IDENTIFICATION AND CHARACTERISATION OF COMPOUNDS WITH ANTIMYCOBACTERIAL ACTIVITY FROM THE LEAVES OF *DOMBEYA ROTUNDIFOLIA* Hochst.

By

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RESEARCH DISSERTATION

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Declaration

I, Matsilane Lethabo Mashilo, hereby declare that the dissertation submitted to the University of Limpopo for the degree of Master of Science in Microbiology is my own work in design and execution and it has not been previously submitted by me or anyone else at this or other University. The materials contained therein have been duly acknowledged.

Signature: ..... Date……

Dedication

I dedicate this study to my mother, Mankopodi Mabel Kgoloko and my siblings, Modinoge Bonny, Matsebe Pontsho, Kholofelo and Dimpho.

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List of abbreviations

^{13}C: Carbon-13

1D NMR: One-dimensional Nuclear magnetic resonance

^{1}H: Hydrogen-1

2 D NMR: Two-dimensional Nuclear magnetic resonance

A: Acetone

AA: Ascorbic acid

B: Butanol

BEA: Benzene, ethanol, and ammonium hydroxide

C: Chloroform

CEF: Chloroform, ethyl acetate and formic acid

CO_2: Carbon dioxide

COSY: Correlation spectroscopy

D: Dichloromethane

DEPT: Distortionless enhancement through polarisation transfer

DMSO: Dimethyl sulfoxide

DNA: Deoxyribose Nucleic Acid

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

DR: *Dombeya rotundifolia*

E: Ethanol

EA: Ethyl acetate

EMW: Ethyl acetate, methanol, and water

FBS: Foetal bovine serum

FRAP: Ferric reducing antioxidant power

GAE: Gallic acid equivalence

H: Hexane

HIV: Human immunodeficiency virus

HMBC: Heteronuclear multiple bond correlation

HMQC: Heteronuclear multiple quantum correlation

HSQC: Heteronuclear single quantum correlation

INT: p-iodonitrotetrazolium violet

M: Methanol

MBC: Minimum bactericidal concentration

MDR: Multi-drug-resistant

MIC: Minimum inhibitory concentration

MS: Mass spectrometry

Mtb: *Mycobacterium tuberculosis*

MTT: 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide

NADPH: Nicotinamide adenine dinucleotide phosphate

NMR: Nuclear magnetic resonance spectroscopy

QE: Quercetin equivalence

RNA: Ribonucleic acid

RPMI medium: Roswell Park Memorial Institute Medium

TB: Tuberculosis

TLC: Thin Layer Chromatography

UV/VIS: Ultraviolet–visible spectroscopy

UV: Ultraviolet light

W: Water.

WHO: World Health Organisation

XDR: Extensively drug-resistant

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Conference presentations

Oral paper

1. Mashilo, M.L. and Masoko, P. Preliminary identification of compounds with antimycobacterial activity from *Dombeya rotundifolia*. Presented during the 11th Annual Faculty of Science and Agriculture Postgraduate Research Day held at Bolivia Lodge, Limpopo, South Africa, October, 2021.
2. Mashilo, M.L. and Masoko, P. Evaluation of phytochemicals, antimycobacterial, antioxidant and cytotoxicity activity of *Dombeya rotundifolia*. Presented during the 24th Annual Indigenous Plant Use Forum (IPUF) Conference held online, July, 2022.
3. Mashilo, M.L. and Masoko, P. Identification and characterisation of compounds with antimycobacterial activity from the leaves of *Dombeya rotundifolia.* Presented during the 12th Annual Faculty of Science and Agriculture Postgraduate Research Day held at Bolivia Lodge, Limpopo, South Africa, September, 2022.
4. Mashilo, M.L. and Masoko, P. In vitro antimycobacterial activity of *Dombeya rotundifolia* used in the management of tuberculosis in Limpopo province, South Africa. Presented during the 2nd African Traditional and Natural Product Medicine Conference held at Protea Hotel The Ranch Resort, Polokwane, Limpopo, South Africa, October, 2022.

Abstract

Plants are not only an important source of medicines, but also play a significant role in drug development for the treatment of diseases such as Tuberculosis (TB). TB is a pulmonary disease that is caused by *Mycobacterium tuberculosis* complex. The aim of the study is to identify and characterise antimycobacterial compounds from *Dombeya rotundifolia*. The plant was collected from the University of Limpopo, dried and ground into fine powder. Extraction was done using different solvents that differ in polarity. The plant was screened and analysed for phytochemicals. Three major phytochemicals were quantified using reagent assays and analysed using standard curves. The antioxidant activity of the plant was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing power assay. The antibacterial activity of the plant extracts was tested against *Mycobacterium smegmatis* using bioautography and serial microplate broth dilution assay. The antibiofilm activity of the plant extracts were evaluated using crystal violet assay. The anti-inflammatory activity of the plant was determined using egg albumin protein denaturation assay. The cytotoxic effects of the extracts were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on THP-1 cell line. The antimycobacterial compounds were isolated and purified using bioassay-guided assay incorporated with column chromatography and preparative TLC. The isolated compounds were analysed and identified using nuclear magnetic resonance spectroscopy (NMR). The results obtained in this study showed that water was the best extractant, extracting 57.6 mg of the plant material, followed by methanol with 38.1 mg and hexane as the least extractant with 12.4 mg. In the phytochemical analysis of TLC plates, there was a separation of compounds in all the mobile systems, and a clear separation of compounds was observed in the BEA mobile system, followed by the EMW system. The plant has all major phytochemicals that are needed for drug development. The plant extracts had antioxidant activity, which was confirmed by the quantitative assays. The high inhibitory activity of the extracts was indicated by low MIC values that inhibited the bacterial growth. The butanol extract had the lowest MIC value (0.13 mg/mL). The plant extracts were able to prevent the formation of biofilm at different concentrations. The plant was proven to have anti-inflammatory properties by having a high inhibition capacity to prevent protein denaturation. The cytotoxicity results showed that the plant was toxic to the cells. The isolated antimycobacterial

compounds were identified as Eicosanoic acid and Docosanoic. They have moderate activity with the MIC value of 0.25 mg/mL. The study indicated that the isolated compounds have antimycobacterial activity, which showed that the plant has a potential to treat TB or symptoms related to TB. However, further studies are needed to evaluate their toxic effects before use.

Chapter 1

1. Introduction

In 1993, the World Health Organisation (WHO) declared TB as a global public health emergency and the major goal was either to eradicate or fully understand TB (Kinnear *et al*., 2017). However, decades later, TB is still a major public health threat globally. According to the 2021 Global TB Report by WHO, there were 10 million new cases and 1,5 million deaths due to TB that were reported worldwide (WHO, 2021). TB is an airborne infectious disease caused by *Mycobacterium tuberculosis* (Mtb) (Singh *et al*., 2020). It mainly affects the respiratory system, which causes mainly pulmonary TB although it may affect other organs of the body causing extra pulmonary TB (Obakiro *et al*., 2020). It is transmitted from person to person through an inhalation of contaminated droplet nuclei (Ahmad and Duse, 2018). The recommended treatment of TB is a combination of four antitubercular drugs, namely, isoniazid, rifampicin, ethambutol, and pyrazinamide for a period of six months (Dalberto *et al*., 2020).

Multidrug-resistance TB (MDR-TB) and extensively drug-resistance TB (XDR-TB) are two types of drug-resistance TB. MDR-TB occurs when the *Mycobacterium* resists both isoniazid and rifampicin, which are the first line drugs while XDR-TB is a rare type of resistance, where the *Mycobacterium* resists the antimicrobial action of isoniazid and rifampicin with the addition of any fluoroquinolone and at least one of the three injected second-line anti-TB drugs such as amikacin, kanamycin, or capreomycin (Allue-Guardia *et al*., 2021). The rise of MDR-TB is primarily due to inappropriate anti-TB drug use, poor anti-TB drug quality, incorrect prescription by healthcare practitioners and premature treatment discontinuation. XDR-TB is a more severe form of drug-resistant TB that is induced by poor MDR-TB care (Tan *et al*., 2020). The drug-resistance of *Mycobacterium tuberculosis* strains continues to pose a threat to TB control. According to the report by the World Health Organisation (WHO), it is estimated that in 2017, 558,000 people worldwide had multidrug-resistant TB, with 8,5% of those cases being extensively drug-resistant (Simbwa *et al*., 2021).

Reducing TB death rates by preventing new infections by *Mycobacterium tuberculosis* and their progression to TB disease is challenging. Wu *et al*. (2020) stated that it will be difficult to end TB by 2030, unless better treatment strategies are used to eliminate

TB within a short period of time. Controlling TB relies on early detection and successful treatment of infectious individuals to preserve lives and prevent the spread of the disease, especially in the case of MDR-TB (Bahizi *et al*., 2021). Medicinal plants have long been utilised because they are inexpensive, easy to obtain and there is no evidence of resistance to whole plant extracts or their effectiveness (Kachmar *et al*., 2021).

Plants have been used for thousands of years as medicine and many modern pharmaceuticals have their origins in traditional applications. Around 80% of the developing world's population still relies on traditional medicines for primary healthcare (Assen *et al*., 2021). Epidemiological studies have revealed that plants are rich in active secondary metabolites with antioxidant and antimicrobial properties that can be used to produce bioactive compounds (Singh *et al*., 2016). One of the top priorities in the search for natural products for the pharmaceutical industry is medicinal plants (Boy *et al.*, 2018).

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Chapter 2

2. Literature review

2.1 Tuberculosis (TB)

TB remains the leading cause of deaths from an infectious illness in the world. It is a long-lasting bacterial infection caused by *Mycobacterium tuberculosis* complex, which includes *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* and is distinguished by the presence of caseous granulomas in the infected tissue. TB was first identified in 1882 by Robert Koch as an aerobic, acid-fast, non-motile; non-encapsulated and non-spore bacillus (Baykan *et al*., 2022). It is known to affect almost all organs such as the intestine, meninges, bones, joints, lymph nodes, skin and other tissues causing extra-pulmonary TB, but generally, it affects the lungs and causes pulmonary TB (Khan *et al.*, 2019). TB can spread through person-to-person transmission by inhaling infected droplet aerosol from coughs or sneezes by the patients with active pulmonary TB and the consumption of raw, unpasteurised dairy products that are contaminated with *M. bovis.* Congenital transmission, sexual transmission, accidental inoculation, immunisation, and therapeutic instillation are among the other uncommon ways of the *Mtb* infection transmission method (Muneer *et al*., 2019).

2.2 Types of TB

TB can affect the pulmonary system, lymphatic circulation system and central neurological. It can harm different body organs such as the spine, intestines, kidneys, and the brain. There are several symptoms associated with TB, which include a persistent cough, chest pain, hemoptysis weakness, weight loss, fever, and sleep hyperhidrosis (Mohammadi *et al*., 2020).

2.2.1 Tuberculous lymphadenitis

Tuberculous lymphadenitis is a common type of extrapulmonary TB and is frequently related to the reactive of latent infection. It is found in the cervical lymph nodes (Mathiasen *et al.*, 2020). Both *Mtb* complex organisms and nontuberculous mycobacteria have the potential to cause it (Sivaratnam *et al*., 2020). The symptoms include malaise, low-grade fever, weight loss, cervical lymphadenopathy, or fistula formation (Mehmood *et al.*, 2019).

2.2.2 Pleural TB

Pleural Tb is a type of extrapulmonary TB that is caused by a number of hypersensitive responses to mycobacterial antigens in the pleural area (Ramirez-Lapausa *et al*., 2015). Clinical features include coughing, pleuritic chest pain and dyspnea as well as fever, weight loss and anorexia (Kumar *et al*., 2010).

2.2.3 Neurological TB

Neurological TB includes TB meningitis and tuberculoma and is caused by *M. tuberculosis.* The disease has a significant death rate, and it affects the central nervous system, specifically the meninges. Its transmission is through a liquid droplet containing bacilli, the *M. tuberculosis* enters the human body and settles in the lung (Khusro and Aarti, 2020). The symptoms include headache, neck stiffness, fever, vomiting, radiculopathy, and coma (Baykan *et al*., 2022).

2.2.4 Osteoarticular TB

Osteoarticular TB is the TB of the bone and joints. The infection starts with the inflammation of the vertebral bodies' anterior surfaces, then spreads to the disc and to nearby bodies by moving behind the anterior ligament and eventually to the nearby soft tissues (Ramirez-Lapausa *et al*., 2015). The clinical signs can vary, but pain and swelling are common (Arathi *et al*., 2013).

2.2.5 Abdominal TB

Abdominal TB is a mycobacterial infection that affects the digestive system, peritoneum, omentum, mesentery, nose, and other solid intra-abdominal organs (Khusro and Aarti, 2020). The common symptoms are pain in the abdomen, loss of weight, anorexia, recurrent diarrhoea, low-grade fever, cough, and distension of the abdomen (Rocha *et al.,* 2015; Khusro and Aarti, 2020).

2.3 Treatment of TB

The control of the global TB pandemic requires the effective use of antibiotics, which, however, is threatened by the global expansion of drug-resistant TB (Rivière *et al*., 2020). To prevent the emergence of drug resistance, the current chemotherapy for drug-sensitive TB lasts for roughly six months; it is the administration of four distinct drugs, namely, isoniazid, rifampicin, ethambutol, and pyrazinamide (Makarov and Mikušová, 2020). Isoniazid (Figure 2.1 A) was first reported in 1952 to treat TB. Due to its strong bactericidal efficacy, the low rate of side effects and inexpensive cost,

isoniazid has been a crucial part of the first-line treatment of active TB and significant medicine in TB control (Karo *et al*., 2019). The mode of action of isoniazid on mycobacteria is the fast loss of acid due to the inhibition of mycolic acid (Unissa *et al*., 2016). In 1971, rifampicin (Figure 2.1 B) was introduced as one of TB drugs because of its effectiveness (Webster *et al*., 2010). Rifampicin effectively kills semi-dormant organisms that are enduring short bursts of metabolic activity (Somoskovi *et al*., 2001). The mechanism of action of rifampicin is to inhibit DNA-directed RNA synthesis of *Mycobacterium tuberculosis* by binding with β subunit of RNA polymerase (Pang *et al*., 2013).

Ethambutol (Figure 2.1 C) was found effective in 1961. It is a first-line bacteriostatic drug that has a strong potent inhibitory effect on drug-susceptible *M. tuberculosis* and other mycobacteria (Xiang *et al*., 2021). Ethambutol targets the cell wall of mycobacteria and disrupts arabinosyl transferase. The enzyme is involved in the production of arabinogalactan and lipoarabinomannan, which is encoded by embCAB operon (Mohammadi *et al*., 2020). Pyrazinamide (Figure 2.1 D) was introduced in the 1970s as a drug to treat TB due to its distinctive sterilising ability in killing *Mycobacterium tuberculosis* when compared to other TB medications and is a key first-line drug that helps to shorten TB therapy from 9 to 12 months to 6 months (Shi *et al*., 2019). There is a lack of clarity about the pyrazinamide mode of action, but pyrazinamide is a prodrug that is hydrolysed to pyrazinoic acid in the mycobacterial cytoplasm by *Mycobacterium tuberculosis* pyrazinamidase/ nicotinamidase (Lamont *et al*., 2020). As a result, it can treat *Mycobacterium tuberculosis* complex infections (Tam *et al*., 2019).

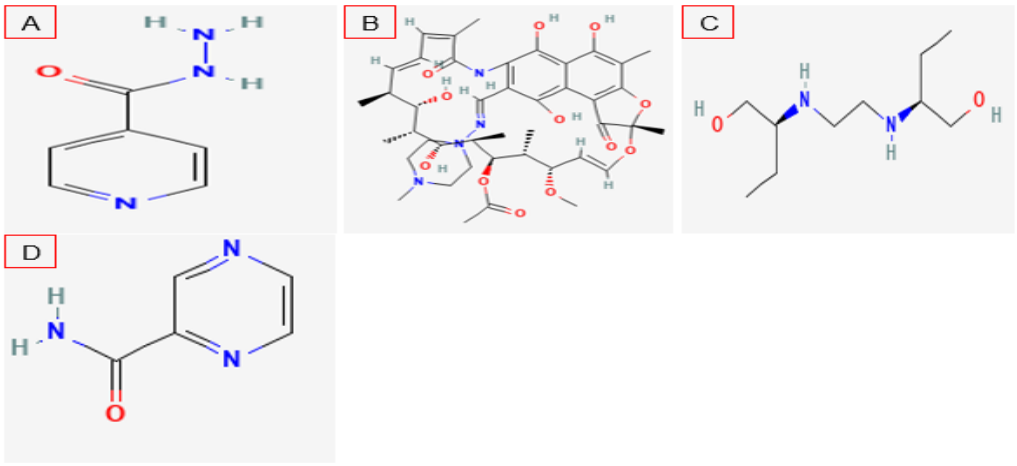

Figure 2. 1: Chemical structures of antitubercular drugs, namely, isoniazid (A), rifampicin (B), ethambutol (C), and pyrazinamide (D) (https://pubchem.ncbi.nlm.nih.gov)

2.4 Drug-resistant TB

One of the main obstacles to TB treatment is the development and spread of antibiotic-resistant strains, particularly multidrug-resistant (MDR) *M. tuberculosis*. A global survey estimated that MDR *M. tuberculosis* affects roughly 0.5 million individuals globally (Mohammadi *et al*., 2020). Furthermore, it has been highlighted that an extensively drug-resistant TB (XDR-TB) is on the rise globally (Webster *et al*., 2010). TB that is resistant to both isoniazid and rifampicin is known as multidrug-resistant TB (MDR-TB). MDR-TB treatment takes about 18 to 24 months of therapy with second-line medications that are more toxic and ineffective than first-line medications (Sun *et al*., 2019). Extensively drug resistant TB is the MDR-TB that is resistant to second line TB drugs fluoroquinolone and at least one of the injectable drugs such as kanamycin, amikacin and capreomycin (Unissa *et al*., 2016). Drug resistance may emerge during the treatment because of patients discontinuing their treatment too soon, low-quality medications or wrong medications prescribed (Vilchèze and Jacobs Jr, 2019). Genetic research revealed that *M. tuberculosis* drug resistance is a result of spontaneous mutations in the genes that either encode the drug's target or enzyme involved in drug activation (Somoskovi *et al*., 2001). Several processes such as compensatory evolution, epistasis, clonal interference, cell envelope impermeability, efflux pumps, drug degradation and modification, target mimicry and phenotypic drug tolerance, contribute to the emergence of drug resistance in *M. tuberculosis* (Singh *et al.*, 2020).

Another key contributor to antimicrobial resistances is biofilm, which is a major threat to health. Up to 80% of infections are caused by biofilms and are challenging to treat due to their significant antibiotic resistance compared to their planktonic counterparts. The bacteria in biofilms cause different infections such as chronic lung, wound and ear infections (De La Fuente-Núñez *et al*., 2012). A biofilm is a sessile microbial population made up of cells that adhere to surfaces and are immersed in matrices. The microcolony may contain between 10 to 25 percent of cells and between 75 to 90 percent of extracellular polymeric substances (EPS) matrix, depending on the species involved. Bacteria are protected by biofilm against antimicrobial and other living cells; so, they are approximately a thousand times resistant to antimicrobial drugs (Miladi *et al*., 2016).

In the past ten years, there has been an increased global effort to find new and effective treatment for TB as the existing ones lose their efficacy, making it impossible to have a world free of TB by 2035 (Obakiro *et al*., 2020). The fight against TB may be aided by a better knowledge of the molecular pathways that result in drug resistance (Safi *et al*., 2008). The use of plants instead of synthetic drugs may help since plants are an important source of medicines and play a significant role in drug development.

2.5 Medicinal plant

According to WHO, traditional medicine is defined as the collection of knowledge, skills and practices based on indigenous theories, beliefs and experiences of various cultures that is used to maintain health as well as to prevent, diagnose, improve, or treat physical and mental illnesses (Mahomoodally, 2013). Traditional remedies are derived from plant parts such as barks, leaves, roots, flowers, and seeds (Ndhlala *et al.*, 2011). Herbs, herbal materials, concoctions and finished herbal products are examples of herbal medicines (Okaiyeto and Oguntibeju, 2021).

People rely on plants for various reasons such as food, medicine, and shelter. People have utilised medicinal plants to fight pandemics in the past and their reliance on medicinal plants may have expanded in recent years all over the world. Medicinal plants can be an alternative to disease prevention (Khadka *et al*., 2021). In the pharmaceutical industry, natural products are among the sources of drugs, and medicinal plants are known to cure a variety of diseases and could be used to develop new drugs (Boy *et al*., 2021).

2.6 Plants as source of drugs

Finding healing powers in plants is an ancient thought that found that plants are a source of molecules with therapeutic potential and as such, they continue to be a valuable resource for the discovery of new drugs (Atanasov *et al*., 2015). Therefore, medicinal plants play an important role in the health sector around the world and in several countries, particularly in rural areas, where medicinal plants are the primary source of healthcare, if not the only source of healthcare (Xu *et al*., 2021). Medicinal plants contain bioactive compounds known as phytochemicals that are found in cereals, vegetables, fruits, and other plant products and play a defensive role against major chronic diseases either in the host metabolic or hereditary dysfunctional disease and infectious disease (Ugboko *et al*., 2020).

Natural goods have always played an important part in the advancement of modern medicine and continue to do so today. Antibiotics, anticancer drugs, anti-inflammatory chemicals, and analgesics are just a few of the key discoveries that have come from the search for novel therapeutic leads from natural resources (Sen and Samanta, 2014). Naturally occurring chemicals have a wide range of in vitro potency against *M. tuberculosis* due to their diverse chemical structures (Abedinzadeh *et al*., 2015). Traditional therapies based on phytochemicals have proved to have significant potential in the treatment of T B, particularly in eradicating *Mycobacterium tuberculosis*, thus enhancing natural immunity, and regulating anti-TB drug adverse effects (Fatima *et al*., 2021).

2.7 Plant metabolites

Plants produce both primary and secondary metabolites. The primary metabolites are the proximate components of the cell, which include sugars, proteins, lipids, and other basic cell metabolites (Marcus *et al*., 2019). Secondary metabolites are small organic molecules produced by an organism but are not required for its growth, development, or reproduction. They are classified according to the pathway by which they are produced (Seca and Pinto, 2018). Secondary metabolites are natural products isolated from a variety of natural sources such as plants, animals, and microorganisms. Phytochemicals are chemical compounds produced during various metabolic processes and have a wide range of therapeutic properties. These include compounds such as flavonoids, steroids, tannins, alkaloids, and triterpenes, which could be used to inhibit or kill disease-causing pathogens (Khajaminya, 2017).

2.7.1 Saponins

Saponins (Figure 2.2) are a type of glycoside with aglycones that can be triterpenes or helical spirostanes (He *et al.,* 2019). They have at least one glycosidic linkage at C-3 between aglycone and a sugar chain and high molecular weight (El Aziz *et al*., 2019). Saponins have been shown to be antimicrobial, antifungal and insect repellent (Saxena *et al.,* 2013). Saponins have a variety of biological functions, including anti-inflammatory, anti-carcinogenic, cell reinforcement, hemolytic, and cell layer permeabilizing properties (Hussain *et al*., 2019).

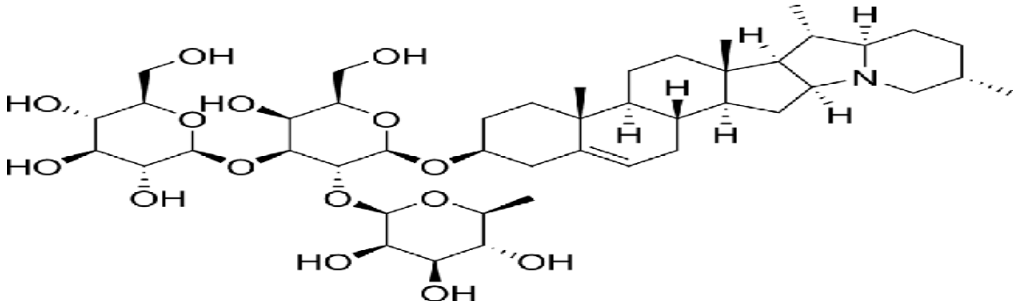

Figure 2. 2: Chemical structure of saponin (Pareek *et al*., 2017).

2.7.2 Terpenes/terpenoids

Terpenoids are the modified terpenes with various functional groups and oxidised methyl groups that have been relocated or removed at various places, whereas terpenes are simple hydrocarbons. Terpenes are the most common type of secondary metabolite and comprise five carbon isoprene units that are linked together in different ways. Depending on the number of carbon units, terpenoids are classified as monoterpenes, diterpenes, triterpenes, sesterpenes and sesquiterpenes (Figure 2.3). They are used to treat different diseases, inhibit any kind of cancer cells, and are used as drugs to treat cancer. Terpenoids are found in a wide range of products, including food, drugs, cosmetics, hormones, and vitamins (Perveen and Al-Taweel, 2018).

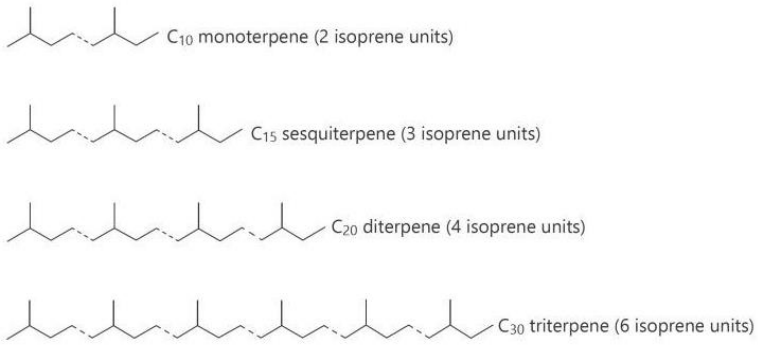

Figure 2. 3: Classification of terpenes (Hanuš and Hod, 2020).

2.7.3 Tannins

Tannins are high molecular weight phenolic compounds found in plants' leaves, bark, and wood, and bound to proteins to form insoluble or soluble tannin-protein complexes (Hassanpour *et al*., 2011). Tannins are classified into four types: gallotannins, ellagitannins, complex tannins and condensed tannins (Figure 2.4). Gallotannins are tannins that contain galloyl units or their meta-depsidic derivatives bound to various polyol-catechin-, or triterpenoid units. Ellagitannins are tannins that have at least two galloyl units that are C–C coupled to each other, but with no glycosidically linked catechin unit. Complex tannins have a catechin unit that is glycosidically bound to a gallotannin or ellagitannin unit. Condensed tannins are oligomeric and polymeric proanthocyanidins that are formed by joining the C-4 of one catechin to the C-8 or C-6 of the next monomeric catechin (Saxena *et al*., 2013). The main role of tannins is to protect the plants against insects, food infections, fungi, or bacteria (Pizzi, 2019).

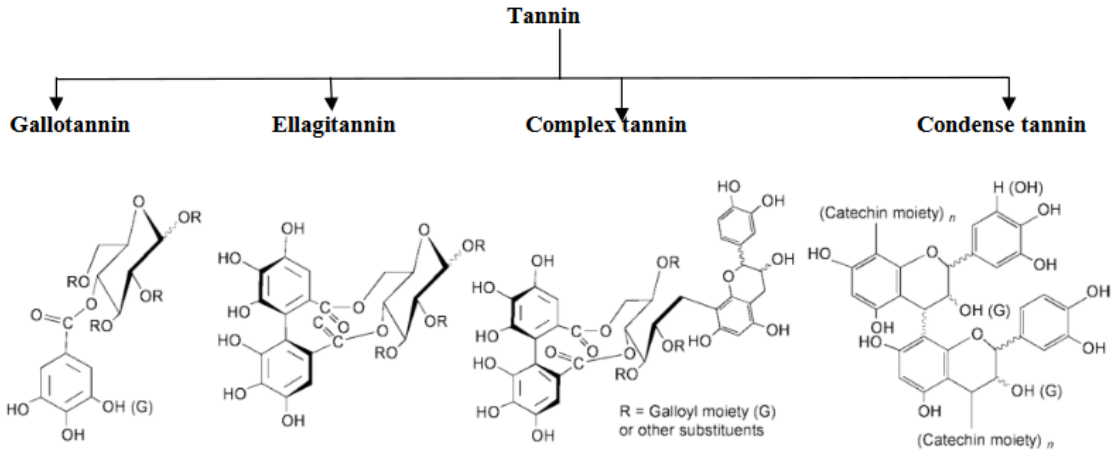

Figure 2. 4: Classification of tannins (Saxena *et al*., 2013)

2.7.4 Cardiac glycosides

Cardiac glycosides (Figure 2.5) are naturally occurring substances produced by a variety of plant and amphibian species and their chemical structure has a steroid ring, a lactone ring with five or six carbons and a sugar moiety (Botelho *et al*., 2019). Their main mechanism of action is to inhibit the membrane sodium-potassium pump, which affects intracellular sodium, calcium and potassium concentrations that result in cardiovascular system disruptions (Malysheva *et al.*, 2020). For medical purposes, cardiac glycosides are used to treat congestive heart failure and to control ventricular rate in people with atrial fibrillation (Oerther, 2010), and protect the plants by poisoning the livestock (Morsy, 2017).

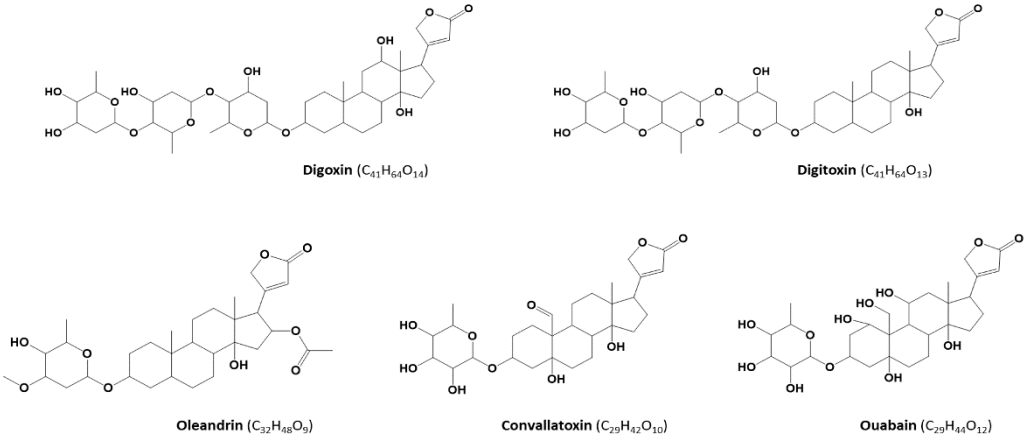

Figure 2. 5: Chemical structures of cardiac glycosides (Malysheva *et al*., 2020)

2.7.5 Flavonoids

Flavonoids (Figure 2.6) are polyphenolic compounds that plants produce and are responsible for their colour, flavour, and pharmacological properties (Kopustinskiene *et al*., 2020). Flavonoids are a group of natural products with C6-C3-C6 skeleton, or more specifically, a phenylbenzopyran function and divided into three classes, namely, isoflavonoids, neoflavonoids and chalcone precursor (Suryawanshi, 2019). Anticancer, antioxidant, anti-inflammatory and antiviral properties as well as neuroprotective and cardioprotective are just a few of the medicinal properties of flavonoids (Ullah *et al.*, 2020).

