INVESTIGATION OF NEUROTOXIC PRINCIPLES OF THE TUMBLEWEED*, TRACHYANDRA LAXA*, RESPONSIBLE FOR LIVESTOCK LOSSES IN SOUTHERN AFRICA

A THESIS

SUBMITTED IN FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE (CHEMISTRY)

OF

THE UNIVERSITY OF NAMIBIA

BY

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MARCH 2020

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ABSTRACT

Livestock losses due to poisonous plants remain a major concern for livestock agriculture in southern Africa. However, for a number of such toxic plants, including *Trachyandra laxa* (family Xanthorrhoeaceae, sub-family Asphodeloideae), the toxic components are still unknown. *Trachyandra laxa* primarily affects the central nervous system when ingested by livestock, causing paralysis. Traditional bioactivity-guided fractionation approaches used for the isolation and identification of toxins are time consuming and laborious. Therefore, in this study, a chemical screening approach involving gas chromatography - mass spectrometry (GCMS) was used. The chemical constituents were extracted from the leaves, inflorescence, flowers, roots and rhizomes of *T. laxa*, using a sequential extraction approach with solvents of different polarity, starting with hexane and ending with methanol. Extracts were screened for cytotoxicity using the MTT-assay, for neurotoxicity using acetylcholinesterase (AChE) inhibition. Results showed no cytotoxicity and the acetylcholinesterase inhibition assay displayed positive results for the dichloromethane (DCM) extract, indicating neurotoxic activity. The chemical screening approach was subsequently pursued on the DCM extracts only. In this approach, the DCM extracts were analysed by GC - MS and their major volatile constituents were tentatively identified from their mass spectra and retention indices. A literature search was conducted to determine which of the tentatively identified compounds are toxic. This led to the discovery that two of the constituents, the α,β-unsaturated lactones, dihydroactinidiolide (DHA) and its hydroxylated analogue, are known to have potent AChE inhibition

properties. It is envisaged that this study will pave the way to further research on specific treatment of animals affected by *T. laxa* poisoning or the development of a preventative agent that can be used to avoid *T. laxa* poisoning.

Keywords: Cytotoxicity, neurotoxicity, Acetylcholinesterase inhibition, Phytochemical Screening, GC-MS, α,β-unsaturated lactone.

CONFERENCE PRESENTATIONS

H M van Wyk, R H Hans, Ed J Pool, E G Kwembeya and S Louw. Identification of the major volatile constituents of the Tumbleweed, *Trachyandra laxa*. University of Namibia 3rd Annual Science Research Conference. 18–19 November 2015, Windhoek, Namibia.

TABLE OF CONTENTS

ABBREVIATIONS

ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
AD		Alzheimer's disease
AE	-	Aqueous extract(s)
Al	-	Alkaloids
An	-	Anthraquinones
AR	-	Analytical Reagent
ATCI	-	Acetylthiocholine iodide
C	-	Coumarins
CHO	-	Chinese Hamster Ovarian
CNS	-	Central nervous system
CoA	-	Coenzyme A
DA		Dihydroactinidiolide
DCM	-	Dichloromethane
DDT	-	Dichlorodiphenyltrichloroethane
DMSO	-	Dimethyl sulphoxide
DTNB	-	5, 5-Dithiobis (2-nitrobenzoic acid)
EI	-	Electron ionization
EI	-	Electron impact (MS)
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol

F	-	Flavonoids
F_{254}	-	Flouorescence at 254 nm
G	-	Glycosides
GABA	-	Gamma aminobutyric acid
GPS	-	Global positioning system
IC_{50}	-	Inhibitory concentration of ligand to equal 50% of the effect produced by a standard
LC	-	Liquid chromatography
MAO		Monoamine oxidase
MeCN	-	Acetonitrile
MeOH	-	Methanol
MET	-	Ministry of Environment and Tourism
m/z	-	Mass per electronic charge
NBRI	-	National Botanical Research Institute
NIST	-	National Institute of Standards and Technology
NT's	-	Neurotransmitters
OD	-	Optical density
OD_{560}	-	Optical density at 560 nm
OE	-	Organic extract
TLC	-	Thin layer chromatography
PREP TLC	-	Preparative thin layer chromatography
RI	-	Retention index

t_R	-	Retention time
SEPASAL	-	Survey of economic plants for arid and semi-arid lands
STLs		Sesquiterpene lactones
SMs	-	Secondary Metabolites
SS	-	Solvent system
T	-	Terpenoids
TIC	-	Total ion chromatogram
TK	-	Traditional Knowledge
TLC	-	Thin Layer Chromatography
UV	-	Ultraviolet
VOCs	-	Volatile Organic Compounds

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

My sincerest gratitude goes to my supervisor Dr Stefan Louw, for motivating and inspiring me throughout the course of my studies. Thank you for your enormous patience and invaluable guidance. Your technical assistance in the laboratory and expert advice into the right direction is much appreciated. You encouraged me in every possible way. I owe you a great debt, thank you!

I would also like to acknowledge my co-supervisor Dr Renate Hans for believing in me. Regardless of her busy schedule, she always lent an ear, rendered advice and extended a helping hand. Thank you for your motivation throughout, I have learned a lot from you. I would also like to extend my gratitude to my colleagues and friends in the Faculty of Science who unselfishly rendered support, motivation and assistance when needed. Prof Edmund Pool needs to be mentioned for allowing me to conduct the neurotoxicity assay in his laboratory at the University of the Western Cape and his guidance during this time.

I would like to thank my family for supporting me during my studies; for their prayers, understanding when I was away from home, putting up with my mood swings many a time and for believing in me.

The Research Foundation of the University of Namibia for their financial support.

DECLARATION

I, Hedwig Maureen van Wyk, declare hereby that this study is a true reflection of my own research, and that this work or part thereof has not been submitted for a degree in any other institution of higher education.

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CHAPTER 1: INTRODUCTION

1.1 Background of the study

The loss of livestock due to poisonous plants is a major concern for livestock agriculture in southern Africa.[1,3] Usually plant poisoning in animals occurs accidentally during unfavourable conditions when pastures are poor due to drought, veld fires, overstocking and trampling of grazing. Under such conditions animals are often forced to eat poisonous plants which would normally be avoided.[4] Poisoned animals move with difficulty, knuckling over at the fetlock and over flexing the hock, with muscle twitching and hypersensitivity. Paresis and paralysis follows, and later sternal recumbency results.[2] Poisonous plants can affect the entire spectrum of organ systems, with some plants having several toxic principles that affect different systems.[1] It is stated that the dominant effect may depend on the condition, growth stage, part of the plant consumed, the amount ingested, and the species as well as the susceptibility of the victim. On consumption of *Trachyandra* spp. (*Tracyandra laxa* and *Trachyandra divaricata*) progressive posterior paresis and paralysis was induced in cattle, sheep, horses and pigs. This is characterized by the accumulation of a brownish pigment in neurons in the brain and spinal cord. These plants occur extensively in overgrazed areas during August to December and become palatable during the growing season.[4] Plants produce secondary metabolites as defence against invasion by microorganisms, viruses and herbivores.[6] Plant toxicity may be due to a diversity of chemical toxins that include alkaloids, triterpenes, steroids, saponins, anthraquinones, glycosides, proteins and amino acids.[7]

1.2 Statement of the problem

Although livestock losses due to poisonous plants are a major concern for livestock agriculture in Southern Africa [2], the toxic components of a number of poisonous plants, including *Trachyandra laxa*, are still unknown.[3] It is known that plants often contain a mixture of compounds that have additive or even synergistic effects thus making it difficult to test and verify their toxicological and pharmacological properties.[6] Hence, knowledge of the complete chemical profile of the plant is necessary.

1.3 Objectives of the study

The objectives of the study were to:

(a) determine the toxicity of the extracts of different plant parts of *T. laxa*;

(b) identify which classes of compounds are present in the extracts and

(c) identify the volatile constituents present in the most toxic extracts.

1.4 Significance of the study

In Namibia, a high mortality of livestock is caused annually by the plant, *T. laxa* (Tumbleweed). Thus far no therapeutic measures have been developed for the prevention or treatment of this poisoning. By identifying the main chemical compounds present in this plant, this research may contribute to the understanding of the mechanism of action by which poisoning takes place when the plant is ingested by livestock. A preventative agent for tumbleweed poisoning of e.g. sheep could potentially be found. This will reduce losses of livestock and ultimately contribute to the economy of Namibia and southern

Africa in general. Since there is no knowledge of the chemistry of *T. laxa*, this study will be the first to identify the major chemical compounds present in this toxic plant.

1.5 Limitation of the Study

Trachyandra laxa occurs only in certain areas of Namibia and only become available during the rainy season and during early spring, thus sufficient sampling had to be done. During periods of drought or no rains these plants are not visible as the leaves dry out or disintegrate if not consumed by animals and the inflorescence dry out and is carried off by the wind for seed dispersal.

CHAPTER 2: LITERATURE REVIEW

It has been reported that poisonous plants cause considerable stock losses in Namibia each year. These losses can translate into many thousands of Namibian dollars, thus it is essential to collect and share information on poisonous plants and their distribution in Namibia. The National Botanical Research Institute (NBRI) works in collaboration with the Directorate of Veterinary Services to acquire such information in order to be able to supply detailed distribution maps as well as precautionary and other information required by individual members of the public, farmers, veterinary surgeons and extension workers.[4] Plant poisonings causing major stock losses in southern Africa have been thoroughly reviewed by Kellerman et al.[9]

2.1 Family Xanthorrhoaceae

Xanthorrhoaceae is a family of flowering plants consisting of three subfamilies: Asphodeloideae, Hemerocallidoideae and Xanthorrhoeoideae. They are diverse but have a few characteristics uniting them. For instance, the flowers of plants from this family are borne on a leafless stalk, the scape, which arises from a basal rosette of leaves. The plant under study, *Trachyandra laxa*, belongs to the subfamily Asphodeloideae.[8]

2.1.1 Botanical description of Trachyandra laxa

Trachyandra laxa is commonly known as the tumbleweed, a terrestrial herb with succulent leaves. It is a perennial geophyte, which grows up to sixty centimetres high. The leaves are arranged in a basal rosette and are linear, fleshy and sometimes sticky, and can grow up to 400 millimetres long, 1-3 mm in diameter and are round in cross section

(**Fig. 2.1** and **Fig. 2.2**). The flowers are carried on the inflorescence, a large branched, diffuse spray. Flowers are small, white in colour, and have six petals curved backwards with a pink-brown stripe down the midline and six stamens, the outer three of which have hairy, yellow filaments (**Fig. 2.2-C**).[4]

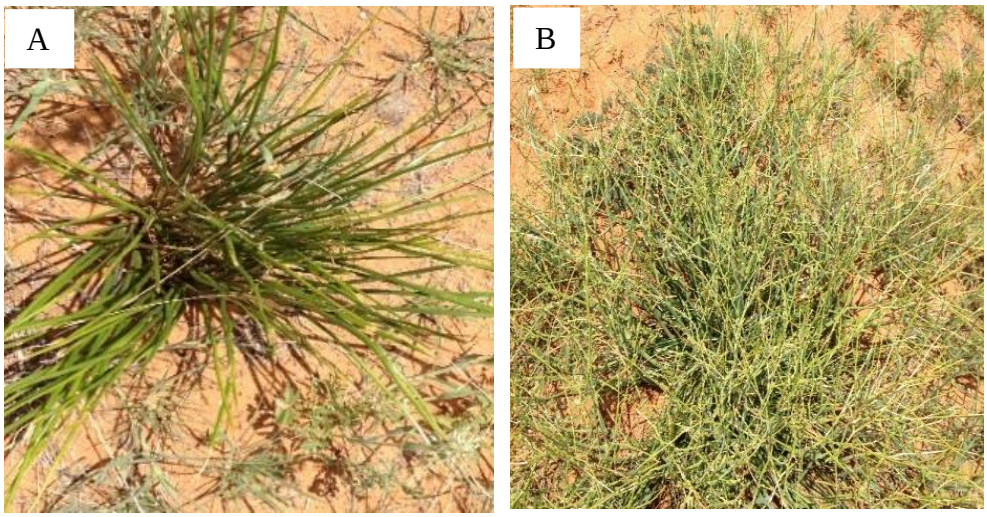


Figure 2. 1: *Trachyandra laxa*, A: young plant, B: inflorescence (Hedwig van Wyk: Photo taken in natural habitat)

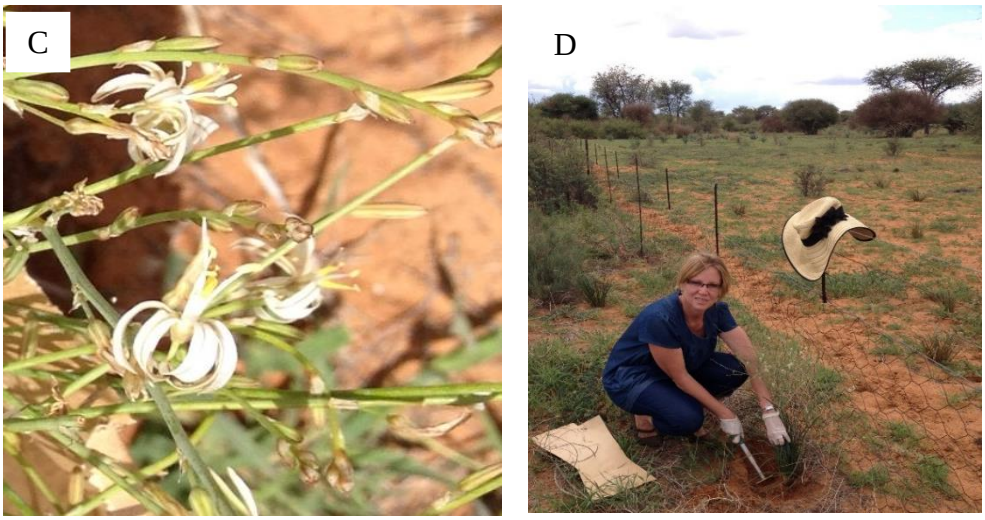


Figure 2.2: *Trachyandra laxa* C: flower, D: (Hedwig van Wyk: Photo taken in natural habitat)

2.1.2 Geographical distribution of Trachyandra laxa

The map in Figure 2.3 indicates the distribution of the *Trachyandra laxa* species in Namibia as retrieved from the specimen database at the NBRI.[4] It shows the distribution of *T. laxa* from the Karas region in the south across the central part in the Khomas, Omaheke, Erongo and up to the Ohangwena regions in the north of the country. *T. laxa* prefer deep sandy soils and is often found in trampled and disturbed places and sometimes in extensive stands.[2]

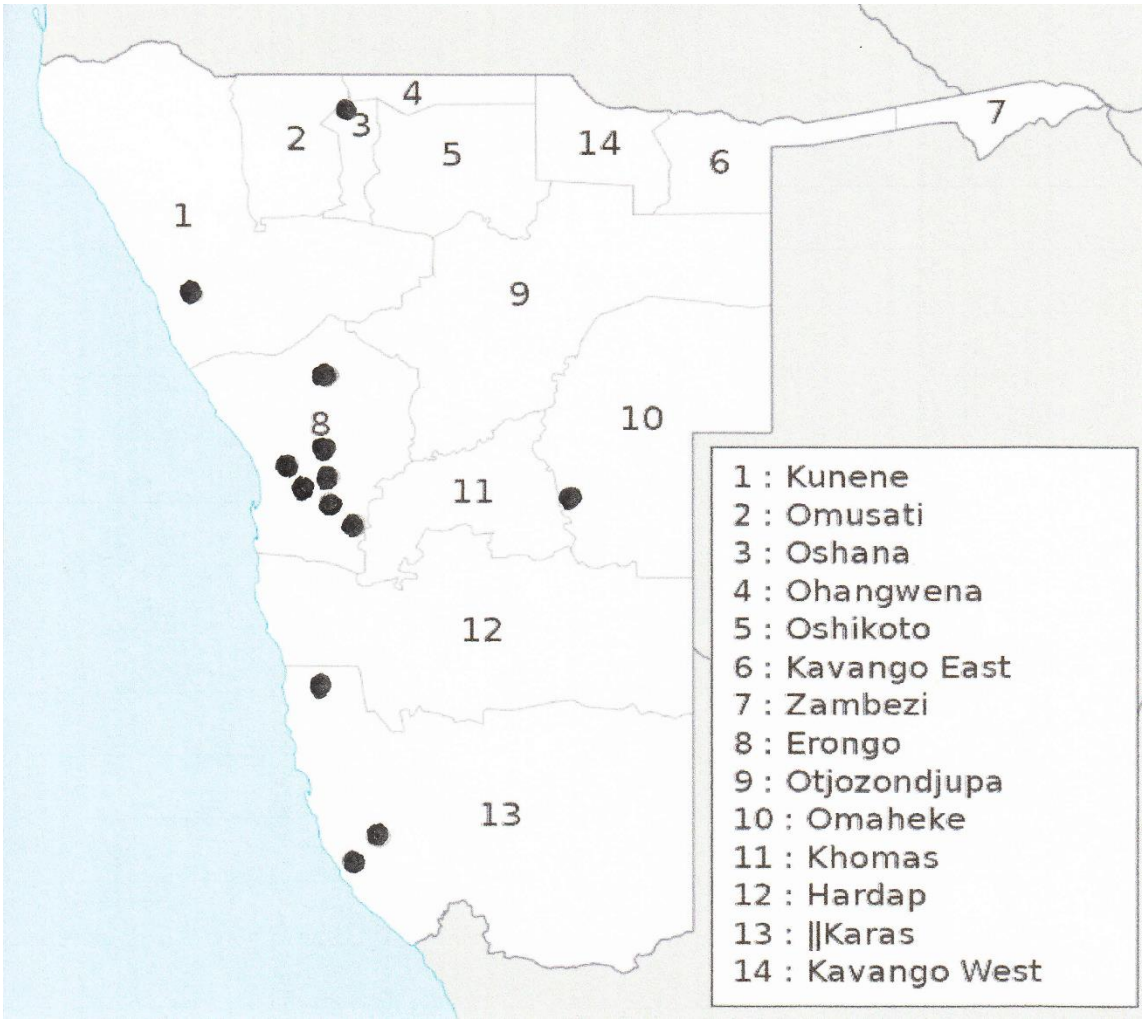


Figure 2. 3: Distribution of *Tracyandra laxa* in Namibia.[4,85]

2.2 Plant toxins and their effects on livestock

Livestock losses due to poisonous plants have a severe impact on the livestock industry of southern Africa.[11] In southern Africa, where livestock are traditionally kept under extreme climatic conditions and animals often dependent entirely on wild plants for their daily intake of food, they are forced to feed on poisonous plants which would otherwise be avoided in favourable conditions. Farm animals often depend entirely on wild plants for their daily intake of food and may regularly nibble on poisonous plants which may often not be sufficiently toxic to cause harm in small quantities.[3] Usually, plant poisoning in animals is accidental and occur under unfavourable conditions when pastures are poor due to drought, veld fires, overstocking and the trampling of grazing.[1] Diagnosis of plant poisonings is not easy and needs good case studies as well as evaluation of clinical signs and laboratory tests where these are available. It seems that prevention, pasture management and supplementary feeding is the best way to avoid animal poisoning. Plants produce a wide range of toxins, which fend off predators or discourage consumption by animals. In the event of ingestion, they may cause poisoning. Kellerman et al. [10] have compiled a wealth of information on plant poisonings and mycotoxicoses of livestock which has been recorded over a period of many years. They reported that the systems of the body which are primarily affected by plant toxins are the liver, heart, central nervous system, gastrointestinal tract, urogenital system, haemopoietic system, respiratory system, the skin and the appendages of organs. The transmission of chemical signals between neurons can also be affected by neurotoxins, which may result in a myriad of problems. Cells may be affected by toxins at any phase of neural transmission or may interact with

neurotransmitters in the synapse. Sekhar and co-workers discussed the classification of toxins based on their structural and chemical properties. They also acknowledged the grouping of plant toxins into alkaloids, glycosides, tannins, proteins, oxalates, enzyme inhibitors, antivitamins, phytoestrogens, volatile etheric layers and photosensitizing substances.[12]

There are a number of poisonous plants in southern Africa which can cause signs of nervous system damage in livestock. Pathological lesions are usually the most sensitive and reliable criterion on which a diagnosis can be made. Neurotoxicoses have been divided into two groups depending on the appearance of notable lesions formed in the central nervous system: neurological disorders without notable pathological lesions and neurological disorders with distinct pathological lesions (under which *T. laxa* is classified). Neuronal lipofuscinosis is clinically characterized by a severe neurologic syndrome, and pathologically by intense lipofuscin storage in neurons in the brain, spinal cord, peripheral ganglion and in some extraneuronal tissues.[14]

The major effect of plant poisoning may depend on the condition and susceptibility of the victim. The growth stage, the plant part as well as the amount of the plant consumed also plays a role. Active principles of many plants and their mode of action are known, however, the mechanism of intoxication of some plants which are also known to induce poisoning, has yet to be determined.[3] Rapid death may be caused by the ingestion of only small amounts of some plants due to their extremely high toxicity. The diagnosis of plant intoxication can be challenging, especially in veterinary medicine where a history of

exposure to a toxic plant is often lacking. Analytical tests are available to detect some plant toxins, although their diagnostic utility is often limited by test availability and timeliness of results. With a few notable exceptions eg. activated charcoal for acute yellow oleander self-poisoning,[13] antidotes for plant toxins are not available as yet.[3] Anti-digoxin Fab fragments have been effective in the treatment of *T. peruviana* poisoning, however, the high cost thereof limits availability. The development of antitoxins will remain a dream in many areas where they are needed until cost and supply issues have been solved.[14]

2.2.1 Livestock poisoning by T. laxa

As mentioned by Naude et.al. and Vahrmeijer, 10-25% of all stock losses in southern Africa can be attributed to plant poisonings.[15] Since 1954 a syndrome of progressive ascending paralysis occurred in Namibia in cattle, sheep, horses and pigs in the Kalahari and the Gobabis area. Areas where the outbreak occurred were overgrazed and vegetation was poor. Grant et al.[16] mentioned the presence of macroscopical pigmentation of the affected animal's brains. Two plants which had readily been eaten by these animals were *Trachyandra laxa* and *Sarcostemma viminale* (Asclepiadaceae). Initially only cattle, pigs and sheep were reported to be affected but later on it was found that goats and horses were also affected. Outbreaks only occurred during August to December and ceased after good rains.[15] Kellerman et al. [17] reported that the different species of animals affected by *T. laxa* have shown similar clinical signs where animals move with difficulty, they knuckle over at the fetlock joints and display various degrees of hypersensitivity and twitching of muscles in the early stages. Some animals may experience over-knuckling and over-

flexion of the hock and carpel joints. Eventually progressive paresis and paralysis follow and animals go down in sternal recumbency. The result is that animals die due to starvation, one to three months after the onset of clinical signs. Yellow-brown to greyish-brown intracytoplasmic pigment have been found present in the larger neurons in the brain, spinal cord and ganglia of animals at necropsy.

Many pasture plants have been reported to have an influence on the development of acquired lipofuscinosis. Neuronal and extra neuronal lipofuscinosis was reported in sheep on pastures in the south of Western Australia with predominantly *Trachyandra divaricata* and in South Africa with *T. divaricata* and *T. laxa*. The pigmentation was also produced experimentally in sheep and horses by feeding them *T. laxa*. Moreover, in a recent study, similar pigmentation associated with consumption of *Asphodelus aestivus* (Asphodelaceae) leaves in western Turkey have also been described. The common clinical signs reported in these publications include neurological signs such as severe tremors, convulsion, and paralysis.[18] Phytochemical investigations performed on Asphodelus species belonging to the same plant family as *T laxa,* Asphodelaceae, have resulted in the isolation of anthranoids, flavonoids and triterpenes.[14]

2.2.2 Toxic principles and their effects

Several phytochemical investigations have been executed on toxic plants. For the sake of survival, plants draw on numerous defences which may be physical or chemical. It has often been found that some secondary metabolites are specific to a particular species or genera and serve to protect the plant from predators. Phenolics, terpenes and steroids,

cyanic compounds and alkaloids have been found to be most prevalent to plant protection.[13] A typical feature of many secondary metabolites is their ability to modulate more than one molecular target, and therefore additive and even synergistic activities can be expected.[19] Secondary metabolites, especially alkaloids have been found to regulate neuronal signal transfer. They do so by interfering with ion channels, ion pumps, neuro-receptors, choline esterase, monoamine oxidase and other enzymes that are related to signal transduction pathways. The table in **appendix 2** summarises the structural types of plant secondary metabolites. It also indicates the abundance of poisons and psychoactive substances as well as those containing nitrogen versus those which do not contain nitrogen.[12]

Alkaloids

Alkaloids, are a diverse group of nitrogen-containing compounds with low molecular weights, and are derived mainly from amino acids.[20] They are mainly stored in tissues which are important for survival, i.e. in the flowers, seeds, roots, stem bark and those tissues which are photosynthetically active.[21] A number of plant species, both native and naturalized, are recognized as toxic for both animals and humans due to their alkaloid content. There are plants containing the hepatotoxic pyrrolizidine alkaloids, neurotoxins such as the indolizidine alkaloid swainsonine and the piperidine alkaloids, coniine and -coniceine as well as tropane alkaloids.[23] The biosynthesis of some compounds, such as tropane alkaloids, which are typical for several genera of the Solanaceae are produced in the roots and then translocated via the xylem to the aerial parts where these neuroactive alkaloids are stored in vacuoles. The synthesis of quinolizidine alkaloids, such as lupanine

or cytisine takes place in chloroplasts of aerial organs, such as leaves of several legumes. These alkaloids are transported to other aerial organs (such as fruits and seeds) and roots via the phloem and accumulate in vacuoles.[12] Mukherjee et al.[24] reported that the majority of studies have focused on the anti-cholinesterase alkaloids, such as physostigmine and galantamine and that more than 35 alkaloids have been reported to have AChE inhibitory activity. Alkaloids are known for their medicinal and toxic effects. One way of classifying alkaloids is according to the structure of the nitrogen-containing ring in the molecule **(Fig. 2.4)**, e.g. pyridine alkaloids have a pyridine ring system while a tropane ring system is prominent in tropane alkaloids. Coniine, an example of a piperidine alkaloid, is an extremely toxic compound found in the hemlock plant, *Conium maculatum* (Apiaceae) and causes paralysis of the motor nerve endings while nicotine, an example of a pyridine alkaloid, acts on the central nervous system, smooth muscle of the intestine and cardiovascular system **(Fig. 2.4)**. Pyrrolizidine alkaloids, quinolizidine alkaloids, steroid alkaloids and terpenoid alkaloids have been classified as toxic.[3] Wink[6] also reported that some alkaloids inhibit enzymes that break down neurotransmitters, such as cholinesterase (AChE) and monoamine oxidase (MAO).

