

**SCREENING, ISOLATION AND PURIFICATION OF BIOACTIVE COMPOUNDS WITH
ANTIBACTERIAL ACTIVITY AGAINST *MYCOBACTERIUM SMEGMATIS***

by

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DECLARATION

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

Initials & Surname (Title)

Date

Student Number: -----

DEDICATION

I dedicate this work to my family; my mother Sophy, my sisters Isabella and Tshidi, and my brothers Neo and Rorisang for having faith in me and for their support and encouragement throughout the project.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BEA	Butanol, ethanol and ammonia
^{13}C	Carbon-13
CEF	Chloroform, ethyl-acetate and formic acid
DCM	Dichloromethane
DPPH	2,2-diphenyl-1-picrylhydrazyl
EMW	Ethyl acetate, methanol and water
ETAC	Ethyl acetate
^1H	Hydrogen-1
HEX	Hexane
INT	ρ -Iodonitro-tetrazolium violet salt
NMR	Nuclear magnetic resonance
MEOH	Methanol
MIC	Minimum inhibitory concentration
MCC	Microbial culture collection
TB	Tuberculosis
TLC	Thin layer chromatography

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ABSTRACT

The leaves of fifteen plant species were collected from the Lowveld Botanical Garden in Nelspruit, Mpumalanga Province, South Africa. The collection was based on a list of plants and their ethnopharmacological information provided by the Phytomedicine Programme at the University of Pretoria. The dried leaves of the plants were powdered and extracted using hexane, dichloromethane, acetone and methanol. The extracts were screened for antibacterial activity against *Mycobacterium smegmatis* and *Rhodococcus erythropolis*. The acetone extract of *Milletia stulhimannii* was the most active, showing activity against *Mycobacterium smegmatis* and *Rhodococcus erythropolis* with MIC values 0.13 and 0.08 mg/ml, respectively. Acetone extracts for all plants had the lowest MIC values ranging between 0.11-1.25 mg/ml and 0.08-1.25 mg/ml for *M. smegmatis* and *R. erythropolis*, respectively. *Milletia stulhimannii*, *Albizia gummifera*, *Xanthocercis zambesiaca* and *Barringtonia racemosa* extracts have shown the greatest potential for anti-tuberculosis agents. These were all active against *M. smegmatis* with an average MIC value of acetone extracts of 0.13 mg/ml. *Apodytes dimidiata* was selected for the isolation of active compounds since its activity on qualitative antibacterial activity assays was highly prominent on TLC plates in comparison to the other plant extracts. Two compounds were isolated from *A. dimidiata* but after purification, their MICs were above 2.5 mg/ml indicating a possible loss of activity during purification. The preliminary NMR spectra analysis suggested that the compounds were a long fatty acid and a triterpene. Future work is required to elucidate the chemical structures of the latter compounds and to test the activity of these compounds against *Mycobacterium tuberculosis*.

Publications from this thesis

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CHAPTER ONE

1.1 Introduction

Plants have provided man with most of his needs in terms of shelter, clothing, food, flavours and fragrances and not least the basis of health care throughout the world since the earliest days of humanity and are still internationally used (Gurib-Fakim, 2006). Plants remain a vital source of medicines for a large proportion of the world's population, particularly in the developing countries (WHO, 2002).

Nature has been a good supply of various medicinal agents for thousands of years and a number of modern drugs have been isolated from natural sources such as plants. A range of medicinal plants have been discovered and used for many years on a daily basis for treatment of different diseases all over the world (Farombi, 2003). Herbal medicine is now globally accepted as a legal, alternative system of therapy for treatment and cure of various diseases and physiological conditions in traditional treatments in the form of pharmaceuticals (Dos Santos-Neto *et al.*, 2006). Various plant species are used by many ethnic groups around the world for the treatment of various ailments ranging from minor infections to dysentery, skin diseases, asthma, and malaria and a range of other indications. Numerous prescriptions, folk drugs, and herbal drugs play an important role in maintaining people's health. These are also the sources for new drug research and development (WHO, 2009).

The healing power of plants is an ancient practice and thousands of indigenous plants have been used by people on all continents as poultices and infusions dating back to prehistory. The use of *Alecea rosea* to control inflammation, to stop bedwetting and as a mouthwash in cases of bleeding gums has been reported 60 000 years ago and is still used around the world today (Cowan, 1999).

Medicinal plants have many industrial uses ranging from traditional medicines, herbal teas, and health foods such as nutraceuticals to galenicals, phytopharmaceuticals and industrially produced pharmaceuticals. Furthermore, these constitute a source of potential chemotherapeutic agents since these are a ready source of drugs such as

quinine and reserpine; of galenicals like tinctures and of intermediates (e.g. diosgenin from *Discorea* sp.) in the production of semi-synthetic drugs (Gurib-Fakim, 2006). Over 350 natural products have been evaluated for their anti-mycobacterial activities (Newton *et al.*, 2002). Herbal remedies play an essential role in traditional medicine in rural areas of South Africa, where they are often the therapeutic treatment of choice and the preparation of herbal medicine may depend on the cultural context.

Most developing countries around the world still rely on the use of indigenous plants for the treatment of various diseases because they are unable to afford pharmaceutical drugs. But the problem is that in most cases very little scientific information is available about the safety, active ingredients and toxicity of such indigenous medicine. There is a great need to harness scientific and clinical research in order to investigate the quality, safety and efficiency of these herbal therapies (Phillipson, 2001).

The use of medicinal plants and their bioactive compounds and scientific knowledge about them should help researchers determine the efficacy, stability, best drug delivery systems and quality controls of plants commonly used in traditional medicine. Through scientific knowledge, various methods have been developed to test, isolate, purify and characterise bioactive compounds from medicinal plants.

Bacterial resistance is the major obstacle to successful treatment of some life threatening illness caused by some bacteria. *Mycobacterium tuberculosis* is already known to be resistant to one out of 100 antibiotics available. It is reported that drug-resistant strains of *Mycobacterium* (MDR) are increasing throughout the world. Hence, the need to discover novel chemical structures as lead compounds from the plant kingdom which can be used for the treatment of individuals suffering from tuberculosis.

Hence in this study, the anti-mycobacterium activity against fifteen medicinal plants was evaluated using a simple *in vitro* screening assay in a 96-well microplate dilution method and bioautographic assays. The plant extracts were screened against rapidly growing and non-pathogenic mycobacteria, *Mycobacterium smegmatis* MC²155 strain

and *R. erythropolis* ATCC 4277 strain with a view of identifying and isolating active compounds which could be later tested against resistant *Mycobacterium tuberculosis*.

1.2 Literature review

1.2.1 Plants as a source of new drug development

For thousands of years, natural products have been the basis of treating and preventing human diseases. Many of the clinically used therapeutic agents are used in their naturally occurring forms or as derivatives or analogues after structural optimization. The influence of natural products upon drug discovery has been well recognized and documented and continues to play an important role in humankind.

The history of using natural products with therapeutic properties dates back to thousands of years and includes plants, minerals and animal products as the main sources of drugs (De Pasquale, 1984). The future of natural products drug discovery will be more holistic and involves wise use of ancient and modern therapeutic skills in a complementary manner to maximise the benefits to the patients and the community.

There has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Mentz and Schenkel, 1989). The interest in drugs of plant origin is due to several reasons such as side effects and ineffective therapy, abusive and/or incorrect use of synthetic drugs, and a large percentage of the world's population (developing countries) does not have access to conventional pharmacological treatment.

The potential use of plants as a source of new drugs is still not thoroughly explored. From the estimated 250,000–500,000 plant species, only a small percentage has been investigated phytochemically and pharmacologically. It is estimated that 5000 plant species have been studied for medical use (Payne *et al.*, 1991).

Scientific reports indicate that natural products as source of bioactive compounds are used to treat 87% of all categorized human diseases (Gurib-Fakim, 2006; Newman *et al.*, 2003; Phillipson, 2001; West and Price, 2004), including being used as antibacterial,

anticancer, anticoagulant and antiparasitic. There was no introduction of any natural products under this seven drug categories: anesthetic, antianginal, antihistamine, anxiolytic, antidote, antidiuretic, and antihypnotic, during the years 1981-2002 (Newman *et al.*, 2003).

Higher plants have a long history of use in the treatment of human diseases. Several well known species, including licorice (*Glycyrrhiza glabra*), myrrh (*Commiphora* species), and poppy capsule latex (*Papaver somniferum*), are still in use today for the treatment of various diseases as ingredients of certified drugs or herbal preparations used in systems of traditional medicine (Newman *et al.*, 2000). Furthermore, morphine, codeine, nicotine, and papaverine isolated from *P. somniferum* were developed as single chemical drugs and are still clinically used.

The majority of traditionally used crude drugs are derived from plant extracts. This has resulted in an inherited pool of information of the healing potential of plant species, thus making them important source of starting material for drug discovery. A different set of metabolites is usually produced in the different anatomical parts of the plant (e.g. root, leaves and flower), and botanical knowledge is crucial also for the correct taxonomical determination of the identified bioactive plants (Newman and Cragg, 2007).

Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Plants with ethnopharmacological uses have been the primary sources of medicines for early drug discovery. In fact, Fabricant and Farnsworth (2001) showed that the uses of 80% of 122 plant-derived drugs were related to their original ethnopharmacological purposes. Current drug discovery from plants has mainly relied on bioactivity-guided isolation methods, e.g., important anticancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminata*. In addition, combretastatin A4, isolated from the South African medicinal tree, *Combretum caffrum*, was developed to combretastatin A4 phosphate (Cirla and Mann, 2003; Pinney *et al.*, 2005) and is in phase II trials (West and Price, 2004).

Current therapeutic applications of metabolites from microorganisms have expanded into immunosuppressive agents (e.g., cyclosporins and rapamycin), cholesterol-lowering agents (eg, lovastatin and mevastatin), antihelmintic agents (eg, ivermectin), an antidiabetic agent (acarbose), and anti cancer agents (eg, pentostatin, peplomycin, and epirubicin) (Butler, 2005; Sneader, 2005).

Plants consist of a complex mixture of a wide variety of compounds which can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells (Cowan, 1999). Substances of this type are considered appropriate candidates for developing new antimicrobial drugs. Many of them are biologically active and have different biological properties which in most cases are not precisely known. Scientists have isolated biologically active substances in plants which have beneficial effects on health (Colegate and Molyneux, 1993). Evidence is growing that these plant constituents may help reduce the risk of development of diseases such as cancer, coronary heart disease, stroke and microbial-based diseases (Adedapo *et al.*, 2008).

In the present situation of increased multiple drug resistant *Mycobacterium* (tuberculosis), there has been an increased search for new antimicrobial substances from other sources including plants (Diamond, 1991). This growing worldwide interest in medicinal plants reflects recognition of the strength of many traditional claims regarding the value of natural products in healthcare (Hamburger and Hostettmann, 1991).

1.2.2 Bioactive compounds in medicinal plants

Plants contain two classes of compounds namely; primary metabolites that are required for the sustenance of the plant (i.e. the plants machinery), and the secondary metabolites that are not a necessity for the plant's survival. The secondary metabolites are mostly compounds which are active for treatment and prevention of diseases in human and animals and are thus termed bioactive compounds. Bioactive compounds are low-molecular weight compounds that do not play a role in primary metabolic functions (photosynthesis, respiration, and carbon fixation) and which vary in their distribution in the plant kingdom (Harborne and Baxter, 1999). Bioactive compounds are generally unique to individual plant species and are species-specific but others may be

found in several or many plant species of a genus, in several related genera, or even families (Kinghorn *et al.*, 2003).

When one considers that a single plant may contain up to thousands of phytoconstituents, the possibilities of making new discoveries become self-evident (Guri-Fakim, 2006). The crucial factor for ultimate success of isolating bioactive plant constituents is the selection of the “right” plant and the relevant part of the plant which contains the active compounds. Many plants concentrate certain secondary metabolites in specific organs and variation in bioactivity is often encountered between different parts of the same plant (O’Neill and Lewis, 1993).

Plants have their own mechanisms of producing secondary metabolites in small to large amounts and concentrations vary. Many of these secondary metabolites are highly toxic and are often stored in specific vesicles or in vacuole. Several studies (Newman and Cragg, 2007; Kinghorn *et al.*, 2003; Rosenthal and May, 2009) indicate that this kind of storage functions on one hand as a detoxification of the plant itself, and on the other hand to protect itself against pathogens which are present in the soil. The importance of these compounds to plants at large is usually of an ecological nature as they are used as defences against predators, parasites and diseases. Van Wyk and Wink, (2004) reported that there are generally three major groups of secondary metabolites which are:

- (i) Nitrogen containing compounds (e.g. alkaloids, and terpenoids)
- (ii) Phenolics (e.g. flavonoids and tannins)
- (iii) Glycosides

1.2.3 Nitrogen containing compounds

1.2.3.1. Alkaloids

Alkaloids are well known for potent pharmacological activities such as analgesics, anti-malarial, anti-spasmodics and treatment of hypertension, mental disorders and tumours (Rajnikant, 2005). Humans have found numerous uses for plant alkaloids, from medicinal (purgatives, pain relievers, tranquilizers, stimulants, muscle paralyzers) to

agricultural (pesticides and herbicides). Some common examples of plant alkaloids include caffeine and cocaine (Robins, 1994). Previous studies indicate that amaryllidaceae alkaloids have antitumor potential and amongst other characteristics, showed *in vivo* activity against various human viruses (Duri *et al.*, 1994; Hutchings *et al.*, 1996). Fig.1.1 shows the chemical structure of a typical alkaloid with the basic unit of nitrogen from the amino acid.

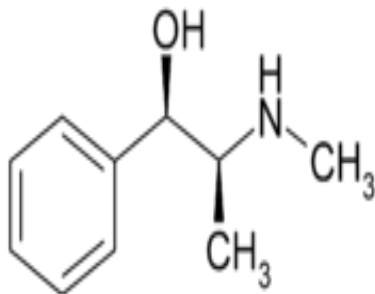


Fig. 1.1. The chemical structure of ephedrine, a phenethylamine alkaloid (Wikipedia, 2010).

1.2.3.2. Terpenoids

Terpenoids are toxins and feeding deterrents to many plant-feeding insects and mammals (Taiz and Zeiger, 2002). Many terpenoids play important roles as plant hormones and in the chemical defences of plants against microbial diseases and insect herbivores (Croteau, 1998). They are reported to have medicinal properties such as anti-carcinogenic, anti-malaria, anti-ulcer, antimicrobial and diuretic activity (Aharoni *et al.*, 2005). Previous studies reported that *Micromeria graeca* has antibacterial activity due to monoterpenes in its essential oil (Marin *et al.*, 2001). Leaves and flowers of *Ludwigia adscendens* have terpenes which possess strong antimicrobial activity (Ahmed *et al.*, 2005). Figure 1.2 shows the chemical structure of a typical terpenoid with the basic 5 carbon skeleton.

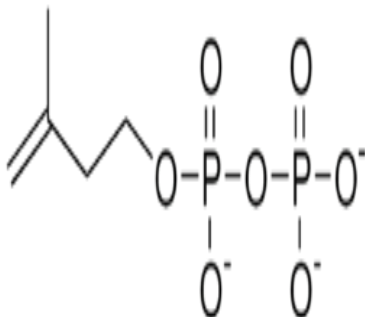


Fig.1.2. The chemical structure of the isopentenyl pyrophosphate, a terpenoid (Wikipedia, 2010).

1.2.3.3. Phenolic compounds

1.2.3.3.1. Flavonoids

Flavonoids are water soluble phenolic molecules containing 15 carbon atoms and are a group of low molecular weight chemical compounds, e.g. the phenylbenzopyrones, found in all vascular plants. They are common constituents of fruit, vegetables, nuts, seeds, stems, flowers, tea, wine and honey (Grange and Davey, 1990). The physiologically active constituents have been used to treat human diseases (Cushnie and Lamb, 2005). These have been reported to possess many useful medicinal properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity (Havsteen, 1983), antiallergic activity (Harborne and Baxter, 1999), antioxidant activity (Middleton and Chithan, 1993), vascular activity and cytotoxic antitumour activity (Harborne and Williams, 2000). Some of the recognized activities of flavonoids include anti-allergic, anti-cancer, antioxidant, anti-inflammatory, anti-viral and many health promoting effects (Harborne, 1996). Figure 1.3 shows the chemical structure of a typical flavonoid with the basic unit of a ketone.

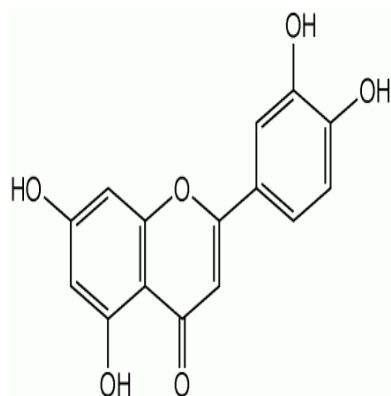


Fig. 1.3. The chemical structure of luteolin, a flavonoid (Wikipedia, 2010).

1.2.3.3.2. Tannins

Tannins are complex group of plant secondary metabolites which are soluble in polar solutions and these are distinguished from other polyphenolic compounds by their ability to precipitate proteins (Silanikove *et al.*, 2001). The amount and type of tannins synthesized by plants vary considerably depending on plant species, cultivars, tissues, stage of development, and environmental conditions (Cornell, 2000). Plant parts containing tannins include the bark, woody part, fruit, fruit pods, leaves, roots and plant galls. These secondary metabolites are evenly distributed in all leaf tissues. Plants containing more than 10% tannins may have potential adverse effects on humans including upset stomachs, renal damage, hepatic necrosis, and an increased risk of oesophageal and nasal cancer (Kemper, 1999). Figure 1.4 shows the chemical structure of a typical tannin with the basic unit of phenol groups.

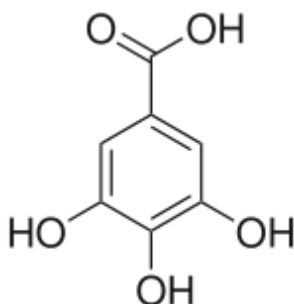


Fig. 1.4. Chemical structure of gallic acid, a tannin (Wikipedia, 2010).

1.3 Identification of bioactive compounds

Numerous methods have been utilized to identify bioactive compounds for drug discovery. Once potential compounds have been identified these can be synthetically made or modified through synthetic chemistry, combinatorial chemistry or molecular modelling to increase potency of the initial compound (Balunas and Kinghorn, 2005). Identification of bioactive compounds involves various steps, as outlined below.

1.3.1 Collection of plant materials

The first step in identifying bioactive compounds is to collect sufficient amount of plant material which is used to isolate compounds from. However, collection of medicinal plants raises a number of complex environmental and social issues that must be addressed locally on a case-by-case-basis. It is acknowledged that these issues vary widely from region to region. In some countries, collection permits and other documents from government authorities and landowners must be obtained prior to collecting any plants from the wild (Woodrow and Miller, 2008).

Collection practices should ensure the long-term survival of wild populations and their associated habitats. The population density of the target species at the collection site(s) should be determined and species that are rare or scarce should not be collected. To encourage the regeneration of source medicinal plant materials, a sound demographic structure of the population has to be ensured. Management plans should refer to the species and the plant parts (roots, leaves, fruits, etc.) to be collected and should specify collection levels and collection practices. It is obligatory on the government or environmental authority to ensure that plant collectors do not place the collected species at risk.

Medicinal plant materials should be harvested during the optimal season or time period to ensure the production of medicinal plant materials of the best possible quality. The time of harvest depends on the plant part to be used. Detailed information concerning the appropriate timing of harvest for some known medicinal plants is often available in

national pharmacopoeias, published standards, official monographs, and major reference books. However, it is well known that the concentration of biologically active constituents varies with the seasons, stage of plants growth and development (Heinrich and Gibbon, 2001). This also applies to non-targeted toxic or poisonous indigenous plant ingredients. The best time for harvest (time of day) should be determined according to the quality and quantity of biologically active constituents rather than the total vegetative yield of the targeted medicinal plant parts. During harvest, care should be taken to ensure that no foreign matter, weeds or toxic plants are mixed with the harvested medicinal plant materials (National Plant Materials Manual, 2010).

Plant collection may involve species with known biological activity for which active compound(s) have not been isolated (e.g. traditionally used herbal remedies) or may involve random collection for a large screening program (Baker *et al.*, 1995). Current strategies for choosing candidate plant species or tissues for isolation of bioactive components are based on ethnobotany.

Ethnobotany is the study of how human beings interact with plants and for what reasons certain plants is used for. It gives information on people's traditional knowledge on plants and helps in the understanding of present uses of plants such as plants for food, medicine, construction, etc. Through the usage of ethnobotany the ethonobotanists are able to relate plants taxonomies and study all the physical and chemical properties of the plants (Minnis, 2000).

Initial screening of plants for possible antimycobacterial activities involves the use of crude extracts extracted by aqueous or organic extraction methods (Houghton and Raman, 1998).

Plant materials can be used fresh or dried. These must be ground to optimize the solvent contact during the extraction process and the weight standardized. Different plant parts (Fig.1.5) may be collected (seeds, leaves, flowers, stem, bark, root, rhizomes, etc.) and dried quickly in drying cabinets to avoid degradation of the components by air or microbes (Willcox *et al.*, 2004). The plant parts most preferred as,

traditional medicines are roots hence the plant part that is essential for the growth of a plant is the most sensitive to harvest is one that is most exploited. Therefore collectors must collect such parts with extreme care to ensure the plant's survival.

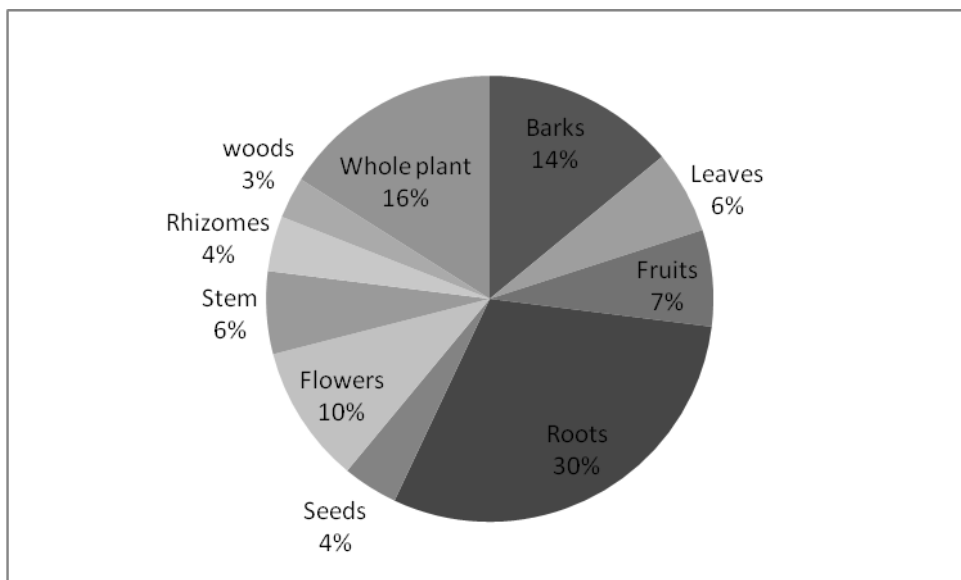


Fig. 1.5. Parts of medicinal plants used in treatment of various diseases (Willcox *et al.*, 2004).

1.3.2 Preparation of plant extracts

In traditional medicinal practice, plant extracts are prepared in various ways including infusions, decoctions and poultices. Application of a remedy is by different routes and methods depending on the perceived cause of the disease and condition (Masika *et al.*, 2000). Since water is a common solvent used by traditional healers, yet most active compounds require solvents of vary polarity, therefore, it would seem unlikely that the traditional healer is able to extract those compounds which are responsible for activity in the non-polar extracts. Water, ethanol, methanol, acetone, chloroform and hexane are some of the common solvents used for extracting bioactive compounds and to determine antibacterial activity from plants (Cowan, 1999). These extracts are complex containing hundreds of different compounds and the isolation of a single bioactive compound is difficult. Except for water, all the other solvents are antimicrobial agents. However, Kotzé and Eloff (2002) showed that the effect of the extractant on subsequent separation procedure is not important, but the extractant should not inhibit the bioassay.

Snyder and Kirkland (1979) tested the efficacy of various extractants which differ in polarity and grouped these ranging from hexane, carbon tetrachloride, di-isopropylether, ethyl ether, methylene dichloride, tetrahydrofuran, acetone, ethanol, ethyl acetate, methanol and water. Eloff (2000) extracted more compounds with higher antibacterial activity with acetone extractant than with sodium bicarbonate. Therefore, acetone was considered as a better solvent than sodium bicarbonate to use when screening a number of plants for antibacterial compounds due to its volatility, miscibility with polar and non-polar solvents and its relatively low toxicity to the test organisms and it is easily removed from the plant material at low temperature (Eloff, 1998b). Generally to extract a wide range of plant compounds four solvents classes are used: non-polar (hexane), slight non-polar (dichloromethane), slightly polar (acetone) and polar (methanol) (Masoko *et al.*, 2006).

There are various extraction methods used and the methods used depend on the target compounds. Below are three of the most commonly used methods of extraction.

1.3.2.1. Solvent extraction

Solvent extraction is the commonly used method to recover a component(s) either from dry or solid plant materials. The sample is contacted with a solvent that will dissolve the solutes of interest. Solvent extraction is of major commercial importance to the chemical and biochemical industries, as it is often the most efficient method of separation of valuable products from complex feedstock or reaction products. Some extraction techniques involve partition separation between two immiscible liquids, others involve either continuous extractions or batch extractions (Gurib-Fakim, 2006).

1.3.2.2. Hot extraction

In hot extraction the plant material are heated with the solvent (water) under reflux. The heating is allowed for some time to extract a number of compounds, mostly insoluble materials such as waxes to lipophilic natural compounds (Gurib-Fakim, 2006).

1.3.2.3 Soxhlet extraction

Soxhlet extraction is used when a compound of low solubility needs to be extracted from a solid mixture. The technique involves the use of a specialised piece of glassware in-between a flask and a condenser. The refluxing solvent repeatedly washes the solid extracting the desired compound into the flask, hence it rely on the polarity gradient of the solvent. Although some component may be destroyed in the process, it is still the best method of extraction used in natural product chemistry (Gurib-Fakim, 2006).

1.3.3 Separation and purification of bioactive compounds

The separation and purification of plant constituents is mainly carried out using one or other, or a combination, of four chromatographic techniques: thin layer chromatography, column chromatography, paper chromatography and high pressure liquid chromatography. The choice of technique or combination depends largely on the solubility, properties and volatilities of the compounds to be separated (Harborne, 1984).

Chromatographic techniques have been instrumental in the separation of natural products. Chromatography is a process whereby a mixture of solutes may be resolved into components by exploiting differences in affinity of the solutes for particles of an insoluble matrix over which a solution of the components is passing. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase (Wagner and Blatt, 1995).

1.3.3.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a fast, relatively cheap and effective method to obtain a characteristic analytical fingerprint of a plant extract (Stoddard *et al.*, 2007). The use of TLC to demonstrate the most characteristic constituents of a plant extract or preparation is favoured for its simplicity, rapidity and affordability (McGaw *et al.*, 2002). Kotzé and Eloff (2002) and Eloff (2001) successfully used TLC analysis on silica gel to compare the chemical composition of various plant parts of some threatened South

African medicinal plants. In certain cases, classes of compounds may be determined by spraying developed plates with stains that give a colour reaction with a particular compound class. Previous studies found that when a vanillin-sulphuric acid spray reagent was applied on TLC-chromatograms more compounds from extract of *Combretum woodii* were visible (Eloff *et al.*, 2005). The same spray reagent was also successfully applied on TLC-chromatograms from several plant extract of *Mikania glomerata*, *Spilanthes acmella*, *Lippia alba*, *Achillea millefolium*, *Piper regnelli*, *Eugenia uniflora*, *Arctium lappa*, *Tanacetum vulgare*, *Erythrina speciosa*, *Psidium guajava*, *Punica granatum*, *Sambucus Canadensis* and *Plantago major* (Holetz *et al.*, 2002).

The TLC method employs glass or aluminium plates pre-coated with the sorbent (e.g. silica gel) to varying thickness depending on the amount of the sample to be loaded. The compound mixture is loaded both in preparative or analytical plates at around 1-2 cm from the bottom of the plate and lowered in a tank containing the solvent. The mixture migrates up the plates and the compound mixture separate according to the polarity of the components. TLC has the advantage of being a highly cost-effective qualitative technique since a large number of samples can be analysed or separated simultaneously. The few drawbacks include poor detection and control compared to high performance liquid chromatography (HPLC) (Ferenczi-Foder *et al.*, 2006).

1.3.3.2. Column chromatography

Column chromatography is a method used to purify individual chemical compounds from mixtures of compounds. It is often used in purification of bioactive compounds from medicinal plants and compounds as small as micrograms to kilograms may be purified for preparative applications. The method requires a glass tube with a diameter from 50 mm and a height of 50 cm to 1 m with a tap at the bottom. The mobile phase with the stationary phase powder (silica gel) is then carefully decanted into the column. It offers a much cheaper and quicker solution to doing multiple injections into prep-HPLC systems (Still *et al.*, 1978).

1.3.3.3. High pressure liquid chromatogram (HPLC)

HPLC is a very popular method and is widely used to separate and quantify compounds for the identification and isolation of bioactive natural products. The method includes the utilization of a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, a UV detector that shows the retention times of the molecules and a fraction collector to collect the mobile phase. The analytical sensitivity can be further enhanced to detect UV molecules by using detection such as a photodiode array (PDA). PDA detection has the advantage of detecting compounds with poor UV characteristics and this is particularly useful in the analysis of natural products such as terpenoids or polyketides, which may not necessarily have chromophores that will rise to a characteristic UV signature (Horváth *et al.*, 1967).

1.4 Bioassays for antimicrobial activity

Bioassay is an evaluation of the biological activity of a substance by testing its static or cidal effects on living organisms such as bacteria, fungi, protozoa, intestinal worms, viruses, etc. and comparing the result with some accepted standards (Feher and Schmidt, 2003). These can range from molecular tissue to whole-organism assays. Bioassays are very crucial stages in assessing the pharmacological actions of plant extracts and their ethnomedical uses. Each has its advantages depending on the objectives. Bioassays commonly used are carried out using the following different methods.

