PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITY STUDIES OF AN EASTERN CAPE MEDICINAL PLANT, *STRYCHNOS HENNINGSII*

BY

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DISSERTATION

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

MAGISTER TECHNOLOGIAE

IN

CHEMISTRY

IN THE

FACULTY OF SCIENCE

AT THE

NELSON MANDELA UNIVERSITY

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DECLARATION

I, Mondeli Mngoma (student number: 211106682), hereby declare that the dissertation for Magister Technologiae (MTech) is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

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Mondeli Mngoma

ACKNOWLEDGEMENTS

I would like to thank the following people;

My supervisor, Dr B. Hlangothi for all her hard work, assistance and motivation. This work would not only be impossible without a supervisor like you, and I would not have discovered my love for the laboratory. I sincerely thank you.

I thank my mother every day, for everything she's ever done for our family. She is and will always be our rock. I also thank my siblings, who made TTUMMS as strong as it is today.

My whole family for cheering me on when I would be caught between a rock and a hard place.

My friends who have helped me try and enjoy this little thing called life.

The BGH research family, who have helped me gain confidence and laugh at my jokes.

The Botany, Biochemistry departments and Dr R. Betz's group for all their assistance.

The Nelson Mandela Metropolitan University for their assistance and opportunities they have presented my way.

The National Research Foundation (NRF) for financially supporting aspiring researchers all over South Africa.

ABSTRACT

This project sees the chemical investigation of an Eastern Cape medicinal plant, *Strychnos henningsii*. The aim of this project was to investigate the phytoconstituents and activity of organic extracts of *S. henningsii* as well as isolation and characterization of single compounds.

S. henningsii is one of the most widely used tree bark in the Eastern Cape in treating a variety of ailments. Evaluation of the traditional herbal use of the *S. henningsii* bark was warranted. Both the ethyl acetate and methanol extract proved to possess non-toxic properties and showed cell growth potential at low concentrations. The anti-inflammatory response of both extracts showed appreciable results, and they did not promote inflammatory due to the alkaloidal presence. Although the extracts both showed the presence of phenols, the anti-oxidant capacity by DPPH, FRAP, and ORAC assays was considerately lower than expected.

The ethyl acetate extract presented a compound thought to be a terpenoid (**4.1**). Three compounds were isolated from the methanol extract; two alkaloidal compounds and one acrylate. The alkaloids were isolated from the methanol extract, stryvomicine (**4.2**) and an isovomicine derivative (**4.3**). Their structures were deduced from NMR (1D and 2D experiments) and HRMS spectra. This acrylate was found to be 3-(4-methylphenyl)acrylic acid via single-crystal XRD, and this is the first report of it being isolated from the *Strychnos* genus.

Profiling of *S. henningsii* was performed via Liquid Chromatography–Mass Spectroscopy. Other spectroscopic techniques utilised in the interpretation of isolated compounds included Nuclear Magnetic Resonance, Infrared, single-crystal X-Ray Diffraction and High Resolution Mass spectroscopy.

Table of Contents

List of Tables

List of Figures

Figure 28: Chemical structure of isovomicine derivative (**4.3**).

Figure 29: The molecular geometry/structure of 3-(4-methylphenyl)acrylic acid, with displacement ellipsoids and atom labels are drawn at 50% probability level.

Figure 30: Energy vs number of steps graphical representation of optimization process of 3-(4-methylphenyl)acrylic acid.

Figure 31: Ball-and-stick models of the chemical structure of 3-(4-methylphenyl)acrylic acid. Ground state (**A**) and the optimized structure *(***B**). The structures are viewed according to the *a*-vector (x-axis) in Mercury software.

List of Abbreviations

CC	column chromatography
$CDCl_3$	deuterochloroform
^{13}C NMR	Carbon Nuclear Magnetic Resonance Cosy Correlation spectroscopy
2D	two dimensional
Dept	Distortionless Enhancement by Polarization Transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGCG	epigallocatechin gallate EtOAc Ethyl acetate
EtOH	ethanol
FRAP	Ferric Reducing Ability of Plasma
^{1}H NMR	Proton Nuclear Magnetic Resonance Hex hexane
HMBC	Heteronuclear Multiple Bond Correlation
HRMS	high-resolution mass spectroscopy
HSQC	Heteronuclear Single-Quantum Correlation
Hz	hertz
IC_{50}	Bioactivity at 50% concentration
IR	Infrared
J	coupling constant
LC-MS	liquid chromatography-mass spectroscopy
m/z	mass-to-charge ratio
MeOH	methanol
MHz	megahertz
NMR	Nuclear Magnetic Resonance
ORAC	Oxygen Reducing Absorbance Capacity
ppm	parts per million
prep TLC	preparative Thin Layer Chromatography
TLC	Thin Layer Chromatography
THM	Traditional herbal medicine
XRD	X-Ray Diffraction

Chapter One: Introduction

1.1 Natural product background

The investigation of traditional medicines has enabled continuous discovery and development of chemotypes and pharmacophores, which are beneficial in medicinal chemistry. These traditional medicines (TM) are of natural origin, in this case being plants. Different cultures have used them for thousands of years for healing ailments.[1] Natural products (of which particular interest in terms of biological activity are secondary metabolites), isolated from these traditional herbal medicines continue to provide unique structural diversity as compared to combinational chemistry.[2]

Sophisticated traditional medicine systems were formed by different cultures throughout the world a long time ago.[3] The ancient people of the world depended on traditional medicine compared to the modern civilization. These various cultural systems founded thousands of years ago, have led to the discovery of various natural products, which are still in use today, of which some are very potent drugs such as morphine and cannabinol (Figure 1). Although the specific chemical structures of the active constituents in traditional herbal medicines (concoctions or not) were known to little extent, the chemical biodiversity of the plants used was acknowledged and recorded.[2]

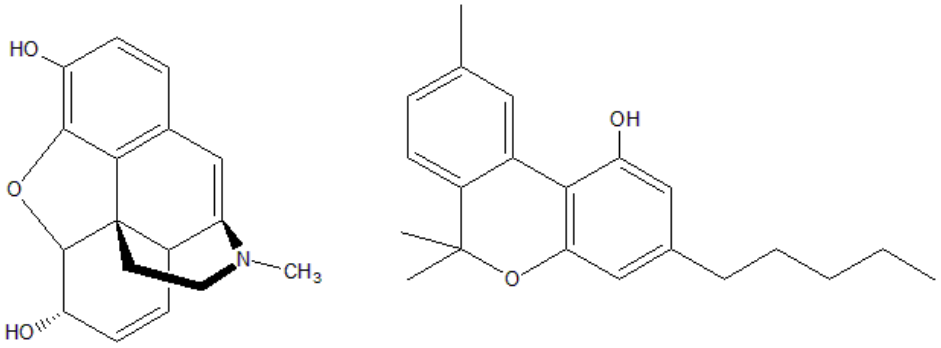

Figure 1: Chemical structures of morphine (*left*) and cannabinol.

The chemical diversity associated with natural products, resulting from plant evolution, among other factors, is thought to be equal, or even superior to that of synthetic combinational chemical libraries.[4] Chemical diversities have also been attributed to the different geographical environments that plants grow in, with a noticeable efficacy in the traditional herbal medicines consisting of plants of vastly different areas. The

special properties of traditional herbal medicine, have led to the development of a wide range of potent drugs that are currently used in orthodox medicine.

Although more than 50% of all drugs used today are natural products or their analogues, much investigation is still to be done, in-order to phytochemically document most of higher plants. It is estimated by the World Health Organization that 80% of the population in developing countries depend on natural remedies like herbs for their daily need.[5] The remedies mainly consist of crude extracts, and these plants become exploited in a very short period. Overexploitation becomes a worry, as the indigenous plant species may be lost forever due to no implementation of conservational harvesting methods.[5,7,8] Thus, scientific documentation of these traditional herbal medicines needs to be done with much haste, as there is a dwindle in the number of plant species in the world.[6]

1.1.1 Phytochemicals

Phytochemicals are secondary metabolites that a plant produces from its own biological pathway, and aids in its survival. These metabolites can be different within the same kind of plant species grown in different environments. Therefore, their chemotype profiles are directly influenced by the environment. Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include saponins, tannins, alkaloids, terpenoids, carbohydrates, steroids, and flavonoids.[4,5] These compounds are synthesized by secondary metabolism of living organisms. Thus, the bioactivity of the plant extract depends largely on the type of metabolites that it may contain. Secondary metabolites are often specific to a plant species. The qualitative phytochemical analysis is a screening of the crude extract(s) to investigate the type of compounds present within the plant. This is done to formally assess chemotype profile of the plant to view the type of compounds thought to be responsible for the plants reported biological activity.

1.2 Purpose of the Study

Indigenous knowledge is one of the basic science areas where the focus should be put in developing countries, such as South Africa.[9,10] Such knowledge, endemic in each region, should be a commodity. Cultures, especially in Africa, still hold to their old ways. Therefore, research in indigenous knowledge, i.e. traditional healing methods, does not only prove the validity of the claims by traditional communities, but also yield broader options for any kind of development in the future. In terms of healthcare, reverting to the more informal administration of medicines such as traditional herbal medicine (THM) to heal an ailment, proves that there is still more to be discovered; to be incorporated into the modern world.

Factors such as the dwindling plant numbers, environmental changes, and alternatives to orthodox healthcare, have prompted many countries and pharmaceutical companies to investigate natural products, with more focus being primarily on traditional herbal medicine.[11] These investigations include botanical, phytochemical, biological and pharmacognosy, amongst other studies. All of them give a better understanding of the plants used in traditional herbal medicine, and further aid in drug development. This study focuses particularly on the phytochemistry and biological activity of plant extracts and single isolated compounds.

The phytochemical investigation of traditional herbal medicine is imperative, as the constitution of chemotypes in any ingested medicine need to be identified. Certain people are allergic to specific types of compounds present in medicines for consumption or external applications, of which in many cases, THMs are of the crude form. Therefore, such investigations are necessary to avoid possible side-effects that may occur.[12-15]

In this study, investigation of the bark of *Strychnos henningsii* medicinal plant from the Eastern Cape region was conducted. Although investigation for its anti-diabetic properties has been reported, this plant may still possess a variety of compounds that might be able to treat other ailments.[7-9] We have found little literature published on the chemistry of *S. henningsii*, especially of the Eastern Cape Province, as a traditional herbal medicine.

Various *Strychnos* species such as *S. icaja, S. nux-vomica, S. axillaris* and *S. potatorum,* have been investigated for their biological activity, with many revealing promising results in terms of drug discovery.[13-21] The biological investigations of this genus show much potential, and, to a certain extent, substantiates their use as traditional herbal medicine. Phytochemical investigations of plants used in traditional herbal medicine, warrants more investigations of the plants used in different regions, because of geographical environment.[22,23] Thus, this study aims to add further knowledge to the perceived biological activity reported by the traditional health practitioners (THPs) of the Eastern Cape, and the southern African region. We anticipate that this manuscript will illuminate some aspects of the chemistry and possibly add scientific knowledge in the use of traditional herbal medicine for the treatment of ailments.[7-9]

1.3 Botanical description of *Strychnos henningsii*

S. henningsii is a small, erect and branched tree, with a height of between 2–12 meters, and a clean green-reddish stem. The tree grows along the coast. In South Africa, it grows along the coastal edges from the Eastern Cape, then Kwa-Zulu Natal and into Mozambique.[8,25] The bark (Figure 2) is peeling, crown compact with a dark green, glossy foliage. The twigs appear pale, ashy, or straw coloured, with a waxy skin splitting lengthwise. It has ovate, opposite, subsessile leaves of 2.5 – 6.5 cm in length and 0.8 – 4.5 cm in width.

The leaves (Figure 2) also have 3 – 5 strongly visible nerves from the base, wide-shaped and are subcordate at the base. The fruit grows up to 1.9 cm in length, and 11 mm in diameter, with a round or oblong shape. They contain 1 – 2 seeds, and are red, orange or brown when ripe.[24-27]

The bark is hard, with a dark brown inner and light, flaky outside. The bark tastes bitter due to the large presence of alkaloids.[26]

Figure 2: The bark (left) and the leaves (right) of *Strychnos henningsii*.

1.3.1 Plant parts used

The bark is the main part of the plant that is used in treating ailments.[25] It can be chewed, or boiled; with the cold-water extract taken in small doses. The wood is also used in fencing, as it has a very durable nature. Various cultures use the plant differently, but the part most used is the bark in THM. [26]

1.3.2 Traditional use

The bark is used in the treatment of various internal ailments, which include nausea and gastrointestinal complaints. It is normally boiled, then taken as a colic remedy, and is reported to be responsive to rheumatic fever and dysmenorrhea. In addition, various *Strychnos* species are used in the treatment of snakebites throughout southern Africa.[22,23,25] To the best of our knowledge, little has been documented about the use of this plant, with only the known literature reporting on *Strychnos henningsii* being a traditional medicine.[24-26]

1.4 Why the *Strychnos henningsii* of the Eastern Cape Province?

The bark of *Strychnos henningsii* collected directly from the forest was chosen in this study due to its wide use in THM; from gastrointestinal problems to snake bites.[24-26] Our hypothesis is that geographical differences, changing environments, among other factors, can alter phytochemical constituents of the same traditional herbal medicine thus altering the application in medicinal use.[26] The aim of this investigation was to investigate the phytochemical constituents and the activity of the tree bark used by traditional health practitioners of the Eastern Cape to treat certain ailments.

The specific objectives of this research were the following:

- Qualitative phytochemical analysis
- Biological screening of crude extracts
- Isolation of active compounds
- Structural evaluation of isolated compounds

1.5 Chapters Overview

In the second chapter, a literature review related to the study is discussed. Chapter three comprises of the experimental methods employed, whilst chapter four elucidates results and discussion of our findings. The summary of the research findings and conclusion, recommendations, and suggestions for future research will be provided in chapter five.

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Chapter Two: Literature Review

2.1 Introduction

Traditional herbal medicine has been a topic of much interest in the past few decades, owing to the diverse array of compounds that can be utilized for drug development.[1,2] Much traditional medicine systems around the world make use of herbs for their therapeutic benefit. Systems such as traditional Chinese medicine (TCM) has received much attention due the ancient documentation of their traditional herbal medicine in the *material medica*, and has been incorporated into the primary healthcare of the country.[3]

As stated earlier in Section 1.1 traditional African medicine is poorly documented, or access to such literature may not be available. Nonetheless, the traditional herbal medicine of various cultures around the world needs to be investigated for their biological activity, whether it be preferred or not. Cases of herbal medicines containing high levels of toxicity have been reported, with much more going undetected in unmonitored settings across the world.[3,4] Since traditional herbal medicines are now being incorporated into the primary healthcare system of many developed and developing countries, such investigations become paramount.

2.2 Traditional herbal medicine in Africa

Traditional medicine has been practiced for a long time, and conventional medicine has a very young history when compared to it, today. The current state of Africa's healthcare systems creates a stronghold for the use of traditional health methods in impoverished areas. With increasing numbers of diseases and lack of proper medical facilities to cater for the increasingly populous, the search for a potent medicine that is cost-effective has made the resorting to traditional herbal medicine more than just an economical choice.

The effectiveness of THMs is being evaluated using biological assays to assimilate the reported human bioactivity. Although modern medicinal methods are used to evaluate the efficacy of THMs, western medicine in its current form will not be able to critically resolve the healthcare issues in Africa's poor countries.[5-8]

Before the advent of conventional medicine, THMs have been practiced by African cultures for a long time. Although much of the development of traditional herbal medicines by ancient cultures came by trial-and-error methods, THMs have become trusted and fairly rigid in terms of specificity of ethnomedicinal plant use. These include prevention, diagnosis, and treatment of mental, social, and physical ailments.[8] Even after the advent of commercial medicine, according to the World Health Organization (WHO), over 80% of the African population has access to THM, of which has shown to be very a large variety of ailments. Traditional health practices continue to thrive in various cultures all over the world.[7,8]

There is a stigma about traditional medicine, as it is thought to be linked to witchcraft and sorcery, especially African traditional medicine. The adaptation of western medicine as the *prima facie* caused the alienation of traditional herbal medicine as a source of healthcare[8,9]. This, has, in turn, enabled the incorporation of foreign beliefs into African traditional medicine. Such that, many African cultures have made Christian praying an integral part of the healing process since many of the medical facilities in Africa were established by missionaries.[8-10]

Many factors drove the wrong attitudes towards THMs, such as politics, big pharmaceutical companies, etc. This has had severe repercussions on the health of the masses before and even after the WHO Alma-Ata declaration of 1978. As 80% of the African population depending on traditional medicine, and inciting factors contributing to the African economic state make orthodox medical health care unreachable.[10]

Therefore, treatment of ailments with the use of traditional herbal medicine enables many developing countries in Africa achieve better healthcare at the primary level.

2.3 Traditional herbal medicine in South Africa

South Africa (S.A), as a developing country in Africa, has problems relating to the health care system of the general population. Many factors drive this problem such as the high unemployment rate, ratios between doctors and patients and high population densities in struggling areas of the country, to mention a few. The state of the health care system is better than, perhaps, all of the African countries. However, the country itself does not have first world status. Costs, availability of medical staff and facilities all contribute

to the degradation of the system.[11]

The use of traditional herbal medicine in S.A is thought to curb such problems. The quick availability of THMs in the rural, semi-, and urban areas is seen to meet the country's demand for a solution to the country's primary health care problems. South Africa (Figure 3) also boasts huge biodiversity of its plant species, some indigenous to the specific biomes of the region. Per Vegetation of southern Africa, biomes mapped in South Africa are the Grassland, Savanna, Succulent Karoo, Nama Karoo, Forest, and Fynbos. These are the sources of the country's large biodiversity.

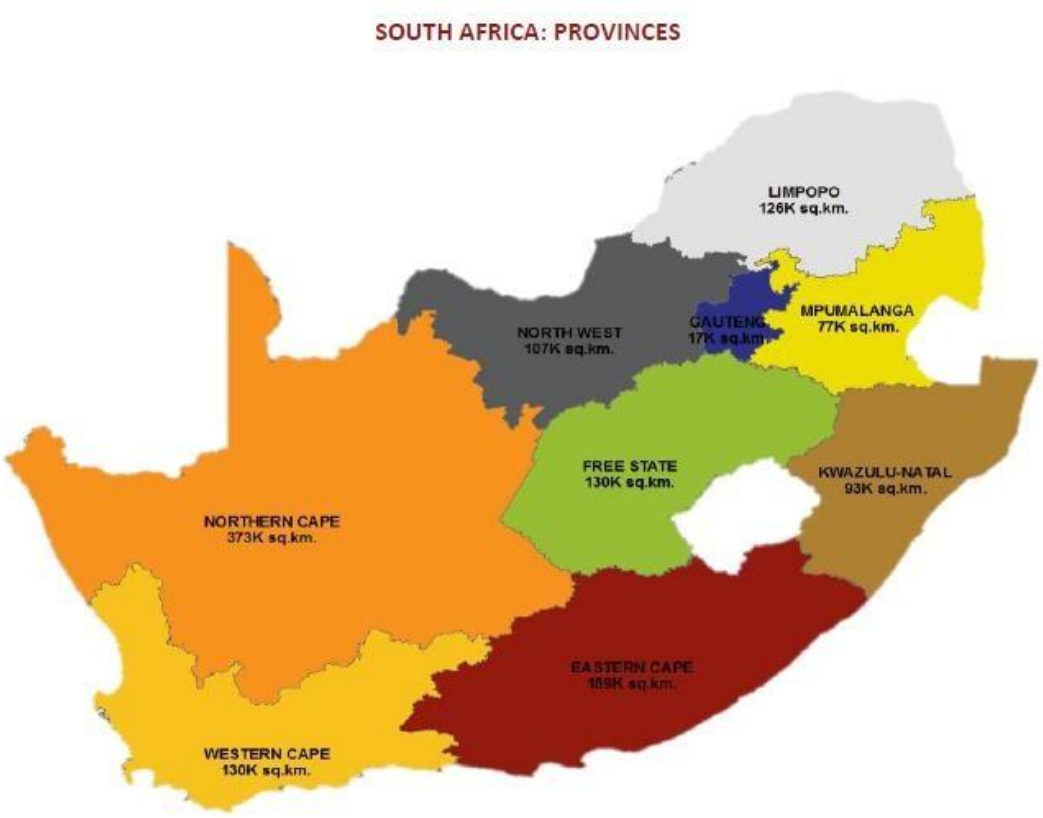


Figure 3: South Africa with labelled provinces and respective measured areas.[12]

With increasing traditional health practitioners (THPs) using plants for their medicinal benefits, the availability of such medicine to the public has increased. Per Mander *et al.*, the traditional herbal medicine market in S.A has become a large and growing industry, with more than 27 million consumers and an estimated economic value of R2.9 billion per year. Consumers trust the efficacy of other plants used, although not investigated. Therefore, the use of traditional herbal medicine, in most areas, is by choice rather than the cost issues associated with conventional medicine.[12]

The plant trade is a vital rural industry and business incubator, especially for the people

employed, mainly coming from very disadvantaged backgrounds. Also, Mander *et al* mention that there are at least 133 000 people employed in the plant trade, of which a large percentage being women.[13] The plant trade has developed itself into a good business, with a large portion of the employed living in rural areas.

The increased demand for healthcare services places more stress on conventional healthcare, but the alternative. The use of these plants as in the primary healthcare level has led to their exploitation, with increasing numbers of people getting involved in the trade.[4] The supply of the approximately 771 plant species is not sustainable. Mass harvesting and lack of replanting initiatives endanger many plants; some species take years to reach maturity. As most are harvested from the wild, local extinction of many plants becomes eminent.

The added use of these plants in cultural ceremonies puts more strain on their bioavailability. With different regions in South Africa inhabited by different ethnic groups (some overlaying each other), the indigenous plant heritage of that region dwindles.

2.4 The Eastern Cape region

The Eastern Cape Province is the second largest province after the Northern Cape, with an area of approximately 169 000 km^2. It is a region occupied largely by the *amaXhosa* tribe. It is divided into eight municipal districts, with the largest being the Cacadu district, and the smallest being the Alfred Nzo (Figure 4).[14] With 88% of the people in the province being black, this, to a certain extent, accounts for the large use of traditional herbal medicine, as an array of cultures are present in the province.[11,14,15]

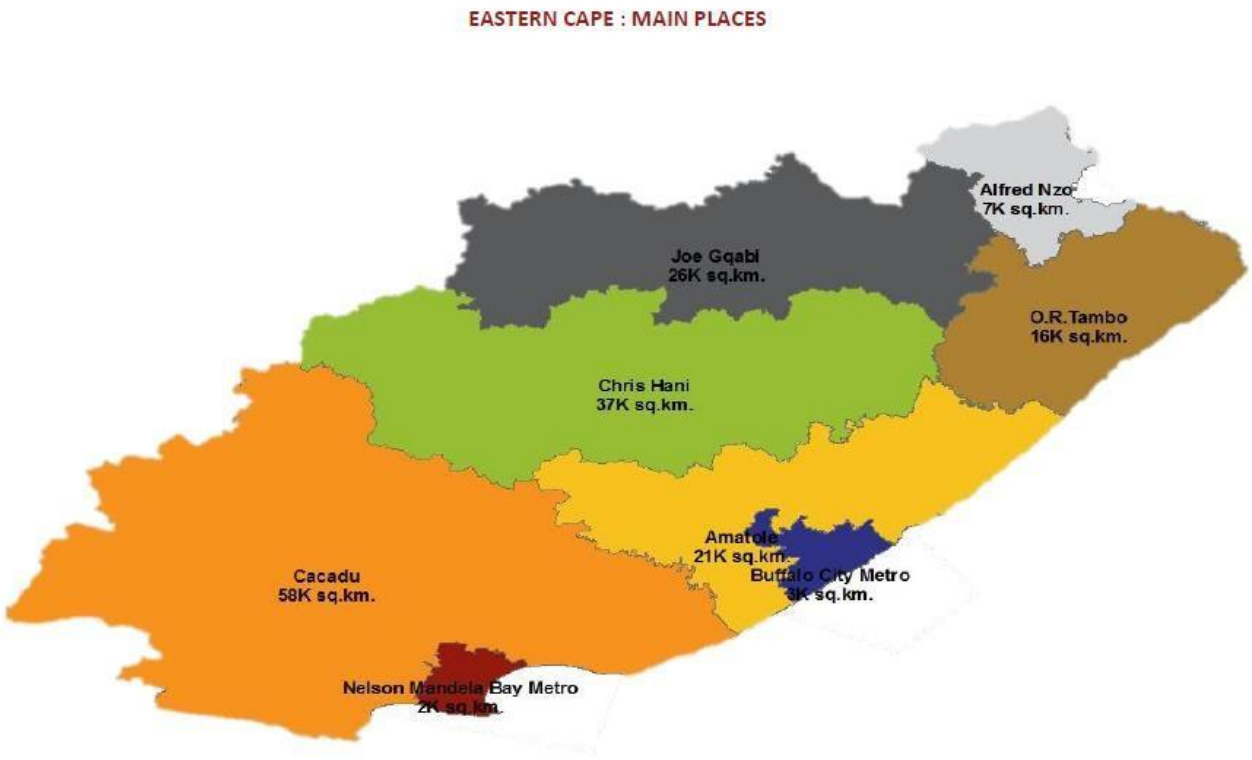


Figure 4: The Eastern Cape Province with its labelled districts.[12]

The current socio-economic state of the province is the result of two centuries of institutionalized racism and underdevelopment, followed by four and a half decades of social engineering during with which the apartheid era has given rise to high levels of poverty and underdevelopment in the Province.[16] Other challenges facing the Province, resulting from the poor development, are extremely high unemployment rates, glaring social inequalities and huge difficulties in accessing basic services like clean water, sanitation, housing, education health facilities and well maintained roads.

Access to healthcare facilities is one of the most fundamental rights to society. The economic state of the province prohibits widespread healthcare facilities, as it has been rendered the poorest province in South Africa. In rural areas, the use of medicinal plants is by choice, as it has been practiced in healing ailments for centuries. Trust between the rural dweller and the traditional healer is thus very high, making the role the traditional healer plays in society a very critical one.[17] Strong moral values tied to culture also increase the value of the traditional healer in the community. There are numerous people that practice TRMs, and these include herbalists, traditional healers, elderly people, and traditional doctors. However, the curative art of herbal medicines is limited to the smaller number of families, with some devotion and secrecy.[18]

There are currently approximately sixty plants species most frequently traded in the Eastern Cape, with a greater number estimated in the overall figure of plants used in traditional herbal medicine in the province[19]. Medicinal use of the leaves dominates any of the other parts of the plants, namely the roots, stems, and barks. Stomach ailments have the most medicinal plant prescriptions.[20] Ailments that follow are skin, fever, purgative, anti-inflammation, nausea, snake bite, etc.[18-20]

Barks, although used less, have grown in popularity as they are used more and more in THM concoctions.[20] Sustainability of tree bark as a source of traditional herbal medicine is thus threatened. The increasing demand for traditional herbal medicine is placing a toll on the natural reserve.[21]

2.5 The Loganiaceae family

The Loganiaceae is a family of flowering plants classified in order Gentianales. According to Leeuwenberg in 1980, the Loganiaceae family has 470 species with 29 genera arranged in ten tribes (i–x; Table 2.1). The Strychneae tribe is the most investigated, with more focus being on the complex alkaloids present in the genera, *Strychnos* more specifically.[22] The complete detailing of the Loganiaceae constitute of ten tribes; 29 genera and 552 species in total. The total number of plants investigated for their phytochemical data in the *Strychnos* tribe is poor. This is believed to be due to the availability of these plants (whether threatened or sampling constraints), chemotype similarity and use as traditional medicine, amongst other issues.

There are limited chemotype distributions of certain compounds within the family, with a large amount of the species having an increased presence of alkaloids, which prompted much investigation into this family. These studies include a variety of biological assays which aim to substantiate or give further insight into the use of the plants as traditional medicine. Genera that have not been reported for any traditional use are still of outmost importance, as the dwindling number of plant species make phytochemical investigations paramount.

Table 2.1: Tribes and genera in Loganiaceae, according to Leeuwenberg (1980), and the total number of species per genus.