Coniine Nicotine

Figure 2.4: Tobacco alkaloids (piperidine-coniine and pyridine-nicotine types).[3]

Alkaloids often originate from the same amino acid precursor as the neurotransmitters serotonin, noradrenalin, dopamine, gamma aminobutyric acid (GABA), glutamic acid or histamine and their lethality in animals correlates with interactions with certain molecular targets; therefore, their structures are able to be superimposed on neurotransmitters.[22]

Cyanogenic glycosides

Cyanogenic glycosides are widely distributed in the plant kingdom in taxa belonging to families Fabaceae, Rosaceae, Linaceae, Compositae and others.[26] They release the lethal gas hydrogen cyanide (HCN), after enzymatic breakdown of the glycoside (**Fig. 2.5**). This is known as prussic acid poisoning.[3] Certain species, eg. *Ruta graveolens* L. (Rutaceae), *Lavandula augustifolia* Miller (Lamiaceae), *Rosmarinus officinalis* L. (Lamiaceae), *Petroselinum crispum* (Mil.) Nym. ex A. W. Hill. (Apiaceae), and *Mentha spicata* L. (Lamiaceae) contain essential oils with terpenes, a group of compounds reported to have AChE inhibitory activity [(2006) cited by Adewusi].[27]

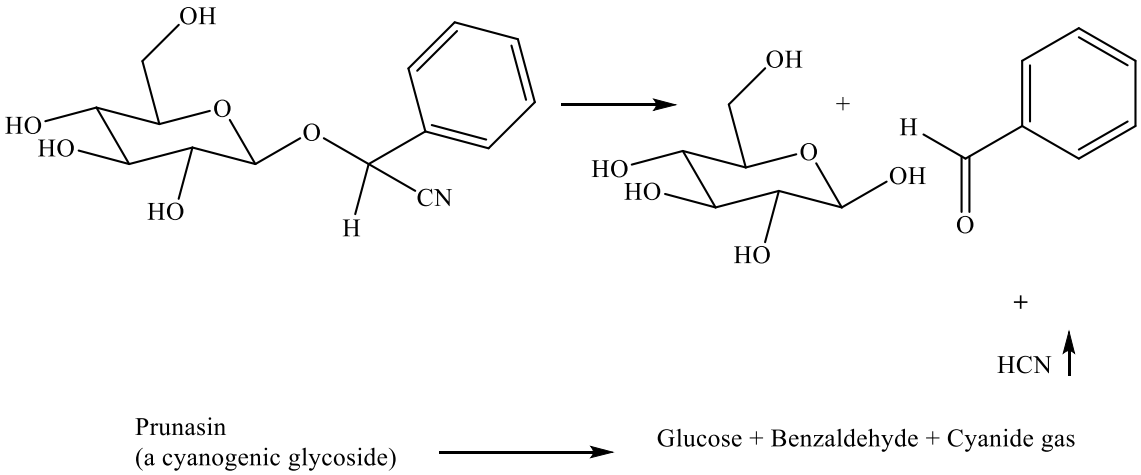


Figure 2.5: Breakdown of prunasin, a cyanogenic glycoside, by enzymatic hydrolysis resulting in the release of cyanide gas.[3]

Terpenoids

In nature terpenoids are formed by isoprene units (five carbon units) that are linked together. They include hydrocarbons and their oxygenated derivatives and are volatile substances which give plants and flowers their fragrance. Monoterpenoids consist of two isoprene units, sesquiterpenoids three units, diterpenoids four units and triterpenoids six units. In nature, terpenoids often occur as glycosides. (**Fig. 2.6**)[3]. Terpenoids represent the largest, most diverse class of chemical compounds produced by plants and are employed for a variety of functions in growth and development. The majority of terpenoids are however used for more specialized chemical interactions. Terpenoids are also used for protection in the abiotic and biotic environment. Plant-based terpenoids have also been used in the food, pharmaceutical and chemical industries. The ecological importance of terpenoids has also increased in the development of strategies in sustainable pest control and protection against abiotic stress. Isoprene, monoterpenoids, sesquiterpenoids and diterpenoids, which are volatile or semi volatile, low-molecular-weight terpenoids, have also been implicated in the protection of plants against abiotic stress as well as in some biotic interactions both above and below ground.[27]

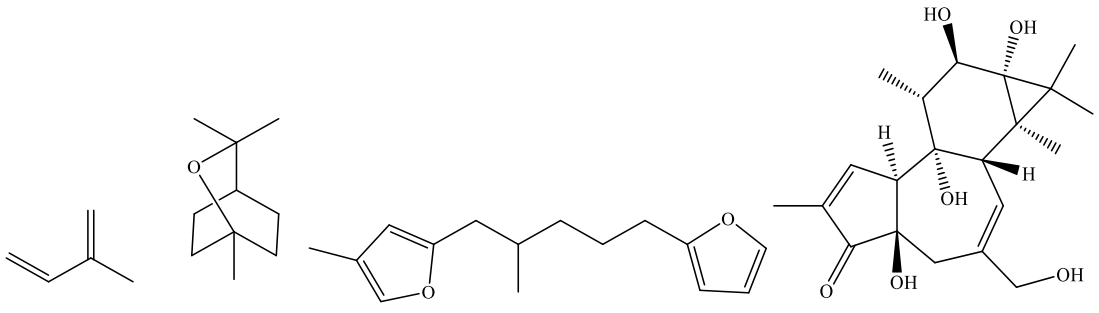


Figure 2.6: Chemical structures of isoprene and terpenoids.[3]

Lectins

A broad range of chemical and protein based toxic compounds like lectins, ribosome-inactivating proteins, protease inhibitors, ureases, arcelins, antimicrobial peptides and pore-forming toxins are synthesised and stored by plants. They have been discovered in different plants and serves as part of their defence system designed to protect them from invading organisms. Due to their biological activities and various degrees of toxicity towards animals, insects, bacteria or fungi, they have also become useful in the protection of crops as well as in biomedical applications like cancer treatment.[29] Lectins are a class of protein which binds specifically to sugars. Agglutination or precipitation of the red blood cells is caused when some proteins and glycoproteins attach themselves to red blood cells.[3] The effect of their toxicity to animals varies from being merely antinutritional to being lethal. The phytohemagglutinin from the bean, *Phaseolus vulgaris* (Fabaceae), is an example of a highly toxic lectin. Their overall toxicity is mainly due to the binding of lectins to specific carbohydrate structures on the epithelial cells of the digestive tract of animals. Due to their biological activities, toxic plant proteins have become useful to crop protection as well as biomedical applications. However, to unravel the mode of action of lectins in their toxicity to predators, still remains a challenge.[29]

Flavonoids

Flavonoids are a large family of important plant secondary metabolites. They are a diverse group of plant phenolics based on a 15-carbon skeleton.[29] The skeleton consists of two phenyl rings (A- and B-rings) connected by a three-carbon bridge (C-ring) at its simplest level (**Fig. 2.7**).[31] More than 9,000 flavonoid compounds have been discovered in various

plants. They are a widespread family of phytochemicals and are the most common distributed group of plant phenolic compounds. They also have diverse biological functions, including antioxidant, antibacterial, anticancer, antiviral, anti-inflammatory and hepatoprotective activities.[32] They are an indispensable factor in plant interactions with the environment and often serve as the first line of defense against pathogen attack and UV irradiation. Flavonoids can be divided into multiple subclasses, including chalcones, flavones, flavonols, flavanones, anthocyanins, and isoflavonoids based on the substitution patterns of the central C ring.[33]

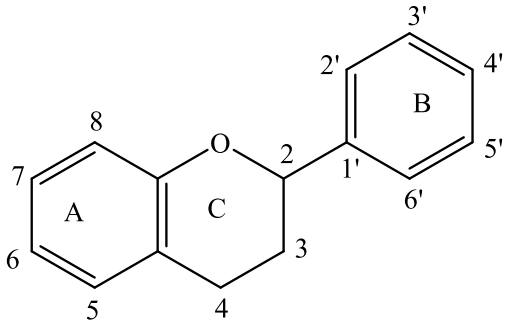


Figure 2.7: Basic structure of flavonoids.[33]

Two new flavonol glycosides (kaempferol-3-*O*-β-$_D$-glucopyranosyl-(1→3)-α-$_L$-rhamnopyranosyl-(1→2)-β-$_D$-glucopyranoside-**(1')**, quercetin-3-*O*-β-$_D$-gluco-pyranosyl-(1→3)-α-$_L$-rhamnopyranosyl-(1→2)-β-$_D$-glucopyranoside **(2')** and a known flavonoid kaempferol **(3')**, have been isolated and elucidated from the methanol leaf extract of *Maytenus robusta* (Celastraceae), (**Fig. 2.8**). Significant acetylcholine esterase (AChE) inhibition was demonstrated for both the above flavonols and this data highlights these compounds as flavonols linked to a trisaccharide chain presenting this activity.[25]

Likewise, acetylcholine inhibition by flavonoids was also confirmed in another study where tiliroside, quercetin, quercitrin and 3-methoxyquercetin were isolated from the ethyl acetate extracts of whole plants of *Agrimonia pilosa ledeb* (Rosaceae).[35] Structure –activity relationship studies revealed that the structural elements required for AChE inhibition by flavonoids are not only the presence of a 4-OMe group but also the presence of a 7-*O*-sugar.[36]

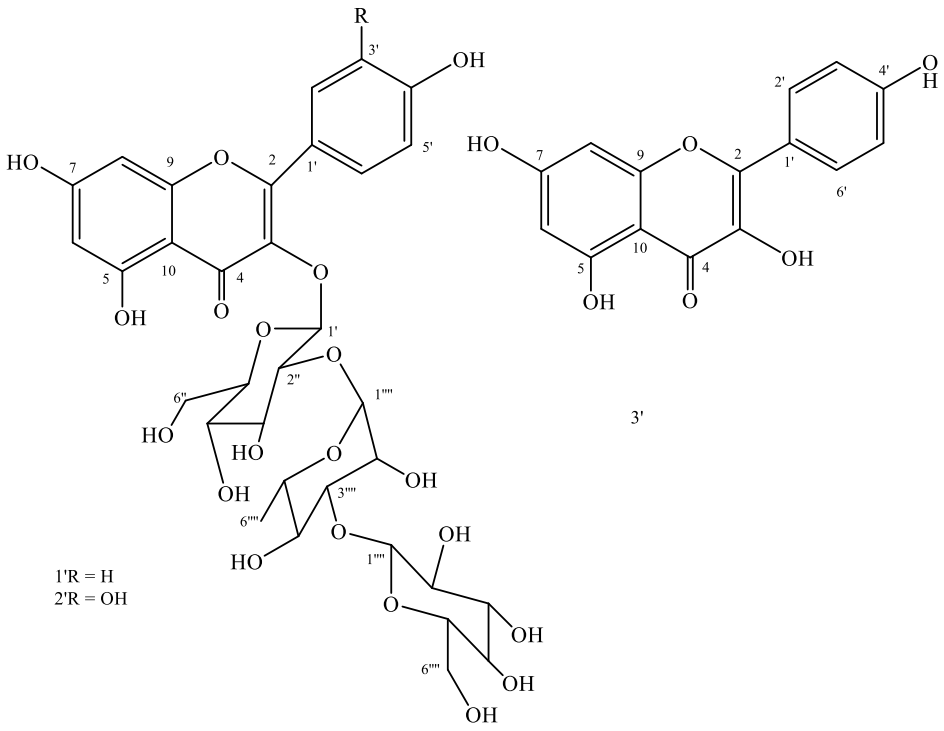


Figure 2.8: Flavonoids isolated from leaves of *M. robusta.*[25]

Lactones

Lactones are cyclic esters and named as 2-oxacycloalkanones in systematic nomenclature. Common names are derived from the name of the carboxylic acid, designating the length of the carbon chain and a Greek letter indicating the carbon to which the carboxyl oxygen is attached. Four-membered ring lactones are β-lactones (carboxyl oxygen is on the β-carbon), five-membered ring lactones are γ-lactones and six-membered ring lactones are δ-lactones.[22] More than 90% of identified lactones were isolated from the plant family Asteraceae. A variety of naturally occurring compounds, (e.g. cardenolides, bufadienolides, actogenins, coumarins and food-flavoring furanones) have the α,β-unsaturated lactones, 2-furanone (5H-furan-2-one) and 2-pyrone (pyran-2-one) as part of their chemical structure. Some of these compounds have a broad spectrum of biological activities, including antimicrobial, anticancer, cardiotonic and toxicological activities.[37]

Sesquiterpene lactones

Sesquiterpene lactones (STL), one of the largest groups of secondary plant metabolites, possess a broad variety of obvious biological activities directed towards all types of predating organisms. Most of these compounds w believed to exert their activities by a common chemical mechanism of action, alkylation of biological macromolecules leading to decisive consequences on cellular function. A number of enzymes and other essential macromolecules are known to be inhibited by STL, even at low concentrations. Even though many STL's possess potentially useful activities, e.g. anticancer, low selectivity of these effects forbids utilization due to high unspecific toxicity. They can thus be

considered non-specific, highly efficient chemical weapons against literally any organism that might be harmful to plants. Some are less common but exert toxicity by very specific molecular mechanisms, such as strong inhibitors of intracellular calcium signalling and some highly potent GABA-antagonistic neurotoxins.[42] The same properties of STLs that make them useful medicines can also cause severe toxicity, thus the toxicological profile of these compounds must be determined.

Sesquiterpene lactones have been isolated from families such as Magnoliaceae, Lauraceae, and Apiaceae. They play an important role in plant defense as antibacterials, antivirals, antifungals and insecticides. The bitter taste of the plants is significant as it rejects herbivorous animals, including insects as well as mammals that would otherwise regard them as potential food. They have displayed allelopathic potential and due to their cytotoxic and anticancer activity there has been and increase interest in sesquiterpene lactones. They belong to the group of C15 terpenoids and are formed from three isoprene units. One of their methyl groups, a part of the isoprene group, was oxidized to lactones. STL's have been isolated from all plant organs but most commonly, they are recorded in leaves and in glandular trichomes on leaves. Due to the presence of α,β-unsaturated γ-lactonic ring they have distinctive biological activities.[39]

STL's have been found to display a variety of activities against numerous organisms, suggesting that individual lactones may play a role in the defence against pathogens and herbivores. The reaction of STLs with the thiol groups of vitally important compounds such as enzymes result in these biological activities. They are the active constituents of a variety of plants frequently used as herbal remedies. The toxicological profile of these

compounds must be thoroughly characterized since the same properties that make useful medicines can also cause several toxic syndromes in farm animals. Recently concerns have been raised regarding their genotoxic potential.[40]

Intoxication may be caused by all plants of the genus *Geigeria* (family Asteraceae), but *Geigeria ornativa* and *Geigeria aspera* are of economic importance in southern Africa.[10] The toxicity induced by these *Geigeria* species is attributed to various sesquiterpene lactones.[41]

It has also been reported that the toxicity of *Geigeria aspera* is ascribed to vermeeric acid, which readily converts to its corresponding lactone, vermeerin. Apart from vermeerin, several other sequiterpenoid lactones are known from *Geigeria* species, such as gingering, geigerinin, greisen and digdrogriesenin. Only vermeerin (**Fig. 2.9**) has been definitely shown to cause vermeersiekte "vomiting disease" (causing symptoms of bloating, stiff muscles, paralysis and vomiting), but the other compounds, particularly those with an α,β-double bond on the lactone ring are also known to be toxic to laboratory animals.[8]

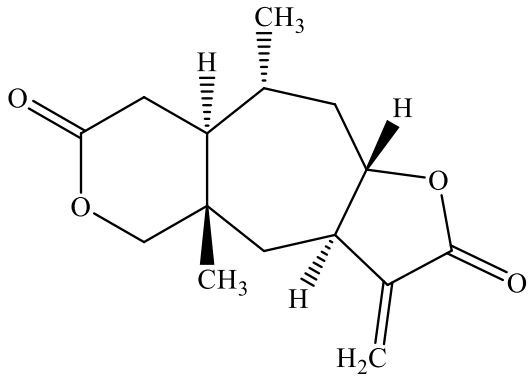


Figure 2.9: Structure of vermeerin.[6]

2.2.3 Mechanism of action of neurotoxins

Extremely hazardous, highly hazardous and moderately hazardous toxins have been reported to interfere with central functions in herbivores. Neurotoxins, affecting the nervous system is the most poisonous followed by cytotoxins and metabolic poisons.[12] Neurotoxins are a group of toxins with highly specific effects on the nervous system of animals, interfering with nerve impulse transmission. Chemically and pharmacologically neurotoxins are a varied group of compounds. They vary in chemical structure and mechanisms of action and produce very distinct biological effects. The complex anatomy of the nervous system, its specialised functions, high metabolic requirements and limited ability to repair itself makes it a target for toxicity. A number of plants cause nervous conditions as the main symptom of poisoning or at least as one of the symptoms.[41] Neurotoxins affecting livestock in southern Africa are listed in **Table 2.1**. Voltage-gated sodium channels are the molecular targets for a range of neurotoxins acting at six or more distinct receptor sites on the channel protein. Sodium conductance is prevented by the physical blocking of the pore by hydrophilic low molecular mass toxins and larger polypeptide toxins. Alkaloid toxins and related lipid-soluble toxins alter voltage-dependent gating of sodium channels via an allosteric mechanism by binding to intramembranous receptor sites. In contrast to this, polypeptide toxins alter channel gating by voltage sensor trapping through binding to extracellular receptor sites.[43] It has long been known that local anaesthetics and anticonvulsant drugs block the sodium channel by occluding its pore.[42] According to Wink and Van Wyk,[6] there are two types of neuro-receptors namely ligand-gated channels (ligands: acetylcholine, glutamic acid, GABA,

serotonin and glycine) and ligand-gated channels G-protein linked neuro-receptors (ligands: acetylcholine, serotonin, dopamine, noradrenaline).[6] In the case where neuronal signal transduction is completely blocked at any stage, especially in the case of ion channels, Na^+, K^+ and Ca^{2+} -ATPase, the central nervous system can no longer control muscular activity, such as that of the heart, respiratory system or skeletal muscles. Consequently, neurotoxins often have paralytic properties causing death from respiratory or cardiac arrest. The CNS itself can be affected and neurotoxins cause numbness, unconsciousness and coma. Where lower concentrations of neurotoxins are concerned, mind-altering properties are often exhibited, especially if neuro-receptors and corresponding enzymes are targeted.[6] The neuroreceptors are a prime target for many alkaloids which structurally resemble the endogenous neurotransmitters that appear in **Figure 2.10**.

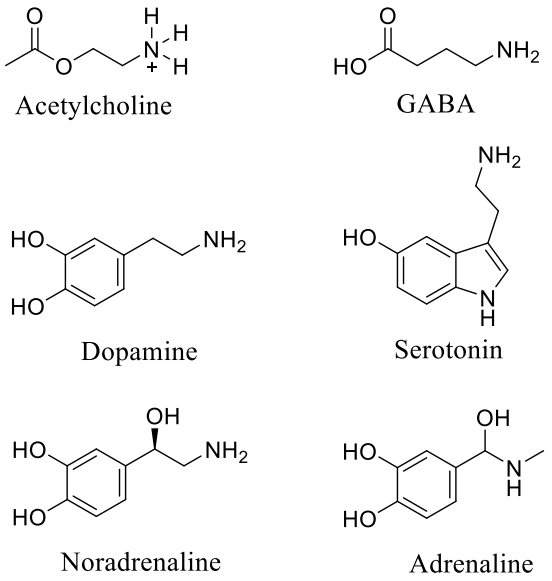
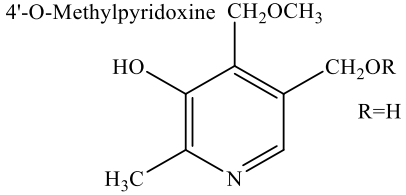
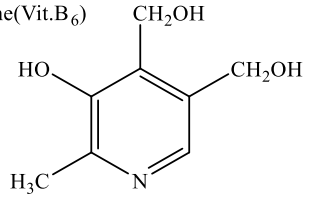
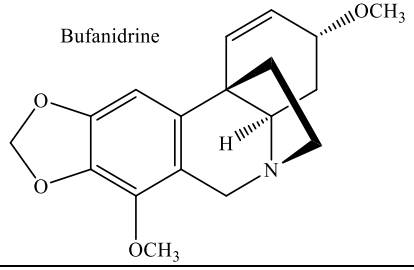
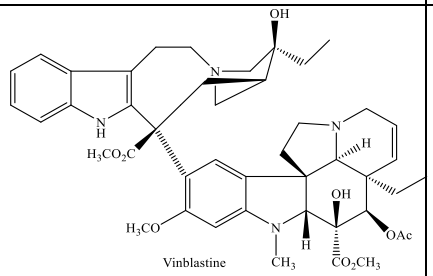
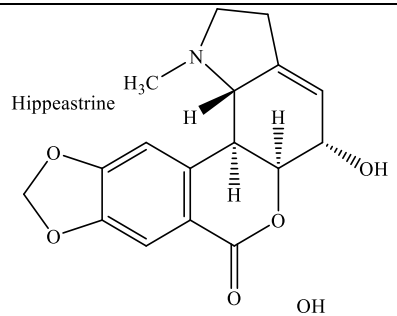
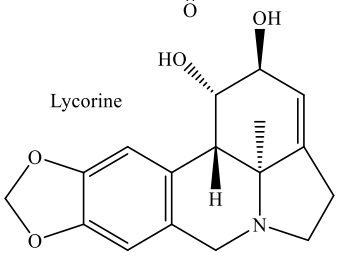
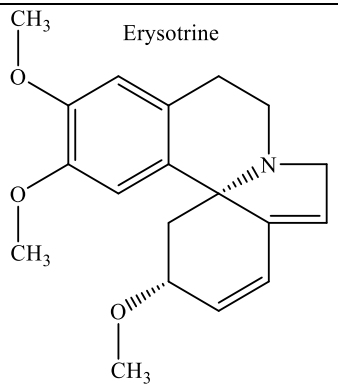
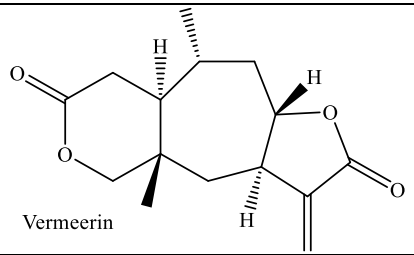


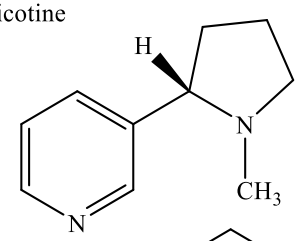
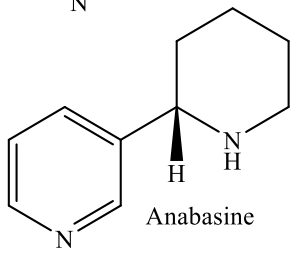
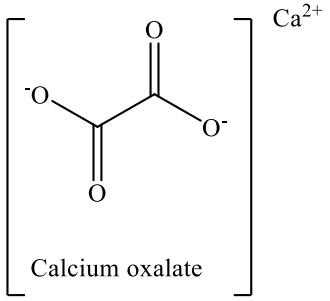
Figure 2.10: Structures of important neurotransmitters.[7]

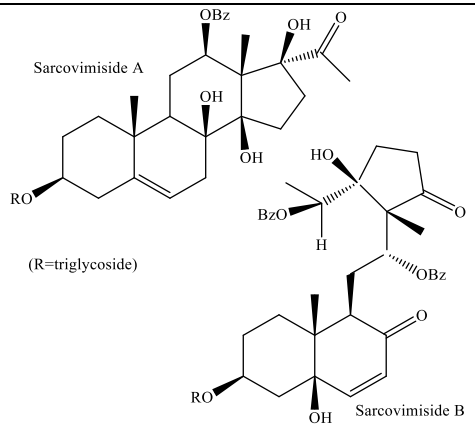
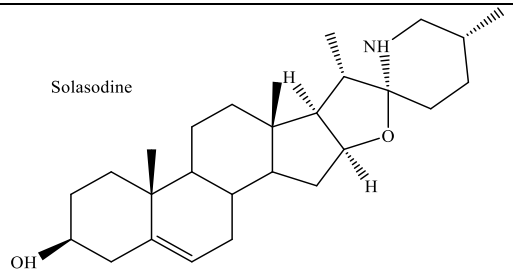
Table 2.1: Neurotoxic Plants found in Southern Africa.[3]

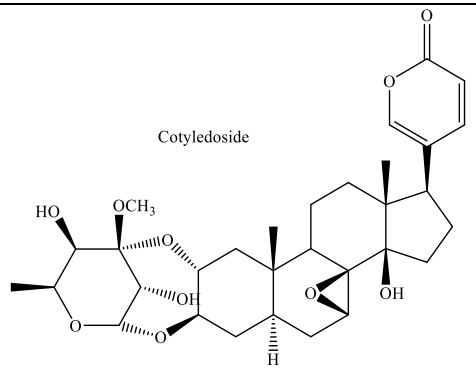
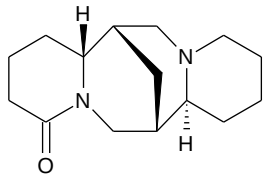
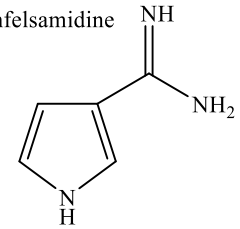
NAME	FAMILY	TOXIN	STRUCTURE	PHARMACOLOGICAL EFFECTS
Albizia versicolkor	Fabaceae	Pyridine alkaloid	4'-O-Methylpyridoxine  Pyridoxine(Vit.B6) 	**Neurotoxicity** caused by presence of methylpyridoxine a pyridoxine (Vit B_6) antagonist. Symptoms can be reversed by dosing with Vit.B_6.
Boophane disticha	Amaryllidaceae	Isoquinoline alkaloid	Bufanidrine 	Buphanidrine-powerful analgesic, hallucinogen & **neurotoxin**.

