A SCIENTIFIC INVESTIGATION OF THE IMMUNOMODULATORY PROPERTIES OF AN INDIGENOUS PLANT, *SUTHERLANDIA FRUTESCENS*

by

ODWA GONYELA

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SUPERVISOR: Prof. J.B. Dewar

CO-SUPERVISOR: Prof. G. Fouchè

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DECLARATION

Name: Odwa Gonyela

Student number: 41254082

Degree: Master's

Exact wording of the title of the dissertation or thesis as appearing on the copies submitted for examination:

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I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SIGNATURE

29/01/2016
DATE

DEDICATION

To my late grandmother Emsie Nosekiwe Gonyela and my late Sister Qiqa Gonyela

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But Jesus looked at them and said, "With man this is impossible, but with God all things are possible." ~ Matthew 19:26 ESV

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Kubo bonke abo bathe baphosa ilitye esi vivaneni kwilinge lam, ndithi ndibamba ngazo zozibini. Ndiswel' intshokotshela yomtshoba mawethu kuba nge ndiyithwala nditshobatshob' okwe nkonyane isiva intlutha yamasi kanina ehlotyeni.

ABSTRACT

Traditional medicines prepared from *Sutherlandia frutescens* are used to manage diseases including HIV and cancer. This study aimed at isolating and identifying biologically active compounds isolated from *S. frutescens*.

Sutherlandia frutescens plants were collected in Petrusburg and Paarl. Powdered plant material was extracted using ethanol or water and their metabolite composition was compared using UPLC-MS. A novel cycloartane, an acetylated variant of this compound as well as a Sutherlandioside B triterpenoid was isolated and characterised using chromatographic and analytical techniques such as NMR and UPLC-MS.

Preliminary biological studies were conducted to assess the activity of plant extracts on cell toxicity, herpes virus replication and cytokine expression. The results of this study suggest that aqueous extracts from *S. frutescens* do not appear to be cytotoxic or show anti-herpetic activity, but may activate the immune system by increasing expression of IL-6, IL-10 and TNFα. Further research should be conducted to confirm and optimise these results.

Key terms

Sutherlandia frutescens, traditional medicine, NMR, UPLC-MS, HIV, AIDS, Cytokines, Immunomodulation, Cytometric bead arrays, flow cytometer.

LIST OF ABBREVIATIONS AND ACRONYMS

1D	One dimensional
2D	Two dimensional
3D	Three dimensional
^{13}C	Carbon 13
^{1}H	Proton
δ NMR	Chemical shift in ppm
δC mult.	Delta carbon multiplicity
δH mult.	Delta proton multiplicity
λmax	Lamda maximum
µg/ml	Micrograms per mililiter
°C	Degrees Celsius
ACN	Acetonitrile
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Complex
BD	Becton Dickinson Biosciences
C-18	18 carbon chain
CBA	Cytometric bead arrays
CD_3OD	Deuterated methanol
$CHCl_3$	Chloroform
COSY	Correlation spectroscopy
CP	Chemically pure grade
CSIR	Council for Scientific and Industrial Research
DBE	Double bond equivalents
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl sulphoxide
d-pyr	Deuterated pyridine
DST	Department of Science and Technology
EtOH	Ethanol
ESI	Electrospray ionisation
ESI^-	Electrospray negative mode
ESI^+	Electrospray positive mode

FBS	Foetal bovine serum
FA	Formic acid
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocynate
g	Grams
GABA	Gamma-aminobutyric acid
H_2O	Distilled water
h	Hour
HCOOH	Formic acid adduct
HIV	Human immunodeficiency virus
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High performance liquid chromatography
HR	High resolution
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IL	Interleukin
IR	Immune response
J	Spin-spin coupling constant in Hz
kg	kilograms
L	Liter
LC	Liquid chromatography
M	Base peak mass
MCF7	Human breast adenocarcinoma cell line
mDa	Milidaltons
MeOH	Methanol
MFI	Mean fluorescent intensity
MHz	Mega hertz
MS	mass spectrometry
m/z	Mass to charge ratio
ng/ml	Nanograms per mililiter
nm	Nanometers
NMR	nuclear magnetic resonance
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PDA	Photo diode array
PE	Phycoerythrin-conjugated anti-human cytokine antibodies
PHA	Phytohemagglutinins
PP	Polypropylene
ppm	Parts per million
QTOF	Quadrupole time of flight
Rf	Retention factor
RF	Radio frequency
RT	Room temperature
RPMI	Roswell Park Memorial Institute
SANBI	South African National Biodiversity Institute
SM	Secondary metabolites
SQD	Single Quadruple Detector
SU1	Sutherlandioside B
SU2	Sutherlandioside A
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
T0	Time zero
TOF	Time of flight
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
UV-VIS	Ultraviolet-visible region

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CHAPTER 1: INTRODUCTION

1.1 Human immunodeficiency virus and immune system

Human Immunodeficiency Virus (HIV) is an agent that causes Acquired Immunodeficiency Syndrome (AIDS). Infection with HIV is epidemic and results in immunosuppression that allows opportunistic infections and malignancies. There are two types of HIV with HIV-1 being the more pathogenic of the two. Consequently, there is a great demand for new therapeutics to this virus (Cos et al., 2002).

Fundamental to the pathogenesis of HIV-1 is the infection and subsequent depletion of CD_4^+ T cells. This leads to a steady drop of peripheral blood CD_4^+ T cell counts during chronic infection, a characteristic of individuals infected with HIV-1. Most of the CD_4^+ T cells are located in the lymphoid tissues, such as the lymph nodes and particularly the mucosal lymphoid tissues within the gastrointestinal tract (Appay & Sauce, 2008). Unlike HIV-1, disease progression in HIV-2-infected individuals is slower and most patients die from HIV-2-unrelated causes (Sousa et al., 2002). However, infection with HIV-2 also shows a decline in both the functionality and the number of CD_4^+ lymphocytes with associated loss of immune competence eventually leading to AIDS (Roshal et al., 2001; Sousa et al., 2002).

The host immune system provides protection against a variety of constantly evolving pathogenic microbes and it also helps the host to get rid of toxic and allergenic substance that enter through mucosal surfaces (Chaplin, 2010). A major role is played by cytokines and chemokines in the host defence against microbes, including HIV (Alfano & Poli, 2005) where cytokines (IFN-γ, IL-6, IL-12, and TNF; alternatively IL-4, IL-10 or IL-13) are released from activated macrophages during early stages of infection or tissue damages (Chaplin, 2010; Parkin & Cohen, 2001).

Both innate and adaptive immune responses combine to form the main two parts of immunity (Parkin & Cohen, 2001). The innate response includes barriers such as skin as well as small bioactive molecule (including cytokines and chemokines) and soluble proteins that are released as the immune response is activated by pathogens

(Chaplin, 2010) and provides an immediate host defence (Parkin & Cohen, 2001). Unlike the innate immune system, the adaptive response is highly specific and has memory of previously encountered microbes (Parkin & Cohen, 2001). It might also take several days or weeks to become prominent after antigen-specific T and B cells have undergone clonal expansion. Synergy between the innate immune system and adaptive immune system is essential for a fully effective immune system (Chaplin, 2010).

Due to the high cost of standard antivirus therapeutics, most Africans resort to traditional medicines as an alternative treatment for the management of HIV/AIDS (Moshi, 2005). In spite of the possibility of harm and relatively little evidence of effectiveness of traditional medicines, such therapeutics are believed to be extensively used among people living with HIV and in many African nations the Ministries of Health endorse the use of traditional medicines for the treatment of HIV and related ailments (Mills et al., 2005a).

1.2 Traditional medicines

In many African countries, more than 80% of the population living in rural areas use traditional medicines in spite of an apparent lack of scientific evidence for their quality, safety and efficacy (Elujoba et al., 2005; Mills et al., 2005b). Due to limitations regarding resources and access to reliable modern medical services, many Africans visit traditional practitioners for their health care needs and traditional medicines are used as a treatment for a wide range of ailments including HIV/AIDS, malaria, sickle cell anaemia, diabetes and hypertension (Kayne, 2009; Okigbo et al., 2006).

In Africa, traditional medicines were the dominant medical system prior to the introduction of western medicines. Traditional medicines form part of the solution for many health challenges as they are considered safe, affordable and have fewer side effects when compared to conventional medicines (Moshi, 2005; Okigbo et al., 2006). A significant proportion of citizens in industrialised countries like Germany (75%), Canada (70%) and England (47%) regularly use traditional medicines

(Kayne, 2009). Unless complemented by traditional medicines, effective health in Africans cannot be achieved with modern medicines alone (Elujoba et al., 2005).

Bioactive compounds found in various parts of plants such as roots, leaves, stems, flowers, seeds or bark play a significant role in the management of diseases and these phyto-compounds are also a good source of plant-derived drugs (Moshi, 2005). Examples include the isoquinoline alkaloid, emetine, which is isolated from an underground part of the plant *Cephaelis ipecacuanha.* This compound is classified as anti-infective and for many years, related species have been used as an amoebicidal drug as well as for treatment of abscesses resulting from the spread of *Entamoeba histolytica* infection (Iwu et al., 1999). While synthetic or chemical drugs may have a greater and faster effect compared to phyto-medicines, they nevertheless may present a higher degree of side effects and risks (Okigbo et al., 2006). Alternatively, drug development from a natural source is often more difficult than synthetic drug development (Elujoba et al., 2005) as there are often several manufacturing problems encountered when designing formulations of herbal medicines. These challenges include failure of good manufacturing practice, misidentification of plants, microbial contamination in the field, lack of standardization, poor packaging, chemical usage and the deleterious effects of environmental conditions such as temperature and exposure to light (Calixto, 2000; Elujoba et al., 2005). In spite of these problems, the South African Ministry of Health endorses the use of two herbal remedies for the management of HIV, *Sutherlandia frutescens (S. frutescens)* and *Hypoxis hemerocallidea* (common name: African potato) (Mills et al., 2005a).

1.3 Biotopes

Standardisation of plant-based pharmaceutical products is affected by varying chemical compositions which may be dependent on geographical location. To assess the metabolite content, Albrecht et al., (2012) studied *S. frutescens* growing in different geographical environments. The use of LC-MS in these studies indicated varying concentrations of sutherlandioside B (SU1) in *S. frutescens* extracts. Of the two locations studied (the arid Karoo and the coastal Gansbaai), virtually

undetectable levels of SU1 were observed in extracts obtained from the Gansbaai populations.

1.4 Problem statement

Sutherlandia frutescens, also known as cancer bush, is a plant species indigenous to South Africa that is well known for its medicinal properties where extracts of the leaves contain many medicinal properties. Nevertheless, little is known about the bioactive compounds existing in such leaf and seed extracts (Shaik et al., 2010).

Pharmacognosy has provided sufficient scientific evidence to the pharmaceutical industry so that phyto-compounds obtained from long-used and purported medical plants have great potential for the development of novel drugs (Phillipson, 2007). The vast majority of the African population still use traditional medicinal plants for healing of many ailments because they are regarded as safe for consumption, are more affordable, are convenient and easy to access and can be used for a long time (Vermani & Garg, 2002).

The bioactive phyto-compounds used in the management of ailments may be found in various parts of the plant, such as roots, leaves, flowers, seed or bark. However, chemical and genetic inconsistency in plants from different geographical biotopes complicates the standardization of commercially available end product (Bourgaud et al., 2001).

Herbal remedies are used for their immune-boosting abilities among HIV-infected individuals but concomitant administration with antiretroviral medication is known to have side effects (Bepe et al., 2012; Brown et al., 2008). Therefore, there is a need to study this species to show its potential as a source of medical phyto-compounds, to identify the bioactive compounds and to compare plants obtained from different geographical areas.

It is necessary, then, to validate claims that have been made about the medicinal properties of *S. frutescens* plant extracts. To this end, extracts were prepared from *S. frutescens* and isolated compounds were identified and screened for immune-boosting, cytotoxicity and antiviral activity.

1.5 Purpose of the study

The aim of this study is to isolate compounds present in various *S. frutescens* plant extracts and to determine the biological activity of these compounds.

1.6 Research Objectives

- To investigate *S. frutescens* collected from at least two different geographical areas
- To use physico-chemical techniques such as liquid chromatography and nuclear magnetic resonance to characterise compounds isolated from these plants.
- To compare the biological activity of compounds isolated from *S. frutescens* plants from the two regions.
- To investigate the effect of such compounds on cytotoxicity, anti-herpes replication and their effect on cytokine production in blood cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal Plants

South Africa is rich in plant diversity and the use of traditional medicines is extensive and endorsed by its Ministry of Health. There are about 200 000 indigenous traditional health practitioners in South Africa and in addition to using modern biomedical services, up to 80% of the population consult these traditional healers (Kasilo et al., 2010; Van Wyk et al., 2005). Medicinal plants are an essential part of the South African cultural heritage and plant-derived medicines form a large part of daily medicine for South African citizens. Due to the rich cultural diversity in South Africa, formal and informal systems of medicines are currently practised in different parts of the country and large volumes of such medicines are sold both in the informal and commercial sectors of the economy as plant material or as extracts (Van Wyk et al., 2005). Medicinal plants consist of mixtures of compounds that may act to improve health and these compounds may act individually, additively or in synergy (Van Wyk & Wink, 2004).

Over 24 000 species of higher plants are found in South Africa, with nearly 9 000 species belonging to the Cape Floral Kingdom. Owing to this bio- and cultural diversity, South Africa has approximately 3 000 plants species used as medicines. Better understanding of the healing power of plants has been gained through treating complicated health conditions. The Khoi-San, the Nguni and the Sotho-speaking people use an informal oral-traditional medicine system which has not yet been documented sufficiently and, hence, knowledge is passed on from one generation to the next by word of mouth (Van Wyk et al., 2005; Van Wyk & Wink, 2004).

In many societies, traditional and allopathic medicines are used side by side to complement each other with the allopathic medicines being used to treat serious and acute health conditions while traditional medicines are mostly used for treating chronic infections to reduce symptoms and improve the quality of life in a cost-effective way (Van Wyk & Wink, 2004).

2.2 *Sutherlandia frutescens*

Sutherlandia frutescens is a South African legume which has been traditionally used as an indigenous medicine for an assortment of diseases (Ojewole, 2004). The genus *Sutherlandia* belongs to the order Galegeae and is closely related to the genera *Astragalus L*. and especially *Lessertia* DC (Van Wyk & Wink, 2004) and belongs to the subspecies: Microphylla, family: Fabaceae/Leguminosa (Fernandes et al., 2004).

Figure 1: *Sutherlandia frutescens* (www.infonet-biovision.org/default/ct/202/medicinalplants)

Physically, *S. frutescens* is a flat to erect, perennial shrub that grows up to 3 m in height. Stems are glabrous, with many leaves near the tips. The leaves are slightly to densely hairy, petiolate, stipulate and pinnate. The *S. frutescens* plant has bright red, tube-like flowers of up to 35 mm which are “markedly compressed laterally, with oblong, boat-shaped keel petals, two tiny wing petals and a large, apically recurved standard petal, typically marked with white lines. The typical fruits are large and seeds are dark brown with a diameter of about 3 mm” (Van Wyk & Wink, 2004).

2.2.1 Medicinal uses of *Sutherlandia*

Sutherlandia frutescens is a variable plant species endemic to southern Africa including the southern parts of Namibia, the southern regions of Botswana, the western, central and eastern parts of South Africa and most of Lesotho (Van Wyk & Wink, 2004). This plant has been used medicinally for many years and it was originally used by the Khoi San and Nama people, who used it mostly as an extract for the washing of wounds (Van Wyk & Albrecht, 2008). Zulu warriors also used the plant to relax themselves after battle (Faleschini et al., 2013; Van Wyk & Albrecht, 2008).

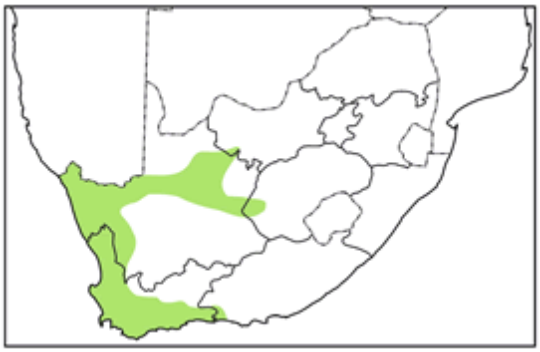


Figure 2: Geographical distribution of *S. frutescens* (Berend, 2000)

Sutherlandia frutescens is a multi-purpose medicinal plant and is used as a supplement for diseases such as diabetes and chronic depression along with HIV-infection management (Van Wyk & Gericke, 2000). It is a useful bitter tonic and is normally taken before meals to help with digestion and to improve the appetite. In addition, tinctures, infusions and decoctions of the leaves and young stems of *S. frutescens* have been used to treat colds, influenza, asthma, tuberculosis, bronchitis, rheumatism, rheumatoid arthritis and osteoarthritis, liver problems, haemorrhoids, piles, bladder, uterus and 'women's complaints', diarrhoea and dysentery, stomach ailments, heartburn, peptic ulcers, backache, diabetes, varicose veins and inflammation (Ojewole, 2004).

Extracts have also been shown to have anti-bacterial, anti-oxidant (Katerere & Eloff, 2005), anti-inflammatory (Kundu et al., 2005) stress-relieving (Prevoo et al., 2008)

blood sugar-reducing (Chadwick et al., 2007), anti-tumour (Stander et al., 2007) and anti-mutagenic (Reid et al., 2006) activities. Ngcobo et al. (2012) showed that the addition of low concentrations of extracts of *S. frutescens* on a primary culture of T cells could stimulate immune responses. Literature reports have also revealed that extracts from *S. frutescens* can also be used in the treatment of HIV/AIDS (Harnett et al., 2005; Mills et al., 2005b), cancer (Chinkwo, 2005; Stander et al., 2007; Tai et al., 2004) as well as to treat diabetes (Chadwick et al., 2007; Sia, 2004).

The anti-cancer and anti-viral properties of extracts from *S. frutescens* have been attributed to the presence of L-canavanine (Green, 1988) because high doses of this compound blocks DNA synthesis *in vitro* and at low concentration affects B-cell function in autoimmune mice. Other *in vitro* studies have shown that L-canaline, a small non-protein amino acid, is cytotoxic to human peripheral blood mononuclear cells (PBMCs) through the disruption of polyamine biosynthesis (Bence et al., 2002). Brown et al., (2008) investigated the effect of aqueous extracts of two traditional medicinal plants, *Hypoxis hemerocallidea* and *S. frutescens,* including L-canavanine isolated from *S. frutescens,* on transportation of nevirapine across human intestinal epithelial cell. A combination of an antiretroviral drug and an extract of *H. hemerocallidea* or of L-canavanine led to inhibition of nevirapine efflux – i.e. these compounds led to a decreased transport in the basolateral (BL) to apical (AP) direction across Caco-2 cell monolayers as compared to movement in the AP to the BL direction (Brown et al., 2008). This study also showed that an extract of *S. frutescens* also has an effect on nevirapine efflux but which was reduced in comparison with that of *H. hemerocallidea* and L-canavanine. However, the high inhibiting effects of L-canavanine were ascribed to the high doses used and an associated increase in the bio-availability of nevirapine was then observed.

The toxicity of *S. frutescens* (SF) was established by examining the effects of extracts in 70% ethanol (SFE) and deionised water (SFW) on normal, isolated human T cells. The toxicity of SF extracts was dose- and time-dependent, with SFE fractions being more potent in inducing cell death over 48 hours. High doses of *S. frutescens* were cytotoxic to the normal T cells following induction of necrotic cell death resulting in exhaustion of cellular ATP, inhibition of caspase-3/-7 activity and induction of DNA fragmentation (Ngcobo et al., 2012).

At low doses, *S. frutescens* influences gene expression and alters metabolic activity. Extracts of *S. frutescens* have anti-diabetic properties in animal models and studies done on a human liver cell cultures and it can also prevent insulin resistance in hepatocytes (Williams et al., 2013). Vorster et al., (2012) reported that *S. frutescens* extracts affect the proliferation, morphology and cell cycle dynamics of both the MCF-7 breast adenocarcinoma and MCF-12A breast epithelial cell lines to varying degrees in a time- and dose-dependent manner. The MCF-7 carcinoma cell line was more susceptible to the cytotoxic effects of aqueous extracts of *S. frutescens* when compared to the MCF-12A cell line. In addition, *S. frutescens* extracts has immune modulating as well as anti-inflammatory activities *in vitro* (Vorster et al., 2012).

2.2.2 Compounds isolated from *Sutherlandia* species

Chemical studies conducted on this plant species have revealed that aerial parts of *S. frutescens* contain several bioactive chemical compounds including L-canavanine, Gamma-aminobutyric acid (GABA), D-pinitol, asparagine, methyl- and propyl-parabens, flavonoids and small amount of saponins including novel triterpernoid glycosides (sutherlandioside A-D) (Avula et al., 2010; Fu et al., 2008; Van Wyk & Gericke, 2000). Compounds such as GABA, L-canavanine, L-arginine, D-pinitol and a glycan have been proposed as being responsible for this bioactivity. Amino acids make up about 10% to 15% (w/w) of dried plant material and the most abundant amino acids are proline, L-asparagine L-canavanine and alanine, which collectively represent approximately 60% of the total amino acid content. However, not all plant samples contained all these amino acids (Mncwangi & Viljoen, 2012).

2.2.2.1 Canavanine

Canavanine, or L-2-amino-4(guanidine-oxy)butyric acid, is a structural analogue of arginine and is a potential competitor for L-arginine. It is often found in seeds of Fabaceae plants (Mills, 2005) and has anti-cancer and anti-viral activity, as well as inhibition of influenza virus and retroviruses (Green, 1988). Between 30 mg and 40 mg of canavanine can be found per dry gram of the *S. frutescens* leaves (Van Wyk &

Gericke, 2000; Gericke et al., 2001). Canavanine is an inhibitor of nitric oxide synthase and this effect may be useful in treating certain forms of heart failure (Van Wyk & Gericke, 2000).

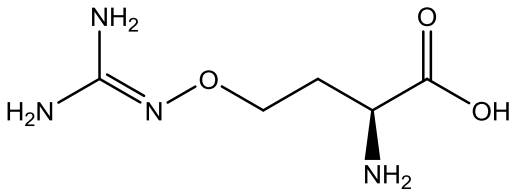


Figure 3: Structural formula of canavanine

2.2.2.2 Gamma Amino Butyric Acid (GABA)

This is a non-essential amino acid found in the leaves of *S. frutescens* and it is an inhibitory neurotransmitter (Ngcobo, 2008). It is synthesized by decarboxylation of glutamate by the enzyme L-glutamic acid-1-decarboxylase (Ebadi, 2007). Gamma amino butyric acid has been used to treat epilepsy and hypertension (Balch, 2006). Together with inositol and niacinamide (vitamin B3), GABA inhibits anxiety- and stress-related neuron messages from reaching the motor centres of the brain by occupying their receptor sites. Consequently it is critical for brain metabolism, where its function is to decrease neuron activity and prevent nerve cells form over-firing (Balch, 2006).

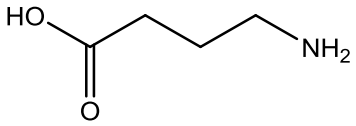


Figure 4: Structural formula of GABA

2.2.2.3 Pinitol

This is a form of sugar found in legumes and is characterised as chiro-inositol. The biological activity of pinitol makes *S. frutescens* a potentially essential traditional remedy in treating diabetes and inflammation (Moshe et al., 1998). It has been used to treat wasting in cancer as well as AIDS and is identified as an anti-diabetic agent (Narayanann et al., 1987; Ostlund & Sherman, 1996). Pinitol possesses an insulin-like effect (hypoglycaemic effect) which lowers blood glucose levels to increase bioavailability of glucose for cell metabolism (Bates et al., 2000). It also increases the retention of creatinine and glucose absorption by muscle cells (Greenwood et al., 2001). Consequently pinitol benefits sporting enthusiasts, who have simultaneous desires for strength and endurance, together with the additional benefits of shortened physiological recovery times.

