

**Isolation and characterization of antibacterial compounds from five
selected plants used against bacteria which infects wounds**

by

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DECLARATION

I Maleho Annastasia Lekganyane declare that the dissertation hereby submitted to the University of Limpopo for the degree Master of Science in Microbiology has not been previously submitted by me for the degree at this or any other University, that it is my own work in design and in execution and that all the materials contained therein have been duly acknowledge.

Lekganyane MA (Miss)

Date

DEDICATION

This dissertation is dedicated to my siblings i.e. Conny, Mabore, Nthabi, Legasa and Naniki.

“Education is the best friend. An educated person is respected everywhere. Education beats the beauty and the youth”: Chanakya

“The roots of education are bitter, but the fruit is sweet”: Aristotle

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90/10/1 v/v/v)
CEF	Chloroform/Ethyl acetate/Formic acid (5/4/1 v/v/v)
CHCl ₃	Chloroform
CLSI	Clinical Laboratory Standards
COSY	Correlated Spectroscopy
DCM	Dichloromethane
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
DPPH	2,2, diphenyl-1-picrylhydrazyl
DQCOSY	Double Quantum Correlated Spectroscopy
EMW	Ethyl acetate/Methanol/Water (40/5.4/4 v/v/v)
EtOAc	Ethyl acetate
FMLP	N-formyl-Met-Leu-Phe
gCOSY	gradient Correlated Spectroscopy
gHSQC	gradient Heteronuclear Single Quantum Coherence
H ₂ DCFDA	2, 7-Dichlorodihydrofluorescein diacetate acetyl ester
HIV	Human Immuno-deficiency Virus

INT	<i>p</i> -Iodinitrotetrazolium salts
LPS	Lipopolysaccharide layer
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NADH	Nicotinamide Adenine Dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Bovine Serum
PMA	Phorbol-12-myristate-13-acetate
R _f	Retardation factor
rpm	revolutions per minute
SEE	Serial exhaustive extraction
TLC	Thin Layer Chromatography
TOSCY	Totally Correlated Spectroscopy
UV	Ultraviolet radiation
v/v	volume per volume
WHO	World Health Organization

CONFERENCES AND PROCEEDINGS

Paper presentations

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ABSTRACT

Five plant species: *Ziziphus mucronata*, *Senna italica*, *Lantana camara*, *Ricinus communis* and *Lippia javanica*, were selected for this study based on their use in traditional medicine. In preliminary screening, crude extracts were prepared using hexane, dichloromethane (dcm), acetone and methanol. Phytochemical profiles on Thin Layer Chromatography plates of the extracts were obtained by developing the plates in mobile phases of varying polarity. Tests for compounds such as tannins, flavonoids, alkaloids, phlobatannins, terpenes, steroids, cardiac glycosides and saponins were carried out. Antibacterial activity of the extracts was carried out using microdilution assay for Minimum Inhibitory Concentration and bioautography against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Antioxidant activity of the extracts was performed using the 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay and Phagoburst test were used to investigate the toxic effects and anti-inflammatory activity of the extracts on mouse Raw 264.7 macrophage cells, respectively. The presence of phytochemicals was observed on the chromatograms after the plates were sprayed with vanillin sulphuric acid reagent. The dcm extracts of the plants showed antibacterial activity against the selected bacterial species on the bioautograms. *Senna italica* and *Z. mucronata* showed the most activity bands on the bioautograms. *Lippia javanica* had the lowest MIC average of 0.56 mg/ml. Antioxidant activity was observed in the extracts of *L. javanica* and *R. communis*. The extracts promoted proliferation of the mouse macrophage cells Raw 264.7 at concentrations ranging from 0.31 mg/ml and 0.08 mg/ml. *Senna italica* leaves were selected for isolation of antibacterial compounds. The isolated compound was analysed on ^1H and ^{13}C nuclear magnetic resonance (NMR) and Mass Spectrometry (MS) for structural analysis. The structure could not be elucidated due to impurities in the compound but the tentative structure is a branched chain alkane with at least one ether linkage per repeating unit. Therefore the study shows that there are plant components with biological activities against wound infecting bacteria and a single lead compound was identified.

CHAPTER 1

1. Introduction

Medicinal plants form part of the major sources of new drugs and other medical products. Sofowora (1982) described medicinal plants as plants which contain substances in one or more of its organs which can be used for therapeutic purposes or substances which can act as precursors for the synthesis of useful drugs. In developing countries, medicinal plants still play an important role in health and in 2002 the World Health Organisation (WHO, 2002) estimated that about 80% of the population in these countries rely on plants for their health care needs. In developing countries there is better acceptance of medicinal plant usage and people believe that the medicine is better compatible with the body and that the plants have fewer side effects as compared to pharmaceuticals (Bansal *et al.*, 2011). Plants have been used to treat diseases such as Human Immuno-deficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS), malaria, diabetes, sickle-cell anaemia, mental disorders and microbial infections among others in developing countries (Elujoba *et al.*, 2005; Okigbo *et al.*, 2005). Most people are integrating traditional medicine with drugs (Fennel *et al.*, 2004).

In South Africa, herbal medicines form part of the culture and tradition of the locals. Most of the people in South Africa both in rural and urban areas rely on herbal medicines for their health care. Herbal medicine is more affordable, accessible and is significant in the locals' cultural beliefs. South Africa has a rich diverse botanical heritage with most of the diversity endemic to the country (Mulholland, 2005). In addition to the botanical diversity, South Africa has a cultural diversity with traditional healing being integral to each ethnic group (van Vuuren, 2008).

The history of medicinal plant use which has accumulated over the years has allowed scientists to isolate a wide variety of drugs which are currently in use. The use of plant derived drugs in modern medicine was as a result of the use of plants as a cure in traditional or folklore medicine. Studies have been carried out around the world on the presence of active compounds in plants and these studies have revealed the presence of antimicrobial compounds in higher plants (Sharma *et al.*, 2012).

Phytochemical research is aimed at studying new plant species and elucidation of the structure. Developments in chromatography methods and spectroscopy led to major breakthroughs in initial phytochemical screening and further isolation, purification and identification of molecular structure (Santana *et al.*, 2012). Screening of plant extracts and isolation of the active compounds require certain steps be taken in order to end up with a pure isolated compound. During the screening process, chromatography is performed. In chromatography, compounds with different characteristics are separated based on their different distribution between two phases, one being the mobile phase and the other being the stationary phase. The stationary phase is the solid phase while the mobile phase can be a liquid or gas. There are several types of chromatographic techniques that can be used and which are named based on the phases used. When screening plant extracts, liquid chromatography is used, where the mobile phase is a liquid and this is called Thin Layer Chromatography (TLC) (Cannell, 1998; Gloppstad, 2010).

For structural analysis of the isolated compound, nuclear magnetic resonance (NMR) spectroscopy is employed. In this technique, the number of specific magnetic atoms in a compound is studied i.e. hydrogen-1 and carbon -13 nuclei. The technique is based on the principle that every nucleus has a charge and spin property, both of which generate a distinct signature magnetic field which is itself affected by the applied magnetic fields (Pavia *et al.*, 2001).

Studies on the antimicrobial properties of plants have helped scientists to identify components responsible for the activity and for development of drugs intended for therapeutic use in human beings (Mahesh and Satish, 2008). A survey has shown that 25% of pharmaceuticals were derived from plant secondary metabolites (Muruganatham, 2012). There is a great diversity of chemical compounds found in higher plants with different biological activities (Muruganatham, 2012). Scientific literature reveals that secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids possess antimicrobial properties (Okpekon *et al.*, 2004).

The random use of commercial antimicrobial drugs used to treat infectious diseases has led to the development of multiple drug resistant strains of some bacteria. Antimicrobial resistance is a global crisis which has created an interest among scientists to find new and alternative drugs from natural sources such as plants. The

chemical diversity of natural products provides an opportunity to find new compounds which can be the basis for formulating new drugs (Sermakkani, 2012).

For antimicrobials to be considered as suitable candidates these should either act as inhibitors of microbes or kill them and at the same time be minimally or non-toxic to the host (Saxena and Sharma, 1999). Organized screening of the plants results in the discovery of new efficient compounds.

Aromatic plants have not only been used as food but also to treat various ailments. Isolated essential oils and natural volatile compounds derived from secondary metabolites are known to have antibacterial and antifungal properties. The oil make-up is based on a balanced composition of various compounds (Bakkali *et al.*, 2008; Edris, 2007). The oil composition can change as a result of differences in environmental, chemical, seasonal, geographical and genetic conditions (Alviano and Alviano, 2009).

Wound healing is a complex process which consists of several steps which are inflammation, proliferation and tissue remodelling (Midwood *et al.*, 2004; Nguyen *et al.*, 2009; Stadelmann *et al.*, 1998). Inflammation is the first step in many pathological conditions and it occurs as a result of the release of inflammatory mediators such as histamine, kinins, serotonin and prostaglandin. The search for anti-inflammatory agents looks for substances which can inhibit the release of inflammatory mediators (Charde *et al.*, 2010). Morshed *et al.* (2011) considers inflammation as a defence mechanism which protects the body against infection, burn, toxic chemicals, allergens or other stimuli. The problem with inflammation is that at times it can become prolonged and if uncontrolled can lead to chronic illnesses (Kumar *et al.*, 2007). The mediators and processes involved with inflammation can induce, stabilize or worsen many diseases (Sosa *et al.*, 2002). A number of plants with medicinal properties were found to have anti-inflammatory activity (Sourabie *et al.*, 2012).

Traditional medicine is now being targeted for affordable, readily available treatment of wounds. Many of the plants used for treatment of wounds in South Africa have not been scientifically validated although there are many plants being used in this regard (Pather and Kramer, 2012). Wound healing starts from the time of injury and continues for varying time depending on the extent of the wound. A number of

antibiotics have been developed to treat different wound types and others such as ointments and wound dressings have also been developed to treat severe skin wounds or other skin conditions such as ulcers, bedsores and bum wounds (Machida *et al.*, 1997). As a result of adulteration, poor drug combination and inactivation of the antibiotics by enzyme producing bacteria, there is increasing bacterial resistance to some of the drugs currently used in wound healing (Momoh *et al.*, 2012).

In South Africa, the prescription and use of traditional medicine is not regulated which may lead to danger of misadministration, in particular of toxic plant extracts. Another concern about the use of herbal medicines is prolonged use which may lead to genotoxic effects. People use plants because they assume that they are safe only based on their long use in treating various diseases according to knowledge accumulated over centuries. However, scientific studies have shown that plants used in traditional medicine are potentially toxic, mutagenic and carcinogenic (Fennel *et al.*, 2004).

The increase in bacterial resistance experienced during treatment of wounds dictates a great need for the search for novel drugs to accelerate the wound healing process. Hence, the need to explore medicinal plants as a rich resource for more effective natural products to improve the wound healing process.

CHAPTER 2

Literature review

2.1. Medicinal plants

For thousands of years plants have provided a source of medicinal agents for humans. Plants have been used for a long time to treat and prevent various ailments. A large number of drugs have been isolated from plants based on their use in traditional medicine. However, the introduction of pharmaceutical medicines and the advancement in science have not stopped the massive use of medicinal plants (Chah *et al.*, 2006).

The World Health Organization (WHO) reported that 80% of the world's population today still depends on the use of traditional medicine for their health needs (WHO, 2002). Rural communities in developing countries are still forced to use traditional medicines for treatment of their daily ailments, because of poverty and unavailability of modern health facilities. In areas where the use of traditional medicine is still in practice, indigenous knowledge preserves information on the use of plants against different ailments (Muthu *et al.*, 2006). The use of ethnomedicine in many African communities is still in practice, mainly because most of them live far from health services and cannot afford the cost of antibiotics (Steenkamp *et al.*, 2004).

South Africa has a rich flora with around 24 000 species consisting more than 10% of the world's vascular plant flora. These plants produce a vast array of secondary metabolites which act as defence against herbivores, diseases and parasites. There is about 250 000 plant species in the world and only 5-15% of these are predicted to have potential biologically active compounds. About 60% of the people in South Africa use traditional medicine, in addition to using pharmaceuticals (McGaw and Eloff, 2008). High unemployment rate and increased HIV infections have resulted in traditional medicines being sold in the market place in South Africa (Shai *et al.*, 2008).

Plants and their extracts have immense potential for the management and treatment of wounds. The phyto-medicines for wound healing are not only cheap and

affordable, but are also safe as hyper-sensitive reactions are rarely encountered with the use of these agents (Raina *et al.*, 2008).

2.17. Medicinal plants as sources of new drugs

The discovery of plants as sources of medicinal products may have been made in prehistoric times when humans observed the behaviour of animals who fed on particular plants (Kay, 1993). The use of plant parts in medicine continued during the middle ages and during this era the treatment was based on the use of the whole plant or some plant parts such as leaves, bark, seeds, berries or fruits (Legoabe, 2004).

At some time during the nineteenth century, compounds isolated from plants were modified in such a way that they had fewer side effects and increased therapeutic effects (Legoabe, 2004). The use of pure synthetic products expanded during the 1950s and is still the most common source of drugs used in therapy. These plant derived compounds are used in different dosage forms and formulations such as tablets, powders, syrups, solutions etc., to treat different diseases (Albanese, 2003).

Scientists study medicinal plants with aims of isolating bioactive compounds to be used directly as drugs and to produce compounds which will serve as a basis for synthesising drugs with high activity and lower toxic effects. An estimated 122 drugs from 94 plant species have already been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001). In South Africa, there is potential for discovering new and useful compounds which may serve as leads for synthesis of new drugs (McGaw and Eloff, 2008).

2.18. Secondary metabolites in medicine

Plants contain secondary metabolites derived from primary metabolites and these secondary metabolites are only present in certain plant species. Secondary metabolites do not play a role in primary metabolism but rather function as defence for the plants or play a role in giving colour to flowers which may attract pollinators (Putnam and Rice, 1983).

The recent discovery of new drugs such as the anticancer drug epothilone, the immunosuppressant rapamycin, or the proteasome inhibitor salinosporamide has made the search for bioactive natural products a fascinating one. Scientists use

secondary metabolites as prerequisites for new drugs and now more than ever finding new secondary metabolites is an urgent task due to the rapid spreading of bacterial resistance and emergence of multidrug resistant strains (Dickschat, 2010). Figure 2.1 is a schematic representation of the steps used by scientists to identify and isolate potential antimicrobial compounds.

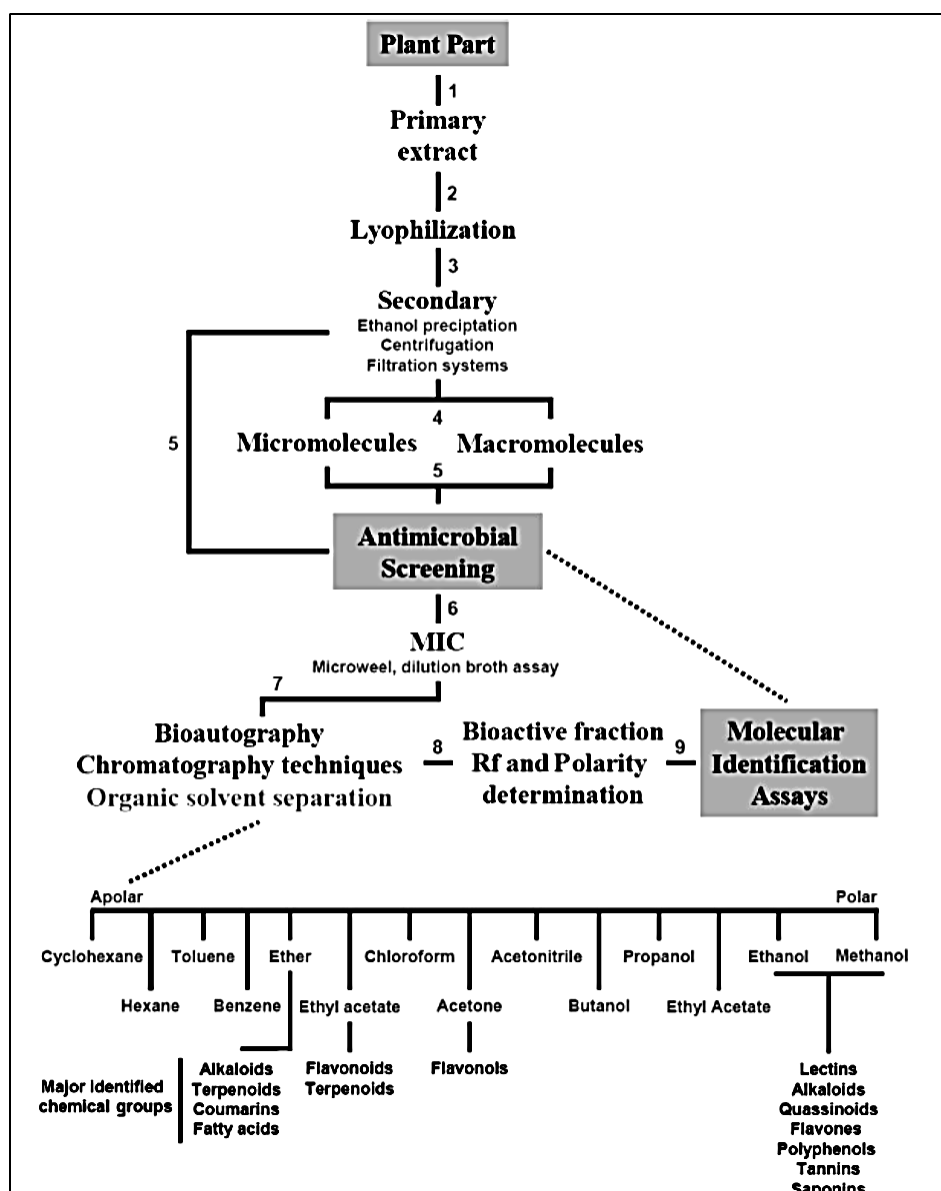


Figure 2.1: Flowchart of the process of drug discovery from medicinal plants (Mendonça-Filho, 2006).

2.18.1. Types of secondary metabolites

2.18.1.1. Tannins

These compounds are of oligosaccharide origin, containing a number of phenolic groups that can react with water to form water insoluble copolymers (Figure 2.2).

Polymerisation of quinone units can also lead to formation of tannins. Cowan (1999) associated tannins with physiological activities such as anti-infective action. In addition, Scalbert (1991) demonstrated toxicity of tannins towards filamentous fungi, yeast and bacteria. Their mechanism of action is associated with the formation of hydrogen bonds, hydrophobic interactions and covalent bonds with protein complexes. Brownlee *et al.* (1990) suggested that tannins can inactivate microbes directly.

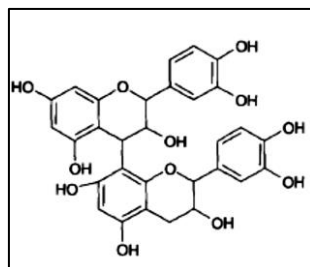


Figure 2.2: Structure of tannin with antimicrobial activity (Legoabe, 2004).

2.18.1.2. Flavonoids

Flavonoids are a group of compounds characterized by a series of C6-C3-C6, i.e. their carbon skeleton consists of two C6 group connected by an aliphatic chain (Figure 2.3). Flavonoids that are more lipophilic in nature may disrupt microbial membranes (Tshuchiya *et al.*, 1996). They are low molecular weight compounds and are widely distributed through the plant kingdom. Flavonoids have a number of bioactivities associated with them including antimicrobial activity (Hernandez *et al.*, 2000). The ability of flavonoids to complex with extracellular and soluble proteins as well as bacterial cell walls are thought to be responsible for their bioactivity (Tshuchiya *et al.*, 1996). In addition to antimicrobial activity, flavonoids have antioxidant, antimutagenic, diuretic and antiviral properties (Peterson and Dwyer, 1998).

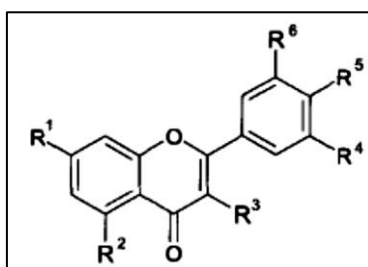


Figure 2.3: Structure of flavonoids with antimicrobial activity (Legoabe, 2004).

2.2.1.3. Terpenoids

Terpenes/terpenoids and their corresponding oxygenated isoprenoid derivatives and analogues are products of medicinal and aromatic plants and together constitute essential oils. Terpenes have diverse structures and they range from relatively simple linear hydrocarbon chains to highly complex ring structures (Figure 2.4). The compounds are essentially in plants because they facilitate plant-plant, plant-insect and plant-pathogen interactions (Back and Chappell, 1996). Terpenes from *Petalostemum purpureum* have shown antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* with little activity against Gram-negative bacteria including *Candida albicans* (Hufford *et al.*, 1993).

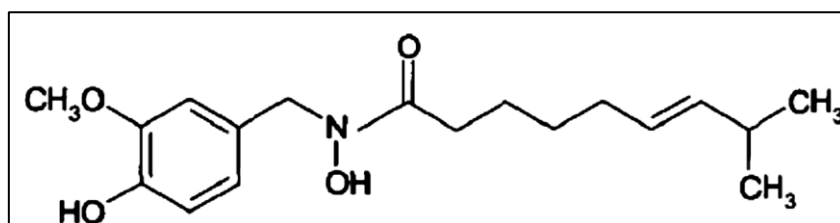


Figure 2.4: General structure of terpenoids which has shown to have antimicrobial activity (Legoabe, 2004).

2.3.1.4. Alkaloids

Alkaloids are nitrogen-containing heterocyclic compounds occurring mainly in plants as salts of common carboxylic acids such as citric, lactic, oxalic etc. (Robinson, 1983). Alkaloids also contain oxygen in addition to carbon, hydrogen and nitrogen (Figure 1.5). In the pharmaceutical industry it is important to know the solubility of alkaloids and their salts (Trease and Evans, 1989). Alkaloids are well known for their pharmacological activities such as analgesic, antihypertensive, anticancer, spasmolytic, antitussive, expectorant, stimulant and antimicrobial activities (Cieřła and Waksmundzka-Hajnos, 2009). They were also found to be active against both Gram-positive and Gram-negative bacteria and fungi (Dweck, 2002).

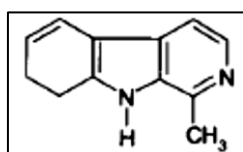


Figure 2.5: Structure of an alkaloid with antimicrobial activity (Legoabe, 2004).

2.3.1.5. Saponins

Saponins are secondary metabolites that occur naturally as glycosides possessing a wide variety of pharmacological properties. Their distribution is mainly in higher plants but they have been also found in animals such as marine invertebrates (Francis *et al.*, 2002). Saponins (Figure 2.6) can occur as one to three straight or branched sugar chains (Vincken *et al.*, 2007).

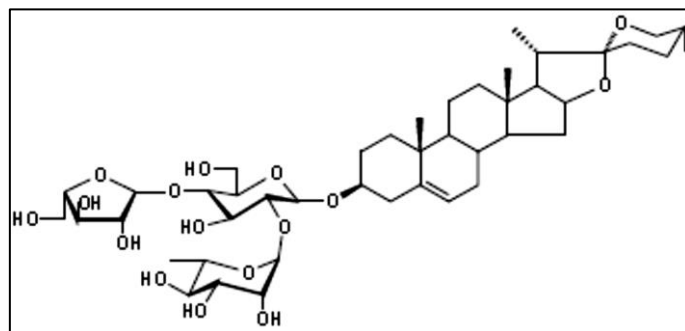


Figure 2.6: Structure of a saponin (Podolak *et al.*, 2010).

2.4. Antimicrobial activity

The introduction of antibiotics in medicine significantly reduced the reliance of western countries on using plants as medicine and also had an impact on African and Asian countries. This meant that pharmaceutical companies then had to rely on bacteria and fungi for the production of antibiotics (Cowan, 1999). When these antibiotics were introduced people believed that infections could be controlled and eventually mastered, but in recent years more and more pathogens have acquired resistance towards the commonly used antibiotics (Williams, 2002). Eloff (1998a) suggested that the problem of drug resistance could be solved by using plant compounds which have different mechanisms of action as compared to the current antibiotics used. Drugs from plants have more therapeutic ability as they can effectively treat infectious diseases while at the same time produce less side effects as compared to synthetic chemical drugs (Tomoko *et al.*, 2002). The presence of antibacterial, antifungal and other biological activities have been demonstrated in extracts of different plant species used in traditional medicine practices (Shai *et al.*, 2008). These include, for example, compounds such as diterpenoid found in *Jatropha zeyheri*, which showed antibacterial activity against *Streptococcus*

pyogenes and some fungi. Tannins, found in a number of plants used in wound healing, were also found to have antibacterial activity (Luseba *et al.*, 2007).

2.5. Bacterial resistance

Bacteria are able to alter their genetic make-up in a way that allows them to transmit and acquire resistance to drugs used as therapeutic agents. In addition to antibacterial resistance, the emergences of new strains which are multidrug resistant have resulted in increased mortality rates in immunosuppressed patients in hospitals (Nascimento *et al.*, 2000). In developing countries, problems such as poverty, lack of knowledge, inaccessibility of health facilities, civil wars and bad leadership in governance have seriously limited the aid of these drugs in treating infectious diseases despite the impact the drugs have made in saving peoples' lives in developed countries (Mendonça-Filho, 2006).

Multi-drug resistant isolates of tuberculosis, acute respiratory infections and diarrhoea often referred to as poverty diseases have emerged especially in developing countries (Mendonça-Filho, 2006). There is an increasing number of patients whose immune system has been compromised by the HIV/AIDS epidemic in developing countries with over 30 million cases (Mendonça-Filho, 2006). Patients with the disease are at a greater risk of acquiring a number of infections and, because of long periods of hospitalization, they are at an even greater risk of acquiring highly resistant organisms (Mendonça-Filho, 2006).

Antibacterial resistance is increasing and it is not certain what the future holds with regard to drug development. In order to deal with drug resistance, systems must be put in place to control and carry out research that will provide better understanding of the genetics behind the resistance, and to develop new drugs either synthetically or naturally with the ultimate goal to provide patients with appropriate and efficient antimicrobials (Nascimento *et al.*, 2000).

2.6. Anti-inflammatory activity

Wounds are accompanied by inflammation, which results in swelling, reddening of the edema and surrounding tissue. Inflammation is a biological process that may occur as a result of the release of hormones such as histamine, kinins, serotonin and prostaglandin. Inflammation is a way to prevent blood loss by forming

aggregation of blood platelets, which leads to coagulation and result in the formation of a thrombus (Chowdhury *et al.*, 2009). The transcription factor NF- κ B is a central protein regulating the transcription of many inflammatory and proinflammatory genes encoding COX-2, cell adhesion molecules or growth factor receptors. Because of its central role in regulating inflammatory responses, pharmacological inhibition of NF- κ B activation *in vivo* may be beneficial in the treatment of inflammation, including those connected to wounds (Schmidt *et al.*, 2009).

Anti-inflammatory agents target the inflammatory mediators such as histamine, serotonin and prostaglandins (Charde *et al.*, 2010). Most plants that are used in wound healing have been found to possess active compounds which have anti-inflammatory activity (Ilavarasan *et al.*, 2006). Procyanidin, that has an antioxidant effect, has been used treating in inflammatory diseases (Grim *et al.*, 2004). This compound has peroxy-nitrite scavenging properties, which condense tannin oligomers and can protect endothelial cells from damage. Flavonoids from leaves, roots and seed oil of *Ricinus communis* have also been reported to have anti-inflammatory activity (Luseba *et al.*, 2007).

2.7. Anti-oxidants

Damaged cells produce free radicals as a result of respiration, thus the wound area would be rich in reactive nitrogen species (RNS) and reactive oxygen species (ROS) with their derivatives. The presence of these free radicals result in oxidative stress, leading to lipid peroxidation, DNA breakage and enzyme inactivation. Evidence has shown that anti-oxidants can be used in the treatment of many diseases caused by accumulation of free radicals. Topical application of compounds with radical scavenging activity has been shown to improve wound healing and protect tissues from oxidative damage (Thiem and Grosslinka, 2003).

Reactive oxygen species are involved in a number of degenerative diseases such as atherosclerosis, cancer, cirrhosis and diabetes and also in wound-healing. Plant-derived antioxidants such as tannins and lignans could delay or prevent the onset of degenerative diseases, because of their redox properties. This allows them to act as hydrogen donors, reducing agents, hydroxyl radicals (OH) or super-oxide radical (O_2^-) scavengers (Marwah *et al.*, 2006). Etsuo (2010) suggested that most

researchers nowadays focus on the role of antioxidants in maintaining good health because it is believed that oxidative stress causes various disorders.

One of the actions of anti-oxidants against free radicals is chain breaking. When a free-radical takes an electron, a second radical is formed. To generate other radicals, the same process takes place and is terminated by stabilization of a free radical by a chain breaking radical such as β -carotene, vitamins C and E. Another action of the antioxidants is prevention of oxidation. The action is carried out by anti-oxidant enzymes, which prevent oxidation by reducing the rate of chain initiation. These antioxidants scavenge initiating radicals, thus stopping an oxidation chain from ever setting in motion (World Cancer Research Fund, AICR, 1997). These can also prevent oxidation by stabilizing transition metal radicals such as copper and iron. The effectiveness of any antioxidant is dependent on the type of free radical involved (Leonard *et al.*, 2002).

2.8. Toxicity of plant extracts

Prolonged use of more popular herbal remedies may potentially lead to genotoxic effects (Fennel *et al.*, 2004). Populations who stay in poor areas who are reliant on medicinal plants are at a great risk of encountering toxicity effects of plants as well as long term effects of the continued use of medicinal plants. Therefore, it is important to evaluate these effects in order to know as to whether people can continue using medicinal plants for their needs without being at risk of the long term toxic effects of these plants (Bagla, 2011).

2.9. Interaction of plant compounds

Plants produce a number of different compounds which they use as defence against different microorganisms and herbivores. Therefore this means that plants are regarded as potential sources of structurally diverse bioactive compounds as well as antimicrobial agents. Plants antimicrobials may be less effective acting on their own but be more effective when working synergistically with other compounds from other plants. This mode of defence is known as synergy where two different compounds are combined to enhance each other's individual activity. In a case where one compound inhibits the action of another compound, the phenomenon is called antagonism (Hemaiswarya *et al.*, 2008; Rani *et al.*, 2009).

Plants contain antimicrobials that are able to function in synergism with commercial antibiotics or they enable antibiotics which were ineffective against bacteria to have activity (Betoni *et al.*, 2006). It is believed that combining antimicrobial agents might promote the effectiveness of each agent, and this may mean that the dose of each drug is reduced (Williamson, 2001).