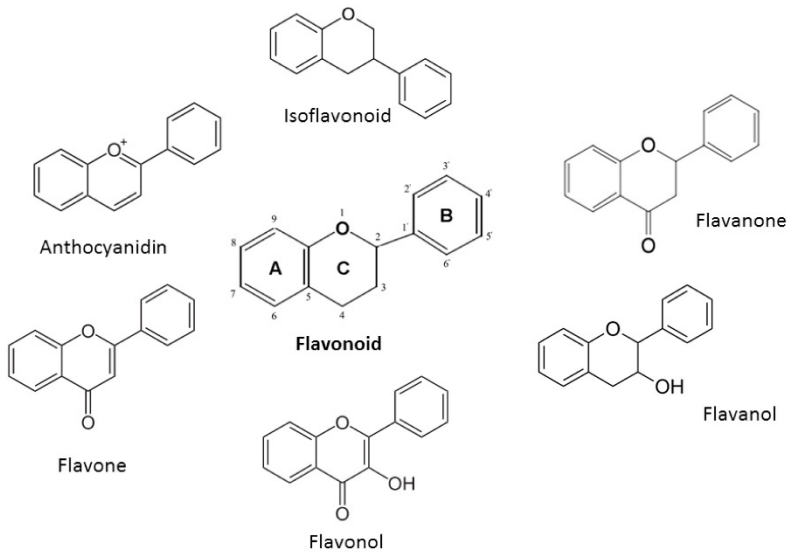

Figure 2. 6: Chemical structures of flavonoids (Kopustinskiene *et al*., 2020)

2.7.6 Alkaloids

Alkaloids (Figure 2.7) are a diverse group of chemical entities that make up one of the largest natural products (Bribi, 2018). The term alkaloids comes from alkaline, which was used to describe any nitrogen-containing base and produced by a wide range of organisms such as bacteria, fungi, plants, and animals (Roy, 2017). They are substances with a lot of promise in the medical field, plant protection, veterinary or toxicology (Adamski *et al*., 2020).

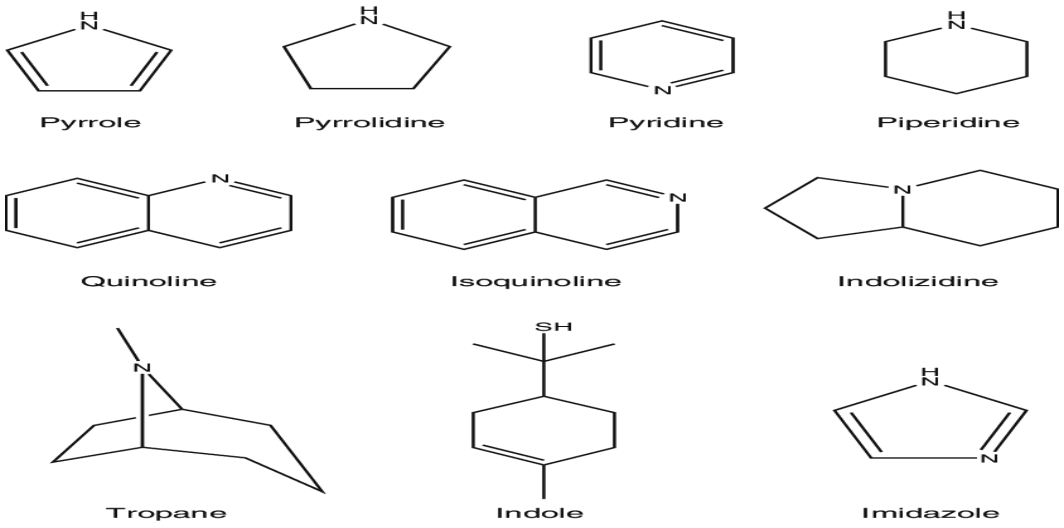

Figure 2. 7: Skeletal structure of true alkaloids (Gutiérrez-Grijalva *et al*., 2020)

2.8 Biological activities of medicinal plants

Plants continue to be the most abundant natural primary source of active medications and are essential in the ethnomedical treatment of a wide range of illnesses. Medicinal plants are typically rich in phytochemicals, some of which are responsible for their biological activities (Ugboko *et al*., 2020). Natural products provide an almost limitless supply of chemicals to aid in the development of drugs. The synergistic and simultaneous action of multiple compounds is usually responsible for the medicinal effectiveness of plant extracts (Thomford *et al*., 2018). Antioxidants, anticancer agents, immunity suppressing agents, detoxifying agents, and neuropharmacological agents are the main functional classes of phytochemicals with therapeutic potential (Ugboko *et al*., 2020).

2.8.1 Antimicrobial activities

Antimicrobial drugs are natural or synthetic substances that inhibit the growth or kill microorganisms such as bacteria, fungi, helminths, protozoa, and viruses. Antimicrobial drugs can be bacteriostatic, which inhibits the growth of the microorganism, or bactericidal, which completely kills the microorganism. The antimicrobial drugs suppress the infection using different modes of action and are classified into three groups' spectrum of activity based on their effect and mode of action (Das and Patra, 2017). Phytoanticipins and phytoalexins are the two types of plant-formed antibiotics. Phytoanticipins are the preformed inhibitory substances and phytoalexins are produced from precursors by a de novo synthesis in response to a microbial attack (Bobbarala, 2012).

Medicinal plants have been used as remedies for human diseases and provide a source of biological active compounds that can be used as antimicrobial agents (Das *et al*., 2010). Polyphenolic compounds are the key components responsible for plants' antimicrobial properties. Flavonols, quinones and flavonoids have antimicrobial properties. The cell wall and cytoplasmic membrane of the microorganisms are destroyed by those compounds because they have lipophilic characteristics (Cioch *et al*., 2017).

2.8.2 Antioxidant activities

Antioxidants are compounds that are present at a low concentration; they prevent or delay the oxidation of cell contents, mainly the macromolecules such as

carbohydrates, and Deoxyribose Nucleic Acid (DNA). They are classified according to their defence, namely; first, second- and third-line defence antioxidants (Gupta and Sharma, 2006). First line defence antioxidants are a group of antioxidants that involve enzymes and metal ions, and their role is to act against any formation of free radical or reactive species in cells. Superoxide dismutase, catalase and glutathione peroxidase are the enzymes that help with the breakdown of hydrogen peroxides and hydroperoxides into harmless molecules. Second line defence antioxidants are known as the scavenging antioxidants. They inhibit initiation chains and break chain propagation reactions by scavenging the active radicals. They perform their role by donating electrons to the free radical to make them less harmful. Lastly, the third line defence antioxidants are a group of enzymes that repair damaged DNA, proteins, and lipids and remove oxidised or damaged macromolecules to avoid the build-up that can be harmful to the body tissues (Ighodaroa and Akinloye, 2018). Molecules like free radicals, reactive oxygen and nitrogen species contain one or more unpaired electrons in the atomic or molecular orbital that characterises free radicals with high activity (Mathew *et al*., 2013).

2.8.3 Anti-inflammatory activities

Inflammation is a process that is initiated in response to injuries, burns, microbial infection, or any stimuli that may threaten the well-being of the host. It is characterised by pain, redness, heat and swelling. Inflammation is reduced by using anti-inflammatory drugs. Systemic mediators like cytokines and chemokines are released when there are inflammatory injuries. Injury to the intestinal tract, acceleration of cartilage destruction or production of liver and kidney toxicities have been shown to be unrelated to the natural anti-inflammatory herbal agents (Apu *et al*., 2012).

2.9 Bioassay for antimicrobial activity

It is important to perform antimicrobial susceptibility tests as they confirm susceptibility to antimicrobial agents chosen or detection of resistance in individual bacterial isolates (Reller *et al.*, 2009). In clinical laboratories, antimicrobial susceptibility tests are done daily on isolated bacteria and rely on phenotypically testing the bacteria isolated (Wheat, 2001).

2.9.1 Thin layer chromatography (TLC)-Direct Bioautography

TLC-Bioautography is a method that uses both TLC and antimicrobial testing to establish the identity of the extracted compound and its antimicrobial activity (Abubakar and Haque, 2020). In this method, a developed TLC plate is dipped in a suspension of microorganisms growing in a nutrient broth and incubated in a humidified environment. The microorganisms grow directly on the surface of a TLC plate excluding antimicrobial spots (Choma and Jesionek, 2015). Tetrazolium salts are commonly used to visualise microbial growth. The salts will be converted into a purple formazan by dehydrogenase enzymes. The most effective detecting reagent is p-iodonitrotetrazolium violet (Balouiri *et al*., 2016). The clear white zones against a purple background show the presence of antimicrobial agents and that region is called the inhibition zone (Choma and Jesionek, 2015).

2.9.2 Micro-dilution Assay

Dilution procedures are the best for determining the MIC because they enable the estimation of the concentration of the tested antimicrobial agent in the agar or broth medium (Balouiri *et al*., 2016). MIC is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation. In the laboratory, MIC is used to confirm an unusual resistance and to give a definitive answer when other methods of testing are used or when disk diffusion methods are not appropriate (Andrews, 2001).

One of the most fundamental antimicrobial susceptibility tests is broth micro- or macro-dilution. The process entails making two-fold dilutions of the antimicrobial agent in a liquid growth medium supplied in tubes with a minimum volume of 2 mL (macro-dilution) or utilising a 96-well microtitration plate with smaller quantities (Balouiri *et al*., 2016). The wells are filled with an equal amount of microbial culture and incubated. The plates are checked for changes in turbidity as a growth indication after incubation. Indicators such as tetrazolium salts or resazurin dye as well as spectrophotometry are used to determine the presence of growth (Das *et al*., 2010).

2.9.3 Agar disk diffusion method

The disc and well diffusion assay is a widely used method for evaluating various antibacterial drugs. The length of a concentration gradient is used to screen a natural resource for its potential to suppress the bacterial growth at an unknown

concentration. The size of the inhibition zone indicates whether a bacterium is susceptible or resistant to antibiotics (Dehyab *et al*., 2020). In this method, the test microorganism is inoculated into agar plates; the filter paper discs containing compounds at the required concentration are inserted on the surface of the agar. This is followed by an incubation of petri dishes under suitable conditions. The antimicrobial agent diffuses into the agar and prevents microbial growth. The diameter of the zone of clearance around the disc is measured (Balouiri *et al*., 2016).

2.10 Bioassay for antioxidant activity

The total antioxidant capacity is the measure of the number of free radicals scavenged by a test solution. This can be measured by using assays that are based on a substance's ability to prevent oxidation (Rubio *et al*., 2016). Antioxidant assays are classified into two types based on their reaction mechanisms, namely; hydrogen atom transfer and single electron transfer. The hydrogen atom transfer methods are based on a hydrogen atom transfer from an antioxidant to a free radical. Single electron transfer methods detect the ability of a potential antioxidant to transfer one electron to reduce any molecule such as metals, carbonyls, and radicals (Hidalgo and Almajano, 2017).

2.10.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

2,2 -Diphenyl-1-picrylhydrazyl radical was introduced by Blois in 1958 to assess the ability of the antioxidant bioactive compounds. Due to its great stability, experimental practicality and low cost, the DPPH radical is an ideal for the analytical application to assess the radical scavenging capability of antioxidants compared to naturally occurring radicals (Yeo and Shahidi, 2019). DPPH assay is an antioxidant activity assay based on the transfer of electrons and produces a violet solution in methanol. Free radicals are stable at room temperature and are reduced in the presence of an antioxidant molecule (Garcia *et al*., 2012). The free radical DPPH with an odd electron gives maximum absorption at 517 nm purple colour. When antioxidants react with DPPH, which is a stable free radical, it becomes paired off in the presence of a hydrogen donor and reduced to the DPPH-H and therefore, the absorbance becomes decreased (Shekhar and Anju, 2014).

2.10.2 Ferric reducing power

The principle behind the reducing power assay method is that substances with reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric- ferrous complex with an absorbance maximum at 700 nm (Bhalodia *et al*., 2013). The method is based on an increase in the absorbance of the solution in the reaction. Increase in the absorbance means that the antioxidant activity has increased (Gulcin, 2020). The reducing power assay consists of the following steps: sample preparation, reaction and lastly, measurement of the absorbance of the sample and standard using the spectrophotometer at a wavelength of 700 nm (Vijayalakshmi and Ruckmani, 2016).

2.11 Bioassay for cytotoxic

Traditional medicine uses plants to treat a variety of infectious and non-infectious diseases; however, medicinal plants can be harmful to one's health (Mounanga *et al*., 2015), due to the risk of side effects from toxic factors that may result in acute toxicity and patient mortality. Poisonous plants can harm a variety of organ systems, with some plants possessing numerous toxic principles that affect multiple systems (Anywar *et al*., 2021). The toxicity of the plant is determined by a variety of factors such as the strength of secondary metabolites, the amount consumed, the time of exposure, different parts of the plant, individual body chemistry, climate and soil, and genetic differences among the species (Mounanga *et al*., 2015).

Cell-based assays are used to screen collections of compounds to see if they affect cell proliferation or have direct cytotoxic effects that cause cell death. Different tetrazolium compounds are used to detect the viability of the cells (Riss *et al*., 2016). The assay's qualities include reliability, rapidity, and simple evaluation (Fotakis and Timbrell, 2006). Toxicological testing is critical for the discovery of novel drugs and the therapeutic potential of existing compounds (Parasuraman, 2011).

2.11.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT assay

The MTT is a colorimetric assay that detects the functional state of mitochondria, which indicates the cell's viability (Vajrabhaya and Korsuwannawong, 2018). Since its creation in the 1980s by Masmann, it has been the gold standard for determining cell viability and proliferation (Van Tonder *et al*., 2015). It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular

oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple colour (Kuete *et al*., 2017). The assay assesses cell viability by measuring reductive activity, which is defined as the enzymatic conversion of a tetrazolium compound to water-insoluble formazan crystals by dehydrogenases (Van Tonder *et al*., 2015).

2.12 Separation and purification of compounds

Chromatography is a technique for the qualitative and quantitative analysis that is used for separation, identification, and purification of the components of a mixture. The method is based on the partition of the sample components between two phases, namely, stationary and mobile phases (Coskun, 2016). The separation of mixtures is important for two reasons: separation techniques are required for analysing any number of complex mixtures and are necessary to purify compounds for further uses (Hademenos *et al.,* 2016).

2.12.1 Thin layer chromatography

TLC is a chromatography technique that was discovered in 1906 by Tswett and used to separate mixtures. This technique can be used to monitor the progress of a reaction, identify compounds and to determine the purity of a compound (Bele and Khale, 2011). In TLC, a thin glass plate coated with aluminium oxide or silica gel serves as a solid phase and the solvent used in the mobile phase is chosen based on the characteristics of the components in the mixture to be examined. The principle of TLC is the distribution of a compound between a solid fixed phase and a liquid mobile phase that is moving over the solid (Ritika, 2018). In the method, the mobile phase travels up through the stationary phase. By capillary action, the solvent travels up the plate soaked with the solvent and after the analytes are separated. Separation depends on the polarity of the materials, solid phase and solvent (Coskun, 2016).

2.13 Structure elucidation of compound

In the chemical sciences, solving the structure of unknown compounds is a major problem that slows down molecule and reaction discovery (Huang *et al*., 2021). However, with several spectroscopic techniques introduced, particularly nuclear magnetic resonance (NMR) and mass spectrometry (MS) and recent computational tools and mathematical modelling, the structural elucidation of phytochemicals is no longer as complex as it once was (Ismail *et al*., 2018). Because of substantial

improvements in NMR sensitivity as well development of 2 D NMR pulse programs, determining molecular composition by NMR is easier and error free (Navarro-Vazquez *et al*., 2018).

The most common method for determining the structure of organic compounds is nuclear magnetic resonance (NMR). NMR spectra provides molecular fingerprints that can be used to determine connectivity and relative stereochemistry by encoding the local environments of the atoms that make up a molecule (Huang *et al*., 2021). It shows peaks for each chemically distinct hydrogen or carbon atom in a molecule, thus giving information about structural elucidation (Reynolds, 2017).

2.14 Microorganism used in this study

2.14.1 Mycobacterium smegmatis

Mycobacterium smegmatis was isolated in 1884 by Lustgarten and named by Lehmann and Neumann in 1889 (Ranjitha *et al*., 2020). *M smegmatis* belongs to Actinobacteria phylum and the *Mycobacterium* genus (Sagong *et al*., 2019). It is a rapidly growing, non-pathogenic, and Gram-positive bacterium with an inner cell membrane and thick cell wall. The organism is widely distributed in soil, water, and plants (Ranjitha *et al*., 2020). This bacterium is a non-motile obligate aerobe that depends on fatty acid biosynthesis for the production of mycolic acids that are found in the cell wall (Megehee and Lundrigan, 2007). *M smegmatis* is used as a model for TB experiments because it is non-pathogenic as compared to *M. tuberculosis* and *M. avium* (Singh, 2016).

2.15 Plant selected for this study

2.15.1 Dombeya rotundifolia

Dombeya rotundifolia (Hochst) is also known as Wild pear (English), *drolpeer* (Afrikaans), *iNhliziyonkhulu* (IsiZulu), *mohlabaphala* (Northern Sotho), *motubane* (Setswana), and *nsihaphukuma* (Xitsonga) (Balogun and Ashafa, 2019). It belongs to the Sterculiaceae and Malvaceae family that consists mainly of trees and shrubs. The genus *Dombeya* is non-monophyletic and consists of about 230 species (Maroyi, 2018). *D rotundifolia* (Figure 2.8) is a deciduous single-stemmed tree with a modest, irregular-shape canopy and grows around 5-6 m tall. The plant grows in the woodland areas such as in Mpumalanga and KwaZulu-Natal, and in warmer and drier habitats (Reid *et al.,* 2001). It is used to treat intestinal ulcers, stomach complaints,

haemorroids, headaches, diarrhoea, nausea in pregnant women and to delay or induce labour, constipation, fever and malaria, infertility, chest complaints and pneumonia, heart problems and in making a love potion (Reid *et al.,*2001; Kudumela and Masoko, 2017; Maroyi, 2018).

Figure 2.8: *Dombeya rotundifolia* (https://www.plantbook.co.za/dombeya-rotundifolia/)

2.16 Aim and objectives

Aim

The aim of the study was to identify and characterise antimycobacterial compounds from the leaves of *Dombeya rotundifolia.*

Objectives

The objectives of this study were to:

i. Analyse phytochemical constituents in *Dombeya rotundifolia* using Thin Layer Chromatography (TLC).

ii. Determine the presence of antioxidant compounds in *D. rotundifolia* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and quantitative total antioxidant activity using ferric reducing power assay and DPPH.

iii. Determine the antimicrobial activity of crude extracts of *D. rotundifolia* against *Mycobacterium smegmatis* using minimum inhibitory concentration (MIC) assay and bioautographic assay.

iv. Determine the antibiofilm property of *D. rotundifolia* against *Mycobacterium smegmatis* using crystal violet assay.

v. Determine anti-inflammatory activity of *D. rotundifolia* using egg albumin protein denaturation assay.

vi. Analyse the effects of *D. rotundifolia* extracts for cytotoxicity on the cell culture (THP-1 cell line) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

vii. Isolate bioactive compounds from *D. rotundifolia* extracts with antimycobacterial activity using column chromatography and preparative TLC.

viii. Characterise isolated compounds from *Dombeya rotundifolia* using Nuclear magnetic resonance spectroscopy (NMR).

2.17 References

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Chapter 3

3. Extraction and preliminary phytochemical analyses

3.1 Introduction

Plants have long been a source of inspiration for the development of innovative medicinal molecules, as plant-derived medications and treatments have contributed significantly to human safety, health, and well-being (Imran *et al*., 2021). The most crucial step in herbal drug formulation preparation is extraction and it can be used for identifying the leading compound by isolating the active compound from the crude extract (Saravanabavan *et al*., 2020). Extraction is the separation of active plant materials or secondary metabolites from inert or inactive material using a suitable solvent and a standard extraction procedure (Abubakar and Haque, 2020). Medicinal plants are a source of therapeutic phytochemicals that may lead to novel drug development. Plants that possess such phytochemicals, namely; phenolics and flavonoids, have a positive impact in that they contribute to cancer prevention, among other benefits (Azwanida, 2015). Plants produce secondary metabolites as a by-product of primary metabolism and as part of their defence mechanisms. Secondary metabolites such as alkaloids, tannins and flavonoids are regarded as sources of plants' healing properties. Due to their ability to neutralise free radicals, phenolics are linked to antioxidant activity (Chigayo *et al*., 2016). Secondary metabolites are responsible for different activities through different mechanisms (Hossain *et al*., 2013). Analysing the key bioactive compounds in plant is an important step in determining the product quality and validating the efficacy and safety of their therapeutic usage (da Silva *et al*., 2015).

Chemical based screening methods are effective in screening because they have a high throughput. Colorimetric reactions are used in the UV/VIS spectrophotometric methods, which are simple to perform, are quick to use in laboratory settings and are acquired at a low cost (Blainski *et al*., 2013). The Folin-Ciocalteu method is an electron transfer-based technique and determines the reducing capacity (Noreen *et al*., 2017). The method is based on the reduction of the Folin-Ciocalteu reagent in the presence of phenolics. A redox reaction occurs between the phenolate anion and the Folin-Ciocalteu reagent (yellow), in which the molybdenum is reduced and the colour of the solution changes from yellow to blue in the dark. The colour change is caused by the

formation of a blue metallic complex ($PMoW_{11}O_4)^{4-}$ and absorbs the light between 620 and 765 nm (Granato *et al*., 2016). The aluminium chloride colorimetric assay is used to determine total flavonoid based on the development of a yellow colour from the reaction between aluminium ion, Al (III) and the carbonyl and hydroxyl groups of flavones and flavonols (Pontis *et al*., 2014). Only flavonols and flavones luteolin are detected in this method, which requires measuring at a wavelength between 410 and 430 nm after an addition of $AlCl_3$ (Pękal and Pyrzynska, 2014).

3.2 Methods and materials

3.2.1 Plant collection

The *Dombeya rotundifolia* was collected in May 2021 at the University of Limpopo with the herbarium voucher number of UNIN 12296. The leaves of the plant were dried at an ambient temperature in the dark for two weeks. The dried leaves were ground into fine powder and stored in an airtight container in the lab at room temperature.

3.2.2 Extraction procedure

3.2.2.1 Preliminary extraction procedure

The dried fine powder (1 g) of the plant was added in each centrifuge tube and this was followed by 10 mL of different solvents (Hexane, Chloroform, Dichloromethane, Ethyl acetate, Acetone, Ethanol, Methanol, Butanol and Water). The mixtures were shaken separately for 10 minutes at a speed of 200 rpm in a shaking incubator (New Brunswick Scientific Co., INC.). The extracts were filtered using cotton wool and a funnel into labelled pre-weighed glass vials and the solvents were evaporated using a fan. The quantity of the plant extracts was determined by subtracting the mass of the empty pre-weighed glass vials from the mass of the dried crude extracts in the vials. The extracts were reconstituted in acetone to a final concentration of 10 mg/mL for subsequent assays.

3.2.3 Phytochemical screening on TLC

The phytochemical profiles of the crude extracts were determined on TLC by loading 10 µL of 10 mg/mL solution. The solutions of the extracts were loaded using a micropipette, at the bottom of the TLC plate, just 1 cm away from the base of the TLC plate. The plates were then put inside the saturated TLC tank that contained the following solvent: Ethyl acetate: Methanol: Water (30:5.4:5) [EMW] (polar), Chloroform: Ethyl acetate: Formic acid: 10:8:2 [CEF] (intermediate polarity: acidic), Benzene: Ethanol: Ammonium hydroxide: 18:2:0.2 [BEA] (nonpolar/basic) (Kotze and Eloff, 2002). Following the development, the plates were observed under the UV light (254 and 365 nm) to view separated compounds. The Vanillin-sulphuric acid reagent (0.1 g vanillin: 28 mL methanol: 1 mL sulphuric acid) was sprayed on the chromatograms and heated at 110°C in an oven to observe the colour development and the visualisation of non-fluorescing compounds.

3.2.4 Preliminary screening for Phyto-constituents

3.2.4.1 Saponins

Exactly 0.5 g of the powdered leaf was suspended in a 5 mL of tap water and thereafter, the mixture was vigorously shaken and heated. The formation of froths indicated the presence of saponins (Odebiyi and Sofowaro, 1978).

3.2.4.2 Terpenes/terpenoids

The powdered leaf (0.5 g) was mixed with 2 mL of chloroform and 3 mL concentrated sulphuric acid. The formation of reddish-brown colour indicated the presence of terpenoids (Odebiyi and Sofowaro, 1978).

3.2.4.3 Phlobatannins

The powdered leaf (0.5 g) was dissolved in 5 mL of distilled water and filtered. The filtrate was boiled with 2 mL of 1% hydrochloric acid (HCL). The formation of a red coloured precipitate indicated the presence of phlobatannins (Borokini and Omotoyo, 2012)

3.2.4.4 Tannins

The powdered leaf (0.5 g) was dissolved in 5 mL of distilled water and then boiled gently and cooled. The solution of 1 mL was added in the test tube as well as 3 drops of 1% ferric chloride solution. The mixture was underplayed with 1 mL of concentrated sulphuric acid (H_2SO_4). The formation of a blue-black, brown-green, green or blue-green colour indicated the presence of tannin (Borokini and Omotayo, 2012).

3.2.4.5 Cardiac glycosides

The powdered leaf (1 g) was treated with 2 mL of glacial acetic acid containing one drop of 1% ferric chloride solution and the mixture was then underplayed with 1 mL of concentrated sulphuric acid (H_2SO_4). The sample was observed for colour change to indicate the presence of cardiac glycosides (Odebiyi and Sofowara, 1978).

3.2.4.6 Flavonoids

The diluted ammonia (5 mL) was added to a portion of the aqueous filtrate of the plant extract, followed by the addition of 1 mL of concentrated sulphuric acid (H_2SO_4). The sample was observed for colour change to indicate the presence of flavonoids (Borokini and Omotayo, 2012).

3.2.4.7 Steroids

Acetic anhydride (2 mL) was added to 0.5 g of powdered leaf, followed by the addition of 2mL of sulphuric acid (H_2SO_4). The sample was observed for colour change to indicate the presence of steroids (Borokini and Omotayo, 2012).

3.2.4.8 Alkaloids

The powdered leaf (0.5 g) was extracted with 95% ethanol in a shaking incubator (200 rpm) for 10 minutes and filtered using cotton wool and funnel. The extract was dried under a fan. The residue was redissolved in 5 mL of 1% of hydrochloric acid (HCL), followed by the addition of 5 drops of Drangendoff's reagent. The colour change was observed to indicate the presence of alkaloids (Odebiyi and Sofowara, 1978).

3.2.5 Quantification of major phytochemicals

3.2.5.1 Total phenolic content

The quantity of phenolics present in each extract was determined by using the Folin-Ciocaltleu reagent method (Humadi and Istudor, 2008), with minor modifications. The plant material (10 mg/mL) was reduced to a concentration of 0.05 mg/mL in a test tube. Exactly 100 µL of 0.05 mg/mL of each plant extracts was transferred to a test tube and diluted with 900µL of distilled water, followed by an addition of 250 µL of Folin-Ciocaltleu reagent. A volume of 1.25 mL of 7% of Sodium carbonate was added to stop the reaction and the mixture was incubated in the dark at a room temperature for 30 min. A 10S UV-VIS spectrophotometer (Thermo Scientific Genesys) was used to determine the absorbance of the mixtures at 550 nm and the results were recorded. A blank was prepared similarly except that the plant extracts were replaced by acetone. The results were obtained from the linear regression formula: $y = 3.025x - 0.1141$. The Gallic acid standard curve was expressed as milligram gallic acid equivalence/ gram of the extract (mg of GAE/ gram extract). The concentrations of the standard used (1.25, 0.63, 0.31, 0.16, 0.08 mg/mL) and the experiment were conducted in triplicates.

3.2.5.2 Total tannin content

The Folin-Ciocalteu method described by Tambe and Bhamber (2014) was used to determine the tannin content in the plant extracts. Briefly, a volume of 50 µL of 7.5 mg /mL of each extract was added to a clean test tube containing 3.8 mL of distilled water. A volume of 0.25 mL of Folin-Ciocalteu reagent was added to the mixture; the mixture

was vortexed and then 0.5 mL of a 35% solution of sodium carbonate was added into the mixture. The mixture was transferred to a 10 mL of a volumetric flask and the volume of the mixture was made up to 10 mL by adding distilled water. The mixture was vortexed and incubated at room temperature for 30 minutes in the dark. A blank was prepared similarly as the test solutions without adding any extract but acetone. The absorbance of the mixture was measured using the A 10S UV-VIS spectrophotometer (Thermo Scientific Genesys) at 725 nm. Gallic acid was used as a standard, and reference standard solutions were prepared, and the absorbance for the solutions was measured against the blank. The Tannin content was expressed as a milligram gallic acid equivalence/ gram of extract (mg GAE/g extract), which was calculated using the Gallic acid standard curve with equation, $y = 3.878x - 0.0023$. The concentrations of the standard used (1, 0.5,0.25, 0.125, 0.0625 mg/mL) and the experiment were conducted in triplicates.

3.2.5.3 Total flavonoid content

The flavonoid content was determined by the aluminium chloride colorimetric assay described by Tambe and Bhambar (2014). Briefly, 100 µL of 10 mg/mL was added to 4.9 mL of distilled water in a clean test tube, followed by an addition of 300 µL of 5% sodium nitrite dissolved into distilled water. The mixture was left at a room temperature for 5 minutes. Thereafter, 300µL of aluminium chloride dissolved in distilled water was added to the mixture. The reaction was incubated at a room temperature for 5 minutes, after 2 mL of sodium hydroxide was added to the solution. The mixture in the test tube was then made up to 10 mL with distilled water. The blank was prepared in the same manner; however, 100 µL of acetone was added instead of the plant extracts. The absorbance of the mixture was determined using the A 10S UV-VIS spectrophotometer (Thermo Scientific Genesys) at a wavelength of 510 nm. The total flavonoid content of the sample was expressed as a milligram quercetin equivalence/gram of extract (mg QE/g extract) using the equation ($y = 0.6522 - 0.0006$) obtained from the quercetin standard curve. The concentrations of the standard used (500, 250, 125, 62.5, 31.25 µg/mL) and the experiment were conducted in triplicates.

3.2.6 Statistical analysis

The experiments were performed in triplicates and the results were presented as an average ± standard deviation. The calculations were carried out using Microsoft Office

Excel, where the calculations were done using the linear equation formula ($y = mx + C$) obtained from each standard curve.

3.3 Results

3.3.1 Preliminary extraction procedure

The figure below shows the mass in milligrams of the plant, extracted from 1 g of powder plant material using nine different solvents. Water was the best extractant, extracting 57.6 mg of the plant material, followed by methanol with 38.1 mg, chloroform with 35.7 mg, and hexane as the least extractant with 12.4 mg (Figure 3.1).

