Chemical studies on some natural products from *Myrothamnus flabellifolius*

By

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Thesis submitted in partial fulfillment of the requirements for the award of the degree of Masters of Science in the Faculty of Natural Sciences University of the Western Cape

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November 2014

ABSTRACT

Crude extracts from *Myrothamnus flabellifolius* were fractionated through various chromatography techniques in order to achieve satisfactory separations. Two compounds, arbutin and lupeol, were isolated from the butanol and ethyl acetate extracts, respectively. Structural elucidation of the compounds was carried out on the basis of ^{1}H and ^{13}C NMR spectroscopy. It was the first time that lupeol had been isolated from *M. flabellifolius*.

The water extract was found to contain heterogeneous pectic polysaccharides. Three polysaccharide fractions were separated from the water extract, namely; MPS, MPS-I and MPS-II. The predominant fraction was found to be MPS, with a molecular weight distribution of 2 X 10^5 Da. The polysaccharides were made up the following monosaccharides; arabinose, rhamnose, xylose, mannose, galactose and glucose. Monosaccharide composition was determined through acid hydrolysis at high temperatures, followed by GC analysis. The linkage analysis was carried out by GC-MS, following partial methylation of the polysaccharides.

The use of human serum albumin (HSA) following its anchoring on magnetic nanoparticles, in order to isolate targeted saponins from *Sutherlandia frutescens* led to successful isolation of four saponins, namely; sutherlandiosides A-D. The saponins showed great affinity to bind to the HSA.

DECLARATION

I, Ndikho Nako, hereby declare that Chemical studies on some natural products from *Myrothamnus flabellifolius* is my own work, that it has not been submitted anywhere else for the award of a degree at any other University, and that all the sources I have used have been acknowledged by complete references.

Date …… Signed ….....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Prof WT Mabusela, for allowing me to be a part of his lab. Thank you Prof, for your tireless efforts, unwavering support, and guidance. Without you, this work would not have been possible.

I would like to thank the Department of Chemistry for affording me this opportunity and for their support.

My sincere gratitude to the MRC for providing the plant material.

I would also like to thank my lab colleagues who have always been there for me whenever I needed help.

TABLE OF CONTENTS

List of abbreviations

ACN	Acetonitrile
AIDS	Acquired immune deficiency syndrome
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
ESI-MS	Electrospray ionization mass spectrometry
EtOAc	Ethyl acetate
FT-IR	Fourier transform infrared spectroscopy
GABA	gamma-Aminobutyric acid
GC	Gas chromatography
GC-MS	Gas chromatography coupled mass spectrometry
HIV	Human immunodeficiency virus
HSA	Human serum albumin
HSA-MNPs	Human serum albumin immobilized magnetic nanoparticles
LC-MS	Liquid chromatography coupled mass spectrometry
MeI	Methyl iodide
MeOH	Methanol
MNP	Magnetic nanoparticles
MRC	Medical Research Council
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
TLC	Thin Layer Chromatography
US	United States
UV	Ultra Violet
WHO	World Health Organization

List of figures

List of schemes

Page

List of tables

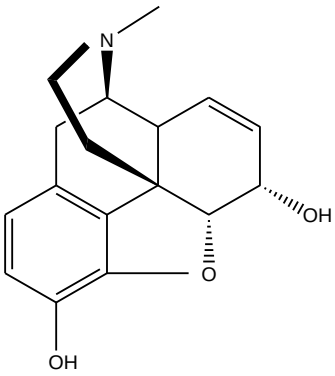
CHAPTER 1

Introduction and Literature review

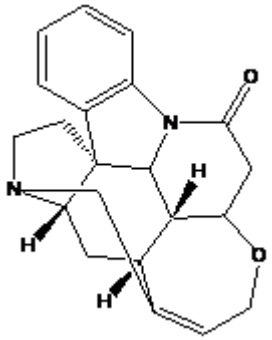
Plant chemistry or phytochemistry is an area of study which focuses on the investigation of the natural product composition of plants. Over the years, phytochemistry has developed as a bridge that connects chemistry and botany, as it also seeks to explain natural product biosynthesis, metabolism and biological function.