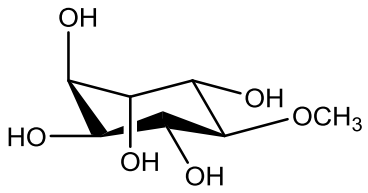
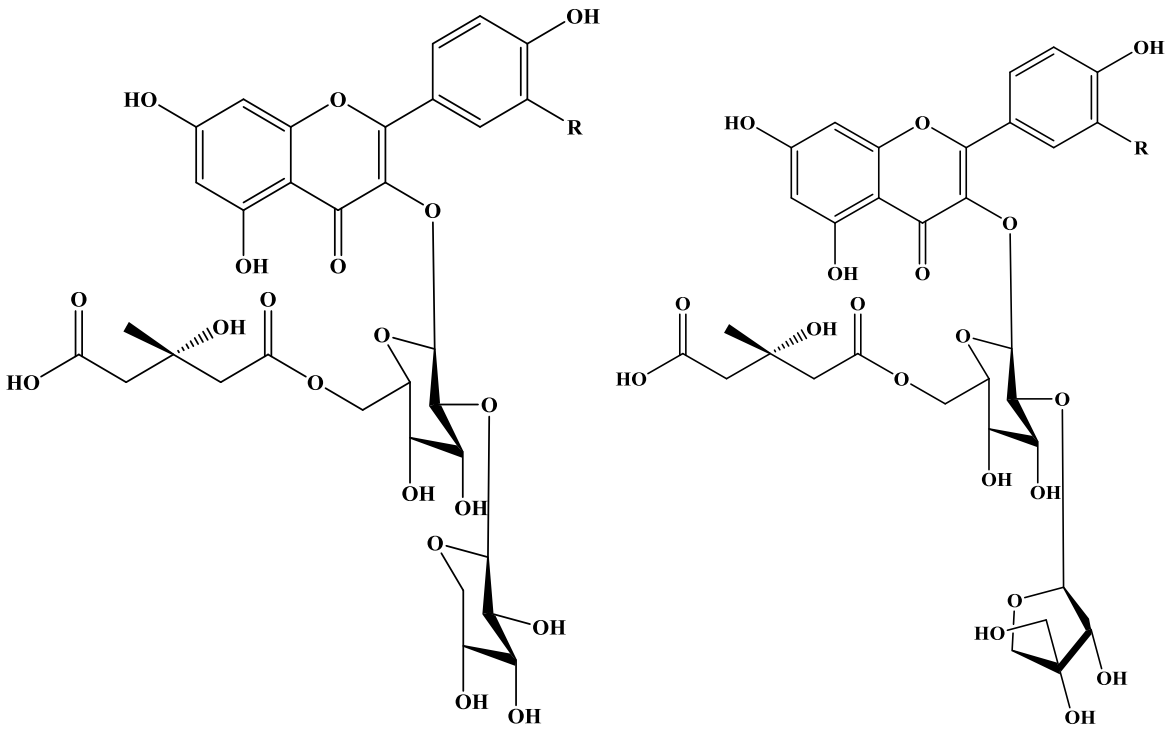


Figure 5 Structural formula of pinitol

2.2.2.4 Flavonoids

Flavonoids are phenolic compounds that occur naturally in fruits, vegetables and certain beverages such as tea and wine and have diverse antioxidant effects. Flavonoids also have both *in vitro* and *in vivo* anti-allergic, anti-inflammatory, antiviral and anticancer activity (Middleton, 1998). Avula et al. (2010) identified four flavonoids (sutherlandins A-D) in an attempt to provide chemical markers for the aerial parts of *S. frutescens*. The four sutherlandins are shown in **figure 6** below.



A: R = OH (**1**)

B: R = OH (**2**)

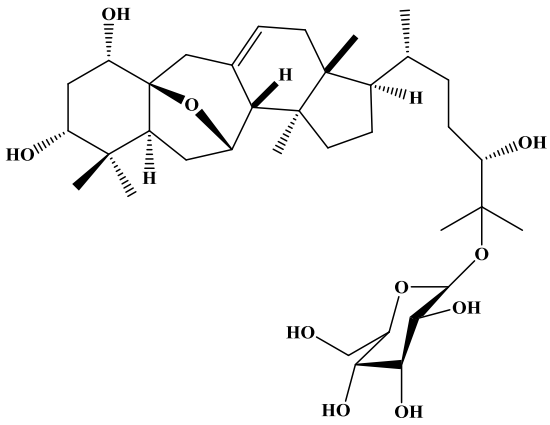
C: R = H (**3**)

D: R = H (**4**)

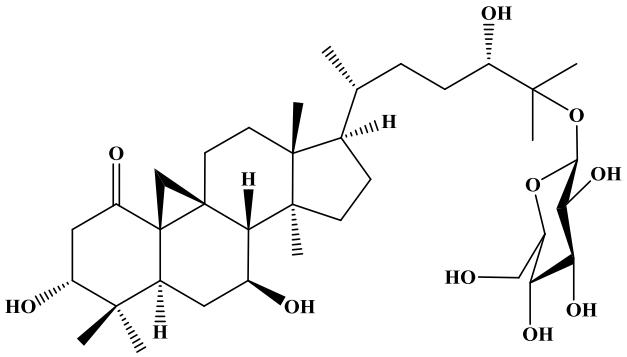
Figure 6: Structural formula of sutherlandins A to D

2.2.2.5 Cycloartane glycosides

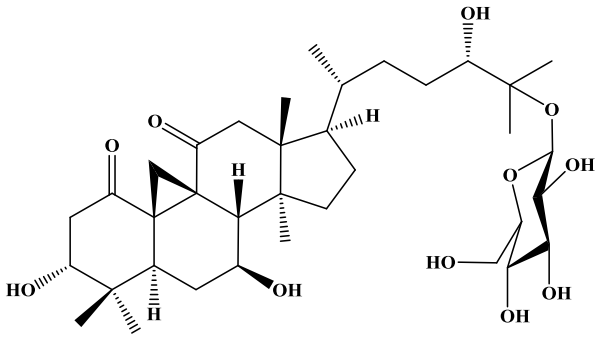
These types of compounds are derived from a 30-carbon compound and are characterised by a complex structural conformation due to their secondary ring formations and the addition of chemical groups such as glucose. Fu et al., (2008) identified four cycloartane glycosides called sutherlandiosides A – D while Olivier et al., (2009) isolated another cycloartane-type triterpene glycoside called SU3 which is the first cycloartane diglycoside isolated from *S. frutescens*.



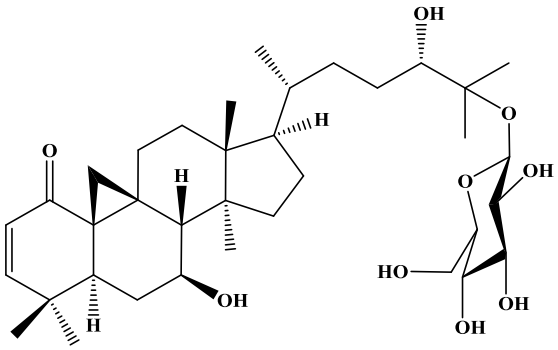
sutherlandioside A (**7**)



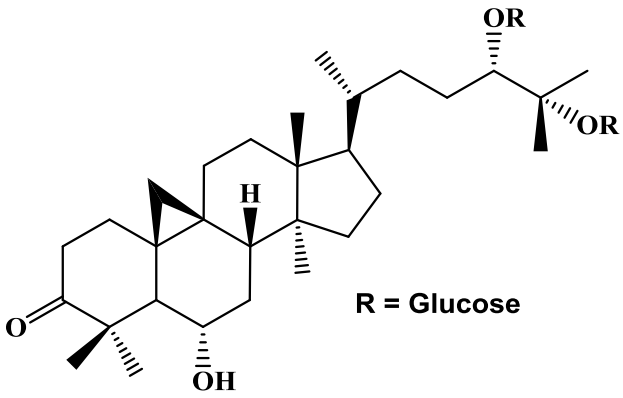
sutherlandioside B (**5**)



sutherlandioside C (**6**)



sutherlandioside D (**8**)



SU3 (**9**)

Figure 7: Structural formulas of sutherlandioside A to D (5-8)

2.2.3 Various activities attributed to *Sutherlandia frutescens* extracts

2.2.3.1 Immunomodulation

The immune system's basic function is to protect an individual against infectious and potential pathogens. Immunomodulation is a modification of the immune response to a particular level by agents that activate or suppress its function. The enhancement of immune responsiveness is known as immunostimulation and the reduction in immune responsiveness is known as immunosuppression (Mukherjee et al., 2014). Cytokines are secreted by cells in response to a stimulus and are the central mediator of inflammation and specific immune responses. Because of the role they play in modulation of HIV gene expression of infected cells they are assumed to be major role players in the pathogenesis of HIV (Estcourt et al., 1997). The type of immune response is determined by three components: the nature of the antigen; by regulatory T cells and the cytokines products of these T cells.

Natural products used for medicinal purposes can serve as alternate immunomodulatory and therapeutic agents that help to boost the body's defence mechanism. Immunostimulatory and immunomodulatory properties are commonly found in plants used in traditional medicine preparations. Such effects play a vital role in modifying the immune response to fight infections such as HIV and AIDS (Bodeker et al., 2006). Faleschini et al., (2013) observed that non-polar compounds from an ethanol extract of *S. frutescens* could amplify the release of specific immune-modulating cytokines, and this was particularly evident in cells which were stimulated by pathogenic microorganisms such as gram negative bacteria.

Three cytokines, namely IL-10, IL-6 and TNF-α, were reported by Stenvinkel et al., (2005) to be the crucial factors in the altered cytokine network of uremia and might also be involved in the development of cardiovascular disease, an inbalance in Th cells and worsening in the uremic milleu. In a study performed by Havlir et al., (2001), these three cytokines as well as others were elevated in HIV-1-infected persons with an advanced immune deficiency. It was then proposed that the elevation of these serum cytokines was due to advanced immune deficiency (Havlir et al., 2001). A focus of the current study involves these three cytokines, viz. IL-10, IL-6 and TNF-α.

2.2.3.2 Anti-oxidant activity

Anti-oxidant micronutrients such as alpha tocopherol and beta carotene improve the production and activity of cytotoxic immune cells that express cytokines that can terminate cancer cells and, hence, these micronutrients can inhibit, prevent or regress cancer *in vitro* (Shklar, 1998). Fernandes et al., (2004) observed that hot water extracts of *S. frutescens* possess potent antioxidant activity and could be compared with some teas such as rooibos where similar activity was also observed.

2.2.3.3 Antibacterial activity

Hexane extracts of *S. frutescens* showed a reasonable activity against both *S. aureus* and *E. faecalis* as compared to other extracts (Eloff et al., 2005). Its activity compares well with other extracts from plants such as *Combretum* species and the high activity of the hexane extract might be attributed to the presence of active compounds in high concentration (Katerere & Eloff, 2005). The activity of *S. frutescens* against *S. aureus* suggests that it could possibly be used in topical applications and reports of its use as a crude form of commercial product have been published (Gericke et al., 2001). A study performed by Katerere & Eloff (2005) showed that non-polar compounds possess an anti-staphylococcal activity and, hence, it may be necessary to use non-polar solvents for optimum therapeutic benefit.

2.2.3.4 Anti-cancer activity

The incidence of cancer has been increasing worldwide despite the substantial progress made in developing anti-cancer therapies. Chemoprevention has been reported in the literature as one of the most rational approaches in reducing the risk of cancer and this strategy is relatively new and promising (Kundu et al., 2005). Chemoprevention is the use of non-toxic substances to block, prevent or to retard tumorigenesis and these substances can be natural, be synthetic in origin or be a

combination of the two. Phytochemical compounds derived from plants can interfere with specific stages of carcinogenesis (Young-Joon, 2003).

Due to the historical use of *Sutherlandia* as an anti-cancer agent, Tai et al., (2004) investigated its anti-proliferative effects on several human tumour cell lines. Different anti-proliferative effects were observed on breast and leukemia cell lines with a higher activity against MDA-MB-468 breast cells compared to Jurkat and HL60 leukemia cells (Tai et al., 2004).

2.2.3.5 Anti-HIV activity

An effective HIV inhibitor requires at least two key activities: inhibiting the interaction between viral and cellular receptors at the surface of a cell and blocking the viral transmembrane gp 41 protein so as to prevent cell fusion and, hence, block cell-to-cell transmission of HIV. Mechanistically, most of the effective natural or natural-based products (e.g. dextran sulphate) interact with the viral gp 120 to then competitively inhibit the binding of gp 120 to the CD_4 recognition site on the T-lymphocytes cell surface (Yang et al.,2001).

As an inexpensive herbal remedy, *S. frutescens* has gained international recognition as a source of agents that may improve the health of HIV/AIDS patients. As a source of antiviral activity, this plant is reported by doctors and healthcare workers to contain compounds that improve CD_4 cell counts, decrease viral loads, improve appetite, improve mood and prompt an increase in patient weight (Chaffy & Stokes, 2002). Harnett, et al., (2005) observed significant inhibition (64%) in a HIV- reverse transcriptase (RT) assay involving *Sutherlandia* and *Lobostemon* extracts, regardless of the loss of inhibition that may have resulted due to the removal of sulphated polysaccharides and the addition of bovine serum albumin (BSA). Hence, the inhibitor may be a novel compound and not a polysaccharide or tannin compound (Harnett et al., 2005).

2.2.3.6 Pharmacokinetics and pharmacology

The pharmacokinetic properties of *S. frutescens* have not been extensively assessed. Thus, when Mills et al., (2005) used *in vitro* studies to assess the effect of *S. frutescens* on CYP3A4, P-gp and PXR, a close to complete inhibition of CYP3A4 (96%) was noted while only moderate activity of P-gp was observed. Comparatively, this showed a 19-31% activity strength relative to verapamil and a more than two-fold activation that was dose-dependent was observed for PXR under exposure to *S. frutescens* ($P < 0.01$) (Mills et al., 2005).

2.2.4 Theory and practice of analytical techniques used in this study

Isolation and structural elucidation of secondary metabolites from traditional medicines is a crucial part of the production of herbal infusions and is achieved through taking advantage of analytical techniques. Secondary metabolites facilitate critical phenomena such as metabolism, signal transduction, mating attraction, and chemical defence (Robinette et al. 2012). In the current study 1D (^{1}H, DEPT, ^{13}C) and 2D (COSY, HSQC, HMBC) nuclear magnetic resonance (NMR) experiments were used for structural elucidation. The UPLC-QTOF-MS also assisted in structure elucidation by providing accurate mass and fragmentation patterns of the isolated compounds.

Nuclear magnetic resonance spectroscopy is a qualitative technique used for quality control, structural elucidation and to assess purity of samples. It uses one to three dimensional approaches, namely one dimension (1D), two dimensions (2D) and three dimensions (3D) for structural elucidation of organic compounds (Fuloria and Fuloria, 2013). It is an analytical technique that takes advantage of the intrinsic magnetic properties that are associated with atomic nuclei with odd numbers of protons and/or neutrons. When a sample of unknown compound with such nuclei is placed in a large magnetic field and subjected to the radio-frequency (rf) field of the oscillator, it then absorbs the rf energy which causes it to align and rotate to lie parallel with the field. The strength of the magnetic field and the rf signal is picked up by a detector (Elad et al., 2012). In this study two Varian NMR instruments allowing analysis at 600 MHz and 400 MHz were used.

Although NMR analysis is the most suitable source of information for structural elucidation it still needs to be complemented by other techniques like the Mass spectroscopy (UPLC-QTOF-MS). Mass spectrometry is made out of three basic components: one that volatilizes and ionises the molecule in a beam of charged particles; another that focuses the beam so that particles of the same mass-to-charge ratio are separated from all others and a third component that is used to detect the particles (Clayden et al., 2001; Robinette et al., 2012).

Mass spectroscopy uses the difference in mass-to-charge ratio (m/z) of ionized atoms or molecules to separate them and structural information is acquired by identifying distinct fragments patterns (Clayden et al., 2001). It operates by creating gas-phase ions followed by separation of ions in space or time based on their mass-to-charge ratio and measures the quantity of ions of each mass-to-charge ratio. The applications in organic chemistry of MS includes determination of molecular mass, characterisation, confirmation of the identity and purity of a unknown samples and providing data on isotopic abundance (Bross-Walch et al., 2005; Clayden et al., 2001).

Flow cytometry was used to assess the immune-potentiating ability of plant extracts, fractions, and isolated compounds. This is a laser-based technique that is used in counting and sorting of cells, in biomarker detection and in protein engineering (Davis, 2007). This technique allows for simultaneous analysis and measurement of multiple physical and chemical characteristics of a single particle (e.g. cell) in a fluid sheath as it flows past a laser beam (Jahan-Tigh et al., 2012; Weaver, 2000). A particle size of about 0.2-150 micrometre is required for analysis. When the particles are fluorescently labelled, they emit light at varying wavelengths when excited by the laser energy (Jahan-Tigh et al., 2012; Weaver, 2000). The dispersed fluorescent light is collected by positioned lenses and directed to the detectors using splitters and filters. Consequently, electronic signals are produced relative to the optical signals striking the detectors (Weaver, 2000).

CHAPTER 3: ANALYSIS OF COMPOUNDS

Traditional medicines form part of the solution for many health challenges and many traditional healers make use of infusions and decoctions of medicinal plants to treat many ailments. As part of proof of concept and to assess the safety and efficacy of medicinal plants, phytochemical studies are usually conducted to scientifically mimic the action of traditional preparations.

3.1 Materials and methods

Column chromatography was used for the isolation of compounds. Isolated compounds were characterised using a variety of analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy (1D and 2D) and ultra-performance liquid chromatography (UPLC)-time of flight (TOF)-mass spectrometry (MS).

3.1.2 Plant Collection

Aerial parts of *S. frutescens* were collected in Petrusburg, Free State, South Africa and in Paarl, Western Cape, South Africa. The plant material was dried at ambient temperature and ground to a powder using a pin mill. For identification, twigs containing flowers were sent to the South African National Biodiversity Institute (SANBI) and the plant species were identified as *Sutherlandia frutescens (L.) R. B.r*. (SANBI Genspec number: 462 and 602).

3.1.3 Chemicals

During the isolation of phytochemical compounds, various organic solvents such as ethanol, hexane, methanol and dichloromethane were used. High quality CP grade solvents were purchased from Merck and distilled before use while for HPLC analysis, solvents were purchased from Microsep (Romil Chemistry, UK). Solid

phase extraction C-18 cartridges (Supelco 140 ml PP tubes) were purchased from Sigma Aldrich. Distilled water was used for all procedures.

3.1.4 Extract preparations

A quantity of 600 g of plant material of *S. frutescens* from Petrusburg was divided into 3 x 200 g amounts and labelled A, C and E, respectively. A different extraction method was used for each of these: for A, absolute ethanol; for C, 70% ethanol and for E, distilled H_2O. Due to a relatively limited amount of material obtained from Paarl, 400 g of plant material was divided into 2 x 200 g amounts and labelled B and D that were then extracted with absolute ethanol and distilled H_2O, respectively.

Plant material from **A** and **B** was extracted three times with absolute ethanol (1.0 L of 99.9% EtOH) overnight and the same procedure was followed for the extraction of **C** with 70% ethanol. For aqueous extraction of **D** and **E**, plant material was boiled for 1 hour in 2.0 L distilled H_2O with occasional stirring. See **Figure 8** below for a flow diagram indicating the extraction methods and percentage yields.

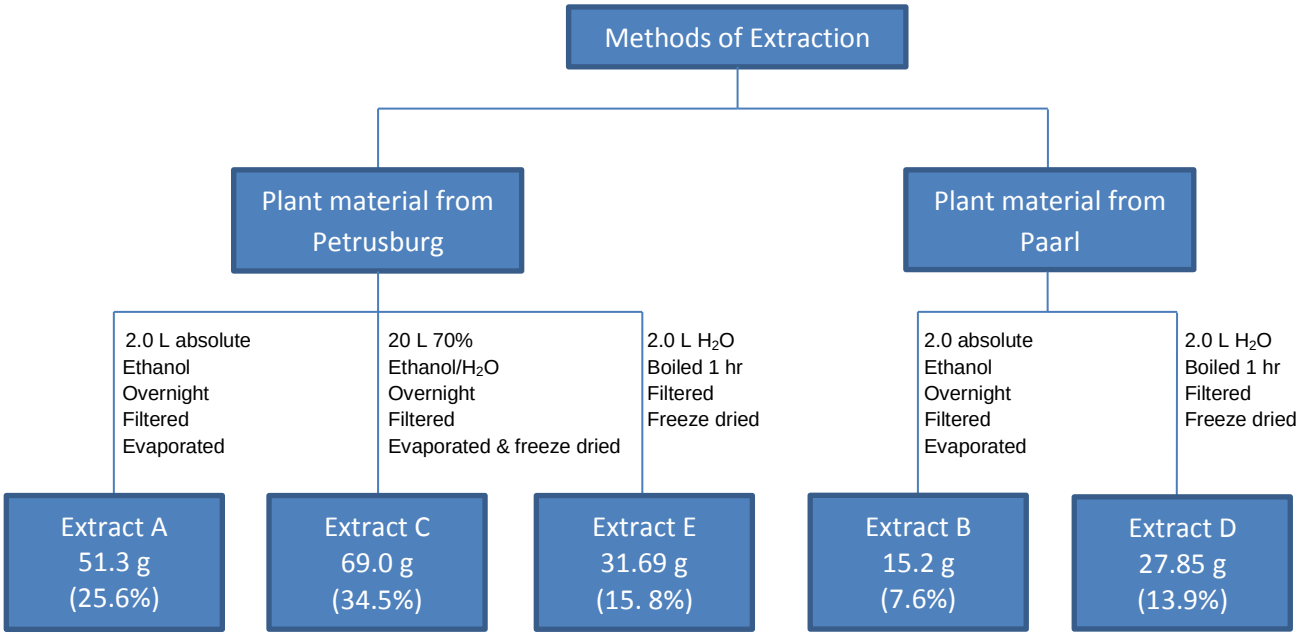


Figure 8: Extraction methods

3.1.5 Liquid-Liquid partitioning

An amount of 10 g of **extract A** was suspended in 100 ml of MeOH/H_2O (ratio 9:1 v/v) and sequentially extracted with hexane, dichloromethane (DCM) and ethyl acetate (EtOAc). Four crude extracts and an emulsion phase were obtained as shown in **Figure 9**. All the resulting organic extracts were evaporated to dryness using a rotary evaporator and the aqueous extracts were freeze-dried to give **extracts A1 to A5**.

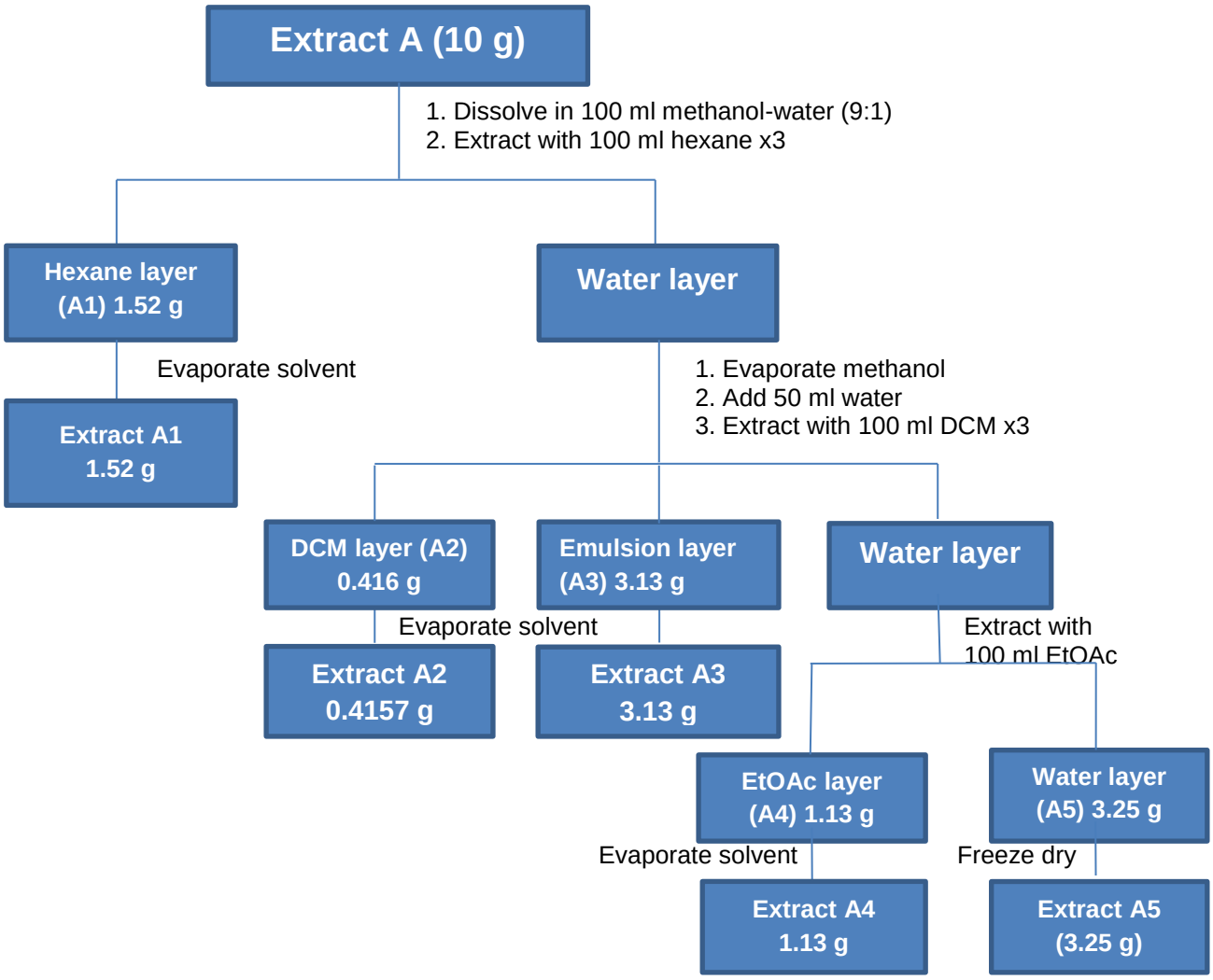


Figure 9: Liquid-liquid flow diagram

3.1.6 Isolation of compounds 5 and 10

Extract A2 (415.7 mg) from the DCM extract was separated on a silica gel column using a stepwise gradient mixture of approximately 500 ml EtOAc/hexane (v/v) in the ratio 4:6, 5:5, 6:4, 8:2, 9:0 as an eluent to give 5 fractions. Fraction 5 was chromatographed using preparative thin layer chromatography (prep-TLC) employing $CHCl_3$/MeOH (9.5:0.5) as the eluent to give **compound 10** (2.2 mg).

