader (BioTek Synergy) at 570 nm. The cytotoxic effects of the plants were expressed as the LC_{50} values, calculated as the concentration of test sample resulting in a 50% reduction of absorbance compared to untreated cells. All the analysis was made in quadruplicate. The selectivity index (SI) was expressed as LC_{50}/ MIC value.

7.2.2. Anti-inflammatory activity assay using DCFHD-A assay

Anti-inflammatory assay was carried out according to the method described by Sekhar *et al.* (2015) with modifications. DCFHD-A assay is a quantitative technique that uses stimulants such as lipopolysaccharide (LPS) to induce oxidative stress. This assay uses dihydrodichlorofluorescein diacetate (H_2DCF-DA) to detect the presence of reactive oxygen species in excess. In the presence of reactive oxygen species H_2DCF-DA is oxidized to fluorescent 2, 7-dichlorofluorescein (DCF). Two hundred microliters of cells (Raw 264.7 macrophages) in RPMI-1640 was seeded in a 96-well plate. The cells were incubated at 37 °C, 5% CO_2 overnight to allow cells to attach. The medium was removed and the cells were washed with PBS. Cells were exposed to 100 µL of acetone extracts (8 mg/mL, 0.64 mg/mL, and 0.32 mg/mL) of *D. rotundifolia* and *S. pinnata* and 20 µL of LPS for 24 hours. Following incubation, the medium was aspirated a new medium without FBS was added and the cells were stained with 100 µL of 20 µM of H_2DCF-DA and incubated for 30 minutes in the dark. The fluorescence was measured at 480 nm. Curcumin (50 µM) was used as a positive control.

7.3. Results

7.3.1. Cell viability assay

The effect of the acetone extracts of the selected plants on cell viability of the African green monkey kidney cells (Vero) was determined using MTT assay. The cytotoxicity was represented as the concentrations of the extracts which reduced cell viability by 50% compared to the untreated cells (LC_{50} values), these value ranged from <25.0 to 466.1 µg/mL. Only *S. pinnata* was highly toxic to the cells with the lowest LC_{50} value of <25.0 µg/mL. The selectivity index was used to relate the toxicity to the observed biological activities. The values were calculated by dividing the LC_{50} with the MIC values and they ranged from 0.02 to 22.06 (Table 7.1). *C. africana* had the highest selectivity index against *E. coli* (22.06), followed by *D. rotundifolia* also against *E. coli* (1.46).

Table 7.1: Cytotoxicity, MIC, and selectivity index (SI) of the acetone extracts of the selected plants.

Plant species	LC_{50} (µg/mL)	MIC value (µg/mL)				Selectivity index (SI)			
		E.c	P.a	E.f	S.a	E.c	P.a	E.f	S.a
S. pinnata	<25.0	320	640	1250	1250	0.08	0.04	0.02	0.02
D. rotundifolia	466.1	320	1250	1250	1250	1.46	0.37	0.37	0.37
E. elephantina	416.4	640	2500	2500	2500	0.17	0.17	0.17	0.17
C. africana	441.1	20	2500	2500	2500	22.06	0.18	0.18	0.18

Key: E.c= *E. coli*; P.a= *P. aeruginosa*; E.f= *E. faecalis*; S.a= *S. aureus*

7.3.2. ROS production inhibition

The anti-inflammatory potential of the acetone extracts of *S. pinnata* and *D. rotundifolia* was determined by measuring their effect on the inhibition of ROS generation in LPS-induced RAW 264.7 macrophage cells. The plant extracts inhibited ROS generation in a dose dependant manner. The inhibition was higher in *D. rotundifolia* than in *S. pinnata* (Figure 7.1). Curcumin (50 µM) was used as a positive control and all the plants had better anti-inflammatory potential than curcumin at the highest concentration tested.

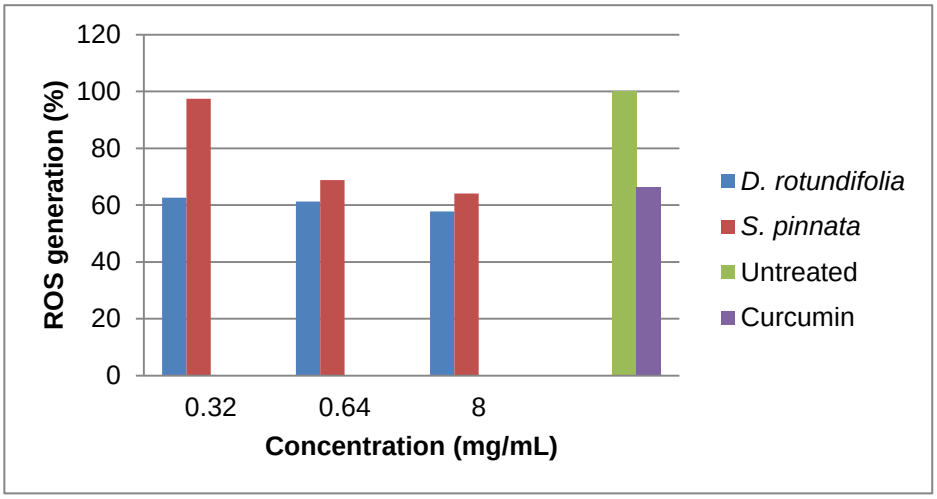

Figure 7.1: The effect of two of the selected plants on ROS generation inhibition activity in LPS-induced RAW 264.7 macrophage cells.

7.4. Discussion

Many of the plants used in ethnopharmacology to treat various aliments are used with no knowledge of their toxic effect (Nondo *et al*., 2015). Thus, following antibacterial efficacy evaluation, the toxic effects of the selected plants were evaluated on African green monkey kidney (Vero) cells using MTT assay. This assay is based on the conversion of MTT to an insoluble purple formazan by the mitochondrial succinate dehydrogenase of viable cells (Fotakis and Timbrell, 2006). The concentrations of the extracts which resulted in 50% reduction of absorbance compared to untreated cells (LC_{50}) are presented in Table 7.1. The cytotoxicity of the acetone extracts of the selected plants ranged from <25 to 466.1 µg/mL. The American National Cancer Institute (NCI) described an LC_{50} <30 µg/mL for plant extracts as a cut off point for cytotoxicity after 72 hours of exposure (Itharat *et al*., 2004). Therefore, *S. pinnata* extract was highly toxic to the monkey Vero cells with the lowest LC_{50} (<25 µg/mL) that is outside the cut-off point. Deutschländer *et al*. (2009) also reported the toxicity of the acetone and ethanol extracts of *S. pinnata* on 3T3-L1 preadipocytes and Chang liver cells. Nevertheless, the rest of the plants were less toxic with high LC_{50} values of 466.1 µg/mL, 416.4 µg/mL, and 441.1 µg/mL for *D. rotundifolia*, *E. elephantina*, and *C. africana*, respectively.

The selectivity indexes were used to relate cytotoxicity and antibacterial activities of plant extracts. These values ranged from 0.02 to 22.06 (Table 7.1). The plant extracts had low selectivity indices with an exception of *C. africana* (22.06). The efficacy of biological activity is considered not to be due to toxicity if the selectivity index is ≥10 (Caamal-Fuentes *et al*., 2011). Therefore, the observed antibacterial activity of acetone leaf extracts of *C. africana* was not due to toxicity.

There are substantial amounts of reports linking ROS production to inflammation and related diseases. As such, the effects of *S. pinnata* and *D. rotundifolia* extracts on the inhibition ROS production were investigated in LPS-induced RAW 264.7 macrophage cells. The tested plants exhibited anti-inflammatory potential and they inhibited the production of ROS in a dose dependant manner. Potent activities were observed in *D. rotundifolia* even at the lowest concentration tested. Nevertheless, *S. pinnata* also exhibited ROS inhibition activity at higher concentrations. However, MTT assay revealed that this plant is toxic at higher doses. Therefore, the anti-

inflammatory efficacy should be evaluated *in vivo* before the plant is recommended for any use. Curcumin was used as a positive control and it also exhibited anti-inflammatory potential at the highest concentration tested (50 µM) (Figure 7.1). However, the plant extracts demonstrated better anti-inflammatory potential than curcumin at the highest concentration tested. The anti-inflammatory potential of these plants could be attributed to flavonoids and tannins, whose presence was demonstrated in the tested plants (Chapter 3). Arts and Hollman, (2005) demonstrated the involvement of flavonoids in the prevention of many oxidative and inflammatory diseases. Meanwhile, tannins were reported to exhibit their anti-inflammatory potential by scavenging free radicals and inhibiting the expression of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenases (COX) (Polya, 2003; Erdèlyi e*t al*., 2005).

7.5. Conclusion

This study has demonstrated the cytotoxic effects of the selected plants on African green monkey (Vero) kidney cells and the anti-inflammatory potential of *D. rotundifolia* and *S. pinnata* acetone extracts. The cytotoxicity of *S. pinnata* highlights the importance of using it with caution and at low doses as an anti-inflammatory agent. *In vivo* studies should be done to evaluate genotoxicity effects and biotransformation of *S. pinnata* before it can be considered for any use. The anti-inflammatory potential of *D. rotundifolia* may be attributed to phenolics, tannins, and flavonoids that were present in high concentrations in this plant.

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Chapter 8: Structural elucidation

8.1. Introduction

Natural products are known to possess complex structures with potent biological activities. However, the aid of modern technologies such as nuclear magnetic resonance (NMR) has allowed successful determinations of these complex structures (Matsumori *et al*., 1999). This technique is useful for determination of chemical structures of natural products, since it provides information about individual hydrogen and carbon atoms in the structure (Cannell, 1998). The advances in technology have enabled development of pulse programs and instrumentation that allows determination of structures of samples even at low quantities (Lekganyane, 2015).

NMR usually applies a number of experiments for structural elucidations of natural products, these include; Proton NMR, carbon 13 NMR, DEPT, COSY, HSQC, HMQC, and HMBC. Proton NRM (1D ^{1}H NMR) gives a quantitative overview of the distribution of hydrogen atoms in a sample, while carbon-13 NMR (1D ^{13}C NMR) provides an overview of the quantitative distribution of carbon atoms. Distortionless enhancement through polarisation transfer (DEPT) (1D ^{13}C NMR) provides information about the number of hydrogen attached to each carbon, while ^{1}H-^{1}H Correlation spectroscopy (COSY) (2D ^{1}H NMR) provides information about coupled protons on adjacent carbons. 2D ^{1}H-^{13}C Heteronuclear single quantum correlation (HSQC) gives information about the H-C correlations by means of a single bond, with protons in one axis and carbons on the other, while heteronuclear multiple quantum correlation (HMQC) (2D ^{1}H- ^{13}C NMR) provides information about correlations of H-C signals using either one bond or longer range couplings. ^{1}H-^{13}C Heteronuclear multiple bond correlation (HMBC) (2D ^{1}H- ^{13}C NMR) gives information about the proton-carbon correlation over 2 and/or 3 bonds (Ahmed, 2012).

The techniques for structural elucidations are not limited to NMR, techniques such as Mass spectroscopy (MS), ultraviolet-visible spectroscopy (UV-vis) and infra-red spectroscopy (IR) can also be used (Sakong, 2012). The aim of this chapter was to elucidate the chemical structures of the isolated compounds using NMR spectroscopy.

8.2. Methods and materials

8.2.1. Structural elucidation of isolated compounds

The structures of the compounds isolated in this study were determined by NMR techniques, mainly 1D (^{1}H, ^{13}C and DEPT 135) and 2D NMR (HMBC, HSQC, and COSY). About 10 mg of each compound was mixed with deuterated acetone (Acetone-d) and this was sent to the Chemistry department at the University of Limpopo for analysis. The prepared samples were ran using 400 MHz NMR Spectrometer (Bruker) at 400 MHz, number of scans were 10240 at a temperature of 295.5 K. Prof Ofentse Mazimba, of the Chemical & Forensic Sciences Department at Botswana International University of Science and Technology assisted with the analysis of the NMR spectra and structure elucidation of the compounds.

8.3. Results

8.3.1. NMR analysis of isolated compound 1

Figure 8.1 to 8.6 represent NMR spectra of compound **1** under different pulse programs i.e. (^{1}H Proton, ^{13}C, DEPT 135, COSY, HSQC, and HMBC). These spectra helped in the characterisation of compound **1**.

