Cape Peninsula
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PHYTOCHEMICAL AND BIOLOGICAL ACTIVITIES OF *PLECTRANTHUS MADAGASCARIENSIS*

by

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DECLARATION

I, Kadidiatou Ndjoubi Ossamy, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed   **Date**

ABSTRACT

Tuberculosis is the leading cause of mortality and morbidity in South Africa due to its association with several chronic illnesses such as psoriasis, diabetes mellitus, and HIV. These chronic diseases weaken the patients resulting in a high risk of progressing from latent to active tuberculosis. Because of side effects associated with synthetic drugs, the attention of the scientific community has shifted to natural products for their use as therapeutic agents to develop effective modern medicines with less adverse effects for treating infectious as well as chronic diseases. Less than 10% of medicinal plant species have been thoroughly investigated and therefore this project focused on exploring one such under-studied medicinal plant namely *Plectranthus madagascariensis.* Phytochemical investigation of *P. madagascariensis* have led to the isolation of five royleanones abietanes and a mixture of two abietane dimers. The five royleanones were identified as; 6*β*,7*α*-dihydroxyroyleanone (**I**), 7*α*-acetoxy-6*β*-hydroxyroyleanone (**II**), horminone (**III**) and coleon U quinone (**IV**) and carnosolon (**V**) and the two the dimers as; grandidone A and 7-epimer grandidone A (VI & VII). All compounds were evaluated for their antitubercular, antidiabetic, antioxidant and cytotoxic activities. Compounds **I-IV** showed activity against the mycobacterial strain $H_{37}Rv$ in 7H9/CAS/Glu/Tx medium only except for compound **II** which showed activity in both 7H9/CAS/Glu/Tx and 7H9/ADC/Glu/Tw medium. Compound **I**, **II**, **IV,** and **VI &VII** displayed inhibitory effects against the intestinal enzyme α-glucosidase with the mixture of **VI & VII** giving the most promising result as an IC_{50} value twenty-one times lower than the positive control acarbose. All compounds exhibited cytotoxic effects against HacaT cell line with compound **V** being the most toxic. Furthermore, the compounds have antioxidant activity through single electron transfer and/or hydrogen atom transfer.

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DEDICATION

I dedicate this thesis to my parents. They have been the strength and the driving force that helped me to never give up through the challenges I have faced during my research studies. Their words of wisdom and encouragement were true motivation and inspiration to me. Thank you for believing in me and teaching me that success comes with hardships and challenges.

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
IPP	Isopentenyl diphosphate
GPP	Geranyl pyrophosphate
FPP	Farnesyl pyrophosphate
DMAPP	Dimethylallyl diphosphate
TLC	Thin layer chromatography
Prep-TLC	Preparative thin layer chromatography
Semi-Prep HPLC	Semi-preparative high-performance liquid chromatography
NMR	Nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
DEPT-135	Distortionless enhancement by polarization transfer
HMBC	Heteronuclear multiple bond coherence
COSY	Correlation nuclear magnetic spectroscopy
J	Coupling constant
s	Singlet
d	Doublet
dd	Doublet of doublets
dt	Doublet of triplets
t	Triplet
td	Triplet of doublet
brs	Broad singlet
m	Multiplet
IR	Infrared spectroscopy
UV	Ultra-violet spectroscopy
$CDCl_3$	Deuterated chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
MeOH	Methanol
H_2O	Deionised water
WHO	World Health Organisation
IUPAC	International union of pure and applied chemistry
Mtb	*Mycobacterium tuberculosis*
HaCaT	Immortalized human skin epithelial keratinocytes cells
DM	Diabetes mellitus
TEAC	Trolox equivalent absorbance capacity
ORAC	Automated oxygen radical absorbance capacity
FRAP	Ferric ion reducing antioxidant power
MIC	Minimum inhibitory concentration
IC	Inhibition Concentration
ADC/	Albumin-Dextrose complex
Glu	D-glucose
Tw	Tween 80
CAS	Casitone
Tx	tyloxapol
GFP	Green fluorescent protein
EDTA	Ethylene diamine tetra-acetic acid

DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
p-NPG	*p*-nitrophenyl-α-glucopyranoside
N/A	Not applicable

CHAPTER ONE
INTRODUCTION

1.1 General introduction

Medicinal plants are plants that possess medicinal or biological properties, i.e., effects related to health, or which have been proven to be useful as drugs or contain organic constituents that are used in the development, discovery, and synthesis of drugs (Farnsworth & Soejarto, 1991). Medicinal plants have traditionally been used as teas, powders, poultices, poisons, spices, tinctures, hallucinogens, polymers, perfumes, waxes, narcotics, oil, fibers, and stimulants (Balick & Cox, 1997; Samuelsson, 2004; Duraipandiyan et al., 2006). Their applications and uses were passed down by healers and their apprentices to the next generation. According to literature, plants are the first medical treatment used by humankind, and about 50 % of modern clinically used drugs are derived from natural products (Matias et al., 2015).

These natural products are used as therapeutic agents to make a remarkable number of effective modern medicines with less adverse effects for treating infectious as well as chronic diseases. Infectious diseases are caused by pathogenic microorganisms namely fungi, bacteria, parasites or viruses (World Health Organization, 2017). These microorganisms are found in almost every habitat present in nature (60 % of the earth's biomass) and they are the main cause of mortality and morbidity, killing nearly 50,000 people per day (Ahmad & Beg, 2001; Silva & Fernandes, 2010).

Among the different infectious diseases present in South Africa, tuberculosis (Tb) is the leading cause of mortality and morbidity in the country. Additionally, due to TB association with several chronic illnesses such as psoriasis, diabetes mellitus (DM), and HIV which weaken the patients, there is an increase risk of progression from latent to active tuberculosis. About 15% of TB patients are likely to develop diabetes due to impaired glucose tolerance caused by TB (WHO, 2014).

Type 2 diabetes is also prevalent in patients with Psoriasis as the inflammatory nature of Psoriasis may affect organic systems, responsible for metabolic diseases (Holm & Thomsen, 2019). Additionally, the treatment of Psoriasis by anti-TNF agents increases the risk of TB infection as anti-TNF agents have the potential to reactivate TB in patients affected by Psoriasis in a period of less than a year (Shaikha, et al., 2012). Furthermore, Wakkeet, et al. (2010) showed that the risk of severe TB infection in patients affected by either diabetes mellitus or Psoriasis is twice as high as the risk for the normal population to contract either disease.

In the following sections, a brief introduction to tuberculosis, diabetes mellitus and psoriasis aetiology, epidemiology, and various chemotherapeutic agents will be detailed.

1.1.1 Tuberculosis

Tuberculosis (TB) is an airborne infectious disease (Yeon & Lee, 2008) caused by a slow-growing bacillus *Mycobacterium tuberculosis* (Njaria, 2017). The bacillus, *Mycobacterium tuberculosis* (*Mtb*), is present in the immune system of approximately 2 billion people (Quan et al., 2016). The majority of the infected people have latent TB infection (LTBI), which can quickly develop to active TB especially when they also suffer from diabetics, psoriasis, alcohol addiction, and human immunodeficiency virus (HIV) (WHO, 2018). Every year, around 10 million new cases of clinical TB encompassing 1.0 million children, 3.2 million women and 5.8 million men were registered (WHO, 2018). Generally, 90% of TB cases occur among adults (aged ≥ 15 years), with 9% being HIV-positive (72% in Africa). Additionally, 60% of TB cases were reported in South Africa (3%), Bangladesh (4%), Nigeria (4%), Pakistan (5%), Philippines (6%), Indonesia (8%), China (9%), and India (27%) (WHO, 2018) as illustrated in Figure 1.1.
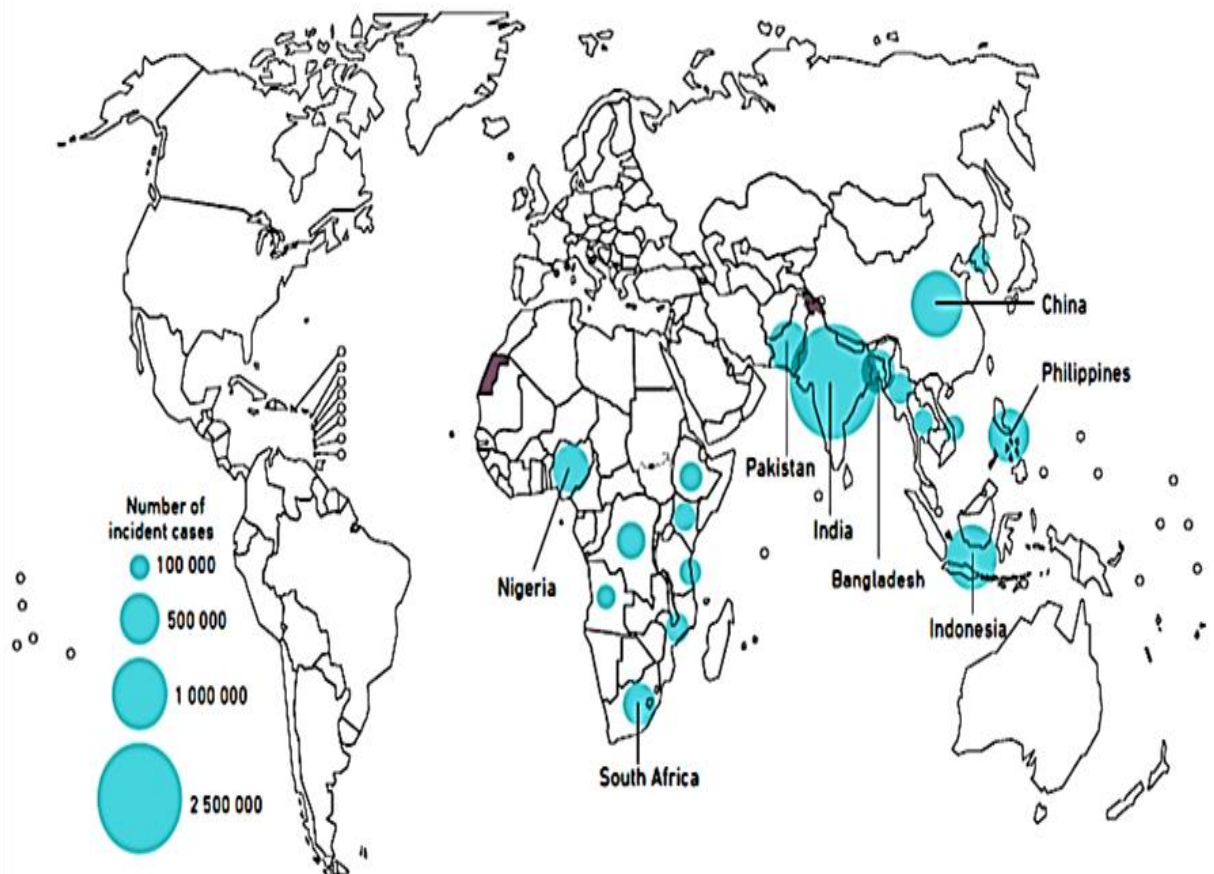
Figure 1.1: Countries with highest TB incident cases in 2017 (WHO, 2018)

The World Health Organisation (2018) and Bordignon et al. (2011) reports that 67% of HIV positive and 18% of psoriasis patients contracted TB while 15% (1.04 million) of TB patients contracted diabetes mellitus. The high prevalence of psoriasis-TB co-infection is due to the suppression of the immune system through psoriasis infection. Additionally, the

rate of TB-DM co-morbidity is becoming higher than TB-HIV co-infection as the percentage of TB-DM reaches at least 20% in TB-DM endemic countries like India and Mexico (Restrepo, 2017).

Worldwide, TB is the second leading cause of mortality and morbidity by infectious diseases, killing 1.3 million of HIV-negative people and 300 thousand of HIV positive patients annually (Srivastava et al., 2007; WHO, 2018). The African and South-East Asia region accounted for 82% of TB deaths in HIV-negative people and 85% of TB deaths in HIV-negative and HIV-positive patients. In South Africa, although the rate of TB cases and deaths has been declining since 2009, the airborne disease remains the primary cause of mortality. South Africa also ranks second highest on the list of reported TB incidence cases with 834 cases of TB per 100000 inhabitants (Churchyard et al., 2014; WHO, 2018).

From 1998 to 2015, The World Health Organisation has classified the high-burden countries (HBCs), which are responsible for 81% of TB incident cases into three categories namely TB, multidrug resistance TB (MDR-TB) and TB/HIV (co-infection) with some overlapping between two or all three groups. South Africa, Zimbabwe, Angola, Thailand, China, Ethiopia, India, Myanmar, Democratic Republic of Congo, Mozambique, Indonesia, Nigeria, Kenya, and Papua New Guinea, are the 14 African and Asiatic countries that appeared in all three categories as shown in Figure 1.1 (WHO, 2018). The highest TB-HIV co-infection incident cases were listed in African countries, with more than 50% of cases occurring in Southern Africa. Among the HBCs, South Africa has the highest number of TB/HIV co-infection incident cases. Furthermore, the second highest number of diagnosed MDR-TB cases as well as the third highest number of TB incident cases are ascribed to South Africa (Churchyard et al., 2014).
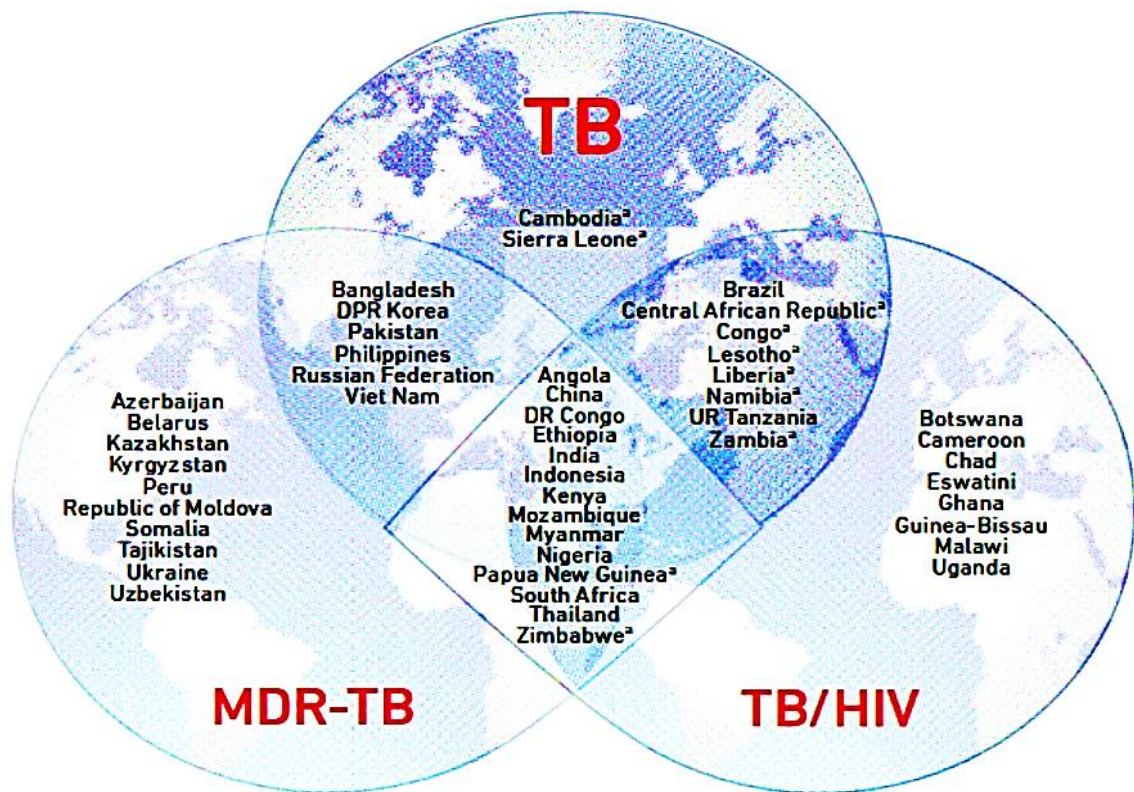
Figure 3.2: The lists for TB, TB/HIV, and MDR-TB high burden countries from 2016-2020 and their areas of overlap, as described in the global tuberculosis report updated by WHO in 2018. "a" shows countries that are included in the list of 30 most affected countries based on the severity of their TB burden as compared to the top 20.

Throughout the years, the spreading of multidrug resistance to almost every antimicrobial drug was observed, and TB strains are no exception. In 2012, about 450 thousand cases of MDR-TB was reported, with 10% of them being classified as extensively Drug-resistant (XDR) (Koch et al., 2014). Although drug resistance against the five frontline anti-tuberculosis drugs rifampin (RIF), streptomycin (SM), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA) were reported before, the emergence of HIV have facilitated the transmission and spreading of MDR-TB and recently XDR-TB (Wade & Zhang, 2004). This is mostly due to the suppression of the immune system through HIV infection and the ability of *Mtb* to readily mutate (Kana & Churchyard, 2013). The XDR-TB which is expressed as MDR-TB with additional resistance to any fluoroquinolone and aminoglycosides antibiotics have severely limited possibility to eradicate TB leading to significant health problems as the number of MDR and XDR is growing every year (WHO, 2018; Kana & Churchyard, 2013). Additionally, the frontline antibiotics RIF, PZA, and INH, also causes painful and disagreeable side effects as tabulated in Table 1.1.

Table 1.1: Rifampin, pyrazinamide and isoniazid side effects

Drug (s)	Side effects
Rifampicin	Anorexia, nausea, abdominal pain, Orange/red-colored urine, Skin itching, rash
Pyrazinamide	Joint pains, Skin itching, rash
Isoniazid	Peripheral neuropathy, Skin itching, rash

1.1.2 Diabetes

Diabetes mellitus (DM) is a chronic disease characterised by hyperglycemia resulting from dysfunction in either insulin action or insulin secretion (Kharroubi & Darwish, 2015). This chronic disease is classified into type 1 and type 2 diabetes with most DM cases being type 2 diabetes (IDF, 2016). Type 1 diabetes which accounts for 5-10% of DM cases is caused by autoimmune destruction of the pancreatic ß cells through T-cell mediated inflammatory response (insulitis); in addition to a humoral (B cell) response (Devendra et al., 2004; Kharroubi & Darwish, 2015). Approximately, 80%-90% of insulin dependent DM cases (type1 DM) are juveniles (Kharroubi & Darwish, 2015; Okur et al., 2017). Type 2 diabetes which is a non-insulin-dependent diabetes resulting from a continuous defect in insulin secretion, accounts for 87-91% of diagnosed DM (Statistics South Africa, 2016; Okur et al., 2017). The number of diagnosed diabetic people has increased from 108 million to 451 million from 1980 to 2016 (WHO, 2016), leading to an estimated 54% (603 million) growth in diabetes cases by 2045 (International Diabetes Federation, 2017). This increase is mostly due to the increased sugar consumption, economic transition, obesity, aging population, genetic factors, autoimmune eradication of the pancreatic β-cells, and urbanization associated with nutrition transition (Okur et al., 2017: 61-65; Pheiffer et al., 2018). In South Africa, DM is ranked as the second most significant cause of mortality and morbidity, with 87% of diagnosed diabetics people (3.35 million) being obese or overweight (Joubert et al., 2007; IDF, 2016; Statistics South Africa, 2016). This is alarming as 69% of South African women and 38% of South African men are obese (Marie et al., 2014).

Glycaemic regulation in the bloodstream for type 2 DM patients is very crucial as it can lead to many macrovascular and microvascular complications such as blindness, renal failure, and stroke. In some cases, a defect in glycaemic regulation can result in an amputation of the lower limb depending on the severity of DM (Okur et al., 2017; Pheiffer et al., 2018). Thus, it is essential to reduce postprandial hyperglycemia which occurs when the blood glucose level is above 180 mg/mL, 1-2h after eating. This is possible by slowing down the rate of dietary carbohydrate or starch ingestion in the small intestine with the help of therapeutic or preventive natural antidiabetics drugs like α-glucosidase inhibitors (Yilmazer-Musa et al., 2012). Moreover, many investigations on DM have shown that DM also caused an increased in free radicals' formation and a reduction in antioxidant capacity to scavenge free radicals. The oxidative stress caused by DM leads to the destruction of

the cell components as well as the genesis and development of many diseases like Alzheimer, cancer, Parkinson, or atherosclerosis (Moharram & Youssef, 2014; Ahmadi et al., 2018). Therefore, it is essential to have potent anti-diabetic drugs with antioxidant properties in order to increase the drug potency and reinforce the defence mechanisms of the organism against the pathologies associated to the attack of free radicals (Moharram & Youssef, 2014).

1.1.3 Psoriasis

Psoriasis is a non-contagious, autoimmune, and incurable skin disease that harms the social and professional life of 2% of the world population(WHO, 2016; Declercq & Pouliot, 2013). This incurable disease is associated with several co-morbidities' illnesses such as Crohn's disease, lymphoma, arthritis, anxiety, diabetes, depression, and cardiovascular disorders (Takeshita et al., 2017). Psoriasis is characterized by symmetrical, sharply demarcated, red plaques, and papules covered with silver or white multi-layered scales (Huang et al., 2019; WHO, 2016). Psoriasis Lesions trigger pain, itching, stinging, and mostly occur on the scalp, trunk, knees, elbows, fingernail, and back with almost an equal distribution among men and women (Jayakar & Kumar, 2016). Chronic dermatosis is most common in Caucasians and Blacks' ethnic groups between 50-69 years old with a more severe form in men compared to women (Huang et al., 2019)

The aetiology of psoriasis is unknown, however the dysregulation of keratinocytes (primary cellular component of the skin) proliferation, differentiation, and death as well as the absence of granular layer results to the hyperproliferation and abnormal differentiation of epidermal keratinocytes (Udensi et al., 2011; Helwa et al., 2015). The hyperproliferation and abnormal differentiation of keratinocytes, which are histological characteristics to psoriasis take 7 to 10 days instead of 28-50 days to renew as compared to normal and healthy skin (Declercq & Pouliot, 2013). Keratinocytes (KCs) play a vital role in the skin permeability barrier, wound repair, as well as in the initiation and perpetuation of skin inflammatory (Corsini & Galli, 1998) and immunological responses, (Colombo et al., 2017; Hanel et al., 2013). The immune system is activated by many environmental factors including smoking, stress, drugs (β-receptor antagonists, non-steroidal anti-inflammatories (NSAIDs), antimalarials, lithium, and angiotensin-converting enzyme (ACE) inhibitors), trauma, excessive alcohol consumption, and streptococcal infection (Raboobee et al., 2010). The presentation of these exogenous antigens to innate natural killer T (NKT) cells, chemokines, cytokines, and dendritic cells and adaptive (CD4+ and CD8+ T lymphocytes) immunity, as well as the formation of the immunological synapse, leads to the differentiation of T cells into effector cells like Th1, Th2, and Th17 (Gaspari, 2006; Declercq & Pouliot, 2013; Engler et al., 2017). The effector Th1 cells which release tumour necrosis factor (TNF-α) and interferon-gamma (IFN-γ) are predominant in psoriasis, whereas the

Th17 cells which release interleukins (IL)-17A, IL-17F and IL-22 play essential roles in psoriasis and vitiligo as it activates the keratinocytes and other cytokines of the innate immune system as shown in Figure 1.3 (Seo et al., 2012; Engler et al., 2017; Nedoszytko et al., 2014). The activation of the keratinocytes results in the upregulation of proinflammatory chemokines and cytokines that are found in psoriatic lesions (Engler et al., 2017; Nedoszytko et al., 2014).
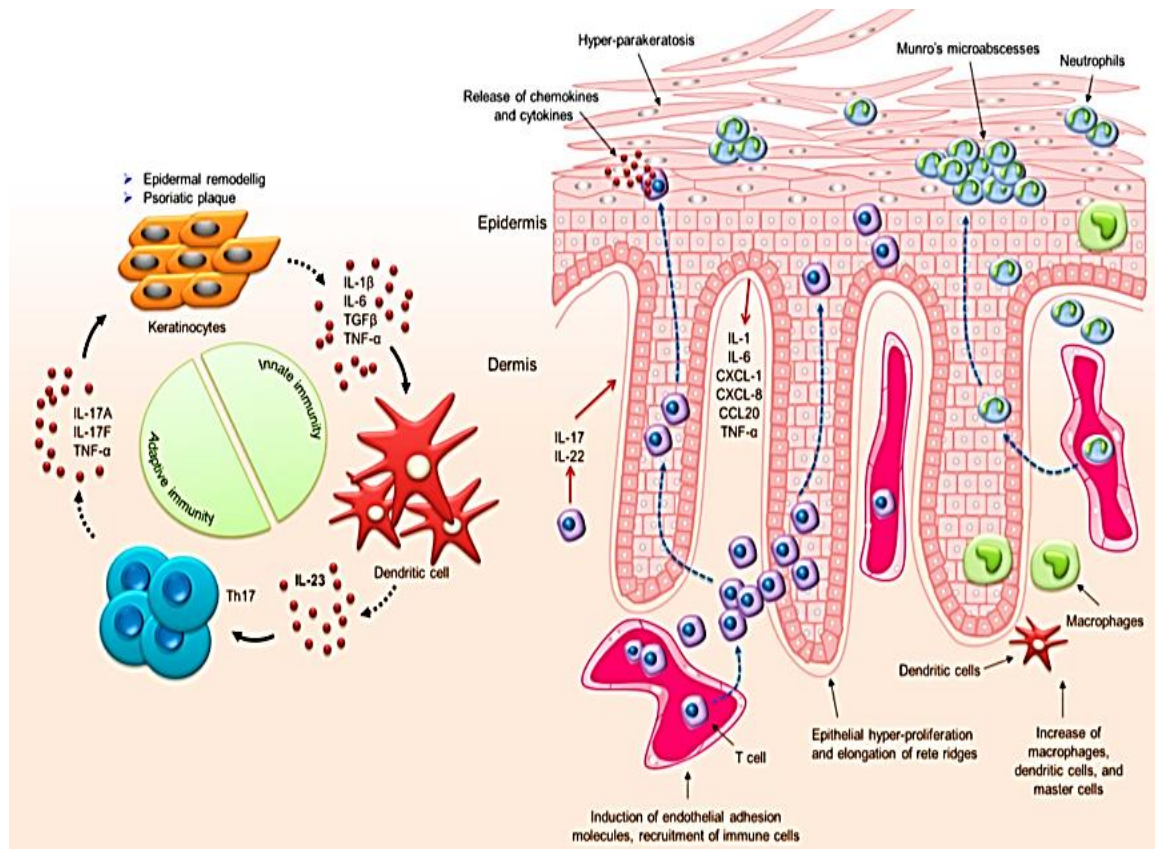
Figure 1.3: Immunopathogenesis of psoriasis (Huang et al., 2019).

Regarding the negative impact the disease has on affected people's lives, many clinical treatments have been in place to attenuate the symptoms of this incurable disease. However, these treatments have some adverse effects like organ toxicity, infection, atrophy, which result in the restriction of long term treatment (Huang et al., 2019). Thus, it is important to find potent anti-psoriatic and antiproliferative keratinocytes agents with less adverse effects.

1.1.4   Rationale for *Plectranthus madagascariensis'* natural products as tuberculosis diabetes and psoriasis therapeutic agents.

The high cost of new chemotherapeutic agents, socioeconomic inequality, and substandard programme management with reference to inadequate case detection, diagnosis, and cure have made tuberculosis, diabetes and psoriasis high burden diseases.

Since these diseases beget significant economic and quality of life burdens, scientists have been looking for medicines from natural resources as they are reported to have fewer side effects compared to chemically synthesized or clinical drugs (Chartone-Souza, 1998; Sasidharan et al., 2011). According to the royal botanic gardens kews there are only 391,000 medicinal plants around the world, only 35,000 of them have been thoroughly studied either chemically or for their physiologist activities. This implies that 356,000 plants with potential and interesting chemical entities could be decisive in the treatment of present and future diseases (Rasool, 2012). Among these under-studied plants, *Plectranthus madagascariensis* (Lamiaceae) has been selected for this research study according to the type of phytochemical constituents biosynthesized by *Plectranthus* species along with their pharmaceutical properties and traditional uses in respiratory and skin infections.

This chapter is divided into nine sections. The importance of medicinal plants in drug discovery, the justification of the study, and problem statement are discussed in sections 1.2, 1.3, and 1.4 respectively. Section 1.5 describes the research questions whereas section 1.6 focuses on the aim of the research. The objectives of the research study are provided in Section 1.7. The limitations of the thesis are presented in section 1.7, followed by the thesis outline in section 1.9.

1.2   The impact of medicinal plants in drug discovery

Medicinal plants are fundamental components of research development in the pharmaceutical industry whose pharmacological properties were first discovered through traditional medicine and ethnomedicinal studies (Singh, 2015). From the ethnomedicinal studies done so far, natural products are the source in the discovery and development of many drugs. For example, nitisinone (**1**) known on the market as orfadin is the lead structure of medicines used to treat the rare inherited disease tyrosinemia (Balunas & Kinghorn, 2005). Moreover, Newman and Cragg (2016) reported that from 1st January 1981 to 31st December 2014, about 33-42 % of approved therapeutic agents were made of natural products and natural product-derived molecules. These natural products' functional groups and diversity have exhibited their impact on drug effectiveness, optimization, safety, and the ability to prevent a disease from spreading. This is mostly due to the natural products' smaller adverse effects, cultural acceptability, and excellent compatibility with the human body (Mustafa et al., 2017; Verma & Singh, 2008). Thus, enhancing the use of about 78 % of these new biological active constituents as a propitious alternative cure for infectious diseases (Lokhande et al., 2007). For instance, various groups of antifungal proteins like glucanase and chitinase used as protection for a developing embryo from many infections are found in the seeds of several medicinal plants (Mustafa et al., 2017).

Several drugs discovered and isolated from plants originated from different continents such as Africa, Australia, Asia, and South America, e.g., atropine (**2**) from *Atropa belladonna*, codeine (**3**) from *Papaver somniferum*, quinine (**4**) from *Cinchona cordifolia*, pilocarpine (**5**) from *Pilocarpus jaborandi* and theobromine (**6**) from *Theobroma cacao* (Figure 1.4, Salim et al., 2008: 1-20). The listed natural products have been used respectively as an anticholinergic, cough suppressant, antimalarial, parasympathomimetic, and anti-arteriosclerosis (Salim et al., 2008). Even though some have been retracted from the pharmaceutical industry due to their lack of efficacy, many of these chemical entities are still in use and possess a similar therapeutic purpose as their first ethnomedical use (Fabricant & Farnsworth, 2001).
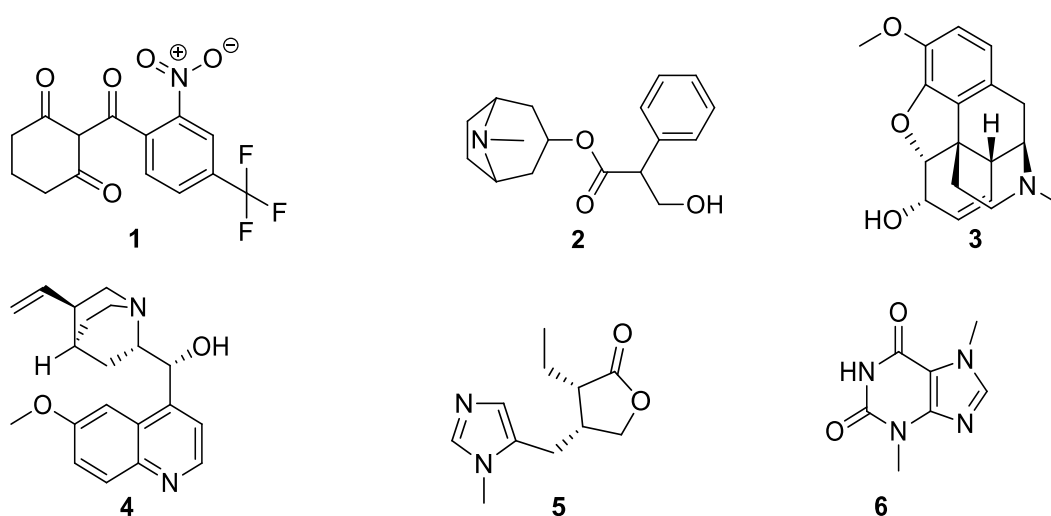
Figure 1.4: Structures of compounds **1**-**6**.

1.3 Justification of the study

South Africa has an astonishing floral diversity used in both formal and informal systems of medicine. About 60-80% of South Africans use medicinal plants as their primary health care due to availability and affordability (Aston et al., 2018). Additionally, the phytoconstituents present in plants represent a significant source of new drugs. Many of the natural compounds have shown potency and usefulness in different industrial sectors such as the pharmaceutical, cosmeceutical, and nutraceutical industries. In the present work, among the phytochemical constituents present in *Plectranthus madagascariensis*, a pronounced interest has been shown towards the abietane diterpenoids due to their antimicrobial, antidiabetic and antiproliferative properties (Kubínová et al., 2014; Diogo et al., 2019).

Furthermore, the increase in multi and extensively *Mtb* resistance towards frontline TB drugs, as well as the different adverse effects caused by the clinically recommended cures,

greatly influence the emergence of tuberculosis and diabetes and psoriasis (Caulfield & Wengenack, 2016).

Regarding this matter, *P. madagascariensis* was chosen due to its history of use in the treatment of scabies, small wounds, colds, cancer, asthma, cough, diabetes, and chest complaints (Rabe & Staden, 1998; Kubínová et al., 2014; Diogo et al., 2019). Furthermore, integrating medicinal plants into modern medicines will stimulate research and might lead to discovery of various efficacious drugs.

1.4   Problem statement

Tuberculosis and diabetes are the top two leading causes of mortality and morbidity in South Africa, accounting for 6.5% and 5.5 % of natural deaths, respectively (Stats SA, 2018). Furthermore, the bacillus *Mtb* being able to adapt to the anti-tuberculosis drugs' mechanism of action results in the formation of *Mtb* multi-drug resistance and extensive drug resistance. Hence, challenging the country's public health as the inhibition or elimination of the bacteria becomes more arduous (Byarugaba, 2004; Chandra et al., 2017). The bad quality of life that comes with having the incurable disease psoriasis and the costly treatment related to the listed diseases is the reason why this study intends to contribute in finding more efficient natural-derived drugs to combat, control and reduce tuberculosis, diabetes and psoriasis pandemic.

1.5   Research questions

The research questions of this study are enumerated below.

i.   What are the active non-volatile secondary metabolites of *P. madagascariensis*?
ii.   What are the chemical structures of these metabolites?
iii. Are *P. madagascariensis* secondary metabolites biologically active?
iv.   Specifically, can they be used to treat tuberculosis, diabetes, and psoriasis or developed into useful drugs?

1.6   Aim of the research

This research aims to isolate, characterize, and evaluate the anti-tuberculosis, antidiabetics, and cytotoxicity properties of the phytochemical constituents of the aerial parts of *P. madagascariensis.*

1.7   Objectives of the research study

The objectives of this study include:

i.   To collect and identify *P. madagascariensis.*
ii.   To extract of the aerial parts of *P. madagascariensis* metabolites

iii. To isolate the bioactive chemical constituents of the total extract using chromatographic techniques such as column chromatography (CC), semi-preparative high performance liquid chromatography (semi-prep-HPLC) and preparative-TLC.

iv. To characterize the isolated compounds using different spectroscopic techniques including 1D and 2D nuclear magnetic resonance (NMR) spectroscopy.

v. To evaluate the biological activities and determine their MIC_{90} and IC_{50} of the isolated compounds.

1.8   Hypotheses

The ethnobotanical records of *Plectranthus* plants have shown that many species of this genera are used in traditional medicine to treat ailments such as Eye infections, diarrhoea, asthma, indigestion, , skin allergies, heart diseases, respiratory infections and fever (Waldiaa et al., 2011). Phytochemical studies of *P. madagascarienis* have revealed the abundant amount of terpenoids and phenolic compounds. Therefore, it is possible that *P. madagascariensis* contains bioactive terpenes or phenolic compounds that can be used in the treatment of tuberculosis, diabetes and psoriasis.

1.9   Delimitations

This research project will focus only on the extraction, isolation, and characterization of the biologically active constituents of *P. madagascariensis* aerial parts. Besides, the isolated secondary metabolites were evaluated for their biological activities.

1.10   Thesis outlines

This thesis is divided into six chapters as detailed below.

CHAPTER ONE: INTRODUCTION

This chapter highlights the different problems and challenges faced by people suffering from tuberculosis, diabetes, and psoriasis in South Africa and worldwide. It also gives an insight into the impact of medicinal plants in drug discovery and development. This section includes the justification of the study, problem statement, aim, objectives, hyphothesis, delimitations, and outlines of the research study.

CHAPTER TWO: DITERPENES

This chapter introduces the various classes of diterpenes and their biosynthetic pathways. The abietane diterpenoids biosynthesis, classes and nomenclatures are the most pointed out in this part of the thesis.

CHAPTER THREE: LITERATURE REVIEW

Chapter 3 gives a detailed literature review on the genus *Plectranthus* and in particular on the Southern African species. A meticulous review, on the phytochemistry, medicinal uses, and pharmacology of the Southern Africa *Plectranthus* species are presented in this chapter.

CHAPTER FOUR: EXTRACTION ISOLATION AND CHARACTERIZATION OF *PLECTRANTHUS MADAGASCARIENSIS* NATURAL PRODUCTS

Chapter four presents the materials and methods used in the isolation and characterization of *P. madagascariensis* components as well as the different chromatography and spectroscopic methods and techniques used in the isolation of the chemical constituents. The structure elucidation of the isolated compounds is also elaborated in the chapter.

CHAPTER FIVE: BIOLOGICAL EVALUATION

This chapter gives details of the efficiency of the isolated natural products as anti-tuberculosis, anti-diabetes, and anti-psoriasis agents.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

This section summarized the outcomes of the investigations and gives some recommendations related to the research study.

CHAPTER TWO
DITERPENOIDS

2.1   Introduction

Diterpenes are naturally occurring compounds of twenty carbon derived from the condensation of four isoprene units via the mevalonate and deoxy-xylulose phosphate pathways (De Sousa et al., 2018). Both pathways are the only route by which diterpenes can be naturally biosynthesized since both pathways produced the biochemical isoprene units, dimethylallyl diphosphate and isopentenyl diphosphate needed for the biosynthesis of diterpenes (Hemmelin et al., 2012; De Sousa et al., 2018). However, some authors stated that the deoxy-xylulose phosphate pathway is the most predominant pathway to naturally biosynthesized diterpenoids, although the mevalonate pathways can also supply different quantities of a molecule (Hemmelin et al., 2012; De Sousa et al., 2018). The condensation of these isoprene units leads to the formation of geranylgeranyl diphosphate GGPP which will undergo some cyclization reactions and produced monocycle, bicyclic, tricyclic, tetracyclic, pentacyclic and macrocyclic diterpenes or diterpenoids (diterpene with functional groups attached to it) (Dewick, 2002)

In the genus *Plectranthus*, only bicyclic (labdanes), tricyclic (abietanes and pimaranes), and tetracyclic diterpenes (kauranes and beyeranes) diterpenoids have been identified so far. A more detailed description of their biosynthesis and cyclization reactions are discussed in section 2.2. The classification, characterization, and nomenclatures of the most diverse type of diterpenes (abietane diterpenoids) biosynthesized by Southern African *Plectranthus* species are given in Section 2.3.

2.2   Biosynthesis of diterpenes

The occurrence, content, amount, and composition of diterpenoids in plants depend upon the plant species. This is due to the presence and activity of the enzymes responsible for their biosynthesis, which take place in three stages (Woźniak et al., 2015). The first stage is the biosynthesis of the activated isoprenes, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The second stage is about the biosynthesis of geranylgeranyl diphosphate (GGPP), whereas the third stage consists of various cyclization reactions required for the biosynthesis of monocyclic, bicyclic, tricyclic and tetracyclic diterpenes from GGPP as described below.