Extract A3 from the emulsion phase was subjected to silica gel column chromatography using a stepwise gradient mixture of approximately 500 ml of $CHCl_3$/MeOH (v/v) in ratio 10:0, 9:1, 7:3, 5:5, 4:6, 1:9 and 0:10 as an eluent. Similar fractions were combined to give 7 fractions. **Fraction 1** was separated on a silica gel flash column and eluted with $CHCl_3$/MeOH (10:0, 9:1, 7:3, 5:5, 0:10) and similar fractions were combined to give 5 fractions (labelled A-E). A C-18 solid phase extraction (SPE) column (Supelco 140 ml PP tubes, Sigma Aldrich) was then used to purify **fraction A** and eluted with acetonitrile (ACN)/MeOH (10:0, 9:1, 7:3). Thereafter, prep-TLC was used to obtain **compound 5** (6.9 mg) with EtOAc/MeOH/Acetic acid (9:1:1 v/v) as solvent system.

3.1.7 Acetylation Procedure

Acetylation of **extract A3** (74.5 mg) samples was achieved by dissolving the extract in a mixture of pyridine-acetic anhydride (1:1) in a round-bottomed flask. The reaction mixture was stirred for 48 h at room temperature and then 3 ml ethanol was added to react with the excess acetic anhydride. The resultant mixture was poured into an ice-chloroform mixture and the acetylated product was extracted three times using liquid-liquid partitioning in a separating funnel. The organic phases were combined and the solvent removed under vacuum. Preparative TLC was used with chloroform-methanol (9:1 v/v) as the solvent system and **compound 12** (2.1 mg) was obtained.

3.1.8 Accurate mass analysis

Accurate mass analysis was performed on an UPLC high-definition quadrupole time-of-flight mass spectrometer (MS) instrument (UPLC-qTOF SYNAPT G1 HDMS system, Waters, Manchester, UK) fitted with an Acquity BEH C8 column (2.1 x 150 mm, 1.7 µm; Waters Corporation).

For all the isolated compounds and fractions, the following method was followed for accurate mass evaluation. A binary solvent system consisting of eluent A: 0.1% formic acid in water and B: 0.1% formic acid in methanol (Romil Chemistry, UK) was used at a constant column temperature of 60°C. A 40 min gradient method at a constant flow rate of 0.4 mL/min was used for compound separation, and the conditions were: 10% B over 0.0-1.0 min, 10%-25% B over 1.0-3.0 min, 25%-30% B over 3.0-9.0 min, 30%-35% B over 9.0-10.0 min, 35%–80% B over 10.0 -35.0 min, 80%-100% B over 35.0-35.1 min and held constant at 100% B over 35.1-37.0 min to wash the column. Thereafter, the column was returned to initial conditions at 38 min and allowed to equilibrate for 2 min. Chromatographic separation was monitored using a photodiode array (PDA) detector with a scanning range set between 200-500 nm, 1.2 nm bandwidth resolution and a sampling rate of 20 points.sec^{-1}.

3.1.5.1 Quadrupole time-of-flight mass spectrometry (Q-TOF-MS)

Post-PDA detection, the metabolites were further characterised with the aid of a SYNAPT G1 high definition mass spectrometer operating in positive and negative ionization modes. The MS conditions were as follows: capillary voltage of 2.5 kV, sample cone voltage of 30 V, extraction cone voltage of 4 V, MCP detector voltage of 1600 V, source temperature of 120ºC, desolvation temperature of 450°C, cone gas flow of 50 L/h, desolvation gas flow of 550 L/h, *m/z* range of 100-1000, scan time of 0.2 sec, interscan delay of 0.02 sec, mode set as centroid, lockmass set as leucine enkephalin (556.2771 / 554.2615 Da), lockmass flow rate of 0.1 mL/min, and mass accuracy window of 0.5 Da. High purity nitrogen gas was used for desolvation, cone and collision gas. The software used to control the hyphenated system was MassLynx Ver. 4.1 (SCN 704).

3.2 Results and Discussion

In order to incorporate traditional medicines into the South African healthcare system, certain precautions need to be considered when verifying claimed medicinal activity. These precautions include toxicity that may prevent immediate use of the claimed medicine (Moshi, 2005). The activity of traditional medicine is attributed to secondary metabolites which are products of secondary chemical pathways in plants that are crucial for the survival of the plants in their natural environment (Hartmann, 2007; Shaik et al., 2010). Secondary metabolites provide a chemical defence against herbivores, insects, microorganisms and viruses (Wink, 2003). These secondary metabolites include a variety of nitrogen-containing compounds including non-protein amino acids, cyanogenic glucosides and alkaloids and non-nitrogen compounds such as terpenoids, phenolics and steroids (Shaik et al., 2010). In plants, secondary metabolites can be found in an active state or as a prodrug which is activated upon infection or wounding of the plant (Wink, 2003).

Different geographic locations may result in a variation in the metabolic profiles of *S. frutescens* (Albrecht et al., 2012). In this chapter the metabolite content of extracts obtained from plants growing in two different geographic environments was also assessed. The metabolic profiles were obtained using UPLC-QTOF-MS

3.2.1 Characterisation of isolated compounds

3.2.1.1 Structural elucidation of 7S,24S,25-trihydroxycycloart-2-en-1-one (compound 10)

At first viewing of the ^{13}C-NMR spectrum (Appendix 1), 30 signals were observed indicating that this compound might be a steroid or triterpene. A closer look at the spectrum showed an oxygen-bearing quaternary carbon signal at δ_C 74.04 (C-25), a carbonyl signal at δ_C 203.49 (C-1) and two oxygen-bearing methine signals at δ_C 69.15 (C-7) and δ_C 79.89 (C-24). It also showed two olefinic carbon signals at δ_C 129.66 (C-2) and 161.31(C-3). The ^{13}C NMR chemical shift of this compound is collated in Table 1.

The ^{1}H spectrum shows the presence of two olefin signals as doublets at δ_H 5.86 (J=9.9 Hz) and δ_H 6.73 (J=9.9 Hz) assignable to H-2 and H-3 of a triterpene moiety. It further revealed two protons on oxygen-bearing carbons at δ_H 3.70 (broad multiplet) and δ_H 3.31 (broad doublet) with a coupling constant of J=9.0 Hz. Six tertiary methyl groups at δ_H 0.91(s, H-30), δ_H 0.96 (s, H-28), δ_H 0.98 (s, H-18), δ_H 1.11 (s, H-26), δ_H 1.12 (s, H-29), δ_H 1.14 (s, H-27), one secondary methyl at δ_H 0.89 (d, H-21, J=6.6 Hz), and cyclopropane methylene signal at δ_H 0.94 and 1.58 (each 1H, J=4.8 and J=4.2) were observed. These cyclopropane methylene signals are characteristics of a cycloartane-type structure.

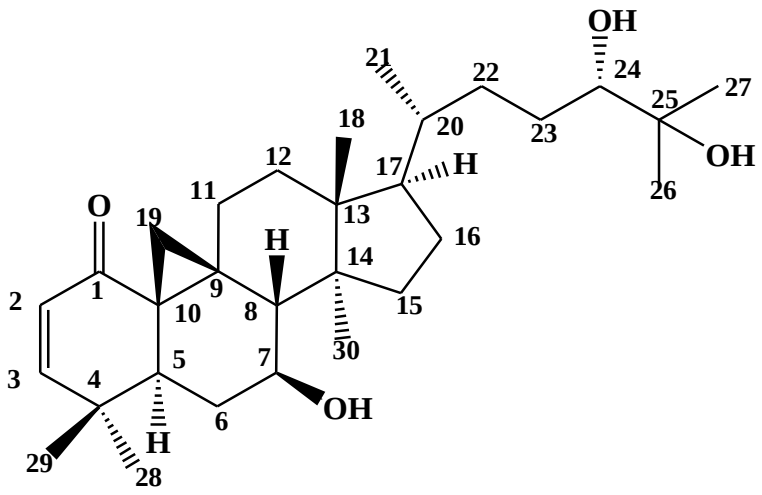


Figure 10: Structure of compound 10

Table 1: ^{1}H- and ^{13}C-NMR data of compound 10 in MeOH (600 MHz)

Position	**Compound 10**	
	δ_C	δ_H (*J=Hz*)
1	203.49	
2	129.66	5.86 d (9.9)
3	161.31	6.73 d (9.9)
4	37.06	
5	42.85	
6	28.85	1.95 m
7	69.15	
8	52.39	
9	50.49	
10	37.71	
11	34.81	
12	33.87	
13	46.52	
14	33.54	
15	26.47	2.00 m, 2.64 m
16	31.06	1.24 m,1.92 m
17	53.19	
18	15.97	0.98 s
19a	29.23	0.94 d (4.5)
19b		1.58 d (4.5)
20	37.47	
21	19.18	0.89 d (6.6)
22	29.04	
23	34.66	1.37 m, 1.61 m
24	79.89	
25	74.04	
26	25.16	1.11 s
27	25.78	1.14 s
28	28.21	0.96 s
29	21.51	1.12 s
30	19.12	0.91 s

The proton-proton correlation spectrum (COSY) revealed that compound 10 has the following partial structures (Figure 11). The short range hydrogen-carbon correlation spectrum (HSQC) displayed the correlation of all protonated atoms with well resolved protons.

These observed correlations were further substantiated by long range hydrogen-carbon 13 correlation (HMBC) (Figure 12). Supplementary information is given in Appendix 1.

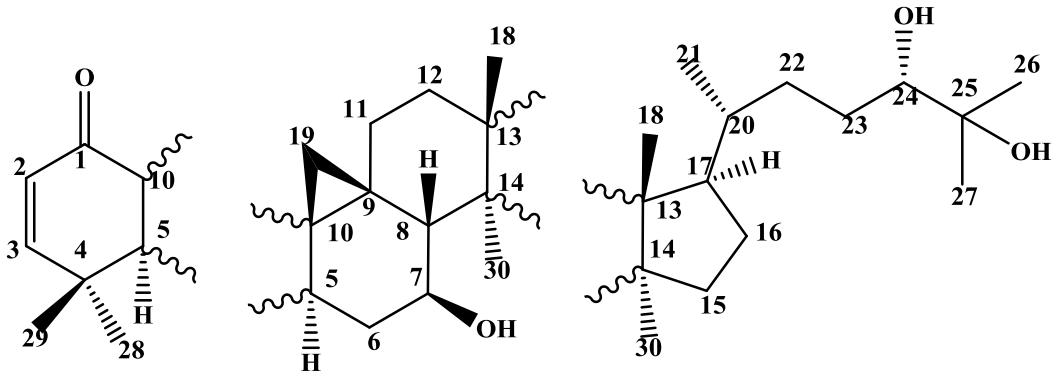


Figure 11: Structural fragments of compound 10

Table 2: ^{1}H-^{13}C HMBC correlations of compound 10

δ_H	δ_C
(H-3)	(C-1), (C-5), (C-28), (C-29)
(H-2)	(C-3)
(H-7)	(C-9)
(H-8)	(C-9), (C-13)
(H-18)	(C-12), (C-17)
(H-19)	(C-5), (C-8)
(H-15)	(C-8), (C-13)
(H-21)	(C-22), (C-17)
(H-24)	(C-22), (C-23),(C-27)
(H-26)	(C-24), (C-27)
(H-27)	(C-24), (C-26)
(H-28)	(C-29)
(H-29)	(C-28)
(H-30)	(C-8), (C-13)

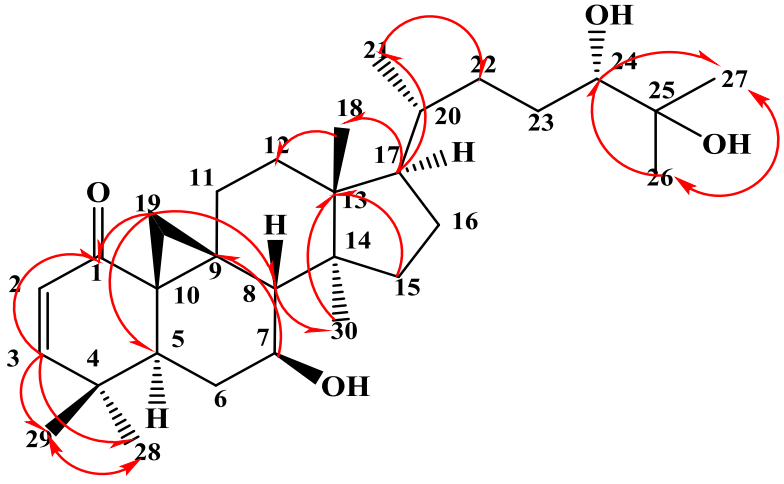


Figure 12: Key HMBC correlations associated with compound 10

The accurate mass obtained from the HRTOFMS spectra (Figure 13) confirmed the structural formula. The ESI^+ (positive mode) showed a pseudo molecular ion at *m/z* 473.3631 (Calculated for $[C_{30}H_{48}O_4 + H]^+$) and a sodium adduct at m/z 495.3450 (calculated for $[C_{30}H_{48}O_4 + Na]^+$). The ESI^- (negative mode) showed a formic acid adduct at m/z 517.3529 (calculated mass for $[C_{30}H_{48}O_4 - H+HCOOH]^-$) due to its presence in the source. Elementary analysis performed on the mass ions gave $C_{30}H_{49}O_4$ and $C_{31}H_{51}O_6$ (corresponding to $[C_{30}H_{48}O_4 + H]^+$ and $[C_{30}H_{48}O_4 - H+ HCOOH]^-$) molecular formulas and both fragments had 0.0 i-FIT value, a mass error of 2.4 mDa and -2.4 mDa and the calculated double bond equivalence (DBE) values were 6.5 and 7.5. The sodium adduct had an i-FIT value of 0.2, a mass error -0.1 mDa, a calculated DBE value of 6.5 and the molecular formula was $C_{30}H_{48}O_4Na$. Supplementary information is given in Appendix 2

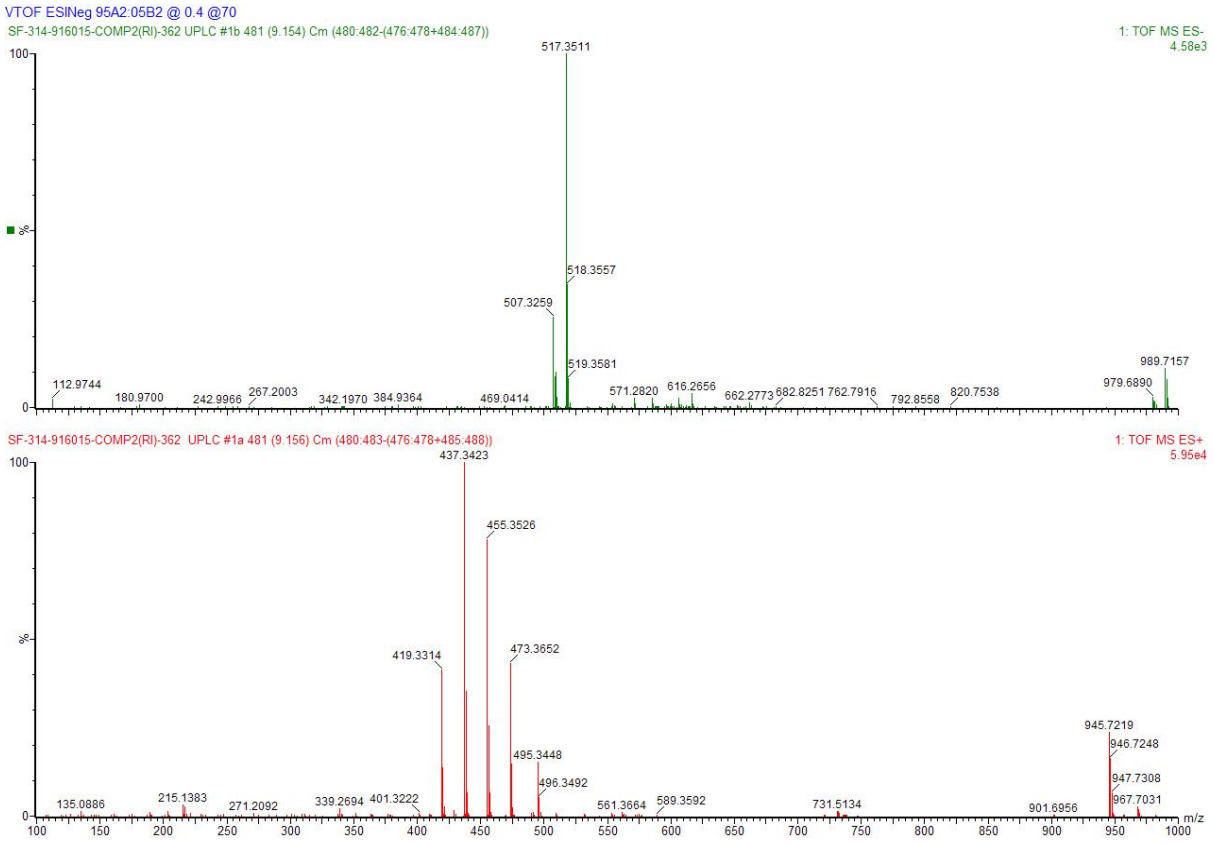


Figure 13: HRTOFMS spectrum (ESI^- and ESI^+) for compound 10

From our literature searches conducted on this compound it appears that this compound has not been previously reported as per review in section 2.2.2.

3.2.1.2 Structural elucidation of the acetylated cycloartane (compound 11)

The proton NMR spectrum revealed the presence of six acetate methyl peaks, a sugar moiety and a triterpene moiety (see appendix 3 for NMR spectra). The six acetate methyl groups suggest that the compound might contain four acetate groups in the sugar moiety and two acetate methyl groups in the triterpene moiety. It further revealed two olefin signals as doublets at δ_H 5.87 (J=9.8 Hz) and δ_H 6.58 (J=9.8 Hz) assignable to H-2 and H-3 of the triterpene moiety; two protons on oxygen-bearing carbons at δ_H 3.66 (broad multiplet) and δ_H 4.85. Six tertiary methyl groups at δ_H 0.88(s, H-30), δ_H 0.91 (s, H-18), δ_H 0.93 (s, H-29), δ_H 1.08 (s, H-28), δ_H 1.15 (s, H-26), δ_H 1.19 (s,H-27), one secondary methyl at δ_H 0.82 (d, H-21, J=6.4 Hz), and cyclopropane methylene signal at δ_H 1.05 and 1.44 (each 1H, J=4.4 and J=4.8) were observed. The presence of the cyclopropane methylene signals also suggested that this compound might be a cycloartane type.

A ^{13}C-NMR spectrum (**Table 1**) displayed 43 resonances with a characteristic oxygen-bearing quaternary carbon signal at δ_C 79.51 (C-25), a carbonyl signal at δ_C 200.58 (C-1), two oxygen-bearing methine signals at δ_C 71.49 (C-7) and δ_C 78.62 (C-24). It also showed two olefinic carbon signals at δ_C 129.25 (C-2) and 158.62 (C-3). Acetate carbon signals were observed in the range of δ_C 169.23 to 170.79 with their methyl carbon signal also ranging from δ_C 20.82 to 20.90.

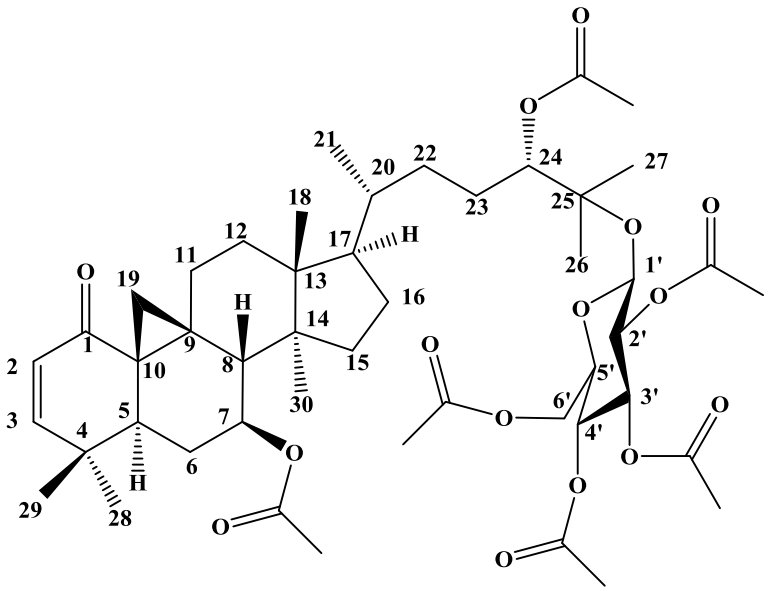


Figure 14: Structure of compound 11

Table 3: 1H- and ^{13}C-NMR data of compound 11 in MeOH (600 MHz)

Position	**Compound 11**	
	δ_C	**δ_H (J=Hz)**
1	200.58	
2	129.25	5.87 d (9.8)
3	158.62	6.58 d (9.8)
4	36.80	
5	41.31	2.33 dd (13.6, 4.0)
6	26.70	
7	71.49	
8	48.16	2.22 d (4.8)
9	31.24	
10	35.37	
11	25.10	
12	25.57	
13	49.15	
14	45.54	
15	33.86	
16	32.54	
17	51.51	
18	15.71	0.91 s
19a	28.32	1.05 d (4.6)
19b		1.44 d (4.6)
20	35.89	
21	18.44	0.82 d (6.4)
22	25.57	
23	27.59	
24	78.62	
25	79.51	
26	21.78	1.15 s
27	24.48	1.19 s
28	28.05	1.08 s
29	21.25	0.93 s
30	18.87	0.88 s
1’	95.72	4.69 d (8.0)
2’	71.79	
3’	73.21	5.185 t (9.4)
4’	69.08	
5’	71.89	
6’a	62.64	4.09 dd (12.0, 2.4)
6’b		4.16 dd (12.0, 5.6)
Acetates		
1(CH_3)	20.82	
2(CH_3)	20.90	
3	169.23	
4	169.67	
5	170.32	
6	170.51	
7	170.79	

The ^{1}H-^{13}C HMBC (Table 4) spectrum revealed the following correlations (Figure 10). The position of the double bond at C2-C3 was supported by COSY correlation of H-2/H-3 and it also supported the presence of cyclopropane methylene by showing splitting hydrogen correlations at C-19 of H-19_a/H-19_b, the correlations of the hydrogen bonded to an oxygenated carbon C-7 and C-8 of H-7/H-8. For the sugar moiety the following COSY correlations of H1'/H2', H2'/H3', H4'/H5', H5'/H6' were observed.

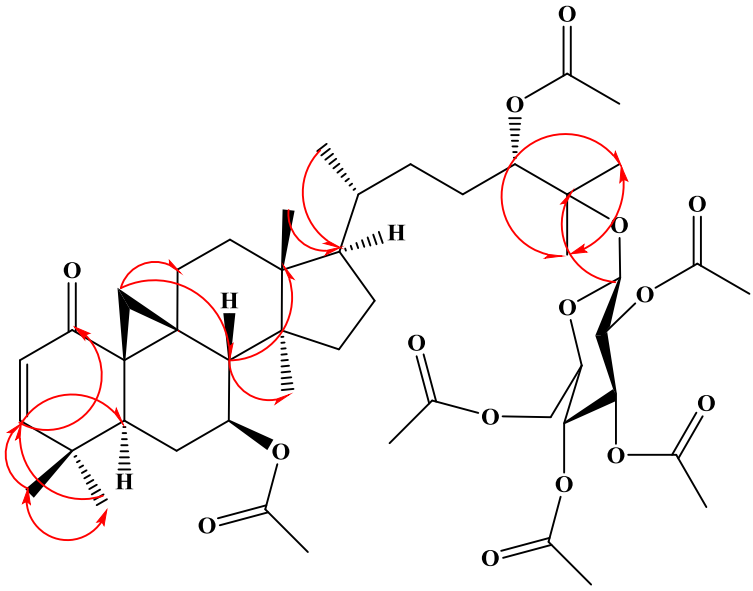


Figure 15: Key HMBC correlations associated with compound 11

Table 4: ^{1}H-^{13}C HMBC correlations of acetylated compound

δ_H	δ_c
(H-3)	(C-5), (C-1)
(H-2)	(C-10), (C-4)
(H-8)	(C-13), (C-10), (C-30)
(H-11)	(C-8)
(H-18)	(C-17)
($H\text{-}19_{a/b}$)	(C-8), (C-11), (C-5)
(H-21)	(C-16)
(H-24)	(C-27), (C-26)
(H-26)	(C-27)
(H-27)	(C-24)
(H-28)	(C-3), (C-29)
(H-29)	(C-3), (C-28)
(H-30)	(C-8), (C-9), (C-13); (C-7)
(H-1')	(C-2'), (C-25)
(H-2')	(C-1')
(H-3')	(C-2')
(H-4')	(C-3'); (C-6')

The structural formula of this acetylated compound was confirmed by HRTOFMS and it also showed a molecular formula of $C_{48}H_{70}O_{15}$. The ESI^+ (positive mode) showed a pseudo molecular ion at m/z 887.4793 (calculated for $[C_{48}H_{70}O_{15} + H]^+$) and a sodium adduct at m/z 909.4612 (calculated for $[C_{48}H_{70}O_{15} + Na]^+$). Elementary analysis performed on the mass ions gave $C_{48}H_{71}O_{15}$ corresponding to $[C_{48}H_{70}O_{15} + H]^+$ molecular formula and fragments had 0.2 i-FIT value, a mass error of 9.9 mDa and the calculated double bond equivalence (DBE) values were 13.5. The sodium adduct had an i-FIT value of 0.1, a mass error 4.4 mDa, a calculated DBE value of 4.5 and the molecular formula was $C_{48}H_{70}O_{15}Na$. HRTOFMS spectra (ESI^+) showed in Appendix 4 (pages 81-83).

