PROFILING OF PLANT EXTRACTS (*CROTON GRATISSIMUS AND LEONOTIS LEONURUS*) FOR THEIR ACTIVITY AGAINST *MYCOBATERIUM TUBERCULOSIS* AND ISOLATION AND CHARECTERISATION OF THE ACTIVE COMPOUNDS

MASTER OF SCIENCE IN CHEMISTRY

B.P MAIFO

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BY

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A RESEARCH DISSERTATION SUBMITTED FOR THE DEGREE, MASTER OF SCIENCE IN CHEMISTRY, SCHOOL OF PHYSICAL AND MINERAL SCIENCES, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO, SOUTH AFRICA

SUPERVISOR: PROF W NXUMALO

2021

Dedication

The work is dedicated to my family; late father, mother, siblings and cousins.

Declaration

I declare that “Profiling of plant extracts (*Croton gratissimus* and *Leonotis leonurus*) for activity against *Mycobacterium tuberculosis* and isolation and characterisation of active compounds” is my own work submitted for the degree Master of Science at University of Limpopo. It has not been submitted for any degree or examination at any other University, and all sources I have used or quoted have been indicated and acknowledged through complete references.

16/04/2021

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Miss BP Maifo Date

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To God who gives me strength, it is because of your grace that the work in this study has become a reality. You deserve all the glory. I want to express my deepest thanks to my supervisor Prof Winston Nxumalo for his support, encouragement, and his admirable patience throughout the study.

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Scientific Contributions
Conferences attended

Oral presentation at:

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Date: 19-20 September 2019

Profiling of *Croton gratissimus* and *Leonotis leonurus* extracts for their activity against *Mycobacterium tuberculosis* and isolation and characterisation of the active compounds.

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Tuberculosis is one of the 10 leading causes of death in the world. However, this problem becomes serious as Mycobacterium tuberculosis (Mtb) developed resistance against both the first line and the second line drugs. Due to this, there is emergence of multi-drug resistant and extensively-drug resistant strands of Mtb all over the world. Medicinal plants have been used for centuries to help traditional healers with the regime to treat various types of diseases as well as TB. The study was carried out to investigate the antitubercular activity of the leave extracts of two plant species. The leaves of Croton gratissimus and Leonotis leonurus were extracted using three different solvent systems. Isolation of active fractions was done using column chromatography and thin layer chromatography. Nine isolated fractions indicated promising activity with minimum inhibitory concentration (MIC_{90}) values ranging from 4.37 to 16,8 µg/mL. In the qualitative antioxidant assay, clear spots against the purple background on TLC plates indicated antioxidant activity of crude extracts. In the quantitative antioxidant assay, all the crude extracts demonstrated good antioxidant activity but not as effective as butylated hydroxytoluene used as positive control antioxidant agent. The structures of the isolated compounds were elucidated using spectroscopic techniques, namely NMR, IR and Mass Spectrometry.

Keywords: *Mycobacterium tuberculosis, Croton gratissimus, Leonotis leonurus.*

Poster presentations at:

- **The African Traditional and Natural Product Medicine conference**

  Date: 16-19 October 2018

  Venue: The Ranch hotel Polokwane, South Africa

- **43RD SACI NATIONAL CONVENTION CONFERENCE**

  Date: 2-7 December 2018

  Venue: CSIR-ICC Pretoria, South Africa

- **THE FRANK WARREN ORGANIC CHEMISTRY CONFERENCE 2019**

  Date: 7-11 July 2019

  Venue: Aha alpine heath resort Drakensburg, South Africa

List of abbreviations

A	
Attp	DNA sequence
AChE	Acetylcholinesterase
ATCC	American Type Culture Collection
B	
BHT	Butylated hydroxytoluene
C	
$CDCl_3$	Chloroform-d
CEM	Centre for Evaluation and Monitoring
CAS	Central Asian Strain
COSY	Correlation Spectroscopy
J	Coupling constant
CNS	Central Nervous System
D	
DMSO	Dimethyl sulfoxide
DCM	Dichloromethane
DSM	Brevibacterium epidermis strain
DPPH	2,2-diphenyl-1-picrylhydrazyl
DEPT	Distortionless enhancement by polarization transfer
d	Doublet
dd	Double of doublet
^oC	Degree Celsius
E	
XDR-TB	Extensively drug resistant tuberculosis
F	
FTIR	Fourier-transformation infrared spectroscopy
FDA	Food and Drug Administration
G	

g	Grams
H	
Hz	Hertz
HIV	Human immunodeficiency virus
$H_{37}RV$	*Mycobacterium tuberculosis* strain
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
HRMS	High resolution mass spectrometry
M	
MIC	Minimun Inhibitory Concentration
$MgSO_4$	Magnesium sulfate
MS	Mass Spectrometry
MDR-TB	Multi-drug resistant tuberculosis
m	Metres
mL	Mille litres
mm	Milli metres
mg	Milli gram
µg	Micro gram
µL	Micro litre
µM	Micro molar
N	
NMR	Nuclear magnetic resonance
P	
ppm	Parts per million
T	
TB	Tuberculosis
TLC	Thin Layer Chromatography
t	Triplet
U	

UCT	University of Cape Town
USDA	United State Department of Agriculture
W	
WHO	World health organization

Abstract

Tuberculosis is one of the top 10 leading causes of death in the world. The development of drug resistant strains of *Mycobacterium tuberculosis* such as Multidrug resistant (MDR) and Extensively drug resistant (XDR) strains further complicate the TB control. Medicinal plants present a possible source for new potential antitubercular drugs. They have played an important role in drug discovery, with many pharmaceutical products originating from them. Isolation and characterisation of new antitubercular compounds from plant extracts is relevant today because of the development of resistant strains.

The aim of the study was to evaluate the antimycobacterial activity of the leave extracts of *Croton gratissimus* and *Leonotis leonorus*. The first step was to extract fine powder leaves of the two plant species using four (dichloromethane, acetone, hexane and ethanol/water) different solvent systems. Isolation of the fractions was done using column chromatography and preparative thin layer chromatography. Minimum inhibitory concentrations were determined using the broth dilution method and the values were recorded in µg/mL.

All the isolated fractions from both plant species were evaluated for preliminary *in-vitro* antimycobacterial activity. Some of the isolated fractions showed an increased activity against the pathogen as compared to the crude extracts. All the crude extracts of the two plants had activity with MIC_{90} values greater than 125 µg/mL. Seven fractions obtained from *Croton gratissimus* showed potential activity against the pathogen with MIC_{90} values ranging from 30.61 to 64.88 µg/mL. *Leonotis leonurus* had three fractions with promising activity with MICs ranging from 1.963 to 62.51 µg/mL.

The crude extracts of the two plant species showed that the two plant species have antioxidant properties. The qualitative antioxidant assay showed that DCM crude extracts had more antioxidants than all other extracts because of more clear zones against the purple background colour on the TLC plates. These was confirmed by the qualitative antioxidant assay where DCM crude extracts was able to inhibit the highest percentage of DPPH at different concentrations than all other solvent extracts. The DCM crude extracts of *L. leonurus* and *Croton gratissimus* inhibited 87 and 93 % of DPPH respectively at 250 µg/mL.

The structures of the compounds within the isolated fractions were elucidated using NMR and confirmed by MS and FTIR spectroscopies. The NMR data showed that the isolated fractions were not pure compounds but mixtures of closely related compounds. The compounds whose structures were elucidated included two labdane diterpenoids (**Croton A** and **Croton B**) and a Cembranolide ((5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate) from *Croton gratissimus* and a phenol (4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol)) from *Leonotis leonurus.*

Chapter 1

1 Introduction

1.1 Medicinal plants

Medicinal plants have been used by traditional healers to treat different types of diseases and remain important part of our natural health. They serve as important therapeutic agents as well as valuable raw materials for manufacturing numerous traditional and modern medicines [1]. The World Health Organisation (WHO) estimate that about 80% of the people in developing countries including South Africa still depend on traditional medicine as their major primary health care [2]. These plants continue to gain popularity due to their advantages such as patient tolerance, few side effects and low cost.

1.1.1 Drug discovery from natural product

In recent years, there has been an increasing interest in natural product-based medicines from plant origin. The plants are an important source of biologically active secondary metabolites which have enormous therapeutic potential [3]. Today, approximately 80% of antimicrobial, cardiovascular, immunosuppressive, and anticancer drugs are of plant origin [4]. It is widely accepted that more than 80% of drug substances are either directly derived from natural products or developed from a natural compound [5]. One way to develop new drugs is by studying the biological activity of natural products, semi-synthesis or the generation of new compounds. Such compounds could be applied, as new drugs with potential antimicrobial activity themselves or by modulating the immune response to enhance the removal of the infective agent.

The great diversity of natural compounds represents a powerful tool for drug discovery. Among 420,000 plant species reported worldwide, very few plants have been screened for biological activity [6]. Therefore, there are tremendous scopes to identify the unexplored plants for biological activity. The knowledge of traditional healers about

medicinal use of plants, which is passed verbally from one generation to another, is one of the important criteria for plant-based discovery program [7,8].

There are reports, reviews and research that have reported about medicines derived from plant sources. Aspirin (**Figure 1**) is one of the many drugs that are available on the market today that were discovered from natural sources. It is so far the world's best known and most universally used medicinal agent. It is originally from the plant genera *Salix spp.* and *Populus spp.* [9].

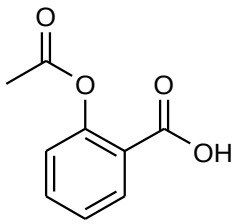

Figure 1. structure of Aspirin (**1**)

Taxol (**2**) is one of the drugs that are used for the treatment of tumors. It was isolated from the stem and bark ethanol extract of *Texas brevifolia* [10]. *Texas brevifolia* is a small growing tree that is mainly found in the coastal regions of the northwest of the United states of America [10]. This compound was screened for antitumor activity and showed 30% activity against a solid tumor known as Walker-256 intramuscular rat carcinosarcoma at a concentration of 23 mg/kg. The cytotoxicity studies of Taxol showed 50% inhibition of a human nasopharyngeal carcinoma cancer cell at a concentration of 3.5×10^{-5} µg/mL [11].

Bicyclol (**3**) is a synthetic drug which originated from *Schisandra chinensis*, used as a herb to treat hepatitis in traditional Chinese medicine [12]. It was approved as a hepatoprotectant by the Chinese Food and Drug Administration (FDA) for treatment of liver injury in 2004 [13]. The *in-vitro* anti-Hepatitis C virus activity of bicyclol showed 70 and 85 % inhibition of the virus at 10 and 2 µmol/L. Bicyclol was found to be effective in improving abnormal liver function and in inhibiting the replication of hepatitis B virus in chronic hepatitis B patients in clinical trials [13].

Artemisinin (**4**) is a sesquiterpene lactone isolated from *Artemisia annua*, a herb that has been long employed in traditional Chinese medicine to remedy fevers [14]. Several derivatives of this drug are currently in use for the treatment of malaria disease. *In-vitro* antimalarial studies showed 60% inhibition of the drug on [^{3}H] hypoxanthine and [^{3}H] isoleucine after 7 hours [15]

Quinine (**5**) is a *cinchona* alkaloid component isolated from the bark of the cinchona (quina-quina) tree used to treat malaria [16]. This alkaloid belongs to the aryl amino alcohol group of drugs. It is an extremely basic compound and is therefore, always presented as a salt [16]. Quinine and other cinchona alkaloids including quinidine, cinchonine and cinchonidine are all effective against malaria. The efficacies of these four alkaloids were evaluated in one of the earliest clinical trials and all four alkaloids were found to be comparable, with cure rates of >98% [17]

Camptothecin (**6**) is a pentacyclic alkaloid that is used in the treatment of tumors. The drug was first isolated from stem wood of *Camptotheca acuminata* by botanists working in the USDA's plant introduction division in the mid-1950s [18]. *Camptotheca acuminate* is a tree native to China and its bark is a recognised Chinese traditional medicine [18]. Monroe and Wani [19] reviewed the co-discovery of Camptothecin and Taxol. Chemical synthesis of **6** in laboratories, and follow-up preclinical and clinical studies were actively conducted in the late 1950s and mid to late 1960s [20].

Camptothecin was investigated in the United States in cancer patients in both Phase I [21] and Phase II [22] clinical trials. About 50% of the patients in Phase I trial showed positive response after two weeks at a dosage of 90-360 mg/m^2 [21]. Only 3% of the patients in Phase II trial showed objective response after two months at a dosage of 90-180 mg/m^2 [22]. Clinical trials for about one thousand patients with colorectal, head- and neck or bladder cancer in China using carboxylate form of Camptothecin (Camptothecin sodium salt) showed positive results [20]. About 60% of the patients showed positive response after 2 weeks at dosage of 90-300 mg/m^2.

Digoxin (**7**) is one of the oldest cardiovascular medications that are currently in use [23]. It is a common agent used to manage atrial fibrillation and the symptoms of heart failure [24]. Digoxin is classified as a cardiac glycoside and was initially approved by

the FDA in 1954 [24]. This drug originates from the foxglove plant, also known as the *Digitalis* plant. Digoxin is used for the treatment of mild to moderate heart failure in adult patients, increase myocardial contraction in children diagnosed with heart failure and maintain control ventricular rate in adult patients diagnosed with chronic atrial fibrillation [25].

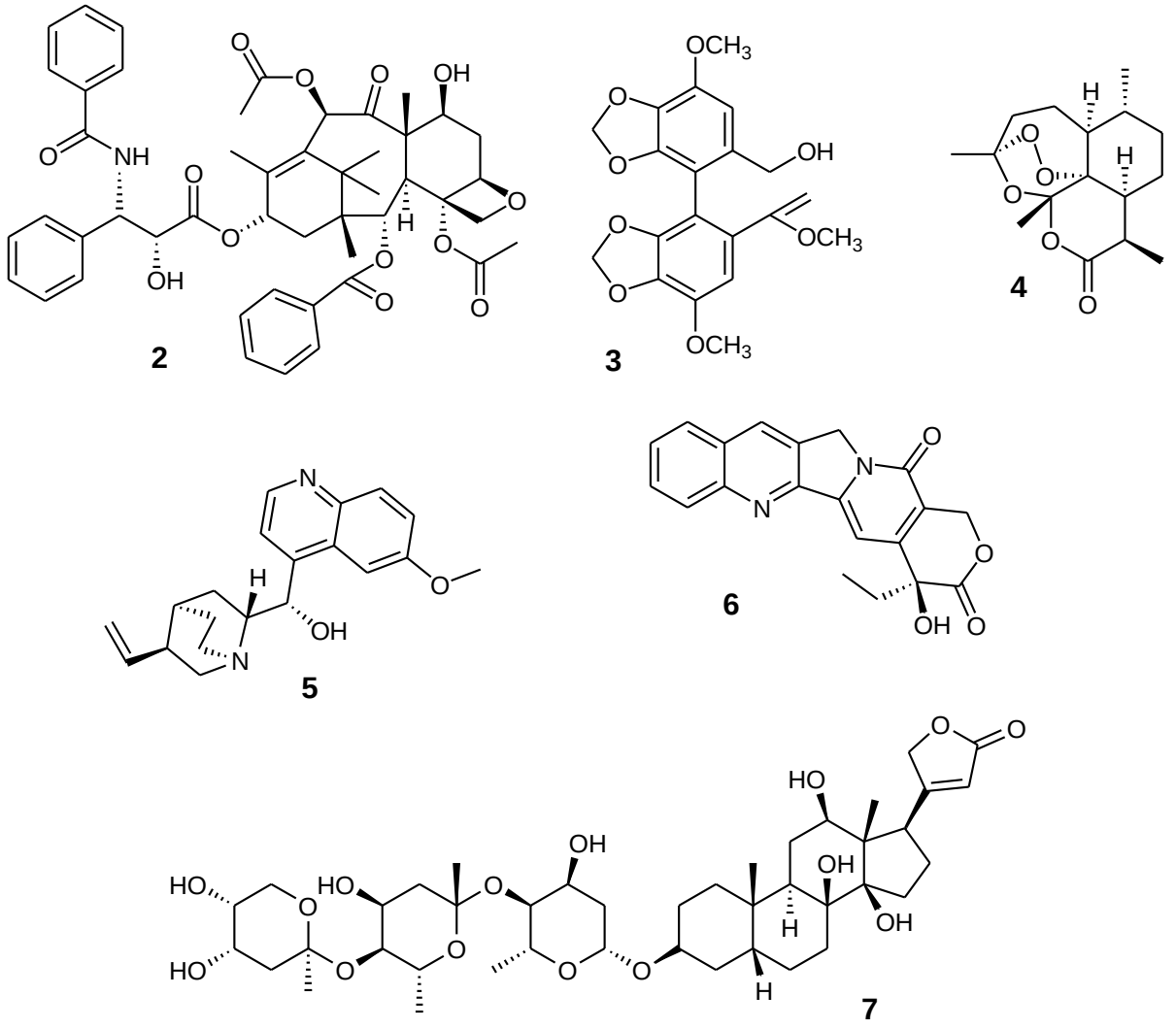

Figure 2. Chemical structures of some of the compounds isolated from natural products.

1.1.2 Medicinal plants as antitubercular agents

Research has been done on the search for new and improved drugs that can inhibit the growth of the pathogen and eventually cure the disease. Different plant extracts

have been screened for activity against *Mycobacterium tuberculosis* and most of them showed promising activity either as crudes extracts or as isolated compounds. The selection of plants to investigate relies largely on ethnobotanical leads. Most of the research has been carried out using fast-growing saprophytic *Mycobacterium* species as test microorganisms.

Few triterpenes and naphthoquinones were isolated from a chloroform root extract of *Euclea natalensis* and evaluated for activity against *Mycobacterium tuberculosis* [26]. The crude extract, diospyrin and 7-methyljuglone gave MIC values of 8.0, 8.0 and 0.5 g/mL respectively against drug-sensitive *Mycobacterium tuberculosis* [26]. Ethanol extract of seven medicinal plants chosen ethnobotanically were investigated for activity against *Mycobacterium tuberculosis* and *Mycobacterium segmatis* [27]. A flavone named 5,7,2-trihydroxyflavone was isolated from *Galenia Africana* and it showed potent activity against both strains with MIC values of 0.10 g/mL against *Mycobacterium tuberculosis* and 0.031 g/mL against *Mycobacterium segmatis* [27].

Joseph *et al.,*[28] reported that the unripe fruits of *Solanum torvum swartz* (Solanaceae) showed potent antimycobacterial activity against the non-pathogenic strains *Mycobacterium bovis bacillus* (ATCC 3574), *Mycobacterium aurum* (ATCC 23366), and the pathogenic strain *Mycobacterium bovis* (ATCC 35720) with MIC values of 19.5 µg/mL, 156.3 µg/mL, and 312.5 µg/mL respectively [28]. In another similar study, Joseph *et al.,* [29] reported that the hydro-ethanolic leave extracts of *Solanum torvum* Sw (commonly known as devil's fig) have potential activity against two strains of *Mycobacterium tuberculosis* (H_{37}Ra and H_{37}Rv). Their minimum inhibitory concentrations were 156.3 µg/mL and 1250 µg/mL, respectively [29].

According to Fyhrquist *et al.,* [30] the bark and root methanol extracts of *Terminalia sambesiaca* and the root methanol extracts of *Terminalia kaiserana* have promising antimycobacterial activity against *Mycobacterium smegmatis* strain [30]. The extracts were able to inhibit 99% of the bacteria at a concentration of 1250 µg/mL [30]. Al-Baadani *et al*., [31] reported the anti-mycobacterial activity of *Acalypha indica* extract against the *Mycobacterium tuberculosis* H_{37}Rv strain. The results revealed that the water leave extracts have exhibited sensitivity at 100 µg/mL concentration when compared with the standard pyrazinamide [31].

Hussein *et al.,* [32] isolated a new labdane diterpene from *Orthosiphon labiatus* ethanol extract and carnosic acid from the ethanol extract of *Salvia africanalutea.* The two compounds exhibited MICs of 157 and 28 µM, respectively, against *Mycobacterium tuberculosis* [32]. After screening several South African *Salvia* species for antimicrobial activity, Kamatou *et al.,* [33] found that the methanol/chloroform (ratio 1:1) of the aerial parts of *Salvia fricana-lutea* showed promising activity against *Mycobacterium aurum* and *Mycobacterium tuberculosis* with MIC values of 2 and 1 mg/mL, respectively.

The hexane fraction of *Chrysophyllum albidum*, butanol fraction of *Mezoneuron. Benthamianum*, ethyl acetate fractions of *Phyllanthus muellerianus* and *Aristolochia fimbriata* showed sensitivity against the *Mycobacterium tuberculosis* H_{37}Rv strain with minimum inhibitory concentration of 0.5 mg/mL [34]. On the hand, Muhammad and colleagues [35] pointed that the methanol leave extract of *Gynura procumbens* at 500 ppm has antimycobacterial activity against the *Mycobacterium tuberculosis* strain H_{37}Rv and multidrug-resistant *Mycobacterium tuberculosis* strain.

1.2 *Croton gratissimus*

Croton gratissimus (C. gratissimus) is a shrub or small tree with corky bark that can grow up to 8 m in height in South Africa but can grow up to 20 m tall further in the northern parts of Africa [36]. It is commonly known as Mologa by Pedi speaking people. The *Croton* genus belongs to the *Euphorbiaceae* family and is a large and complex group of plants ranging from shrubs and herbs to trees [37]. The family Euphorbiaceae is made up of 317 genera and about 7,500 species. The genus *Croton* consist about 700 species [38]. The species occurs in tropical regions of the world. In South Africa, the species is found in Limpopo, Mpumalanga, North West, Kwazulu-Natal and Northern Cape. It is also found in other African Countries such as Botswana, Zimbabwe and Mozambique [37]. All parts of the plant have reputed medicinal value [39].

Figure 3. Leaves of *C. gratissimus* [40]

1.2.1 Medicinal uses of *C. gratissimus*

Different parts of the plant have different traditional medicinal uses. The leaves are considered strengthening and have medicinal value [40]. They are highly aromatic and often used as infusion for coughs. Dried and pulverized leaves are used to make a pleasant perfume [41]. A leaf-decoction is used as a wash/soap for body rashes. The bark is most frequently used to treat bleeding gums, abdominal disorders, skin inflammation, earache and chest complaints [40]. Roots decoctions have been used to treat chest complaints, coughs, fever and sexually transmitted diseases such as syphilis [41]. The combinations of roots and bark to treat respiratory disorders have also been reported [40]. The oil extracted from the seed of *Croton gratissimus* has recently been reported to be a promising feedstock in the large-scale production of biodiesel [42].

1.2.2 Biological activity screening of *C. gratissimus* extracts

Several studies have been taken in order to investigate the biological activity of different parts of *Croton gratissimus*. Crude extracts, fractions and individual compounds have showed potential activity against different pathogens that causes life threatening diseases.

Emmanuel *et al*., [37] reported the potential anti-inflammatory and anticancer activity of the acetone leave extracts of *C. gratissimus* with inhibitory concentrations (IC_{50}) of 2.58 µg/mL against 5-lipoxygenase and 0.57 µg/mL against Hela cell, respectively. Ashwell *et al.,* [43] indicated moderate acetylcholinesterase (AChE) inhibitory effect of ethyl acetate and butanol crude leave extracts of *C. gratissimus* with IC_{50} of 66.9 and 64.5 µg/mL respectively.

In their study, van Vuuren and co-workers [44] reported the antimicrobial activity of extracts of different parts of *C. gratissimus.* The bark extract showed potential activity against several microbes with MIC_s of 0.4 mg/mL against *Staphylococcus aureus* (ATCC 12600), 0.9 mg/mL against *Bacillus cereus* (ATCC 11778) and 0.8 mg/mL against *Staphylococcus epidermidis* (ATCC 2223) [35]. The root extract had potential effect against *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 12600) with MIC_s of 0.8 and 0.4 mg/mL, respectively. Combination of the roots, bark and leaves extracts showed promising activity with MIC of 0.3 mg/mL against both microbes [44]. Leave extracts showed moderate activity against most of the microbes with MIC_s that were ranging from 1.5 to 6.0 mg/mL [44].