2.2.1   Biosynthesis of IPP and DMAPP

Plants produce IPP and DMAPP either via the mevalonate pathway (MVA) located in the plant's cytosol or the methylerythritol phosphate pathway (MEP), also known as the 1-deoxy-D-xylulose (DOX) pathways which occurs in the plant's chloroplast (Rijo, 2010).

Sometimes plants produced IPP and DMAPP using both paths simultaneously. These activated isoprenes are the introductory material for the biosynthesis of GGPP.

2.2.1.1 Mevalonate pathways

In this step, two molecules of acetyl-coenzyme A (acetyl-CoA) will react and be catalyzed by thiolase to yield acetoacetyl-CoA. The produced acetoacetyl-CoA reacts with another molecule of acetyl-CoA by Claisen condensation to produce β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). This is followed by the production of the intermediate mevalonic acid by reduction of HMG-CoA carbonyl groups to mevalonic acid with nicotinamide adenine dinucleotide phosphate (NADPH). Subsequently, the phosphate groups from two of adenosine triphosphate (ATP) molecules are transferred to mevalonic acid to produce 5-diphosphomevalonic acid. The 5-diphosphomevalonic acid is converted to IPP by diphosphomevalonic acid decarboxylase. Afterward, IPP is isomerized to yield DMAPP, as shown in Figure 2.1.
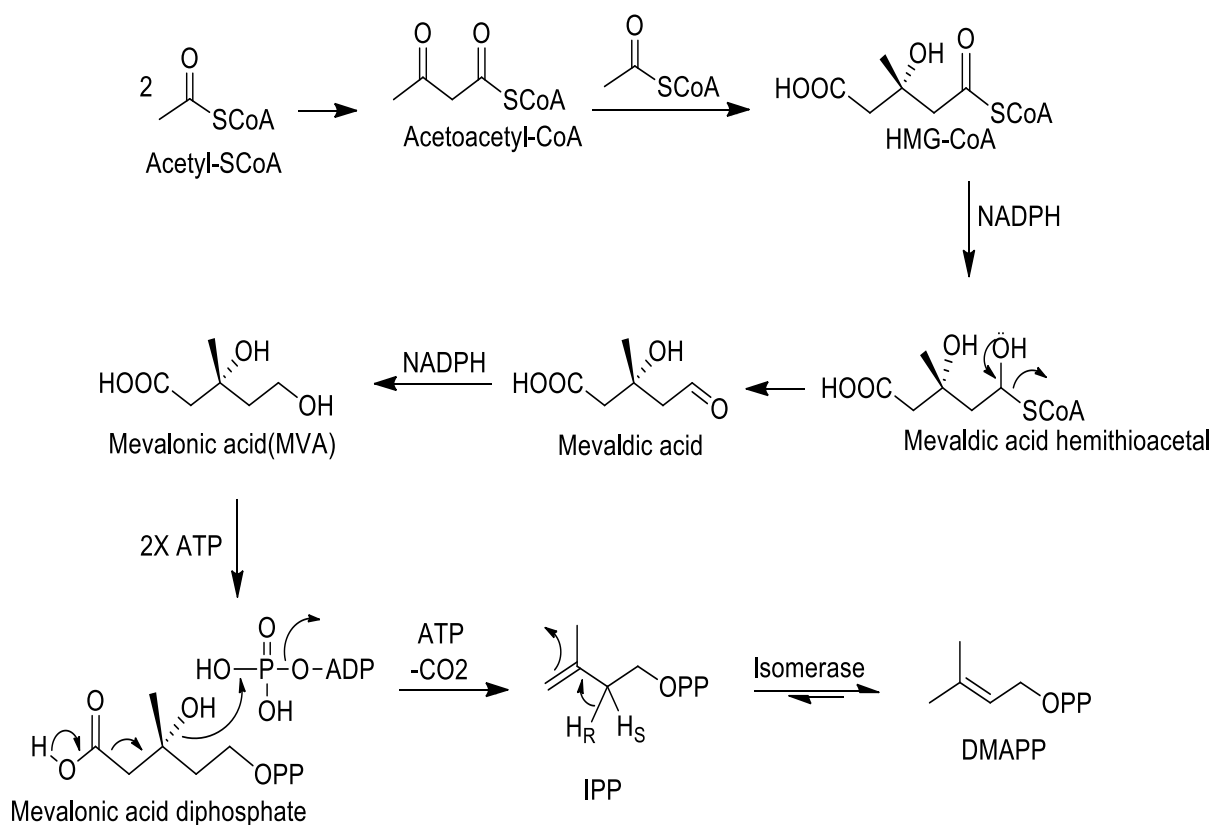
Figure 2.1: Biosynthesis of IPP and DMAPP via the mevalonate pathway (Dewick, 2002).

2.2.1.2 Methylerythritol phosphate pathway (MEP)

This step is initiated by the condensation of glyceraldehyde 3-phosphate and pyruvic acid (Figure 2.2). These two compounds undergo a condensation that leading to the decarboxylation of pyruvate to form 1-deoxy-D-xylulose 5-phosphate (DXP).

Intramolecular rearrangement of DXP is assumed to give a hypothetical rearranged intermediate, 2-C-methylerythrose 4-phosphate, which is then reduced to 2-C-methyl-D-erythritol-4-phosphate (MEP) by NADPH (Kuzuyama, 2002). MEP reacts with cytidine triphosphate (CTP) to form 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME), which is phosphorylated with ATP and cyclase to yield 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) which is reduced to IPP and DMAPP by an unspecified and unclear reduction process (Rijo, 2010).
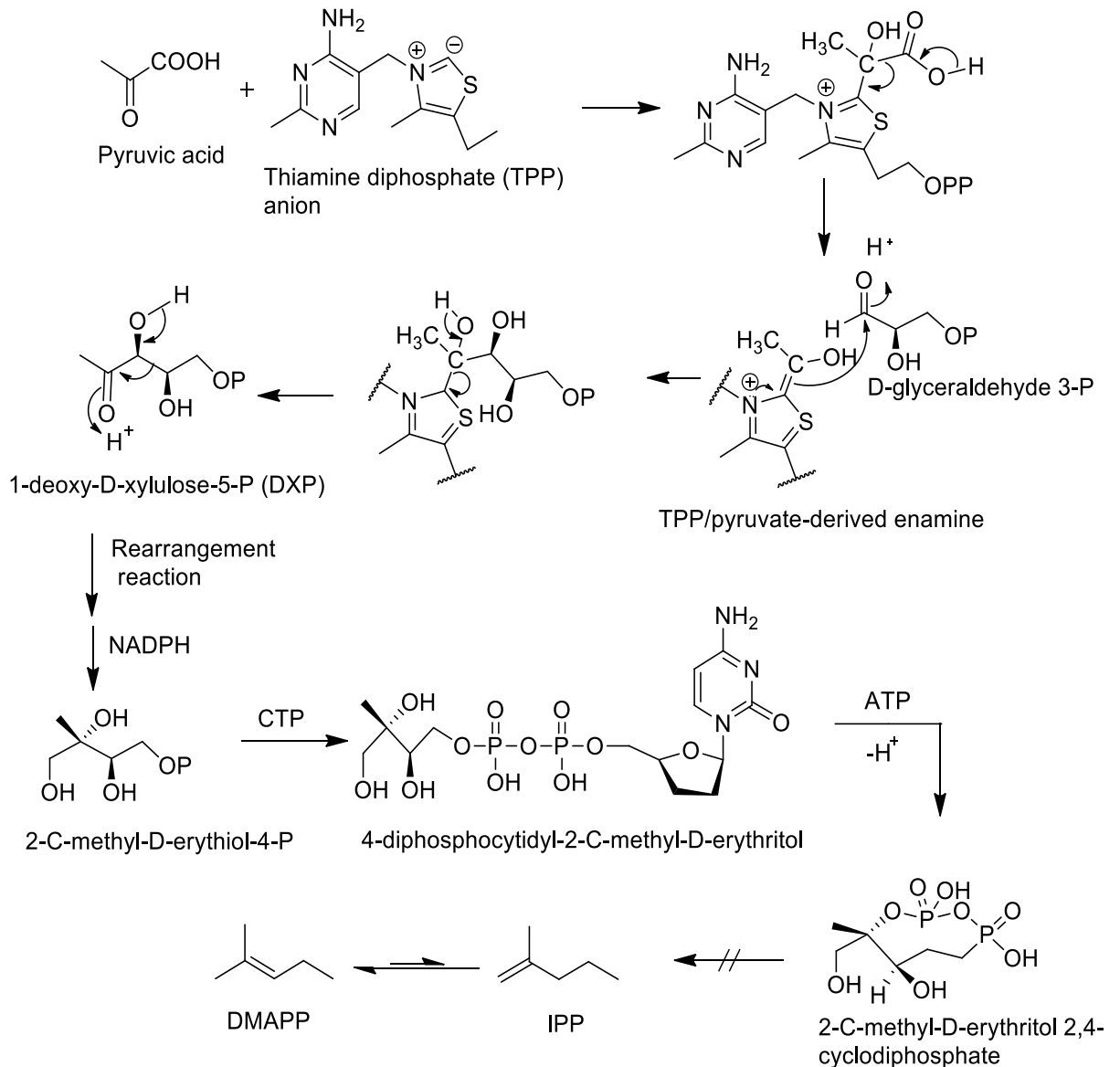
Figure 2.2: Biosynthesis of IPP and DMAPP via the methylerythritol phosphate pathway (Dewick, 2002).

2.2.2 Biosynthesis of GGPP

This stage involves a head to tail coupling of IPP with DMAPP to generate geranyl pyrophosphate (GPP). The tail of the geranyl pyrophosphate is condensed with the head of IPP to yield farnesyl pyrophosphate (FPP). Hereafter, IPP is added to FPP in a head to

15

tail arrangement that results in the formation of geranylgeranyl diphosphate (GGPP). Figure 2.3 shows the representation of the biosynthesis of GGPP.
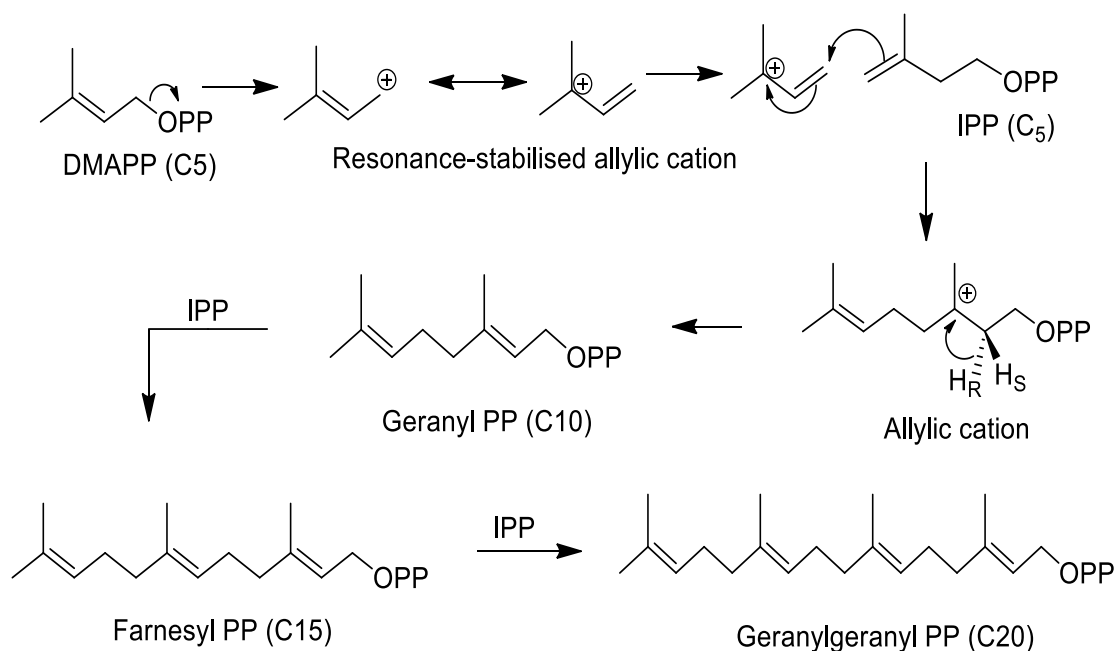
Figure 2.3: Biosynthesis of GGPP (Dewick, 2002).

2.2.3   Biosynthesis of monocyclic, bicyclic, tricyclic, and tetracyclic diterpenes

2.2.3.1 Biosynthesis of monocyclic diterpenes

After the biosynthesis of GGPP, the double bond on the isopropylidene unit located at the head of the GGPP chain is protonated to form the tertiary carbocation resulting in the first concerted cyclization and formation of monocyclic type diterpenes (Figure 2.4).
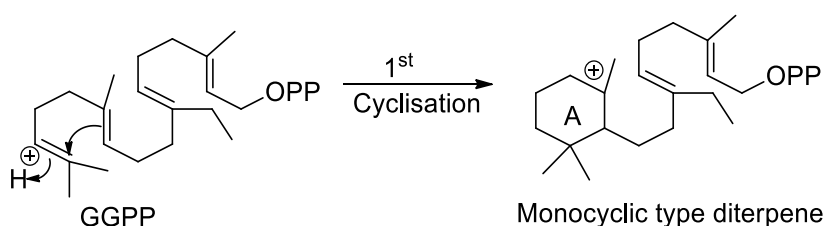
Figure 2.4: Formation of monocyclic type diterpenes from GGPP (reproduced from Dukhea, 2010).

2.2.3.2 Biosynthesis of bicyclic diterpenes

The sequential cyclization of GGPP in a chair-chair "normal" conformation followed by a loss of a proton from a methyl yields the bicyclic type diterpenes copalyl [(+)-copalyl PP], also known as labdadienyl PP. A rearrangement of GGPP in a chair-chair "antipodal"

conformation generates its stereoisomer ent-copalyl PP [(-)-copalyl PP]. The loss of PP from labdadienyl gives the labdane type diterpenes (Figure 2.5).

GGPP (Chair-chair "antipodal")

Cyclisation

Ent- copapyl

GGPP (Chair-boat "normal")

Cyclisation

(+)-Copalyl (labdadienyl PP)

Labdadienyl
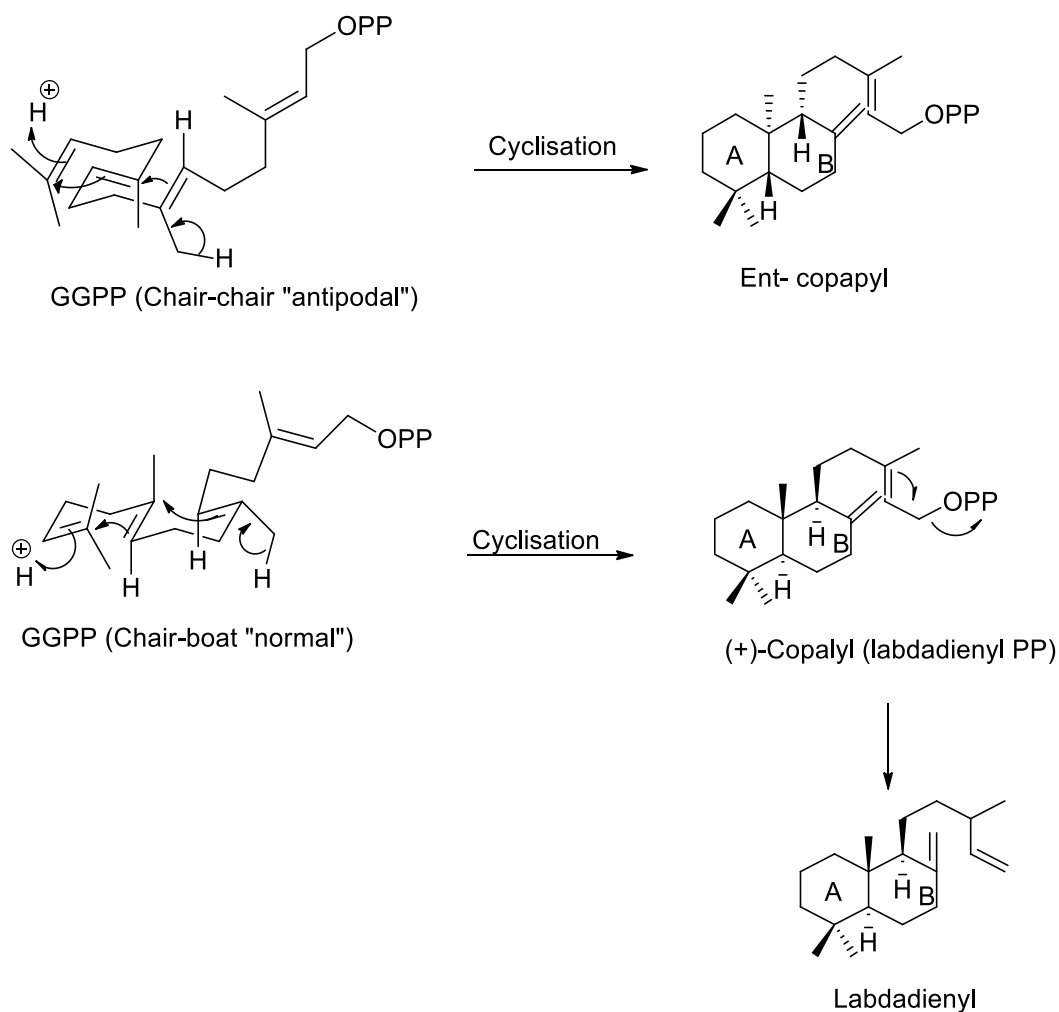
Figure 2.5: Formation of copalyl diphosphate stereoisomers (bicyclic diterpenes) from GGPP (De Sousa et al., 2018).

2.2.3.3 Biosynthesis of tricyclic diterpenes

The removal of the diphosphate from copalyl PP generates primary allylic cation, and further cyclization of labdadienyl leads to the formation of tricyclic type diterpenes like the intermediate sandaracopimarenyl cation. The gain of proton of sandaracopimarenyl cation from C-14 generates a double bond at ring C between C-8 and C-14, resulting in the formation of the second intermediate sandaracopimaradiene. The intermediate undergoes a methyl migration from C-17 to C-15, which yields an abietenyl cation. The loss of a proton at C-7 produces the diene abietadiene. Sequential oxidation of abietadiene 4α methyl to carboxylic acid via alcohol and aldehyde yields abietic acid (Figure 2.6).
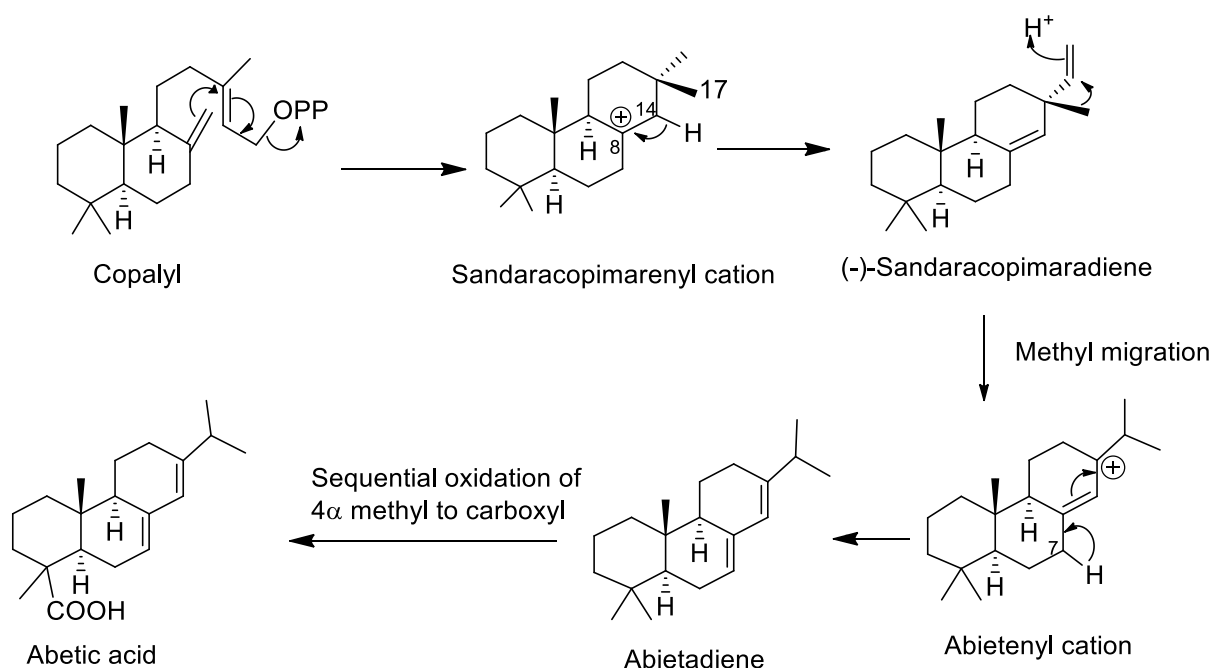
Figure 2.6: Biosynthesis pathway of abietic acid (Dewick, 2002).

2.2.3.4 Biosynthesis Tetracyclic diterpenes

The tertiary carbocation on Sandaracopimarenyl obtained from ent-copalyl is attacked by the double bond to yield the fourth ring system and a secondary cation. The secondary cation on the fourth ring was stabilized by converting the secondary cation to a tertiary cation by 1,2 alkyl migration, which is followed by the loss of the proton from the methyl group results in the formation of ent-kauranes and its exocyclic double bonds. The sequential oxidation reactions of ent-kauranes results in the formation of various oxygenated kauranes like ent-kaurenoic acid (Figure 2.7).
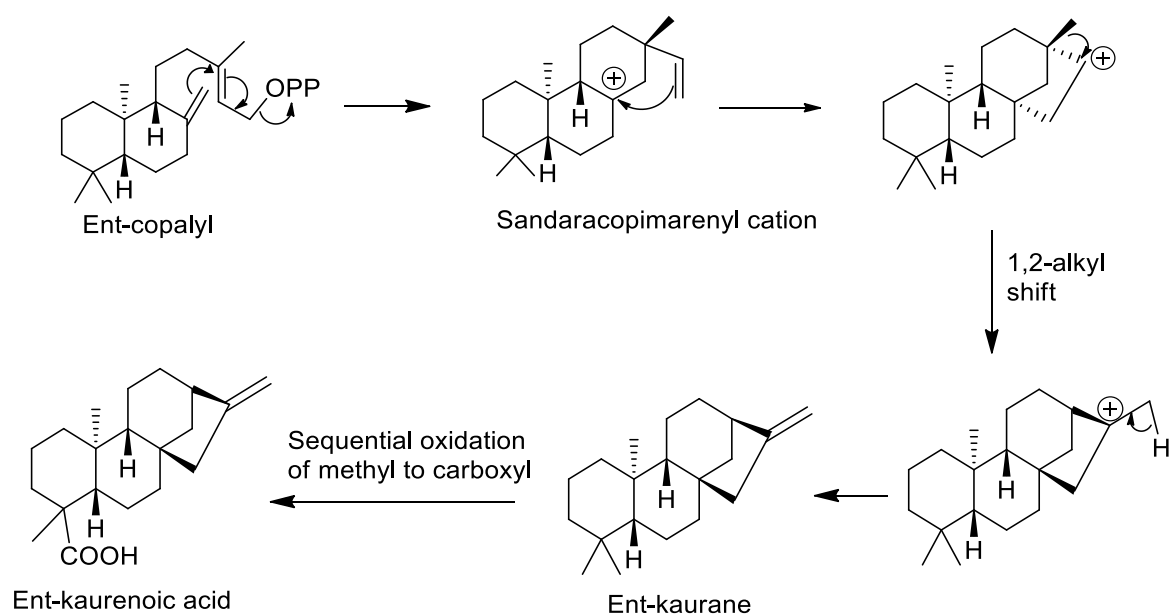
Figure 2.7: Biosynthesis pathways of ent-kaurane (Dewick, 2002).

The protonation of sandaracopimarenyl cation by the double bonds at C-16 with loss of the methyl at C-13 form the beyeranes type diterpenoids (Figure 2.8).

Ent-copalyl   Sandaracopimarenyl cation   Beyerane
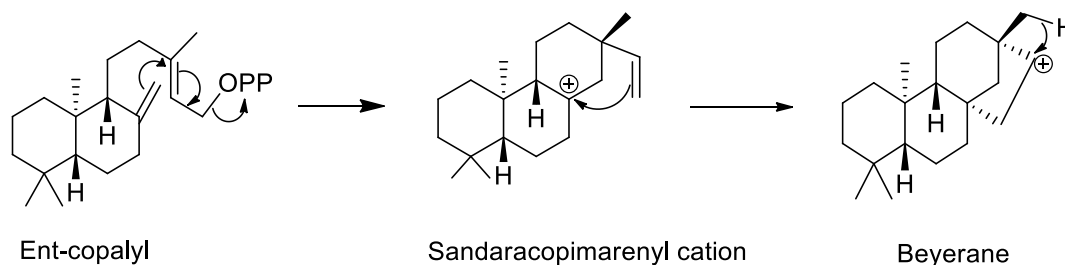
Figure 2.8: Biosynthesis of beyerene (Manito, 1981).

2.3   Classification, characterization, and nomenclature of the abietane diterpenoids

Among the different types of diterpenoids biosynthesized by *Plectranthus* species, Abietane diterpenoids are the most diverse type of diterpenoids found in the aerial parts and leaves of Plectranthus species. Abietane diterpenoids skeleton consists of two methyl groups at C-4, one methyl group at C-10, and one isopropyl group at C-13. The numbering system of this highly modified diterpenes was established in 1993 by the International Union of Pure and Applied Chemistry (IUPAC) as illustrated in Figure 2.9
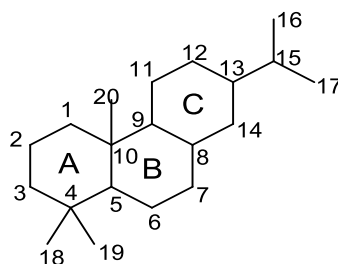
Figure 2.9: Numbering system of abietane diterpenes.

According to Abdel-Mogib et al. (2002), abietane diterpenoids can be classified into ten class, namely: phenolics abietanoids, dimeric abietanoids, royleanones, 1,4-phenanthraquinones, acylhydroquinones, spirocoleons, vinylogous quinones, seco-abietanoids, quinone methides, and abeo-acylhydroquinones. Each class of abietane diterpenoids exhibits a wide range of oxygenated groups allocated at different positions varying from one class to another. For example, the royleanones are characterized by a 12-hydroxy, 1,4-benzoquinone ring C, whereas the acylhydroquinones type abietane has a trihydroxybenzene ring C, a carbonyl group at C-7 and a double bond at C-5 - C-6. The spirocoleons have a cyclopropane attached at C-13, and 2-Cyclohexene-1,4-dione ring C. Vinylogous quinone is characterized by a tri- or di- hydroxycyclohexa-1,4-diene with double bonds at C-5 - C-6 and C-7 -C-8 or carbonyl groups at C-6 and C-7. In the case of vinylogous quinone with dihydroxycyclohexa-1,4-diene ring C, the carbon at position 12 is

attached to a carbonyl group. The 1,4-phenanthraquinones type abietane is characterized by aromatic ring A, B, and C. Quinone methides are bearing a p-quinone methide with a hydroxide group at C-11 and a cyclohexadiene ring corresponding to ring B. Furthermore, alkyl, alkenyl, carboxyl, cycloalkyl, hydroxyl and carbonyl, and hydroxyl groups can be substituted at position 2, 3, 6, 7, 16, 15 but rarely at position 20, 18 and 19 depending on the class of abietane diterpenoids.

The terms "nor", "abeo" and "seco" are usually used in the nomenclature of some abietanes (Figure 2.10). The prefix "nor" is used when a methyl group is removed from a particular ring in the structure while the term "seco" is used to designate the removal of a side chain or broken bond within the parent structure. The prefix "abeo" or the prefix X(Y→ Z) abeo point out the migration of a single bond from its initial position in a fundamental parent structure to a new position; where, X is the locant of the position of the unchanged, end of the migrating bond; Y is the locant of the position of the moving end of the migrating bond and Z is the locant of the position of the moving end of the migrating bond in the final structure (IUPAC, 1999)

Abietane (fundamental parent structure)   **18-nor-abietane**

Abietane (fundamental parent structure)   **(3αH)-5(4 → 3)-abeo-abietane**

Kaurane (fundamental parent structure)   **Seco-kaurane**
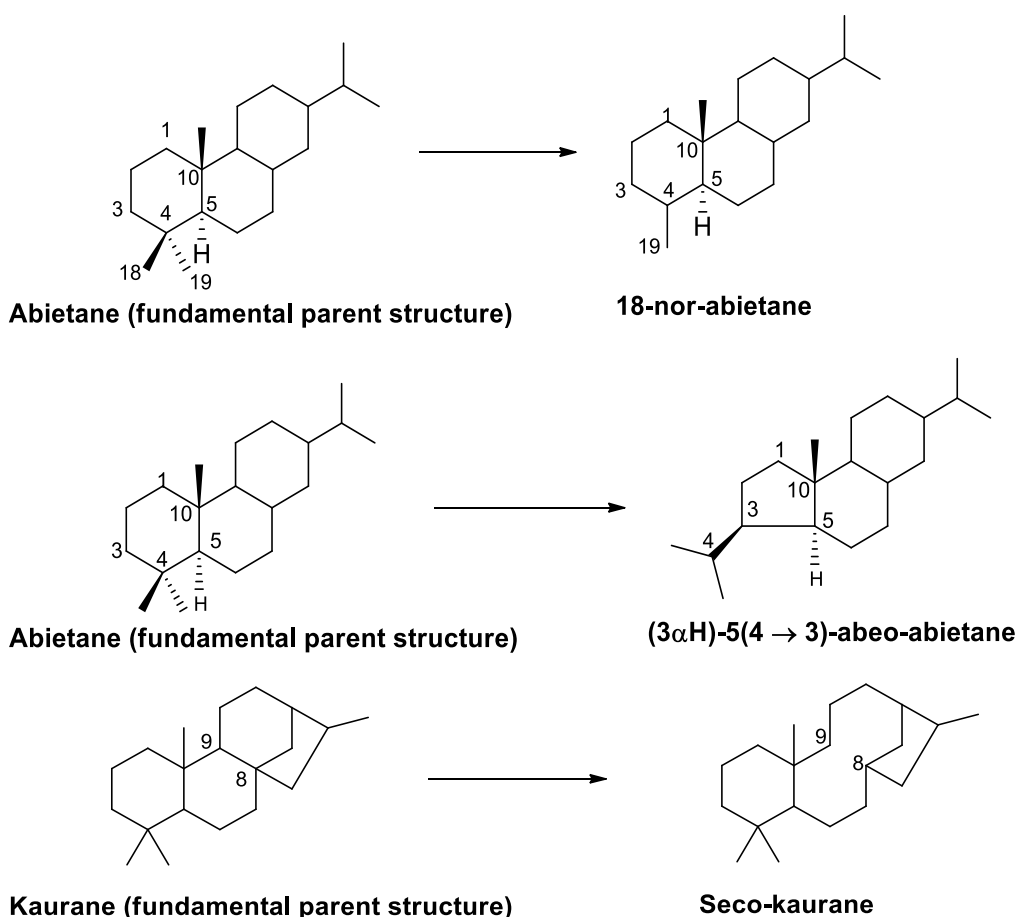
Figure 2.10: Illustration of the uses of the terms "nor", "abeo" and "seco".

CHAPTER THREE
LITERATURE REVIEW

3.1   Introduction

Plants are abundant resources of secondary metabolites, which play a very crucial role in traditional and modern medicine. Secondary metabolites, also referred to as natural products are biologically active organic compounds that have been identified as therapeutic agents with economic and culinary importance. Among the various plant species belonging to the Cape flora of Southern Africa, the genus *Plectranthus* (section 3.2) and more specifically the Southern Africa *Plectranthus* species were selected and are being reviewed in this chapter. The botanical characteristics (section 3.3), phytochemistry (section 3.4), ethnobotanical uses, ethnopharmacological, pharmacological, and others uses (sections 3.5-3.7) of the Southern African *Plectranthus* species are described in detail.   Regarding their phytochemistry, an emphasis is made on abietane diterpenoids since they are the most diverse type of bioactive compounds produced by *Plectranthus* species. Furthermore, the botanical identification of *P. madagascariensis* is given in section 3.3.2

3.2   *Plectranthus* genus

The genus *Plectranthus* consists of 350 species of various sizes, foliage, and scent that belongs to the sage family Lamiaceae, subfamily Nepetoideae, and the tribe Ocimeae (Waldiaa et al., 2011).   *Plectranthus* species are characterized by the two-lipped corolla and the aromatic leaves arranged in opposite pairs (Van Jaarsveld, 1987). Most of the 350 attractive and decorative *Plectranthus* species are semi-succulent to succulent shrubs or herbs growing in tropical and subtropical regions as well as in regions with warm weather like Africa, Australia, Asia and a few Pacific islands (Rice et al., 2011). In Southern Africa (South Africa, Lesotho, Namibia and Swaziland), over 53 species are reported to be endemic to the region with a few used to treat ailments and diseases like *P. ambigus*, *P.amboinicus*, *P. barbatus*, *P. caninus*, *P. eckloni*, *P. elegan*, *P. ernstii*, *P. fructicosus, P. grandidentatus*, *P. herereonsis*, *P. madagascariensis*, *P. ornatus*, *P. porcatus*, *P. saccatus*, and *P. strigosus* (Codd 1985; Rabe & Staden 1998). In South Africa, the largest concentration of species is found in the Southern KwaZulu Natal and the North-Eastern coastal part of the Eastern Cape (Van Wyk & Smith, 2001). The genus *Plectranthus* has six subgenera, namely *Calceolanthus*, *Plectranthus*, *Burnatastrum*, *Coleus*, *Xerophilus*, and *Nodiflorus* and two significant clades, coleus and *Plectranthus* clades (Paton et al., 2004; Van Jaarsveld 2006). However, the *Plectranthus* clade which includes the genera *Aeollanthus*, *Tetradenia*, *Capitanopsis*, *Dauphinea*, *Thorncroftia,* and some species of the genus Plectranthus like *P. fruticosus* has below 50% of bootstrap support (Paton et al.,

2018). The *Coleus* clade which encompassed the remaining species of the genus *Plectranthus* (including the species that used to be called Coleus) and the genera Anisochilus and *Pycnostachys,* is the most recognised one (Paton et al., 2013). Paton et al. (2009; 2013) investigations on how to differentiate these two clades stated that *Plectranthus* species in the *Plectranthus* clade have more or less equal posterior and anterior corolla lobes and calyces with a centrally fixed pedicel (Paton et al., 2018). While those in the *Coleus* clade possess a wider anterior and shortened posterior corolla lip and calyces with a pedicel connecting the opposite posterior lip of the calyx (Paton et al., 2018).

3.3 Botanical identification

3.3.1 General description

Plectranthus species are aromatic, annual or perennial shrub, well-branched herbs with different and attractive foliage and flowers like *P. argentatus, P. oertendali,* and *P. madagascariensis* (Figure 3.1, Rabe & Staden, 1998)*.*

| *A* | *B* | *C* |
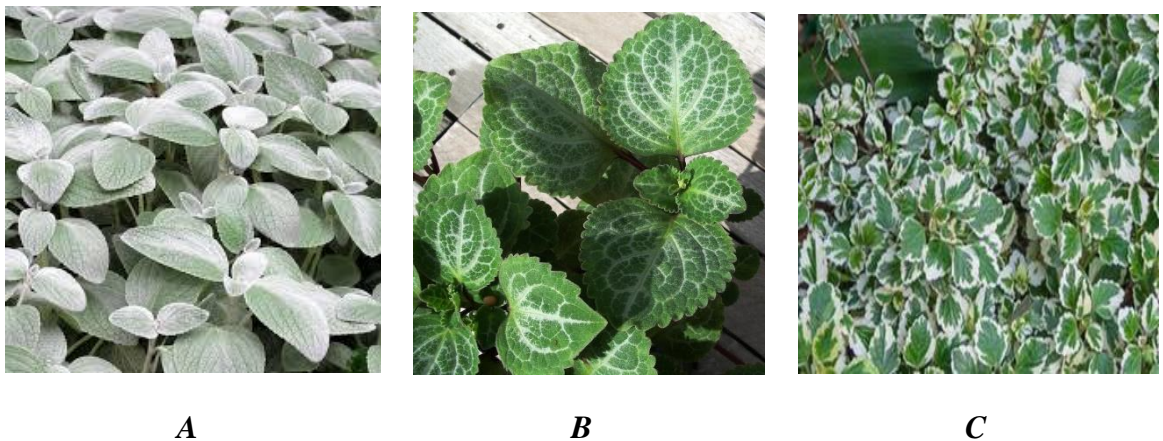
Figure 3.1: Leaves of *P. argentatus* (**A**), *P. oertendali* (**B**) and *P. madagascariensis* (**C**)

These evergreen herbaceous species are approximately 100 cm high and have opposite, oval leaves of length 4 cm and with 3-7 pairs of rounded teeth that are slightly hairy to the touch (Harrower, 2014). The roots of *Plectranthus* species are thick, tuberous, spindle-shaped, conical, and strongly aromatic (Soni & Singhai, 2012). The tubular flowers of 7-18 mm in length are either pale lavender, white, or light yellow. The inflorescence is a terminal, erect raceme, composed of 4 -6 flowers at each node (Harrower, 2014). The calyx is 2-4 mm long, elongating to 5 mm after the flower drops and holds the smooth, roundish, flattened, pale-brown seeds (nutlets) of 0.5-0.7 mm in diameter (Harrower, 2014). The flowers grow from February to May in Southern Africa. These botanical descriptions are common to the seventeen *Plectranthus* species that are endemics to Southern Africa regions. The differences between them lie in their foliage, size and local names. Thus, a

more detailed description of the research study plant *P. madagascariensis* is done in the next section.

3.3.2 Plectranthus madagascariensis

P. madagascariensis is commonly known as a thicket spurflower, candle plant, and Madagascar spurflower (Harrower, 2014). Locally the plant is named muishondblaar in Afrikaans and iboza lehlathi or ilozane in isiZulu (Harrower, 2014). The Madagascar spurflower is a hardy, small (54 cm) and fast-growing groundcover perennial herb. The plant has variegated evergreen leaves with whitish tooth edges. The creamy-white flowers appear from February to May. The general characteristics of *Plectranthus* species detailed in section 3.3.1 are also applied to *P. madagascariensis.*

3.4   Phytochemistry

Species of the genus *Plectranthus* are recognized as ornamental and medicinal plants with economic importance since they are rich sources of essential oil and diterpenoids with broad applications in pharmaceutical and cosmeceutical industries (Khan et al., 2016). The phytochemical analysis of *Plectranthus* constituents demonstrated the presence of many abietanes (**7-66**), labdanes (**67-99**), *ent*-kaurane (**100-120**), phyllocladane (**121-127**), neoclerodane (**128-135**), halimane (**136**), pimarane (**137-138**), and beyerene (**139-141**) diterpenoids (Rijo et al., 2007: 215-221). The diterpenoids isolated from the Southern African *Plectranthus species* are tabulated in Table 3.1 to 3.15.

3.4.1   Abietane diterpenoids isolated from Southern African *Plectranthus* species

In phytochemical studies, the highly oxidized abietane diterpenoids isolated from *Plectranthus* aerial parts, stem or roots are classified as royleanones (**7-18**), spirocoleons (**19-30**), acylhydroquinones (**31-35**), quinone methides (**36-43**), vinylogous quinones (**44-51**), dimeric abietanes (**52-58**), phenolics abietanes (**59-63**), seco abietanes (**64** and **65**), and dioxin quinone abietanes (**66** and **67**).

The paraquinone abietane, also known as royleanones are abundantly found in Southern Africa *Plectranthus* species. Furthermore, most of the royleanones isolated from Southern African *Plectranthus* species are 6,7 unsaturated compounds (**7-16,** and **18**). However, some of them are classified as 6,7 saturated molecules like compound **17** or 6,7-unsaturated compounds with external double bonds and some rearrangements like compounds **18** and **12,** which are also categorized as nor-abietane and bisabeo-abietane diterpenoids respectively (Figure 3.2, Table 3.1).