3.2.1.3 Structural elucidation of compound 5

Compound 5 exhibited 36 signals in the ^{13}C NMR (Table 5) spectrum indicating a possibility of an aglycone moiety attached to a triterpene moiety. Supplementary information is given in Appendix 5.

In the ^{1}H-NMR spectrum, three protons on oxygen-bearing carbons at δ_H 3.35 triplet, δ_H 3.68 triplet (H-7) and δ_H 3.68 triplet (H-24) with coupling constants J=4.8, 4.8, 8.7 Hz were observed. It further revealed six tertiary methyl groups at δ_H 0.89 (H-27), δ_H 0.94 (s, H-30), δ_H 0.95 (s, H-18), δ_H 0.98 (s, H-28), δ_H 1.18 (s, H-29), δ_H 1.23 (s, H-26), one secondary methyl at δ_H 0.89 (d, H-21, J=6.6 Hz). Interestingly, in the spectrum, splitting cyclopropane methylene signals at δ_H 0.73 and 1.41 (each 1H, d, J=4.8 and J=4.2) were observed and these cyclopropane methylene signals suggest that the triterpene is a cycloartane type.

On the ^{13}C NMR spectrum, characteristic oxygen bearing quaternary carbon signal was observed at δ_C 81.7 (C-25), a carbonyl signal at δ_C 213.5 (C-1), three oxygen-bearing methine signals at δ_C 69.5 (C-7), δ_C 78.4 (C-3) and δ_C 78.9 (C-24). ^{13}C NMR indicated that **compound 5** is a Sutherlandioside B triterpenoid also known as SU1 as described by Olivier et al., (2009)(Figure 16 and discussed in section 2.2.2.5).

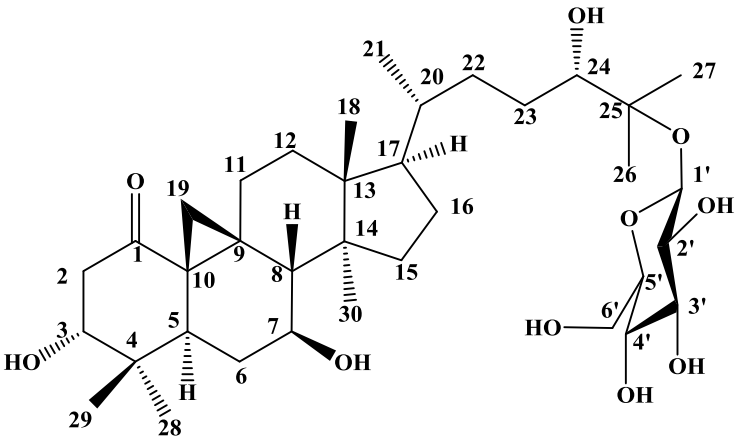


Figure 16: Structure of compound 5

Table 5: ^{1}H- and ^{13}C-NMR data of compound 5 in MeOH (600 MHz)

	Experimental		**Literature (Olivier et al. 2009)**.	
Position	**13C**		**13C**	
1	213.5		213.0	
2	48.9	a: 2.96 dd (14.4, 5.4)	48.7	a: 3.01 dd (14.3, 4.5)
		b: 2.37 dd (14.1,4.5)		b: 2.41 dd (14.3, 4.5)
3	78.4	3.68 t (4.8)	78.8	3.75 t (4.8)
4	40.2		40.1	
5	38.8	2.44 dd (13.5, 3.3)	38.6	2.48 dd (14.0, 3.6)
6	31.0		30.8	
7	69.5	3.68 t (4.8)	69.3	3.75 m
8	51.4	2.08 d (4.2)	51.2	2.12 d (3.6)
9	30.8		30.6	
10	40.6		40.4	
11	29.1	1.86 m	29.0	1.88 m
12	34.0		33.8	1.67(m), 1.53 d (3.0)
13	46.6		46.4	
14	50.6		50.5	
15	34.5	1.36 m	34.4	1.39 td (10.5, 2.1)
16	28.9	1.95 m	28.7	1.90 m
17	53.2	1.64 m	53	1.68 td (9.3, 2.2)
18	15.8	0.98 (s)	15.6	1.01 s
19	24.9	a: 075 d (4.5)	24.6	a:0.78 d (4.5)
		b: 1.43 d (4.5)		b:1.47 d (4.5)
20	37.5	1.47 m	38.0	1.48 m
21	19.2	0.91 d (6.0)	19.3	0.96 d (6.3)
22	34.8	1.95 m	35.1	1.90 m
23	29.1	1.64 m	29.3	1.68 m
24	78.9		79.5	3.36 br d (9.3)
25	81.7		81.9	
26	23.2	1.25 s	23.8	1.29 s
27	21.9	1.21 s	21.3	1.26 s
28	24.9	1.00 s	24.8	1.04 s
29	22.7	0.97 s	21.7	0.97 s
30	19.1	0.98 (s)	18.9	1.01 s
1'	98.2	4.52 d (7.8)	98.6	4.56 d (7.8)
2'	75.5	3.17 br t (8.4)	75.1	3.21 br t (7.8)
3'	78.1	3.28 m	78.1	3.35 t (8.6)
4'	71.8	3.37 m	71.6	3.38 m
5'	77.9	3.28 m	77.7	3.32 m
6'	62.9	a: 3.65 dd (11.7, 5.1)	62.6	a: 3.69 dd (11.9, 5.1)
		b: 3.82 dd (12.0, 1.8)		b: 3.87 dd (11.7, 2.1)

The accurate mass obtained from the HRTOFMS spectra (Figure 17) is similar to that reported in the literature (Fu et al., 2008b; Olivier et al., 2009). The ESI^+ (positive mode) showed a pseudo molecular ion at m/z 653.4265 (calculated mass for $[C_{36}H_{60}O_{10} + H]^+$). The ESI^- (negative mode) showed a pseudo molecular ion at m/z 651.4108 (calculated mass for $[C_{36}H_{60}O_{10} - H]^-$) and a formic acid adduct (calculated mass for $[C_{36}H_{60}O_{10} - H+ HCOOH]^-$) at m/z 697.4163 due to its presence in the source. Elementary analysis performed on the mass ions gave $C_{36}H_{61}O_{10}$, $C_{36}H_{59}O_{10}$ and $C_{37}H_{61}O_{12}$ corresponding to $[C_{36}H_{60}O_{10} + H]^+$, $[C_{36}H_{60}O_{10} - H]^-$ and $[C_{36}H_{60}O_{10} - H+ HCOOH]^-$) molecular formulas and for all the fragments i-FIT values were 0.0, a mass error of -3.9 mDa, -4.2 mDa and 2.5 mDa and the calculated double bond equivalence (DBE) values were 6.5, 6.5 and 7.5. Supplementary information is given in Appendix 6

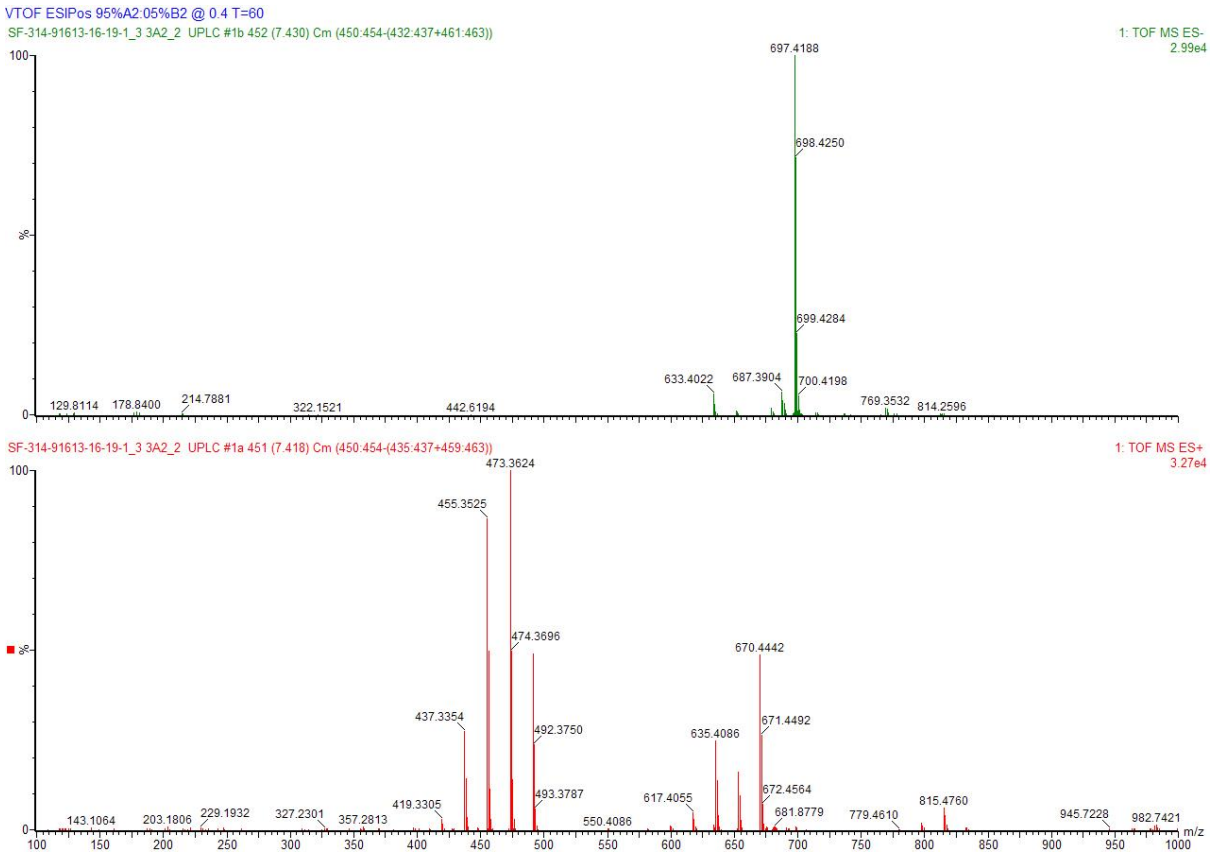


Figure 17: HRTOFMS spectrum (ESI^- and ESI^+) for compound 5

3.3 Comparison of metabolite content of plant extracts.

When the extracts from plants obtained in Paarl were compared with extracts obtained from plants from Petrusburg, the LC-MS elution profiles were similar with noticeable variation as to quantities of some of the metabolites. This is shown in figure 19 below. The profiles of extracts A and B were obtained from Petrusburg and Paarl, respectively while the profiles D and E involved plant material from Paarl and Petrusburg, respectively. Elution profiles A and B involved ethanol extraction while D and E involved water extraction. Comparison of A and B shows that compounds 1, 2, 3 and 4 eluted separately and at higher relative concentration from Paarl plant material (B) relative to the corresponding metabolites from plant material obtained in Petrusberg (A). Conversely, the plant profiles obtained from water extraction from Petrusburg plant material showed an increased diversity and concentration relative to the Paarl plant material.

The metabolites were more visible on the LC-ESI-MS negative mode and only five out of the eight biomarkers published by Avula et al. (2010) could be positively identified on the LC-MS spectra (Figure 19). All the biomarkers isolated from *S. frutescens* are showed on Table 6. Comparison of the electrospray ionisation negative mode with literature is presented on Table 7. One triterpenoid was observed, namely the SU1 (compound 5) and it appeared to be the most dominant metabolite with varied quantities as compare to the other metabolites (see Table 6 below for compound identification). The SU1 elutes at approximately 10 min (Figure 19). The other four metabolites were flavanoids namely sutherlandins A to D. Sutherlandins A and B were found to co-elute at 7.71 min for the Petrusburg extracts and at approximately 7.6 min for the Paarl extracts. Sutherlandins C and D were only observed from the Paarl extracts and they also co-eluted at 6.65 for the 99.9% ethanol and at 7.01 for the aqueous extracts. In the aqueous extracts greater quantities of metabolites were observed in the polar region.

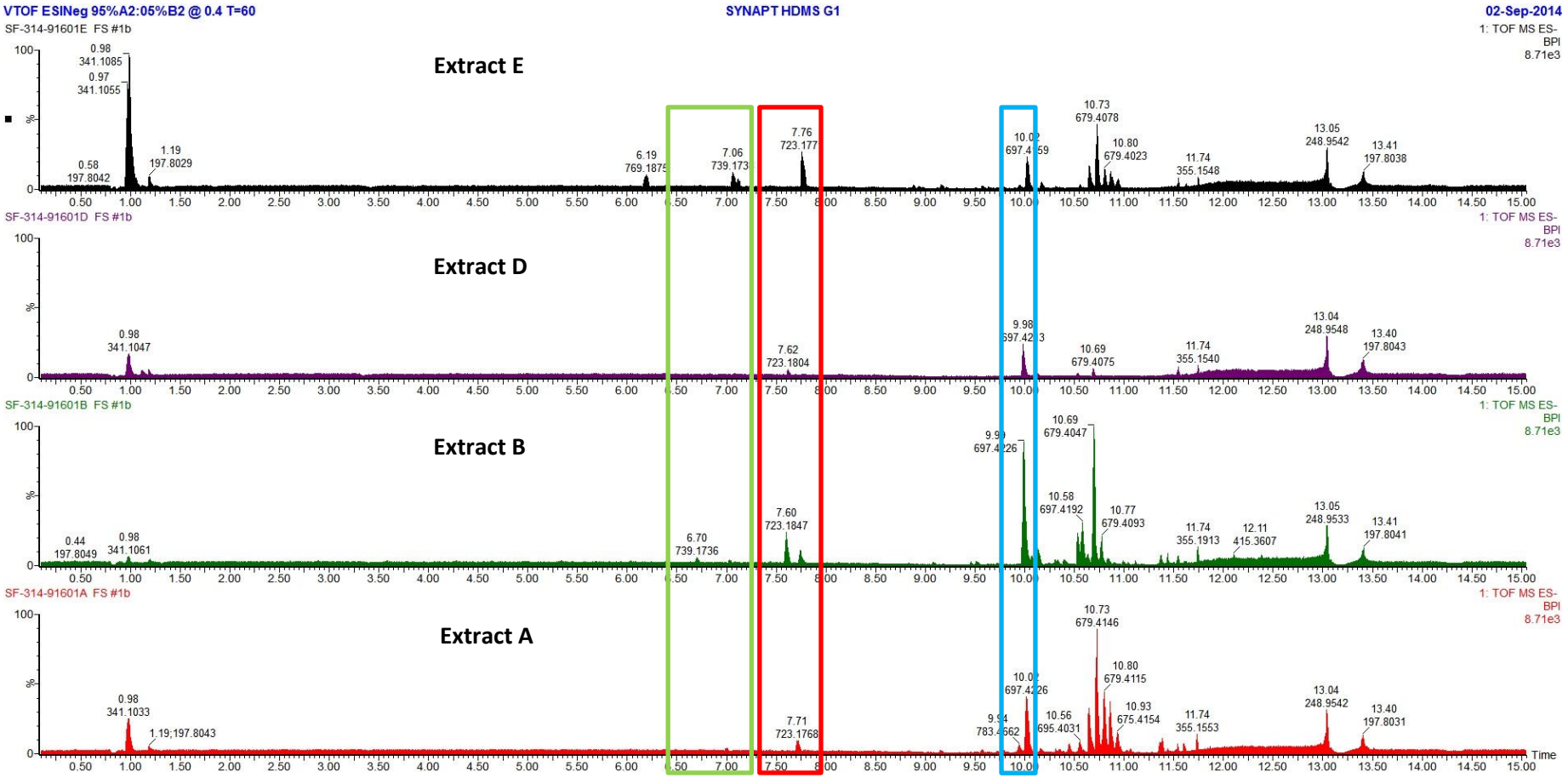
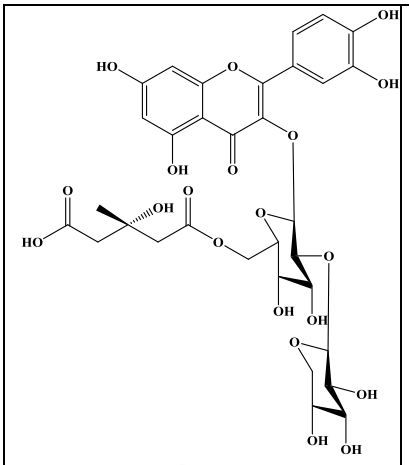
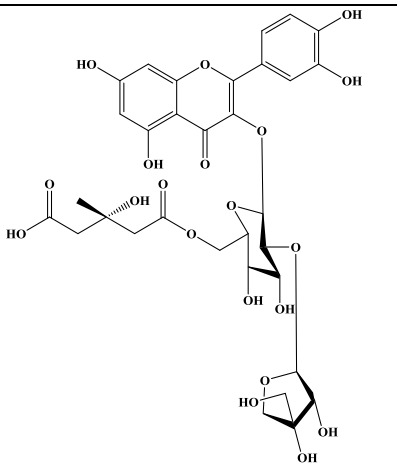
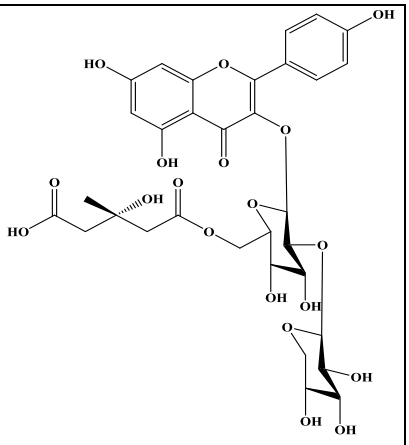
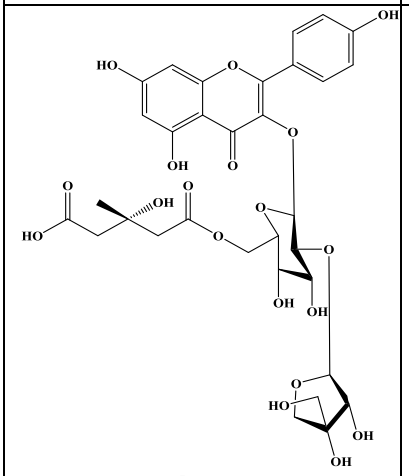
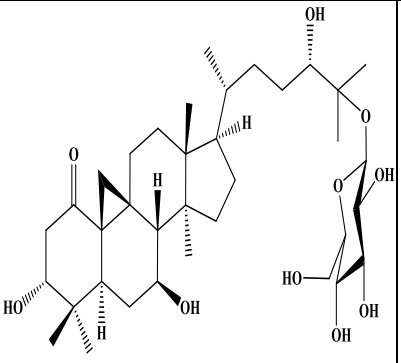
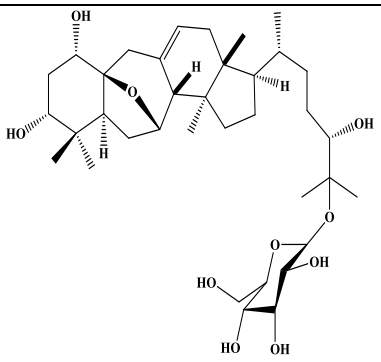
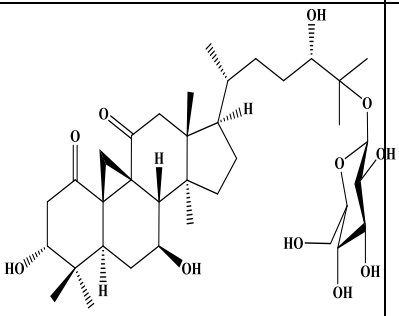
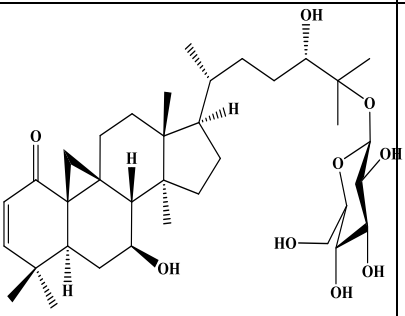
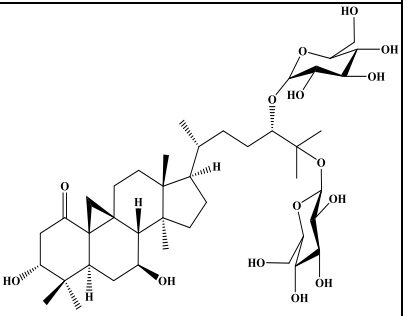


Figure 18: LC-MS metabolite profiles of extracts with the green ring representing compound 1 and 2; red ring representing compound 3 and 4; blue representing compound 5. Extract A & B: 99% ethanol; extract D & E: distilled H_2O.

Table 6: Compound Identification

 **Compound 1:** Sutherlandin A	 **Compound 2:** Sutherlandin B	 **Compound 3:** Sutherlandin C
 **Compound 4:** Sutherlandin D	 **Compound 5:** sutherlandioside B or SU1	 **Compound 6:** sutherlandioside A
 **Compound 7:** sutherlandioside C	 **Compound 8:** sutherlandioside D	 **Compound9:** SU3

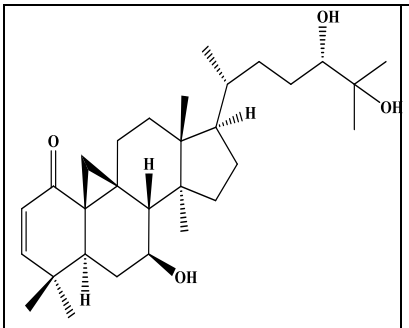
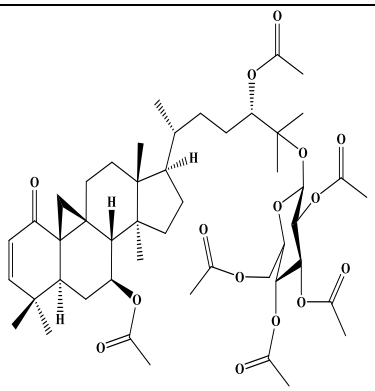
 **Compound 10**	 **Compound 11**	

Table 7: Comparison of the UPLC-MS data with literature (Avula et al., 2010b).

Extract	Compound	ES^-	ES^+	Accurate mass ES^-	Molecular Formular
A	3 and 4	723.1745 $[M-H]^-$, 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$, 747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$, 747.1791 $[M+Na]^+$	723.1773 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5	651.4108 $[M-H]^-$, 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$, 653.4223 $[M+H]^+$	697.4215 $[M-H+ HCOOH]^-$ $C_{37}H_{61}O_{12}$	$C_{36}H_{60}O_{10}$
B	1 and 2	739.1705 $[M-H]^-$ 739.1725 $[M-H]^-$	741.1887 $[M+H]^+$, 763.1739 $[M+Na]^+$ 741.1941 $[M+H]^+$, 763.1749 $[M+Na]^+$	739.1722 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	3 and 4	723.1745 $[M-H]^-$ 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$, 747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$, 747.1791 $[M+Na]^+$	723.1816 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5 and 11	651.4108 $[M-H]^-$, 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4197 $[M-H+ HCOOH]^-$	$C_{36}H_{60}O_{10}$
C	1 and 2	739.1705 $[M-H]^-$ 739.1725 $[M-H]^-$	741.1887 $[M+H]^+$, 763.1739 $[M+Na]^+$ 741.1941 $[M+H]^+$, 763.1749 $[M+Na]^+$	739.1803 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	3 and 4	723.1745 $[M-H]^-$ 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$, 747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$, 747.1791 $[M+Na]^+$	723.1835 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4152 $[M-H+ HCOOH]^-$ $C_{37}H_{61}O_{12}$	$C_{36}H_{60}O_{10}$
D	3 and 4	723.1745 $[M-H]^-$ 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$, 747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$, 747.1791 $[M+Na]^+$	723.1780 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4222 $[M-H+ HCOOH]^-$ $C_{37}H_{61}O_{12}$	$C_{36}H_{60}O_{10}$
E	1 and 2	739.1705 $[M-H]^-$ 739.1725 $[M-H]^-$	741.1887 $[M+H]^+$, 763.1739 $[M+Na]^+$ 741.1941 $[M+H]^+$, 763.1749 $[M+Na]^+$	697.4157 $[M-H+ HCOOH]^-$ $C_{37}H_{61}O_{12}$	$C_{36}H_{60}O_{10}$
	3 and 4	723.1745 $[M-H]^-$ 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$, 747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$, 747.1791 $[M+Na]^+$	723.1782 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4155 $[M-H+ HCOOH]^-$	$C_{36}H_{60}O_{10}$
A4	3 and 4	723.1745 $[M-H]^-$ 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$,747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$,747.1791 $[M+Na]^+$	723.1757 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4180 $[M-H+ HCOOH]^-$	$C_{36}H_{60}O_{10}$

A3	3 and 4	723.1745 $[M-H]^-$, 723.1784 $[M-H]^-$	725.1918 $[M+H]^+$, 747.1773 $[M+Na]^+$ 725.1961 $[M+H]^+$, 747.1791 $[M+Na]^+$	723.1765 $[M-H]^-$ $C_{36}H_{35}O_{19}$	$C_{36}H_{36}O_{19}$
	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4194 $[M-H+ HCOOH]^-$	$C_{36}H_{60}O_{10}$
A2	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4158 $[M-H+ HCOOH]^-$	$C_{36}H_{60}O_{10}$
Fraction 1	5	651.4108 $[M-H]^-$ 697.4188 $[M-H+ HCOOH]^-$	653.4257 $[M+H]^+$, 675.4117 $[M+Na]^+$ 653.4223 $[M+H]^+$	697.4133 $[M-H+ HCOOH]^-$	$C_{36}H_{60}O_{10}$

3.4 Conclusion

Sutherlandia frutescens plant material was collected from two different regions for comparison, namely from Petrusburg and Paarl. Two different extraction methods (water and ethanol) were performed and five extracts (extract A-B) were obtained. Extract A was then subjected to further purification. A novel compound (compound 10), known compound (compound 5) and synthesised compound (compound 11) were isolated and characterised using chromatographic techniques and the chemical structures were confirmed by NMR and UPLC-MS. Compound 10 was isolated from the DCM layer and shown to be an aglycone-type of cycloartane triterpenoid in contrast to the other two cycloartane glycosides (compound 5 and 11) isolated from the emulsion layer. Compound 11 was an acetylated cycloartane glycoside.

