Microbiological and Biochemical Studies of Traditional Medicinal Plants used in Limpopo Province for Anti-*Mycobacterium Tuberculosis* Activity

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[redacted]

THESIS

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DECLARATION

I, Nancy Patience Motlalepula Komape, declare that **Microbiological and Biochemical Studies of Traditional Medicinal Plants used in Limpopo Province for Anti-*Mycobacterium Tuberculosis* Activity** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other institution.

…….. **…….**

Nancy Patience Motlalepula Komape **Date**

DEDICATION

I dedicate this work to my two awesome sons: Neo Shuga Miguel and Ofentse Skattie Blessings.

“*Bana baka thuto ke lesedi*”.

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LIST OF ACRONYMS AND SYMBOLS

^{13}C	Carbon- 13
^{1}H	Hydrogen- 1
ACN	Acetone
ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonium hydroxide
Chl	Chloroform
CEF	Chloroform/Ethyl acetate/Formic acid
DMEM	Dubleco's Modified Essential Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EMW	Ethyl acetate/Methanol/Water
Hex	Hexane
INT	p- iodonitrotetrazolium violet
Met	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectroscopy
MTT	3-(4,5- dimethylthiazol-2-yl)- 2,4- diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance spectroscopy
R_f value	Retention factor value
TLC	Thin layer chromatography

UV	Ultraviolet light

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PUBLICATIONS AND CONFERENCES

Publications

Komape N. P. M., Bagla V. P., Kabongo- Kayaoka P. and Masoko P. “Antimycobacteria and Synergistic Effects of Plants used to treat Tuberculosis and Related Symptoms by the Bapedi People in Limpopo Province, South Africa” 2017. (Full citation, APPENDIX A).

Conferences

Komape N. P. M., Aderogba M., Bagla V. P., Masoko P. and Eloff J.N. “Antibacterial and Antioxidant Activities of *Combretum vendae* leaves” and won 1st prize for the best PhD Student Presenter held at Bolivia Lodge, Polokwane. 2013: University of Limpopo, Faculty of Science and Agriculture Research Day [Podium presentation].

Komape N. P. M., Bagla V. P. and Masoko P. “Antimicrobial Properties of Selected Plants used by Bapedi Traditional Healers to treat Tuberculosis and Related Symptoms in Limpopo Province, South Africa” 2016: University of Limpopo, Faculty of Science and Agriculture Research Day [Podium presentation].

Komape N. P. M., Bagla V. P., Kabongo- Kayaoka P. and Masoko P. “Antimycobacteria and Synergistic Effects of Plants used to treat Tuberculosis and Related Symptoms by the Bapedi People in Limpopo Province, South Africa” 2017: at Indigenous Plant Use Forum (IPUF) Conference held in Pretoria. [Podium presentation].

Komape N. P. M., Bagla V. P. and Masoko P. “Anti-mycobacterial Activity of the Butanol Sub-fraction of *Apodytes dimidiate* against *M. tuberculosis* H37Rv strain and MDR- TB strain” 2017: University of Limpopo, Faculty of Science and Agriculture Research Day [Podium presentation].

Komape N. P. M., Bagla V. P., Kabongo- Kayaoka P. and Masoko P. “Isolation of Ursolic acid and Oleanolic acid from *Apodytes dimidiata* leaves used traditionally to treat Tuberculosis and Related Symptoms in Limpopo Province, South Africa” 2018: at Indigenous Plant Use Forum (IPUF) Conference held in Oudsthoorn. [Poster presentation].

Komape N. P. M., Bagla V. P. and Masoko P. “Isolation of Ursolic acid and Oleanolic acid from *Apodytes dimidiata* leaves used traditionally to treat Tuberculosis and Related Symptoms in Limpopo Province, South Africa” 2018: University of Limpopo, Faculty of Science and Agriculture Research Day [Podium presentation].

ABSTRACT

Tuberculosis (TB) is one of the top ten diseases that causes morbidity and mortality worldwide. Although TB is curable, the main problem currently with TB is development resistance to the current chemotherapy. Medicinal plants, as a source of drugs, have been found to cause less or no resistance. Medicinal plants are studied and considered for their efficacy and safety because they possess bioactive compounds with various biological activities. The aim of the study was to isolate and characterise bioactive compounds from selected seven plant species [*A. dimidiata* (LNBG 1969/46)*, A. afra* (LNBG 2010/27)*, Z. capense* (LNBG 1969/100)*, C. herorense* (LNBG 1977/71)*, L. javanica* (LNBG 1969/460), *E. camaldulensis* and *C. lemon* (UNIN 12330)] with activity against *Mycobacterium smegmatis,* Multi- drug resistant tuberculosis starain and H37Rv *Mycobacterium tuberculosis* strain. It was also imperative to determine whether crude extracts, sub- fractions of the extracts and the isolated bioactive compounds are cytotoxic or not.

Leaves of the seven selected plants were collected from South African National Botanical Institute (SANBI) at Nelspruit, Mpumalanga Province, South Africa. The leaves were dried and milled to fine powder. The leaves of each plant were extracted using solvents of varying polarity (i.e. hexane, dichloromethane, acetone and methanol). Phytochemical screening was done using Thin Layer Chromatography (TLC) developed in three mobile phases varying in polarity and then sprayed with vanillin sulphuric acid in methanol heated at 110^{o}C for optimal colour development. Qualitative antioxidant activity was determined by using 1,2- diphenylpicryl hydrazyl (DPPH) assay on TLC plates. Antimycobacterial activity for all the plant extracts was done using bioautography assay in qualitative analysis of the active compounds and for quantitative analysis, the microplate dilution assay was used. The plants which showed better activity (*C. lemon, C. hereroense* and *A. dimidiata*) with the microplate dilution assay and bioautography were further subjected to solvent- solvent fractionation as the first step towards isolation of bioactive compounds. Synergistic, additive, indifferent and antagonistic effects of the crude extracts combinations of the three selected plants was determined. The combinations where *A. dimdiata* was also part of the combinations frequently showed synergistic effect. On the

other hand, with the combinations of *C. hereroense* and *A. dimdidata* (CH-AD) there was no antagonistic effect observed. The combinations of crude extracts of *C. lemon* and *A. dimidiata* all showed synergistic effect, except for only three combinations. Based on the synergistic effect observed and the bioactivity on the bioautography and microplate dilution assay of the sub- fractions, *A. dimidiata* was chosen for further analysis for antimycobacterial activity using the MDR- TB strain and *M. tuberculosis* H37Rv strain. The sub- fractions of *A. dimidiata* with the most activity were hexane and butanol. Hexane and butanol fractions both showed good MIC activity against the TB isolated M. tuberculosis field strain and H37Rv strain of 0.47 and 031 mg/ml, respectively. Butanol fraction was further taken for isolation using open colum chromatography doing bioassay guided isolation. The isolated compounds, together with the crude were tested for their biological activity using MTT assay to determine their cytotoxicity and antimycobacterial activity assay to confirm their activity against *M. smegmatis* and *M. tuberculosis*. Cytotoxicity assay showed that the crude extracts of *A. dimidiate* were toxic against the Vero kidney cells and the subfractions (i.e. butanol and hexane) became moderate to non-toxic and one compound (oleanolic acid) from the butanol sub-fraction was non-cytotoxic. This indicates that the isolation of the crude extracts tends to become non- toxic to the cells. The study suggests the use of pure compounds to fight against TB as compared to crude extracts since they are both bioactive and non- cytotoxic. Crude extracts combinations were effective in killing Mycobacterium as compared to single crude extracts. The present study recommends the use of *A. dimidiata* plant leaves crude extracts combinations as they mostly exhibit synergistic effect. Furthermore, Mycobacterium and also contain non- cytotoxic antimycobacterial compound (oleanolic acid). The study serves as a scientific proof for the use of this plant in traditional medicine for TB treatment.

CHAPTER 1

1. INTRODUCTION

Tuberculosis is an infectious, communicable disease and the causative agent of the disease has, over the years, developed resistance to streamline chemotherapeutic agents with dire consequences. In 1882 March the 24th, Robert Koch discovered a tubercle bacillus which causes tuberculosis (TB). Hence to this day, the 24th of March is named World TB day. Tuberculosis has been one of the diseases worldwide which causes the high mortality rate even though it is curable. In South Africa, 55000 people die annually due to TB. The World Health Organization (WHO) data suggest that the global case detection rates of TB are disappointing because about three million cases fail to be notified each year (McNerney *et al*., 2015). Africa, in particular, has the lowest case detection rate of 52% (McNerney and Daley, 2011; McNerney *et al*., 2015; WHO, 2014a). Globally, about 2 million people die annually due to TB (Gautam *et al*., 2007).

Tuberculosis has been regarded as the number one killer in South Africa. This is partly attributed to the TB-HIV/AIDS co-infection. The number of TB cases in sub-Saharan Africa has increased substantially in the past decade, fuelled by the HIV epidemic (Raviglione *et al*., 1997; Ghandi *et al*., 2006). The virulence of the causative agent (*Mycobacterium tuberculosis*) is extraordinarily complicated and multifaceted. The organism does not produce any toxins but possesses a huge repertoire of structural and physiological properties that have been recognised for their contribution to their virulence and to pathology of TB (Todar, 2015). There are a number of medicinal plants that have been screened for anti- mycobacterial activity in the past few years (Cantrell *et al*., 2001; Copp and Pearce, 2003; Okunade *et al*., 2004; Luo *et al*., 2011; Soejarto *et al*., 2012; Singh *et al*., 2013).

In many countries, especially developing countries like South Africa, people have been using medicinal plants as the source of bioactive constituents for the cure of various diseases as opposed to western medicine. Through periods of trial, error and success, herbalists and their apprentices have accumulated a large body of knowledge about

medicinal plants (Iwu *et al*., 1999). Southern Africa has a rich diversity of plants (van Wyk *et al.*, 2008).

According to Iwu *et al.* (1999), the first generation of plant drugs were usually simple botanicals employed in more or less their crude form. Based on this, several effective medicines used in their natural state were selected as therapeutic agents based on empirical study of their application by traditional societies from different parts of the world. Currently, the global market for medicinal plants has been estimated to be approximately $62 billion (US dollars) and the demand is growing rapidly (Indian Council of Medical research, 2003). These different medicinal plants have been used for decades to treat different kinds of ailments, including infectious diseases.

1.1. Drugs used to treat Tuberculosis

Tuberculosis has over the years been treated using four first line drugs, namely; rifampicin, isoniazid, ethambutol and pyrazinamide for about 6 months. Although this treatment is effective, the usage of this regimen has resulted in the rise of strains that are resistant to some or all of the first-line or second-line TB drugs (Loddenkemper and Hauer, 2010; Nguta *et al*., 2015). An emerging strain has also been found, which is not only resistant to isoniazid and rifampicin (MDR tuberculosis), but also to at least three classes of the second-line drugs, termed the Extensively Drug-Resistant (XDR) Tuberculosis. In 2006, 347 patients infected with XDR-TB were identified worldwide (Ghandhi, 2006; Anon, 2006) and the number is still on the rise.

Currently, efforts in TB drug development resulted in the discovery of new therapeutics, including delamanid (previously known as OPC 67683) and bedaquiline (previously known as TMC 207), which are active against MDR and XDR tuberculosis strains (Nguta *et al*., 2015). Tuberculosis drugs are chosen by a stepwise selection process through five groups of antituberculosis drugs listed in table 1.1 below, on the basis of efficacy, safety and cost (Caminero *et al*., 2010). One of the major assessments before starting treatment regimen of patients is to do Drug Susceptibility Testing (DST), which is a very critical component of TB treatment (WHO, 2014b).

Table 1.1: Categories of antituberculosis drugs (Caminero *et al*., 2010).

	Daily dose
Group 1: First line oral antituberculosis drugs (use all possible drugs)	
Isoniazid	5 mg/kg
Rifampicin	10 mg/kg
Ethambutol	15- 25 mg/kg
Pyrazinamide	30 mg/kg
Group 2: Fluroquinolones (use only one because they share genetic targets)	
Ofloxacin	15 mg/kg
Levoflaxacin	15 mg/kg
Moxifloxacin	7.5- 10 mg/kg
Group 3: injectable antituberculosis drugs (use only one because they share very similar genetic targets)	
Streptomycin	15 mg/kg
Kanamycin	15 mg/kg
Amikacin	15 mg/kg
Capreomycin	15 mg/kg
Group 4: less-effective second line antituberculosis drugs (use all possible drugs if necessary)	
Ethionamide/ Prothionamide	15 mg/kg
Cycloserine/ Terizidone	15 mg/kg
P- aminosalicylic acid (acid salt)	150 mg/kg
Group 5: less-effective drugs or drugs o which clinical data are sparse (use all necessary drugs if there are less than four from the other groups)	
Clofazimine	100 mg
Amoxicillin with clavulanate	875/125 mg every 12 h
Linezolid	600 mg
Imipenem	500- 1000 mg every 6 h
Clarithromycin	500 mg/ 12 h
High- dose isoniazid	10- 15 mg/kg
Thioacetazone	150 mg

1.2. Problems associated with the use of anti-TB drugs

In South Africa, poor communities are the ones which are mostly affected, particularly by the drug resistance strains, due to lack of adherence. The Multi-Drug Resistance TB (MDR- TB) strain, is defined as the strain that is not susceptible to at least two major anti-TB second-line drugs of higher toxicity (Zager and McNerney, 2008) and that for Extremely Drug Resistant TB (XDR- TB) involves resistance to at least rifampicin, isoniazid, a second-line drug (capreomycin, kanamycin or amikacin) and a fluroquinolone (CDC, 2006). The treatment of TB involves an extended period requiring patients to take their prescriptions for over eight weeks. Patients tend to abandon treatment within this period assuming they are feeling better whereas the *Mycobacterium* is still in the body. The problem of TB drug inaccessibility is due to the fact that most people live in rural areas in most parts of Africa and in South Africa, particularly in Limpopo Province (where 60% of the province is rural), and transportation is a problem.

1.3. Resistance development in TB

Resistance development in TB is as a result of various factors like the long length of time of treatment of the current TB regimens which does not ensure clearance of the infection without relapse (Lenaerts *et al.*, 2007). Patients on TB treatment tend to leave treatment when they see that the symptoms of TB have disappeared, which leads to resistance development to TB drugs. This development of drug resistance is commonly referred to as “resistance due to lack of adherence”. Microbial resistance is recognised at present as a major public health problem (Iwu *et al*., 1999). The major problem lies in an uncontrolled use of drugs used to treat TB resulting in the development of resistant strains. Resistant bacterial strains have emerged and have spread throughout the world because of the remarkable genetic plasticity of the microorganisms, heavy selective pressures and the mobility of the world population. The main and most common way of bacterial strain resistance development is through the exchange of genetic material; pathogens can transmit resistance material through the same species and even across species (Lewis *et al*., 2000).

Resistance to antimicrobial agents occurs due to changes in bacterial chromosome or exchange of genetic material via plasmids and transposons among other adaptations (Famakin, 2002). As a result of this, drugs which could be used on a less frequent basis but with prolonged effect should be developed. This could alleviate or reduce the number of mortality cases caused by infections due to bacterial resistant strains. Resistance development limits the useful lifespan of antibiotics and results in requirement of a development of new drugs with less or no resistance (Spellberg and Shlaes, 2014).

1.4. Possible solution to counter resistance to antibiotics

In recent years, plants have been used as a source of medicine both by scientists and most people in developing countries. Furthermore, medicinal plants contribute tremendously to the development of conventional western medicines. Slightly over 25% of the prescription drugs used in USA and Canada are plant derived or modelled around phytochemicals (Farnsworth, 1988). There is a worldwide green revolution, which is reflected in the starting molecules of plant origin, for example digoxin/digitoxin, the vinca alkaloids, reserpine and tubocurarine (Iwu *et al*., 1999).

Plants play a vital role in combating resistance. There are several advantages of using plants as compared to antibiotics. One of the major reasons is that plants are easily available synthetic alternatives and they yield profound therapeutic benefits and are cheap (Masoko and Nxumalo, 2013). Plant extracts are usually used instead of antibiotics because they contain active compound. These plant extracts can each contain more than one active compound making them potently relevant.

1.5. The Economic Importance of TB

Each year, there are about 100 million TB people who fall below the poverty line because of the financial burden caused by living with the disease (WHO, 2012; Tanimura *et al*., 2014). Many countries around the world, including South Africa, aim to provide TB diagnosis and treatment free of charge in the public health services which do not come without financial implications to national budget. In the past twenty years, through national efforts and global financial support, access to free TB care has been expanded

substantially (WHO, 2013; Tanimura *et al*., 2014). Even in an effort to provide free healthcare services, many TB patients and families are still facing very high direct and indirect costs due to TB illness and care seeking, hampering access and putting people at risk of financial ruin or further impoverishment (WHO, 2009; Mauch *et al*., 2013; Tanimura *et al*., 2014).

Patients suffering from TB face various problems that are social and economic in nature (Dhuria *et al*., 2008). Tuberculosis and poverty are closely linked. Overcrowding, malnutrition, poor air circulation and sanitation factors associated with poverty, both increase the probability of becoming infected and developing the clinical disease. There is a vicious cycle of TB and poverty. Poor people go hungry and live in close vicinities and unhygienic places where TB flourishes. Tuberculosis then decreases people's capacity to work. Patients who receive TB treatment add to their expenses, increasing their poverty. In addition, the poor receive inadequate health care, preventing even the diagnosis of the disease. The poor are also less likely to seek and receive care from medical practitioners when ill and they tend to self-medicate, which in turn encourages the emergence of drug resistant TB strains.

According to the most recent WHO estimates, out of 8.4 million people infected with TB, of which most of them are potential wage earners, there is about 30% decline in average productivity, which costs a country approximately one billion dollars yearly. The number of deaths annually also increases the average loss of income and adds an additional deficit. Tuberculosis causes somewhere near 12 billion dollars loss to the global economy. The burden of the disease also affects the formal and informal economies, as well as within households due to the lack of productivity by those affected. When a person gets diagnosed with TB, there tends to be three to four months' work time lost annually to the disease and household income loose earnings of between 20 to 30%. Loss of productivity increases due to the economic difficulties that put pressure on the state health budgets (WHO, 2017).

The disease increases costs as percentage of income among poor people and those with MDR-TB strains. Tanimura *et al.* (2014), stated that "income loss often constitutes the largest financial risk for patients". Besides paying for the healthcare services and

delivering them in a way that minimises direct or indirect costs, there is a need to ensure that the affected families as well as TB patients themselves receive appropriate income replacement and other social protection interventions (Tanimura *et al*., 2014).

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CHAPTER 2

2. LITERATURE REVIEW

2.1. Medicinal Plants in the Treatment of Infectious Diseases

Medicinal plants are those plants that can elicit therapeutic properties and induce health towards man and animals. The same plants can include any plant parts such as roots, stems, leaves, bark, seeds, fruits and flowers, which can be composed of the right constituents to restore health. Medicinal plants have always played a very important role in the traditional health care system of most parts of the world. In 1994, South Africa had an estimate of between 12 and 15 million or 60% of the people using medicinal plant remedies from as many as 700 indigenous plant species (Moeng, 2010). Many of these plants are used for infectious diseases.

2.2. Medicinal plants as a source of antimycobacterial agents

There is a decline in success regarding the use of common and cheap TB medicine (Tabuti *et al*., 2010). Between the years 1950 and 1970, there was a discovery of effective drugs used to treat TB, *viz*., ethambutol, isoniazid, pyrazinamide, rifampicin and streptomycin. At first, the development of these drugs reduced the number of TB cases, especially in developing countries. In the 1980s, the number of TB cases increased rapidly worldwide due to the emergence of Multidrug Resistant TB (MDR-TB) strain (Chan and Isemann, 2002; Gautam *et al*., 2007), and eventually the emergence of the Extremely Drug Resistant TB (XDR- TB). It is very important to develop new drugs which cause less or no resistance. Hence, drugs derived from natural sources can be effective in treating TB. This can be achieved by using medicinal plants. Medicinal plants are currently considered as prospects for development of new drugs to treat TB (Fabricant and Farnsworth, 2001; Balunas and Kinghorn, 2005).

Medicinal plants have always played a significant role in the traditional health care system of South Africa (Moeng, 2010). Approximately 60% of world's population still rely on medicinal plants for their primary health care (Gautam *et al*., 2007). Various scientists have researched and discussed plant-derived compounds and plant-derived

antimycobacterial natural compounds (Mitscher and Baker, 1998; Newton *et al*., 2000). An extensive review by Newton *et al.* (2000) describes the antimycobacterial activity of extracts and compounds from 123 plant species. One other important study was conducted by Tabuti *et al* (2010) in Uganda where they discovered that traditional medical practitioners have the knowledge to identify and treat TB using plants species. Their study predominantly provided the evidence that some plant species that are used by traditional medical practitioners are efficient against TB and that has been validated (Tabuti *et al*., 2010).

2.3. Common classes of compounds found in plants

In nature, many plants are autotrophs, where the implication is that they use “primary” metabolites like carbon dioxide, minerals, water and sunlight to synthesize all the metabolites necessary for their growth and development. There are large varieties of compounds that are produced by plants that have no direct function in growth and development of plants (Mazid *et al*., 2011). These compounds have no well-known functions in solute transport, photosynthesis, respiration, nutrient assimilation, translocation and differentiation (Mazid *et al*., 2011). The common classes of compounds found in plants are called secondary metabolites. These are useful in the agrochemical, pharmaceutical, aroma and flavour industries and are produced by plants on a continuous basis with no seasonal limitation (Nemudzivhadi, 2014).

Plants have limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives (Geissman, 1963). Many of the synthesized compounds are secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have been found in vitro to have antimicrobial properties. Plants also provide a good source of anti-infective agents; emetine, quinine and berberine to fight against microbial infections. Plants containing protoberberines and related alkaloids, priclima type indole alkaloids and garcinia biflavonones used in traditional African system of medicine, have been found to be active against a wide variety of micro-organisms (Bansal *et al*., 2010).

2.3.1. Phenolic compounds

There are two major classes of phenolic compounds, namely; phenolic acids and cell wall phenolics. Phenolic acids are one of the main classes of compounds within the Plant Kingdom and they occur in the form of esters, glycosides or amides, but rarely in a free form. The phenolic acids vary in the number and location of the hydroxyl groups on the aromatic ring (Khoddami *et al*., 2013; Pereira *et al*., 2009). The two major groups of cell wall phenolics are lignins and hydroxycinnamic acids. As the plant grows, it protects the cell wall against infections and harsh conditions (Khoddami *et al*., 2013).

Phenolics act as antioxidants in a number of ways. Their hydroxyl groups are good hydrogen donors. Their antioxidant capacity is also attributed to their ability to chelate metal ions involved in the production of free radicals (Pereira *et al*., 2009; Yang *et al*., 2001). Due to their hydrophobic benzenoid rings and the hydrogen bonding potential of the phenolic hydroxyl groups, the structure of the phenols often has a potential to strongly interact with proteins (Pereira *et al*., 2009).

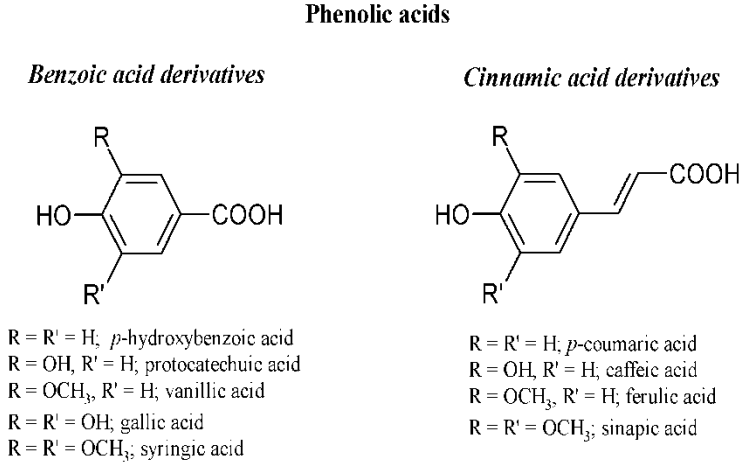

Figure 2.1: General structures of phenolic acid derivatives (Pereira *et al*., 2009)

2.3.2. Alkaloids

This class of compounds constitutes a ring structure and a nitrogen atom located in the heterocyclic ring structure of a molecule. These compounds are one of the most effective bioactive compounds found in plants. They are used as lead compounds for new drug

discoveries and are classified into different categories due to their biosynthetic pathways. This class of compounds is widely used as an anticancer agent. Alkaloids like camptothecin, topoisomerase inhibitor and vinblastine are the ones used as anticancer agents. Examples of alkaloids from plants include: berberine, evodiamine, matrine, sanguinarine, piperine and tetradrine (Lu *et al*., 2012).

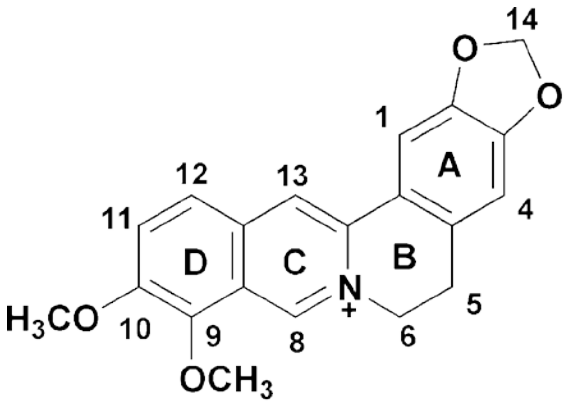

Figure 2.2: Structure of berberine. Example of an alkaloid (Schmeller *et al.,* 1997)

2.3.3. Flavonoids

They are phenolic compounds found in plant tissues and have different colours such as blue, yellow, orange, purple and red which play a major role in plant carotenoids and chlorophylls. This class has a phenylbenzopyran chemical structure (Pereira *et al*., 2009). They are derivatives of aromatic amino acids, phenylalanine and tyrosine. Their structures are different based on their scale and form of hydroxylation, prenylation, alkanisation and glycosylation reactions that change the general structure. They can be grouped into 4 classes: major flavonoids (2- phenylbenzopyrans), isoflavonoids (3-benzopyrans), neoflavonoids (4- benzopyrans) and minor flavonoids (Pereira *et al*., 2009). There are various examples of flavonoids, which include: flavones, flavonols, iso-flavonols, anthocyanins, anthocyanidins, proanthocyanidins and catechins (Khoddami *et al*., 2013). In plants, flavonoids have Ultraviolet (UV) light screening properties which constitute protection for the plant. These compounds occur in nearly all the plant species (Bruneton, 1999; Pereira *et al*., 2009). They have useful properties which include: anti-

inflammatory, oestrogenic, enzyme inhibition, antimicrobial and antiallergic (Pereira *et al*., 2009).

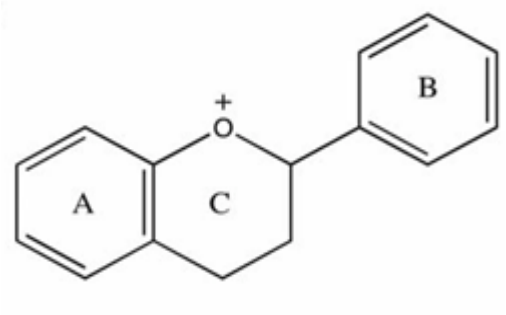

Figure 2.3: General structure of flavonoids (Khoddami *et al*., 2013)

2.3.4. Terpenes/ terpenoids

This class of secondary metabolites is responsible for the medicinal activity of some plants. Most of the terpenoids have a similar structure which differs only in their functional groups and carbon skeletons. Terpenoids have various roles in mediating antagonistic and beneficial interactions among organisms. They also defend many species of plants, animals and microbes against predators, pathogens and competitors. They are involved in giving messages to conspecifics and mutualists regarding the presence of food, mates and enemies (Gershenzon and Dudareva, 2007).

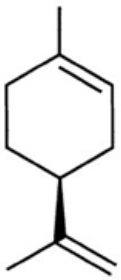

Figure 2.4: Representative terpenoid biosynthesized by plants (Trapp and Croteau, 2001)

2.3.5. Tannins

Tannins are made of various groups of phenolic compounds joined together to form reversible and irreversible complexes of proteins, polysaccharides and nucleic acids. This class of secondary metabolites has defensive properties and is found mostly in various medicinal plants, and also in fruits such as grapes and blueberries, in chocolates and also in tea (Mazid *et* al., 2011; Saxena *et al*., 2013). They have been used both in food and the pharmaceutical industries. They are also used as antioxidants in beer, fruit juice and wines and play a significant role as caustic for cationic dyes in dyestuff industry and in ink production (Saxena *et al*., 2013).

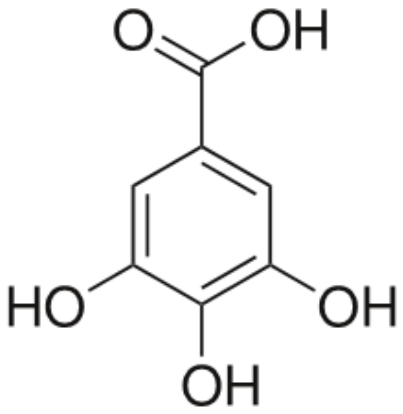

Figure 2.5: Structure of gallic acid from plants (Haslam and Cai, 1994)

2.3.6. Steroids

This class of secondary metabolite plays a very important role in plant cell membranes and plasma membranes as regulatory channels of plant cells. Again, they protect the plants from herbivores, for example, triterpene and limenoid are some of the bitter steroids produced by citrus fruits to fight against herbivores. They also allow movement of small molecules from the outside to the inside of the cell by decreasing motion of fatty acid chains (Mazid *et al*., 2011).

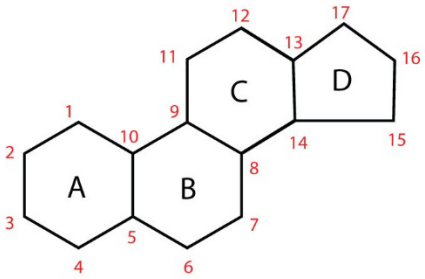

Figure 2.6: Basic chemical structure (skeleton) of steroids (Moss, 1989)

2.3.7. Saponins

The Saponins are secondary metabolites found in natural sources like plants. There are three groups of saponins, viz., triterpenoids, glycosylated steroids and steroid alkaloids. They play a crucial role in traditional medicinal plants by bringing forth immunostimulants, hypocholesterolaemic, anticarcinogenic, antifungal, antiviral and antioxidant properties. They are particularly known to have antimicrobial activity and also for their ability to protect plants from insect attack (Saxena *et al*., 2013).

