PHYTOCHEMICAL ISOLATION OF COMPOUNDS FROM THE PLANT *SCELETIUM TORTUOSUM*

by

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DEDICATION

To everyone who believed in my success when all I saw was a dark cloud. I now know what they mean when they say “you haven’t found yourself crying on the pavement until you do an MSc or PhD.”

DECLARATION

Student number: 45951012

I, Itumeleng Bridgette Setshedi, hereby declare that the phytochemical isolation and biological exploration of *Sceletium tortuosum* is my own work and that all sources that I have used and/or quoted have been indicated and acknowledged by means of complete references. The dissertation has not been submitted or will not be submitted to any other university or institution for the award of a degree.

Signed (Author): Date:

Signed (Supervisor): Date:

Signed (Co-supervisors) 1. Date:

2. Date:

ABSTRACT

Traditionally, *Sceletium tortuosum* has been used as a medicine and for social and spiritual purposes. The genus is distributed in the south-western parts of South Africa. This project phytochemically analysed and characterised *Sceletium* plant extracts and determined if any extract showed anti-malarial properties. Extracts were prepared in ethanol and methanol and various compounds were purified using column chromatography with hexane and ethyl acetate as mobile phase. The structure of isolated compounds, including mesembrine, pinitol, sucrose, mesembrenone and obtusalin, was confirmed using NMR. The *Plasmodium* Lactate dehydrogenase assay was used to screen all extracts and mesembrine to show that four extracts showed anti-malarial activity with activity values ranging between 1.47 µg/ml and 7.32 µg/ml, well below the 10 µg/ml cut off value. The study recommends extracting compounds from fresh plant material and further research as to anti-malarial activity of compounds isolated from *Sceletium tortuosum.*

KEY TERMS:

Sceletium tortuosum, Mesembryanthemaceae, San hunter-gatherers, traditional medicine, traditional preparaton, phytochemistry, *Plasmodium* Lactate DeHydrogenase (pLDH) assay, Nuclear Magnetic Resonance, compound stability, obtusalin, pinitol, sucrose

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"Oh my God, I never thought this day will come. I always lived by the following words – success is not a result of spontaneous combustion, one has to first set themselves on fire to reach their destiny. I now know the difference between speaking because you can and burning in order to succeed"

There were so many people who helped me along the way and my gratitude is beyond measure. I have had bad and good times while experiencing rough storms and triumphant moments in my life studies. There were misunderstandings as with any project that involves more than one person, but we always found a balance and regained understanding and the best solutions ever. For that I will always be grateful to my supervisor and co-supervisors. Through them, I have learnt to be more patient, strategic, and most important of all, I have learnt that just because I did the work does not mean I understand and know everything; nor does it make me an expect. I have learnt to accept criticism and use it to my advantage. Each and every single one of them instilled a piece of them in me and I am proud to say "I have taken shape from the rough diamond I was before they carved me into perfection as a fragile piece in their caring and delicate hands.

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ABBREVIATIONS AND ACRONYMS

1D	one dimensional
2D	two dimensions
α	alpha
β	beta
^{13}C	carbon 13
^{o}C	degree Celsius
Δ	delta
δ	delta
%	percentage
^{1}H	proton
+	positive
AR	analytical reagent
APAD	3-acetylpyridine adenine dinucleotide
BP	British pharmaceutical
brb	broad doublet
brs	broad singlet
CC	column chromatography
CDC	Center of Disease Control
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
Cl	chloroquine
cm	centimetre
CM	complete medium
CO_2	carbon dioxide gas
CP	chemical pure
CSIR	Council forScientific and Industrial research
DEED	N,N-diethyl-m-toluamide
dd	doublet of a doublet
ddd	doublet of a doublet of a doublet
DMSO	dimethylsulfoxide

ECD	Enterprise Creation for Develpment
EtOH	ethanol
Et_2N	diethyl amine
EtOAc	ethyl acetate
ESI^+	electrospray positive mode
F	frequency
FC	flash chromatography
g	gram
H	hydrogen
H_2O	water
HEPES	hydroxyethyl-piperazine-ethanesulfonic acid
Hz	Hertz
Hex	hexane
hrs	hours
HPLC-MS	high performance liquid chromatography – mass spectrometer
HPR	histidine rich protein
IC_{50}	inhibition concentration at 50% concentration
ICTs	immunochromatographic tests
J	coupling constant
k’	capacity factor / capacity ratio
kg	kilogram
$KHCO_3$	potassium hydrogen carbonate
L	left
L	liter
LC	liquid chromatography
m	multiplet
min	minutes
Med	medical
MeOH	methanol
MDR	multiple drug resistance
MHz	mega Hertz
ml	milligram
m/z	mass to charge ration

NAD	adenine dinucleotide
neg	negative
NBT	nitro-blue tetrazolium
nm	nano meter
NMR	nuclear magnetic resonance
NPs	natural products
N_2	nitrogen gas
O_2	oxygen gas
PDA	photo diode array
Pf	*Plasmodium falciparum*
pH	hydrogen ion concentration / measure of alkalinity or acidity
pos	positive
pLDH	*Plasmodium* lactate dehydrogenase / parasite lactate dehydrogenase
ppm	parts per million
Prep	preparative
RDTs	rapid diagnostic tests
rpm	revolutions per minute
RSA	Republic of South Africa
Rt/(t_R)	retention time
s	singlet
SANBI	South African National Biodiversity Institute
SCE	*Sceletium*
t	triplet
Ti	relaxation time
TLC	thin layer chromatography
t_o	hold-up time
UNISA	University of South Africa
UV	ultraviolet
XDR	extreme drug resistance

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Chapter 1:

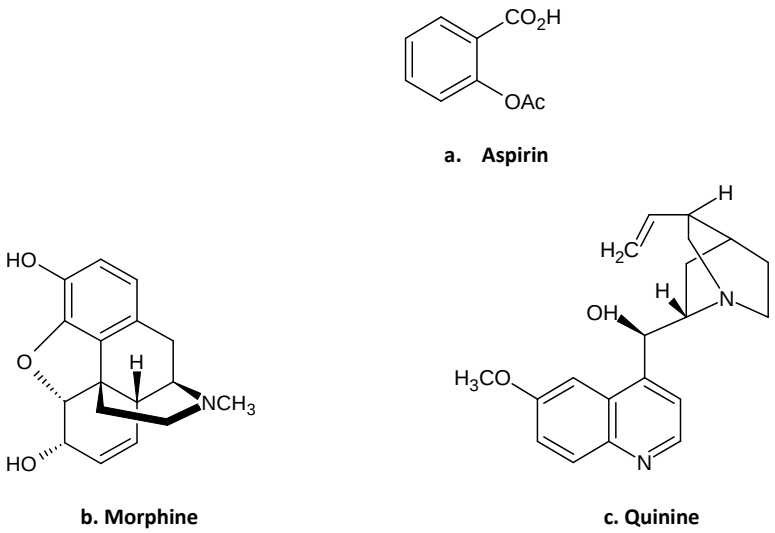
Introduction

1.1 Background

Globally, there are estimated to be 250 000 plant species including approximately 24 000 species that make up South Africa's rich botanical diversity. Furthermore, about 4 000 plant species are used in traditional medicine to remedy different diseases, yet with so much indigenous knowledge at our disposal, less than 10% of the world's biodiversity has been tested for biological activity (Harvey, 2000). Natural products (NPs) include plants, animals, marine life and minerals. They have been the basis of treatment of human diseases dating back to the existence of human civilization. The use of plants, in particular to control human diseases, is a centuries' old practice that has led to the discovery of more than half of all modern pharmaceuticals (Patwardhan *et al*., 2004; Clark, 1996). Indigenous people have sourced medicine and poison from thousands of plants. Unfortunately, many of these systems of medicine have almost completely broken down and disappeared, particularly in developed countries and in some developing countries where the indigenous population has been marginalized. These latter countries include many in Africa, where indigenous systems have been fragmented along with fragmented tribal and geographical areas (Patwardhan *et al*., 2004).

The medicinal value of plants has been recognized by almost every society worldwide in recent years. Most developing countries have relied on, and will continue to rely on, traditional natural medicines due to the limiting effect of high costs associated with developing modern allopathic medicines. Current estimates indicate that about 80% of people in developing countries still rely for their primary healthcare on traditional medicines that are based largely on various species of plants (Patwardhan *et al*., 2004). The science connected with drug discovery is essentially multidisciplinary, involving sub-disciplines which include chemistry, pharmacology and clinical science. While based on the indigenous use of medicinal plants, the main goal of drug discovery is to characterise novel, plant-derived compounds that are biologically active. Clinical, pharmacological and chemical studies of traditional medicines derived from plants formed the basis of drug discovery a few decades ago. These include medicines such as aspirin **(a)**, morphine **(b)**,

quinine **(c**), the structures of which are shown in **Figure 1** below (Newman, et al., 2000; Butler, 2004). A recent analysis of a number of NP-derived drugs amongst the total drugs launched from 1981 to 2002 concluded that NPs were still a significant source of new drugs (Newman *et al*., 2003).



a. Aspirin

b. Morphine

c. Quinine

Figure 1: Early medicines predominantly derived from plants (Butler, 2004).

One of the features of NPs is that they provide a greater variety of structural diversity compared to that obtained with standard combinatorial, synthetic chemistry. As such, the NP approach offers many more opportunities for finding novel, low molecular weight structures that might have biological activity against a wide range of assay targets. In general, various compounds that have been shown to have activity in bio-assays are usually small molecules capable of being absorbed and metabolized by the body (Harvey, 2000). Over the past few decades, NPs have featured extensively in the chemotherapy of parasitic diseases, particularly where quinine, emetine and berberine were used to treat malaria, amoebiasis and leishmaniasis, respectively, while santonin was used to treat helminthic infections. In addition, with the discovery by Chinese scientists in the early 1970s, of artemisinin, an antimalarial component contained in the plant *Artemisia annua*,

attention was rekindled regarding the potential source of plant extract products to treat an assortment of parasitic diseases (Croft, 2001).

It is common knowledge that medicine and NPs have been closely linked through the use of traditional medicine and natural poisons over the years. However, even with this knowledge, many more plant species are awaiting analysis for NP discovery (Butler, 2004). Natural products are the most consistently successful sources of drug leads, but despite this their use in drug discovery has fallen out of favour. As mentioned above, less than 10% of the world's biodiversity has been tested for biological activity and, thus, many more useful natural lead compounds are awaiting discovery, and the challenge facing scientists is how to access and harness this natural chemical diversity (Harvey, 2000). Although traditionally NPs have played an important role in drug discovery, in the past few years most multinational pharmaceutical companies have either terminated or considerably scaled down their natural product operations (Butler, 2004). This shows that despite the success of natural products in drug discovery, there is still little belief in the power of this indigenous knowledge. However, NPs are still providing their fair share of new clinical candidates and drugs despite the development of highly competitive drug discovery methods in the industry (Newman *et al*., 2003).

The *Sceletium* genus is amongst taxa that have been extensively researched, but never in terms of an anti-malarial drug. The genus is distributed in the south-western parts of South Africa and has an affinity for arid environments. Traditionally, plants of the genus *Sceletium* (Mesembryanthemaceae) have been used to relieve thirst and hunger, to combat fatigue, as medicines and for social and spiritual purposes by San hunter-gatherers and Khoi pastoralists (Gericke and Viljoen, 2008). Although most of the traditional uses have been scientifically validated, this plant may offer up many more NPs. Malaria is one of the world's most deadly infectious diseases and continues to be associated with a considerable burden of disease and death and thus, has a significant social and economic impact on developing countries and societies (Alshawsh *et al*., 2007). An increasingly serious problem associated with malaria parasites, is the development of resistance to quinoline-containing drugs, such as chloroquine, making the disease a serious worldwide health problem (Kaur *et al*., 2009; Gelb, 2007). Individuals must take anti-malarial drugs as prophylaxis about one to two weeks before travelling to a malaria-infested area

and for four weeks after leaving the area. Travellers are advised by Centres for Disease Control (CDC) to avoid malarial outbreak areas. Wearing appropriate clothing is very important in minimizing skin exposure. Where there is no adequate air conditioning or accommodation is not adequately screened, bed nets are a necessity to provide protection. Bed nets are most effective when they are treated with an insecticide or repellent such as permethrin. Insecticides can help to clear rooms or areas of mosquitoes, but optimum protection can be provided by applying repellents containing at least up to 50% N,N-diethyl-m-toluamide (DEET) and not more than 10% for children (www.doh.gov.za/docs/policy/2011/malaria_prevention.pdf).

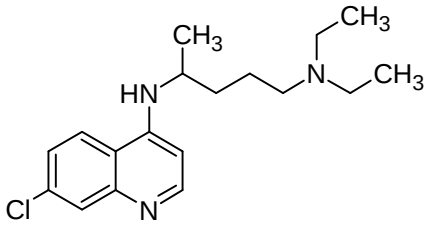


Figure 2: Structure of chloroquine (Read *et al*., www.bank.ca/drugs/DB00608)

Africa is amongst several continents that are significantly affected by drug-resistant malarial parasites and this is reflected in the doubling of malaria-attributable child mortality in Southern Africa (White, 2004). It is a developing continent that is neither well established nor equipped for this battle. In reality, most malaria cases occur in remote areas, where adequate resources are limited, if not completely unavailable (Hommel, 2008). This infection could cripple some of these developing countries and continent's economy, especially in Africa where approximately 50% (if not more) of its current population is poverty stricken (Wanjala, 2008). Resistance to all known anti-malarial drugs (i.e. multidrug resistance (MDR) or extreme drug resistance (XDR) has developed to various degrees in several countries. Thus, there is an urgent need to counteract MDR/XDR malaria strains by developing new classes or compounds of anti-malarials (Bloland, 2001). While many countries and their populations still rely on traditional medicine as a resource for the treatment of this disease, there is reason enough to support studies of combined traditional medicine with the western approach of scientific research and technology to produce a reliable anti-malarial drug.

1.2 Problem statement

Even with competition from other drug discovery methods, natural products still provide new clinical candidates and drugs (Newman et al., 2003). The chemistry and pharmacology of many of these medicinal plants, such as *Sceletium tortuosum*, have not yet been thoroughly investigated. South Africa can benefit from the scientific evaluation of this indigenous plant and its knowledge base (Harvey, 2000). With alkaloids being one of the most important classes of natural products providing drugs since ancient times (Kaur et al., 2009), this study will focus on the chemical characterisation, together with some biological evaluation, of substances isolated from *Sceletium tortuosum*.

1.3 Aims and Objectives

1.3.1 Aim of the study:

To perform phytochemical analysis of extracts of *Sceletium tortuosum* in order to characterise secondary metabolites and also to evaluate these together with crude extracts and fractions for anti-malarial activity.

1.3.2 Objectives:

- To scientifically investigate the traditional extraction method from *Sceletium tortuosum (S. tortuosum)* plant material.
- To phytochemically isolate secondary metabolites from *S. tortuosum* extracts.
- To process and structurally elucidate compounds from *S. tortuosum* extract, using Nuclear Magnetic Resonance (NMR) and High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS).
- To profile and compare different extracts from *S. tortuosum* using HPLC-MS.
- To screen extracts from *S. tortuosum* for biological activity using an anti-malarial assay system.

Chapter 2
Literature review

2.1 Introduction

The mesembryanthemaceae (leaf succulent plants) are one of the largest succulent plant families constituting a major part of the South African succulent flora. This family is almost exclusively confined to southern Africa, with less than 2% of the species occurring naturally outside this region. The family is sometimes included in the Aizoaceae group and the family name commonly used in several articles is Mesembryanthemaceae Fenzl. Within this group, the Mesembryanthemaceae family is the second largest family, with approximately 2000 species and 116 genera, but more than three-quarters of its species have never been studied (Chesselet *et al*., 1995; Ihlenfeldt, 1994). Originally, all species of this family were united in a single genus *Mesembryanthemum L*., as proposed by Breyne in 1689 in Ihlenfeldt (1994). With the growing number of known species, it became necessary to split this genus and several attempts using mainly life-form characters were made (Ihlenfeldt, 1994). The word *Mesembryanthemum* originates from the name 'midday flower', which refers to the opening of the flowers around noon. Other common names include ice plant, fig-marigold, iqina (Xhosa) and mesemb. Plants that belong to the Mesembryanthemaceae family are known by most South Africans as *vygies* (Chesselet *et al*., 2002).

The genus *Sceletium* N.E. Br. is classified under the family Mesembryanthemaceae (Aizoaceae) and belongs to the sub-family Mesembryanthemoideae (Patnala and Kanfer, 2009; Smith *et al.,* 1996). The genus name is derived from the word 'sceletus' which means 'skeleton', referring to the prominent vein-like lines (See **Figure 2.1** below) which are most easily visible in the old, dry and withered leaves (Gericke and Viljoen, 2008). Of the twenty two *Sceletium* species described by Brown (1926) in Gericke and Viljoen, (2008), eight species are currently recognized i.e. *Sceletium crassicaule* (Haw.) L. Bolus, *S. emarcidum* (Thunb.) L. Bolus ex H.J. Jacobson, *S. exalatum* Gerbaulet, *S. expansum* (L.) L. Bolus, *S.rigidum*, L. Bolus, *S.strictum,* L. Bolus, *S. tortuosum* and *S. varians* (Haw.) Gerbaulet (Smith *et al.,* 1998). Of the eight, only *S. tortuosum* is well known and is used in commercial herbal products (Van Wyk and Wink, 2004).

Figure 2.1: *Sceletium* leaves showing the distinctive idioblasts on the leaf surface and the characteristic skeletonised old leaves (Gericke and Viljoen, 2008).

The general application of *Sceletium* (Aizoaceae, subfamily Mesembryanthemoideae) had been revised by various authors since the genus was established in 1925 by N.E. Brown. This group of plants is characterised by the skeletonised leaf venation pattern visible in dried leaves. In 1986, Bittrich argued for a broader application of *Phyllobolus* which included *Sceletium* as one of five subgenera (Gericke and Viljoen, 2008). Since Gerbaulet (1996) was unable to find a synapomorphy (a unique derived character) for Bittrich's broad concept of *Phyllobolus*, she reinstated *Sceletium* as a genus (Gericke and Viljoen, 2008). Species of this genus are distinguished on the basis of vegetative, flower, fruit and seed characteristics. Some species are reduced to synonymy including *Sceletium joubertii* L. Bol., and *S. namaquense* L. Bol. now considered part of *S. tortuosum*. *Sceletium* exhibits a climbing or decumbent habit and has characteristic succulent leaves with "bladder cells" or idioblasts (**Figure 2.1**). The flowers range from white, yellow to pale pink. These plants were commonly known by their vernacular names which include *kanna* (Khoi) and *kougoed* (Afrikaans), the latter referring to the use of the plant material by chewing (Gericke and Viljoen, 2008).

Figure 2.2: Representative of the characteristics mentioned in the above paragraph (Gericke and Viljoen, 2008).

Kougoed usually grows hidden under other bushes, but it occasionally grows out in the open. The small size of seeds is adapted for wind dispersal, whereupon landing in a suitable area, the plant will grow. The plant occurs in winter rainfall areas (**Figure 2.3**) and is at its best in the summer months, when the growing season is over and the leaves appear yellowish and wilted. The plant still retains its activity when it is green, but a lot of its vigour is lost through fluid that drains from the plant (Gericke and Viljoen, 2008).

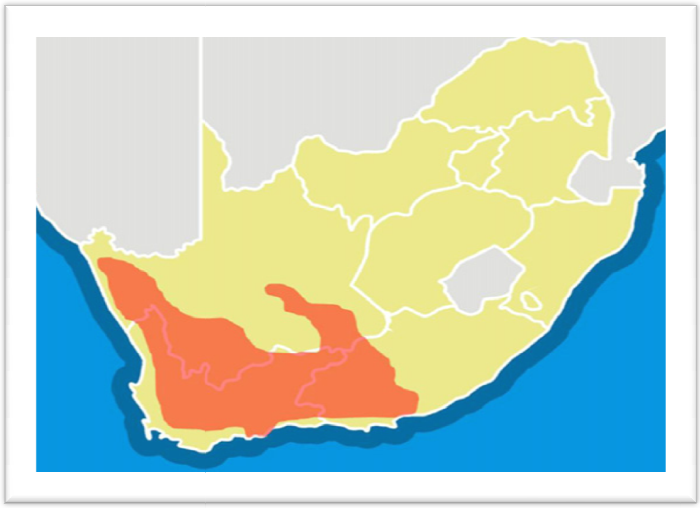


Figure 2.3: Geographical distribution of *Sceletium* in South Africa (Gericke and Viljoen, 2008)

2.2 Traditional preparation, dosage and uses

One of the co-authors in Smith *et al.,* (1996) undertook a field trip to Namaqualand to collect these plants using traditional methods of preparation for commercial re-sale. An informant reported that historically, a skin or canvas bag was used as a fermentation vessel, but that these have been replaced by plastic bags (Smith *et al.,* 1996). According to this method of preparation, whole plant material is crushed in the plastic bag between two stones, after which the tightly sealed bag of crushed '"*kougoed"* is then left in direct sunlight to ferment. The bag is then opened after 2-3 days, its contents re-mixed and then the bag is tightly closed again. The bag is opened on the eighth day, after which the crushed. *kougoed* is spread out to dry in the sun, as shown in **Figure 2.4**. It is claimed that eating fresh plant is not beneficial (as the plant doesn't have powers in its fresh form); therefore, one must follow the whole process of fermentation to unlock powers from the plant (Smith *et al*., 1996; Gericke and Viljoen, 2008; Patnala and Kanfer, 2009).

Figure 2.4: Dried fermented plant material ready for use (Van Wyk and Wink, 2004).

Traditionally, the fermented and dried herb is chewed, hence, the Afrikaans name "*kougoed"*. It can also be used as a tea, decoction or tincture. A dose of 100-200 mg of the dried powdered herb, either in tablet and capsule form, also contains around 1-4 mg of alkaloids, can be taken two or three times a day. *Sceletium tortuosum* can be used as a mood enhancer, as well as to counteract anxiety, stress and tension. Decoctions of this herb have reportedly been used to treat colic in infants, as well as forming part of replacement therapy for alcoholics, where, therapeutically, hypnotic and sedative properties have also been attributed to this plant. The apparent absence of

physical and psychological dependency, even after many years of habitual use, is one of the interesting features of this unique plant. There has not been a single record of any negative side effects arising out of using material from this plant, although it has been reported that high doses may cause euphoria, although the plant is not hallucinogenic (Van Wyk and Wink, 2004).

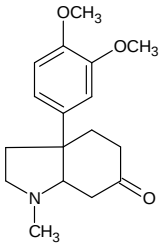
2.3 General background on the chemistry of alkaloids

For over 4 000 years, humans have used drugs such as medicine, potions, teas and poison that have since been determined to contain alkaloids. Alkaloids are diverse, secondary compounds derived from amino acids. These compounds are classified according to the amino acids that provide their nitrogen atom and part of their carbon skeleton. As such, alkaloids are not the end product of metabolism but can be degraded to serve as a nitrogen source when needed. Purine alkaloids are the ones most commonly ingested as a daily dose acquired in a cup of tea or coffee (Aniszewski 2007; van Wyk et al., 2008). In general, alkaloids are bitter tasting, white solids, with nicotine being an exception, occurring as it does as a brown liquid. There are three main types of alkaloids: true alkaloids, proto-alkaloids and pseudo-alkaloids. True alkaloids form water-soluble salts mostly occurring as well-defined crystalline substances. In addition, these compounds have decarboxylated amino acids that are condensed with a non-nitrogenous structural moiety occurring in limited number of species and families. In the class “pro-alkaloids”, however, there is an exception for those amine compounds, like the mescaline amines, that have a nitrogen atom placed in a ring structure (Robinson, 1974). Although proto alkaloids do not have a heterocyclic ring containing a nitrogen atom, they are, nevertheless, also derived from amino acid sources. These alkaloids have a very simple structure with closed rings and are considered a minority group amongst the alkaloids. Pseudo alkaloids contain a heterocyclic ring with nitrogen, but their basic carbon skeletons are not derived from amino acids. These alkaloids are connected to amino acid pathways being derived from the precursors, or postcursors, of amino acids and of non-amino acid precursors (Aniszweski, 2007; Cherney and Baran, 2011). Alkaloids can be classified in terms of their biological activity, chemical structure (nucleus-containing nitrogen) and by their biosynthetic pathway (Roberts & Wink 1998; Hegnauer, 1988).

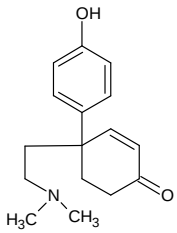
2.3.1 Chemistry of alkaloids applied to the genus *Sceletium*

Van Wyk and Wink (2004) have indicated that alkaloid toxicity and pharmacological activity in animals and humans appears to correlate with interactions involving particular molecular targets. Alkaloids are undoubtedly multipurpose compounds that, depending on the situation, may be variously active, e.g. as defence compounds against herbivorous animals, bacteria, fungi, viruses or competing plants. Knowledge of alkaloidal variation in plants is important in the production of plant medicine, because it allows the plant to be collected and harvested at the right time and the right growth stage (Van Wyk and Wink, 2004).It is believed that the phytochemical exploration of the genus *Sceletium* commenced in 1898 when Meiring isolated a crude alkaloid mixture from *Sceletium tortuosum*. This was followed by the work of Zwicky in 1914 who apparently isolated several alkaloids including mesembrine and mesembrenine (Gericke and Viljoen, 2008). It was suggested that mesembrine isolated by Zwicky was not pure, meaning that the molecular formula ($C_{16}H_{19}NO_4N$) that was first proposed was incorrect. The correct formula ($C_{17}H_{23}NO_3$) was later confirmed in the early 1960's by the well-known German pharmaceutical company C.F. Boehringer and Soehne, and also by S.B. Penick in New York (Gericke and Viljoen, 2008).

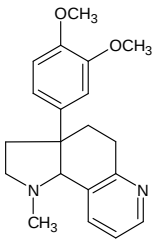
The number of *Sceletium* species within the Aizoaceae family that have been examined for the presence of alkaloids has been restricted by their geographical inaccessibility. Within the *Sceletium* genus, a number of alkaloids are produced which mainly belong to the crinane class of compounds. The structure of four different alkaloids are indicated in **Figure 2.5 (a to d)**: (a) the 2a-aryl-*cis*-octahydroindole class (e.g. mesembrine), (b) the ring C-secomesembrine alkaloids (e.g. joubertiamine), (c) a tetracyclic pyridine alkaloid class with only two reported members, (e.g. *Sceletium* alkaloid A_4), and (d) a ring C-seco*Sceletium* alkaloid A_4 (tortuosamine) (Jeffs *et al*., 1982).



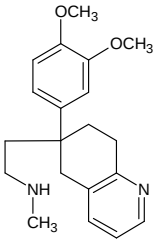
a. Mesembrine



b. Joubertiamine



c. *Sceletium* alkaloid A_4



d. Tortuosamine

Figure2.5: Structure of alkaloids from the genus *Sceletium* (Jeffs, 1982).

2.4 Phytochemistry:

Phytochemistry is the study of the chemical makeup of medicinal plants (Saroya, 2011) and, being a sub-discipline founded on both organic as well as biochemistry, focuses on the extraction and subsequent characterisation of phytochemical entities that have been used by herbalists, but with an added dimension of employing a number of chromatographic techniques to isolate pure compounds for structural elucidation and pharmacological testing (Macías *et al*., 2007).This sub-discipline also plays a fundamental role as an analytical technique for quality control and standardisation of phytotherapeuticals. Column chromatography (CC) was introduced at the beginning of the twentieth century to separate plant pigments and is now routinely used to separate various compounds (targeted and non-targeted, polar or non-polar) from any plant extraction of choice (Ettre, 2003). Thin layer chromatography (TLC) offers the option of presenting the result as a series of banded images which can be photographed, particularly under UV light, and is an ideal screening method for biological and chemical analysis. In general, the TLC methodology provides for chemical identification with either qualitative or quantitative results, or just with semi-quantitative determination (Marston, 2007).

2.5 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) is a highly versatile, chemically non-destructive tool used in analytical chemistry to determine, amongst other things, content and purity of a sample (Moco *et*

al., 2007). In addition, this technique allows for the investigation of structural and dynamic aspects of macromolecules (Griesinger *et al*., 2012). As such, NMR analysis provides an added benefit of being able to accommodate quality control tracking of sample preparation process methodologies. As such, NMR analysis is considered to be one of the most powerful techniques for solution conformational analysis of organic (and inorganic) compounds and bio-macromolecules. NMR spectroscopy, thus, gives detailed information about molecules and their environment based on the interaction of nuclear magnetic moments with electromagnetic radiation (Corcoran and Spraul, 2003). In essence, sample material from fractionated extracts derived from plant material can be analyzed when the material is placed in a strong magnetic field and irradiated with radio waves to cause the entire proton (^{1}H) and carbon 13 (^{13}C) nuclei to occupy the higher energy – $^{1}/_{2}$states. As the nuclei in the sample material preparation relax back to the + $^{1}/_{2}$ state, they release radio waves corresponding to the energy of the difference between the two spin states. The radio waves are recorded and analyzed by computer to give intensity versus frequency plot of the sample. This information can then be used to determine the structure of various compounds present in the sample material (Havsteen, 2002).

2.6 Malaria Bioassay

2.6.1 Bioassay approach to screen for biological activity of various compounds isolated from *S tortuosum*.

It is not a norm to have reports on the medicinal plant use as a key source of health care. It is reported that 80% of the world's population relies on traditional herbs for wellness and reliable health care source. A further estimated 80% of Africans are said to rely on traditional remedies (Karunamoorthi and Tsehaye, 2012). An elevated prevalence of malaria as a disease and its parasitic resistance to treatments resulted in the search for novel anti-malarial compounds from plants used in traditional medicine (Bero *et al*., 2009). Twelve different plants were gathered, extracted and evaluated for their anti-plasmodial activity *in vitro* against a chloroquine-sensitive strain of *Plasmodium falciparum* 3D7 and the *Plasmodium* lactate dehydrogenase (pLDH) technique was used to measure parasite viability according to the method described by Makler *et al*., (1998). Some of the plants screened in this study included *Papalialappacea*, *Byrsocarpuscoccines*, *Trichilia emetic* and *Acanthospermumhispidum*. The J774 macrophage-like murine cells, W138 human normal fibroblast cells, W2 chloroquine resistant strain of pLDH were

also assayed for both cytotoxicity, and anti-plasmodial activity. From this study, Bero and colleagues (2009) found that out of the 42 extracts yielded from the 12 plants, 5 were considered effective with IC_{50} values ≤20 µl/ml. Most extracts were considered not to be toxic. Only two extracts extracted with dichloromethane were found to be toxic (Bero *et al*., 2009). A similar study, where 170 plant species reported to be commonly used as traditional medicine for various pharmacological properties including anti-parasitic activities, was undertaken on indole alkaloid-containing plants possessing anti-plasmodial and cytotoxic activities (Girardot *et al*., 2012). Anthropologically based studies on plant usage to treat malaria have also been undertaken as described by Stangeland *et al*., (2011).

2.6.2 Malaria Rapid Diagnostic Tests (RDTs)

The *in vitro* diagnostic assay is based on the specific detection of *Plasmodium* Lactate DeHydrogenase (pLDH) activity. This assay exploits a panel of monoclonal antibodies that capture the parasite enzyme and allow for the quantitation and species identification of human malaria infection. The pLDH enzyme is produced by live parasites and is easily detectable when they are in late trophozoite and schizont phase. Based on the detection of this enzyme, relative drug resistance and activity can also be detected for all human *Plasmodium* infections (Piper *et al*., 1999).

Chapter 3

Research Methodology: Part A

3.1. Plant collection and initial extraction protocols

3.1.1 Plant collection

Nineteen kg of *Sceletium* plant material was harvested on 11 – 12 June 2009 under sunny and dry conditions at Kamieskroon in the Northern Cape of South Africa. The plant material was supplied to the Council for Scientific and Industrial Research (CSIR) for this study by Enterprise Creation for Development (ECD) and collected by Mr Jonny Burger, an independent botanist. This study required harvesting of whole plant material by the supplier. The mass of the plant material thus supplied depended on the availability of the plants themselves. Plant material was submitted to the South African National Biodiversity Institute for positive identification and confirmation. The plant material was identified as *Sceletium tortuosum* N.E. Br. Storage, transportation and handling were carried out by following standard operating procedures to allow the material to be used in product development.

A few months before the study ended, Parceval supplied two batches of dried, ground plant material to the CSIR for the stability test required for this study. It is not known if they were harvested at the same time or place. The first batch was ground, but could be visibly identified as *Sceletium* species (spp.). The total mass supplied and used was 1.280 kg. The second batch, although not ground to a fine powder, could not visibly be identified as *Sceletium* spp. and because of this, extractions and experiments on this batch of plant material were carried out separately. The original mass received was 2.423 kg, but only 0.423 kg was used for this study and the rest was used by CSIR researchers for an independent study.

3.1.2 Ethical consideration

Permission to carry out this study was requested from the Research and Ethics Committee of The University of South Africa (UNISA) (Muckleneuk campus). Accompanying the application form were copies of: the Bio-risk assessment Bioprospecting CSIR research check list; the Harvesting of plant material certificate; the plant identification certificate issued by South African National Biodiversity Institute (SANBI) and the research proposal. Permission was granted by the UNISA Research and Ethics Committee. In terms of CSIR regulations, a Research Ethics check list was used to identify whether a full application for ethics approval needed to be submitted before the work commenced - according to the check list this was deemed not to be necessary.

3.2 Plant preparation and extraction

3.2.1 Plant preparation

The experimental process was divided into 3 groups designed to accommodate:-

- freeze drying of wet plant material;
- fermentation in a conventional oven at 35^{o}C under natural conditions and;
- drying plant material in a conventional oven at 60^{o}C.

From the 19.8 kg of plant material received, 10 kg of fresh *Sceletium tortuosum* plant material was retained at -20^{o}C, while the remaining 9.8 kg was used for the experiment. The experimental stock was divided into 14 batches of 250 g each and the remaining material of 6.3 kg was placed in a 60^{o}C oven to dry. The dry plant material was not ground to a fine powder, but was broken and crushed into smaller pieces. The first 250 g of plant material was crushed and extracted on the day of arrival. The second 250 g was placed at -20^{o}C and then freeze dried the following day. After 6 months, from the 10 kg of specimen that had been stored at -20^{o}C, 250g was weighed out and then freeze-dried for approximately 36 hrs. After freeze drying, the plant material was crushed and broken into smaller pieces.

3.2.1.1 Extraction

Plant material was transferred to 1000 ml glass beakers (Schott-Duran, Germany). Two magnetic stirrers and sufficient ethanol solvent (96% Rectified A.R., Radchem, E1315X) was added to cover the plant material which was placed into each beaker during the extraction process. The mixture was covered tightly with a piece of aluminium foil before being placed on a stirring heating plate (Labotec, Heidolph, MR 3002) and heated at 40^{o}C for 2 days (48 hrs). The temperature was monitored throughout the extraction process with a mercury thermometer. After extraction, the contents were mixed thoroughly and then filtered through filter paper into a 1000 ml conical flask connected to a vacuum pump (KnF Neuberger, Laboport). The extract was filtered twice to remove any debris remaining in the suspension. The ethanol in the filtrate was evaporated at 45^{o}C (Med + low BP Mixture) using the GeneVac® Personal evaporator.

The following step applies only to the last sample mentioned in plant preparation part A. Following evaporation of the ethanol, 100 ml of water was added to the dry extract that was then dissolved and freeze dried. From the dry plant material, 2.3 kg was extracted in the same manner as above. For the remaining 4 kg, the same method was followed as described above, but only in this case chemically pure (CP) grade methanol (Merck, RSA) was used as the solvent of choice.

3.2.2 Plant preparation - Fermentation

Of the 12 remaining experimental 250 g samples, 2 x 250 g was prepared for 7 days' fermentation under natural conditions. The first 250 g was placed in a thermal plastic bag and tightly sealed, while the duplicate 250 g amount was placed in an open aluminium tray. These samples were crushed and placed under direct sunlight and left for seven days. On the third day of the experiment, the plant material was thoroughly mixed, re-sealed and left to further ferment till the seven days had elapsed. The following process was used for the remaining 10 x 250 g samples. Of these, 5 x 250 g amounts were placed in thermal plastic bags that were then sealed and labelled as follows: Day 2 (48 hrs), Day 4 (96 hrs), Day 6 (144 hrs), Day 8 (192 hrs) and Day 10 (240hrs). Additional duplicates were prepared with the remaining 5 x 250 g amounts that were placed in aluminium open trays and labelled appropriately. While in the baggage state, plant material was crushed and subsequently placed in an oven for fermentation at 35^{o}C, for an

appropriate period specified on the labels. In parallel, but only after 6 months had elapsed, 4 kg of the original plant material stored at-20^{o}C, was divided into 10 x 400 g. The method of fermentation described above was subsequently followed for these new samples. To better understand the process above, please view **Figure 3.1** and **Figure 3.2** on pages 28-29.

3.2.2.1 Extraction of Fermentation Products

After 2 days of fermentation, the samples labelled Day 2 (A - thermal bag) and (B - open tray) were removed from the oven and transferred to 1800 ml glass beakers for extraction. Two magnetic stirrers were placed in the beakers, one was placed at the bottom of the beaker under the plant material and the other stirrer was placed on top of the plant material. Approximately 600 ml of 96% Rectified Ethanol (A.R.) was poured into the beakers to cover the plant material which was then tightly covered with a piece of aluminium foil. The beakers were then placed on a stirring heating plate that was adjusted to 40^{o}C and set at 1250 revolutions per min (rpm) for the duration of the extraction process. The plant material in the two beakers was extracted for 2 days (48 hrs). During this extraction period, the plant material was manually stirred and crushed, while the temperature was monitored with a mercury thermometer. Following extraction, the contents were thoroughly mixed before being filtered through a clean paper filter into a 1000 ml conical flask. The extracts were filtered twice to remove any debris remaining in the suspension. Filtrates were evaporated at 45^{o}C (Med + low BP Mixture) using the GeneVac® Personal evaporator connected to a vacuum pump (KnF Neuberger, Laboport).Of the 4 kg fermented plant extraction that was carried out 6 months after the initial fermentation process, the following steps were added to the process described above. Specifically, filtered suspensions were further divided into two equal volumes before solvent evaporation. After the ethanol was evaporated, one of the dry extracts was dissolved in 100 ml of water and freeze dried. The other dry extract was dissolved in methanol and evaporated at 45^{o}C using the rotary vacuum evaporating system, after which it was stored at 4^{o}C. Apart from these additional steps, the extraction processes for both stored and directly extracted sample lots were identical.

3.2.3 Parceval plant extraction

Parceval dried plant material was extracted with ethanol according to the extraction process described in section 3.2.2, except that this extraction took place at room temperature. These extracts were stirred continuously and temperature was monitored throughout the process. Concentration/evaporation was also carried out as described in section 3.2.2, but none of the extracts were freeze dried. These extracts were labelled as follows: SCE-187-46311 and SCE-187-46312.[1]

[1] To further describe the processes mentioned above, the fermentation processes are shown in **Figure 3.1** and **3.2**. **Figure 3.1** summarizes extraction of all samples from the initial 9.8 kg plant material, while figure 3.2 summarizes extraction process of the 4.25 kg sample that had been stored for 6 months at -20^{o}C.

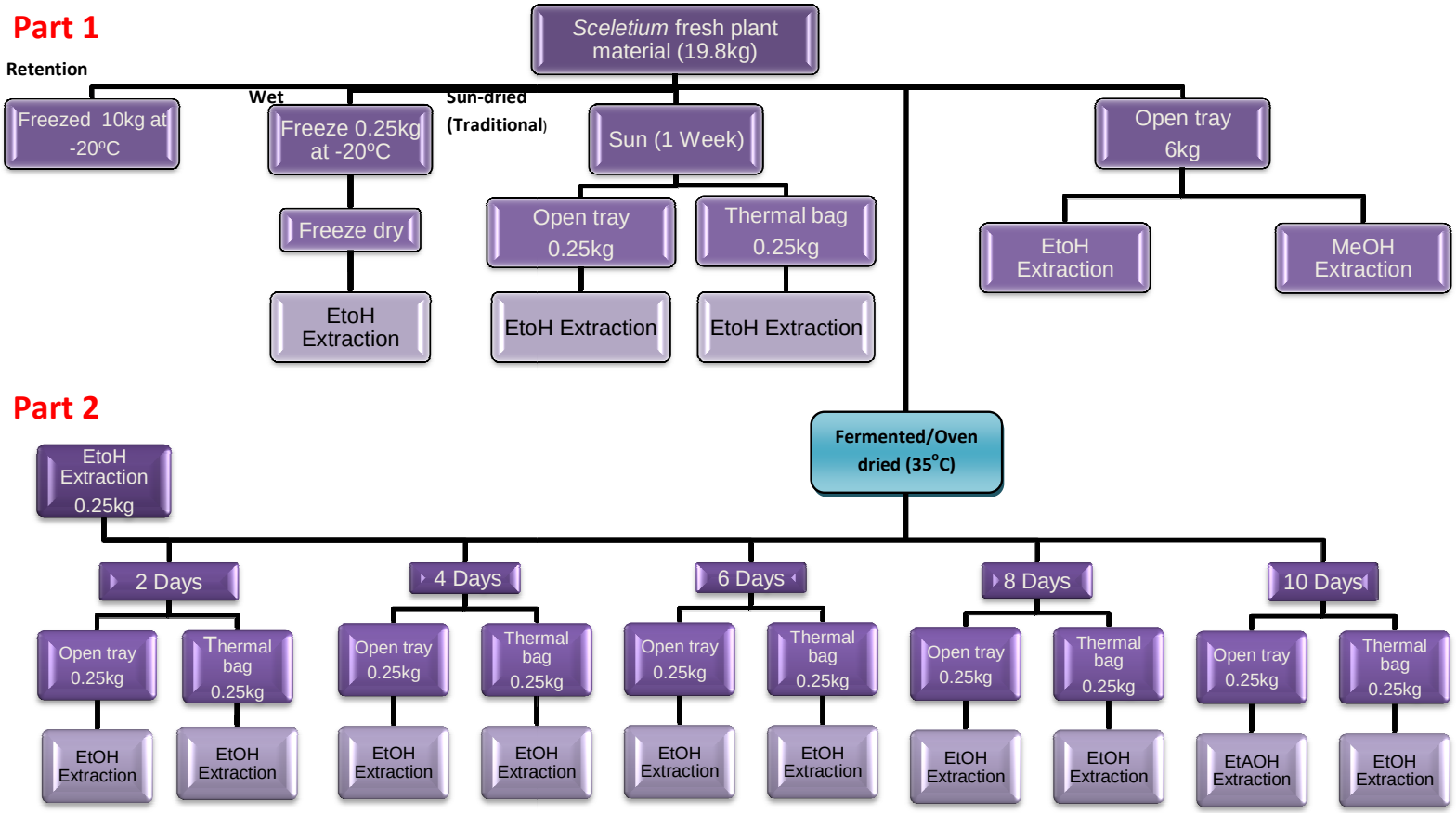


Figure 3.1: Extraction process followed for *S.tortuosum* fresh plant material.[2]

[2] Whole plant was used in all preparations.