2.10. Isolation of bioactive compounds

Plants contain a large diversity of natural products which may serve as potential drugs of the future (Cos *et al.*, 2006). Isolation of compounds such as morphine in 1861, from the opium poppy, *Papaver somniferum* led to many other drugs which were effective pain killers (Benyhe, 1994). Recent studies by Mathur, *et al.* (2010; 2011) have shown that plant derived drugs have beneficial aspects as good sources of antibiotics, antioxidants and anti-inflammatory agents.

During the isolation process, natural plant chemists can work together with microbiologists to develop bioassays for monitoring and purification of new compounds. These bioassays help in early de-selection of already known compounds. The activity spectrum which is carried out during the isolation process helps in de-selecting toxic-compounds which then allows one to focus on biologically active compounds. Therefore, instead of focusing only on novelty, one can use novelty and potential when isolating biologically active compounds (Houghton, 2001). Bioassays such as bioautography can be used during bioassay guided isolation of bioactive compounds as this method requires less sample material and it simplifies the identification and isolation processes (Rahalison *et al.*, 2007).

In order to isolate bioactive compounds, one first needs to select the plant species of interest. The problem with the selection is the unavailability of the plant extracts as most of them are found in rain forests. And today, rain forests and their ecosystems are disappearing because of economic factors such as industrialisation and urbanisation. People living in tropical countries rely on plant extracts as sources of medicine as it is readily available and because of the high price of modern medicine. It is therefore important to investigate and isolate these traditional medicinal plants before the knowledge of traditional healers disappears (Adou, 2005).

Excessive collection and exploitation of medicinal plants has resulted in researchers focusing on plants with economic value and importance when carrying out their ethnobotanical and ethnomedicinal investigations with the aim of meeting the demand for herbal products (Laloo *et al.*, 2000). Plants are affected by factors such as climate change, specificity in terms of land for growth, grazing, land use disturbances et cetera, some of which may lead to genetic drift (Kala, 2000, 2003; Oostermeijer *et al.*, 2003). Kala and Sajwan (2007) found that the population decline of the majority of high valued medicinal plants is caused by exploitation and loss of habitats.

2.11. Bacteria associated with wounds

Wounds are open skin areas which are prone to infection. Some of the bacteria most commonly associated with wound infections, especially nosocomial infections include the following:

2.11.1. *Escherichia coli*

Escherichia coli (Figure 2.7) is a Gram-negative, rod-shaped, facultative anaerobic bacterium. It is non-spore forming and motile, and lives in the intestines. Most sub-species are harmless. However, virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections and neonatal meningitis. In rare cases these virulent strains are also responsible for haemolytic-uremic syndrome and mastitis. *E. coli* has been associated with a number of postoperative wound infections (Todar, 1996).

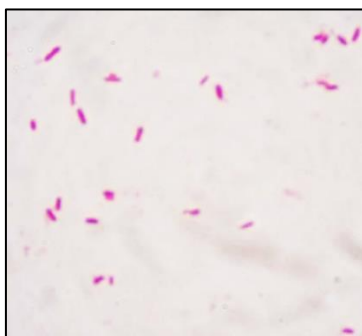


Figure 2.7: *E. coli* cells viewed under conventional light microscope (Todar, 2009).

2.11.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (Figure 2.8) is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. It is a free-living bacterium, commonly found in soil

and water, and also inhabits the surfaces of plants and animals. This bacterium has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance (Todar, 2005). Several different epidemiological studies track its occurrence as a nosocomial pathogen (Martineau and Davis, 2007). It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections and bacteraemia (Todar, 1996).



Figure 2.8: *P. aeruginosa* cells viewed under conventional microscope (Todar, 2009).

2.11.3. *Staphylococcus aureus*

Staphylococcus aureus (Figure 2.9) is a Gram-positive coccus, non-motile, non-spore forming facultative anaerobe. It is frequently part of the skin flora found on the nose and on skin. This bacterium can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome and septicaemia. It is still one of the four most common causes of nosocomial infections, often causing post-surgical infections (Todar, 2005).

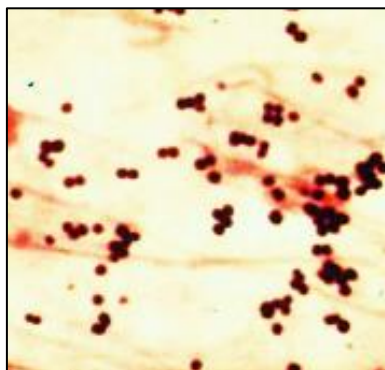


Figure 2.9: *S. aureus* cells viewed under conventional light microscope (Todar, 2009).

2.11.4. *Enterococcus faecalis*

Enterococcus faecalis (Figure 2.10) is a Gram-positive, non-motile and facultative anaerobic commensal bacterium. It inhabits the gastrointestinal tracts of humans and other mammals. *Enterococcus faecalis* can cause life-threatening infections in humans, especially in the nosocomial environment where the natural high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity (Ryan and Ray, 2004). It can also cause endocarditic as well as bladder, prostate and epididymal infections (Pelletier, 1996).

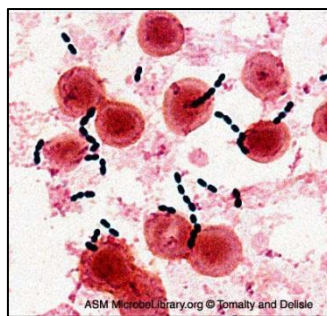


Figure 2.10: *E. faecalis* cells viewed under conventional light microscope (Todar, 2009).

2.12. Wound healing process

A wound is defined as the disruption of the cellular and anatomic continuity of a tissue (Sharma *et al.*, 2013). A wound may be produced by physical, chemical, thermal, microbial or immunological effects on the tissue. The process of wound healing consists of integrated cellular and biochemical events, leading to reestablishment of structural and functional integrity with regain of strength of injured tissue. Clinically, one often encounters non-healing, under-healing or over-healing. Therefore, the aim of treating a wound is to either shorten the time required for healing process or to minimize the undesired consequences (Myers *et al.*, 1980).

Wound healing is an important biological process involving tissue repair and regeneration. This can be classified into any of three types – healing by first intention, healing by second intention or healing by third intention, depending on the nature of the edges of the healed wounds. In wounds healed by the first intention, the edges are smoothly closed so that no scar is left. On the other hand, wound

healing by second intention involves formation of granulation tissues, which fill up the gaps between the wound edges and is associated with significant loss of tissue, leaving little scars. Wounds healed by third intention, are usually those left open for three to five days until granulation bed falls before they are stitched, generally resulting in extensive scar formation (Esimone *et al.*, 2005).

There are four distinct stages involved in wound healing, namely: inflammatory stage, debridement stage, proliferation stage/maturation or remodelling stage (Suresh Reddy *et al.*, 2002). When an injury occurs, the vascular integrity of the injured area is disrupted leading to extravasations of blood into the surrounding tissue or plasma when the damage is minor. The inflammatory stage is directed at preventing further loss of blood by platelet adhesion/accumulation at the site, leading to coagulation that result in the formation of thrombus. The debridement stage occurs from the third to the sixth day after injury and involves the appearance of neutrophils to clear contaminating organisms (Quinn, 1998). The proliferation or repair stage is characterized by endothelial budding in the nearby blood vessels, forming new capillaries that penetrate and nourish the injured tissue. The maturation stage commences from the tenth day to several months, depending on wound severity, during which the number of capillaries decreases and wound changes from pink to white (Panduraju *et al.*, 2010).

2.13. The use of plants in wound healing

Medicinal plants in wound healing are responsible for disinfecting the wound, for debridement and providing a moist environment that resemble the environment for natural healing process (Purna and Babu, 2000). In a study carried out by Karodi *et al.* (2009), the application of *Rubia cordifolia* extract, accelerated the wound healing and repair and this was explained by the anti-inflammatory effects of the plant. The study showed that there was an enhanced rate of wound contraction and a noticeable reduction in healing time than the control without the extract. Several drugs of plant origin were found to increase healing of different types of wounds (Biswas and Mukherjee, 2003).

There has been an increased interest in wound healing agents by biomedical scientists and traditional healers especially in countries like China and India where there is little knowledge on which plants are used in wound healing (Kumar *et al.*,

2007). In Africa and Asia, scientists have been investigating potential traditional medicine for the treatment of wounds and related disorders (Krishman, 2006). A number of medicinal plants have been found to be effective when used to treat wounds. Such plants have provided a basis on which new medicinally useful compounds can be isolated and their structure elucidated. In this regard, combining modern and traditional knowledge, better drugs for wound healing with fewer side effects can be produced (Nagori and Solanki, 2011).

2.14. The need for scientific validation

The use of plants as medicine is based on folklore knowledge passed on from one generation to another. The usages are based on instructions from traditional doctors and herbalists without any scientific validation (Kumar *et al.*, 2007). In today's world there is a need for scientific validation, standardization and safety evaluation of plants used traditionally as medicine (Raina *et al.*, 2008). Scientists are now faced with the problem of trying to prove if indeed there are active components within plants which are useful, active and safe (Rukangira, 2003). In an attempt to prove their effectiveness, scientists are carrying out studies on plants' biological activity and isolate the active compounds. Evaluating plant extracts for pharmacological activity allows for the development of cheap drugs to be used in poor regions (de Souza *et al.*, 2004).

Scientists have found a number of compounds with inhibitory effects during *in vitro* screening methods against various types of microorganisms. These methods are significant in research as they provide insight in terms of validating the plant use in traditional medicines (Mendonça-Filho, 2006). Using scientific methods to validate the medicinal properties of plants will form a basis for their ultimate use as an alternative form of medicine or when Western medicine is failing (Hutt and Houghton, 1998).

2.15. Plants selected for this study

The following plants were selected based on their use by traditional healers for wound healing.

2.15.1. *Ziziphus mucronata*

Buffalo thorn (Figure 2.11) as it is commonly known, is distributed in summer rainfall areas throughout sub-Saharan Africa, extending from South Africa northwards. The plant grows in areas dominated by thorny vegetation in both temperate and tropical climates. It is also found in a wide range of habitats such as woodlands and open scrubland. It reaches its largest size on the margins of scrub forests and on deep, alluvial soils near water. Its presence is said to indicate the presence of underground water. A decoction of the glutinous roots is commonly administered as a painkiller for all sorts of pains, as well as dysentery. A concoction of the bark and the leaves is used for respiratory ailments and other septic swellings of the skin. Root and leaf pastes can be applied to treat boils, swollen glands, wounds and sores. In East Africa, roots are used for treating snake bites (Hutchings *et al.*, 1996). All of the above can be attributed to the peptide alkaloids and antifungal properties isolated from the bark and leaves (Van Wyk *et al.*, 1997).



Figure 2.11: Leaves and flowers of *Ziziphus mucronata* (http://www.ukzn.ac.za/bcs/images/Medicinal/Ziziphus_mucronata).

2.15.2. *Lantana camara*

Lantana camara L. (Figure 2.12) (Verbenaceae) is a native shrub of America and Africa, and has been cultivated as an ornamental plant in other countries. The plant has been reported to possess a number of medicinal properties (Kurian, 1995). Various parts of the plant are used in the treatment of a number of ailments, including itches, cuts, ulcers, swellings, eczema and wound healing (Mahmood *et al.*, 2009). The juice extract from the leaves of the plant has been found to enhance wound contraction (Kurian, 1995). *Lantana camara* has some toxicity associated with it, which includes nephrotoxicity, hepatotoxicity, and photosensitization (Raina *et al.*, 2008).



Figure 2.12: Leaves and flower of *Lantana camara* (www.fao.org).

2.15.3. *Lippia javanica*

Lippia javanica (Figure 2.13) is a woody shrub, which stands erect and is multi-stemmed. The stems have a square appearance when looked at in cross-section. The leaves are hairy with noticeable veins and when crushed gives off a strong lemon-like smell. It is one of the most aromatic of South Africa's indigenous shrubs. The small cream flowers can be found on the shrub from summer to autumn in some areas and in others are produced all year. These flowers are arranged in dense, rounded flower heads. The fruit are rather inconspicuous, small and dry. Skin disorders such as heat rash and other rashes, as well as scratches, stings and bites can also be treated. In such instances fever tea is usually cooled and then applied like a lotion (van Wyk *et al.*, 1997). Fever tea contains high concentrations of terpenoids which are released as volatile oils. Seven compounds from leaves, stems and flowers have been identified through chemical analysis with the predominant one being the monoterpene 3-methyl-6-(1-methylethylidene-cyclohex-2-en-1-one (piperitenone) (van Wyk *et al.*, 1997).



Figure 2.13: Leaves and flowers of *Lippia javanica* (Maharaj *et al.*, 2008).

2.15.4. *Ricinus communis*

Ricinus communis (Figure 2.14) also known as castor bean is cultivated for the seeds, which yield fast-drying, non-yellowing oil and is used mainly in industries and medicines. The oil and seed have been used as folk remedies for warts, cold tumours, indurations of the abdominal organs, whitlows, lacteal tumours, indurations of the mammary gland, corns, and moles, etc. Castor-oil is a cathartic and has labour-inducing properties. Ricinoleic acid has served in contraceptive jellies. Ricin, a toxic protein in the seeds, acts as a blood coagulant. Oils are used externally for dermatitis and eye ailments. Seeds also contain the alkaloids ricinine and toxalbumin ricin, and are considered to be purgative, a counter-irritant in scorpion stings and as a fish poison. Leaves are made into paste applied to the head to relieve headache and can be used as a poultice for boils (Dweck, 2002; Duke and Wain, 1981).



Figure 2.14: Leaves and fruits of *Ricinus communis* (Worbs *et al.*, 2011).

2.15.5. *Senna italica*

Senna italica (Figure 2.15) is native to many African countries. The leaves, pods and mature seeds of *Senna italica* are used as a purgative. They are taken, usually as a decoction or maceration, to cure stomach complaints, fever, jaundice, venereal diseases, biliousness, and as a treatment against intestinal worms. The leaves, either fresh or dried and pulverized, are used as a dressing for skin problems such as burns and ulcers. A tea made from the flowers is used as a purgative and to induce labour. A maceration of the roots is taken to cure influenza, and boiled roots are used as wound dressing. A root infusion is used as eye drops for sore eyes. The roots also enter in treatments of indigestion, liver complaints, and gall bladder disorders, nausea, vomiting and dysmenorrhoeal (<http://www.database.prota.org>).



Figure 2.15: Leaves and flowers of *Senna italica* ([database.prota.org](http://www.database.prota.org)).

2.16. Purpose of study

2.16.1. *Aim*

The aim of this study the biological properties of the selected plants described above against bacteria which are associated with wounds and to isolate and characterize compounds with antibacterial activity.

2.16.2. *Objectives*

The objectives of this study are to:

- i. obtain crude extracts of the five plants using four solvents of varying polarity;
- ii. analyze the chemical profile of the extracts using vanillin/ sulphuric acid reagent;
- iii. investigate the antibacterial activity of the extracts using the microplate method for inhibitory concentration and bioautography;
- iv. study the antioxidant activity of the extracts using the Thin Layer Chromatography- 2,2-diphenyl-2-picrylhydrazyl (TLC-DPPH) assay;
- v. study the cytotoxic effects of the plants on Raw 264.7 cell line using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay;
- vi. study the anti-inflammatory activity of the extracts using Phargoburstt kit;
- vii. isolate the bioactive compounds using column chromatography;
- viii. identify the isolated compounds using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS).

CHAPTER 3

Phytochemical screening of the plant extracts

3.1. Introduction

After selection of the plants of interest, certain phytochemicals such as alkaloids, flavonoids, tannins, steroids, terpenoids or terpenes, phlobatannins and saponins, are screened using selective tests. Although it is easy to perform these tests, one can also get false results (Farnsworth, 1966; Segelman *et al.*, 1968). During screening, scientists cannot relate one compound to a certain biological activity as compounds falling within the same class have varying activities (Fabricant and Farnsworth, 2001).

During extraction, medicinally active portions of a plant are separated by use of selective solvents through standard procedures. The method for extraction involves separating active compounds from inactive ones using selective solvents. The solvents in the extracts diffuse into the material and solubilize the compounds of the same polarity (Ncube *et al.*, 2008). Although traditional healers use water for extraction, research has revealed that extracts from organic solvents have more consistent antimicrobial activity when compared with the water extracts (Das *et al.*, 2010).

Thin Layer Chromatography (TLC) is a separation technique that has been used for ages in laboratories all over the world. Thin layer chromatography (TLC) can be used for quantitative, qualitative as well as preparative separation. Although TLC has set backs when used as an analytical technique, it cannot be substituted by any other technique when it comes to working with plant extracts. The technique offers an advantage over the other separation techniques as it involves less laborious sample preparation and it is less time-consuming. In addition, there is less sensitivity loss, a number of samples can be analysed at once because of the easy assays involved and it is less expensive (Handa *et al.*, 2008).

Phytochemical methods are used as a tool to study the different secondary metabolites present in the plants which may be responsible for the plants' efficacy (Ayodele, 2003). Secondary metabolites include saponins, tannins, essential oils,

alkaloids, flavonoids and other biologically active components (Sofowora, 1993). Plants produce secondary metabolites for repair processes and protection in their natural environment (Bako *et al.*, 2005).

3.2. Methodology

3.2.1. Plant collection

The selected plants (*Ziziphus mucronata*, *Lippia javanica*, *Lantana camara*, *Senna italica* and *Ricinus communis*) were collected from the University of Limpopo's Turfloop Campus and identification was validated at the University of Limpopo's Herbarium. The plants were stored at room temperature in a well-ventilated room for drying. After drying the leaves were ground to fine powder which was then stored in airtight containers.

3.2.2. Extraction procedure

The ground leaves (1 g) were separately extracted with 10 ml of different solvents of varying polarity (hexane, dichloromethane (DCM), acetone and methanol), using 50 ml centrifuge bottles. Extraction on each of plant material was carried out three times per solvent. The extracts were filtered into universal bottles. The filtrates were placed under a fan to evaporate the solvents.

3.2.3. Phytochemical analysis

The plant extracts were dissolved in acetone to give a final concentration of 10 mg/ml. For each plant, 10 ml of 10 mg/ml was loaded on aluminium-backed TLC plate and the plate was developed in three solvent systems i.e. benzene/ethanol/ammonium solution (18:2:0.2) [BEA] (non-polar/basic): chloroform/ethyl acetate/formic acid (10:8:2) [CEF] (intermediate polarity/acidic): ethyl acetate/methanol/water (10:1.35:1) [EMW] (polar neutral) (Kotze and Eloff, 2002). The plates were viewed under UV light (254 and 365 nm) for compounds which are fluorescent and later sprayed with vanillin-sulphuric acid reagent and heated to visualize colours of the different compounds from each plant.

3.2.4. Tests for compounds

The leaves of the selected plants were examined for the presence of alkaloids, tannins, saponin, steroids, terpenes, flavonoids, phlobatannin and cardiac glycosides.

3.2.4.1. Alkaloids

The Drangendoff's reagent method described by Harborne (1973) was used. Ground and powdered leaves (0.2 g) were extracted with 95% ethanol in a Soxhlet extractor for six hours and the ethanolic extract was evaporated to dryness using a vacuum evaporator at 45°C. The residue was redissolved in 5 ml of 1% HCl and 5 drops of Drangendoff's reagent was added. Colour change was observed to draw inference.

3.2.4.2. Saponin

The persistent frothing test for saponin described by Odebiyi and Sofowora (1978) was used. One gram of the powdered leaf sample was suspended in 30 ml tap water. The mixture was vigorously shaken and heated. The sample was observed for the formation of froth to draw inference.

3.2.4.3. Phlobatannin

The powdered leaf sample (0.2 g) was dissolved in 10 ml of distilled water and filtered. The filtrate was boiled with 2% HCl solution. The sample was observed for the formation of a coloured precipitate to draw inference (Borokini and Omotayo, 2012)

3.2.4.4. Tannins

The method of Trease and Evans (1989) was adopted. Powdered leaf samples (0.5 g) were dissolved in 5 ml of distilled water, then boiled gently and cooled. The solution (1 ml) was put in a test tube and 3 drops of ferric chloride solution was added. The sample was observed for a blue-black, green or blue-green colour to draw inference.

3.2.4.5. *Terpenes/ terpenoids*

The Salkowski test was used to test for presence of terpenes. The powdered leaf sample was mixed in 2 ml of chloroform, and 3 ml concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. The sample was observed for a colour change to draw inference (Sofowora, 1982).

3.2.4.6. *Steroids*

Acetic anhydride (2 ml) was added to 0.5 g powdered leaf of each plant sample, and was followed by the addition of 2 ml of sulphuric acid. The sample was observed for a colour change to draw inference (Borokini and Omotayo, 2012).

3.2.4.7. *Cardiac glycosides*

The Keller-Killani test was used. The powdered leaf sample (5 mg) studied was treated with 2 ml of glacial acetic acid, containing one drop of ferric chloride solution. This was underplayed with 1 ml of concentrated sulphuric acid. The sample was observed for colour changes to draw inference (Sofowora, 1982).

3.2.4.8. *Flavonoids*

Diluted ammonia (5 ml) solution was added to a portion of the aqueous filtrate of each plant extract, followed by addition of concentrated sulphuric acid. The sample was observed for colour changes to draw inference (Borokini and Omotayo, 2012).

3.3. Results

3.3.1. Extraction process

The selected plants (1 g) were extracted with four solvents of varying polarity. The graph (Figure 3.1) represents the mass of the extracts in different solvents. Methanol was the best extractant which yielded more compounds from all plant extracts followed by hexane and DCM extracts with the least yield.

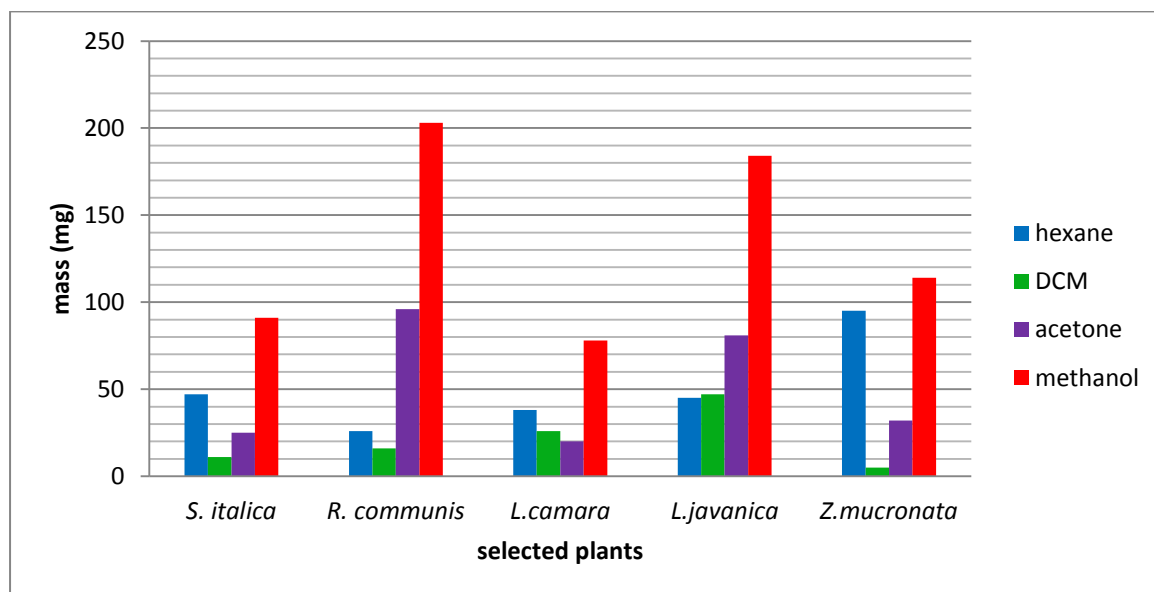


Figure 3.1: Histograms showing the mass of the plants extracted using different solvents.

3.3.2. Phytochemical analysis

Thin layer chromatography was used to analyze the phytochemical composition of the extracts. The different colours on the plates (Figure 3.2) were observed after spraying the plates with vanillin-sulphuric acid reagent. More bands were observed on the plates for BEA followed by bands separated by EMW while CEF had the least bands.

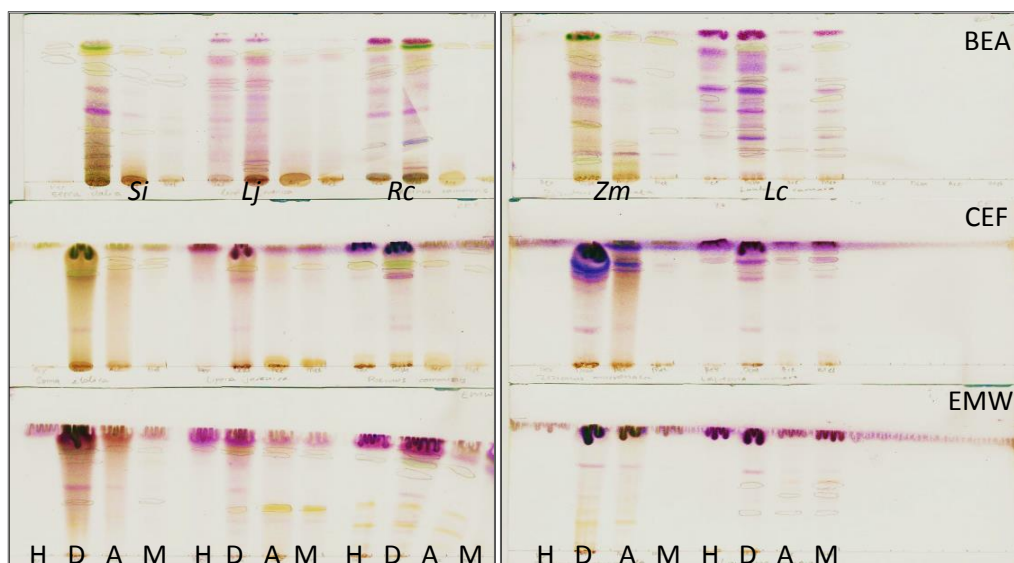


Figure 3.2: Chromatogram showing the different compounds in the plants after the plates were sprayed with vanillin-sulphuric acid reagent. Key: H-hexane; D-dichloromethane; A- acetone: M- methanol. Si- *Senna italica*; Lj- *Lippia javanica*; Rc- *Ricinus communis*; Zm- *Ziziphus mucronata*; Lc- *Lantana camara*.

3.3.3. Tests for compounds

Different tests were conducted to test for the presence of different compounds. Table 3.1 below represents the different compounds which were tested. *Senna italica* and *Lippia javanica* tested positive for most of the compounds. Compounds such as steroids, flavonoids, tannins, and cardiac glycosides were present in all the plants, while none of the plants tested positive for phlobatannins.

Table 3.1: Presence of different phytochemical constituents of the selected plants

Compounds	RC	LJ	SI	LC	ZM
Steroids	+	+	+	+	+
Terpenes	+	+	+	-	-
Flavonoids	+	+	+	+	+
Saponins	-	-	-	-	+
Phlobatannin	-	-	-	-	-
Tannins	+	+	+	+	+
Alkaloids	-	+	+	+	-
Cardiac glycosides	+	+	+	+	+

Key: RC: *Ricinus communis*; LJ: *Lippia javanica*; SI: *Senna italica*; LC: *Lantana camara*; ZM: *Ziziphus mucronata*. (+): presence; (-): absence.

3.4. Discussion

Plants have been used traditionally for years to treat various ailments. The plants used in this study were selected because they are used by traditional healers for treatment of wounds. Extraction of the active compounds from the selected plants was carried out using different solvents even though the majority of traditional healers use water because it is a readily available solvent and it is non-toxic. Researchers avoid using water as an extractant because it does not extract non polar active compounds (Masoko *et al.*, 2008). In addition to that, researchers prefer to use solvents of varying polarity because they will collect compounds of varying polarity from the crude material (Eloff, 1998; Kotze and Eloff, 2002). After extraction, all the extracts were redissolved in acetone as it was found to be non-toxic to bacteria (Eloff, 1998b) and fungi (Masoko *et al.*, 2007). Methanol was found to be the best extractant with all the plants extracting more polar compounds (Figure 3.1). This may be due to the polar compounds present in the leaves or the season in which the leaves were collected wherein the leaves contained more carbohydrates (Bagla, 2011).

The extracts were loaded on TLC plates and developed in different mobile phases of varying polarity i.e. BEA, CEF and CEF to separate the compounds based on their polarity. The different colours separated on the plates in Figure 3.2 represent the diversity of compounds within the leaves. The different colours developed after the plates were sprayed with vanillin-sulphuric reagent. This means that the plants contain steroidal compounds since the spray reagent used detects steroidal compounds (Manana, 2003). The number of bands on each plate shows the quantity and polarity of the compounds depending on the mobile phase used, for example more bands were separated on the BEA plate meaning that these compounds are non-polar. Techniques such as TLC are important during the earliest stages of screening because they allow for localization and identification of compounds which can be targeted for isolation (Hostettmann *et al.*, 1999).

Plants contain many biomolecules which play different roles in the plants. In addition, these biomolecules may be distributed in different plant parts and may also differ in distribution among plant species (Bako *et al.*, 2005). The presence of phytochemicals within extracts depends on the time of cultivation, and the time of

harvest (Nalawade and Tsay, 2003), and this means that phytochemical screening must be done frequently even on the plants whose phytochemical composition is known (Borokini and Omotayo, 2012). Table 3.1 shows the different compounds that were tested in the selected plants. A number of the selected plants contained phytochemicals such as tannins, flavonoids, steroids and cardiac glycosides. The results show that the presence of phytochemicals varies from plant to plant. *Ziziphus mucronata* was the only plant which tested positive for saponins. The presence of saponins was confirmed by the presence of a consistent froth when boiled (Odebiyi and Sofowora, 1978). Saponins are believed to be responsible for anti-inflammatory activity and wound healing activity (Onike, 2010). A study conducted by Onwuliri and Wonang (2003) revealed that saponins have antifungal properties. When saponins interact with cells, they act as surface active agents and disrupt or change the penetrability of the cell wall which then facilitates entry of poisonous materials or cause vital constituents of the cell to leak (Onwuliri and Wonang, 2003).

The presence of alkaloids was observed by the formation of an orange precipitate and was detected in *L. javanica*, *S. italica* and *L. camara*. Alkaloids have pharmacological activity on malignant diseases, malaria and diuretic diseases (Trease and Evans, 1989). Alkaloids have been ranked as one of the most effective and medicinally substantial plant components (Harborne, 1973). All the plants tested positive for steroids and their presence was confirmed by the formation of a blue colour (Borokini and Omotayo, 2012). A study by Singh *et al.* (2012) revealed the antimicrobial activity of steroidal compounds. In addition to antimicrobial activity, steroids can control carbohydrate metabolism and also have anti-inflammatory activity (Borokini and Omotayo, 2012).