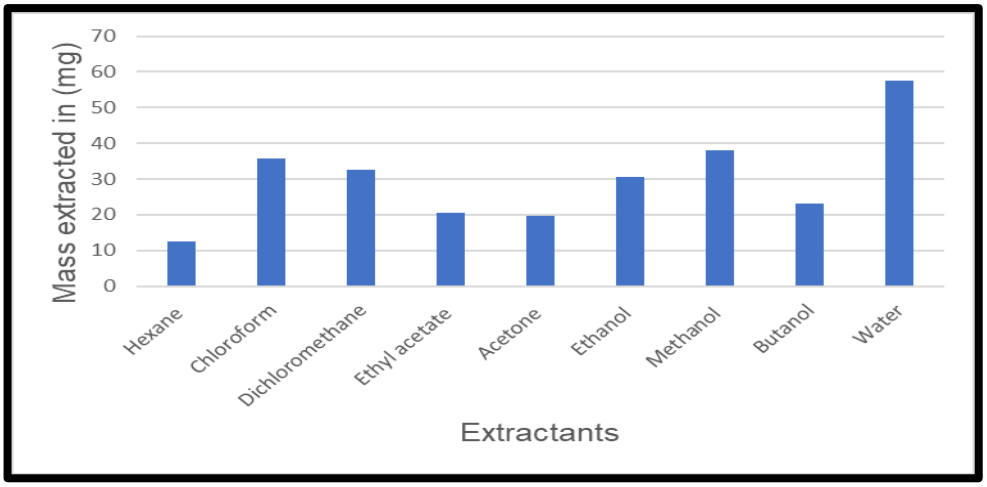

Figure 3. 1: Mass of *Dombeya rotundifolia* extracts, extracted using different solvents with an increase in polarity.

3.3.2 Phytochemical analysis on TLC plates

TLC was used to analyse the phytochemical constituents in the plant (*Dombeya rotundifolia*). The fluorescencing compounds were observed under the UV light (Figure 3.2 and 3.3), and then the Vanillin-sulphuric acid was sprayed on the plates to visualise the compounds that were not visible under the UV light (Figure 3.4). All the compounds were present in all the mobile systems, but in BEA, there was a clear separation, and more compounds were there. This means that the compounds in the plants are non-polar (Figure 3.3).

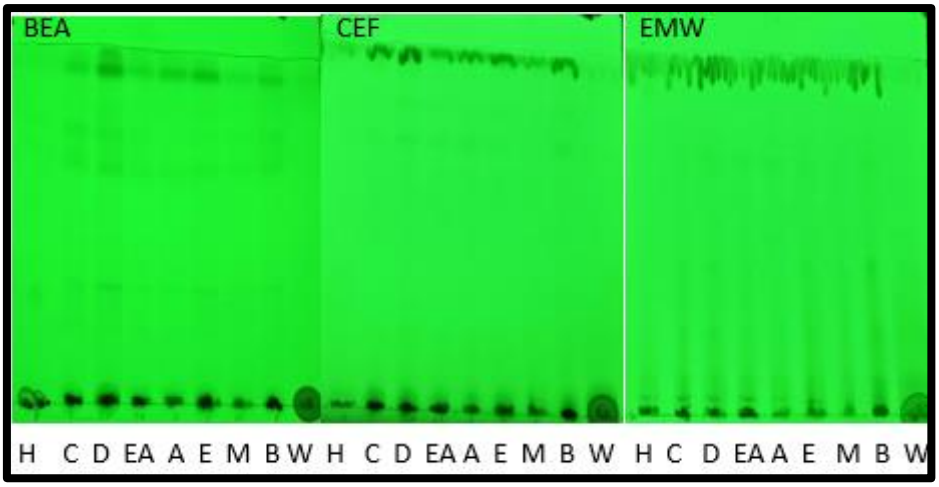

Figure 3. 2: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and observed under the UV light of 254 nm.

Key: H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol and W- Water, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

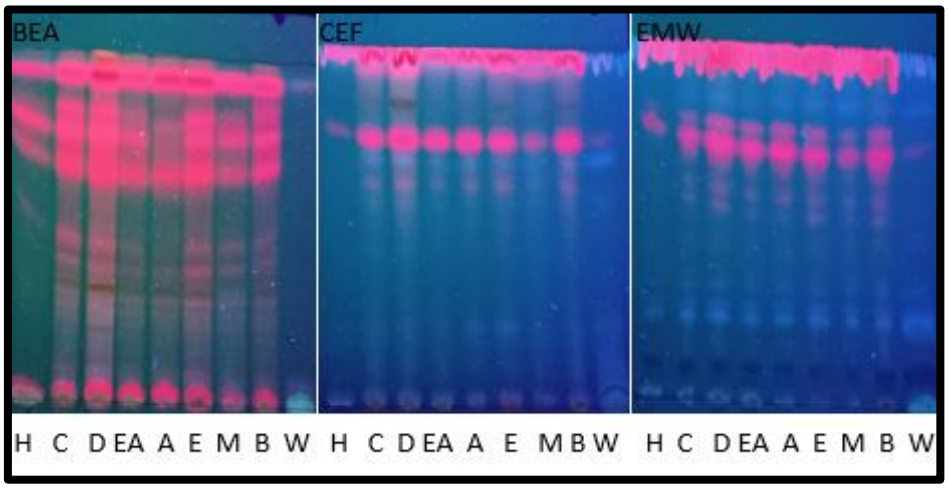

Figure 3. 3: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and observed under the UV light of 365 nm.

Key: H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol and W- Water, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

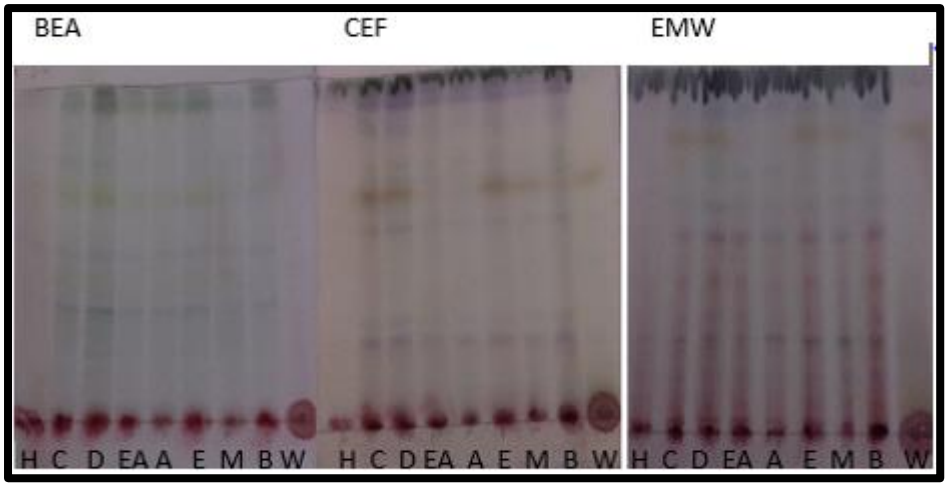

Figure 3. 4: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C.

Key: H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol and W- Water, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

3.3.3 Screening of phytochemicals

The powdered plant was exposed to different solvents to determine the presence of different phytochemicals in the plant. The plant had all phytochemicals, except phlobatannins and alkaloids (Table 3.1).

Table 3. 1: The phytochemicals that were tested in the plant material.

Phytoconstituents	*Dombeya rotundifolia*
Saponins	+
Terpenes/ terpenoids	+
Phlobatannins	-
Tannins	+
Cardiac glycosides	+
Flavonoids	+
Steroids	+
Alkaloids	-

Key: + = present, - = absent

3.3.4 Standard curves used for estimation of phyto-constituent contents

Below are the standard curves that were used to estimate the total phenolic, tannin and flavonoid. The curves depict the standard curves of gallic acid (Figure 3.5 and Figure 3.6) and quercetin (Figure 3.7). The curves indicate a positive linear correlation between the concentration and absorbance.

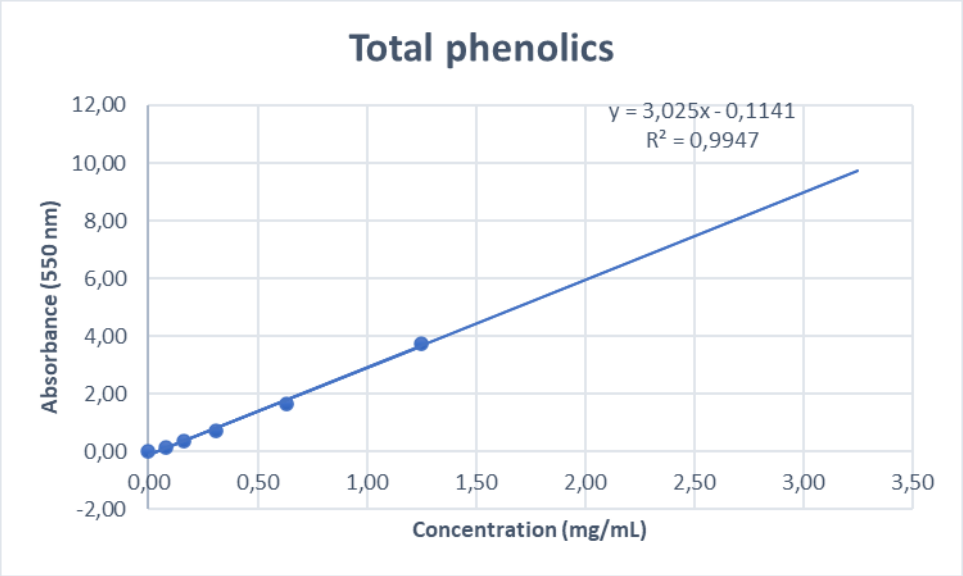

Figure 3. 5: The gallic acid standard curve for determining the total phenolic content.

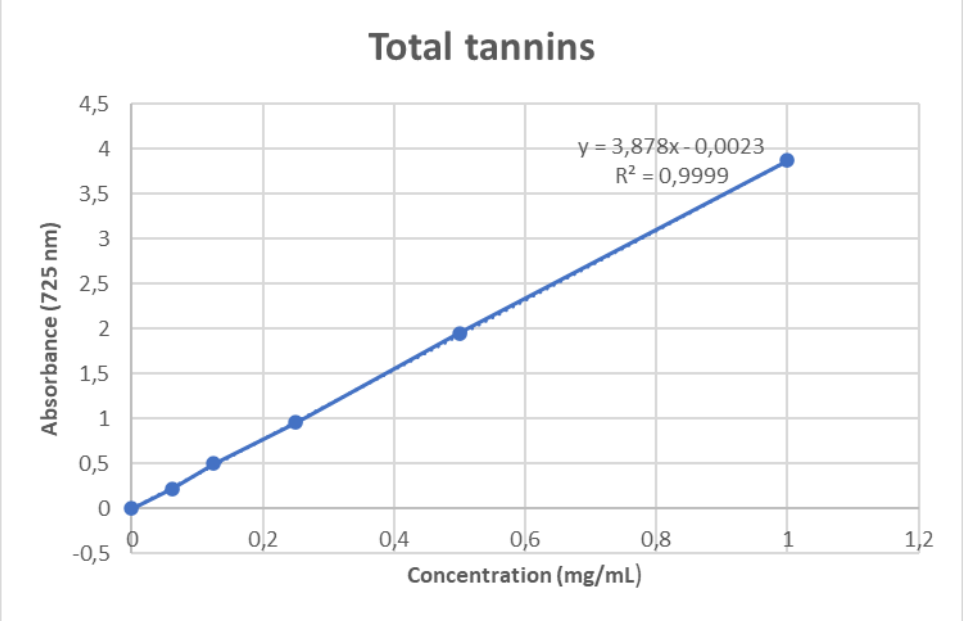

Figure 3. 6: The gallic acid standard curve for determining the total tannin content.

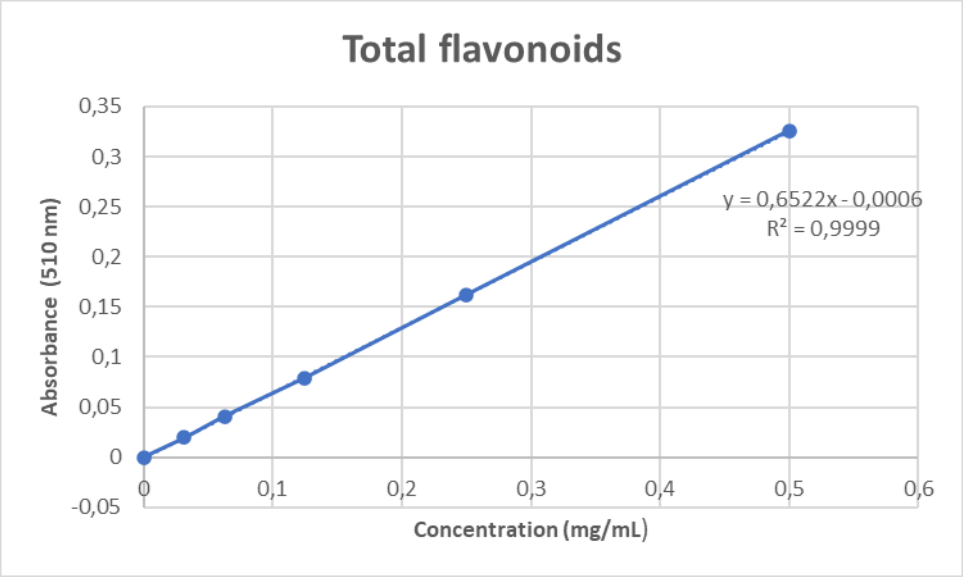

Figure 3. 7: The quercetin standard curve for determining the total flavonoid content.

3.3.5 Total phenol, tannin, and flavonoid contents

The total content of the compound in the plant extracts were quantified using different reagent assays. The concentrations were obtained from the standard curves. For both the total phenolic content and total tannin content, the Gallic acid (mg of GAE/g extract) was used as a standard curve and for the total flavonoid content, quercetin (mg QE/g extract) was used as a standard curve. For the total phenolic content, the hexane extract had the highest total phenolic content while the acetone extract had the least content. For the tannin content, the ethyl acetate extract had the highest total tannins content whereas the methanol extract had the least content. For the flavonoids content, the hexane extract had the highest total flavonoids content whereas the methanol extract had the least content. Overall, the plant had the notable phenolic content meaning that the plant had the higher total phenolic content when compared to total tannin and flavonoid content (Table 3.2).

Table 3. 2: Total phenolic, tannin and flavonoid contents of the different extracts of *Dombeya rotundifolia*.

Extractants	Total phenolic content (mg GAE/g extract	Total tannin content (mg GAE/g extract	Total flavonoid content (mg QE/g extract
Hexane	186.29 ± 0.70	24.60 ± 0.27	122.91 ± 2.82

Chloroform	108.97 ± 0.47	15.83 ± 1.29	53.30± 3.47
Dichloromethane	132.67 ± 0.70	16.51 ± 0.69	65.87 ± 0.87
Ethyl acetate	135.84 ± 0.33	25.59 ± 0.36	65.95 ± 6.40
Acetone	49.03 ± 0.70	9.76 ± 0.22	53.07 ± 1.19
Ethanol	115.08 ± 0.05	19.95 ± 0.42	90.40 ± 5.64
Methanol	54.72 ± 1.82	9.69 ± 0.89	28.23 ± 1.19
Butanol	119.74 ± 0.94	15.05 ± 0.64	64.95 ± 6.69
water	99.48 ± 1.64	24.48 ± 0.36	55.29 ± 1.95

The results are represented as an average of the triplicates ± standard deviation.

Key: GAE = gallic acid equivalence, QE = quercetin equivalence

3.4 Discussion

The aim was to investigate the phytochemical properties of *Dombeya rotundifolia*. The extraction was done using different solvents that varied in polarity. The plant material was screened and analysed for phytochemicals using standard methods. The major phytochemicals were further quantified and analysed. Plant derived medications make a significant contribution to human health and well-being, and have been a major source of inspiration for the development of new drugs. They are generally safe and provide therapeutic benefits at a lower cost (Roy, 2017). Extraction is a process of separating medically active parts of the plant using selective solvents. The purpose of extraction for crude drug is to obtain the therapeutically required portions while removing the unwanted material (Swami *et al*., 2008). For extraction, plant leaves were dried to avoid the contamination of bacteria and fungi since microorganisms are found on plant leaves due to the existing mutualism relationship between plants and microorganisms (Kudumela and Masoko, 2018); and fresh leaves are fragile, deteriorate faster; hence, a fine powder material was used for an efficient extraction to occur. Lowering the particles size increases contact between the plant material and the extraction solvents for a better extraction of the target analytes (Colvin, 2018). Figure 3.1 shows that water was the best extractant, followed by methanol and lastly, hexane was the least extractant. Water is generally used by traditional healers as their extractant because it is cheap, nontoxic, non-flammable and is a most polar solvent; so, it extracts a wide range of polar compounds. The second best extractant was methanol, which is the simplest alcohol. Alcohol is not toxic at a low concentration and polar; so, it extracts polar compounds even though it does not dissolve other substances such as fats. Hexane is nonpolar; thus, it generally extracts nonpolar compounds (Abubakar and Haque, 2020).

TLC was used for the analysis of phytochemicals. Figure (3.2) shows that there was a separation of compounds in all the mobile systems, but a clear separation of compounds was in the BEA mobile system, followed by the EMW system. Figure (3.3) shows a clear separation of compounds in all the mobile systems. The bands represent the compounds in the plant. According to Figure (3.2 and 3.3), the compounds were visualised under the UV light because they have UV absorption. The TLC plates have a fluorescent indicator that causes the plate to glow green when exposed to the UV light with a wavelength of 254 nm. The substances that absorb the

UV light at the wavelength of 254 will quench the green fluorescence, yielding dark spots on the plate (Cai, 2014). The number of coloured bands on a chromatogram indicate the number of fluorescent compounds present. The fluorescence show that the compounds have conjugated double bonds and /or extended pi (π) electron configurations. The fluorescing compounds may be due to the polyphenolic compounds' presence, which have long conjugated aromatic systems in their chemical structures (Matotoka and Masoko, 2018). The plate was further screened by spraying it with the Vanillin-sulphuric acid which allows the visualisation of non-fluorescing compounds (Figure 3.4). The reaction on the plate causes the colourless compounds to change to colour compounds for easy detection. Vanillin-sulphuric acid is used for the detection of steroids, higher alcohols, phenols, and essential oils. The coloured bands on the plates represent a group of compounds. Purple or bluish-purple bands represent terpenoids, pinkish, yellow, or orange bands represent flavonoids and pink bands represent proanthocyandins (Matotoka and Masoko, 2018).

The phytochemicals were screened using a standard method. Table 3.1 shows that *D. rotundifolia* had the following phytochemicals: saponins, terpenes/terpenoids, tannins, cardiac glycosides, flavonoids, steroids, except phlobatannins and alkaloids. These results are supported by the studies conducted by Reid *et al*. (2001) and Kudumela and Masoko (2018), who reported the same results. Medicinal plants are a rich source of a wide range of phytochemicals that are needed; hence, it is important to do a phytochemical screening to find and develop innovative therapeutic agents with increased efficacy (Yadav *et al*., 2014). The secondary metabolites were known to protect the plant, but studies show that they are responsible for preventing disease and promoting health due to their biological activity (Saxena *et al*., 2013). Studies also show that plants with bioactive compounds such as saponins, tannins and alkaloids have therapeutic potential such as antioxidants, anticancer, immunity potentiating, detoxifying and neuropharmacological agents (Ugboko *et al*., 2020). Phenolic and flavonoids are known for their antioxidant activity because of their free radical scavenging activities (Chigayo *et al*., 2016), and are also known to lower the risk of cancer, heart diseases, hypertension, and stroke (Maroyi, 2016).

The major phytochemicals were quantified and analysed using colorimetric assays. Table 3.2 shows that the plant extracts had more total phenolic content, followed by flavonoids and then tannins. The total phenolic content was determined using gallic

acid as standard and expressed as milligram gallic acid equivalence/ gram of extract. The method is based on the reaction between the Folin-Ciocalteu reagent and phenolic compounds, which results in the formation of a blue colour complex. The total phenolic content ranges from 49 to 186 mg GAE/g extract, where the hexane extract had the highest phenolic content, followed by the ethyl acetate extract whereas the acetone extract had the least content. The total flavonoid content was determined using quercetin as a standard and expressed as milligram quercetin equivalence/ gram of extract. The method is based on the reaction of flavonoids and $NaNO_2$-AI(III)-NaOH. The total flavonoid contact ranges from 28 to 122 mg QE/g extract. Hexane extract had the highest flavonoid content, followed by the ethanol extract while the methanol extract had the least content. The total tannin content was determined using gallic acid as a standard and expressed as milligram gallic acid equivalence/ gram of extract. The total tannin content ranged from 9 to 25 mg GAE/ g extract, where ethyl acetate extract had the highest content, followed by the hexane extract whereas the methanol extract had the least content. Phenolics are related to antioxidant activity. Table (3.2) showed that the phenolic content was higher, indicating that the plant extracts are beneficial to human health because they can quench reactive free radicals or primary oxidants. Flavonoids are important for fighting against diseases and when coupled with phenols, they have a high antioxidant activity (Chigayo *et al*., 2016). Other important activities associated with flavonoids include being anti-viral, anti-cancer, anti-inflammatory and anti-allergic (Suryawanshi, 2019). Tannins are known for their bactericidal effects since they react with proteins irreversibly. They bind within the bacterial membranes and inhibit their activities (Pizzi, 2019). The higher phenolic content in hexane extract is responsible for bioactivity. This extract is expected to perform well in antioxidant and antibacterial activities. The redox characteristics of phenolic chemicals found in plants enable them to act as antioxidants (Johari and Khong, 2019). Water and methanol were good extractive yields but have low total phenolic, tannin and flavonoid contents, it is because the outcome of the extraction process is influenced by the polarity of the solvent, pH level, the temperature, the duration of the extraction and the makeup of the substance being extracted (Do *et al*., 2014) while the total contents does not depend on the polarity of the solvent (Abubakar *et al*., 2022).

3.5 Conclusion

Medicinal plants are important in drug discovery and contribute immensely to the human health due to their therapeutic effects. Plants have secondary metabolites, which are significant to the biological activities of the plant such as being antimicrobial, antioxidant, anticarcinogenic, anti-inflammatory, antidiabetic and anti-allergic. The plant (*Dombeya rotundifolia)* possesses important phytochemical compounds such as phenolic, tannins and flavonoids. Therefore, a further analysis of antioxidant and antimicrobial activity may prove that the plant has a potential for drug development against TB.

3.6 References

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Chapter 4

4. Antioxidant activity of *Dombeya rotundifolia*

4.1 Introduction

The study of antioxidant molecules, particularly flavonoids, vitamins, synthetic chemicals, micronutrients, and phenolic compounds, began in the 18th century, where the qualities, features, source, and applications of antioxidant substances were investigated. The investigation paved the way for a wide range of use of antioxidant chemicals to achieve and maintain optimal human health (Ahmed and Iqbal, 2018). Research has shown that antioxidants in natural sources can be used to protect humans from oxidative stress damage that can occur during physiological activities (Bizuayehu *et al*., 2016). Medicinal plants are known as natural antioxidants because they have a low toxicity and are rich in pharmaceutical chemicals (Singh *et al*., 2016). Antioxidants are group of chemicals in the cell that eliminate free radicals and reactive species (Zehiroglu and Ozturk Sarikaya, 2019). Antioxidants provide protection against oxidative and free radical damage. They work as free radical scavengers, slowing down the oxidation of radical scavengers and the harmful impact they have on the body (Gupta and Verma, 2011). Many pathophysiological disorders have been linked to oxidative stress, which is defined as an imbalance between reactive oxygen, nitrogen species and antioxidant defence (Abderrahim *et al*., 2016). Because of the unpaired electrons, free radicals are a mediator of several diseases with a high reactivity such as cancer, inflammation, and cardiovascular diseases (Sulastri *et al*., 2018).

Antioxidants are classified into different groups, namely, primary and secondary antioxidants. Primary antioxidants are the molecules that break the chain of lipid radicals and convert them into more stable molecules while doing the secondary work by capturing the radicals and blocking the chain reaction from occurring (Moharram and Youssef, 2014). Antioxidant activity can be measured using a variety of methods such as hydrogen atom transfer, single electron transfer, reduction power and metal chelation (Shahidi and Zhong, 2015). Antioxidant efficiency is influenced by structural properties, temperature, the characteristics of the oxidizable substrate, concentration, as well as the presence of synergistic and pro-oxidant compounds and the system's physical state (Munteanu and Apetrei, 2021).

The analytical methods that use free radical scavenging activity to quantify antioxidant activity are quick, easy, and simple. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay is used to evaluate antioxidant activity and to examine the ability of substance to behave as a free radical scavenger or hydrogen donor (Gupta and Verma, 2011). The DPPH scavenging assay is based on antioxidants reducing the violet DPPH radical through a hydrogen atom transfer mechanism, which results in a colour shift to stable pale-yellow DPPH molecules. The discoloration of DPPH can be detected at 515 to 520 nm (Sirivibulkovit *et al*., 2018), and the discoloration serves as a sign of antioxidant efficacy (Shahidi and Zhong, 2015). Ferric reducing power is based on the transfer of single electrons and measures the reduction of the complex of ferric ion (Fe^{3+}) to blue ferrous complex (Fe^{2+}) in the presence of antioxidants. Antioxidant activity is evaluated at 593 nm as the absorbance increases. It takes place in an acidic pH environment to maintain iron solubility. Low pH causes a shift in the reaction mechanism by lowering the ionisation potential, which drives electron transfer and increases the redox potential. The product, Prussian blue, can be formed in two ways: antioxidants either reduce Fe^{3+} to Fe^{2+} which binds the ferricyanide to produce Prussian blue, or they reduce ferricyanide to ferrocyanide, which bind to the free Fe^{3+} to produce Prussian blue (Munteanu and Apetrei, 2021). The method is proven to be effective in determining antioxidant capabilities and comparing the efficacy of various compounds (Spiegel *et al*., 2020).

4.2 Method and materials

4.2.1 Qualitative DPPH antioxidant activity assay on TLC plates

TLC coupled with DPPH were used to screen for the presence of the antioxidant activity of the plant extracts. The amount of 10 µL of each crude extract was loaded using a micropipette, at the bottom of the TLC plate, just 1 cm away from the base of the TLC plate. The plates were put inside a saturated TLC tank that contained the following solvents: EMW, CEF and BEA, as mentioned in section 3.3. This was then followed by spraying the plate with the DPPH solution (0.2 % w/v) in methanol. The development of yellow spots against a purple background on the TLC plates indicated the antioxidant activities of the extracts (Deby and Margotteaux, 1970).

4.2.2 Quantitative antioxidant activity assay

4.2.2.1 DPPH free radical scavenging assay

The free radical scavenging activity of the plant extracts were determined using the DPPH method described by Chigayo *et al*. (2016). The concentrations (250-15.63 µg/mL) were prepared to the volume of 1 mL of the solution. L-Ascorbic acid was used as a standard by preparing the same concentration range as the concentrations of the extracts. To these 1 mL solutions, 2 mL of 0.2 mmol/L DPPH solution dissolved in methanol was added and vortexed thoroughly. To prepare a blank, 0.5 mL of acetone and 0.5 mL of methanol were added into the test tube. The control solution was prepared by adding 2 mL of the 0.2 mmol/L DPPH to 1 mL of acetone. All the prepared mixtures were left to stand in the dark for 30 minutes. After the elapsed time, the solutions were analysed with a UV-VIS spectrophotometer (Thermo Scientific Genesys 10S). The absorbance of the solutions was read at 517 nm and the percentage antioxidant potential of the solution was calculated using the formula:

$$\%Inhibition = \frac{Ac - As}{Ac} \times 100$$

Where, AC is the absorbance of the control solution and AS is the absorbance of plant extract. The experiment was performed in triplicates.

4.2.2.2 Ferric reducing power assay

The ferric reducing power of the plant extracts was determined using the methods of Vijayalakshmi and Ruckmani (2016) and Ahmed *et al.* (2012). Five different concentrations of the plant extracts (625-39 µg/mL) were prepared by serially diluting

a stock solution of 1250 µg/mL. The different concentrations (625 to 39 µg/mL), 2.5 mL from each concentration was mixed with a 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v in distilled water), in a test tube. The mixtures were vortexed after the addition of the solutions. The mixtures were incubated for 20 minutes at 50 °C. Two millilitres of trichloroacetic acid (10% w/v in distilled water) were added to the test tubes after incubation, after that 5 mL of the supernatant was transferred to a clean test tube. To this solution, 5 mL of distilled water and 1 mL of ferric chloride (0.1% w/v in distilled water) were added consecutively with thorough vertexing after each addition. UV-VIS spectrophotometer (Thermo Scientific Genesys 10S) was used to read the absorbance of the solution at a 700 nm wavelength. The blank for this procedure was prepared in the same manner; however, the plant extracts were replaced by an equal amount of acetone. L-Ascorbic acid (625-39 µg/mL) was used as a positive control and was prepared similarly to the plant extracts. The experiments were performed in triplicates.

4.3 Results

4.3.1 Qualitative antioxidant activity assay

The antioxidant activity of the plant was screened using DPPH on the TLC plates which were developed in different separation systems. The yellow spots on the purple

background indicated the presence of antioxidant compounds. The plant extracts have antioxidant compounds but separated well in the EMW mobile system (Figure 4.1). Most of the antioxidant compounds were visible in the EMW mobile system.

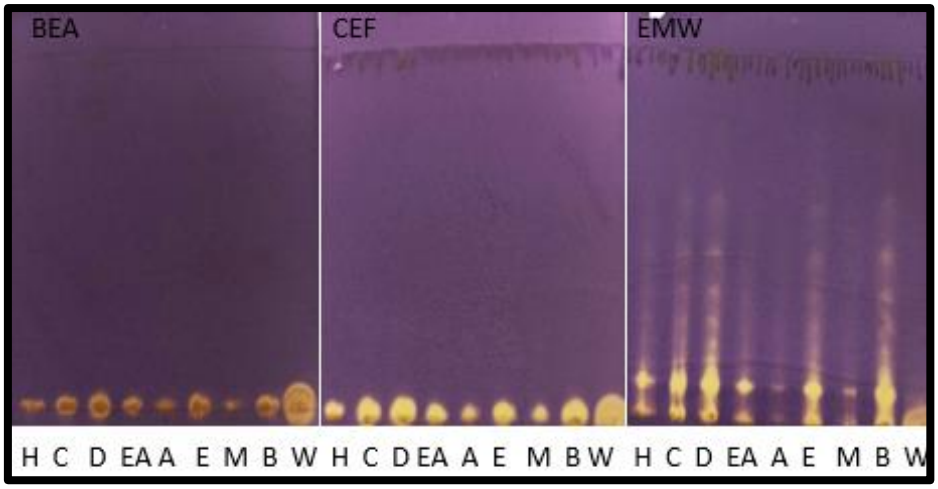

Figure 4. 1: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and sprayed with 0.2% DPPH.

Key: H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol, W- Water, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

4.3.2 Quantitative antioxidant activity assay

The antioxidant activity of the plant was quantified using the DPPH method. The graph below shows the quantitative antioxidant activity, which is represented by the percentage scavenging activity of the plant extracts. The increases in scavenging activity increase with the increase in the concentration of the plant extracts. Ascorbic acid (control) had the highest scavenging activity compared to the plant extracts. The dichloromethane extract had the highest scavenging activity while acetone and methanol extracts had the lowest scavenging activity (Figure 4.2). The antioxidant activity of the plant was quantified using the ferric reducing power method. The increase in the absorbance increases with the concentration in the plant extracts. Ascorbic acid (control) had the highest reducing capacity compared to the plant extracts. In the plant extract, hexane had the highest reducing capacity while acetone had the least (Figure 4.3).