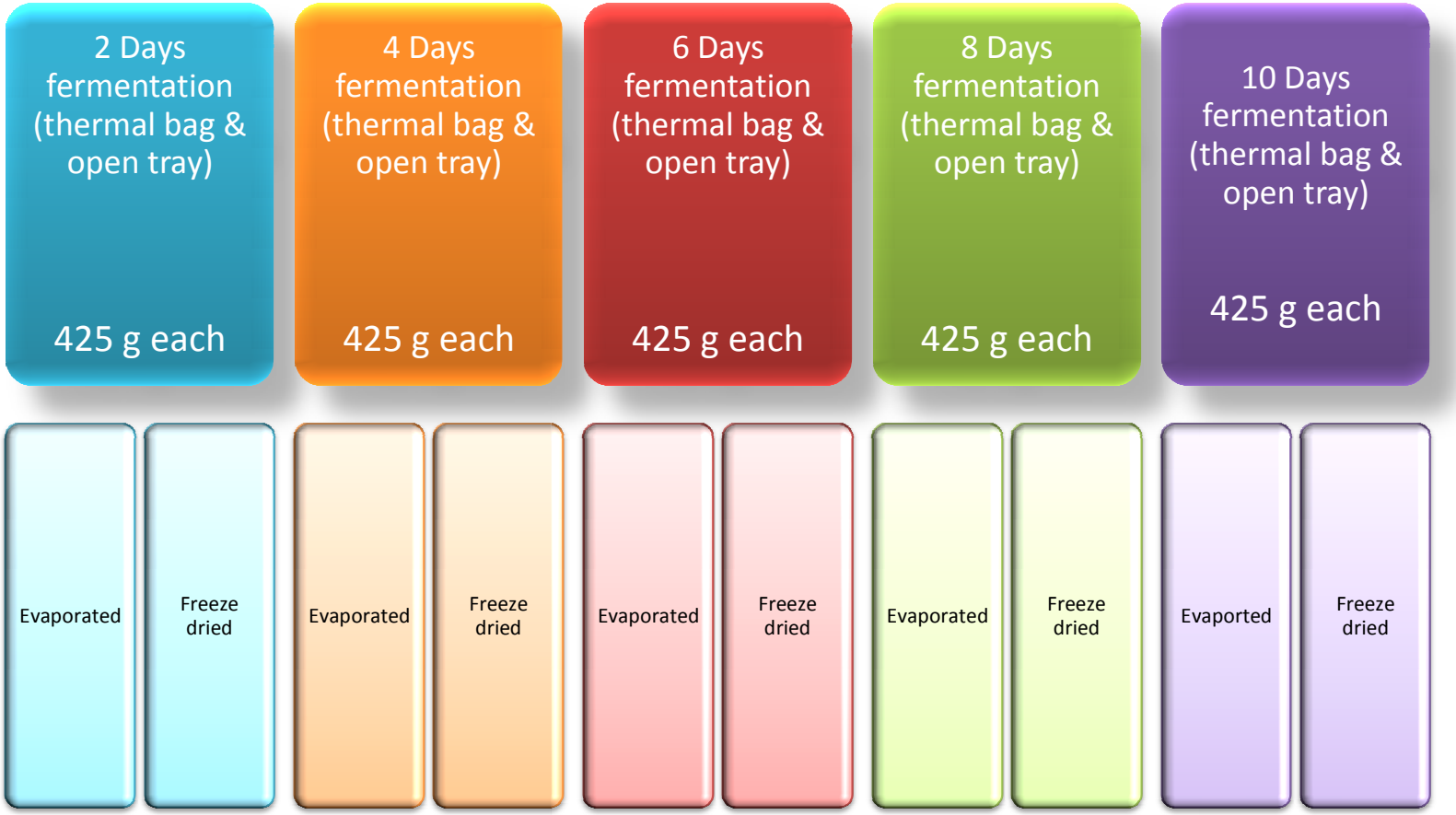


Figure 3.2: Extraction process followed for *S. tortuosum* plant material retained at -20°C.[3]

[3] This is the same as part 2 of **Figure 3.1** page 30.

Chapter 3 (Continued)

Research Methodology: Part B

3.3 Chemical analysis:

3.3.1 Chromatography

3.3.1.1 High Performance Liquid Chromatography (HPLC)

The word 'chromatography' was originally applied to a process to separate coloured (chromatogram) chemical entities via differential migration through an immobile (stationary phase) using a suitable solvent (the mobile phase). When the migration is in a liquid phase, percolating through a column filled with a stationary material, the technique is called liquid chromatography (LC). The stationary phase can be an adsorbent or a liquid-impregnated adsorbent. The mobile phase is a liquid. When the mobile phase is under increased hydraulic pressure, this allows for more efficient column separation of closely sized/electronically charged sample components and LC is referred to as high pressure liquid chromatography (HPLC). Efficiency of HPLC is expressed in the reduced plate height form and can be obtained with similar particles and, therefore, higher pumping pressures and by using good column technology. Thus, it can be argued that HPLC is that form of chromatography when high pressure is applied, regardless of efficiency (Verzele and Dewaele, 1986).

On a chromatogram, the peaks are recorded on a time axis from the time that the sample is introduced into the column. The peaks are represented in a form of a (quasi) Gaussian curve. The mean time, at the maximum of this curve that a peak spends in the column, is the retention time for the compound (t_R/Rt). When reporting or referring to a peak, its Rt is important to state as different compounds elute at different times due to, for example, the difference in molecular masses. The Rt is what makes it easy to distinguish between two different compounds, although it is possible to find two different compounds eluting or presenting with the same Rt. Retention time is usually displayed at the top of every peak in the chromatogram, but it can be calculated using the following formulae $t_R = t_o$ (1=k') where t_o is the "hold up" time of the column where non-retained and non-

adsorbed molecules are eluted; and k' is the ratio of the concentrations, weight or number of moles in the two phases of the chromatographic two phase system (Verzele and Dewaele, 1986).

High performance liquid chromatography (HPLC) is used routinely in phytochemistry to pilot the preparative isolation of natural products. Essentially, this involves the optimization of the experimental conditions by paying particular attention to the consistency of the yield of different fractions throughout the separation, in order to control the final purity of the isolated compounds (Marston, 2007). Other chromatographic techniques employed in the purification of fractions and crude extracts during this study included flash chromatography (FC), column chromatography (CC), TLC, preparative-TLC and use of the chromatotron. Depending on the complexity of the crude extract or fraction being purified, CC employed different column sizes ranging between 11 – 60 cm in length and 1.5 - 6 cm in diameter with, 10 - 1000 ml capacity. Silica gel 60 (particle size ranging from 0.063 – 0.2 mm, 70 – 230 mesh ASTM) (Sigma – Aldrich Logistic GmbH) was used as a stationary phase. Monitoring of the progress of the CC technique was done by using 0.2 mm thick silica pre-coated TLC aluminium or glass plates (F_{254} UV active) viewed under short (254 nm) and long (365 nm) wavelength ultraviolet fluorescence within an analysis cabinet (Spectroline model CM-10, Spectronics corporation, Westbury, New York U.S.A).

3.3.1.2 Isolation and purification of compound 1

Approximately 2 g of ethanol (EtOH) extract was weighed (Sartorius AG GŐTTINGEN weighing instrument, Max 200 g – d=0.1 mg, Germany) and dissolved in 10 ml of methanol (MeOH), sufficient to cover and dissolve the extract. Sufficient silica gel was added to the suspension and the solvent was evaporated. A flash column was prepared as follows: silica 60 was mixed in chloroform ($CHCl_3$, CP Grade, Merck, RSA), sufficient to create a mobile phase for column packing, and then poured into the 500 ml column whose base was blocked with a cotton wool plug that allowed the solvent to flow but prevented silica from escaping with the solvent. The excess solvent was removed from the column until just about 20 ml remained above the silica (this method of preparation was applied to all columns). The solvent was allowed to run out of the column to just above the silica bed before the sample was loaded on top of the prepared column filled with silica gel before eluting (the volume of the silica gel differed according to the size of the column being used. This was then covered with a thick layer of cotton wool to prevent disturbance and layer

distortion when adding the solvent. Solvent systems used were $CHCl_3$/MeOH 9:1, $CHCl_3$/MeOH 8:2 and $CHCl_3$/MeOH 7:3 and finally 100% MeOH. Twelve fractions of between 300 – 400 ml were collected into 500 ml Erlenmeyer flasks. These were placed in the fume hood to evaporate the solvent. When spotting on TLC plates, the fractions were dissolved with 1 ml MeOH. The plates were run in 9:1 $CHCl_3$/MeOH.

Compound 1:

Fraction 7 indicated that it was a suitable fraction for further purification. This was as a result after spraying the TLC plate with the spray reagent and target compounds were identified in large quantities in this fraction. Since alkaloids were compounds being targeted, a spray reagent that could react with nitrogen- or amine-containing compounds was essential. Ninhydrin (2.2-dihydroxyindan - 1,3-dione, $C_9H_6O_4$, Merck, RSA) was used to spray all plates in this experiment. A second column was run with the same solvent systems as the first column and 35 fractions were collected. Fractions 2 – 21 were combined, evaporated and purified using preparative TLC (Prep-TLC) using the same solvent as above.

3.3.1.3 Isolation and purification of compounds 2 and 3

Approximately 5 g of methanol extract (sample no. SCE-187-46222) was weighed out and dissolved in methanol (MeOH CP grade, Merck, RSA). Silica gel 60 (particle size ranging from 0.063 – 0.2 mm, 70 – 230 mesh ASTM) (Sigma – Aldrich Logistic GmbH) was added to the suspension and the solvent subsequently evaporated. A gravity column (with reference to that described in section 3.3.2.1) was then prepared and fractions eluted with $CHCl_3$/MeOH (9.5:0.5). Fractions of about 50 ml were collected into 100 ml bottles. Solvent ratios were increased by 0.5% MeOH after every 1 L of solvent system used. The results of the TLC were used as an additional guide line to determine solvent system change. When spotting a fraction on TLC, one or several constituents may elute at different retention times (the distance at which the compound has travelled along the plate when exposed to a solvent system). In a serial fashion, when no more constituents are detectable, either UV positive or non-UV positive, the solvent system is changed and the polarity increased to elute more polar compounds than those previously isolated. A total of 497 of the 50 ml fractions were collected, concentrated and dissolved in 1 ml methanol. The fractions were spotted on TLC plates and resolved with $CHCl_3$/MeOH (8:2). Since there were many

fractions collected, many samples were combined, based on the similarities portrayed on the TLC plates. The 18 new fraction combinations were named group A – R. Forty eight hours after the column was completed, all new groups were dissolved in methanol and crystals were observed in a few of the vials from fraction I - N. Crystals observed in fraction I through to fraction K looked similar in colour, texture and morphology and these crystals were different from those noted in fractions L and M.

Compound 2:

Crystals formed in fractions K, L and M were combined and washed with methanol. As they did not dissolve in MeOH, dimethylsulfoxide (DMSO) was used to dissolve the crystal in preparation for TLC spotting. The solvent system used to run the plates was acetone, butanol, H_2O and acetic acid (40:10:5:1). As these compounds were found to be non-UV active, they were provisionally assumed to be a sugar connected to a nitrogen-containing compound, and subsequently sprayed with both vanillin and ninhydrin spray reagents to help prove their identity.

Compound 3:

The column was washed with $CHCl_3$/MeOH (6:4 – 100%), concentrations were increase by 1% MeOH for every 500 ml used. These Erlenmeyer flask fractions were covered with a cotton wool plug and placed in an open space at room temperature and allowed to stand until complete evaporation had occurred. Some fractions (view section **3.3.1.3 Isolation and purification of compounds 2 and 3**~~**3.3.1.3 Isolation and purification of compounds 2 and 3**~~; **Figure3.4**, page 40) produced similar crystals and so the fraction with the most crystals was selected for further analysis. These crystals were dissolved in 1 ml H_2O and analysed on TLC in the same solvent system used for compound 2, before being sprayed with vanillin. Unfortunately, the oil in these samples could not be separated from the crystals and this led to the compound streaking on the TLC plate. The sample was evaluated against table sugar as a control, as it was suspected to be sucrose. To prove if this compound was sucrose, NMR analyses were performed on these samples.

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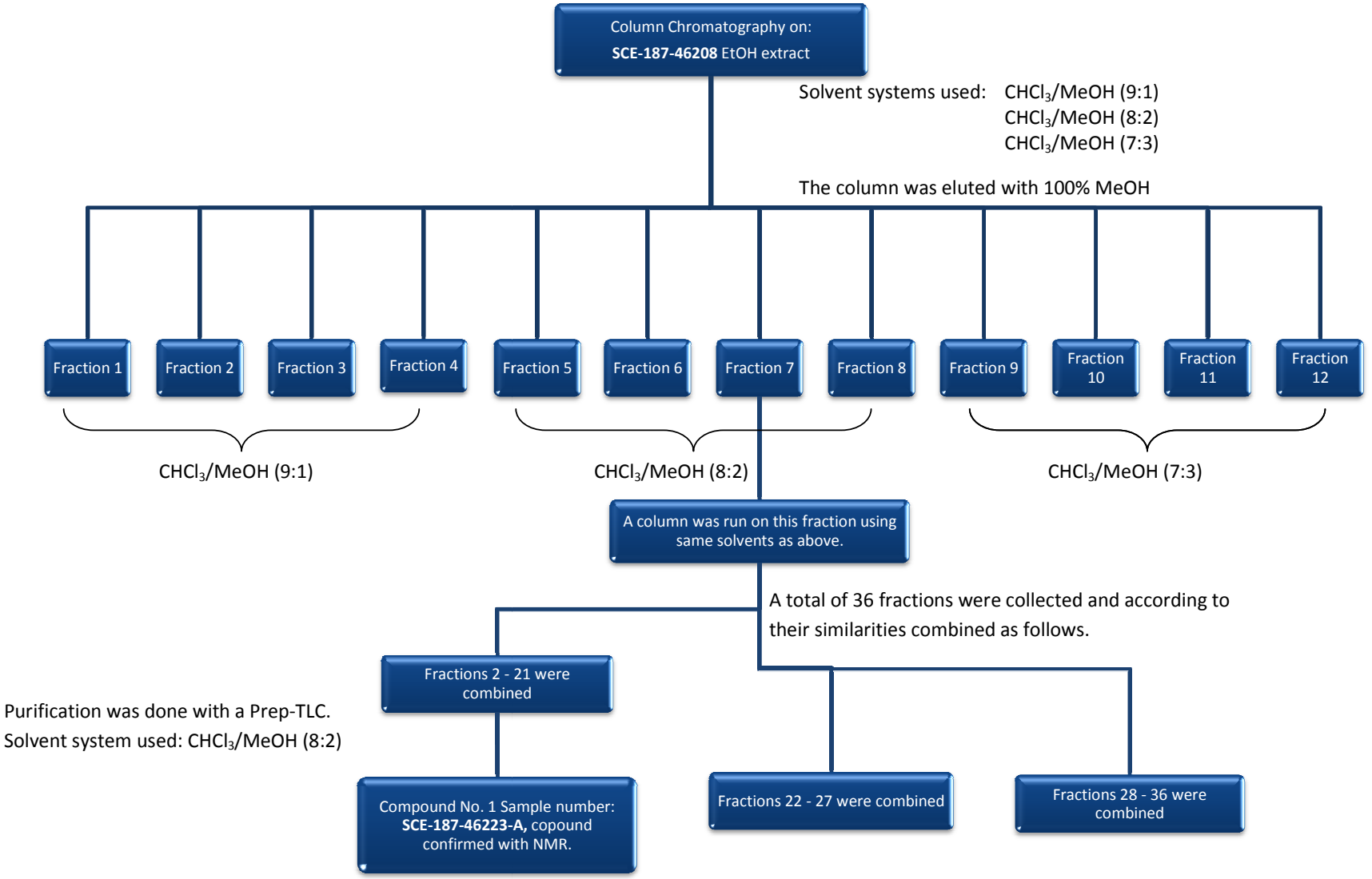

Figure. 3.3: Column chromatography: Isolation and purification of compound 1 and compound 2.

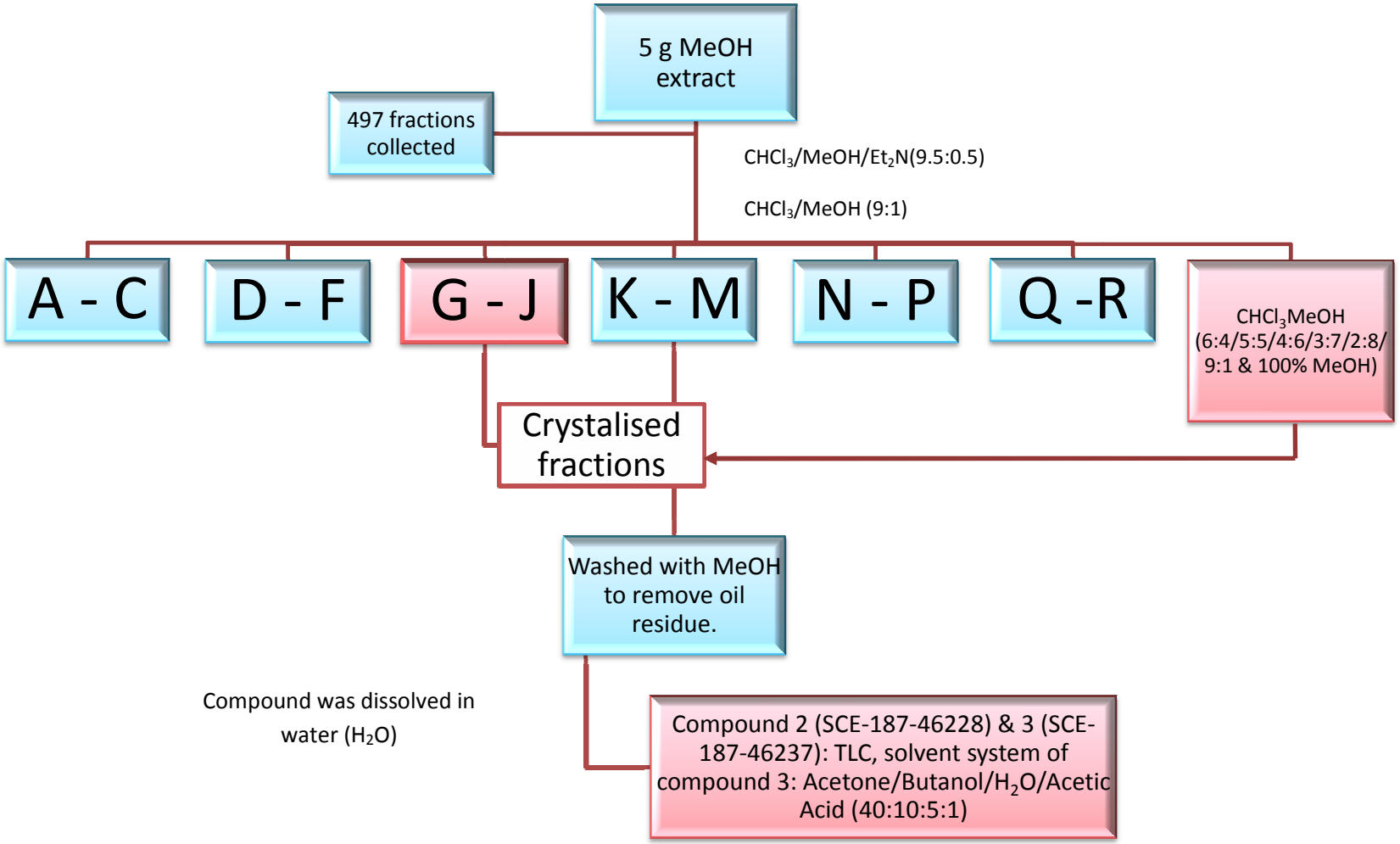

Figure. 3.4: Column chromatography: Isolation and purification of compound 3 and compound 4.

3.3.1.4 Isolation and purification of compound 4

Approximately 1 g of EtOH crude extract (SCE-187-46208) was dissolved in 15 ml MeOH. The solution was divided into two (7.5 ml each) and applied onto two separate aluminium TLC plates. Hex/EtOAc/Et_2N (hexane/ethyl acetate/diethylamine) (6:4:1) was the solvent system used to develop the plates. UV active bands were circled and removed from the plate. Similar bands on the TLC plate were combined and cut into small pieces and placed in a 10 ml beaker. The compound was extracted with MeOH. The solutions was filtered and concentrated by solvent pervaporation under a cool stream of air. The compound was then dissolved in 1 ml MeOH and analysed using TLC to confirm that it was a pure compound for further analysis by NMR. The compound was dissolved in deuteratedCHCl$_3$ ($CDCl_3$), filtered and analysed.

3.3.1.5 Isolation and purification of compound 5

About 1 g of MeOH crude extract (SCE-187-46222) was dissolved in 10 ml MeOH. About 5 ml of the mixture was applied on each of the two prep-TLC glass plates and eluted with Hex/EtOAc/Et_2N (6:4:1). All UV active bands were circled and similar bands were combined in one beaker. The compounds were extracted from the silica using MeOH, filtered and concentrated. As such, the volume of MeOH used to dissolve the compounds varied from beaker to beaker, as the amount of silica in the beakers was not identical. The dry compounds were then dissolved in 1 ml MeOH and analysed by TLC using the same solvent system as above. Preparative TLC was repeated on individual compounds to further purify them for NMR analysis. Two compounds were successfully purified, one of these being compound 1, while a second compound was also identified and confirmed with NMR experiments. This compound was then labelled as SCE-187-46311-B.

3.3.2 HPLC analysis

The HPLC technique was used to chemically profile the plant extracts, while mass spectrometry was used to verify the molecular weight of some of the isolated compounds including mesembrine, Δ4mesembrenone and obtusalin. Analytical (chromatographic) methods were developed by Mr. Nial Harding, who, together with Dr Paul Steenkamp, generated the data for subsequent analysis.

About 25 mg of the ethanol extract (sample number: SCE-187-46208 ECD-MP-0054) was weighed into a 5 ml vial. To this 5ml of methanol was added. The vial was then placed in an ultrasonic bath (Integral systems, Main 220 V. RSA) until the extract was dissolved. The sample was filtered through a 0.2 µm filter membrane GMP using a 2 ml syringe (Norm-jectLuer Lock) and collected into an HPLC vial.[4] About 2.0 g of pre-dried plant material provided by ECD (sample numbers: TTSI-MP-0013 and TTSI-MP-0015) was weighed out and ground into powder. Each was extracted into 5 ml of ethanol and left to stand for four hours, before being placed in the ultrasonic bath (no heat applied) for a further one hour. The extract was then filtered and transferred into an HPLC vial as described above. Two capsules of *S. tortuosum* (kana) were emptied into a 5 ml volumetric flask; the powder was extracted into 5 ml of ethanol as described above. The sample was then filtered and transferred into an HPLC vial as described for the first sample. The tincture was not treated before use.

3.3.2.1 HPLC-UV-MS analysis

Extracts and compounds were analysed with a Waters Alliance 2695 HPLC linked to a Waters XBridge C18 3.5 µm, 2.1x150 ml dimension column. Separation required a pH of approximately 10, thus 10 mM ammonium bicarbonate buffer (pH 10) was used. The sample injection volume was 5 µl. UV detection was done on a Waters 996 PDA scanning from 210-400 nm. Running conditions are summarised in **Table 3.1.**

[4] Note: The method was followed for all extracts analysed.

Table 3.1: HPLC gradient timetable/method

Time (min)	Flow rate (ml/min)	% B	% C	%D
0.00	0.3	20.0	80.0	0.0
10.00	0.3	20.0	80.0	0.0
12.00	0.3	25.0	75.0	0.0
40.00	0.3	30.0	70.0	0.0
48.00	0.3	45.0	55.0	0.0
80.00	0.3	70.0	30.0	0.0
100.00	0.3	0.0	0.0	10.00
107.00	0.3	0.0	0.0	10.00
110.00	0.3	20.0	80.0	0.0
120.00	0.3	20.0	80.0	0.0

Mobile phase used

Solvent B – 20.0% Methanol

Solvent C – 80.0% 10 mM $(NH_3)_2HCO_3.H_2O$

3.4 NMR sample preparation

Compounds were purified in the laboratory as described in Section 3.3. The compounds were concentrated by solvent pervaporation under a stream of cold air. Deuterated solvents (MeOH, $CHCl_3$ and DMSO) were used to dissolve the compounds according to their solubility. Once dissolved, the compounds were filtered through compressed cotton wool before being added into the NMR tubes. ^{1}H, ^{13}C, and other two dimension (2D) experiments required were conducted using the Varian 600 MHz NMR instrument. Time duration of these experiments and the quality of the results depended on the sample concentration.

3.5 Biological Assay:

3.5.1. Bioassay approach to screen for biological activity of various compounds isolated from *S. tortuosum*.

3.5.1.1 Malaria Rapid Diagnostic Tests (RDTs)

The malaria rapid diagnostic tests (RDTs) include immunochromatographic tests (ICTs) that target antigens on one or more *Plasmodium* species (Maltha *et al*., 2010). As such, these RDTs were chosen as one of the bioassay options, but not specifically directed towards finding anti-malarial activity of any of the compounds isolated from *S. tortuosum*.[5] Positive ICT results would be indicated by two to three test lines that turn cherry-red to purple with one of these lines being a control line (to indicate if the test was performed correctly). The original two band ICT generates a test line that targets *P. falciparum* by detecting either histidine-rich protein 2 (HPR-2) or *P. falciparum*-specific parasite lactate dehydrogenase (Pf-pLDH) (Piper, 2011; Maltha *et al*., 2010).

The more recently developed three band ICTs include a second target that is common to the four *Plasmodium* species, including aldolase or pan-specific parasite lactate dehydrogenase (pan-pLDH). A concern with the three-band ICTs is that this test detects a *P. falciparum*-specific antigen and a pan-*Plasmodium* antigen that cannot be used to distinguish between a *P. falciparum* infection and a mixed infection with *P. vivax*, when both test lines are simultaneously observed. Discrimination between the two species is important; since both *P. falciparum* and *P. vivax* require different treatment scenarios (Maltha *et al*., 2010).

3.5.2. *Plasmodium* lactate dehydrogenase (pLDH) screening

The enzyme lactate dehydrogenase (LDH) is found in all cells (prokaryotes and eukaryotes), being a catalyst in the formation of pyruvate from lactate (Vander Jagt *et al*., 1990). It was then reported that parasite lactate dehydrogenase (pLDH) activity is distinguishable from host LDH activity on the basis of the unique epitopes found in the pLDH protein, as well as on the enzymatic characteristics i.e. by its capability to use the 3-acetyle pyridine adenine dinucleotide (APAD) analog of nicotinamide adenine dinucleotide. Such an observation has led to the development of an enzymatic method for the evaluation of anti-malarial compounds (Makler et al., 1993; Makler *et al*.,

[5] Nevertheless, a detailed account on the pathogenesis and distribution of malaria infections is included in Appendix 5.

1998). By way of contrast, usage of the pLDH assay has been extended to the examination stage, to determine the presence of pLDH antigen in lysed whole blood (Cooke *et al*., 1999). In the process, LDH reduces nicotinamide adenine dinucleotide (NAD) co-enzyme to NADH. The NAD analogue APAD is reduced to APDH, which reduces the chromogenic substrate Nitro Blue Tetrazolium (NBT) using the enzyme diaphorase and changing the substrate from a yellow colour to a purple formazan product (see **Figure 3.5).** The host erythrocyte lactate dehydrogenase activity can easily be distinguished from the pLDH, as it is incapable of using APAD as a co-factor. To quantitate the formazan formation, a spectrophotometer is used to read light absorbance of each well at 620 nm. Formazan levels are used to measure parasite survival in the presence of the test compound. Formazan levels, thus, depend on pLDH activity that also acts as an indicator of parasite concentration in the culture (Piper *et al*., 1999; B.P.R.C., 2010).

Schematic representation:

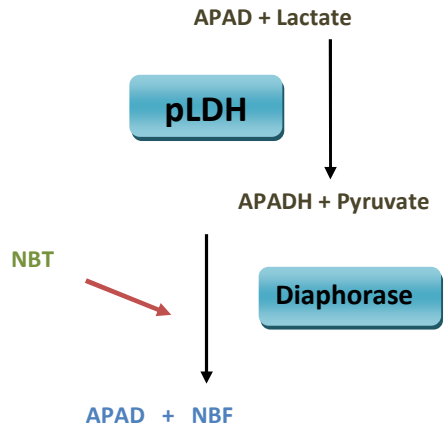

Figure 3.5: Schematic representation of the principle behind the pLDH assay.

3.5.3. Preparation of test samples and standards

The following protocol is a standard method which was followed for all experiments undertaken for anti-malarial screening. A stock solution of 20 mg/ml in 100% MeOH/DMSO/H_2O was prepared. This was followed by 1:10 dilution of the stock solution in complete medium (CM), to obtain the desired starting concentration (200 µg/ml, 20 µg/ml or 2 µg/ml). The final concentration and solvent percentages were, therefore, 100 µg/ml (0.5%), 10 µg/ml (0.05%) and 1 µg/ml (0.005%). Wells treated with chloroquine was used as the reference standard.

3.5.4. Preparation of micro-titre plates (96 wells)

The assays were performed when the parasites were in their trophozoite stages. Thus, blood with a 2% haemotocrit and 2% parasitaemia (3D7 *falciparum* strain) was prepared. All 96 well experimental plates were labelled, divided and prepared as indicated in **Figure 3.6**: In column 1, 100 µl of 2% haematocrit blood and 100 µl of complete medium (CM), (prepared with RPMI 1640 Media 500 ml containing L-Glutamine and 25 mM HEPES, 2 g Glucose, 0.044 g Hypoxanthine, 2.5 g Albumax II and 0.6 ml Gentamicin, (supplied by Whitehead Scientific) was added. In column 2, 100 µl of 2% parasitaemic blood and 100 µl of CM were added. Column 3 represented test samples, and to each of three wells was added 150 µl of test sample in triplicate. A volume of 100 µl CM was added in column 4 right through to column 12. Three-fold serial dilutions of test samples were prepared using CM from column 3 – 12 (Serial dilutions: 50 µl CM was added to 100 µl in Compound 5 (column 4), mixed, then 50 µl was taken from column 4 into column 5. The process was repeated until column 12 was reached, with the remaining 50 µl in tubes being discarded. About 100 µl of 2% parasitaemic blood was added to columns 3– 12, bringing the final volume in each well up to 200 µl. The plates were then covered with a lid and placed in a chamber, where they were gassed (Fresh line Mixture Inert gas heavier than air CO_2, 5% O_2 balance N_2, Air products, K360C) for approximately 1 minute, before being placed in a 37^oC incubator for 48 hours.

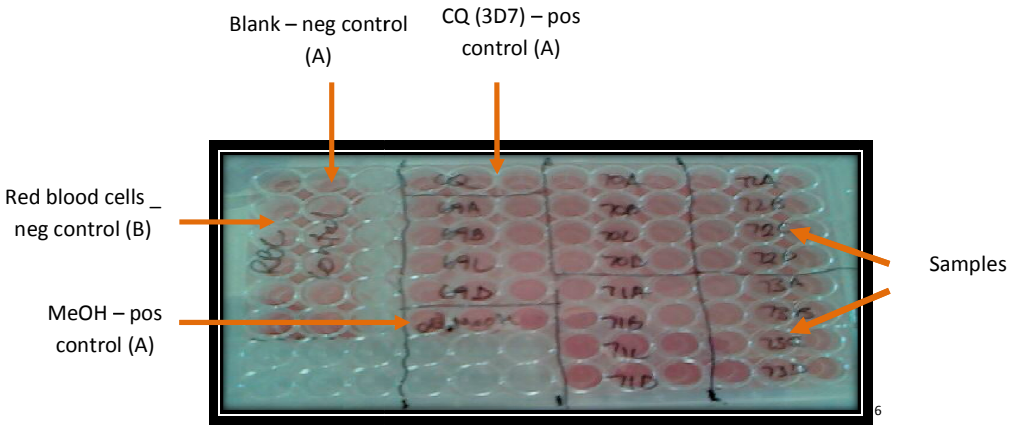

Figure 3.6: Photograph of one of the single concentration experimental 96 well plates with labelled wells. RBC - red blood cells. Negative Control –blank, CQ –Chloroquine (3D7), MeOH– Methanol (positive control) and those with numbers i.e. 69A are number of samples. (Vertical columns 1 through to 12 run sequentially from L to R)

3.5.5. Preparation of reagents for plate development

Malstat: 400 µl Triton X-100

4 g L–lactate

1.32 g Tris buffer

Add 200 ml mH_2O. Stir until dissolved.

Add 22 mg of APAD and pH titrated down to 9.

NBT/PES: 160 mg Nitro blue tetrazolium salt

8 mg phenazineethosulphate

Add 100 ml mH_2O. Cover the bottle with foil.

Store at 4˚C

[6] This plate shows screening of extracts done in triplicate as a selection criterion. These are done in single concentrations before moving to the next step in section 3.5.4.

3.5.6. Developing plates (Non-sterile)

For each experiment, fresh microtitre plates were prepared and to each well was added 100 µl of malstat. About 20 µl of cell suspension was added to the wells, which was followed by the addition of 25 µl of nitro blue tetrazolium (NBT, Merck, RSA) chloride. As the reagents used in this step were light sensitive, plates were kept in a dark area and covered with foil for 2-3 minute incubation periods. The absorbance of the individual wells in each plate was then measured at 620 nm (TecAN – Manufacturer, Infinite F 500). All of these experiments were done in triplicate.

Chapter 4

Results and Discussion

Amongst other compounds, *S. tortuosum* is known to contain alkaloids. Alkaloids are complex molecular structures containing heterocyclic-bound nitrogen atoms, with more or less expressed basic character associated with pronounced physiological action of being found in both plants and animals. These highly bioactive plant compounds were commonly used in all major human cultures since ancient times (Zenk and Juenger, 2007).

Sceletium has been known as a traditional sedative of the Khoi-San people of the Cape Province of South Africa, being a plant that is widely used for its mood elevating properties, as well as its ability to reduce anxiety and stress (Gericke & Viljoen, 2008). Currently, *Sceletium* is marketed through health shops and on the internet as dried plant powder and in capsules, tinctures and tea bags. This plant is alleged to be useful in the treatment of psychological disorders and studies were required to identify compounds present within the extract. Since there were no available analytical methods in the literature and no commercially available standards of relevant alkaloids for use in the analysis and quality control of *Sceletium* products and dosage forms, Patnala and Kanfer (2010) took it upon themselves to isolate and characterize appropriate analytical markers for use in assays and as well as markers for fingerprinting by HPLC. The main objectives of their study were the development of an efficient HPLC method for the separation and quantitative analysis of the relevant alkaloid components in *Sceletium*, as well as to reduce the RT of epimesembranol, in particular, from >30 min.

In this particular study, the identity of the peaks in the standard chromatogram was confirmed by the respective spectral data generated from several analytical techniques, including ^{1}H, ^{13}C and 2D NMR and mass spectroscopy. The accuracy of the methods used was found to be between 96.9% - 100% for all five compounds. The recovery of all five identified alkaloids was seen to be in the range of 95% - 100%. Almost 80% of known natural substances are non-volatile and thermolabile, making HPLC with UV detection the most commonly used analytical technique for natural products (Abian, 1999). Photo diode array (PDA) detectors enhance the versatility of analysis, by allowing

multi-wavelength detection of compounds based on their distinct chromophore active regions, thus making PDA a very useful analytical technique, particularly for multi-component sample analysis and/or compound identification (Patnala & Kanfer, 2010). In the present study, nuclear magnetic resonance spectroscopy of isolated compounds was performed using a Varian-NMR (600 MHz) instrument. The spectra were recorded at 25^{o}C, with chemical shifts of the different molecular components being recorded along the ppm-axis coupled to hertz. Deuterated solvents, such as $CDCl_3$, D_2O and DMSO were used, depending on the polarity of the compound and the relaxation times (Ti) required, generating spectral data of the components of each molecule of interest.

4. Analysis of Compound 1

The history of the phytochemical analysis of the genus *Sceletium* is believed to go back to 1898 when Meiring isolated a crude alkaloid mixture from *Sceletium tortuosum*. Meiring's research was reportedly followed by the work done by Zwicky (quoted in: Smith *et al*., 1998) who isolated a number of alkaloids including mesembrine and mesembrenone. Later in 1967, Popelak and Lettenbauer (1968) suggested that the "mesembrine" Zwicky isolated was probably not a pure compound. Therefore, they corrected the molecular formula for mesembrine from $C_{16}H_{19}NO_4N$ to $C_{17}H_{23}NO_3$ which was later confirmed by the well-known German pharmaceutical company C.F. Boehringer and Soehne in the early 1960's and also by S.B. Penick in New York (Gericke & Viljoen, 2008).

While some believe the structural elucidation of mesembrine commenced in 1967 by Zwicky, other researchers believe the structural investigation began in 1975 when it was revealed that mesembrine contains a ketone, a tertiary amine and two methoxy substituents. It is, however, agreed that Popelak and co-workers completed the structural elucidation through a combination of degradation and synthetic methods (Denark and Marcin, 1997). The alkaloids of *Sceletium* species are said to be derived from the aromatic amino acids phenylalanine and tyrosine by a complex series of reactions that are only partly understood (Jeffs *et al*., 1977). It is believed that mesembrine is the main compound or alkaloid responsible for mood elevating and the stress- and anxiety-reduction effects of the plant (Geriken & Viljoen, 2008). Jeffs (*et al*., 1977) disagrees with this and argues that mesembrenone could well be the psychoactive constituent of "*kougoed*". He based this conclusion on the elevated quantity of mesembrenone observed following fermentation

of the fresh *Sceletium* material he was working on. It was also found that although the intoxicating doses can be euphoric, the plant is not hallucinogenic, but is a narcotic-anxiolytic (Smith *et al*., 1995). Research shows that pharmacologically, mesembrine acts as a serotonin re-uptake inhibitor (Gericke & Viljoen, 2008).

4.1 Identification of Compound 1 as Mesembrine

Mesembrine is a sticky, yellow compound and in the current study, **compound 1**, upon spraying a TLC plate with ninhydrin, presented with a pink centre with a yellow outer circle. The structural elucidation of the compound was based on ^{1}H and ^{13}C experiments. The ^{1}H and ^{13}C chemical shifts for the unknown compound were compared to those found in the literature for mesembrine (Gu and You, 2011). **Table 4.1** and **Table 4.2** shows that the NMR spectral data for compound 1 agrees with published values for mesembrine (Gu and You, 2011). The proton spectrum shows three conspicuous proton signals at δ_H 3.89, 3.87 and 2.30 ppm. These three signals resonate with the two methoxy groups in the aromatic ring and one N-methyl group (**Appendix 6**). Also present are aromatic protons between δ_H 6.92 and 6.83 ppm. The relics of the rest of the peaks resonate between δ_H 2.60 and 2.00 ppm. These signals are highly congested and, thus, present with signal overlapping, thereby making it impossible to individually analyze each signal. When analyzing the carbon 13 spectrum of **compound 1**, a carbonyl carbon peak is observed downfield resonating at $\delta^{13}C$ 211.4. There are six peaks in the aromatic region, resonating between $\delta^{13}C$ 100.0 and 160.0. These peaks signify the presence of a benzene ring. The mass spectrum of compound **1** observed below (**Figure. 4.1**) showed a peak at *m/z* 290.1757 (ESI^{+}), as was shown for mesembrine by Patnala and Kanfer (2009).

Table 4.1: ^{1}H NMR spectral data of Compound 1 ($CDCl_3$) in comparison with that found in the literature (Gu & You, 2011).

Compound 1	
δH (*J* in Hz)(Isolated)	**δH (*J* in Hz)(Literature)**
2.09 – 2.22 (m, 5H)	2.08 – 2.25 (m, 5H)
2.30 (s, 3H)	2.33 (s, 3H)
2.24 (s, 2H)	2.30 (m, 2H)
2.58 (brs, 2H)	2.62 (d, *J* = 3.3, 2H)
2.93 (brs, 1H)	2.96 – 2.98 (m, 1H)
3.11 – 3.13 (brt, 1H, *J* = 7.7)	3.13 – 3.18 (m, 1H)

3.89 (s, 3H)	3.89 (s, 3H)
3.91 (s, 3H)	3.19 (s, 3H)
6.83 – 6.92 (m, 3H)	6.84 – 6.93 (m, 3H)

Table 4.2: ^{13}C NMR spectral data of compound 1 ($CDCl_3$) in comparison to that found in the literature (Gu & You, 2011).

