ISOLATION AND STRUCTURE ELUCIDATION OF BIOACTIVE COMPOUNDS FROM

RAUVOLFIA CAFFRA SOND

BY

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DECLARATION

I, Bafedile Dorcas Tlhapi, hereby declare that the dissertation for the Master of Science (MSc) Degree in Chemistry, submitted at the University of Venda, is an original research document and has not been submitted previously for a degree at this or any other university, that it is my own work in design and in execution, and that all reference material contained therein has been duly acknowledged.

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(Signature of Candidate) Date

DEDICATIONS

I would like to dedicate this dissertation to my mom (Maria Tlhapi), my dad (Molefe Moses Tlhapi) and my younger sister (Moleboheng Thabia Tlhapi), who have always been my source of inspiration through difficult times. I would also like to express my genuine gratitude to everyone who played a role in my upbringing, my undergraduate and postgraduate studies. Thank you all for your encouragement, patience and support. I hope that this research will justify that hard work, discipline, and perseverance yield good results.

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Abstract

Rauvolfia caffra Sond, a species of evergreen trees and shrubs in the dogbane family, (Apocynaceae), is used as a medicinal plant among traditional communities in many countries for the treatment of malaria, diabetes, coughs, gastrointestinal disturbances, skin infections, impotence, insomnia, diarrhoea, dysentery, scabies, worm infections, and both parasitic and microbial infections. Phytochemical studies have revealed that indole alkaloids are the major constituents of the stem bark. However, there are limited studies linking the compounds with the ethnomedicinal uses. The aim of this study is to isolate and characterize bioactive compounds from *Rauvolfia caffra* Sond.

The highest phenolic content found in a fraction was 16.06±0.125 mg GAE/g, while the highest flavonoid content measured was 9.453±0.081 mg QE/g. In the DPPH free radical scavenging activity and reducing power tests, a lowest IC_{50} value of 0.022±0.003 µg/mL and $IC_{0.5}$ value 0.518±0.044 µg/mL, respectively, was found. Six compounds were isolated from the stem bark, including lupeol, a pentacyclic tri-terpenoid isolated for the first time from the genus *Rauvolfia*; raucaffricine, a rare glycoalkaloid of the monoterpenoid indole class; *N*-methylsarpagine, an indole alkaloid isolated for the time from *R. caffra* and spegatrine, an indole alkaloid isolated for the first time from *R. caffra*, respectively.

Concerning antimicrobial activity, the highest activity of a fraction was against *B. cereus* with MIC values as low as 12.5 mg/mL. One fraction at the tested concentration (250 µg/mL) decreased the viability of *Plasmodium falciparum* (4.149±6.979 %) with an IC_{50} value of 6.533 µg/mL. The crude extract and some fractions affected the viability of the *Trypanosomes* at the tested concentration (250 µg/mL), giving -0.133 ± 0.206 %, 11.334 ± 2.692 %, 1.026 ± 0.143 % and 20.769 ± 9.054 % with IC_{50} values of 18.50 µg/mL, 14.15 µg/mL, 15.58 µg/mL and 34.71 µg/mL, respectively. Furthermore, the fractions did not show significant cytotoxic effects at a concentration of 50 µg/mL.

Key words: bioactive compounds, polyphenolic content, *Rauvolfia caffra* Sond, antioxidant, antimicrobial, antiplasmodial, antitrypanosomal and cytotoxic activities.

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List of Symbols and Abbreviations

% w/v	Percent of weight of solution in the total volume of solution
AAT	African animal trypanosomiasis
Abs	Absorbance
ABTS	(2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid)
AIDS	Acquired immune deficiency syndrome
ANOVA	One-way analysis of variance
ATP	Adenosine Triphosphate
BHI	Brain-heart infusion
n-C_6H_{12}	*n*-Hexane
CH_2Cl_2	Dichloromethane
CTA	The cell toxicity assay
δ	Chemical shift
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-1-picrylhydrazyl radical
E.M.W.	Ethyl acetate/Methanol/Water (81:11:8)
EtOAc	Ethyl acetate
HAT	Hydrogen atom transfer
HAT	Human African trypanosomiasis
HeLa	Human cervix adenocarcinoma
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
IC_{50}	50 % inhibitory concentration
INT	Iodo-nitro-tetrazolium
IR	Infrared spectroscopy
m/z	Mass to charge ratio
MeOH	Methanol

mg GAE/g	Milligram Gallic Acid Equivalent per gram
mg QE/g	Milligram Quercetin Equivalent per gram
MIC	Minimum inhibitory concentration
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PA	Proanthocyanidin
PEG	Polyethylene glycol
PES	Phenazine ethosulphate
pLDH	Parasite lactate dehydrogenase
ppm	Parts per million
PTLC	Preparative thin layer chromatography
RSA	Radical scavenging activity
SD	Standard deviation
STD's	Sexually transmitted diseases
TC_{50}	50 % toxic concentration
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TIAs	Terpenoid indole alkaloids
TLC	Thin layer chromatogram
TPC	Total phenolic content
TRAP	Total peroxyl radical-tapping antioxidant parameter
UPLC-MS	Ultra-performance liquid chromatography - mass spectrometry
UV-B	Ultraviolet-B radiation (short wave)

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Chapter 1

General introduction

1.1. Background

Traditional medicine has a very deep rooted and rich cultural heritage in South Africa with approximately 200 000 traditional healers being consulted by about 30 million people for their healthcare needs.[1,2] The main reason for this is that most of the population today in urban South Africa as well as in smaller rural communities are reliant on herbal medicine for their health care needs. Apart from the cultural significance of herbal medicine, it is also used based on accessibility and affordability.[3] As a consequence, their use is increasing worldwide, leading to integration of traditional medicine with primary health care.

For decades large numbers of naturally occurring plant species have been investigated by chemists and biologists as a source of biologically active compounds. These compounds exist in a wide variety of chemical classes and exhibit medicinal properties that are even today still used in folk medicine by 70 % to 80 % of people in rural and indigenous communities throughout Africa for treatment of many different types of diseases.[4] Plant infusions used to cure a range of diseases or relieve pain are common in African folk medicine and are frequently found to substitute the use of modern medicines.[5] Herbal medicines are an important part of the culture and tradition of African people.[6]

Traditional medicinal plants and plant-derived medicines are also becoming increasingly popular in modern societies as natural alternatives to synthetic chemicals. More of these natural remedies are being discovered through scientific research to determine their biological activities that make them useful as folk medicines by identifying their most active ingredients, pharmacological effects, and efficacy. According to Van Wyk and Wink, numerous medicinal plants are being described every day in the scientific literature.[7]

Medicinal plants are commonly known to affect the human system by increasing the proficiency of the body to clear an infection.[8] Herbal medicines have physiologically active components which over the years have been exploited in traditional medical practices for the treatment of many diseases.[9] Plants are useful because they are natural materials containing chemical substances produced by living organisms found in nature and have biological activities that may be useful in pharmaceutical drug discovery and drug design. A natural product can be considered as such even if it can be prepared by total synthesis. Natural products and their derivatives represent more than 50 % of all drugs that are used clinically worldwide; however, higher plants contribute less than 20 % to the total.[10] In South Africa, medicinal plants or plant extracts are traded easily and are an increasing commercial trend.[11]

African traditional medicine is diverse due to the cultural and sociological diversity of about 2000 language/cultural groups. Because of this diversity, many different plants and their parts (leaves, roots, bark, fruits and seeds) are used to treat different health disorders and in different preparations. Depending on the conditions, the whole plant or part of the plant can be used; however, in some plants one part can be found to be toxic and the other harmless, therefore selectivity is highly required.[7]

1.2. Problem Statement

Rauvolfia caffra is known for the treatment of depression, hypertension, diarrhoea, inflammation, rheumatism and pneumonia. It is also known to have sedative action. *R. caffra* has been reported to contain bioactive compounds such as alkaloids, terpenoids, phenolics, tannins, flavonoids, saponins, glycosides and steroids. Much is not known about the biological activities of isolated compounds from *R. caffra*. More so, many of the reports on the biological activities have been limited to crude extracts. Against this backdrop, we decided to isolate bioactive compounds from the stem bark.

1.3. Aim

The aim of this study is to isolate and characterize bioactive compounds from *Rauvolfia caffra* Sond.

1.4. Objectives

i. To extract bioactive compounds from the stem bark of *Rauvolfia caffra*.
ii. To isolate and purify bioactive compounds.
iii. To elucidate the structure of isolated compounds.
iv. To test the biological activities of the crude extract and isolated compounds.

1.5. Dissertation Outline

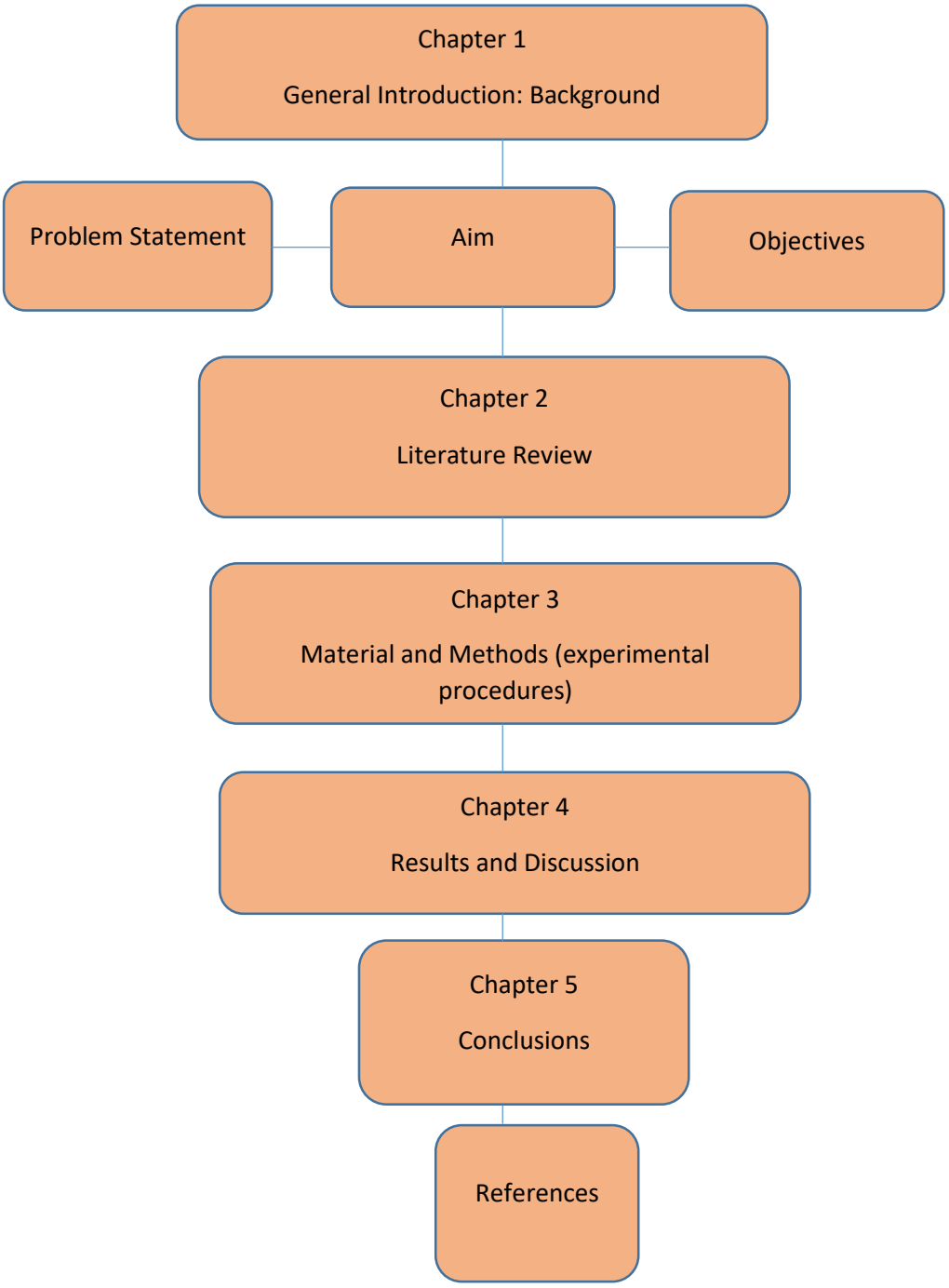


Figure 1.1: Dissertation outline.

Chapter 2

Literature Review

2.1. Medicinal plants for pharmacological purposes

Medicinal plants remain the dominant form of medicine in many countries worldwide. About 80 % of the population of Asian and African countries depend primarily on raw plant products as aspects of their daily health care needs.[12] Plant parts (leaves, stems, roots, bark, twigs, tubers, bulbs, exudates, flowers and fruits) produce different kinds of chemical constituents for performing different pharmacological activities and defence against insects, fungi, animals, and microbes. In South Africa, Uganda and Namibia, traditional medicine is used in the treatment of diseases such as headaches, pneumonia, inflammation, mental illnesses, sexually transmitted diseases and management of HIV/AIDS related infections.[13,14] Many plants used as traditional medicines are now being authenticated through scientific research by isolation of bioactive constituents for direct use in medicines. Currently, more than 12 000 of these bioactive constituents have already been isolated, a number which is estimated as below 10 % of the total. Plants manufacture a bewildering range of phytochemicals, though most are derivatives of a couple of biochemical motifs.[15,16]

2.2. Oxidative stress and antioxidant activity

Oxidative stress is a condition that occurs when the level of free radicals in the body increases beyond the scavenging ability of the antioxidants in the body, resulting in severe consequences such as DNA damage, protein oxidation or lipid peroxidation.[17,18] Catalase and hydroperoxidase enzymes convert hydrogen peroxide and hydroperoxides to nonradical forms and function as natural antioxidants in the human body. Due to depletion of the immune system's natural antioxidants in different maladies, consuming antioxidants as free radical scavengers may be necessary. Currently available synthetic antioxidants like butylated hydroxyanisole, butylated hydroxytoluene, tertiary butylated hydroquinone and gallic acid esters, have been suspected to cause or prompt negative health effects.[19]

Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity.[19]

Antioxidants are molecules that inhibit the oxidation of other molecules, and they are of two types: Synthetic and Natural antioxidants. Synthetic antioxidants are commercially produced while natural antioxidants such as vitamin A, C and E are obtained from herbs, grains, spices, fruit and vegetables.[17,20-23] Synthetic antioxidants such as butylated hydroxytoluene (BHT) and propylgallate are associated with carcinogenesis.[24] Whereas natural antioxidants are used as preventive medicine, most have been isolated and incorporated as ingredients in dietary supplements for management of various diseases such as cancer, coronary heart disease and others.[24] Thus identification of more phytochemicals with antioxidant activity is still deemed pertinent.

The total antioxidant capacity cannot be measured directly but rather by the effects of the antioxidant in controlling the amount of oxidation. The analytical methods of evaluation of antioxidant activity include: The hydrogen atom transfer reaction (HAT) assay, total peroxyl radical-trapping antioxidant parameter (TRAP) assay, the 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay, oxygen radical absorbance capacity (ORAC) assay and the electron transfer reaction assay that includes trolox equivalent antioxidant capacity (TEAC/ ABTS) assay. DPPH, ORAC and ABT are widely used.[25]

2.3. The use of medicinal plants as antibacterial agents

Traditional medicinal plants have always been considered as a natural source of new drugs of high value to mankind. Natural medicinal plants are known to have therapeutic properties and have been used globally for healing numerous diseases.[26-28] Medicinal plants are a rich source of secondary metabolites and have been used for their antibacterial, antifungal and antiviral activities for many years.[29] There are a number of antibacterial bioassays that are used to test for bioactive compounds with antimicrobial activity, which includes agar diffusion, micro-broth dilution and bioautography.[30]

These methods have been employed for many years to screen medicinal plants for antibacterial constituents based on their traditional use in rural regions for similar purposes. Plants which displayed the presence of antibacterial compounds provided information which led to the development of new antimicrobial drugs.[31] About 80 % of the world's population depends on traditional medicinal plants for their primary health care.[32] Over 50 % of all modern remedies have been derived from natural sources including plants, and many of these medication were isolated based on the use of such plants traditionally.[33]

2.4. Cytotoxic activity

Large numbers of plants are being collected and sold for treatment of diseases and not much is known about how poisonous these plants are. One has to be careful before using these plants for treatment of diseases. While some of these plants are useful for the treatment of various diseases others will cause adverse health problems and others may even cause death. There is an increase in the number of studies dealing with activity and uses of plants; unfortunately, most of the studies deal with antimicrobial activities of medicinal plants and not the toxicities of the plant extracts. Very few studies deal with medicinal plant extract toxicity.[34] There is a need to study the medicinal plants for their toxicity and develop a cell culture assay. A cell culture assay is any method which is used to assess the cytotoxicity of a material. This refers to the *in vitro* assessment of material to determine whether it releases toxic chemicals in sufficient quantities to kill cells either directly or indirectly through the inhibition of cell metabolic pathways. Cell culture evaluations are the precursor to whole animal studies and are a way to determine if significant cytotoxicity exists for the given material.[34]

There are 3 basic parameters upon which cytotoxicity measurements are based. Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as Trypan-blue are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components.[35] Among the methods of testing cytotoxicity is to check if the membrane has allowed substances normally found inside the cell to the outside e.g. lactate dehydrogenase. Cytotoxicity can also be monitored using the metabolic test MTT or

MTS assay. This assay measures metabolic function by measuring cellular ATP levels or mitochondrial activity. Viable cells will reduce the MTS reagent to a coloured formazan product. The third type of assay is the direct measure of cell number, since dead cells normally detach from a culture plate, and are washed away in the medium. Cell number can be measured by direct cell counting. TC_{50} represents the concentration causing a 50 % cytotoxic effect.[35]

2.5. Medicinal plants as antimalarial agents

Plasmodium falciparum is a protozoan parasite, one of the species of *Plasmodium* that cause malaria in humans. Malaria caused by this species is the most dangerous (deadly) form of malaria, followed by malaria caused by *P. vivax*, both with the highest rates of complications and mortality.[36] Other *Plasmodium* species that cause malaria in humans except *P. falciparum* and *P. vivax* are *P. malariae* and *P. ovale*.[36] In South Africa, Kenya, Zimbabwe, Congo, Brazil, Nigeria and Cameroon herbal plants are generally used in the treatment of various diseases, such as malaria and its associated symptoms.[37] Various medicinal plants used as alternative drugs are indicative of the vital role that plants play in various developing countries, and are also a sources of novel plant-derived constituents which could be leads for treatments against numerous ailments. Malaria can be treated using antimalarial drugs, such as quinine and artemisinin, which are derived from herbal plants. For many years, more than 200 isolated compounds (alkaloids, quassinoids, terpenes, sesquiterpenes, triterpenoids, chalcones, flavonoids, xanthones, quinones, coumarins, limonoids and peptides) from medicinal plants displayed antiplasmodial activity.[38-42]

2.6. Antitrypanosomal activity of medicinal plants

Trypanosomiasis is a parasitic disease caused by blood-dwelling genus *Trypanosoma*, affecting humans and animals in many African countries. *Trypanosoma*, *Trypanosoma vivax* (*T. vivax*), *Trypanosoma congolense* (*T. congolense*) and *Trypanosoma brucei brucei* (*T. b. brucei*) are the main species responsible for African animal trypanosomiasis (AAT) while *T. b. rhodesiense* and *T. b. gambiense* cause sleeping sickness (human African trypanosomiasis,

HAT). The parasites are predominantly transmitted by the bite of infected flies in the Tabanidae family, such as horse flies and tsetse flies.[43]

Both human and animal trypanosomiases negatively affect the whole economy of Africa by weakening both human and animal health.[44] The modern chemotherapy of HAT depend on only six drugs (suramin, pentamidine, melarsoprol, eflornithine, arsobal and mel B), five of which were developed more than 30 years ago. Others such as homidium, isometamidium and diminazene aceturate are used in animal infections. These drugs show highly toxic effects and are expensive, therefore the development of cost-effective new drugs in the treatment of sleeping sickness is urgently required in order to control the disease.[45] However, it has also been observed that natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity.[46]

2.7. Phytochemical constituents

Phytochemicals (natural products) are typically secondary plant metabolites causing pharmacological or toxicological effects in man and animals. They are mostly produced for chemical protection against attack by animals, insects, microorganisms (fungi and bacteria), and other plants competing for nutrients and light, but can also serve as a form of chemical store for the plant.[47] Secondary metabolites are made within the plants, and are the final products of the primary biosynthetic and metabolic pathways for compounds related with plant development. Some of them are found to hold numerous types of significant functions in the living plants, such as protection and attraction, and since many natural products show some pharmacological activity, they are often used in the treatment of ailments.[48]

Natural products can be grouped into two major classes: primary (produced by all living organisms) and secondary (produced by plants only). Chemically, the secondary plant metabolites can be classified broadly as glycosides, flavonoids, tannins, alkaloids, terpenes and phenolic compounds.[49] Medicinal plant parts such as roots, bark, stem, leaves, flowers, and fruits are normally rich in phenolic compounds such as flavonoids, phenolic acid, stilbenes, tannins, coumarins, lignans and lignins, which protect cells against oxidative

damage and reduce the risk of developing certain types of cancer.[50] Medicinal plants constitute an effective source of antimicrobial natural products. The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into the African continent.[51]

2.7.1. Glycosides

Glycosides are found in many medicinal plants and can have high medicinal efficacy or toxicity, depending on the plant of origin and the amount of plant product consumed. Numerous groups of secondary metabolites are bound to a mono- or oligosaccharide or to a uronic acid to form glycosides. The saccharide or uronic acid part is called glycone, and the other part the aglycone. The main groups of glycosides are cardiac glycosides (e.g., oleandrin **1**), cyanogenic glycosides (e.g., amygdalin **2**), glucosinolates **3**, coumarin glycosides (e.g., apterin **4)** and anthraquinone glycosides (e.g., anthraquinone **5**) (Figure 2.1).[49]

2.7.2. Flavonoids

Flavonoids are the largest class of polyphenolic compounds having a substance that has two substituted benzene rings (A and B as shown in Figure 2.2) linked by a heterocyclic pyran ring (C), and usually occurring glycosides. They contain phenolic groups imparting general antioxidant activity to these compounds. Flavonoids also act as a secondary antioxidant defence system in plant tissues subjected to diverse abiotic and biotic stresses.[52]

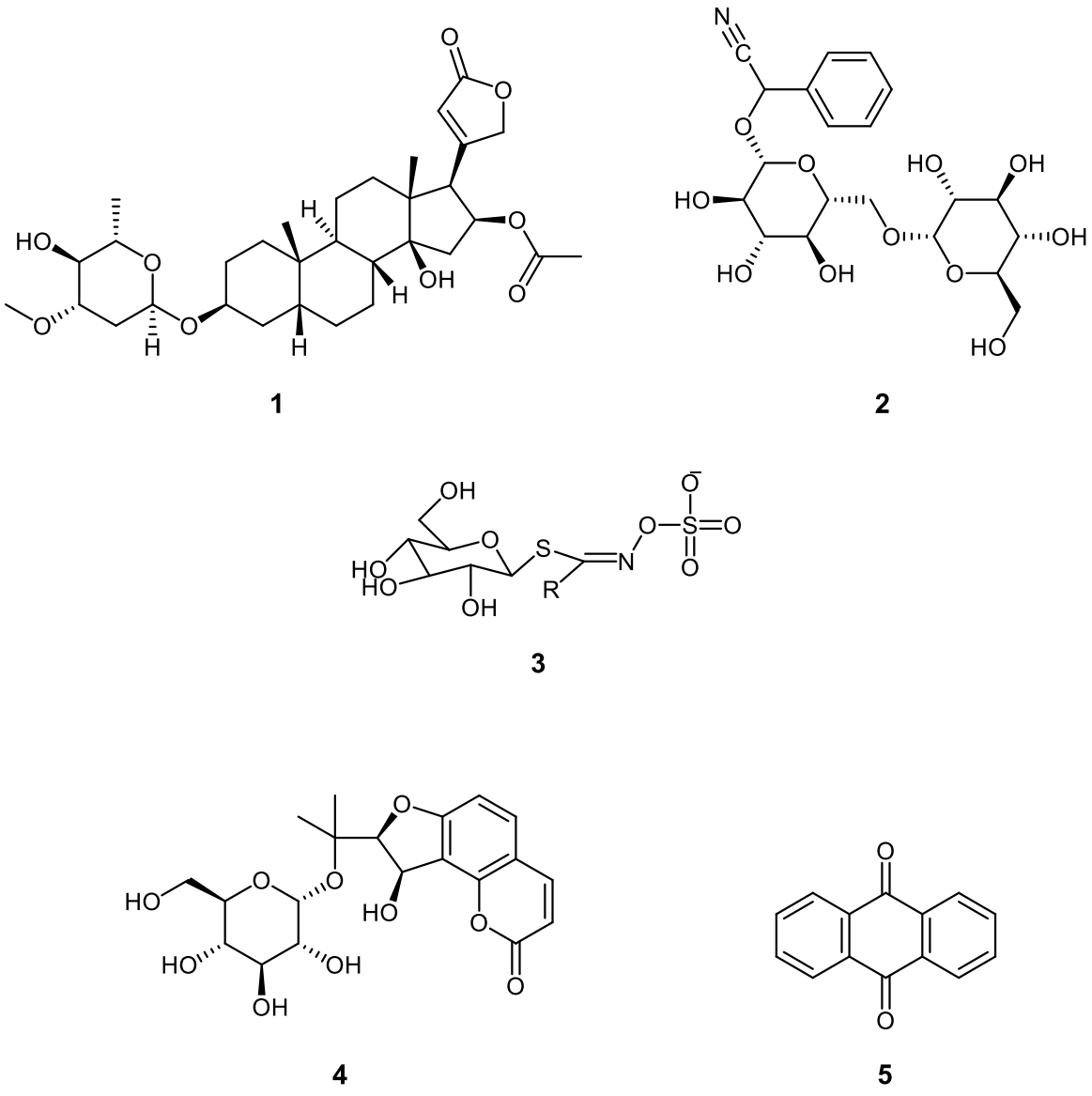


Figure 2.1: Examples of major glycoside classes.

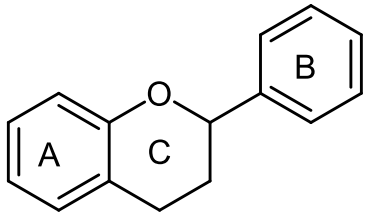


Figure 2.2: Basic flavonoid structure.

The major subgroups of flavonoids include flavones **6** (e.g., flavone, apigenin, and luteolin), flavonols **7** (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones **8** (e.g., flavanone, hesperetin, and naringenin), chalcones **9** (e.g., phloridzin, arbutin, phloretin and chalconaringenin), catechins **10** (e.g., epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), anthocyanins **11** (e.g., cyanidin, delphinidin, malvidin, pelargonidin and peonidin), and isoflavonoids **12** (e.g., genistein, daidzein, genistin, glycitein and daidzin) (Figure 2.3).[52]

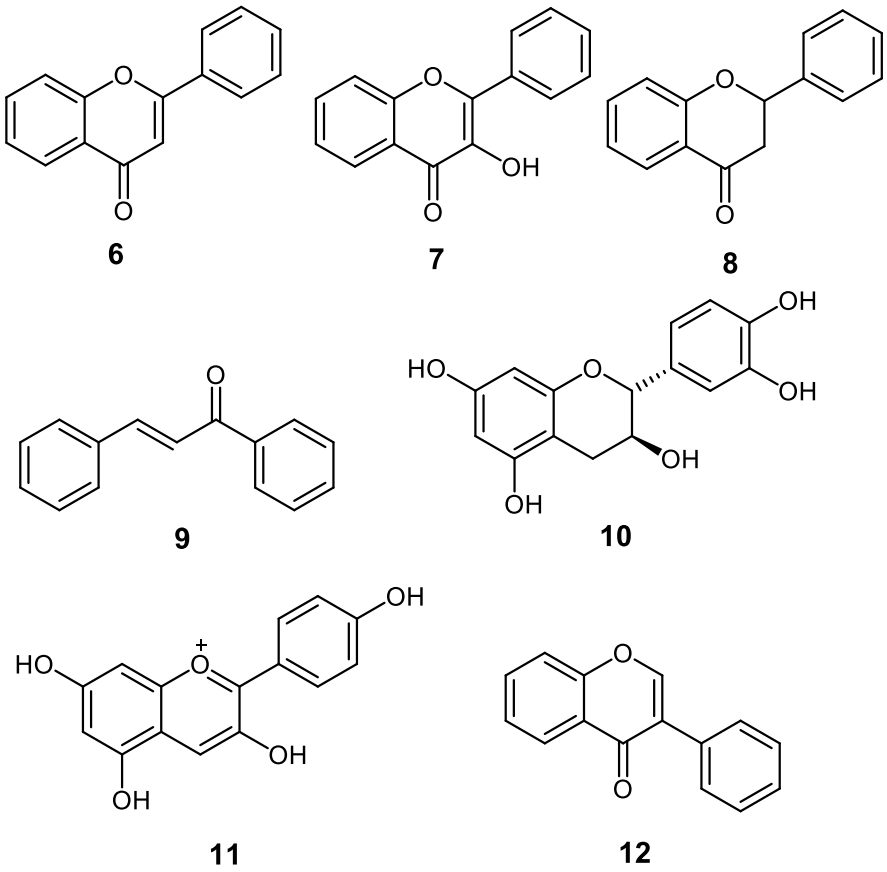


Figure 2.3: Examples of flavonoids.

Flavonoids are widely distributed in fruit, vegetables, grains, bark, roots, stems, flowers, beverages and consequently form an important part of the human diet.[53] They have numerous functions, including protection against UV-B radiation, pathogen attack, cardiovascular disease, cancer and age-related diseases such as dementia.[54]

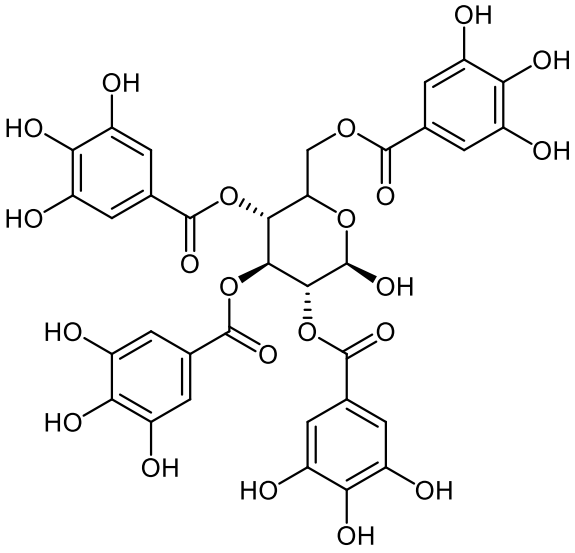
2.7.3. Tannins

Tannins are complex chemical substances derived from phenolic acids (sometimes called tannic acid) that bind to and precipitate proteins and numerous organic compounds, including amino acids and alkaloids, for their phytochemical activity.[55] The tannin compounds are widely distributed in bark of trees, wood, leaves, buds, stems, fruits, seeds, and roots, and occur in many species of plants, where they play a vital role in the defence against predation, pesticides, and plant growth regulation.[56] They exhibit a variety of pharmacological activities and form a constant protein-tannin complex and inhibit enzymatic activities considered as very effective. There are two distinct types of tannins: Hydrolysable tannins **13** and condensed tannins **18**. Hydrolysable tannins **13** are compounds hydrolysed by acids or enzymes having a central core of glucose, and the products of hydrolysis are gallic acid **15** in gallotannins **14** or ellagic acid **17** in ellagitannins **16**. Condensed tannins **18** are large polymers of flavan-3-ol (catechin) units associated through an interflavan carbon bond, and are much resistant to hydrolysis. The two types of tannins have most properties in common, but hydrolysable tannins are less stable and have greater potential to be toxic (Figure 2.4).[57]

2.7.4. Alkaloids

Alkaloids are a large, naturally occurring and structurally varied group of chemical compounds that have basic nitrogen atoms and are produced by a large diversity of organisms including bacteria, fungi, plants, and animals. They can be purified from crude extracts of these organisms by acid-base extraction. Many alkaloids are poisonous and often have an extensive variety of biological activities including antimalarial, anticancer, antibacterial and antihyperglycemic activities, which makes them interesting for use as medicine.[58]

Alkaloids are derived from amino acids, but many result from modification of numerous classes of molecules including polyphenols, terpenes, or steroids. They are usually soluble in aqueous ethanolic media and they usually occur as salts (e.g., chlorides or sulphates) and/or as *N*-oxides in the plants. Most of them contain a heterocyclic nitrogenous ring or ring system and have a basic (alkaline) character.[59] Plant-derived alkaloids currently in clinical use include morphine **19**, scopolamine **20** and sanguinarine **21** (Figure 2.5).



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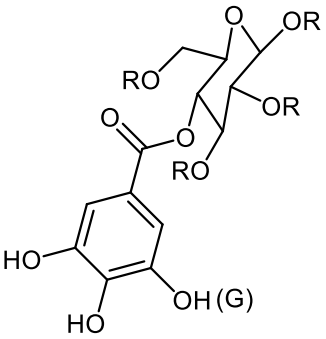

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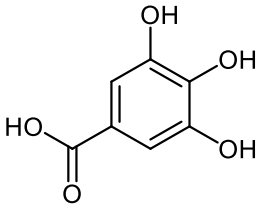

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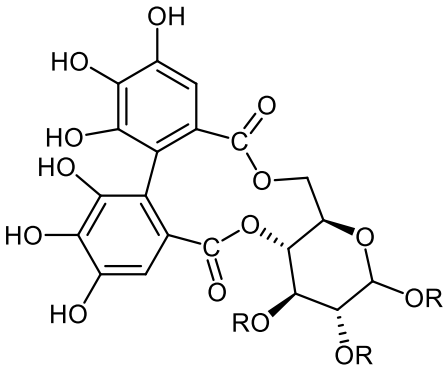

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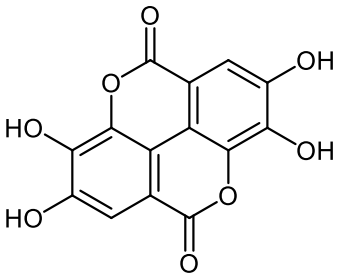

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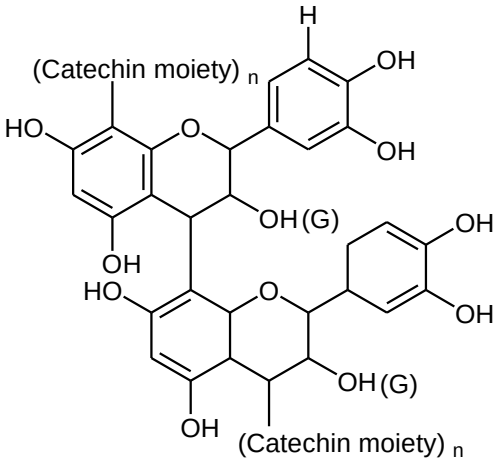

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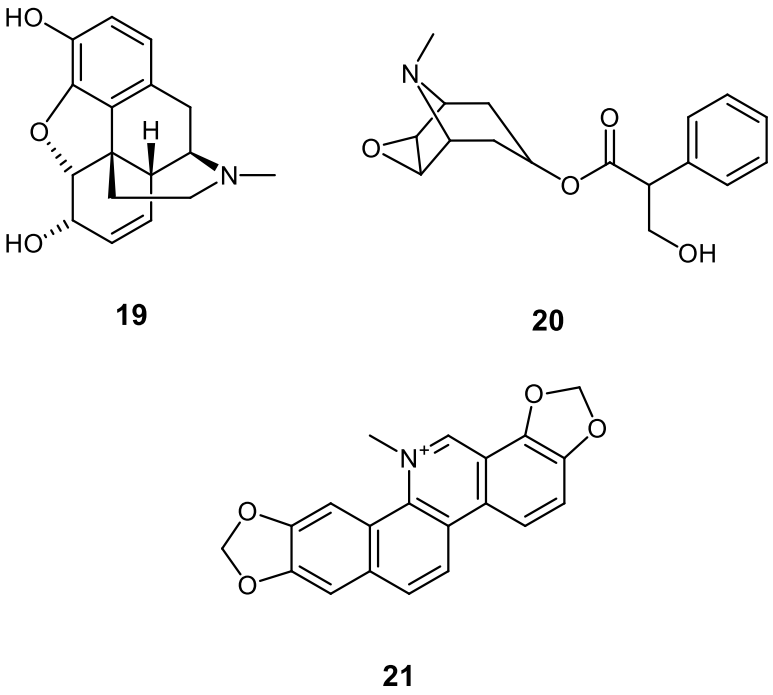
Figure 2.4: Examples of condensed and hydrolysable tannins.



19 **20** **21**

Figure 2.5: Examples of alkaloids.