NAME	FAMILY	TOXIN	STRUCTURE	PHARMACOLOGICAL EFFECTS
Catharanthus roseus	Apocynaceae	Indole alkaloid	 Vinblastine	Vinblastine has central and peripheral **neurotoxic** effects which is very poisonous.
Clivia miniata	Amaryllidaceae	Isoquinoline alkaloid (lycoranan type)	 Hippeastrine  Lycorine	Lycorine highly toxic (LD_{50}) in dogs 41mg/kg). Symptoms like salivation etc. leading to **paralysis** and collapse.

NAME	FAMILY	TOXIN	STRUCTURE	PHARMACOLOGICAL EFFECTS
Erythrina caffra	Fabaceae	Isoquinoline alkaloid	 Erysotrine	Erysotrine, is known to have curare-like **neuromuscular blocking** effects. Curare general term used for substances that have **paralysing** effect on **skeletal muscles**, however, not toxic when ingested.
Geigeria ornativa	Asteraceae	Sesquiterpenoid lactone	 Vermeerin	*Geigeria* poisoning results in cough, diarrhoea, bloating, followed by stiffness and **paralysis** (choking, respiratory paralysis).
Helichrysim argyrosphaerum	Asteraceae	UNKNOWN	UNKNOWN	Symptoms-weakness, blindness, paresis, **paralysis**. Symptoms similar to vermeersiekte (caused by *Geigeria*).

NAME	FAMILY	TOXIN	STRUCTURE	PHARMACOLOGICALEFFECTS
Nicotiana glauca	Solanaceae	Pyridine alkaloid	Nicotine   Anabasine	Symptoms in extreme cases, paralysis of respiratory muscles, nausea, weakness, headaches, salivation, convulsions, confusion, difficulty in breathing, hypertension.
Oxalis pecaprae	Oxalidaceae	Oxalic acid	 Calcium oxalate	Abdominal pain, vomiting are typical & other disorders resulting from **paralysis of nervous system.**

NAME	FAMILY	TOXIN	STRUCTURE	PHARMACOLOGICAL EFFECTS
Sarcostemma viminale	Asclepidaceae	Steroid glycoside		Symptoms similar to cynanchosis- hypersensitivity, seizures & **paralysis.**
Solanum incanum	Solanaceae	Steroid alkaloid		Salivation, vomiting, diarrhoea, bloat, rapid pulse & breathing, cramps, **paralysis**.

NAME	FAMILY	TOXIN	STRUCTURE	PHARMACOLOGICAL EFFECTS
Tylecodon wallichii	Crassulaceae	Cardiac glycoside (bufadienolide)	Cotyledoside 	Affects nervous system & muscular system with fatal results.
Lupinus angustifolius	Fabaceae	Quinolizidine alkaloid	Lupanine 	Tremours, convulsions, **paralysis** & death. Acetic acid is used as emergency treatment.
Nierembergia hippomanica	Solanaceae	Amidine	Brunfelsamidine 	Causes nervous signs in cattle, **partial paralysis**, ataxia, salivation and irregular heartbeat.

These neuroactive alkaloids can function either as agonists (overstimulating a neuroreceptor) or as antagonists (blocking certain neuroreceptors). On the other hand, some alkaloids inhibit enzymes like AChE and monoamine oxidase (MAO) which break down neurotransmitters and display similar toxic properties as neuroreceptor agonists.[12] Initiation and propagation of action potentials in almost all excitable cells is due to voltage-gated sodium channels. They are well known as the primary target of dichlorodiphenyltrichloroethane (DDT), and of naturally occurring pyrethrins found in extracts of the flowers of *Chrysanthemum* (family Asteraceae) species and modern synthetic pyrethroids which are structural derivatives of pyrethrins. This large functionally complex channel protein is furthermore known to possess at least nine independent target sites for a variety of neurotoxins produced by plant and animals for defence or predation, such as tetrodotoxin, alpha and beta-scorpion toxins and batrachotoxin. Where neuronal signal transduction is completely blocked at any stage, especially in the case of ion channels, Na^+, K^+ and Ca^{2+} -ATPase, the central nervous system (CNS) is no longer able to control muscular activity (i.e. the heart, respiratory system or skeletal muscles). Neurotoxins thus often have paralytic properties which may cause death from respiratory or cardiac arrest. The CNS itself can be affected and neurotoxins can thus cause numbness, unconsciousness and coma. In the case of lower concentrations of neurotoxins, mind-altering properties are often displayed, especially when neuro-receptors and their corresponding enzymes are targeted.[6] Acetylcholine (ACh) was the first discovered neurotransmitter which transfers neural signal at all autonomic ganglia. This includes neuromuscular junction as well as synapses in the central nervous system. The neurotransmission of signal is governed by ACh between the preganglionic sympathetic and parasympathetic neuron in the autonomic nervous

system and is also responsible for the stimulation of muscles. During the process of neuronal transmission, ACh breaks down to form acetate and choline by the enzyme acetylcholinesterase (AChE) on the post-synaptic membrane. During the degradation of ACh, the normal nervous transmission in the synaptic cleft is broken.[41] Impulses are chemically mediated across synapses through the release of neurotransmitters from the pre-synaptic terminal after which they move across the synaptic cleft where they bind to their post-synaptic target receptor resulting in either an excitatory or inhibitory response in the post-synaptic neuron or muscle.

ACh, one of the common neurotransmitters involved in veterinary neurotoxicoses, mediates effects at the neuromuscular junction at the preganglionic neurons of the parasympathetic and sympathetic nervous systems and at many of the post-ganglionic neurons of the parasympathetic nervous system. An action potential is converted into a chemical signal in the synapse and at neuromuscular junctions in neuronal signal transduction. The enzyme AChE, attached to the post synaptic membrane removes acetylcholine from the synaptic cleft by degrading ACh to choline and acetic acid.[27]

2.3 Determination of toxicity

In the analysis of plants, extraction is the first crucial step necessary to extract the desired chemical components from the plant material in order to do further separation and characterization. A bioassay is a test method to measure responses of living animal or plant tissue to toxicity of chemical contaminants. Individuals can be exposed to different concentrations of contaminants for different periods in order to examine toxic effects. Bioassays can be used to determine if extracts are active (pre-screening). Hereafter, those which responded positively will be subjected to further testing. They

can also be used to prioritize active extracts, where extracts are tested over a range of concentrations and their responses quantified and used as a monitoring tool. After this the tested fractions are submitted for further separation and monitoring and the less active fractions set aside. This ensures the isolation of the most active compound. Bioassays should correlate to the problem and should be simple, fast, reliable, inexpensive and reproducible. However, no bioassay can meet all these criteria. *In vivo* testing can provide more valid data than *in vitro* cellular testing but is often complicated and expensive, whereas cellular assays can be fast and inexpensive. There is no absolute or specific test that will accurately predict toxicity of any particular plant or chemical substance. The LD_{50} toxicity test can be performed, thus determining the lethal dose for 50% of a certain group of animals. This however has become controversial due to humanitarian and ethical concerns.[3]

2.3.1 Traditional methods used to test for toxicity

In the past the only available tools for compound isolation were distillation (for liquids), and sublimation or fractional recrystallization for solids. Tests are usually done in several animal species (*in vivo*) and in laboratory modes (*in vitro*) where human cells are grown in suspension cultures and used. A solution of suspended live cells is usually transferred into 96-well plates and various concentrations of the chemical compound or extract being studied added to the wells. After a certain time period, a colour reagent that interacts with live cells only is then added. A colour reaction usually shows up only in those wells where the cells are still alive and the absence of colour indicates the cells are dead. The LD_{50} toxicity test can be done among others to determine toxicity, thus determining the lethal dose for 50% of a

certain group of animals. This test however, has become controversial due to humanitarian and ethical concerns. Even so, carefully researched animal studies still provide valuable information regarding toxic effects as well as potential beneficial effects. Specialised tests are conducted on animals to evaluate special forms of toxicity, e.g. carcinogenicity (potential to induce cancer), mutagenicity (potential to induce cellular damage) and teratogenicity (potential to damage the foetus or offspring). The disadvantage is that *in vitro* studies do not necessarily give an accurate picture of what would happen in the human body. There is however still a place for this type of testing which provides valuable toxicological and pharmacological knowledge.[3]

2.3.2 Cytotoxicity assays

When a substance has the ability to adversely affect and potentially kill living cells, we refer to the cytotoxicity of that substance. In the event of cells being exposed to a cytotoxic chemical or substance, it can lead to reduced cell viability (reduced or no cell growth and division), cell death due to necrosis and programmed cell death (apoptosis).[45]

Choosing a cell viability or cytotoxicity assay from among the numerous different options available can be a challenging task. One needs to understand what the assay is measuring as an endpoint, how the measurement correlates with cell viability and what the limitations of the assay chemistries are. Assays are available to measure a variety of different markers that indicate the number of dead cells (cytotoxicity assay), the number of live cells (viability assay), the total number of cells or the mechanism of cell death. A basic understanding of the changes occurring during different

mechanisms of cell death will assist in the decision of the endpoint to choose for a cytotoxicity assay. If the information sought is simply whether there is a difference between "no treatment" negative controls and "toxin treatment" of experimental wells, the choice between measuring the number of viable cells or the number of dead cells may be irrelevant. If, however, more detailed information on the mechanism of cell death is sought, the duration of exposure to toxin, the concentration of the test compound and the choice of the assay endpoint becomes critical.[46]

2.3.3 Neurotoxicity assays

Neurotoxicity refers to the effects that exposure to toxins may have on both the nervous system structure and its function. It may result in cell death, structural damage or disruption of signalling processes.[45] For instance, when compounds inhibit acetylcholinesterase enzymes and cause a disruption of neurotransmission, they are essentially acting as neurotoxins. Reversible and irreversible acetylcholinesterase inactivating compounds are of interest for their specific and nonspecific toxic effects. Neurotoxicity testing is used to identify potential neurotoxic substances and at times is considered as a component of target organ toxicity; the central nervous system (CNS) being one of the major target organ systems. Neurotoxicity, like other target organ toxicities, can result from different types of exposure; oral, dermal or inhalation to a substance. Neurotoxicity may be observed after a single dose or after repeated dosing. A decrease in the ability of cholinesterase to break down acetylcholine, causing very high acetylcholine levels, results in cholinesterase inhibitor toxicity. Cholinesterase inhibitors fall into two classes, organophosphorus compounds, and carbamates of which the first class generally is more toxic. Organophosphorus

compounds also display longer duration of action and are more commonly responsible for causing CNS toxicity.[47]

2.4 Isolation and characterisation of toxins in toxic plants

To extract phytochemicals, multiple solvents have commonly been used with dried powder of plants to extract bioactive compounds and at the same time eliminate the interference of water. Solvents used for the extraction of biomolecules from plants are selected based on the polarity of the solute of interest as a solvent of similar polarity to the solute will properly dissolve the solute. To limit the amount of analogous compounds in the desired yield, multiple solvents can be used sequentially The polarity, from least polar to most polar, of a few common solvents is as follows: Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water.[48]

In the analysis of plants, extraction is the first crucial step as it is necessary to extract the desired chemical components from the plant materials in order to do further separation and characterization.[49] Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. Separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is common practice to use different separation techniques such as thin layer chromatography (TLC), column chromatography, flash-chromatography, ion exchange chromatography on Sephadex and high performance liquid chromatography (HPLC), to obtain pure compounds in the isolation of these bioactive compounds. The pure compounds are then used for the determination of structure and biological activity. Non-chromatographic techniques

such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay and Fourier-transform infrared spectroscopy (FTIR), can also be used jointly to obtain and aid in the identification of the bioactive compounds.[50] The technique of choice for the identification of volatile chemical components in natural mixtures or extracts is gas chromatography mass spectrometry (GC-MS). This is done by matching measured spectra with the spectra in a reference library, however, the mass spectrum-based compound identification cannot differentiate isomers.[51] In this pursuit, gas chromatography equipment can be directly coupled with rapid scan mass spectrometer of various types. When chromatography is combined with mass spectrometry, mixtures can be separated into individual compounds or elements. Due to the low flow rate from the capillary column, output can be fed directly into the ionization chamber of MS. In the ion trap detector, ions are created from the eluted sample by chemical ionization and stored in a radio frequency field. From here the trapped ions are ejected from the storage area to an electron multiplier detector. Ejection is regulated, making scanning on the basis of mass-to-charge possible.[52] GC-MS is the primary technique used for identification of volatile chemical components in natural mixtures or extracts by matching measured spectra with the spectra in a reference library. It has also become one of the important techniques for phytochemical analysis.[53] In the article by Zhang et.al.[54] it is said that to date, spectral library search is still the most commonly used method for tentative compound identification. The disadvantage is that it only works for compounds in the library and since many similar mass spectra exist in the reference library, we always have a hit list of candidates with similar numerical similarity indices. This makes it difficult to select the correct candidate as the first candidate is not necessarily the correct choice, taking

into consideration noise, background and co-eluting components. Due to this it is still necessary to extract more information from GC-MS data to achieve accurate identification. The retention index (RI) calculation of chemical compounds and comparison with literature is usually followed in the identification of compounds. In the applications of GC-MS and mass spectrum matching–based compound identification, isomers cannot be differentiated from each other. The retention index (RI) system was thus introduced in 1985 by Kováts to allow the results measured in one laboratory to be used in other laboratories. The efficiency of the process of identification of compounds based on their RIs is due to recent advances in the RI database development and the accumulation of experimental retention measurements in computerized libraries. More accurate identification of isomers with very similar mass spectra has become possible with the availability of libraries of retention data. With the use of retention data, filtering of MS hits substantially lower the number of possible candidates.[55,56]

CHAPTER 3: MATERIALS AND METHODS

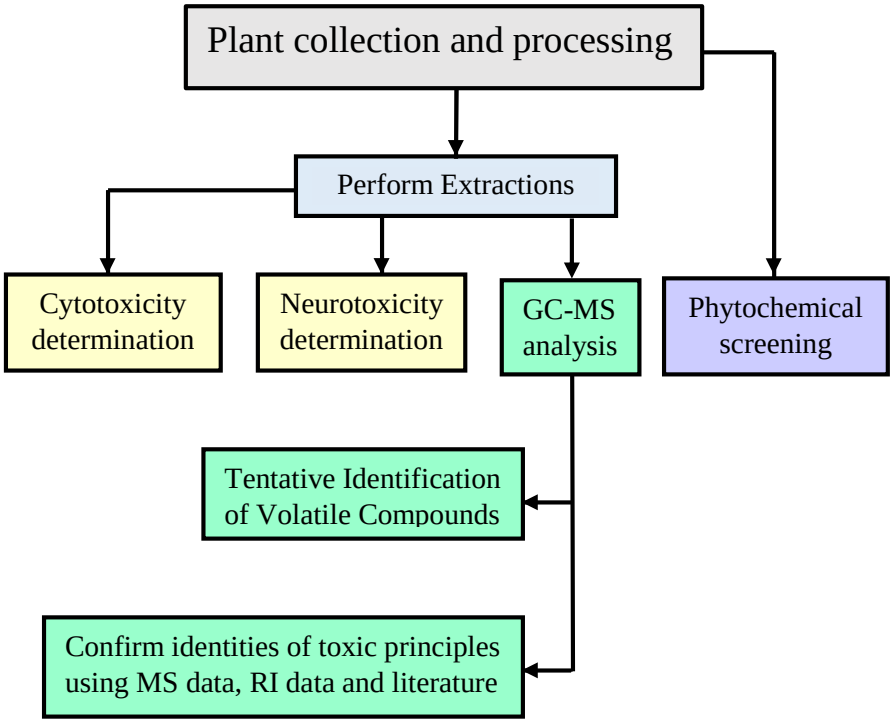


Figure 3. 1: Research methodology

3.1 Chemicals and materials

Merck high-performance liquid chromatography-grade dichloromethane (DCM; Biodynamics, Windhoek, Namibia) was used to prepare extracts and standard solutions. An alkane mixture [C_{10}-C_{40}; all even] used for retention index determinations was of analytical grade and purchased from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals and reagents (methanol, hexane etc.) were of analytical reagent grade (AR). Purified water used for dilutions was obtained using a Milli-Q Integral 3 water purification system. For the cytotoxicity assay, tetrazolium salt (MTT), 100% DMSO and emetine were used to screen for *in vitro* cytotoxicity against a mammalian cell-line, Chinese Hamster Ovarian (CHO).

Greiner 96-well microtiter plates were used for the AChE assay and HPLC vials used for storage of sample solutions. Blood was collected at the University of the Western Cape from a healthy male volunteer who was not on any medication. Blood was collected in sterile heparin vacutainer tubes (Lasec, South Africa) and stored at room temperature. Blood samples were used for AChE inhibition assays within 30 min of collection.

3.2 Collection and processing of plant material

Plant material was collected near farm Dakota 35 (23°04'119"S; 18°28'813"E) in the Omaheke region (Research/collection permit number: 1869/2014, **Appendix 3**). Samples of the plant were harvested after the rainy season in February 2014, as they were abundantly available at the time. Plants were randomly selected, and uprooted. A voucher specimen of the plant was deposited at the National Botanical Research Institute (NBRI), and correctly identified as *T. laxa* from the family Xanthorrhoeaceae, sub-family Asphodeloideae (National Herbarium of Namibia, identification report number: 2014/348, **Appendix 4**). For consistency, only mature flowers and fresh green leaves were considered. The collected plants were carefully separated into their different parts, roots, rhizomes, leaves, inflorescence, and flowers (**Fig. 3.2**). These were cut into smaller pieces and left to dry at room temperature in a well-ventilated room. During the drying of the plant material it was closely monitored for any microbial growth.

3.3 Preparation of crude extracts

The constituents of the different plant parts were extracted using sequential extractions with solvents of increasing polarity (**Appendix 1**) during each step; starting with

hexane, which was followed by dichloromethane and finally methanol. Hexane (200 mL) was added to 10g of finely ground plant material (leaves, flowers, roots, inflorescence and rhizomes) in separate Erlenmeyer flasks. The flasks were sealed

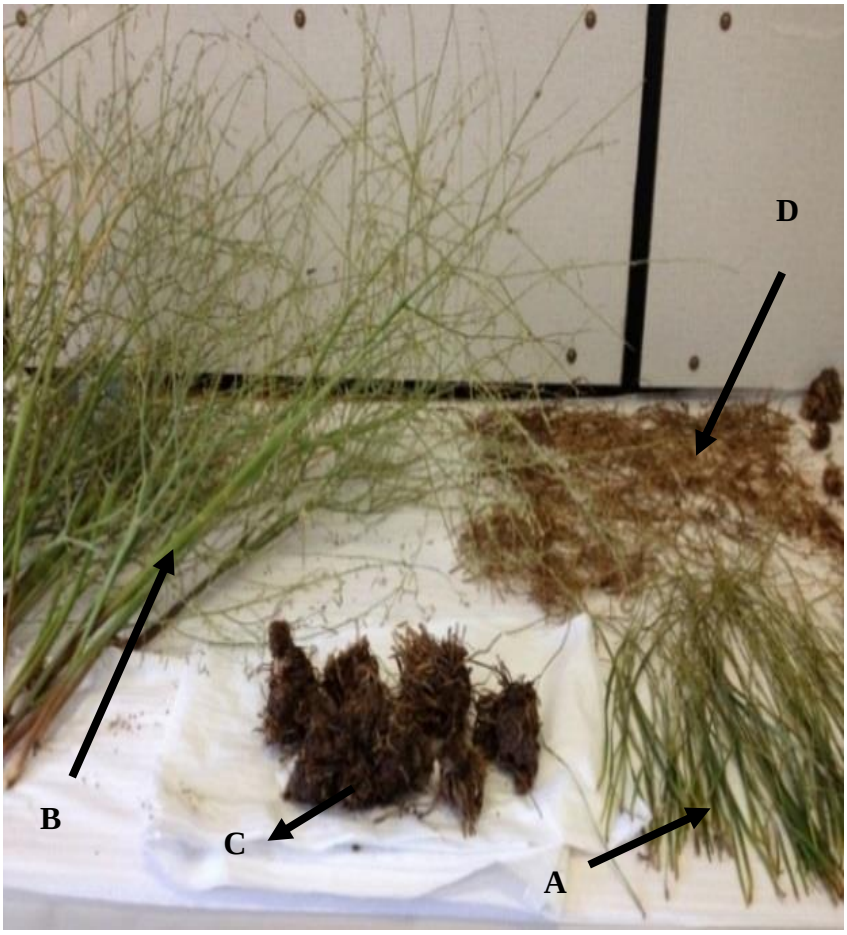


Figure 3.2: The different plant parts of *T. laxa* , (A) leaves, (B) inflorescence, (C) rhizomes and (D) roots, drying at room temperature.

with aluminum foil and placed on an orbital shaker for 24 hours. Extraction was performed at 200 rpm and room temperature (approximately 25°C). A Buchner funnel with filter paper was used to filter each extract and the filtrate was transferred to a 250 mL round bottom flask. The retained dry plant residue was transferred to another Erlenmeyer flask and 200 mL dichloromethane (DCM) was added for the next

extraction. The extraction was performed in the same way as the previous extraction. The resulting dry plant residue was then used for the preparation of the methanol extract, following the same procedure. The filtrates were then concentrated individually in round bottom flasks using a rotary evaporator at approximately 150 rpm under reduced pressure and a water bath temperature of 20-25°C for the DCM extract, 40°C for the hexane extract and 40°C for the methanol extract to a volume below 10 mL. Extracts were then transferred to pre-weighed 10 mL vials. The solvent was then further evaporated in a nitrogen atmosphere in order to avoid loss of volatile compounds and to obtain dry extracts for storage (**Table 3.1**). The dried extracts were then stored at -20°C.

Table 3.1: Yield of crude extracts produced from different plant parts of *T. laxa*

Plant part	Mass of material (g)	Mass of dried extract (g)		
		Hexane	DCM	Methanol
Leaves	10	0.0969	0.2739	0.8735
Flowers	5	0.0919	0.1211	0.4571
Inflorescence	10	0.0934	0.0876	0.7871
Rhizomes	10	0.0689	0.4644	0.8415
Roots	5	0.0249	0.1433	0.4678

3.4 Bioassays

3.4.1 Cytotoxicity assay

Test samples were screened for *in vitro* cytotoxicity against a mammalian cell-line, Chinese Hamster Ovarian (CHO) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays[5]. The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The

test samples were tested in triplicate on one occasion. The test samples were prepared to a 20 mg/ml stock solution in 100% DMSO. Stock solutions were stored at -20ºC. Further dilutions were prepared in complete medium on the day of the experiment. Emetine was used as the reference drug in all experiments. The initial concentration of emetine was 100 µg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/ml. Test samples were tested at 50 µg/ml. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability. The 50% inhibitory concentration (IC_{50}) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

3.4.2 Acetylcholinesterase inhibition assay

In this study, all samples were tested in triplicate. Samples of the hexane, DCM and methanol extracts of the leaves, roots, inflorescence, rhizomes and flowers of *T. laxa* (**Table 3.2**) were screened for general neurotoxicity, using an acetylcholinesterase inhibition assay procedure. Phosphate buffer (0.1 M) was prepared by adding 1.7 g sodium hydrogen phosphate in 100 mL distilled water and the pH was adjusted to 7.5 using a pH meter. Stock solutions of the extracts were prepared in dimethyl sulphoxide (DMSO), at concentrations of 10 mg/mL. A dilution of each extract was prepared by adding 5 µL of the sample to 495 µL phosphate buffer to have final concentrations of 0.1 mg/mL. The positive control consisted of 10 µL of 1 mM chlorpyrifos, an organophosphate insecticide that inhibits acetylcholinesterase, added to 90 µL buffer. As negative control 10 µL DMSO was added to 90 µL buffer.