$\delta\,^{13}C$ (Isolated)	$\delta\,^{13}C$ (Literature)
56.0	55.9
36.2	36.1
47.5	47.4
35.3	35.2
40.6	40.5
211.4	211.5
38.8	38.7
70.4	70.3
40.1	40.0
140.2	139.9
111.7	110.7
149.0	148.8
147.5	147.3
110.0	109.6
117.9	117.8
54.8	54.8
55.9	55.8

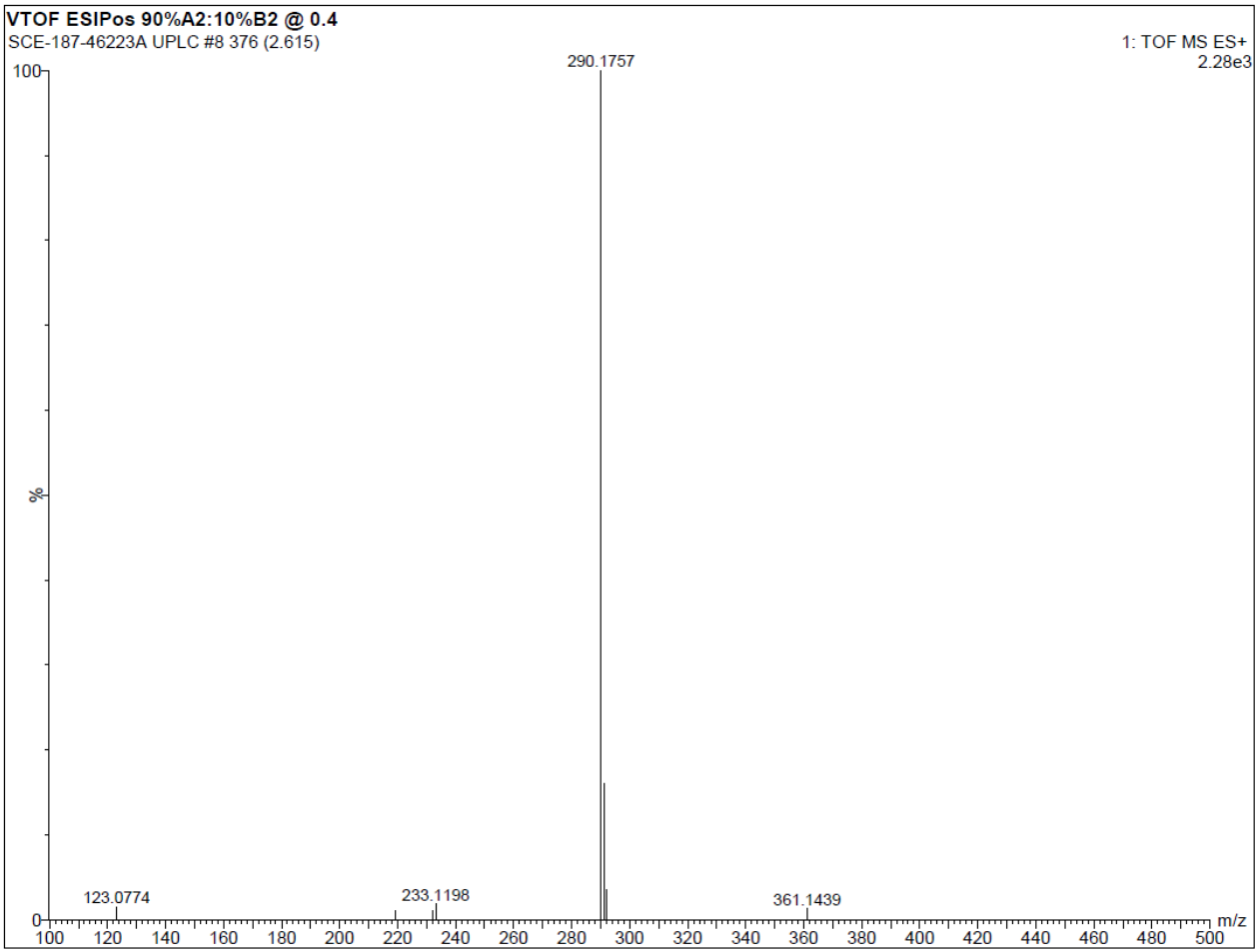

Figure 4.1: ESI$^+$ TOF mass spectrum of isolated mesembrine

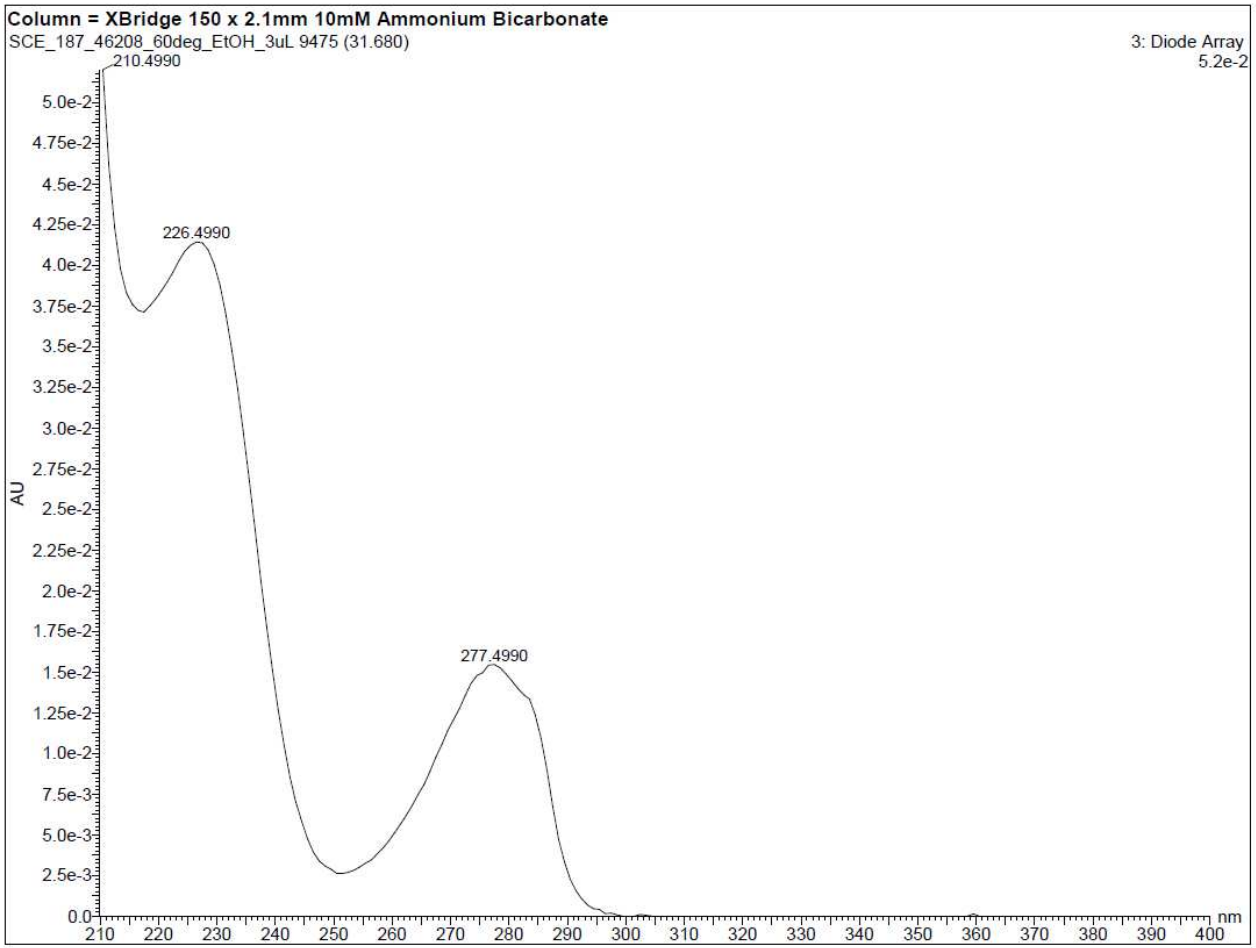

Figure 4.2: UV absorption spectrumof mesembrine detected in crude SCE-187-46208

HPLC is used routinely in phytochemistry to provide efficient separation of metabolites and is a valuable quality control in standardization of sample preparation techniques (Marston, 2007). Patnala and Kanfer (2010) performed quantitative determination of relevant alkaloids within *S. tortuosum* extracts. They then obtained UV peaks (**Appendix 29**) of each alkaloid of interest present in their plant stock. A UV spectrum of Compound **1** is depicted in **Figure 4.3** to show that this UV data and the MS data compares to that presented by Patnala and Kanfer (2009) for mesembrine. Taken together, these data confirm that **compound 1** is mesembrine with the following structure:

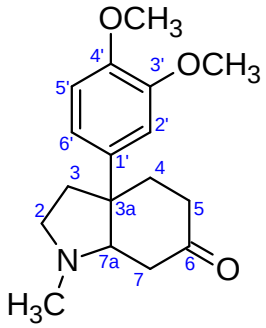

Figure 4.3: Structure of Compound 1 (Mesembrine)

4.1.1. Characterization of Compound 2

This compound was isolated by column chromatography. It then crystallised as different shapes and colours ranging from white to light brown in separate fractions. Analysis of this compound by column chromatography followed by TLC and staining with ninhydrin and vanillin indicated a bright pink streak when sprayed with ninhydrin and with a dark black streak when sprayed with vanillin. Structural elucidation of this compound was based on ^{1}H and ^{13}C NMR experiments.

Table 4.3: ^{1}H NMR spectral data of compound 2(dissolved in deuterated DMSO) in comparison with that described in the literature for pinitol (Blanco, 2008).

Compound 2		
Proton number:	**δH (*J* in Hz)(Isolated)**	**δH (*J* in Hz)(Literature)**
1	3.61 (s)	3.62 (m)
2	3.48 (m)	3.50 (m)
3	2.98 (t, *J* = 9.4)	3.00 (t)
4	3.33 (brs)	3.32 (dd)
5	3.42 (brs)	3.41 (m)
6	3.61 (brs)	3.62 (m)
OCH_3	3.42 (s)	3.44 (s)
OH – 1	4.31 (d, *J* = 5.3)	4.34
OH – 2	4.44 (d, *J* = 6.3)	4.46 (d)
OH – 4	4.48 (d, *J* = 4.4)	4.51
OH – 5	4.60 (brs)	4.63 (brs)
OH – 6	4.69 (brs)	4.72 (brs)

Table 4.4: ^{13}C NMR spectral data of compound 2 (dissolved in deuterated DMSO) in comparison to that in literature (Pyridine deutorated) (Misra & Siddiqi, 2004).

Compound 2		
Carbon number:	**δ^{13}C (Isolated)**	**δ^{13}C (Literature)**
1	83.8	85.9
2	71.0	73.1
3	72.4	74.2
4	70.1	72.3
5	73.6	74.7
6	72.0	73.8
OCH_3	59.6	60.8

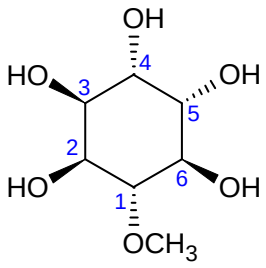

Figure 4.4: Structure of Compound 2 (Pinitol)

Pinitol or *O*-methyl inositol (1-methoxy-2,3,4,5,6-penta-hydroxyl cyclohexane) is a natural product of a cyclitol group occurring mainly in its positive (+) from. It has been shown to be a feeding stimulant for larvae of the yellow butterfly *Euremahecabemandarina* and also inhibits larval growth of *Heliothiszea* on soybeans (Ley and Sternfeld, 1989; Misra and Siddiqi, 2004). Pinitol also exerts some sort of insulin-like effect due its involvement downstream of the cellular signal transduction mediate by the insulin receptor (Kim *et al*., 2005). Pinitol has been isolated from the leaves of *Bougainvillea spectabilis* and was found to have anti-diabetic and hypoglycaemia-inducing properties (Misra & Siddiqi, 2004). Misra and Siddiqi (2004) agree that spectra supporting data of (+)-pinitol are not readily available, particularly data involving ^{13}C NMR. For that reason, a carbon spectrum of (+)-pinitol using pyridine as deuterated solvent of choice was utilized as reference for **compound 2**.

The NMR proton spectrum indicated a methyl proton peak resonating at $\boldsymbol{\delta}_H$ 3.42. The peak is shifted more downfield than would normally appear and this is due to the presence of an oxygen molecule attached to the methyl group. This peak is situated in the region between $\boldsymbol{\delta}_H$ 2.90 and 3.60 where all explicit protons being H-1 to H-6 are saturated. Furthermore, all five oxygenated hydrogens are clustered between $\boldsymbol{\delta}_H$4.30 and 4.00, with protons OH-5, OH-4 and OH-2 presenting as doublets (**Appendix 11**). In the carbon 13 spectrum, the methyl carbon resonates at $\boldsymbol{\delta}^{13}$C 59.6. The rest of the carbons (C-2 to C-6) resonate between $\boldsymbol{\delta}^{13}$C70.00 and 72.60 ppm. These peaks are clustered in that region, indicating the presence of a ring structure. C-1 resonates at $\boldsymbol{\delta}^{13}$C83.80, more downfield than other carbon peaks and this is due to the oxygen molecule attached to it.

4.1.2. Characterization of Compound 3

Sucrose was isolated from one of the fractions after washing the column using highly polar solvents (**Figure. 3.4**). The crystallized compound consisted of clear square crystals. Brown oil was mixed with the crystals and could not be completely removed by washing. Although the compound could not be identified by TLC, it stained black after spraying with vanillin. To confirm the identity of this compound, the structure was elucidated using NMR in comparison to commercially available sucrose (table sugar). ^{1}H and ^{13}C spectra of both samples were obtained and the resulting chemical shifts were compared to one another and to those found in the literature (SDBS – riodb01.ibase.aist.go.jp; Popov *et al.,* 2006). See **Table 4.5** and **Table 4.6** in this regard.

Table4.5: ^{1}H NMR spectral data of Compound 3 (D_2O) and commercially available (D_2O) sucrose in comparison to that found in literature (D_2O) (SDBS – www.aist.go.jp).

Compound 3			
Proton	**δH(*J* in H_z)**	**δH(*J* in H_z)**	**δH(*J* in H_z)**
1	5.30 (d, 1H, *J* = 3.5)	5.27 (d, 1H, *J* = 3.5)	5.41
2	3.44 (dd, 1H, *J* = 3.0, 10)	3.42 (dd, 1H, *J* = 3.5, 10.1)	3.56
3	3.64 (t, 1H, *J* = 9.7)	3.61 (t, 1H, *J* = 9.4)	3.76
4	3.35 (t, 1H, *J* = 9.4)	3.32 (t, 1H, *J* = 9.4)	3.47
5	3.72 (m, 1H)	3.70 (m, 1H)	3.86
6	3.70 (brs, 2H)	3.67 (brs, 2H)	3.81
1'	3.56 (brs, 2H)	3.53 (s, 2H)	3.67
2'			
3'	4.06 (d, 1H, *J* = 8.8)	4.07 (d,1H, *J* = 8.8)	4.21
4'	3.93 (t, 1H, *J* = 8.9)	3.90 (t, 1H, *J* = 8.5)	4.05
5'	3.77 (m, 1H)	3.75 (m, 1H)	3.89
6'	3.70 (brs, 2H)	3.68 (brs, 2H)	3.83

Table 4.6: ^{13}C NMR spectral data of Compound 3 (D_2O) and commercially available (D_2O) sucrose to that found in literature (D_2O) (Popov *et al.,* 2006).

Compound 3			
Carbon numbers:	**$\delta^{13}C$ (Isolated)**	**$\delta^{13}C$ (Table sugar)**	**$\delta^{13}C$ (Literature)**
1	92.2	92.1	92.3
2	71.0	71.0	71.1
3	72.5	72.5	72.5
4	69.2	69.1	69.3
5	72.4	72.3	72.3
6	60.1	60.0	60.2
1'	61.4	61.3	61.4
2'	103.7	103.6	103.5
3'	76.5	76.3	76.6
4'	74.0	73.9	74.1
5'	81.3	81.3	81.3
6'	62.4	62.3	62.4

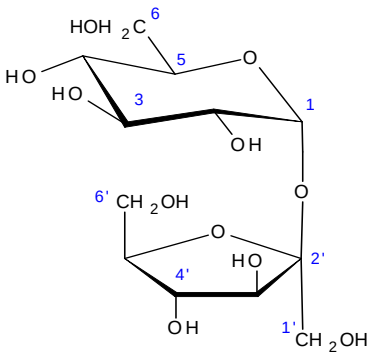

Figure 4.5: Structure of Compound 3

Sucrose (β-D-fructofuranosyl-α-D-glucopyranoside) is one of the most common sugars, occurring widely in plants, fruits and honey. Both of the sucrose rings behave together as a single rigid entity, bound by intramolecular hydrogen bonds and reorienting isotropically in an aqueous solution. Thus, a dissociation of one hydroxyl-group in some way affects the entire molecule (Popov *et al*., 2006).

In the proton spectrum, proton peaks resonated between δ_H 3.2 and 5.30 ppm. This is due to the absence of methylated protons in this compound and to the oxygen molecules binding to eight of the fourteen protons resulting in the protons being shifted downfield. H- 6' and H-6 resonated at δ 3.70 ppm. These two signals overlap because H-6 presented with a broader peak masking the resonance of H-6'. H-5' presented as a multiplet due to the effects of the multiple protons in the field and the oxygen molecules present. H-1 resonated at δ 5.30 ppm and the reason for this proton to be moved further downfield compared to the rest of the protons is the fact that C-1 is directly bound to the two oxygen molecules and to C-2 that has an OH substituent. Twelve peaks were observed between δ 60.0 and 104.0 ppm of the ^{13}C spectrum. Typically of the sucrose carbon spectrum, the CH_2 carbons (C-6, C-1', C-6') resonate between 60.0 ppm and 63.0 ppm. These carbons are also bound to an OH group. A group of seven CH molecules resonates further downfield between 69.0 and 80.0 ppm region. There is one anomeric carbon signal resonating at 92.2 ppm which is typical of an α-orientation hydroxyl group attached to C-1 position. Sucrose is one of the most common disaccharides with a quaternary acetal carbon signal resonating at 103.7 ppm. The NMR data obtained for this compound were in close agreement with that published in literature of the sucrose molecule (Popov *et al.,* 2006)

4.1.3. Characterization of Compound 4

As noted by Patnala and Kanfer (2009), some publications suggest that unspecified mesembrenone and other alkaloidal changes resulted from crushing and bruising of the plant material. These authors subsequently carried out a further study which revealed that the fermentation process undeniably transformed mesembrine to Δ^4mesembrenone. They stated that this process required the presence of an aqueous environment together with the presence of light to facilitate such a transformation. In this particular study, this was not the case, as the plant material was only crushed and bruised before extraction. Nevertheless, change of, or gradual

reduction of mesembrine was noticed in various extracts, together with those that resulted from fermentation of the plant material itself. From this plant, Δ^4mesembrenone was isolated using prep-TLC, appearing as a white solid film that adhered to glassware. Although this compound was found to be UV active, there was no colour change after spraying with ninhydrin. The structural elucidation of the compound was based on ^{1}H and ^{13}C experiments. The ^{1}H and ^{13}C chemical shifts were compared to those of Bastida *et al*., (1989) and Jeffs *et al*., (1974).The 3a-aryl-cis-octahydronindone skeleton is a constituent of the major subgroup of mesembrine alkaloids which includes Δ^4 and Δ^7mesembrine-type series, based on the two double bonds at either position 4 - 5 or 7 - 7a, respectively (Patnala and Kanfer, 2008). Like mesembrine, this compound has been isolated and published in the literature and, therefore, structural elucidation depended solely on comparison of the ^{1}H and ^{13}C NMR experimental data, with that found in the literature.

Table 4.7: ^{1}H NMR spectral data of compound 4 ($CDCl_3$) in comparison with that found in literature ($CDCl_3$) (Bastida *et al*., 1989).

Compound 4		
Proton number	**δH, (J in H_Z) (Isolated)**	**δH, (J in H_z) (Literature)**
2α	3.32 (1-H, brt; *J* = 5.4)	3.34 (1-H, m)
2β	2.51 (1-H)	2.50 (H-7)
3α	2.44 (1-H, brd; *J* = 6.5)	2.42 (1-H, dd; *J* = 8.4, 2.4)
3β	2.20 (1H-, brdd; *J* = 8.8, 4.7)	2.21 (1-H, ddd, *J* = 12.6, 8.4, 4.2)
4	6.73 (1-H, brd; *J* = 8.8)	6.74 (1-H, d; *J* = 10.1, 2.0)
5	6.11 (1-H, brd; *J* = 10.6)	6.11 (1-H, dd; *J* = 10.1, 0.8)
7α	2.57 (1-H, brs)	2.55 (1-H, brd)
7β	2.51 (1-H, brs)	2.50 (1-H, dd; *J* = 8.4, 4.8)
7a	2.67 (1-H, m)	2.65 (1-H, m)
NCH_3	2.32 (3-H, s)	2.32 (3-H, s)
2'	6.88 (1-H, s)	6.88 (1-H, s)
5'	7.03 (1-H, d; *J* = 8.2)	6.89 (1-H, d; *J* = 8.0)
6'	6.84 (1-H, d;*J* = 8.0)	6.82 (1-H, d; *J* = 8)
O CH_3	3.88 (3-H, s)	3.89 (3-H, s)
O CH_3	3.89 (3-H, s)	3.90 (3-H, s)

Table 4.8: ^{13}C NMR spectral data of compound 4 ($CDCl_3$) in comparison with that found in literature ($CDCl_3$) (Jeffs et al., 1974).

Compound 4		
Carbon number	**$\delta^{13}C$ (Isolated)**	**$\delta^{13}C$ (Literature)**
2	56.1	56.2
3	37.8	38.3
3a	50.9	50.9
4	153.7	153.3
5	126.5	126.2
6	197.4	196.9
7	38.7	38.6
7a	73.8	73.8
NCH_3	40.1	40.1
1'	135.8	135.2
2'	111.2	110.9
3'	149.1	148.6
4'	148.2	147.7
5'	110.2	110.0
6'	118.1	118.8
O CH_3	55.8	55.8
O CH_3	55.9	55.9

Sceletium's Δ^4 mesembrenone and Δ^7mesembrenone are some of the compounds that have been isolated from *S. tortuosum* as reported in the literature. These compounds are similar in structure, with the only difference being in the stereochemistry of the double bond found in the six membered ring. In Δ^4mesembrenone, the double bond is found between C-4 and C-5, whereas the double bond in Δ^7mesembrenone is found between C-7 and C-7a. Although the difference between the two structures is negligible, in essence it is possible to compare the NMR parameters of one with the other. From an NMR perspective, the difference in stereochemistry between the two compounds has a huge impact on the proton and carbon resonance spectra. Carbon 13 NMR and proton NMR spectral data are in agreement with that found in the literature (Bastida *et al*., 1989

and Jeffs *et al*., 1974). There are proton signals found in the ^{1}H spectra of mesembrenone that are unique to this compound. A singlet at δ_H 2.32 represents the N-methyl group. The two signals at δ_H 3.89 and δ_H 3.90 represent the methoxy groups. There is broad doublet at δ_H 6.73 with a coupling constant of 8.8 Hz. The two olefinic protons H-4 and H-5 are represented by a broad doublet with a coupling constant of 10.6 Hz. The two unique doublets representing the aromatic protons resonated at δ_H 6.84 and δ_H 7.03. In the analysis of the ^{13}C spectra, the NMe group resonated at $\delta_C{}^{13}$ 40.1. The C-3a quaternary carbon resonated at δ_H 50.9. The two methoxy groups are represented by peaks at δ_H 55.8 and δ_H 55.9. Due to the presence of the oxygen, the carbonyl group shifts more downfield away from the rest of the carbons. This peak is usually found around δ 200. C-6 carbonyl group of this compound resonated at δ_H 197.4.

Standards of the *S. tortuosum* compounds similar to those described by Patnala and Kanfer (2008) were isolated from the plant material, while some standards were purchased for comparative purposes. An HPLC profile of the four standards (Δ^7mesembenone, mesembranol, mesembrenone, Δ^4mesembrine and epimesembranol) was compiled (see below – **Appendix 29**) as well as an LC-MS chromatogram of the Δ^7 mesembenone peak (**Appendix 21)**. These data (Patnala and Kanfer, 2008) were used to compare with the LC-MS of the laboratory-isolated Δ^4 mesembrenone (isolated in this study – **Figure 4.8**, 288 *m/z*). The information seemed to be in direct agreement with the major peak resonating at 288 *m/z* on the chromatogram. View **Figures. 4.6** and **4.7** for the results obtained by Patnala and Kanfer (2009). Although Patnala and Kanfer (2009) reported on Δ^7mesembrenone, the data are still applicable and can be used for comparison as these two compounds are of the same mass.

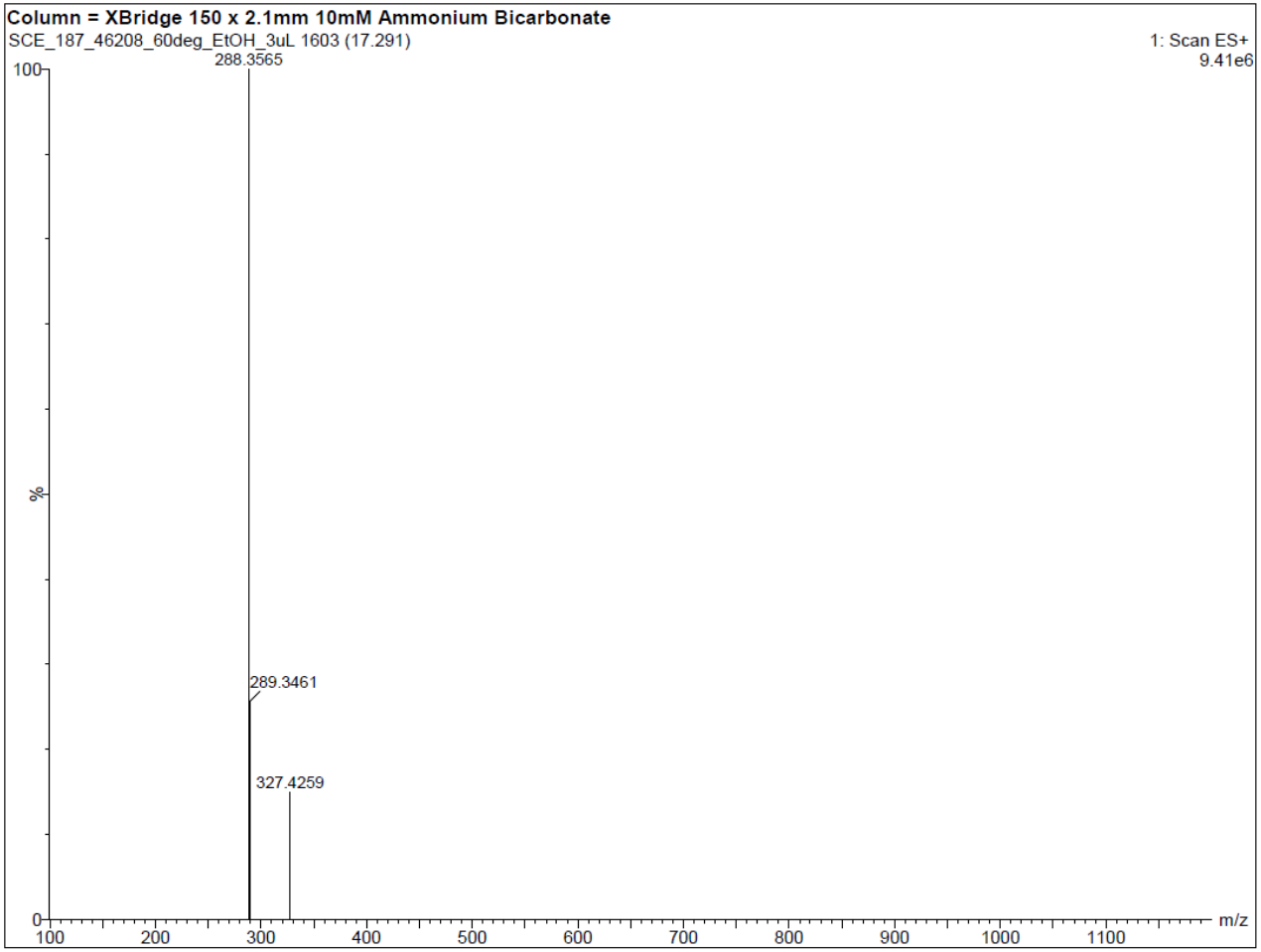

Figure 4.6: Mass spec of Mesembrenone isolated from crude SCE-187-46208. Showing a peak at 299.35 *m/z* that is a representative of mesembrenone. In this case, Δ^4 mesembrenone and Δ^7 mesembrenone share the same molecular weight of which the stereo chemistry of the structure is used to determine which the correct compound.

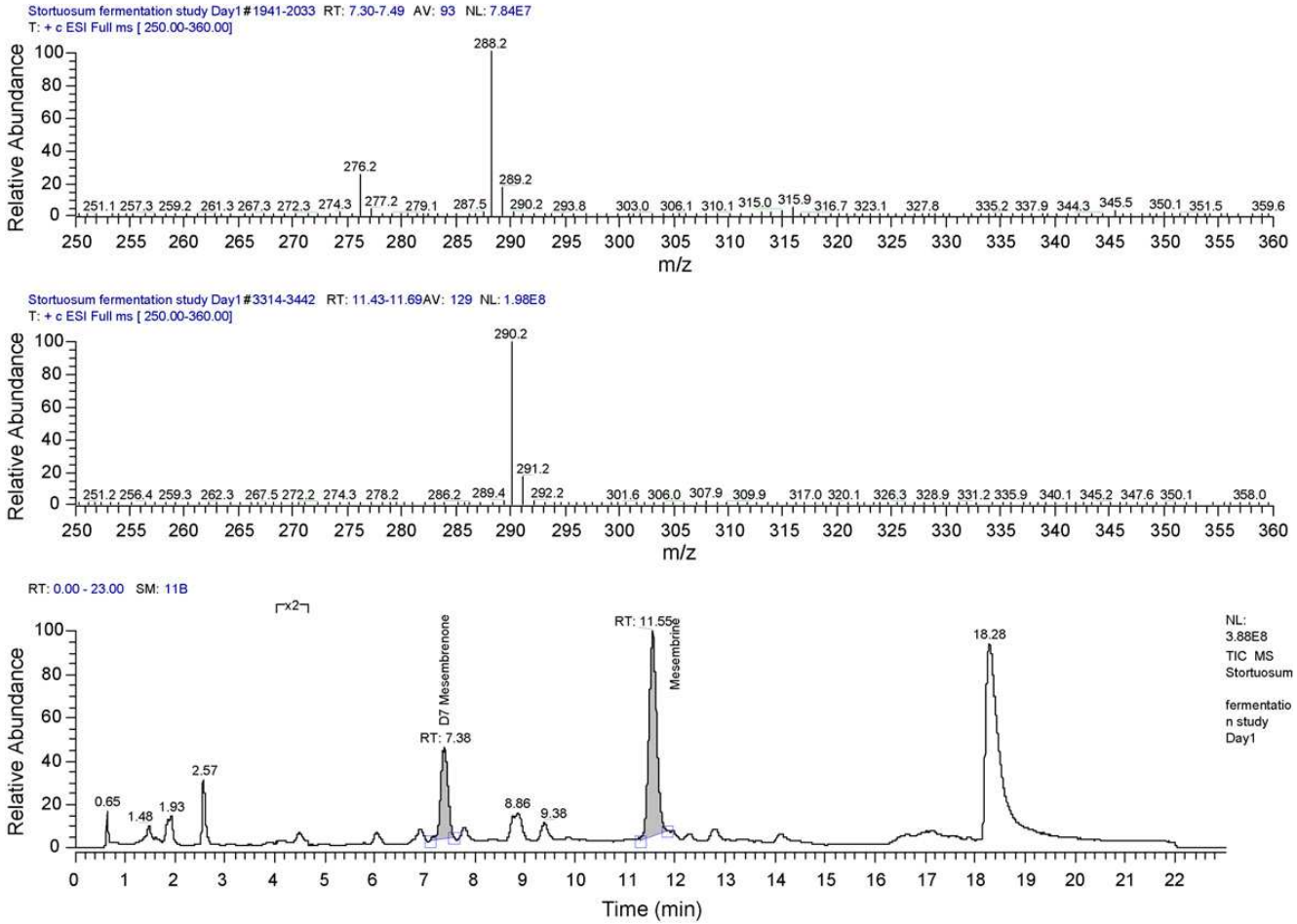

Figure 4.7: LC-MS chromatogram of crushed plant material on day 10. MS Ion spectra of Δ^7 mesembrenone *m/z* 288.2 (top), mesembrine *m/z* 290.2 (middle) and TIC (bottom) (Patnala and Kanfer, 2009). An HPLC representative of *S. tortuosum* purchased standards used in the experiments carried out by Patnala and Kanfer, 2009 (bottom spectrometer) as well as MS ion peaks of two compounds as forth mentioned.

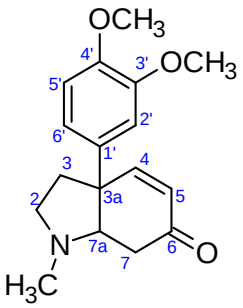

Figure 4.8: Structure of Compound 4

4.1.4. Characterization of compound 5

According to the literature, no triterpenoid has been previously isolated from phytochemical investigations on *S. tortuosum*. Triterpenoids are commonly found in most plants and are produced by arrangements of squalen epoxide in a chair-chair-chair-boat arrangement subsequently followed by condensation (Patočka, 2003). These compounds are isopentenoids composed of thirty carbon atoms and may possess acyclic, mono, di-, tri-, tetra- or pentacyclic carbon skeletons. Pentacyclic triterpenoids are dominant constituents of this class and have been widely investigated (Mahato and Kundu, 1994).

Obtusalin[7] is a pentacyclic triterpene belonging to the lupane class of compounds (Zheng *et al*., 2010) that was first isolated by Siddiqui and colleagues in 1989 from the leaves of *Plumeria obtusa*. Different parts of this plant have been used as a laxative, menstrual flow enhancer and as a fever-reducing medicine (Siddiqui *et al*., 1989). Obtusalin is an addition to the few naturally occurring pentacyclic triterpenoids possessing a C-27-hydroxyl group in conjunction with a double bond at C-12 in the lupine chain of triterpenoids (Begum *et al*., 1994 and Siddiqui *et al*., 1989). Pentacyclic triterpenes are reported to possess a wide spectrum of biological activities, where some may be used as medicines (Patočka, 2003). The genus *Plumeria* has a medicinal value in indigenous systems, particularly where some of its species have shown antibiotic effects. While different parts of *P. obtusa* have presented with certain biological activity (Siddiqui *et al*., 1989), obtusalin was reported to have no intrinsic biological properties (Sharma *et al*., 2011). *P. obtusa*

[7] This compound is novel to *S. tortuosum* and to the family as a whole.

has no physical, chemical or medicinal properties or attributes in common with *S. tortuosum*, which is what makes this finding so interesting and unique.

Table 4.9: ^{1}H NMR spectral data of compound 5 ($CDCl_3$) in comparison to that found in literature ($CDCl_3$) (Siddiqui et al., 1989)

Compound 5		
Proton	**δH (*J* in H_Z)(isolated)**	**δ^{13}H(*J*in H_Z) (Literature)**
3α	3.16 (br*d*, *J* = 12.69)	3.21 (*dd*, *J* = 10.8; 4.9)
5α	0.68 (br*t*, *J* -= 12.1)	0.72 (*dd*, *J* = 11.6; 1.5)
9	1.54 (m)	1.54 (*dd*, *J* = 10.0, 3.4)
11α	1.85 (br*dd*, *J* = 8.8, 6.4)	1.84 (*ddd*, *J* = 13.2, 3.6,
11β	1.63 (m)	1.61 (*ddd*, *J* = 13.2, 10.0,
12	5.07 (*brs*)	5.13 (*t*, 3.6)
23	0.94 (*s*)	1.01 (*s*)
24	0.93 (s)	0.98 (*s*)
25	0.72 (s)	0.78 (*s*)
26	0.88 (s)	0.94 (*s*)
27a	3.46 (brd, *J* = 11.2)	3.52 (*d*, *J* = 10.9)
27b	3.14 (*brd, 11.1*)	3.18 (*d*,10.9)
28	1.03 (*s*)	1.10 (*s*)
29/30	0.87 (*brd*, *J* = 5.2)	0.93 (*d*, *J* = 5.8)
30/29	0.75 (*brd, J* = 7.0)	0.80 (*d*, *J* = 5.9)

Table 4.10: ^{13}C NMR spectral data of compound 5 ($CDCl_3$) in comparison to that found in literature ($CDCl_3$) (Siddiqui *et al*., 1989 and Begum *et al*., 1994)

Compound 5		
Carbon Number	**$\delta^{13}C$ (Isolated)**	**$\delta^{13}C$ (Literature)**
1	38.8	38.8
2	27.2	27.3
3	79.0	79.1
4	38.0	38.0
5	55.1	55.2
6	18.3	18.4
7	32.8	32.9
8	40.0	40.1
9	47.6	47.7
10	36.9	36.9
11	23.4	23..4
12	125.0	125.1
13	138.7	138.8
14	42.0	42.1
15	23.4	23.4
16	26.0	26.0
17	38.8	38.8
18	54.0	54.0
19	39.4	39.5
20	39.3	39.4
21	30.6	30.7
22	35.2	35.2
23	28.1	28.2
24	16.8	16.8
25	15.6	15.6
26	15.7	15.7
27	69.9	69.9
28	23.3	23.3
29	21.3	21.3
30	17.3	17.3

^{1}H NMR, ^{13}C NMR and MS spectral data of **compound 5** are in agreement with those reported in the literature for obtusalin (Siddiqui *et al*., 1989 and Begum *et al*., 1994). Olefenic protons were observed in the proton NMR spectrum (**Table 4.9)** and resonated at δ_H 5.07,while two secondary methyl groups resonated at δ_H 0.87 (*J*= 5.2Hz) and δ_H 0.75 (*J*= 7.0Hz), five three-proton singlets indicating tertiary methyls that resonated at δ 0.94, 0.93, 0.72, 0.88 and 1.03.The ^{13}C NMR spectrum (**Table 4.10**) showed the presence of 30 carbon signals: olefinic carbons at δ_C^{13} 125.0 and δ_C^{13} 138.7; oxygenated carbons at δ 79.0 and 69.9 and seven methyls at δ_C^{13} 15.6, 15.7, 16.8, 17.3, 21.3, 23.3 and 28.1. Accordingly, the structure of **compound 5** was assigned as obstusalin. The HRTOFMS (ESI$^+$) spectrum (**Figure 4.10**) of **compound 5** showed a pseudo-molecular ion signal $[M]^+$at *m/z*441 which corresponds to the molecular formula $C_{30}H_{50}O_2$. There are significant fragments (**Figure 4.11**) at *m/z* 191.1800 [234 – isopropyl group]$^+$. This peak strongly indicates that the compound is of a lup-12-ene type. Furthermore, the base peak at *m/z* 441.3689 [234 – $CH_2OH)^+$] represents the location of the second hydroxyl group at C – 27 as one is present at C - 3.

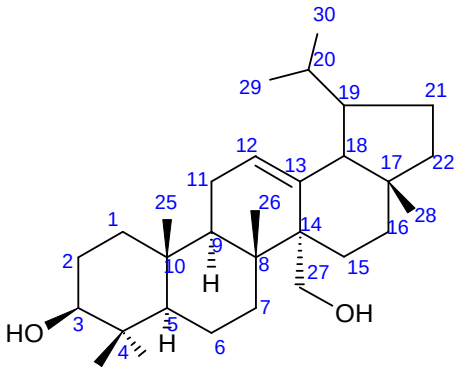

Figure 4.9: Structure of Compound 5

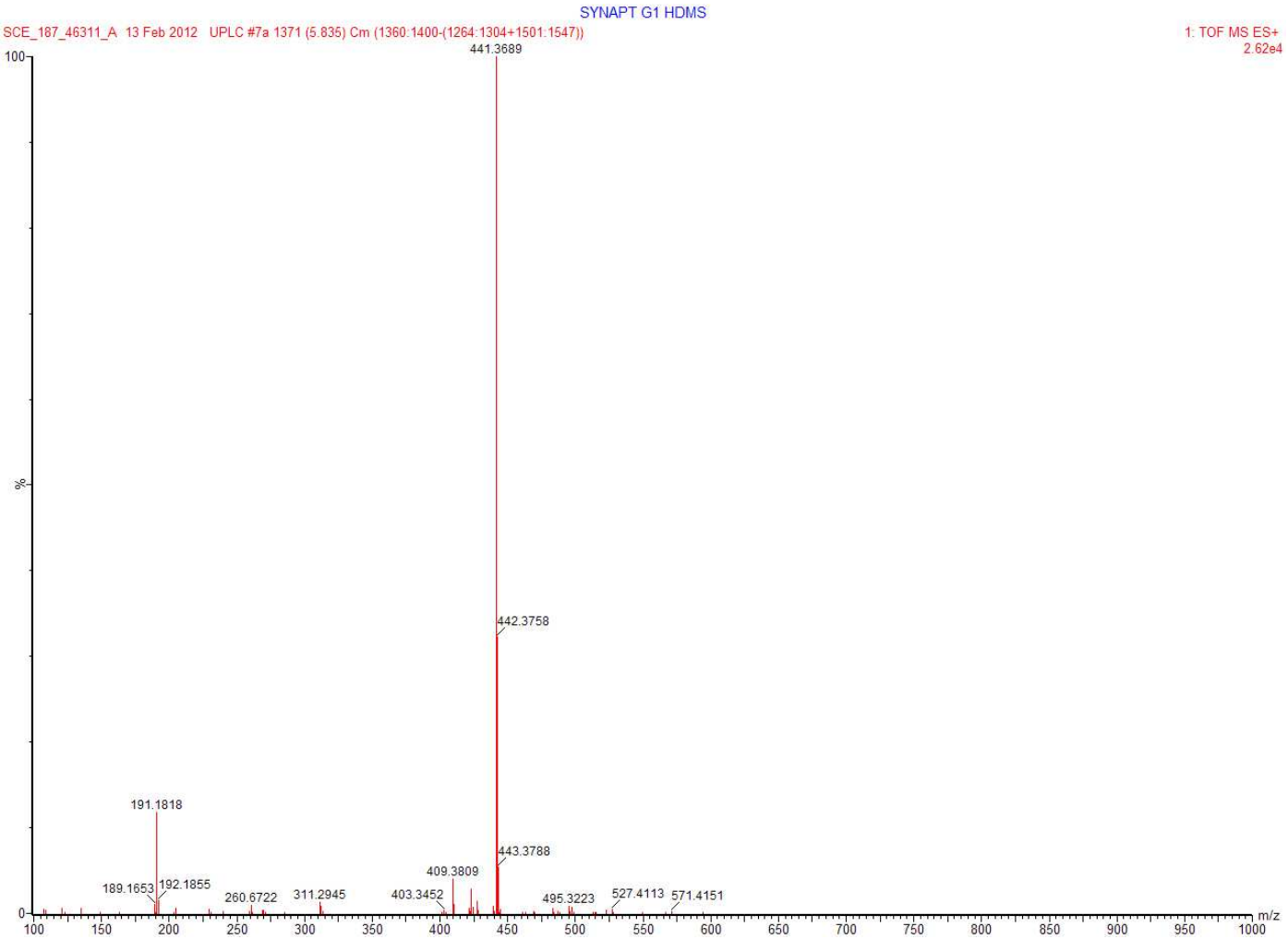

Figure 4.10: HRTOFMS (ESI^+) chromatogram of compound 5

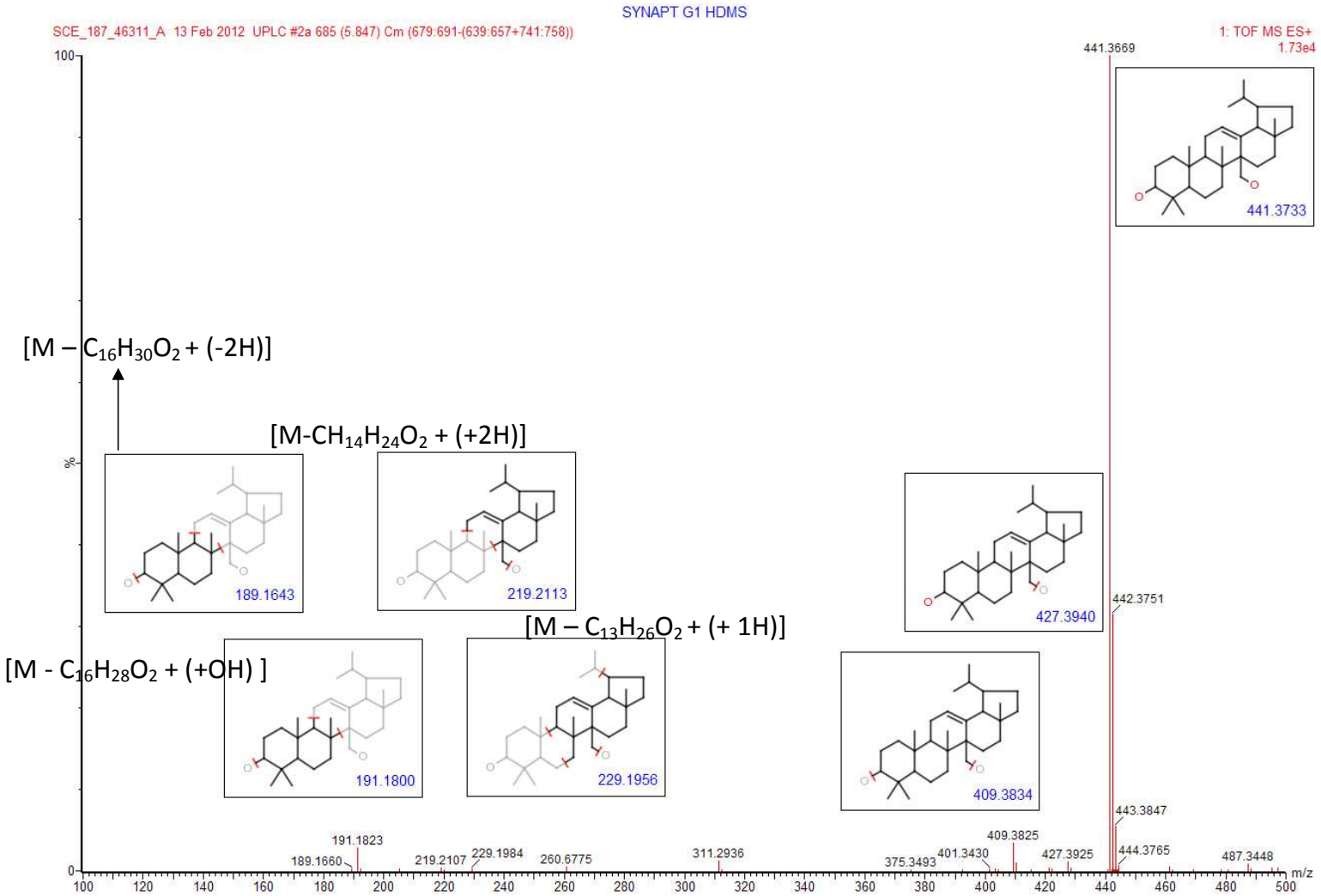

Figure 4.11: HRTOFMS (ESI$^+$) fragmentation for compound 5. Showing peak fragmentation correlating to those of $C_{30}H_{50}O_2$ with significant fragments which strongly suggests that the compound is of a lup-12-ene type.