2.7.5. Terpenes

The terpenes are the largest and most diverse classes of metabolites, ranging in structure from linear to polycyclic molecules. They are synthesized from isoprenoid units, and share metabolic pathways with fatty acids. Terpenes exist in diverse chemical forms as an array of linear functionalized hydrocarbons or chiral, carbocyclic skeletons with diverse chemical modifications such as hydroxyl, carbonyl, ketone, aldehyde and peroxide groups. Terpenes and terpenoids have been found to possess antimicrobial activity.[60]

The mechanism of action of terpenes on microbes involves membrane disruption by the lipophilic compounds. Cantrell *et al.*[61] reported that the addition of a methyl group on kaurene diterpenoids drastically reduced their antimicrobial activity; more lipophilic compounds were significantly more antibacterial than their more polar analogues. Terpenes occur as hemiterpenes (e.g., isoprenol **22** and isovaleric acid **23**), monoterpenes (e.g., (-)-menthol **24**, α-terpineol **25** and (-)-limonene **26**), sesquiterpenes (e.g., humulene **27**, (*E*,*E*)-α-farnesene **28** and farnesol **29**), diterpenes (e.g., cembrene A **30**, retinol **31** and phytol **32**), triterpenes (e.g., lanosterol **33** and squalene **34**), tetraterpenes (e.g., monocyclic gamma-carotene **35**, bicyclic alpha-carotene **36**), (Figure 2.6).[60]

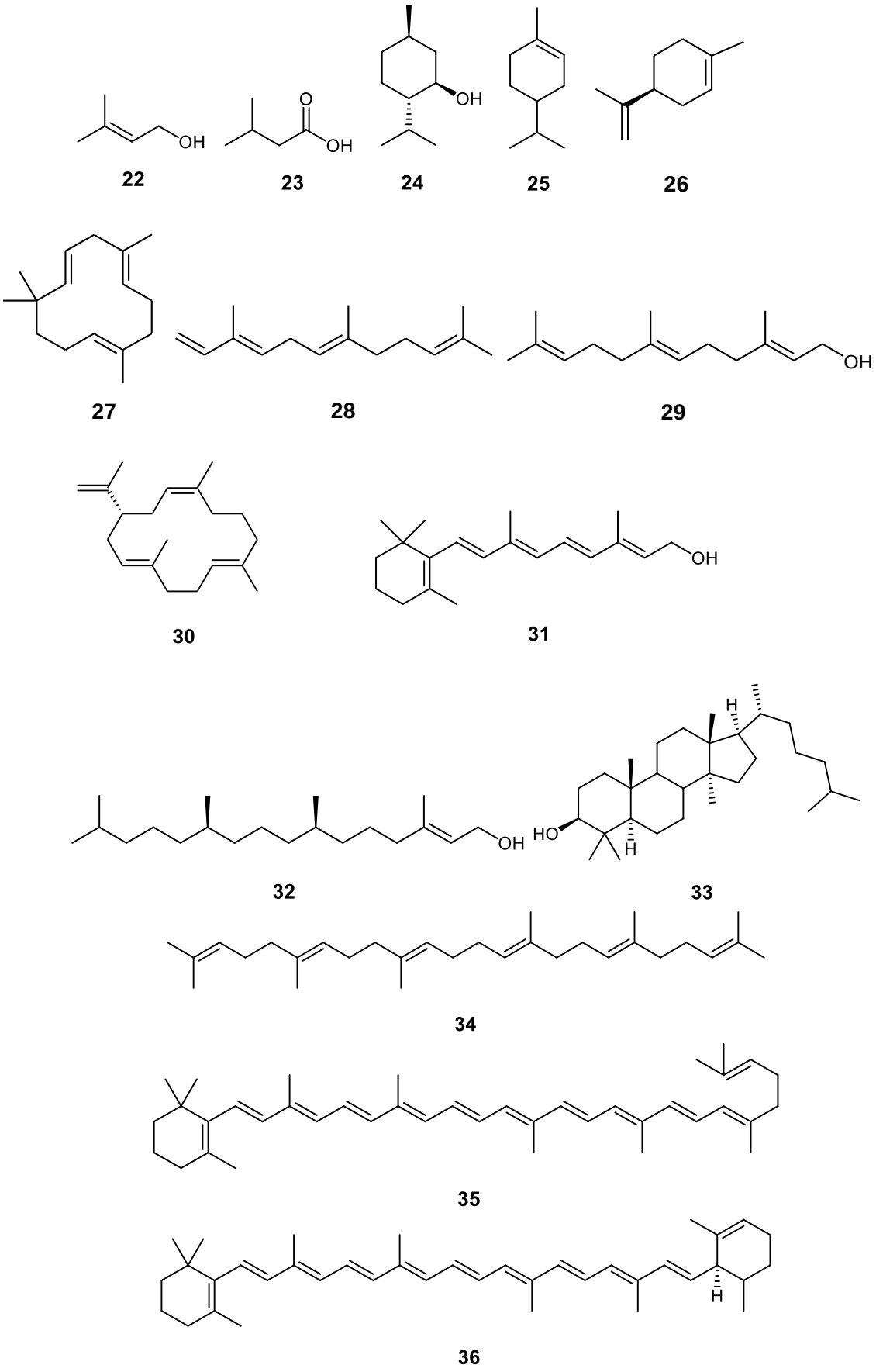
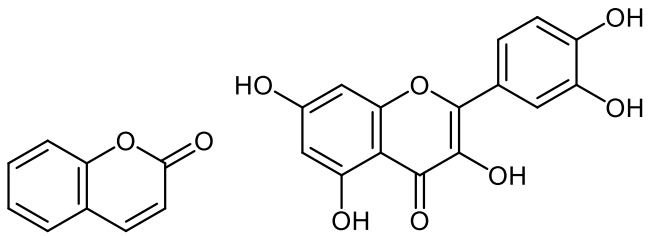


Figure 2.6: Examples of terpenes.

2.7.6. Phenolics

Phenolics are compounds having one or more aromatic rings with one or more hydroxyl groups. The simplest of the class is phenol, which is also called carbolic acid. Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule. Among these compounds there are coumarins **37** and quercetin **38**.[62] They are extensively distributed secondary metabolites of plants, with approximately 8 000 phenolic structures presently known, alternating from simple molecules such as phenolic acids to highly polymerized substances such as tannins.

Natural phenolic compounds occur widely in leaves, fruits, bark and beverages and can accumulate in large amounts in particular organs or tissues of the plant.[63] Depending on their structure, they can be classified into phenolic acids, flavonoids and tannins. De Pascual-Teresa *et al*. reported that phenolics have various beneficial properties for human health, including anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumor activity, but the most significant action of phenolics is their antioxidant activity (Figure 2.7).[64]



37 **38**

Figure 2.7: Chemical structures of phenolic compounds.

Phenolic compounds have a distinctive taste, flavour, and health-promoting properties as found in vegetables and fruits.[65] They are vital for plant growth and reproduction, and are formed as a response to environmental factors (light, chilling and pollution) and to protect damaged plants.[66] Phenolics and flavonoids have varied pharmacological activities, for example, antiulcer, anti-inflammatory, antioxidant, cytotoxic, antitumor, antispasmodic and

antidepressant activities.[67] The phenolic acids can be divided into two classes: derivatives of hydroxybenzoic acid, such as gallic acid **15**, and derivatives of hydroxycinnamic acid, such as coumaric, caffeic **39** and ferulic acid **40** (Figure 2.8).[68]

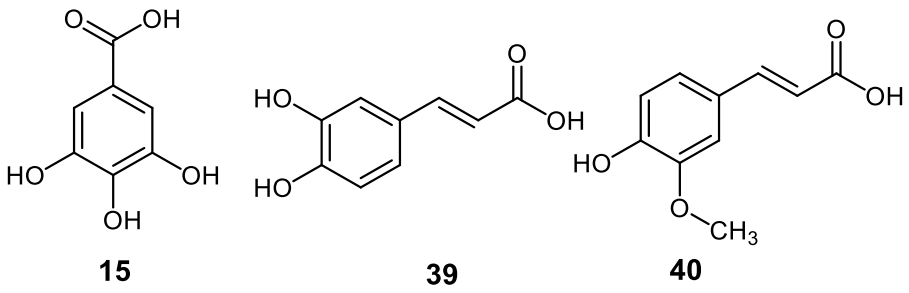


Figure 2.8: Derivatives of hydroxybenzoic acid and hydroxycinnamic acid.

Hydroxycinnamic acids are formed as simple esters with glucose or hydroxycarboxylic acids. Plant phenolic compounds are varied in molecular structure, and are categorized by hydroxylated aromatic rings. In numerous plants the phenolics are polymerized into greater molecules such as the proanthocyanidins (PA; condensed tannins) and lignins. Furthermore, phenolic acids can arise in food plants as glycosides or esters with other natural compounds such as sterols, alcohols, glucosides and hydroxyl fatty acids.[69]

2.8. The subject of this study: *Rauvolfia caffra*

Rauvolfia caffra (Figure 2.9) is a plant species belonging to the family Apocynaceae. It is generally known as "quinine tree". *Rauvolfia* is a genus consisting of about 60 species. Seven of these are found in Africa, three in Madagascar and one is restricted to southern Africa. *R. caffra* is limited to coastal forests in east, central and Southern Africa. The species occurs in seven provinces in South Africa and in Swaziland. In South Africa, it is found along the coastal belt, in the eastern regions of South Africa, from the Eastern Cape to KwaZulu-Natal, spreading further north and inland to Mpumalanga, Limpopo, Gauteng and North-West. *R. caffra* is generally used in Sub-Sahara Africa by communities as a medicine.[70]

Figure 2.9: *Rauvolfia caffra* Sond.

2.8.1 The genus *Rauvolfia*

The genus *Rauvolfia* was named after Leonhart Rauwolf, a 16th century German physician, botanist and a collector of drug plants. Examples of species in this genus are *R. serpentina*, *R. caffra*, *R. vomitoria* and *R. stricta*.[71]

2.8.1.1. *Rauvolfia serpentina*

Rauvolfia serpentina L. Benth. Ex Kurz. is an evergreen, woody, glabrous and perennial shrub with maximum height up to 60 cm. The plant possesses tuberous roots with pale brown cork and elliptic to lanceolate or obovate leaves in whorls of three.[72] The plant belongs to the family Apocynaceae and occurs in habitats of tropical and subtropical regions. The family includes 50 species, distributed worldwide in the region of the Himalayas, Indian peninsula, Burma, Indonesia and Sri Lanka and is indigenous to India, Bangladesh and other regions of Asia.

The roots, leaves and stem bark are used in the treatment of various disorders, including hypertension, anxiety, insomnia and insanity due to the presence of alkaloids, carbohydrates, flavonoids, glycosides, phlobatannins, phenols, resins, saponins sterols, tannins and terpenes.[73,74]

2.8.1.2. *Rauvolfia vomitoria*

Rauvolfia vomitoria belongs to the family Apocynaceae. The plant is a shrub or tree which grows over 10 m tall of the forest and has oval leaves with straight venation and clusters of small flowers.[75] *R. vomitoria* is common in secondary growth throughout most of African regions and the plant is of different species: the Indian species (*Rauvolfia serpentina*) and the African species (*R. vomitoria*).[76] The roots, leaves and stem-bark are used in herbal medicine for the treatment of many diseases, e.g., snake bites, fever and nervous disorders. Major phytochemical constituents of this plant include alkaloids, glycosides, polyphenols, and reducing sugars.[77]

2.8.1.3. *Rauvolfia stricta*

Rauvolfia stricta, also known as *Rhazya stricta* Decne, is a member of the Apocynaceae family. The genus *Rhazya* is a traditional medicinal plant in the Middle East and South Asia and produces a large amount of terpenoid indole alkaloids (TIAs), some of which have essential biological activities. *R. stricta* is an evergreen small, glabrous, erect, toxic shrub, which is commonly distributed in the Middle East and Indian sub-continent and has been used as a traditional medicine to treat numerous ailments such as fever and chronic rheumatism.[78,79] *Rauvolfia stricta* grows in depressions with silty and sandy soils and has been reported that it increased in abundance along gradient of sand. Soils supporting dense stand of *R. stricta* have also been reported to be relatively rich in available magnesium.[80]

2.8.2. Taxonomy

R. caffra is known by several common names, such as quinine tree (English), munadzi (Venda), nchongo (Tsonga), Kinaboom (Afrikaans), umJelo, umThundisa (isiXhosa), umHlambamoanzi, umKhadluvungu (isiZulu). Its full botanical classification is:

- **Kingdom :** Plantae
- **Division** : Magnoliaphyta
- **Class** : Magnoliopsida **(unranked)** : Angiosperms
- **Order** : Gentianales
- **Family** : Apocynaceae (The Oleander family)
- **Genus** : *Rauvolfia*
- **Species** : *R. caffra*

2.8.3. Ecology and physiology

Rauvolfia caffra is an evergreen tree up to 30 m tall with a roundish crown (Figure 2.10). The main stem is straight, tall, and bare. Bark is grey to brown, rough with protuberant leaf scars on younger branches, becoming yellowish brown, thinly corky and cracked into lesser squares with age. The leaves are simple in whorls of 3–6, crowded at the ends of the branchlets, slightly leathery and tapering to both ends about 120–280 mm long and 30–60 mm broad. Furthermore, the leaf blade is shiny green above, paler green below with midrib raised and margins smooth. The leaf stalk is up to 35 mm long.[81] *R. caffra* occurs at an altitude from 0 to about 1 500 m on sandy and loamy soils. It is typically found along wooded streams, on river banks, at margins of evergreen forest and in swamp forest where it can reach up to 30 m in height. When growing away from rivers and streams the species is always related with existing ground water, and is therefore regarded as an indicator of water, and classified as a hydrophyte.[70]

(a) (b)

Figure 2.10: *Rauvolfia caffra* flowers (a) and fruit (b).

(www.plantzafrica.com/plantqrs/rauvolfiacaffra.htm, accessed on 10/03/2016).

2.8.4. Ethnomedicinal uses

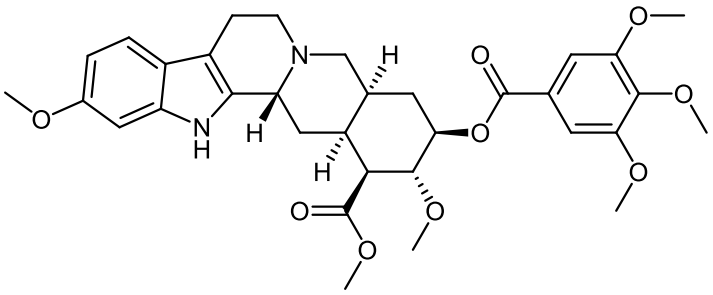
In East Africa the bark is usually used in traditional medicine. The crushed bark is applied on measles or itching rash.[82] A bark decoction is taken as a constricting, laxative or emetic to treat fever, swellings, abscesses, rheumatism, hepatitis, pneumonia, abdominal pain and as a sedative.[83] A piece of bark is chewed for the treatment of sexually transmitted diseases (STD's), coughs and toothaches. In South Africa a decoction of the bark is used to treat insomnia, and is taken as a sedative for hysteria. The Chagga and Meru of North Eastern Tanzania inhale the vapour of the bark decoction to treat epilepsy and eye diseases. In Zambia the bark is used to treat STD's. The crushed bark is applied on skin wounds, measles or itching rashes.[82] Zulus use *R. caffra* bark for the therapy of skin rashes caused by measles, urticaria and other forms of rashes. The bitter bark is intensely purging and produces severe abdominal pains; nonetheless, the Pondo's of South Africa use the bark for abdominal disorders.[84] The Venda people of South Africa regard the plant as insecticidal and use the powdered bark as a bandage for sores and the infusion is used to kill maggots in wounds. The latex is used to treat diarrhoea and other stomach diseases.[85]

Dried pulverized leaves are sniffed to cure headaches, and in Tanzania a root decoction is taken to treat abdominal pain, constipation, irregular periods or hypertension, fever, swollen legs, insomnia and palpitation of the heart. The root of the plant is also used for treating insomnia, insecurity, restlessness, hardened swellings and anxiety. The root bark can be used as an acaricide and the pounded unopened inflorescences are applied to wounds. A root bark decoction is used for treatment of internal parasites, such as roundworm and tapeworm.[83] In Zimbabwe the sap of crushed fruits is used as ear drops to treat earache.

Apart from the medicinal uses, the wood is also used to make household utensils, drums, fruit boxes, kitchen furniture and shelving. In Kenya the thickened latex is used as a bird lime, and poles are used in hut building as well as making bee hives. *Rauvolfia caffra* is used as a shade tree in coffee plantations, and the tree is a good source of fuel wood. In Cameroon and Gabon the bark is used to make crossbow strings.[85]

2.8.5. Phytochemistry of the genus *Rauvolfia*

Erasto *et al.*[86] reported that *R. caffra* Sond is rich in indole alkaloids, most of which have been isolated and identified. According to these researchers' report, the roots and stem bark extract possess high concentrations of reserpine **41**, ajmaline **42** and ajmalicine **43**, which are used as antihypertensive and anti-inflammatory agents. They also determined that the extracts and derived alkaloids possess high antimicrobial activity due to the prevention of some redox pathways and other chemical processes in the bacterium cell. These alkaloids possess numerous biological activities, including antimalarial, antitumor and antidiabetic efficiency.[87,88]



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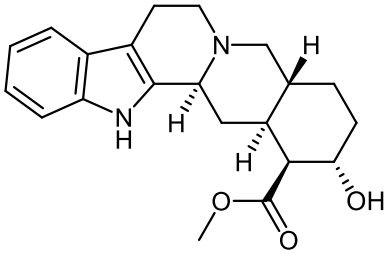

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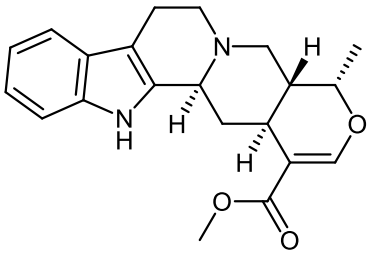

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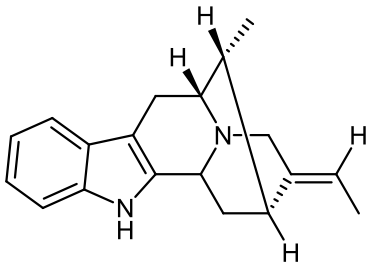
Thirty-two alkaloids were isolated from the stem bark of *Rauvolfia caffra* and 28 were identified. The alkaloids represented sarpagan **44**, akuammicine **45**, dihydroindole **46**, indolenine **47**, peraksine **48**, oxindole **49** and yohimbine **50** types. The anhydronium base serpentine **51** was detected but not isolated. The principal alkaloids from the stem bark are ajmaline **42**, ajmalicine **43** and geissoschizol **52**.[86]



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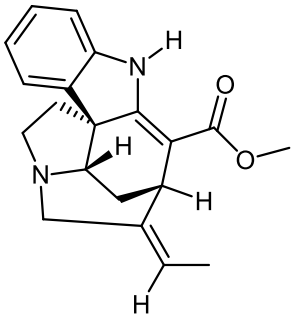

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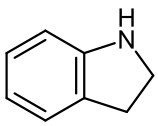

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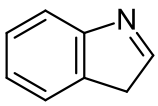

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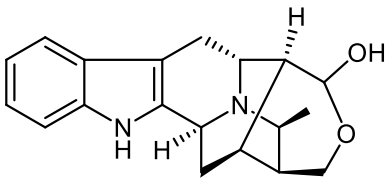

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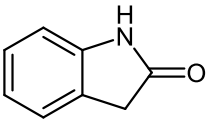

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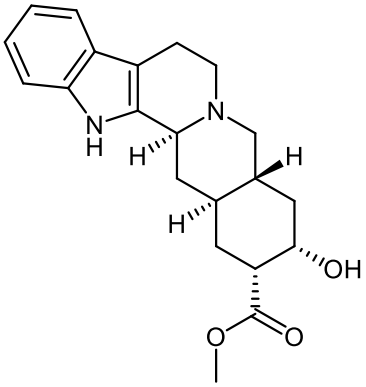

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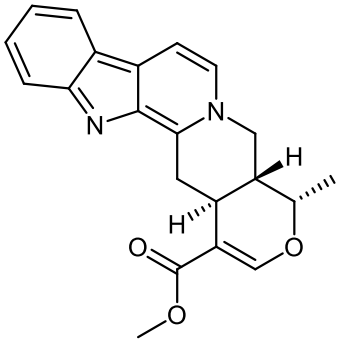

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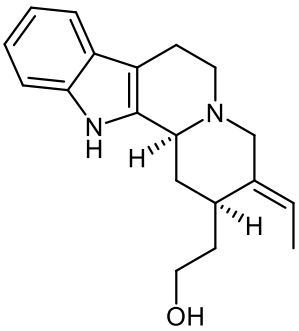

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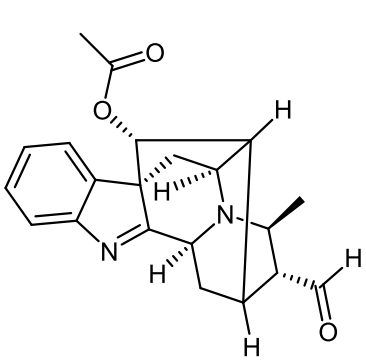
The main alkaloids isolated from the leaves are from the less common indolenine type (perakine **53** and vomilenine **54**) and from the peraksine type (peraksine **48**, dihydroperaksine **55** and deserpidine **56**). Reserpine **41**, ajmalicine **43**, reserpinine **57**, deserpidine **56**, and ajmaline **42** are used in western medicine. Reserpine **41** is an antihypertensive, antipsychotic, and tranquillizing drug, and causes depression. Ajmalicine **43** is mostly used to treat stroke, head injuries and reduces the rate of depolarization of atrial and ventricular cells. Reserpinine **57** and deserpidine **56** can be used to treat the same disorders as reserpine **41**.[89]



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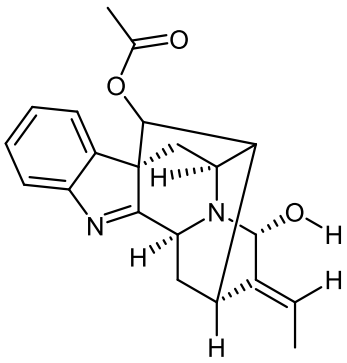

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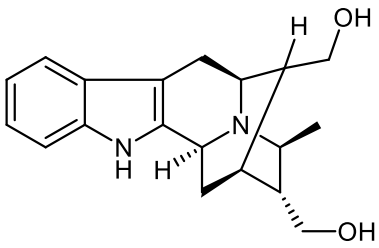

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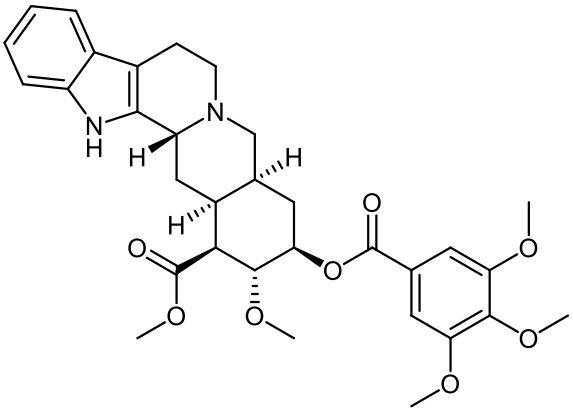

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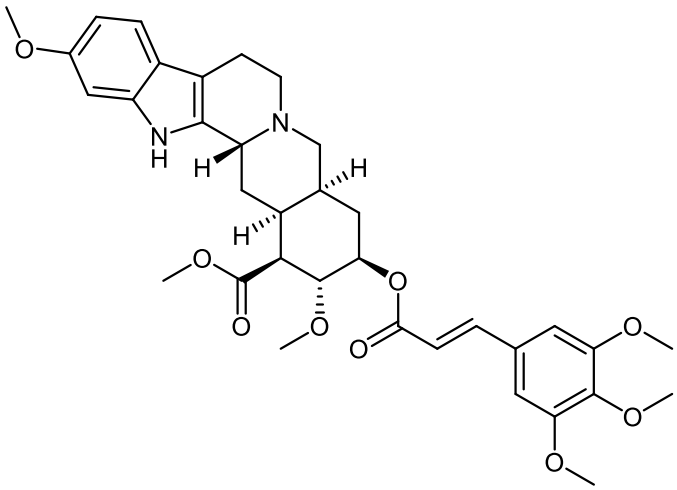

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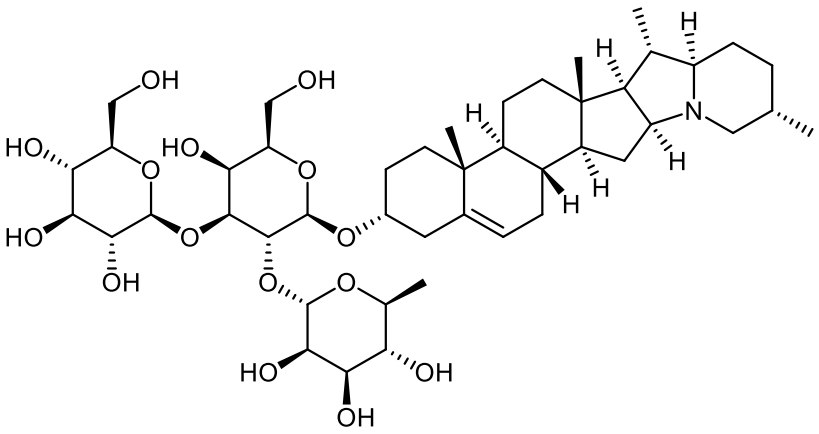
Francis *et al.*[90] discovered that *R. caffra* contains saponins **58**. Saponins **58** are compounds with a wide range of biological functions, including antimicrobial, antiviral, antioxidant and cytotoxic properties. However, these compounds are known to inhibit the ingestion of protein and uptake of vitamins and minerals. Therefore, they are mostly used as detergents and externally active agents in industries.



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2.9. Pharmacological activities

2.9.1. Antibacterial activity

Elisabetsky and Costa-Campos[91] reported that *Rauvolfia* species have anti-microbial and antibacterial activities. They demonstrated that the aqueous and methanolic extracts of the stem bark, root and leaf of *R. caffra* display antibacterial activities against the microbial species *Escherichia coli* (Gram-negative), *Staphylococcus aureus* (Gram-positive), and *Enterococcus faecalis*. However, the methanolic extracts of the root and stem barks exhibited more antibacterial activity against Gram-negative bacteria (*E. coli*) than the Gram-positive bacteria. The ethanolic aqueous and alkaloid extracts of the roots of *R. caffra* displayed moderate to high antimicrobial activity against the tested bacterial strains. As a result, the alkaloid extracts of the roots of *R. caffra* were more active than all test samples with minimum inhibitory concentration (MIC) values ranging from 0.625 mg/mL to 1.25 mg/mL and 5.00 mg /mL against *S. aureus*, *E. coli*, and *E. faecalis*, respectively.

Schmelzer and Gumb-Fakin[92] reported that the stem bark of *R. caffra* has more medicinal uses than roots and leaves. They reported that the stem bark and the root extracts exhibited no significant differences in antibacterial activities with root and leaf extracts at $p > 0.05$, but an important difference was observed in the antibacterial activities of the stem bark and leaf extracts against *E. coli* at $p < 0.05$. Usually, the root bark extract was observed to be more potent than the stem bark and the leaf extracts.

2.9.2. Antioxidant activity

Several *Rauvolfia* species have been reported to have antioxidant activities.[86,93] Extracts from the stem bark of *R. caffra* revealed a strong dose-dependent radical scavenging activity (RSA) against DPPH. The percentage radical scavenging activity (% RSA) of *R. caffra* stem bark extracts was 93.15 % at 0.1 mg/mL, whereas that of the standard quercetin was 96.39 % at 0.1 mg/mL, suggesting *R. caffra* stem bark extract is a comparatively strong antioxidant. However, Hamilton *et al.*[94] reported that the extracts from stem bark samples of *R. caffra* had a free radical inhibition of 79.65 % ± 1.86, while the leaves showed 70.55 % ±1.26 inhibition. Therefore, statistically, there was no major difference between the free radical scavenging activity of *R. caffra* stem bark extracts and the standard quercetin ($p > 0.05$). The activity of the standard quercetin was higher but similar to that of the extract of *R. caffra*.

Chapter 3

Materials and Methods

3.1. Plant collection and preparation

The stem bark of *Rauvolfia caffra* was collected at the University of Venda Campus in January 2016. Botanical identification was provided by Prof. Tshisikhawe, a botanist in the Department of Botany at the University of Venda, and a voucher specimen (BD 001) was deposited. The plant samples were air-dried for two months and the dry samples were ground to fine powder using an industrial blender (mill).

3.2. Extraction of plant materials (bioactive compounds) from the stem barks of *R. caffra*

About 1.7 kg ground stems of *Rauvolfia caffra* were soaked with 2 L of methanol for 48 hours at room temperature. The methanol extract was filtered, and then concentrated using a rotary evaporator to obtain 49.2 g of dried extracts. The extraction procedure is shown in Figure 3.1.

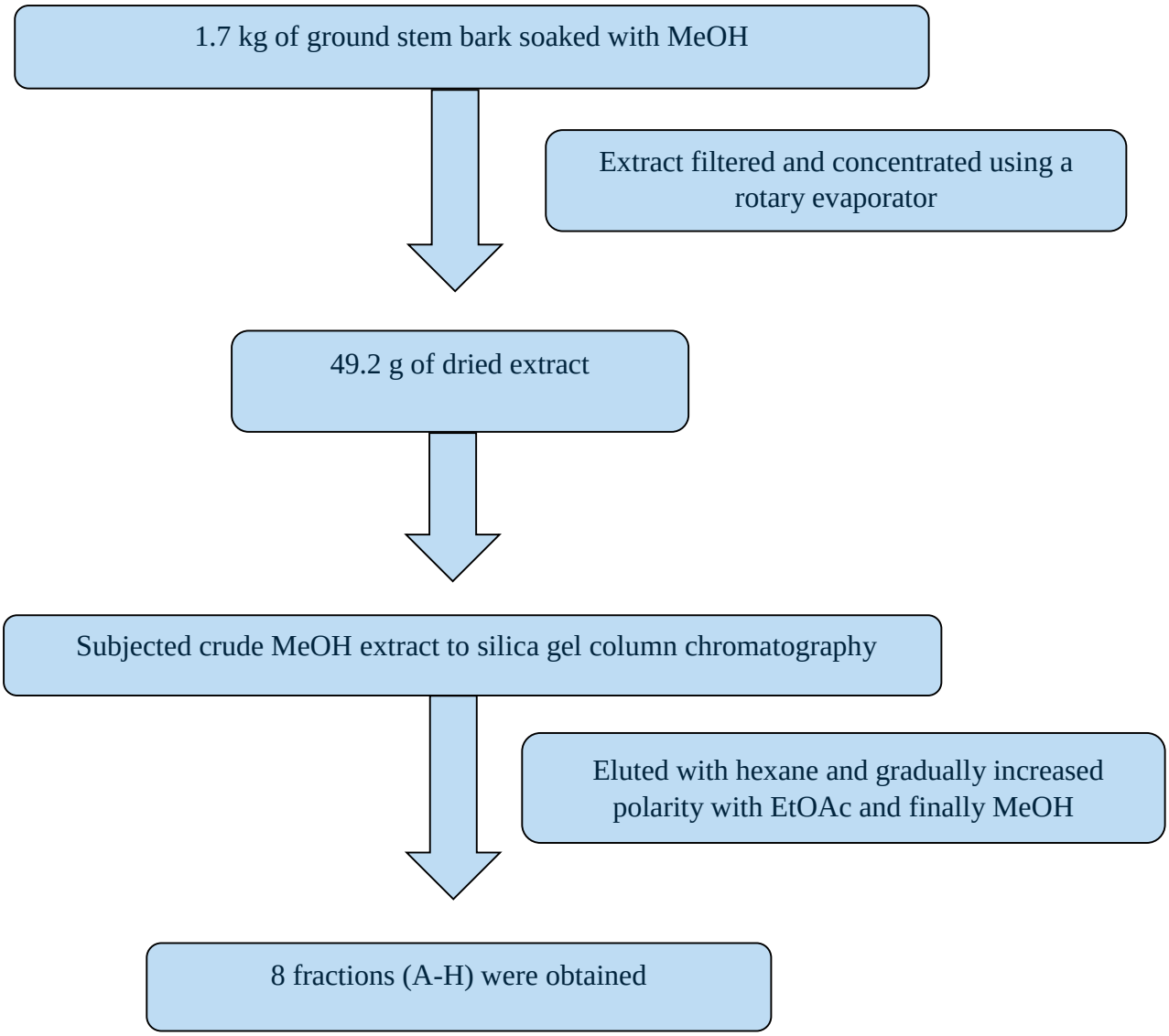


Figure 3.1: Extraction protocol of crude extract.

The crude methanol extract (49.2 g) was subjected to column chromatography over silica gel. The extract was eluted initially with hexane and the polarity was gradually increased with ethyl acetate and finally methanol, yielding 17 fractions. Fractions with similar TLC profile were combined and concentrated to dryness on a rotary evaporator giving a total of 8 fractions coded as F_A-F_H. F_A was obtained with hexane/ethyl acetate (70:30); F_B-F_C were obtained with hexane/ethyl acetate (30:70); F_D was obtained with ethyl acetate (100 %); F_E-F_F were obtained with ethyl acetate/methanol (70:30); and F_G-F_H were obtained with ethyl acetate/methanol (30:70). The collected fractions were monitored on TLC plates.

3.3. Isolation of fractions using column chromatography

Fractions F_B, F_E, F_F and F_G were further fractionated (Figure 3.2) using column chromatography since they contained a large amount of material compared to fractions F_A, F_C, F_D and F_H.

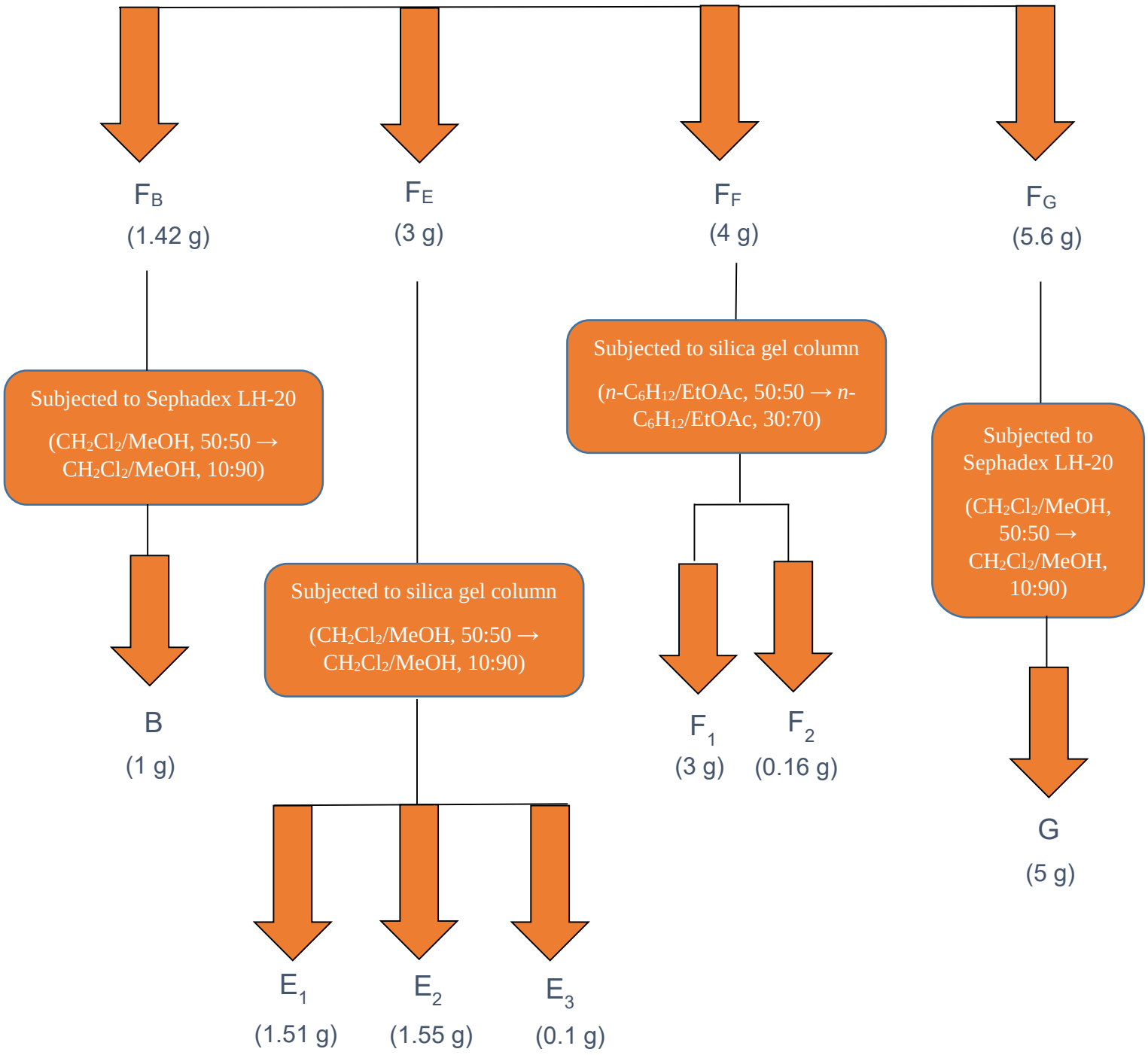


Figure 3.2: Isolation of fractions using column chromatography.

Fraction F_B (1.42 g) was subjected to Sephadex LH-20 column chromatography; the column was eluted with CH_2Cl_2/MeOH (50:50) followed by an increasing gradient of CH_2Cl_2/MeOH (up to 10:90) to obtain fraction B (1 g). Fraction F_E (4 g) was subjected to silica gel column chromatography; the column was eluted using CH_2Cl_2/MeOH (50:50) followed by an increasing gradient of CH_2Cl_2/MeOH (up to 10:90) to obtain 3 subfractions, E_1 (1.51 g), E_2 (1.55 g), and E_3 (0.1 g). Fraction F_F (4 g) was also subjected to silica gel column chromatography and the column was eluted using *n*-C_6H_{12}/EtOAc (50:50) followed by an increasing gradient of *n*-C_6H_{12}/EtOAc (up to 30:70) to obtain 2 subfractions, F_1 (3 g) and F_2 (0.16 g). Fraction F_G (5.6 g) was subjected to Sephadex LH-20 column chromatography; the column was eluted using CH_2Cl_2/MeOH (50:50) followed by an increasing gradient of CH_2Cl_2/MeOH (up to 10:90) to obtain fraction G (5 g). The collected fractions were monitored on TLC plates. The thin layer chromatograms were developed in a solvent system of ethyl acetate/methanol/water (EMW 81:11:8). A natural product staining solution (1 g methanolic diphenylboric acid, 100 mL methanol, 5 mL PEG 400 and 95 mL ethanol) was used to visualize compounds on a TLC plate. The procedure for the isolation of fractions is summarized in Figure 3.2.

3.4. Purification of isolated compounds

Fractions E_3 (0.1 g) and F_2 (0.16 g) were not further purified since they had insufficient amounts of material.

Fraction B (0.5 g) was subjected to preparative TLC (normal phase) to obtain compound **59** (0.230 g). Fraction E_1 (0.5 g) was also subjected to preparative TLC (reversed phase) to obtain compound **60** (0.2102 g). Fraction E_2 (0.5 g) was further purified using semi-preparative HPLC to yield two compounds **61** (0.019 g) and **62** (0.012 g). Fraction F_1 (0.5 g) was also subjected to preparative TLC (reversed phase) to obtain compound **63** (0.1238 g). Fraction G (1 g) was further purified using semi-preparative HPLC to obtain compound **64** (0.086 g). The method for the purification of the isolated compounds is shown in Figure 3.3.

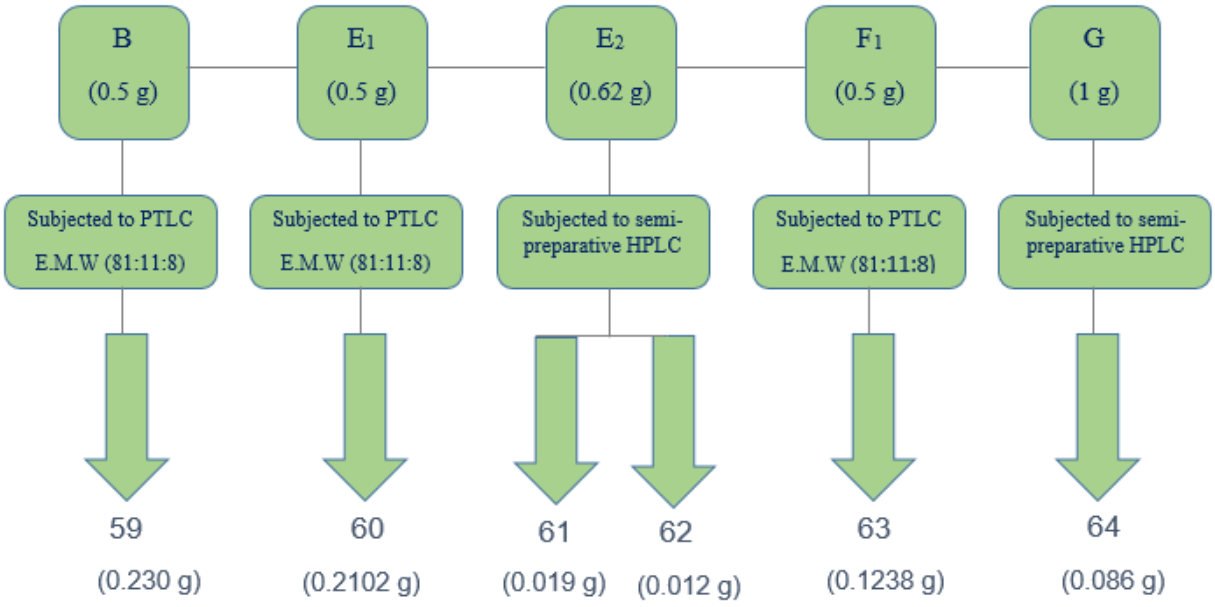


Figure 3.3: Purification of isolated compounds.