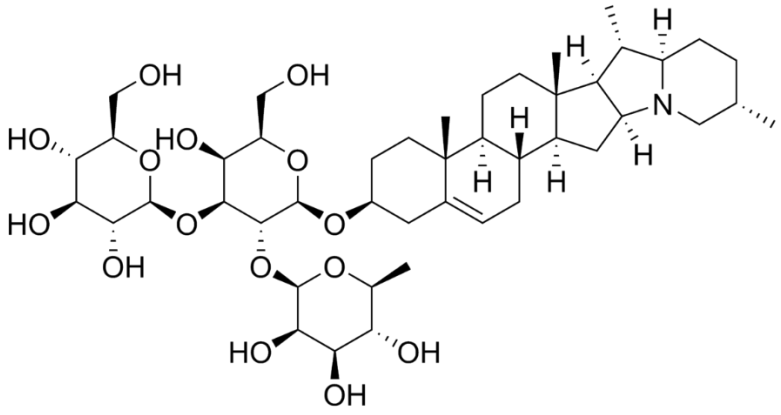

Figure 2.7: Basic structure of saponin (Hostettmann and Marsden, 1995)

2.4. Relevance of toxicity studies in drug development

Testing whether a drug is toxic to humans during drug development is of paramount importance. Toxicity studies were done in the early 1960s after the thalidomide

catastrophe. This involved thousands of children born worldwide with severe birth defects. The incidence led to many countries of the world doing toxicity studies (Agrawal and Paridhavi, 2007; Arome and Chinedu, 2013). Arome and Chinedu (2013) mentioned that "the essence of toxicity testing is not just to check how safe the test substance is, but to characterize the possible toxic effects it can produce".

Toxicity studies are important because of the five major reasons. The first one is that they establish a dose response curve. Secondly, they ensure the safety of new chemicals for use as pesticides, drugs, or food additives before they are registered for general use in industries or hospitals. Thirdly, they also establish the mode of action or mechanism for a toxic effect that may have been noted in other studies. The fourth reason is that they produce epidemiological studies to explain observations in the population. And lastly, they validate new methods of testing or investigation, particularly those conducted in vitro rather than in animals (Woolley, 2008).

2.5. Role of antioxidants in the treatment of infectious diseases

Oxygen is an essential element of life. It is used to generate energy and free radicals are produced as a result of energy (ATP) production by the mitochondria. Free radicals can cause microbial infections due to oxidative stress. There are a number of diseases associated with free radicals. The risk of diseases due to oxidative stress is increased by an unhealthy lifestyle, exposure to chemicals, pollution, cigarette smoking, drugs, illness and stress, etc. (Sen and Chakraborty, 2011).

Antioxidants are defined as substances which at low concentration significantly stop or delay the process of oxidation while often being oxidized themselves. Antioxidants are required to scavenge free radicals and assist in decreasing the incidence of oxidative stress induced damage. Antioxidants can produce their protective role against free radicals by different mechanisms. The potential beneficial effects of antioxidants in protecting against disease have been well established. Phytochemicals or plant phytoconstituents are the major source of antioxidants (Sen and Chakraborty, 2011). The phytoconstituents are mostly secondary metabolites. Among these compounds are

phenolic acids, flavonoids, terpenes, tocopherols, vitamin C and carotenoids, which are well distributed in several medicinal plants (Ozkan *et al*., 2016).

Many studies have indicated that medicinal plants have been used to treat human diseases for thousands of years owing to their antimicrobial and antioxidant activities. Medicinal plants have the ability to reduce the oxidative stress in plants and prevent a variety of diseases (Ozkan *et al*., 2016). Traditional herbal medicines and dietary foods were the main source of antioxidant for ancient people that protected them from damage caused by free radicals.

Infectious diseases caused by microorganisms such as fungi, bacteria, viruses and parasites, can be spread directly or indirectly from one person to another. One of the most common infectious disease worldwide, particularly in developing countries, TB, has been linked with high resistance to the current chemotherapy. There have been many studies (Askun *et al*., 2013) that prove that medicinal plants can be used as a source of natural anti-tuberculosis drugs. Hence, showing that medicinal plants are used against infectious diseases (Ozkan *et al*., 2016).

2.6. A Review of the Plants used in the Study

The plants in this study were selected based on the article written by Semenya and Maroyi (2012) in which they tabulated the plants used in the treatment of tuberculosis by the Bapedi traditional healers in the three districts of the Limpopo Province, namely; Capricorn, Sekhukhune and Waterberg. The three plants selected were amongst the mostly used plants used by the Bapedi traditional healers to treat TB, which are *Artemisia afra*, *Myothamnus flabellifoius* and *Lippia javanica*. The other plants that were selected were only used by two or three healers (Semenya and Maroyi, 2012).

2.6.1. *Artemisia afra*

This plant belongs to the family of Asteraceae (daisy family) and it is commonly known as *lengana* (Sepedi). *Artemisia afra* is a common species in South Africa with a wide distribution from the Cederberg Mountains in the Cape, northwards to tropical Cederberg

Mountains in the Cape, northwards to tropical East Africa and stretching far north as Ethiopia (figure 2.8) (Van Wyk, 2008). It grows in thick, bushy, slightly untidy clumps and it usually grows up to 2 m high and as low as 0.6 m. A wide range of its diverse uses has been recorded. In South Africa, it is used by people from all cultural backgrounds to treat different ailments which range from coughs, colds, fever, loss of appetite, colic, headache, earache, and intestinal worms to malaria (Department of Agriculture, Forestry and Fisheries, 2012).

Figure 2.8: *Artemisia afra* (Department of Agriculture, Forestry and Fisheries, 2012)

2.6.2. *Apodytes dimidiata*

Apodytes dimidiata belongs to the family Icacinaceae and is commonly known as *sephopha–madi* (Sepedi) (figure 2.9). It is an ideal tree for the home garden as it does not have messy fruits and it is safe to plant near the house. This plant occurs in coastal evergreen bush, at the margins of medium altitude evergreen forests, in open woodlands and on grassy mountain slopes, often among rocks. It is a constituent of the forest such as Knysna, George, Tsitsikama, Alexandria, Amatola, Umgoye and Dukuduku. It is a small bushy tree of about 4 to 5 m tall and can reach a height of about 20 m when growing in the forest. It has evergreen, glossy, bright green leaves that appear pale and dull underside (Killick, 1973). This tree is valued by the Zulu people in traditional medicine. They use it as an enema, for intestinal parasites and also to treat ear inflammation (Pretorius *et al*., 1991).

Figure 2.9: *Apodytes dimidiata* (Pretorius *et al*., 1991)

2.6.3. *Zanthoxyllum capense*

It belongs to the family Rutaceae. It grows in the eastern region of Southern Africa, from the vicinity of Knysna, Western Cape to the Zimbabwean granite shield and coastal Mozambique. It bears clusters of compound leaves on the tips of their branches. The leaves and fruits are noticeably citrus-scented (figure 2.10). The fruits are round capsules of about 5 mm in diameter, fully covered with glands. It is used to treat ailments such as coughs (Van Wyk *et al.,*1997).

Figure 2.10: *Zanthoxyllum capense* (Pooley, 1998)

2.6.4. *Lippia javanica*

The plant belongs to the family Verbenaceae and is commonly known as *bokhukhwane*. It is named after Augustin Lippi, 1678- 1701. It is widespread throughout large parts of South Africa, with the exception of the Western Cape. It grows from the Eastern Cape northwards extending into tropical Africa including Botswana, Swaziland, Mozambique, Malawi, Tanzania, Zambia and Kenya. It is a 1 to 2 m high woody shrub which stands erect and is multi-stemmed. The leaves are hairy with noticeable veins and when crushed, it gives off a strong lemon-like smell (figure 2.11). The Xhosa people are known to drink it as a weak infusion, substituting it for tea and in a stronger infusion for the treatment of coughs, colds and bronchial problems in general (Le Roux, 2004).

Figure 2.11: *Lippia javanica* (Le Roux, 2004)

2.6.5. *Citrus lemon*

Belongs to the family Rutaceae (Citrus family) that originated in Asia (probably India and Pakistan) and is now commercially grown worldwide in tropical, semi-tropical and warm temperate countries. The tree grows to 6 m tall and has stout spines. The leaves are dark green, leathery and evergreen, oblong, elliptical, or oval and up to 14 cm long (figure 2.12). Lemon fruits can be highly acidic and are high in citric acid and vitamin C. Their tart flavour is popular in beverages (lemonades and iced teas as well as many cocktails).

Traditional uses for the fruit, peels and oil obtained from seeds include treating fever and colic and as astringent and diuretic (Moore, 2001).

Figure 2.12: *Citrus lemon* (Van Wyk and Gericke, 2000)

2.6.6. *Combretum heroroense*

The plant belongs to the Combreteceae family and it is widely distributed in Uganda, Kenya, Namibia, Botswana and South Africa. It has dark green to grey green leaves. Flowers are creamy white to pale yellow, in dense, axillary and terminal spikes. Fruits are rich, reddish brown in the centre with golden brown wings (figure 2.13). *Combretum* have a range of uses for problems ranging from heart and worm remedies to wound dressings, treatment of the mentally ill and scorpion stings (Fyhrquist *et al*., 2004; Hedberg *et al.*, 1982; Watt and Breyer- Brandwijk, 1962).

Figure 2.13: *Combretum heroroense* (Van Wyk, 2005)

2.6.7. *Eucalyptus camaldulensis*

It belongs to the family Eucalypt. The tree is about 45 m tall and the bark has smooth to small branches or with a few rough loose grey basal slabs; smooth bark, white, cream and pale grey with yellow, pink or brown patches (figure 2.14). *Eucalyptus camaldulensis* is used traditionally to treat wounds, boils and other microbial infections (Babayi *et al*., 2003).

Figure 2.14: *Eucalyptus camaldulensis* (Van Wyk, 2005)

2.7. Medicinal Plants and Synergism activity in antimicrobial infections

Synergism is the cooperative action of discrete agencies such that the total effect is greater than the sum of the effects taken independently (Slinker, 1998; Webster, 2008). Most scientists, when referring to synergism mean that "the whole is greater than the sum of the parts" (Slinker, 1998). Synergistic combinations of antimicrobial agents with different mechanisms of action have been introduced as more successful strategies to combat infections (Farooqui *et al*., 2015). Much of the current synergistic effect that has been studied with regard to medicinal plants involves the combination of plant extracts with antibiotics. This is because of the resistance caused by the use of antibiotics in treatment of microbial infections. Very few studies involve plant extract-plant extract combinations to study synergism.

2.8. Hypothesis

Traditional medicinal plants have been shown to have constituents with immense antimicrobial potential in the treatment of various disease conditions. Based on this knowledge, the researcher hypothesizes that the selected medicinal plants in this study have antimicrobial compounds which may be purified as single entities, used as fractions or crude extracts that may act as an affordable treatment regimen for TB.

2.9. Purpose of the Study

2.9.1. Aim

To evaluate the anti-mycobacterial activity of selected traditional medicinal plants against *M. tuberculosis*.

2.9.2. Objectives

i) Extract the chemical constituents of the selected plants using hexane, dichloromethane, ethanol and methanol.
ii) Determine the phytochemical profiles of the extracts using Thin Layer Chromatography (TLC).
iii) Determine the presence of various phytoconstituents using chemical methods and evaluate the antioxidant activities of the crude extracts of selected plants using qualitative and quantitative assays.
iv) Determine the Minimum Inhibitory Concentration (MIC) against *Mycobacterium smegmatis* using extracts, fractions or isolated compounds
v) Evaluate for the presence of anti-mycobacterial compounds in crude extracts, fraction or isolated compound(s) of selected plants against *M. smegmatis* by bioautography
vi) Select the most active plant with anti-mycobacterial activity against *M. smegmatis*
vii) Evaluate activity of the most active extract or fraction against *M. tuberculosis*
viii) Isolate compounds from the most active fraction on bioautography against *M. smegmatis*
ix) Evaluate the activity of the isolated compound(s) against *M.* smegmatis using MIC
x) Elucidate the structure of the isolated compound(s)
xi) Evaluate the cytotoxic potential of the most active extract, fraction or compound(s) isolated from the most active plant.

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CHAPTER 3

EXTRACTION, PHYTOCHEMICAL ANALYSIS, PHYTOCOSTITUENT AND QUANTIFICATION OF TOTAL PHENOLICS

3.1. Introduction

Medicinal plants play a significant role in the traditional health care system of South Africa. Different plant parts (i.e. barks, roots, leaves, etc.) are used in the preparation of concoctions used to treat various diseases. The preferred plant part widely used in the preparation of medicines is dried leaves. Using dried leaf material from plants is preferred because there are fewer challenges when using it than fresh plant material (Eloff, 1998).

Different solvents varying in polarity can extract different compounds. The choice of solvent used for extraction depends on a number of factors, one of which involves the ability of the solvent to extract the largest quantities of material while also extracting material with high antimicrobial activity (Eloff, 1998). Another important factor governing the choice of solvents used in extraction is the type of phytochemical groups that are to be extracted (Houghton and Raman, 1998). Different phytochemicals are screened for so as to have an idea of the phytoconstituents or secondary metabolites present in the plant extracts.

There are different solvents with varying polarity that are used for extracting compounds from plants (Vlietinck *et al.,* 1995). Water, methanol, ethanol, chloroform, methylene dichloride and acetone have been used to isolate antimicrobial compounds from various plants (Cowan, 1999). Hence, different solvents varying in polarity were used to extract a wide variety of compounds varying in polarity.

The phytochemicals present in plants are produced in response to abiotic and biotic stress for protection and survival of plants in harsh conditions, such as high temperature, low water content, Ultraviolet degradation and exposure to herbivores (Vaghasiya *et al*., 2011). Phytochemicals have a number of biological activities which are responsible for the plants' therapeutic properties. They are potential precursors of new valuable drugs such as quinine, artemisin and shikonin, which are extracted from traditional medicinal

plants (Vaghasiya *et al*., 2011). The Thin Layer Chromatography (TLC) is used in testing various phytochemicals. Testing for phytochemicals has been aided enormously by the development of rapid and accurate methods for screening. Phytochemicals of the plant extracts can be screened or done by using standard phytochemical methods. These methods are used to validate the presence of phytochemical constituents from various plant extracts. Phytochemicals are screened using the methods described by Trease and Evans (1989), Harborne (1973), Odebiyi and Sofowora (1978) and Borokini and Omotayo (2012). The methods described by the above-mentioned authors can be used to determine the presence of various compounds such as alkaloids, flavonoids, tannins, saponins and terpernoids. Determining the presence of various phytoconstituents in plant extracts gives a clear guide on the types of secondary metabolites present in a plant. The screening of plant extracts gives an idea of where the bioactive compounds are and their actual composition, for example, the types of phenolic compound present that exhibit bioactivity. It also gives an idea of the different types of compounds present in the various extracts. Hence, it is important to screen plants to check for the presence of various phytochemicals and to do various bioassays to determine whether those phytochemicals present exhibit any biological activity.

The Thin Layer Chromatography (TLC) is a technique that is simple, affordable and a quick separation technique that gives researchers an idea of the number of components that are present in a mixture (Sasidharan, 2011). It is a method of choice for mainly separating all lipid soluble components of the plant, i.e. lipids, steroids, carotenoids, simple quinones and chlorophylls (Harborne, 1973). The phytochemicals present in plant extracts are visualised by spraying TLC plates developed in mobile phase varying in polarity with chemical reagents such as anisaldehyde or vanillin sulphuric acid. The different compounds present in plant extracts can be identified using the retention factor (R_f) value for each particular compound using TLC technique. Thin Layer Chromatography is one of the separation and identification techniques that is used in this study.

3.2. Materials and methods

3.2.1. Plant collection and storage

Seven medicinal plants were selected based on their ethnopharmacological information provided by traditional healers in the Sekhukhune, Waterberg and Capricorn districts on TB in Limpopo Province. The plants selected for this study were: *A. dimidiata* (LNBG 1969/46)*, A. afra* (LNBG 2010/27)*, Z. capense* (LNBG 1969/100)*, C. herorense* (LNBG 1977/71)*, L. javanica* (LNBG 1969/460), *E. camaldulensis* and *C. lemon* (UNIN 12330). Leaves of the plants were used in this study. Samples of *A. dimidiata, A. afra, Z. capense, C. herorense* and *L. javanica* were collected from the Lowveld National Botanical Garden. Voucher specimens in the garden herbarium and tree labels were confirmed by Mr Willem Froneman (Control Horticulturist) as well as the provision of plant accession details. The collected leaves were transported in sterile sealed, labelled containers to the laboratory where they were allowed to dry completely at room temperature. *E. camaldulensis* and *C. lemon* were collected from various areas surrounding Limpopo. The names of the collected plants were verified by Dr Egan Brownyn of the University of Limpopo-Turfloop Campus Science Centre Herbarium. The dried leaves were ground into fine powder using Jankel and Kunkel Model A10 mill. The samples were stored in airtight containers in a dark place to prevent oxidation until the extraction stage.

3.2.2. Preparation of crude extracts

The crude extracts were prepared according to the method of Kotze and Eloff (2002), where plant materials from each species were individually extracted by weighing 1 g each of the finely ground samples and each extracted with 10 ml of a different solvents varying in polarity, namely; n-hexane, dichloromethane, acetone and methanol in polyester centrifuge tubes, respectively. The tubes were vigorously shaken for 10 minutes in a series 25 shaking incubator machine (New Brunswick Scientific Co., Inc) at 100 rpm. After centrifugation at 959 xg for 10 min, the supernatant was decanted into pre-weighed labelled bottles. The extraction process was repeated three times to thoroughly extract the compounds. The solvents were air dried in a fume cupboard at room temperature and quantified.

3.2.3. Phytochemical screening

The plant extracts were re-dissolved in acetone to give a final concentration of 10 mg/ml. The compounds of the plant extracts were analysed by Thin Layer Chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F254) (Sigma) according to the method of Kotze and Eloff (2002). For each plant extract, 10 µl (or 10 µg) was loaded on aluminum TLC plate. The TLC plates were developed under saturated conditions with each of the three mobile phases differing in polarity *viz.* (1) ethyl acetate: methanol: water (10:1.4:1), [EMW] (polar/neutral); (2) chloroform: ethyl acetate: formic acid (5:4:1), [CEF] (intermediate polarity/acidic); and (3) benzene: ethanol: ammonia hydroxide (9:1:0.1): [BEA] (non-polar/basic). The separated compounds on the chromatograms were visualized under UV light (254 and 365 nm) for compounds which are fluorescing and later sprayed with vanillin-sulphuric acid reagent (0.1 g vanillin (Sigma): 28 ml methanol: 1 ml sulphuric acid) and heated at 110°C for optimal color development. The sprayed plates were scanned with a laser scanner and analysed.

3.2.4. Preliminary biochemical analysis of phytochemicals

Extracts of each plant were tested for the presence of saponins, phlobatannins, tannins, terpenes/terpenoids, steroids, cardiac glycosides and flavonoids using standard procedure as described by Borokini and Omotayo (2012).

3.2.4.1. Flavonoids

The presence of flavonoids was tested in the aqueous extracts of the selected plant leaves by adding 5 ml of diluted ammonia solution to a portion of the aqueous filtrate of plant extracts, followed by the addition of 1 ml of concentrated sulphuric acid. To draw inferences, a yellow coloration that disappears on standing indicated the presence of flavonoids (Borokini and Omotayo, 2012).

3.2.4.2. Terpenes/terpenoids

The ethanol extracts of the selected plants were tested for the presence of terpenoids by weighing 0.5 g of the extract and dissolving it in 2 ml of chloroform. Thereafter, 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish brown

colouration of the interface indicated the presence of terpenoids (Borokini and Omotayo, 2012).

3.2.4.3. Cardiac glycosides

To test for the presence of cardiac glycosides, the Keller- Killiani test was used. Plant extract (0.5 g) was weighed and diluted in 5 ml of water. The mixture of 2 ml of glacial acetic acid containing one drop of 0.1% ferric chloride solution was added to diluted plant extracts. The mixture was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxy sugar characteristic of cardenolides (Borokini and Omotayo, 2012).

3.2.4.4. Tannins

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

3.2.4.5. Alkaloids

To determine the presence of alkaloids, Drangensdorff's reagent was used. Ground leaf material (0.2 g) of the selected plants was weighed and extracted with 95% ethanol using soxhlet extractor. The extracting solvent was evaporated to dryness using a vacuum evaporator at 45^{o}C. The plant residues were dissolved in 5 ml of 1% hydrochloric acid and 5 drops of Drangendorff's reagent was added. To draw inferences, a reddish-brown colour change was observed (Harborne, 1973).

3.2.4.6. Saponins

Saponins were tested by performing the persistent frothing test whereby 1 g plant powdered leaf material of the chosen plants was mixed with 30 ml of tap water. The mixture was vigorously shaken and heated at 100 ^{o}C. To draw inferences, the mixture was observed for the formation of persistent froth (Odebiyi and Soforwa, 1978).

3.2.4.7. Steroids

The presence of steroids was tested by adding 2 ml of acetic anhydride to 0.5 g of selected plant extracts, followed by the addition of 2 ml of sulphuric acid to the mixture. To draw inference, blue or green colour change was observed (Borokini and Omotayo, 2012).

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

3.2.5.1. Total phenolic content

Total phenolic content of *A. dimidiata*, *C. hereroense* and *C. lemon* extracts was determined spectrophotometrically using a method described by Singleton *et al.* (1999). Follin-ciocaltau method was used in determination of the total phenolic content of *A. dimidiata*, *C. hereroense* and *C. lemon* leaf extracts. One millilitre of plant extracts was mixed with 9 ml of distilled water in a 25 ml volumetric flask. Thereafter, 1 ml of follin-ciocaltau phenol reagent was added to the mixture and it was well shacked. After 5 minutes, 1 ml of 7% sodium carbonate (Na_2CO_3) solution was added to the mixture and distilled water was added to make a final volume of 2.5 ml. Gallic acid (0.0625, 0.125, 0.25 and 1 mg/ml) was obtained using the same procedure used for plants mixture. The extracts and gallic acid mixture were allowed to stand for 90 minutes at room temperature in the dark. The absorbance of the mixtures was spectrophotometrically recorded at 550 nm. The total phenolic content was determined using a linear regression analysis from a gallic acid calibration standard curve.

3.2.5.2 Total flavonoid content

The aluminium chloride colorimetric method was used for determination of total flavonoid content from *A. dimidiata*, *C. hereroense* and *C. lemon* leaf extracts (Tambe and Bhambar, 2014). One millilitre of plant extracts was added to 4 ml of distilled water in a 10 ml volumetric flask. Thereafter, 0.3 ml of 10% aluminium chloride was added to the mixture. After 5 minutes, 2 ml of 1 M sodium hydroxide was added and the flask was filled with distilled water to make a final volume of 10 ml. A set of reference standard mixtures of quercetin in various concentrations was prepared in the same manner as for extracts. The absorbance of the mixtures was recorded against the reagent blank at 510 nm using

UV / visible spectrophotometer. The total flavonoid content was determined by linear regression analysis from a quercetin calibration standard curve.

3.2.5.3. Total tannin content

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

3.3. Results

3.3.1. Extraction

From the seven indigenous plants, the methanol extract of *E. camaldulensis* extracted the most as compared to the other plant species followed by that of *C. heroroense* and *A. dimidiata,* whereas *L. javanica* extracted the least. This indicates that on average, the most extracted compounds in all the plants were polar since methanol extracted the most as compared to other solvents. The intermediate to non-polar compounds were extracted the most by dichloromethane of *A. dimidiata* followed by *L. javanica* while C*. lemon* extracted the least (Figure 3.1).

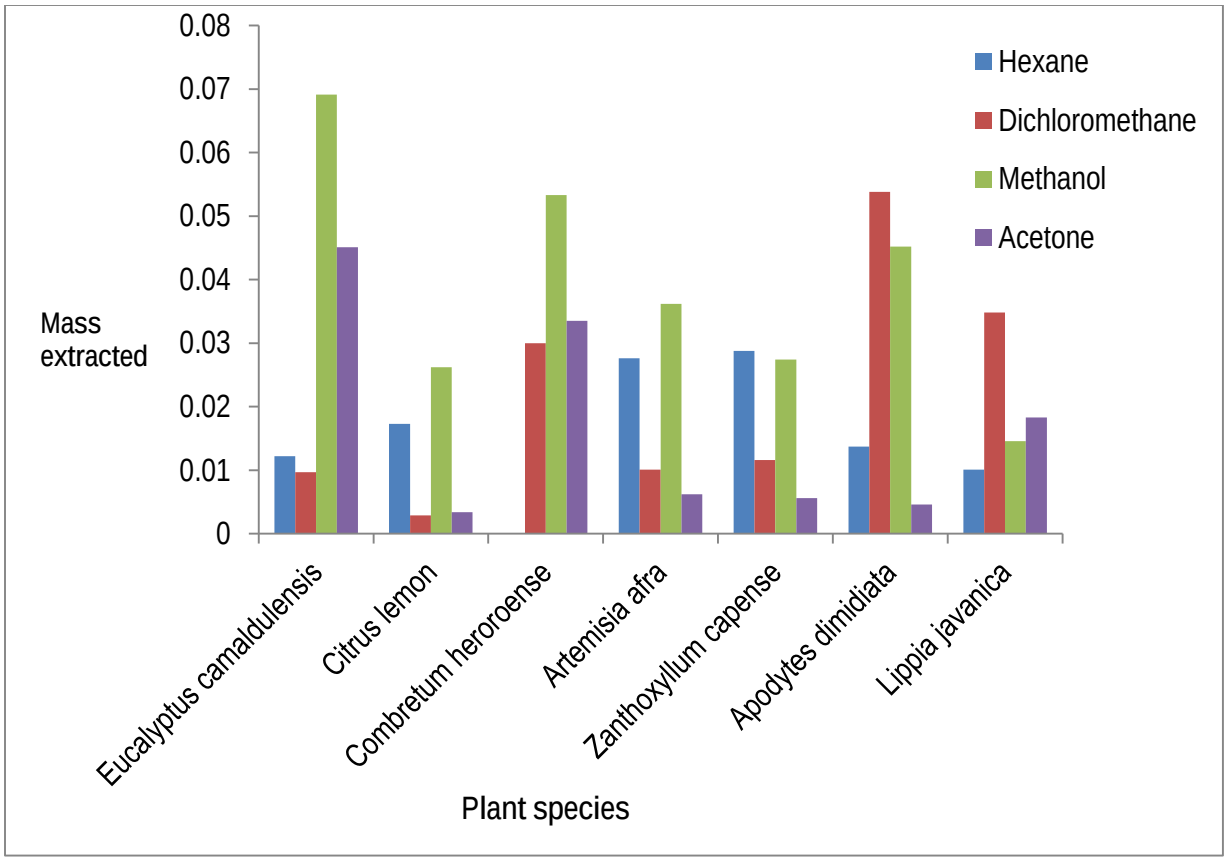

Figure 3.1: Mass extracted (g) of the leaves of the seven selected plants using four different solvents varying in polarity.

Methanol extracted the most material (3.90 %) in terms of mass among all the plant species, followed by dichloromethane with an average percentage mass of 1.80 %. Percentage average mass extracted by acetone (1.67 %) was almost within the same range with that of hexane (1.57 %). The highest average mass extracted by all the solvents was for E*. camaldulensis* (3.40 %) followed by *A. dimidiata* (2.93 %). The lowest yield was from *C. lemon* (1.25 %) followed by *Z. capense* (1.84 %) having the least percentage yield (Table 3.1).

Table 3.1: The percentage mass (%) of different plants extracted using four extractants from dried powdered leaves.

Plant species	Percentage mass residue extracted (%)				
	Hexane	**DCM**	**Methanol**	**Acetone**	**Average**
E. camaldulensis	1.22	0.97	6.91	4.51	**3.40**
C. lemon	1.73	0.29	2.62	0.34	**1.25**
C. heroroense	0.01	0.30	5.33	3.35	**2.25**
A. afra	2.76	1.01	3.62	0.62	**2.00**
Z. capense	2.88	1.16	2.74	0.56	**1.84**
A. dimidiate	1.37	5.38	4.52	0.46	**2.93**
L. javanica	1.01	3.48	1.46	1.83	**1.95**
Average	**1.57**	**1.80**	**3.90**	**1.67**	

DCM= Dichloromethane

3.3.2. Phytochemical analysis

3.3.2.1. Presence of phytochemicals

Tannins, terpenes/terpenoids, steroids and flavonoids were present in all the plants while phlobatannins were absent, whereas saponins were only present in *A. dimidiata*, *A. afra* and *Z. capense* (Table 3.2).

Table 3.2: Shows the phytochemical constituents present in various plants.

	EC	CL	CH	AA	ZC	AD	LJ
Saponins	-	-	+	-	+	+	+
Phlobatannins	-	-	-	-	-	-	-
Tannins	+	+	+	+	+	+	+
Terpenoids/ terpenes	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+
Cardiac glycosides	+	-	+	+	+	-	-
Flavonoids	+	+	+	+	+	+	+

EC= *E. camaldulensis*, CL= *C. lemon*, CH= *C. hereroense,* AA= *A. afra,* ZC= *Z. capense,* AD= *A. dimidiata* and LJ= *L. javanica*

- = absence, + = presence

The plant extracts spotted on TLC plates were sprayed with vanillin spray reagent to visualize the different compounds present in the extracts.

There were several compounds on the chromatograms visible under the UV light and others became visible after the plates were sprayed with vanillin sulphuric acid. There was a difference in the composition of the extracts. The CEF system had a better separation of non-polar compounds than the EMW system which separated polar compounds. In general, TLC plates developed in BEA had many colourful bands for all the plants, followed by CEF and finally EMW had the least. Overall, for all the mobile systems, *L. javanica* had many colourful bands when the plates were sprayed with vanillin sulphuric acid, followed by *E. camaldulensis* and *Z. capense* had the least bands. The solvent system that separated different compounds the best was BEA (Figure 3.1a to Figure 3.1c).

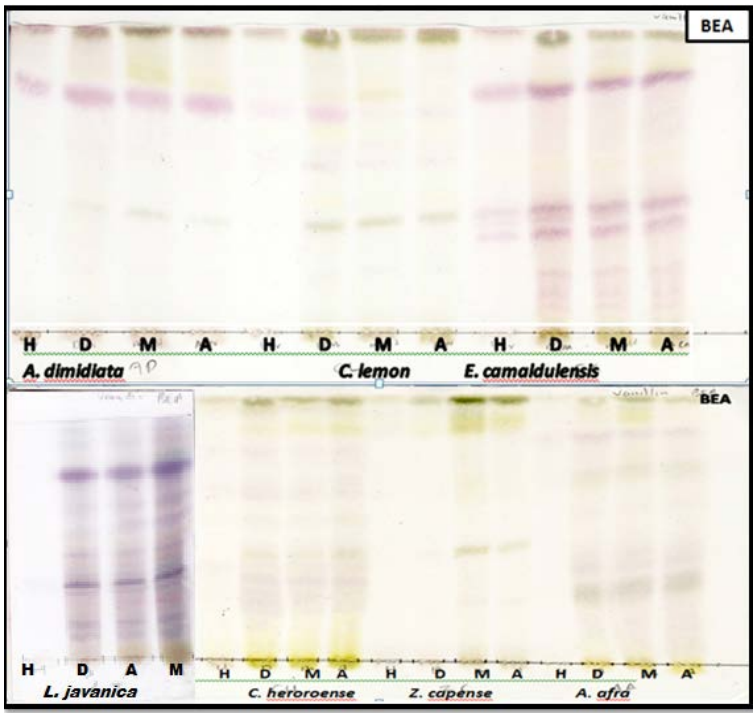

Figure 3.2a: Chromatogram of *A. dimidiata, C. lemon, E. camaldulensis* (above), *L. javanica, C. heroroense, Z. capense* and *A. afra* (below) respectively, developed in BEA solvent system and sprayed with vanillin-sulphuric acid to show compounds extracted with hexane (H), dichloromethane (D), methanol (M) and acetone (A) in lanes from left to right for each plant.