1.4.1. Agar diffusion assays

Agar diffusion involves the inoculation of microorganism on a solid matrix and investigating the movement of molecules (plant extract) through the matrix that is formed by the gelling of agar and its interaction with the target microorganism. When performed under controlled conditions, the degree of the molecule's movement can be related to the concentration of the molecule. This phenomenon forms the basis of the agar diffusion assay that is used to determine the susceptibility or resistance of a microorganism to an antibacterial agent present in the plant extract. The resistance or

susceptibility is determined by the absence or presence of a clearing around the test organism.

1.4.2. Disc diffusion

The disc diffusion method commonly known as Kirby-Bauer is widely used for antibacterial activity tests (Kelmanson *et al.*, 2000). In this method, one species of bacteria is uniformly swabbed onto a nutrient agar plate while plant extract is applied to sterile filter paper discs which are allowed to dry before being placed onto the top layer of the agar plates (Rasoanaivo and Ratsimamanga-Urveg, 1993). Each extract is tested in quadruplicates. The relative effectiveness of a compound is determined by comparing the diameter of the zone of inhibition of bacterial growth around the discs with values in a standard table.

1.4.3. Well diffusion

The agar well diffusion assay uses a similar method as disc diffusion assay, except that the extracts are placed into wells made on the solid agar after the inoculation with standardized bacterial culture. The antibacterial activity is also measured as the diameter (mm) of clear zone of growth inhibition around the well (Hufford *et al.*, 1975).

Most researchers use agar diffusion assays to determine the antibacterial activity of extracts (Eloff, 1998b) however there are limitations to this method, mainly because of the sensitivity of the test to change in operator technique and also in the subsequent interpretation of zone diameter.

The antimicrobial effect of plant extracts may be inhibited or increased by external factors or contaminants. The agar type, salt concentration, incubation temperature and molecular size of the antimicrobial component influence results obtained with agar diffusion assays. This technique does not distinguish between bactericidal and bacteriostatic effects and the minimum inhibitory concentration cannot be determined and it only detects toxicants that can pass through agar (Eloff, 1998b). The technique works well with defined inhibitors but when examining extracts containing unknown

components, there are problems leading to false positive and false negative results. In recent years there has been a move towards a more quantitative method, namely, the measurement of an antimicrobial agent's minimum inhibitory concentration (MIC) (Eloff, 2000).

1.4.4. Dilution assays (Minimum Inhibitory Concentration)

1.4.4.1. Agar-dilution method

The method involves mixing of the plant extract with nutrient agar and allowing it to set (Grierson and Afolayan, 1999). The test organism is streaked in radial patterns on the agar plate. The minimum inhibitory concentration is expressed as the lowest concentration of plant extracts that inhibit bacterial growth. The advantage of Agar-dilution method over serial dilution method is that, the concentration of the plant extract to be used should be known at the beginning before streaking the bacteria. However, it is difficult to prepare sterile plant extract without the use of autoclaving or other aseptic conditions (Mitscher *et al.*, 1972). Furthermore, a larger volume of plant extract is required when using agar-dilution whereas a small amount of volume is needed for serial dilution method.

1.4.4.2. Serial dilution method

A plant extract is mixed with nutrient broth in a microtitre plate and then the cultures are added into the wells. The approximate concentration is known at the start of the experiment before the appropriate number and amount of dilutions are made. Serial dilution method is preferable in that it eliminates a lot of the uncertainty and impreciseness involved in making very small concentrations relative to the stock solution. This method is not cost effective but it also allows for small aliquots to be diluted instead of using large quantities of materials. Some researchers use the microtitre plate to detect the presence of antibacterial activity in plant extracts (Eloff, 1998b; McGaw *et al.*, 2002; Rabe *et al.*, 2002; Eloff *et al.*, 2005). Different plant extracts in solvents such as: ethanol, water, acetone and hexane, can be used for the bioassay. Antibiotic and solvents are included as positive and negative controls. An

equal volume of sterile distilled water and plant extract are added and serially diluted before the bacterial culture is added to each well. The culture mixture is incubated for 24 hours. Iodotetrazolium salt (INT) is added to the microtiter plate to indicate if there is growth (pink colour) or no growth (no colour change). Minimum inhibitory concentration is recorded as the lowest concentration of extract resulting in complete inhibition of bacterial growth (no colour change).

1.4.5. Bioautography

Bioautography is a useful method for bioassay guided fractionation of compounds with antimicrobial activity. It is a very convenient and simple way of testing plant extracts and pure substances for their effects on both human and plant pathogenic microorganisms. It can be employed in the target-directed isolation of active constituents. The antibacterial activity of fractions resulting from each purification stage is tested using the bioautographic assay. An inoculated layer of agar is poured over a developed thin layer chromatography (TLC) plate, and lack of bacterial growth in certain areas identifies the presence and location of antibacterial compounds on the TLC plate. However, some compounds show poor migration through the agar overlay and may not be detected (Gibbons and Gray, 1998). In such cases, it is often better to use a liquid broth medium. The inhibition of bacterial growth by compounds separated on the TLC plate is visible as white spots against a deep red background (Begue and Kline, 1972). The red background is as a result of INT reduction by bacteria into formazan. Eloff (2001) used bioautographic assay to screen antibacterial compounds from *Sclerocarya birrea*. Rabe *et al.* (2002) also screened antibacterial compounds from *Vernonia colorata* and isolated and identified active compounds.

1.5 Identification of chemical structures

The elucidation of the chemical structure is an important task, and for a long time it remained the most time-consuming step in natural product drug discovery (Mahler and Thomason, 2005). New methods have been applied in this field, thus making the task easier and faster. Spectroscopic methods coupled with good separation techniques like

chromatography have contributed to the phenomenal success of natural product chemistry over the past 50 years. Sound strategies have helped in the isolation and characterization of many bioactive molecules (Rios *et al.*, 1991). In particular, mass spectrometry (MS) has contributed to the enhanced ease of structure determination. MS is a method in which individual compounds are identified based on their mass/charge ratio after an artificial ionization. Natural compounds mainly exist as mixtures (when extracted from their origin) so the combination of liquid chromatography and mass spectrometry are often used to separate the individual compounds and determine their mass/charge ratios online. Databases of mass spectra's for known natural compounds are available thus allowing the referencing of the unknown with known compounds so as to determine the nature of the unknown compound. Besides MS, nuclear magnetic resonance (NMR) spectroscopy is another important technique for determining chemical structures of natural products. NMR yields information about individual hydrogen and carbon atoms in the structure allowing detailed reconstruction of the molecule's architecture (Cannell, 1998).

1.6 The classification, distribution, description and uses of the fifteen plants used in this study

1.6.1 *Albizia gummifera*

This plant belongs to the Fabaceae family and its traditional name is fuwem. It is geographically distributed from the northern parts of the eastern parts of South Africa and northern Limpopo throughout the tropical countries up into Senegal in the west and Ethiopia in the east of Africa. The tree can reach a height of over 40 m. The bark is grey to reddish brown and rough. The leaves are bipinnate, each with 8–21(–23) pairs of leaflets with the terminal pair moderately smaller than the rest, 7–21 mm. long, 3.5–8.5 mm (ILDIS, 2005). In traditional medicine the plant has been reported to be used for the treatment of coughs, gonorrhoea, fever, skin diseases, malaria and stomach pains (Kokwaro, 1976).



Fig. 1.6. *Albizia gummifera* (Lemmens, 2007).

1.6.2. *Annona senegalensis*

Annona senegalensis belongs to the family Annonaceae and is found widely distributed in sand forest and dry woodlands on the central, western and eastern side of Africa from Tanzania southwards to the Maputaland region shared by Mozambique and Kwazulu-Natal. Traditionally the plant possesses several medicinal uses including being used as an anthelmintic by local livestock farmers in Nigeria (Ibrahim *et al.*, 1984). The stem, root and bark are used to treat diarrhoea and gastrointestinal troubles (Burkill, 1985), while the stem bark and leaves are used for the treatment of skin cancer and leukemia (Abubakar *et al.*, 2007).



Fig 1.7. *Annona senegalensis* (Medicinal Plants in Nigeria, 2010).

1.6.3. *Antidesma venosum*

Antidesma venosum belongs to the family Euphorbiaceae and it's widespread from Senegal to West Cameroons, and elsewhere in tropical and Southern Africa. The plant is a tree which can grow up to 10 m high. Leaf decoctions are used for abdominal cramps and dysentery (Hutchings *et al.*, 1996).



Fig. 1.8. *Antidesma venosum* (Hyde and Wursten, 2010).

1.6.4 *Apodytes dimidiata* subspecies *dimidiata*

This plant belongs to the family Icacinaceae and its common name is white pear. It is one of the best-known forest trees in Southern Africa as it is found from Table Mountain in the Cape Peninsula, along the coast through to Kwa-Zulu Natal, Gauteng, Swaziland and Kenya. This is a small bushy tree 4 to 5 m tall but reaching a height of 20 m when growing in a forest. It has glossy, bright green leaves which have a paler green, dull underside (Killick, 1973). This tree is valued by the Zulu nation in traditional medicine. An infusion from the root bark is used as an enema for intestinal parasites. The leaves are used in the treatment of ear inflammation. Root and stem bark have the highest molluscicidal activity (Pretorius *et al.*, 1991).



Fig.1.9. *Apodytes dimidiata* (*The World Botanical Associates*, 2007).

1.6.5. *Barringtonia racemosa*

Barringtonia racemosa belongs to the family Lecythidaceae and it's commonly known as powderpuff tree. *Barringtonia racemosa* is the only indigenous species of this genus occurring in South Africa and the plant is found in very humid, moist conditions. It is common along tropical and subtropical coasts in the Indian Ocean, starting at the east coast of South Africa. It is also common in Mozambique, Madagascar, India, Sri Lanka, Malaysia, Thailand, Laos, and Southern China. The leaves are alternate and carried in clusters at the ends of branches and are 180-320 x 55-145 mm, with petioles 5-12 mm long. It has a straight, unbranched stem that leads to a rounded crown and is usually 4-8 m tall, but occasionally reaches 15 m. The bark is greyish brown to pink with white blotches and raised dots and lines (Strey, 1976). The seeds, bark, wood and roots contain the poison saponin and is used to stun fish. Extracts from the plant are effective insecticides and are also used medicinally in South Africa. The Zulus use the fruit to treat malaria. The fruits are effective in cough, asthma and diarrhea, and the seeds are aromatic and useful in colics and ophthalmia (Nadkarni, 1982).



Fig. 1.10. *Barringtonia racemosa*.

1.6.6. *Kigelia africana*

It belongs to the family Bignoniaceae and it's also known as the sausage tree. The genus *Kigelia* has one species and occurs only in Africa. The tree is found on riverbanks, where it may reach 20 m, along streams and on floodplains and also in open woodland, from KwaZulu-Natal to Tanzania. The short, squat trunk has light brown, sometimes flaky bark and supports a dense rounded to spreading crown (18 m high, 20 m wide) of leathery, slightly glossy foliage (deciduous). The huge, grey-brown fruits, 800 x 120 mm. hang from long stalks, from December to June and weigh anything up to 9 kg. Traditional remedies prepared from crushed, dried or fresh fruits are used to deal with ulcers, sores and syphilis - the fruit has antibacterial activity. Powdered fruit are applied to sores and wounds used for rheumatism (Van Wyk and Wink, 2004). The bark is used to treat dysentery and stomach ailments (Van Wyk and Wink, 2004).



Fig. 1.11. *Kigelia africana*.

1.6.7. *Kirkia acuminata*

Kirkia acuminata belongs to the family Simaroubaceae with a vernacular name white syringe. The plant is distributed throughout Southern Africa and extends from Gauteng, Botswana, Namibia and to the north in Tanzania. It's a tree with a straight-stem and round. It grows from 6 to 18 m high with a trunk diameter of 0.8 m. The leaves are sticky when young, colouring splendidly to gold and red in autumn. The leaf is compound with 6-10 leaflets and one terminal one. The narrowly ovate leaflets are 20-80 x 10-25 mm, with or without hairs. In South Africa the wood is made into furniture and floor blocks. An infusion of the bark is taken against vomiting and abdominal pain and an infusion of the root is taken to treat cough (Van Wyk and Wink, 2004).



Fig. 1.12. *Kirkia acuminata* (Kirstenbosch NBG, 2004).

1.6.8. *Macaranga capensis*

Macaranga capensis belongs to the genus *Macaranga* and it's the largest genus of Old World tropical trees of the family Euphorbiaceae. The genus comprises over 300 different species widely distributed throughout Africa, Australasia, Asia and the South Pacific. The associated species *M. Peltata* and *M. Indica* are used for treatment of venereal sores (Kirtikar and Basu, 1984); cuts, wounds and stomach-ache (Jain *et al.*, 2004).



Fig 1.13. *Macaranga capensis* (Hyde and Wursten, 2010).

1.6.9 *Maytenus* species

The genus *Maytenus* consists of \pm 150 species that occur mainly in the tropics and subtropics of both the northern and southern hemispheres. In southern Africa there are \pm 11 species, widespread throughout the region. *Maytenus senegalensis* and *Maytenus udanta* belongs in the spike-thorn family, Celastraceae, a large, cosmopolitan and diverse family of trees, shrubs and woody climbers. Their leaves are hard and leathery, bright green with a bluish bloom. They are simple, alternate and egg-shaped to oval with a narrowed base, the midrib is ridged above, the margins are untoothed and rolled back and the apex is rounded, sometimes notched. The leaves of *M. senegalensis* are used for treatment of diarrhoea and intestinal worms in calf, dog bites (Koné and Kamanzi, 2008); tuberculosis (Lall and Meyer, 1999).



Fig. 1.14. (A) *Maytenus udanta* and (B) *Maytenus senegalensis*(Hyde and Wursten, 2010).

1.6.10. *Millettia stuhlmannii*

Millettia stuhlmannii belongs to family Fabaceae and it's found throughout the Congo and southern regions of Africa, Tanzania and Mozambique. This species is reported to be rather secure with very little threat to its existence within most of its growth range including the Congo, but it is officially classified as extinct, endangered or rare in Cameroon. The tree grows to heights of 60 to 90 feet with a trunk diameter of 3 to 4 feet. The bark from the tree is used for its toxins to stun fish for harvest. It is also used in parts of Africa for mask carving. The plant is often used for treatment of toothache and spleen disorders (Jain, 1991).



Fig. 1.15. *Millettia stuhlmannii* (Hyde and Wursten, 2010).

1.6.11. *Sclerocarya birrea*

Sclerocarya birrea is commonly known as the marula tree and it belongs to the family Anacardiaceae (mango family). The marula tree is widespread in Africa from Ethiopia in the north to KwaZulu-Natal in the south and is abundant in the in Limpopo Province (South Africa). It occurs naturally in various types of woodland, on sandy soil or occasionally sandy loam. It is a medium-sized to large deciduous tree with an erect trunk. A decoction of the bark is used to treat dysentery, diarrhoea, rheumatism and it has a prophylactic effect against malaria. The bark is an excellent remedy for haemorrhoids. Roots and bark are also used as laxatives. A drink made from marula leaves is used for the treatment of gonorrhoea (Van Wyk and Wink, 2004).



Fig. 1.16. *Sclerocarya birrea*.

1.6.12. *Vangueria infausta* subspecies *infausta*

Vangueria infausta subspecies *infausta* belongs to the family Rubiaceae and has an English common name wild medlar. The plant is distributed from the Eastern Cape, Free State, KwaZulu-Natal, Swaziland, Mpumalanga, Gauteng, Limpopo, and North-West to Northern Cape and it is common in open, exposed grassland. This is a deciduous shrub that varies in height from 3-7 m depending on the habitat. It can be single or multi-stemmed but usually the latter. The bark is greyish to yellowish brown, smooth and peeling in irregular small strips. The branchlets are covered with short, woolly hairs, especially when young. The leaves are light green in colour, covered with soft, velvety short hairs when young (Steel and Behr, 1996). It is fed to cattle suffering

from East Coast Fever, and people take it as a cure for parasitic worm infections in the form of a decoction (De Boera *et al.*, 2005), anthelmintic action (Teichler, 1935) and antiplasmodial activity (Nundkumar and Ojewole, 2002)



Fig. 1.17. *Vangueria infausta* subsp. *infausta*.

1.6.13. *Warburgia salutaris*

Warburgia salutaris belongs to the family Canellaceae with a common name pepper-bark tree. This is a tropical forest tree that extends southwards as far as KwaZulu-Natal, eastern and northern Gauteng and across Swaziland. Its growth habitat is forests and kloofs. This is an evergreen, slender tree that grows from 5 to 10 m tall. The dark green, glossy leaves are paler green below with entire margins, and are simple, alternately arranged, elliptic to lanceolate. The midrib is slightly off-centre with the tapering apex and base. The leaves have a bitter, burning, aromatic taste. The stem is covered by a rich brown bark that is also bitter and peppery. Medicinally, the pepper-like, bitter stems and root bark are used as a remedy for common colds (Hutchings *et al.*, 1996). Dried and ground, these make a snuff used to clear the sinuses. Taken orally these are believed to cure spots in the lungs, sores in the mouth and as a natural antibiotic to treat chest infections (Van Wyk and Gericke, 2000).



Fig. 1.18. *Warburgia salutaris* (Van Wyk and Gericke, 2000).

1.6.14. *Xanthocercis zambesiaca*

Xanthocercis is a tree genus in the family Fabaceae and there are two species: *Xanthocercis madagascariensis* and *Xanthocercis zambesiaca*. *Xanthocercis zambesiaca* is commonly known as Nyala tree. The plant is commonly found in Malawi, Mozambique, South Africa, Zambia and Zimbabwe. It is an evergreen tree that branches low down and can grow as high as 30 cm. The leaves are dark green and shiny, with about 7 pairs of leaflets with a terminal leaflet. It is rough, does not peel, but is cracked into small, irregular squares. The flowers are small with a prominent stamen. Sweet-scented, white to cream sprays grow at the end of the branches.



Fig. 1.19. *Xanthocercis zambesiaca* (Hyde and Wursten, 2010).

1.7. Microbiology and pathogenicity of *Mycobacterium*

There are more than 70 species of *Mycobacteria* and two of these are major pathogens, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The remaining *Mycobacteria* are environmental organisms (non-tuberculous mycobacteria) and are responsible for opportunistic infections, especially in people with HIV/AIDS (Ghosh *et al.*, 2009). *Mycobacterium tuberculosis* and *Mycobacterium leprae* are most common in developing countries and cause tuberculosis and leprosy, respectively. These organisms have cell walls of unusually low permeability, which contribute to their resistance to therapeutic agents (WHO, 2000).

Mycobacterium tuberculosis is a pathogen which enters the human host through inhalation of small sputum particles aerosolized by the coughing of a patient of positive pulmonary tuberculosis. The bacterium infects the lung by multiplying within alveolar cells and induces the formation of granulomas (tubercles) (WHO, 2000). As with other bacterial pathogens, surface and secreted proteins of *Mycobacterium tuberculosis* contribute significantly to its virulence. The organism is difficult to treat because it can colonize their host without showing any serious clinical symptoms during the initial infection, hence billions of people contract it every year (Parish and Brown, 2009). Its cell wall is neither truly Gram negative nor positive. Mycobacterial cells are naturally resistant to a number of antibiotics which disrupt cell-wall biosynthesis due to their unique cell wall and can survive long exposure to acids, alkalis, detergents, oxidative bursts and many antibiotics (Parish and Brown, 2009).

1.7.1. Treatment of *Mycobacterium tuberculosis*

Tuberculosis (TB) is the most common disease which affects approximately one third of the human population and is the largest cause of death among the black population in South Africa (Lall and Meyer, 1999). Tuberculosis is currently treated with antibiotics for extensive time periods (6 months or longer) since the organism grows slowly and may become dormant. The treatment involves the use of two or more antibiotics (including rifampin and isoniazid) to reduce the possibility of resistance developing during this

extended time. Isoniazid and rifampin which are part of this combination medication may cause severe (sometimes fatal) liver problems and hepatitis can develop with use of isoniazid drug at any time during treatment (Hardman *et al.*, 2001). Isoniazid inhibits the synthesis of mycolic acid and rifampin prevents transcription and translation of RNA to proteins and both drugs are bactericidal (Ghosh, *et al.*, 2009). Some of the side effects are fatigue, weakness, loss of appetite, nausea, vomiting, dark urine, yellowing of the eyes or skin or abdominal pain but some of the side effects are rare. While the current drugs can cure TB it nevertheless still kills people who do not have access to these drugs because of costs but more seriously because of the prolonged treatment regime people do not complete the course of treatment. This serious public health problem has led to a renewed initiative to search for other effective novel drugs including structural compounds obtained from higher plants (Cantrell *et al.*, 2001). The idea is to find compounds which can be developed into drugs which act within a shorter period (less than 6 months) to cut cost of treatment and also reduce the side effects of the current treatment.

1.7.2. Medicinal plants as a potential source of anti-*Mycobacterium tuberculosis*

While plants have been used successfully for treatment of various infectious diseases, there is limited scientific information available on the use and success of bioactive compounds from plants for the treatment of *Mycobacterium tuberculosis*. Some terpenes, such as citronellol, nerol and geraniol have shown moderate antimycobacterial activity (Cantrell *et al.*, 2001).

Aromatic plants have been used since ancient times for their preservative and medicinal properties, as well as to impart aroma and flavor to food (Edris, 2007). The pharmaceutical properties of aromatic plants are partially attributed to essential oils. Essential oils are natural, complex, multi-component systems composed mainly of terpenes along with a few non-terpene components (Edris, 2007). The ancient Egyptians used aromatic plants in embalming to stop bacterial growth and prevent decay, an effect largely attributed to their essential oil content. Strong *in vitro* evidence

indicates that essential oils can act as antimycobacterial agents against a wide spectrum of pathogenic bacterial strains (Edris, 2007).

Gupta *et al.* (2010) screened five plants extracts against one of the multi drug resistant (MDR) strain of *Mycobacterium tuberculosis* H37RV. Their results showed that all tested plants exhibited activity against MDR *Mycobacterium tuberculosis* H37RV while *Acalypha indica* and *Allium sativum* had high inhibition 95% and 72%, respectively. They concluded their study by isolating and identifying of active substances from the extracts which exhibited promising activities as part of their next publication (Gupta *et al.*, 2010). Buwa and Afolayan, (2009) screened three plants against *Mycobacterium aurum*. DCM extract of *T. violaceae* exhibited MIC value of 0.780 mg/ml indicating good activity when compared to other plant extracts.

Since *Mycobacterium tuberculosis* grows slow and it is pathogenic to humans the initial approach is to test plant extracts on *Mycobacterium smegmatis* and only once a compound has been purified to then test it on *Mycobacterium tuberculosis*. This approach cuts down time, costs and reduces the chances of the researcher being infected with *Mycobacterium tuberculosis*. *Rhodococcus erythropolis* was also included since its cell membrane is made up of long fatty acids (mycolic acids) similar to *mycobacterium species*. Mycolic acids of mycobacterium have functional groups whereas *Rhodococcus* does not have functional groups (Sutcliffe, 1998). The rationale for the study was to identify and isolate compounds which may be potential drugs for treatment of TB hence the use of *Mycobacterium smegmatis* and *Rhodococcus erythropolis*. The two species were selected based on previous study by Chaturvedi *et al.* (2007) where the species give reliable results and other than that they are non-pathogenic, fast-growing. When used in cell viability based screen, they serve as an alternate for multi-drug resistant (MDR) *Mycobacterium tuberculosis*. A brief overview of their microbiological properties and pathogenicity are discussed below:

1.7.3. *Mycobacterium smegmatis* and *Rhodococcus erythropolis*

Mycobacterium smegmatis is 3.0 to 5.0 μm long bacilli, aerobic and nonmotile bacterium. *Mycobacterium smegmatis* is an acid-fast bacterial species and is generally considered a non-pathogenic microorganism. However, in some very rare cases it may cause disease in an immunocompromised animal. Its major component of the cell wall is made up of long fatty acid chain (mycolic acids) with functional groups attached to it. The percentage of carbon atoms differs from species to species and the presence of mycolic acids gives *Mycobacterium tuberculosis* many characteristics that resist medical treatment (Steck *et al.*, 1978). Mycolic acids contribute to the organism's increased resistance to chemical damage and dehydration, and prevent the effective activity of hydrophobic antibiotics. In addition mycolic acids are important for survival and pathogenesis by allowing the bacterium to grow readily inside macrophages and effectively hiding from the host's immune system hence it is difficult to treat because of its asymptotic characteristics (Bhatt *et al.*, 2007).

Mycobacterium smegmatis is commonly used in research analysis of other *mycobacterium* species in laboratory experiments due to its fast growing capability and being non-pathogenic (McGaw *et al.*, 2008). Figure 1.20 shows typical cells of *Mycobacterium smegmatis* stained with the acid fast stain method.

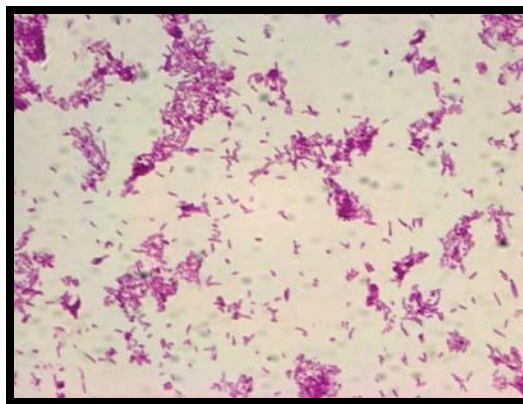


Fig.1.20. *Mycobacterium smegmatis* (Reynolds *et al.*, 2009).

Rhodococcus erythropolis is an aerobic, non-sporulating, non-motile gram-positive bacterium closely related to *Mycobacteria* and *Corynebacteria species*. They are grouped within the same phylum Actinobacteria and shares the same characteristics of the cell wall structures (Van der Geize and Dijkhuizen, 2004). Few species of *Rhodococcus* are pathogenic but most are non-pathogenic such as *Rhodococcus erythropolis* and have been found to thrive in a broad range of environments, including soil and water. *Rhodococcus erythropolis* is also an experimentally advantageous organism to use due to its relatively fast growth rate and simple developmental cycle (Goodfellow & Alderson, 1979). The genus *Rhodococcus* is closely related to *Mycobacterium* and includes the species *Rhodococcus equi*, a facultative intracellular pathogen of macrophages in different animals (Hondalus, 1997). Figure 1.21 shows Gram stained cells of *Rhodococcus erythropolis*.

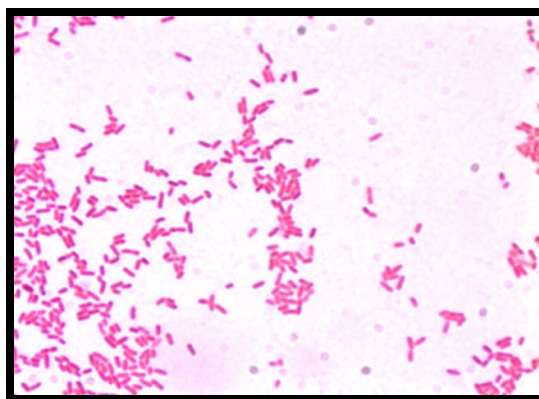


Fig. 1.21. *Rhodococcus erythropolis*.

1.8. Rationale and hypothesis for the study

The structural diversity of plant-derived antimycobacterial compounds makes plants suitable for alternative lead chemical structures for drug development. Several recent reviews (Newman and Cragg, 2007; Newton *et al.*, 2000; Kinghorn *et al.*, 2003; Rosenthal and May, 2009) emphasize the potential of plant species and natural products as sources of antimycobacterial extracts and chemicals is growing. Medicinal plants are used in many parts of Southern Africa to treat TB-related symptoms including chest complaints and coughing. Under section 1.6.2 we referred to some scientific studies in which some plants showed anti-microbial activities against mycobacterium.

Based on these observations it was reasonable to assume that plants traditionally used for treatment of TB-related symptoms may contain bioactive compounds which can be scientifically identified and isolated for treatment of TB. Hence, fifteen plants which have been reported to be used by some traditional healers for treatment of chest and coughing related diseases were screened for potential bioactive compounds for tuberculosis. The approach was to first screen the plants against non-pathogenic bacterial species closely related to *Mycobacterium tuberculosis*, the aetiological agent of TB. It was hoped that once good potential bioactive compounds were identified and purified these could then, in a separate study, be tested against *Mycobacterium tuberculosis*.

1.9. Aim and objectives

1.9.1. Aim

The aim of the study was to scientifically evaluate the antimycobacterial activities of selected indigenous medicinal plants, so as to see if we could identify and purify good lead compounds which may be used for the treatment of humans infected with *Mycobacterium tuberculosis*.

1.9.2. Objectives

The objectives of the study were to:

- (i)** Extract compounds from the leaves of 15 selected plants using hexane, dichloromethane, acetone, and methanol as extractants;
- (ii)** Analyze the phytochemical properties of each extract;
- (iii)** Evaluate the antioxidant properties of each extract;
- (iv)** Determine the minimum inhibitory concentration of each plant extracts against *Mycobacterium smegmatis* and *Rhodococcus erythropolis*;
- (v)** Screen for antimycobacterial compound(s) which may be present in the plant extracts using bioautography;
- (vi)** Isolate and purify active compound(s) which has the best antimycobacterial activities.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Collection of medicinal plants

Fifteen medicinal plants were selected based on a list of plants which were crudely screened by the Phytomedicine Programme at the University of Pretoria, (Prof. J.N. Eloff's laboratory). Plants which demonstrated minimal inhibitory concentrations of <0.1 mg/ml against *Mycobacterium smegmatis* and whose ethnopharmacological information were known were selected for this study. The plants which were selected for this study were *Albizia gummifera* (J.F. Gmel.) C.A. Sm., *Annona senegalensis* Pers, *Macaranga capensis* Baill. Benth. ex Sim, *Maytenus undata* (Thunb.) Blakelock, *Antidesma venosum* E Mey. ex Tul., *Maytenus senegalensis* (Lam.) Excell, *Xanthocercis zambesiaca* (Baker) Dumaz-le Grand., *Sclerocarya birrea* (A.Rich.) Hochst., *Millettia stuhlmannii* Taub., *Barringtonia racemosa* (L.) Roxb., *Kigelia Africana* (Lam.) Benth., *Kirkia acuminata* Oliv., *Warburgia salutaris* (Bertol. f.) Chiov., *Vangueria infausta* subsp *infausta* Burch., *Apodytes dimidiata* subsp *dimidiata* E.Mey. ex Arn. Only the leaves were used in this study because they are easy to work with and their harvesting does not adversely affect the plant. Leave samples of the referenced plants were collected at the University of Limpopo and at the Nelspruit Botanical Garden.