Table 3.1: Royleanones isolated from Southern Africa Plectranthus species

Plants	Isolated compounds	References
Common to *Plectranthus* genus	7α-acetoxy-6β-hydroxy-7α-acetoxy-royleanone (**7**) Horminone (**8**)	Teixeira et al., 1997; Yulianto et al., 2016.
P. herereonsis	6β-hydroxyroyleanone (**9**) 16-acetoxy-7α, 12-dihydroxy-8, 12-abietadiene-11, 14-dione (**10**) 16-acetoxyhorminone (**11**) 3β-acetoxy-6β,7α, 12-trihydroxy-17(15→16) 18(4→3)-bisabeo-abieta-4(19),8,12,16-tetraene-11,14-dione (**12**)	Batista et al., 1995; Batista et al., 1996.
P. madagascariensis	6β,7α-dihydroxyroyleanone (**13**) 6β,7β-dihydroxyroyleanone (**14**) 7β-acetoxy-6β-hydroxyroyleanone (**15**) 7α-formyloxy-6β-hydroxyroyleanone (**16**) Coleon U quinone (**17**)	Kubínová et al., 2014; Diogo et al., 2019.
P. barbatus	Plectranthone J (**18**)	Alasbahi & Melzig, 2010.

	R₁	R₂
7	β-OH	α-OAc
8	H	β-OH
9	β-OH	H
13	β-OH	α-OH
14	β-OH	β-OH
15	β-OH	β-OAc
16	β-OH	α-CHO

10 R: CH₃
11 R: Ac

12

18   **17**
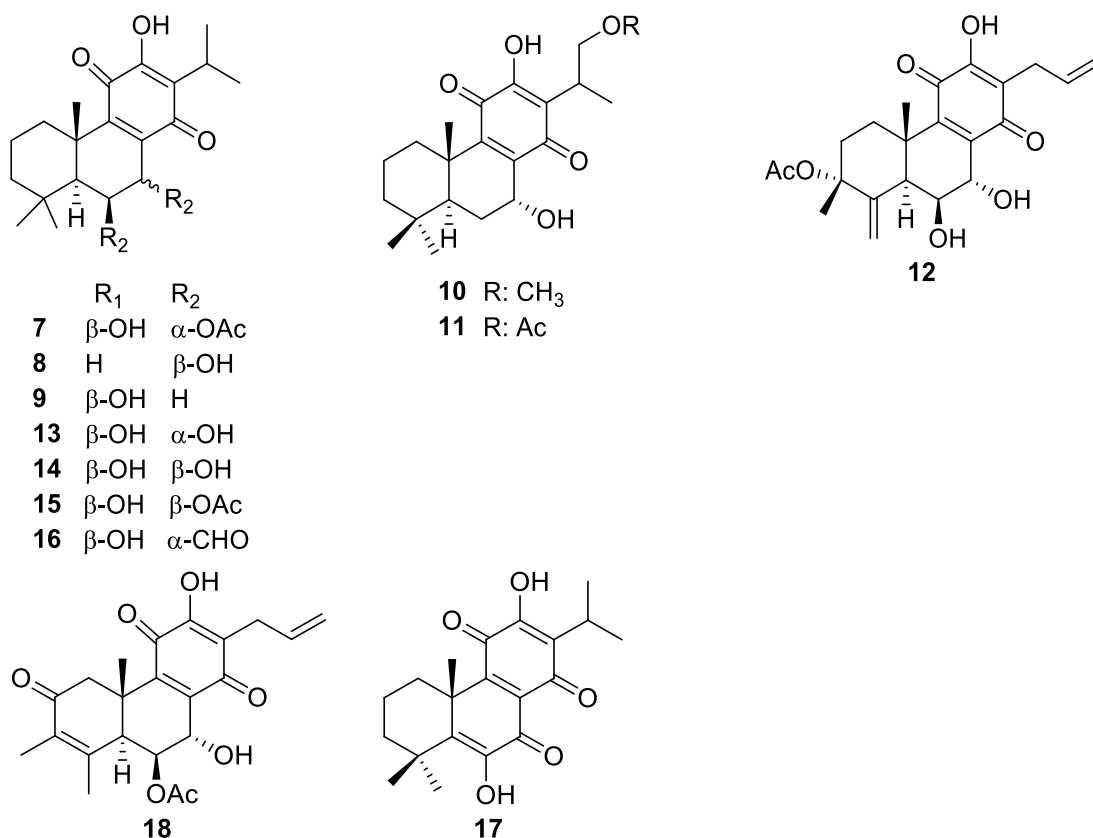
Figure 3.2: Structures of compounds **7-18**

P. caninus and *P. barbatus* are the primary sources of spirocoleons from Southern Africa *Plectranthus* species as they account for ten out the twelve isolated compounds with compound **21** being common to both plants. *P. ambinoicus*, P. *ornatus*, and *P. porcatus* yielded one compound each. The isolated spirocoleons are characterized by a cyclopropyl ring at C-13 linked to a cyclohexene quinone or cyclohexane ring, as shown in Figure 3.3. Moreover, the alkyl, hydroxyl or acetate group attached at C-3, C-6, C-7, and C-12 of the isolated spirocoleons are either in α or β position (Table 3.2, Figure 3.3).

Table 3.2: Spirocoleons isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. ambinoicus *P. ornatus*	3β-hydroxy-3-deoxybarbatusin (**19**)	Silva et al., 2017.
P. barbatus	Coleon O (**20**) Coleon T (**21**) Cyclobutatusin (**22**) 7β-acetyl-12-deacetoxycyclobutatusin (**23**) Plectrin (**24**)	Alasbahi & Melzig, 2010.
P. caninus	7,12-diacetylcoleon J (**25**) Coleon M (**26**) Coleon N (**27**) Coleon R (**28**) Coleon T (**21**) Barbatusin (**29**)	Arihara et al., 1977; Arihara et al., 1983.
P. porcatus	(13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy- 13β,16-cyclo-8-abietene-11,14-dione (**30**)	Simões et al., 2010.
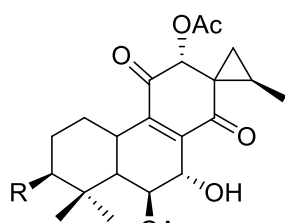
	R:			
19	R: OH
28	R: OAc
29	R: =O
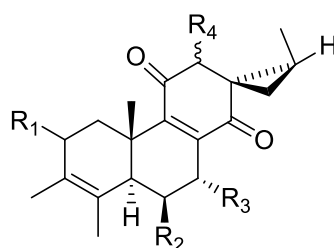
	R₁	R₂	R₃	R₄
20	H	OH	OAc	α-OH
21	H	OH	OH	β-OH
24	=O	OAc	OH	α-OH
26	H	OH	OAc	α-OAc

	R₁	R₂
22	α-OH	α-OAc
23	β-OAc	β-OH

25 α-CH₃
27 β-CH₃
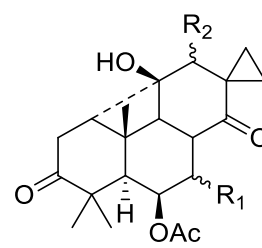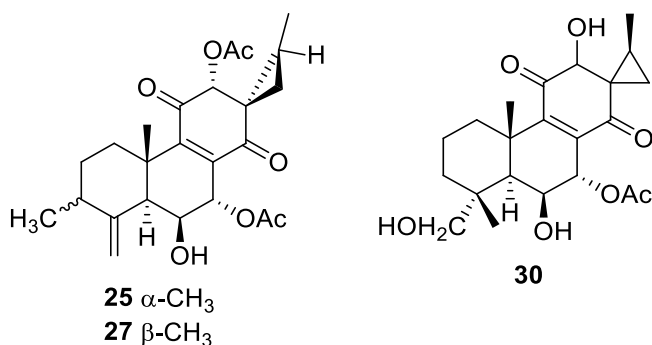
Figure 3.3: Structures of compounds **19**-**30**.

About five acylhydroquinones abietanes (**31**-**35**) were isolated from the Southern Africa *Plectranthus* specie *P. barbatus.* The isolated compounds are bearing one to two double bonds on C-3 and C-4 or C-5 and C-6 or both, in addition to the trihydroxy-benzene ring and the carbonyl group at C-7 as shown in Table 3.3 and Figure 3.4.

Table 3.3: Acylhydroquinones isolated from Southern Africa *Plectranthus* species

Plant	Isolated compounds	References
P. barbatus	(16*R*)-Plectrinon A (**31**) Plectrinon B (**32**) Coleon C (**33**) Coleon S (**34**) 14-Deoxycoleon U (**35**)	Alasbahi & Melzig, 2010.
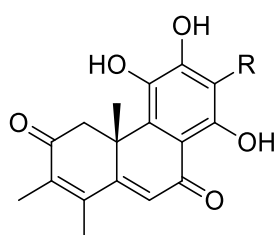
31 R: (*R*)-CH₂CH(OH)CH₃
32 R: CH₂CHCH₂
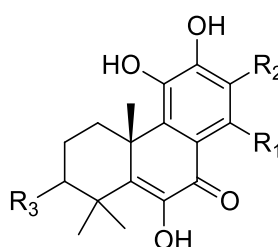
	R₁	R₂	R₃
33	OH	CH(CH₃)CH₂OH	H
34	OH	CH(CH₃)₂	OH
35	H	CH(CH₃)₂	H

Figure 3.4: Structures of compounds **31**-**35.**

Ten quinone methides abietanes (Table 3.4, Figure 3.5) were isolated from *P. ambigus, P. eckloni,* and *P. strigosus,* with eight of them isolated from *P. strigosus.* Compounds **36**-**43** are bearing a p-quinone methide with a hydroxy group at C-11 and a cyclohexadiene ring corresponding to ring B. Compounds **36**-**43** have their substituents attached to C-1 (**39**, **41**

and **42**) or C-18 (**37**, **38** and **40**) and sometimes to C-1 and C-18 like compounds **37** and **43**.

Table 3.4: Quinone methides abietanes isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. ambigus *P. strigosus*	Parviflorone A (**36**)	Liu & Ruedi, 1995. Alder et al., 1984.
P. strigosus	Parviflorone B (**37**) Parviflorone C (**38**) Parviflorone D (**39**) Parviflorone E (**40**) Parviflorone F (**41**) Parviflorone G (**42**) Parviflorone H (**43**)	Alder et al., 1984; Gaspar-Marques et al., 2008.
P. eckloni *P. strigosus*	Parviflorone D (**39**) Parviflorone F (**41**)	Uchida et al., 1981; Van Zyl et al., 2008
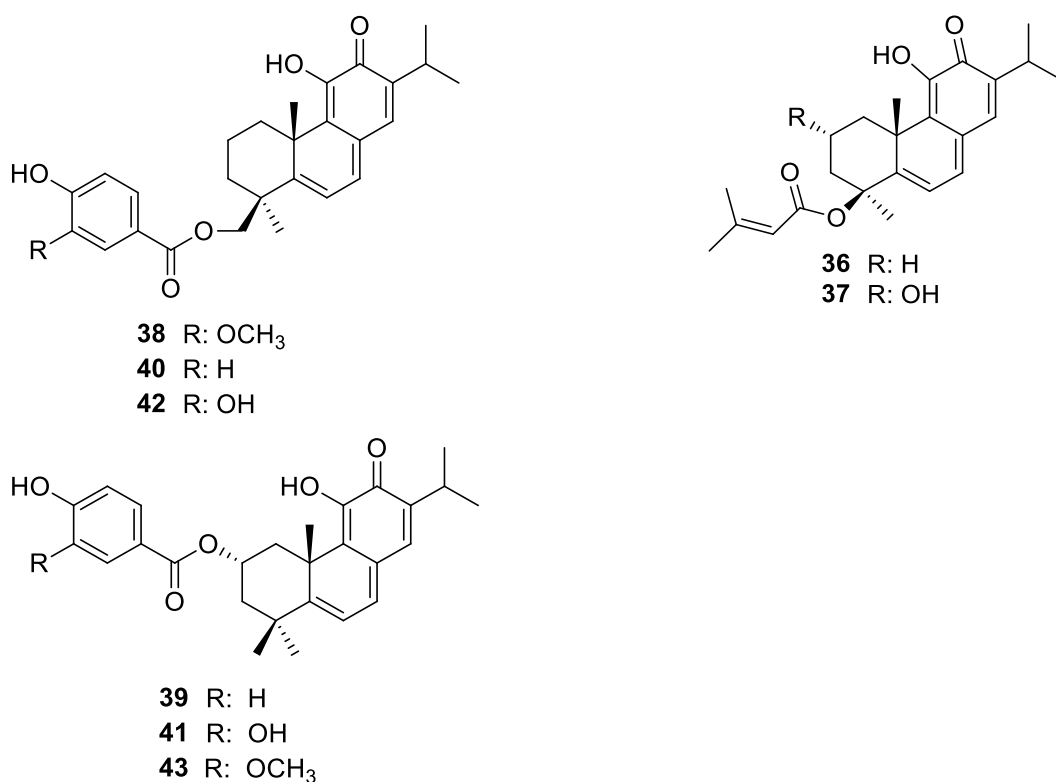
Figure 3.5: Structures of compounds **36-43**

A total of eight vinylogous quinone abietane (Table 3.5, Figure 3.6) were isolated from *P. grandidentatus* (**44-51**), two from *P. elegans* (**42** and **43**) and one each from *P. barbatus*

(**39**), *P. caninus* (**40**), and *P. ambinoicus* (**41**). The main differences between compounds **44**-**51** hinge on the substituents attached to C-6, C-13, and C14. For example, compound **48** has an oxolane group attached at C-13 and C-14, whereas compound **50** has an isopropyl group at C-13 and a hydroxy group at C-14. Compounds **44** and **45** showed similarity, except that compound **44** has a carbonyl group at C-6, while compound **45** has a hydroxy group attached to the quaternary carbon at position 6. Compounds **47** and **49** are the only compounds with one methyl group at C-4.

Table 3.5: Vinylogous quinone abietanes isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. grandidentatus	Coleon V (**44**) Coleon U (**45**) 14-O-acetyl-coleon U (**46**)	Rijo et al., 2007.
P. barbatus	Coleon F (**47**)	Alasbahi & Melzig, 2010.
P. caninus	Coleon P (**48**)	Arihara et al., 1983.
P. ambinoicus	(16S)-coleon E (**49**)	Silva et al., 2017.
P. elegans	11-hydroxy-12-oxo-7,9 (11),13-abietatriene (**50**) 14-hydroxytaxodione (**51**)	Dellar et al., 1996.
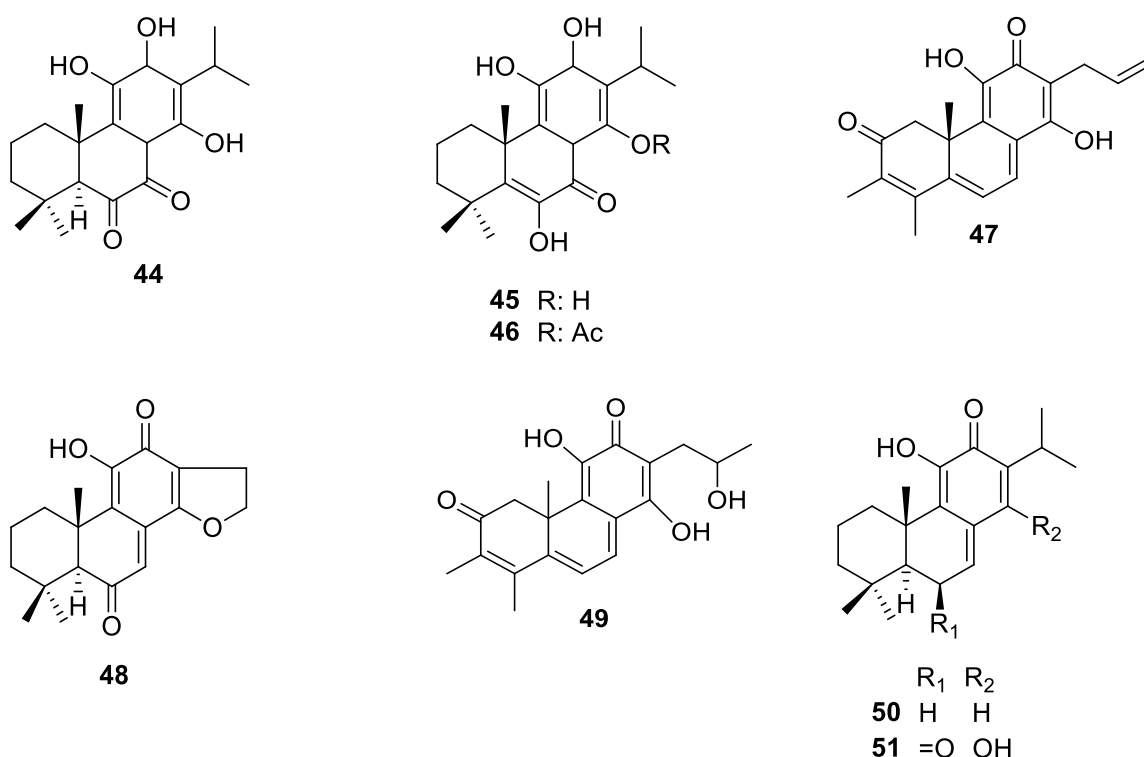
Figure 3.6: Structures of compounds **44**-**51**

The seven royleanone type abietane dimers (**52-58**) isolated from *P. grandidentatus* consist of linking two abietanes either by carbon atoms (**54**) or oxygen atoms (**52**, **53**, **55**, **56**, **57** and 59). The two molecules are linked from C-7 to C-7' (**54**), or C-7 to C11' and C-12' (**52**, **53**, **56** and **57**) or C-7 to C-14' (**55** and **58**). Additionally, compounds **56**, **57,** and **58** are respectively epimers of compounds **52**, **53**, and **55** as shown in Table 3.6 and Figure 3.7.

Table 3.6: Dimers isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. grandidentatus	Grandidone A (**52**)	Teixeira et al., 1997.
	Grandidone B (**53**)	
	Grandidone C (**54**)	
	Grandidone D (**55**)	
	7-epigrandidone A (**56**)	
	7-epigrandidone B (**57**)	
	7-epigrandidone D (**58**)	

52 β-C(7)-O-C(11')
56 β-C(7)-O-C(12')

53 β-C(7)-O-C(11')
57 β-C(7)-O-C(12')
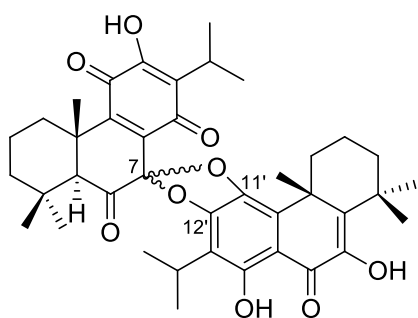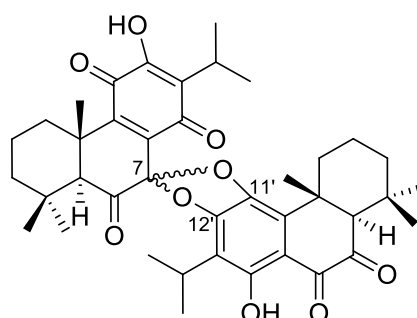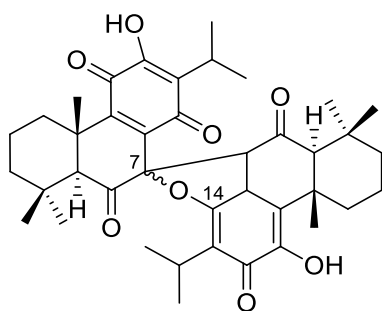
55 β-C(7)-O-C(14)
58 β-C(7)-O-C(14)
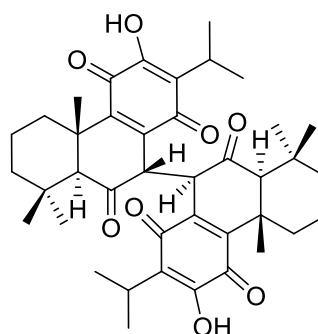
54

Figure 3.7: Structures of compounds **52-58**

Six phenolic abietanes (**59-63**) were isolated from *P. elegans* (**59**) and *P. barbatus* (**60-63**). The abietatriene **59** bears a hydroxy group at C-6 and C-11 as well as a methoxy group at C-12. The difference between compounds **61** and **62** lies in the substitution at C-7 while the dissimilarity between the abietatriene bearing the 6, 20 epoxy group (**62** and **63**) lies on their substituents at C-6 and C-20 (Table 3.7, Figure 3.8).

Table 3.7: Phenolic abietane isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. elegans	7α,11-dihydroxy-12-methoxy-8,11,13-abietatriene (**59**)	Dellar et al., 1996
P. barbatus	Ferruginol (**60**) Sugiol (**61**) 20-deoxocarnosol (**62**) 6β-hydroxycarnosol (**63**)	Alasbahi & Melzig, 2010.
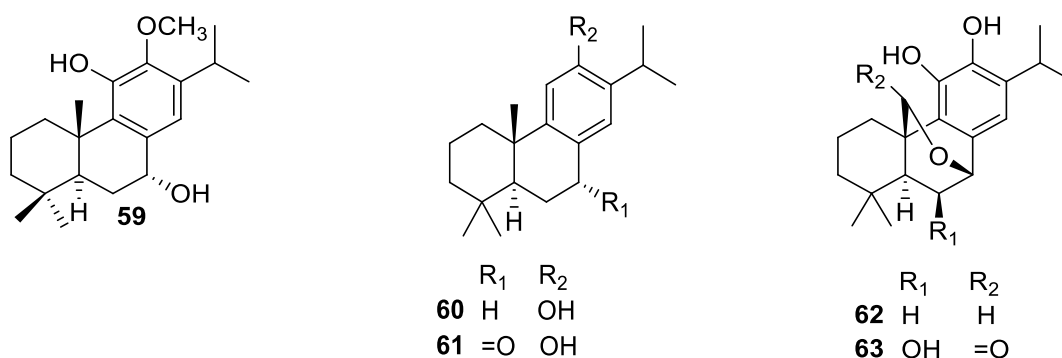
Figure 3.8: Structures of compounds **59-63**

From *P. barbatus,* two 6,7 seco-abietane diterpenoids (**64** and **65**) were isolated, as shown in Table 3.8 and Figure 3.9.

Table 3.8: Seco-abietane isolated from Southern Africa *Plectranthus* species

Plant	Isolated compounds	Reference
P. barbatus	6,7-secoabietane diterpene I (**64**) 6,7-secoabietane diterpene II (**65**)	Alasbahi & Melzig, 2010.
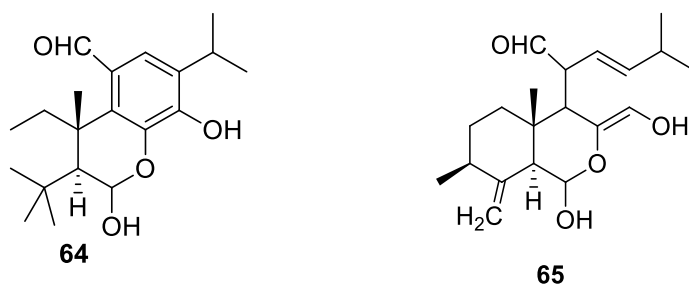
Figure 3.9: Structures of compounds **64** and **65**

Compounds **66** and **67** isolated from *P. ecklonii* are referred as dioxin quinone abietanes. Both compounds have a dibenzo-p-dioxin quinone ring, as well as an isopropanoate group at C-2, and alkyl groups at C1 and C-4 as substituents.

Table 3.9: Dioxin quinone abietanes isolated from Southern Africa Plectranthus species

Plants	Isolated compounds	References
P. ecklonii	Ecklonoquinone A (**66**)	Uchida et al., 1980.
	Ecklonoquinone B (**67**)	

	R$_1$	R$_2$
66	CH(CH$_3$)$_2$	CH$_3$
67	CH$_3$	CH(CH$_3$)$_2$
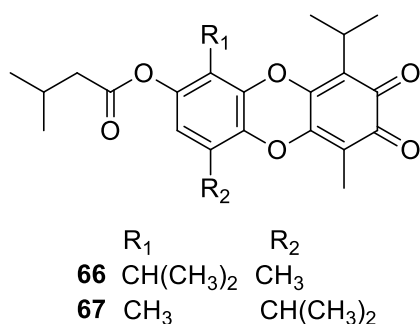
Figure 3.10: structures of compounds **66** and **67**

3.4.2 Labdane diterpenoids isolated from Southern Africa *Plectranthus* species

Thirty-two labdane diterpenoids (Table 3.10, Figure 3. 11) were isolated from four Southern Africa *Plectranthus* species, namely *P. barbatus* (**68-87**), *P. ernstii* (**70**), *P. fruticosus* (**73-79**) and *P. ornatus* (**117-120**). Compounds **68-87**, as well as compounds **118** and **119**, have a δ-lactone group at C-8 and C-9 with an acetyl or a hydroxyl or an alkyl group at C-1, C-6, C-7, and C-9. The dissimilarity between these compounds lies in their stereochemistry, which is either *R* or *S*. Compounds **73-79** are differentiated by the orientation (*Z* or *E*) of the 3-methylpenta-1.3-diene at C-12. Compounds **117** and **120** are labdanes with a carboxylic group at C-15 and an olefinic group at C-13 and C-14.

Table 3.10: Labdanes isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. barbatus	Forskolin (**68**)	Alasbahi & Melzig, 2010.
	9-deoxyforskolin, (**69**)	
	1,9-dideoxyforskolin (**70**)	
	1,9 dideoxy-7-deacetylforskolin (**71**)	
	Deacetyl-1-deoxyforskolin (**72**)	
	6-acetyl-1-deoxyforskolin (**73**)	
	6-acetyl-1,9-dideoxyforskolin (**74**)	
	1,6-di-O-acetyl-forskolin (**75**)	
	Isoforskolin (**76**)	
	1,9-dideoxycoleonol B (**77**)	
	7-deacetyl-forskolin (**78**)	
	Forskolin E (**79**)	
	Forskolin F (**80**)	
	Forskolin G (**81**)	
	Forskolin H (**82**)	
	Forskolin I (**83**)	
	Forskolin J (**84**)	
	Forskolin K (**85**)	
	Forskolin L (**86**)	
	Coleosol (**87**)	
P. ernstii	$1R,11S$-dihydroxy-$8R,13R$-epoxylabd-14-ene (**88**)	Stavri et al., 2009.
P. fruticosus	*Ent*-2α-acetoxylabda-8(17),12*Z*,14-trien-3β-ol (**89**)	Gaspar-Marques et al., 2003; Gaspar-Marques et al., 2004.
	Ent-labda-8(17),12*Z*,14-trien-2α-ol (**90**)	
	3β-acetoxylabda-8(17),12*E*,14-trien-2α-ol (**91**)	
	Ent-2α-acetoxylabda-8(17),12*Z*,14-triene (**92**)	
	Ent-labda-8(17),12*Z*,14-triene-$2\alpha,3\beta$-diol (**93**)	

	Ent-3β-acetoxylabda-8(17),12*Z*,14-trien-2α-ol (**94**) *Ent*-labda-8(17),12*Z*,14-triene-2α,3β-dibenzoate (**95**)	
P. ornatus	Labd-13-en-8β-hydroxy-15-oic acid (**96**) Plectrornatin B (**97**) Plectrornatin C (**98**) Rhinocerotinoic acid (**99**)	Oliveira et al., 2005; Rijo et al., 2002; Rijo et al., 2007.
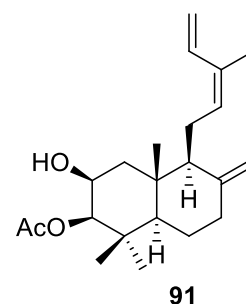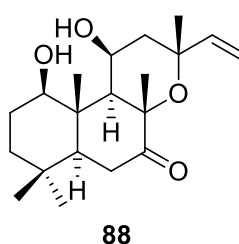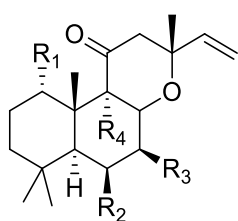
	R$_1$	R$_2$	R$_3$	R$_4$
68	OH	OH	OAc	OH
69	OH	OH	OAc	H
70	H	OH	OAc	H
71	H	OH	OH	H
72	H	OH	OH	OH
73	H	OAc	OAc	OH
74	H	OAc	OAc	H
75	OAc	OAc	OAc	OH
76	OAc	OH	OAc	OH
77	H	OAc	OH	H
78	OH	OH	OH	OH
79	OAc	OH	OAc	H
80	H	OH	OAc	OH
81	OH	OAc	OAc	H
82	OAc	OAc	H	H
83	OAc	OAc	OH	OH
84	OH	OAc	OAc	OH
85	OAc	OAc	OAc	H
86	H	OH	H	H
87	H	OH	H	OH
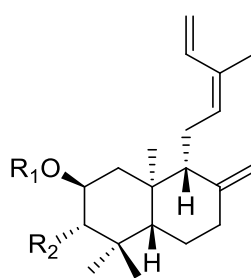
	R$_1$	R$_2$
89	Ac	OH
90	H	H
92	Ac	H
93	H	OH
94	H	OAc
95	COC$_6$H$_5$	COC$_6$H$_5$

97 R: H
98 R: Ac
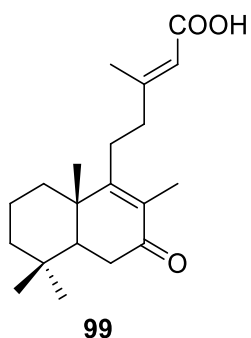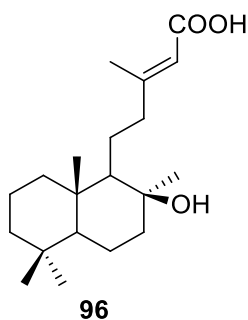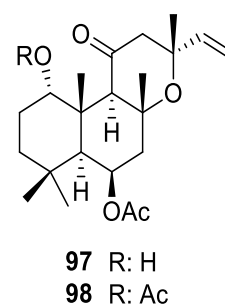
Figure 3.11: Structures of compounds **68-99**

3.4.3 Kaurane diterpenoids isolated from Southern Africa *Plectranthus* species

Twenty-one *ent*-kauranes diterpenes are isolated from *P. fructicosus* (**100**-**116**) and *P. strigosus* (**117**-**120**). The differences between these compounds lie on the substituents and functional group attached to the carbons at positions 15 and 16.

Table 3.11: Ent-kauranes isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	References
P. fruticosus	*Ent*-15*β*,16*β*-epoxykauran-19-oic acid (**100**)	Gaspar-Marques et al., 2003; Gaspar-Marques et al., 2004.
	Ent-15*β*,16*β*-epoxykauran-19-ol (**101**)	
	Ent-12*β*-acetoxy-16-kauren-19-oic acid (**102)**	
	Ent-12*β*-acetoxy-15-kauren-19-oic acid (**103**)	
	Methyl *ent*-12*β*-acetoxy-16-kauren-19-oate (**104**)	
	Methyl *ent*-12*β*-acetoxy-15-kauren-19-oate (**105**)	
	Methyl *ent*-15*β*,16*β*-epoxykauran-19-oate (**106**)	
	Ent-12*β*-acetoxy-15*β*-hydroxykaur-16-en-19-oic acid (**107**)	
	Ent-7*β*-hydroxykaur-15-en-19-oic acid (**108**)	
	Ent-12*β*-acetoxy-17-oxokaur-15-en-19-oic acid (**109**)	
	Ent-12*β*-acetoxy-7*β*-hydroxykaur-16-en-19-oic acid (**110**)	
	Ent-7*β*-Hydroxy-15*β*,16*β*-epoxykauran-19-oic acid (**111**)	
	Ethyl *ent*-12*β*-hydroxykaur-16-en-19-oate (**112**)	
	Methyl-*ent*-12*β*-acetoxy-7*β*-hydroxykaur-15-en-19-oate (**113**)	
	Methyl-*ent*-12*β*-acetoxy-15*β*,16*β*-epoxykauran-19-oate (**114**)	
	Methyl-*ent*-12*β*-acetoxy-15*β*-hydroxykaur-16-en-19-oate (**115**)	
	Methyl *ent*-12*β*-acetoxy-17-oxokaur-15-en-19-oate (**116**)	
P. strigosus	*Ent*-16-kauren-19-ol (**117**)	Gaspar-Marques et al., 2008.
	Ent-16-kauren-19-oic acid (**118**)	
	Xylopic acid (**119**)	
	Xylopinic acid (**120**)	

	R_1	R_2	R_3	R_4
103	H	H	OAc	CH_3
105	CH_3	H	OAc	CH_3
108	H	OH	H	CH_3
109	H	H	OAc	CHO
113	CH_3	OH	OAc	CH_3
116	CH_3	H	OAc	CHO

	R_1	R_2	R_3
100	COOH	H	H
101	CH_2OH	H	H
106	$COOCH_3$	H	H
111	COOH	OH	H
114	$COOCH_3$	H	OAc

	R_1	R_2	R_3	R_4
102	H	H	OAc	H
104	CH_3	H	OAc	H
107	H	H	OAc	OH
110	H	OH	OAc	H
112	CH_3	H	H	H
115	CH_3	H	OAc	OH

	R_1	R_2	R_3
117	H	H	CH_2OH
118	H	H	COOH
119	H	OAc	COOH
120	OAc	=O	COOH

Figure 3.12: Structures of compounds **100-120**

Seven phyllocladanes (13β-kauranes) were isolated from *P. ambigus* (Table 3.12, Figure 3.13). Compounds **121-125** have esters group at C-2 and C-3 and sometimes at C-16 like compounds **122** and **124**. The only difference between compounds **126** and **127** is oriented to the substituent at C-2, which is an isobutanoate for compound **126** and a CH_2 for compound **127**.

Table 3.12: Phyllocladanes isolated from Plectranthus species

Plants	Isolated compounds	References
P. ambigus	(16*R*)-2α-(3-methyl-2-butenoyloxy)-3α- acetoxy-phyllocladane-16,17-diol (**121**)	Liu & Ruedi, 1996: 1563-1568.
	(16*R*)-2α-(3-methyl-2-butenoyloxy)-3α,17-diacetoxy-16-hydroxyphyllocladane (**122**)	
	(16*R*)-2α-(3-methylbutanoyloxy)-3α-acetoxy-phyllocladane-16,17-diol (**123**)	

	(16R)-2α-(3-methylbutanoyloxy)-3β,17-diacetoxy-16-hydroxyphyllocladane (**124**)	
	(16R)-3α-acetoxyphyllocladane-16,17-diol (**125**)	
	(16R)-2α-(3-methyl-2-butenoyloxy)-16,17-dihydroxyphyllo-cladane-3-one (**126**)	
	Calliterpenone (**127**)	

	R₁	R₂	R₃
121	OCOCHC(CH₃)₂	OAc	H
122	OCOCHC(CH₃)₂	OAc	Ac
123	OCOCHC(CH₃)₂	OAc	H
124	OCOCHC(CH₃)₂	OAc	Ac
125	H	OAc	H
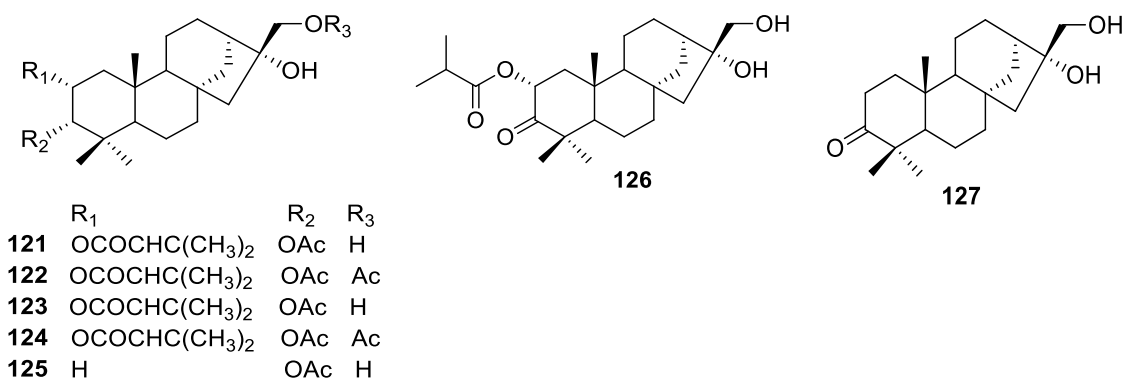
Figure 3.13: Structures of compounds **121**-**127**

3.4.4   Clerodanes and halimane isolated from Southern Africa *Plectranthus* species

About Eight neoclerodane diterpenoids (**128**-**135**) and a halimane (**136**) diterpenoids were isolated from *P. ornatus* (Table 3.13, Figure 3.14). Compounds **128**, **130**, and **133** have the decalin skeleton while compounds **129**, **131**, and **135** have a cyclohexene skeleton as a ring A and cyclohexane as ring B. compound **132** and **134** are similar except that compound **132** has a cyclopentane skeleton as ring A whereas compound **134** has cyclopentene group as ring A.

Table 3.13: Clerodanes and halimanes isolated from Southern Africa *Plectranthus* species

Plants	Type of diterpenes	Isolated compounds	References
P. ornatus	Neocleraodanes	11*R*-acetoxy-3β-hydroxycleroda-4(18), 13*E*-dien-15-oic acid (**128**)	Oliveira et al., 2005; Ávila et al., 2017.
		11-acetoxy-2-oxo-ent-cleroda-3,13*E*-dien-15-oic acid (**129**)	
		Ent-3α-hydroxycleroda-4(18),13*E*-dien-15-oic acid (**130**)	
		Ornatins A (**131**)	
		Ornatins B (**132**)	
		Ornatins C (**133**)	
		Ornatins D (**134**)	

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		Ornatins E (**135**)	
	Halimane	11*R*-acetoxyhalima-5,13*E*-dien-15-oic acid (**136**)	Rijo et al., 2007.
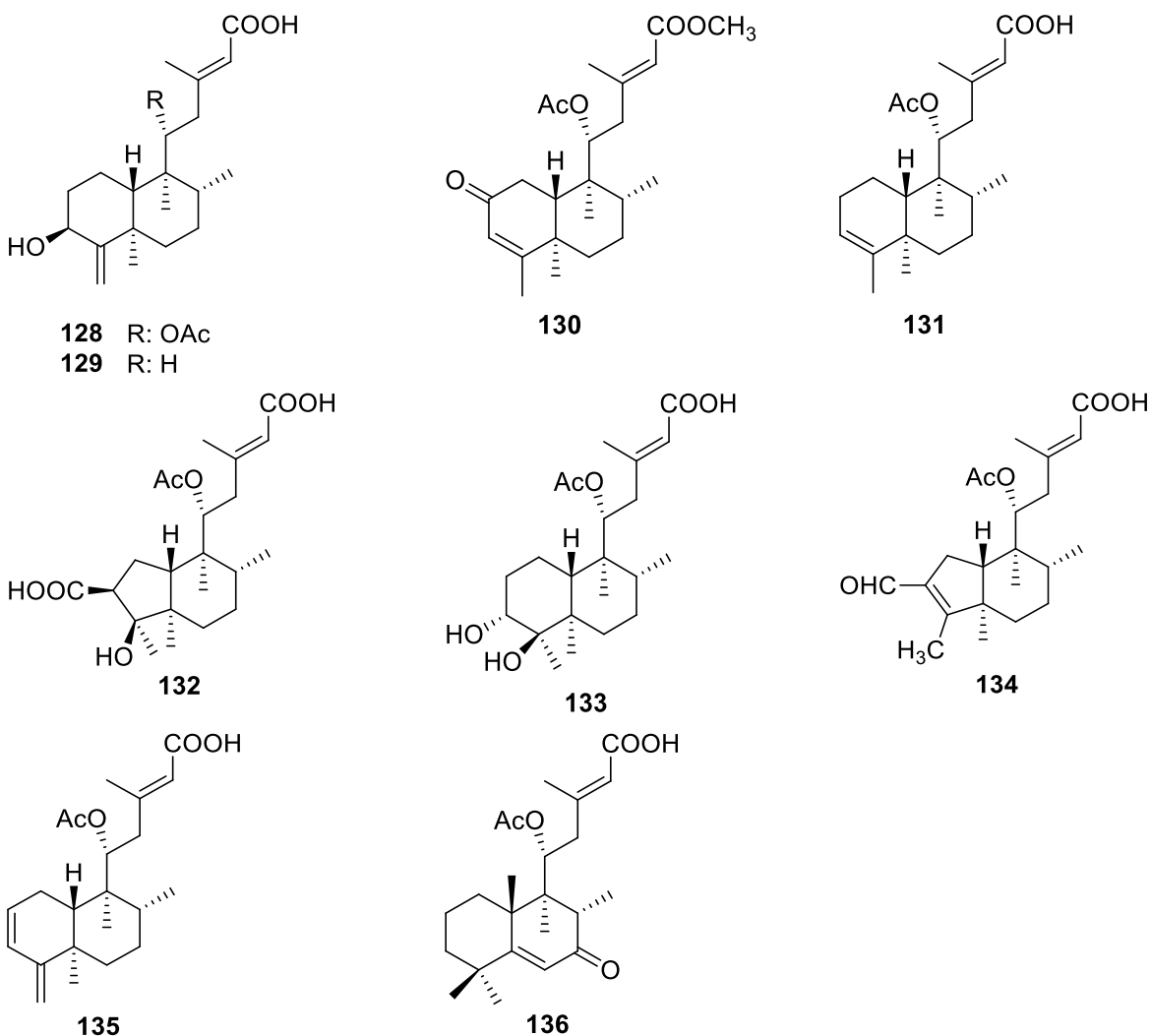
Figure 3.14: Structures of compounds **128**-**136**

3.4.5   Pimaranes and beyranes isolated from Southern Africa *Plectranthus* species

The pimarane diterpenoids **137** and **138** isolated from *P. ernstii* have an oxirane group attached to cyclohexene ring. Compound **137** has a hydroxy group at C-7, whereas compound **138** has a carbonyl group at that position (Table 3.14, Figure 3.15).