3.5. Structure elucidation of isolated compounds

The different interactions of electromagnetic radiation with organic compounds based on their structural features form the basis of the application of spectroscopy in the structural elucidation of organic compounds. The identification of compounds involves a diverse range of analytical techniques and methods such as nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, high resolution mass spectroscopy (HRMS) and ultra-violet liquid chromatography (UPLC-MS). In this study, structure elucidation of the isolated compounds (**59**, **60**, **61**, **62**, **63** and **64**) from *R. caffra* was attempted by a combination of nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and high resolution mass spectrometric (HRMS) analysis. The structures of compounds **59, 61, 63** and **64** were confirmed unambiguously. However, structure elucidation of compound **60** and **62** was not possible due to the lingering presence of impurities in the samples.

3.5.1. General Experimental Procedure

One-dimensional and two-dimensional NMR (^{13}C-NMR and ^{1}H-NMR) experiments were performed using a Bruker Avance 400 MHz NMR spectrometer. CD_3OD and DMSO-d_6 were used as solvents for preparation of the NMR samples. Residual protonated solvent was used as a reference compound for both ^{13}C-NMR and ^{1}H-NMR. Two-dimensional spectra were obtained by recording a series of conventional NMR spectra in which two parameters are changed incrementally. The most readily established connections between the individual carbon atoms were derived from connectivities through couplings between the protons as compiled from a COSY spectrum. From these correlations, the main parts of the proton spin systems were outlined and several structural fragments were identified. Heteronuclear multiple bond correlation (HMBC) experiments provided information on the direct ^{13}C and ^{1}H heteronuclear connectivity. The method relies on the indirect detection of the ^{13}C by observing their effects on the more sensitive proton nuclei to which they are coupled. It not only shows the connection of unprotonated carbon atoms to the proposed elements, but also indicates vicinal proton relationships not resolved in the COSY spectrum.

Fourier-transform infra-red (FTIR) spectra were recorded on a Bruker Alpha Fourier Transform IR spectrometer, with absorption maxima reported in terms of wavenumbers (cm^{-1}). The high-resolution mass spectrometry (HRMS) were measured on a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC).

Column chromatography was prepared by using Silica Gel 60F, 70-230 mesh and Sephadex (LH-20). Analytical Thin Layer Chromatography (TLC) was performed on commercially aluminium supported silica gel 60 pre-coated TLC sheets. Further purification of isolated compounds was performed on reverse phase preparative TLC (RP-18W/UV_{254}) and normal phase preparative TLC (SIL G-100 UV_{254}).

The multiplicities of the signals are characterized using the following notation: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet and m = multiplet.

3.5.2. Spectroscopic data

3.5.2.1. 3β-Lup-20(29)-en-3-ol (59)

Lupeol (**59**): White powder

^{1}H-NMR (400 MHz, CD_3OD): δ_H 4.7 (1H, s, 29a-H), 4.6 (1H, s, 29b-H), 3.18 (1H, m, 3-H), 2.38 (1H, m, 19-H), 1.91 (1H, m, 21-H), 1.70 (3H, s, 30-H), 1.60 (1H, m, 2-H), 1.39 (1H, m, 18-H), 1.30 (1H, m, 9-H), 1.04 (3H, s, 26-H), 1.0 (3H, s, 23-H), 0.97 (3H, s, 27-H), 0.86 (3H, s, 25-H), 0.77 (3H, s, 24-H) ppm.

^{13}C-NMR (100 MHz, CD_3OD): δ_C 150.95 (S, C-20), 108.54 (T, C-29), 78.27 (D C-3), 55.48 (D, C-5), 50.64 (D, C-9), 49.16 (D, C-18), 42.17 (S, C-14), 40.53 (S, C-8), 38.67 (S, C-4), 38.54 (T, C-1), 38.17 (D, C-13), 37.00 (S, C-10), 36.91 (T, C-16), 34.21 (T, C-7), 30.43 (T, C-21), 29.52 (Q, C-23), 29.27 (T, C-2), 27.19 (T, C-15), 25.54 (T, C-12), 22.79 (T, C-11), 20.70 (Q, C-30), 18.17 (T, C-6), 18.03 (Q, C-28), 15.32 (Q, C-26), 14.69 (Q, C-24), 13.67 (Q, C-27) ppm.

HRMS: *m/z* 455.3633 $[M+H]^+$.

IR: ν_{max} (KBr): 3000 (O-H) cm^{-1}.

3.5.2.2. (16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate (61)

Raucaffricine (**61**): brown amorphous solid

^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 7.55 (1H, d, *J* = 7.6 Hz, 9-H), 7.49 (1H, d, *J* = 7.2 Hz, 12-H), 7.39 (1H, dd, *J* = 7.6 Hz and *J* = 7.6 Hz, 10-H), 7.23 (1H, dd, *J* = 7.2 Hz and *J* = 7.6 Hz, 11-H), 5.65 (1H, q, *J* = 6.8 Hz, 19-H), 5.17 (1H, s, 21-H), 5.03 (1H, d, *J* = 4.4 Hz, 1'-H), 4.57 (1H, s, 17-H), 4.42 (2H, dd, *J* = 7.6 Hz and *J* = 8.8 Hz, 6'-H), 3.9-3.6 (1H, m, 5'-H), 3.18 (1H, dd, *J* = 5.6 Hz and *J* = 6 Hz, 5-H), 3.08 (1H, m, 15-H), 2.68 (1H, dd, *J* = 4.8 Hz and 4.4 Hz, 6-H_β), 2.34 (1H, dd, *J* = 6 and *J* = 6.4 Hz, 16-H), 2.16 (3H, s, $OCOCH_3$), 1.87 (1H, dd, *J* = 4 Hz and *J* = 12 Hz, 14-H_α), 1.77 (1H, dd, *J* = 4.8 Hz and *J* = 4.8 Hz, 14-H_β), 1.67 (3H, d, *J* = 11.6 Hz, 18-CH_3), 1.45 (1H, d, *J* = 6.8 Hz, 6-H_α) ppm.

^{13}C-NMR (100 MHz, DMSO-d_6): δ_C 184.42 (S, C-2), 170.13 (S, C-22), 156.86 (S, C-13), 137.94 (S, C-20), 137.09 (S, C-8), 127.06 (D, C-11), 125.81 (D, C-10), 124.29 (D, C-9), 122.76 (D, C-19), 120.85 (D, C-12), 99.32 (D, C-1'), 88.22 (D, C-21), 77.48 (D, C-3',5'), 77.21 (D, C-17), 74.26 (D, C-2'), 70.58 (D, C-4'), 65.19 (D, C-7), 61.56 (T, C-6'), 55.24 (T, C-5), 50.45 (D, C-3), 48.49 (D, C-16), 37.32 (T, C-6), 27.47 (D, C-15), 24.67 (T, C-14), 21.32 (Q, C-23), 13.28 (Q, C-18) ppm.

HRMS: *m/z* 513.2241 $[M+H]^+$

IR: ν_{max} (KBr) 3421.51 (OH), 1662.02 (C=O) cm^{-1}.

3.5.2.3. 10,17-dihydroxy-1,4-dimethylsarpaganium (63)

N-methylsarpagine (**63**): brown amorphous solid.

^{1}H-NMR (400 MHz, CD_3OD): δ_H 7.11 (1H, d, *J* = 8.6 Hz, 12-H), 6.77 (1H, d, *J* = 2.0 Hz, 9-H), 6.68 (1H, dd, *J* = 8.9, 2.2 Hz, 11-H), 5.59 (1H, q, *J* = 6.8 Hz, 19-H), 4.41 (1H, d AB, *J* = 15.6 Hz, 21-H$_\alpha$), 4.16 (1H, d AB, *J* = 15.6 Hz, 21-H$_\beta$), 3.48 (2H, d, *J* = 7.2 Hz, 17-H), 3.28 (3H, s, N-CH_3), 3.15 (1H, dd, *J* = 12.4, 4.8 Hz, 6-H$_\beta$), 3.0 (3H, s, N^+-CH_3), 2.95 (1H, dd, *J* = 2.0, 10.4 Hz, 15-H), 2.9 (1H, d, *J* = 15.2 Hz, 6-H$_\alpha$), 2.4 (1H, dd, *J* = 11.6, 10.8 Hz, 14-Hα), 2.12-2.0 (2H, m, 16-H + 14-H$_\beta$), 1.82 (s, OH), 1.63 (3H, d, *J* = 6.7 Hz, 18-H) ppm.

^{13}C-NMR (100 MHz, CD_3OD): δ_C 150.91 (S, C-10), 132.10 (S, C-20), 131.68 (S, C-13), 127.67 (S, C-8), 126.83 (S, C-2), 120.72 (D, C-19), 112.34 (D, C-12), 111.75 (D, C-11), 102.14 (D, C-9), 99.77 (S, C-7), 65.45 (D, C-5), 64.39 (T, C-17), 62.40 (T, C-21), 61.07 (D, C-3), 43.63 (D, C-16), 32.01 (T, C-14), 26.01 (D, C-15), 23.87 (T, C-6), 11.57 (Q, C-18) ppm.

HRMS: *m/z* 369.1750 $[M+H]^+$.

IR: ν_{max} (KBr): 3312.90 (O-H), 2942.44 (C-H), 2831.65 (C-H) cm^{-1}.

3.5.2.4. (15α,19*E*)-10,17-dihydroxy-4-methylsarpagan-4-ium (64)

Spegatrine (**64**): brown amorphous solid.

^{1}H-NMR (400 MHz, CD_3OD): δ_H 7.24 (1H, d, *J* = 8.8 Hz, 12-H), 6.90 (1H, d, *J* = 2.0 Hz, 9-H), 6.78 (1H, dd, *J* = 2.2, 8.8 Hz, 11-H), 5.68 (1H, q, *J* = 6.8 Hz, 19-H), 4.45 (1H, d AB, *J* = 15.6 Hz, 21-H_α), 4.23 (1H, d AB, *J* = 15.6 Hz, 21-H_β), 3.58 (2H, d, *J* = 7.6 Hz, 17-H), 3.27 (1H, dd, *J* = 12.4, 4.8 Hz, 6-H_β), 3.15 (3H, s, N^+-CH_3), 3.12 (1H, dd, *J* = 2.0, 10.4 Hz, 15-H), 3.04 (1H, d, *J* = 17.2 Hz, 6-H_α), 2.54 (1H, dd, *J* = 11.6, 10.8 Hz, 14-Hα), 2.23-2.13 (2H, m, 16-H + 14-H_β), 1.97 (s, OH), 1.74 (3H, d, *J* = 6.8 Hz, 18-H) ppm.

^{13}C-NMR (100 MHz, CD_3OD): δ_C 150.93 (S, C-10), 132.10 (S, C-20), 131.66 (S, C-13), 127.68 (S, C-8), 126.83 (S, C-2), 120.71 (D, C-19), 112.35 (D, C-12), 111.73 (D, C-11), 102.13 (S, C-7), 99.77 (D, C-9), 65.46 (D, C-5), 64.38 (T, C-21), 62.40 (T, C-17), 61.08 (D, C-3), 46.67 (Q, N^+- CH_3), 43.62 (D, C-16), 32.00 (T, C-14), 26.01 (D, C-15), 23.87 (T, C-6), 11.56 (Q, C-18) ppm.

HRMS: *m/z* 325.1912 $[M+H]^+$

IR: ν_{max} (KBr): 3352.1 (O-H), 1638.8 (N-H) cm^{-1}.

3.6. Bioactivities of *Rauvolfia caffra*

3.6.1. Determination of the content of polyphenol compounds

From the crude extract and fractions (B, E_1, E_2, F_1 and G), a 10 mg/mL of DMSO/water (80:20) solution was prepared to be used in the testing.

3.6.1.1. Total phenolic content (TPC)

This method was executed as described by Singleton and Rossi.[95] The 96-well plates were filled with 80 µL of water, thereafter 100 µL of the crude extract and each fractions (B, E_1, E_2, F_1 and G) were added in triplicates. Twenty microlitres of 10 % Folin-Ciocalteau reagent and 60 µL of 7 % sodium carbonate (Na_2CO_3) solution were added to the mixture respectively. The absorbance was read at 760 nm using VersaMax™ tuneable microplate reader. Gallic acid standard curve was used to convert the absorbance of the crude extract and fractions to total phenolic content expressed in milligram gallic acid equivalent per gram of the crude extract and fractions (mg GAE/G).

3.6.1.2. Total flavonoid content (TFC)

Flavonoid content was quantified as described by Boulanonuar *et al*.[96] The 96-well plates were filled with 80 µL of water per well, then 100 µL of the crude extract and each fraction (B, E_1, E_2, F_1 and G) was added in triplicate. Hundred microlitres of 2 % aluminium chloride ($AlCl_3$) solution was added to the mixture, and the mixture was allowed to stand at room temperature for 1 hour. The absorbance was read at 420 nm using a VersaMax™ tuneable microplate reader. Quercetin was used as standard to convert the absorbance of the crude extract and fractions (B, E_1, E_2, F_1 and G) to total flavonoid content expressed in milligram quercetin equivalent per gram of the extract (mg QE/g).

3.6.2. Antioxidant activities

For determination of antioxidant and reducing power the concentration of the crude extract, fractions (B, E_1, E_2 F_1 and G) and compound **64** was 5 mg/mL of DMSO/water (80:20).

3.6.2.1. Free radical scavenging assay (DPPH)

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl) method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When antioxidants react with DPPH, which is a stable free radical, it becomes paired off in the presence of a hydrogen donor (e.g. a free radical scavenging antioxidant) and is reduced to the DPPH-H. The absorbance is reduced significantly from the DPPH radical to the DPPH-H form, resulting in decoloration (yellow colour) with respect to the number of electrons captured. The amount of decoloration gives a quantitative indication of the reducing ability. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, this gives rise to the reduced form (diphenylpicrylhydrazine; non radical) with the loss of this violet colour.[97]

The DPPH free radical scavenging ability of the crude extract and fractions B, E_1, E_2, F_1 and G was determined according to the spectrophotometric method of Anokwuru *et al.*[98] A 125 mM DPPH/methanol solution was prepared by dissolving 10 mg DPPH in 200 ml of methanol. The 96-well plates were filled with 100 µL of distilled water per well. One hundred µL of the crude extract, fractions B, E_1, E_2, F_1 and G, compound **64** and 100 µL methanol were added in triplicate into the first three wells followed by serial dilution. Two hundred µL of 0.3 M DPPH/methanol solution was added to each well containing the mixtures. The 96-well plate was kept in the dark for 30 minutes and the absorbance was measured using a VersaMax™ tuneable microplate reader at 517 nm.

The percentage radical scavenging was calculated by the following formula:

$$\% \text{ Free radical scavenging activity} = \frac{\text{Absorbance of DPPH} - \text{Absorbance of sample}}{\text{Absorbance of DPPH}} \times 100$$

3.6.2.2. Reducing power

Substances with reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric ferrous complex that has an absorption maximum at 700 nm.

The reducing power was determined according to the method of Anokwuru *et al.*[98] A 0.2 M (pH 6.6) sodium phosphate buffer (50 µL) and 50 µL of the crude extract, fractions B, E_1, E_2, F_1 and G, and compound **64** were added in triplicate in the first three wells of a 96-well plate, followed by serial dilution. Fifty µL of a 1 % aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution was added to each well.

The 96-well plates were placed in an incubator for 20 minutes at 50 °C. After incubation, 50 µL of 10 % trichloroacetic acid solution was added to each well. Eighty µL of each mixture was transferred to another 96-well plate and 80 µL of distilled water, then 16 µL ferric chloride (0.1 % w/v) was added. Absorbance was measured using a VersaMax™ tuneable microplate reader at 700 nm.

3.6.3. Antimicrobial activity

The concentration of the crude extract and each fraction (B, E_1, E_2, F_1 and G) used was 200 mg/mL.

3.6.3.1. Test microorganisms

The microorganisms *Staphylococcus aureus* (gram-positive, ATCC 25923); *Escherichia coli* (gram-negative, ATCC 25922); *Enterococcus faecalis* (gram-positive, ATCC 29212) and *Bacillus cereus* (gram-positive, ATCC 10876) were obtained from the University of Venda, Department of Microbiology. They were cultured in a petri dish containing nutrient agar. Before testing, a 0.5 McFarland standard was prepared and used in the micro-dilution assay.

3.6.3.2. Determination of the minimal inhibitory concentration (MIC)

The two-fold serial dilution microplate method of Eloff[99] was used with some modification to determine the MIC values of the crude extract and fractions B, E_1, E_2, F_1 and G. One hundred 100 µL brain-heart infusion medium (BHI) was added in all the wells of a 96-well plate microplate, followed by addition of 40 µL of the crude extract and fractions B, E_1, E_2, F_1 and G in all the wells of the first row. A two-fold serial dilution was performed in triplicate. Then 100 µL of medium brain-heart infusion medium (BHI) was added in all wells, followed by an addition of 100 µL of the microorganisms.

The plates were incubated at 37 °C for 24 hours. After incubation, 40 µL of iodo-nitro-tetrazolium (INT) was added in each well. Gentamicin®, a standard antibacterial drug was used as a positive control and sterile deionised water was used as a negative control. Results were read after 30 minutes. They were observed by the colour change, and the MIC was recorded as the lowest concentration of the crude extract and fractions that inhibited visible growth.[99]

3.6.4. Cytotoxic activity

To assess the overt cytotoxicity of the compounds, they were incubated at a fixed concentration of 20 µM for pure compounds (**61**, **62** and **64**) and 50 µg/mL for crude extract and fractions B, E_1, E_2, F_1 and G in 96-well plates containing HeLa (human cervix adenocarcinoma) cells for 48 hours. The numbers of cells surviving drug exposure were determined using the resazurin based reagent and reading resorufin fluorescence in a multiwell plate reader. (All samples were tested at Rhodes University).

3.6.5. Antiplasmodial activity

Malaria parasites (*Plasmodium falciparum* chloroquine-sensitive strain 3D7) were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 25 mM Hepes (Lonza). The medium was further supplemented with 5 % Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 µg/mL gentamycin and 2-4 % hematocrit human red blood cells. The parasites were cultured at 37 ^{o}C under an atmosphere of 5 % CO_2, 5 % O_2 and 90 % N_2 in sealed T25 or T75 culture flasks. For screening compounds against malaria parasites, 20 µM for pure compounds (**61**, **62** and **64**) or 25 µg/mL for the crude extract and fractions B, E_1, E_2, F_1 and G were added to parasite cultures in 96-well plates and incubated for 48 h in a 37 ^{o}C CO_2 incubator. After 48 h the plates were removed from the incubator. Twenty µL of culture was removed from each well and mixed with 125 µL of a mixture of Malstat solution and NBT/PES solution in a fresh 96-well plate. These solutions measured the activity of the parasite lactate dehydrogenase (pLDH) enzyme in the cultures. A purple product was formed when pLDH was present, and this product was quantified in a 96-well plate reader by absorbance at 620 nm (Abs_{620}). The Abs_{620} reading in each well was thus an indication of the pLDH activity in that well and also the number of parasites in that well. (All samples were tested at Rhodes University).

3.6.6. Antitrypanosomal activity

Trypanosoma brucei (T. b.) parasites are the causative agents of African sleeping sickness (human African trypanosomiasis) in humans and Nagana (animal African trypanosomiasis) in cattle. The subspecies responsible for Nagana (*T. b. brucei*) is not infective to humans and is commonly used for drug screening.

To assess trypanocidal activity, compounds were added to *in vitro* cultures of *T. b. brucei* in 96-well plates at a fixed concentration of 20 µM for pure compounds (**61**, **62** and **64**) or 25 µg/mL for the crude extract and fractions B, E_1, E_2, F_1 and G. After an incubation period of 48 hours, the numbers of parasites surviving drug exposure was determined by adding a resazurin based reagent. The reagent contained resazurin which was reduced to resorufin by living cells. Resorufin is a fluorophore (Exc_{560}/Em_{590}) and can thus be quantified in a multiwell fluorescence plate reader. (All samples were tested at Rhodes University).

3.6.7. Single concentration screening

The % cell viability at the concentration of the crude extract, fractions B, E_1, E_2, F_1 and G, and compounds **61**, **62** and **64** was calculated. The compounds were tested in triplicate wells, and a standard deviation (SD) was derived. For comparative purposes, chloroquine (an anti-malarial drug), pentamidine (an existing drug for treatment of trypanosomiasis) and emetine (which induces cell apoptosis) were used as a positive control drug standard for antimalarial, antitrypanosomal and cytotoxicity activities, respectively.

3.6.8. Dose-response relationships

The percentage viability of the crude extract, fractions B, E_1, E_2, F_1 and G, and compounds **61**, **62** and **64**, was plotted against log (compound concentration) and the IC_{50} (50 % inhibitory concentration) obtained from the resulting dose-response curve by non-linear regression. For comparative purposes, chloroquine, pentamidine and emetine were used as a drug standard according to the type of test performed, and as a result they yielded IC_{50} values in the range 0.001-0.05 µM.

3.6.9. Statistical analysis

The collected data were entered on an Excel spreadsheet and statistical analysis was done using the SPSS package. The absorbance and reduced concentrations were used to calculate the linear regression. One-way analysis of variance (ANOVA) was used to compare mean values of the crude extract, fractions B, E_1, E_2, F_1 and G, and compound **64** obtained in each test (antioxidant and content of polyphenol), $p < 0.05$ was considered statistically significant.

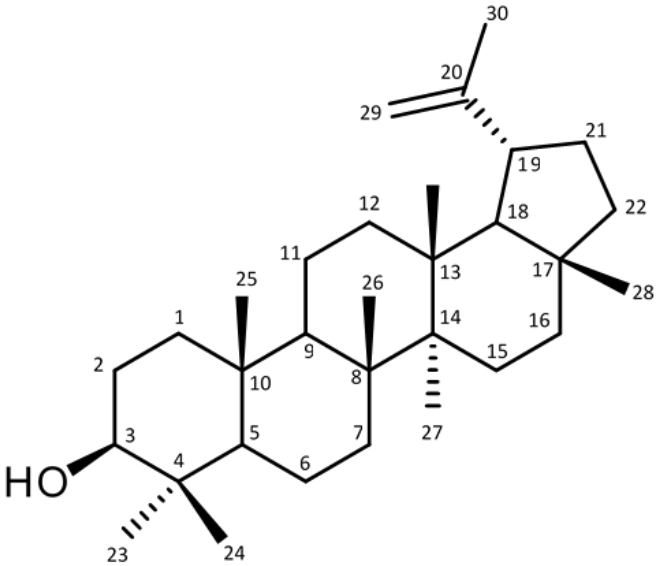
Chapter 4

Results and Discussion

This chapter discusses in detail the structures of compounds isolated by column chromatography from the crude methanol extracts of *R. caffra*. The polyphenol content, antioxidant, antimicrobial, antiplasmodial, antitrypanosomal and cytotoxic properties of the crude extract, fractions B, E_1, E_2, F_1 and G, and compounds **61**, **62** and **64** are also discussed and supported by the relevant literature. The conclusions of this chapter not only demonstrate the success of the methodology employed, but also show the shortcomings of our experimental procedures. Recommendations for future research are also suggested.

4.1. Structure Elucidation

4.1.1. 3β-Lup-20(29)-en-3-ol (59)



59

The HRMS spectrum (Appendix 1) showed a $[M+H]^+$ ion peak at *m/z* 455.3633 matched with the molecular formula of $C_{30}H_{50}O$. The IR spectrum (Appendix 2) of lupeol (**59**) showed peaks corresponding to hydroxyl (3000 cm^{-1}, O-H).

The ^{1}H-NMR spectrum (Appendix 3B) of lupeol (**59**) revealed the presence of six tertiary methyl protons at δ_H 0.77 ppm (3H, s), δ_H 0.86 ppm (3H, s), δ_H 0.97 ppm (3H, s), δ_H 1.0 ppm (3H, s), δ_H 1.04 ppm (3H, s) and δ_H 1.70 ppm (3H, s), corresponding to protons at positions 24, 25, 27, 23, 26 and 30, respectively. A multiplet of one proton at δ_H 2.38 ppm (1H, m) assigned to 19-H is characteristic of lupeol. The 3-H proton displayed a multiplet at δ_H 3.18 ppm (1H, m) while a pair of broad singlets at δ_H 4.7 ppm (1H, s) and δ_H 4.6 ppm (1H, s) was indicative of olefinic protons at (29a-H and 29b-H), (Appendix 3A). These assignments are in good agreement for the structure of lupeol (**59**).

The structural assignment of lupeol (**59**) was further confirmed by the ^{13}C-NMR (Appendix 4A-C) and DEPT 135 (Appendix 5 A and B) experiments which exhibited six methyl carbons at δ_C 29.52 (Q, C-23), 20.70 (Q, C-30), 18.03 (Q, C-28), 15.32 (Q, C-26), 14.69 (Q, C-24) and 13.67 (Q, C-27); the signals due to an exomethylene group at δc 108.54 (T, C-29) and 151.95 (S, C-20); eleven methylenes at δ_C 108.54 (T, C-29), 38.71 (T, C-22), 38.54 (T, C-1), 36.91 (T, C-16), 34.21 (T, C-7), 30.43 (T, C-21), 29.27 (T, C-2), 27.19 (T, C-15), 25.54 (T, C-12), 22.79 (T, C-11) and 18.17 (T, C-6); five methines at δ_C 78.27 (D, C-3), 55.48 (D, C-5), 50.64 (D, C-9), 49.16 (D, C-18) and 38.17 (D, C-13); and five quaternary carbons at δ_C 150.95 (S, C-20), 42.17 (S, C-14), 40.53 (S, C-8), 38.67 (S, C-4) and 37.00 (S, C-10). The deshielded signal at δc 78.27 was due to C-3 with a hydroxyl group attached to it.

In the HMBC spectrum (Appendix 6), the methyl proton peak at 23-H (δ_H 1.0) showed correlations with C-3 (δ_C 78.27), C-4 (δ_C 38.67), C-5 (δ_C 55.48) and C-24 (δ_C 14.69). In addition, the HMBC spectrum (Appendix 6) also revealed correlations of methyl proton signals at 24-H (δ_H 0.77) with C-3 (δ_C 78.27), C-4 (δ_C 38.67), C-5 (δ_C 55.48) and C-23 (δ_C 29.52). The pair of broad singlets of olefinic proton at 29b-H (δ_H 4.6) and 29a-H (δ_H 4.7) showed correlations with a methyl carbon signal C-30 (δc 20.70). Furthermore, the HMBC spectrum (Appendix 6) showed correlations of 30-H (δ_H 1.70) and 26-H (δ_H 15.32) with C-21 (δ_C 30.43) and C-9 (δ_H 50.64) and C-14 (δ_H 42.17), respectively (See Scheme 1).

The HSQC spectrum (Appendix 7) exhibited correlations between 29a-H (δ_H 4.7) and 29b-H (δ_H 4.6) with C-29 (δ_C 108.54); 3-H (δ_H 3.18) with C-3 (δ_C 78.27) and C-18 (δ_C 49.16) and 27-H (δ_H 0.97) with C-23 (δ_C 29.52). Moreover, the HSQC spectrum (Appendix 8) indicated the presence of five quaternary carbons at δ_C 150.95 (S, C-20), 42.17 (S, C-14), 40.53 (S, C-8), 38.67 (S, C-4) and 37.00 (S, C-10) which is in agreement with DEPT 135 (Appendix 5) spectra.

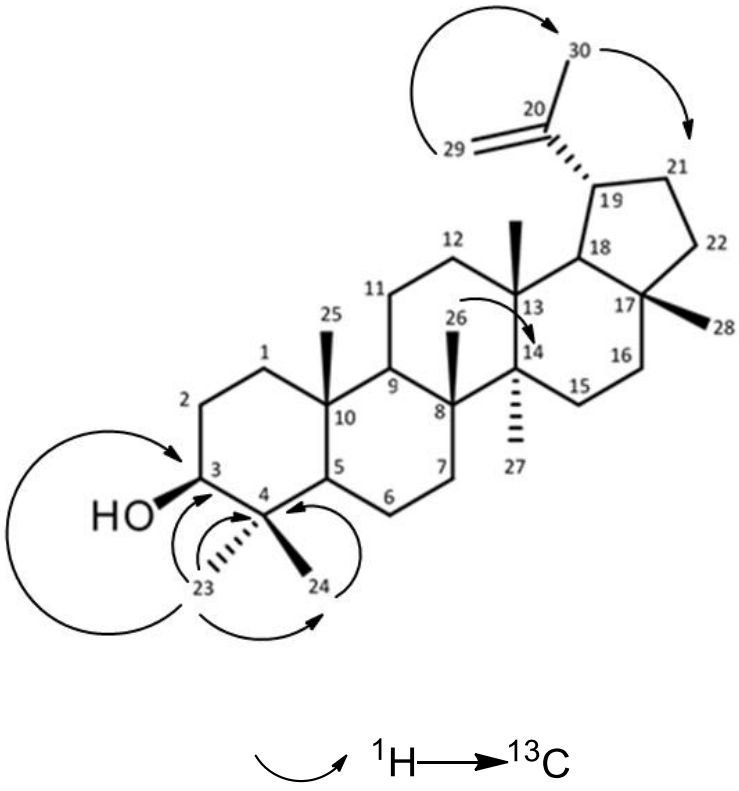
The COSY spectrum (Appendix 8) of lupeol (**59**) revealed some cross signals between 19-H (δ_H 2.38) and one methylene proton signal 21-H (δ_H 1.91) and another methine proton signal 18-H (δ_H 1.39); and between oxygenated methine proton signal 3-H (δ_H 3.18) and methylene signal 2-H (δ_H 1.60).

Finally, data comparison with the literature confirmed the isolation of lupeol (**59**), a pentacyclic triterpenoid which was previously isolated from *Wrightia tinctoria* and *Centaurea omphalotricha* (As shown in Table 1). This is the first report of the isolation of lupeol from *R. caffra*.[100]

Table 1: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of lupeol (**59**) in CD_3OD (δ in ppm, *J* in Hz)

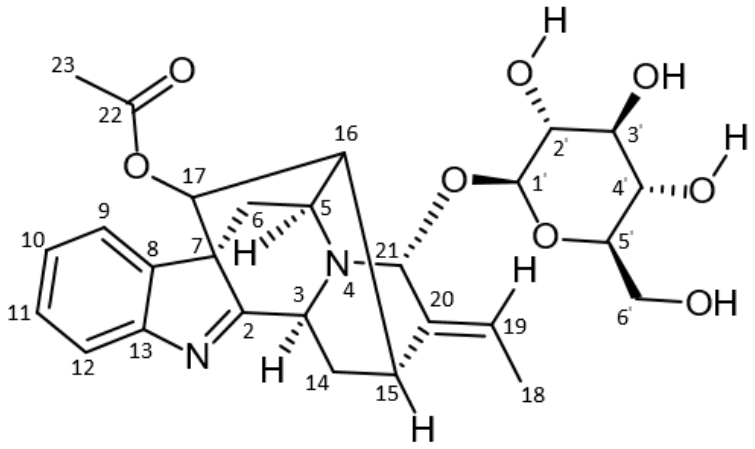
Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)	^{13}C-NMR (δ ppm) (Mouffok *et al.*, 2012)
1	-	38.54 (CH_2)	38.7 (CH_2)
2	1.60 (1H, m)	29.27 (CH_2)	27.5 (CH_2)
3	3.18 (1H, m)	78.27 (CH)	79.3 (CH)
4	-	38.67 (C)	39.8 (C)
5	-	55.48 (CH)	55.5 (CH)
6	-	18.17 (CH_2)	19.0 (CH_2)
7	-	34.21 (CH_2)	34.2 (CH_2)
8	-	40.53 (C)	41.1 (C)
9	1.30 (1H, m)	50.64 (CH)	50.9 (CH)
10	-	37.00 (C)	37.2 (C)
11	-	22.79 (CH_2)	21.2 (CH_2)
12	-	25.54 (CH_2)	25.3 (CH_2)
13	-	38.17 (CH)	38.5 (CH)

14	-	42.17 (C)	42.8 (C)
15	-	27.19 (CH_2)	27.2 (CH_2)
16	-	36.91 (CH_2)	35.9 (CH_2)
17	-	-	43.2 (C)
18	1.39 (1H, m)	49.16 (CH)	48.5 (CH)
19	2.38 (1H, m)	-	47.8 (CH)
20	-	150.95 (C)	151.2 (C)
21	1.91 (1H, m)	30.43 (CH_2)	30.1 (CH_2)
22	-	-	40.3 (CH_2)
23	1.0 (3H, s)	29.52 (CH_3)	28.4 (CH_3)
24	0.77 (3H, s)	14.69 (CH_3)	15.6 (CH_3)
25	0.86 (3H, s)	-	16.2 (CH_3)
26	1.04 (3H, s)	15.32 (CH_3)	16.1 (CH_3)
27	0.97 (3H, s)	13.67 (CH_3)	14.8 (CH_3)
28	-	18.03 (CH_3)	18.1 (CH_3)
29	4.7(1H, s, 29a-H) & 4.6 (1H, s, 29b-H)	108.54 (CH_2)	109.5 (CH_2)
30	1.70 (3H, s)	20.70 (CH_3)	19.8 (CH_3)



Scheme 1: The HMBC correlations of lupeol (**59**)

4.1.2. (16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate (61)



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Raucaffricine (**61**) was identified mainly by ^{1}H-NMR, ^{13}C-NMR and MS analysis. In the HRMS the molecular ion peak appeared at *m/z* 513.2241 $[M+H]^{+}$ (see Appendix 9), which is consistent with the molecular formula $C_{27}H_{32}N_2O_8$. Evidence of the presence of 27 carbons is in good agreement with the ^{13}C-NMR spectrum (Appendix 10). The IR spectrum (Appendix 11) suggested the presence of a strongly H-bonded OH group with ν_{O-H} at 3421.5 cm^{-1} as well as a carbonyl group with $\nu_{C=O}$ at 1662.0 cm^{-1}.

The ^{1}H-NMR spectrum (Appendix 12A) of raucaffricine (**61**), showed an *ortho*-disubstituted aromatic ring with δ_H 7.57 to 7.2 ppm, which included two sets of doublets at δ_H 7.55 and 7.49 ppm corresponding to protons at positions 9 and 12, a doublet of doublets at δ_H 7.39 ppm corresponding to a proton at position 10 and another doublet of doublets at δ_H 7.23 ppm corresponding to a proton at position 11. The spectrum also showed the existence of an ethylidene group with a doublet at δ_H 1.67 ppm assigned to position 18 and a quartet at δ_H 5.65 ppm assigned to a proton at position 19.

The ^{1}H-NMR spectrum (Appendix 12 B and C) also revealed several other important features, including one methyl signal at δ_H 2.16 (s) corresponding to $OCOCH_3$ and three methylene signals at δ_H 4.42 ppm, δ_H 1.87 and δ_H 1.77 ppm, and δ_H 2.68 and δ_H 1.45 ppm, corresponding to 6'-H, 14-H (alpha and beta), and 6-H (beta and alpha), respectively. Moreover, a glucopyranosyl moiety was indicated by the signal of the anomeric proton at δ_H 5.03 (1H, d, J = 4.4 Hz, 1′-H) and in agreement with carbon signals at δ_C 99.32 (CH, C-1'), 77.48 (CH, C-3', C-5'), 74.26 (CH, C-2'), 70.58 (CH, C-4') and 61.56 (CH_2, C-6').

The ^{13}C-NMR (Appendix 10) and DEPT 135 (Appendix 13) spectra indicated the presence of six quaternary carbons at δ_C 184.42 (S, C-2), 170.13 (S, C-22), 156.86 (S, C-13), 137.94 (S, C-20), 137.09 (S, C-8) and 65.19 (S, C-7); fifteen methines: δ 127.06 (D, C-11), 125.81 (D, C-10), 124.29 (D, C-9), 122.76 (D, C-19), 120.85 (D, C-12), 99.32 (D, C-1'), 88.22 (D, C-21), 77.48 (D, C-3',5'), 77.21 (D, C-17), 74.26 (D, C-2'), 70.58 (D, C-4'), 50.45 (D, C-3), 48.49 (D, C-16), and 27.47 (D, C-15); four methylenes: 61.56 (T, C-6'), 55.24 (T, C-5), 37.32 (T, C-6), and 24.67 (T, C-14); and two methyl groups at δ 21.32 (Q, C-23) and 13.28 (Q, C-18), respectively.

The HMBC (Appendix 14) spectrum revealed evidence for the presence of an *ortho*-disubstituted aromatic ring with correlations between 10-H (δ_H 7.39) and C-9 (δ_C 124.29), 11-H (δ_H 7.23) and C-12 (δ_C 120.85), 9-H (δ_H 7.55) and C-8 (δ_C 137.09), 12-H (δ_H 7.49) and C-13 (δ_C 156.86), 10-H (δ_H 7.39) and C-13 (δ_C 156.86), 11-H (δ_H 7.23) and C-13(δ_C 156.86) and 11-H (δ_H 7.23) and C-8 (δ_C 137.09), respectively. Furthermore, the HMBC spectrum (Appendix 14) displayed correlations between $OCOCH_3$ (δ_H 2.16) and C-22 (δ_C 170.13). The existence of the ethylidene group was confirmed by correlations between 19-H (δ_H 5.65) and C-18 (δ_C 13.28), 19-H (δ_H 5.65) and C-15 (δ_C 27.47) and 19-H (δ_H 5.65) and C-21 (δ_C 88.22) (See Scheme 2).

The HSQC spectrum (Appendix 15) also confirmed the existence of the aromatic ring by the correlations between 9-H (δ_H 7.55) and C-12 (δ_C 120.85), 12-H (δ_H 7.49) and C-9 (δ_C 124.29), 10-H (δ_H 7.39) and C-11 (δ_C 127.06), and 11-H (δ_H 7.23) and C-10 (δ_C 125.81), respectively. Moreover, one methyl signal at δ_H 2.16 (s) corresponding to $OCOCH_3$ couples with C-23 (δ_C 21.32).