2.2. Preparation of crude extracts

The leaves were allowed to dry by natural evaporation at room temperature by spreading the leaves on a flat surface. The names of the collected plants were verified by the University of Limpopo Herbarium (Ms B Egan) where the reference specimens are kept. The dried leaves were ground into fine powder using Waring commercial electric blender (model 32BL80) and the samples were stored in airtight glass containers in a dark place to prevent oxidation until the extraction stage.

The crude extracts were prepared according to the method of Kotzé 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 either hexane, dichloromethane (DCM), acetone or methanol in 50 ml polypropylene tubes. The tubes were vigorously shaken for 10 min at high speed. After centrifugation at 959 g for 10 min, the supernatants were decanted into pre-weighed 50 ml Erlenmeyer flasks. The extraction process was repeated three times to thoroughly extract the plant material. The solvents were evaporated in the fume cupboard at room temperature and the remaining matter was quantified.

2.3. Test organisms

Antimycobacterial activity was tested against *Mycobacterium smegmatis* mc²155 and *R. erythropolis* ATCC 4277. The bacterial strains were a gift from the School of Molecular and Cell Biology, University of Witwatersrand. *Mycobacterium smegmatis* was maintained on Middlebrook 7H9 broth containing 0.05% Tween 80 and 10% v/v ADC supplement (Albumin Fraction V, Dextrose and Catalase) and *R. erythropolis* on Luria Bertani broth (LB) at 37°C. The purity of the cultures were ensured by means of the Ziehl-Neelsen staining and Gram staining, respectively, before being used in the antimicrobial assays.

2.4. Screening of plants

2.4.1. Phytochemical screening

The main compounds of the plant extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F₂₅₄) according to the method of Kotzé and Eloff (2002). The TLC plates were developed under saturated conditions with each of the three mobile phases viz. (1) ethyl acetate: methanol: water (40:5.4:4), [EMW] (polar/neutral); (2) chloroform: ethyl acetate: formic acid (30:24:6), [CEF] (intermediate polarity/acidic); and (3) benzene: ethanol: ammonia hydroxide (72:8:0.8): [BEA] (non-polar/basic). The separated compounds on the chromatograms were visualized under UV light and sprayed with vanillin-sulphuric acid and heated at 110°C for colour development. The sprayed plates were scanned with a laser scanner and analysed.

2.4.2. Antioxidant assay

The chromatograms were prepared as in Section 2.4.1. The chromatograms were sprayed with 0.2% 2,2-diphenyl-2-picrylhydrazyl (DPPH) to visualize any potential antioxidant compounds within the separated plant extracts. Their presence was detected by yellow spots against a purple background on TLC plates. The bands/compounds showing the antioxidant properties were compared to the bands showing the antimycobacterial activity to determine whether the observed antimicrobial properties were as a result of antioxidant property of the extracts or other activities.

2.4.3. Minimal inhibitory concentration

The lowest concentration of active extracts which inhibits the test organisms was determined using the micro-dilution assays in a 96 well micro-plates, as described by Eloff (1998a). One hundred microliters (100 μ l) of sterile distilled water was dispensed in all the wells of a 96 well microtiter plate. Methanol-, acetone-, hexane- and dichloromethane-extracts were re-dissolved in acetone to a concentration of 10 mg/ml, following which 100 μ l of each were added into the first well of a 96 well plate and serially diluted. One hundred microliters of standardized broth cultures were dispensed in all the wells and incubated at 37°C for 24 hrs. After incubation, 40 μ l of 0.2 mg/ml of *p*-iodonitro-tetrazolium violet (INT) were added to all the wells to determine the presence of bacterial growth in the plates. After the addition of INT, the plates were further incubated at 37°C for 45 minutes to allow colour change (during the active growth of bacteria, INT is reduced from a colourless colour to pink-red colour indicating growth). The MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth after 24 hrs and each extract was tested in triplicate. The experiment was repeated two times and the results were recorded as the mean from two triplicates of independent experiments.

2.4.4. Bio-autographic assays

Bio-autographic analyses were 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 μ l of 10 mg/ml solution of each extract as detailed under

phytochemical analysis (section 2.4.1.). The plates were developed in EMW, CEF, and BEA solvent systems. Bio-autograms were left to fan-dry for 3-5 days to completely evaporate the mobile phases and each bioautogram was sprayed with each of the bacterial strains and then incubated at 37°C for 24 hrs in humid conditions. After incubation the bioautograms were sprayed with a visualization stain (INT), and incubated further at 37°C for 2-4 hrs in sealed plastic boxes to allow the pink colour to develop. The appearances of clear zones/white spots on the bioautograms were considered as areas of growth inhibition whereas a pink-red colour indicated growth.

2.5. Isolation of bioactive compounds

2.5.1. Preparation of crude extracts

Exhaustive extraction method was used to extract ground leaves powder (2 kg) of *Apodytes dimidiata* subsp *dimidiata* with acetone. Plant material was extracted for three to four hours with 6 litres of acetone. The extracts were then filtered and concentrated using a Büchi rotary evaporator (Labotec) under reduced pressure, rotating at 100 rpm and the water bath temperature of 40°C. The concentrated extracts were transferred into pre-weighed beakers, dried under fan and weighed.

2.5.2. Column chromatography

The solvent-solvent fractionation was selected to simplify extracts by fractionating the chemical compounds into broad groups based on their solubilities. A Bucher funnel (13 cm x 5.7 cm, 150 g) was packed with 1.13 kg silica gel 0.04-0.063 mm (MERCK). Eighty five grams of finely ground acetone extract was thinly spread on top of the overnight packed silica gel and then covered with cotton wool and eluted with 100% DCM to 100% methanol (MEOH). The column was eluted with 4 L of 100% DCM, 2 L of 90:10 DCM: MEOH, 2 L of 70:30 DCM: MEOH, 2 L of 50:50 DCM: MEOH, 2 L of 30:70 DCM: MEOH and 1 L of 100% MEOH and sub fractions (250 ml) of each fraction were collected in Erlenmeyer flasks.

2.5.3. Analysis and bioassays of fractions

A total of 49 sub-fractions were collected and concentrated using the rotary evaporator (Büchi Rotavapor R-210/215) under reduced pressure, rotating at 100 rpm and with water bath at 40°C. The 100% DCM and 90:10 DCM: MEOH fractions were phytochemically analysed using thin layer chromatography (10 µl of 10 mg/ml extract) on Merck TLC F254 plates with HEX: ETAC 90:10. The 70:30 DCM: MEOH fractions were eluted with HEX: ETAC 80:20 and 50:50, 30:70 DCM: MEOH and 100 MEOH fractions with HEX: ETAC 70:30. The separated components were visualised under visible and ultraviolet light (254 and 360nm) plates were then sprayed with vanillin-sulphuric acid and slightly heated (Carr and Rogers, 1986). A qualitative assay of extracts was done using the method described under section 2.2.4. The fractions with the compound(s) of interest were pooled and re-eluted.

2.5.4. Purification of pooled and unseparated compounds

To select the best mobile phase for eluting the pooled fractions and unseparated compounds, 10 µl of 10 mg/ml extract was spotted on TLC and ran with combination of solvents systems as to determine which system gave the best separation of compounds.

Fractions A-(F4 - F6), B-(F8 - F10) and C-(F13 - F16) of 100% DCM with mass of 9.9 g, 1.22 g and 2.06 g, respectively were pooled based on the profile given by results from section 2.5.3. A column for fraction A was packed with 300 g silica gel (Kieselgel 60, 0.015-0.04) suspended in DCM and left for 2 hrs to swell after which it was poured into the column. The fraction was added to the top of the column using a pipette with great care so as not to disturb the top of silica gel in the column. The column was eluted with 100% HEX, 98:2 HEX: ETAC, 96:4 HEX: ETAC and 80:20 HEX: ETAC using gravitational force to facilitate elution. Fractions of 25 ml were collected and analysed as described in section 2.5.3. Sub-fraction (1-5) for 100% HEX, 97.5:2.5 HEX: ETAC and 95:5 HEX: ETAC were pooled based on the results obtained from section 2.5.3; the same applies to 90:10 HEX: ETAC AND 85:15 HEX:ETAC sub-fractions.

The sub-fractions of 90:10 and 85:10 were further re-eluted in a silica gel column using 3:1 DCM:ACE to reduce the impurities in the fraction.

2.5.4.1. Preparative TLC

Both fractions were dissolved in small amounts (c. 2 ml) of acetone in which they would dissolve and applied in a band across the preparative TLC plates (Silica gel 60 F₂₅₄). The plates for the first fraction was developed with 100 % hexane and the second fraction with 3:1 DCM:ACE. The bands were visualized under ultraviolet light (254 and 360 nm) before a small part on the side of the plates were sprayed with vanillin-sulphuric acid and heated with a heating gun. The rest of the bands/compounds on the plates were protected with glass and an aluminium foil against the damage by spray and heat. The visualised bands on the side were used as the reference line for scraping the remaining compound/band on the plate with a glass rod. The components were collected into separate beakers and crushed into fine powders using a glass rod. The silica powder was eluted with hexane and acetone for compound A and B, respectively. The volume of solvent was dependent on the quantity and pigment recovered by filtration through a cut glass pipette plugged with cotton wool to facilitate the removal of impurities. The process was repeated thrice or until the silica gel powder regained its original colour. Each purified sample was then evaporated and weighed and thereafter transferred into separate brown vials.

2.5.5. Characterization of pure compounds by nuclear magnetic resonance (NMR)

The clean samples were sent to Mr F. H. Makhubela of the Chemistry department, University of Limpopo (Medunsa Campus). ¹H NMR and ¹³C NMR spectra of the two purified components were run with Varian Gemini 300 MHz NMR Spectrometer (Merck) at 300 MHz using acetone as the solvent signal reference. The spectra analyses and structure elucidation was performed by Dr L. Mdee of the Pharmacy department, University of Limpopo (Turfloop Campus).

CHAPTER THREE

RESULTS

3.1. Mass of plant extracts

The amount of sample extracted was measured in mg as shown in Figures 3.1. Different quantities of extracts were obtained as indicated by the histogram. Plant materials extracted with methanol yielded more extract than the plant material extracted with the other solvents.

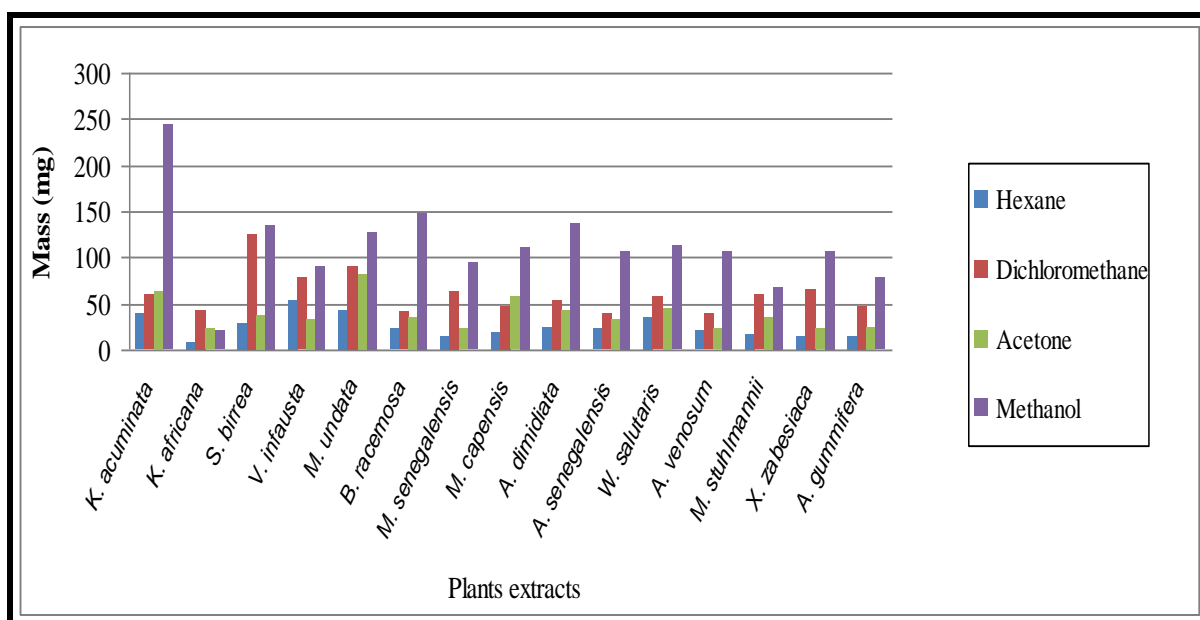


Fig. 3.1. Mass of samples extracted by different solvents with varying polarity from 1 g of starting material.

3.2. Phytochemical analysis of plant extracts

TLC fingerprinting was used for the phytochemical analysis of the plant materials extracted using hexane, DCM, acetone and methanol. A number of distinct bands were observed on TLC plates indicating the different phytoconstituents which reacted with vanillin-sulphuric acid (Fig. 3.2 – Fig. 3.6). The TLC plate's shows the separation of different compounds when developed in BEA, EMW and CEF with most of the bands on TLC plates developed with BEA. Fluorescing and quenching bands were circled with a pencil. Some of the compounds could not be moved from the base of the TLC plates.

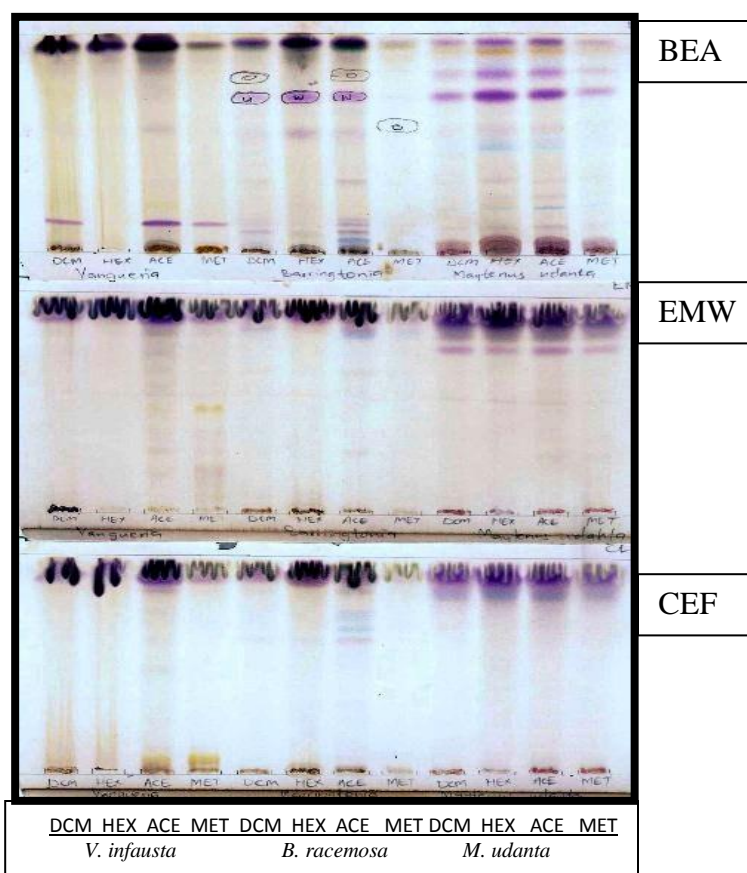


Fig. 3.2. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with vanillin-sulphuric acid to indicate separated compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

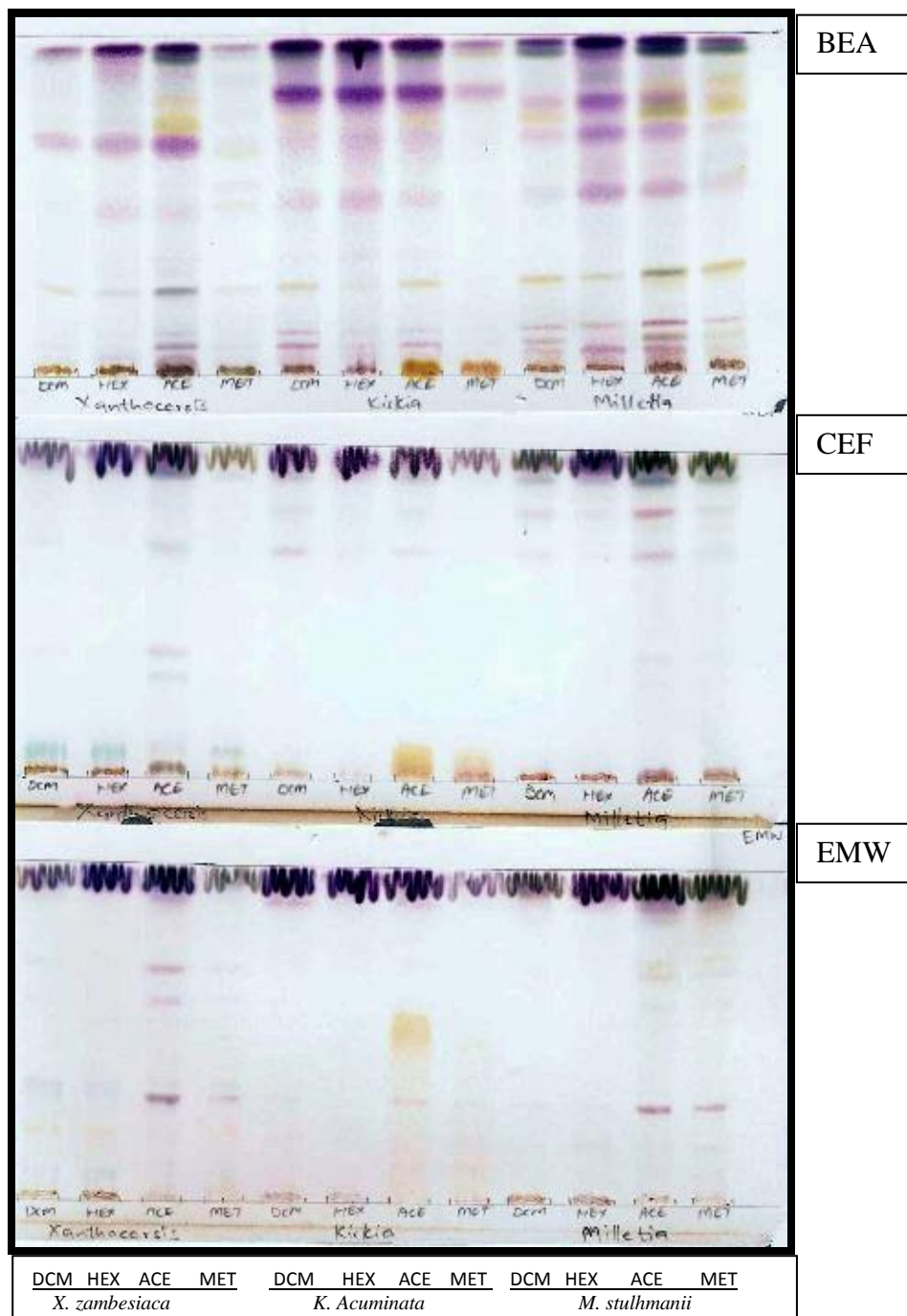


Fig. 3.3. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with vanillin-sulphuric acid to indicate separated compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

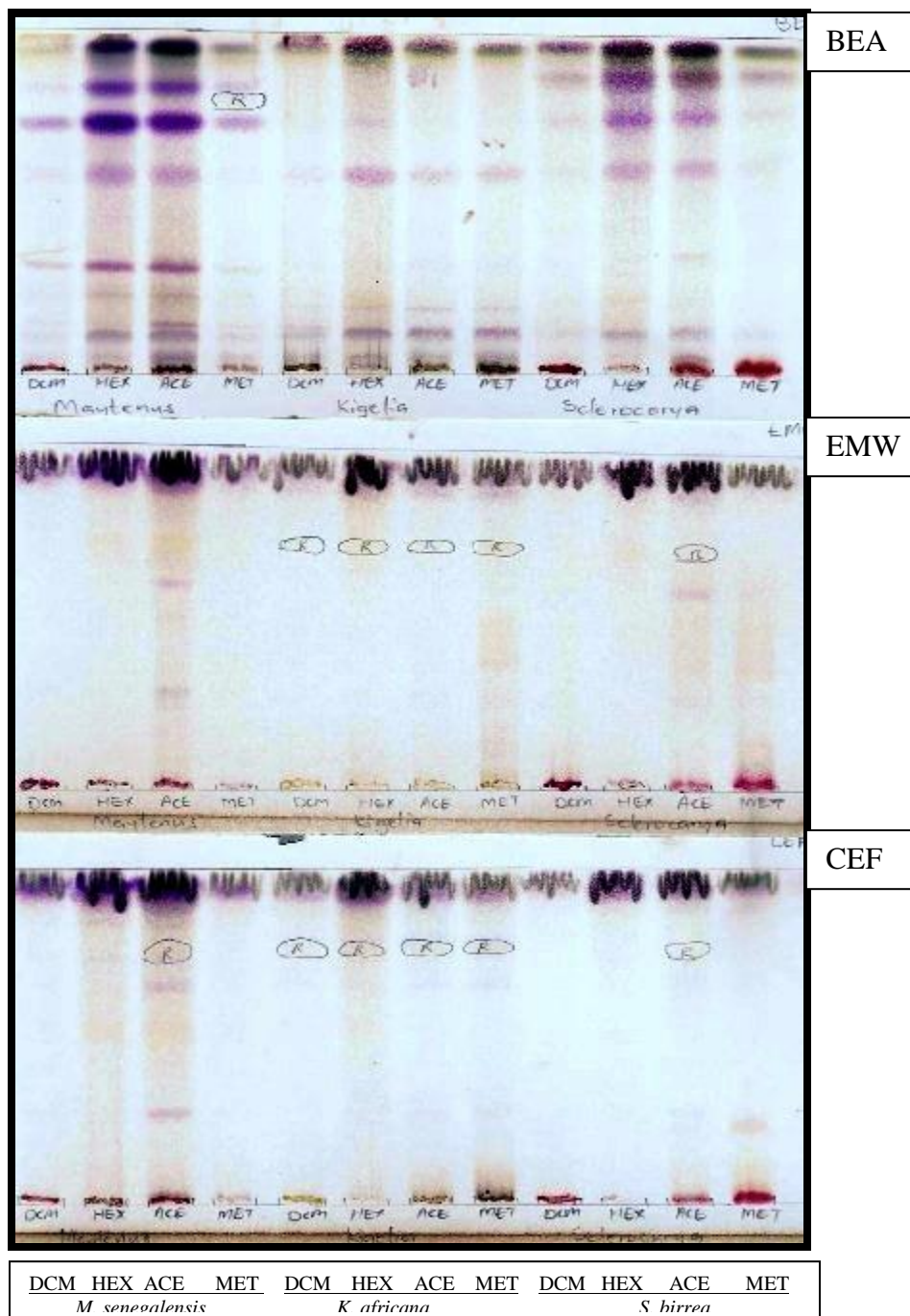


Fig. 3.4. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with vanillin-sulphuric acid to indicate separated compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

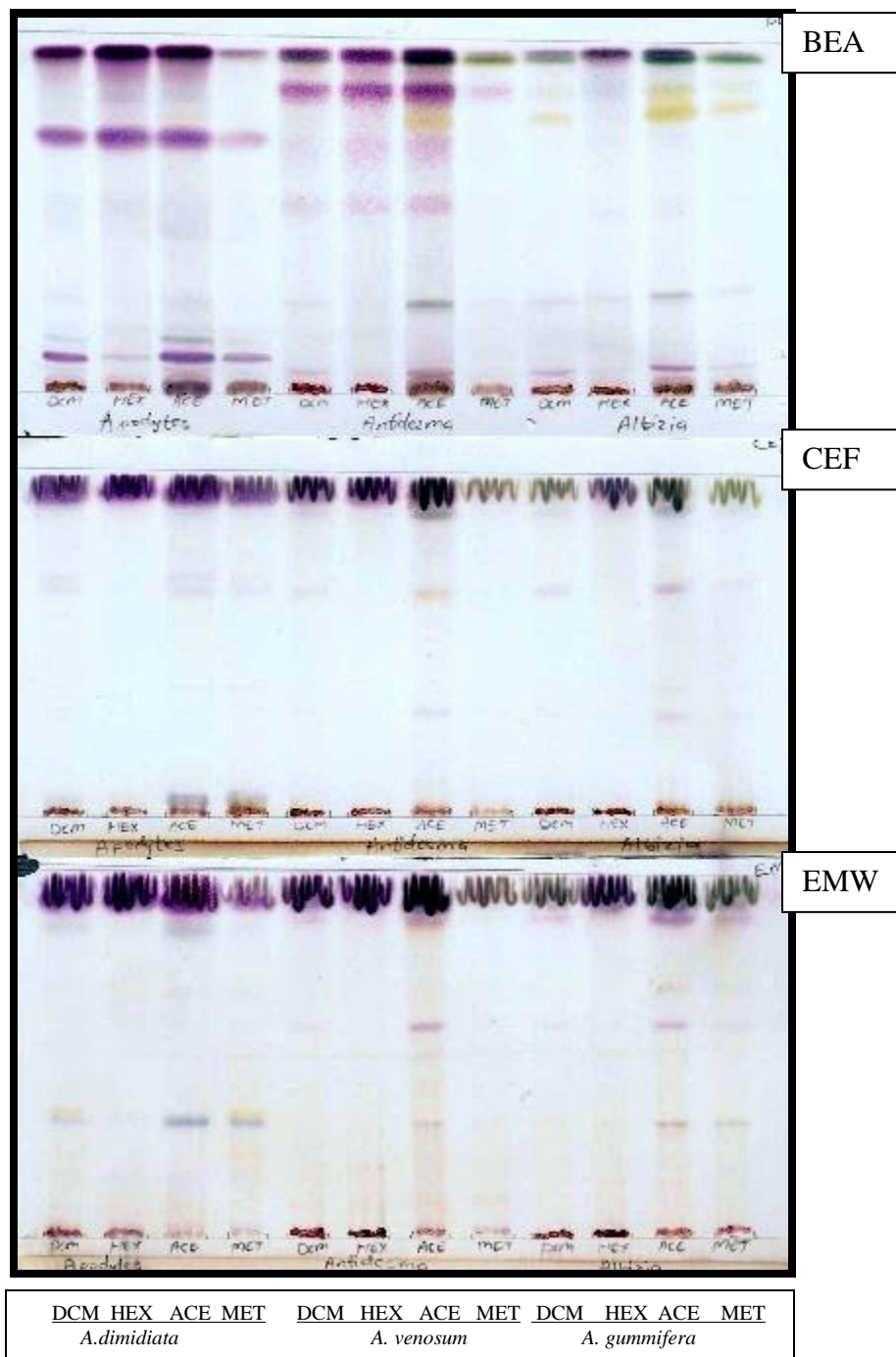


Fig. 3.5. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with vanillin-sulphuric acid to indicate separated compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

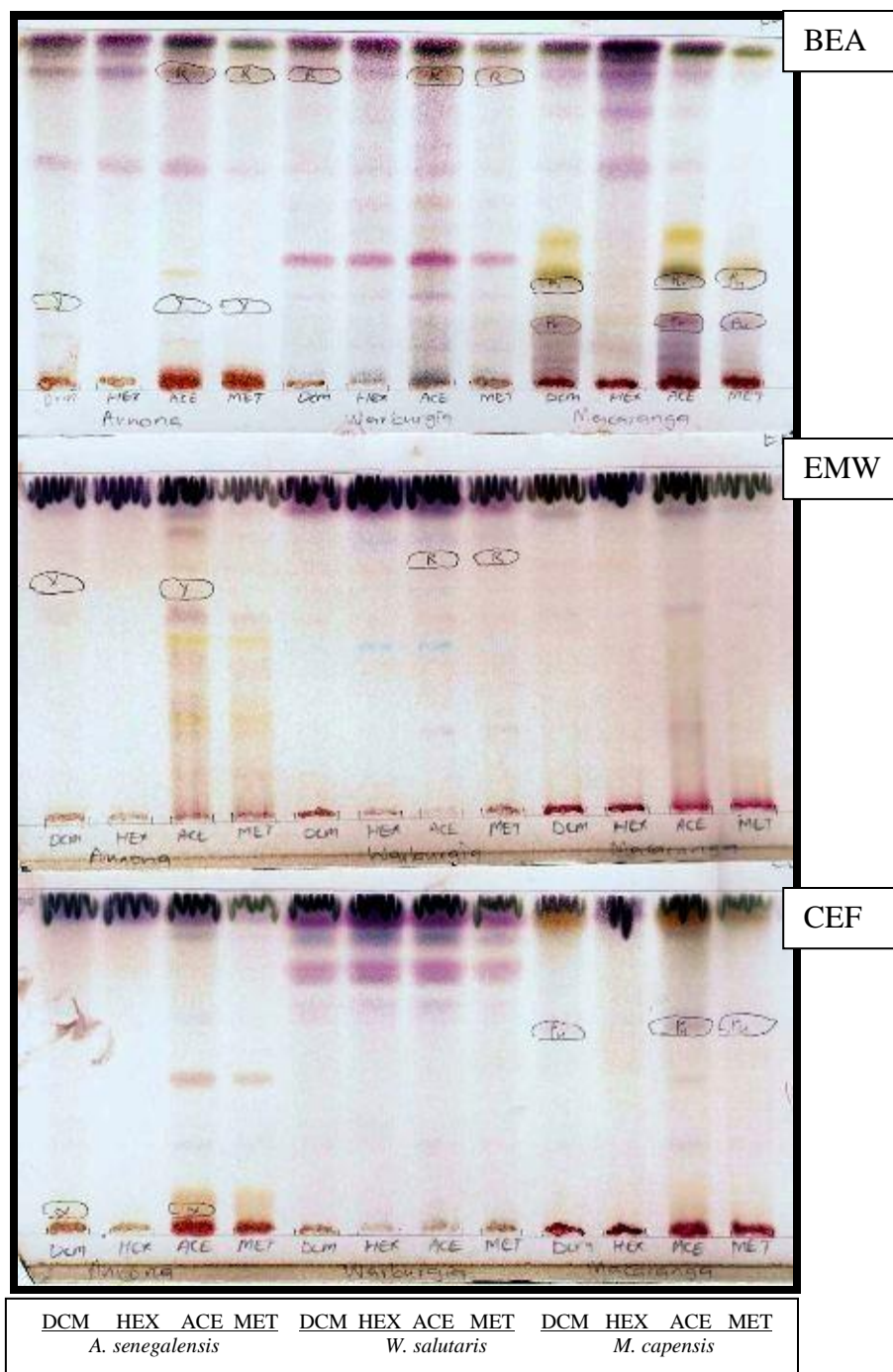


Fig. 3.6. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with vanillin-sulphuric acid to indicate separated compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

3.3. Qualitative (DPPH) assay on TLC (Antioxidant activity)

Qualitative DPPH assay on TLC plates was used to screen the plant extracts for the presence of antioxidant compounds. For the visualization of compounds with antioxidant activity, 0.2% DPPH was used to spray the developed TLC plates as shown in Fig. 3.7- Fig. 3.11. The presences of antioxidant compounds with scavenging activities are indicated with yellowish bands on a purple background. The compounds with antioxidant properties seem to be highly polar since they could not be separated on the TLC plates by the non-polar BEA mobile phase, except for *M. capensis* which had better separation when compared to all plant extracts (Fig. 3.11).