Table 3.14: Pimaranes isolated from Southern Africa *Plectranthus* species

Plants	Isolated compounds	Reference
P. ernstii	15(ζ),16-epoxy-7α-hydroxypimar-8,14-ene (**137**) -15(ζ),16-epoxy-7-oxopimar-8,14-ene (**138**)	Stavri et al., 2009.
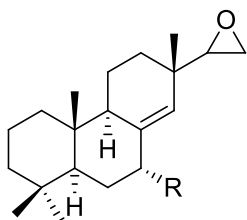
137 R: OH
138 R: =O

Figure 3.15: Structures of compounds **137** and **138**

Three beyerane diterpenoids were isolated from *P. saccatus.* The compounds **139** and **140** have an ester group at C-3, and a carboxyl group at C-19, whereas compound **141** has a methanol group at C-18 (Table 3.15, Figure 3.16).

Table 3.15: Beyeranes isolated from Southern Africa species

Plants	Isolated compounds	References
P. saccatus	*Ent*-3β-(3-methyl-2-butenoyl)-oxy-15-beyeren-19-oic acid (**139**) *Ent*-3β-(3-methyl-butanoyl)-oxy-15-beyeren-19-oic acid (**140**) *Ent*-7α-acetoxy-15-beyeren-18-oic acid (**141**)	Wellsow et al., 2006; Simões et al., 2010.
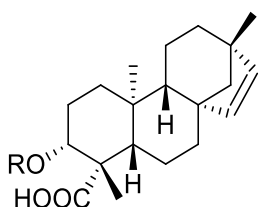
139 R: COCHC(CH₃)₂
140 R: COCH₂CH(CH₃)₂
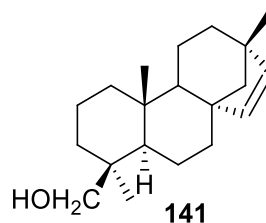
Figure 3.16: Structures of compounds **139-141**

3.4.6 Essential oils

The genus *Plectranthus* is abundant in essential oils mainly made of monoterpenes and sesquiterpenes. Such volatile components are responsible for the herbaceous *Plectranthus* aromatic as well as therapeutic properties. For example, essential oils extracted from *Plectranthus* species are used in the making of perfume, shampoos, cream, soap, and deodorant due to their firm, pleasant and long-lasting smell, along with their antimicrobial, antioxidant and nematocidal properties (Ali et al., 2012; Marwah et al., 2007). The phytochemical analysis of *Plectranthus* essential oils via gas chromatography- flame-ionisation detectors (GC-FID) distinguished and identified the oil's major components or

types of terpenes present in them. Analysis of the essential oil of *P. amboinicus* collected in Crato and Fortaleza by Santos et al. (2016) showed that the composition of the plant oil differs from one region to another due to climate, type of soil, and the surrounding environment. For instance, the essential oil of *P. amboinicus* from Crato was mainly composed of germacrene (38.60%), *E*-caryophyllene (18.91%), and copaene (8.03%) whereas, the essential oil from the one collected in Fortaleza was rich in carvacrol (90.55%) and *β*-caryophyllene (3.09%).

Ascensao et al. (1998) investigations on *P. madagascariensis* essential oils extracted via distillation extraction and hydrodistillation from flowers and leaves revealed the presence of the orange-to-reddish crystalline diterpenoid 6,7-dehydroroyleanone. On the other hand, the essential oil of *P. fruticosus* is rich in sabinyl acetate (>50%), sabinene (20.28%) and bornyl acetate (12.81%) (Pages et al., 1991; Maistry, 2007).

Waldia et al. (2011) reviewed the volatile components and reported that *α*-pinene, camphene, terpinen-4-ol, *α*-cubebene, caryophyllene, germacrene D, *γ*-cadinene, thymol, ethyl salicylate, neral, chavicol, eugemol, *β*–phellandrene, geranyl acetate, farnesene, 1,8-cineole, isobornyl acetate, (*Z*)- *β*–ocimene, carvacrol acetate, and piperitone epoxide are also present in other *Plectranthus* species. However, their abundance or presence in a plant depends on the genus plant's flowering and vegetative phases, as well as the parts harvested and provenance.

3.5   Ethnobotanical uses of Southern African *Plectranthus* species

A review on the ethnobotanical applications of the genus *Plectranthus* revealed that the stems, leaves, roots, and tubers of the *Plectranthus* species are used in folklore medicine in the treatment of wounds, skin infections, gastric disorders, infections and fever, genito-urinary conditions, muscular-skeletal conditions and pain as summarized in Table 3.18 (Maree et al., 2014; Lukhoba et al., 2006). The gastric disorders are the most common ailments healed by *Plectranthus* species as 21 out of the 62 studied *Plectranthus* species are used to treat gastric disorders (Brito et al., 2018).

Plectranthus madagascariensis is mostly used to treat respiratory and skin diseases, whereas the most widely used species of the genus Plectranthus are *P. barbatus* and *P. amboinicus,* which account for the 68% ethnobotanical usage of this genus. These two species have been used in thirteen categories as described in the Economic Botany Data Collection Standard (Rice et al., 2011; Cook, 1995). *P. laxiflorus* is used in the treatment of ten categories, while other Southern Africa *Plectranthus* species are applied in one to four groups as shown in Table 3.16 (Lukhoba et al., 2006).

Table 3.16: Summary of Southern Africa Plectranthus species' medicinal uses and biological activities.

Species	Ethnomedicinal uses	Pharmacological properties	References
P. ambiguus	Respiratory		Potgieter et al., 1999.
P. amboinicus	Respiratory veterinary	Antimicrobial Antiviral Anti-HIV inhibition Anti-tumour Anti-cancer Anti-expectorant	Rice et al., 2011; Lukhoba et al., 2006 Arumugam et al., 2016; Roshan et al., 2014.
P. barbatus	Digestive Liver complaints Skin Respiratory Infections/fevers Genito-urinary Pain Muscular-Skeletal Circulation and Blood Nervous Sensory Poison treatment Inflammation	Antibacterial Antiviral Antifungal Antiprotozoal Anti-tumour Anti-inflammatory Anti-feedant Anti-candida	Alasbahi & Melzig, 2010; Rice et al., 2011; Lukhoba et al., 2006.
P. caninus	Digestive Respiratory	Anti-tumour	Painuly & Tandon, 1983; Lukhoba et al., 2006.
P. ecklonii	Pain Fever Skin	Anti-bacterial Anti-fungal Anti-fever	Waldia et al., 2008; Rice et al., 2011.
P.elegans	Digestive Respiratory Urinary	Anti-bacterial Anti-fungal Anti-helmintic	Rice et al., 2011; Waldia et al., 2008.
P. esculent	Digestive Pain	Antibacterial	Lukhoba et al., 2006.
P. fruticosus	Skin	Antimicrobial	Gaspar-Marques et al., 2003.
P. hadiensis	Respiratory Digestive Skin Inflammatory	Antioxidant Antiplasmodial Anticancer	Hutchings et al., 1996; Muthukumarana & Dharmadasa, 2014; Laing et al., 2006; Van Zyl et al.,2008.
P. hereroensis	Digestive	Anti-viral Anti-bacterial	Batista et al., 1995.
P. laxiflorus	Digestive Skin Respiratory Infections/Fever Inflammation	Antimicrobial Antioxidant	Hutchings et al., 1996; Rabe & Van Staden, 1998; Lukhoba et al., 2006.

	Genito-urinary Pain Mouthwash Muscular-skeletal Nervous Sensory Veterinary (Cattle)		
P. madagascariensis	Respiratory Skin	Antibacterial Antioxidant	Rabe & Van Staden 1998; Lukhoba et al., 2006.
P. Ornatus	Skin	Antibacterial	Lukhoba et al., 2006.
Saccatus	N/A	Antifeedant	

3.6   Ethnopharmacological and pharmacological uses of Southern African *Plectranthus* species

3.6.1   *Plectranthus amboinicus*

P. amboinicus is a medicinal plant known for its antioxidant, antiepileptic, diuretic, antimutagenic, antimicrobial, cytotoxic, radioprotective, anticlastogenic, anti-tumorigenic, antifeedant and antigenotoxic properties (Arumugam et al., 2016; Kazdan et al., 1981). An *in vivo* investigation done by Chiu et al. (2012) revealed that the plant possessed anti-inflammatory and analgesic properties while Gurgel et al. (2009) demonstrated that besides the anti-inflammatory activity, the plant possessed anti-tumor activity against Sarcoma-180 and Ehrlich ascites carcinoma. The *in vitro* work on this medicinal plant showed its antibacterial, antifungal, antioxidant, and antidandruff activities (Yulianto et al., 2016: 28-34). According to Yulinato et al. (2006), 7-acetoxy-6-hydroxyroyleanone is the compound responsible for the inhibition of MCF-7 cells leading to the cytotoxic activity.

The essential oil extracted from the leaves was reported to have larvicidal inhibitory effect against *Anopheles gambiae* after 48 h (Prudent et al., 1995; Arumugam et al., 2016). Its ethyl acetate extract exhibited vigorous larvicidal activity against *Anopheles stephensi, Culex quinquefasciatus*, and *Aedes aegypti* (Baranitharan & Dhanasekaran, 2014). Whereas, the ethanolic extract showed antibacterial activity against *Proteus mirabilis, Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Proteus mirabilis.* In Cuba, a decoction of the leaves was used to inhibit the growth of *Mycobacterium tuberculosis* activity (Frame et al., 1998). Studies done on some of the *P. amboinicus* crude extracts have shown anti-HIV inhibition activity (Kusumoto et al., 1995) and antiviral activity against Herpes Simplex Virus-1 (Hattori et al.,1995).

3.6.2 Plectranthus barbatus

P. barbatus is the most studied and used *Plectranthus* species in both formal and informal medical system. This edible medicinal plant is used as food supplements, and the main components of its aqueous extracts viz rosmarinic acid, (16*S*)-Coleon E (**49**) and scutellarein 4'-methyl ether 7-*O*-glucuronide impact the crude antioxidant and antiacetylcholinesterase activities (Falé et al., 2009). Compound (**49**) is also known to prevent dental caries by inhibiting the glucosyltransferase activity and reduce biofilm formation of *Streptococcus mutans* and *Streptococcus sobrinus* that are the crucial factors for dental plaque formation or oral diseases (Figueiredo et al., 2010).

In Brazil, it is used as a substitute for *Peumus boldus* to treat gastritis and intestinal spasms as well as hepatic disorders (Alasbahi & Melzig, 2010). For instance, Silva et al. (2017) investigation on 3β-hydroxy-3-deoxybarbatusin (**19**) and barbatusol isolated from *P. barbatus* leaves showed their gastric effect in rats where oral administration (10mg/Kg) reduce the lesions by 96% and 76% respectively.

In Brazil, concoctions of *P. barbatus* leaves are used to treat the unpleasant psychological and physiological effects experienced by people in hangover state (Brito et al., 2018). In Central Africa, this plant is also used in the treatment of syphilis and as an emmenagogue, an aphrodisiac, and a contraceptive (Lukhoba et al., 2006).

3.6.3 Plectranthus caninus

The diterpenes and phenolic compounds isolated from the plant are responsible for the plant cytotoxic, diuretic, and antitumor properties (Painuly & Tandon, 1983; Aswal et al., 1984). Tadesse et al. (2011) investigations on P. *caninus* essentials oils showed the antibacterial, anti-inflammatory, anti-infective, and free radical scavenging activities of the volatile components of this medicinal plant.

3.6.4 Plectranthus ecklonii

The quinone methides abietane diterpenoids parvifloron D (**39**) and parvifloron F (**41**) isolated from *P. ecklonii* ethyl acetate extract displayed an inhibitory effect against *Listeria monocytogenes, Mycobacterium smegmatis, Mycobacterium tuberculosis, S. aureus, E. coli, E. faecalis and P. aeruginosa* (Nyila et al., 2009). Both compounds also demonstrated their anti-tyrosinase activity as well as their cytotoxic activity against Vero monkey kidney cell lines (Nyila et al., 2009). Compound (**41**) is also utilized as an antimalarial compound (Van Zyl et al., 2008). A study by Grayer et al. (2003) revealed the antifungal activities of the caffeic acid nepetoidin A and B against *Aspergillus niger*.

3.6.5 Plectranthus elegans

The chloroform crude extract of *P. elegans* reduces the growth of the spore germination of the fungus *Cladosporium cucumerinum* (Dellar et al., 1996). 11-hydroxy-12-oxo-7,9(ll),13-abietatriene (**50**), and 7*α*,11-dihydroxy-12-methoxy-8,11,13-abietatriene (**59**) showed antibacterial activity against *B. subtilis*, *S. aureus,* and *Streptomyces scabies*.

3.6.6 Plectranthus ernstii

The pimarane 15(ζ),16-epoxy-7*α*-hydroxypimar-8,14-ene (**137**) showed moderate inhibition against methicillin-resistant and different multidrug-resistant strains of *S. aureus* (Stavri et al., 2009). Additionally, 1*R*,11*S*-dihydroxy-8*R*,13*R*-epoxylabd-14-ene (**88**), 15(ζ),16-epoxy-7α-hydroxypimar-8,14-ene (**137**) and *rel*-15(ζ),16-epoxy-7-oxopimar-8,14-ene (**138**) exhibited antimycobacterial activity against *M. fortuitum*, *M. phlei,* and *M. smegmatis*.

3.6.7 Plectranthus esculents

In Eastern and Southern Africa, *P. esculentus* is used to alleviate headaches and fever (Rice et al., 2011; Roshan et al., 2014). It is also stated to have anthelmintic activity (Kokwaro, 1993: 325-380).

3.6.8 Plectranthus fruticosus

P. fruticosus is used in Rome to treat burns (Lukhoba et al., 2006). Sabinyl acetate, being the main component of *P. fruticosus* essential oil, gives the rich oil a highly foetotoxic and teratogenic effect (Fournier et al., 1986). From all the kauranes isolated from the plant, only *ent*-15β,16*β*-epoxykauran-19-oic acid (**100**), and methyl *ent*-12*β*-acetoxy-17-oxokaur-15-en-19-oate (**109**) showed moderate activity against *S. aureus* (Gaspar-marques et al., 2003).

3.6.9 Plectranthus grandidentatus

Review done by Cerqueira et al. (2004) on the genus *Plectranthus*, indicated that the coleon and royleanones abietane diterpenoid isolated from *P. grandidentatus* are responsible for the plant's antifungal, antibacterial, and antitumor activities. For example, coleon U (**45**), 7*α*-acetoxy,6*β*-hydroxyroyleanone (**7**), and horminone (**8**) reduce the growth of vancomycin-resistant *Enterococcus faecalis* (VRE) and methicillin-resistant *S. aureus* (MRSA) bacteria (Gaspar-Marques et al., 2006). These abietane diterpenoids also isolated from the genus *Salvia* were proven to have antitumor, cytotoxic, antioxidant, and antibacterial activities (Kinouchi et al., 2000; Mei et al., 2002). Cerqueira et al. (2004) investigations on *P. grandidentatus*, demonstrated the high capacity of compounds (**45**)

and (**7**) to inhibit the spreading of B and T-lymphocytes induced by mitogens, inhibiting preferentially T-cell population.

3.6.10 Plectranthus hadiensis

The hexane extract of *P. hadienis* showed a good inhibitory effect against the *fungus Rhizoctonia solani* and *Sclerotinia sclerotiorum,* whereas the dichloromethane extract exhibited moderate activity against the fungi *S. sclerotiorum*, the bacteria *B. subtilis* and *Xanthomonas campestris* (Laing et al., 2006). The royleanones **7** and **13** isolated from the dichloromethane extract of the plant's aerial parts showed anticancer activity against MCF-7, NCI-H460, TK-10, UACC-62, and SF-268 cells lines (Gaspar-Marques et al., 2002). However, these two compounds were less potent than Cyclosporin A (Positive control). Compounds **7** and **16** were reported to possess antiplasmodial activity against FRC-3 with MIC of 3.11μM and 4.6μM respectively (Van Zyl et al., 2008; Bero & Fre, 2009; Amoa Onguéné et al., 2013).

3.6.11 Plectranthus hereroensis

The royleanones 16-acetoxy-7α,12-dihydroxy-8,12-abietadiene-ll,14-dione (**10**), exhibited bacterial inhibition effect against *Vibrio cholerae* and *S. aureus* (Batista et al., 1995: 167-169). Compound (**10**) was revealed to be an active antiviral agent against Herpes simplex type II (Batista et al., 1995). Compound **8** isolated from the acetone extract of the root of *P. hereroensis* was stated to be antimicrobial agents against *Candida albicans, P. aeruginosa, S. aureus*, and *V. cholerae*.

3.6.12 Plectranthus laxiflorus

P. laxiflorus is rubbed onto the skin in the treatment of leprosy, rheumatism, and abdominal pain (Lukhoba et al., 2006: 1-24). In South Africa, tea made from the leaves is used to cure coughs and colds (Bhatt et al., 2010; Rabe & Van Staden, 1998). *P. laxiflorus* and *P. barbatus* are both used as laxatives and mouthwash for teeth and gum disorders (Bhatt et al., 2010; Alasbahi & Melzig, 2010). Traditionally, the Pokot people in Kenya applied it as an enema for the treatment of influenza (Lukhoba et al., 2006), abdominal pain, and feverishness (Rabe & Van Staden, 1998). Some local people used *P. laxiflorus* as a contraceptive (Lukhoba et al., 2006). Infusion of its crushed root is used to treat gall illness and as a mouthwash for loose and bleeding teeth (Rice et al., 2011; Hulme, 1954). Hutchings et al. (1996) investigations revealed that crushed leaves and stems of the plant are used to treat eye complaints and to repel mosquitoes. A combination of the bark and root concoction is used against redwater (Masika & Afolayan, 2003).

3.6.13 Plectranthus madagascariensis

In folklore medicine, *P. madagascariensis* is used for scabies and small wounds as well as in the treatment of colds, asthma, cough, and chest complaints (Rabe & Staden, 1998). Kubínová et al. (2014) reported that the plant extract was an effective inhibitor of α-glucosidase and butyrylcholinesterase (BuChE) due to the presence of 7*β*-acetoxy, 6*β*-hydroxyroyleanone (**15**), and rosmarinic acid. The extract containing the compound (**15**) was stated to have antibacterial activity against *S. aureus* and *E. faecalis,* as well as antifeedant effects (Wellsow et al., 2006). Coleon U quinone (**17**) isolated from *P. madagascariensis* leaves was revealed to be efficacious against the gram-negative bacteria *Pseudomonas syringae* and the gram-positive bacteria *Bacillus subtilis* (Wellsow et al., 2006). Compounds (**7**) and (**13**) exhibited high selectivity for lung cancer cells (Diogo et al., 2019).

3.6.14 Plectranthus Ornatus

In Brazil, *P. ornatus* leaves were indigenously utilized to treat skin infections (Brasileiro et al., 2006). *Ent*-3*α*-hydroxycleroda-4(18),13*E*-dien-15-oic acid (**130**), ornatin C (**133**), ornatin D (**134**), and coleon R (**28**) showed mild activity against *S. aureus* (Ávila et al., 2017). Ornatin E (**135**) inhibited *Staphylococcus epidermidis* (Ávila et al., 2017); whereas, Plectrornatin B and C (**97 and 98**) moderately reduced the growth of the bacteria *candida albicans* (Rijo et al., 2002). The halimane **136** possessed antibacterial activities against five *S. aureus* strains and two *E. faecalis* strains (Rijo et al., 2011). Compound **136** was also effective against *Enterococcus flavescens* and *enterococcus faecium* (Rijo et al., 2011), whereas the labdane rhinocerotinoic acid (**99**) is known as an anti-inflammatory compound (Dekker et al., 1988: 33-35).

3.6.15 Plectranthus porcatus

The cycloabietane isolated from *P. porcatus* (13*S*,15*S*)-6*β*,7*α*,12*α*,19-tetrahydroxy-13*β*,16-cyclo-8-abietene-11,14-dione (**30**) exhibited feeble antibacterial activity against *S. aureus* (Simões et al., 2010).

3.6.16 Plectranthus Saccatus

The beyerane diterpenoid *ent*-3*β*-(3-methyl-2-butanoyl)-oxy-15-beyeren-19-oic acid (**139**), was revealed to have antifeedant activity against *Spodoptera littoralis* (Wellsow et al., 2006).

3.6.17 Plectranthus strigosus

The kaurane *ent*-16-kauren-19-ol (**117**) and *ent*-16-kauren-19- oic acid (**118**), were revealed to have an antiherpetic property (Gasper-Marques et al., 2008).

3.7 Other uses

Some of the Southern African *Plectranthus* species have also been used as food, food additives, insect repellent, material to drive away evil spirits and for horticultural purposes, as presented in Table 3.17.

Table 3.17: Other uses of Southern African *Plectranthus* species

Plectranthus species	Uses	References
P. amboinicus	Food (Vegetables) Food additives (Food stuffings; mask the odour of strong smells associated with fish, goat, and shellfish; and spices dishes containing tomato) Insect repellent	Morton, 1992; Prudent et al., 1995; Lukhoba et al., 2006. .
P. barbatus	Horticulture Food (vegetables)	Fleurentin et al., 1983; Lukhoba et al., 2006.
P. ecklonii *P. elegans* *P. madagascariensis*	Horticulture	Lukhoba et al., 2006.
P. esculentus	Food (starch, minerals and vitamin A) Food additives (to sweeten porridge)	Lukhoba et al., 2006.
P. fruticosus	Fly repellent	Roberts, 1990.
P. hadiensis	Fodder (to feed rock rabbits in Tanzania) Fish poison Charm	Hutchings et al., 1996; Lukhoba et al., 2006.
P. laxiflorus	Food (vegetable) Mosquito repellent Materials (to drive away evil spirits in India, Kenya, and Tanzania)	

CHAPTER FOUR

EXTRACTION ISOLATION AND CHARACTERIZATION OF *PLECTRANTHUS MADAGASCARIENSIS* NATURAL PRODUCTS

4.1   Introduction

The phytochemical study of Plectranthus *madagascariensis* extract was carried out using various chromatographic methods such as thin layer chromatography (TLC), Sephadex LH-20, Preparative TLC (prep-TLC), semi-preparative high performance liquid chromatography (semi-prep HPLC) as well as silica gel 60 column chromatography. The solvent system used for these chromatography techniques were determined according to the extracts constituent's migration over the TLC plates or on how the solvent and the solutes interact in the column in the case of semi-prep HPLC. The characterization and structure elucidation were conducted using spectroscopy techniques and methods such as nuclear magnetic resonances spectroscopy (NMR), infrared spectroscopy (IR), ultraviolet spectroscopy (UV), X-ray spectroscopy and optical rotation spectroscopy.

This chapter is organized into eight sections. The materials and methods used in the extraction, isolation, and characterization of the *P. madagascariensis* components are given in section 4.2. Section 4.3 details the sample collection parameters, while section 4.4 emphasizes the extraction of the bioactive compounds. Sections 4.5 and 4.6 focus respectively on the isolation and characterisation of *P. madagascariensis* natural products. The spectroscopic data characteristics of *P. madagascariensis* constituents are discussed in section 4.7. A brief conclusion of the chapter outcomes is given in section 4.8.

4.2   Materials and Methods

4.2.1   General Reagents

The solvents used in the research study were purified via distillation except for the deuterated and HPLC grade solvents (Table 4.1). Different solvents and reagents used are tabulated in the following Table.

Table 4.1: Solvent and reagents used in the research study

Solvents	Supplier
Hexane	Kimix, Cape Town, SA
Ethyl acetate	Kimix, Cape Town, SA
Methanol	Kimix, Cape Town, SA
HPLC acetonitrile	Sigma, Aldrich Cape Town, SA

HPLC methanol	Merck, Cape Town, SA
Methanol	Kimix, Cape Town, SA
Dichloromethane	Kimxi, Cape Town, SA
Deuterated chloroform	Merck, Cape Town, SA
Sulphuric acid	Sigma, Aldrich, Cape Town, SA
Vanillin	Sigma, Aldrich, Cape Town, SA
Deuterated methanol	Merck, Cape Town, SA

4.2.2 Chromatographic methods

4.2.2.1 Thin-layer chromatography (TLC)

TLC analysis was done on a precoated silica gel 60 F_{254} aluminum plates (Merck, Germany). A small amount of each sample was diluted in methanol/dichloromethane (MeOH/DCM) and spotted on the TLC plate before their development in the mobile phase solvent system of different polarities (Table 4.2). Afterward, the plate was exposed to ultraviolet (UV, CAMAG) light at wavelengths 254 and 366 nm to detect the compounds with conjugated double bonds before spraying with vanillin/sulphuric acid reagent and heating at 100 ℃ for five minutes (Wagner et al., 1984). By heating the plate, the compounds with and without conjugated bonds were visualized and identified.

Table 4.2: TLC solvent systems used in the study

Solvent systems	Ratio	TLC Solvent system code
Hexane: Ethyl acetate	90:10	A
	85:15	B
	80:20	C
Dichloromethane (DCM): Methanol (MeOH)	99:1	D
	98:2	E
	95:5	F

4.2.2.2 Preparation of the spray reagent

The vanillin/sulphuric acid reagent spray was prepared by dissolving 15 g of vanillin in 250 mL of absolute ethanol, and 2.5 mL of concentrated sulphuric acid was carefully added.

4.2.2.3 Preparative thin-layer chromatography (prep-TLC)

Preparative TLC analysis was done by spotting the sample across a glass TLC plate coated with silica gel 60 F_{254} (Merck, Germany). The target components were visualized

and identified under the UV lamp as bands, scraped from the TLC plates, and recovered from the silica using a MeOH and DCM mixture.

4.2.2.4 Column chromatography

Glass columns of different diameters and lengths were packed with either silica gel 60 [(0.040-0.063mu), (230-400 mesh)], or with Sephadex LH-20. The Sephadex LH-20 was used in the case of size exclusion chromatography, whereas the silica gel 60 was used for normal phase chromatography. Different solvent systems with different polarities were used as mobile phase "eluent". During the process, the volume of eluent collected depended on the quantity of the starting material.

4.2.2.5 Semi-preparative high-performance liquid chromatography (semi-prep HPLC)

A Shimadzu HPLC was used to profile crude extracts and purify their chemical constituents. The purification process was conducted by filtering the sample dissolved in 100% HPLC grade methanol with 0.2-micron Whatman GD/X syringe filters to remove insoluble residue or particles. 50 µL of the dissolved sample was injected in an HPLC grade acetonitrile and deionized water of increasing polarity and pumped into a Supelco C18 column (25 x 1 cm, 5 µm) by LC-20AB binary pump at a flow rate of 1.5 mL/min for an hour. For more proficient purification, the column temperature was regulated by the column oven CTO-10AD VP at approximately 40 ℃. The target compounds were detected by the highly sensitive SPD-M20A UV/VIS detector that operated at wavelengths 254, 272, and 366 nm. The collection of the compound(s) peak(s) was done manually.

4.2.3   Spectroscopic methods

4.2.3.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR analysis was recorded on a Bruker 400 MHz FT-NMR spectrometer. The chemical shifts were reported in part per million (ppm) relative to tetramethylsilane (TMS) as standard. The splitting pattern of the isolated compounds protons are presented in terms of broad (*br*), singlet (*s*), doublet (*d*), triplet (*t*), quartet (*q*), multiplet (*m*), double of triplets (*dt*), etc. The coupling constants (*J*) are reported in Hz.

4.2.3.2 Single crystal X-ray analysis

The phi scan and omega scan techniques were used to generate the Single-crystal X-ray intensity data of compound **II** which was collected on a Bruker Kappa Duo Apex II diffractometer using graphite monochromated Mo Kα radiation with a wavelength of 0.71073 Å at 173K (Bruker, 2005). An oxford cryostream cooling system with N_2 gas at a flowrate of 20 cm^3/min, was used to control the temperature. The Bruker SAINT-Plus

Software (Madidon , USA) was used to scale and reduce the data while the multi-scan method (SADABS) was used to correct data for absorption effects (Sheildrick, 1997).

The Sheldrick (2008; 2015) software, namely SHELXS was operated within the X-seed interface (Barbour, 2001) and used to solve and refine the structure. The crystal system was established by observing the Laue symmetry of the diffraction pattern, while the space group was determined by utilizing the predetermined cell parameters and collected intensities as inputs to the program XPREP (Bruker, 1997). The hydrogen atoms were geometrically constrained except for those involved in hydrogen bonding, which were found in the electron density map and refined isotropically.

4.2.3.3 Fourier Transform Infrared Spectroscopy (FTIR)

A Perkin-elmer fourier Transform infrared spectrophotometer (UATR two) was used to support compound structures and confirm the absence or presence of specific functional groups in the studied extract.

4.2.3.4 Optical rotation measurements

Anton Paar MCP 200 Polarimeter was used to measure the optical activity of the isolated compounds (0.1 g/mL in DCM) with a wavelength of 589 nm at 25 ^{o}C.

4.2.3.5 Melting point

The melting point apparatus, analogue SMP 11, was used to measure the melting point of the isolated crystals. The samples were heated up to 10 ^{o}C/min to the samples melting temperatures which were measured with a thermometer ranging from 1-300 ^{o}C.

4.2.3.6 Ultraviolet (UV) Spectroscopy

A SPECTRO star Nano Absorbance Reader (BMG LABTECH) was used to determine the UV-visible absorbance maxima of each compound from wavelengths 200 to 950 nm.

4.3 Plant sample collection

The aerial parts of *P. madagascariensis* were collected at the Cape Peninsula University of Technology (Bellville Campus) student center in January 2018 and identified by Prof. Christopher Cupido of Fort Hare University.

4.4 Extraction of plant sample

The fresh aerial parts of *P. madagascariensis* (2448.3 g) were cut into pieces and macerated in about 2 L of a solution made of hexane, dichloromethane, and acetone (2:2:1) for one hour under frequent swirling at room temperature. The mixture underwent vacuum filtration using Whatman filter paper yielding the dark orange filtrate which was

concentrated to dryness using Buchi Rotavapor R-300 at 45 ℃ and 14 mbar. The dark orange solid (K4-1-Hex) was weighed (13 g) and transferred into a sample vial which was kept in the fridge to prevent decomposition of compounds until ready for further analysis.

4.5 Isolation of *P. madagascariensis* constituents

4.5.1 Column chromatography of the total extract

The total extract (13 g) was subjected to silica gel (Merck 60, 0.040-0.063 mm) column chromatography using hexane and ethyl acetate gradient of increasing polarity resulting in the collection of 61 fractions of 250 mL each (Table 4.3).

Table 4.3: The solvent systems used during the total extract fractionation

Volume of solvent	Solvent system	Fractions (250 mL)
1 L	Hexane 100	1-4
2 L	Hexane: Ethyl acetate 95:5	5-12
2 L	Hexane: Ethyl acetate 93:7	13-21
1.5 L	Hexane: Ethyl acetate 89:11	22-28
1.5 L	Hexane: Ethyl acetate 95:15	29-35
1.5 L	Hexane: Ethyl acetate 80:20	36-42
1 L	Hexane: Ethyl acetate 75:25	43-46
1 L	Hexane: Ethyl acetate 70:30	47-50
1 L	Hexane: Ethyl acetate 60:40	51-54
1 L	Hexane: Ethyl acetate 50:50	55-58
1 L	Methanol 100	59-61

The collected fractions were concentrated to 2 to 3 mL using rota evaporator and were monitored with TLC using solvent systems A, B, and C. Fractions with similar retention factors, color, and UV characteristics were combined and given codes with roman numbers. Thus, generating 16 combined fractions which were weighed and subjected to TLC using solvent systems B and E, as shown in Figures 4.1, 4.2, and 4.3 as well as in Table 4.4.

Table 4.4: Summary of the main fractions code, weights, and TLC solvent systems

Fractions (250 mL)	Combined fractions code	Weight of portions (g)	TLC Solvent systems
1-5	K4-3-I	0.0052	B
6-10	K4-3-II	0.1264	
11-14	K4-3-III	0.0908	

15-21	K4-3-IV	0.2099	
22-24	K4-3-V	0.3097	
25-28	K4-3-VI	1.2397	
29-32	K4-3-VII	1.3729	
33-35	K4-3-VIII	0.9761	
36-39	K4-3-IX	0.4564	B and E
40-42	K4-3-X	0.3693	
43-47	K4-3-XI	0.6221	
48-53	K4-3-XII	0.3772	
54-56	K4-3-XIII	0.1577	
57-59	K4-3-XIV	0.3799	
60	K4-3-XV	0.4963	
61	K4-3-XVI	0.8345	

Figures 4.1, 4.2, and 4.3 are representative of the main fractions TLC profile after spraying with vanillin/sulphuric acid under UV light at wavelength 254 nm and 366 nm.
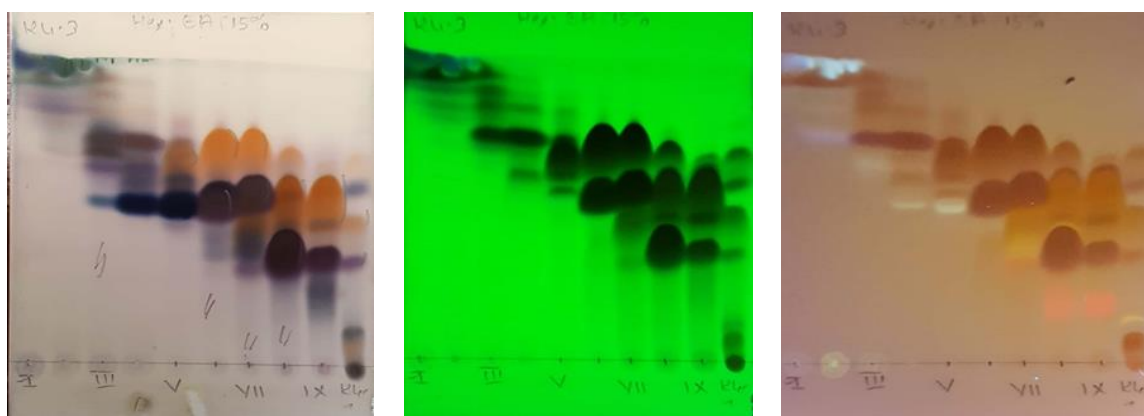
Figure 4.1: TLC profile of K4-3-I to K4-3-IX fractions using solvent system B as mobile phase
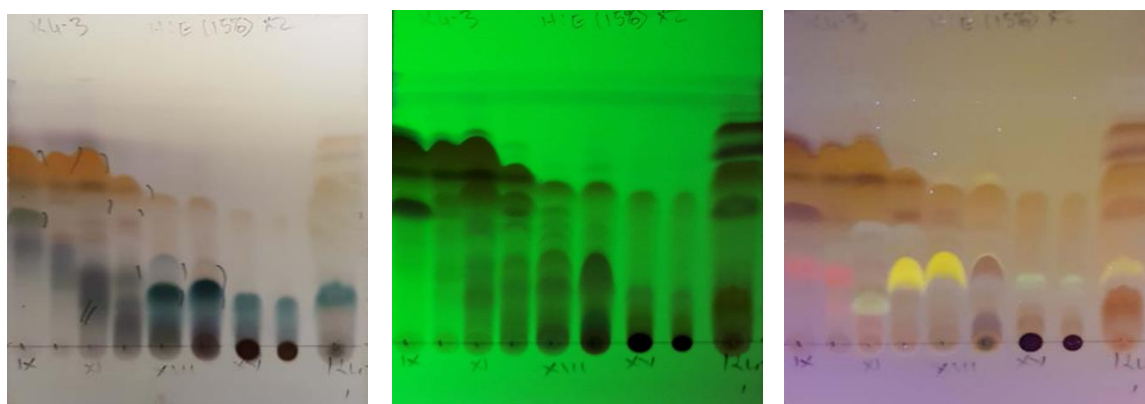
Figure 4.2: TLC profile of K4-3-IX to K4-3-XVI fractions using solvent system B as mobile phase
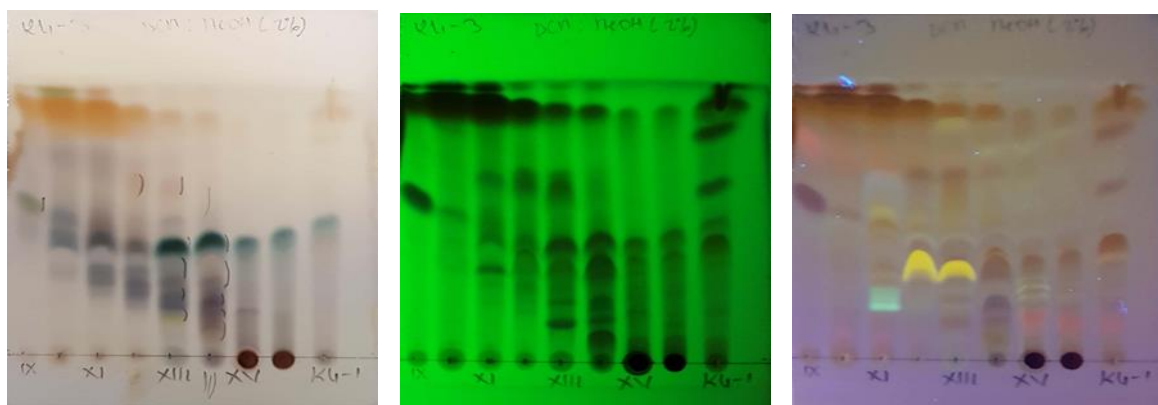
Figure 4.3: TLC profile of K4-3-IX to K4-3-XVI fractions using solvent system E as mobile phase

4.5.2   Isolation of *P. madagascariensis* secondary metabolites

From the 16 main fractions, only fractions K4-3-III, K4-3-IV, K4-3-VI, K4-3-VIII, K4-3-XI, K4-3-XIII and K4-3-XIV were further purified. The remaining fractions were not considered due to their similarities with the selected subfractions except fractions K4-3-I and K4-3-II which did not show interesting bioassay results.