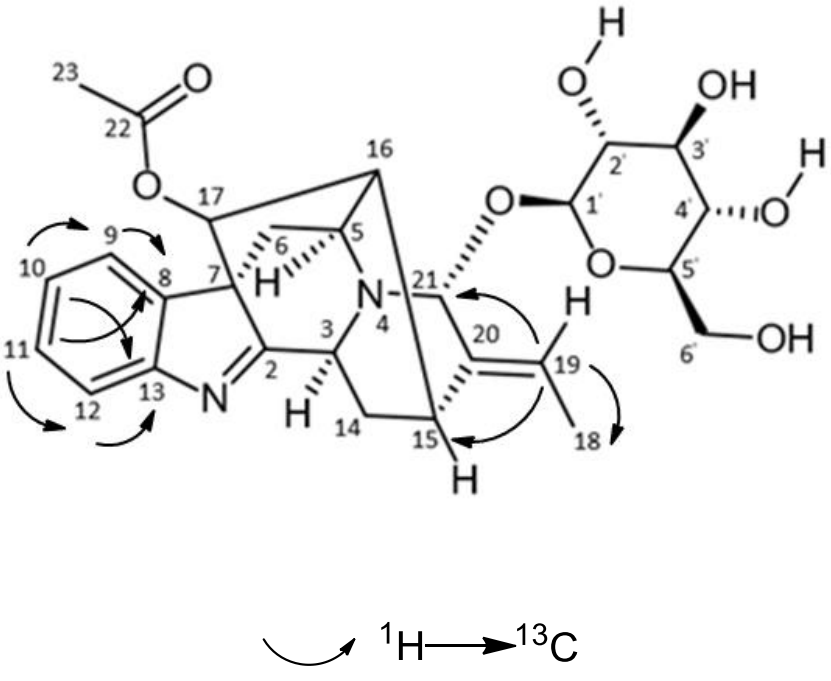
The HSQC spectrum (Appendix 15) indicated the presence of six quaternary carbons at δ_C 184.42 (S, C-2), 170.13 (S, C-22), 156.86 (S, C-13), 137.94 (S, C-20), 137.09 (S, C-8) and 65.19 (S, C-7) which is in agreement with DEPT 135 (Appendix 13) spectra.

Finally, data comparison with the literature confirmed the isolation of raucaffricine (**61**), one of the rare glycoalkaloids of the monoterpenoid indole series which was previously isolated from *Rauvolfia caffra* Sond (As shown in Table 2).[101]

Table 2: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of raucaffricine (**61**) in DMSO-d_6 (δ in ppm, *J* in Hz)

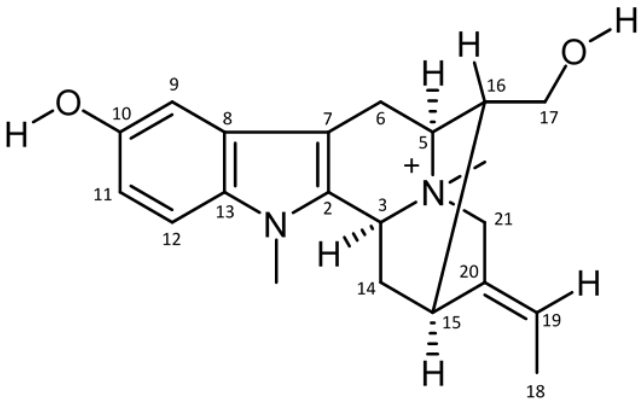
Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)	^{13}C-NMR (δ ppm) (Khan *et al*., 1965)
2	-	184.42 (C)	184.6 (C)
3	-	50.45 (CH)	51.5 (CH)
5	3.18 (1H, dd, *J* = 5.6 Hz and *J* = 6 Hz)	55.24 (CH_2)	56.1 (CH_2)
6	2.68 (1H, dd, *J* = 4.8 Hz and 4.4 Hz, 6-H_β) & 1.45 (1H, d, *J* = 6.8 Hz, 6-H_α)	37.32 (CH_2)	37.6 (CH_2)
7	-	65.19 (CH)	65.7 (CH)
8	-	137.09 (C)	137.6 (C)
9	7.55 (1H, d, *J* = 7.6 Hz)	124.29 (CH)	124.5 (CH)
10	7.39 (1H, dd, *J* = 7.6 Hz and *J* = 7.6 Hz)	125.81 (CH)	125.9 (CH)
11	7.23 (1H, dd, *J* = 7.2 Hz and *J* = 7.6 Hz)	127.06 (CH)	129 (CH)
12	7.49 (1H, d, *J* = 7.2 Hz)	120.85 (CH)	121.5 (CH)
13	-	156.86 (C)	157.8 (C)
14	1.87 (1H, dd, *J* = 4 Hz and *J* = 12 Hz, 14-H_α) & 1.77 (1H, dd, *J* = 4.8 Hz and *J* = 4.8 Hz, 14-H_β)	24.67 (CH_2)	25.5 (CH_2)
15	3.08 (1H, m)	27.47 (CH)	28.1 (CH)

16	2.34 (1H, dd, *J* = 6 and *J* = 6.4 Hz)	48.49 (CH)	48.9 (CH)
17	4.57 (1H, s)	77.21 (CH)	78.1 (CH)
18	1.67 (3H, d, *J* = 11.6 Hz)	13.28 (CH_3)	13 (CH_3)
19	5.65 (1H, q, *J* = 6.8 Hz)	122.76 (CH)	123 (CH)
20	-	137.94 (C)	139 (C)
21	5.17 (1H, s)	88.22 (CH)	90.6 (CH)
22	-	170.13 (C)	170.1 (C)
23	-	21.32 (CH_3)	21 (CH_3)
1'	5.03 (1H, d, *J* = 4.4 Hz)	99.32 (CH)	102.2 (CH)
2'	-	74.26 (CH)	90.6 (CH)
3'	-	77.48 (CH)	78.7 (CH)
4'	-	70.58 (CH)	71.6 (CH)
5'	3.9-3.6 (1H, m)	77.48 (CH)	78.7 (CH)
6'	4.42 (2H, dd, *J* = 7.6 Hz and *J* = 8.8 Hz)	61.56 (CH_2)	62.8 (CH_2)
$OCOCH_3$	2.16 (3H, s, $OCOCH_3$)	-	-



Scheme 2: The HMBC correlations of raucaffricine (**61**)

4.1.3. 10,17-dihydroxy-1,4-dimethylsarpaganium (63)



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The HRMS spectrum (Appendix 16) showed a $[M+H]^+$ ion peak at *m/z* 369.1750 matched with the molecular formula of $C_{21}H_{27}N_2O_2$. The IR spectrum (Appendix 17) of *N*-methylsarpagine (**63**) showed characteristic absorption frequencies at 3312.90, 2942.44 and 2831.65 cm^{-1} typical of the O-H, C-H and C-H bond vibrations, respectively.

The ^{1}H-NMR spectrum (Appendix 18A) of *N*-methylsarpagine (**63**) showed the presence of signals of three aromatic protons which included a doublet at δ_H 7.11 ppm (1H, d, *J* = 8.6 Hz), corresponding to a proton at position 12, another doublet at δ_H 6.77 ppm (1H, d, *J* = 2.0 Hz) corresponding to a proton at position 9 and a doublet of doublets at δ_H 6.68 ppm (1H, dd, *J* = 22. Hz and *J* = 8.9 Hz) corresponding to a proton at position 11. The spectrum also showed the existence of an ethylidene group with a doublet at δ_H 1.63 ppm assigned to position 18 and a quartet at δ_H 5.59 ppm assigned to a proton at position 19. The ^{1}H NMR spectrum (Appendix 18B) of *N*-methylsarpagine (**63**) also displayed an active hydroxyl proton at δ_H 1.82 ppm, attached to C-10 (δ_C 150.91) and two methyl groups corresponding to N_1-Me at δ_H 3.28 ppm and N^+_4-Me at δ_H 3.0 ppm.

The ^{13}C-NMR (Appendix 19) and DEPT 135 (Appendix 20) spectra exhibited nineteen carbon signals, consisting of eight methine carbons at δ_C 120.72 (D, C-19), 112.34 (D, C-12), 111.75 (D, C-11), 102.14 (D, C-9), 65.45 (D, C-5), 61.07 (D, C-3), 43.63 (D, C-16), and 26.87 (D, C-15); four methylenes at δ_C 64.39 (T, C-17), 62.40 (T, C-21), 31.01 (T, C-14), and 23.87 (T, C-6); one methyl at δ_C 11.57 (Q, C-18); and six quaternary carbons at δ_C 150.91 (S, C-10), 132.10 (S, C-20), 131.68 (S, C-13), 127.67 (S, C-8),126.83 (S, C-2) and 99.77 (S, C-7).

For the three protons displayed in the aromatic region, the HMBC spectrum (Appendix 21) revealed correlations of 12-H (δ_H 7.11) with C-10 (δ_C 150.91), C-8 (δ_C 127.67) and C-9 (δ_C 102.14); 9-H (δ_H 6.77) with C-10 (δ_C 150.91), C-13 (δ_C 131.68) and C-12 (δ_C 112.34) and 11-H (δ_H 6.68) with C10 (δ_C 150.91), C-13 (δ_C 131.68) and C-9 (δ_C 102.14) between aromatic protons and carbons, respectively. In addition, the HMBC spectrum (Appendix 21) also displayed correlations of 17-H (δ_H 3.48) with C-16 (δ_C 43.63) and C-15 (δ_C 26.01); 6β-H (δ_H 3.15) with C-5 (δ_C 65.45) and C-21(δ_C 62.40) and 18-H (δ_H 1.63) with C-19 (δ_C 120.72). Furthermore, the HMBC (Appendix 21) correlations from H-17 (δ_H 3.48) to C-5 (δ_C 65.45) permitted the assignment of a hydroxyl group at C-17 (See Scheme 3).

The HSQC spectrum (Appendix 22) revealed correlations between 19-H (δ_H 5.59) with C-19 (δ_C 120.72); H-12 (δ_H 7.11) with C-12 (δ_C 112.34); 11-H (δ_H 6.68) with C-11 (δ_C 111.75); 9-H (δ_H 6.77) with C-9 (δ_C 102.14); 18-H (δ_H 1.63) with C-18 (δ_C 11.57); 21α,β-H (δ_H 4.41 and 4.16) with C-21 (δ_C 62.40); 17-H (δ_H 3.48) with C-17 (δ_C 64.39); 15-H (δ_H 2.95) with C-15 (δ_C 26.01) and 6α,β-H (δ_H 2.9 and 3.15) with C-6 (δ_C 23.87). Moreover, the HSQC spectrum (Appendix 22) indicated the presence of six quaternary carbons at δ_C 150.91 (C-10), δ_C 132.10 (C-20), δ_C 131.68 (C-13), δ_C 127.67 (C-8), δ_C 126.83 (C-2) and δ_C 99.77 (C-7) which is in agreement with DEPT 135 (Appendix 20) spectra.

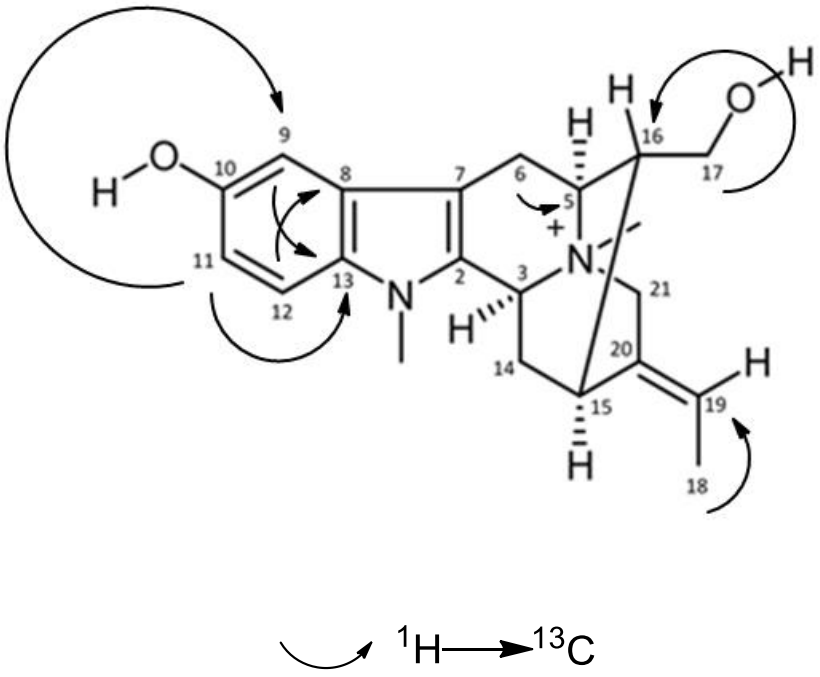
The COSY spectrum (Appendix 23) showed the coupling between protons 19-H (δ_H 5.59) and 18-H (δ_H 1.63) and 21α,β-H (δ_H 4.41 and 4.16) associated with the ethylidene group. Furthermore, the COSY spectrum (Appendix 23) displayed the coupling between protons 21α,β-H (δ_H 4.41 and 4.16) and 18-H (δ_H 1.63), 17-H (δ_H 3.48) and OH (δ_H 1.82), 15-H (δ_H 2.95) and 14α-H (δ_H 2.4), 12-H (δ_H 7.11) and 9-H (δ_H 6.77) and 12-H (δ_H 7.11) and 11-H (δ_H 6.68).

The forgoing data, led us to propose the structure of compound **63** as *N*-methylsarpagine, an indole alkaloid. The data for compound **63** are similar to published NMR data of another isolated *N*-methylsarpagine alkaloid that was first isolated from *Rauvolfia vomitoria* (As shown in Table 3). Therefore, these data led to the conclusion that compound **63** is the known compound 10,17-dihydroxy-1,4-dimethylsarpaganium (**63**). This is the first report of the isolation of *N*-methylsarpagine from *R. caffra*.[102]

Table 3: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of *N*-methylsarpagine (**63**) in CD_3OD (δ in ppm, *J* in Hz)

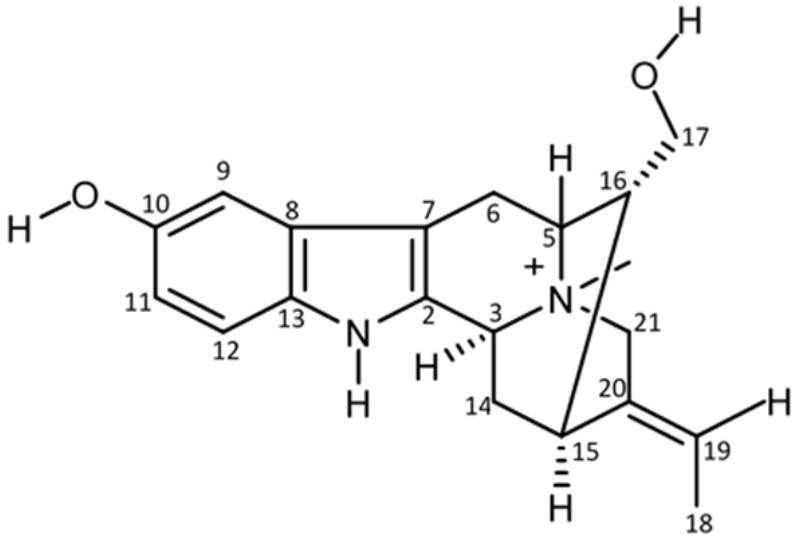
Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)	^{13}C-NMR (δ ppm) (Khan *et al*., 1972)
2	-	126.83 (C)	131.8 (C)
3	-	61.07 (CH)	51.7 (CH)
5	-	65.45 (CH)	53.8 (CH)
6	3.15 (1H, dd, *J* = 12.4, 4.8 Hz, 6-Hβ) & 2.9 (1H, d, *J* = 15.2 Hz, 6-Hα)	23.87 (CH_2)	24.9 (CH_2)
7	-	99.77 (C)	101.3 (C)
8	-	127.67 (C)	126.1 (C)
9	6.77 (1H, d, *J* = 2.0 Hz)	102.14 (CH)	102.1 (CH)
10	-	150.91 (C)	150.5 (C)
11	6.68 (1H, dd, *J* = 8.9, 2.2 Hz)	111.75 (CH)	111.6 (CH)
12	7.11 (1H, d, *J* = 8.6 Hz)	112.34 (CH)	112.0 (CH)
13	-	131.68 (C)	126.9 (C)
14	2.4 (1H, dd, *J* = 11.6, 10.8 Hz, 14-Hα) & 2.12-2.0 (2H, m, 16-H + 14-Hβ)	32.01 (CH_2)	31.2 (CH_2)
15	2.95 (1H, dd, *J* = 2.0, 10.4 Hz)	26.01 (CH)	26.1 (CH)
16	2.12-2.0 (2H, m, 16-H + 14-Hβ)	43.63 (CH)	42.5 (CH)
17	3.48 (2H, d, *J* = 7.2 Hz)	64.39 (CH_2)	62.4 (CH_2)

18	1.63 (3H, d, *J* = 6.7 Hz)	11.57 (CH_3)	11.9 (CH_3)
19	5.59 (1H, q, *J* = 6.8 Hz)	120.72 (CH)	121.8 (CH)
20	-	132.10 (C)	132.4 (C)
21	4.41 (1H, d AB, *J* = 15.6 Hz, 21-H_α) & 4.16 (1H, d AB, *J* = 15.6 Hz, 21-H_β)	62.40 (CH_2)	57.2 (CH_2)
N-CH_3	3.28 (3H, s)	-	-
N^+-CH_3	3.0 (3H, s)	-	-
OH	1.82 (s)	-	-



Scheme 3: The HMBC correlations of *N*-methylsarpagine (**63**).

4.1.4. (15α,19*E*)-10,17-dihydroxy-4-methylsarpagan-4-ium (64)



64

The HRMS spectrum (Appendix 24) obtained for spegatrine (**64**) gave a molecular ion at *m/z* 325.1912, corresponding to the molecular formula $C_{20}H_{25}N_2O_2^+$. The IR spectrum (Appendix 25) suggested the presence of OH (3352.1 cm^{-1}) and NH (1638.8 cm^{-1}) functional groups.

The ^{1}H-NMR spectrum of spegatrine (**64**) (Appendix 26B) showed an active hydroxyl proton at δ 1.97 ppm, attached to C-10 (δ_C 150.93). The ^{1}H-NMR spectrum (Appendix 26A) was further characterized by a doublet at δ_H 7.24 ppm (1H, d, *J* = 8.8 Hz), corresponding to a proton at position 12, another doublet at δ_H 6.90 ppm (1H, d, *J* = 2.0 Hz) corresponding to a proton at position 9 and a doublet of doublets at δ_H 6.78 ppm (2H, dd, *J* = 2 Hz and *J* = 2.4 Hz) corresponding to a proton at position 11. Furthermore, the ^{1}H-NMR spectrum (Appendix 26B) showed an ethylidene group at δ_H 1.74 ppm (3H, d, *J* = 6.8 Hz) associated with position 18 and a quartet at δ_H 5.68 ppm (1H, q, *J* = 6.8 Hz) assigned to a proton at position 19. A N^+-Me group at δ_H 3.12 ppm (s) which is a strong electron withdrawing group let the 21-H protons appear at δ_H 4.45 ppm (1H, d AB, *J* = 15.6 Hz) and at δ_H 4.23 ppm (1H, d AB, *J* = 15.6 Hz), respectively. Another signal shifted downfield was found at δ_H 3.58 ppm (1H, d, *J* = 7.6 Hz) corresponding to two protons at C-17 (δ_C 62.40). This condition may be due to the presence of a hydroxyl group attached to C-17 (δ_C 62.40).

The ^{13}C-NMR (Appendix 27) and DEPT 135 (Appendix 28) spectra exhibited nineteen carbon signals, consisting of eight methine carbons at δ_C 120.71 (D, C-19), 112.35 (D, C-12), 111.73 (D, C-11), 102.13 (D, C-7), 99.77 (D, C-9), 65.46 (D, C-5), 61.08 (D, C-3), 43.62 (D, C-16), and 26.01 (D, C-15); four methylenes at δ_C 64.38 (T, C-21), 62.40 (T, C-17), 32.00 (T, C-14), and 23.87 (T, C-6); one methyl at δ_C 11.56 (Q, C-18); and six quaternary carbons at δ_C 150.93 (S, C-10), 132.10 (S, C-20), 131.66 (S, C-13), 127.68 (S, C-8), 126.83 (S, C-2) and 102.13 (C-7).

The COSY spectrum (Appendix 29) showed the coupling between protons 19-H (δ_H 5.68) and 18-H (δ_H 1.74) and 21α,β-H (δ_H 4.45 and 4.23) associated with the ethylidene group. Furthermore, the COSY spectrum (Appendix 29) displayed the coupling between protons 21α,β-H (δ_H 4.45 and 4.23) and 18-H (δ_H 1.74), 12-H (δ_H 7.24) and 11-H (δ_H 6.78), 17-H (δ_H 3.58) and 16-H (δ_H 2.23-2.13), 15-H (δ_H 3.12) and 14α-H (δ_H 2.54) and 6α-H (δ_H 3.04) and 16-H (δ_H 2.23-2.13).

For the three protons displayed in the aromatic region, the HMBC spectrum (Appendix 30) revealed correlations of 9-H (δ_H 6.90) with C-13 (δ_C 131.66) and C-7 (δ_C 102.13); 12-H (δ_H 7.24) with C-8 (δ_C 127.68); 14α-H (δ_H 2.54) with C-2 (δ_C 126.83); 18-H (δ_H 1.74) with C-19 (δ_C 120.71) and 16-H (δ_H 2.23-2.13) with C-17 (δ_C 62.40) between aromatic protons and carbons, respectively. In addition, the HMBC spectrum (Appendix 30) also displayed correlations of 15-H (δ_H 3.12) with C-2 (δ_C 126.83), 14-H$_\alpha$ (δ_H 2.54) with C-2 (δ_C 126.83). Furthermore, the HMBC (Appendix 30) correlations from H-17 (δ_H 3.58) to C-5 (δ_C 65.46) permitted the assignment of a hydroxyl group at C-17 (See Scheme 4).

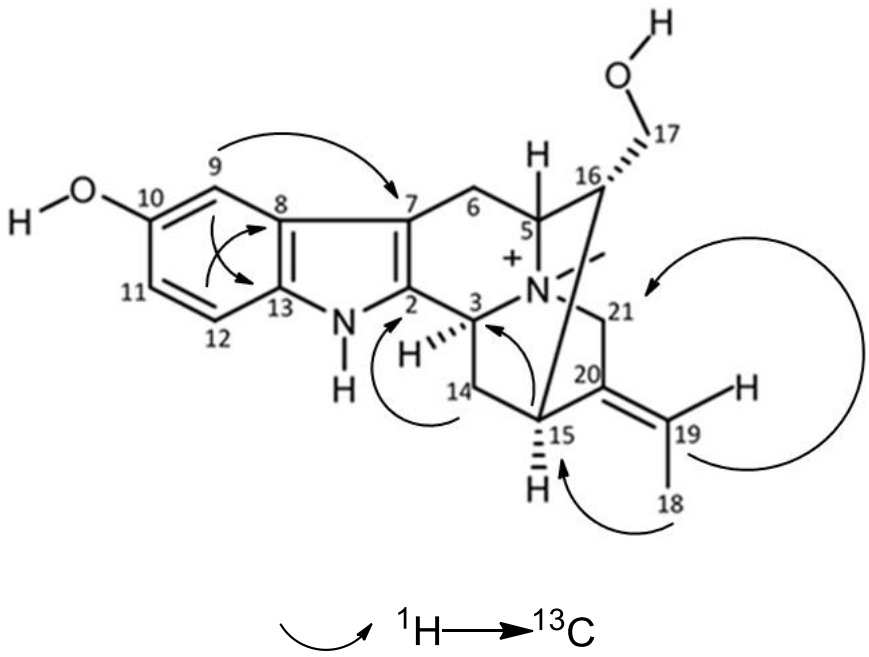
The presence of an aromatic ring is shown on the HSQC spectrum (Appendix 31) by correlations between 12-H (δ_H 7.24) and C-11 (δ_C 111.73), 9-H (δ_H 6.90) and C-7 (δ_C 102.13) and 11-H (6.78) and C-12 (112.35). Moreover, the HSQC (Appendix 31) revealed correlations between 17-H (δ_H 3.58) and C-21 (δ_C 64.38). The HSQC spectrum (Appendix 31) indicated the presence of five quaternary carbons at δ_C 150.93 (C-10), δ_C 132.10 (C-20), δ_C 131.66 (C-13), δ_C 127.68 (C-8), δ_C 126.83 (C-2) and δ_C 102.13 (C-7) which is in agreement with DEPT 135 (Appendix 31) spectra.

Combined analysis of the ^{1}H-NMR and ^{13}C-NMR data indicated that compound **64** had the carbon framework of a spegatrine-type alkaloid.[101] The data for compound **64** are similar to published NMR data of another isolated spegatrine alkaloid that was first isolated from *Rauvolfia verticillata* (As shown in Table 4). Therefore, these data led to the conclusion that compound **64** is the known compound (15α,19*E*)-10,17-dihydroxy-4-methylsarpagan-4-ium (**64**).[103]

Table 4: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral data of spegatrine (**64**) in CD_3OD (δ in ppm, *J* in Hz)

Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)	^{13}C-NMR (δ ppm) (Naranjo *et al.*, 1985)
2	-	126.83 (C)	126.3 (C)
3	-	61.08 (CH)	60.7 (CH)
5	-	65.46 (CH)	64.6 (CH)
6	3.27 (1H, dd, *J* = 12.4, 4.8 Hz, 6-Hβ) & 3.04 (1H, d, *J* = 17.2 Hz, 6-Hα)	23.87 (CH_2)	23.4 (CH_2)
7	-	102.13 (C)	100.3(C)
8	-	127.68 (C)	126.3 (C)
9	6.90 (1H, d, *J* = 2.0 Hz)	99.77 (CH)	102.7 (CH)
10	-	150.93 (C)	149.1 (C)
11	6.78 (1H, dd, *J* = 2.2, 8.8 Hz)	111.73 (CH)	121.6 (CH)
12	7.24 (1H, d, *J* = 8.8 Hz)	112.35 (CH)	112.8 (CH)
13	-	131.66 (C)	131.9 (C)
14	2.54 (1H, dd, *J* = 11.6, 10.8 Hz, 14-Hα) & 2.23-2.13 (2H, m, 16-H + 14-Hβ)	32.00 (CH_2)	31.3 (CH_2)
15	3.12 (1H, dd, *J* = 2.0, 10.4 Hz)	26.01 (CH)	25.5 (CH)
16	2.23-2.13 (2H, m, 16-H + 14-Hβ)	43.62 (CH)	43.1 (CH)
17	3.58 (2H, d, *J* = 7.6 Hz)	64.38 (CH_2)	64.6 (CH_2)
18	1.74 (3H, d, *J* = 6.8 Hz)	11.56 (CH_3)	12.0 (CH_3)

19	5.68 (1H, q, *J* = 6.8 Hz)	120.71 (CH)	112.3 (CH)
20	-	132.10 (C)	132.6 (C)
21	4.45 (1H, d AB, *J* = 15.6 Hz, 21-H_α) & 4.23 (1H, d AB, *J* = 15.6 Hz, 21-H_β)	62.40 (CH_2)	62.0 (CH_2)
N^+-CH_3	3.15 (3H, s)	-	-
OH	1.97 (s)	-	-



Scheme 4: The HMBC correlations of spegatrine (**64**).

4.2. Ultra-performance liquid chromatography mass spectrometry analysis

The chemical profile (Figure 4.1) of the crude extract was obtained by ultra-performance liquid chromatography mass spectrometry (UPLC-MS). The chemical profile of Fraction E_2 (Figure 4.2) and Fraction G (Figure 4.3) revealed that the major constituents of *R. caffra* are found in fractions E_2 (*m/z* 327.2, *m/z* 513.2 and *m/z* 349.2) and G (*m/z* 325.2 and *m/z* 335.1). Both fractions (E_2 and G) were further purified using semi-preparative HPLC to obtain raucaffricine (**61**), a glycoalkaloid and spegatrine (**64**), an indole alkaloid isolated for the first time in *R. caffra*, respectively.

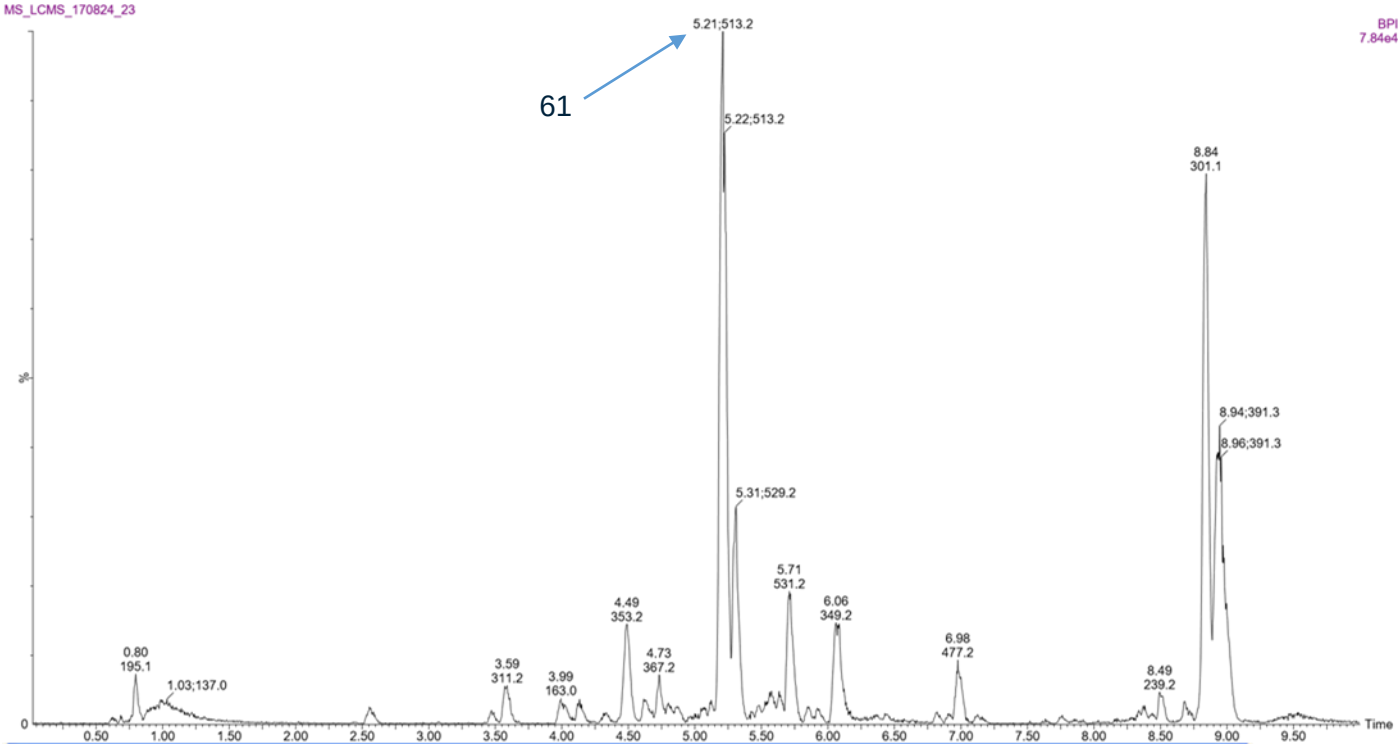


Figure 4.1: UPLC-MS of the crude extract.

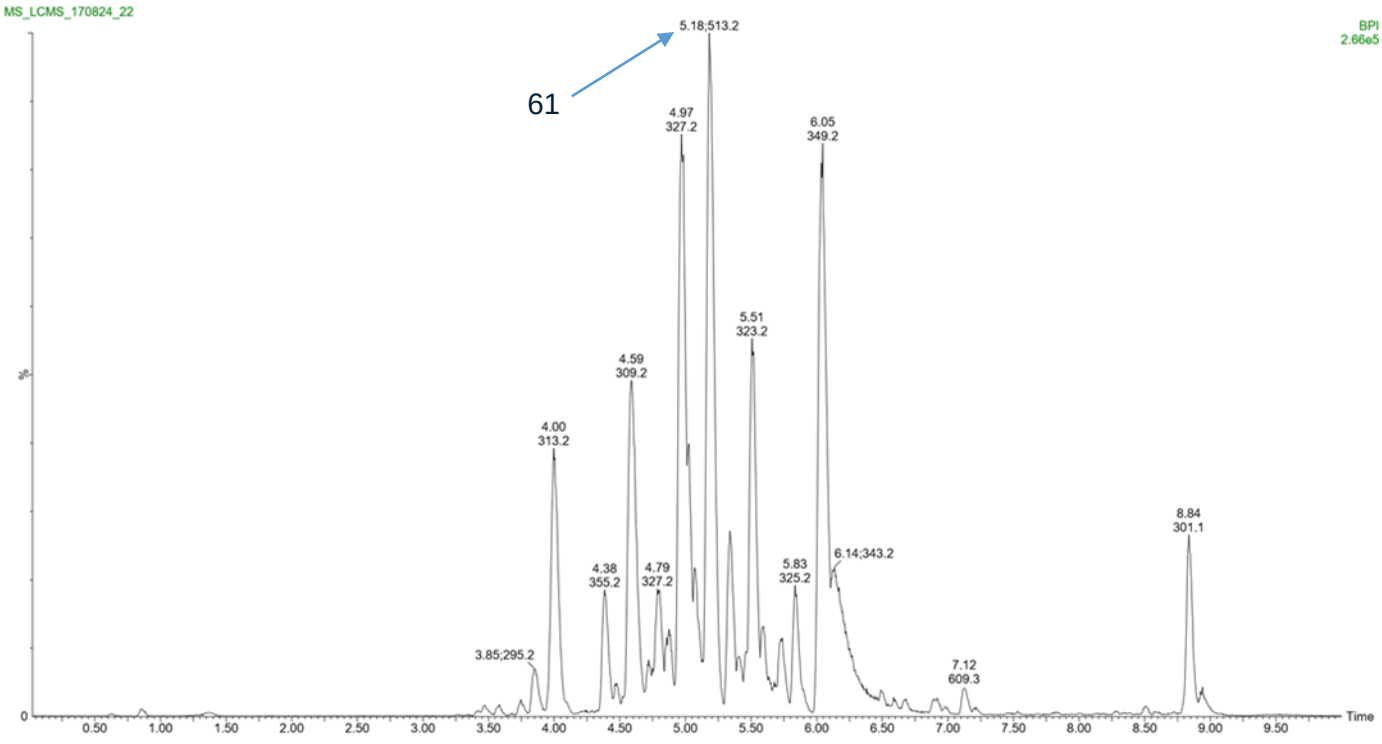


Figure 4.2: UPLC-MS of Fraction E_2.

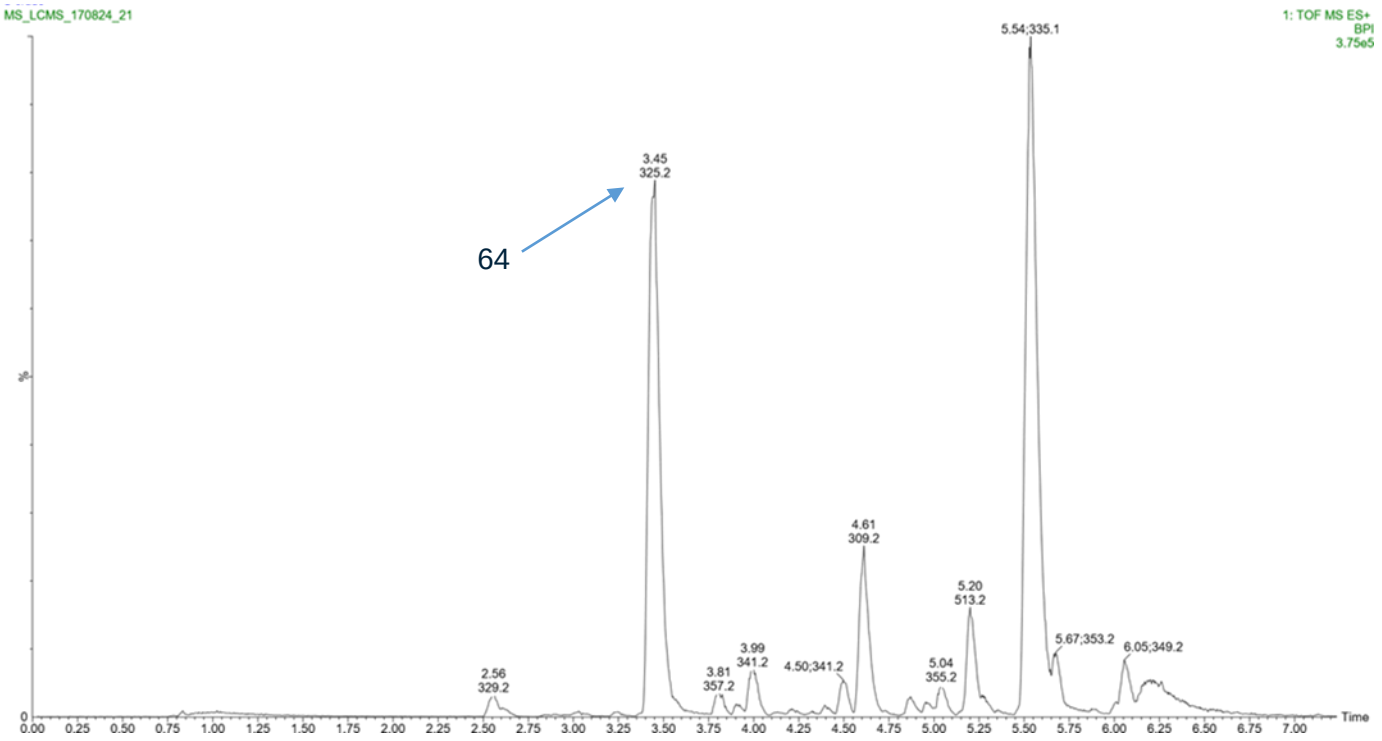


Figure 4.3: UPLC-MS of Fraction G.

The chemical profile of the crude extract (see Figure 4.1) revealed two major peaks which had molecular ions *m/z* 513.2 and *m/z* 301.1, respectively. The molecular ion *m/z* 513.2 was identified as raucaffricine (**61**), a rare glycoalkaloid of the monoterpenoid indole class. However, the molecular ion at *m/z* 301.1 was not identified due to the fact that the compound was lost during column fractionation. The chemical profile of fraction E_2 (see Figure 4.2) revealed three major constituents which had molecular ions *m/z* 327.2, *m/z* 513.2 and *m/z* 349.2, where raucaffricine (**61**), with the molecular ion *m/z* 513.2 was found to be the major constituent of both the crude extract and Fraction E_2. The chemical profile of Fraction G (see Figure 4.3) revealed two major constituents, which had molecular ions *m/z* 325.2 and *m/z* 335.1, respectively, where the molecular ion *m/z* 325.2 was identified as that of spegatrine (**64**), an indole alkaloid isolated for the first time in *R. caffra*.