Mthethwa and co-workers [45] investigated the bioactive properties of the methanol leave extracts of *C. gratissimus*. The extract was able to inhibit 17 and 5 % *Staphylococcus aureus* and *Staphylococcus epidermidis* multidrug resistant strains respectively at a concentration of 0.2 µg/mL. The methanol extracts also exhibited abilities to inhibit HIV-1_{IIIB} with significant selectivity indexes (SI). The EC_{50} was estimated to be 9.6 and 3.5 µg/mL with SI of 10.4 and 57.1 indicating anti-HIV-$1_{IIIB.}$ [45].

After assessing eighteen plants for antimicrobial activity against pathogens associated with sexually transmitted infections, Naidoo and van Vuuren [46] reported that the essential oil of *C. gratissimus* was active against most of the microorganisms. The dichloromethane/methanol (ratio1:1) oil extract was active against *Oligella ureolytica* (ATCC 43534), *Ureaplasma urealyticum* (clinical strain), *Neisseria gonorrhoeae* (ATCC 19424) and *Gardnerella vaginalis* (ATCC 14018) with minimum inhibitory concentrations of 1.0, 4.0, 1.0 and 2.0 mg/mL, respectively [46]. The leave extract of

the same species was active against *Gardnerella vaginalis* (ATCC 14018) with MIC value of 1.0 mg/mL [46].

In another study, van Vuuren *et al*., [47] investigated the antimicrobial activity of 41 oils against *Brevibacterium* species which causes bromodies (foot odour). The results revealed that essential oil from *C. gratissimus* displayed noteworthy activity against two *Brevibacillus* strains. The MIC values were 0.13 mg/mL against *Brevibacillus agri* (ATCC 51663) and 0.50 mg/mL against *Brevibacterium epidermidis* (DSM 20660) [47].

1.2.3 Phytochemical screening of *C. gratissimus*

Various reports on the phytochemical screening of *C. gratissimus* leaves and stem bark confirm the presence of diterpenes, cembranolids, essential oils and flavonoids [48]. **Figure 4** shows the structures of some of the compounds isolated from *C. gratissimus*.

Sadgrove *et al.,* [49] isolated two new volatile diterpenes named 12-β-furanyl-halima-5,9-dien-4-methylcarboxylate (**8**) and *ent*-abiet-8(14), 13(15)-dien-3-one (**9)**, and two known non-volatile diterpenes named crotohalimaneic acid (**10**) and hardwickiic acid (**11**) from the water leave extracts of *C. gratissimus*. Compounds **8** and **9** were subjected to antimicrobial testing against Gram-negative bacterial strains*; Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive strains; *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Bacillus cereus* ATCC 11175 and did not show any antimicrobial activity.

When studying the anti-oxidative and cholinesterase inhibitory effects of the leaf extracts of two closely related croton species, Ndhlala and co-workers [50], isolated Apigenin-6-*C*-glucoside (**12)** and Tiliroside (**13)** from the leave extracts of 20% aqueous methanol of *C. gratissimus*. The two compounds were tested for Cholinesterase Inhibitory Effects. Apigenin-6-*C*-glucoside inhibited 52.1% of Acetylcholinesterase (AChE) Enzyme at IC_{50} of 189.5 µg/mL while Tiliroside was able to inhibit 31.8 % of the same enzyme at IC_{50} of 391.3 µg/mL.

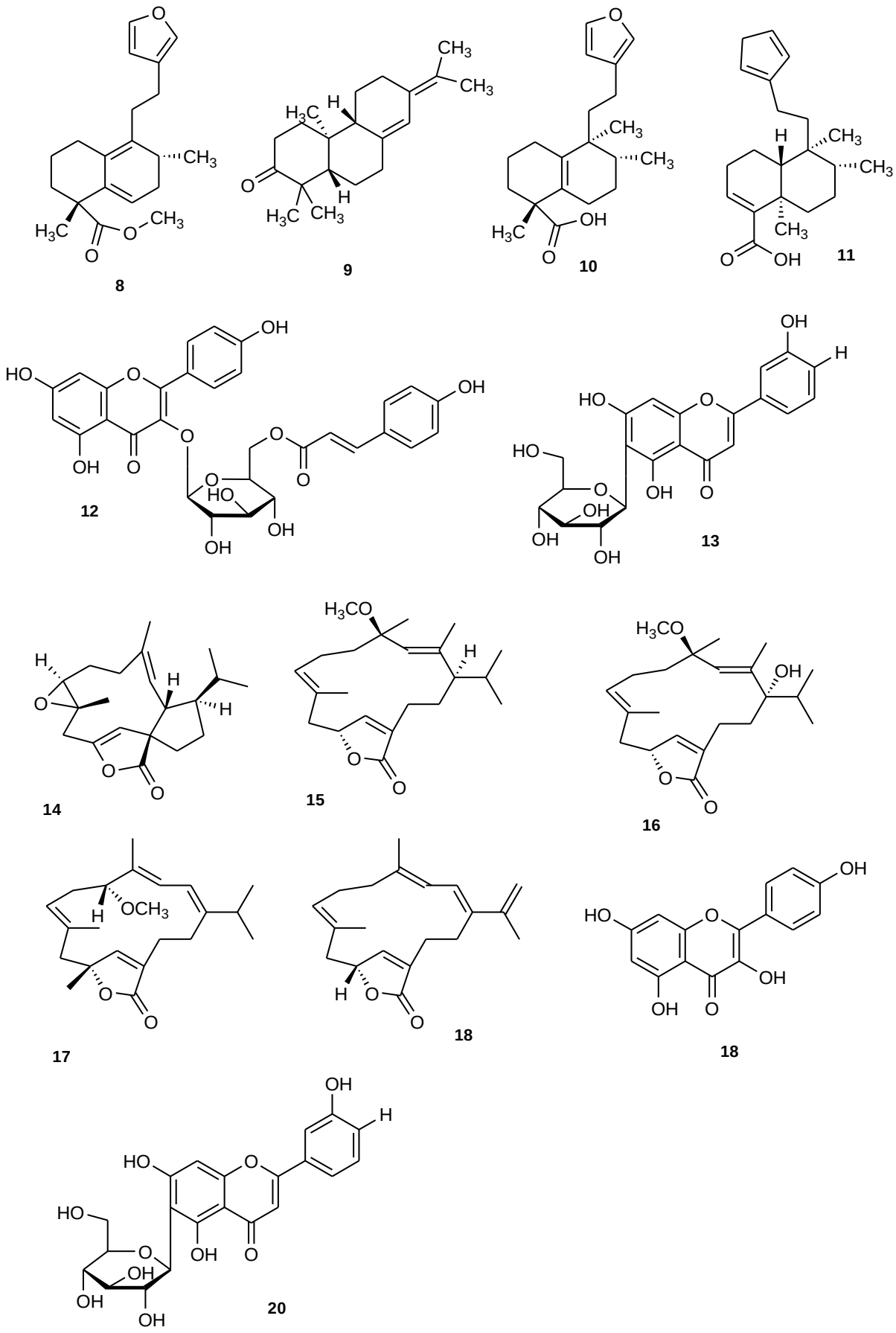

Figure 4. chemical structures of the compounds isolated from *C. gratissimus*.

Mulholland and workers [51] isolated four cembranolides from the DCM bark extracts of *C. gratissimus* including (+)-[1*R**,2*S**,7*S**,8*S**,12*R**]-7,8-epoxy-2,12-cyclocembra-3*E*,10*Z*-dien-20,10-olide (**14**) which was first reported. This compound showed moderate activity against the resistant PEO1TaxR ovarian cancer cell lines with an IC_{50} of 200 nM.

From the hexane leave extracts of *C. gratissimus*, Langat *et al.,* [52] isolated ten cembranolides including (−)-(1*R**,4*R**,10*R**)-4-methoxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide (**15**), (−)-(1*S**,4*R**,10*R**)-1-hydroxy-4-methoxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide (**16**), (+)-(1*R**,4*S**,10*R**)-4-hydroxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide (**17**) and (+)-(1*S**,4*S**,7R*,10*R**)-1,4,7-trihydroxycembra-2*E*,8(19),11*Z*-trien-20,10-olide (**18**). Compounds **17** and **18** evaluated against a chloroquine-sensitive strain of *Plasmodium falciparum* (D10) but the data was not given.

When studying the HPTLC fingerprinting of *C. gratissimus*, Van Wyk and co-workers [53] isolated Isoorientin (**19**) and kaempferol-3-*β*-D-(6″-*O*-*trans*-*p*-coumaroyl) glucopyranoside (**20**) from the acetone leave extracts. The two compounds were never investigated for any biological activity.

1.3 *Leonotis leonurus*

Leonotis leonurus (L. leonurus), commonly known as Wild dagga or Lion's ear is a very hardy, drought and frost resistant and evergreen perennial shrub belonging to the *Lamiaceae* (mint) family which comprises of about 3,200 species and 200 genera [54]. The genus *Leonotis* consist of about 1300 species. *L. leonurus* can grow up to 2-3 m tall and 1.5 m wide and grows on rocky hillsides, riverbanks and grassland of tropical Southern Africa, Asia and Southern India [55]. In South Africa the species is found in Limpopo, Kwazulu-Natal, Eastern Cape and Western Cape provinces. The plant produces orange, apricot or white flowers in clusters and the hairy flowers resemble a lion’s ears, hence the name *“leonurus* (lion coloured) [56]. *L. leonurus* has many reputed traditional medicinal applications and is mainly taken orally or per rectum and as a topical application [57].

Figure 5. leaves of *L. leonurus* [58]

1.3.1 Medicinal uses of *L. leonurus*

Leonotis leonurus boasts many recorded traditional uses. The Literature surveyed shows that the plant has been used for many years both topically and orally for treatment of many ailments such as a remedy for hypertension, coughs and headaches [59]. Powdered leaves are compounded into an ointment and applied topically for relief of pain above the eye [60]. The fresh stem juice is an infusion drank for 'blood impurity' [60]. The use of *L. leonurus* leaves and flowers as a remedy for snake bites and to treat other bites and stings, such as bee and scorpion stings is widespread in South Africa [61].

Watt and Breyer-Brandwijk reported that a decoction of the powdered stem or seed is administered orally for the relief of haemorrhoids and used topically as a lotion for sores on the legs and head [62]. Khoisan people were particularly fond of smoking it instead of tobacco and used the leave decoction as a strong purgative and as an emmenagogue [63]. Infusions of the leaves and stems are widely used as purgatives and tonics to treat influenza, tuberculosis, jaundice, muscular cramps, skin diseases and sores [64]. Early colonialists employed it in the treatment of leprosy [64].

1.3.2 Biological activity screening of *L. leonurus* extracts

A range of biological activities have been reported for extracts and isolated molecules obtained from *L. leonurus*, including anti-bacterial, antifungal, antiprotozoal, enzyme inducing, anti-inflammatory activities and modulation of immune cell functions.

The dichloromethane/methanol (1:1) of the twigs and leave extracts have been reported to have antimalarial activity against the chloroquine-sensitive strain (D10) of *Plasmodium falciparum* with IC_{50} = 5.2 ug/mL [65]. A compound (Luteolin 7-O-β-glucoside) isolated from the ethanol extract showed antimalarial activity against the chloroquine resistant (W2) strain (1.8 mg/mL) and the D6 clone (2.2 mg/mL) [66]. Klos *et al*., [67] reported that ethanolic extracts of *L. leonurus* leaves exhibited inhibitory activity in an antiviral assay. NKR-CCR5 (a humanT-lymphoblastic cell line) cell cultures, showed a 33% reduction in HIV-1 p24 core protein. The aqueous extract also showed activity against HIV-1 protease inhibition, with an IC_{50} of 120.6 µg/mL [67].

The extracts of the leaves and flowers are traditionally used to treat hypertension and diabetes [68]. A leaf extract of *L. leonurus* has been reported to possess hypoglycaemic effects in a streptozotocin (STZ)-induced diabetic rat model by reducing the blood glucose while increasing high density lipoprotein levels [69]. The anti-diabetic activity of the extract was due to the presence of different flavonoids, diterpenoids, polyphenolics [70], however a study by Mnonopi *et al*., [71] shows that Marrubin was identified as the antidiabetic active constituent of the organic extracts. The anti-amoebic and anthelmintic activities of *L. leonurus* have been studied.

McGaw *et al.,* [72] indicated that either the aqueous or the ethanolic leave extracts *of L. leonurus* showed anti-amoebic activity against *Entamoeba histolytica*. In another study, Maphosa *et al.,* [73] reported that the leave extract exhibited anthelmintic activity against, *Caenorhabditis elegans*, a free-living nematode. When evaluated for reproductive ability and mortality of the parasite, the hexane, ethanolic and aqueous leave extracts showed activity against the parasite at concentrations of 1 and 2 mg/mL, the standard treatment levamisole was used as a control at 5 µg/mL [73, 74].

Studies on the anti-inflammatory and cardiovascular properties of the leave extracts have also been reported. El-Ansari *et al.,* [75] indicated that the flowering parts of the ethanolic and chloroform extracts showed strong anti-inflammatory activity in rats. The 70% methanol/chloroform extracts (500 mg/100 g BW) caused a significant reduction (20 and 41 %, respectively) in the paw edema test as compared to Voltarin (0. 7 mg/100 g BW), utilized as a reference anti-inflammatory, that produced inhibition of 26% [75].

Obekize *et al.,* [76] investigated the cardiovascular activity of the leave extracts of *L.leonorus*. The methanol extracts exhibited cyclooxygenase enzyme inhibitory activity and antihypertensive effect [76]. The aqueous extract has positive inotropic and negative chronotropic activity at low concentrations, while at higher doses (> 2 mg/mL) it has toxic effects on isolated perfused rat heart [76,77].

1.3.3 Phytochemical screening of *L. leonurus*

The phytochemical studies on *L. leonurus* have reported the isolation and characterization of labdane diterpenes, acyclic diterpenes, iridoid glycosides, alkaloids, dicarboxylic acid and flavonoids [78]. In total about 37 metabolites have been identified which largely consist of labdane diterpenes [78]. The labdane diterpenes presence in *L. leonurus* and other species are a chemotaxonomic marker for the genus and the mint family, *Lamiaceae* [79,80]. Other compounds were isolated; however they were not given names because of the similarities in structure to other labdane diterpenoids and were named Leoleorines [79] while other compounds from the leave water extracts were named as Leonurenones [81].

Wu and co-workers [82] isolated eight new labdane diterpenoids from the acetone leave extracts of *L. leonurus* which they called leoleorins. The isolated leoleorins included Leoleorin H (**21),** Leoleorin I (**22**) and Leoleorin J (**23**). The compounds were screened against different G-protein-coupled receptors. Compounds **21** and **22** demonstrated 50.0 and 61.9 % inhibitions respectively, at the serotonin 5-HT_{1A} receptor while compound **23** showed 57.0% inhibition at the serotin $5HT_3$ receptor.

Agnihotri and colleagues [83] isolated five metabolites from the methanol flowering top extracts of *L. leonurus*. The metabolites included two known metabolite, succinic acid (**24**) and uracil (**25**) and a new metabolite 1,2,3-Trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate (**26**). The metabolites were not active against chloroquine resistant (W2) strain and the D6 clone strain. A novel labdane diterpenoid, leonurun (**27**) and a known labdane marrubin (**28**) were isolated from the acetone leave extracts of *L. leonurus* [84, 85]. The two labdane diterpenoids were never screened for any biological activity.

Chen *et al.,* [86] isolated a compound called leonurine (**29**) from the methanol leave extracts of *L. leonurus*. When studying the phytochemical and pharmacological studies on *L. leonurus,* Mohamed *et al.,* [87] isolated ten flavonoids from the flowering aerial parts. The isolated flavonoids included two monoglycoside flavones, apigenin 7-*O*-β-glucoside **(30)** and luteolin 7-*O*-β-glucoside **(31)**. Compound **31** was screened for antimalarial activity and showed activity against chloroquine resistant (W2) strain (1.8 mg/mL) and the D6 clone (2.2 mg/mL).

In another study, He and colleagues [88] isolated three compounds they called leonurenones from the water leave extracts of *L. leonurus*. The isolated leonurenones were leonurenone A (**32**), leonurenone B (**33**) and leonurenones C (**34**). Leonurenones A and B were screened for CNS receptor binding assays at the GABAA site. The two compounds showed 50% inhibition at a concentration of 10 μM.

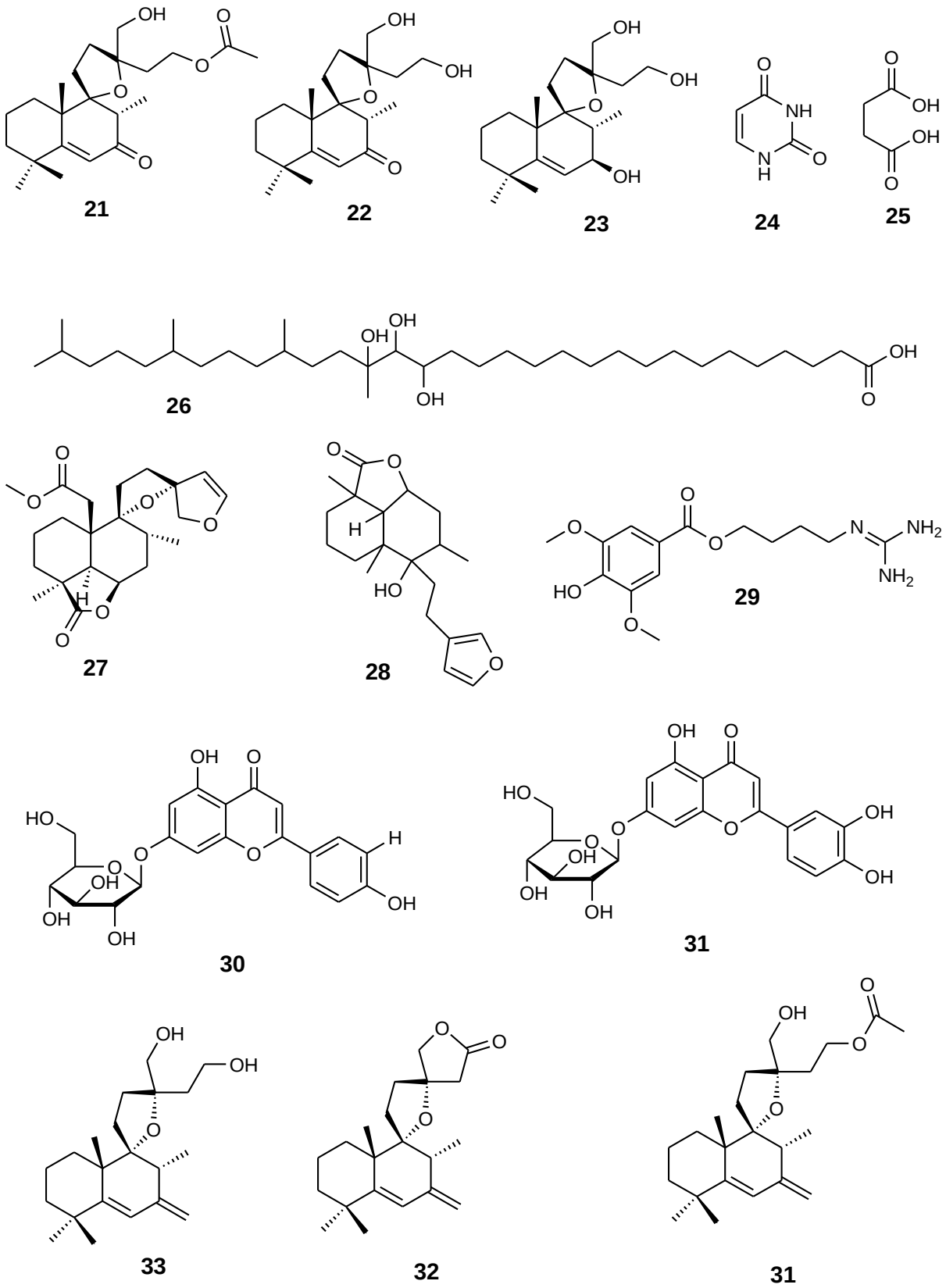

Figure 6. Chemical structures of some of the compounds isolated from *Leonotis leonurus.*

C. gratissimus and *L. leonurus* are known to be used by traditional healers to treat TB, coughs and headaches [41, 42, 59, 64]. Biological activity screening of the two plant extracts showed good activity against most microbes. After doing deep search on Sci-finder search and google search, there was no published work done on the antimycobacterial investigations of the two plant species against the *Mycobacterium tuberculosis* $H_{37}R_V$ strain. Therefore, the present study is aimed at investigating the antimycobacterial activity of the leave extracts of the two plants species against the *Mycobacterium tuberculosis* $H_{37}R_V$ strain.

1.4 Tuberculosis

Tuberculosis (TB), caused by a pathogen called *Mycobacterium tuberculosis* is a contagious airborne disease that mainly infects the lungs [89]. The World Health Organisation (WHO) declared TB as a global health threat and as one of the leading causes of death all over the world [90]. An estimated 10 million people were infected with TB and about 1.5 million people died from the disease in 2018 according to WHO. South Africa is one of the countries with the highest burden of TB [91]. In 2017, WHO estimated incidence of 322,000 cases of active TB in South Africa and out of the estimated number, about 60% of the people were HIV positive [92]. TB is transmitted from one person to another through droplets from the throat and lungs of people with the active disease [93]. The disease may however affect different parts of the body [93].

1.4.1 Types of TB

WHO revealed that there are four types of TB's [93]. The first type of TB is Latent TB where the bacteria can live in human body without making the person sick, since the body is able to fight the bacteria from growing [94]. The second type of TB is called the TB disease or active TB diseases which results when the bacteria becomes active within the human body and it becomes difficult for the immune system to fight or inhibit the growth of the bacteria. The third type is the Multi drug resistant (MDR) TB that arises by self-generation of the chromosomal mutations occurring within short period of time [89, 93]. It is known to resist two powerful first line drugs used for treatment of TB. The Last type of TB is called Extensive drug resistant (XDR) TB, which also arises

from self-generation of chromosomal mutation within a brief period of time. It is known to resist almost all the drugs used for TB treatment [94].

1.4.2 Current TB treatment

The main aim of TB treatment it to cure the patient. Other aims are to prevent the spread of TB, and to prevent the development of drug resistant TB [95]. The current TB regime is a combination of five drugs that includes Isoniazid, Rifampicin, Pyrazinamide, Ethambutol and Streptomycin [86]. These drugs generally have the greatest activity against *Mycobacterium tuberculosis* and are used for patients with active TB disease who have not had TB drug treatment before. The duration takes about six to nine months. While new drugs such as bedaquiline and delamanid have recently been discovered and approved for use in MDR TB, they are expensive and mostly unavailable in poor resourced countries [96].

1.4.3 Challenges of the current treatment

There are few problems that are associated with the current and available TB treatment. The complexity and the long duration of the treatment result in nonadherence of the patients to the treatment. This leads to the development of resistant strains such as MDR and XDR strains and continuous spread of the disease [97]. Since *Mycobacterium tuberculosis* is a slow growing organism, it requires administration of a combination of drugs for extended periods to achieve effective therapy and to prevent the emergence of resistant strains. The risk of adverse reactions therefore becomes a challenge [98]. According to WHO, success rate of regime used for treatment of drug resistant TB are much lower [99]. Drug toxicity coupled with the problem of mycobacterial persistence highlights the need to develop novel TB drugs that are active against drug resistant bacteria and kill persistent bacteria as well as shorten the length of TB treatment.

1.5 Aim and objectives

1.5.1 Aim

The aim of the study was to evaluate the antimycobacterial activity of the leave extracts of *Croton gratissimus* and *Leonotis leonorus* and isolate and characterise the active compounds.