Table 3.2: Samples of *T. laxa* used in the acetylcholinesterase assay

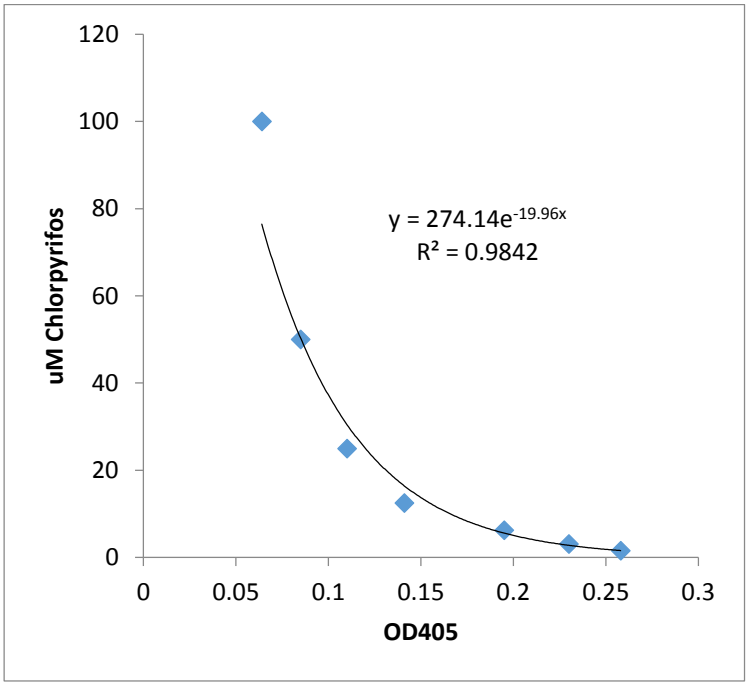
SAMPLE NO	PLANT PART	EXTRACT
1	LEAVES	DICHLOROMETHANE
2	ROOTS	
3	INFLORESCENCE	
4	RHIZOMES	
5	FLOWERS	
6	LEAVES	HEXANE
7	ROOTS	
8	INFLORESCENCE	
9	RHIZOMES	
10	FLOWERS	
11	LEAVES	METHANOL
12	ROOTS	
13	INFLORESCENCE	
14	RHIZOMES	
15	FLOWERS	
BLANK		

Both the positive and negative controls were used to prepare a series of dilutions (**Table 3.3**). An acetylcholinesterase extract was prepared by mixing heparinised human blood, distilled water and 0.1 M phosphate buffer (in a ratio of 3:20:97). A volume of 25 µL of each sample was added in triplicate to a flat bottom 96-well microtiter plate followed by 25 µL of blood solution. A blank was also prepared with no sample which was used to compare enzyme inhibition. The plate was subsequently left to incubate for two hours. Thereafter 50 µL of substrate solution was added to each well containing sample solution. The substrate solution was a mixture of 200 µL 5, 5-dithiobis (2-nitro-benzoic acid) (DTNB), 6 µL phosphate buffer and 40 µL of freshly prepared acetylthiocholine iodide (ATCI). After preparation of the microtiter

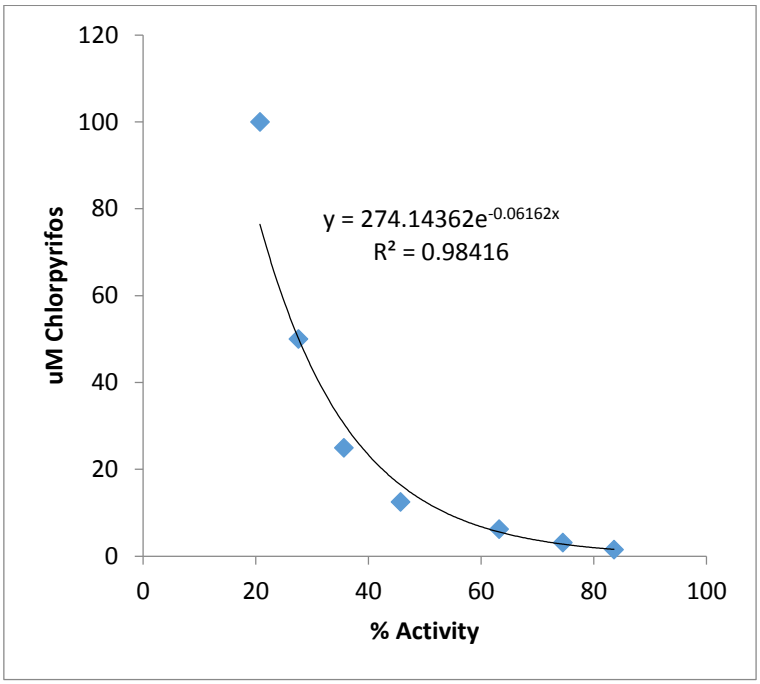
Table 3.3: Template for preparation of the Greiner 96-well microtiter plate.[55]

+ve Inhibition (µM Pyrifos0	-ve Inhibition (% DMSO)	SAMPLES 1-15					
100	10	1	1	1	9	9	9
50	5	2	2	2	10	10	10
25	2.5	3	3	3	11	11	11
12.5	1.25	4	4	4	12	12	12
6.25	0.625	5	5	5	13	13	13
3.125	0.3125	6	6	6	14	14	14
1.5625	0.1563	7	7	7	15	15	15
0	0	8	8	8	BLANK	BLANK	BLANK

plate it was left on a mechanical stirrer to incubate for two hours. A volume of 50 µL of the prepared substrate solution was added to each well and the optical density (OD) was immediately measured at 405 nm and then every 15 minutes for one hour, to check activity (activation or inhibition).[55] Subsequently a graph of difference of OD vs. positive control concentration was constructed.



The positive control, chlorpyrifos, inhibits acetylcholinesterase. From this graph it is noted that a decrease in the chlorpyrifos concentration resulted in an increase in optical density difference. The curve below depicts the percentage enzyme activity in the different concentrations of chlorpyrifos, showing that the lower the chlorpyrifos concentration, the higher the percentage activity. This curve was used to calculate the percentage enzyme activity in the samples.



3.5 Phytochemical Screening

Qualitative chemical tests were carried out on the powdered leaves, inflorescence, rhizomes, roots and flowers of *T. laxa* to detect the presence or absence of selected classes of compounds. Tests were performed in duplicate, based on the methods described by Harborne and Farnsworth.[58] The phytochemical screening was also

carried out with a negative control in order to compare colour changes. Weak coloration was assigned a + while strong coloration was indicated by +++.

(i) Alkaloids

An amount of 5 mL of 2% hydrochloric acid was boiled with 0.2 g of the finely ground plant material for 5 minutes and then filtered. The filtrate was divided into two test tubes, A and B (1 mL in each test tube). Two drops of Dragendorff's reagent was added in tube A and 2 drops of Mayer's reagent in tube B. A red precipitate forming in tube A indicated the presence of alkaloids and a creamy-white precipitate forming in tube B indicated the presence of alkaloids.

(ii) Saponins

A portion of finely ground plant material (1 g) was added to 15 mL of water in a test tube and heated on a water bath for five minutes. The solution was filtered using Whatman No. 1 filter paper and left to cool to room temperature. The filtrate (10 mL) was shaken in a 16x160 mm test tube for ten seconds. The height of the honeycomb froth which persisted was measured. Froth higher than one centimeter confirmed the presence of saponins.

(iii) Anthraquinones

Finely ground plant material (1 g) was extracted with ether-chloroform (1:1) for 15 minutes. It was then filtered using Whatman No.1 filter paper and 1 mL of the filtrate treated with 1 mL of 10% sodium hydroxide solution. A red coloration indicated the presence of anthraquinones. A weak coloration was assigned + while a strong/intense coloration was assigned +++.

(iv) Anthranoids

Finely ground plant material (0.2 mg) was boiled for two minutes with 1 mL 0.5 N potassium hydroxide and 0.5 mL of 5% hydrogen peroxide. After cooling the mixture was filtered and the filtrate treated with 6 drops of acetic acid and the resulting solution mixed with 5 mL of toluene. The toluene (upper layer) was separated with a pipette and transferred to a test tube, to which 2 mL of 0.5 N potassium hydroxide was added. If a red color appeared in the aqueous layer, it indicated the presence of anthranoids.

(v) Terpenes

Chloroform (3 mL) was added to 0.5 g finely ground plant material. The mixture was shaken and filtered using Whatman No. 1 filter paper. Ten drops of acetic anhydride were added to the filtrate followed by two drops of concentrated sulphuric acid. A reddish brown coloration at the interface was looked out for to show a positive result for the presence of terpenes.

(vi) Polyphenols

Finely ground plant material (1 g) was added to 15 mL water and heated on a water bath at temperature 65°C for fifteen minutes. The mixture was then filtered using Whatman No. 1 filter paper. Three drops of ferric cyanide solution (1 mL of 1% $FeCl_3$ and 1 mL of freshly prepared potassium ferricyanide) to 2 mL of the filtrate. The formation of a blue-green colour indicated the presence of polyphenols.

(vii) Flavonoids

One gram of finely ground plant material was mixed with 1 0 mL water and 5mL

methanol. The mixture was filtered and a few magnesium turnings were added to

3 mL of the filtrate. Concentrated hydrochloric acid was then added drop-wise (cyanide reaction). Development of colors indicates the presence of flavonoids: flavones = orange, flavonols = red color and flavanones = pink color.

(viii) Leucoanthocyanins

A portion of 1 g of finely ground plant material was put in 20 mL water, 0.5 mL of the mixture was mixed with 2 mL of 2 N hydrochloric acid and heated on a water bath. A slow development of red to violet color is indicative of a positive reaction. Faint coloration is indicated as +, medium coloration as ++ and strong/intense coloration is indicated as +++.

(ix) Tannins

A portion of 2 g of finely ground plant material was added to a test tube with 15 mL water and heated on a water bath at temperature of 65°C for five minutes. After cooling the mixture was filtered to remove any suspension and 5 mL of a 2% sodium chloride solution was added. Then 5 mL of 1% gelatin was added. Precipitation confirmed the presence of tannins.

3.6 GC-MS Analysis

GC-MS analysis were performed on a Thermo Scientific Focus GC coupled to an ITQ 700 MS. The flow rate of the carrier gas, helium, was kept constant at 1.0 mL/min and a split ratio of 10 was used. A SGE BP5MS capillary GC column (30 m x 0.25 mm i.d), with a 5% diphenyl, 95% dimethyl polysiloxane stationary phase (0.25 μm film thickness) was used for the separations. The GC injector temperature was maintained at

220°C. Ion source and interface temperatures of 200 and 250°C, respectively, were used for the analysis. The oven was programmed at a rate of 2°C/min from 40 to 300°C to ensure optimal separation of the volatile constituents.[62] Electron ionization-Mass Spectrometry (EI-MS) data was acquired at 70 eV and a mass range of *m/z* 25 to 600 was scanned. Data acquisition and processing was performed using Xcalibur Software, version 2.1. A mixture of alkanes (C_{10}-C_{40}: all even numbers of carbons) was initially analysed under the same conditions for experimental retention index (RI) determination.

Samples were prepared for GC-MS analysis by dissolving each extract in DCM with concentrations of 11.1 mg/mL for the leaf DCM extract, 11.8 mg/mL for the inflorescence DCM extract, 10.0 mg/mL for the flower DCM extract. A volume of 1 µL of each solution was analysed. Since the EI mass spectra of the compounds detected in the analyses did not contain peaks above *m/z* 500, only a mass range of *m/z* 25–400 is displayed. Similarly, no peaks were observed after 180 minutes in the total ion chromatograms and hence the chromatograms are only displayed up to a retention time of 180 minutes.

3.7 Retention index determination

Constituents of the analysed samples were tentatively identified based on their MS data and their retention indices (RIs) where the RI of a compound is its retention time (tR) normalised to the retention times of closely eluting *n*-alkanes. Although retention varies between individual chromatographic systems, the derived RIs are independent of these parameters. This allows the comparison of values measured by different analytical laboratories under varying conditions, thus experimentally determined RI

values can be compared to published RIs in order to aid in the confirmation of compound identities.[59] The mixture of alkanes (C_{10}-C_{40}) and sample solutions were analysed under the same conditions. Subsequently linear Kővats RIs of the compounds from *T. laxa* were calculated using the following equation:[61]

$$I = 100\left[n + (N - n)\frac{\log t_r'(unknown) - \log t_r'(n)}{\log t_r'(N) - \log t_r'(n)}\right]$$

Where: *I* is the retention index

N is the number of carbon atoms in the larger alkane

n is the number of carbon atoms in the smaller alkane

$t_r'(unknown)$ is the adjusted retention time of the unknown

$t_r'(n)$ is the adjusted retention time of the small alkane

$t_r'(N)$ is the adjusted retention time of the bigger alkane

t_r' is the adjusted retention time $= t_r - t_m$, where $t_r$is the actual retention time and t_m is the time that it takes for un-retained compound to move through the column

CHAPTER 4: RESULTS AND DISCUSSION

As the nature of plant poisons is seldom known, it makes the detection of poisons a very difficult part of the analytical toxicology process.[7] A general screening approach is thus essential for the investigation of toxins which are unknown. The assay-guided analytical approach was employed in this study whereby hexane, DCM and methanol extracts of the leaves, roots, inflorescence, rhizomes and flowers of *T. laxa* were subjected to cytotoxicity and neurotoxicity assays. Thereafter, phytochemical screening was performed in order to determine which major chemical classes would test positive in the different extracts. This was done as a guiding step to subsequent characterisation of the major metabolites in *T. laxa*. Subsequently, GC-MS was employed in the characterisation of the major organic compounds found to be present in search for the toxic principles.

4.1 Toxicity determination

Preliminary cytotoxicity tests on all the *T. laxa* samples (leaf, root, inflorescence, rhizome and flower) were performed in order to identify those displaying cytotoxicity before determining the IC_{50}. These results are displayed in **Table 4.1**. None of the samples revealed cytotoxicity to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay at concentrations < 50 µg/mL. Concentration values above this are not measured as one starts seeing non-specific effects. Hence, the IC_{50} values must be regarded as >50 µg/mL. Perhaps this is not surprising, since ingestion of *T. laxa* is known to affect the CNS. Reports indicate progressive posterior paresis and paralysis in cattle, sheep, horses and pigs on consumption of *Trachyandra* species[1], therefore a toxic principle that would affect the

CNS would presumably have acetylcholinesterase inhibition. Hence, it was deemed necessary to test the extracts for neurotoxicity. Consequently, the AChE inhibitory activity of the extracts was tested.

Table 4.1: IC_{50}-values of samples tested for cytotoxicity

VIAL Number	**Estimated IC_{50} (µg/ml)**
1*A	>50
1**A	>50
1***A	>50
1****A	>50
2*A	>50
2**A	>50
2***A	>50
2****A	>50
4*A	>50
4**A	>50
4***A	>50
5*A	>50
5**A	>50
5***A	>50
5****A	>50
10*A	>50
10**A	>50
10***A	>50
10****A	>50
Emetine	0.084 µg/ml

Key: 1-Leaves; 2-Roots; 4-Inflorescence; 5-Rhizomes; 10-Flowers
*DCM extraction **Hexane extraction ***Methanol extraction
****Acetone extraction

The *in-vitro* AChE inhibition assay was performed on the different extracts by measuring optical densities (ODs) at different time intervals to monitor the change in acetylcholine. The preparation was left to incubate for a period of two hours after the substrate was added. Subsequently the OD values were measured every 15 minutes for a period of 60 minutes.

The results in **Figure 4.1** indicate that high enzyme activities are observed for the DMSO blank (negative control) as well as a number of samples, since no inhibition of AChE occurs. On the other hand, the lowest enzyme activities are observed for samples 1, 3 and 4, hence they are the ones that inhibit AChE the most. These samples are the DCM extracts of the leaves and roots.

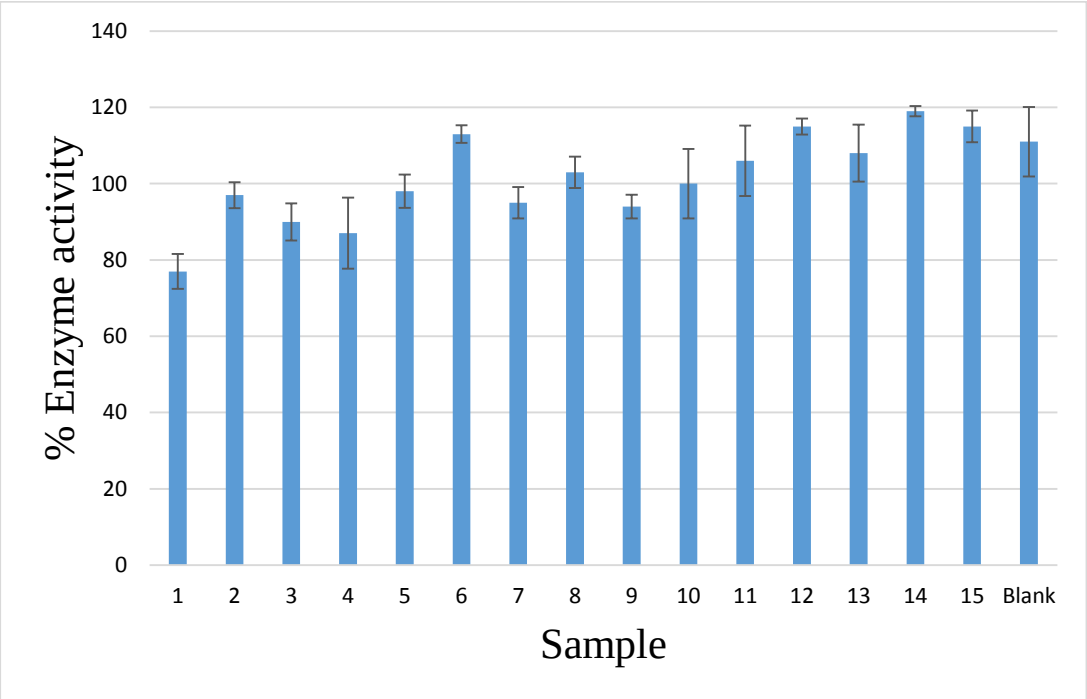


Figure 4.1: Percentage AChE activity of the samples.

4.2 Phytochemical Screening

Phytochemicals are bioactive compounds of which the most important constituents in plants are alkaloids, tannins, flavonoids and phenolic compounds.[64] They are also known to exert therapeutic effects on many pathological conditions by modulating diverse signaling pathways.[65] For instance, extracts of Fumariaceae, Papaveraceae and Ericaceae families have shown strong inhibition of AChE which could be attributed to their high alkaloid content.[27] In this study phytochemical screening was performed as a guiding step to identify the classes of compounds that are abundant in the different parts of the plant. Finely ground plant material (leaves, inflorescence, flowers, roots and rhizomes) were screened separately for the presence of the classes of the compounds listed in **Table 4.2**. All the plant parts except the flowers tested positive for saponins. Leaves, seeds and sprouts of the *Aesculus hippocastacanum* (Sapindaceae) plant have been found to be toxic due to the presence of glycoside aesculin and saponin aescin. Symptoms were muscle weakness and paralysis, among others. Anthraquinones were found to be present in all the plant parts except for the roots, while the leaves were the only plant part which tested positive for flavonoids (**Table 4.2)**. In fact, the presence of anthraquinones have been reported to be a common characteristic of the Xanthorrhoaceae family.[65] All the plant parts tested positive for polyphenols and tannins. In the test for tannins no precipitate formed. However, upon the addition of 10 drops of 1% $FeCl_3$ a colour change was observed. Tannins present various pharmacological activities. Several tannins, particularly the hydrolysable tannins, act as antioxidants in cellular pro-oxidant states and inhibit different enzymatic activities by precipitating proteins and peptides.[66] They generally induce a negative response when ingested by animals as they act as a defense

mechanism in plants. It was reported by Wink and Van Wyk that most saponins and anthraquinones are toxic as well as some tannins (see **Appendix 2**). The most abundant phytochemicals were found to be saponins, anthraquinones, tannins and polyphenols, while alkaloids, anthranoids, cardenolides, terpenes and leucoanthocyanins were not detected in any plant parts (**Table 4.2**).

Table 4.2: A summary of the phytochemical screening results for the finely ground plant material for the different plant parts of *T.laxa*

	LEAVES	INFLORE-SCENCE	RHIZOMES	ROOTS	FLOWERS
ALKALOIDS	-	-	-	-	-
SAPONINS	+++	+++	++	+++	-
ANTHRAQUINONES	+	++	++	-	+
ANTHRANOIDS	-	-	-	-	-
CARDENOLIDES	-	-	-	-	-
TERPENES	-	-	-	-	-
POLYPHENOLS	+	+	++	++	+++
FLAVONOIDS	+	-	-	-	-
LEUCOANTHOCYANINS	-	-	-	-	-
TANNINS	++	+	+++	++	+++

Key: - not detected; + present in small quantities; ++ present in medium quantities; +++ present in large quantities.

4.3 Identification of the major volatile constituents in the DCM extracts of T. laxa plant material by GC-MS

Based on the results of the phytochemical screening and the acetylcholine inhibition analysis, the DCM extracts of the aerial parts of the plant were considered for further investigation. However, since the leaves are the plant part that is most abundant and accessible to animal consumption, only the DCM extract of this plant part was studied. A solution of the DCM extract of the leaves of *T. laxa* (10 mg/mL) was analysed by GC-MS .

Compounds were tentatively identified by comparing their mass spectra to those reported in literature and those in the National Institute of Standards and Technology (NIST) 11 mass spectra database as well as the experimentally determined retention indices (RIs) with RI values reported in literature, seeing to it that they do not differ by more than 10 index units (provided that the authors reporting the values determined the RIs experimentally using authentic reference standards). Using this identification strategy, 14 compounds (**Figure 4.2**) were tentatively identified, which included lactones, alcohols, alkanes, esters, a diterpene and a phytosterol (**Table 4.3**). A detailed account of how individual compounds were identified is presented in the following subsections.

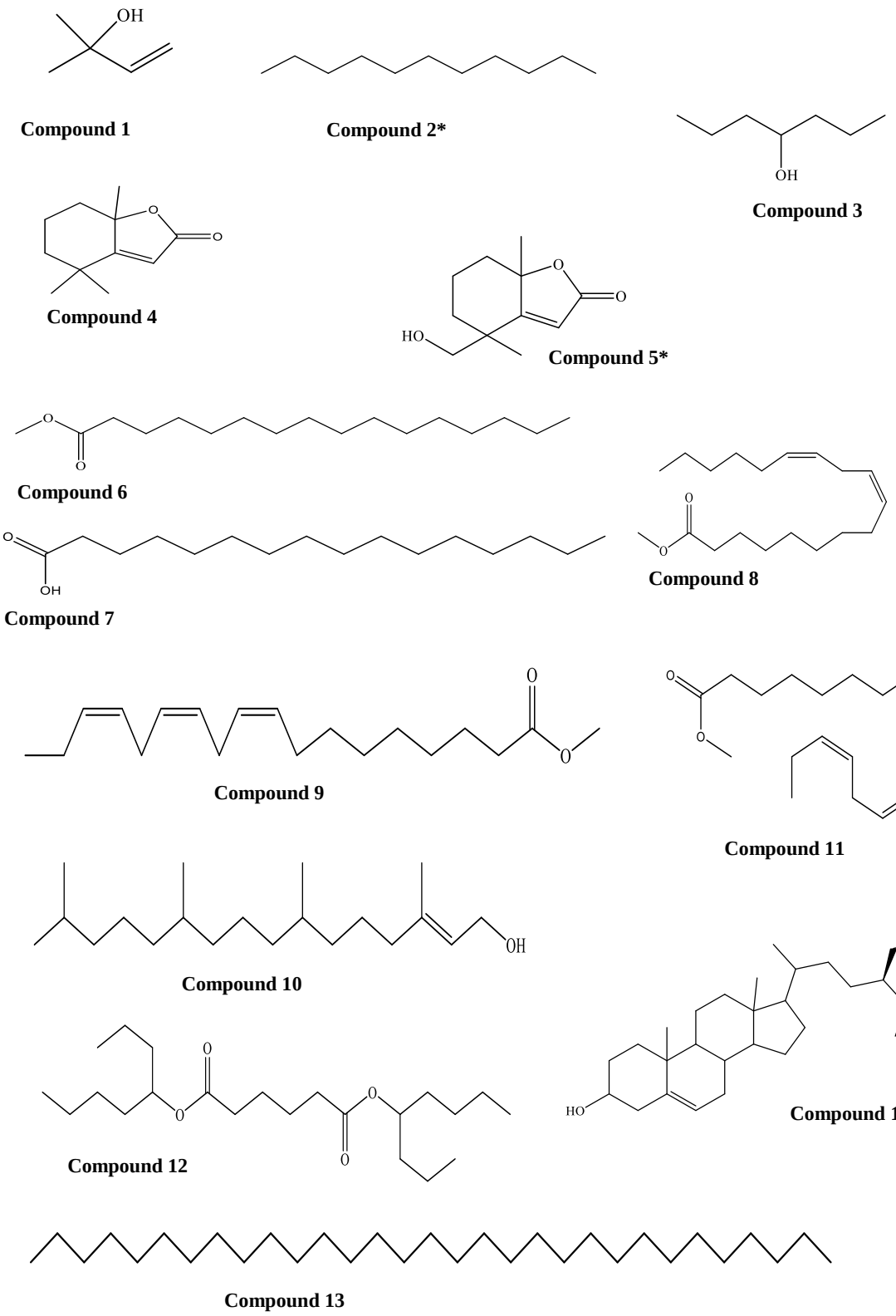
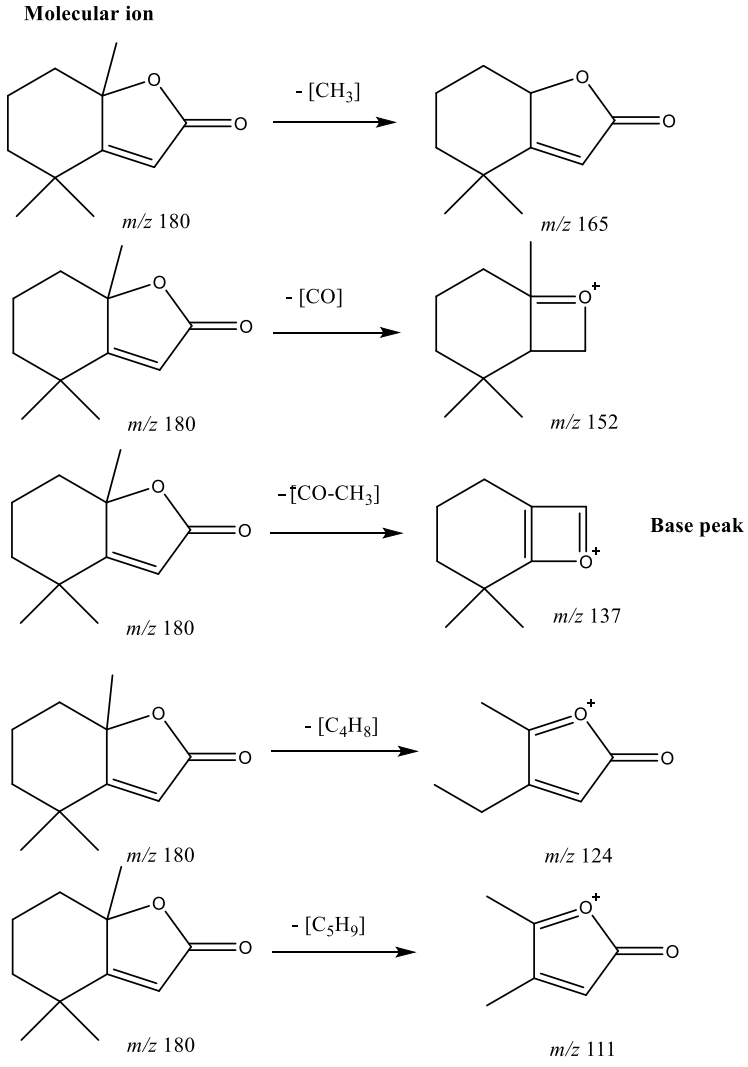


Figure 4. 2: Structures of the 14 identified compounds (*proposed structures).