1.1 Natural Products

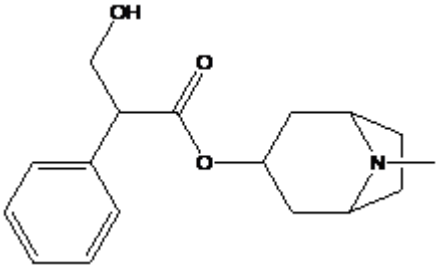
Natural products derived from plants are known to have different uses that are important to humans. Some of these natural products possess pharmacological or biological activities which may be exploited in pharmaceutical drug discovery and drug design. Others may be used in the preparation of food supplements, antioxidants, insecticides, etc. Given the above mentioned benefits, scientists began to do research on natural products in the early nineteenth century. Friedrich Sertürner isolated morphine (see Figure 1) from *Papaver somniferumin* in 1806, and since then natural products have been extensively screened for their medicinal purposes. Among the first natural products to be isolated, were atropine and strychnine (see Figure 1). In 1826, E. Merck manufactured the first commercially pure natural product, morphine (Newman *et al*., **2000**).



Morphine



Strychnine



Atropine

Figure 1: Some of the first natural products to be isolated from plants.

In modern medicine, about 41% of new and approved drugs have natural products as their origin, either directly or indirectly as shown in Figure 2 (Cragg et al, **1997**).

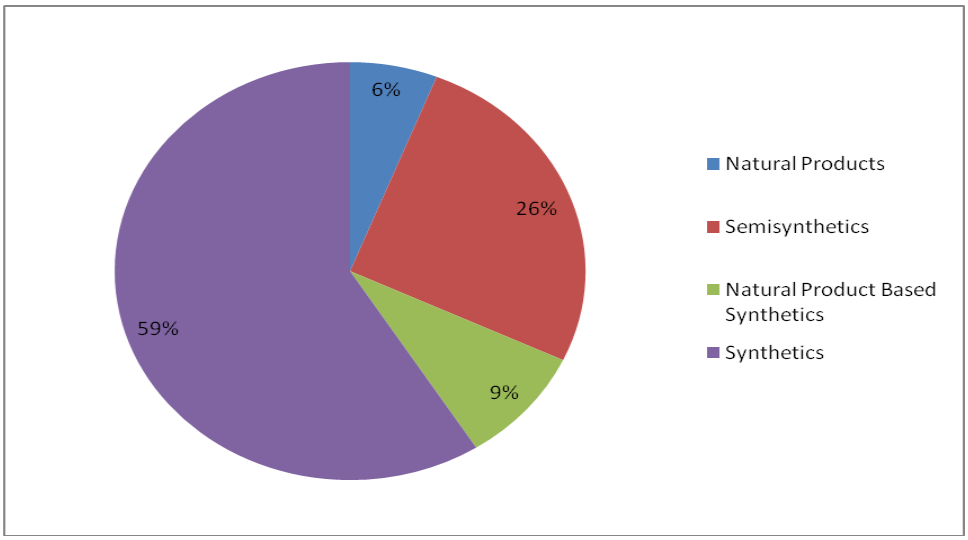


Figure 2: **The role of natural products in modern medicine.**

A close look at the anticancer drugs on the market today (Figure 3), shows that the percentage of new anticancer drugs which originated from natural products increased to about 60% (Cragg *et al*, **1997**). Between the years 1981-2006, about a hundred anticancer agents have been developed, of which twenty five are semisynthetics, eighteen are natural product based synthetics and twenty are pure natural products (Newman *et al*, **2007**).