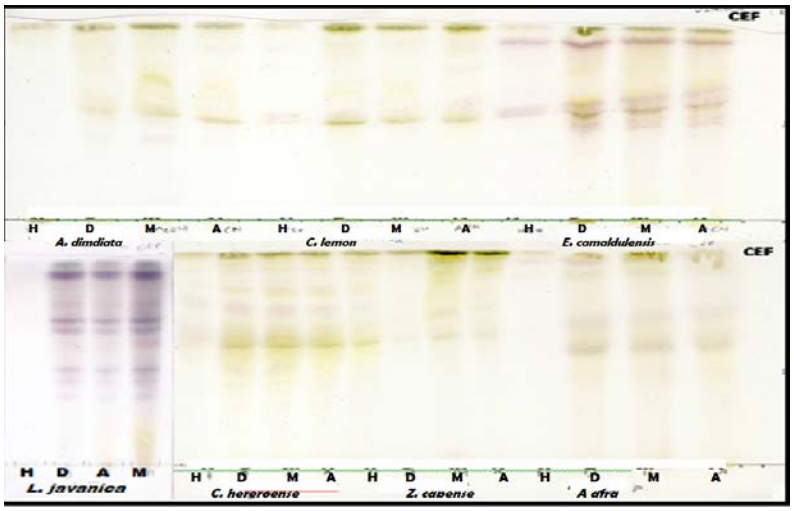

Figure 3.2b: Chromatogram of *A. dimidiata, C. lemon* and *E. camaldulensis* (above) *L. javanica, C. heroroense, Z. capense* and *A. afra* (below) respectively, developed in CEF solvent system and sprayed with vanillin-sulphuric acid to show compounds extracted with hexane (H), dichloromethane (D), methanol (M) and acetone (A) in lanes from left to right for each plant.

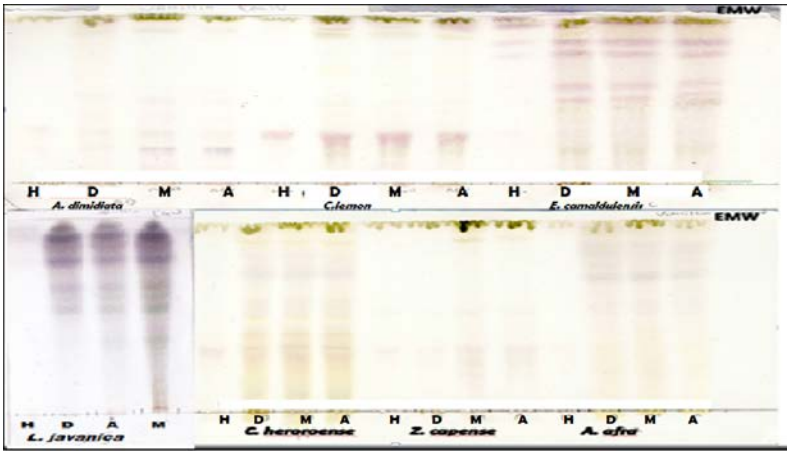

Figure 3.2c: Chromatogram of *A. dimidiata, C. lemon* and *E. camaldulensis* (above) *L. javanica, C. heroroense, Z. capense* and *A. afra* (below) developed in EMW solvent system and sprayed with vanillin-sulphuric acid to show compounds extracted with hexane (H), dichloromethane (D), methanol (M) and acetone (A) in lanes from left to right for each plant.

3.3.3. Quantification of total phenolics

Three plants, namely *A. dimidiata*, *C. hereroense* and *C. lemon* were selected for further studies based on the results from preliminary screening of the seven plants. The three plants were selected because they exhibited potent effect on the inhibition of the growth of *M. smegmatis* (which is clearly illustrated in the next chapters).

The calibration curves below (figures 3.3a to figure 3.3c) indicate the linear regression of the total phenols concentration and their absorbance, total tannin concentration and its absorbance as well as total flavonoid content and its absorbance.

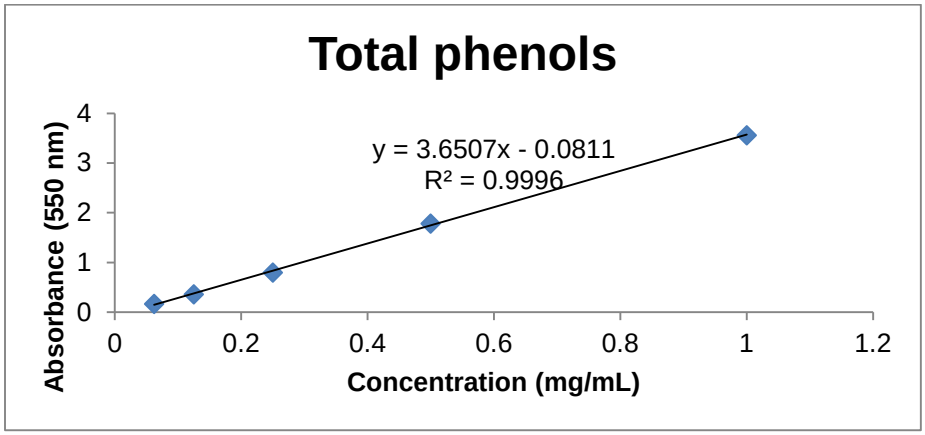

Figure 3.3a: Calibration standard curve of gallic acid for the determination of total phenols.

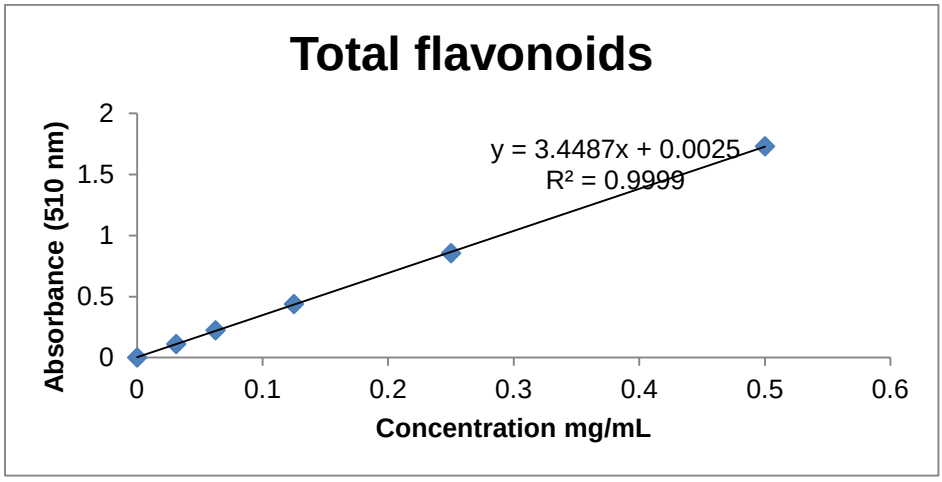

Figure 3.3b: Calibration standard curve of quercetin for the determination of total flavonoids.

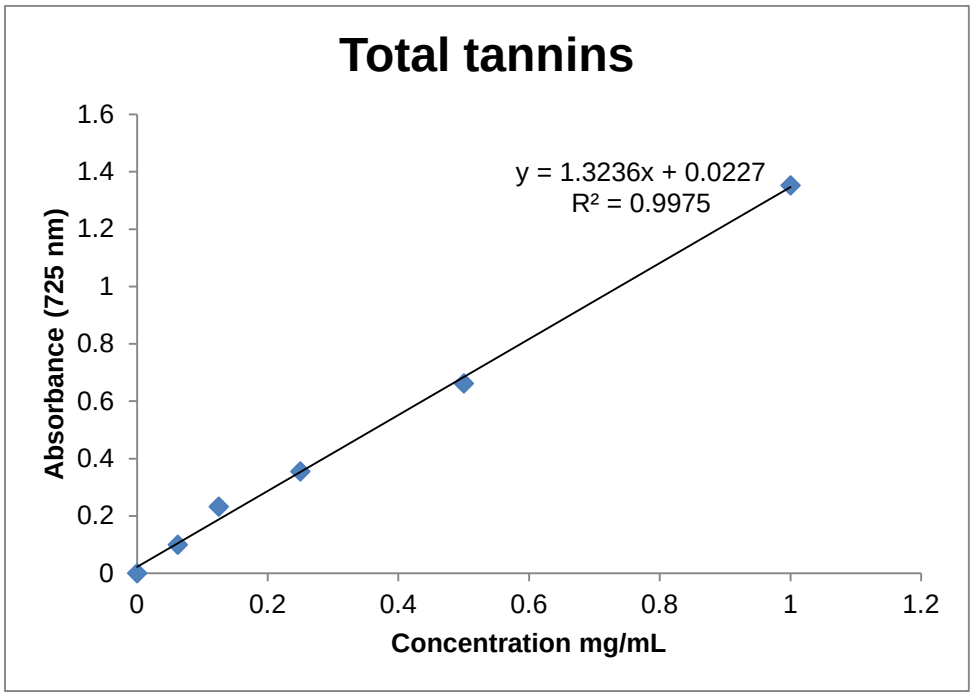

Figure 3.3c: Calibration standard curve for the determination of total tannins.

The quantitative measure of total phenolic, tannin and flavonoid content are presented in Table 3.3. The quantity of these phytochemicals varied between the plant species. The highest concentration of phenolic (135± 7.05 mg GAE/g of sample) was with *A. dimidiata*, followed by *C. heroroense* with total phenols of 114.8± 7.36 mg GAE/g of sample and *C. lemon* had the least (44.95± 0.724 mg GAE/g of sample) of the three plants. Tannin content of *C. hereroense* (73.76 ± 5.57 mg GAE/g of sample) amongst the three plants, followed by *A. dimidiata* (48.83 ± 6.99 mg GAE/g of sample) and *C. lemon* had the least (35.48 ± 1.57 mg GAE/g of sample). Flavonoid content of *A. dimidiata* (57.46 ± 1.65 mg QE/g of sample) was the highest, followed by *C. hereroense* (38.61 ± 3.35 mg QE/g of sample) and *C lemon* (15.23 ± 0.31 mg QE/g of sample) had the least flavonoid content of the three plants (table 3.3).

Table 3.3: Total phenol, tannin and flavonoid content concentrations of the 70% aqueous acetone extract of *A. dimidiata* (AD)*, C. hereroense* (CH) and *C. lemon* (CL) plant leaves

Sample	Total phenols (mg of GAE/g of sample)	Tannins (mg of GAE/g of sample	Flavonoids (mg of QE/mg of sample)
CL	44.95 ± 0.724	35.48 ± 1.57	15.23 ± 0.31
CH	114.8 ± 7.36	73.76 ± 5.57	38.61 ± 3.35
AD	135.44 ± 7.05	48.83 ± 6.99	57.46 ± 1.65

Abbreviations: GAE, gallic acid equivalent; QE, quercetin equivalent.

Each value is the average of 3 analyses ± standard deviation

3.4. Discussion

The four solvents, namely; hexane, dichloromethane, acetone and methanol, were used to extract a wide range of plant compounds. The most common solvent used by traditional healers is water which is limited by its inability to extract non-polar compounds. Water frequently does not dissolve the intermediate polar to nonpolar components of a dried material. The success of determining the biologically active compounds largely depends on the type of solvent used in extraction. Therefore, it is important to use solvents that will extract all compounds, that is, covering all ranges of polarity. Methanol was the best extractant resulting in a greater yield of plant material extracted and hexane was the least (figure 3.1). Masoko *et al.* (2008) reported that methanol extracts had the best extract yield, although their study was not based on determining the best extractant. *C. hereroense* had the best extracted material, and *Z. capense* had the least extracted plant material. Following extraction, extracts were re-dissolved in acetone as reported by Eloff (1999) as it has the ability to dissolve many hydrophilic and lipophilic compounds and miscible with water and is not toxic to bacterial and fungi species.

Traditional medicinal plants have a variety of phytochemicals which can be classified as primary and secondary metabolites (Vhaghasiya *et al*., 2011). The TLC chromatograms showed the presence of a variety of compounds for all the plants used in this study. Finger print profiling using TLC separates compounds with different R_f values in different solvent

systems. The R_f values of the phytochemicals provide a very important clue in understanding the polarity of different compounds present in plants and also in understanding their polarity. It also assists in the selection of appropriate solvent system for separation of pure compounds by column chromatography (Sharma *et al.,* 2017). By analyzing the R_f values of the compounds in different solvents, one can be able to select the appropriate solvent system for a particular plant extract (Gujjeti and Mamidala, 2013). The TLC chromatograms developed in BEA exhibited good separation of the various metabolites present in all the plants, followed by the plates developed in CEF. In this study, *A. dimidiata* and *C. heroroense* had 5 out of the 7 phytochemicals investigated, whereas *C. lemon* had 4 out 7 phytochemicals investigated. Masoko and Nxumalo (2013) reported similar results for all the investigated plants, emphasizing the validity of the number of phytochemicals present in the plants. Flavonoids were found to be present in all the plants and they have been reported to possess antimicrobial and antioxidant activities. On the other hand, terpenes/ terpenoids, which are also present in all the plants, have been reported to have medicinal properties such as anti-carcinogenic, antimalarial, antiulcer, antimicrobial and diuretic activities (Aharoni *et al*., 2005).

Babayi *et al.* (2004) have reported the presence of saponins in *E*. *camaldulensis* which was not the case in this study. The difference observed may be due to the variation in harvesting time of plant material and also the difference in the location in which the plant was harvested. The presence of tannins in all the plants shows that the plant may be useful in various industries such pharmaceutical and leather industries (Nguji, 1988; Daziel, 1995; Babayi *et al*., 2004), although in this study the focus on this group of compounds relates to their role in the treatment of TB or related symptoms. More bands were observed in BEA, followed by CEF and EMW, which indicates that in the selected plant there is an abundance of non-polar compounds. There were bands with various colours observed after spraying with vanillin sulphuric acid, which indicated the presence of certain phytochemicals in the plants. The was a blue, bluish violet or purple colour present, which indicated the presence of terpenoids in the plant extracts. The presence of blue violet colour in various extracts indicated the presence of saponins (Karthika and Paulsamy, 2015). Some extracts had a yellow colour which indicated the presence of

steroids in those extracts. There was also a greenish colour present in the extracts which is an indication of the presence of chlorophyll in the plant leaves.

Phenolic compounds have redox properties, which allow them to act as antioxidants (Soobrattee *et al*., 2005; Baba and Malik, 2015). As the free radical scavenging ability of phenolic compounds is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity in plants. Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo (Shimoi et al., 1996; Geetha et al., 2003; Baba and Malik, 2015).* The total phenols displayed by the three plants corresponded very well with the percentage scavenging potential of the plants (which is clearly explained in the next chapter). *Apodytes dimidiata* had the highest total phenols indicating that it may be the best plant to use for isolation of bioactive compounds since it had more phenols than the other selected two plants. The high phenolic content in the plants was the basis for screening antioxidant activity because they are natural harbours of antioxidants (Matotoka and Masoko, 2018).

3.5. Conclusion

A large number of phytochemical compounds were extracted from all the seven plants. Overall, methanol was the best extractant for all the plants, resulting in a greater yield of plant extract. In this study, BEA was the best solvent system in separating various phytochemicals.

The findings of this study also indicate that various phytochemical compounds such as alkaloids, tannins, steroids, flavonoids, terpenoids and cardiac glycosides are present in most of the plants used. This could suggest that these phytochemicals are responsible for the therapeutic effect of the plants in the treatment of various ailments.

3.6. References

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CHAPTER 4

ANTIOXIDANT ACTIVITY ASSAYS

4.1. Introduction

Antioxidants are compounds capable of either inhibiting or delaying the oxidation processes that occur under the influence of atmospheric oxygen or reactive oxygen species. Antioxidants are used for the stabilization of polymeric products, of petrochemicals, foodstuffs, cosmetics and pharmaceuticals (Pisoschi and Negulescu, 2011). They are involved in the defence mechanism of the organisms (for example, *Mycobacterium tuberculosis*) against the pathologies associated with the attack of free radicals. Endogenous antioxidants are enzymes, like superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds, such as uric acid, bilirubin, albumin, metallothioneins. When endogenous factors cannot ensure a rigorous control and a complete protection of the organism against the reactive oxygen species, the need for exogenous antioxidants arises, as nutritional supplements or pharmaceutical products, which contain as an active principle, an antioxidant compound. Amongst the most important exogenous antioxidants are: vitamin E, vitamin C, β-carotene, vitamin E, flavonoids, etc. Exogenous antioxidants can be derived from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc. (Litescu *et al*., 2011; Pisoschi and Negulescu, 2011).

Many infectious diseases produce hydrogen peroxide which in turn gives out oxygen free radicals. One of the major effects of most diseases process, is the production of oxygen free radicals by the cells in the body. These free oxygen radicals cause damage to the cells. Antioxidants are considered important nutraceuticals because of their many health benefits (Droge, 2002; Lee *et al*., 2004; Valko *et al*., 2007). There are a variety of standard antioxidant assays that are very important because they assist in comparing the results of different laboratories and validation of the conclusions. 1,1-Diphenyl-2-picryl- hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge (Eklund *et al*., 2005). The scavenging of DPPH radical is the basis of the

popular DPPH antioxidant assay (Alma, Mavi *et al*., 2003; Karioti *et al*., 2004; Kordali *et al*., 2005).

The basis of antioxidant activity assay is scavenging of DPPH free radicals (Sharma and Bhat, 2008). There are numerous assays that have been introduced for the measurement of total antioxidant of body fluids (Wayner *et al*., 1985; Miller *et al*., 1993), food extracts (Salah *et al*., 1995) and pure compounds (Arnao *et al*., 1990; *Miller et al*., 1994; Kono *et al.*, 1995; Rice- Evans *et al*., 1996). Every method relates to the generation of a different radical, acting through various mechanisms and the measurement of a range of end points at a fixed time point or over a range (Miller *et al*., 1994; Miller *et al*., 1996). There are currently two main methods for determining antioxidant activity. The first one is the inhibition assay, in that the extent of the scavenging by hydrogen- or electron- donation of a pre-formed free radical is the marker of the antioxidant activity. The second one is the assay involving the presence of antioxidant system during generation of free radical (Re *et al*., 1998).

1, 1 Diphenyl 2- Picryl Hydrazyl is a stable (in powder form) free radical with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as:

$$(DPPH) + (H\text{-}A) \rightarrow DPPH\text{-}H + (A)$$

Antioxidants react with DPPH and reduce it to DPPH-H and as a consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

The reducing agent becomes oxidized by transferring electrons to another substance. Since the reducing agent gives electrons, it is also called an electron donor. Electron donors can also form charge transfer complexes with electron acceptors. There are various reductants in Biochemistry. For example, ferric ions (Fe(3+)) are good reducing agents. Also, different bioanalytical reduction methods are available such as Fe(3+)-

ferrous ions (Fe(2+)) reduction method, ferric reducing antioxidant power reducing assay (Gülcin, 2015)

The methods are chosen as a function of the nature of the sample and the comparison is valid only on the same sample types. The advantages of the analytical techniques can refer to the complexity of the necessary tools, to the simplicity of the applied procedure, to the duration of the analysis, to the biological relevance and the performances of the method (sensitivity, precision, accuracy, detection limit). Determinations relying on photometric measurements (DPPH, ABTS and FRAP assays) are simple and rapid and need only a UVV is spectrophotometer to perform, which probably explains their widespread use in antioxidant screening. Most methods can be rapidly automatized and some can be applied in vivo (e.g. ABTS assay). Nevertheless, the analytical signal is sometimes difficult to measure and does not account for all antioxidants.

4.2. Materials and methods

4.2.1. Qualitative antioxidant assay

The potential antioxidant activity of the plant extracts was determined on the basis of the scavenging activity of stable 1, 1- diphenyl- 2-picrylhydrazyl (DPPH) (Sigma®). The chromatograms were prepared as in section 3.2.4.1. The chromatograms were sprayed with 0.2% DPPH to visualize any potential antioxidant compounds within the separated plant extracts (Deby and Margotteaux, 1970).

4.2.2. Quantitative antioxidant assay

The scavenging activity of the leaves samples of the three selected plants for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described (Velazquez *et al.,* 2003) with some modifications. Leaves samples for each plant were dissolved in methanol at a concentration of 2.65–170 mg/ml, and 0.75 ml of each sample was mixed with 1.5 ml of DPPH (Fluka Chemie, Switzerland) in methanol (0.02 mg/ml), with methanol serving as the blank sample. The mixtures were left for 15 min at room temperature and the absorbances then measured at 517 nm. Ascorbic acid (Labosi, Paris, France) (0–40 mg/l) was used as a positive control. The radical scavenging activity was calculated as

follows as: % Inhibition = [(blank absorbance - sample absorbance)/blank absorbance] · 100. The mean of three IC_{50} (concentration causing 50% inhibition) values of each extract was determined graphically. The antioxidant content was evaluated as described by Chen *et al.,* (2012), with some modifications. Plant leaves samples were dissolved in methanol (0.02 or 0.04 g/ml) and 0.75 ml of each was mixed with 1.5 ml of a 0.02 mg/ml solution of DPPH in methanol. The mixtures were left for 15 min at room temperature and the absorbances then measured (517 nm). The blank sample consisted of 0.75 ml of a honey solution with 1.5 ml of methanol. The antioxidant content was determined using standard curves for ascorbic acid (0–10 mg/ml). The means of three values were obtained, expressed as mg of ascorbic acid equivalent antioxidant content (AEAC) per 100 g of leaves sample for each plant.

4.2.3. Ferric reducing power

The antioxidant capacity was evaluated using the reducing power assay described by Oyaizu (1986) with modifications. A set of concentrations ranging from 0.0625 mg/ml to 1 mg/ml of the 70% aqueous acetone extracts of the selected plants was made. Two milliliters of each of the prepared concentration was added into a test tube, to this 2 ml of sodium phosphate buffer (1 M, pH 6.6) and 2 ml of potassium ferricyanide (1% w/v in distilled water) were added and mixed well. This mixture was incubated in a water bath at 50^0C for 20 minutes. Following incubation, 2.5 ml of trichloroacetic acid (10% w/v in distilled water) was added and the mixture was centrifuged at 650 rpm for 10 minutes. About 3 mL of the supernatant was added into a test tube. To this, 10 ml of distilled water and 1 ml of ferric chloride (0.1%w/v in distilled water) solution was added and mixed well. The absorbances of the solutions were measured at 700 nm against a blank prepared as described above but replacing the plant extract with an equal volume of a solvent (Sacho and Schoub, 1993).

4.3. Results

4.3.1. Qualitative antioxidant activity

Antioxidant activity assay was done to determine the free radical scavenging ability of the compounds present in the different extracts. Chromatograms developed in BEA had activity with all the extracts for *C. hereroense*, with *A. dimidiata* and *L. javanica,* the compounds which had prominent activity were retained at the bottom of the TLC plate. *E camaldulensis* and *A. afra* had distinctive bands of antioxidant activity with the intermediate polar to polar extractants, i.e. dichloromethane, methanol and acetone extracts. No activity was observed with *C. lemon* when plates were developed in BEA. Chromatograms developed in CEF exhibited distinctive yellowish bands for the dichloromethane, methanol and acetone for *E.* camaldulensis. For C*. hereroense* and *L. javanica*, all the extracts exhibited antioxidant activity although the extracts that showed clear antioxidant activity did not show clear separation. All the other plants had antioxidant activity with all the extracts even though it was not very clear. Chromatograms EMW showed antioxidant activity with better separation as compared to BEA and CEF for all the plant extracts that exhibited antioxidant activity, indicating that extracts with antioxidant are intermediate polar to polar. Overall, *L. javanica*, followed by *C. heroroense* and *E. camaldulensis* exhibited better antioxidant activity as compared to other plants, with better separated antioxidant compounds (figure 4.1a to figure 4.1c).

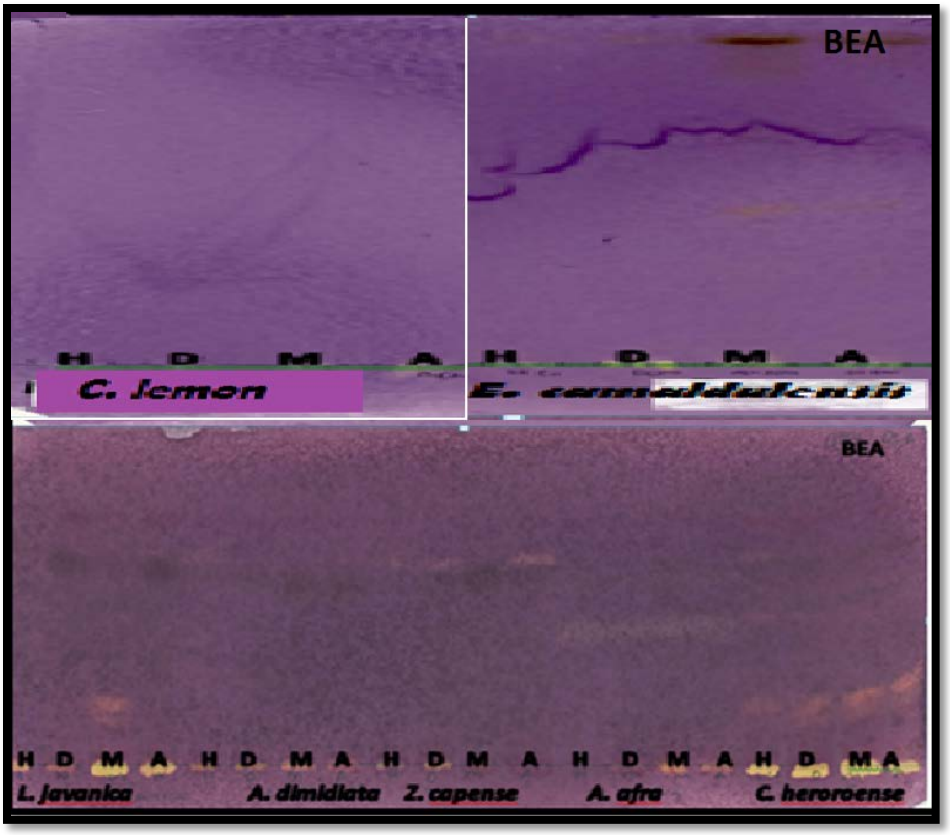

Figure 4.1a: TLC profile of the serial leaf extracts of *C. lemon* and *E. camaldulensis* (above), *L. javanica, A. dimidiata, Z. capense, A. afra* and *C. heroroense* (below), respectively; developed in BEA and sprayed with 1,2- diphenylpicryl hydrazyl (DPPH). Lanes from left to right: H (hexane), D (dichloromethane), M (methanol) and A (acetone) extracts.

Figure 4.1b: TLC profile of the serial leaf extracts of *L. javanica, A. dimidiata, Z. capense, A. afra* and *C. heroroense* (above), *C. lemon* and *E. camaldulensis* (below) respectively; developed in CEF and sprayed with 1,2- diphenylpicryl hydrazyl (DPPH). Lanes from left to right: H (hexane), D (dichloromethane), M (methanol) and A (acetone) extracts.

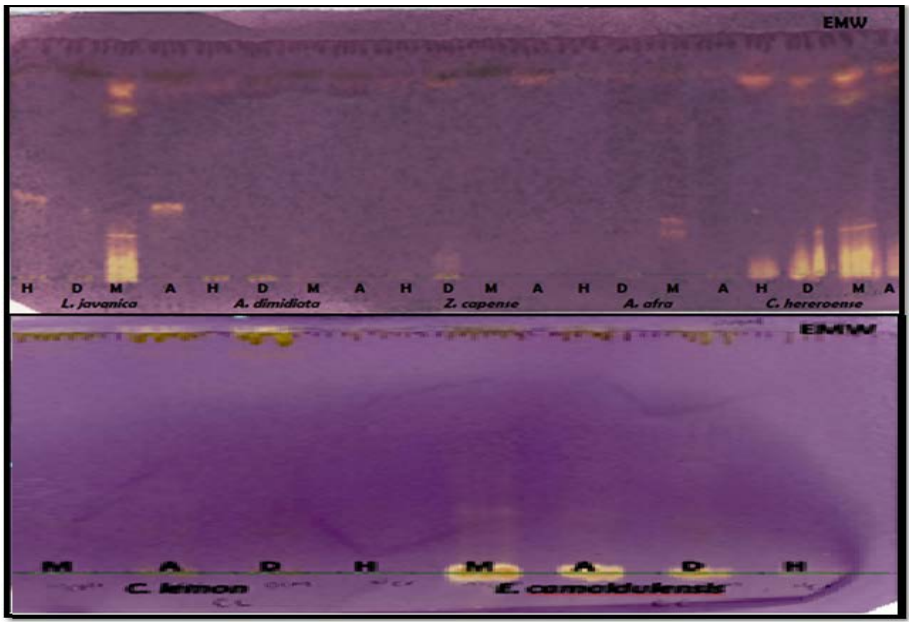

Figure 4.1c: TLC profile of the serial leaf extracts of *L. javanica, A. dimidiata, Z. capense, A. afra* and *C. heroroense* (above), *C. lemon* and *E. camaldulensis* (below) respectively; developed in EMW and sprayed with 1,2- diphenylpicryl hydrazyl (DPPH). Lanes from left to right: H (hexane), D (dichloromethane), M (methanol) and A (acetone) extracts.

Overall, *E. camaldulensis*, *C. heroroense*, *L. javanica* and *A. afra* were found to exhibit antioxidant effects, based on the scavenging activity of the stable 1,1 –diphenyl- 2-dipicrylhydrazyl (DPPH) free radical.

Since three plants (A. dimidiata, C. hereroense and C. lemon) were selected for further analysis, their individual qualitative antioxidant activity was determined using TLC plates sprayed with DPPH (figure 4.2a to figure 4.2c). The various compounds of the plants were viewed under UV to verify whether the same compounds which exhibited antioxidant activity also had UV activity.