4.3.1 Dihydroactinidiolide and its hydroxylated analogue

Comparison of the mass spectrum of compound **4** (**Figure 4.14**) to those in the NIST MS database, revealed that this compound may be the α,β-unsaturated lactone, dihydroactinidiolide (DHA). Hence it was assumed that the ion observed at *m/z* 180 is the molecular ion and that the base peak at *m/z* 137 corresponds to the fragment ion $[M\text{-}CH_3CO]^+$.[44] Other prominent peaks corresponding to the fragment ions $[M\text{-}CO]^+$, $[M\text{-}C_4H_8]^+$, and $[M\text{-}C_5H_9]^+$ were observed at *m/z* 152, 124 and 111, respectively. These peaks were also reported by Chen and co-workers to be present in the mass spectrum of dihydroactinidiolide:[67]



The RI was experimentally determined to be 1516 while the closest RI value reported in the literature is 1529.[68] Based on this analysis, compound **4** was tentatively identified as DHA. It is an α,β-unsaturated lactone, commonly found in nature as a degradation product of carotenoids. When β-carotene is cleaved it gives rise to various compounds like β-ionone, 5,6-epoxy-β-ionone and DHA.[66,69] The pathway of DHA production, proposed by Bosser et. al.[69] appear in **Figure 4.3**. They have reported that DHA is a potent AChE inhibitor and thus a potential candidate for anti-Alzheimer's disease (AD) therapy.[69] In a study conducted by Das et al.[70] DHA and its anti-AD potential was investigated and it was found that DHA, synthesized from β-ionone was a potent AChE inhibitor with IC_{50} value of 34.03 nM. The cytotoxicity and hemolysis profiles of DHA were also found to be safe, thus it could be safely used for the treatment of AD. In addition, it was reported that DHA is responsible for the aroma of black tea and tobacco. DHA displays free radical scavenging, antioxidant activity as well as anticancer activity against four human tumour cell lines. In a study of the leaves of *Vallisneria. Spiralis* (family Hydrocharitaceae), dihydroactinidiolide, an important flavour component from tobacco, tea, coffee, juice, and macrophytes was also found to be an active allelochemical which could strongly inhibit the growth of algae and other aquatic macrophytes.[71] Of interest to this study are reports that α,β-unsaturated lactones have been reported to be toxic to animals.[3]

The mass spectrum of compound **5 (Figure 4.15)** has a similar fragmentation pattern to that of compound **4**, but with prominent ions at e.g. *m/z* 178 and *m/z* 135, instead of *m/z* 180 and *m/z* 137, respectively. If it is assumed that the ion observed at *m/z* 196, is the molecular ion, then the ion at *m/z* 178 is the $[M-H_2O]^{+\bullet}$ ion and hence it may be

inferred that compound **5** is a hydroxylated analogue of dihydroactinidiolide (compound **4**). The ion observed at *m/z* 163 will then correspond to a $[M-CH_3-H_2O]^+$ ion, while the base peak, observed at *m/z* 135, will then be formed by the loss of CO from the ion observed at *m/z* 163.

The RI of compound **5** was experimentally determined to be 1751. If the group contribution method for the estimation of the Kováts RI, as reported in the article by Stein et al.[71] is used, the experimental RI value of compound **5** can be explained by using the RI value of DHA and subtracting group increments, i.e. 1516 minus 112 ($-CH_3$) plus 99 (CH_2) plus 225 (1-OH). This brings us to a RI value of 1758, which is within 10 units of its experimentally determined value. Based on this analysis, we can tentatively identify compound **5** as a hydroxylated analogue of dihydroactinidiolide, with the hydroxyl group located on a methyl group. The compounds loliolide, aeginetolide, dihydroactinidiolide and actinidiolide (**Figure 4.4**) are structurally similar C11-terpene lactones which arise from biological or oxidative degradation of carotenoids.

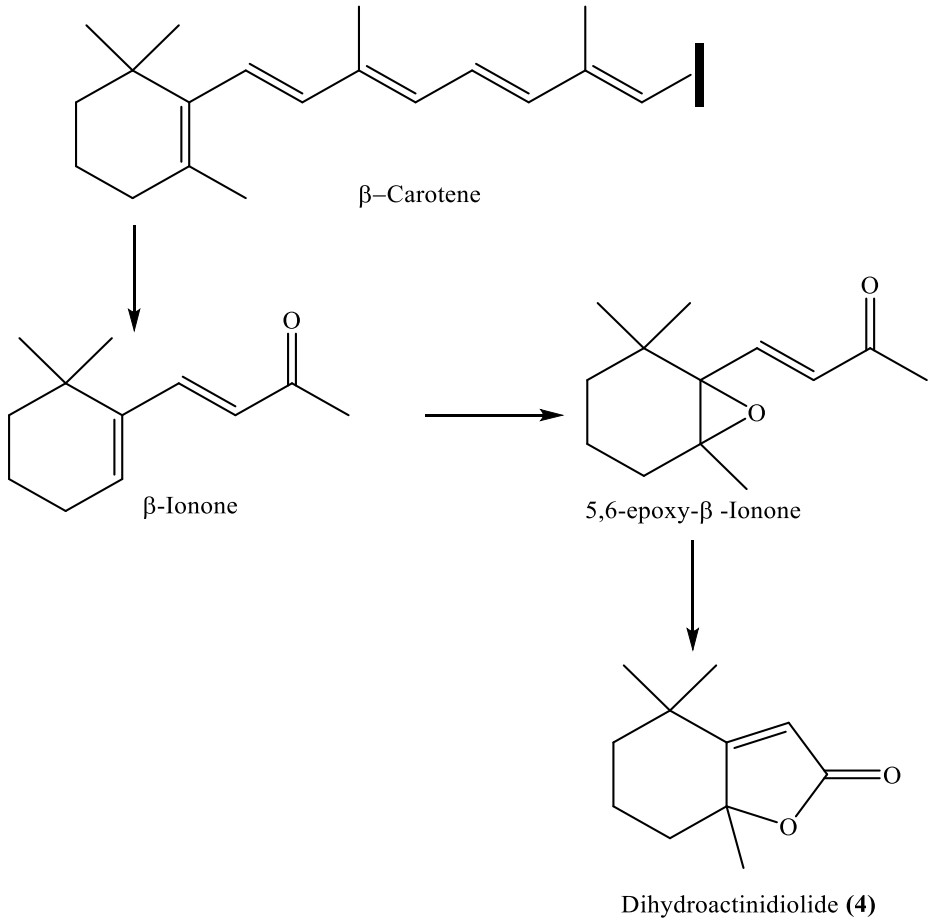


Figure 4.3: The pathway of the formation of dihydroactinidiolide from β-carotene.[69]

Loliolide is found in a number of plants, including tobacco and tea while dihydroactinidiolide and actinidiolide have been identified as flavor molecules in tea and tobacco. DHA has also been reported to be a very active allelochemical which could strongly inhibit the growth of many aquatic macrophytes, especially algae.[71]

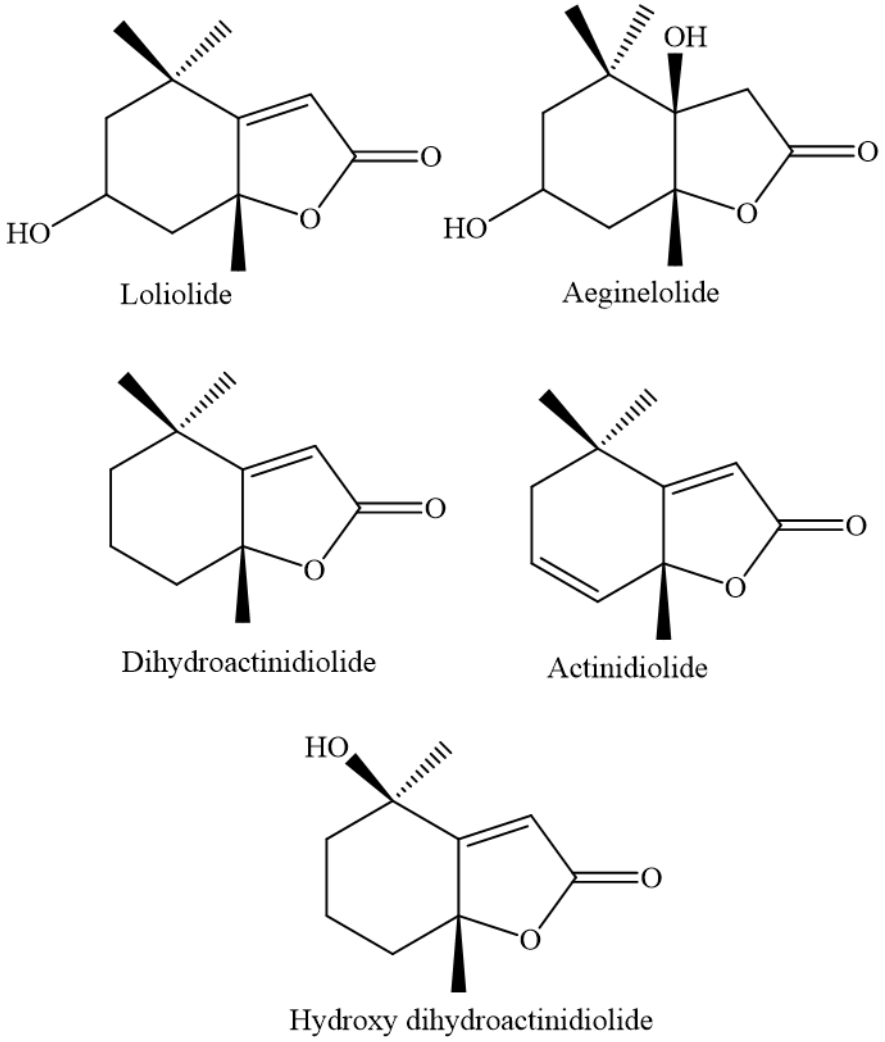
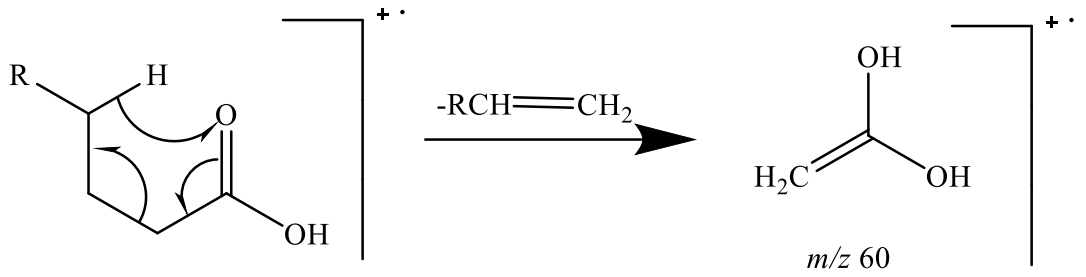


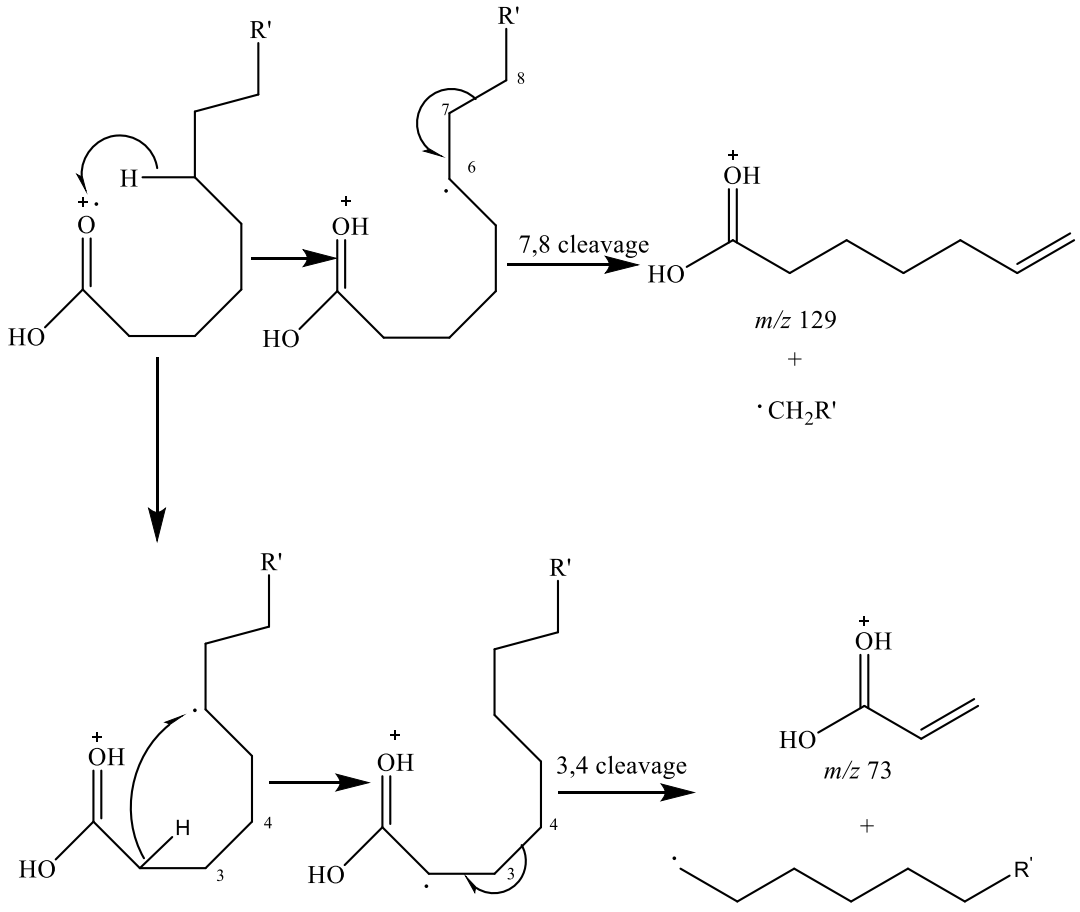
Figure 4.4: A series of structurally similar C11-terpene lactones.[70]

4.3.2 Hexadecanoic acid and its methyl ester

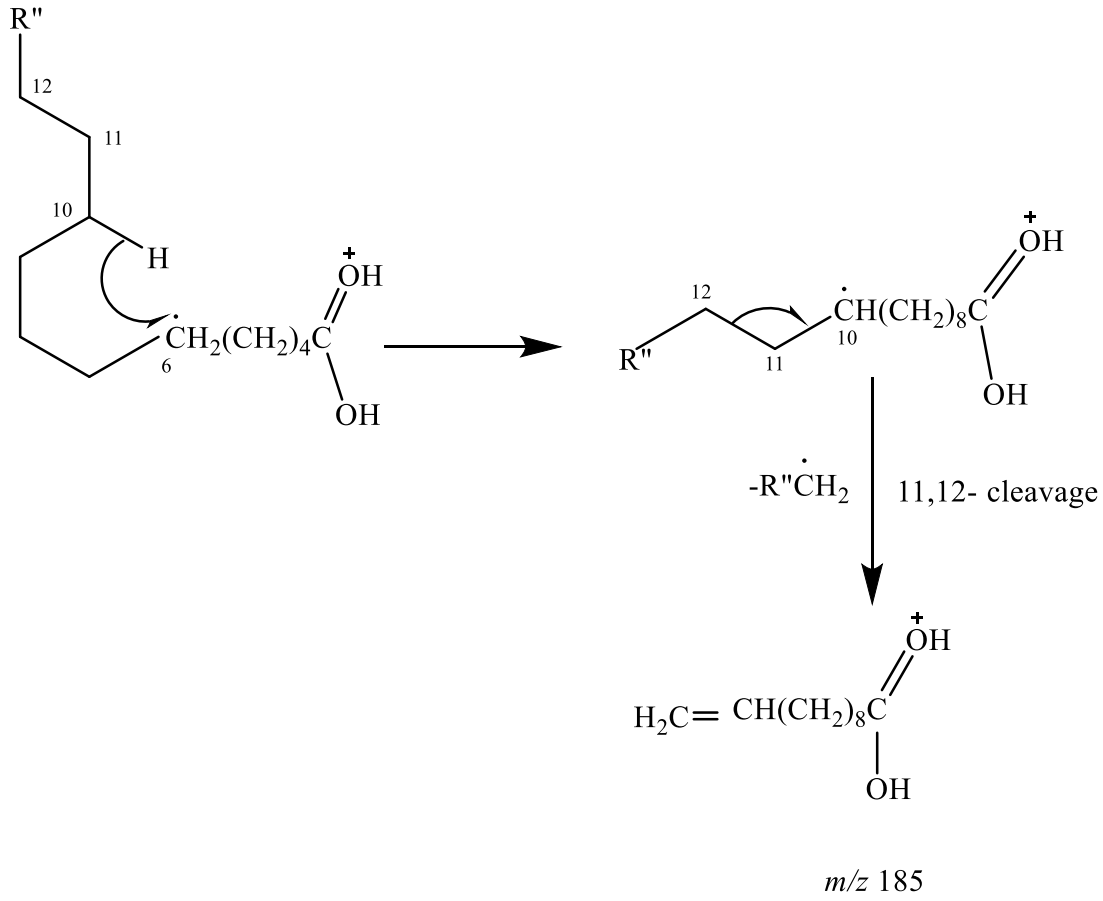
The EI mass spectra of compounds **7** and **8**, identified as methyl hexadecanoate and hexadecanoic acid, contain prominent ions at *m/z* 55 and 87 (**Figures 4.17** and **4.18**). These ions are characteristic of the mass spectra of long chain carboxylic acids. In addition, an ion at *m/z* 60 indicates that the carboxylic acid is not α-branched (compound **8**). After ionization of a carboxylic acid in the mass spectrometer, a McLafferty rearrangement takes place, resulting in the following ion:



Long chain fatty acids yield stable ions of the general formula $[CH_3(CH_2)_nCOOH]^+$, with n = 2, 6, 10, etc. Strong peaks thus occur at *m/z* 73, 129 and 185 in these spectra. A hydrogen radical is transferred from C6 to the ionized oxygen, after which the 7,8 bond is cleaved to form the ion at *m/z* 129. Another alternative is that after the said hydrogen radical transfer, a further hydrogen radical is transferred from C2 to C6, after which the C3,C4-bond is cleaved to form the ion at *m/z* 73.[44]

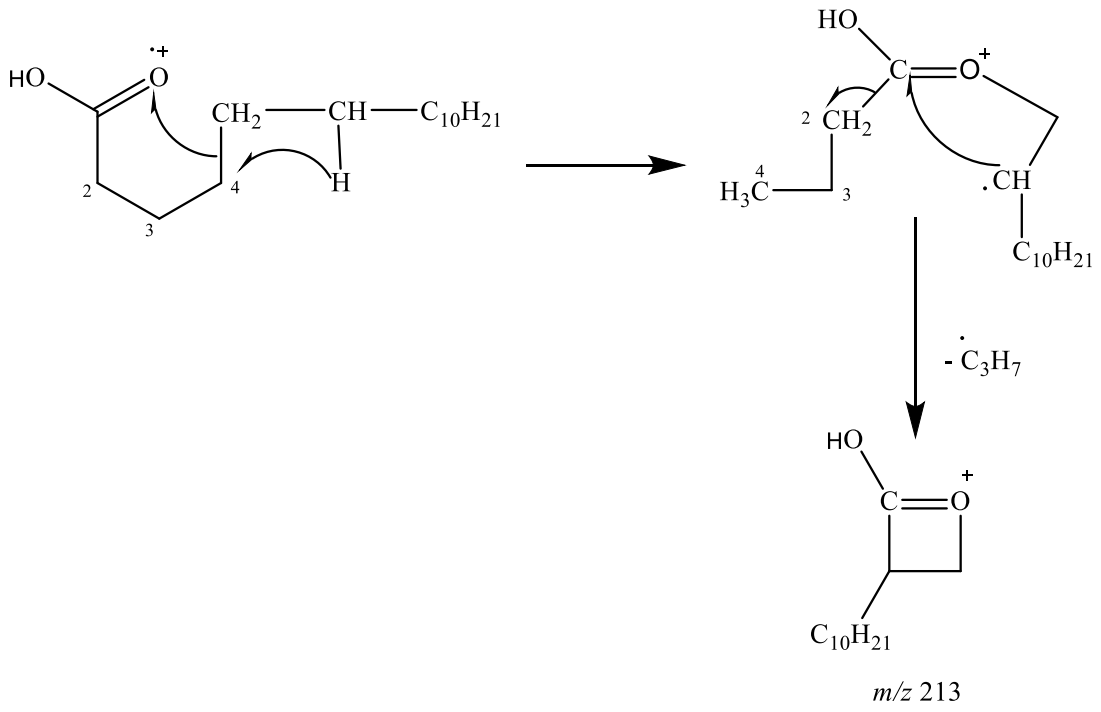


The ion at *m/z* 185 is formed by cleavage of the C11, C12 - bond, after a radical is formed on C10.



m/z 185

In these spectra, the (M-43) ion is also prominent, for example at *m/z* 213 in the mass spectra of methyl hexadecanoate and hexadecanoic acid (compounds **7** and **8**). These ions are formed by the elimination of C2, C3 and C4, together with an additional hydrogen atom.[44]



Prominent molecular ions are also apparent in the mass spectra of the aliphatic carboxylic acids. Taking into account the M-43 ion, it can be deduced how many carbon atoms the acid contains. By comparison with the NIST MS database and the NIST RI database, compound **7** and **8** were therefore tentatively confirmed to be methylhexadecanoate and hexadecanoic acid. The calculated RI values of compounds **7** and **8** were 1927 and 1974, which matched with those found in literature as 1924 and 1968 respectively, thus comfortably confirming them to be methylhexadecanoate and hexadecanoic acid respectively.[56]

Methylhexadecanoate has been reported to cause autolysis of membranous structures, inhibit phagocytic activity and induce aortic dilation.[74] Sermakkani and co-workers reported hexadecanoic acid to have antioxidant, hypochloesterolemic, nematicide, pesticide, antandrogenic, flavor, and haemolytic, and 5-alpha reductase inhibition properties. They also reported methylhexadecanoate having antioxidant,

hypocholesterolemia, pesticide, flavour, and haemolytic, 5α reductase inhibition properties.[75]

4.3.3 Unsaturated carboxylic acid esters

The EI mass spectrum of compound **9** (**Figure 4.19**) showed the molecular ion peak at *m/z* 294. A base peak was observed at *m/z* 67. Other prominent peaks were observed at *m/z* 95, 79 and 55. The experimentally determined RI value of compound **9**, which was calculated to be 2090, was found to be very close to one of the values reported in the literature, i.e. 2091.[55] Therefore, compound **9** was tentatively identified as methyl linoleate. The mass spectra of compound **10** (**Figure 4.20**) and **12** (**Figure 4.22**) closely matched those of methyl linolenate, an omega-3 fatty acid ester, and methyl (ZZZ)-9,12,15-octadecantrienoate in the NIST-MS database, respectively. The ion observed at *m/z* 149 in the mass spectra of compounds **10** and **12** is assumed to be formed from the hydrocarbon tail of the molecule involving β-cleavage (**Figure 4.5**). A base peak was observed at *m/z* 79 in both cases and can be ascribed to the presence of hydrocarbon ions of the general formula $[C_nH_{2n-5}]^+$. The peak at *m/z* 108, which is an omega ion, is a diagnostic peak for methyl ester of the fatty acids of the n-3 family.[75] Rout et al.[77] have successfully identified methyl linoleate and methyl linolenate from *Murrary paniculata* (Jack flowers) (Rutaceae). They reported RI values of 2092 and 2096 respectively, which compared well to the experimentally determined RI values of 2090 and 2095 calculated in the study of *T. laxa*. Thus compounds **9** and **10** could comfortably be identified as methyl linoleate and methyl linolenate, respectively. Methyl linolenate has been reported to have hypocholesterolemic, nematicide, anitarthric, hepatoprotective, antiandrogenic, 5-alpha reductase inhibition,

anticoronary, insectifuge and antieczemic properties.[76] Salah et al. [78] also reported anti-inflammatory and anti-atherogenic properties of methyl (ZZZ)-9,12,15-octadecantrienoate. Sermakkani and co-workers reported on its cancer preventative, antihistaminic and antiacne activity.[75] As reported by Hehana et al.[80] Ganesh and co-workers reported methyl (ZZZ)-9,12,15-octadecantrienoate to have anti-inflammatory, hypocholesterolemic, cancer preventative, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary and insectifuge properties.

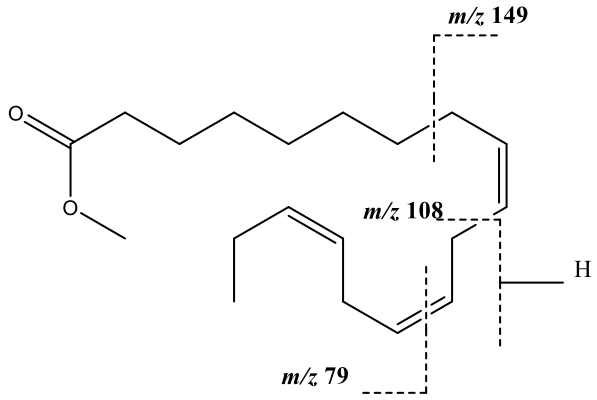


Figure 4. 5: Structure and characteristic fragmentations of compound 10, tentatively identified as methyl linolenate in the DCM extract of the leaves of *T.laxa*.

4.3.4 Alcohols

A comparison of the mass spectrum of compound **1** (**Figure 4.11**) to those in the NIST MS database, revealed it to be an alcohol, 2,3-dimethyl-3-buten-2-ol. The expected molecular ion peak (*m/z* 100) is not clearly observed as alcohols lose water readily upon ionization. The base peak observed at *m/z* 85 in the mass spectrum of this compound corresponds to $[M\text{-}CH_3]^+$. Other prominent peaks were observed at *m/z* 67, corresponding to a loss of water and a methyl group. Peaks observed at *m/z* 59 and 41

corresponds to the radical cations with the formulas $[C_3H_7O]^+$ and $[CH_3CCH_2]^+$, respectively (**Figure 4.6**).

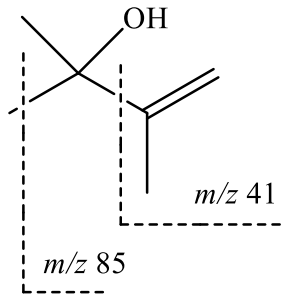


Figure 4.6: Chemical structure of 2,3-Dimethyl-3-buten-2-ol tentatively identified in the DCM extract of *T. laxa* and its characteristic fragmentations.