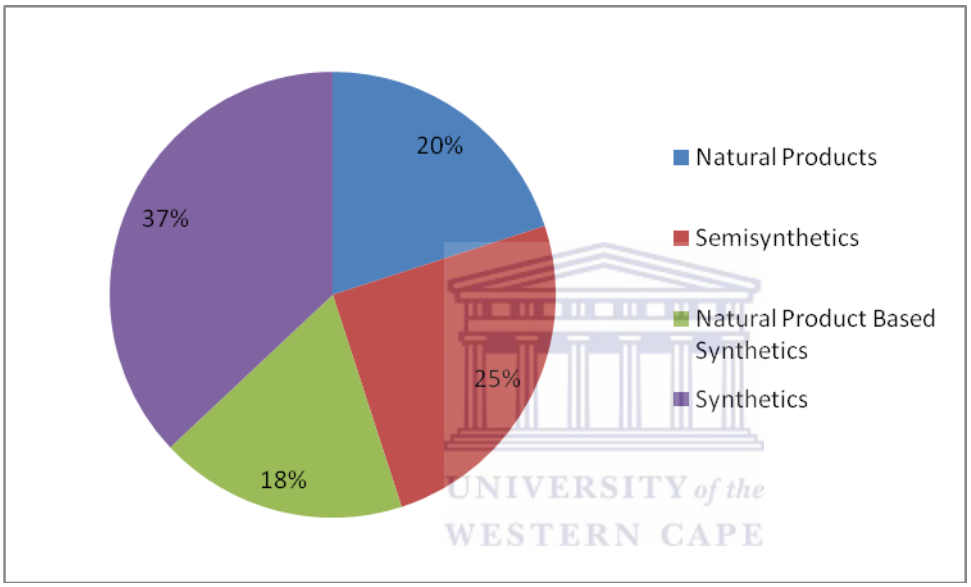


Figure 3: **The role of natural products in anticancer drugs.**

According to Van Wyk *et al.* (**2000**), the use of natural products in medicine is justified since they are the main active substances in medicinal plants that act in the treatment or prevention of diseases, either directly or indirectly (e.g. see Table 1). Over the years, more studies carried out on natural products have led to the discovery of diverse compounds with unique properties. Furthermore, advances in technology have guaranteed great improvement in relation to extraction, isolation, purification and structural elucidation of these compounds.

Table 1: **Some examples of prescription drugs from the plant kingdom.**

Name of drug	Plant source	Medical use
Morphine	*Papaver somniferum*	Analgesic
Betulinic acid	*Betula alba*	Anticancer
Taxol	*Taxus brevifolius*	Anticancer
Quinine	*Cinchona pubescences*	Antiprotozoal/malaria
Cocaine	*Erythoxylum coca*	Anaesthetic
Hyoscine	*Datura stramonium*	Sedative
Atropine	*Datura stramonium*	Analgesic
Vincristine	*Catharanthus roseus*	Leukaemia

Some of these compounds have important applications in other fields such as cosmetics and food industries, organic synthesis, etc. For example, quinine from *Cinchona pubescences* is known to give tonic water that bitter taste. In addition, herbs and spices have long been known to provide flavour to foods.

1.2 Classification of natural products

Natural products consist of all organic compounds which are produced as a result of the natural processes that plants are subjected to. In the initial stages, these processes are driven by light energy (from the sun) which is then stored for further use by the plants. Natural

products may be divided into two categories: primary and secondary metabolites. Primary metabolites are compounds that are mostly present in every living cell, and which posses structural, reproductive and metabolic functions. Some examples of primary metabolites include: carbohydrates, cellulose, amino acids and proteins. On the other hand, secondary metabolites are not present in all living organisms. These compounds usually have diverse biological effects on other organisms. Some various classes of secondary metabolites that are known include: terpenoids, glycosides, tannins, alkaloids, phenolics, etc.

1.3 Some natural products isolated from the plant kingdom

1.3.1 Glycosides

Glycosides are compounds which have a carbohydrate that is attached to a non-carbohydrate moiety through an acetal linkage. Upon hydrolysis, glycosides release a saccharide unit (glycone) and a non-carbohydrate moiety (aglycone). When the carbohydrate part is glucose, the compound is usually called a glucoside or a fructoside in cases where it is fructose. Aglycones may be any class of natural products from sterols, phenolics, triterpenoids, etc. Glycosides are very important because of the various biological activities they posses, e.g. saponins (steroidal glycosides) from *Dioscorea nipponica* are believed to be the active ingredients in the plant's use in the treatment and prevention of coronary heart disease (Qing *et al*., **2010**). A well known glycoside, rutin, from *Carpobrotus edulis* is responsible for the plant's antibacterial (Van der Watt and Pretorius, **2001**) and antioxidant properties (Ibtissem *et al*., **2012**). In the market, rutin is available as a drug used in the treatment of veins fragility.