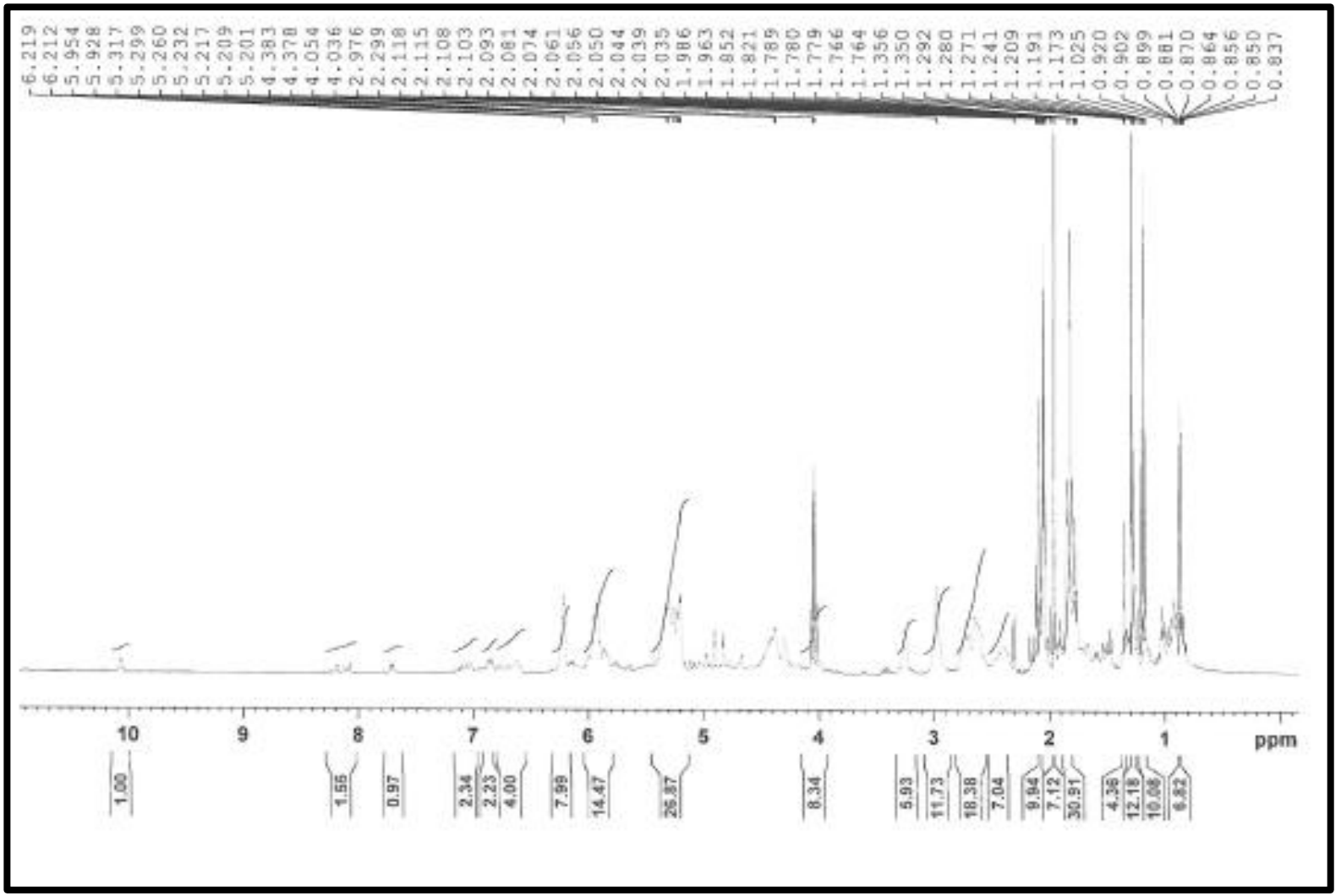

Figure 8.1: ^{1}H NMR spectrum of isolated compound **1**

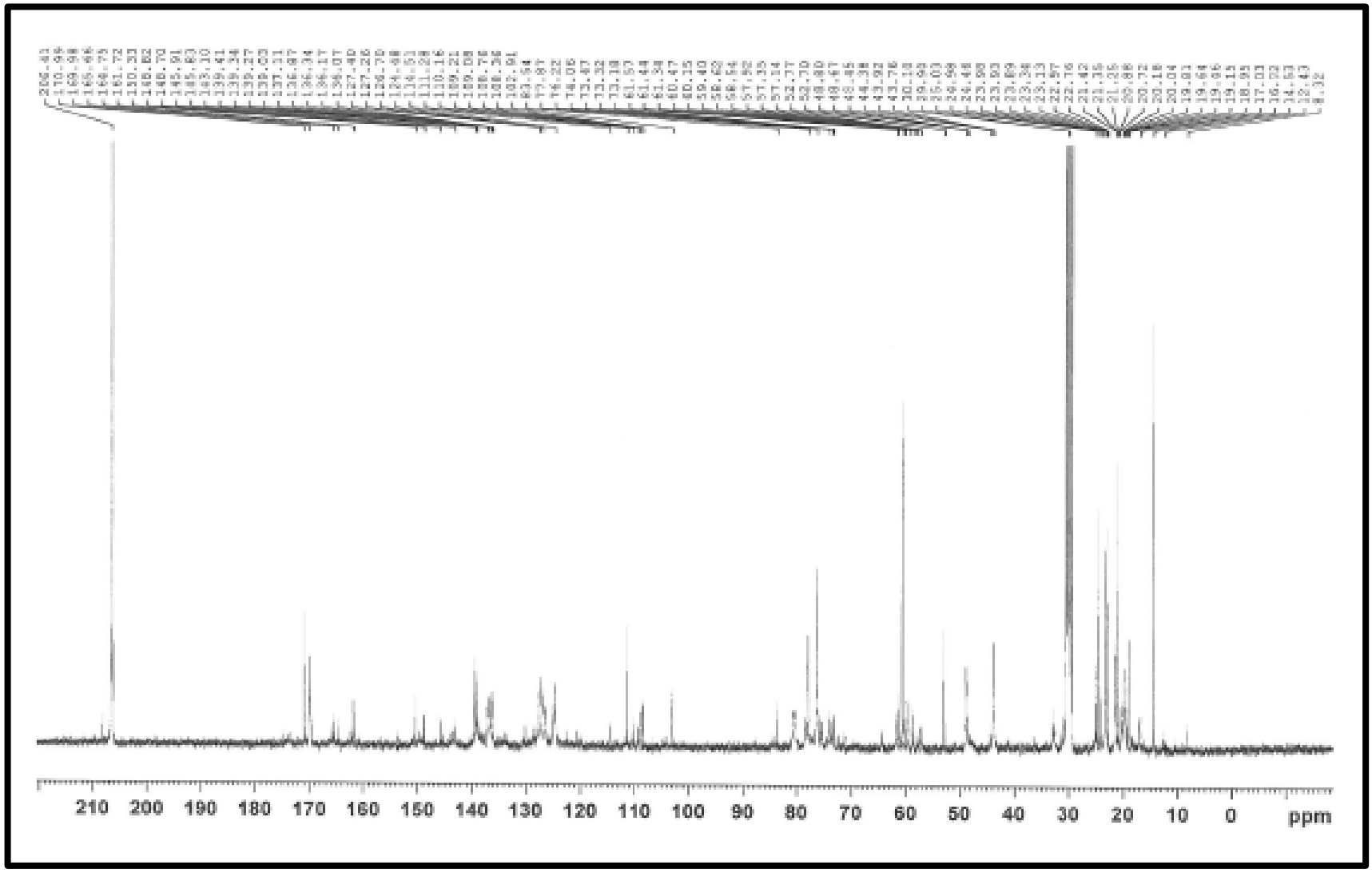

Figure 8.2: ^{13}C CPD NMR spectrum of compounds **1**

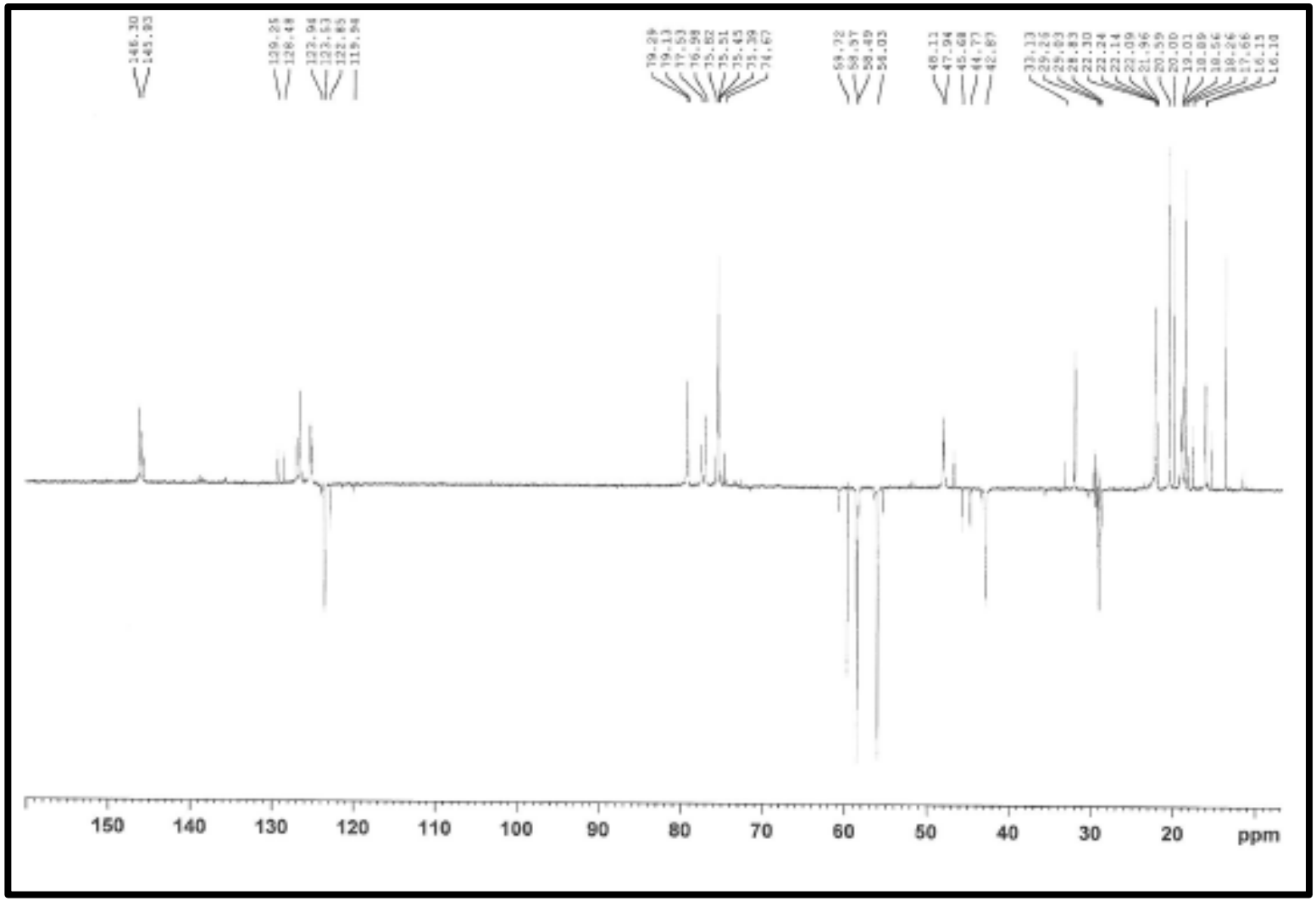

Figure 8.3: DEPT 135 NMR spectrum of compound **1**

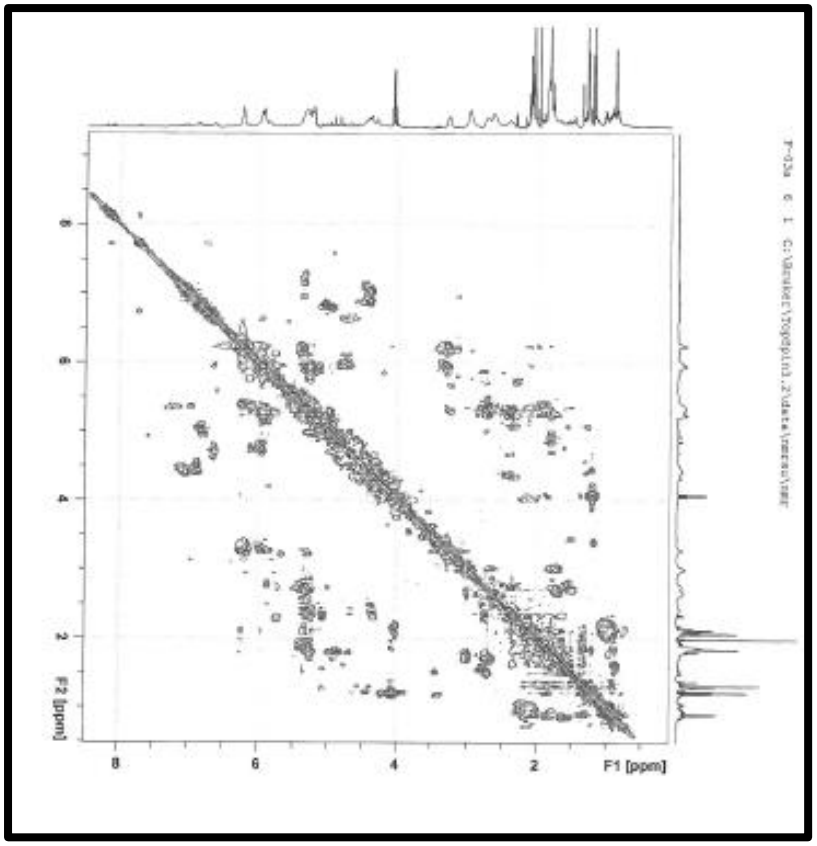

Figure 8.4: COSY NMR spectrum of compound **1**

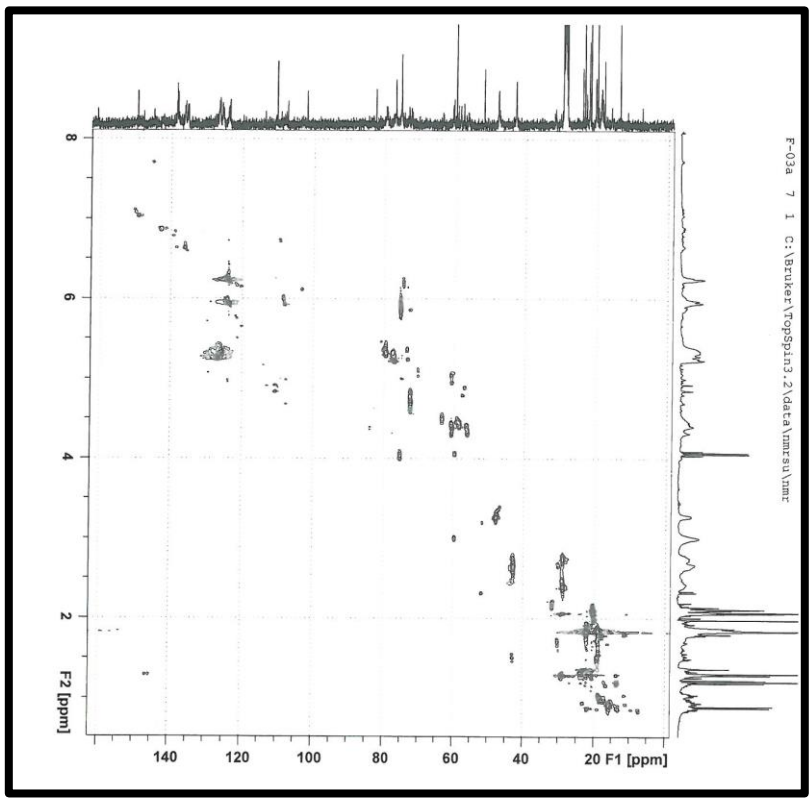

Figure 8.5: HSQC NMR spectrum of compound **1**

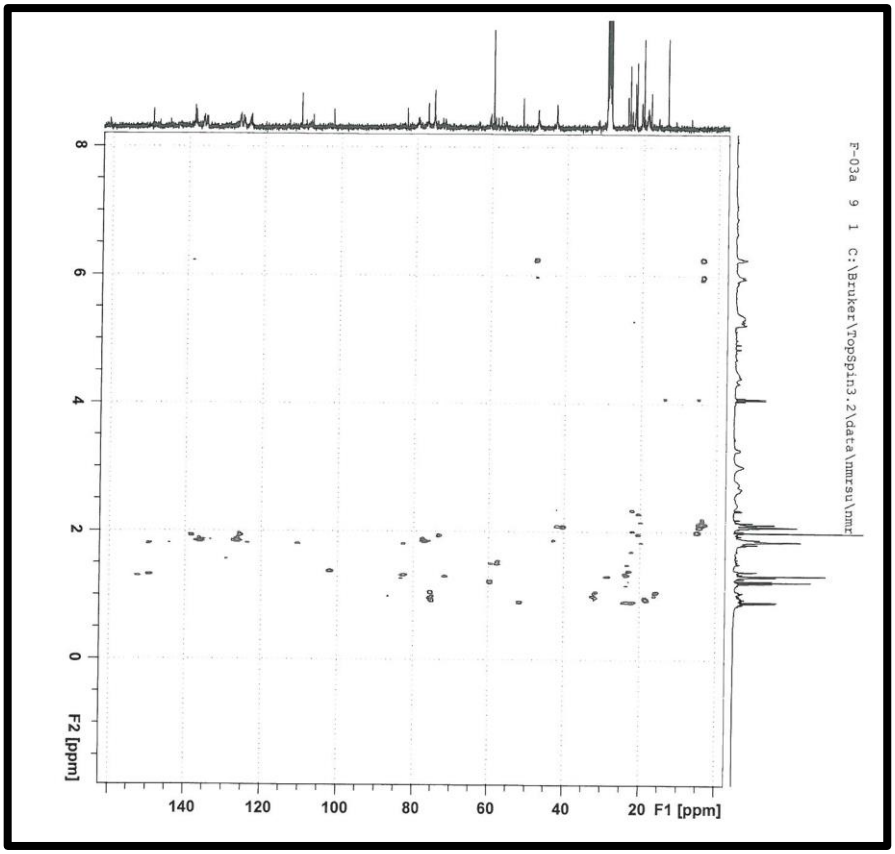

Figure 8.6: HMBC NMR spectrum of compound **1**

8.3.2. Spectroscopic data

The data below summarises all the NMR spectroscopic data of compound 1, this was compared with guanolides from literature. Compound 1 was identified as a mixture of two compounds (minor and major) which are isomers of each at carbons 9 and 10 (Table 8.1).

Table 8.1: Summarised spectroscopic data of ^{13}C shifts for major and minor peaks of compound **1** and guanolides from literature.

Positions	6α-Hydroxyinuviscolide (Cheng *et al*., 2011)		Compound 1 (major)	4α,6α-Dihydroxy-1β,5α,7αH-guaia-9(10),11(13)-dien-12,8α-olide (Cheng *et al*., 2011)		Compound 1 (minor)
1	40.15	CH	48.6	38.7	CH	48.6
2	25.3	CH_2	29.7	22.8	CH_2	29.7
