ANTIMYCOBACTERIAL EVALUATION, PRELIMINARY PHYTOCHEMICAL AND CYTOTOXICITY STUDIES OF *CASSIA PETERSIANA*

BY

RAMOKONE FLORAH MOTHUPI

[redacted]

RESEARCH DISSERTATION SUBMITTED IN FULFILMENT FOR THE DEGREE

Of

MASTER OF SCIENCE

In

MICROBIOLOGY

In the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life sciences)

At the

UNIVERSITY OF LIMPOPO

SUPERVISOR: PROF P. MASOKO

July 2022

Declaration

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

……. …….

Signature Date

Dedication

This work is dedicated to my parents, Mr and Mrs Mothupi; my siblings, Maphuti, Simon, Thebe, Machoene; my niece, Bokamoso, and nephew, Mahlatse.

Acknowledgments

I thank GOD for giving me the courage, strength, and wisdom to pursue and complete this study.

I would like to thank my supervisor, Prof P Masoko, for the opportunity to study under his supervision. I am grateful for his support, guidance, and encouragement throughout the study.

My gratitude also goes to Ms Mutshidzi Malada, Mr Mash Matotoka, and my other colleagues at the Department of Biochemistry, Microbiology and Biotechnology for assistance in the laboratory.

I thank Ms Tselane Ramakadi for her assistance with the NMR analysis.

I also thank Prof Ofentse Mazimba from Botswana International University of Science and Technology for assistance with the structure elucidation of the isolated compound.

I thank my family for their undying support and encouragement throughout this study.

My sincere gratitude goes to my partner for the constant support throughout the good and the bad times I faced during the study. Thank you for your love and care.

I thank the CSIR for providing financial and psychological support towards the completion of this study.

My sincere gratitude goes to the University of Limpopo for offering me the opportunity to study for my Master's degree at their facility.

Conference presentations

Mothupi, R.F. and Masoko P. Antimycobacterial evaluation, preliminary phytochemical and cytotoxicity studies of *Cassia petersiana*. Presented during the 11th annual Faculty of Science and Agriculture Postgraduate Research Day held at Bolivia Lodge, Polokwane, Limpopo, South Africa, October 2021.

Mothupi, R.F. and Masoko P. Antimycobacterial evaluation, preliminary phytochemical and cytotoxicity studies of *Cassia petersiana*. Presented during 23rd Indigenous Plant Use Forum (IPUF) conference held virtually, July 2021.

Table of contents

List of abbreviations

^{13}C	Carbon- 13
^{1}H	Hydrogen- 1
A	Acetone
B	Butanol
BEA	Benzene: Ethanol: Ammonium hydroxide (18:2:0.2)
C	Chloroform
CEF	Chloroform: Ethyl acetate: Formic acid (10:8:2) E Ethyl acetate
D	Dichloromethane
DPPH	2, 2- diphenyl-1-picrylhydrazyl radical
EMW	Ethyl acetate: Methanol: Water (40:5.4:5)
E	Ethanol
EA	Ethyl acetate
GAE	Gallic acid equivalence
g	Gram
H	n-Hexane
INT	p- iodonitrotetrazolium violet
M	Methanol
MIC	Minimum inhibitory concentration
QE	Quercetin equivalence
rpm	Revolutions per minute
TB	Tuberculosis
TLC	Thin Layer Chromatography
UV	Ultraviolet
W	Water

WHO	World Health Organization

List of figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

List of tables

Chapter 3

Chapter 5

Chapter 7

Chapter 8

Chapter 9

Abstract

This study aimed to investigate antimycobacterial and cytotoxic compounds from *Cassia petersiana*. *Cassia petersiana* was selected for the current study based on its traditional use for treating tuberculosis (TB) symptoms. Extraction is an important step in the use of medicinal plants; hence, solvents of varying polarity were employed to extract a wide range of compounds where chloroform was the best extractant (67 mg). As there is no relation between the amount of plant material extracted and the bioactivity of the extracts, standard tests were used to determine the presence of different phytochemical constituents from *Cassia petersiana* and the total phenolic, flavonoid, and tannin contents were quantified using colorimetric assays. It was revealed that all the tested phytochemical constituents were present, and it was proven that phenolic compounds were the most abundant, followed by the tannins, while the flavonoids were the least among the common phytochemical constituents quantified. The phytochemical compounds were further profiled on thin-layer chromatography (TLC) and developed in BEA, CEF, and EMW solvent systems. Colourful compounds which indicated diverse phytochemicals were visualised with both vanillin-sulphuric acid and ultraviolet light on the phytochemical chromatograms and good separation of the compounds was from the BEA solvent system. The qualitative and quantitative antioxidant activity and antimycobacterial activity assays were used to evaluate the extracts from *Cassia petersiana*. Minimal antioxidant activity was observed on the qualitative antioxidant activity profile. These findings correlated with the minimal quantity of antioxidants from extracts of *Cassia petersiana* from the quantitative antioxidant assays; ferric reducing power and DPPH scavenging activity assays. *Cassia petersiana* extracts had bioactivity against *Mycobacterium smegmatis* as indicated by the lowest MIC value. The cell viability effects of the acetone crude extract from *Cassia petersiana* were evaluated against the tryptophan hydroxylase-1 *(*TPH-1) macrophage cells. Large scale extraction procedure was employed to extract a sufficient amount of plant material in preparation for the isolation of the bioactive compound. Bioassay-guided fractionation combined with column chromatography and TLC were used to isolate and purify the bioactive compound from the n-hexane extract of *Cassia petersiana*. The purified isolated compound was elucidated as β-sitosterol, which showed remarkable bioactivity against *Mycobacterium smegmatis* only on the TLC-bioautographic assay, while the quantitative antimycobacterial activity was higher

with the MIC value of 2.5 mg/mL. Although β-sitosterol is known as a good antioxidant, it showed no antioxidant activity on the qualitative antioxidant activity assay. Therefore, further studies, including *in vivo* assay, are recommended on the isolated compound to evaluate its biological activities before consideration of its use in the development of alternative drugs.

CHAPTER 1: Introduction

1.1 General Introduction

Tuberculosis (TB) remains a global health threat and the major cause of annual deaths (Aro *et al*., 2015; Harding, 2020). Drug-resistant TB caused by the emergence of resistance of the Mycobacterium complex towards the first-line TB drugs (rifampicin and isoniazid) is the leading drawback to the management and treatment of TB (Nunes *et al.,* 2020; Pitso *et al.,* 2019*;* Salehi *et al*., 2018; Seaworth and Griffith*,* 2017). In addition, the resistance of the causative agents of TB towards the second-line TB drugs led to the emergence of extensively drug-resistant TB, which complicates the management and treatment of TB even further. This is because second-line TB drugs often result in prolonged antibiotic therapy, further posing a risk of toxicity to TB patients (Vikrant, 2011; WHO, 2018).

The drawback to the management and treatment of TB is even higher in developing countries due to limited access to the costly treatment whose efficacy is gradually decreasing (Vikrant, 2011). According to the World Health Organisation (WHO) (2020), South Africa is amongst the top 30 countries with a high burden of TB. The TB burden is appalling to the less privileged South Africans due to limited access to costly TB antibiotics (Pai and Memish, 2015; Semenya and Maroyi, 2013). As a result, the discovery of alternative TB antibiotics with improved efficacy at a lower cost is of great importance.

To account for this, medicinal plants are considered imperative to the development of alternative TB drugs because of their traditional use. From ancient times, medicinal plant remedies were a key health care service for the population in developing countries, particularly in Africa (Fomogne-Fodjo *et al*., 2014; Fouotsa *et al*., 2013). During the investigations to determine the safe dosage of the traditional medicinal plant remedies, compounds of great potency against the causative agents of TB were discovered. For example, the bioactivity of the acetone fraction from the stem bark of *Combretum molle* against *Mycobacterium tuberculosis* was discovered (Arunkumar and Muthuselvam, 2009).

The therapeutic properties of medicinal plants are attributed to the phytochemical compounds that are produced naturally by these plants as a defence mechanism against unfavourable conditions (Cowan, 1999*;* Gómez-Cansino *et al*., 2017).

Some phytochemicals with anti-tubercular activity discovered over the years include tannins, alkaloids, phenolics, glycosides, sterols, triterpenoids, xanthones and quinones (Fauziyah *et al*., 2017; Nwokeji *et al., 2016;*). Bioactive antimycobacterial compounds discovered can either be used as crude extracts where they act synergistically or as isolated compounds (Dzoyem *et al.*, 2016; Gupta *et al.*, 2018; Hett and Rubin, 2008; Luo *et al.*, 2011; Nguta *et al.*, 2016).

As medicinal plants are gaining recognition as sources of new drugs, limited literature is available on some of these plants, such as *Cassia petersiana*. The bark of this plant is used to treat TB-related diseases and their symptoms (Green *et al*., 2010). Therefore, the current study investigates and isolates the antimycobacterial compounds from *Cassia petersiana.* The importance of the isolated compounds may be highlighted as a new lead in TB drug development.

1.2 References

Aro, A., Dzoyem, J., Hlokwe, T., Madoroba, E., Eloff, J. and McGaw, L., 2015. Some South African Rubiaceae tree leaf extracts have antimycobacterial activity against pathogenic and non-pathogenic Mycobacterium species. *Phytotherapy Research*, 29(7), pp.1004-1010.

Arunkumar, S. and Muthuselvam, M., 2009. Analysis of phytochemical constituents and antimicrobial activities of *Aloe vera* L. against clinical pathogens. *World Journal of Agricultural Sciences*, 5(9), pp.572-576.

Cowan, M., 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12(4), pp.564-582.

Dzoyem, J., Aro, A., McGaw, L. and Eloff, J., 2016. Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal plants used in Southern Africa to treat tuberculosis and respiratory ailments. *South African Journal of Botany*, 102, pp.70-74.

Fauziyah, P., Sukandar, E. and Ayuningtyas, D., 2017. Combination effect of antituberculosis drugs and ethanolic extract of selected medicinal plants against multi-drug resistant *Mycobacterium tuberculosis* isolates. *Scientia Pharmaceutica*, 85(1), p.14.

Fomogne-Fodjo, M., van-Vuuren, S., Ndinteh, D., Krause, R. and Olivier, D., 2014. Antibacterial activities of plants from Central Africa used traditionally by the Bakola pygmies for treating respiratory and tuberculosis-related symptoms. *Journal of Ethnopharmacology*, 155(1), pp.123-131.

Fouotsa, H., Mbaveng, A., Mbazoa, C., Nkengfack, A., Farzana, S., Iqbal, C., Marion Meyer, J., Lall, N. and Kuete, V., 2013. Antibacterial constituents of three Cameroonian medicinal plants: *Garcinia nobilis*, *Oricia suaveolens* and *Balsamocitrus camerunensis*. *BMC Complementary and Alternative Medicine*, 13(1), pp.1-10.

Gómez-Cansino, R., Guzmán-Gutiérrez, S., Campos-Lara, M., Espitia-Pinzón, C. and Reyes-Chilpa, R., 2017. Natural compounds from Mexican medicinal plants as potential drug leads for anti-tuberculosis drugs. *Anais da Academia Brasileira de Ciências*, 89(1), pp.31-43.

Green, E., Samie, A., Obi, C., Bessong, P. and Ndip, R., 2010. Inhibitory properties of selected South African medicinal plants against *Mycobacterium tuberculosis*. *Journal of Ethnopharmacology*, 130(1), pp.151-157.

Gupta, V., Kaushik, A., Chauhan, D., Ahirwar, R., Sharma, S. and Bisht, D., 2018. Anti-mycobacterial activity of some medicinal plants used traditionally by tribes from Madhya Pradesh, India for treating tuberculosis related symptoms. *Journal of Ethnopharmacology*, 227, pp.113-120.

Harding, E., 2020. WHO global progress report on tuberculosis elimination. *The Lancet Respiratory Medicine*, 8(1), p.19.

Hett, E. and Rubin, E., 2008. Bacterial growth and cell division: a mycobacterial perspective. *Microbiology and Molecular Biology Reviews*, 72(1), pp.126-156.

Luo, X., Pires, D., Aínsa, J., Gracia, B., Mulhovo, S., Duarte, A., Anes, E. and Ferreira, M., 2011. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. *Journal of Ethnopharmacology*, 137(1), pp.114-120.

Nguta, J., Appiah-Opong, R., Nyarko, A., Yeboah-Manu, D., Addo, P., Otchere, I. and Kissi-Twum, A., 2016. Antimycobacterial and cytotoxic activity of selected medicinal plant extracts. *Journal of Ethnopharmacology*, 182, pp.10-15.

Nunes, J., Duque, M., Freitas, T., Galina, L. and Ducati, R., 2020. *Mycobacterium tuberculosis* Shikimate Pathway. *Molecules Journal*, 25(6), pp.1-36.

Nwokeji, A., Appiah-Opong, R., Nyarko, A., Yeboah-Manu, D., Addo, P., Otchere, I. and Kissi-Twum, A., 2016. The chemistry of natural product: plant secondary metabolites. *International Journal of Technology enhancements and emerging engineering research*, 4(8), pp.1-8.

Pai, M. and Memish, Z., 2015. Global tuberculosis control requires greater ambition and resources. *Journal of Epidemiology and Global Health*, 5(1), pp.1-2.

Pitso, L., Potgieter, S. and Van der Spoel van Dijk, A., 2019. Prevalence of isoniazid resistance-conferring mutations associated with multidrug-resistant tuberculosis in Free State Province, South Africa. *South African Medical Journal*, 109(9), p.659.

Salehi, B., Kumar, N., Şener, B., Sharifi-Rad, M., Kılıç, M., Mahady, G., Vlaisavljevic, S., Iriti, M., Kobarfard, F., Setzer, W., Ayatollahi, S., Ata, A. and Sharifi-Rad, J., 2018. Medicinal plants used in the treatment of Human Immunodeficiency Virus. *International Journal of Molecular Sciences*, 19(5), p.1459.

Seaworth, B. and Griffith, D., 2017. Therapy of multidrug-resistant and extensively drug-resistant tuberculosis. *Microbiology Spectrum*, 5(2).

Semenya, S. and Maroyi, A., 2013. Medicinal plants used for the treatment of tuberculosis by Bapedi traditional healers in three districts of the Limpopo Province, South Africa. *African Journal of Traditional, Complementary and Alternative Medicines*, 10(2).

Vikrant, A., 2011. A Review on anti-tubercular plants. *International Journal of PharmTech Research*, 3(2), pp.872-880.

WHO, Global Tuberculosis Report World Health Organization, Geneva, 2018.

World Health Organization, 2020. *Global tuberculosis report 2020*. [online] Who.int. Available at: <https://www.who.int/publications-detail-redirect/9789240013131> [Accessed 17 June 2021].

CHAPTER 2: Literature review

2.1 Traditional medicinal plants

Traditional medicinal plants have been an integral part of Africans' culture since time immemorial (Sheng-Ji, 2001; van-Wyk *et al*., 1997; Zhang *et al*., 2018). Medicinal plants are those plants that possess therapeutic properties and bring about health benefits to humans and animals. Any part of the plant which has the desired compounds with health benefits can be used, including the leaves, barks, roots, stems, fruits, flowers and seeds (Tang and Halliwell, 2010). Folk medicine is commonly used as dried plant material because of their increased shelf-life (Mativandlela *et al*, 2008).

A study conducted in Nigeria discovered that local communities regularly consult traditional healers because they are familiar with the culture of the patients and are found within a short distance, with the cost of treatment being fair (Rinne, 2001). Similarly, medicinal plants are vital in the lives of South Africans, particularly those from rural areas, because medicinal plants are their source of primary health, form part of their religion and are also a source of income from ancient times (Borokini and Omotayo, 2012; Liu *et al*., 2007).

For instance, it was reported in 2013 that communities in the rural areas of Limpopo Province, South Africa, depend on traditional medicine for the treatment and management of TB (Semenya and Maroyi, 2013). It was estimated that about 80% of the South African population uses traditional medicine for their healthcare needs at some stage of their life (Brandt and Muller, 1995). Furthermore, most of the people from rural areas in South Africa depend on the use of folk medicine for the treatment of diseases due to their limited access to formal healthcare services (Hossan, 2010).

Plant species exceeding 30 000 are indigenous to South Africa; among those, 3000 are used in traditional medicine across the country (van-Wyk *et al*., 1997). Although South Africa is abundant with plant biodiversity, with most of them being vital for medicinal uses, this diversity is continually declining based on an estimation that about half a million people are involved in the trading of medicinal plants in South Africa, which puts them at risk of being extinct (Afolayan and Adebola, 2005).

For example, *Solanum aethiopicum* is threatened with extinction in that it is the most traded medicinal plant in the Limpopo Province; in fact, it is already extinct in KwaZulu

Natal (Moeng and Potgieter, 2011). Furthermore, the standard harvesting method for most of the medicinal plants from the wild involves the removal of the rhizomes and roots, resulting in the complete eradication of the plant; hence, their depletion (Bodeker, 1997).

Documentation of the research findings on the traditionally used medicinal plants is a possible solution to help prevent the depletion and extinction of these plants, mainly because indigenous knowledge is passed orally from generation to generation. The use of medicinal plants in the health sector has several benefits as opposed to modern health systems. A survey conducted to determine the effectiveness of plant-based remedies in folk medicine had a great outcome as it discovered that they have minimal side effects in addition to being cheap. Medicinal plants have biological agents which are promising bioactive pharmacological compounds (Borokini and Omotayo, 2012).

Traditional medicine had a remarkable effect on treating diseases, including TB, malaria, HIV/AIDS, diabetes, mental disorders, and sickle-cell anaemia, thus reducing mortality, morbidity, and disability resulting from these diseases (Elujoba, 2004; Mathibela, 2013). Although biologically active compounds from medicinal plants can treat a variety of diseases and are assumed to be safe based on their long history of use, a lack of knowledge on the safe dosage given to adults and children poses a risk of toxicity to the patients (Fennelly *et al*., 2004; Gakuba, 2009). Some of the risks associated with the toxicity of medicinal plants on patients include the destruction of the red blood cells, irritation of the gastrointestinal tract, damage of the kidney and heart (Nondo *et al*., 2015).

It is, therefore crucial that cytotoxicity studies are conducted on all medicinal plants used in ethnopharmacology to establish their safe dosage as a way of appreciating their phytochemistry (Kudumela *et al*., 2018).

2.2 Phytochemistry

Plants have mechanisms that enable them to maintain their growth and protect themselves from unfavourable environmental conditions that may arise due to seasonal changes, infections from pathogens such as bacteria, fungi, and viruses, and predators that prey on the plants and insects, among other factors (Li *et al.*, 1993; Wink, 2003; Yang *et al*., 2018). They produce primary metabolites that are essential for their growth and development and secondary metabolites whose primary role is to

protect plants from factors that may harm them (Fernie and Pichersky, 2015; Kroymann, 2011).

Due to this, secondary metabolites are produced in low concentrations because they are produced occasionally to cater to the stressful conditions that may arise in the plants to allow for their survival (Ncube and Van Staden, 2015). The use of plants for traditional medicinal purposes is due to the remarkable biological activities ascribed to plant secondary metabolites (Balandrin *et al*., 1985; Bates, 1985; Payne, 1991).

Some of the common secondary metabolites produced by plants include large molecules such as flavonoids, steroids, alkaloids, phenolic acids, and terpenoids (Bourgaud *et al.,* 2001*;* Walton and Brown, 1999*)*. Flavonoids have gained increased interest in medical research, where properties such as enzyme inhibition, anti-inflammatory, antiallergic, antioxidant, and antimicrobial activities have been reported. Almost all plants possess flavonoids that can protect them because of their ultraviolet screening properties (Aron and Kennedy, 2008; Cushnie and Lamb, 2005).

Phenolic compounds have antioxidant capabilities that are attributed to the chelate metal ions that are involved in the production of free radicals. They also have phenolic hydroxyl groups, which enable the phenolics to act as antioxidants by inhibiting some of the enzymes that are involved in radical generation (Parr and Bolwell, 2000).

Antioxidant activity from the tannins is noteworthy given their ability to scavenge free radicals, chelate trace metals, and suppression of enzymatic activity by binding to the proteins (Fingerman and Nagabhushanam, 2006). The secondary metabolites produced by medicinal plants are believed to have little or no toxic effects that are capable of either inhibiting or killing microorganisms (Cowan, 1999).

The secondary metabolites of plants used in the pharmacological sector to produce antibiotics have shown very promising activity against most of the causative agents for diseases that displayed resistance to common antibiotics.

2.3 Antibiotic resistance

Antibiotics are the major sources of the control and management of infectious diseases globally. They have reduced the number of deaths caused by infectious diseases over the years. For instance, they have contributed to the reduction of TB-related deaths following the introduction of kanamycin and streptomycin. Antibiotics

are classified into six categories based on their mechanism of action: cell wall synthesis inhibitors, protein synthesis inhibitors, DNA intercalators, DNA synthesis inhibitors, RNA synthesis inhibitors (Arenz and Wilson, 2016; Voorhees and Ramakrishnan, 2013).

Antibiotic resistance is the tolerance of disease-causative agents to the drugs that are synthesised to either inhibit the growth of or kill these agents. It presents a financial threat to the global health system due to the continual emergence of antimicrobial resistance (World Bank, 2017). Martinez *et al*. (2015) suggest that a strain is resistant to an antibiotic if its Minimum Inhibitory Concentration (MIC) is higher than that of the corresponding parental wild-type strain. The evolution of the resistant strains towards existing antibiotics is due to the over-reliance and extensive use of antibiotics while undesirable side effects like the depression of the bone marrow, nausea, agranulocytosis, and thrombocytopenic result from the use of some antibiotics (Marchese and Schito, 2000; Mundy *et al*., 2016).

Furthermore, a resistant gene was defined as one whose presence allows for a bacterium to withstand a higher antibiotic concentration or whose absence increases the susceptibility of the antibiotic (Bengtsson-Palme *et al*., 2017; Martínez *et al*., 2007). The main route of exposure for resistant microorganisms to the humans is the same as the route for infectious bacteria in general, which occurs by infection from person to person either in community settings or in clinics (Levin *et al*., 2014; Livermore, 2000).

Given the abundance of plant biodiversity in South Africa, the synthesis of antibiotics with improved efficacy from medicinal plants is quite promising (Afolayan and Adebola, 2005). Possible antibiotics with improved efficacy can be synthesised from the diversity of medicinal plants in South Africa for the treatment of TB as one of the leading causes of death in developing countries such as South Africa.

2.4 Tuberculosis

TB is an infectious bacterial disease caused by an airborne, acid-fast bacillus, *Mycobacterium* complex, which consists of *Mycobacterium tuberculosis, Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti,* and *Mycobacterial bovis* Bacille Calmette-Guerin (BCG) (Fennelly *et al.*, 2004). *Mycobacterium tuberculosis* is a non-motile, non-spore-forming, rod-shaped

bacterium that has no capsule and has considerably slow growth. It has a generation time of about 24 hours on both the infected animals and synthetic medium (Cole *et al*., 1998).

The laboratory growth of *M*. *tuberculosis* on a solid media requires about 3 to 4 weeks to form visible colonies whose surface is dry and wrinkled (Bloom and Murray, 1992). TB is transmitted between humans through inhalation of droplets expelled from either a cough or sneeze from an infected person (the respiratory route) (Fennelly *et al*., 2004). The infection of TB is either latent TB infection or active TB disease. Latent TB infection is when the causative agent of TB remains in an infected person in an inactive state where there is no bacterial metabolism, but; it is not eradicated and does not cause TB and cannot be transmitted. However, the bacterium remains in the body of the infected person, and it is reactivated into active TB when the immune system is compromised even after decades after the initial infection (Parrish, 1998).

Active TB is the infectious form of TB that weakens the immune system causing the occurrence of TB symptoms (Parrish, 1998; WHO, 2015). TB is one of the leading causes of death annually and in 2017, it was estimated that approximately 1.3 million people died from it (WHO, 2018). Although TB was first identified in 1882 by Dr. Robert Koch, its effective antibiotics were only discovered and tested in clinical trials between the 1940s and 1950s as isoniazid, streptomycin, and *para*-aminosalicylic acid (Fox *et al*., 1999). The causative agent for TB developed resistance against the first-line TB antibiotics (rifampicin and isoniazid) over the years, which led to the emergence of multidrug-resistant TB (WHO, 2018).

The global health care system is burdened by the resistance of the causative agents of a variety of diseases towards existing antibiotics, and TB is no exception (Mabhula and Singh, 2019). To compensate for the resistance towards the first-line TB antibiotics, second-line TB antibiotics are used as treatment. However, resistance towards the second-line TB antibiotics has led to the emergence of extensively drug-resistant TB (XDR-TB) (WHO, 2018). The effective use of the current TB drugs requires months of combined therapy, which usually leads to compliance issues as well as notable side effects (Vikrant, 2011).

Furthermore, the shifting of healthcare resources towards the coronavirus disease poses an alarming risk to the control and prevention of TB, which may lead to a spike

in TB-related infections and deaths (Evans, 2020; Togun *et al*., 2020). There are three major drawbacks to the elimination of TB: firstly, the continual emergence of resistance of the causative agent of TB towards first-line TB antibiotics (isoniazid and rifampicin) and second-line TB antibiotics (levofloxacin, moxifloxacin, bedaquiline, delamanid, and linezolid) leading to multidrug-resistant, extensive drug-resistant and extreme drug-resistant TB (WHO, 2020).

Secondly, the complications associated with TB control and treatment are ascribable to HIV and other comorbidities, which attack the weakened immune system with a high burden in Sub-Saharan Africa and Asia (Granich and Gupta, 2018). Thirdly, the prevalence of non-communicable diseases with their combined risk factors, which include indoor pollution, alcohol, diabetes, smoking, drug abuse and malnutrition (Liu *et al.*, 2017; Noubiap *et al.*, 2019). WHO implemented the “End TB Strategy” with the aim to improve the early diagnosis of the disease, reduce the global number of TB-associated deaths, and develop enhanced treatment options, such as the synthesis of new TB drugs (Uplekar *et al*., 2015).

The treatment of drug-sensitive TB involves the use of multiple first-line drugs such as rifampicin, isoniazid, ethambutol, and pyrazinamide, which are used in combination for a minimum of six months (WHO, 2017). TB patients face the challenge of taking many antibiotics daily as part of their treatment, which usually leads to subsequent side-effects. The side-effects reported by the patients include nausea, fatigue, heartburn, skin rash, weight loss, numbness in the legs or arms, and loss of appetite.

This challenge is increased in the patients with multi-drug resistant TB because their treatment is longer and is often increased to up to 24 months, where the injectable drugs are included (Hansel *et al*., 2004; Marra *et al.*, 2004; Ting *et al*., 2020; WHO, 2010). In some cases, the side-effects caused by the treatment of TB become severe to a point where the treatment must be paused because the drugs are potentially toxic and an adjusted treatment regimen is required (Ting *et al*., 2020; Yee *et al*., 2003).

There is an urgent need for the development of novel TB drugs to keep up with the continual emergence of drug-resistant *Mycobacterium tuberculosis* (Anochie, 2018). Medicinal plants are routinely screened to discover new bioactive compounds as they are considered a great source of drugs for the traditional healthcare system as well as synthetic pharmaceutical drugs (Ncube *et al*., 2008).

Traditionally used medicinal plants displayed promising bioactive compounds against resistant strains of TB over the years; hence, the wide interest in screening for antimycobacterial compounds from medicinal plants. For instance, a wide range of traditional medicinal plants prescribed to TB patients by the Bapedi traditional healers has been proven to have potential antimicrobial activity for several infectious pathogens (Semenya and Maroyi, 2013).

In the continual studies towards the discovery of alternative TB drugs from medicinal plants, a variety of promising antimycobacterial compounds have been discovered based on their traditional use as medicine for different diseases. For example, *Knowltonia vesicatoria* is a plant traditionally used to treat TB in South Africa. This plant demonstrated antimycobacterial activity from the isolated compounds, namely, 5-(hydroxymethyl) furan-2(5H)-one and 5-(hydroxymethyl) dihydrofuran-2(3H)-one (Efange, 2002).

Phytochemical constituents which show antimycobacterial activity include tannins, phenolics, alkaloids, sterols, glycosides, quinones, xanthones, triterpenoids, etc. (Anochie, 2018) It was previously reported that the extracts from *Tabernaemontana elegans*, which is used by the Zulu and Vhavenḓa people of South Africa showed antimycobacterial activity against *M. smegmatis* (Mehta *et al*., 2013).

Triterpene euscaphic, which exhibits antimycobacterial activity against the sensitive strain of *M*. *tuberculosis*, is a phytochemical compound isolated from the *Lippia javanica* (Mohamad *et al*., 2011). Another plant used traditionally in South Africa to treat TB is *Knowltonia vesicatoria*, which was used to investigate synergy by combining its extracts with isoniazid. The compounds isolated from this plant also demonstrated antimycobacterial activity against the sensitive strain of *M. tuberculosis* (Efange, 2002). However, some of the compounds and affective plant parts with potential bioactivity against targeted microorganisms may present health risks to humans due to cytotoxicity (Ricci and Zong, 2006). As a result, low-toxicity antimicrobial agents are desired for the development of novel drugs for a variety of diseases (Komape *et al*., 2014).

There is a variety of approaches and techniques involved in the screening of bioactive compounds from medicinal plants to synthesise alternative drugs for diseases. These

include extraction methods, antimicrobial activity assays, separation, purification and characterisation techniques.

2.5 Extraction techniques for medicinal plants

Extraction is a process by which the medicinal plant material is subjected to a selective solvent that diffuses into the plant to attain portions of the plant compounds that are therapeutically desired while eliminating unwanted material. The extract obtained is composed of a mixture of complex plant secondary metabolites which have medicinal uses and are referred to as crude plant material (Handa *et al*., 2008). A variety of factors such as the extraction procedure, the plant part used as a starting material, and the solvent used for extraction, must be taken into consideration before research is conducted on the medicinal plants because they affect the quality of medicinal plants (Ncube *et al*., 2008).

Various techniques can be used to extract bioactive compounds from medicinal plants, including maceration, digestion, infusion, decoction, sonication, and serial exhaustive extraction (Handa *et al*., 2008). Maceration is the type of extraction where either the whole or powdered plant material is mixed with a specific solvent and then agitated for a defined period so that soluble compounds from the plant material can dissolve into the solvent (Ncube *et al*., 2008). Digestion is the type of maceration where gentle heat is applied to the plant material to increase the efficiency of the plant material. The type of extraction where the plant material is macerated for a short period using either cold or boiling water is referred to as infusion (Remington and Beringer, 2006).

Sonication is the type of extraction that involves the subjection of the plant material to an ultrasound which permeates the cell wall of the plant material. However, the ultrasound causes the damage of the bioactive compounds on the medicinal plants through the formation of free radicals, leading to undesirable changes in the bioactive compounds (Handa *et al*., 2008). The decoction is the extraction method that is suitable for the bioactive compounds that are heat stable and water-soluble. It involves boiling the constituents for a defined period, followed by cooling, staining and then passing sufficient cold water through the bioactive compounds to produce the desirable volume (Remington and Beringer, 2006).

In serial exhaustive extraction, the constituents from the plant material are successively extracted by solvents of varied polarity, which range from non-polar

(hexane) to polar (methanol) to extract a wide range of compounds (Das *et al*., 2010). Among the mentioned extraction techniques, maceration is the commonly used method for medicinal plant research, given its ability to extract and expose a variety of compounds depending on the extraction solvents of choice. Separation techniques such as TLC are of utmost importance in profiling the variety of compounds from the plant material to explore the phytochemical constituents, antioxidants, and antimicrobial activity.

2.6 Chromatographic separation techniques

Chromatography is a highly effective and simple technique that separates molecules in mixtures. It consists of two immiscible phases: the stationary phase, which is in a fixed position, and the mobile phase, which flows through the interstices of the stationary phase along with molecules in mixtures (Porath, 1997). The stationary phase is always made of either a solid phase or a layer of liquid absorbed on the surface of solid support while the mobile phase is composed of either a gaseous or liquid component.

The movement of the mobile phase, which can either be a liquid or gas, is caused by either capillarity, gravity, or pressure (Gerberding and Byers, 1998). The chromatography technique accounts for a sufficiently good separation of molecules within a suitable time interval. Some of the chromatographic techniques include TLC column chromatography, and high-pressure liquid chromatography (Harwood and Moody, 1989).

2.6.1 Thin Layer Chromatography

The stationary phase of TLC is a solid adsorbent substance. The mobile system in this technique moves upwards through the stationary phase. The movement of the mobile system is through capillary action (Ryder, 1991). TLC allows access to the separated sample for post-chromatographic evaluation within a short period. This technique is inexpensive and ensures minimal sample preparation steps while also suitable for an analysis of those substances with poor detection characteristics which require post-chromatographic treatment for detection (Fried and Sherma, 1996).