Tribe		**Genus** (number of species in brackets)
i.	Spigelieae	*Polypremum* (1)
		Spigelia (50)
		Mitreola (6)
		Mitrasacma (40)
ii.	Loganieae	*Geniostoma* (20)
		Labordia (20)
		Logania (15)
iii.	Strychneae	*Strychnos* (190)
		Gardneria (5)
		Neuburgia (12)
iv.	Plocospermeae	*Plocospermum* (1)
v.	Gelsemieae	*Mostuea* (8)
		Gelsemium (3)
vi.	Antonieae	*Bonyunia* (4)
		Antonia (1)
		Norrisa (2)

		Usteria (1)
vii.	Buddlejeae	*Peltanthera* (1)
		Sanango (1)
		Nuxia (15)
		Androya (1)
		Gomphostigma (2)
		Buddleja (100)
		Emorya (1)
viii.	Retzieae	*Retzia* (1)
ix.	Potalieae	*Potalia* (1)
		Anthocleista (14)
		Fagraea (35)
x.	Desfontaineae	*Desfontania*

2.6 *Strychnos* species

The *Strychnos* genus has about 190 species, and are mainly found in the tropic areas. These include *Strychnos angustiflora, axillaris, henningsii, icaja, moandaenis, nux-vomica, potatorum, pseudoquina, usambarensis* and *vanprukii,* just to mention a few.

Numerous investigations have been taken into this genus. These investigations have enabled further insight into natural product chemistry, and help gain more understanding of the metabolic pathways followed by plants. Phytochemical analysis of these plants also helps to document threatened medicinal plants.

Approximately 7% of the *Strychnos* species have been investigated. Those that have undergone investigations, have been for anti-ulcerogenic, anti-plasmodial, anti-tumor, anti-hyperglycemia (antidiabetic) and anti-oxidant potentials, hepatoprotective and toxicity actions[23-27]. The biological assays have not been applied to all the species that were of interest. This is largely due to the similarity in chemotype profiling observed in a relatively few number of species investigated.[23]

The most biologically active and diverse class of compounds reportedly present in the *Strychnos* species are alkaloids. These alkaloids have been of much interest in the last 40 years.[28] Alkaloids consist of varying structures, and have been reported to be largely responsible for the respective plant's bioactivity in traditional medicine.[29-31]

2.6.1 Known compounds from *Strychnos* species

Many *Strychnos* species have been investigated for their biological activity, a large number have undergone isolation of various chemotypes. The chemotype mostly investigated in this genus are alkaloids. They are present in large concentrations in various *Strychnos* species, and are thought to be responsible for the plants' use in traditional medicine. They are investigated for several biological benefits, which include antiplasmodial activity.[32]

One of the most noticeable characteristics of the *Strychnos* species is the presence of strychnine (Figure 5), an alkaloid notoriously known as a poison, in a huge majority of the species.

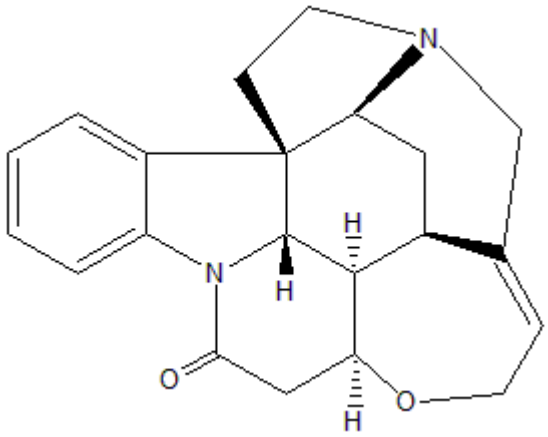


Figure 5: Chemical structure of strychnine.

The *Strychneae* alkaloids have a similar skeletal structure, suggesting that the compounds follow a similar biological synthesis pathway. This suggestion is strongly justified by the fact that stryvomicine was isolated from the seeds of *Strychnos nux-vomica*, and *N*-methyl-*sec*-isopseudostrychnine from the matured bark of *Strychnos icaja.*[33,34]

There are numerous other alkaloids isolated from various *Strychnos* species, which bear a similar skeletal structure. The following compounds, in Figure 6, display this statement clearly; brucine (**A**), diaboline (**B**), *N*-methyl-sec-isopseudostrychnine (**C**) and stryvomicine (**D**) to mention just a few. These compounds were isolated using various chromatographic techniques such as vacuum liquid chromatography, column chromatography and prepTLC.[33,34]

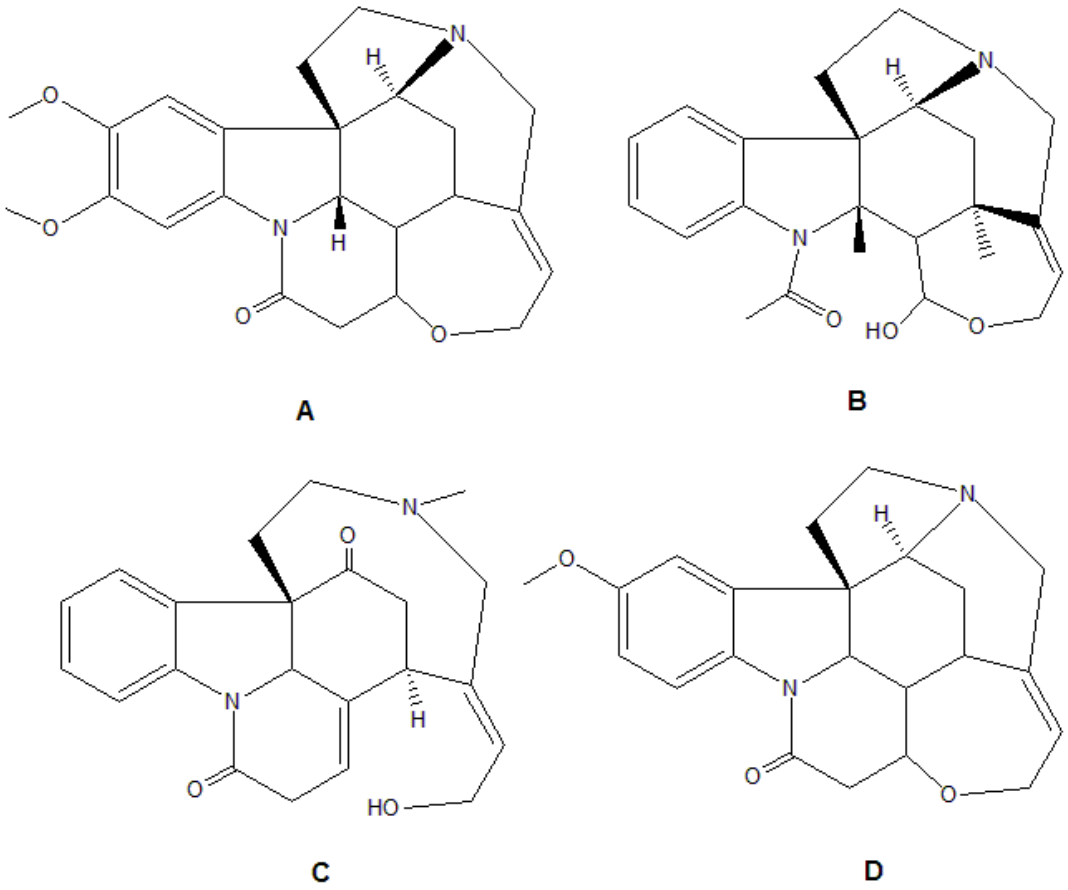


Figure 6: Chemical structures of alkaloids isolated from various *Strychnos* species include the following; (**A**) brucine, (**B**) diaboline, (**C**) *N*-methyl-*sec*-isopseudostrychnine and (**D**) β-colubrine.[33,34]

The remarkable resemblance in the chemical structure of these four aforementioned compounds (Figure 6) to that of strychnine allows the focus on the different functional groups and stereochemistry to that of the compounds' biological activity in drug discovery. Strychnine derivatives allow for better understanding of the structure-activity relationships within the *Strychnos* species.

The chemical diversity of chemotypes present in *Strychnos* species is huge, with many compounds present in trace amounts. Other alkaloids, perhaps with novel structures, are yet to be discovered.[30]

Other chemotypes that have been of much interest are phenolic and iridoid glycosides. In East Asia, plants such as *Strychnos axillaris* have been used in the preparation of arrow poisons. A chemical study of *S. axillaris* resulted in the isolation of a number of compounds, such as 6'-*O*-β-D-apiofuranosylcalleryanin (**E**), 7-*O*-*trans*-caffeoylvanillosiden (**F**) and axillaroside (**G**).[35]

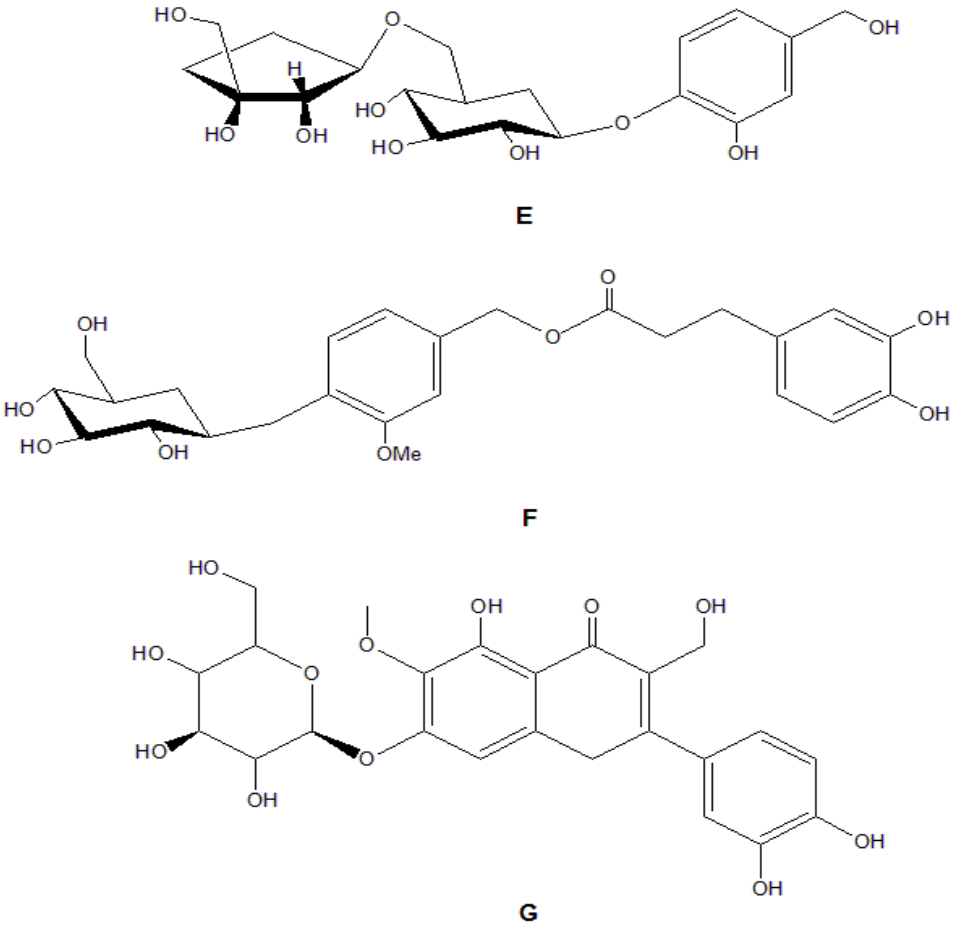


Figure 7: Chemical structures of 6'-*O*-β-D-apiofuranosylcalleryanin (**E**), 7-*O*-*trans*-caffeoylvanillosiden (**F**) and axillaroside (**G**).[35]

The phenolic iridoids in Figure 7-G were elucidated using NMR and Mass Spectroscopy. Elucidation of chemical structures such as axillaroside (**G**), was achieved, and units of secologanoside (**H**) and loganin (**I**) (Figure 8) were deduced. These two units have specific *m/z* values (e.g. secologanoside has a fragment ion peak at *m/z* 389), and when incorporated with ^{1}H and ^{13}C NMR spectra, the chemical structure proposed was verified.

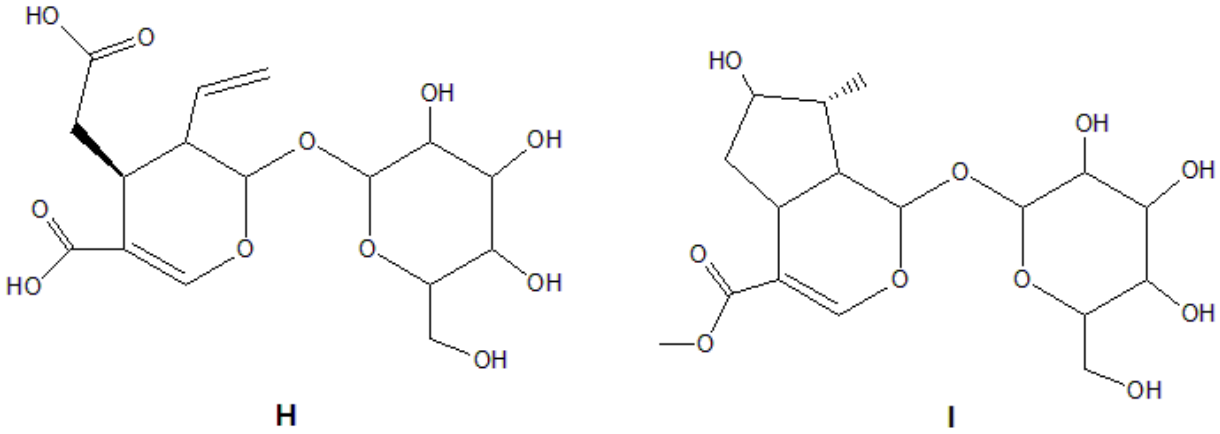


Figure 8: Chemical structures of secologanoside (**H**) and loganin (**I**).[35]

Terpenoids are another class of compounds synonymous with the *Strychnos* species. This is a large group of organic compounds which consists of sub-group such terpenes, diterpenes and sesquiterpenes, to mention a few. The difference between terpenoids and terpenes is that terpenes are hydrocarbons, whereas terpenoids consist of additional functional groups. This distinction is not always adhered to in literature, with the two terms used interchangeably in many cases. An example of a triterpenoid, lanost-9(11)-enyl acetate, is given in Figure 9.[36]

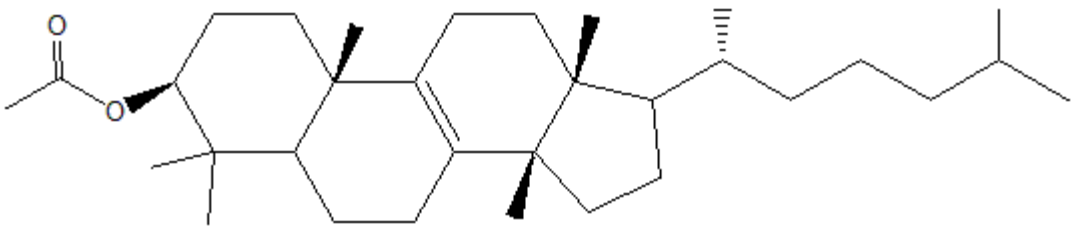


Figure 9: Chemical structure of lanost-9(11)-enyl acetate.[36]

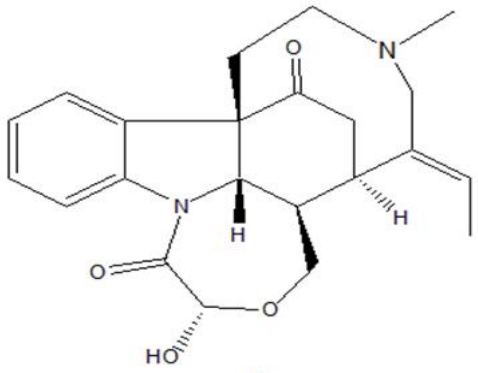
There are numerous other chemotypes present in *Strychnos* species, which is why the genus has been subject to many investigations of its biologically active constituents. Much of the focus of such studies on the genus have, although there is the aspect of synergistic effects, primarily focused more on bioactivity screening of the crude extracts than single isolated compounds. Isolation of these compounds not only benefits understanding plant chemistry, but also plant metabolic systems and expanding secondary metabolite libraries. Screening of isolated compounds is thus fundamental in the assessment of bioactivity of traditional herbal medicine.

2.7 *Strychnos* henningsii

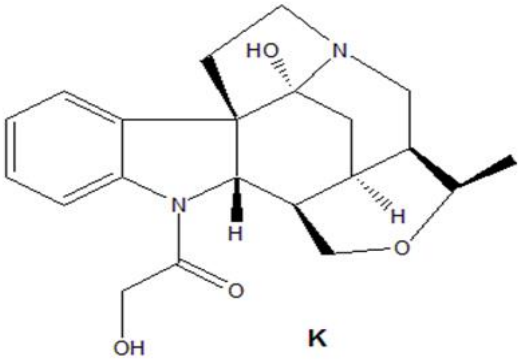
Strychnos henningsii was named in honour of Professor Paul Christoph Henning. *S. henningsii* belongs to the family Loganiaceae. Common names include Red bitter and Henning's Strychnos.[38,37] Various cultures around South Africa that use this tree for medicinal purposes, call it by various names; *uMnonono* (Xhosa), *uMqalothi* (Zulu), *koffiehardpeer* (Afrikaans), coffee bean (English), etc., according to World Agroforestry Center.[38]

2.7.1 Chemical investigations of *Strychnos henningsii*

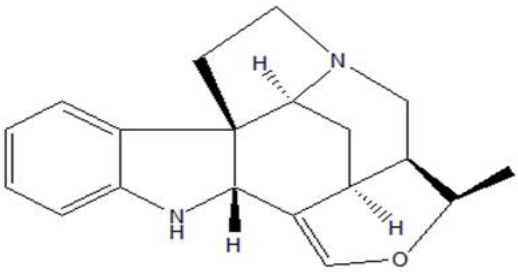
There are numerous compounds isolated from *Strychnos henningsii*, and although not all have been investigated for their respective biological activity, a large majority have subject to many investigations.[32] Holstiine (**J**) is a compound first isolated from *Strychnos holstii*, a species closely related to *S. henningsii*. Splendoline (**K**) was first isolated from *S. splendens*, which grows in western African regions of Sierra Leone, Ghana up to Senegal all along the coast. From *S. psilosperma*, spermostrychnine (**L**) was initially reported, and re-isolated from *S. henningsiii*. Other alkaloids reported (see Figure 10) include 23- hydroxyspermostrychnine (**L**), 19-*epi*-23-hydroxyspermostrychnine (**M**), retuline (**N**), henningsiine (**O**), dehydroxyacetylhenningsiine (**P**), *O*-acetylhenningsiine (**Q**), 3- hydroxyhenningsiine (**R**), henningsiiine-*N*(4)-oxide (**S**), 23-hydroxyspermostrychnine- *N*(4)-oxide (**T**), 17,23-dihydroxyspermostrychnine (**U**), henningsamide (**V**), *O*- acetylhenningsamide (**W**), deshydroxyacetylhenningsamide (**X**), cyclostrychnine (**Y**), henningsoline (**Z**).[39]



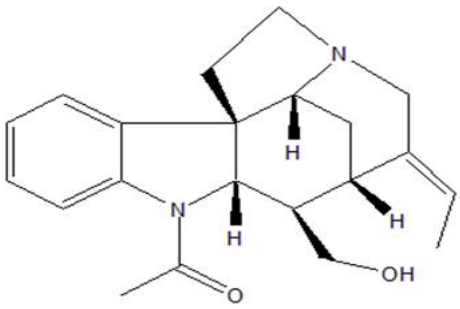
J



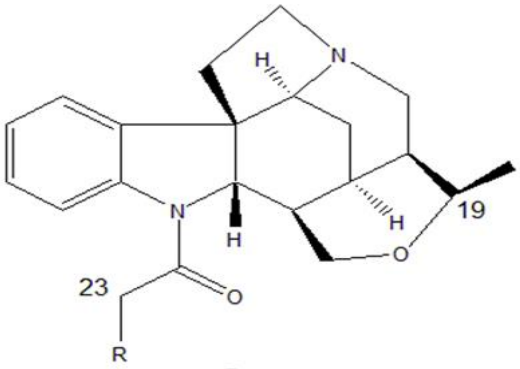
K



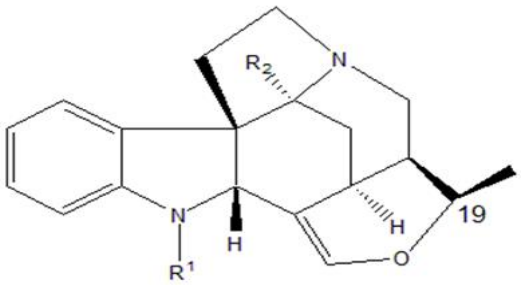
L



O



	R		
M	OH	19	H
N	OH	19	H
U	OH	19	H N(4)-O



	R^1	R^2
P	$COCH_2OH$	H
Q	H	H
R	$COCH_2OAc$	H
S	$COCH_2OH$	OH
T	*N*(4)-oxide	

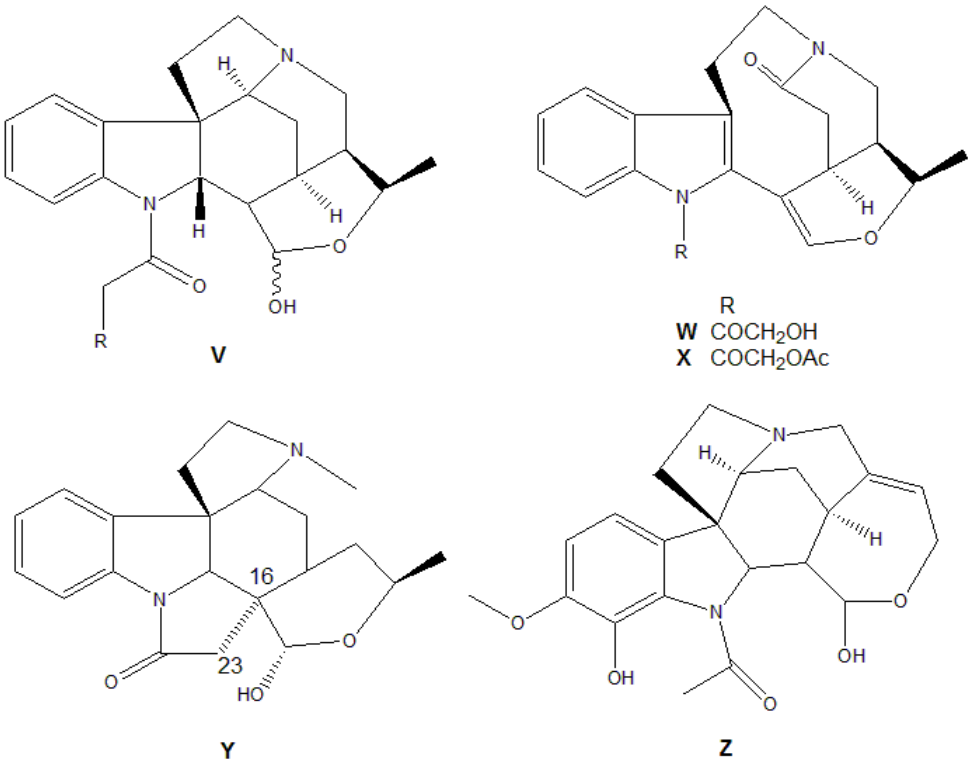


Figure 10: Chemical structures of the compounds isolated from *Strychnos henningsii*; holstiine (**J**), splendoline (**K**), spermostrychnine (**L**), 23-hydroxyspermostrychnine (**M**), 19-*epi*-23-hydroxyspermostrychnine (**N**), retuline (**O**), henningsiine (**P**), dehydroxyacetylhenningsiine (**Q**), *O*-acetylhenningsiine (**R**), 3-hydroxyhenningsiine (**S**), henningsiiine-*N*(4)-oxide (**T**), 23-hydroxyspermostrychnine-*N*(4)-oxide (**U**), 17,23-dihydroxyspermostrychnine (**V**), henningsamide (**W**), *O*-acetylhenningsamide **(X),** cyclostrychnine (**Y**), henningsoline (**Z**).[39]

There are other compounds isolated from *S. henningsii* (flavonoids, glycosides, iridoids, etc.).[28-36] The Strychneae tribe has unique, complex alkaloids, hence the focus on the compounds (see Figure 10). The alkaloids isolated from the *Strychnos* species thus far, share a common indole alkaloidal carbon framework.

2.7.2 Biological assays

Biological assays investigated with *S. henningsii* include antiplasmodial, antidiabetic, anti- hyperglycemia and anti-oxidant assays.[27,32,40] These concluded that although the single isolated compounds showed moderate antiplasmodial activity, the aqueous extract of *S. henningsii* bark improved of glycemic control of diabetes in an animal model (Wistar rats) i.e. it has antidiabetic properties. The anti-hyperglycemic assay showed that an overuse of *S. henningsii* in the treatment of diabetes might cause side-effects.[41]

The results from the biological assays of *Strychnos henningsii* substantiate the use of the *S. henningsii* bark as a traditional herbal medicine. Further investigation into the phytochemical profile and possible chemotype variations among such species, for reasons mentioned before, is of utmost importance when such dependency has been placed on traditional herbal medicines.

Many investigations have been conducted on the *Strychnos* genus, with most being performed on crude extracts.[23-27] Other classes of compounds such as phenols and glycosides have also been investigated.[35]

The class of compounds mostly focused on are alkaloids, with much investigations orientating around their structural configuration. Their biological activity in *Strychnos henningsii*, however, has not been broadly studied. The large presence of alkaloids in the plant surely influence the bioactivity, and individual compound analysis should be performed in-order to evaluate their roles in the overall bioactivity of the crude extracts. Therefore, concentration-activity relationships should also be assessed for traditional herbal medicines, as to ascertain efficacy levels.

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Chapter Three: Experimental

3.1 Plant material

Strychnos henningsii bark (otherwise known as 'Mnonono' by the Xhosa people of the Eastern Cape) was collected from the Kwelerana forest of the Pirie Mission village, located in King Williams Town, Eastern Cape. The exact GPS coordinates of the collection site are -32.780580, 27.244648. The plant was authenticated as *Strychnos henningsii* by Prof E. Campbell of the Department of Botany at the Nelson Mandela Metropolitan University, with the specimen given the identification code PEU 6856 and stored at the university's herbarium.

A local guide was made use of to pinpoint the exact trees the locals picked for harvesting the plants. The herbal remedy is normally prepared by soaking 3 g of the plant material in 750 mL of water and half a cup is taken orally three times a day for treatment.

3.2 Preparation of bark extracts

The material collected was dried in an oven at 40 ºC over two days. This was done as to accelerate the drying process as barks normally retain moisture, and high temperatures tend to denature natural products. The material (512.610 g) was then crushed using a hammer and subjected to polarity gradient extraction; whereby the bark was extracted with multiple solvents of increasing polarity. The extraction process was performed by macerating the plant material with the respective solvents in 5 litres of solvent in total, under constant stirring. Hexane was used first (3 x 700 mL), then ethyl acetate (3 x 700 mL), and lastly methanol (4 x 600 mL). This was done to combine extraction and fractionation process into a single step. All solvents used were of analytical grade from Merck.

The hexane extract consisted mainly of fatty acids, which were of no interest in this project. The extracts of interest were the other two, particularly the methanol extract, as it had a larger concentration of compounds from the thin layer chromatography (TLC) profiles under different solvent systems.

3.3 Chromatography

3.3.1 Analytical thin-layer chromatography

Small amounts of the respective crude extracts, ethyl acetate and methanol, were dissolved in their respective solvents, and spotted on cut out pieces of TLC plates (20 cm x 20 cm, TLC Silica gel 60 F_{254}, Merck), and developed at varying solvent systems. For the ethyl acetate extract, the TLC plates were developed from 9.5:0.5 to 5:5 Hex:EtOAc, dried, inspected under UV light at 254 and 365 nm, and then sprayed with the respective reagent for phytochemical analysis. The same was done with the methanol extract, with the only change being the solvent system composition, $CHCl_3$:MeOH.

3.3.2 Liquid chromatography

Vacuum liquid chromatography (VLC) was performed for both crude extracts. The stationary phase was silica gel (Fluka Analytical Silica gel 60, 70-230 mesh, 63-200 µm). The stationary phase was dry packed. VLC was performed using a Buchner setup (500 mL Buchner flask with a 12 cm x 15 cm Buchner funnel). The extracts were introduced into their respective setups by dissolving the extracts in a minimal solvent and adding small amounts of silica gel. The latter was done to aid the transition between the sample and the stationary phase. The ethyl acetate extract was fractionated using 9:1 to 4:6 Hex:EtOAc (100 mL) in a step-wise manner, with each solvent system performed in duplicate. The same was performed on the methanol extract, with the solvent system being $CHCl_3$:MeOH, and hot solvent wash being performed at the end of the fractionation process to wash out the remaining polar compounds.