4.2 HPLC chemical profiling of crude extracts

Crude extracts of identical plant material were prepared with two different solvents and chemically profiled to determine the presence and quantity of known compounds. This was also done to establish if these solvents eluted a similar compound, or if the polarity of the solvents determined which compounds were extracted and the quantity thereof. The two solvents used were 96% EtOH and MeOH (view **chapter 3** for process manufacturing details). Crude SCE-187-46208 is an EtOH extract and crude SCE-187-46222 is a MeOH extract. ECD provided two samples of dried plant material that were both extracted in EtOH. In addition, two ECD samples, commercially sourced *Sceletium tortuosum* capsules and a tincture were also chemically profiled as shown in **Figure 4.12 and Figure 4.14**.

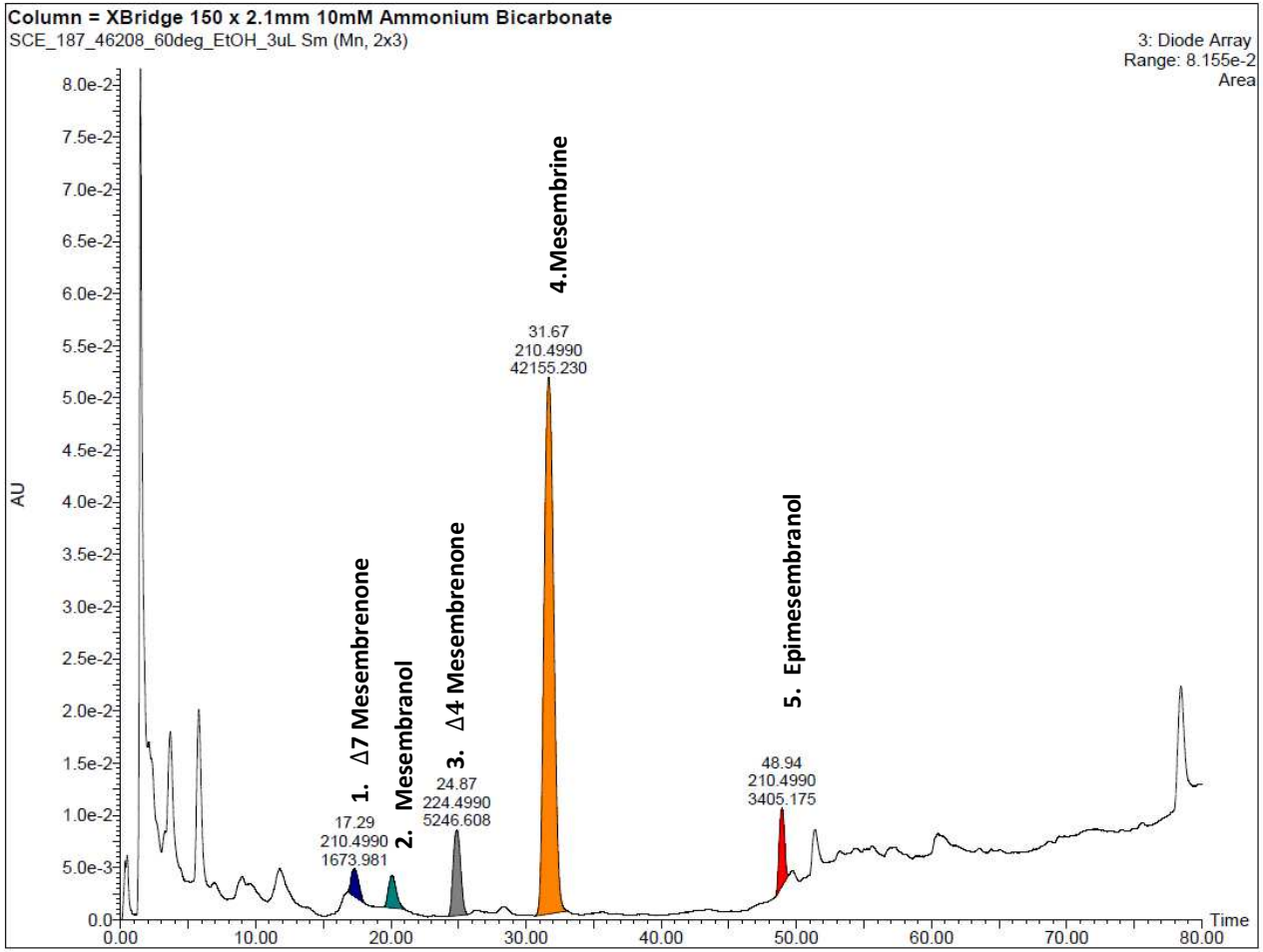

Figure 4.12: HPLC chemical profile of SCE-187-46208 (Ethanol extract). Showing the presence of mostly isolated and reported compounds of *S. tortuosum* with mesembrine being the major compound present in this sub-species.

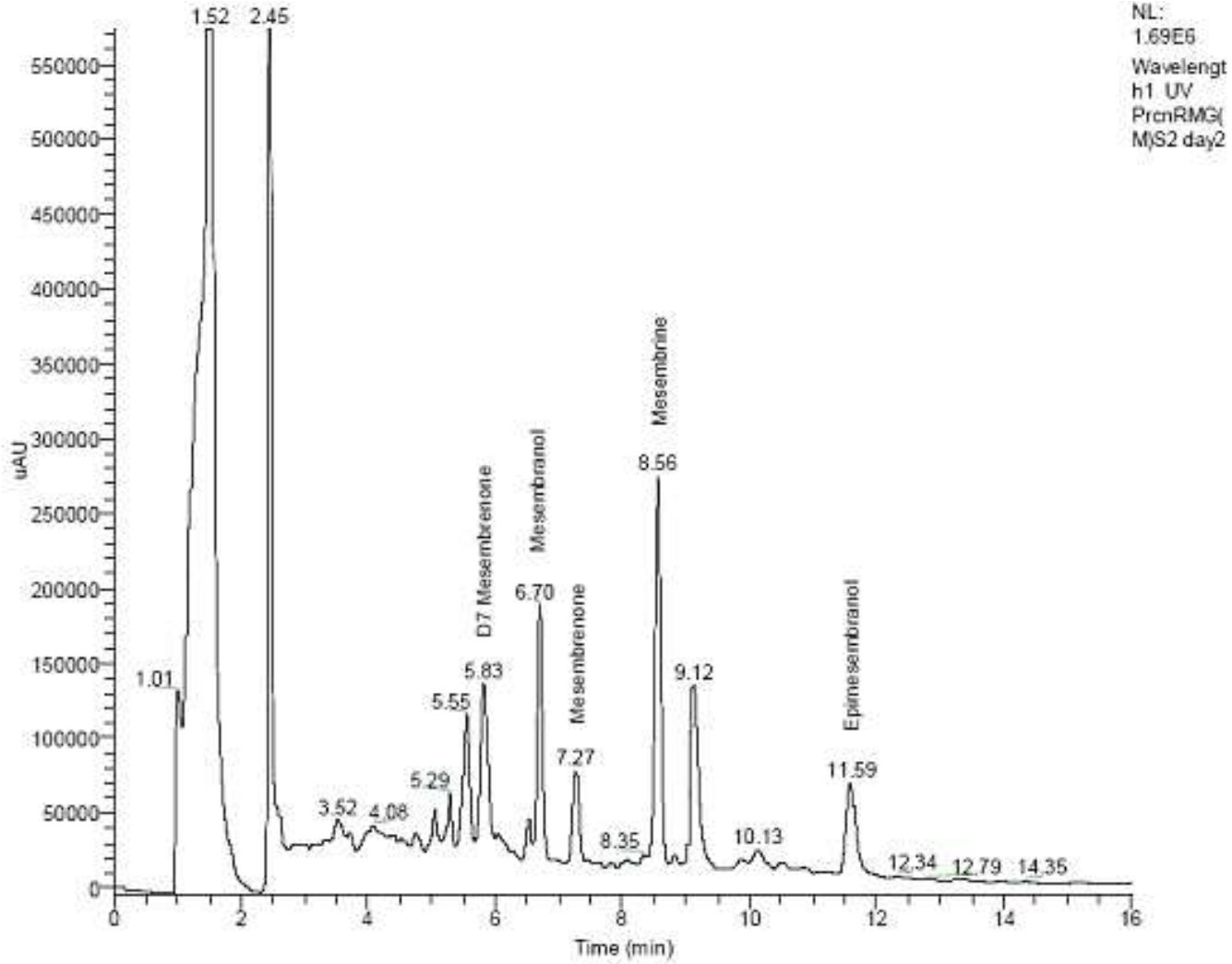

Figure 4.13: HPLC chromatogram of *Sceletium* plant material (Patnala & Kanfer, 2010).

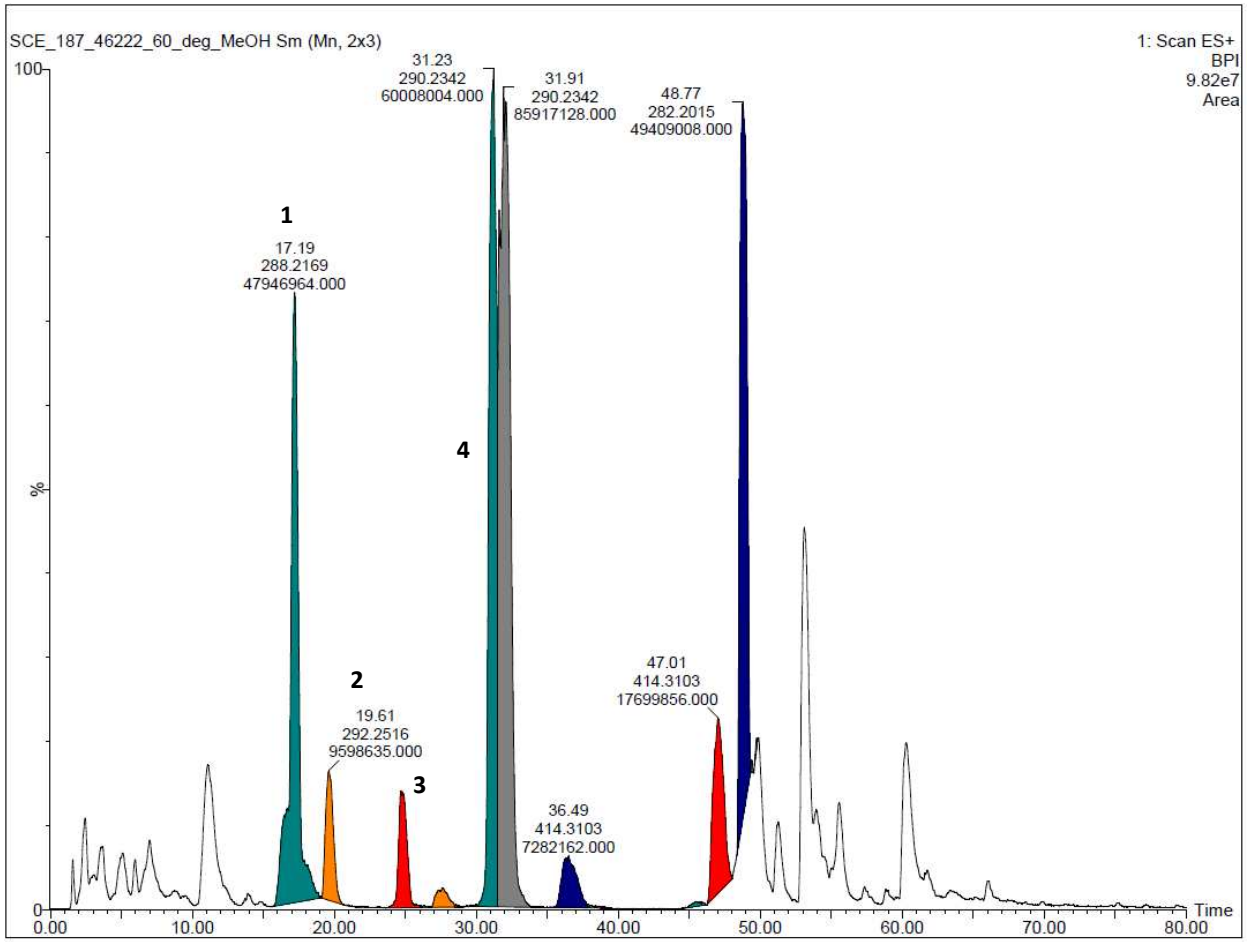

Figure 4.14: HPLC chemical profile of SCE-187-46222 (Methanol extract). Representing some of the commonly reported and isolated compounds of *S. tortuosum*. This spec shows more compounds isolated in the alkaloidal region as compared to that of EtOH extraction (Figure 4.12). View Figure 4.15 bellow to view the spectral regions.

The *S. tortuosum* methanol extract (SCE-187-46208) was analyzed using the same conditions discussed and as reflected in **Table 3.1** (page 43) together with a PDA-UV detector for improved analysis. The abovementioned conditions separated Δ^7mesembrenone (RT 17.29 min), mesembranol (RT 19.61), Δ^4mesembrenone (RT 24.87 min), mesembrine (RT 31.67 min) and epimesembranol (RT 48.94 min) (**Figure. 4.12**). Due to high sample concentrations, the PDA-UV data of the methanol extract (SCE-187-46622) were not used. Instead, ESI MS data were used. In the ESI MS of the methanol extract, Δ^7 mesembrenone was detected at 17.19 RT, while mesembranol eluted at 19.61 min and Δ^4 mesembrenone in the ethanol extract detected at 24.94 min. Mesembrine, on the other hand, presented in one broad peak and split into two peaks when integrated, this was due to the high concentration of the crude injected in the experimental run. An average retention time of the two peaks was 3.51 min.

ECD provided two packs of dried plant material labelled only with sample numbers. Samples TTSI-MP-0013 and TTSI-MP-0015 were extracted in ethanol (5 hrs, **Section 3.3.2** page 40) before profiling using HPLC. Once again, concentrating on the intermediate region (15.00 min – 40.00 min) and a small part of the non-polar region of the HPLC chromatogram, the aim of the experiment was to identify major alkaloids present in the plants and to identify any similarities or differences between compounds within these plants, as well as the tincture, commercially available capsules or the experimental plant (the plant being phytochemically analyzed in this study). It has been claimed that *S. tortuosum* has identified sub-species that can be differentiated at a molecular level by concentration and/or quantity of alkaloids or compounds present. The major alkaloid contained within the plant appears to belong within the 2a-aryl-*cis*-actehydroindole class (mesembrine type) (**Section. 2.3**). One explanation for this finding is that *S. tortuosum* has sub-species that can only be differentiated by determining the alkaloids within the plant. Thus, not all *S. tortuosum* plants have mesembrine as their major compound and some plants within the species have Δ^7mesembrenone, mesembranol, Δ^4mesembrenone or epimesembranol as a major compound (Prof. A. Viljoen, (IPUF, 2011) (*presentation and pers comm*). It should be taken into consideration that levels of secondary plant metabolites, including the presence of alkaloids, are stongly infuenced by season, growing conditions and age of the plant.

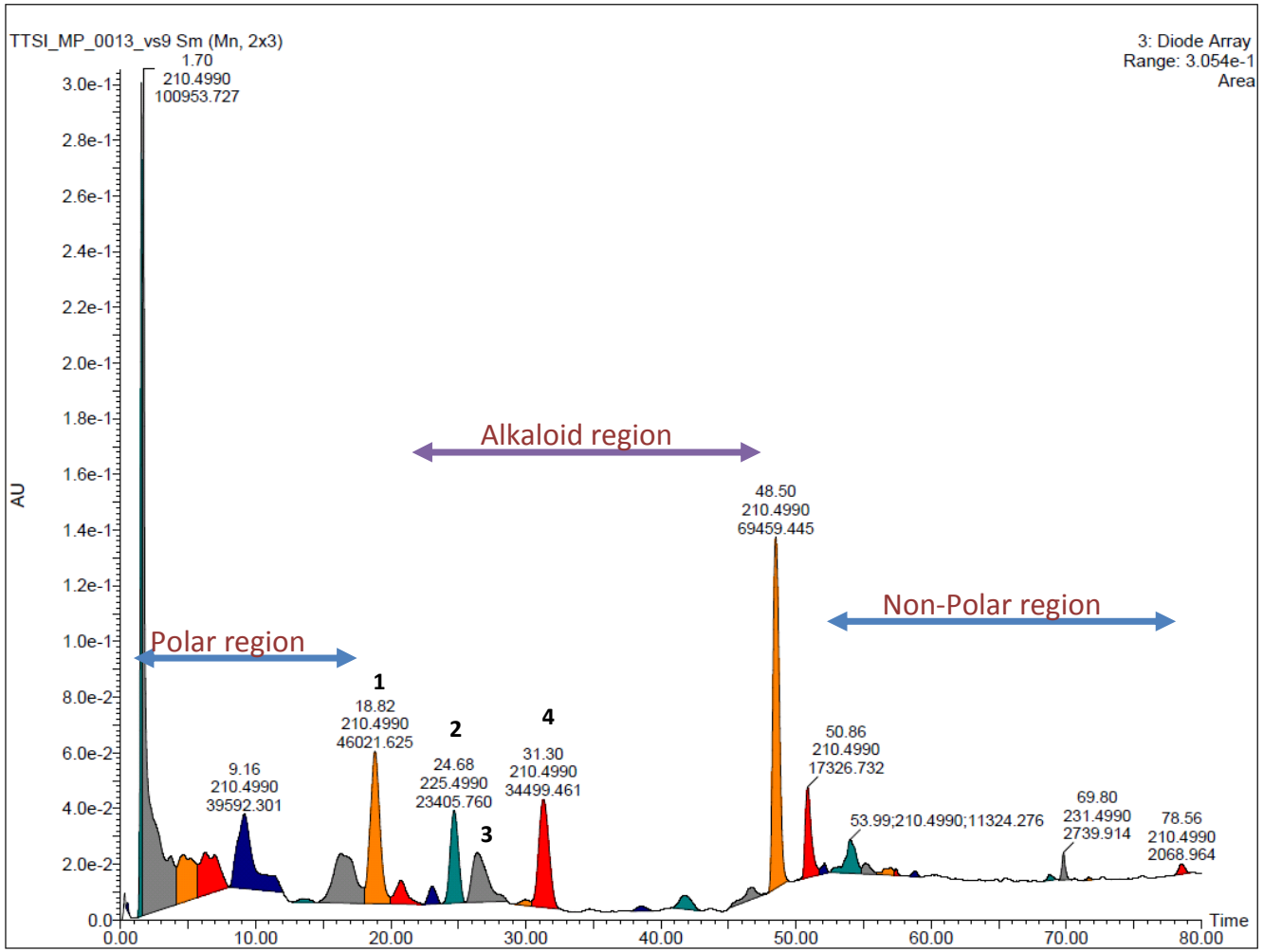

Figure 4.15: HPLC profile of ECD dried plant material, TTSI-MP-0013

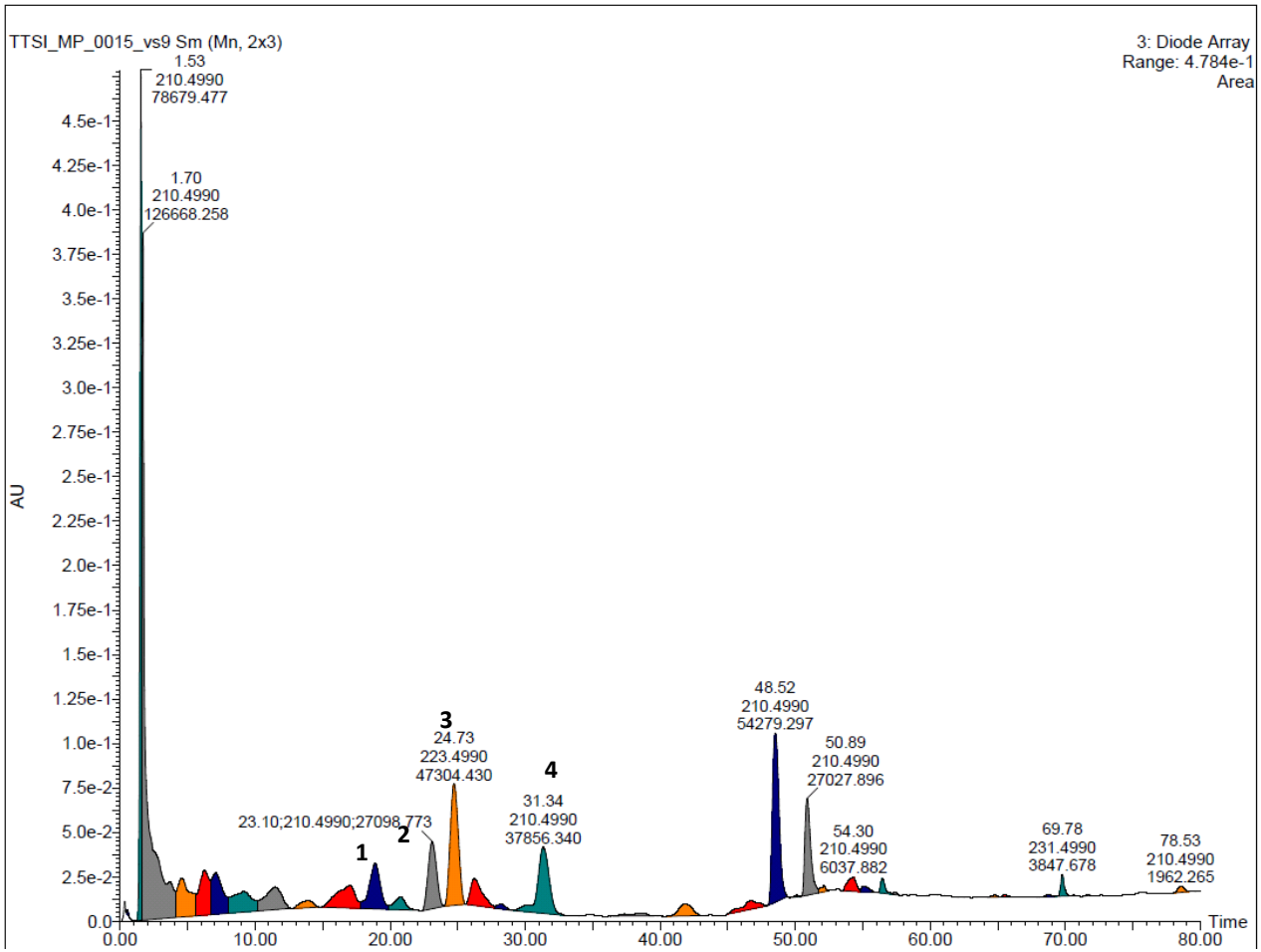

Figure 4.16: HPLC profile of ECD dried plant material, (TTSI-MP-0015). TSS-MP-0015 showed a similar compound profile as TSS-MP-0013 with epimesembranol being the major compound as compared to the laboratory profiled *S. tortuosum* which had mesembrine as its major compound.

TTS-MP-0013 presented with epimesembranol (RT 48.50 min), mesembranol (RT 18.82 min), mesembrine (RT 31.30 min) and mesembrenone (RT 24.68 min). Δ^7 Mesembrenone was detected at very low concentration compared to the other alkaloids present in this extract. TSS-MP-0015 showed a similar compound profile with epimesembranol being the major compound (eluting at RT 48.52 min). In this particular plant extract, mesembrenone (RT 24.73 min), together with mesembranol (RT 23.10 min), manifested at a slightly higher quantity when compared to mesembrine, which eluted at RT 31.34 min. Δ^7 Mesembrenone was also present at a lower concentration and similar to TTSI-MP-0013.

4.2.1 Profiling of Tincture

It is reported that mesembrine, amongst other alkaloids, effects the central nervous system following intake of *S. tortuosum*. Based on this, tinctures of *S. tortuosum* and capsules are available in health shops to be ingested by individuals manifesting with stress, anxiety, depression and other mood-related conditions. A tincture as well as capsules (dried *S. tortuosum* plant material) were sourced and analyzed to identify if mesembrine was the main ingredient of the concoction. See **Figures 4.17** and **4.18** (pages 79 and 80) for more details of results

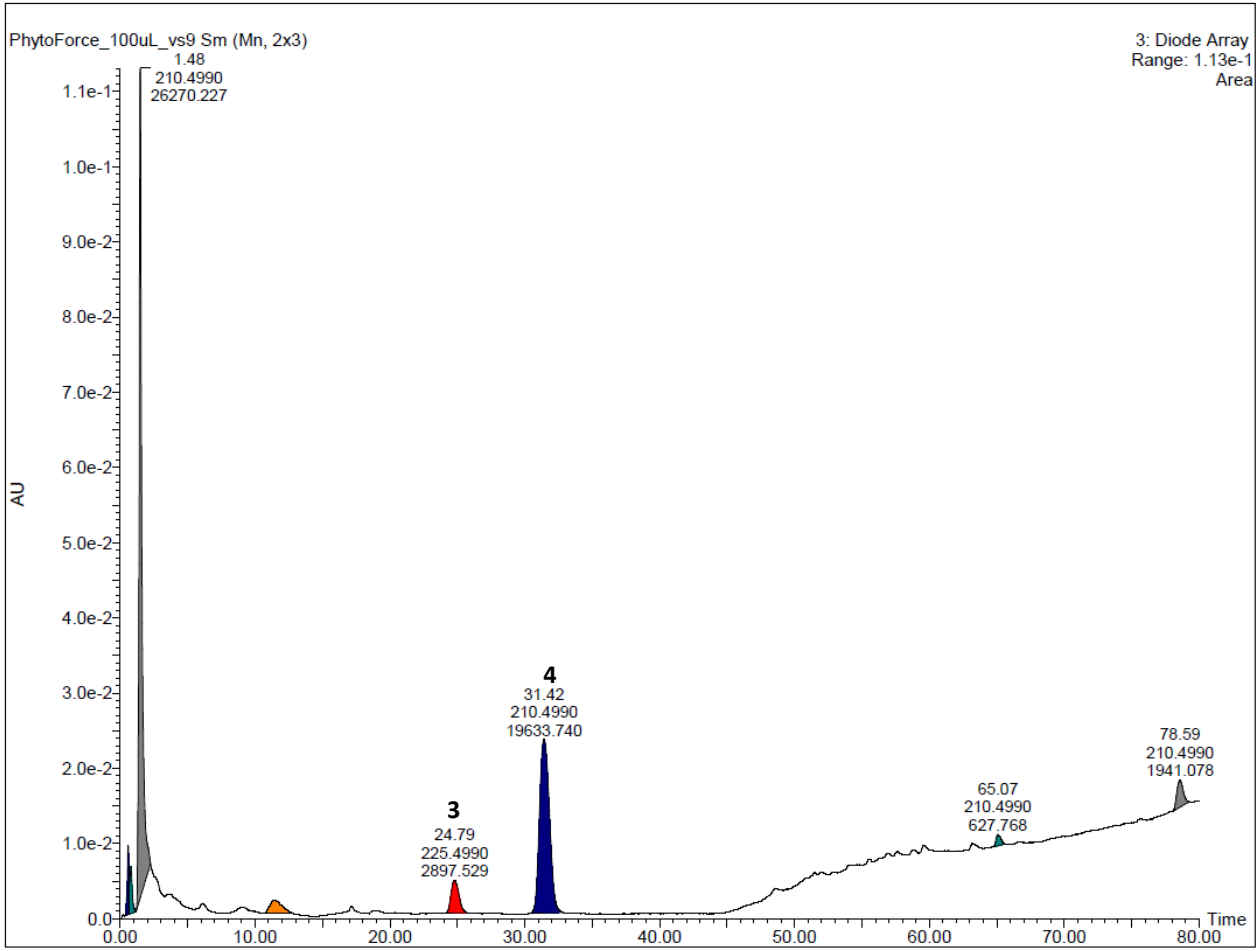

Figure 4.17: HPLC PDA profile of a commercially available tincture. Used to investigate the constituent profile of one of the market available tinctures to determine which compounds are available in abundance resulting in the proclaimed properties.

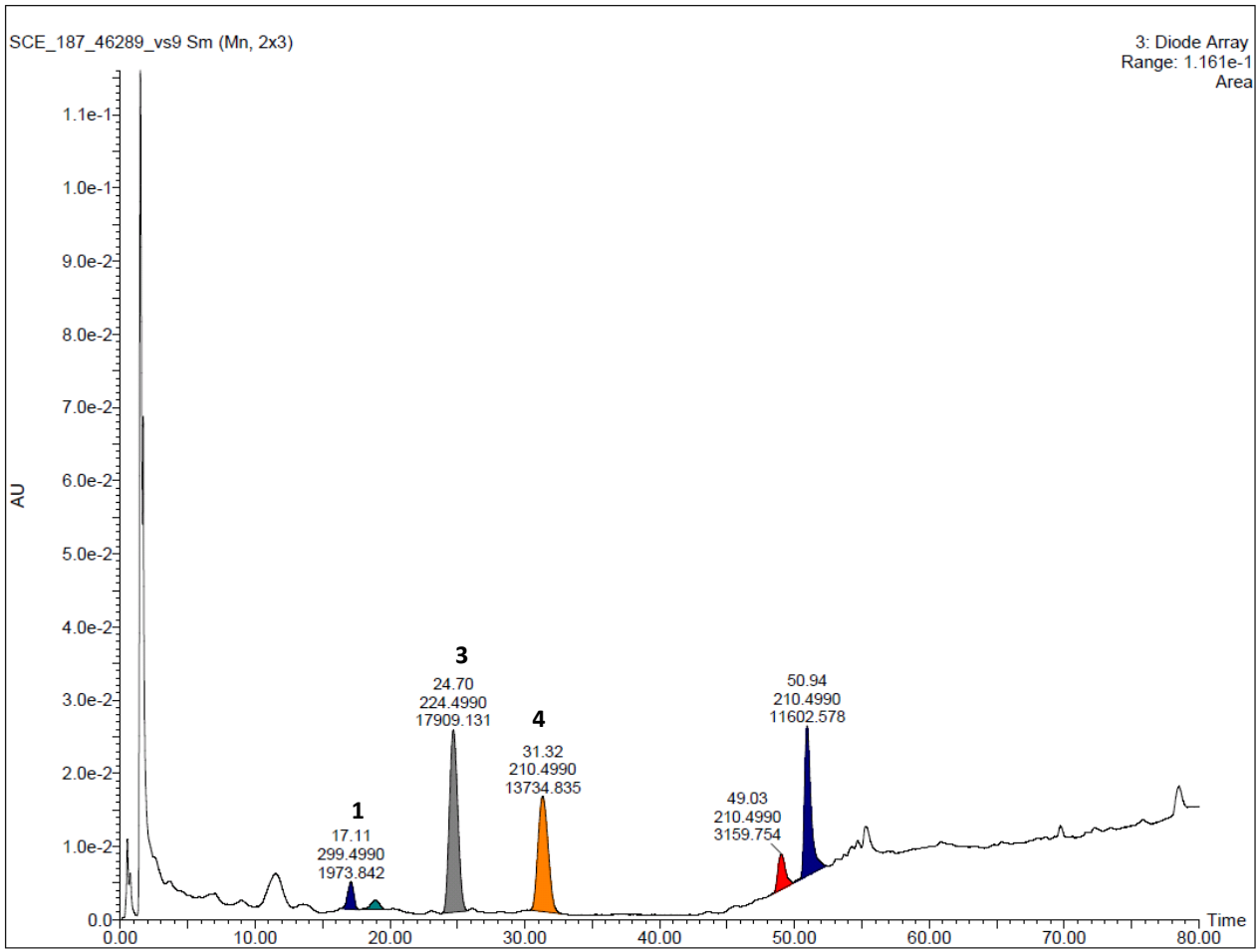

Figure 4.18: HPLC PDA profile of SCE-187-46289(commercially available capsules).

From the chromatogram spectrum (**Figure 4.17**), the tincture was a mixture of two compounds, with mesembrine being the major compound eluting at RT 31.42 min, while mesembrenone was found to be present at lower concentration and eluting at RT 24.79 min. Analysis of the chromatographic spectra of the Kanna capsule (SCE-187-46289 **Figure 4.18**) indicates that there are two minor and three major compounds present. Mesembrenone appears to be the major compound of this particular extract, eluting at RT 24.70 min, being present at a slightly higher concentration compared to mesembrine (eluting at RT 31. 32 min).In addition, there was also an unidentified compound suspected to be epimesembranol, which was observed at RT 50.94 min, being present in quantities higher than that of mesembrine. Again, it is possible that these compounds (mesembrenone and mesembrine) have the same health benefiting properties, as suggested by Smith *et al*., (1998).

4.3 Plasmodium Lactate Dehydrogenase screening (pLDH):

4.3.1 First round pLDH screening of *Sceletium tortuosum*:

Vector resistance to synthetic insecticides, as well as and the hazardous environment resulting from contamination by toxic non-biodegradable residues resulting from these insecticides, calls for innovative strategies, including that of natural product usage, to control destructive disease-causing vectors. In the latter instance, extracts from herbal/medicinal plant material constitutes a rich source of biodegradable bioactive compounds which break down into non-toxic products, making them an ideal source for mosquito control measures (Elango et al., 2010). The genus *Sceletium* is rich in alkaloids, which constitute one of the most important classes of naturally occurring products for drug usage since ancient times. Although alkaloids have been successfully used to treat parasitic infections, extracts from *Sceletium* have not been sufficiently researched as a treatment for various ailments of biological importance (Kaur *et al*., 2009).

In order to supplement research performed on (extracts of) *S. tortuosum* by other workers, the approach taken in this study was focussed towards a possible outcome of using this plant, or extracts thereof, for treatment of selected medical conditions, such as malaria infection, or against the vector responsible for transmission of this disease. Due to the high quantity of alkaloids present in this plant, a *Plasmodium* lactate dehydrogenase (pLDH) biological assay was used to detect whether this plant showed any anti-malarial properties.

Table 4.7 shows the *in vitro* anti-malarial screening of 19 extracts from *S. tortuosum*. Activity from these samples ranged from 1.47 to >100 µg/ml. For an extract to be regarded as an active anti-malarial, a resultant IC_{50} value of less than or equal to 10 µg/ml is required while any result greater than 10 µg/ml is considered non-active or a negative result. Of the 19 samples, four were found to be active. These are shown in red in **Table 4.7.**

Table 4.11: IC_{50} of test samples from a screening assay of fermentation of *S. tortuosum*.

	Test sample	IC_{50} (µg/ml
1	SCE-187-46201A	37.96
2	SCE-187-46201B	>100
3	SCE-187-46202A	12.10
4	SCE-187-46202B	32.74
5	SCE-187-46204A	>100
6	SCE-187-46204B	31.07
7	SCE-187-46205A	1.50
8	SCE-187-46205B	1.47
9	SCE-187-46205FD	18.14
10	SCE-187-46206A	19.94
11	SCE-187-46206B	15.65
12	SCE187-46207A	7.32
13	SCE187-46207B	2.68
14	SCE-187-46208	>100
15	SCE-187-46208A	33.38
16	SCE-187-46208B	32.28
17	SCE-187-46222	69.18
18	SCE-187-46223	>100
19	SCE-187-46245	>100
20	MeOH (Positive control)	>100
21	Blank-no drug (negative control)	>100
22	Reference strain – Cl 3D7	11.79

Samples were tested in triplicate while using chloroquine as a reference drug and MeOH as solvent control. Samples 7 and 8 (**Table 4.7)** were fermented in a conventional oven at 35^{o}C for 6 days, whereas samples 12 and 13 were fermented under natural conditions exposed to direct sunlight for 7 days. Sample 7 and 12 were both fermented in thermal bags, whereas sample 8 and 13 were fermented in open trays.[8,9] SCE-187-46208 and SCE-187-46222 were the extracts tested for biological activity, with the former being the EtOH extract and the latter being the MeOH extract. When reference to original plant specimen (SCE-187-46223) is made, isolated compounds from this material have been characterised as the **compound mesembrine** (see pages 55), while SCE-187-46245 was similarly characterised as the **compound pinitol** (see pages 57).

In contrast, extracts prepared from other plant material, such as specimen SCE187-46205, involved fermentation in an oven at constant temperature, and consistently gave very positive, low assay readings (1.50 and 1.47, respectively). Similarly, the extracts prepared from specimen SCE187-46207, involved fermentation under varying temperature (day and night) in sunlight, resulting in positive, but varied, results (7.32 and 2.68, respectively). These results may support the hypothesis that particular plant specimens may require preparation at a relatively constant and high temperature, as experienced in the incubator, while the appropriate decomposition of other plant material may require various stresses, such as by varying temperature and duration of exposure time to sunlight.

Another factor which needs to be taken into consideration, is dehydration of plant material during fermentation. When organic material or any sample containing water is enclosed in a plastic bag and exposed to sunlight or heat, decomposition of the organic compounds may occur to a greater or lesser extent. As the specimens 7, 8, 12 and 13 were screened soon after receipt, these results support the observation that effective, medicinal use of *S. tortuosum* extracts are best achieved with fresh plant material. While significant results have been achieved in this study, there may be a number of additional (variable factors) that will contribute synergistically or antagonistically towards the isolation of a medically active compound. As such, these results must be verified by further analysis.

[8] To view the preparation of test samples and standards go to Section 3.5.3.
[9] To view what other samples are and how they were prepared and what the alphabet A and B stand for, please read Section 3.2.2 – 3.2.2.1.

Samples showing a low IC_{50} result are indicated in **Table 4.7**. **Figures 4.19, 4.20, 4.21**, and **4.22** indicate the HPLC profiles of *Sceletium* preparations (extracts dissolved in MeOH).The spectra above indicate commonly isolated alkaloids from this plant as Δ^7 mesembrenone, mesembranol, Δ^4 mesembrenone and mesembrine. As such, it is very difficult to identify which of these compounds within these extracts is responsible for the anti-malarial activity, particularly as HPLC profiling of extracts from plant material which were negative for anti-malarial activity showed similar peaks as in the four profiles.

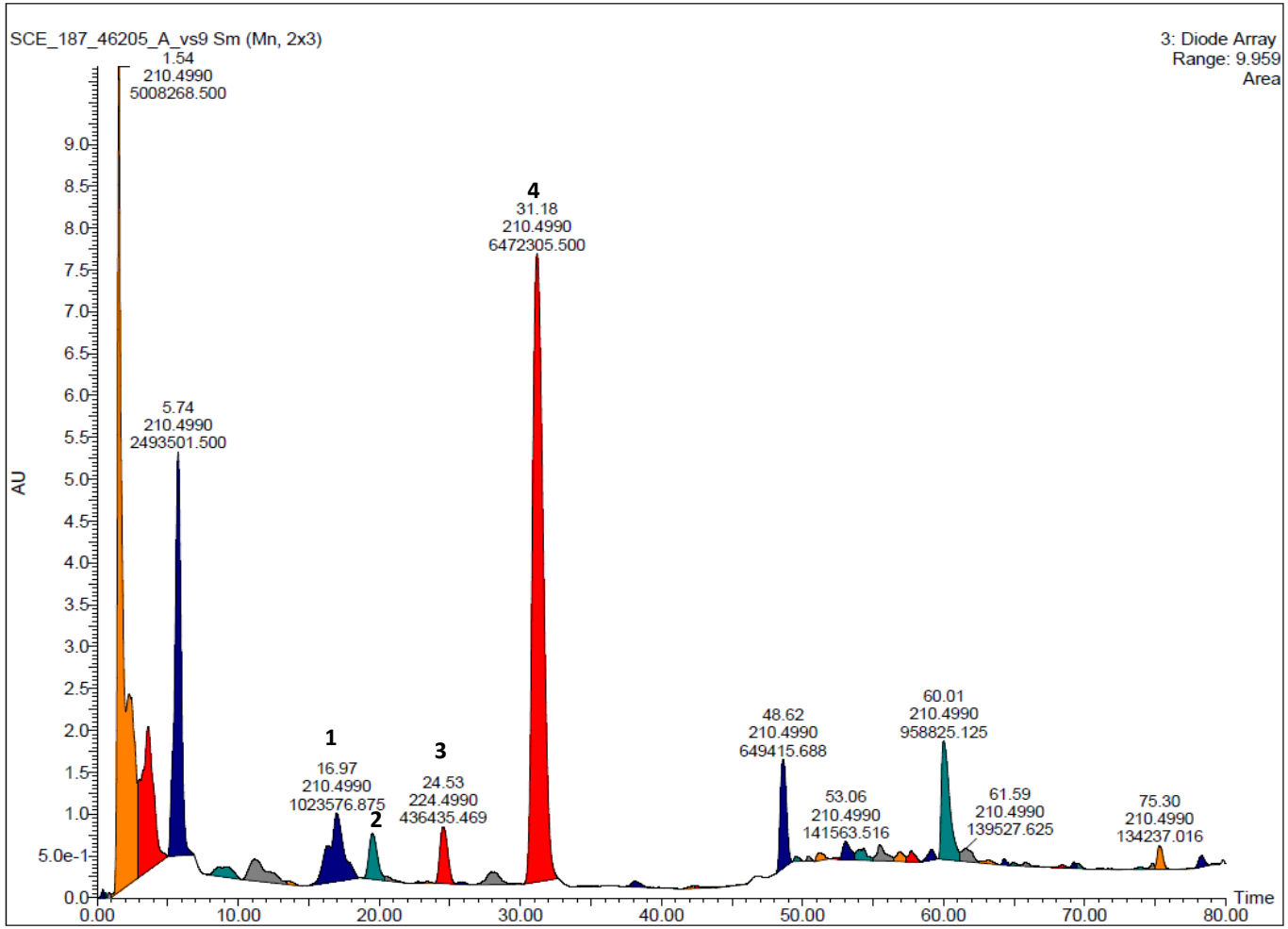

Figure 4.19: HPLC profile of SCE-187-46205A associated with IC_{50} = 1.50µg/ml. Showing a profile of one of the extracts that presented with excellent biological value suggesting that this extract could as well have anti-*plasmodial* properties. This profile does not in any way show which compound is responsible for the observed activity.