2.6.2 Column chromatography

Column chromatography is suitable for the purification of biomolecules as it allows the simple and quick isolation of the desired compounds from a mixture. The stationary phase of column chromatography is a solid adsorbent which can either be silica gel aluminium or cellulose that is placed in a vertical glass column. The mobile phase is a liquid that is added at the top of the column after the stationary phase and flows down through the column by gravity. The compounds in the column are separated through the varying absorption and interaction between the stationary and mobile phases. The purified sample is collected at the bottom of the column in a time-volume dependant manner (Das and Dasgupta, 1998).

2.6.3 High-Pressure Liquid Chromatography (HPLC)

The utmost important components of HPLC device are commercially prepared column, high-pressure pump, solvent depot, detector and recorder. In this technique, the mobile phase passes through columns that have atmospheric pressure under 10-400 and a high flow rate of 0.1-5 cm/sec. The fact that the analysis of samples with HPLC occurs within a short time is attributed to the use of small particles as well as the application of high pressure on the rate of solvent flow, which also increases separation power (Regnier, 1983).

2.7 Antimicrobial activity bioassays

2.7.1 Bioautography

Bioautography is a method suitable for the detection of microbial growth inhibition by the application of TLC (Nyiredy, 2001). It is a cheap, simple, and effective method, which is good for the identification of bioactive leads from plant extracts. It is competent for the chemical and biological screening of complex plant extracts before bioassay-guided isolation. This technique is suitable not only for small research laboratories with minimal access to advanced types of equipment, but also for those highly developed laboratories (Simões-Pires *et al*., 1990).

TLC-based bioautography was first introduced in 1961 by Fisher and colleagues (Fischer and Lautner, 1961; Nicolaus *et al*., 1961). There are three types of bioautography methods, which include agar diffusion or contact bioautography,

immersion or agar overlay bioautography, and direct TLC bioautography (Rios *et al*., 1988; Wagman and Bailey, 1969).

Contact bioautography (figure 2.1) involves the diffusion of antimicrobial agents separated on a TLC plate to an inoculated agar by placing the TLC plate upside down on the agar for a specific period. This is followed by the removal of the TLC plate on the agar, and thereafter, the agar is incubated. Antimicrobial activity is indicated by the zones of inhibition on the agar after the incubation period (Meyers and Smith, 1964; Narasimhachari and Ramachandran, 1967; Sherma, 2008). However, it is difficult to obtain complete contact between the agar and the TLC plate, and in addition to this, the varied ways by which components from the TLC plate diffuse to the agar, especially those not soluble in water, is problematic (Wagman and Bailey, 1969).

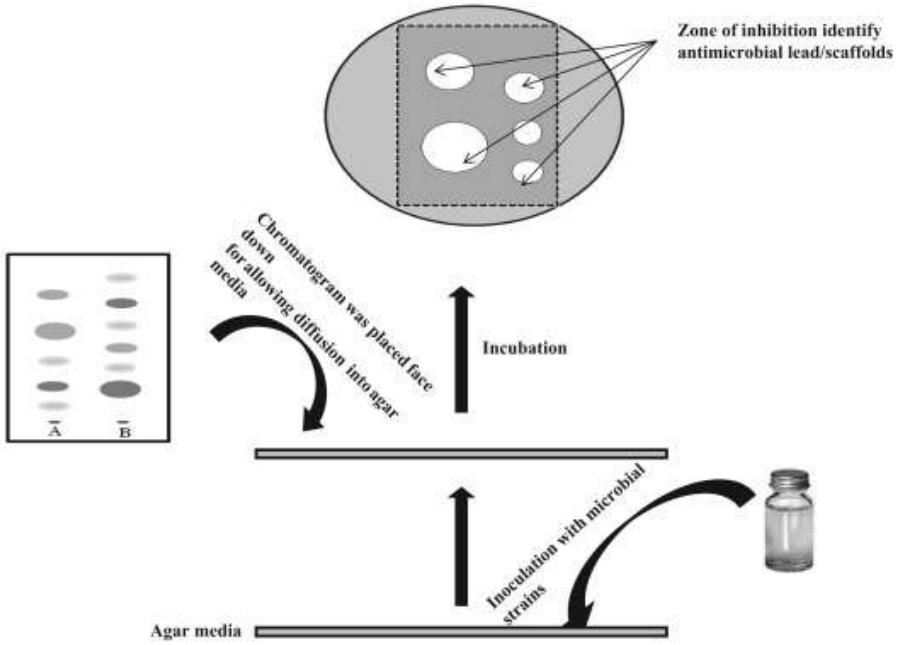

Figure 2.1: Schematic representation of contact bioautography (Dewanjee *et al.*, 2015).

In immersion bioautography (figure 2.2), the developed TLC plate is covered with molten, seeded agar medium and left for solidification. This is followed by incubation after which the medium is stained to visualise the zones of inhibition.

Figure 2.2: Schematic representation of immersion bioautography (Dewanjee *et al.*, 2015).

Direct bioautography (figure 2.3) involves spraying or dipping the developed TLC plate in the test fungal or bacterial suspension, followed by incubation of the plate in humid conditions. After the incubation period, the plate is sprayed or dipped in a growth indicator and further incubated for a specific period to visualise the zones of inhibition as bands (Meyer and Dilika, 1996; Schmourlo *et al*., 2004; Silva *et al*., 2005). Direct bioautography is suitable for rapid chemical and biological screening of plant extracts and it is the most used bioautographic technique amongst the listed three (Choma and Grzelak, 2011; Masoko *et al*., 2005; Paxton, 1991).

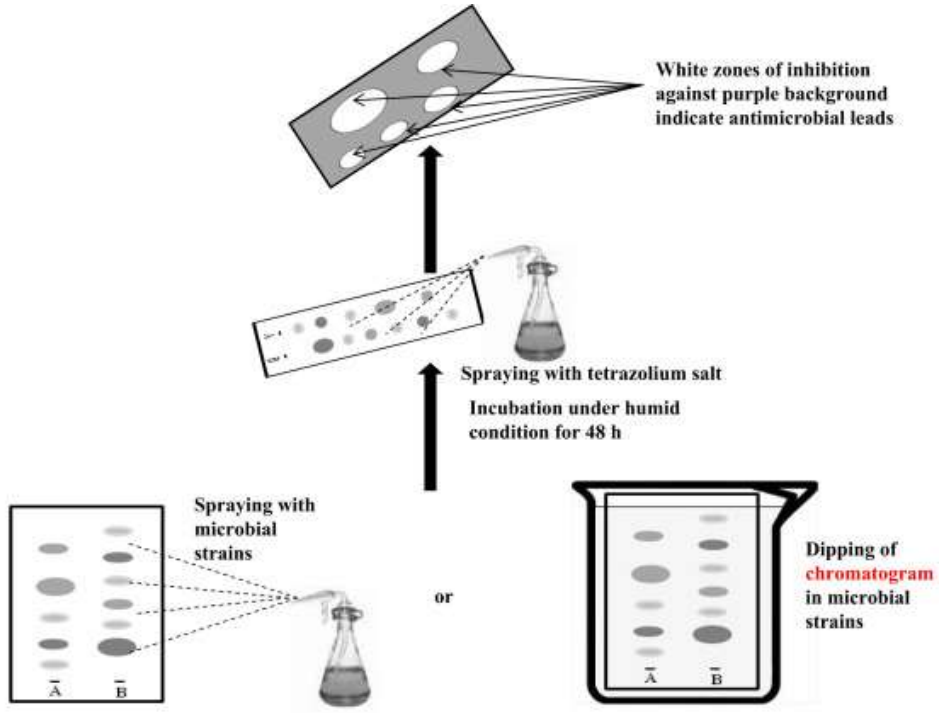

Figure 2.3: Schematic representation of direct bioautography (Dewanjee *et al.*, 2015).

2.7.2 Broth-dilution assays

The most common antimicrobial susceptibility tests are the broth-microdilution and macro-dilution procedures. They are both suitable for the determination of the minimum concentration of the antimicrobial agent that can inhibit the growth of microorganisms. Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that can completely inhibit the growth of the test microorganism as indicated by the observation of the growth or inhibition indication in the tubes or microdilution wells with the naked eye (Pfaller, 2004).

Micro-dilution assay involves the use of smaller volumes of the broth culture and antimicrobial agents in 96 well micro-titration (figure 2.4) plates while macro-dilution assay uses larger volumes in test tubes and are both incubated under defined temperature normally without agitation. However, the drawbacks of the macro-dilution assay are the increased risk of errors when preparing the antimicrobial solutions for each test, manual undertaking, large amounts of reagents, and space required (Jorgensen and Ferraro, 2009).

Figure 2.4: A 96-well microtiter plate used for microdilution assay (Balouiri *et al*., 2016)

Therefore, micro-dilution assay is the most commonly used technique in these procedures given the careful management of reagents, the space, and the reproducibility that occurs when this technique is used (Balouiri *et al*., 2016; Cockerill, 2012). Hence, the use of the micro-dilution assay by several researchers for the determination of the MIC of the antimicrobial agents for a variety of test microorganisms over the years (Kudumela *et al*., 2018; Masoko and Makgapeetja, 2015; Matotoka and Masoko, 2017).

2.8 Bioassay-guided isolation

Bioassay-guided fractionation is the advanced method for the identification of bioactive natural products. It comprises repetitive preparative-scale fractionation and evaluation of biological activity until the isolation of pure constituents with the targeted biological activity is achieved (Brusotti *et al*., 2014). The main steps followed to utilise bioactive compounds from plant sources include extraction, pharmacological screening, isolation and characterisation of toxicological and clinical evaluations (Sasidharan *et al*., 2011; Wall *et al*., 1996). Extraction targets the high priority extracts, specifically those with a wide range of polarity or those that are enriched with common secondary metabolites such as saponins and alkaloids (Keller, 1983; Wall *et al*., 1996).

Phytochemical screening is vital for the investigation of possible bioactive compounds from plant extracts so that data on them is obtained. Isolation and identification of bioactive compounds procedures are coupled with purification and separation processes to track down the phytochemical compounds with potential pharmacological activity (Brusotti *et al*., 2014). The identification and characterisation of bioactive compounds from plant sources is a challenge based on the norm that plant extracts often act synergistically or have different polarities.

Furthermore, the isolation of bioactive compounds requires the integration of different separation methods (Sticher, 2008). These include TLC, Column Chromatography, Flash Chromatography, Sephadex Chromatography, and High-Pressure Liquid Chromatography (HPLC) (Sasidharan *et al*., 2011). The biological activity of plant extracts can be evaluated by the antimicrobial and antifungal assays, which includes TLC bioautographies that are easy to use. These are broadly exploited in small laboratory settings based on their simplicity in giving an idea of the presence or absence of bioactive compounds (Valgas *et al*., 2007). The technique of TLC bioautography can only profile bioactive molecules on the TLC plate; hence, it is important that follow-up tests are conducted for the determination of the specific components that play a role in the bioactivity observed. The standard techniques used to determine the structure of the bioactive compounds include Nuclear Magnetic Resonance (NMR), Electron Ionisation Mass Spectrometry (EI-MS), and Electrospray Ionisation Mass Spectroscopy (ESI-MS) (Dewanjee *et al*., 2015). Nuclear Magnetic Resonance technique is the preferred follow-up technique for determining bioactive compounds because it has been previously reported that TLC bioautography with NMR technology produce fruitful information (Wang *et al*., 2021).

Once the bioactivity of a compound has been established from the crude extracts, it is isolated from the complex mixture by purification. Although bioassay-guided fractionation ensures the isolation of targeted bioactive compounds from complex extracts, it is perceived to be labour-intensive, time-consuming, and expensive (Bero *et al*., 2011; Manvar *et al*., 2012).

Plant extracts are rich in chemical diversity when either utilised as standardised extracts or as pure compounds. This feature accounts for the unlimited drug discovery potential of plant extracts (Cos *et al*., 2006). However, plant extracts may be potentially toxic at a certain dosage. As a result, cytotoxicity evaluation of the plant extracts is key to ensuring the quality, efficacy and safety of their preparations (Schimmer *et al*., 1994).

WHO recommends that the practice of herbal medicine as a source of healthcare is advised only after establishing the non-toxicity of the herbal preparations (Akintonwa *et al*., 2009). Nuclear Magnetic Resonance (NMR) spectroscopy is commonly used for the characterisation of pure bioactive compounds.

2.9 Characterisation of bioactive compounds

NMR spectroscopy is an analytical technique used for the characterisation of purified bioactive compounds. It is the preferred technique because it is non-destructive and completely non-invasive (Gadian, 1996). The non-specificity of this technique allows for the detection of unexpected compounds. This explanation is authenticated by the identification of previously unrecognised phosphate-containing metabolites from extracts of both normal and dystrophic muscles by NMR techniques. The prime restriction of NMR spectroscopy is the lack of sensitivity and the requirement of prolonged recordings (Newman, 1984). The choice of a nucleus in NMR spectroscopy is important. The protons (^{1}H) nucleus are employed in most NMR experiments because of their sensitivity. The ^{1}H NMR spectroscopy results in the recording of spectra with a broad number of resonances due to the use of high molecular weight molecules. The ^{13}C NMR has applications to the investigation of intermediary metabolism (Alger *et al*., 1981). NMR spectroscopy is a powerful technique in the medical sciences and has been used by chemists and physicists for a long time because of its uniqueness.

The basic principle of NMR spectroscopy is that nuclei of some of the atoms, including hydrogen (^{1}H), carbon (^{13}C), phosphorus (^{31}P), sodium (^{23}Na), and fluorine (^{19}F), are magnetic dipoles. NMR spectroscopy is a preferred technique because it is non-invasive, non-destructive; in addition, it is thought to be free from biological hazards. NMR spectroscopy also gives rise to intracellular environmental information of the metabolites, which is normally lost during the freeze clamping process. According to the literature, any nucleus with an odd mass number, such as ^{1}H, ^{13}C, ^{23}Na, and ^{17}O, can be studied using NMR (Ackerman *et al*., 1980).

In this technique, the nuclei of atoms are used to align with or misalign with the field in the presence of a strong magnetic field. Subsequently, the nuclei are tipped away from their orientation along the magnetic field lines using a pulse of perturbing electromagnetic energy at the resonant frequency. The termination of the perturbing radiofrequency pulse leads to the return of the nuclei to their initial lower energy orientations where they emit energy. This energy can be picked up by a radiofrequency receiver, which transforms it into a spectrum with the assistance of a computer. The different nuclei resonate with the same magnetic field at very different

frequencies. The differences in the chemical bonding of individual nuclei give rise to the individual peaks of the spectrum. There is proportionality between the areas of the resonance peaks and concentrations of the individual nuclei resonating at that certain frequency (Shulman *et al*., 1984).

NMR is relevant in all four stages of drug discovery, which are: target identification, high-throughput screening of hit compounds, hit validation, and lead optimisation (Torres and Orts, 2018).

2.10 Medicinal plant selected for this study

Cassia petersiana (figure 2.5) is a small to medium-sized (shrub) tree from the Fabaceae family, which mostly grows on sandy soil up to a height of about 12 m with an altitude of up to 1050 m above sea level. It is commonly known as the Monkeypod in English, *Munembenembe* in Venda (Tshidzumba, 2018), and its common scientific name is *Senna petersiana* (Steenkamp, 2003). Its flowers are yellow, and it has pinnate leaves, which are used for the treatment of typhoid fever in Cameroon and the cure of skin diseases in Southern Africa. It is also used as a febrifuge, treatment of fever, skin infections and gonorrhea (Djemgou *et al*., 2007). Its roots are also used for the treatment of arthritis by consumption of the boiled ground dried roots (Tshidzumba, 2018).

Figure 2.5: *Cassia petersiana* plant (Burrows *et al*., 2018).

It has a brown, fissured, and rough bark. It is thin, hairy, and its long pods which are often slightly constricted between the seeds are either eaten raw or cooked as gruel. Its roots are used as a treatment for colds, coughs, stomach aches and syphilis (Msonthi, 1984). The isolated compounds from the crude leaf extracts of *Cassia petersiana* showed antibacterial activity against *Salmonella typhi*, *Salmonella paratyphi* A and *Salmonella paratyphi* B (Djemgou *et al*., 2007).

Cassia petersiana commonly occurs along rivers and streams. The root bark of this plant is used to treat a dog injured from the horns of an antelope given in either soup or milk. The same concoction can also be used to increase the appetite for a dog to make it more eager to hunt (Coetzee *et al*., 2000). The leaves and roots of this plant are used as a purgative to treat constipation, stomach ache, and intestinal worms (Hutchings *et al*, 1996; van-Wyk *et al*, 1997). The flavonoid compound, luteolin, was previously isolated from this plant and proven to show antibacterial activity against *Bacillus cereus*, *Bacillus pumilus*, and *Staphytococcus aureus* (Tshidzumba, 2018). Luteolin from Senna petersiana was also proven to have anti-HIV activity (Tshikalange *et al*., 2008).

2.11 Microorganism used in the study

Mycobacterium smegmatis is closely related to the causative agent of TB, *Mycobacterium tuberculosis* (Hentschel *et al*., 2017). *Mycobacterium* complex entails pathogenic microorganisms that pose several limitations to antimycobacterial research even though they are of greater clinical importance (Nash, 2003).

Although it is not clinically important, *Mycobacterium smegmatis* is a suitable and model organism for mycobacterial research because of its non-pathogenicity, fast-growth as used by most researchers in antimycobacterial studies over the years (Lelovic *et al*., 2020; Smith, 2003; Sundarsingh *et al*., 2020). Vonmoos *et al*. (1983) were the first to discover the pathogenicity of *Mycobacterium smegmatis* to humans, and several reports followed reporting on its cause of skin and soft-tissue infections (Best and Best, 2009; Newtown *et al*., 1993). *Mycobacterium smegmatis* has been found to be non-pathogenic and fast-growing than other *Mycobacterium* species (Newton and Fahey, 2002).

Aim

The aim of the study is to investigate the antimycobacterial and cytotoxic compounds of *Cassia petersiana*.

Objectives

The objectives of this study were to:

i. Evaluate the phytochemical constituents of *C. petersiana* using TLC.
ii. Determine the presence of antioxidant compounds in C. *petersiana* using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and quantify total antioxidant activity using ferric ion reducing antioxidant power (FRAP) procedure and DPPH scavenging activity assay.
iii. Evaluate the antimycobacterial activity of crude extracts in *C. petersiana* against *Mycobacterium smegmatis* using serial broth dilution and bioautographic assays.
iv. Isolate bioactive compounds with antimycobacterial and antioxidant activity using column chromatography and preparative TLC.
v. Characterise isolated compounds from *C. petersiana* using spectroscopy**.**
vi. Analyse the effects of the crude extracts for cytotoxicity on tryptophan hydroxylase-1 *(*TPH-1) macrophage cells using cell viability assay.

2.12 References

Ackerman, J., Grove, T., Wong, G., Gadian, D. and Radda, G., 1980. Mapping of metabolites in whole animals by 31P NMR using surface coils. *Nature*, 283(5743), pp.167-170.

Afolayan, A. and Adebola, P., 2005. *In vitro* propagation: a biotechnological tool capable of solving the problem of medicinal plants decimation in South Africa. *African Journal of Biotechnology*, 3(12), pp.683-687.

Akintonwa, A., Awodele, O., Afolayan, G. and Coker, H., 2009. Mutagenic screening of some commonly used medicinal plants in Nigeria. *Journal of Ethnopharmacology*, 125(3), pp.461-470.

Alger, J., Sillerud, L., Behar, K., Gillies, R., Shulman, R., Gordon, R., Shaw, D. and Hanley, P., 1981. *In Vivo* Carbon-13 Nuclear Magnetic Resonance studies of mammals. *Science*, 214(4521), pp.660-662.

Anochie, P., 2018. African Medicinal plants that can control or cure tuberculosis. *International Journal of Pharmaceutical Sciences and Developmental Research*, 4(1), pp.001-008.

Arenz, S. and Wilson, D., 2016. Bacterial protein synthesis as a target for antibiotic inhibition. *Cold Spring Harbor Perspectives in Medicine*, 6(9), pp.a025361.

Aron, P. and Kennedy, J., 2008. Flavan-3-ols: nature, occurrence and biological activity. *Molecular Nutrition & Food Research*, 52(1), pp.79-104.

Balandrin, M., Klocke, J., Wurtele, E. and Bollinger, W., 1985. Natural plant chemicals: sources of industrial and medicinal materials. *Science*, 228(4704), pp.1154-1160.

Balouiri, M., Sadiki, M. and Ibnsouda, S., 2016. Methods for *in vitro* evaluating antimicrobial activity: a review. *Journal of Pharmaceutical Analysis*, 6(2), pp.71-79.

Bates, D., 1985. Plant utilization: patterns and prospects. *Economic Botany*, 39(3), pp.241-265.

Bengtsson-Palme, J., Kristiansson, E. and Larsson, D., 2017. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology Reviews*, 42(1), pp.68-80.

Bero, J., Hannaert, V., Chataigné, G., Hérent, M. and Quetin-Leclercq, J., 2011. *In vitro* anti-trypanosomal and anti-leishmanial activity of plants used in Benin in traditional medicine and bio-guided fractionation of the most active extract. *Journal of Ethnopharmacology*, 137(2), pp.998-1002.

Best, C. and Best, T., 2009. *Mycobacterium Smegmatis* infection of the hand. *HAND*, 4(2), pp.165-166.

Bloom, B. and Murray, C., 1992. Tuberculosis: commentary on a reemergent killer. *Science*, 257(5073), pp.1055-1064.

Bodeker, G., Bhat, K., Burley, J. and Vantomme, P., 1997. Medicinal plants for forest conservation and health care. 11th ed. Rome: Food and Agriculture Organization of the United Nations, pp.1-158.

Borokini, T. and Omotayo, F., 2012. Phytochemical and ethnobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research*, 6(7), pp.1106-1118.

Bourgaud, F., Gravot, A., Milesi, S. and Gontier, E., 2001. Production of plant secondary metabolites: a historical perspective. *Plant Science*, 161(5), pp.839-851.

Brandt, H. and Muller, G., 1995. Traditional medicines and acute poisoning. *Chicago Mercantile Exchange*, 13, pp.1053-1060.

Brusotti, G., Cesari, I., Dentamaro, A., Caccialanza, G. and Massolini, G., 2014. Isolation and characterization of bioactive compounds from plant resources: the role of analysis in the ethnopharmacological approach. *Journal of Pharmaceutical and Biomedical Analysis*, 87, pp.218-228.

Burrows, J., Burrows, S., Schmidt, E., Lotter, M. and Wilson, E., 2018. Trees and shrubs Mozambique. 1st ed. Cape Town: Publishing Print Matters, p.1052.

Choma, I. and Grzelak, E., 2011. Bioautography detection in thin-layer chromatography. *Journal of Chromatography A*, 1218(19), pp.2684-2691.

Cockerill, F., 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 9th ed. Wayne, Pa: Clinical and Laboratory Standards Institute, pp.16-43.

Coetzee, J., Mciteka, L., Malan, E. and Ferreira, D., 2000. Structure and synthesis of the first procassinidin dimers based on epicatechin, and gallo- and epigallo-catechin. *Phytochemistry*, 53(7), pp.795-804.

Cole, S., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S., Eiglmeier, K., Gas, S., Barry, C., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M., Rajandream, M., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J., Taylor, K., Whitehead, S. and Barrell, B., 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393(6685), pp.537-544.

Cos, P., Vlietinck, A., Berghe, D. and Maes, L., 2006. Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. *Journal of Ethnopharmacology*, 106(3), pp.290-302.

Cowan, M., 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12(4), pp.564-582.

Cushnie, T. and Lamb, A., 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, 26(5), pp.343-356.

Das, K., Tiwari, R. and Shrivastava, D., 2010. Techniques for evaluation of medicinal plant products as antimicrobial agent: current methods and future trends. *Journal of Medicinal Plant Research*, 4(2), pp.101-111.

Das, M. and Dasgupta, D., 1998. Pseudo-affinity column chromatography based rapid purification procedure for T7 RNA polymerase. *Preparative Biochemistry and Biotechnology*, 28(4), pp.339-348.

Dewanjee, S., Gangopadhyay, M., Bhattacharya, N., Khanra, R. and Dua, T., 2015. Bioautography and its scope in the field of natural product chemistry. *Journal of Pharmaceutical Analysis*, 5(2), pp.75-84.

Djemgou, P., Gatsing, D., Kenmogne, M., Ngamga, D., Aliyu, R., Adebayo, A., Tane, P., Ngadjui, B., Seguin, E. and Adoga, G., 2007. An antisalmonellal agent and a new

dihydroanthracenone from *Cassia petersiana*. *Research Journal of Medicinal Plant*, 1(2), pp.65-71.

Efange, S., 2002. Natural products: a continuing source of inspiration for the medicinal chemist. *Advances in Phytomedicine*, 1, pp.61-69.

Elujoba, A., Odeleye, O. and Ogunyemi, C., 2004. Traditional medicine development for medical and dental primary health care delivery system in Africa. *African Journal of Traditional, Complementary and Alternative Medicines*, 2(1), pp.46-61.

Fennelly, K., Martyny, J., Fulton, K., Orme, I., Cave, D. and Heifets, L., 2004. Cough-generated aerosols of *Mycobacterium tuberculosis*. *American Journal of Respiratory and Critical Care Medicine*, 169(5), pp.604-609.

Fernie, A. and Pichersky, E., 2015. Focus issue on metabolism: metabolites, metabolites everywhere. *Plant Physiology*, 169(3), pp.1421-1423.

Fingerman, M. and Nagabhushanam, R., 2006. Biomaterials from aquatic and terrestrial organisms. Enfield, United States of America: Science Publishers, pp.115-174.

Fried, B. and Sherma, J., 1996. Practical thin-layer chromatography-a multidisciplinary approach. 1st ed. Boca Raton: CRC Press, pp.1-288.

Gadian, D., 1996. *Nuclear magnetic resonance and its applications to living systems*. 2nd ed. Oxford: Clarendon Press, pp.1-300.

Gerberding, S. and Byers, C., 1998. Preparative ion-exchange chromatography of proteins from dairy whey. *Journal of Chromatography A*, 808(1-2), pp.141-151.

Evans, M., 2020. Covid's color line — Infectious disease, inequity, and racial justice. *New England Journal of Medicine*, 383(5), pp.408-410.

Fischer, R. and Lautner, H., 1961. Zum papierchromatographischen Nachweis von Penicillinpräparaten. *Archiv der Pharmazie*, 294(1), pp.1-7.

Fox, W., Ellard, G. and Mitchison, D., 1999. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. *International Journal of Tuberculosis and Lung Disease*, 3(10 Suppl 2), pp.S231-79.

Gakuba, E., 2009. Isolation and characterisation of secondary metabolites of two Asteraceae species, *Artemisia Afra* and *Elytropappus rhinocerottis.* Thesis, University of KwaZulu-Natal, Pietermaritzburg, South Africa.pp.1-91.

Granich, R. and Gupta, S., 2018. Two diseases, same person: moving toward a combined HIV and tuberculosis continuum of care. *International Journal of STD & AIDS*, 29(9), pp.873-883.

Handa, S., Khanuja, S., Longo, G. and Rakesh, D., 2008. Extraction technologies for medicinal and aromatic plants. Trieste (Italy): earth, environmental and marine sciences and technologies, pp.21-25.

Hansel, N., Wu, A., Chang, B. and Diette, G., 2004. Quality of life in tuberculosis: patient and provider perspectives. *Quality of Life Research*, 13(3), pp.639-652.

Harwood, L. and Moody, C., 1989. Experimental organic chemistry. Oxford: Blackwell Scientific, pp.180-185.

Hentschel, J., Burnside, C., Mignot, I., Leibundgut, M., Boehringer, D. and Ban, N., 2017. The complete structure of the *Mycobacterium smegmatis* 70S ribosome. *Cell Reports*, 20(1), pp.149-160.

Hossan, S., Agarwala, B., Sarwar, S., Karim, M., Jahan, R. and Rahmatullah, M., 2010. Traditional use of medicinal plants in Bangladesh to treat urinary tract infections and sexually transmitted diseases. *Ethnobotany Research and Applications*, 8, pp.61-74.

Jorgensen, J. and Ferraro, M., 2009. Antimicrobial Susceptibility Testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases*, 49(11), pp.1749-1755.

Keller, W., 1983. Reviews: introduction to alkaloids—a biogenetic approach. *Journal of Pharmaceutical Sciences*, 72(3), p.328.

Komape, N., Aderogba, M., Bagla, V., Masoko, P. and Eloff, J., 2014. Anti-bacterial and anti-oxidant activities of leaf extracts of *Combretum vendee* (Combretecacea) and the isolation of an anti-bacterial compound. *African Journal of Traditional, Complementary and Alternative Medicines*, 11(5), pp.73-77.

Kroymann, J., 2011. Natural diversity and adaptation in plant secondary metabolism. *Current Opinion in Plant Biology*, 14(3), pp.246-251.

Kudumela, R., McGaw, L. and Masoko, P., 2018. Antibacterial interactions, anti-inflammatory and cytotoxic effects of four medicinal plant species. *BMC Complementary and Alternative Medicine*, 18(1), pp.1-7.

Lelovic, N., Mitachi, K., Yang, J., Lemieux, M., Ji, Y. and Kurosu, M., 2020. Application of *Mycobacterium smegmatis* as a surrogate to evaluate drug leads against *Mycobacterium tuberculosis*. *The Journal of Antibiotics*, 73(11), pp.780-789.

Levin, B., Baquero, F. and Johnsen, P., 2014. A model-guided analysis and perspective on the evolution and epidemiology of antibiotic resistance and its future. *Current Opinion in Microbiology*, 19, pp.83-89.

Li, J., Ou-Lee, T., Raba, R., Amundson, R. and Last, R., 1993. Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. *The Plant Cell*, 5(2), pp.171-179.

Liu, F., Vind, J., Promchote, P. and Ly, P. 2007. Medicinal plants, its condition and socio economic impacts− a case study in Makomereng and Pepela, South Africa. Interdisciplinary land use and natural resource management. Faculty of Life Sciences

Liu, Q., Li, W., Xue, M., Chen, Y., Du, X., Wang, C., Han, L., Tang, Y., Feng, Y., Tao, C. and He, J., 2017. Diabetes mellitus and the risk of multidrug resistant tuberculosis: a meta-analysis. *Scientific Reports*, 7(1), pp.1-7.

Livermore, D., 2000. Epidemiology of antibiotic resistance. *Intensive Care Medicine*, 26(1), pp.S014-S021.

Mabhula, A. and Singh, V., 2019. Drug-resistance in *Mycobacterium tuberculosis*: where we stand. *MedChemComm*, 10(8), pp.1342-1360.

Manvar, D., Mishra, M., Kumar, S. and Pandey, V., 2012. Identification and evaluation of antiHepatitis C virus phytochemicals from Eclipta alba. *Journal of Ethnopharmacology*, 144(3), pp.545-554.

Marra, C., Marra, F., Cox, V., Palepu, A. and Fitzgerald, J., 2004. Factors influencing the quality of life in patients with active tuberculosis. *Health and Quality of Life Outcomes*, 2(1), pp.1-10.

Marchese, A. and Schito, G., 2000. Resistance patterns of lower respiratory tract pathogens in Europe. *International Journal of Antimicrobial Agents*, 16(1), pp.25-29.

Martínez, J., Baquero, F. and Andersson, D., 2007. Predicting antibiotic resistance. *Nature Reviews Microbiology*, 5(12), pp.958-965.

Martínez, J., Coque, T. and Baquero, F., 2015. What is a resistance gene? Ranking risk in resistomes. *Nature Reviews Microbiology*, 13(2), pp.116-123.

Masoko, P., Picard, J. and Eloff, J., 2005. Antifungal activities of six South African *Terminalia* species (Combretaceae). *Journal of Ethnopharmacology*, 99(2), pp.301-308.

Masoko, P. and Makgapeetja, D., 2015. Antibacterial, antifungal and antioxidant activity of *Olea africana* against pathogenic yeast and nosocomial pathogens. *BMC Complementary and Alternative Medicine*, 15(409), pp.1-9.

Mathibela, K.M. 2013. An investigation into aspects of medicinal plant use by traditional healers from Blouberg Mountain, Limpopo Province, South Africa. MSc thesis, South Africa, pp.1-152.

Mativandlela, S., Meyer, J., Hussein, A., Houghton, P., Hamilton, C. and Lall, N., 2008. Activity against *Mycobacterium smegmatis* and *M. tuberculosis* by extract of South African medicinal plants. *Phytotherapy Research*, 22(6), pp.841-845.

Matotoka, M. and Masoko, P., 2017. Evaluation of herbal concoctions sold at Ga Maja (Limpopo Province) in South Africa and *in vitro* pharmacological evaluation of plants used to manufacture the concoctions. *Journal of Evidence-Based Complementary & Alternative Medicine*, 22(4), pp.805-815.