3.3.3 Column chromatography

Column chromatography was also performed using the same stationary phase. Due to the huge amount of methanol extract, a 60 cm x 7.5 cm glass column, with the silica gel being introduced in a hexane slurry. The glass column was plugged with a small piece of cotton wool, packed with approximately 2 cm of washed sea sand, and then 50 cm of stationary phase. On top of the stationary phase was the methanol extract

dissolved in a minimal solvent, with a little bit of silica added. Another 2 cm layer of washed sea sand was added in order to prevent any disturbance of the sample by addition of the mobile phase, and then capped with a small amount of cotton wool. The rest was left as the solvent reservoir. Gradient elution started from 9.5:0.5 to 5:5 $CHCl_3$:MeOH, in a stepwise manner (0.5 decrease in $CHCl_3$), in 100 mL additions to the reservoir.

The fractions collected (each approximately 20 mL, filled at 10 drops per 5 seconds) were constantly monitored by TLC, and those presenting almost similar profiles were combined and dried in a fume cupboard.

3.4 Phytochemical analysis

Two different phytochemical methods of analysis were used, with one being the TLC and the other being the test-tube method. The TLC method is visualizing the developed TLC plate under UV light, both short and long wavelength (256 and 365 nm, respectively). The TLC plate would then be sprayed with a freshly prepared reagent and detecting the colour change after heating in an oven in some cases. The test-tube method is subjecting the specific solvent extract with reagent(s) and the physical changes are indications of either a positive or negative presence of the chemotype tested.

The following tests were used in the chemotype

analysis: TLC method

The respective TLC plates at various solvent systems of increasing polarity were each sprayed with the respective reagent after development. After spraying, each colour change, or lack of, would indicate the presence or absence of the respective chemotype.

The reagent sprays were prepared as follows;

Screening of steroids – a vanillin solution (0.8 g of *p*-vanillin was dissolved in 80 mL of sulphuric acid and 20 mL ethanol) was sprayed over developed TLC plates. Pink spots were indicative of the presence of steroids.[1,3]

Screening of sulfonamides, amides, indoles and ergot alkaloids – developed TLC plates

were sprayed with a van Urk reagent (2 g of *p*-dimethylaminobenzyldehyde, 50 mL 95% ethanol and 50 mL concentrated HCl). The following colour changes were indicative of the presence of the respective chemotypes; pink-purple = sulfonamides, green = amides, blue = indoles, and blue-green = ergot alkaloids.[1-4]

Screening of Vitamin A &D and carotenoids – an antimony chloride solution (25 g antimony (III) chloride dissolved in 75 mL chloroform) was sprayed over developed TLC plates. A pink colour change on the TLC plate indicates the presence of vitamin A & D, with carotenoids indicated by a yellow colour change.[2-3]

Reaction method

Screening of flavonoids – dilute sodium hydroxide (2 mL) was added to 2 mL of both extracts (separate). The appearance of a yellow colour indicates the presence of flavonoids.[4,5]

Screening of phenols – equal volumes (1 mL) of both extracts and Iron (III) chloride were mixed. A deep bluish green solution gave an indication of the presence of phenols[3-5].

Screening of tannins – a portion of the extracts were dissolved in water in separate vials, after which the solution was clarified by filtration. A 10% ferric chloride solution was added to the resulting filtrate. The appearance of a bluish colour indicates the presence of tannins.[2-6]

Screening of phlobatannins – a few drops of 1% hydrochloric acid was added to 1 mL of the extracts and boiled. A red precipitate is an indication of the presence of phlobatannins.[1,2]

Screening of terpenoids – Acetic anhydride (0.5 mL) was mixed with 1 mL of both extracts and a few drops of concentrated sulfuric acid. A blue-green precipitate indicates the presence of terpenes.[1,3]

Screening of alkaloids – the filtered extracts were treated with small doses of Dragendorff's reagent (a potassium bismuth iodide solution). Formation of a red precipitate is an indication of alkaloids.[1-5]

Screening of coumarins – the crude extracts were evaporated, re-dissolved in approximately 2 mL of distilled water and each subsequent solution divided into two equal volumes. One volume of each extract taken as the reference (without ammonium hydroxide), and the other one doped with 0.5 mL of 10% ammonium hydroxide

(NH_4OH). Each extract was spotted with its reference onto a filter paper and viewed under UV light. The doped extract samples exhibited intense fluorescence as compared to their reference samples, which is indicative of the presence of coumarins.[3-6]

3.5 *In vitro* assays

Cytotoxicity, anti-inflammatory, and anti-oxidant assays were undertaken at the Department of Biochemistry, Nelson Mandela Metropolitan University.

3.5.1 Cytotoxicity

Cell culture conditions: human cervical cancer cells (HeLa; Highveld Biological, South Africa) were routinely maintained in 10 cm culture dishes without antibiotics in RPMI cell culture medium containing 25 mM HEPES and L-glutamine (Cyclone Laboratories Inc., South Logan, Utah, USA) and 10% fetal bovine serum (Bio west, Nubile, France) in a humidified incubator and 5% CO_2 at 37 ºC.

Sample preparation: crude extracts were reconstituted in DMSO to give a final concentration of 50 mg/mL. Samples were stored at 4 ºC until required.

Screening protocol: HeLa cells were seeded into 96-well microtiter plates at a cell density of 60 000 cells/mL; using 100 µL of cell suspension per well (6000 cells per well). The microtiter plates were incubated for 24 hours in a humidified incubator and 5% CO_2 at 37
ºC prior to addition of the extracts. The crude extracts were diluted to double the desired final maximum test concentration with complete medium and filter sterilized. Working concentrations were prepared by dilutions ranging between 25 and 250 µg/mL. Aliquots of 100 µL of these different extracts were added to the appropriate microtiter wells already containing 100 µL of the medium, resulting in the required final extract concentrations (ranging between 12.5 and 125 µg/mL). Dimethyl sulfoxide (DMSO) was used as the positive control. Melphalan was used as the negative control, with final concentrations of 50 and 100 µM. Plates were incubated for 48 hours at 37 ºC and 5% CO_2 in a humidified incubator.[6]

MTT assay: after 48 hours, treatments were removed via aspiration, 100 µL MTT [0.5 mg/mL dissolved in complete medium] added to each well, and incubated for 3 hours

at 37 ºC.[6] After 3 hours, MTT was removed via aspiration, 100 µL DMSO added to each well to dissolve the formazan crystals, and the absorbance read at 540 nm using a BioTek® PowerWave XS spectrophotometer.

Data analysis: cell viability was determined using four replicate wells for each concentration. Untreated cells were considered to have 100% cell viability (i.e. the mean OD of the untreated wells is equal to 100% viability). Cell viability in test cells was calculated relative to the untreated control and expressed as a percentage.

3.5.2 Anti-inflammatory

The method followed in for the anti-inflammatory assay is as follows;

The RAW cells were seeded in 96-well plates at a density of 50 000 cells per well and allowed to attach overnight. The spent culture medium was removed and replaced with fresh medium containing the samples of interest (50 µl per well). Aminoguanidine was used as the positive control.

To activate the macrophages, 50 µl of LPS (lipopolysaccharide) containing 100 ng/mL was added to all wells except the ones assigned as blank representatives and those used to determine pro-inflammatory activity. They were then incubated for 18-20 hours. Then 50 µl of the medium was removed from each well and transferred to a new a microplate. Griess reagent (50 µl) was added and incubated at room temperature for 10-15 min, and then read at 510 nm.[7]

To the remaining cells, 100ul MTT solution was added to obtain a final concentration of 0.5mg/mL. The cells were returned to the incubator for approximately 15-20 min. The spent culture medium was removed and 100 µl DMSO was added to solubilize the MTT crystals and then read at 540 nm.[7]

3.5.3 Anti-oxidant

3.5.3.1 DPPH radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable radical because of the paramagnetism conferred by is odd electron (delocalization of the spare electron over the whole molecule). This assay sees the decrease in the absorption as the anti-oxidant power of the sample, measured at 517 nm when compared to the DMSO (blank)

sample. The 0.1 mM DPPH reagent was prepared by dissolving 4 mg of DPPH in 100 mL ethanol.[8,9]

Different masses of samples (0.2 – 1 mg) were dissolved in the reagent and analyzed in increasing concentration. The control used was quercetin, a known anti-oxidant. The concentration ranges were the same as for the samples.

3.5.3.2 FRAP (Ferric Reducing Ability of Plasma)

Reagent preparation: the reagents involved were 0.3 M acetate buffer at pH 3.6 (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$ (BDH Laboratory Supplies, England) in 1 litre distilled water) The TPTZ (2,4,6-tripyridyl-*s*-triazine) solution was prepared by dissolving 10 mol TPTZ (Fluka Chemicals, Switzerland) in 100 mL of 40 mmol/L HCl (BDH). The 20 mmol/L ferric solution was prepared using $FeCl_3.6H_2O$ (BDH). The working FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$ solution, the ratio being 10:1:1 respectively. The reagent was prepared daily and warmed to 37 ºC in a water bath prior to use.[8,10]

Stock solutions (20 mg/mL) of the samples were prepared, and from that, the sample concentrations were derived (50 – 250 µg/mL). The absorbance of the reaction mixture was then read after 5 minutes at 593 nm. The measurements were taken in triplicate, and a standard curve drawn from the results. The control was epigallocatechin gallate (EGCG).

3.5.3.3 ORAC (Oxygen Radical Absorbance Capacity)

In this assay, AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) is used as the free- radical initiator, as it forms free radicals at a constant rate. Fluorescence decay is monitored, as anti-oxidants quench the oxygen radicals generated by APPH. Fluorescein the sodium salt is preferred in the place of β-phycoerythrin, because of it less expensive, does not interact with other compounds and does not photobleach. A Vitamin E derivative, Trolox ((±)-6hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), is used as the baseline in assessing the anti-oxidant activity of a sample. The ORAC assay was conducted using the following procedure;

AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) is prepared at a concentration of 79.65 mmol/L by adding 216 mg to 10 mL phosphate buffer (75 mM, pH 7.4). The

fluorescein solution was prepared by dissolving 22.5 mg into 50 mL PBS to make the first stock solution. A second stock solution was prepared by adding 50 µL of the first fluorescein stock solution to 10 mL phosphate buffer.

Finally, 320 µL of the second fluorescein stock solution is added to 20 mL phosphate buffer. The experimental samples and control samples, including Trolox, were prepared at various concentrations. The samples were dissolved in water. The working fluorescein solution, 400 µL, was added to the samples and control and made in increasing concentrations.[11]

The phosphate buffer was used as the blank, with the Trolox concentrations were 20, 40, 60, 80 and 100 µM. The concentration range of the experimental samples was the same as Trolox, 20 to 100 µM, in the same steps. An aqueous green tea extract was used as a positive control, at concentrations 10, 20, 30. 40 and 50 µM. When the aliquots were added to each well, fluorescence was taken for $time_0$. During cycle 4, the reaction is initiated with the injection of 150 µL of AAPH into the respective wells. Fluorescence readings were taken every 1:40 min and the experiment had 54 cycles. Fluorescence readings were then used to calculate areas under the curves (AUC) and Trolox Equivalents (TE). The assay was allowed to run for 90 min, with an excitation wavelength of 480 nm and an emission wavelength of 525 nm.[11]

3.6 Analytic instruments

3.6.1 Nuclear Magnetic Resonance (NMR)

A Bruker ULTRASHIELD 400 MHz/54 mm magnet system NMR instrument was used for the accumulation of ^{1}H, ^{13}C, Cosy, Dept-135, HSQC and HMBC spectra. The internal standard was tetramethylsilane (TMS). The respective spectra were processed using Topspin 3.0 software. Deuterated NMR solvents were used, namely $CDCl_3$ and MeOD unless otherwise stated.

Physical data of isolated compounds

SHB.E-Y (4.1)

^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 0.46 (s), 0.61 (s), 0.82 (m), 0.94, 1.03, 1.11, 1.19, 1.38, 1.55 (m), 1.79 (m), 1.95 (m), 2.14 (s), 2.28 (t), 3.448 (s), 5.28 (s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 10.24, 11.86, 14.09, 30.91, 31.52, 31.59, 31.92, 32.67, 33.57, 33.76, 34.80, 36.14, 36.39, 37.25, 38.23, 39.78, 42.29, 44.33, 45.07, 45.85, 50.14, 51.24, 56.77, 56.87, 71.83, 77.19, 118.14, 119.59, 121.72, 130.02, 134.14; HRMS(+), m/z 465.2991 $[M + H]^+$, (calculated. for $C_{30}H_{41}O_4$: 465.3005).

Stryvomicine (4.2)

^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 1.04 (1H, m, *HH*-6), 1.19 (1H, s, H-16), 1.68 (1H, m, *HH*-14), 1.91 (3H, s, NC<u>H</u>$_3$) 2.12 (1H, s, *HH*-14), 2.27 (1H, s, *HH*-6), 2.35 (1H, m, *HH*-5), 3.10 (1H, m, H-15), 3.37 (1H, m, *HH*-5), 3.43 (1H, d, *J*=7, *HH*-24), 3.70 (1H, m, *HH*-18), 3.73 (1H, s, *HH*-18), 4.47 (1H, s, H-2), 4.75 (1H, s, O<u>H</u>) 6.67 (1H, d, *J*=9, H-19), 7.12 (1H, t, *J*=14.5 Hz, Ar<u>H</u>)), 7.24 (1H, m, Ar<u>H</u>) 7.29 (1H, m, Ar<u>H</u>); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 23.01 (N<u>C</u>H$_3$), 30.76 (C-15), 34.56 (C-24), 40.54 (C-14), 46.29 (C-6), 47.83 (C-5), 56.15 (C-7), 60.00 (C-2), 62.38 (C-21), 67.70 (C-18), 71.30 (C-17), 73.87 (C-3), 111.77 (C-11), 117.50 (C-9), 126.56 (C-10), 128.33 (C-13), 130.28 (C-19), 136.36 (C-8), 146.89 (C-20), 150.82 (C-12), 163.66 (C-23); HRMS(+), m/z 381.1817 $[M + H]^+$, (calculated. for $C_{22}H_{25}N_2O_4$: 381.1814).

Isovomicine derivative (4.3)

^{1}H-NMR (400 MHz, MeOD) δ_H: 1.30 (1H, m, *HH*-6), 1.46 (1H, m, H-16), 1.68 (1H, m, *HH*-14), 1.91 (3H, s, NC<u>H</u>$_3$), 2.12 (1H, s, *HH*-14), 2.29 (1H, m, *HH*-6), 2.47 (1H, d, *J*=19, *HH*-5), 3.10 (1H, m, H-15), 3.35 (1H, d, *J*=15.2, *HH*-5), 3.43 (1H, d, *J*=7, *HH*-24), 3.73 (1H, s, H-18), 4.35 (1H, d, *J*=11, H-2), 4.75 (1H, s, O<u>H</u>), 6.67 (1H, d, *J*=9, H-19) 7.14 (1H, m, Ar<u>H</u>), 7.25 (1H, m, Ar<u>H</u>) 7.86 (1H, d, *J*=6.9, Ar<u>H</u>); ^{13}C-NMR (100 MHz, MeOD) δ_C: 30.76 (C-15), 34.59 (C-24), 38.82 (N<u>C</u>H$_3$),40.54 (C-14), 49.88 (C-6), 53.88 (C-5), 57.11 (C-7), 60.48 (C-2), 62.38 (C-21), 67.70 (C-18), 68.15 (C-3), 107.54 (C-9), 11.77 (C-11), 123.28 (C-17), 126.53 (C-8), 127.33 (C10), 128.33 (C-13), 130.28 (C-19), 135.70 (C-16), 142.61 (C-20), 144.27 (C-12), 163.66 (C-23); IR ν_{max} (cm^{-1}): 3382.97 (OH), 2919.45, (CH), 1638 (C=C), 1445.61 (CH_3), 1391 (CH_3); HRMS(+), m/z 383.1979

$[M + H]^+$, (calculated. for $C_{22}H_{27}N_2O_4$: 383.1979).

3.6.2 Liquid Chromatography–Mass Spectroscopy (LC-MS)

All the LC-MS spectra were ran using a Waters Synapt G2 instrument at the Central Analytical Facilities (CAF) at Stellenbosch University. Electron spray ionization (ESI) was used in the sample introduction method and a 15 volts cone voltage. Leucine encephalin was used as the lock mass. Mainly electrospray positive (ES+) was used in the analyses as opposed to electrospray negative (ES-). All the samples were dissolved in methanol. The exact run time was 15 min, with the mobile phase composition being water:acetonitrile at a gradient elution with all the samples. The ESI probe was injected into a stream of acetonitrile.

3.6.3 High-Resolution Mass Spectroscopy (HRMS)

The HR-MS spectra were also achieved on the Waters Synapt G2, with the same parameters as the in the acquisition of the LC-MS spectra.

3.6.4 X-Ray Diffraction (XRD)

The XRD instrument used was the Bruker kappa Apex II, working with the conventional ω-2θ scan mode with graphite monochromatic Mo-Kα radiation (λ = 0.71073 angstrom).

3.6.5 Infrared (IR)

A Bruker Tensor 27 Platinum ATR was used for the assessment of the respective sample's IR spectrum.

3.6 References

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Chapter Four: Results and Discussion

4.1 Phytochemical analysis

This chapter sees the results obtained from both crude extract and isolated compound analyses. Various techniques were employed for such assessment, which includes phytochemical qualitative analysis, spectroscopic analysis and bioassay inspection. All these methods have been highlighted in the previous chapter, with their protocols clearly stated.

The phytochemical analysis is the investigation of chemotypes present in a mixture by subjecting the mixture to a specific reaction, of which a specific colour change will be indicative of the presence of that chemotype. This can be done in two ways; TLC and reaction method as mentioned in section 3.4.

Table 4.1 represents the results obtained from the methods described in section 3.4. These results include both the ethyl acetate and methanol crude extracts.

The phytochemical test results indicate that the biological activity reported in literature might be due to the presence of alkaloids, catechins tannins, phenols, and terpenoids. These classes of natural products are known to have some biological activity. Synergistic effects of these chemotypes complicate the allocation of bioactivity by chemotype profiles, but with such analyses, the activity can be narrowed down to certain chemotypes.[1]

The large presence of alkaloids in the methanol extract was expected, as *Strychnos* species are known to possess a large number of alkaloids. The bitter taste of *Strychnos hennings*ii as a traditional herbal medicine is attributed to this. The presence of ergot and indole alkaloids show the similarity within the chemotype present. Alkaloids are known to have antimicrobial activity, and the use of *S. hennings*ii in wound healing might be aided by their presence.[1]

Table 4.1: Phytochemical results for the ethyl acetate and methanol crude extracts.

Phytochemical test	Ethyl acetate crude	Methanol crude
Alkaloids	-	+++
Ergot alkaloids	-	++
Ergot alkaloids	-	++
Phenols	++	++
Flavonoids	-	-
Carotenoids	+	++
Vitamin A & D	-	++
Coumarins	-	+++
Sulfonamides	+++	+
Tannins	-	+++ (catechins)
Phlobatannins	-	-
Terpenoids	+	++
Steroids	+	++

NB: '+' indicates the slight presence of the chemotype, '++' indicates the moderate presence and '+++' indicates a very significant presence.

The presence of catechin tannins in the plant material may explain the biological activity of the plant material to a certain degree. This is because tannins are a non-specific class of compounds in terms of medicinal benefit.[1-3] They are detected in well-known plant extracts like green tea and grapes and of great significance as they show positive results *in vivo* and *in vitro* tests. Tannins also intensify the positive result of phenols in the plant material since they are phenolic compounds as well.[4,5]

Oyedemi *et* al have reported on the antioxidant activity of the aqueous crude extract of *Strychnos* henningsii from the Eastern Cape.[1] This activity could be due to the presence of phenols, which act as proton donators or electron acceptors in the interaction with the radical (2,2-diphenyl-1-picrylhydrazyl) in the DPPH assay as well as coumarins. This is the mode of action of compound(s) thought to have anti-oxidant potentials since they have also been reported to have radical scavenging properties.[1-6]

Phenols, on the other hand, have long been recognized to possess anti-oxidant properties, having been largely focused on tea extracts.[7] One of the most known groups of polyphenols are flavonoids and are categorized as follows: flavones, flavonols, flavanones, dihydroflavonols and their carbohydrate derivatives. They are also known to have anti-inflammatory, anti-allergic, hepatoprotective, anti-thrombotic, anti-carcinogenic and antiviral activities.[8,9] Although flavonoids are reported of showing slight presence in the *Strychnos* species, in literature, they were not detected in both the ethyl acetate and methanol crude extracts.[1] Many phenolic compounds function as free radical scavengers to help decrease formation of both advanced lipid peroxidation and advanced glycation end products.[6-8] Most phenolic compounds undergo metabolism and are limited in terms of bioavailability., the question whether the anti-oxidant properties of these small molecule phytochemicals really act as free radical scavengers *in vivo* has not yet been answered. Once again, the synergistic properties of the compounds present in the form of mixtures better explain the bioactivity reported.[10]

Other chemotypes reported positive in in this section do have significant biological activity, however, for the sake of current investigation the scope of the biological activity was narrowed down to anti-oxidant and anti-inflammatory. This was done in order to corroborate the results found by Oyedemi *et al.*[10] Other chemotypes such as tannins are thought to be non-specific in bioactivity and modes of action are difficult to deduce.[3,5]

4.2 Screening of crude extracts

Biological activity evaluation of the crude extracts is crucial in determining their use as traditional herbal medicine. We mentioned in our introduction that traditional herbal practitioner's claim activity of extract and also there are so many reports of activity from different plant species from other regions, as well as with Eastern Cape plant species. However, there are not many reports on *Strychnos hennings*ii. Therefore, the plant's medicinal benefit need to be evaluated and confirmed in order to create a solid library as anticipated. This can be made possible by means of *in vitro* and *in vivo* studies.

The first step would be *in vitro* assays, as they assimilate biological processes outside of the human body. Herewith, the cytotoxicity, anti-inflammatory and anti-oxidant *in vitro* assays are reported on both ethyl acetate and methanol extracts. Also, we report on the anti-oxidant activity of isolated single compounds later in this chapter.

4.2.1 Cytotoxicity screening

Traditional herbal medicines may, although they are reported to be potent in healing ailments, possess chemotypes that, in significant amounts, ultimately cause damage to tissue cells. Thus, evaluation of the toxicity of such medicines becomes a crucial aspect of efficacy evaluation. Synergistic effects may mask the overall effect of the compounds *in vivo*, but the use of sensitive cell lines *in vitro* allows for detection beforehand of such instances.[11-14]

Both ethyl acetate and methanol extracts were submitted for cytotoxicity screening – to evaluate their potential damage to human body cells. The results represented in Figures 11 and 12 are for concentration ranges 10 – 125 µg/ml.

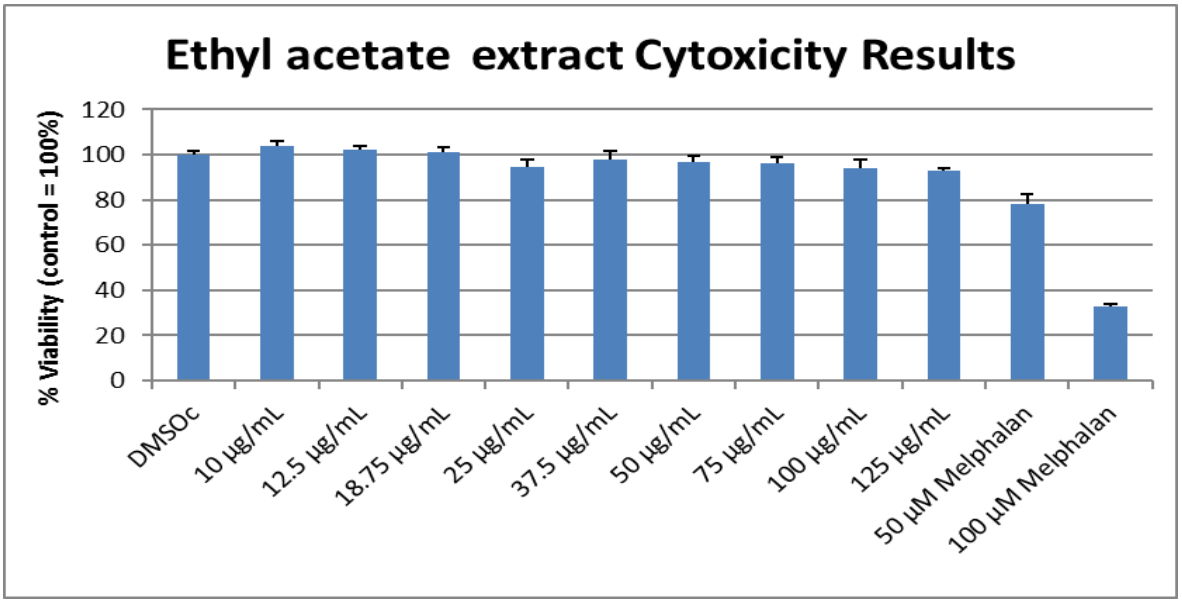

Figure 11: Cytotoxicity results of ethyl acetate extract.

The ethyl acetate extract showed a non-toxic result, as all the concentrations are above the 50 µM melphalan reading (Figure 11). As melphalan was used as the control, any value of percent viability control below its reading at 50 µM would indicate significant cytotoxicity and any value below 100 µM would signify potent toxicity. The extract, at lower concentrations, actually has cell growth benefits. This means that the extract itself does not promote any cell damage, and has cell-count boosting properties. This may be due to the fact that the extract has a majority of less to moderately polar compounds that may not affect the biological processes to a significant degree. More polar compounds contain more functional groups than less polar compounds.

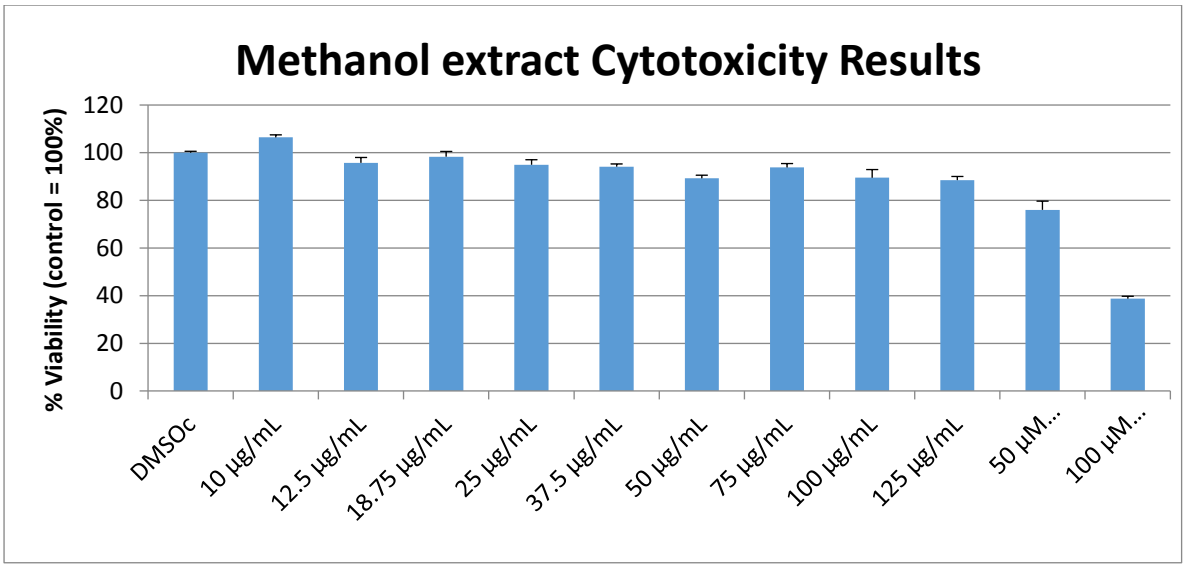

Figure 12: Cytotoxicity results of methanol extract.

The cytotoxicity results of the methanol extract follow the pattern of the ethyl acetate extract results. The methanol extract also showed cell-boosting properties at 10 µg/mL, with the highest concentration of 125 µg/mL having a percent viability control higher than that of 50 µM melphalan (Figure 12). This means that the methanol extract is also proven to be non-toxic.