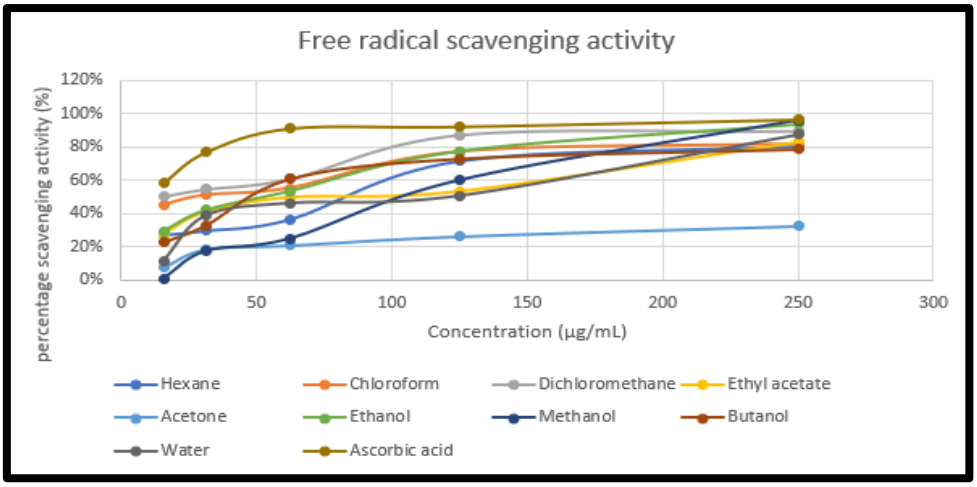

Figure 4. 2: Percentage free radical (DPPH) scavenging of the plant extracts compared with the ascorbic acid as a standard at 517 nm.

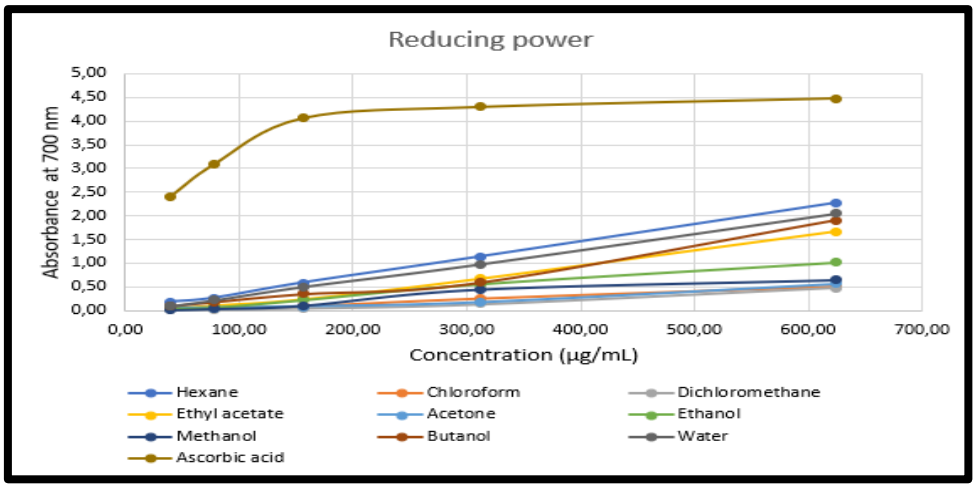

Figure 4. 3: The ferric reducing power of the plant extracts compared with the ascorbic acid as a standard at 700 nm.

4.4 Discussion

The aim was to investigate the antioxidant activity of *Dombeya rotundifolia*. Plants are a key source of natural antioxidants and produce a variety of secondary metabolites with antioxidative properties that may be used therapeutically. Polyphenols are the most common antioxidant chemical found in plants. The antioxidant activity is based on the redox characteristics that allow them to work as reducing agents, hydrogen donors, singlet oxygen quencher and metal chelators (Stanković *et al*., 2016). The antioxidant activity of the plant was evaluated using DPPH and the ferric reducing power since one cannot conclude based on one method, because antioxidant activity occurs through different mechanisms and is influenced by numerous factors (El Jemli *et al*., 2016). The yellow spots against the purple background indicated the presence of antioxidant compounds (Figure 4.1). This means that the plant extracts have hydrogen donating antioxidant compounds because they were able to reduce violet DPPH to a yellow DPPPH-H. All the plant extracts had the antioxidant compounds represented by a yellow spot, but they did not separate in all the systems due to their polarity to the solvent in the system. There was a clear separation in the EMW mobile system and most of the antioxidant compounds were visible in the EMW mobile system. The chromatograms (Figure 4.1) are similar to the study of Kudumela and Masoko (2017), who noted a separation in one mobile system (EMW) whereas in other systems the compounds were found at the bottom.

The quantification of antioxidants was done using the DPPH assay and the ascorbic acid was used as control. The method is based on the reduction of violet DPPH into a yellow DPPH molecule, which represents the free radical reducing activity of antioxidant. Ascorbic acid had the highest scavenging activity compared to the plant extracts (Figure 4.2). Vitamins, phenolic and carotenoids are the important types of natural antioxidants that provide protection (Bajalan *et al*., 2016); hence, the ascorbic acid had the highest activity. In plant extracts, dichloromethane had the highest scavenging activity while acetone and methanol extracts had the least activity. Analysing the results for each concentration, dichloromethane had the highest scavenging activity for all the concentrations except at 250 µg/mL where the methanol extract had the highest activity. The acetone extract had the least activity at all the concentrations except at 31 and 15 µg/mL, where methanol had the lowest activity at those concentrations. When relating the total content and the antioxidant activity,

acetone and methanol as extracts both had lower content in phenolic, tannin and flavonoid; hence, they have the lower antioxidant activity. The absorbance of DPPH radical decreases as the concentration increases. This is because of the reaction between antioxidant and radical molecules, which results in the radical being scavenged by hydrogen donation (Bhalodia *et al*., 2013). Antioxidants offer an alternative method for both treating and preventing chronic disorders. Due to their oxidative activity, phenolic compounds have the potential to prevent and treat a wide range of oxidative stress-related disorders (Sofowora *et al*., 2013). Phytochemicals with antioxidant properties are needed so that plant products can be effective in treating human diseases such TB (Kumar *et al*., 2021).

The quantification of antioxidants was done using the ferric reducing power assay and the ascorbic acid was used as control. The assay was based on the reduction of Fe^{3+}/ ferricyanide complex to the Fe^{2+}/ ferrous form, which acts as a potential indicator of antioxidant activity. In the plant extract, hexane had the highest reducing capacity while acetone had the least due to their hydrogen donating ability (Figure 4.3). The hexane extract acts as an electron donor which reacts with free radicals to convert them into more stable molecules and end the free radical chain reactions (Irshad *et al*., 2012). The absorbance increases with an increase in concentrations which indicate the antioxidant activity; so, the reducing power of the plant extracts and ascorbic acid increase as the concentration increases. There is a relationship between the phenolic content and the antioxidant activity; hence, the extract that had the highest phenolic content had the highest reducing capacity, which indicated the antioxidant activity. Kudumela and Masoko (2018) evaluated antioxidant activity using the same method, where *D rotundifolia* had the highest reducing power when compared to other plants. The antioxidant activity was due to the flavonoids and phenolics that had been identified from the leaves.

4.5 Conclusion

An increase in demand for natural antioxidants has necessitated greats effort towards finding new drugs because of their numerous effects such as antioxidant, anti-aging, and anti-inflammation. The antioxidant analysis showed that the plant extracts had antioxidant properties due the major phytochemicals such phenolics present in the plant. Since the plant has antioxidant properties, the evaluation of antimycobacterial activity is a dire necessity.

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Chapter 5

5. Antimycobacterial activity and antibiofilm assays

5.1 Introduction

Due to increases in microbial resistance, there has been an increasing interest in discovering and producing novel antimicrobial drugs from different sources. More focus has been on antimicrobial activity screening and evaluation methodologies (Balouiri *et al*., 2016). Natural compounds derived from microorganisms and plants have a wide range of biological activity and drugs properties; therefore, they are a valuable source of drugs in the pharmaceutical industry (Santhosh and Suriyanarayanan, 2014). Plants with therapeutic properties have been used in medicine since ancient times. Studies provide evidence to ascertain the usefulness of medicinal plants and provide insight into the synthesis of plant-based compounds for therapeutic use. Phytochemicals can show antimicrobial activity through a variety of methods such as the inhibition of enzymes and toxins, bacterial membrane damage, suppression of virulence factors, biofilm development, protein synthesis inhibition and quorum quenching (Ugboko *et al*., 2020). Antimicrobial therapy aims to kill or supress the infectious organism while causing no harm to the host. An antimicrobial testing for substance is important to determine their effectiveness against infectious organisms. Depending on the MIC value, the degree of drug effectiveness is classified as ‘susceptible’, ‘intermediate’ or ‘resistant’. ‘Susceptibility’ is when a bacterial strain is inhibited by a concentration of the drug that is associated with the high likelihood of therapeutic success. ‘Intermediate’ is when bacterial strain is inhibited by a concentration of the drug that has an unknown therapeutic effect. ‘Resistant’ is when a bacterial strain is inhibited by a concentration of the drug that is linked with a high risk of treatment failure (Rodloff *et al*., 2008).

An antimicrobial susceptibility test is an important tool used in pathology to test microbial resistance to various antimicrobials and in pharmacology research to determine the effectiveness of antimicrobials derived from a biological extract against different microorganisms (Das *et al*., 2010). The choice of a bioassay method is important for determining the active compounds in plant extracts; therefore, the bioassay test must be sensitive, reliable, easy to use and quick (Valle *et al*., 2016). Bioautography and diffusion methods are classified as qualitative methods because

they indicate the presence or absence of antimicrobial compounds while the dilution methods are regarded as quantitative methods because they involve determining the MIC (Valgas *et al*., 2007). TLC-bioautography was introduced by Fischer and Lautner as a combination technique of TLC with biological and chemical detection methods. It is used to screen organic extracts for antibacterial and antifungal activity (Balouiri *et al*., 2016). The principle of the method is dipping the developed TLC plate in a suspension of microorganisms growing in a suitable broth and incubating it in a humid environment. The tetrazolium salt is sprayed onto the bioautogram to visualise the microbial growth. The tetrazolium salt is converted to intense coloured formazan by the dehydrogenase of live bacteria. As a result, the white spot against the pink background indicates the presence of antibacterial agents (Choma and Grzelak, 2011). The dilution methods are the best for determining the MIC and minimum bactericidal concentration (MBC). The MIC is the lowest antimicrobial agent concentration required to inhibit the growth of microorganism while MBC is the lowest antimicrobial concentration required to kill microorganism (Owuama, 2017). The method involves a two-fold serial dilution of the extracts that were prepared directly in a microtiter plate and the bacterial broth that was added in each well. The plate was covered and incubated (Manandhar *et al*., 2019). After incubation, *p*-iodonitrotetrazolium violet (INT) was added in each well as an indication of bacterial growth and the plate was further incubated to determine the MIC visually. The MIC value was determined by taking the lowest concentration of each extract that showed the inhibition of bacterial growth (Mogana *et al*., 2020).

Microplates are crucial instruments for biofilm research because they enable a high-throughput testing of strains that generate biofilms or the evaluation of anti-biofilm medicines. Biofilm is quantified using a microtiter plate-based crystal violet assay which was introduced in 1985 (Shukla and Rao, 2017). Cells grow in the microtiter plate for a certain time and then the plankton bacteria are removed from the wells by washing the wells. The remaining adherent cells are then dyed to make it possible to see the pattern of attachment. The surface with dye is then evaluated for the biofilm developed (Merritt *et al*., 2011).

5.2 Methods and materials

5.2.1 Microorganism used in this study

The test organism, *Mycobacterium smegmatis* was obtained from Prof Green, University of Johannesburg, at the Department of Biotechnology and Food Technology. The culture was maintained on a Middlebrook 7H9 agar base with glycerol and the Middlebrook OADC growth supplement. The *M. smegmatis* culture was inoculated into the Middlebrook 7H9 broth base with glycerol and Middlebrook OADC growth supplement and incubated at 37°C for 24 hours.

5.2.2 Bioautography

The anti-mycobacterial activities of the plant extracts were determined using the bioautography procedure according to Begue and Kline (1972). The amount of 20 µL of each crude extract was loaded using a micropipette, at the bottom of the TLC plate and separated, as described in section 3.2.3. These plates were then air-dried for five days to allow the solvent to evaporate. The actively growing culture was sprayed on the plates until they were completely wet, and the plates were incubated at 37°C for 24 hours. Following incubation, the plates were sprayed with 2 mg/mL of *p*-iodonitrotetrazolium violet (INT) (sigma) dissolved in water and further incubated for 3 hours. The bioautograms were observed for bacterial growth, and clear zone against the red-pink background indicated growth inhibition by the compounds with antimycobacterial activity.

5.2.3 Micro-dilution Assay

The MIC values (2.5-0.019 mg/mL) were determined using the serial microplate broth dilution method developed by Eloff (1998). The plant extracts were dissolved in acetone to give a final concentration of 10 mg/mL. A volume of 100 µL of distilled water was added in each well of a 96 well microtiter plate using a multi-channel micropipette. Extracts of 100 µL were added into the first well respectively and were serially diluted (50%) with sterile distilled water in 96 well microtiter plates and then a 100 µL of the *Mycobacterium* culture was added into each well. Acetone was used as a negative control while Rifampicin was used as a positive control. The microtiter plates were covered and incubated at 37°C for 24 hours. Following the incubation, 40 µL of 0.2 mg/mL of *p*-iodonitrotetrazolium violet (INT)(sigma) dissolved in water was added to each well as an indicator of growth. The covered plates were incubated for 30 minutes at 37°C and relative humidity. The plates were observed for clear wells (activity), which

resulted from the reduction of the purple colour and the MIC values were recorded as the lowest concentration that inhibited bacterial growth. The tests were done in triplicates. The total activity was determined by dividing the mass of the plant extracts by the MIC value.

5.2.4 Antibiofilm activity assay

A 100 µL aliquot of standardised concentration of cultures with OD_{600} =0.02 of *Mycobacterium smegmatis*, was added into individual flat-bottomed 96-well microtiter plates and incubated at 37 °C for 48 hours to allow the mature development of the biofilm. The plates were incubated without shaking. Following incubation periods, 100 µL aliquots of plant extracts (20 mg/mL) were added into the wells of a 96-well microtiter plates to give a concentration range between 5-0.31 mg/mL. The plates were incubated further at 37°C for 24 hours. Rifampicin at the same concentration as the extracts served as a positive control while Dimethyl sulfoxide (DMSO) served as a negative control (Famuyide, 2019). The biofilm biomass was assayed using the modified crystal violet (CV) staining assay. The assay was done as previously described with some modifications. Briefly, the 96-well microtiter plates were washed three times with sterile distilled water, air dried and then oven-dried at 60 °C for 45 minutes. The wells were then stained with 100 µL of 1% crystal violet and incubated at room temperature for 15 minutes, after which the plates were washed thrice with sterile distilled water to remove unabsorbed stain. At this point, the biofilms were observed as purple rings at the side of the wells. The semi-quantitative assessment of biofilm formation was done by adding 100 µL of Dimethyl sulfoxide (DMSO) to distain the wells. A 100 µL aliquot of the distaining solution was transferred to a new sterile plate and the absorbance was measured at 595 nm using a microplate reader (Bio-Rad). The mean absorbance of the samples was determined, and the percentage inhibition of biofilm was determined using the equation below (Djordjevic *et al*., 2002).

$$\%\ inhibition = \frac{OD\ control - OD\ experimental}{OD\ control} \times 100$$

5.2.5 Statistical analysis

Statistical analysis was performed through the Prism software package (GraphPad Software). The results were analysed using two-way ANOVA to examines the effect of different factors on a dependent variable (Antibiofilm activity data), Dunnett's

multiple comparisons test ($p < 0.05$). p values lower than 0.05 were considered to be statistically significant.

5.3 Results

5.3.1 Bioautography

The Bioautography technique was used to screen for antimycobacterial activity and INT was used as an indicator. The bioautograms showed the presence of antimycobacterial activity in all the systems, which is indicated by the zone of inhibition (white colour). There were more antimycobacterial compounds in the CEF and EMW system (Figure 5.1).

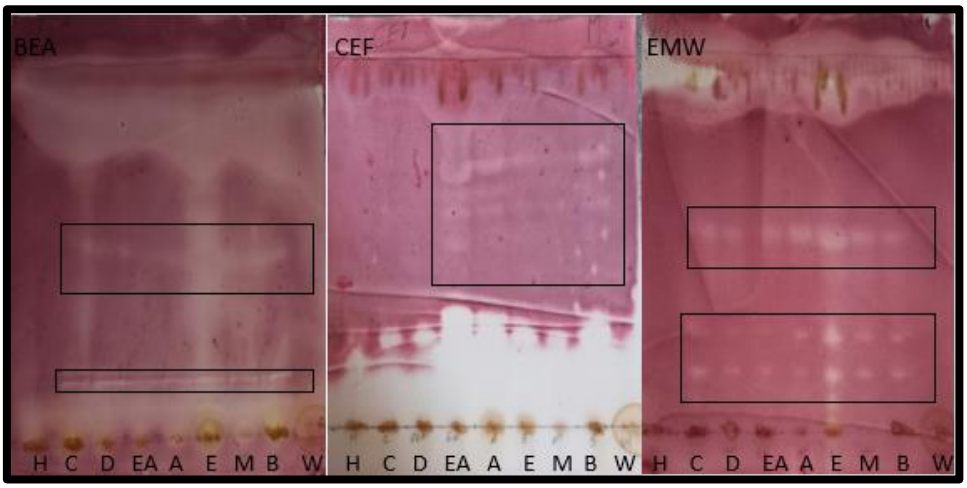

Figure 5. 1: The chromatograms of *Dombeya rotundifolia* plant extracts separated in BEA, CEF and EMW and sprayed with *M. smegmatis.*

Key: H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol, W- Water, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

5.3.2 Micro-dilution assay

The MIC values were determined using the serial broth dilution microplate method. The results showed that the plant had antimycobacterial activity, the extract that had the lowest MIC value was butanol with MIC value of 0.13 mg/mL and the extract that had the highest MIC value was water with value of 0. 42 mg/mL (Table 5.1). The total activity was determined by dividing the mass of the plant extracts by the MIC value. The dichloroethane extract had the highest total activity (203.13 mL/g), followed by the butanol extract (176.92 mL/g) while the hexane extract had the least total activity (59.05 mL/g) (Table 5.2).

Table 5. 1: The MIC at which the plant extracts inhibited the growth of *M. smegmatis* after 24 hours of incubation.

MIC values (mg/mL)									
DR	H	C	D	EA	A	E	M	B	W
	0.21	0.37	0.16	0.31	0.16	0.26	0.26	0.13	0.42

Key: DR- *Dombeya rotundifolia,* H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M-Methanol, B- Butanol and W- Water, Rifampicin (positive control) = 0.08mg/mL, Acetone (negative control) = greater than 2.5 mg/mL

Table 5. 2: The total activities of the plant extracts after 24 hours incubation.

Total activity (mL/ g extract)									
DR	H	C	D	EA	A	E	M	B	W
	59.05	96.49	203.13	66.13	123.75	118.08	146.54	176.92	137.14

Key: DR- *Dombeya rotundifolia,* H- Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M-Methanol, B- Butanol and W- Water.

5.3.3 Antibiofilm activity assay

The antibiofilm activity of the plant extracts was evaluated using the crystal violet assay. The results showed that the plant extracts have antibiofilm activity. The hexane (ranged from 61% to 56%), dichloromethane (ranged from 67% to 61%), and acetone (ranged from 69% to 65%) plant extracts showed a good activity while the methanol extract (ranged from 58% to 43%) at a certain concentration did not have a good activity (Figure 5.2).

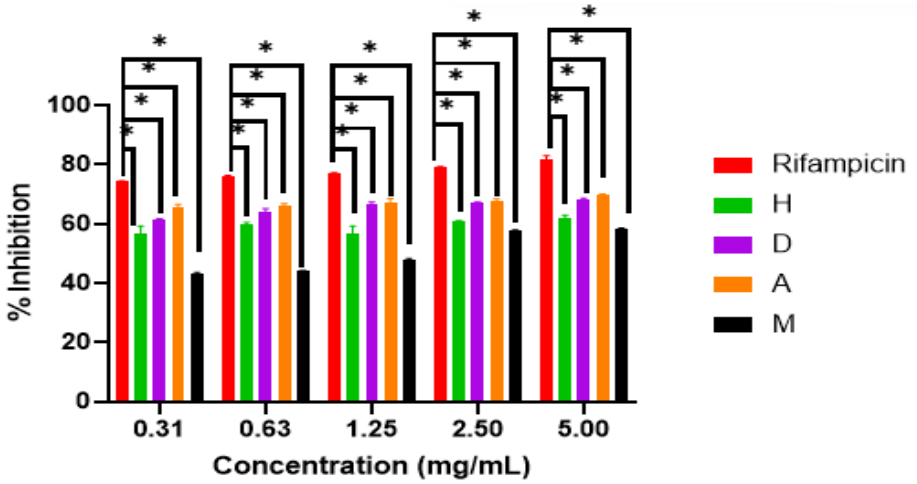

Figure 5. 2: Anti-biofilm of *Dombeya rotundifolia* extracts at different concentrations.

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol and (*) = significant difference (between Rifampicin and plant extracts at different concentrations)

5.4 Discussion

The aim was to investigate the antimycobacterial and antibiofilm activities of *Dombeya rotundifolia*. Despite the success of antibiotic discovery, infectious diseases continue to be the second biggest cause of mortality worldwide and resistance remains one of the most serious concerns of the twenty-first century (Ugboko *et al*., 2020). The resistance to antimicrobial treatments increases when bacteria enter biofilms. Antibiotic resistance is higher in biofilm-forming bacteria than in planktonic forms (Mohammadi *et al*., 2019). According to the National Institutes of Health (NIH), biofilm is responsible for more than 80% of all illnesses in the body. Biofilms are linked to about 80% of all chronic diseases and 65% of all microbiological infections (Jafri *et al*., 2019). The emergence of diseases and the expansion of scientific understanding about herbal medicines as an important alternative or complementary treatment of diseases justify the use of plant derived medicinal agents. The bioactive compounds in plants are used as a starting point for the production of antibiotics which are used to treat infection illnesses (Kebede *et al*., 2021). The antibacterial activity of an agent has two mechanisms: interfering with the production or function of key components of bacterial resistance, and/or evade the antibacterial resistance mechanisms (Khameneh *et al*., 2019). The antimycobacterial activity was evaluated using the broth micro-dilution assay and bioautography. The antibiofilm was evaluated using crystal violet assay.

The qualitative antimycobacterial activity of the extracts was evaluated using the bioautography method, where INT was used as an indication of bacterial growth. The pinkish colour developed indicated the viability of the microorganism, which means that the tetrazolium salts were converted to formazan while the white colour (zone of inhibition) indicated the presence of antimycobacterial compounds. Figure 5.1 shows that the antimycobacterial compounds were present in all the mobile systems, that is, in EMW and BEA, all the extracts contained antimycobacterial compounds except for water and the hexane extracts while in CEF, all the extracts had antimycobacterial compounds except for the hexane, chloroform, dichloromethane and water extracts. In the CEF, the white colour against the pink background at the bottom may not indicate the presence of antimycobacterial compounds but bacterial growth inhibition due to the formic acid residues left on the chromatogram which prevented the growth (Masoko *et al*., 2008). The extracts that did not have activity are not effective against

M. smegmatis or lack activity due to the active compound that evaporated, photo-oxidation, or a small amount of active compound (Masoko and Eloff, 2005).

The antimycobacterial activity was evaluated in the broth micro-dilution assay. The antimycobacterial of the plant extracts was evaluated by determining the MIC against *M. smegmatis*. Rifampicin was used as a positive control and acetone was used as a negative control. In 1971, rifampicin was added to the antituberculosis medication making treatment shorter and more effective (Webster *et al*., 2010). Acetone was used as a negative control because it dissolves a wide range of hydrophilic and lipophilic components, miscible with water, and is less harmful to bacteria (Eloff, 1998). The results showed that the plant had antimycobacterial activity. The extract that had the lowest MIC value was butanol with the MIC value of 0.13 mg/mL, followed by acetone and dichloromethane with the MIC value of 0.16 mg/mL while the extract that had the highest MIC value was water with the value of 0. 42 mg/mL (Table 5.1). The lowest concentration of extracts that inhibited bacterial growth (low MIC value) indicates the high inhibitory action of the extracts (Masoko, 2017). Phytochemicals are usually classified as antimicrobials based on susceptibility tests that produce MICs in the range of 100 to 1000 µg/mL (.Simoes *et al*., 2009). The MIC of the extracts is known to be good when the values are less than 0.1 mg/mL, moderate when the values are between 0.1 and 0.625 mg/mL, and weak when the values are greater than 0.625 mg/mL (Kuete, 2010). All the extracts in this study were within the moderate category, which they ranged from 0.13 to 0.42 mg/mL. The total activity (Table 5.2) was determined by dividing the mass of the plant extracts by the MIC value. Dichloromethane had the highest total activity of 203.13 mg/L followed by the butanol extract (176.92 mg/L) while the hexane extract had the least total activity of (59.05 mg/L). It is important to determine the total activity of the plant extracts when assessing their potential use for treating diseases or infections. The total activity unit (mL/g) indicates the degree to which active compounds in one gram of the plant material can be diluted and still inhibit the growth of the microorganism (Eloff, 2004).

The antibiofilm was evaluated using the crystal violet assay. Despite the limitations, such as the need for repeated washings which may result in cell loss and biofilm disruption, the crystal violet assay is widely used to determine the anti-biofilm potentials of natural compounds (Danquah *et al*., 2022). Rifampicin was used as a positive control while Dimethyl sulfoxide (DMSO) served as a negative control.

Rifampicin is a broad-spectrum antimicrobial agent that effectively kills microorganisms, and it is one of the rare antimicrobial agents that can penetrate biofilms and eliminate microorganisms during their stationary phase of growth (Perlroth *et al*., 2008). DMSO has been used in toxicology and experimental pharmacology and is recommended *in vitro* and *in vivo* research and standard protocols for the antimicrobial screening of natural compounds (Summer *et al.*, 2022). DMSO is an organic polar solvent that is used in the manufacture of medications and antibiotics (Yaacob *et al.,* 2021). The extracts with an inhibition above 50% were considered to have good activity while those with an inhibition lower than 50% were considered to have poor activity. The results (Figure 5.2) showed that hexane, dichloromethane, and acetone plant extracts had a good activity while methanol extracts at certain concentration did not have a good activity. Rifampicin had the highest activity when compared with the plant extracts. At a concentration of 5 mg/mL, it had the percentage inhibition of 81%. It is expected for Rifampicin to have a high antibiofilm activity since it is the one of the first-line drugs used to treat TB. It was followed by the acetone extract with the percentage of 69% at a concentration of 5 mg/mL while methanol had the least activity (58% at the concentration of 5 mg/mL). At a higher concentration (5mg/mL), the plant extracts have potential to prevent the formation of biofilm. Substances that disrupt the biofilm's structure show a great deal of promise in treating biofilm-mediated illnesses (Chung and Toh, 2014). The statistical analysis using GraphPad software showed that there is a significant difference between rifampicin and plant extracts at different concentrations.

5.5 Conclusion

The plant extracts of *D. rotundifolia* had potential antimicrobial and antibiofilm activity against *M. smegmatis.* The extracts may be used to control the formation of biofilm or model for new drug developments. With these noteworthy activities, the plant has the potential to treat TB, but further investigation is needed to check the toxicity of the plant before developing it as a drug.

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Chapter 6

6. Cytotoxicity and anti-inflammatory activity assay

6.1 Introduction

Medicinal herbs have been utilised in traditional medicine since many years ago. In recent years, focus in scientific research has been on the possible side-effects of some plants or their interaction with other medications (Paul *et al.,* 2015). Herbs used in traditional medicine are thought to have low toxicity due to their long-term use by humans. Many plants contain cytotoxic and genotoxic chemicals, and green plants in general are a key source of antimutagens and natural toxic agents (Aşkin Çelik and Aslantürk, 2010). The ability of a material to have negative effects on living things is referred to as toxicity and such toxicity can be classified as acute, sub-acute or chronic toxicity (Mensah *et al*., 2019). Acute toxicity is the term used to describe the harmful effect induced by a single exposure to drugs for a brief period. The primary goal of acute toxicity study is to find a single dose that causes a life-threatening toxicity, which involves an estimation of the minimal dose that causes mortality. A sub-acute toxicity study involves the administration of the sub-lethal dosages of a drug daily for 14 to 21 days. It is used to assess how the drug affects the biochemical and hematogical parameters of the blood and the histopathological effect. A chronic toxicity study involves the administration of a drug in various doses for 90 days to a year to assess the drug's potential to cause cancer and mutagenesis (Chanda *et al*., 2015). Toxicity testing is critical in the evaluation of novel medications before they are approved for use in people. The purpose of toxicity testing is to characterise the potential hazardous consequences of a test material, not only to determine its safety (Arome and Chinedu, 2013). Toxins can be assessed in both qualitative and quantitative ways. A quantitative analysis provides information about the chemistry of toxins and their concentrations, whereas a qualitative analysis provides information about their nature (Parasuraman, 2011).

Plant-derived natural compounds have demonstrated strong anti-inflammatory effectiveness and have advanced from preclinical investigations to clinical trials. There are three types of anti-inflammatory medications that are currently prescribed, namely, non-steroidal anti-inflammatory drugs, cyclooxygenase, and steroidal anti-inflammatory drugs (Akhtar, 2022). However, a continuous use of those medications

may be toxic or have secondary side-effects which lead to damage to liver, gastrointestinal tract, cardiovascular and renal failure (Bailey-Shaw *et al*., 2017). There is a need to develop plant-based compounds with anti-inflammatory activity, which are not toxic to humans.

Medicinal plants continue to provide new treatments to humanity; as such, the safety, quality, toxicity, suitable amount of plant materials to use, and effectiveness of medicinal plants should all be investigated (Nemudzivhadi and Masoko, 2014). 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is superior because it is simple to use, it is safe, and reliable, and is frequently used to measure cell viability and cytotoxicity (Tomar *et al*., 2021). The MTT assay uses water-soluble yellow salt (MTT), which is converted into an insoluble purple formazan due to the dehydrogenase enzyme found in metabolically active cells and then quantified using the spectrophotometer. The number of active mitochondria in the living cell is proportional to the reduction of the MTT (Sharif *et al*., 2017).

When assessing the anti-inflammatory effects of a medicinal plant using the denaturation approach, the egg albumin method offers a cheap alternative method. Inflammation has been linked to protein denaturation. Inflammation occurs when living tissues are harmed and is characterised by swelling, heat, pain, redness, and loss of function in the affected area (Dharmadeva *et al*., 2018). Protein denaturation is a process in which external stress or chemicals cause proteins to lose their quaternary, tertiary, and secondary structure. When biological proteins are denatured, they lose their biological function (Ruiz-Ruiz *et al*., 2017). When testing for anti-inflammatory characteristics, the heat induced egg albumin denaturation method is used because it is widely used, validated, sensitive, quick, and reliable method. During the experiment, the temperature is increased from 37°C to 70°C to avoid the formation of irregular clumps caused by protein coagulation when water molecules evaporate from the egg white and thermal denaturation of egg protein (Heendeniya *et al*., 2018), then the mixture is allowed to cool, and the absorbance is read.