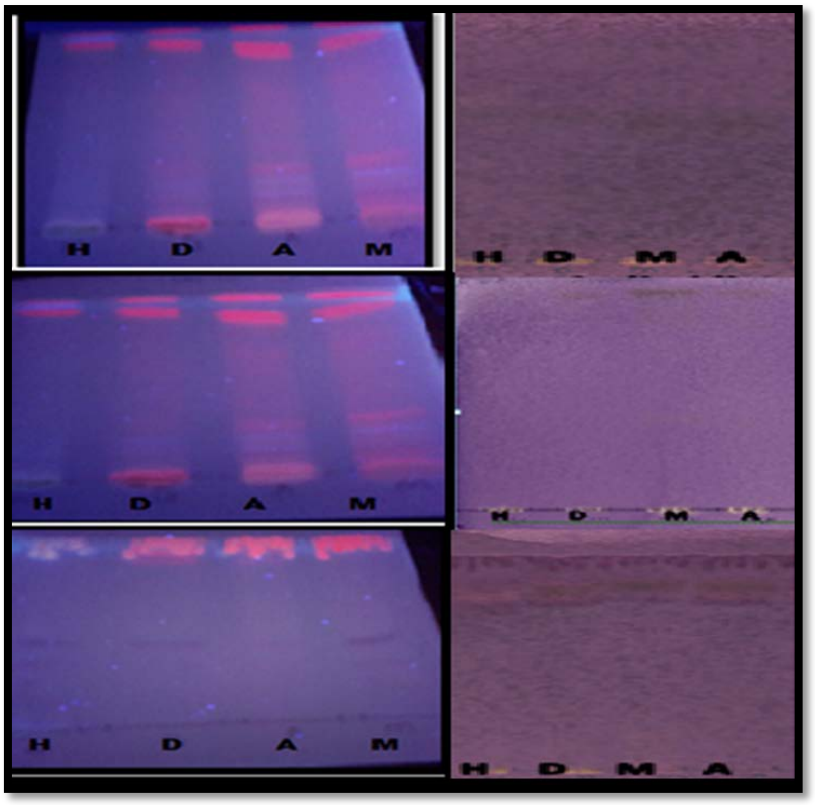

Figure 4.2a: Thin Layer Chromatography plates viewed under UV (left) and sprayed with 1,2 diphenylpicryl hydrazyl (right) of the four extracts hexane (H), dichloromethane (D), acetone (A) and methanol (M) of *Apodytes dimidiata* developed in BEA (top), CEF (middle) and EMW (bottom).

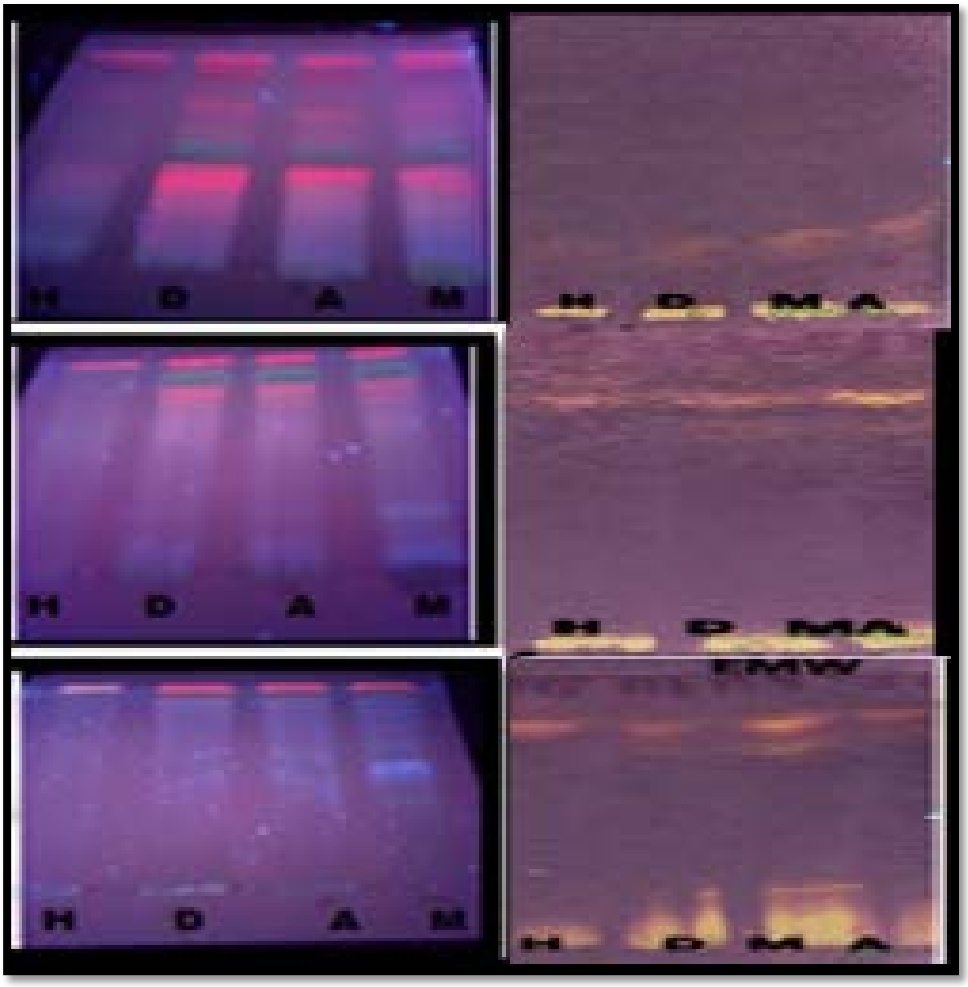

Figure 4.2b: Thin Layer Chromatography plates viewed under UV (left) and sprayed with 1,2 diphenylpicryl hydrazyl(right) of the four extracts hexane (H), dichloromethane (D), acetone (A) and methanol (M) of *Combretum hereroense* developed in BEA (top), CEF (middle) and EMW (bottom).

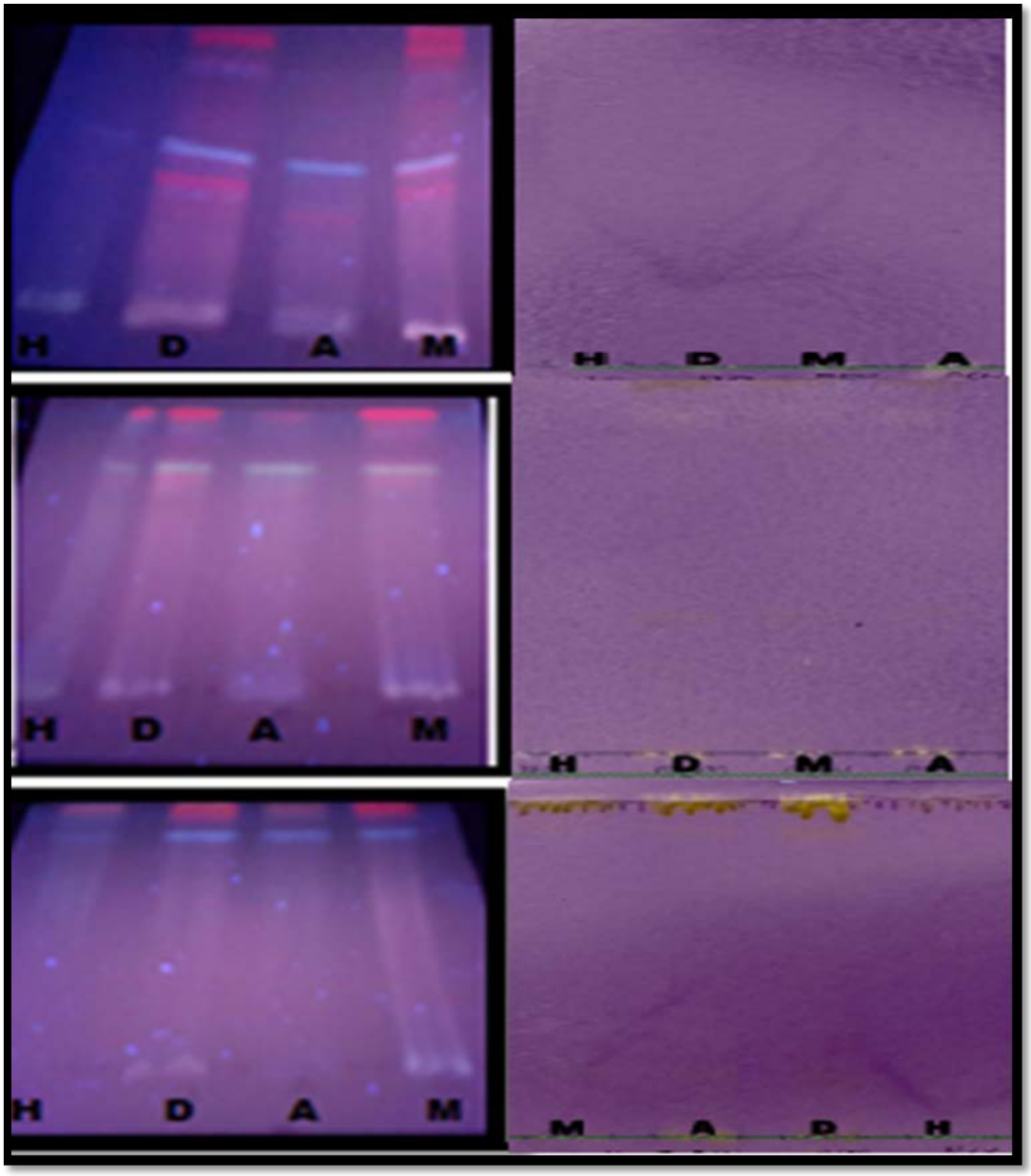

Figure 4.2c: Thin Layer Chromatography plates viewed under UV (left) and sprayed with 1,2 diphenylpicryl hydrazyl (right) of the four extracts hexane (H), dichloromethane (D), acetone (A) and methanol (M) of *Citrus lemon* developed in BEA (top), CEF (middle) and EMW (bottom).

The three selected plants were further subjected to the first step of isolation, which is solvent-solvent fractionation (as described in detail in the next chapters). Qualitative antioxidant assay of the subfractions of the three plants was evaluated (figure 4.3). All the subfractions of *C. hereroense* had antioxidant compounds as compared to the other plants, followed by *C. lemon* subfractions (butanol, hexane, ethylacetate and second water) and lastly, *A. dimidiata* subfractions (butanol, ethylacetate and methanol). The antioxidant subfractions were well separated in the intermediate polar solvent system, CEF. The most antioxidant activity was observed with EMW, the most polar mobile system in all the plants, particularly *C. hereroense*, although separation of the compounds was not clear (figure 4.3).

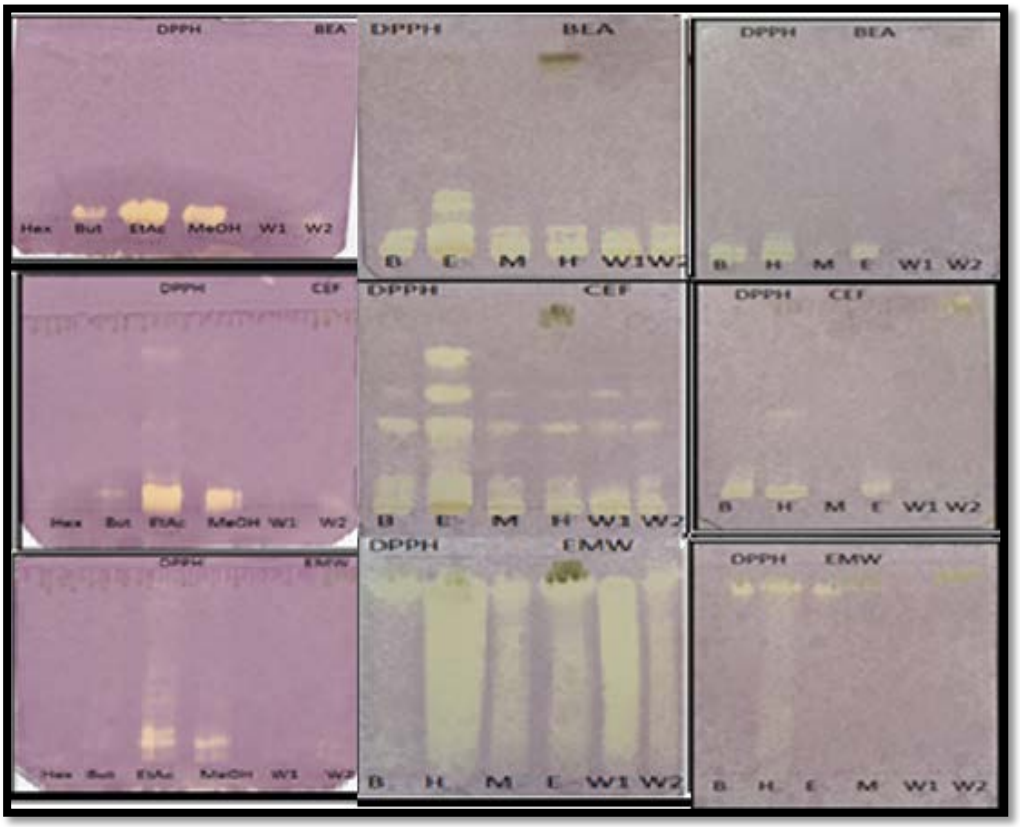

Figure 4.3: Chromatograms of solvent-solvent fractions [Hex (hexane), But (butanol), EtAc (ethylacetate), MeOH (methanol), W1 (water fraction no. 1) and W2 (water fraction no. 2)] of *A. dimidiata* (left), *C. hereroense* (middle) and *C. lemon* (right) developed in BEA (top row), CEF (middle row) and EMW (bottom row) and sprayed with 1,2-diphenylpicryl hydrazyl.

4.3.2. Quantitative antioxidant activity

Quantitative antioxidant activity of *A. dimidata*, *C. hereroense* and *C. lemon* was evaluated. The percentage scavenging potential of the plants corresponded very well with their total phenolic content.

Table 4.1: Scavenging activity of *C. lemon* (CL), *C. hereroense* (CH) and *A. dimidiata* (AD).

Sample	DPPH scavenging potential EC_{50} mg/ml
CL	3,40 ± 0,0252
CH	2,30 ± 0,0153
AD	1,32 ± 0,006
ASC ACID	0,25±0,0091

ASC ACID= Ascorbic acid

4.3.3. Ferric reducing power

The reducing power capacity of all the plants increased in a dose-dependent manner (Kudumela and Masoko, 2018). This method is based on the ability of antioxidants in the plants to reduce the ferric complex to its ferrous form (Brand-Williams *et al*., 1995). The plant which had the highest total phenolic, tannin, and flavonoid content also displayed the highest free radical scavenging and ferric reducing capacity. This correspondence may be attributed to the well-known fact that phenolics and flavonoids possess high antioxidant potential (Kudumela and Masoko, 2018; Brand- Williams, 1995) as free radical scavengers and metal ion reducers (Masoko *et al*., 2010). The reducing power method of antioxidant capacity assay revealed that *A. dimidiata* had the highest reducing power while the other plants showed almost equal efficacy that is lower (Figure 4.4).

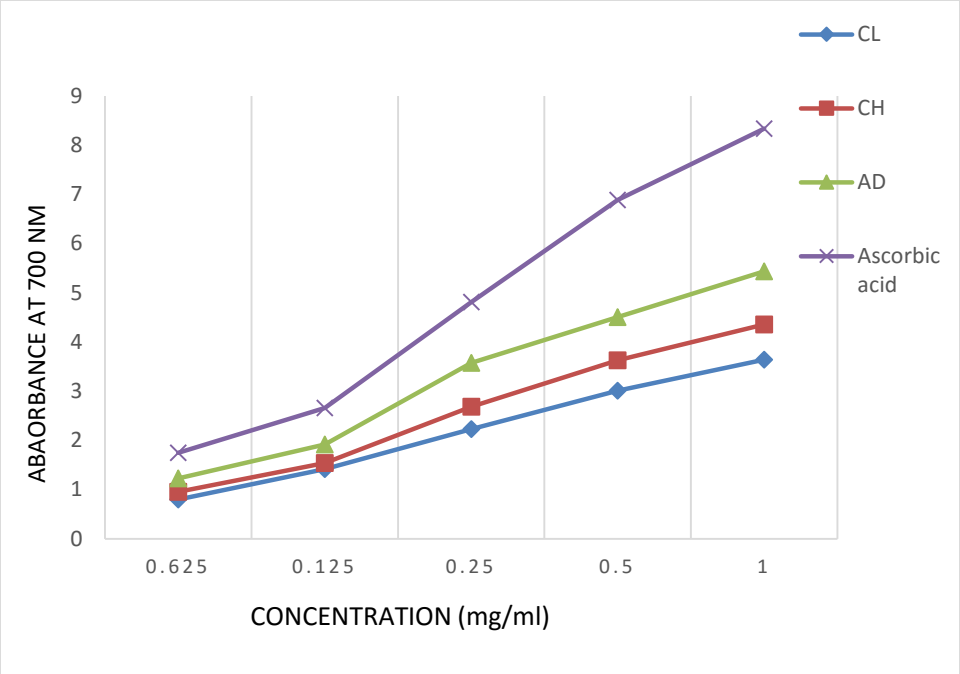

Figure 4.4: The reducing power potential of the selected plants, *C. lemon* (CL), *C. hereroense* (CH) and *A. dimidiata* (AD), and ascorbic acid, with absorbance increasing with increasing concentration.

4.4. Discussion

Some extracts had more antioxidants when compared to others. This is due to the polarity of the components of the compounds present in the extractants reacting with DPPH. The presence of antioxidant constituents in the extracts of *L. javanica*, *E. camaldulensis* and *C. heroroense* that are intermediate polar to polar were shown as yellow spots against the purple background, even though they were not well separated when developed in CEF and EMW solvent systems (figure 4.1b and figure 4.1c). Lekganyane *et al*., (2012) reported antioxidant activity in *L. javanica,* and Pretorius (2010) also reported that *L. javanica* shows great potential as a medicinal plant with antioxidant activity and may therefore be beneficial in decreasing the negative oxidative effects caused by free radicals. The significance of evaluating antioxidant activity in the plant extracts was to discover any link or relationship between the antioxidant activity and the therapeutic property being investigated. The presence of the compounds within these plants suggests that the extracts contain hydrogen-donating antioxidant compounds, which were able to

reduce DPPH to a yellow DPPH-H. The observed antioxidant compounds were not separated probably because of their polarity relative to the solvent systems used. Altering the ratio of the solvent can assist in achieving a good separation (Kudumela and Masoko, 2018).

Antioxidant compounds are typically polyphenolic compounds like flavonoids, proanthocyanidins and coumarins. These compounds are generally polar compounds because of the presence of many hydroxyl groups. Various assays have been used to test for antioxidant activity but the most widely used methods are those that involve generation of free radical species, which are neutralized by antioxidant compounds (Trease and Evans, 1989). The potential beneficial effects of antioxidants in protecting against disease have been well established (Auddy *et al*., 2003). In process of ageing as well as neurodegenerative diseases, free radicals play an important role in a complex interplay of different mechanisms (Beal, 1995; Auddy *et al*., 2003). During an infection, antioxidants in plants have the ability to reduce the oxidative stress in plants and prevent a variety of diseases (Ozkan *et al*., 2016). Reactive oxygen species and free radicals are simultaneously degraded to non-reactive forms by enzymatic and non-enzymatic antioxidant defence. Plants produce significant amounts of antioxidants to prevent oxidative stress caused by photons and oxygen, and they represent a potential source of new compounds with antioxidant activity (Auddy *et al*., 2003).

The qualitative and quantitative evaluations did not match. The plants that exhibited the most scavenging potential when quantitative analysis was done, *A. dimidiata* (1,32 ± 0,006 EC_{50} mg/ml), had fewer yellow bands (that indicates the presence of antioxidants) against the purple background on the TLC plates sprayed with DPPH. This may be due to the separation of the antioxidant compounds with the mobile system used. Kudumela and Masoko (2018) mentioned that altering the ratio of the mobile system may have an effect on the observed antioxidant activity.

Many plants that possess antioxidant activity have been known to have numerous therapeutic properties. The reducing power capacity of all the plants increased in a dose-dependent manner. This method is based on the ability of antioxidants in the plants to reduce the ferric complex to its ferrous form (Brand-Williams *et al*., 1995; Kudumela and

Masoko, 2018). *Apodytes dimidiata* plant which had the highest total phenolic, tannin, and flavonoid content also displayed the highest free radical scavenging and ferric reducing capacity. This correspondence may be attributed to the well-known fact that phenolics and flavonoids possess high antioxidant potential as free radical scavengers and metal ion reducers (Guleria *et al*., 2013).

4.5. Conclusion

The study demonstrated that the extracts of the selected plants have antioxidant potential. The extracts of *A. dimidiata* had free radical scavenging and reducing power antioxidant potential, which may be attributed to the highest amount of phenolics and flavonoids observed in the former plant. Therefore, further phytochemical and pharmacological studies of these plants are essential and significant for possible application as natural plant–based drugs in the pharmaceutical industry.

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CHAPTER 5

5. ANTIMYCOBACTERIAL ASSAYS, CYTOTOXIC ASSAY AND SYNERGISTIC EFFECT

5.1. Introduction

The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* underscores the need for continuous development of new and efficient methods to determine the susceptibility of isolates of *Mycobacterium tuberculosis* in the search for novel antimycobacterial agents. Natural products constitute an important source of new drugs, and design and implementation of antimycobacterial susceptibility testing methods are necessary to evaluate the different extracts and compounds (Abuzeid *et al*., 2014). Over 350 natural products, mainly plant species, which have been used in traditional medicine, have been assessed for their antimycobacterial activities (Newton *et al.,* 2000). Various plant species have been studied for antimycobacterial activity (Abuzeid *et al*., 2014), like *Psoralea corylifolia* and *Sanguinaria canadensis* (Newton *et al*., 2000). Abuzeid *et al*. (2014)'s study confirmed a significant inhibitory effect on an avirulent strain of *Mycobacterium tubercluosis* (H37Ra) at the initial screening doses (125 and 6.25µg/ml) on the bark and leaf extracts. These were bark and leaf extracts of *Khaya senegalensis* and the leaf extract of *Rosmarinus officinalis* L. Plant extracts are tested whether they are effective against a certain bacterium by doing various antimicrobial tests. There are many other plants that were studied for their antimycobacterial activity and had a good response (Abuzeid *et al*., 2014).

Another important factor is to evaluate medicinal plants for their safety, quality, toxicity, to check if the appropriate amount of plant material is used and also to verify their efficacy (Shai *et al*., 2008). Traditional medicinal plants are acclaimed to be non-toxic and they are regarded as safe, because they are from nature and have been used for years to treat various diseases (Chen *et al*., 2011; Fennell *et al*., 2004). However, scientific studies on safety and efficacy of some of these medicinal plants have shown that there are many phytochemicals that are found in plants that have cytotoxic, genotoxic and carcinogenic effects (Ahmed *et al*., 2012). Since traditional medicines are used worldwide, it is vital

that these plants are tested for toxicity and efficiency in treating various ailments. During drug development, one of the essential steps is to determine the cytotoxicity of the test substance. This assists in determining whether the developed drug is cell toxic or not, as well as its level of cell toxicity. The test looks at the various cell functions such as the enzyme activity, cell membrane permeability, cell adherence, ATP production, and nucleotide uptake activity. Cytotoxicity assays of the plants studied by Abuzeid *et al*. (2014) revealed that the chloroform fraction of *Khaya senegalensis* bark was non-toxic to human monocyte-derived macrophages and other cell types at the concentrations used and hence, further analysis, including assessment of IC50 and intracellular activity was done with this fraction (Abuzeid *et al*., 2014).

In light of the development of resistance in the infectious diseases (like TB) with existing drugs, one strategy employed in traditional herbal medicine to overcome this phenomenon is the combination of herbal remedies. To this effect, some authors have attempted the combination of antibiotics with plant extract (Dawoud *et al*., 2013) while others have focused on plant extract combinations to achieve a more potent antimicrobial activity (Ncube *et al*., 2012). While many studies have focused on either antimicrobial screening or the phytochemistry, very little is reported on plant –plant combinations, in spite of the traditional use (Smith,1895; Hutchings,1996; Felhaber,1997). Although some studies have been conducted to evaluate medicinal plant interactions from southern African species (Kamatou *et al*., 2006; Van Vuuren, 2008; Suliman *et al*., 2010; Ncube *et al*., 2012; Mabona *et al*., 2013), the use of plant combinations to treat TB has been neglected, although a few studies were done (Mabona *et al*., 2013; Komape *et al*., 2017).

Three plants, namely; *A. dimidiata*, *C. hereroense* and *C. lemon* were selected for further studies based on the results from preliminary screening of the seven plants. The three plants were selected because they exhibited potent effect on the inhibition of the growth of *M. smegmatis.*

5.2. Materials and methods

5.2.1. Preparation of crude extracts

The crude extracts were prepared according to the method of Kotze and Eloff (2002), where plant materials from each species were individually extracted by weighing 1 g each of the finely ground samples and each extracted with 10 ml of a different solvents varying in polarity, namely; n-hexane, dichloromethane, acetone and methanol in polyester centrifuge tubes, respectively. The tubes were vigorously shaken for 10 minutes in a series 25 shaking incubator machine (New Brunswick Scientific Co., Inc) at 100 rpm. After centrifugation at 959 xg for 10 min, the supernatant was decanted into pre-weighed labelled bottles. The extraction process was repeated three times to thoroughly extract the compounds. The solvents were air dried in a fume cupboard at room temperature and quantified.

5.2.2. Test organisms

5.2.2.1. *Mycobacterium smegmatis*

Antimycobacterial activity was tested against *Mycobacterium smegmatis* (ATCC 1441) in the Microbiology laboratory at the University of Limpopo. The organisms were maintained on Middlebrook media (Fluka M7H9) with Middlebrook ADC growth supplement at 37°C. The purity of the cultures was checked by means of the Ziehl-Neelsen stain before use in antimicrobial assays.

5.2.2.2. *Mycobacterium tuberculosis* multidrug resistant isolates (MDR-TB)

A clinical isolate of Multidrug Resistant *Mycobacterium tuberculosis* (MDR-TB) was used. The clinical isolate was obtained from patients admitted to the MDR-TB ward at Tshepong hospital in Klerksdorp, North West Province of South Africa in December 2012. Samples of sputum were submitted to the National Health Laboratory Services (NHLS) in Pretoria for culture in liquid medium and PCR/Line Probe Assay. The isolate was found to be resistant to isoniazid and rifampicin.

5.2.2.3. Rapidly growing mycobacteria

The *Mycobacterium tuberculosis* H37Rv which is routinely used as a reference strain at NHLS was obtained from the American Type Culture Collection (ATCC) number 25177.

5.2.2.4. Maintenance of cultures of pathogenic isolate

Fresh culture was used in the relevant assays. The pathogenic isolate of *Mycobacterium* spp. kept at room temperature on Lowenstein-Jensen (LJ) slants supplemented with glycerol was used within a month. Prior to each assay, culture was revived in liquid medium, Middlebrook 7H9, using MGIT 960 tubes which were incubated at 37 °C in the BACTEC MGIT 960 instrument, in which it was automatically monitored each hour for fluorescence development for 42 days or until a positive signal developed. Bacterial suspensions from MGIT tubes were then subcultured on solid medium LJ slants with glycerol for *M. tuberculosis*. Löwenstein Jensen tubes were then incubated in a walk-in incubator at 37 °C for 4 to 6 weeks. A stained Ziehl Neelsen smear was made from the sediment of the MGIT tube and the slant of LJ medium. The reference culture of *M. tuberculosis* H37Rv (ATCC 25177) was used as a positive control for growth of microorganisms.

5.2.2.5. Minimum Inhibitory Concentration (MIC) determination using *M. smegmatis*

Minimum Inhibitory Concentration values were determined using the serial micro-dilution method described by Eloff (1998). The MIC is described as the lowest concentration of the compounds inhibiting the growth of the microorganisms. Dried extracts were re-dissolved in acetone to a concentration of 10 mg/ml. The test was carried out in triplicates. The plant extracts were combined 50 µl each and the final mixture was 100 µl. The mixture of combined plant extracts was then serially diluted 50% with water in a 96-well microtitre plates. Bacterial cultures were sub-cultured and transferred into fresh Middlebrook 7H9 broth and 100 µl of the culture was transferred into each well. Acetone blanks were included and rifampicin was used as a positive control. The microtitre plate was incubated at 37 °C for 24 h. After incubation, 20 µl ofp- iodonitrotetrazolium violet (Sigma) (INT) dissolved in water was added to each of microplates wells as an indicator

for growth. The plates were covered and further incubated for 30 min at 37 °C and 100% relative humidity for colour development. Purple-red c olour indicates microbial growth and clear wells indicate inhibition of microbial growth by extracts.

5.2.2.6. Minimum inhibitory concentration (MIC) determination using pathogenic strain

The MIC values were determined using the serial microplate method developed by Eloff (1998), slightly modified for mycobacteria by (McGaw *et al*., 2008). Mycobacterial suspensions were prepared from a pure culture of fresh colonies from solid medium and suspended in Middlebrook 7H9 (M7H9) liquid medium supplemented with 10% OADC. These colonies were transferred into a sterile screw capped tube containing 3 ml of M7H9 broth and homogenized by placing the tube on a Vortex mixer for 5 min. After the larger particles had settled, the mycobacterial suspension was adjusted to McFarland no.1 turbidity standard by adding more broth (Lall and Meyer, 1999). The assay was performed using sterile 96-well microplates with round bottoms. The sample to be tested was prepared at a concentration of 10 mg/ml prior to serial dilution. One hundred µl of M7H9 broth was added to all the wells from column 1 to 12 and then 100 µl of the sample to be tested were added to the relevant wells in the first row. A two-fold serial dilution was carried out leaving 100 µl of different concentrations of diluted tested samples in each well starting with a concentration of 2.5 mg/ml in the first wells. Then 100 µl of the relevant bacterial suspension were added to the relevant wells. Each test was triplicated (3 wells). Tested samples also included acetone, pure broth as negative control and reference drug isoniazid including rifampicin and streptomycin as positive controls starting with a concentration of 100 µg/ml. The microplates were covered and sealed in plastic bags, placed in humid chambers to minimize the evaporation of the culture medium and incubated at 37 °C for a period of 7 to 15 days. At the end of incubation, a volume of 40 µl of 0.2 mg/ml of iodonitrotetrazolium chloride (INT) was added to each well, plates were incubated for 30 min or longer at 37 °C and the development of colour observed. A coloured red purple formazan or pink colour indicated the reduction of INT by metabolizing organisms whereas a yellow colour or decrease in colour indicated the

inhibition of bacterial growth (Eloff, 1998). If the colour development was not strong enough for slow growing organisms, plates were incubated much longer and monitored.

5.2.2.7. Bio-autographic assays

Bio-autography was carried out on TLC plates according to Beque and Kline (1972) to detect the main bioactive compounds within the crude extracts. TLC plates were loaded with 10 µl of 10 mg/ml density of the solution of each extract as described under phytochemical analysis (section 3.2.3). The plates were developed in EMW, CEF, and BEA solvent systems. Bioautograms were left to fan-dry for three days to completely evaporate the mobile phases and each bioautogram was sprayed with *Mycobacterium smegmatis* and then incubated at 37°C for 24 hours under humid conditions. After incubation, the bioautograms were sprayed with visualization stain (iodonitrotetrazolium salt), and incubated further at 37°C for 24 hours in sealed plastic containers to allow the pink colour to develop. The appearances of clear zones/white spots on the bioautograms were considered as areas of growth inhibition.