Flavonoids were present in all the plants and they were confirmed by the formation of a yellow precipitate. This group of compounds are produced by the plant in response to microbial infection and have shown activity against a number of microbes (Harborne, 1973). Manikandan *et al.* (2006), reported biological activity such as antioxidant, antimicrobial and anticarcinogenic properties associated with flavonoids.

Phytochemicals such as flavonoids and tannins are common in plant parts such as leaves, flowers, stems as well as barks (Larson, 1988). These compounds are

responsible for development and as defence against contagion and damage. The colours that are seen on the flowers, leaves and fruits are provided by flavonoids (Heldt, 1997). The presence of phytochemicals may account for their medicinal efficacy (Ashafa *et al.*, 2010).

3.5. Conclusion

The results from the preliminary screening show the phytochemical diversity present within the plant extracts. The presence of these compounds serves as a foundation for further studies on these to determine their specific biological activities and the identification and isolation of potential wound healing compounds.

CHAPTER 4

Biological assays

4.1. Introduction

The use of plants is a practice all over the world and it was revealed by Verpoorte in 2000 that only 6% was studied for biological activity while 15% has been studied phytochemically using trial and error method (Verpoorte, 2000). Researchers who are working with traditional healers to identify medicinally useful plants, were able to discover 122 drugs from 94 plant species (Fabricant and Farnsworth, 2001). Many plant species have been studied and it was found that they have medicinal importance. Plants are used as alternative forms of medicine but their use is not being monitored for safety by health professionals therefore it is relevant to investigate their biological activities (Houghton *et al.*, 2005).

The right bioassays are required to screen for natural products that are biologically active. The reliability and sensitivity of the test system selected can be used to detect biologically active compounds in plant extracts. In activity-guided fractionation, bioassays are important in that they allow for monitoring of the active substances in the fractions until the active compounds are isolated and purified (Hostettman *et al.*, 1995).

A number of assays are used to study biological activity of potential natural products *in vitro* first and later be evaluated *in vivo*. These biological activities include antibacterial, anti-inflammatory and antioxidant activity etc. During *in vitro* testing, a standard drug is usually included in the tests to check the effectiveness of the natural product. Recommendation and usage of traditional medicine in South Africa is not regulated which leads to improper use, in particular those with toxic effects (Fennel *et al.*, 2004).

The rising bacterial resistance towards antibiotics as a result of abuse and over prescription, means that new drugs are needed (Leggadrio, 1995). Plants have provided a medicinal source for years and are potential sources of antimicrobial agents (Cowan, 1999). Instead, there has been success in developing plant extracts, especially those that can be used to treat skin infections (Eloff *et al.*, 2005). Bacterial organisms that have mostly been used in bioassays are *Staphylococcus aureus*,

Pseudomonas aeruginosa, *Enterococcus faecalis* and *Escherichia coli* species because they are the major cause of nosocomial infections in hospitals (Sacho and Schoub, 1993), and are mainly the strains recommended for use by the National Committee for Clinical Laboratory Standards (NCCLS, 1992).

Tests used for evaluating antimicrobial activity must be simple, quick, reproducible and cheap and increase the throughput so that one can cope with different number of extracts and fractions (Hostettman *et al.*, 1997). Methods employed are based on broth dilutions for inhibitory determinations which were standardized and recommended by Clinical Laboratory Standards (CLSI) (NCCLS, 2003c). A quantitative method developed by Eloff (1998a) to obtain the minimum inhibitory concentration (MIC) of the plant extracts is used to determine their antibacterial activity. Serial two-fold dilutions of the extracts are prepared in a 96 well microtiter plate and the bacterial culture is added before overnight incubation. Bacterial growth is observed by addition of a tetrazolium salt usually p-iodinitrotetrazolium violet (INT) which turns to a coloured formazan by respiring cells. The lowest concentration with decreased colour intensity is recorded as the MIC value (Eloff, 1998a). This microdilution method can be used for both screening and bioassay guided isolation of antimicrobial compounds (Eloff *et al.*, 2008). Microdilution assay for minimum inhibitory concentration can also be used to determine the MIC values of plant extracts against fungi (Masoko *et al.*, 2005). After obtaining the MIC value, one gram of the dried plant material is divided by the MIC value to give the total activity of the plant. Total activity written in ml/g, shows the largest volume to which the bioactive compounds in one gram of plant material can be diluted and still inhibit bacterial growth (Eloff, 2000).

The Thin Layer Chromatography (TLC) technique called bioautography is also used in bioassays to determine the antimicrobial properties of plant compounds. The method was described by Begue and Kline in 1972 where plant extracts are separated by TLC and the plates are placed under a stream to evaporate the eluent. The chromatograms are sprayed with actively growing cultures and incubated overnight. The chromatograms are sprayed with tetrazolium violet after incubation. Areas on the plate where there is active growth of the organism are observed is purple or red in colour and inhibition of growth is represented by clear zones on the

plates. Masoko and Eloff (2005) discovered that it is difficult to use bioautography for fungi as they grow slowly and there is a risk of contamination. Bioautography allows for localization of active compounds and is also effective during fractionation for bioassay-guided isolation of active compounds present in an extract (Eloff *et al.*, 2008).

People use medicinal plants because they believe that they are safe based on the history of usage. Contrary to that, scientific studies have shown that most of the plants used in traditional medicine can be deadly, mutagenic and cancerous (Schimmer *et al.*, 1994). Therefore, it is important to evaluate both bioactivity and safety of plants if they are to be prescribed for human purposes (McGaw and Eloff, 2008). Plant extracts are tested for toxicity towards mammalian cells by a method described by Mosmann (1983) where 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used. Toxicity is observed by a colour change from yellow MTT solution to dark blue formazan crystals that cannot permeate through cell membranes (Mosmann, 1983).

Medicinal plants can be screened for radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay method (Blois, 1958; Deby and Margotteaux, 1970). The DPPH method relies on the electron donating ability of plant compounds i.e. the antioxidant molecule decolourises the DPPH molecule by donating an electron to it (Krishnaiah *et al.*, 2011). A number of inflammatory diseases may arise as a result of active oxygen-induced and free-radical-mediated oxidation of biomolecules, membranes, and tissues. As soon as free radicals are formed, a series of chain reactions take place unless antioxidants or other free-radical scavengers quench them. Compounds that react with free radicals and protect adjacent structures from oxidation damage are called antioxidants (Jukić *et al.*, 2012).

Inflammation is one of the immune responses to bacterial infection. Free radicals are produced by cells as a defence mechanism. These free radicals act by killing bacteria but because they are released into the surrounding environment they often cause damage to cells, usually acute inflammation. Methods that are used to detect anti-inflammatory activity of molecules usually involve growing mammalian cells in the presence of bacterial cells or using lipopolysaccharide layer as stimulants of free

radical production. Molecules with antioxidant activity will be able to scavenge free radicals to prevent damage to cells and consequently inflammation (Jukić *et al.*, 2011).

4.2. Methods and materials

4.2.1. Antibacterial activity

4.2.1.1. Quantitative antibacterial activity assay by minimum inhibitory concentration (MIC)

Four bacterial species were used for the assay, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922. Each organism were inoculated into 100 ml of nutrient broth, incubated for 24 hrs at 37°C and was used as the stock culture. The organisms (10 ml from stock culture) were further inoculated into 100 ml of nutrient broth and incubated at 37°C for 24 hrs. The plant residues were dissolved in acetone to give a final concentration of 10 mg/ml and 100 µl of the plant extract was serially diluted (50%) with water in a 96 well microtitre plate. Hundred microlitres of bacterial culture was added to each well. The plates were incubated at 37°C for 24 hrs. Similar dilution of ampicillin was used as the positive control. Another well with only water and the test organism was used as the negative control. After incubation, 40 µl of p-iodinitrotriazolium violet (INT) dissolved in water was added to each of the microplate wells to detect growth. Bacterial growth inhibition is indicated by the reduction of the purple colour (Eloff, 1998a).

4.2.1.2. Qualitative antibacterial activity assay by Bioautography

The bioautography method by Begue and Kline (1972) was used. Thin Layer Chromatography plates were cut and loaded with 20 µl (10 mg/ml) of the plant extract dissolved in acetone. The plates were developed in the solvent systems as used above in phytochemical analysis (section 3.2.3.). After development, the plates were placed under a stream of air for a period of five days to allow the solvents to evaporate. The four bacterial species used in microdilution assay were used as the test organisms. The plates were sprayed with the cultures until they were just wet and then incubated at 37°C for 24 hrs. After incubation, the plates were sprayed with

INT and incubated for 30 minutes. The plates were observed for bacterial growth inhibition.

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

Thin Layer Chromatography plates were used to separate extracts as described earlier (section 3.2.3). The plates were dried in the fumehood. To detect antioxidant activity, chromatograms were sprayed with 0.2% 2,2-diphenyl-2-picrylhydrazyl (DPPH) in methanol, as an indicator. The presence of antioxidant compounds was detected by yellow spots against a purple background on TLC plates sprayed with 0.2% DPPH in methanol as described by Deby and Margotteaux, 1970.

4.2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Raw 264.7 cells in 100 µl of Dulbecco's Modified Essential Medium (DMEM) (growth medium) were plated in 96 well plates. The cells were treated with 50 mM of plant extracts dissolved in dimethyl sulfoxide (DMSO). The treated cells were incubated at 95% humid air for 24 hrs at 37°C. A 20 µl solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/ml in PBS) was added to each of the wells. The plates were incubated for 4 hrs at 37°C in the dark. The growth medium was removed or aspirated and 100 µl of acidic DMSO was added and mixed. The plate was incubated for an additional 1 hr at 37°C in the dark. The plate was read using a microtiter plate reader and the OD was measured at 570 nm at a background wavelength of 630 nm.

4.2.4. Phagoburst test for inflammatory activity

Bursttest kit (ORPEGEN Pharma) for the quantification of the oxidative burst activity of Raw 264.7 cells was used to test for inflammatory activity. The cells were mixed and aliquoted on the bottom of 5 ml tubes. The cells were incubated in an ice bath for 10 minutes to cool them down to 0°C before adding the lipopolysaccharide (LPS). For activation, the precooled lipopolysaccharide was well mixed and 20 µl of the LPS was added to cells in tube #1. In tube #2 (negative control), 20 µl of washing solution was added. In tube #3 (low control), 20 µl of *N*-formyl-Met-Leu-Phe (FMLP) working solution was added. Phorbol-12-myristate-13-acetate (PMA) working solution (20 µl)

was added in tube (#4) for high control. All the tubes were mixed once more. The samples were incubated for 10 minutes at 37°C in a water bath. After incubation, 20 µl of substrate solution was added and vortexed thoroughly. The tubes were incubated for another 10 minutes at 37°C in a water bath. At the end of incubation, all the samples were taken out of the water bath simultaneously. The cells were lysed and fixed with 2 ml pre-warmed 1× lysing solution. The sample was vortexed and incubated for 20 minutes at room temperature. The tubes were centrifuged for 5 minutes at 250×g and 4°C and the supernatant was discarded. The samples were washed once with 3 ml washing solution for 5 minutes at 250×g and 4°C. The supernatant was decanted. For DNA staining, 200 µl of DNA staining solution (2, 7-Dichlorodihydrofluorescein diacetate acetyl ester (H₂DCFDA)) was added. The samples were vortexed and incubated at 0°C. The cell suspension was measured within 30 minutes. The cells were analysed by flow cytometer using the blue-green excitation light (488 nm, argon-ion laser).

4.3. Results

4.3.1. Antibacterial activity

4.3.1.1. Microdilution assay

The values in the tables represent MIC (Table 4.2) and total activity (Table 4.3) values of the plant extracts against the selected bacteria. *Lippia javanica* was the most active plant and *S. aureus* was the most sensitive microorganism.

Table 4.1: MIC values of plant extracts in mg/ml.

Organisms	<i>S. i</i>	<i>L. j</i>	<i>R. c</i>	<i>Z. m</i>	<i>L. c</i>	Amp(µg/ml)
<i>E.coli</i>	1.25	0.64	1.25	0.53	-	0.16
<i>E. faecalis</i>	1.25	0.64	0.84	1.05	-	0.16
<i>P.aeruginosa</i>	0.84	0.32	-	-	-	0.13
<i>S. aureus</i>	0.63	0.64	-	0.53	-	0.08
Average	0.99	0.56	1.05	0.70	-	0.13

Key: --→ no activity. *S.i*- *Senna italica*; *L.j*- *Lippia javanica*; *R.c*- *Ricinus communis*; *Z.m*- *Ziziphus mucronata*; *L.c*- *Lantana camara*.

Table 4.2: Total activity of the plant extracts (ml/g).

Organism	<i>S. italica</i>	<i>L. javanica</i>	<i>R. communis</i>	<i>Z. mucronata</i>	<i>L. camara</i>
<i>E.coli</i>	20	127	77	60	-
<i>E. faecalis</i>	20	127	114	30	-
<i>P.aeruginosa</i>	30	253	-	-	-
<i>S. aureus</i>	40	127	-	60	-
Average	28	159	96	50	-

Key: --→no activity.

4.3.2.2. Bioautography

Clearing bands observed on the bioautograms (Figure 4.1 to Figure 4.4) indicate zones of bacterial growth inhibition. For all the bacteria, BEA plates had the most bands, with no bands or a few bands observed in CEF and EMW. *Ziziphus mucronata* and *S. italica* were the most active against the selected bacteria.

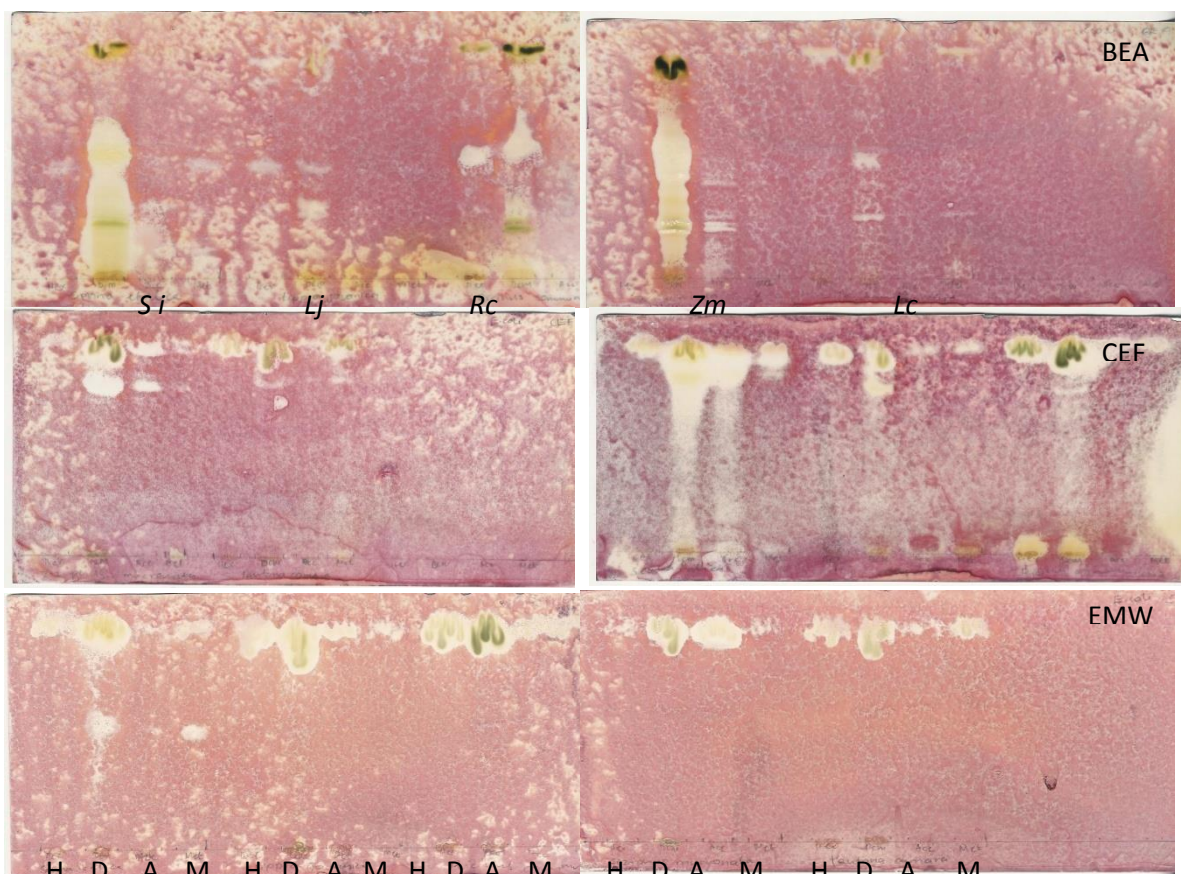


Figure 4.1: Bioautogram of different leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *E.coli*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of a compound(s) that inhibited the growth of *E. coli*.

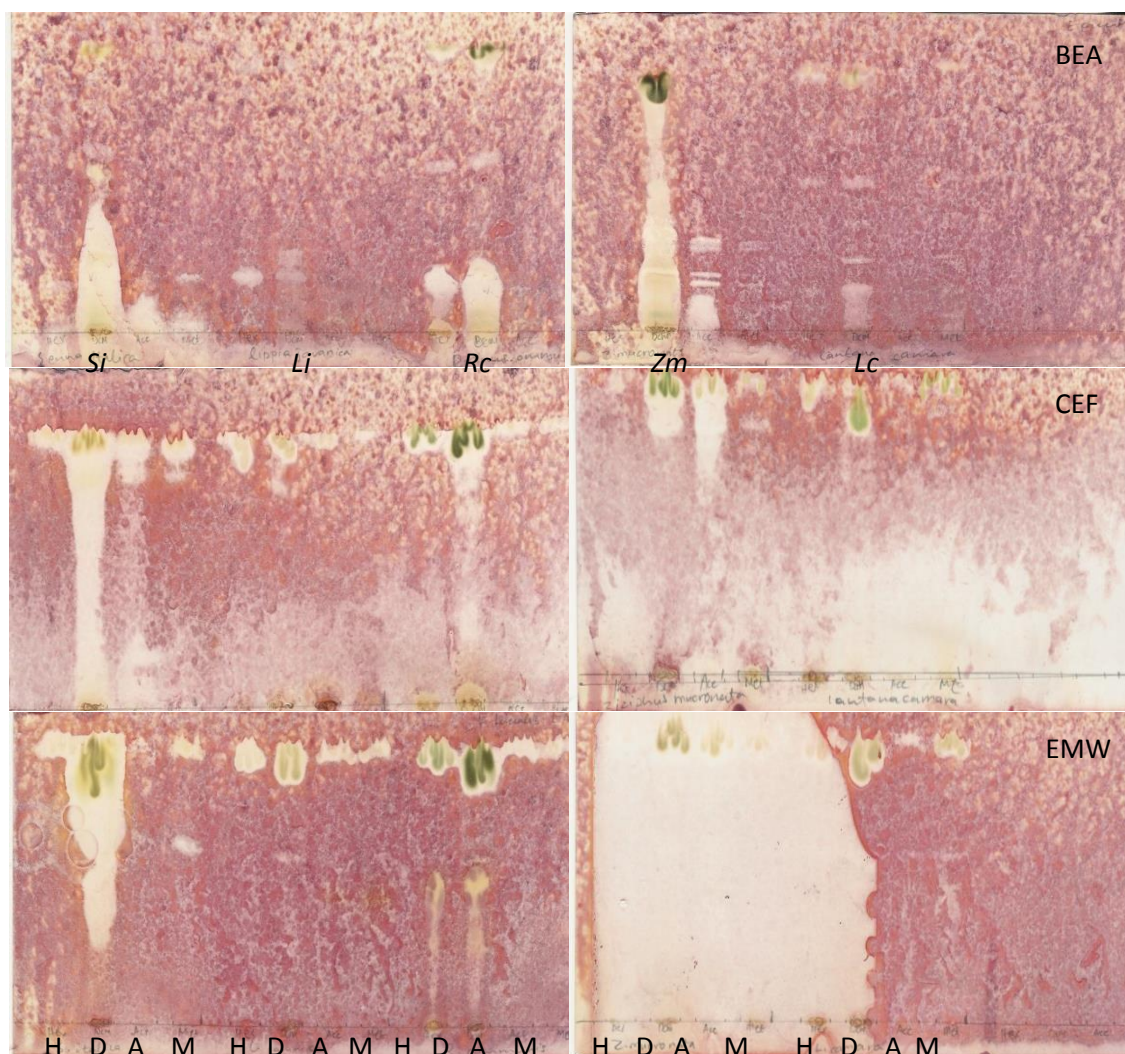


Figure 4.2: Bioautogram of different leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *E. faecalis*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of a compound(s) that inhibited the growth of *E. faecalis*.

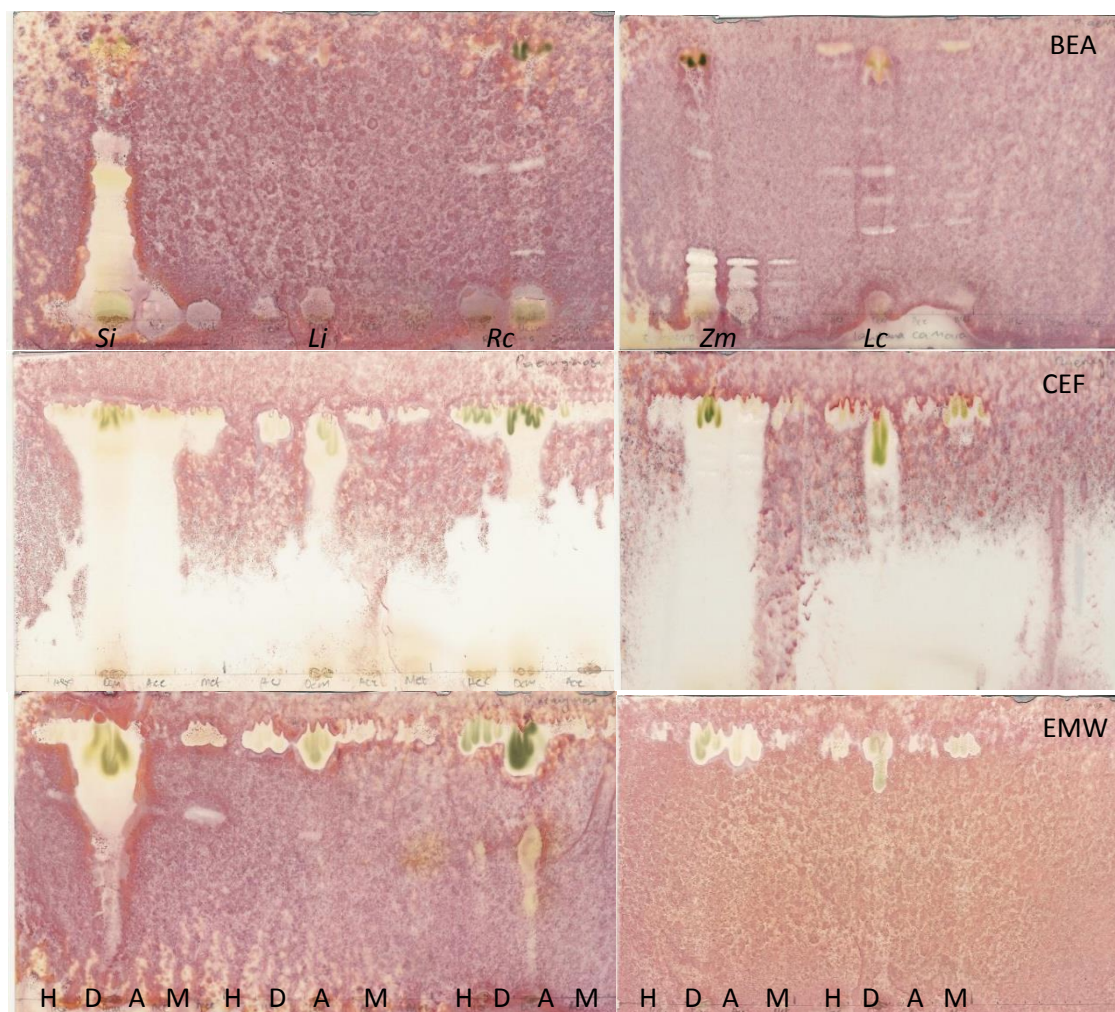


Figure 4.3: Bioautogram of different leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *P. aeruginosa*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of a compound(s) that inhibited the growth of *P. aeruginosa*.

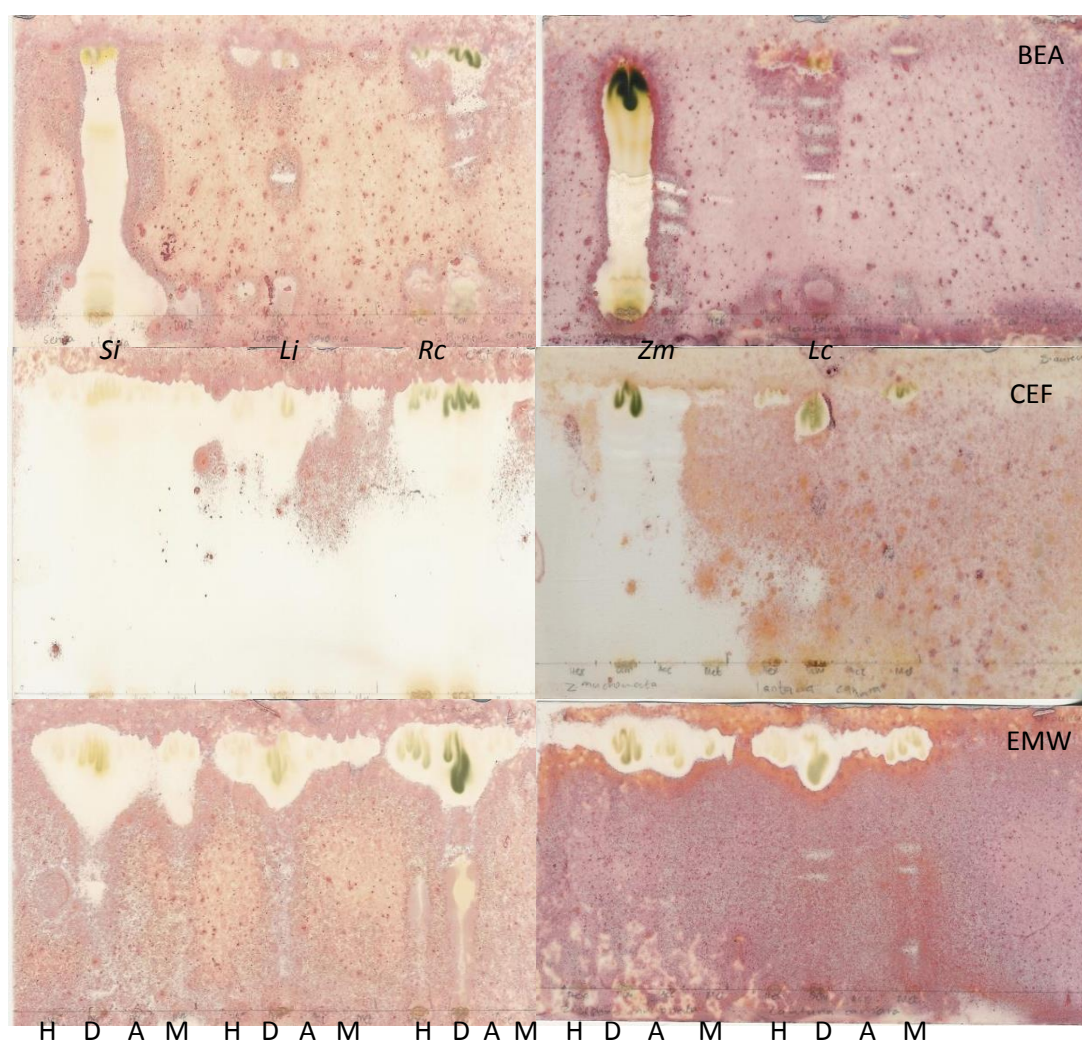


Figure 4.4: Bioautogram of different leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *S. aureus*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of a compound(s) that inhibited the growth of *S. aureus*.

Table 4.3: Inhibition of bacterial growth by bioautography of the extracts of *S.italica*, *L. javanica* and *R. communis* separated by TLC with BEA as eluent. R_f values and relative degree of inhibition are shown.

R _f values	BEA/ Hexane				BEA/DCM				BEA/ Acetone				BEA/ Methanol			
	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a
<i>Senna italica</i>																
0.77								X								
0.72							XXX	XXX				X				
0.56					XXX	XXX	XXX	XXX								
0.46	XX				XXX				X				XX	XXX		
0.31					XXX	XXX										
0.21					XXX		XXX	XXX								
<i>Lippia javanica</i>																
0.56							XXX									
0.46	XX				XX			XX								
0.31		X	XX		XX	X	XX	X								
0.21		XXX				X										
<i>Ricinus communis</i>																
0.77				X			XXX	X								
0.69							XX	X								
0.51		XX		XX		XX	XX	XXX								
0.46					XX											
0.38					XXX											
0.31	XXX	X			XXX	XXX		X								
0.21		XXX	X			XXX	X	X		X						

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

Relative degree of inhibition: X = slight inhibition, XX = moderate inhibition, XXX = high inhibition. BEA = benzene: ethanol: ammonium solution.

Table 4.4: The inhibition of bacterial growth by bioautography of the extracts of *Z. mucronata* and *L. camara* separated by TLC with BEA as eluent. R_f values and relative degree of inhibition are shown

	BEA/ Hexane				BEA/DCM				BEA/ Acetone				BEA/ Methanol			
R_f value	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a
<i>Ziziphus mucronata</i>																
0.81								XX								
0.7								XX								
0.57								XX								
0.48					XXX		XXX		X		XXX					
0.42					XXX				XX							
0.38						XXX	XXX			XXX				X		
0.35					XXX	XXX				XXX						
0.26					XXX	XXX	XXX	XXX		XXX		XXX				
0.19					XXX			XXX			XX	XXX				X
0.16					XXX		XXX	XXX								
0.13					XXX	XXX		XXX			XX	XXX		X		XX
<i>Lantana camara</i>																
0.97			X				X									
0.86							X									
0.63		X				XXX	X	X						X		
0.51				X		XX	X	XX						X		X
0.48				X	XXX			XX					X			XX
0.38				X	X			XX				X	X			X
0.26					X									X		
0.13		X	X			XXX	X									

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

Relative degree of inhibition: X = slight inhibition, XX = moderate inhibition, XXX = high inhibition. BEA = benzene: ethanol: ammonium solution.