Mehta, A., Srivastva, G., Kachhwaha, S., Sharma, M. and Kothari, S., 2013. Antimycobacterial activity of *Citrullus colocynthis* (L.) Schrad. against drug-sensitive and drug-resistant *Mycobacterium tuberculosis* and MOTT clinical isolates. *Journal of Ethnopharmacology*, 149(1), pp.195-200.

Meyer, J. and Dilika, F., 1996. Antibacterial activity of *Helichrysum pedunculatum* used in circumcision rites. *Journal of Ethnopharmacology*, 53(1), pp.51-54.

Meyers, E. and Smith, D., 1964. Bioautography of antibiotic spread-layer chromatograms. *Journal of Chromatography A*, 14, pp.129-132.

Moeng, E. and Potgieter, M., 2011. The trade of medicinal plants by muthi shops and street vendors in the Limpopo Province, South Africa. *Journal of Medicinal Plants*, 5(4), pp.558-564.

Mohamad, S., Zin, N., Wahab, H., Ibrahim, P., Sulaiman, S., Zahariluddin, A. and Noor, S., 2011. Antituberculosis potential of some ethnobotanically selected Malaysian plants. *Journal of Ethnopharmacology*, 133(3), pp.1021-1026.

Mundy, L., Pendry, B. and Rahman, M., 2016. Antimicrobial resistance and synergy in herbal medicine. *Journal of Herbal Medicine*, 6(2), pp.53-58.

Msonthi, J., 1984. Nor-diterpene oxides: colensanone and colensenone from *Cassia petersiana*. *Planta Medica*, 50(1), p.114.

Narasimhachari, N. and Ramachandran, S., 1967. A simple bioatuographic technique for identifying biologically active material on thin-layer chromatograms. *Journal of Chromatography A*, 27, p.494.

Nash, K., 2003. Intrinsic macrolide resistance in *Mycobacterium smegmatis* is conferred by a novel erm gene, erm (38). *Antimicrobial Agents and Chemotherapy*, 47(10), pp.3053-3060.

Ncube, B. and van-Staden, J., 2015. Tilting plant metabolism for improved metabolite biosynthesis and enhanced human benefit. *Molecules*, 20(7), pp.12698-12731.

Ncube, N., Afolayan, A. and Okoh, A., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, 7(12), pp.1797-1806.

Newman, R., 1984. Clinical applications of nuclear magnetic resonance spectroscopy: a review. *Journal of the Royal Society of Medicine*, 77(9), pp.774-779.

Newton, G. and Fahey, R., 2002. Mycothiol biochemistry. *Archives of Microbiology*, 178(6), pp.388-394.

Newton, J., Weiss, P., Bowler, W. and Oldfield, E., 1993. Soft-tissue infection due to *Mycobacterium smegmatis*: report of two cases. *Clinical Infectious Diseases*, 16(4), pp.531-533.

Nicolaus, B., Coronelli, C. and Binaghi, A., 1961. Mikrobiologischer nachweis von antibiotika auf dünnschichtchromatogrammen. *Experientia*, 17(10), pp.473-474.

Nondo, R., Moshi, M., Erasto, P., Zofou, D., Njouendou, A., Wanji, S., Ngemenya, M., Kidukuli, A., Masimba, P. and Titanji, V., 2015. Evaluation of the cytotoxic activity of extracts from medicinal plants used for the treatment of malaria in Kagera and Lindi regions, Tanzania. *Journal of Applied Pharmaceutical Science*, 5(4), pp.007-012.

Noubiap, J., Nansseu, J., Nyaga, U., Nkeck, J., Endomba, F., Kaze, A., Agbor, V. and Bigna, J., 2019. Global prevalence of diabetes in active tuberculosis: a systematic review and meta-analysis of data from 2·3 million patients with tuberculosis. *The Lancet Global Health*, 7(4), pp.e448-e460.

Nyiredy, S., 2001. Planar Chromatography: a retrospective view for the third millenium. 4th ed. Budapest: Springer Scientific Publisher, pp.489-503.

Parr, A. and Bolwell, G., 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture*, 80(7), pp.985-1012.

Parrish, N., Dick, J. and Bishai, W., 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends in Microbiology*, 6(3), pp.107-112.

Paxton, J., 1991. Assays for antifungal activity, in: K.Hostettmann (Ed.), Methods in plant biochemistry—assays for bioactivity, vol.6, London: Academic Press.

Payne, S., 1991. Lay representations of breast cancer. *Psychology & Health*, 5(1), pp.1-11.

Pfaller, M., Sheehan, D. and Rex, J., 2004. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. *Clinical Microbiology Reviews*, 17(2), pp.268-280.

Porath, J., 1997. From gel filtration to adsorptive size exclusion. *Journal of Protein Chemistry*, 16(5), pp.463-468.

Regnier, F.E., 1983. High-Performance Liquid Chromatography of biopolymers. *Science*, 222(4621), pp.245-252.

Remington, J. and Beringer, P., 2006. The science and practice of pharmacy. 21st ed. Philadelphia: Lippincott Williams and Wilkins, pp.773-774.

Ricci, M. and Zong, W., 2006. Chemotherapeutic approaches for targeting cell death pathways. *The Oncologist*, 11(4), pp.342-357.

Rinne, E., 2001. Water and healing - experiences from the traditional healers in Ile-Ife, Nigeria. *Nordic Journal of African Studies*, 10(1), pp.41-65.

Rios, J., Recio, M. and Villar, A., 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology*, 23(2-3), pp.127-149.

Ryder, D., 1991. Handbook of Thin-Layer Chromatography. *Journal of Labelled Compounds and Radiopharmaceuticals*, 29(9), pp.1097-1097.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. and Latha, L., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, 8(1), pp.1-10.

Schimmer, O., Krüger, A., Paulini, H. and Haefele, F., 1994. An evaluation of 55 commercial plant extracts in the Ames mutagenicity test. *Pharmazie*, 49(6), pp.448-451.

Schmourlo, G., Mendonça-Filho, R., Alviano, C. and Costa, S., 2004. Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. *Journal of Ethnopharmacology*, 96(3), pp.563-568.

Semenya, S. and Maroyi, A., 2013. Medicinal plants used for the treatment of tuberculosis by Bapedi traditional healers in three districts of the Limpopo province, South Africa. *African Journal of Traditional, Complementary and Alternative Medicines*, 10(2), pp.1-8.

Sheng-Ji, P., 2001. Ethnobotanical approaches of traditional medicine studies: some experiences from Asia. *Pharmaceutical Biology*, 39(sup1), pp.74-79.

Sherma, J., 2008. Planar chromatography. *Analytical Chemistry*, 80(12), pp.4253-4267.

Shulman, G., Alger, J., Prichard, J. and Shulman, R., 1984. Nuclear Magnetic Resonance spectroscopy in diagnostic and investigative medicine. *Journal of Clinical Investigation*, 74(4), pp.1127-1131.

Simões-Pires, C., Hmicha, B., Marston, A. and Hostettmann, K., 1990. A TLC bioautographic method for the detection of α- and β-glucosidase inhibitors in plant extracts. *Phytochemical Analysis*, 20(6), pp.511-515.

Silva, M., Simas, S., Batista, T., Cardarelli, P. and Tomassini, T., 2005. Studies on antimicrobial activity, *in vitro*, of *Physalis angulata* L. (Solanaceae) fraction and physalin B bringing out the importance of assay determination. *Memórias do Instituto Oswaldo Cruz*, 100(7), pp.779-782.

Smith, I., 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical Microbiology Reviews*, 16(3), pp.463-496.

Steenkamp, V., 2003. Traditional herbal remedies used by South African women for gynaecological complaints. *Journal of Ethnopharmacology*, 86(1), pp.97-108.

Sticher, O., 2008. Natural product isolation. *Natural Product Reports*, 25(3), p.517.

Sundarsingh, J., Ranjitha, J., Rajan, A. and Shankar, V., 2020. Features of the biochemistry of *Mycobacterium smegmatis*, as a possible model for *Mycobacterium tuberculosis*. *Journal of Infection and Public Health*, 13(9), pp.1255-1264.

Tang, S. and Halliwell, B., 2010. Medicinal plants and antioxidants: what do we learn from cell culture and Caenorhabditis elegans studies? *Biochemical and Biophysical Research Communications*, 394(1), pp.1-5.

Ting, N., El-Turk, N., Chou, M. and Dobler, C., 2020. Patient-perceived treatment burden of tuberculosis treatment. *PLOS ONE*, 15(10), pp.1-13.

Togun, T., Kampmann, B., Stoker, N. and Lipman, M., 2020. Anticipating the impact of the COVID-19 pandemic on TB patients and TB control programmes. *Annals of Clinical Microbiology and Antimicrobials*, 19(1), pp.1-6.

Torres, F. and Orts, J., 2018. Nuclear Magnetic Resonance structure-based drug design. *Future Medicinal Chemistry*, 10(20), pp.2373-2376.

Tshidzumba, P.W., 2018. An inventory and pharmacological evaluation of medicinal plants used as anti-diabetes and anti-arthritis in Vhembe District Municipality. Limpopo Province. M.Sc. Dissertation, University of Venda, South Africa.

Tshikalange, T., Meyer, J., Lall, N., Muñoz, E., Sancho, R., Van de Venter, M. and Oosthuizen, V., 2008. *In vitro* anti-HIV-1 properties of ethnobotanically selected South African plants used in the treatment of sexually transmitted diseases. *Journal of Ethnopharmacology*, 119(3), pp.478-481.

Uplekar, M., Weil, D., Lonnroth, K., Jaramillo, E., Lienhardt, C., Dias, H., Falzon, D., Floyd, K., Gargioni, G., Getahun, H., Gilpin, C., Glaziou, P., Grzemska, M., Mirzayev, F., Nakatani, H. and Raviglione, M., 2015. WHO's new end TB strategy. *The Lancet*, 385(9979), pp.1799-1801.

Valgas, C., Souza, S., Smânia, E. and Smânia Jr., A., 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, 38(2), pp.369-380.

van-Wyk, B., van-Oudtshoorn, B. and Gericke, N., 1997. *Medicinal plants of South Africa*. 1st ed. Pretoria: Briza Publications, p.304.

Vikrant, A., 2011. A Review on anti-tubercular plants. *International Journal of PharmTech Research*, 3(2), pp.872-880.

Vonmoos, S., Leuenberger, P., Beer, V. and de Haller, R., 1986. Pleuropulmonary infection caused by *Mycobacterium smegmatis*. Case description and literature review. *Schweizerische medizinische Wochenschrift*, 116(52), pp.1852-6.

Voorhees, R. and Ramakrishnan, V., 2013. Structural basis of the translational elongation cycle. *Annual Review of Biochemistry*, 82(1), pp.203-236.

Wagman, G. and Bailey, J., 1969. Use of silicic acid—glass fiber sheets for bioautography of antimicrobial substances. *Journal of Chromatography A*, 41(1), pp.263-264.

Wall, M., Wani, M., Brown, D., Fullas, F., Olwald, J., Josephson, F., Thornton, N., Pezzuto, J., Beecher, C., Farnsworth, N., Cordell, G. and Kinghorn, A., 1996. Effect of tannins on screening of plant extracts for enzyme inhibitory activity and techniques for their removal. *Phytomedicine*, 3(3), pp.281-285.

Walton, N. and Brown, D., 1999. *Chemicals from plants*. London: Imperial College Press, pp.1-25.

Wang, M., Zhang, Y., Wang, R., Wang, Z., Yang, B. and Kuang, H., 2021. An evolving technology that integrates classical methods with continuous technological developments: Thin-Layer Chromatography bioautography. *Molecules*, 26(15), p.4647.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry*, 64(1), pp.3-19.

World Bank. Drug resistant infections: a threat to our economic future (vol. 2): final report. 2017. Report Number 114679

World Health Organization. The end TB strategy. Geneva (Switzerland): World Health Organization; 2015.

World Health Organization (WHO). Meeting report of the WHO expert consultation on the definition of extensively drug-resistant tuberculosis. Geneva: World Health Organization; 2020. Available from: https://apps.who.int/iris/handle/10665/338776

World Health Organization. Guidelines for treatment of drug-susceptible tuberculosis and patient care. Geneva: World Health Organization 2017.

World Health Organization, Stop TB Initiative. Treatment of tuberculosis: guidelines. Geneva: World Health Organization; 2010.

Yang, L., Wen, K., Ruan, X., Zhao, Y., Wei, F. and Wang, Q., 2018. Response of plant secondary metabolites to environmental factors. *Molecules*, 23(4), p.762.

Yee, D., Valiquette, C., Pelletier, M., Parisien, I., Rocher, I. and Menzies, D., 2003. Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, 167(11), pp.1472-1477.

Zhang, L., Zhuang, H., Zhang, Y., Wang, L., Zhang, Y., Geng, Y., Gou, Y., Pei, S. and Wang, Y., 2018. Plants for health: An ethnobotanical 25-year repeat survey of traditional medicine sold in a major marketplace in North-west Yunnan, China. *Journal of Ethnopharmacology*, 224, pp.119-125.

CHAPTER 3: Extraction and phytochemical evaluation

3.1 Introduction

Traditional medicinal plants are the principal source of healthcare for the population in the rural areas of developing countries, including South Africa (Amoo *et al*., 2009). These plants show a great capability for the discovery of novel bioactive compounds; hence, they possess the potential of alternative drug leads, given their abundance and variety for those indigenous to South Africa as they indicate chemical diversity (Amoo *et al*., 2009; Newman and Cragg, 2007). Extraction is the key to the analysis of medicinal plants. It is the process through which selective solvents are employed to separate medicinally active portions of plants from the medicinally inactive components (Ncube *et al*., 2008).

In this process, selective solvents diffuse through the plant material to solubilise the compounds of similar polarity. However, traditional healers mostly use water to extract bioactive compounds from medicinal plants due to their limited access to other solvents; hence, restricted bioactive compounds are obtained (Buwa and van-Staden, 2006; Kelmanson *et al*., 2000). Solvents of varying polarity must be used to obtain a wide range of compounds from medicinal plants. The extraction solvents are carefully selected with more preference to those that are unable to cause the extract to complex or dissociate, display low toxicity, evaporation at low heat, and show no interference to subsequent bioassays when the extraction aims to screen for antimicrobial activity of the extracts (Das *et al*., 2010; Ncube *et al*., 2008).

Several extraction procedures exist, these include but are not limited to maceration, digestion, infusion, and decoction. Amongst these procedures, maceration is the preferred extraction procedure in laboratory settings based on its suitability for heat-sensitive compounds as compared to other extraction procedures where heat is used in the processes (Ncube *et al*., 2008). Any plant part can be used for the extraction as either fresh or dried plant material. However, dried plant material is the most preferred form based on its use by traditional healers and due to the varying water content within different plant tissues (Das *et al*., 2010; Masoko and Nxumalo, 2013; Ncube *et al*., 2008).

The medicinal plants' therapeutic properties result from secondary metabolites produced by the plants during unfavourable conditions. The secondary metabolites,

also known as phytochemical compounds, are the main target in drug development from medicinal plants. Phytochemical compounds are screened to identify and track bioactive compounds from medicinal plants using techniques such as TLC in the drug discovery process (Brusotti *et al*., 2014; Cai, 2014).

TLC is the type of chromatography suitable for analysing extracts from medicinal plants because it is inexpensive, quick, sensitive, and only requires a few micrograms of the sample for a successful analysis. TLC has several uses, which include monitoring the progress of a reaction, determination of the number of solvents in a mixture, verification of the identity and purity of a compound, analysis of the fractions obtained from column chromatography, and the determination of the solvent composition for preparative separations (Cai, 2014).

After TLC, specific chemical substances, fluorescence, or radioactivity can be used to produce a visible coloured product for the colourless molecules separated on TLC to identify their positions on the chromatogram (Pavia *et al*., 2006). The focal point of this chapter was to screen for phytochemical constituents using standard tests, profiling of the phytochemical compounds by TLC and quantification of common phytochemical compounds from *Cassia petersiana* extracts.

3.2 Materials and method

3.2.1 Plant collection and storage

The leaves of *Cassia petersiana* were collected at the Lowveld National Botanical Garden in Nelspruit, Mpumalanga, South Africa. The voucher specimen (voucher number SSS511) was deposited at Larry Leach Herbarium (UNIN) for identification. The plant material was dried at room temperature, ground into a fine powder using a blender and was then stored in the dark in an airtight bottle.

3.2.2 Extraction procedures

3.2.2.1 Preliminary extraction

An amount of 1 gram of the finely powdered plant material was subjected to solvents ranging from non-polar to polar in 9 polyester centrifuge tubes to extract crude extracts from *Cassia petersiana*. The solvents used include n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water. The mixtures were agitated in a shaking incubator at 200 rpm for 30 minutes, and then

filtered into pre-weighed vials using cotton wool. The filtrates were put under a fan to evaporate the solvents before reconstituting the extracts with acetone to a concentration of 10 mg/mL.

3.2.2.2 Serial exhaustive extraction

To extract more compounds from *Cassia petersiana*, 5 g of the plant material was mixed and agitated with 50 mL of n-Hexane for 1 hour at 200 rpm. The filtrate was collected into a pre-weighed beaker before the fresh n-Hexane was added to the same plant material. The mixture was agitated for 2 hours with the collection of the filtrate after each hour. The same procedure was repeated using chloroform, dichloromethane, ethyl acetate, acetone, ethanol, and methanol with the same plant residue.

3.2.3 Profiling of phytochemicals

TLC was used to profile the compounds preliminarily extracted from *Cassia petersiana* (Kotzé *et al*., 2002). The quantity of 10 µL of each of the reconstituted extracts was loaded at the bottom of the labelled aluminium-backed TLC plate. The TLC plates were developed into three different mobile systems: benzene/ethanol/ammonium hydroxide (BEA), which is non-polar/basic with the ratio of 9:1:0.1, chloroform/ethyl acetate/formic acid (CEF) of intermediate polarity/acidic with the ratio of 5:4:1 and ethyl acetate/methanol/water (EMW), which is polar/neutral with the ratio of 40:5.5:5. Ultraviolet light at wavelengths 254 and 365 nm was used to visualise the fluorescing phytochemicals before spaying the separated TLC plates with vanillin-sulphuric acid (0.1 g vanillin: 28 ml methanol: 1 mL sulphuric acid) for visualisation of non-fluorescing compounds. To visualise the non-fluorescing phytochemicals, the TLC plates were sprayed with vanillin-sulphuric acid and heated for about 1 minute at 110°C for colour development.

3.2.4 Screening for phytochemical constituents

A variety of chemical tests were conducted to screen for the presence or absence of the common phytochemical constituents from *Cassia petersiana*, which include the tannins, saponins, phlabotannins, flavonoids, terpenoids, alkaloids, cardiac glycosides and steroids.

3.2.4.1 Tannins

An amount of 0.5 g of the powdered *Cassia petersiana* plant material was dissolved in 5 ml of distilled water. The solution was boiled, cooled and 1 mL of it was transferred to a new test tube followed by the addition of a few drops of 1% ferric chloride. The presence of tannins was indicated by the blue-black or the brown-green colour change (Trease and Evans, 2009).

3.2.4.2 Saponins

To test for saponins, an amount of 0.5 g of the plant material of *Cassia petersiana* was mixed with 5 mL of tap water by shaking vigorously. The mixture was heated at 100 °C. Formation of froth was an indication of the saponins (Odebiyi and Sofowora, 1978).

3.2.4.3 Phlabotannins

A quantity of 0.5 g of the *Cassia petersiana* powdered leaf material was dissolved in 5 mL of distilled water followed by filtration of the mixture. An amount of 2 mL of 1% hydrochloric acid was added to the filtrate and boiled at 100 °C. The presence of phlabotannins was indicated by the formation of the red-coloured precipitate (Borokini and Omotayo, 2012).

3.2.4.4 Flavonoids

The presence of flavonoids was determined by adding diluted ammonia (5 mL) to a portion of the aqueous filtrate of the plant extract (Borokini and Omotayo, 2012). Concentrated sulphuric acid (H_2SO_4) was then added before observing for the colour change to draw an inference. A yellow colouration that disappears on standing indicates the presence of flavonoids.

3.2.4.5 Terpenoids

The Salkowski test was followed to test for the presence of terpenoids. An amount of 0.5 g of the plant material was subjected to 2 mL of chloroform and 3 mL of concentrated sulphuric acid (H_2SO_4) at a later stage. To draw the inference, the mixture was observed for a reddish-brown colour change (Borokini and Omotayo, 2012).

3.2.4.6 Alkaloids

To test for the presence of alkaloids, 95% ethanol was used to extract 0.5 g of the powdered plant material for 10 minutes in a shaking incubator at the speed of 200 rpm. The extract was filtered using Whatman no. 1 filter paper using a Buchner funnel and evaporated under vacuum using Buchi rotavaporator. The dried extract was dissolved with 5 mL of 1% hydrochloric acid (HCL) then 5 drops of Drangendoff's reagent were added before observing for the colour change to draw inference (Harborne, 1984).

3.2.4.7 Cardiac glycosides

To test for the presence of cardiac glycosides, the plant material was subjected to 2 mL of glacial acetic acid, which contained one drop of ferric chloride solution. An amount of 1 mL of concentrated sulphuric acid (H_2SO_4) was added to the mixture before observing the sample for colour change to draw inference (Borokini and Omotayo, 2012).

3.2.4.8 Steroids

The presence of steroids was determined following the method by Borokini and Omotayo (2012), by adding 2 mL of acetic anhydride to 0.5 g of the powdered plant material. An amount of 2 mL of sulphuric acid (H_2SO_4) was added before observing the mixture for the colour change to draw an inference.

3.2.5 Quantification of total phenolic, flavonoid, and tannin contents

3.2.5.1 Total phenolic content

The Follin-Ciocaltleu reagent method was followed to quantify the total phenolic content from *Cassia petersiana* extracts with minor modifications (Velioglu *et al*., 1998). The initial concentration of the plant extracts (10 mg/mL) was reduced with acetone to 5 mg/mL in a test tube. An amount of 100 µL of the 5 mg/mL plant extracts was diluted with 900 µL of distilled water in a new test tube. Follin-Ciocaltleu reagent (100 µL) was added to the mixture, followed by an addition of 1.25 mL of sodium carbonate to stop the reaction. The mixture was then incubated in the dark room for 90 minutes. The same procedure was followed to prepare a blank, however; the extracts were replaced with acetone. Gallic acid was used as standard at a range of concentrations (1.25, 0.63, 0.31, 0.16, 0.08 mg/mL). An ultraviolet/visible (UV/VIS)

spectrophotometer was used to determine the absorbance of all the solutions at a wavelength of 550 nm. The formula ($y = 2.6561x + 0.2988$, $R^2 = 0.9988$) obtained from the gallic acid standard was used to calculate the total phenolic content, which was expressed as milligram gallic acid equivalence/gram of extract (mg of GAE/g extract). The experiment was conducted in triplicates, independently with three times repetition for each plant extract.

3.2.5.2 Total flavonoid content

The total flavonoid content was quantified by following the aluminium chloride colorimetric assay procedure (Tambe and Bhamber, 2014). This was done by the addition of 100 µL of the 10 mg/mL plant extracts to 4.9 mL of distilled water in a clean test tube. Following this, an amount of 300 µL of 5% sodium nitrite ($NaNO_2$), which was dissolved in distilled water, was added to the mixture in the test tube. The solution was left at room temperature for 5 minutes. After that, an amount of 300 µL of 10% aluminium chloride ($AlCl_3$), which was dissolved in distilled water, was added to the mixture, and then the solution was incubated at room temperature for 5 minutes. After the incubation period, an amount of 2 mL of 1 M sodium hydroxide (NaOH) was added to the mixture before topping up the mixture to 10 mL with distilled water. The standard for this experiment was quercetin, which was prepared following the same method as the plant extracts by preparing different concentrations of quercetin (500-31.5 µg/mL). A spectrophotometer (UV/VIS) was used to determine the absorbance of the experimental samples as well as the standard at a wavelength of 510 nm. The same procedure was followed for preparing the standard, but the plant extracts were replaced with 100 µL of distilled water. The equation ($y = 3.2798x + 0.036$, $R^2 = 0.9967$) from the quercetin standard curve was used to calculate the total flavonoid content and expressed as milligram quercetin equivalence/gram of extract (mg QE/g extract). The experiment was done in triplicates and independently repeated three times for each plant extract.

3.2.5.3 Total tannin content

The tannin content in the *Cassia peterisana* extracts was quantified according to the Follin-Ciocalteu method (Tambe and Bhambar, 2014). An amount of 3.8 mL of distilled water was added to a clean test tube before 50 µL of 10 mg/mL of plant extract was added. This was followed by the addition of 0.25 mL of Follin-Ciocalteu reagent, then

the mixture was vortexed to mix. An amount of 0.5 mL of 35% sodium carbonate (Na_2CO_3) solution was added to the mixture before transferring the mixture to a 10 mL volumetric flask. Distilled water was used to top up the volume of the mixture to 10 mL. The mixture was then vortex mixed and incubated in the dark at room temperature for 30 minutes. The same procedure was followed to prepare the blank, but the plant extract was replaced with distilled water. Gallic acid was used as the standard. Different concentrations of the standard (1-0.625 mg/mL) were prepared. A spectrophotometer (UV/VIS) was used to measure the absorbance of the mixtures against the blank at a wavelength of 725 nm. The total tannin content was calculated using the equation ($y= 1.2697x + 0.1128$, $R^2 = 0.9983$) from the gallic acid standard curve and expressed as milligram gallic acid equivalence /gram of extract (mg GAE/g extract).

3.3 Results

3.3.1 Preliminary extraction

The amount of mass extracted from 1 g of the leaves of *Cassia petersiana* by solvents of varying polarity is shown in figure 3.1. These preliminary extraction results revealed that chloroform is the best extractant (67 mg), followed by distilled water (56 mg), while n-hexane and butanol extracted the least of the plant material (20 mg).

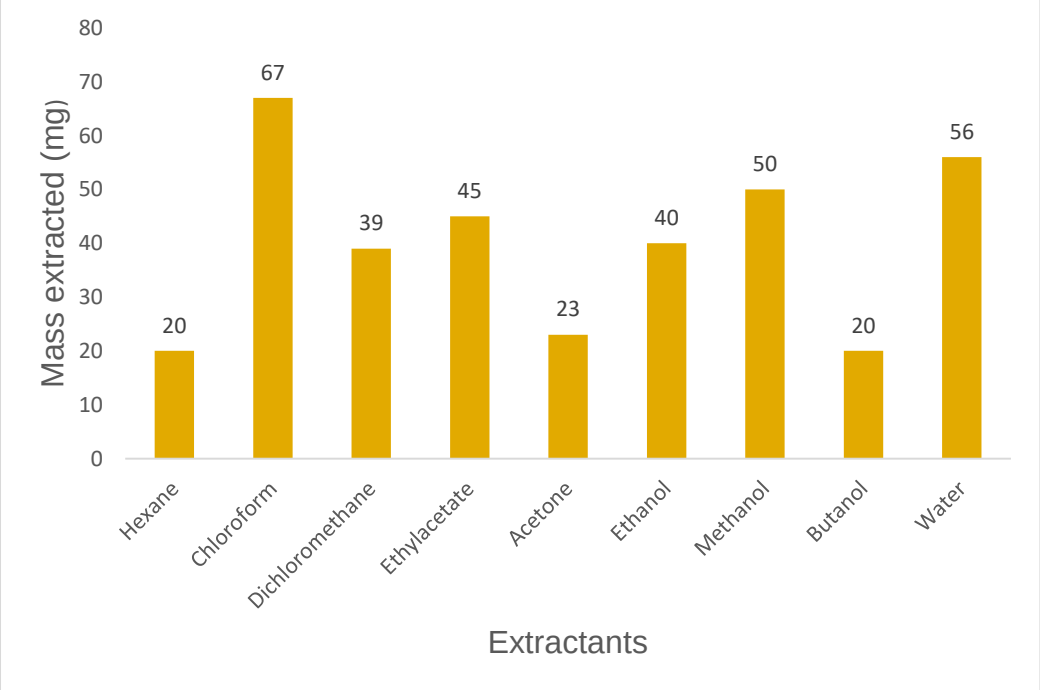

Figure 3.1: The mass of *Cassia petersiana* extracted preliminary with solvents of varying polarity; n-Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water.

3.3.1.1 Phytochemical analysis

Fingerprints of the phytochemical compounds from *Cassia petersiana* extracts were generated through the TLC technique (figure 3.2). The aluminium-backed TLC plates were developed in BEA, CEF, and EMW solvent systems, which differ in polarity. The non-fluorescing phytochemical compounds were visualised by spraying the TLC plates with vanillin sulphuric acid (A), while the fluorescing phytochemicals were visualised with ultraviolet light at 254 (B) and 365 nm (C). BEA showed great separation of the phytochemical compounds followed by EMW with 365 nm ultraviolet light.

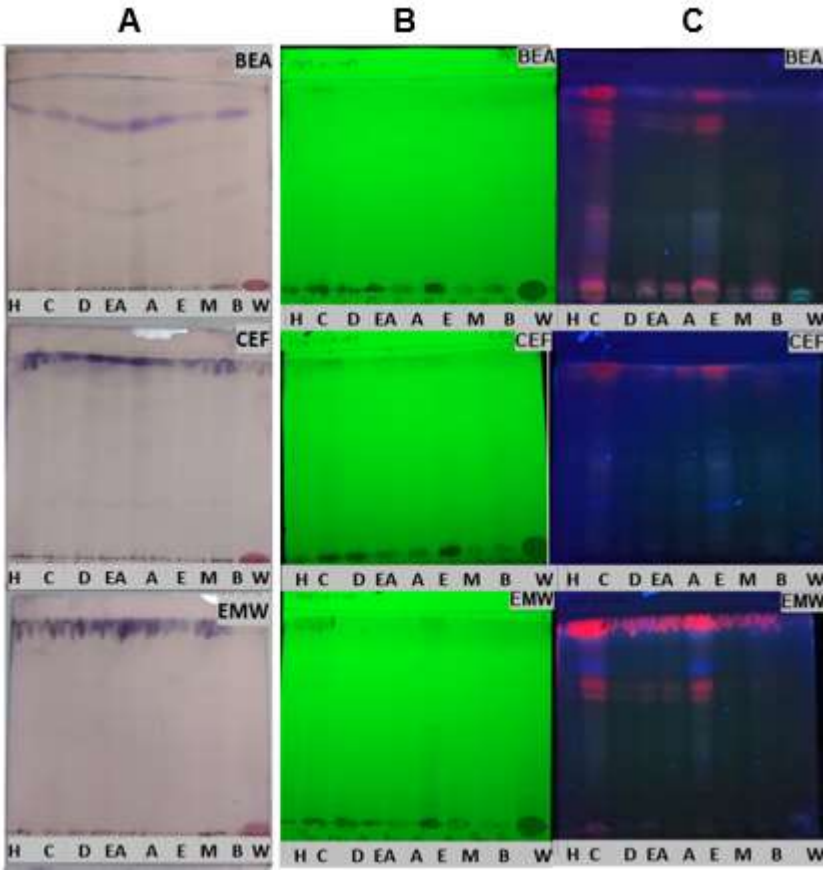

Figure 3.2: The phytochemical constituents of *Cassia petersiana* extract profiled on TLC in solvent systems of varying polarity; BEA (non-polar), CEF (intermediate

polarity), and EMW (polar). Vanillin sulphuric acid (A) and ultraviolet light at 254 (B) and 365 nm (C) were used to visualise the phytochemicals.

Key: H- n-Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol, and W- Distilled water.

3.3.2 Exhaustive extraction

An exhaustive extraction procedure was conducted to expose more phytochemical constituents from *Cassia petersiana* for further analysis. n-hexane extracted most of the plant material (539.9 mg). It was followed by methanol with 225.4 mg, while acetone extracted the minimal amount (18.8 g).