5.2.3. Cytotoxic assay against Vero monkey kidney cells

The tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) and modified by McGaw *et al*. (2007) was used to investigate cytotoxicity of four crude extracts of *A. dimidiata*, sub fractions (i.e. butanol and hexane sub fractions) from solvent- solvent fractionation of *A. dimidiata*. The samples were tested for cytotoxicity against Vero monkey kidney cells. The cells were maintained in Minimal Essential Medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell suspensions were prepared from confluent monolayer cultures and plated at 0.5×10^3 cells per well (200 µl) into each well of a 96-well microtitre plate, except for columns 1 and 12 which contained MEM only. Plates were incubated overnight at 37 ºC in a 5% CO_2 incubator for the cells to attach to the plate. Stock solutions of the isolated compounds (20 mg/ml) were prepared by dissolving in DMSO. A range of dilutions of each isolated compound was prepared in growth medium. Berberine chloride (Sigma) was used as a positive control. The viable cell growth after 120 hours incubation with isolated compounds (200 µl in each well) was

determined. After incubation, the wells were rinsed with 150 µl phosphate buffered saline (PBS) and fresh medium (200 µl) was placed in each well. Then, 30 µl of MTT (Sigma) dissolved in PBS (5 mg/ml stock solution) was added to each well and the plates were incubated at 37 ºC for 4 h. The medium was removed and the purple MTT formazan crystals dissolved in DMSO (50 µl per well). The absorbance was measured on a Versamax microplate reader at 570 nm wavelength. In the cytotoxicity assay, each concentration of each compound was tested in quadruplicate, and the assay in its entirety was repeated three times. Berberine chloride was used as a positive control.

5.2.4. Determination of the synergistic effect

The synergistic effect of the crude extract was done using microplate assay developed by Eloff (1998) with modifications. Hundred microliters (100 µl) of sterile distilled water was added into all the wells of the 96 well microtitre plates. Methanol, acetone, hexane and dichloromethane extracts for *A. dimidiata*, *C. hereroense* and *C. lemon* leaves were re-dissolved in acetone to a concentration of 10 mg/ml, following which 50 µl of each crude extract combination was added into each of the first well of 96 well plate microtitre plates and serially diluted up to 50%.

All the wells were filled with 100 µl of standardized broth culture organisms and incubated at 37°C for 24 hrs. After incubation, 40 µl of 0.2 mg/ml of *p*-iodonitro-tetrazolium violet (INT) (Sigma®), was added to all the wells to determine the presence of bacterial growth in the plates. After adding INT, the plates were further incubated at 37°C for 45 min to allow colour change (where there was active bacterial growth, INT was reduced from a colourless to pink-red, indicating growth). The MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth after 24 hours. Each combination of the extracts was tested in triplicates and the results were recorded as the mean from two triplicates of independent experiments. The MIC of the extracts were compared with reference antibiotic rifampicin (as a positive control).

The Fractional Inhibitory Concentration (FIC) was calculated for each combination. Antimicrobial analysis was carried out by determining the Fractional Concentration Index (ΣFIC) as defined by Van Vuuren and Viljoen (2011) using the following equation;

Equation 5.1

FIC (i) = MIC (a) in combination with (b)/ MIC (a) independently

FIC (ii) = MIC (b) in combination with (a)/MIC (b) independently

FIC (i) and FIC (ii) in the equations represent the different plants in combination, while MIC (a) and MIC (b) represent the individual plant extracts. The sum of the FIC, known as the FIC index was thus calculated as ΣFIC= FIC (i) + FIC (ii), and was used to determine the interaction between the two plants. This may be classified as either synergistic (<0.50), additive (>0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00) (Suliman *et al*., 2010; Van Vuuren and Viljoen, 2011). Table 5.6 below shows the calculated fractional inhibitory concentration of the combined crude extracts of the three selected plants.

5.3. Results

5.3.1. Minimum Inhibitory Concentrations (MIC)

5.3.1.1. Minimum Inhibitory Concentrations (MIC) against *M. smegmatis*

To observe the sensitivity of *M. smegmatis* to the plant extracts, MIC values were determined for all the extracts. Minimum inhibitory concentration is the lowest concentration of the test sample expressed in mg/ml that leads to the inhibition of growth. There were substantial differences between the MICs for the different extracts (Table 5.1a).

E. camaldulensis had the lowest average MIC value of 0.45 mg/ml followed by *A. dimidiata* (0.66 mg/ml). The acetone extract of *E. camaldulensis* and *C. lemon* showed lowest MIC value of 0.08 mg/ml and that for the dichloromethane extract of *Z. capense*. *A. dimidiata* had the second lowest MIC value of 0.16 mg/ml for the methanol extract *C.*

heroroense had an MIC value of 0.31 mg/ml with the dichloromethane and methanol extracts (Table 5.1a).

Table 5.1a: Minimum Inhibitory Concentration (mg/ml) values of the extracts of the selected plants against *M. smegmatis*.

	Hex	**DCM**	**MeOH**	**ACN**	**Average**
E. camaldulensis	1.25	0.31	0.16	0.08	0.45
C. lemon	1.25	0.08	1.25	0.63	0.80
C. heroroense	1.25	0.31	0.31	1.25	0.78
A.afra	0.62	1.25	1.25	0.47	0.90
Z. capense	1.25	0.63	0.63	1.25	0.94
A.dimidiata	1.25	0.63	0.16	0.63	0.67
L. javanica	0.62	1.25	0.47	1.25	0.90
Average	1.07	0.64	0.60	0.80	

Hex= hexane, DCM= dichloromethane, MeOH= methanol and ACN= acetone

Total activity is calculated by dividing the mass of 1g of plant extract by the MIC value of the plant extract. The total activity of the extracts was calculated to determine which plant or extract had the most activity.

E. camaldulensis showed the highest average total activity of 329 indicating that it has the most bioactive compounds as compared to the other extracts; followed by *A. dimidiata* (97), *C. heroroense* (76) and *C. lemon* (48). *A. afra* showed the least average total activity as compared to the other plant extracts. The extract with the highest total activity was methanol (average total activity of 141, followed by dichloromethane extracts (average total activity of 103 for all the plants (Table 5.1b).

Table 5.1b: Total activity ($ml^{-1)}$ of the crude extracts of the seven selected plants.

	Hex	DCM	MeOH	ACN	Average
E. camaldulensis	10	313	432	563	329
C. lemon	14	150	21	5	48
C. heroroense	8	97	171	27	76
A.afra	45	8	29	13	24
Z. capense	23	46	43	5	29
A.dimidiata	11	86	281	8	97
L. javanica	13	23	10	7	13
Average	18	103	141	90	

hexane (Hex), dichloromethane (DCM), methanol (MeOH) and acetone (ACN)

5.3.1.2. Minimum Inhibitory Concentrations (MIC) against pathogenic strains

The plant with the highest average total activity was *A. dimidiata*. Based on the bioautography results and MIC results, the hexane and butanol fractions of *A. dimidiata* were further tested for activity against Tuberculosis (TB) strain and Multi-Drug Resistance (MDR) -field strain of TB. The table below (Table 5.2) shows the activity of the butanol and hexane fractions against the MDR field strain and the H37Rv TB strain. The extracts showed good activity against the MDR field and H37Rv strains with MIC values of 0.47 mg/ml and 0.31 mg/ml respectively. The extracts show more potent activity against MDR field strains than the positive controls (isoniazid, rifampicin, streptomycin).

Table 5.2: Minimum inhibitory concentration (mg/ml), values of the butanol and hexane sub-fractions of *A. dimidiata* (AD) against MDR field strain and H37Rv strain of *M. tuberculosis*

Fraction/control	MDR field strain	H37Rv (ATCC)
AD Butanol	0.47	0.31
AD Hexane	0.47	0.31
Isoniazid (INH)	>2.5	<0.02
Rifampicin	>2.5	<0.08
Streptomycin	>2.5	<0.02
Acetone	>2.5	>2.5

5.3.2. Bioautography

Bioautography indicates the number of antimicrobial compounds in the different extracts. The tree solvent systems used were: CEF, BEA and EMW. Figures 5.1a to 5.1c below show the bioautographic plates.

A. dimidiata had prominent antimycobacterial activity with the dichloromethane, acetone and methanol extracts followed by *E. camaldulensis* showing activity with the same extracts with TLC plates developed in BEA (Figure 5.1a). Chromatograms developed in CEF and EMW had little or no activity for all the plants (Figures 5.1b and 5.1c). This may be due to the polarity of the mobile systems used.

The R_f values of the plant extracts developed in BEA which had activity for *A. dimidiata* were the same for dichloromethane, methanol and acetone (R_f value of 0.9). *E. camaldulensis* also had activity with the same extracts as *A. dimidiate*, that is, dichloromethane, methanol and acetone extract but with different R_f value of 0.4 for all the three extracts (i.e. dichloromethane, methanol and acetone). For the bioautograms developed in CEF, the two plants, *A. dimidiata* and *E. camaldulensis* showed activity against *M. smegmatis*. For *A. dimidiata* all the four extracts had activity with hexane extract having a compound with activity of R_f value of 0.7 and dichloromethane, methanol and acetone extracts fraction having the same R_f value of 0.1. All the other plants had no visible activity on the bioautograms.

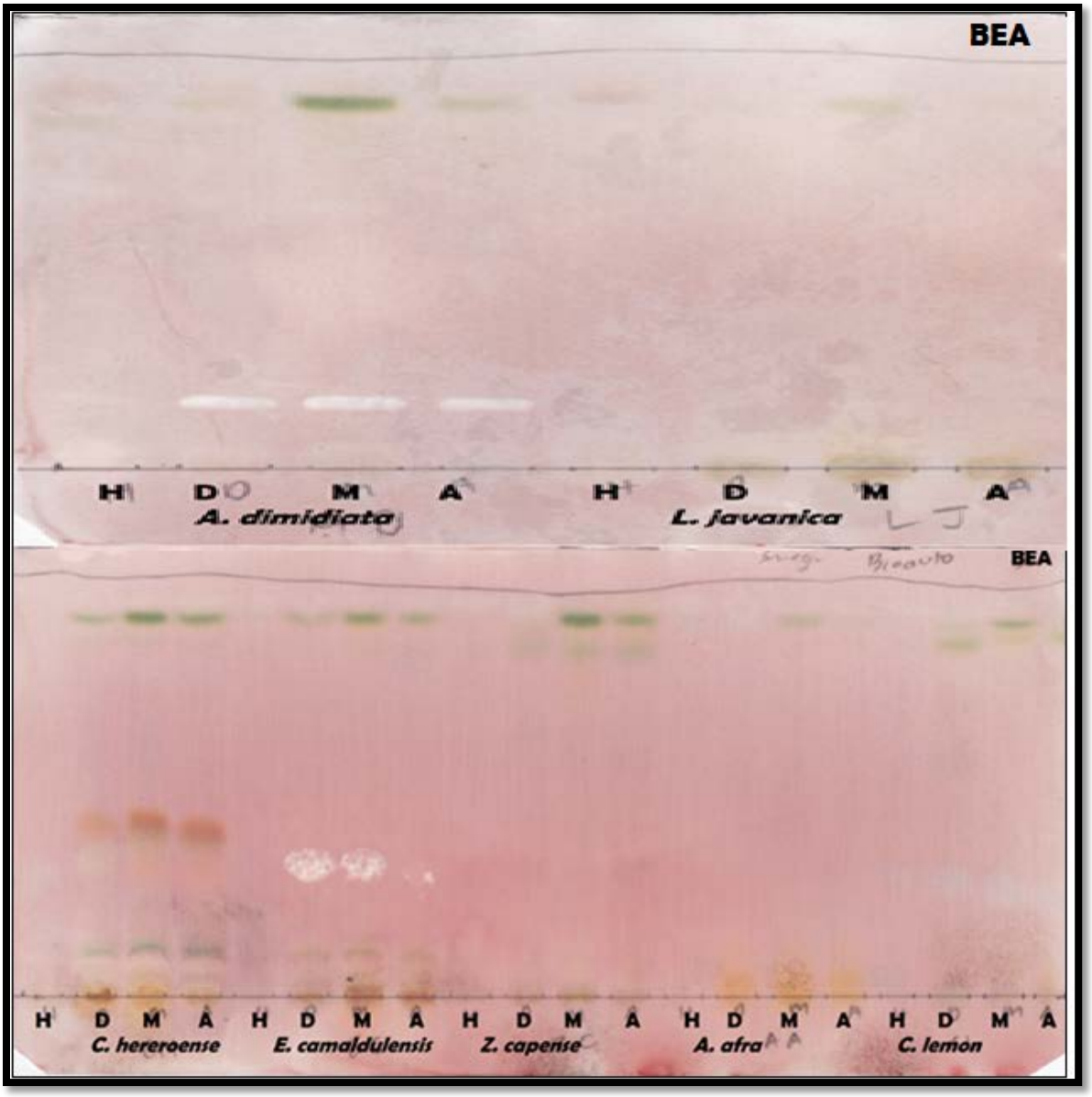

Figure 5.1a: Chromatogram of *A. dimidiata* and *L. javanica* (above), *C. heroroense, E. camaldulensis, Z. capense, A. afra* and *C. lemon* (below) respectively; developed in BEA solvent system and sprayed with *M. smegmatis* and then p-iodonitrotetrazolium (INT) salt to show compounds extracted with hexane (H), dichloromethane (D), methanol (M) and acetone (A) in lanes from left to right for each plant.

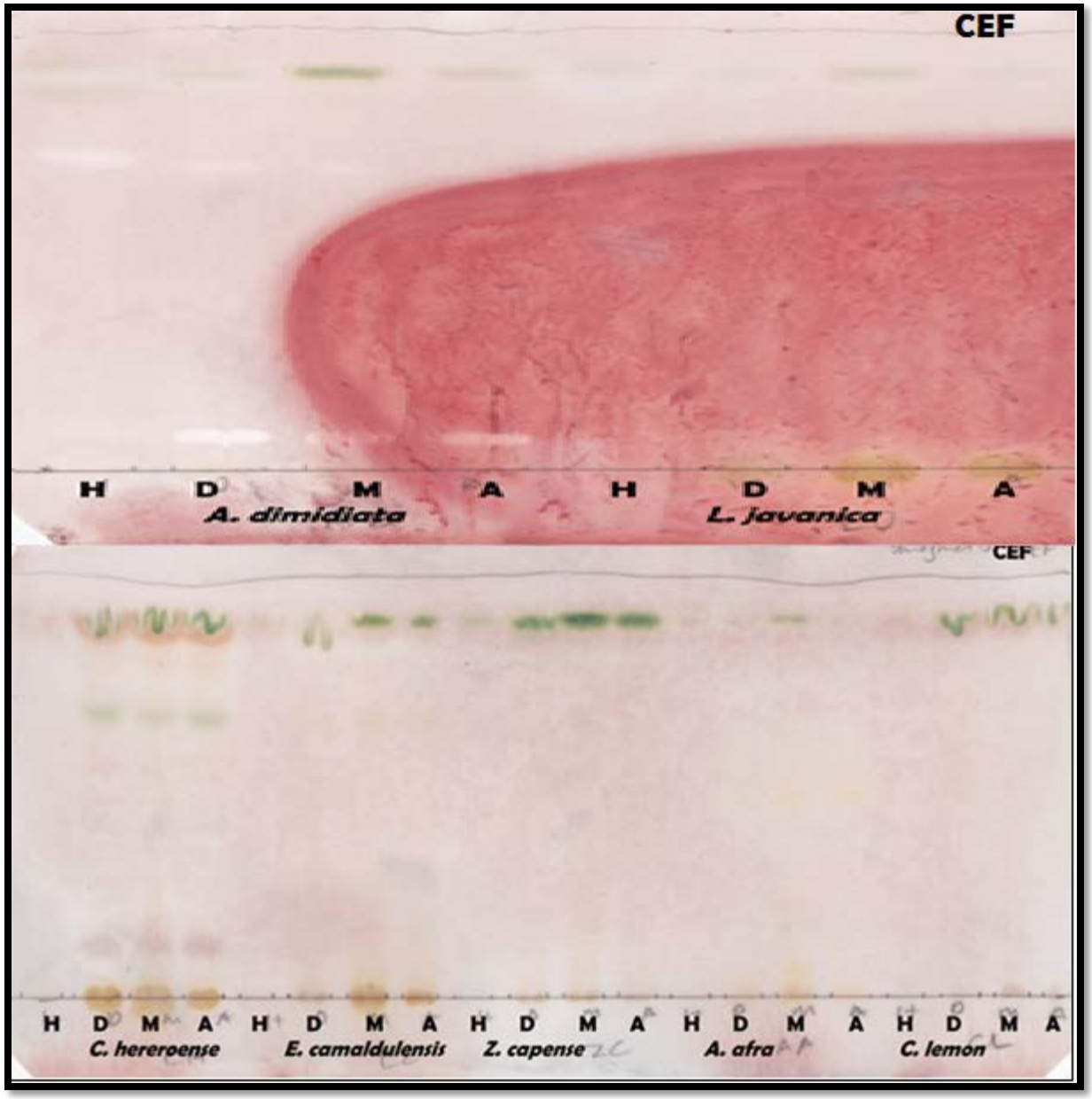

Figure 5.1b: Chromatogram of *A. dimidiata* and *L. javanica* (above), *C. heroroense, E. camaldulensis, Z. capense, A. afra* and *C. lemon* (below), respectively; developed in CEF solvent system and sprayed with *M. smegmatis* and then p-iodonitrotetrazolium (INT) salt to show compounds extracted with hexane (H), dichloromethane (D), methanol (M) and acetone (A) in lanes from left to right for each plant.

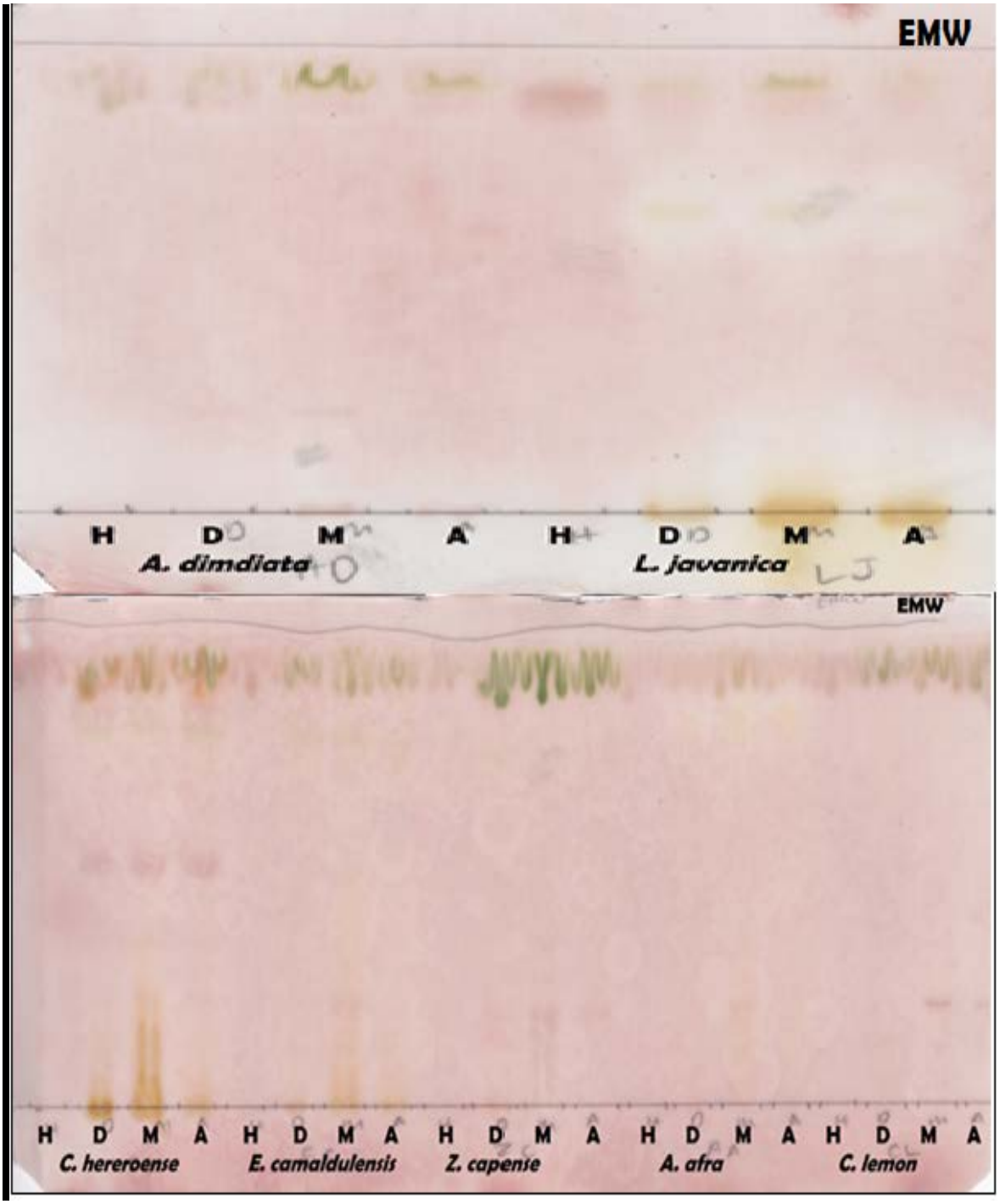

Figure 5.1c: Chromatogram of *A. dimidiata* and *L. javanica* (above), *C. heroroense, E. camaldulensis, Z. capense, A. afra* and *C. lemon* (below) respectively; developed in EMW solvent system and sprayed with *M. smegmatis* and then p-iodonitrotetrazolium (INT) salt to show compounds extracted with hexane (H), dichloromethane (D), methanol (M) and acetone (A) in lanes from left to right for each plant.

5.3.3. Cytotoxicity evaluation

Cytotoxicity effect of the crude leaf extracts (i.e. hexane, dichloromethane, acetone and methanol), subfractions (hexane and butanol) from solvent-solvent fractionation that showed activity against *M. tuberculosis* of *A. dimidiata* were investigated against Vero kidney cell line. All the crude extracts were toxic against Vero kidney cells. The subfraction from solvent-solvent fractionation, hexane had the highest LC_{50} value (lowest toxicity) of 0.14 mg/ml while the other subfraction (butanol) showed moderate toxicity with LC_{50} value of 0.04 mg/ml (table 5.3). Selectivity index values of the samples were calculated by dividing LC_{50} (mg/ml) values by MIC (mg/ml) values (table 5.3). The higher the SI value, the safer the extract. The average SI value for all the crude extracts

Table 5.3: The LC_{50} of the crude extracts, subfractions from solvent-solvent fractionation of *A. dimidiata* leaves and their selective index.

Sample	LC_{50} (mg/ml)	MIC (mg/ml)	Selective Index (SI)
Butanol subfraction	0.040	0.16	0.25
Hexane subfraction	0.14	0.04	3.50
Hexane	0.024	1.25	0.019
Dichloromethane	0.022	0.63	0.035
Acetone	0.036	0.63	0.057
Methanol	0.040	0.16	0.25

5.3.4. Test for synergistic effect

Same and different extracts of the selected plants were combined to test for their synergistic effect against *M. smegmatis* (Table 5.4). The combination of different extracts of the plants viz the hexane and acetone, and the dichloromethane and methanol extracts of *C. heroroense* and *A. dimidiata* showed excellent activity with MIC value of 0.04 mg/ml, followed by the acetone and methanol, and hexane and methanol extracts of the same plants (MIC 0.08 mg/ml). A similar MIC value (0.08 mg/ml) was also obtained for the combination of the dichloromethane and acetone extracts of *C. lemon* and *C. heroroense*. With same extracts of the different plants, potent activity was shown by the combination of the acetone-acetone and

dichloromethane-dichloromethane extracts of *C. heroroense* and *A. dimidiata* (MIC 0.08 mg/ml). Moderate activity which ranged from MIC 0.1.2 to 0.63 mg/ml was shown with the different and same combination of extracts of the selected plants while the lowest activity was obtained at MIC 1.25 mg/ml for the hexane and dichloromethane extracts combination of *C. heroroense* and *A. dimidiata*.

Table 5.4: Crude plant extracts combinations of CL, CH and AD against *M. smegmatis* to show the synergistic effects (MIC recorded in mg/ml).

Extracts	CL+CH	CH+AD	AD+CL
H+H	0.31	0.12	0.18
D+D	0.63	0.08	0.63
M+M	0.16	0.12	0.12
A+A	0.16	0.08	0.12
H+D	0.16	1.25	0.63
D+A	0.08	0.16	0.31
A+M	0.16	0.08	0.31
M+H	0.16	0.08	0.63
H+A	0.16	0.04	0.63
D+M	0.16	0.04	0.16

Rifampicin = 0.13 mg/mL

H= Hexane; D= Dichloromethane; M= Methanol; A= Acetone

CH= *C. heroroense*, CL = *C. lemon*, AD= *A. dimidiata*

From Table 5.5, *C. lemon and C. hereroense* (CL-CH) combination of dichloromethane and dichloromethane, showed an antagonistic effect (∑FIC= 9.91) and an additive effect with the methanol + methanol (∑FIC= 0.65), hexane + dichloromethane (∑FIC= 0.65), acetone+ methanol (∑FIC= 0.77), and an indifferent effect with the dichloromethane +

acetone (∑FIC= 1.06) and acetone+ methanol (∑FIC= 0.77) combinations. The rest of the combination for CL-CH showed synergistic effect. For *C. hereroense* and *A. dimdidata* (CH-AD) combination, there was no antagonistic effect observed. However, an additive effect with dichloromethane+ acetone (∑FIC= 0.77) and acetone+ methanol (∑FIC= 0.56) was observed. All the other combinations for CH-AD exhibited synergistic effect and an indifferent effect with methanol+ methanol (∑FIC= 1.14) and hexane+ dichloromethane (∑FIC= 2.98) combination. For *A. dimidiata* and *C. lemon* (AD-CL) combination, there was an antagonistic effect with the dichloromethane+ dichloromethane (∑FIC=8.90) and methanol+ hexane (∑FIC= 4.44). An Indifferent was observed with hexane + acetone (∑FIC= 1.50) and an additive effect with the methanol + methanol (∑FIC= 0.85), dichloromethane+ acetone (∑FIC= 0.98) and the acetone+ methanol (∑FIC= 0.74) combination. The other three combinations showed synergistic effect.

Table 5.5: Fractional Inhibitory Concentrations (FIC) of the combined crude extracts of *C. lemon* (CL), *C. heroroense* (CH) and *A. dimidiata* (AD) against *M. smegmatis* to show the synergistic, additive, indifferent or antagonistic effects of the 3 plants.

	CL+CH			**CH+AD**			**AD+CL**		
Extracts	**FIC (i)**	**FIC(ii)**	**∑FIC**	**FIC (i)**	**FIC (ii)**	**∑FIC**	**FIC (i)**	**FIC (ii)**	**∑FIC**
H+H	0.25	0.25	0.50	0.096	0.096	0.19	0.14	0.14	0.28
D+D	7.88	2.03	9.91	0.26	0.13	0.39	1.00	7.90	8.90
M+M	0.13	0.52	0.65	0.39	0.75	1.14	0.75	0.096	0.85
A+A	0.25	0.13	0.38	0.064	0.13	0.19	0.19	0.19	0.38
H+D	0.13	0.52	0.65	1.00	1.98	2.98	0.50	7.88	8.38
D+A	1.00	0.064	1.06	0.52	0.25	0.77	0.49	0.49	0.98
A+M	0.25	0.52	0.77	0.064	0.50	0.56	0.49	0.25	0.74
M+H	0.13	0.13	0.26	0.26	0.064	0.32	3.94	0.50	4.44
H+A	0.13	0.13	0.26	0.032	0.063	0.095	0.50	1.00	1.50
D+M	2.00	0.52	2.52	0.13	0.25	0.38	0.25	0.13	0.38

H= hexane, D= dichloromethane, A= acetone and M= methanol

5.4. Discussion

For all the extracts, the average MIC value of *E. camaldulensis* was the lowest followed by *A. dimidiata*. The results were similar to the findings from Masoko and Nxumalo (2013) where they found that acetone extracts of *A. afra* had the MIC value of 0.39 mg/mL against *M. smegmatis* while that for C. *hereroense* and *L. javanica* had MIC value of 0.47 mg/ml.

The dichloromethane, methanol and acetone extracts of *A. dimidiata* and *E. camaldulensis* demonstrated an inhibition of growth of *M. smegmatis* on the BEA bioautogram with R_f values of 0.4 and 0.8, respectively. Overall, the bioautography results obtained in this study differ slightly with those found by Masoko and Nxumalo (2013) because they found that the acetone extracts of *A. dimidiata* and *C. hereroense,* and dichloromethane extract of *L. javanica* demonstrated an inhibition of the growth of *M. smegmatis* on BEA bioautogram. *C. hereroense* displayed one band that inhibited growth in the EMW bioautogram. A total of 4 bands were observed that inhibited the growth of *M. smegmatis*. The values were 0.12, 0.63, and 0.87 for *A. dimidiata, C. hereroense,* and *L. javanica* respectively in the BEA chromatogram. The value R_f for *C. hereroense* in EMW chromatogram was 0.73. The poor antibacterial activity of Z. *capense* shown in this study agrees with the study conducted by McGaw *et al.,* (2000) and Adamu *et al*., (2014) although their studies were both on Gram positive and Gram negative bacteria.

Some of the extracts that showed activity on bioautography also exhibited the lowest MIC value. However, there were some extracts that exhibited a good activity in the microplate assay which did not show any clear bands on bioautograms. This may be because some of the active compounds were volatile and evaporated during the drying period of the TLC chromatograms prior to bioautography. Also, it may be due to residues of formic acid or ammonia on the TLC plates following evaporation. Another possible reason may be the biological activity synergism between different compounds in the extracts (Mahlo *et al*., 2016). All of the crude extracts of *A. dimidiata,* were toxic to the Vero monkey kidney cell line. But then, as the isolation of the bioactive compound was done, the sub-fraction of the acetone crude extract (i.e. the hexane), was found to be non-toxic. This may be due to the fact that as isolation was done, toxicity of the crude extract was reduced.

Plant-plant combinations had an overall synergistic effect and very few antagonistic effects. Overall, the *A. dimidiata* and *C. hereroense* combinations of extracts exhibited a good synergistic effect. Hence, the two plants were noted for isolation of bioactive compounds.

5.5. Conclusion

Plant therapy has many potentially significant advantages associated with the synergistic interactions, like increased efficiency and reduction of undesirable effects. The plant combinations used in this study had potent synergistic effect, additive effect and very few indifferent and antagonistic effects. All the extracts of plants tested exhibited some degree of antimycobacteria activity, which was enhanced when extracts of the same plant or combination of plants in the study were employed. Sub-fraction in some cases can reduce activity of extracts especially where the desired effect is synergy dependent. The hexane and butanol sub-fractions of *A. dimidiata* exhibited potent anti-mycobacteria activity more than the positive control against the MDR-field strain.

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CHAPTER 6

6. ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS

6.1. Introduction

Plants are capable of synthesizing bioactive compounds that are beneficial to humankind. There are challenges in isolating and purifying these compounds because there are usually, large numbers of phytochemicals present in plants. Since plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, separating them still remains a big challenge for the process of identification and characterization of bioactive compounds (Sasidharan *et al*., 2011).