1.3.2 Terpenoids

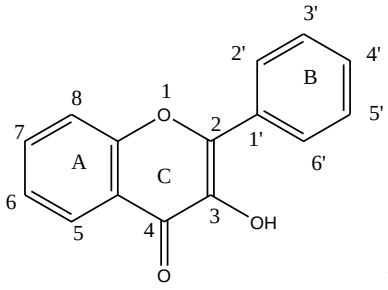
Terpenoids represent a major class of natural products, with over ten thousand compounds which are derived from an isoprene unit (C_5H_8). Depending on the number of isoprene units, terpenoids may be classified into: hemiterpenoids (1 unit), monoterpenoids (2 units), sesquiterpenoids (3 units), diterpenoids (4 units), sesterpenoids (5 units), triterpenoids (6 units), tetraterpenoids (8 units), and polyisoprenes (more than 8 units). Terpenoids play a very important role, therapeutically, in traditional herbal remedies due to their pharmacological activities, e.g. a monoterpenoid called menthol is a very popular pain reliever. Another example is a popular anticancer drug called taxol, a diterpenoid first identified in *Taxus brevifolia*. Terpenoids are mostly found to exist in the triterpenoid form. Some interesting triterpenoids occur in *Sutherlandia frutescens*, a very popular medicinal plant indigenous to South Africa, where they act as protection against viral infections. Katerere *et al*. (**2003**) reported that some pentacyclic triterpenes isolated from members of the African Combretaceae expressed antimicrobial activity against *Mycobacterium fortuitum* and *Staphylococcus aureus.* Lee *et al*. (**1989**) reported that triterpenes and their derivatives expressed *in vitro* cytotoxicity to tumor cell lines. Fujioka *et al*. (**1994**) reported that a triterpenoid, betulinic acid, isolated from the leaves of *Syzigium claviflorum* expressed anti-HIV activity.

1.3.3 Essential oils

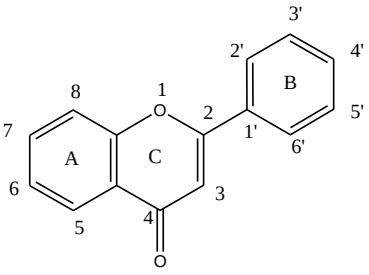
Essential oils are a mixture of aromatic, volatile compounds which are insoluble in water, such as monoterpenoids, sesquiterpenoids and their derivatives. Plants containing essential oils are traditionally known to express anticarcinogenic, astringent and anti-inflammatory effects (Lovkova *et al.*, **2001**).

1.3.4 Phenolic compounds

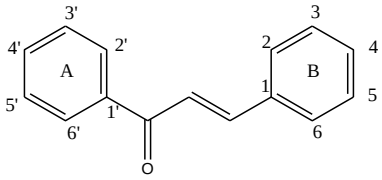
Phenolic compounds are a major group of compounds found in plants, which may be classified according to their water solubility. There is a class of soluble phenolic compounds which includes flavonoids, quinones, anthocyanins, etc. Another class consists of water-insoluble phenolic compounds such as tannins. Flavonoids are a major group within the phenolics class of compounds (see Figure 4), and they are synthesized via the shikimic acid pathway. Flavonoids are constituents of vegetables, fruits and other plant types and parts. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) suggest that many animals, including humans, ingest significant quantities in their diet. Therefore, flavonoids are often used as dietary substances for health promotion, anti-ageing and disease prevention (Fernandes *et al.*, **2004**). Flavonoids are known to produce the colour pigmentation in flowers and fruits, which is sometimes regarded as a defensive mechanism against attacks from insects and microbes. Some of the major types of flavonols such as quercetin have been isolated in plants such as *Butea frondosa*. Cancer researchers have reported that study participants who consumed foods containing certain flavonoids appeared to be protected from developing lung cancer (David, **2007**). Some of the flavonoids that seemed to offer the most protection include quercetin, and only small amounts of these flavonoids are required to provide health benefits. Flavonoids have also been reported to be present in *Sutherlandia frutescens*. Due to the large variety of phenolic compounds, medicinal plants exhibit a variety of pharmacological and therapeutic effects, e.g. flavonoids act as antioxidants (Sigh *et al.*, **2005**), among many others.