Table 4.5: The inhibition of bacterial growth by bioautography of the extracts of the selected plants separated by TLC with CEF as eluent. R_f values and relative degree of inhibition are shown

R_{fval}	CEF/Hexane				CEF/DCM				CEF/Acetone				CEF/Methanol			
	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a
<i>Senna italica</i>																
0.73					XXX				XXX				X			
0.62						XXX				XXX						
0.21						XXX				XXX				XXX		
<i>Lippia javanica</i>																
0.73					XX				X				XX			
0.69								XXX								
0.62						XX										
<i>Ricinus communis</i>																
0.72						XXX										
0.62						XXX										
<i>Ziziphus mucronata</i>																
0.82						XXX				XXX				XX		
0.72							XXX				XXX					
0.67							XXX				XXX					
0.62							XXX				XXX				X	
0.56					XXX											
<i>Lantana camara</i>																
0.72							X									
0.56					XX											

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

Relative degree of inhibition: X = slight inhibition, XX = moderate inhibition, XXX = high inhibition. CEF = chloroform: ethyl acetate: formic acid.

Table 4.6: The inhibition of bacterial growth by bioautography of the extracts of the selected plants separated by TLC with EMW as eluent. R_f values and relative degree of inhibition are shown

R_f values	EMW/Hexane				EMW/DCM				EMW/Acetone				EMW/Methanol			
	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a
<i>Senna italica</i>																
0.71					XXX				X				XXX			
0.63				XX		XXX		XXX		X		X		XXX		XXX
0.4							XXX									
<i>Lippia javanica</i>																
0.56						XX	XX	XX								
<i>Ricinus communis</i>																
0.53						XX	XX	XX								
0.46		XX	XX	XX		XXX	XXX	XXX								
<i>Lantana camara</i>																
0.44						X	XX			X				X	XX	
0.31							XX							XX	XX	
0.18															XX	

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

Relative degree of inhibition: X = slight inhibition, XX = moderate inhibition, XXX = high inhibition. EMW = ethyl acetate: methanol: water.

Table 4.7: Number of antibacterial bands at different R_f values in all the five selected plants separated by BEA

R _f values	BEA/Hexane				BEA/DCM				BEA/Acetone				BEA/Methanol				Total
	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	
0.97				1			1										2
0.86							1					1					2
0.81								1									1
0.77				1			1	2									4
0.72							1	1				1					3
0.7								1									1
0.69							1	1									2
0.63		1				1	1	1							1		5
0.57								1									1
0.56					1	1	2	1									5
0.51		1		2		2	2	2							1	1	11
0.48					2		1	1	1		1					1	7
0.46	2				3			1	1				1	1			9
0.38				1	2			1			1		1	1		1	8
0.35					1	1				1		1					4
0.31	1	2	1		3	3		2	1								13
0.26					1												1
0.21		2	1		1	2	2	2		1							11
0.19					1			1			1	1					4
0.16					1		1	1									3
0.13		1	1								1	1					4
Total	3	7	3	5	16	10	14	20	3	2	4	5	2	2	2	3	
Grand total	18				60				14				9				

Table 4.8: Number of antibacterial bands at different R_f values in all five selected plants separated by CEF

R_f values	CEF/Hexane				CEF/DCM				CEF/Acetone				CEF/Methanol				Total
	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	
0.82						1				1				1			3
0.73					2				2				2				6
0.72						1	1				1						3
0.69								1									1
0.67							1				1						2
0.62						3	1			1	1				1		7
0.56					1												1
0.21						1				1				1			3
Total	0				3	6	3	1	2	3	3	0	2	2	1	0	
Grand total	0				13				8				5				

Table 4.9: Number of antibacterial bands at different R_f values in all five selected plants separated by EMW

R_f values	EMW/Hexane				EMW/DCM				EMW/Acetone				EMW/Methanol				Total
	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	E.c	E.f	S.a	P.a	
0.71					1				1				1				3
0.63				1		1		1		1		1		1		1	7
0.56						1	1	1									3
0.53						1	1	1									3
0.46		1	1	1		1	1	1									6
0.44						1	1			1				1	1		5
0.4							1										1
0.31							1							1	1		3
0.18															1		1
Total	0	1	1	2	1	5	6	4	1	2	0	1	1	3	3	1	
Grand total	4				16				4				8				

4.3.4. Antioxidant activity

Antioxidant activity of the extracts was investigated using the DPPH assay method. Clear bands on the plates (Figure 4.5) indicate antioxidant activity. No clear bands were observed when the plates were resolved with BEA and CEF respectively. On the EMW plate, distinct bands were observed with one band in *L. javanica* and two bands in *R. communis*.

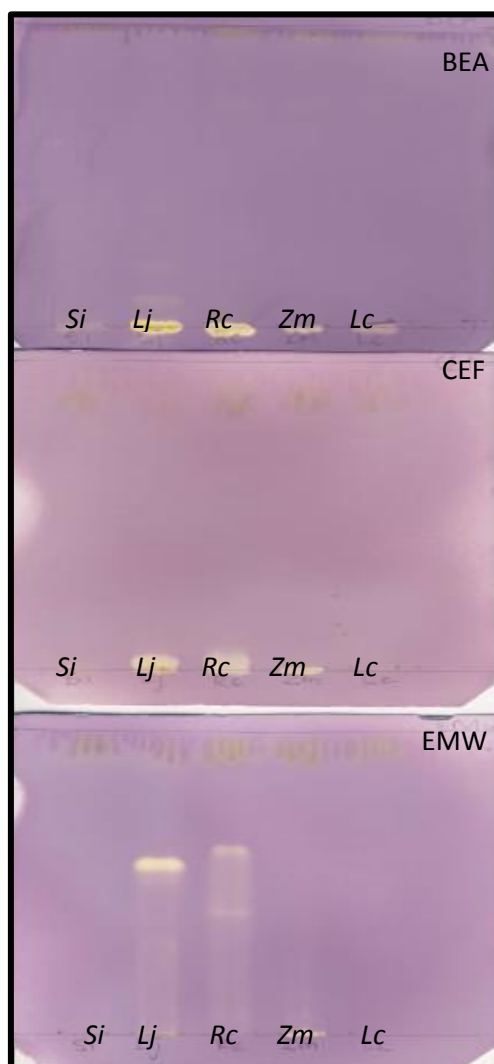


Figure 4.5: Chromatograms of different plants developed in BEA, CEF and EMW solvent system sprayed with 0.2% DPPH in methanol. Clear zones indicate antioxidant activity of compounds. Key: Si- *Senna italica*; Lj- *Lippia javanica*; Rc- *Ricinus communis*; Zm- *Ziziphus mucronata*; Lc- *Lantana camara*.

4.3.3. Toxicity assay

The graph below (Figure 4.6) represents toxic effects of the selected plants on the Raw 267.4 macrophages. The results show that as the concentration of the extracts increased the cells viability was reduced.

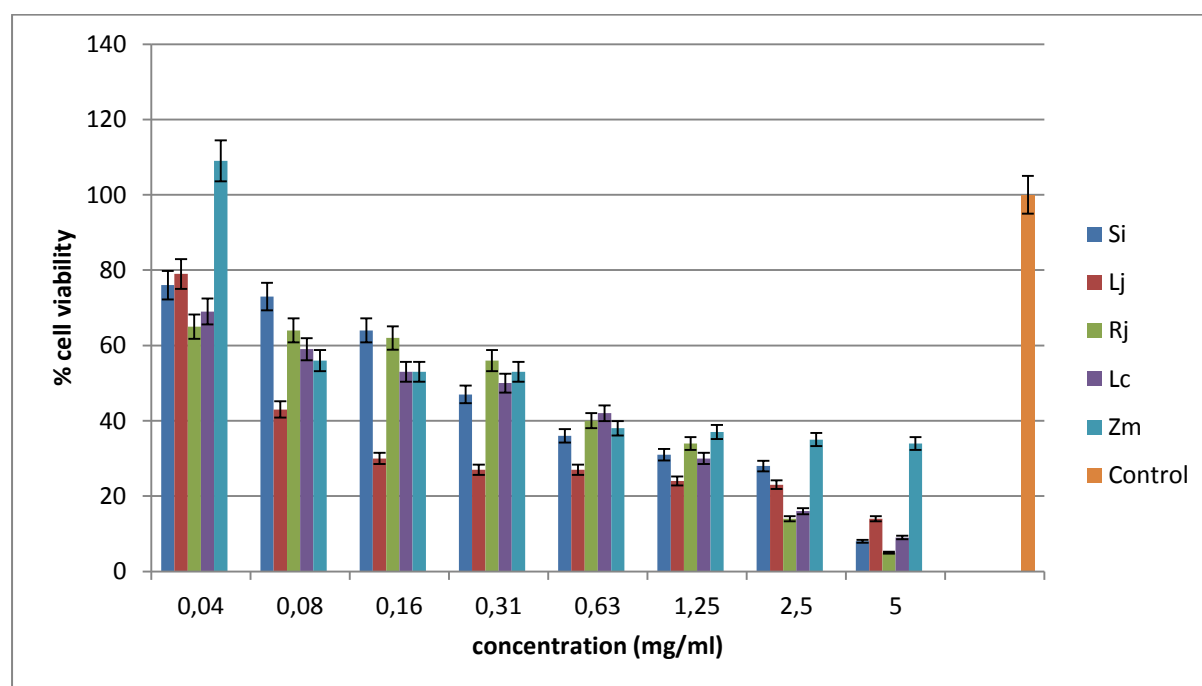


Figure 4.6: Percentage cell viability of Raw 267.4 macrophages after they were treated with different concentrations of plant extracts dissolved in DMSO. *Key:* Si- *Senna italica*; Lj- *Lippia javanica*; Rc- *Ricinus communis*; Lc- *Lantana camara*; Zm- *Ziziphus mucronata*.

4.3.4. Anti-inflammatory activity

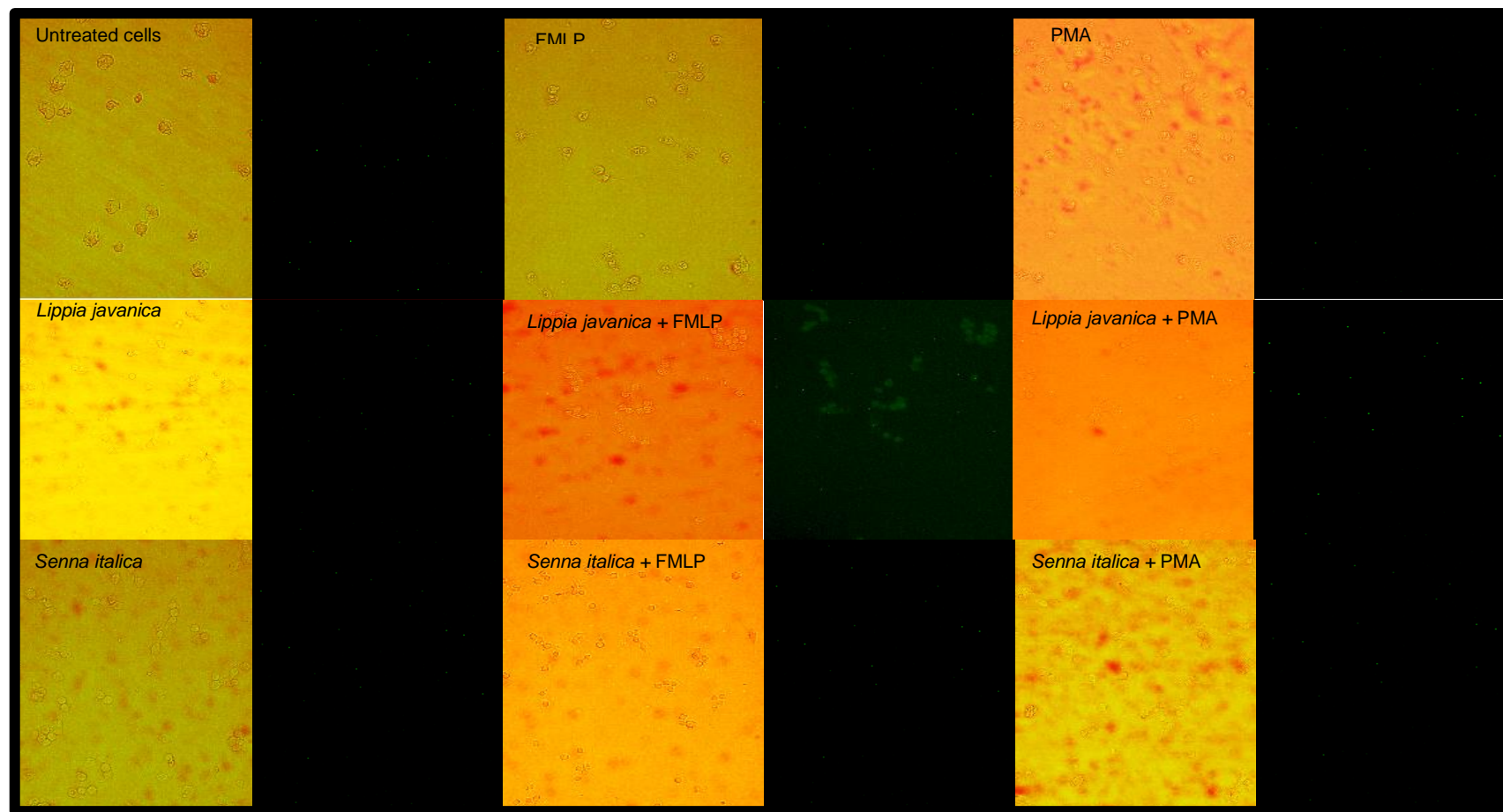


Figure 4.7: Anti-inflammatory effects of the selected plants on Raw 267.4 cells carried out using PMA and FMLP as stimulants. Cells treated with *L. javanica* and FMLP fluoresced green. Cells undergoing inflammation fluoresce green when stained with H₂DCFDA. Key: PMA- Phorbol-12-myristate-13-acetate; FMLP- N-formyl-Met-Leu-Phe.

4.4. Discussion

The selected plants were screened for various biological activities using various assays. In order to determine the antimicrobial activity of plants, it is often necessary to use the extracts against the target organisms. However in order to avoid working with pathogens, representative microorganisms are used instead. In this study, the test bacteria used were recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1992). Antibacterial activity of the extracts was evaluated using microplate method and bioautography. The solvents used for extraction are toxic to microbes, and in order to avoid toxicity, the solvents were removed by evaporation and the residue re-dissolved in acetone which is harmless (Eloff *et al.*, 2007). In the microplate method, the MIC values were recorded as the lowest concentration with activity. *Lippia javanica* and *Senna italica* were active against all the test organisms. *Lantana camara* showed no activity on any to the test organisms.

In Bioautography (Figure 4.1 to Figure 4.4) antibacterial activity is recognised by the presence of clear areas on the bioautograms against a purple background. The DCM extract of the selected plants had the most activity against all the test organisms, implying that they are good candidates for isolating antibacterial compounds. The most active bands are observed on the BEA plates which imply that non-polar mobile phases separate more active compounds than polar mobile phases. Some of the bioautograms which were developed in CEF had slight growth or no growth at all: that might be due to formic acid which was still present on the plates and it is toxic to the bacterial cells hence no growth (Masoko and Eloff, 2006). Tables 4.3 to 4.6 represent the R_f values of the compounds which showed antibacterial activity. The tables confirm what was observed on the bioautograms with regard to the DCM extract i.e. the tables show that the bands which showed high inhibition are the ones present in the DCM extract. High inhibition of the compounds can also be observed on the bioautograms by observing the intensity of each band. The intensity of the bands can also be attributed to the concentration of the compounds in the extracts. The difference in antimicrobial activity observed in microplate method and bioautography could be due to the active compounds being too dilute, and

antagonistic effects of one compound on another rather than the compounds being inactive (Taylor *et al.*, 2001).

Tables 4.7 to 4.9 represent the number of compounds which had antibacterial activity. In Table 4.7, a compound with an R_f value of 0.31 was the most abundant as the antibacterial bands of the compound were observed 13 times on the BEA plate. The compound was active against all the test organisms which imply that the compound has a broad spectrum of activity. The compound was also present on the EMW chromatogram and its absence on the CEF plate might have been as a result of poor growth on the plate, where one cannot distinguish whether the inhibition was due to formic acid or the active compounds. Sixty compounds present in the DCM extract were able to show antibacterial activity. Twenty of the sixty compounds with activity inhibited the growth of *P. aeruginosa*.

Antioxidant activity of the plants was investigated by spraying TLC plates with the extracts with DPPH reagent. The method measures the radical scavenging ability of plant compounds. Figure 4.5 shows that *Lippia javanica* and *Ricinus communis* had antioxidant activity and this was observed by clear bands on the TLC plates against a purple background. This means that the two plants have compounds which were able to donate electrons which resulted in the scavenging of the DPPH hence the clear/yellow band. Phytochemical tests for different compounds were carried out on the selected plants. The results (Table 3.1) revealed the presence of compounds such as flavonoids, alkaloids, saponins, terpenes etc. in the plants. The presence of secondary metabolites such as flavonoids with known antioxidant activity (Manikandan *et al.*, 2006) may be responsible for the activity observed in this study.

In order to assess the safety of plants used in traditional medicine, *in vitro* toxicity assays are carried out using mammalian cells. In this study Raw 267.4 macrophages were used in MTT assay to investigate the toxic effects of the extracts. The method measures the production of a purple colour which is indicative of live cells. The MTT assay results (Figure 4.6) show that as the concentration of the extracts increased, the viability of the cells decreased. The cells showed the best growth when grown in the presence of *Z. mucronata* at 0.04 mg/ml. All the plants showed increased viability at lower concentrations.

Inflammation plays an important role in wound healing but at times, inflammation may be prolonged as a result of the presence of compounds such as reactive oxygen species (ROS) which may result in chronic inflammation (Kahn and Solomon, 2007). In order to accelerate the healing process, researchers are trying to find ways in which the inflammatory stage can be bypassed or shortened (Eksioglu-Demiralp *et al.*, 2001). The Phargoburst test was used to evaluate the anti-inflammatory capabilities of the selected plant extracts. N-formyl-Met-Leu-Phe (FMLP) and Phorbol-12-myristate-13-acetate (PMA) were used as stimulants of inflammation. The stimulants trigger cells to produce reactive oxygen species which will lead to inflammation. 2, 7-Dichlorodihydrofluorescein diacetate acetyl ester (H₂DCFDA) dye was used for detecting the presence of ROS. When stimulated cells are viewed under fluorescence microscope, they fluoresce green. The dye becomes fluorescent when the acetate groups are oxidized by cellular esterases during cellular oxidation (Wang *et al.*, 2010).

4.5. Conclusion

Bioassays such as the ones used in the study provide a foundation for the use of plants in traditional medicine. Even though the biological activity of the extracts observed during *in vitro* evaluation does not necessarily imply that the plants are effective medicines, they however provide a basis for further evaluation of the active compounds. Therefore evaluation of the biological activity of plants extracts is a crucial step in identifying biological capabilities of plants.

CHAPTER 5

Isolation of bioactive compounds

5.1. Introduction

The major path for antibiotic development is to isolate and identify biologically active compounds. These active compounds from crude extracts act as building blocks for synthesis of new drugs with new mechanisms of action to treat various ailments (Jothy *et al.*, 2011). In that regard, ethnomedicine provides a source of information about which plants can be targeted (Kunwar *et al.*, 2003). As such, a number of pharmaceutical have been manufactured using compounds from medicinal plants (Gilani and Rahman, 2005). It has been established that 25% of drugs that are currently in use were synthesized from plant secondary metabolites (Hamburger and Hostettman, 1991). The compounds found in plants have antimicrobial activity against both plants and human pathogens (Hasegawa *et al.*, 1995). What scientists are looking for is a compound that has activity but is not harmful to host cells (Kapoor *et al.*, 1969). And, isolation of compounds may result in pure compounds that are both active and non-toxic (McGaw and Eloff, 2008).

In order to isolate, crude extracts of the selected plants must be collected using Serial Exhaustive Extraction (SEE). This method of extraction involves successive extraction using solvents of increasing polarity from non-polar hexane to polar methanol (Das *et al.*, 2010). One has to bear in mind that when selecting the conditions for extraction with the aim of isolating compounds; the properties of both the compound and the source must be taken into consideration. After the extracts are collected, the eluent is removed from the soft extract using evaporation. This is usually carried out using vacuum evaporation at temperatures not exceeding 55°C for quality results, especially, for constituents that are volatile and thermolabile (Handa *et al.*, 2008).

For purification of plant compounds, a number of steps are taken and these includes extraction, pre-purification and one or several chromatographic steps. In the chromatography step, preparative TLC can be used for small samples but large samples column chromatography is used (Braga *et al.*, 1996). Thin layer

chromatography in purification can be used to check for purity or used as a purification method (Lafont *et al.*, 1994).

5.2. Methods and materials

5.2.1. Preparation of crude extracts

The exhaustive extraction method was used to extract ground leaf powder (1 kg) of the *Senna italica*, *Lippia javanica*, *Ziziphus mucronata*, *Lantana camara* and *Ricinus communis* with hexane, DCM acetone and methanol. Plant material was extracted three times with 4L of the selected solvents. The extracts were then filtered and concentrated using a Buchi rotary evaporator under reduced pressure, rotating at 100 rpm and the water bath temperature of 40°C. The concentrated extracts were transferred into pre-weighed beaker, dried under a fan and weighed. Phytochemical analysis was performed as described in section 3.2.3.

5.2.2. Column chromatography (solvent-solvent fractionation)

The solvent-solvent fractionation was selected to simplify extracts by fractionating the chemical compounds into broad groups based on their solubility. A Büchner funnel was packed with silica gel to a height of 30 cm with a diameter of 7 cm. The finely ground DCM extract was thinly spread on top of the overnight packed silica gel and then covered with cotton wool and eluted with DCM: hexane (7:3; 8:2; 9:1, v/v) and 100% DCM. To increase polarity of DCM: MeOH (9:1; 8:2; 7:3; 6:4; 5:5, v/v) and 100% MeOH solvent system was used. The sub fractions of each fraction were collected in test tubes.

5.2.3. Analysis and bioassays of fractions

The sub fractions were collected and concentrated using the rotary evaporator (Buchi Rotavapor R-210/215) under reduced pressure, rotating at 100 rpm and with the water bath at 40°C. All the collected fractions were phytochemically analysed using thin layer chromatography (10 µl of 10 mg/ml extract) with different solvents. The separated components were visualised under visible and ultraviolet light (245 and 360 nm); the plates were then sprayed with vanillin-sulphuric acid reagent and slightly heated. A qualitative assay of extracts was done using the method described under section 3.2.3. The fractions with the compound(s) of interest were pooled and re-eluted.

5.2.4. Purification of pooled and unseparated compounds

The best mobile phase for eluting the pooled fractions and unseparated compounds was selected by running 10 µl of 10 mg/ml extract spotted on TLC with a combination of solvent systems. The fractions collected were pooled based on the profile given by the results from section 5.2.3. Column chromatography for each fraction was performed. The column was eluted with different solvents using gravitational force to facilitate elution. Fractions were collected and analysed as described in section 5.2.3.

5.2.5. Preparative TLC

The fractions were dissolved in small amount of 100% chloroform and applied in bands across the preparative TLC plates. The plates were developed in 100% chloroform. The bands were visualized under ultraviolet light (254 and 360 nm) before a small part on the side of the plates was sprayed with vanillin-sulphuric acid and heated with a heat gun. The rest of the bands or compounds were covered with glass and an aluminium foil to prevent damage of the compounds from spraying and heat. The visualised bands on the side were used as the reference line for scraping the remaining compounds from the plate with a sterile razor blade. The components were collected into separate beakers and crushed into fine powders using a glass rod. The silica powder was eluted with different solvents based on the compound. The volume of the solvent was dependent on the quantity and pigment recovered by filtration through a cut glass pipette plugged with cotton wool to facilitate the removal of impurities. The process was repeated three times or until the silica gel powder regained its original colour. Each purified sample was evaporated and weighed and thereafter transferred into separate vials.

5.3. Results

5.3.1. Serial exhaustive extraction

Senna italica was selected for isolation of antibacterial compounds. The leaves were extracted exhaustively using hexane, DCM, acetone and methanol. Table 5.1 below represents the mass extracted with each solvent. The greatest mass was extracted with methanol.

Table 5.1: Total mass yield of the extracts

Extract solvent	Yield (g)	Total yield (g)	Yield (%)
Hexane I	31.1	80.9	8.09
Hexane II	31.3		
Hexane III	18.5		
DCM I	14.4	24.6	2.46
DCM II	6.2		
DCM III	4.0		
Acetone I	8.5	15.3	1.53
Acetone II	6.2		
Acetone III	0.6		
Methanol I	17.3	85.4	8.54
Methanol II	25.7		
Methanol III	42.4		

5.3.2. Phytochemical analysis

The extracts obtained from exhaustive extraction of the leaves *Senna italica* were separated on TLC plates and sprayed with vanillin sulphuric acid reagent for visualization. Figure 5.1 below shows that mostly non-polar compounds were separated on the BEA plate.

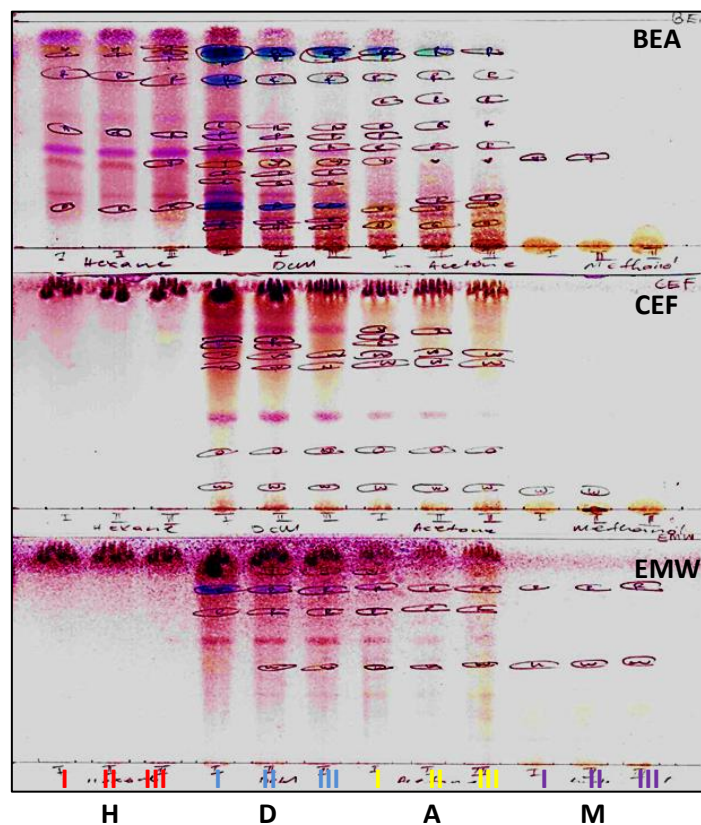


Figure 5.1: Chromatogram showing the different compounds in the plant after the plates were sprayed with vanillin-sulphuric acid reagent. Key: H-hexane; D-dichloromethane; A-acetone; M-methanol.

5.3.3. Qualitative antibacterial assay

Antibacterial activity of the extracts was carried out by Bioautography. Figure 5.2 to Figure 5.5 represent the bioautograms obtained after spraying the plates with different organisms. Most active bands were observed on the bioautograms for *E. coli* while no activity was observed on the *P. aeruginosa* plates.

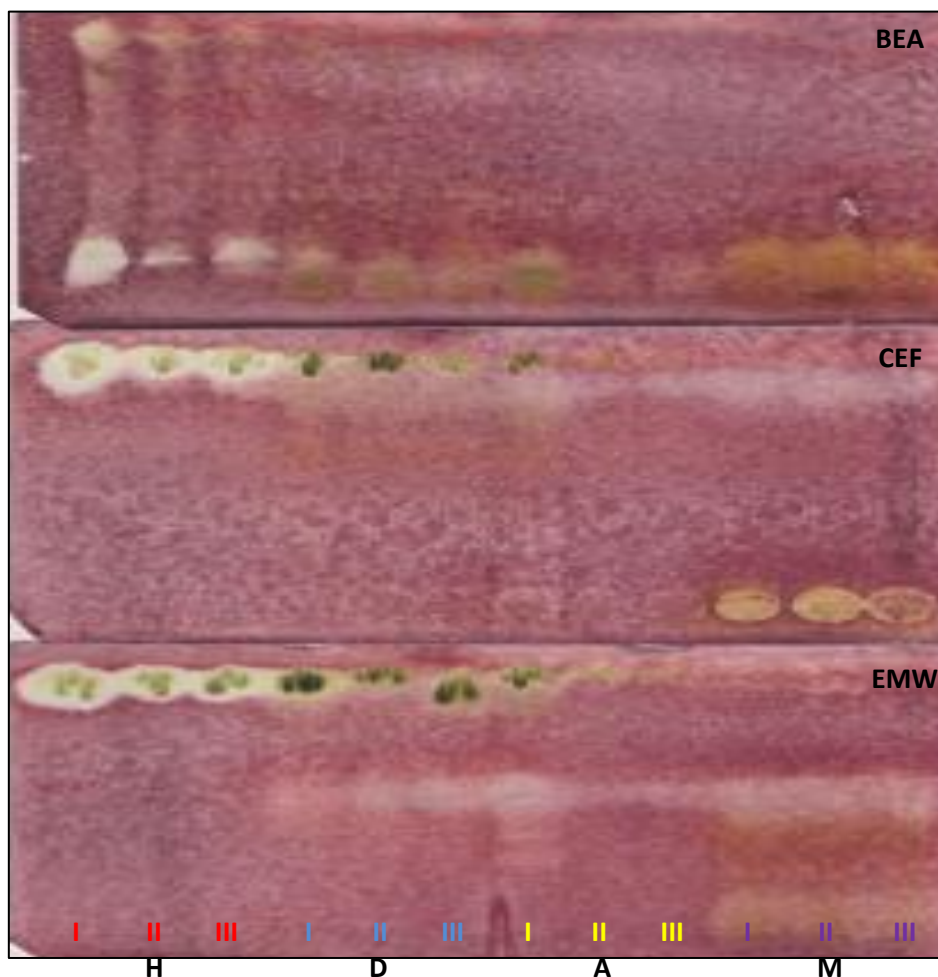


Figure 5.2: Bioautogram of *Senna italica* leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *S. aureus*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compound that inhibited the growth of *S. aureus*.