The methanol extract has more polar compounds than in the ethyl acetate extract. The large presence of alkaloids reported in *Strychnos henningsii*, as well as in the majority of other *Strychnos* species, one would expect significant toxicity levels. Perhaps the toxicity levels in the crude extract were dampened by the presence of other chemotypes, such as vitamin A and D, polyphenols and other chemotypes. The specific biological activity could be explained, only to a certain extent, by isolation of a huge number of compounds in the extract, and examine the bioactivity of them all.[13-15]

The use of *Strychnos henningsii* as a traditional herbal medicine, has, to a limited extent, been substantiated as an active traditional medicine. The presence of diverse classes of compounds play different, yet effective roles. Thus, this study features preliminary investigations of the compounds present in the plant's bark.

4.2.2 Anti-inflammatory screening

Various modes of *in vitro* analyses were made use of to fully probe the anti-inflammatory benefit of the crude. Both the methanol (SHB.M) and ethyl acetate (SHB.E) crude extracts were chosen for analysis. Figure 13 represents the cell viability investigation of the extract in unstimulated macrophage cells; an anti-inflammatory cytotoxicity assay on the RAW cells.[16] The values have been normalized for the differences in cell numbers.

Sample-assay bioactivity investigation in terms of the nitrate production in unstimulated macrophage cells, in which lipopolysaccharide (LPS) serves as the positive control is illustrated by Figure 13. The bioactivity is presented by the ability of the sample to inhibit the inducible nitric oxide (iNOS) and subsequent nitric oxide (NO) production.[16-19] Figure 13 shows the nitrate production inhibition in LPS activated macrophage cells, in which DMSO is used as the positive control, as it attenuates the induction of iNOS expression and subsequent NO production.

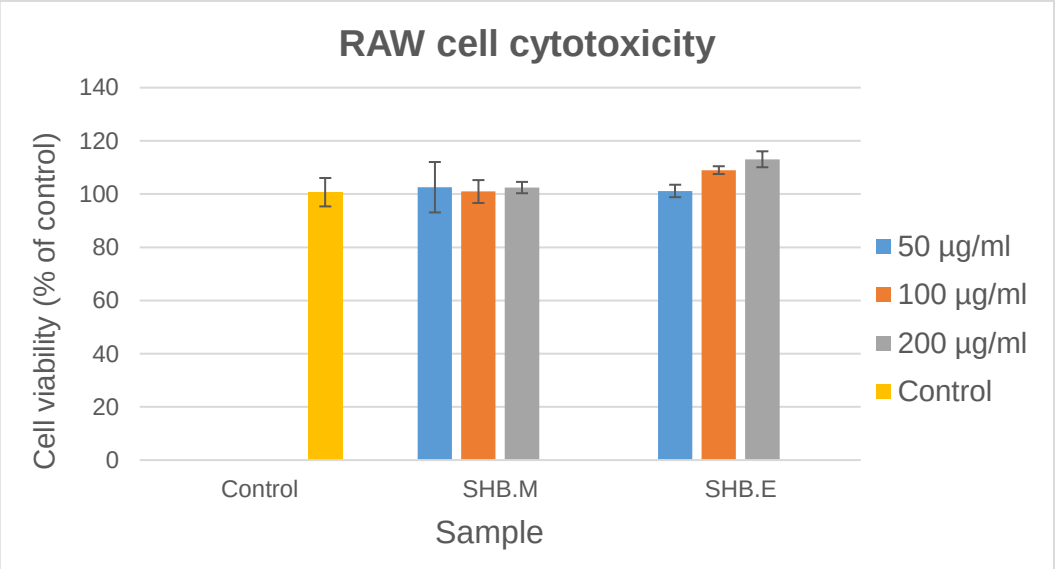

Figure 13: RAW cell viability cytotoxicity results of crude extracts.

The extracts did not exhibit any cytotoxicity on the RAW cells (Figure 13). The ethyl acetate extract, again, proved to have cell growth properties, as visible by the increase in the cell viability of the extracts.

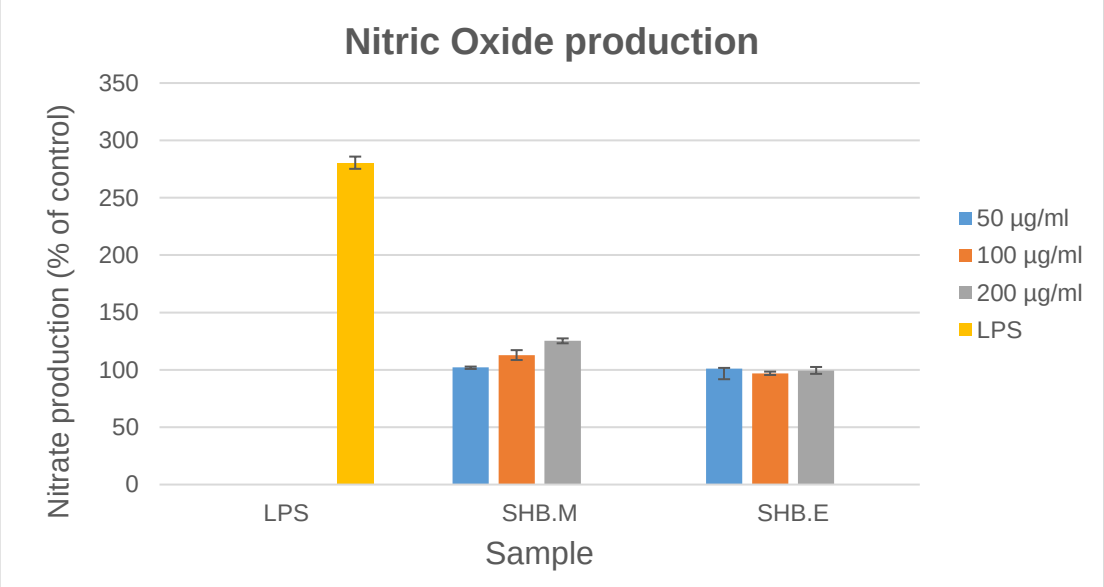

Figure 14: Nitric oxide (NO) production in unstimulated macrophage cells to assess the pro-inflammatory potential of both extracts.

LPS serves as a positive control to illustrate macrophage activation as it induces the expression of iNOS and subsequent nitric oxide production. This represents the pro-inflammatory potential of the tested extracts. The extracts did not produce much inflammation as compared to LPS. This means that the ethyl acetate and methanol extract are not prone to induce any significant inflammation in the cells. The methanol extract at 200 µg/mL was shown to have slightly induced more nitrate oxide (Figure 14). This may be due to the presence of alkaloids in the extract, which are known to be quite reactive. The *Strychneae* alkaloids associated with the *Strychnos* species are thought to be responsible for the activity at 200 µg/mL because of the presence of the tertiary amides.

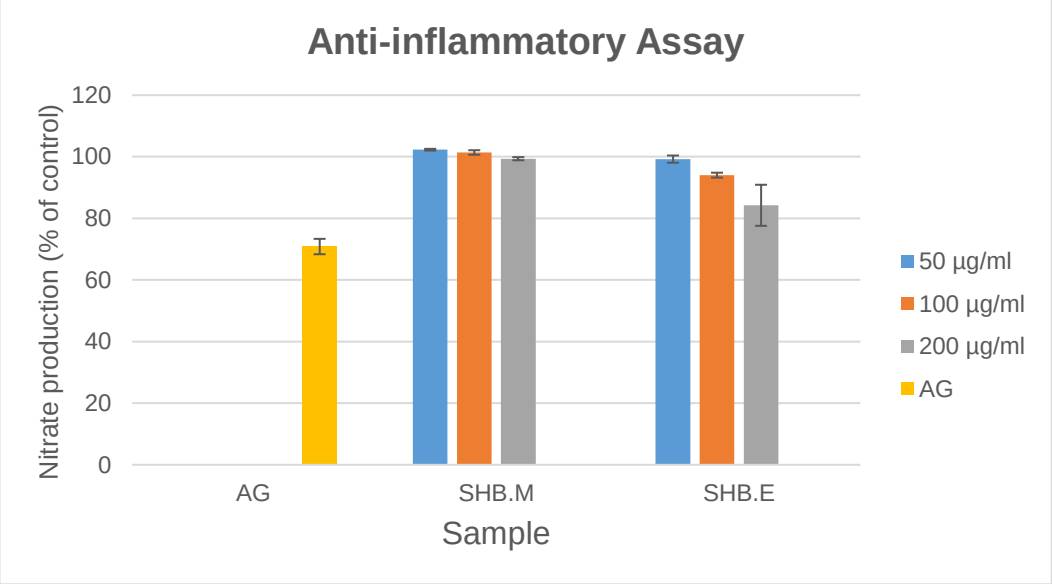

Figure 15: Inhibition of nitrate production in LPS activated macrophage cells.

The results show the reference sample, aminoguanidine (AG), possesses better control of NO production. The presence of alkaloids in the crude extracts might help inhibit NO production, especially in induced conditions as in *in vitro* assays. Follow up studies of these results are underway in our laboratories. Both extracts do not show better anti-inflammatory activity when compared to aminoguanidine, a known drug that inhibits NO production in LPS activated macrophage cells. Therefore, the extracts are shown to maintain the nitrate production.

4.2.3 Anti-oxidant assays

Following the phytochemical screening of the extracts, the anti-oxidant potential was relevant in the bioactivity profiling of the plant material. The presence of phenols and coumarins, as evaluated in Section 4.1, warrants investigation of possible anti-oxidant potential. This activity might help in substantiating the use of the medicinal plant in healing wounds, perhaps due to the presence of phenols.[20,23,26] The chosen biological assays, i.e. DPPH, FRAP and ORAC, are known to be sensitive, and in increasing manner. [20-29]

The DPPH assay is a reaction between DPPH (2,2-diphenyl-1-picrylhydrazyl) and the sample. The activity is seen as the readiness of the sample to donate a hydrogen to the radical. A change in colour is indicative of the reaction, which is monitored by absorption.

The FRAP assay is based on the ability of the sample to reduce Fe^{3+} to Fe^{2+}, which is an important parameter for a compound to be considered a good anti-oxidant. This can be achieved by donation of an electron. The ORAC assay is based on the downward change of the monitored fluorescence intensity due to the radicals produced by heating 2,2- azobis(2-amidinopropane) dihydrochloride (AAPH).[24,27]

The following are the results obtained from the screening processes, which are described in Section 3.5.3.

4.2.3.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The crude extracts were subjected to anti-oxidant analysis, with the method stated in Section 3.5.3.1. Each absorbance of a specific concentration (50 – 250 µg/ml) was recorded in quadruplicates (Figure 16). The %inhibition was calculated from the following formula;

$$\%\ inhibition = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\ x\ 100$$

Where Abs_{blank} = absorbance value of blank and Abs_{sample}= absorbance value of sample (Figure 17).

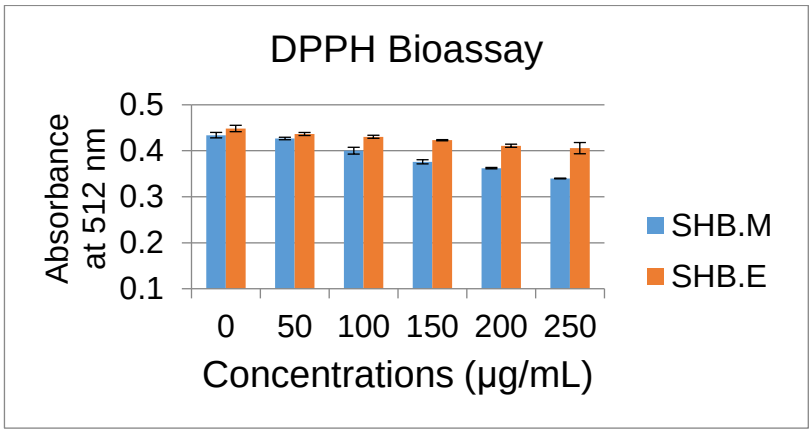

Figure 16: DPPH assay results of crude extracts with error bars at 95% certainty.

The IC_{50} values of both ethyl acetate and methanol extracts, as well as the control, are 462.72 µg/ml, 317 µg/ml and 179.54 µg/ml respectively. Both extracts have weaker biological activity in terms of anti-oxidant potentials when compared to quercetin, a known anti-oxidant (Figure 16).

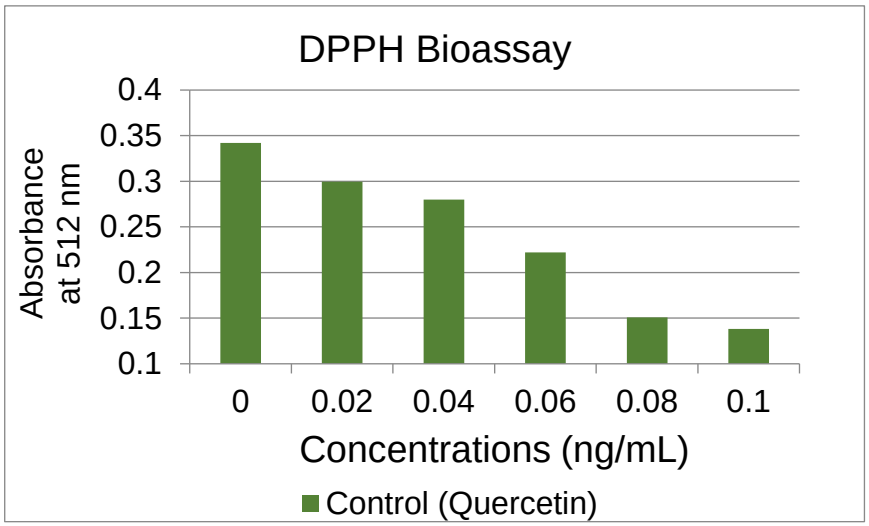

Figure 17: Absorbance bar graph of quercetin as the positive control

The difference in the IC_{50} values of both crude extracts, although they have similar TLC profiles, is due to the presence of the different chemotypes that make each extract unique. The methanol extract proved to possess more anti-oxidant potential than the ethyl acetate extract. This activity is thought to aid the synergistic activity in the *in vitro* assay, as the presence of a huge number of potentially bioactive chemotypes helps the radical scavenging potential of the samples.

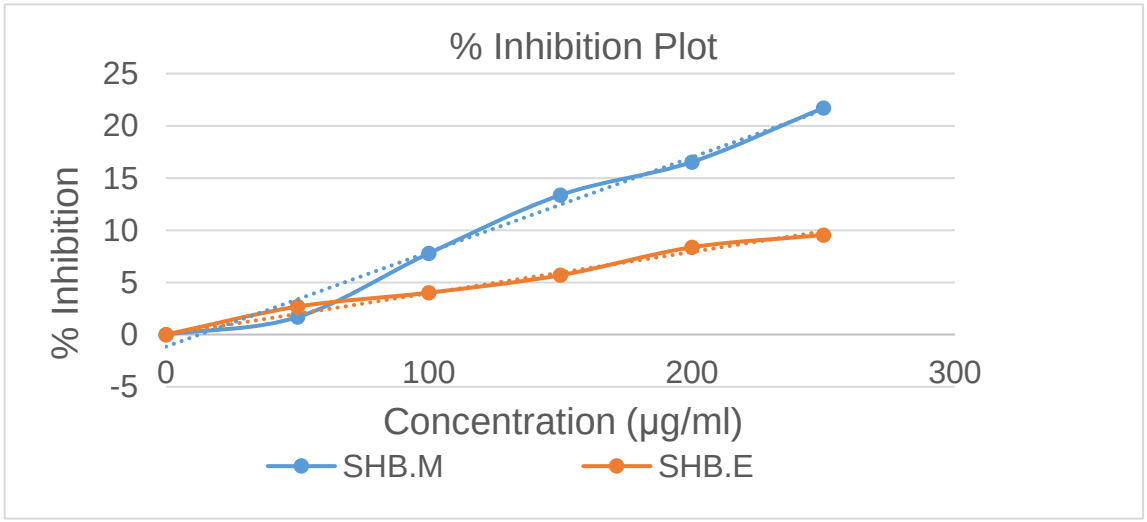

Figure 18: % Inhibition plot of DPPH assay of crude extracts from 50 – 250 µg/mL.

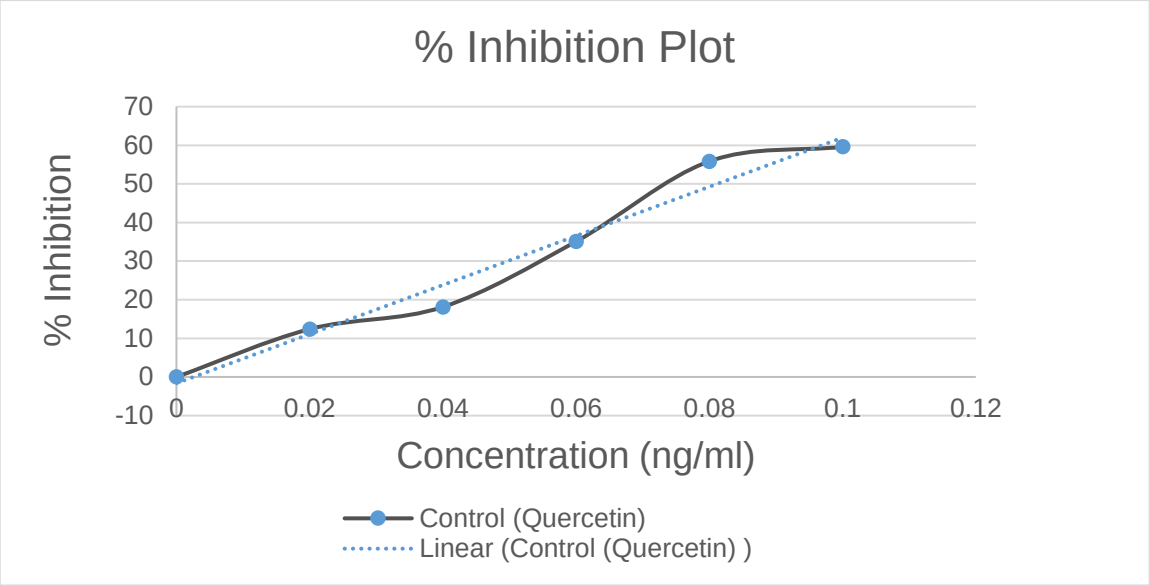

Figure 19: % Inhibition plot of DPPH assay of quercetin from 0.02 – 0.1 ng/mL

The results displayed in the previous diagrams, Figure 18 and Figure 19, exhibit the anti-oxidant potential from highest to lowest, in the following order; quercetin, methanol extract, and lastly the ethyl acetate extract. This is expected as quercetin is one of the most potent anti-oxidants. The methanol extract slope in Figure 18 is 0.0907, whilst the ethyl acetate extract is 0.0396, as compared to quercetin's 636.09. Although the positive control's concentration was in ng/mL, and both the methanol and ethyl acetate extracts in µg/mL, the extracts do possess anti-oxidants, that, if isolated in considerable amounts, could give more conclusive data on the anti-oxidant potential of *S. hennigsii*.

4.2.3.2 FRAP (Ferric Reducing Ability of Plasma)

Both crude extracts were then subjected to a FRAP anti-oxidant assay. The method followed is clearly stated in Section 3.5.3.2. Each absorbance reading of each concentration was recorded in quadruplicate. The results are represented in graphically in Figure 20.

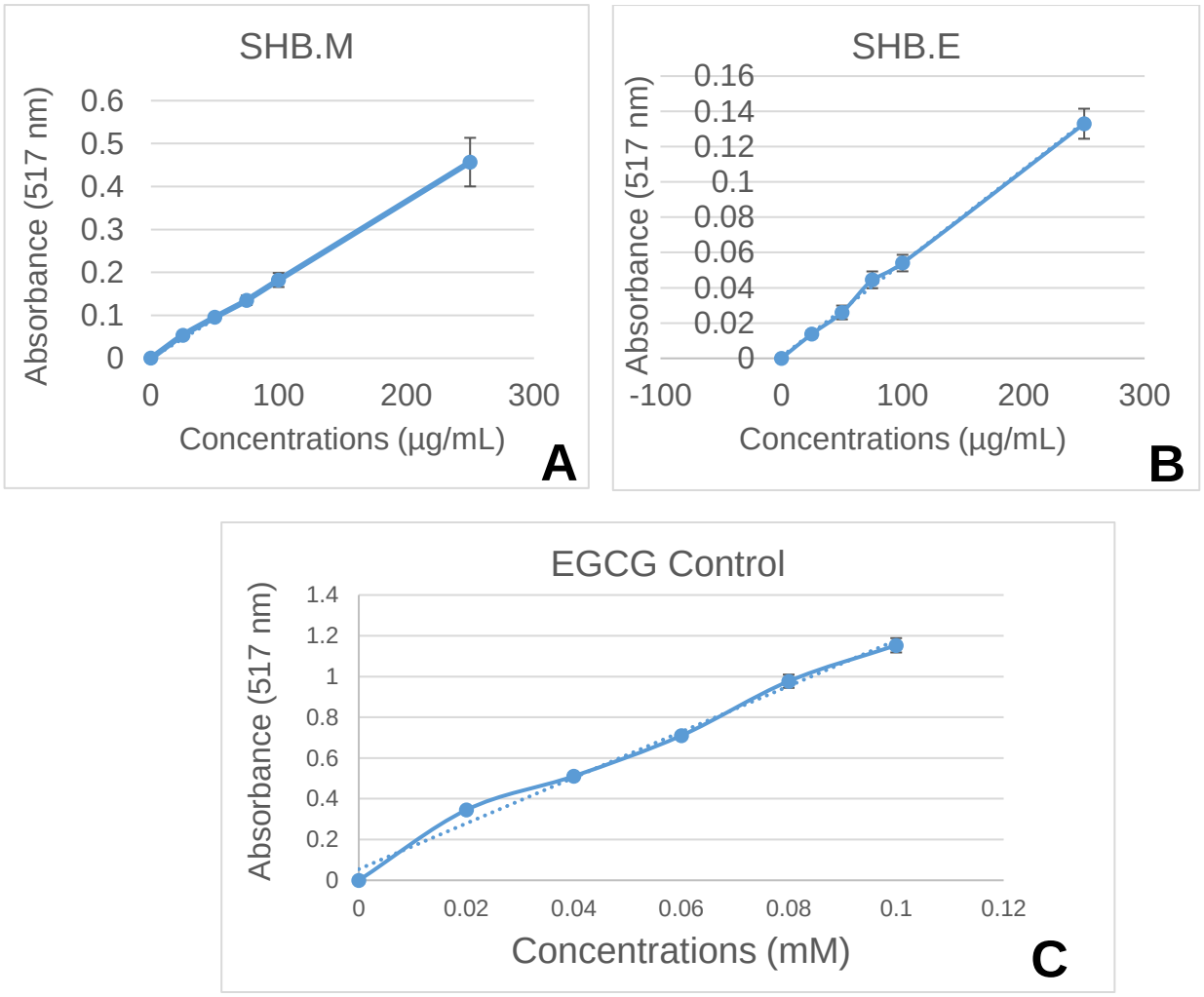

Figure 20: FRAP assay results of crude extracts and ECGC (epigallocatechin gallate) as positive control; (**A**) = methanol crude extract, (**B**) = ethyl acetate crude extract, and

(**C**) = EGCG.

The IC_{50} values of the extracts, methanol (SH.M) and ethyl acetate (SH.E), are 126.87 µg/ml and 131.2 µg/ml respectively. The control, EGCG, had an IC_{50} value of 0.046 mM. According to the assay, both extracts possess poor anti-oxidant potential when compared to that of a known anti-oxidant.

It can be seen from the graph (Figure 20B) that the ethyl acetate does not have much anti-oxidant potential containing compounds. The difference in the activities of both extract, again, is due to the chemotype profiles of each. The methanol extract, containing more polar compounds due to the presence of multiple hydroxyl groups, exhibits better anti-oxidant activity when compared to the ethyl acetate extract.

4.2.3.3 ORAC (Oxygen Radical Absorbance Capacity)

In this assay, fluorescence intensity decreases as oxidative degradation increases, with the fluorescence typically recovered around 100 min from the addition of the oxygen radical initiator. When an anti-oxidant, experimental sample, is mixed with the fluorescent molecule and the oxygen radical initiator, however, it can protect the fluorescent molecule from degradation and thus fluorescent intensity is maintained over time. Therefore, the longer the fluorescent molecule maintains its intensity, the less the degradation caused by the oxygen radicals present, or the more it is protected by the anti-oxidant compound.[24,28,29]

Only the methanol extract was chosen for this assay, as the previous assays were used for preliminary assessment purposes. The sensitivity of this assay, time, as well as the availability of the reagents, were also determinative factors. The method used has been described in section 3.5.3.3.

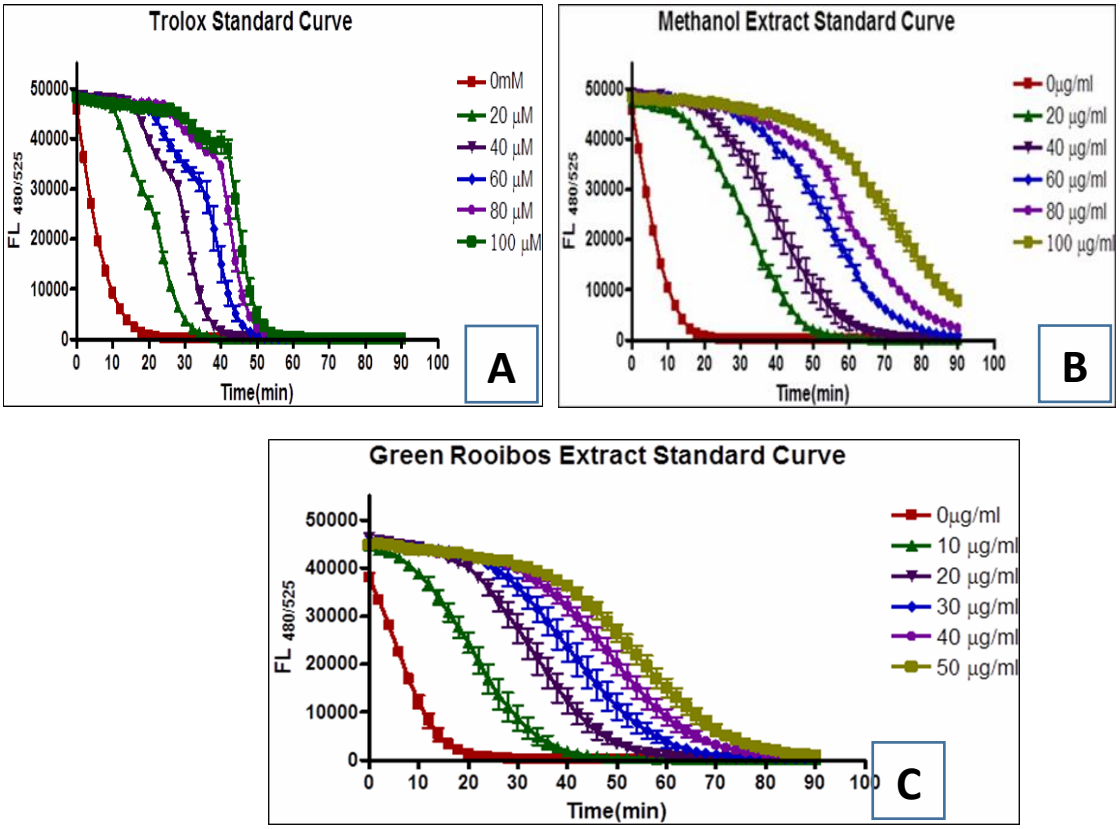

Figure 21: ORAC assay results; (**A**) = Trolox (positive control), (**B**) = Methanol crude extract, and (**C**) = Green tea extract.

From Figure 21, the Trolox sample, at the same concentrations as the methanol crude extract, exhibited less activity because Trolox only managed to protect fluorescein until around the 50th min. The assay was allowed to completion as to record the full experiment for comparison reasons.The methanol extract exhibited better anti-oxidant capacity as compared to Trolox. This is evident in the 100 µg/mL sample still retaining fluorescence even at 90 min, at the end of the assay time (see Figure 22).

The green tea extract (rooibos = red bush) exhibited far superior anti-oxidant capacity than Trolox, which as expected. The tea extract was obtained by hot water extraction. This is due to a large amount of polyphenols present in the tea extract. The presence of a diversified polyphenolic group attributes to the tea's anti-oxidant activity. Even at the concentration range of 10 to 50 µg/mL, it clearly shows the dramatic difference between the mixtures altogether (Figure 20). Synergistic effects are thought to have an effect on the tea's bioactivity.