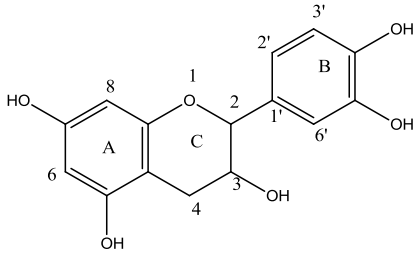
Flavonol



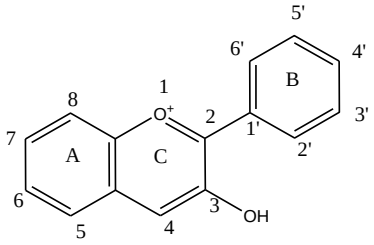
Flavones



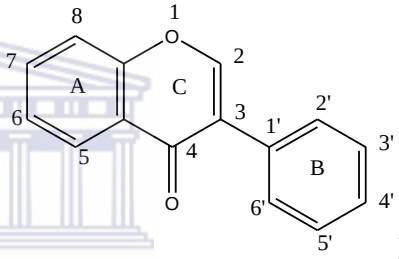
Chalcones



Catechins



Anthocyanidins



Isoflavones

Figure 4: Some classes of flavonoids found in medicinal plants.

1.3.5 Polysaccharides

Polysaccharides are polymers that yield many monosaccharide molecules when hydrolyzed, e.g. starch yields glucose molecules. Starch is the main source of energy in plants. Starch is composed of two types of polysaccharides, amylose and amylopectin. Amylose is a linear polymer composed of glucose residues with (1→4) linkages, while amylopectin consists of (1→4) linkages and ~5% (1→6) branch linkages. Polysaccharides are important natural products because they offer great benefits to human health. Kouakou *et al*. (**2013**) reported that polysaccharides from an African plant *Clerodendrum splendens* expressed

immunomodulatory activity. A β-glucan found in mushrooms was reported to exhibit some antitumor properties (Ren *et al*., **2012**), and so were the polysaccharide-protein complexes from mushrooms (Ooi and Liu, **2000**). Zhao *et al*. (**2007**) reported that polysaccharides islated from *Dendrobium chrysotoxum* expressed antioxidant and anti-hyperglycemic activity.

Some polysaccharides are composed of acidic monosaccharides, i.e. monosaccharides that contain the carboxylate ion, COO^-. Pectins fall within this type of polysaccharides. Pectins have a characteristic backbone in which 1,4-linked galacturonic acid units have a "kink" due to rhamnogalacturonan regions made up of a backbone of alternating 1,2-linked rhamnose and 1,4-linked galactose units (Van Soest, **1994**). Neutral side chains are mostly substituted at O-4 of the rhamnose units, and consist of arabinose and galactose units.

There is also a class of polysaccharides known as arabinogalactans. Arabinogalactans are generally divided into two structural types. Type I is mainly pectic arabinogalactans which consist of a principal chain of 1,4-linked galactose units with arabinosyl units occurring as substituents (Clarke *et al*., **1979**; Stephen, **1983**). The arabinosyl units are linked to galactose units through O-3. Type II arabinogalactans are highly branched polysaccharides made up of 1,3-linked and 1,6-linked galactose units, with the 1,6-linked units mostly found in the exterior and the 1,3-linked found in the interior of the main chain. The arabinose units are sometimes attached to the through O-3 of the 1,6-linked galactose units.

Pectic polysaccharides are known to exhibit some important biological activities. In cosmetic and food industries, pectin extracts from fruits such as apples and citrus peels are known to function as stabilizing and gelling agents. Krachanova *et al.* (**2010**) reported that the pectic polysaccharides isolated from *Allium porrum* exhibited immunostimulating activities. Pectic polysaccharides isolated from *Mosla chinensis* have been reported to show considerable

antitumor activity, as well as inhibition of NO production (Li *et al*., **2014**). As part of the daily fibre intake, pectins have been reported to lower blood cholesterol levels (Sriamornsak, **2001**), and remove toxic cations from the respiratory organs and gastrointestinal tract (Kohn, **1982**). Thakur *et al*. (**1997**) have reported some antimicrobial activity of pectins towards *Echerichia coli*.