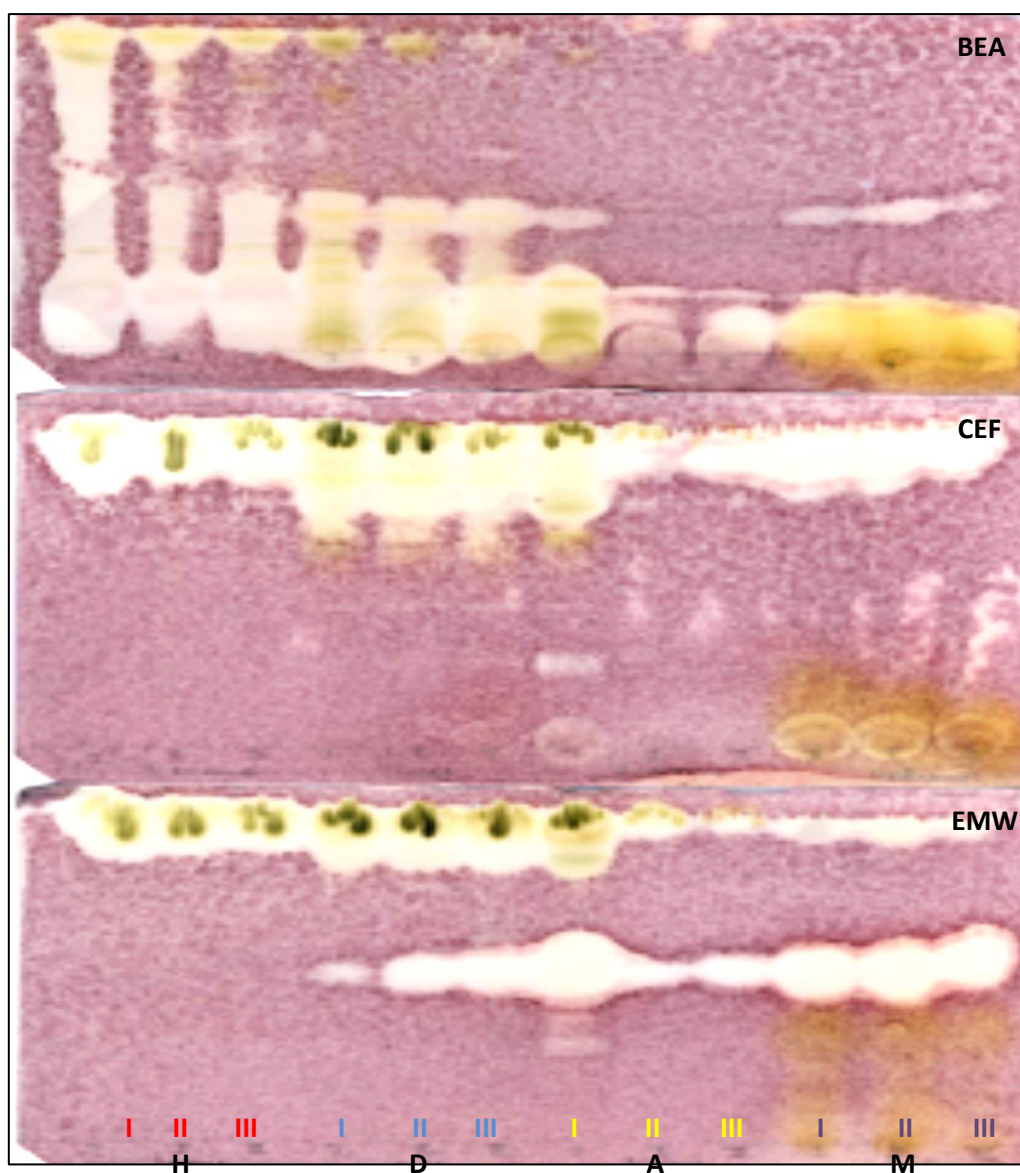


Figure 5.3: Bioautogram of *Senna italica* leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *E. coli*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compound that inhibited the growth of *E. coli*.

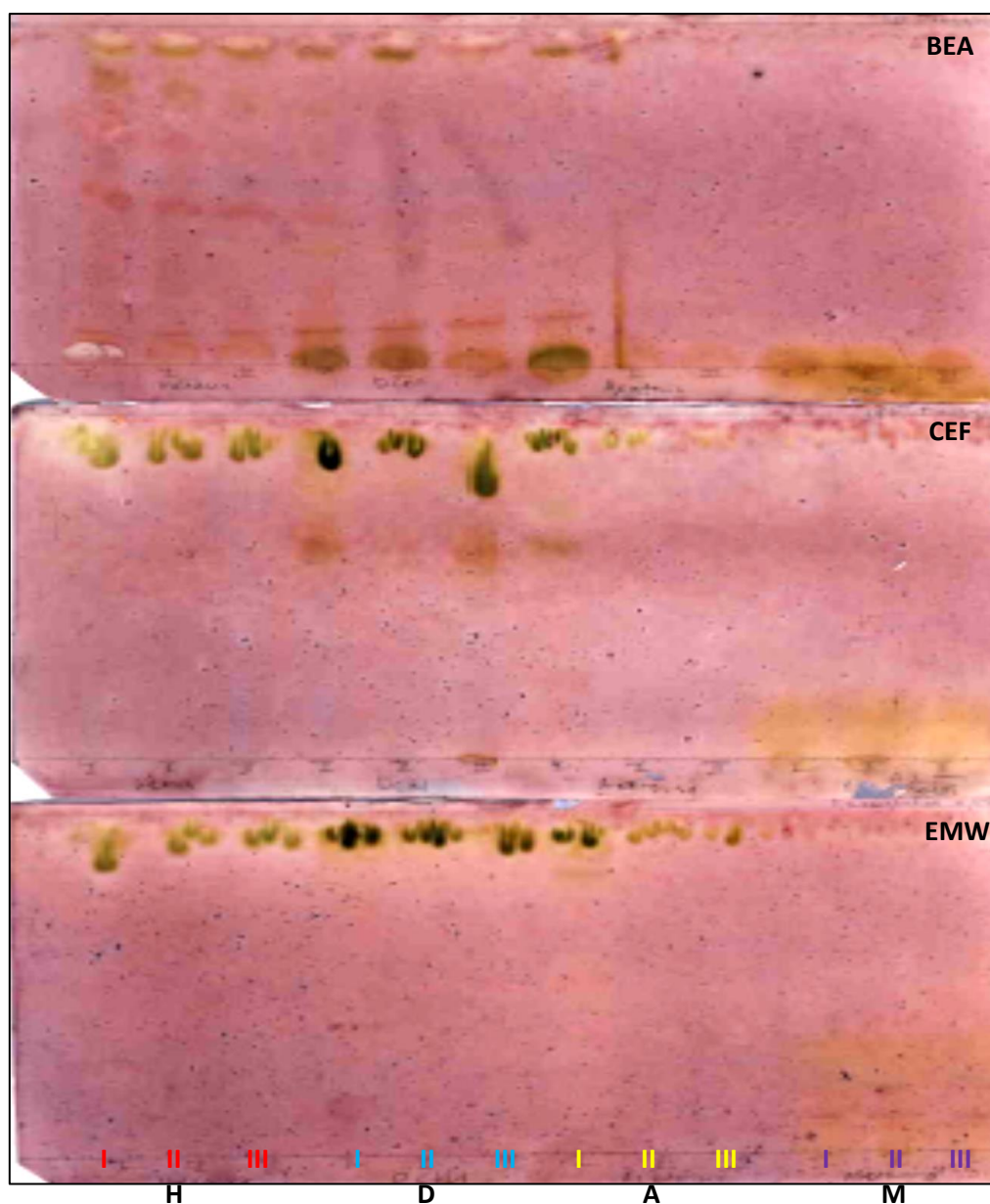


Figure 5.4: Bioautogram of *Senna italica* leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *P. aeruginosa*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compound that inhibited the growth of *P. aeruginosa*.

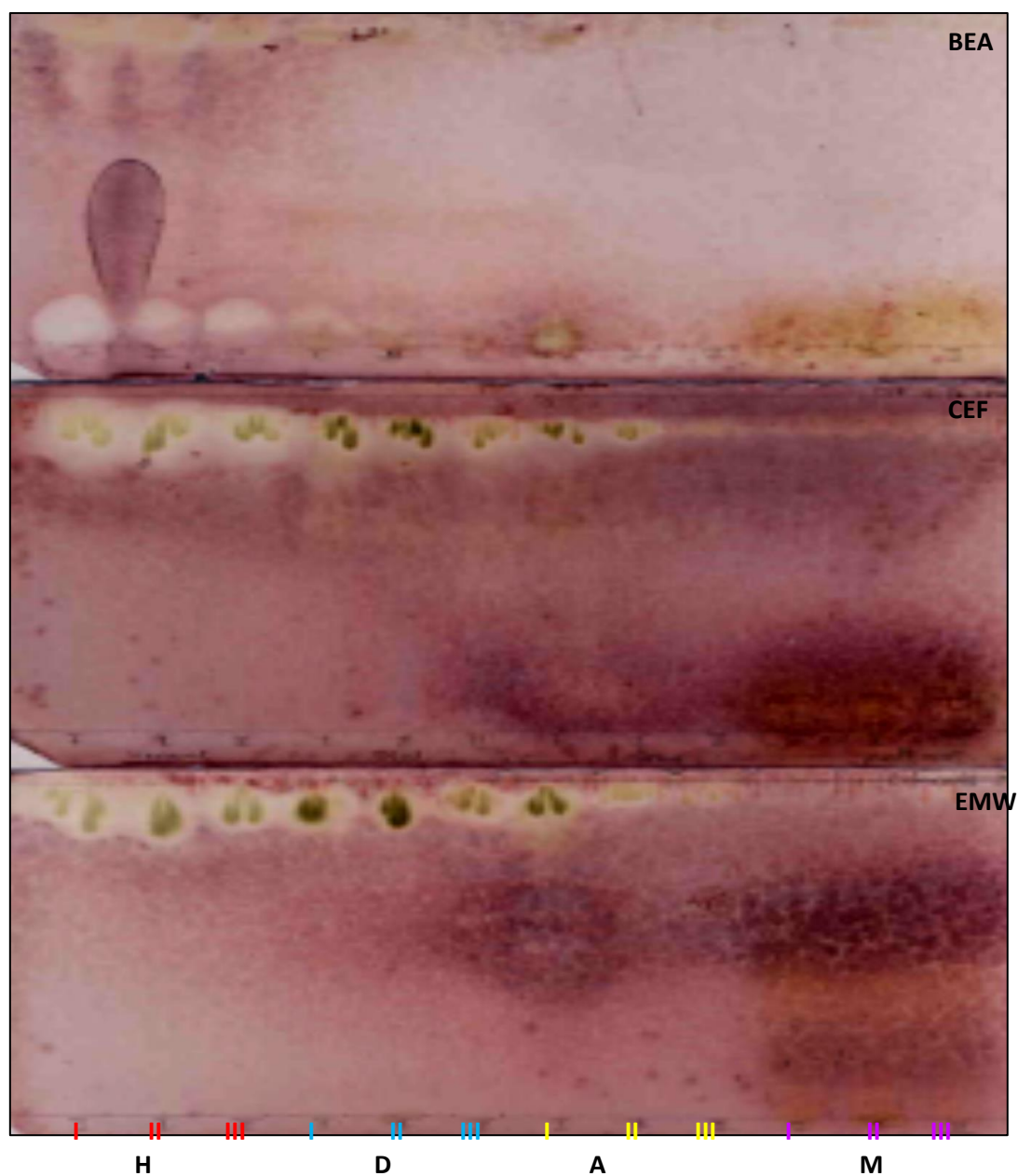


Figure 5.5: Bioautogram of *Senna italica* leaves extracted with hexane (H), dichloromethane (D), acetone (A) and methanol (M), separated by BEA, CEF and EMW sprayed with *E. faecalis*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compound that inhibited the growth of *E. faecalis*.

5.3.4. Microdilution assay

Microdilution assay for Minimum Inhibitory Concentration of the extracts was carried out on the selected bacterial species (Table 5.2). The lowest concentrations were observed with the DCM and acetone extracts. The extracts had more activity against Gram positive *S. aureus* and *E. faecalis*. Table 5.3 represents the total activity of the extracts, where the highest total activity is observed in the DCM extract.

Table 5.2: Minimum inhibitory concentrations (MICs) in mg/ml values of the *Senna italica* leaf extracts tested on various bacteria

Organisms	H				D				A				M				Amp (µg/ml)
	I	II	III	Ā	I	II	III	Ā	I	II	III	Ā	I	II	III	Ā	
<i>E. coli</i>	1.25	-	-	1.25	0.31	0.31	0.31	0.31	0.31	0.31	0.16	0.26	1.25	1.25	-	1.25	0.16
<i>P. aeruginosa</i>	-	-	-	-	0.16	0.31	0.31	0.26	0.16	0.63	0.63	0.47	0.63	0.31	0.63	0.52	0.13
<i>S. aureus</i>	0.31	0.31	0.31	0.31	0.08	0.16	0.16	0.13	0.08	0.08	0.16	0.11	0.63	0.63	-	0.63	0.08
<i>E. faecalis</i>	0.63	0.63	0.63	0.63	0.16	0.31	0.31	0.26	0.16	0.08	0.08	0.11	0.16	0.16	-	0.16	0.16

Key: H= Hexane; D= dichloromethane; A= Acetone; M= Methanol; Amp= ampicillin; -→ no activity; Ā= Average

Table 5.3: Total activity (ml/g) of different *Senna italica* leaf extracts

Organisms	H				D				A				M			
	I	II	III	Ā	I	II	III	Ā	I	II	III	Ā	I	II	III	Ā
<i>E. coli</i>	25	-	-	25	46	20	13	26	27	20	4	17	14	21	-	18
<i>P. aeruginosa</i>	-	-	-	-	90	20	13	41	53	10	1	21	27	83	67	59
<i>S. aureus</i>	100	101	60	87	180	39	25	81	106	78	4	63	27	41	-	34
<i>E. faecalis</i>	49	50	29	43	90	20	13	41	53	78	8	46	108	161	-	135

Key: H= Hexane; D= dichloromethane; A= Acetone; M= Methanol; Amp= ampicillin; -→ no activity; Ā= Average

5.3.5. Determination of suitable mobile phase

Different solvent combinations were used in order to select the suitable mobile phase for elution of the compound of interest. Figure 5.5 below represents TLC profiling and bioautograms of the crude DCM extract developed in different solvent combinations of hexane and ethyl acetate (Figure 5.6), followed by DCM in ethyl acetate (Figure 5.7), DCM in MeOH (Figure 5.8 and 5.9) and DCM in hexane (Figure 5.10). Bioautography was used to guide the identification of the active compounds on the plates. The mobile phase which had good separation of the compounds was 70% DCM in 30% hexane and it was selected as the eluent.

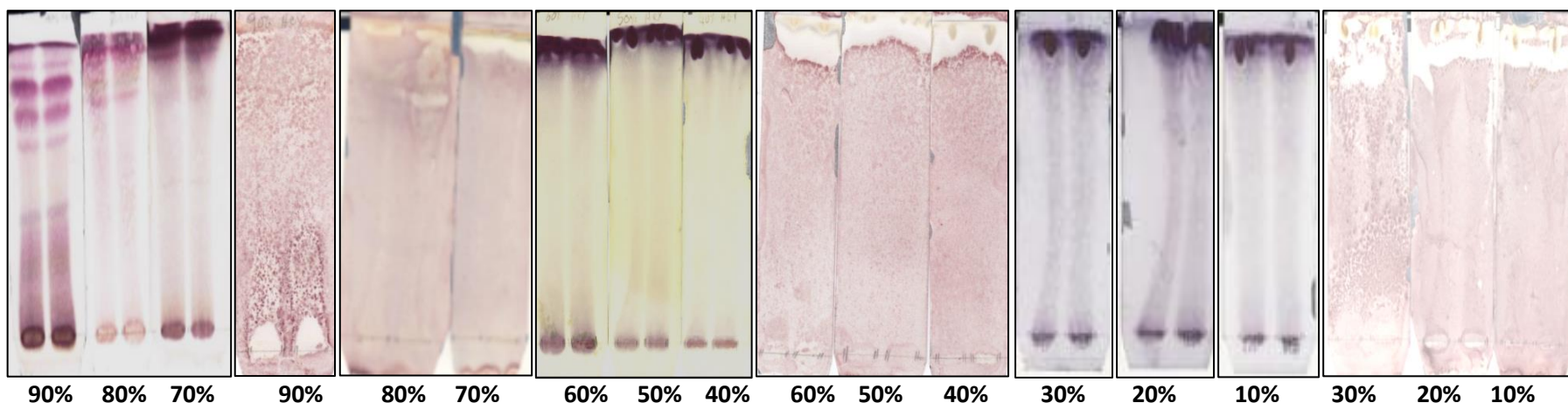


Figure 5.6: TLC profiling of *Senna italica* DCM extract developed in 90% to 10% hexane in ethyl acetate and bioautograms against *E. coli*. The colours on the TLC plates represent the different compounds in the extract and the clear areas against a purple background on the bioautograms are zones of *E. coli* inhibition.

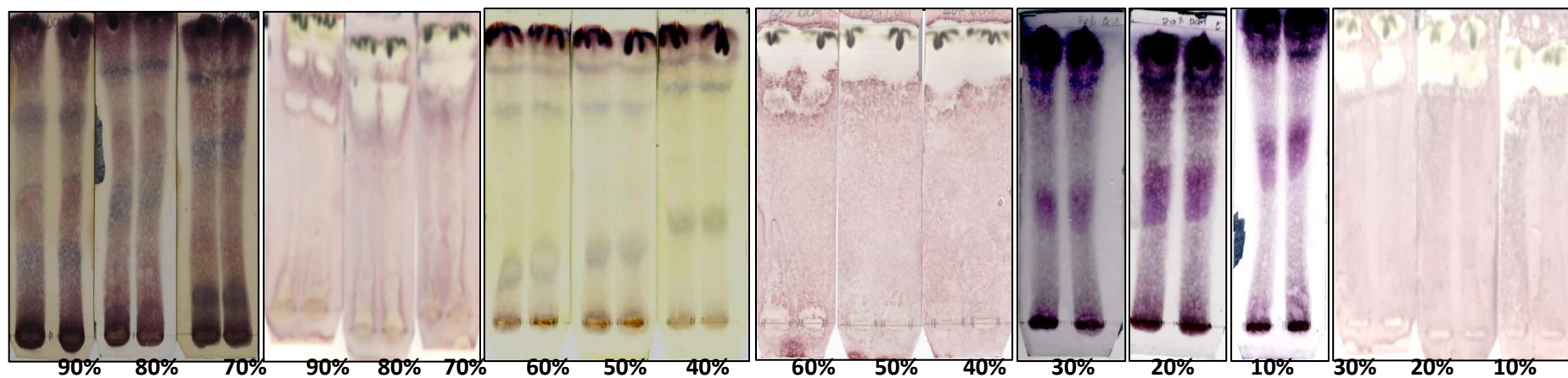


Figure 5.7: TLC profiling *Senna italica* DCM extract developed in 90% to 10% DCM in ethyl acetate and bioautograms against *E. coli*. The colours on the TLC plates represent the different compounds in the extract and the clear areas against a purple background on the bioautograms are zones of *E. coli* inhibition.

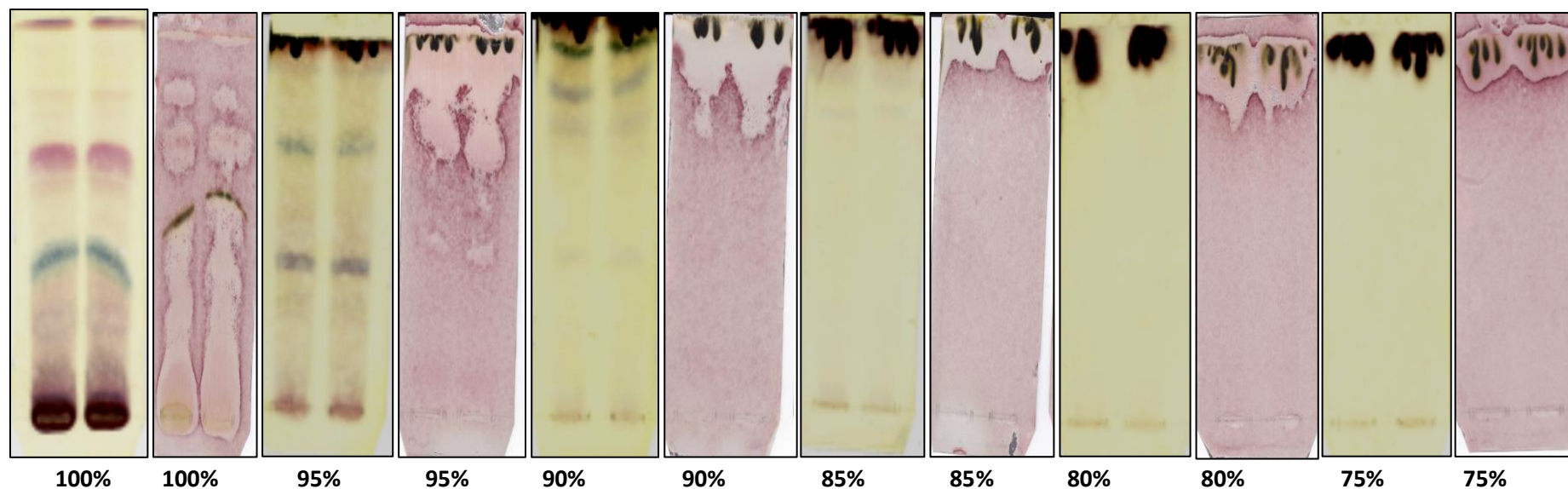


Figure 5.8: TLC profiling of *Senna italica* DCM extract developed in 100% to 75% DCM in MeOH and bioautograms against *E. coli*. The colours on the TLC plates represent the different compounds in the extract and the clear areas against a purple background on the bioautograms are zones of *E. coli* inhibition.

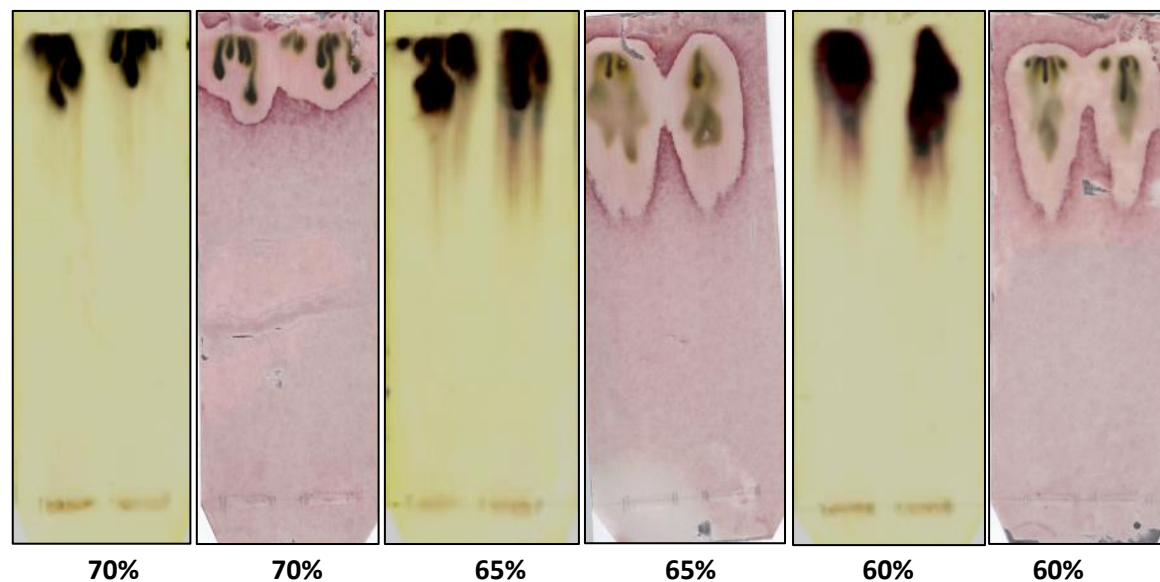


Figure 5.9: TLC profiling of *Senna italica* DCM extract developed in 70% to 60% DCM in MeOH and bioautograms against *E. coli*. The colours on the TLC plates represent the different compounds in the extract and the clear areas against a purple background on the bioautograms are zones of *E. coli* inhibition.

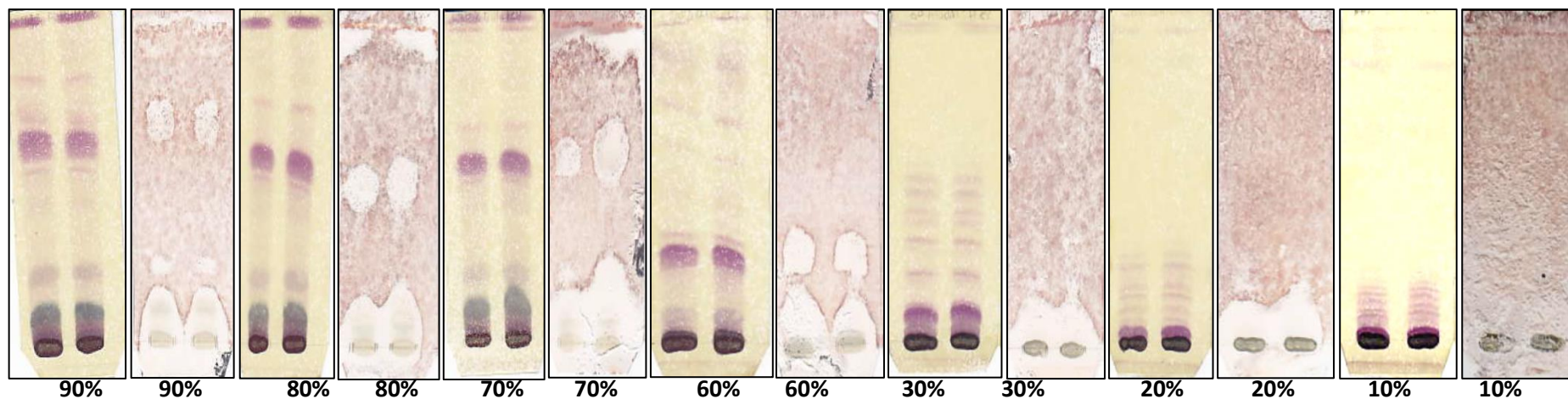


Figure 5.10: TLC profiling of *Senna italica* DCM extract developed in 90% to 10% DCM in hexane and bioautograms against *E. coli*. The colours on the TLC plates represent the different compounds in the extract and the clear areas against a purple background on the bioautograms are zones of *E. coli* inhibition.

5.3.6. Antioxidant activity of the selected fraction

The DPPH assay was used to determine the radical scavenging ability of the DCM extract. The plate was sprayed with 0.2% DPPH in methanol. Antioxidant activity was observed by the development of yellow areas on the chromatogram against a purple background. In Figure 5.11, the compound of interest did not show antioxidant activity.

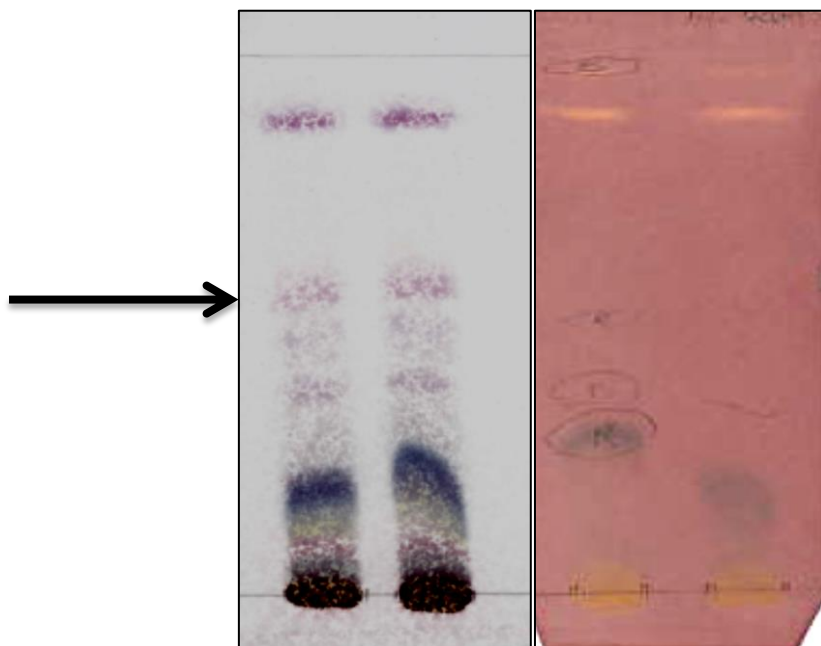


Figure 5.11: TLC profiling and antioxidant activity of the DCM extract in DCM: Hexane (7:3, v/v). The compound of interest marked with an arrow did not have antioxidant activity.

5.3.7. Isolation of bioactive compounds by column chromatography

Column chromatography of the DCM fraction was carried out using DCM:Hexane (v/v) as the mobile phase. The eluents were collected in tubes and spotted on TLC plates to observe their chemical profile. Figure 5.12-5.20 represents the column chromatography results which were run with different percentages of DCM:Hexane and DCM:MeOH. Figure 5.29 shows the flow diagram of the isolation of the active compounds.

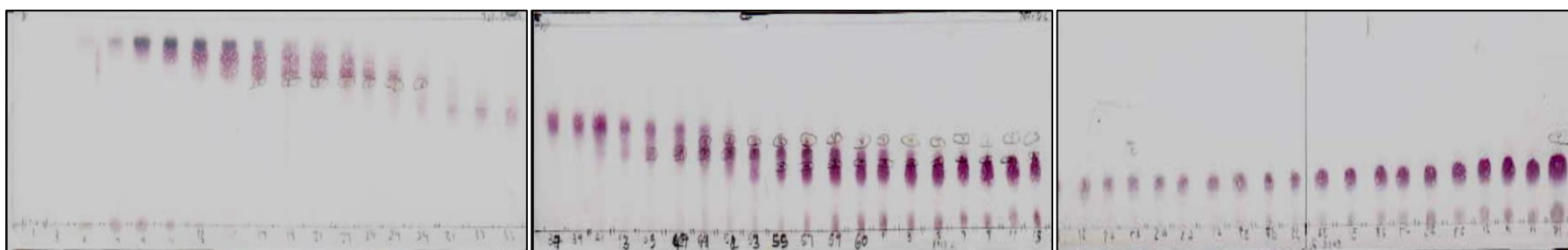


Figure 5.12: TLC profile of compounds collected from elution of DCM extract with DCM: Hexane (7:3, v/v) from a silica gel column.