Most purifications were carried out with Sephadex LH-20 using 95% MeOH and 5% deionised water (H_2O). However, the purification process of some fractions also required the use of prep-TLC, semi-prep-HPLC as well as deactivated silica gel 60 (prepared with silica gel and 10% w/v of H_2O) column chromatography. All the chromatography methods used during the purification process of the isolated compounds were monitored with TLC.

4.5.2.1 Processing of fraction K4-3-VIII and isolation of compound I

Fraction K4-3-VIII (97 mg) was subjected to Sephadex LH-20 and eluted with MeOH and H_2O (95:5). The fractionation of K4-3-VIII produced seven subfractions (K4-7-1 to K4-7-7). Subfraction K4-7-3 (29.8 mg) was further purified by going through an isocratic silica gel column chromatography using Hexane and ethyl acetate (95:5). The column chromatography process results in the pure single compound (**I,** 25.7 mg) (Figure 4.4).
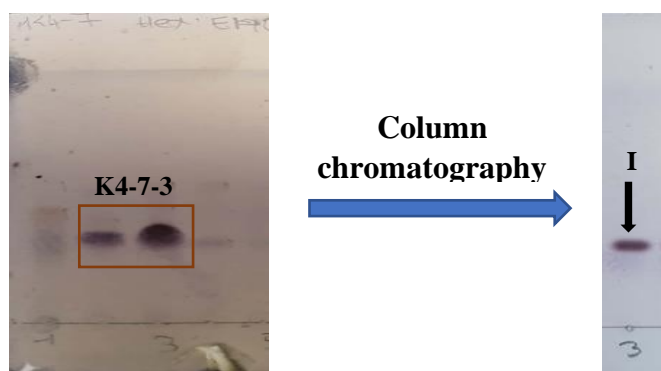
Figure 4.4: TLC profile of compound **I**

4.5.2.2 Processing of fraction K4-3- VI and isolation of compound II as well as the Compound VI &VII

Fraction K4-3-VI (200 mg) was subjected to Sephadex LH-20 using 95% MeOH and 5% H_2O producing in the process seven sub-fractions (K4-5-1 to K4-5-7). Subfractions K4-5-2 and K4-5-3 as well as subfractions K4-5-6 and K4-5-7 were combined and underwent isocratic silica gel column chromatography using hexane and ethyl acetate (98:2). The isocratic column chromatography of K4-5-2 & 3 (74 mg) resulted in the isolation of the pure compound **II** (61.9 mg). Whereas the isocratic column chromatography of K4-5-6 & 7 (64.2 mg) yielded the mixture (**VI** & **VII**) which appeared as a single spot on TLC (Figure 4.5).
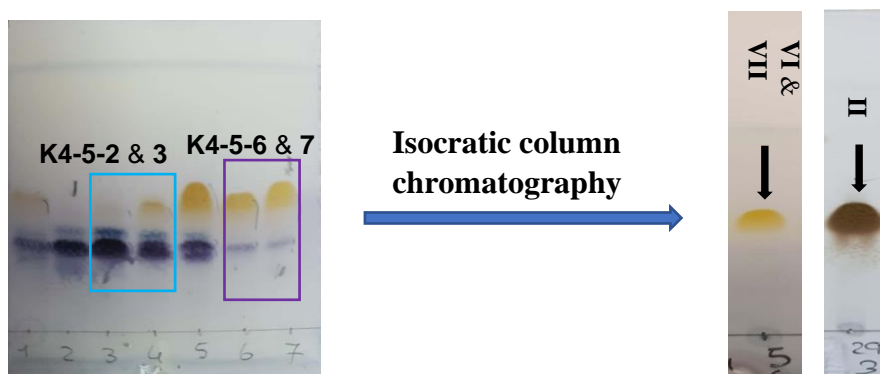
Figure 4.5: TLC profile of subfractions K4-5, compound **II** and the mixture **VI** & **VII**

4.5.2.3 Processing of fraction K4-3-III & IV and isolation of compound III

Main fractions K4-3-III (0.0908 g) and K4-3-IV (0.01982 g) were merged (K3-3-III & IV) and subjected to isocratic column chromatography using deactivated silica gel 60 and Hexane: ethyl acetate (90:10) as eluent. The fractionation of the main fraction K4-3-III & IV (0.2890 g) yielded 38 sub-fractions which were collected (50 mL each) and pooled together according to their TLC profiles to finally generate six subfractions (K4-19-1 to K4-19-6) as pointed out in Figure 4.4. Sub-fraction K4-19-4 (60 mg) was applied to four prep-TLC plates and developed twice in Hexane: ethyl acetate (90:10) solvent system to yield compound **III** (Figure 4.6).
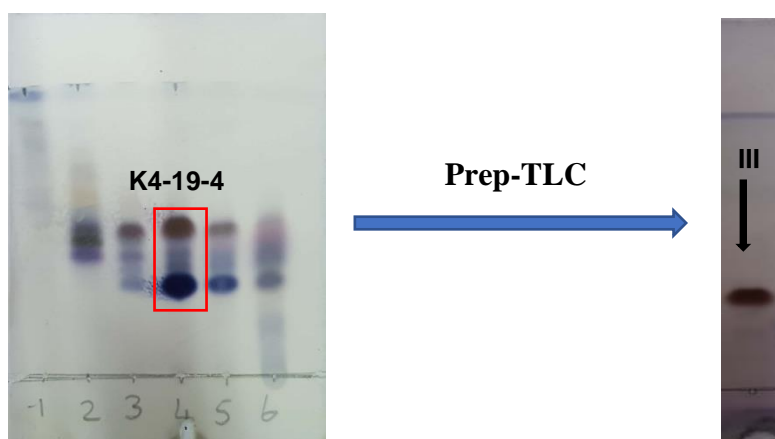
Figure 4.6: TLC profile of subfraction K4-19, and compound **III**

4.5.2.4 Processing of fraction K4-3-XI and Isolation of compound IV

Fraction K4-3-XI (0.6221 g) was subjected to sequential Sephadex LH-20 using MeOH: H_2O (95:5), which yielded nine subfractions (K4-55-1 to K4-55-9). Subfraction K4-55-1 was injected into the semi-prep HPLC and eluted to acetonitrile: deionized water of increasing polarity (60% to 80% acetonitrile in 30 minutes, then 80% to 100% acetonitrile for 15 minutes, Table 4.5). Two major peaks were observed at 28 min and 39.5 min (Figure 4.7). The peak at 39.5 minutes (Figure 4.7) was collected and found to be pure compound **IV** (6 mg), whereas the peak at 28 minutes was a mixture of compound **IV** and a derivative (not determined).
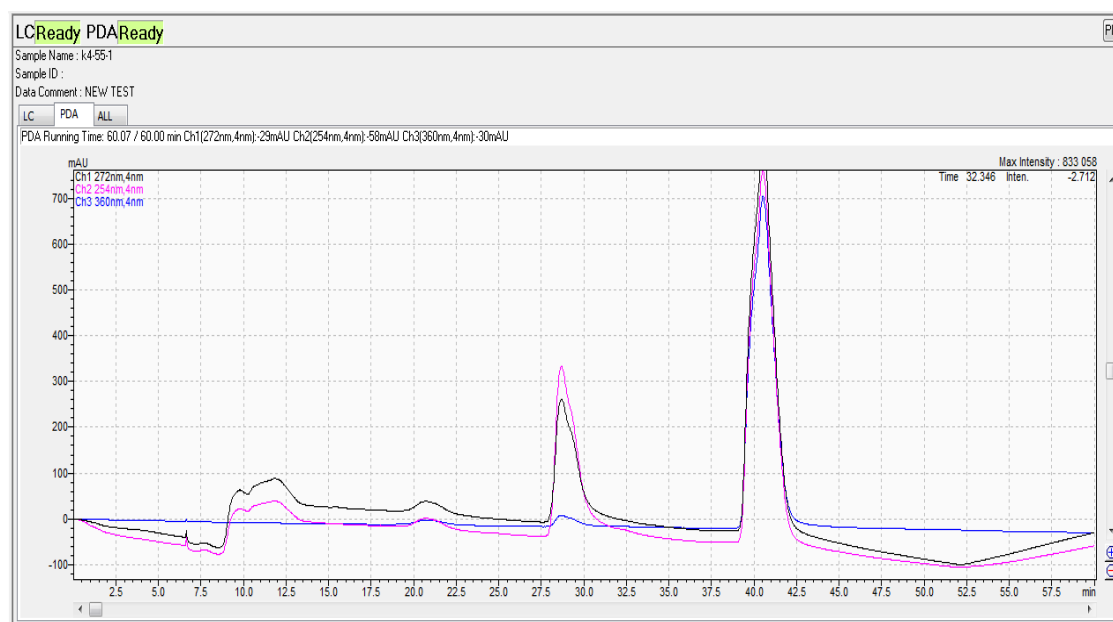
Figure 4.7: semi-prep HPLC profile of subfraction K4-55-1

Table 4.5: Conditions of the semi-prep HPLC

Solvent	Acetonitrile: deionised water (60 to 100% acetonitrile)
Column	SUPELCO, RP18 (25X2.1 cm)

Flow rate	1.5 mL/min
Detection	UV at λ 272; 254 and 360 nm

4.5.2.5 Processing of fraction K4-3-XIII & XIV and isolation of compound V

Fraction K4-3-XIII and XIV (400 mg) were combined and subjected to sequential Sephadex LH-20 using MeOH: H_2O (95:5) to yield subfractions K4-9 and K4-13. The subfractions constituents were screened with TLC (Figure 4.8). From the TLC results, subfractions K4-9-2 (18.7 mg) and K4-13-3 (44.2 mg) were pooled together and purified with Sephadex LH-20 and elute with MeOH: H_2O (95:5) which resulted in the isolation of compound **V** (26.7 mg).
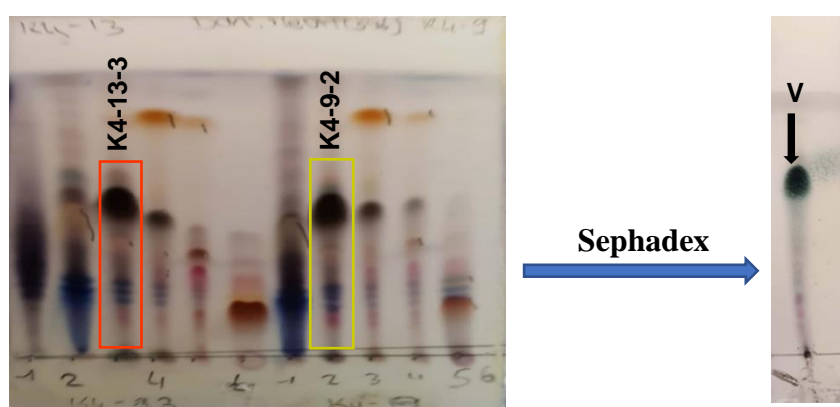
Figure 4.8: TLC profile of subfractions K4-13-3, K4-9-2, and compound **V**

4.6   Results and discussions

P. madagascariensis collected from the greenhouse of the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic has been studied, and four compounds were identified *viz* rosmarinic acid, 6*β*,7*β*-dihydroxyroyleanone (**14**), 7*β*-acetoxy-6*β*-hydroxyroyleanone (**15**) and coleon U quinone (**17**) (Kubínová et al., 2014).

To the best of our knowledge the isolated compounds **14** and **15** which contain 6β, 7β stereochemical configurations have not been identified before from Lamiaceae and especially *Plectranthus*, instead (and more common) 6*β*, 7*α* orientations have been documented and identified. Additionally, a recent study was published by Diogo et al. (2019) on the bioactive compounds of the plant material collected from Portugal, and they identified rosmarinic acid, 6*β*,7*α*-dihydroxyroyleanone (**13**), 7*α*-formyloxy-6*β*-hydroxyroyleanone (**16**), 7*α*-acetoxy-6*β*-hydroxyroyleanone (**7**), and coleon U (**45**). This publication did not mention the isolation and/or identification of 6*β*, 7*β* related structures, but stated that compounds **14** and **15** have 6β, 7α configurations without further explanations. The situation becomes unclear, as Kubínová et al. (2014) did not report the spectroscopic data of the two compounds in their study. This is the reason why it was

essential to conduct a careful study for the explicit identification of the secondary metabolites from these species and determined their biological activities.

P. madagascariensis aerial parts were collected from the Cape Peninsula University of Technology (Bellville campus) in January 2018 and extracted with dichloromethane hexane and acetone in a 2:2:1 ratio. The chromatographic isolation led to the isolation of five pure compounds (**I-V**) and the inseparable mixture of two epimeric dimers (**VI & VII**). The isolated compounds were identified using NMR. The ^{1}H and ^{13}C NMR results of compounds **I-V** are tabulated in Table 4.6, whereas the dimers **VI & VII** NMR results are summarized in Table 4.6.

Table 4.6: NMR data of compounds **I-V** in CDCl₃

No (C)	I		II		III		IV		V	
	¹³C	¹H, *m*, (*J* = Hz)	¹³C	¹H, *m*, (*J* = Hz)	¹³C	¹H, *m*, (*J* = Hz)	¹³C	¹H, *m*, (*J* = Hz)	¹³C	¹H, *m*, (*J* = Hz)
1	38.4	2.59, *dt* (12.7, 3.0) 1.19*	38.3	2.58, *dt* (12.7, 3.0) 1.22*, *m*	35.8	1.16* 2.70, *dt* (12.7, 3.0)	30.8	2.66, *m* 1.60*	29.6	2.13, *td* (12.7, 3.0) 2.74*
2	19.0	1.61, *m* 1.83, *m*	18.9	1.59, *dt* (12.7, 3.0) 1.80, *m*	18.9	1.54, *m* 1.74, *m*	17.7	1.89, *m* 1.58, *m*	18.5	1.79* 1.69, *m*
3	42.3	1.25* 1.50*	42.3	1.40, m 1.24, *m*	41.1	1.46, *m* 1.25, *m*	36.3	1.99*, 1.49*	41.3	1.26, *m* 1.43*, *m*
4	33.7		38.6		33.0		36.4		32.4	
5	49.5	1.47, *s*	49.7	1.27, *s*	45.8	1.60, *s*	143.3		58.2	1.63, *s*
6	69.3	4.46, br*s*	66.4	4.24, *s*	25.8	1.96, *d*	146.8		105.2	
7	69.1	4.53, *d*, (1.5)	69.0	5.60, *d*, (1.8)	63.2	4.73, *d* (1.5)	177.5		192.8	
8	140.9		137.0		143.2		126.8		121.4	
9	147.6		150.1		147.8		155.1		137.7	
10	38.6		33.5		39.1		41.4		51.5	
11	183.5		183.3		183.9		183.6		140.5	
12	151.2		151.2		151.3		150.7		148.3	
13	124.3		124.3		124.2		126.0		133.3	
14	189.1		186.0		189.1		184.3		120.1	7.65, s
15	24.0	3.18, *septet* (7.1)	24.1	3.09, *septet* (7.08)	24.0	3.16, *septet* (7.1)	24.4	3.22, *septet* (7.0)	27.1	3.02, *septet* (7.1)
16	19.8	1.23, *d* (7.1)	19.6	1.11, *d* (7.08)	19.9	1.20, *d* (7.1)	19.8	1.24, *d* (7.0)	22.4	1.16, *d* (7.1)
17	19.9	1.23, *d* (7.1)	19.8	1.13, *d* (7.08)	19.8	1.21, *d* (7.1)	19.8	1.25, *d* (7.0)	22.5	1.17, *d* (7.1)
18	33.5	1.06, *s*	33.5	0.92, *s*	33.2	0.98, *s*	27.2	1.43, *d* (3.9)	33.7	1.04, *s*
19	24.3	1.27, *s*	23.6	1.16, *s*	21.7	0.90, *s*	29.1	1.42, *d* (3.9)	22.2	1.31, *s*
20	21.6	1.62, *s*	21.3	1.55, *s*	18.4	1.22, *s*	27.5	1.64, *s*	72.3	3.37, *d* H_{α,} (7.5) 4.29, *d* H_{β} (7.5)
7-OCOC**H**₃			21.0	1.98, *s*						
7-O**C**OCH₃			170.1							
6-OH		5.31, *s*						7.09		5.21, *s*
7-OH						3.03 *br*				
12-OH				7.23, *s*		7.26, *s*		7.08, *s*		7.23, *s*

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4.6.1 Compound I (6β,7α-dihydroxyroyleanone)

The ^{1}H NMR spectrum (Figure 4.10) showed signals of five methyls at δ_H 1.62 (*s*, Me-20); 1.27 (*s*, Me-19); 1.06 (*s*, Me-18);1.23 (*d*, *J* = 7.1 Hz, Me-17); 1.23 (*d*, *J* = 7.1 Hz, Me-16). The latter two methyl groups with the proton signal at δ_H 3.18 (*septet*, *J* = 7.1 Hz, H-15) indicate the presence of an isopropyl group. Furthermore, the ^{1}H NMR showed signals of two low field two protons attached to two oxygenated carbons at δ_H 4.53 (*d*, *J* = 1.5 Hz, H-7) and 4.46 (*brs*, H-6); in addition to a cluster of proton signals between δ_H 1.20 to $\square_H$ 1.50 (see Table 4.6 for more details). The fact that the two protons at C-6β and C-7α have negligible coupling between them is directly reflected from the distortion chair form of ring B, as a result of the direct effect of the planar structure of ring C and affected the stereochemistry of all ring B proton directly (Figure 4.9) directly.
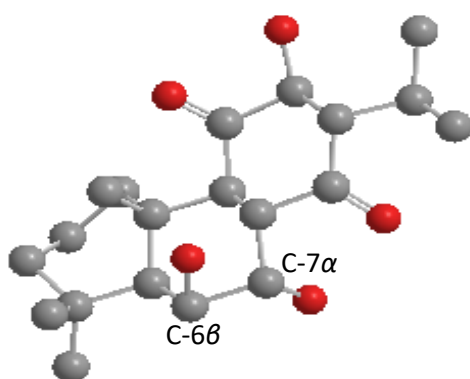
Figure 4.9: 3D structure of compound I (protons were omitted for clarity, generated by CHEM-DRAW (version 12)
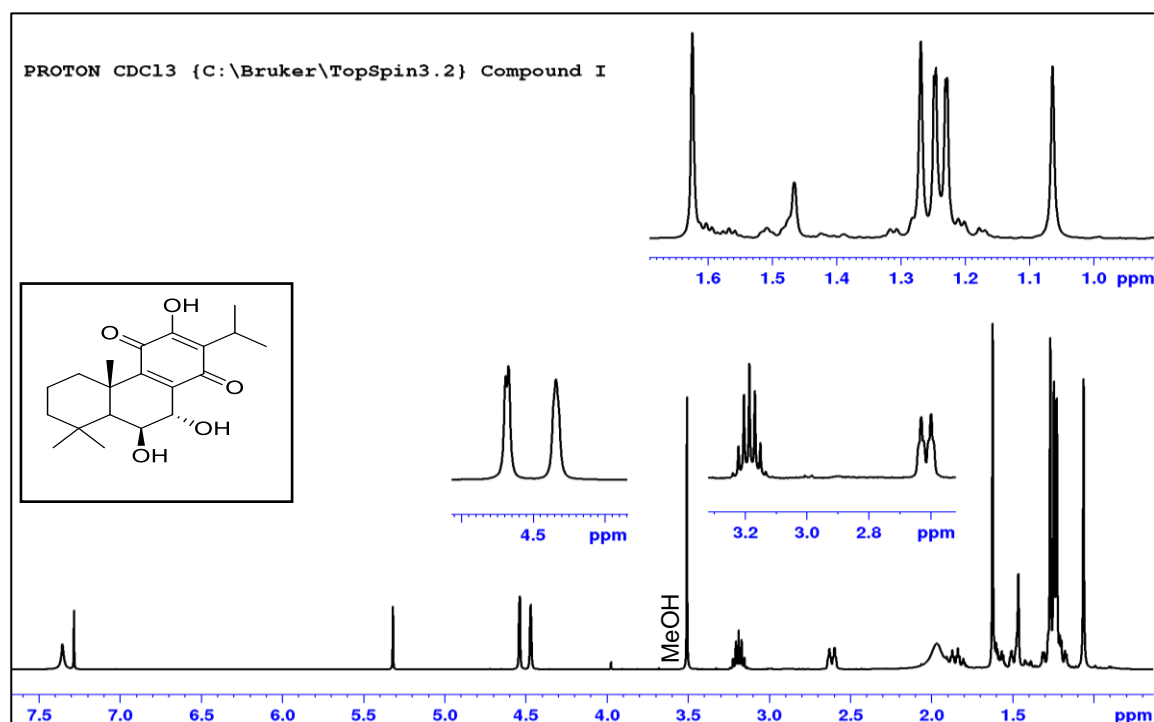
Figure 4.10: ^{1}H NMR spectrum of the 6β,7α-dihydroxyroyleanone (**I**)

The ^{13}C NMR spectrum (Figure 4.11) showed twenty signals which were classified according to DEPT-135 (Figure 4.12) to five methyls, three methylenes; four methines groups and eight quaternary carbons with six of them forming quinonoidal structure (ring C), at δ_C 140.9 (C-8), 147.6 (C-9), 183.5 (C-11), 151.2 (C-12), 124.3 (C-13), and 189.1 (C-14), in addition to the two oxygenated carbons at δ_C 69.3 (C-6), and 69.1 (C-7).

C13CPD CDCl3 {C:\Bruker\TopSpin3.2} Compound I
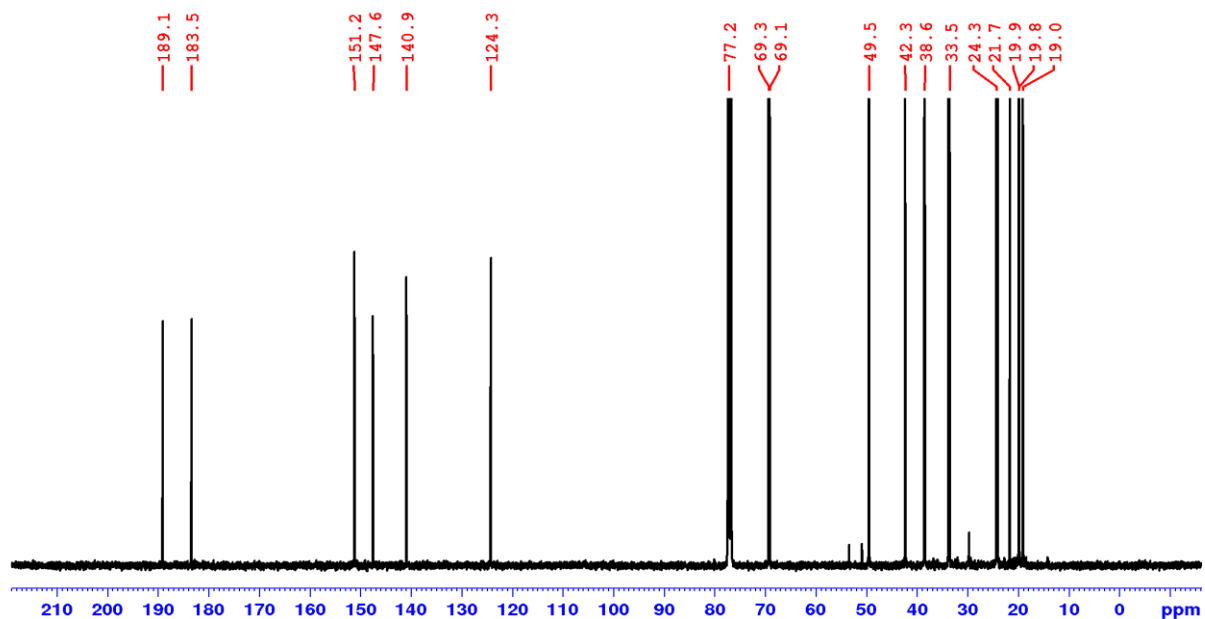
Figure 4.11: ^{13}C NMR spectrum of 6*β*,7*α*-dihydroxyroyleanone (**I**)

C13DEPT135 CDCl3 {C:\Bruker\TopSpin3.2} Compound I
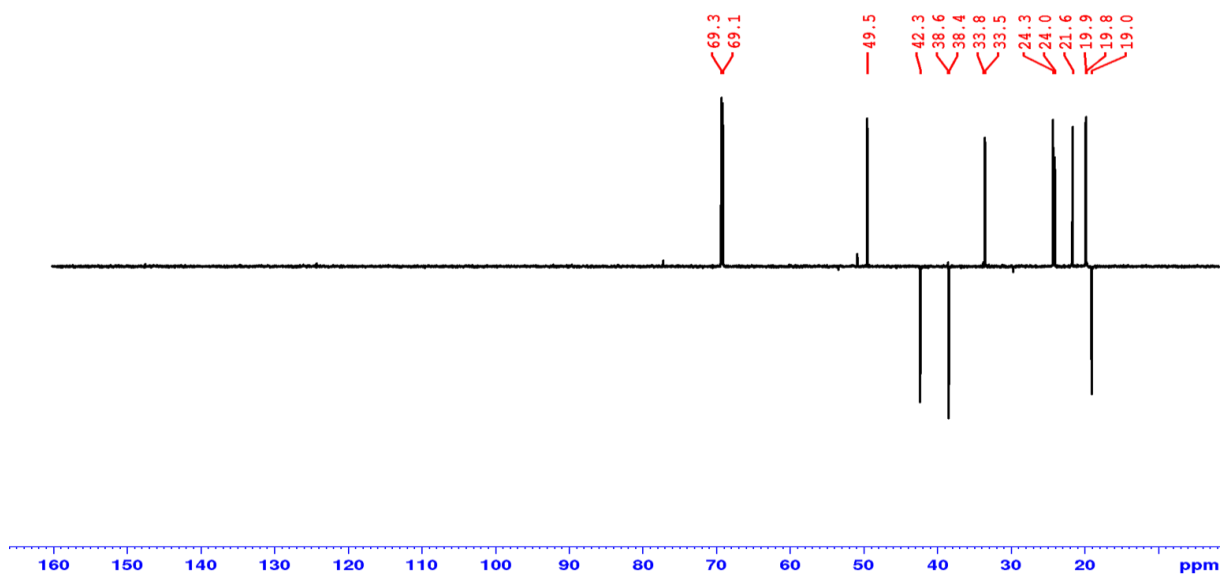
Figure 4.12: DEPT-135 NMR spectrum of 6*β*,7*α*-dihydroxyroyleanone (**I**)

The above mentioned data were identical to the royleanone 6*β*,7*α*-dihydroxyroyleanone isolated by   Rasikari (2007), and Diogo et al. (2019) from   *P. fasciculatus* and   *P. madagascariensis*, respectively.

4.6.2   Compound II (7*α*-acetoxy-6*β*-hydroxyroyleanone)

The ^{1}H NMR spectrum (Figure 4.13) showed signals of five upfield methyl groups at δ_H 1.55 (*s*, Me-20); 1.16 (*s*, Me-19); 0.92 (*s*, Me-18); 1.13 (*d*, *J* = 7.08 Hz, Me-17); 1.11 (*d*, *J* = 7.08 Hz, Me-16); as well as the signal of a downfield methyl attached to a carbonyl group at δ_H 1.98 (OCO<u>C</u>H$_3$). The ^{1}H NMR showed signals of a downfield proton attached to the two methyls (Me-16 and Me-17) at δ_H 3.09 (*septet*, *J* = 7.08 Hz, H-15). Two signals of deshielded protons attached to an oxygenated carbon were observed in the ^{1}H NMR spectrum at δ_H 4.24 (H-6α) and 5.60 (H-7β). Moreover, a cluster of protons signals between δ_H 1.10 to 1.70 was observed (see Table 4.6 for more details). The compound shows a similar ^{1}H NMR profile like I, except the low field shift of H$_7\alpha$ (from 4.53 to 5.60) which indicates the link of an acetoxy group to this position.
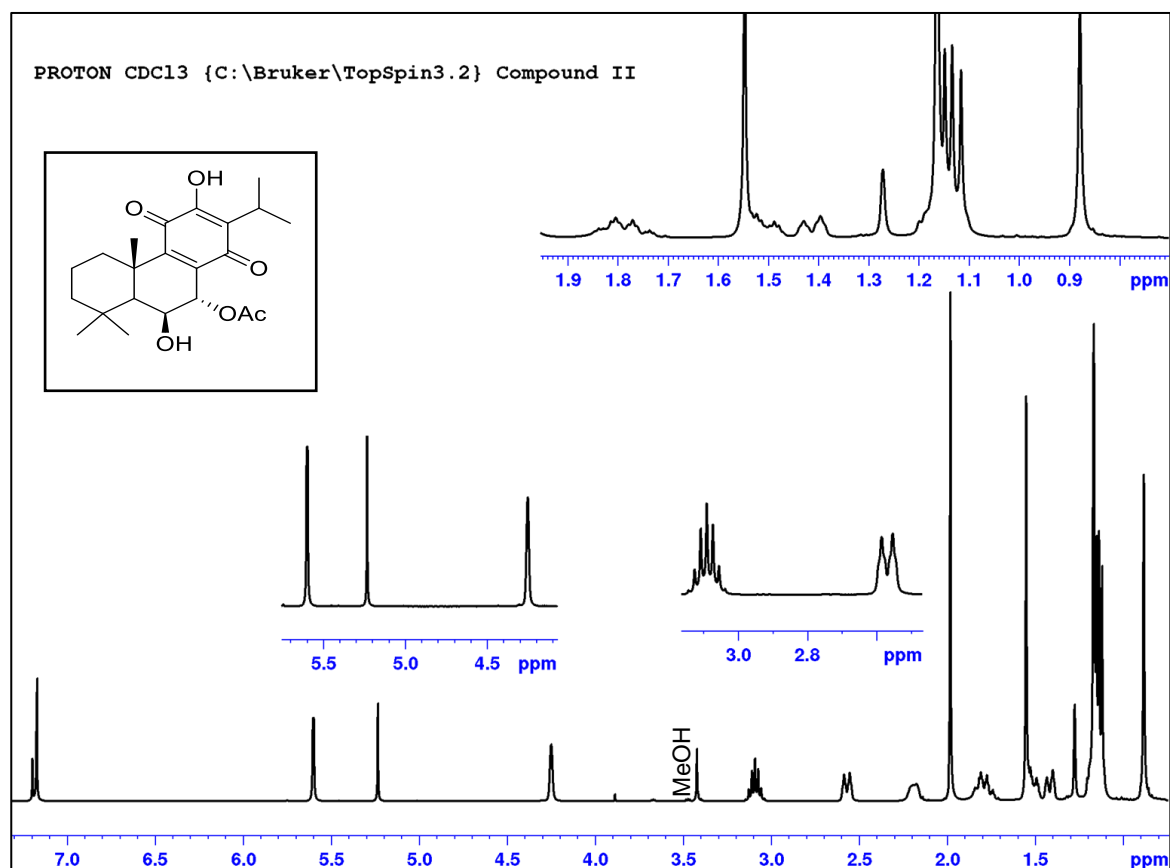
Figure 4.13: ^{1}H NMR spectrum of 7*α*-acetoxy-6*β*-hydroxyroyleanone (**II**)

The ^{13}C NMR spectrum (Figure 4.14) showed twenty-two signals which were classified according to DEPT-135 (Figure 4.15) to six methyls, three methylenes; four methines and

nine quaternary carbons with six of them forming quinonoidal structure (ring C), at 137.0 (C-8), 150.1 (C-9), 183.3 (C-11), 151.2 (C-12), 124.3 (C-13), and 186.0 (C-14). Moreover, two oxygenated carbons at δ_C 66.4 (C-6), and 69.0 (C-7) and an acetoxy group at δ_C 21.0 (7-OCOC$\underline{H}_3$) and 170.1 (7-O$\underline{C}$OCH$_3$) were identified.

X: Ethyl acetate solvent peaks
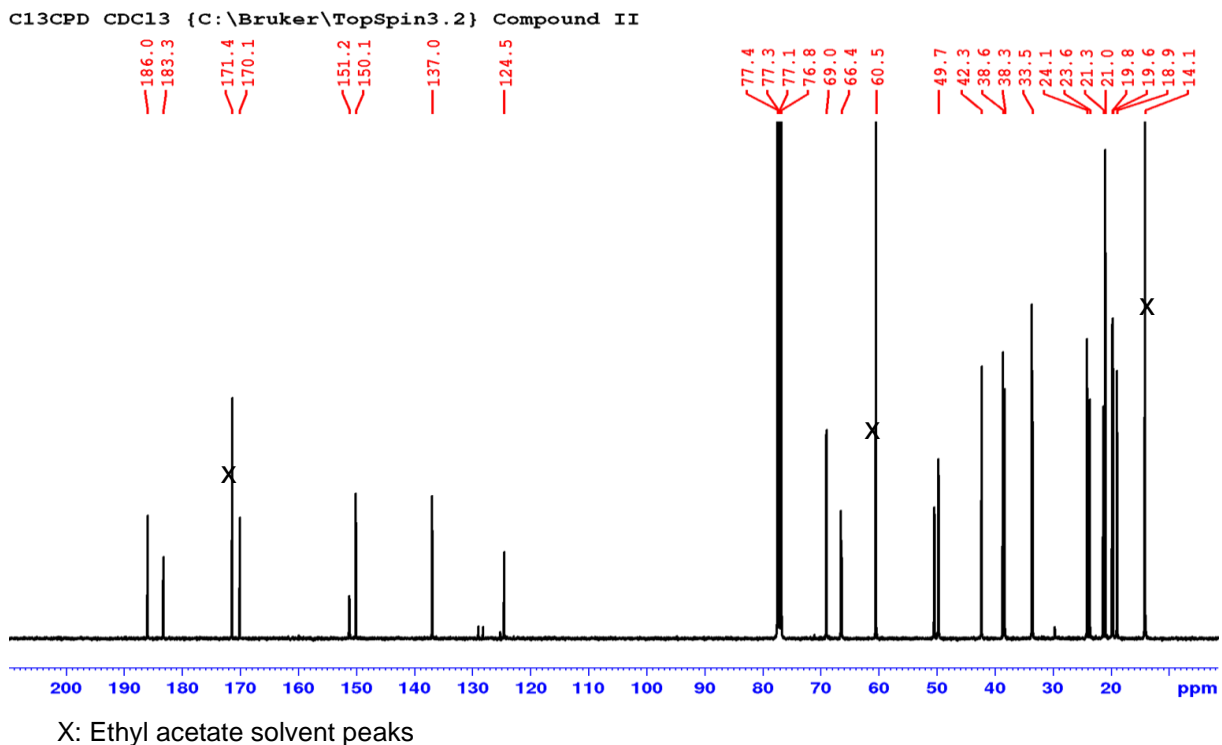
Figure 4.14: ^{13}C NMR spectrum of 7α-acetoxy-6β-hydroxyroyleanone (**II**)

X: Ethyl acetate solvent peaks
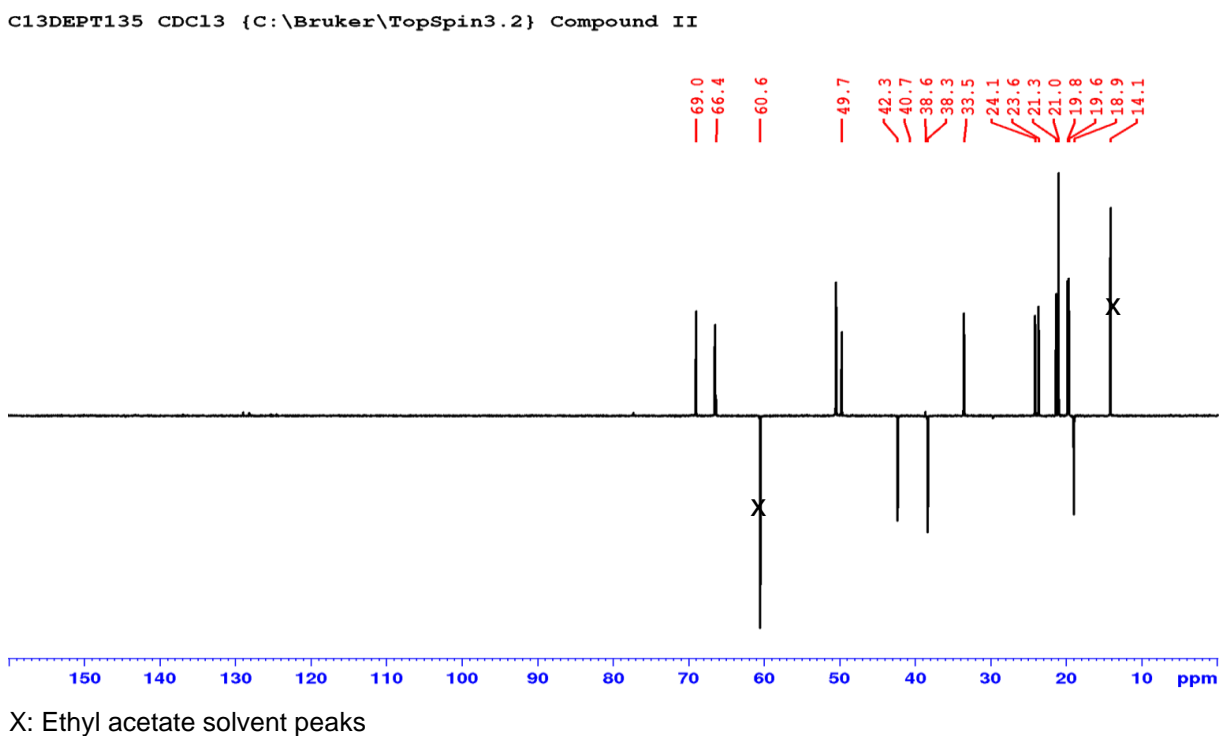
Figure 4.15: DEPT-135 NMR spectrum of 7α-acetoxy-6β-hydroxyroyleanone (**II**)

The above-mentioned data are identical to the royleanone 7α-acetoxy-6β-hydroxyroyleanone isolated by Rasikari (2007) and Diogo et al. (2019) from *P. actites* and *P. madagascariensis*, respectively. Compounds **I** and **II** isolated in this study correspond to compounds **13** and **7** isolated from *P. madagascariensis* by Diogo et al. (2019). From Diogo et al. (2019) publication, we assume that compounds **14** and **15** isolated by Kubínová et al. (2014) should correspond to compounds **13** and **7** which have different stereochemical orientation for C-6 and C-7. Thus, for clarification about the exact configuration of these compounds isolated from *P. madagascariensis*, the configuration of compound **II** was analyzed using X-ray spectroscopy as it formed an excellent crystal adequate for X-ray analysis. The results of the X-ray analysis (Figure 4.16) showed that compound **II** consists of the typical three fused cyclohexane rings with two of them are trans fused cyclohexane rings (C-5-C-10 and C-8-C-9/C-11-C-14) which is endemics to royleanone type abietane diterpenoids. Although the temperature of analysis (173K) of compound **II** is slightly higher than the one of Bernades et al., 2018 (165K, Table 4.7), it was noticed that the crystalline packing have similar R_6^6 synthon motif (Figure 4.17). This motif involved the intramolecular (O2- H(2O)…O1) and intermolecular (O2-H(2O)…O3 and O6-H(6O)…O5) hydrogen bonds. All these parameters confirmed that compound **II** is indeed a typical 6β, 7α configuration as reported by Diogo et al. (2019).
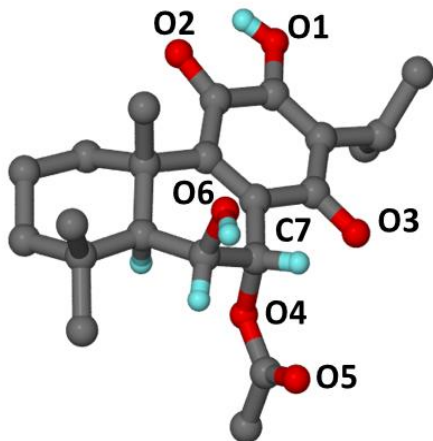
Figure 4.16: X-ray crystallographic structure of compound **II**. The hydrogens atoms have been omitted for the structure clarity purpose.
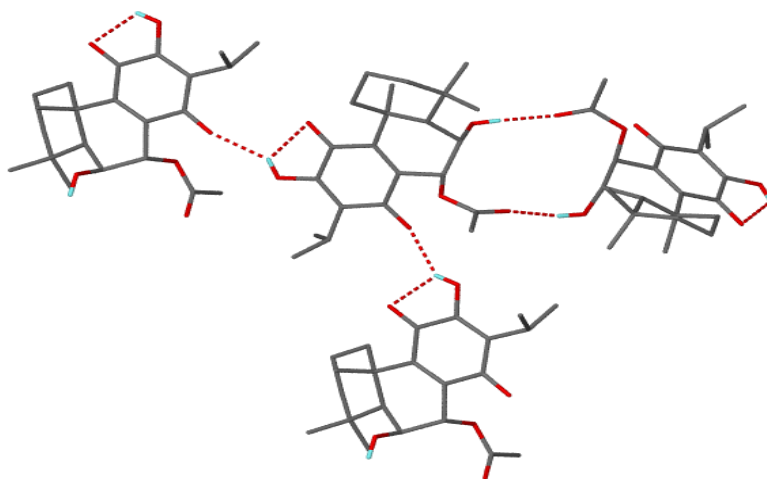
Figure 4.17: Synthon motif of compound **II**