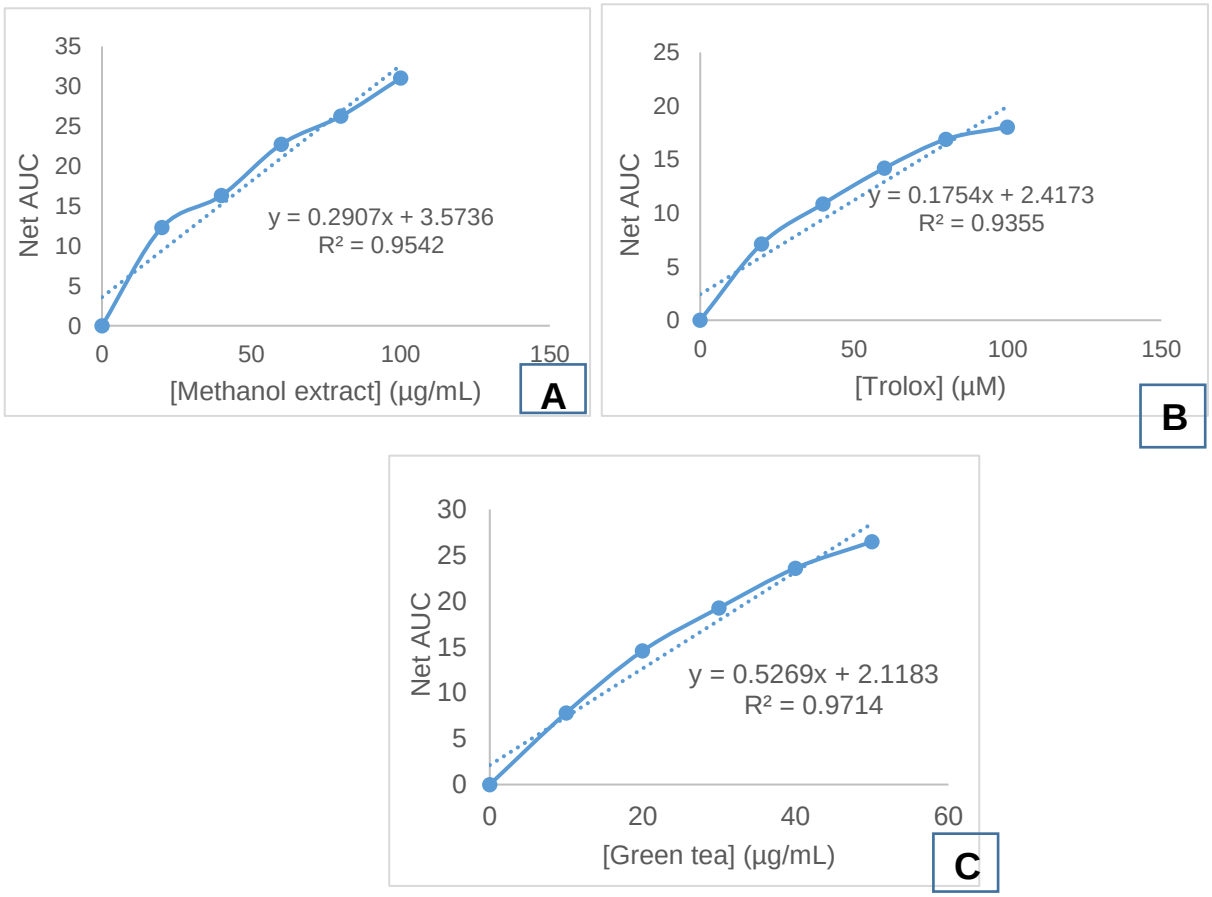

Figure 22: Anti-oxidant dose-response curves. Net AUC (area under the curve) plot

of samples of known anti-oxidant properties. Each curve subjected to linear regression analysis for Trolox comparison.

The Trolox equivalent is calculated using the following equation;

$$TE_{(concentration\ ranges)} = m_{sample} - m_{Trolox}$$

Table 4.2: Trolox equivalent (TE) determination of extracts.

Anti-oxidant Capacity		
	Slope	TE
Methanol extract	$0.2907 \pm 9.634 \times 10^{-3}$	0.1153
Green tea	$0.5269 \pm 10.001 \times 10^{-3}$	0.3515

From Figure 22, *Strychnos henningsii* is seen to have significant anti-oxidant potential. The results obtained from the ORAC assay see the comparison of the methanol extract with two samples of known anti-oxidant activity, Trolox and green tea extract. The ORAC assay results corroborate those obtained from the previous anti-oxidant assays, that is DPPH and FRAP. *Strychnos henningsii*, thus, is shown to have considerable anti-oxidant properties. The Trolox equivalent (TE) of the methanol extract also corroborates *S. henningsii* possessing anti-oxidant properties, with a value of 0.1153. Although it is small when compared to other known anti-oxidants, the possibility of other chemotypes present in the methanol extract dampening the anti-oxidant potential of the plant extract cannot be ignored.

The methanol extract has shown to be better than Trolox, which was used as the baseline sample for anti-oxidant capacity in this assay. However, the anti-oxidant capacity of the methanol extract is attributed to the synergistic effects of the compounds present in the extract. It shows that the *S. henningsii* does have biological activity, although not in the same capacity as green tea, a mixture of known anti-oxidant properties.

The methanol extract either contains chemotypes that protected fluorescein from degradation, reacted with the oxygen radicals, preventing them from targeting fluorescein, or both processes. The mode of action cannot be deduced since the extract contains numerous chemotypes. However, from the phytochemical analysis,

chemotypes such as phenols would seem to be largely responsible for the activity in such cases because of the proton donation to the oxygen radicals. Structure-activity relationships have been formulated for optimization of bioefficacy of compounds.[29-31]

The biological assays conducted on *S. henningsii* have substantiated its use as a traditional medicine, and further studies are, however, warranted to understand the mode of action of the chemotypes present. The phytochemical tests revealed the present of flavonoids, among others, which are known for their anti-oxidant and anti-inflammatory benefits. Other chemotypes may have hydroxyl groups attached, which enable the compound(s) in radical scavenging.

4.3 Spectroscopic analysis of isolated compounds

4.3.1 Analysis of isolated compounds

The crude ethyl acetate extract was fractionated by vacuum liquid chromatography (VLC). Six fractions were collected using Hex:EtOAc (2 x 50 mL) solvent system. For this process, the solvent system was increased in polarity steps of 10% overall, starting from 9:1 to 4:6 (Hex:EtOAc). The fractionation process of the crude ethyl acetate extract is reported in Figure 23, which also indicated the masses of the fractions collected.

Isolation and purification of these compounds by preparative TLC afforded few compounds. It is worth reporting that fraction 2 looked similar to fraction 3 when spotted on TLC, therefore the two fractions were combined. Fractions 1, 4, 5 and 6 did not yield a significant amount of sample for analysis thus they were not investigated further.

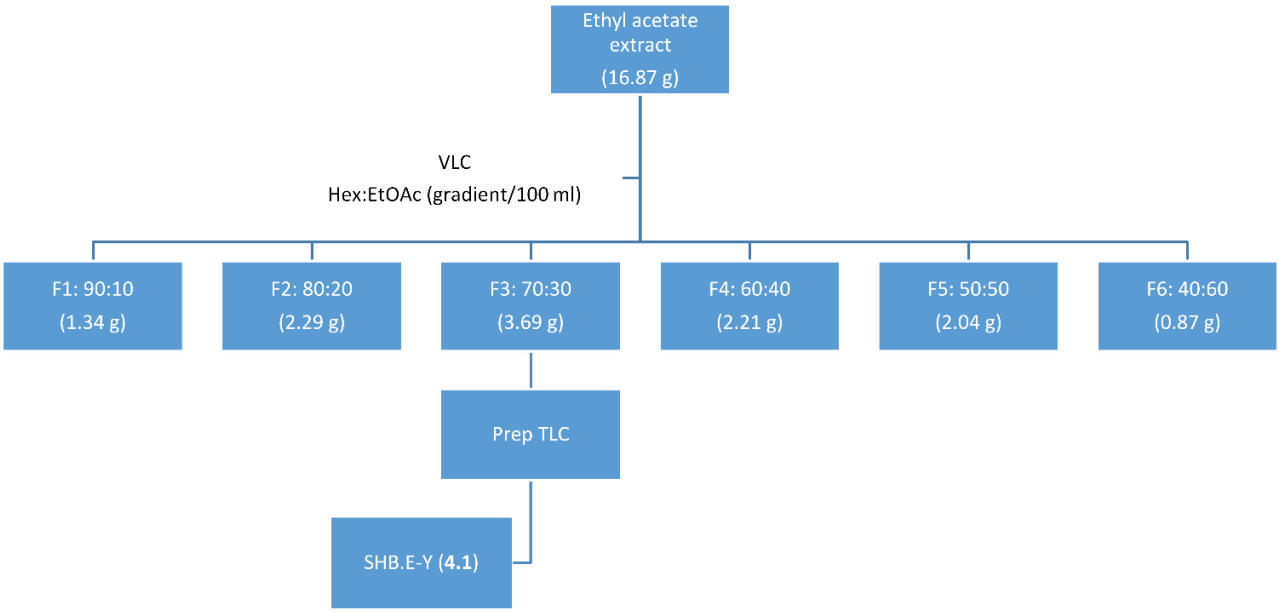

Figure 23: Isolation procedure of the ethyl acetate extract scheme.

The LC-MS spectra of both positive mode (ES+) (Figure 23A), and negative mode (ES-) (Figure 23B), show the relative quantity of compounds present in the ethyl acetate crude extract that are reactive to both modes. In both spectra in Figure 23, there are at least three sets of what appear to be diastereomers in the extract; the three sets of 'doublets' displaying stereoisomeric features. There are also sharp single peaks in the ES- spectrum. The poor separation could be due to compounds being of the same chemotype, as they exhibit strong affinity towards each other.[35]. It has also been found that the ES- mode possesses higher signal/noise ratios, of which would be one of the contributing factors of increased reactivity by $[M - H]^-$ molecules.[36] The use of the ES- mode enabled the detection of negatively charged compounds like phenols and carboxylic acids (typically with a [M – H] or [M + Cl] observation).[36]

The significant peaks indicating the presence of some stereoisomers and/or similarities in molecular weight from ES- mode. Since there were no standards available for these compounds, it was not possible at the time of analysis to identify which specific candidate compounds were present. However, phytochemical analysis by LC-MS was able to confirm a few alkaloidal candidates. Two potentially alkaloidal compounds, with the same molecular weight of 399.2 (molecular formula $C_{27}H_{25}N_2O_5$), eluted at 2.05 and 2.69 min. The other compounds eluted at 3.75 min (ES+, molecular mass 397.2, $C_{22}H_{25}N_2O_5$), and 2.13 min (ES-, molecular mass 353.1, $C_{21}H_{25}N_2O_3$). On the ES+ spectrum, two compounds (retention times 9.53

and 10.30 min) consist of similar molecular formulae of $C_{30}H_{41}O_5$ and $C_{29}H_{41}O_5$, respectively. The compound eluting at 9.51 min (ES-, molecular weight 479.3) has the most intense peak, with a molecular formula of $C_{30}H_{39}O_5$. The aforementioned compound's molecular mass is synonymous with those of triterpene. This information allowed us to show some evidence of the relationship of compounds present in the crude extract from the Eastern Cape *Strychnos henningsii*. The data further showed that these (whether as a single compound of a specific molecular weight or as a combination of compounds of similar molecular weight) were present in different amounts.

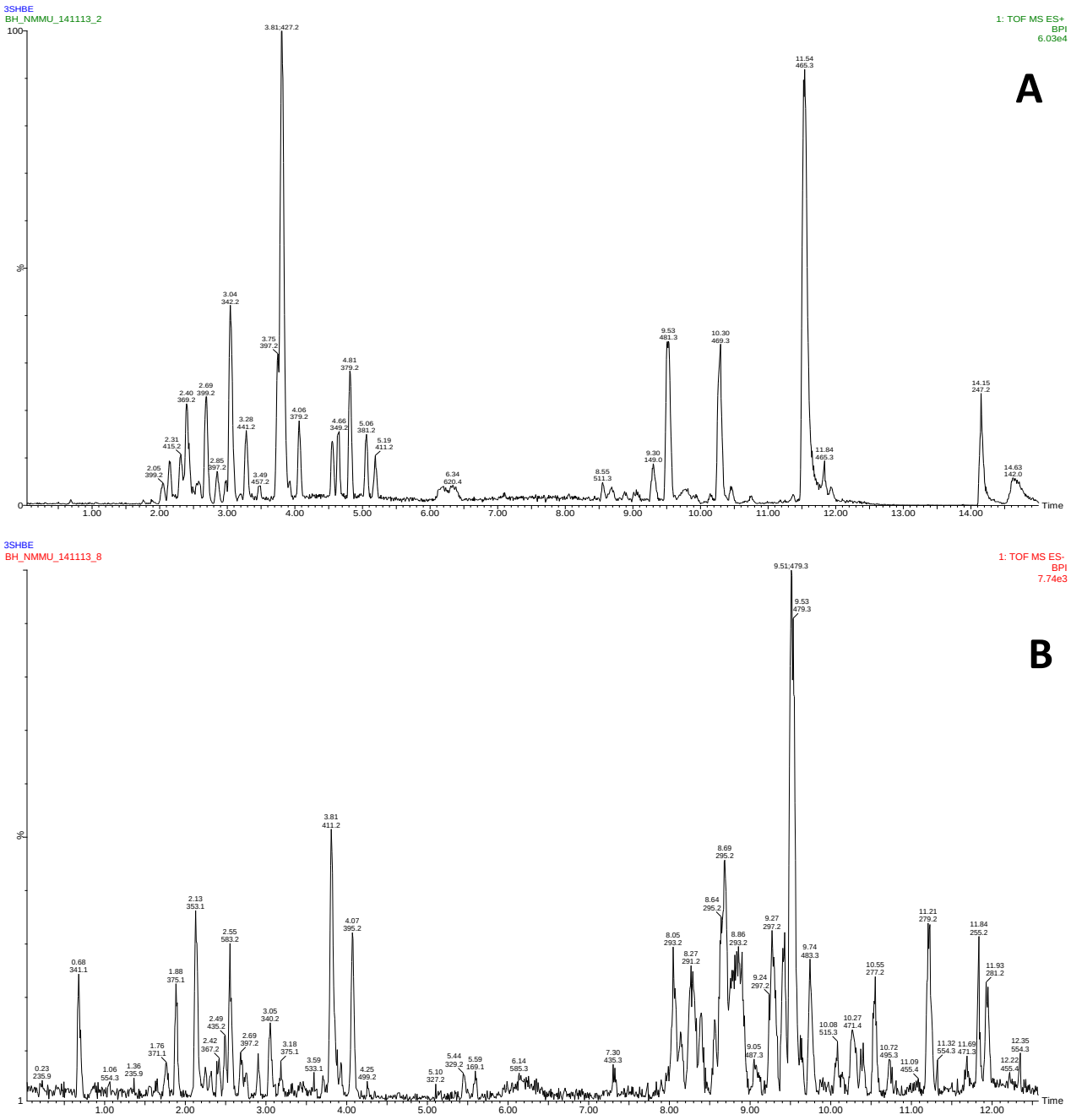

Figure 24: LC-MS chromatograms of ethyl acetate extract *via* positive (**A**) and negative (**B**) ion modes.

From the ethyl acetate extract, a single compound coded SHB.E-Y (**4.1**) was isolated from combined fractions (2 and 3) through prep TLC (yellow rectangle in Figure 25). The aluminum coated with silica prep TLC plate was developed in Hex:EtOAc (85:15). After elution, the plate was viewed under UV to evaluate the bands after which the band of interest was cut-off, extracted with ethyl acetate multiple times and subsequently rinsed with methanol (Figure 25). The solvent was removed under vacuum to afford a yellow oil. The isolated compound (**4.1**), was identified as a terpenoid based on the colour of

its spot on TLC. The TLC stained pink when treated with vanillin. A molecular ion peak at *m/z* of 465.3005 $[M + H]^+$ observed in the HRMS spectrum was in agreement with the molecular formula $C_{30}H_{41}O_4$. The molecular formula counted for 11 degrees of unsaturation. This molecular mass (465.3005) at R_t 11.54 minutes was observed in the LC-MS (ES+) chromatogram as a major compound detected in the crude ethyl acetate extract. The IR spectrum of **4.1** showed a weak -OH absorption (3367.93 cm^{-1}) alkane C-H stretch (2923.20 and 2853.24 $cm^{-1)}$, alkane C-H bending (1456.57 cm^{-1})), C=O (1731.39 cm^{-1}), C=C (1638.09 cm^{-1}) functional groups (Appendix A7).

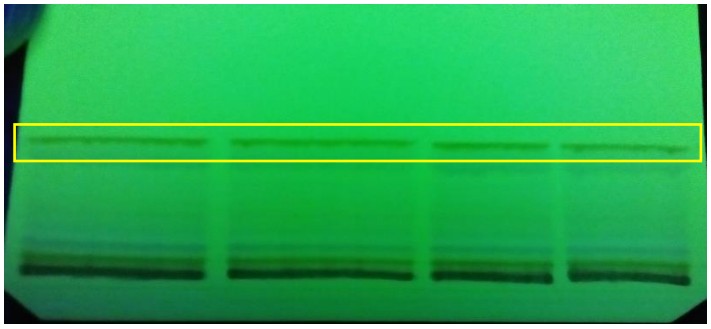

Figure 25: Prep TLC plate of fraction 3 developed using Hex:EtOAc (85:15) solvent system.

The ^1H and ^{13}C NMR spectra of **4.1** revealed an impure compound (Appendix A1 to A6). 30 carbons that would suggest a triterpenoid were expected from the ^{13}C NMR as indicated above (m/z of $C_{30}H_{41}O_4$) However, due to impurities, more than 30 carbons were observed and these included a carbonyl characteristic peak at □c 206.9. Characterization of resonances in the ^1H NMR spectrum was not attempted since the ^1H-^{13}C correlation in the HSQC spectrum was not clear, thus unidentified structure. To the best of our knowledge, a type of compound with characteristics of **4.1** has not been reported in the literature of *S. henningsii species,* therefore, this information is taken into consideration for future studies.

The methanol extract, on the other hand, was subjected to a gradient fractionation by VLC, yielding 8 fractions. The flow diagram for the isolation of compounds is shown in Figure 26.

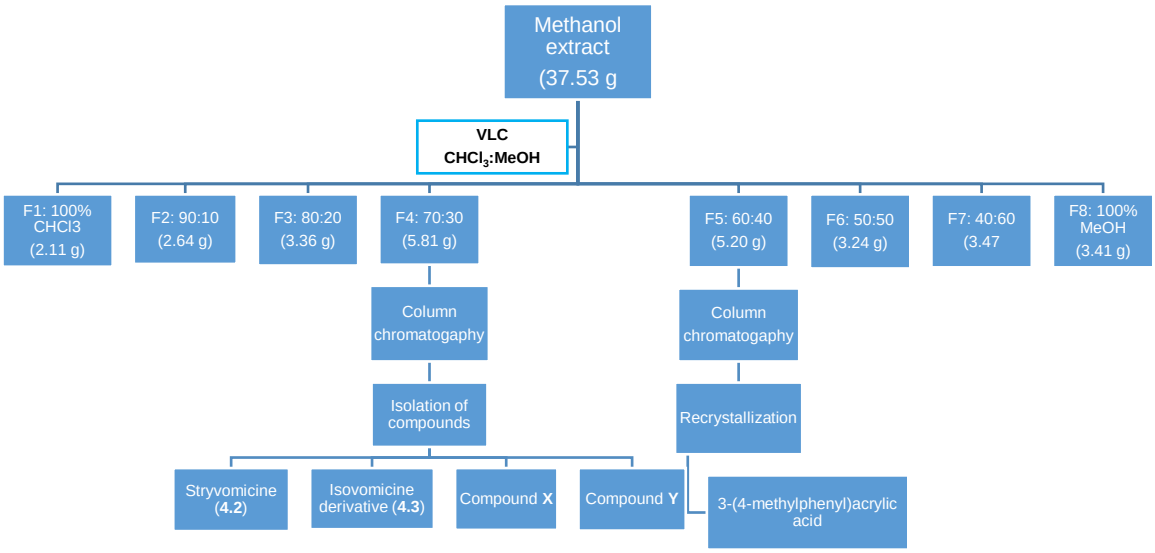

Figure 26: Isolation procedure of the methanol extract scheme.

After normal TLC profiling, fractions 4 and 5 (Figure 26) were subjected to column chromatography, in different column set-ups. The two fractions were chosen due to the ease of separation they displayed in their TLC profiles. Typical silica gel column chromatography protocols were followed which included constant TLC tracking and the combination of fractions of similar TLC profiles. From fraction 4, four compounds were purified and isolated via prep TLC. These compounds were found in the mid-polar region. The main purification technique employed was prep TLC, as it is a relatively less time-consuming, easy to handle small amounts of fractions and minimizes the loss of material as would occur in the case of silica gel column chromatography. In the prep TLC, use of trimethylamine (not more than two drops) allowed for the proper separation of bands where striking of compounds was observed. This process eventually led to the isolation of stryvomicine (**4.2**), isovomicine derivative (**4.3**) and 2 unidentified compounds labelled **X** and **Y** in the figure above. Upon careful inspection, **4.2** and **4.3** showed similarities when compared to those reported in the literature. The ^{1}H NMR spectra of these compounds strongly resembled indole alkaloids. The two compounds are discussed below.

4.3.1.1 Stryvomicine (**4.2**)

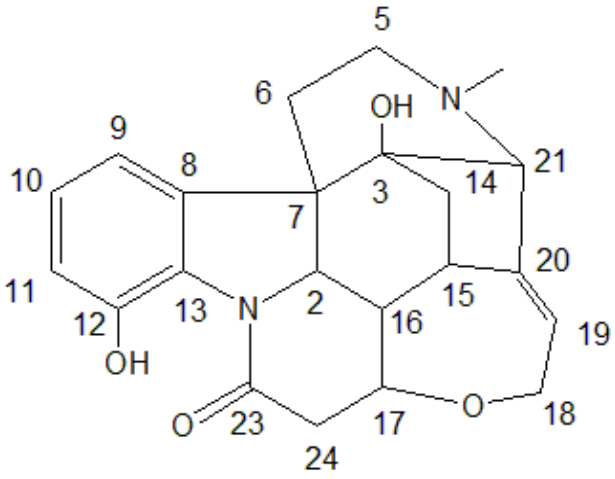

Figure 27: Chemical structure of stryvomicine (**4.2**).

Compound **4.2** (Figure 27) was obtained as a yellow-brown solid after solvent evaporation. In the (ES+) HRMS spectrum, the molecular ion peak observed at *m/z* 381.1817 (calcd 381.1814) $[M + H]^+$ is in agreement with the molecular formula $C_{22}H_{25}N_2O_4$, with 12 degrees of unsaturation. The NMR spectra (Appendix B1 to B6) closely resembles that published by Zhao *et. al.*[37] In the ^{1}H NMR, three aromatic protons assignable to a benzene moiety at δ_H (m, 7.29), δ_H (m, 7.24) and δ_H 7.12 (t, *J* = 12 Hz) were observed. The ^{1}H NMR also shown one olefinic proton and one hydroxyl proton downfield as shown in table 4.3 below. The hydroxyl proton, however, was shouldered on the solvent peak, as CD_3OD was used. In the Dept-135 spectrum of **4.2**, a few significant CH_2 peaks in the negative y-axis were observed. The CH_2 peaks caused by C-5, C-6, C-14, C-18, and C-21 appeared at various chemical shifts (Table 4.3). In addition, the ^{13}C NMR spectrum showed quaternary carbons appearing downfield, which were assigned to C-7, C-8, C-12, C-13, C-20, and C-23, respectively. The ^{1}H and ^{13}C NMR data of isolated compound **4.2** was in agreement with the reported data. The complexity in the indole alkaloids' exact structural conformation is another cause of unidentified peaks. Minor differences were noticed, which may be caused by use of difference solvents. Some of the peaks in our study were not well resolved or accounted for in comparison due to different solvents used for NMR experiments, as well as purity of the compound.

Table 4.3: Summarized NMR data of stryvomicine (**4.2**) and structure published by Zhao *et. al*.[37]

Carbon Number	**Isolated stryvomicine (4.2)[a]**		**Published data for Stryvomicine[b]**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
2	4.47, s	60.00	4.42, d (9)	58.9
3	-	73.87	-	78.8
5α	2.35, m	47.83	2.52, overlap	43.7
5β	3.37, m		3.37, m	
6α	2.27, s	46.29	2.52, overlap	34.7
6β	1.04, m		1.11, overlap	
7	-	56.15	-	49.9
8	-	136.36	-	140.2
9	7.29, m	117.50	7.99, dd (6.5, 2.0)	119.2
10	7.24, m	126.53	7.17, overlap	127.9
11	7.12, t (12)	111.77	7.15, overlap	118.4
12	-	150.82	-	147.4
13	-	128.33	-	129.0
14α[b]	1.68, m	40.54	1.63, d (12.0)	41.1
14β[b]	2.12, s		2.07 dd (12.0, 6.5)	
15	3.10, m	30.76	3.13, m	37.2
16	1.19, s	*	1.14, overlap	51.0
17	*	71.30	4.26, m	76.2
18α	3.73, s	67.70	4.17, dd (14.0, 7.0)	64.3
18β	3.70, m		4.06, dd (14.0, 6.5)	
19	6.67, d (9)	130.28	6.17, dd (6.5, 7.0)	123.6
20	-	146.89	-	145.5
21α	*	62.38	3.85, s	73.8
21β	*		*	
23	-	163.66	-	172.2
24α	3.43, d (7)	34.59	3.25, dd, (10.5, 8.0)	43.7
24β	*		2.85, overlap	
N-CH_3	1.91, s	23.01	2.86, s	44.3
12-OH	4.75, s		12.60, s	

[a] Data was recorded at 400 MHz for proton and at 100 MHz for carbon in CD_3OD for strvomicine (**4.2**)
[b] Data was recorded at 500 MHz for proton and at 125 MHz for carbon in pyridine-d_5 for the published structure.

- There are no protons at the carbon positions
* Missing signals could not be allocated due to high signal-to-noise, overlapping signals and unfavorable relaxation times

4.3.1.2 Isovomicine (**4.3**)

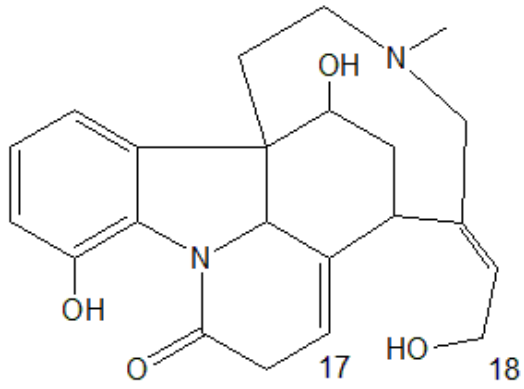

Figure 28: Chemical structure of isovomicine derivative (**4.3**).

Compound **4.3** (Figure 28), on the other hand, showed a molecular ion peak at *m/z* of 383.1959 $[M + H]^+$, (calcd 383.1959, $C_{22}H_{27}N_2O_4$) indicating a chemical structure resemblance to that of stryvomicine ($C_{22}H_{24}N_2O_4$, *m/z* 381.1816). Compound **4.3** had 11 degrees of unsaturation. The absence of C-3 carbonyl peak in the ^{13}C NMR spectra, deviates from what is reported in the literature.[37,47] The HSQC spectra revealed that there is a hydroxyl group at C-3. Therefore it was suggested that **4.3** resembles isovomicine as seen reported by Zhang *et. al*.[48] In the ^{13}C NMR spectrum, detection of the C-24 signal was made extremely difficult due to small quantity of **4.3** and as well as the choice of solvent system. The complexity of the indole alkaloids' structural framework led to some of the peaks unidentified. The ^{1}H and ^{13}C NMR data comparison of **4.3** and published date of isovomicine is given in Table 4.4.

The carbon framework is similar to that of stryvomicine, except for the absence of the C3-C21 bond, a hydroxyl group being attached to C-18 instead of the ether oxygen, and a C16-C17 double bond. This conclusion was validated by C-17 correlating to a proton via the HSQC experiment, and C-16 correlating to three protons via the HMBC experiment. Also, there was a correlation between C-18 and the hydroxyl proton. The proposed assignments fitted with the molecular formula of *m/z* 383.1959.