1.5.2 Objectives

The objectives were to:

- Isolate the active compounds from the plant extracts.
- Test the isolated compounds for activity against *Mycobacterium tuberculosis*.
- Elucidate the structures of the isolated compounds.
- Determine the active dosage required to inhibit *Mycobacterium tuberculosis.*
- Determine the safety of the isolated compounds.

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Chapter 2

2 Results and discussion

2.1 Extraction

The plant materials were extracted as described in section 4.2.1. The amount (in grams) of the extracted leave materials of *C. gratissimus* and *L.leonorus* using four different solvents of varying polarities are displayed in **Figure 7** and **Figure 8** respectively.

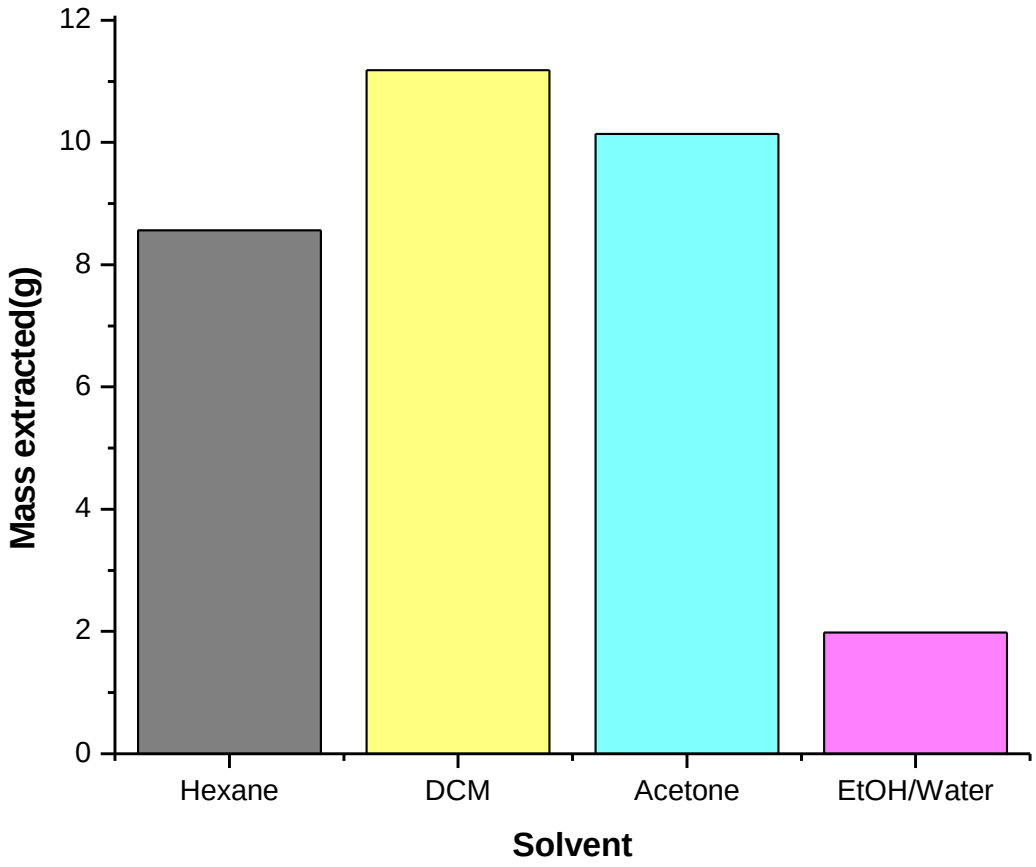

Figure 7. Amount in grams extracted from *C. gratissimus* leaves using solvents of varying polarities.

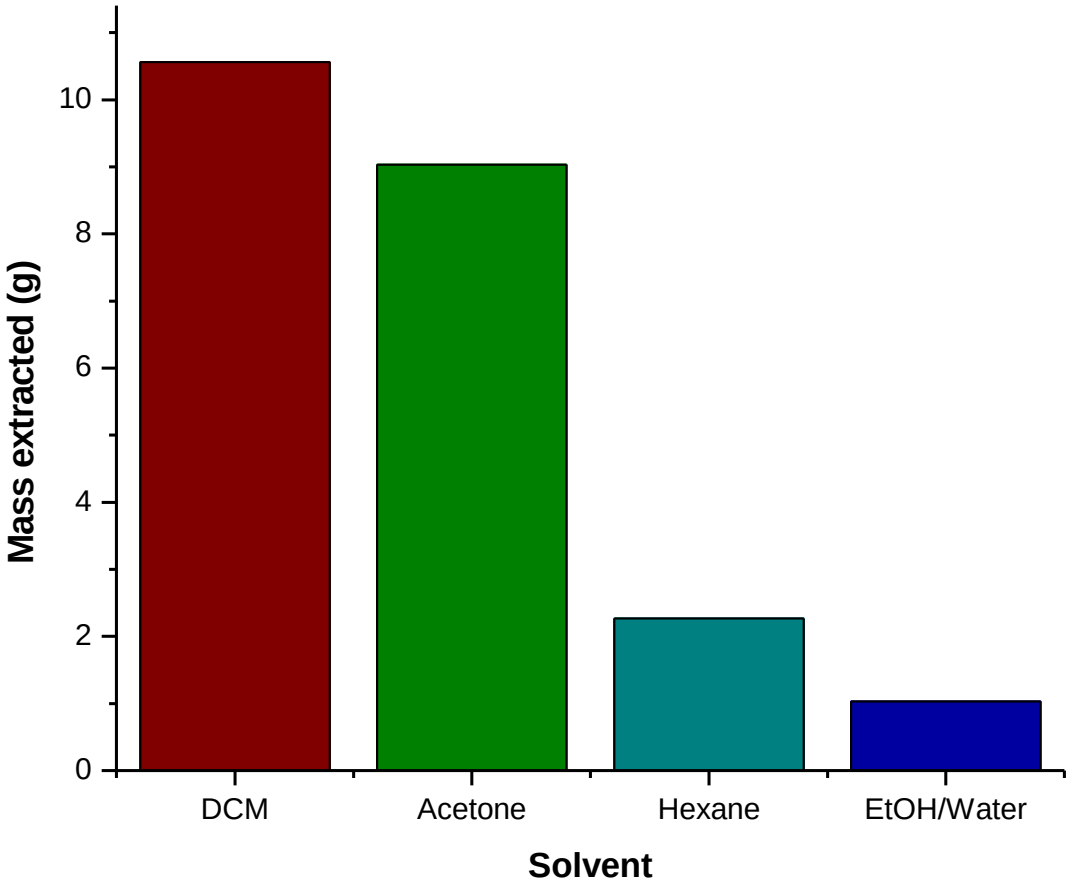

Figure 8. Mass in grams of the extracted material from *L.leonorus leaves* using solvents of varying polarities.

After extracting 100 g of *C. gratissimus* and *L. leonurus* materials using different solvents separately, DCM was able to extract most of the components in terms of mass (11.18 g from *C. gratissimus* and 10.56 g from *L. leonurus*) followed by acetone and hexane. Acetone was able to extract 10.14 and 9.03 g while hexane extracted 8.56 and 8.27 g from *C. gratissimus* (**Figure 7**) and *L. leonurus* (**Figure 8**) respectively. Extraction with combination of ethanol and water (35% EtOH/water) gave the lowest quantities of 1.98 and 1.03 g from *C. gratissimus* and *L. leonurus,* respectively. Extraction with EtOH/water was done using an espresso machine [1] while normal extraction was done with all other solvents.

2.2 Antioxidant assay

Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that may damage the cells of organisms [2]. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol [3]. This free radical that is stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colourless ethanol solution.

DPPH method was developed by Blois [4] with the viewpoint to determine the antioxidant activity using DPPH. The assay is based on measurement of the scavenging capacity towards it [5]. Butylated hydroxytoluene (BHT) is well known as an artificial antioxidant which may be added to meat and meat products for preventing oxidation and extending the storage life of meat products [6,]. This artificial antioxidant was used as a standard oxidant comparing its scavenging activity towards the free radical (DPPH) with the plant extracts.

2.2.1 Qualitative antioxidants analysis

Qualitative analysis of the antioxidants within the plant extracts was determined as described in section 4.5.1. **Figure 9** and **Figure 10** shows the TLC bioautograms of crude extracts of *C. gratissimus* and *L. leonurus* respectively after development in Ethyl acetate/methanol/water (EMW), Chloroform/ethylacetate/formicacid (CEF) and Benzene/ethanol/ammonia hydroxide (BEA).

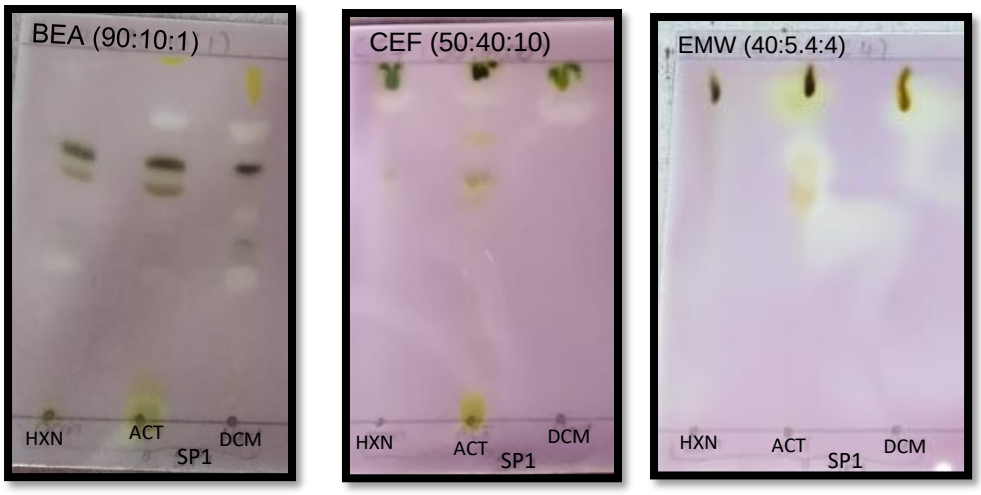

Figure 9. TLC bioautograms of *C. gratissimus* extracted with DCM, acetone and hexane. Chromatograms were developed in BEA, CEF and EMW and sprayed with DPPH.

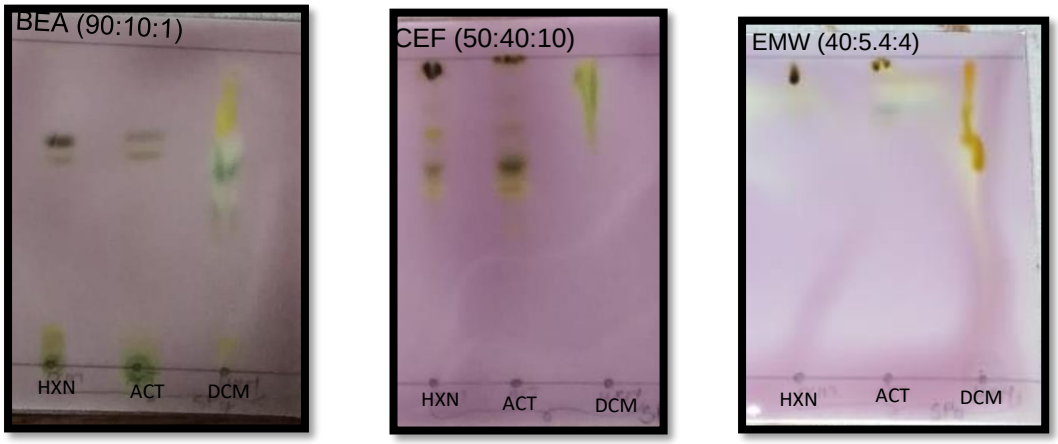

Figure 10. TLC bioautograms of *L.leonorus* extracted with DCM, acetone and hexane. Chromatograms were developed in BEA, CEF and EMW and sprayed with DPPH.

The TLC bioautograms of *C. gratissimus* and *L. leonurus* in **Figures 9 and 10** respectively shows that DCM extracts had more clear zones in different mobile systems as compared to all other solvents. The DCM extract from *C.grattissimus* showed 3 clear spots in the BEA mobile system while acetone and hexane extracts showed only one clear zone each. Even though there was no clear separation of the compounds in CEF and EMW mobile systems clear zones were observed from all the extracts which indicate the presence of antioxidants within the extracts.

The DCM extract of *L. leonurus* in **Figure 10** also showed more clear zones as compared to acetone and hexane extracts which did not show any clear zones. While clear separation of the acetone and hexane extracts was observed in the CEF mobile system, these extracts did show any clear zone, but the DCM extract showed a clear zone around the unseparated fraction which was moved to the top of the plate. The extracts in the EMW solvent did not separate but clear zones were observed which indicated the presence of the antioxidants.

2.2.2 Quantitative antioxidant analysis

Quantitative antioxidant analysis was done to measure the ability of the plants extracts to scavenge DPPH radicals. These was done by comparing the percentages at which the plant extracts and BHT inhibited DPPH at different concentrations as shown in **Figures 11 and 12**.

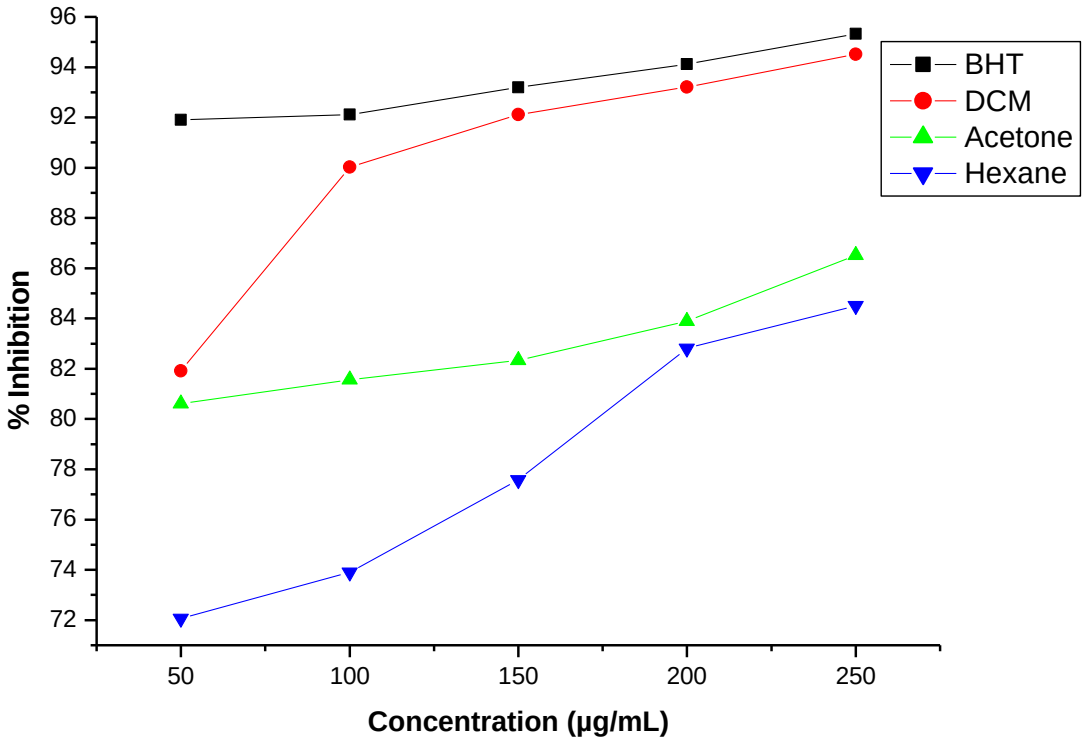

Figure 11. A line graph showing the percentage inhibition of different solvents (DCM, acetone and hexane) extracts of *C. gratissimus* against DPPH compared to BHT (standard antioxidant).

The percentage at which *C. gratissimus* extracts and BHT inhibit DPPH increases as the concentration increases as shown in **Figure 11.** BHT had the highest inhibition at varying concentrations as compared to the three extracts. It was able to inhibit 91.1% of the DPPH at 50 µg/mL followed by DCM and acetone extracts which inhibited 81.9 and 80.6 % respectively while hexane inhibited the lowest quantity of 72%. The %inhibition of BHT and the extracts increased with concentration until at 250 µg/mL where BHT inhibited 95.3% followed by acetone and DCM with 93.3 and 86.2 % respectively with hexane again inhibiting the lowest percentage (84.5%) compared to other extracts. The results are consistent with the qualitative analysis results where

DCM showed a greater number of clear zones followed by acetone then hexane with the least number of clear zones.

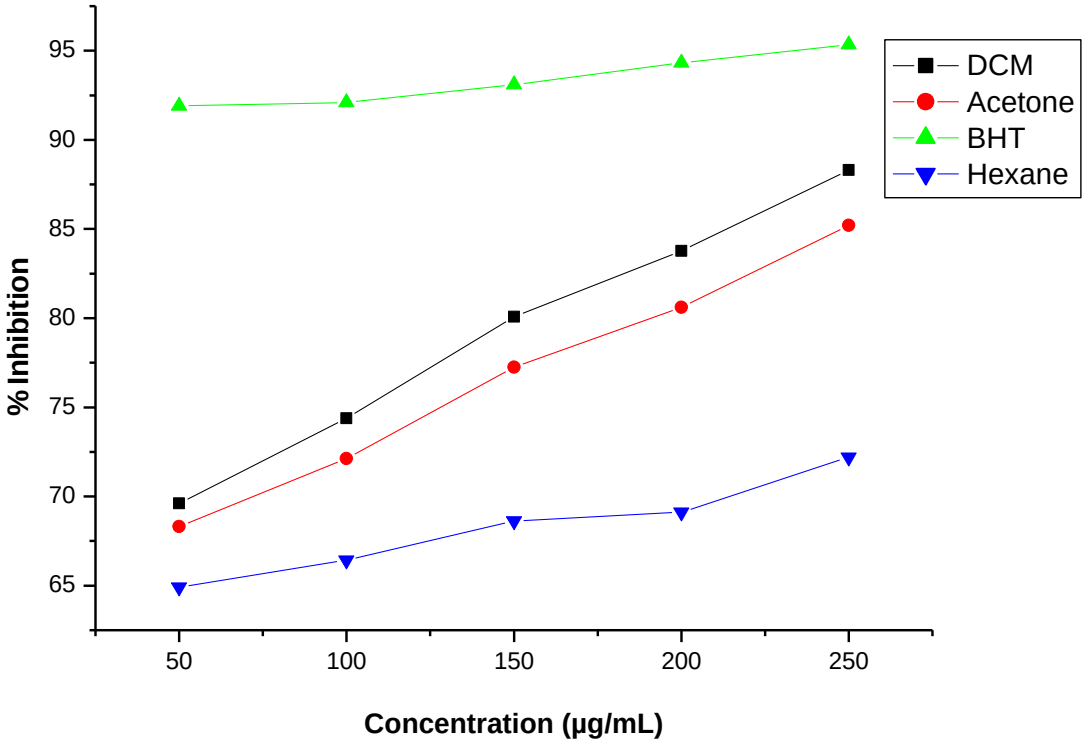

Figure 12. A line graph showing the percentage inhibition of different solvents (DCM, ACT and HXN) extracts of *L. leonorus* against DPPH compared to BHT (standard antioxidant).

The percentage at which BHT and the extracts from *L. leonurus* inhibit DPPH is directly proportional to the concentration. BHT had the highest inhibition percentages at different concentrations than all the three extracts with hexane having the least inhibition percentages. BHT was able to inhibit 94.1 and 95.3 % of DPPH at 50 and 250 µg/mL respectively with hexane inhibited lower percentages of 64.92 and 72.21 % at the same concentrations respectively. The percentage inhibitions of DCM at different concentrations is higher than those of hexane and acetone which is consistent with the qualitative analysis results were the two extracts showed less number of clear zones of inhibition.

2.3 Isolation and minimum inhibitory concentrations

TLC finger printing of *C. gratissimus* and *L. leonurus* showed that DCM extracted more compounds than all other solvents, with the plates showing a greater number of spots than all other solvents as shown in **Figure 13**. Some of the spots had similar R_f values as some spots in the extracts of other solvents which suggested that DCM extracted the same compounds that were extracted by other solvents and more. Therefore, purification and isolation were done on the DCM extracts only for both plant species.

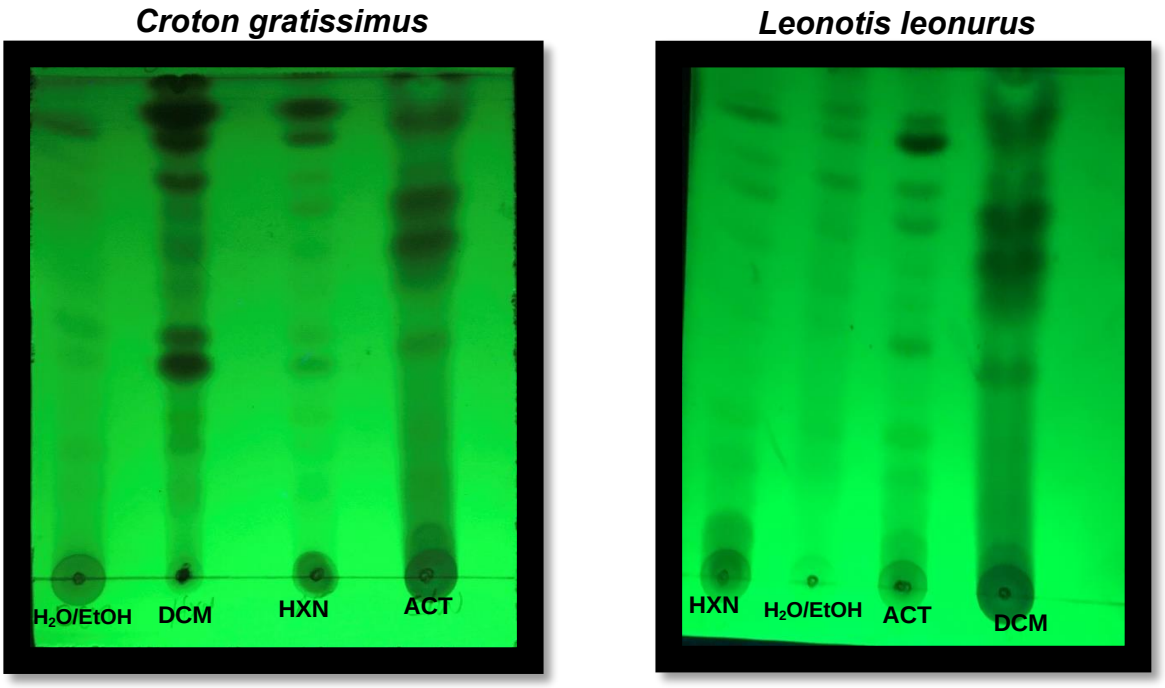

Figure 13. TLC fingerprinting of components extracted by different solvents from *C. gratissimus* and *L. leonurus*.

Isolation of the components within the DCM extracts of the two plant species was done following the bioassay-guided fractionation protocol, meaning step-by-step separation of the extracted components based on their differences in the R_f values and assessing the biological activity followed by the next round of separation and assessing [7]. The components within the crude extracts were separated using column chromatography and sent for *in-vitro* antimycobacterial activity. The second round of separation was done using preparative thin layer chromatography. All the isolated fractions/components are displayed in **Figure 14** and **Figure 15** for *C. gratissimus* and *L. leonurus* respectively.