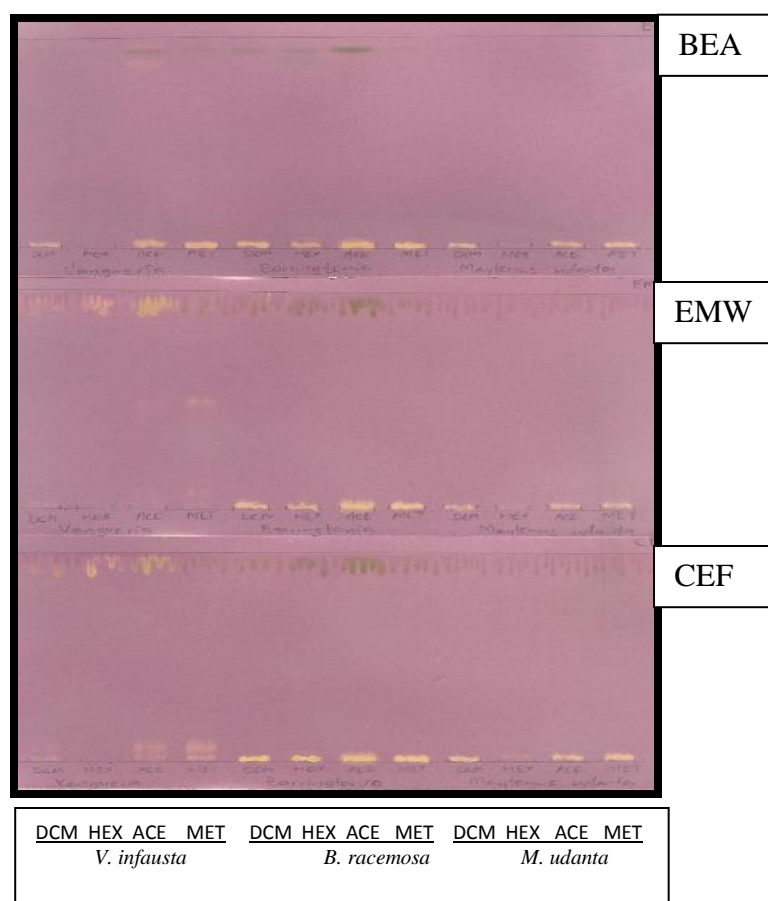


Fig. 3.7. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with 0.2% DPPH to indicate antioxidant compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

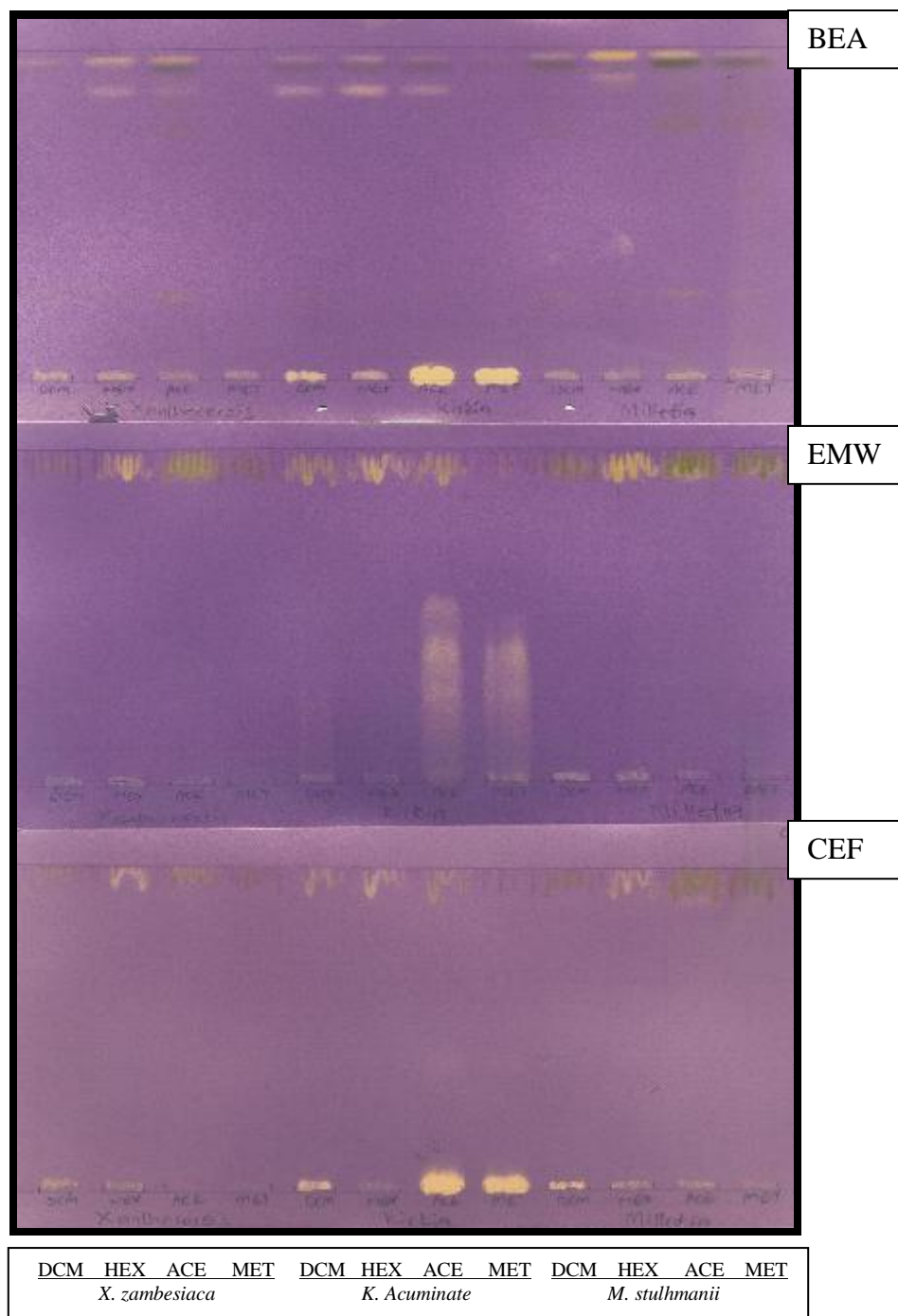


Fig. 3.8. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with 0.2% DPPH to indicate antioxidant compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

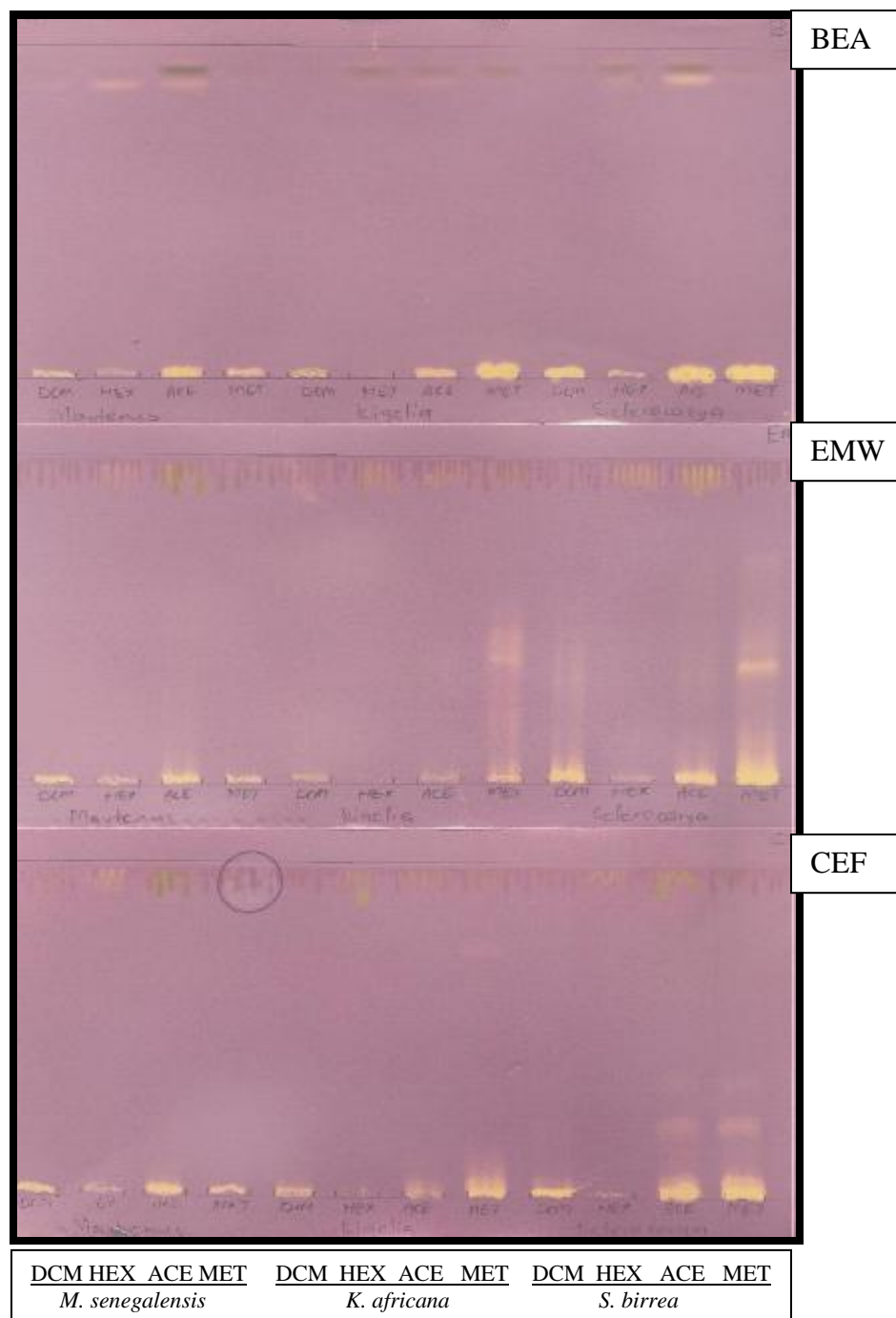


Fig. 3.9. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with 0.2% DPPH to indicate antioxidant compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

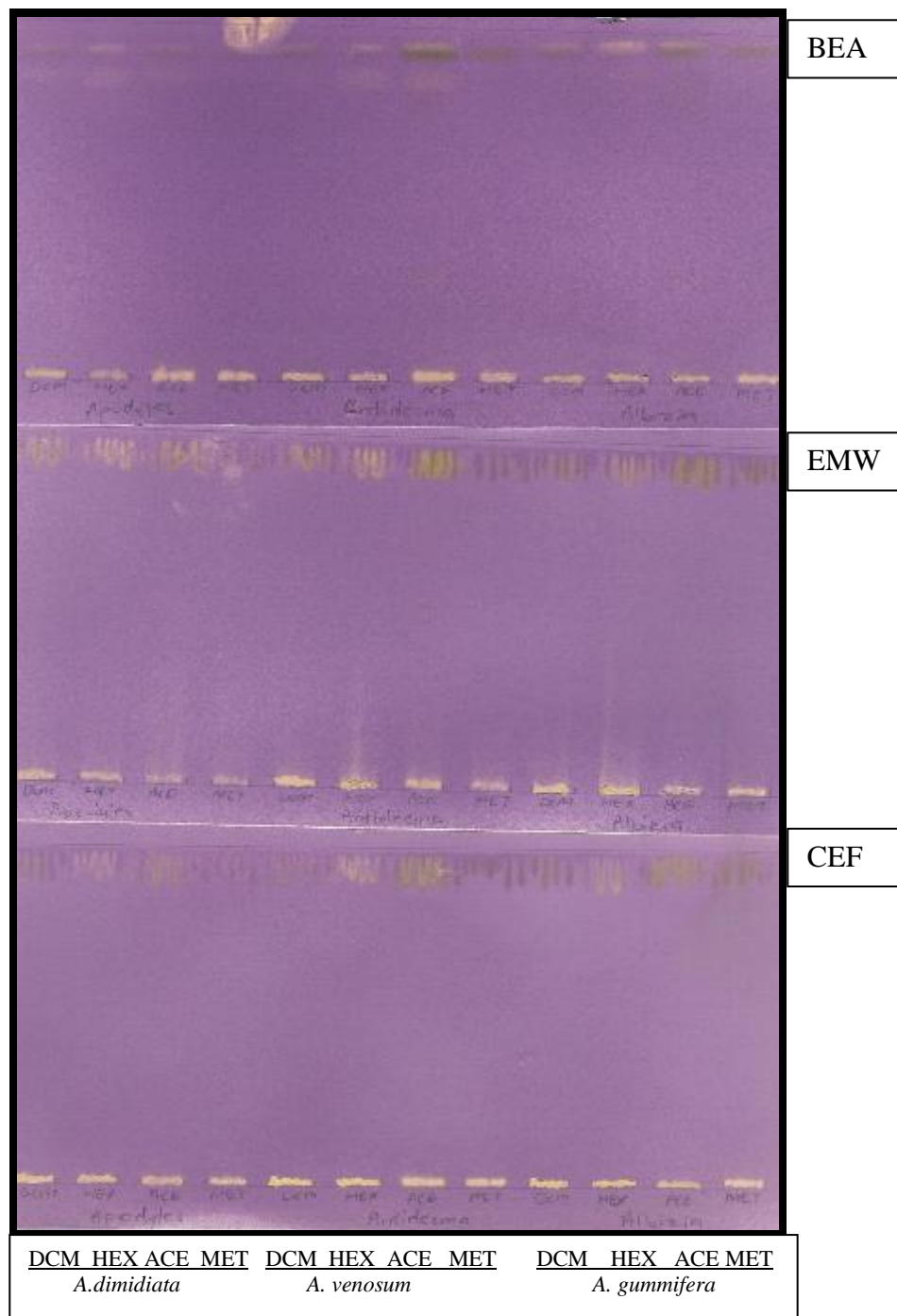


Fig. 3.10. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with 0.2% DPPH to indicate antioxidant compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

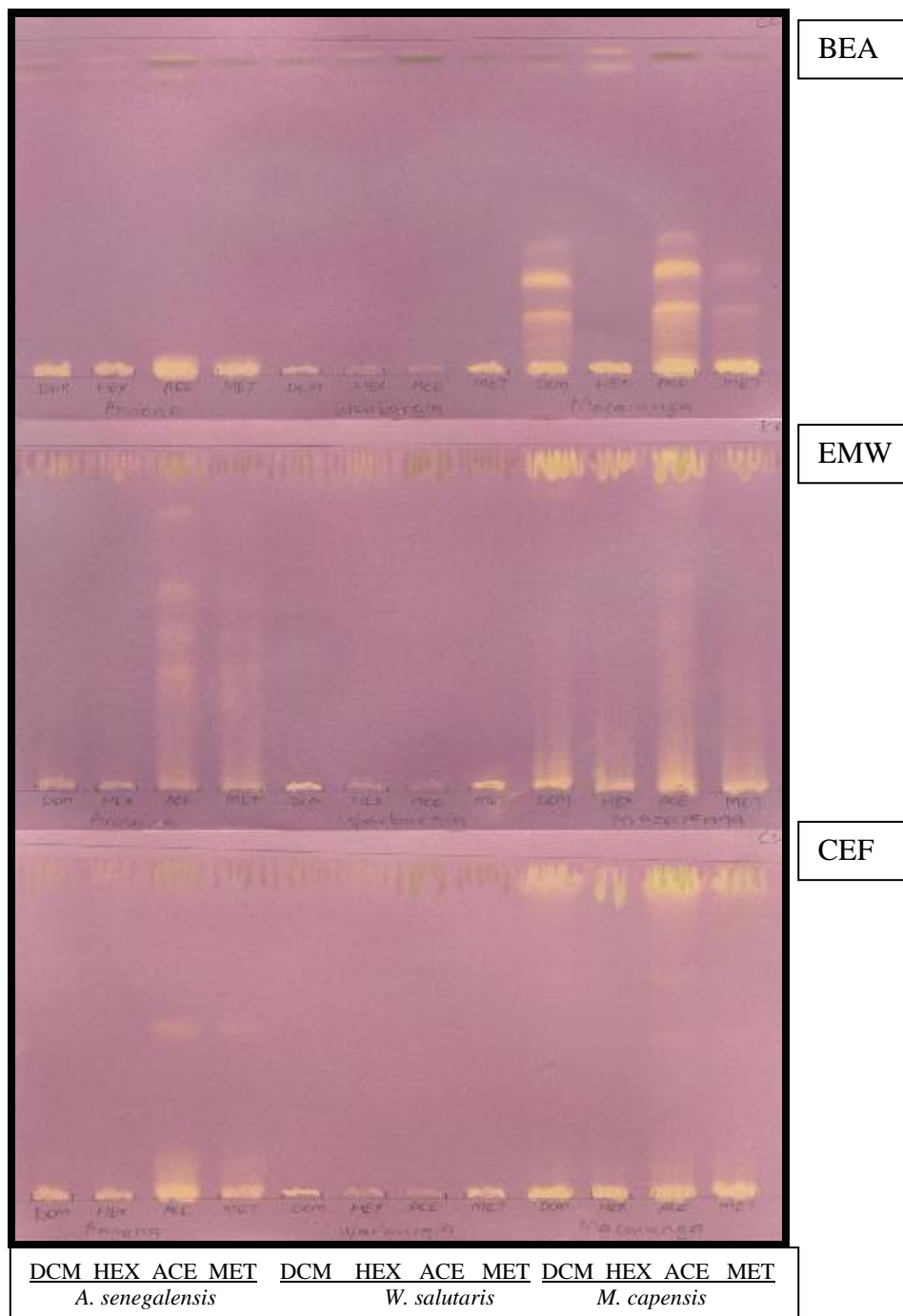


Fig 3.11. Chromatograms of crude extracts developed in solvent systems (BEA, EMW and CEF) and sprayed with 0.2% DPPH to indicate antioxidant compounds extracted with dichloromethane, hexane, acetone and methanol in lanes from left to right for each plant.

3.4. Minimum inhibitory concentrations (MIC) and total activities of the extracts

Quantitative antibacterial activity assay by serial microdilution (Eloff, 1998b) was used to determine the minimum inhibitory concentration (MIC) of the plant extracts extracted with hexane, DCM, acetone and methanol. INT was used as an indicator of growth and the MIC was recorded as the lowest concentration of the plant extract that inhibited the growth of the test organisms as shown in Table 3.1 and Table 3.2. From the MIC total activity was calculated and the total activity indicates the degree to which the active extracts in one gram of the plant material can be diluted with water and still inhibit the growth of the test organism. Table 3.1. shows the MICs and the corresponding total activity for all the plant extracts against *M. smegmatis*. Table 3.2. shows the MICs and the corresponding total activity for *R. erythropolis*. On average the acetone extracts had the lowest MIC values ranging between 0.11- 1.25 mg/ml and 0.08-1.25 mg/ml for *M. smegmatis* and *R. erythropolis*, respectively.

Table 3.1. Average MIC (mg/ml) and total activity (ml/g) of selected plant species after 24 hr incubation at 37°C.

<i>Mycobacterium smegmatis</i> mc²155																
Minimal inhibitory concentration (mg/ml)																
Extractant	<i>Albizia gummifera</i>	<i>Kirkia acuminata</i>	<i>Xanthocercis zambesica</i>	<i>Milletia stuhlmannii</i>	<i>Barringtonia racemosa</i>	<i>Sclerocarya birrea</i>	<i>Maytenus udantia</i>	<i>Vangueria infausta</i> subsp. <i>infausta</i>	<i>Macaranga capensis</i>	<i>Maytenus senegalensis</i>	<i>Apodytes dimidiata</i> subsp. <i>dimidiata</i>	<i>Kigelia Africana</i>	<i>Antidesma venosum</i>	<i>Annona senegalensis</i>	<i>Warburgia salutaris</i>	Average
Hexane	1.25	0.63	1.67	0.31	0.63	0.52	1.25	1.25	0.63	0.14	1.25	0.63	1.25	1.25	1.25	0.92
DCM	0.31	0.31	1.25	0.31	0.63	0.63	1.25	1.25	0.52	na	1.04	1.67	1.25	1.25	1.25	0.92
Acetone	0.16	0.31	0.11	0.13	0.11	0.26	1.25	0.52	0.26	0.52	0.63	0.63	0.26	0.42	0.42	0.40
Methanol	0.31	0.63	0.63	0.26	0.84	0.42	1.25	1.04	0.41	1.25	na	0.63	0.84	na	1.25	0.75
Average	0.51	0.47	0.91	0.25	0.55	0.46	1.25	1.02	0.46	0.63	0.97	0.89	0.90	0.97	1.04	
Total activity (ml/g)																
Hexane	11.2	63.5	8.40	54.8	34.9	53.5	34.4	43.2	30.2	144.2	20	14.3	16	17.6	27.2	38.5
DCM	151.6	190.3	52	190.3	65.1	200	72.8	62.4	91.8	na	51.8	26.4	31.2	17.6	46.4	89.3
Acetone	150	203.2	207.6	263.2	327.1	146.2	66.4	61.2	219.2	44	68.3	36.5	88.5	79.1	107.9	137.9
Methanol	254.8	387.3	168.3	257.7	176.8	326.1	101.6	87.3	266..2	75.2	na	33.3	126.6	na	90.4	173.8
Average	141.9	211.1	109.1	191.5	151.0	181.5	80.3	63.5	113.7	87.8	46.7	27.6	65.6	38.1	68.0	

na = No activity at 2.5 mg/ml

Rifampicin= 0.125 mg/ml

Table 3.2. Average MIC (mg/ml) and total activity (ml/g) of selected plant species after 24 hr incubation at 37°C.

<i>Rhodococcus erythropolis</i> ATCC 4277																
Minimal inhibitory concentration (mg/ml)																
Extractant	<i>Albizia gummifera</i>	<i>Kirkia acuminata</i>	<i>Xanthocercis zambesiaca</i>	<i>Milletia stuhlmannii</i>	<i>Barringtonia racemosa</i>	<i>Sclerocarya birrea</i>	<i>Maytenus udanta</i>	<i>Vangueria infausta</i> subsp <i>infausta</i>	<i>Macaranga capensis</i>	<i>Maytenus senegalensis</i>	<i>Apodytes dimidiata</i> subsp <i>dimidiata</i>	<i>Kigelia Africana</i>	<i>Antidesma venosum</i>	<i>Annona senegalensis</i>	<i>Warburgia salutaris</i>	Average
Hexane	1.25	0.63	0.84	0.31	0.84	1.25	1.25	na	0.63	0.63	na	0.31	na	na	1.25	0.83
DCM	0.31	0.31	0.63	0.31	1.25	2.5	1.25	1.67	0.21	1.04	0.84	0.63	na	na	1.25	0.94
Acetone	0.13	0.31	0.11	0.08	0.13	0.21	1.25	0.63	0.11	0.31	0.63	0.52	0.31	0.63	0.31	0.38
Methanol	0.31	0.51	0.31	0.16	0.84	0.84	1.25	1.25	0.78	0.84	0.63	0.52	0.63	1.25	0.84	0.73
Average	0.50	0.44	0.47	0.22	0.76	1.20	1.25	1.18	0.43	0.71	0.70	0.50	0.47	0.94	0.91	
Total activity (ml/g)																
Hexane	11.2	63.5	16.7	54.8	26.8	22.4	34.4	na	30.2	60.4	na	29	na	na	34.2	34.9
DCM	151.6	190.3	103.2	190.3	32.8	50.4	72.8	46.8	228.6	60.4	64.5	69.8	na	na	100.6	104.8
Acetone	184.6	203.2	207.6	437.5	269.2	190	66.4	50.8	537.7	74.2	68.3	44	74.2	52.4	173.7	175.6
Methanol	254.8	475.6	341.9	418.8	176.8	162.5	101.6	72.8	142.3	112.3	217.5	40.2	168.3	84.8	193.7	197.6
Average	150.6	233.2	167.4	275.4	126.4	106.3	80.3	56.8	234.7	76.8	116.8	45.8	121.3	68.6	125.6	

na = No activity at 2.5 mg/ml

Rifampicin= 0.125 mg/ml

3.5. Qualitative antibacterial activity assay by bioautography

Bioautography reveals the diversity of antibacterial compounds present in different extracts. Inhibition zones are observed as white bands on a pink background (White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of bioactive compounds that inhibit the growth of the test organisms (Fig. 3.12 – Fig. 3.21). There are distinct bands on bioautograms loaded with extracts of *A. dimidiata*, *V. infausta* and *M. udanta* showing antimycobacterial activities (Fig. 3.13 and Fig. 3.14).

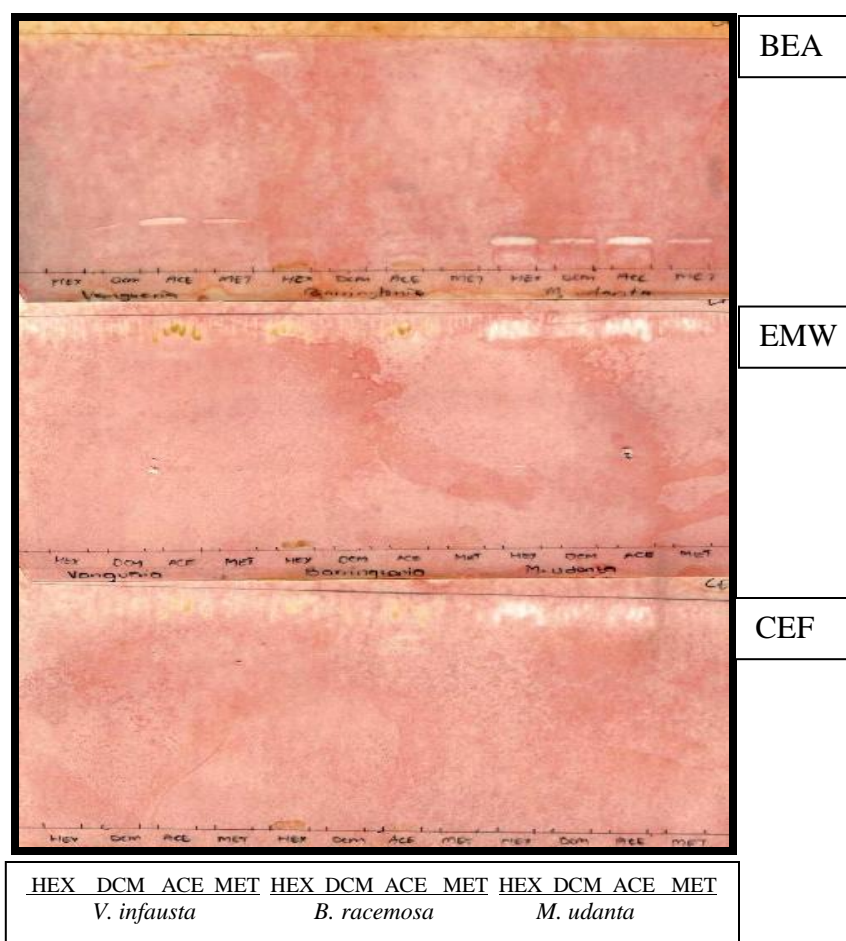


Fig. 3.12. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *M. smegmatis*.

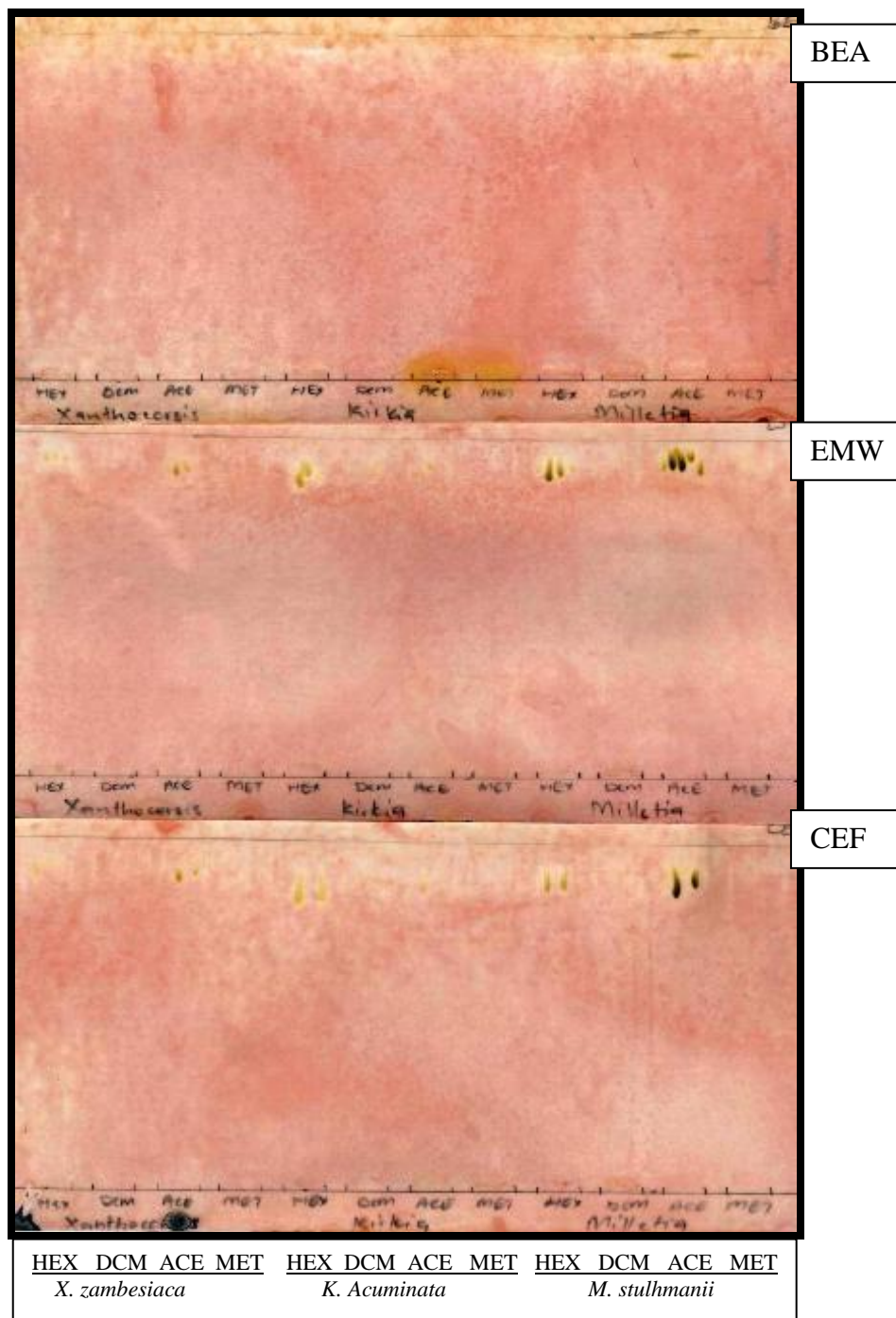


Fig. 3.13. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *M. smegmatis*.