Table 4.4: Summarized NMR data of (**4.3**) and isovomicine.[48]

Number	**Isolated isovomicine der.**		**Published data for Isovomicine[b]**	
	δ_H (J in Hz)	**δ_C**	**δ_H (J in Hz)**	**δ_C**
2	4.35, d (11)	60.48	4.91, d (2.4)	65.50
3	-	68.15	-	187.0
5α	2.47, d (19)	53.88	2.84, dt (12.9, 3.3)	49.8
5β	3.35, d		2.54 dd (12.9, 6.0)	
6α	2.29, m	49.88	1.85, dd (13.2, 3.3)	46.9
6β	1.30, m		3.02, dt (13.2, 6.0)	
7	-	57.11	-	54.2
8	-	126.53	-	125.8
9	7.86, d	107.54	7.13, dd (1.2, 7.5)	116.9
10	7.14, m	127..33	7.04, t (7.5)	127.1
11	7.25, m	111.77	6.76, dd (1.2, 7.5)	117.4
12	-	144.27	-	144.4
13	-	128.33	-	135.3
14α[b]	1.68, m	40.54	1.63, d (12.0)	41.0
14β[b]	2.12, s		2.07 dd (12.0, 6.5)	
15	3.10, m	30.76	3.13, m	39.4
16	1.46, m	135.70	1.14, overlap	138.3
17	*	123.28	4.26, m	123.4
18	3.73, s	67.70	4.24, d (6.0)	57.8
19	6.67, d (9)	130.28	5.61, t (6.0)	130.10
20	-	142.61	-	137.2
21α	*	62.38	3.31, m	64.5
21β	*	-	3.35, m	
23	-	163.66	-	168.7
24α	3.43, d (7)	34.59	3.25, dd, (10.5, 8.0)	35.3
24β	*		2.85, overlap	
N-CH_3	1.91, s	38.82	2.06, s	38.6
12-OH	4.75, s		12.60, s	

[a] Data was recorded at 400 MHz for proton and at 100 MHz for carbon in CD_3OD for isovomicine derivative (**4.3**)
[b] Data was recorded at 300 MHz for proton and at 75 MHz for carbon in $CDCl_3$ for the published structure.

- There are no protons at the carbon positions
* Missing signals could not be allocated due to high signal-to-noise, overlapping signals and unfavorable relaxation times

The most well-known *Strychnaea* compound, strychnine, was not isolated from this study. It was suggested that this due to various factors like geographical environments, sampling time, and detection of similar strychnine-type alkaloids. As stated in section 1.3, geographical influences play a significant role in the production of secondary metabolites.[45] Non-detection of strychnine does not necessarily mean the absence of the compound in the plant, other factors such as solvent affinity and procedure used directly affects compounds detection. In literature there are reports where aqueous or ethanolic extraction has been performed; the use of ethyl acetate and methanol in this study allowed for the isolation of other strychnine-like compounds as reported in other strychnos species.[37-46]

4.3.1.3 3-(4-methylphenyl)acrylic acid

X-ray diffraction (XRD) is a fast analytical technique, which is primarily used for identification of crystalline materials. It can provide in-depth information of the crystalline material such as the unit cell dimensions, crystal lattice, orientation, and so on. XRD is based on the constructive interference of monochromatic X-rays, which are generated by a cathode ray tube. These X-rays are then filtered to produce monochromatic radiation, collimated to concentrate and directed at the sample. The incident rays are diffracted off the sample to produces a diffraction pattern, which is then processed. Collection of data at low temperatures help maintain crystallinity and reduce thermal vibrations in the atoms. The conversion of the diffraction peaks to d-spacings allow identification of the crystalline material because each crystalline substance has its own set of d-spacings. As with many other spectroscopic instruments, this is achieved by comparison with standards.[47,48]

Factors that, collectively, give reliability of the structures generated after the processing of XRD data are called Rietveld indices and employ the least squares mean approach. The least squares method is usually used a statistical tool for comparison of a specific value with certainty that a solution or model is agreeable with the experimental data obtained. In short, it is a method of statistical analysis of how well the structural model fits the experimental data. The refinement process continually configures the orientation of the atoms to relative atomic displacement parameters of the calculated model, to provide a better ‘fit’ with the experimental data. The process, ultimately, tries

to minimize the difference between the observed structure factors and the calculated factors. This is represented by the goodness of fit (GooF) and the residual index (R). The closer the GooF is to 1, and the lower the R, the better.[47]

The column chromatography of fraction 5 afforded needle-like crystals after evaporation of the solvent in sample vials. Recrystallization was attempted using a 1:1 ($CHCl_3$:MeOH) solvent systems under reduced pressure. The crystal was identified as 3-(4-methylphenyl)acrylic acid (Figure 30). Their 3-dimensional structures are displayed in the following figures.

3-(4-methylphenyl)acrylic acid (Figure 29) crystal closely resembles a crystal published by Lasri *et. al*., except for minor differences in vector values.[51] It is thought to be one of the active constituents in the anti-rheumatic properties of *Terminalia chebula* Retz.[52] It may be one of the compounds responsible for the reported biological activity of *Strychnos henningsii*.

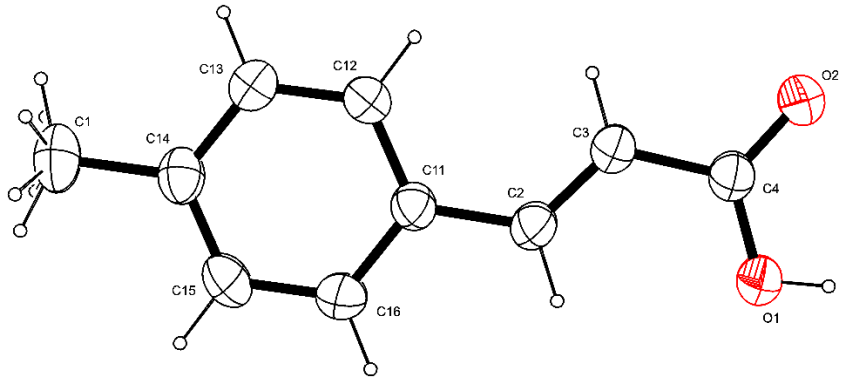

Figure 29: The molecular geometry/structure of 3-(4-methylphenyl)acrylic acid, with displacement ellipsoids and atom labels are drawn at 50% probability level.

The compound, 3-(4-methylphenyl)acrylic acid, has a molecular formula of $C_{10}H_{10}O_2$ that crystallizes in a triclinic type of crystal system with a space group of P1 along the cell dimensions of *a* = 7.1244(6) Å, *b* = 7.7449(6) Å, *c* = 9.1359(7) Å and a volume of 63.899(2) $Å^3$. Data collection was at 200 K, with the Residual factor/index (R) = 0.0170, as well as wR2 = 0.01495. The experimental values obtained for 3-(4-methylphenyl)acrylic acid are summarized in Table 4.5.

Table 4.5: Crystal data and parameters for structure refinement of 3-(4-methylphenyl)acrylic acid.

Features	**Data**
	3-(4-methylphenyl)acrylic acid
Molecular formula	$C_{10}H_{10}O_2$
Molecular weight	162.18
Crystal system	Triclinic
Space group	P1
***a* (Å)**	7.1244(6)
***b* (Å)**	7.7449(6)
***c* (Å)**	9.1359(7)
α (º)	73.767(3)
β (º)	74.201(3)
γ (º)	63.899(2)
V	428.06(6)
Z	2
D_{cal} (g/cm^3)	1.258
Crystal dimension (mm)	0.12 x 0.37 x 0.61
μ (/mm)	0.087
Radiation (Å)	0.7173
D min (Mo)	0.75
I/σ	133.3
R_{int}	1.70%
Shift	0.000
Max Peak	0.32
Min Peak	-0.21
GooF	1.04
R1	4.90%

The experimental data of 3-(4-methylphenyl)acrylic acid] was subjected to optimization calculation via the Centre for High-Performance Computing, to show the structural model at the best fit at to experimental data at the overall energy minimum of the crystal. This is known as convergence. Structural optimization calculations were performed using the B3LYP DFT method, at the Hartree-Fock level, using the basis set 6-31G(d). The specific basis command used was 6-311++G(2d,2p), of which '++' calculated for diffuse functions and '(2d,2p)' improves the polarization of the basis set. For 3-(4-methylphenyl)acrylic acid, convergence was achieved after 59 steps (Figure 31). The experimental and optimized structures are given in Figure 36A and B, respectively.

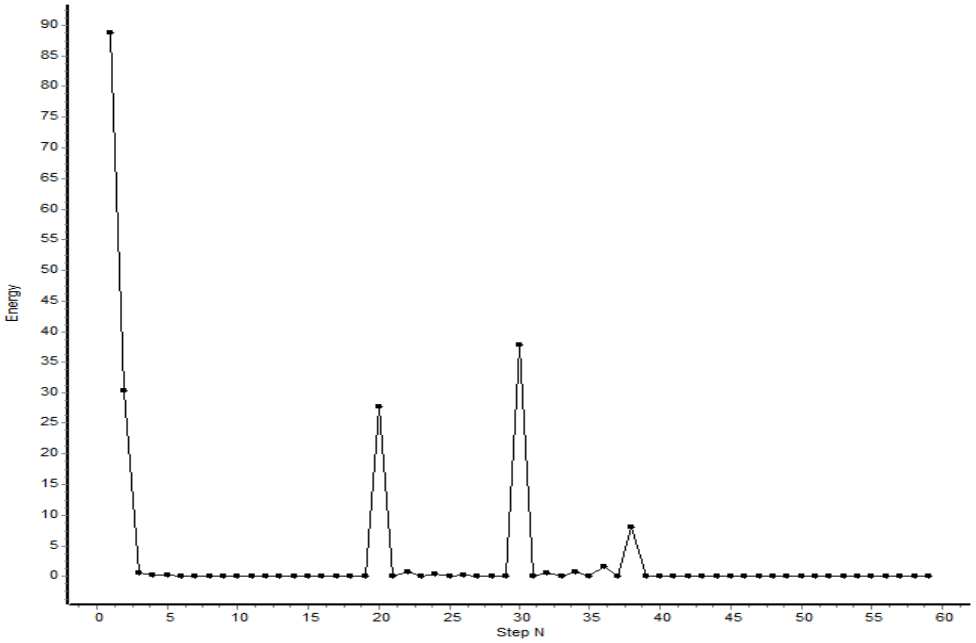

Figure 30: Energy vs number of steps graphical representation of optimization process of 3-(4-methylphenyl)acrylic acid.

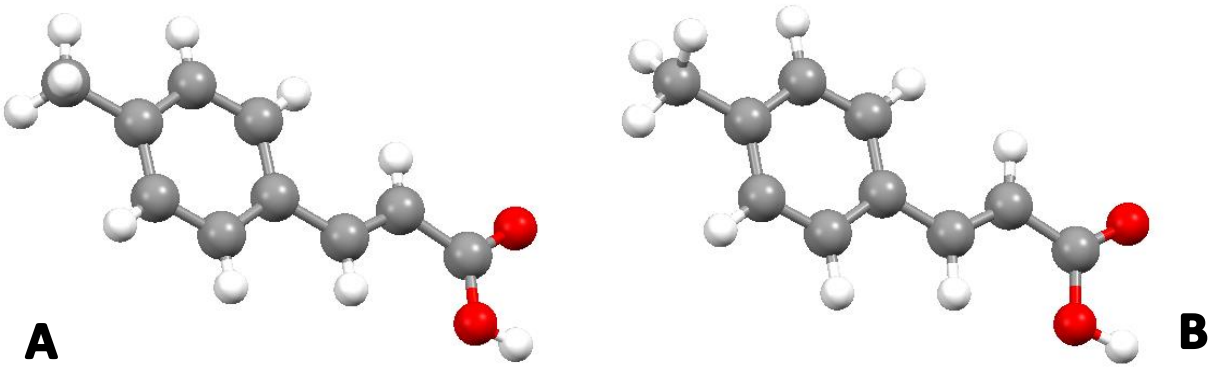

Figure 31: Ball-and-stick models of the chemical structure of 3-(4-methylphenyl)acrylic acid. Ground state (**A**) and the optimized structure *(***B**). Both structures are viewed according to the *c*-vector (x-axis) in Mercury software.

The orientation both structures are displayed in is the c-vector, otherwise known as the z-axis. The arrangement of the methyl protons of structure **B** is more rotated as compared to those of structure **A**, which may be due to their interaction with the aromatic ring. Structure **B** (optimized) is more planar, with the exception of the methyl protons. The structure orientation given, theoretically, allows for more ordered crystal packing, thus a much more stable compound.

Due to the amounts used in the structural determination of 3-(4-methylphenyl)acrylic acid, Infrared spectroscopy couldn't be made use of with the remaining crystals.

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Chapter Five: Conclusion

The preliminary biological activity profiling of the ethyl acetate and methanol extracts of *Strychnos henningsii* was achieved by *in vitro* bioassay analysis of their cytotoxicity, anti- inflammatory and antioxidant potentials. Separation of the compounds from both extracts was achieved by use of various chromatographic techniques. Chemical structures were deduced by means of spectroscopic techniques.

Phytochemical analyses revealed the presence of a variety of chemotypes such as alkaloids, phenols, carotenoids, coumarins and tannins (Table 4.1). Contrary to the large presence of indole alkaloids initially thought to make the extract more toxic, cytotoxicity assay evaluation showed cell-boosting properties at 10 µg/mL. At varying concentrations of both extracts (Figures 11 and 12), they were shown to possess insignificant cytotoxicity levels, but actually possess cell growth properties at concentrations of 10 µg/mL in both extracts. The significant difference between the highest and lowest concentrations, according to the error bars as they do not overlap, and that the activity is concentration dependent to some degree.

The antioxidant potentials of the extracts evaluated using DPPH, FRAP and ORAC assays showed that the methanol extract exhibited better potential than the ethyl acetate extract. More polar extracts are then thought to possess more biologically active constituents. However, from the anti-inflammatory assay, the ethyl acetate extract exhibited better activity. Diverse chemotype profiles within a plant material enable better healing ability.

The chemical structures of the isolated compounds were evaluated against those from literature, of which one lead the conclusion of it being stryvomicine and another being an isovomicine derivative. Unfortunately, other isolated compounds' structures were not concluded (Coumpounds **X** and **Y**), due to complexity of the NMR spectra. Concentration issues hindered investigation of other compounds. In addition to the complexity in identifying *Strychneae* alkaloids, there should be updated structural names as some compounds were isolated when NMR spectroscopy was in the development stage.

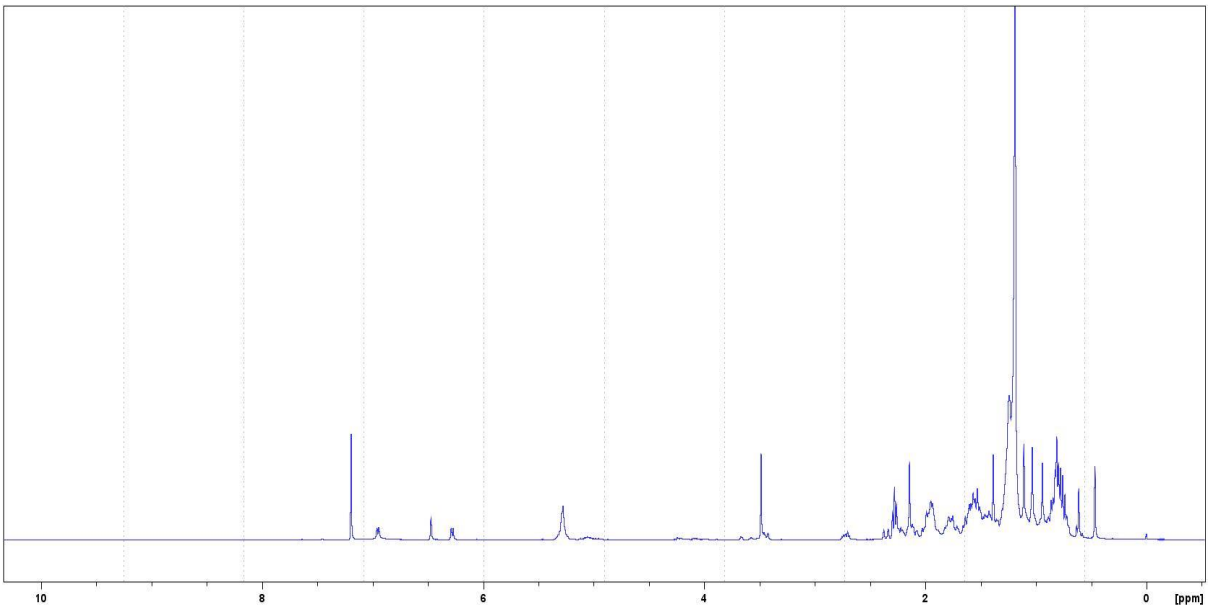
In general, we can conclude that the Eastern Cape *S. henningsii* show similarities with the extracts reported from other different geographical areas. The polymeric

compound, which crystallized by surprise during the run of column chromatography is reported for the first time here. Therefore, we strongly believe that the data presented and discussed here will add to scientific knowledge.

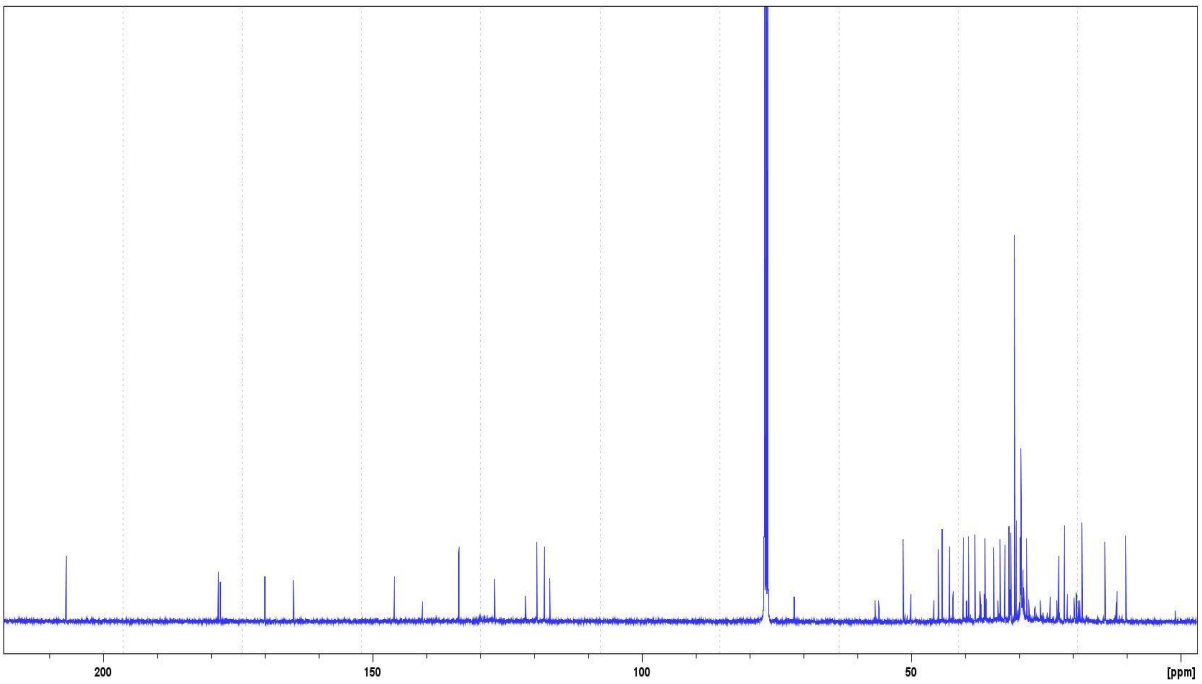
Future work on this study include isolation of compounds of diverse nature and subjecting them to the biological screening. Thus, to investigate compound(s) largely responsible for the plant's bioactivity. In addition, biological activity of the plant should be evaluated via investigation of concoctions prepared with it, although a much more complicated work and the mechanism of action is always a mystery.

APPENDICES

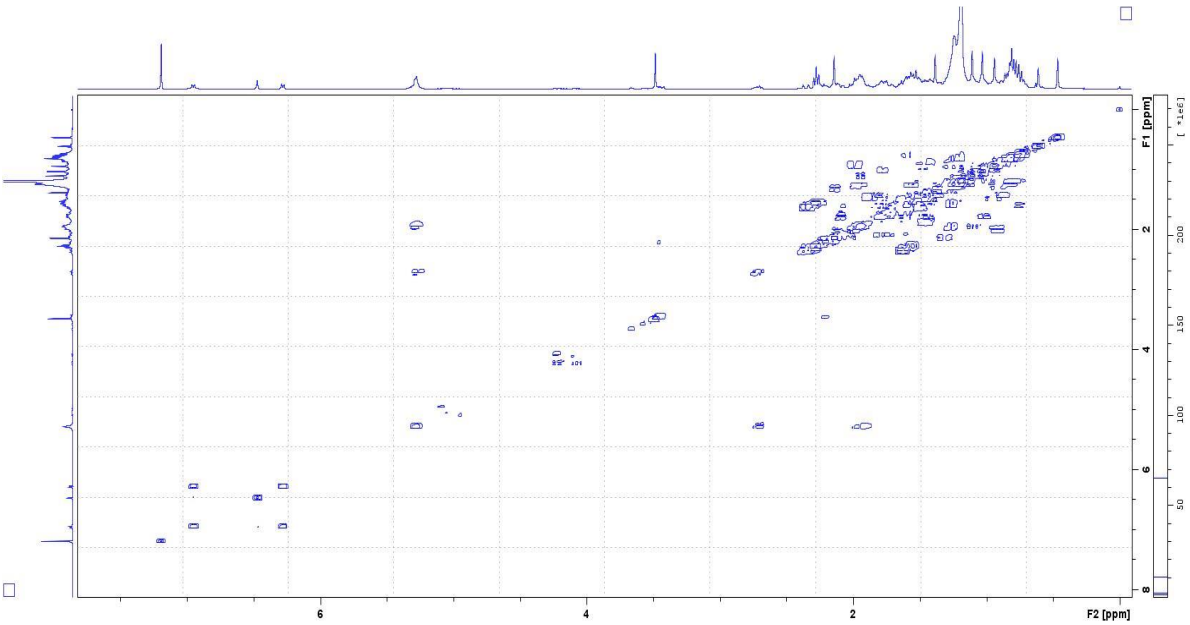
Appendix A1: ^{1}H NMR spectrum of SHB.E-Y (4.1) in $CDCl_3$.



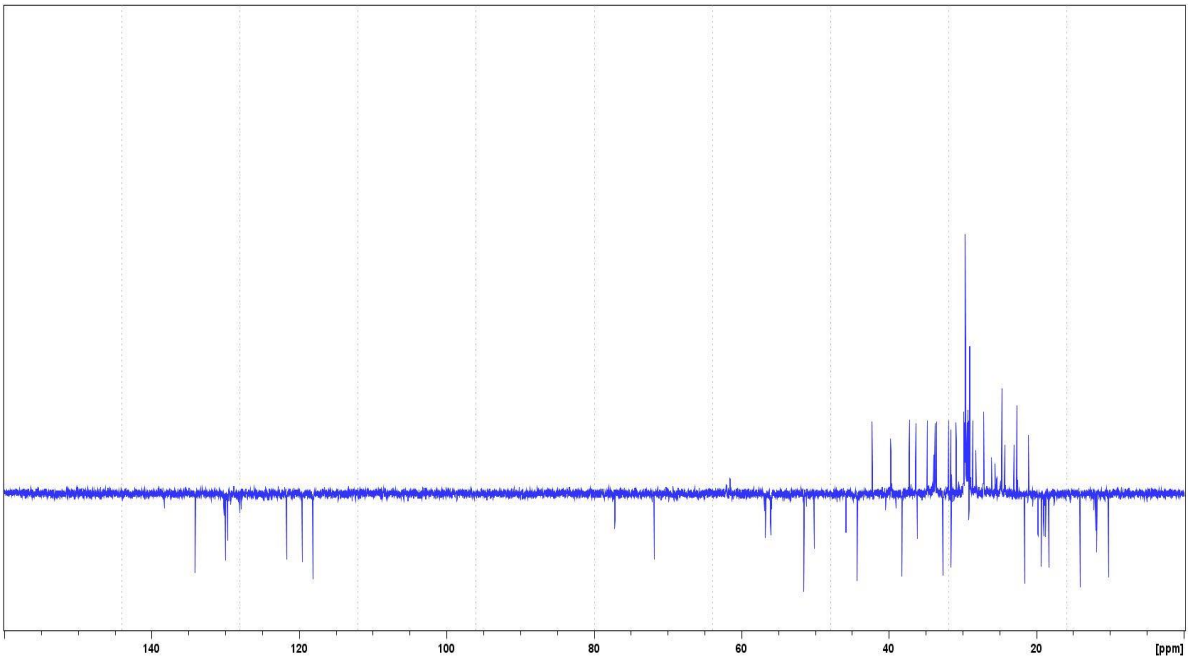
Appendix A2: ^{13}C NMR spectrum of SHB.E-Y (4.1) in $CDCl_3$.



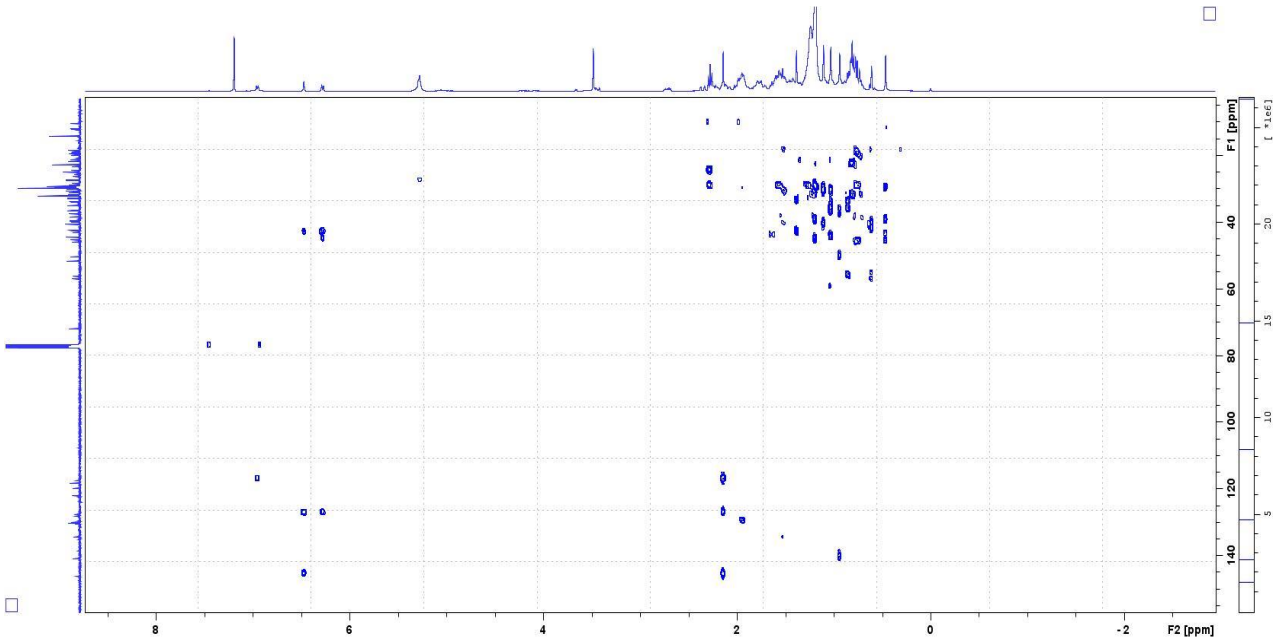
Appendix A3: Cosy NMR spectrum of SHB.E-Y (4.1) in $CDCl_3$.