2.3.1 Isolation and minimum inhibitory concentrations of fractions from *C. gratissimus*

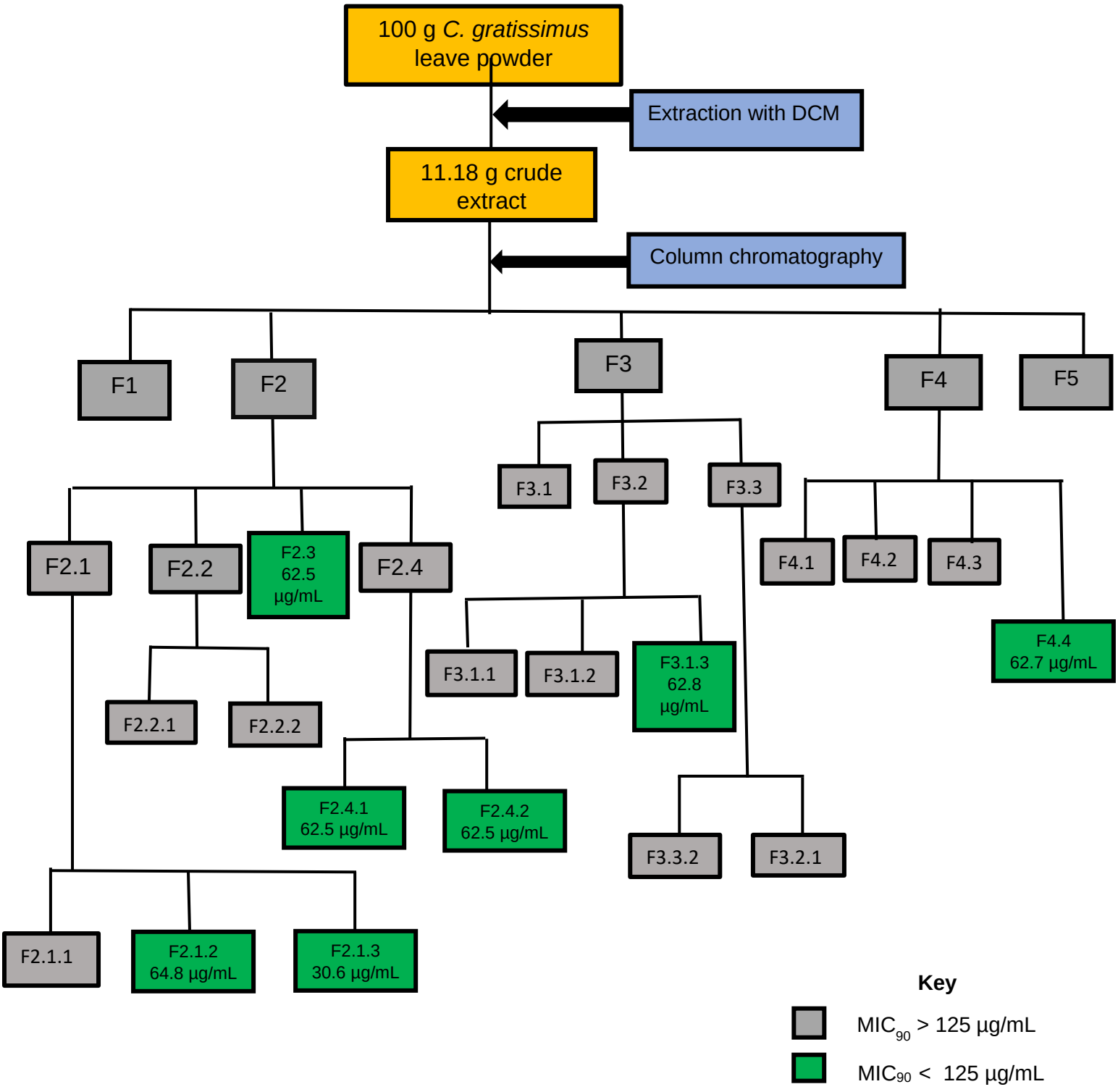

Figure 14. Flow chart diagram of the isolated fractions from *C. gratissimus* with MIC_{90} values of the fractions with potential activity.

After fractionation of the crude extract of *C. gratissimus* using column chromatography, eighty fractions were collected in test tubes. The fractions were allowed to concentrate under a stream of cold air in a fume hood. Fractions containing similar constituents were combined (monitored by TLC finger printing) and made a total of five fractions which were labelled F1, F2, F3, F4, and F5 as shown in **Figure 11**. All the five fractions were able to inhibit the *Mycobacterium* with MIC_{90} >125 µg/mL. Fractions F1 and F5 were collected as single components and were not further purified. F1 was collected as a yellow oily fraction while F5 was a dark coloured fraction with masses of 16.3 and 20 mg, respectively. Fractions F2, F3 and F4 had masses of 1, 0.6, and 1.88 g, respectively.

Fractionation of F2 gave four more fractions which were labelled F2.1, F2.2, F2.3 and F2.4. All the fractions also had MIC_{90} >125 µg/mL except for F2.3 which was isolated as a single component and gave MIC_{90} of 62.5 µg/mL. Three fractions (F2.1.1,F2.1.2 and F2.1.3) were isolated from Fraction F2.1 and two of them (F2.1.2 and F2.1.3) showed an increased activity with MIC_{90} of 64.88 and 30.61 µg/mL respectively as compared to the mother fraction (F2.1) which had MIC_{90} that was >125 µg/mL. Fraction F2.2 gave two more fractions which were labelled F2.2.1 and F2.2.2 and both had MIC_{90} >125 µg/mL. Two fractions (F2.4.1 and F2.4.2) with increased activity were isolated from F2.4 and both had MIC_{90} of 62.5 µg/mL.

Three fractions (F3.1, F3.2 and F3.3) resulted from fractionation of fraction F3 and all of them gave MIC_{90} >125 µg/mL. While F3.2 was isolated as a single component, Purification of F3.1 and F3.2 resulted in three (F3.1.1, F3.1.2 and F3.1.3) and two fractions (F3.3.1 and F3.3.2) respectively. Fraction F3.1.3 showed an increased activity with MIC_{90} of 62.8 µg/mL while F3.1.1 and F3.1.2 had MIC_{90} >125 µg/mL. Fraction F4 was fractionated into four fractions (F4.1, F4.2, F4.3 and F4.4) and all of them had MIC_{90} >125 µg/mL except for F4.4 which had MIC_{90} of 62.71 µg/mL. A total of 28 fractions were isolated from the DCM crude extract of *C. gratissimus*. All the fractions showed activity against the *Mycobacterium tuberculosis* at concentrations greater than 125 µg/mL except for seven fractions that showed potential activity with MIC_{90} values ranging from 30.61 to 64.88 µg/mL.

2.3.2 Isolation and minimum inhibitory concentration of the fraction from *L. leonurus*.

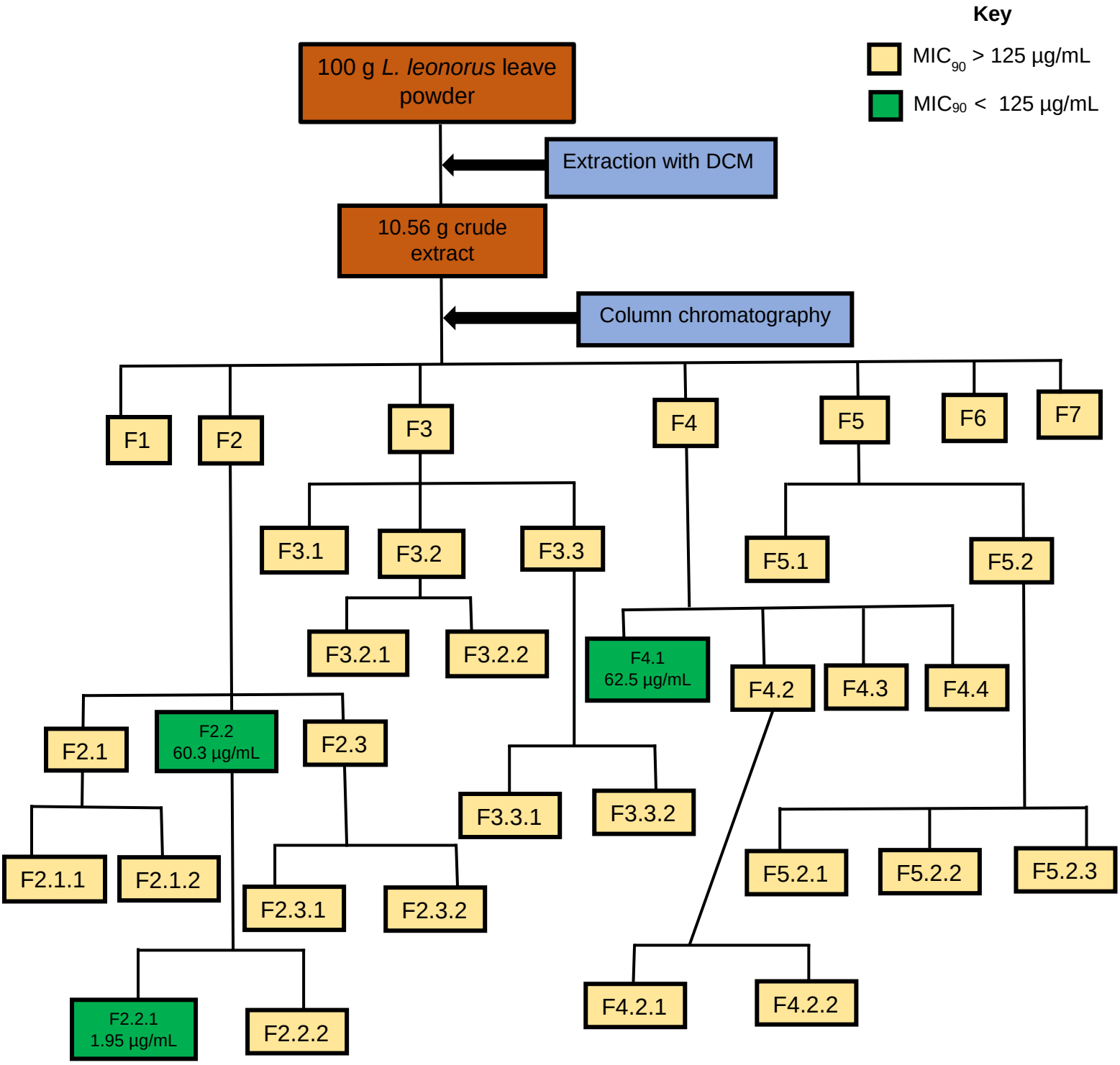

Figure 15. Flow chart diagram of the bioassay purification of fractions isolated from *L.leonorus* and the MIC_{90} values of the fractions with potential activity.

Fractionation of DCM crude extract of *L.leonorus* resulted in a total of seven fractions which were labelled F1-F7 as shown in **Figure 14**. The fractions were able to inhibit 90% of *Mycobacterium tuberculosis* at a concentration that was greater than 125 µg/mL. Fractions F1, F6 and F7 were isolated as single components (according to TLC finger printing) and were not further purified/fractionated. F1 fraction was obtained as yellow oily extract while F6 and F7 were obtained as light green extracts.

Fraction F2 was further purified using Prep TLC and gave three more fractions labelled F2.1, F2.2 and F2.3. Fractions F2.1 and F2.3 showed the same activity (MIC_{90}>125 µg/mL) as their mother fraction (F2) while F2.2 showed an increase activity with MIC_{90}= 60.3 µg/mL. Fractionation of F2.2 resulted in two fractions (F2.2.1 and F2.2.2) where F2.2.1 had an increase activity as compared to F2.2 with MIC_{90} = 1.95 µg/mL while F2.2.2 had MIC_{90} greater than 125 µg/mL.

Further purification of F2.1 and F2.3 gave two fractions each (F2.1.1 and F2.1.2; F2.3.1 and F2.3.2 respectively) with MIC_{90}>125 µg/mL. Three fractions (F3.1. F3.2 and F3.3) obtained from F3 had MIC_{90}>125 µg/mL. F3.1 was isolated as a single component and was not further purified but further purification of F3.2 and F3.3 gave two fractions each (F3.2.1 and F3.2.2; F3.3.1 and F3.3.2 respectively) with MIC_{90}>125 µg/mL. Fractionation of F4 led to one fraction (F4.1) with an increased activity (MIC_{90} = 62.5 µg/mL) and three other fractions (F4.2, F4.3 and F4.4) with MIC_{90} >125 µg/mL. All the fractions obtained from F5 (F5.1 and F5.2) showed the same activity as F5 (MIC_{90} >125 µg/mL). Further purification of F5.2 gave three more fractions (F5.2.1, F5.2.2 and F5.2.3) which also had the same activity as F5. A total of 31 fractions were successfully isolated from DCM leave extracts of *L. leonurus.* Three fractions showed potential activity with MIC_{90} values ranging from 1.963 to 60.51 µg/mL. The other fractions had MIC_{90} values that were greater than 125 µg/mL

2.4 Structure elucidation of the isolated compounds

Combination of 1D (^{1}H and ^{13}C) and 2D (COSY, DEPT HSQC and HMBC) NMR spectroscopy was used for the chemical structural elucidation of the compounds that were contained within the isolated fractions. The data showed that the isolated fractions were not pure compounds but mixtures of closely related compounds that had the same R_f values on the TLC plates. HPLC-MS and FTIR were used to determine the molecular weights and the functional groups within the compounds respectively.

2.4.1 Structure elucidation of compound from fraction F4.1 of *L. leonurus.*

Fraction F4.1 was isolated as a dark coloured paste with about 25 mg of mass. The fraction showed potential activity against the *Mycobacterium* with MIC_{90} value of 62.5 µg/mL. Even though the TLC fingerprinting showed only one spot when the fraction was isolated, NMR spectra showed that the fraction is a mixture of isomeric compounds that had the same R_f values. The major compound was identified by the appearance of tall peaks on the ^{13}C NMR and ^{1}H spectra and its structure was elucidated. **Figure 16** shows the chemical structure of the major compound while **Table 1** shows the NMR data of these compound.

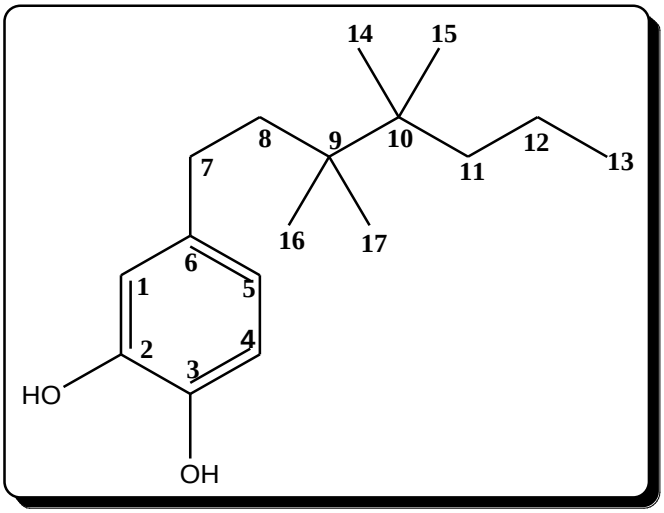

Figure 16. The chemical structure of a 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L.leonorus*.

All seventeen carbons of the compound could be identified on the ^{13}C NMR spectrum (**Figure 18**). The carbons included two phenol carbons (δ_C 146.9, C-2 and 147.5, C-3), four olefinic carbons (δ_C 119.0, 123.9, 124.4 and 138.5), four methylene carbons (δ_C 31.9, 29.3, 29.6 and 22.6), five methyl carbons (δ_C 14.0, 29.6, 30.1, 30.2 and 31.3) and two quaternary alkane carbons (34.3, C-9 and 34.5,C-10). The ^{1}H spectrum (**Figure 17**) showed the presence of three aromatic protons (δ_H 7.13 (dd J=2.6, 8.7), 7.34 (d J=2.6) and 7.53 (d J = 8.7)), overlapping methylene protons (δ_H 1.24 (s) and 1.27 (s)), terminal methyl protons at δ_H 0.86 (s), three overlapping methyl protons at δ_H 1.32 (s) and other methyl protons at δ_H 1.27 (s) overlapping with methylene protons.

The proton at δ_H 7.34 showed HSQC correlation with a methine carbon at δ_C 119.0 (C-1) and was then assigned H-1(δ_H 7.34). Two other methine carbons at δ_C 123.9 (C-4) and 124.4 (C-5) showed HSQC correlations with the protons at δ_H 7.53 and 7.13 and these protons were assigned H-4 (δ_H 7.53) and H-5 (δ_H 7.13), respectively. The methine carbon at δ_C 123.9 further showed HMBC correlation with the protons δ_H 7.34 (H-1) and 7.13 (H-5). Two quaternary carbons at δ_C 147.9 (C-2) and 147.5 (C-3) showed HMBC correlation with the aromatic carbons at δ_H 7.13, 7.34 and 7.53. These carbons further showed HMBC correlation with the methylene protons at δ_H 1.24 (H-8) and 1.27 (H-7). A quaternary carbon at δ_C 138.98 (C-6) showed HMBC correlation with overlapping protons at δ_H 1.24, 1.27 and 7.53 (H-4).

The quaternary carbons resonating at δ_C 34.25 (C-9) and 34.45 (C-10) showed HMBC correlation with the protons at δ_H 1.24, 1.27 and 1.32. The two carbons further showed HMBC correlations with the three aromatic protons at δ_H 7.13, 7.34 and 7.53. The protons at δ_H 1.27 showed HSQC relations with the two methylene carbons resonating at δ_C 31.86 (C-7) and 22.64 (C-12). The carbon at δ_C 22.64 further showed HMBC correlation with the methyl protons at δ_H 0.86 which led to assigned 2H-12 (δ_H 1.27). Two other methylene carbons at δ_C 29.31 (C-8) and 29.60 (C-11) showed HSQC correlations with the protons at δ_H 1.24 and these protons were then assigned to H-8 (δ_H 1.24) and H-11 (δ_H 1.24). The two methylene carbons above are equivalent.

The methyl protons resonating at δ_H 0.86 showed HSQC correlation with the carbon at δ_C 14.0 (C-13). This methyl resonance was then assigned to 3H-13 (δ_H 0.86). Three methyl carbons resonating at δ_C 29.60 (C-14), 30.10 (C-15) and 30.23 (C-16) showed

HMBC correlations with the protons at δ_H 1.32 and these carbons are equivalent. Another methyl carbon resonating at δ_C 31.3 (C-17) showed HSQC correlation with the protons at δ_H 1.27. This carbon is equivalent to the three methyl carbons mentioned above.

The aromatic proton at δ_H 7.13 showed COSY correlation with the aromatic protons at δ_H 7.34 and 7.53. While the methyl protons at δ_H 0.86 showed COSY correlation with the protons at δ_H 1.24 and 1.27.

The elucidated structure was a one membered aromatic ring attached to a branched alkane chain. The ^{13}C and ^{1}H NMR chemical shifts of the elucidated were assigned in comparison with the chemical shifts of similar structures on Sci-Finder search. The FTIR spectrum gave an adsorption broad band at 3405.25 cm^{-1} that is consistent with the two alcohol functional groups that are present in the molecule. The spectrum also gave sharp adsorptions bands at 962.44 and 2924.03 cm^{-1} that are consistent with the double bonds (C=C-H) of the disubstituted alkene ring and the C-H bonds of the alkane branched chain respectively. The structure had a molecular formula of $C_{17}H_{28}O$ that correspond to the molecular weight of 264.2089 g/mol. The LC-MS and HRMS spectra did not give any molecular ion peak corresponding to the elucidated structure.

Table 1. NMR spectral data (δ) of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) (400 MHz, CDCI3, room temperature)

Position	^{13}C	DEPT	^{1}H(mult)	COSY	HMBC
1	119.0	CH	7.34 (d J=2.6)	7.13; 7.53	
2	147.6	C			1.27; 1.24; 7.11 7.34;7.53
3	147.5	C			1.27;1.24;7.11 7.34;7.53
4	123.9	CH	7.53 (d J=8.7)	7.13	7.11; 7.34

5	124.4	CH	7.13 (dd J= 2.6, 8.7)	7.34	1.32
6	138.3	C			1.32
7	31.9	CH_2	1.27 (s)		
8	29.3	CH_2	1.24 (s)	0.86	
9	34.5	C			1.27;1.24;7.13, 7.34;7.53
10	34.8	C			7.13;7.34;7.53 1.32
11	29.7	CH_2	1.24 (s)	0.86	
12	22.6	CH_2	1.27 (s)		0.86
13	14.1	CH_3	0.86 (m)	1.24; 1.32	
14	29.6	CH_3	1.32 (s)	0.86	1.32
15	30.1	CH_3	1.32 (s)	0.86	
16	30.2	CH_3	1.32 (s)	0.86	
17	31.4	CH_3	1.27 (s)	0.86	1.24; 1.27