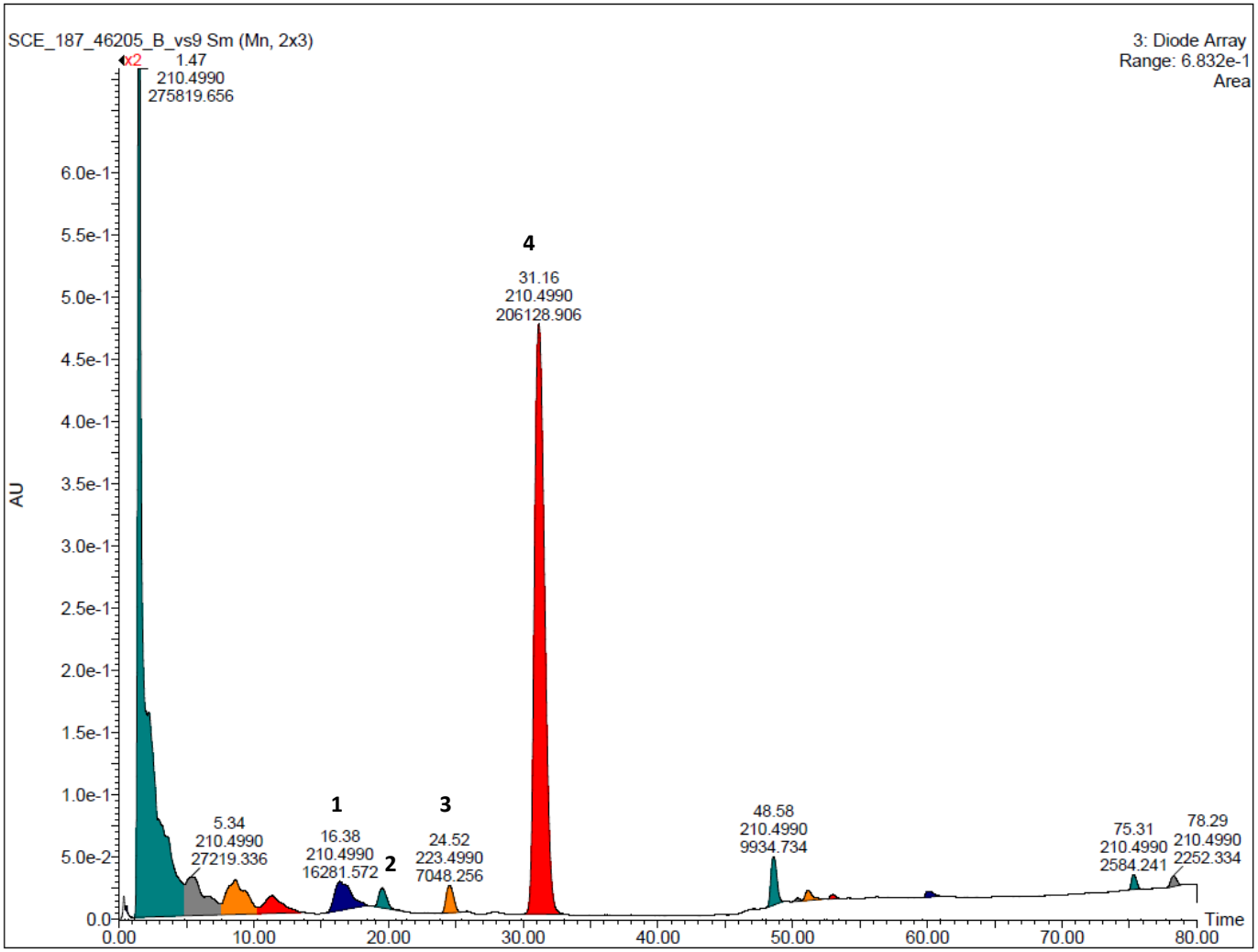

Figure 4.20: HPLC profile of SCE-187-46205B associated withIC_{50} = 1.47 µg/ml

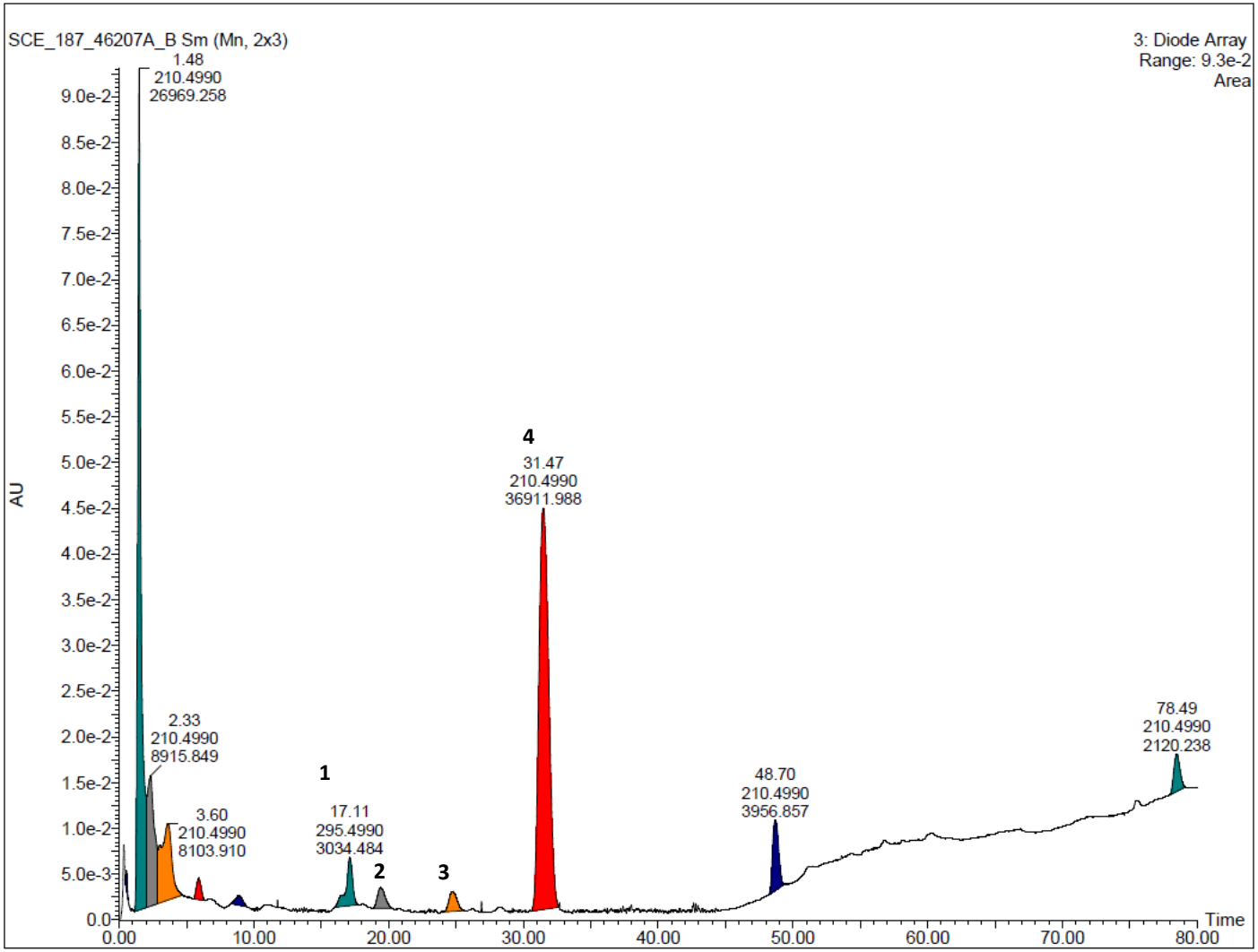

Figure 4.21: HPLC profile of SCE-187-46207A associated with IC_{50} = 7.32µg/ml

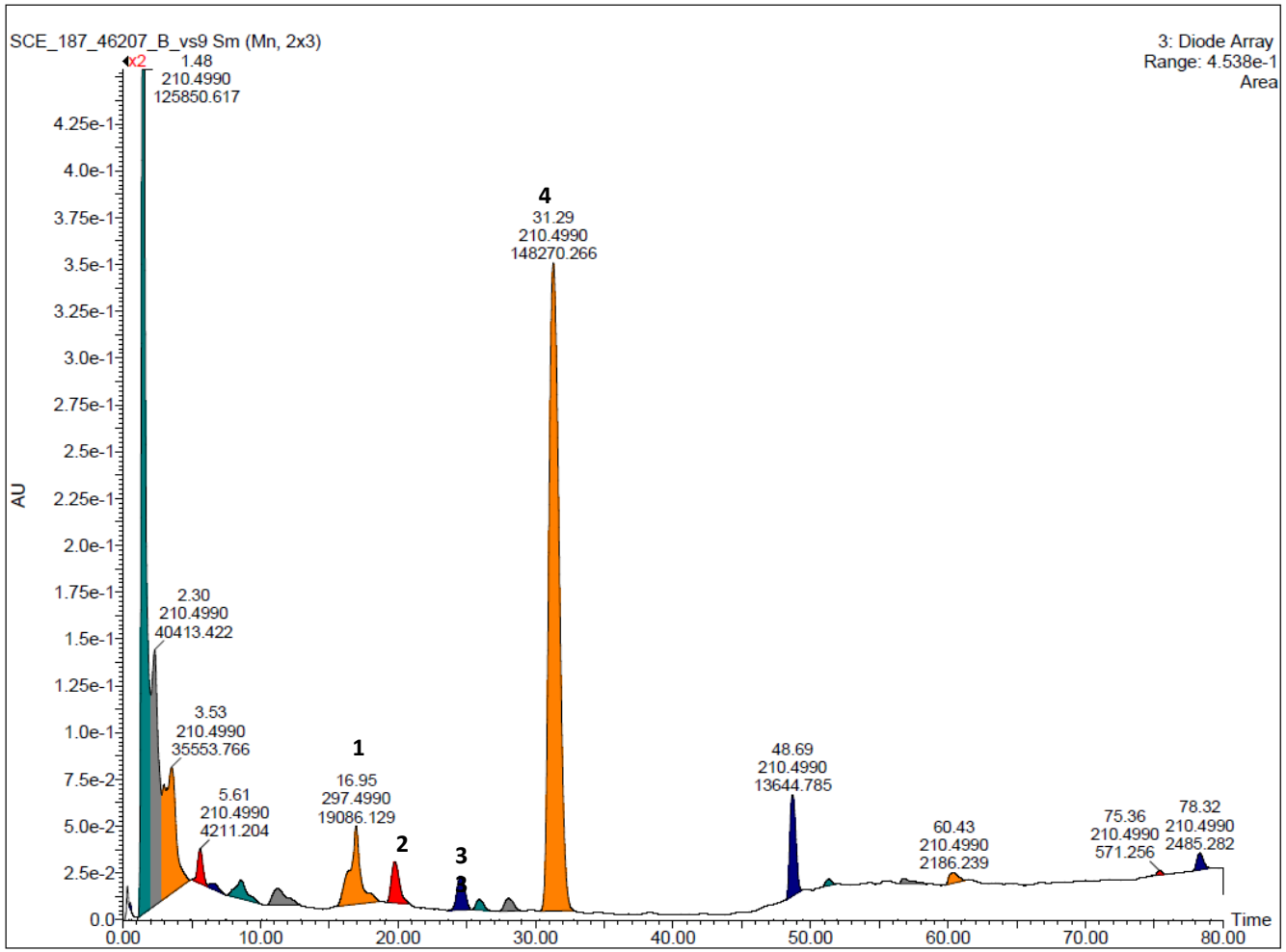

Figure 4.22: HPLC profile of SCE-187-46207B associated with IC_{50} = 2.68 µg/ml

As far as **Figures 4.19** to **4.22** are concerned, it might be the presence of other compounds in the extract that are responsible for the observed anti-malarial activity. Also note that the HPLC profiles of the four anti-malarial extracts shared similar peaks, particularly in the polar regions, but also in the non-polar regions of the chromatograms as well. Overall, no peak(s) appeared to be uniquely linked to anti-malarial activity. Although mesembrine appeared to be present in high quantities in all sample extracts tested, subsequent screening of this compound for biological activity did not show any anti-malarial properties. Alternatively, another compound/molecule may be attached to one or more of the compounds indicated in the HPLC profile, possibly forming a coordination complex with one of the above compounds. The proposed coordination complex may be destabilised during fermentation or during the extraction process prior to bio-assaying. This explanation may be supported by a study done by Mbeunkui and colleagues (2012) involving the stem bark of *Geissospermum vellosii,* where it was concluded that the bark contained alkaloids associated with lead, which subsequently formed molecular structures that could together be responsible for their observed anti-malarial activity.

As mentioned in **section 4.1.4**, mesembrine and other related compounds may be structurally and functionally labile, which prompted the need to rescreen the four anti-malarial samples (presented in **Table 4.7**).The extracts that were subsequently shown to have anti-malarial activity, were stored at 4^{o}C in the cold room. These extracts were dissolved in (5 ml) DMSO as it was found that other extracts no longer completely dissolved in methanol. As with MeOH, DMSO was included as one of the controls in the bioassay. The results of this rescreening experiment are presented below in **Figure 4.23** where the status of the compounds is indicated as “Freshly prepared”, “Old samples” and “Freeze dried”. The results of the anti-malarial activity of these extracts were compared to the original anti-malarial screening results and are presented on the same scale. The same procedure for plant material preparation was followed as previously described (Section 3.2.1). The originally active samples (SCE-187-46205A, SCE-187-46205B, SCE-187-46207A and SCE-187-46207B) were stored at 4^{o}C. These were also re-tested for activity and are presented as “Old samples” in the **Figure 4.23** below.

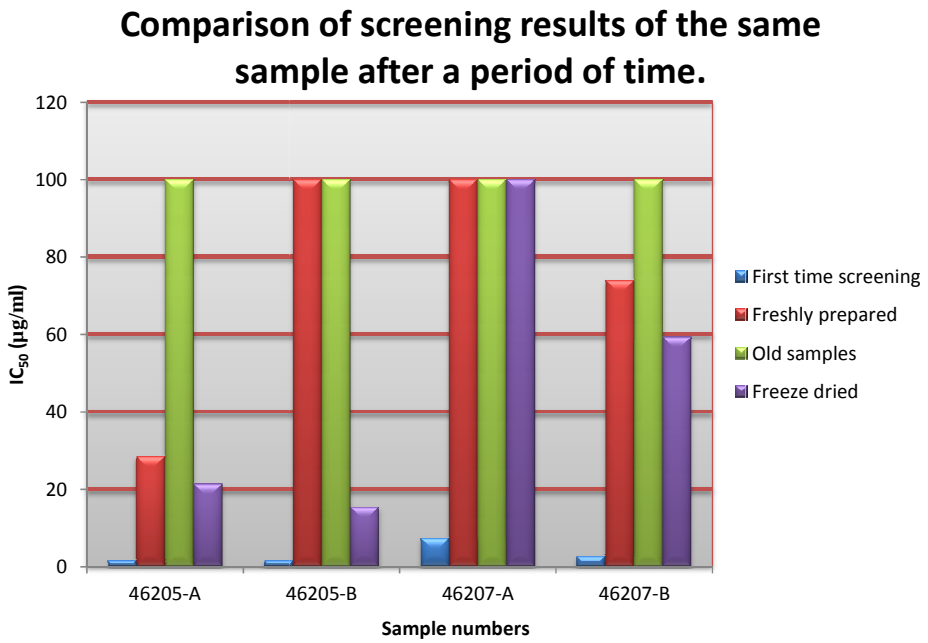

Figure4 .23: Results of rescreening for anti-malarial activity of samples 7,8,12 and 13 after 6 months' storage at 4°C compared to the initial bioassay (in blue).

A qualitative analysis of **Figure 4.23** indicates substantial loss of anti-malarial activity following any form of storage of the extracts. Compared to the original anti-malarial results (in blue), the storage of active anti-malarial extracts resulted in a complete loss in anti-malarial activity, as indicated by the green bars. These originally active extracts had been stored for six months at 4°C, by which time any anti-malarial activity was lost. When the solvent was removed from an original extract, followed by reconstitution in methanol and subsequent re-assaying, complete loss of activity was observed in two of specimens, together with substantial loss of activity in the remaining two extracts. These results are indicated as red bars. It appears that dehydration, resulting from freeze drying of the extracts, might have improved the anti-malarial activity of stored material. These results are indicated in **Figure 4.23** as purple bars, where a potential improvement in anti-malarial activity was noted in three of four extracts, compared to the red bars.

Due to the controversial results thus obtained, a stability test was designed and carried out, which lasted over a period of two months. The extracts were then chemically profiled, from the initial time of experiment, after a month of incubation at different temperatures and after 2 months of incubation of storage, at a respective temperature. No plant extracts were screened for anti-malaria activity immediately after extraction. The plant material received from Parceval pharmaceuticals was dry ground plant material and therefore never identified before use, like that supplied by ECD. Extracts were divided into duplicates of 2g and placed at different temperatures. This was to see if there would be any changes in constituents of the extracts at different temperatures being 4 ^{o}C, -20 ^{o}C, $^{+}$/$_{-}$ 25 ^{o}C, 35 ^{o}C, and 60 ^{o}C. For a summarised presentation of results, please view **Appendix 40**.

Chapter 5:

Conclusion and recommendations

5.1. Conclusion:

This study complements previous studies on *S. tortuosum* carried out by Smith *et al*., (1996), Gericke & Viljoen (2008), Patnala & Kanfer (2009) and Shikanga *et al*., (2011) as well as several other researches. The abovementioned researchers extracted plant material from this species according to the traditional method of preparation, as mentioned in Chapter 1. Although the traditional method of preparation was followed in the present study, a few steps were adjusted to accommodate the conditions and location, where this process was taking place. For example, all steps were followed as described in Chapter 1, except that the plant material was not buried under ground, but was directly exposed to sun light. The aim of changing those steps was to optimize a scaled-up method of preparation/fermentation which differs somewhat from that used traditionally, but is still a reliable method to produce consistent results / effects and /or compounds of interest.

It can be argued that this method of fermentation was successfully optimized, as compounds of interest were not compromised in the process. As discussed in Chapter 4, alkaloids of interest, similar to those described in the literature, were identified in all extracts, with each being analyzed and chemically profiled for various constituents using HPLC methodology. The results of this study were then compared to those of Patnala & Kanfer (2009), with commercial standards of *S. tortuosum* alkaloids being used as a reference when performing HPLC-based chemical profiling. According to the information gathered from the San people by Smith *et al*., (1996), it is believed that if not fermented, *S. tortuosum* will not possess 'biologically active' powers. It is assumed, that in this particular instance, they were referring to psychotic powers, as the San people believe this plant to have spiritually enhancing powers. It has also been noted, that unspecified mesembrenone and alkaloidal changes result from crushing and bruising of the plant material. Patnala and Kanfer (2009) stated that "the fermentation process undeniably transforms mesembrine to Δ^7mesembrenone. This would obviously result in the degradation of mesembrine, when stored during and after fermentation. The same phenomenon where mesembrine quantities were observed to have decreased was observed during this study and also shown in the previously mentioned HPLC profiling results. Thus; it can confidently be concluded that, plant preparation and fermentation processes were successfully optimized.

Overall, it was found that various chromatographic techniques including TLC and CC that were, used in this study proved to be useful and reliable. Column chromatography (CC) was employed for the isolation of chemical constituents from the plant *S. tortuosum*. This method was considered a method of choice by Smith *et al*., (1996) sixteen years ago and is still useful in this day and age, as proven by Shikanga *et al*., (2011) who successfully used column chromatography, as well as other techniques, for the isolation of compounds from this species.

Other chromatographic methods have proven to be better than others, especially where compound isolation is concerned. In this regard, when compared to CC, preparative TLC proved to be very useful when it came to isolation of alkaloids. In this study, compounds were isolated and purified by CC, which turned out to be time consuming. Preparative TLC was found to be tedious, but to be the best method for isolation of alkaloids from *Sceletium* as well as in the subsequent purification of compounds. Knowing which solvent system to use, for the isolation of a targeted alkaloid, was not only saved time but also proved to be beneficial in the long run.

All four major alkaloids were successfully isolated and purified by both CC and prep-TLC. The compounds of this plant material were unfortunately found to be unstable, as discussed in Chapter 4. As all four alkaloids degraded and regrettably could not easily be re-accumulated, the ultimate acquisition of mesembrine and Δ^4 mesembrenone thus obtained occurred with low yield. Sucrose and pinitol were isolated using CC, whereas obtusalin was isolated using prep-TLC. Since no other phytochemical method was available, or had to be employed for the isolation and purification of the last of the four mentioned compounds, it was therefore concluded that the two methods were sufficiently reliable in this project for the isolation of compounds of *S tortuosum*. The confirmation of these isolated compounds depended on NMR spectroscopic results. Since all compounds isolated in this project were known and had previously been reported in the literature, only ^{1}H and ^{13}C experiments were performed on purified plant extracts. The results were compared to those in literature as referenced in Chapter 4 and presented in the Appendices. NMR successfully validated the compounds thus isolated.

As stated by Marston (2007), HPLC is the best technique for an efficient separation of crude extracts and thus was a good technique to apply in this study. HPLC methodology played an important role in the confirmation of the presence of targeted alkaloids. Patnala & Kanfer, (2009) profiled the constituents of *S. tortuosum*, but due to the differences in geographical region, as well as differences in the harvesting season, a different method for HPLC profiling of *S. tortuosum*had to be developed. Fortunately, the two researches used the same standards of alkaloids that were present in the species used in this project. As such, eluted compounds which were observed in the intermediate region of the chromatogram of this study, were similar to those presented by Patnala & Kanfer (2009), thereby not only corroborating the presence of the same compounds, but that this plant was indeed *S. tortuosum* and that the method developed was correctly optimised.

HPLC was also used in the quantification[10] of mesembrine (**Appendix 41**) after a ten day fermentation process. In addition, a laboratory isolated and purified mesembrine was used as a reference standard in this experiment. Smith *et al*., (1996) reported on the differential distribution of alkaloidal chemicals in the leaves (0.3%) and stem (0.86%) of *S. tortuosum*. Popelak & Lettengauer (1968) reported on the presence of mesembrine in "*kougoed*" (fermented *S. tortuosum*) to be around 0.7% in both leaves and stems of this plant. In this study, mesembrine was not quantified before fermentation, but the amount of mesembrine in plant material fermented in a thermal bag was found to be in agreement with the 0.7% quantity found in fermented *S. tortuosum* mesembrine material. Therefore, despite this plant being fermented under different conditions, compared to that of the traditional preparation and optimization of the method of preparation and fermentation was effective in agreement with values provided in literature.

HPLC was then used for the evaluation of extracts set up for the stability test of *S. tortuosum*, as well as to track changes occurring in these extracts over time. The extracts were analytically profiled using HPLC before storage, after a month of storage and after two months of storage at different temperatures, as previously mentioned in Chapter 4. There were no major differences observed in the UV Diode Array spectra, but significant changes were observed in the ESI^+ mode (**Appendix 40, Figure i and ii**), where it was noticed that there was a differential increase in the concentration of some compounds at the expense of others. This differential trend was

[10] Summary of quantified mesembrine in samples fermented from the second batch (plant material stored at -20°C for six months) fermentation – Appendix 41.

observed throughout all the stored extracts, irrespective of the temperature of storage. This observation therefore supports the previously observed changes and confirms the notion that decomposition of certain alkaloids does in fact occur within the stored extracts over time. **Table i.** as well as **Figure iv** of **Appendix 41** further indicates the changes in the quantity of mesembrine in older crude material, originally extracted from the same plant batch (material).

The isolation of the compound obtusalin was a highlight of this study, as no other triterpenoid, or structurally related compound has been reported in the literature to have ever been isolated from *S. tortuosum*, or the genus in general. Triterpenoids are generally very stable and, unlike the alkaloids of this plant material, do not decompose but were rather found in very low concentrations. Since mesembrine was screened for anti-malarial properties and was found to be deficient of any activity, it might be assumed that other compounds unknown to this plant, like obtusalin could be responsible for some of the biological activity observed in this study. Since obtusalin was isolated at the latter stage of the study, it was not screened for anti-malarial activity; therefore; no such conclusion can be made at this stage.

In essence, the isolation of this particular compound indicates that there are likely to be more compounds which still have to be discovered for the *Sceletium* genus, which might also have the potential for biological activity.

5.2. Recommendations

Significant compound decomposition in extracts was noticed after a period spent in storage. Extracts also seemed to develop two layers, where the oil separates from the extract paste. Constituent instability was also observed in the fresh (not dried) plant material stored at -20^{o}C for six months. In this particular case, it is recommended that one should store plant material in a dried powder form, or as a dried whole plant. This will help preserve the plants constituents for however long is required. Extracts must be freeze-dried and stored in a temperature stable, dry and dark place, to prevent chemical changes. It is also recommended that isolated compounds are dried before storage, to prevent them from breaking down.

In retrospect, it would be wise to obtain the HPLC profiles of the fresh plant material before storage or usage, and then to compare or use as reference material in future experiments.

HPLC was used for the possible detection of compounds responsible for the activity observed from the pLDH screening assay results, but no specific compound was identified or connected with the observed activity. Thus, it is recommended, where future studies of this kind are planned, that a biological assay-guided fractionation approach be followed. In terms of phytochemistry, compounds of this plant tend to bond with silica and elutes slowly from silica columns. It is therefore advisable to always use 1% base (in this case Et_2N) when isolating basic compounds and acetic acid when targeting very polar compounds as part of the solvent system.

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APPENDICES

COMPANY CONFIDENTIAL
Report No. CSIR/BIO/ACT/SD/2008/0002/B

CSIR our future through science BIOSCIENCES	Programme: Agro-processing and Chemical Technology	ACT Number: 313
	Effective Date: 17 April 2008	Revision: Rev. 00
	Document type: GENERIC STANDARD OPERATING PROCEDURE	Page 4 of 7
Title: Harvesting of plants for research and development studies		

CSIR			
RAW MATERIAL			
GEOGRAPHIC AREA	KAMIESKROON, NORTHERN CAPE		
ITEM	SCELETIUM		
SUPPLIER	ECD		
BATCH No	ECD-MP-0054	DATE HARVESTED	
COLLECTOR'S NAME		JOHNNY BURGER	
COLLECTOR'S SIGNATURE			
CONTAINER No			
WEIGHTS	TARE		
	GROSS	19-8	
	NETT		
QUARANTINED			

Master Copy Stamp		Controlled Copy Stamp	
	Originator	Checked By	Authorizer
Initials			

If not stamped in red as a "Master Copy" or "Authorized Copy": valid for 1 month from effective date

Appendix 1: Harvesting details of Sceletiumtortuosum raw material by the CSIR

CSIR our future through science BIOSCIENCES	Programme: Agro-processing and Chemical Technology	ACT Number: 313
	Effective Date: 17 April 2008	Revision: Rev. 00
	Document type: GENERIC STANDARD OPERATING PROCEDURE	Page 5 of 7
Title: Harvesting of plants for research and development studies		

Certificate for harvesting of plants

I, Johnny Burger, hereby agree that I have harvested plants for CSIR and have abided by the following specifications during my collection:

Harvest date start: 11-June-09 Harvest date end: 12-June-09

Area of collection: KAMIESKROON, NORTHERN CAPE (NAMAQUA)

Name of plant: SCELETIUM **Sample Number:** ECD-MP-00614

Macroscopic description of plant material:

.....

.....

.....

Method of harvesting:

Hand picked

.....

.....

Storage:

Paper box

In my capacity as a Botanist/ Project Leader for cultivation site, I confirm that the identification of the plants as SCELETIUM

Signed by: .....

Name: J.W. BURGER

Date: 16-6-2009

Master Copy Stamp		Controlled Copy Stamp	
	Originator	Checked By	Authorizer
Initials			

If not stamped in red as a "Master Copy" or "Authorized Copy": valid for 1 month from effective date

Appendix 2: Certificate of harvesting of *Sceletiumtuortosum* plant material.

COMPANY CONFIDENTIAL
Report No. CSIR/BIO/ACT/SD/2008/0002/B

CSIR our future through science BIOSCIENCES	Programme: Agro-processing and Chemical Technology	ACT Number: 313
	Effective Date: 17 April 2008	Revision: Rev. 00
	Document type: GENERIC STANDARD OPERATING PROCEDURE	Page 6 of 7
Title: Harvesting of plants for research and development studies		

Plant Species: Sceletium

Plant Part: Aerial

Sample Number: ECD - MP - 0054

Approximate mass of sample:

Area of collection: Noupivies, Kamieskroon, N-Cape.

Number of plants harvested:

Date of Harvest: 11 - 12 June 2009.

Weather conditions: Sunny & Dry

Other comments:

Harvest completed by: 12 - JUNE - 2009

GPS co-ordinates:

Signature:

Master Copy Stamp		Controlled Copy Stamp	
	Originator	Checked By	Authorizer
Initials			

If not stamped in red as a "Master Copy" or "Authorized Copy": valid for 1 month from effective date

Appendix 3: Part 2 of the certificate of harvesting of *Sceletiumtortuosum* plant material.

South African National Biodiversity Institute

Ref:
Addr: 605

PLANT IDENTIFICATION DISPATCH LIST

Final List

Batch no: 10025 **Date received:** 21 April 2010 **Date:** 29 July 2010

To: Dr V.J. Maharaj
CSIR Food Science and Technology
P.O. Box 395
0001 Pretoria

From : The Director
National Botanical Institute
Private Bag X7
Claremont, 7735
South Africa
Tel: (021) 762-1166
Fax: (021) 761-4151

ID CODES:
1 = Specimen too poor to ID
2 = Label information inadequate
3 = Cannot match specimen in Compt
4 = Specialist not available to do ID
5 = Genus requiring/under revision
6 = Specimen closest to name listed (cf)
7 = Please send more material
8 = Please refer to attached note/letter
9 = New record

	SPECIMEN NO	GENSPEC NO	PLANT NAME	ID CODE
Dr. Fouche				
*	ECDMP- 100	22	Sceletium tortuosum (L.) N.E.Br.	

SUMMARY: **Received** = 1 **(Requested back (*)** = 2 **)** **Completed** = 1
Insystem = 0

Appendix 4: SANBI plant identification certificate.

Background on Malaria Infection

Malaria is one of the world's deadliest diseases and is caused by the protozoan parasites belonging to the genus *Plasmodium*. There are four species known to account for almost all human infections thereby being responsible for most morbidity and mortality. These are "*P. falciparum, P. vivax, P. malariae, and P. ovale*", with *P. falciparum* being the commonest of the four human malaria parasites found across much of Africa especially sub-Saharan Africa (Greenwood *et al*., 2005). Malaria can be transmitted by several species of female anopheline mosquitoes that differ in behaviour, thus leading to various epidemiological patterns of the disease observed worldwide. *Anophelise gambiae* is the most common and widespread in Africa. *P. vivax* can develop in mosquitoes at a lower temperature than *P. falciparum*, hence its wider geographical range (Greenwood *et al*., 2005).

Malaria is usually an endemic disease, but it may also occur as an outbreak. This may occur in areas with low seasonal transmission. There are several factors that may course outbreaks and these include an increase in vector breeding sites, migration of infected people into a vector-rich area, populated with susceptible individuals, arrival of new efficient vectors, breakdown of vector control measures, resistance of the parasites to treatment, and resistance of the vector to insecticides (Elango *et al*., 2010). The life cycle, immunological defence mechanisms and clinical development of malaria is complicated. This is reflected by aspects of clinical malaria that is characterized by periodic fever, which follows the lysis of infected erythrocytes, caused mainly by stimulation of cytokines interleukin-1 and tumor necrosis factor. *P. falciparum* infection can have serious effects, for example, anaemia, cerebral complications (from coma to convulsion), hypoglycaemia and glomerulonephritis. The disease is most serious in non-immune individuals, including children, pregnant women and tourists (Kaur*et al*., 2009).

Malaria transmission

Epidemiology

Burden of disease

Malaria is the world's most important tropical disease. It is prevalent in about 100 countries and is estimated that 247 million clinical episodes of malaria occur globally per annum, of which 86% are in sub-Saharan Africa. More than 767 000 Africans were estimated to have died from malaria in 2008, and 88% were children under five years of age, equivalent to a child dying of malaria every 45 seconds (Bagavan *et al*., 2010 and Diap*et al*., 2010). Although most malarial-associated deaths occur in Africa, evidence suggests that the number of clinical episodes of *P. falciparum* malaria is even higher than reported (including that due to *P. vivax* infections) and that morbidity due to malaria in Asia has been greatly under-estimated (Greenwood *et al*., 2005). In South East Asia alone, 100 million malaria cases occur every year and 70% of these are reported from India (Bagavan *et al*., 2010).

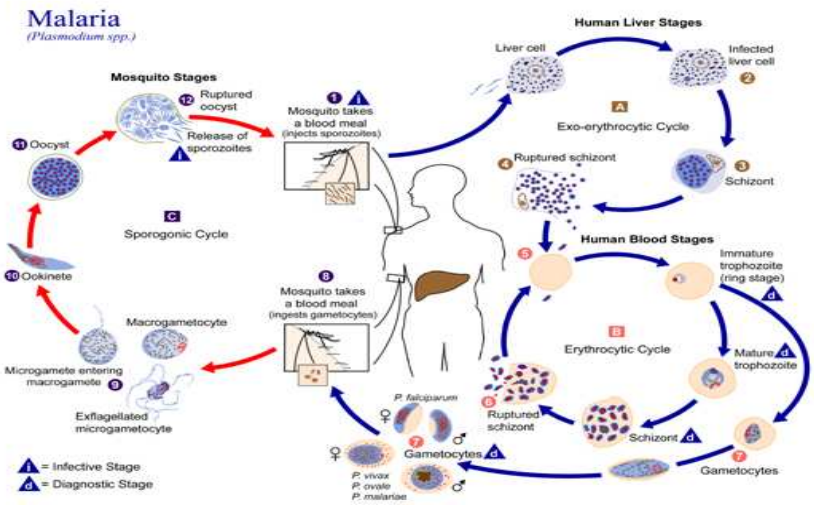
In malaria-endemic areas, it is believed that malaria infections in pregnant women account for up to a quarter of all cases of severe maternal anaemia and for 10-20% of low birth weight in babies. In addition to its direct effect in infants, as based on its effect on birth weight, the disease could account for an additional 5-10% of neonatal and infant deaths. The effect of malaria extends far beyond the superficial measures and observation of mobility and mortality, and is associated with intellectual development impairment and persisting developmental abnormalities (Greenwood *et al*., 2005).

Pathogenesis

Malaria has a complex life cycle which consists of sexual and asexual phases. The sexual cycle occurs in mosquitoes and the asexual cycle takes place in the human host. The parasite depends on both hosts to complete its deadly life cycle. The parasite enters into the blood stream through injection as the mosquito bites the human host. The parasite starts reproducing rapidly in the liver. Some parasites lie dormant in the liver and become activated perhaps years after the initial infection has occurred. The parasite enters the bloodstream within 30 minutes, invades liver cells and starts to multiply. They then lyse the liver cells and migrate to the bloodstream, where they attach and enter red blood cells and further reproduce. Infected red

blood cells lyse and allow the parasites to infect other blood cells and this becomes a continuous, and rapid, process in the blood stream.

This repeating cycle depletes the body of oxygen and also causes fever and chills. After release, a dormant version of malaria travels through the host's blood stream waiting to be ingested by another mosquito to carry it to a new host. Inside the mosquito itself, female and male sporozoites form a zygote-oocyte, cell division and multiplication takes place at a phenomenal rate. The zygote-oocytes migrate to the salivary gland and wait for re-infection to take place (JrSacci*et al.,* 2006).



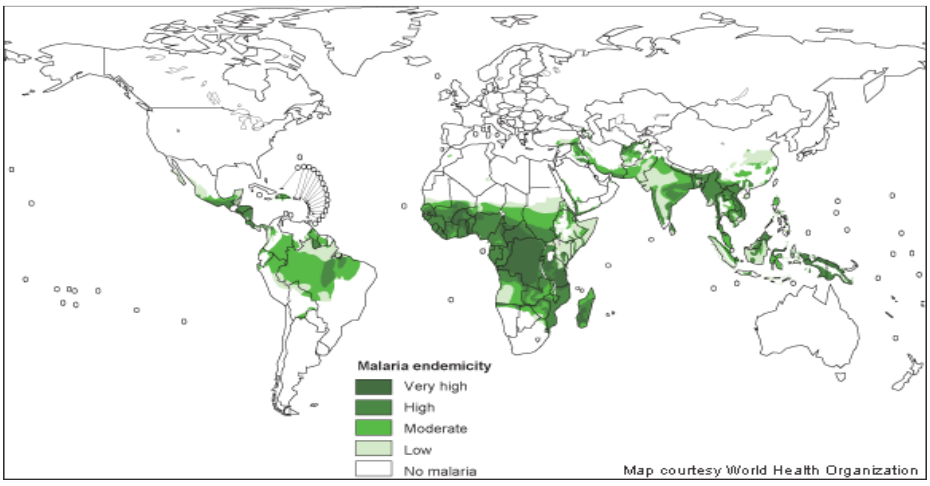
Malaria life cycle Source: http://dpd.cdc.gov/dpdx/html/Malaria.htm

Malaria has an incubation period of around three weeks (seven to twenty one days). This is the period between the time the mosquito bites the human and the onset of the malaria illness. The initial time is somewhat unpredictable as reports suggest that the extent of incubation periods may range from four days to one year. Since some parasites remain dormant in the liver cells, the patient can relapse even after they have received treatment (Davis, 1996).

Distribution

Tropical diseases such as malaria have a serious impact on economic welfare and on public health problems in developing countries situated in the tropical belts of Asia, Africa and Latin

America and are less common in other parts of these continents (De Ridder*et al*., 2008 and Breman *et al*., 2004). Climatic conditions over a large part of Africa favour malaria transmission and global warming, together with changes in land use, are extending the areas of transmission (Pillay *et al*., 2008). Limpopo, KwaZulu Natal and Mpumalanga are the three provinces in South Africa to where malaria transmission is currently restricted. These provinces are situated in the north-eastern parts of the country along the borders of Mozambique and Swaziland. Transmission is associated with higher rainfall and an increase in temperature between September and May each year with a substantial inter-annual variation in the number of malaria cases. Low intensity seasonal transmission precludes the development of acquired immunity (Pillay *et al*., 2008 and Mehta *et al*., 2007).

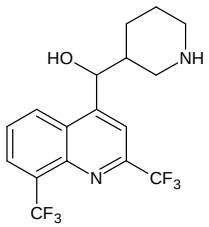


Global distribuiton map of malaria

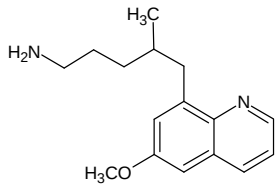
Malaria treatment

The world's first anti-malarial drug, quinine, was isolated in 1820 from *Cinchona* bark. Chloroquine, the most widely used anti-malarial drug was subsequently synthesized in 1940. The structure of a number of anti-malarial drugs are shown in Figure i. (a to i) below. Mefloquine **(a)** is one of the synthetic anti-malarial drugs with a mode of action similar to that of chloroquine. Primaquine **(b)** is given as a follow up drug after treatment with chloroquine to eradicate the liver hypnozoites of *P. vivax* and *P. ovale*. Atovaquone **(c)** and proguanil **(d)** are used together to treat uncomplicated *P. falciparum* malaria whereas halofantrine **(e)** is administered in cases of uncomplicated multi-resistant*P. falciparum* malaria. Artemisinin **(f)** is

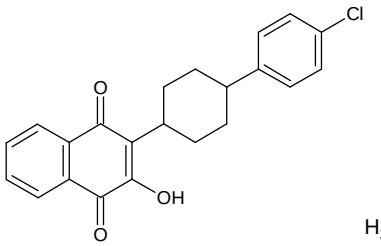
an endoperoxide sesquiterpene lactone that was isolated from Chinese herb *Artemisia annua* in 1972. Artemisinin and its derivatives are used for both uncomplicated and severe *P. falciparum* malaria. Derivatives most commonly used are artemisinin artemether **(g)**, arte-ether **(h)** and artesunate **(i)** (Breman *et al*., 2004, Rosenthal, 2003 and Mital, 2007). The chemical structures of these compounds are shown in Figure i.



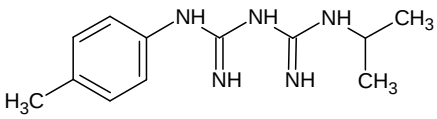
a. Mefloquine



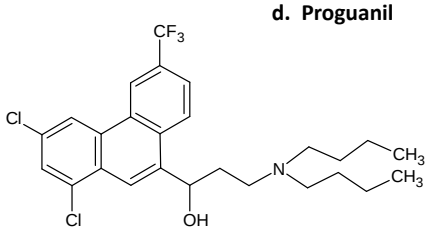
b. Primaquine



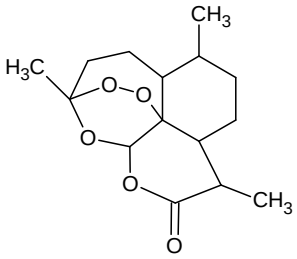
c. Atovaquone



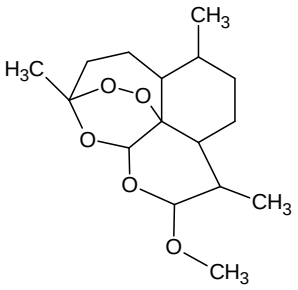
d. Proguanil



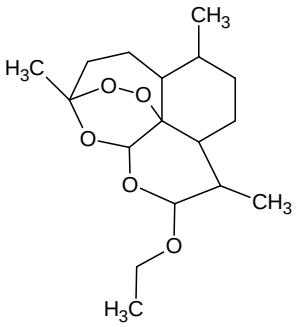
e. Halofantrine



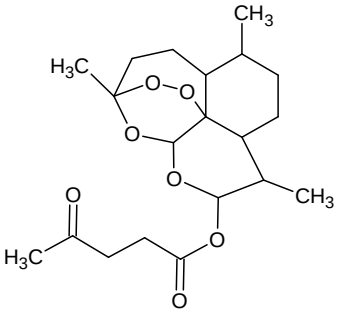
f. Artemisinin



g. Artemether



h. Arteether



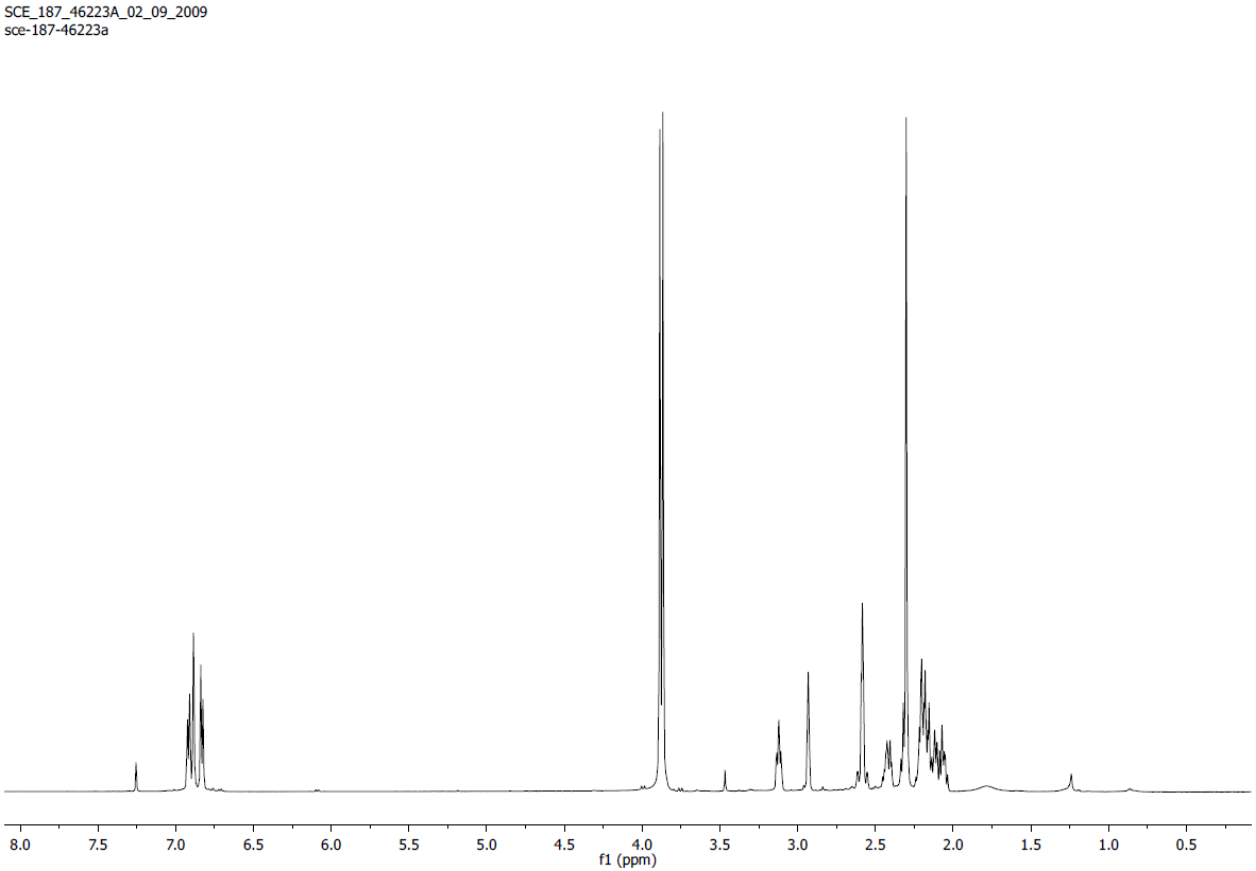
i. Artesunate

Figure i: (a to i) Structures of anti-malarial drugs.