There are a number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, that can be used to obtain pure compounds. The pure compounds are used for the determination of structure and biological activity. Other than that, non-chromatographic techniques such as immunoassay, which use Monoclonal Antibodies (MAbs), phytochemical screening assay, Fourier-Transform Infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds (Sasidharan *et al*., 2011).

The leaves of *A. dimidiata* were selected based on the screening results obtained showing their antimicrobial activity against *M. smegmatis* and *M. tuberculosis* strains. The first towards isolation of bioactive compounds was solvent-solvent fractionation. Fractionation makes it possible to separate more than two components in a mixture in a single run. It is carried out by suspending each extract in water separately and partitioning with different organic solvents, such as hexane, chloroform, ethyl acetate, and methanol in order of increasing polarity by using separating funnel. Solvent-solvent fractionation is a separation and purifying technique that involves using solvents varying in polarity to separate compounds based on their polarity. This method simplifies separation in that before one does column chromatography as a method of separating bioactive compounds already they have an idea of the polarity of the target bioactive compound. Thin layer chromatography, column chromatography and preparative TLC techniques, on the other hand, are also used for further separation of phytochemical compounds. For fingerprinting

of the plant extracts, TLC is used as a first step because it is a fast and a cheap method that displays a number of compounds present in the plant extracts and the distance travelled by the compounds on the plates. It can also indicate the type of secondary metabolites that are present in the plant extracts by the colour displayed on the TLC plate, for example, purple colour indicates the presence of terpenoids in the plant extract. Another important role of TLC in the isolation and purification process of bioactive compounds is to confirm purity of isolated compounds (Sasidharan *et al*., 2011).

For further purification of the desired compounds, column chromatography can be employed using one or two solvents in various ratios. Preparative TLC can also be used for further purification, based on the number of compounds present. The final step involves the use of TLC to confirm purity of the isolated compounds (Visht and Chaturvedi, 2012). Bioactive compounds obtained from the separation methods discussed above needs to be characterised. Different varieties of spectroscopic techniques like UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy can identify the purified compounds (Popova *et al*. 2009). Nuclear Magnetic Resonance spectroscopy is one of the instruments that can be used in characterising the bioactive compounds. The NMR gives an idea of the positions of the carbon (e.g. using DEPT) and hydrogen atoms present in the compound isolated. This is because many isolated compounds are organic compounds. In cases where not enough information is obtained from NMR, the other instrument that can also be used is Mass Spectrophotometer (MS). This instrument also simplifies things because it uses the mass of the isolated compound to determine the compound. It has a library of isolated compound which can either be references of the isolated compound or be the match of the isolated compound. There are many other instruments, such as HPLC, that can also be used in both isolation and determining the structure of the isolated compound. In this study, NMR was used in structure elucidation. There are different types of NMR, for example, ^{13}C, ^{1}H and ^{31}P Nuclear Magnetic Resonance (^{13}C, ^{1}H and ^{31}P NMR) (Altemimi *et al*., 2017).

Samdumu (2006) mentioned that in determining the structure of an isolated compound, there are two key points for solving complex problems are that no single spectrum will entirely solve a problem and that all information must be used simultaneously and also

that it is preferable to get key structural facts from each spectrum and then assemble the pieces.

6.2. Materials and methods

6.2.1. Bulk extraction

Ground leaf powder of *A. dimidiata* (166.5 g) was exhaustively extracted using acetone (3 litres). The mixtures of acetone and leave extracts were shaken for 60 minutes at 150 rpm on the Thermo Scientific MaxQ 3000 shaker. Then the supernatant was filtered from the mixture using Whatman No. 1 filter paper on a funnel into 1000 ml Erlenmeyer flask. The extract was concentrated using the Bűchi rotary evaporator and further dried under the fan in a pre- weighed beaker. Extraction with acetone was repeated several times until the supernatant was clear.

6.2.2. Solvent- solvent fractionation

The solvent-solvent fractionation method was used to obtain sub-fractions of the plant material of *A. dimidiata*, *C. hereroense* and *C. lemon*. The solvent-solvent fractionation method was used to separate the fractions of different extracts. The procedure as outlined by the USA National Cancer Institute (Suffiness and Douros, 1982) was followed with minor modifications. The extracts were fractionated using solvents of different polarities as indicated in the flow chart below (figure 6.1).

From the solvent-solvent fraction, the n-butanol sub-fraction (38.7 g) had better antimycobacterial activity as compared to the other sub-fractions and it was further subjected to column chromatography. Although there were other subfractions from *A. dimidiata* that had antimycobacterial activity, n-butanol sub-fraction had the best antimycobacterial activity.

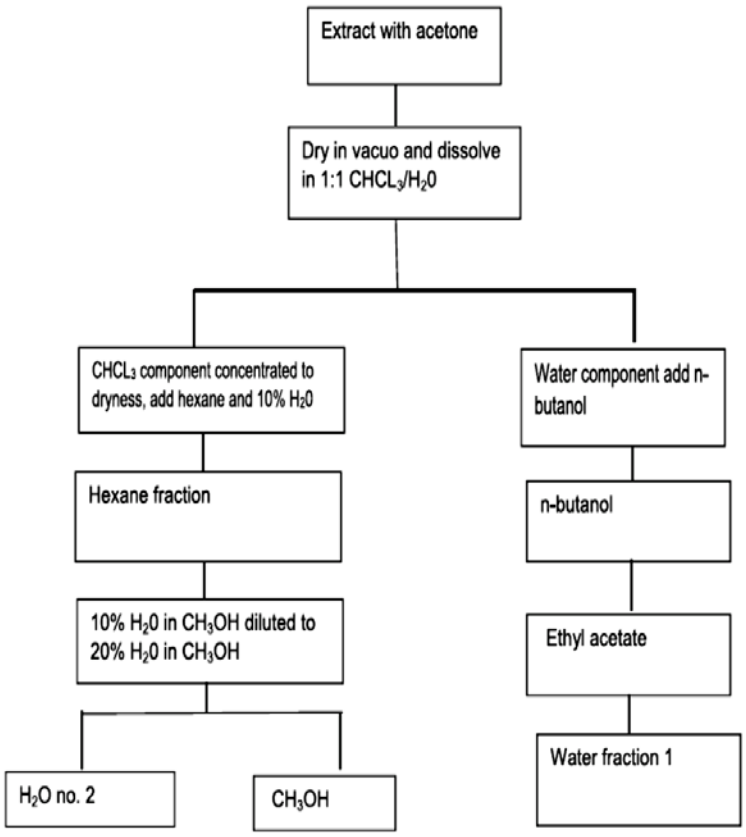

Figure 6.1: A Schematic representation of solvent-solvent fractionation method

6.2.3. Column chromatography

6.2.3.1. Open column chromatography

The n-butanol (38.7 g) sub-fraction of *A. dimidiata* from solvent-solvent fractionation was further subjected to column chromatography for further separation of bioactive compounds. Column chromatography (40 x 60 cm) with silica gel 60 (particle size 0.063-0.200 mm) (Fluka), was packed using 100% hexane. The sample (38.7 g) was mixed with small amount of silica gel and thereafter subjected to column chromatography. Constituents of the n-butanol subfraction were eluted through an open column chromatography using solvents in table 6.1 at different ratios. Each of the different fractions (column collection) were assayed to indicate which of the collected fractions has

the compound of interest using bioautography. The fractions with the compound of interest was pooled together and re-eluted until a pure compound was obtained.

Table 6.1: Various percentages ratios of solvents used for eluting open column chromatography.

Solvent	Percentage ratios (%)
Hexane	100
Hexane: Ethyl acetate	90:10
	80:20
	70:30
	60:40
	50:50
	40:60
	30:70
	20:80
	10:90
Ethyl acetate	100
Ethyl acetate: Methanol	90:20
	80:20
	60:40
	40:60
Methanol	100

6.2.3.2. Small column chromatography

Fractions from the open column (section 6.2.3.1) with bioactive compounds were combined for further isolation using a small open column. The small open column chromatography (37 x3 cm) was packed with silica gel 60. The column was eluted with 70% hexane in ethyl acetone (table 6.2). The eluents were collected in small test tubes

and placed under a stream of air to concentrate the fractions for further analysis using TLC.

Table 6.2: Various percentages ratios of solvents used for eluting small open column chromatography.

Solvent	Percentage ratios (%)
Hexane	100
Hexane: Acetone	90:10
	80:20
	70:30
	60:40
	50:50
	40:60
	30:70
	20:80
	10:90
Acetone	100

6.2.4. Preparative thin layer chromatography

Bioactive fractions from the small column chromatography were further separated on TLC silica gel glass plates (Merck Silica gel 60 F_{254}) using 70% chloroform in ethyl acetate as eluent. The plates were visualized under Ultraviolet light (254 and 360 nm) to locate targeted bands on the TLC plates. A small portion on the side of the plate was sprayed with vanillin-sulphuric acid reagent while the rest of the plate was covered with aluminium foil. Visualized bands on the side were used as reference for scraping off active bands from developed TLC plates. The active compounds were scrapped off from the plate into acetone. The active compounds in the solvent were filtered and separated compounds from silica gel. To remove silica gel completely, filtrates were passed through cotton wool packed in a glass Pasteur pipette. Purity of isolated compounds was confirmed by

spraying TLC plates with vanillin- sulphuric acid and heated at 110^{o}C until colour developed.

6.2.5. Determination of TLC fingerprint of the isolated fractions

The phytochemical constituents of the fractions were analysed by Thin Layer Chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F254) according to the method of Kotze and Eloff (2002) as explained in chapter 3 (section 3.2.3).

6.2.6. Bioautography assay

Bioautography was carried out on TLC plates according to Beque and Kline (1972) to detect the main bioactive compounds within the fractions from the columns as described in chapter 5 (section 5.2.2.7).

6.2.7. Antioxidant assay

Qualitative antioxidant activity of the solvent-solvent subfractions was determined on the basis of the scavenging activity of stable 1, 1- diphenyl- 2-picrylhydrazyl (DPPH) (Sigma®). The chromatograms were prepared as in section 3.2.4.1. The chromatograms were sprayed with 0.2% DPPH to visualize any potential antioxidant compounds within the separated plant extracts (Deby and Margotteaux, 1970).

6.2.8: Structure elucidation

The structure of the isolated compound was determined by using Nuclear Magnetic Resonance **(**NMR) in collaboration with Prof. L Mdee (Department of Pharmacy, University of Limpopo, Turfloop, South Africa). The structure was also confirmed by comparison of the spectral data with that from literature. The NMR spectra were recorded on a Bruker 600 Avance II NMR at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C and Distortionless Enhancement through Polarisation Transfer (DEPT) NMR. Comprehensive structure determination involved ^{1}H-NMR and ^{13}C-NMR spectroscopic analyses, including Correlated Spectroscopy (COSY), Hetero nuclear Multiple Quantum Coherence (HMQC). Samples were re-dissolved in acetone solvent.

6.3. Results

6.3.1. Solvent- solvent fractionation

The selected plants were solvent-solvent fractionated into the butanol, hexane, ethyl acetate, methanol and water fractions no.1 and 2. The fractions of each of the selected plants were evaluated for the presence of antioxidant and antimycobacterial constituents on TLC. A representative bioautogram depicting fingerprint profile, antioxidant and antimycobacterial activity of *A. dimidiata* is shown in Figure 6.2. All the fractions for the three plants namely: *A. dimidiata*, *C. heroroense* and *C. lemon* contained constituents with different colours indicating the presence of a variety of compounds. With *A. dimidiata* sub-fractions, constituents on plates eluted in BEA were best resolved with antioxidant constituents being of high polarity. Similar compounds with anti-mycobacterial activity were present in all the sub-fractions although the concentration in the hexane extract was low (Figure 6.2). Sub-fractions of *C. heroroense* showed presence of compounds with antioxidant activity that were best resolved in the CEF eluent system and that for anti-mycobacterial activity contained in the ethyl acetate fraction in the EMW eluent system (Figure 6.3). *C. lemon* on the other hand, did not show antimycobacterial activity with the sub-fractions (Figure 6.4).

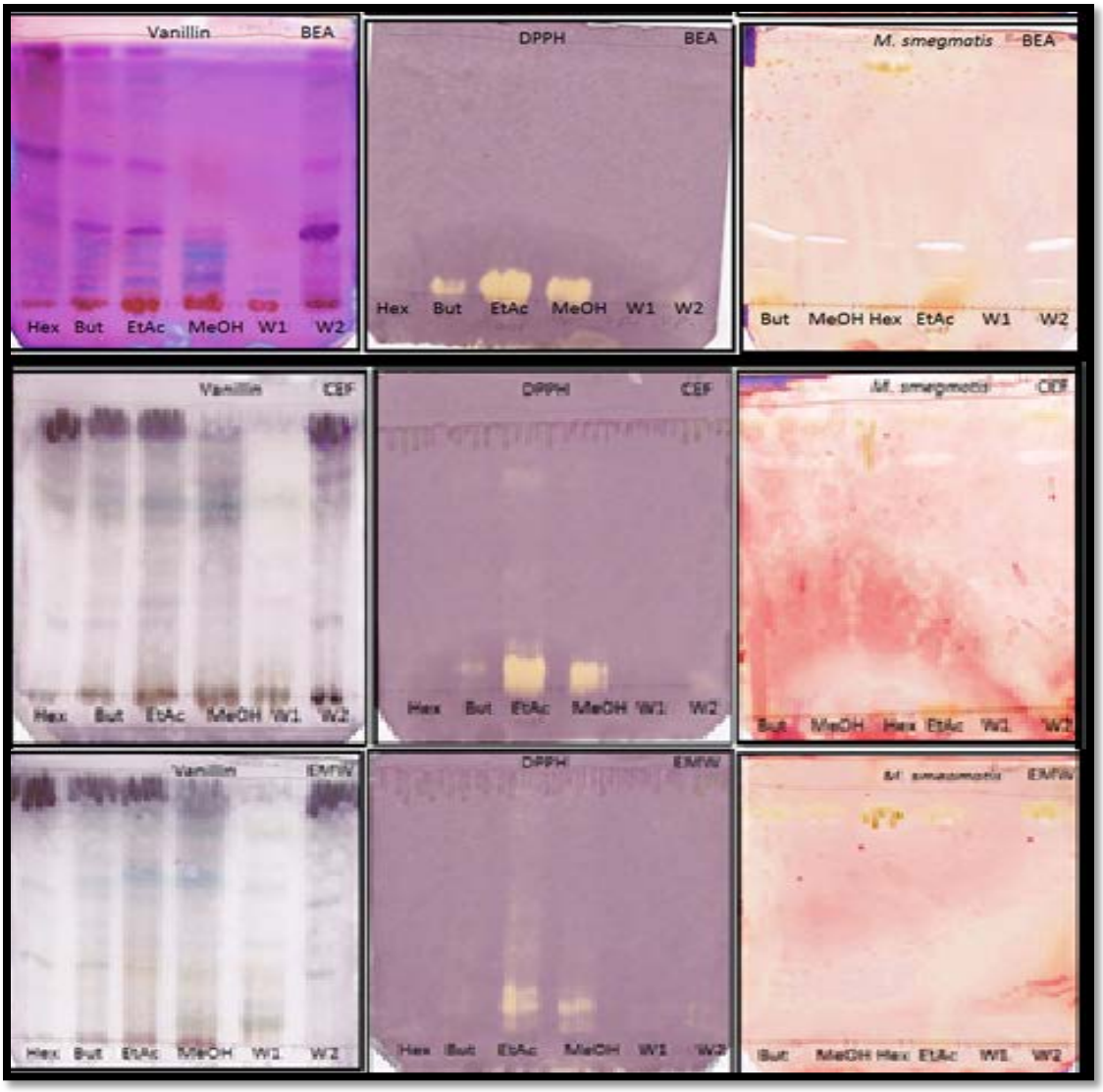

Figure 6.2: Chromatograms of solvent-solvent fractions [Hex (hexane), But (butanol), EtAc (ethylacetate), MeOH (methanol), W1 (water fraction no. 1) and W2 (water fraction no. 2)] of *A. dimidiata* developed in BEA (top row), CEF (middle row) and EMW (bottom row) and sprayed with vanillin sulphuric acid (left), 1, 2-diphenylpicryl hydrazyl (middle) and *M. smegmatis* (right).

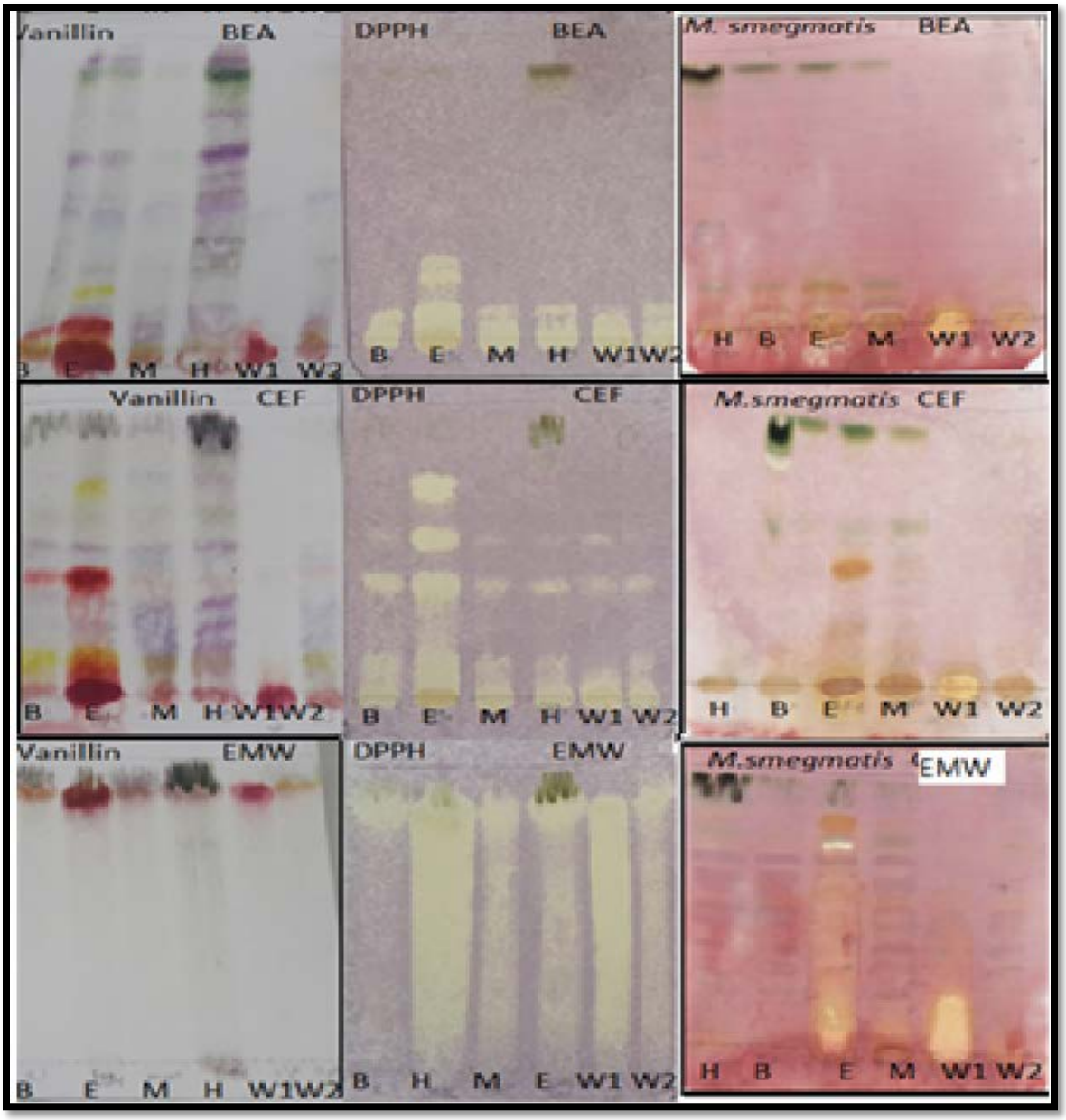

Figure 6.3: Chromatograms of solvent-solvent fractions [H (hexane), B (butanol), E (ethylacetate), M (methanol), W1 (water fraction no. 1) and W2 (water fraction no. 2)] of *C. heroroense* developed in BEA (top row), CEF (middle row) and EMW (bottom row) and sprayed with vanillin (left), 1, 2-diphenylpicryl hydrazyl (middle) and *M. smegmatis* (right).

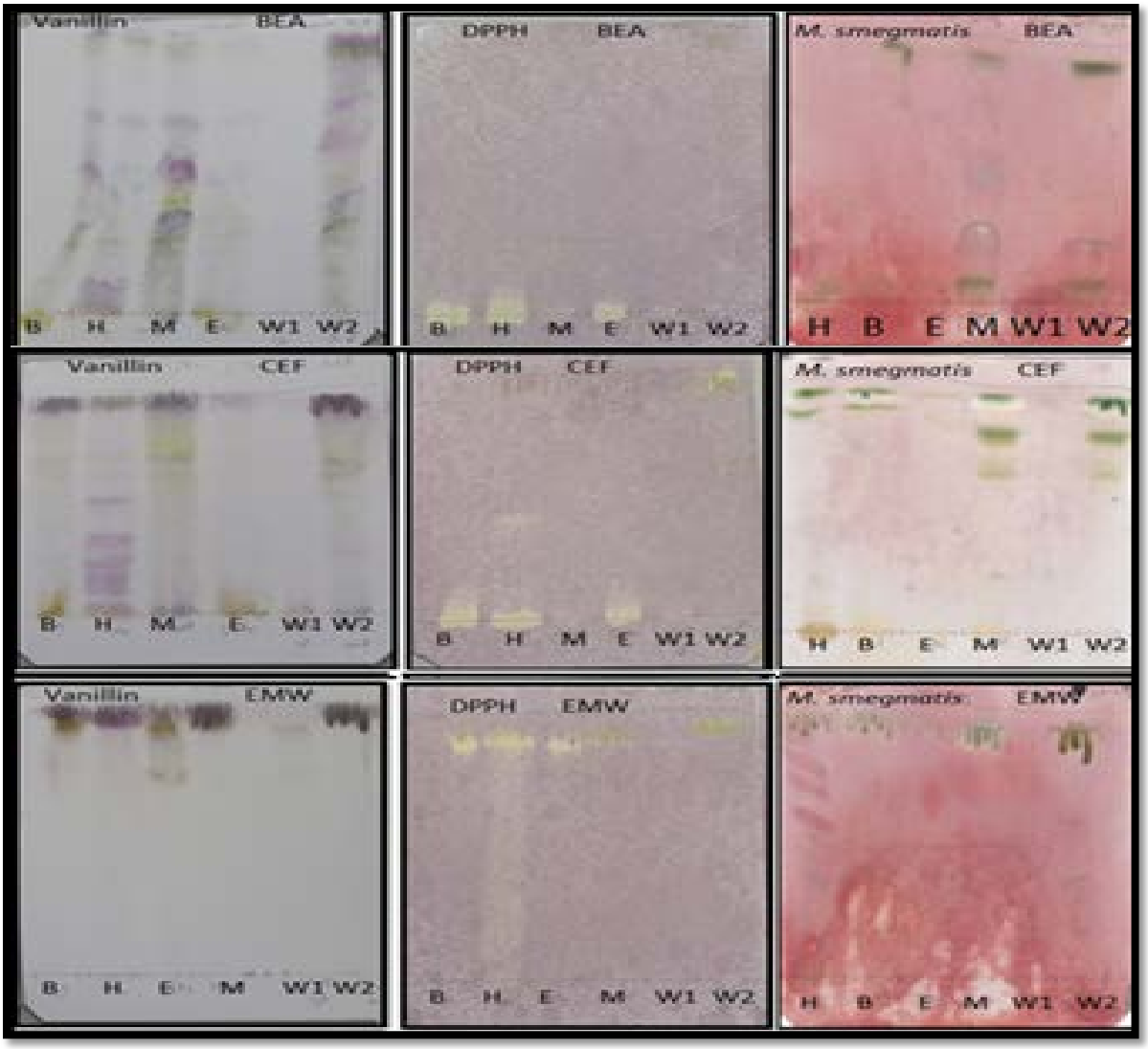

Figure 6.4: Chromatograms of solvent-solvent fractions [H (hexane), B (butanol), E (ethylacetate), M (methanol), W1 (water fraction no. 1) and W2 (water fraction no. 2)] of *C. lemon* developed in BEA (top row), CEF (middle row) and EMW (bottom row) and sprayed with vanillin (left), 1, 2-diphenylpicryl hydrazyl (middle) and *M. smegmatis* (right).

6.3.1.1. Minimum Inhibitory Concentration of sub-fractions against *M. smegmatis*

Table 6.3 represents MIC values obtained for sub-fractions of the selected plants tested against *M. smegmatis*. In general, potent to moderate activity was shown for sub-fractions between the ranges of MIC 0.04 to 0.63 mg/ml. The butanol fraction of selected plants showed moderate activity with average MIC value of 0.16 mg/ml followed by the hexane

and methanol sub-fraction (0.19 mg/ml). The hexane sub-fraction of *A. dimidiata* showed the most potent activity (MIC 0.04 mg/ml) as well as moderated activity with the butanol, ethyl acetate, methanol fractions (MIC 0.16 mg/ml).

Table 6.3: Minimum Inhibitory Concentration (MIC) in mg/ml of the solvent-solvent fractions of *C. lemon* (CL), *C. heroroense* (CH) and *A. dimidiata* (AD) against *M. smegmatis*

Plant species	But	Hex	EtAc	MeOH	H_2O no 1	H_2O no 2
C.lemon	0.16	0.24	0.63	0.16	0.31	0.31
C.heroroense	0.16	0.31	0.31	0.24	0.16	0.63
A.dimidiata	0.16	0.04	0.16	0.16	1.25	1.25
Average	0.16	0.19	0.37	0.19	0.57	0.73

But= butanol, Hex= hexane, EtAc= ethylacetate, MeOH= methanol, H_2O no. 1= water fraction no. 1 and H_2O no. 2= water fraction no. 2

6.3.2. Column chromatography

6.3.2.1. Determination of suitable mobile phase

Thin Layer Chromatography (TLC) profiling and bioautography of the n-butanol sub-fraction of *A. dimidiata* was done using different solvent combinations of varying polarity as an initial step to validate the presence of bioactive compound and also as a guidance of the polarity of the target bioactive compound (figure 6.2).

Since the compound of interest was clearly visible in the BEA fraction, after solvent-solvent fractionation, the n-butanol fraction of *A. dimidiata* was subjected to TLC. Chromatograms were developed in BEA and sprayed with *M. smegmatis* and vanillin sulphuric acid (figure 6.2). The R_f values of the compounds in the bioactive n-butanol sub fraction that showed clear zones when TLC developed in BEA was sprayed with *M. smegmatis* was found to be 0.29 and 0.19, respectively.

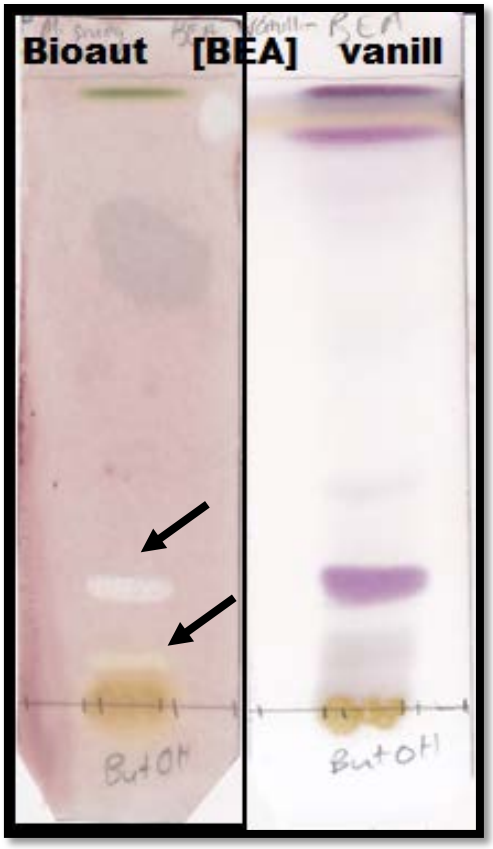

Figure 6.5: Chromatograms of butanol fraction from solvent-solvent fractionation of *A. dimidiata* developed in BEA, sprayed with *M. smegmatis* and vanillin sulphuric acid, respectively.

Before the n-butanol fraction of *A. dimidiata* was subjected to open column chromatography, mobile systems that separate the compound of interest from the other compounds was determined. From the profiling in figure 6.3, it can be clearly seen that 70:30 ratio of hexane and ethyl acetate separated the best. This eluent system was thus chosen for silica gel column chromatography. Hexane and ethyl acetate at the ratio 70:30 was the best eluent as compared to the other ratios. Hence, it was chosen for further analysis.

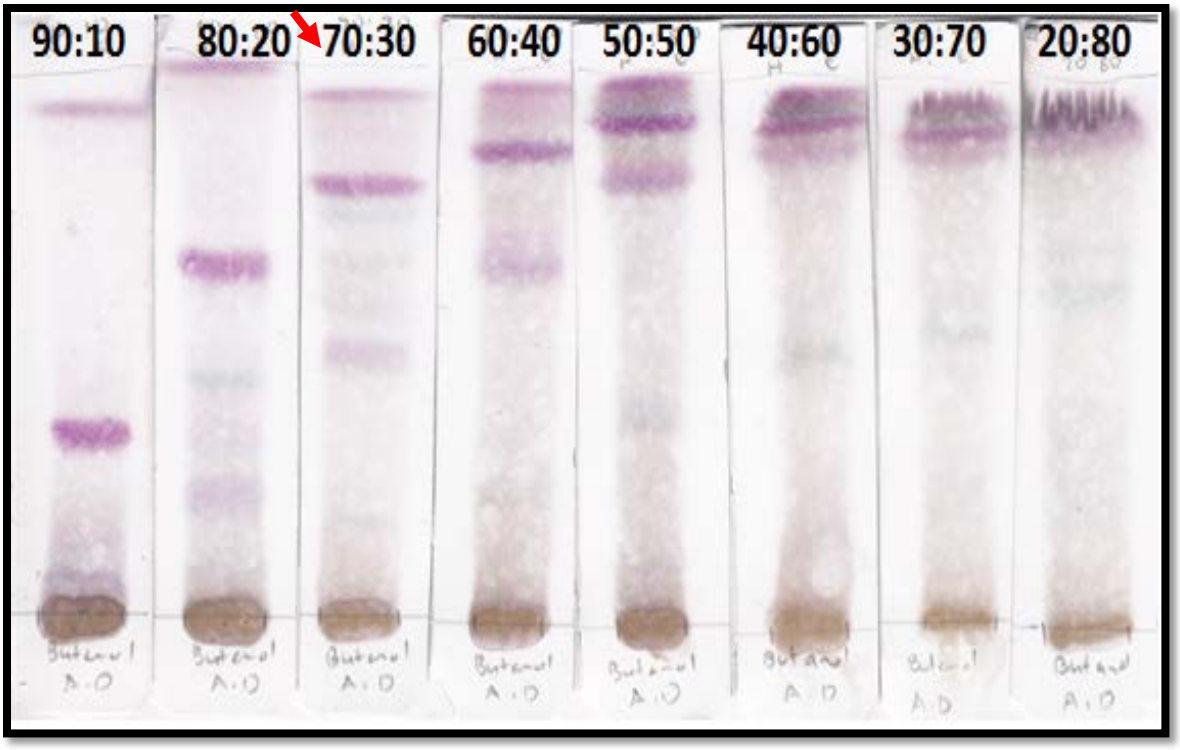

Figure 6.6: Chromatograms of n-butanol fraction from solvent-solvent fractionation developed in different ratios (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80) of hexane and ethyl acetate and sprayed with vanillin sulphuric acid.