Comparison of the chemical profiles of Fractions E_2 and G with that of the crude extract showed that the molecular ions *m/z* 327.2, *m/z* 349.2 and (*m/z* 335.1) observed in the UPLC-MS of fractions E_2 and G, respectively were not visible in the UPLC-MS of the crude extract. This was because the high raucaffricine (**61**) content suppressed observation in the UPLC-MS of the crude extract of all the other compounds.

4.3. Bioactivities of *Rauvolfia caffra*

4.3.1. Polyphenol content of *R. caffra*

The quantity of total phenolic and flavonoids content of the crude extract and fractions B, E_1, E_2, F_1 and G of *R. caffra* were determined using spectrophotometric methods.

4.3.1.1. Total Phenolic Content (TPC)

Natural antioxidants from plants are usually found in the form of phenolic compounds such as flavonoids, phenolic acids, tocopherols etc.[104] Numerous studies have revealed that the higher antioxidant ability associated with tradition or medicinal plants is attributed to the total phenolic compounds.[105] Therefore, the total phenolic content in crude extract and fractions B, E_1, E_2, F_1 and G in stem bark of *Rauvolfia caffra* Sond were estimated using the Folin-Ciocalteau´s reagent. The total phenolic content of the crude extract and fractions B, E_1, E_2, F_1 and G was calculated and expressed as GAE through the manipulation of regression equation of gallic acid calibration curve ($Y= 3.6537 X + 0.4455$, $R^2 = 1$, see Figure 4.4) to facilitate the comparisons.

In this study, the results of this assay ranged from 16.06±0.125 mg GAE/g to 11.467±0.9441 mg GAE/g. Fraction B was the most interesting one with the highest phenolic content (16.06±0.125 mg GAE/g). The highest total phenol content was found in Fraction B (16.06±0.125 mg GAE/g) followed by the crude extract (14.393±1.013 mg GAE/g), while Fraction F_1 had the lowest content (11.727±0.913 mg GAE/g) as shown in Figure 4.5. This shows that Fraction B has more polyphenolic compounds compared to the other tested fractions (E_1, E_2, F_1 and G). There was no significant difference ($p < 0.005$) between Fractions F_1 and E_2.

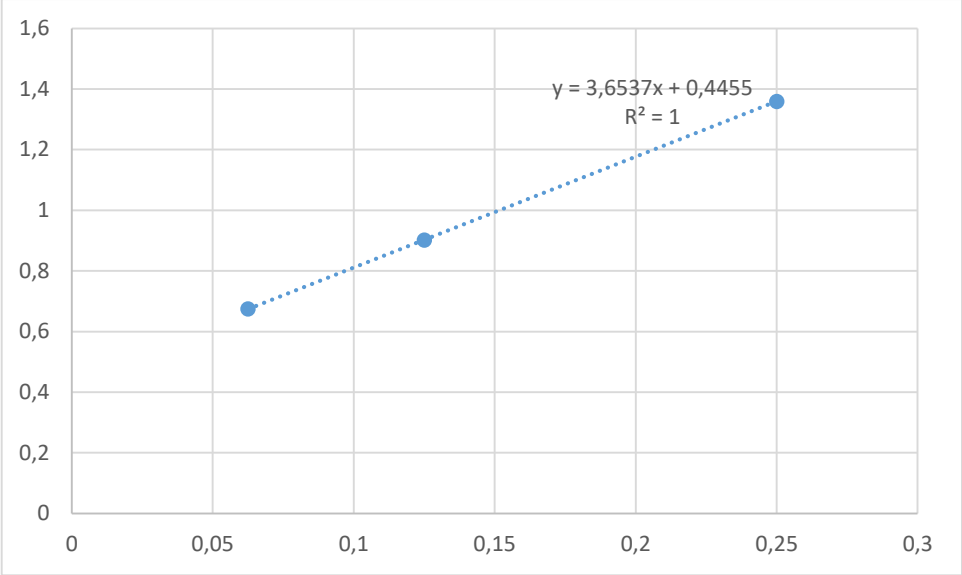


Figure 4.4: Regression equation of gallic acid calibration curve.

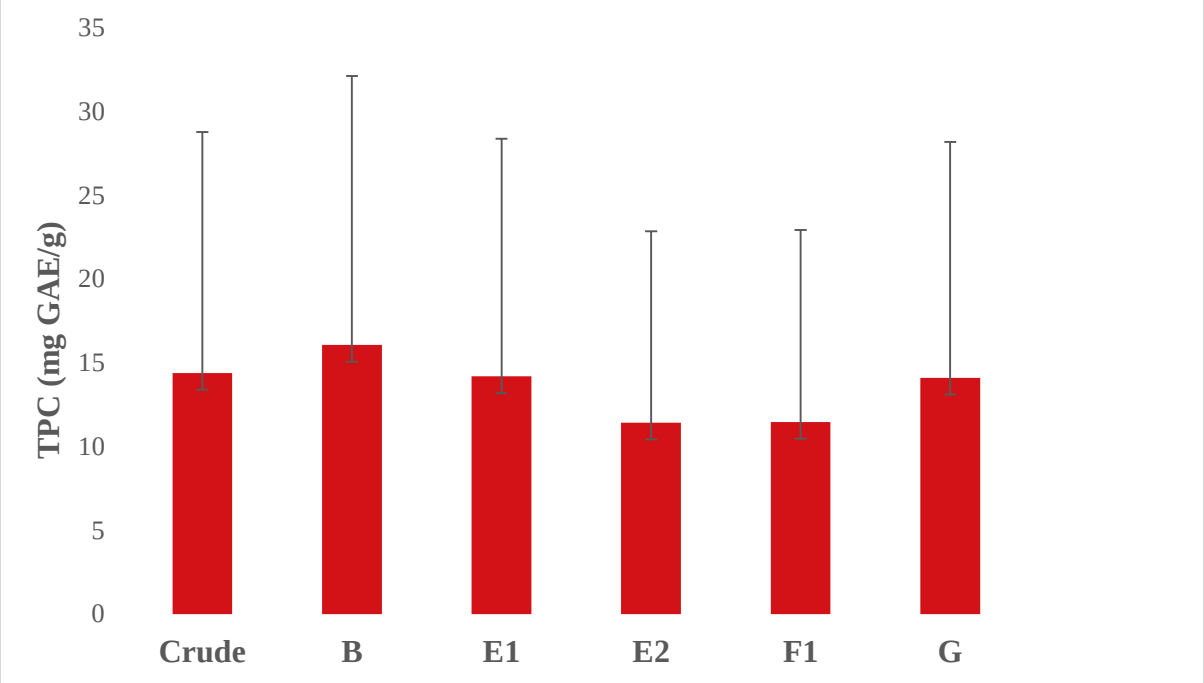


Figure 4.5: Total phenolic content of crude extract and fractions expressed as mean ± standard error.

Phenolic compounds are a class of antioxidant agents which act as free radical terminators.[19] Phenolic compounds inhibit lipid oxidation by scavenging free radicals, chelating metals, activating antioxidant enzymes, reducing tocopherol radicals and inhibiting enzymes that cause oxidation reactions. Total phenol contents of plant species were analysed with Folin-Ciocalteu reagent. Phenolic compounds are also reported to be effective hydrogen donors, making them very good antioxidants.[106] Thus, it was reasonable to determine their total amount in *Rauvolfia caffra* Sond.

These results suggest that phenolics are not the only compound class responsible for the antioxidant activity. Indole alkaloids have been reported to possess antioxidant activities.[106] In the literature, total phenolic assay of methanol crude extracts from the stem bark of *Rauvolfia caffra* agrees with our results.[107] However, isolated compounds from this plant have never been examined for their total phenol content in the literature before. That makes our study the first survey to detect important phenolic content of *R. caffra* in literature.

4.3.1.2. Total Flavonoid content (TFC)

The antioxidant activity of medicinal plants could be attributed to its flavonoid content. Flavonoids are large compounds naturally occurring in food plants, they are compounds that normally display various antioxidant abilities. They are hydroxylated phenolic substances which are synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide range of microorganisms. Their activity is said to be due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall.[108] In higher plants, they are required predominantly for UV fractionation.[109] Flavonoid act as scavengers of various oxidizing species i.e. superoxide anion, hydroxyl radical or peroxy radicals, they also act as quenchers of singlet oxygen.[110]

Total flavonoid content of the crude extract and each fraction (B, E_1, E_2, F_1 and G) was evaluated by the aluminium chloride ($AlCl_3$) colorimetric method, with quercetin as a standard, constructed standard curve of equation Y= 5.5211 X + 0.5862 (see Figure 4.6). The total flavonoid content was found to vary from 1.853±0.079 to 9.453±0.081 mg QE/g with the ascending order: F_1 < E_1 < G < Crude < B < E_2 (see Figure 4.7).

Therefore, the assay showed the highest flavonoid content of Fraction E_2 (9.453±0.081 mg QE/g) while Fraction F_1 (1.853±0.079 mg QE/g) had the lowest content in comparison with the other fractions, as shown in Figure 4.7. This shows that there are more polyphenolic compounds distributed in Fraction E_2 compared to the other fractions (B, E_1, E_2, F_1 and G). Fraction E_2 was significantly different ($p < 0.005$) from all the fractions (B, E_1, F_1 and G) and crude extract.

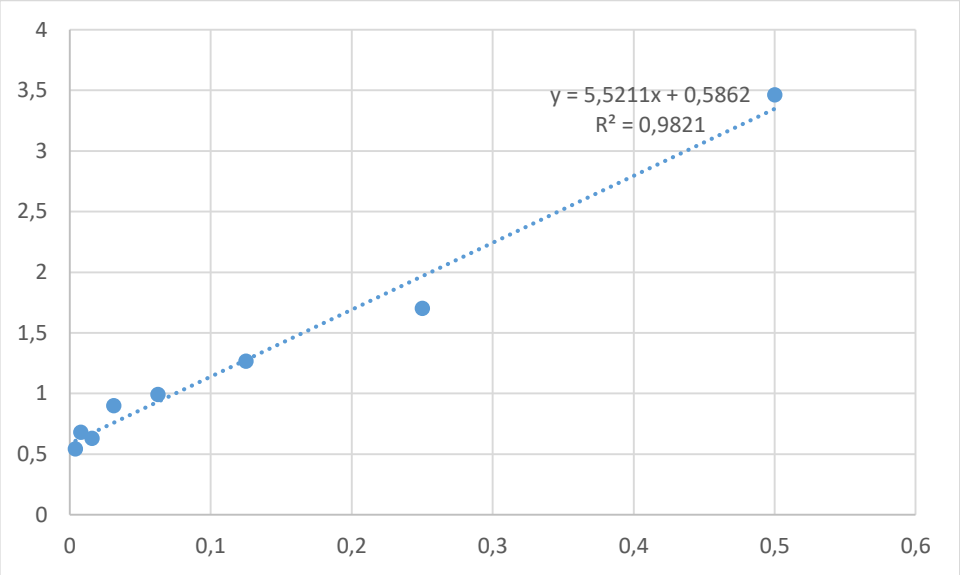


Figure 4.6: Regression equation of quercetin calibration curve.

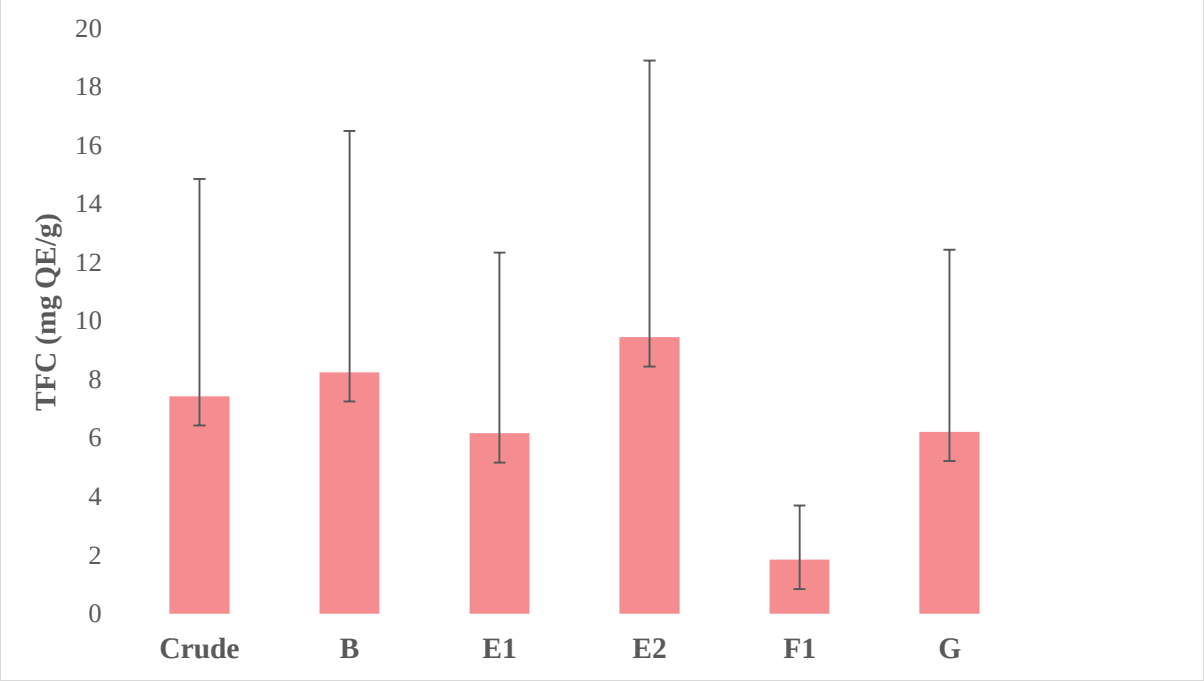


Figure 4.7: Total flavonoid of crude extract and fractions expressed as mean ± standard error.

4.3.2. Antioxidant activities

The DPPH free radical scavenging assay was used to measure the antioxidant activity of fractions and crude extract on the basis of free radical scavenging efficacy, while reducing power was measured on the basis of reduction potential. The results were expressed as IC_{50} or $IC_{0.5}$ (µg/mL) value which indicates the total amount of antioxidants in the crude extract, fractions (B, E_1, E_2, F_1 and G) and spegatrine (**64**) that is required to reduce the initial concentration of DPPH by half (50 %). The present study revealed that the crude, fractions B, E_1, E_2, F_1 and G, and spegatrine (**64**) have free radical scavenging ability, reductive capability and contain good amount of indole alkaloid compounds.

The DPPH radical scavenging assay is regularly used for determination of the antioxidant activity. DPPH is a stable free radical, even at room temperature, and shows strong absorbance at 517 nm. The DPPH radical reacts with antioxidants which have the ability to donate protons to free radicals.[111]

In this assay, the colour change was observed indicating reduction from purple DPPH colour to yellow DPPHH, indicating that the tested compounds have antioxidant activity.

The highest IC_{50} value indicates the lowest antioxidant activity while the lowest IC_{50} values imply the highest activity. In the DPPH free radical scavenging activity test, positive results were obtained from this assay, with Fraction F_1 exhibiting the lowest IC_{50} value of 0.022±0.003 µg/ml, followed by Fraction G (0.036±0.007 µg/mL), and Fraction E_2 showed the highest IC_{50} value of (1.143±0.478 µg/mL), as shown in Table 5. The DPPH free radical scavenging activity of fraction F_1 (0.022±0.003 µg/mL) > G (0.036±0.007 µg/mL) > Crude (0.213±0.068 µg/mL) > spegatrine (**64**) (0.119±0.067 µg/mL) > E_1 (0.413±0.195 µg/mL) > B (0.653±0.307 µg/mL) > E_2 (1.143±0.478 µg/mL).

The antioxidant activity of the crude extract, fractions B, E_1, E_2, F_1 and G, and spegatrine (**64**) was also evaluated by ferric reducing power assay. In this method, the yellow test solution changed to various shades of green and blue when the existence of reducers transformed the Fe^{3+} ferricyanide complex to its ferrous form (Fe^{2+}). As a result, Fraction E_2 showed the lowest $IC_{0.5}$ value of 2.151±0.372 µg/mL while Fraction F_1 had the highest $IC_{0.5}$ value (0.518±0.044µg/mL). The reducing power of Fraction F_1 (0.518±0.044 µg/mL) > spegatrine (**64**) (0.712±0 µg/mL) > G (1.076±0.136 µg/mL) > Crude (1.226±0.443 µg/mL) > E_1 (1.282±0.036 µg/mL) > B (2.036±0.266 µg/mL) > E_2 (2.151±0.372 µg/mL), as shown in Table 5. Therefore, Fraction F_1 exhibited the highest antioxidant activity against DPPH free radical scavenging assay and reducing power assay. Spegatrine (**64**) also exhibited antioxidant activity against DPPH free radical scavenging assay and reducing power assay, the antioxidant activity may be due to the presence of hydroxyl groups.

Table 5: Antioxidant activity of crude extract, fractions, pure compound and control (gallic acid) against DPPH. Values in the same column followed by the same letter are significantly different ($p < 0.05$).

Samples	DPPH IC_{50} (µg/mL)	Reducing power $IC_{0.5}$ (µg/mL)
Crude	0.213 ± 0.068^{a}	1.226 ± 0.443^{a}
B	0.653 ± 0.307^{a}	$2.036\pm0.266^{b,c}$
E_1	0.413 ± 0.195^{a}	1.282 ± 0.036^{d}
E_2	1.143 ± 0.478^{a}	$2.151\pm0.372^{e,f}$
F_1	0.022 ± 0.003^{a}	$0.518\pm0.044^{c,e}$
G	0.036 ± 0.007^{a}	1.076 ± 0.136^{g}
Spegatrine (**64**)	0.119 ± 0.067^{a}	0.715 ± 0^{h}
Gallic acid	0.045 ± 0.018^{a}	$0.115\pm0.007^{b,f}$

These results were not unexpected since research by Erasto *et al.*[86] and Gbonjubola[93] showed that indole alkaloids from the stem bark of *R. caffra* contain comparatively strong antioxidants. Furthermore, they also exhibited good electron donating ability, which implies that indole alkaloids reduce the level of free radicals. These results suggests that the free radical scavenging compounds are highly polar while the reducing compounds are less polar.

4.3.3. Antimicrobial activity

4.3.3.1. Determination of the minimal inhibitory concentration (MIC)

Generally, the initial crude extracts show less activity compared to the fractionated extracts. In some of the cases observed, the antimicrobial activity is only present in the fractionated extracts and not in the initial crude extracts. This is probably due to the active compounds in the crude extracts being in a low concentration and thus, not able to be detected by the antimicrobial assay. There is also a possibility that interactions with other components in the crude extracts antagonize its effect.

The antimicrobial activity in an extract should be greater in the purified compound. However, it is not unusual for the initial extract to show greater activity than any of the fractions obtained with bioassay-guided fractionation. In cases like this, the possibility of synergy or loss of highly active minor constituents should be considered and if possible, checked by testing the bioactivity of the combined fractions.[112]

The micro-dilution method was used to determine the susceptibility of organisms of the crude and fractions, as described in Table 6. Gentamicin was used as a positive control with the MIC value of 0.30 mg/mL. A current definition of the Minimum Inhibitory Concentration (MIC) is "the lowest concentration which resulted in the reduction of inoculum viability".[113] As a result, Fractions B and G were found to be the most active against *B. cereus* with the MIC values of 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL, respectively. Fractions E_1 and E_2 both had lower activity against *E. faecalis* and *S. aureus*, with MIC values of 100 mg/mL, respectively. In the *E. coli* strain, Fraction E_2 exhibited weak activity, with MIC values of 50 mg/mL and 25 mg/mL. In conclusion, all the tested fractions (B, E_1, E_2, F_1 and G) had higher activity against *B. cereus* compared to the other organisms (*E. coli*, *S. aureus* and *E. faecalis*).

Table 6: Antimicrobial activity (MIC) of the crude extract, fractions and control (gentamicin).

MIC (mg/mL)				
Fractions	*E. coli*	*S. aureus*	*E. faecalis*	*B. cereus*
Crude extract	-	-	-	-
B	-	-	-	100
	-	-	-	50
	-	-	-	25
	-	-	-	12.5
E_1	-	100	100	100
E_2	50	100	100	100
	25	-	-	50
	-	-	-	25
	-	-	-	12.5
F_1	-	100	-	100
	-	-	-	50
G	-	100	-	100
	-	-	-	50
	-	-	-	25
Positive control (Gentamicin)	0.3	0.3	0.3	0.3

Based on the results of the assay, Fraction E_2 exhibited lower activity, with MIC values of 50 mg/mL and 25 mg/mL against *E. coli* (Gram-negative bacteria) strain. This is probably due to the cell envelope of the bacteria. Gram-positive bacteria have a relatively simple cell envelope that contains of two or three layers. Whereas in Gram-negative bacteria, the cell envelope is a highly complex, multi-layered structure.[114] This makes the active compounds from the crude extract and fractions harder to penetrate through the cell envelope of Gram-negative bacteria. Therefore, Gram-negative bacteria are harder to destroy compared to Gram-positive bacteria.

Among all the tested samples, the study showed that the crude extract and Fractions B, E_1, E_2, F_1 and G from the methanolic extract of the stem bark of *R. caffra* contained significant inhibitory properties against tested bacterial species, namely *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Bacillus cereus*. However, the Fractions (B, E_1, E_2, F_1 and G) from the methanolic extracts of the stem bark showed higher antibacterial activity against gram positive bacteria (*B. cereus)* than the gram negative bacteria (*E. coli*). Fractions B and G were the most active against *B. cereus* with the MIC values of 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL, respectively (As shown in Table 6).

These results are probably due to the isolated fractions (B, E_1, E_2, F_1 and G) containing active indole alkaloids which are more soluble in polar solvent and these were accountable for the activity. The presence of these indole alkaloids in the crude extract and Fractions (B, E_1, E_2, F_1 and G) has been related to their activities against microorganisms that cause disease and infection by pathogenic microorganisms.[115,116]

4.3.4. Cytotoxic activity

Toxic chemicals usually affect the basic cellular metabolic activities and can also cause cell death. Monitoring the changes in metabolic activity is the best way to evaluate cellular damage. In addition to beneficial bioactive compounds, medicinal plants contain certain toxic chemicals which are almost always toxic at high doses, and pharmacology is simply toxicology at lower doses or vice versa. It is therefore very essential to evaluate the cytotoxicity of medicinal herbs during the screening process. The toxicity of the herbal extracts can be measured by using several *in vivo* tests such as MTT assay, Alamar blue assay, DNA, MTS, and membrane integrity tests.[117] These *in vitro* tests help to screen a large number of herbs for their toxicity and also help to evaluate the required dosage.

Cytotoxic evaluation of natural products is an important part of discovering new drugs. In the present study, the toxicity of the crude extract, fractions and pure compounds of *R. caffra* was determined. In efforts to screen for cytotoxic compounds, the cell toxicity assay (CTA) was used to determine the cytotoxic abilities of the crude extract, Fractions B, E_1, E_2, F_1 and G, raucaffricine (**61**), compound **62** (unidentified) and spegatrine (**64**) against HeLa (human cervix adenocarcinoma) cells.

As a result, the standard drug emetine displayed an IC_{50} value of 0.02557 µM, whereas all the tested samples did not cause significant cytotoxic effects at a concentration of 50 µg/mL (reduced the viability of Hela cells to below 50 %), as shown in Figure 4.8. The observed results were due to the fact that cancerous cells used stopped growing and dividing, and therefore not activating a genetic program of controlled cell death. Perhaps if normal cells were used, the crude, fractions and pure compounds could or might have exhibited a significant cytotoxic activity.

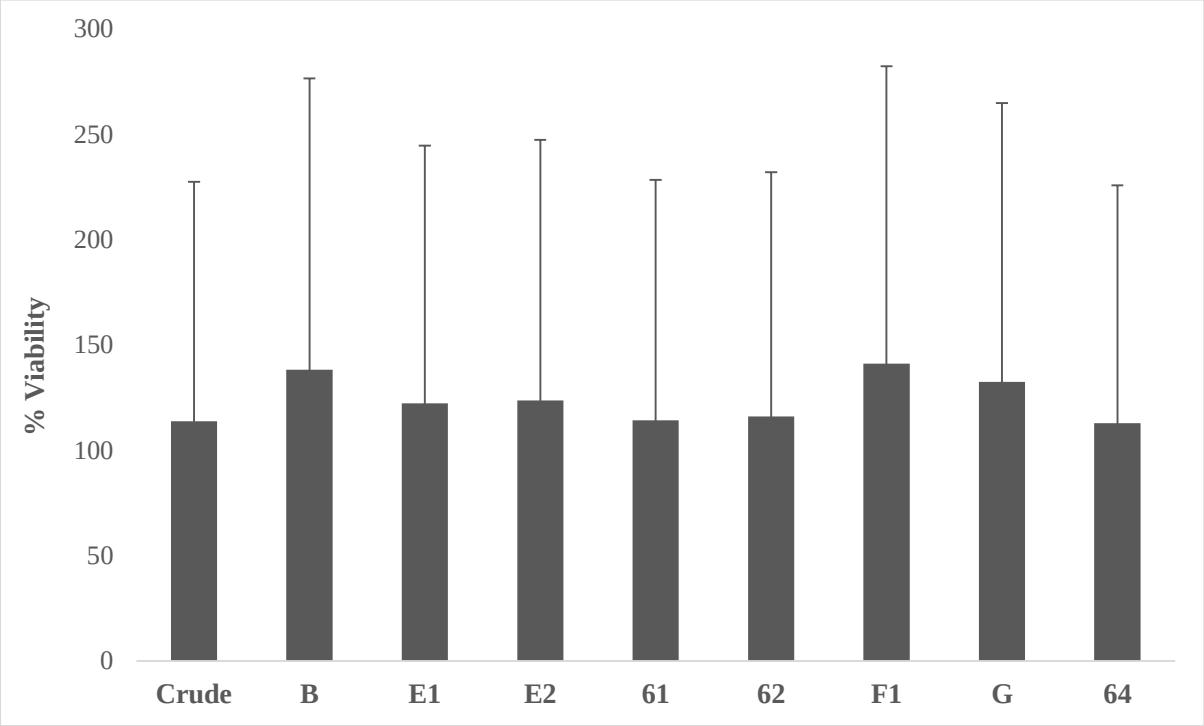


Figure 4.8: Cytotoxic activity of crude extract, fractions and pure compounds expressed as % HeLa cell viability ± SD obtained for the individual compounds.

Moreover, the results obtained in this study were in agreement with the results obtained by Milugo.[118] In their study, anti-proliferative activity of alkaloids were assessed using crystal violet assay where human hepatocellular carcinoma (Liver cancer; Hep-G2) and rhabdomyosarcomas (Muscle cancer; RD) were used as model cell lines, while Vero cells were used as control to test for possible cytotoxicity to normal cells. As a result, the alkaloids from the MeOH/CH_2Cl_2 extract of the stem bark of *R. caffra* did not show significant activity against proliferation of RD and Hep-G2 cells, but it exhibited activity against proliferation of Vero cells.

Comparison of the obtained results from this study with that obtained from literature indicates that the cytotoxicity activity of alkaloids from the stem bark of *R. caffra* are more active against normal cells than cancerous cells.[118]

4.3.5. Antiplasmodial activity

Malaria is one of the world's most deadly infectious diseases. The existence of chloroquine-resistant strains and the emergence of the artemisinin- resistant strains of *P. falciparum* have stirred up the urgency to find alternative and efficient drug for malaria treatment. This study aimed to find compounds from extracts with *P. falciparum* activity by screening the stem bark of *Rauvolfia caffra* Sond, because *R. caffra* contains secondary metabolites such as alkaloids, terpenoids and flavonoids which have various pharmacological properties including antimalarial, antitumor and antidiabetes efficacy. [119,120]

The antimalarial activities of the crude extract, fractions B, E_1, E_2, F_1 and G, raucaffricine (**61**), compound **62** (unidentified) and spegatrine (**64**) against *Plasmodium falciparum* strain 3D7 were determined using the parasite lactate dehydrogenase (pLDH) assay. As shown in Figure 4.9, Fraction E_2 caused a significant decrease in pLDH at the tested concentration (50 µg/mL), whereas its pure compounds raucaffricine (**61**) and compound **62** (unidentified) did not cause a significant decrease in pLDH at the tested concentration (50 µg/mL). This may be the result of the removal of a more active compound from the fraction, or because of a synergistic effect of several compounds in the fraction.

In contrast, at the same concentration, the crude extract had the lowest antiplasmodial activity compared to Fractions B, E_1, F_1, G, raucaffricine (**61**), compound **62** (unidentified) and spegatrine (**64**). Fraction E_2 was not found to be cytotoxic, therefore it was put forward for pLDH IC_{50} assay since it reduced pLDH activity to less than 20 %. Fraction E_2 was found to be more active compared to Fractions B, E_1, F_1, G, crude extract, raucaffricine (**61**), compound **62** (unidentified) and spegatrine (**64**). In contrast, at the same concentration, its pure compounds raucaffricine (**61**) and compound **62** (unidentified) were active and could be responsible for the activity of fraction E_2. In literature, the antiplasmodial activity of alkaloids from *Rauvolfia caffra* agrees with our results.[119]

However, isolated compounds from this plant has never been studied for their antiplasmodial activity in the literature before. Therefore, that makes our study the first survey to detect significant antiplasmodial activity of *R. caffra* in literature.

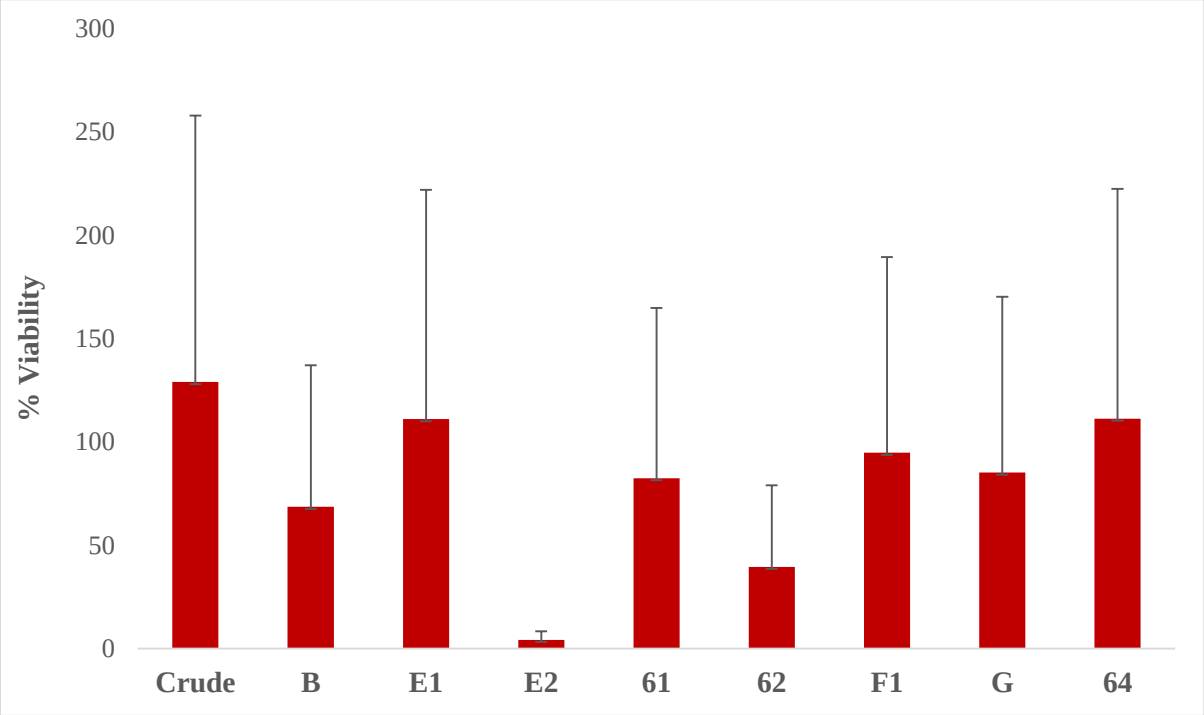


Figure 4.9: Antimalarial activity of crude, fractions and pure compounds expressed as % parasite viability ± SD obtained for the individual compounds.

4.3.5.1. Dose-response curve for antimalarial assay

As shown in Figure 4.10, Fraction E_2 at a concentration 250 µg/mL decreased the viability of *Plasmodium falciparum* (4.149 ± 6.979 %) with an IC_{50} value of 6.533 µg/mL, whereas the reference drug chloroquine showed an IC_{50} value of 0.0102 µM.

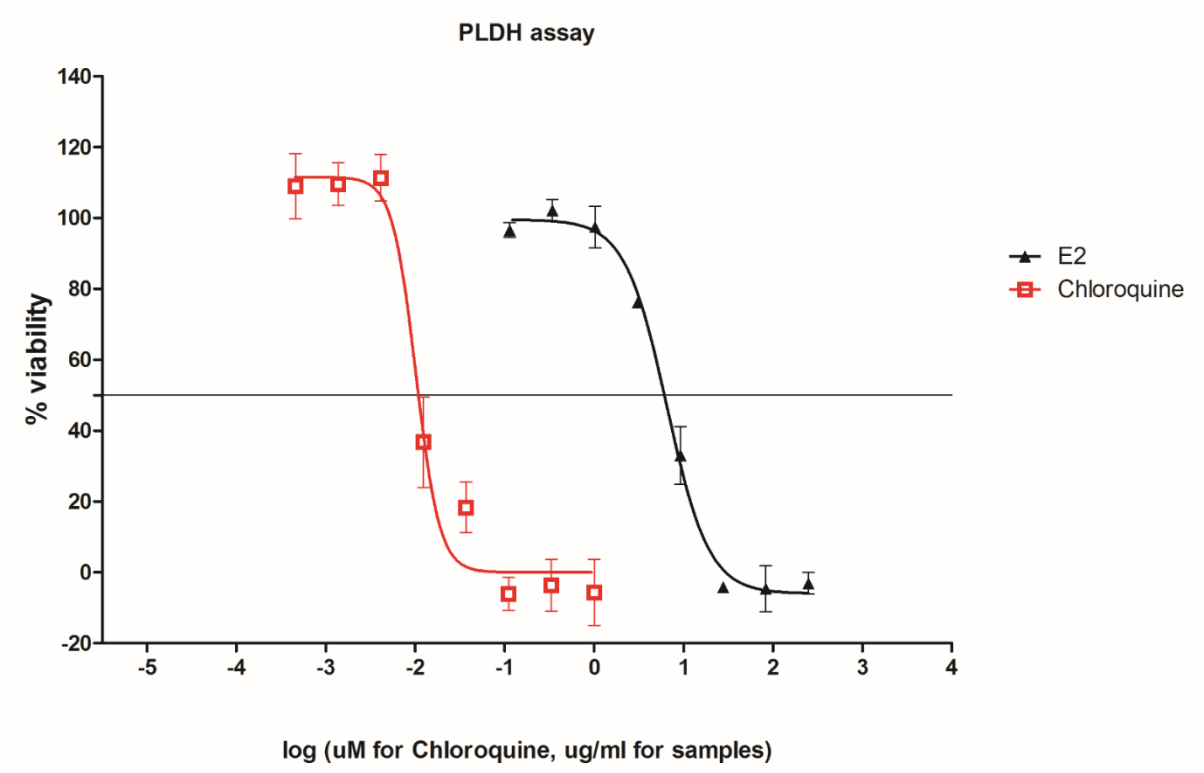


Figure 4.10: Dose-response curve for antimalarial assay.

4.3.6. Antitrypanosomal activity

Trypanosomiasis is a parasitic disease caused by haemoprotozoan belonging to the genus, *Trypanosoma* of the family *Trypanosomitidae*. African animal trypanosomiasis (AAT) is most common disease of domestic livestock covering about 37 sub-Saharan countries in Africa.[121,122] For several decades trypanosomiasis has continued to contribute adversely to economic and social well-being of sub-Saharan Africans.[123] Despite the enormity of health and economic implication of African trypanosomiasis current chemotherapeutic options are very limited and far from ideal for both human and livestock.[124,125] So the need for safer, cheaper, available sources of medication cannot be overemphasized.

Literature studies and field survey have shown that a number of medicinal plants and their secondary metabolites have been screened for anti-trypanosomal activity and quite a number of them have been reported to have significant antitrypanosomal activity.[126,127]

This is due to the fact that plants contain active natural products such as alkaloids, phenolics, saponins, cardiac glycosides, terpenoids, and polyacetylenes which are responsible for this activity.[128-,130]

In this regard, this study tried to evaluate the antitrypanosomal activity of the crude extract, Fractions B, E_1, E_2, F_1, G, raucaffricine (**61**), compound **62** (unidentified) and spegatrine (**64**) using the Trypanosome assay against *Trypanosoma brucei (T. b.)* parasites. As a result, Fraction B, crude extract, Fraction E_2 and its pure compound **62** (unidentified) displayed the highest activity at the tested concentration (50 µg/mL), as shown in figure 4.11. This was due to their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defences against oxidative stress.[126] This is because medicinal plants have structures capable of generating radicals that may cause peroxidative damage to trypnothione reductase that is very sensitive to alterations in redox balance. The crude extract, Fractions B and E_2 and pure compound **62** (unidentified) were not found to be cytotoxic, therefore they were put forward for pLDH IC_{50} assay since they reduced pLDH activity to less than 20 %.

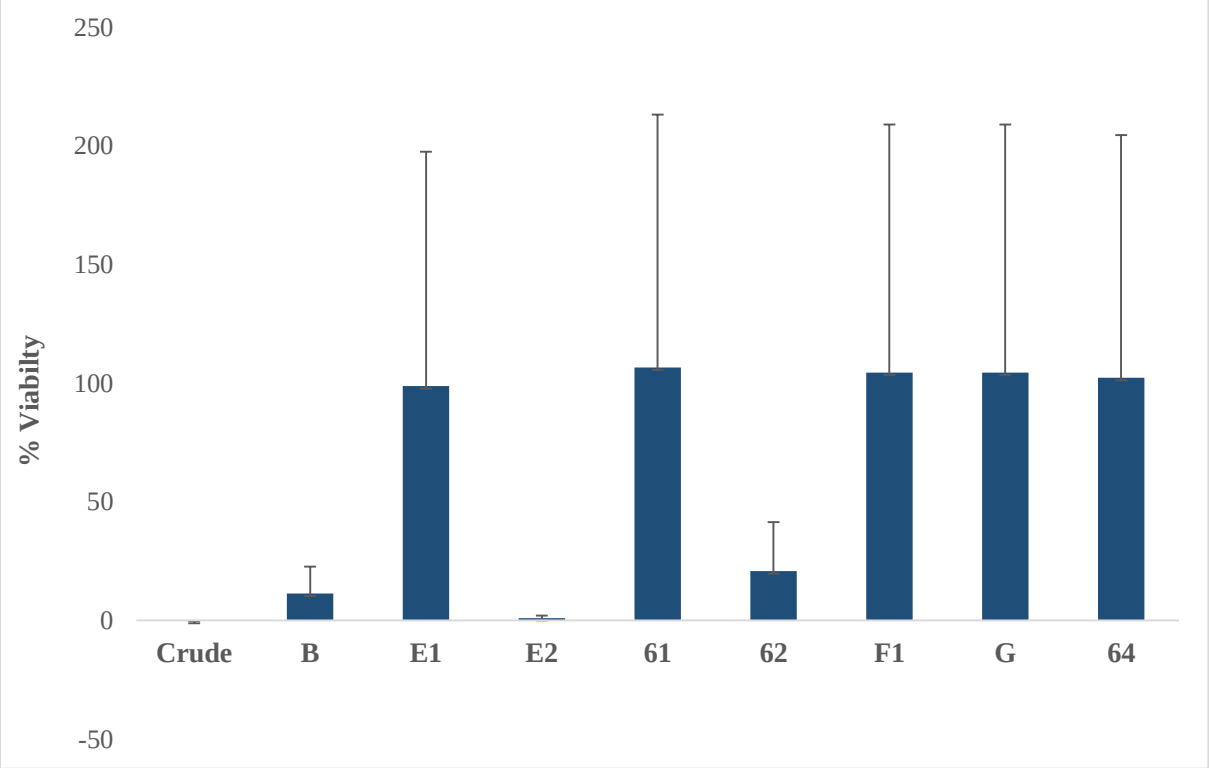


Figure 4.11: Antiparasitic activity of crude extract, fractions and pure compounds expressed as % parasite viability ± SD obtained for the individual compounds.