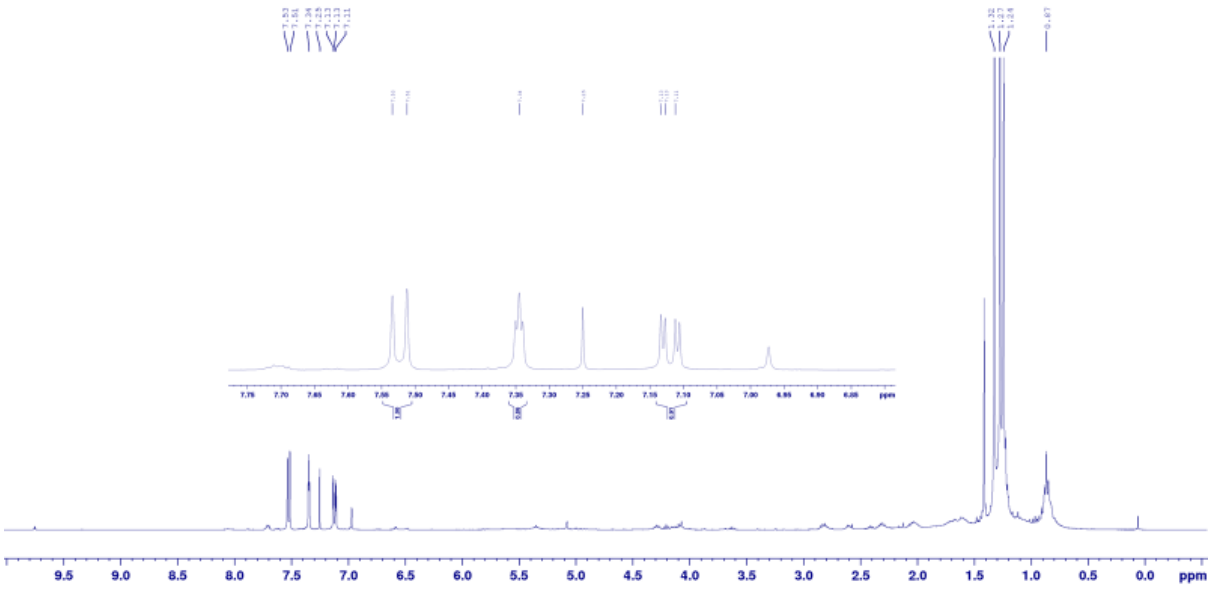

Figure 17. ^{1}H NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

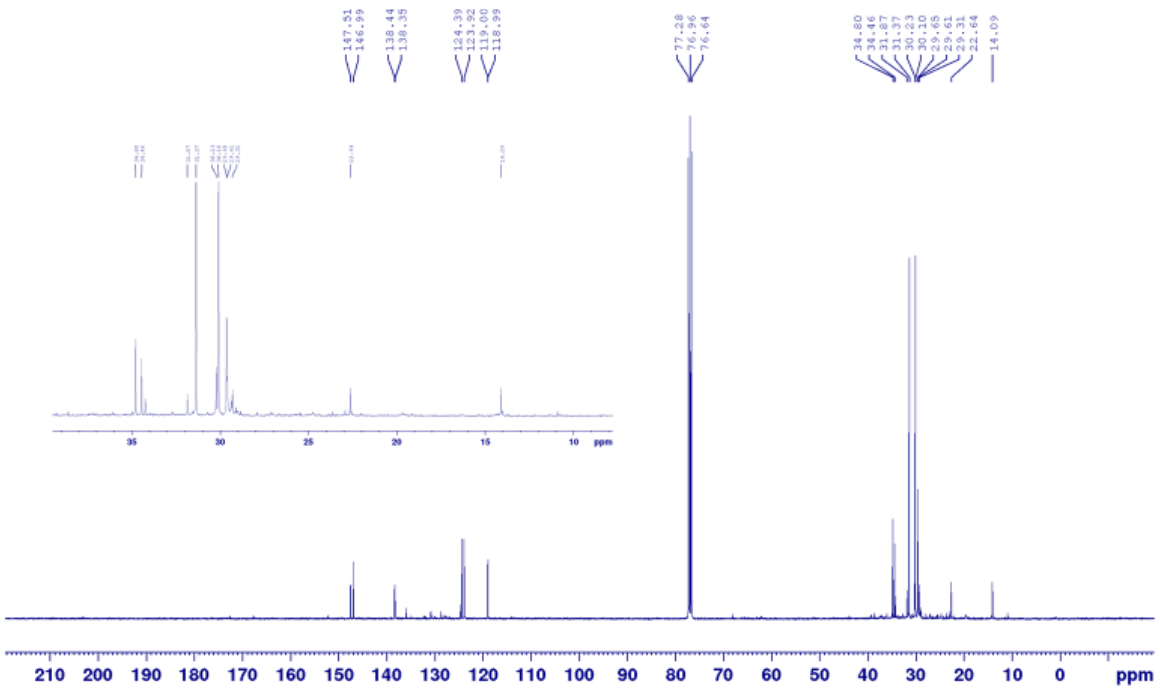

Figure 18. ^{13}C NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

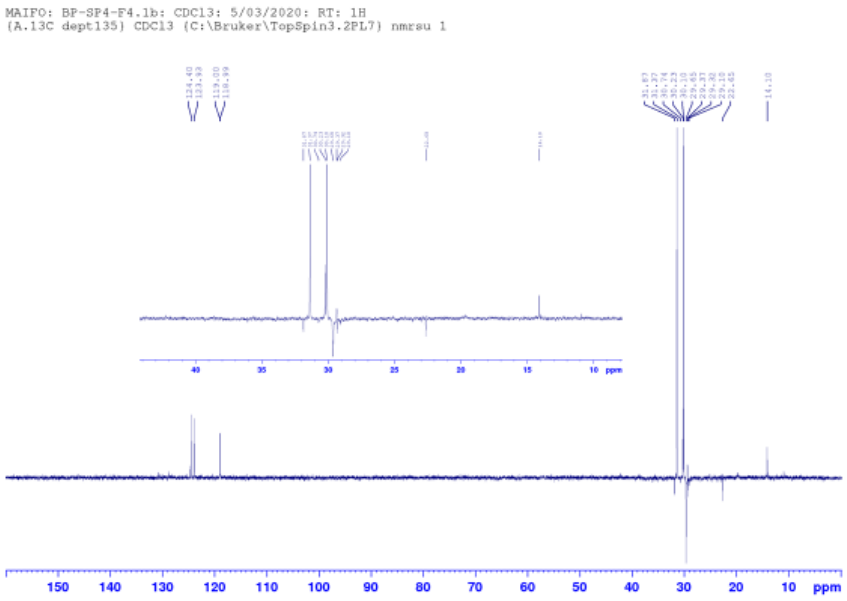

Figure 19. DEPT NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

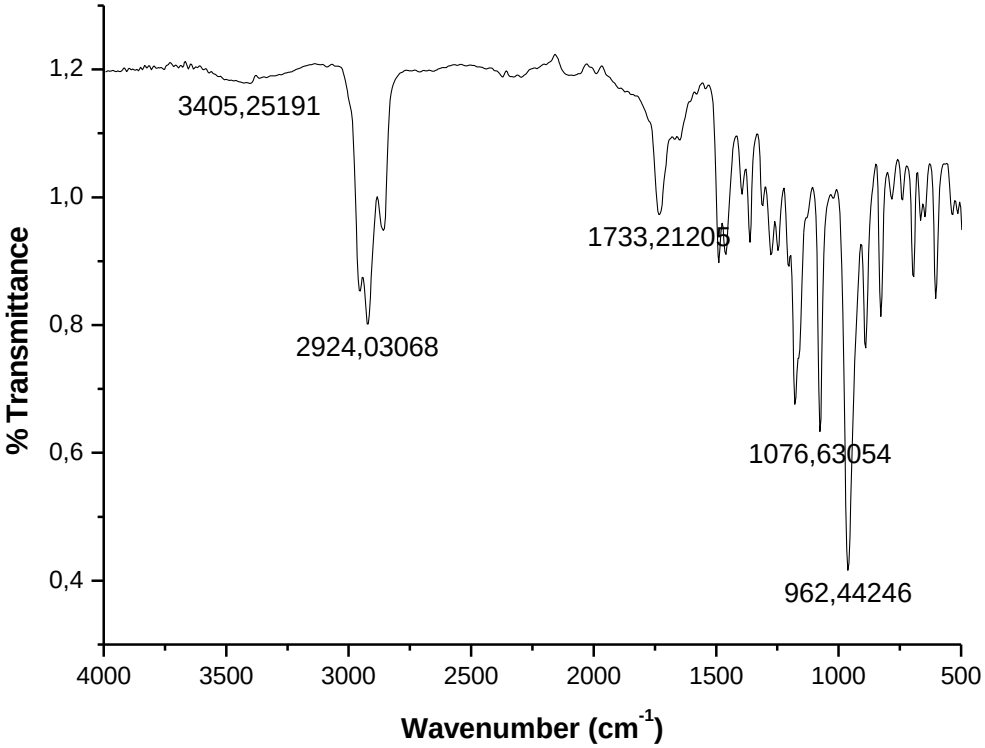

Figure 20. FTIR spectrum of NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

2.4.2 Structure elucidation of compound from fraction F5 of *C. gratissimus*

Fraction F5 from *C. gratissimus* was isolated as a colourless oil with about 20 mg of mass. The fraction had an MIC_{90} value that was greater than 125 µg/mL. NMR analysis of this fraction showed that the fraction is not pure, but it contained a mixture of cembranolides that had the same R_f value on the TLC plates. The ^{13}C NMR data of the cembranolide that was present in large quantities (major compound) matched closely with the ^{13}C NMR data of the cembranolide isolated from the leaves of the same species by Langat and co-workers [8]. Langat and his colleagues stated that the purified cemranolides are unstable on standing but appear to be stable in the impurified form [8].

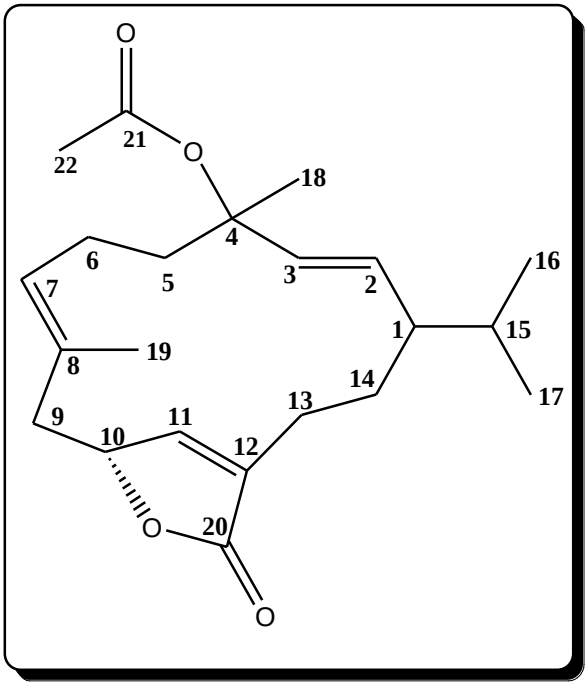

Figure 21. Chemical structure of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

The 22 carbons of the compound (**Figure 21**) could be identified on the ^{13}C NMR spectrum (**Figure 23**) including two carbonyl carbons (δ_C 174.1, C-20 and 180.6, C-21), five methyl group carbons (δ_C 20.0, 20.0, 23.0,15.7 and 18.5), six olefinic carbons (δ_C 127.7, 135.5, 131.7, 130.6, 147.7 and 133.7), an oxymethine at δ_C 77.5 (C-10) and five methylene carbons (δ_C 42.0, 29.0, 41.8, 23.4 and 32.2).

The ^{1}H NMR spectrum (**Figure 22**) indicated the presence of four olefinic proton resonances (two overlapping resonances at δ_H 5.77 (s), one at δ_H 5.20 (s) and another one at δ_H 6.34 (s)), five methyl group proton resonances at δ_H 0.91 (s), 0.82 (s), 1.25 (s), 3.64 (m) and a vinylic methyl group at 1.61 (m), three methine protons (two overlapping methine protons at δ_H 1.60 (m) and an oxymethine proton at δ_H 4.68 (m)).

The carbonyl carbon at δ_C 174.1.1 (C-20) showed correlations in the HMBC spectrum with a methine proton at δ_H 4.68 (H-10) and one of the methylene protons at δ_H 1.95 (H-14). The methyl protons at δ_H 1.25 (H-18) showed HMBC correlations with the carbons at δ_C 42.0 (C-5), 29.0 (C-6), 180.6 (C-21) and 135.5 (C-3). The carbons at δ_C 135.5 (C-3) and δ_C 179.5 (C-21) further showed HMBC correlations with the methyl protons at δ_H 0.87 (H-17) and δ_H 3.63 (H-22), respectively.

The identified cembranolide in **Figure 21** had molecular formula of $C_{22}H_{32}O_3$ which correspond to the molecular weight of 360.2309 g/mol. The HRMS (**Figure 26**) indicated the M^+ peak m/z at 317.2127 corresponding to molecular formula of $C_{20}H_{29}O_2$ which resulted from the loss of C_2H_3O fragment of the ester group of the identified molecule. The spectrum also indicated the m+1 molecular peak at 318.2123 with 22% relative abundance to the M^+ ion peak with 20 carbons. FTIR gave sharp absorption bands at 1737.29 and 2936.26 cm^{-1} consistent with the carbonyl and the olefinic functional groups respectively that are present in the isolated cembranolide.

Table 2. Comparison of ^{13}C NMR spectral data (δ) of (Methyl (5S,8R,9S,10R)-2-oxo-ent-clerod-3,13-dien-15-oate) and (4R,5E,7R,10E,13R)-4-isopropyl-7-methoxy-5,7,11-trimethyl-14-oxa-bicyclo[11.2.1]hexadeca-5,10-dien-15-one both isolated from the leaves *C.grattisumus*.

Position	^{13}C (ppm) Literature [8]	^{13}C (ppm) isolated	^{1}H (mult)	HMBC C-H
1	48.9 CH	51.5 CH	1.61 (m)	
2	134.0 CH	126.7 CH	5.79 (s)	

3	135.8 CH	135.5 CH	5.79 (s)	0.87; 1.25
4	77.2 C	75.8 C		
5	43.7 CH_2	40.4 CH_2	2.21 (m) 1.53 (m)	0.87, 1.25
6	25.5 CH_2	28.6 CH_2	2.48 (m) 2.46 (m)	
7	131.7 CH	131.7 CH	5.20 (s)	
8	129.4 C	130.6 C		
9	44.8 CH_2	40.0 CH_2	2.19 (m)	
10	80.3 CH	76.8 CH	4.68 (m)	
11	149.6 CH	147.7 CH	6.34 (s)	
12	133.5 C	133.7 C		
13	22.0 CH_2	22.3 CH_2	2.15 (m) 2.23 (m)	
14	34.0 CH_2	31.6 CH_2	1.95 (m) 1.50 (m)	
15	28.5 CH	29.9 CH	1.60 (m)	0.87; 1.25
16	20.7 CH_3	20.5 CH_3	0.91 (s)	
17	19.5 CH_3	20.5 CH_3	0.87 (s)	
18	22.3 CH_3	30.4 CH_3	1.25 (s)	0.87; 1.25
19	16.3 CH_3	17.5 CH_3	1.68 (m)	
20	174.2 C	173.1 C		4.68; 1.95
21		179.5 C		1.25; 3.63
22		19.5 CH_3	3.64 (m)	

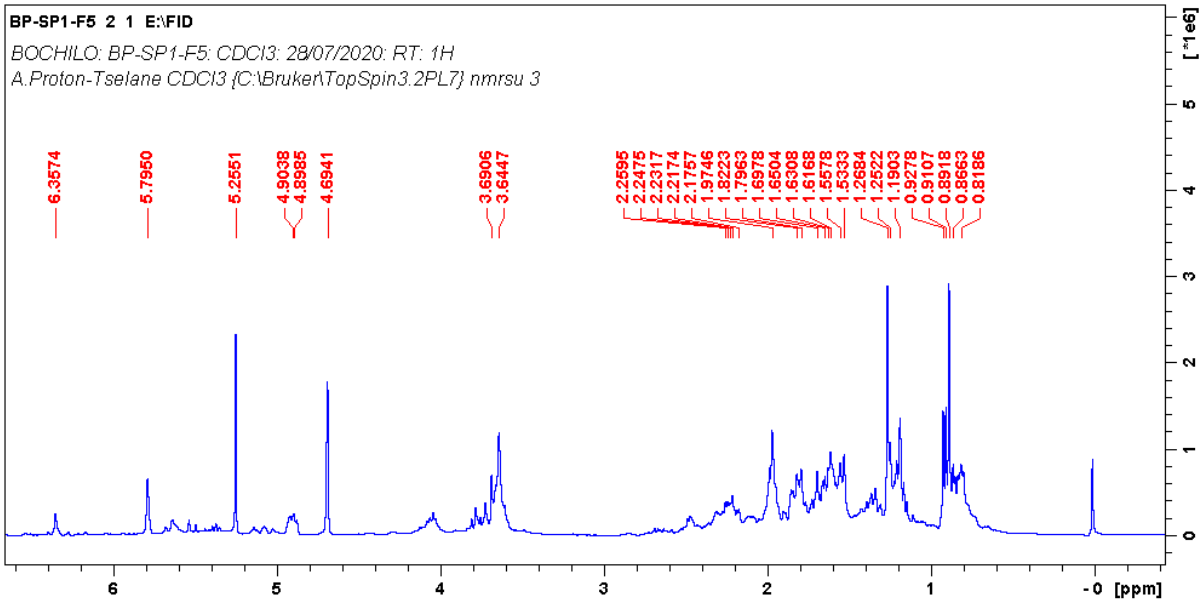

Figure 22. [1]H NMR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

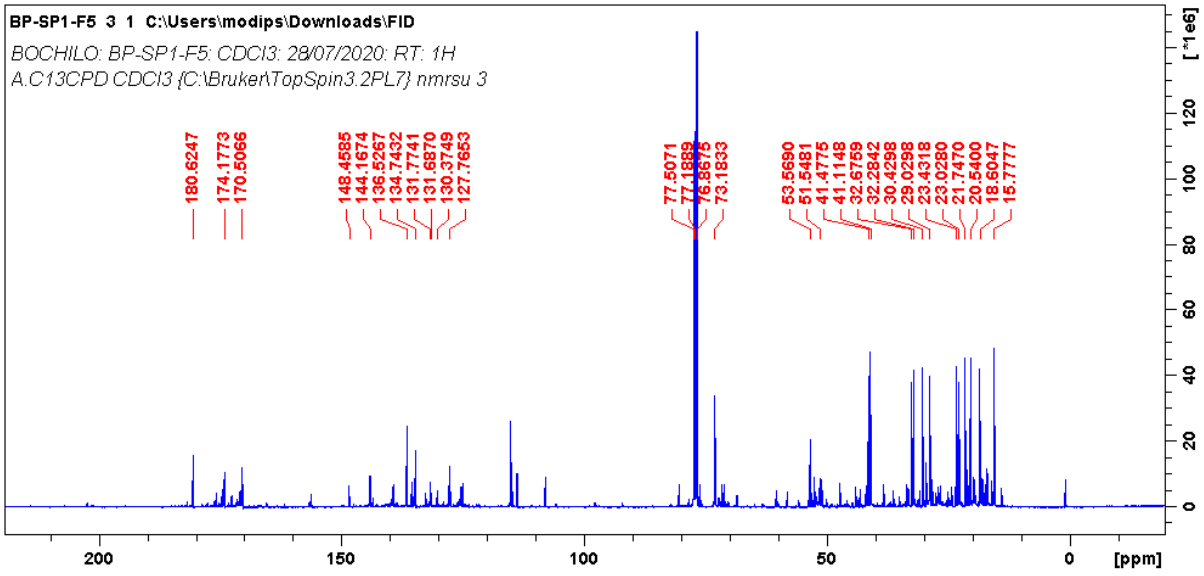

Figure 23. [13] C NMR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

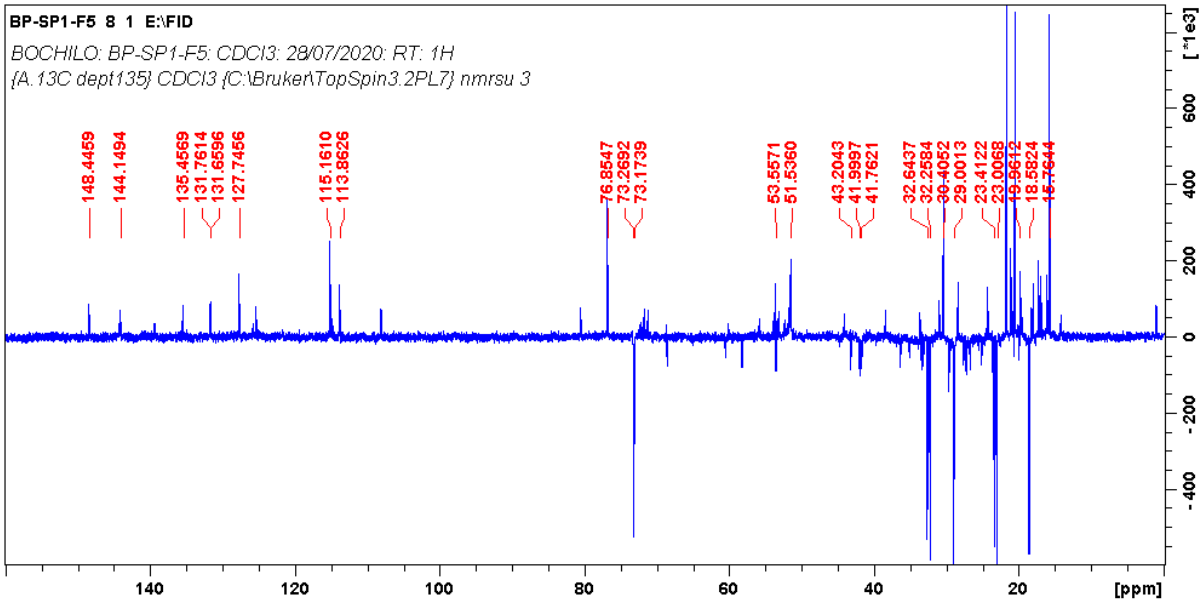

Figure 24. DEPT NMR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

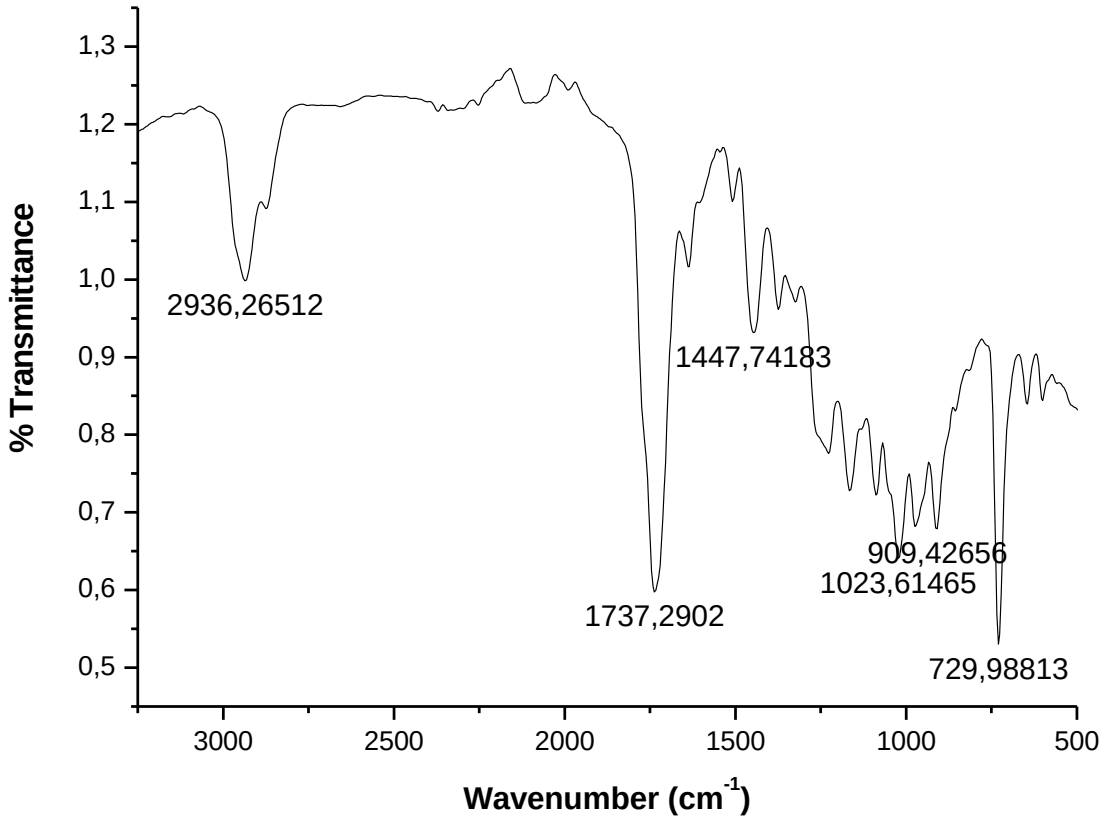

Figure 25. FTIR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-

bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

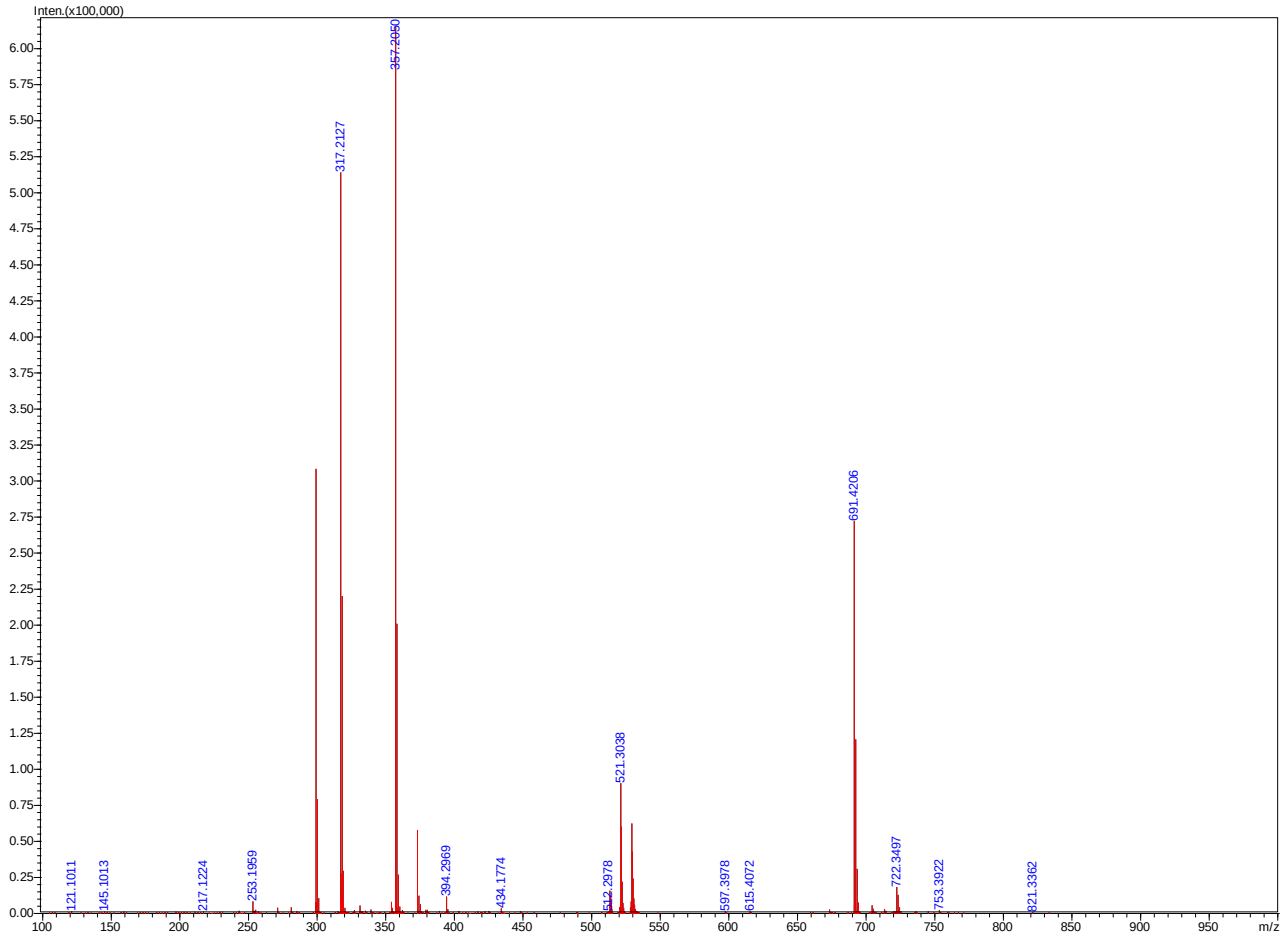

Figure 26. HRMS spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

2.4.3 Structure elucidation of compounds fraction F1 of *C. gratissimus*

Fraction **F1** of *C. gratissimus* was obtained as a yellowish oil with 16.3 mg of mass and MIC_{90} greater than 125 µg/mL. Although, TLC fingerprinting showed only one spot when fraction **F1** was isolated, NMR data showed that the fraction was not a pure compound but a mixture of inseparable clerodane diterpenoids isomers that had the same R_f value even when different mobile systems were used to separate them. The elucidated clerodane diterpenoids were named **Croton A** and **Croton B**.