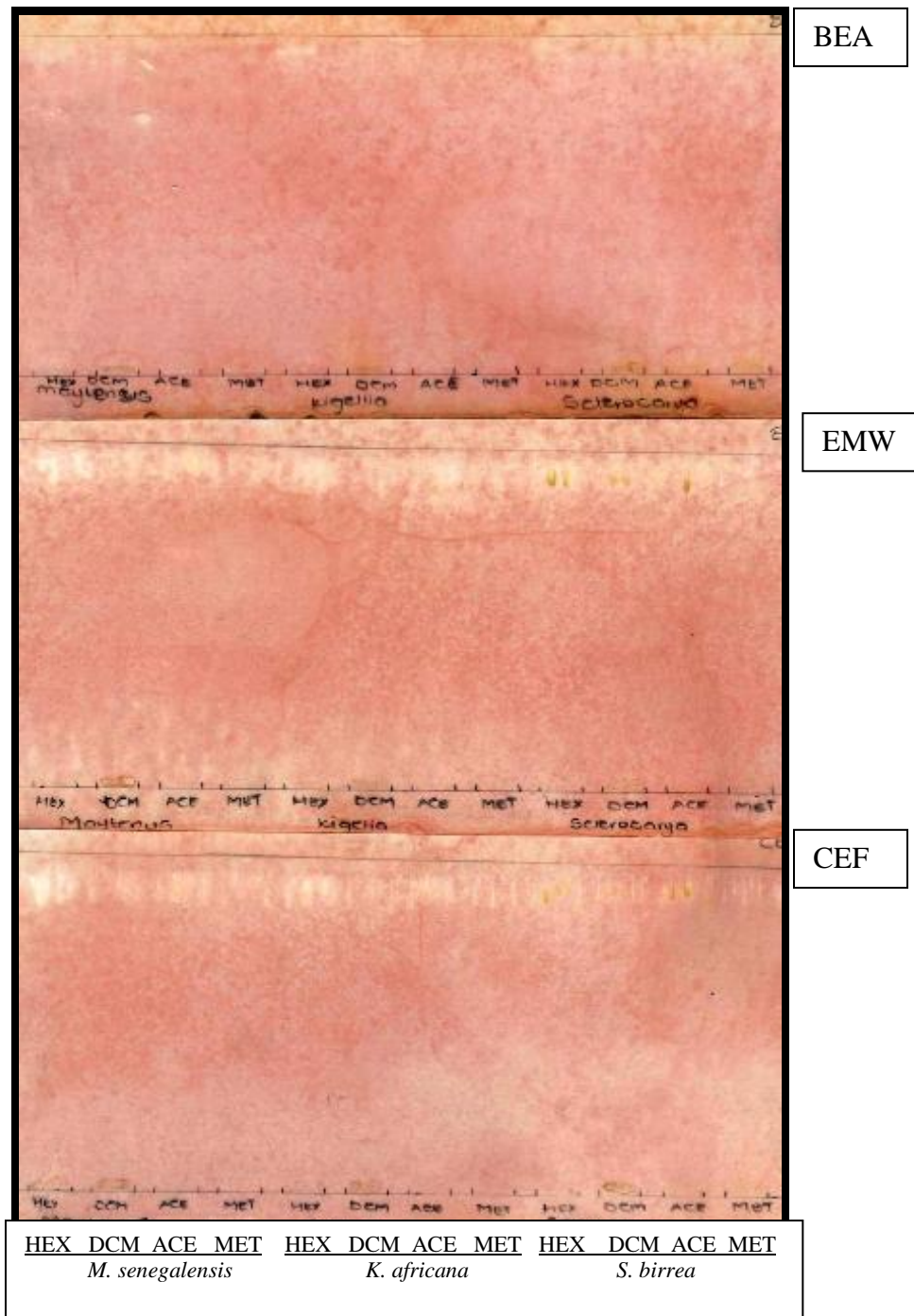


Fig. 3.14. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *M. smegmatis*.

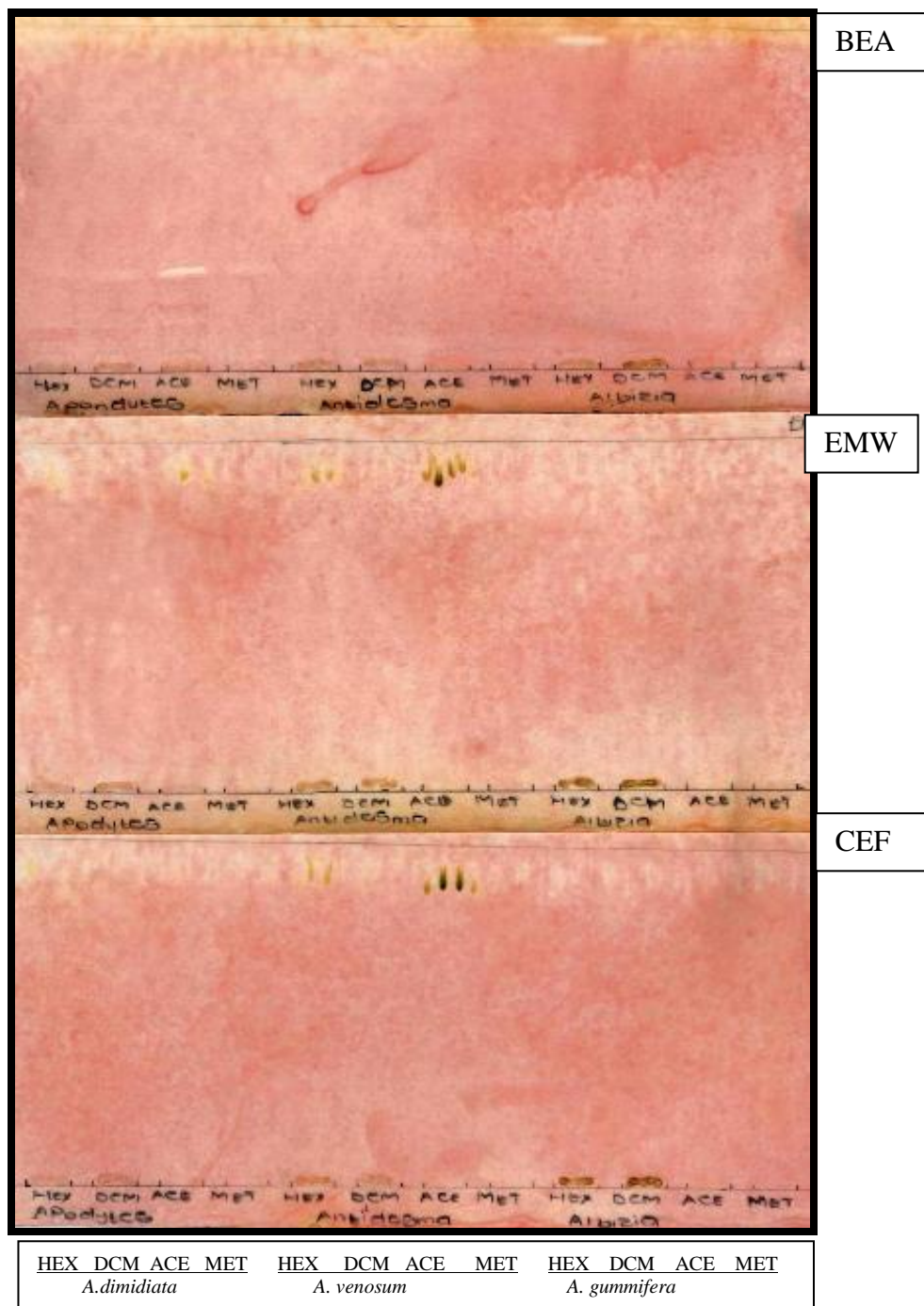


Fig 3.15. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *M. smegmatis*.

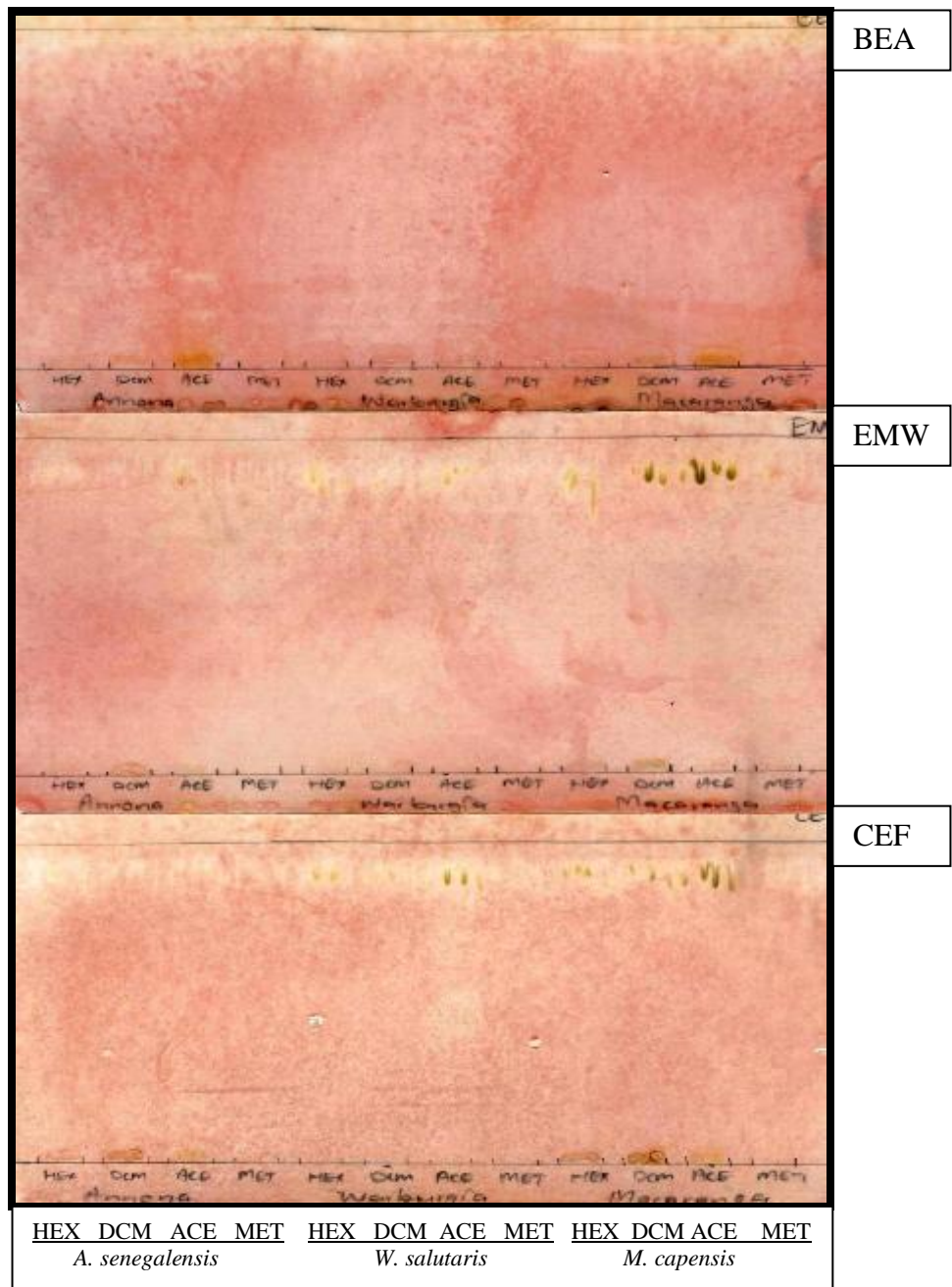


Fig 3.16. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *M. smegmatis*.

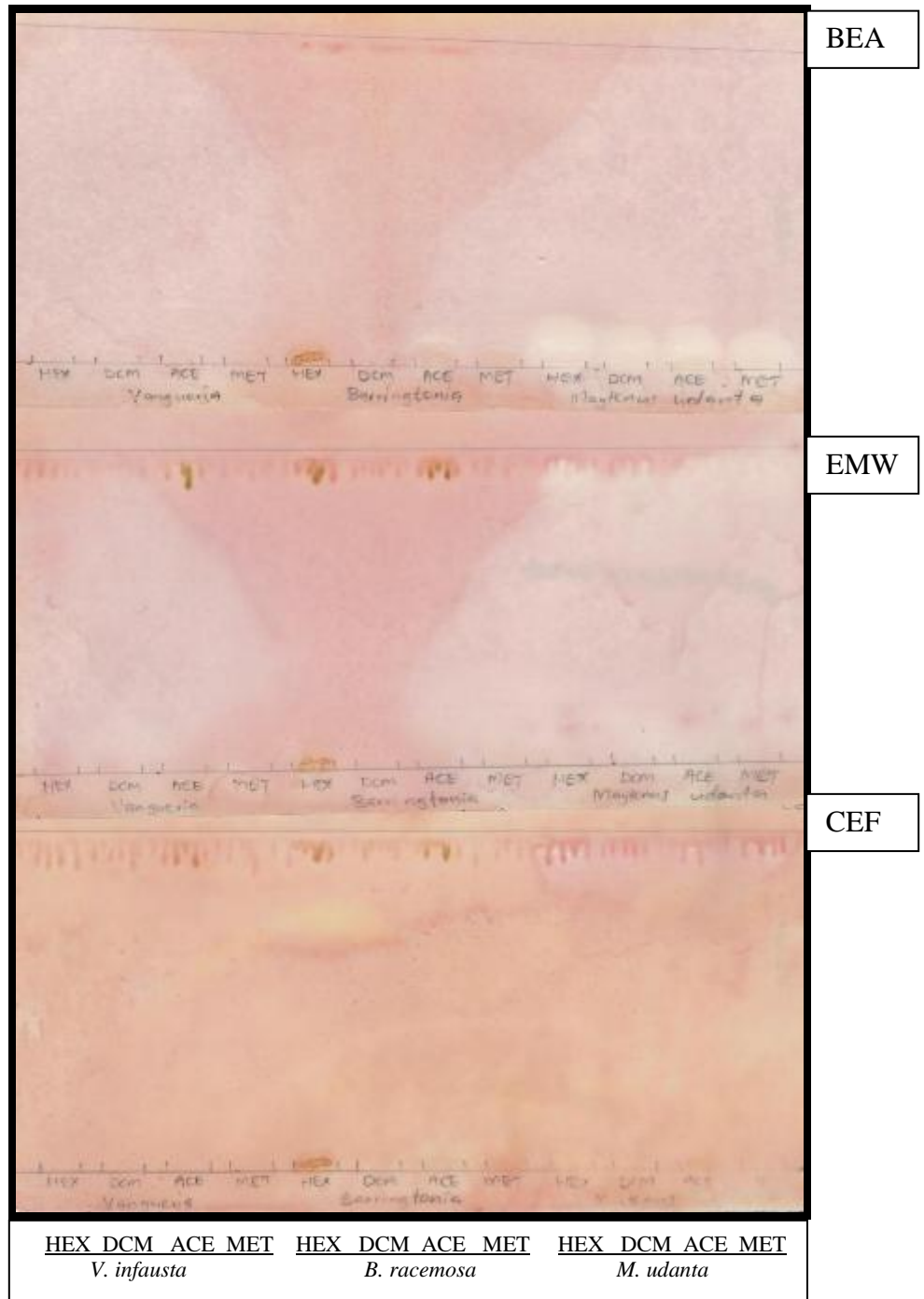


Fig. 3.17. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *R. erythropolis*.

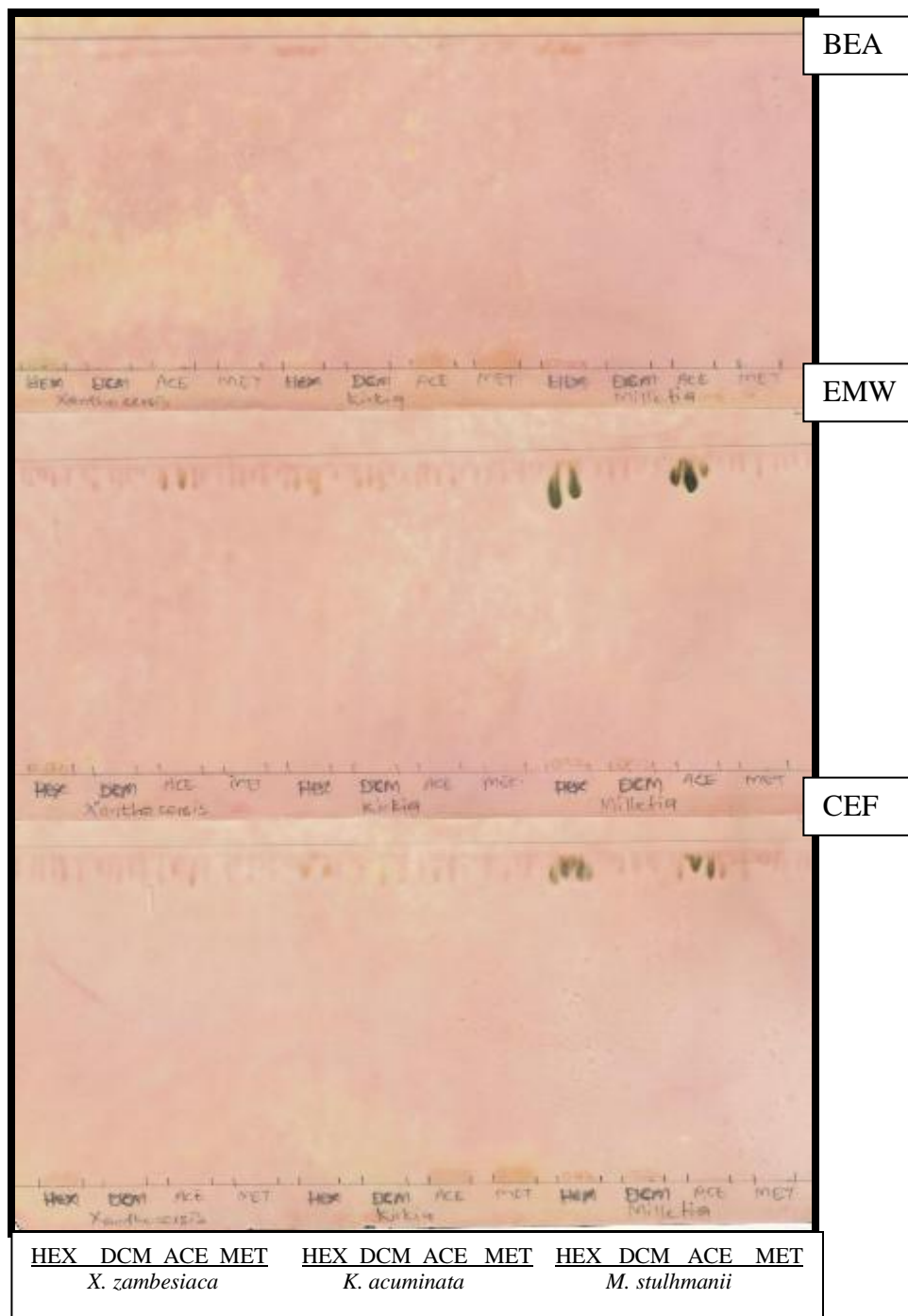


Fig. 3.18. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *R. erythropolis*.

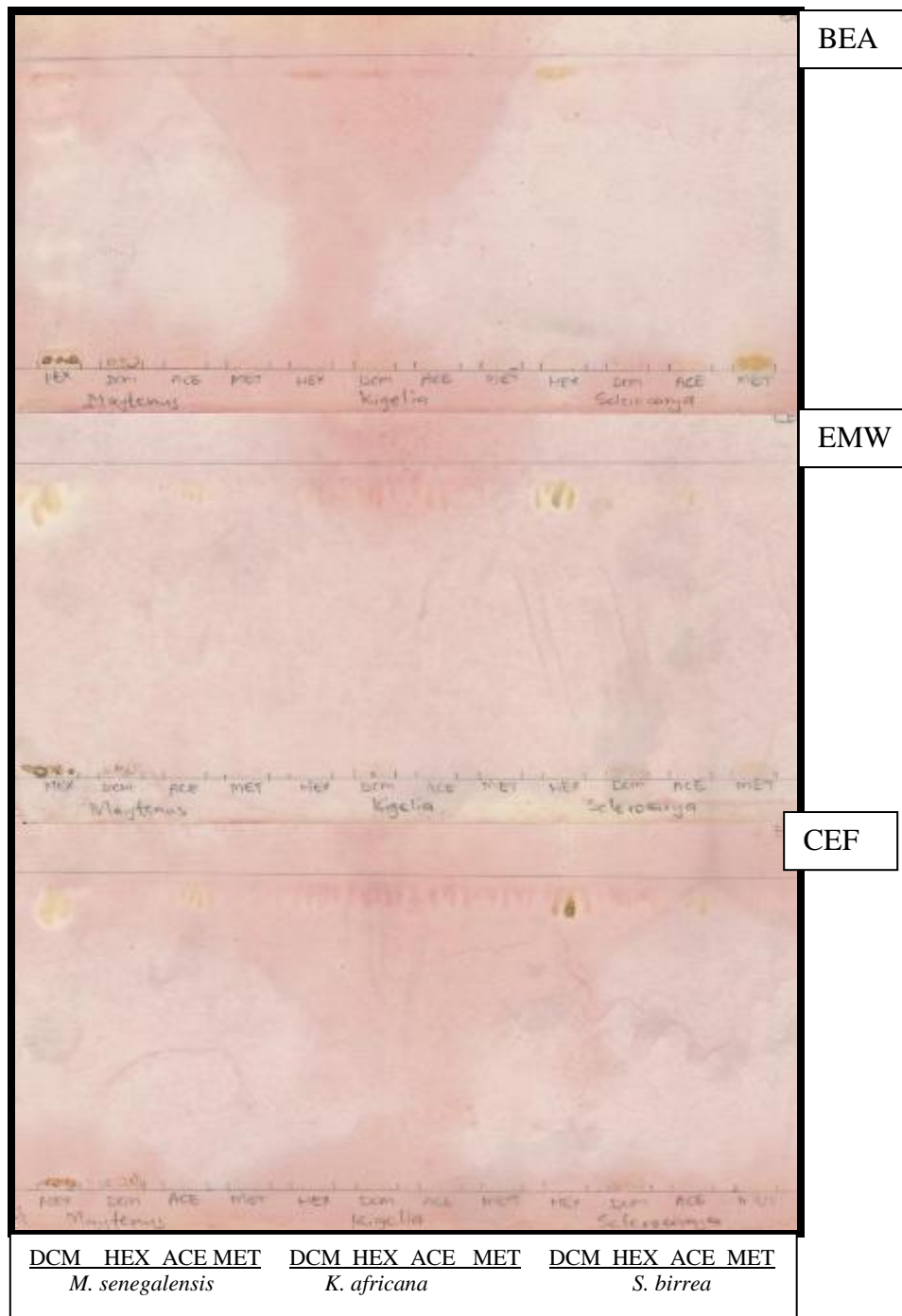


Fig. 3.19. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *R. erythropolis*.

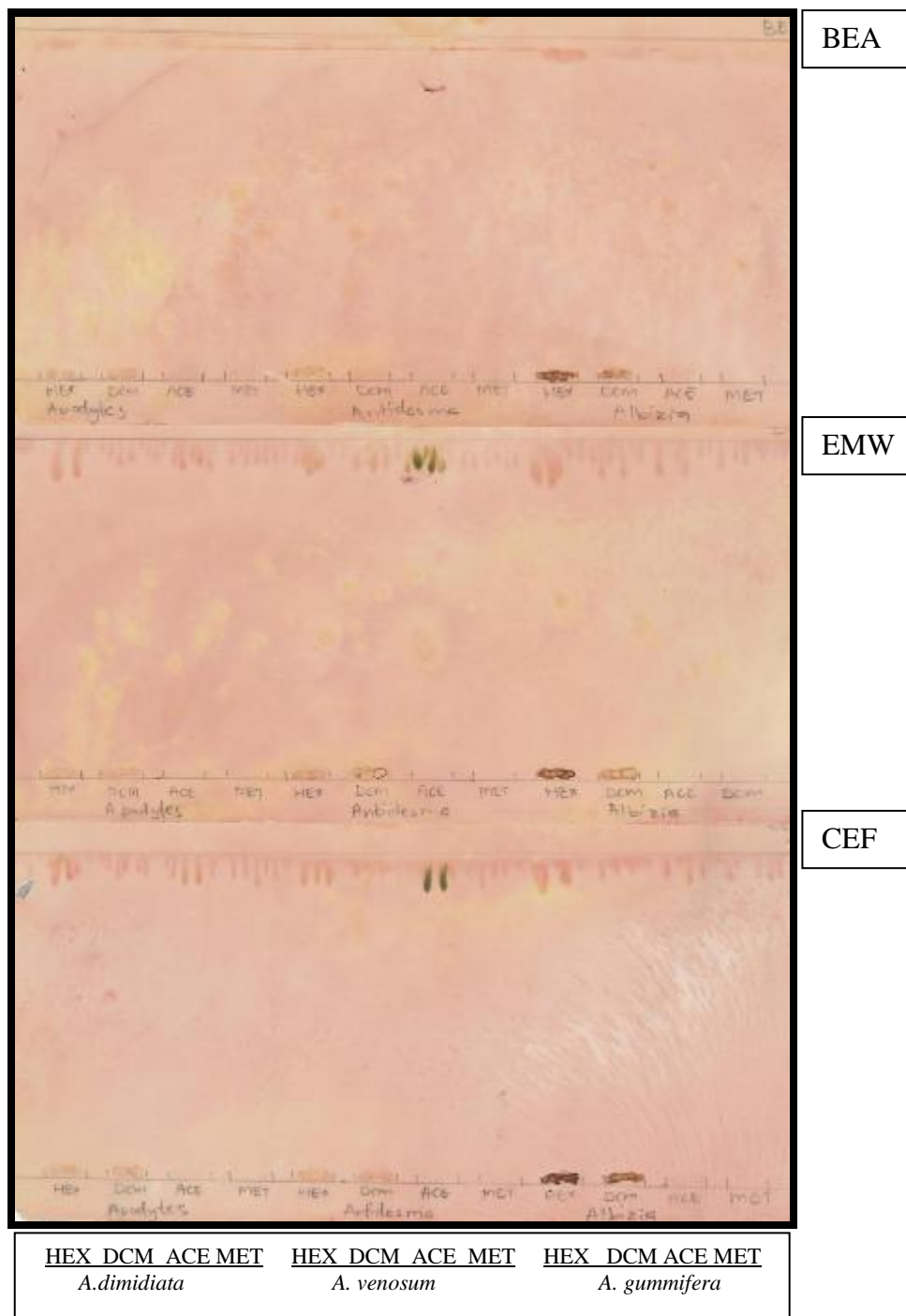


Fig. 3.20. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *R. erythropolis*.