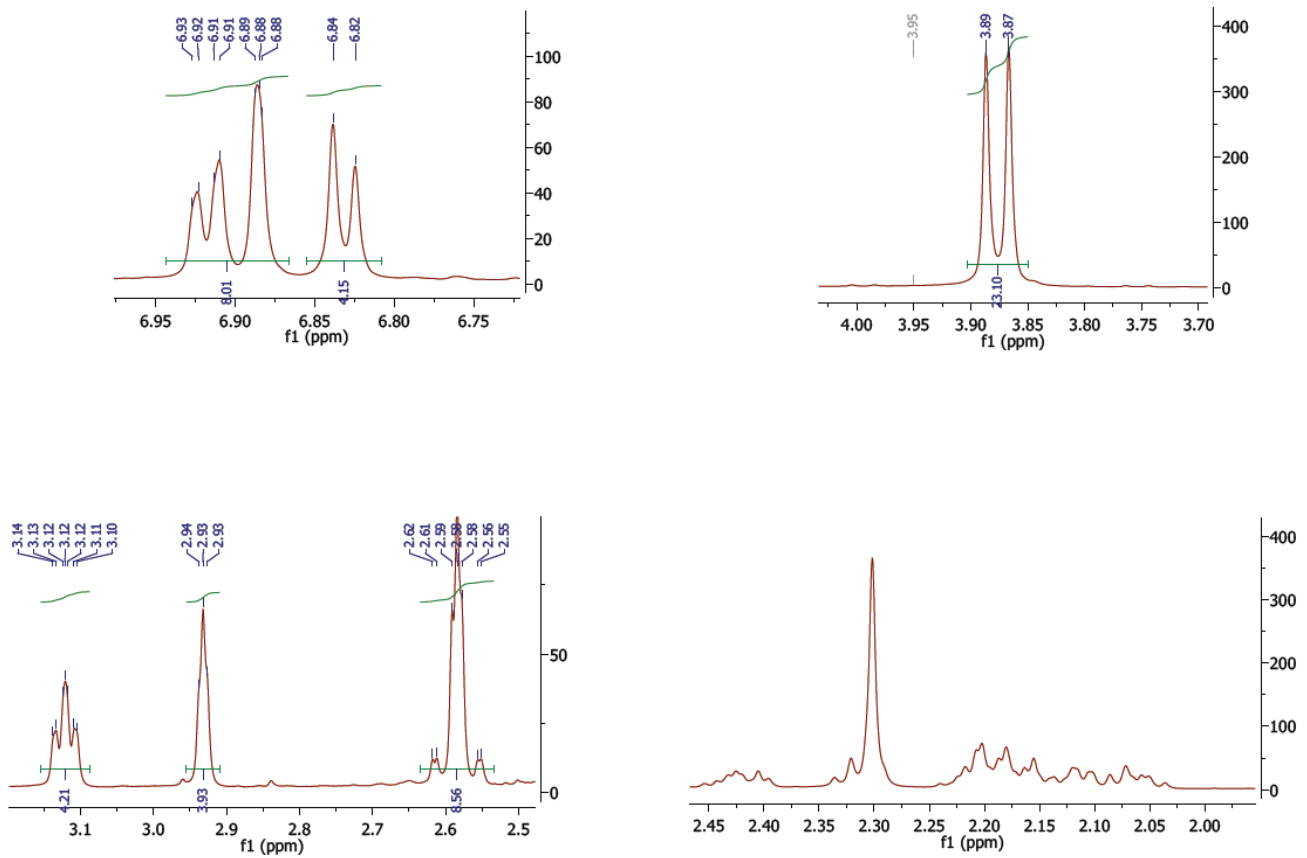
Drug resistance

Antimalarial drug resistance was defined as “the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance”. Resistance appears to occur through natural mutation that prompts reduced sensitivity to a given drug or class of drugs (Bloland, 2001). Drug resistance was noticed in the late 1950’s and early 1960’s and expanded to become a worldwide problem. Multi-drug and extreme-drug resistance is a growing challenge in Africa and South-East Asia that resulted in a paradigm shift in the search for new and more effective anti-malarial drugs (Kian, 1995). Synergistic use of drugs has been useful, thus far, with some resistance being reported in recent years. Treatment combination reduces treatment failure, recrudescence and gametocyte carriage and increases drug efficacy. Resistance is also associated with reduced drug half-life, and, therefore, the longer the half-life of a drug, the

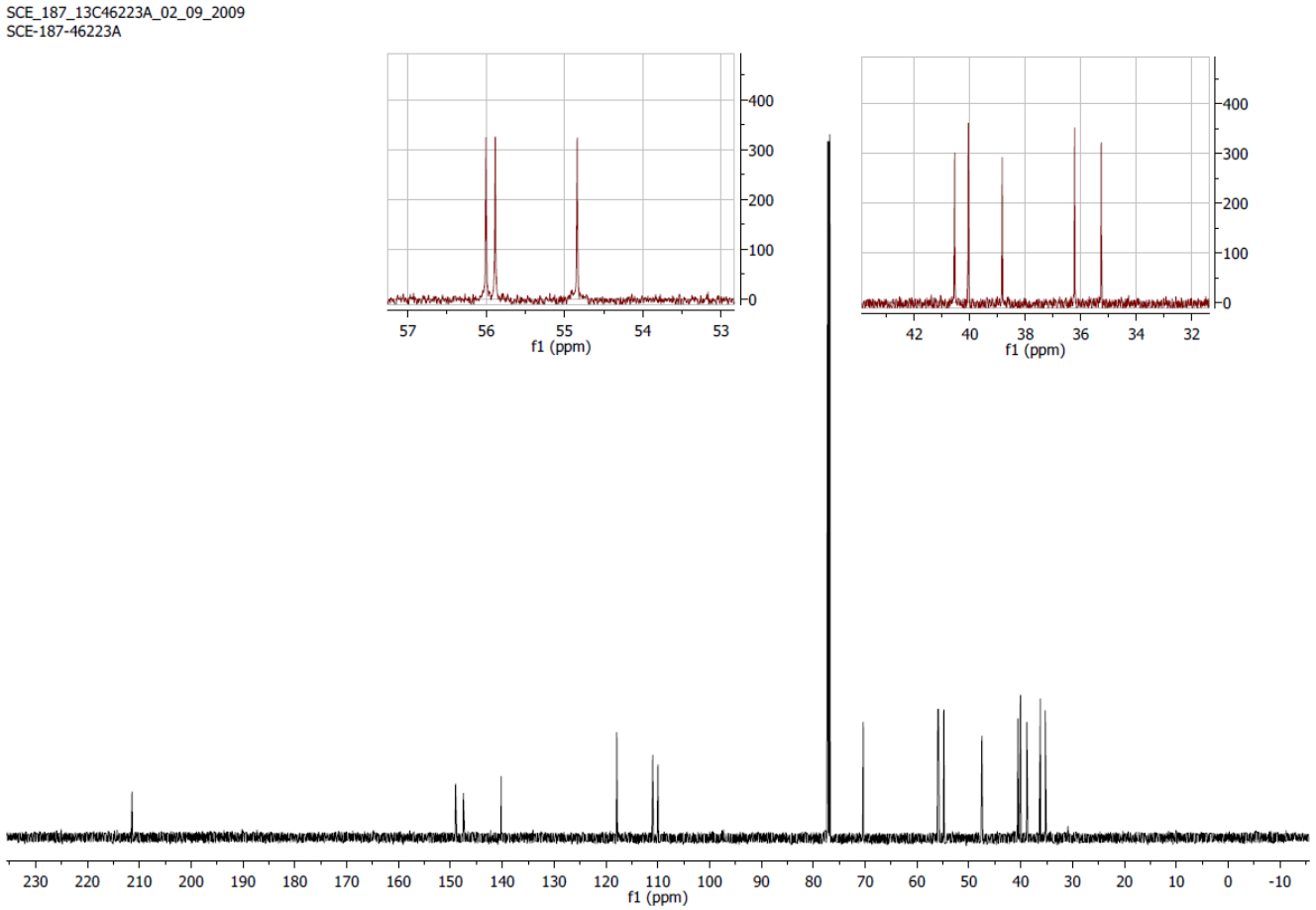
greater are the chances that drug resistance development will be delayed (May and Meyer, 2003).In all four malaria parasites that naturally infect humans, resistance to antimalarial drugs has been reported for only two species, *P. falciparum* and *P. vivax*. *P. falciparum* has developed resistance to nearly all antimalarials in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly. It has been shown that *P. vivax* infections acquired in some areas are resistant to chloroquine and/or primaquine. Sulfadoxine-pyrimethamine (SP) is becoming more prevalent in Africa and is increasingly being relied on as a replacement for chloroquine (Bloland, 2001).



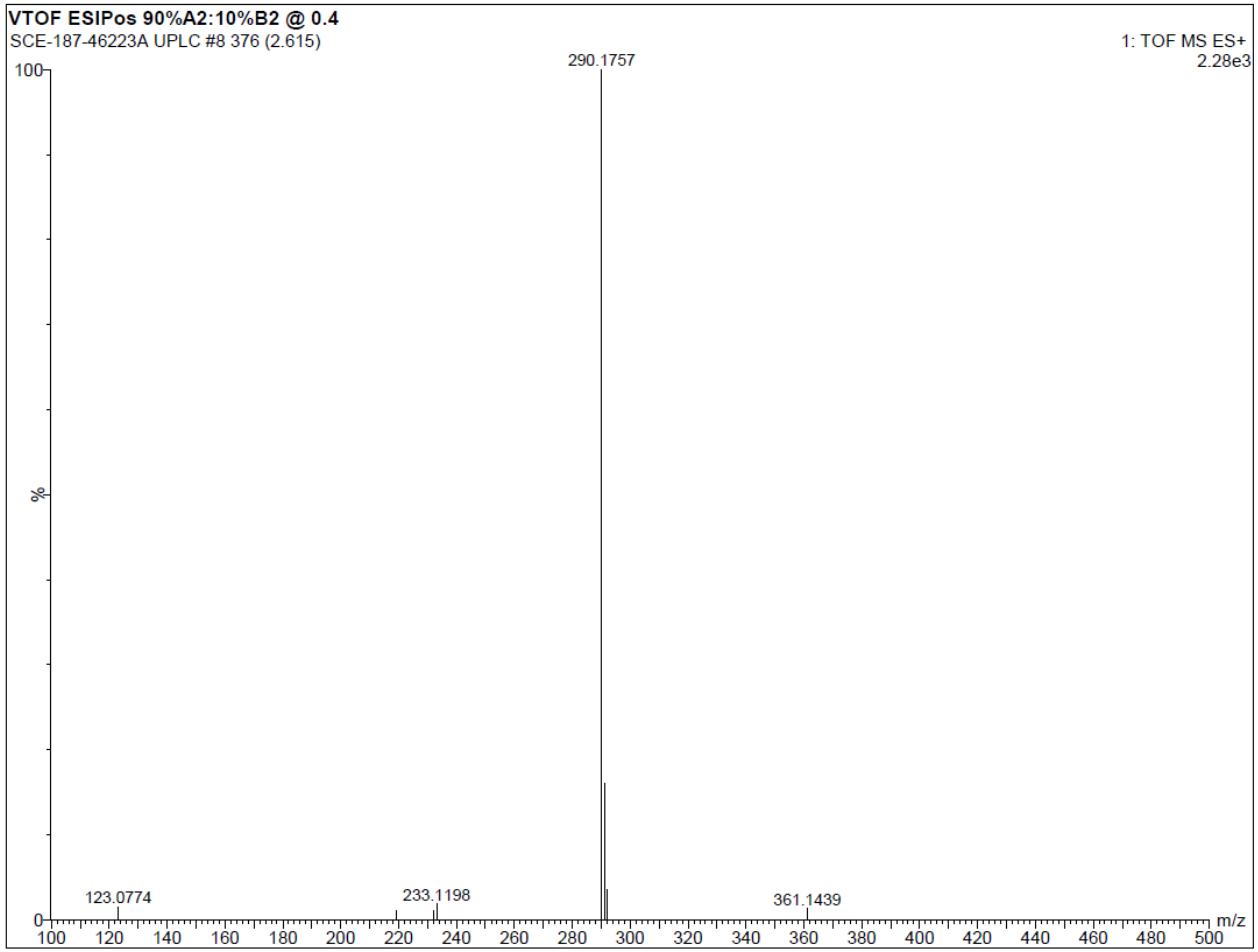
Appendix 6: Mesembrine proton spectrum (processed using MestReNova software version 8). Refer to page 50 and page 54 to view the structure.



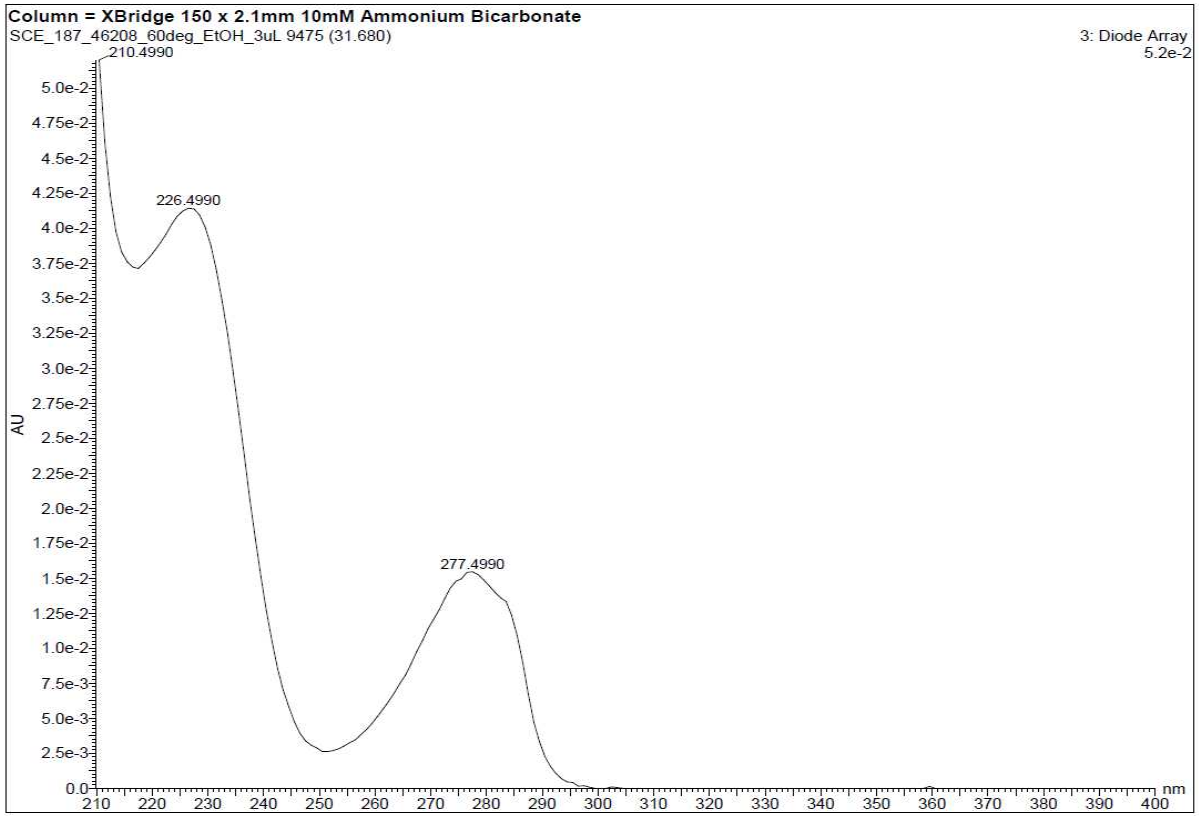
Appendix 7: Expansions of proton peaks fromt the mesembrine parameters (processed using MestReNova software version 8)



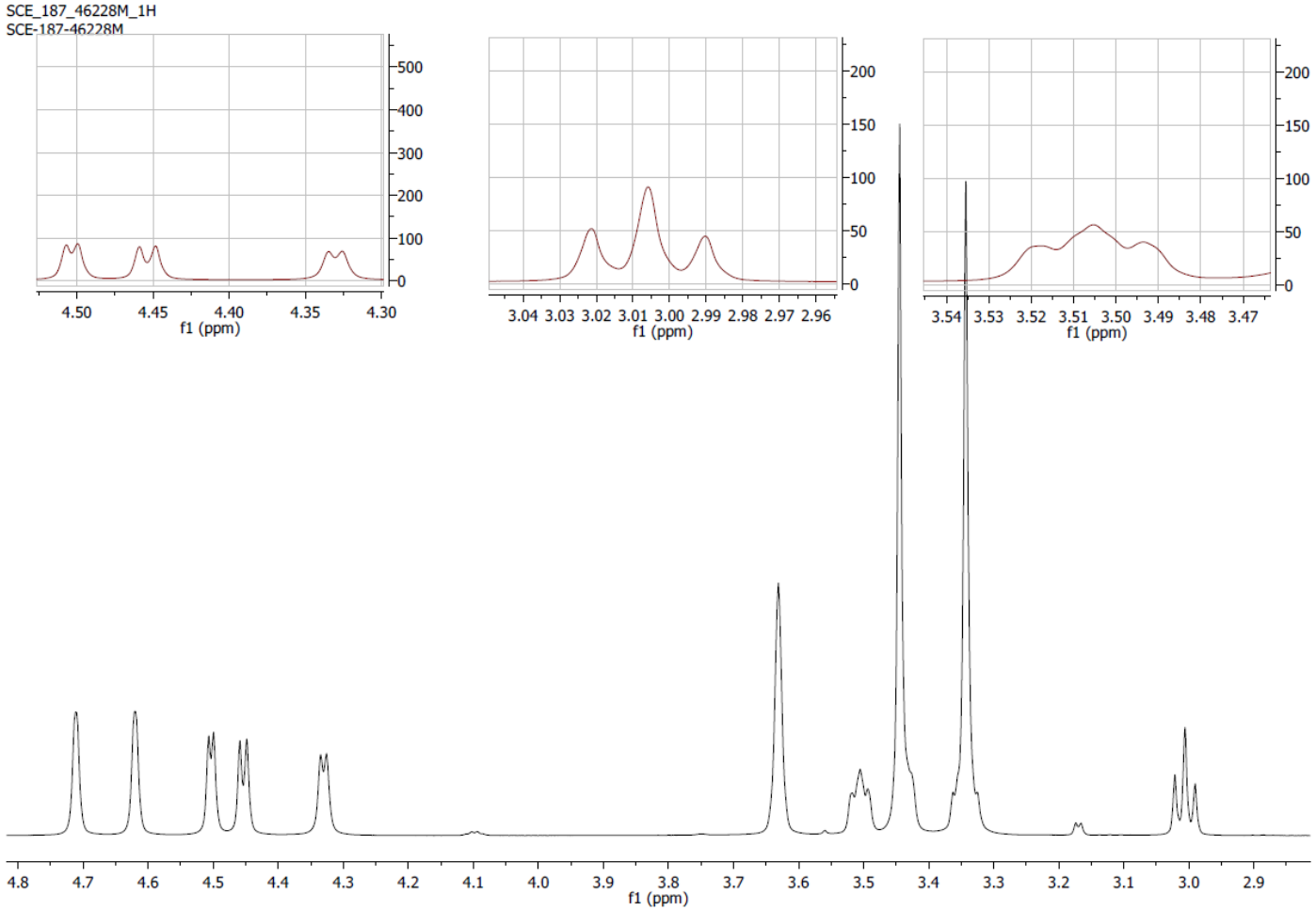
Appendix 8: Mesembrine carbon 13 spectrum (processed using MestReNova software version 8). Refer to page 51 and page 54 to view the structure.



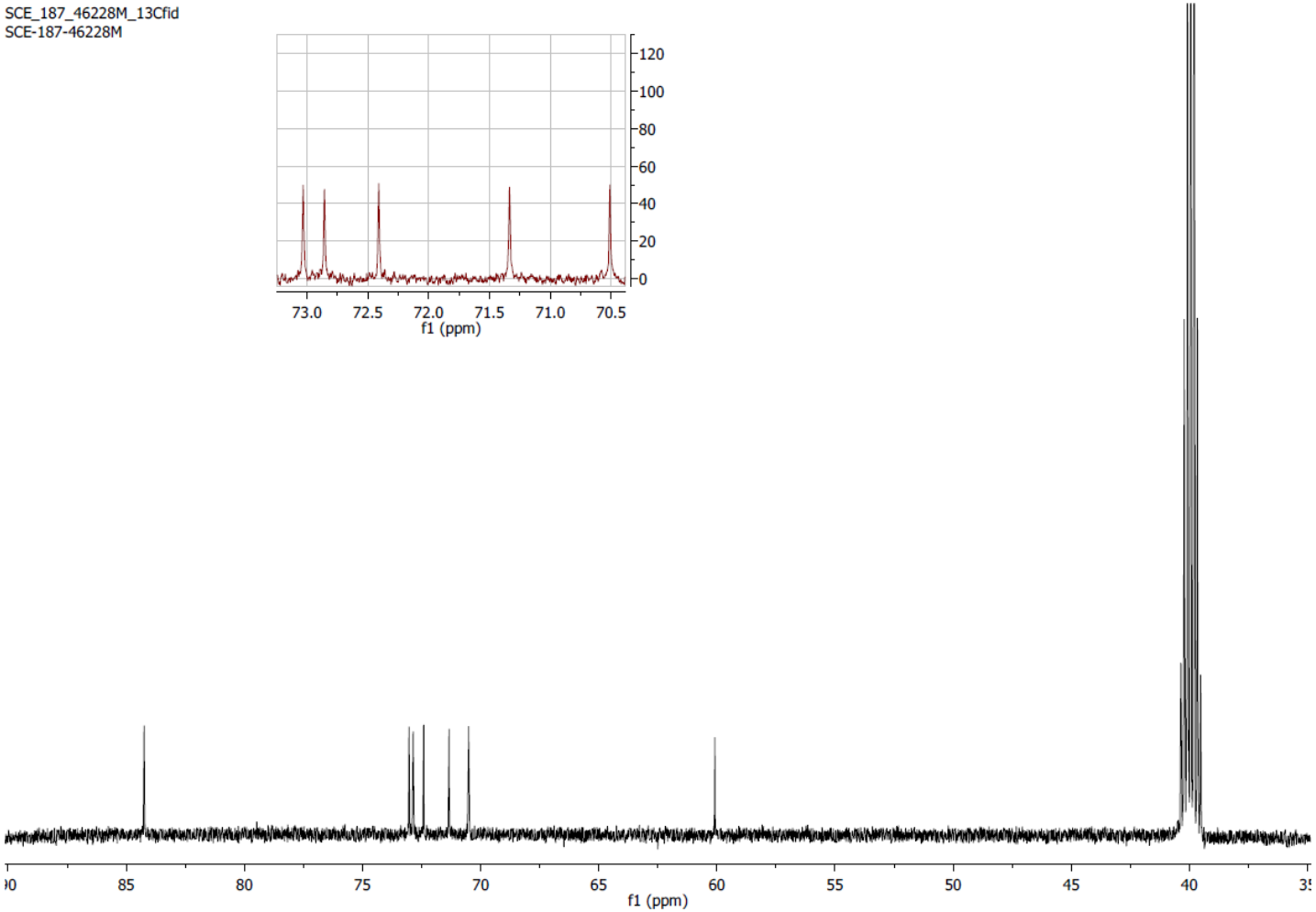
Appendix 9: ESI$^+$ TOF mass spectrum of isolated mesembrine. Refer to page 52.



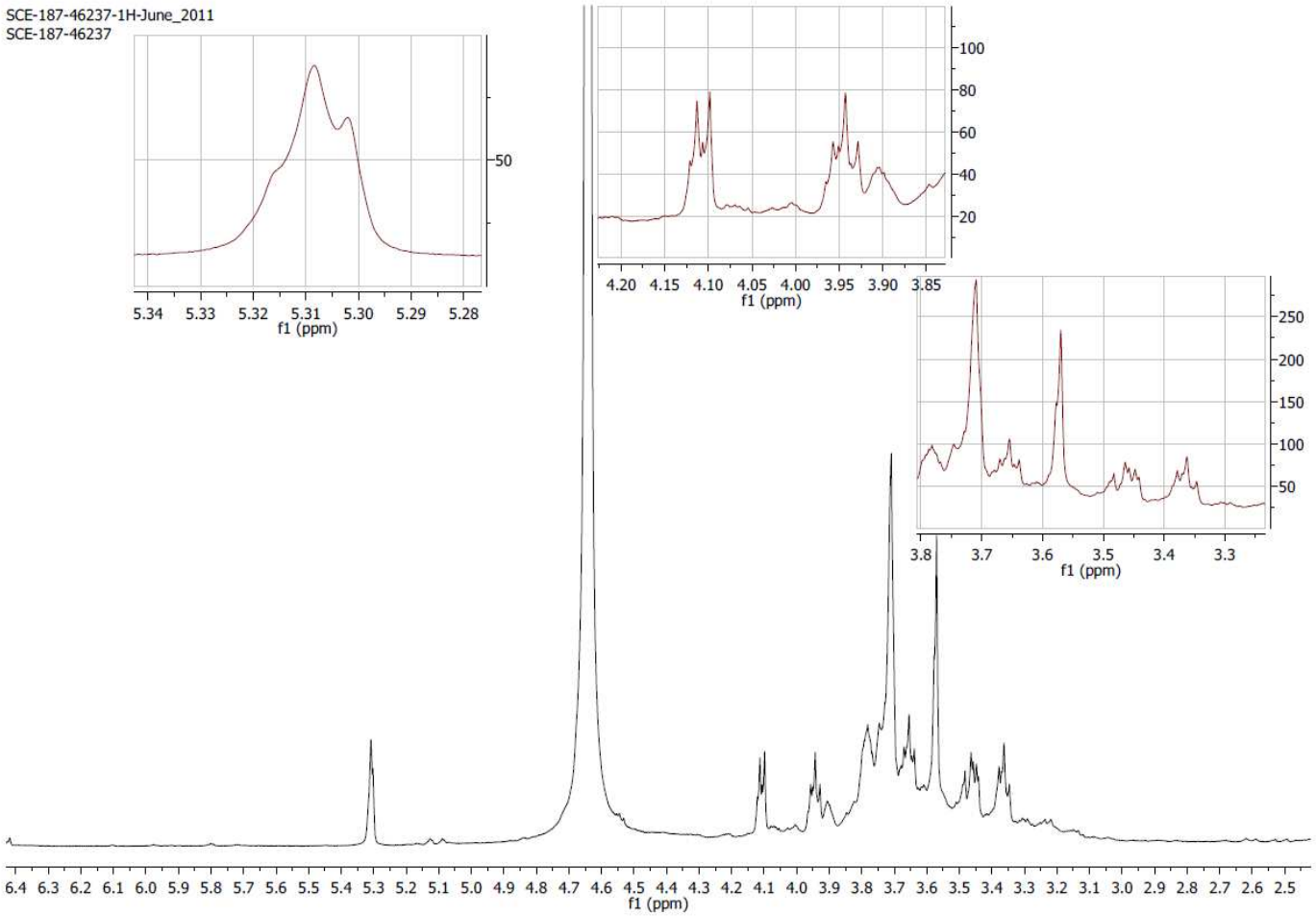
Appendix 10: UV absorption spectrum of Mesembrine detected in crude SCE-187-46208. Refer to page 53.



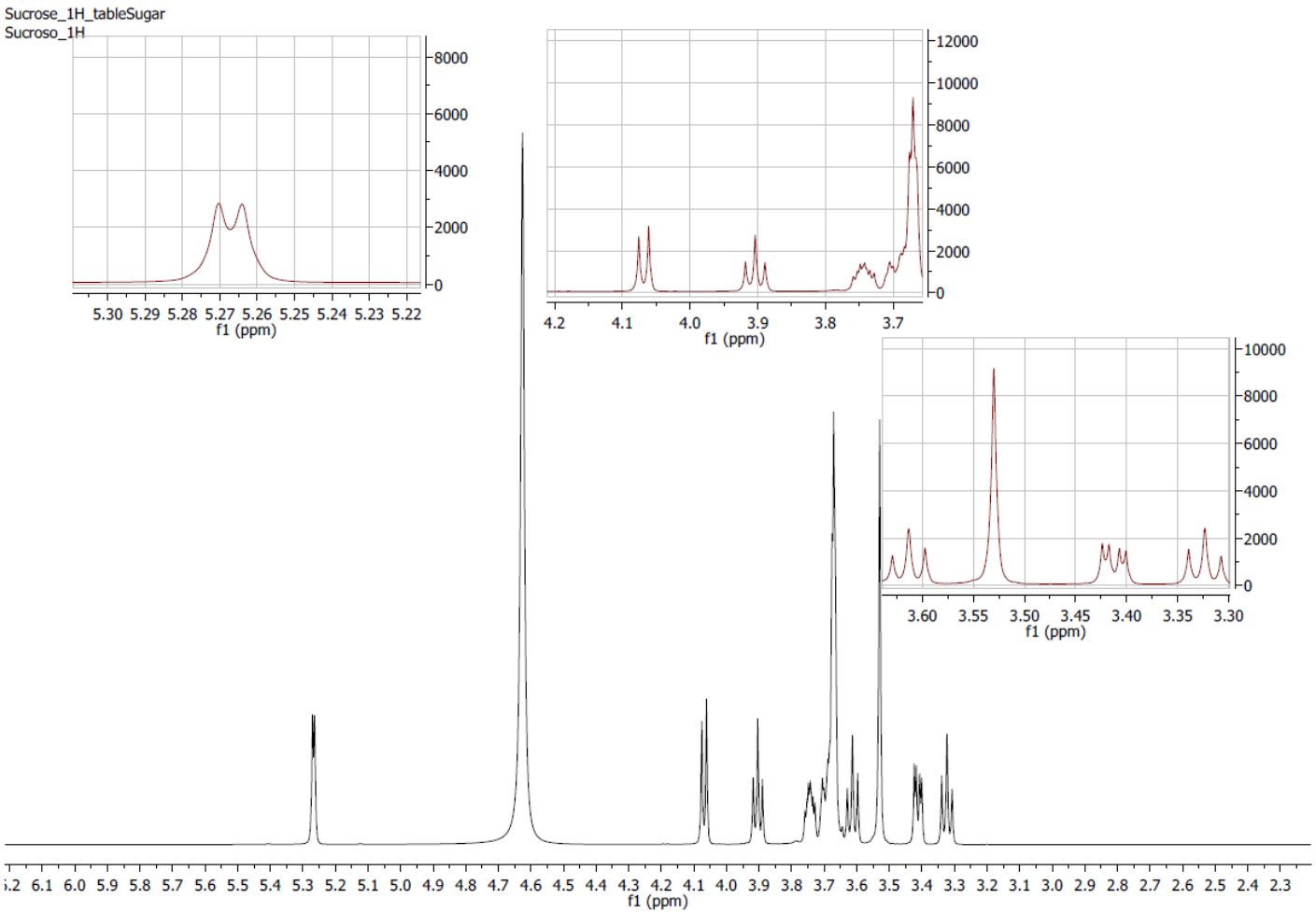
Appendix 11: Proton spectra of pinitol (processed using MestReNova software version 8). Refer to page 55 and page 56 to view the structure.



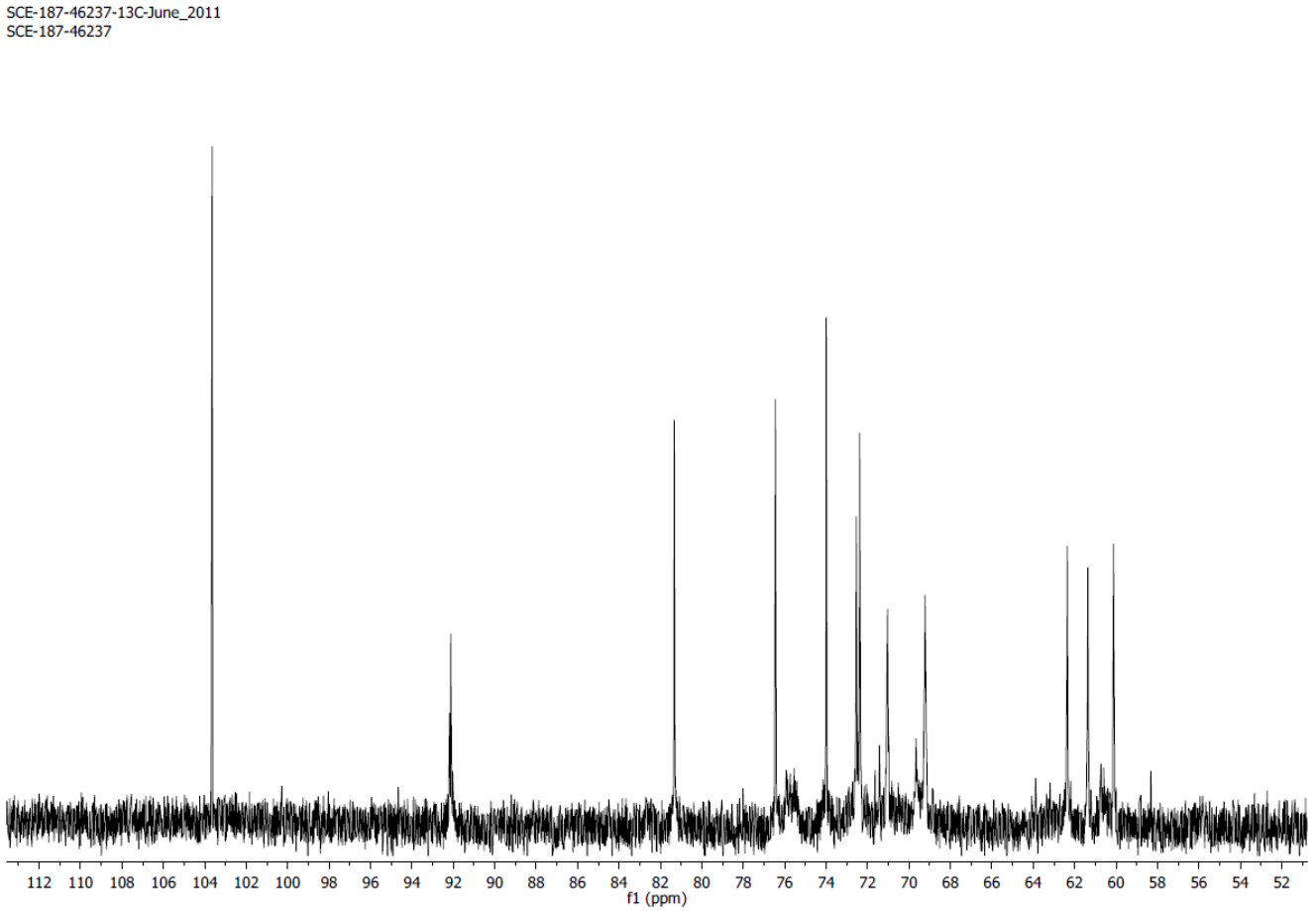
Appendix 12: Carbon 13 spectra of pinitol (processed using MestReNova software version 8). Refer to page 55 and page 56 to view the structure.



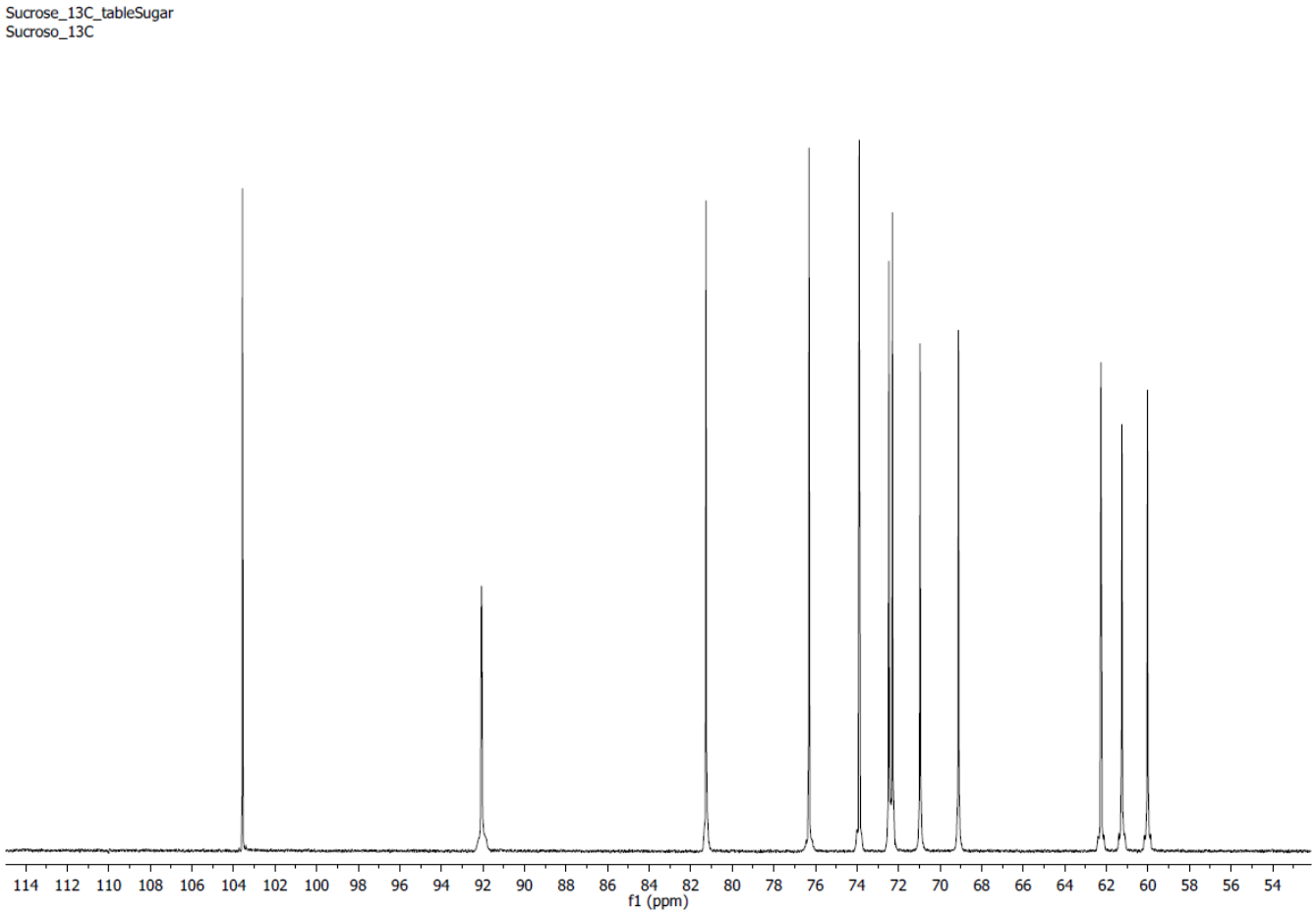
Appendix 12~~13~~: Laboratory isolated sucrose proton spectra with expansions (processed using MestReNova software version 8). Refer to page 57 and page 58 to view the structure.



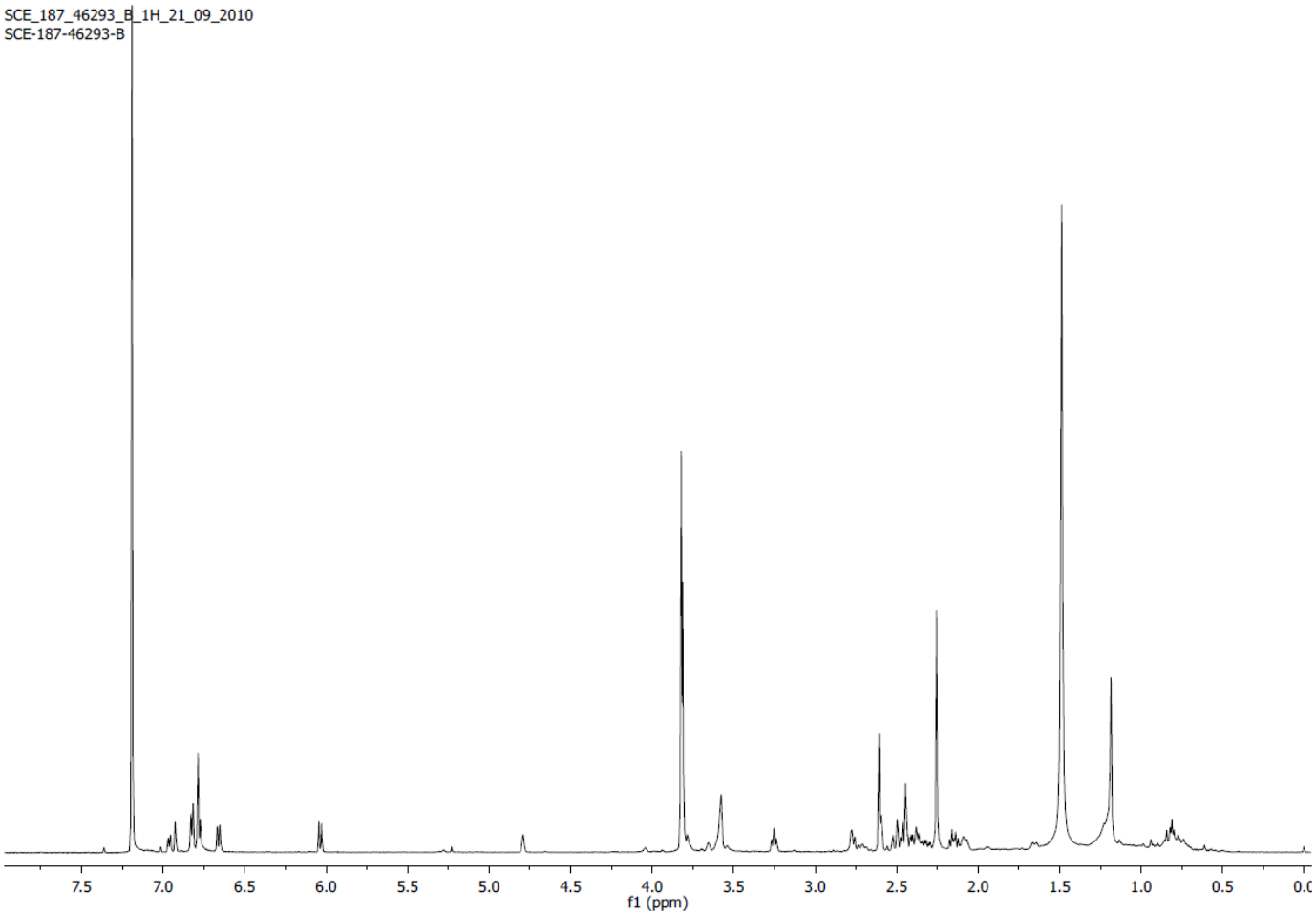
Appendix 13~~14~~:Cormetial sucrose (table sugar) proton spectra with expansions (processed using MestReNova software version 8). Refer to page 57 and page 58 to view the structure.