The EI mass spectrum of compound **3** (**Figure 4.13**), was compared with the NIST data base and tentatively identified as an unknown alcohol. Its mass spectrum showed a base peak at *m/z* 55 in addition to prominent peaks at *m/z* 74 and 41. The base peak at *m/z* 55 corresponds to $[M\text{-}CH_3\text{-}CH_2\text{-}CH_2]^+$ and the peak at *m/z*, 74 resulted from the addition of CH_2 moieties, which is characteristic of *n*-alkane fragmentations. The peak at *m/z* 98 could be mistaken as the molecular ion but it is formed due to the loss of H_2O, resulting in the $[M\text{-}H_2O]^+$ ion that is observed. The fragmentation pattern of alkanes is characterised by clusters of peaks, 14 mass units (CH_2) apart.[44] Based on the comparison of its experimentally determined RI value of this compound with those in the NIST RI database, compound **3** was tentatively identified as an unknown alcohol.

The mass spectrum of compound **11** (**Figure 4.21**) was compared to those in the NIST MS database which revealed that this compound may be 3,7,11,15-tetramethyl-hexadecen-1-ol. The EI mass spectrum of compound **11** exhibits a base peak at *m/z*

81 in addition to other prominent peaks at *m/z* 43, 71, 95 and 123 [M-CH_2]. Rout and co-workers have successfully identified compound **11** from *Murrary paniculata* (Jack flowers), (Rutaceae) and reported an RI value of 1949.[77] The experimentally determined RI value in this study was 2111. This is a good match compared to the 2116 RI value reported in literature.[56] Compound **11** was therefore tentatively identified as 3,7,11,15-tetramethyl-2-hexadecen-1-ol. Sermakkani et al.[75] and Ganesh [79] have reported antimicrobial, anticancer, cancer preventative, diuretic and anti-inflammatory activity of this compound. It has also shown to display anti-inflammatory activity.[78,82,83]

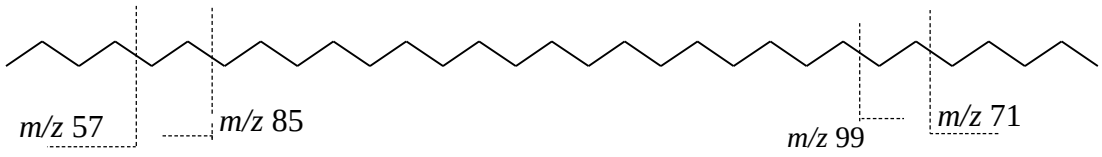
The mass spectrum of compound **6** (**Figure 4.16**) was compared to those in the NIST MS database revealed it to be a mono-unsaturated alcohol. Its mass spectrum displayed a base peak at *m/z* 95 and other prominent peaks at *m/z* 250, 109, 81 and 67 which clearly displays fragmentation pattern of alkanes, i.e. clusters of peaks, 14 mass units (CH_2) apart. Subsequently compound **6** was tentatively identified as a mono-unsaturated alcohol. The experimentally determined RI value was found to be 1841. Thus compound **6** could only tentatively be identified as a mono-unsaturated alcohol.

4.3.5 Alkanes

Comparison of the mass spectrum of compound **2** (**Figure 4.12**) to those in the NIST database, revealed that this compound may be an alkane. Although the mass spectrum of compound **2** matched well with a number of alkanes in the NIST MS database, a good match could not be found in the NIST RI database. Since its experimental RI value of 1019 is more than 10 units higher than that of decane (ie. 1000), compound **2**

could only be tentatively identified as a branched undecane, because a branched C_{11} alkane is expected to have a RI value between those of decane and undecane (1000<RI<1100).

The EI mass spectra of compound **13** (**Figure 4.23**) showed a base peak at *m/z* 57 in addition to prominent peaks at *m/z* 71, 85, 43 and 99. Based on the comparison of their retention times to the alkane series of (C_{10}–C_{40}) and the experimentally determined RI value of compound **13** to that in the NIST-RI database, it was definitely identified as a long chain alkane. The base peak is at *m/z* 57 $[M\text{-}CH_3\text{-}CH_2\text{-}CH_2]^+$ and the peaks at *m/z* 71, 85 and 99 resulted from the addition of a CH_2 moiety, which is characteristic of *n*-alkane fragmentation. Comparison of the experimentally determined RI of compound **13** with those in the NIST RI database confirmed that this compound is most definitely hentriacontane, a C_{31} alkane.



n hentriacontane

Figure 4.7: Chemical structure of hentriacontane identified in the DCM extract of *T.laxa* and its characteristic fragmentations.

Plants produce different types of *n*-alkanes, with the odd alkanes dominating. Due to the fact that long *n*-alkanes are stable and long lived, they contribute to the hydrophobic properties of the leaf wax and function as part of the plant's first barrier from the external environment thus guarding the leaf from water loss via evaporation.[81]

4.3.6 Phytosterol

Comparison of the mass spectrum of compound **14** (**Figure 4.24**) to those in the NIST MS database, revealed that this compound may be β-sitosterol. The EI mass spectrum of compound **14** suggest a molecular ion peak at *m/z* 414. This spectrum showed fragment ions at *m/z* 396 $[M–H_2O]^{+\cdot}$, 381 $[M–CH_3–H_2O]^+$, 329 $[M–C_6H_{13}]^+$, 303 $[M–C_7H_{11}O]^+$, 273 $[M–\text{side chain}]^+$, 255 $[M–\text{side chain}–H_2O]^+$, 231[M–side chain–ring D cleavage–CH_3]$^+$ and 213 [M–side chain–ring D cleavage–CH_3–H_2O]$^+$ (**Figure 4.8**). Based on the MS data, comparison of the RI values and the characteristic fragmentations, compound **14** was tentatively identified as the phytosterol, β-sitosterol. The reported biological activities of sitosterol include anti-inflammatory, anti-diabetic and anti-peroxidation properties. It also contributes to the reduction of blood cholesterol and is cytotoxic against Hela, Caski, MCF-7 and A546 cancer cell lines. The main source of phytosterols for humans is vegetables. Phytosterols from these sources are bioavailable and a high intake of this group of compounds result in the reduction of breast cancer. Sitosterol has a reported bioavailability of approximately 4%. This has been estimated from the bioactivity of dietary sterols. Phytosterols have been found to be absorbed less than cholesterol by animals and humans due to their structural differences and remain in the gastrointestinal tract.[83]

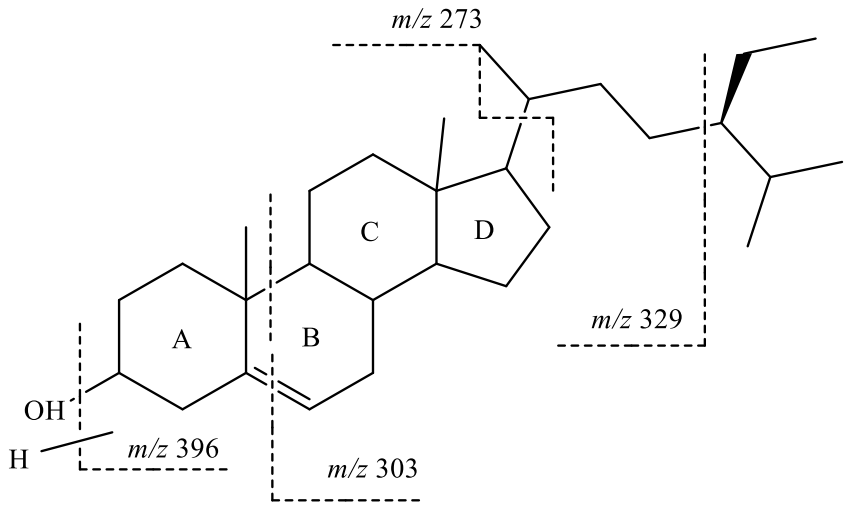


Figure 4.8: Structure of compound 14 tentatively identified as β-sitosterol in the DCM extract of the leaves of *T.laxa*, and its characteristic fragmentations.

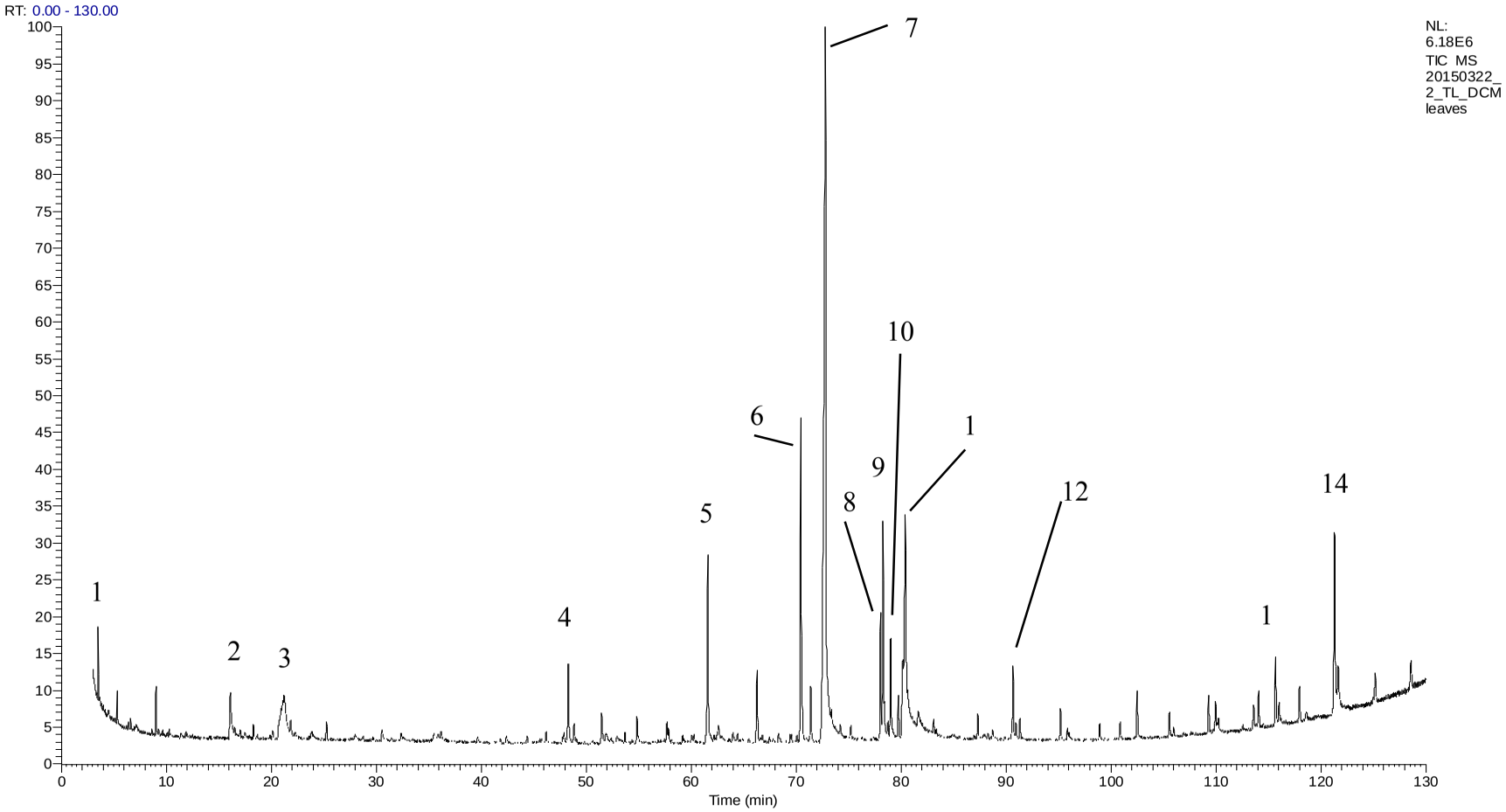


Figure 4.9: Total ion chromatogram of the dichloromethane extract of *T. laxa* leaves.

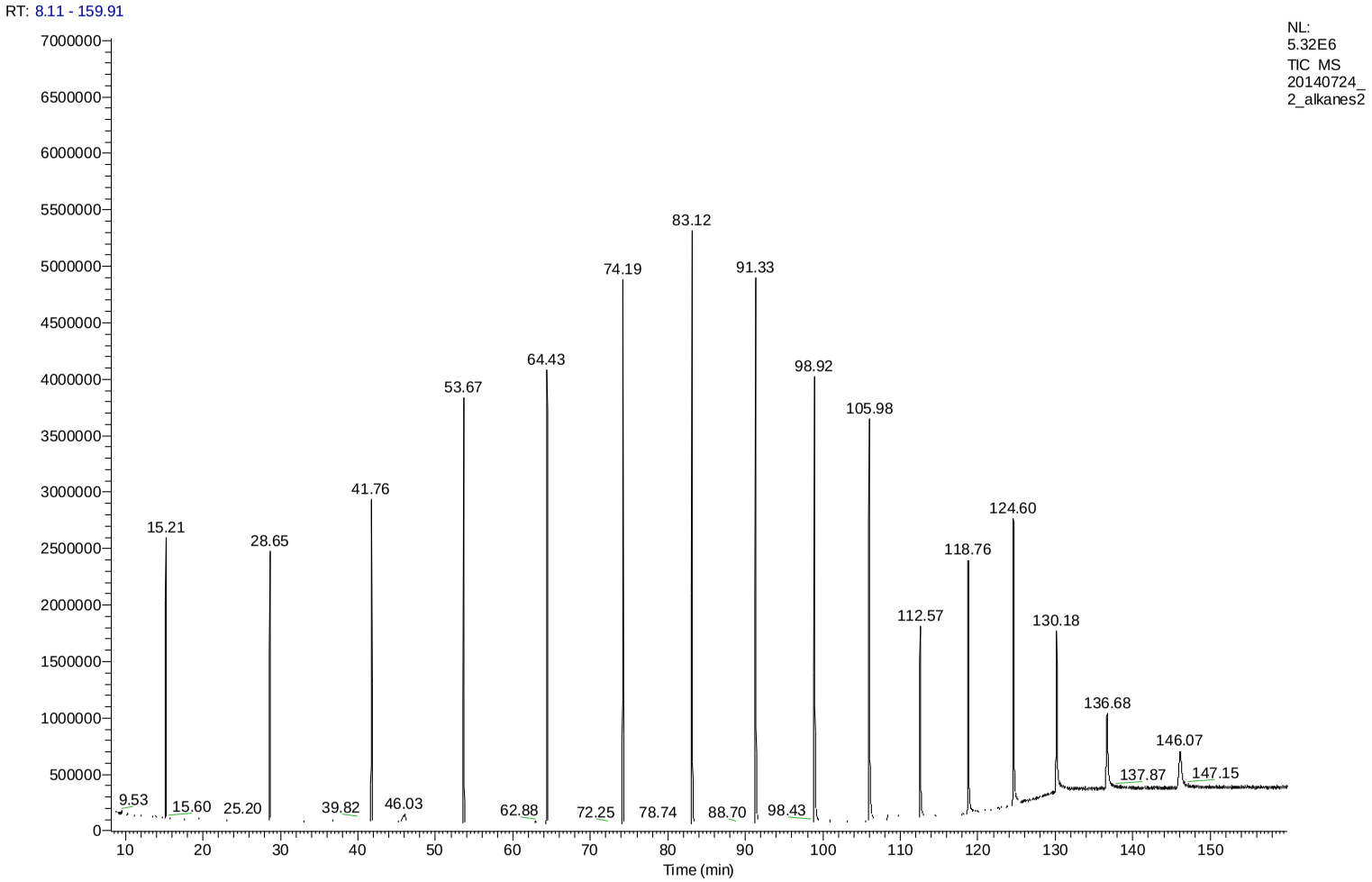


Figure 4.10: Total ion chromatogram of a mixture of C10-C40 *n*-alkanes

Table 4. 3: Compounds identified in the DCM extracts of *T.laxa* leaves in order of elution from a non-polar column (DB-5 equivalent).

Peak no.	t_R (min)	RI (Exp)[a]	RI (Literature)[b]	Component	Formula	MW	Figure	Id. method	Compound type
1	3.53	736	677-746	2,3-Dimethyl 3-buten-2-ol	$C_6H_{12}O$	100	4.10	A,B	Alcohol
2	16.11	1019	1069	Branched C_{11} alkane (undecane)	$C_{11}H_{24}$	190	4.11	A	Branched alkane
3	21.23	1106	880	Unknown alcohol	$C_7H_{16}O$	116	4.12	A,B	Alcohol
4	48.30	1516	1529	Dihydroactinidiolide	$C_{11}H_{16}O_2$	180	4.13	A,B	α,β- unsaturated lactone
5	61.61	1751	1413	Hydroxy dihydroactinidiolide	$C_{12}H_{18}O$	178	4.14	A,B	α,β- unsaturated lactone
6	70.47	1927	1924	Methylhexadecanoate	$C_{17}H_{34}O_2$	270	4.15	A,B	Ester
7	72.82	1974	1968	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	4.16	A,B	Fatty acid
8	78.07	2090	2091	Methyl linoleate	$C_{19}H_{34}O_2$	294	4.17	A,B	Ester
9	78.30	2095	2082-2125	Methyl linolenate	$C_{19}H_{32}O_2$	292	4.18	A,B	Ester
10	79.03	2111	2116	3,7,11,15 Tetramethyl 2-hexadecen-1-ol	$C_{20}H_{40}O$	296	4.19	A,B	Diterpene alcohol
11	80.42	2142	2086	Methyl (ZZZ)-9,12,15 octadecatrienoate	$C_{19}H_{32}O_2$	292	4.20	A,B	Ester
12	90.68	2385	2414	Dioctan-4-yl- hexanedioate	$C_{22}H_{42}O_4$	370	4.21	A,B	Ester
13	115.70	3102	3100	Hentriacontane	$C_{31}O_{64}$	478	4.22	A,B	n-alkane
14	121.32	3289	3187	β-sitosterol	$C_{29}H_{50}O$	414	4.23	A,B, C	Phytosterol

a: Experimentally determined RI values, *b*: RI values from literature, A: Comparison of mass spectrum with NIST database and published data, B: Comparison of RI with NIST database values, C: mass spectrum interpretation.

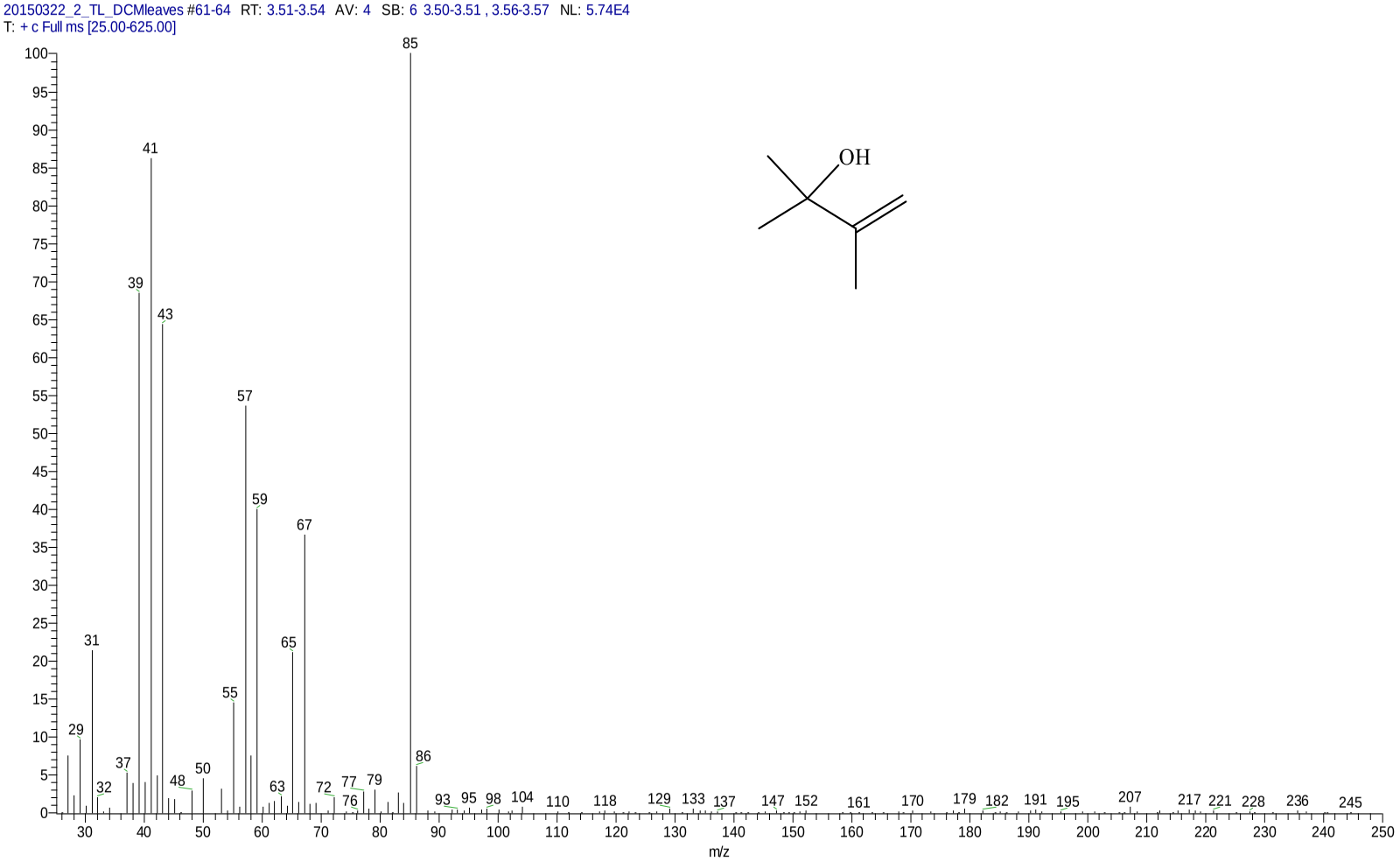


Figure 4.11: Mass spectrum of compound **1**, tentatively identified as 2,3-dimethyl-3-buten-2-ol- in the DCM l extract of *T. laxa* leaves

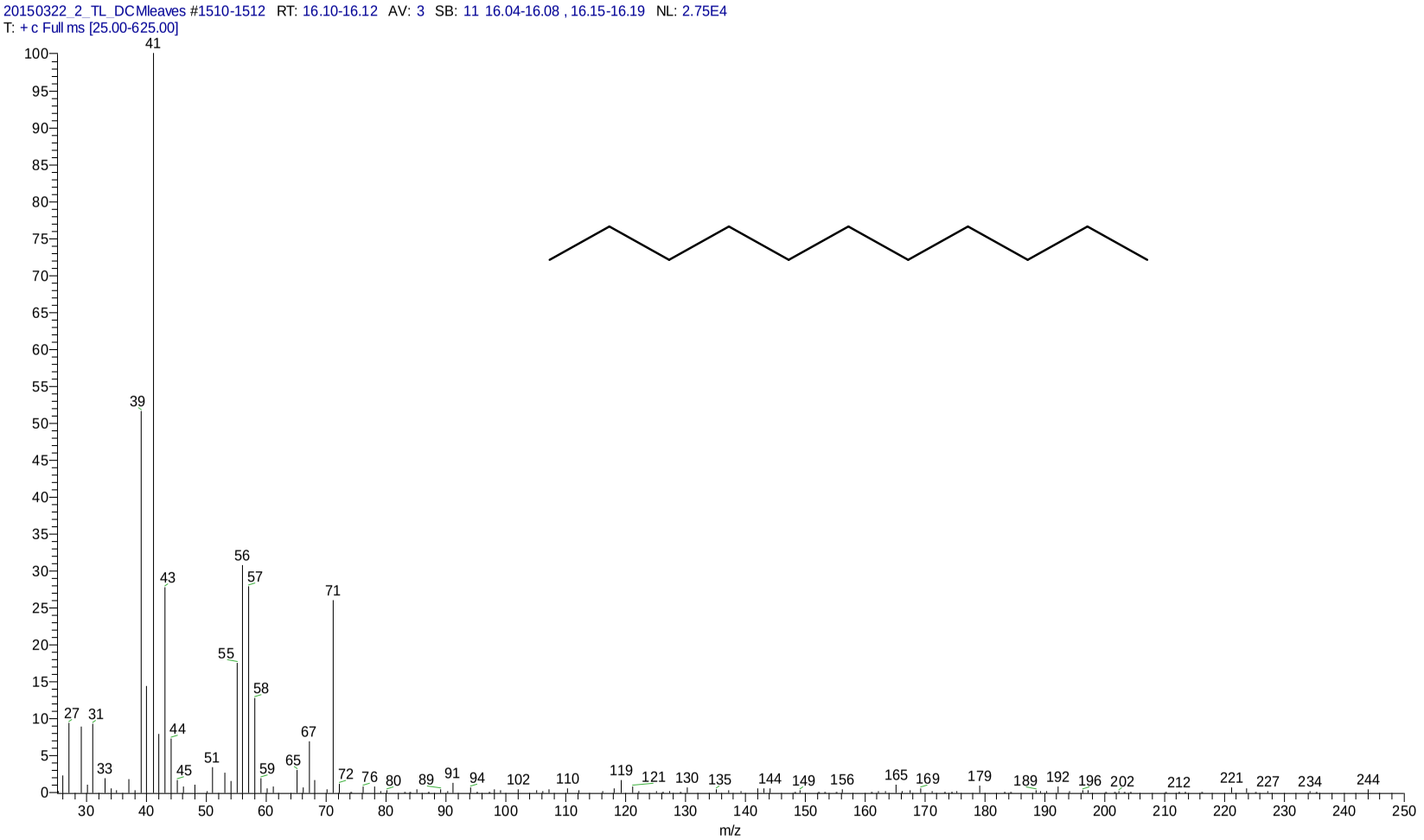


Figure 4.12: Mass spectrum of compound **2**, tentatively identified as a branched alkane in the DCM extract of *T. laxa* leaves