6.2 Methods and materials

6.2.1 Cell viability assay

The MTT colorimetric assay, described by Mosmann (1983), was performed with modifications. The cell culture (THP-1 cell line) was maintained in a flask with RPMI 1640 medium (Whitehead scientific) supplemented with 10% foetal bovine serum (FBS) (Adcock-Ingram). Trypan blue was used to dye the cells and an automatic cell counter (model) was used to quantify viable cells. The cells were diluted with the Roswell Park Memorial Institute (RPMI) complete media to obtain 5×10^4 cells/mL cell suspension. In a 50 mL vial, the growing THP-1 culture was diluted to 2×10^5 cell/mL. The THP-1 cells were pre-treated with 25 µL of 100 µg/mL PMA to give a final concentration of 50 ng/µL in the 50 mL vial and the cells were seeded in flat bottom 69 well plates (per well) at 2×10^5 cell/mL for 72 hours in 5% CO_2 at 37°C to induce the maturation of the monocytes into macrophage-like adherent cells. THP-1 cells per well were seeded in 96 well plates to a final volume of 100 µL. It is important to arrest the differentiation of the cell line after 48 hours treatment with PMA to increase macrophage markers (Chanput *et al*., 2014). The spend media was removed after the treatment with PMA and the cells were washed with pre-warmed 1X PBS. Fresh media was added and then incubated for 24 hours (Safar *et al.*, 2019). The stock solutions of the extracts were prepared to a concentration of 250 mg/mL dissolved in dimethyl sulfoxide (DMSO). The extracts were diluted to 1 mg/mL with complete media and a 0.25% of DMSO was maintained. One hundred microliters of the extracts (1000, 500, 100 µg/mL) prepared in a separate 95 well plate was transferred to the plate containing the cell cultures. The microtiter plates were incubated at 37 ºC in a 5% carbon dioxide incubator for 24 hours. Following incubation, 20 µL of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dissolved in 1X PBS was added to each well and the plates were further incubated for 4 hours. After incubation, the media was removed from the plates and 100 µL of DMSO was added to each well. The plates were carefully swirled to dissolve the purple formazan crystals. Purple formazan crystals are formed when MTT is reduced by metabolically active cells. Thus, the amount of formed formazan products produced provides an indication of the number of viable cells. A microtitre plate reader (promega) was used to measure the absorbance of the purple colour at 540 nm. Cells treated with the extracts were compared with untreated cells.

6.2.2 Anti-inflammatory activity

The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of 0.05 M Phosphate buffer (pH 6.6) and 2 mL of varying concentrations (2,1 and 0.5, mg/mL) of the plant extracts and standard drug (aspirin) (2, 1, and 0.5 mg/mL). The product control solution (5 mL) consisted of 3 mL of 0.05 M Phosphate buffer and 2 mL of each extract solution to consider the colour of the extracts. Egg albumin solution (0.2 mL) and 4.8 mL Phosphate buffer were used as the test solution's positive control while for negative control, 0.2 mL of egg albumin, 2.8 mL of 0.05 M Phosphate buffer (pH 6.6) and 2 mL of DMSO were used. The mixtures were incubated at 37 ± 2°C in a biochemical oxygen demand (BOD) incubator for 15 minutes and then heated at 70°C for 5 minutes. After heating, the solutions were allowed to cool at a room temperature for 30 minutes. After cooling, their absorbance was measured using the A 10S UV-VIS spectrophotometer (Thermo Scientific Genesys) at 660 nm. The Phosphate buffer was used as a blank (Uttra and Alamgeer, 2017; Rahman *et al*., 2015). The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\%\ Anti-denaturation = \frac{Absorbance\ of\ control - Absorbance\ of the\ test\ sample}{Absorbance\ of\ control} \times 100$$

*Absorbance of test sample = absorbance of test solution – product control - absorbance of negative.

6.2.3 Statistical analysis

Statistical analysis was performed with the Prism software package (GraphPad Software). The results were analysed using one-way ANOVA, Dunnett's multiple comparisons test ($p < 0.05$). p values lower than 0.05 were considered to be statistically significant.

6.3 Results

6.3.1 Cell viability assay

The plant was assessed for cytotoxicity using the MTT assay on THP-1 macrophages. The cytotoxicity was represented as a percentage cell viability when comparing the plant extract (acetone) and untreated cells. The percentage cell viability for acetone extract ranged from 59% to 49% at 100 µg/mL to 1000 µg/mL. The number of the cells decreased as the concentration increased. 0.25% of DMSO was not toxic to the cells because there was no significant change in cell viability while the plant extracts at different concentrations were toxic to the macrophages (Figure 6.1).

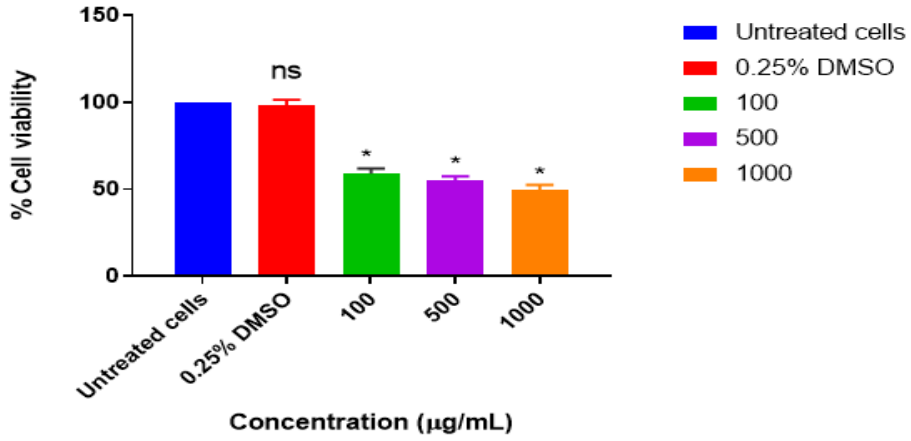

Figure 6. 1: Cytotoxic effect of *Dombeya rotundifolia* (acetone extract) against THP-1 macrophages.

Key: ns - non-significant difference (between untreated cells and DMSO), (*) - significant difference (between concentrations 100, 500 and 1000 µg/mL, and untreated cells).

6.3.2 Anti-inflammatory activity

The anti-inflammatory activity of the plant was determined using the egg albumin protein denaturation assay. The results showed that the inhibition capacity ranged from 85% to 97% for the plant extracts where the dichloromethane extract had the highest inhibition (97.54%), followed by the acetone extract with (97.1%) while the hexane extract had the lowest inhibition capacity of 85.59% (Figure 6.2).

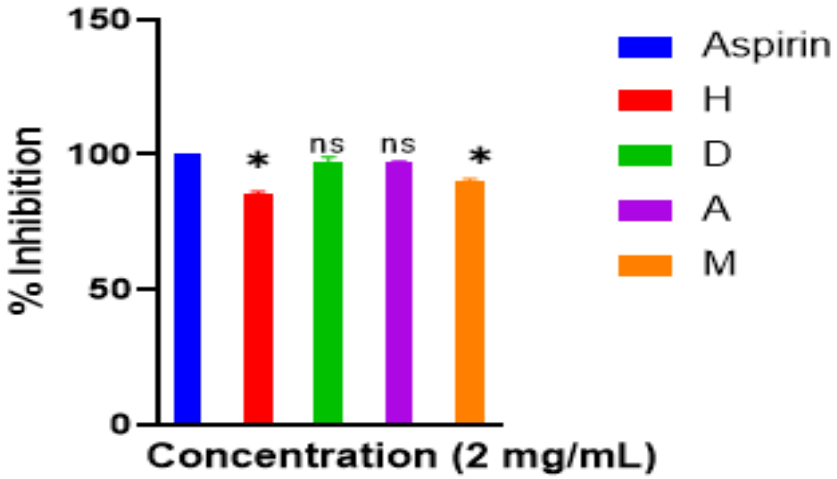

Figure 6.2: Anti-inflammatory activity of *Dombeya rotundifolia* extracts at a concentration of 2 mg/mL.

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol, ns = non-significant difference (aspirin and plant extracts), (*) = significant difference (between aspirin and plant extracts)

6.4 Discussion

The aim was to investigate the cytotoxic and anti-inflammatory effect of *Dombeya rotundifolia*. Many medicinal plants have intriguing therapeutic characteristics and yet are not tested, and their safety and efficacy have not been properly evaluated (Kmail *et al.*, 2015). Human health and life are in danger due to a lack of information on the safety of medicinal plants; hence, the use of in vitro toxicity testing as a method for assessing the safety of various medications and substances has grown significantly (Sharif *et al*., 2017).

The cytotoxic effect of the acetone extract of *D rotundifolia* on THP-1 macrophages at a concentration ranging from 100-1000 µg/mL using MTT assay, which is a colorimetric assay that determines the functional state of mitochondria, indicates cell viability. The percentage cell viability was calculated by measuring the absorbance of the purple colour (formazan), which is formed from the reduction of MTT solution by dehydrogenases in the mitochondria. DMSO was used as a positive control. It is a frequently used solvent in pharmacology and toxicology to improve drug delivery and dissolve a wide range of medications and herbal extracts. It is less toxic at a concentration less than 10% (Sangwen *et al*., 2021).The cell viability is proportional to the reduction of the MTT solution. When concentration increases, cell viability decreases. The results (Figure 6.1) show that the percentage cell viability for the acetone extract ranged from 59% to 49% at 100 µg/mL to 1000 µg/mL. With those percentage ranges, the plant may be toxic to THP-1 macrophages, especially at a higher concentration. Macrophage is one of the innate immune cells that perform first line defence during infection initiates and plays a vital role in immune reactions like phagocytosis (Kim *et al.*, 2019). Depending on the chemical compounds that the plant contains and their quantity, different plants have different threshold doses at which they can cause harm. The degree of toxicity is related to the dose supplied, meaning the more the dose, the more significant the lesions are, if intake is frequent (Mounanga *et al*., 2015). It was found that the plant is toxic to the THP-1 macrophages, which indicates that the plant does not have immunomodulatory properties. Other tests are needed on different cells since some studies proved that the plant is not toxic. Kudumela and Masoko (2017) tested the plant on the African green monkey kidney (Vero) cell using the MTT assay and showed that the plant was not toxic. The statistical analysis using the GraphPad software showed that there is a significant difference

between the treated cells at different concentrations when compared with the untreated cells, while the difference between the DMSO and untreated cells is non-significant. The MTT assay is often performed to evaluate the drug sensitivity and IC50 dose. The IC50 dosage is known as the amount of plant extract at which cell growth can be inhibited by 50% (Paul *et al*., 2015). The extract is considered as nontoxic if the LC_{50} value exceeds the concentration of 0.1 mg/mL, poisonous if the value is less than 0.1 mg/mL and slightly toxic if the value is between 0.1 and 0.5 mg/mL (Dougnon *et al*., 2021). The benefit of the MTT assay is that it is the gold standard method for cytotoxicity testing, but it produces numerous known interferences since the conversion to formazan crystals depends on the metabolic rate and mitochondrial number (Kuete *et al*., 2017).

The anti-inflammatory activity of the plant was determined using the egg albumin protein denaturation assay and aspirin was used as a positive control. The concentrations ranged from 2 to 0.5 mg/mL to represent the percentage inhibition of protein denaturation, but the results showed for the concentration of 2 mg/mL because lower concentrations had an undetectable activity. The results (Figure 6.2) showed that the inhibition capacity ranged from 85% to 97% for the plant extracts where the dichloromethane extract had the highest inhibition (97.54%), followed by the acetone extract (97.1%) while the hexane extract had the lowest inhibition capacity of 85.59%. The statistical analysis using GraphPad software showed that there is significant difference between the hexane and methanol extracts when compared to aspirin, while the difference is non-significant when compared to dichloromethane and acetone extracts. There is a relationship between protein denaturation and the inflammatory drug where a substance that can inhibit the denaturation of protein have the potential to be an anti-inflammatory drug (Osman *et al.*, 2016). The obtained results showed that the plant has the potential to be an anti-inflammatory drug due to the presence of phenolic, tannins and flavonoids in it. Other phytochemicals that contribute to anti-inflammatory drugs in plants are alkaloids, saponins and phytosterols (Oguntibeju, 2018). The results are in support with the studies done on *D. rotundifolia* extracts, such as that of Reid *et al* (2001) on anti-inflammatory using prostaglandin-synthesis inhibitor assay, where ethanol and dichloromethane showed higher anti-inflammatory activity. Another study was done by Kudumela and Masoko (2017) on the effects of *D.*

rotundifolia extract (acetone) on the inhibition of ROS production in LPS-induced RAW 264.7 macrophage cells, where the plant showed anti-inflammatory activity.

6.5 Conclusion

The plant extract of *D. rotundifolia* was toxic to the cells, but some of the cells were able to survive and had higher inflammatory activity. This is important when isolating antimicrobial compound/s from plants that have no or low toxicity to the host cells. Thus, safety is crucial when developing new drugs. More tests are needed before the plant can be used and recommended amounts of the plant are taken. With the promising results from cytotoxicity and antimycobacterial assays, the plant can then be subjected to isolation.

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Chapter 7

7. Bioactivity guided isolation of antibacterial compounds

7.1 Introduction

Natural products have contributed significantly to the development of drugs in the past and hold great a potential for the future. The search is based on a biological and ecological rationale. Many efficient medications have been made from natural products (Malviya and Malviya, 2017). There are numerous secondary metabolites that are found in the medicinal plants that can be utilised to treat or prevent diseases or to improve overall human health and well-being (Mfotie Njoya *et al*., 2014). *Dombeya rotundifolia* is a deciduous single-stemmed tree with modest, irregular-shape canopy and grows around 5-6 m tall. There are two variants of *D. rotundifolia*: *D. rotundifolia var. rotundifolia*, which is found throughout most of the species' distribution range. *D. rotundifolia var. velutina I. Verd* has only been observed in Namibia's Naukluff mountains near Rehoboth. It is primarily used to induce labour and as a natural remedy for fever, chest pains, headache, heart problems, pneumonia, and others. Studies reveal that the plant the plant has the following pharmacological properties: anthelmintic, anti-hypertensive, acetylcholinesterase inhibitory, anti-inflammatory, antibacterial, and antioxidant effects (Maroyi, 2018). To evaluate the medicine made from a plant, plant extracts must be screened, the active chemicals must be isolated and identified, their mechanisms of action must be studied, and the drug must also be proven to be non-toxic to human cells (Mfotie Njoya *et al*., 2014).

Modern and effective methods for isolating bioactive compound include the bioassay-guided assay. This method might speed up the entire process and lower some of the costs (Mroczek *et al*., 2020). The bioassay-guided fractionation involves sequentially separating extracted compounds based on differences in their physicochemical properties and evaluating the biological activity. It is a common protocol for isolating a pure chemical agent from a natural source. During the fractionation process, each fraction that is generated is tested in a bioassay system, and only those that exhibit activity are further fractionated (Malviya and Malviya, 2017).

Serial exhaustive extraction is a technique that comprises a sequential extraction with solvents of increasing polarity, moving from a non-polar, e.g., hexane to a more polar solvent to ensure that a wide range of polarity of compounds could be extracted (Pandey and Tripathi, 2014). It was created to prepare crude extracts and a liquid-liquid partition, which is used to isolate active compounds (Ngouana *et al*., 2021). Based on how the solvent flows down the column, column chromatography is divided into two groups, namely, gravity column and flash chromatography. Gravity column chromatography is the process of allowing the solvent to flow down the column by gravity or percolation while flash is the process in which a solvent is pushed down in the column by positive air pressure (Ayare *et al*., 2014). The purification of organic synthesised products such as those produced in the process of discovering new drugs or from natural extracts, can be accomplished quickly and affordably using flash chromatography (Bhusal *et al*., 2017).

In column chromatography, the mixture is loaded into a column that is filled with finely powdered adsorbent, and the mixture is then developed with a solvent followed by eluting the separated components in the mixture by passing a solvent through the column (Bhore and Khanvilkar, 2019). One of the most popular and important organic chemistry purification methods is preparative chromatography and is used in laboratories all over the world (Primdahl *et al*., 2022). On a small semi-preparative scale, preparative TLC can be used to separate combinations containing up to a few hundred milligrams. Instead of “spotting” the mixture as spots on the TLC plate, the mixture is placed to the plate as a thin and even layer that extends to and just above the solvent level. Instead of horizontally separated spots, the compounds separate in horizontal bands when developed with a solvent. The desired band is scraped off one at a time (Bele and Khale, 2011).

7.2 Method and materials

7.2.1 Serial exhaustive extraction

The serial exhaustive extraction was used to extract bioactive compounds from the *Dombeya rotundifolia* plant material since it had promising antimycobacterial activity. About 1 kg of the plant material was weighed and dissolved into 5 litres of n-hexane in a bottle. The bottle was vigorously shaken for over a night at the speed of 200 rpm. The supernatant was filtered, concentrated using rotary evaporator at 67 °C, and transferred into pre-weighed labelled beakers. The same process was repeated two times at different time intervals of 3 hours and 1 hour, respectively. The same plant's residues were extracted as mentioned above in the following order with 5 litres of dichloromethane, acetone, and methanol. Solvents were evaporated at room temperature under the fan and the masses of the crude extracts were determined.

7.2.2 Phytochemical analysis

The chemical profiles of *D. rotundifolia* extracts were analysed on aluminium backed TLC plates (Merck, silica gel 60 F254) using a method developed by Kotze and Eloff (2002), as described in section 3.2.3.

7.2.3 TLC-DPPH assay

The qualitative DPPH assay was done using TLC according to the method described by Deby and Margotteaux (1970), as explained in section 4.2.1.

7.2.4 Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972), as described in section 5.2.2.

7.2.5 Broth micro-dilution assay

The broth micro-dilution method described by Eloff (1998) was used to determine the MIC values of crude extracts against *M. smegmatis,* as explained in section 5.2.3.

7.2.6 Isolation of antimycobacterial compounds

7.2.6.1 Open column chromatography

Column chromatography was used for isolation, separation, and purification of the active compounds with antibacterial activities from the dichloromethane (D1-3) and acetone (A1-3) extracts. An open column (47 cm height * 3 cm radius) was packed with silica gel 60 (particles size 0.063 - 0.200 mm) (Fluka) using 100% n-hexane. The

extracts (21.26 g) were mixed with small amounts of silica gel and subjected to column chromatography. The constituents of the extracts were eluted through an open column using 1.2 L of the solvent systems in Table 7.1 from non-polar (hexane) to polar (methanol). The fractions were collected and concentrated using a rotary evaporator. The solvents were evaporated under a fan at room temperature and the masses of the crude extracts were determined. The fractions were then tested for antioxidant and antibacterial activity using TLC-DPPH (Section 7.2.3), bioautography (Section 7.2.4) and serial broth micro-dilution (Section 7.2.5)

Table 7. 1: Solvent systems used in the first column.

Elution solvent	Percentage (%)
Hexane	100
Hexane: Ethyl acetate	90:10
	80:20
	70:30
	50:50
	30:70
	10:90
Ethyl acetate	100
Ethyl acetate: Methanol	90:10
	80:20
	70:30
	50:50
	30:70
	10:90
Methanol	100

7.2.6.2 Second open column chromatography

The biological activities of the fractions obtained from the first column revealed that the hexane: ethyl acetate (70:30 and 50:50) in the BEA mobile phase, had potential antibacterial activities. Therefore, they were chosen for further separation and purification of the bioactive compounds. The combined fractions were subjected to an open column chromatography (60 * 2 cm) packed with silica gel 60. The column was

eluted with 80% hexane: 20% ethyl acetate. The eluents were collected in small test tubes and placed under the fan to concentrate. The phytochemical profile of the fractions was analysed on the TLC (Section 7.2.2), and those with similar chemical profiles were combined.

7.2.6.3 Preparative TLC

The sub-fractions from the test tube 6(142-151) and 7(154-178) from the second open column chromatography were further separated on TLC silica gel glass plates (Merck Silica gel 60 F254) using chloroform as mobile system. To detect UV reactive bands, the plates were visualised under UV light at 360 and 254 nm to locate the bands on the TLC plates bands and the compounds of interest were scraped off from the developed TLC plates. To separate the compound from the silica, the compound was immersed separately in ethyl acetate and filtered using cotton wool. The compounds were analysed for antimycobacterial activity using bioautography and broth micro-dilution assay, with *M. smegmatis* as the test organism.

7.3 Results

7.3.1 Serial exhaustive extraction

The serial exhaustive extraction of *D. rotundifolia* (1kg) with different solvents (hexane, dichloromethane, acetone, and methanol) which yield total mass of 137.77 g (Table 7.2) was done. Methanol was the best extractant which extracted 98.29 g, followed by hexane (18.21 g) while acetone extracted the least (9.88 g).

Table 7. 2: The quantity of plant material extracted from *Dombeya rotundifolia*.

Extracts		Mass residue (g)	
		Mass	Total
Hexane	H1	13.62	18.21
	H2	3.15	
	H3	1.44	
Dichloromethane	D1	4.97	11.39
	D2	2.99	
	D3	3.43	
Acetone	A1	1.88	9.88
	A2	5.46	
	A3	2.54	
Methanol	M1	70.27	98.29
	M2	20.04	
	M3	7.98	
Total			137.77

7.3.2 Phytochemical analysis on TLC plates

TLC was used to analyse the phytochemical constituents in the plant (*Dombeya rotundifolia*). Fluorescencing compounds were observed under the UV light at 254 nm (Figure 7.1) and 365 nm (Figure 7.2), and then the plate was sprayed with Vanillin-sulphuric acid to visualise the bands that were not visible under the UV light (Figure 7.3) All compounds were present in all mobile systems, but there was a clear separation in the following extracts: hexane, dichloromethane, and acetone (Figure 7.3).

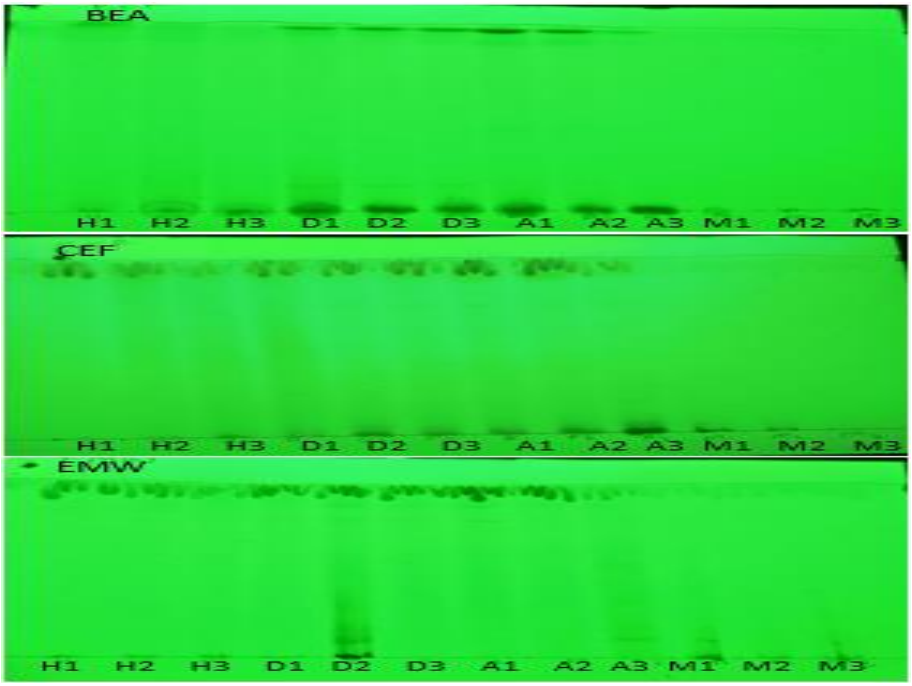

Figure 7. 1: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and observed under the UV light of 254 nm.

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

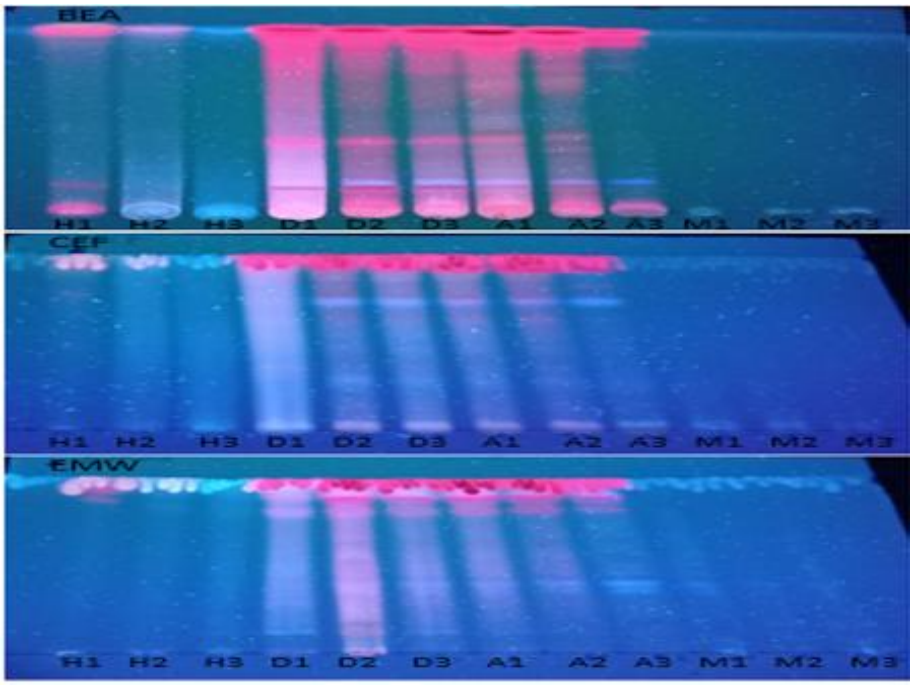

Figure 7. 2: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and observed under the UV light of 365 nm.

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

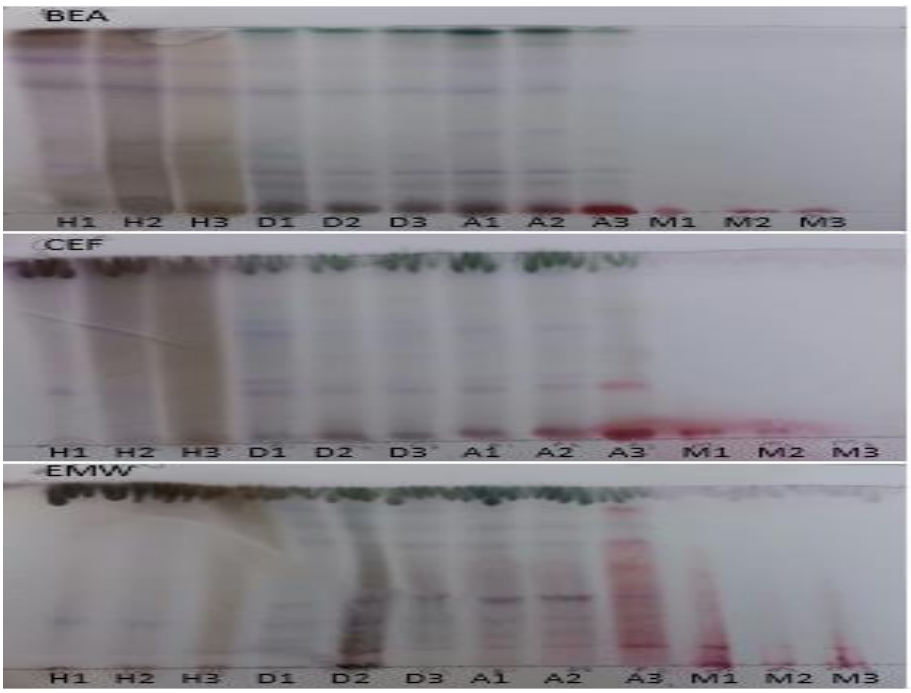

Figure 7. 3: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C.

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

7.3.3 Antioxidant activity assay

7.3.3.1 Qualitative antioxidant activity assay

The antioxidant activity of the plant was screened using DPPH on the TLC plates developed in different separation systems. The yellow spots on the purple background indicated the presence of antioxidant compounds. Figure 7.4 shows that the plant extracts have antioxidant compounds, but they separate well in the EMW and CEF mobile systems. The following extracts: dichloromethane, acetone and methanol, have antioxidant compounds in all the mobile systems.

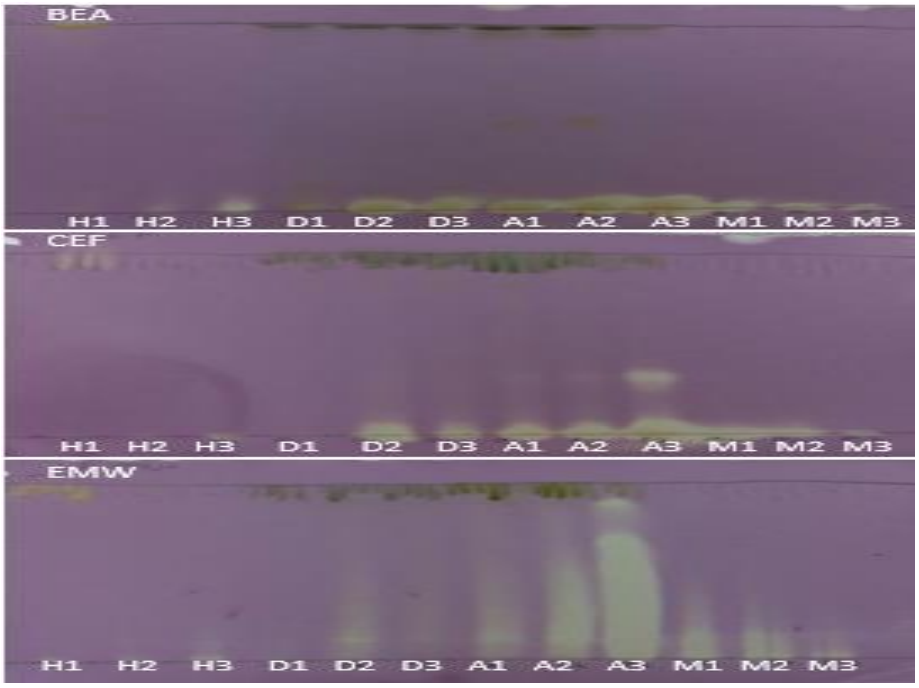

Figure 7. 4: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and sprayed with 0.2% DPPH.

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

7.3.4 Antibacterial activity assays

7.3.4.1 Bioautography

The Bioautography technique was used to screen for antimycobacterial activity and INT was used as an indicator. After incubation time, the pinkish colour was developed, which indicates the viability of the microorganism while the white colour represents the zone of inhibition. Chromatograms (Figure 7.5) showed the presence of antimycobacterial activity in BEA and EMW systems, which is indicated by the zone of inhibition (white colour).