The LC-MS data showed similar metabolic profiles of the two regions with varied quantities of metabolites. The profile obtained from the Paarl material extracted with ethanol showed higher quantities in comparison to the Petrusburg extracts whereas the profile of the Petrusburg material extracted with distilled water had more compounds at higher concentrations than the Paarl water extract. Only five out of the eight biomarkers published by Avula et al. (2010) could be positively identified on the LC-MS spectra namely SU1 (compound 5) and sutherlandins A to D. The above metabolites were more visible on the LC-ESI-MS negative mode.

CHAPTER 4: PRELIMINARY BIOLOGICAL STUDIES

4.1 Cell viability assay

Sutherlandia frutescens gained its popularity in Africa due to the relatively long history of its usage. However, it is also known to possess side effects such as dry mouth, mild diarrhoea, mild diuresis and dizziness in cachectic patients (Gericke et al., 2001; Mills et al., 2005b). The recommended dose is two *Sutherlandia* tablets containing 300 mg (dried leaf powder) per day (Seier et al., 2002). The current assay evaluates the cell viability of aqueous extracts and ethanol extracts obtained from *S. frutescens* plant material collected at two different locations.

4.1.1 Methodology

The cell viability assay was conducted at the CSIR Biosciences, Molecular Biology Platform and screened by Dr Lindiwe Thete. To evaluate cytotoxicity of the plant compounds, $1x10^4$ Vero cells/well were seeded in a 96 well plate for 24 hours to obtain 90% confluence. Thereafter, the growth medium was removed and the cell monolayer was washed twice with phosphate buffered saline, pH 7,4 (PBS). Each cell monolayer was then treated with an aliquot of 100 ug/ml of a plant extract benchmarked against a positive control (emetine) known to inhibit eukaryotic protein synthesis (Dimitrijevic and Duncan, 1998) included in duplicate wells at a concentration at 50 ug/ml. The plate was then incubated for 3 days to evaluate potential cytotoxicity. Cell viability was measured by using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium salt (MTS) assay kit (Promega Corporation, Madison, USA) according to the manufacturer's instructions. In this assay, the compound tetrazolium is converted into a blue formazan dye by metabolically active mitochondria of viable cells (Mosmann, 1983). At the end of the incubation period, cells were incubated with MTS solution for 2 h, and the absorbance was measured at 492 nm using a spectrophotometric microtitre plate reader (Infinite, F500, Tecan Group Ltd, Mannedorf, Switzerland). The cell viability was expressed as a percentage relative to the control using the following equation:

Cell viability (%) = 100* (ODcmp/ODcntrl)

where OD_{cmp} is the optical density of the test compound and OD_{cntrl} of the control (untreated cells).

4.1.2 Results

Extracts were toxic to the cells when initially tested for antiviral activity at a concentration of 1 mg/ml (data not shown). The concentration of the extracts was then reduced to 100 ug/ml. Two compounds (extracts A and B) showed similar levels of cytotoxicity as shown by the control cells treated with emetine. The remaining compounds (extracts C, D and E) were not cytotoxic (Figure 20).

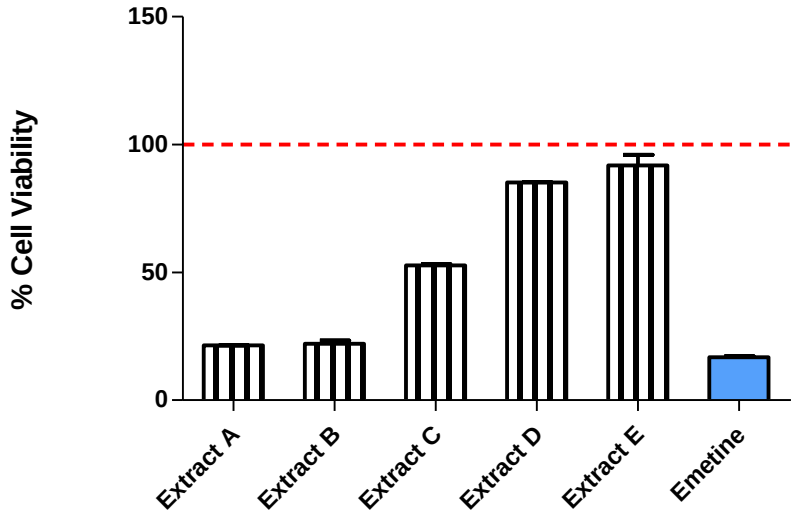


Figure 19: Cytotoxicity of compounds at 100 ug/ml on Vero cells. Data expressed relative to control (untreated cells), mean, st dev, n=3. Extract A & B: 99% ethanol; Extract C: 70% ethanol; Extract D & E: distilled H_2O.

From the above figure it is evident that aqueous extracts (extracts C, D and E) appear not to be toxic when compared to the cytotoxic effects of 99% ethanol extracts (A and B). The toxicity of the latter two compounds was similar to that of the included emetine control. The toxicity of extract C (extracted with 70% ethanol) is

intermediate between the cytotoxicity of extracts A and B and extracts D and E. This confirms that cytotoxicity appears to decrease from non-aqueous to aqueous which might mean that the non-polar compounds are more likely to be toxic compared to the polar compounds.

4.2 *In vitro* antiviral assay

Herpes simplex virus (HSV) is an epitheliotropic virus infectious to children and adults (Danaher et al., 2011; Smith & Robinson, 2002). During infection, HSV replicates and destroys cells at the portal entry and infection found in the epithelial cells results in de-envelopment, rapid replication and spread to adjacent cells and nerve endings (Danaher et al., 2011; Zhou et al., 2013). In this study the antiviral potential of *S. frutescens* was evaluated by screening plant extracts against HSV-1.

4.2.1 Methodology

4.2.1.1 Solubility of compounds

The solubility profiles and sample identification is presented on Table 1 below. All compounds were dissolved and sonicated for an hour prior to experimental execution.

Table 8: Sample identification and solubility profile of compounds

Extract number	**Extract type**	**Final concentration of solvent used**	**Solubility**
SF-314-91601A	100% Ethanol	2.5% Ethanol	Partly
SF-314-91601B	100% Ethanol	2.5% Ethanol	Soluble
SF-314-91601C	70% Ethanol	1.75% Ethanol	Soluble
SF-314-91601D	H_2O	H_2O	Soluble
SF-314-91601E	H_2O	H_2O	Soluble

4.2.1.2 Antiviral assay using Herpes simplex virus 1

The antiviral assay was conducted at the CSIR Biosciences, Molecular Biology Platform and screened by Dr Lindiwe Thete. Plant extracts were evaluated for antiviral activity using a cytopathic inhibitory method previously described by Schmidtke et al., (2001) to test the potency of plant extracts against herpes simplex virus 1. Briefly, Vero cells (African green monkey kidney cell line, Cat No. CCL-81) purchased from ATCC (Manassas, VA) were seeded at $0.5x10^5$ cells/well in 12 well plates for 24 hours to obtain 80%-90% monolayer confluence. The culture media (199, Lonza, Cat no. BE12-117F) was supplemented with 10% fetal bovine serum (The Scientific Group, Cat. No. BC/S0615-HI, South Africa), 100 U/ml penicillin, and 100 ug/ml streptomycin was removed and the monolayer was washed three times with PBS, pH 7,4 (Lonza, Cat. No. BE12-516F). Then herpes simplex 1 MacIntyre strain (ATCC, Cat No. VR-539) was allowed to adsorb to the cells for 1 hour at a multiplicity of infection of 0.05 particle forming units. Then, unadsorbed virus was removed and each of the cell monolayers was treated separately with 100 ug/ml of extracted compounds. The positive control used in this assay was acyclovir at a concentration of 5 ug/ml, a drug that is used to treat symptomatic herpes simplex virus infection (Whitley & Gnann, 1992). The plate was then incubated for 5 days to allow for the formation of virus-cytopathic effects (CPE). Any CPE were measured by using an MTS assay kit (Promega Corporation, Madison, USA) according to the manufacturer's instructions and as described above.

The percentage of CPE inhibition was calculated by subtracting the mean value of virus-infected cell control (0%) from the measured absorbance, and the resulting number was divided by the measured absorbance of uninfected cell control (100%) using the equation below:

CPE inhibition (%) = 100* [(ODcmp- ODvc/ODcmp)/ ODcc]

where OD_{cmp} is the optical density of the test compound, OD_{vc} of the virus control and ODcc of cell control (untreated).

4.2.2 Results

4.2.2.1. Antiviral activity

None of the compounds showed significant activity (CPE inhibition >50%) against herpes simplex 1 virus (Figure 21).

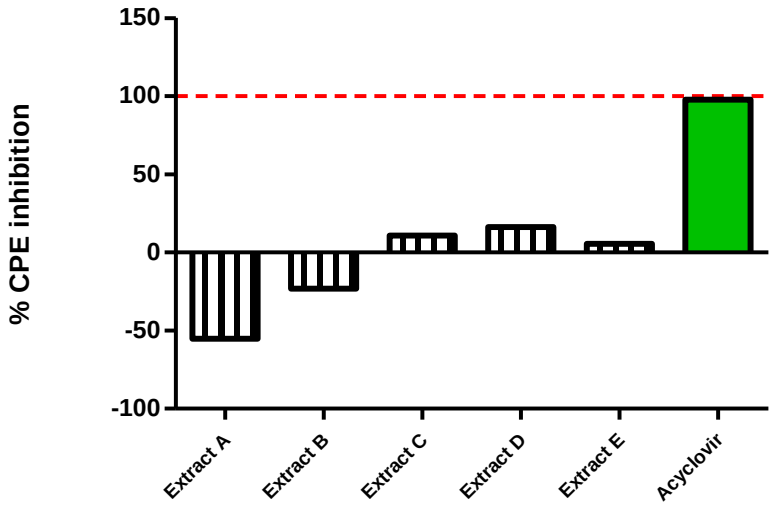


Figure 20: Antiviral activity of compounds (100 ug/ml) and acyclovir (5 ug/ml) tested against Herpes simplex virus 1. Data expressed relative to virus control. Extract A & B: 99% ethanol; extract C: 70% ethanol; extract D & E: distilled H_2O.

The results presented in figure 21 correlate to an extent to the results of the cytotoxicity screening performed on the same extracts (section 5.1), where extract A and B were highly cytotoxic, similar in activity to the positive control (Figure 20). The results of the experiment indicated values below zero after treating the cells and viruses with the two extracts (A and B). This suggests that the two extracts had a cytocidal effect rather than an effect on virus replication (data not shown).

The aqueous extract D provided limited protection against HSV infection around 20% compared to the acyclovir control and this warrants further analysis, particularly by testing cell protection against HSV-1 in various concentrations of this extract.

4.2.3. Conclusions and recommendations

None of the extracts showed acceptable antiviral activity. Figure 21 above showed that extracts A and B do not appear to have antiviral activity but, rather, may well contribute to cytotoxicity as was shown in figure 20. Also the concentration of compounds in extracts is lower than the positive control which might be a contributing factor to the loss of activity. In comparison to the acyclovir control, a relatively minor antiviral effect was shown by extracts C, D and E with extract D showing around 20% anti-HSV when compared to the control. A future, closer investigation of the plant extraction process, including a determination of the concentration of each of the extracts, into the antagonistic and/or synergistic effects of the extracts relative to effect on virus replication might provide further insights into the antiviral activity of *S. frutescens* extracts.

4.3 Immunomodulation assay

The immune-potentiating ability of compounds isolated from *Sutherlandia frutescens* was assessed using a flow cytometry-based, multiplex cytokine bead assay (CBA) system. The BD CBA Human Th1/Th2/Th17 Cytokine kit was employed as it has more sensitive than the conventional ELISA kit and, together with flow cytometry, allows the researcher to rapidly quantify the expression of multiple cytokines from single samples in the presence of plant extracts and to generate a standard curve for each analyte. The current study investigated the following interleukins (IL): IL-6 and IL-10 and tumour necrosis factor alpha (TNF-α).

4.3.1 Materials and methods

4.3.1.1 Blood sample preparation

To test the effects of extracts of *S. frutescens* on the activity of immune cells, plant extracts were incubated with aliquots of whole blood and then tested for the expression of cytokines. Briefly, aliquots of whole blood (125 µl) were separately introduced into a 15 ml conical polypropylene (PP) tube and then 100 µg/ml of sample (plant extract, fraction or pure compounds) dissolved in 0.1% DMSO were added to each separate tube followed by growth medium (RPMI 1640) to make a final volume of 1250 µl. A positive control was included in these experiments and consisted of 0.3 µg/ml phytohaemagglutin A (PHA). Negative controls consisted of unstimulated controls containing the same amount of blood as other samples followed by the addition of an equal volume of solvent that had been used to dissolve the plant extract samples. Then, RMPI 1640 was added to the experiment cells and the control tubes to make up a volume of 1250 µl. The tubes were incubated at 37℃ in the presence of 5% carbon dioxide (CO_2) and at 100% relative humidity for 3 days. On day 4, 500 µl of supernatant medium was removed from each tube and transferred into separate labelled Sarsted tubes and stored at -80℃.

To harvest the cells, 2 mM EDTA was used and 15 ml sterile polypropylene Sterilin tubes were vortexed and incubated at room temperature (RT) for 10 min before being centrifuged at 450 *g* (1400 rpm) for 7 min at room temperature and the

supernatant decanted. This was followed by the addition of 5 ml of phosphate buffer saline (PBS) to the pellet. Then the tubes were centrifuged again and the supernatant discarded. For cell (white blood cells) staining, a fixable viability dye was used, vortexed and incubated at room temperature for 20 minutes in the dark after which all steps were performed in the dark. Then 5 ml of PBS with 1% fetal calf serum was added into the tubes followed by centrifugation at 450 *g* (1400 rpm) for 7 minutes at RT and the supernatant was discarded. Blood samples were lysed with 1X FACSlyse solution in a ratio of 1:10 to the sample volume and then the tubes were vortexed well and incubated for 10 min at room temperature (RT). The tubes were then centrifuged at 600 *g* (1600 rpm) for 7 min at RT followed by decanting of the supernatant. Cell pellets were re-suspended in 0.5-1 ml cryo-solution and transferred into appropriately labelled cryovials. These tubes were then transferred into Mr Frosty containers and stored in a -80℃ freezer.

4.3.1.2 Cytokine determination

This assay was performed according to the BD CBA Cytokine Kit Assay procedure. A series of 0 to 5000 pg/ml standards were prepared by serial dilution in order to generate standard curves. Then 50 µl of mixed captured beads, 50 µl of the human Th1/Th2/Th17 Cytokines Standard dilutions and 50 µl human Th1/Th2/Th17 Phycoerythrin (PE) detection reagents were added consecutively into each sample tube and these were incubated for 3 h at room temperature in the dark. After incubation, each sample was washed with 1 ml of wash buffer followed by centrifuging at 200 *g* for 5 minutes. The supernatant was discarded and 300 µl wash buffer was added to each tube to resuspend the bead pellet.

The experiment involving the flow cytometric analysis of the cytokines was performed in duplicate using a BD LSR Fortessa^{TM} flow cytometer at the University of the Witwatersrand. Assistance was kindly provided by Mrs Patti Kay. This flow cytometer was set up using cytometer setup beads (cat: 51-9005731; 1.5 ml) to obtain a target mean fluorescent intensity (MFI) of 72 for Allophycocyanin (APC), 69 for PE and 71 for Fluorescein Isothiocyanate (FITC). Data were acquired using BD

FACSDiva software following which fcs files were transferred to FCAP Array (version 3) software for analysis.

4.3.2 Results and discussion

Preliminary studies were conducted to assess any immune potentiation by *S. frutescens* extracts, fractions and compounds. It was noted that some of the fractions and compounds only partially dissolved in 0.1% DMSO – these included extracts A3, A3Ac, B and Fraction 1, each at a concentration of 100 ug/ml. Blood samples showed *de novo* elevated concentrations (pg/ml) of IL-6, IL-10 and TNF-α (Figure 22). The elevation of the TNF-α confirms an observation reported by Estcourt et al., (1997) where a comparison was made between healthy blood donors and HIV-infected patients, and where the healthy donors had high concentration of TNF-α. TNF-α and IL-6 have pro-inflammatory and pro-atherosclerotic actions that induce IL-10 which acts as an anti-atherosclerosis factor and as well as a pro-inflammatory inhibitor. This then suggests that cytokines may be counterbalanced by inhibitors or other cytokines with opposing effects (Stenvinkel et al., 2005). The test subject in the current study tested negative for HIV.

4.3.2.1 Analysis of figure 22.

IL-6 analysis:

There was not a large increase in IL-6 expression when comparing the positive control with the negative controls. Thus, most of the samples showed little or no increase (immunopotentiation) or decrease (suppression) in IL-6 expression (Figure 22A). However, extracts C and D and compounds 10 and 11 prompted a relative increase in IL-6 expression while Fraction 1 and, to a lesser extent, compound 5 showed a reduced IL-6 expression compared to the controls.

IL-10 analysis:

Figure 22B shows widespread suppression of IL-10 expression as indicated by the addition to the blood cells of many of the samples (extracts, fractions and

compounds. The effect of addition of samples can be divided into three groups according to their ability to effect IL-10 expression: Fraction 1 and compound 10 are effective suppressors of IL-10 expression; extracts A and B, extract A3 and A3 Ac and compound 5 and compound 11 show intermediate suppression of IL-10 expression; significantly, extracts C, D and E appear to show an immunopotentiation effect.

TNFα analysis:

Compared to the control, most of the 11 test samples showed an immunosuppressive effect. Only two (extract A and compound 10) showed no impact on the expression of TNFα as compared to the controls. In determining relative immunosuppressive effects, four samples (extract B, extract A3 and A3 Ac, and fraction 1) appeared to exert the greatest suppressive effect on TNFα expression while the remaining 5 samples showed an intermediate effect on TNFα suppression.

Figure 22C showed a relative increase in suppression of TNF-α compared to the suppression of IL-10 and this suppression was significantly greater than suppression of IL-6. The only really consistent result was that fraction 1, and possibly compound 5, inhibited the expression of all three of these cytokines.

In themselves, these results don't appear to be very informative. However, the regulation of expression of interleukins and factors associated with control mechanisms of the immune system is immensely complicated. To make sense of the results of this part of this study perhaps requires correlation between the dynamics of interleukin expression and specific clinical data. Such a correlation between *in vitro* serological results and the clinical picture may provide the key to insight into the immune response and contribute to a meaningful discussion of results such as these.

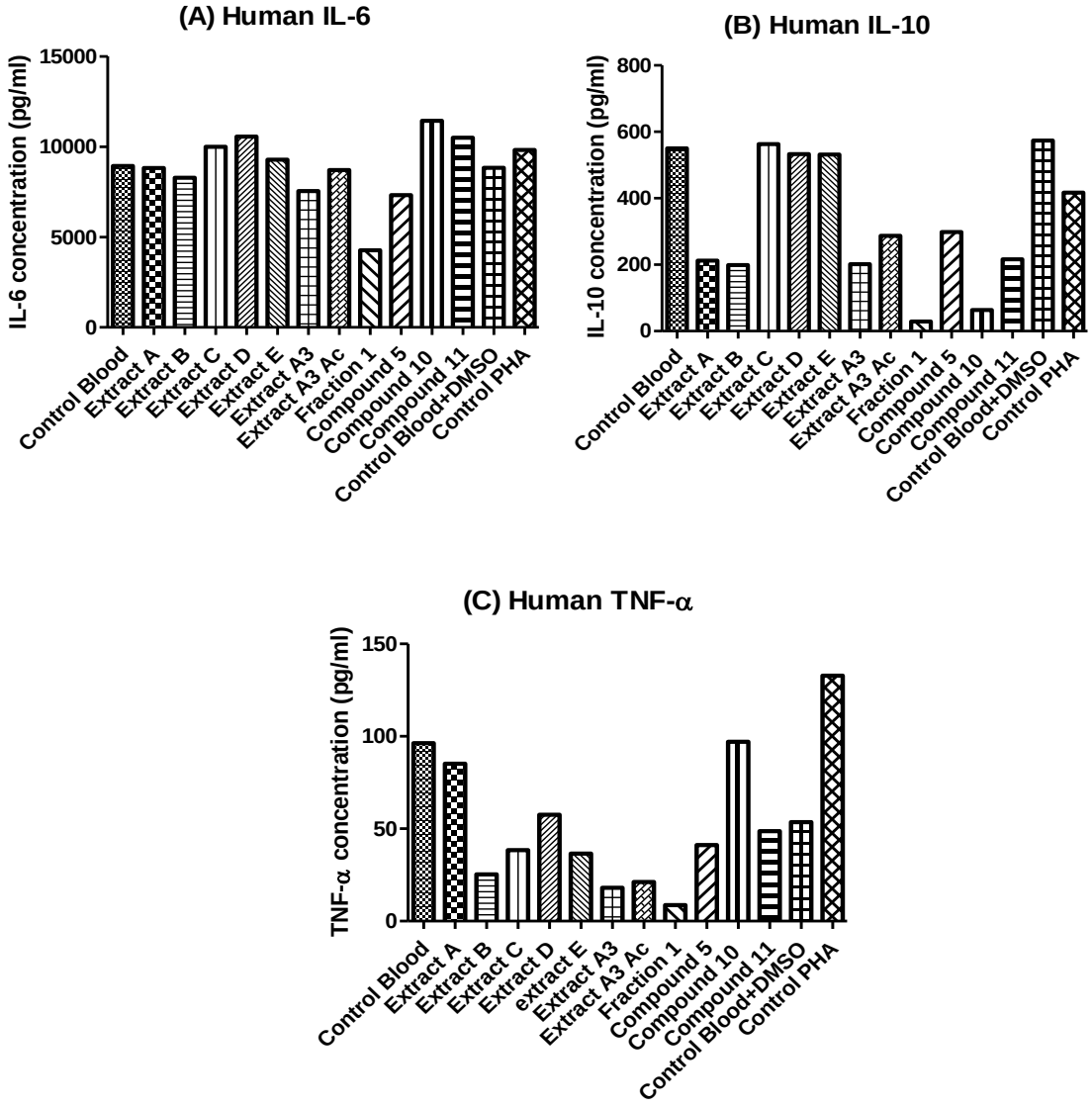


Figure 21: Flow cytometric evaluation of IL-6, IL-10 and TNF-α. Extract A & B: 99% ethanol; extract C: 70% ethanol; extract D & E: distilled H_2O; extract A3: emulsion layer; extract A3 Ac: acetylated emulsion layer.

4.3.3 Conclusion

At a point in the homeostasis of any cell, differentiated or not, a dynamic profile of cytokines should be evident that reflects gene expression within the cell at that point in time. This profile should adjust according to changes in homeostasis and to the cellular macro- and microenvironment, be these changes in response to normal physiology or due to pathology.

Of the three cytokines analysed in detail, IL-6 showed consistently high levels of expression except when in the presence of fraction 1, and to a lesser extent, compound 5. The plant compounds had a much greater impact on the other two cytokines, particularly TNFα, and their action appeared to be directed at reducing expression of these cytokines. These cytokine profiles might result in a counterbalancing of cytokines by other cytokines or inhibitors (Stenvinkel et al., 2005).

The study did not provide sufficient evidence to support immune potentiating effects associated with *de novo* expression of cytokines in the blood cells. An in-depth analysis of the three cytokines needs to be performed to assess any such relationship between the plant extracts, fractions or compounds and the dynamics of cytokine expression/repression. Variation in data from flow cytometric analysis of the cytometric profiles against plant sample treatment might be attributed to partial solubility of some of the plant samples, the differences in the extraction methods used for each extract and, thus, differences in the polarities of the extraction solvents. The other confounding factor is the ability or otherwise to correlate the results obtained in these *in vitro* experiments: plant extracts, fractions or compounds may be immunopotentiating *in vivo* regardless of the observed *de novo* expression of cytokines in the experimental blood cells.

CHAPTER 5: GENERAL CONCLUSIONS

Different extraction methods were perform using absolute ethanol (extract A and B), 70% ethanol (extract C) or distilled H_2O (extract D and E) in which only two extraction methods were used for plant material obtain in Paarl as compared to three extraction methods used for Petrusburg due to the amount of material obtained.