3	40.19	CH_2	43.7	40.5	CH_2	43.9
4	81.6		83.5	80.5		77.8
5	64.5	CH	59.3	56.6	CH	64.4
6	75.5	CH	76.2	74.9	CH	74.1
7	49.7	CH	48.7	51.5	CH	48.0
8	76.9	CH	77.8	76.8	CH	80.5
9	40.4	CH_2	52.7	125.3	CH	126.5
10	146.0		145.9	140.9		145.9
11	136.9		139.4	137.6		139.2
12	170.6		169.8	170.9		169.9
13	125.8	CH_2	124.6	124.1	CH_2	124.6
14	111.7	CH_2	111.4	21.2	CH_3	20.8
15	23.2	CH_3	23.1	22.2	CH_3	22.7
1’			170.9			169.8
2’			139.2			136.1
3’		CH	143.4		CH	139.4.4
4’		CH_2	59.8		CH_2	58.5
5		CH_2	61.5		CH_2	60.1

8.3.3. The structure of compound 1

Figure 8.7 a and b below illustrates the elucidated structures of compound **1**. Based on the peaks obtained from NMR, compound **1** was found to be a mixture of compound **2** and **3** represented as major (a) and minor (b) peaks.

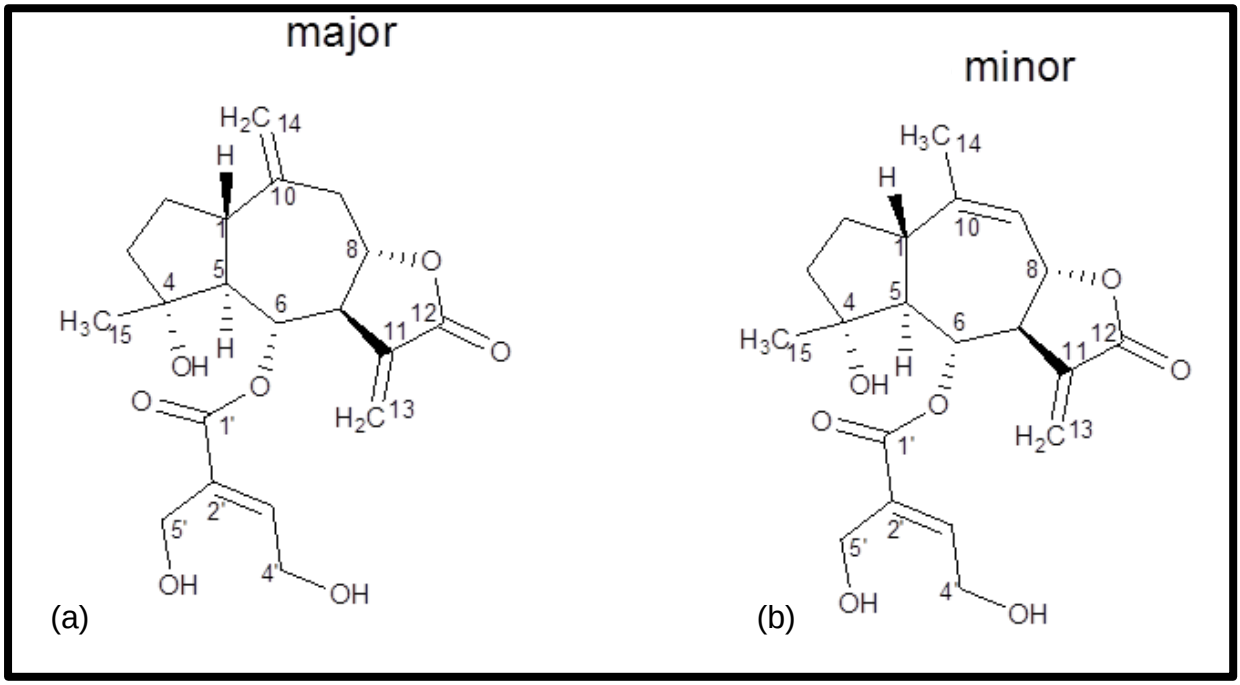

Figure 8.7: The structure of compound **1** ((a) = 6-(2, 3-dihydroxyangeloylory) -6α-hydroxyinuviscolide (b)= C-9 and C-10 isomers of (a))

8.3.4. NMR analysis of compound 2

Figure 8.8 to 8.12 represent NMR spectra of compound **2** under different pulse programs i.e. (^{1}H Proton, ^{13}C, DEPT 135, COSY, and HMBC). The peaks from these spectra helped in the elucidation of compound **2**.