4.3.6.1. Dose-response curve for trypanosome assay

Fraction B and the crude extract affected the viability of the Trypanosomes at 250 µg/mL concentration, giving (11.334 ± 2.692 % and -0.133 ± 0.206 %, as shown in Figure 4.12) with IC_{50} values of (14.15 µg/mL and 18.50 µg/mL), respectively. Fraction E_2 and its pure compound **62** (unidentified) also decreased the viability of *T. b. brucei* at the same concentration, with a percentage of viable parasites of (1.026 ± 0.143 % and 20.769 ± 9.054 %, as shown in Figure 4.12) with IC_{50} values of (15.58 µg/mL and 34.71 µg/mL) respectively, therefore displaying an antitrypanosomal activity. The pentamidine used as reference drug showed an IC_{50} value of 0.003114 µM. In literature, the antitrypanosomal activity of fractions and isolated bioactive constituents from *Rauvolfia caffra* have never been evaluated. Therefore, that makes our study the first survey to detect important antitrypanosomal activity of *R. caffra* in literature.

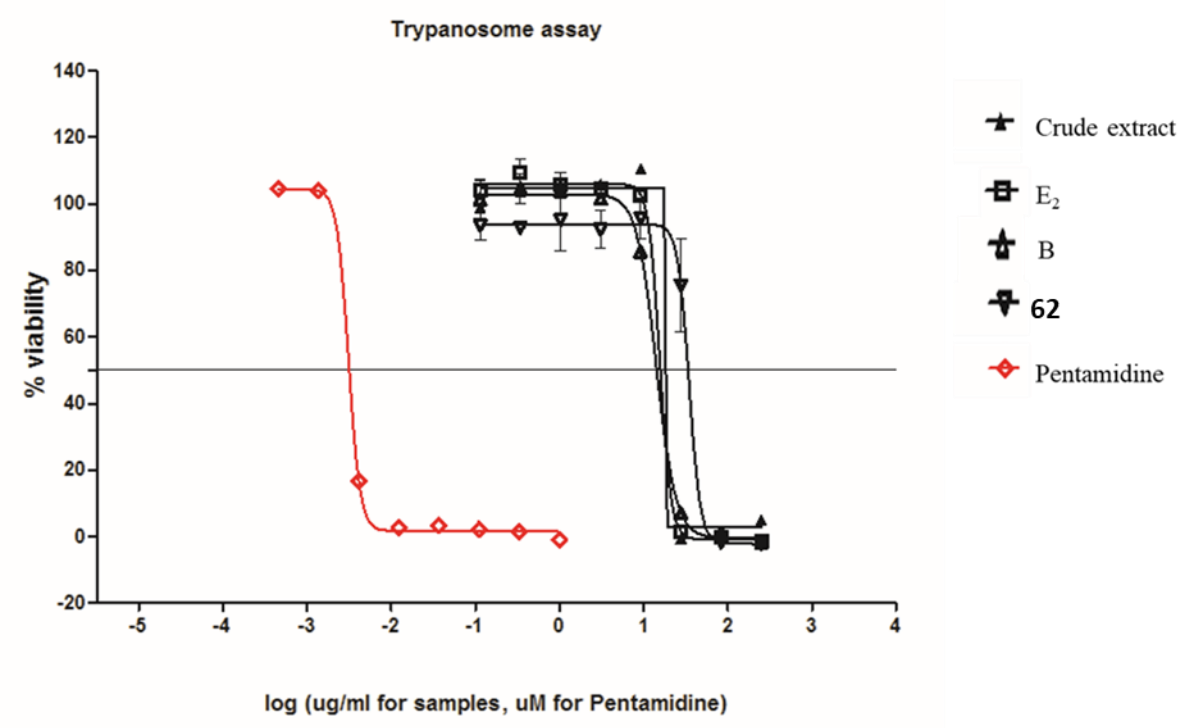


Figure 4.12: Dose-response curves for the trypanosome assay.

Chapter 5

Conclusion

The core objectives of this project were achieved to a certain extent, which were to isolate and characterize bioactive compounds from *Rauvolfia caffra* Sond, as well as to test for their antioxidant, antimicrobial, antiplasmodial, antitrypanosomal, and cytotoxic activities. Furthermore, the polyphenol content of the crude extract and fractions (B, E_1, E_2, F_1 and G) were evaluated. On the basis of the results obtained, it may be concluded that the stem bark of *R. caffra* can act as an important source of phenolic and flavonoid constituents.

Six compounds (**59**, **60**, **61**, **62**, **63** and **64**) were isolated from the stem bark methanol extract and compounds **61** and **64** were identified using the UPLC-MS as raucaffricine, a rare glycoalkaloid of monoterpenoid indole class and spegatrine, an indole alkaloid isolated that was first isolated from *Rauvolfia verticillata*, respectively. This is the first report of the isolation of spegatrine from *R. caffra*. Furthermore, compounds **59** and **63** were characterised and identified using a combination of various spectroscopic techniques such as nuclear magnetic resonance (NMR), infra-red (IR) spectroscopy and high resolution mass spectroscopy (HRMS) as lupeol, a pentacyclic tri-terpenoid which was previously isolated from *Centaurea omphalotricha* and *N*-methylsarpagine, an indole alkaloid that was first isolated from *Rauvolfia vomitoria*. This is the first report of the isolation of lupeol and *N*-methylsarpagine from the *Rauvolfia* genus and *R. caffra*, respectively.

The structures of compounds **59**, **61, 63** and **64** were confirmed unambiguously. However, the structure elucidation of compounds **60** and **62** was not possible due to the lingering presence of impurities in the samples. As a consequence of this, it is recommended that detailed chemical dissection of these plant species should be continued under optimized conditions.

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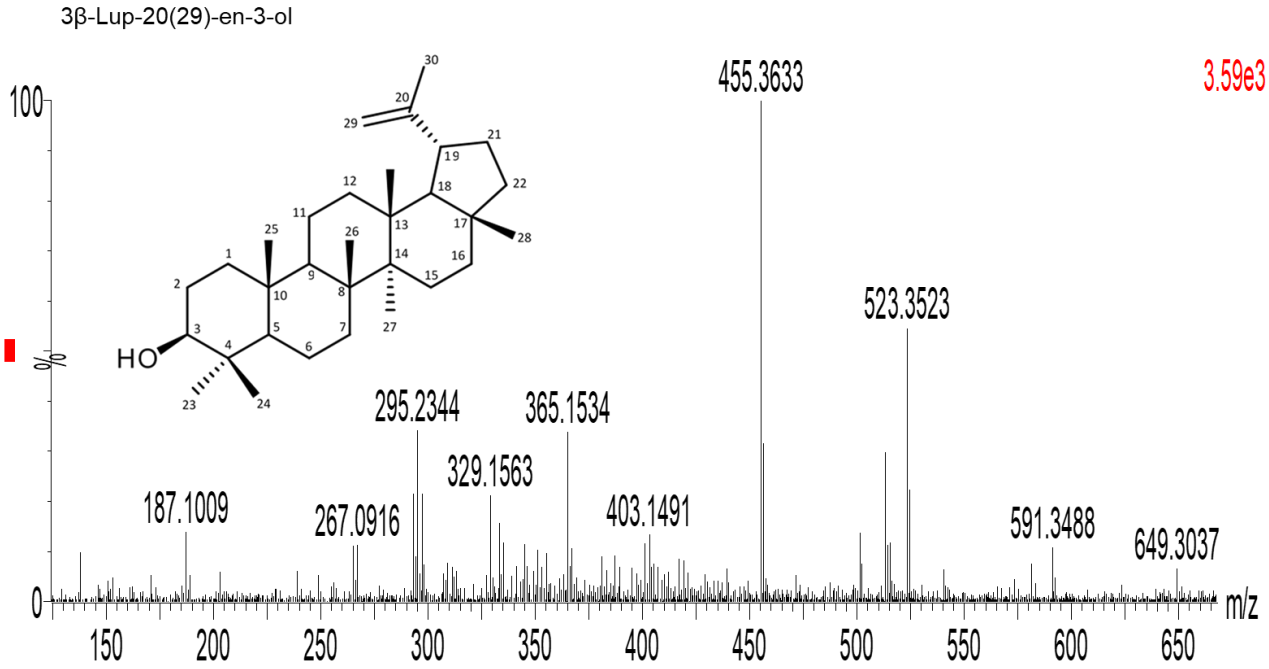
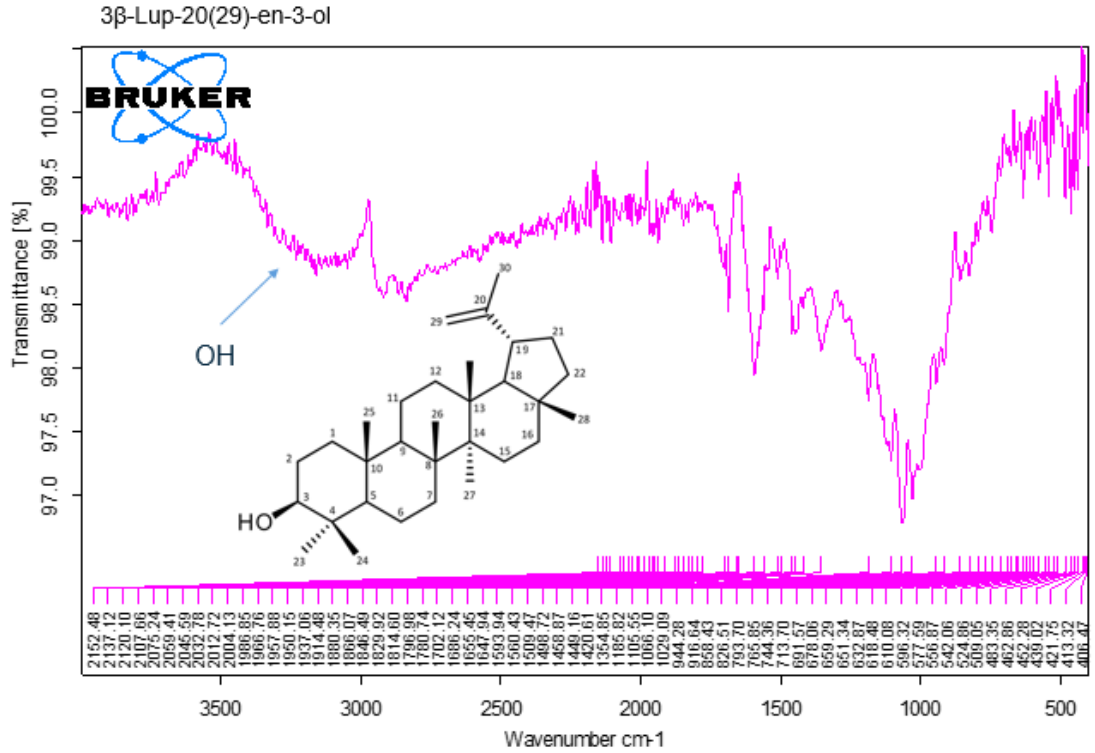
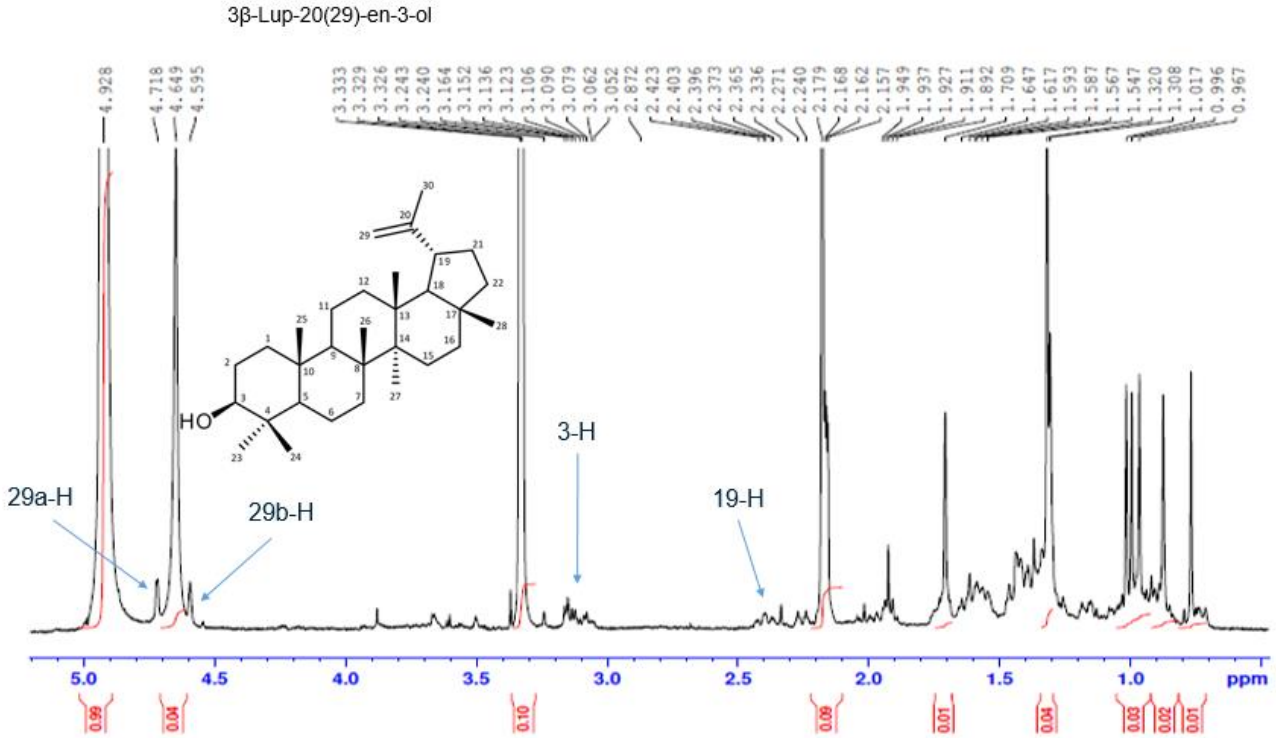
Appendix



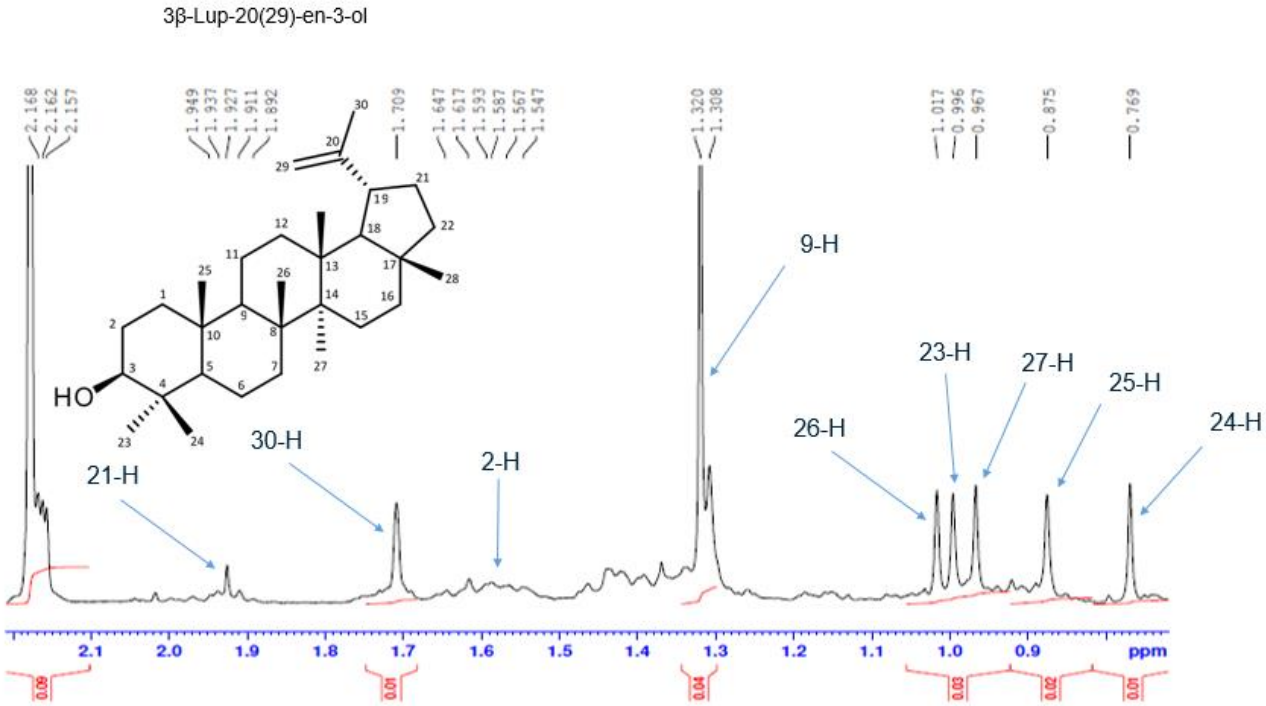
Appendix 1: Mass spectrum of lupeol (**59**).



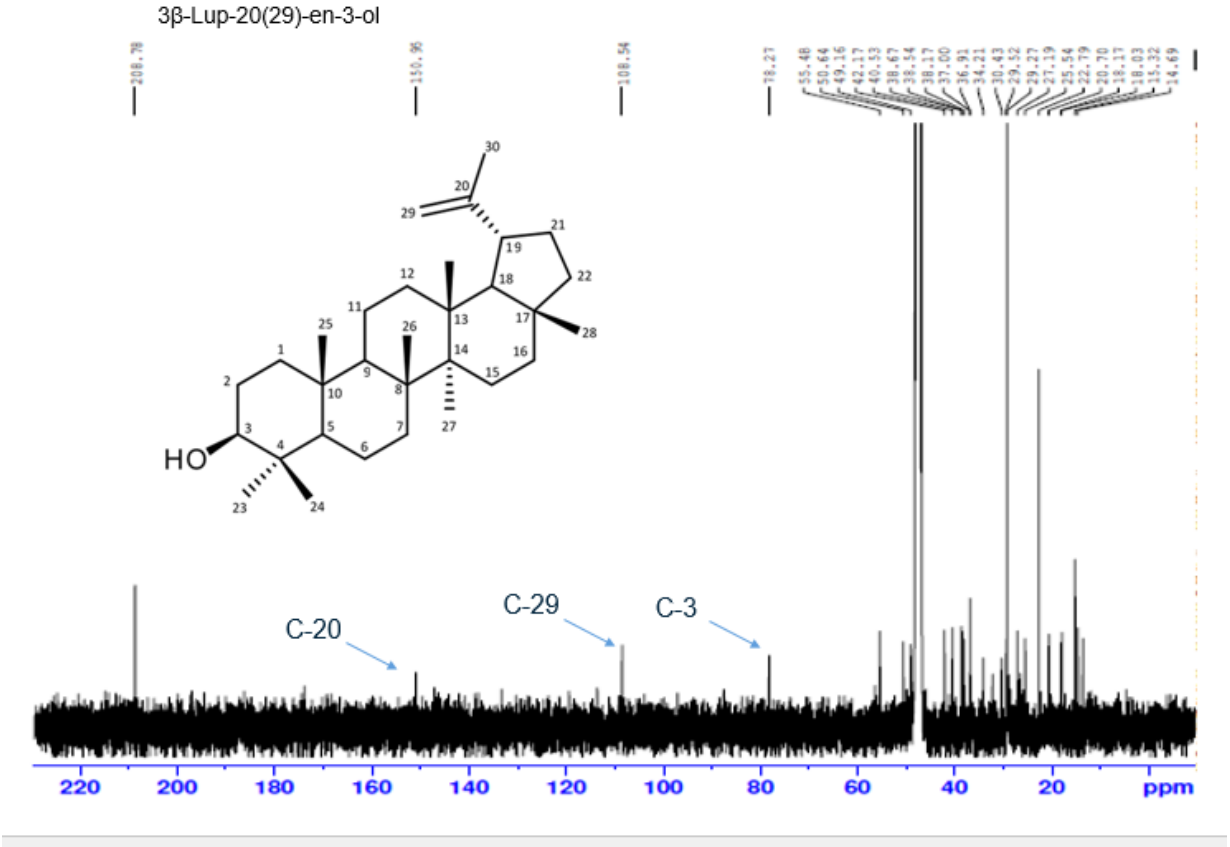
Appendix 2: IR spectrum of lupeol (**59**).



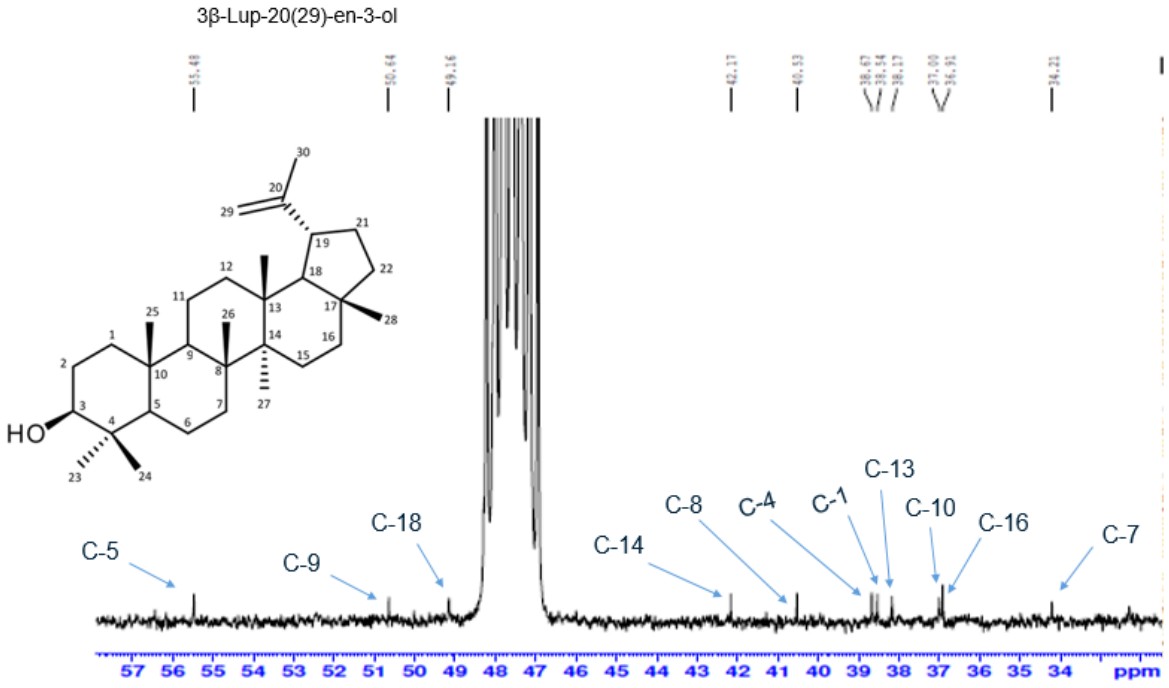
Appendix 3A: Expanded ^{1}H-NMR spectrum of lupeol (**59**).



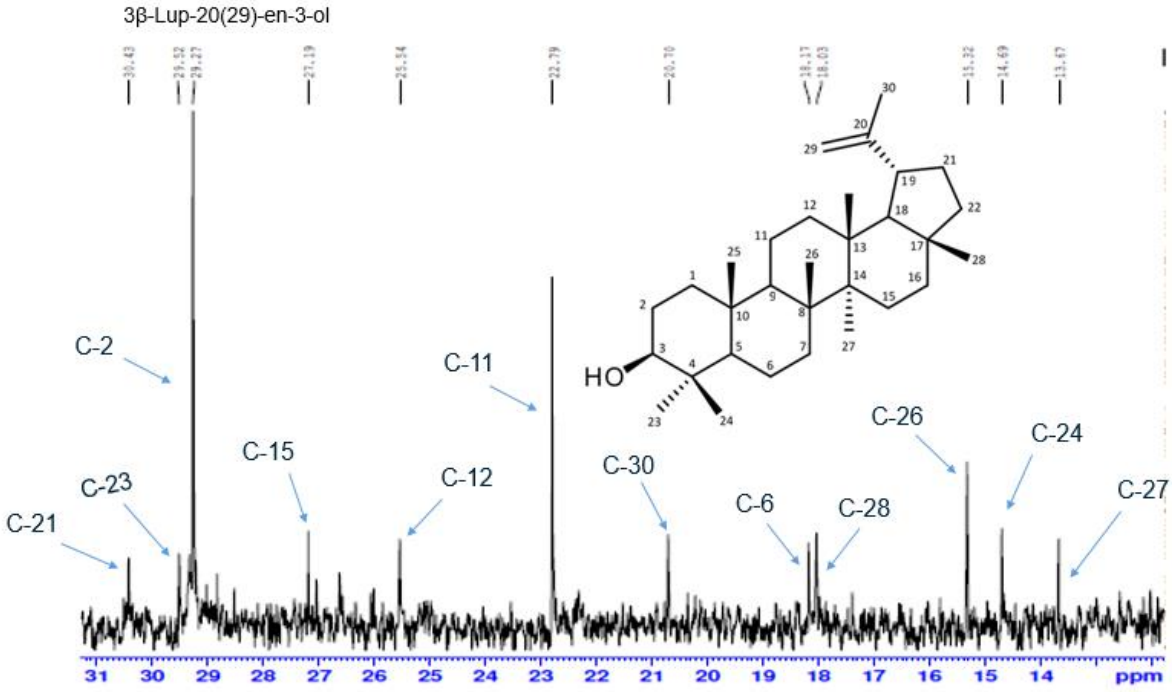
Appendix 3B: Expanded ^{1}H-NMR spectrum of lupeol (**59**).



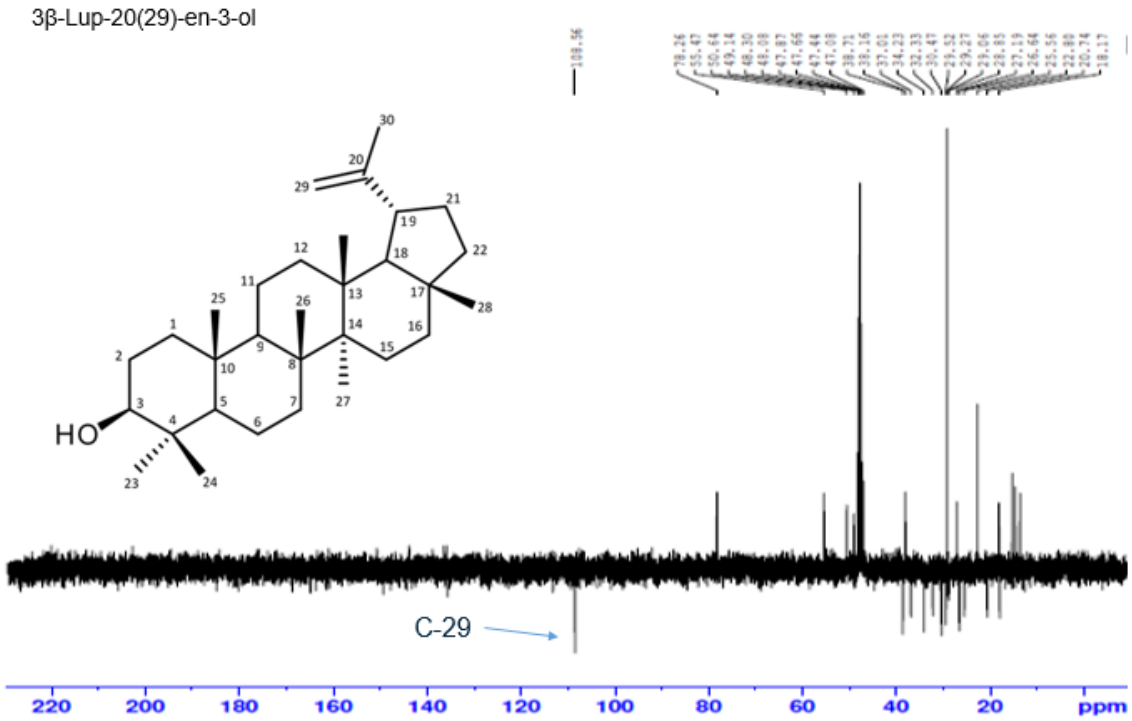
Appendix 4A: ^{13}C-NMR spectrum of lupeol (**59**).



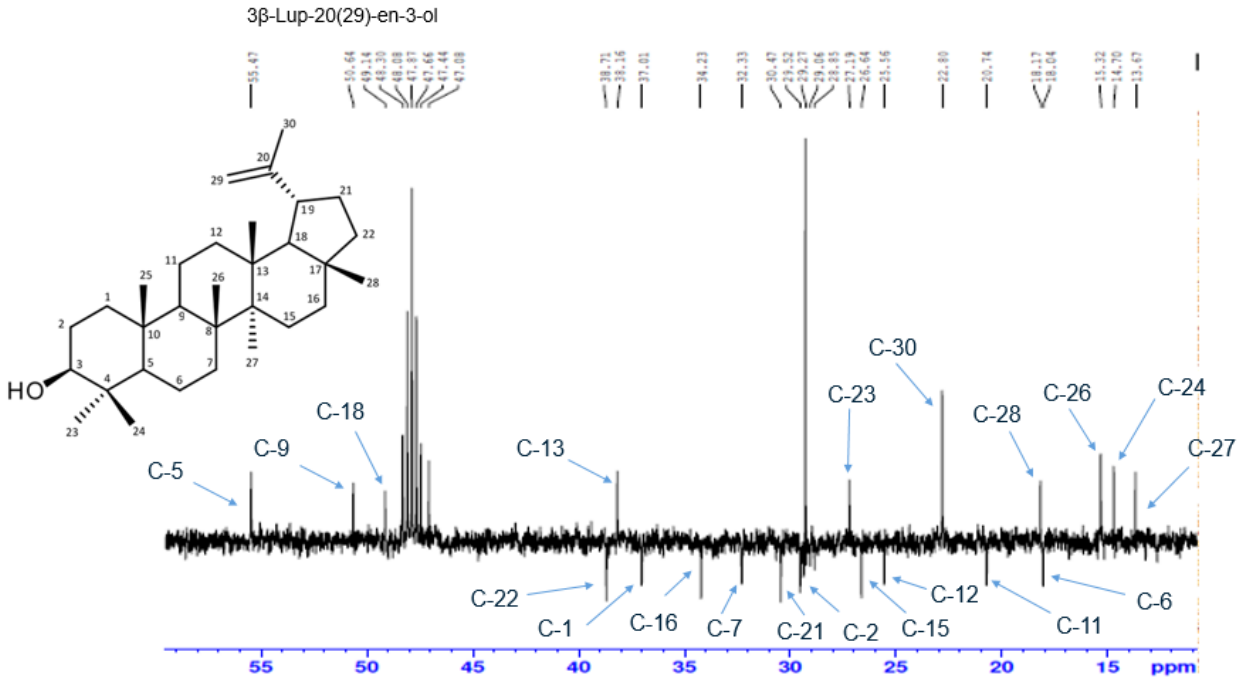
Appendix 4B: ^{13}C-NMR spectrum of lupeol (**59**).



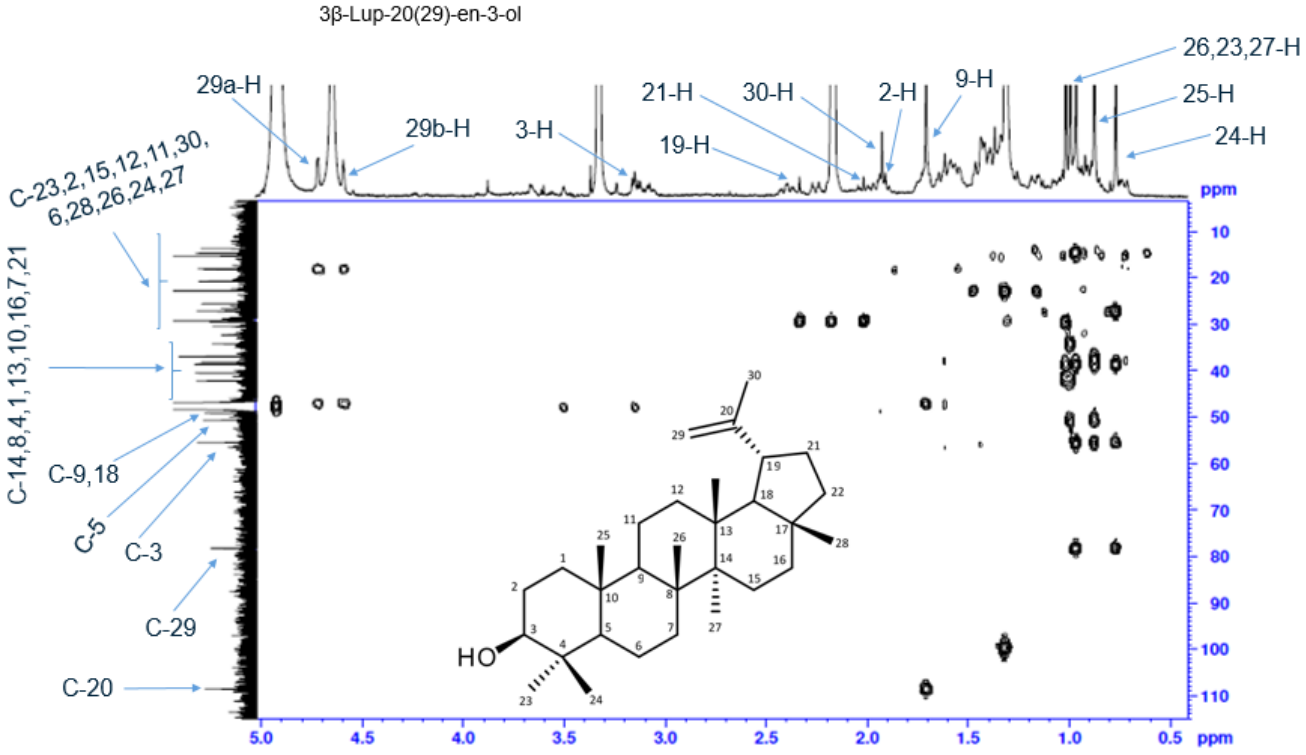
Appendix 4C: ^{13}C-NMR spectrum of lupeol (**59**).



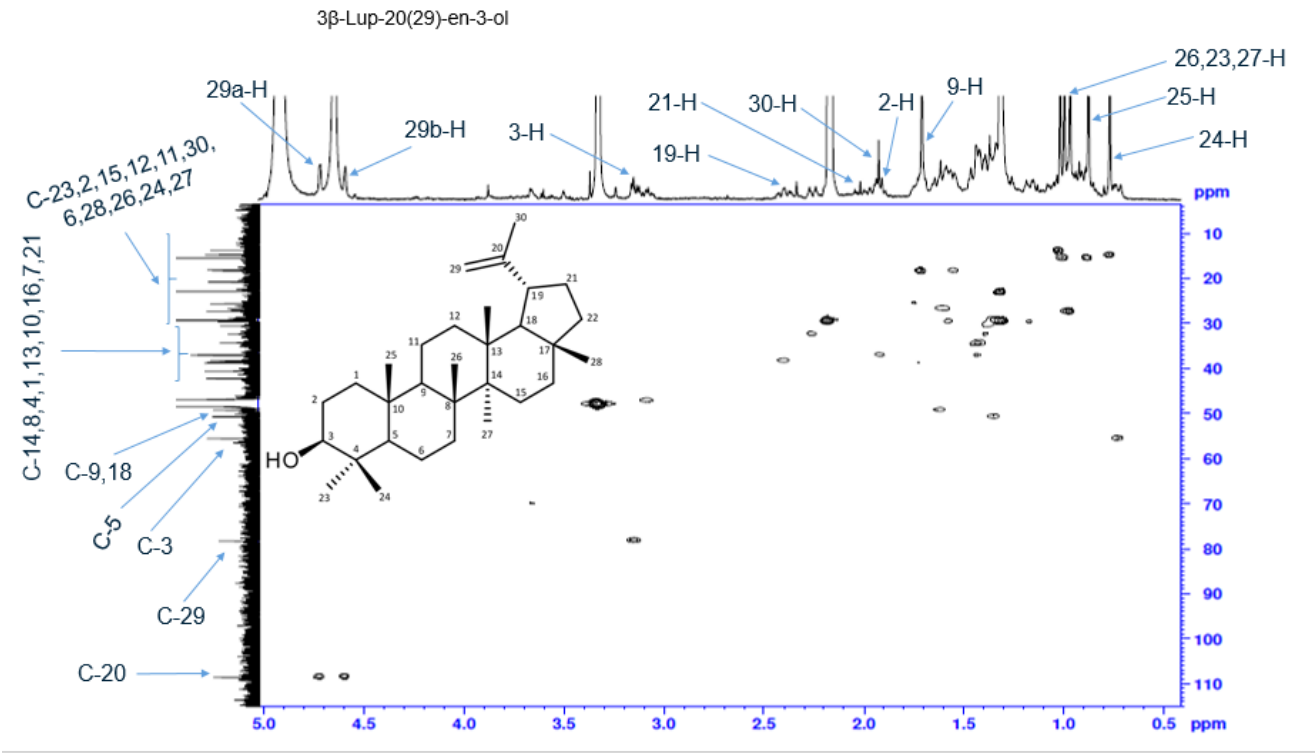
Appendix 5A: DEPT 135 spectrum of lupeol (**59**).