The ^{13}C NMR chemical shifts of the minor compound which was named **Croton A** matched closely with the ^{13}C NMR chemical shifts of a clerodane type diterpenoid (Methyl (5S,8R,9S,10R)-2-oxo-ent-clerod-3,13-dien-15-oate) isolated from the leaves of *Aristolochia brasiliensis* by Lopes *et al* [9]. This led to the proposed structure with the proposed configuration at C-17 and C-18 and the *cis*-stereochemistry at the junction of the two rings. **Figure 27** shows the structure of the isolated clerodane diterpene (**Croton A**) while **Table 3** compares the ^{13}C NMR spectral data of both **Croton A** and the clerodane diterpene from literature. Even though the chemical shifts of the two structures matched, there were some resonances which differed by up to 4 ppm because of the different methods used in the elucidation of the two structures.

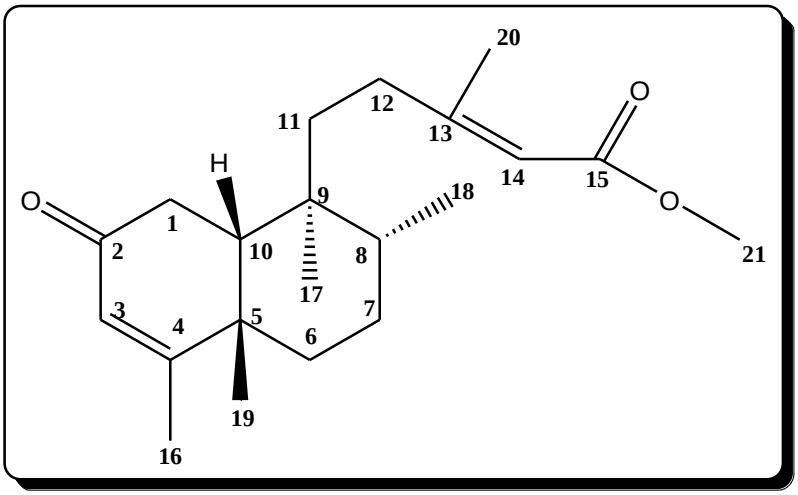

Figure 27. Structure of minor clerodane diterpenoid (Methyl (5S,8R,9S,10R)-2-oxo-ent-clerod-3,13-dien-15-oate) (**Croton A**) isolated from the leaves of *C. gratissimus*.

Croton A (**Figure 27**) consists of 21 carbons which were identified on the ^{13}C NMR spectrum (**Figure 30**). The carbons included a methoxy carbon resonating at δ_C 51.0 (C-21), five methyl carbons (δ_C 19.0, 14.1, 22.7, 31.9 and 17.0), six quaternary carbons (δ_C 199.0, 166.2, 42.7, 40.6, 164.3, and 167.2), two methine carbons (δ_C 128.1 and 114.1) and five methylene carbons(δ_C 31.0, 32.0, 29.0, 34.2 and 38.6). ^{1}H spectrum (**Figure 29**) confirmed the presence of five methyl protons (δ_H 1.81, 0.91, 1.73, 1.44 and 0.86), a methoxy at δ_H (3.22), methylene protons (δ_H 2.51,1.16, 1.23, 1.62 and 2.51) and methine protons at 5.79 (d J=1.2) and 4.96 (t).

Methine protons at δ_H 5.79 and 4.76 showed HSQC correlations with the methine carbons at δ_C 128.1 and 114.1 respectively, which were then assigned as H-3 (δ_H 5.79) and H-14 (δ_H 4.76). Methylene protons resonating at δ_H 1.62 showed HMBC correlations with a methylene carbon at δ_C 34.2 (C-11) and further showed HMBC relations with two quaternary carbons at δ_C 166.2 (C-4) and 167.2 (C-15). The methyl proton resonances at δ_H 0.86 (d J=7.6) and 1.44 (m) showed HSQC correlation with methyl carbons at δ_C 17.0 (C-20) and 31.9 (C-19) which were then assigned as 3H-20 and 3H-19 respectively. The methyl resonances at δ_C 19.0 (C-16), 14.1 (C-17) and 22.7 (C-18) are characteristics of clerodane type diterpenoid and they showed HSQC correlation with protons at δ_C 1.81, 1.73 and 0.91 respectively.

The elucidated structure (**Figure 27**) had a molecular formula of $C_{21}H_{32}O_3$ corresponding to molecular weight of 332.2351 g/mol. The HRMS spectra (**Figure 33**) indicated an M^+ molecular ion peak m/z 301.1413 corresponding to molecular formula of $C_{20}H_{29}O_2$ which resulted from the loss of CH_3O fragment of the ester group in the molecule. The m+1 molecular ion peak had relative abundance of 22% to the M^+ ion peak with 20 carbons. The FTIR spectrum indicated adsorption bands at 1659.64 and 2916.60 cm^{-1} that are consistent with the carbonyl and the alkane functional groups present in **Croton A**.

Table 3. Comparison of ^1H and ^{13}C NMR spectral data (δ) of (Methyl (5S,8R,9S,10R)-2-oxo-ent-clerod-3,13-dien-15-oate) isolated from *Aspergillus brasiliensis* and *C.grattisumus*.

Position	^{13}C (ppm) Isolated	$^{13}C^*$ (ppm) Literature [9]	^1H (ppm)	HMBC C-H
1	31.0 (CH_2)	34.34	1.62 (m)	
2	199.0 (C)	200.3		
3	128.1 (CH)	128.5	5.79 (d J=1.2)	
4	166.2 (C)	167.5		1.62
5	42.7 (C)	38.6		
6	32.0 (CH_2)	36.7	2.51 (m)	

7	29.0 (CH_2)	28.9	1.16 (s) 1.23 (br s)	
8	31.0 (CH)	36.6	1.51 (m)	
9	42.7 (C)	39.9		
10	40.9 (CH)	45.7	1.81 (d J=1.2)	
11	34.2 (CH_2)	34.0	1.62 (m)	1.62
12	38.6 (CH_2)	36.8	2.51 (m)	
13	164.3 (C)	160.3		
14	114.1 (CH)	115.2	4.96 (t)	
15	167.2 (C)	167.0		1.62
16	19.0 (CH_3)	19.1	1.81 (d J=1.2)	
17	14.1 (CH_3)	15.9	0.91 (d J=7.6)	
18	22.7 (CH_3)	20.5	1.73 (s)	
19	31.9 (CH_3)	32.1	1.44 (m)	
20	17.0 (CH_3)	17.8	0.86 (d J=7.6)	
OMe	51.0 (CH_3)	50.7	3.22 (m)	

The chemical structure of **Croton B** was elucidated in comparison with **Croton A**. The ^{13}C NMR chemical shifts of **Croton B** were similar with the ^{13}C NMR chemical shifts of **Croton A** except for the differences at carbons C-5, C-10, C-11 and C-12. The assignment at these carbons for **Croton B** was done in comparison with literature [10].

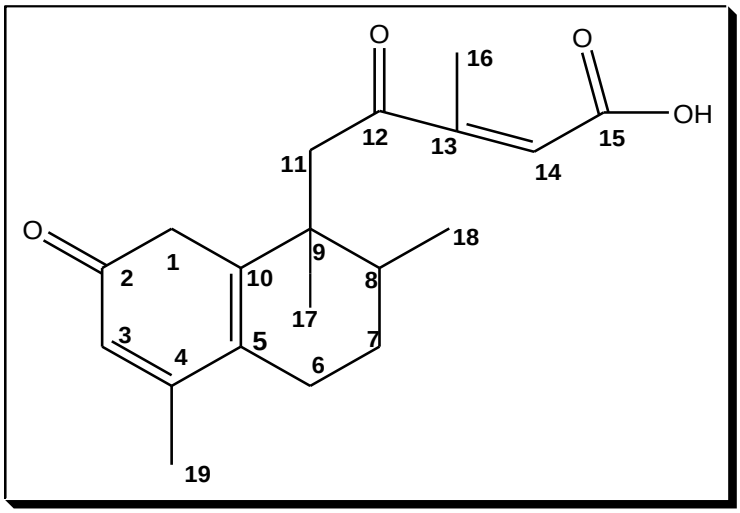

Figure 28. structure of major clerodane diterpenoid (**Croton B**) isolated from the leaves of *C. gratissimus*.

All 19 carbons of the compound (**Figure 28**) could be identified on the ^{13}C NMR (**Figure 30**) spectrum. The ^{13}C NMR spectrum confirmed the presence of 8 (δ_C 42.7, 143.7, 144.0, 166.2, 164.3, 167.2, 191.7, 207.1) quaternary carbons. The resonance at δ_C 167.2 is a characteristic of a carboxylic carbon and was assigned C-15 after comparison with available ^{13}C NMR data for neo-clerodane diterpenoid isolated from *Baccharie macraei* by Gambaro *et al* [10]. The resonances at δ_C 191.7 and 207.1 with less intense peaks are characteristics of carbonyl carbons for C-2 and C-15 respectively. The carbons resonating at δ_C 143.7 and 144.0 were assigned to C-5 and C-10 respectively, after comparison with literature.

DEPT NMR spectrum (**Figure 31**) confirmed the presence of four methyl carbons (δ_C 13.7, 16.3, 19.1, and 24.0), four methylene carbons (δ_C 29.7, 31.5, 34.2 and 38.5) and two methine carbons at δ_C 128.1 and 131.0.

^1H NMR (**Figure 29**) spectrum confirmed the presence of four methyl protons at δ_H 0.87 (d, J=7.6), 0.91 (d, J=7.6), 1.16 (s) and 1.23 (s). The methyl resonances at δ_H 1.16 and δ_H 0.91 are characteristics of clerodane type diterpenoid and were therefore assigned as 3H-17 (1.16) and 3H-18 (δ_H 0.91) upon comparison with literature values. The protons at δ_H 0.91(H-18) showed HMBC correlation with a quaternary carbon at δ_C 42.7 (C-9) and COSY coupling with a methine proton at δ_H 2.16 (H-8). Two other methine protons resonated at δ_H 5.89 (d J=1.2) and 5.79 (d J=2.0) showed HSQC correlations with the carbons at δ_C 131.0 (C-14) and 128.1 (C-3) and were then assigned H-14 (δ_H 5.89 J=1.2) and H-3 (δ_H 5.79 J= 2.0)

The presence of five methylene protons (δ_H 1.82 (m), 2.10 (d J=1.8), 2.60 (m), 2.36 (m) and 2.51 (br,s)) was also confirmed by ^1H NMR spectrum. Two methylene protons at δ_H 2.36 (H-1) showed HMBC correlation with quaternary carbons at δ_C 166.2 (C-4), 42.7 (C-9) and 191.7 (C-12). Methylene proton at δ_H 1.82 (H-7) showed COSY coupling with another methylene proton at δ_H 2.10 (H-6) and futher showed HMBC correlations with two quaternary carbons at δ_C 144.0 (C-5) and 143.7 (C-10) and a methine carbon at δ_C 128.1 (C-3).

The resonance at δ_H 2.60 (H-11) indicated HMBC correlations with a methine carbon at δ_C 131.0 (C-14) and a quaternary carbon at δ_C 143.7 (C-10) while the resonance at δ_H 2.51 (H-1) indicated HMBC correlation with a methylene carbon at δ_C 38.5 (C-11).

Carbons resonating at δ_C 166.2 (C-4) and 164.3 (C-13) respectively are examples of quaternary alkene carbons with methyl carbons attached to them. The resonance at δ_C 42.7 is a characteristic of an alkane for a quaternary carbon for C-9

The elucidated structure was a two membered ring diterpene with molecular formula $C_{19}H_{24}O_4$ corresponding to molecular weight of 315.38 g/mol. The molecular ion peaks of the elucidated structure could not be found on the HRMS or the LC-MS spectra. The FTIR spectrum indicated the adsorption bands at 1659.64 and 2916.60 cm^{-1} that are consistent with the carbonyl and the alkane functional groups present in **Croton B**.

Table 4. NMR spectral data (δ) of **Croton B** (400 MHz, CDCI3, room temperature)

Position	^{13}C (δ,ppm)	DEPT(δ,ppm)	^{1}H(mult)	COSY H-H	HMBC(δ,ppm) C-H
1	34.2	CH_2	2.51 (br s) 2.36 (m)		2.16
2	207.1	C			2.16
3	128.1	CH	5.79(d J=2.0)		1.82; 2.10; 2.36
4	166.2	C			1.82; 2.36
5	144.0	C			1.82; 2.10; 2.36
6	31.5	CH_2	2.10(d J=1.8)	1.82	1.82; 2.16
7	29.7	CH_2	1.82 (m)	2.10;2.16	5.89;2.16; 2.16
8	40.9	CH	2.16 (m)	0.91;1.82	
9	42.7	C			0.91; 2.36
10	143.7	C			1.82; 2.60
11	38.5	CH_2	2.60 (s) 2.36 (m)		2.51

12	191.7	C			2.36
13	164.3	C			
14	131.0	CH	5.89(d J=1.2)		1.82; 2.60
15	167.2	C			2.16;
16	13.7	CH_3	0.87(d J=7.6)		
17	19.1	CH_3	1.16 (s)		
18	16.3	CH_3	0.91(d J=7.6)	2.16	2.16
19	24.0	CH_3	1.23 (s)		

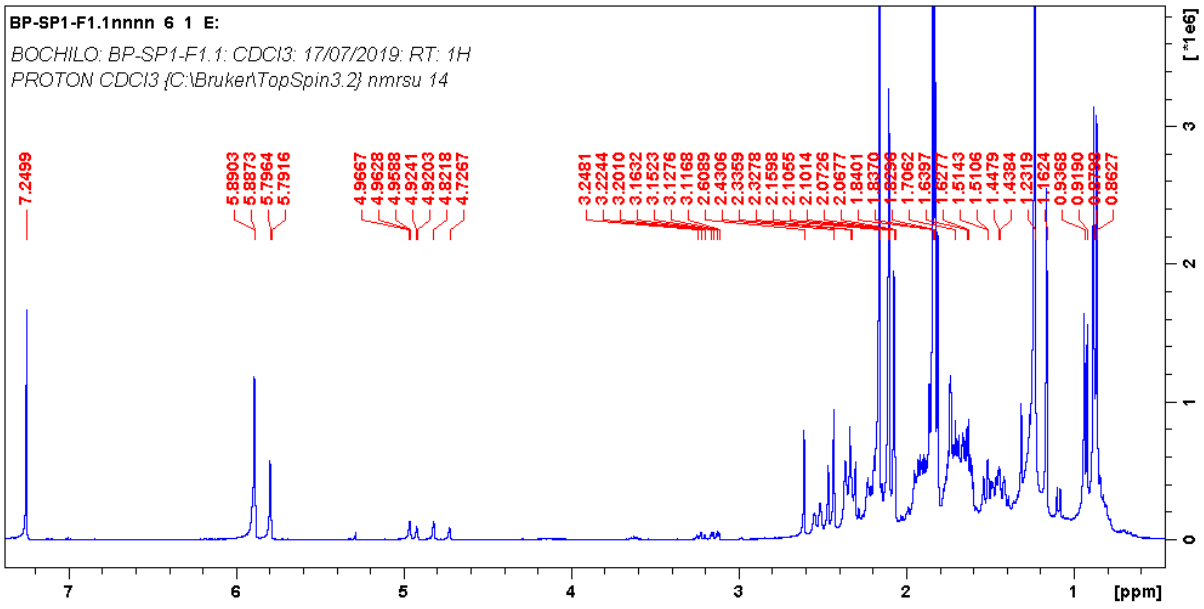

Figure 29. ^{1}H NMR spectrum of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus*.

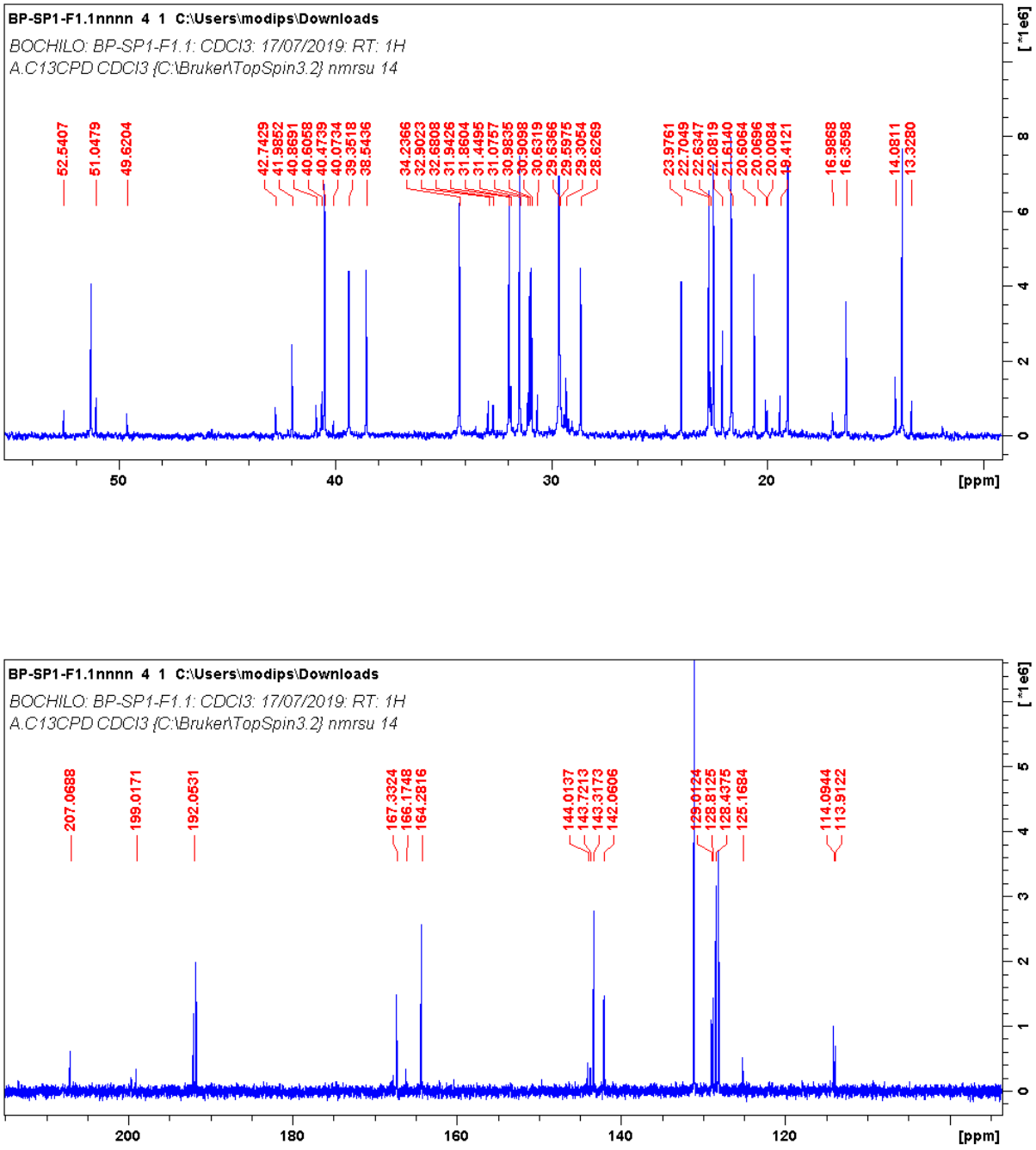

Figure 30. Expansions of ^{13}C NMR spectra of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus*.

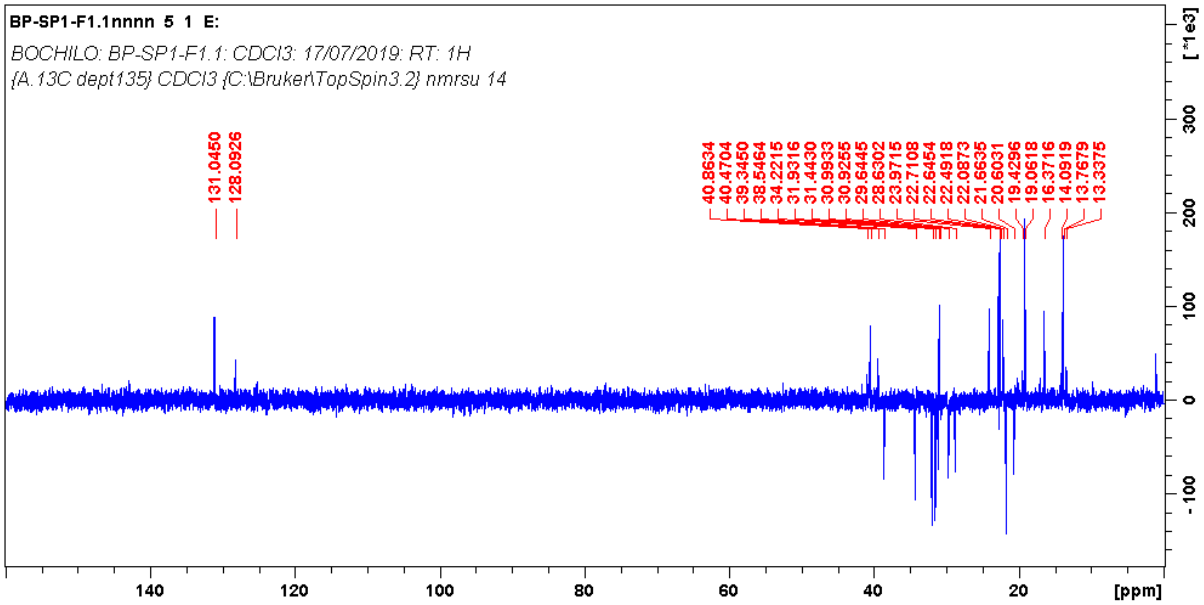

Figure 31. DEPT NMR spectrum of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus*.