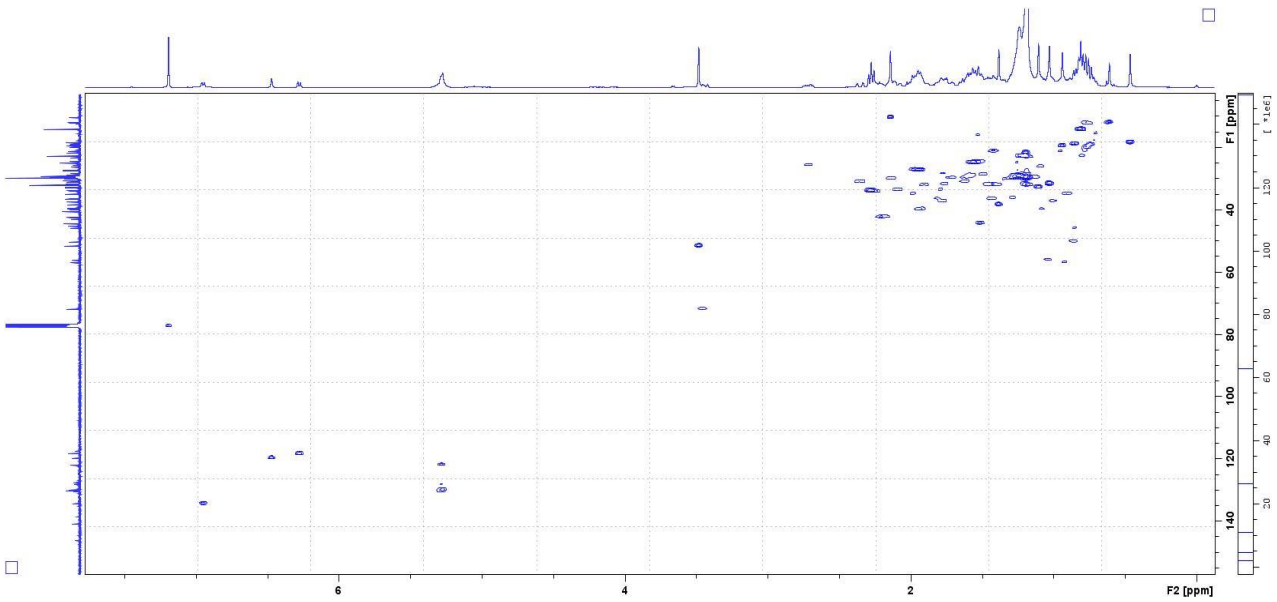
Appendix A4: Dept-135 NMR spectrum of SHB.E-Y (4.1) in $CDCl_3$.



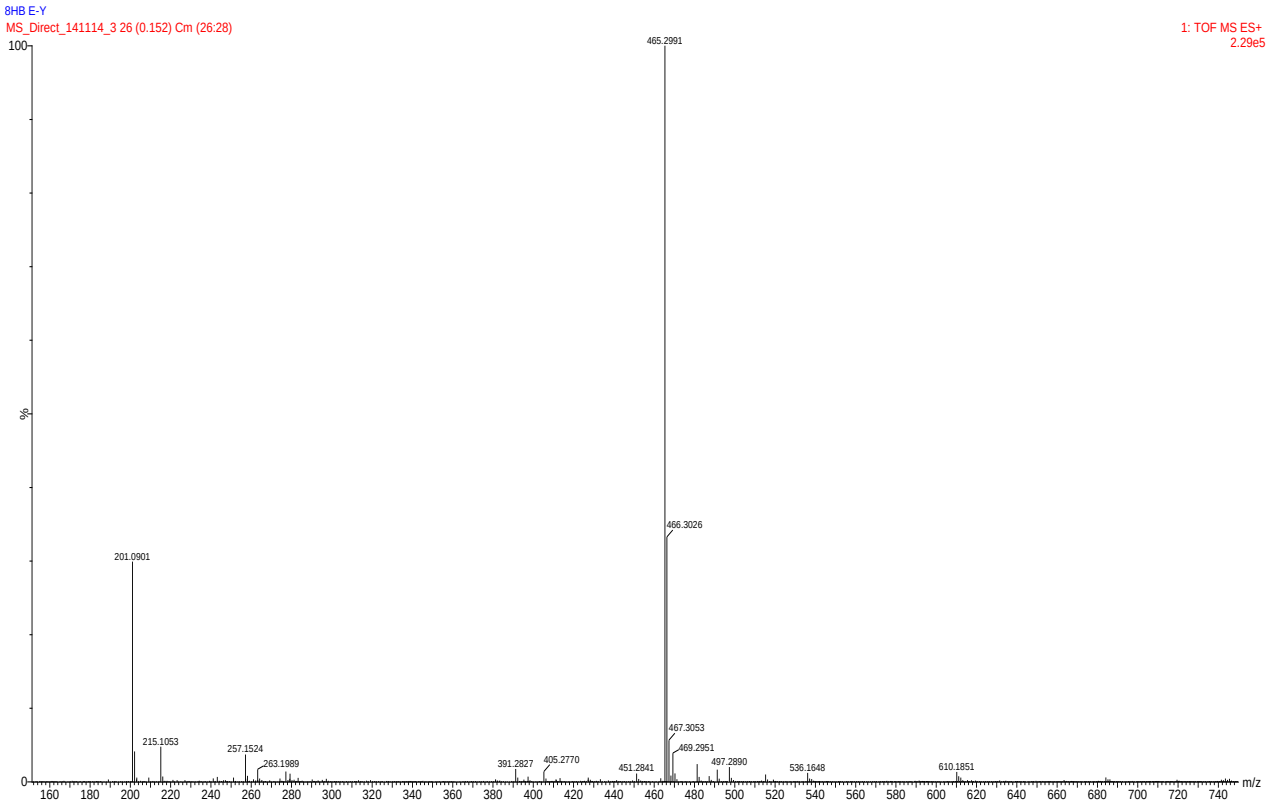
Appendix A5: HSQC NMR spectrum of SHB.E-Y (4.1) in $CDCl_3$.



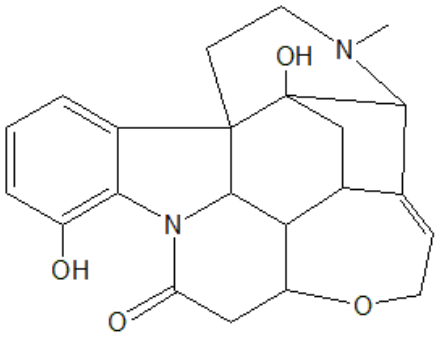
Appendix A6: HMBC NMR spectrum of SHB.E-Y (4.1) in $CDCl_3$.



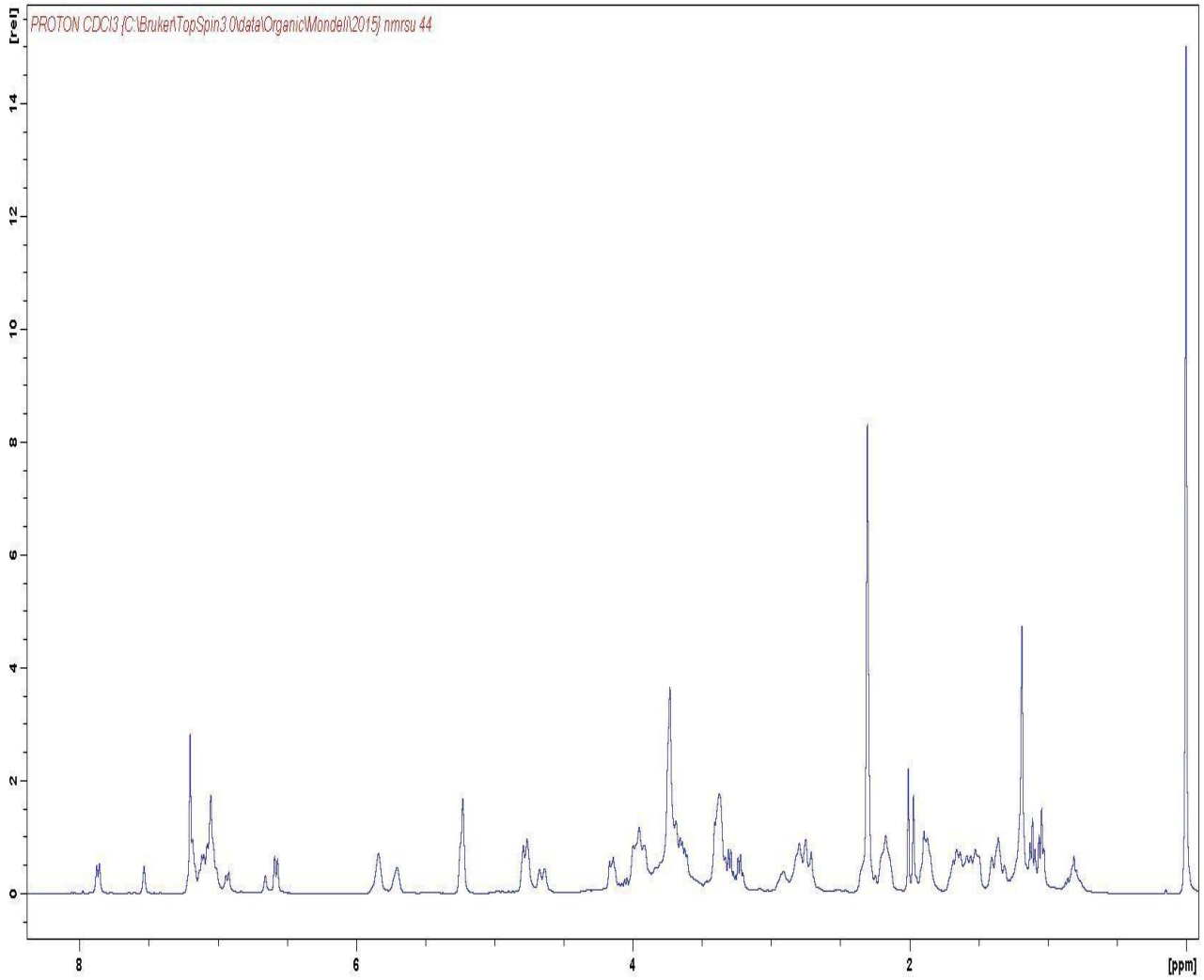
Appendix A7: High resolution mass spectrum SHB.E-Y (4.1) via ES+ mode.



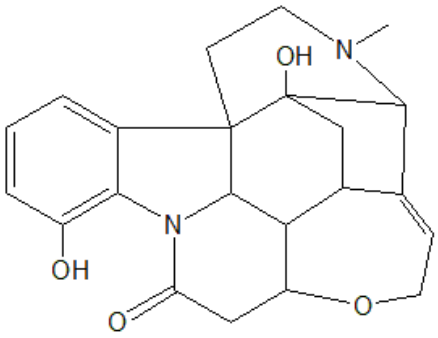
Appendix B1: ^{1}H NMR spectrum of stryvomicine (4.2) in $CDCl_3$.



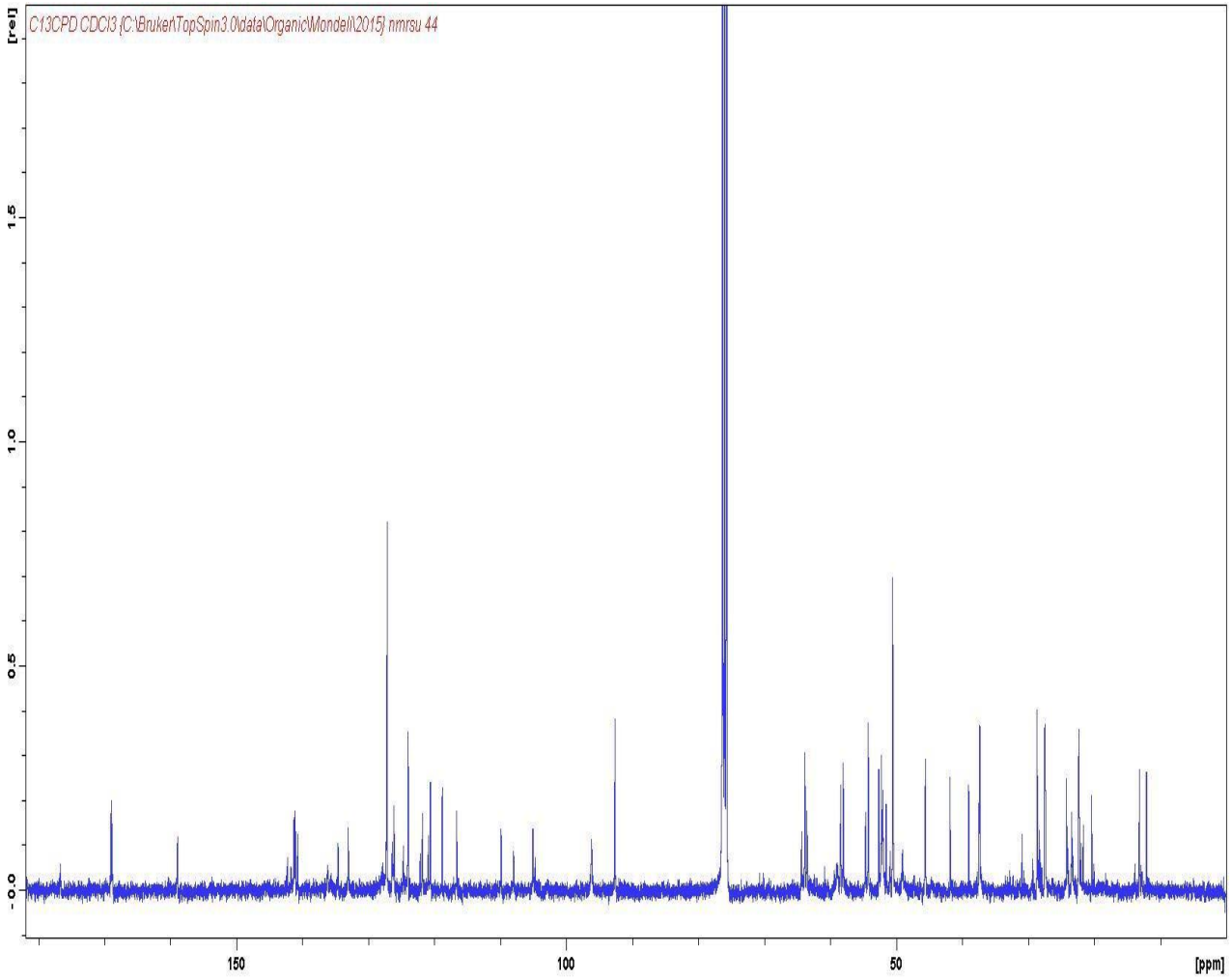
4.2



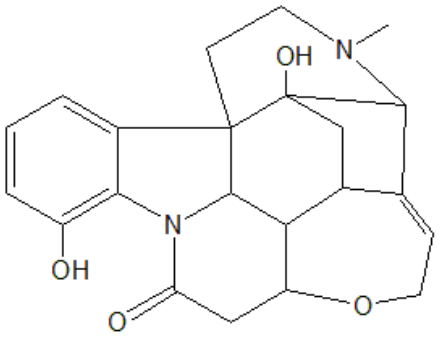
Appendix B2: ^{13}C NMR spectrum of stryvomicine (4.2) in $CDCl_3$.



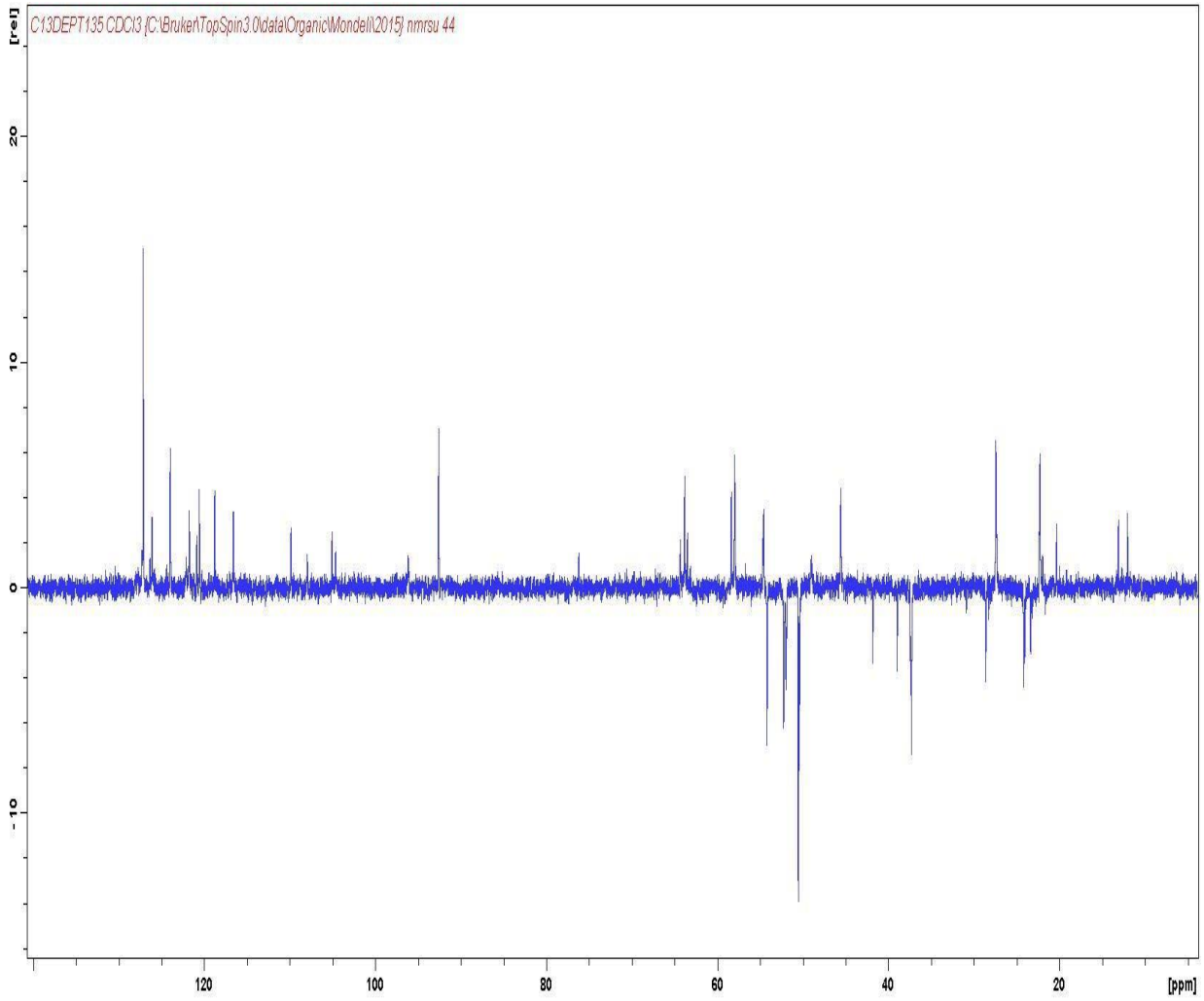
4.2



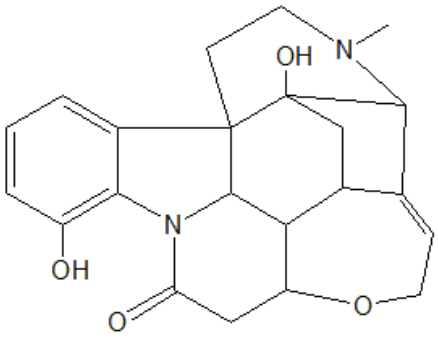
Appendix B3: Dept-135 NMR spectrum of stryvomicine (4.2) in $CDCl_3$.



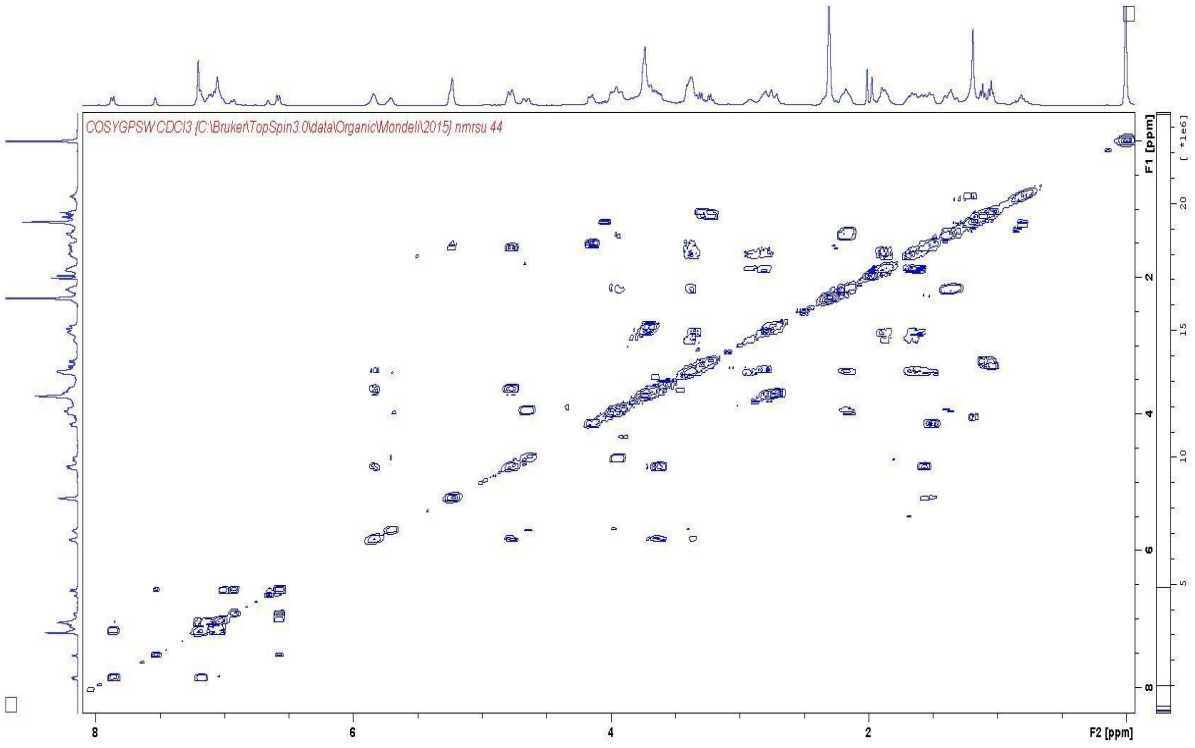
4.2



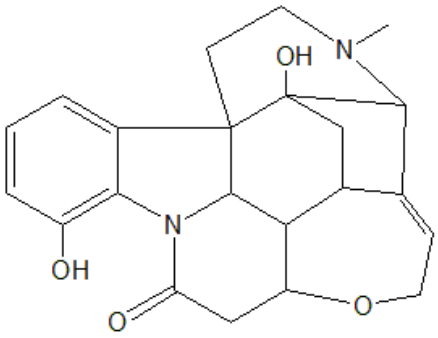
Appendix B4: Cosy NMR spectrum of stryvomicine (4.2) in $CDCl_3$.



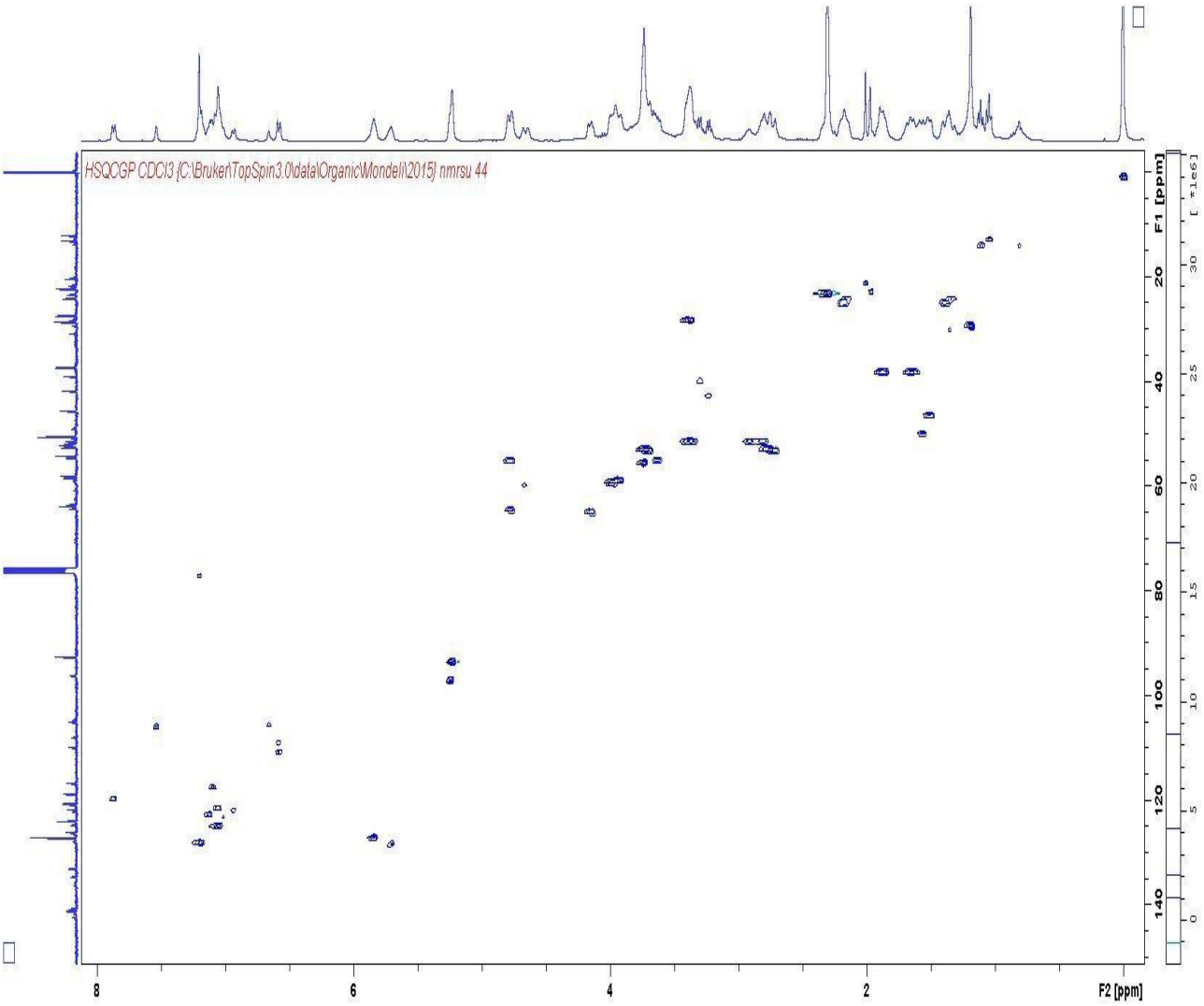
4.2



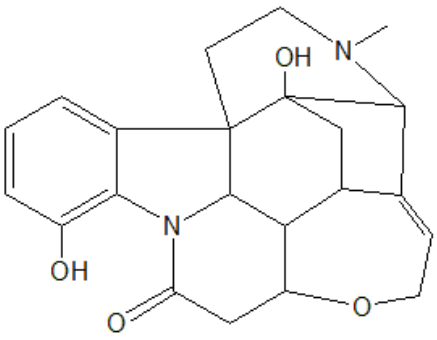
Appendix B5: HSQC NMR spectrum of stryvomicine (4.2) in $CDCl_3$.