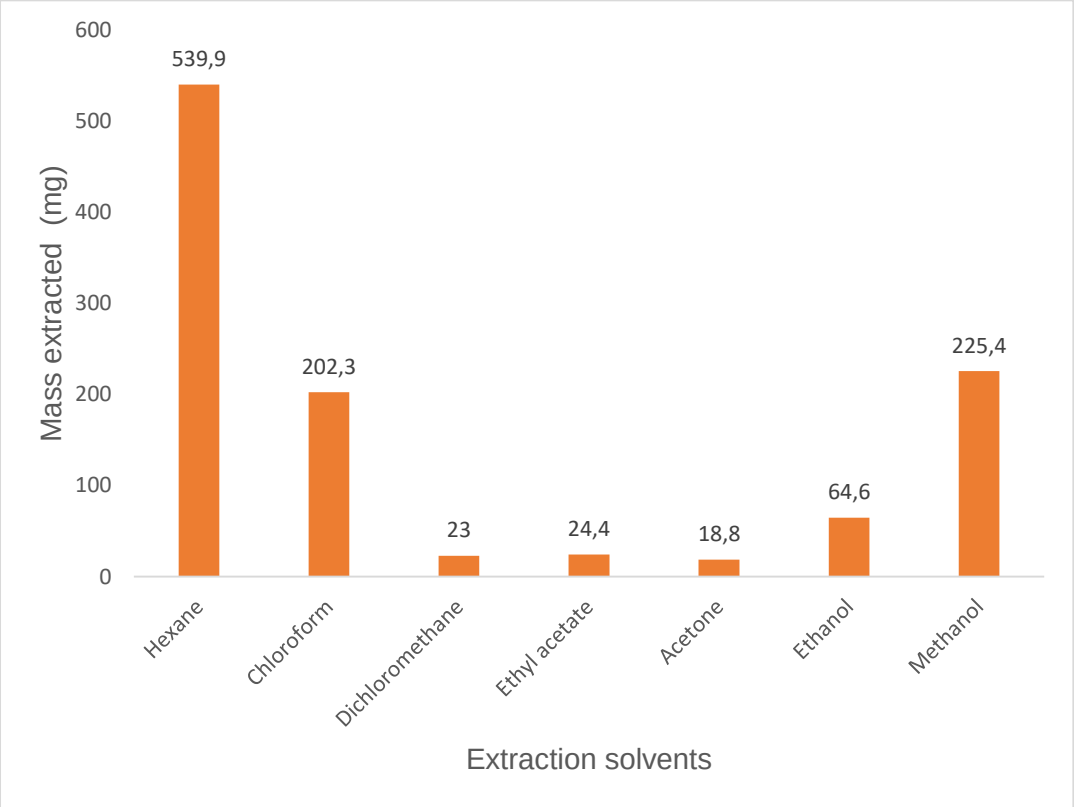

Figure 3.3: The mass extracted from *Cassia petersiana* through the exhaustive extraction procedure using solvents that differ in polarity.

3.3.2.1 Phytochemical analysis

The profiled phytochemical compounds were visualised with vanillin-sulphuric acid (figure 3.4, A) and ultraviolet light at 254 (figure 3.4, B) and 365 nm (figure 3.4, C). The phytochemical constituents from the exhaustive extraction procedure (figure 3.5),

which were profiled on TLC, revealed that BEA was the great solvent system for separation. The phytochemical constituents were colourful in all three solvent systems.

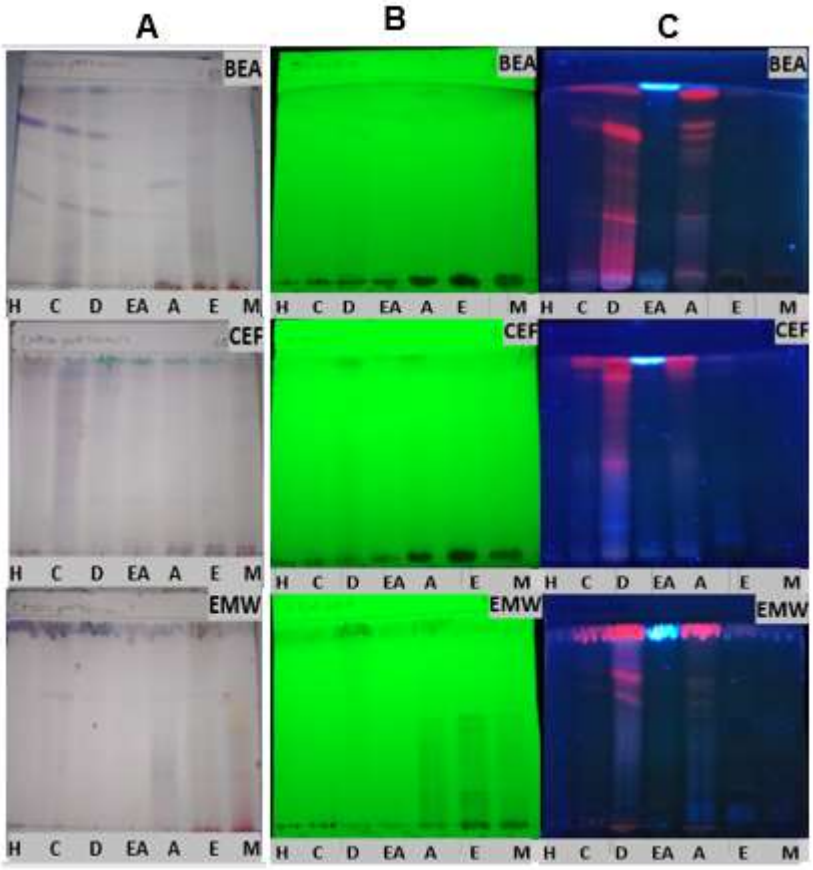

Figure 3.4: Phytochemical fingerprints of extracts from *Cassia petersiana* visualised by spraying with vanillin-sulphuric acid (A) and under ultraviolet light at 254 (B) and 365 nm (C). Colourful bands indicate phytochemical compounds.

Key: H- n-Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, and M- Methanol.

3.3.4 Screening of phytochemical constituents

Standard tests were followed to screen for the common phytochemical compounds where it was found that all the screened phytochemical constituents were present in *Cassia petersiana* as shown in table 3.1.

Table 3.1: The tested phytochemical constituents of *Cassia petersiana*.

Phytochemical constituent	Reaction
Tannins	+
Saponins	+
Phlabotannis	+
Flavonoids	+
Terpenoids	+
Alkaloids	+
Cardiac glycosides	+
Steroids	+

Key: + = present

3.3.5 The quantification of the total phenolics, tannins, and flavonoids.

The quantitative analysis of the three most common phytochemical compounds was conducted by the colorimetric assays. Standard curves (figures 3.7, 3.8, and 3.9) were prepared for the quantification of all the phytochemicals, and the formulae were used in the determination of the total phenolic, tannin, and flavonoid contents (Table 3.2). Phenolic contents were abundant, followed by tannin content, while the flavonoids were the least in all the extracts (table 3.2).

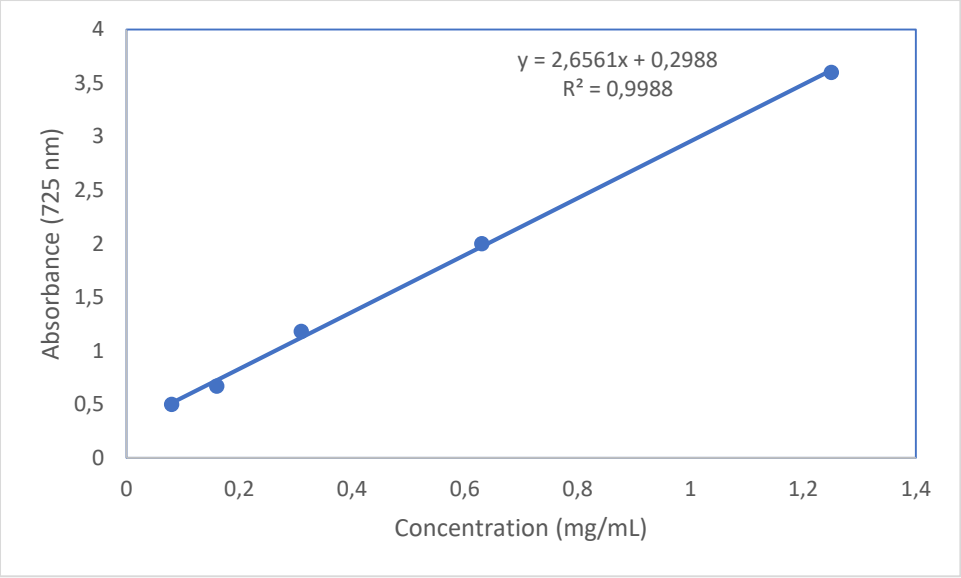

Figure 3.5: The gallic acid standard curve for the determination of total phenolic content.

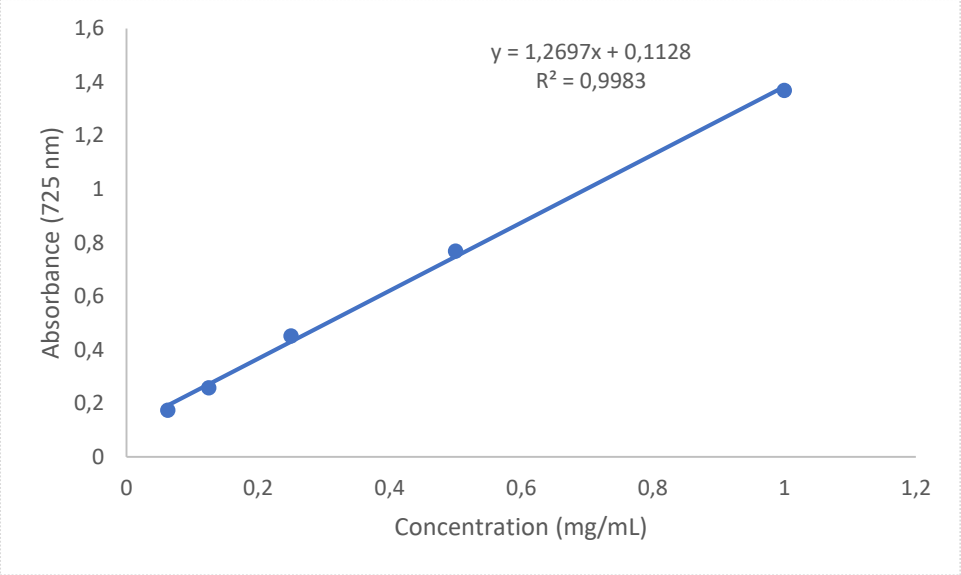

Figure 3.6: The gallic acid standard for determination of total tannin content.

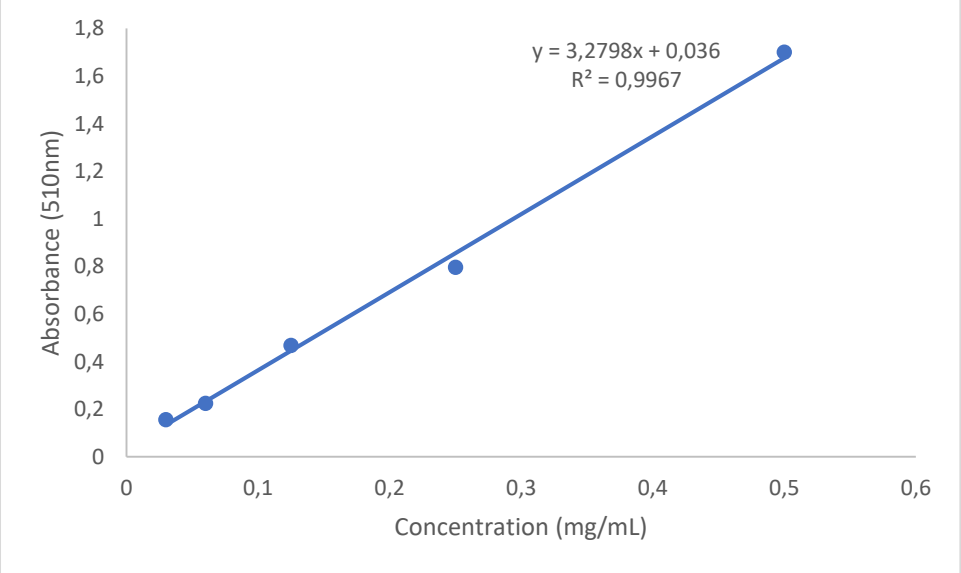

Figure 3.7: Quercetin standard curve for the determination of total flavonoid content.

Table 3.2: The quantification of the total phenolic, flavonoid, and tannin contents from *Cassia petersiana*.

Extraction solvent	Phenolic content (mg of GAE/g extract)	Flavonoid content (mg of QE/g extract)	Tannin content (mg of GAE/g extract)
Hexane	34.68 ±3.39	3.69 ± 2.05	7.81 ± 0.50
Chloroform	44.21 ± 3.21	1.87 ± 0.23	5.90 ± 3.34
Dichloromethane	4.98 ± 0.97	3.70 ± 0.43	4.22 ± 3.05
Ethyl acetate	37.83 ± 2.27	1.47 ± 0.31	1.46 ± 1.0
Acetone	25.62 ± 1.39	1.52 ± 0.35	3.51 ± 0.24
Ethanol	51.67 ± 10.13	4.27 ± 1.62	15.4 ± 4.08
Methanol	51.29 ±3.90	2.56 ± 0.54	13.27 ± 5.85
Butanol	71.10 ±7.36	0.26 ± 0.25	12.30 ± 3.09
Water	156.98± 19.56	1.11 ± 0.23	33.73 ± 1.80

3.4 Discussion

It is essential to extract the targeted compounds from medicinal plants for further analysis and characterisation. Extraction is the critical first step in the study of medicinal plants (Sasidharan *et al*., 2010). Although traditional healers use only water for extraction and preparation of herbal concoctions, solvents ranging from non-polar to polar were selected for the extraction procedure based on the target to extract a wide range of bioactive compounds. The solvents of varying polarity employed in the extraction procedure were n-hexane (non-polar), chloroform, dichloromethane, ethyl acetate, acetone (intermediate-polar), ethanol, methanol, butanol, and distilled water (polar). Maceration was the type of extraction procedure chosen for this study because it does not require heat; hence, it is suitable for heat-sensitive compounds which may be targeted for further analysis (Sasidharan *et al*., 2010).

Preliminary extraction results revealed that chloroform (67 mg) is the best extractant while butanol and n-hexane extracted the least plant material (20 mg) as shown in figure 3.1. Other authors identified methanol as the most effective solvent for extraction given its ability to extract polar and non-polar compounds (Blondeau *et al*.,

2019; Truong *et al*., 2019). However, n-hexane extracted most of the plant material (539.9 mg), while the least was from acetone (18.8) in the exhaustive extraction (figure 3.3). The increase in the mass extracted from the exhaustive extraction as compared to preliminary extraction may be due to the exposure of more plant material for extraction as the time for extraction was increased while the same solvents were used three times on the same plant residue. BEA was the best solvent system for the separation of phytochemical constituents from both preliminary (figure 3.2, A) and exhaustive (figure 3.4, A) extraction. The purple colour of the phytochemical constituents in Figure 3.2, A was also common in Figure 3.4, A, which had more colourful bands.

Figures 3.2B and 3.4B suggest that phytochemical compounds from *Cassia petersina* do not fluoresce at 254 nm, while great separation and visualisation of phytochemicals were evident in the 365 nm (figures 3.2C and 3.4C). Common phytochemicals, which were visible with vanillin sulphuric acid and ultraviolet light at 365 nm, were taken note of. The expected outcomes of the qualitative analysis of phytochemical compounds from preliminary and exhaustive extraction suggest that most of the phytochemical compounds from *Cassia petersiana* are non-polar as they were visualised and separated better in BEA solvent system.

The phytochemical screening of medicinal plants is considered a helpful approach in the discovery of bioactive compounds as it assists in the identification and tracking of bioactive compounds (Brusotti *et al*., 2014). Standard tests followed to screen for the presence of common phytochemical constituents revealed the presence of all the tested phytochemicals (Table 3.1). This outcome correlates with the findings of Gatsing and Adoga (2007), who discovered the presence of all the tested phytochemicals in *Cassia petersiana* leaves. During the quantification of the total phenolic, tannin, and flavonoid contents, it was found that *Cassia petersiana* is abundant with phenolics, followed by the tannins, while the flavonoids were at minimal content (Table 3.2). Furthermore, distilled water extract had the highest phenolic content (78.49 ± 9.78 mg of GAE/g extract) while dichloromethane extract had the lowest (2.49 ± 0.48 mg of GAE/g extract), ethanol extract had the highest flavonoid content (4.27 ± 1.62 mg of QE/g extract) while butanol had the lowest (0.26 ± 0.25 mg of QE/g extract), and distilled water had the highest tannin content (33.73 ± 1.80 mg of GAE/g extract) while the lowest was from ethyl acetate (1.46 ± 1.0 mg of GAE/g

extract). These results made *Cassia petersiana* a good candidate for the screening of bioactive compounds as it may possess a range of bioactive compounds.

3.5 Conclusion

It was possible to reveal diverse phytochemical compounds from crude extracts of *Cassia petersiana* through the standard tests, TLC, and colorimetric assays. Although the quantity of the phenolic, tannin and flavonoid contents was significantly lower, the diversity of phytochemical constituents illustrated in *Cassia petersiana* suggests the use of this plant for the screening of antimycobacterial compounds as some of the bioactive compounds in the extracts may act synergistically to show bioactivity.

3.6 References

Amoo, S., Ndhlala, A., Finnie, J. and Van Staden, J., 2009. Antibacterial, antifungal and anti-inflammatory properties of *Burchellia bubalina*. *South African Journal of Botany*, 75(1), pp.60-63.

Blondeau, D., St-Pierre, A., Bourdeau, N., Bley, J., Lajeunesse, A. and Desgagné-Penix, I., 2019. Antimicrobial activity and chemical composition of white birch (*Betula papyrifera* Marshall) bark extracts. *MicrobiologyOpen*, 9(1), pp.1-23.

Borokini, T. and Omotayo, F., 2012. Phytochemical and ethnobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research*, 6(7), pp.1106-1118.

Brusotti, G., Cesari, I., Dentamaro, A., Caccialanza, G. and Massolini, G., 2014. Isolation and characterization of bioactive compounds from plant resources: the role of analysis in the ethnopharmacological approach. *Journal of Pharmaceutical and Biomedical Analysis*, 87(2014), pp.218-228.

Buwa, L. and van-Staden, J., 2006. Antibacterial and antifungal activity of traditional medicinal plants used against venereal diseases in South Africa. *Journal of Ethnopharmacology*, 103(1), pp.139-142.

Cai, L., 2014. Thin Layer Chromatography. *Current Protocols Essential Laboratory Techniques*, 8(1), pp.8.

Das, K., Tiwari, R. and Shrivastava, D., 2010. Techniques for evaluation of medicinal plant products as antimicrobial agent: current methods and future trends. *Journal of Medicinal Plants Research*, 4(2), pp.104-111.

Gatsing, D. and Adoga, G., 2007. Antisalmonellal activity and phytochemical screening of the various parts of *Cassia petersiana* Bolle (Caesalpiniaceae). *Research Journal of Microbiology*, 2(11), pp.876-880.

Harborne, J., 1984. Phytochemical methods. 2nd ed. London: Springer, Dordrecht, pp.1-36.

Kelmanson, J., Jäger, A. and van Staden, J., 2000. Zulu medicinal plants with antibacterial activity. *Journal of Ethnopharmacology*, 69(3), pp.241-246.

Kotzé, M., Eloff, J. and Houghton, P., 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). *South African Journal of Botany*, 68(1), pp.62-67.

Masoko, P. and Nxumalo, K., 2013. Validation of antimycobacterial plants used by traditional healers in three districts of the Limpopo Province (South Africa). *Evidence-Based Complementary and Alternative Medicine*, 2013, pp.1-7.

Ncube, N., Afolayan, A. and Okoh, A., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, 7(12), pp.1797-1806.

Newman, D. and Cragg, G., 2007. Natural products as sources of new drugs over the last 25 years. Journal of Natural Products, 70(3), pp.461-477.

Odebiyi, O. and Sofowora, E., 1978. Phytochemical screening of Nigerian medicinal plants II. *Lloydia*, 41(3), pp.234-246.

Pavia, D., Lampman, G., Kritz, G. and Engel, R., 2006. Introduction to organic laboratory techniques. 4th ed. Mason, Ohio: Thomson Brooks/Cole, pp.797–817.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. and Latha, L., 2010. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, 8(1), pp.1-10.

Tambe, V. and Bhambar, R., 2014. Estimation of total phenol, tannin, alkaloid and flavonoid in *Hibiscus tiliaceus* Linn. wood extracts. *Journal of Pharmacognosy and Phytochemistry*, 2(4), pp.2321–6182.

Trease, G. and Evans, W., 2009. Trease and Evans' pharmacognosy*:* a physician's guide to herbal medicine. 16th ed. London: Baillière Tindall, pp.225-228.

Truong, D., Nguyen, D., Ta, N., Bui, A., Do, T. and Nguyen, H., 2019. Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and *in vitro* anti-inflammatory activities of *Severinia buxifolia*. *Journal of Food Quality*, 2019, pp.1-9.

Velioglu, Y., Mazza, G., Gao, L. and Oomah, B., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry*, 46(10), pp.4113-4117.

CHAPTER 4: Antioxidant activity assays

4.1 Introduction

Oxidative balance is key to the survival of cells in the human body. During oxidation, free radicals (Gulcin, 2020) as well as other species that can react to oxygen are produced (Bae *et al*., 1999) and are involved in chain reactions which cause oxidative changes that may damage and affect cells (Kasote *et al*., 2013; Winrow *et al*., 1993). These changes are managed by antioxidants (Gutteridge, 1994), whose roles are to capture and stabilise free radicals, along with the delay or inhibition of the occurrence of free radical reactions due to the presence of relative oxygen (Huyut *et al*., 2017; Kim and Byzova, 2014; Leong and Shui, 2002). However, oxidative stress (Apak *et al*., 2016) may occur due to the imbalance between reactive oxygen species and antioxidant defences (Antolovich *et al*., 2001; Krishnaiah *et al*., 2011).

Oxidative changes are further controlled by several enzymes, including catalase, dismutase, peroxidase, superoxide, glutathione, serum albumins, vitamin E, and uric acid. However, an excess in free radicals leads to the damage of these enzymes (Bae *et al*., 1999). Moreover, various illnesses and chronic diseases may arise if free radicals are not treated at the right time (Cetin Cakmak and Gülçin, 2019; Young and Woodside, 2001). These include diabetes, Alzheimer's disease, cardiovascular disease, cancer, arteriosclerosis, eye diseases, and Parkinson's disease, which causes cell damage (Kasote *et al*., 2013).

Furthermore, the human body naturally produces a great number of free radicals during its different physiological processes (Foyer and Noctor, 2005) while limited antioxidants are also produced naturally in the body (Kasote *et al*., 2013). As a result, naturally occurring antioxidants are usually outnumbered by free radicals. Consequently, the continual supply of the external antioxidants sources are essential to maximise the benefits of antioxidants (Kim and Byzova, 2014; Young and Woodside, 2001).

The increase of antioxidant intake in the human body is beneficial as it provides added protection for the body against heart, immune system, eye, and memory problems as well as protection against mood disorders (Sena and Chandel, 2012). The wide use of synthetic antioxidants is common in the food industry (Antolovich *et al*., 2001), and these are also included in the human diet (Leclercq *et al*., 2000; Maziero *et al*., 2001).

However, safety concerns of synthetic antioxidants (Iverson, 1999) led to the promotion of naturally occurring antioxidants that possess antioxidants of similar or even higher levels than synthetic antioxidants (Velioglu, 1998). In addition, natural antioxidants are known to be multifunctional (Arnao, 2000).

As a result, the search for natural antioxidants is of utmost importance to overcome degenerative disease problems (Leong and Shui, 2002). The health benefits of most plant extracts are attributed to the diversity of free radical scavenging molecules, which include vitamins, anthocyanins, carotenoids, and phenols (Gunathilake *et al*., 2018). Phenolic acids and flavonoids are antioxidants that can be involved in the adsorption and neutralisation of free radicals (Tlili *et al*., 2013). They have additional therapeutic properties such as antiviral, anti-allergic, antibacterial, anti-inflammatory and anticancer (Wang *et al*., 2018).

The analysis of antioxidants comprises DPPH free radical scavenging activity assay as one of the important techniques. In this assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is reduced to DPPH when it receives an electron from an antioxidant compound. The gaining of an electron from an antioxidant compound by the DPPH radical results in its reduction to DPPH, which changes its purple colour to yellow as an indication of antioxidant activity (Pavithra and Vadivukkarasi, 2015). Ascorbic acid, used as a positive control for antioxidant assays, is an antioxidant of great importance as it contains thiols which enable it to terminate the chain reactions initiated by the free radicals (Padayatty *et al*., 2003). The goal of this chapter was to evaluate the antioxidant capacity of the *Cassia petersiana* extracts qualitatively, by DPPH free radical scavenging assay profiled on TLC as well as quantitatively, using DPPH free radical scavenging and ferric reducing power assays.

4.2 Materials and methods

4.2.1 Qualitative DPPH free radical scavenging assay on TLC

Evaluation of the qualitative antioxidant activity of the plant extracts was carried out by the method by Deby and Margotteaux (1970). This method comprised the coupling of TLC with 2.2-Diphenyl-1-picrylhydrazyl (DPPH). The aluminium-backed TLC plates were prepared and developed in three solvent systems, according to this study's subsection 3.2.4 of chapter 3. The developed aluminium-backed TLC plates were air-dried at room temperature to allow the solvent systems to evaporate before their

exposure to 0.2 % DPPH solution (0.2 g DPPH dissolved in 100 mL methanol) by spraying. The development of the yellow spots against the purple colour of DPPH indicated antioxidant activity.

4.2.2 Quantitative DPPH free radical scavenging activity assay

The initial concentration (10 mg/mL) of the extracts was reduced to different concentrations (250-15.63 µg/mL) to a total volume of 1 mL using acetone. An amount of 2 mL of 0.2 mmol/L DPPH solution dissolved in methanol was added to the 1 mL solutions, followed by a vortex mix. The mixtures were then incubated at room temperature in the dark for 30 minutes. The same procedure was followed for the preparation of the control solution with acetone replacing the extracts. L-Ascorbic used as a standard was prepared following the same procedure for the preparation of extracts. Following incubation, all the solutions were analysed with a UV/VIS spectrophotometer at 517 nm. The experiment was done in duplicates and repeated three times. The absorbance readings were used to calculate the percentage of antioxidants potential using the formula below, where Ac and As are the absorbance of the control and experimental samples (Chigayo *et al*., 2016).

$$\% \text{ inhibition} = \frac{\text{Ac} - \text{As}}{\text{Ac}}$$

4.2.3 Ferric ion reducing power assay

The potential of *Cassia peterisana* extracts to reduce ferric ion was evaluated by the ferric ion reducing power assay (Ahmed *et al*., 2012; Vijayalakshmi and Ruckmani, 2016). A range of concentrations of the plant extracts (625-39 µg/mL) was prepared by a serial dilution of the stock solution of 1250 µg/mL into 5 clean test tubes. An amount of 2.5 mL of each of the concentrations was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of (1% w/v in distilled water) potassium ferricyanide in test tubes. The solutions were vortex mixed before incubation at 50°C for 20 minutes. After the incubation time had elapsed, 2 mL of (10% w/v in distilled water) trichloroacetic acid was added to the test tubes followed by centrifugation of the mixtures at 3000 rpm for 10 minutes. Clean test tubes were prepared to which 5 mL of the resulting supernatant was transferred and mixed with 5 mL of distilled water and 1 mL of (0.1% v/v in distilled water) ferric chloride, which were added successively with vortex mixing after every addition. A UV/VIS spectrophotometer was used to read

the absorbances of the solutions at a wavelength of 700 nm. L-Ascorbic acid used as a positive control was prepared by following the same procedure with the same range of concentrations (625-39 µg/mL). The blank sample was prepared by following the same procedure. However, the plant extracts were replaced by an equal amount of acetone. The experiments were carried out in triplites and repeated three times.

4.3 Results

The antioxidant activity of extracts from *Cassia petersiana* was evaluated qualitatively and quantitatively by the DPPH free radical scavenging activity assay as well as ferric ion reducing power assay. Extracts from preliminary and exhaustive extraction procedures were profiled on TLC for the evaluation of DPPH free radical scavenging activity (figure 4.1 and 4.2). Minimal antioxidants were observed in both figures 4.1 and 4.2 with no separation of the antioxidants as depicted by the yellow colour behind the purple background. Antioxidant activity from the distilled water extract was higher, as indicated by the more intense yellow bands behind the purple background in all the solvent systems compared to other extracts (figure 4.1). The acetone, ethanol and methanol extracts from the exhaustive extraction showed improved antioxidant activity compared to other extracts (figure 4.2). The antioxidants from *Cassia petersiana* also showed to be lower when compared to the ascorbic acid, which was used as the positive control in the quantitative analysis for antioxidant activity (figure 4.3 and figure 4.4). Furthermore, the distilled water extract showed higher antioxidant activity in both the quantitative antioxidant assays, while methanol had the lowest antioxidant activity.

Figure 4.1: DPPH free radical scavenging activity evaluation of *Cassia petersiana* extracts from preliminary extraction. The yellow colour behind the purple background of DPPH indicates antioxidant activity.

Key: H- n-Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, M- Methanol, B- Butanol, and W- Distilled water.

Figure 4.2: The DPPH free radical scavenging chromatograms for extracts from *Cassia petersiana* from exhaustive extraction. The presence of antioxidant activity is shown by the yellow colour behind the purple background.

Key: H- n-Hexane, C- Chloroform, D- Dichloromethane, EA- Ethyl acetate, A- Acetone, E- Ethanol, and M- Methanol.

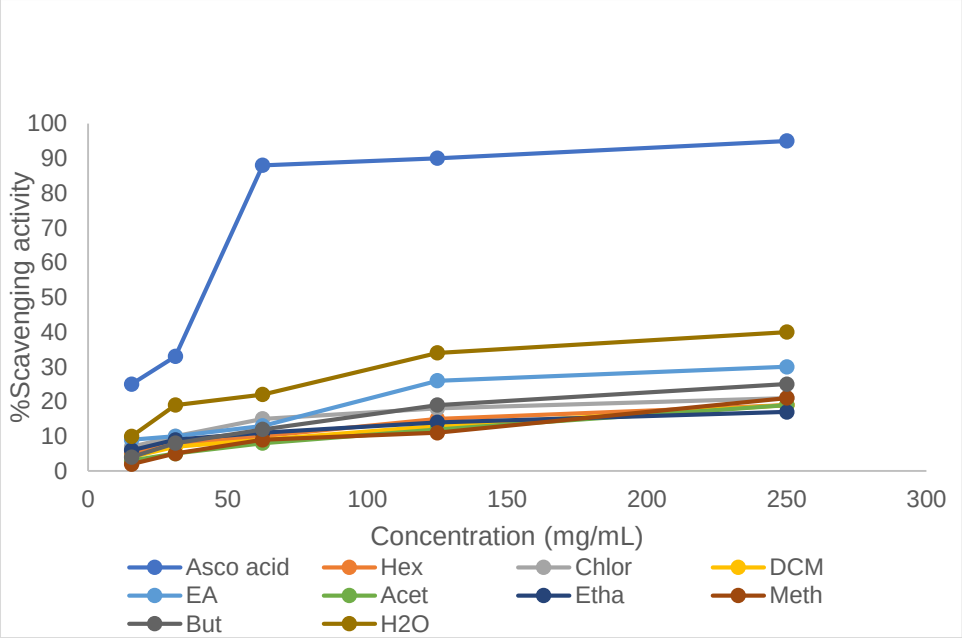

Figure 4.3: DPPH free radical scavenging activity of *Cassia petersiana* extracts at different concentrations. Ascorbic acid was used as a positive control.

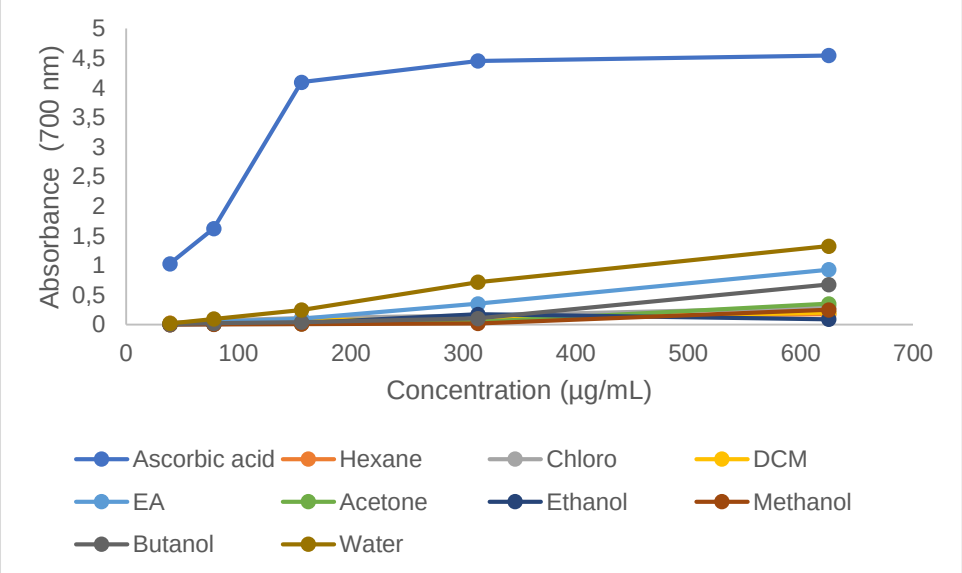

Figure 4.4: The ferric reducing power of *Cassia petersiana* extracts at different concentrations. Ascorbic acid was used as a positive control.