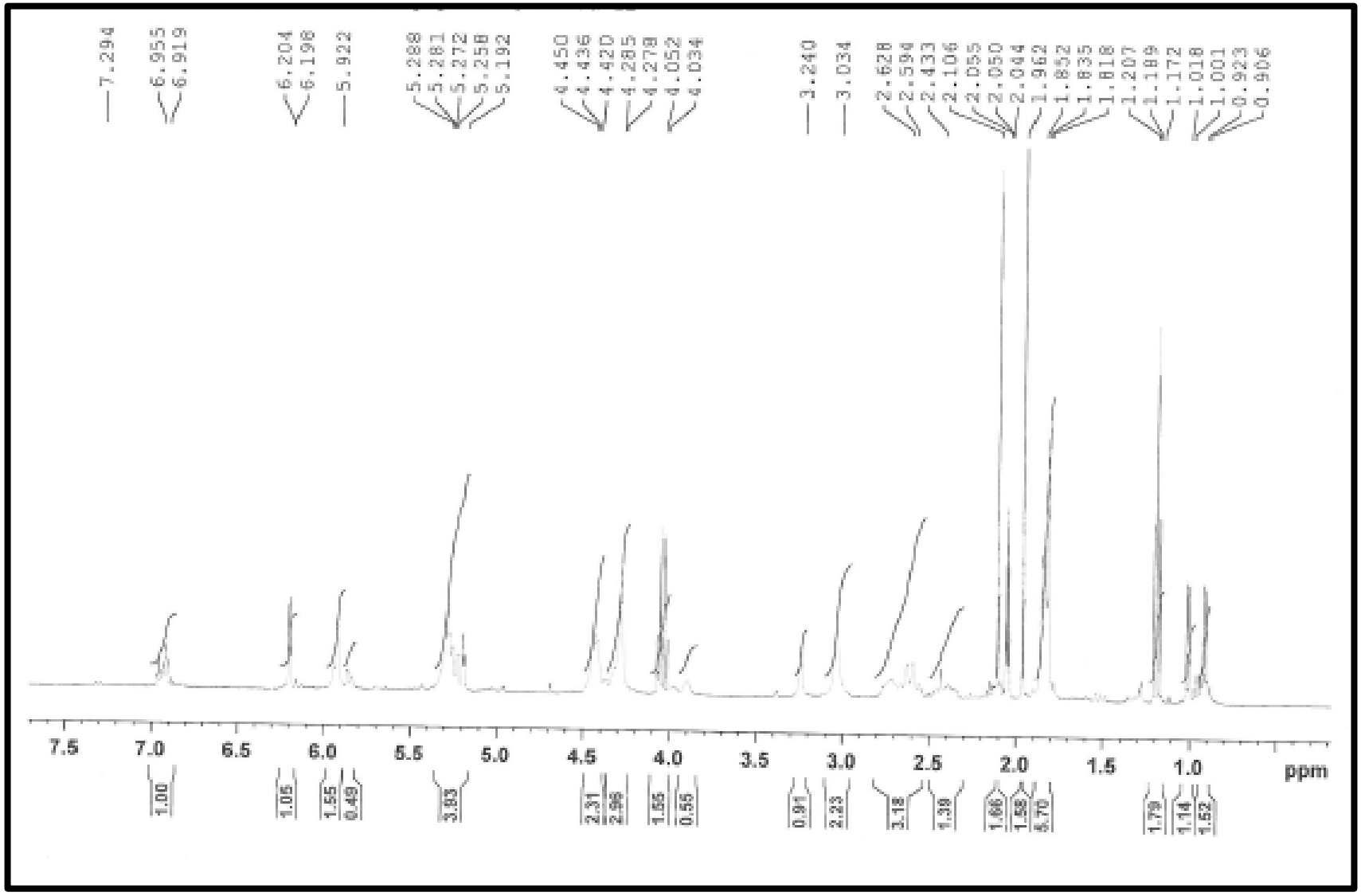

Figure 8.8: ^{1}H NMR spectrum of compound **2**

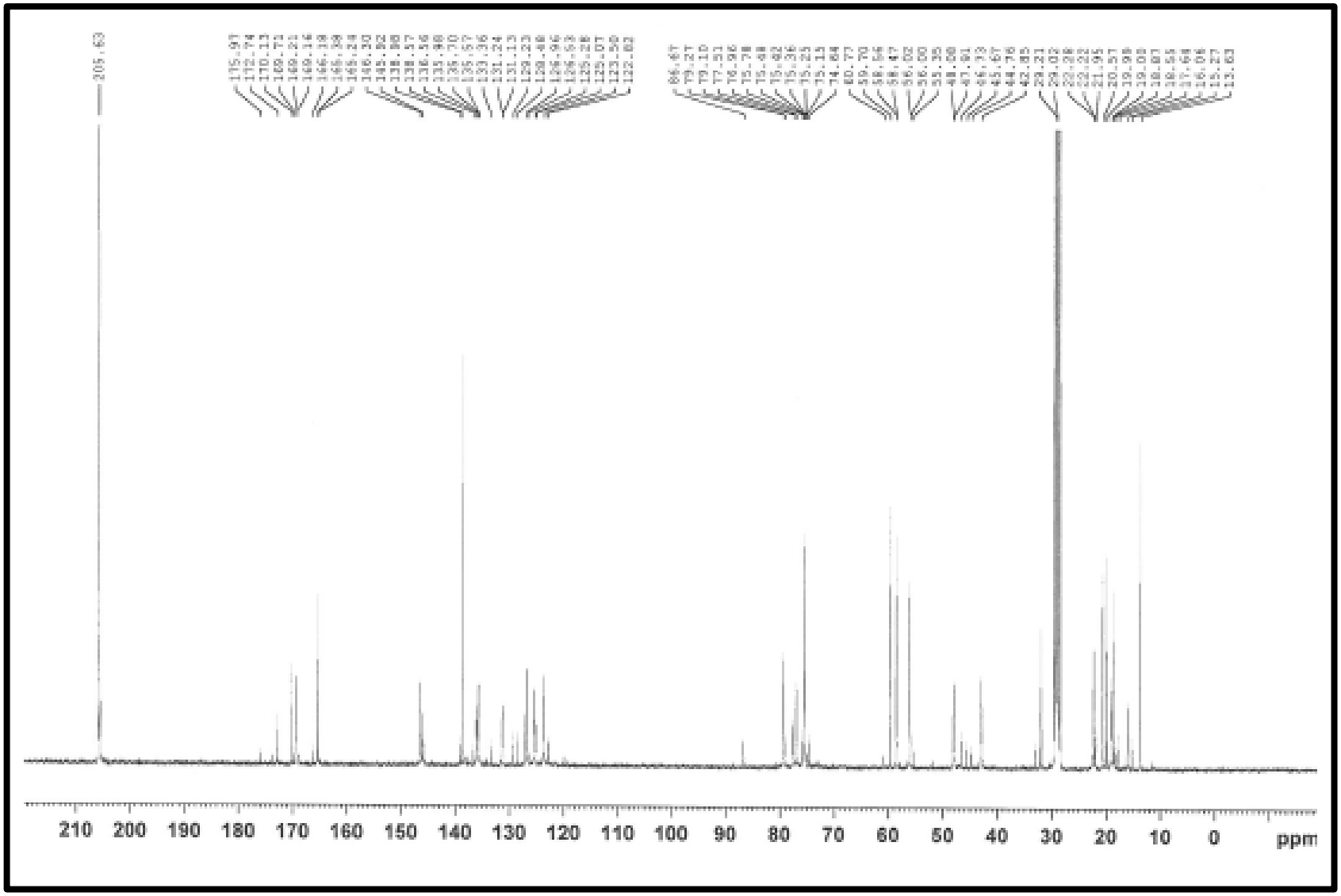

Figure 8.9: ^{13}C CPD NMR spectrum of compound **2**

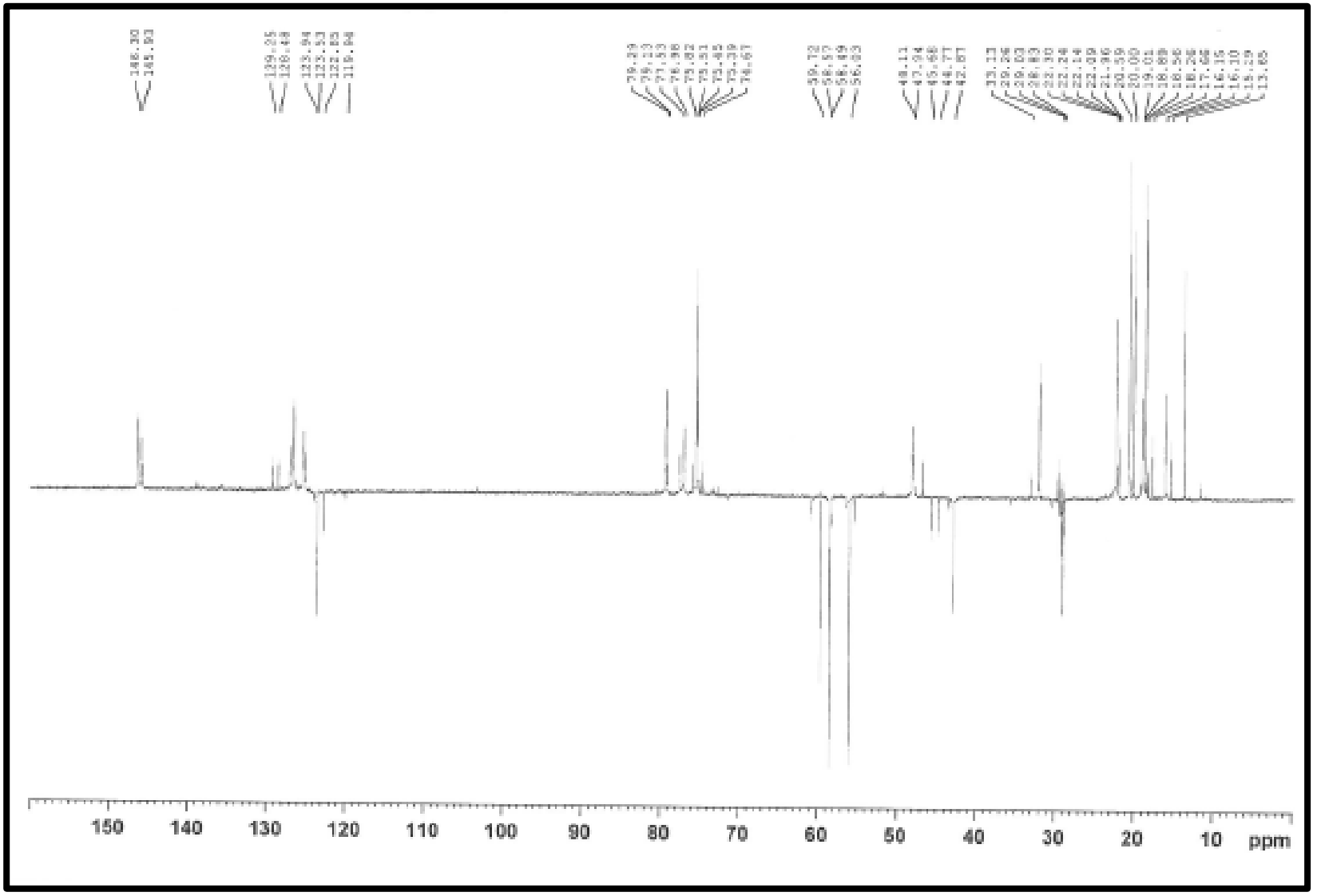

Figure 8.10: DEPT 135 NMR spectrum of compound **2**

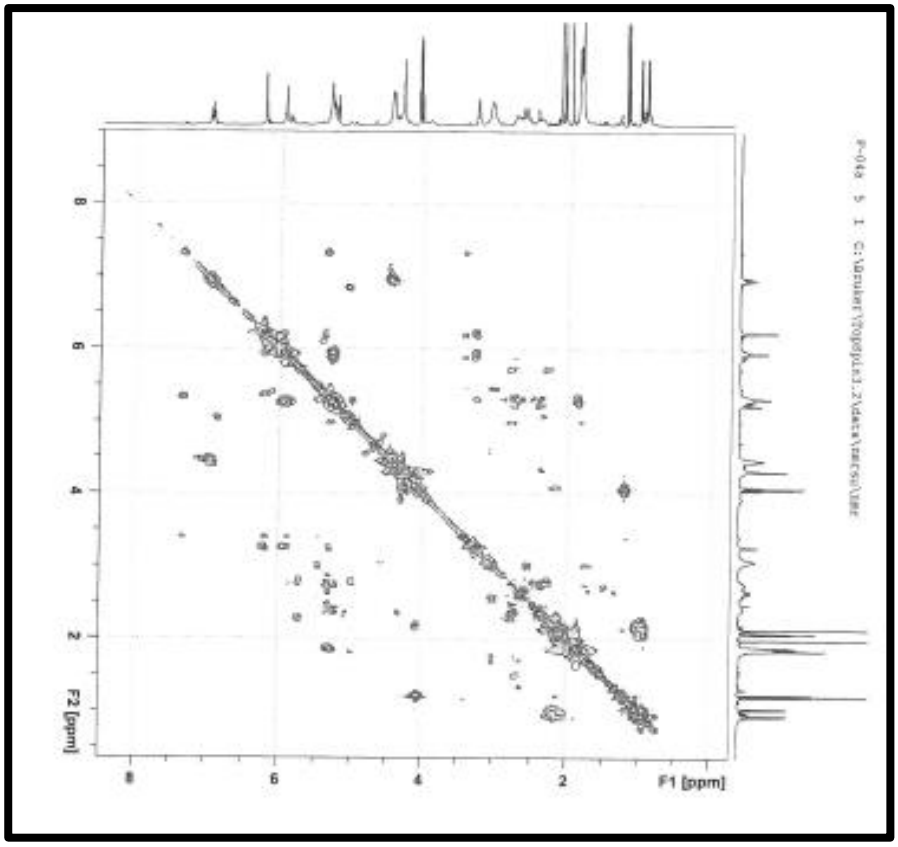

Figure 8.11: COSY NMR spectrum of compound **2**

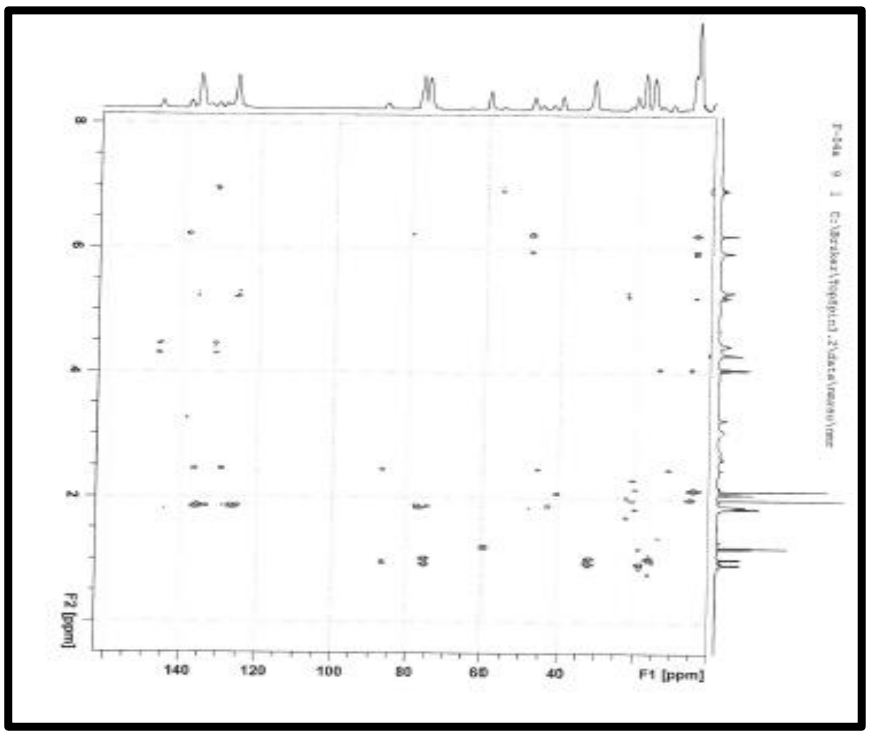

Figure 8.12: HMBC NMR spectrum of compound **2**

8.3.5. Spectroscopic data for compound 2

Table 8.2 summarises the NMR spectroscopic data of compound 2 and compares it with a heliangolide in literature. There is a peak that is present in carbon 2'' of compound 2 and absent in the heliangolide in literature.