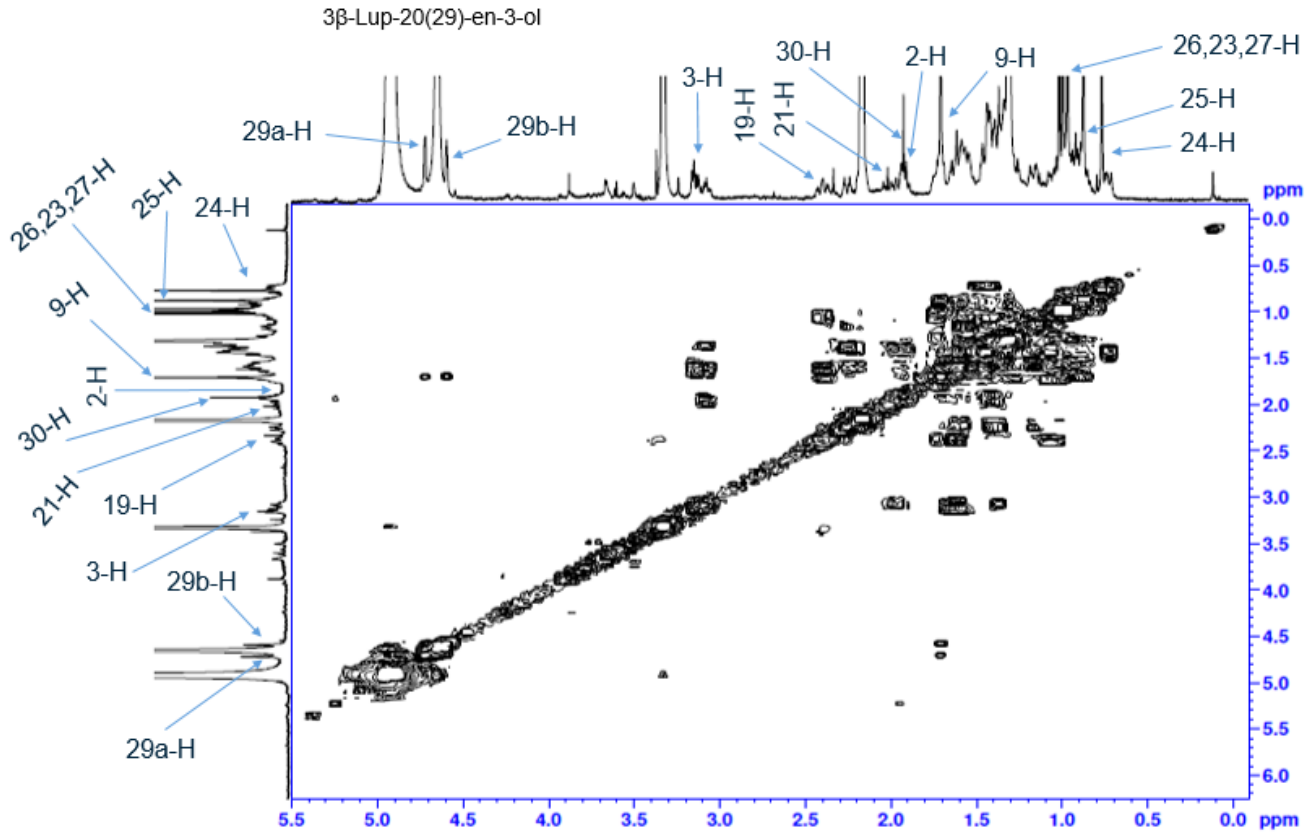
Appendix 5B: DEPT 135 spectrum of lupeol (**59**).



Appendix 6: HMBC spectrum of lupeol (**59**).

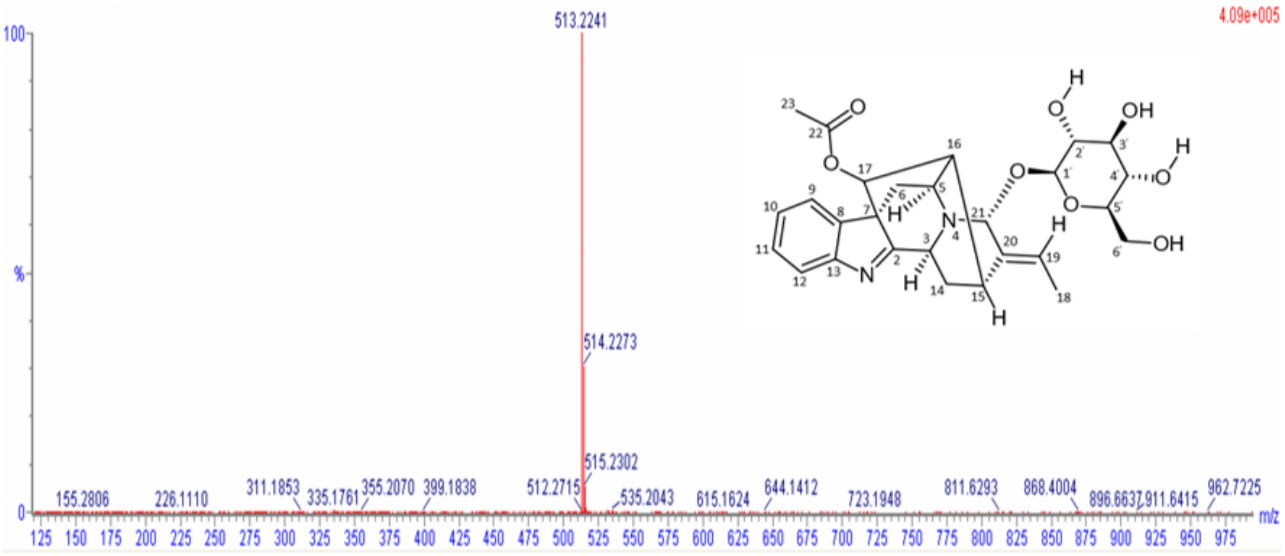


Appendix 7: HSQC spectrum of lupeol (**59**).



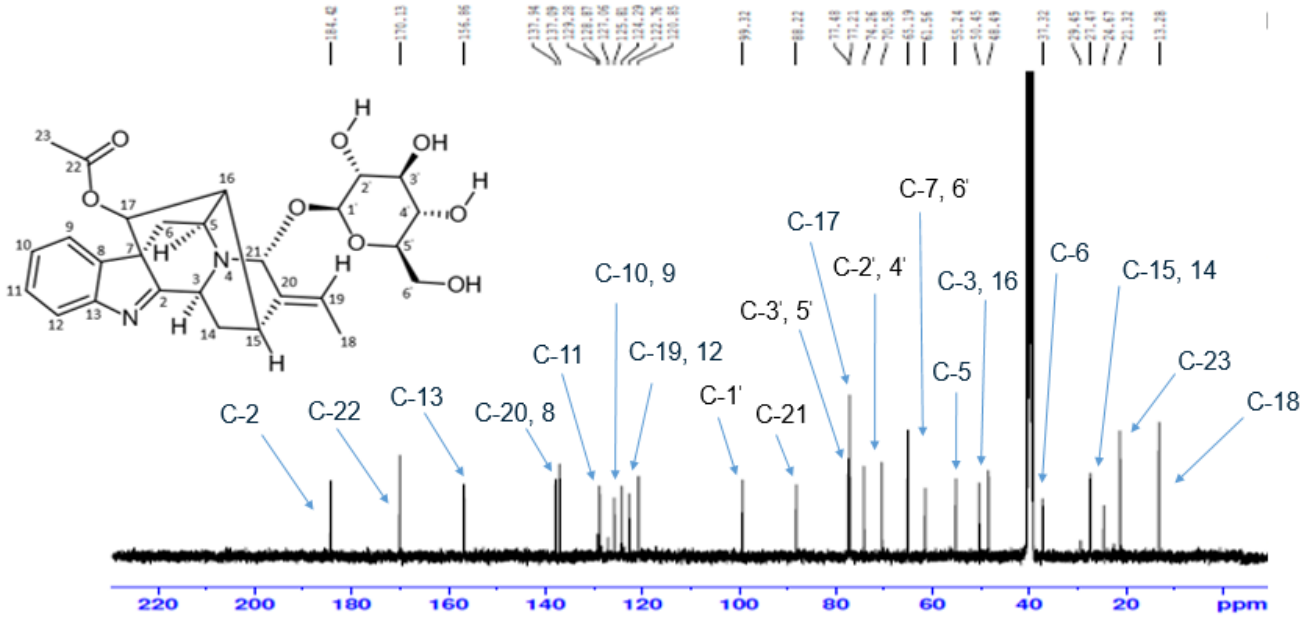
Appendix 8: COSY spectrum of lupeol (**59**).

(16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate



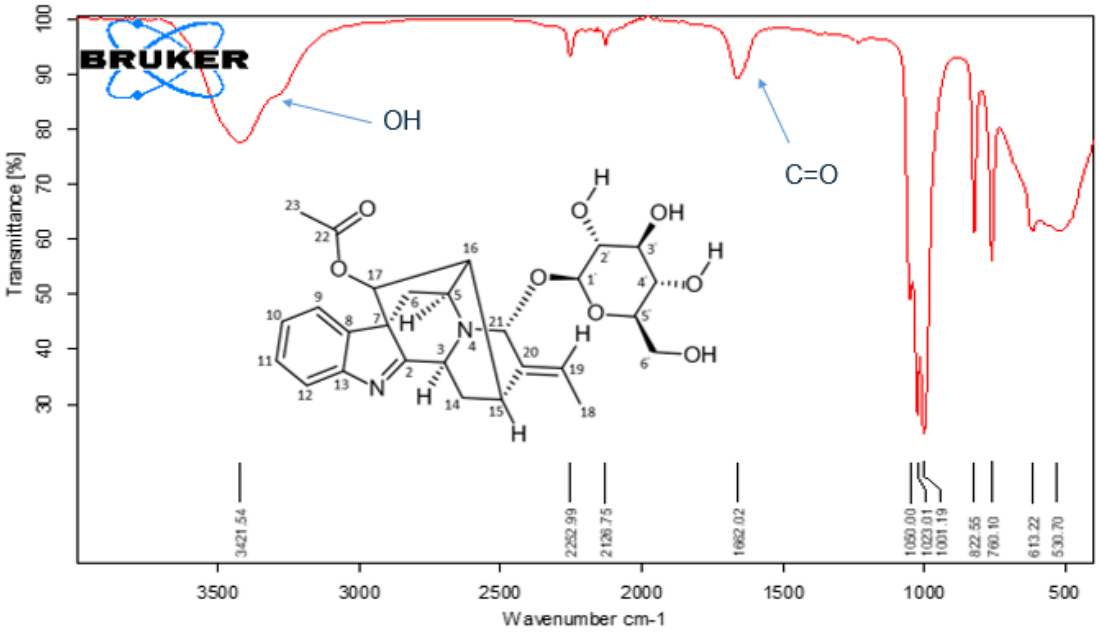
Appendix 9: Mass spectrum of raucaffricine (**61**).

(16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate



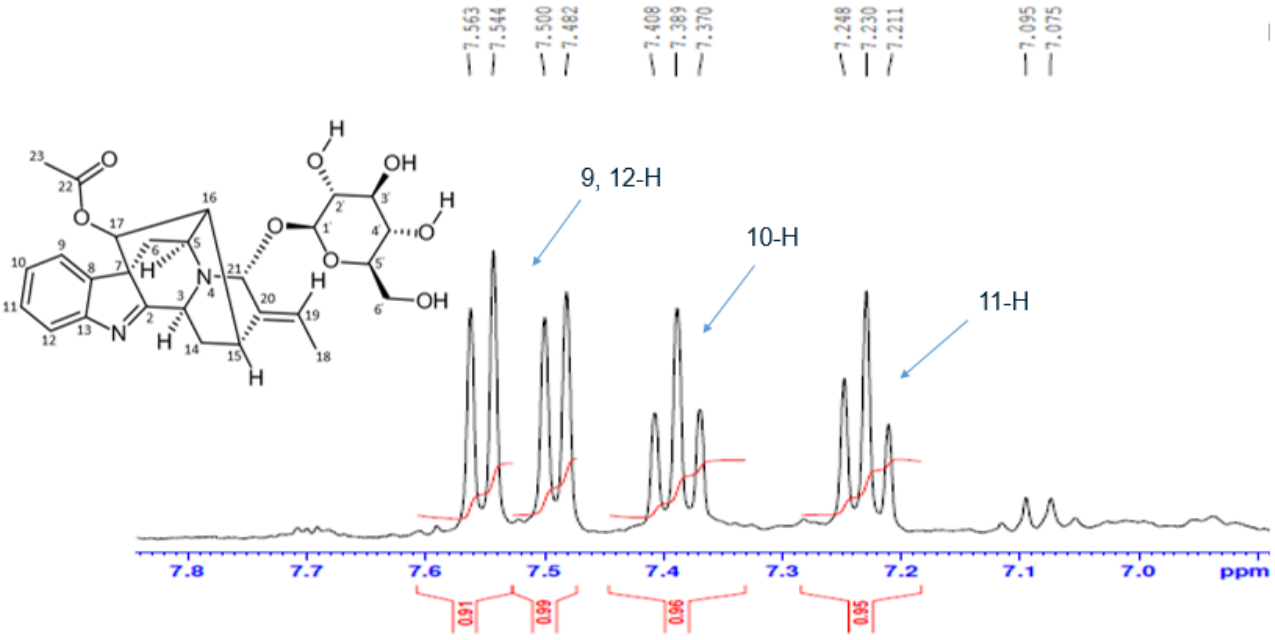
Appendix 10: ^{13}C-NMR spectrum of raucaffricine (**61**).

(16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate



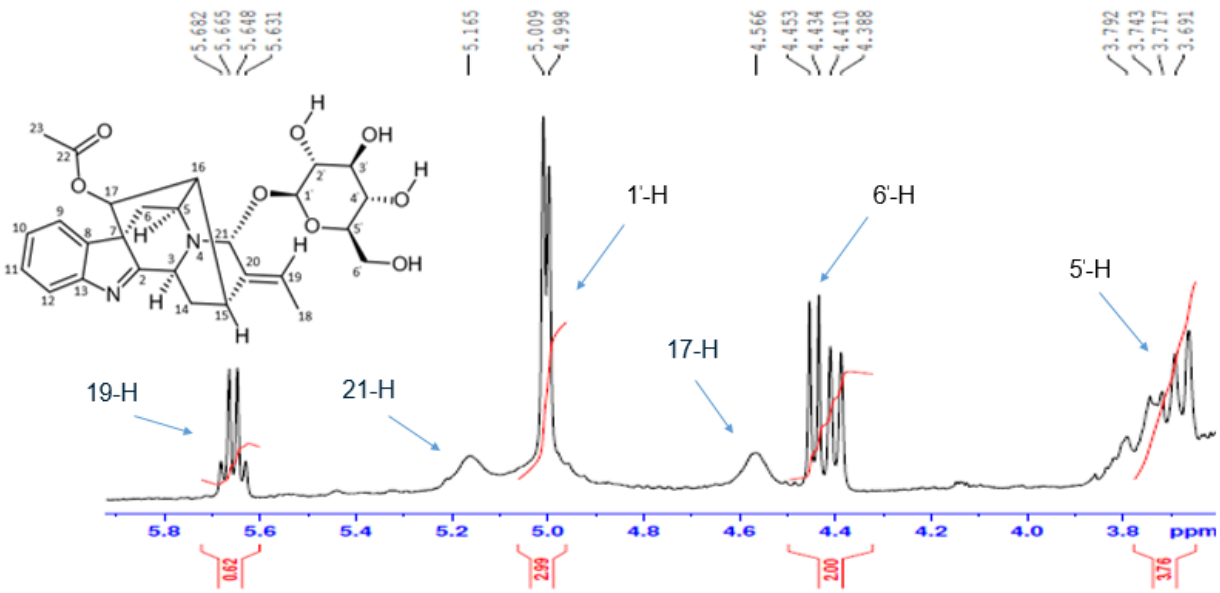
Appendix 11: IR spectrum of raucaffricine (**61**).

(16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate



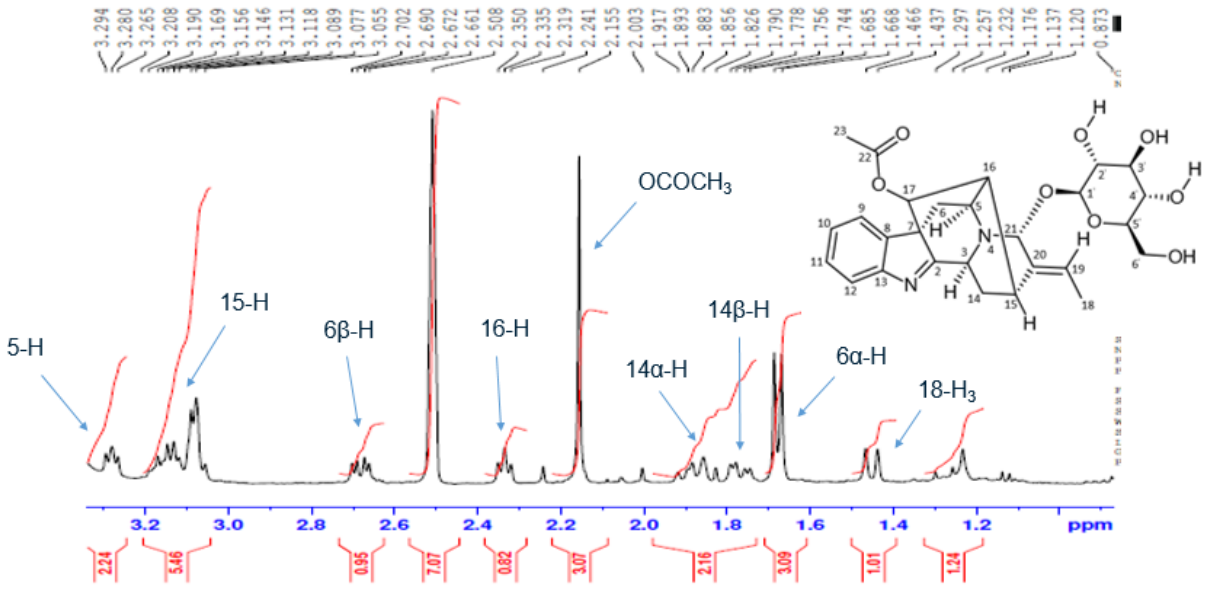
Appendix 12A: Expanded ^{1}H-NMR spectrum of raucaffricine (**61**).

(16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate

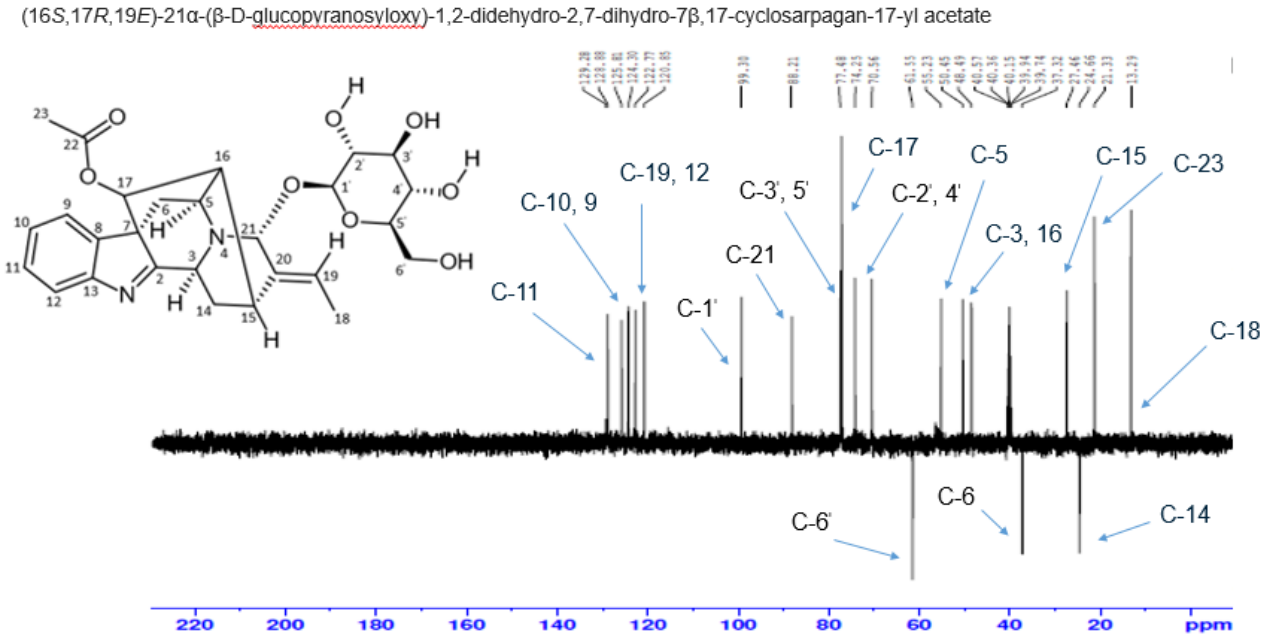


Appendix 12B: Expanded ^{1}H-NMR spectrum of raucaffricine (**61**).

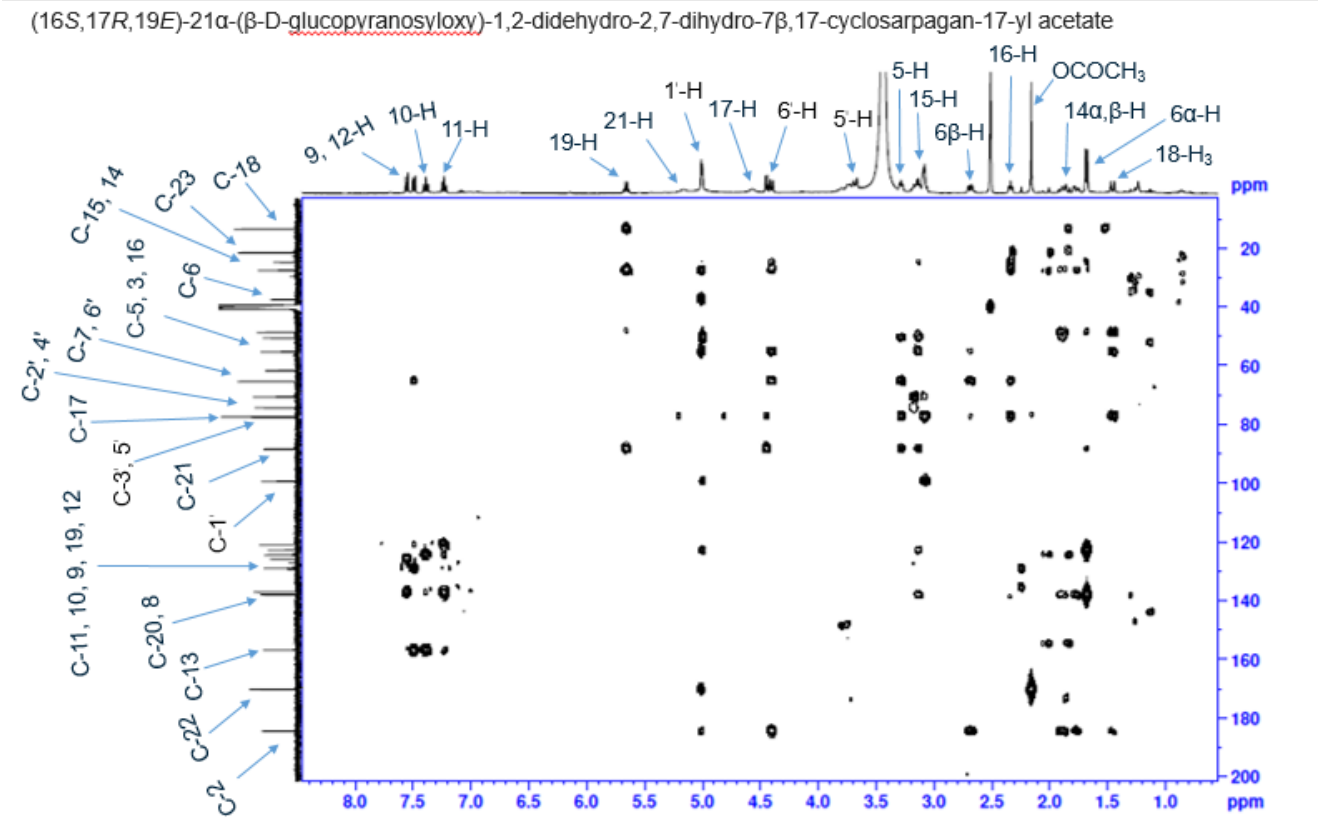
(16*S*,17*R*,19*E*)-21α-(β-D-glucopyranosyloxy)-1,2-didehydro-2,7-dihydro-7β,17-cyclosarpagan-17-yl acetate



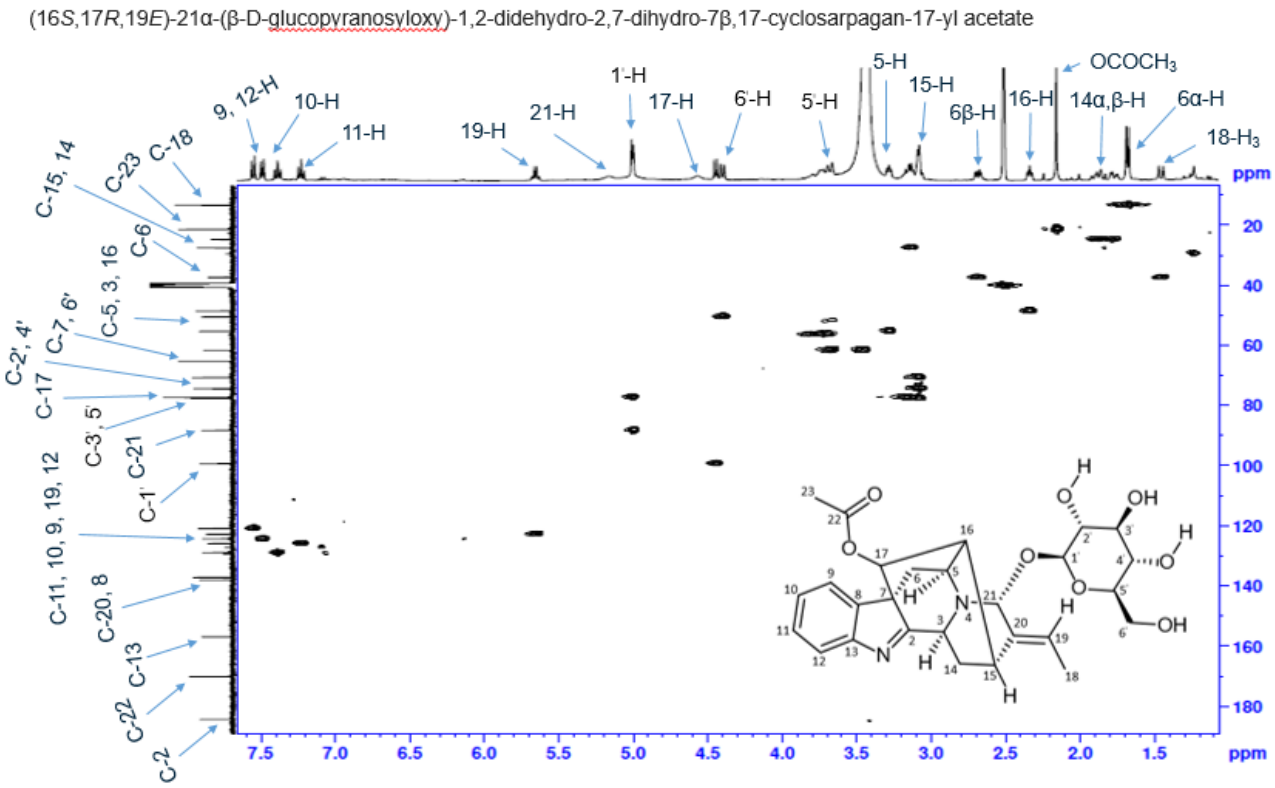
Appendix 12C: Expanded ^{1}H-NMR spectrum of raucaffricine (**61**).



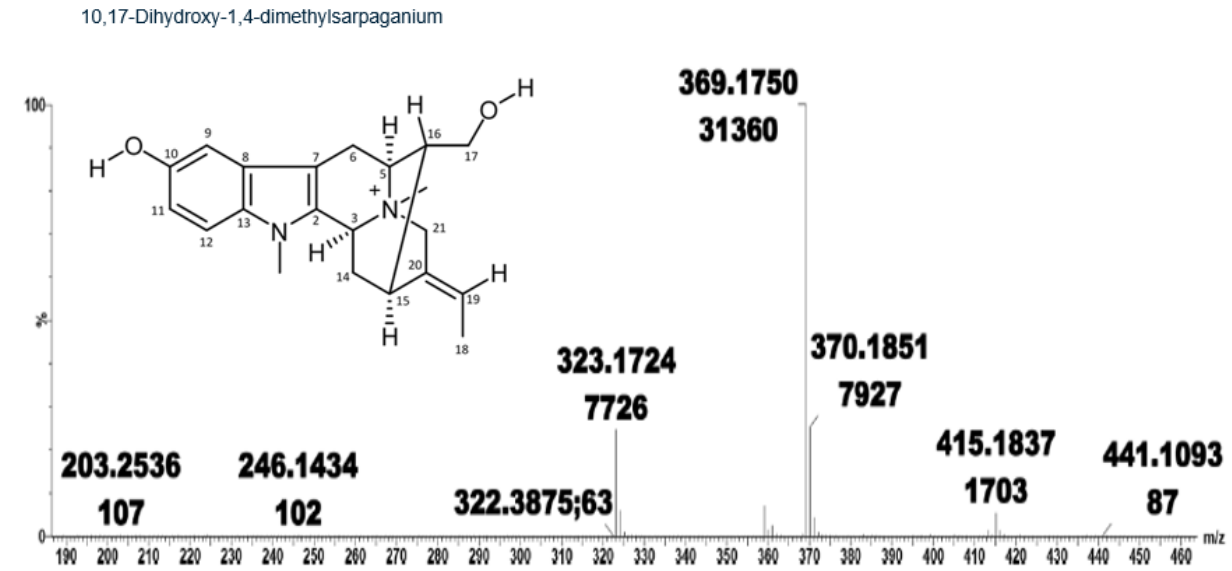
Appendix 13: DEPT 135 spectrum of raucaffricine (**61**).



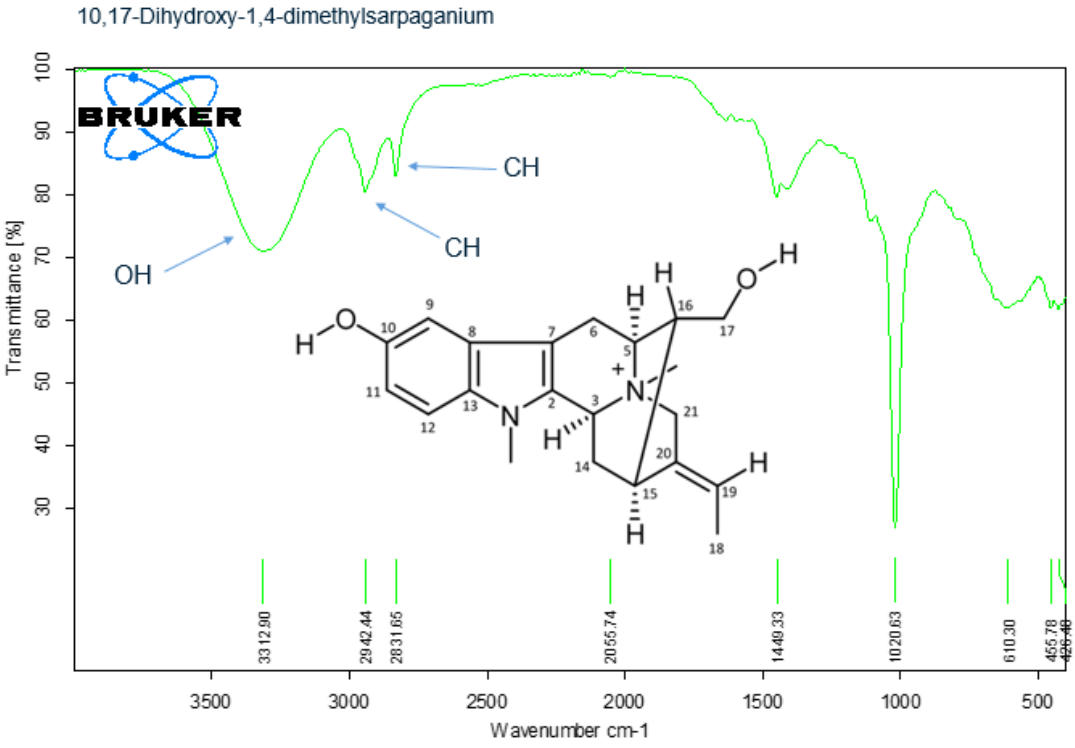
Appendix 14: HMBC spectrum of raucaffricine (**61**).



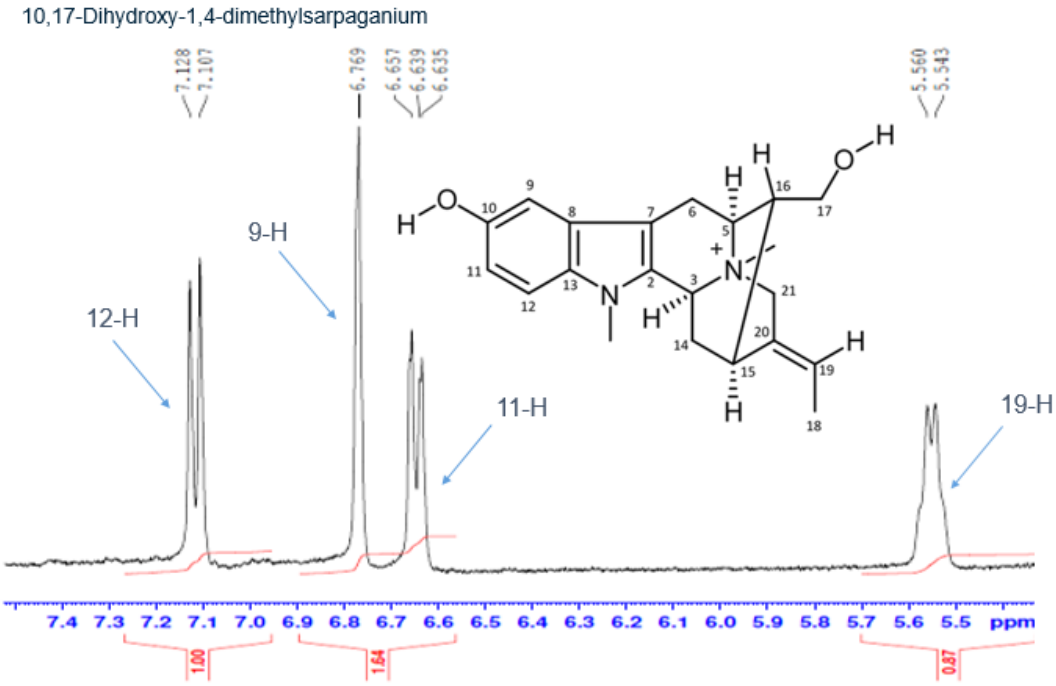
Appendix 15: HSQC spectrum of raucaffricine (**61**).



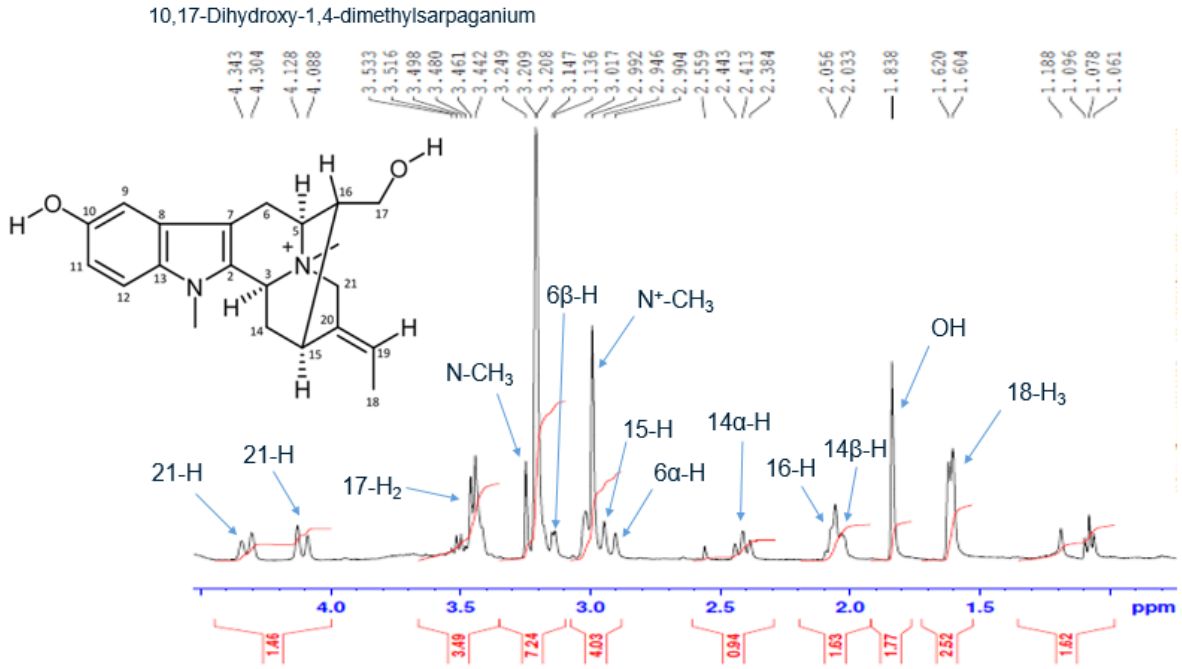
Appendix 16: Mass spectrum of *N*-methylsarpagine (**63**).



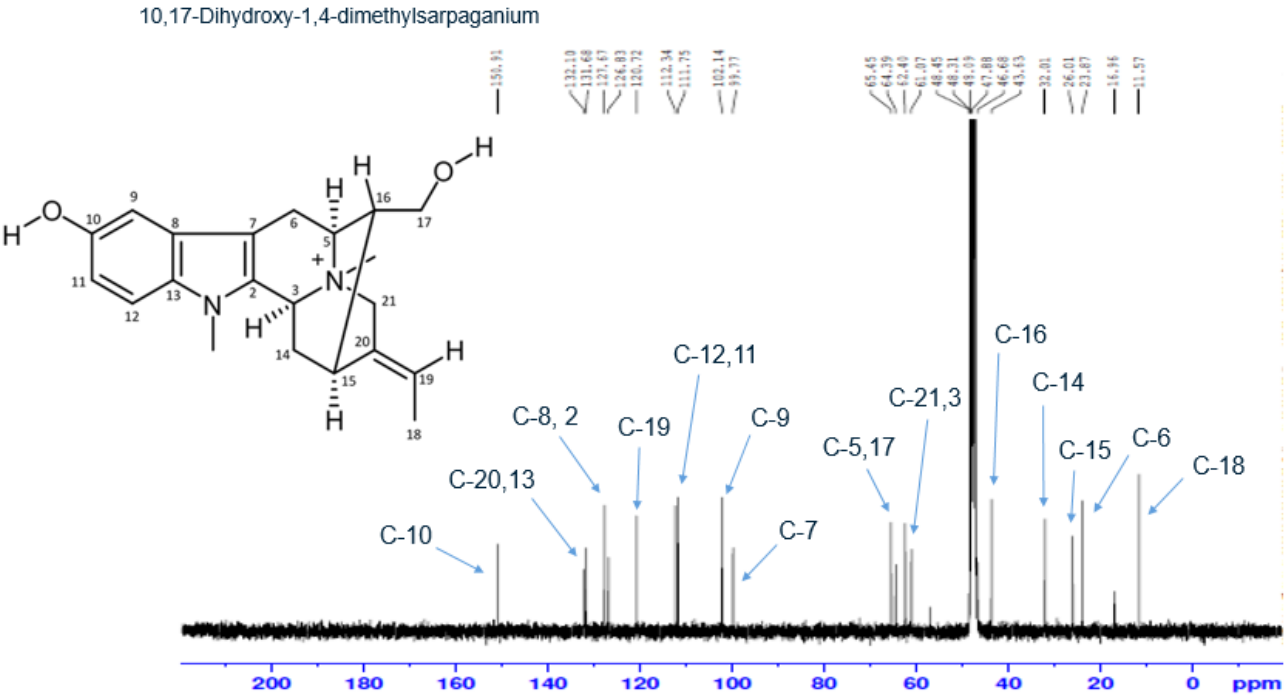
Appendix 17: IR spectrum of *N*-methylsarpagine (**63**).