Table 4.7: Single-crystal X-ray parameters of 7*α*-acetoxy-6*β*-hydroxyroyleanone (**II**)

Parameters	Values	
Identification code	**II**	Bernades et al., 2018
Molecular formula	$C_{20}H_{30}O_6$	$C_{20}H_{30}O_6$
Temperature	173 K	165 K
Crystal size	0.290 x 0.360 x 0.400 mm	0.25 X 0.20 X 0.500 nm
Crystal system	Orthorhombic	Orthorhombic
Unit cell dimensions		
A	7.3893(4) Å	7.3873(7) Å
B	14.1145(8) Å	14.0964 (12) Å
C	20.6199(12) Å	20.5705 (18) Å
Volume	2150.6(4) Å^3	2142.1 (3) Å^3
Reflections collected	32427	10019

4.6.3 Compound III (horminone)

The ^{1}H NMR spectrum (Figure 4.17) showed signals of five methyls at δ_H 1.22 (*s*, Me-20); 0.90 (*s*, Me-19); 0.98 (*s*, Me-18); 1.21 (*d*, *J* = 1.7 Hz, Me-17); 1.20 (*d*, *J* = 1.7 Hz, Me-16). The ^{1}H NMR signal at δ_H 3.16 (*septet*, *J* = 7.1 Hz, H-15) is representative of the presence of the isopropyl group. Whereas the low field ^{1}H NMR signal at δ_H 4.73 (*d*, *J* = 1.5 Hz) indicates the presence of a proton attached to an oxygenated carbon. Several proton signals between δ_H 1.20 to 1.60 are also observed (see Table 4.6 for more details).
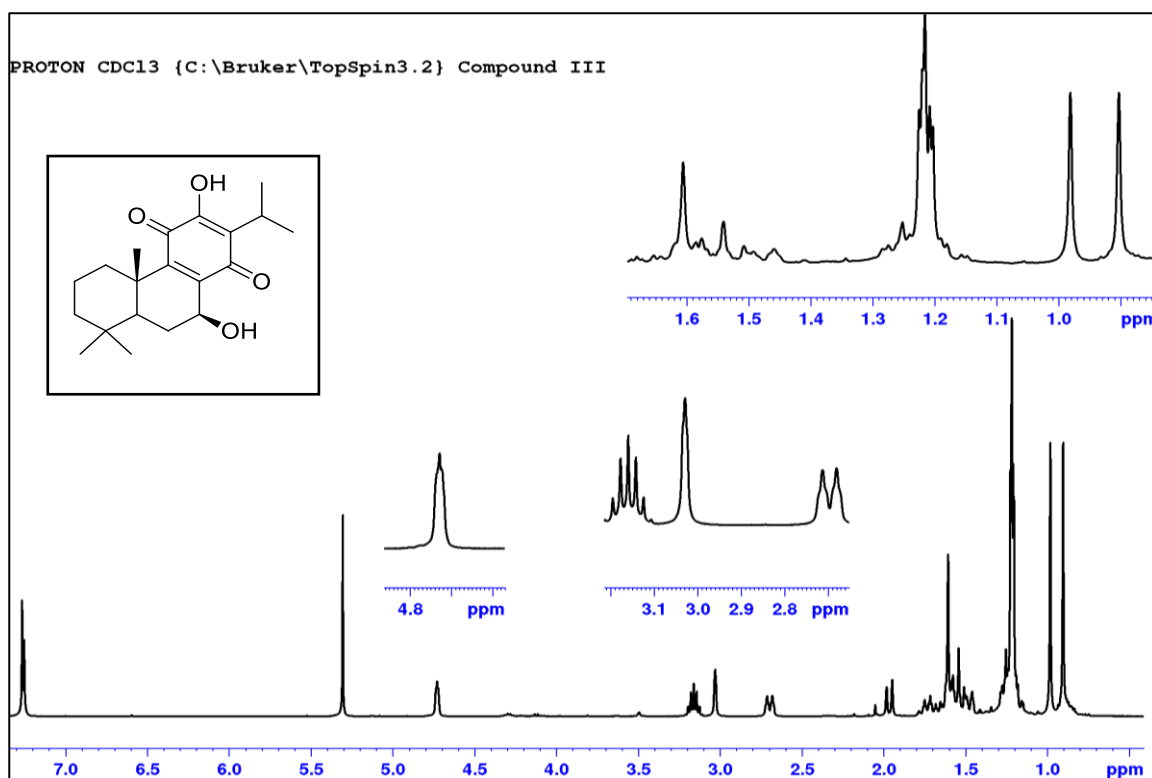
Figure 4.18: ^{1}H NMR spectrum of the horminone (**III**)

The ^{13}C NMR spectrum (Figure 4.18) showed twenty signals which were classified according to DEPT-135 (Figure 4.19) to five methyls, four methylenes; three methines and eight quaternary carbons with six of the quaternary carbons forming the quinonoidal structure (ring C), at δ_C 140.9 (C-8), 147.5 (C-9), 183.1 (C-11), 151.2 (C-12), 124.3 (C-13), and 189.5 (C-14). Additionally, an oxygenated carbon at δ_C 63.2 (C-7α) was detected.
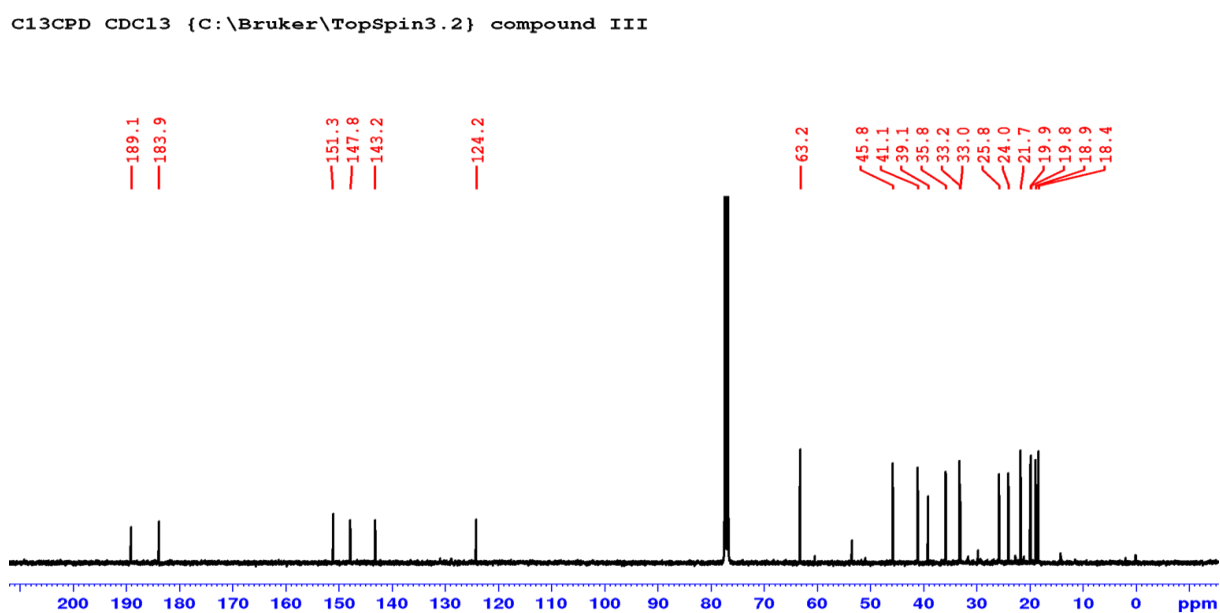
Figure 4.19: ^{13}C NMR spectrum of horminone (**III**)
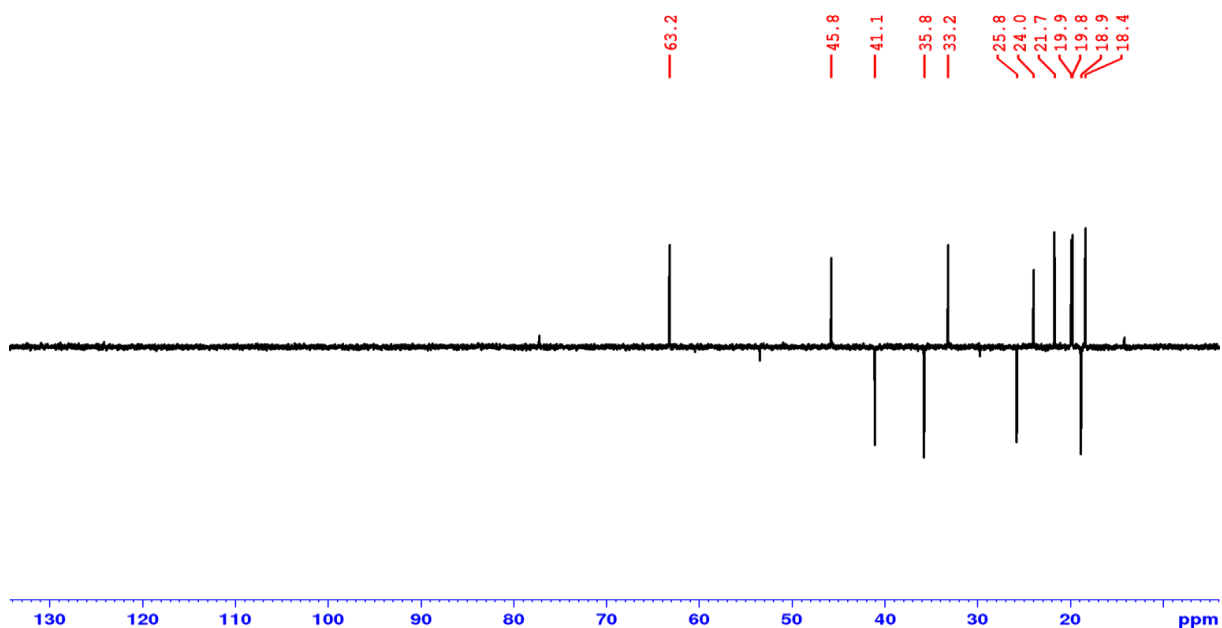
Figure 4.20: DEPT-135 NMR spectrum of horminone (**III**)

The above-mentioned data were identical to the royleanone 7α-hydroxyroyleanone (Horminone) isolated by Naman (2015) from *Plectranthus species.*

4.6.4 Compound IV (coleon U quinone)

The ^{1}H NMR spectrum (Figure 4.20) showed signals of five methyls at δ_H 1.64 (*s*, Me-20); 1.42 (*d*, *J* = 3.9 Hz, Me-19); 1.43 (*d*, *J* = 3.9 Hz, Me-18); 1.25 (*d*, *J* = 6.8 Hz, Me-17); 1.24 (*d*, *J* = 6.8 Hz, Me-16). The proton signal at δ_H 3.22 (*septet*, *J* = 7.0 Hz, H-15) indicate the presence of an isopropyl group, in addition to a cluster of proton signals between δ_H 1.22 to 1.80 (see Table 4.6 for more details).
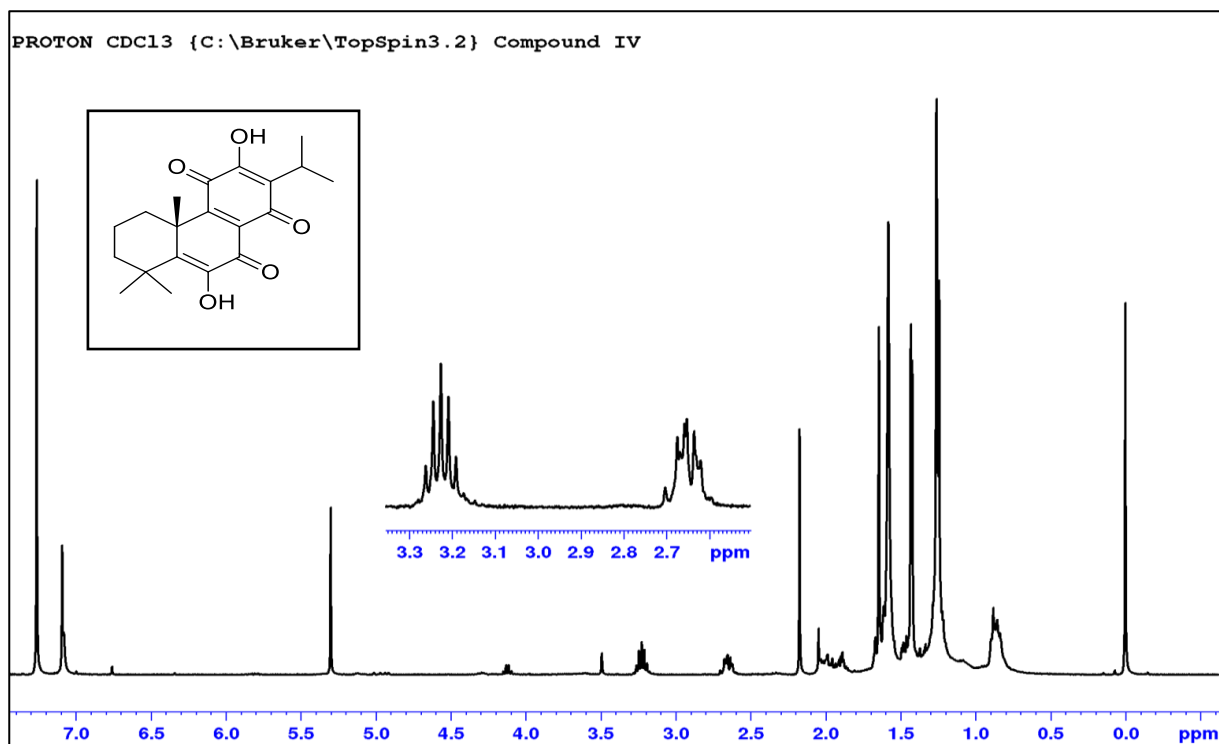
Figure 4.21: ^{1}H NMR spectrum of coleon U quinone (**IV**)

The 13C NMR spectrum (Figure 4.21) showed 20 signals which were classified according to DEPT-135 (Figure 4.22) to five methyls, three methylenes; one methines and ten quaternary carbons with Six of them forming quinonoidal structure (ring C), at δ_C 126.8 (C-8), 155.1 (C-9), 183.6 (C-11), 150.7 (C-12), 126.0 (C-13) and 184.3 (C-14).
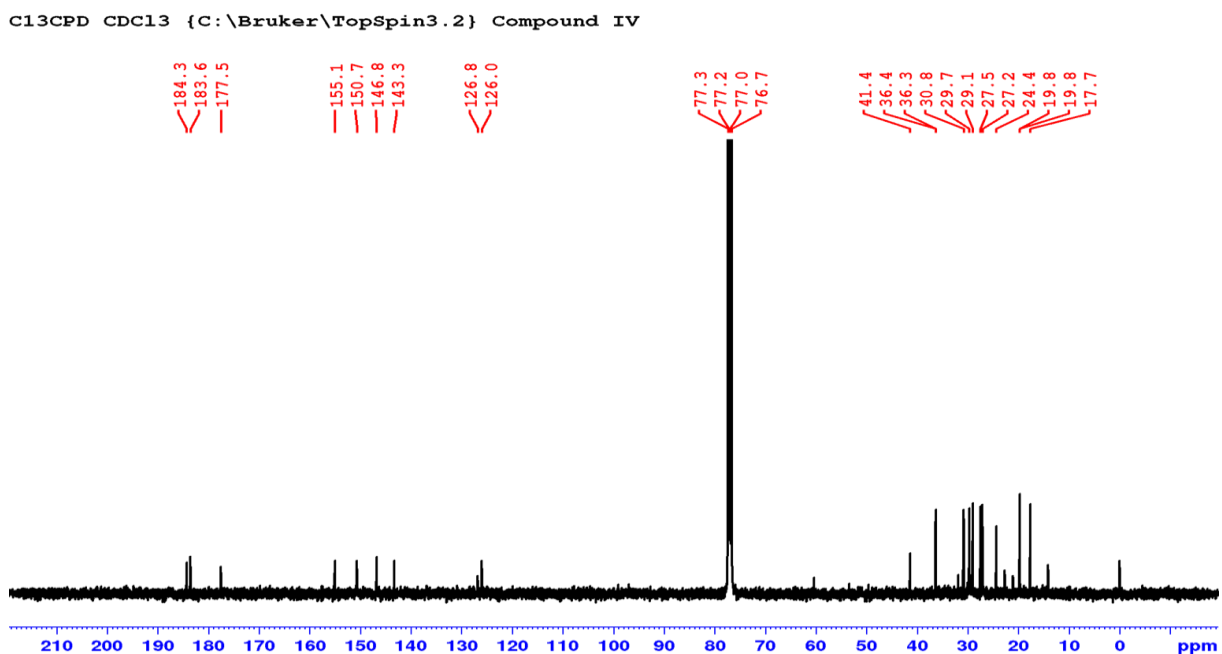
Figure 4.22: ^{13}C NMR spectrum of coleon U quinone (**IV**)
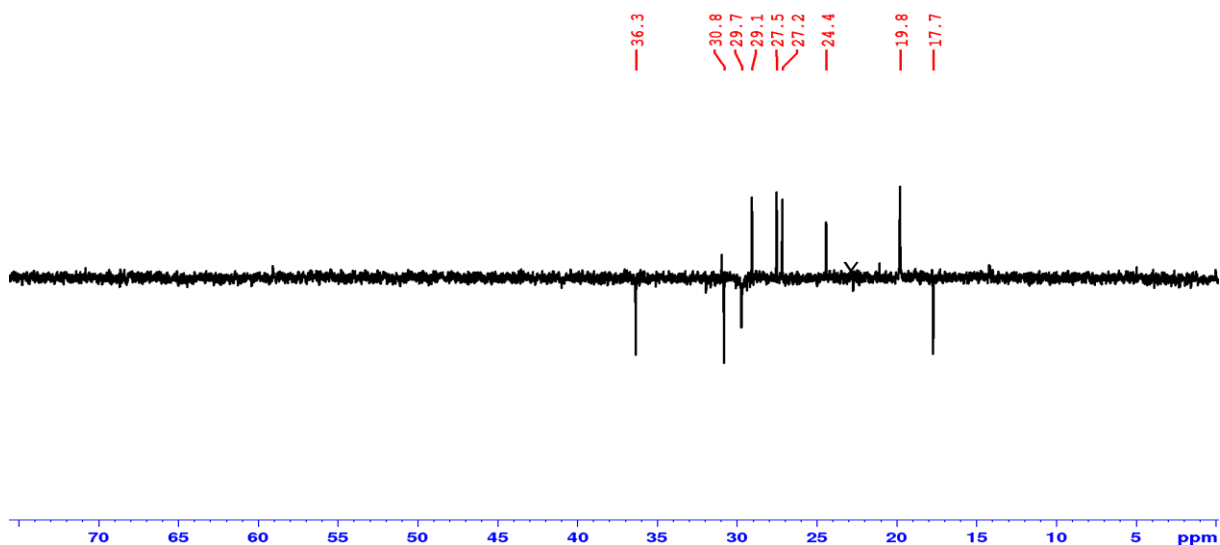
X: Impurity

Figure 4.23: DEPT-135 NMR spectrum of coleon U quinone (**IV**)

The above-mentioned data were identical to the coleon U quinone isolated by Alder et al. (1984) from *P. argentatus*

4.6.5 Compound V (carnosolon)

The ^{1}H NMR spectrum (Figure 4.23) showed signals of four methyl groups at δ_H 1.31 (*s*, Me-19); 1.06 (*s*, Me-18); 1.17 (*d*, *J* = 7.1 Hz, Me-17); 1.16 (*d*, *J* = 7.1 Hz, Me-16). It also showed a signal of a downfield proton attached to two methyls at δ_H 3.0 (*septet*, *J* = 7.1 Hz, H-15). The ^{1}H NMR signals showed a downfield proton attached to a benzene ring at δ_H 7. 65 (*s*, H-14), indicating the presence of a penta-substituted benzene ring. Signals of two low field non-equivalent protons attached to an epoxy group at δ_H 3.37 (*d*, *J* = 7.5 Hz, H$_\alpha$-20) and δ_H 4.29 (*d*, *J* = 7.5 Hz, H$_\beta$-20); in addition to a cluster of proton signals between δ_H 1.20 to δ_H 1.80 were identified (see Table 4.6 for more details).
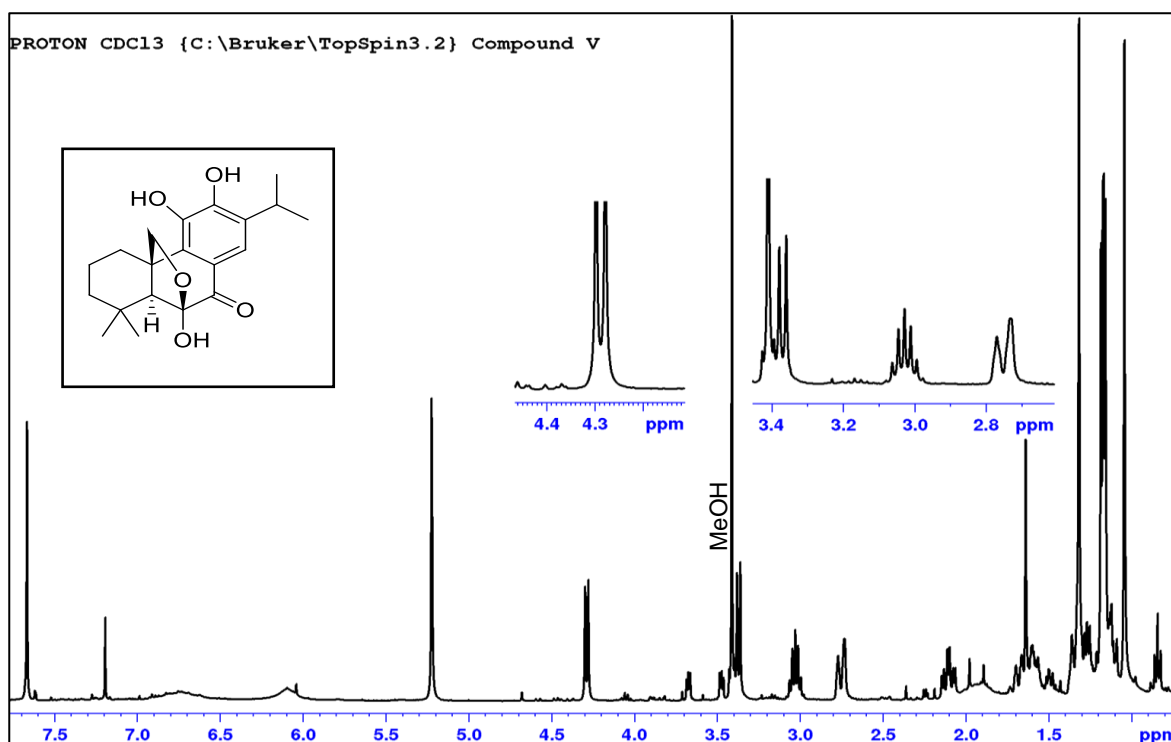
Figure 4.24: ^{1}H NMR spectrum of carnosolon (**V**)

The ^{13}C NMR spectrum (Figure 4.24) showed twenty signals and could be classified according to DEPT-135 (Figure 4.25) to four methyls, four methylenes; three methines and nine quaternary carbons with six of them forming the benzene structure (ring C), at δ_C 121.4 (C-8), 137.8 (C-9), 140.5 (C-11), 148.3 (C-12), 133.3 (C-13), and 120.1 (C-14). Furthermore, two oxygenated carbon forming an epoxy group was observed on the ^{13}C NMR data at δ_C 105.2 (C-6) and 72.3 (C-20), while the chemical shift of the C-6 (105.2) indicated the dioxygenated carbon present.

C13CPD CDC13 {C:\Bruker\TopSpin3.2} Compound V
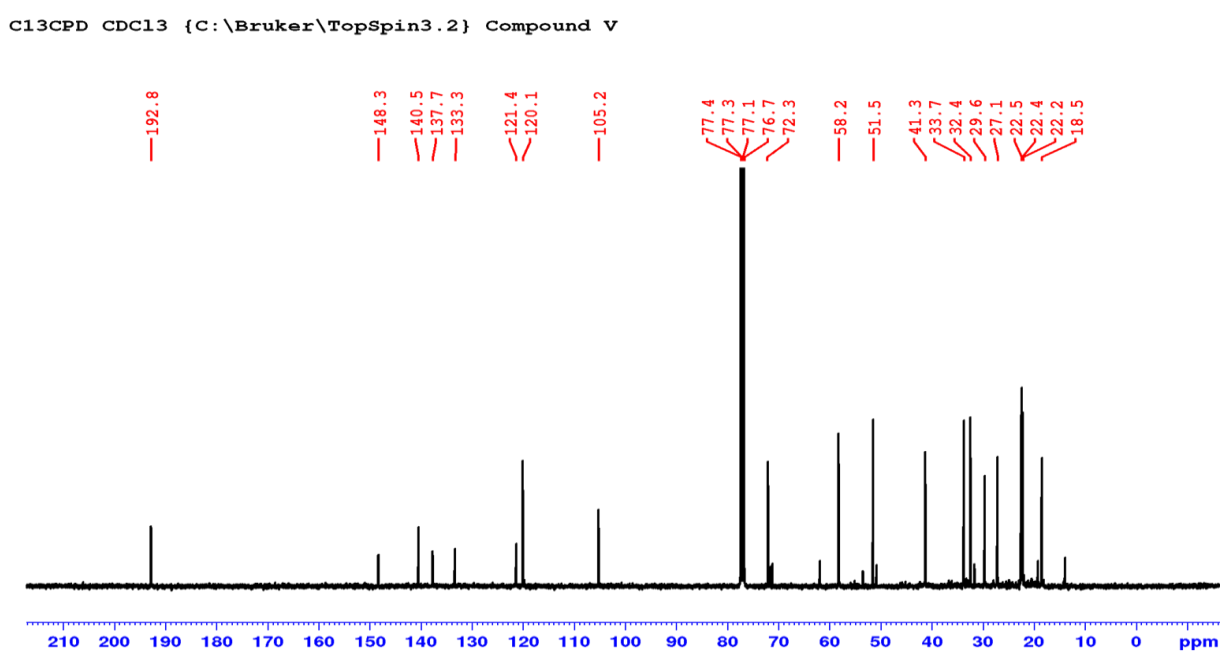
Figure 4.25: ^{13}C NMR spectrum of carnosolon (**V**)
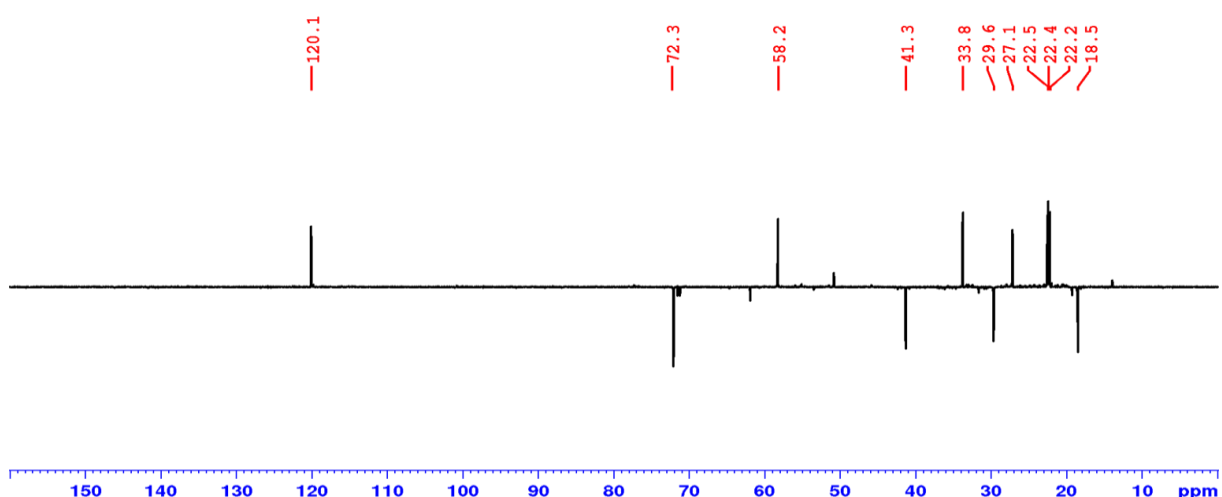
Figure 4.26: DEPT-135 NMR spectrum of carnosolon (**V**)
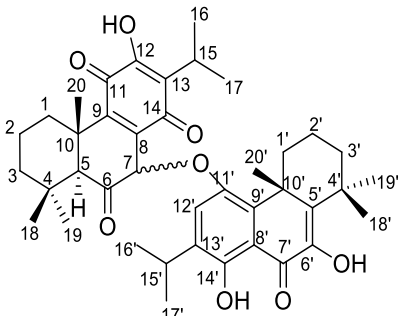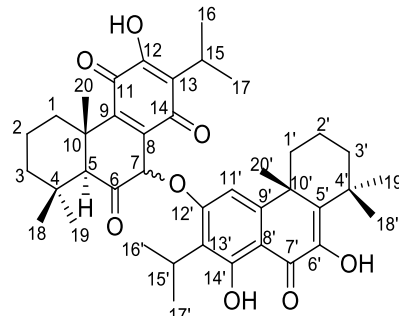
The above mentioned data were identical to carnosolon isolated by Horvath et al. (2004) from *P. cyaneus*.

4.6.6 Compounds VI (grandidone A) and VII (7-epimer grandidone A)

The isolated compound showed a single spot on the TLC (see Figure 4.5), and complex NMR signals suggesting the compound is a mixture and many trials to purify it further has been conducted; however, the same NMR signal profile remained.

The ^{1}H NMR spectrum (Figure 4.26) showed signals of two isopropyl group at δ_H 3.04 (m, H-15) and δ_H 3.47 (m, H-15') indicating the presence of a dimer. However, the ^{13}C NMR spectrum (Figure 4.27) showed eighty signals which were classified according to DEPT-135 (Figure 4.28) to twenty methyls, twelve methylenes, six methines, and twenty quaternary carbons (Table 4.8). Furthermore, it was observed that each signal came in duplicate, implying that the sample might be a mixture of dimers. Additionally, the ^{13}C NMR signals of the sample were compared to the ^{13}C NMR signals of grandidone A and 7-epimer grandidone A isolated by Uchida et al. (1981) from *P. grandidentatus*. Grandidone A and 7-epimer grandidone A ^{13}C NMR signals were similar to the sample ^{13}C NMR signals, implying that the sample is a mixture of grandidone A and 7-epimeric grandidone A (**VI & VII**). The sample was also characterised using 2D NMR spectroscopy (Figure 4.29 and Figure 4.30) but because the dimers are epimers only the ^{13}C NMR data was used to identify them as the only difference between the dimers ^{1}H NMR signals lies in their C-11' and C-12' ^{1}H NMR signals.

Table 4.8: ^{13}C NMR data of compounds **VI** and **VII** in CDCL$_3$

Position	VI	Uchida et al., 1981 (VI)	VII	Uchida et al., 1981: (VII)
		^{13}C		^{13}C
1	36.7	36.9	36.7	36.9
2	17.2	17.3	17.4	17.4
3	41.2	41.4	41.2	41.4
4	36.1	36.2	36.1	36.1
5	60.0	60.1	60.0	60.1
6	196.6	196.6	196.1	196.2
7	106.2	106.4	106.0	106.0
8	137.1	137.4	135.2	135.3
9	151.5	151.8	153.0	151.7
10	39.9	40.0	39.8	39.7
11	183.0	150.9, d	183.0	150.8,s
12	150.7	183.2	150.6	183.3
13	126.0	126.2	126.1	126.2
14	183.3	183.4	183.2	183.4
15	24.0	24.1	24.1	24.1
16	19.6	19.7	19.7	19.5
17	20.7	20.7	20.4	20.5
18	32.0	32.0	32.2	32.3
19	21.8	21.8	21.7	21.7
20	28.5	28.6	30.0	29.9
1'	29.7	29.9	30.7	30.7
2'	18.5	18.5	18.5	18.5
3'	35.9	36.1	35.9	36.0
4'	44.3	44.4	44.1	44.2
5'	142.2	142.3	142.1	142.3
6'	141.9	142.1	141.3	141.3
7'	182.5	182.7	182.3	182.5
8'	105.9	106.0	105.3	105.5
9'	130.5	130..0	130.6	130.7
10'	32.4	32.4	32.5	32.5
11'	133.7	133.9	133.6	133.8
12'	150.7	150.9	150.6	150.8
13'	116.3	116.4	116.3	116.4
14'	158.2	158	158.7	158.9
15'	24.3	24.1	24.3	24.3
16'	19.8	19.8	19.8	19.8
17	20.7	20.7	20.4	20.5
18'	27.3	27.5	27.2	27.3
19'	21.0	21.0	21.1	21.1
20'	27.9	28.0	27.9	27.9

PROTON CDCl3 {C:\Bruker\TopSpin3.2} Compound VI & VII

Figure 4.27: ^{1}H NMR spectrum of grandidone A and 7-epimer grandidone A

C13CPD CDCl3 {C:\Bruker\TopSpin3.2} Compound VI & VII
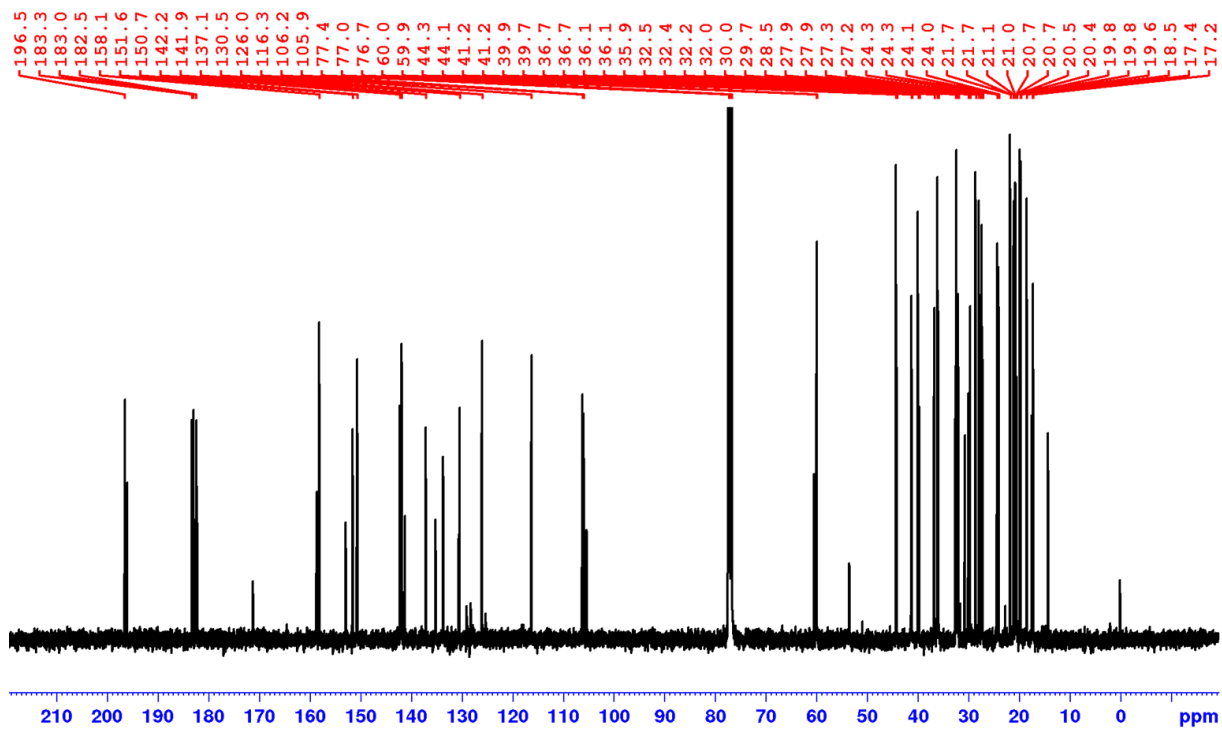
Figure 4.28: ^{13}C NMR spectrum of grandidone A and 7-epimer grandidone A
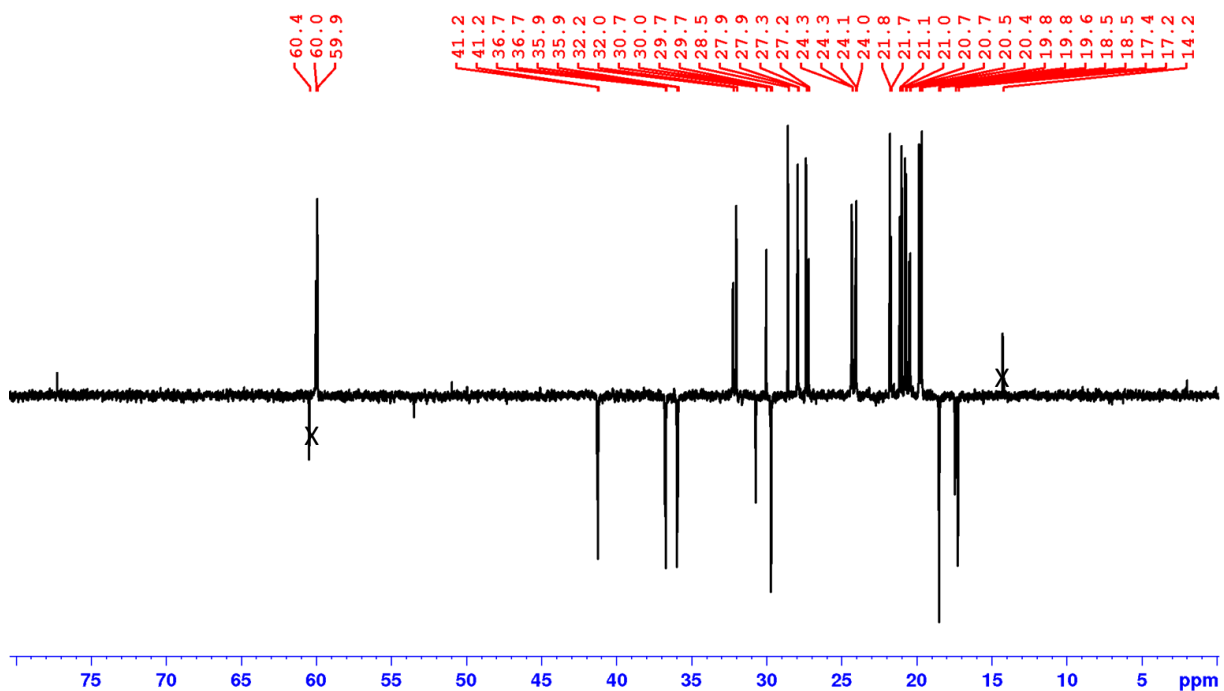
X: Ethyl acetate solvent peaks