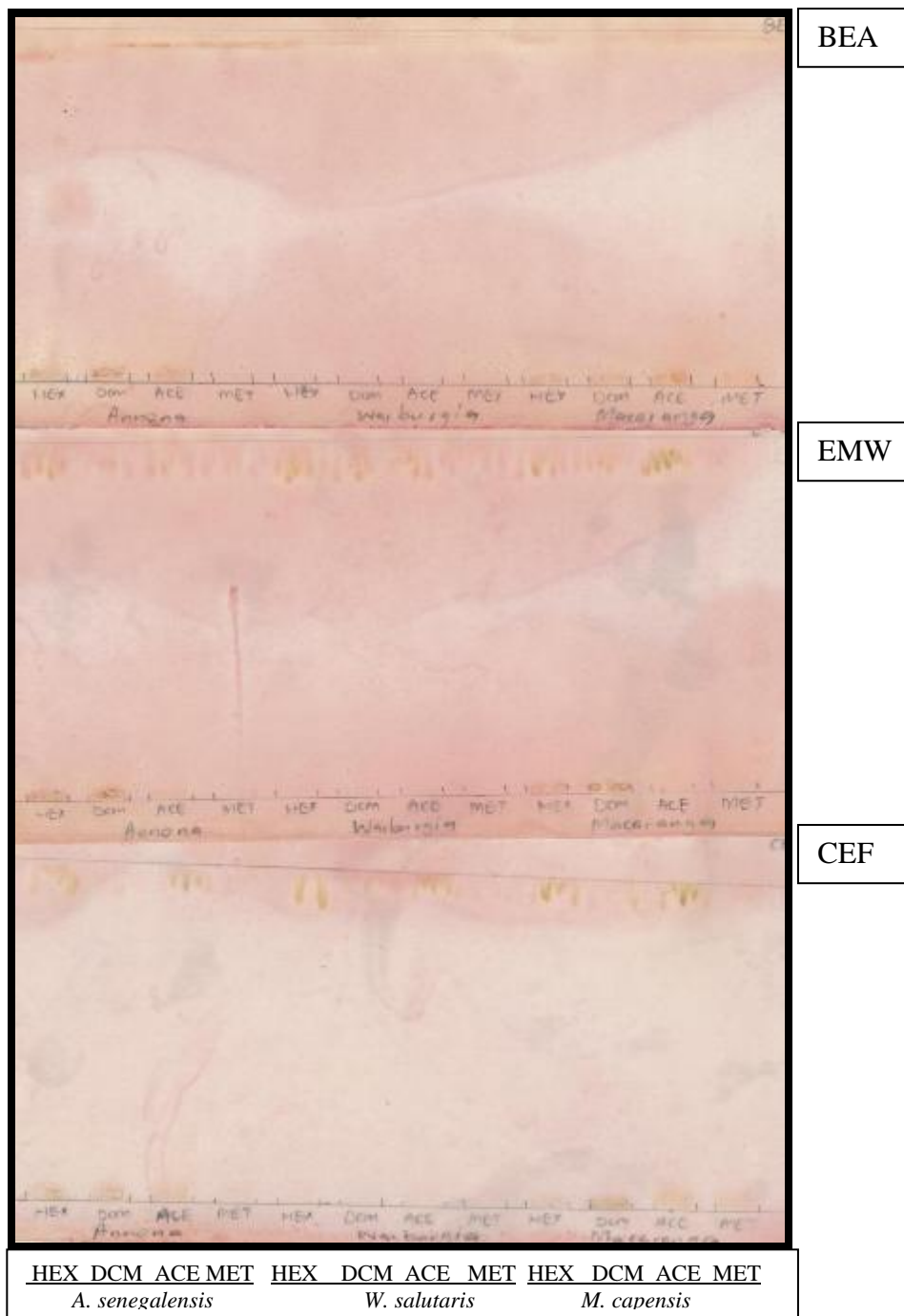


Fig. 3.21. Bioautograms of crude extracts extracted with hexane, dichloromethane, acetone and methanol in lanes from left to right for each plant, separated with BEA, CEF, EMW, and sprayed with *R. erythropolis*.

3.6. Isolation of bioactive compounds from acetone fraction of *A. dimidiata*

Isolation of bioactive compounds from acetone fraction was performed through the use of bioassay-guided fractionation. The compounds observed as white bands on a pink background are active antibacterial compounds. From the bioutography results (Fig. 3.12 and Fig. 3.15) there were distinct bioactive bands on the bioautograms loaded with extracts of *A. dimidiata*, *V. infausta* and *M. udanta*. Fig. 3.22 indicates the phytoconstituents of the selected plant (*A. dimidiata*) used for isolation of bioactive compounds.

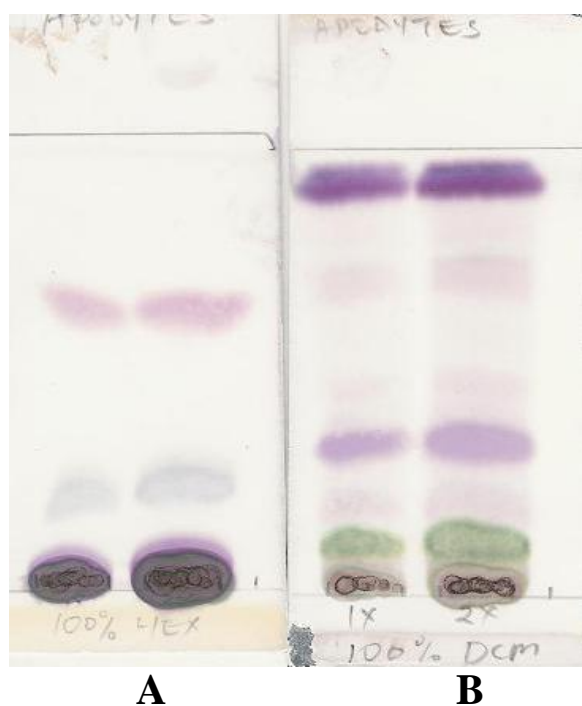


Fig. 3.22. The components profiling of *Apodytes dimidiata* developed in 100% HEX (A) and 100% DCM (B). The TLC plates were sprayed with vanillin-sulphuric acid to indicate the best solvent system to be used in separation of extracts in column chromatography.

Of the two solvent systems used from Fig. 3.22 it shows that DCM was the best solvent for further isolation since it had a better separation (more distinct separated bands) than hexane. Figure 3.23 shows the phytochemical analysis results of the first column chromatography which was ran with 100 % DCM and slightly increasing polarity with methanol, whereas Fig. 3.24 indicates the corresponding bioautography of the fraction. The 100 % DCM and 90:10 DCM:MEOH fractions are showing antimycobacterium activity indicated by clear zones. Another column chromatography was run with hexane to further purify the active compounds in Fig. 3.24. The first fractions in Fig. 3.25 (F1 100%HEX – F5 95:5 HEX:ETAC) were pooled and recorded as compound 1. The corresponding Fig. 3.26 indicates the bioactive compounds and fractions F1- F3 85:15 HEX:ETAC were further targeted for purification since they had a different R_f value from compound A. Figure 3.27 shows the phytochemical and bioassay results for the pooled fractions in fig. 3.25 (compound A) with less activity indicating reduced clear zone intensity. The active compounds from Fig.3.28 were further purified by running a column with 3:1 HEX:DCM (Fig. 3.29–Fig. 3.31). Figure 3.32 shows the pooled fractions which were purified and labelled as compound B.

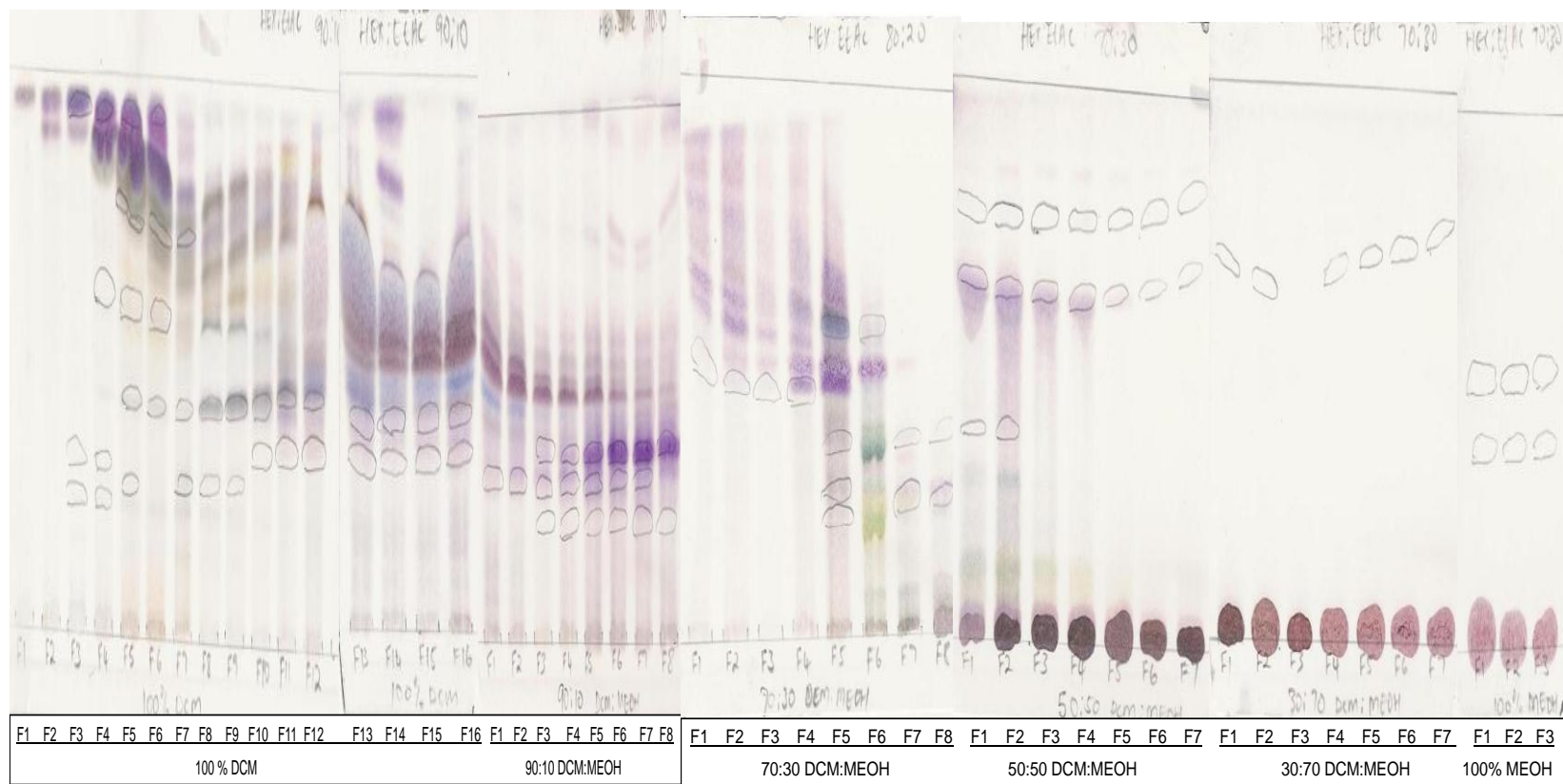


Fig. 3.23. TLC profiles of fractions obtained from eluting acetone extract from *A. dimidiata* with 100% DCM, 90:10, 70:30 50:50 30:70 and 100% MEOH. The TLC plates are indicating the phytoconstituents of the sub fractions after sprayed with vanillin-sulphuric acid.

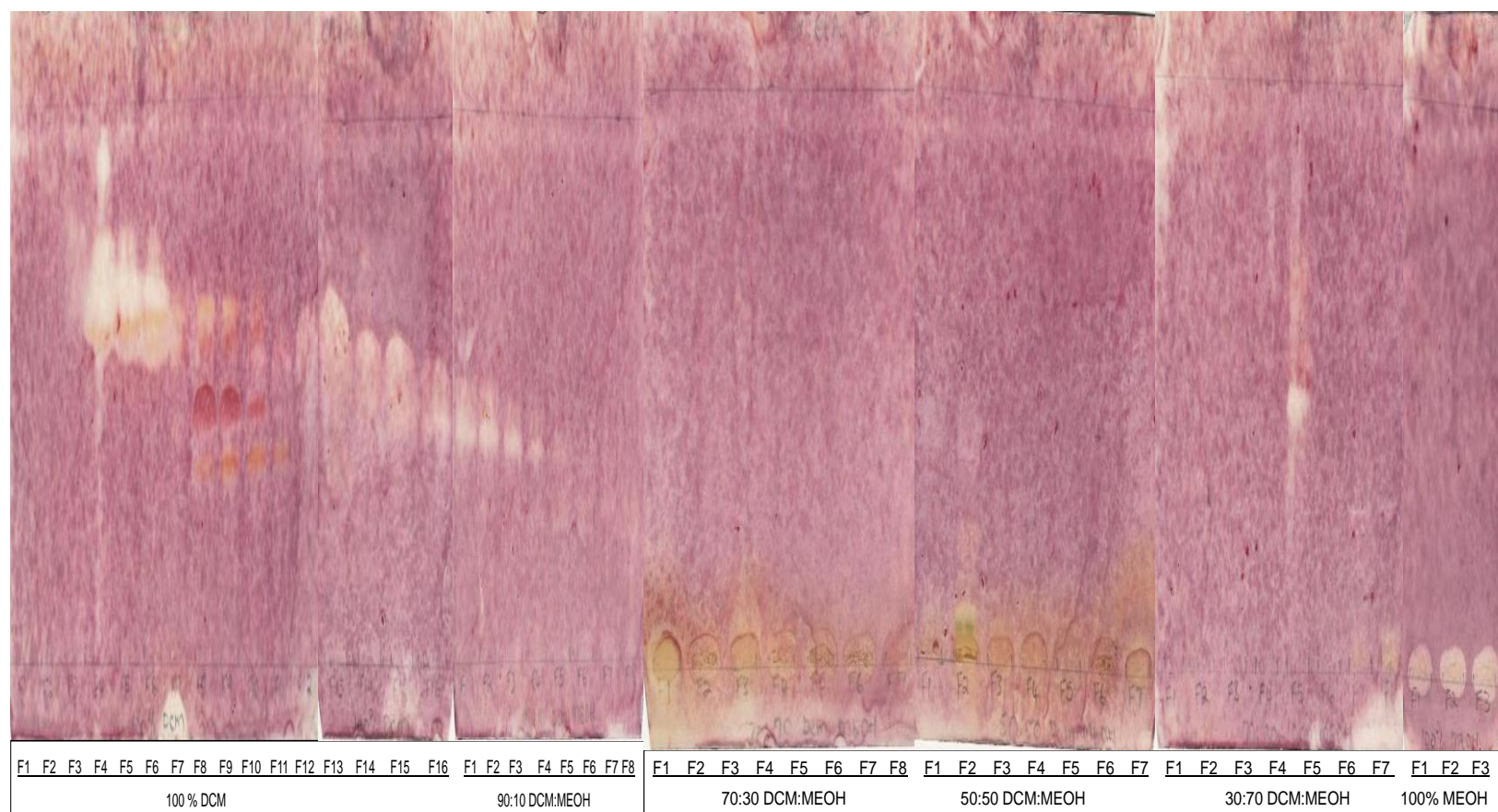


Fig. 3.24. Bioautography of fractions obtained from eluting acetone extract from *A. dimidiata* with 100% DCM, 90:10, 70:30 50:50 30:70 and 100% MEOH. The bioautograms are indicating the bioactive-components of the sub fractions after being sprayed with INT as white bands on pink background.

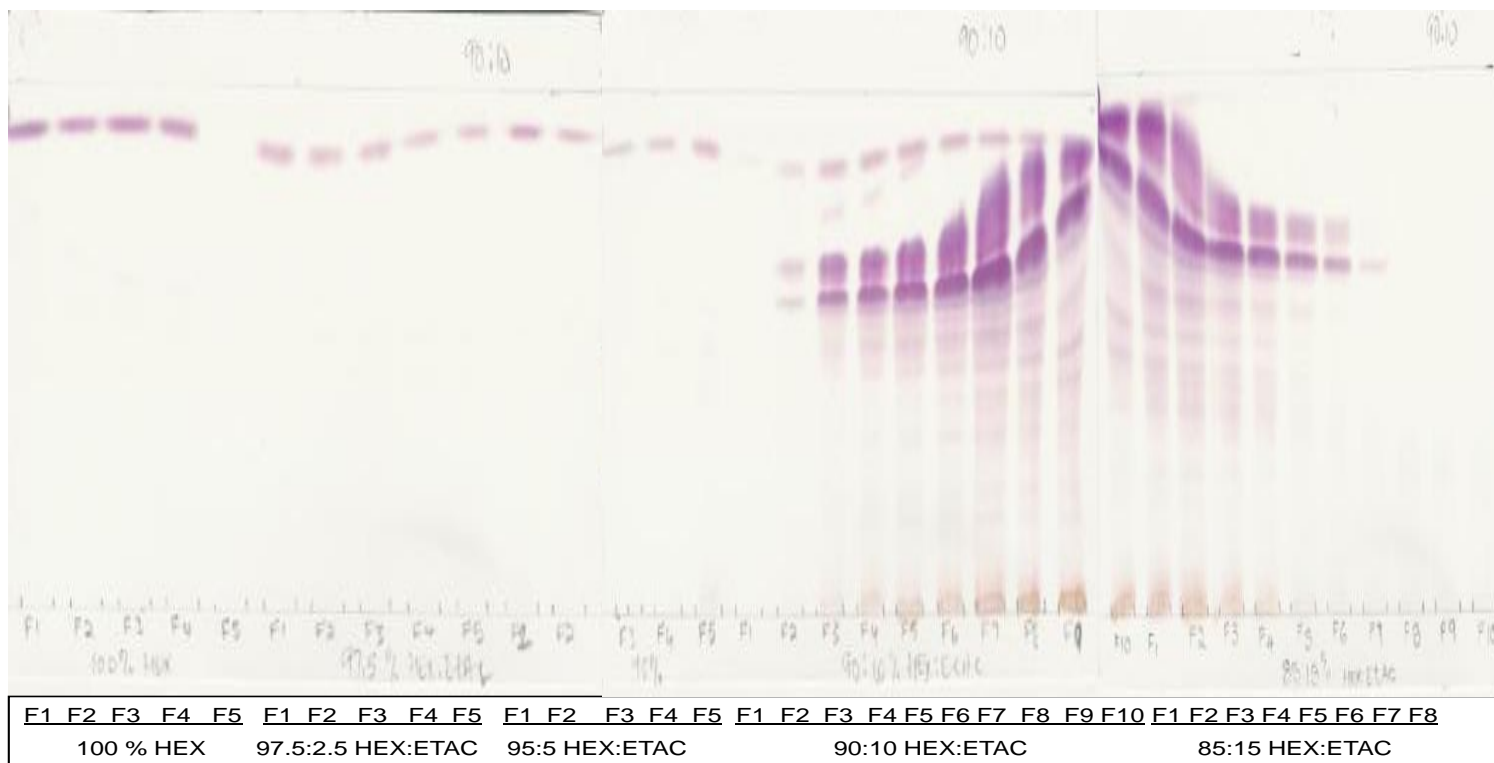


Fig. 3.25. TLC profile of the pooled fractions (A) obtained from Subfraction (F2-F6) of 100% DCM after re-elution with 100% HEX, 97.5:2.5, 95:5, 90:10 and 85:15 indicating the phytoconstituents of the sub fractions. The first fractions indicates compounds A (F1 100% HEX - F5 95:5 HEX:ETAC).



Fig. 3.26. Bioautography of the pooled fractions (A) obtained from Sub fraction (F2-F6) of 100% DCM after re-elution with 100% HEX, 97:2.5, 95:5, 90:10 and 85:15 indicating the bioactive components of the sub fractions. The first fractions indicate compounds A (F1 100% HEX - F5 95:5 HEX:ETAC) with bio-activity indicated by white bands with pink background.

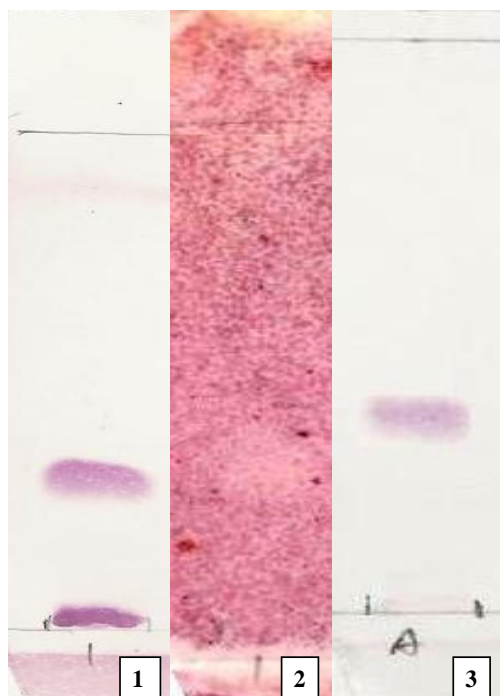


Fig. 3.27. TLC plates showing phytochemical and bioautography analysis of compound A from fraction A (Fig 3.2.6) developed with Hexane (1 and 3). TLC plate number 3 indicates compound A after further purification on the preparative TLC plate.

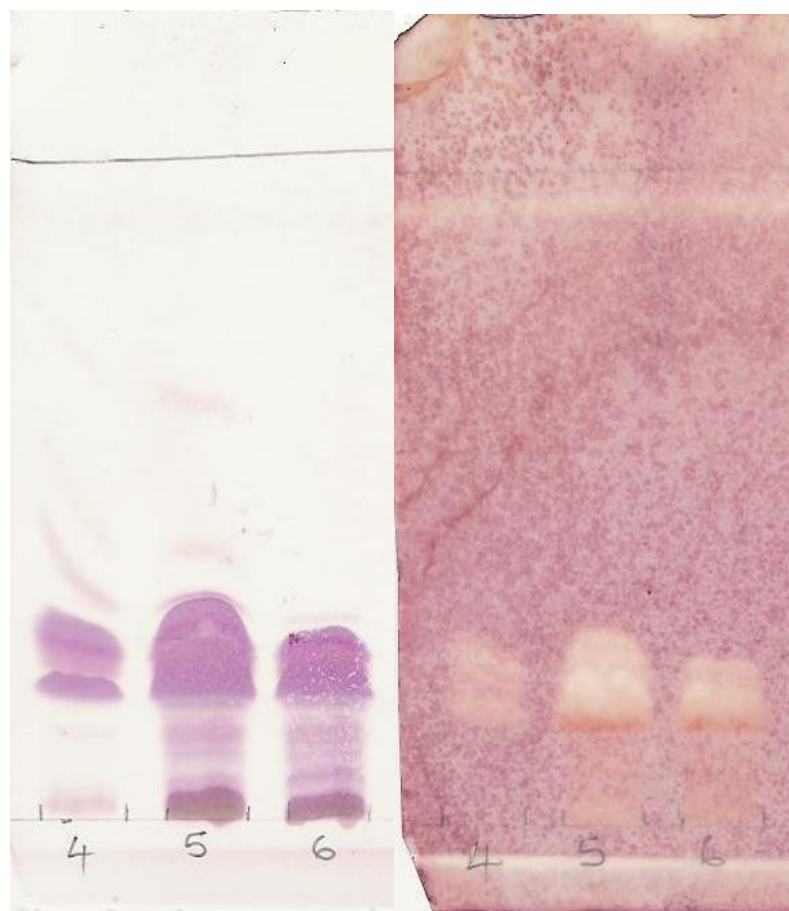


Fig. 3.28. TLC profile showing the phytochemical and bioautography analysis of the pooled fractions (A) obtained from Subfraction (F1 of 90:10 HEX:ETAC - F7 of 85:15HEX:ETAC) after developing with hexane.

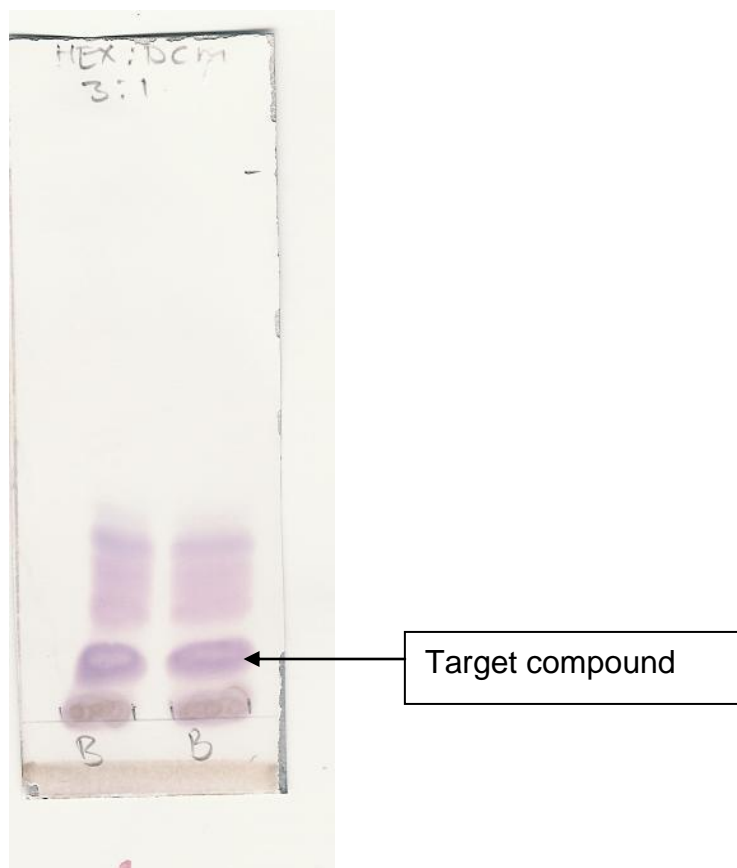


Fig. 3.29. Screening for mobile phase to isolate compound 2 from fraction A and HEX:DCM (3:1) gave better separation of the compounds.

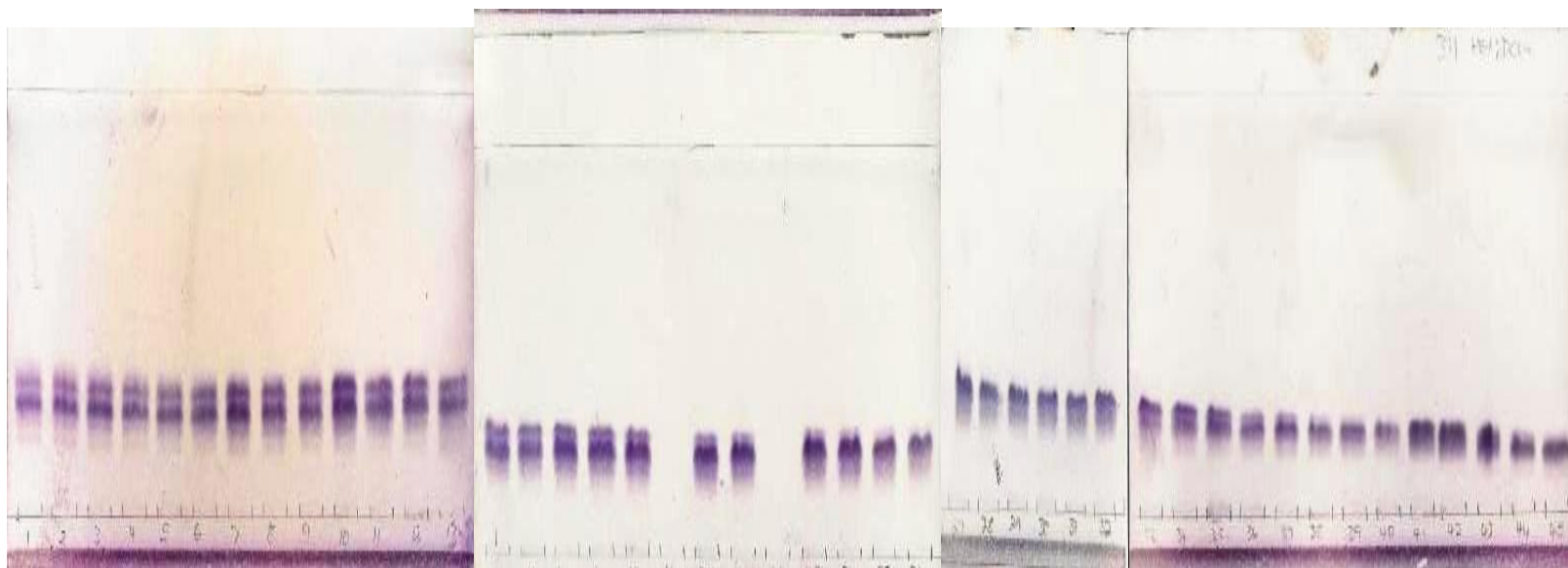


Fig. 3.30. TLC profile of fractions (A) after re-elution with HEX:DCM 3:1 indicating the phytoconstituents of the sub-fractions.

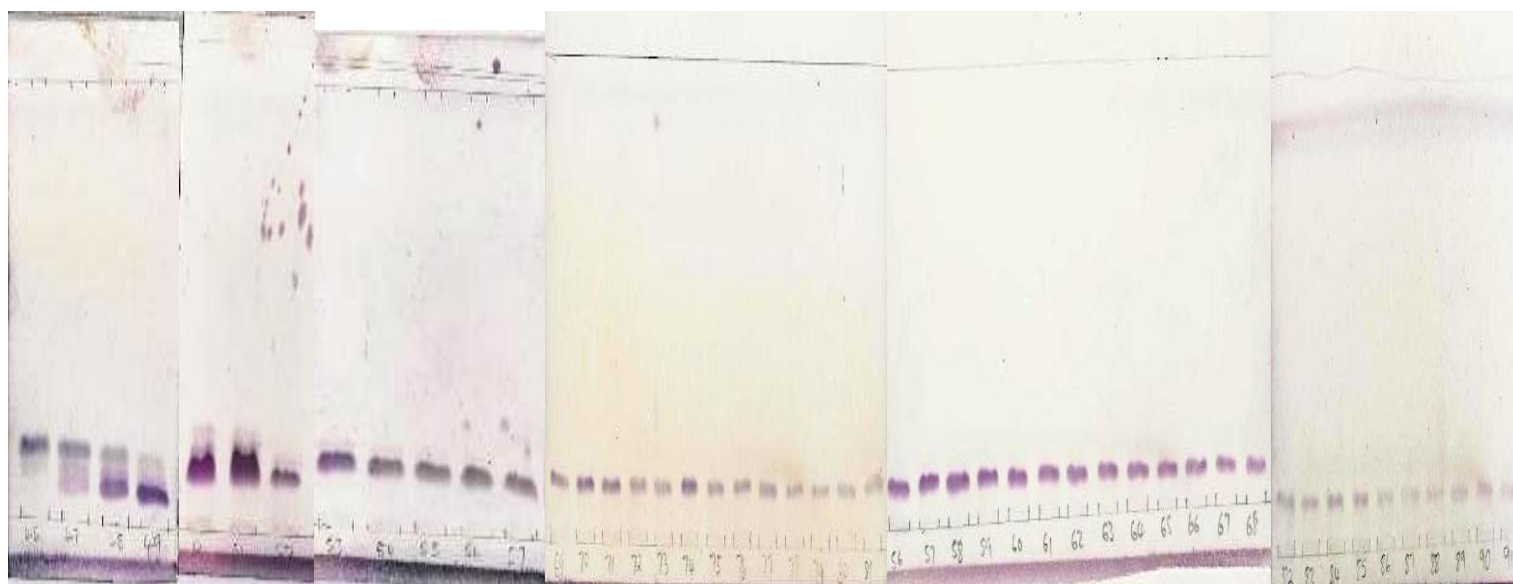


Fig. 3.31. TLC profile of fractions (A) after re-elution with HEX:DCM 3:1 indicating the phytoconstituents of the sub fractions. From sub-fraction 53 to 90 the compounds of interest was showing to be free from contaminants.