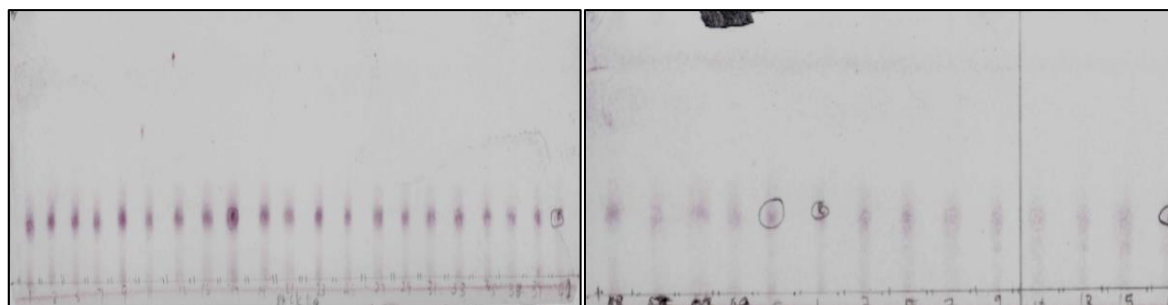


Figure 5.13: TLC profile of compounds collected from elution of DCM extract with DCM: Hexane (8:2, v/v) from a silica gel column.

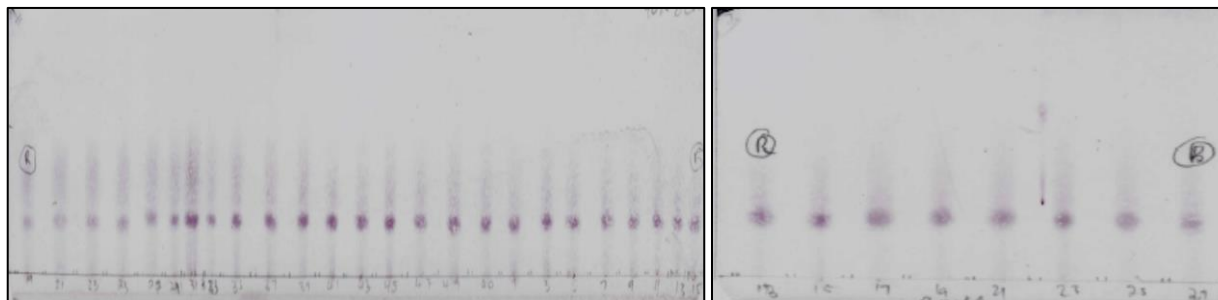


Figure 5.14: TLC profile of compounds collected from elution of DCM extract with DCM: Hexane (9:1, v/v) from a silica gel column.

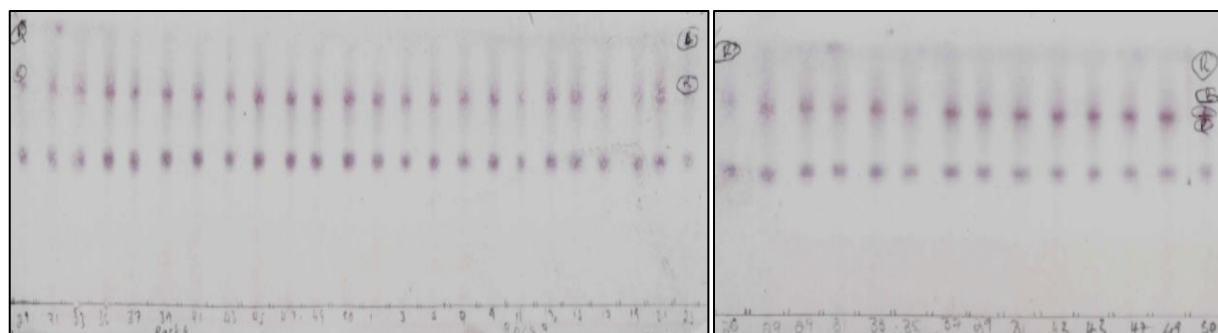


Figure 5.15: TLC profile of compounds collected from elution of DCM extract with DCM: Hexane (9.5:5, v/v) from a silica gel column.

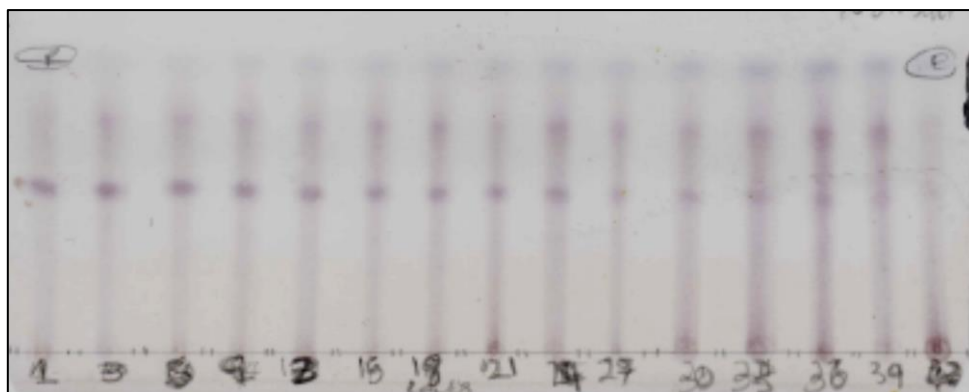


Figure 5.16: TLC profile of compounds collected from elution of DCM extract with DCM: Hexane (9.5:5, v/v) from a silica gel column.

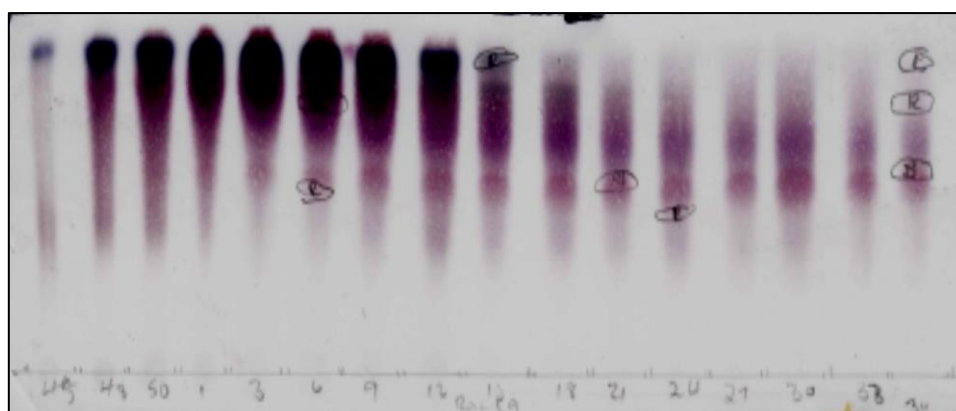


Figure 5.17: TLC profile of compounds collected from elution of DCM extract with DCM: MeOH (9.5:5, v/v) from a silica gel column.

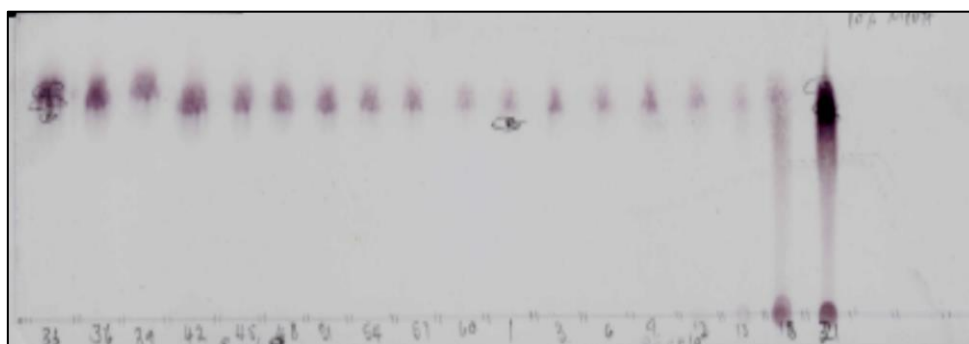


Figure 5.18: TLC profile of compounds collected from the elution of DCM extract with DCM: MeOH (9:1, v/v) from a silica gel column.

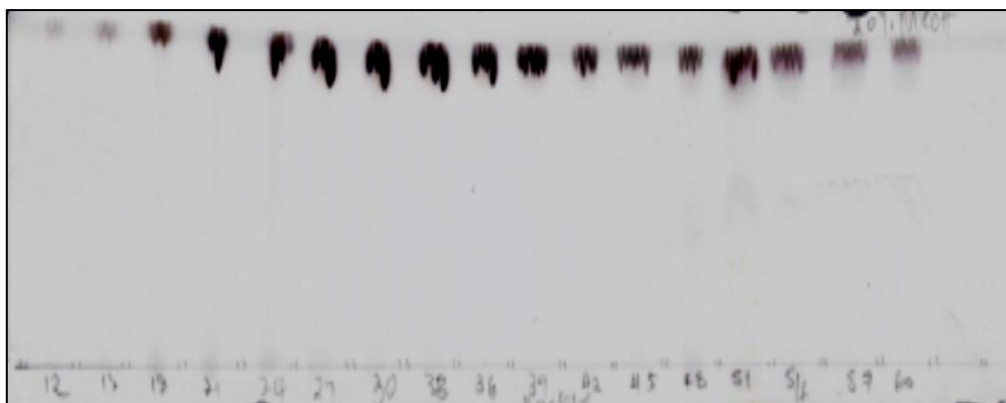


Figure 5.19: TLC profile of compounds collected from the elution of DCM extract with DCM: MeOH (8:2, v/v) from a silica gel column.

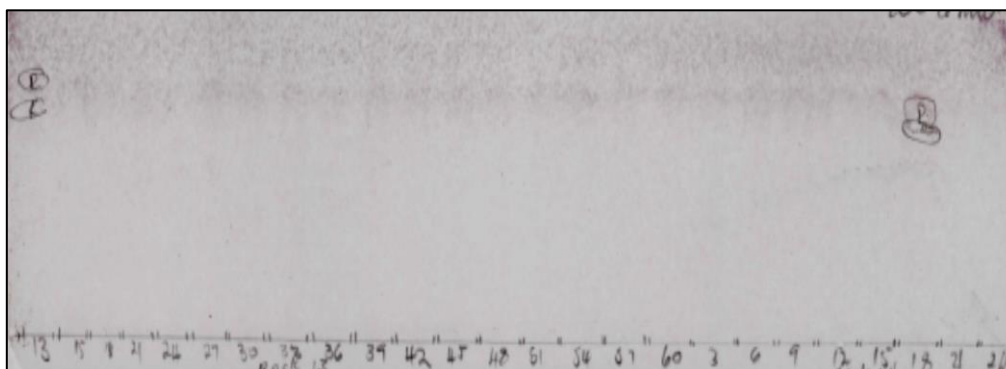


Figure 5.20: TLC profile of compounds collected from the elution of DCM extract with MeOH (100%, v/v) from a silica gel column.

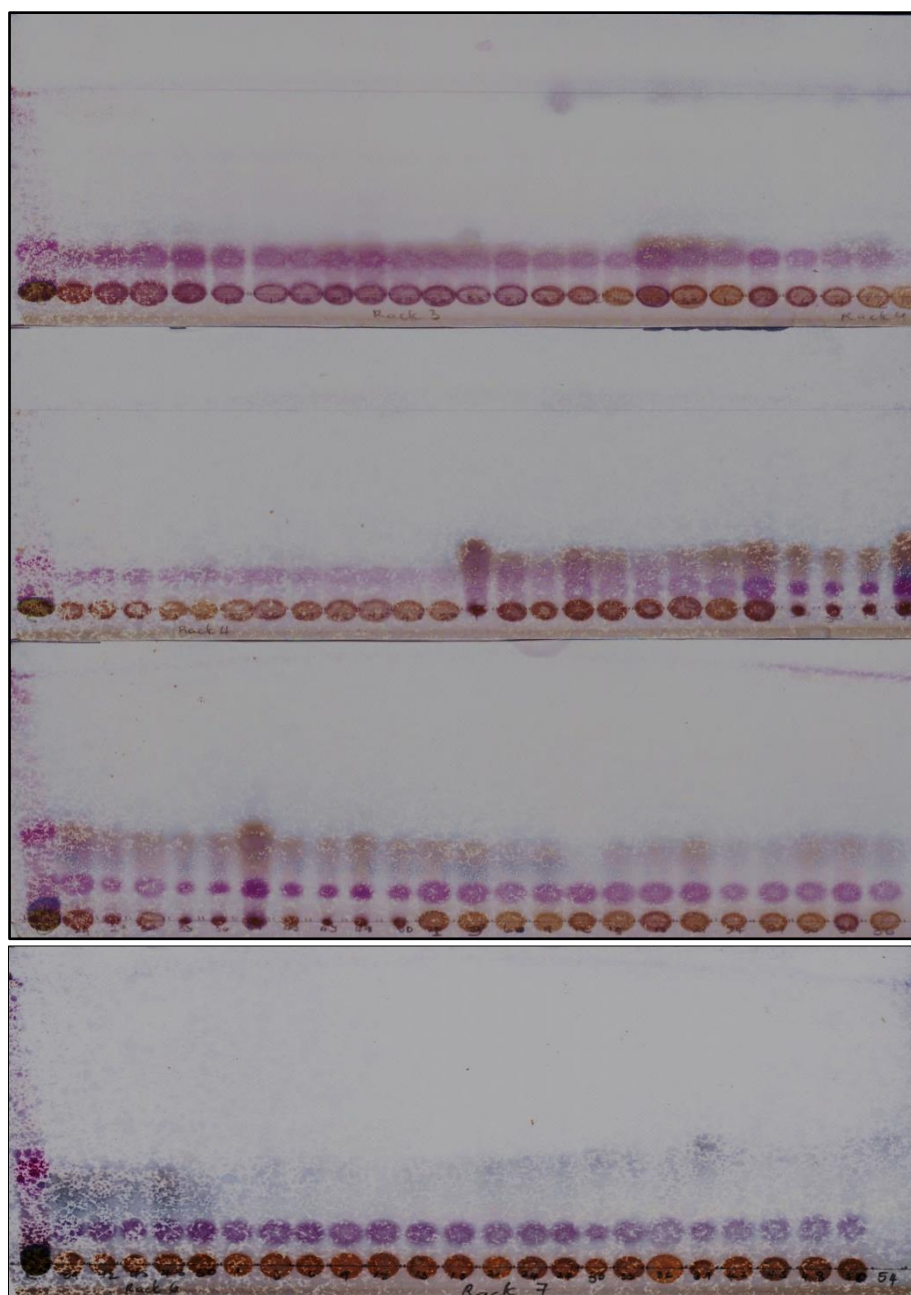


Figure 5.21: TLC profile of tubes that were pooled together from the elution of DCM extract developed in DCM: Hexane (5:5, v/v) from a silica gel column.

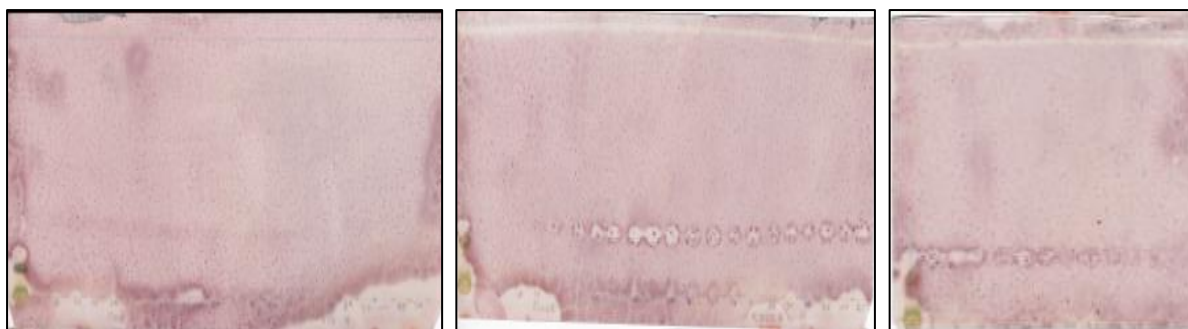


Figure 5.22: Bioautograms of tubes that were pooled together from the elution of DCM extract developed in DCM: Hexane (1:1, v/v) from a silica gel column.

5.3.8. Biological activity of the pooled tubes

Phytochemical analysis, antioxidant and antibacterial activities of the contents of the pooled tubes were performed to assess whether the isolates have activity. Figure 5.22 below represents the antibacterial and antioxidant activity of the isolates. A number of clear bands can be observed both on the plate sprayed with DPPH and the bioautogram.

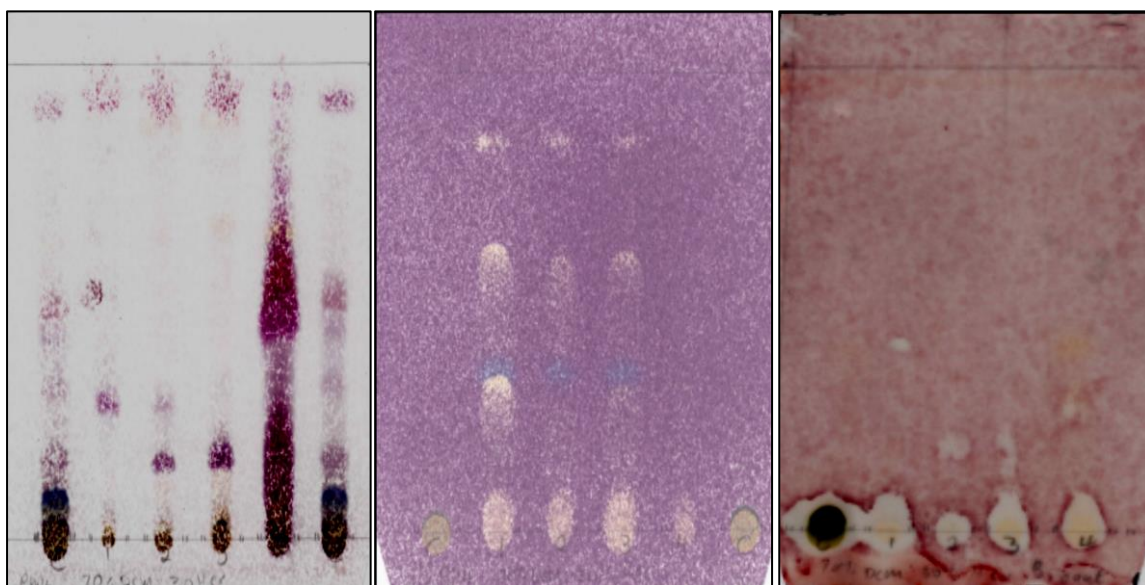


Figure 5.23: TLC profiling, antibacterial and antioxidant activity of the pooled tubes developed in 7:3 v/v of DCM: hexane.

5.3.9. Determination of mobile phase for second elution

The mobile phase for the second elution was determined by developing the fractions from first elution in DCM: hexane (9:1; 8:2; 7:3) (Figure 5.24). The mobile phase of 80% DCM in hexane was selected for the second elution.

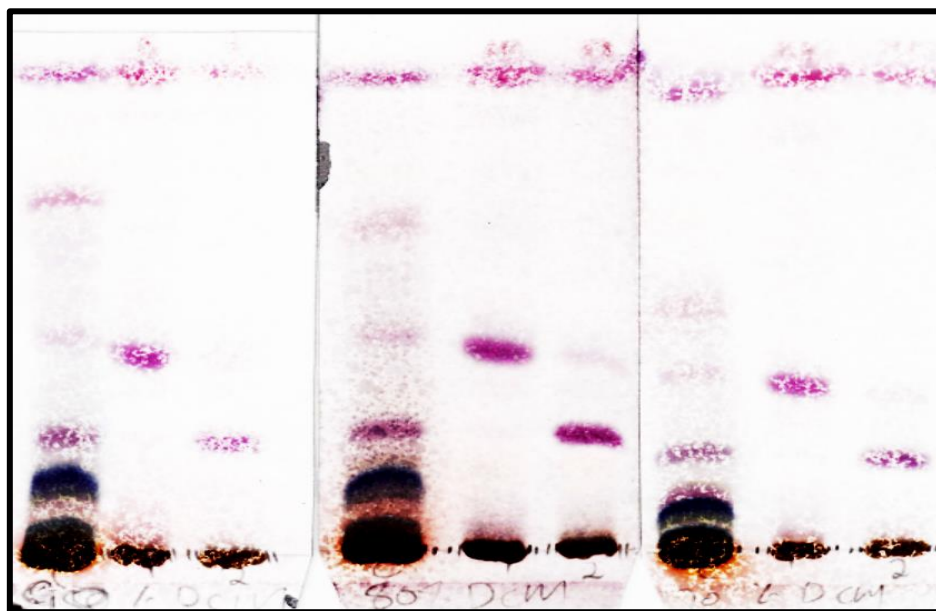


Figure 5.24: Chromatogram of the fractions from first elution in different concentrations of DCM and hexane.

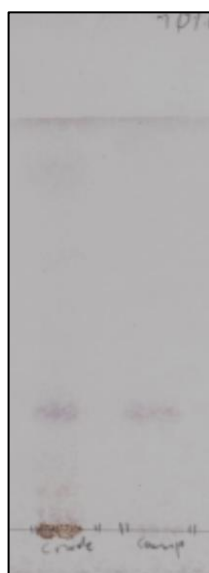


Figure 5.25: Chromatogram of the compound after the second elution in 7:3 DCM: hexane (v/v).

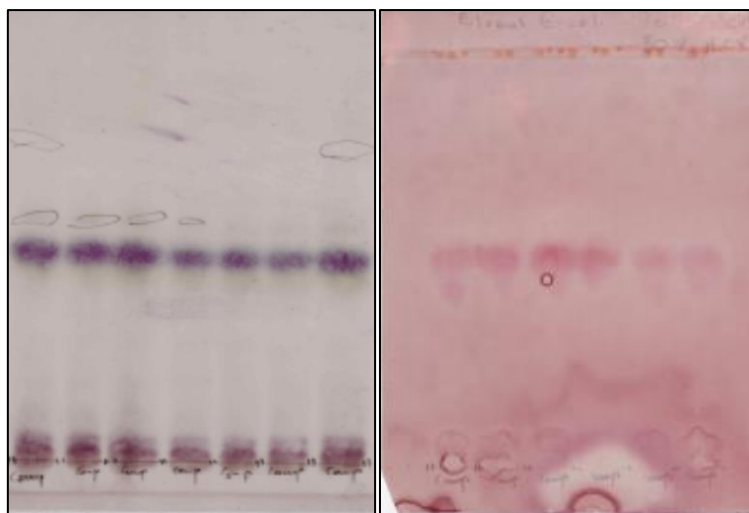


Figure 5.26: Phytochemical and antibacterial analysis of the collected compounds in 7:3 DCM: hexane.

5.3.10. Preparative TLC

Figure 5.27 below represents the TLC profiling of the compound developed in 100% chloroform in an attempt to separate the compound of interest from other compounds. Preparative TLC was performed using 100% chloroform as the mobile phase. Figure 5.28 represents the purified compound.



Figure 5.27: TLC profiling of the compound in 100% chloroform.



Figure 5.28: TLC profiling of the target compound purified by preparative TLC analysis in 100% chloroform.

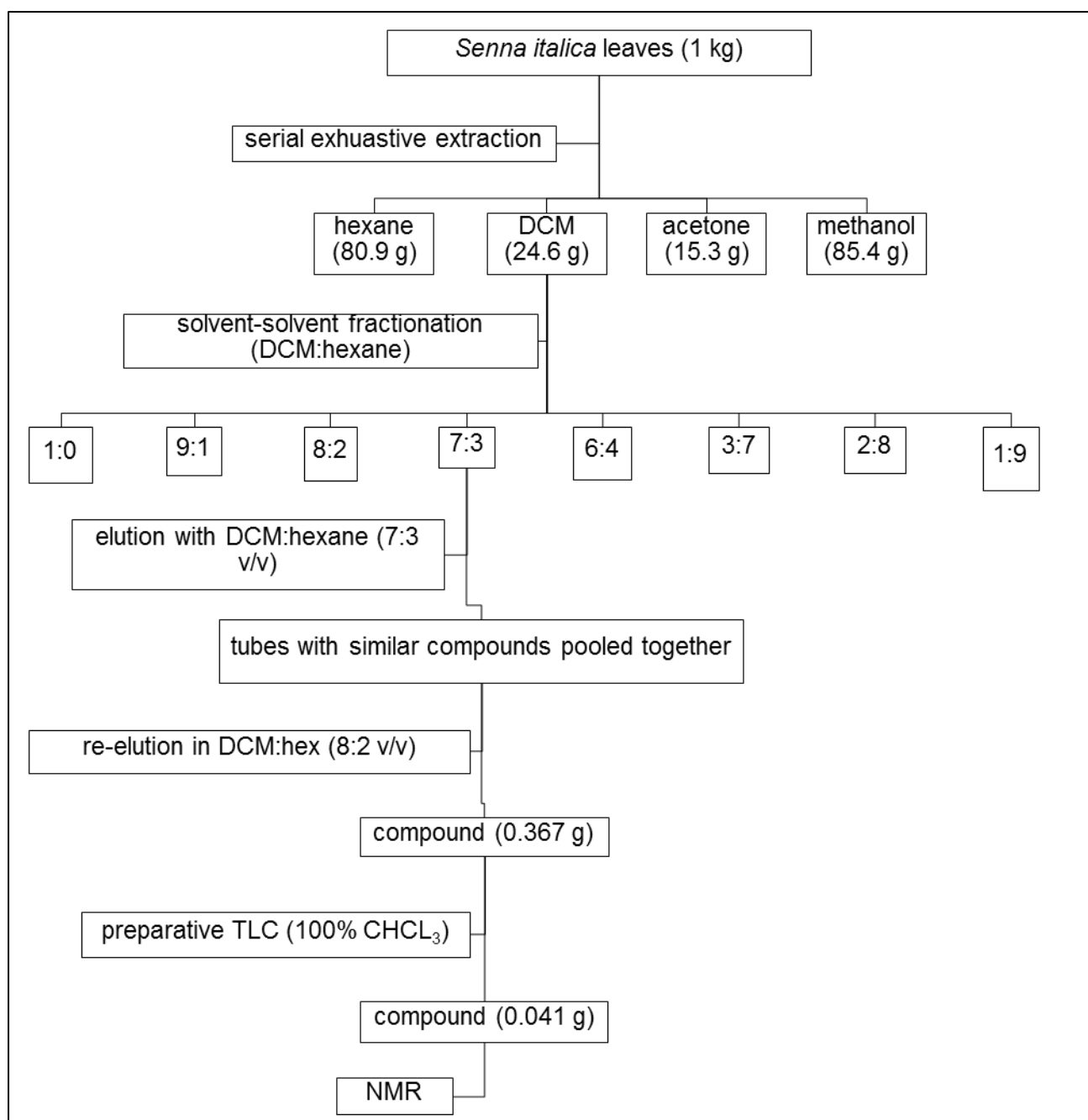


Figure 5.29: Flow diagram of isolation of the antibacterial compounds from *Senna italica* leaves.

5.4. Discussion

The leaves of *Senna italica* had interesting antibacterial activity against the test organisms in the preliminary bioautography assays that were reported in Chapter 4. Therefore, *S. italica* was selected for further isolation of the active compounds. In order to obtain enough mass for isolation, large scale extraction in the form of serial exhaustive extraction was carried out. The greatest mass was obtained when the leaves were extracted with methanol. Phytochemical analysis was performed to obtain the TLC fingerprint of the extracts. Figure 5.1 represents the TLC profile of the extract and the results show that more compounds were separated on the BEA plate which means that the compounds are non-polar. Although the greatest mass was obtained in the methanol extract, no bands were visible on the plate where the methanol extracts were loaded; only the pencil markings of UV active compounds were observed on the plates. This may be because the compounds do not react with the spray reagent used. The solvent used during extraction determines the type of compounds or secondary metabolites that will be isolated. For example, in most cases, extractions are carried out using organic solvents instead of water. This is because the extracts obtained using organic solvents show more antimicrobial activity than those obtained from water. Most studies reported that different solvents extract different compounds. Acetone and methanol extract saponins which were found to have antimicrobial activity. In addition, solvents such as dichloromethane and chloroform extract terpenes (Tiwori *et al.*, 2011).

Bioautography and microdilution assays were used for detecting antibacterial activity. Bioautography results (Figure 5.2 to 5.5) showed good antibacterial activity against *E. coli* (Figure 5.3). The DCM and acetone extracts had the lowest MIC values and had good activity against all the test organisms. Because the DCM extract showed good activity in both bioautography and microdilution assays, it was selected for isolation. The use of bioassays during isolation helps to localize and identify the potential active compounds (Masoko and Eloff, 2007).

Column chromatography requires that a suitable solvent system that will be able to elute the compound of interest be selected. Different solvent combinations were prepared in an attempt to identify a good mobile phase for elution of the compounds. Phytochemical analyses were performed in conjunction with bioautography in different mobile phases in order to observe the separation pattern of the compounds

and the activity. In Figure 5.6, the plates were developed in varying concentration of hexane in ethyl acetate. The profiles show that some of the active compounds were moving with the solvent front while some remained at the loading line. Figure 5.7 represent separation of the extract in varying concentrations of DCM in ethyl acetate. The profile shows that the compounds were localized just below the solvent front. In another attempt, a combination of DCM and methanol was used. The results in Figure 5.8 until Figure 5.9 had a good separation when 100% DCM was used as the mobile phase. As the polarity was increased by high concentrations of methanol, the compounds were moving with the solvent because methanol dissolves both polar and non-polar compounds. In another attempt to find the suitable mobile phase, DCM was combined with hexane. The TLC profiles in Figure 5.10 show the results for the separation in DCM: hexane and a good separation was observed on the plate developed in 7:3 v/v of DCM: hexane. The mobile phase was selected for elution of the active compounds in column chromatography. Choice of solvents during extraction is important as it determines the success of isolation of active compounds (Masoko and Eloff, 2006). When the DCM fraction was tested for antioxidant activity using the DPPH assay, the compound which had antibacterial activity did not show any antioxidant activity (Figure 5.11).

Column chromatography of the DCM fraction was performed using 7:3 (v/v) DCM: hexane as the eluent. Figure 5.12-5.20 represents the compounds which were eluted with different concentrations of DCM in hexane. The results show that as the polarity of the eluent increases, different compounds are being eluted based on their polarity. Phytochemical analysis and bioautography of the collected tubes were carried out in 5:5 v/v of DCM: hexane in order to see which tubes contained similar compounds. With the results in Figure 5.21, tubes with similar compounds were observed and the tubes were pooled together. The bioautogram in Figure 5.22 had clear zones against a purple background which meant antibacterial activity of the compounds. The tubes were pooled into three separate vials depending on the tube's contents. The vials' contents were evaluated for phytochemical and biological activities using TLC, bioautography and DPPH assay. Yellow bands against a purple background and clear zones against a purple background were observed for antioxidant and antibacterial activity respectively, (Figure 5.23). Since there was antibacterial activity, the next step in this isolation process was to find a suitable mobile phase for the

second elution and 8:2 v/v (DCM:hexane) was selected by developing plates loaded with the sample (Figure 5.24). After elution, TLC of the collected compound had a single band on the plate (Figure 5.25). Although a single band was observed on the plate, there was some brown material remaining where the sample was loaded. Bioautography was performed to see if the compound still possessed its antibacterial activity. On the bioautogram (Figure 5.27), there seemed to be overgrowth over the compound. It seemed as if there was something inhibiting the activity of the compound because the colour around the overgrowth was lighter than the rest of the plate. Chloroform (100%) was used to separate the compound from the other compound blocking its activity (Figure 5.28). Therefore, the compound was further purified using preparative TLC with 100% chloroform as the solvent system since it was able to separate the two compounds. Figure 5.29 shows the purified compound developed in 100% chloroform. The compound was sent for nuclear magnetic resonance (NMR) structural analysis.