Table 8.2: Summarised NMR spectroscopic data of ^{13}C shifts for compound **2** and a heliangolide from literature.

Positions	Pacciaroni *et al*., 1995		Compound 2
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

8.3.6. The structure of compound 2

Figure 8.13 below represents the structure of compound **2** which was isolated as a clear oily substance; the spectrum obtained from NMR aided the elucidation.

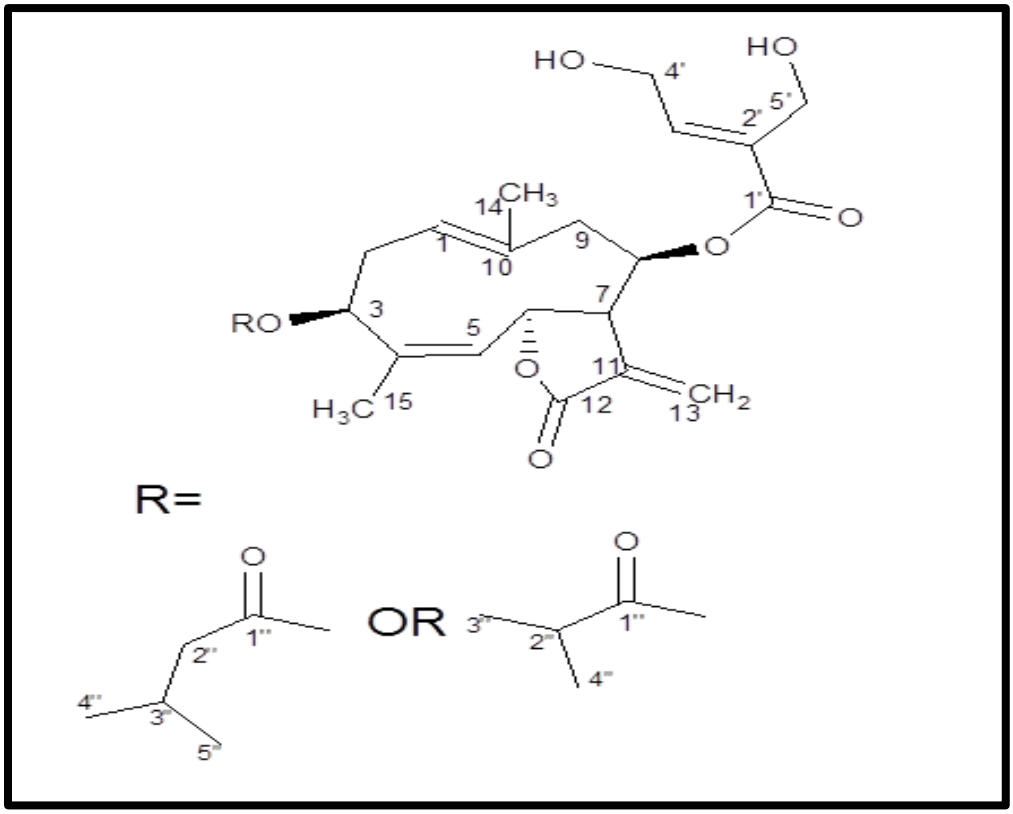

Figure 8.13: The structure of compound **2** (Heliangolide).

8.3.7. NMR analysis of compound 3

The spectra below (Figure 8.14 to 8.18) represent the peaks obtained after NMR experiments i.e. ^{1}H, ^{13}C, DEPT, COSY, and HMBC NMR for characterisation of isolated compound **3.**