4.4 Discussion

Free radicals that are produced naturally by the human body can be lethal when in excess and not managed accordingly (Cetin Cakmak and Gülçin, 2019). They are managed by antioxidants, which are also produced naturally by the body, but at lower amounts that cannot manage all the free radicals present; hence, putting the human

body at risk of diseases (Huyut *et al*., 2017). To account for this, external antioxidants, particularly antioxidants from natural sources such as plants, are considered based on their promising activity (Arnao, 2000). It is of great importance to evaluate the antioxidant capacity of the extracts from medicinal plants with the goal of their use as external antioxidant sources (Leong and Shui, 2002).

This study deployed three techniques for the analysis of the antioxidant activity of extracts from *Cassia petersiana*, of which one technique was the qualitative analysis on TLC (DPPH free radical scavenging activity on TLC) while the remaining were quantitative analysis (DPPH free radical scavenging activity and ferric reducing power assays). The presence of antioxidants was observed at a relatively low intensity (figure 4.1) on the DPPH-TLC profile, as indicated by the yellow colour behind the purple background from the extracts obtained from preliminary extraction.

The same trend was also observed after extracting the material exhaustively, where the intensity of the yellow colour was relatively low with no separation of the antioxidants (figure 4.2). The intensity of the yellow colour on the TLC-DPPH profile indicates antioxidant capacity (Praptiwi *et al*., 2018). This suggests that the antioxidant capacity of extracts from *Cassia petersiana* is relatively low. Ascorbic acid was used as a positive test for the scavenging activity because it is a good antioxidant as it contains thiols which enable it to terminate the chain reactions initiated by the free radicals (Padayatty *et al*., 2003).

The percentage scavenging activity of the extracts from *Cassia petersiana* was low as compared to the ascorbic acid. The higher percentage scavenging activity observed from the water extract was not surprising because of the higher yellow intensity in all the solvent systems in figure 4.1. The lower percentage scavenging activity of all other extracts accounts for the faint yellow bands in figure 4.1. However, the exhaustive extraction procedure promoted the exposure of antioxidants from acetone, ethanol, and methanol extracts as the intensity of the yellow colour behind the purple background from figure 4.2 are higher than that in figure 4.1.

Ascorbic acid was also used as a positive control in the evaluation of the ferric reducing power of extracts from *Cassia petersiana*. The ferric reducing power capacity of the extracts is relative to a higher absorbance value observed. Ascorbic acid had a higher ferric reducing power capacity as compared to the test extracts. Once more, the higher

ferric reducing capacity of the water extract was higher than other extracts. Although the antioxidants capacity from *Cassia peterisana* is low, water extract showed to possess higher antioxidants as compared to other solvents.

4.5 Conclusion

The antioxidant activity of the extracts from *Cassia petersiana* was evaluated by means of qualitative analysis on TLC, whereas quantitative analysis was done by ferric reducing power and DPPH scavenging activity assays. These assays all revealed the lower antioxidant activity from the extracts from *Cassia petersiana*. Although the distilled water extracts showed higher antioxidant activity than other extracts, the activity is not significantly promising. Therefore, the results observed in this study suggest that *Cassia petersiana* extracts cannot be a good candidate for synthesising antioxidants from natural sources.

4.6 References

Ahmed, A., Elgorashi, E., Moodley, N., McGaw, L., Naidoo, V. and Eloff, J., 2012. The antimicrobial, antioxidative, anti-inflammatory activity and cytotoxicity of different fractions of four South African *Bauhinia* species used traditionally to treat diarrhoea. *Journal of Ethnopharmacology*, 143(3), pp.826-839.

Antolovich, M., Prenzler, P., Patsalides, E., McDonald, S. and Robards, K., 2001. Methods for testing antioxidant activity. *The Analyst*, 127(1), pp.183-198.

Apak, R., Özyürek, M., Güçlü, K. and Çapanoğlu, E., 2016. Antioxidant activity/capacity measurement. 1. classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *Journal of Agricultural and Food Chemistry*, 64(5), pp.997-1027.

Arnao, M., 2000. Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. *Trends in Food Science and Technology*, 11(11), pp.419-421.

Bae, G., Seo, D., Kwon, H., Lee, H., Hong, S., Lee, Z., Ha, K., Lee, H. and Han, J., 1999. Hydrogen peroxide activates p70s6k signaling pathway. *Journal of Biological Chemistry*, 274(46), pp.32596-32602.

Cetin Cakmak, K. and Gülçin, İ., 2019. Anticholinergic and antioxidant activities of usnic acid-an activity-structure insight. *Toxicology Reports*, 6, pp.1273-1280.

Chigayo, K., Mojapelo, P., Mnyakeni-Moleele, S. and Misihairabgwi, J., 2016. Phytochemical and antioxidant properties of different solvent extracts of *Kirkia wilmsii* tubers. *Asian Pacific Journal of Tropical Biomedicine*, 6(12), pp.1037-1043.

Deby, C. and Magotteaux, G., 1970. Relationship between essential fatty acids and tissue antioxidant levels in mice. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales*, 164(12), pp.2675-2681.

Foyer, C. and Noctor, G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell*, 17(7), pp.1866-1875.

Gulcin, I., 2020. Antioxidants and antioxidant methods: an updated overview. *Archives of Toxicology*, 94(3), pp.651-715.

Gunathilake, K., Ranaweera, K. and Rupasinghe, H., 2018. Change of phenolics, carotenoids, and antioxidant capacity following simulated gastrointestinal digestion and dialysis of selected edible green leaves. *Food Chemistry*, 245, pp.371-379.

Gutteridge, J., 1994. Biological origin of free radicals, and mechanisms of antioxidant protection. Chemico-Biological Interactions, 91(2-3), pp.133-140.

Huyut, Z., Beydemir, Ş. and Gülçin, I., 2017. Antioxidant and antiradical properties of selected flavonoids and phenolic compounds. *Biochemistry Research International*, 2017, pp.1-10.

Iverson, F., 1999. *In vivo* studies on butylated hydroxyanisole. *Food and Chemical Toxicology*, 37(9-10), pp.993-997.

Kasote, D., Hegde, M. and Katyare, S., 2013. Mitochondrial dysfunction in psychiatric and neurological diseases: cause(s), consequence(s), and implications of antioxidant therapy. *BioFactors*, 39(4), pp.392-406.

Kim, Y. and Byzova, T., 2014. Oxidative stress in angiogenesis and vascular disease. *Blood*, 123(5), pp.625-631.

Krishnaiah, D., Sarbatly, R. and Nithyanandam, R., 2011. A review of the antioxidant potential of medicinal plant species. *Food and Bioproducts Processing*, 89(3), pp.217-233.

Leclercq, C., Arcella, D. and Turrini, A., 2000. Estimates of the theoretical maximum daily intake of erythorbic acid, gallates, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in Italy: a stepwise approach*. Food and Chemical Toxicology*, 38(12), pp.1075-1084.

Leong, L. and Shui, G., 2002. An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chemistry*, 76(1), pp.69-75.

Maziero, G., Baunwart, C. and Toledo, M., 2001. Estimates of the theoretical maximum daily intake of phenolic antioxidants BHA, BHT and TBHQ in Brazil. *Food Additives and Contaminants*, 18(5), pp.365-373.

Padayatty, S., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J., Chen, S., Corpe, C., Dutta, A., Dutta, S. and Levine, M., 2003. Vitamin C as an antioxidant: evaluation of

its role in disease prevention. *Journal of the American College of Nutrition*, 22(1), pp.18-35.

Pavithra, K. and Vadivukkarasi, S., 2015. Evaluation of free radical scavenging activity of various extracts of leaves from *Kedrostis foetidissima* (Jacq.) Cogn. Food Science and Human Wellness, 4(1), pp.42-46.

Praptiwi, R., Wulansari, D., Fathoni, A. and Agusta, A., 2018. Antibacterial and antioxidant activities of endophytic fungi extracts of medicinal plants from Central Sulawesi. *Journal of Applied Pharmaceutical Science*, 8(8), pp.69-74.

Sena, L. and Chandel, N., 2012. Physiological roles of mitochondrial reactive oxygen species. *Molecular Cell*, 48(2), pp.158-167.

Tlili, N., Elfalleh, W., Hannachi, H., Yahia, Y., Khaldi, A., Ferchichi, A. and Nasri, N., 2013. Screening of natural antioxidants from selected medicinal plants. *International Journal of Food Properties*, 16(5), pp.1117-1126.

Velioglu, Y., Mazza, G., Gao, L. and Oomah, B., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry*, 46(10), pp.4113-4117.

Vijayalakshmi, M. and Ruckmani, K., 2016. Ferric reducing anti-oxidant power assay in plant extract. *Bangladesh Journal of Pharmacology*, 11(3), pp.570-572.

Wang, T., Li, Q. and Bi, K., 2018. Bioactive flavonoids in medicinal plants: structure, activity and biological fate. *Asian Journal of Pharmaceutical Sciences*, 13(1), pp.12-23.

Winrow, V., Winyard, P., Morris, C. and Blake, D., 1993. Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Medical Bulletin*, 49(3), pp.506-522.

Young, I. and Woodside, V., 2001. Antioxidants in health and disease. *Journal of Clinical Pathology*, 54(3), pp.176-186.

CHAPTER 5: Antimycobacterial activity assay

5.1 Introduction

The gradual emergence of resistance of most infectious agentsposes a clinical threat to the public globally as the efficacy of existing antibiotics is declining (Cushnie *et al*., 2020). As a result, the investigation and discovery of novel bioactive compounds is vital. The use of bioactive compounds produced by plants, animals and microorganisms (Lage *et al*., 2018) for health benefits has always been part of the history of mankind (Offiah *et al*., 2011). Consequently, natural sources such as plants are of interest for the screening of novel bioactive compounds as they possess a wide range of complex and structurally diverse compounds (Sasidharan *et al*., 2010).

The increased interest on the application of TLC in pharmaceutical analysis led to the employment of TLC-bioautography for the analysis of bioactive compounds from natural sources by most researchers (Pyka, 2014). This was influenced by the great advancement in TLC instrumentation such as TLC combined with densitometer or mass spectrometry, IR (Pyka, 2014) or NMR spectroscopy (Owen *et al*., 2019) as well as the ability of TLC to evaluate samples of low purity and concentration, as compared to gas chromatography and HPLC (Pyka, 2014). Although TLC-bioautography shows poor efficiency for water-insoluble compounds, it is a fast, simple, effective, and cheap technique (Lage *et al*., 2018; Shahverdi *et al*., 2007) that facilitates the determination of small amounts of drug components, especially when it is combined with densitometer or MS. TLC-bioautography enables the evaluation of separated complex mixtures from extracts with detection (Pyka, 2014) and localisation of compounds with antimicrobial activity on the TLC plate (Móricz *et al*., 2018).

Techniques used for the detection of bioactive compounds developed on TLC-bioautography include the physical method where an individual colour of a substance is taken note of, or fluorescence of a substance is evaluated with ultraviolet light, the chemical method where the visualising agents are used to detect the coloured reactions of separated substances, the physicochemical method, which include the application of isotopes as visualising agents and biological where the bio detectors are applied (Pyka, 2014).

Another technique used for the antibacterial bioassay of bioactive compounds is the dilution technique, which is used for determining the minimum inhibitory concentration

(MIC). It comprises broth medium and agar dilution methods (Lage *et al*., 2018). Broth dilution, which was employed in this study, can be done in a 96-well microtiter plate and the MIC outcome is recorded as the lowest concentration of antimicrobial agent that can completely inhibit growth after incubation (Monteiro *et al*., 2012). The dilution method offers an easy interpretation of quantitative results that are suitable for MIC calculation. It is also suitable for fastidious and non-fastidious bacteria, yeast, and filamentous fungi. However, this technique is labour-intensive, time-consuming, and results in poor efficiency for water-insoluble compounds (Lage *et al*., 2018).

Succeeding the determination of biological activity, the crude/complex plant extracts may be subjected to purification to allow for the isolation of bioactive compounds of interest (Sticher, 2008). The objective of this chapter was to preliminarily screen for the antimycobacterial compounds from *Cassia petersiana* extracts to allow for the subsequent purification and isolation of bioactive compounds against *Mycobacterium smegmatis*.

5.2 Method and materials

5.2.1 Test microorganism

The antimycobacterial activity of extracts from *Cassia petersiana* was evaluated against *Mycobacterium smegmatis* (ATCC 607). The stock culture of *M. smegmatis* was prepared by inoculation of the microorganism from the nutrient agar into 225 mL of Middlebrook broth, followed by incubation in a shaking incubator at 200 rpm speed at 37°C for 24 hours. The culture for antimycobacterial analysis was prepared by inoculation of 10 mL from the stock culture into 225 mL of freshly prepared Middlebrook broth media. The culture was incubated at 37°C for 24 hours for optimal growth of the microorganism.

5.2.2 Qualitative antimycobacterial assay

5.2.2.1 TLC-Bioautographic assay

The antimycobacterial activity of the extracts was evaluated by TLC-bioautography assay, according to Begue and Kline (1972). The TLC plates were loaded with 20 µL of the plant extracts and developed in solvent systems, as described in section 3.2.3. The TLC plates were placed under a fan for 5 days to evaporate the solvent systems before spraying with the test microorganism. The sprayed TLC plates were placed in

a container with humid conditions and incubated at 37°C for 24 hours. Following incubation, the plates were sprayed with 2 mg/mL of p-iodonitrotetrazolium violet (INT) dissolved in distilled water and incubated further for 2-3 hours to visualise the growth of the sprayed test microorganism. The clear zones behind the pink background were indicative of growth inhibition.

5.2.3 Quantitative antimycobacterial assay

5.2.3.1 Broth microdilution assay

The serial microbroth dilution method adopted from Eloff (1998) was employed to determine the MIC of the plant extracts against the test microorganism. The dried plant extracts were reconstituted to a concentration of 10 mg/mL with acetone. An amount of 100 µL of sterile distilled water was aseptically added to each well of the 96 well microtiter plate followed by a serial dilution of the plant extracts (100 µL) across all the wells. After a serial dilution, 100 µL of the test microorganism (prepared according to section 5.2.2.1) was added to the 96 well microtiter plate wells. Acetone was used as a negative control, while rifampicin was used as a positive control. The microtiter plates were covered with plastic and incubated for 24 hours at 37°C. After incubation, an amount of 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) was added to each well of the microtiter plate, followed by incubation for 30 minutes to allow the microbial growth indicator to develop colour. The microtiter plates were then observed for clear zones, which indicate microbial growth inhibition as compared to the violet-purple zones, which indicate microbial growth. The MIC values were recorded as the lowest concentration that inhibited microbial growth. The assay was done in triplicates and repeated twice. The total activity of the extracts was determined by division of the MIC values with the mass extracted from 1 gram of the plant material. The total activity of the plant extracts was determined by dividing the MIC values with the mass (in milligrams) extracted from 1 gram of the plant material. The values obtained indicate the volume to which the amount obtained from 1 g of the plant material could be diluted to and still inhibit the growth of the test microorganisms (Eloff, 2000).

5.3 Results

The antimycobacterial activity of *Cassia petersiana* extracts against *Mycobacterium smegmatis* was discovered, as shown by the bioautography and microbroth dilution assays below. The noteworthy antimycobacterial activity from the preliminary

extraction procedure was the faint white bands against the pink background in BEA mobile system in figure 5.1. The lowest MIC (0.13 mg/mL) was from the chloroform extract in table 5.1. The total activity which represents the volume to which the amount obtained from 1 g of the plant material could be diluted to and still inhibit the growth of *Mycobacterium smegmatis* was determined, as represented in table 5.2. The highest total activity was from chloroform extract (307). Extracts from the exhaustive extraction procedure displayed antimycobacterial activity as indicated by several white bands against the pink background observed only in the BEA mobile system in figure 5.2, while dichloromethane and acetone showed the lowest MIC (0.21 mg/mL) in table 5.3.

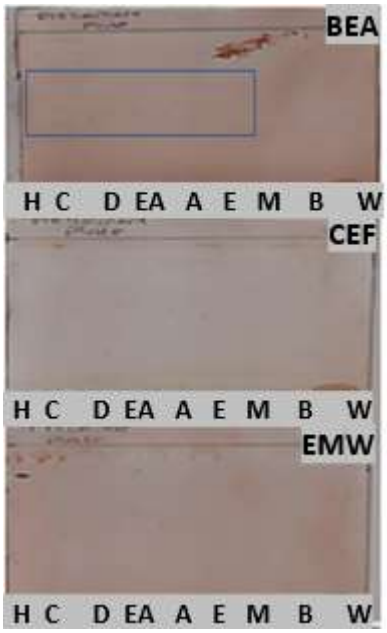

Figure 5.1: Bioautograms of the extracts from the leaves of *Cassia petersiana* separated on aluminium-backed TLC plates. The faint white bands behind the pink background on the BEA TLC plate are indicative of antimycobacterial activity against *Mycobacterium smegmatis*, which resulted from n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), and acetone (A) extracts.

Key: H= n-Hexane, C= Chloroform, D= Dichloromethane, EA= Ethyl acetate, A= Acetone, E= Ethanol, M= Methanol, B= Butanol, H_2O= Distilled water.

The noteworthy findings were that from the chloroform extract, as highlighted in Table 5.1 below, which had the lowest MIC and highest total activity (308 mL/g) as compared to other extracts. However, positive control (rifampicin) had the lowest antimycobacterial activity of all the extracts.

Table 5.1: Minimum inhibitory concentration (MIC) of extracts from *Cassia petersiana* obtained with preliminary extraction procedure.

Plant extract and MIC values (mg/mL)									
H	C	D	EA	A	E	M	B	H_2O	Rifampicin
0.41	0.13	0.83	0.63	1.04	0.31	0.83	0.52	1.04	0.08

Key: H= n-Hexane, C= Chloroform, D= Dichloromethane, EA= Ethyl acetate, A= Acetone, E= Ethanol, M= Methanol, B= Butanol, H_2O= Distilled water.

Table 5.1: Total activities (mL/g) of the extracts from *Cassia petersiana*.

H	C	D	EA	A	E	M	B	H_2o
73	308	108	111	87	129	169	58	77

Key: H= n-Hexane, C= Chloroform, D= Dichloromethane, EA= Ethyl acetate, A= Acetone, E= Ethanol, M= Methanol, B= Butanol, H_2O= Distilled water.

Figure 5.2: Bioautograms of *Cassia petersiana* extracts from exhaustive extraction procedure. The n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), and acetone (A) extracts showed antimycobacterial activity against *Mycobacterium smegmatis* as indicated by the white bands behind the pink background in BEA solvent system.

Table 5.2: MICs (mg/mL) of *Cassia petersiana* extracts from exhaustive extraction procedure. Dichloromethane and acetone extracts showed the lowest MICs as compared to other extracts. Rifampicin was used as a positive control.

Plant extract and MIC values (mg/mL)							
H	C	D	EA	A	E	M	Rifampicin
0.37	0.42	0.21	1.04	0.21	1.04	1.04	0.08

Key: H= n-Hexane, C= Chloroform, D= Dichloromethane, EA= Ethyl acetate, A= Acetone, E=Ethanol, and M= Methanol.

5.4 Discussion

The decline in the efficacy of existing antibiotics for diseases accounted for the urgency to produce alternative drugs with improved features (Cushnie *et al*., 2020). As synthetic drugs continue to pose major drawbacks in the management and treatment of several diseases, drugs from plant sources remain the possible potential candidates. This is based on the chemical diversity of the plants due to secondary metabolites that they produce (Sasidharan *et al*., 2010). Based on the traditional use of medicinal plants for the treatment of diseases, extraction is a vital step. Solvents of varying polarities were used to obtain a wide range of bioactive compounds from the ground plant material from *Cassia petersiana*. Before the extracts are considered for the isolation of bioactive compounds, it is important that biological tests are conducted to determine the availability of the targeted bioactivity against the tested microorganisms.

Bioautographic and serial broth micro-dilution assays were used in this study to evaluate the antimycobacterial activity of the extracts from *Cassia petersiana*. Direct TLC-bioautography was the preferred method for the screening of antimycobacterial compounds from *Cassia petersiana* as it is an effective technique to track down bioactive compounds while showing their location directly on the TLC absorbent (Móricz *et al*., 2018). Extracts from the preliminary extraction were spotted on the aluminium-backed TLC plates for the qualitative evaluation of antimycobacterial activity by TLC-bioautography. The TLC plates were sprayed with *Mycobacterium smegmatis* and incubated before spraying with INT, which is an indicator of microbial growth. The pink colour on the bioautograms represents microbial growth, while the

white bands indicate zones of inhibition. However, the bioactivity of the extracts against the tested microorganism was very slight, as a very faint band was observed on the chromatogram from the BEA mobile system (figure 5.1).

Interestingly, the extracts showed significant antimycobacterial activity against *Mycobacterium smegmatis* after the serial broth micro-dilution assay which evaluated the quantitative antimycobacterial activity of the extracts (table 5.1). The lowest MIC observed was from the chloroform extract (0.13 mg/mL). The chloroform extract was considered the more bioactive extract from preliminary extraction as it was previously reported that the extract with the lowest MIC value represents the most bioactivity against the tested microorganism (Eloff, 2000). It is important to consider the total activity of the extracts altogether with their MIC values during the assessment of the bioactivity of the extracts (Eloff, 2000). As a result, the total activity of the extracts was determined and represented in table 5.2. The highest activity was from the chloroform extract (308 mL/g), followed by the ethanol extract (129 mL/g), while the least was from the butanol extract (58 mL/g). These findings reveal that the quantity of the chloroform extract can be diluted to 308 mL/g and still inhibit the growth of *Mycobacterium smegmatis*. The possible explanation for the non-correlation of antimycobacterial activity from the TLC-bioautographic and serial broth micro-dilution assays may be because some of the compounds with antibacterial activity on serial broth micro-dilution assay acted synergistically, and the separation of these compounds by TLC may have resulted in the decreased bioactivity. Another possible reason for the two assay not to correlate may be due to the loss of bioactive compounds during the air-drying process that occurred before bioautography assay (Komape *et al*., 2014).

As the findings in table 5.1 indicated, the significant bioactivity against *Mycobacterium smegmatis*, it was important that exhaustive extraction was considered to expose more bioactive compounds from *Cassia petersiana*. The extraction procedure resulted in the increased period of exposure of the bioactive compounds to extraction solvents. The antimycobacterial activity of the extracts in figure 5.2, indicated by the white bands behind the pink background, was evidence that more bioactive compounds were exposed following an exhaustive extraction procedure. The lowest MIC of the extracts from *Cassia petersiana* against *Mycobacterium smegmatis* was from dichloromethane and acetone extracts (0.21 mg/mL), as shown in table 5.3.

5.5 Conclusion

The antimycobacterial activity of the extracts from *Cassia petersiana* was evaluated qualitatively by the TLC-bioautographic assay and quantitatively by the serial broth micro-dilution assay. Although the qualitative antimycobacterial assay from preliminary extraction showed minimal bioactivity, the quantitative antimycobacterial activity assay showed promising bioactivity, which motivated an alternative extraction procedure to obtain more bioactive compounds. The exhaustive extraction resulted in clear zones of inhibition following the TLC-bioautographic assay, and the serial broth micro-dilution assay also showed antimycobacterial activity. The antimycobacterial activity observed suggests the consideration of the extracts from *Cassia petersiana* for assays leading to the isolation of antimycobacterial compound(s).

5.6 References

Begue, W. and Kline, R., 1972. The use of tetrazolium salts in bioauthographic procedures. *Journal of Chromatography* A, 64(1), pp.182-184.

Cushnie, T., Cushnie, B., Echeverría, J., Fowsantear, W., Thammawat, S., Dodgson, J., Law, S. and Clow, S., 2020. Bioprospecting for antibacterial drugs: a multidisciplinary perspective on natural product source material, bioassay selection and avoidable pitfalls. *Pharmaceutical Research*, 37(7), pp.1-24.

Eloff, J., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, 64(08), pp.711-713.

Eloff, J., 2000. A proposal on expressing the antibacterial activity of plant extracts – a small first step in applying scientific knowledge to rural primary health care in South Africa. *South African Journal of Science*, 96, pp.116-118.

Komape, N., Aderogba, M., Bagla, V., Masoko, P. and Eloff, J., 2014. Anti-bacterial and anti-oxidant activities of leaf extracts of *Combretum vendae* (Combretecacea) and the isolation of an anti-bacterial compound. *African Journal of Traditional, Complementary and Alternative Medicines*, 11(5), p.73.

Lage, O., Ramos, M., Calisto, R., Almeida, E., Vasconcelos, V. and Vicente, F., 2018. Current screening methodologies in drug discovery for selected human diseases. *Marine Drugs*, 16(8), p.279.

Monteiro, M., de la Cruz, M., Cantizani, J., Moreno, C., Tormo, J., Mellado, E., De Lucas, J., Asensio, F., Valiante, V., Brakhage, A., Latgé, J., Genilloud, O. and Vicente, F., 2012. A new approach to drug discovery. *Journal of Biomolecular Screening*, 17(4), pp.542-549.

Móricz, Á., Szeremeta, D., Knaś, M., Długosz, E., Ott, P., Kowalska, T. and Sajewicz, M., 2018. Antibacterial potential of the Cistus incanus L. phenolics as studied with use of thin-layer chromatography combined with direct bioautography and *in situ* hydrolysis. *Journal of Chromatography A*, 1534, pp.170-178.

Offiah, N., Makama, S., Elisha, I., Makoshi, M., Gotep, J., Dawurung, C., Oladipo, O., Lohlum, A. and Shamaki, D., 2011. Ethnobotanical survey of medicinal plants used in

the treatment of animal diarrhoea in Plateau State, Nigeria. *BMC Veterinary Research*, 7(1), pp.36-45.

Owen, L., White, A. and Laird, K., 2019. Characterisation and screening of antimicrobial essential oil components against clinically important antibiotic-resistant bacteria using thin layer chromatography-direct bioautography hyphenated with GC-MS, LC-MS and NMR. *Phytochemical Analysis*, 30(2), pp.121-131.

Pyka, A., 2014. Detection progress of selected drugs in TLC. *BioMed Research International*, 2014, pp.1-19.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. and Latha, L., 2010. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, 8(1), pp.1-10.

Shahverdi, A., Abdolpour, F., Monsef-Esfahani, H. and Farsam, H., 2007. A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. *Journal of Chromatography B*, 850(1-2), pp.528-530.

Sticher, O., 2008. Natural product isolation. *Natural Product Reports*, 25(3), p.517.

CHAPTER 6: Cytotoxicity assay

6.1 Introduction

Cytotoxicity is described as the extent to which an exposed tissue is damaged by a chemical substance where the effect covers a whole organism or sub-structural component of an organism, such as a cell (Faqi, 2017). A cytotoxic substance causes interference with cell attachment, alters cell growth, and proliferation or induces cell death (Riss *et al*., 2011). It is very important to determine the cytotoxicity of substances to discover compounds or affective parts that may present health risks to humans. Cytotoxic substances cause damage to living cells by either accidental cell death or programmed cell death (Ricci and Zong, 2006).

Traditional medicinal plants are continually used to meet the healthcare needs of individuals based on their ancient use and the diversity of their bioactive compounds. However, the diversity of phytochemical constituents in medicinal plants, such as terpenoids, alkaloids, and glycosides, may be useful or harmful to health given their varied mechanisms of interactions, which can be additively or synergistically (Dhiman and Builders, 2020). It is important to assess the cytotoxicity of the commonly used traditional medicinal plants to ensure the quality, efficacy, and safety of their preparations. Cytotoxicity studies on medicinal plants are critical in the development of new therapeutic products for ensuring the safety of individuals who use herbal medicine. It is also recommended that all bioactive medicinal plants are evaluated for toxicity at a cellular level (Fernandes de Sá Ferreira and Ferrão Vargas, 1999; Kassie *et al*., 1996; Schimmer *et al*., 1994).

The fatalities due to the toxicity of medicinal plants have been minimised in many cultural settings owing to the systematic selection of medicinal plants for use (Nasri and Shirzad, 2013). Relatively toxic medicinal products can be exploited for health benefits only below their toxic levels because they barely result in any fatality when administered this way by professional practitioners or experienced persons (Dhiman and Builders, 2020).

Deshpande (2002) and Hill (2020), suggest that the dosage of any substance, including herbal remedies, is relative to its toxicity, implicating that a very toxic substance can be regarded as safe at a low dosage while a non-toxic substance can be toxic at a high dosage. Using human cell lines for in vitro cytotoxicity assays is vital

for predicting human acute toxicity as opposed to acute lethality studies in rodents (Hayes, 2013).

According to the WHO, the practice of herbal medicine as a source of healthcare system is advised only after establishing the non-toxicity of herbal preparations (Akintonwa *et al*., 2009). *In vitro* toxicity testing is a remarkable tool for the evaluation of the safety of different chemicals and drugs (Boada *et al*., 2016). The cytotoxic potential of several environmental pollutants, chemicals, drugs, and plant extracts is determined by the assays, which include MTT colorimetric assay (Boncler *et al*., 2014; Bunel *et al*., 2014). This assay determines the viability of cells upon exposure to toxicants and serves as an indicator of anti-proliferative activation, cell activation, and cytotoxicity (Khasawneh *et al*., 2011).

The principle of the MTT assay is based on the measurement of formazan colour, which is formed by the oxidoreductase enzymes present in the mitochondria of living cells (Stockert *et al*., 2012). The MTT assay is relevant for the cytotoxicity evaluation of natural products, which include those that are volatile (Khadir *et al*., 2016). During the MTT assay, the metabolically active cells reduce the yellow water-soluble tetrazolium salt into blue/magenta (MTT) formazan crystal which is water-insoluble (Morgan, 1998). This allows for the quantification of the concentration of the dissolved formazan crystal by the use of a spectrophotometer which correlates directly to the number of metabolically active cells (Wang *et al*., 2010). In this essay, the 96-well microtiter plates are employed; hence, they allow for the analysis of many experiments with several variables examined (Cole, 1986).

6.2 Method and materials

The 3- [4.5-dimethylthiazol- 2-yl]-2.5 diphenyl tetrazolium bromide (MTT) assay was employed for the analysis of the cytotoxicity effects of the acetone extract from *Cassia petersiana* on tryptophan hydroxylase-1 *(*TPH-1) macrophage cells (Mosmann, 1983). The cells were maintained in Minimal Essential Medium (MEM), which was supplemented with 0.1% gentamicin and 5% fetal calf serum were the negative control comprised untreated cells. A sterile 96-well microtiter plate was used where an amount of 200 µL of the cell suspension (5 × 104 cells/mL) was added into each well. To allow cells to attach, the microtiter plates were incubated for 24 hours at 37°C in a 5% CO_2 incubator. After the incubation time elapsed, the cells were washed with 150 µL

phosphate-buffered saline. The cells were subsequently treated with 200 µL of a range of concentrations (1- 0.025) from plant extracts prepared in MEM before the incubation of the microtiter plates in the conditions described above for 24 hours. An amount of 30 µL of MTT (5 mg/mL) in phosphate-buffered saline was added to each well after the incubation period. This was followed by further incubation of the microtiter plates for 4 hours at 37°C, after which the medium was removed and replaced with 50 µL of dimethyl sulfoxide. The microtiter plates were then gently swirled to dissolve the MTT formazan crystals. The reduction of MTT was measured by the detection of absorbance using a microplate reader at 570 nm. The experiment was conducted independently, in quadruplets and repeated twice.

6.3 Results

The acetone extract of the dried leaves of *Cassia petersiana* was evaluated for cytotoxicity on the tryptophan hydroxylase-1 *(*TPH-1) macrophage cells using the 3-[4.5-dimethylthiazol- 2-yl]-2.5 diphenyl tetrazolium bromide (MTT) assay (figure 6.1). The viability of cells displayed in percentages is indicative of metabolic reactions occurring in the cells. The untreated cells had the highest percentage of cell viability as cytotoxicity was not expected; in contrast, the percentage of cell viability varied at different concentrations on the cells treated with acetone extracts from *Cassia petersiana*. The highest percentage of cell viability from the treated cells was from 100 µg/mL, while the least was from 1000 µg/mL.

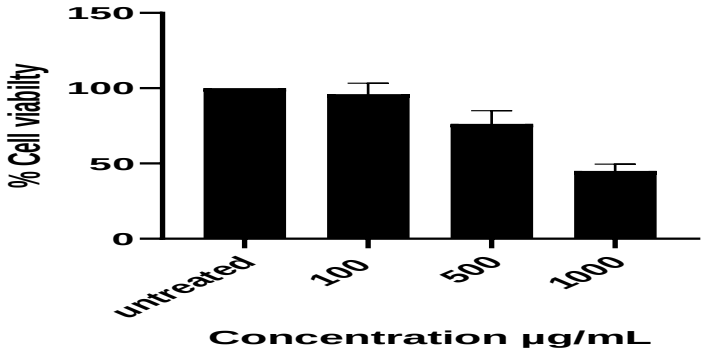

Figure 6.1: The cytotoxicity effects of *Cassia petersiana* acetone extract on the tryptophan hydroxylase-1 *(*TPH-1) macrophage cells.