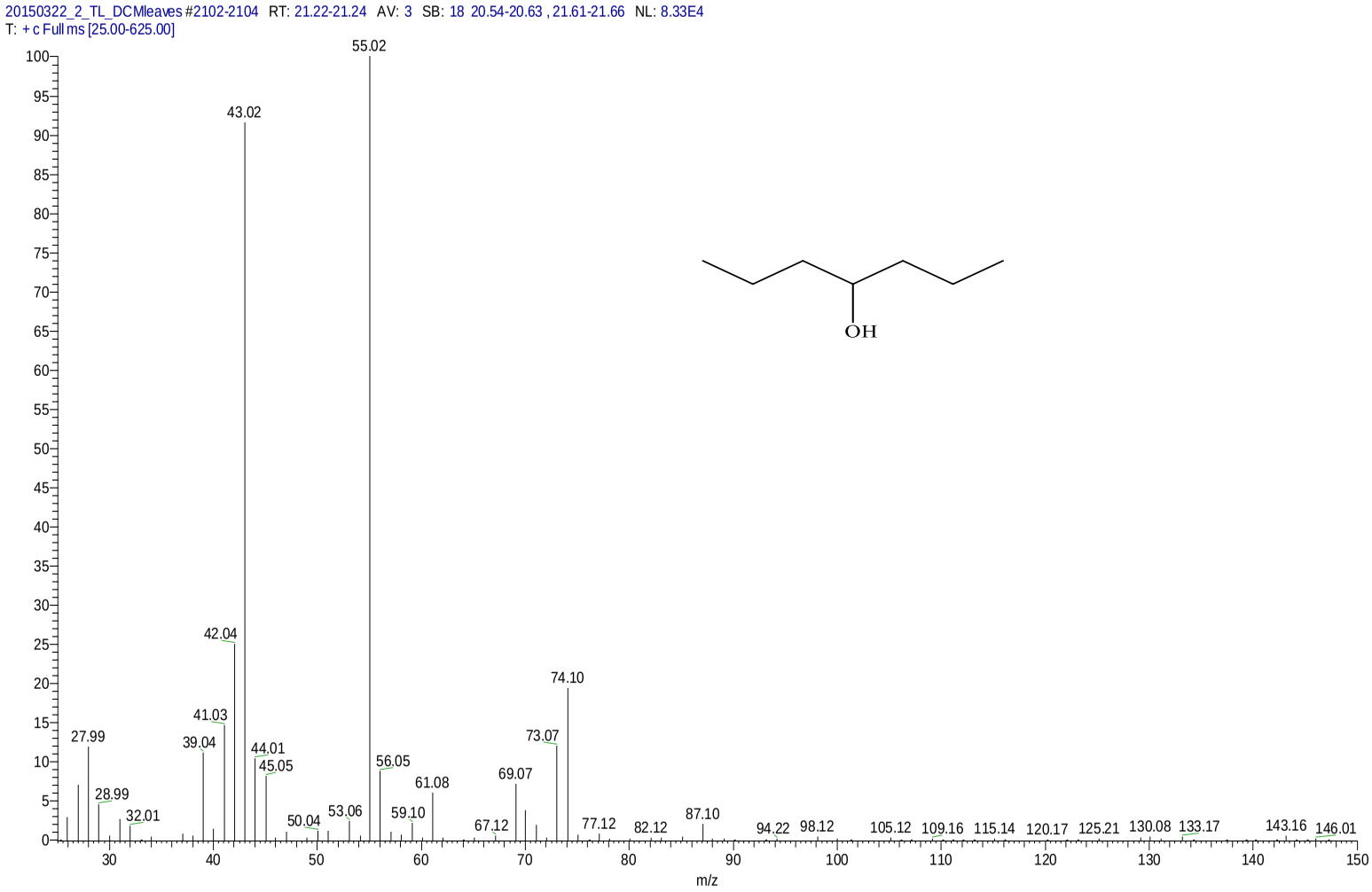


Figure 4.13: Mass spectrum of compound **3**, as an unknown alcohol in the DCM extract of *T. laxa* leaves

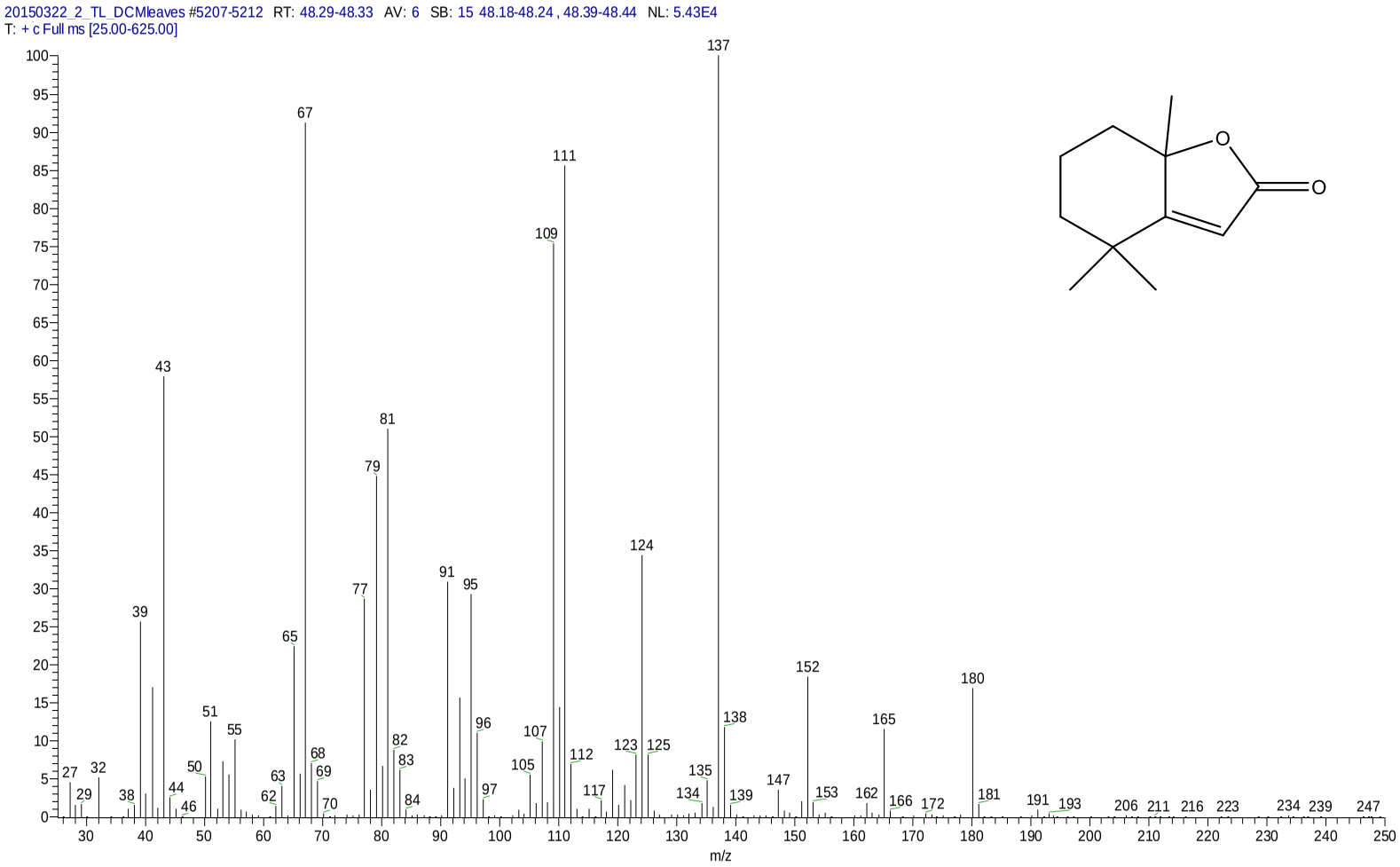


Figure 4.14: Mass spectrum of compound **4**, tentatively identified as Dihydroactinidiolide in the DCM extract of *T. laxa* leaves

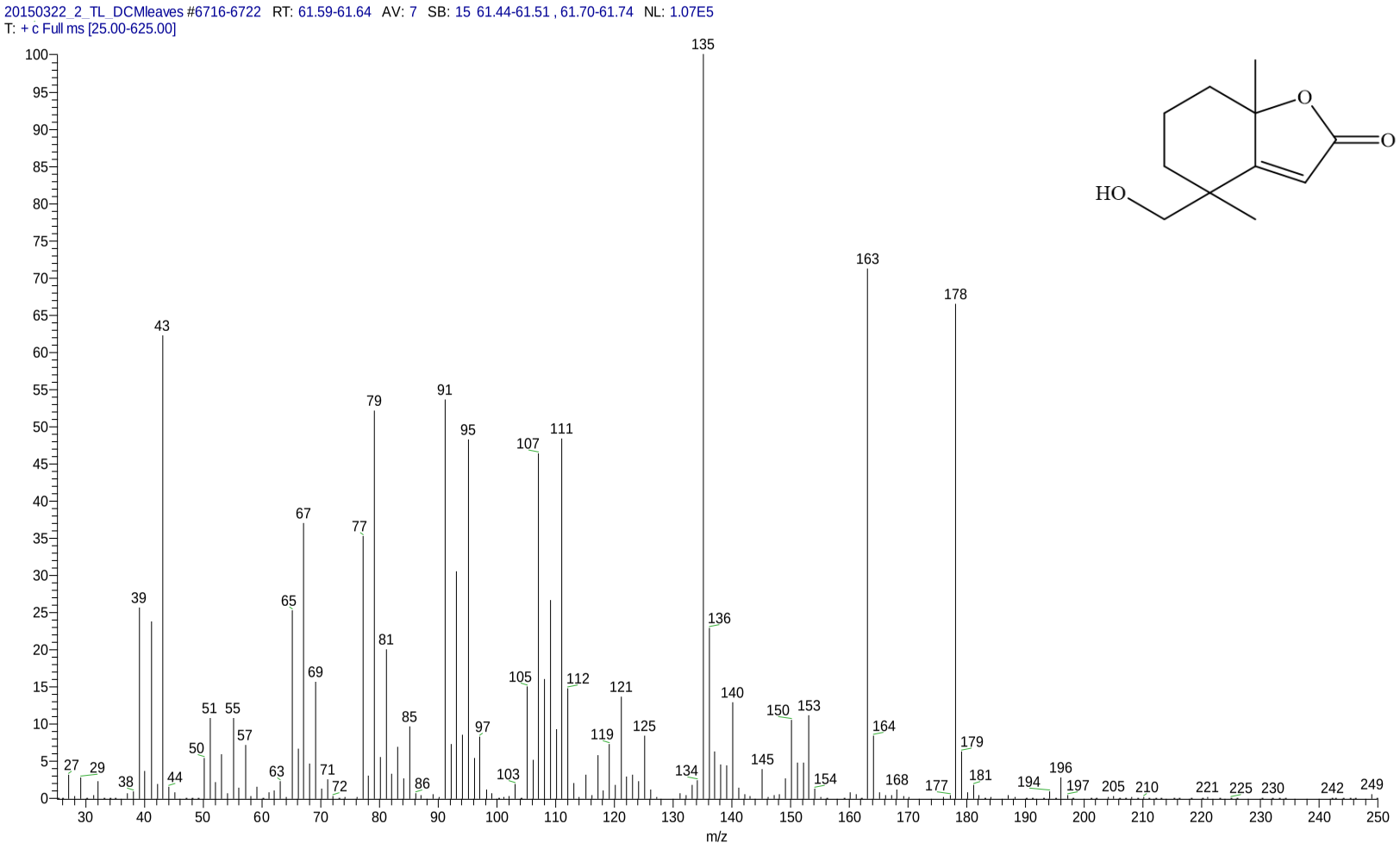


Figure 4.15: Mass spectrum of compound **5,** tentatively identified as Hydroxy dihydroactinidiolide in the DCM extract of *T. laxa* leaves (with a possible proposed structure).

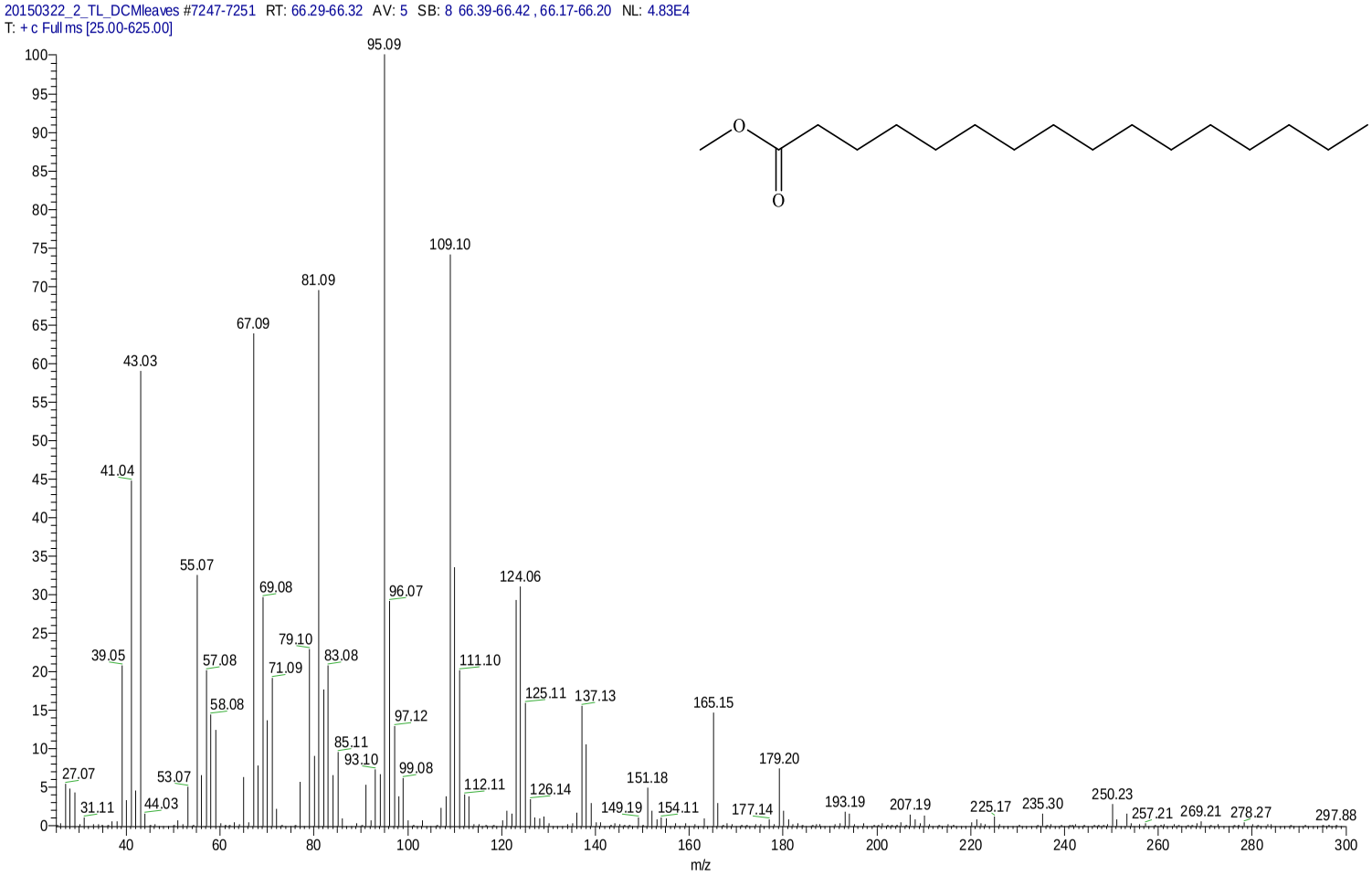


Figure 4.16: Mass spectrum of compound **6**, tentatively identified as Methylhexadecanoate in the DCM extract of *T. laxa* leaves

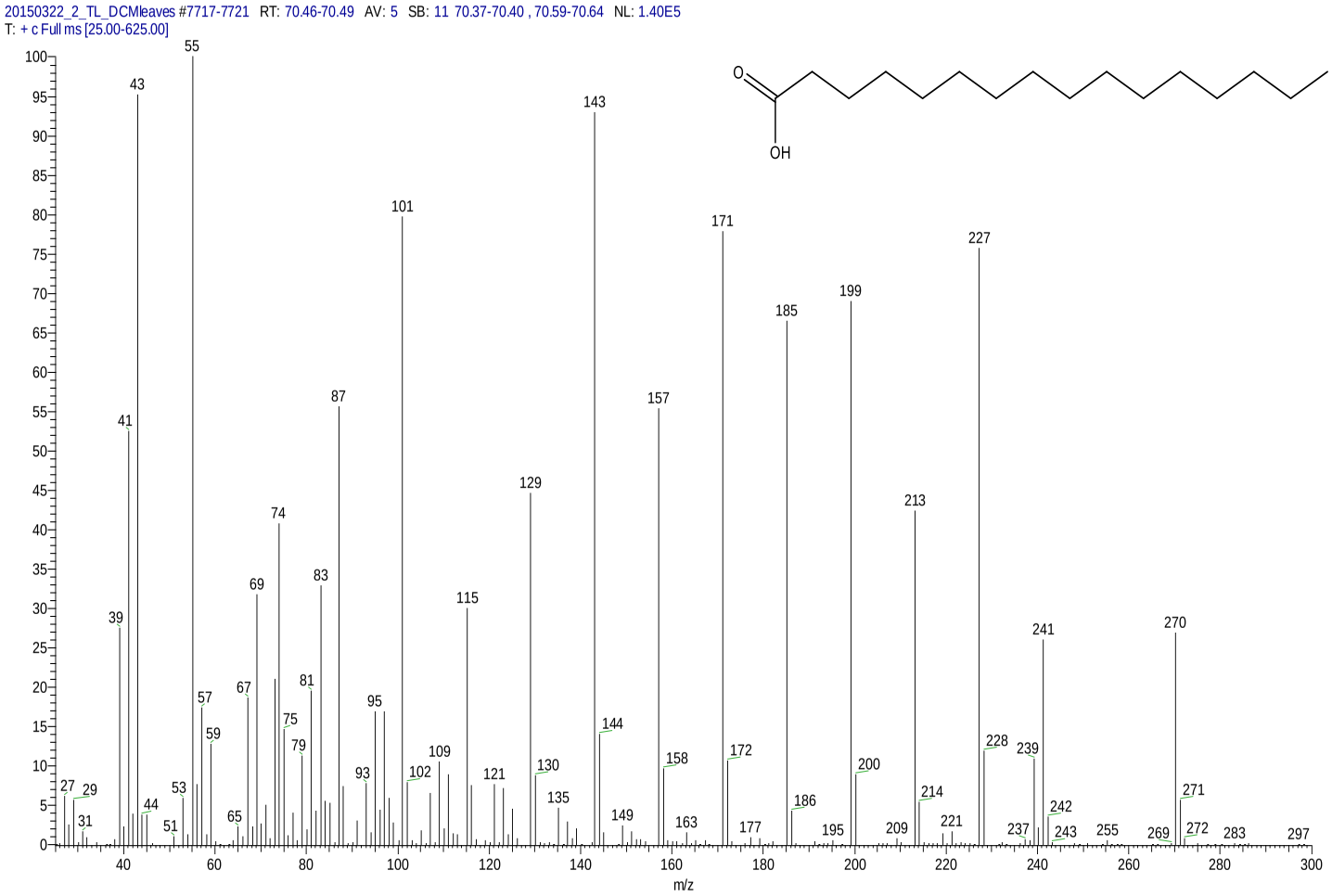


Figure 4.17: Mass spectrum of compound **7**, tentatively identified as Hexadecanoic acid in the DCM extract of *T. laxa* leaves

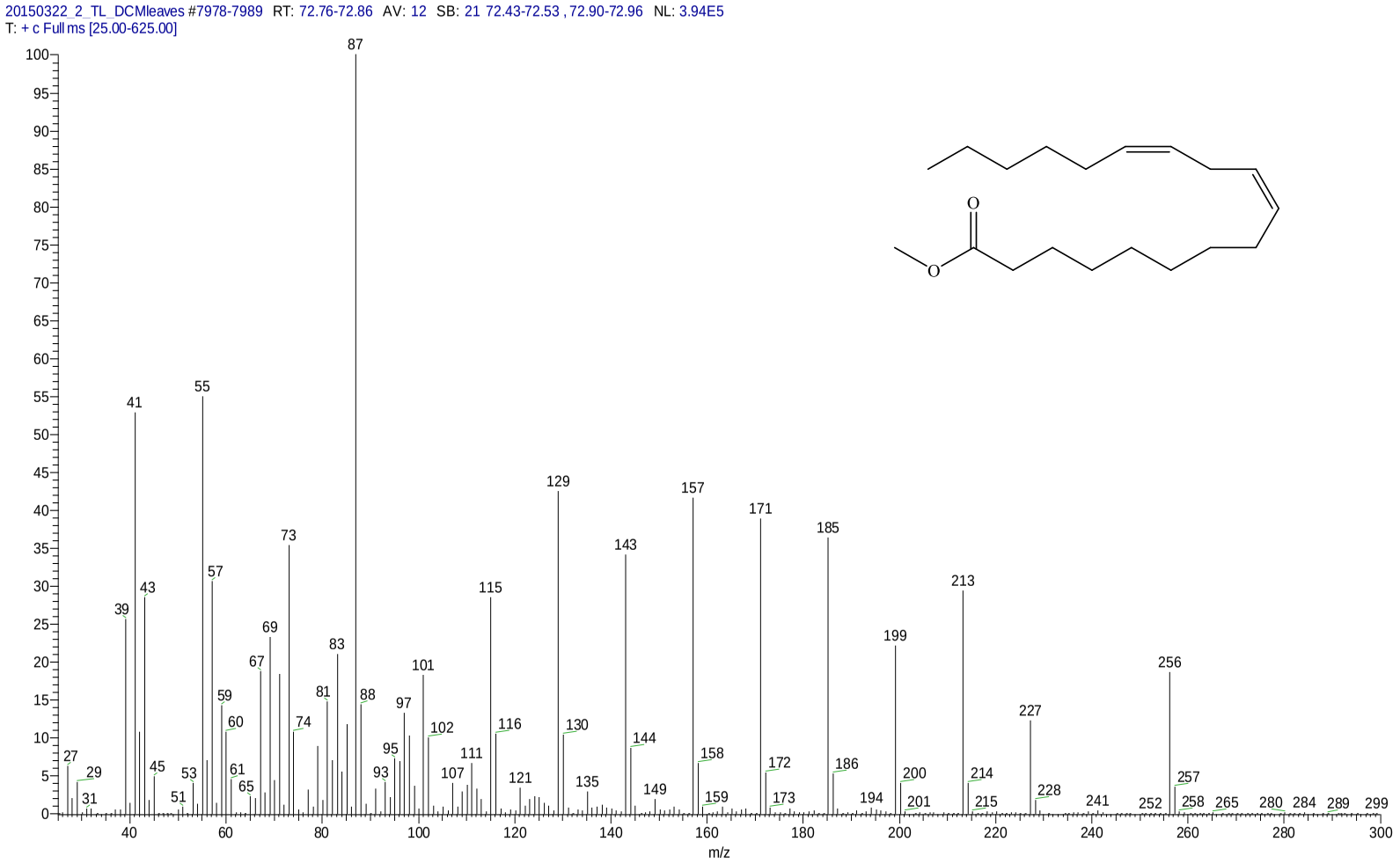


Figure 4.18: Mass spectrum of compound **8**, tentatively identified as Methyl linoleate in the DCM extract of *T. laxa* inflorescence

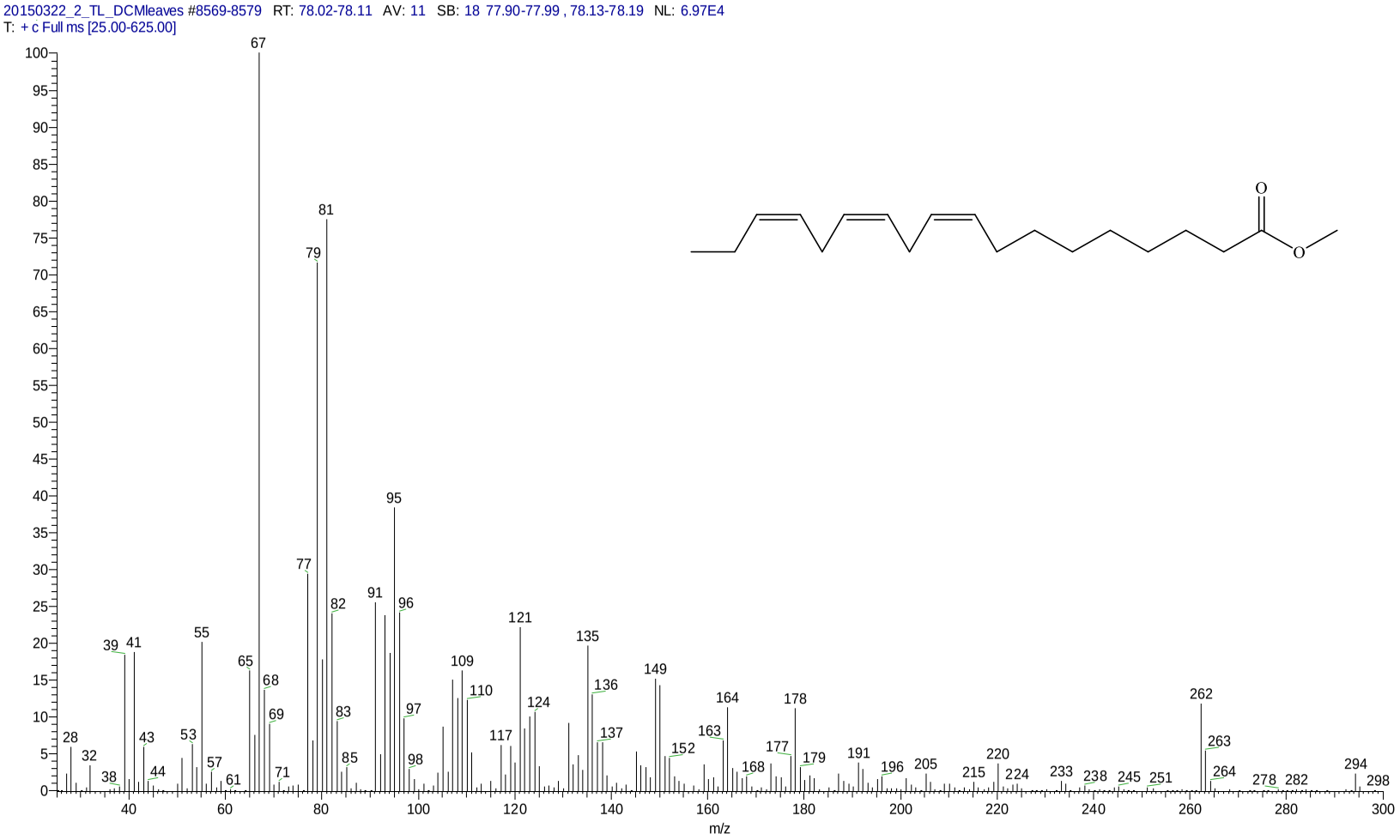


Figure 4.19: Mass spectrum of compound **9**, tentatively identified as Methyl linolenate in the DCM extract of *T. laxa*