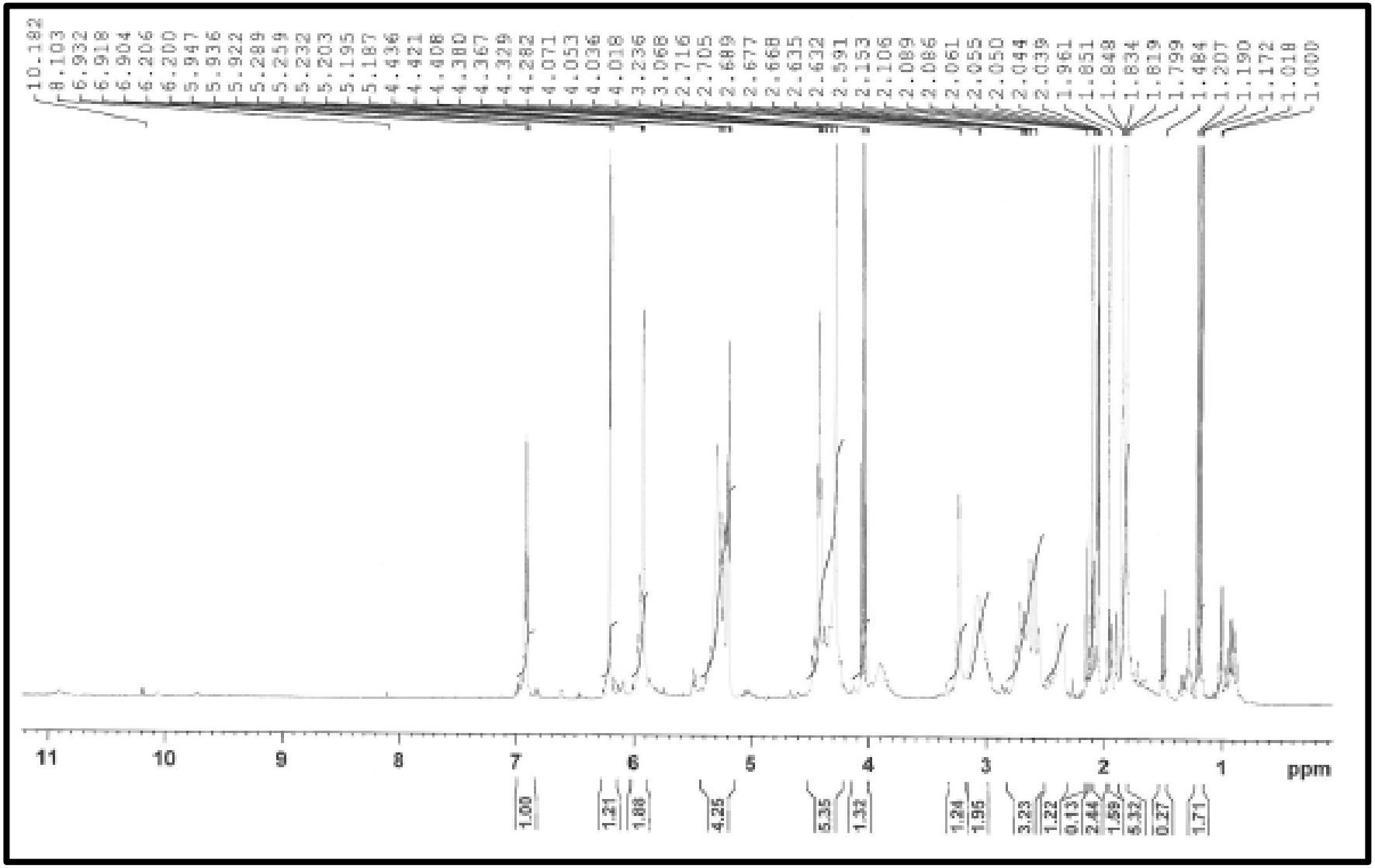

Figure 8.14: ^{1}H NMR spectra of compound **3**

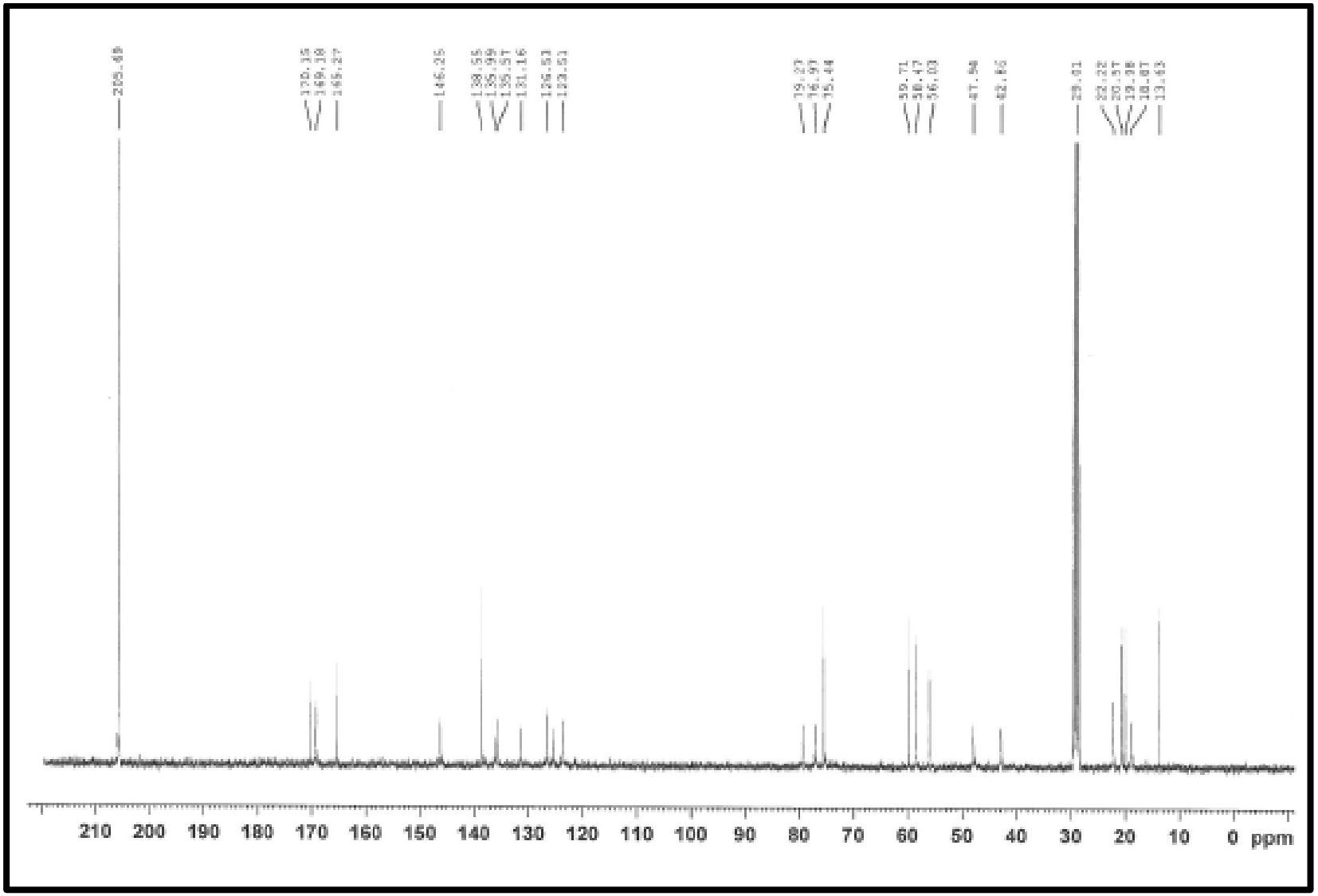

Figure 8.15: ^{13}C NMR spectrum of compound **3**

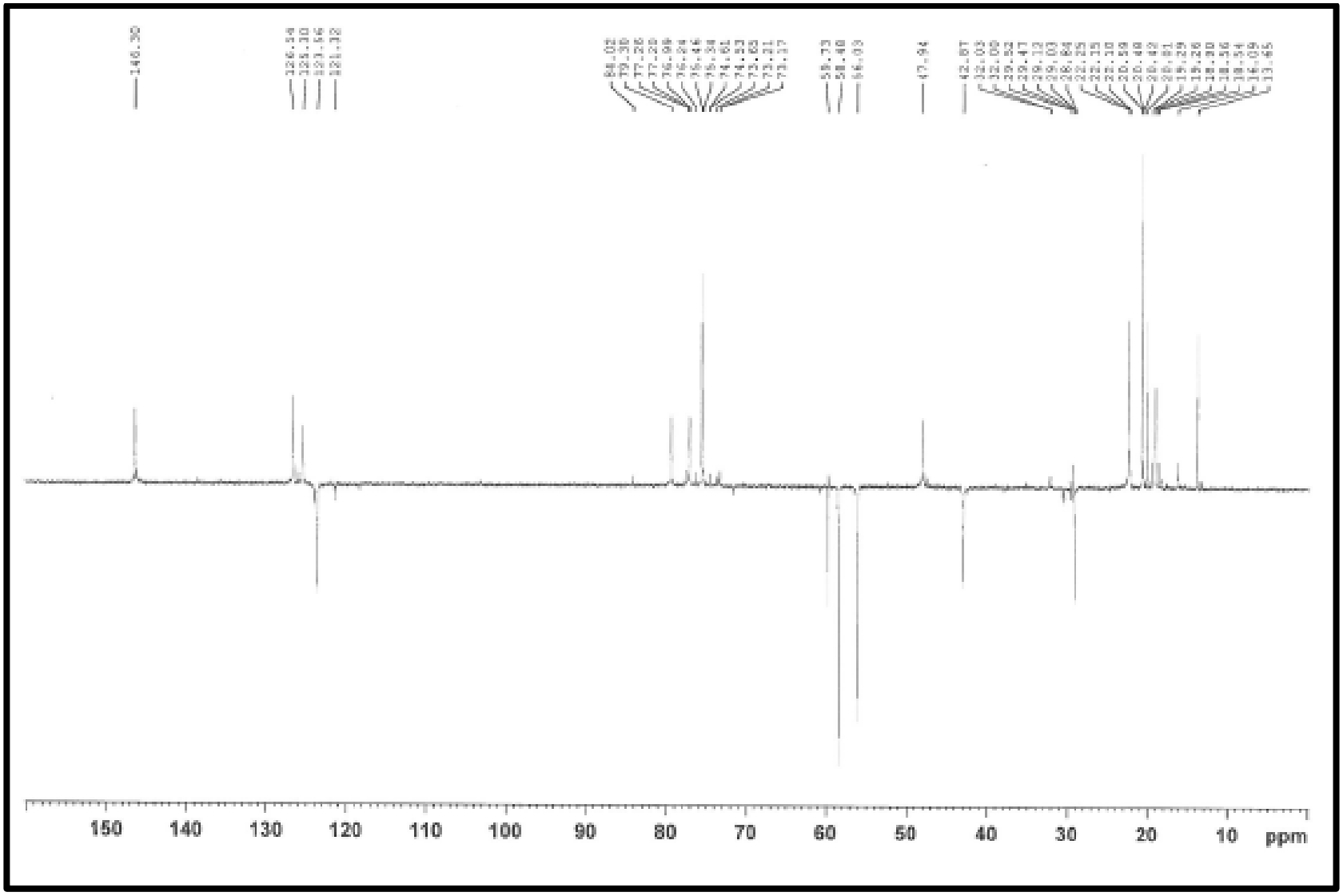

Figure 8.16: ^{13}C DEPT 135 NMR spectrum of compound **3**

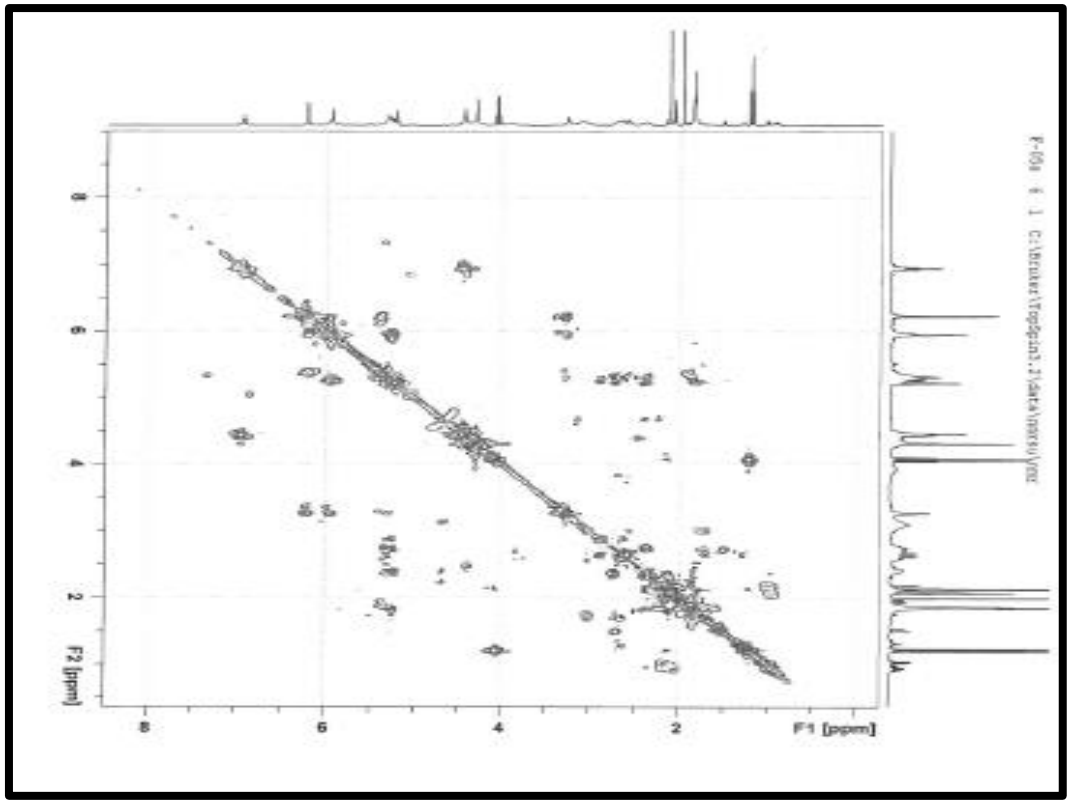

Figure 8.17: COSY NMR spectrum of compound **3**

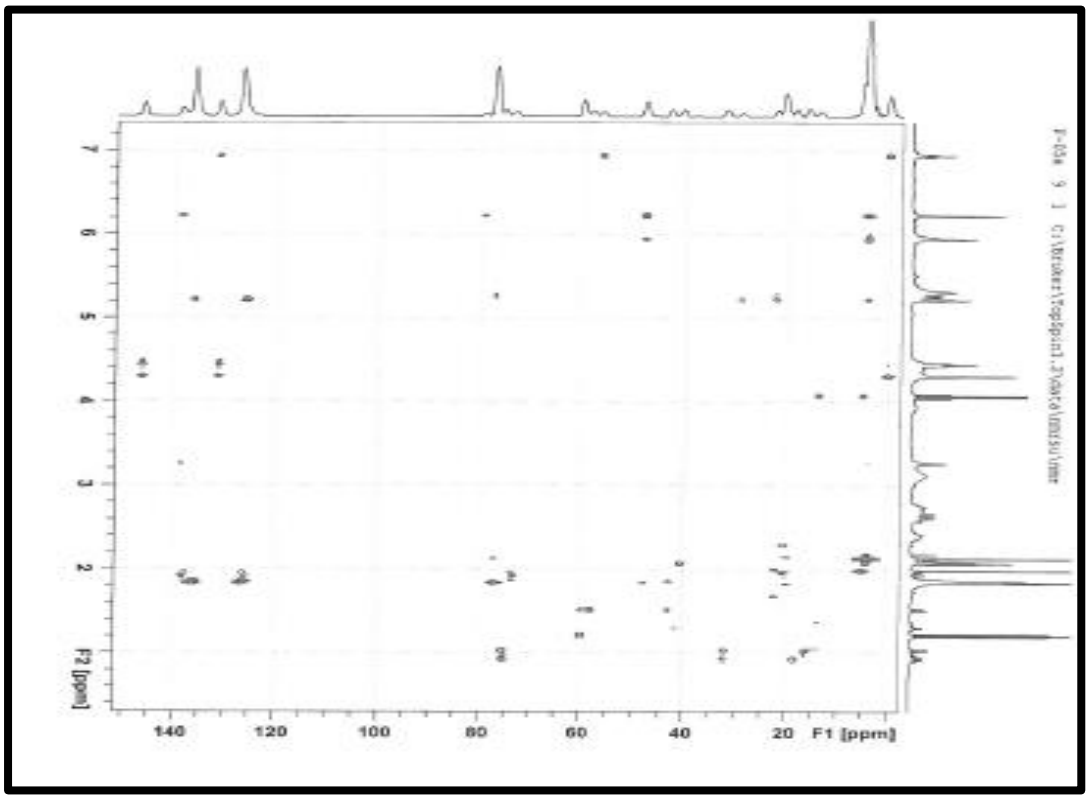

Figure 8.18: HMBC NMR spectrum of compound **3**

8.3.8. Spectroscopic data for compound 3

The data below summarises the NMR spectroscopic data of compound 3 in comparison with a eucannabiolide in literature. Both these compounds have a similar peaks and number of carbon atoms (Table 8.3).

Table 8.3: Summarised spectroscopic data of ^{13}C shifts compound **3** and a eucannabiolide from literature.

Positions	Herz and Govindan, 1980		Compound 3
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

8.3.9. The structure of compound 3

Structure below (Figure 8.19) represents compound **3** which was isolated as an oily matter from the third column. The NMR spectra above aided the elucidation of this structure.

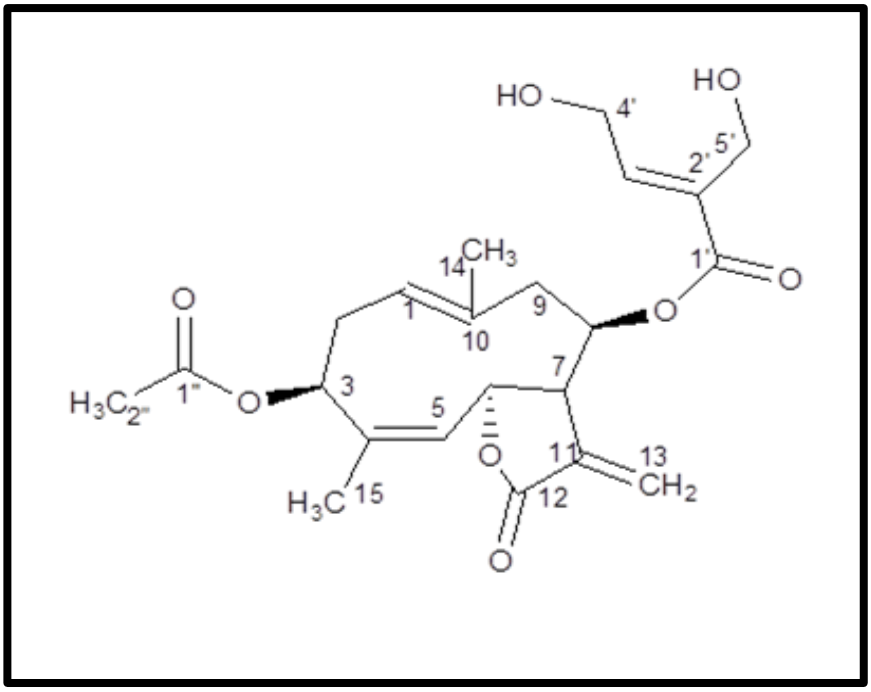

Figure 8.19: The structure of compound **3** (Derivative of Eucannabinolide).

8.4. Discussion

Repeated fractionation of dichloromethane extracts of *S. pinnata* (Lam.) Kuntze ex Thell on column chromatography afforded isolation of three compounds labelled compound **1**, **2** and **3**. The proton, carbon 13, DEPT, COSY, HQSC, and HMBC NMR spectra of these compounds were generated and these aided their structural elucidations. The isolated compounds were identified as members of groups from sesquiterpene lactones class of phytochemicals. These are common constituents in species from the Asteraceae family (de Vivar e*t al*., 1980; Zhang *et al*., 2014). They are said to have a bitter sensory quality (Zidorn, 2008), they are probably responsible for the bitter taste of *S. pinnata*.

Compound **1** (675.6 mg) was isolated as yellowish oil and was identified as a mixture of two sesquiterpene lactones (guanolides) i.e. 6– (2, 3- dihydroangeloylory) -6α-hydroxyinuviscolide (minor) and C-9 and C-10 isomers of the minor compound (major) (Figure 8.7). Figure 8.1 to 8.6 gave the information about the number of protons, carbons, hydrogen attached to carbons, coupled hydrogen atoms on adjacent carbons and proton-carbon correlations present in the compound respectively. Some parts these minor and major compounds were similar to 6α-

hydroxyinuviscolide and 4α,6α-dihydroxy-1β,5α,7αH-guaia-9(10),11(13)-dien-12,8α-olide isolated from ethanolic extracts of *Inula falconeri* (Cheng *et al*., 2011). The information about the ^{13}C chemical shifts of the isolated minor and major compounds of compound **1** in comparison with the one in literature is summarised in Table 8.1.