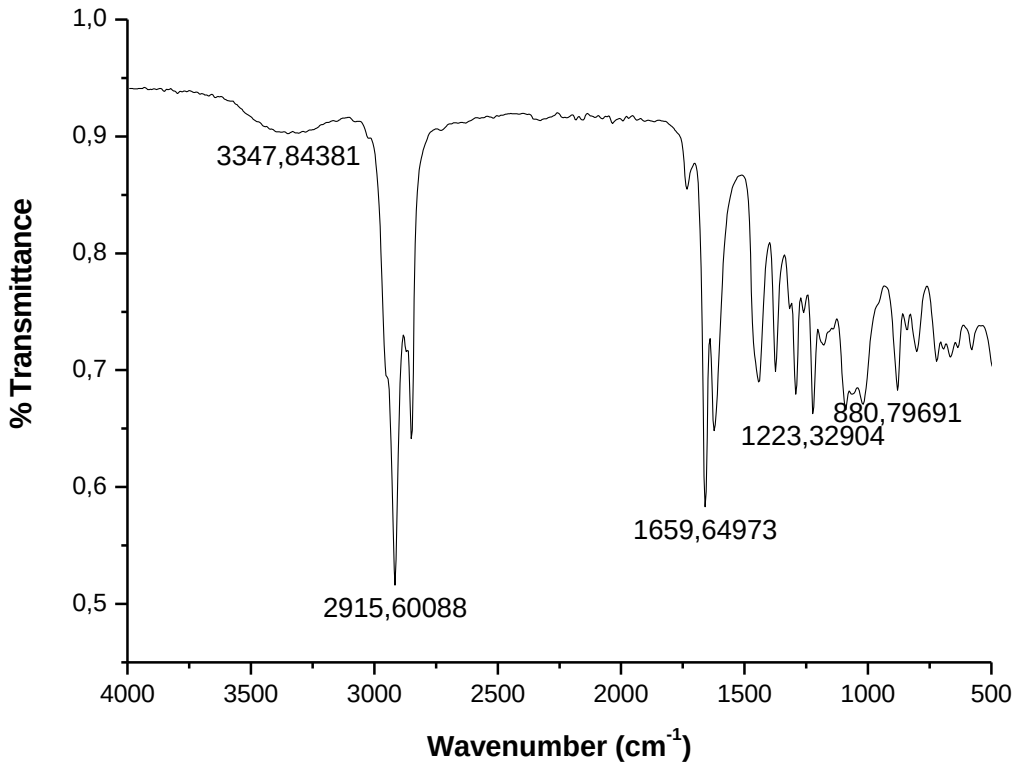

Figure 32. FTIR NMR spectrum of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus.*

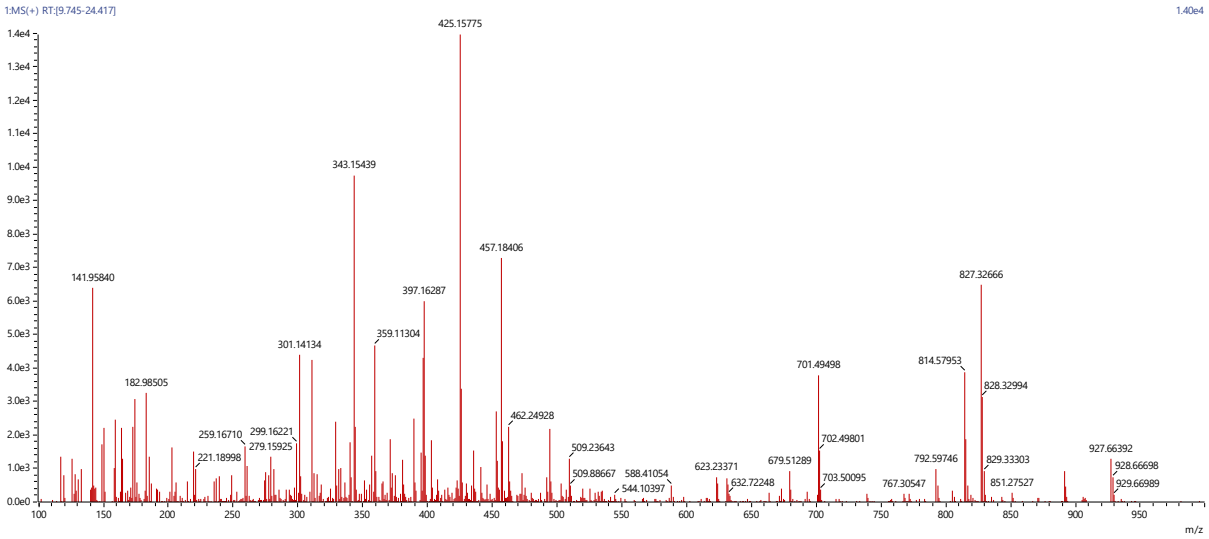

Figure 33. HRMS spectrum of clerodane diterpenoids (Croton A and Croton B) isolated from the leaves of *C. gratissimus*.

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Chapter 3

3.1 Conclusion

From the four different solvent systems used to extract the leaves of both C. gratissimus and *L. leonurus*, DCM was able to extract more material in terms of mass. This was confirmed by TLC fingerprinting where DCM extracts showed a greater number of spots than hexane, acetone and a mixture of ethanol/water. A number of fractions were successfully isolated from the DCM leave extracts of both *C. gratissimus* and *L. leonurus*. The successfully isolated fractions from both plant species together with the crude extracts were evaluated for *in-vitro* antimycobacterial activity against *Mycobacterium tuberculosis* $H_{37}R_v$ strain.

Five fractions were obtained after using column chromatography to purify the DCM crude extract of *C. gratissimus*. The five fractions were further purified using preparative thin layer chromatography to give 23 more fractions. A total of 28 fraction were successfully isolated from the DCM extract of *C. gratissimus*. Seven fractions showed potential activity against *Mycobacterium tuberculosis* with MIC_{90} values ranging from 30.61 µg/mL to 64.88 µg/mL. The crude extract together with the other 21 fractions gave MIC values that were greater than 125 µg/mL.

DCM crude extract of *L. leonurus* gave seven fractions after purification with column chromatography. All seven fractions together with the crude extract had MIC_{90} greater than 125 µg/mL. Further purifications of these fractions using preparative thin layer chromatography led to isolation of 27 more fractions. only three fractions from the 27 fractions indicated potential activity against the *Mycobacterium tuberculosis* with MIC_{90} values ranging from 1.963 to 62.51 µg/mL. A total of 34 fractions were successfully isolated from DCM crude extract of *L. leonurus*.

Antioxidant analysis of the two plant extracts indicated that the two plant species have antioxidants properties. In the qualitative antioxidant analysis, clear zones against the purple background colour indicated the presence of antioxidants. DCM crude extracts from both plant species had a greater number of clear spots than all other solvent extracts suggesting more antioxidants within the extracts. BHT which was used as a

standard antioxidant in the quantitative antioxidant analysis. DCM extracts inhibited the highest percentage of DPPH at different concentrations following BHT. DCM crude extract of *C. gratissimus* was able to inhibit 81.9 and 93.3 % of DPPH at 50 and 250 µg/mL respectively, while *L. leonurus* DCM extract inhibited 69 and 87 % of DPPH at the same concentrations respectively.

The chemical structures of four compounds were elucidated using NMR technique and confirmed by MS and FTIR spectroscopies. NMR data showed that the isolated fractions were not pure compounds but a mixture of closely related compounds that had the same R_f values even when different mobile systems were used to separate them. The chemical shifts were assigned in comparison with the chemical shifts of similar structures which were isolated before.

The elucidated structures were two labdane terpenoids (**Croton A** and **Croton B**) and a cembranolide ((5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate) from *C. gratissimus* and a phenol (4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol)) from *L. leonurus*. **Croton A** and **Croton B** were elucidated from fraction F1 (16.3 mg) of *C. gratissimus* which was isolated as a yellowish oil. The fraction had an MIC_{90} greater than 125 µg/mL. Cembranolide ((5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate) was elucidated from fraction F5 (20 mg) of *C.gatissimus* with MIC_{90} greater than 125 µg/mL. Fraction F4.1 (25 mg) was isolated as a dark coloured paste and gave a phenolic 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol)) compound.

Elucidation of the structures was based on the purity of the isolated fractions. Even though some of the fractions showed potent activity than others, their chemical structures could not be elucidated because they were not clean enough to make any elucidations.

Based on the good antioxidant activity of the two plant extracts and the good activity of some of the isolated fractions against the *Mycobacterium*, the claims made by the traditional healers that the plant extracts can treat TB may be true since they use the extracts in large amounts. The antioxidants will boast the immune system while other compounds fight the bacterium directly.

3. 2 Future work

Future work will involve further purification of the fractions showing promising activity against the *Mycobacterium tuberculosis* using advanced techniques to obtain pure compounds. The pure compounds obtained will be screened for activity against the *Mycobacterium* to check if the activity increases or decreases. Furthermore, the fractions that will show potent activity will be combined to check if the compounds work better in a group (synergistic effect) or they work better as individuals (antagonistic effect).

Cytotoxicity studies will be done on the compounds that will show potential activity against the bacterium to check if the compounds are not toxic to human cells. Synthesis of the compounds that will show potent activity and not toxic to the human cells will be attempted. Total antioxidant determination methods will be used to determine the type of antioxidants within the plant extracts.

Chapter 4

4 Experimental Procedure

4.1 General information

Commercially available reagents and solvents were purchased from Sigma Aldrich, Merck and Rochelle chemicals (South Africa). All chemicals were used directly as received unless stated otherwise. All measurements were done at room temperature unless otherwise specified. Plant material was ground using Sunbeam coffee bean grinder. Column chromatography followed by preparative Thin Layer chromatography (Prep TLC) were used as purification techniques to isolate individual fractions. Column chromatography was performed using Merck silica gel 60 on a particle size of 0.04-0.063 mm (230- 400 mesh ASTM). Preparative Thin Layer chromatography (Prep TLC) plates were prepared using Prep silica gel (200 g) and water (500 mL), the mixture was poured on the glass plates and were dried at room temperature overnight and were activated in the oven for 6 hours.

4.1.1 Physical and spectroscopic properties of compounds

The structures of the isolated compounds were elucidated and confirmed using nuclear magnetic resonance (NMR) (Bruker Ascend 400 MHz Topspin 3.2), IR (Agilent technologies cary 600 series FTIR spectroscopy) and HPLC-MS (Shumadzu). ^{1}H NMR and ^{13}C spectra were referenced internally using solvents signals. The signals were 7.25 and 77.00 ppm $CDCl_3$ for ^{1}H NMR and ^{13}C NMR spectra respectively. Chemical shifts were expressed in δ-values parts per million (ppm) and the coupling constants (J) in Hertz (Hz). Multiplicity of the signals is given as follows: s = singlet, d = doublet, t = triplet, and m = multiplet.

4.2 Plant collection

The leaves of *C. gratissimus* and *L.leonorus* were collected at university of Limpopo (Sovenga hill). The old yellow/brown leaves were separated from the fresh green leaves and were discarded. Fresh green leaves were left to dry at room temperature for a period of two weeks. A commercial coffee grinder was used to grind the leaves

into fine powder. The powdered material was stored in sealed jars at room temperature until needed.

4.3 Extraction

4.3.1 Maceration extraction

Maceration method of extraction was used to extract plant materials for both plants. The plant samples were extracted by weighing 100 g of fine powder followed by addition of 400 mL of the appropriate solvent (dichloromethane (DCM), hexane, acetone) into a 500 mL Erlenmeyer flask. The mixture was left on a magnetic stirrer for 24 hours. The undissolved material was then filtered, and the supernatant was transferred into a pre weighed one neck round bottom flask. The process was repeated three times and the resultant supernatants were then combined. A rotary evaporator was used to remove the solvent and the crude extracts were weighed and the mass of each crude was recorded. An amount of 1 mg was taken from each crude extract for TB screening. The crude extracts were stored in a refrigerator at -5 ^{0}C until needed. The solvents used for extraction were n-hexane, acetone and dichloromethane.

4.3.2 Espresso machine extraction

Fine powder of the plant material (10 g) was mixed with 2 g of sand and placed into the portafilter (sample compartment) of an espresso machine and then extracted with 35% EtOH/H_2O (300 mL of a hot solution). Extraction was done three times and the extracts were combined and concentrated to a volume of about 100 mL on a rotary evaporator in order to remove EtOH (40^{0}C water bath temperature). The ensuing mixture was transferred into a separating funnel and extracted with DCM (3× 50 mL). followed by drying with $MgSO_4$. The undissolved material was filtered, and the extracts were transferred into a pre weighed round bottom flask. The process was repeated until 100 g of the plant material was extracted. The mass of the crude extracts was then recorded, and the extracts were placed in a refrigerator at -5^{0}C until needed.

4.4 Isolation/purification

Small TLC plates were used to check the chemical composition of the crude extracts. The TLC plates were loaded with crude extract (10 µg) of different solvents and developed into different percentages (10, 20, 30, 40, 50, and 60 %) of ethyl acetate/ hexane to determine which percentage best separate the compounds within the extracts. Chromatograms were visualised under ultraviolet light at 254 nm. After determining which solvent extracted more compounds and the best mobile system to separate individual fractions, column chromatography was used to isolate different fractions. n-hexane was used to pack the column with silica gel.

An amount of 7 g of dissolved DCM crude extract was loaded on a packed column and 30% ethyl acetate was used as the best mobile system to separate compounds within the extracts. Individual fractions were collected in separate test tubes. Small TLC plates were again used to check the best mobile system in order to purify the individual fractions further. The collected fractions were then transferred into pre-weighed round bottom flasks and a rotary evaporator (at 50 ^{0}C) was used to remove the solvent. The mass of each fraction was recorded. Further purification of the fractions was performed using Prep TLC plates. Dissolved extract was spread thinly on Prep TLC plates and developed into the best mobile system. The plates were dried at room temperature and the fractions were collected and the yields were recorded. The process was repeated until the TLC plates showed only one fraction/compound. Each fraction was sent for TB screening before further purification. The structures of some of the fractions showing potential activity against the Mycobacterium were elucidated.

The DCM leave extracts of *C. gratissimus* led to the isolation of the following compounds: Fraction F5 (20 mg) isolated as a colourless oil gave a cembranolide: (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide), ^{1}H NMR (CDCl3, 400 MHz): δ_H 0.87(s), 0.91 (s), 1.25 (s), 1.50 (m), 1.53 (m), 1.60 (m), 1.61 (m), 1.68 (m), 1.95 (m), 2.15 (m), 2.19 (m), 2.21 (s), 2.23 (m), 2.46 (s), 2.48 (m), 3.64 (m), 4.68 (m), 5.20 (s), 5.79 (s) and 6.34 (s). ^{13}C NMR (CDCl3, 400 MHz): δ_C 15.7, 18.5, 20.0, 20.0, 23.0, 23.4, 29.0, 30.4, 32.2, 41.8, 42.0, 51.5, 77.5, 76.8, 127.7, 130.4, 131.7, 133.7, 135.5, 148.4, 174.1, and

180.6. FTIR: v_{max} 1737.29 and 2936.26 cm^{-1}. HRMS $[M]^+$ m/z 317.21 calculated from $C_{20}H_{29}O_2$.

Fraction F1 (16.3 mg) isolated as yellowish oil gave two labdane diterpenoids named **Croton A,** ^1H NMR (CDCl3, 400 MHz): δ_H 0.87 (d J=7.6), 0.91 (d J=7.6), 1.16 (s), 1.23 (br s), 1.62 (m), 1.44(m), 1.51(m), 1.73(s), 1.81(d J=1.2), 2.51 (m), 3.22 (m), 4.96 (t), 5.79 (d J=1.2). ^{13}C NMR (CDCl3, 400 MHz): δ_C 14.1, 17.0, 19.0, 22.7, 29.0, 31.0, 34.2, 32.0, 36.8, 38.6, 40.9, 42.7, 50.1, 114.1, 128.1, 164.3, 166.2, 167.2, 199.0. FTIR: v_{max} 1659.64 2916.60 cm^{-1}. HRMS $[M]^+$ m/z 301.1413 Calculated from $C_{20}H_{29}O_2$.

Croton B, ^1H NMR (CDCl3, 400 MHz): δ_H 0.87 (d J=7.6), 0.91 (d J=7.6), 1.16 (s), 1.23 (s), 1.82 (m), 2.16 (s), 2.36 (m), 2.51 (br s), 2.60 (s), 5.79 (d J=2.0) 5.89 (d J=1.2). ^{13}C NMR (CDCl3, 400 MHz): δ_C 13.1, 16.3, 19.1, 24.0, 29.7, 31.5, 34.2, 40.9, 42.7, 128.1, 131.0, 143.7, 144.0, 164.3, 166.2, 167.2, 191.7, 207.1. FTIR: v_{max} 1659.64, 2916.60 3347.84 cm^{-1}.

DCM leave extracts of *L. leonurus.* gave fraction F4.1 (25 mg) which was isolated as dark coloured paste. This fraction led to the isolation of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol). ^1H NMR (CDCl3, 400 MHz): δ_H 0.86 (m),1.24 (s), 1.27 (s), 1.32 (s), 7.13 (dd J=2.6, 8.7), 7.34 (d J=2.6), 7.53 (d J=8.7). NMR (CDCl3, 400 MHz): δ_C 14.1, 22.6, 29.3, 29.6, 29.7, 30.1, 30.2, 31.4, 31.9, 34.5, 34.8, 119.0, 123.9, 124.4, 138.3, 147.5, 147.6.

4.5 Antioxidant activity

Qualitative and quantitative analysis of the antioxidants within the extracts were done using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as free radical and butylated hydroxytoluene (BHT) as a standard antioxidant. Antioxidants are compounds that inhibit oxidation.

4.5.1 Qualitative antioxidant analysis

In the qualitative assay, a volume of the crude extract was loaded on the TLC plates. using Merck silica gel 60 on a particle size of 0.04-0.063 mm (230- 400 mesh ASTM) The plates were then developed into mobile phases of Ethyl acetate/methanol/water

40:5.4:4 (EMW) (polar /neutral);(v/v/v), Chloroform/ethyl acetate/formic acid 5:4:1 (CEF); (v/v/v); (intermediate polarity/acidic) and Benzene/ethanol/ammonia hydroxide 90:10:1 (BEA); (v/v/v); (non-polar/basic). After air drying the plates at room temperature and visualising them under UV light (254 nm), the plates were sprayed with 0.2% DPPH in methanol and dried in a fumehood carboard. The presence of antioxidants compounds was detected as clear/yellow spots against the purple background colour of DPPH on the TLC plates.

4.5.2 Quantitative antioxidant analysis

Methods that are used for measuring antioxidative activity involve the generation of free radicals. Such free radicals are neutralised by antioxidant compounds [6]. The ability of *C. gratissimus* and *L. leonurus* to scavenge the DPPH radicals was assessed

by using the Blois method. About 0.2 mmol/L solution of DPPH in methanol was prepared, amount of 3mL of this solution was added to extracts of different concentrations (50-250 µg/mL). The mixture was then shaken vigorously and allowed to stand at room temperature for 30 minutes. A control was prepared as above but without the sample extracts. Methanol was used for baseline correction and the changes in the absorbance of the plant samples were measured using a spectrophotometer at 517 nm. Results were compared with the standard antioxidant (BHT). The ability of DPPH radical scavenging activity was calculated using the following formula:

DPPH scavenging effect (% inhibition) = $(A_0 - A_1) \times 100 / A_0$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample extracts.

4.6 Minimum inhibitory concentration

The crude extracts and the isolated fractions were evaluated for *invitro* antimycobacterial activity against *Mycobacterium tuberculosis* $H_{37}R_V$ strain at the university of Cape Town, drug discovery and development centre (H3D). The minimum inhibitory concentrations (MICs) values were obtained following the broth dilution method [7]. The method allows a range of antibiotic concentrations to be tested on a single 96-well microtitre plate in order to determine the minimum inhibitory

concentration (MIC). Briefly, a 10 mL culture of a mutant *Mycobacterium tuberculosis* ($H_{37}R_V$) strain constitutively expressing recombinant alamar blue assay of a plasmid integrated at the *attB* locus is grown to an OD600 of 0.6–0.7. The *Mycobacterium tuberculosis* $H_{37}R_V$ strain culture is then diluted 1:100 in 7H9 GLU CAS TX. In a 96-well microtitre plate, 50 µL of 7H9 GLU CAS TX medium is added to all wells from Rows 2-12. The compounds to be tested are added to Row 2-12 in duplicate, at a final concentration of 640 µM (stocks are made up to a concentration of 12.8 mM in DMSO and diluted to 640 µM in 7H9 GLU CAS TX medium). A two-fold serial dilution is prepared, by transferring 50 µL of the liquid in Row 1 and 2 to mix. 50 µL of the liquid in Row 2 is then transferred to Row 3 and aspirated. The procedure is repeated until Row 12 is reached, from which 50 µL of the liquid is discarded to bring the final volume in all wells to 50 µL. Finally, 50 µL of the 1:100 diluted *Mycobacterium tuberculosis* cultures are added to all wells in Rows 2-12. Row 1 serves as a contamination control which includes media, 5% DMSO and rifampicin. The microtitre plate is stored in a secondary container and incubated at 37 °C with humidifier to prevent evaporation of the liquid. The lowest concentration of compounds which inhibit growth of more than 90% of the bacterial population is considered to be the MIC_{90}. The pellet data is reported as visual score and calculated MIC during day 14 post inoculation.