5.5. Conclusion

The use of bioassays during isolation of compounds plays an important role because they enable one to track the compound of interest and also to see if the compound is still active or not. Thin layer chromatography can be used as one of the purification steps or just to assess the purity of the isolated compound with the aid of some visualization steps (Dinan *et al.*, 2001).

CHAPTER 6

Structure elucidation of the active compound

6.1. Introduction

Mass spectrometers can identify many compounds at the same time even if they are co-eluting. This is possible because individual compounds are quantified by their molecular masses and their fragmentation pathways resulting in selectivity and identification. Perfect ionization of all compounds is essential for the quantification of the molecular mass and detection on the chromatogram. But it is not always possible to achieve complete ionization of all compounds since compounds tend to obstruct each other especially when they are closely related and elute simultaneously. As such, the compounds have to be separated in order to achieve strong quantification (Tolstikov *et al.*, 2002).

Nuclear Magnetic Resonance (NMR) is a powerful technique used for investigation of structural and other properties of molecules. One of its major applications is elucidation of structures of new compounds. Even though NMR requires samples in large amounts, developments made in NMR instrumentation, pulse programs and computing power in the last decade have allowed for detection of proton and carbon signals with samples weighing as much as 1 mg (Fossen and Andersen, 2005). The One dimensional NMR determines the frequency of resonance of each ^1H or ^{13}C nuclei in the molecule (Pinheiro and Justino, 2012). The ^1H NMR is used to determine the relative number of protons and their type by comparing their chemical shifts while the ^{13}C NMR is used to compliment the data provided by the ^1H NMR. However, the ^{13}C NMR is less sensitive due to the abundance of ^{13}C (Claridge, 1999).

A pulse program called Correlated Spectroscopy (COSY), where cross-peaks at the intersection of the frequencies of the protons which are coupled together are displayed is used in two dimensional NMR. A variation of the COSY called gradient COSY (gCOSY) displays the cross-peaks present in protons that have 3-bond proton-proton scalar coupling. Another variation of the program called Double Quantum COSY (DQCOSY) discloses the proton-proton interactions that the proton spectrum cannot reveal (Sperry *et al.*, 2004).

Another pulse program called gradient Heteronuclear Single Quantum Coherence (gHSQC) is a two dimensional NMR technique which reveals the H-C correlation. In this technique one axis is the proton while on the other axis is the carbon. Totally Correlated Spectroscopy (TOSCY) is another pulse program which detects the cross-peaks between all of the protons within a coupling network especially proton-proton correlation in protein experiments (Kurosawa *et al.*, 2006).

6.2. Methods and materials

A clean sample was sent to CSIR for NMR spectral analysis. The sample was characterized using ^1H NMR and ^{13}C NMR spectra and processed using INOVA 400 spectrometer at 5353 Hz with chloroform as the solvent signal reference. Dr. L. Mdee of the Department of Pharmacy, University of Limpopo (Turfloop Campus), assisted with structure determination.

6.3. Results

Nuclear Magnetic Resonance of isolated compounds was run under different pulse sequences i.e. ^1H ; gCOSY; gDQCOSY; gHSCQTOXY 1,2,4,5 and ^{13}C in chloroform. The spectra (Figure 6.1 to Figure 6.11) below show the NMR chromatograms under the different pulse sequences. Although Figure 6.1 and Figure 6.11 show prominent peaks, there is some impurity which is related to the compound and can be observed on Figure 6.6 to Figure 6.10.

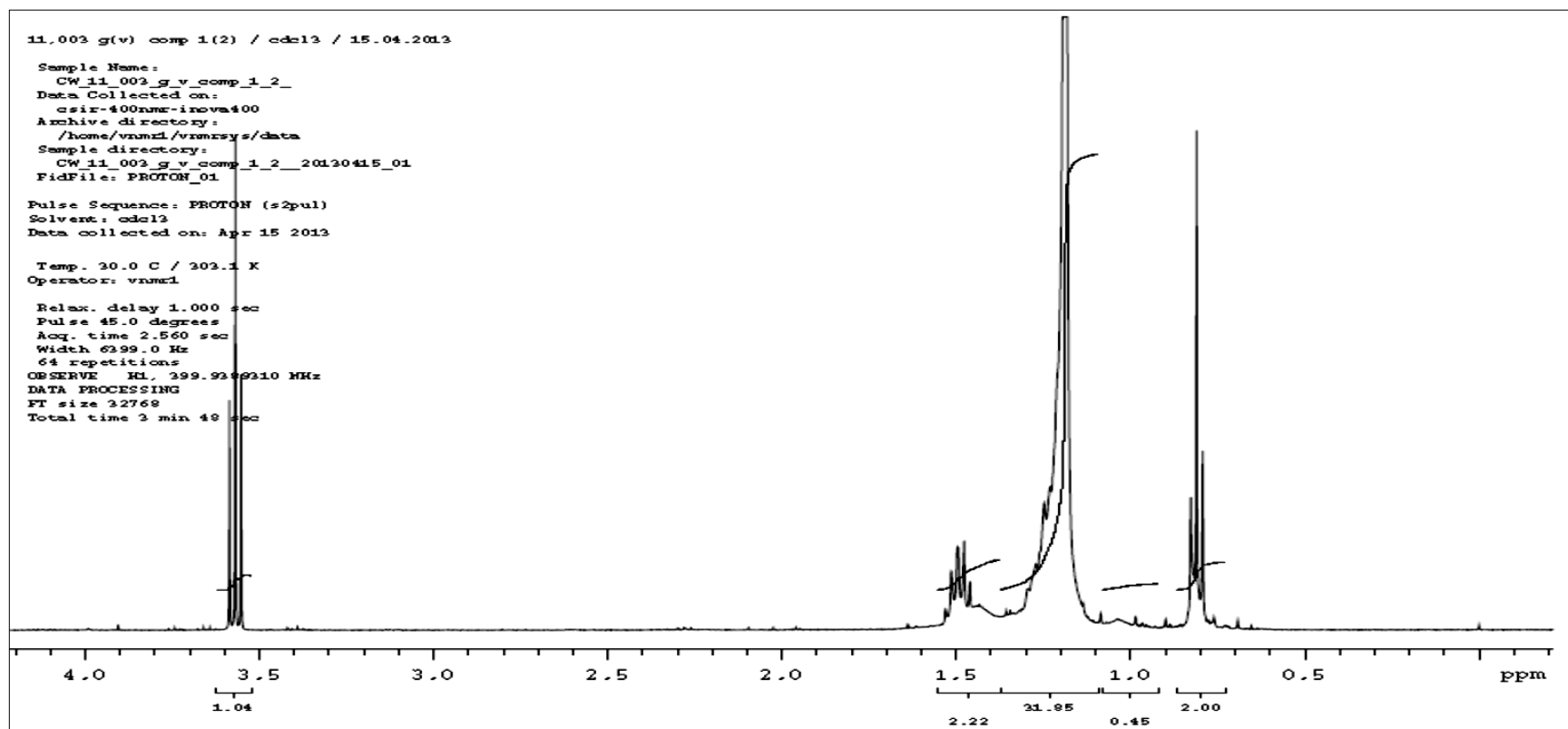


Figure 6.1: ^1H NMR spectrum of isolated compound in CDC 13.

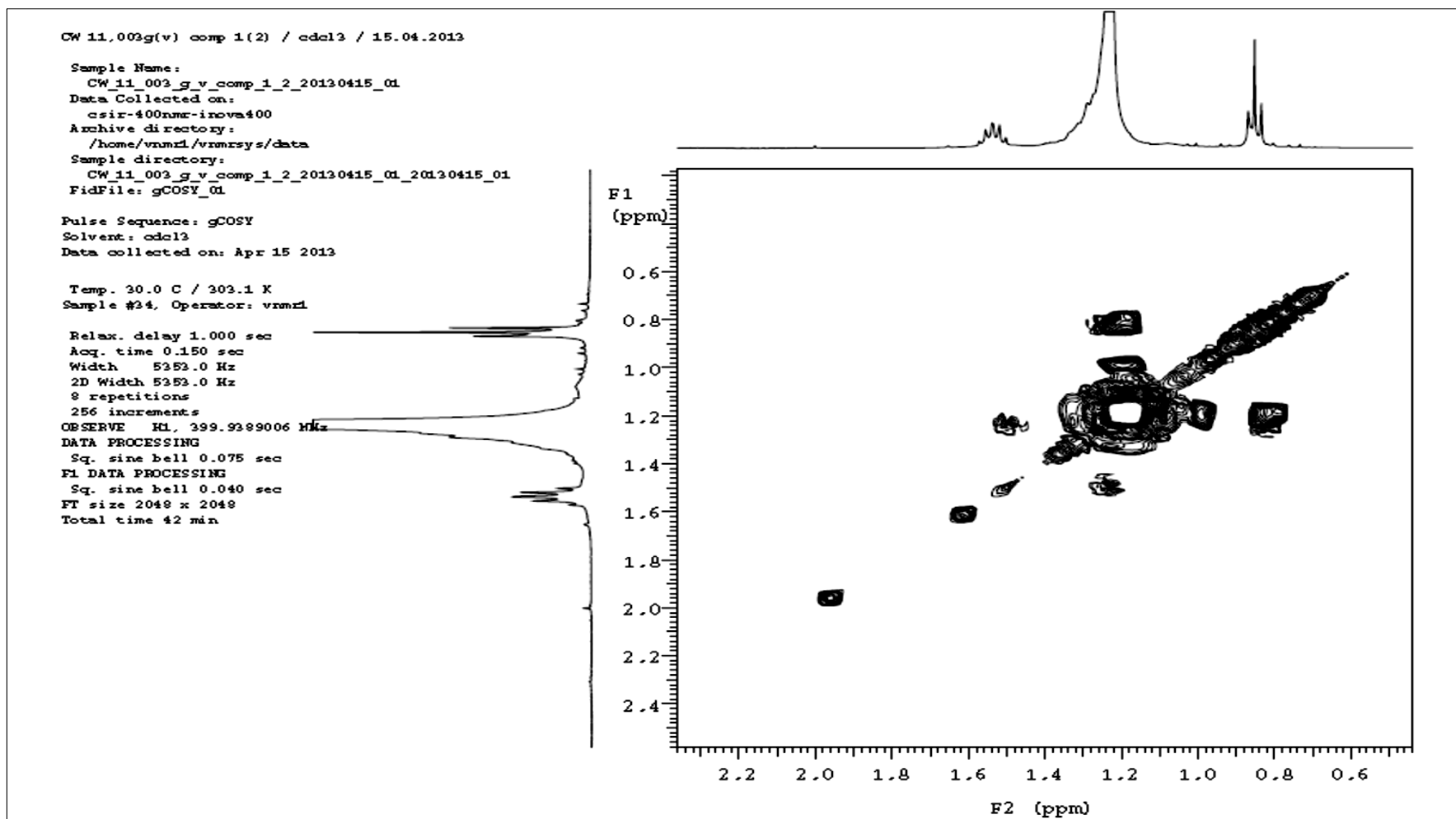


Figure 6.2: gCOSY spectrum of isolated compound in CDC 13.

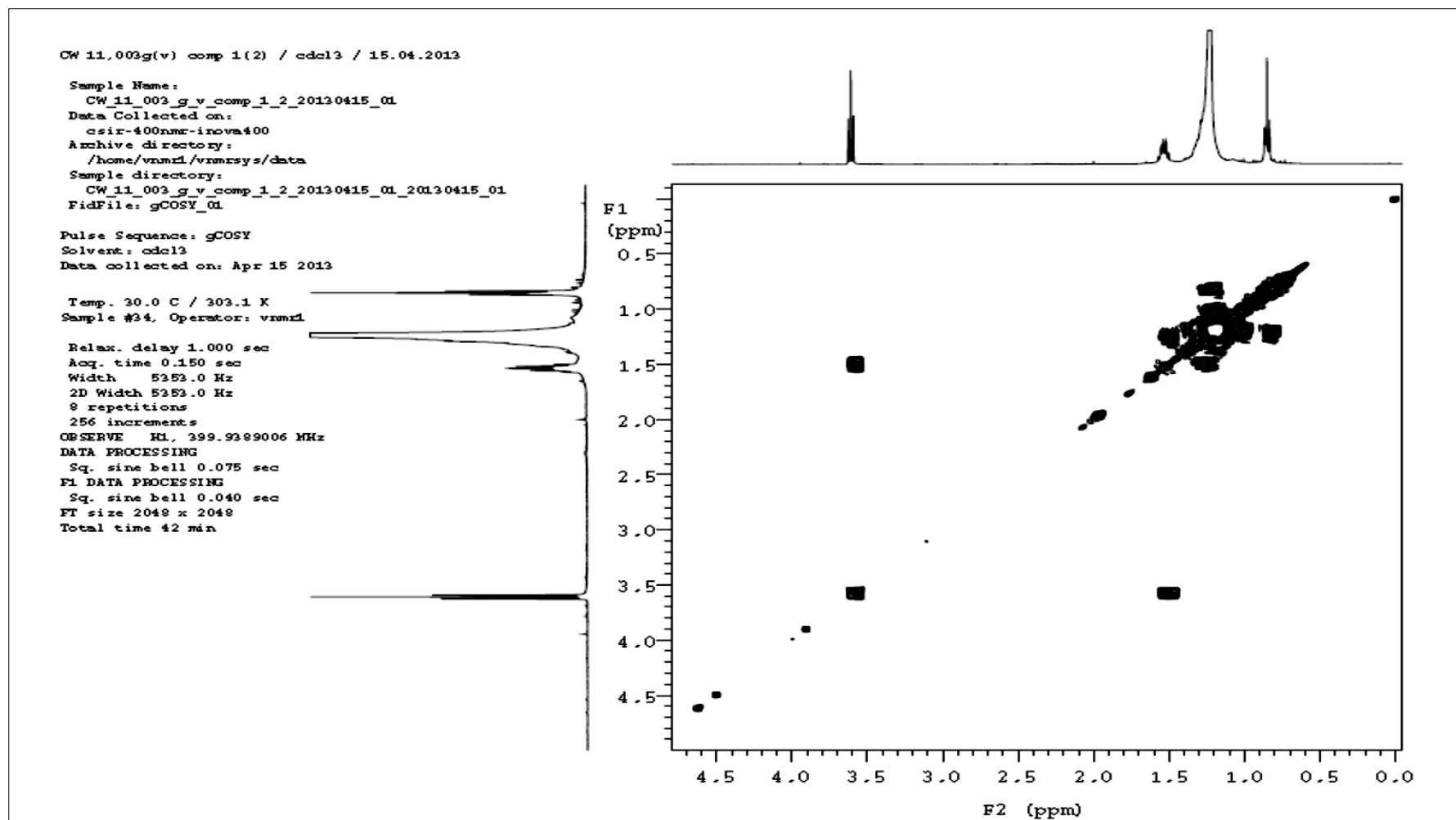


Figure 6.3: gCOSY spectrum of isolated compound in CDC 13.

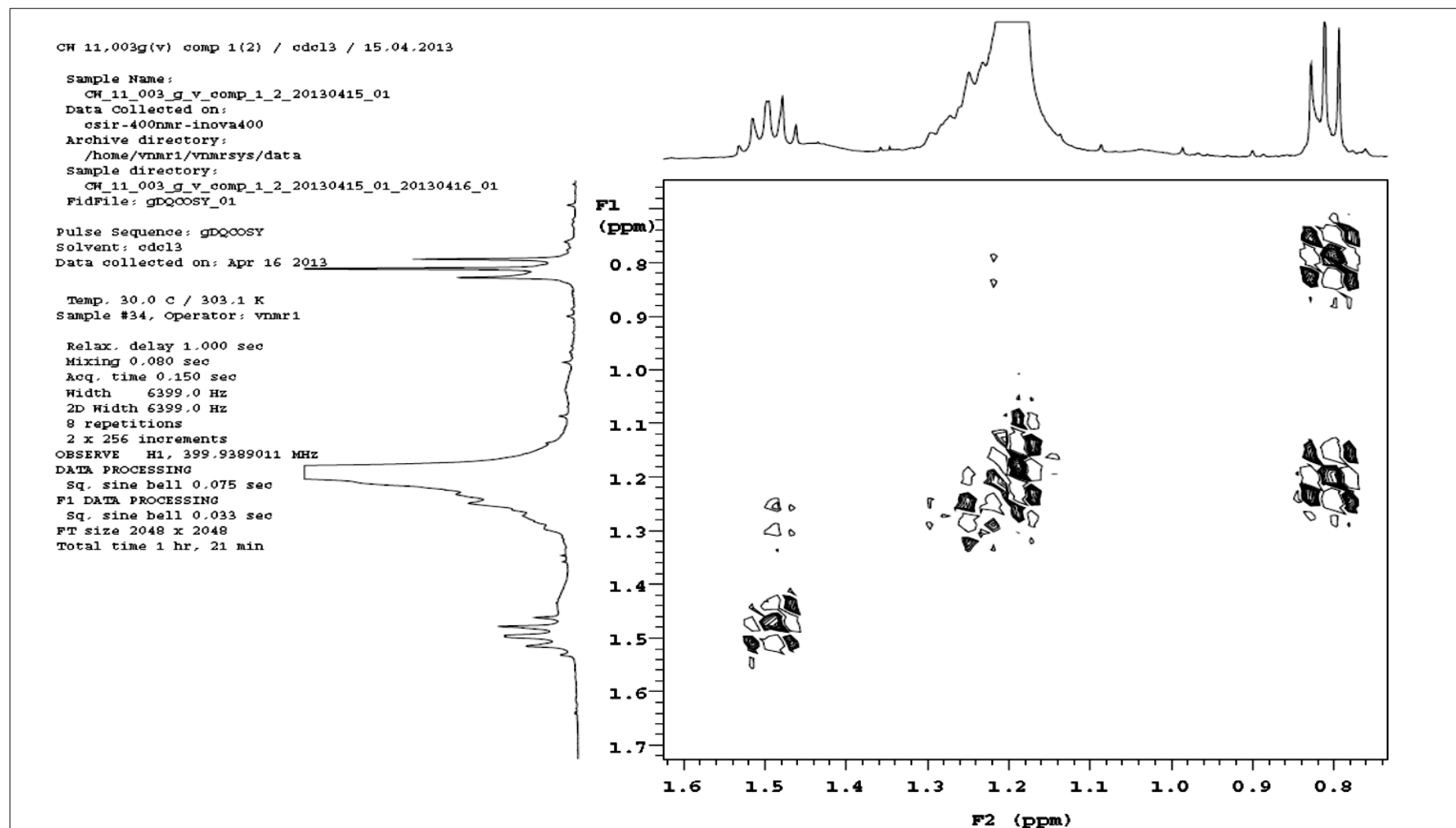


Figure 6.4: gDQCOSY spectrum of isolated compound in CDC 13.

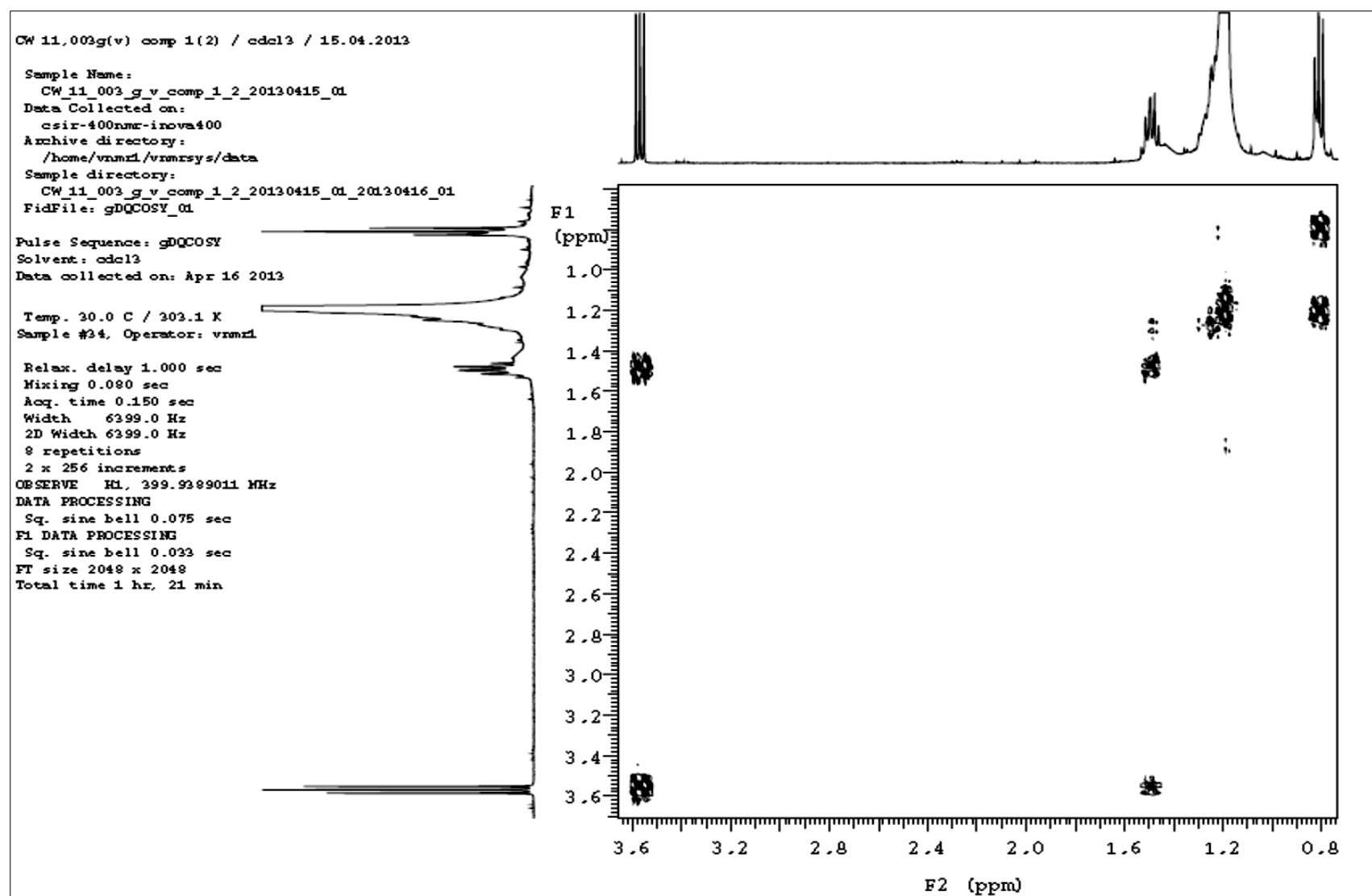


Figure 6.5: gDQCOSY spectrum of isolated compound in CDC 13.

11.003 g(v) comp 1(2) / cdcl3 / 19.04.2013

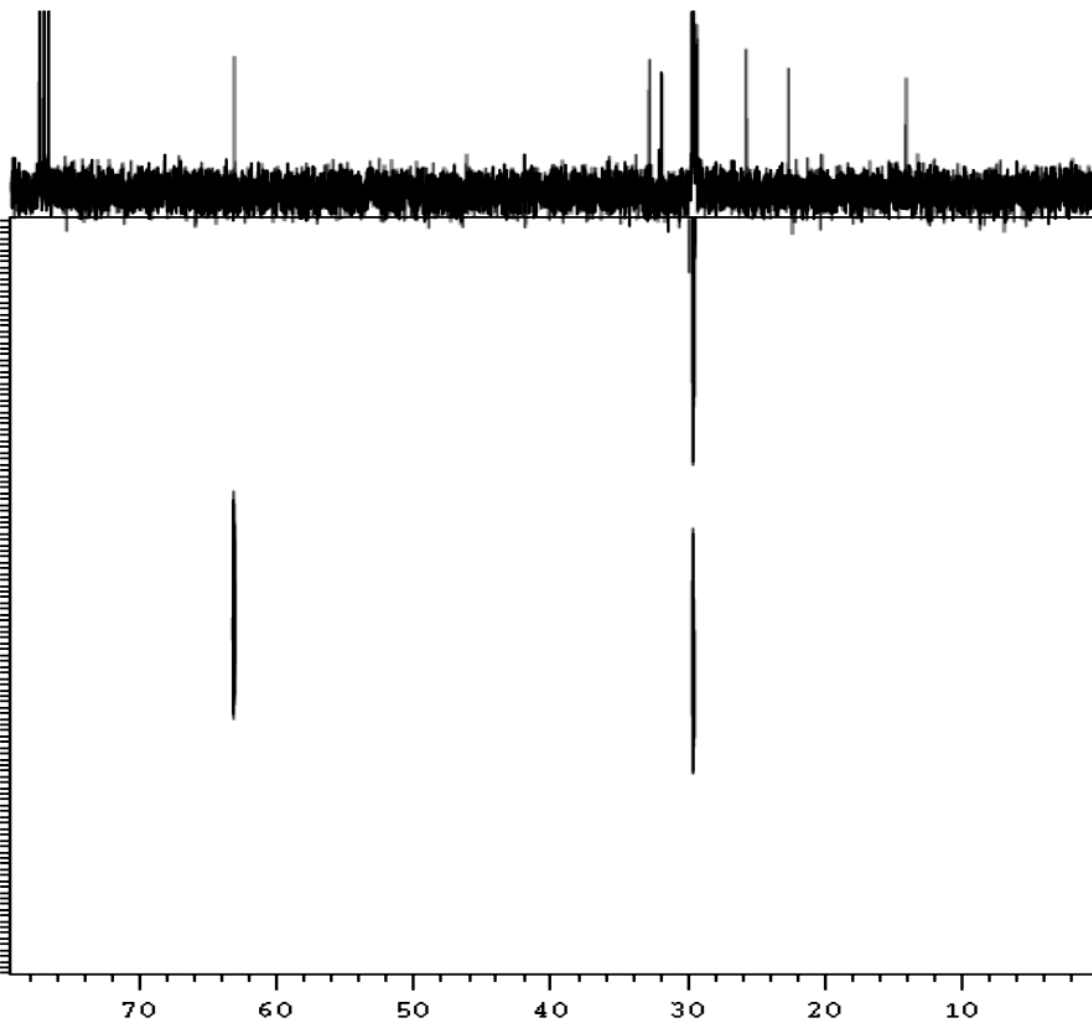
Sample Name:
CW_11_003_g_v_comp_1_2_
Data Collected on:
csir-400nmr-inova400
Archive directory:
/home/vnmr1/vnmrsys/data
Sample directory:
CW_11_003_g_v_comp_1_2_20130419_01
FidFile: gHSCQTOXY_01

Pulse Sequence: gHSCQTOXY
Solvent: cdcl3
Data collected on: Apr 19 2013

Temp. 30.0 C / 303.1 K
Sample #34, Operator: vnmr1

Relax. delay 1.000 sec
Acq. time 0.150 sec
Width 5351.5 Hz
2D Width 17097.7 Hz
40 repetitions
2 x 2048 increments
OBSERVE H1, 399.9388932 MHz
DECOUPLE C13, 100.5722605 MHz
Power 41 dB
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.069 sec
F1 DATA PROCESSING
Gauss apodization 0.055 sec
FT size 2048 x 4096
Total time 61 hr, 21 min

F2
(ppm)
1.39
1.40
1.41
1.42
1.43
1.44
1.45
1.46
1.47
1.48
1.49
1.50
1.51
1.52
1.53
1.54
1.55
1.56
1.57
1.58
1.59
1.60
1.61
1.62



F1 (ppm)

Figure 6.6: gHSCQTOXY 4 spectrum of isolated compound in CDC 13.

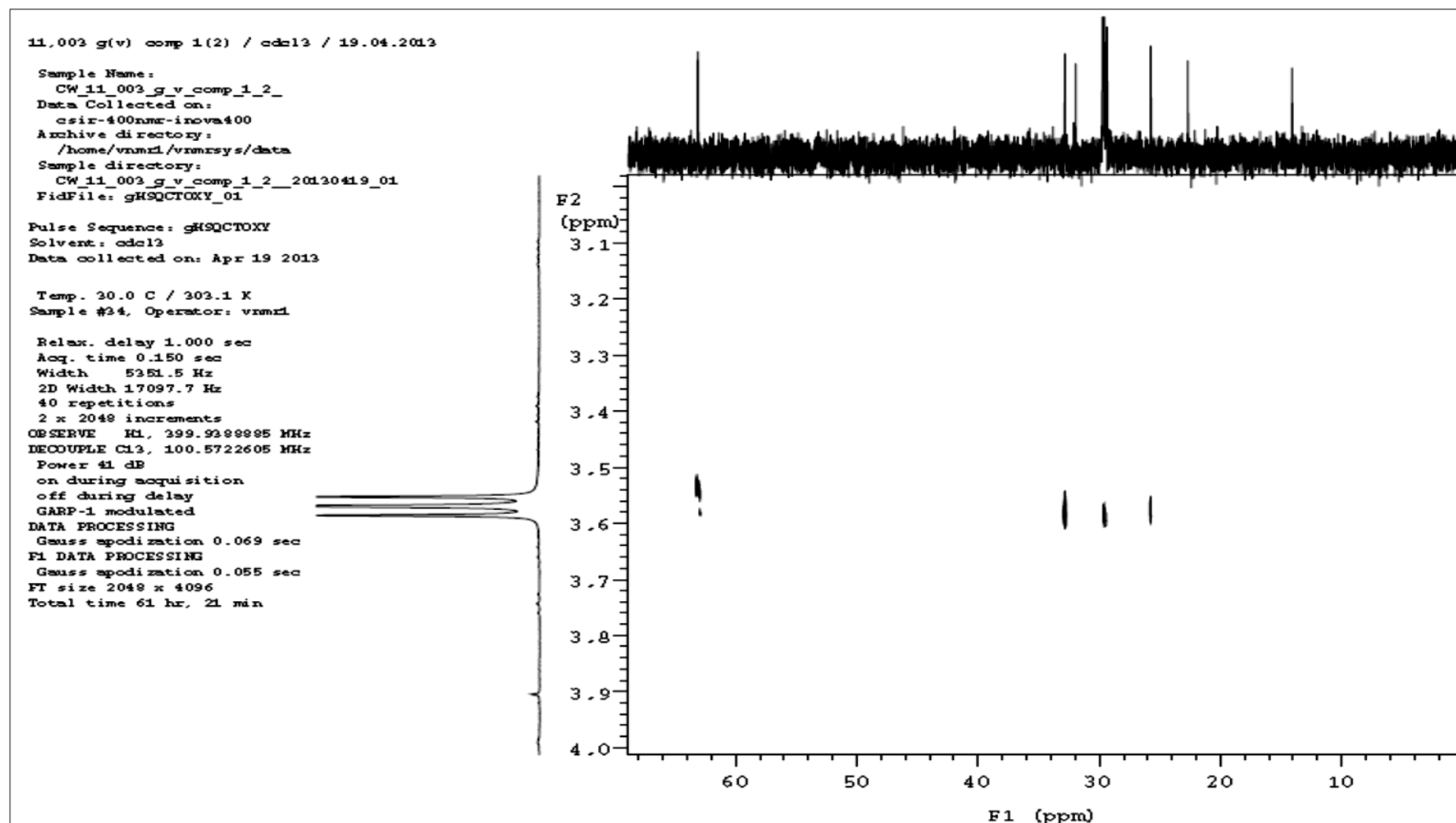


Figure 6.7: gHSCQTOXY 1 spectrum of isolated compound in CDC 13.