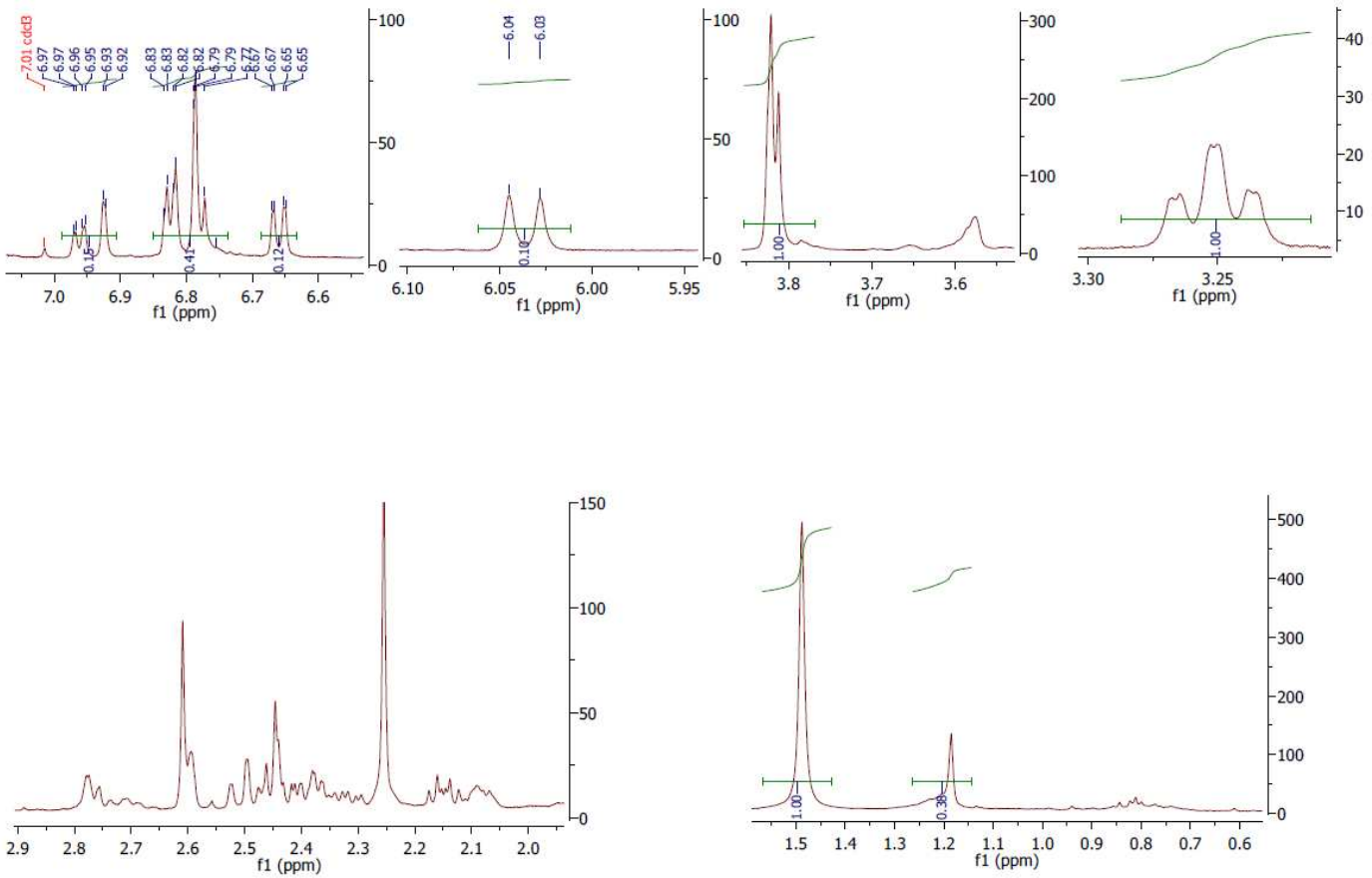
Appendix 14~~15~~: Carbon 13 spectra of laboratory isolated sucrose (processed using MestReNova software version 8). Refer to page 58 and page 58 to view the structure.



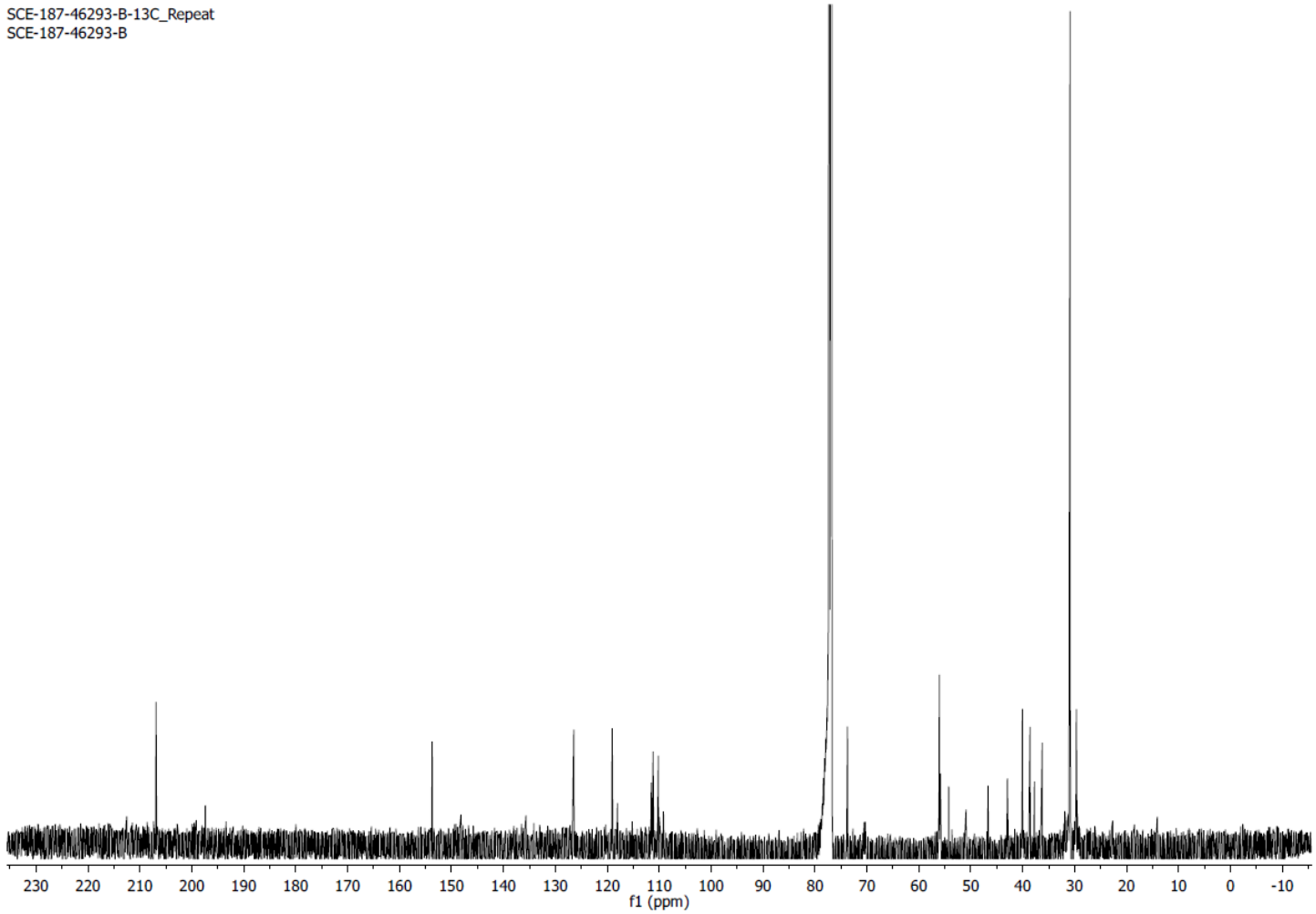
Appendix 15~~16~~: Carbon 13 spectra of comercial sucrose (table sugar) (processed using MestReNova software version 8). Refer to page 58 and page 58 to view the structure.



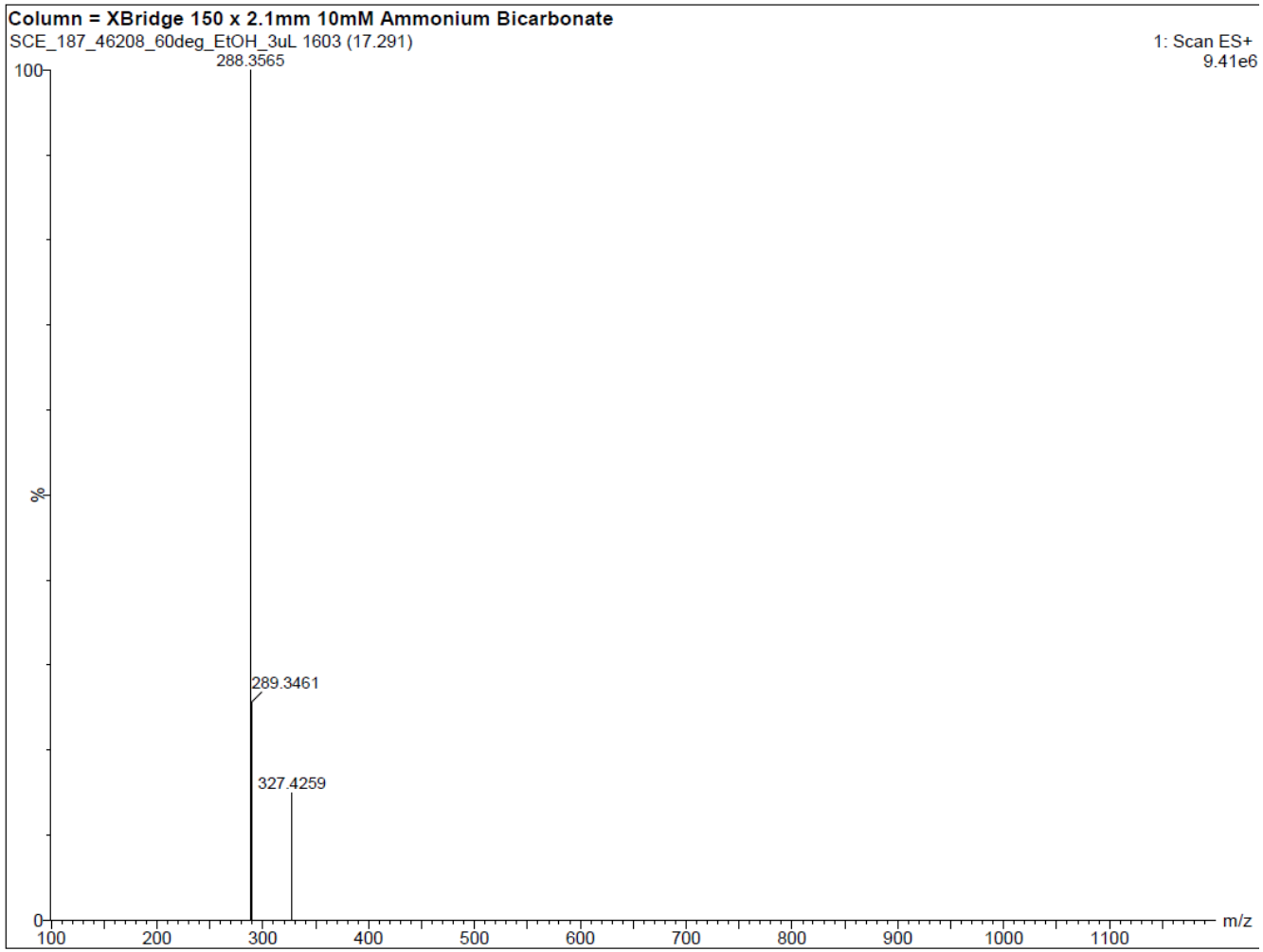
Appendix 16~~17~~:Mesembrenone proton spectrum (processed using MestReNova software version 8). Refer to page 60 and page 65 to view the structure.



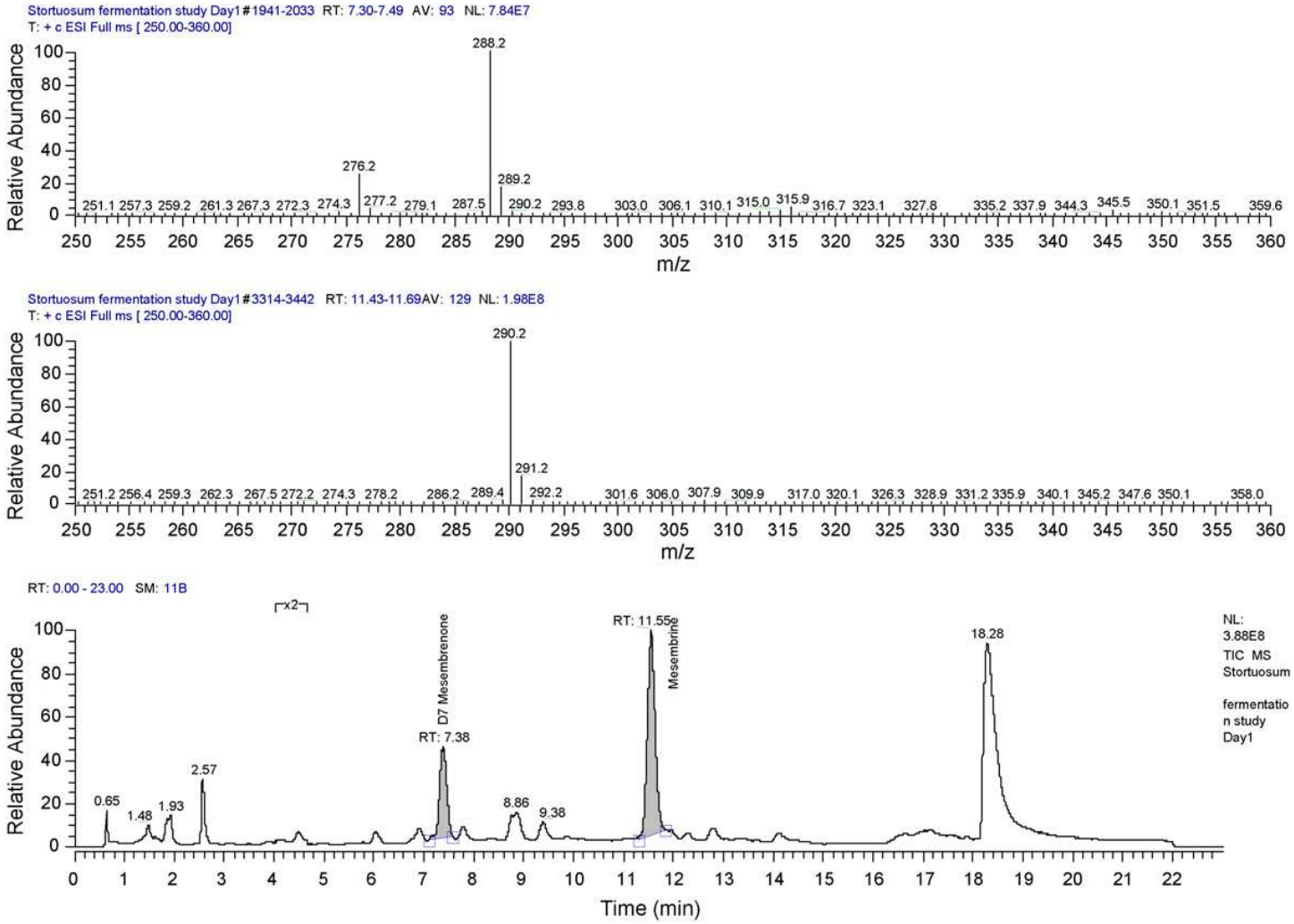
Appendix 17~~18~~: Expansions of proton peaks from the mesembrenone parameters (processed using MestReNova software version 8)



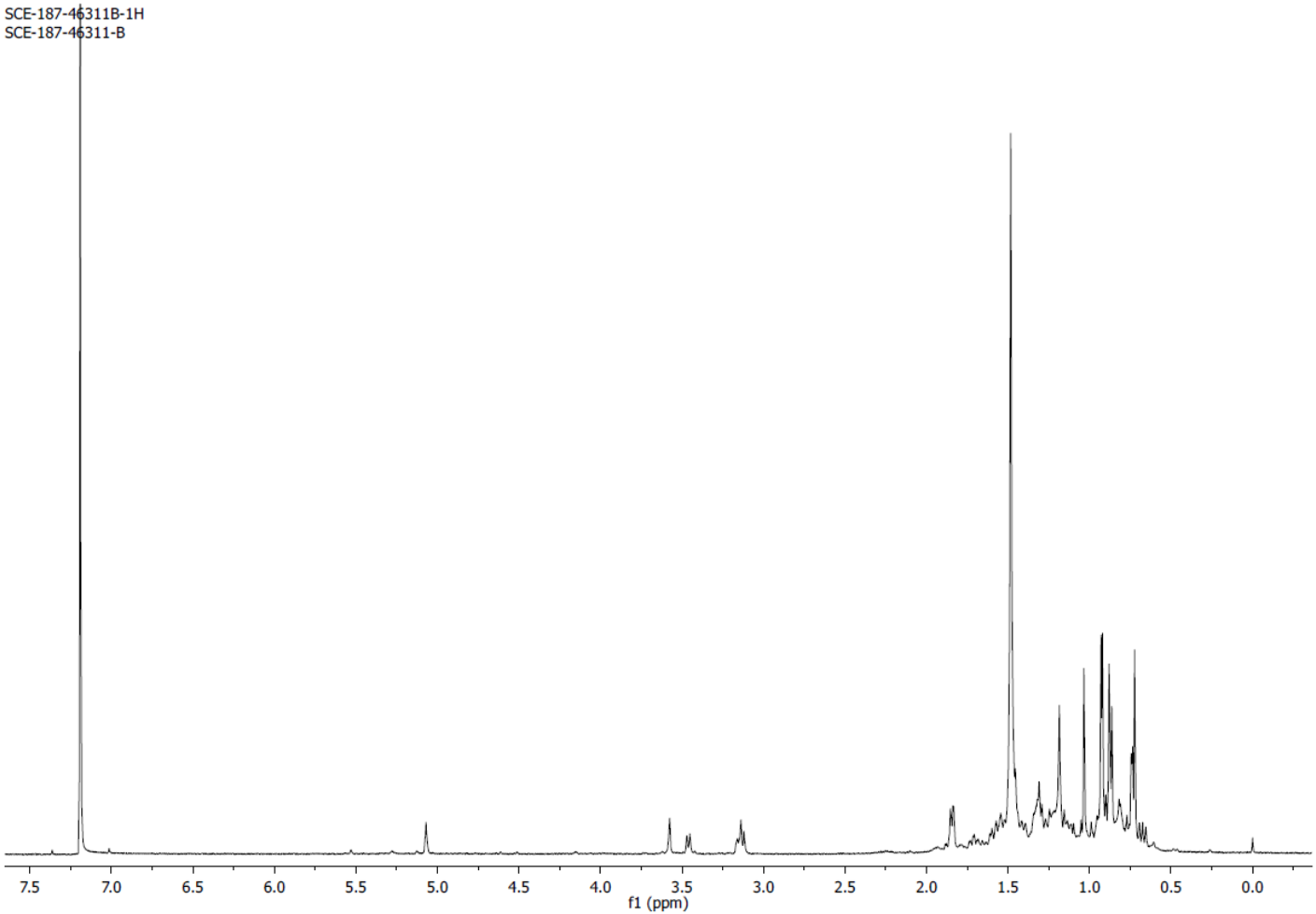
Appendix 18~~19~~: Carbon 13 spectra of mesembrenone (processed using the MestReNova software version 8). Refer to page 61 and page 65 to view the structure.



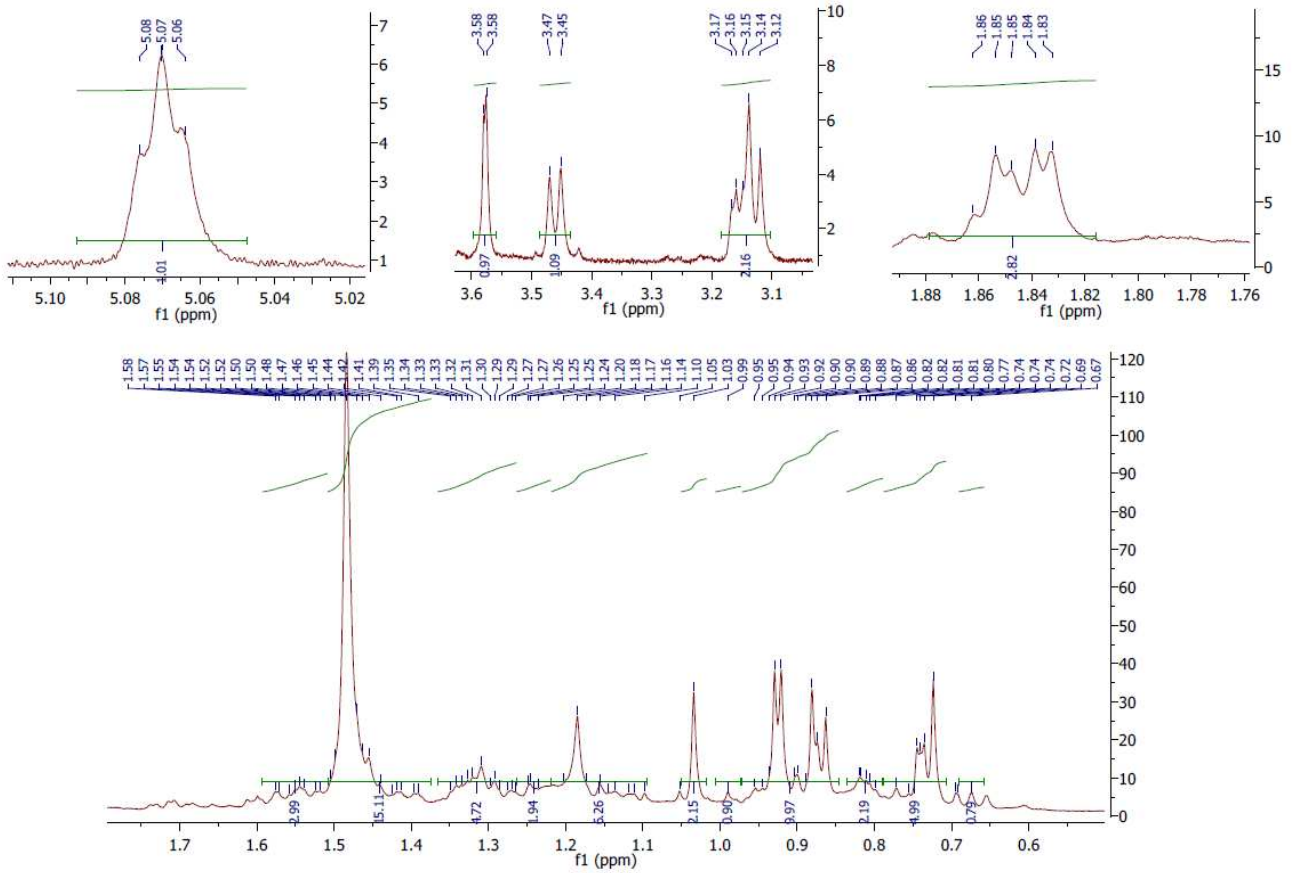
Appendix 20: ESI$^+$ TOF mass spectrum of isolated mesembrenone. Refer to page 63 and page 65 to view the structure.



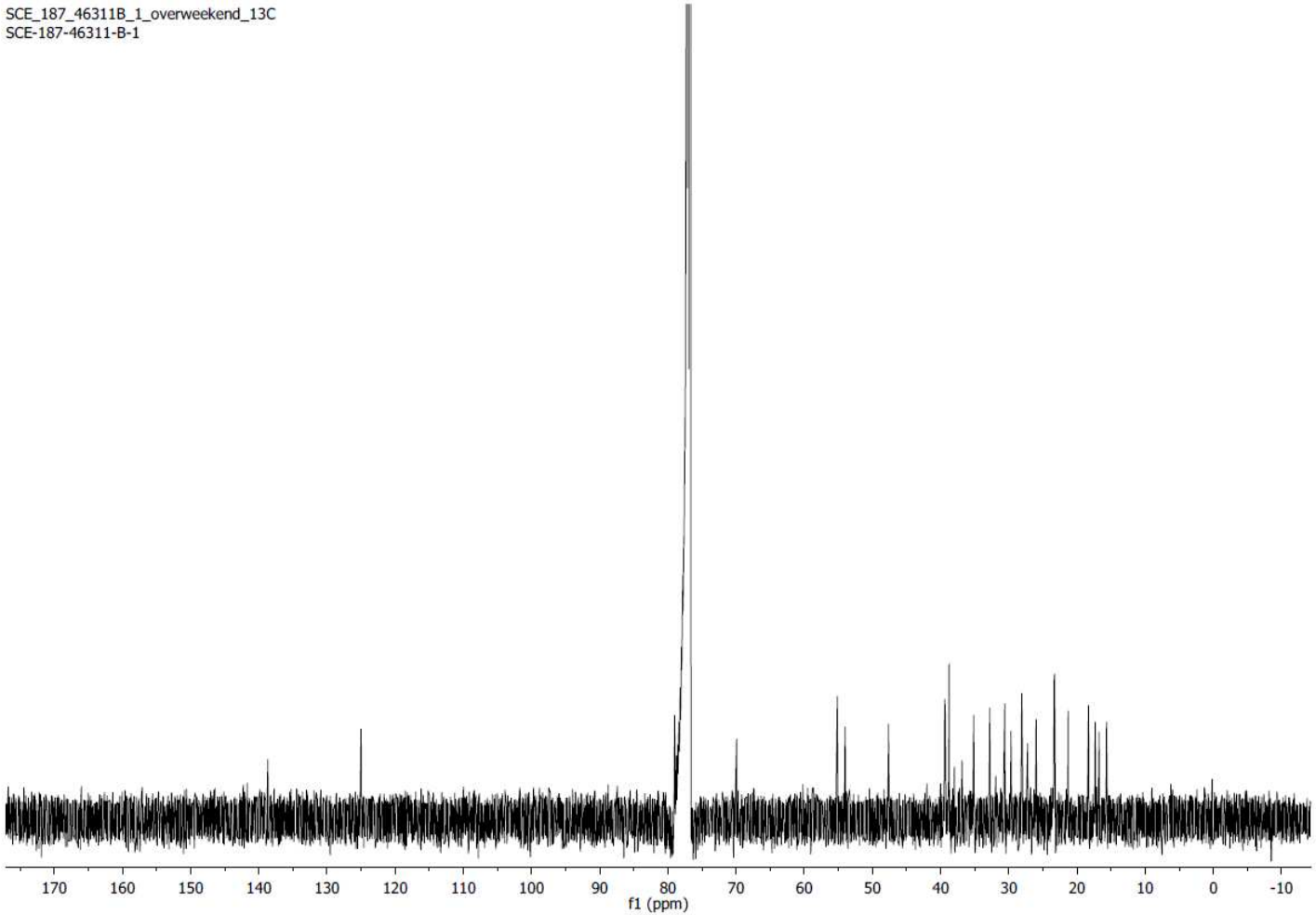
Appendix 19~~21~~: LC-MS chromatogram of crushed plant material on day 10 MS IUon spectra of mesembrenone m/z 28802 (top). Refer to page 64.



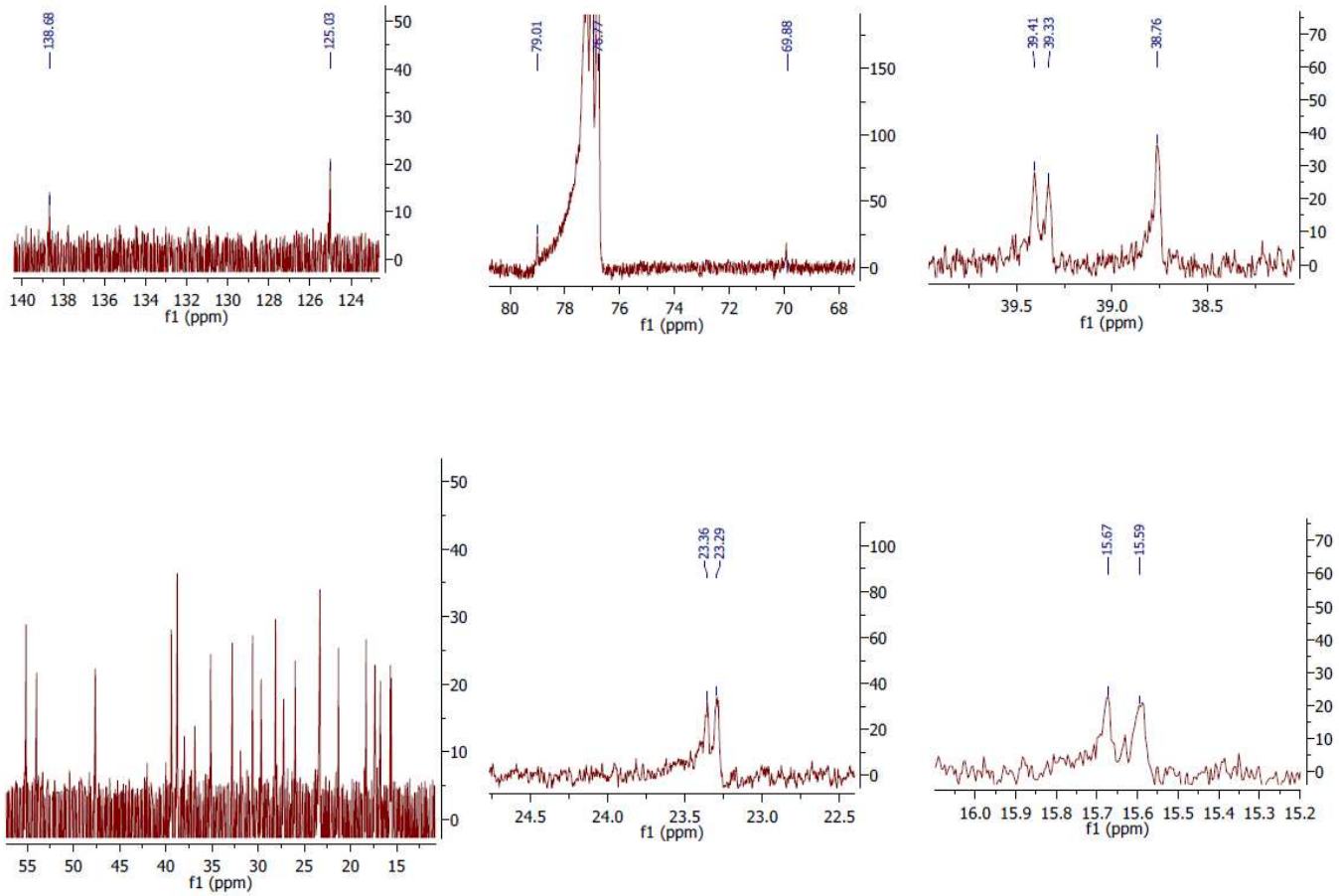
Appendix 20~~22~~: Obtusalin proton spectrum (processed using MestReNova software version 8). Refer to page 66 and page 66 to view the structure.



Appendix 21~~23~~: Expansions of proton peaks from the obtusalin parameters (processed using MestReNova software version 8)

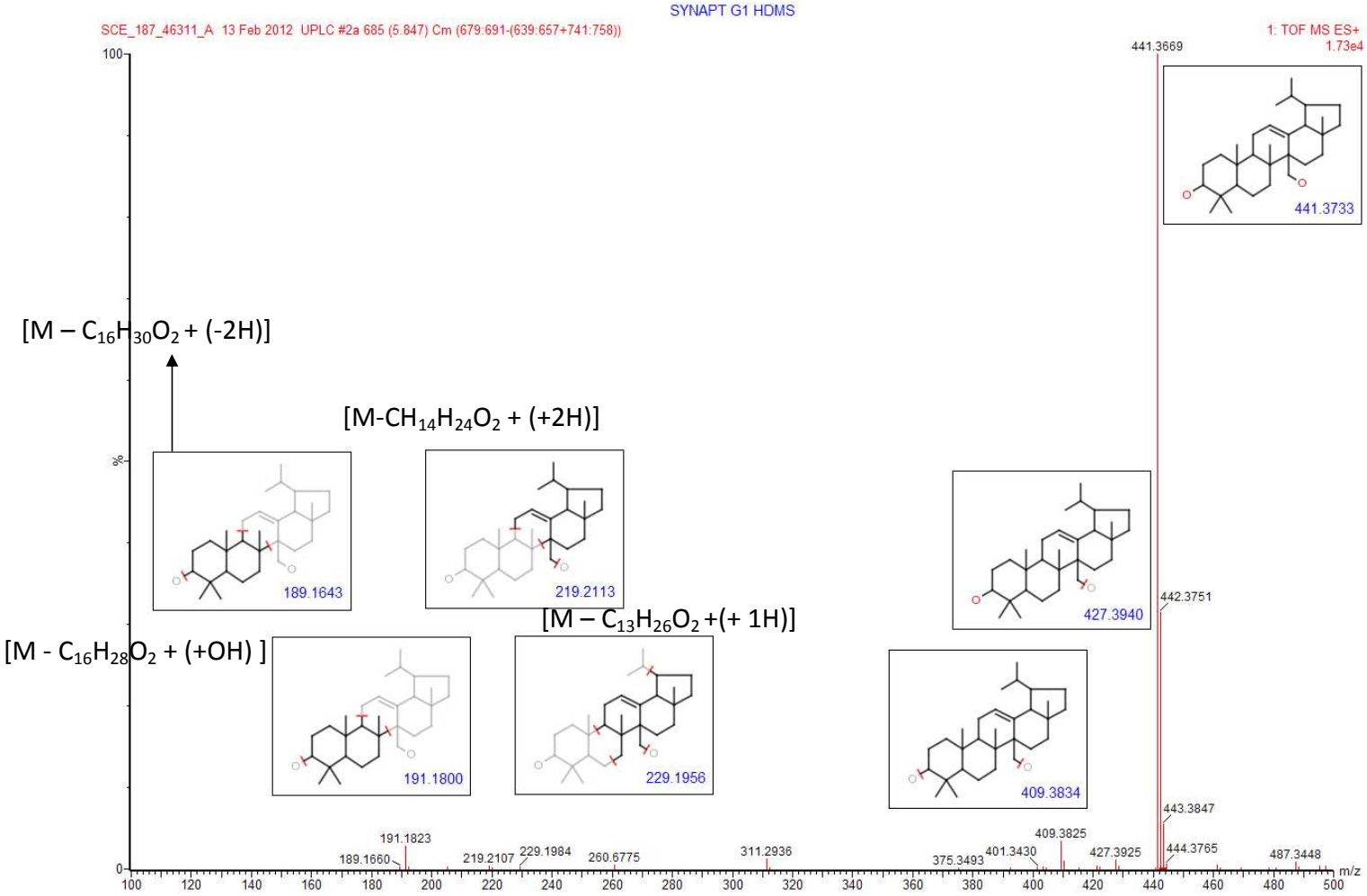


Appendix 2224: Carbon 13 spectra of obtusalin (processed using the MestReNova software version 8). Refer to page 66 and page 67 to view the structure.

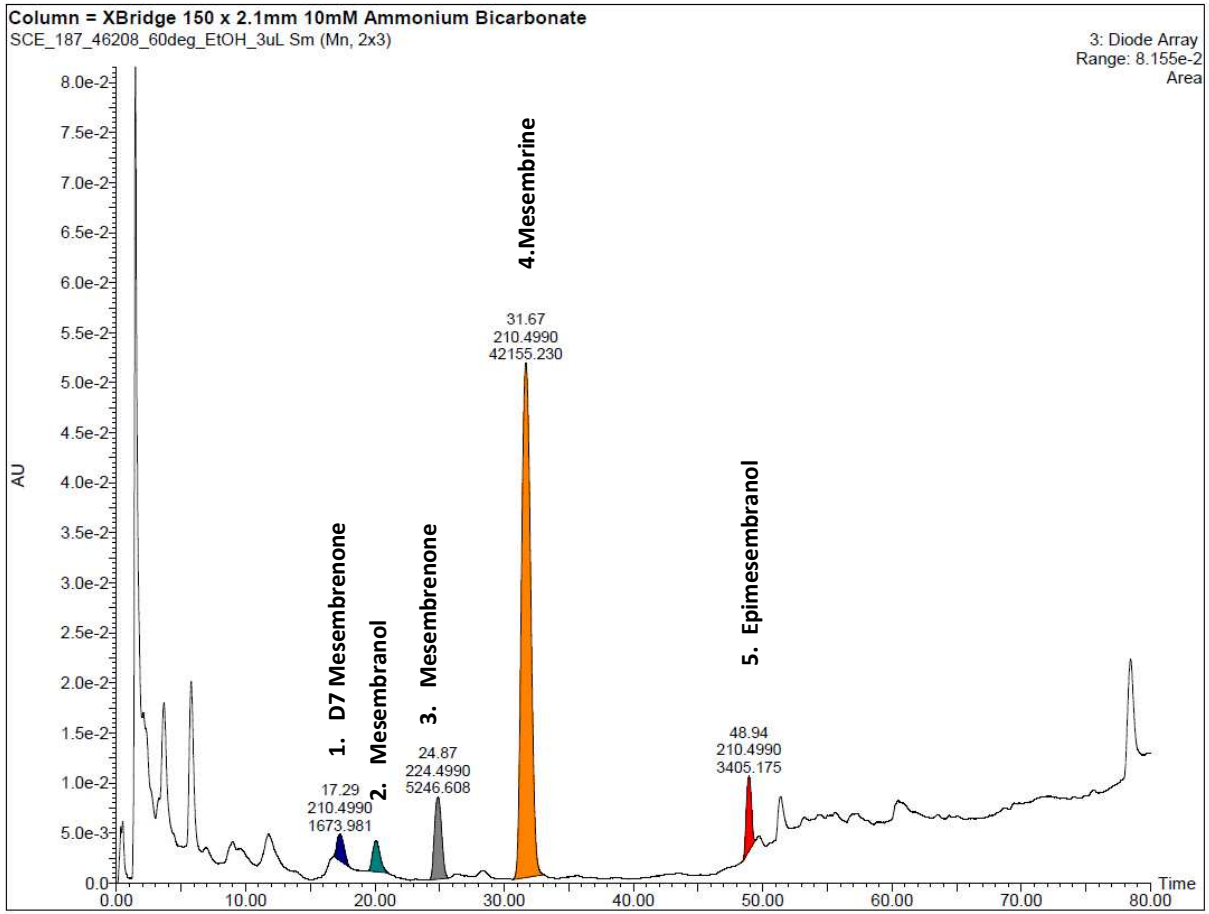


Appendix 2325: Carbon 13 expansions of obtusalin (processed using the MestReNova software version 8)

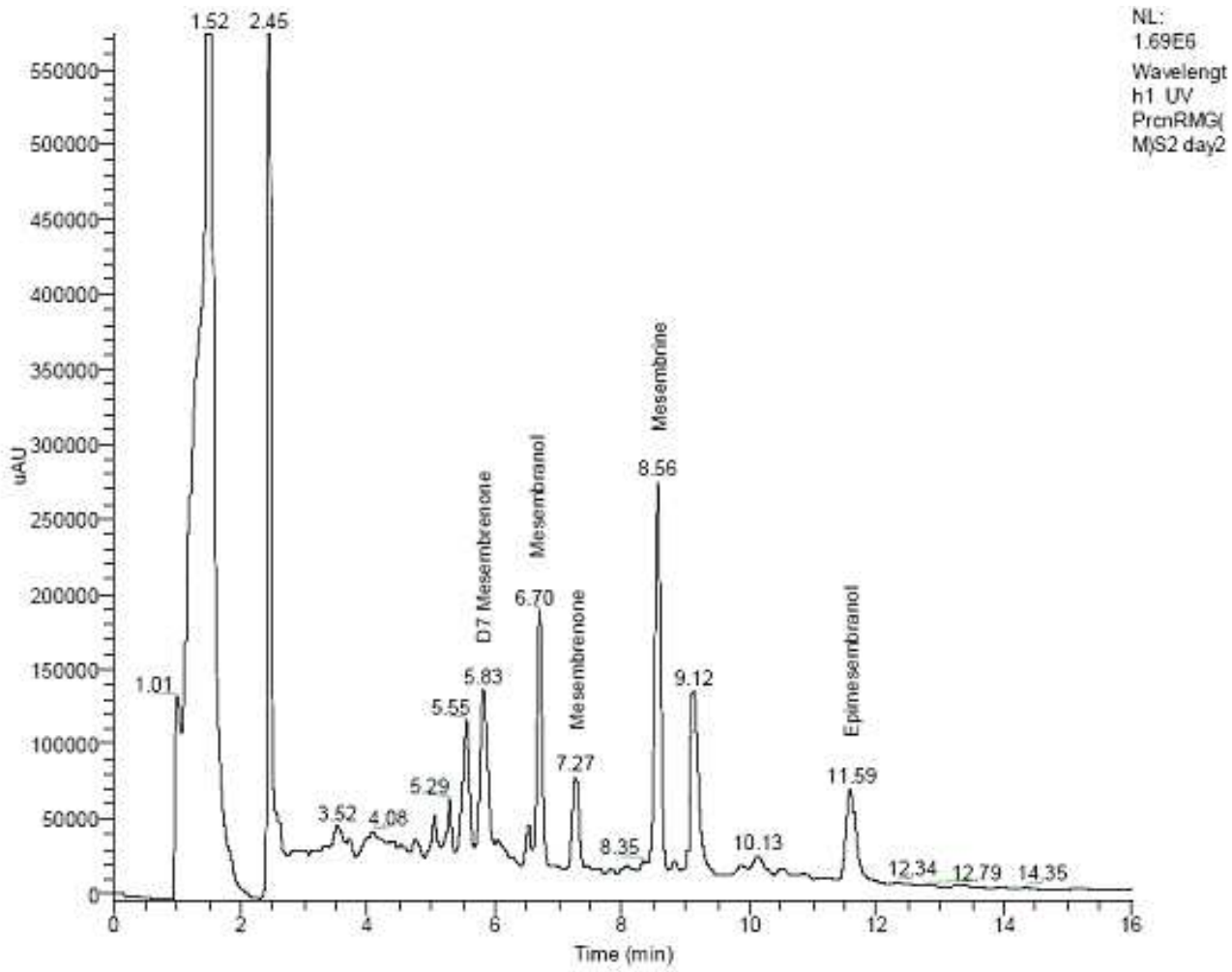
Appendix 24~~26~~: HRTOFMS (ESI^+) chromatogram of compound 5. Refer to page 69 and page 66 to view the structure.