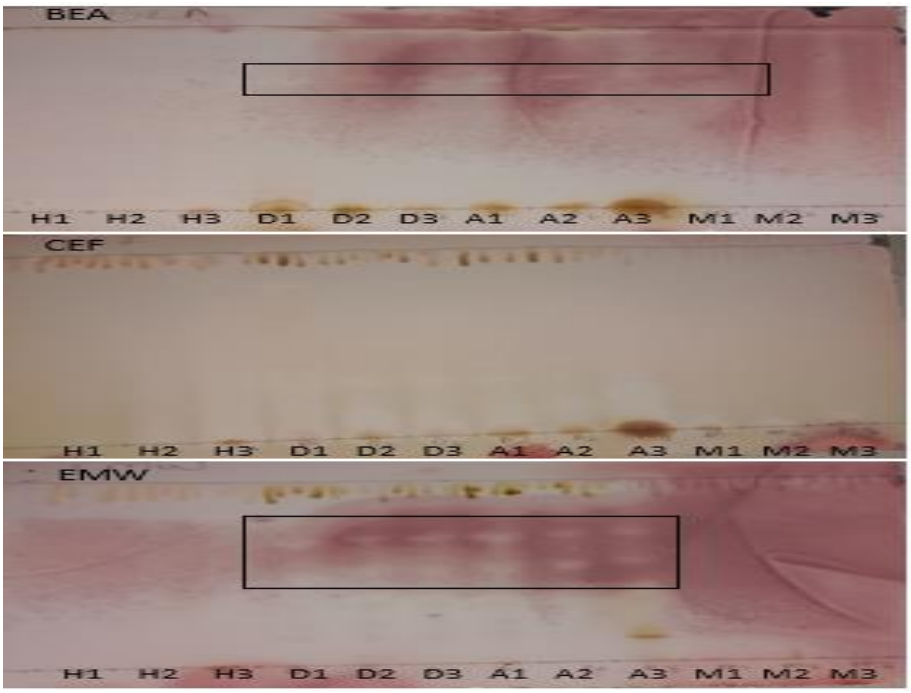

Figure 7. 5: The chromatograms of *Dombeya rotundifolia* plant extracts separated in BEA, CEF and EMW and sprayed with *M. smegmatis.*

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

7.3.4.2 Micro-dilution assay

The MIC values were determined using the serial broth dilution microplate method. The results (Table 7.3) showed that the plant had antimycobacterial activity, the extract that had the lowest MIC value was acetone 1 with the MIC value of 0.21 mg/mL and the extracts that had the highest MIC value were methanol and hexane 1 and 2.

Table 7. 3: The MIC at which the plant extracts inhibited the growth of *M. smegmatis* after 24 hours of incubation.

Antimycobacterial activity of *Dombeya rotundifolia*												
MIC (mg/mL)	H1 >2.5	H2 >2.5	H3 0.52	D1 0.26	D2 0.26	D3 0.42	A1 0.21	A2 0.42	A3 0.42	M1 >2.5	M2 >2.5	M3 >2.5

Key: H- Hexane, D- Dichloromethane, A- Acetone, M-Methanol

Rifampicin (positive control) = 0.08mg/mL, Acetone (negative control) = greater than 2.5 mg/mL

7.3.5 Isolation of active compounds

7.3.5.1 First open column chromatography

7.3.5.1.1 The masses of the collected fractions

The dichloromethane (D1-3) and acetone (A1-3) extracts resulting from the serial exhaustive extraction exhibited a high antibacterial activity and were therefore selected for the isolation of antibacterial compounds. The combined extracts (21.27 g) were subjected first to open column chromatography where they were eluted with solvents of varying percentages. The total mass of 16.9 g was obtained from the fractions. The plant extract that extracted the highest mass was 70% Ethyl acetate: 30% Methanol with 3 g followed by 50% Hexane: 50% Ethyl acetate with 2.95 g while 90% Hexane: 10% Ethyl acetate extracted the least (0.08 g) (Table 7.4).

Table 7. 4: The masses (g) of the fractions from fractionation of a combination of dichloromethane (D1-3) and acetone (A1-3).

Elution solvent	Percentage (%)	Mass (g)
Hexane	100	0.29
Hexane: Ethyl acetate	90:10	0.08
	80:20	0.33
	70:30	1.26
	50:50	2.95
	30:70	0.84
	10:90	0.95
Ethyl acetate	100	0.93
Ethyl acetate: Methanol	90:10	0.33
	80:20	1.68
	70:30	3
	50:50	1.08
	30:70	1.76
	10:90	1.02
Methanol	100	0.4
Total		16.9

7.3.5.1.2 Phytochemical analysis on TLC plates

TLC was used to analyse the phytochemical constituents in the plant (*Dombeya rotundifolia*). The fluorescencing compounds were observed under the UV light at 254 nm (Figure 7.6) and 365 nm (Figure 7.7), and then the plate was sprayed with Vanillin-sulphuric acid to visualise the bands that were not visible under UV (Figure 7.8).

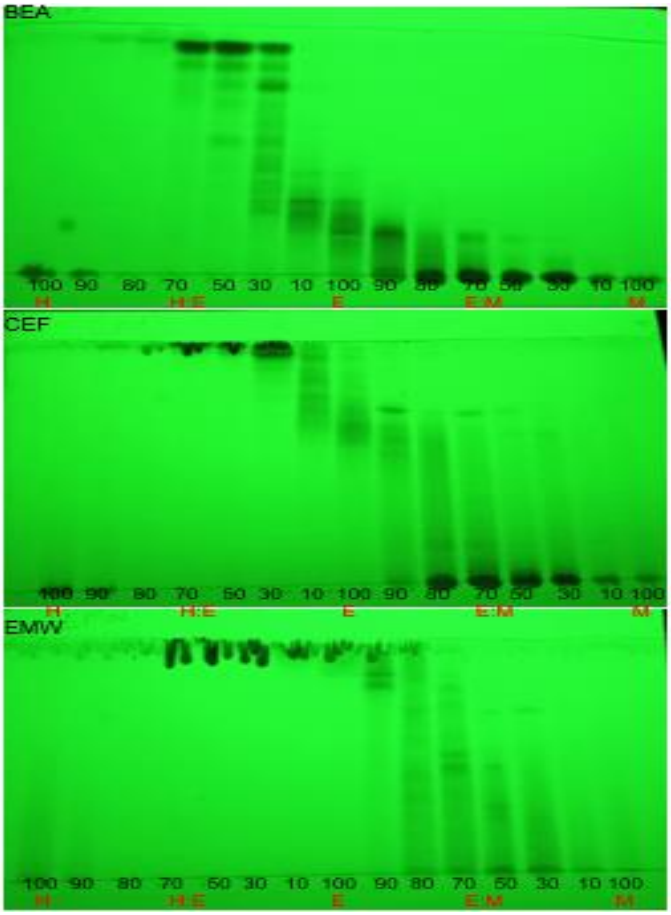

Figure 7. 6: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and observed under the UV light of 254 nm.

Key: H- Hexane, H: E- Hexane: Ethyl acetate, E- Ethyl acetate, E: M- Ethyl acetate: Methanol, M- Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

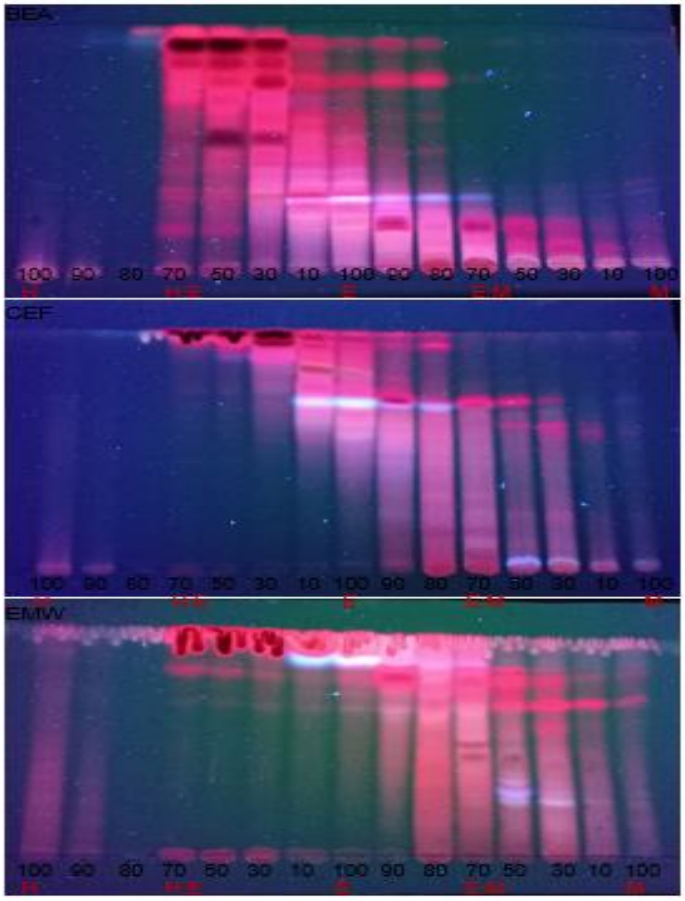

Figure 7. 7: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and observed under the UV light of 365 nm.

Key: H- Hexane, H: E- Hexane: Ethyl acetate, E- Ethyl acetate, E: M- Ethyl acetate: Methanol, M- Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

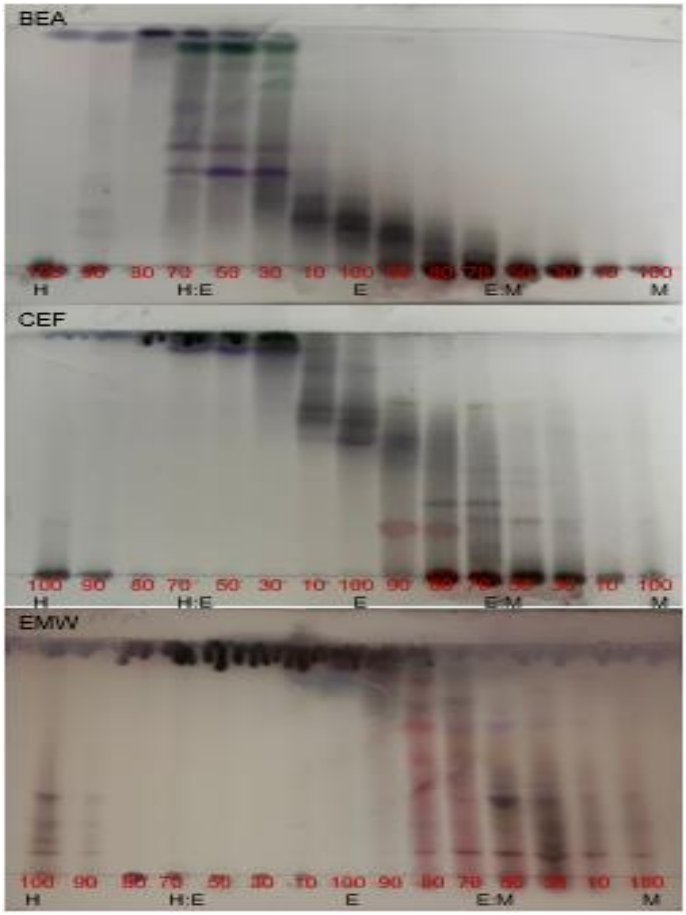

Figure 7. 8: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C.

Key: H- Hexane, H: E- Hexane: Ethyl acetate, E- Ethyl acetate, E: M- Ethyl acetate: Methanol, M- Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

7.3.5.1.3 Antioxidant activity assay

7.3.5.1.3.1 Qualitative antioxidant activity assay

The antioxidant activity of the plant was screened using DPPH on the TLC plates developed in different separation systems. The yellow spots on the purple background indicated the presence of antioxidant compounds. The plant extracts had antioxidant compounds for E: M- Ethyl acetate: Methanol (90 to 30) in all the mobile systems (Figure 7.9).

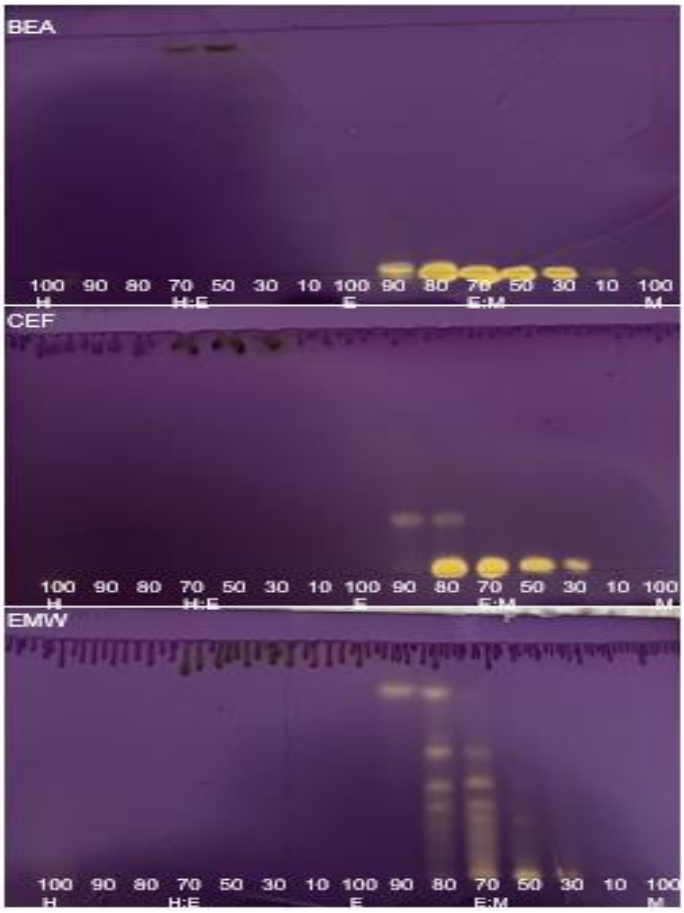

Figure 7. 9: Chromatograms of *Dombeya rotundifolia* developed in BEA, CEF and EMW and sprayed with 0.2% DPPH.

Key: H- Hexane, H: E- Hexane: Ethyl acetate, E- Ethyl acetate, E: M- Ethyl acetate: Methanol, M- Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

7.3.5.1.4 Antibacterial activity assays

7.3.5.1.4.1 Bioautography

The Bioautography technique was used to screen for antimycobacterial activity and INT was used as an indicator. Chromatograms showed the presence of antimycobacterial activity in BEA and CEF systems, which is indicated by the zone of inhibition (white colour) (Figure 7.10).

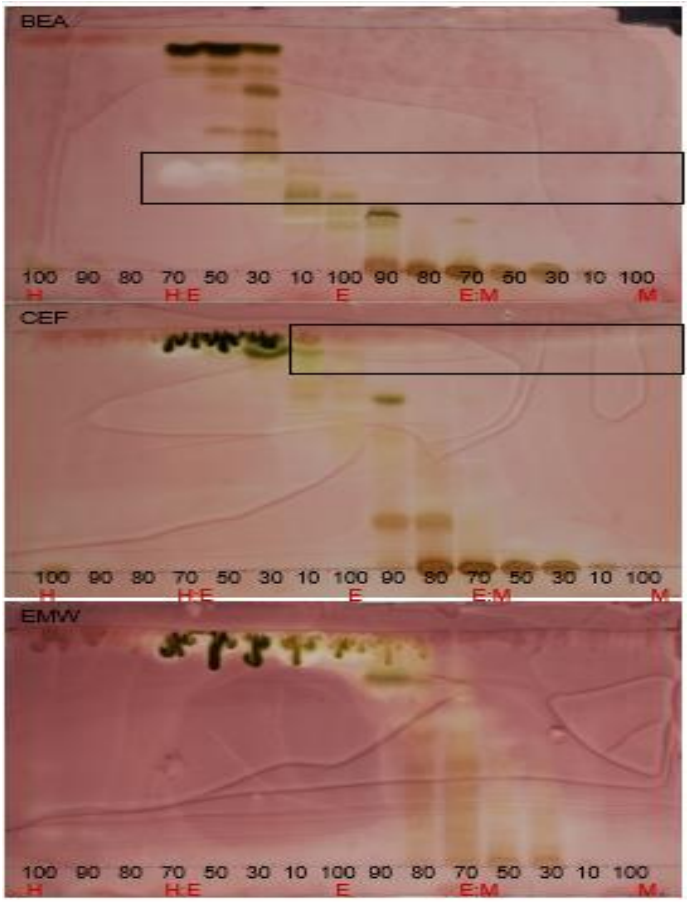

Figure 7. 10: The chromatograms *of Dombeya rotundifolia* plant extracts separated in BEA, CEF and EMW and sprayed with *M. smegmatis.*

Key: H- Hexane, H: E- Hexane: Ethyl acetate, E- Ethyl acetate, E: M- Ethyl acetate: Methanol, M- Methanol, EMW- Ethyl acetate: Methanol: Water, CEF- Chloroform: Ethyl acetate: Formic acid and BEA- Benzene: Ethanol: Ammonium hydroxide.

7.3.5.1.4.2 Micro-dilution assay

The MIC values were determined using the serial broth dilution microplate method. The extracts that had the lowest MIC value were 70% Hexane: 30% Ethyl acetate, 50% Hexane: 50% Ethyl acetate, 30% Hexane: 70% Ethyl acetate with MIC value of 0.26 mg/mL while 100% Hexane, 90% Hexane: 10% Ethyl acetate, 80% Hexane: 20%

Ethyl acetate,10% Ethyl acetate: 10% Methanol and 100% Methanol had the highest MIC, which is greater than 2.5 mg/mL (Table 7.5).

Table 7. 5: The MIC (mg/mL) of fractions from the first column chromatography against *M. smegmatis*.

Antimycobacterial activity of *Dombeya rotundifolia*															
MIC (mg/mL)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	>2.5	>2.5	>2.5	0.26	0.26	0.26	0.52	0.42	0.52	0.52	0.63	0.63	0.63	>2.5	>2.5

Key:1= 100% Hexane, 2= 90% Hexane: 10% Ethyl acetate, 3= 80% Hexane: 20% Ethyl acetate, 4= 70% Hexane: 30% Ethyl acetate, 5= 50% Hexane: 50% Ethyl acetate, 6= 30% Hexane: 70% Ethyl acetate, 7= 10% Hexane: 90% Ethyl acetate, 8= 100% Ethyl acetate, 9= 90% Ethyl acetate: 10% Methanol, 10= 80% Ethyl acetate: 20% Methanol,11= 70% Ethyl acetate: 30% Methanol, 12= 50% Ethyl acetate: 50% Methanol, 13= 30% Ethyl acetate: 70% Methanol, 14= 10% Ethyl acetate: 10% Methanol and 15= 100% Methanol.

Rifampicin (positive control) = 0.08mg/mL, Acetone (negative control) = greater than 2.5 mg/mL

7.3.5.2 Determination of solvent system for second column

The results from the first column chromatography showed that Hexane: Ethyl acetate (70: 30 and 50:50) had higher antibacterial activity and they were selected for further analysis. The plates were developed in different mobile systems to determine the best solvent system to use when running the second column chromatography and 80% Hexane: 20% Ethyl acetate was the best system. The fluorescencing compounds were observed under the UV light at 254 nm (Figure 7.11) and 365 nm (Figure 7.12), and then the plate was sprayed with Vanillin-sulphuric acid to visualise the bands that were not visible under the UV light (Figure 7.13).

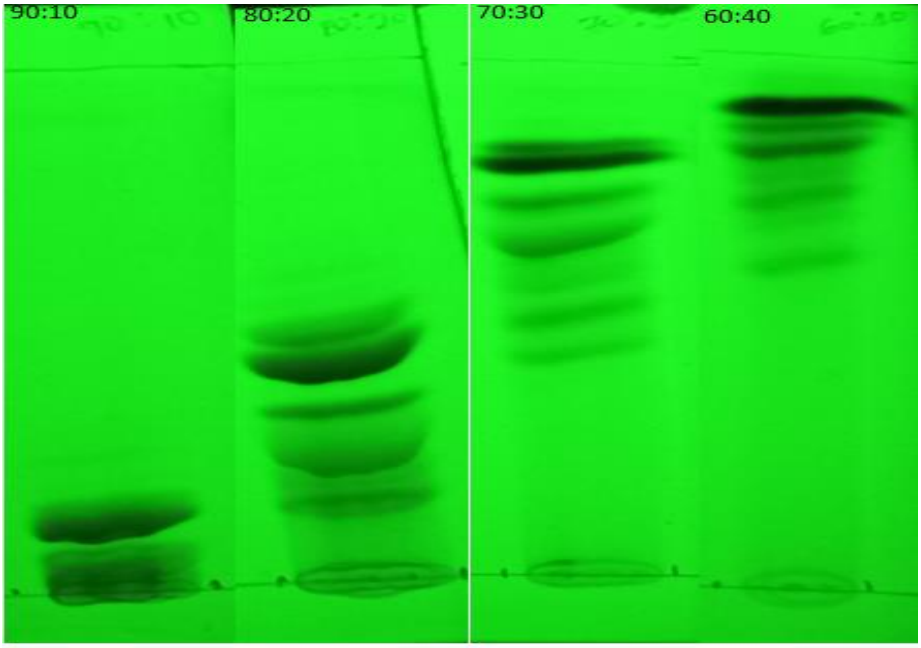

Figure 7. 11: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate and observed under the UV light of 254 nm.

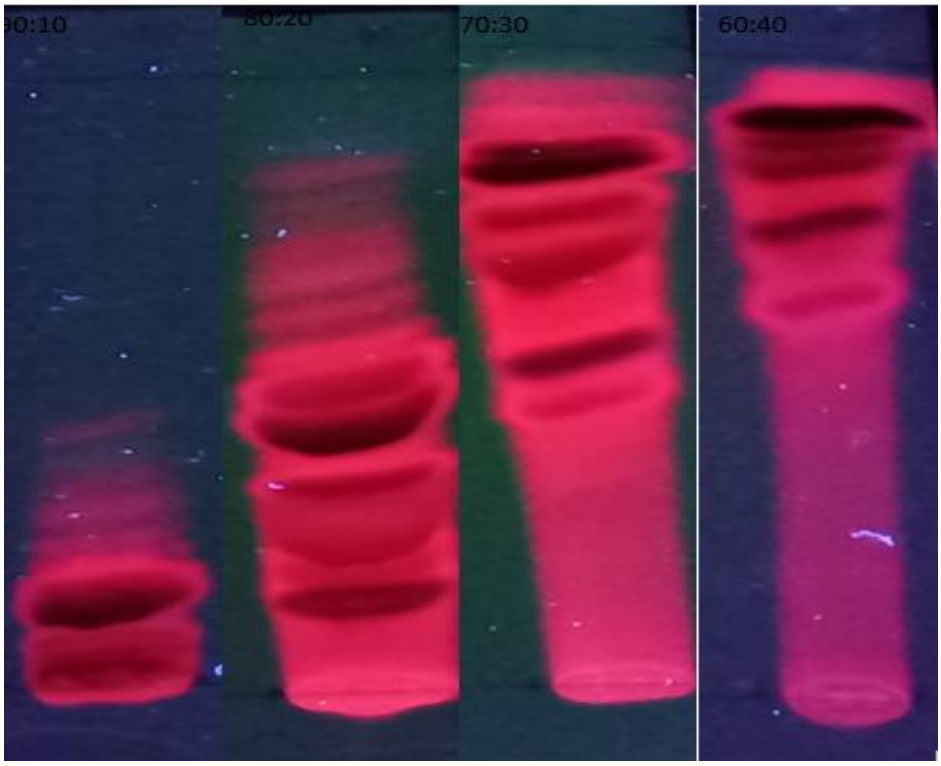

Figure 7. 12: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate and observed under the UV light of 365 nm.

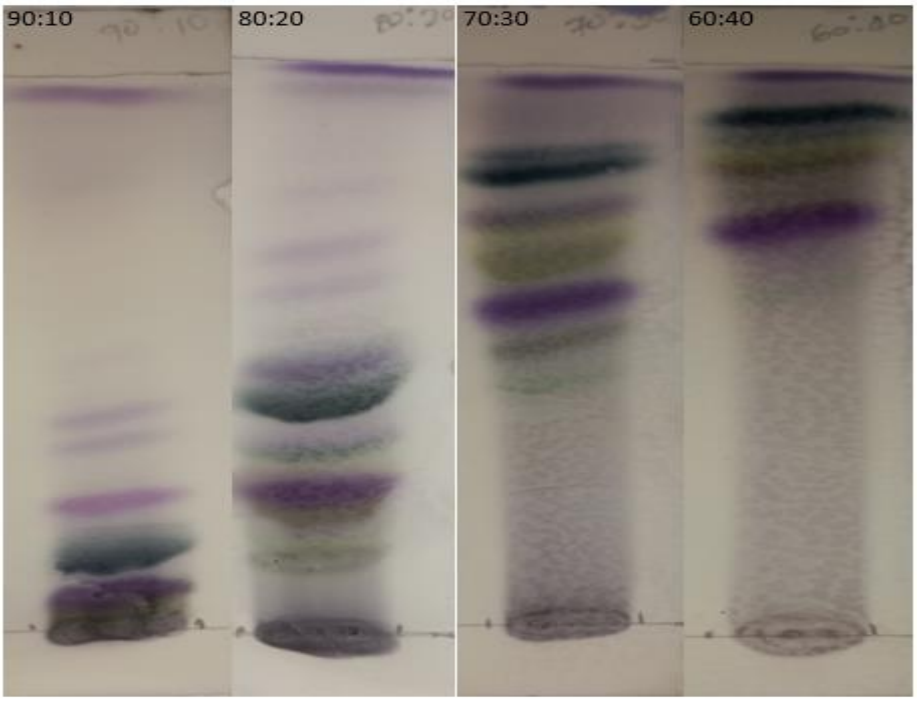

Figure 7. 13: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate, and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C.

7.3.5.3 Second column

Fraction (70% hexane: 30% ethyl acetate and 50% hexane: 50% ethyl acetate) were subjected to second column chromatography, eluted with 80% hexane: 20% ethyl acetate and collected in tests tubes (540). After evaporating the solvents under the fan, the fractions were spotted on TLC, observed under the UV at 254nm (Figure 7.14) and 365 nm (Figure 7.15) and sprayed with vanillin sulphuric acid (Figure 7.16) to determine their profile.

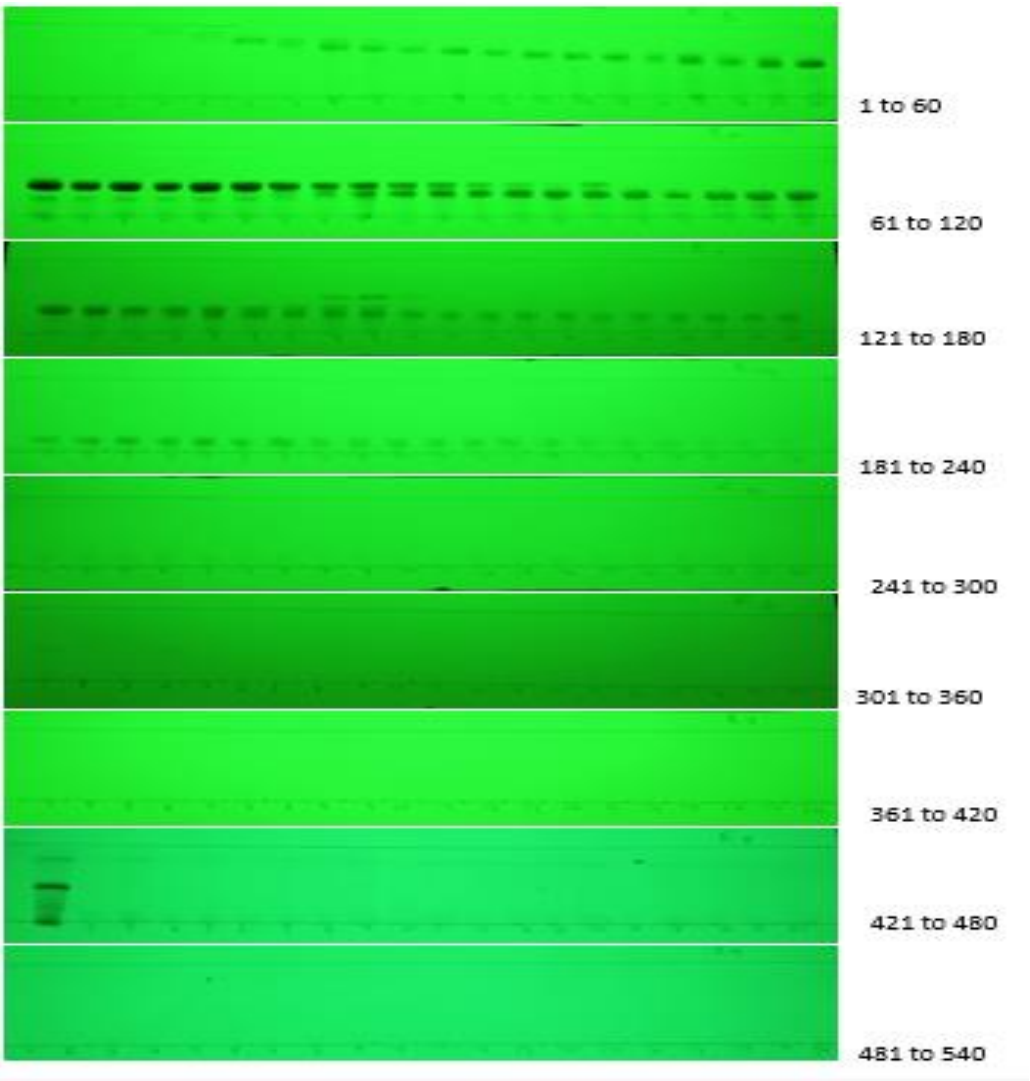

Figure 7. 14: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate (80:20) and observed under the UV light of 254 nm.

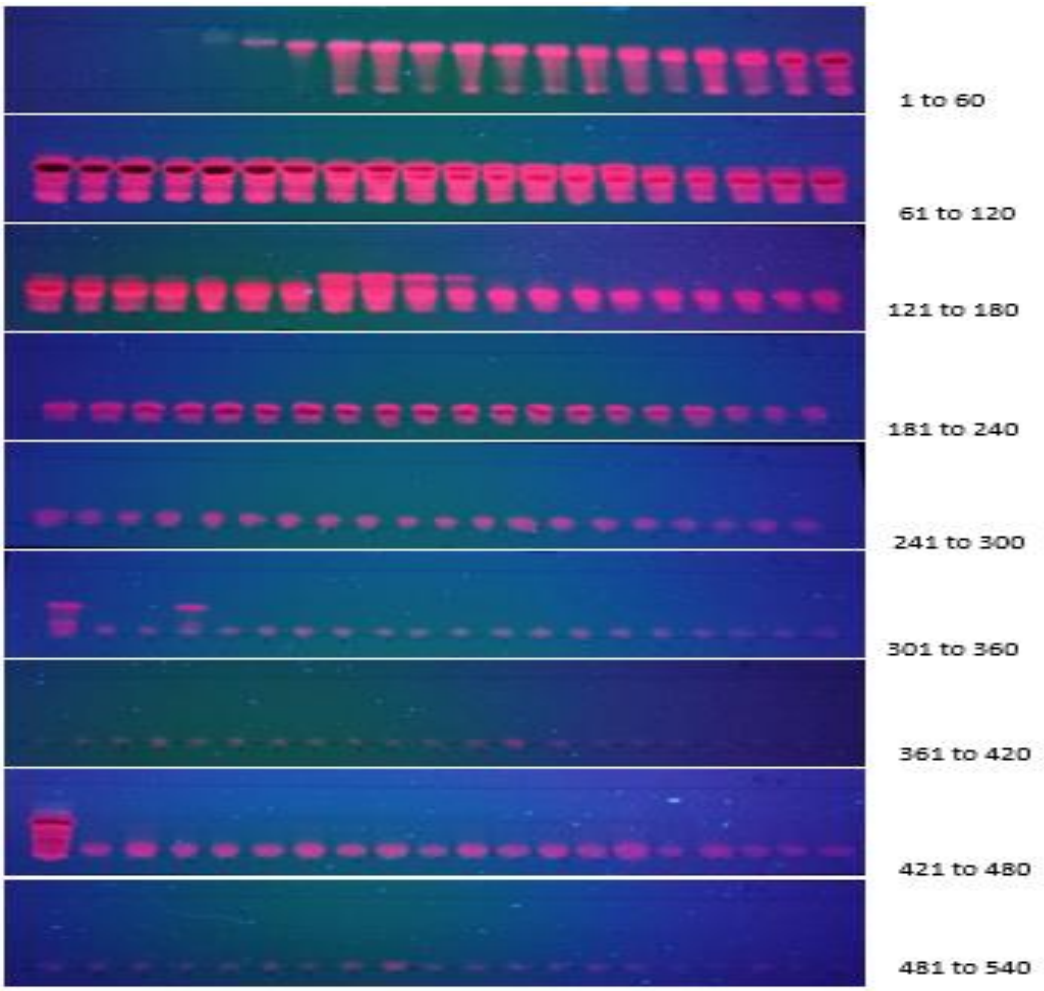

Figure 7. 15: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate (80:20) and observed under the UV light of 365 nm.