6.4 Discussion

The use of traditional medicinal plants to meet the healthcare needs of individuals is common in Africa (Fomogne-Fodjo *et al*., 2014). They are preferred by many due to their ancient use as they form an integral part of their history and religion (Borokini and Omotayo, 2012; Liu *et al*., 2007). Furthermore, remarkable bioactive compounds for a variety of diseases have been established from traditionally used medicinal plants. However, cytotoxicity effects of herbal preparations on humans is a very critical aspect as it may affect the livelihood of individuals (Fennelly *et al*., 2004).

Herbal preparations may either be toxic or non-toxic to the cells at a defined dosage. The administration of cytotoxic herbal preparation may lead to the death of an individual; hence, the importance of evaluating the cytotoxic effects of traditionally used medicinal plants. WHO states that herbal preparations are only used once their cytotoxicity effects have been evaluated (Akintonwa *et al*., 2009).

This study employed the MTT assay for the analysis of the cytotoxic effects of the acetone extracts from *Cassia petersiana* on tryptophan hydroxylase-1 *(*TPH-1) macrophage cells. The MTT assay is a simple and rapid colorimetric assay that yields quantitative data by the quantification of the cellular activity by measuring the absorbance of the solution at a specific wavelength (Chanlalit *et al*., 2012). During this assay, the metabolically active cells reduce the yellow tetrazolium salt to formazan crystal, which is blue/magenta (Morgan, 1998; Stockert *et al*., 2012). Formazan accumulates in healthy cells and can be measured by a spectrophotometer and its colour intensity correlates with the metabolically active cells (Fotakis and Timbrell, 2006; Wang *et al*., 2010).

The cytotoxicity effects of the acetone extract from *Cassia petersiana* were evaluated on the tryptophan hydroxylase-1 *(*TPH-1) macrophage cells at different concentrations ranging from 100 to 1000 µg/mL (figure 6.1). It was noteworthy that percentage cell viability decreased with an increase of the concentration of the acetone extract from *Cassia petersiana*. The lowest percentage cell viability (45%) was from 1000 µg/mL. These findings suggest that the acetone extracts from *Cassia petersiana* may be potentially toxic to the cells at higher concentrations. The importance of dosage during the preparation of herbal medicine was well demonstrated in this study by the varied percentage cell viability at different concentrations of acetone extracts.

6.5 Conclusion

The cytotoxicity effects of the acetone extract of *Cassia petersiana* on tryptophan hydroxylase-1 *(*TPH-1) macrophage cells were successfully evaluated by the MTT assay. This study suggests that the herbal preparations from *Cassia petersiana* may be potentially toxic at higher concentrations; hence, it recommends the preparation of herbal extracts at a lower concentration. However, it is important to use the human cell lines for *in vitro* cytotoxicity assays for the prediction of human acute toxicity.

6.6 References

Akintonwa, A., Awodele, O., Afolayan, G. and Coker, H., 2009. Mutagenic screening of some commonly used medicinal plants in Nigeria. *Journal of Ethnopharmacology*, 125(3), pp.461-470.

Boada, L., Henríquez-Hernández, L. and Luzardo, O., 2016. The impact of red and processed meat consumption on cancer and other health outcomes: epidemiological evidences. *Food and Chemical Toxicology*, 92, pp.236-244.

Boncler, M., Różalski, M., Krajewska, U., Podsędek, A. and Watala, C., 2014. Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. *Journal of Pharmacological and Toxicological Methods*, 69(1), pp.9-16.

Borokini, T. and Omotayo, F., 2012. Phytochemical and ethnobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research*, 6(7), pp.1106-1118.

Bunel, V., Ouedraogo, M., Nguyen, A., Stévigny, C. and Duez, P., 2014. Methods applied to the *in vitro* primary toxicology testing of natural products: state of the art, strengths, and limits. *Planta Medica*, 80(14), pp.1210-1226.

Chanlalit, C., Shukla, D., Fitzsimmons, J., A.K. and O'Driscoll, S., 2012. Stress shielding around radial head prostheses. *The Journal of Hand Surgery*, 37(10), pp.2118-2125.

Cole, S., 1986. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemotherapy and Pharmacology*, 17(3), pp.259-263.

Deshpande, S., 2002. Handbook of food toxicology. 18th ed. New York: Marcel Dekker, pp.1-5.

Dhiman, A. and Builders, P., 2020. Herbal medicine. 1st ed. Ljubljana: Salem Press, pp.63-86.

Faqi, A., 2017. A comprehensive guide to toxicology in nonclinical drug development. 2nd ed. London: Elsevier Science, pp.109-127.

Fennelly, K., Martyny, J., Fulton, K., Orme, I., Cave, D. and Heifets, L., 2004. Cough-generated aerosols of *Mycobacterium tuberculosis*. *American Journal of Respiratory and Critical Care Medicine*, 169(5), pp.604-609.

Fernandes de Sá Ferreira, I. and Ferrão Vargas, V., 1999. Mutagenicity of medicinal plant extracts in Salmonella/microsome assay. *Phytotherapy Research*, 13(5), pp.397-400.

Fomogne-Fodjo, M., van-Vuuren, S., Ndinteh, D., Krause, R. and Olivier, D., 2014. Antibacterial activities of plants from Central Africa used traditionally by the Bakola pygmies for treating respiratory and tuberculosis-related symptoms. *Journal of Ethnopharmacology*, 155(1), pp.123-131.

Fotakis, G. and Timbrell, J., 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, 160(2), pp.171-177.

Hayes, A., 2013. Principles and methods of toxicology. 5th ed. Boca Raton: Chemical Rubber Company Press, pp.52-62.

Hill, M., 2020. Understanding environmental pollution. 4th ed. Cambridge: Cambridge University Press, pp.44-66.

Kassie, F., Parzefall, W., Musk, S., Johnson, I., Lamprecht, G., Sontag, G. and Knasmüller, S., 1996. Genotoxic effects of crude juices from *Brassica* vegetables and juices and extracts from phytopharmaceutical preparations and spices of cruciferous plants origin in bacterial and mammalian cells. *Chemico-Biological Interactions*, 102(1), pp.1-16.

Khadir, A., Sobeh, M., Gad, H., Benbelaid, F., Bendahou, M., Peixoto, H., Sporer, F., Ashour, M. and Wink, M., 2016. Chemical composition and biological activity of the essential oil from *Thymus lanceolatus*. *Zeitschrift für Naturforschung* C Journal of Biosciences, 71(5-6), pp.155-163.

Khasawneh, M., Elwy, H., Hamza, A., Fawzi, N. and Hassan, A., 2011. Antioxidant, anti-lipoxygenase and cytotoxic activity of *Leptadenia pyrotechnica* (Forssk.) decne polyphenolic constituents. *Molecules*, 16(9), pp.7510-7521.

Liu, Q., Li, W., Xue, M., Chen, Y., Du, X., Wang, C., Han, L., Tang, Y., Feng, Y., Tao, C. and He, J., 2017. Diabetes mellitus and the risk of multidrug resistant tuberculosis: a meta-analysis. *Scientific Reports*, 7(1), pp.1-7.

Morgan, D., 1998. Tetrazolium (MTT) assay for cellular viability and activity. *Polyamine Protocols*, 79, pp.179-184.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1-2), pp.55-63.

Nasri, H. and Shirzad, H., 2013. Toxicity and safety of medicinal plants. *Journal of Herbmed Pharmacology*, 2(2), pp.21-22.

Ricci, M. and Zong, W., 2006. Chemotherapeutic approaches for targeting cell death pathways. *The Oncologist*, 11(4), pp.342-357.

Riss, T., Moravec, R. and Niles, A., 2011. Cytotoxicity testing: measuring viable cells, dead cells, and detecting mechanism of cell death. *Methods in Molecular Biology*, 740, pp.103-114.

Schimmer, O., Krüger, A., Paulini, H. and Haefele, F., 1994. An evaluation of 55 commercial plant extracts in the Ames mutagenicity test. *Pharmazie*, 49(6), pp.448-451.

Stockert, J., Blázquez-Castro, A., Cañete, M., Horobin, R. and Villanueva, Á., 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica*, 114(8), pp.785-796.

Wang, H., Cheng, H., Wang, F., Wei, D. and Wang, X., 2010. An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of *Escherichia coli* cells. *Journal of Microbiological Methods*, 82(3), pp.330-333.

CHAPTER 7: Bioassay guided fractionation

7.1 Introduction

Drug discovery and development are among the topics of interest for most researchers due to the gradual emergence of antibiotic resistance of most infectious agentst. This has led to the decreased efficacy of current drugs for diseases such as TB. The current treatment for TB involves the combinations of rifampicin, isoniazid, ethambutol, and pyrazinamide, which are taken from twice weekly to daily for a period of six to nine months (Quan *et al*., 2019). This treatment has proven to be less effective due to its toxicity and poorer efficacy. As a result, the discovery of alternative drugs for TB and other infectious diseases is vital (Ndjeka *et al*., 2018). It is known that drug discovery requires chemical diversity. Fortunately, nature provides for the chemical diversity needed for drug development through natural products such as plants (Alvin *et al*., 2016).

Over the years, natural products have proven to be highly significant in the drug discovery and development processes (Cragg *et al*., 2009), as they possess diverse lead compounds, drug candidates, and pharmaceuticals (Alvin *et al*., 2016). The interest in the use of natural products as potential drugs is motivated by the discovery that secondary metabolites contained in complex plant extracts work synergistically and biological activity observed is commonly not from a single molecule/compound (Brusotti *et al*., 2014). Furthermore, a high percentage of new highly effective drugs that are introduced to the market are from natural products or derivatives from natural products (Alvin *et al*., 2016).

Before natural products are considered for use in drug discovery and development, the biological activity against the chosen test microorganism must be determined by defined procedures. Thereafter, the complex mixture will be purified to isolate the bioactive compounds (Sticher, 2008). The steps followed to extract bioactive compounds from plant sources to be used as drugs include extraction, pharmacological screening, isolation, characterisation, toxicological evaluation, and clinical evaluation of the bioactive compounds (Sasidharan *et al*., 2011).

During extraction, solvents of varying polarity are employed to extract possible compounds ranging from non-polar to polar. In this chapter, a large scale exhaustive extraction procedure was used to obtain a sufficient amount of candidate bioactive

compounds. The extracts were screened for phytochemical, antioxidant as well as antimycobacterial evaluation to track down the bioactive compounds for isolation. The extracts with promising bioactivity were subjected to purification and isolation. However, since plant extracts occur as a combination of different bioactive compounds or phytochemicals with varying polarities, their separation and purification require different isolation techniques such as TLC, Column Chromatography, Flash Chromatography, Sephadex Chromatography, and HPLC (Sasidharan *et al*., 2011). The combination of Column Chromatography and TLC was used in this study for the isolation of bioactive compound(s). The aim of this chapter was to use bioassay-guided fractionation to isolate antimycobacterial compounds from *Cassia petersiana* active against *Mycobacterium smegmatis*.

7.2 Method and materials

7.2.1 Serial exhaustive extraction

The serial exhaustive extraction procedure was used to extract bioactive compounds from *Cassia petersiana* ground leaf material. An amount of 555 g of the plant material was subjected to 4 litres of n-hexane, followed by vigorous shaking overnight at 200 rpm speed. After filtration of the supernatant, the same plant material was further subjected to the same amount of n-hexane with shaking period of 3 hours. The filtrate was concentrated with rotary evaporator and transferred into pre-weighed labelled beakers. The beakers were placed under a stream of cold air at room temperature to evaporate the solvents before the masses of the crude extracts were determined. The same procedure was repeated on the same plant material using dichloromethane, acetone, and methanol.

7.2.2 Phytochemical evaluation

The phytochemical profiles of the dried crude extracts from *Cassia petersiana* were analysed on aluminium-backed TLC plates, as described in section 3.2.3.

7.2.3 Qualitative antioxidant activity assay

Antioxidant evaluation of the crude extracts from *Cassia petersiana* was analysed with TLC-DPPH, as described in section 4.2.1.

7.2.4 Antimycobacterial activity assays

Bioautography and serial micro-broth dilution assays of the crude extracts from *Cassia petersiana* against *Mycobacterium smegmatis* were evaluated, as described in sections 5.2.2 and 5.2.3, respectively.

7.2.5 Isolation of antimycobacterial compounds

7.2.5.1 Open column chromatography

Crude n-hexane extracts from serial exhaustive extraction were selected to be subjected to column chromatography for isolation of antimycobacterial compounds based on the significantly high antimycobacterial activity observed. An open column (30 cm height x 7 cm radius) was packed with silica gel (particles size 0.063 - 0.200 mm) using 90% n-hexane/ethyl acetate. The extracts were mixed with a trace amount of silica gel and subjected to column chromatography. The fractions were collected into clean test tubes followed by a concentration of the fractions under a stream of air. The concentrated fractions were evaluated for phytochemical analysis to track down compounds with similar chemical profiles. The fractions with similar chemical profiles were combined and concentrated further. The combined fractions were evaluated for phytochemical analysis (section 7.2.2), antioxidant activity using TLC-DPPH (section 7.2.3) and antimycobacterial activity assays (section 7.2.4)

7.3 Results

7.3.1 Serial exhaustive extraction

7.3.1.1 Mass of plant material extracted from *Cassia petersiana*.

An amount of 555 g of the *Cassia petersiana* plant material was subjected to solvents of varying polarity for extraction. From this amount, a total of 65.07 g was obtained from all the solvents, where methanol extracted most of the plant material (37.34 g), followed by n-hexane (12.19) while the least was from acetone (4.57), as shown in table 7.1.

Table 7.1: The mass exhaustively extracted from *Cassia petersiana* using the solvents of varying polarity.

Extraction solvents		Mass of plant extract (g)	
		Mass	Total
n-Hexane	1	8.53	12.19
	2	3.66	
Dichloromethane	1	7.88	10.97
	2	3.09	
Acetone	1	2.91	4.57
	2	1.66	
Methanol	1	20.40	37.34
	2	16.94	
	Total:		**65.07**

Key: 1- overnight and 2- first three hours.

7.3.1.2 Phytochemical evaluation on TLC

The extracts obtained from exhaustive extraction using n-hexane, dichloromethane, acetone and methanol were subjected to TLC to evaluate their phytochemical profiles. The aluminium-backed TLC plates were developed in solvent systems of varying polarity ranging from non-polar to polar to separate phytochemicals with a wide range of polarities. The phytochemicals were visualised under ultraviolet light at 254 and 365 nm (figure 7.1A and B) before spraying with vanillin sulphuric acid (figure 7.1C) to visualise the non-fluorescing phytochemicals. Most of the phytochemical compounds were visualised in the BEA solvent system with an ultraviolet light at 365 nm and with vanillin-sulphuric acid. This suggests that more non-polar compounds were extracted from *Cassia petersiana*.

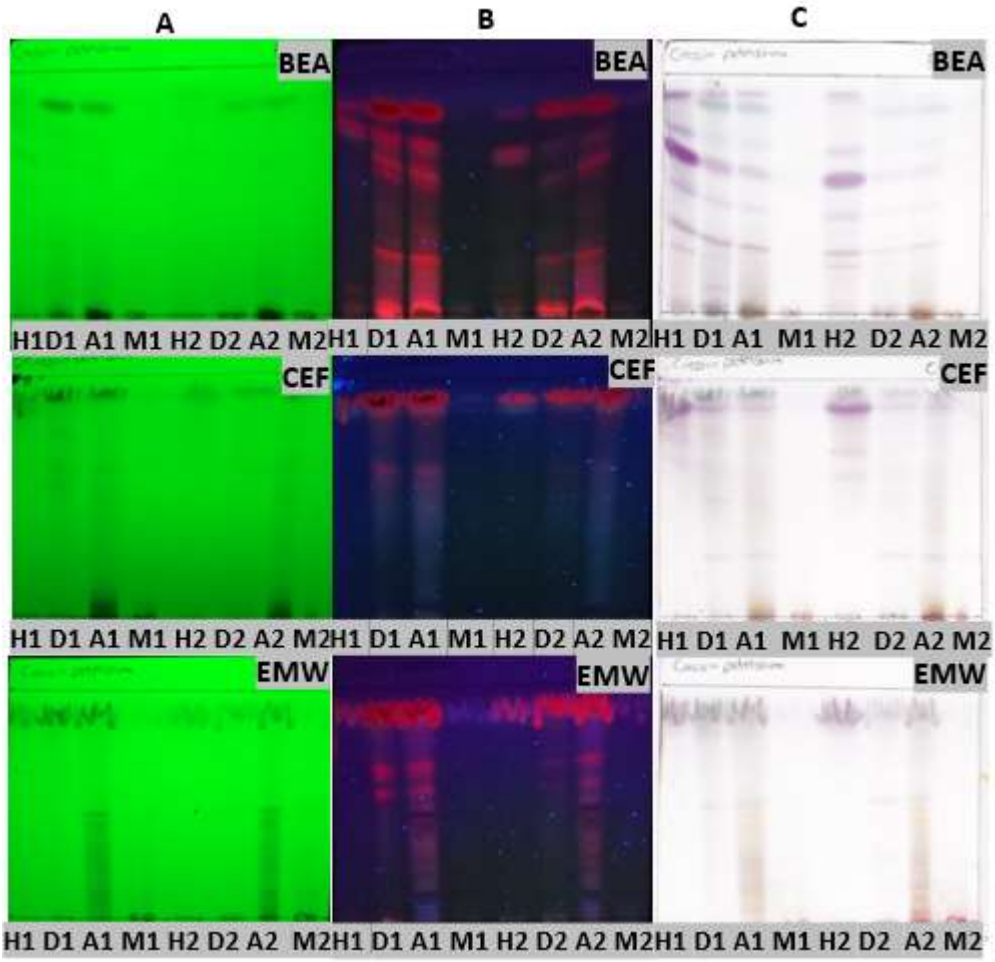

Figure 7.1: The phytochemical profile of extracts from *Cassia petersiana* exhaustively extracted with solvents of varying polarity. The fluorescing phytochemical compounds were visualised with an ultraviolet light at 254 (**A**) and 365 nm (**B**) while the non-fluorescing phytochemical compounds were visualised by spraying the aluminium-backed TLC plates with vanillin-sulphuric acid (**C**).

Key: H= n-hexane, D= dichloromethane, A= acetone and M= methanol.

7.3.1.3 Antioxidant activity evaluation by TLC-DPPH assay

The TLC-DPPH procedure was employed to evaluate the antioxidant activity of the extracts obtained from *Cassia petersiana*. The developed aluminium-backed TLC plates were sprayed with 0.2% DPPH in methanol to observe the antioxidant activity indicated by the yellow bands against the purple background (figure 7.2). It was observed that the antioxidant activity was present at minimal amounts with the separation of the antioxidants only from acetone extracts in the EMW solvent system.

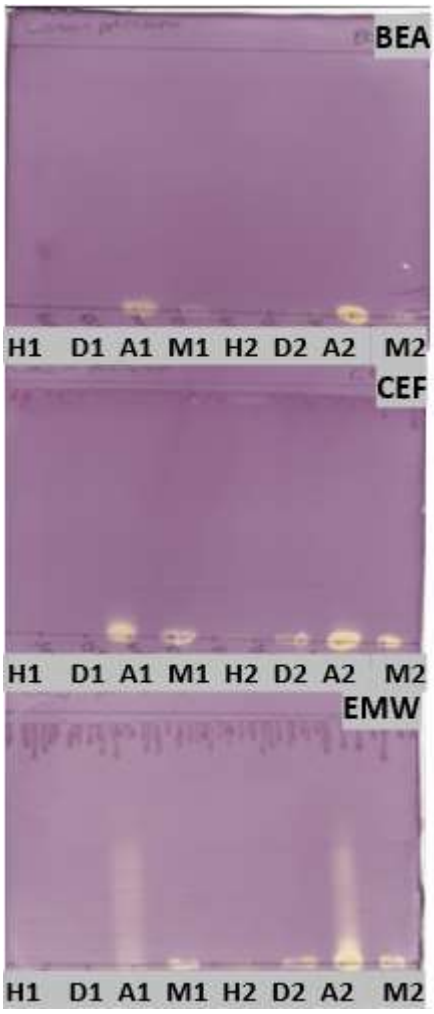

Figure 7.2: Antioxidant profile of extracts from *Cassia petersiana* obtained from exhaustive extraction procedure. The yellow bands against the purple background indicate the presence of antioxidant activity.

Key: H= n-hexane, D= dichloromethane, A= acetone and M= methanol.

7.3.1.4 Bioautography assay

The antimycobacterial activity of the extracts from *Cassia petersiana* against *Mycobacterium smegmatis* was evaluated by the TLC-bioautographic assay. The developed aluminium-backed TLC plates were sprayed with the test microorganism after the evaporation of the solvents systems, followed by the spraying of INT as an indicator for microbial growth. Antimycobacterial activity was noteworthy from the n-hexane extracts, as indicated by the white bands behind the pink background (figure 7.3).

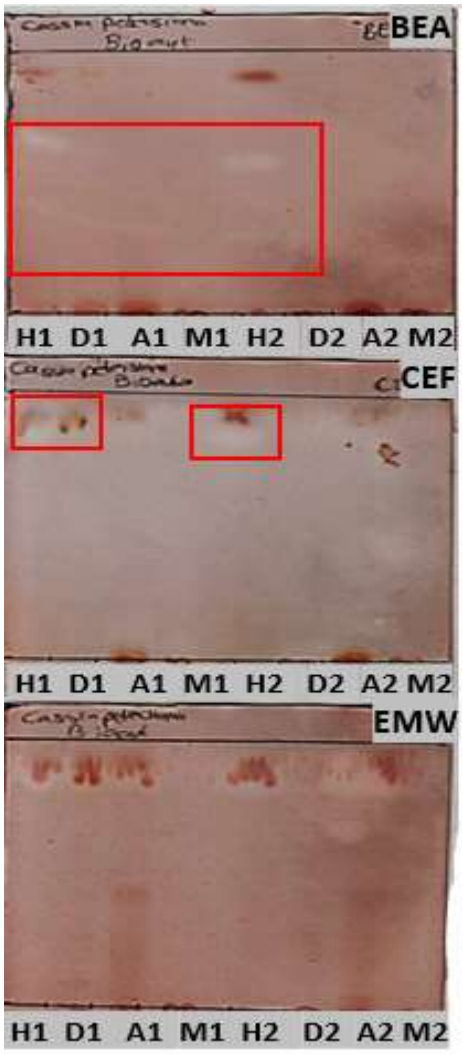

Figure 7.3: The bioautograms of *Cassia petersiana* extracts sprayed with *Mycobacterium smegmatis* as the test microorganism for antimycobacterial activity of the extracts from *Cassia petersiana*. The white bands behind the pink background indicate antimycobacterial activity.

Key: H= n-hexane, D= dichloromethane, A= acetone and M= methanol.

7.3.1.5 Serial broth micro-dilution assay

Antimycobacterial activity of the extracts exhaustively extracted from *Cassia petersiana* was determined quantitatively by the serial broth micro-dilution assay. The assay evaluated the MIC of the extracts against *Mycobacterium smegmatis*. Dichloromethane 1 and acetone 1 were found to have the lowest MIC (0.415 mg/mL) against the tested microorganism (table 7.2).

Table 7.2: The MIC values of the extracts exhaustively extracted from *Cassia petersiana* against *Mycobacterium smegmatis*.

Plant extracts and MIC values (mg/mL)								
H1	H2	D1	D2	C1	C2	M1	M2	Rifampicin
2.08	2.08	0.415	1.25	0.415	0.52	2.5	2.5	0.08

Key: H= n-hexane, D= dichloromethane, A= acetone and M= methanol

7.3.2 Isolation of antimycobacterial compounds from n-hexane extracts

7.3.2.1 Selection of elution solvents for column chromatography

n-Hexane extracts were selected for subjection to column chromatography due to the outstanding antimycobacterial activity observed in the bioautogram (figure 7.3). Fraction 1 and 2 of n-hexane extracts were combined and diluted with minimal n-hexane and spotted on the aluminium-backed TLC plates. Each of the TLC plates were developed in varied percentages of n-hexane and acetone to determine the combination that separates the phytochemicals better. It was observed that the phytochemicals moved near the solvent front in the n-hexane/ acetone combinations (figure 7.4). This resulted in the consideration to develop the spotted TLC plates in different combinations of n-hexane and ethyl acetate starting from 90% n-hexane. The movement of the phytochemicals was easily trackable in the combinations of n-hexane and ethyl acetate, where 90% was selected as the appropriate solvent combination for the isolation of compounds with column chromatography (figure 7.5).

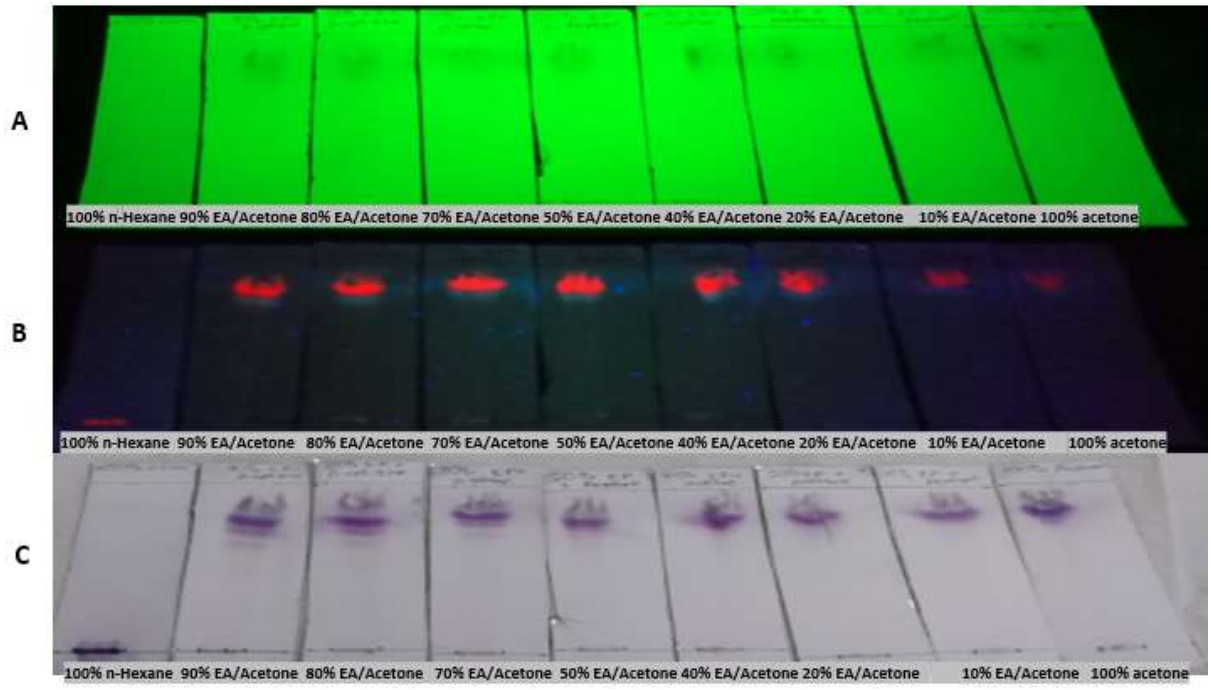

Figure 7.4: The fluorescing phytochemical compounds from n-hexane extract developed in different combinations of n-Hexane, ethyl acetate and acetone and visualised with ultraviolet light at 254 nm.

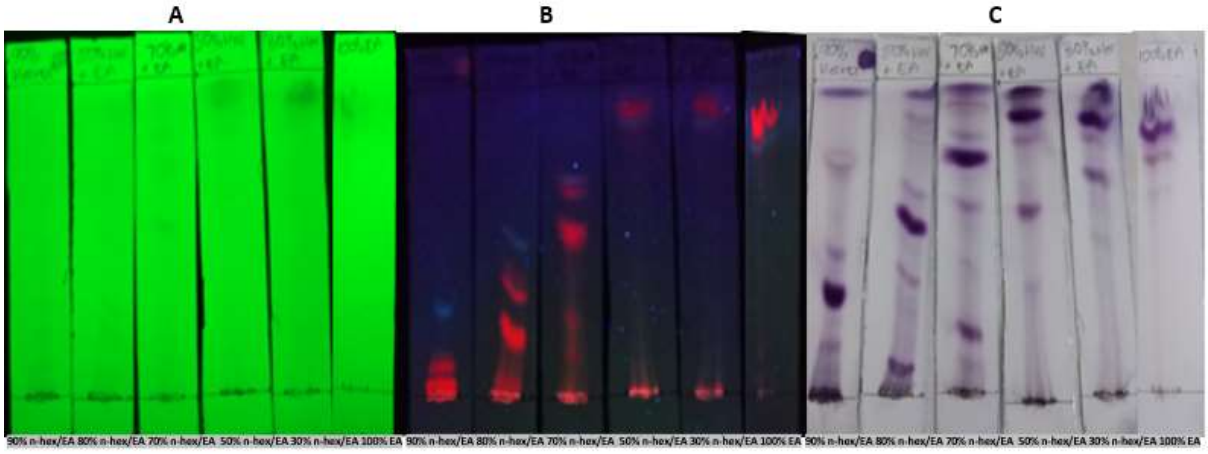

Figure 7.5: The chromatograms of n-hexane extract from *Cassia petersiana* visualised with ultraviolet light at 254 nm (**A**), 365 nm (**B**) and with vanillin-sulphuric acid (C).

7.3.2.2 Open column chromatography

Based on the separation between the compounds in 90% n-hexane/ethyl acetate compared to other combinations, this combination was selected as the eluent system for open column chromatography. The fractions were collected into clean test tubes followed by the concentration of the fractions under a stream of air before spotting

onto the aluminium-backed TLC plates. The TLC plates were developed in 90% n-hexane/ ethyl acetate prior to visualisation of the fluorescing phytochemicals under the ultraviolet light at 254 (figure 7.6A) and 365 nm (figure 7.6B). The non-fluorescing phytochemicals were visualised by spraying the aluminium-backed TLC plates with vanillin-sulphuric acid (figure 7.7). A range of phytochemicals which displayed similar properties were observed in both figures 7.6 and 7.7.

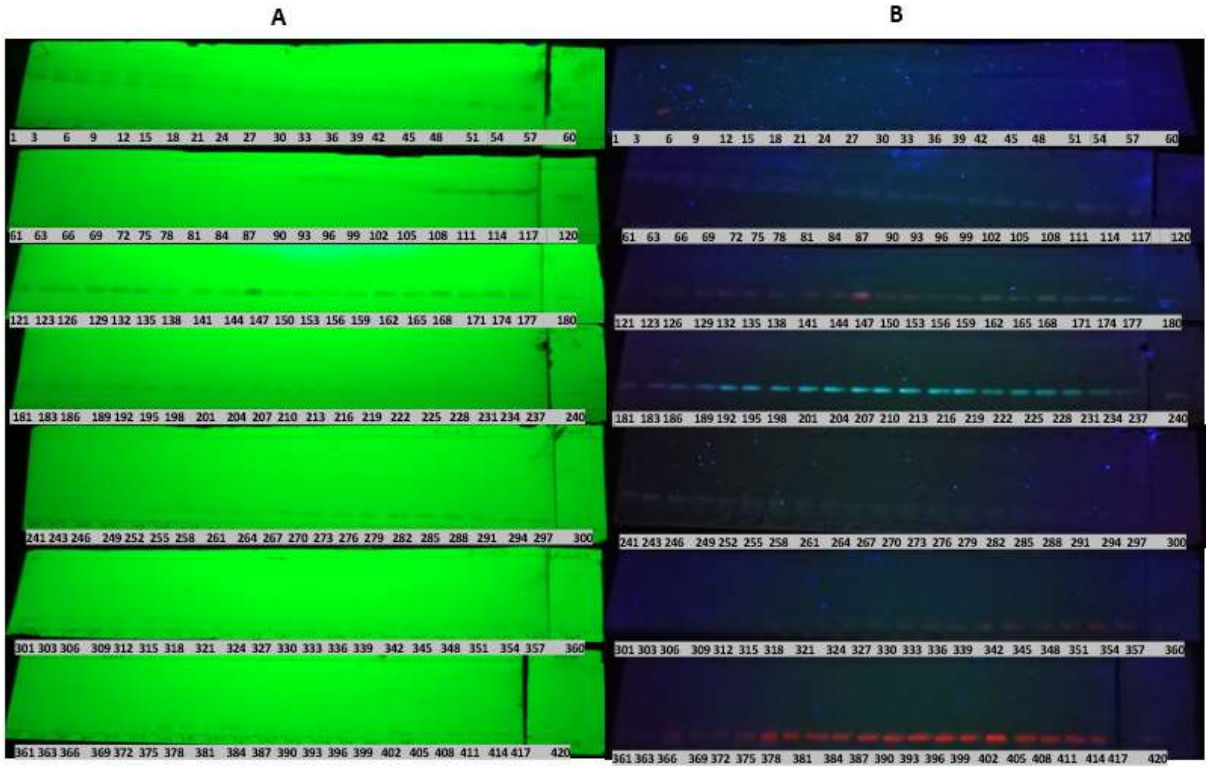

Figure 7.6: Chromatograms of phytochemical compounds of the collected fractions from column chromatography developed in 90% n-hexane and chloroform and visualised with ultraviolet light at 254 nm (**A**) and 365 nm (**B**).