Figure 4.29: DEPT-135 NMR spectrum of grandidone A and 7-epimer grandidone A
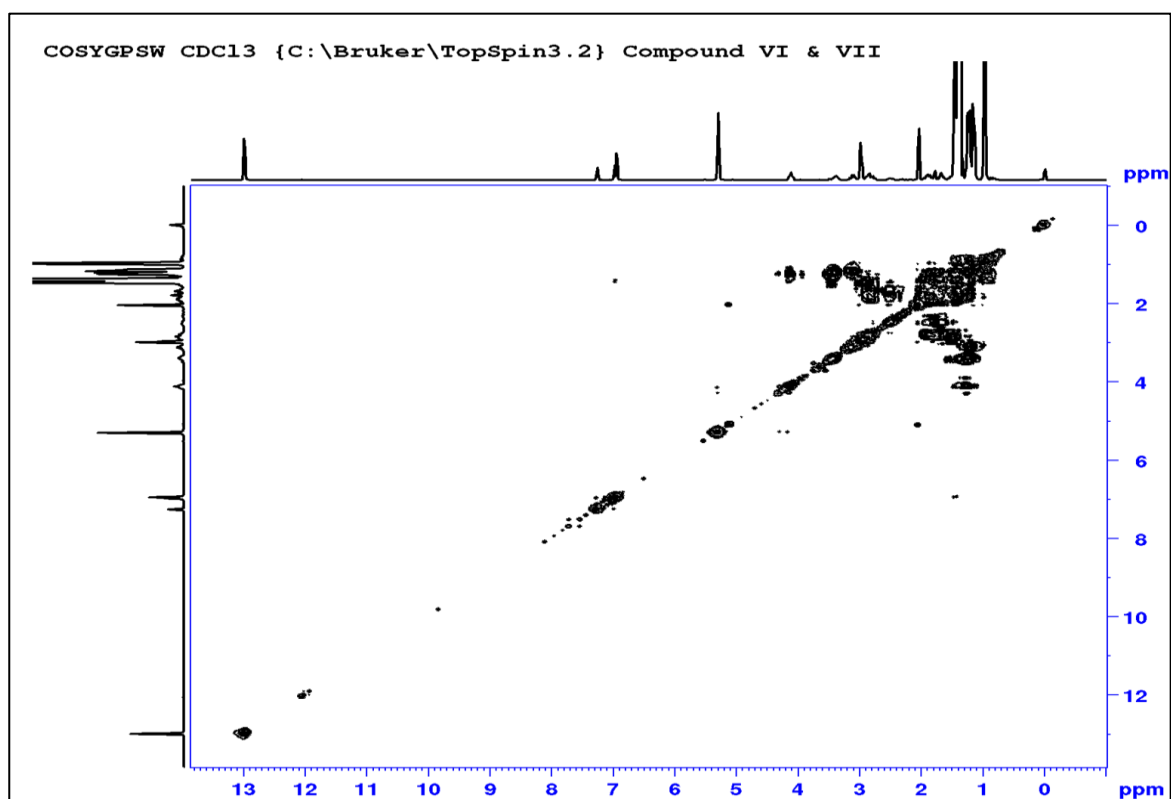
Figure 4.30: COSY NMR spectrum of grandidone A and 7-epimer grandidone A
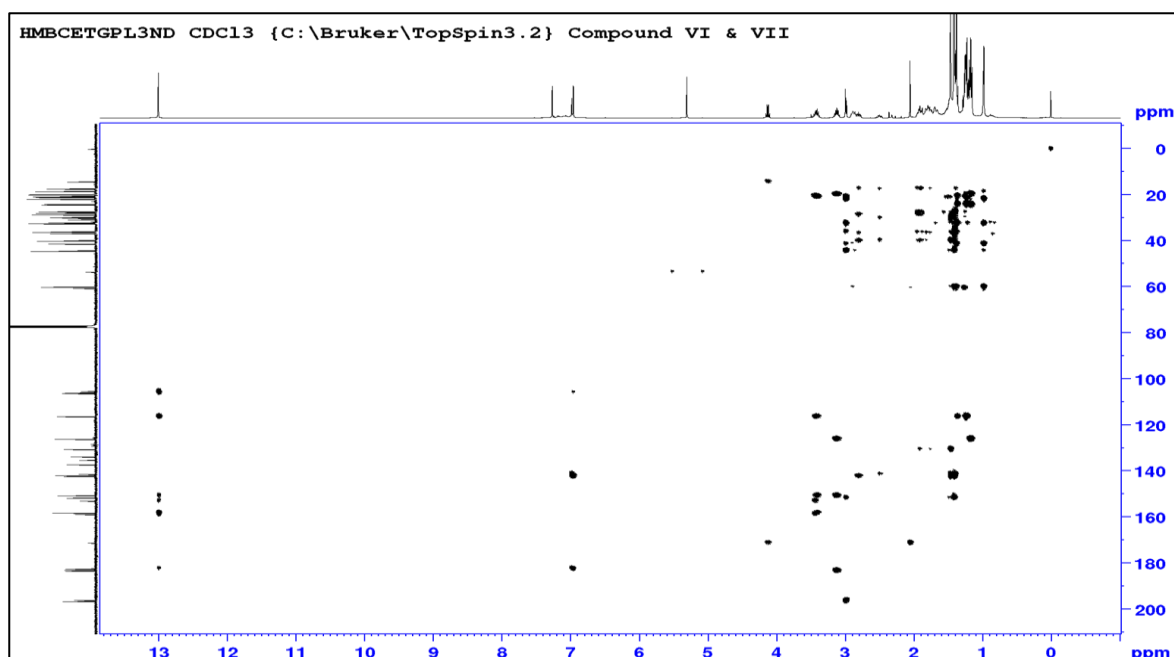
Figure 4.31: HMBC NMR spectrum of grandidone A and 7-epimer grandidone A

4.7 Spectroscopic data of the isolated compounds

6β,7α-dihydroxyroyleanone (C$_{20}$H$_{28}$O$_5$): Yellow crystal; melting point: 200°C; $[\alpha]_D^{20}$ = -53; IR: 3326 cm-1 (OH), 2938 cm-1 (CH), 1649 cm-1 (C=O), 1422 cm-1 (C=C), 1117 cm-1 (C-O); UV: 270 nm and 400 nm; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$): see Table 4.6.

7α-acetoxy, 6β-hydroroyleanone (C$_{22}$H$_{30}$O$_6$): Yellow crystal, melting Point: 209°C; $[\alpha]_D^{20}$ = +24; IR: 3292 cm-1 (OH), 2938 cm-1 (CH), 1674 cm-1 (C=O), 1457 cm-1 (C=C), 1209cm-1 (C-O); UV: 264 nm and 408 nm; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$): see Table 4.6.

Horminone (C$_{20}$H$_{27}$O$_4$): Yellow crystal, melting point: 180 °C; $[\alpha]_D^{20}$ = -130; IR : 3301 cm-1 (OH), 2947 cm-1 (CH), 1451 cm-1 (C=C), 1016 cm-1 (C-O); UV: 262 nm and 410 nm; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$): see Table 4.6.

Coleon U quinone (C$_{20}$H$_{24}$O$_5$): Yellow orange solid; IR: 3324 cm-1 (OH), 2946 cm-1 (C-H), 1649 cm-1 (C=O), 1000 cm-1 (C-O); UV: 260 nm and 424 nm; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$): see Table 4.6.

Carnosolon (C$_{20}$H$_{26}$O$_5$): Green solid yellow crystal; $[\alpha]_D^{20}$ = +62; IR: 3339 cm-1 (OH), 2934 cm-1 (C-H), 1594 cm-1 (C=O), 1440 cm-1 (C=C), 1299 cm-1 (C-O); UV: 278 nm, 404 nm ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$): see Table 4.6.

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Grandidone A ($C_{40}H_{48}O_9$) and 7-epimer grandidone A ($C_{40}H_{28}O_9$): Brown solid; $[\alpha]_D^{20} = +81$; IR: 3318 cm^{-1} (OH), 2947 cm^{-1} (CH), 1657 cm^{-1} (C=O), 1420 cm^{-1} (C=C), 1117 cm^{-1} (C-O); UV: 258 nm and 402 nm; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$): see Table 4.10.

4.8   Conclusion

The sequential chromatographic methods and techniques used on the main fractions K4-3-III & IV, K4-3-VI, K4-3-VIII, K4-3-XI, and K4-3-XIII & XIV resulted in the isolation of five pure known royleanone type abietane diterpenoids viz 6β,7α-dihydroxyroyleanone (**I**), 7α-acetoxy, 6β-hydroroyleanone (**II**), horminone (**III**), coleon U quinone (**IV**) and carnosolon (**V**). In addition to the isolated pure compounds, a mixture of epimeric dimers, namely grandidone A and 7-epimer grandidone A (**VI & VII**) was also isolated from the plant's extract, as illustrated in Figure 4.31. The weights of the compounds isolated from the main fractions (K4-3-III & IV, K4-3-VI, K4-3-VIII, K4-3-XI, and K4-3-XIII & XIV) is shown in Table 4.9. The structure elucidation and characterization of the isolated compounds were done by ^{1}H NMR, ^{13}C NMR, IR, UV, and optical rotation spectroscopic techniques, and the NMR data were compared with literature values. The X-ray crystallographic analysis of compound **II** and the 3D structure of compound **I** established the stereochemistry of compounds **I** and **II** as β and α at C-6 and C-7 positions respectively.

Table 4.9: Compounds isolated from main fractions (K4-3-III & IV, K4-3-VI, K4-3-VIII, K4-3-XI, and K4-3-XIII & XIV) and their weight

Main fractions	Isolated compounds and code	Weight of compounds (mg)
K4-3-III & IV	Horminone (**III**)	13.5
K4-3-VI	7α-acetoxy-6β-hydroxyroyleanone (**II**)	61.9
	Grandidone A and 7-epimer-grandidone A (**VI & VII**)	16.9
K4-3-VIII	6β,7α-dihydroxyroyleanone (**I**)	25.7
K4-3-XI	Coleon U quinone (**IV**)	6.0
K4-3-XIII & XIV	Carnosolon (**V**)	26.7
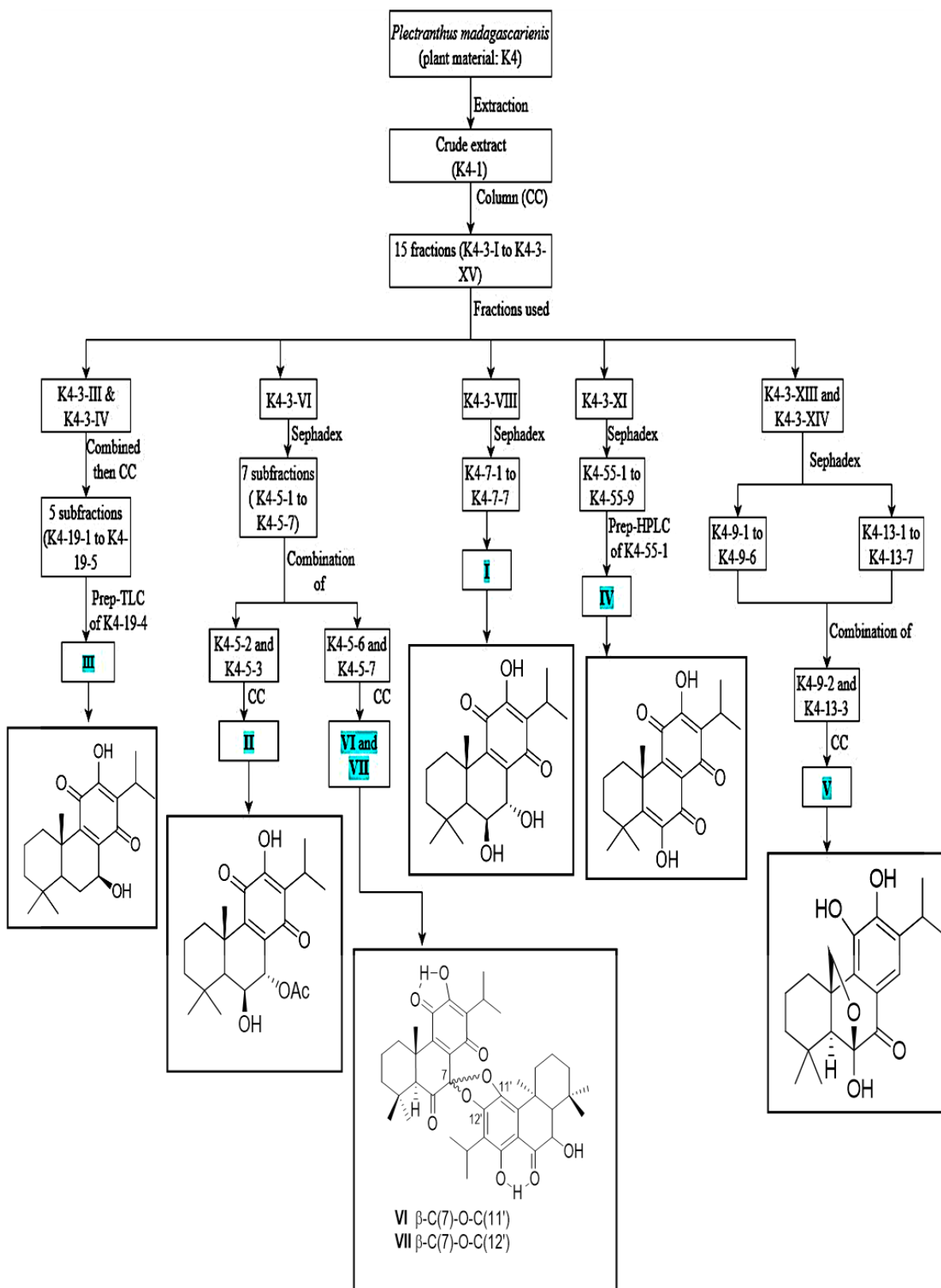
Figure 4.32: Methodology steps used in the isolation of bioactive compounds

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CHAPTER FIVE
BIOLOGICAL EVALUATION

5.1   Introduction

P. madagascariensis total extract and natural products were tested for their anti-diabetics, anti-tuberculosis, anti-psoriasis, and antioxidant activities. Some of the isolated compounds such as 6*β*,7*α*-dihydroxyroyleanone (**I**), 7*α*-acetoxy-6*β*-hydroxyroyleanone (**II**), and horminone (**III**) previously isolated from *P. grandidentatus* showed activity against *Mycobacterium tuberculosis* H_{37}Rv (Rijo et al., 2010). The 7*β* isomers of compound **I** and **II**, as well as coleon U quinone (**IV**) isolated from the study plant by Kubínová et al. (2014: 749-752), showed moderate to poor activity against α-glucosidase inhibitor. Compounds **I-VII** were never evaluated for their anti-psoriasis activity. However, compounds **I-IV** were found to possess cytotoxic activities against NCI-H460 (lung cancer), NCI-H460/R (lung cancer), MCR-5 (healthy lung) and MCF-7 (breast cancer) cell lines. Moreover, the epimeric dimers grandidone A (**VI**) and 7-epimer grandidone A (**VII**) previously isolated from *P. grandidentatus* are known for their cytotoxicity properties against human cancer cell lines TK-10, MCF-7, UACC-62, NCI-H460, and SF-268 (Gaspar-Marques et al., 2002: 839-840; Cerqueira et al., 2004: 217-223). Compound **V** (carnosolon) isolated before from *P. cyanus* is an efficient antioxidant agent and is the only compound amongst those isolated which has been assessed for its antioxidant activity (Naman, 2015: 93-97). To our knowledge, the cytotoxicity activity of the isolated compounds against HaCaT cells was evaluated for the first time. Moreover, compounds **V**, **VI** & **VII** were also assessed for their α-glucosidase and antimycobacterial activities for the first time.

In this chapter, the chemicals used during the biological studies are listed in section 5.2. Sections 5.3 to 5.6 describes the bioassays experimental procedures, cell cultures, data analysis, and interpretations of results. A conclusion about the tested compounds antituberculosis, antidiabetics, antioxidant, and anti-psoriasis inhibitory effects are given in section 5.7.

5.2   Chemicals

The chemicals used for the different bioassays are tabulated in Table 5.1

Table 5.1: Bioassays chemicals reagents

Chemicals	Suppliers
α-Glucosidase (*Saccharomyces cerevisiae)*	Sigma, Aldrich Cape Town, SA
P-nitro-phenyl-α-glucopyranoside	Sigma, Aldrich Cape Town, SA
Sodium carbonate	Kimix, Cape Town, SA

Sodium dihydrogen phosphate	Kimix, Cape Town, SA
Dimethyl sulfoxide	Kimix, Cape Town, SA
Green fluorescent protein	Sigma, Aldrich Cape Town, SA
Tyloxapol	Sigma, Aldrich Cape Town, SA
Albumin-Dextrose Complex	Sigma, Aldrich Cape Town, SA
D-glucose	Sigma, Aldrich Cape Town, SA
Casitone	Sigma, Aldrich Cape Town, SA
Tween 80	Sigma, Aldrich Cape Town, SA
7H9 broth medium	Sigma, Aldrich Cape Town, SA
Dibasic sodium phosphate	Sigma, Aldrich Cape Town, SA
Monobasic sodium phosphate	Sigma, Aldrich Cape Town, SA
Trypsin-EDTA	Sigma, Aldrich Cape Town, SA
Dulbecco's Modified Eagle Medium	Sigma, Aldrich Cape Town, SA
Fetal bovine serum	Sigma, Aldrich Cape Town, SA
Penicillin-streptomycin solution	Sigma, Aldrich Cape Town, SA
2,2′-azobis(2-amidinopropane) dihydrochloride	Sigma, Aldrich Cape Town, SA
Trolox	Sigma, Aldrich Cape Town, SA
L-ascorbic	Sigma, Aldrich Cape Town, SA

5.3   Antimycobacterial assay

The in vitro antimycobacterial activities of *P. madagascariensis* secondary metabolites, main fractions, and total extract against the green fluorescent protein (GFP) tagged *Mtb* $H_{37}Rv$ strain were carried out according to the standard broth microdilution method developed by Collins and Franzblau (1997), and Collins et al. (1998). In this assay, the mycobacterial strain $H_{37}Rv$ was cultured in the Middlebrook 7H9 broth medium supplemented with either Albumin-Dextrose complex, D-glucose, and tween 80 (7H9/ADC/Glu/Tw) or casitone, D-glucose, and tyloxapol (7H9/CAS/Glu/Tx). The minimum inhibition concentration at 90% (MIC_{90}) of the tested samples were scored visually at one week and two weeks' post-inoculation using the microplate Alamar blue dye assay (MABA) and expressed in µg/mL. The media (7H9/ADC/Glu/Tw and 7H9/CAS/ Glu/Tx), as well as 5% dimethyl sulfoxide (DMSO) were used as a negative control, and rifampicin was used as a positive control.

5.3.1   Cells culture

10 mL culture of *Mtb* $H_{37}Rv$ was grown to an optical density of 0.6-0.7 at 600nm (OD 600) at 37 ℃. The culture was then diluted with either 7H9/ADC/Glu/Tw or 7H9/CAS/Glu/Tx medium to a ratio of 1:100.

5.3.2 Experimental procedure

12.8 mM of stock solutions were prepared in DMSO and diluted to 640 mM in two 7H9 mediums (7H9/ADC/Glu/Tw and 7H9/CAS/Glu/Tx). In a 96-well microtiter plate, 100 µL of the diluted stock solution was added in duplicate to the wells in row 1 while 50 µL of 7H9 medium supplemented with ADC/Glu/Tw or with CAS/Glu/Tx was added to the wells in rows 2-12. Afterward, a two-fold serial dilution was done by transferring 50 µL of the solution in row 1 to row 2 using a multichannel pipette and aspirated to mix. The two-fold serial dilution procedure was replicated until row 12 was reached, from which 50 µL was discarded in order to bring the final volume in all the wells to 50 µL. Then, 50 µL of the 1:100 diluted *Mtb* culture is added to all wells in rows 2-12, except row 1, as this serves as a contamination control evaporation. The microtiter plate was sealed in a zip lock bag and incubated at 37 °C alongside a humidifier to prevent liquid evaporation.

5.3.3 Data analysis

The MIC_{90} values of the tested samples were determined using the dose-response curve (% inhibition) analysis of the relative fluorescence (excitation wavelength at 485 nm and emission wavelength at 520 nm) which were measured on the FLUOstar OPTIMA microplate reader. The dose-response curve was obtained by normalizing data to the minimum and maximum inhibition controls using the Levenberg-Marquardt algorithm method, from which the MIC_{90} was calculated.

5.3.4 Results and discussions

The MIC_{90} values of the tested substances after 7 and 14 days of post inoculation are tabulated in Table 5.2 and classified according to their potency.

Compounds and extracts with $MIC_{90} \leq 10$ µg/mL were stratified as active; those with MIC_{90} varying between 10-20 µg/mL were indicated as having moderate activity, and those with MIC_{90} between 20-125 µg/mL were indicated with poor activity. Any MIC_{90} values >125 µg/mL were considered as inactive.

Table 5.2: The antimycobacterial activities of the isolated compounds, main fractions, and total extract at 90% inhibition in 7H9/ADC/Glu/Tw and 7H9/CAS/ Glu/Tx media.

	Identification code of samples	7H9/CAS/ Glu/Tx (µg/mL)		7H9/ADC/Glu/Tw (µg/mL)	
		7 days	14 days	7 days	14 days
Isolated compounds	I	62.5	60.62	>125	>125
	II	15.22	15.63	14.36	14.64
	III	14.34	3.96	>125	>125
	IV	15.62	1.93	>125	>125
	V	>125	>125	>125	>125
	VI & VII	>125	>125	>125	>125

Positive Control	Rifampin	0.015	0.035	0.001	0.002
Main fractions	K4-3-III & IV	>125	>125	>125	>125
	K4-3-VI	31,25	31,864	63,095	>125
	K4-3-VIII	16,189	14.71	>125	>125
	K4-3-XI	16,043	7.318	>125	>125
	K4-3-XIII & XIV	64,241	14.375	>125	>125
Total extract	K4-1-Hex	31.42	16.04	>125	>125

The antimycobacterial evaluation of the isolated compounds against *Mtb* H_{37}Rv showed that the media in which the cells were cultured impinged on the potency of the tested substances. As observed, the isolated compounds showed activities only in the presence of 7H9/CAS/Glu/Tx medium except for compound **II** and main fraction K4-3-VI that displayed moderate activities in both medium (7H9/ADC/Glu/Tw and 7H9/CAS/Glu/Tx) from 7-14 days. This result may imply that the acetate group at the position 7α in ring B of compound **II** is liable to the inhibitory activity in 7H9/ADC/Glu/Tw medium. This statement is verified as only the main fraction (K4-3-VI) containing compound **II** showed little activity against *Mtb* H_{37}Rv in 7H9/ADC/Glu/Tw media at 7 days. Moreover, it was observed that the antimycobacterial potency of the isolated compounds increased from 7-14 days especially for compounds **III** and **IV** with MIC_{90} values varying from 15.62-1.93 µg/mL and 14.34-3.93 µg/mL, respectively. Considering that the structure of the compounds **I** differs from that of compound **II** only by the presence of β-hydroxy group at C-6 and compound **I** (60.62 µg/mL) had a poor antimycobacterial activity than compound **III** (3.96 µg/mL), it was inferred that the 6β-hydroxy group reduces the compound's antimycobacterial potency. This observation can imply that *p*-benzoquinone ring C responsible for the antimycobacterial activities of several quinone compounds, as well as the substituents at C-6 and C-7 in ring B influence considerably the activity (Rijo et al., 2010). Compounds **V** and **VI & VII** did not show activity against the *Mtb* H_{37}Rv in both mediums with MIC_{90} values higher than 125 µg/mL. However, main fractions K4-3-XIII & XIV and K4-3-VI comprising these bioactive compounds (**V** and **VI & VII**) showed weak to moderate activities between 7-14 days. This suggests that the isolated compounds are not responsible for the main fractions' inhibitory effects. The total extract antituberculosis activity went from poor to moderate activity with an MIC_{90} that was reduced by half from 7-14 days showing the synergy between the bioactive compounds present in the total extract.

5.4   *In vitro* α-glucosidase assay

The *in vitro* α-glucosidase inhibition assay was conducted according to Telagari and Hullatti (2015) method with acarbose as a positive control. However, the half maximal inhibition concentration (IC_{50}) of acarbose was above 610.4 µg/mL. In this assay, the IC_{50} of the tested substances was determined by measuring the release of *p*-nitrophenol from the *p*-

nitrophenyl-α-glucopyranoside (*p*-NPG) substrate. This method helps in determining the compounds and extracts capable of slowing down and regulate glucose absorption (Li et al., 2018).

5.4.1 Phosphate buffer

20.209 g of dibasic sodium phosphate and 3.394 g of monobasic sodium phosphate were mixed and added to 800 mL of deionised water. The pH of the solution was adjusted to 6.8 using sodium hydroxide and topped up to 1L with distilled water.

5.4.2 Experimental procedure

The isolated compounds and total extract were dissolved in MeOH to produce a stock solution of 1 mg/mL, while the total extract was dissolved in MeOH to make a stock solution of 2 mg/mL. In 96 well plates, 50 µl phosphate buffer (100mM), 10 µl alpha-glucosidase (1 U/mL), and 20 µL of the tested samples were added and pre-incubated at 37 °C for 15 min. Different concentrations of the isolated compounds (500-3.25µg/mL), and total extract (1000-100 µg/mL) were tested. The enzyme reaction was started by quickly adding 20 µL of the substrate *p*-NPG (5 mM) before incubation at 37 °C for 20 min. Then, the enzyme reaction was ceased by adding 50 µL of sodium carbonate (0.1 M). The change of absorbance was measured at wavelength 405 nm using a microplate reader.

5.4.3 Data analysis

The inhibitory effect of the isolated compounds, main fractions, and total extract were calculated according to the following formula:

$\frac{A_s - A_c}{A_c} \times 100$; where A_s is the absorbance of the tested samples and A_c is the absorbance of the positive control.

The IC$_{50}$ of each sample was obtained by performing a nonlinear regression using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA).

5.4.4 Results and discussions

The inhibitory effects of *P. madagascariensis* total extract and isolated compounds against the intestinal enzyme α-glucosidase are summarized in Table 5.3. The positive control acarbose (IC$_{50}$ = 610.4 µg/mL) had the lowest IC$_{50}$ among the tested samples implying that all the tested compounds have inhibitory effects against the enzyme α-glucosidase (Table 5.3).

Table 5.3: Activity profile of the IC_{50} values of *P. madagascariensis* isolated compounds and total extract against α-glucosidase

	Identification code of samples	IC_{50} (µg/mL)
Isolated compounds	I	128.50 ± 7.6
	II	94.06 ± 2.4
	III	280.6 ± 3.5
	IV	72.58 ± 0.8
	V	208.3 ± 6.5
	VI & VII	4.22 ± 0.4
Positive Control	Acarbose	610.4
Total extract	K4-1-Hex	156.4 ± 1.3

The inhibitory activities of the bioactive compounds viz 6*β*,7*α*-dihydroxyroyleanone (**I**), 7*α*-acetoxy-6*β*-hydroxyroyleanone (**II**) and coleon U quinone (**IV**) against the enzyme α-glucosidase were similar to the one obtained by Kubínová et al. (2014) except for compound **IV** which showed greater activity with an IC_{50} of 72.58 µg/mL. The epimeric dimers grandidone A and 7-epimer grandidone A (**VI & VII**) as well as compounds **II** and **IV** showed significant inhibition potencies against α-glucosidase with IC_{50} of 4.22 µg/mL (**VI & VII**), 94.06 µg/mL (**II**) and 72.58 µg/mL (**IV**) respectively. Based on these results, these two pure compounds (**II** and **IV**) and the epimeric dimers (**VI & VII**) can slow down the hydrolysis or digestion of the starch by α-glucosidase enzymes which will result in a suppression of hyperglycaemia and better regulation of blood glucose level (Figure 5.1 and Figure 5.2). Furthermore, the mixture of dimers which was never reported as α-glucosidase inhibitors, displayed a strong inhibitory effect against α-glucosidase. Compounds **I, III** and **V** showed activity with an IC_{50} of 128.50 µg/mL, 280.6 µg/mL, and 208.3 µg/mL respectively. The total extract K4-1-Hex showed moderate activity against α-glucosidase with IC_{50} of 156.4 µg/mL.
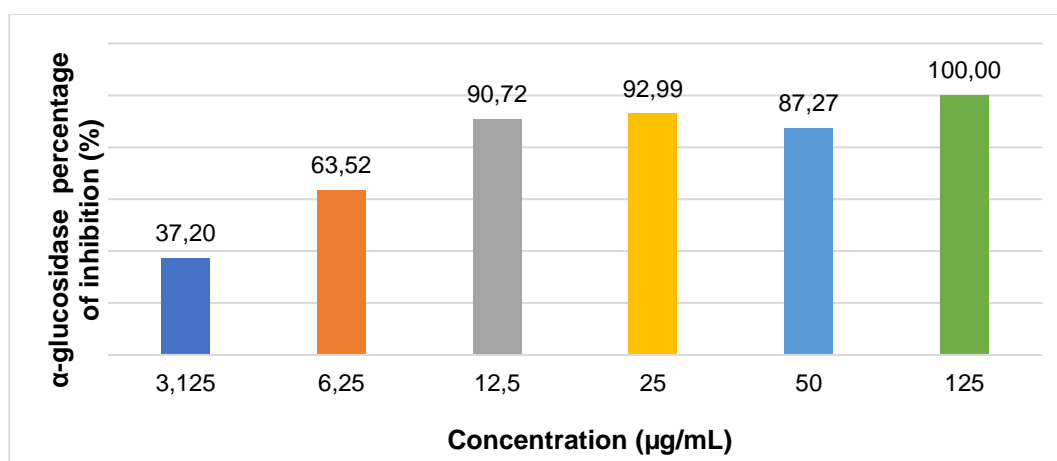
Figure 5.1: α-glucosidase percentage inhibition of the grandidone A and 7-epimer grandidone A (**VI & VII)**.
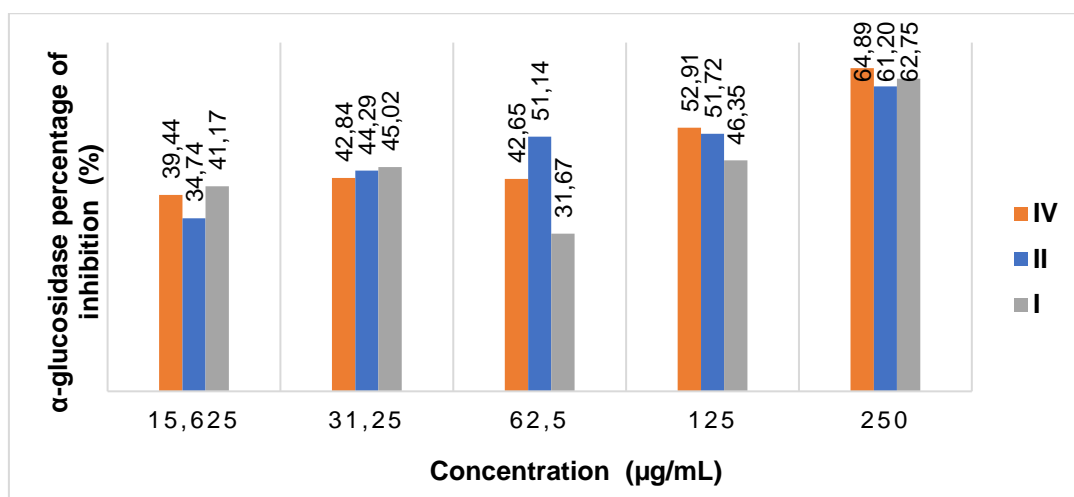
Figure 5.2: α-glucosidase percentage of inhibition of compounds **I**, **II,** and **IV**.

5.5   *In vitro* Cytotoxicity Assay

The immortalized human skin epithelial keratinocytes (HaCaT) cell proliferation after treatment with *P. madagascariensis* total extract and isolated compounds was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reported by Mossmann (1983). The assay was used to determine the *in vitro* cytotoxicity activity of the isolated abietane diterpenoids and total extract against the HaCaT cells with tamoxifen as a positive control.

5.5.1   Cells Culture

HaCaT cells were cultured in Dulbecco's modified eagle medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (penstrep). The HaCaT cells were grown for 4-5 days at 37 ◦C in a 5% carbon dioxide atmosphere. The cells were removed from the culture using trypsin-ethylenediaminetetraacetic acid solution (trypsin-EDTA) and transferred into a 96 well plate.

5.5.2   Experimental procedure

Stock solutions of 1 mg/mL of each sample were prepared in 10% DMSO and diluted to 100 µg/mL in the complete DMEN medium. It was followed by ten-fold serial dilution (100-0.001 µg/mL) and two-fold serial dilution (100-25 µg/mL) of the stock solutions. The positive control tamoxifen was also subjected to comparable dilutions process. The tested samples were applied to the cells and incubated at 37 °C for 24 hours. Thiazolyl blue (MTT, SIGMA) dissolved in phosphate buffered saline solution (PBS) at a final concentration of 0.8 mg/mL, was added to the cells that were exposed to the isolated compounds and total extract before incubation at 37°C in the dark for 4 hours. After observing the MTT change of colour (blue to purple), the media was separated and washed with PBS. The produced formazan

salts were dissolved with DMSO, and their concentrations were obtained by measuring their absorbance at 570 nm in a spectrophotometer.

5.5.3   Data analysis

All experiments were performed in triplicate, and recorded absorbances were used to determine the compounds (**I-VII**) and total extract cytotoxic activities against HaCaT cells. The IC_{50} values of the tested compounds were determined using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). The dose-response curve (Figure 5.3) was obtained by normalizing data to the minimum and maximum inhibition controls using the Levenberg-Marquardt algorithm method, from which the IC_{50} was calculated.

5.5.4   Results and discussions

The percentage of proliferation of the isolated compounds and total extract against HaCaT cells is presented in terms of the log dose-response curve (Figure 5.3) with their IC_{50} values summarised in Table 5.4.

From the results obtained, the total extract and isolated compounds inhibit HaCaT cells in a dose-response dependent manner. Compounds **IV** and **V,** as well as the total extract (K4-1-Hex), were the most active test substances with IC_{50} of 33.88 µg/mL, 27.52 µg/mL, and 28.18 µg/mL, respectively. The royleanone 7*α*-acetoxy-6*β*-hydroxyroyleanone, 6*β*,7*α*-dihydroxyroyleanone, and horminone were moderately active against HaCaT cells whereas the dimers grandidone A and 7-epimer grandidone A showed poor antiproliferation inhibitory effect. The total extract (IC_{50} = 28.18 µg/mL) which contains all the isolated compounds showed a good inhibitory effect as well as the synergetic effect of the bioactive compounds to suppress HaCaT cells. Furthermore, Diogo et al. (2019) stated that the difference between the cytotoxicity effect of royleannone type abietane lies on the polarity and lipophilicity of the substituent at 7α position as well as on the presence of an electron-donating group at position 6. It was clearly proven by the IC_{50} values of compounds **I-III** which demonstrated that compound **II** is more potent than compounds **I** and **III** because the acetate group at position 7α of compound **II** is more polar than the hydroxyl group at position 7α of compounds **I** and **III**. It was also observed that compound **III** is more active than compound **I**, which implies that the substituents at position 6 of royleanones type abietane does not really affect the compounds cytotoxicity effects against HaCaT. González (2014) stated that aromatic abietane diterpenoids with catechol containing molecules and a carbonyl group at C-7 as well as coleons type abietane with diosphenol moiety in ring B displayed significant cytotoxicity activity. This statement was corroborated by compounds **IV** and **V** which were found to be the most active isolated compounds with an IC_{50} of 33.88 µg/mL and 27.52 µg/mL. Grandidone A, which was reported to have cytotoxicity activity showed weak antiproliferation activity when combined with 7-epimer

grandidone A. It can be suggested the combination of these epimeric dimers can barely reduce the proliferation of lymphocyte apoptosis in HaCaT cells or they have antagonistic effect when combined.

Table 5.4: The cytotoxicity activities of the *P. madagascariensis* isolated compounds and total extract against HaCaT cells.

Samples	IC$_{50}$ (µg/mL)
I	28.18 ± 2.42
II	60.25 ± 3.95
III	42.66 ± 1.22
IV	53.70 ± 0.67
V	33.88 ± 1.43
VI & VII	75.86 ± 1.31
K4-1-Hex	28.18 ± 0.84
Positive control (Tamoxifen)	22.00
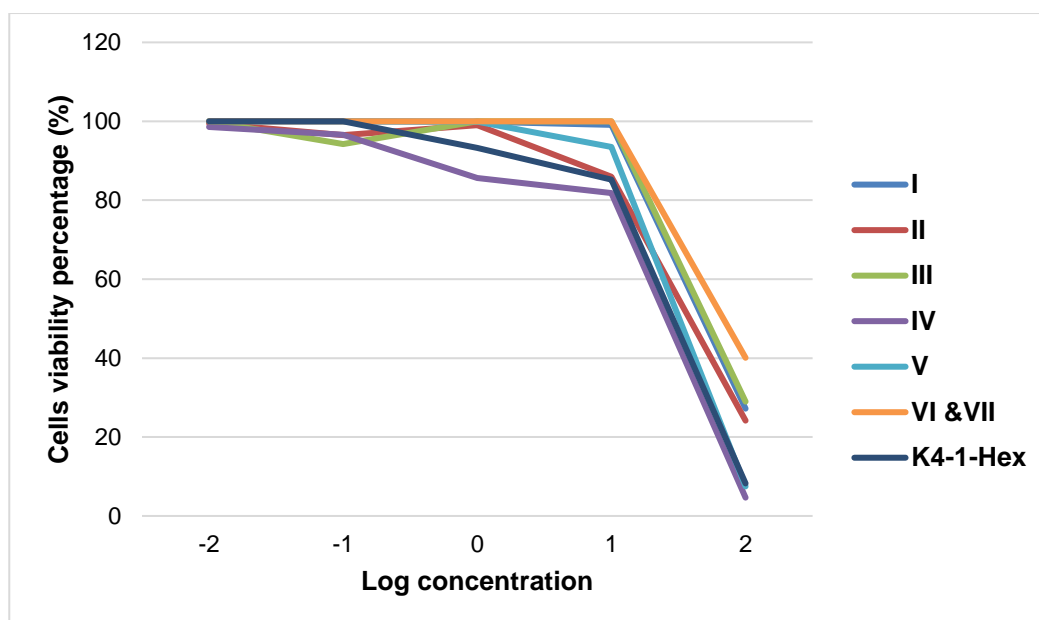
Figure 5.3: Dose-response curve of *P. madagascariensis* constituents and total extract against HaCaT cells

5.6 Antioxidant assay

The antioxidant activities of compound **I-VII** was assessed using three different free radical scavenging assays, namely ferric ion reducing antioxidant power **(**FRAP), automated oxygen radical absorbance capacity (ORAC), and Trolox equivalent absorbance capacity (TEAC).

The FRAP assay was conducted according to Benzie and Strain's (1996) method, which consists of finding the compounds' antioxidant potential in terms of µM ascorbic acid equivalents per milligram dry weight (µM AAE/g).

The ORAC assay was conducted according to Prior et al. (2003) method, which is a modified version of the original ORAC assay developed by Cao and Prior (1998: 50-62). In this assay, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) underwent thermal decomposition to generate peroxyl radicals resulting in the reduction of fluorescent from fluorescein. The antioxidant potential of the isolated compounds was measured by their ability to prevent any loss of fluorescent from fluorescein by neutralizing peroxyl radicals and assessing the sample's fluorescence area under the curve (AUC). ORAC values were expressed as µM Trolox equivalents (TE) per milligram of test samples (µM TE/L).

The TEAC assay was done according to the method developed by Pellegrini et al. (1999), which consists of determining the activities of the isolated compounds in terms of µM Trolox equivalents per milligram dry weight (µM TE/g) of the test samples.

L-ascorbic was used as a standard in FRAP assay with concentrations varying between 0 and 1000 µM (Figure 5.4). On the other hand, Trolox was used as the standard for ORAC and TEAC assays with concentrations ranging from 0-500 µM (Figure 5.5). The antioxidant epigallocatechingallate (EGCG) was used as a positive control.