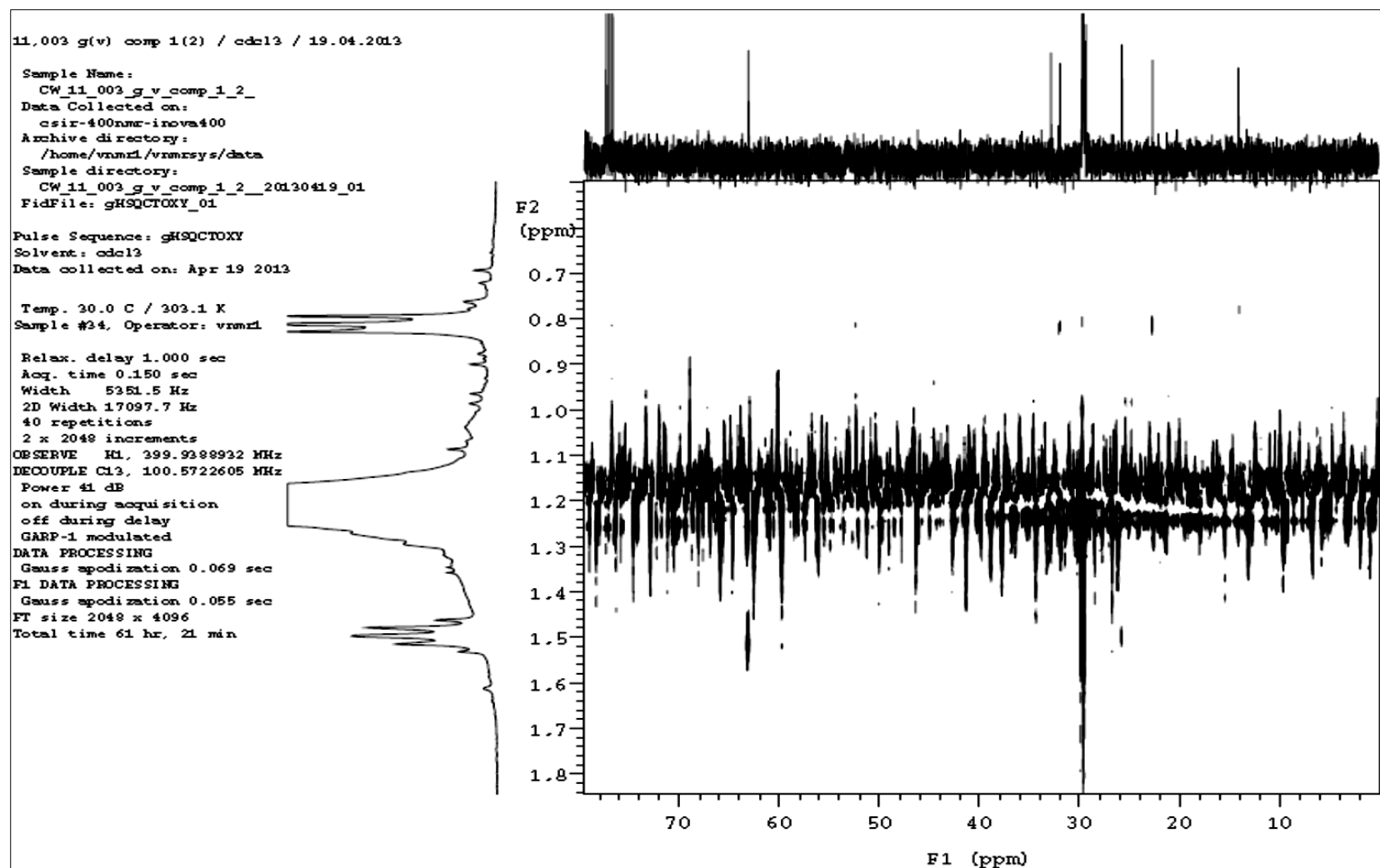


Figure 6.8: gHSCQTOXY 2 spectrum of isolated compound in CDC 13.

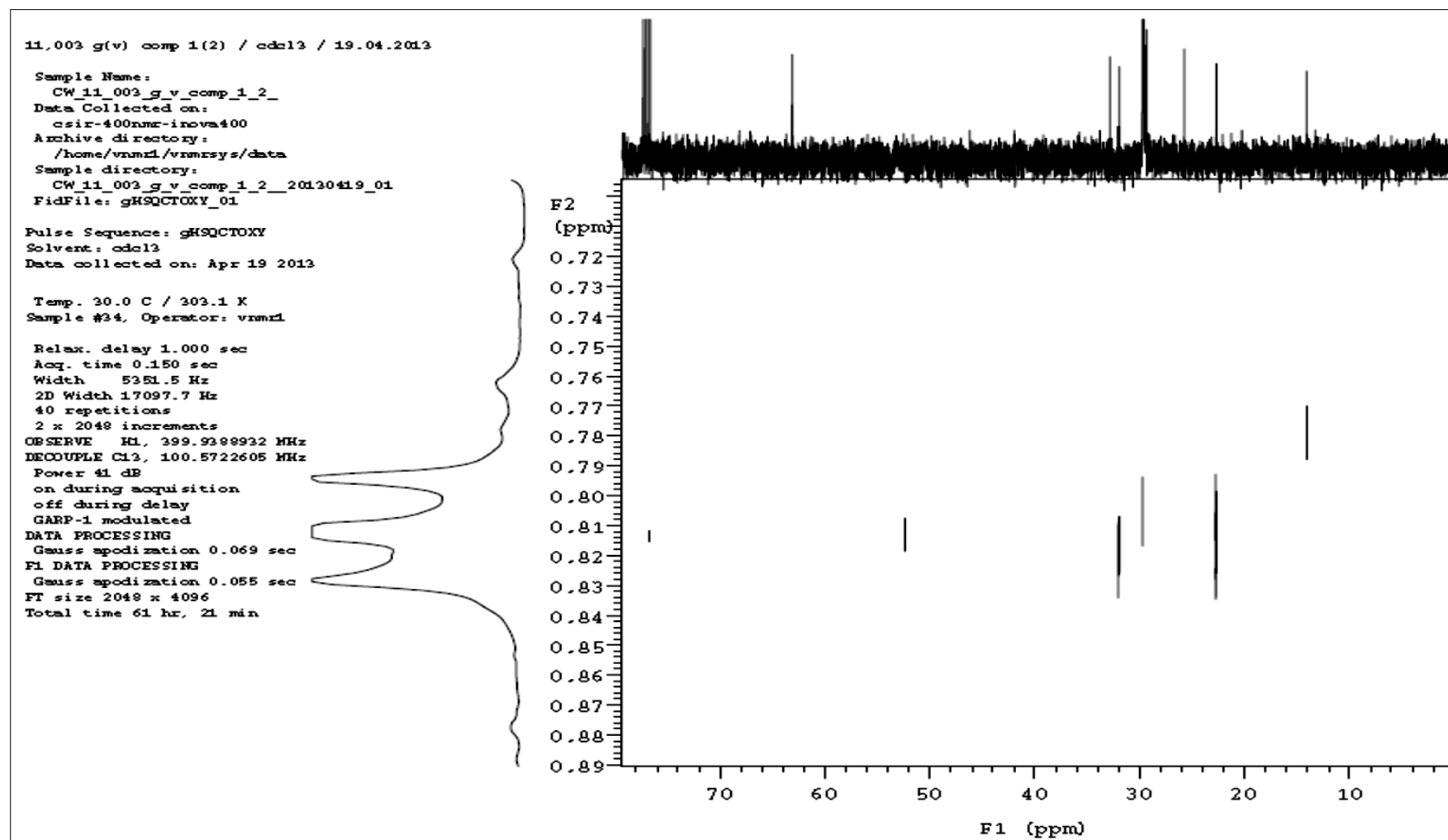


Figure 6.9: gHSCQTOXY 5 spectrum of isolated compound in CDC 13.

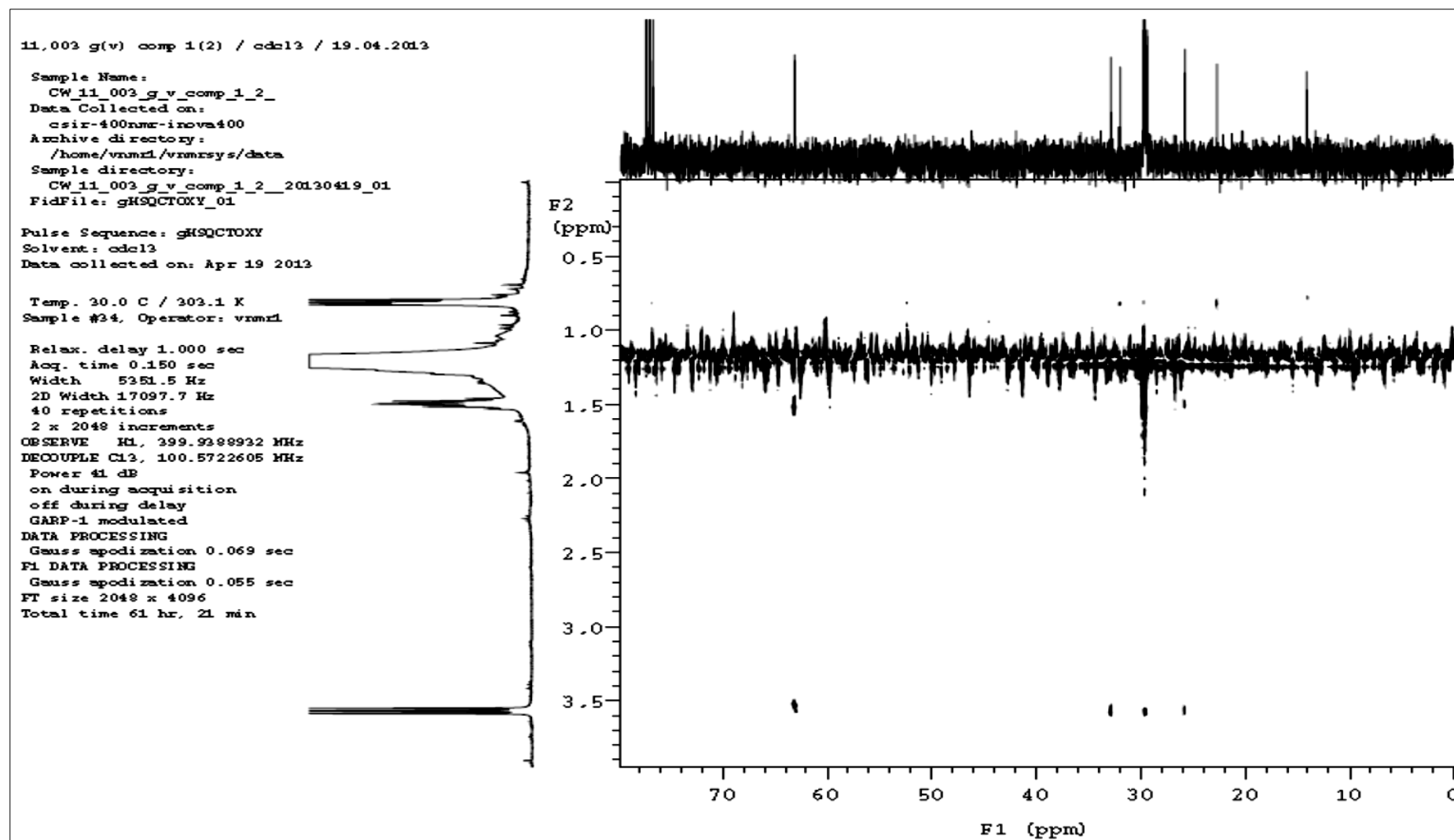


Figure 6.10: gHSQCTOXY total spectrum of isolated compound in CDC 13.

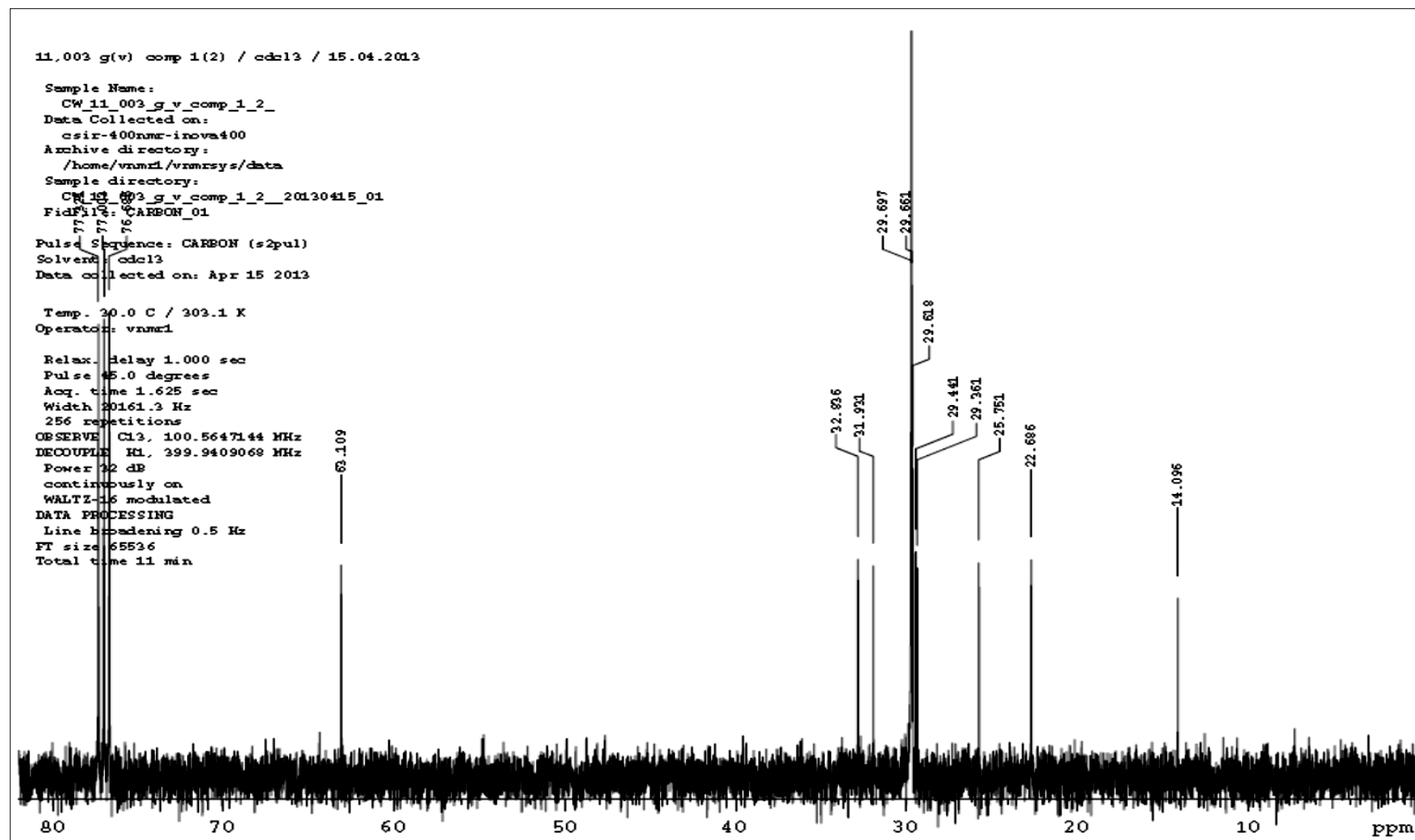


Figure 6.11: ^{13}C NMR spectrum of isolated compound in CDCl_3 .

Other researchers have isolated several compounds like 1,1,8,8 -tetrahydroxy-7 '-methoxy-3,3 '-dimethyl-10,10 '-bianthracen-9,9 '-dione (Figure 6.12) from *S. italica* and the University of Limpopo Medicinal Plants Research Group has isolated 3,4-5-trihydroxystilbene (Figure 6.13) from the roots of *S. italica*. Based on NMR results and although not included, it was concluded that the tentative structure of the compound isolated is an alkane. Possible structures of alkanes are presented in Figure 6.14.

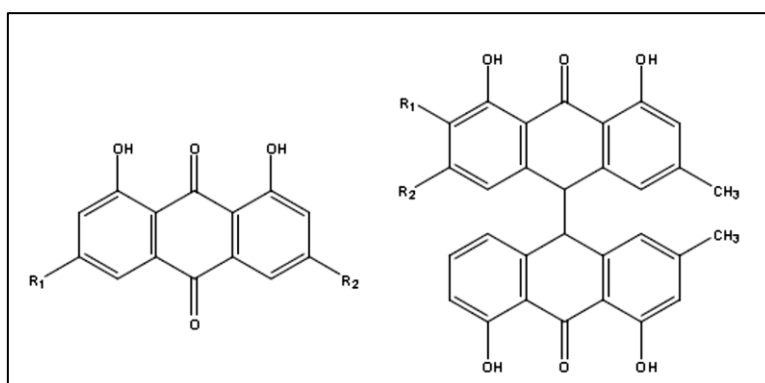


Figure 6.12: Structures of compounds isolated from *Senna italica* (Yagi *et al.*, 2013).

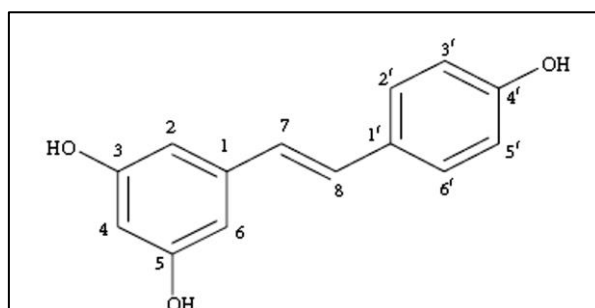


Figure 6.13: The compound identified as 3,4,5-trihydroxystilbene ($C_{14}H_{12}O_3$) from 1H - and ^{13}C -NMR spectra peak assignment (Mokgotho *et al.*, 2013).

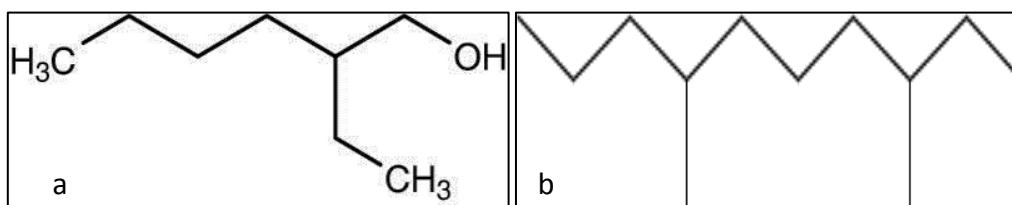


Figure 6.14: 2-ethyl-1-hexanol (a) and 3,7-dimethyl decane (b) are possible alkane structures of the isolated compound.

6.4. Discussion

Isolation of antibacterial compounds from Senna leaves was carried out using column chromatography and bioautography as a guide. The TLC profile showed a purple compound which fluoresced red when viewed under ultraviolet light. Preparative TLC was used to purify the isolated compound with 100% chloroform as the mobile phase. The collected compound (0.04 g) appeared as a white powder with a smooth texture.

The compound was sent to the Centre for Scientific and Industrial Research (CSIR) in Pretoria for NMR and Mass Spectrometry analysis. Nuclear Magnetic Resonance was run on inova400 machine at 30°C in chloroform. Although the compound appeared to be pure on the TLC plate, a large quantity of the sample did not dissolve in chloroform, acetone or methanol, at room temperature or even when heated. When chloroform was added to the sample, a scummy layer formed from the turbid suspension on standing and precipitated out with time. This impurity is common in purifications and extractions where commercial-grade hexane that has not been fractionally distilled is used. This impurity related to the compound itself can be seen from the prominent signal at 1.19 ppm on the ^1H spectra (Figure 6.1) and 29.70 ppm on the ^{13}C spectra (Figure 6.11). According to the observations made, the impurity can be removed through pTLC because of its lipophilic nature.

A significant part of the spectra is obscured by the signal in both spectra, and these resulted in thwarted attempts to cross-correlate atoms or establish linkages between atoms. As many as 5 or six 'other' carbon signals seemed to be obscured under this major contaminant in the carbon and carbon-cross-correlated spectra. The impact of these carbon signals cannot be determined from this sample, as their similarity to the signal obscuring them seems to suggest a common origin. These carbon signals could be genuine, or this was simply detecting the smaller outliers as part of the polymeric distribution of the contaminant in at least some of the cases. There were genuine carbon signals under this substance seen from a clear cross-correlation to the area in the gDQCOSY experiment (Figure 6.4 and Figure 6.5) between the up-field signals at 0.8 ppm and the obscured region.

Some HSQC correlations were evident in the 2D gHSQCTOXY experiment (Figure 6.6 to Figure 6.10), the most visible being typical 1J C-H correlations. Only one clear

spin system could be detected related to the proton at 3.9 ppm, but it was hardly sufficient data for structural elucidation. The tentative structure for the compound is a branched alkane structure, with at least one ether linkage per repeating unit based on the signals at 63.10 ppm and 3.57 ppm (Figure 6.11). Mass Spectrometry of the compound was unsuccessful because the quantity of the sample was small and no observable peaks were present on the spectra.

Several alkanes such as 2,6-di-*sec*-butylphenol, di-*n*-octylphthalate, eicosane, tetratriacontane and 2,2'-methylenebis[6-(1,1-dimethyl)-4-methylphenol] were isolated from the pod oil of *Senna italica* by Yagi *et al.* (2013). Figure 6.12 is 1,1,8,8-tetrahydroxy-7'-methoxy-3,3'-dimethyl-10,10'-bianthracen-9,9'-dione which was isolated by Yagi *et al.* (2013). In a study by Mokgotho *et al.* (2013) 3,4,5-trihydroxystilbene commonly known as Resveratrol was isolated from the roots of *Senna italica* (Figure 6.13). One of the above mentioned compounds could possibly be the compound isolated in this study. Figure 6.14 shows two compounds which could be possible structures of the isolated compound. These compounds which had antibacterial activity were isolated in a study by Dehpour *et al.* (2012).

6.5. Conclusion

The NMR results suggest that the structural elucidation of the isolated compound cannot be carried due to the presence of impurities which were obscuring the visibility of the cross-correlation and linkages between atoms. Therefore these results suggest that isolation of the compound be repeated for the structure to be elucidated. And since the impurity is thought to be coming from the solvents used during the isolation, the quality of the solvents to be used must be taken into great account. Furthermore the biological activities of the isolated compound could not be performed due to insufficient sample after purifying the compound.

CHAPTER 7

General discussion and conclusions

7.1. Discussion

The present study was carried out to investigate the biological properties of five medicinal plants with potential use in wound healing by isolating and identifying the antimicrobial compounds in their leaves. The leaves were extracted with solvents with varying polarities to obtain a diversity of the compounds in terms of their polarity. The extraction of more compounds using methanol solvents may be indicative of large amounts of polar compounds present in the leaves of these medicinal plants. The season in which the leaves were collected could also have an influence on the amount of polar compounds present in leaves since leaves produce more carbohydrates in certain seasonal periods (Bagla, 2011). The solubility of the solvent also plays an important role in the mass transfer between the active ingredients and the solvent (Patil *et al.*, 2009) and as such this implies that methanol is more soluble than the rest of the solvents employed in the extraction process because of the mass extracted.

The diversity of the compounds is further observed on the chromatograms where the compounds were separated in mobile phases of varying polarity from non-polar BEA to EMW (Kotze and Eloff, 2002). The different compounds can be observed as coloured bands on the chromatograms. Furthermore, the diversity of the compounds is confirmed by phytochemical tests for biomolecules such as tannins, steroids, alkaloids etc. where the plants tested positive for several of the biomolecules. Phytochemical studies pave the way for the identification of different biomolecules which have different biological functions and because of their localization in the different plant parts; they play different functions in the plant. However parameters such as biological activity and chemical composition of plant extracts may vary as a result of the time and season of collection, location and the part of the plant analysed. In most cases traditional healers tend to use the stem bark because it is available throughout all the seasons (Tagwireyi *et al.*, 2002) but in this study leaves were used because that is where the metabolic reactions that lead to the synthesis of biomolecules take place. Studies by Borokini and Omotayo (2012) have shown

that plant compounds do play a role in medicine and while some of the compounds are responsible for merely the colours and taste of flowers and fruits, some of the compounds act as a defence against pathogens (Larson, 1988; Heldt, 1997).

The extracts were redissolved in acetone because it was found not to be harmful to organisms such as fungi (Masoko *et al.*, 2007) and bacteria (Eloff, 1998). Biological assays are used to evaluate the biological activities of plants. The microplate method (Eloff, 1988a) and bioautography (Begue and Kline, 1972) were used to study antibacterial activity; while DPPH, MTT and Phargoburstt assays were used to study the antioxidant, toxicity and anti-inflammatory activities of the plants respectively. The microplate method indicates the lowest concentration with inhibitory activity while bioautography shows the individual compounds with inhibitory activity towards the test organisms. The microplate results and the bioautography results did not correspond due to synergistic and antagonistic properties of the compounds or their concentration within the extracts (Taylor *et al.*, 2001). The biological activity observed in these plants may be due to the presence of compounds such as alkaloids, saponins, tannins, terpenoids, phlobatannins, cardiac glycosides, steroids and flavonoids which were tested. The antimicrobial activity of *L. javanica*, *S. italica*, and *L. camara* observed on the bioautograms could be as a result of alkaloids because they are believed to be responsible for biological activities such as antibacterial activity (Evans, 2002). *Ziziphus mucronata* contains saponins which may be responsible for its antimicrobial activity as they are known to prevent microbial attacks in plants, which imply that they are natural antibiotics (Okwu and Emenike, 2006). Therefore the antimicrobial activity observed in the plants may be attributed to the presence of saponins (Di maro *et al.*, 2007). A study by Quiroga *et al.* in 2001 suggested that tannins present in plants that are used in wound healing are responsible for their wound healing efficacy (Quiroga *et al.*, 2001).

Thin layer chromatography (TLC) plates were developed and sprayed with 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol for antioxidant screening. Visualization of separated bands exhibiting antioxidant activities enabled the localization and the subsequent identification of the potential active compounds. Antioxidant compounds were identified by the yellow bands on the plates against a purple background (Brand-Williams *et al.*, 1994). BEA and EMW showed the most antioxidant bands

though not clear in BEA and CEF. Natural compounds such as tannins, saponins and flavonoids have been reported by Baravka *et al.* (2008) to have antioxidant activity. Tannins and flavonoids present in *Lippia javanica* and *Ricinus communis* are responsible for the antioxidant activity of the plants. Masoko *et al.* (2010) found compounds with antioxidant activity in the roots of *Senna italica*, but no antioxidant activity was observed in the leaves. This may be due to the localization and function of the antioxidant compound in the roots of the plant. At times when working with bioassays of plant extracts the observed results may be as a result of a mixture of biologically active plant compounds and the sensitivity may vary from batch to batch of the collected material (McLaughlin and Rogers, 1998).

Substances such as free radicals and other reactive oxygen species (ROS) which are by products of metabolic pathways in the body can act as factors which delay the healing process. Since ROSs are unstable, they bind to cellular molecules such as proteins, DNA, cell membranes and lipids which then leads to severely damaged tissues. A plant with antioxidants will be able to scavenge the free radicals and subsequently reduce tissue damage (Thakur *et al.*, 2011). Therefore plants with both antioxidant and antimicrobial activities are good candidates for the treatment of infected wounds.

Lippia javanica showed inhibitory activity against the test organisms in the microplate assay and bioautography and antioxidant activity assay. Anti-inflammatory activity of the plant was carried out on *L. javanica* using the Phargoburstt test kit. This kit measures the reduction of ROSs produced by Raw 264.7 macrophages. In the presence of ROSs, the cells fluoresce green when viewed under fluorescence microscope. The cells which were treated with *L. javanica* were fluorescent under the microscope which means that the extract was unable to prevent the synthesis of the ROSs. It was expected since *L. javanica* had significant antioxidant activity it will reduce ROS and result in no fluorescent or significant reduction in fluorescent, however, this was not the case. It is still unclear why this happened but one possibility may be that the speed and amount of ROS produced outweighed the amount of antioxidants produced by the plant extracts. This is an interesting observation which requires further study.

Senna italica was selected for isolation of antibacterial compounds based on the microplate assay and bioautography results which showed antibacterial activity of the plant towards the test organisms. After Serial Exhaustive Extraction and bioautography, the DCM extract had the most activity. Column chromatography of the extract resulted in the isolation of one compound purified using preparative TLC in 100% chloroform. The compound was sent for NMR analysis and it was suggested that the tentative structure was a branched chain alkane but due to the presence of impurities eluting with the compound, the structure of the compound could not be elucidated. Mass Spectrometry of the compound was not carried out due to the loss of material during purification of the sample. Other researchers have isolated biologically active compounds isolated from *Senna italica*; Yagi *et al.* (2013) isolated 1,1,8,8-tetrahydroxy-7'-methoxy-3,3'-dimethyl-10,10'-bianthracen-9,9'-dione and Mokgotho *et al.* (2013) isolated 3,4,5-trihydroxystilbene. In this study the compound isolated is a branched chain alkane.

7.2. Conclusion

Phytochemical studies carried out on the selected plants have demonstrated a wide variety of the biomolecules present in the plants which may be responsible for their use in traditional medicine. Through various bioassays, different biologically active compounds were identified. Since bioautography was used to monitor the antibacterial activity of the compound of interest during isolation, this shows that bioassays can be used as a guide during isolation. Although an alkane compound isolated in this study was not identified due to a contaminant, this study shows that there is a biologically active compound belonging to the alkane group which may be unique. Further work is required to identify the specific alkane and to determine if it is indeed unique and a good candidate for the treatment of infected wounds.

CHAPTER 8

References

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