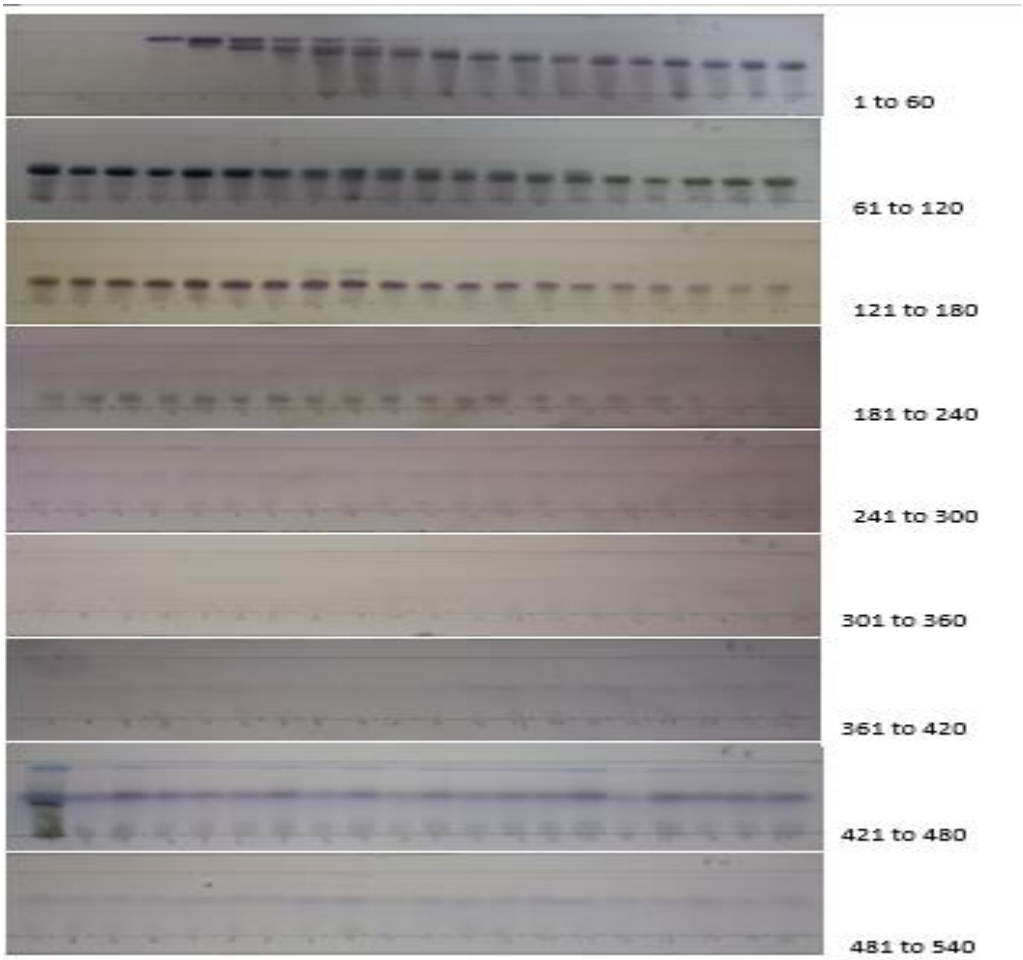

Figure 7. 16: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate (80:20), and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C.

7.3.5.4 Combination of fraction

The fractions with similar profiles were combined to give a total fraction of 13. The groups were 1(4), 2(13-31), 3(37-58), 4(61-120), 5(121-139), 6(142-151), 7(154-178), 8(181-240), 9(241-300), 10(313-360), 11(373-420), 12(424-480) and 13(481-540). The fractions were spotted on TLC, observed under the UV light at 254nm (Figure 7.17) and 365 nm (Figure 7.18) and sprayed with vanillin sulphuric acid (Figure 7.19) to determine their profile.

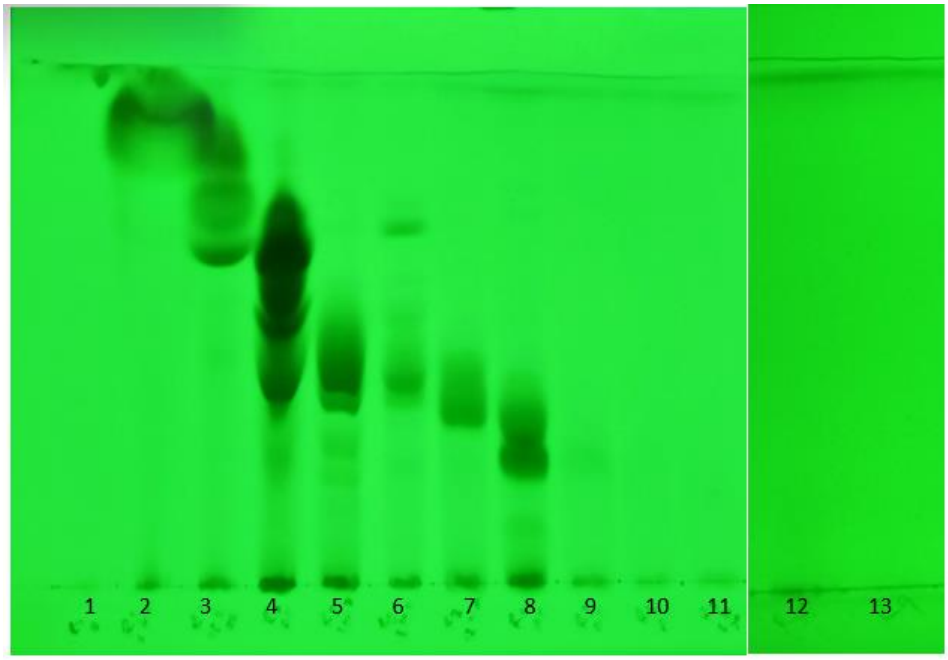

Figure 7. 17: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate (80:20) and observed under the UV light of 254 nm.

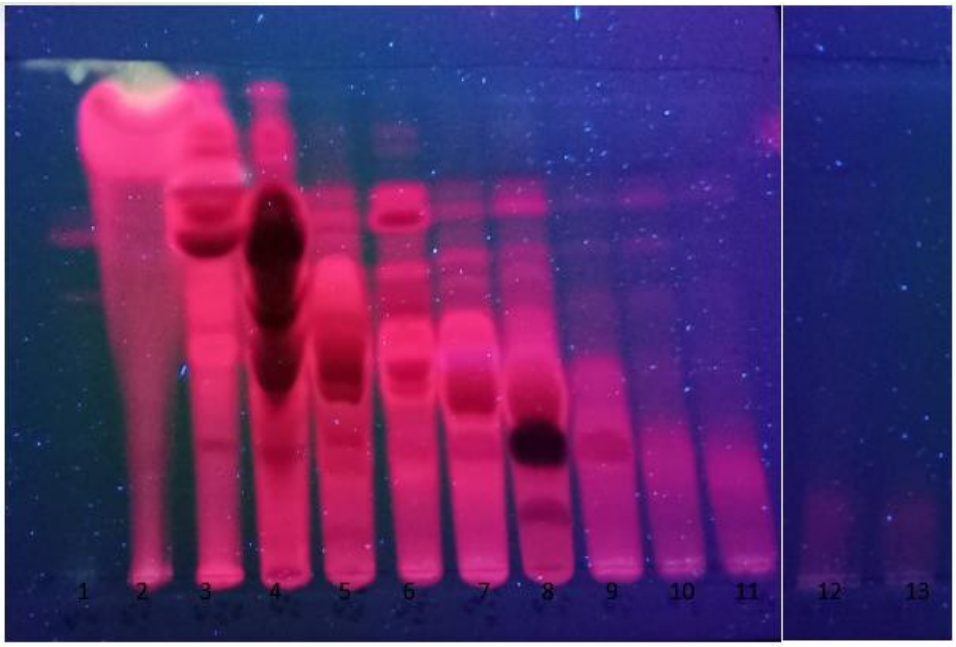

Figure 7. 18: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate (80:20) and observed under the UV light of 365 nm.

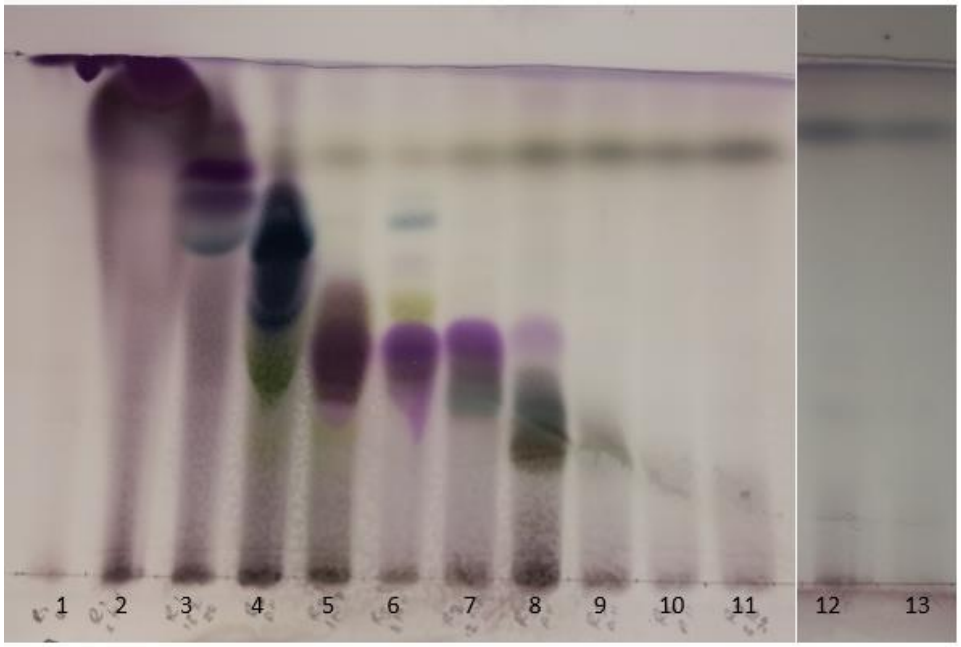

Figure 7. 19: Chromatograms of *Dombeya rotundifolia* developed in Hexane and Ethyl acetate (80:20), and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C.

7.3.5.5 Determination of solvent system for preparative TLC

The results obtained from combination fractions showed that fraction 6, 7 and 8 had a similar profile and were selected for preparative TLC. The plates were developed in different mobile systems, namely, chloroform and dichloromethane, to determine the best solvent system. Dichloromethane was the best system (Figure 7.21).

Chloroform

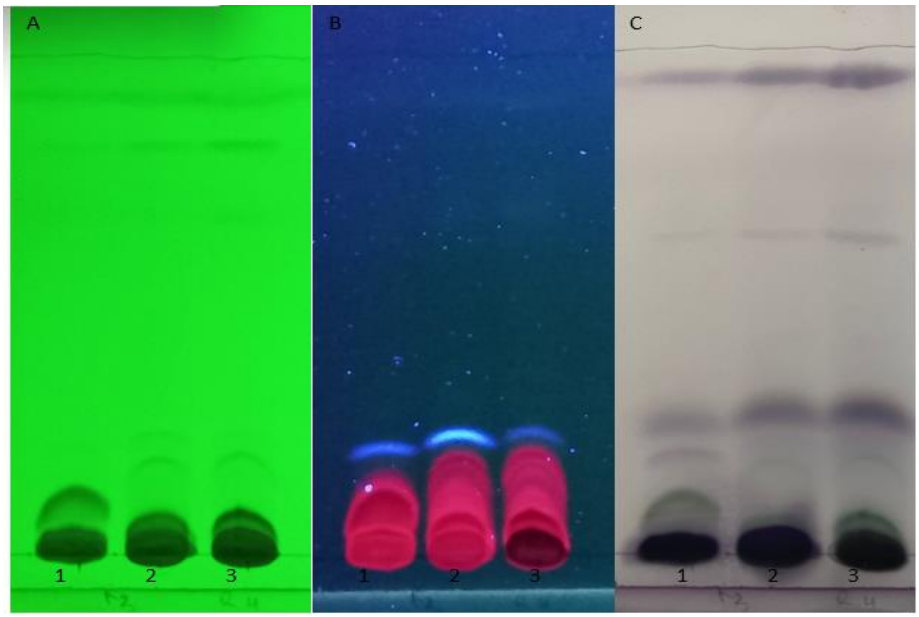

Figure 7. 20: Chromatograms of *Dombeya rotundifolia* developed in Chloroform and was observed under the UV light of 254 nm (A), 365 nm (B) and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C (C).

Dichloromethane

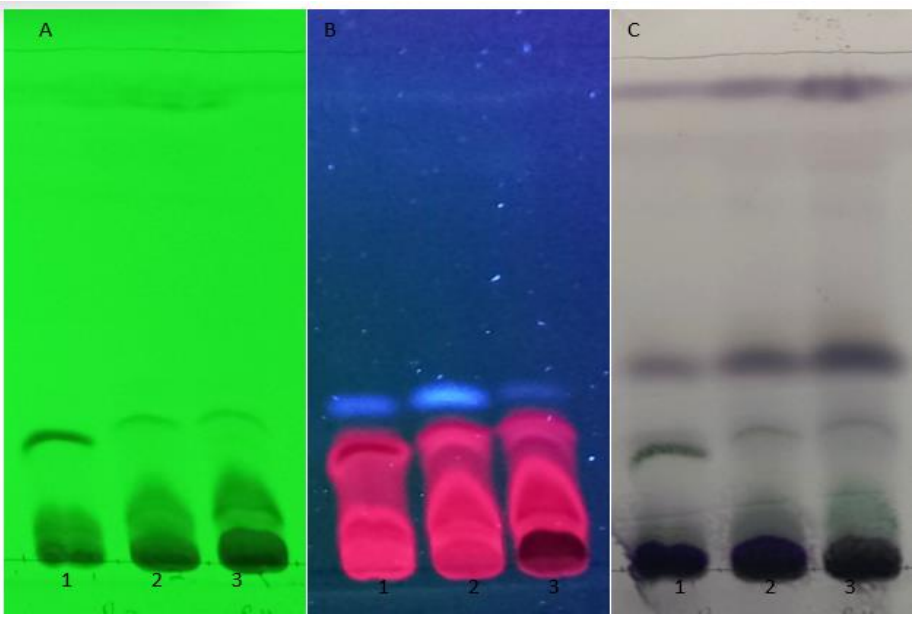

Figure 7. 21: Chromatograms of *Dombeya rotundifolia* developed in Dichloromethane and was observed under the UV light of 254 nm (A), 365 nm (B) and sprayed with vanillin-sulphuric acid reagent then heated in the oven at 110°C (C).

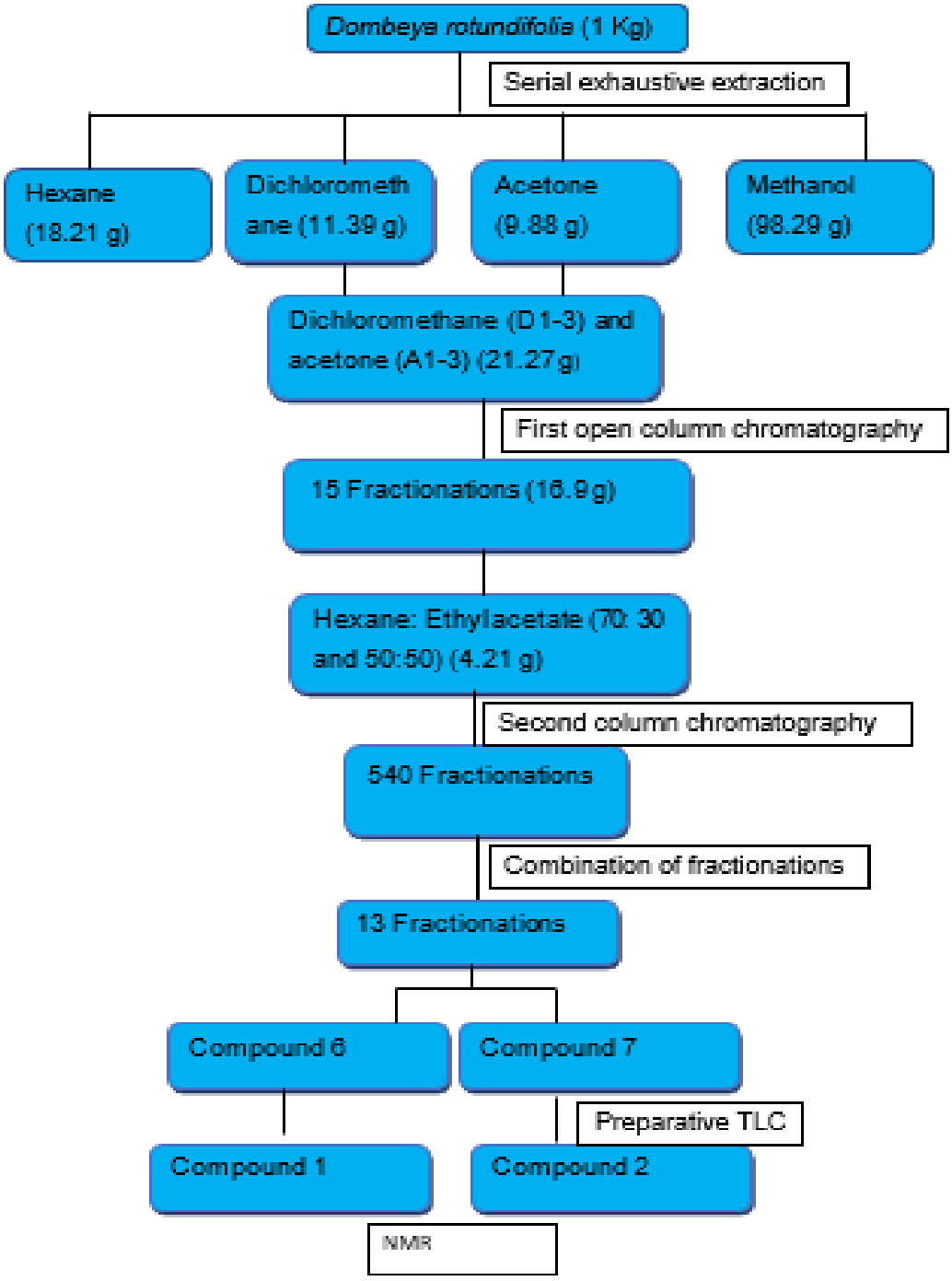

Figure 7. 22: A schematic diagram of the summary of isolation process.

7.4 Discussion

The aim was to isolate and purify compounds with antimycobacterial activity from *Dombeya rotundifolia.* Drugs should be standardised so that their identification, quality, and purity can be determined. The references provided in pharmacopoeia are used to determine the validity, quality, and purity of herbal medications. The pharmacopeia prescriptions (numerical value) such as structural, analytical, and physical standards for medications (Pandey and Tripathi, 2014). Due to their greater safety compared to conventional medications, plant-derived medications are frequently used. The development of new and innovative drugs relied heavily on medicinal plants, which were viewed as valuable and highly beneficial natural resources. Medicinal plants were known for treating numerous diseases brought by microorganisms (Abdallah *et al*., 2021). Anti-infectives like vincristine, vinblastine, aspirin, cocaine, digitoxin, quinine, and morphine, some of which are still used today, were isolated through the drug discovery process from medicinal plants (Adefuye and Ndip, 2013).

The isolation and purification of compounds with antimycobacterial activity from *Dombeya rotundifolia* were achieved using Bioassay-guided fractionation, which is based on bioassay tests. The Bioassay-guided fractionation method is used to identify the bioactive chemicals in plant extracts and evaluating them for possible use as novel bio-based medicines. The fractionation procedure is used to screen natural products in the plant extracts better (Abdallah *et al*., 2021). The serial exhaustive extraction was used to extract bioactive compounds from *Dombeya rotundifolia* plant material since it had promising antimycobacterial activity. The serial exhaustive extraction was done using different solvents (hexane, dichloromethane, acetone, and methanol) with different polarity. Table 7.2 shows masses extracted where methanol was the best extractant, which extracted 98.29 g, followed by hexane (18.21 g) while acetone extracted the least (9.88 g). To separate the desired natural products from the raw materials, extraction is the first step. The extraction efficiency rises as the extraction duration is increased (Zhang *et al*., 2018). In this method, extraction took place in different time intervals to ensure that bioactive compounds were extracted well. In the phytochemical analysis of TLC plates, fluorescencing compounds were observed under the UV light at 254 nm (Figure 7.1) and 365 nm (Figure 7.2), and then the plate was sprayed with Vanillin-sulphuric acid to visualise the bands that were not visible

under the UV light (Figure 7.3). All compounds were present in all mobile systems, but there was a clear separation in the following extracts: hexane, dichloromethane, and acetone (Figure 7.3). The antioxidant analysis using DPPH on the TLC plates (Figure 7.4) and the yellow spots on the purple background indicated the presence of antioxidant compounds. The results showed that the plant extracts have antioxidant compounds, but they separated well in the EMW and CEF mobile systems. The following extracts: dichloromethane, acetone and methanol, had antioxidant compounds in all the mobile systems. For the antimycobacterial analysis, the Bioautography technique was used to screen for antimycobacterial activity. After incubation time, the pinkish colour was developed, which indicates the viability of the microorganism while the white colour represents the zone of inhibition. Chromatograms (Figure 7.5) showed the presence of antimycobacterial activity in BEA and EMW systems, which is indicated by the zone of inhibition (white colour). The serial broth dilution microplate method was used to determine the MIC values. The results (Table 7.3) shows that the plant had antimycobacterial activity, with the extract that had the lowest MIC value being acetone 1 with the MIC value of 0.21 mg/mL whereas the extracts that had the highest MIC value were methanol and hexane 1 and 2.

In the first open column chromatography, the serial exhaustive extraction results showed that dichloromethane and acetone had higher antimycobacterial activity, as such, they were combined and subjected to column chromatography. The fractions were collected and gave a total of 16.9 g. The plant extract that extracted the highest mass was 70% Ethyl acetate: 30% Methanol with 3 g followed by 50% Hexane: 50% Ethyl acetate with 2.95 g while 90% Hexane: 10% Ethyl acetate extracted the least (0.08 g). In the phytochemical analysis of TLC plates, the fluorescencing compounds were observed under the UV light at 254 nm (Figure 7.6) and 365 nm (Figure 7.7), and then the plate was sprayed with Vanillin-sulphuric acid to visualise the bands that were not visible under the UV light (Figure 7.8). There is separation in the BEA system, especially in the following extracts: Hexane: Ethyl acetate (70, 50 and 30) in all the Figures (7.6, 7.7 and 7.8). In the antioxidant analysis, the yellow spots on the purple background indicated the presence of antioxidant compounds. Figure 7.9 showed that the plant extracts have antioxidant compounds in E: M- Ethyl acetate: Methanol (90 to 30) in all the mobile systems. In the antimycobacterial analysis, the Bioautography

method was used to screen for antimycobacterial activity, and the serial broth dilution microplate method was used to determine the MIC. The chromatograms (Figure 7.10) showed the presence of antimycobacterial activity in BEA and CEF systems, which is indicated by the zone of inhibition (white colour), but in CEF, it is not that clear. The extracts 70% Hexane: 30% Ethyl acetate, 50% Hexane: 50% Ethyl acetate and 30% Hexane: 70% Ethyl acetate showed antimycobacterial activity when compared to other extracts in the BEA system. The results (Table 7.5) showed that the plant had antimycobacterial activity, with the extracts that had the lowest MIC value being 70% Hexane: 30% Ethyl acetate, 50% Hexane: 50% Ethyl acetate, 30% Hexane: 70% Ethyl acetate with MIC value of 0.26 mg/mL while 100% Hexane, 90% Hexane: 10% Ethyl acetate, 80% Hexane: 20% Ethyl acetate,10% Ethyl acetate: 10% Methanol and 100% Methanol had the highest MIC greater than 2.5 mg/mL. The results obtained from the antimycobacterial activity support each other in that the extracts: 70% Hexane: 30% Ethyl acetate, 50% Hexane: 50% Ethyl acetate and 30% Hexane: 70% Ethyl acetate have antimycobacterial compounds so they can be tested further.

In the second column chromatography, fractions with high antimycobacterial activity namely 70% hexane: 30% ethyl acetate and 50% hexane: 50% ethyl acetate were subjected to second column chromatography, eluted with 80% hexane: 20% ethyl acetate and collected. In the phytochemical analysis, the fractions were spotted on TLC, observed under the UV light at 254nm (Figure 7.14) and 365 nm (Figure 7.15) and sprayed with vanillin sulphuric acid (Figure 7.16) to determine their profile. All the figures showed that there were different compounds present, but there were those with similar characteristics which could be combined to make them a compound. The combination of fractions with similar profiles gave a total fraction of 13. The fractions were spotted on TLC, observed under the UV light at 254nm (Figure 7.17) and 365 nm (Figure 7.18) and sprayed with vanillin sulphuric acid (Figure 7.19) to determine their profile. Based on the results from (Figure 7.19), compounds: 6, 7 and 8 were chosen for further purification using preparative TLC due to their similar purple band.

In the determination of the solvent system for preparative TLC, the plates were developed in different mobile systems, namely, chloroform and dichloromethane to determine the best solvent system. Dichloromethane was the best system (Figure 21). Compounds: 6 and 7 were isolated using preparative TLC since they were not pure. The obtained pure compounds were sent for structural analysis.

7.5 Conclusion

The bioassay guided fractionation was used to isolate and purify compounds with antimycobacterial activity from *Dombeya rotundifolia* using column chromatography and preparative TLC. The isolated compounds have potential to develop a new drug that can be used to treat TB because of their antimycobacterial activity against *M. smegmatis*. The following chapter focuses on the identification of the isolated compounds.

7.6 References

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Chapter 8

8. Structural elucidation

8.1 Introduction

Drug development relies on the identification of chemical structures in mixtures of natural products, but it is a difficult problem since structural elucidation takes a long

time and is constrained by the mass spectra of existing natural products (Su *et al*., 2017). There have been significant modifications in the methods used for the structural elucidation of natural products. It used to take years of investigation and a significant amount of material to fully understand the structures of even relatively simple natural items, but with today's advanced and sensitive spectroscopic techniques, complex structures can be quickly identified, including the sub-milligram quantities of material (Hanson, 2017). For the analytical examination of molecules and complicated mixtures, mass spectrometry is a typical technique. It is crucial for figuring out a molecule's elemental make-up and for getting a partial picture of its structure from mass spectral fragmentations. A variety of independent techniques such as one- and two-dimensional nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy, X-ray crystallography, and other spectroscopic techniques are always used to confirm the ultimate structure of an unknown organic chemical (Kind and Fiehn, 2010).

An essential cornerstone of organic chemistry is the identification of the structural characteristics of both natural and manufactured compounds. NMR spectroscopy is now the most popular and widely used method for carrying out the work (Marcarino *et al*., 2020). NMR has been used frequently to determine the qualitative structure of natural products and to quantify purified target analyses (Gödecke *et al*, 2012). NMR involves two techniques, namely, one-dimensional and two-dimensional techniques. One-dimensional spectra were employed to provide a chemical shift while two-dimensional correlation spectra gave more details on homo- and hetero-nuclear connectivity, allowing the identification of the sub-structure of natural products (Hanson, 2017).

NMR uses the following experiments to determine structures such as Proton NMR (1D ^{1}H NMR), carbon- 13 (1D ^{13}C NMR), Distortionless enhancement through polarisation transfer (DEPT) (1D ^{13}C NMR), ^{1}H-^{1}H Correlation spectroscopy (COSY) (2D ^{1}H NMR), Heteronuclear single quantum correlation (HSQC) (2D ^{1}H-^{13}C), heteronuclear multiple quantum correlation (HMQC) (2D ^{1}H-^{13}C NMR), and Heteronuclear multiple bond correlation (HMBC) (2D ^{1}H-^{13}C NMR). The ^{1}H- and ^{13}C-NMR spectra provide information about the qualitative and quantitative composition of an unknown compounds and are used to determine the molecular formula (Elyashberg, 2015). The DEPT spectra divides a compound's carbon atoms into primary (CH3), secondary

(CH2), tertiary (CH), and quaternary (C) spectra (Ahmed, 2012). The COSY spectrum indicates homonuclear correlations (spin couplings) between vicinal hydrogens separated by three bonds (3JHH). This allows for the identification of the nearby carbon atoms linked by a chemical bond. The HMBC spectrum shows heteronuclear correlations between 1H and 13C (15N) nuclei that are separated by two or three chemical bonds, which enables users to identify “fuzzy” pieces surrounding a specific C or N atom (Elyashberg, 2015). The HSQC and HMQC spectra show heteronuclear correlations (^{1}H- ^{13}C 1 bond correlation). Cross peaks represent carbon chemical shifts in one dimension and proton chemical shifts in the other dimension (Ahmed, 2012).

8.2 Method and materials

The pure isolated compounds were sent to the Department of Chemistry at the University of Limpopo for the structural identification using NMR techniques. The compounds were analysed using 1-dimensional NMR (1H, 13C and DEPT 135) and 2-dimensional NMR (HSQC). About 5 mg of a sample (compound 1 and compound 2 separately) dissolved in chloroform and run using 400 MHz NMR Spectrometer

(Bruker) at 400 MHz. Prof Ofentse Mazimba, from Botswana International University of Science and Technology, Department of Chemical and Forensic Sciences, assisted with the analysis of the NMR spectra and structure elucidation of the compounds.

8.3 Results

8.3.1 NMR analysis of isolated compound 1

The isolated compound run under different pulse programs (^{1}H Proton, ^{13}C, DEPT 135 and HSQC) using NMR. Figure 8.1 to Figure 8.3 represent the NMR spectra of compound 1. Table 8.1 shows the ^{1}H and ^{13}C Proton shift values of the isolated

compound compared to the values obtained from literature. Those spectra helped with the characterisation of compound 1 (Figure 8.4).