Thin Layer Chromatography (TLC) plates developed in BEA solvent system had good resolution of phytochemical compounds present in *A. dimidiata* leaves, followed by CEF and the least were in EMW. Therefore, the fractions from column chromatography were developed in BEA as the main mobile system. The TLC plates were sprayed with vanillin sulphuric acid. Different colours appeared on the TLC plates confirmed the bioactive compounds of *A. dimidiata* column fractions reacted with vanillin-sulphuric acids (figure 6.4).

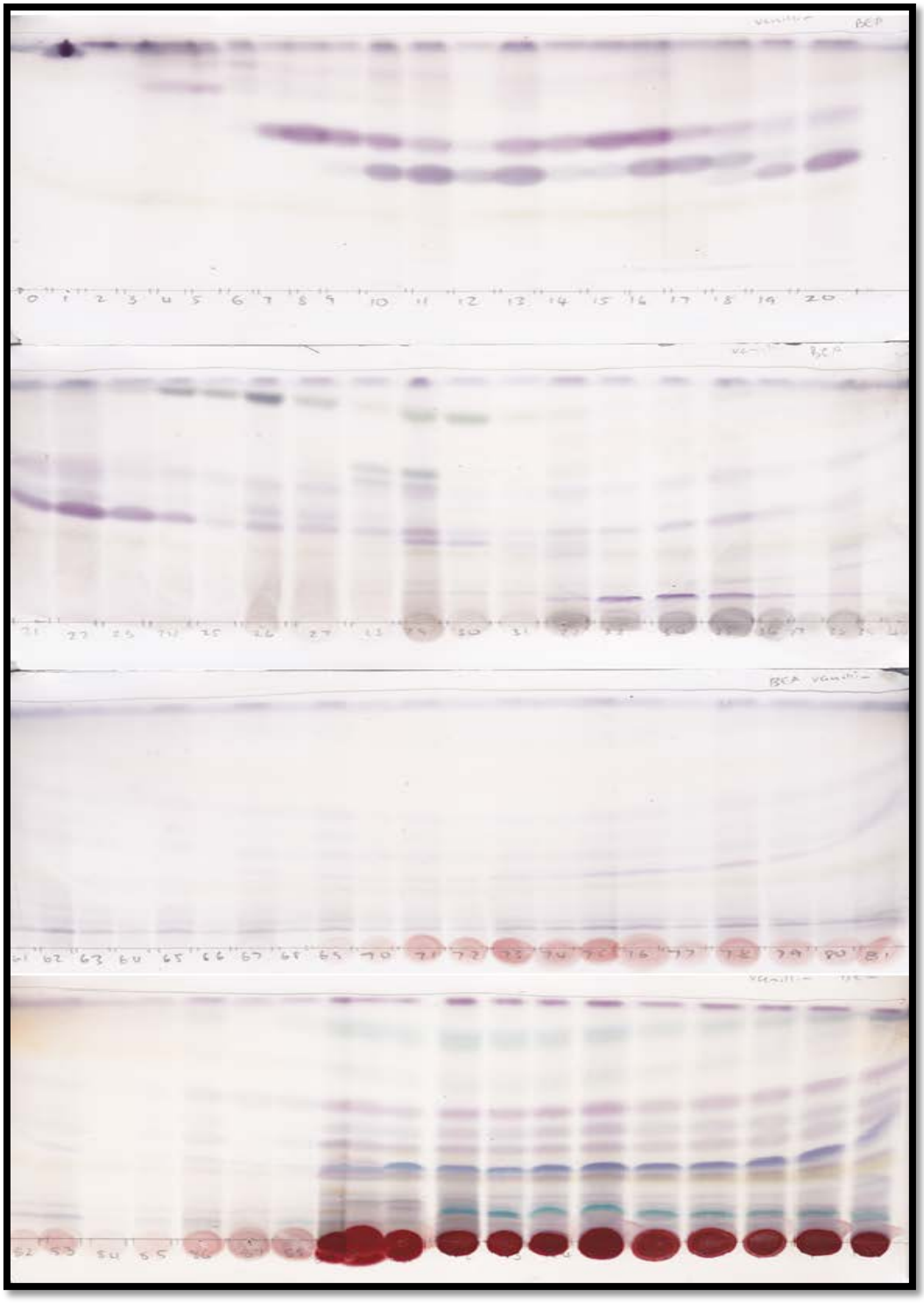

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Figure 6.7: Chromatograms of n-butanol fraction from open column combined and sprayed with vanillin sulphuric acid developed in BEA mobile sysytem.

The column fractions were also developed in the best mobile phase H: A (70:30) to have a clearer view of the compounds and thereafter sprayed with vanillin sulphuric acid (figure 6.5).

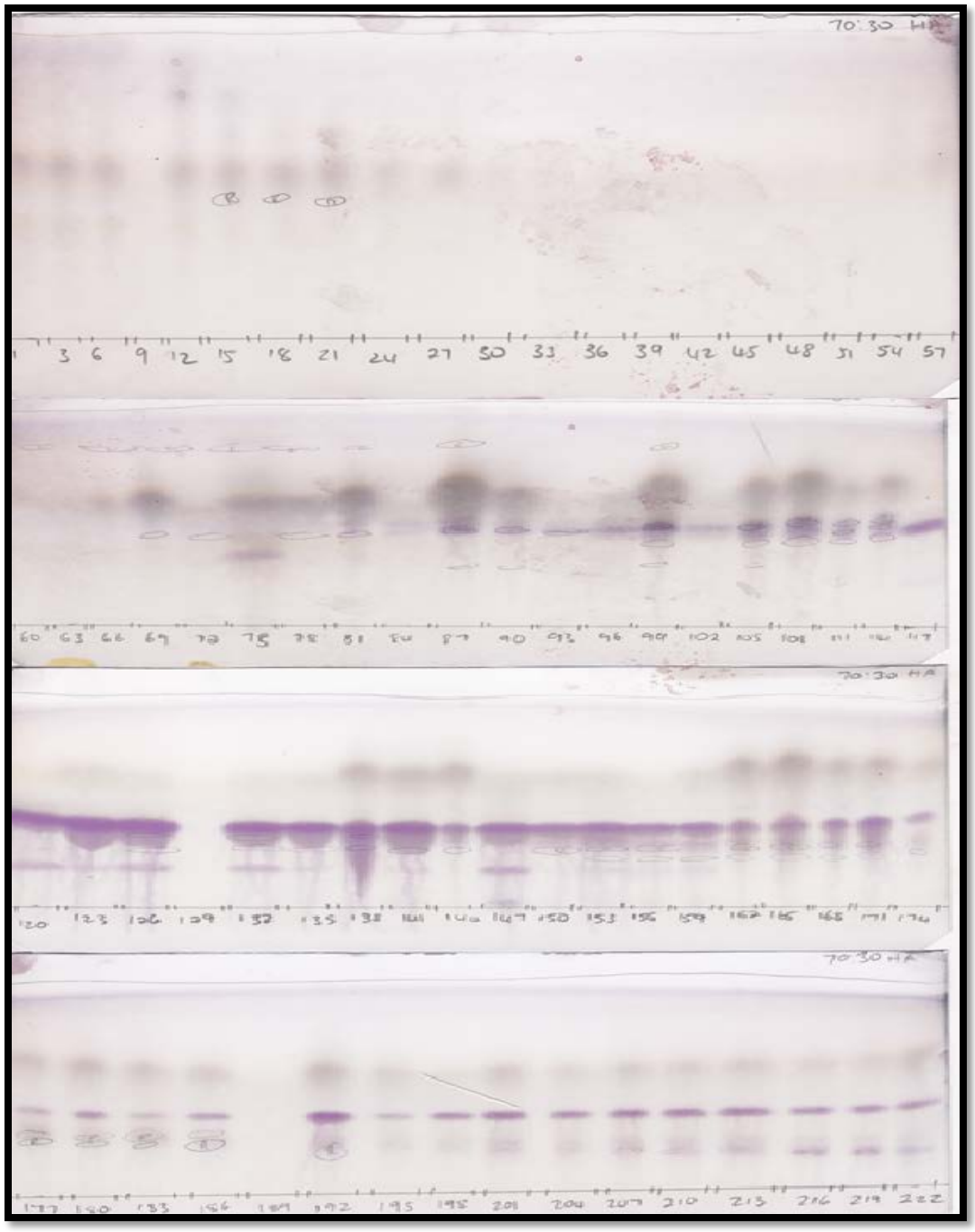

Figure 6.8: Chromatograms of n-butanol fraction from open column combined and sprayed with vanillin sulphuric acid developed in H: A (70:30) mobile system.

From the open column chromatography, fractions were obtained and combined. Fractions that possessed antimycobacterial compounds were pooled together (figure 6.6). Fractions 7 to 12 and fractions 27 to 39 were combined so they can be subjected to another column to ultimately get pure compounds. The total mass of fractions 7 to 12 was 5.1 g and for fractions 27 to 39 was 6.7 g.

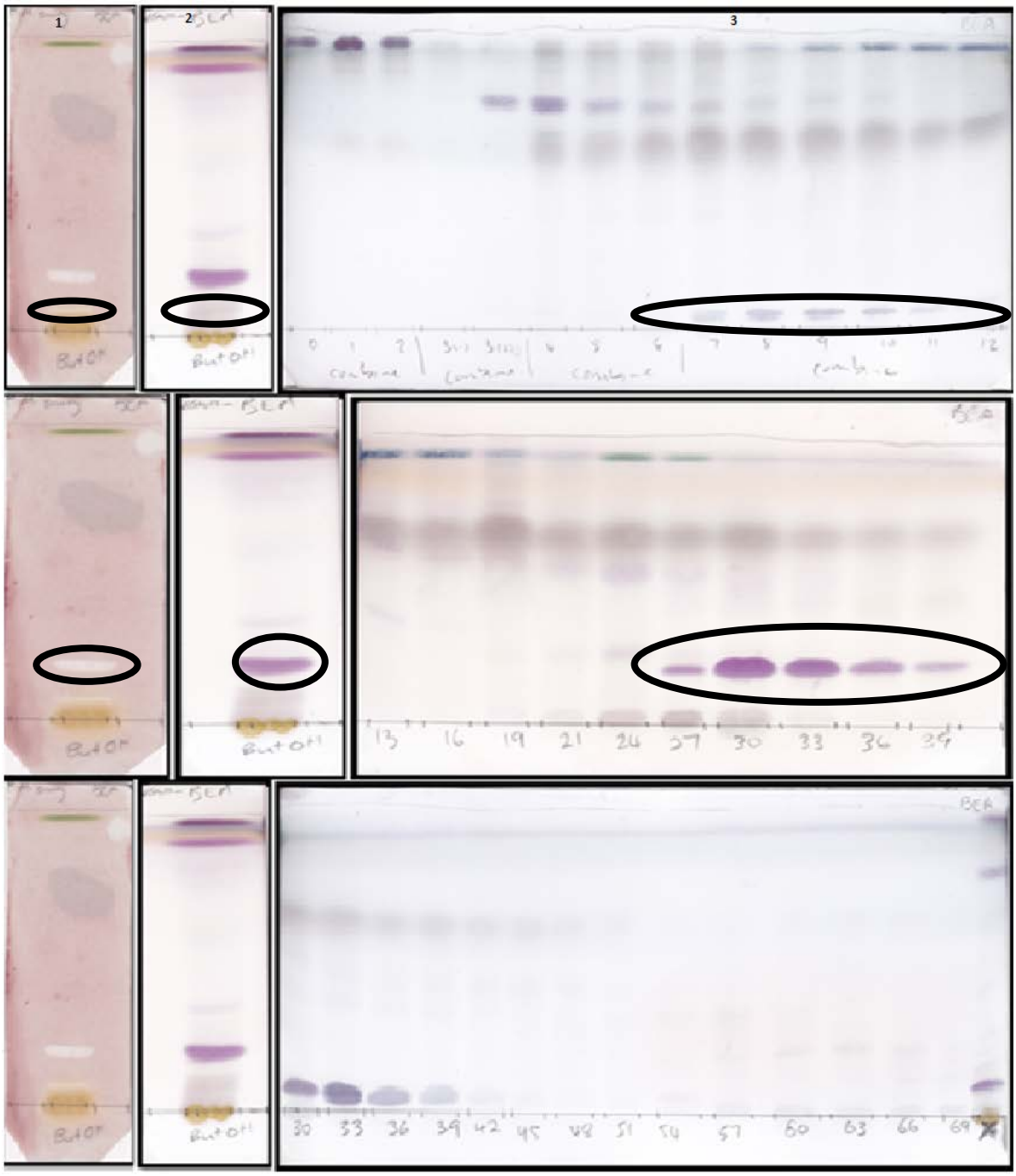

Figure 6.9: Chromatograms of n-butanol fraction from open column combined and sprayed with *M. smegmatis* (plates in row 1) and vanillin sulphuric acid (plates in rows 2 and 3) developed in BEA mobile sysytem. To determine the best eluent system, for the combined fractions from open column chromatography, Hexane: Acetone combinations of different ratios were used as a mobile system for the fractions. Hexane: Acetone (70:30) was found to be the best mobile phase to separate the target compounds (figure 6.7). The combined fractions (7- 12 and 27- 39) were further subjected to open column chromatography.

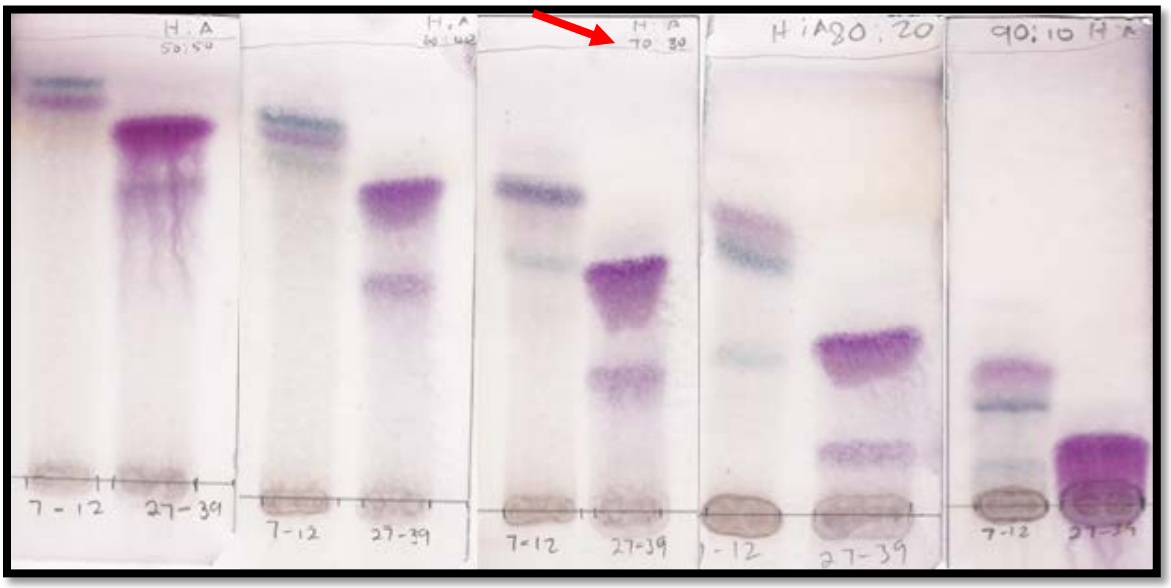

Figure 6.10: Chromatograms of n-butanol fraction from solvent-solvent fractionation developed in different ratios (50:50, 60:40, 70:30, 80:20, 90:10) of hexane and acetone and sprayed with vanillin sulphuric acid.

The R_f value of the desired compounds with bioactivity was 0.5 and 0.4 respectively when the TLC plate was developed in BEA. The same compounds with bioactivity were well separated in H: A (70:30) solvent system (figure 6.7).

The flow diagram below (figure 6.8) shows the masses and the fractions obtained from extraction of the acetone leaves material of *A. dimidiata,* the masses obtained, of fraction and pure compounds. The overview flow chart of isolation of antimycobacterial

compounds from acetone extracts respectively is shown in Figure 6.8. Pure compounds were further characterised using Nuclear Magnetic Resonance spectroscopy for structure elucidation.

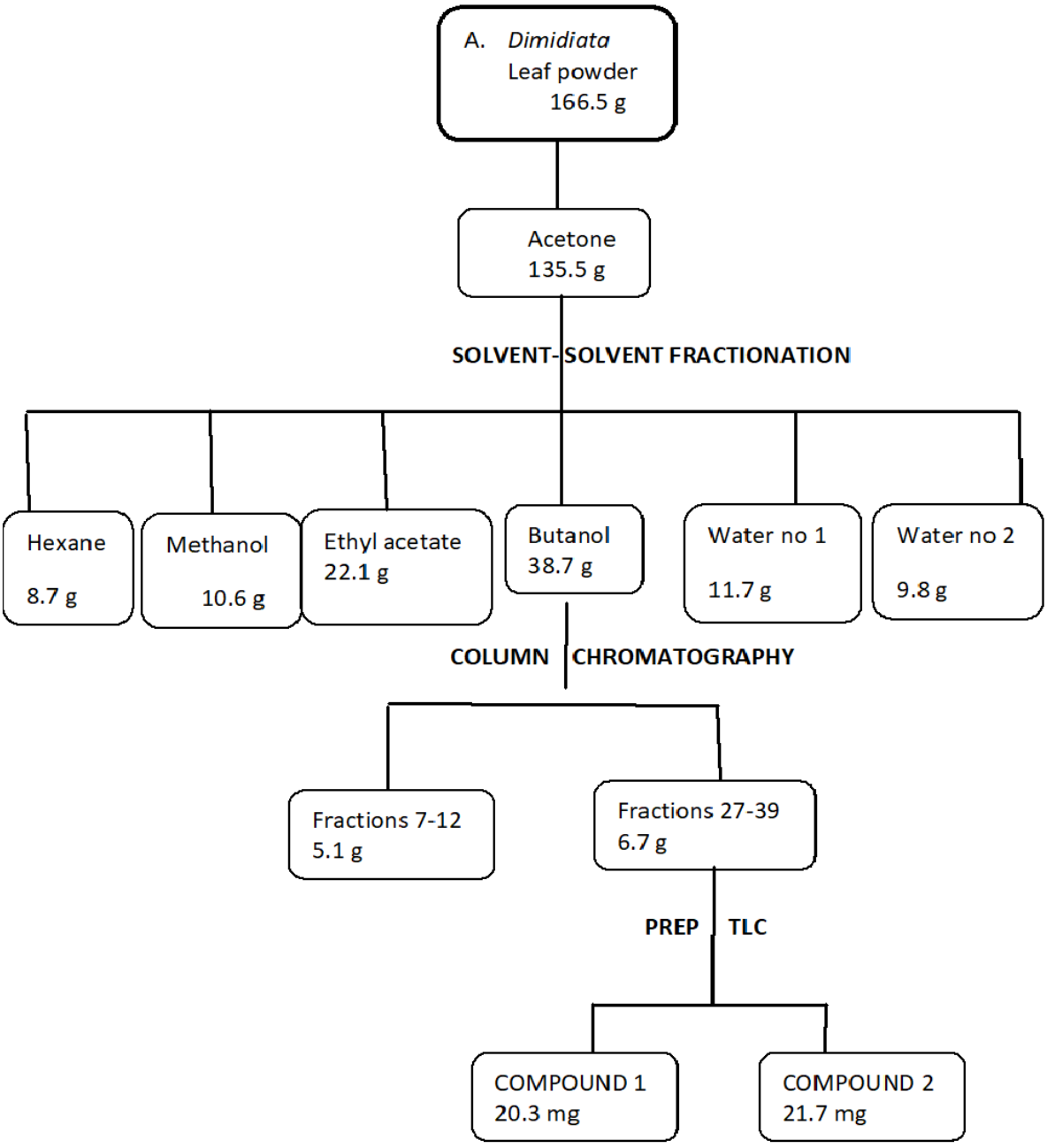

Figure 6.11: Flow diagram of isolation of compound 1 and 2

Pure compounds were further characterised using Nuclear Magnetic Resonance spectroscopy. The spectral data for the isolated compounds is presented in figures 6.9 and 6.12.

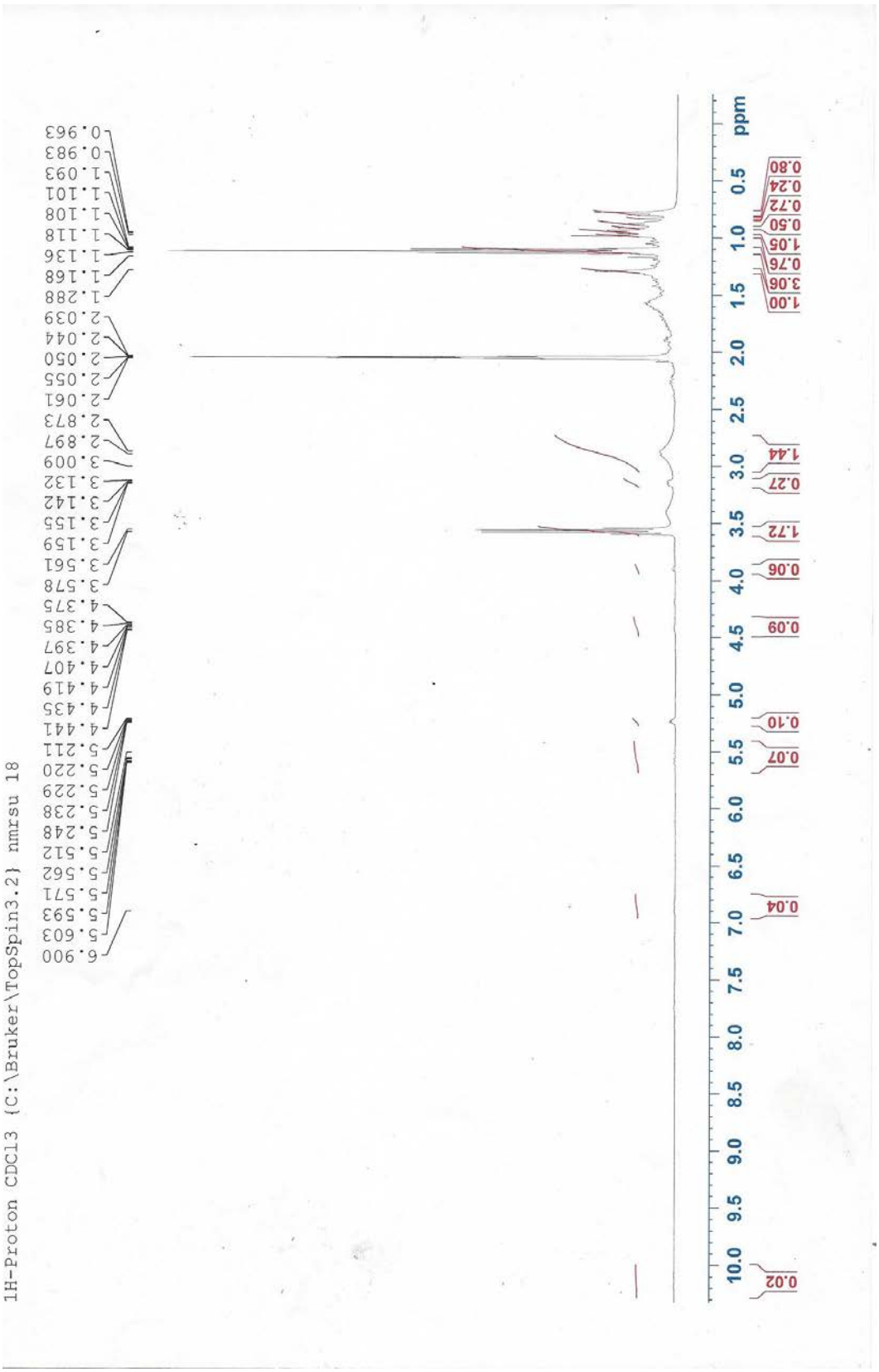

Figure 6.12: ^{1}H- NMR spectrum of compound 1.

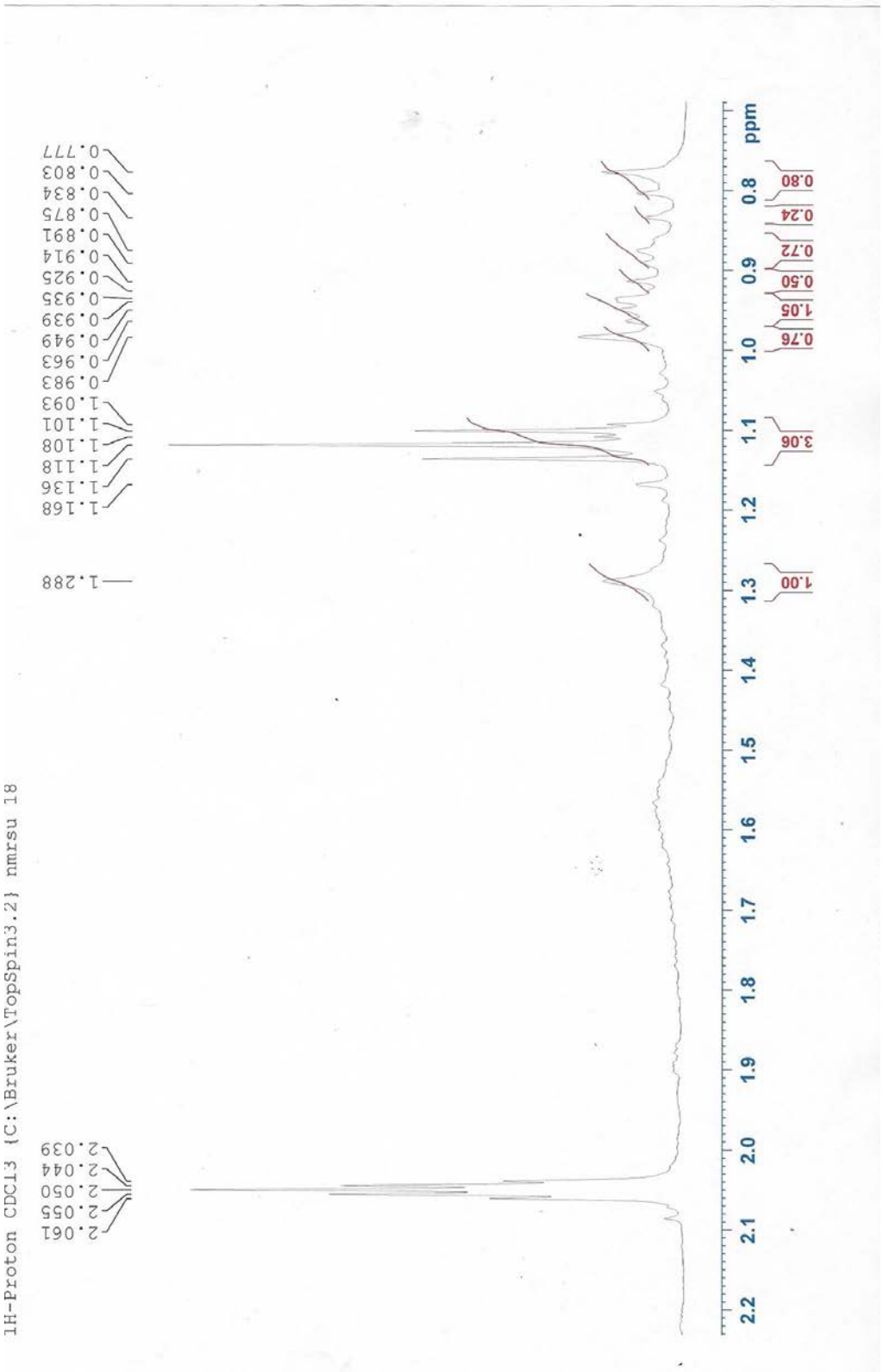

Figure 6.13: ^{1}H- NMR spectrum of compound 2.

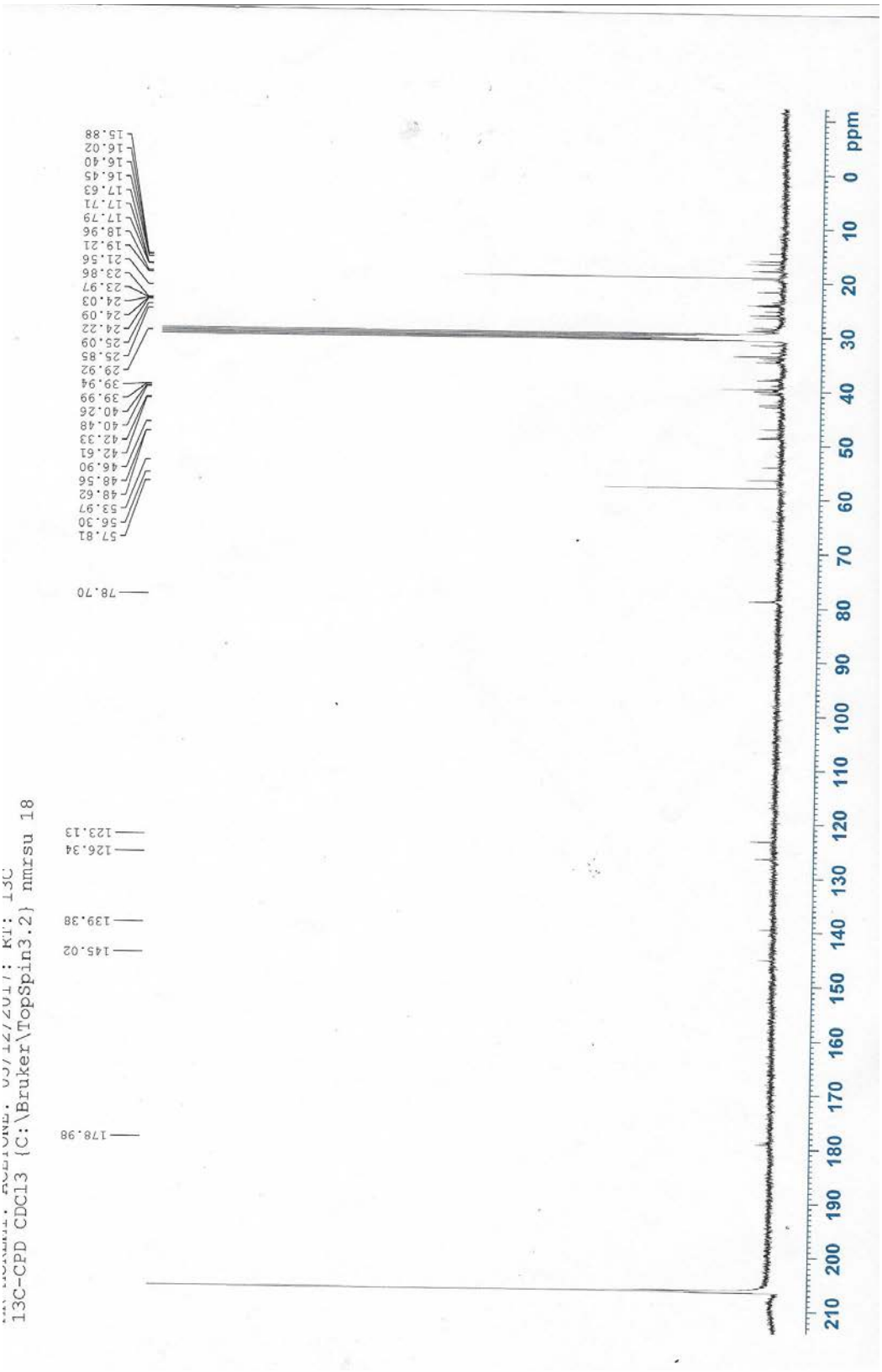

Figure 6.14: 13C- NMR spectrum of compound 1.