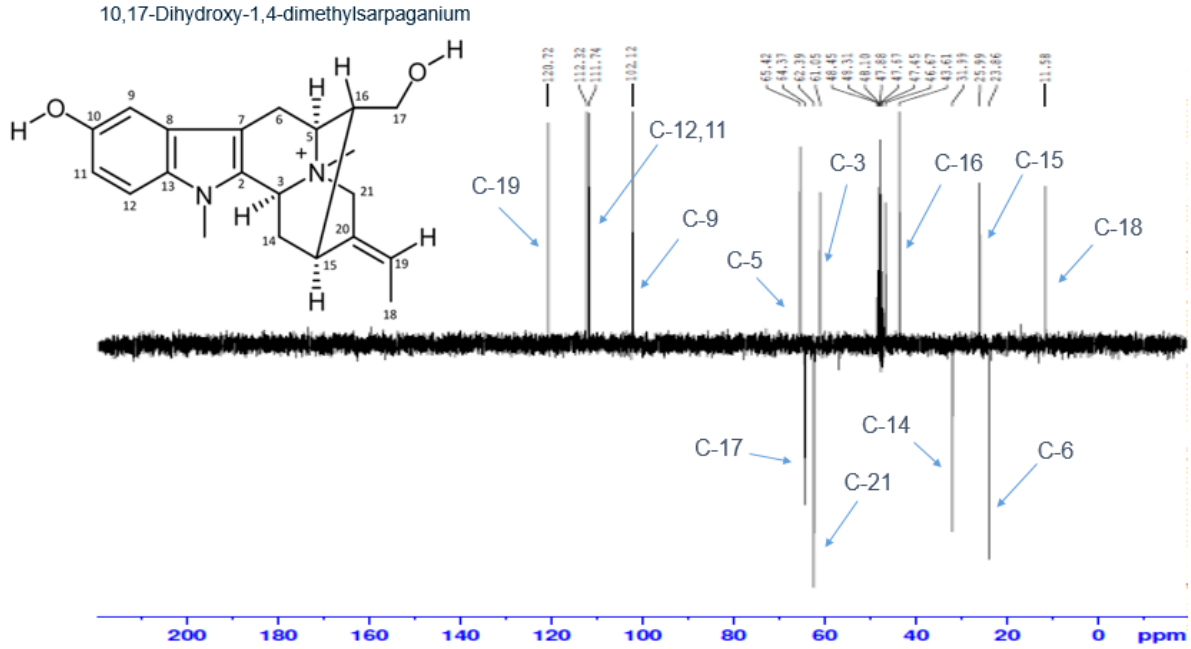
Appendix 18A: Expanded ^{1}H-NMR spectrum of *N*-methylsarpagine (**63**).



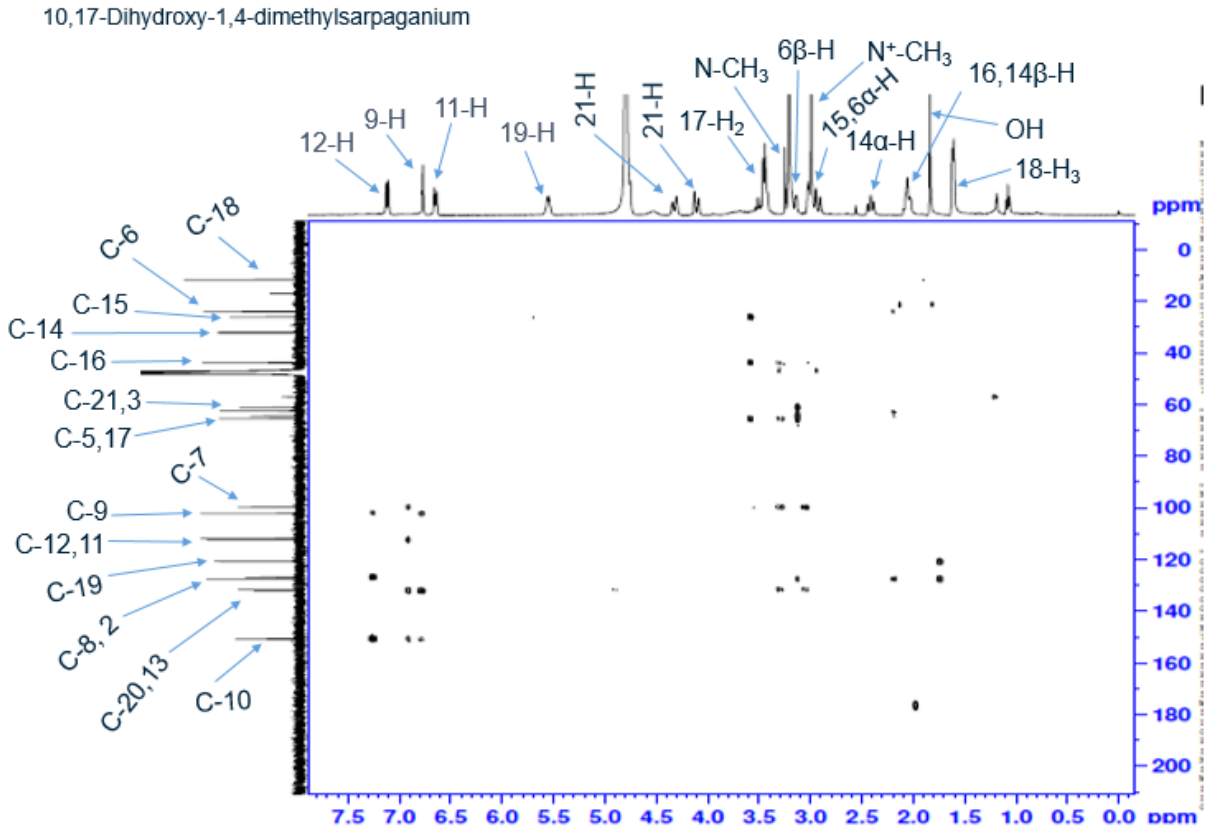
Appendix 18B: Expanded ^{1}H-NMR spectrum of *N*-methylsarpagine (**63**).



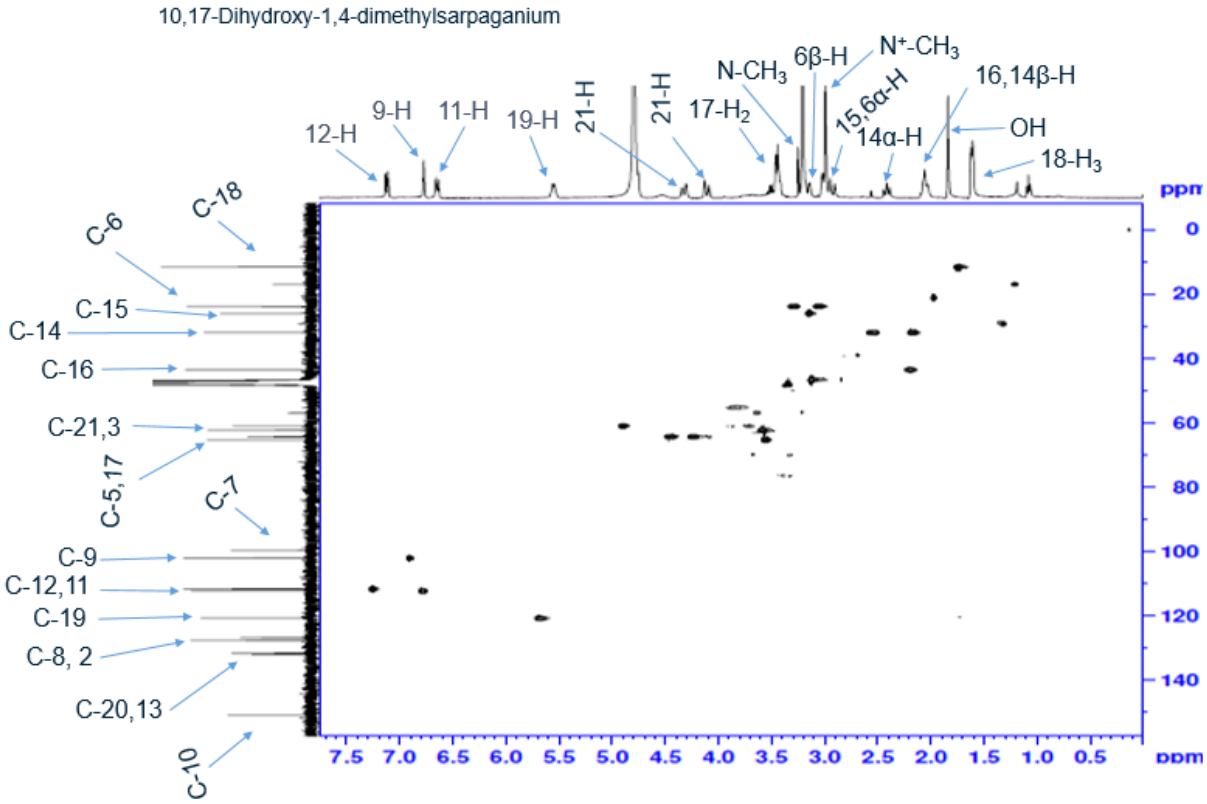
Appendix 19: ^{13}C-NMR spectrum of *N*-methylsarpagine (**63**).



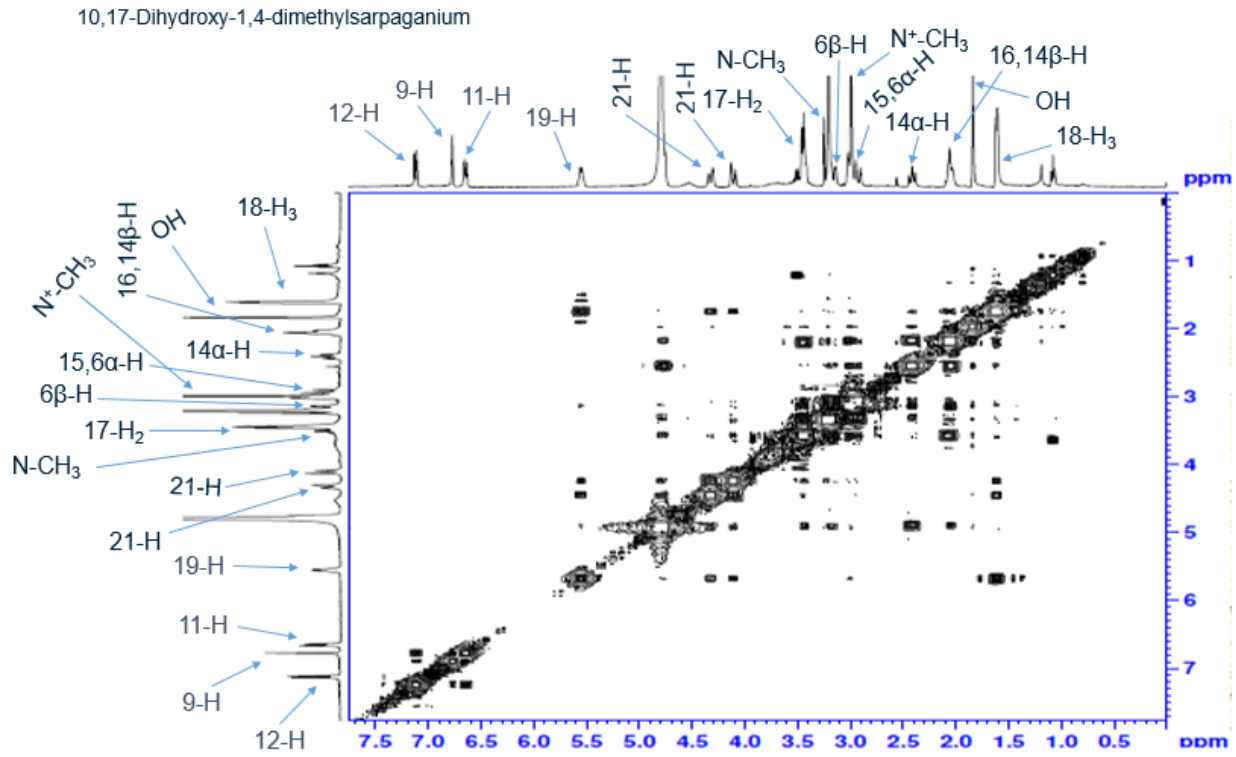
Appendix 20: DEPT 135 spectrum of *N*-methylsarpagine (**63**).



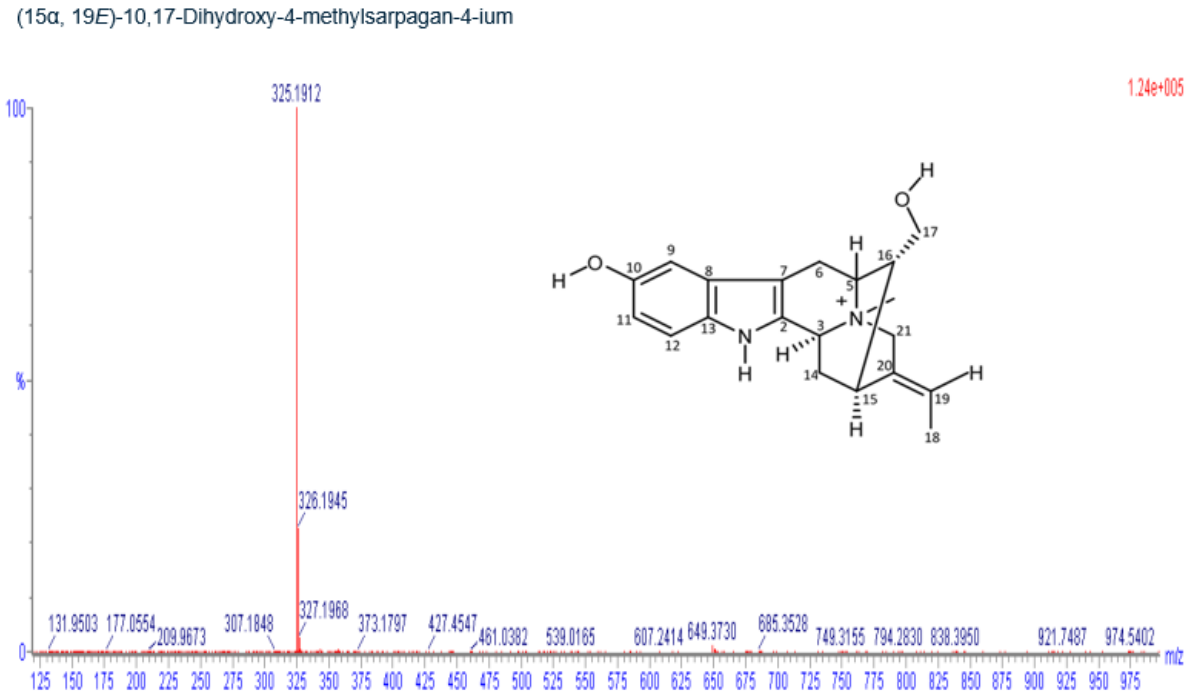
Appendix 21: HMBC spectrum of *N*-methylsarpagine (**63**).



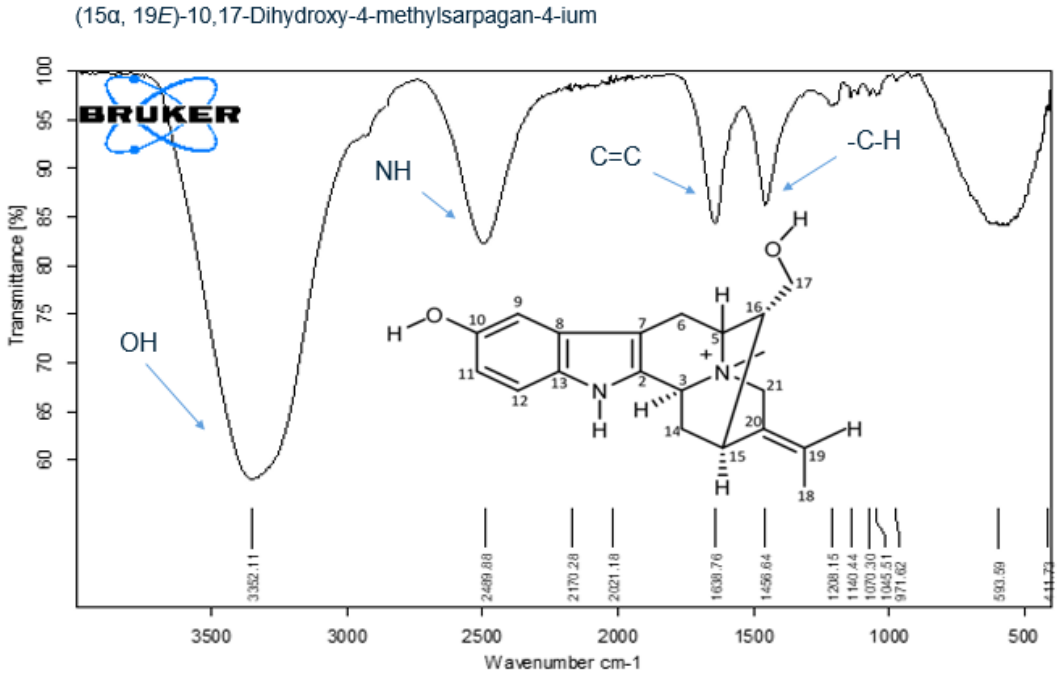
Appendix 22: HSQC spectrum of *N*-methylsarpagine (**63**).



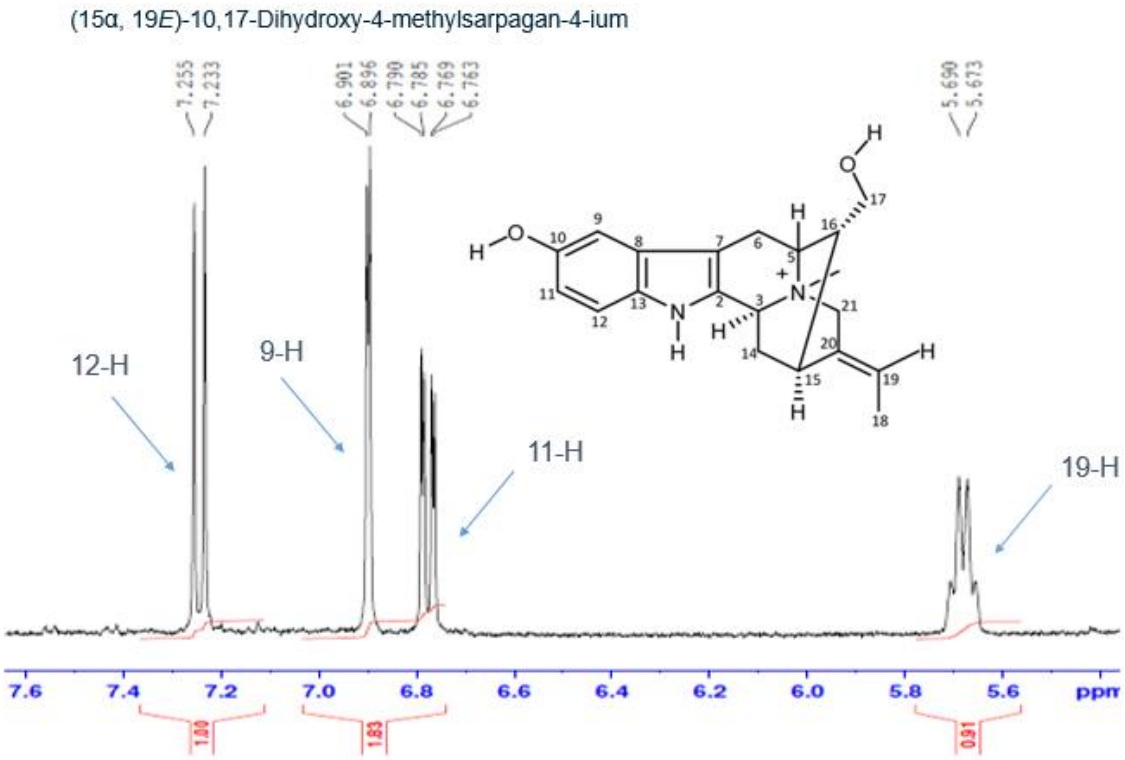
Appendix 23: COSY spectrum of *N*-methylsarpagine (**63**).



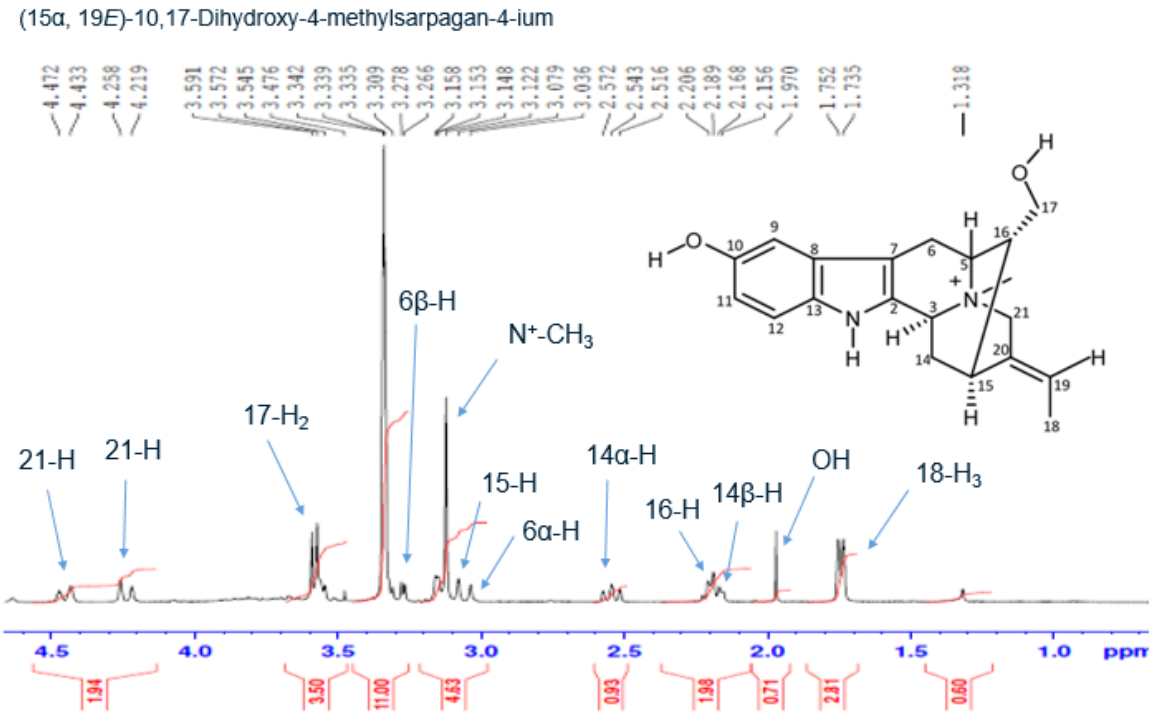
Appendix 24: Mass spectrum of spegatrine (**64**).



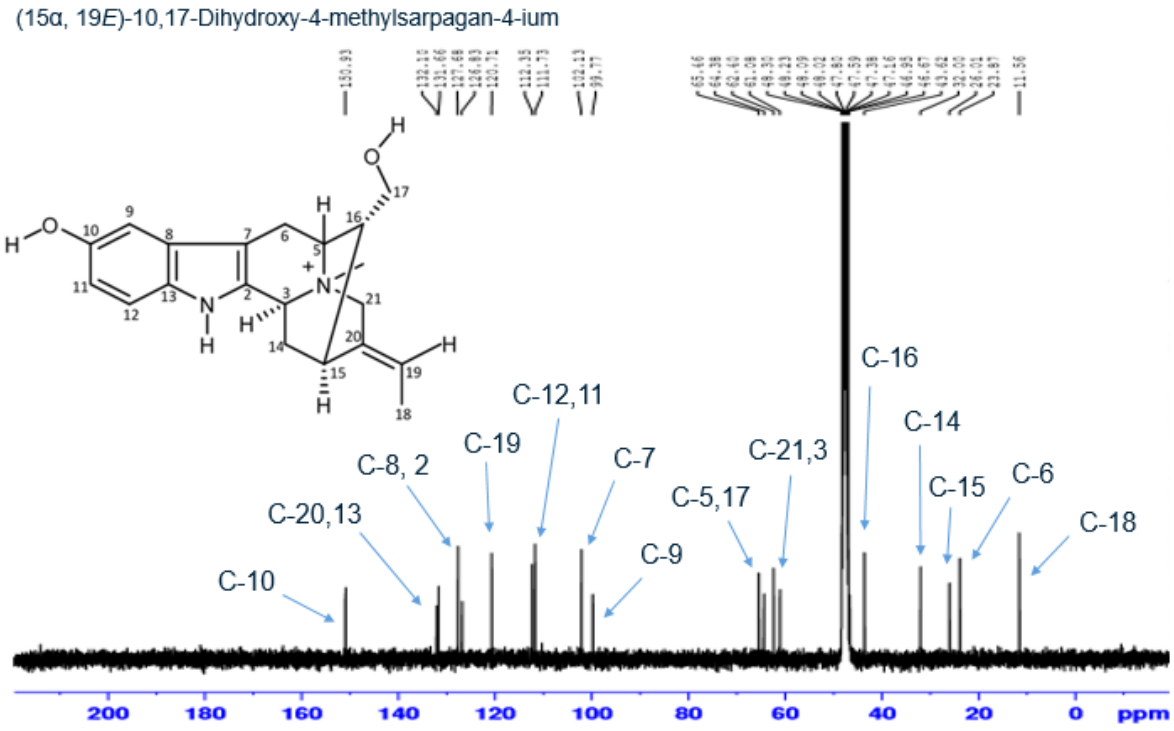
Appendix 25: IR spectrum of spegatrine (**64**).



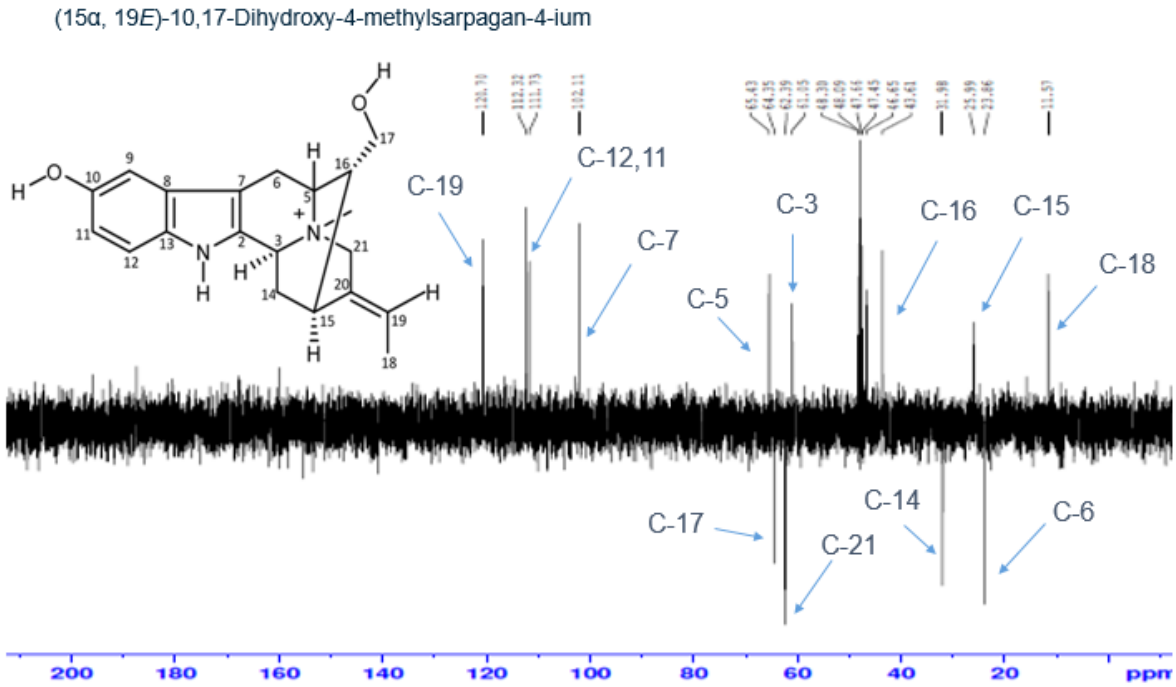
Appendix 26A: Expanded ^{1}H-NMR spectrum of spegatrine (**64**).



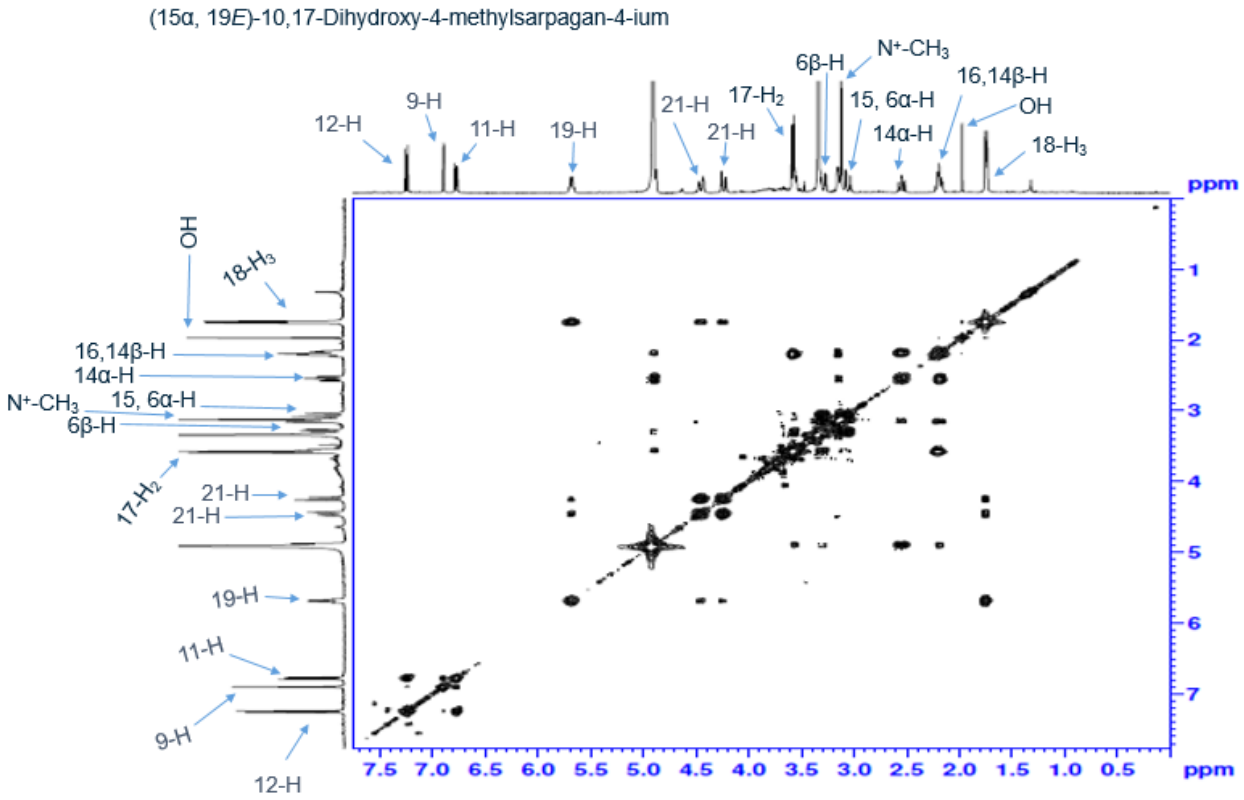
Appendix 26B: Expanded ^{1}H-NMR spectrum of spegatrine (**64**).



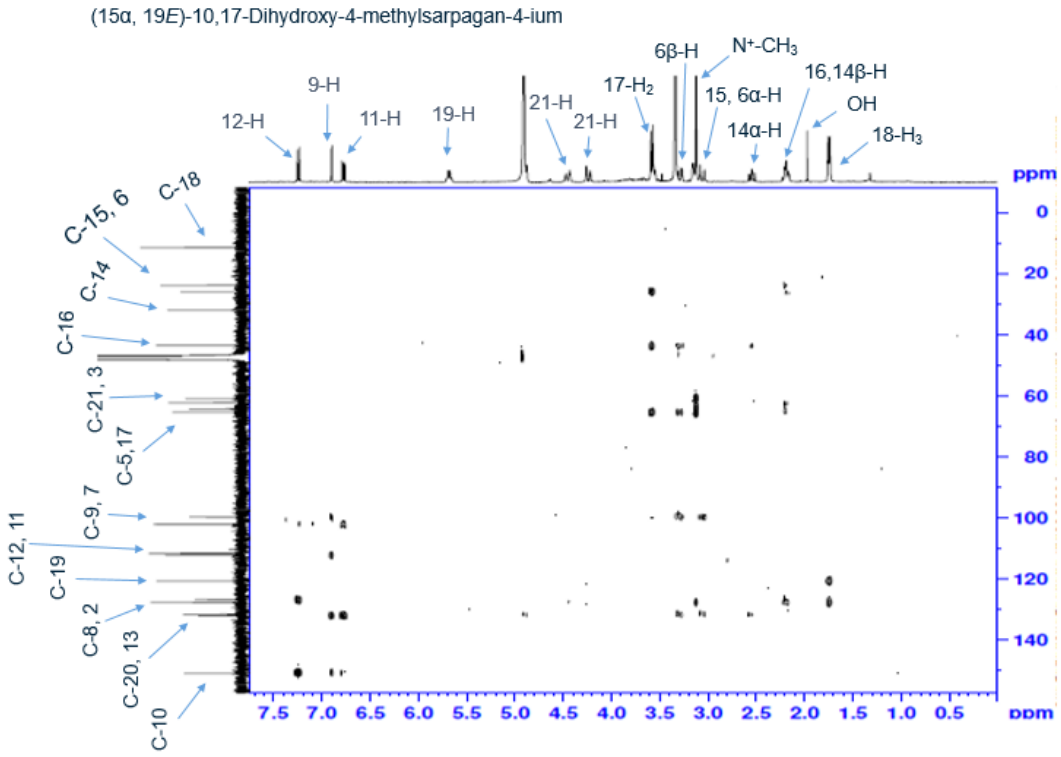
Appendix 27: ^{13}C-NMR spectrum of spegatrine (**64**).



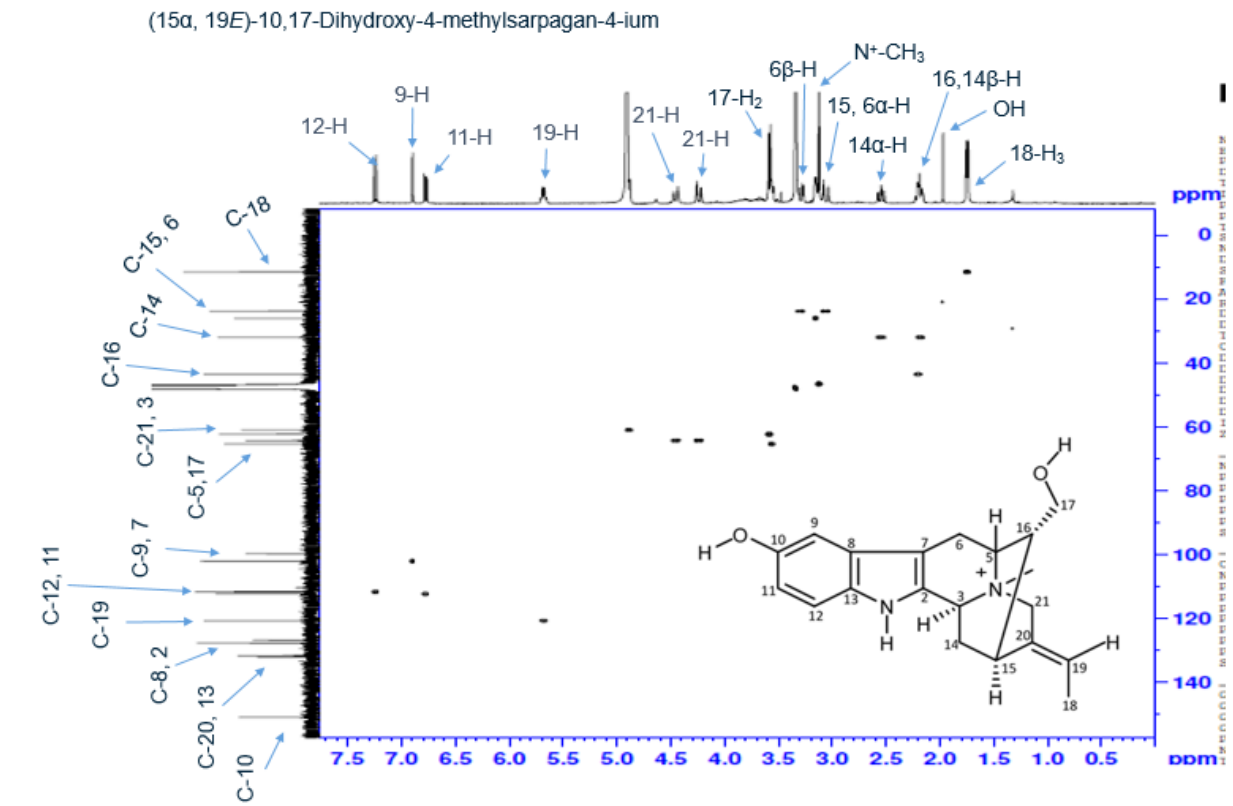
Appendix 28: DEPT 135 spectrum of spegatrine (**64**).



Appendix 29: COSY spectrum of spegatrine (**64**).



Appendix 30: HMBC spectrum of spegatrine (**64**).



Appendix 31: HSQC spectrum of spegatrine (**64**).