Fig. 3.32. TLC profile of pooled sub-fraction 53 to 90 which are free from contaminants after developed with HEX:DCM 3:1 indicating the compound interest.

3.7. Nuclear magnetic resonance of purified compounds

The two isolated and purified compounds from section 3.6 were analysed with a 300 MHz NMR instrument (Merck) to test for purity and structure elucidation. The ^{13}C and ^1H analysis were performed and the results are shown in Fig. 3.33-3.37 for compound A and Fig. 3.38-3.44 for compound B. NMR spectroscopy is a technique used to obtain physical, chemical, electronic and structural information about molecules due to either their chemical shift present on the resonant frequencies of the nuclei present in the sample. The ^{13}C and ^1H spectra allow the identification of carbon and hydrogen atoms in an organic molecule by detecting only the ^{13}C isotope of carbon and ^1H isotope of hydrogen. The various series of lines are named according to the lowest energy level involved in the transitions that give rise to the lines.

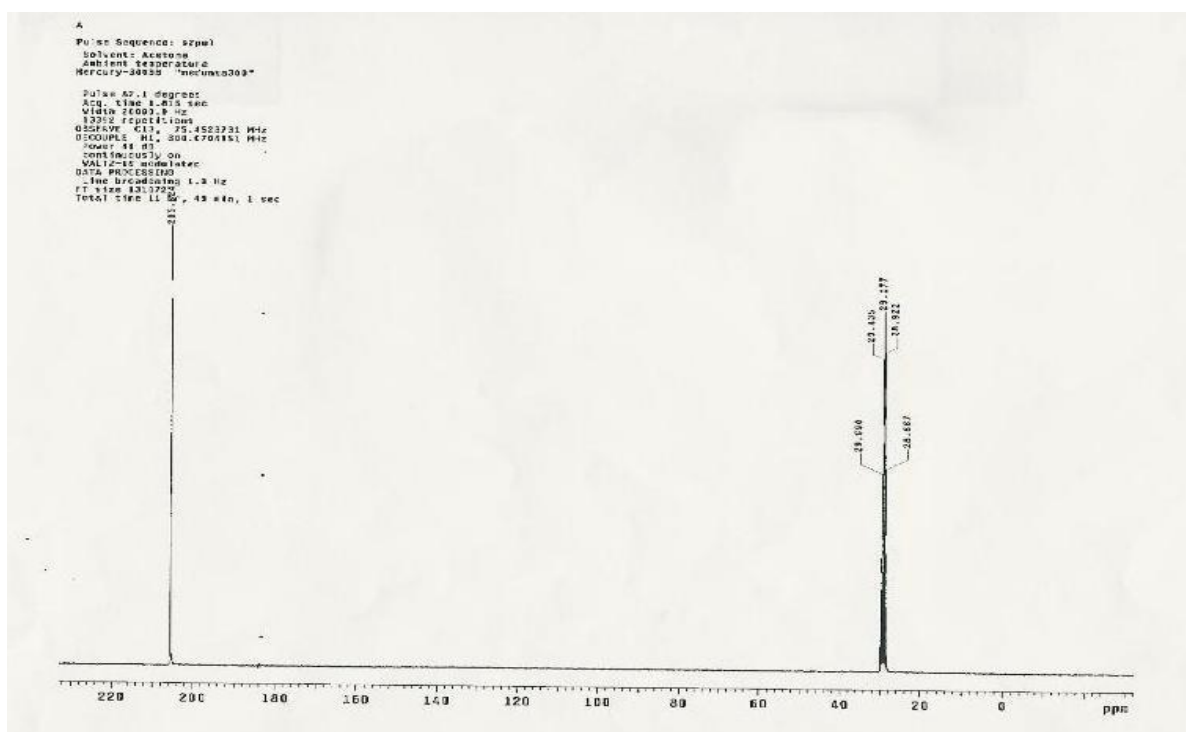


Fig. 3.33. ^{13}C NMR spectra of compound A.

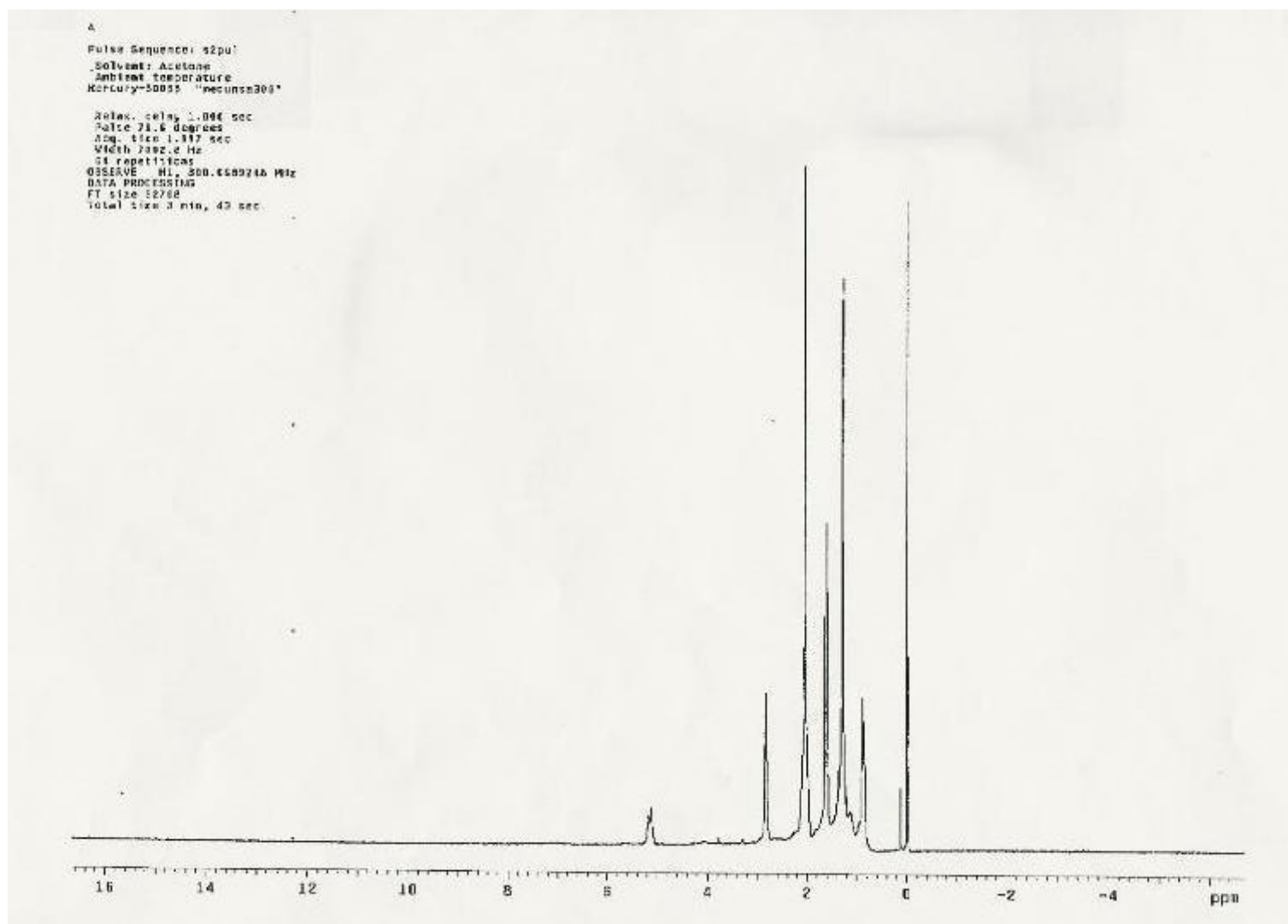


Fig. 3.34. ^1H NMR spectra of compound A.

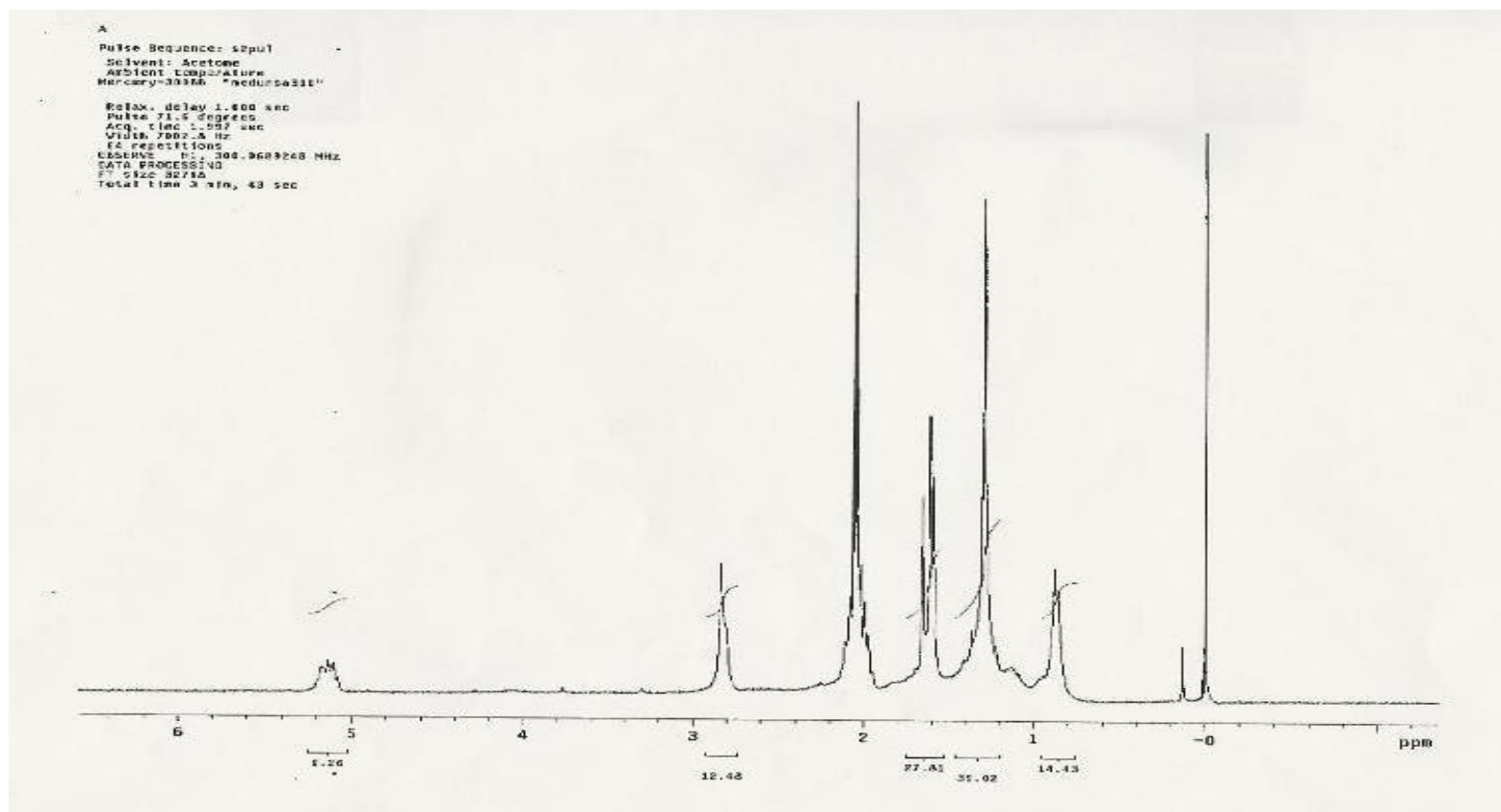


Fig. 3.35. ^1H NMR spectra of compound A.

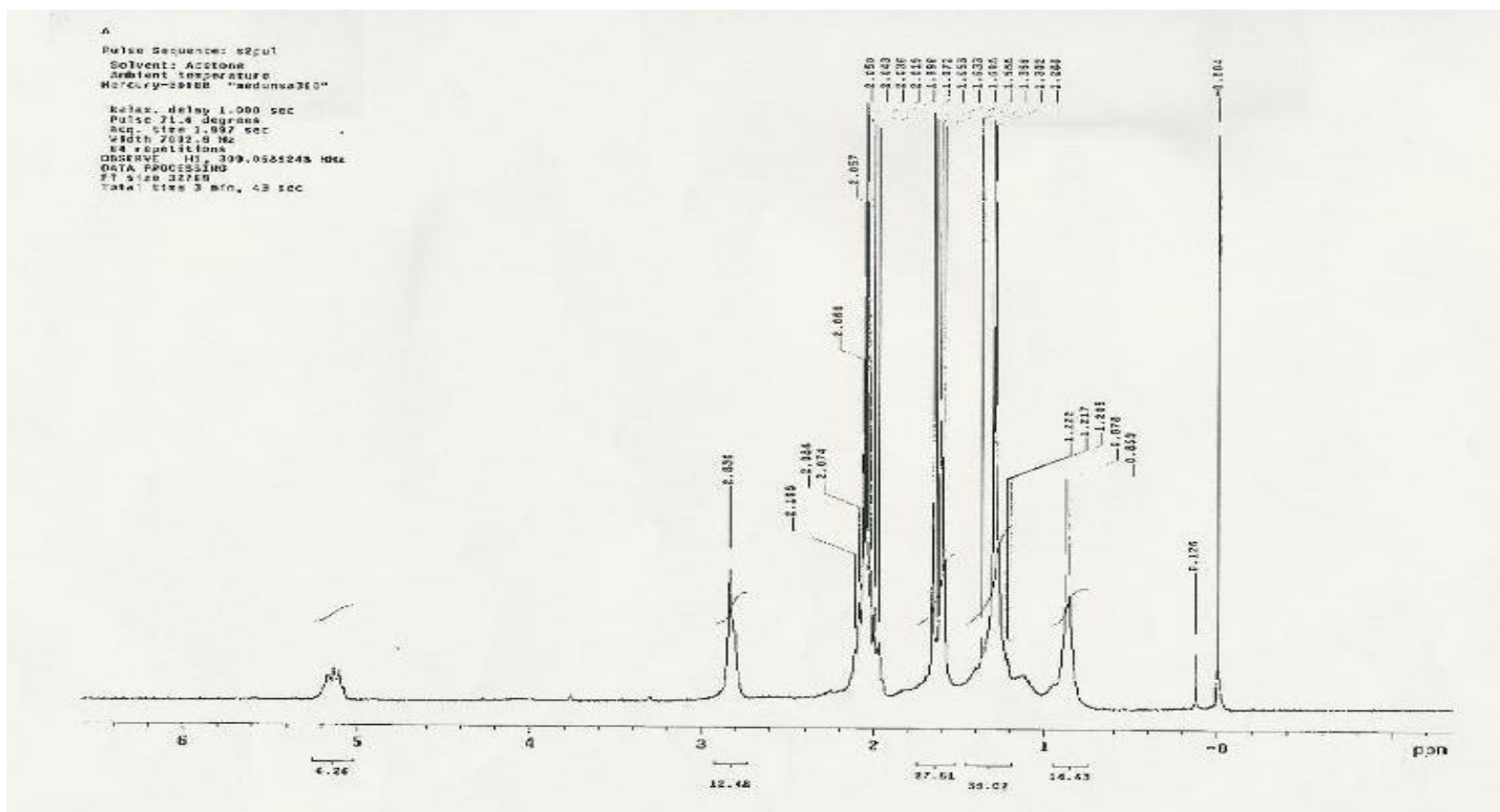


Fig. 3.36. ^1H NMR spectra of compound A.

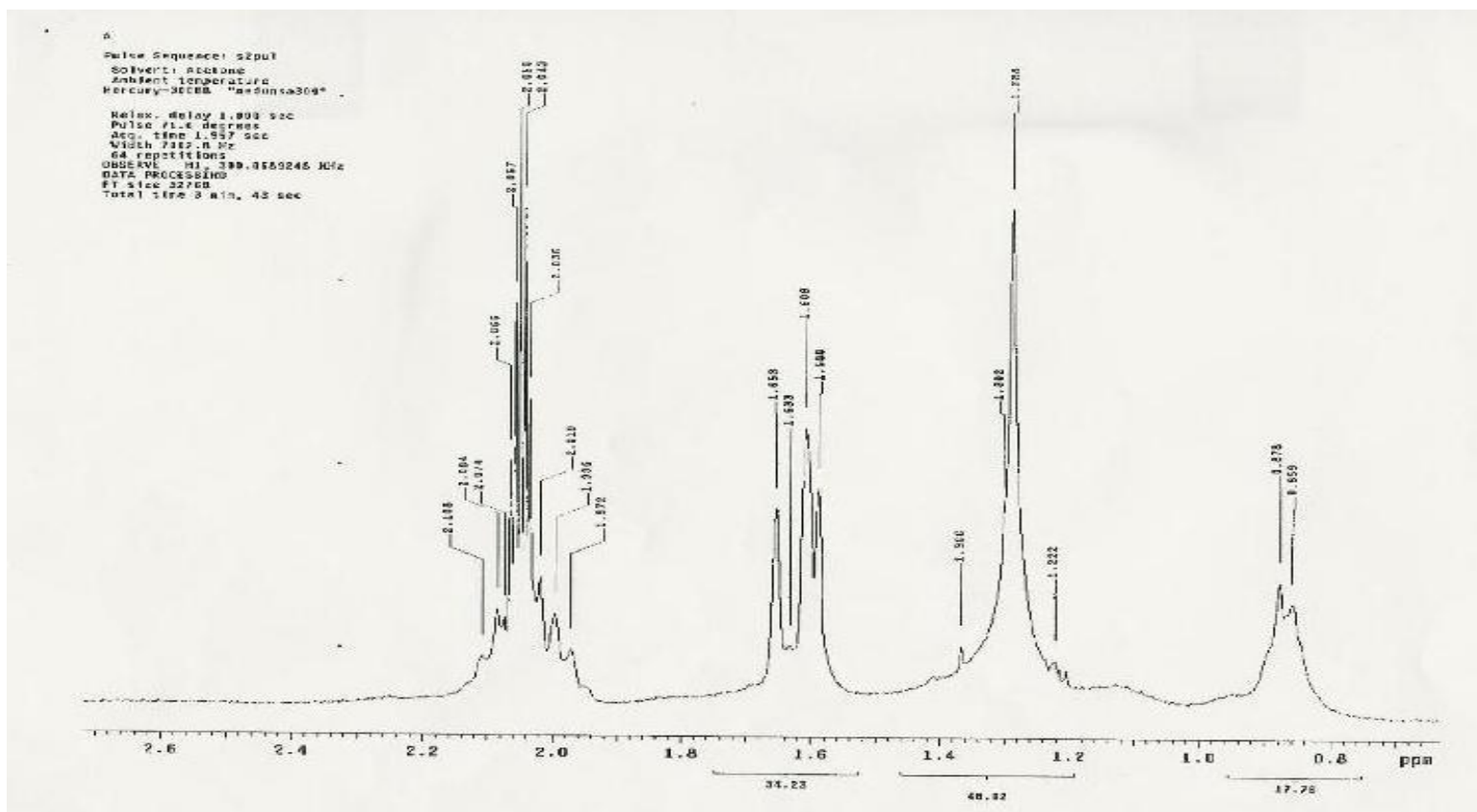


Fig. 3.37. ¹H NMR spectra of compound A.

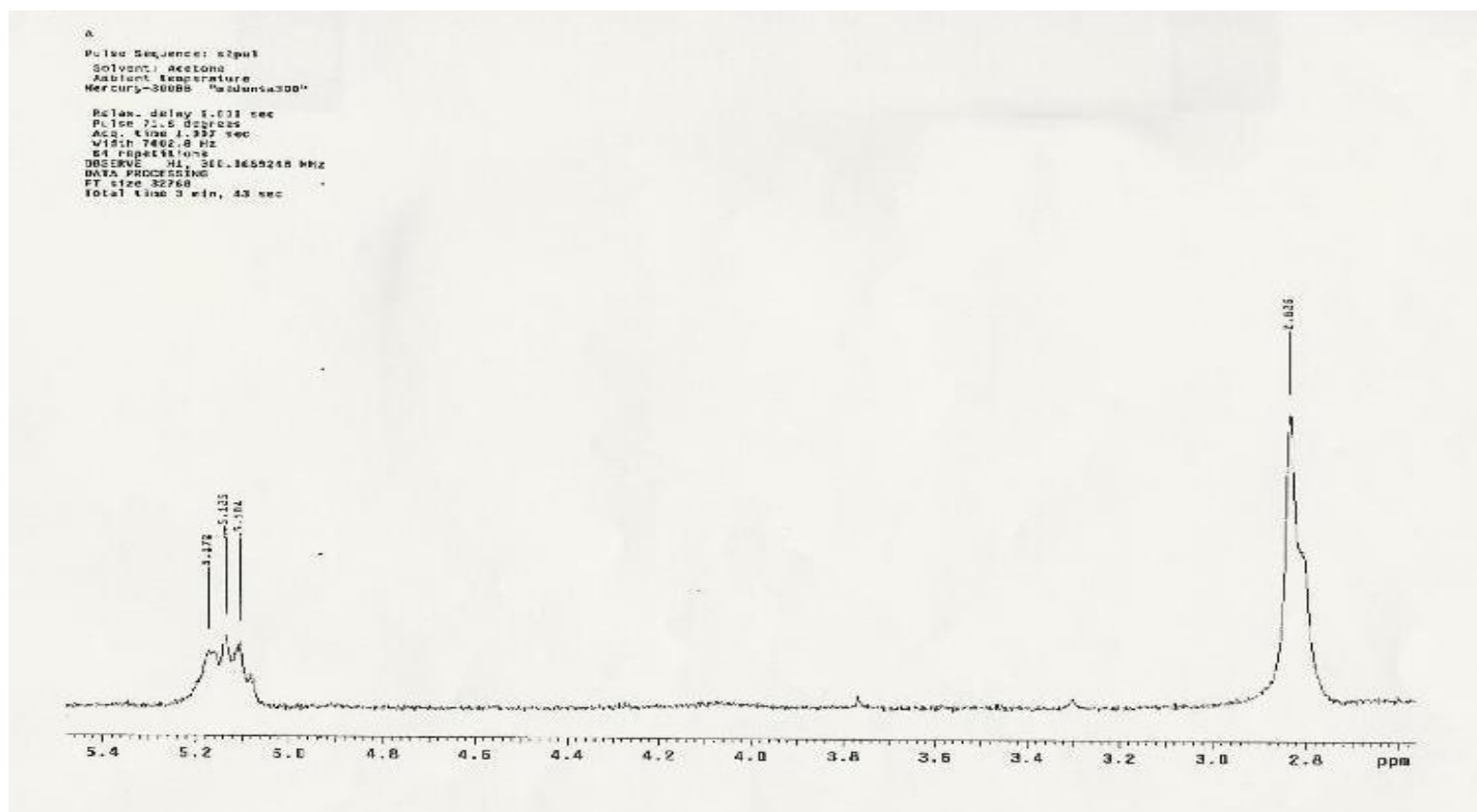


Fig. 3.38. ^1H NMR spectra of compound A.

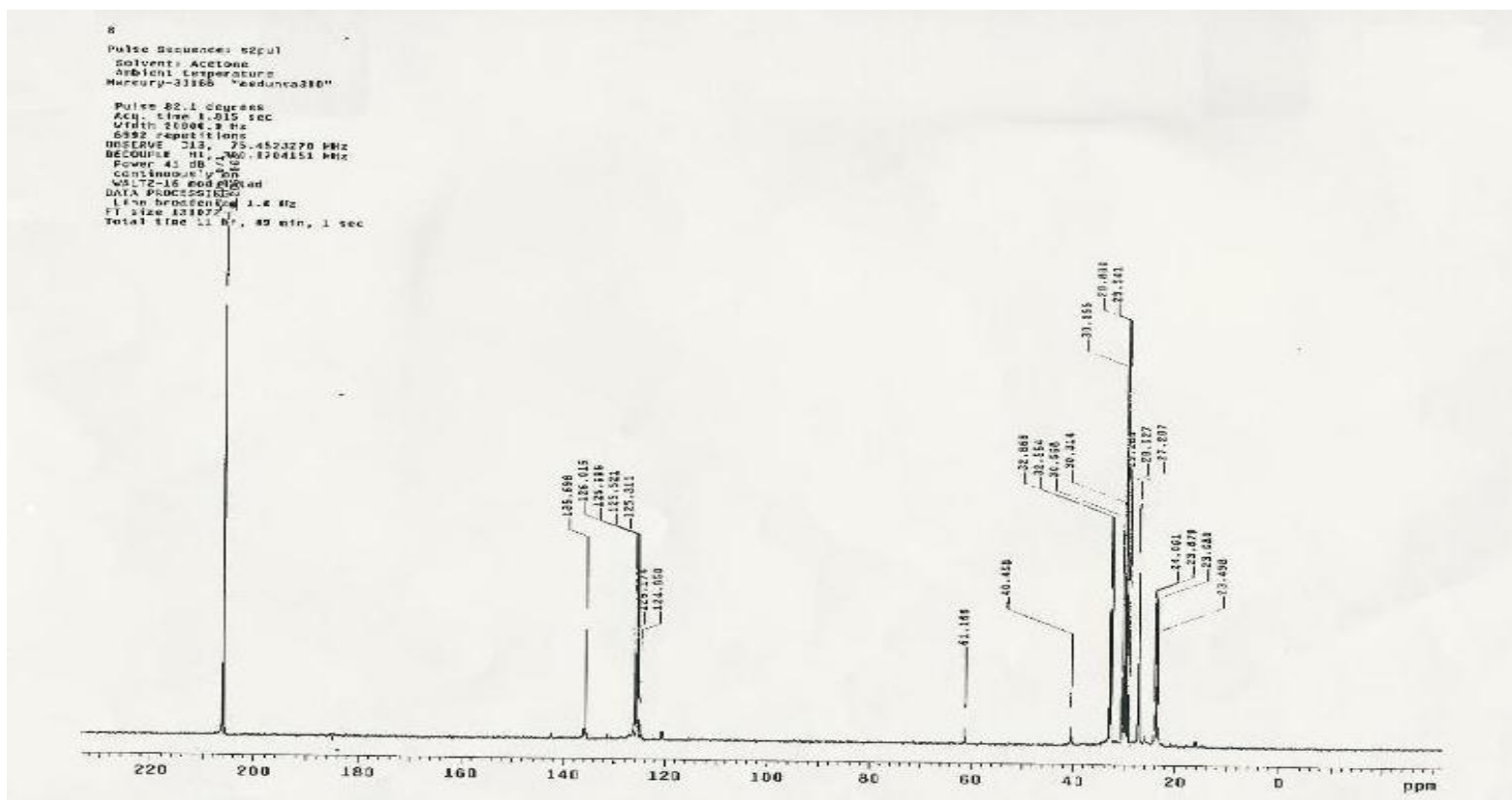


Fig. 3.39. ¹³C NMR spectra of compound B.