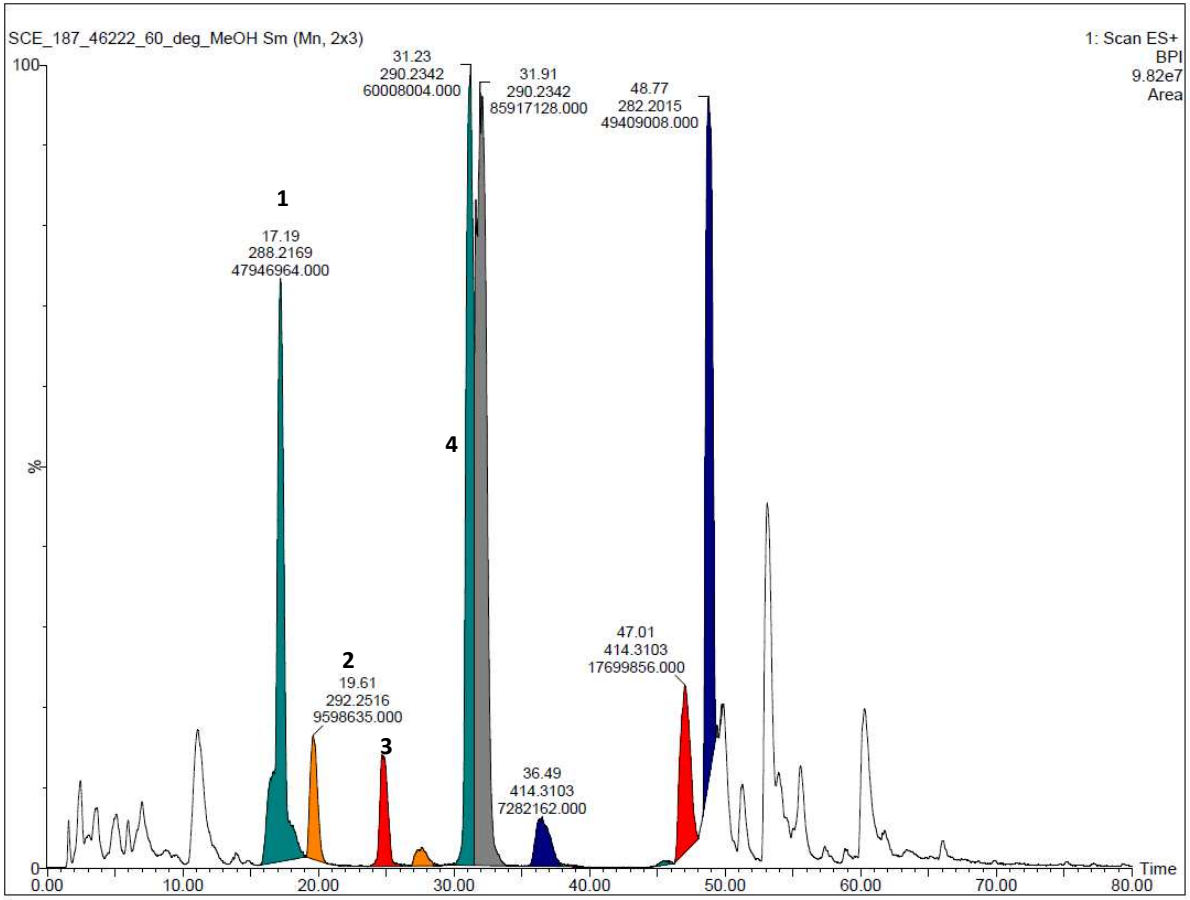
Appendix 2527: HRTOFMS (ESI$^+$) fragmentation of compound 5. Refer to page 70 and page 66 to view the structure.



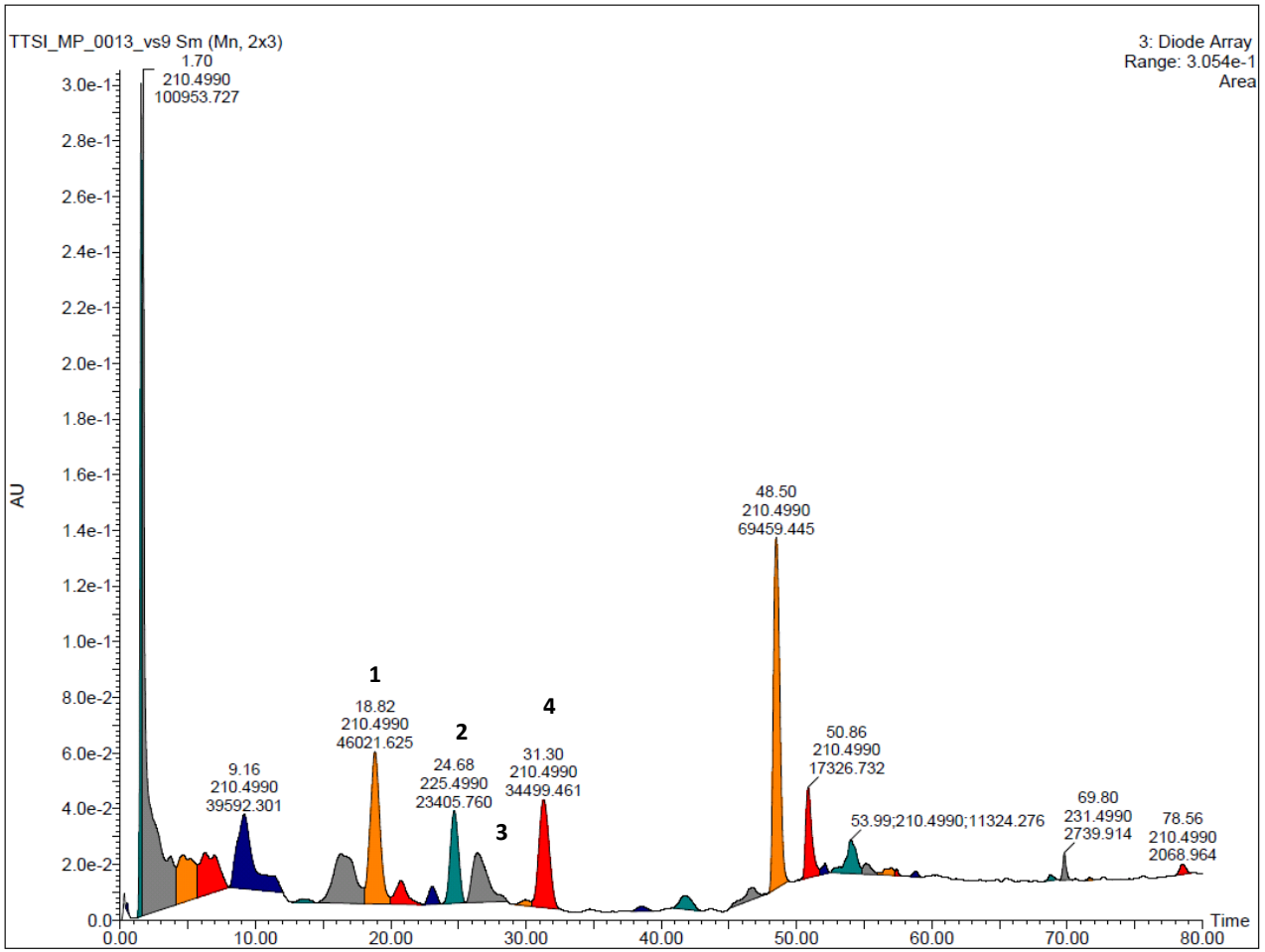
Appendix 28: HPLC chemical profile SCE-187-46208 (ethanol extract). Refer to page 72.



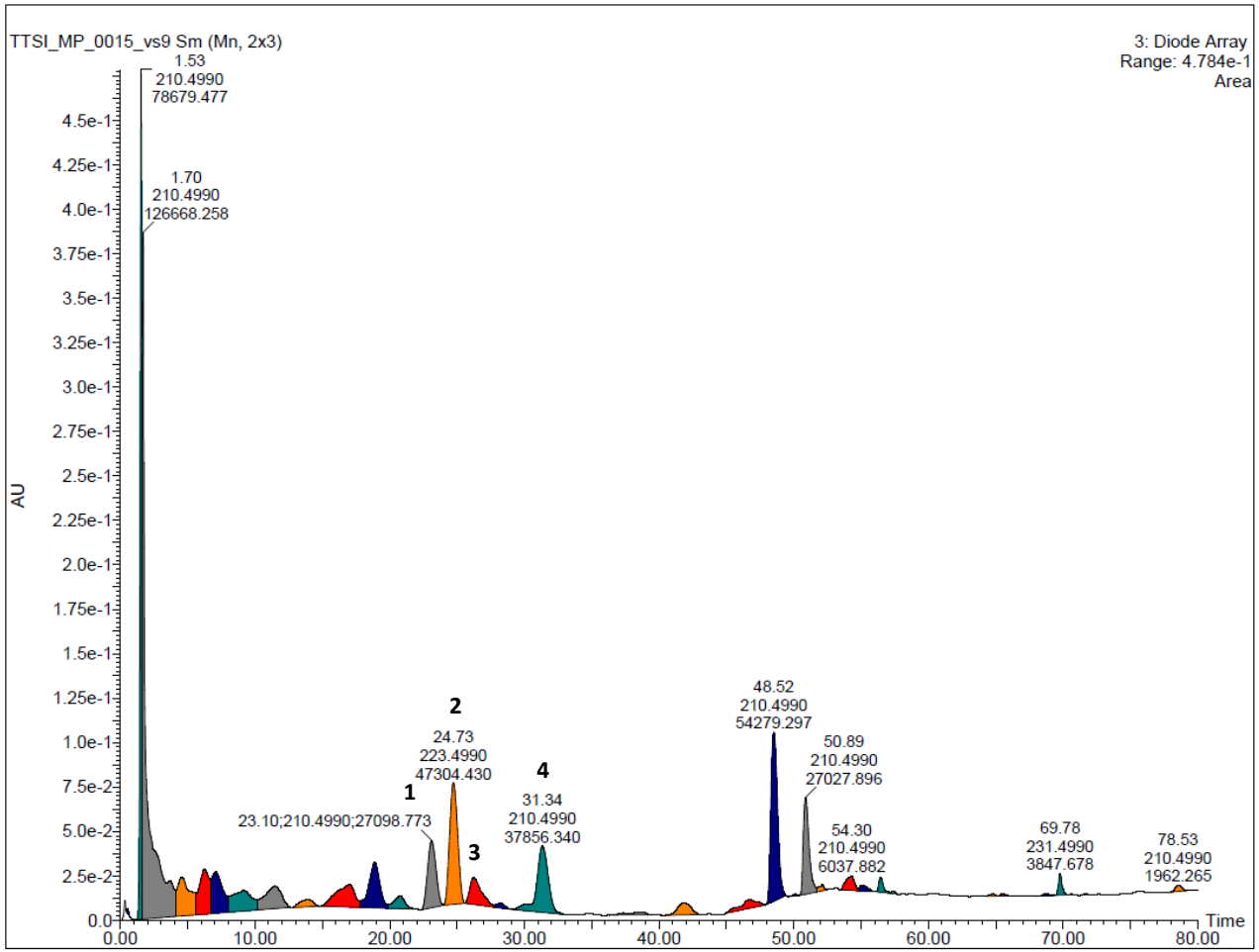
Appendix 27~~29~~: HPLC chromatogram of Sceletium plant material (Patnala&Kanfer, 2010). Refer to page 73.



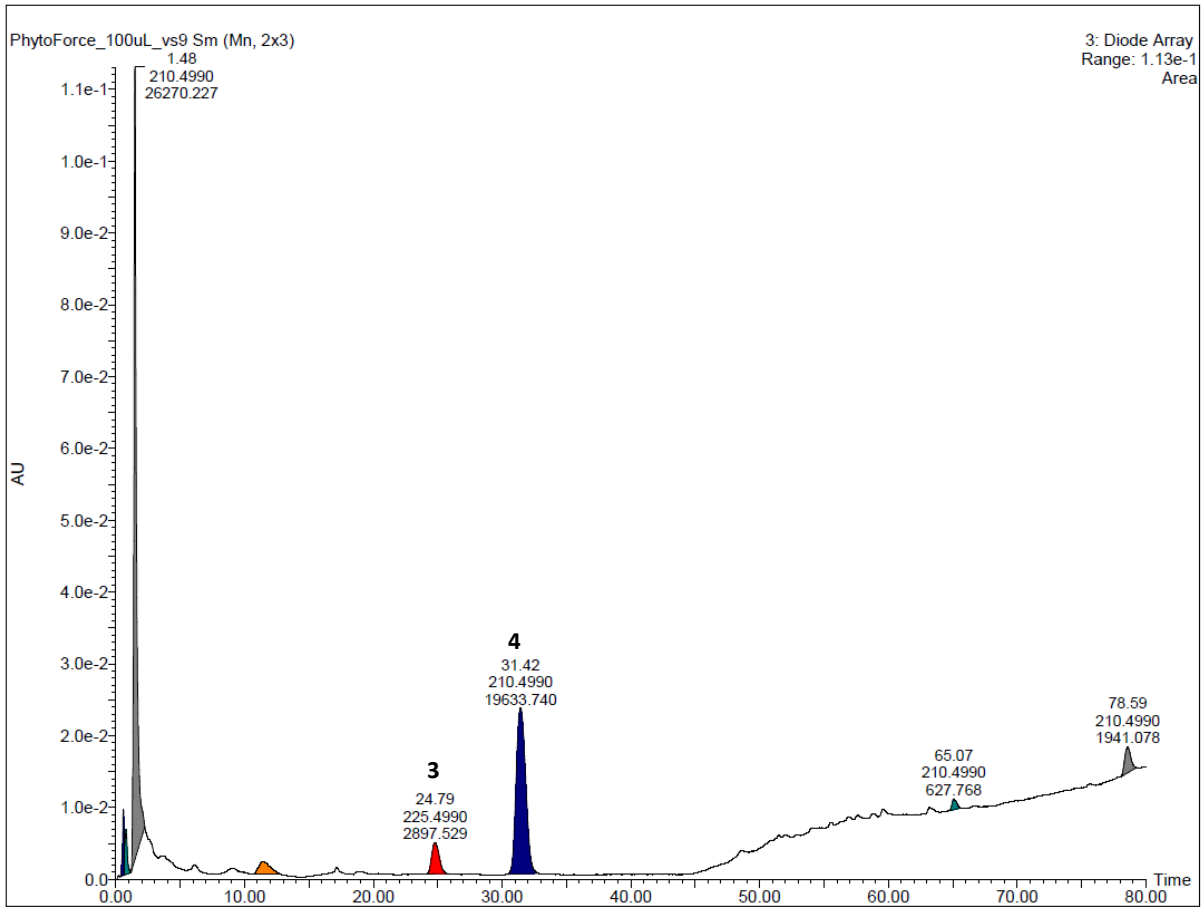
Appendix 2830: HPLC chemical profile of SCE-187-46222 (methanol extract). Refer to page 74.



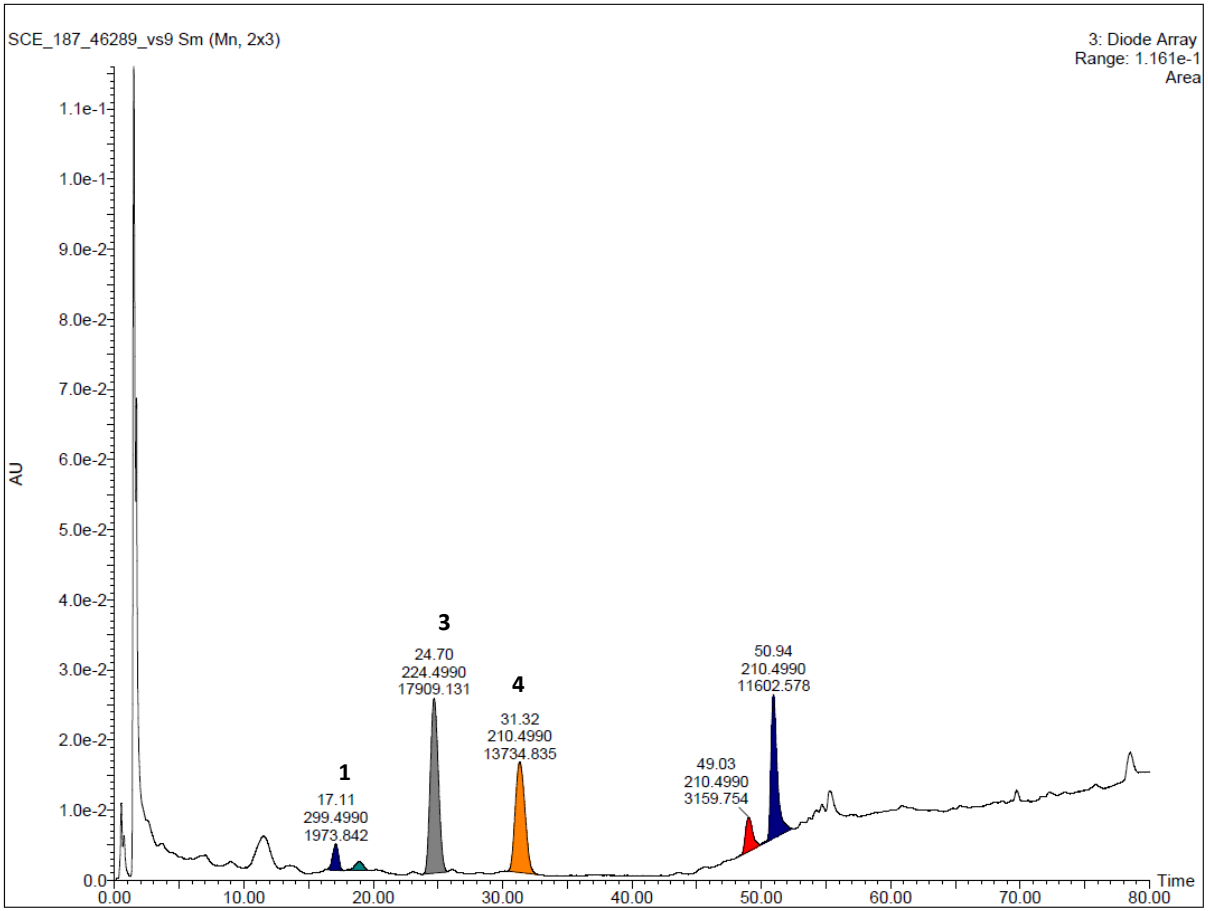
Appendix 29~~31~~: HPLC chemical profile of ECD^{+}dried plant material, TTSI-MP-0013. Refer to 76, discussed on page 75.



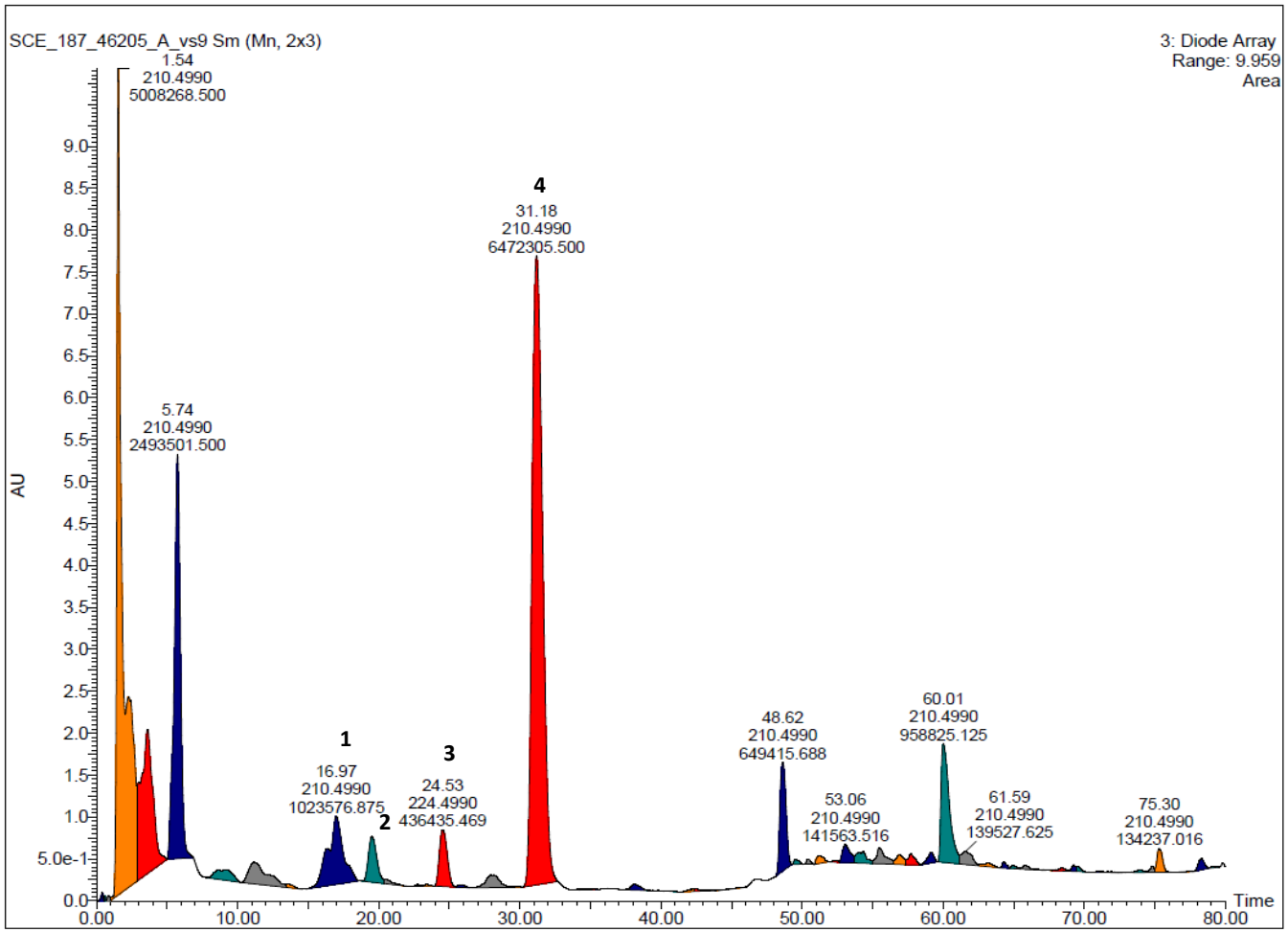
Appendix 30~~32~~: HPLC chemical profile of ECD^{+} dried plant material, TTSI-MP-0015. Refer to page 77, discussed in page 75.



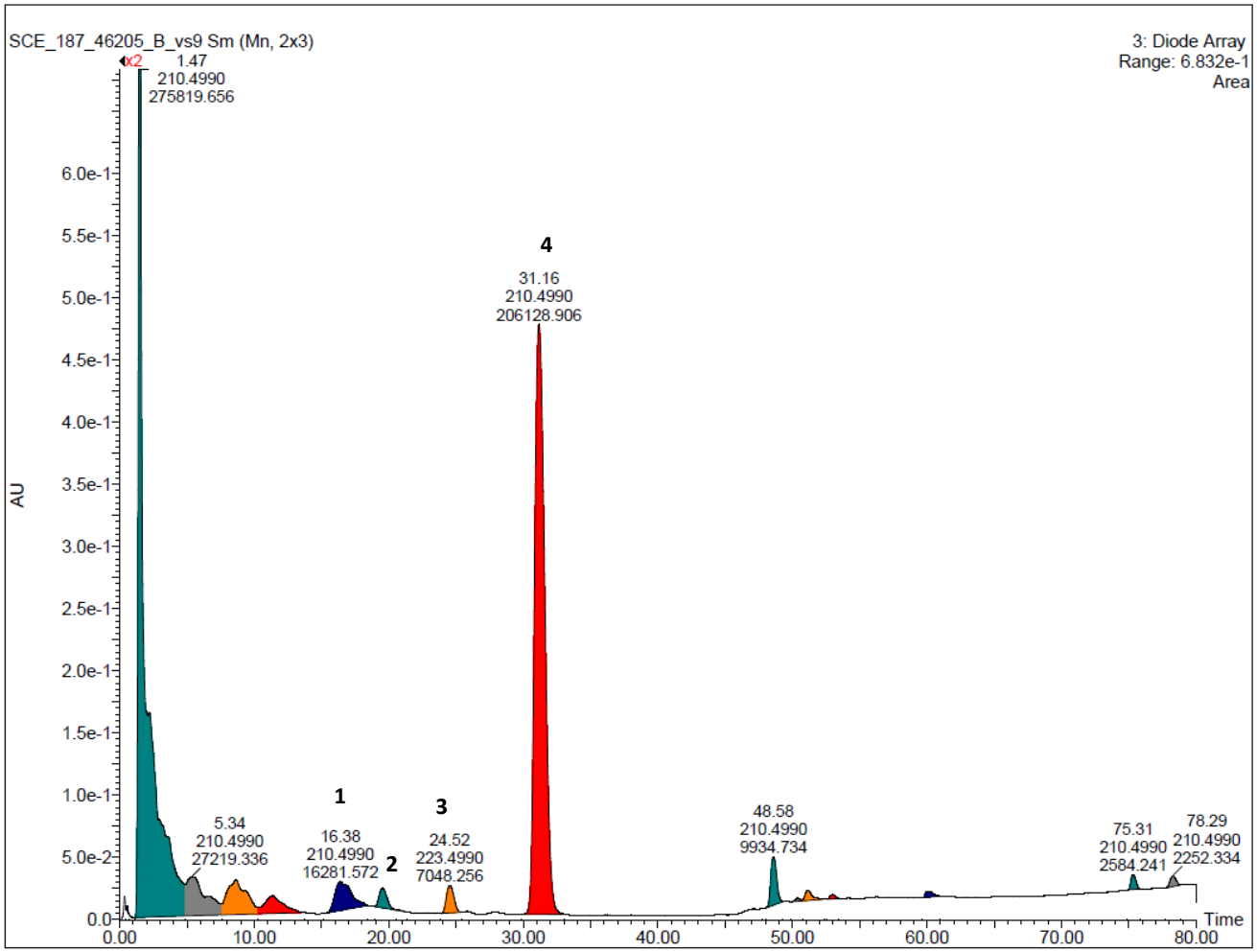
Appendix 31~~33~~: HPLC PDA profile of a commercially available tincture. Refer to page 79, discussed in page 78.



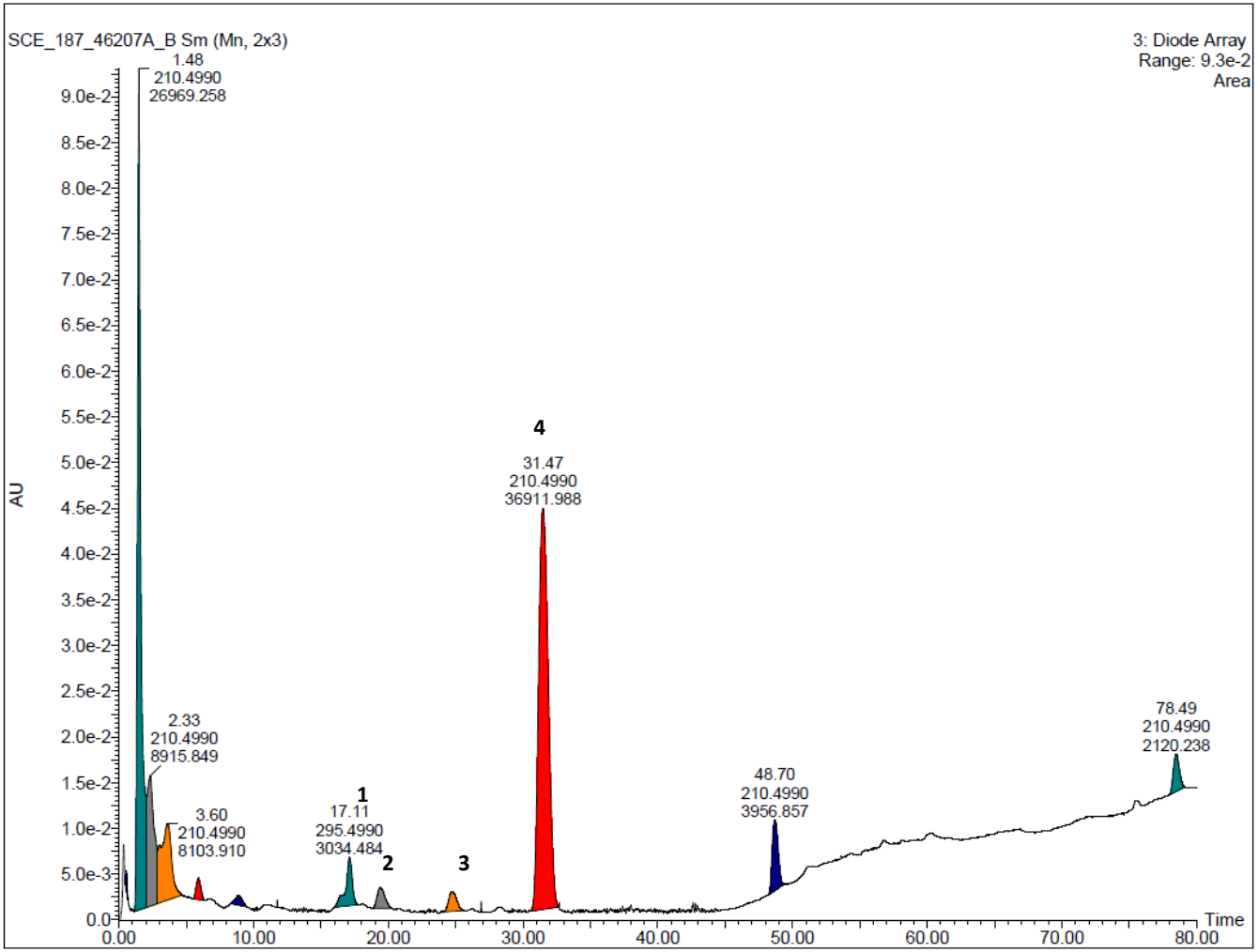
Appendix 3234: HPLC PDA profile of a commercially available capsule (SCE-187-46289). Refer to page 80, discussed in page 78.



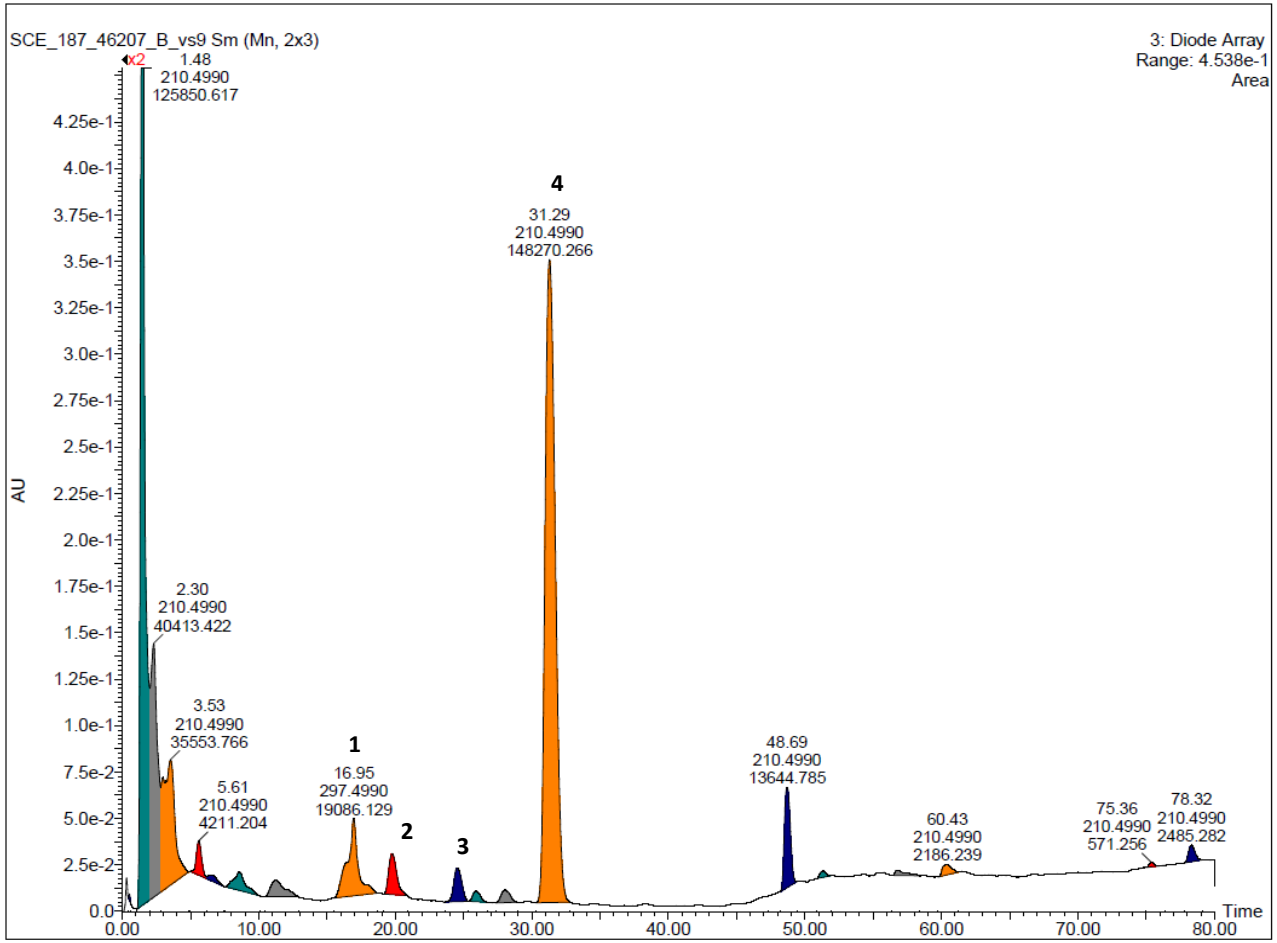
Appendix 3335: HPLC profile of SCE-187-46205A associated with IC_{50} = 1.50µg/ml. Refer to page 85, discussion and table in pages 81-88.



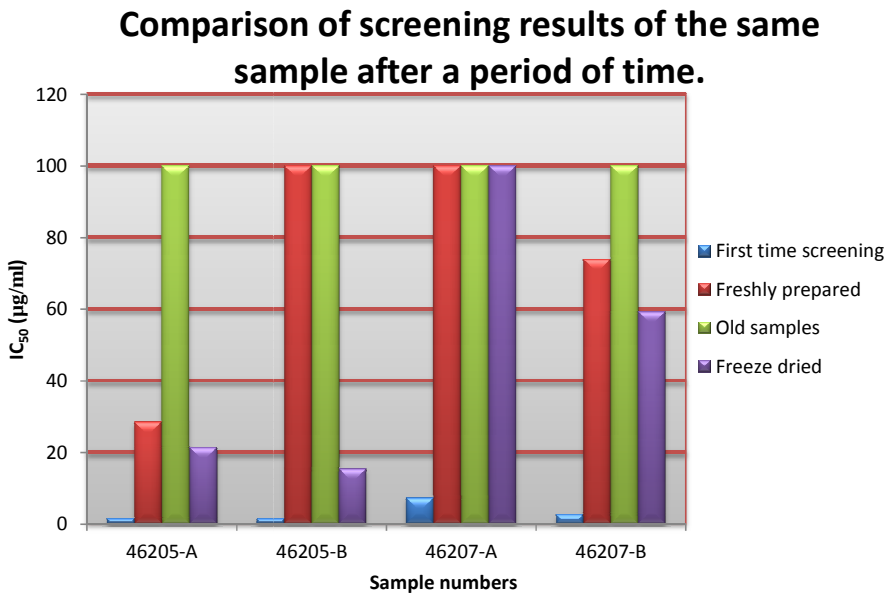
Appendix 3436: HPLC profile of SCE-187-46250B associated with IC_{50} = 1.47µg/ml. Refer to page 86, discussion and table in pages 81-88.



Appendix 35~~37~~: HPLC profile of SCE-187-46207A associated with IC_{50} = 7.32µg/ml. Refer to page 87, discussion and table in pages 81-88.



Appendix 3638: HPLC profile of SCE-187-46205B associated with IC_{50} = 2.68µg/ml. Refer to page 88, discussion and table in pages 81-88.



Appendix 37~~39~~: Results of rescreening for anti-malaria activity of samples 7, 8, 12 and 13 after 6 month's storage at 4C compared to the initial bioasay (in blue). Refer to page 90.

Appendix 40: A summary of stability test procedure, results and background.

Summary of stability testing of *S. tortuosum* constituents

As explained in chapter 4, this experiment was carried out over a two month period. The aim of this experiment was to see if there were any changes that were occurring within the extracts during the period of fermentation. Changes were tracked by using HPLC to chemically profile the constituents of the extracts and identify any changes be it an increase or decrease of compound concentrations or appearance of new peaks and disappearance of other peaks. Figure ii. represents UV chromatograms of a single extract (SCE-187-46311-A) from initial time after extraction to the end of the experimental period (2 months). The bottom chromatogram has peaks higher in intensity, but the quantity of injection is the same as in the other two chromatograms. Figure i. represents the ESI^+ of the same extract discussed above. Unlike in the UV chromatograms where no specific or particular changes were observed, it is evident that there are changes where some peaks seem to increase in intensity over time and some peaks seem to have disappeared or decreased resulting in a possible decomposition of certain compounds observed in the profile.

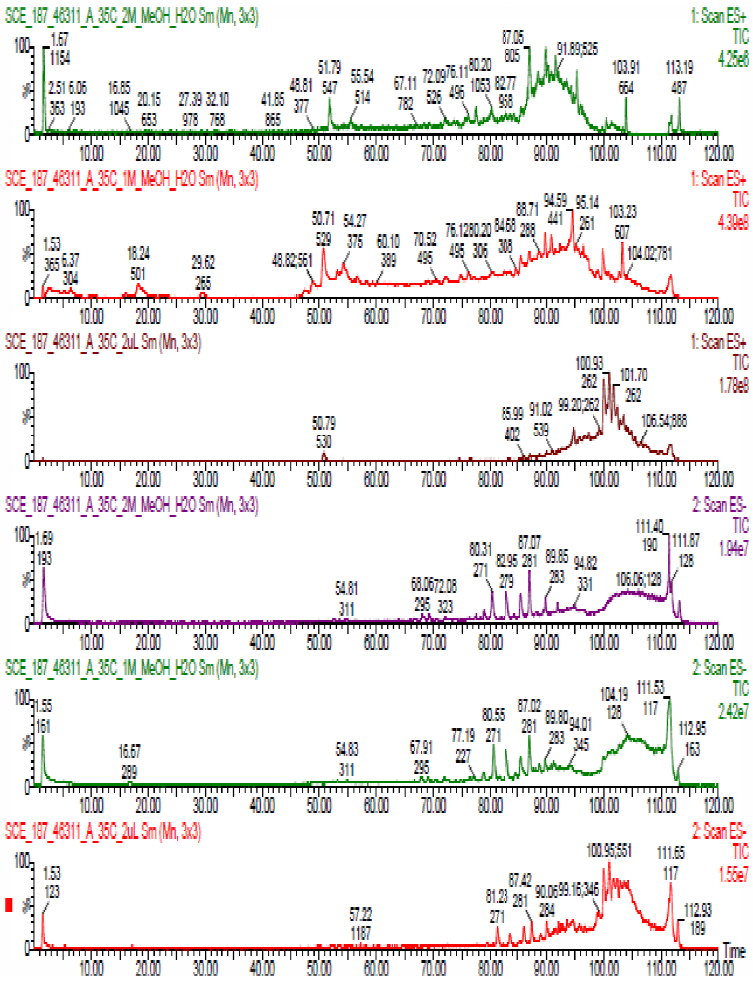


Figure ii: ESI^{+}mode results of an extract stored at 35^{o}C over a 2 month period.

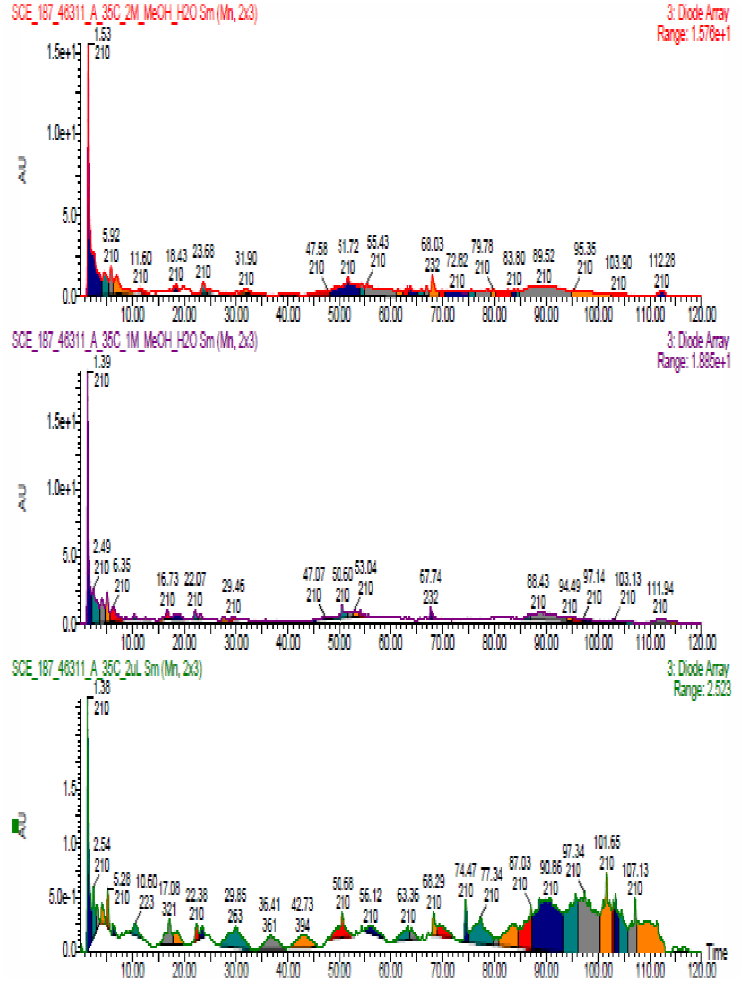


Figure iii: UV chromatogram of extract stored at 35 ^{o}C over a two period of time.

Appendix 41: Below is a summarized table and bar graph of mesembrine quantification of second batch of fermented plant material (that stored at -20^{o}C for six months)

Table i: Comparison of mesembrine quantities in plant material freeze-dried and fermented in a thermal bag to those fermented in an open tray over a period of 10 day.

Open tray fermentation	% Mesembrine
SCE-187-46269-A	1.7
SCE-187-46269-B	1.7
SCE-187-46269-C	2.5
SCE-187-46269-D	2.4
SCE-18746270-A	0.7
SCE-18746270-B	1.1
SCE-187-46270-C	1.9
SCE-187-46270-D	2.4
SCE-18746271-A	0.8
SCE-18746271-B	1.4
SCE-187-46271-C	1.5
SCE-187-46271-D	1.3
SCE-187-46272-A	0.5
SCE-187-46272-B	2.1
SCE-187-46272-C	1.4
SCE-187-46272-D	1.6
SCE-187-46273-A	0.8
SCE-187-46273-B	0.3
SCE-187-46271-C	1.6
SCE-187-46271-D	1.7
SCE-187-46275 freeze-dried	0.6

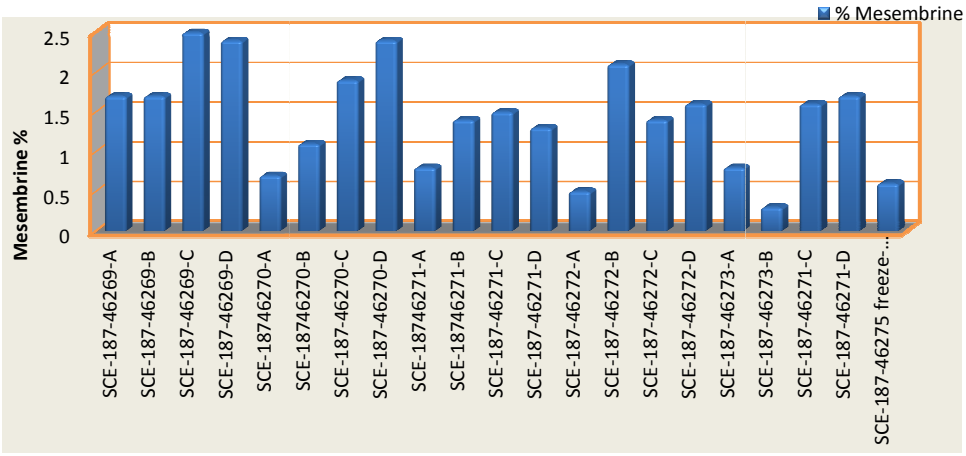


Figure iv: Mesembrine percentage detected in freeze-dried and fermented plant material, in both thermal bags and open trays. (Plant material in retention).