APPENDIX

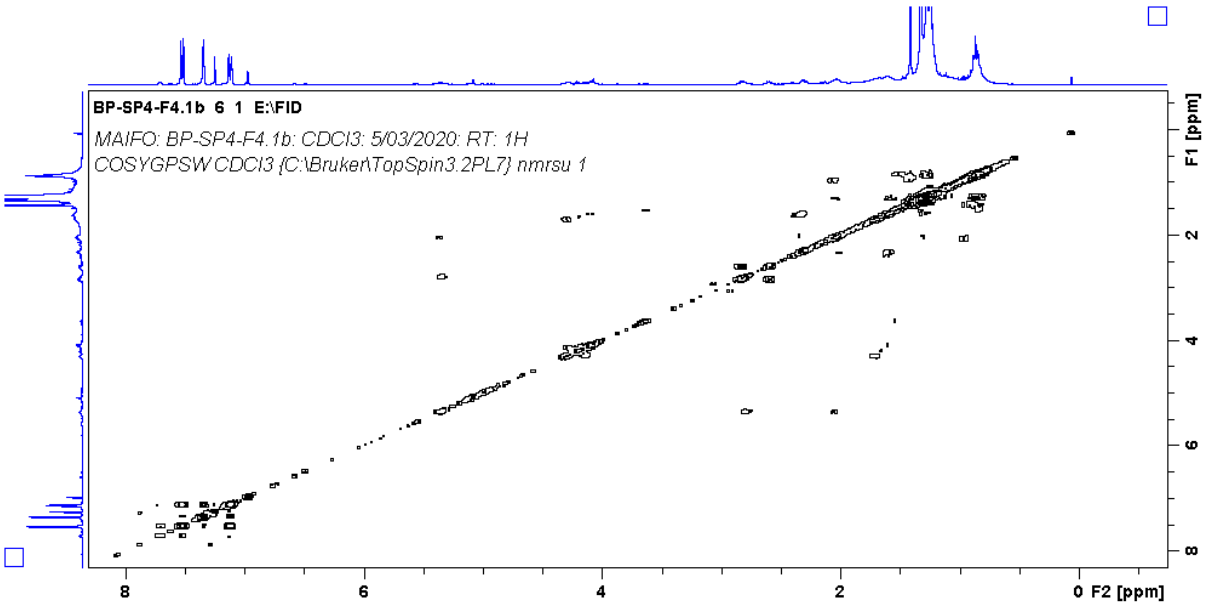

Figure 34. COSY NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

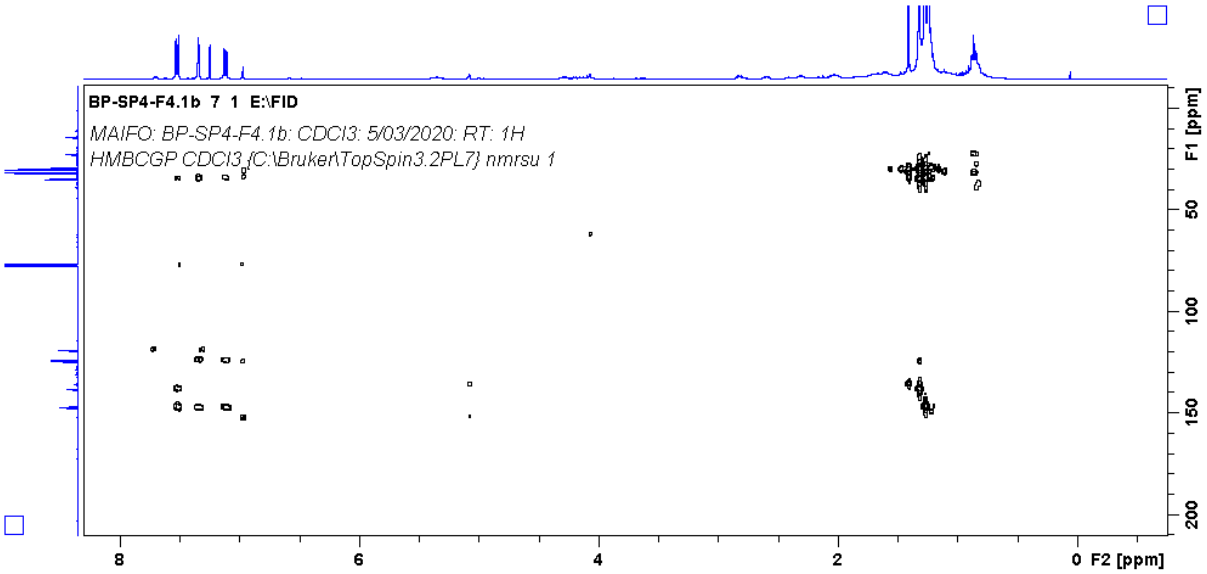

Figure 35. HMBC NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

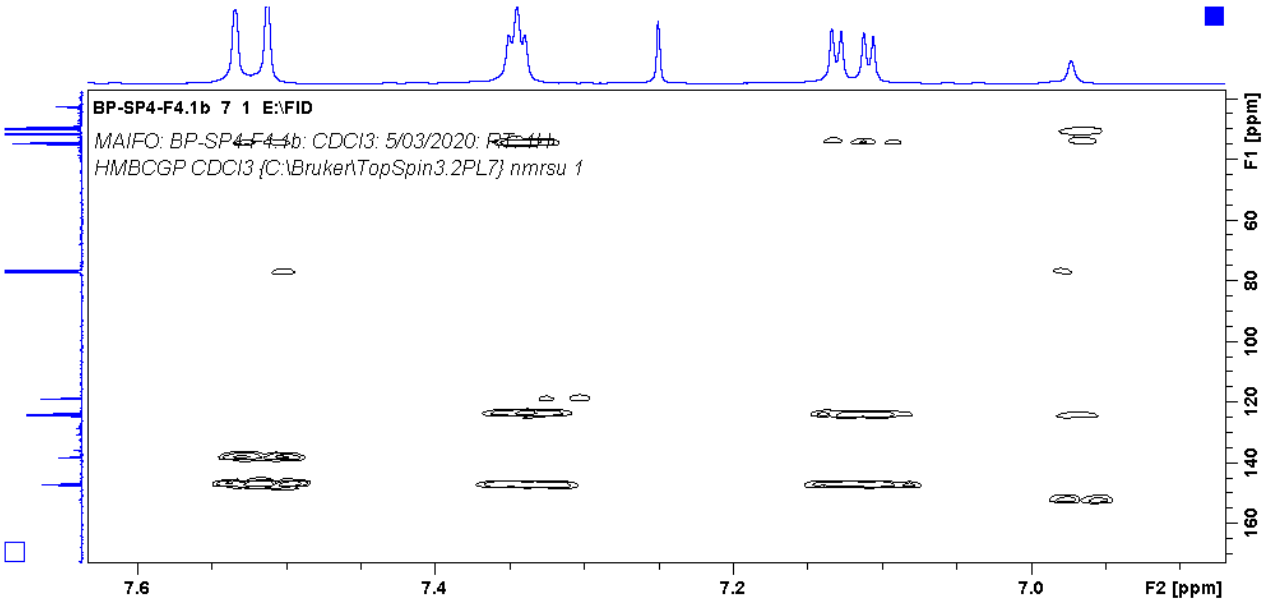

Figure 36. Expanded region of HMBC NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

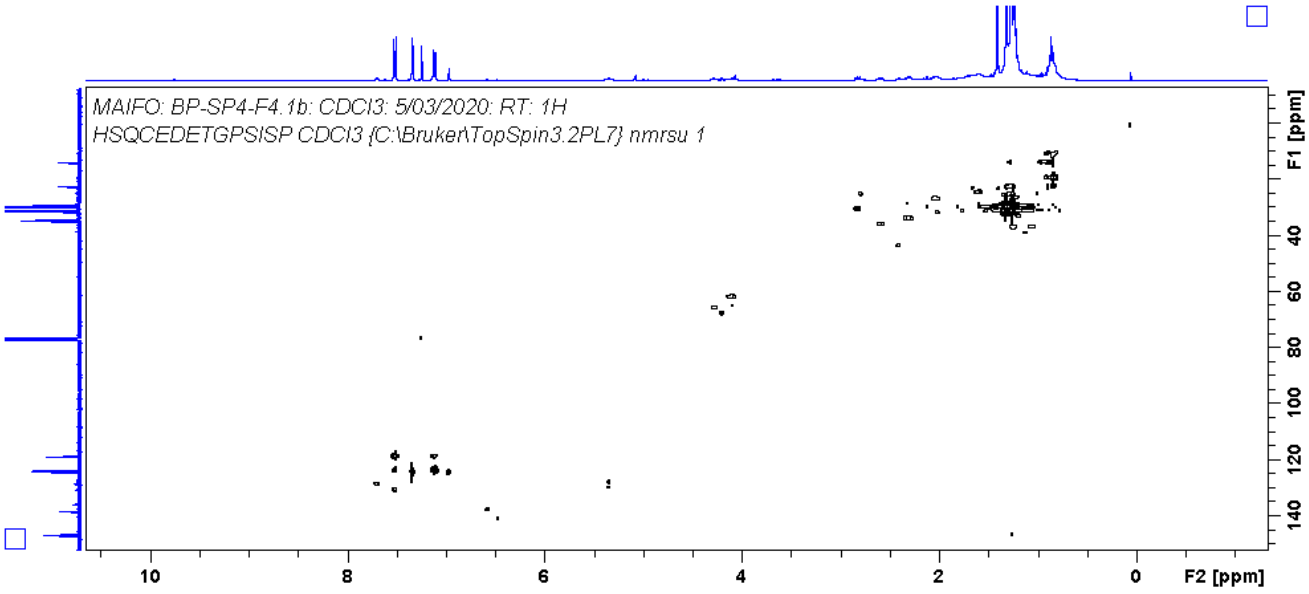

Figure 37. HSQC NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

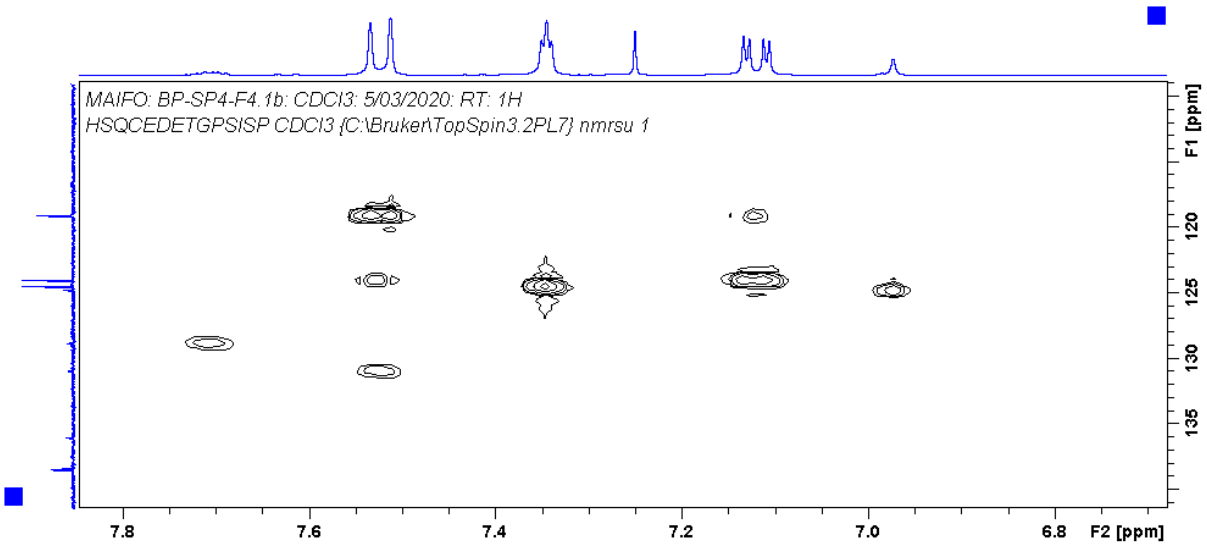

Figure 38. Expanded region of HSQC NMR spectrum of 4-(3,3,4,4-tetramethylheptyl) benzene-1,2-diol) isolated from the leaves of *L. leonurus.*

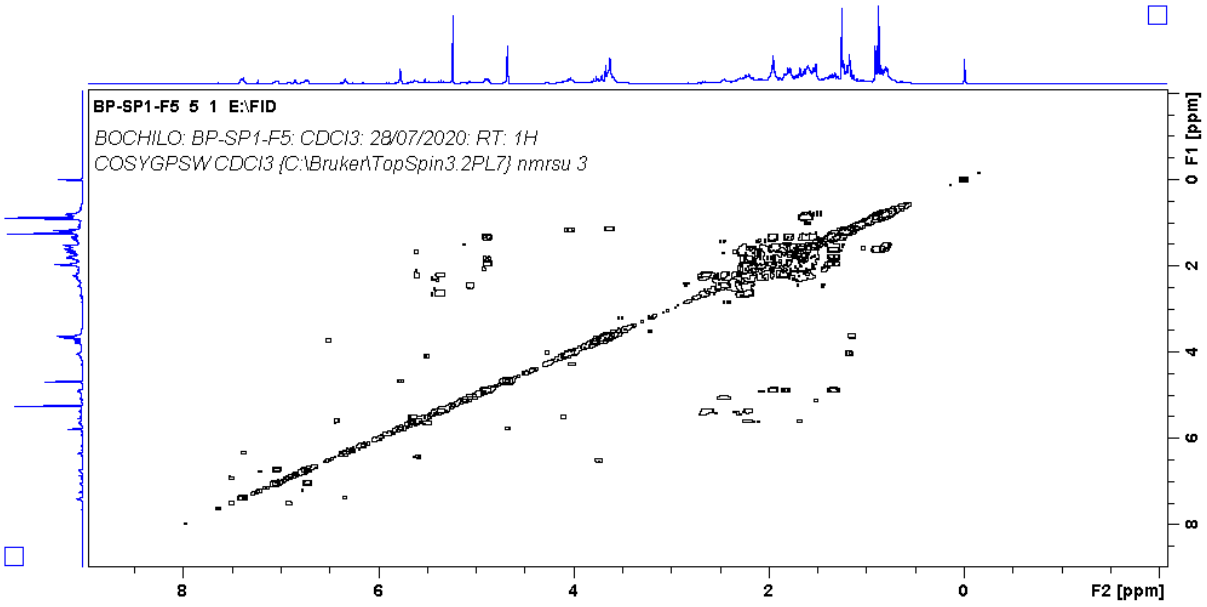

Figure 39. COSY NMR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

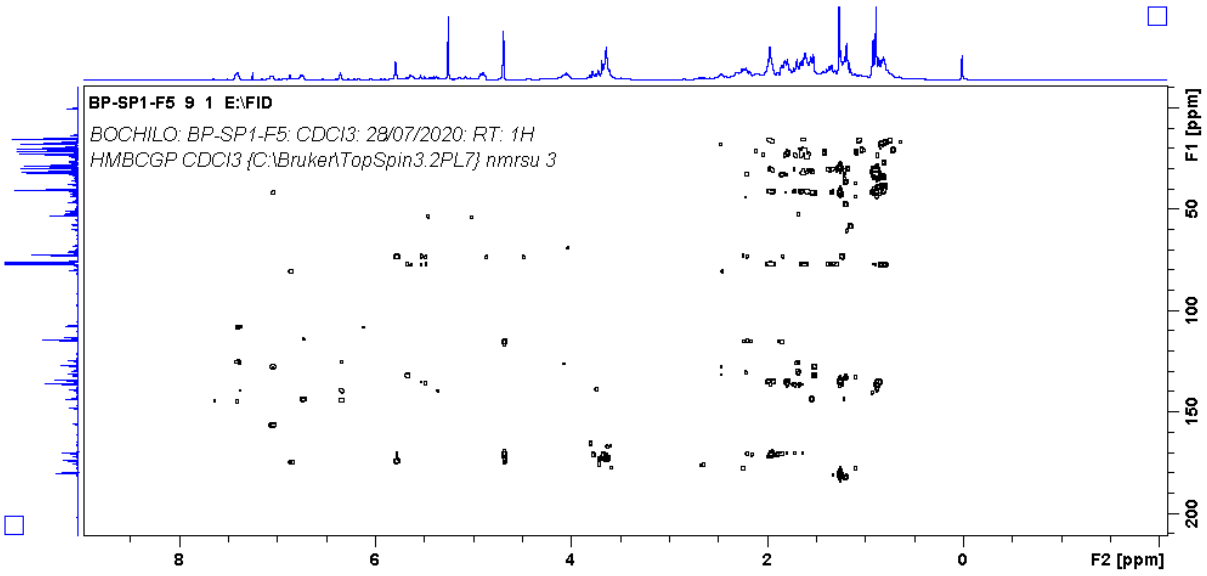

Figure 40. HMBC NMR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

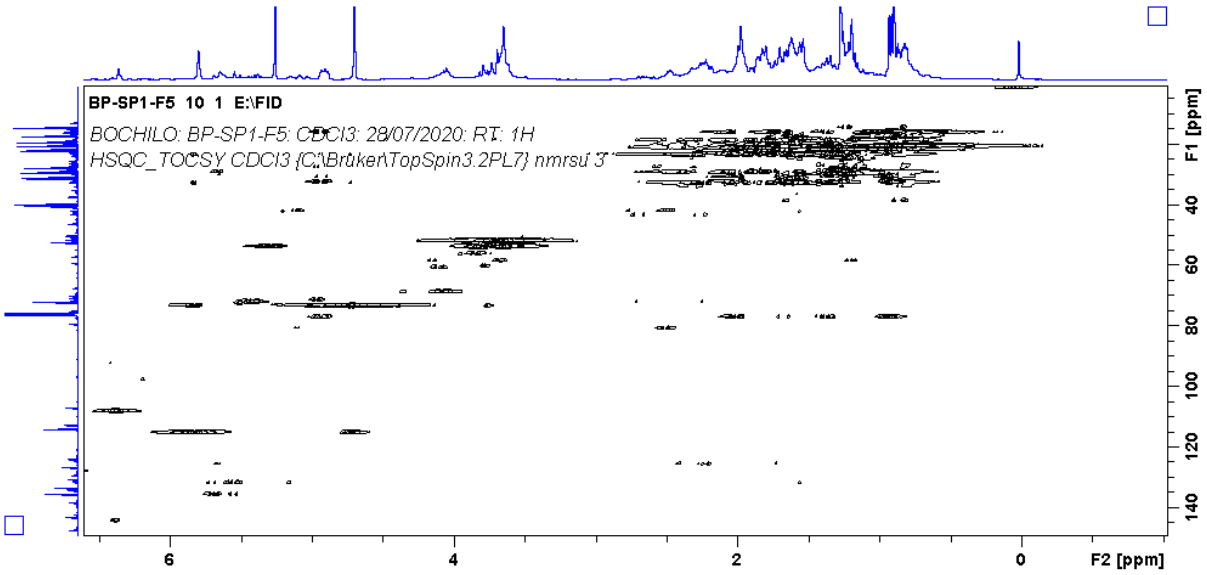

Figure 41. HSQC NMR spectrum of (5E,10E,13R)-4-isopropyl-7,11-dimethyl-15-oxo-14-oxa-bicyclo [11.2.1] hexadeca-5,10-dien-7-yl acetate (cembranolide) isolated from the leaves of *C. gratissimus.*

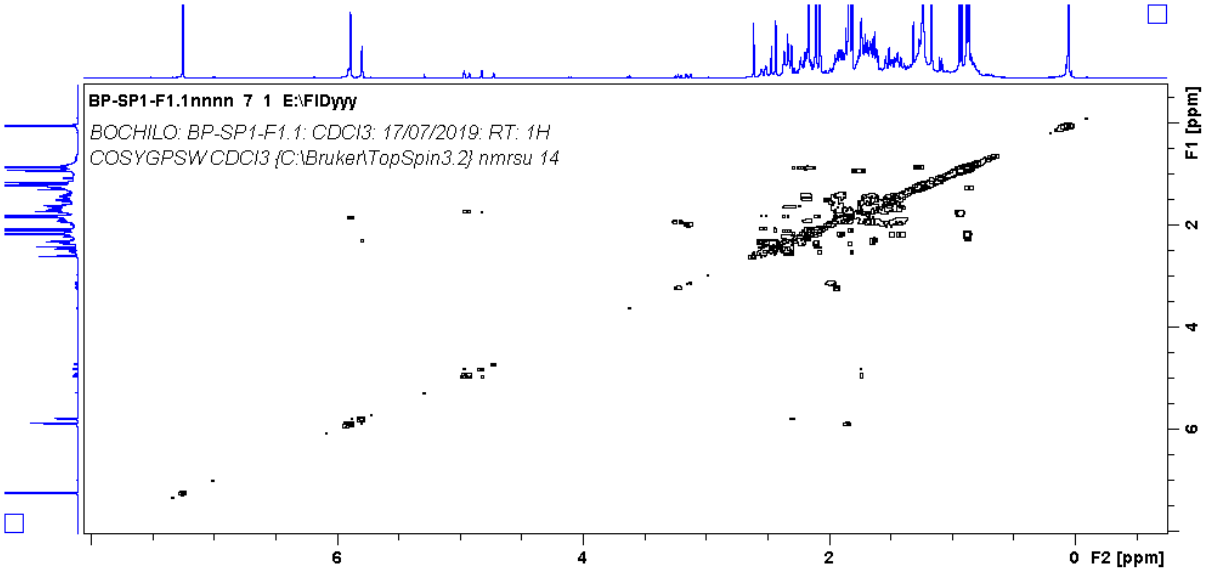

Figure 42. COSY NMR spectrum of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus*.

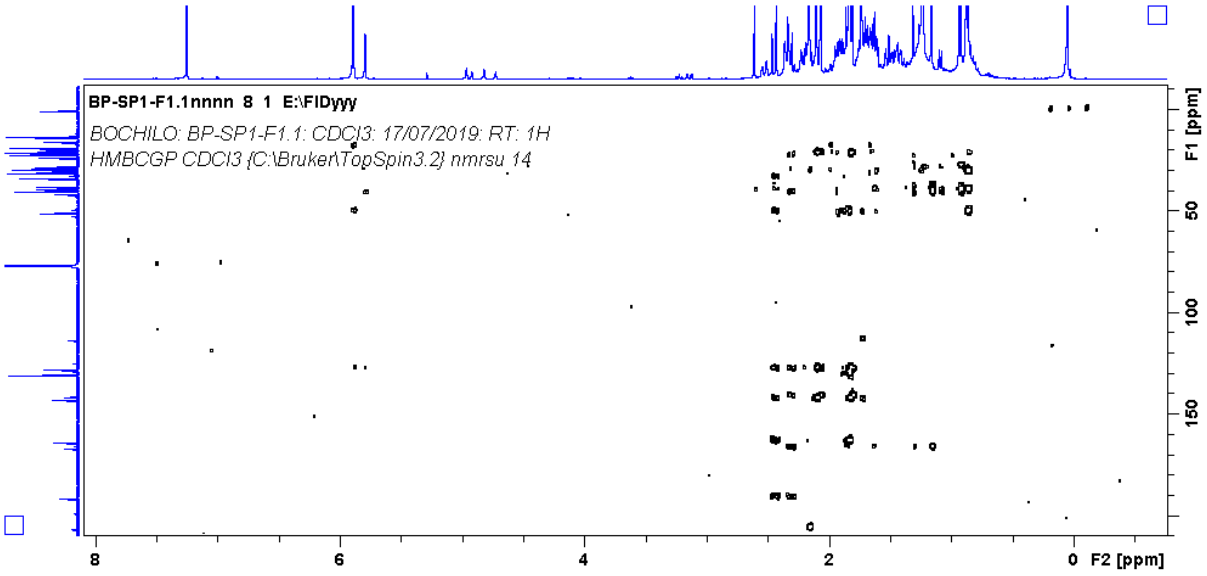

Figure 43. HMBC NMR spectrum of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus*.

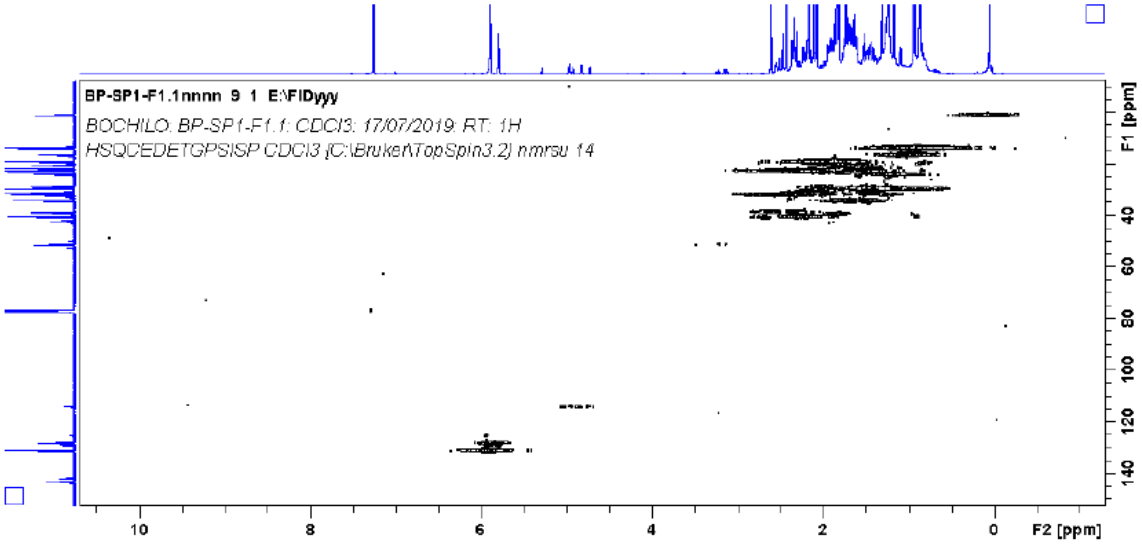

Figure 44. HSQC NMR spectrum of clerodane diterpenoids (**Croton A** and **Croton B**) isolated from the leaves of *C. gratissimus*.