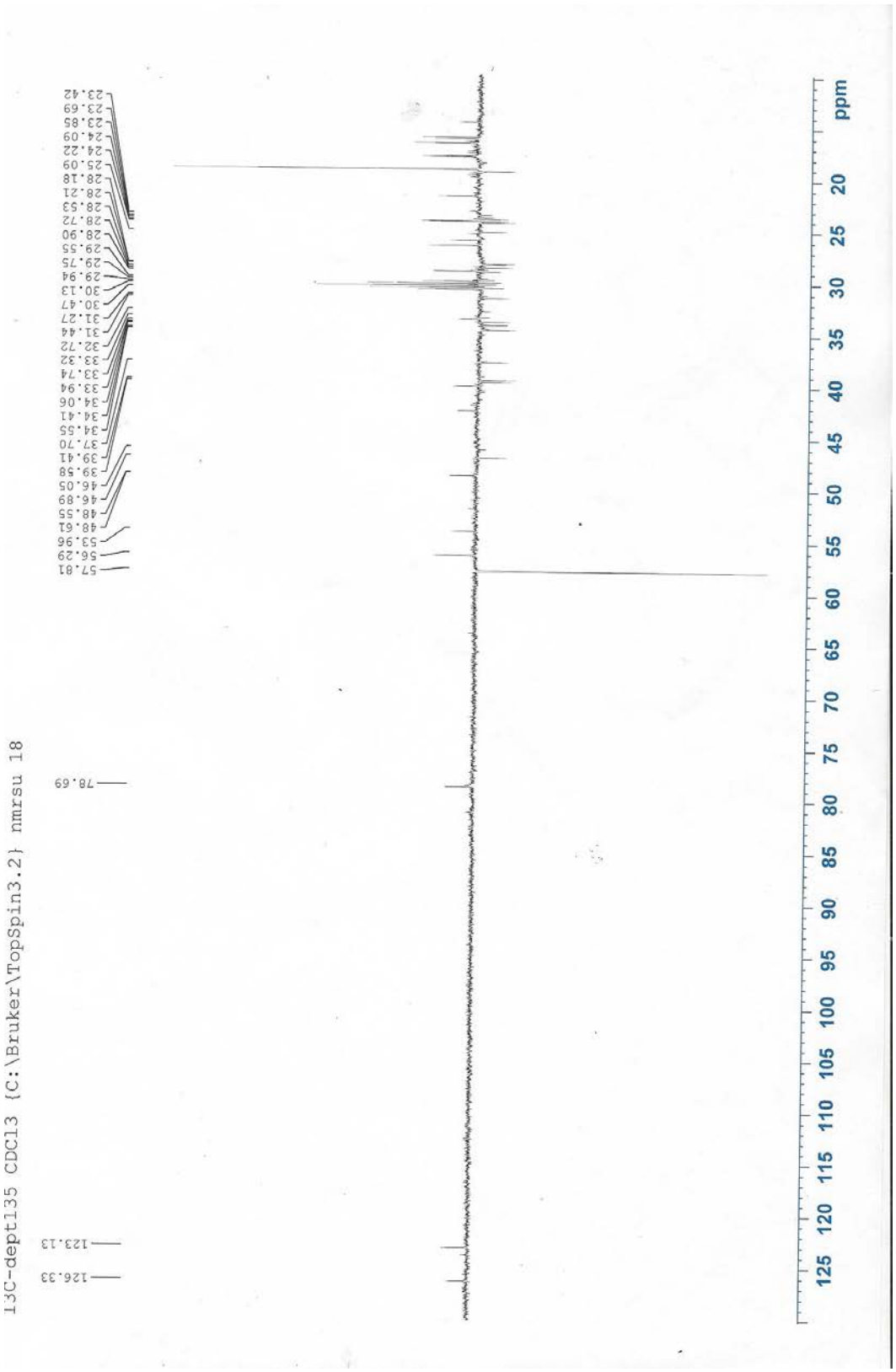

Figure 6.15: ^{13}C NMR spectrum of compound 2**.**

The elucidated structures of the isolated compounds (figures 6.13) are illustrated below.

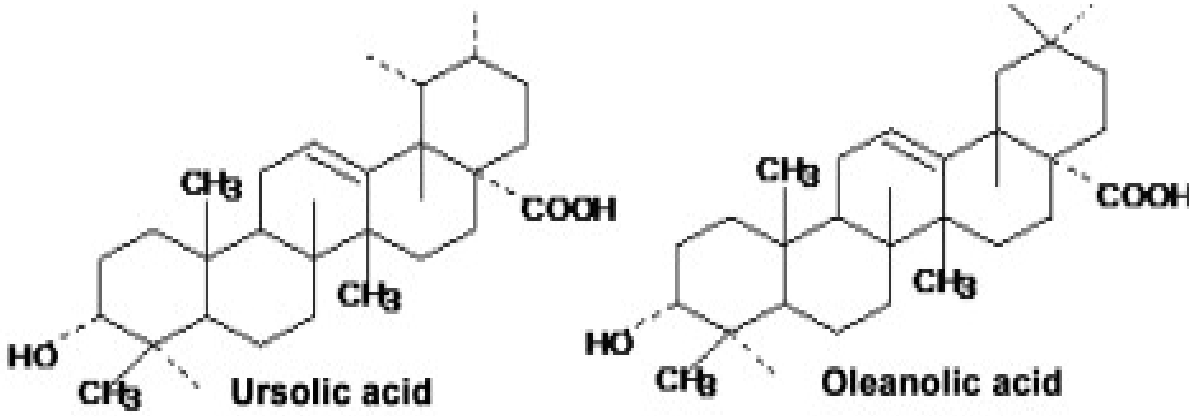

Figure 6.16: Structures of the isolated compounds

6.4. Discussion

The three selected plants were further sub-fractionated into the hexane, butanol, ethyl acetate, methanol, water fraction no. 1 and water fraction no. 2 and assessed for the presence of antioxidant activity against *M. smegmatis* on TLC. All the fractions of the three plants namely *A. dimidiata*, *C. heroroense* and *C. lemon* contained constituents with different colours indicating the presence of a variety of compounds. Sub-fractions of *A. dimidiata*, were best resolved in BEA with antioxidant constituents with a high polarity. All the sub-fractions contain compounds with anti-mycobacterial activity, although the concentration in the hexane extract was low. Sub-fractions of *C. heroroense* showed the presence of compounds with antioxidant activity that were best resolved in the CEF eluent system and in the antimycobacterial activity contained in the ethyl acetate fraction in the EMW eluent system. *Citrus lemon* on the other hand, did not show anti-mycobacterial activity with the sub-fractions suggestive of a synergistic effect of compounds contained in the crude extract (Ghaemi *et al*., 2011) with a possible loss of activity through sub-fractionation. Compounds that exhibited anti-mycobacterial activity on bio-autograph were not shown to correspond to those with antioxidant activity. It can be asserted, therefore, with some degree of certainty that the compounds contained in the fractions with antioxidant activity when acting alone are not responsible for the observed antimycobacterial effects recorded in this study. The antimycobacterial activity observed

in the bioautograms will assist in bioassay guided isolation of pure compounds. In general, apart from the butanol fraction of all the plants and hexane fraction of *A. dimidiata*, subfractionation of the crude plants, were not shown to markedly result in a more potentiated activity going by MIC values when compared to the crude itself. This finding was not surprising, since recent reports have shown fractionation to reduce cytotoxicity rather than enhance the anti-helminthic activity of *Heteromorpha arborescens* (Adamu *et al*., 2016). The butanol sub-fractions of selected plants showed moderate activity with average MIC value of 0.16 mg/ml followed by the hexane and methanol sub-fraction (0.19 mg/ml). The hexane sub-fraction of *A. dimidiata* showed the most potent activity (MIC 0.04 mg/ml), followed by the butanol, ethyl acetate and methanol fractions (MIC 0.16 mg/ml) against M. smegmatis. Solvent-solvent fractionation was a very important first step towards isolation. The isolation of bioactive compounds on silica gel 60 (particle size 0.063- 0.200 mm) in open column chromatography resulted in the successful isolation of antimycobacterial compounds from *A. dimidiata*. Active compounds were extracted, isolated and purified using column chromatography and preparative TLC.

Although some compounds lost their activity with each step of the purification process, this is a proof that some compounds work synergistically to produce various biological activity. In other instances, some phytochemical compounds hindered the activity of other compounds, i.e. had an antagonistic effect, and showed their own activity once they were separated (Van Vuuren and Viljoen, 2011).

The isolated compounds were found to be the ursolic acid and oleanolic acid. These compounds have been isolated before in the bark of *A. dimdiata,* but this is the first report on the isolation of these compounds on the leaves of *A. dimidiata*. Oleanolic acid has been previously isolated from various plants (Eloff *et al*., 2017) and has been reported to have anti-allergic, anti-atherosclerotic, antibacterial, anti-inflammatory, anti-malarial, anti-viral, anti-oxidant, anti-septic, anti-carsonomic, anti-plaque, anti-plasmodial, anti-tumor and anti-ulcer properties (Dr Duke's Phytochemical and Ethnobotanical Databases; Eloff *et al*., 2017). Uroslic acid and oleanolic acid have been isolated in plants like *Baccharis uncinella* (but not in *A. dimidiate*).

6.5. Conclusion

Solvent-solvent sub-fractions, in some cases, can reduce activity of extracts especially where the desired effect is synergy dependent. Column chromatography and preparative TLC were important methods in isolation of bioactive compounds. Nuclear magnetic spectroscopy is the best tool for characterisation of isolated bioactive compounds. Hence, the compounds, ursolic acid and oleanolic acid were successfully isolated from the n-butanol sub-fraction of *A. dimidiata* leaves. Based on the findings in this study, *A. dimidiata* is highly recommended as a source of potent antimycobacterial activity and this serves as a scientific proof for the use of this plant in traditional medicine to treat tuberculosis and related symptoms. In the next chapter, the cytotoxicity activity and further bioassays on the isolated compounds will be evaluated.

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CHAPTER 7

7. BIOLOGICAL ASSAYS OF ISOLATED COMPOUNDS

7.1. Introduction

Reactive Oxygen Species (ROS), that are referred to as free oxygen radicals, have hydroxyl radical, superoxide and singlet oxygen. They can yield to cellular harm leading to disease that cause DNA damage. Free radicals play a role in aging and in diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1999). Plant extracts containing antioxidant compounds may protect patients indirectly by stimulating the immune system (Masoko, 2007).

The body's defense systems of the antioxidants, can only protect the body when the quantity of the free radicals is within the normal physiological level. When there is a shift in balance in the body towards more free radicals, that is, increasing their burden in the body either due to environmental condition or infections, it will lead to oxidative stress, and that may result in tissue injury and eventually diseases (Finkel and Holbrook, 2000). Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial, or anti-viral activities to a greater or lesser extent (Owen *et al*., 2000 and Sala *et al*., 2002; Masoko, 2007).

During drug development, one of the essential steps is to determine the cytotoxicity of the test substance. This assists in determining whether the developed drug is cell toxic or not, as well as its level of cell toxicity. The test looks at the various cell functions such as the enzyme activity, cell membrane permeability, cell adherence, ATP production, and nucleotide uptake activity.

Traditional medicinal plants are acclaimed to be non-toxic and they are regarded as safe, because they are from nature and have been used for years to treat various diseases (Chen *et al*., 2011; Fennell *et al*., 2004). However, scientific studies on safety and efficacy of some of these medicinal plants have shown that there are many phytochemicals that are found in plants that have cytotoxic, genotoxic and carcinogenic effects (Ahmed *et al*.,

2012). Since traditional medicines are used worldwide, it is vital that these plants are tested for toxicity and efficiency in treating various ailments.

A cytotoxicity assay is a rapid and cost-effective tool to determine whether a compound is entered into the costly development process and to help choose the optimal candidate (Wallin and Arscott, 1998). Testing for cytotoxicity of a compound is rapid, standardised, sensitive and inexpensive means to determine whether a compound contains significant quantities of biologically harmful extractables. The test has high sensitivity due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body (Ricerca, 1998; Samdumu, 2006). Antimycobacterial assays are done to verify the bioactivity of the compound against various mycobacterial strains. In this study, the isolated compounds, oleanolic acid and ursolic acid were investigated for antimycobacterial activity against *M. smegmatis* and *M. tuberculosis* using MIC and bioautography assays.

7.2. Materials and methods

7.2.1. Qualitative antioxidant assay

The potential antioxidant activity of the plant extracts was determined on the basis of the scavenging activity of stable 1, 1- diphenyl- 2-picrylhydrazyl (DPPH) (Sigma®). The chromatograms were prepared as in section 3.2.4.1. The chromatograms were sprayed with 0.2% DPPH to visualize any potential antioxidant compounds within the separated plant extracts (Deby and Margotteaux, 1970).

7.2.2. Minimum Inhibitory Concentration (MIC) assay

7.2.2.1. Minimum Inhibitory Concentration (MIC) determination using *Mycobacterium smegmatis*

Minimum Inhibitory Concentration is the lowest concentration of the isolated compounds. Minimum Inhibitory Concentration was determined using the micro-dilution assays in a 96 well micro-plates, as described by Eloff (1998), indicated in section 5.2.2.5. Hundred microliters (100 µl) of sterile distilled water was added into all the wells of the 96 well

microtitre plates. Oleanolic acid and ursolic acid were re-dissolved in acetone to the concentration of 10 mg/ml.

7.2.2.2. Minimum Inhibitory Concentration (MIC) determination using pathogenic strain

The MIC values were determined using the serial microplate method developed by Eloff (1998), slightly modified for mycobacteria by (McGaw *et al*., 2008) as described in section 5.2.2.3 and 5.2.2.4.

7.2.2.3. Bio-autographic assays

Bio-autography was carried out on TLC plates according to Beque and Kline (1972) to detect the main bioactive compounds. TLC plates were loaded with 10 µl of 10 mg/ml density of the solution of each extract as described under phytochemical analysis (section 5.2.2.7).

7.2.3. Cytotoxic assay against Vero monkey cells

The tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) and modified by McGaw *et al.* (2007) was used to investigate cytotoxicity of the two compounds isolated from *A. dimidiata* in sufficient quantity as described in section 5.2.3.

7.3. Results

7.3.1. Antioxidant assay

Despite the fact that the isolated compounds had a promising good antimycobacterial activity, there was no antioxidant activity observed with both ursolic acid and oleanolic acid (figure 7.1). This was when qualitative scavenging ability of the isolated compounds was done. The yellowish spots against the purple background after spraying with 1.2-diphenylpicryl hydrazyl was not seen on the developed chromatograms.

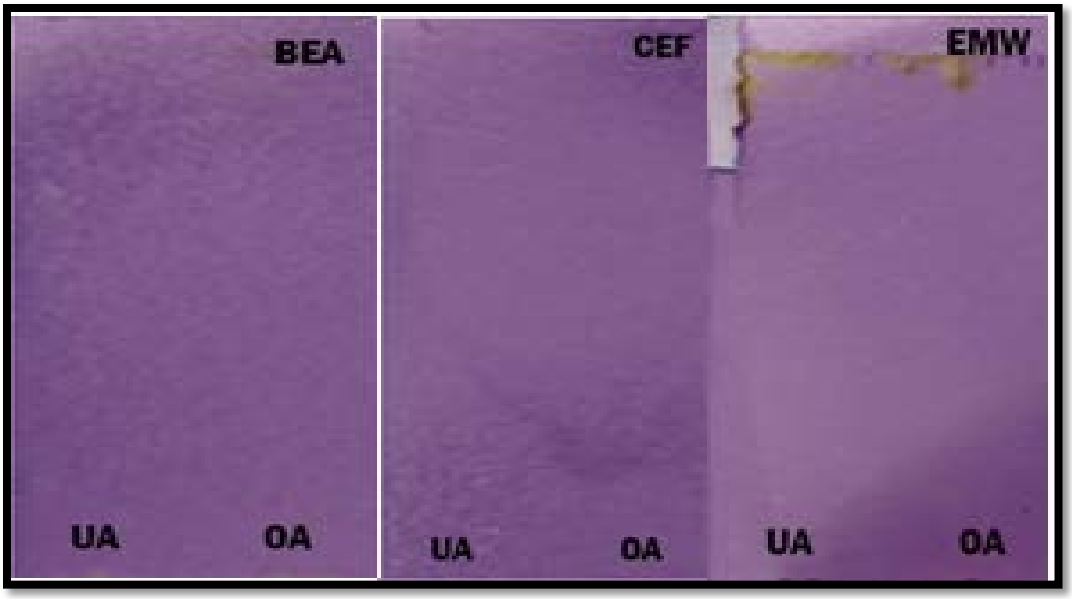

Figure 7.1: Chromatograms developed in BEA, CEF and EMW of ursolic acid (UA) and oleanolic acid (OA) sprayed with 0.2% of DPPH in methanol.

7.3.2. Minimum Inhibitory Concentration (MIC) of isolated compounds against *M. smegmatis* and *M. tuberculosis* (H37Rv)

Oleanolic acid (OA) had the best MIC value of 0.12 mg/ml and ursolic acid (UA) had a better MIC value of 0.23 mg/ml against *M. smegmatis*. Both compounds had a fairly high total activity of 88.26 ml^{-1} (UA) and 180.83 ml^{-1} (OA) when the test organism used was *M. smegmatis* (Table 7.1).

Table 7.1: Mass (mg) of isolated compounds, Minimum Inhibitory Concentration (MIC) against *M. smegmatis* in mg/ml and their total activity (TA) in ml^{-1}.

Compound	Mass (mg)	MIC (mg/ml)	TA (ml^{-1})
Ursolic acid	20.3	0.23	88.26
Oleanolic acid	21.7	0.12	180.83

When using the pathogenic strain, H37Rv, oleanolic acid, still had a lower MIC value (0.31 mg/ml) than ursolic acid (0.62 mg/ml). The total activities of ursolic acid and oleanolic acid was found to be 32.74 ml^{-1} and 70 ml^{-1}, respectively (table 7.2).

Table 7.2: Mass (mg) of isolated compounds, Minimum Inhibitory Concentration (MIC) against *M. tuberculosis* in mg/ml and their total activity (TA) in ml^{-1}.

Compound	Mass (mg)	MIC (mg/ml)	TA (ml^{-1})
Ursolic acid	20.3	0.62	32.74
Oleanolic acid	21.7	0.31	70.00

7.3.3. Bioautography assay

Antimycobacterial activity of usrolic acid and oleanolic acid was clearly observed in a non-polar mobile system BEA. Mobile system had an influence on whether the bioactive components on the bioautograms could be seen or not. Increase in polarity of the mobile system made the antimycobacterial activity of the isolated compounds to disappear (figure 7.2).

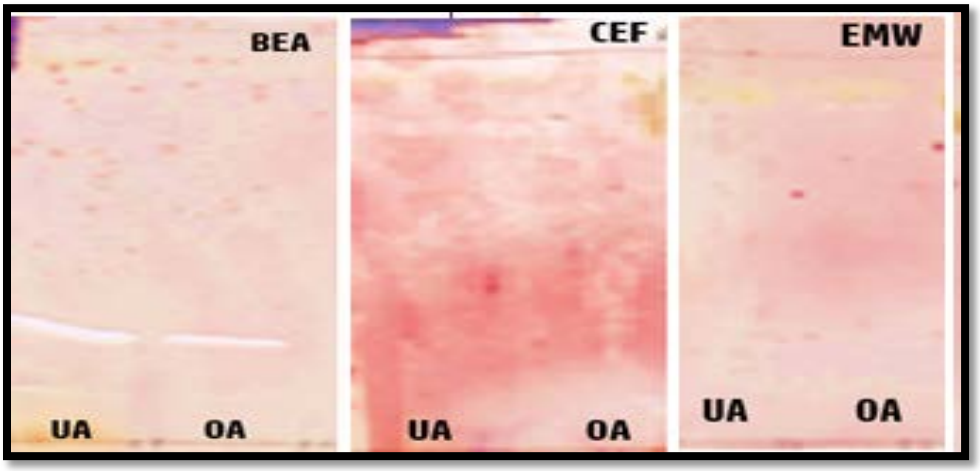

Figure 7.2: Bioautograms of ursolic acid (UA) and oleanolic acid (OA) developed in BEA, CEF and EMW

7.3.4. Cytotoxicity evaluation of the isolated compounds

Cytotoxicity effect of the two isolated compounds (ursolic acid and oleanoic acid) of *A. dimidiata* were investigated against Vero kidney cell line. Compound 1 (ursolic acid) was cytotoxic and compound 2 (oleanoic acid) showed non- cytotoxic effect (table 7.3). Selectivity index values of the samples were calculated by dividing LC_{50} (mg/ml) values by MIC (mg/ml) values (table 7.3). The higher the SI value, the safer the extract/ compound. In the case of the compounds isolated in this study, the average SI value for oleanolic acid was higher (1384.17) than that of ursolic acid (19.17) indicating that it is a safer compound to use against mycobacteria as compared to ursolic acid.

Table 7.3: The LC_{50} of the crude extracts, subfractions from solvent-solvent fractionation and the isolated compounds of *A. dimidiata* leaves and their selective index.

Sample	LC_{50} (mg/ml)	MIC (mg/ml)	Selective Index (SI)
Ursolic acid	4.14	0.23	19.17
Oleanolic acid	166.1	0.12	1384.17

7.4. Discussion

Both compounds had no significant antioxidant activity when their qualitative antioxidant activity was evaluated. This may be due to the fact that their concentration was not enough when they were spotted on the TLC plate when doing the antioxidant activity test. Otherwise, they are terpenoids, and triterpenoids are known to possess antioxidants. Triterpenoids like ursolic acid have been found to play a prominent role in the prevention and therapy of a variety of ailments and some have already entered Phase I clinical trials. Ursolic acid has attracted great attention of late for its potential as a chemopreventive and chemotherapeutic agent in various types of cancer. Ursolic acid has been shown to target multiple proinflammatory transcription factors, cell cycle proteins, growth factors, kinases, cytokines, chemokines, adhesion molecules, and inflammatory enzymes. These targets can potentially mediate the chemopreventive and therapeutic effects of ursolic acid by inhibiting the initiation, promotion and metastasis of cancer (Shanmugam *et al*., 2013).

Compounds with MIC value of 0.1 mg/ml are regarded as being significantly active; those with MIC values >0.1 to 0.625 mg/ml are considered moderately active and when the MIC value is >0.625 mg/ml the activity is weak (Aro *et al*., 2015; Eloff, 1998; Sánchez and Kouznetsov, 2010). Since oleanolic acid had an MIC value of 0.12 mg/ml, it can be regarded as a significantly active compound against *M. smegmatis.* Ursolic acid, on the other hand, can be regarded as a moderately active compound against *M. smegmatis* since it had an MIC value of 0.23 mg/ml. The two compounds also showed clear antimycobacterial activity on bioautograms when the plate was developed in BEA (a non-polar mobile system) as compared to when developed in the other two mobile phases (i.e. CEF and EMW). In the study of Jiménez-Arellanes *et al*., (2013), the in Vitro assay of ursolic acid and oleanolic mixture exhibited synergistic activity. The intracellular activity of these compounds against *M. tuberculosis* H37Rv and a MDR clinical isolate in a macrophage cell line showed that both compounds, alone and in combination, were active against intracellular mycobacteria even at low doses. Also, when both compounds were used to treat BALB/c mice with TB induced by H37Rv or MDR bacilli, they showed a significant reduction of bacterial loads and pneumonia compared to the control used in their study. But then, animals treated with UA and OA showed a higher expression of IFN-γ and TNF-α in their lungs, than control animals (Jiménez-Arellanes *et al*., 2013). The sub-fraction of the acetone crude extract (i.e. the hexane), was found to be non-toxic. This may be due to the fact that as isolation was done, toxicity of the crude extract was reduced. One of the isolated compounds, oleanolic acids also had no cytotoxicity and the other, ursolic acid was toxic.

7.5. Conclusion

The oleanolic acid which has been isolated from different plants before but not *A. dimidiata*, showed a significant non- cytotoxic effect as compared to ursolic acid. This indicates that the oleanolic acid can be used in the treatment of TB and related symptoms since it is safe and it can be highly effective. On the other hand, ursolic acid, even if it has antimycobacterial properties, it cannot be safely used to treat TB due to its cytotoxicity.

7.6. References

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CHAPTER 8

8. GENERAL DISCUSSION AND CONCLUSION

Tuberculosis is a re-emerging disease of global importance. The burden of TB is so high that it is estimated that one third of the global population is infected with *M. tuberculosis*. The projections of WHO (2015) show that by the year 2020 (the next coming 3 years), nearly one billion people will be newly infected with TB, 200 million will develop TB and 35 million mortalities due to TB will occur (WHO, 2015). Such statistics raise a great concern and the need to combat this threat to human existence. The current chemotherapy for TB treatment is not purely effective against resistant strains of *M. tuberculosis*. Hence, there is a need to find new drugs for the treatment of TB from natural sources. Drugs from natural sources such as plants cause less or no resistance against TB. In this study, 7 plants were selected based on their usage traditionally for the treatment of TB and related symptoms in the three districts of Limpopo Province (Semenya and Maroyi, 2012). The plants were screened for anti-mycobacterial activity so as to eventually isolate and purify compounds that can be developed into drugs that can be used to treat TB. When extracting with four solvents (*viz*., hexane, dichloromethane, acetone and methanol) varying in polarity, overall, methanol was the best solvent since it extracted a greater quantity of plant material when compared to the other solvents.

In South Africa, traditional methods of treatments based on medicinal plants are still an important part of social life and culture. The acceptability of these plants as claimed to be effective remedies is quite high among people living in South Africa. The claimed therapeutic value of the reported plant species calls for modern scientific studies to establish their safety and efficacy and to preserve and document the plants (Nguta *et al*., 2010). Traditional knowledge on the use of medicinal plants must be conserved because of its vital role for human well-being. Traditional knowledge is considered reliable for the exploitation of herbal remedies in that indigenous people, through a period of long experimentation with herbal medicines, are likely to have retained those plants that are effective and tolerably safe whilst discarding preparations with low efficacy or acute toxicity (Newton *et al*., 2000).

Information on efficacy and safety of widely used TB remedies is also lacking. These gaps have made inclusion of traditional TB treatments in the national primary health care package difficult (Tabuti *et al*., 2010). This study, to some extent, addressed efficacy of the traditional medicines used to treat TB and also the safety, by conducting cytotoxic assay of the crude fractions of the bioactive plants. There is a need for pharmacological evaluation of the species to determine which species are efficacious and which ones are safe to use.

Tabuti *et al.* (2010) mentioned that traditional medicine knowledge is eroding in many human populations. Limited availability and accessibility of some medicinal species restrict their use and this leads to the loss of important traditional knowledge such as species identification. Some plants have shown promising results, but very few active molecules have been purified from medicinal plants. So far, few plants have been tested against mycobacteria (Gupta *et al*., 2010). Of all the plants selected for antimycobacterial activity, *A. dimidiata* and *C. hereroense* displayed effective activity towards *M. smegmatis* and *A. dimidiata* also towards *M tuberculosis* and MDR- TB strains, demonstrating their potential as a source of anti-TB drug leads. Further phytochemical and pharmacological studies of these plants are essential and significant.

The result of this finding is significant, considering the increase in the number of MDR-TB affected countries, including South Africa, and the need for development of more potent antimycobacteria drugs against drug resistant strains of the pathogen. This is the first report of ursolic acid and oleanolic acid isolation from *A. dimidiata* leaves. Oleanolic acid, since it has both antimycobacterial activity for pathogenic strain and anti- cytotoxic, has the potential to function as an effective antimycobacterial chemotherapeutic agent.

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APPENDIX A

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RESEARCH ARTICLE Open Access

Anti-mycobacteria potential and synergistic effects of combined crude extracts of selected medicinal plants used by Bapedi traditional healers to treat tuberculosis related symptoms in Limpopo Province, South Africa

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Abstract

Background: Tuberculosis is an infectious communicable disease and the causative agent of the disease has over the years developed resistance to streamline chemotherapeutic agents with dire consequences and there is a need for development of new and more potent alternatives.

Methods: Constituents of leaves material of *Combretum heroroense*, *Citrus lemon* and *Apodytes dimidiata* were serially extracted using solvents of varying polarity. TLC finger print profile of the different extracts were determined by spraying eluted plates with vanillin sulphuric acid and 2, 2- diphenylpicryl hydrazyl (DPPH) for the presence of antioxidant constituents. Presence of different phytochemicals was determined using standard chemical test. Bioautography was used to determine the number of compounds present in sub-fractions active against *Mycobacterium smegmatis*. Minimum inhibitory concentration (MIC) values extract and sub-fractions were determined using serial microplate dilution method against *M. smegmatis* (ATCC 1441), *M. tuberculosis* (ATCC H37Rv) and multi-drug resistant TB (MDR-TB) field strain. Synergy of the crude extracts of the three plants was determined using microplate dilution method against *M. smegmatis*.

Results: Mass extracted by different solvents was less than 6% dry weight for all the plants. Phlobatannins were not detected in *A. dimidiata*, *C. heroroense* and *C. lemon* as well as cardiac glycosides in *C. lemon* and *A. dimidiata*, and saponins in *C. heroroense*. Sub-fractions of the different plants were shown to contain constituents with antioxidant activity with the highest number detected in *C. heroroense*. Bioautography results reveal the presence of a compound(s) in the ethyle acetate sub-fraction of *C. heroroense* and butanol, methanol/water, ethyl acetate and water no.2 subfractions of *A. dimidiata*, active against *M. smegmatis* that were not shown to have antioxidant capacity. MIC results for different crude extracts of the three plants against *M. smegmatis* ranges from 0.1 to 3 mg/ml. The average MIC for the synergistic effect of the plants ranged from 0.04 mg/ml to 1.25 mg/ml. An activity greater than that obtained for the reference drugs was shown for the butanol and hexane fractions of *A. dimidiata* (0.47 mg/ml) against the field strain of MDR-TB while that obtained for the *M.TB* (ATCC H37Rv) was 0.31 mg/ml.

(Continued on next page)

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