Extract A (10 g, absolute ethanol extract from Petrusburg) was subjected to further purification using liquid-liquid partitioning, TLC and column chromatography with a variety of solvents (MeOH, distilled H_2O hexane, DCM and EtOAC). One novel, one known and one synthesised compound was isolated and characterised using chromatographic techniques and the chemical structures were confirmed by NMR and UPLC-MS.

Preliminary biological studies were conducted to assess the activity of extracts, fractions or compounds. Cell viability studies showed that the aqueous extracts were not toxic in comparison to the ethanol extracts which were highly toxic. Extract C confirmed the observation that cytotoxicity decreases from non-aqueous to aqueous which might mean that the non-polar compounds are more likely to be toxic compared to the polar compounds. *In vitro* antiviral studies also confirmed the results obtained in the cell viability studies and extracts A and B had no antiviral effect and might contribute to cytotoxicity. *De novo* elevation of three cytokines was observed in the immune modulation assay. Of all of the cytokines tested, three cytokines - Interleukin 6 (IL-6), Interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF-α) – showed elevated expression. A great variation in the cytokine profiles of the samples was also observed from the flow cytometric analysis. This variation suggested that extracts from *Sutherlandia* may be immunopotentiating regardless of the *de novo* expression of cytokines in the blood cells.

CHAPTER 6: RECOMMENDATIONS

Further studies involving the purification, isolation and characterisation of bioactive compounds from all the extracts need to be conducted. This may be followed by an in-depth analysis of cytokines to confirm the results obtained above and assess any relationship between the plant extracts, fractions and compounds, and also the dynamics of cytokine expression/repression. It may be useful to perform a study that involves a meta-analysis of published data regarding the expression/suppression of cytokines in cells and to develop software that may be used to statistically determine relationships linking variously reported cytokine expression data so as to provide insights into the dynamics behind cytokine expression/repression.

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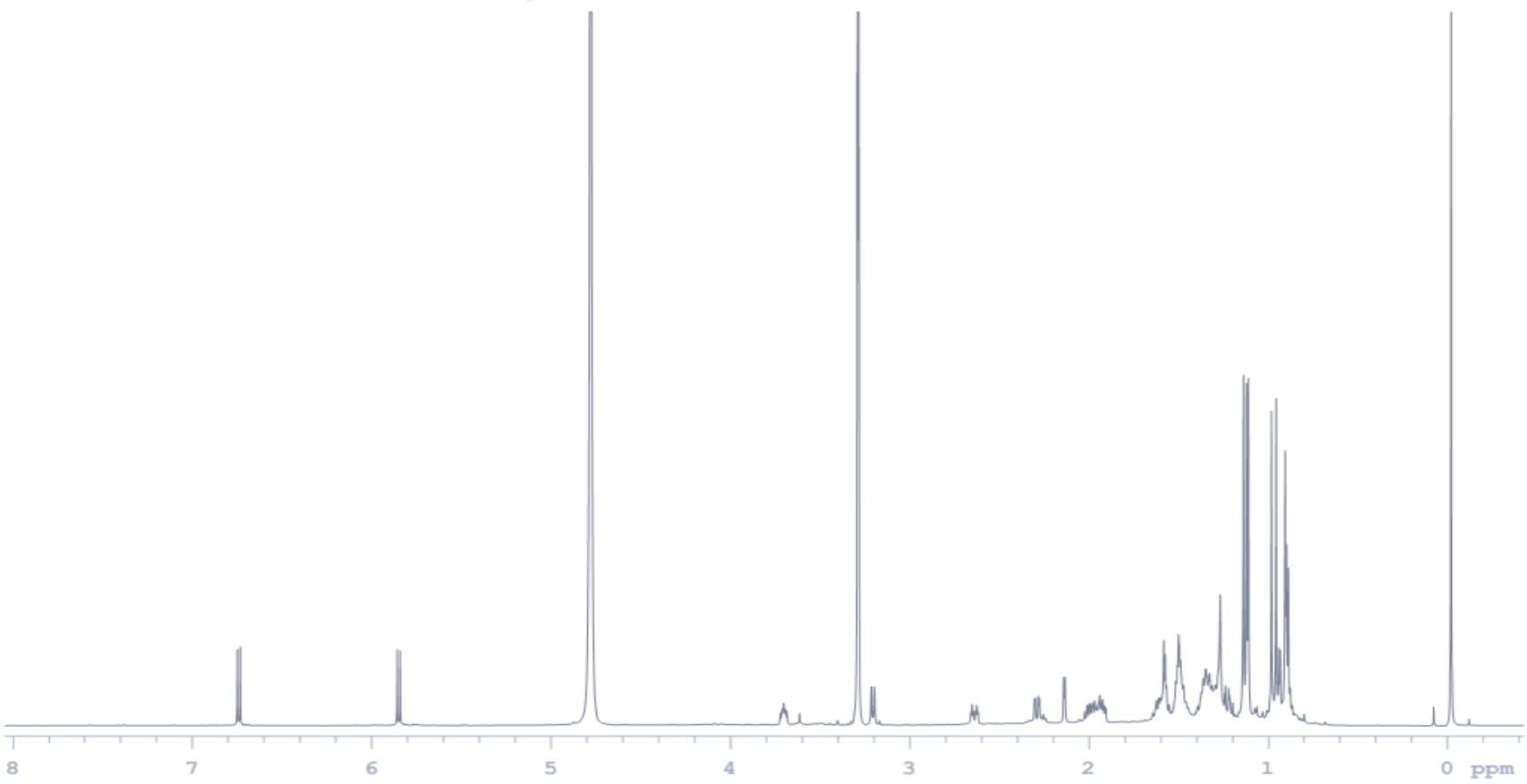
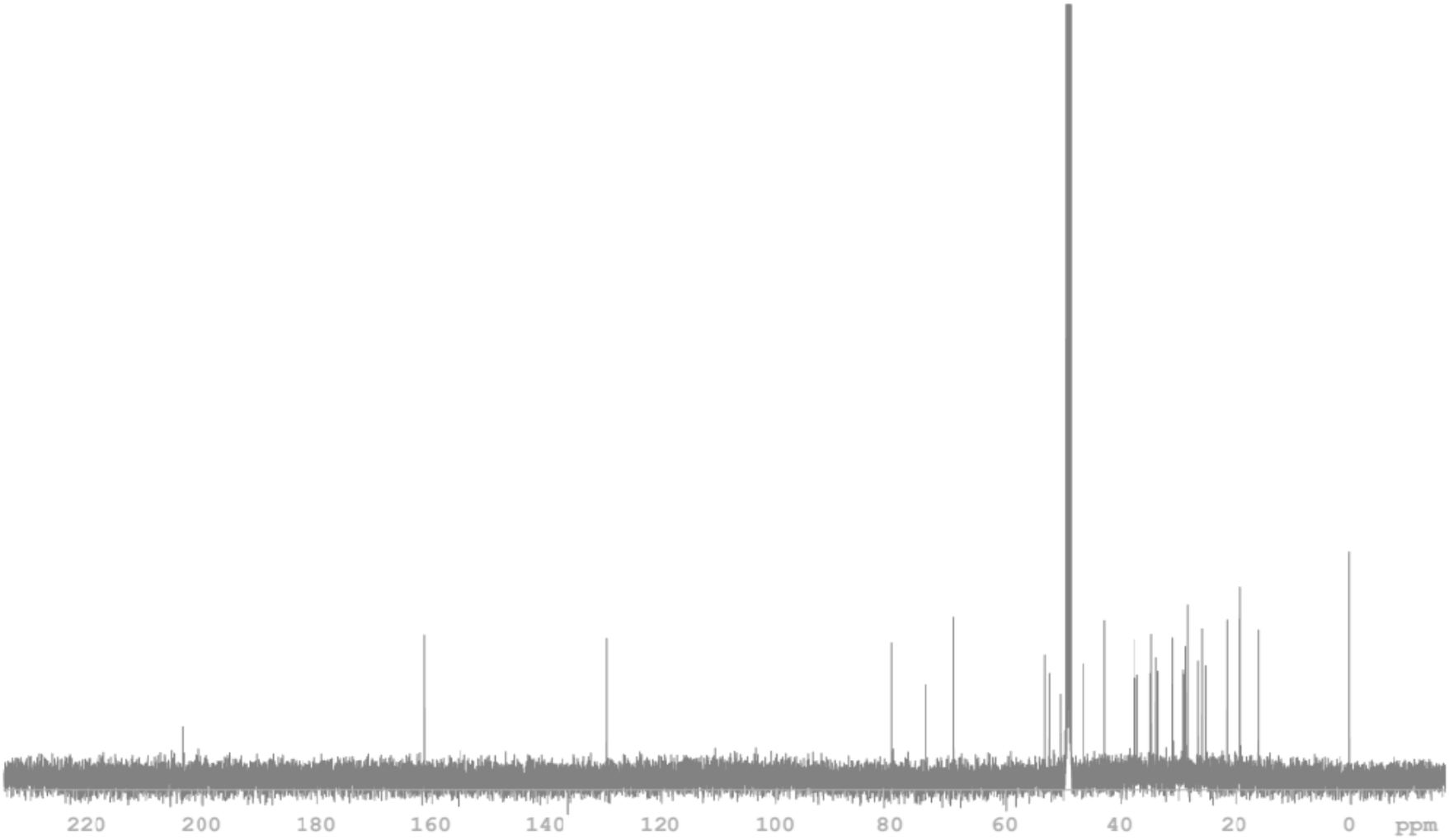
APPENDICES

APPENDIX 1

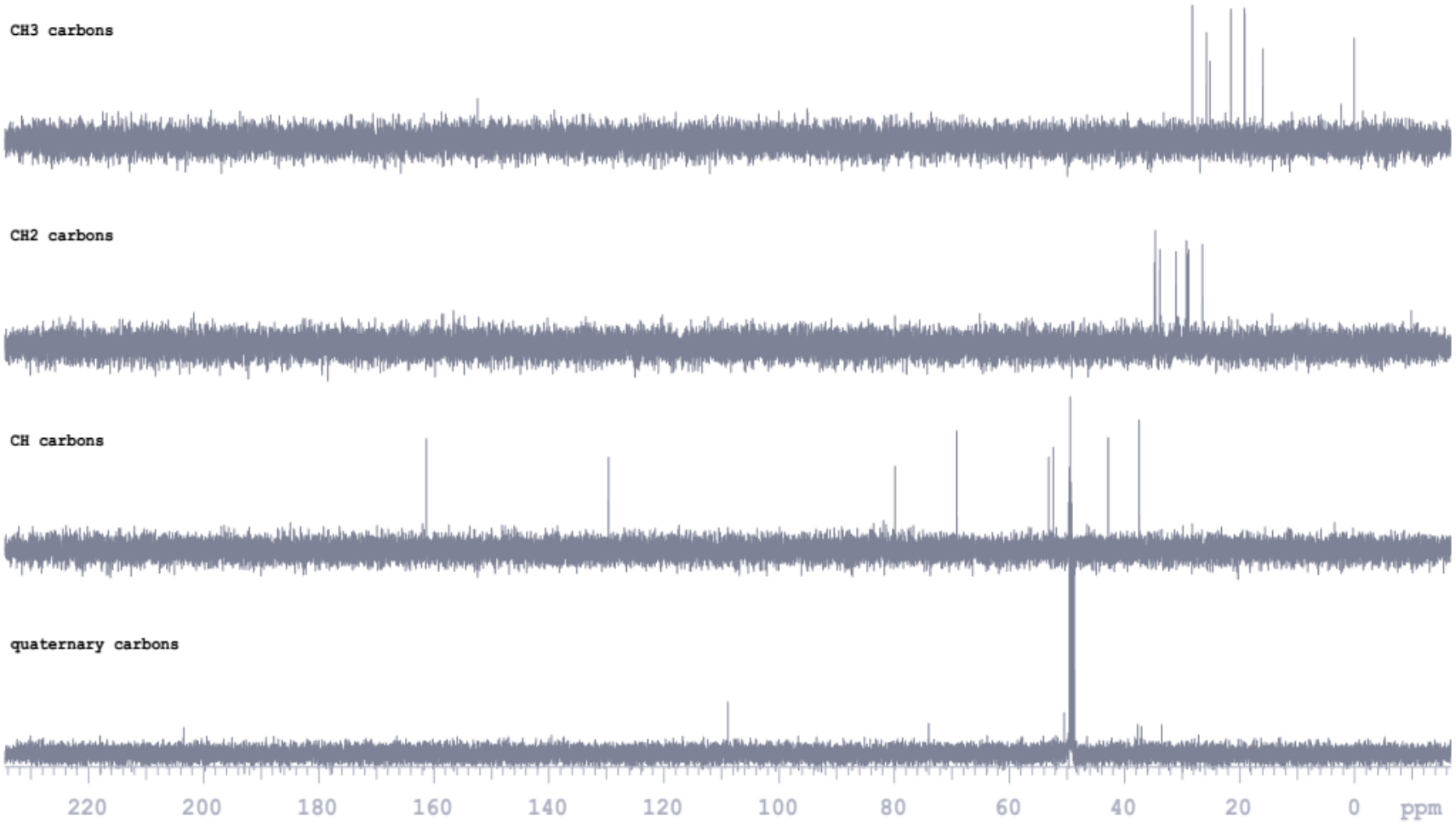
1.1 Compound 10 ^{1}H-NMR



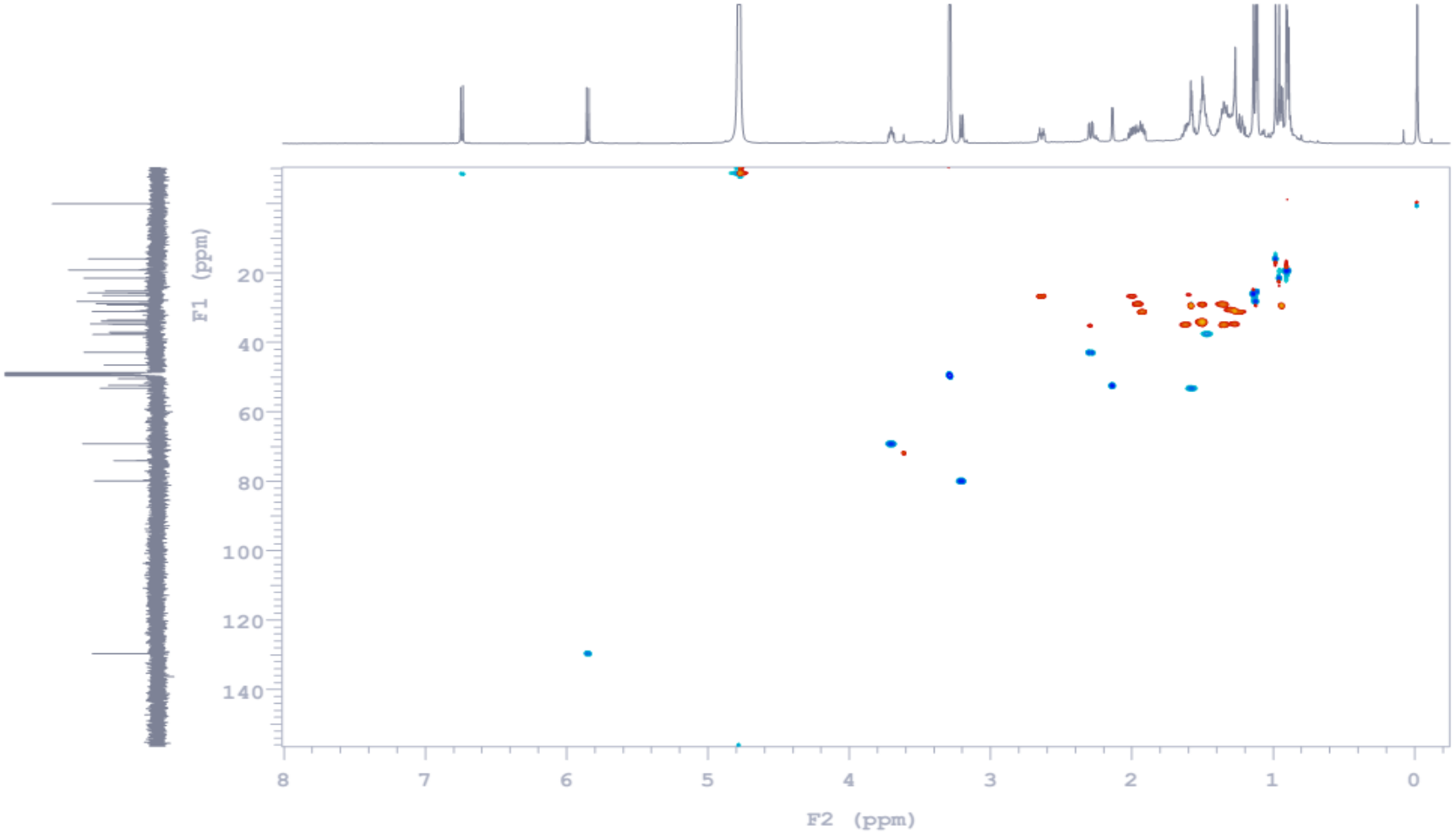
1.2 Compound 10 ^{13}C-NMR



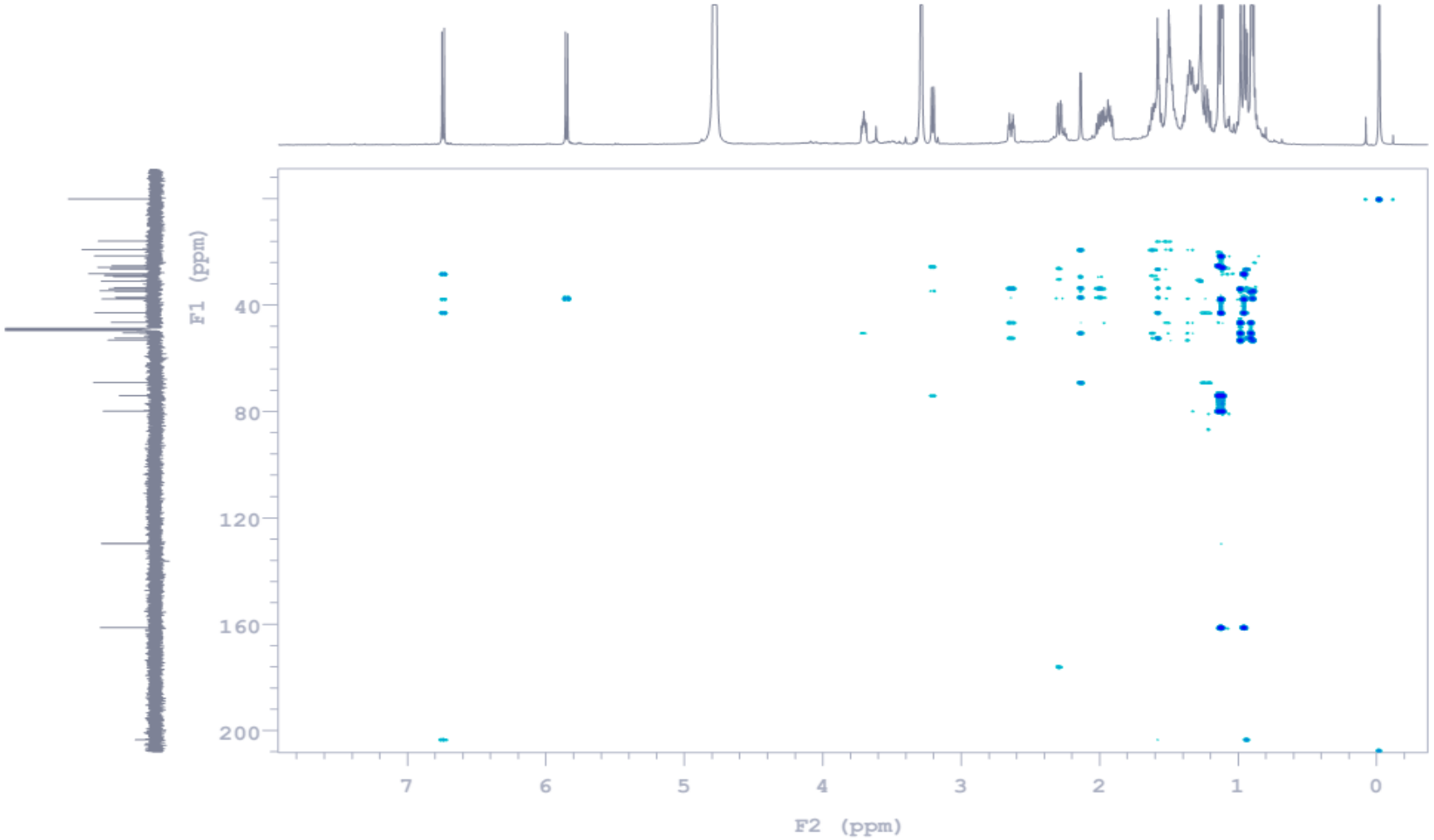
1.3 Compound 10 Dept



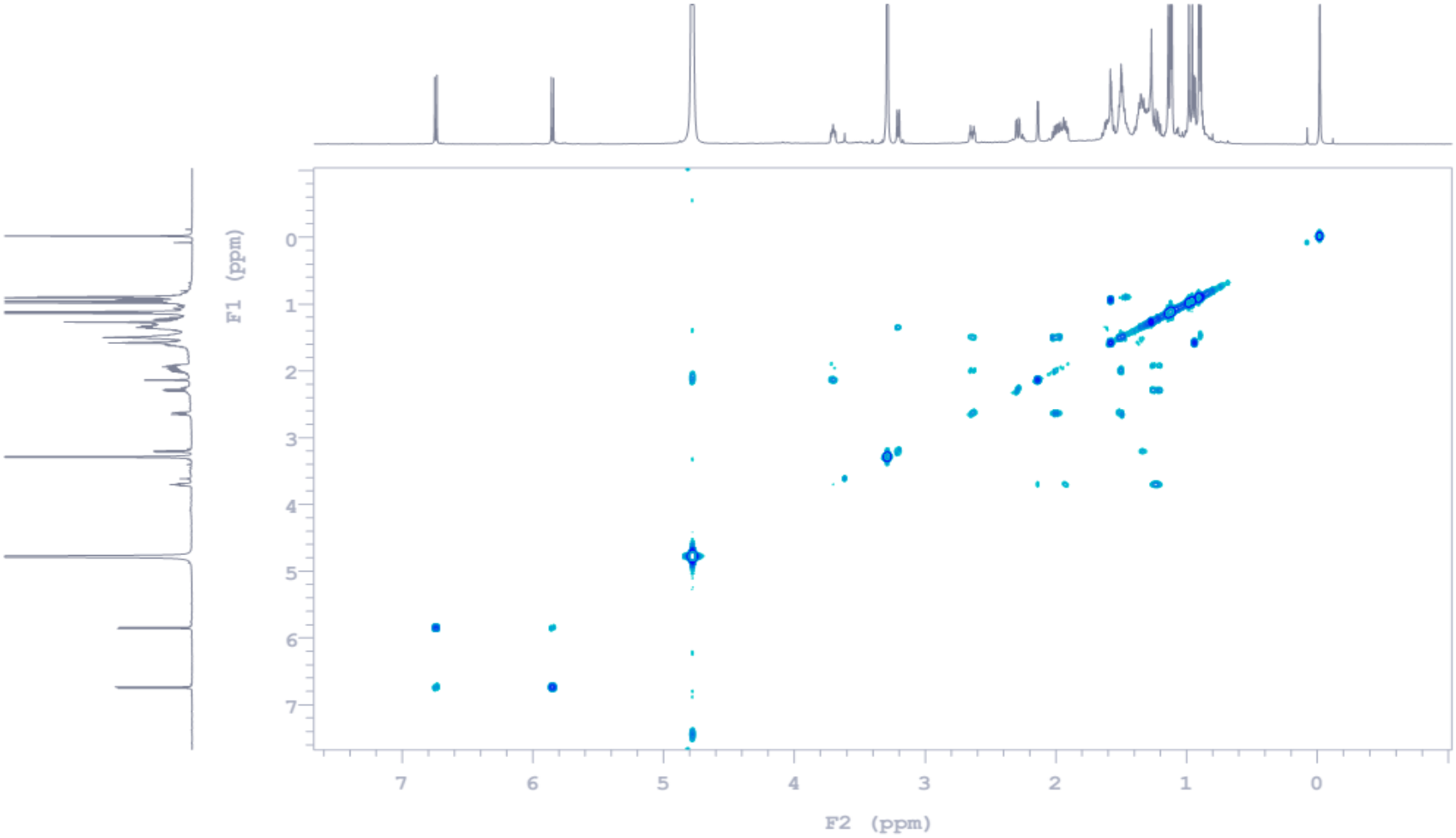
1.4 Compound 10 HSQC



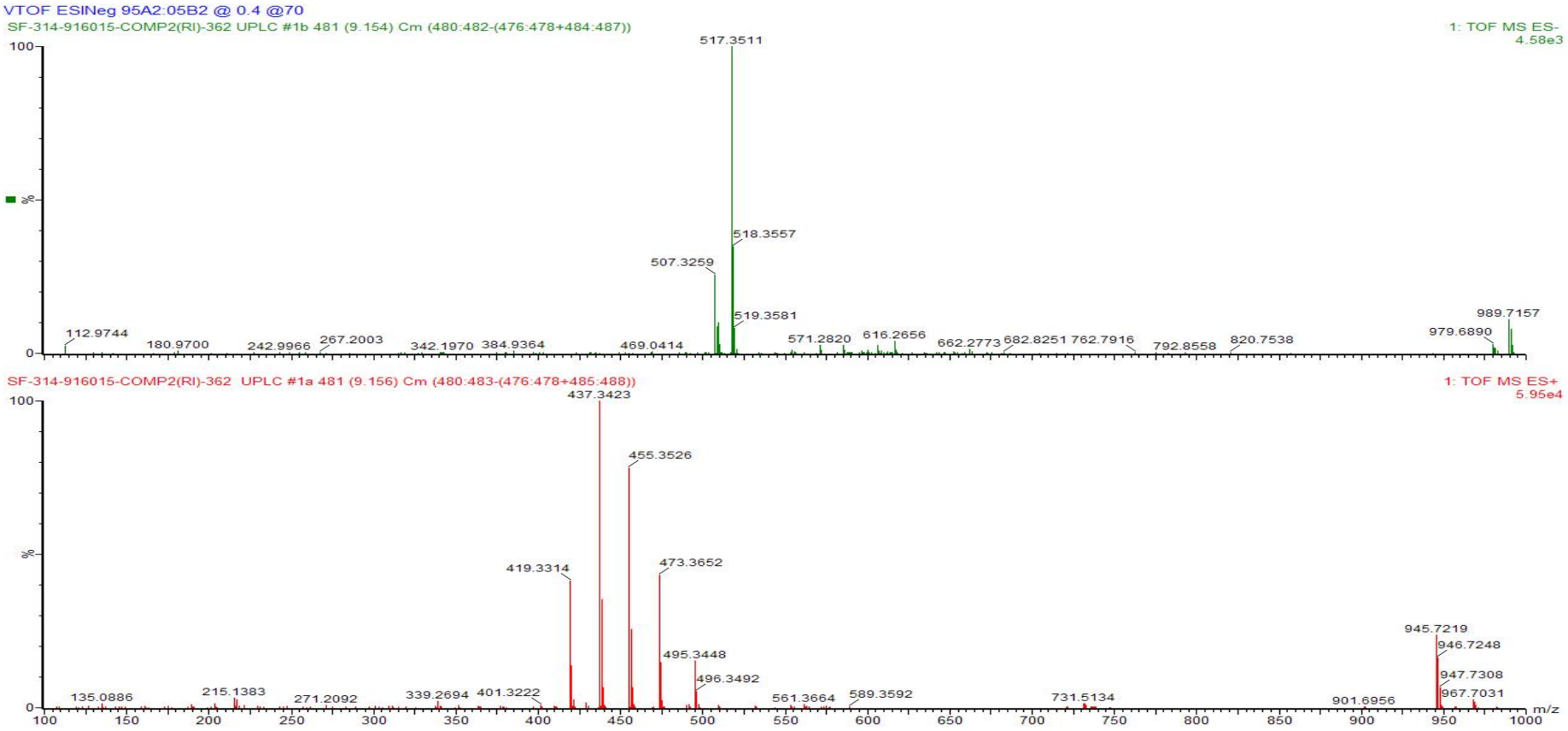
1.5 Compound 10 HMBC



1.6 Compound 10 COSY



APPENDIX 2: Compound 10 Mass analysis



Appendix Figure 22: HRTOFMS spectrum (ESI^- and ESI^+) for compound 10 (refer to page 29)