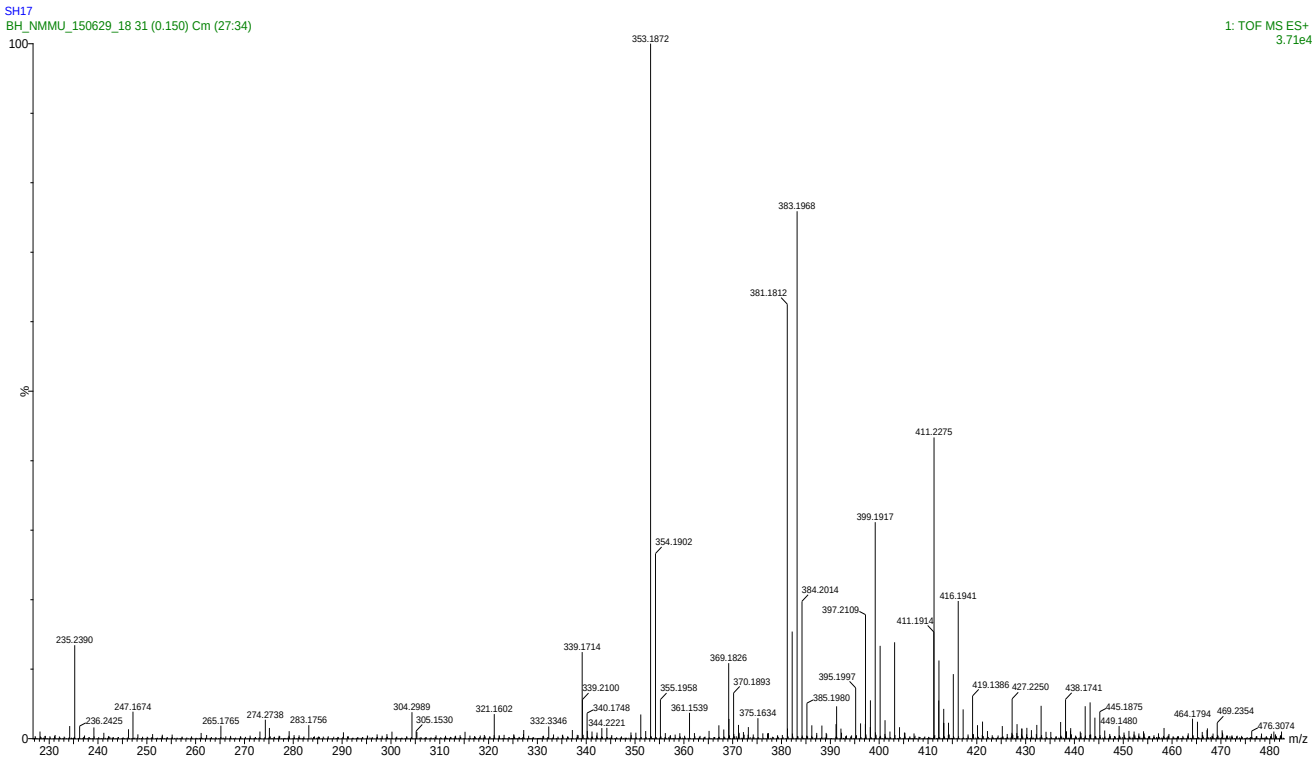
4.2



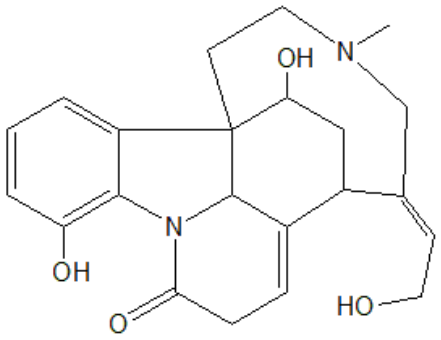
Appendix B7: High resolution mass spectrum of stryvomicine (4.2) via ES+ mode.



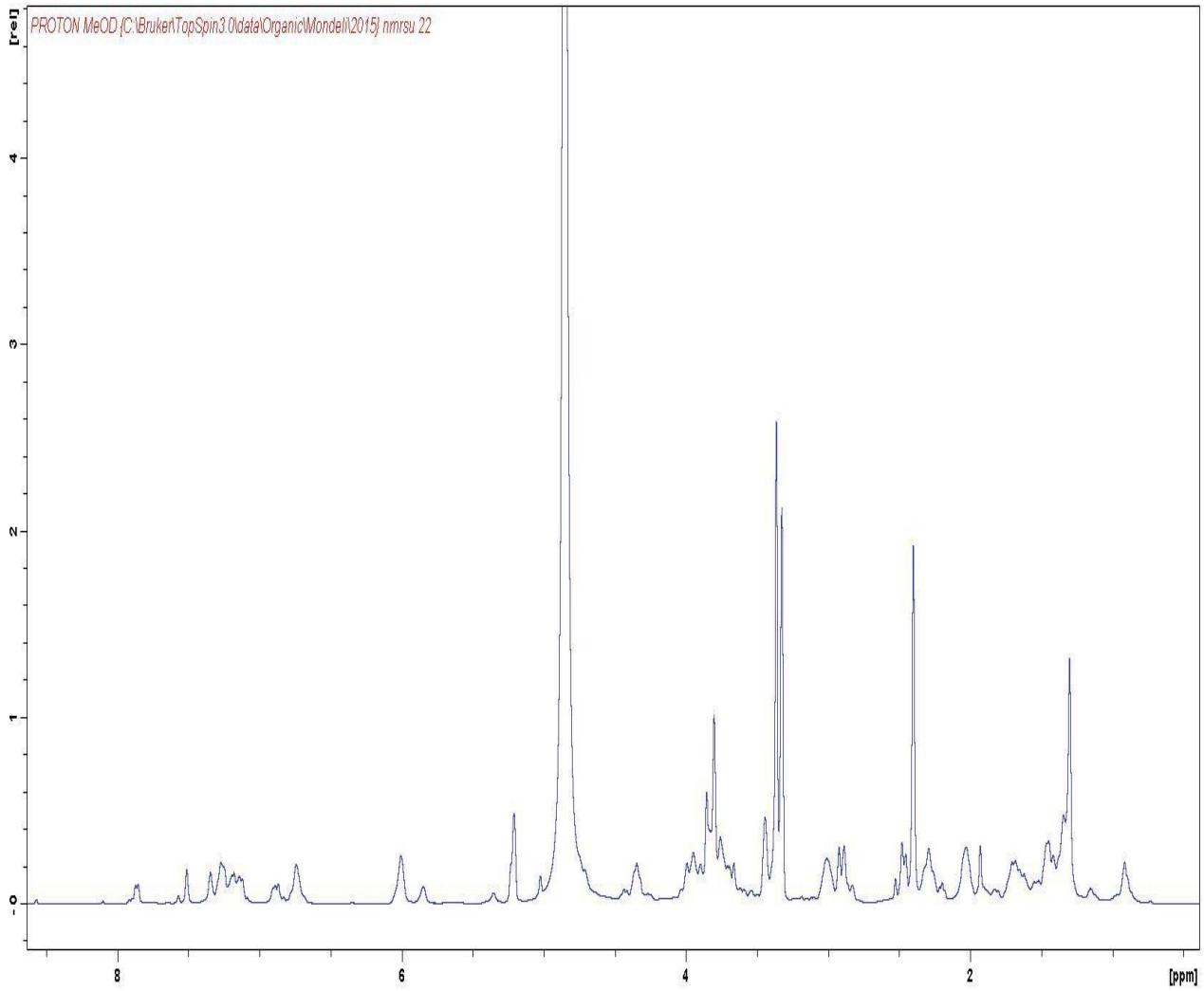
4.2



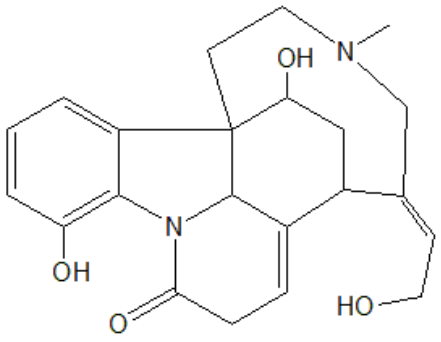
Appendix C1: ^{1}H NMR spectrum of isovomicine derivative (4.3) in MeOD.



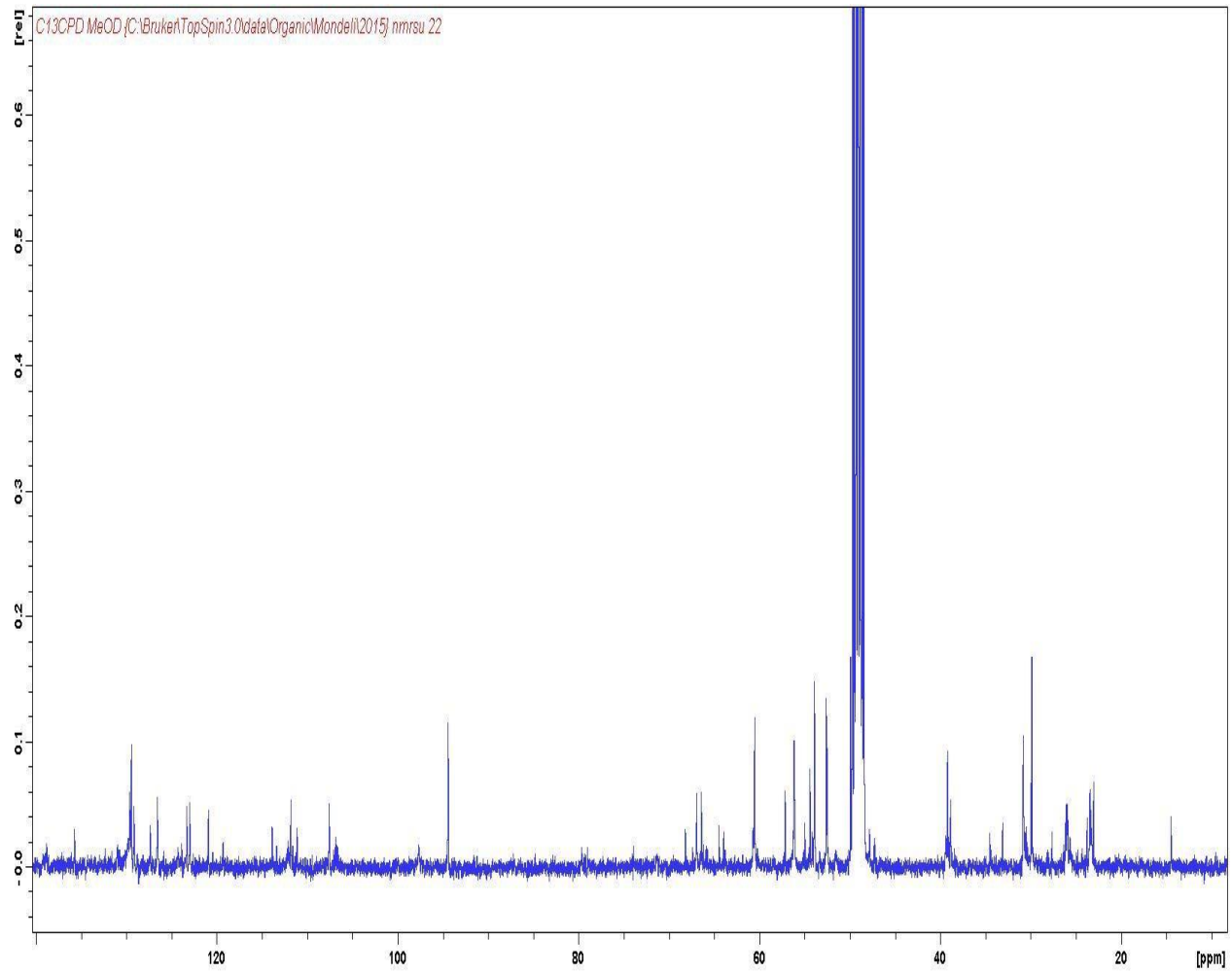
4.3



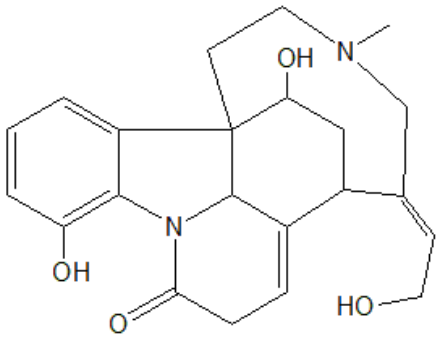
Appendix C2: ^{13}C NMR spectrum of isovomicine derivative (4.3) in MeOD.



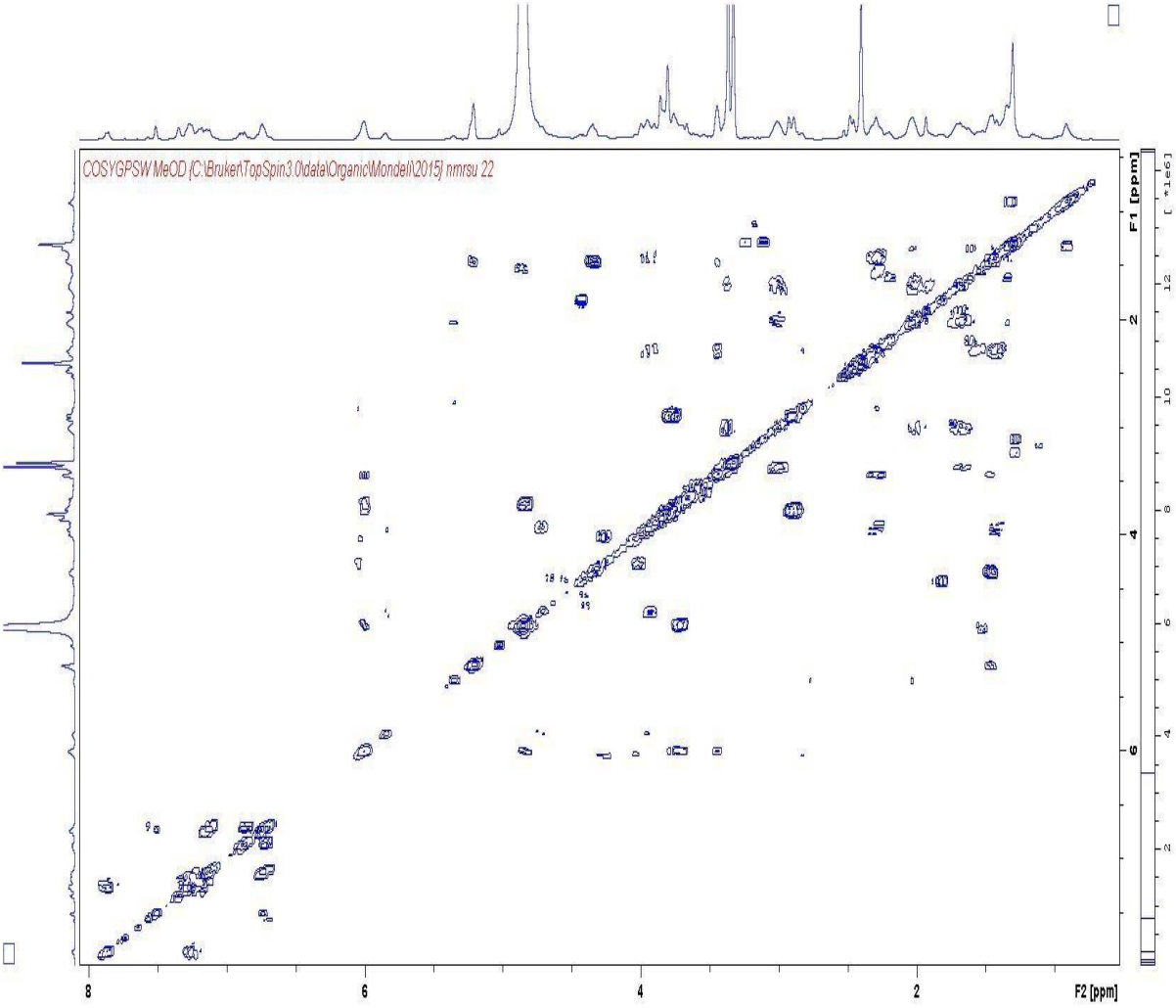
4.3



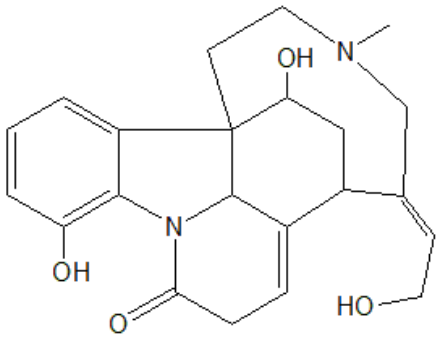
Appendix C3: Cosy NMR spectrum of isovomicine derivative (4.3) in MeOD.



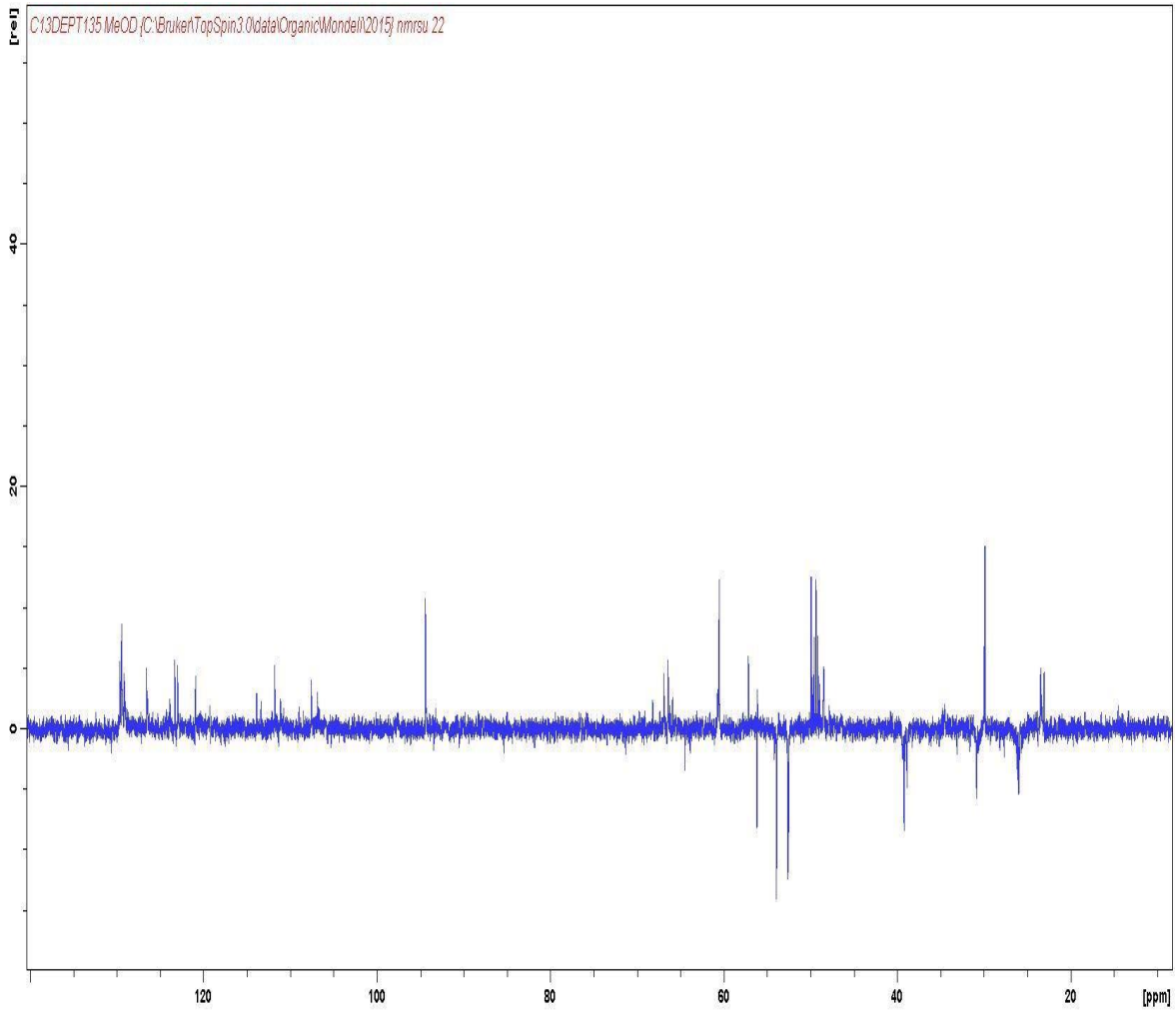
4.3



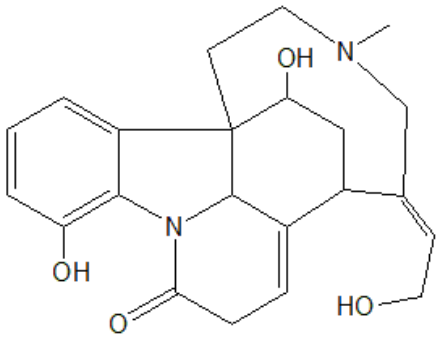
Appendix C4: Dept-135 NMR spectrum of isovomicine derivative (4.3) in MeOD.



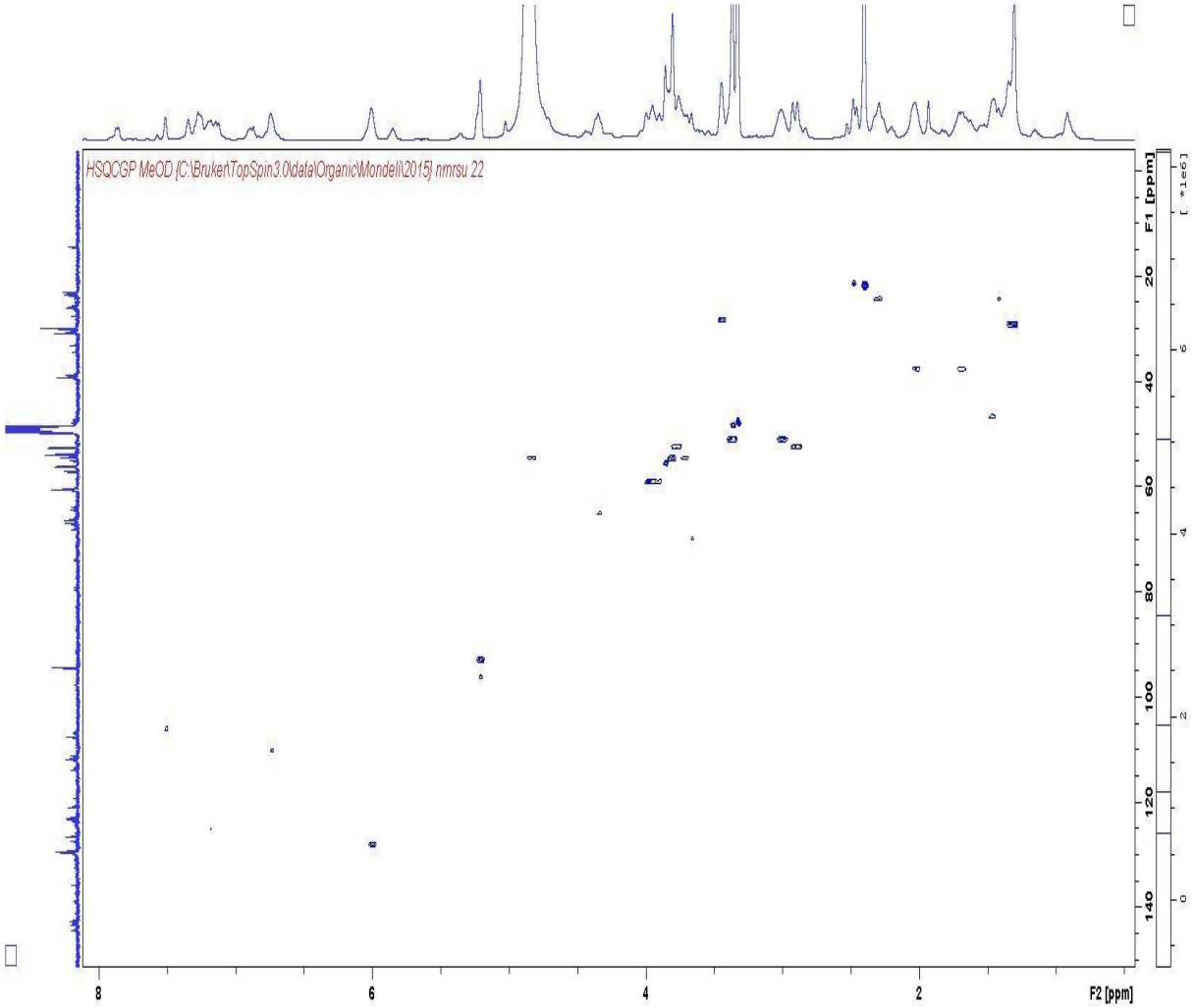
4.3



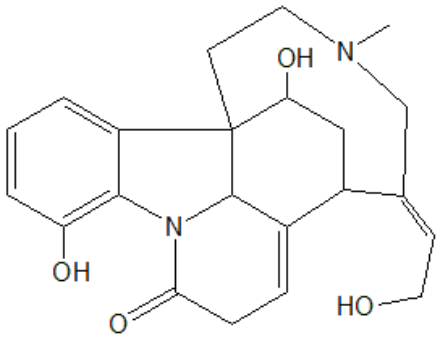
Appendix C5: HSQC NMR spectrum of isovomicine derivative (4.3) in MeOD.



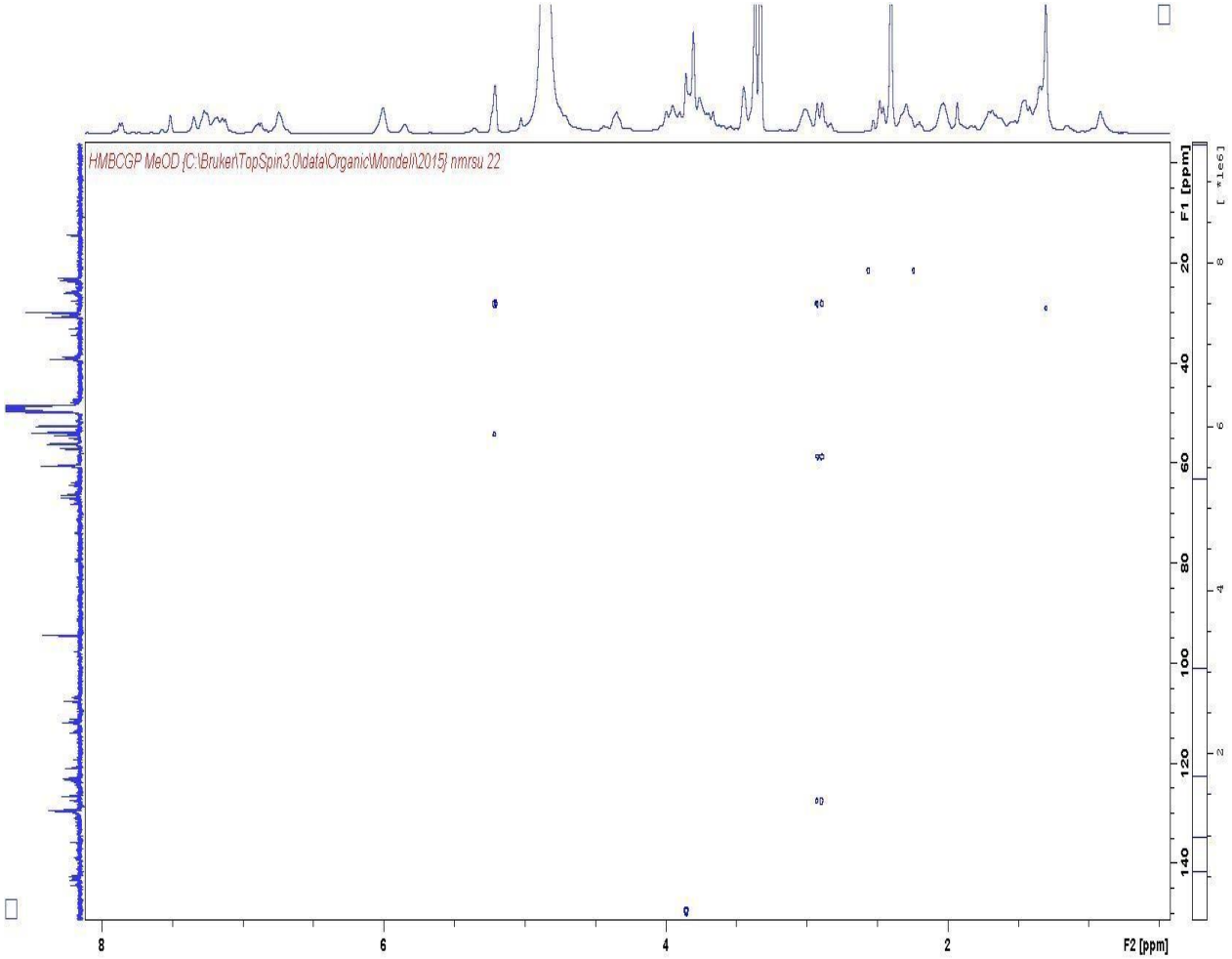
4.3



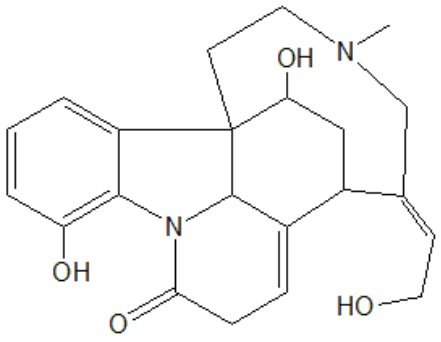
Appendix C6: HMBC NMR spectrum of isovomicine derivative (4.3) in MeOD.



4.3



Appendix C7: High resolution mass spectrum of isovomicine derivative (4.3) via ES+ mode.



4.3