5.6.1   FRAP assay

In a 96 well plate, 10 µL of the 100 µg/mL of the isolated compounds (**I-VII**) and total extract (K4-1-Hex) stock solutions were added to 300 µL of FRAP reagent. The FRAP reagent was prepared by mixing 30 mL of acetate buffer (300 mM, pH 3.6), 3mL of tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and 3mL of $FeCl_3.6H_2O$ (20 mM) in a 50 mL conical tube. After the addition of the samples' stock solutions to the FRAP reagent, the 96 well plate was incubated at room temperature for 30 minutes. The antioxidant potential was read at a wavelength of 593 nm in a Multiskan (Thermo Fisher Scientific) spectrum plate reader.

5.6.2   ORAC assay

An illuminated 96 well plate was used to mix 12 µL of 100 µg/mL of each sample and 138 µL of a fluorescein working solution. This was followed by the addition of 50 µL of AAPH prepared by adding 6 mL phosphate buffer to 150 mg of AAPH. The Fluoroskan spectrum plate reader was programmed to record fluorescein's fluorescence every 2 minutes after the addition of AAPH with the excitation wavelength at 485 nm and the emission wavelength at 530 nm. The Trolox stock solution was used as standard with concentration varying between 0-417 µM. The ORAC values were calculated using a regression equation ($Y = mx + c$) between Trolox concentration (Y in µM) and the net area under the fluorescence decay curve (X).

5.6.3 TEAC assay

The stock solution used is a mixture of 88 µL of 140mM of potassium peroxodisulphate ($K_2S_2O_8$) and 5 mL of 7 mM of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in a 15 mL screw cap. The prepared ABTS solution was kept in a dark place at room temperature for 24 hours before use. The following day the prepared ABTS solution was diluted with ethanol (about 1 mL of ABTS solution mixed with 20 mL of ethanol) to be able to read the absorbance of approximately 2.0 (± 0.1). Trolox was used as the standard with concentrations ranging between 0-500 µM. In a 96 well plate, 25 µL of the standards and isolated compounds were added to 300 µL of ABTS mixture and incubated at room temperature for 30 minutes. The absorbance was recorded by a Multiskan spectrometer plate reader at wavelength 734 nm.

5.6.4 Results and discussions

The antioxidant activities of compounds **I-VII** were assessed using different free radical scavenging assays, namely ORAC, TEAC, and FRAP as summarised in Table 5.5.

Table 5.5: Antioxidant activity of the isolated compounds

Compounds	TEAC (µM TE/g)	FRAP (µM AAE/g)	ORAC (µM TE/L)
I	5080.8 ± 0.04	541.5 ± 1.59	23625 ± 1.64
II	3700.8 ± 1.50	345.5 ± 0.77	21404.1 ± 4.35
III	Inactive	296.5 ± 6.72	12443.3 ± 3.61
IV	225.5 ± 1.47	2109.6 ± 2.78	21857.8 ± 5.85
V	4876.3 ± 0.49	13772.2 ± 2.76	29287.4 ± 4.75
VI & VII	3981.9 ± 0.16	1154.1 ± 2.60	11803.2 ± 1.50
Positive control (EGCG)	4722.5 ± 2.22	10455.1 ± 0.81	14970 ± 5.53

Based on the results obtained, compound **V** is the most potent antioxidant compound as it displayed a remarkable antioxidant inhibitory effect in all assays. Compound V ability to suppress radicals chain reactions via hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms is due to the catechol groups in the benzene ring. Compound **I** showed very little activity in FRAP assay. However, in TEAC and ORAC assays, compound **I** displayed good antioxidant activities suggesting that compound **I** neutralize the radical cation generated by ABTS as well as the peroxyl radicals generated from the thermal decomposition of AAPH by HAT mechanism (Zhong & Shadidi, 2015). Compounds **II** and **IV** showed good activity in ORAC assay and weak antioxidant activities in TEAC and FRAP assays. At last, none of the isolated compounds showed significant activity in the FRAP assays classifies as SET methods except for compound **V**.

5.7   Conclusions

The antimycobacterial, anti-psoriasis, antidiabetic and antioxidant evaluation of the isolated compounds showed that compound **IV** is the most remarkable one due to its ability to inhibit *Mtb* H$_{37}$Rv in 7H9/CAS/Glu/Tx medium as well as HaCaT cells. Moreover, compound **IV** can prevent peroxyl free radical formation by the HAT mechanism as well as slowing down glucose hydrolysis by the intestinal enzyme α-glucosidase.

Compounds **I** showed little activity in all assays except for the antioxidant assay TEAC and ORAC. This implies that compound **I** is an antioxidant agent that can only suppress free radicals by HAT mechanisms. Compound **II** displayed mild activity against *Mtb* H$_{37}$Rv in both medium (7H9/CAS/Glu/Tx and 7H9/ADC/Glu/Tw). Like compound **I**, the antioxidant activity of compound **II** is pronounced only in assays that suppress free radicals by the HAT mechanism. Compound **II** also showed moderate inhibitory effects against the enzyme α-glucosidase and HaCaT cells. Compound **III** exhibited good antimycobacterial property in 7H9/CAS/Glu/Tx media, but it has a poor antioxidant and cytotoxicity properties. Compound **V** is the most potent antioxidant and cytotoxic agents among the isolated compounds. Furthermore, compound **V** can stop the free radical chain reaction by SET and HAT mechanisms. The mixture of dimers (**VI & VII**) displayed strong α-glucosidase inhibitory effect and mild antioxidant activity in assays classified as HAT methods. However, the mixture (**VI & VII**) cytotoxic inhibitory effect against HaCaT cells is weak.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1   Conclusions

This research study aimed to extract, isolate, characterize, and evaluate the antitubercular, antidiabetic, and anti-psoriasis properties of the phytochemical constituents of the aerial parts of *P. madagascariensis*. Through the phytochemical screening of P. madagascariensis semi-polar extract via chromatographic methods, it was found that the plant is rich in abietane diterpenoids, but it also possesses triterpenoids and caffeic acids. The bioactive compounds of this medicinal plant were isolated from the plant extract using chromatographic techniques such as column chromatography, prep-TLC, Sephadex LH-20, and semi-prep HPLC. The chemical study of *P. madagascariensis* led to the isolation of seven compounds consisting of five pure compounds and a mixture of two dimers. The structural elucidation of the seven bioactive compounds isolated from *P. madagascariensis* was done using the spectroscopic techniques namely NMR, X-ray, UV, and FTIR. From the NMR, X-ray, UV and FTIR spectrums, compounds **I, II, III, IV**, **V** were determined to be royleanone type abietane diterpenoids, whereas compounds **VI** and **VII** are abietane dimers. The royleanones $6\beta,7\alpha$-dihydroxyroyleanone (**I**), 7α-acetoxy-6β-hydroxyroyleanone (**II**), horminone (**III**) and coleon U quinone (**IV**) and carnosolon (V), as well as the dimers grandidone A and 7-epimer grandidone A (**VI & VII**), were evaluated for their antitubercular, antidiabetic, antioxidant and cytotoxic activities. The X-ray crystallography of compound II showed that compound I and compound II have the 6β, 7α stereochemical configurations

The antitubercular activity of compounds **I-V** and the mixture **VI & VII** showed that the medium, in which the *Mtb* bacillus is cultured, affects the percentage of inhibition of the compounds. Moreover, the *p*-benzoquinone ring C responsible for the antimycobacterial activities of several quinone compounds, as well as the substituents at C-6 and C-7 in ring B, influence the activity considerably. The royleanone 7α-acetoxy-6β-hydroxyroyleanone is the only isolated compound to show activity against *Mtb* H_{37}Rv in 7H9/ADC/Glu/Tw media due to the acetate group at position 7α in ring B. Coleon U quinone and horminone effectively inhibit the *Mtb* H_{37}Rv in the 7H9/CAS/Glu/Tx medium, after 14 days with MIC_{90} values of 1.93 µg/mL and 3.96 µg/mL respectively. The MIC_{90} values of compound **I-III** demonstrated that the hydroxy group at position 6β reduced the compound's antimycobacterial activities. Carnosolon and the epimeric dimers grandidone A and 7-epimer grandidone A are inactive against *Mtb* H_{37}Rv in both mediums with MIC_{90} values higher than 125 µg/mL.

The cytotoxicity activity of the isolated natural products showed that compounds **IV** and **V** are good cytotoxicity agents against HaCaT cells with IC_{50} of 33.88 µg/mL and 27.52

µg/mL. Compounds **I-III** displayed moderate activity against HaCaT cells, whereas the mixture **VI & VII** showed poor antiproliferation activities. The epimeric dimers (**VI & VII**) showed little to no activity in antioxidant, antituberculosis, and cytotoxicity assays. However, they exhibited a strong inhibitory effect against the intestinal enzyme α-glucosidase with IC_{50} 4.22 µg/mL. The roylenaone **I**, **II,** and **IV** showed mild activity with IC_{50} of 128.50 µg/ml, 94.06 µg/mL, 72.58 µg/mL respectively. Compound **I**, **III,** and **V** exhibited weak (**II**) to no activity (**III** and **V**) against the enzyme α-glucosidase.

The free radical scavenging assays (ORAC, TEAC, and FRAP) done on *P. madagascariensis* natural products showed that the isolated compounds suppress free radicals' formation or chain reactions by HAT mechanism except for compound **V** which operate by the SET and HAT mechanisms. Compounds with the ability to inhibit free radicals by both mechanisms showed strong antioxidant activity compared to the ones that operate by HAT or SET only.

Furthermore, it was also noticed that compounds with good cytotoxicity activity like compounds **II**, **IV,** and **V** are good antioxidant agents. Moreover, compounds **II** and **IV** are the only isolated abietane diterpenoids that displayed good to moderate activities in all assays.

The findings of this research study establish *P. madagascariensis* as an important medicinal plant rich in diterpenoids which may be useful for the treatment of tuberculosis, diabetes, and psoriasis. It is envisaged that this study will contribute to a better understanding of the phytochemistry of *P. madagascariensis* and contribute to the development of potential antitubercular, antidiabetic, and anti-psoriasis compounds with improved activities.

6.2   Recommendation

The recommendations that can be made are to derivatize the isolated compounds and re-assess their antitubercular, antidiabetic, and cytotoxicity properties to study the structure-activity relationship in order to improve the biological activities.

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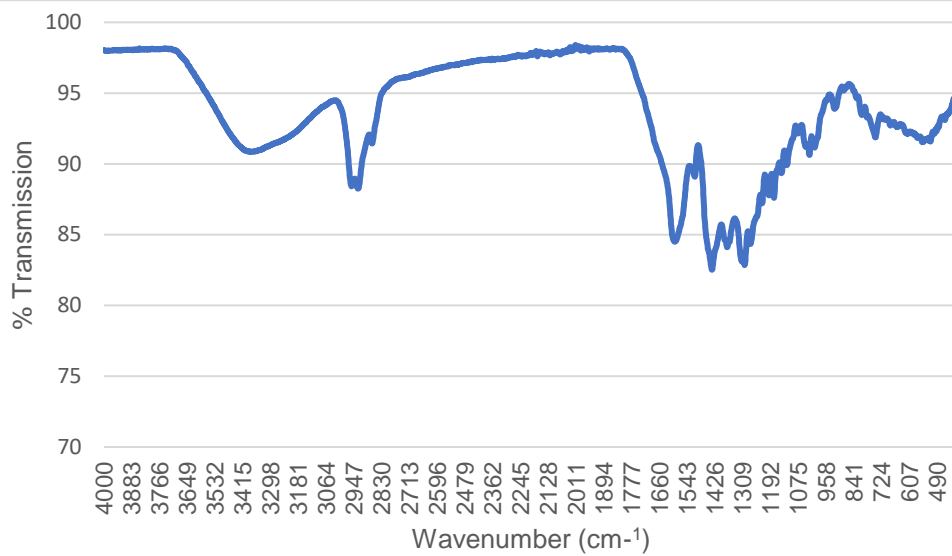**Appendix A:** IR and UV spectrum of *P. madagascariensis* Isolated natural products
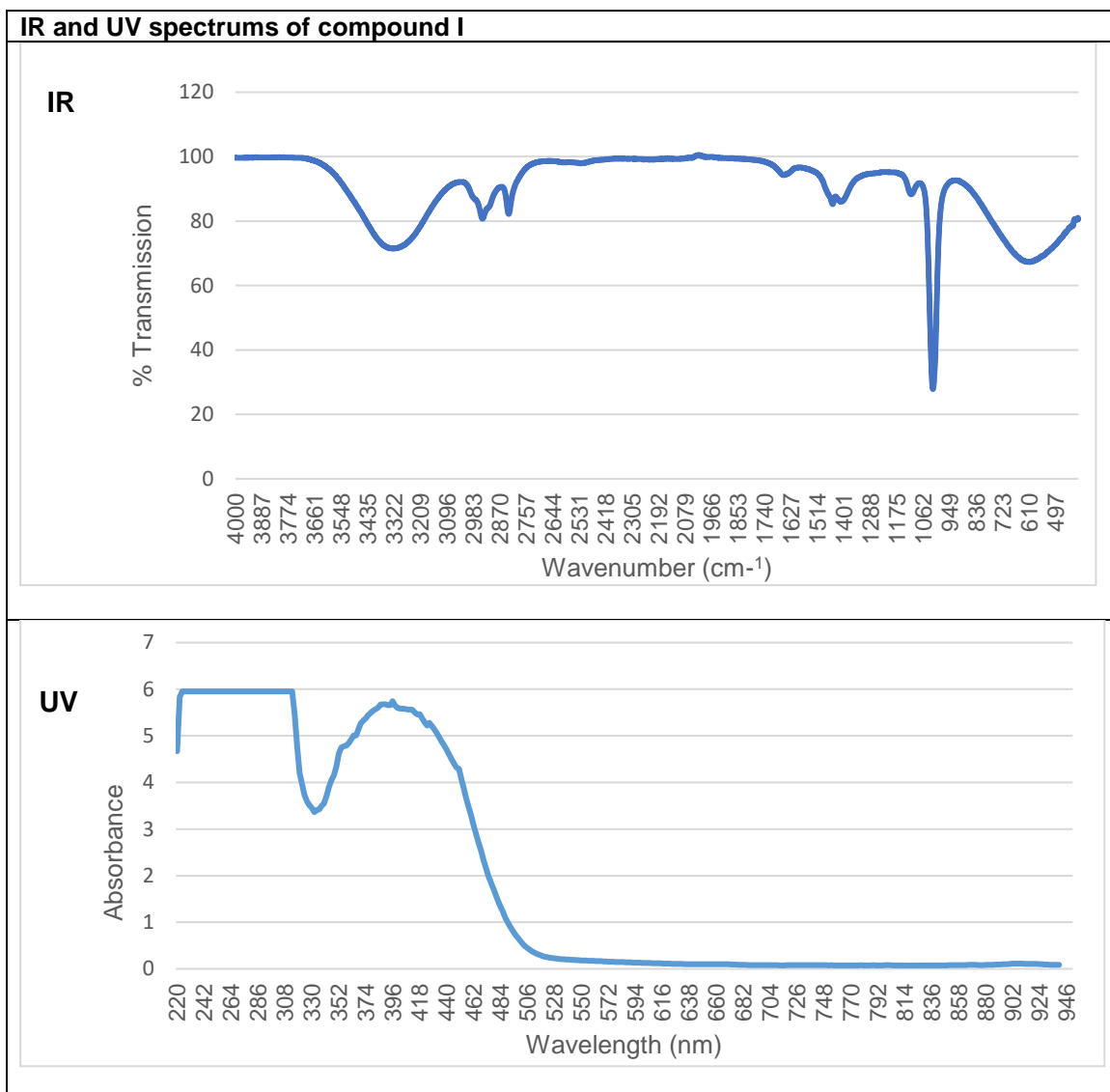
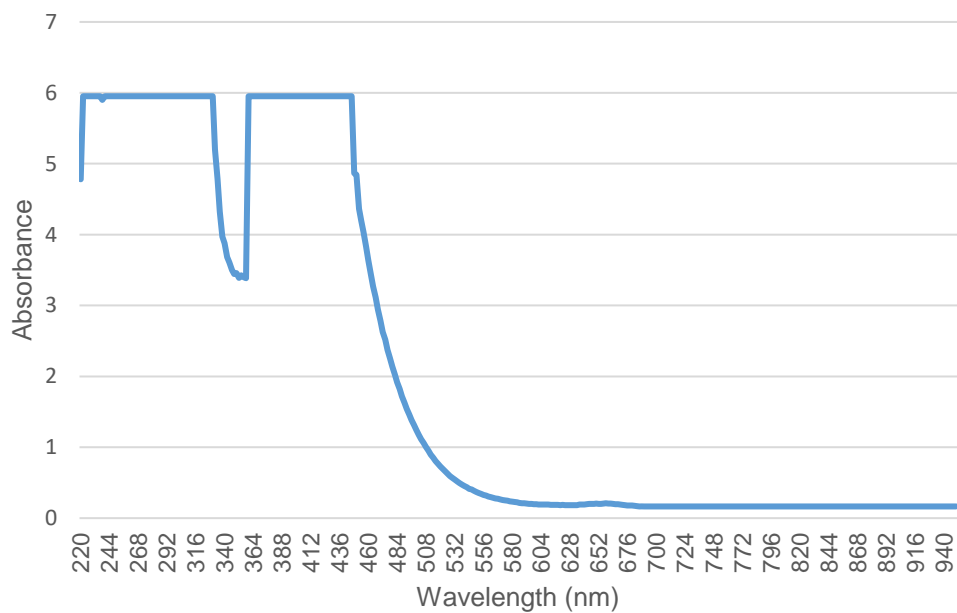**IR and UV spectrums of compound I**

IR and UV spectrums of compound II

IR

% Transmission vs Wavenumber (cm-1)

UV

Absorbance vs Wavelength (nm)

IR and UV spectrums of compound III

IR

% Transmission vs Wavenumber (cm⁻¹)

UV

Absorbance vs Wavelength (nm)

IR and UV spectrums of compound IV

IR spectrum — % Transmission vs Wavenumber (cm^{-1})

UV spectrum — Absorbance vs Wavelength (nm)
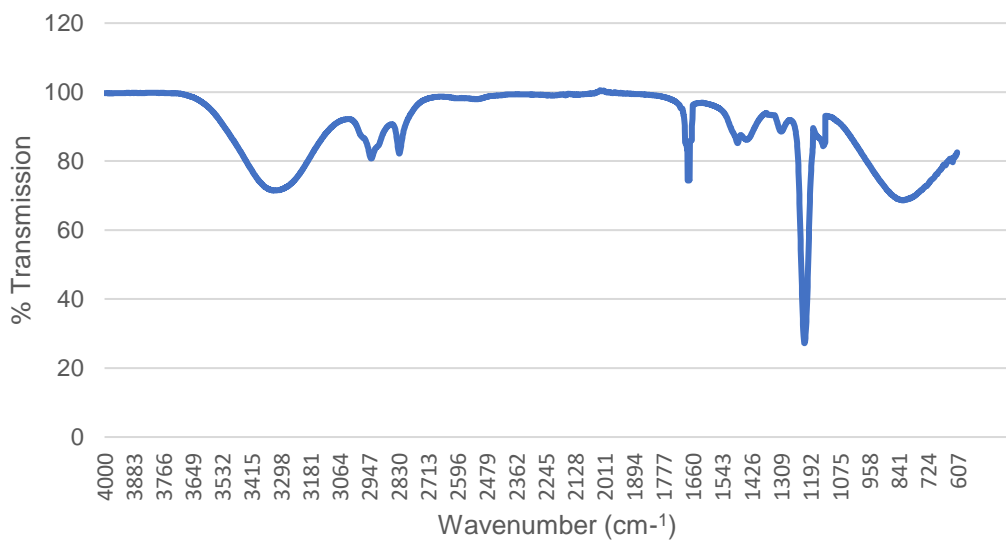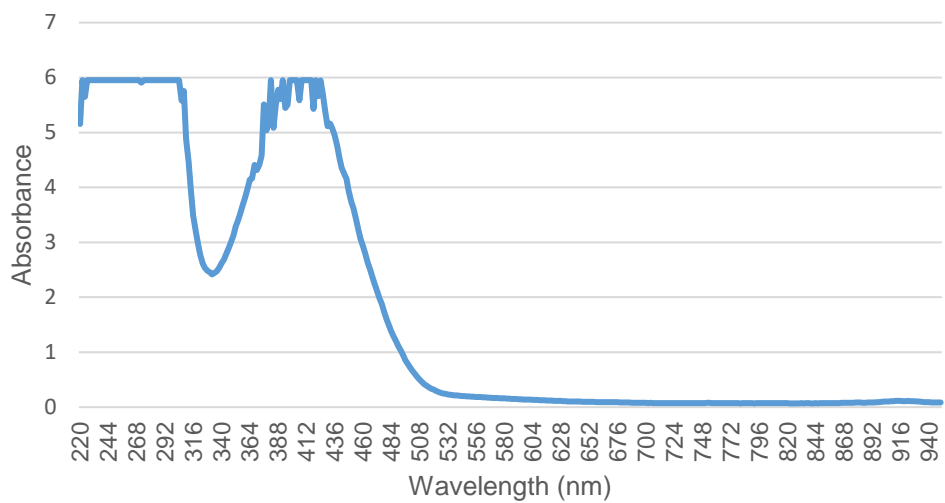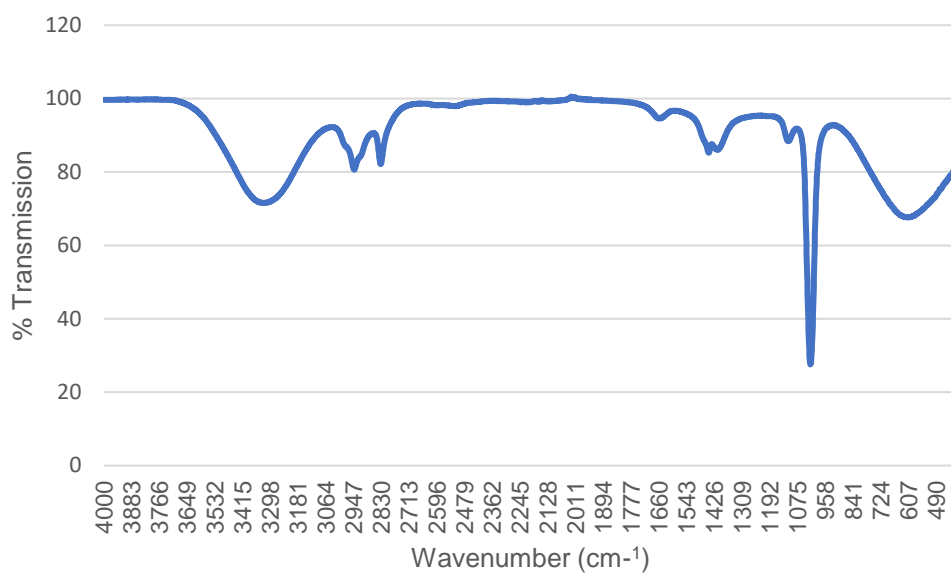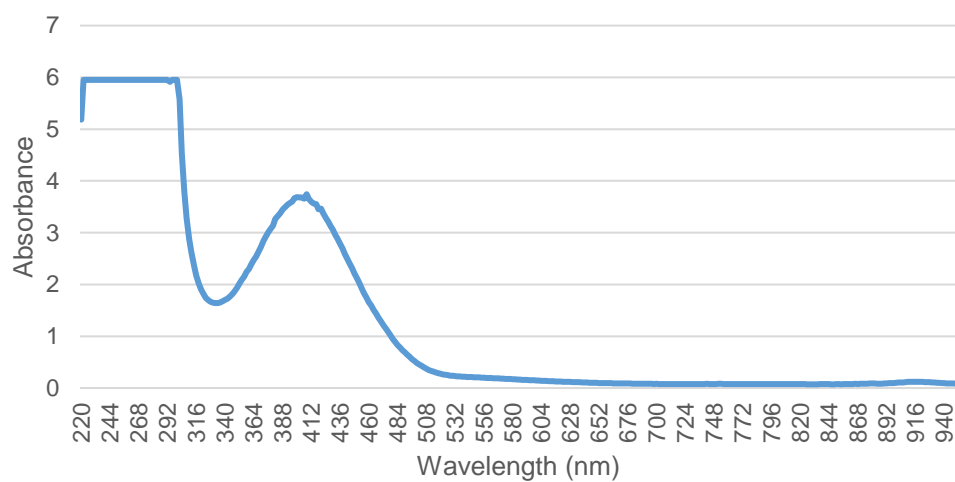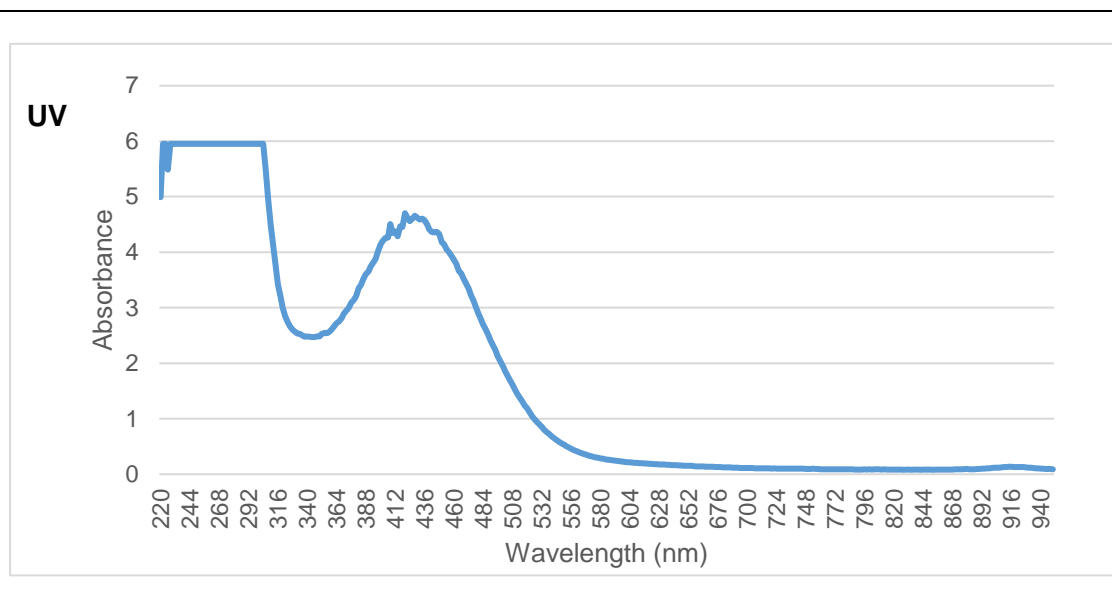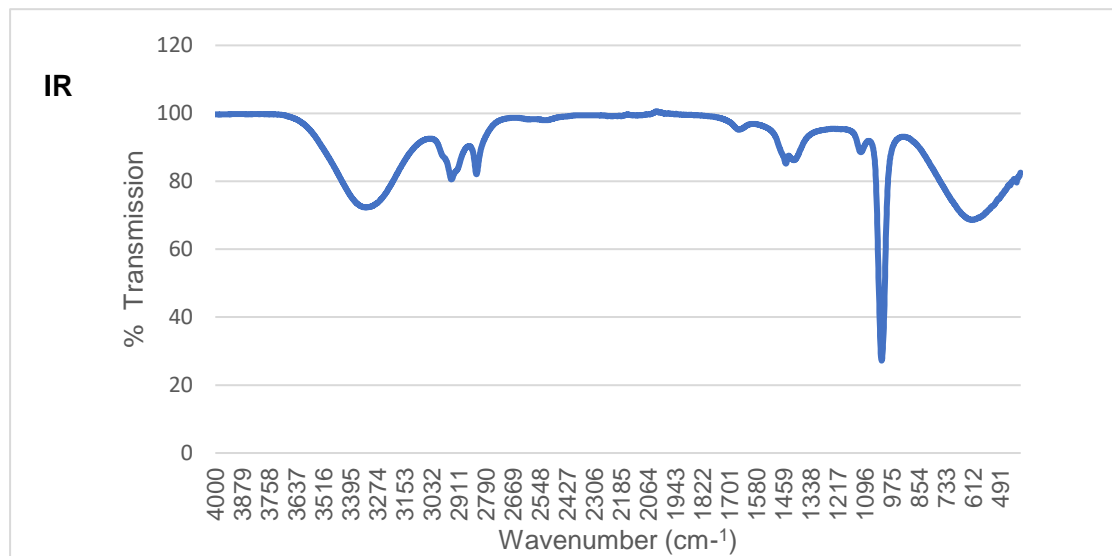
IR and UV spectrums of compound V

IR and UV spectrums of compound VI & VII

IR

% Transmission vs Intensity (cm-1)

UV

Intensity vs Wavelength (nm)
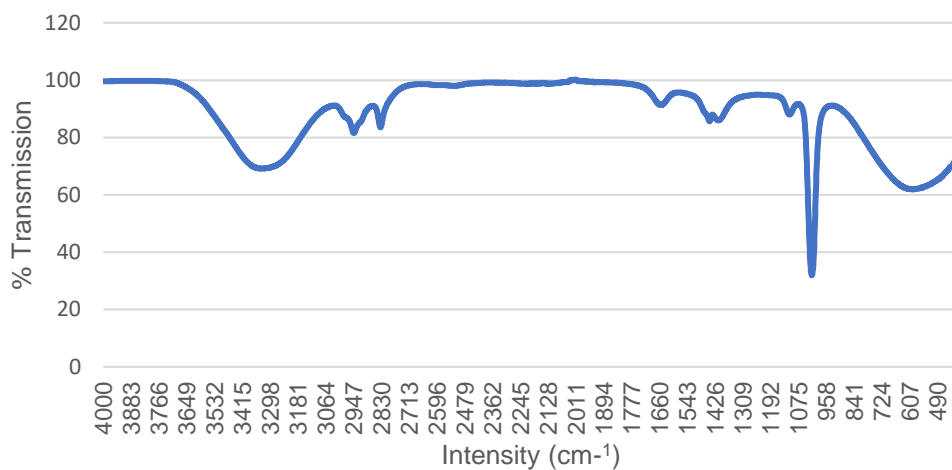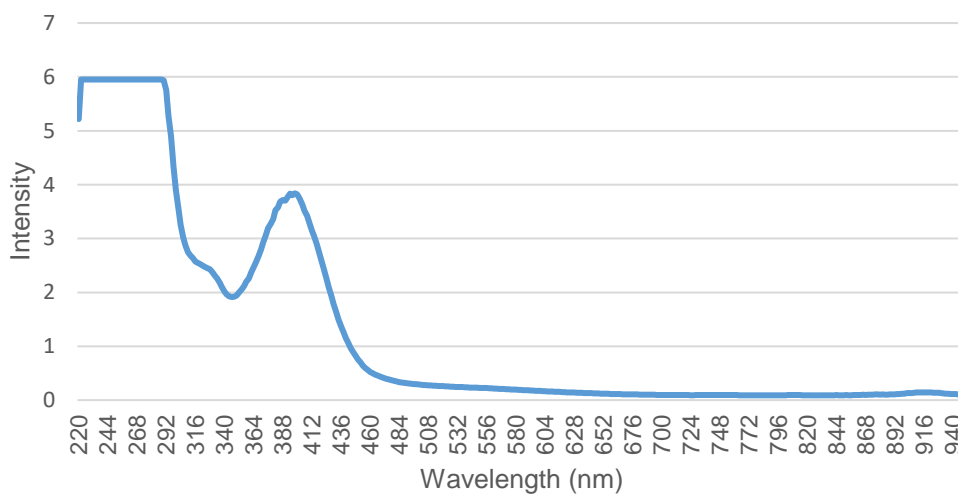
Appendix B: GFP tagged *Mtb* Assay - 10pt DR: Dose-response Plot of *P. madagascariensis* isolated compounds, main fractions and total extract

Compound ID	GFP Assay - 10pt DR: Dose-response Plot [Media: 7H9 CAS GLU Tx, Day:7]	GFP Assay - 10pt DR: Dose-response Plot [Media: 7H9 CAS GLU Tx, Day:14]
I		

II

UKN3__ConcentrationResponseCurve

● Unknown3 (Unknown3: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A-D}{1+\left(\frac{x}{C}\right)^B}$

Unknown3
$R^2 = 1.000$
$EC50 = 13.96$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	-6.60e-6	0.002	[-0.005, 0.005]
B	25.39	198.6	[-460.6, 511.4]
C	13.96	12.28	[-16.10, 44.02]
D	100.0	0.003	[99.99, 100.0]

UKN3__ConcentrationResponseCurve

● Unknown3 (Unknown3: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A-D}{1+\left(\frac{x}{C}\right)^B}$

Unknown3
$R^2 = 0.999$
$EC50 = 6.872$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	-0.077	0.901	[-2.283, 2.129]
B	9.800	7.494	[-8.536, 28.14]
C	6.872	0.679	[5.210, 8.534]
D	98.73	0.903	[96.52, 100.9]

III

UKN4__ConcentrationResponseCurve

● Unknown4 (Unknown4: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A-D}{1+\left(\frac{x}{C}\right)^B}$

Unknown4
$R^2 = 0.977$
$EC50 = 7.448$

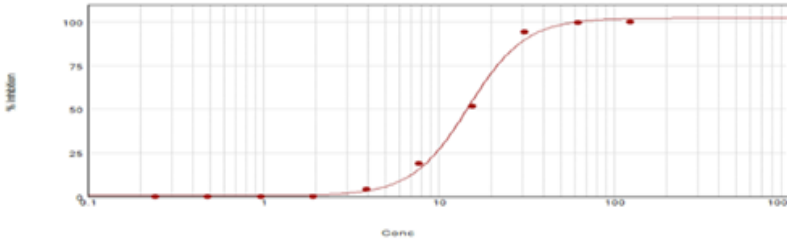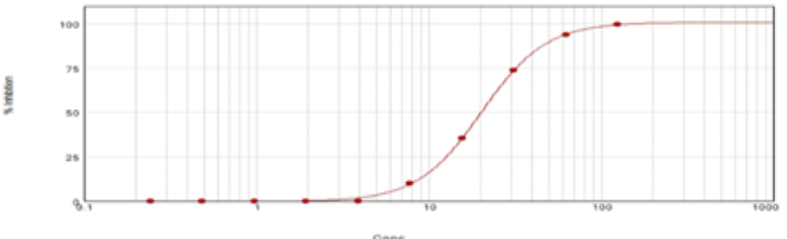| Parameter	Estimated Value	Std. Error	Confidence Interval
A	36.47	2.939	[29.28, 43.66]
B	3.354	1.277	[0.230, 6.478]
C	7.448	0.841	[5.391, 9.505]
D	99.62	3.301	[91.54, 107.7]

UKN4__ConcentrationResponseCurve

● Unknown4 (Unknown4: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A-D}{1+\left(\frac{x}{C}\right)^B}$

Unknown4
$R^2 = 0.690$
$EC50 = 3.772$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	22.42	0.610	[20.93, 23.91]
B	44.94		
C	3.772		
D	99.02	14.18	[64.32, 133.7]

IV	UKN6__ConcentrationResponseCurve %Inhibition vs Conc • Unknown6 (Unknown6: %InhibEdit vs Concentration) Weighting: Fixed Curve Fit Results ▲ Curve Fit : 4-Parameter Logistic $\quad y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$	UKN6__ConcentrationResponseCurve %Inhibition vs Conc • Unknown6 (Unknown6: %InhibEdit vs Concentration) Weighting: Fixed Curve Fit Results ▲ Curve Fit : 4-Parameter Logistic $\quad y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

IV (left)

Unknown6
$R^2 = 0.918$
$EC50 = 7.821$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	27.52	5.840	[13.23, 41.81]
B	53.97		
C	7.821		
D	99.33	6.529	[83.36, 115.3]

IV (right)

Unknown6
$R^2 = 0.607$
$EC50 = 1.845$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	40.23	0.000	[40.23, 40.23]
B	46.26		
C	1.845		
D	98.74	12.76	[63.32, 134.2]

V (left)

UKN2__ConcentrationResponseCurve

• Unknown2 (Unknown2: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $\quad y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

Unknown2
$R^2 = 1.000$
$EC50 = 127.1$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	-4.79e-10	6.18e-18	[-4.79e-10, -4.79e-10]
B	34.59	1.25e+8	[-3.05e+8, 3.05e+8]
C	127.1	8.91e+8	[-2.18e+9, 2.18e+9]
D	198.8	3.06e+10	[-7.49e+10, 7.49e+10]

V (right)

UKN2__ConcentrationResponseCurve

• Unknown2 (Unknown2: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $\quad y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

Unknown2
$R^2 = 1.000$
$EC50 = 127.1$

Parameter	Estimated Value	Std. Error	Confidence Interval
A	-5.87e-10	7.57e-18	[-5.87e-10, -5.87e-10]
B	34.57	0.193	[34.09, 35.04]
C	127.1		
D	241.1		

VI & VII		
Rifampicin		

K4-3-III & IV		
K4-3-VI		

K4-3-VIII		

UKN3__ConcentrationResponseCurve (left)

● Unknown3 (Unknown3: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Unknown3 $R^2 = 1.000$ EC50 = 13.93	A	-0.004	0.116	[-0.288, 0.281]
	B	14.59	18.95	[-31.77, 60.95]
	C	13.93	2.083	[8.830, 19.02]
	D	99.74	0.150	[99.37, 100.1]

UKN3__ConcentrationResponseCurve (right)

● Unknown3 (Unknown3: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Unknown3 $R^2 = 1.000$ EC50 = 13.60	A	-3.28e-6	0.009	[-0.021, 0.021]
	B	27.87	1540	[-3741, 3796]
	C	13.60	104.5	[-242.2, 269.4]
	D	99.98	0.011	[99.95, 100.0]

K4-3-XI		

UKN4__ConcentrationResponseCurve (left)

● Unknown4 (Unknown4: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Unknown4 $R^2 = 0.964$ EC50 = 7.832	A	30.67	2.973	[23.39, 37.94]
	B	43.88		
	C	7.832		
	D	99.83	2.394	[93.98, 105.7]

UKN4__ConcentrationResponseCurve (right)

● Unknown4 (Unknown4: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Unknown4 $R^2 = 1.000$ EC50 = 6.756	A	-5.80e-6	0.016	[-0.039, 0.039]
	B	27.48	1788	[-4348, 4403]
	C	6.756	63.89	[-149.6, 163.1]
	D	99.98	0.016	[99.94, 100.0]

K4-3-XIII & XIV		
K4-1-Hex		

Compound ID	GFP Assay - 10pt DR: Dose-response Plot [Media: ADC GLU Tw, Day:7]	GFP Assay - 10pt DR: Dose-response Plot [Media: ADC GLU Tw, Day:14]
II		
K4-3-VI		

| Rifampicin | | |

ConcentrationResponseCurve

● Reference   (ReferenceDrugControl: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A-D}{1+\left(\frac{x}{C}\right)^{B}}$

Reference
$R^2 = 1.000$
EC50 = 6.69e-4

Parameter	Estimated Value	Std. Error	Confidence Interval
A	68.46	0.231	[67.90, 69.03]
B	2.282	0.031	[2.307, 2.357]
C	6.69e-4	6.06e-6	[6.54e-4, 6.84e-4]
D	100.2	0.033	[100.2, 100.3]

ConcentrationResponseCurve

● Reference   (ReferenceDrugControl: %InhibEdit vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 4-Parameter Logistic $y = D + \dfrac{A-D}{1+\left(\frac{x}{C}\right)^{B}}$

Reference
$R^2 = 0.996$
EC50 = 8.51e-4

Parameter	Estimated Value	Std. Error	Confidence Interval
A	2.307	3.186	[-5.489, 10.10]
B	5.276	0.659	[3.665, 6.889]
C	8.51e-4	3.91e-5	[7.56e-4, 9.47e-4]
D	98.91	1.143	[96.11, 101.7]