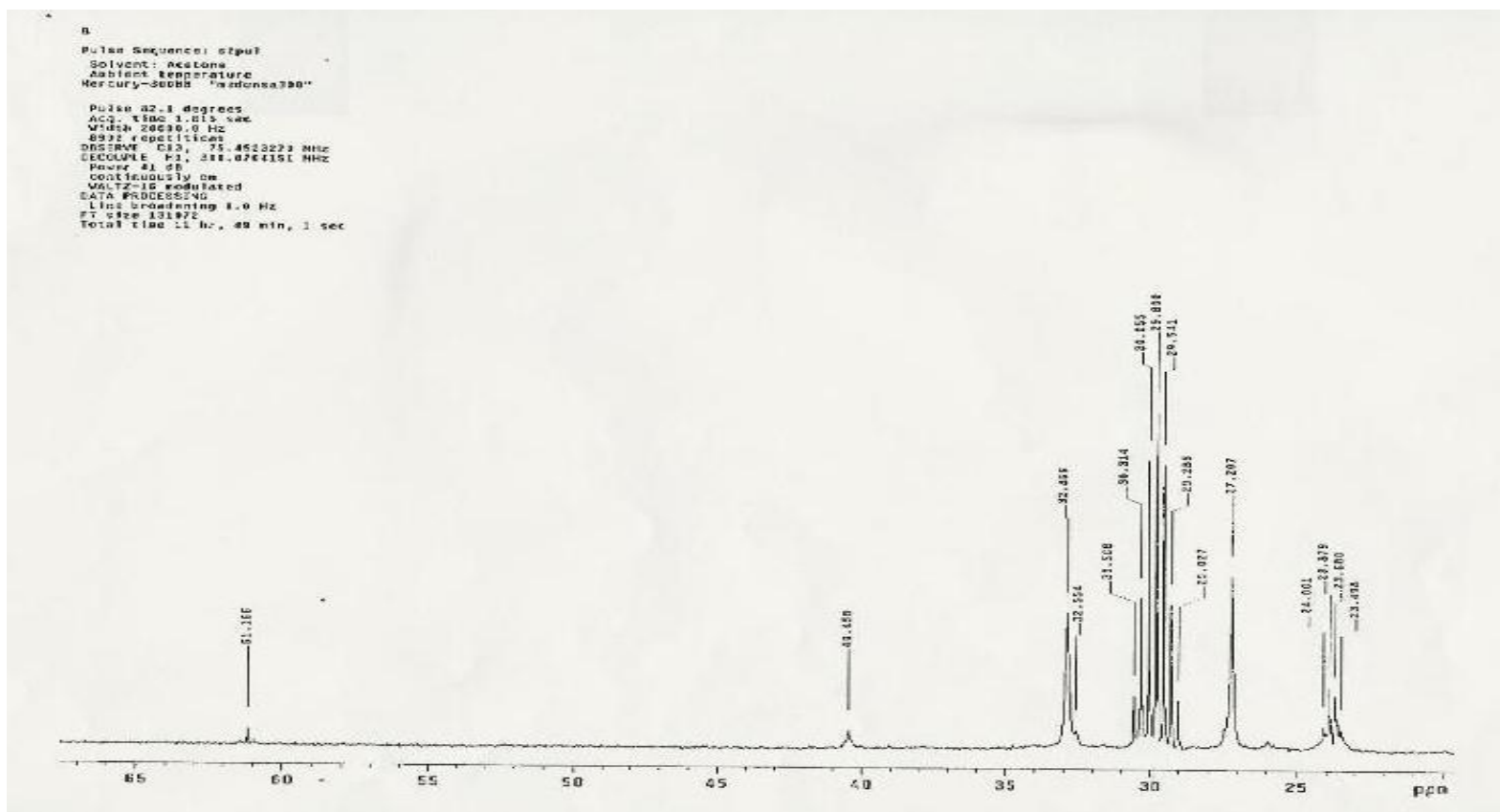


Fig. 3.40. ^{13}C NMR spectra of compound B

B
Pulse Sequence: zgpg3
Solvent: Acetone
Ambient Temperature
Nucleus: 13C
Pulse 82.1 degrees
Acq. time: 7.015 sec
Width: 20039.0 Hz
2022 repetitions
OBSERVE CH: 75.4523273 MHz
DECODE CH: 306.0704151 MHz
Power: 41 dB
Continuity: on
WALTZ-16 modulated
DATA PROCESSING:
Line Broadening: 1.0 Hz
FT Size: 131072
Total time: 11 hr, 49 min, 1 sec

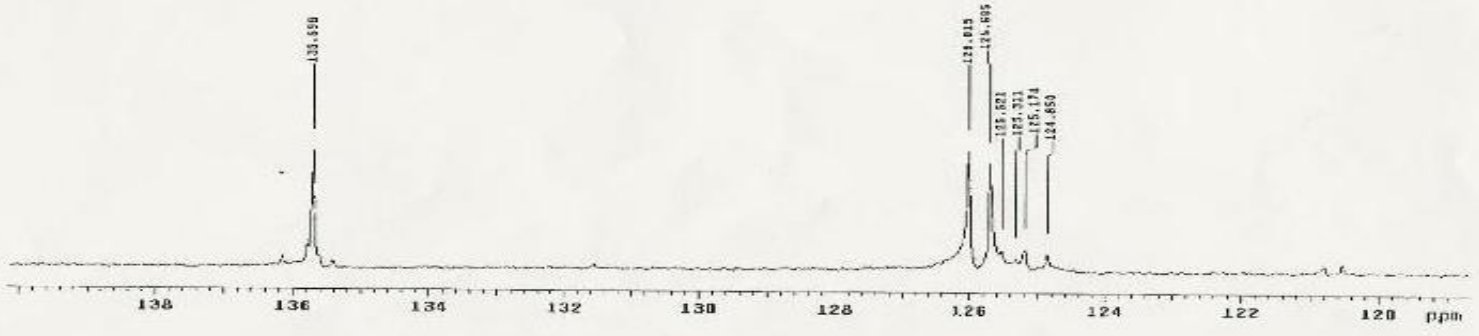


Fig 3.41. ¹³C NMR spectra of compound B

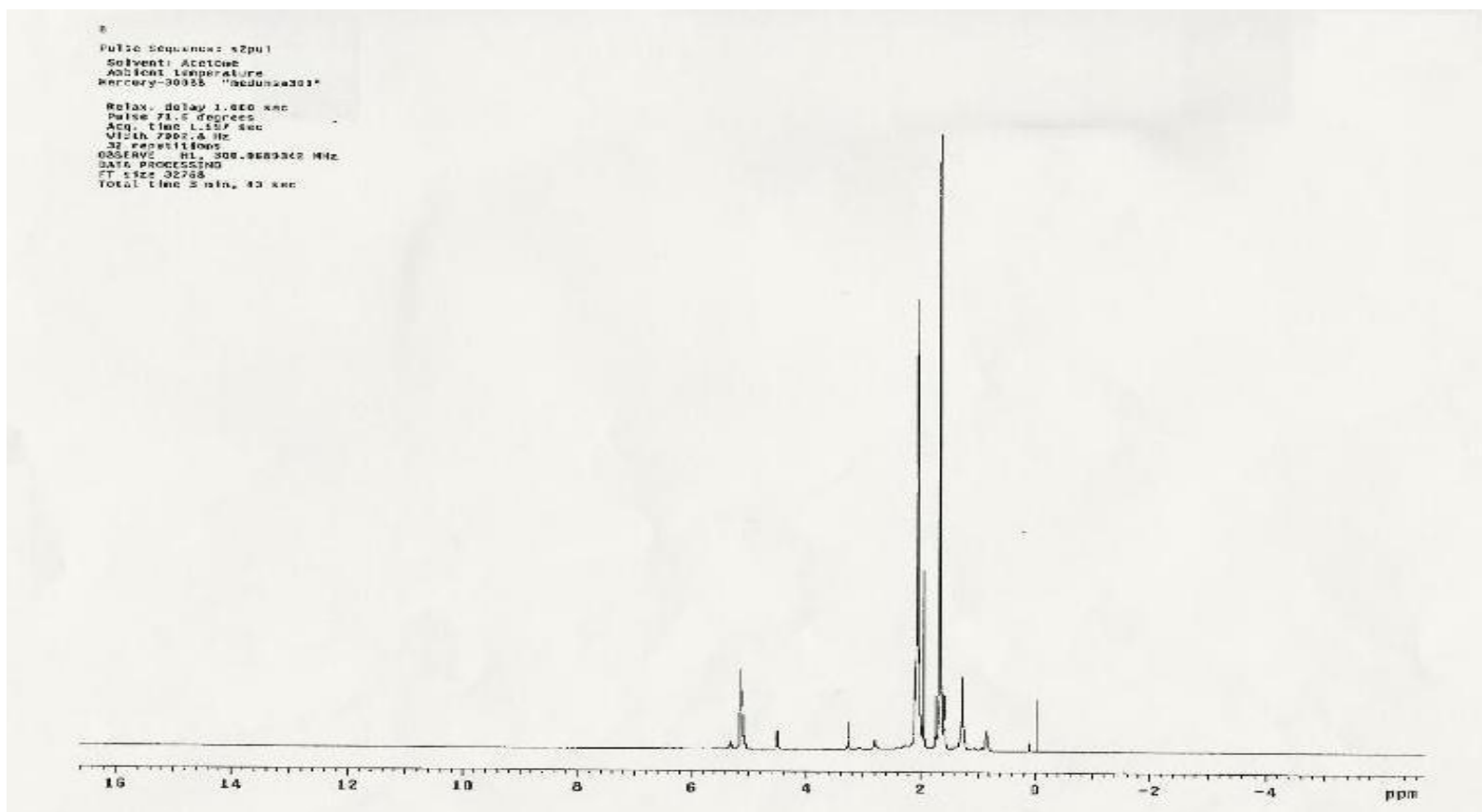


Fig. 3.42. ^1H NMR spectra of compound B.

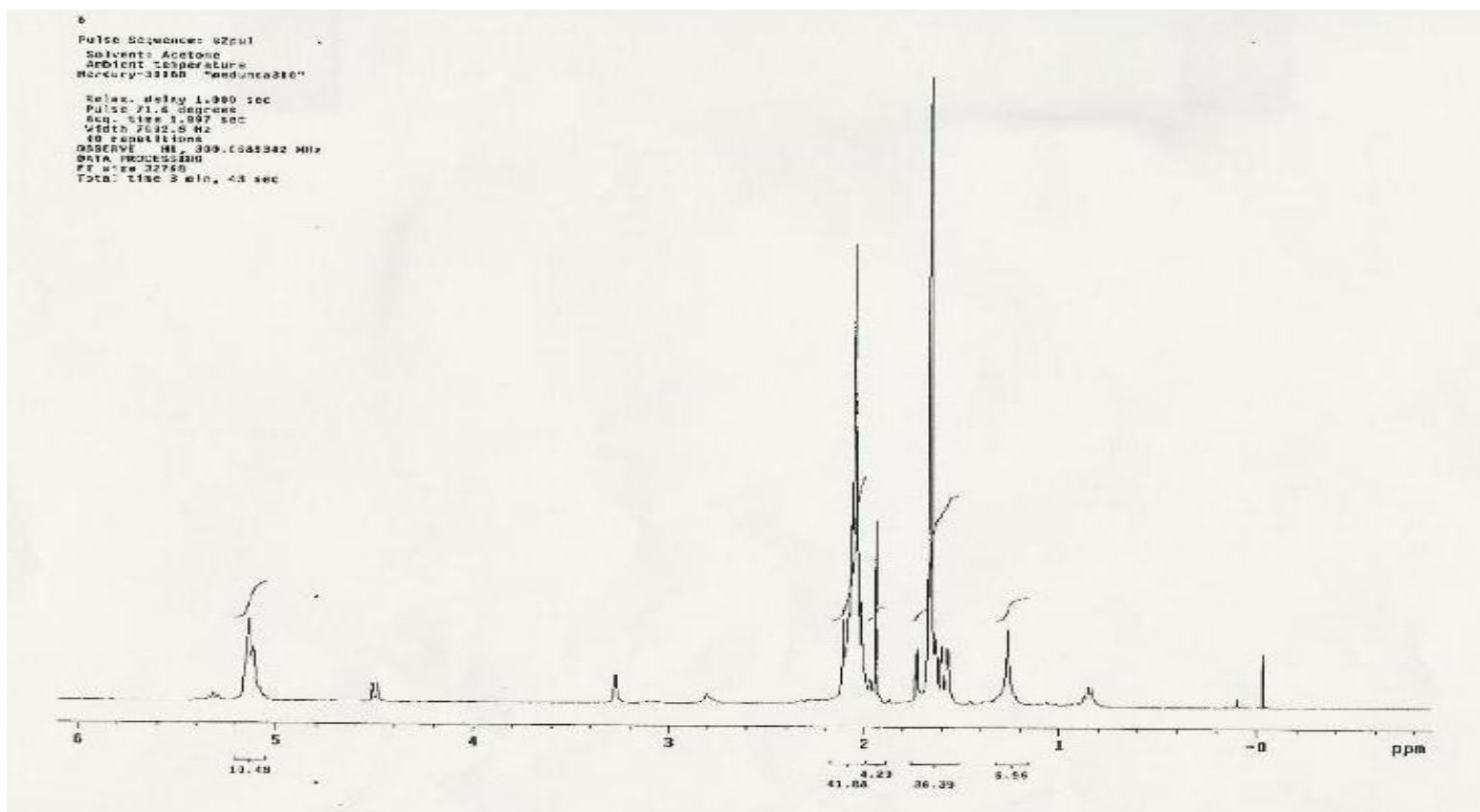


Fig. 3.43. ^1H NMR spectra of compound B.

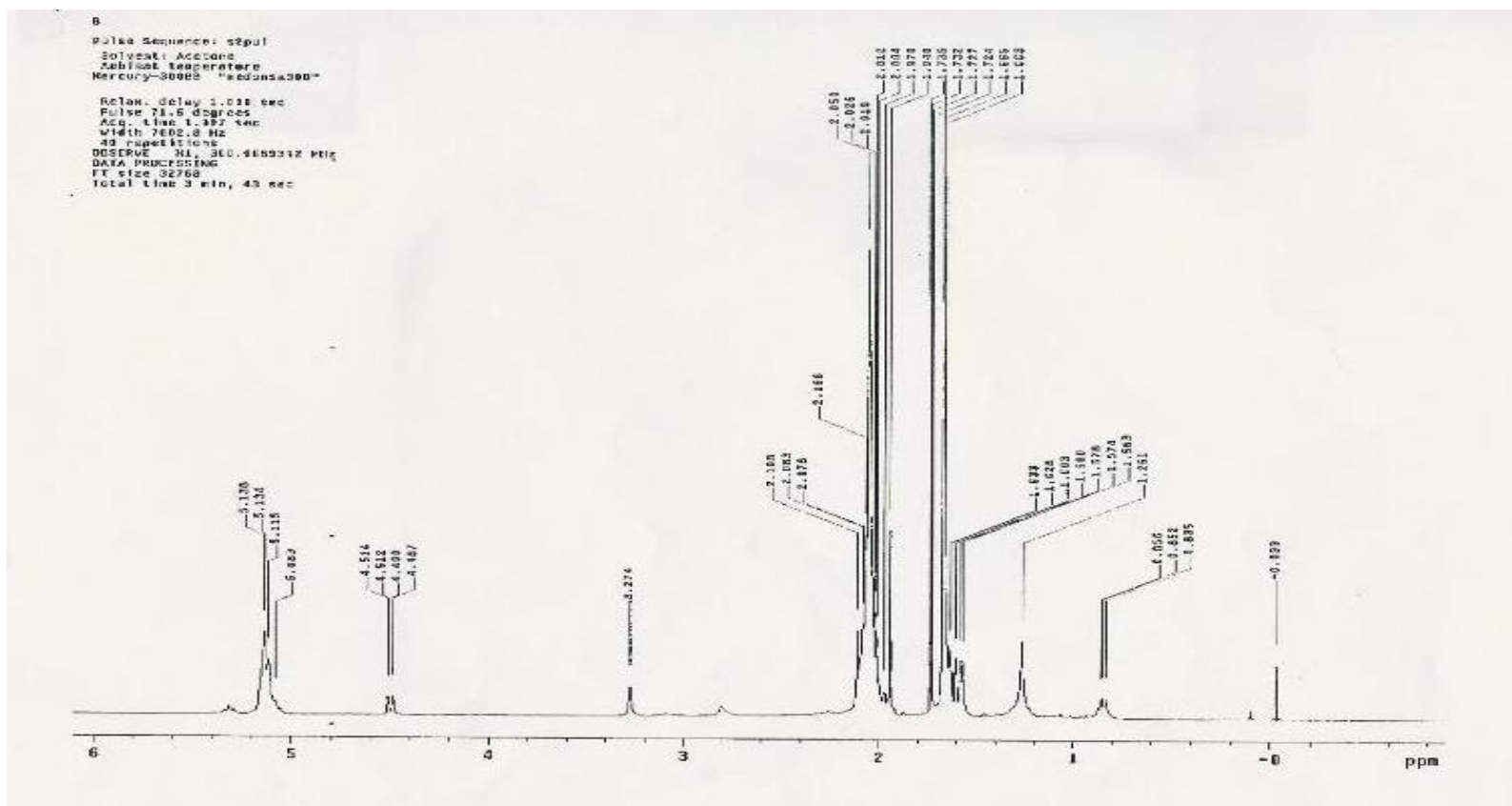


Fig. 3.44. ^1H NMR spectra of compound B.

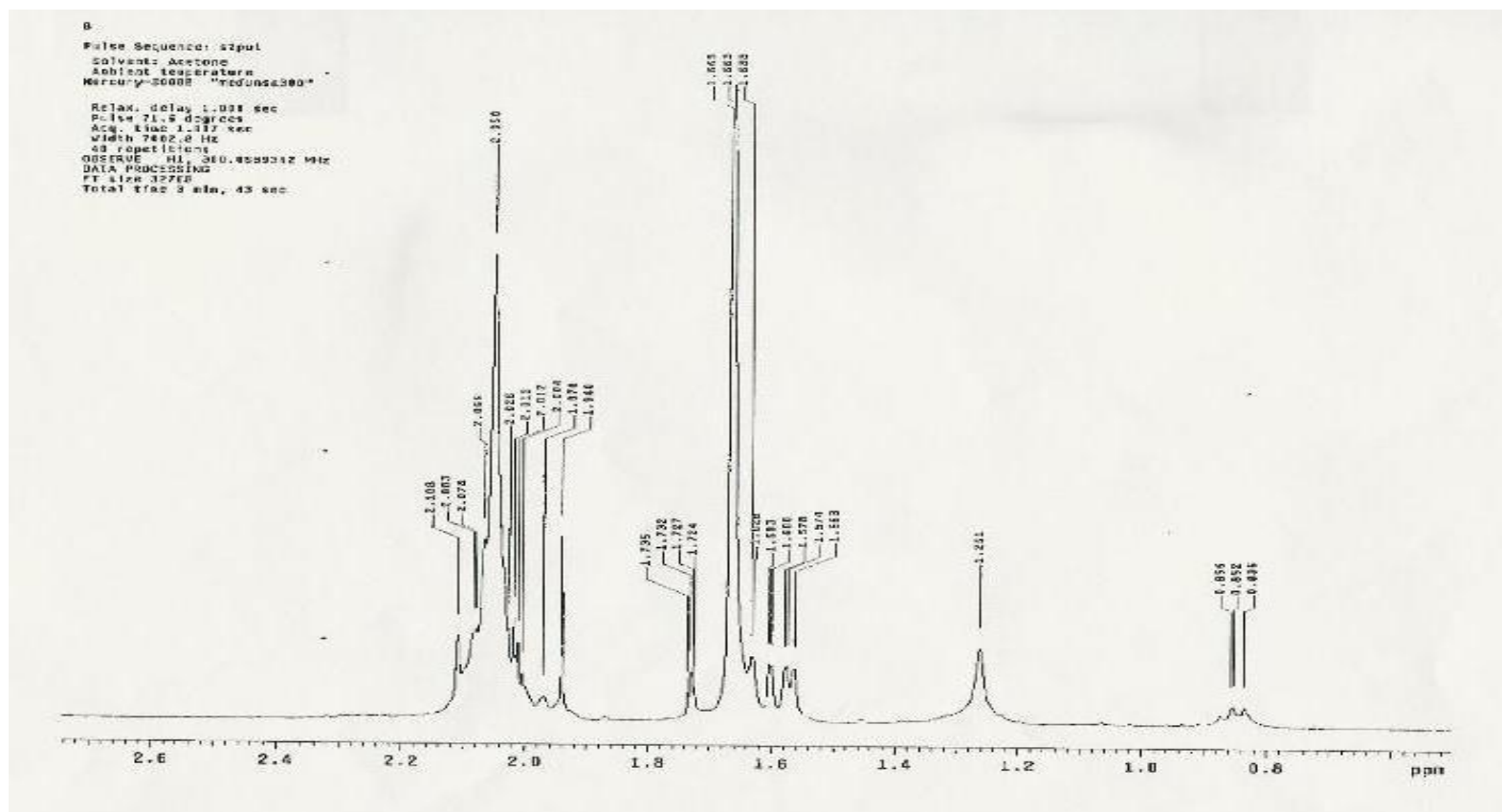


Fig. 3.45. ^1H NMR spectra of compound B.

CHAPTER FOUR

DISCUSSION

Tuberculosis has always been a serious public health challenge and is still one of the most devastating diseases of mankind. There has been no new anti-TB drug for treatment in the past 30 years and the available drugs are ineffective against resistant strains of *M. tuberculosis* hence there is a need to find new drugs for the treatment of TB. Plants have been a source of some important drugs against certain infectious diseases, thus the current study was aimed to investigate the possibility of discovering a chemical compound(s) from plants which could be used as a cure for TB. To this end 15 plants species were selected based on their usage in traditional medicine and screened for antimycobacterial activity with a hope of eventually isolating and purifying compound(s) that could be developed into a drug for the treatment of TB.

Of the four solvents used in the study methanol was the best solvent since it extracted a greater quantity of plant material from leaves than any of the other solvents. Hexane extracted the least amount of material. Masoko *et al.* (2008) also found that methanol was a better solvent compared to the others. The strong extractability of methanol is expected since methanol has been shown to efficiently penetrate cell membrane of plants, permitting the extraction of high amount endocellular components (Britton, 1991). This suggested that methanol extracts contained higher concentration of compounds compared to the other solvents used in this study.

When the extraction from the different plant leaves are compared, the *M. undata* leaves yielded almost equivalent mass from all solvents and its acetone extract was the highest amongst all the acetone extracts from the other plants. Overall, different mass yields were extracted from the different plants species using different solvents thus suggesting that different plant leaves have different phytoconstituents.

Thin layer chromatography was used to analyse the phytoconstituents of the plant crude extracts since it is a rapid and effective method to obtain the fingerprints of plant extracts (McGaw *et al.*, 2002). The BEA system showed a good separation for

compounds for all plant extracts, indicating that the extracts are rich in slightly and non-polar compounds (Figs. 3.2-3.6). The benzene and ammonia within the BEA solvent system contributes the non-polar parts as non-polar solvent separates non-polar compounds better (Kotzé and Eloff, 2002).

Macaranga capensis had yellow bands appearing on the TLC plate against a purple DPPH background signifying compounds with antioxidant activity. The high antioxidant activity of *M. capensis* was also observed by Kaikabo, (2009) at the University of Pretoria. The reason for determining the antioxidant activity of plant extracts is to ascertain if the antimicrobial activity observed is due to the antioxidant activity or due to some specific compounds.

Acetone is a known antimicrobial agent, so to eliminate the possibility that the MIC results were due to acetone it was included as negative control in the study. Acetone showed no inhibition of growth of *M. smegmatis* and *R. erythropolis* at concentrations of 25% and below. Following this observation the plant extracts were dissolved to yield final concentration of 25% acetone.

The reference antibiotic, rifampicin, had an MIC value of 0.125 mg/ml against both *M. smegmatis* and *R. erythropolis*.

Milletia stulhimannii showed the greatest antimicrobial effect when compared to the other plants extracts with an average MIC values as low as 0.25 mg/ml against *Mycobacterium smegmatis* and 0.22 mg/ml against *R. erythropolis* (Tables 3.1 and 3.2). Overall acetone extracts for all plants had the lowest MIC value of 0.40 mg/ml for *M. smegmatis* and 0.38 mg/ml for *R. erythropolis* after 24 hrs incubation (Tables 3.1 and 3.2). Acetone extracts of *Milletia stulhimannii*, *Albizia gummifera*, *Xanthocercis zambesiaca* and *Barringtonia racemosa* showed promising anti-mycobacterial activities against *M. smegmatis* with an average MIC value of 0.13 mg/ml (Table 3.1). Some other crude extracts had higher MIC (low potency) and a high MIC value does not necessarily mean that a particular plant is not as active as a plant with lower MIC values. Firstly, this low MIC values might be indicative of other agents inhibiting the desired compounds active, in a process known as antagonistic effect. Secondly, it could

be that active compounds may be in very low concentration and hence exhibit low activity (Amoo *et al.*, 2009).

In general the MIC values, as compared to other studies, showed that acetone is the best solvent for extracting antibacterial activity. Eloff (1998a) used acetone for extracting plant material and found that greater antibacterial activity were obtained than in the case of methanol, ethanol and water.

Total activity indicates the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the bacteria (Eloff, 1998b). Extracts with higher total activity values were considered the best for isolation of potential compounds. The extracts of *K. acuminata* exhibited the highest total activity, followed by *M. stuhlmannii* when compared to the others. Thus one gram of *K. acuminata* and *M. stuhlmannii* acetone extract diluted to 211.1 ml and 191.5 ml, respectively, with water still inhibited the growth of *M. smegmatis*. The correlation between MIC and total activity does not always exist since most plants exhibiting low MIC do not have high total activity (Eloff, 1998b), hence further tests are required to establish the real activity of an extract. Biological activity using chromatograms provides a more direct method of establishing the real activity of an extracts.

The chromatograms developed in BEA, CEF and EMW were used for the bioautography even though EMW and CEF systems demonstrated poor separation of compounds during initial chromatographic analyses. The motivation for using these two systems was to establish whether unseparated compounds had biological activity or not. Antimycobacterial activity was observed only in TLC plates developed in BEA suggesting that the active compounds were slightly non-polar. The TLC confirmed that the other extracts separated with EMW and CEF systems did not exhibit any biological activity. Plant extracts from *M. undanta*, *V. infausta* and *A. dimidiata* were the only extracts which had biological activity against *M. smegmatis* and *R. erythropolis*.

From the literature review it was established that *Apodytes dimidiata* was a plant that was not studied for antimicrobial compounds compared to the other two plants.

Secondly, it's clear and marked biological activity made it a good candidate for further study. The plant has been reported to have molluscicidal activity and only two compounds were isolated which are iridoid genipin and its 10-monoacetate derivative (Drewes and Kayonga, 1996).

From the six sub-fractions obtained from acetone extract only three had activity with retention factor ranging between 0.40-0.67 (Fig. 3.24). Bioassay-guided fractionation was followed to isolate the active compound.

Results shown in Figures 3.24, 3.26 and 3.27 demonstrated that the extracts were losing activity with each step of purification. The findings were similar for both compounds. Loss of activity is a common problem associated with attempts to purify single compound from crude plant extracts. For example, Nwodo *et al.*, (2010) made similar observations and one reason for lost of activity has been advanced. It has been argued that since crude extracts contains several compounds these compounds may act synergistically to give elevated activity. Therefore when attempts are made to obtain a single compound the other compounds are lost and hence a drop in activity. These findings suggest that it might be better to use the crude extracts for anti-mycobacterial application than the purified compounds. This might explain why people in developing countries prefer to use crude extracts for antimicrobial therapy. The use of crude extracts for antimicrobial therapy may act to reduce the development of resistant microbes. It is easier for a pathogenic microbe to develop resistance against a single purified compound than a mixture of compounds (extract) which target different compounds or sites responsible for pathogenicity of an organism.

Conclusion and future work

As noted, *A. dimidiata* is highly valued by the Zulu in their traditional medicine (Van Wyk and Wink, 2004). The leaves are used in the treatment of ear inflammation. The stem barks have the highest molluscicidal activity (Pretorius *et al.*, 1991) and also its infusion is used as an enema for intestinal parasites. The current research found that out of the 15 plants investigated, *A. dimidiata* was active against both *M. smegmatis* and *R. erythropolis*. So this research, to some extent, supports the indigenous traditional use of

this plant. However, as can be seen the MIC value of the acetone extracts of *A. dimidiata* was 0.63 mg/ml and this MIC value increased to more than 2.5 mg/ml with the purified compound. It is uncommon to find that the initial crude extract has a lower MIC and as it is fractionated the MIC increases (Jean *et al.*, 2001). Various researchers have cited the following reasons for this increase. Firstly, the crude extract may have several compounds which act simultaneously on the test organism. Alternatively, several compounds may act synergistically to inhibit growth. Therefore, as fractionation takes place some of constituent compounds may be lost. When one compares the MIC values for the two purified compounds against rifampicin they were found to be 10 fold weaker.

The two compounds were isolated in relatively small amounts (25 mg and 39 mg, respectively). However the compounds were inactive at MIC value of 2.5 mg/ml against all the tested organisms, (results not shown). The data obtained from nuclear magnetic resonance (NMR) results suggested that the compounds isolated were long fatty acids chain (compound A) and a triterpene (compound B). We could not establish this conclusively as more plant materials were required to enable further testing and there was not sufficient time to redo the purification of these compounds. There is certainly a need to increase the amount of purified compounds to enable further mass spectroscopy analysis in order to identify the compounds conclusively. The cytotoxicity and mode of action of the bioactive compounds has to be ascertained.

CHAPTER FIVE

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CHAPTER SIX

APPENDICES

6.1. Appendix A: Solutions

6.1.1. Preparation of Vanillin sulphuric acid

0.1 g Vanillin (Sigma-Aldrich, Batch No. 086K3676)

28 ml Ethanol (Reidel-de Haen, Batch No. 32221)

1 ml Sulphuric acid 95-97% (Fluka, Batch No. 84720)

Vanillin powder of 0.1 g was weighed into a dark brown coloured bottle, 28 ml of ethanol was added into the same bottle, and 1 ml of sulphuric acid was lastly added slowly into the bottle and shaken the mixture. The recipe was followed in this order with the acid. The mixing was done at room temperature. Vigorously shaking of the mixture insured complete dissolving of the ingredients and resulting in a homogenous mixture of vanillin sulphuric acid.

6.1.2. Preparation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution

0.2 g DPPH (Sigma-Aldrich, Cat.: D9132)

100 ml Methanol (Sigma-Aldrich, Batch No. 34860)

DPPH of 0.2 g was weighed and added it into a brown bottle and added 100ml of methanol into the same brown bottle. The mixture was shaken to dissolve the DPPH. The DPPH solution was stored in the fridge until needed.

6.1.3. Preparation of *p*-iodonitrotetrazolium chloride (INT) solution

0.2 g *p*-iodonitrotetrazolium chloride (Sigma-Aldrich, Batch No. 18377-5G)

100 ml distilled water

Preparation

p-iodonitrotetrazolium chloride of 0.2 g was weighed and added to a dark brown bottle and 100 ml of distilled water to the bottle. The mixture was vigorously shaken until all the *p*-iodonitrotetrazolium chloride has dissolved in the distilled water. The INT solution was stored in the fridge until needed.

6.2. Appendix B: Media preparation

6.2.1. Preparation of 1000 ml Middlebrook 7H9 broth

4.7 g Middlebrook 7H9 Broth (Difco Cat. No. 271310

100 mL of Middlebrook ADC Enrichment (Sodium Chloride - 0.85 g, Bovine Albumin (Fraction V) - 5.0 g, Dextrose - 2.0 g, Catalase - 3 mg)

0.5 ml Polysorbate 80 or Tween 80

Preparation

Middlebrook 7H9 Broth powder of 4.7 g was suspended in 900 mL of purified water containing 0.5 ml polysorbate 80. Medium was sterilized by autoclaving for 15 minutes at 121°C and 1 atm. Aseptically 100 mL of filter sterilized Middlebrook ADC Enrichment was added to the medium when cooled to 45°C. For preparation of agar medium 19 g of agar bacteriological (Biolab, Batch No. 1020577) was included before sterilization.

6.2.2. Preparation of 1000 ml Luria Bertani broth

10 g Tryptone

5 g Yeast extract

5 g NaCl

Preparation

All the components were added in one liter of distilled water and left to boil to dissolve the components completely. Medium was sterilized by autoclaving for 15 minutes at

121°C and 1 atm. For preparation of agar medium 19 g of agar bacteriological (Biolab, Batch No. 1020577) was included before sterilization.

6.3. Appendix C: Mobile phases in 100 ml

6.3.1. EMW

40 ml Ethyl acetate (Fluka, Batch No. 45760)

5.4 ml Methanol (Sigma-Aldrich, Batch No. 34860)

1 ml Distilled water

6.3.2. BEA

72 ml Benzene (Fluka, Batch No. 12552)

8 ml Ethanol (Reidel-de Haen, Batch No. 32221)

0.8 ml Ammonia 85% (Saarchem, Batch No. 10839)

6.3.3. CEF

30 ml Chloroform (Saarchem, Batch No. 1017468)

24 ml Ethyl acetate (Fluka, Batch No. 45760)

6 ml Formic acid 98% (Fluka, Batch No. 06440)

Preparation of all the mobile phases was done by adding in the same order in a glass container and shaken to ensure homogenous mixture.

6.4. Appendix D: TLC plates and other equipment

6.4.1. TLC silica gel/TLC-cards (Fluka, Batch No. 60778)

6.4.2. Bench top centrifuge machine (Hettich zentrifugen, Rotina 38R D785322 Tuttlingen)

6.4.3. Incubator (IncoTherm^s Labotec)

6.4.4. Incubator shaker (New Brunswick Scientific, series 25)

6.4.5. Shaking machine (Optalabor)