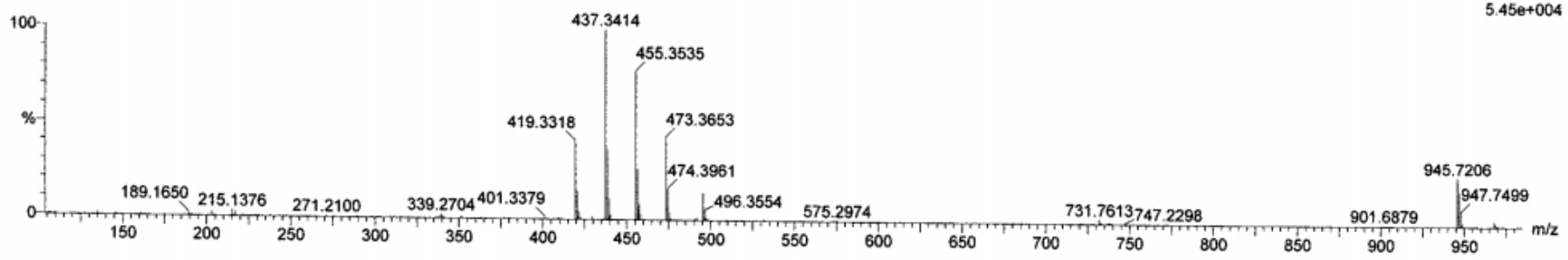
Elemental Composition Report

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.0, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
63 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 10-40 H: 1-100 O: 0-10
VTOF ESIPos 95A2:05B2 @ 0.4 @70

SYNAPT HDMS G1

1: TOF MS ES+
5.45e+004



Minimum:				-1.0			
Maximum:		5.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
473.3653	473.3631	2.2	4.6	6.5	28.0	0.0	C30 H49 O4

Elemental Composition Report

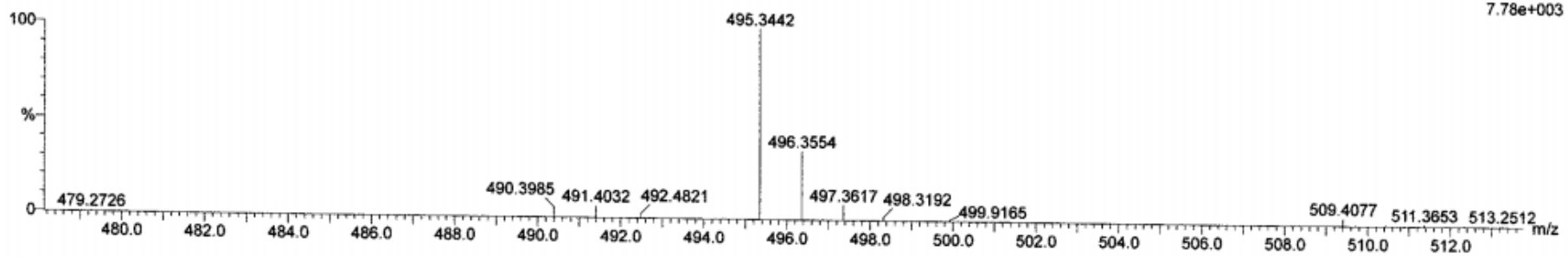
Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.0, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
59 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 10-40 H: 1-100 O: 0-10 Na: 1-1
VTOF ESIPos 95A2:05B2 @ 0.4 @70

SYNAPT HDMS G1

1: TOF MS ES+
7.78e+003



Minimum:				-1.0			
Maximum:		5.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
495.3442	495.3450	-0.8	-1.6	6.5	18.1	0.0	C30 H48 O4 Na

Elemental Composition Report

Single Mass Analysis

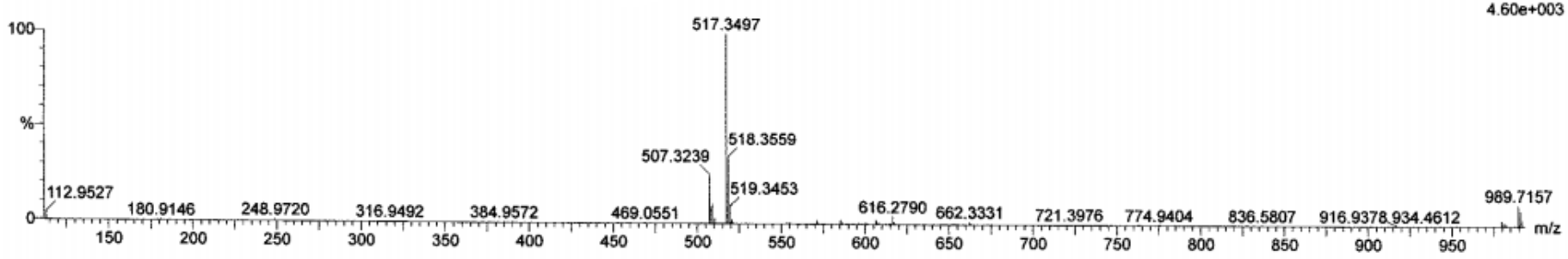
Tolerance = 5.0 mDa / DBE: min = -1.0, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
64 formula(e) evaluated with 2 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 10-40 H: 1-100 O: 0-10

VTOF ESINeg 95A2:05B2 @ 0.4 @70

SYNAPT HDMS G1

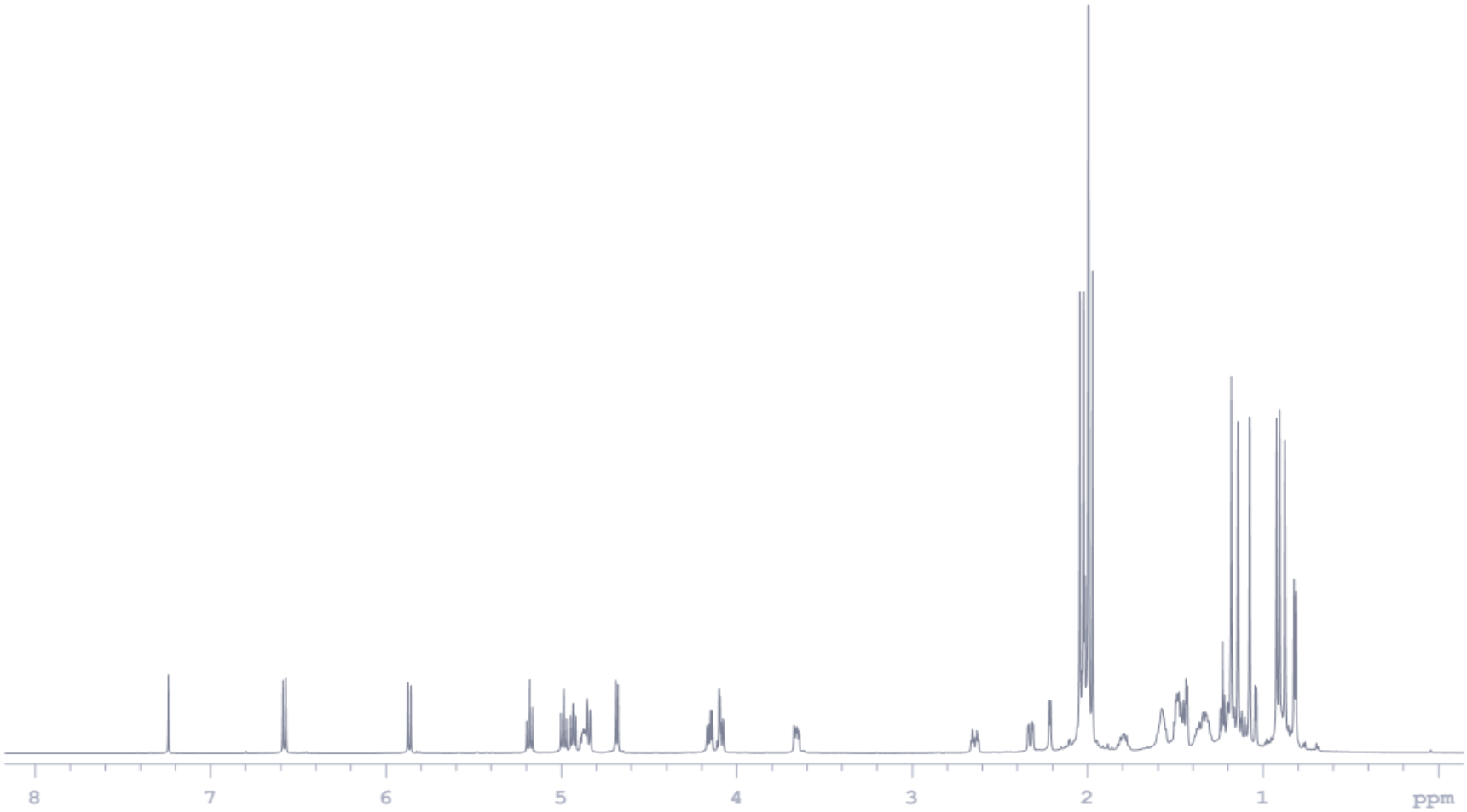
1: TOF MS ES-
4.60e+003



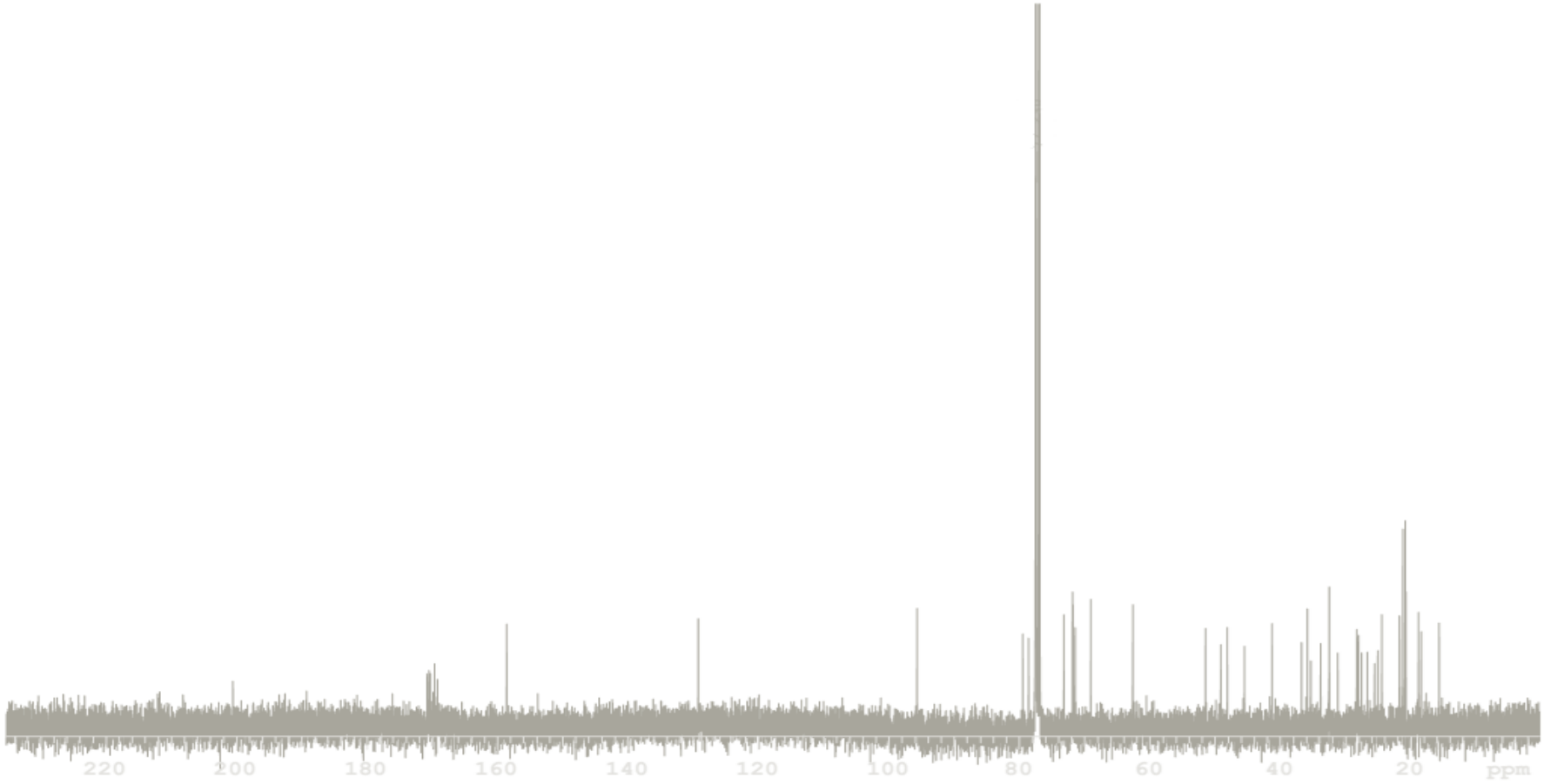
Minimum:				-1.0			
Maximum:		5.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
517.3497	517.3529	-3.2	-6.2	7.5	15.7	0.0	C31 H49 O6
	517.3470	2.7	5.2	16.5	19.2	3.5	C38 H45 O

APPENDIX 3:

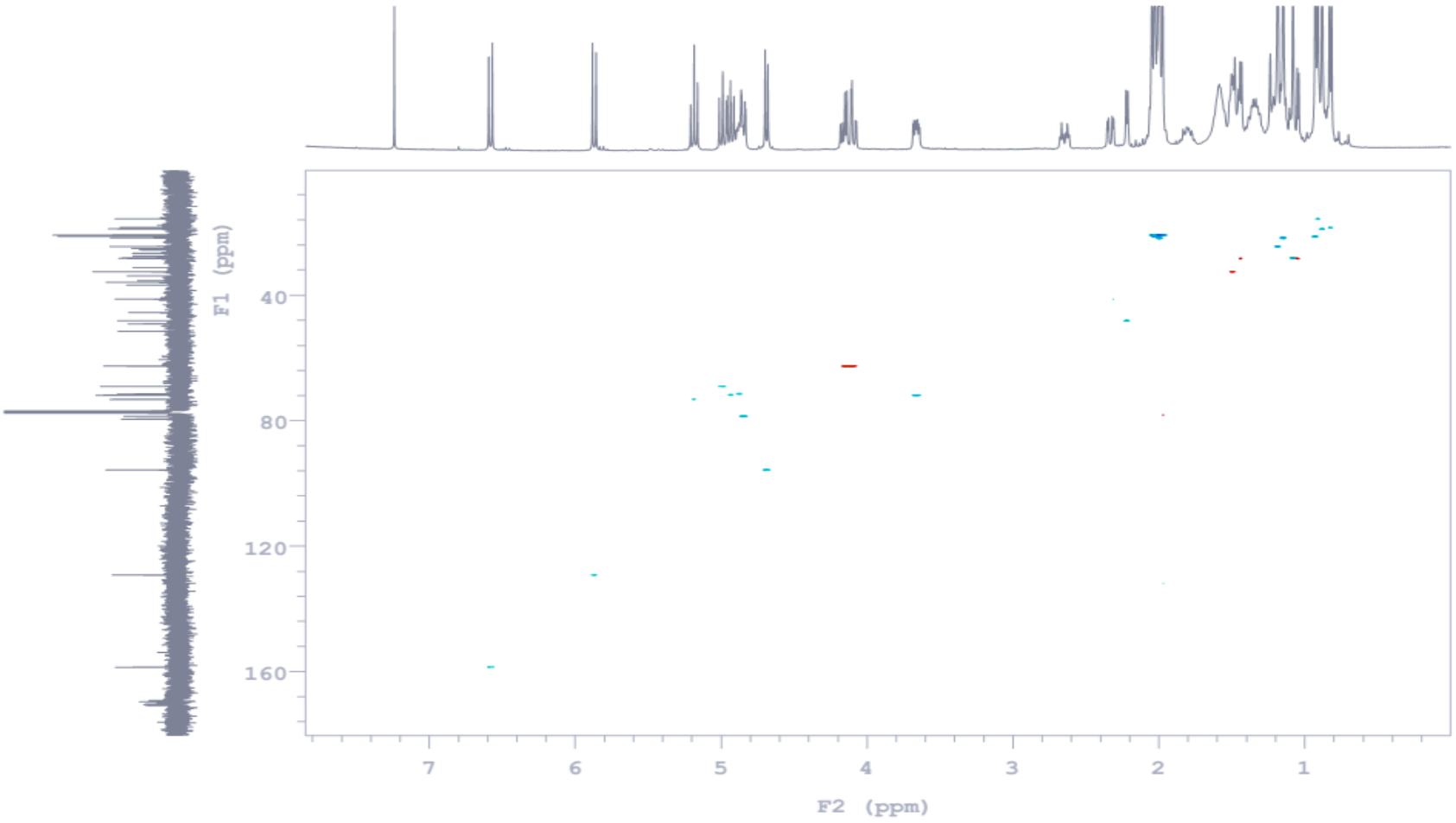
3.1 Compound 11 ^{1}H-NMR



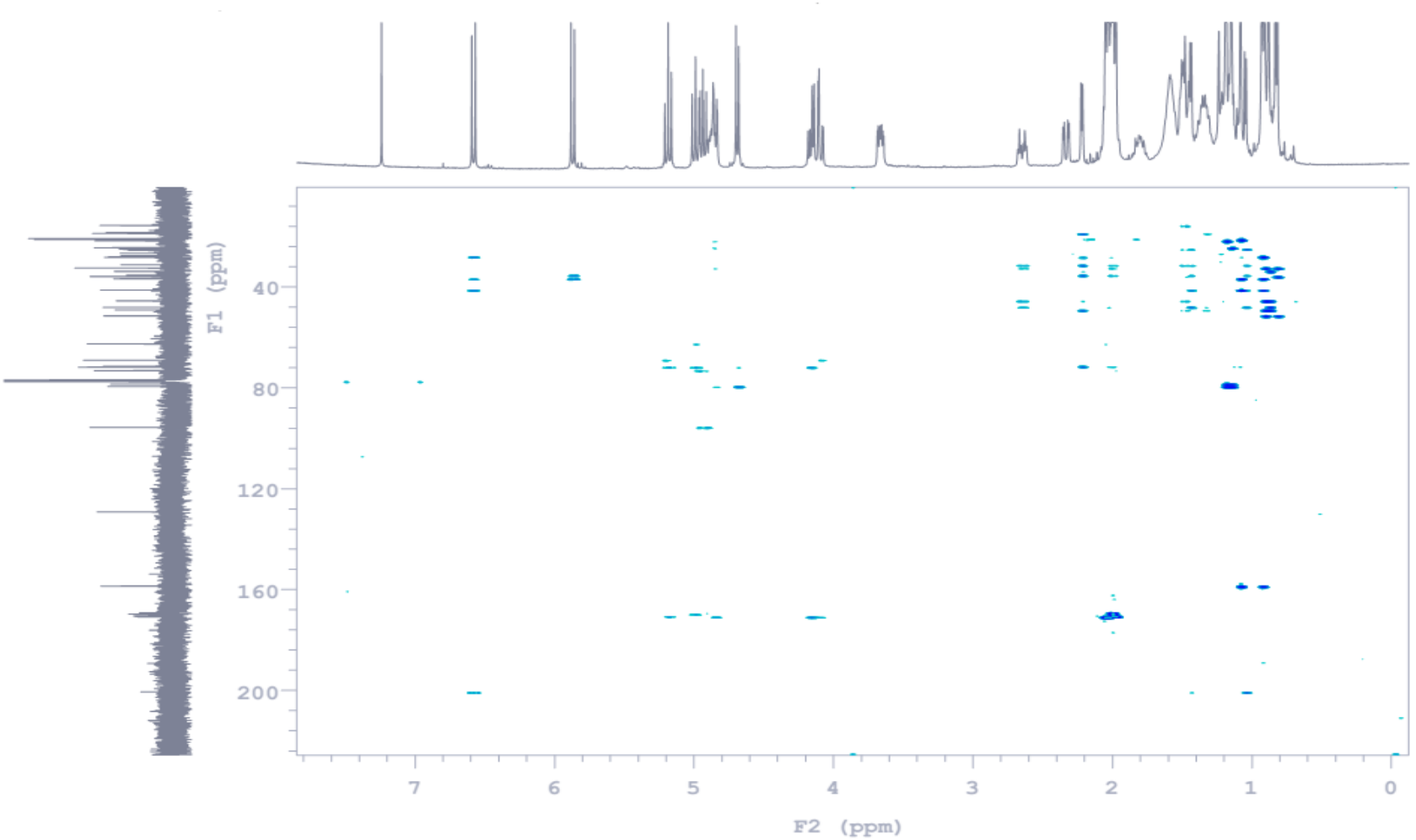
3.2 Compound 11 ^{13}C-NMR



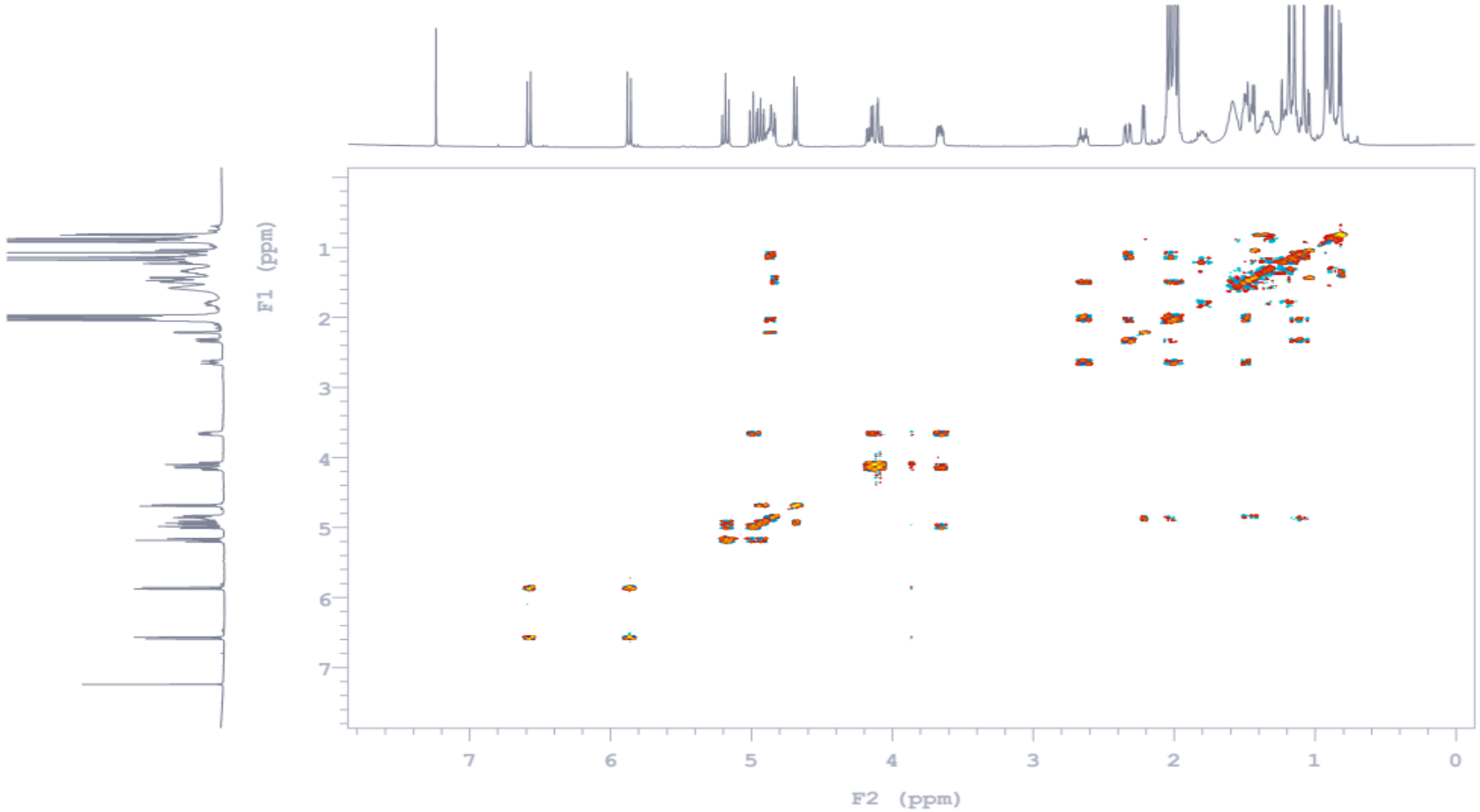
3.3 Compound 11 HSQC



3.4 Compound 11 HMBC

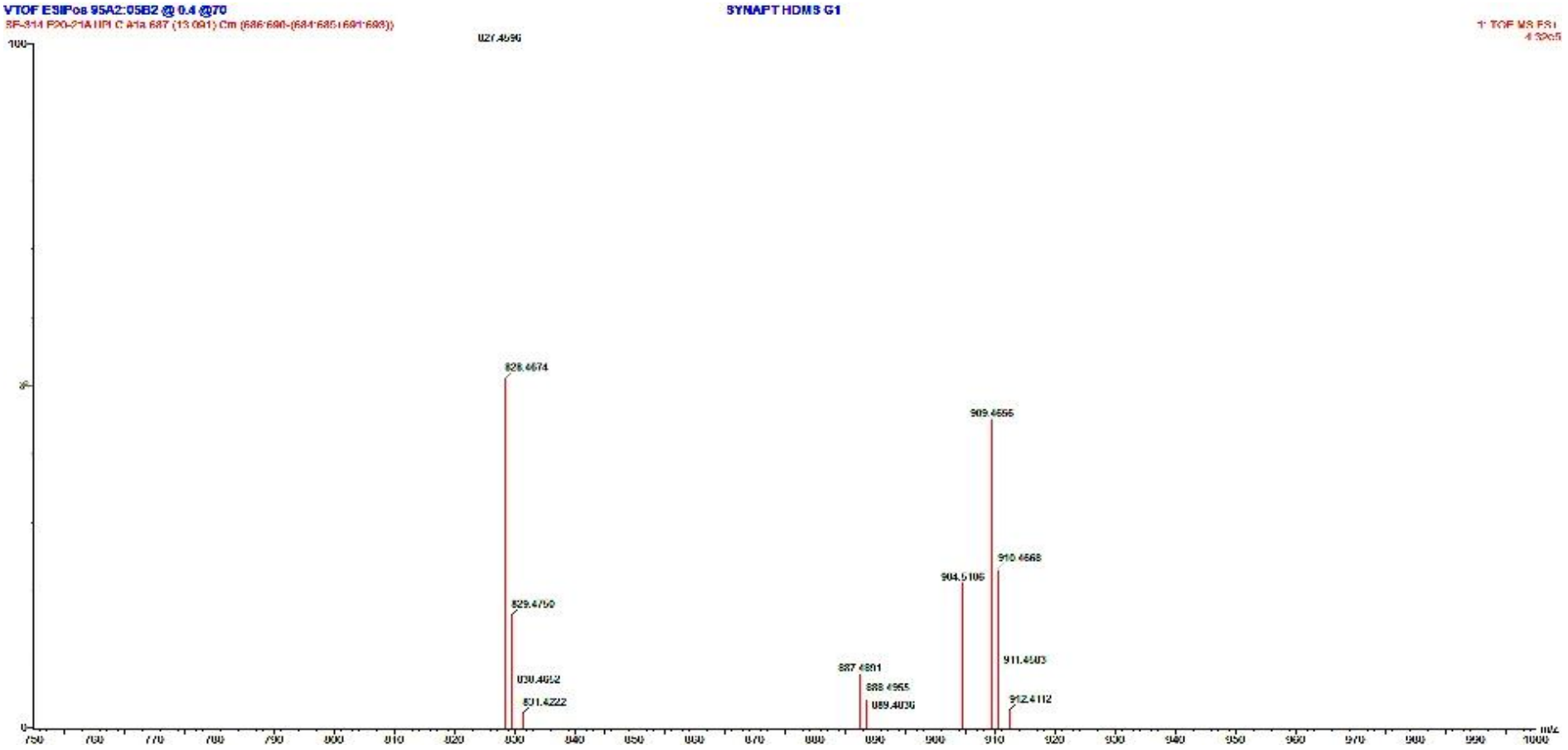


3.5 Compound 11 COSY



APPENDIX 4: Compound 11 Mass Analysis

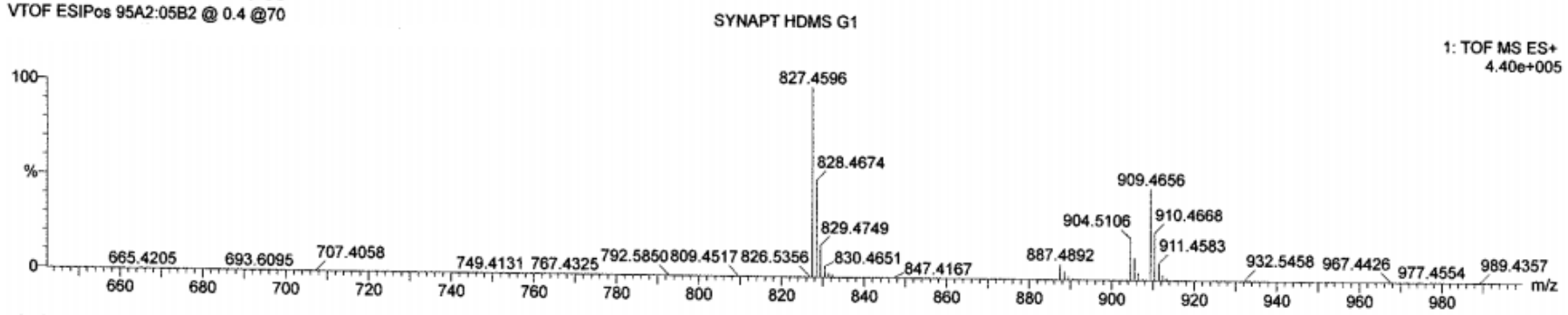
HRTOFMS spectrum (ESI+) for compound 11



Elemental Composition Report

Single Mass Analysis
Tolerance = 10.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
118 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 40-60 H: 0-100 O: 10-30
VTOF ESIPos 95A2:05B2 @ 0.4 @70



Minimum:				-1.5			
Maximum:		10.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
887.4892	887.4793	9.9	11.2	13.5	24.3	0.2	C48 H71 O15
	887.4946	-5.4	-6.1	17.5	26.2	2.1	C52 H71 O12
	887.4852	4.0	4.5	4.5	27.0	2.9	C41 H75 O20

Elemental Composition Report

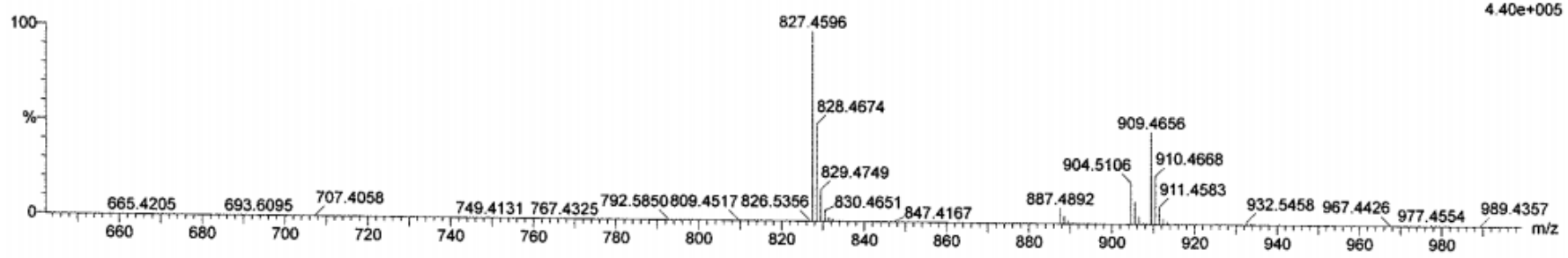
Single Mass Analysis
Tolerance = 10.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
118 formula(e) evaluated with 2 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 40-60 H: 0-100 O: 10-30 Na: 1-1

VTOF ESIPos 95A2:05B2 @ 0.4 @70

SYNAPT HDMS G1

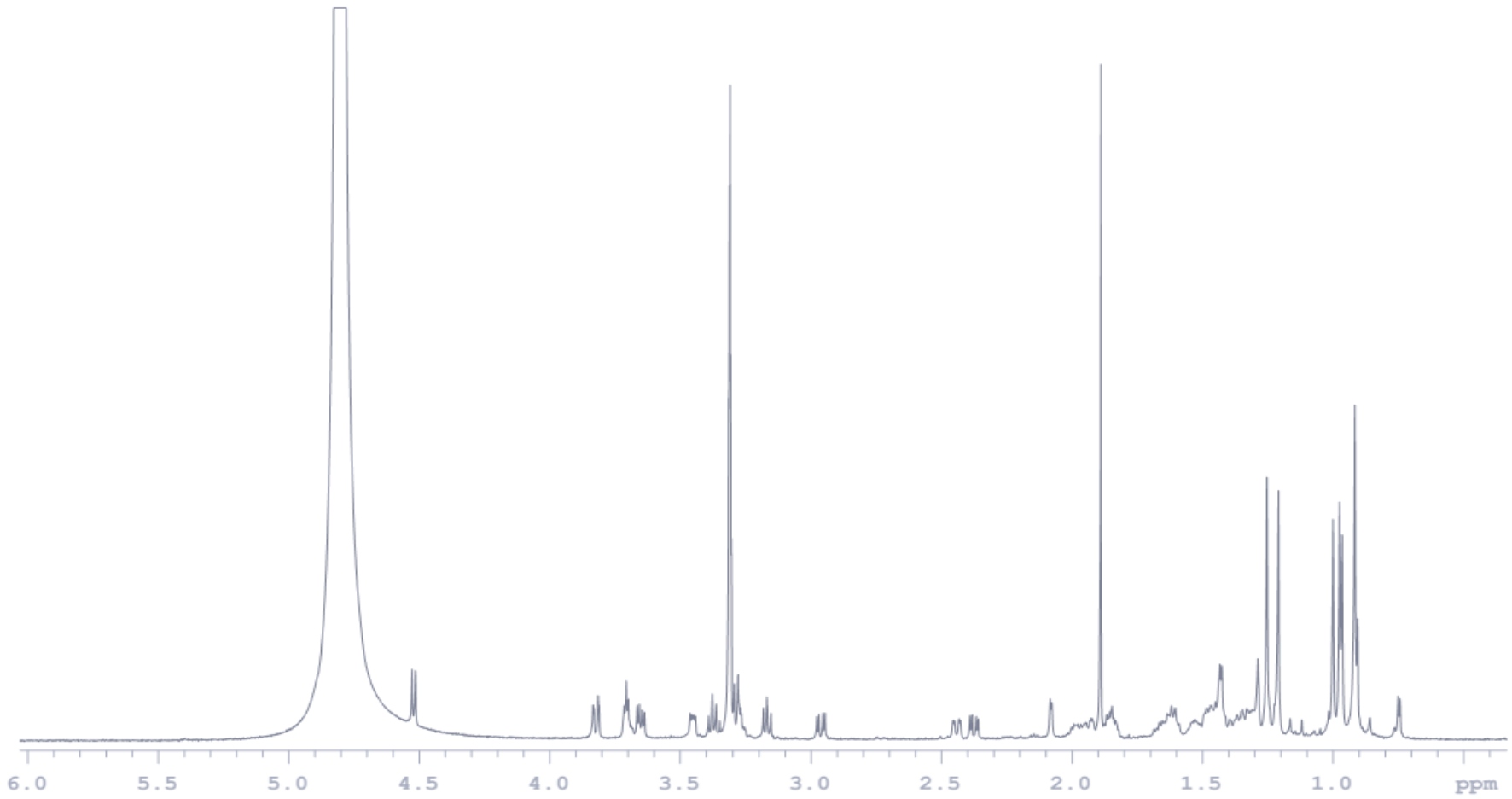
1: TOF MS ES+
4.40e+005



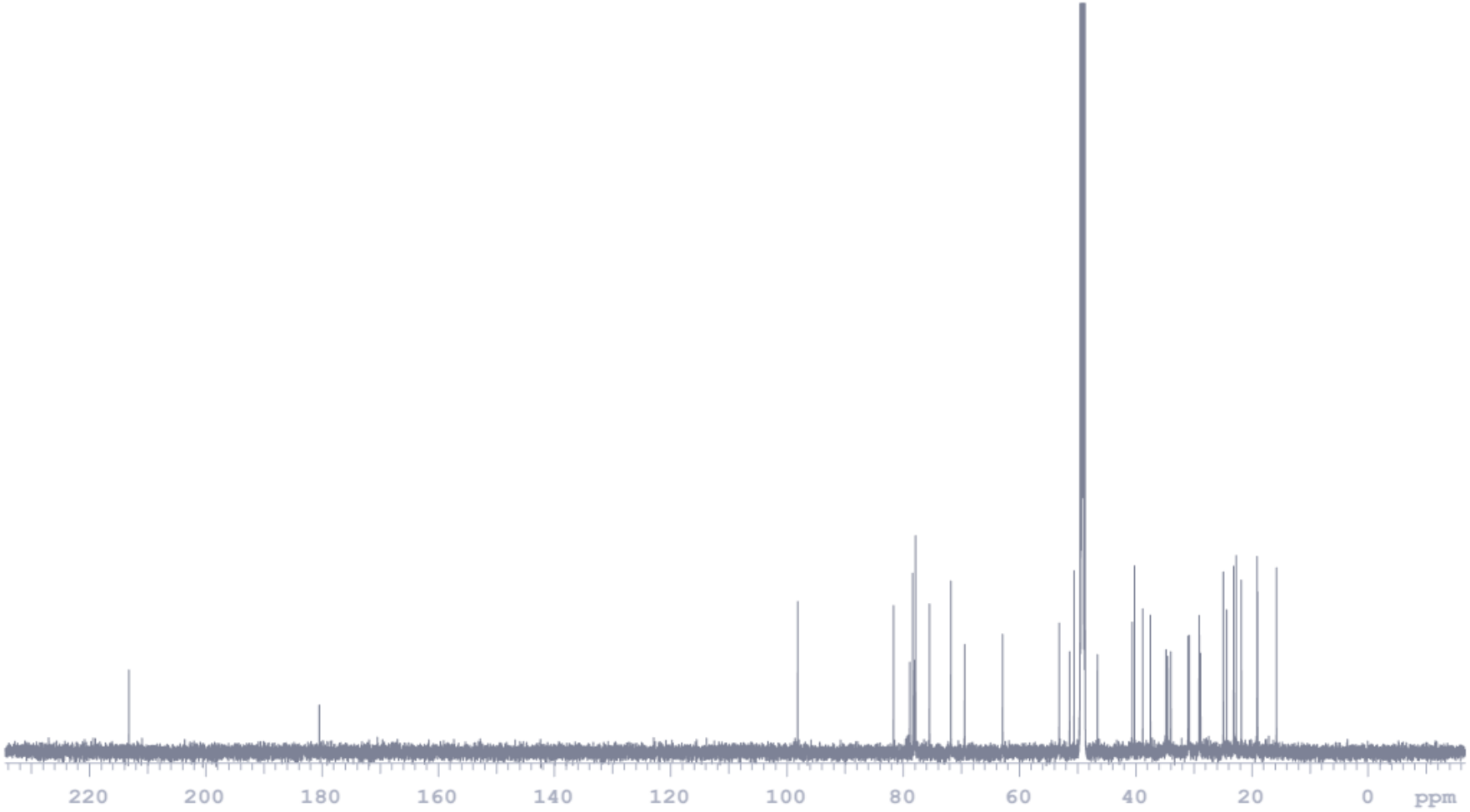
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
Minimum:				-1.5			
Maximum:		10.0	10.0	50.0			
909.4656	909.4612	4.4	4.8	13.5	29.4	0.1	C48 H70 O15 Na
	909.4671	-1.5	-1.6	4.5	31.6	2.3	C41 H74 O20 Na

APPENDIX 5:

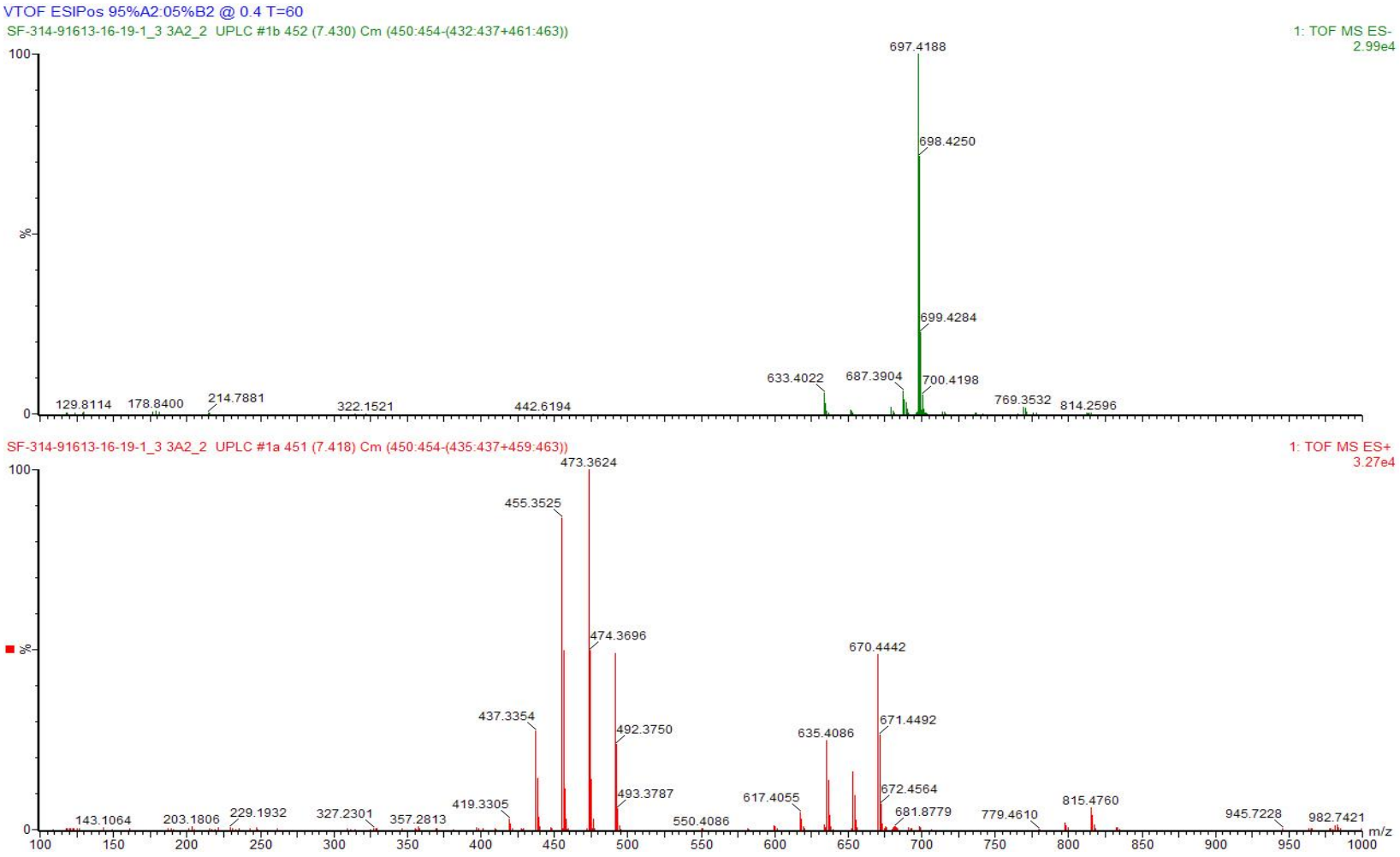
5.1 Compound 5 ^{1}H-NMR



5.2 Compound 5 ^{13}C-NMR spectra



APPENDIX 6: Compound 5 Mass analysis

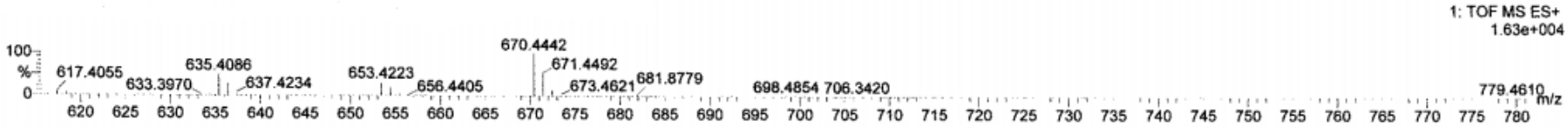


Appendix Figure 23: HRTOFMS spectrum (ESI^- and ESI^+) for compound 5 (refer to page 36)

Elemental Composition Report

Single Mass Analysis
Tolerance = 10.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
83 formula(e) evaluated with 1 results within limits (up to 10 closest results for each mass)
Elements Used:
C: 5-40 H: 1-100 O: 0-20
VTOF ESIPos 95%A2:05%B2 @ 0.4 T=60

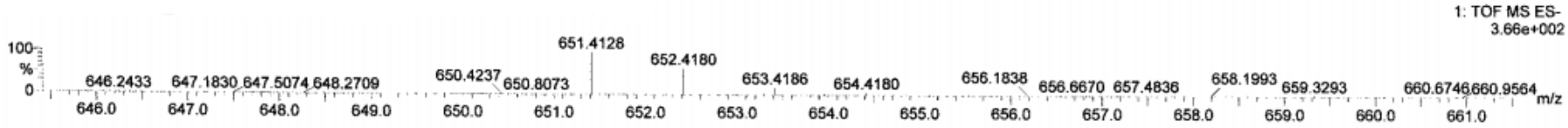


Minimum:				-1.5			
Maximum:		10.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
653.4223	653.4265	-4.2	-6.4	6.5	18.6	0.0	C36 H61 O10

Elemental Composition Report

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
82 formula(e) evaluated with 1 results within limits (up to 10 best isotopic matches for each mass)
Elements Used:
C: 5-40 H: 1-100 O: 0-20
VTOF ESINeg 95%A2:05%B2 @ 0.4 T=60

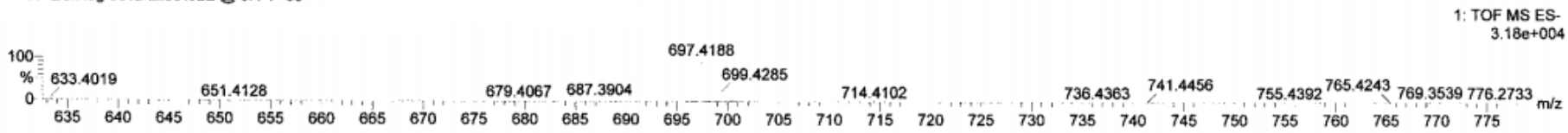


Minimum:				-1.5			
Maximum:		3.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
651.4128	651.4108	2.0	3.1	7.5	71.6	0.0	C36 H59 O10

Elemental Composition Report

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
71 formula(e) evaluated with 1 results within limits (up to 10 closest results for each mass)
Elements Used:
C: 5-40 H: 1-100 O: 0-20
VTOF ESINeg 95%A2:05%B2 @ 0.4 T=60



Minimum:				-1.5			
Maximum:		3.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
697.4188	697.4163	2.5	3.6	7.5	248.3	0.0	C37 H61 O12

APPENDIX 7: Conference presentation

7.1 SACI Annual Young Chemist Symposium at Melville, Johannesburg, 13th September 2013 (oral presentation).

7.2 The 5th CSIR conference at CSIR ICC , 1 Meiring Naude Rd, Pretoria, 0001, 8-9 October 2015 (poster presentation).

APPENDIX 8: Articles published.

8.1 The contents of this work will be published.