Compound **2** (4303.9 mg) was obtained as clear oil and was identified as a heliangolide sesquiterpene lactone (Figure 8.13). The information about the number of protons, carbons, hydrogen attached to carbons, coupled hydrogen atoms on adjacent carbons and proton-carbon correlations present in the compound were represented by Figure 8.8 to 8.12, respectively. Similarly, Table 8.2 summarises the ^{13}C chemical shifts of this compound in comparison with a heliangolide (previously isolated as diacetate) isolated from the ethanolic extract *of Schkuhria pinnata* var. *abrotanoides* (Roth) CaBr (Pacciaroni *et al*., 1995).

Compound **3** (2008.6 mg) was isolated as yellowish oil and was identified as a derivative of the eucannabiolide sesquiterpene lactone (Figure 8.19). Figure 8.14 to 8.19 gave the information about the number of protons, carbons, hydrogen attached to carbons, coupled hydrogen atoms on adjacent carbons and proton-carbon correlations present in the compound respectively. Meanwhile, Table 8.3 summarises the ^{13}C shifts within the compounds and comparing it to the eucannabiolide in literature. This identified compound was found to be similar to the one isolated from aerial parts of *Schkuhria virgata* (La Llave *et* Lex) DC. by Herz and Gorvindan (1980).

8.5. Conclusion

This study demonstrated the isolation and identification of three sesquiterpene lactones from the guanolides, heliangolides, and eucannabinolide subtypes which are similar to those isolated previously from different species of *Schkuhria* and *Inula* specie. Nuclear overhauser effect spectroscopy (NOESY) and Mass spectroscopy (MS) of these compounds should be performed to determine their 3-D structures and confirm molecular weights respectively. The biological activities of these sesquiterpene lactones are investigated in the next chapter.

8.6. References

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Chapter 9: Biological activities of isolated compounds

9.1. Introduction

Schkuhria is a small genus from the Asteraceae family of angiosperms; it consists of about 15 species (Ganzer and Jakupovic, 1990). A number of compounds have been isolated from members of this genus, these include; sesquiterpene lactones (Ganzer and Jakupovic, 1990) such as eucannabinolides (Herz and Govindan, 1980), heliangolides (Bohlmann and Zdero, 1981), schkuhriolides, and melampolide schkuhrioidin (de Vivar *et al*., 1980) and some acyl phenylpropanoids (León *et al*., 2009) .

Sesquiterpene lactones are a large class of secondary metabolites mainly isolated from members of the Asteraceae family (Zhang *et al*., 2014). These are cyclic fifteen-carbon compounds with isoprene units and *cis*- or *trans*-fused lactone classified in into groups based on their carboxylic skeleton. The groups include; the ten-ringed germacranolides, the 6/6-bicyclic compounds (eudesmanolides and eremophilanolides), the 5/7-bicyclic compounds (guaianolides, pseudoguaianolides, heliangolides and hypocretenolides). The biological activities of sesquiterpene lactones include; cytotoxicity to certain cancer cell lines, induction of detoxifying enzymes (Zidorn, 2008), anticancer, anti-inflammatory and immunomodulatory effects, anti-ulcer, antimicrobial and antiviral activity (Ivanescu *et al*., 2015). Therefore, the aim of this chapter was to determine the anti-inflammatory and antibacterial activities of the isolated compounds using DCFHD-A, bioautography and broth micro-dilution assays respectively.

9.2. Methods and materials

9.2.1. Phytochemical analysis

The chemical profiles of the isolated compounds were analysed on aluminium backed TLC plates (Merck, silica gel 60 F_{254}) using a method developed by Kotze and Eloff, (2002) as described in **section 3.2.3**.

9.2.2. Bioautography

Bioautography was done according to the method described by Begue and Kline (1972) as described in **section 5.2.2.1** and the bacterial species were maintained as outlined in **section 5.2.1**.

9.2.3. Broth micro-dilution assay

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

9.2.4. Cell viability assay

The effect of the isolated compounds the viability of African green monkey (Vero) cells was analysed using tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) as outlined in **section 7.2.1**.

9.2.5. Anti-inflammatory activity assay using DCFHD-A assay

The effect of the isolated compounds on reactive oxygen species generation was measured using the DCFHD-A assay by Sekhar *et al.* (2015) with modicications as outlined in **section 7.2.2**. This time the cells were exposed to 100 µL of the compounds (80 µg/mL, 31.25 µg/mL and 1.95 µg/mL) and 20 µL of LPS for 24 hours.

9.3. Results

9.3.1. Phytochemical analysis

Figure 9.1 below represent TLC fingerprint of three isolated compounds. The chromatogram revealed three single bands all with a blue colour suggesting similar profiles.

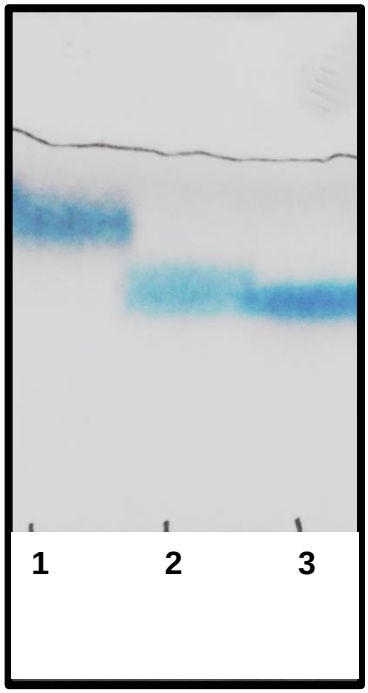

Figure 9.1: Chromatogram of the isolated compounds developed in ethyl acetate: acetone (70:30) and sprayed with vanillin-sulphuric acid. The different colours represent 6-(2, 3-dihydroxyangeloylory) -6α- hydroxyinuviscolide (**1**), Heliangolide (**2**) and Eucannabinolide (**3**).

9.3.2. Bioautography

Bioautography was used to evaluate the antibacterial potential of the three compounds against *P. aeruginosa* (P. a), *E. coli* (E. f), *E. faecalis* (E. f), and *S. aureus* (S. a). All the compounds were active against *P*. *aeruginosa* and *S. aureus*, while none of them was activate against *E. c*oli. Only compound **1** was active against *E*. *faecalis* (Figure 9.2).

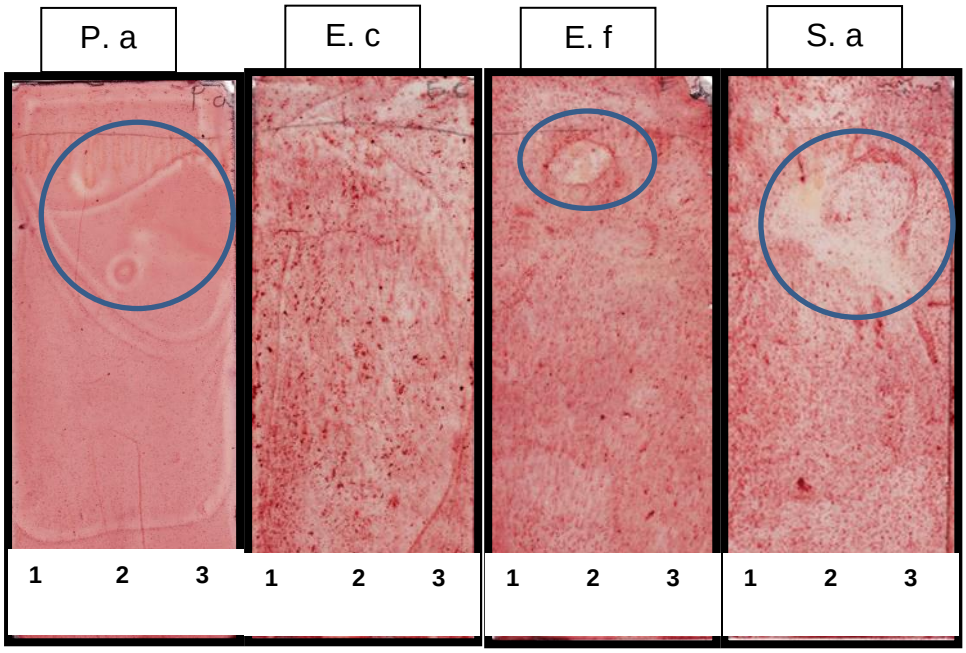

Figure 9.2: Bioautograms of the isolated compounds developed in ethyl acetate: acetone (70:30) and sprayed with *P. aeruginosa* (P. a), *E. coli* (E. f), *E. faecalis* (E. f), and *S. aureus* (S. a). For 6-(2, 3-dihydroxyangeloylory) -6α- hydroxyinuviscolide (**1**), Heliangolide (**2**) and Eucannabinolide (**3**).

9.3.3. Broth micro-dilution assay

The minimum inhibitory concentrations (MIC) values of the isolated compound were determined using the broth micro-dilution assay. The MIC values ranged from 46.8 to >250 µg/mL (Table 9.1). Compound **1** was the most active with the lowest average MIC value (89.8 µg/mL), while compound **2** and **3** were the least with equal MIC values (>250 µg/mL).

Table 9.1: MIC (µg/mL) values of isolated compounds and ampicillin against the four tested bacteria.

Microorganisms	MIC values (µg/mL)				Amp
	1	2	3	Average	(µg/mL)
E. coli	125	>250	>250	**>187.5**	62.5
P. aeruginosa	46.8	250	>250	**>182.3**	31.3
E. faecalis	125	>250	>250	**>208.3**	>250
S. aureus	62.5	>250	>250	**>187.5**	>250
Average	**89.8**	**>250**	**>250**		

Key: 1= 6-(2, 3-dihydroxyangeloylory) -6α- hydroxyinuviscolide; 2= Heliangolide; 3= Eucannabinolide; Amp= ampicillin.

9.3.4. Cell viability

The effect of the isolated compounds on the viability of African green monkey kidney (Vero) cells was determined using MTT assay. Cytotoxicity was represented as the concentrations of the compounds which reduced cell viability by 50% compared to the untreated cells (LC_{50} values) and these values ranged from <10 to 14.2 µg/mL. The selectivity index was used to relate cytotoxicity to the observed biological activities. The values were calculated by dividing the LC_{50} with the MIC values and they ranged from 0.05 to 0.21 (Table 9.2). The values greater than >250 µg/mL were assumed as 250 µg/mL. The compounds were highly toxic to the Vero cells with very low LC_{50} and selectivity index values.

Table 9.2: The LC_{50}, MIC and selectivity index values of the isolated compounds.

Compounds	LC_{50}	MIC values (µg/mL)				Selectivity indexes (SI)			
	(µg/mL)	E.c	P.a	E.f	S.a	E.c	P.a	E.f	S.a
Compound 1	<10	125	46.88	125	62.5	0.08	0.21	0.08	0.16
Compound 2	13.5	>250	250	>250	>250	0.05	0.05	0.05	0.05
Compound 3	14.2	>250	>250	>250	>250	0.06	0.06	0.06	0.06

Key words: E.c= *E. coli*; P.a= *P. aeruginosa*; E.f= *E. faecalis*; S.a= *S. aureus*.

9.3.5. Anti-inflammatory activity assay

The effect of the isolated compounds on the inhibition of the production of ROS was determined to evaluate the anti-inflammatory potential of the compounds on LPS-induced RAW 264.7 macrophage cells. The inhibition of ROS increased in a dose-dependent manner. All the compounds had anti-inflammatory potential, with compound 1 and 3 exhibiting similar effects. Curcumin was used as positive control and it had anti-inflammatory potential at the highest concentration tested (50 µM) (Figure 9.3).