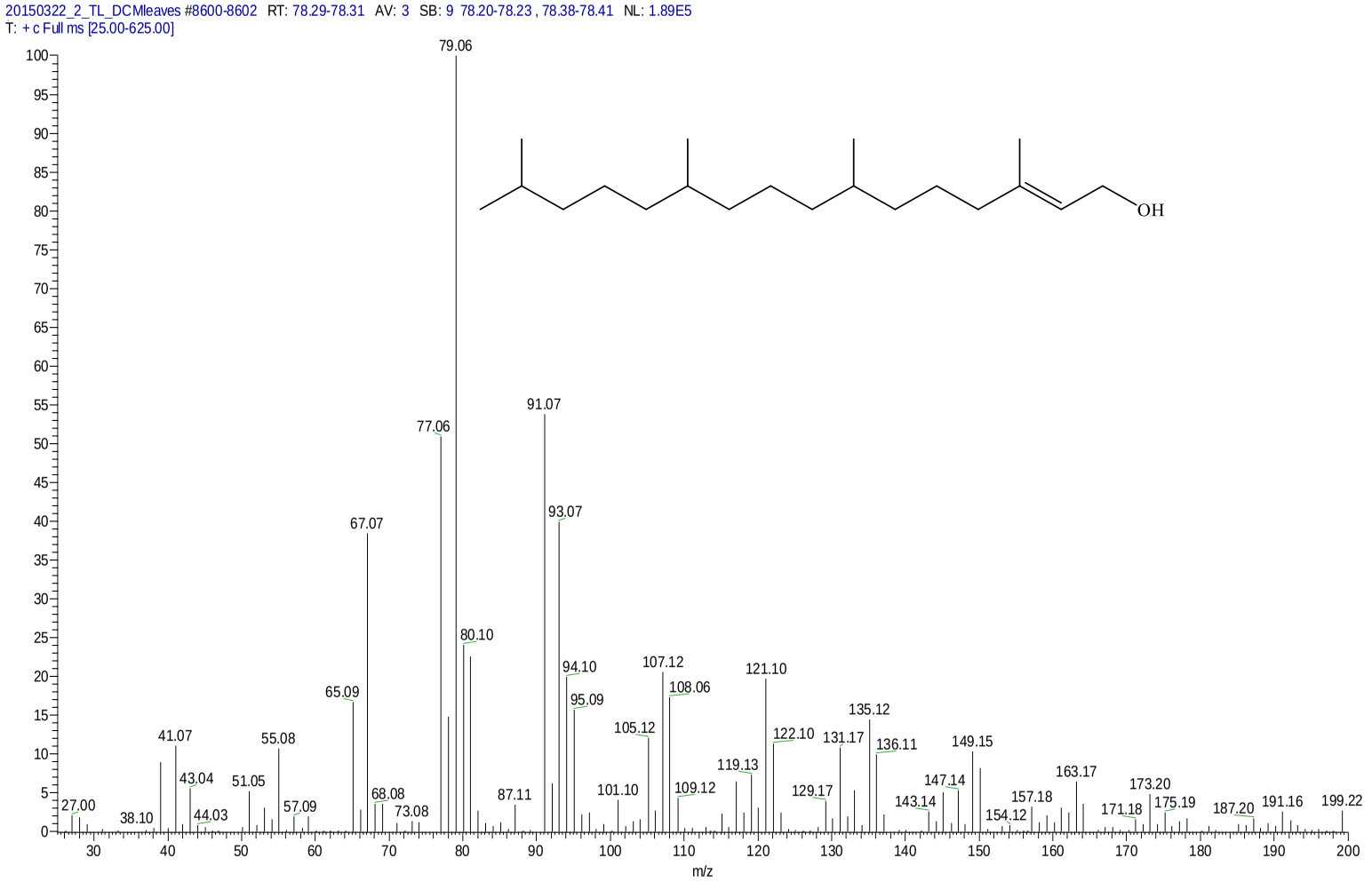


Figure 4.20: Mass spectrum of compound **10**, tentatively identified as 3,7,11,15-Tetramethyl-2-hexadecen-1-ol in the DCM lextract of *T. laxa* leaves

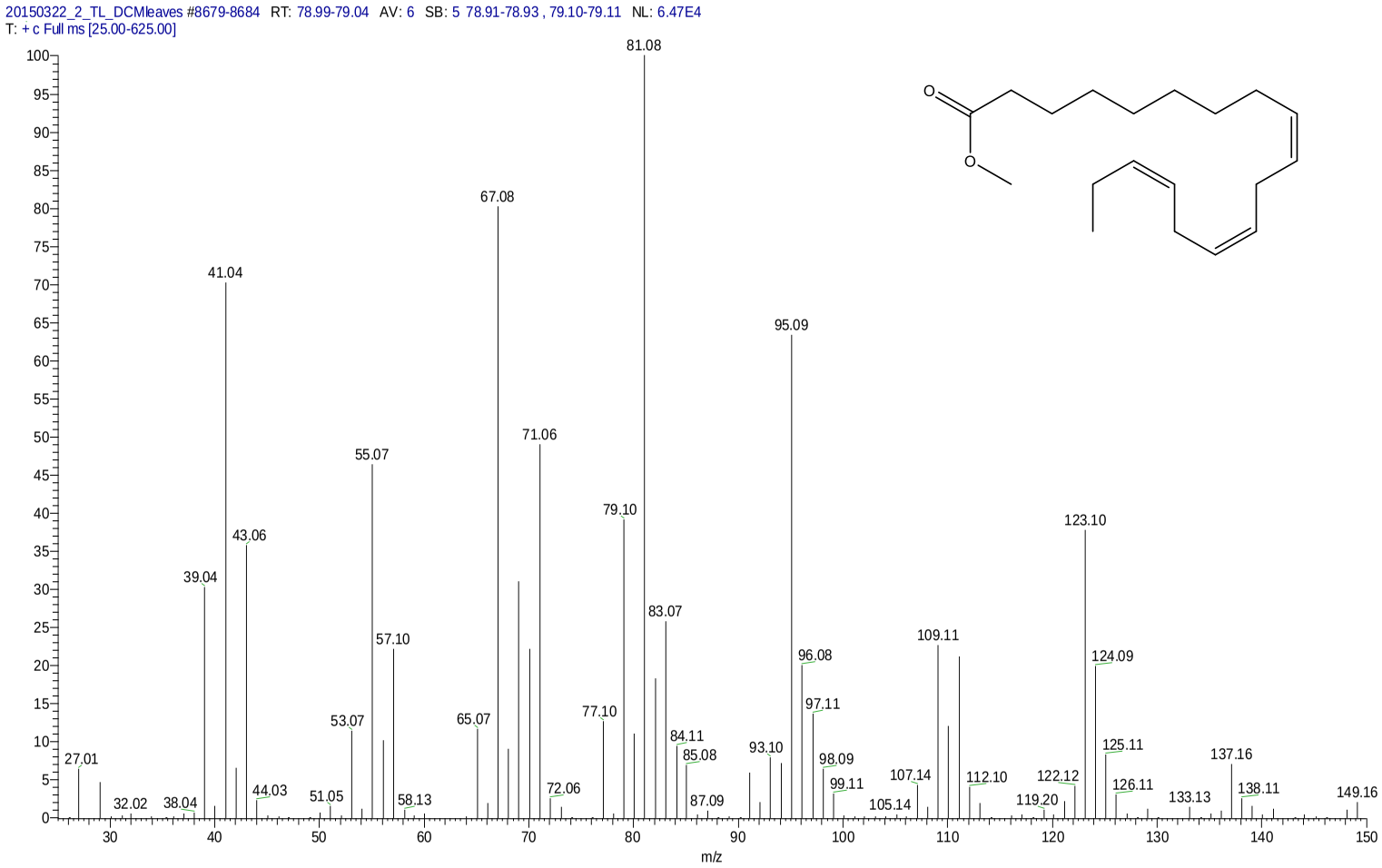


Figure 4.21: Mass spectrum of compound **11**, tentatively identified as Methyl(ZZZ)-9,12,15 octadecatrienoate in the DCM extract of *T. laxa* leaves

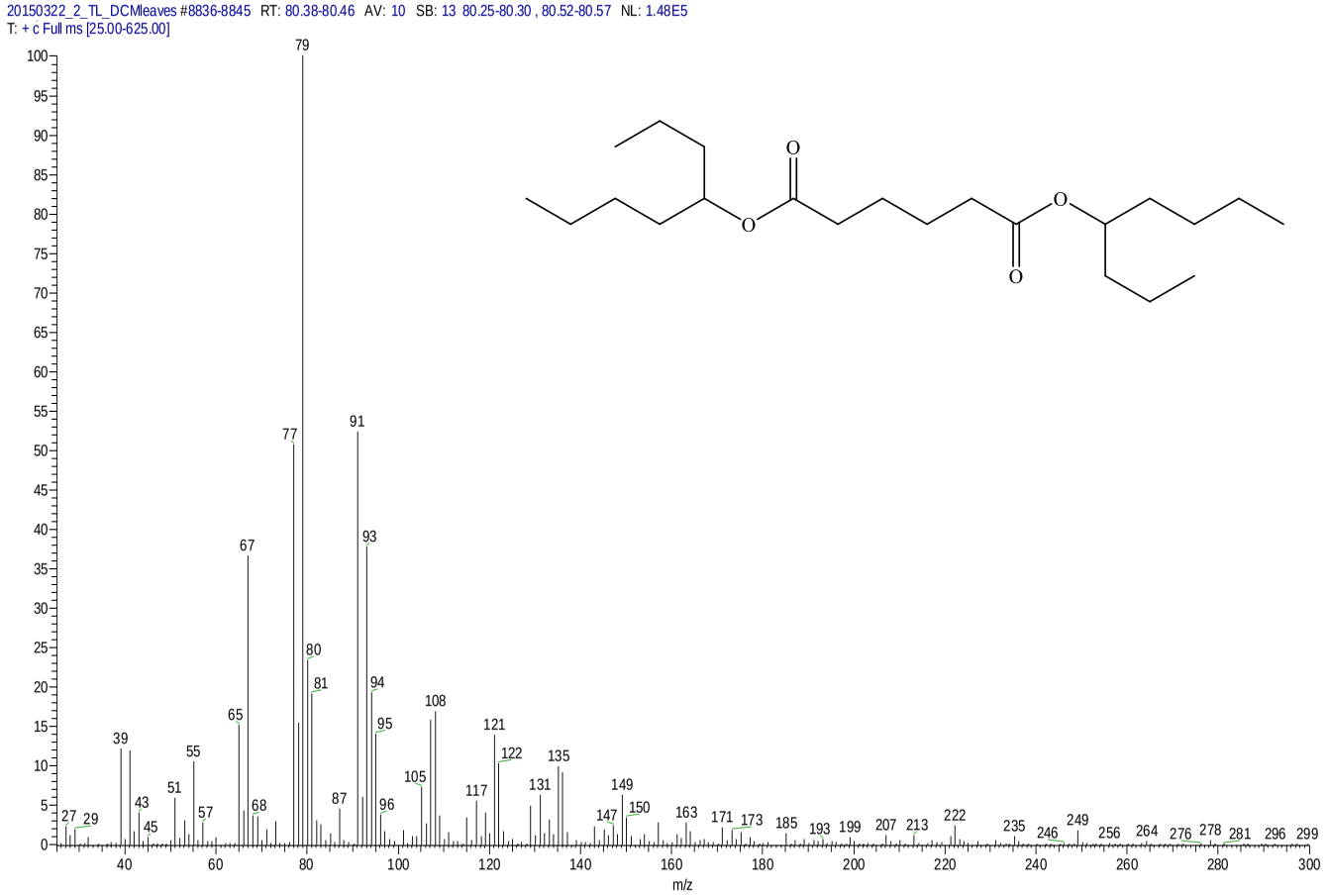


Figure 4.22: Mass spectrum of compound **12**, tentatively identified as Dioctan-4-yl-hexanedioate

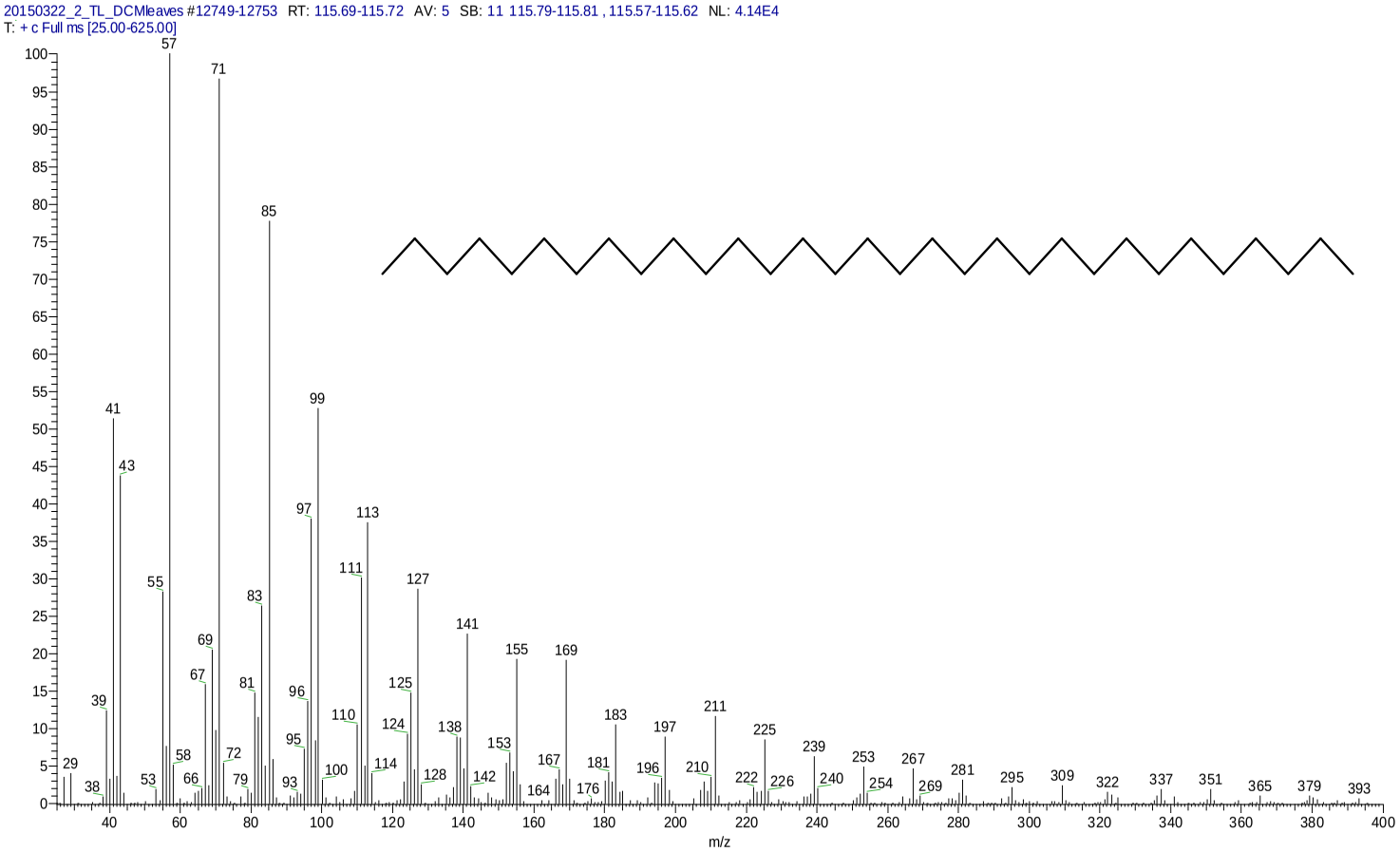


Figure 4.23: Mass spectrum of compound **13**, tentatively identified as Hentriacontane in the DCM extract of *T. laxa* leaves

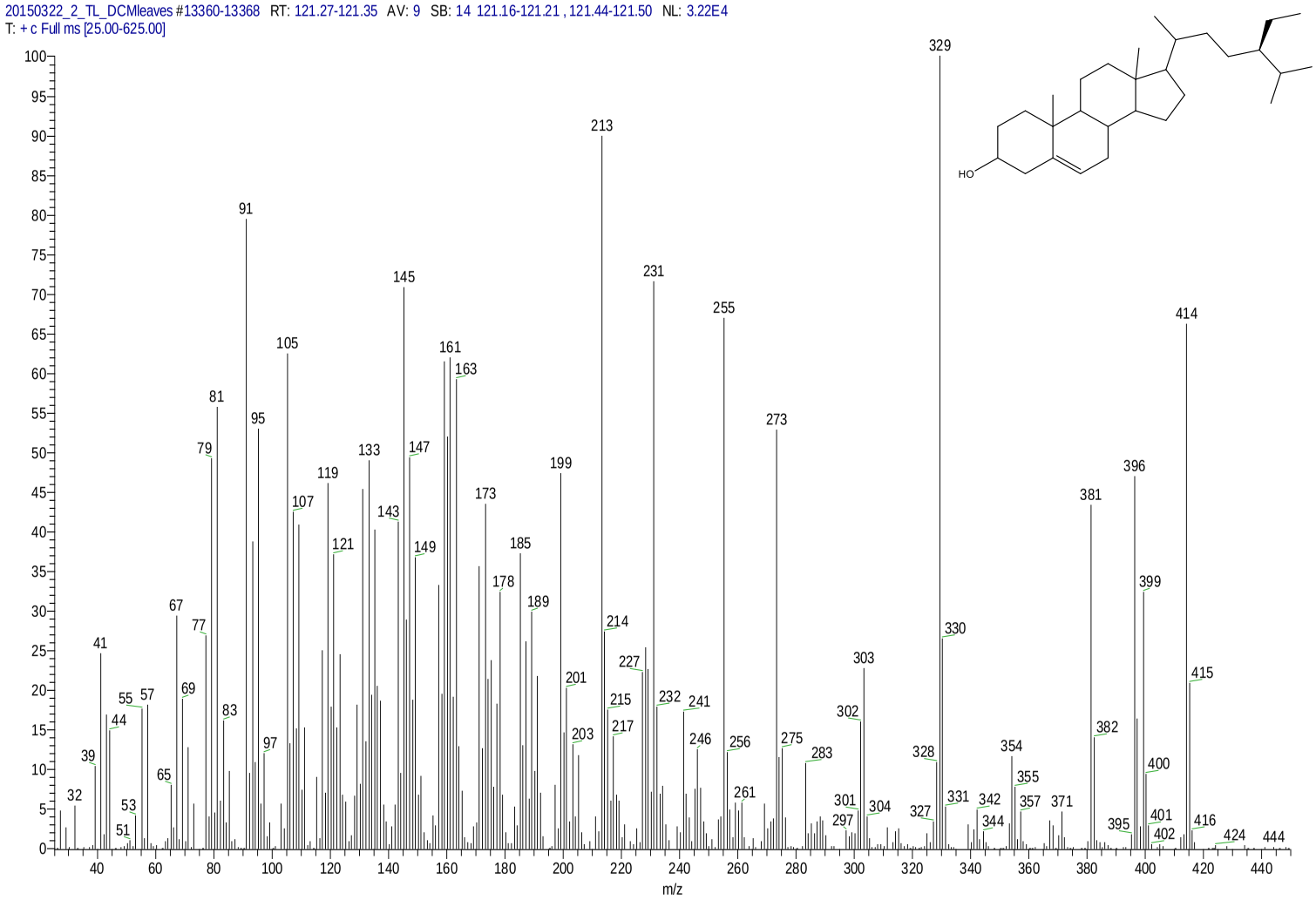


Figure 4.24: Mass spectrum of compound **14**, tentatively identified as β-sitosterol in the DCM extract of *T. laxa* leaves

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

The chemistry of *T. laxa*, a plant responsible for loss of livestock in southern Africa, is reported here for the first time. This study entailed the characterization of compounds from the aerial parts and has added to the chemical knowledge about the plant. None of the samples revealed cytotoxicity to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay. AChE inhibition is known to cause neurotoxicity in animals, resulting in flaccid paralysis. The DCM extracts of the aerial parts of *T. laxa* were subjected to AChE inhibition assay which revealed inhibitory activity. Subsequently fourteen of the major volatile constituents of the DCM extract of the leaves were tentatively identified using GC-MS. The 14 constituents included α,β-unsaturated lactones, alcohols, alkanes, esters, a diterpenoid and a phytosterol. The phytochemical screening did not pick up any terpenes present in *T.laxa*, however, phytochemical tests are qualitative tests thus it is possible to detect false positives or false negatives. Terpenes could have been present in small quantities only, or masked. The α,β-unsaturated lactones were identified as dihydroactinidiolide and its hydroxylated analogue. The former compound has been reported to possess potent AChE inhibition activity. This compound and potentially also its analogue is therefore presumably one of the main contributors to the neurotoxicity of *T. laxa.*

Toxicity of *T. laxa* and its influence on livestock mortality, greatly impacts the GDP of Namibia. Thus further studies should be conducted on this plant to isolate chemical compounds not identified in this study and to further investigate the presence of any other possible α,β-unsaturated lactones. Since compounds occur in plant material as

multi-component mixtures their separation and identification is still challenging. Most of them have to be purified using a combination of different chromatographic techniques and various other purification methods need to be employed to isolate bioactive compound(s). The use of NMR spectroscopic technique, a gold standard in structure elucidation is recommended for future studies on compounds of interest isolated from *T. laxa*. Furthermore, investigations are required to isolate the active constituents responsible for toxicity and to elucidate their exact mechanisms of action that could lead to the development of effective remedial therapies. Ultimately, knowledge on the mechanism of action of these compounds will aid in the design of an antidote to treat affected livestock or to the development of a preventative agent.

Researchers should be obliged to share information from their research with the NBRI and the Directorate of Veterinary services in order to work towards finding antidotes for poisonous/toxic plants and ultimately empowering people (farmers) at grass-root level. Agriculture contributes five percent towards the GDP of Namibia and employs about thirty percent of the total labor force thus the need for further investigation to ultimately boost the economy of Namibia.[83]

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85. Katangais -Namibia, administrative divisions - https://commons.wikimedia.org/w/index.php?curid=55275250

Appendix 1: Chromatographic solvents

CHROMATOGRAPHIC SOLVENTS

Petroleum ether

Cyclohexane

Carbon tetrachloride

Benzene

Methylene chloride

Chloroform (alcohol free)

Ethyl ether

Ethyl acetate

Pyridine

Acetone

1-Propanol

Ethanol

Methanol

Water

Acetic acid

Increasing

Polarity

Mixtures of two or more solvents can be used as developing solvents in chromatographic separations.

Appendix 2: Structural types of plant secondary metabolites

	Structural types of plant secondary metabolites and abundance of poisons and psychoactive substances		
	Class	**Number of structures**	**Poisons**
With Nitrogen	Alkaloids	20 000	Most
	Non-protein amino acids (NPAAs)	700	Some
	Amines	100	Some
	Cyanogenic glucosides	60	Most
	Glucosinolates	100	Some
	Alkamides	150	Most
	Lectins, peptides, polypeptides	2000	Most
Without Nitrogen	Monoterpenes (including iridoids)	2500	Some
	Sesquiterpenes	5000	Many
	Diterpenes	2500	Many
	Triterpenes,Steroids, Saponins	5000	Most
	Tetraterpenes	500	None
	Phenylpropanoids, Coumarins, Lignans	2000	Some
	Flavonoids, Tannins	4000	Some
	Polyacetalenes, Fatty acids, Waxes	1500	Some
	Polyketides (Anthraquinones)	750	Most
	Carbohydrates	200	Few

Reproduced from Wink and Van Wyk (2008)[5]

Appendix 3: Research/Collection Permit

MINISTRY OF ENVIRONMENT AND TOURISM

RESEARCH/COLLECTING PERMIT

Permit Number 1869/2014
Valid from 29 January 2014 to 31 December 2014

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:

Name: **Dr. S. Louw**
Address: **Department of Chemistry and Biochemistry**
University of Namibia
Private Bag 13301
Windhoek
Namibia
Coworkers: **Dr. R.H. Hans, Dr. E.G. Kwembeya, and Ms. H.M. Van Wyk**

To conduct a study on identification of toxic compounds in indigenous plants responsible for livestock poisoning in Southern Africa allover Namibia excluding protected areas, subject to attached conditions.

IMPORTANT: This permit is not valid if altered in any way.

MINISTRY OF ENVIRONMENT AND TOURISM
REPUBLIC OF NAMIBIA
2014 -01- 27
WINDHOEK
Private Bag 13306, Windhoek
Tel: 2842111 • Fax: 258861

Authorising Officer

IMPORTANT
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.

Enquiries: Conservation Scientist, email imatheus@met.na
Private Bag 13306, Windhoek, Namibia

Appendix 4: Identification report of T. laxa

Ministry of Agriculture, Water and Forestry

National Herbarium of Namibia (WIND)

Identification Report

Report No.: 2014/348

2 June 2014

Collector/s: Mrs. H. M. Van Wyk

Address: UNAM
P/Bag 13301
Pionierspark
Windhoek

Number	Identification
01	Trachyandra laxa (N.E.Br.) Oberm. var. rigida (Suess) Roessler
02	Oxygonum alatum Burch. var. alatum

Comment:

Curator
National Herbarium of Namibia (WIND)

Private Bag 13184, Windhoek Tel: +264 - 61 - 202 - 2021 Fax: +264 - 61 - 259 - 153 e-mail: essiek@nbri.org.na

Appendix 5: Ethical Clearance Certificate

UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SEC/FOS/58/2014 **Date: 29 September, 2014**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

Title of Project: **ISOLATION AND CHARACTERISATION OF TOXINS FROM THE TUMBLEWEED, *TRACHYANDRA LAXA*, RESPONSIBLE FOR LIVESTOCK LOSS IN NAMIBIA**

Nature/Level of Project: MASTERS

Principal Researcher: HEDWIG MAUREEN VAN WYK (Student No: 8623236)

Host Department & Faculty: Chemistry& Biochemistry, Faculty of Science.

Main Supervisor (s): Dr Stefan Louw (Main), (Co) Dr Renate Hans

Take note of the following:

(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.

(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.

(c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.

(d) The UREC retains the right to:

(i). withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

(ii). request for an ethical compliance report at any point during the course of the research.

UREC wishes you the best in your research.

Prof. I. Mapaure
UNAM Research Coordinator
ON BEHALF OF UREC