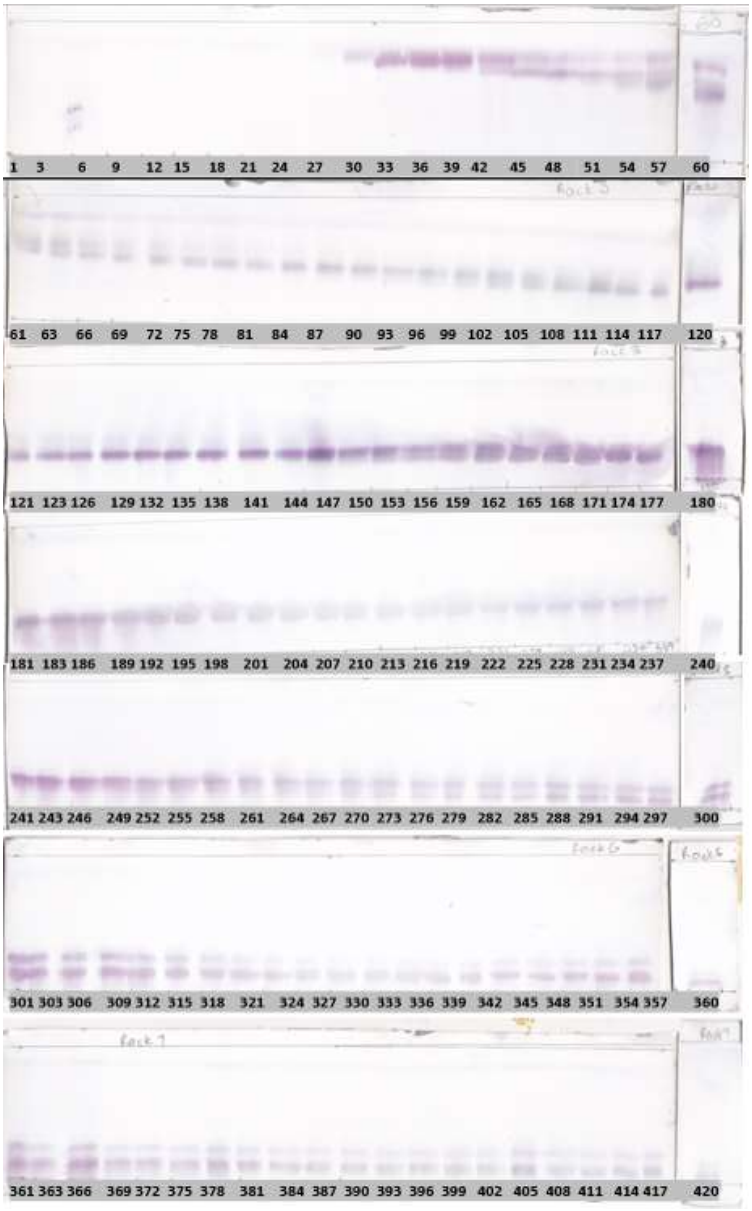

Figure 7.7: Chromatograms of phytochemical compounds of the collected fractions from column chromatography developed in 90% n-hexane and chloroform and visualised by spraying the aluminium-backed TLC plates with vanillin-sulphuric acid.

After careful analysis of the phytochemical profiles in figures 7.6 and 7.7, groups of fractions with similar phytochemical profiles were selected and combined to make a total of 8 groups. Group 1 (121-141), group 2 (144-168), group 3 (171-177), group 4 (180-189), group 5 (195-237), group 6 (241-297), group 7 (301-360) and group 8 (361-420). The combined groups were evaluated for antioxidant activity (figure 7.8), phytochemical evaluation (figure 7.9) as well as antimycobacterial activity evaluation (figure 7.10 and table 7.2).

Figure 7.8: TLC-DPPH assay for antioxidant activity of the combined fractions from column chromatography. Antioxidant activity, which is indicated by the yellow bands behind the purple background, was not observed.

Key: group 1= fractions 121-141, group 2= fractions 144-168, group 3= fractions 171-177, group 4= fractions 180-189, group 5= fractions 195-237, group 6= fractions 241-297, group 7= fractions 301-360 and group 8= fractions 361-420.

The evaluation of the combined fractions from column chromatography on TLC for phytochemical analysis revealed two fluorescing phytochemicals at 365 nm (figure 7.9 B) while a number of colourful phytochemicals were visualised after spraying the developed TLC plate with vanillin-sulphuric acid solution (figure 7.9C).

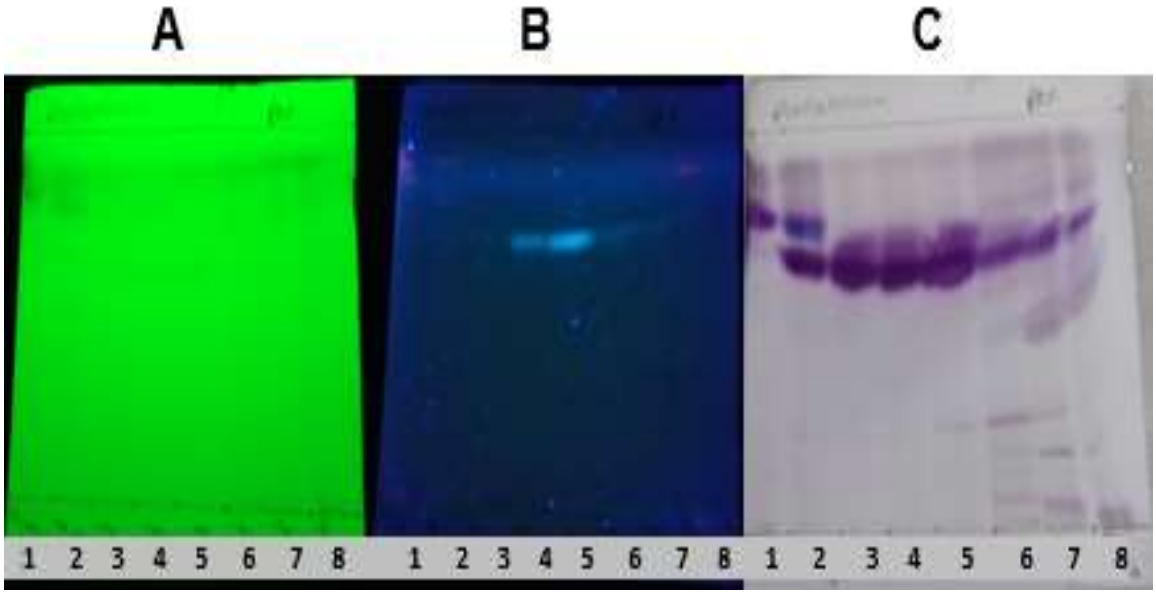

Figure 7.9: Phytochemical profiles of the combined fractions from column chromatography. The spotted TLC plates were developed in BEA solvent system and visualised with the ultraviolet light at wavelengths 254 (**A)** and 365 nm (**B**), and vanillin-sulphuric acid (**C**).

Key: group 1= fractions 121-141, group 2= fractions 144-168, group 3= fractions 171-177, group 4= fractions 180-189, group 5= fractions 195-237, group 6= fractions 241-297, group 7= fractions 301-360 and group 8= fractions 361-420.

The presence of similar compounds visualised in figure 7.9C following further analysis of the combined fractions from column chromatography for antimycobacterial activity on TLC was noteworthy. The clear bands against the pink background indicate antimicrobial activity against *Mycobacterium smegmatis*.

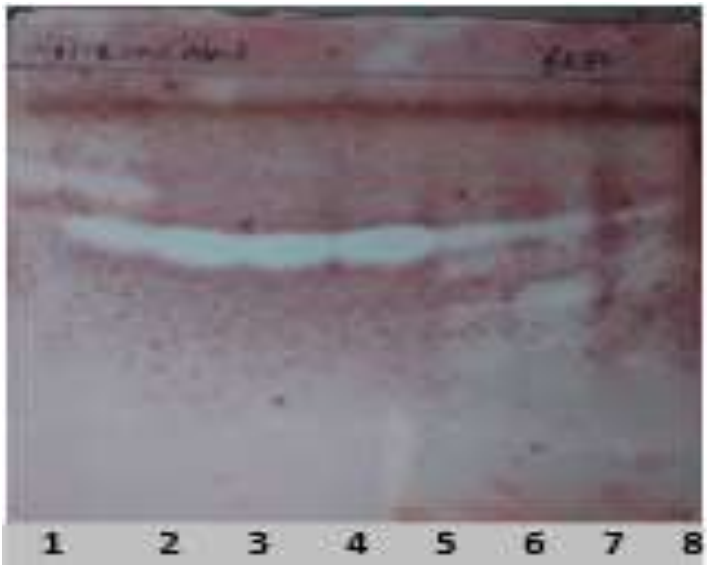

Figure 7.10: Bioautogram of the combined fractions from column chromatography developed in BEA solvent system. Following the incubation of the TLC plates sprayed with the test microorganism, INT was sprayed to serve as an indicator for microbial growth, which is indicated by the pink colour of the TLC profile. The white bands indicate zones of inhibition for growth of the test microorganism.

Key: group 1= fractions 121-141, group 2= fractions 144-168, group 3= fractions 171-177, group 4= fractions 180-189, group 5= fractions 195-237, group 6= fractions 241-297, group 7= fractions 301-360 and group 8= fractions 361-420

To quantitatively determine the antimycobacterial activity of the combined fractions from column chromatography, serial micro-broth dilution was employed. However, even though the qualitative evaluation of antimycobacterial activity of the collected fractions was evident with clear white bands against the pink background, the MIC of the fractions was at maximal, 2.5 mg/mL (table 7.2).

Table 7.3: The MIC values of the combined fractions from column chromatography against *Mycobacterium smegmatis*.

Groups of fractions	MIC values (mg/mL)
1	2.5
2	2.5
3	2.5
4	2.5
5	2.5

6	2.5
7	2.5
8	2.5
Average	2.5
Positive control	0.08

Key: group 1= fractions 121-141, group 2= fractions 144-168, group 3= fractions 171-177, group 4= fractions 180-189, group 5= fractions 195-237, group 6= fractions 241-297, group 7= fractions 301-360, group 8= fractions 361-420, positive control- Rifampicin.

The figure below (figure 7.11) represents the summary of the isolation process from the serial exhaustive extraction of the ground plant material from *Cassia petersiana* with solvents of varying polarity. It shows the masses of plant material used from the start of the extraction process; the amount subjected to column chromatography to the mass of the collected compound which was further characterised by NMR for structural elucidation.

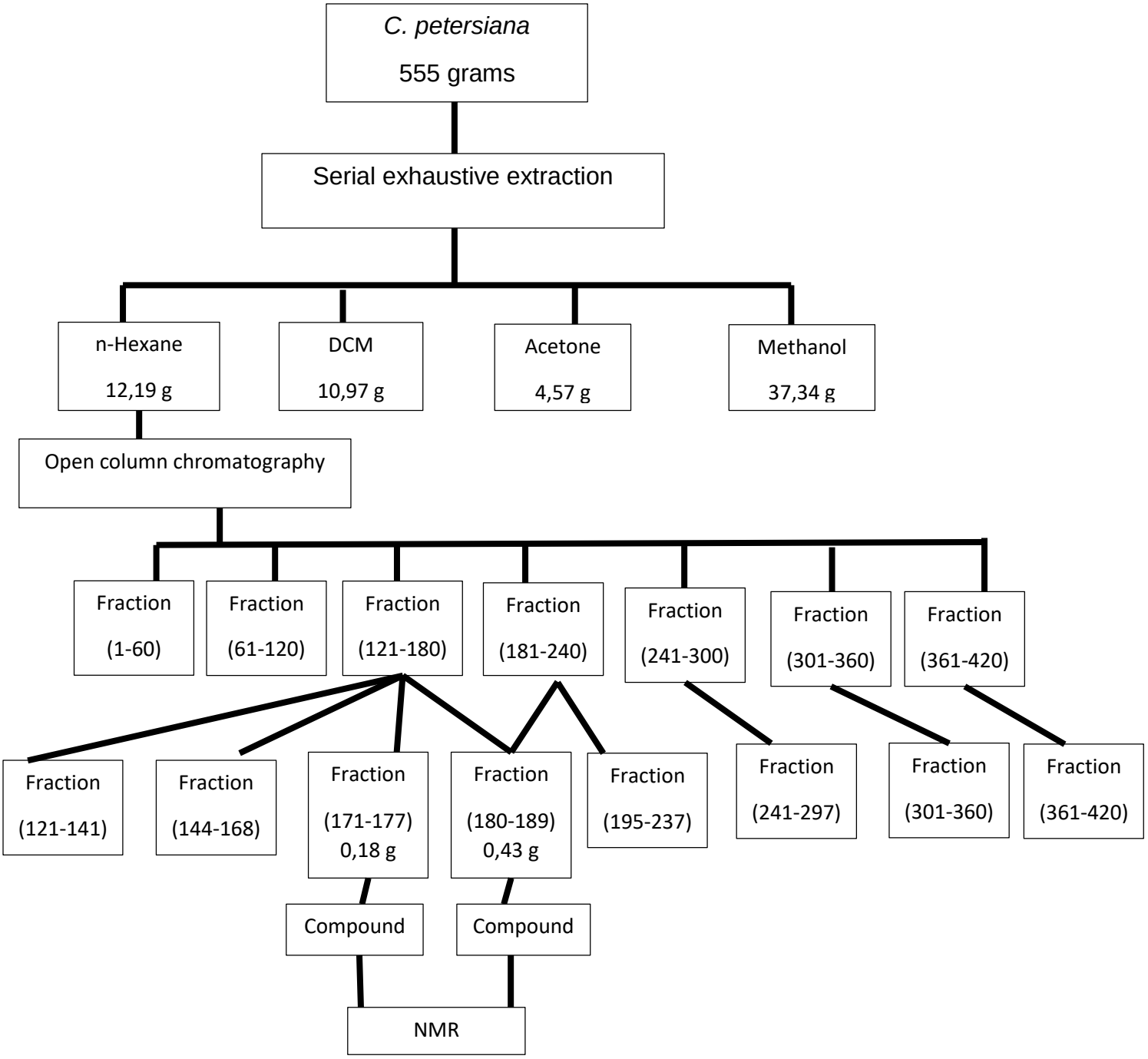

Figure 7.11: Flow diagram of the process followed to isolate bioactive compound from *Cassia petersiana* leaves.

7.4 Discussion

Infectious diseases such as TB continue to pose a clinical health threat to the world population. This is due to the gradual emergence of resistance of the disease-causing agents towards existing drugs (Bloom and Cadarette, 2019). As a result, drug development research is key for the protection of humankind against the negative effects of antibiotic resistance. Extraction is the first vital step in drug development investigations. Solvents used for extraction are carefully selected based on the desired end use of the products from extraction. In this study, four solvents (n-hexane, dichloromethane, acetone, and methanol) of increasing polarity were selected for extraction using the serial exhaustive extraction procedure to obtain a sufficient amount of extracts with a wide range of polarity.

A total amount of 65.07 grams was extracted from the 555 grams of the *Cassia petersiana* ground plant material (table 7.1). Methanol showed to be the best extractant as it extracted most of the mass (37.34 grams), followed by n-hexane with 12,19 grams while acetone extracted the least plant material (4.57 grams). Other studies also found methanol to be the best solvent for extraction. However, since there is no direct relation between the amount of plant material extracted by the solvent and desired bioactivity, it is important that the extracts are evaluated for antioxidant, phytochemical as well as antimicrobial activity using defined assays to screen for the desired bioactive compounds for purification and isolation.

During the phytochemical evaluation of the extracts with TLC, the aluminium-backed TLC plates were first observed under the ultraviolet light to visualise the fluorescing phytochemicals at wavelengths 254 and 365 nm before spraying with vanillin-sulphuric acid to visualise the non-fluorescing phytochemicals. Most phytochemicals were visible under 365 nm as compared to 254 nm and the similar compounds were also visible after spraying the aluminium-backed TLC plates with vanillin-sulphuric acid with additional compounds observed particularly in the BEA mobile system (figure 7.1). Less phytochemicals were visualised in the CEF and EMW mobile systems. This suggests that potential bioactive compounds from *Cassia petersiana* may be non-polar as it is known that a great number of drugs are active in the range of the ultraviolet light and can be directly detected and determined on the chromatographic plate (Mody *et al*., 1998). Antimycobacterial activity was evaluated by the TLC-bioautography

technique, where the bioactivity was noteworthy in the n-hexane extracts, as highlighted in figure 7.3. Furthermore, it was very interesting to notice that the compounds with antimycobacterial activity in figure 7.3 were also observed in figure 7.1. These findings have drawn much interest to the theory by Mody *et al*. (1998), as mentioned previously. To quantitatively evaluate the antimycobacterial activity of the extracts from *Cassia petersiana*, serial broth micro-dilution assay was conducted to determine the MIC of the extracts against *Mycobacterium smegmatis*. It was observed that although dichloromethane and acetone extracts displayed very faint bands on the chromatograms (figure 7.3), they had the lowest MIC (0.415 mg/mL) against *Mycobacterium smegmatis*.

The n-hexane extracts were chosen to be subjected to column chromatography based on the potential bioactive compounds observed in figure 7.3. Suitable solvent combination for the column chromatography was carefully selected by screening for the combination which best separates phytochemicals from the n-hexane extract on TLC. Different combinations of solvents were used at different concentrations. The criteria for choosing a desired solvent combination were based on the observation that the targeted potential bioactive compound was from n-hexane extract, which is non-polar, and the bioactivity of the extract was also from a non-polar mobile system. Hence, non-polar solvents such as n-hexane, ethyl acetate, and acetone, were selected for screening. Different combinations of n-hexane and acetone were used to screen good separation of the phytochemicals indicated by the easily trackable movement of the separated compounds at different percentages of the combinations. However, these combinations showed undesirable outcomes as most phytochemicals were near the solvent front (figure 7.4). The desired separation of the phytochemical was only observed with the combination of n-hexane and ethyl acetate (figure 7.5). From these combinations, 90% n-hexane/ethyl acetate was chosen as the solvent combination for column chromatography based on the wide distance observed between the separated phytochemicals compared to other combinations where the phytochemicals were closer to each other.

The column chromatography was packed with and eluted with 90% n-hexane/ethyl acetate. A total of 420 fractions were collected into clean test tubes, subjected under the stream of air to concentrate until the test tubes were half-full and spotted on TLC for phytochemical analysis. More fluorescing phytochemicals were visualised at 365

nm as compared to 254 nm (figure 7.6), while even more non-fluorescing phytochemicals were visualised after spraying with vanillin sulphuric acid (figure 7.7). Fractions with similar phytochemical profiles were carefully selected, combined, and spotted on TLC for phytochemical evaluation. A total of 8 fractions (fractions 121-141, 144-168, 171-177,180-189, 195-237, 241-297, 301-360 and 361-420) were selected from the combinations in figure 7.6 and 7.7, developed in BEA mobile system and further evaluated for antioxidant, phytochemical and antimycobacterial activity assays based on their similar phytochemical profiles. The selected combinations showed to have no antioxidant activity as there was no yellow colour that was observed against the purple colour of the DPPH (figure 7.8). These findings were not surprising as antioxidant activity from the crude extracts of *Cassia petersiana* showed to be at minimal throughout the assays that led to the isolation of the bioactive compound (chapter 4, figures 4.1-4.4.4). A few faint bands indicating phytochemicals were visualised under 254 nm, while two clear phytochemicals were visualised under 365 nm, and even clearer phytochemicals were visualised after spraying with vanillin-sulphuric acid (figure 7.9). Antimycobacterial activity was evident, as indicated by the white bands behind the pink background on the bioautogram in figure 7.10. It was very interesting to observe the similarity in the compounds from phytochemical evaluation (figure 7.9, C) and qualitative antimycobacterial activity evaluation (figure 7.10).

However, the microbroth dilution assay results revealed that the bioactivity of the selected compounds from *Cassia petersiana* was at a relatively higher MIC of (2.5 mg/mL). High MIC values indicate low potency for the compounds to be used as potential drugs against the causative agents of diseases as this may be an indication that the bioactive agents of these compounds is at a very low concentration (Amoo *et al*., 2009). The fractions 121-141, 171-177 and 180-189 were selected for further analysis with NMR for the characterisation and structural elucidation as they were potentially promising pure compounds based on the phytochemical (figure 7.9) and antimycobacterial evaluations (figure 7.10).

7.5 Conclusion

The isolation of promising compounds with bioactivity against *Mycobacterium smegmatis* was possible with bioassay-guided fractionation by column chromatography combined with TLC separation techniques. Although a clear

indication of antimycobacterial activity was evident on the bioautogram shown, the microbroth dilution assay suggests that the bioactivity observed is possible at a higher MIC. These findings were alarming since it was previously indicated in Chapter 6, that the acetone extracts from *Cassia petersiana* can be potentially cytotoxic at higher concentrations. This concern was based on the possible explanation that when the MIC value of a compound is higher, the possibility is that the bioactive agents in the compound are at a very low concentration. The structural elucidation of the isolated compounds will be analysed in the next chapter.

7.6 References

Alvin, A., Kalaitzis, J., Sasia, B. and Neilan, B., 2016. Combined genetic and bioactivity-based prioritization leads to the isolation of an endophyte-derived antimycobacterial compound. *Journal of Applied Microbiology*, 120(5), pp.1229-1239.

Amoo, S., Ndhlala, A., Finnie, J. and Van Staden, J., 2009. Antibacterial, antifungal and anti-inflammatory properties of Burchellia bubalina. *South African Journal of Botany*, 75(1), pp.60-63.

Bloom, D. and Cadarette, D., 2019. Infectious disease threats in the twenty-first century: strengthening the global response. *Frontiers in Immunology*, 10(549), pp.1-12.

Brusotti, G., Cesari, I., Dentamaro, A., Caccialanza, G. and Massolini, G., 2014. Isolation and characterization of bioactive compounds from plant resources: the role of analysis in the ethnopharmacological approach. *Journal of Pharmaceutical and Biomedical Analysis*, 87(2014), pp.218-228.

Cragg, G., Grothaus, P. and Newman, D., 2009. Impact of natural products on developing new anti-cancer agents. *Chemical Reviews*, 109(7), pp.3012-3043.

Mody, V., Pandya, K., Satia, M., Modi, I., Modi, R. and Gandhi, T., 1998. High performance thin-layer chromatographic method for the determination of sparfloxacin in human plasma and its use in pharmacokinetic studies. *Journal of Pharmaceutical and Biomedical Analysis*, 16(8), pp.1289-1294.

Ndjeka, N., Schnippel, K., Master, I., Meintjes, G., Maartens, G., Romero, R., Padanilam, X., Enwerem, M., Chotoo, S., Singh, N., Hughes, J., Variava, E., Ferreira, H., te Riele, J., Ismail, N., Mohr, E., Bantubani, N. and Conradie, F., 2018. High treatment success rate for multidrug-resistant and extensively drug-resistant tuberculosis using a bedaquiline-containing treatment regimen. *European Respiratory Journal*, 52(6), pp.1-9.

Quan, D., Nagalingam, G., Luck, I., Proschogo, N., Pillalamarri, V., Addlagatta, A., Martinez, E., Sintchenko, V., Rutledge, P. and Triccas, J., 2019. Bengamides display

potent activity against drug-resistant *Mycobacterium tuberculosis*. *Scientific Reports*, 9(1), pp.1-10.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. and Latha, L., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, 8(1), pp.1-10.

Sticher, O., 2008. Natural product isolation. *Natural Product Reports*, 25(3), p.517.

CHAPTER 8: Structural elucidation

8.1 Introduction

New drug discovery and development are important aspects of pharmacology as they aim to create new drugs to meet the health requirements caused by antibiotic resistance (Koh *et al*., 2019). Before new drugs are developed, new molecules with disease preventative actions must be discovered either from natural products or a library with countless chemical compounds (Hughes *et al*., 2011). Following the evaluation and determination of the targeted diseases, preventative actions of the molecules, the identity and structure of the molecules of interest must be determined. A combination of analytical techniques and methods such as NMR, high-performance liquid chromatography (HPLC), ultraviolet (UV), infrared (IR) spectroscopy and mass spectroscopy are involved in the structural elucidation of compounds (Breton and Reynolds, 2013).

NMR, which was employed in this study, is a very powerful tool in drug discovery (Li and Kang, 2020). It is intrinsically quantitative while able to provide several different approaches that are routinely utilised to identify and structurally elucidate molecules of interest. In addition, compared to mass spectrometry, NMR is a non-invasive, non-destructive technique with extremely high reproducibility, which permits researchers to acquire measurements under different conditions, often while the sample is inside the magnet (Samai *et al*., 2016; Silva *et al*., 2019). The inherent quantitative nature of the signal of NMR spectroscopy is one of its major advantages, as the measured signals can be directly proportional to the number of investigated nuclei in the same environment. These signals can be obtained for nuclei such as ^{1}H, ^{13}C, ^{19}F, and ^{31}P, which are most relevant to the pharmaceutical industry.

Amongst these nuclei, the ^{1}H is mostly commonly used in quantitative NMR spectroscopy because of its presence in most drugs, higher sensitivity, and short relaxation times (Zloh, 2019). The 1-dimensional NMR (^{1}H, ^{13}C and DEPT 135) and 2-dimensional NMR (HMBC and HSQC) were used for the structural elucidation of the compound isolated from *Cassia petersiana*.

The 1-dimensional ^{1}H-NMR is highly effective in drug design studies due to its relatively high sensitivity, its non-destructive properties and also because the hydrogen atoms are extremely abundant in most molecules of interest. In comparison with ^{1}H, ^{13}C has a low natural abundance but has a higher chemical shift dispersion; however, the ^{13}C spectra are more challenging to obtain, especially for less concentrated samples (Zloh, 2019). Better structural information about the structurally elucidated compounds is obtained through the incorporation of the ^{13}C-NMR spectra as compared to when the ^{1}H-NMR spectra is used alone as the ^{13}C-NMR spectra has the added signal resolution. The 2-D COSY-NMR shows the homonuclear coupling of the nuclei, which are separated by up to seven covalent bonds, while the 2-D HSQC-NMR help to resolve spectral overlap while providing the ^{13}C information without the inherent loss of sensitivity involved in ^{13}C direct detection (Fontaine *et al*., 1987; Reynolds and Enríquez, 2002; Tsujimoto *et al*., 2018). The aim of this chapter was to structurally elucidate the purified compound isolated from the n-hexane extract of *Cassia petersiana* using NMR.

8.2 Method and materials

The purified compound isolated from the n-hexane extract of *Cassia petersiana* was sent to the Chemistry Department, University of Limpopo, for structural elucidation and characterisation using NMR. An amount of 20 mg of the compound was dissolved in chloroform and characterised using 1-dimensional NMR (^{1}H, ^{13}C and DEPT 135) and 2-dimensional NMR (HMBC, and HSQC). The prepared sample was run using 400 MHz NMR spectrometer at 400 MHz, with chloroform-d as a reference signal solvent at a temperature of 295.5 K. Professor Ofentse Mazimba, situated at the Department of Chemical and Forensic Sciences, Botswana International University of Science and Technology, assisted with the structural elucidation using the spectroscopic data provided.

8.3 Results

The 1-dimensional NMR (^{1}H, ^{13}C and DEPT 135) and 2-dimensional NMR (HMBC and HSQC) techniques were used to deduce the NMR spectra, which led to the structural elucidation of the isolated compound (figures 8.1- 8.6). The summary of ^{13}C and ^{1}H proton shift values of the isolated compound in comparison to the values obtained from literature was depicted in table 8.1.

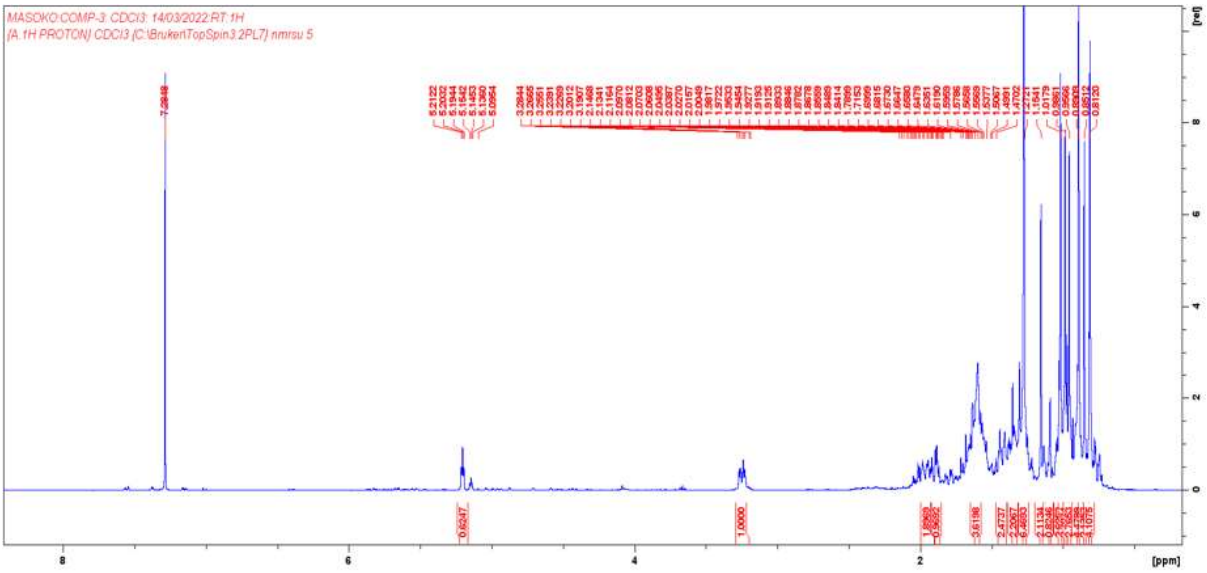

Figure 8.1: The ^{1}H-NMR spectrum of the compound isolated from n-hexane extract of *Cassia petersiana*.

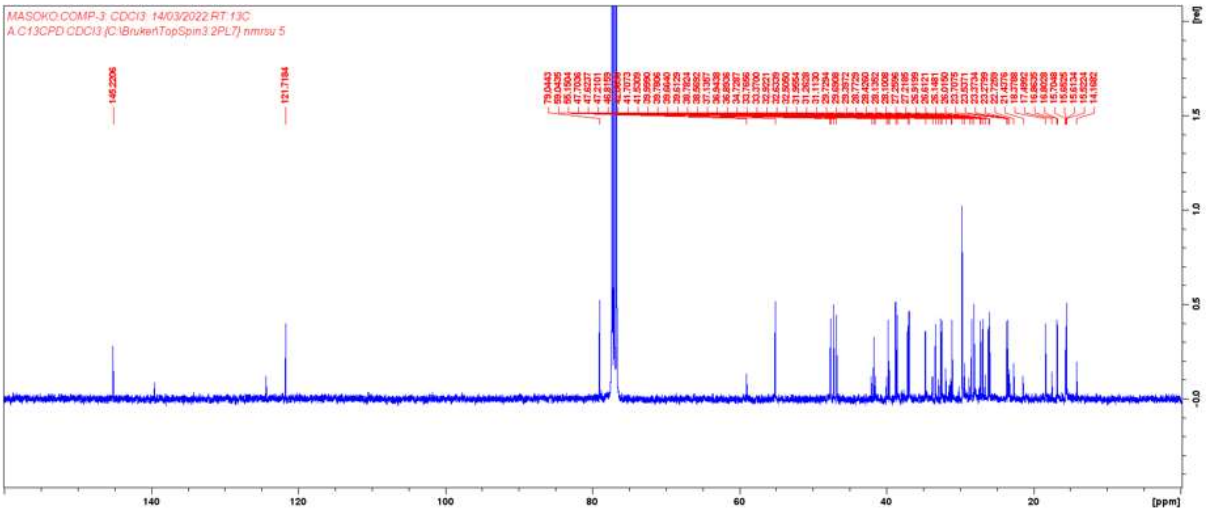

Figure 8.2: The ^{13}C-NMR spectrum of the compound isolated from n-hexane extract of *Cassia petersiana*.