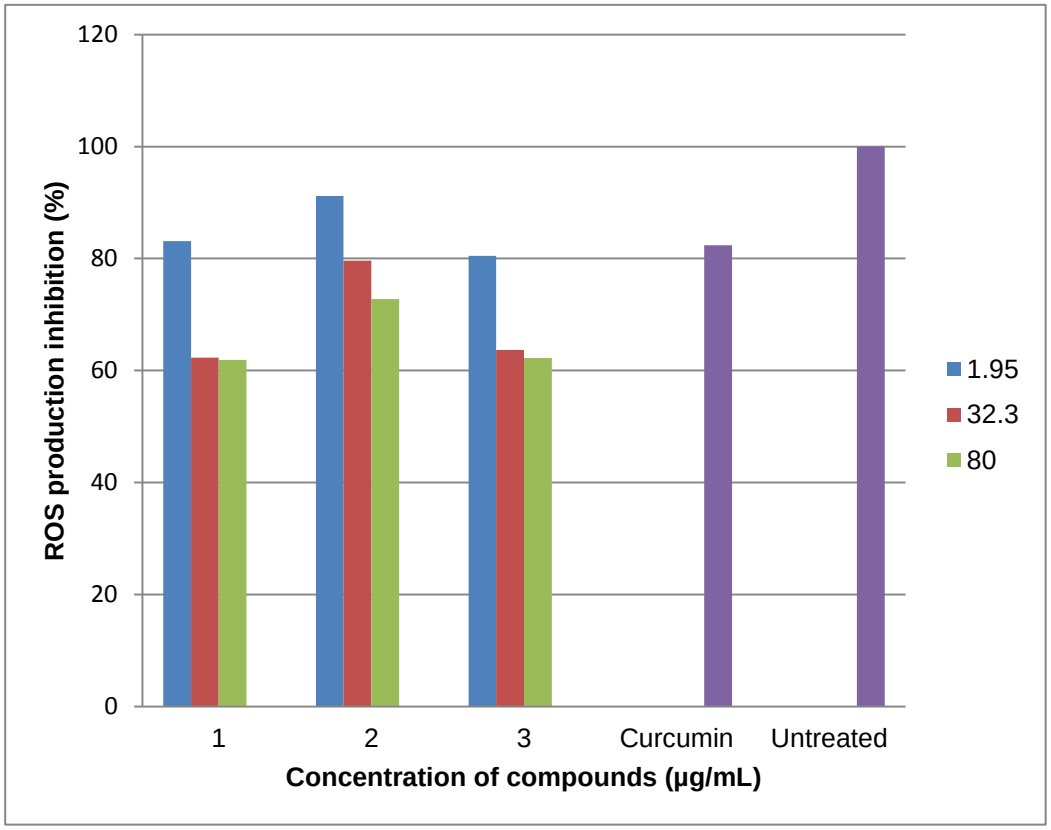

Figure 9.3: The effect of the three isolated compounds on the inhibition of ROS production activity in LPS-induced RAW 264.7 macrophage cells. 6-(2, 3-dihydroxyangeloylory)-6α-hydroxyinuviscolide (**1**), Heliangolide (**2**) and Eucannabinolide (**3**).

9.4. Discussion

The isolated compounds were analysed on thin layer chromatography. The acidic vanillin reaction of all three compounds resulted in different shades of blue (Figure 9.1) indicating compounds with similar profiles. The antibacterial potential of the compounds was evaluated using bioautography and broth dilution methods against *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus.* Although not abundant, this study has demonstrated the antibacterial activity of the three sesquiterpene lactones i.e. guanolide (6-(2, 3-dihydroxyangeloylory)-6α-hydroxyinuviscolide), heliangolide and eucannabinolide against *P. aeruginosa* and *S. aureus*. Meanwhile all of the compounds exhibited no activity against *E. coli*, only 6-(2, 3-dihydroxyangeloylory)-6α-hydroxyinuviscolide exhibited activity against *E. faecalis* (Figure 9.2). Secotanapartholides A and B; sesquiterpene lactones isolated from *Artemisia princeps* var. *orientalis* were also reported to have antibacterial potential against *S. aureus* and no effect against *E. coli* (Ivanescu *et al*., 2015).

The minimum inhibitory concentrations of the compounds ranged from 46.8 to <250 µg/mL (Table 9.1). The most active compound was 6-(2, 3-dihydroxyangeloylory)-6α-hydroxyinuviscolide (**1**) with the lowest average MIC (89.8 µg/mL) against all the tested bacteria, while the least active were heliangolide (**2**) and eucannabinolide (**3**) with equal MIC values (<250 µg/mL). The most susceptible bacteria to the compounds was *P. aeruginosa* (<182.3 µg/mL) followed by *E. coli* and *S. aureus* with equal average MIC values (<187.5 µg/mL). The least susceptible was *E. faecalis* with the highest average MIC value (<208.3 µg/mL). Ampicillin was used as a positive control and its MIC values ranged from 62.5 to <250 µg/mL.

In vitro cytotoxicity assay of the isolated compounds was determined on African green monkey (Vero) cells using MTT assay. This was to ascertain the safety of these compounds for their potential use. MTT assay revealed the cytotoxic effects of the isolated compounds with LC_{50} values ranging from <10 to 14.2 µg/mL. An extract or compound is considered toxic if its LC_{50} is <30 µg/mL after 72 hours of exposure (Itharat *et al*., 2004). Therefore, all the isolated compounds were highly toxic to the monkey Vero cells, with the most toxic compound being compound **1** (<10 µg/mL), followed by compound **2** (13. 5 µg/mL), and compound **3** (14.2 µg/mL). These compounds have a likelihood of exhibiting anticancer properties, as such, this

study recommends that the compounds be screened for anticancer properties. Although the compounds were highly toxic they had a higher LC_{50} compared to doxorubicin (2.29 µg/mL) (Dzoyem *et al*., 2014). It should be noted that cytotoxicity observed *in vitro* is not always encountered *in vivo*. This is probably because when in the biological system, some toxic compounds have the ability to undergo metabolic transformations which leads to the formation of less toxic end products (Nchu *et al*., 2011). As such, this study recommends *in vivo* studies on the isolated compounds so as to evaluate their toxic effects *in vivo* and their genotoxicity on important biomolecules.

The selectivity indexes were used to relate cytotoxicity and antibacterial activities of the isolated compounds. The efficacy of biological activity is considered not to be due to toxicity if the selectivity index is ≥10 (Caamal-Fuentes *et al*., 2011). These values ranged from 0.05 to 0.21 (Table 9.2). As such the observed antibacterial activities of the isolated compounds are likely to be due to toxicity because their selectivity index values were very low. However, McGaw *et al*. (2007) reported that plants containing toxic compounds may have useful biological activities, since toxicity at low doses can be associated with pharmacological activity. Nevertheless, these compounds could be modified chemically and structurally, such that their toxicity is reduced and their biological activities are enhanced to increase their potential for use (Mokoka *et al*., 2013).

All the compounds had anti-inflammatory potential; this was seen by their ability to inhibit the production of ROS in a dose dependant manner on LPS-induced RAW 264.7 macrophage cells (Figure 9.3). Ivanescu *et al*. (2015) also demonstrated the anti-inflammatory potential of guanolides through the inhibition of the expression of nuclear factor κB (NF- κB). As such, these compounds could serve as lead structures for development of anti-inflammatory drugs. Curcumin is a well-known anticancer drug, it was used as a positive control and its effect on ROS production was also demonstrated at the highest concentration tested (50 µM).

9.5. Conclusion

This study has demonstrated the cytotoxicity, anti-inflammatory potential and antibacterial activity against four problematic bacteria of the three sesquiterpene lactones isolated from dichloromethane extracts of *S. pinnata* (Lam.) Kuntze ex Thell. *In vivo* cytotoxicity and more anti-inflammatory activity assays should be done to evaluate the genotoxicity and anti-inflammatory efficacy and possibly determine the mechanism of action exerted by these compounds. This study recommends that anti-cancer activity assays be done on these compounds since literature highlighted the anti-cancer activities of some members of the sesquiterpene lactone class of phytochemicals. Further pharmacological studies of these compounds are essential and significant for possible application as natural plant-based drugs and lead compounds in the pharmaceutical industry.

9.6. References

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Chapter 10: General discussions and conclusions

Discussions

Infectious diseases, oxidative stress, and inflammatory and related disorders are amongst the problematic areas of the healthcare system, because the treatment of the above-mentioned diseases poses challenges. This is due to the constant emergence of resistant pathogenic strains, the toxicity, and adverse side effects associated with the drugs currently used for treatment of such diseases. Screening, isolation and identification of compounds from the plants used in folklore medicine have led to the synthesis of important drugs. Hence, the aim of this study was to isolate compounds with antibacterial and antioxidant activity from selected medicinal plant species and evaluate their cytotoxicity. Although not achieved to satisfaction the following objectives were set to achieve the aim.

10.1. Antibacterial assays to determine bioactive compounds

Commelina africana, *Dombeya rotundifolia*, *Elephantorrhiza elephantina,* and *Schkuhria pinnata* were selected for the purpose of this study based on their traditional uses. The plants were extracted with different solvents of varying polarities to obtain a diversity of compounds. The extracts were examined for the presence of different phytoconstituents and phytochemicals, and antibacterial activities. Terpenoids, flavonoids, tannins, and saponins are amongst the important phytoconstituents which were present in these plants. The total phenolic, tannin, and flavonoid contents present in each plant were determined and were all observed to be high in *D. rotundifolia*. These constituents were reported to have a number of biological activities including antibacterial, antioxidant and anti-inflammatory activities. As such the antibacterial activity of all the selected plants against *E. coli* and *P. aeruginosa* could be as a result of these constituents. Antibacterial activity was demonstrated against all the tested bacteria with the Gram-negative bacteria being more susceptible than Gram-positives.

10.2. Evaluation of interactions in exhibiting antibacterial activity

The plants were evaluated for any interaction in exhibiting antibacterial activities using broth micro-dilution assay. When all the plants were combined in a 1:1:1:1 the efficacy against all the tested bacteria was enhanced with average MIC value (0.28 mg/mL) that is lower than the average MIC values of the plants independently against all the tested bacteria. Nevertheless, potent antibacterial activity was observed against *P. aeruginosa* when *S. pinnata*, *C. africana*, and *D. rotundifolia* were combined in a 1:1:1 (0.06 mg/mL) and when *D. rotundifolia*, *S. pinnata*, and *E. elephantina* were combined in the same ratio (0.07 mg/mL). The combinations of *S. pinnata* and *C. africana* (A+B), *D. rotundifolia* and *S. pinnata* (C+A), and *D. rotundifolia* and *E. elephantina* (C+D) had synergistic effects against *P. aeruginosa*. Meanwhile, Combination A+C and B+C had antagonistic effects against *E. coli*.

10.3. Determination of antioxidant, anti-inflammatory and toxic effects

The plants were evaluated for antioxidant activities using both qualitative and quantitative methods. Both methods demonstrated potent free radical and reducing power antioxidant properties of compounds in *D. rotundifolia* while no significant activity was observed with the other plants. The same plant had potent anti-inflammatory effects against LPS-induced RAW 264.7 macrophage cells by inhibiting the production of reactive oxygen species (ROS) even at the lowest concentrations tested. This plant had the highest concentration of phenolics, tannins, and flavonoids; therefore, the observed activities may be attributed to these phytochemicals. Meanwhile cell viability assays revealed that only *S. pinnata* had toxic effects against African green monkey kidney (Vero) cells with the lowest LC_{50} value (25 μg/mL).

10.4. Characterisation, identification, biological and toxic effects of bioactive compounds

Bioassay guided fractionation on column chromatography aided the isolation and identification of three sesquiterpene lactones from dichloromethane extracts of *S. pinnata*. The compounds were labelled **1**, **2**, and **3** and were identified as guanolides,

heliangolide, and eucannabinolide subtypes of sesquiterpene lactones respectively. Compound **1** was identified as a mixture of two compounds i.e. 6-(2, 3-dihydroxyangeloylory)-6α-hydroxyinuviscolide (major) and an isomer of the major compound at C-9 and C10 (minor). The compounds had potent anti-inflammatory activity and inhibited the production of ROS in a dose dependant manner. Meanwhile, potent antibacterial activities were observed with compound **1** against some of the tested bacteria. The compounds were all toxic to the Vero cells with LC_{50} values that are way below the cut-off point (<30 µg/mL). Their selectivity index values are also poor since they are all below 1. However, the toxicity observed *in vitro* may not always be encountered *in vivo*, since toxic compounds go through biotransformations to yield non-toxic end products (Nchu *et al*., 2011).

10.5. Isolation of antioxidant compounds

Even after countless efforts the separation of the antioxidant compounds in *D. rotundifolia* extracts was not successful. No optimal separation was obtained even when the polarities of the mobile phases were increased (results not shown). Meanwhile the amount/concentration of antioxidant compounds present in *S. pinnata* extracts were too low to be separated on column chromatography. As such, no antioxidant compounds were isolated from any of the selected plants this study.

Conclusions and recommendations

This study has demonstrated the isolation, characterisation, and identification of three antibacterial sesquiterpene lactones. This study demonstrated cytotoxic effects of *S. pinnata* and the isolated compounds on African green monkey kidney (Vero) cells. Therefore, more *in vitro* cytotoxicity assays of both *S. pinnata* acetone extracts and the isolated compounds using different cell lines is recommend. Genotoxicity and *in vivo* studies of these are also recommended to evaluate their toxic effects on important macromolecules before they can be recommended for any use. This study recommends the evaluation of anti-cancer properties of the isolated compounds since members of the sesquiterpene lactones were reported to have anti-cancer properties. The potent anti-inflammatory activities demonstrated should also be evaluated with more *in vitro* and *in vivo* assays to possibly determine the mechanism

of anti-inflammation effect. No antioxidant compounds were isolated because of poor separation. The antibacterial properties of *S. pinnata* and the isolated compounds against the tested bacteria validate the use of this plant for treatment of bacterial infections in traditional medicine. Although, the extracts had better antibacterial activity compared to the isolated compounds, isolation of bioactive compounds is recommended, as these compounds may serve as leads for synthesis and production of less toxic potent antibacterial compounds.

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Nchu, F., Githiori, J.B., McGaw, L.J. and Eloff, J.N., 2011. Anthelmintic and cytotoxic activities of extracts of *Markhamia obtusifolia* Sprague (Bignoniaceae). *Veterinary Parasitology*, *183*(1), pp.184-188.