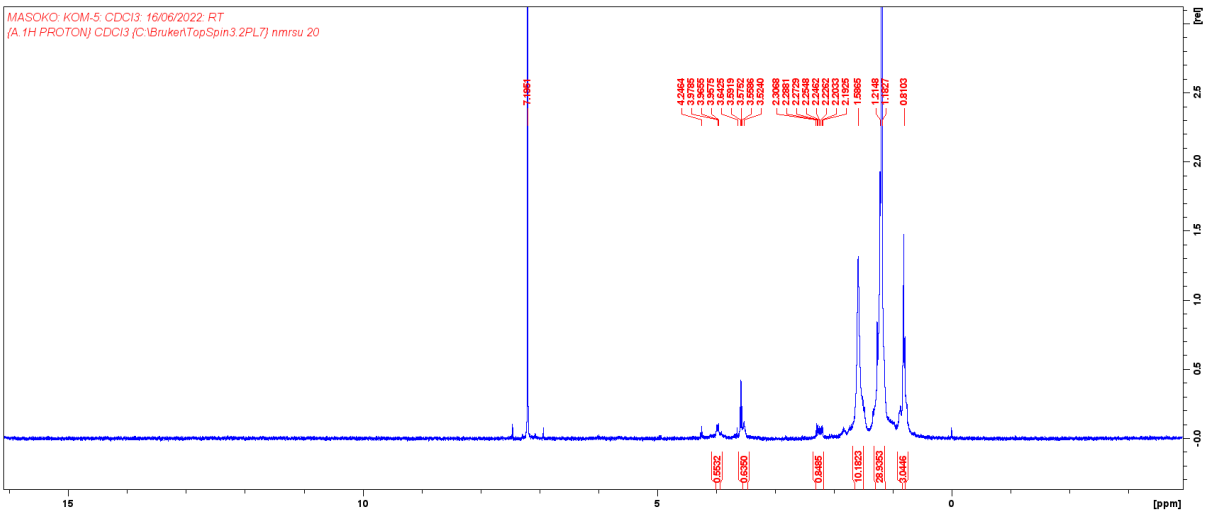

Figure 8. 1: ^{1}H NMR spectrum of isolated compound 1.

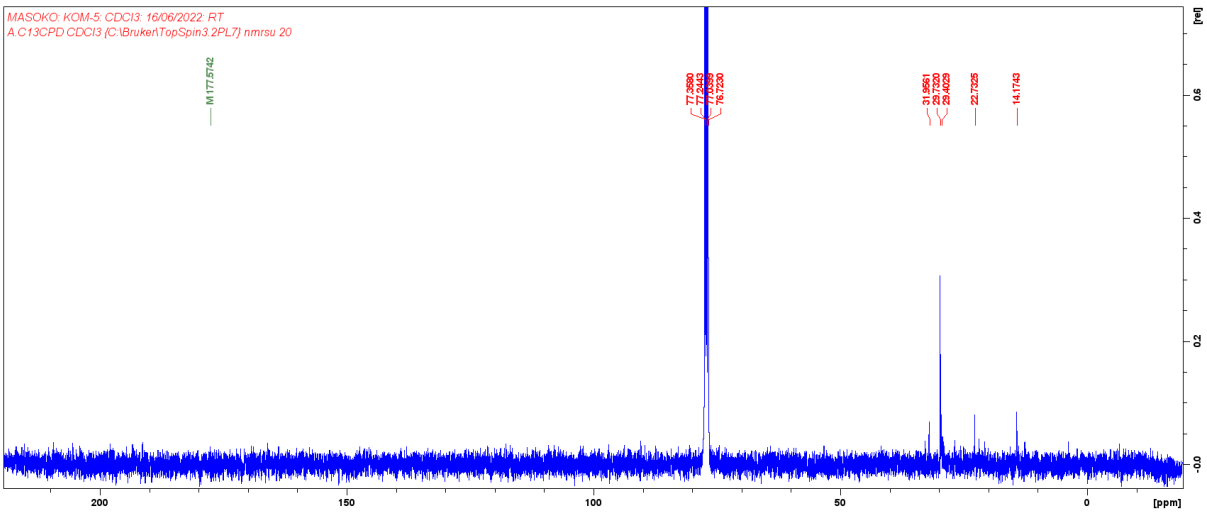

Figure 8. 2: ^{13}C CPD NMR spectrum of isolated compound 1.

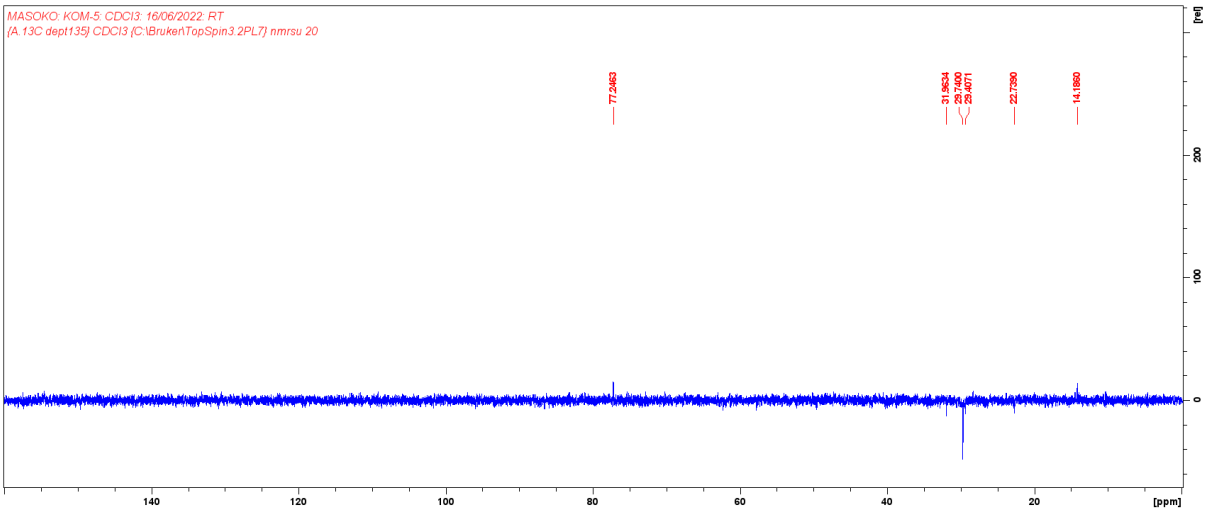

Figure 8. 3: ^{13}C DEPT 135 NMR spectrum of compound 1.

Spectroscopic data

Table 8. 1: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) data of isolated compound 1.

Position	δ_H	δ_C	δ_H	δ_C
	literature		**Isolated compound**	
1		179.4 (*s*)		177.5
2	2.28, 2H, *t*, *J* = 7.8 Hz	33.9 (*t*)	1.58, 2H, brs	31.9
3-19	1.19, 36H, *br s*	22.7, 29.1-29.7 & 31.9 (*t*)	1.18, 28H, brs 1.58, 8H, brs	22.7, 29.4-29.7
20	1.56, 2H, *m*	14.1 (*q*)	0.81, 3H, t, J=6.6Hz	14.1

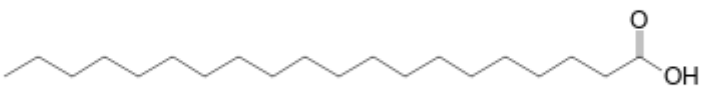

Figure 8. 4: Structure of compound 1 (Eicosanoic acid)

8.3.2 NMR analysis of isolated compound 2

The isolated compound was run under different pulse programs (^{1}H Proton, ^{13}C, DEPT 135 and HSQC) using NMR. Figure 8.5 to Figure 8.8 represent NMR spectra of compound 1. Table 8.2 shows the ^{1}H and ^{13}C Proton shift values of the isolated compound compared to the values obtained from literature. Those spectra helped with the characterisation of compound 2 (Figure 8.9) .

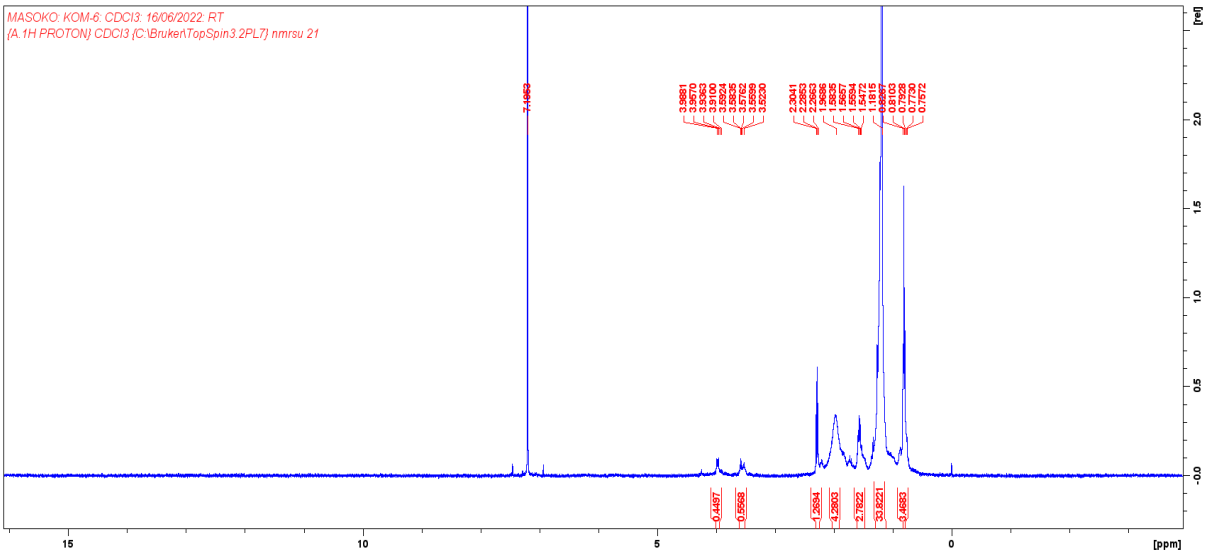

Figure 8. 5: ^{1}H NMR spectrum of isolated compound 2.

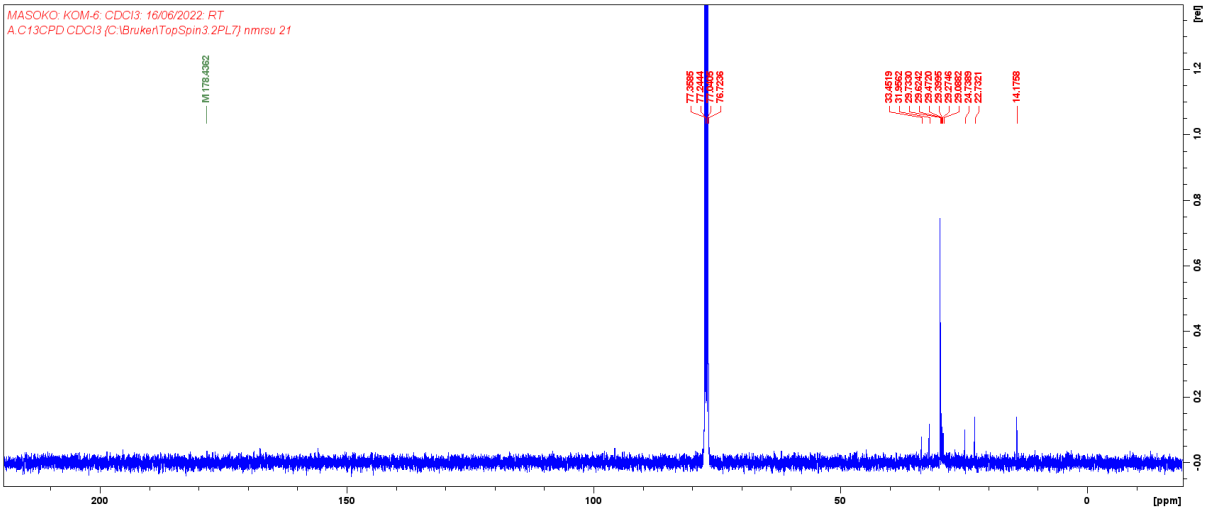

Figure 8. 6: ^{13}C CPD NMR spectrum of isolated compound 2.

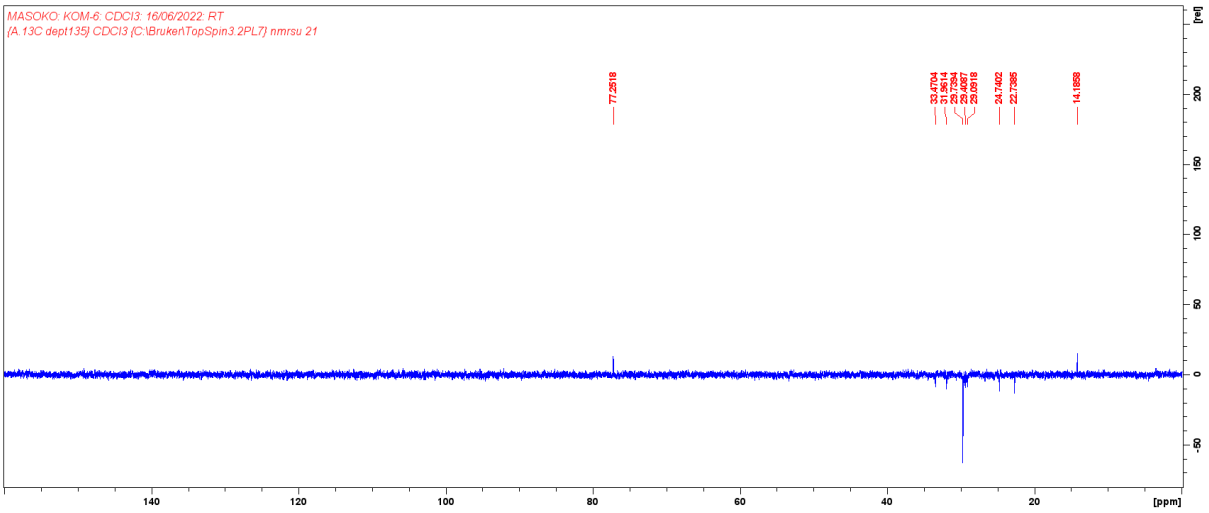

Figure 8. 7: ^{13}C DEPT 135 NMR spectrum of compound 2.

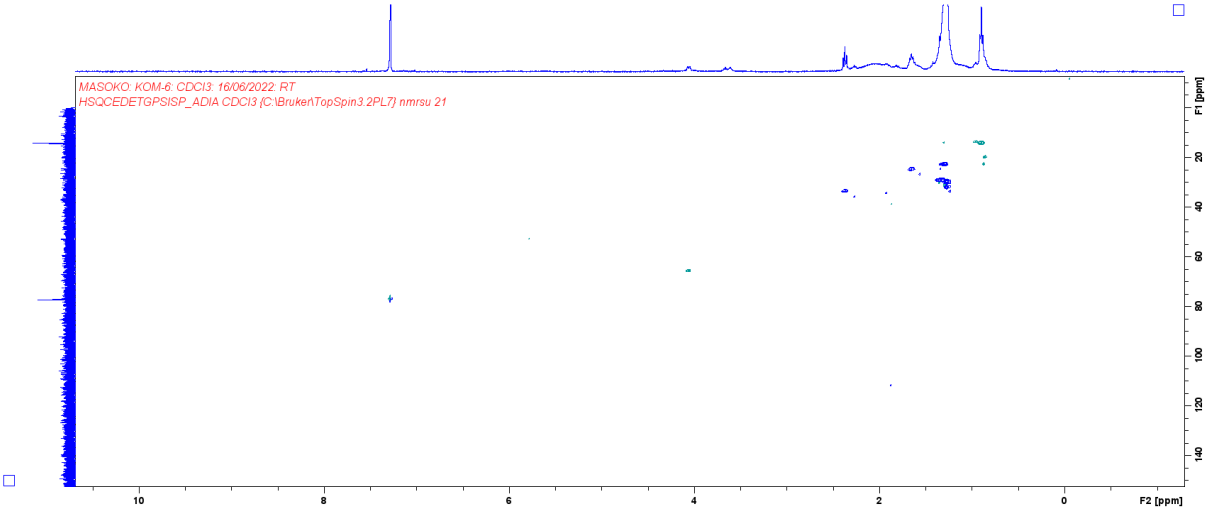

Figure 8. 8: HSQC NMR spectrum of compound 2.

Spectroscopic data

Table 8. 2: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) data of isolated compound 2.

Position	δ_H	δ_C	δ_H	δ_C
	literature		**Isolated compound**	
1		179.4 (*s*)		178.4
2	2.28, 2H, *t*, *J* = 7.8 Hz	33.9 (*t*)	1.96, 4H, brs	33.4

3-20	1.19, 36H, *br s*	22.7, 29.1-29.7 & 31.9 (*t*)	1.18, 34H, brs	22.7, 29.0-29.7 31.9
21	1.56, 2H, *m*	24.7 (*t*)	1.56, 2H, t, J=7.4Hz	24.7
22	0.81, 3H, *t*, *J* = 3.6 Hz	14.1 (*q*)	0.81, 3H, t, J=6.6Hz	14.1

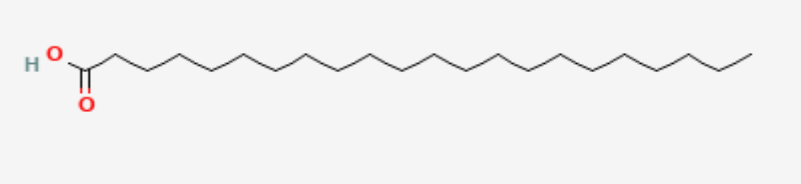

Figure 8. 9: Structure of compound 2 (Docosanoic acid (Behenic acid)).

8.4 Discussion

The aim of this chapter was to identify the isolated compounds from *Dombeya rotundifolia* using NMR spectra. Medicinal plants play an important role in drug discovery. Nearly 98% of the substances discovered in the human metabolome are natural products, and more than 66% of all medications are derived from natural products. For a very long time, NMR spectroscopy has been regarded as the “gold standard” for determining the structures of novel natural products and metabolites. NMR not only enables the identification of 3D molecule structures, but also the absolute configuration of chiral substances and frequently, the relative configuration of diastereomers. This knowledge is essential for comprehending the biological function and metabolic genesis of many natural products (Wishart *et al*., 2022).

Structure elucidation refers to *de novo* structural identification, which results in a comprehensive molecular connection table with proper stereochemical assignments (Kind and Fiehn, 2010). The structure was elucidated using the following techniques from NMR: ^{1}H Proton, ^{13}C, DEPT 135 and HSQC. The isolated compounds were found to be Eicosanoic acid (Figure 8.4) and Docosanoic acid (Behenic acid) (Figure 8.9), which are saturated fatty acid. The identity of the structures were verified by comparing them with literature by using ^{1}H (300 MHz) and ^{13}C (75.4 MHz) (Table 8.1 and 8.2). Fatty acids are carbon chains that have methyl groups and carboxyl groups at opposite ends, respectively. Most saturated fatty acids have 12 to 22 carbon atoms (Rustan and Drevon, 2001). Some of isolated compounds from *Dombeya rotundifolia* with antibacterial activity are lauric, myristic, stearic, and palmitic acids (Balogun and Ashafa, 2019). It has been reported that lupeol and beta-sitosterol have been isolated from the stem’s bark (Ndwigah *et al*., 2005). Most of antibacterial compounds isolated from the Sterculiaceae species are fatty acids. Compounds isolated from Sterculiaceae species with antibacterial activity are Eicosane, Myristic acid, Palmitic acid, Stearyl alcohol, Lauric acid, Stearic acid (Reid *et al*., 2005). The compounds isolated in this study were firstly isolated from *Dombeya* rotundifolia. It is known that fatty acids have antibacterial properties and are significant components of plants (McGaw *et al.,* 2002).

8.5 Conclusion

NMR was used to elucidate the structure of the isolated compounds from *Dombeya rotundifolia*. The structures were identified as Eicosanoic acid and Docosanoic acid.

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Chapter 9

9. Biological activities of isolated compounds

9.1 Introduction

There are several natural products with antimycobacterial bioactive compounds that are found from plants and those plants derived antimycobacterial compounds belong to the following classes: alkaloids, terpenoids, coumarins, peptides, and phenolics (McGaw *et al*., 2008). *Dombeya rotundifolia* is a member of the Malvaceae family. It is a deciduous, fast-growing, frost and drought-resistant tree that usually grows between 3 and 6 metres. It is used to treat intestinal ulcers, stomach troubles, and sharp stomach pains, fever, diarrhoea, palpitation, nausea, enemas for dyspepsia, and to induce labour. It possesses the following biological activities: being antibacterial, anti-inflammatory, anthelminthic, antidiarrheal, and abortifacient (Balogun and Ashafa, 2019).

A bioactive compound is a substance with biological activity that can influence one or more metabolic processes, which promotes the improvement of health problems (Angiolillo *et al*., 2015). The isolated bioactive compounds are Eicosanoic acid and Docosanoic acid (Behenic acid). Lauric, myristic, stearic, and palmitic acids are some of the isolated compounds with the antibacterial activity from *Dombeya rotundifolia* (Balogun and Ashafa, 2019). There are influential biological activities identified in fatty acids such as antibacterial, antifungal, antiprotozoal, antiviral, and cytotoxic. Fatty acids' biological activity contributes to the host's ability to defend itself from potentially harmful or opportunistic bacteria (Agustini and Wijayanto, 2020).

9.2 Method and materials

9.2.1 Phytochemical analysis

The chemical profiles of *D. rotundifolia* extracts were analysed on aluminium backed TLC plates (Merck, silica gel 60 F254) using a method developed by Kotze and Eloff, (2002), as described in section 3.2.3.

9.2.2 Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972), as described in section 5.2.2.

9.2.3 Broth micro-dilution assay

The broth micro-dilution method described by Eloff (1998) was used to determine the MIC values of crude extracts against *M. smegmatis*, as explained in section 5.2.3.

9.3 Results

9.3.1 Phytochemical analysis on TLC plates

TLC was used to analyse the phytochemical constituents in the plant (*Dombeya rotundifolia*). Fluorescencing compounds were observed under the UV light at 254 nm and 365 nm (Figure 9.1). The single bands represent the isolated compounds.

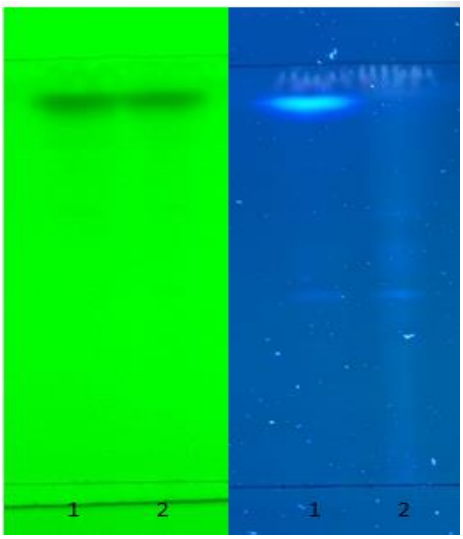

Figure 9. 1: Chromatograms of isolated compounds observed under the UV light of 254 nm and 365 nm.

9.3.2 Bioautography

The Bioautography technique was used to screen for antimycobacterial activity and INT was used as an indicator. There was the presence of antimycobacterial activity, as indicated by the zone of inhibition (white colour) (Figure 9.2).

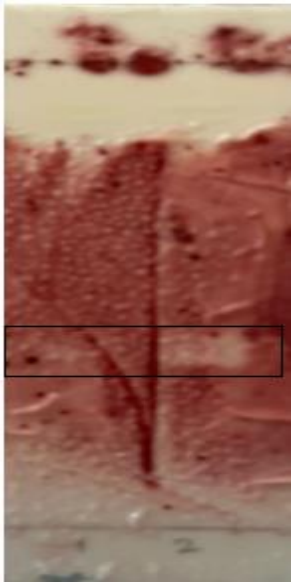

Figure 9. 2: The chromatogram of isolated compounds and sprayed with *M. smegmatis.*

9.3.3 Micro-dilution assay

The MIC values were determined by using the serial broth dilution microplate method. The results (Table 9.1) showed that the isolated compounds had antimycobacterial activity with the MIC value of 0.25 mg/mL.

Table 9. 1: The MIC at which the isolated compounds were able to inhibit the growth of *M. smegmatis* after 24 hours of incubation.

Microorganism	MIC values (mg/mL)	
	Compound 1 (Eicosanoic acid)	Compound 2 (Docosanoic acid)
M. smegmatis	0.25	0.25

Rifampicin (positive control) = 0.002 mg/mL, Acetone (negative control) = greater than 0.25 mg/mL

9.4 Discussion

The aim of this chapter was to investigate the antimycobacterial activity of isolated compounds from the leaves of *Dombeya rotundifolia*. To reduce drug-resistant TB cases and end of the TB epidemic, the newly discovered antitubercular drugs require novel mechanisms of activity, shorter treatment duration and minimal toxicity (Madikizela *et al*., 2014). Plant-derived antimicrobial compounds may suppress bacteria by a different mechanism than currently used antibiotics and may be useful in treating pathogenic strains that are resistant to antibiotics (Eloff,1998).

The active compounds were isolated by the use of bioassay-guided fractionation and identified by spectroscopic analysis as Eicosanoic acid and Docosanoic acid. Their profiles were analysed using the TLC plates method and their antimycobacterial activities were evaluated using bioautography and Broth micro-dilution assay. Figure 9.1 shows the analyses that were done under the UV light of 254 nm and 365 nm, where the single band represents the isolated compounds. The isolated compounds are almost in the same line, and this may be due to the fact that those compounds are similar in structure. Eicosanoic acid has 20 carbons while Decosanoic acid has 22 carbons. Figure 9.2 shows that there is an activity, which is indicated by the white colour against the pinkish colour (zone of inhibition) but is not that good. This may be due to the low concentration of the isolated compounds (1 mg/mL); so, they do not work at their best at a low concentration against *M. smegmatis* or they need to be combined with other active compounds. The combination antimicrobial therapy is successful when employed with some synergistic effects (Mundy *et al.,* 2016). Table 9.1 shows that the isolated compounds have the MIC value of 0.25 mg/ML. According to Kuete (2010) (discussed in Chapter 5), when a compound has the MIC value between 0.1 and 0.625 mg/mL, it has moderate activity. As such, the compounds have moderated activity. The effectiveness of the isolated antimycobacterial compound may or may not depend on the resulting MIC values. There is a possibility that there is an extract with the low MIC value, meaning high activity may have a large amount of only a few moderate active compounds, while a moderate active extract may have minor constituents with a high activity. The antimycobacterial activity can be increased by combining the moderate active compounds with the synthetic analog (Nguta *et al*., 2015). Further studies are needed to determine if the antimycobacterial activity of

isolated compounds is not due to toxicity. Safety is important when developing a new drug.

9.5 Conclusion

The isolated compounds were found to have antimycobacterial activity, which is indicated by the MIC value of 0.25 mg/ML (moderate activity). The isolated compounds are worthwhile because they form a good preliminary basis for the selection of a candidate drug that might be part of TB treatment, but further investigation is needed to validate the plant for pharmacological use, especially in treating TB. Medicinal plants play a role in the development of antimycobacterial drugs due to their interesting chemical structure and high antimycobacterial activity.

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Chapter 10

10. General discussion, conclusion, and recommendations

10.1 General discussion

Currently, antimicrobial resistance is a significant global problem. There is currently a shortage of effective drugs in the medical field to treat diseases, particularly those brought by the dreadful multidrug-resistant strains, as the rate of the emergence of antimicrobial resistance has outpaced the rate of drug discovery and the introduction of new effective treatments (Teh *et al*., 2017). The World Health Organization (WHO) proclaimed TB as a global emergency in 1993, which was the first infectious disease to be declared as such. After decades of having been declared as a global emergency, TB is still one of the leading killer diseases in the world. In 2013, WHO estimated that there were 9.0 million new cases and 1.5 million cases of mortality (WHO, 2014). In WHO's 2022 report, the death rate was still the same, with additional 1 million new cases. With those statistical reports, there is a need for new effective drugs to treat TB because we are still far from ending TB by 2030.

Ethnobotanical studies are among the most trustworthy sources for humans when it comes to exploiting the herbal resources that nature has to offer (Getasetegn and Tefera, 2016). Due to a lack of medical facilities, the efficacy of traditional medicines, cultural priorities, and choices, traditional medicines serve as an affordable alternative for basic health care in poor nations. Traditional medicine use is rapidly expanding in developed countries. The traditional knowledge of medicinal plants has helped modern pharmaceutics in the creation of significant medications. Many therapeutic plants still need to have their traditional uses verified, despite some of them having undergone phytochemical and pharmacological testing. As a result, more focus is put on conducting phytochemical, pharmacological, microbiological, toxicological, preclinical, and clinical investigations to confirm their safety and effectiveness (Aziz *et al*., 2018).

The first and most important step in the study of medicinal plants is extraction since it is important to separate and characterise the desired chemical components from the plant materials (Sasidharan *et al*., 2011). In this study, extraction was done using different solvents that vary in polarity. Extraction depends on the type of method and solvent/s used. Depending on their polarity, various solvents extract different amounts of components from raw plants that may be beneficial or detrimental to biological

systems (Mokgotho *et al*., 2013). The plant extracts were analysed on TLC plates to determine the type of compounds (polar, non-polar or intermediate) that are present in the plant depending on their affinity toward the mobile systems. The plant was screened for phytochemicals. It showed that *D. rotundifolia* had the following phytochemicals: saponins, terpenes/ terpenoids, tannins, cardiac glycosides, flavonoids, steroids except phlobatannins and alkaloids. These phytochemicals present are linked to their ability to prevent or treat diseases. Since the plant has major phytochemicals (phenolic, flavonoids and tannins), they were further quantified. Those phytochemicals are important in the pharmaceutical field because they have biological activities such as being antimicrobial, antioxidant, anticarcinogenic, anti-inflammatory, antidiabetic, anti-allergic, which are required when developing a drug.

The biological activity of the plant was evaluated for its antioxidant, antimycobacterial, antibiofilm, anti-inflammatory, and cytotoxicity activity/effect. The plant proved to have antioxidant compounds when analysed qualitatively using DPPH. There was a clear separation in the EMW mobile system, which may suggest that the active antioxidant compounds are polar. The antioxidant activity was further quantified using DPPH and ferric reducing power assay. It is important for the plant to have antioxidant compounds to maintain the defence system of the host since patients with TB have different health problems and weak immune systems due to high oxidative stress (Pandey *et al*., 2022). The plant extracts were evaluated for antimycobacterial activity against *Mycobacterium smegmatis*, the plant extracts showed to have moderate activity. The plant extracts were evaluated for antibiofilm against *Mycobacterium smegmatis*. The extracts were able to prevent the formation of biofilm at various concentrations, but it was the best at a higher concentration (5mg/mL). The plants extracts were evaluated for anti-inflammatory activity, and it was showed that the plant extracts have the best inhibition capacity, which ranged from 85% to 97% when compared with positive control (aspirin) with inhibition capacity of 100%.The plant was also evaluated for cytotoxicity, and it was found that it was toxic to the cells, but some of the cells were able to survive after the treatment. The plant proved to have antimycobacterial compounds and as such, it was further selected for isolation and purification of bioactive antimycobacterial compounds using bioassay-guided assay and identified using NMR. The isolated antimycobacterial compounds were found to be Eicosanoic

acid and Docosanoic acid with notable activity. They were the first to be isolated from *D. rotundifolia*.

10.2 Conclusion/ recommendations

The results obtained from the study are important considering the rise in TB deaths in developing and developed countries. There is a need for effective antimycobacterial drugs. The results of this study contribute to the knowledge of South African pharmacopoeia of medicinal plants with potential anti-TB activities. It is recommended that further investigation should be done on the isolated antimycobacterial compounds such as cytotoxicity and *in vivo* studies should be conducted to evaluate their toxic effects before use. In addition, the anti-mycobacterium activity of the plant extracts should be tested on the *Mycobacterium tuberculosis* H37Rv and clinical isolates to further validate the activity.

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