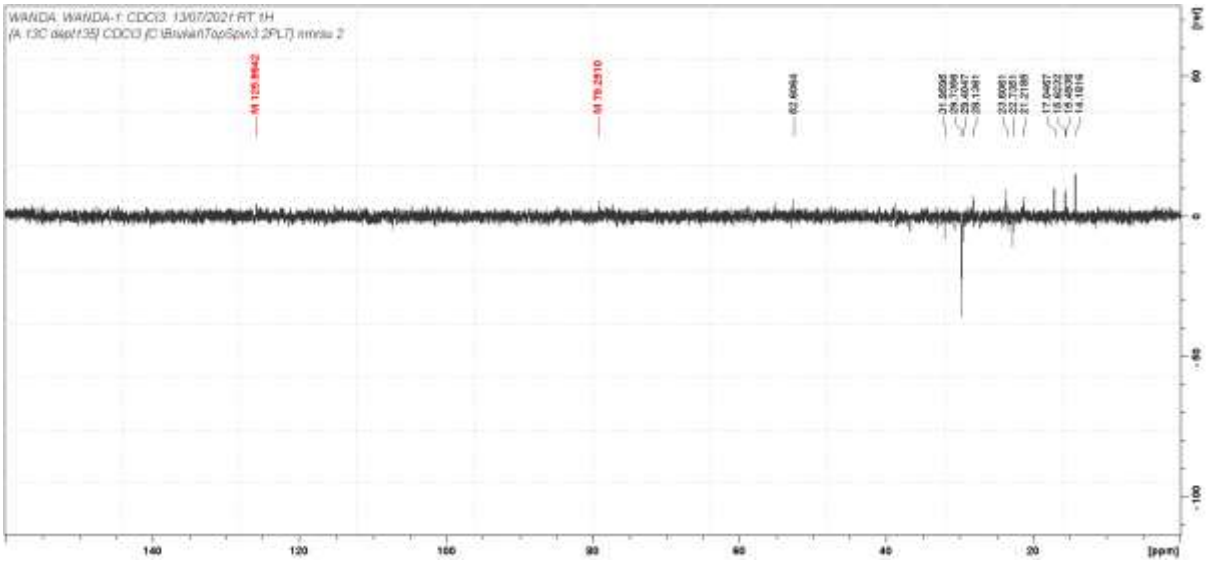

Figure 8.3: The DEPT 135-NMR spectrum of the compound isolated from n-hexane extract of *Cassia petersiana*.

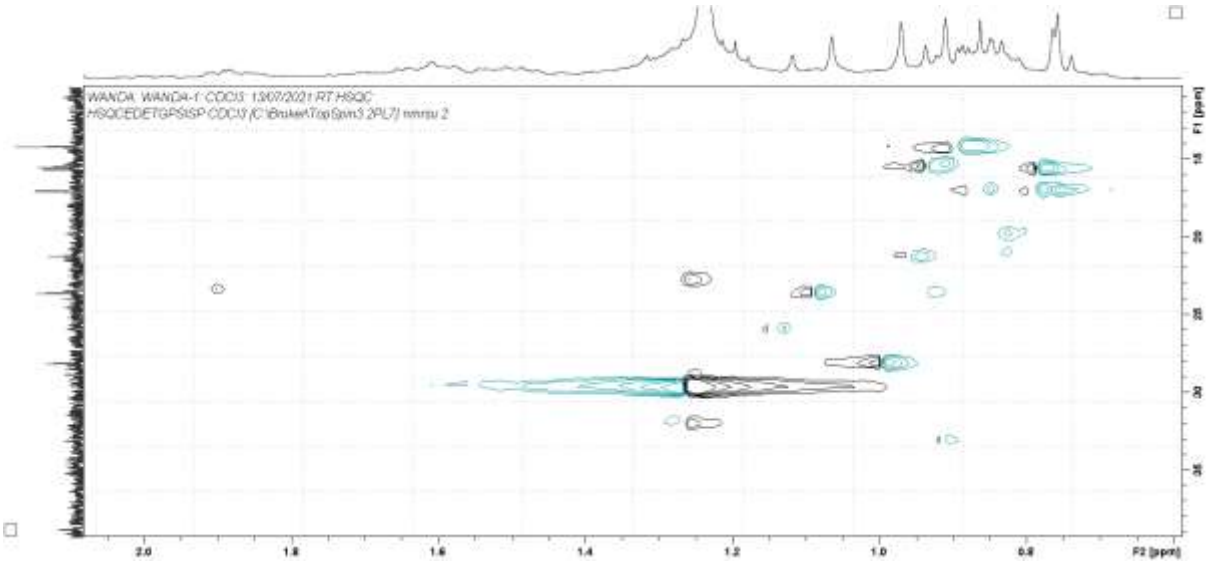

Figure 8.4: The HSQC-NMR spectrum of the compound isolated from n-hexane extract of *Cassia petersiana*.

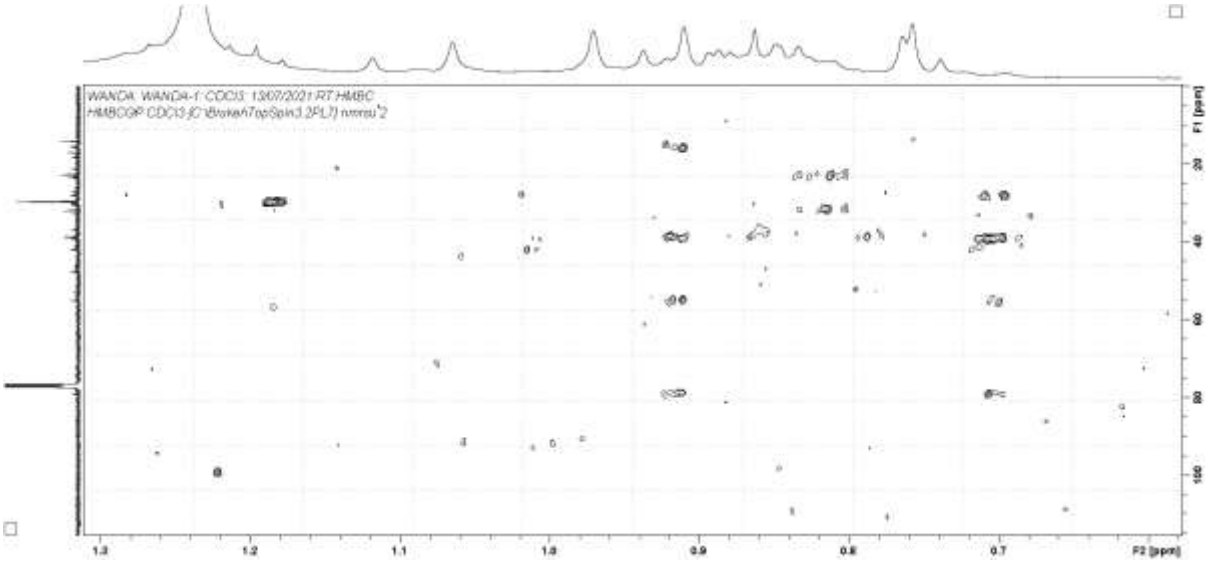

Figure 8.5: The HMBC-NMR spectrum of the compound isolated from n-hexane extract of *Cassia petersiana*.

Table 8. 1: The ^{1}H and ^{13}C NMR data of the isolated compound.

Position	Type	Chemical shift, δ(ppm) value (Ododo *et al*., 2016)	
		^{13}C NMR ^{1}H NMR	^{13}C NMR ^{1}H NMR

1	CH_2	37.28	1.46 (m)	38.77	1.65-1.68
2	CH_2	31.69	1.56 (m)	30.5	119-1.85
3	CH(OH)	71.82	3.54 (m)	79.01	3.21, m
4	CH_2	42.33	2.32 (m)	41.92	2.32, m
5	QC(=)	140.77	-	137.97	-
6	CH(=)	121.73	5.37 (t)	125.79	5.23,b(J=3.5Hz)
7	CH_2	31.93	2.04 (m)	31.91	2.15 m
8	CH	31.93	1.69 (m)	31.91	2.12 m
9	CH	50.16	1.55 (m)	47.86	191-1.85
10	QC	36.51	-	36.66	-
11	CH_2	21.11	1.52 (m)	21.16	1.65-1.60
12	CH_2	39.80	1.51 (m)	39.41	1.65-1.60
13	QC	42.34	-	41.57	-
14	CH	56.79	1.50 (m)	55.13	1.65-1.60, 8H
15	CH_2	24.33	1.58 (m)	23.55	1.74-1.67, 3H
16	CH_2	28.27	1.85 (m)	27.94	1.99, m, 2H
17	CH	56.08	1.45 (m)	52.57	1.65-1.60, 8H
18	CH_3	11.89	0.70 (s)	15.29	0.91, s
19	CH_3	19.42	1.03 (s)	15.44	1.12, s
20	CH	36.17	1.60 (m)	36.94	1.74-1.67
21	CH_3	18.84	0.94 (d)	15.57	1.06
22	CH_2	33.98	0.93 (m)	32.88	0.89, m
23	CH_2	26.11	1.15 (m)	27.15	1.52-1.46, m,4H
24	CH	45.86	1.38 (m)	47.4	1.58, m ,1H
25	CH	29.19	1.57 (m)	29.35	1.74-1.67, 3H
26	CH_3	19.84	0.84 (d)	18.24	0.91, s
27	CH_3	19.06	0.86 (d)	17.0	0.97, s
28	CH_2	23.10	1.10 (m)	22.68	1.52-1.46, 4H
29	CH_3	12.01	0.82 (t)	14.12	0.86, s
-	OH	-	1.98 (s)	-	

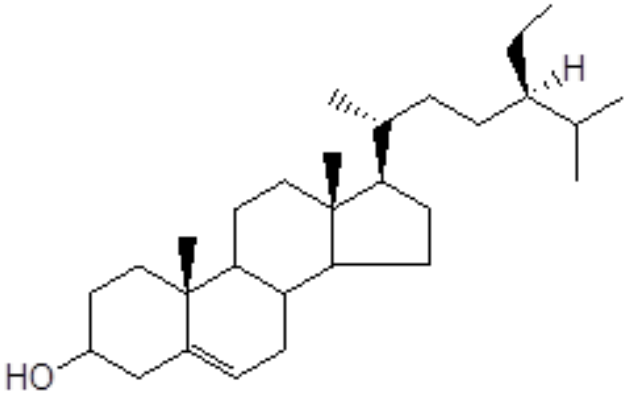

Figure 8.6: The structure of β-sitosterol isolated from n-hexane extract of *Cassia petersiana*.

8.4 Discussion

NMR is a powerful tool in drug discovery as it comprises various approaches for the structural elucidation of molecules of interest. It is also more advantageous compared to other analytical techniques used for the structural elucidation of compounds from natural sources (Li and Kang, 2020; Silva *et al*., 2019). The 1-dimensional NMR (^{1}H, ^{13}C and DEPT 135) and 2-dimensional NMR (HMBC and HSQC) approaches were employed to structurally elucidate the compound isolated from n-hexane extract of *Cassia petersiana*. The NMR spectral data obtained for the ^{1}H, ^{13}C, DEPT 135, HMBC, and HSQC NMR allowed for the elucidation of the structure of the isolated compound. Furthermore, the ^{1}H and ^{13}C proton shift of the isolated compound was compared with the data from the literature (Ododo *et al*., 2016) to determine their similarities, and the values were found to be very close to that from the literature. As a result, the structure of the isolated compound was elucidated as β-sitosterol. It is a typical white powdered compound, which is found in cells and membranes of natural sources such as plants, vegetables, fruits, trees, grains, and seeds that can produce oil (Sen *et al*., 2012). β-sitosterol has been previously isolated from various plants of the genus Cassia. These include the methanol extract of the leaves of *Cassia nigricans* (Ayo *et al*., 2009), leaves of *Cassia javanica* (Chaudhuri and Chawla, 1987), ethanol extract of flowers of *Cassia reticulata* (Messmer *et al*., 1968), n-hexane fractions of seeds of *Cassia fistula* (Misra *et al*., 1996), methanol extract of the roots of *Cassia obstusifolia* (Guo *et al*., 1998), stems of *Cassia tora*, seeds of *Cassia reingera* (Ledwani and Singh, 2005), and the

n-hexane extract of the stem bark of *Cassia racemose* (Mena-Rejóna *et al*., 2002). Although β-sitosterol has been previously isolated from a number of plants of the genus Cassia, which was the first report on the isolation of β-sitosterol from *Cassia petersiana*. Interestingly, during the isolation and purification process of β-sitosterol from the n-hexane extract of *Cassia petersiana*, it was observed that this compound formed a white crystal during the concentration of the compound under a stream of air in the test tubes. This observation was not surprising as it was previously reported that β-sitosterol usually forms a white crystal (Erwin *et al*., 2020; Ododo *et al*., 2016).

8.5 Conclusion

The structural elucidation of β-sitosterol isolated from the n-hexane extract of *Cassia petersiana* was possible through the NMR. The structure was successfully elucidated by the spectral data of the ^{1}H, ^{13}C, DEPT 135, HMBC and HSQC NMR, and the comparison of the ^{1}H and ^{13}C proton spectral shift with that from literature. Although β-sitosterol was previously isolated from the plants of the genus Cassia, this is the first report on the isolation of β-sitosterol from *Cassia petersiana*.

8.6 References

Ayo, R., Amupitan, J., Ndukwe, I. and Audu, O., 2009. Some chemical constituents of the leaves of *Cassia nigricans* Vah. *African Journal of Pure and Applied Chemistry*, 3(11), pp.208-211.

Breton, R. and Reynolds, W., 2013. Using NMR to identify and characterize natural products. *Natural Product Reports*, 30(4), pp.501-524.

Chaudhuri, K. and Chawla, H., 1987. Anthraquinones and terpenoids from *Cassia javanica* leaves. *Journal of Natural Products*, 50(6), p.1183.

Erwin, Pusparohmana, W., Safitry, R., Marliana, E., Usman and Kusuma, I., 2020. Isolation and characterization of stigmasterol and beta-sitosterol from wood bark extract of *Baccaurea macrocarpa* Miq. Mull. Arg. *Rasayan Journal of Chemistry*, 13(04), pp.2552-2558.

Fontaine, X., Kennedy, J., Shaw, B. and Vila, J., 1987. Determination of the relative signs of $^{2J}(^{31}P–^{31}P)$ in complexes of tungsten(0) and molybdenum(0) using two-dimensional [$^{31}P–^{31}P$]-COSY-45 Nuclear Magnetic Resonance chemical shift correlation. *Journal of the Chemical Society., Dalton Trans.*, (10), pp.2401-2405.

Guo, H., Chang, Z., Yang, R., Guo, D. and Zheng, J., 1998. Anthraquinones from hairy root cultures of *Cassia obtusifolia*. *Phytochemistry*, 49(6), pp.1623-1625.

Hughes, J., Rees, S., Kalindjian, S. and Philpott, K., 2011. Principles of early drug discovery. *British Journal of Pharmacology*, 162(6), pp.1239-1249.

Koh, J., Gottfried, D., Lee, D. and Navarrete, S., 2019. Understanding and addressing regulatory concerns in research, basic methods handbook for clinical orthopaedic research. 1st ed. Heidelberg: Springer, pp.515-531.

Ledwani, L. and Singh, M., 2005. Anthraquinone glycoside from stem bark, fatty acids and sterols from seeds of *Cassia reingera*. *Indian Journal of Chemistry*, 44(9), pp.1970-1971.

Li, Q. and Kang, C., 2020. A practical perspective on the roles of solution nmr spectroscopy in drug discovery. *Molecules*, 25(13), p.2974.

Mena-Rejóna, G., Pérez-Rivas, K., Sansorez-Peraza, P., Rios, T. and Quijano, L., 2002. Racemochrysone, a dihydroanthracenone from *Senna racemosa*. *Zeitschrift für Naturforschung C*, 57(9-10), pp.777-779.

Messmer, W., Farnsworth, N., Persinos, G. and Wilkes, J., 1968. Phytochemical investigation of the flowers of *Cassia reticulata* Willd. (Leguminosae). *Journal of Pharmaceutical Sciences*, 57(11), pp.1996-1998.

Misra, T., Singh, R., Pandey, H. and Pandey, R., 1996. Chemical constituents of hexane fraction of *Cassia fistula* pods. *Fitoterapia*, 67(2), pp.173-174.

Ododo, M., Choudhury, M. and Dekebo, A., 2016. Structure elucidation of β-sitosterol with antibacterial activity from the root bark of *Malva parviflora*. *SpringerPlus*, 5(1), pp.1-11.

Reynolds, W. and Enríquez, R., 2002. Choosing the best pulse sequences, acquisition parameters, postacquisition processing strategies, and probes for natural product structure elucidation by NMR spectroscopy. *Journal of Natural Products*, 65(2), pp.221-244.

Samai, S., Sapsanis, C., Patil, S., Ezzeddine, A., Moosa, B., Omran, H., Emwas, A., Salama, K. and Khashab, N., 2016. A light responsive two-component supramolecular hydrogel: a sensitive platform for the fabrication of humidity sensors. *Soft Matter*, 12(11), pp.2842-2845.

Sen, A., Dhavan, P., Shukla, K., Singh, S. and Tejovath, G., 2012. Analysis of IR, NMR and antimicrobial activity of β-sitosterol isolated from *Momordica charantia*. *Science Secure Journal of Biotechnology*, 1(1), pp.9-13.

Silva, M., Silva, L., Quintela, A., dos Santos, A., Silva, F., de Oliveira, F., Alves Filho, E., de Brito, E., Canuto, K., Pessoa, C. and Zocolo, G., 2019. UPLC-HRMS and NMR applied in the evaluation of solid-phase extraction methods as a rational strategy of

dereplication of Phyllanthus spp. aiming at the discovery of cytotoxic metabolites. *Journal of Chromatography B*, 1120, pp.51-61.

Tsujimoto, T., Yoshitomi, T., Maruyama, T., Yamamoto, Y., Hakamatsuka, T. and Uchiyama, N., 2018. 13C-NMR-based metabolic fingerprinting of Citrus-type crude drugs. *Journal of Pharmaceutical and Biomedical Analysis*, 161, pp.305-312.

Zloh, M., 2019. NMR spectroscopy in drug discovery and development: evaluation of physico-chemical properties. *ADMET and DMPK*, 7(4), pp.242-251.

CHAPTER 9: Biological activities of the isolated compound

9.1 Introduction

Traditional medicinal plants cannot only serve as potential antimicrobial crude drugs, but also as sources of new anti-infective and chemotherapeutic agents (Babalola *et al*., 2013). Bioassay-guided isolation of the bioactive compounds from these plants is consequently important to give a clear understanding of the mechanism of the compounds from medicinal plants that results in bioactivity against tested microorganisms. The current study resulted in the isolation of β-sitosterol from the n-hexane extracts of *Cassia petersiana*.

β-sitosterol is a phytosterol common in the plant kingdom. It has been previously isolated and purified by various chromatographic methods from diverse families (Saeidnia *et al*., 2014), including various species from the genus Cassia (Ledwani and Singh, 2005; Mena-Rejóna *et al*., 2002). The compounds reported to have been isolated from *Cassia petersiana* are the derivatives of chromone, which were elucidated as petersone A and petersone B (Gatsing *et al*., 2007). β-sitosterol was proven by Sen *et al.* (2012) to be a nontoxic, safe, and effective nutritional supplement with noteworthy potential health benefits in many applications such as antibacterial activity. β-sitosterol is a highly active compound with noteworthy potential health benefits in medicine and food. These include antioxidant, antifungal, antibacterial, anti-inflammatory activity in human systems, anti-cancer activity and antidiabetic properties (Gogoi *et al*., 2018; Saeidnia *et al*., 2014).

Furthermore, it was reported that β-sitosterol can be used as an antimicrobial agent for agricultural pathogens, with more emphasis on *Ralstonia solanacearum* (Xie *et al*., 2021). The aim of this chapter was to evaluate the antioxidant activity, phytochemical and antimycobacterial activity of β-sitosterol isolated from the n-hexane extract of *Cassia petersiana*.

9.2 Method and materials

9.2.1 Antioxidant assay

The antioxidant activity of the isolate compound was evaluated with TLC-DPPH assay, according to section 4.2.1

9.2.2 Phytochemical analysis

The phytochemical analysis of the isolated compound was evaluated with TLC, according to section 3.2.3.

9.2.3 Antimycobacterial activity assay

9.2.3.1 Bioautographic assay

Bioautographic assay was employed to evaluate the antimycobacterial activity of the isolated compound against *Mycobacterium smegmatis*, according to section 5.2.1.

9.2.3.2 Broth microdilution assay

The quantitative antimycobacterial analysis of the isolated compound was evaluated by serial broth microdilution assay, according to section 5.2.2.

9.3 Results

9.3.1 Antioxidant activity analysis

The antioxidant activity of β-sitosterol was evaluated by TLC-DPPH assay. No antioxidant activity was evident, as it must be indicated by the yellow bands behind the purple background of the DPPH (figure 9.1).

Figure 9.1: Qualitative antioxidant activity profile of β-sitosterol on aluminium-backed TLC plate. Antioxidant activity depicted by the yellow spots against the purple background was not observed.

9.3.2 Phytochemical evaluation

The phytochemical profile of β-sitosterol was evaluated by TLC. The compound was visualised with ultraviolet light at 254 and 365 nm, as well as vanillin-sulphuric acid. It

was revealed that β-sitosterol could fluoresce at 365 nm, and it was also visible after spraying the aluminium-backed TLC plate with vanillin-sulphuric acid (figure 9.2).

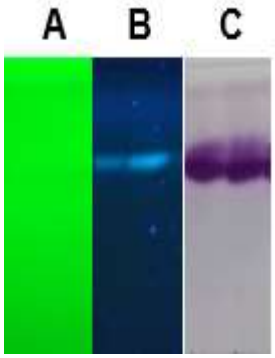

Figure 9.2: Chromatograms of β-sitosterol developed in BEA solvent system. The compound was visualised under the ultraviolet light at 254 (A) and 365 nm, and also visualised with vanillin-sulphuric acid (C).

9.3.3 Qualitative antimycobacterial activity assay

Qualitative antimycobacterial activity of β-sitosterol was evaluated by the TLC-bioautographic assay. The spotted aluminium-backed TLC plate was sprayed with *Mycobacterium smegmatis* following incubation and further spayed with INT as an indicator for microbial growth after optimum incubation with the test microorganism. The pink colour on the chromatogram indicates microbial growth, while the white bands indicate microbial growth inhibition (figure 9.3).

Figure 9.3: Bioautogram of β-sitosterol developed in BEA solvents and sprayed with *Mycobacterium smegmatis*. The white bands behind the pink background indicate bioactive of β-sitosterol against the tested microorganism.

9.3.4 Quantitative antimycobacterial activity assay

Serial broth microdilution assay was employed to determine the MIC of β-sitosterol against *Mycobacterium smegmatis*. Rifampicin was used as a positive control.

Table 9.1: MIC value of β-sitosterol

	MIC (mg/mL)
β-sitosterol	2.5
Rifampicin	0.08

9.4 Discussion

The antioxidant activity of β-sitosterol was evaluated with TLC-DPPH assay, where it was revealed that no antioxidant activity was evident as it must be indicated by the yellow bands behind the purple background (figure 9.1). Although β-sitosterol isolated from the n-hexane extract of *Cassia petersiana* showed no antioxidant activity, Vivancons and Moreno (2005) reported that β-sitosterol can protect against oxidative stress through the modulation of the antioxidant enzymes. β-sitosterol fluoresced under the ultraviolet light at 365 nm and was also visible after spraying the aluminium-backed TLC plate with vanillin-sulphuric acid (figure 9.2). This observation was very interesting as it was previously reported that many drugs are active in the range of the ultraviolet light allowing their direct detection on the chromatographic plate. As a result, phytochemical evaluation performed to investigate the purity of β-sitosterol is possible through the profiling of the compound on aluminium-backed TLC followed by visualisation of the compound under the ultraviolet light.

β-sitosterol also indicated antimycobacterial activity against *Mycobacterial smegmatis* as indicated by the white bands behind the pink ground on the bioautographic chromatogram (figure 9.3). However, quantitative antimycobacterial evaluation of β-sitosterol by serial broth microdilution revealed the MIC of 2.5 mg/mL, which is not scientifically significant (table 9.1). According to (Amoo *et al*., 2009), high MIC values indicate low potency for the compounds to be used as potential drugs against the causative agents of diseases as this may be an indication that the bioactive agents of these compounds is at a very low concentration.The possible explanation for the high MIC value of β-sitosterol against *Mycobacterium smegmatis* may be the low

concentration of the bioactive agents in the compound. Furthermore, the lower MIC value of a compound is directly proportional to the higher bioactivity against the tested microorganism (Eloff, 2000); hence, the high bioactivity of β-sitosterol not being scientifically significant. A promising bioactivity of β-sitosterol was observed against *Mycobacterium smegmatis* on the bioautographic TLC plate (figure 9.3), with the quantity of the bioactivity being relatively high (table 9.1). β-sitosterol was previously proven to be a good candidate in the treatment of TB. β-sitosterol was previously tested in the treatment of patients with culture proven pulmonary TB by the use of blinded randomised placebo-controlled trials. In this study, patients were divided into two groups where one group consisted of a sitosterol, and placebo treatment was set upon the other group. As part of this research, the patients were hospitalised for the duration of the treatment with monthly check-ups with regards to the sputum culture positivity, weight gain, liver function and other important test response. This trial revealed that patients placed on the sitosterol treatment marked a greater weight gain and white blood count, which contributed to a faster recovery as compared to the other group (Donald *et al*., 1997). The reported findings warrant β-sitosterol an important compound in the treatment of TB.

9.5 Conclusion

The evaluation of the biological activity of β-sitosterol by antioxidant activity, phytochemical evaluation, and antimycobacterial activity assays was possible. Based on the qualitative antioxidant activity results observed and the literature reported on β-sitosterol regarding antioxidant activity, it is recommended that the quantitative antioxidant activity of the compound is evaluated. The antimycobacterial activity observations highlight that more studies are conducted on β-sitosterol to evaluate its bioactivity against *Mycobacterium tuberculosis* before consideration for further studies that may lead to the synthesis of TB drugs with this compound.

9.6 References

Amoo, S., Ndhlala, A., Finnie, J. and Van Staden, J., 2009. Antibacterial, antifungal and anti-inflammatory properties of Burchellia bubalina. *South African Journal of Botany*, 75(1), pp.60-63.

Babalola, I. and Adelakun, E., 2013. Isolation of stigmast-5-en-3β-ol (β-sitosterol) from dichloromethane extract of *Sterculia setigera* leaves (Sterculiaceae). *Scholars Research Library*, 5(5), pp.16-19.

Donald, P., Lamprecht, J., Freestone, M., Albrecht, C., Bouic, P., Kotze, D. and van-Jaarsveld, P., 1997. A randomised placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis. *The International Journal of Tuberculosis and Lung Disease*, 1(6), pp.518-522.

Eloff, J., 2000. A proposal on expressing the antibacterial activity of plant extracts – a small first step in applying scientific knowledge to rural primary health care in South Africa. *South African Journal of Science*, 96, pp.116-118.

Gatsing, D., Djemgou, P., Garba, I., Aliyu, R., Tchuendem, M., Tane, P., Ngadjui, B., Seguin, E. and Adoga, G., 2007. Dihydronaphtalenone and chromone from *Cassia petersiana* Bolle and the antisalmonellal activity of its crude extract. *Research Journal of Phytochemistry*, 1(1), pp.40-45.

Gogoi, D., Pal, A., Chattopadhyay, P., Paul, S., Deka, R. and Mukherjee, A., 2018. First report of plant-derived β-sitosterol with antithrombotic, *in vivo* anticoagulant, and thrombus-preventing activities in a mouse model. *Journal of Natural Products*, 81(11), pp.2521-2530.

Ledwani, L. and Singh, M., 2005. Anthraquinone glycoside from stem bark, fatty acids and sterols from seeds of *Cassia reingera*. *Indian Journal of Chemistry*, 44(9), pp.1970-1971.

Mena-Rejóna, G., Pérez-Rivas, K., Sansorez-Peraza, P., Rios, T. and Quijano, L., 2002. Racemochrysone, a dihydroanthracenone from *Senna racemosa*. *Zeitschrift für Naturforschung C*, 57(9-10), pp.777-779.

Saeidnia, S., Manayi, A., Gohari, A. and Abdollahi, M., 2014. The story of Beta-sitosterol- a review. *European Journal of Medicinal Plants*, 4(5), pp.590-609.

Sen, A., Dhavan, P., Shukla, K., Singh, S. and Tejovath, G., 2012. Analysis of IR, NMR and antimicrobial activity of β-sitosterol isolated from *Momordica charantia*. *Science Secure Journal of Biotechnology*, 1(1), pp.9-13.

Vivancos, M. and Moreno, J., 2005. β-Sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages. *Free Radical Biology and Medicine*, 39(1), pp.91-97.

Xie, Y., Peng, Q., Ji, Y., Xie, A., Yang, L., Mu, S., Li, Z., He, T., Xiao, Y., Zhao, J. and Zhang, Q., 2021. Isolation and identification of antibacterial bioactive compounds from *Bacillus megaterium* L2. *Frontiers in Microbiology*, 12, pp.1-10.

CHAPTER 10: General discussions, conclusions and recommendations

10.1 General discussions

The healthcare system remains highly burdened with the treatment and management of various infectious diseases. The factors that are the major drawbacks in the treatment and management of diseases include antibiotic resistance, toxicity because of the use of current antibiotics, and the decrease in the efficacy of existing antibiotics. This has resulted in the urgent need for the development of alternative drugs where natural products such as medicinal plants are the promising possible candidates that may target bioactive compounds with improved features. The consideration of medicinal plants for the screening of alternative bioactive compounds was based on the ancient use of traditional medicine for the treatment of various diseases. Alternative drugs with improved features have been previously synthesised from compounds isolated from medicinal plants. Hence, the screening, evaluation, and isolation of bioactive compounds from medicinal plants with targeted biological activity remain a subject of interest for the development of alternative drugs for different diseases. Therefore, the aim of this study was to investigate the antimycobacterial and cytotoxic compounds from *Cassia petersiana*.

Extraction is an important step in the use of plants for medicinal purposes and scientific evaluations as it influences the type of compounds extracted. Solvents of varying polarity ranging from polar to non-polar were used to extract compounds from *Cassia petersiana* to allow for the extraction of compounds of diverse polarity. Preliminary studies on the extracted compounds are important to investigate the biological activity of the extracts and to determine the presence of bioactive compounds against the test microorganism before the extracts are considered for isolation. During the preliminary studies, standard tests were followed to investigate the presence of common phytoconstituents where it was revealed that all the tested phytochemicals were present. The most common phytoconstituents (phenols, flavonoids, and tannins) were further quantified and found to be at minimal content. The profiling of phytochemical compounds present in the crude extracts of *Cassia petersiana* revealed the presence of most compounds in the BEA solvent system, which is non-polar. These findings suggested that most compounds extracted were non-polar as it is known that during extraction solvents extract compounds of similar polarity.

The qualitative and quantitative analysis of antioxidant activity of the extracts revealed the presence of antioxidant activity at a minimal amount as compared to the ascorbic acid, which was used as the positive control. These results suggested crude extracts from *Cassia petersiana* may not be good candidates for the development of alternative antioxidants. Since the extracts from preliminary extraction showed minimal antimycobacterial activity against *Mycobacterium smegmatis* on the bioautograms, exhaustive extraction was considered to increase the period of exposure of the bioactive compounds to the extraction solvents. This has resulted in the improved observation of the biological activity of the extracts against the tested microorganism, as displayed in the bioautogram. Based on the confirmed targeted biological activity observed, large-scale extraction was considered to increase the amount of bioactive compounds extracted using solvents of varying polarity.

Standard biochemical tests were further conducted to evaluate the antioxidant activity, phytochemical evaluation as well as bioactivity of the isolated extracts. Similar observations were noted as the antioxidant activity was minimal with no separation of the antioxidant compounds, the phytochemicals were visualised better in the BEA mobile system, and biological activity was further observed in the BEA mobile system. From these findings, n-hexane extracts were chosen for the isolation of the bioactive compounds with column chromatography based on the clearer bands on the bioautogram even though the MIC value was not the lowest as compared to other extracts. During the isolation process, similar fractions collected were combined and developed in the BEA mobile system, where they showed the presence of few compounds in the phytochemical profile and the bioautogram. This has resulted in the isolation of a phytosterol named β-sitosterol. The biological evaluation of the compound showed an impressive activity on the bioautogram; however, the MIC value was not significantly impressive as it was higher.

10.2 Conclusions and recommendations

The current study demonstrated the antimycobacterial activity potential of the crude extracts and isolated compound from *Cassia petersiana*. However, further studies, including *in vivo* assay, are recommended on β-sitosterol to evaluate its biological activities before consideration in the use for the development of alternative drugs.