1.4 Medicinal plants

According to Sofowora (**1982**), a medicinal plant is defined as any plant that can be used in the treatment of diseases or ailments because it contains, in any of its parts, natural products that have biological activity against such diseases or ailments. Medicinal plants also play a significant role in the development of medical drugs since most of the modern medicines owe their origins to plants. Natural products extracted or isolated from plants are used as precursors or blueprints for the synthesis of synthetic or partially synthetic drugs (Simpson and Ogorzaly, **2001**).

Despite the technological advances in modern medicine, the majority of the world's population still depend on the use of medicinal plants for health care purposes. For example, in South Africa, about 80% of the total population rely fully or partly on medicinal plants for their health care needs (Balick *et al.*, **1994**). This reliance on medicinal plants may be attributed to the cultural and religious practices of some ethnic groups, which the indigenous plants form an integral part thereof. Additionally, this sort of health care system is easily the most accessible and affordable to those poor communities who have difficulties accessing commercial drugs. Across the first world countries, about 1500 medicinal plants are used in the form of herbal remedies and drugs (Hoareau and DaSilva, **1999**).

Over the recent years, there has been a noticeable increase in the consumption of herbs and drugs from medicinal plants. The World Health Organization (WHO) has reported that, in Africa, more than 60% of infants are entirely dependent on medicinal plants for the treatment of malaria and fever (Diarra, **1999**). This has prompted the South African Ministry of Health to embark on a promotional campaign for the consumption of medicinal plant extracts. Two of the most championed medicinal plant species, currently, are *Sutherlandia frutescens* and *Hypoxis hemerocallidea*. These plant species have shown a great deal of potential in the management or treatment of diseases such as cancer and HIV (Mills *et al.*, **2005**). *S. frutescens*, a plant which has been used in this thesis as a model to explore one of the newer approaches to the study of medicinal plants, is a plant which has a wide range of medical uses without any reported toxicity or side effects associated with its use. The powdered or encapsulated *S. frutescens* is distributed by Phyto Nova, a South African pharmaceutical company. Another company, Parceval Pharmaceuticals, manufactures "Power-Your-Life" tablets and an immune-boosting syrup, both of which are derived from *S. frutescens* extracts (www.parceval.co.za).

A complete contrast to what has long been seen as a primitive and unconventional practice, the use of medicinal plants has now become more commercialized. More and more pharmaceutical companies have undertaken to the processing, distribution and selling of herbs or natural products, with the assistance of some media coverage (Brevoort, **1998**). Inevitably, both the local and international trades have increased due to the demand of medicinal plant based products. Medicinal plants are not only cheaper than synthetic drugs, but they also have far greater value in the export trade. WHO has estimated that pharmaceutical companies around the world attract the equivalent of billions of US dollars in annual gross profits. According to Aarts (**1998**), the sales of *Hypericum perforatum*, a

medicinal plant used as a treatment for depression, went up exponentially in the space of a year. Large quantities of a highly popular medicinal plant in the food industry, *Agathosma spp.* (Buchu), are being exported around the world.

1.5 Ligand fishing

Ligand fishing is a recently introduced methodology, which relies on the ability of bioactive molecules contained in crude extracts to selectively and reversibly bind to certain biological macromolecules, which in most cases represent the so-called protein receptors. This process is generally designed for the purpose of triggering off a cascade of biochemical reactions in the relevant organism, or to facilitate the transportation of certain molecules in the blood stream, depending on the location of the receptor in question. Given that most plant extracts are mostly likely to contain compounds with these characteristics and capacity, it should be considered worthwhile to include this method as one of the routine ways of studying medicinal plant extracts

In drug discovery and drug development, the receptor theory accounts for mechanism of drug action, and the relationship of drug action to its molecular structure (Brenner and Stevens, **2006**). Ligand fishing is a method, therefore, that uses the principles of the receptor theory to screen cellular and botanical extracts for potential ligands. The ligands may be of orphan or known receptors. Ligand fishing is thus very useful in detecting bound ligands (bioactive substances) from botanical extracts (Catimel *et al.*, **2000**).

In a study by Qing *et al*. (**2010**), saponin ligands from *Dioscorea nipponica* were investigated for their binding capacity to human serum albumin (HSA), using HSA functionalized magnetic particles (MNPs). HSA is understood to be the most abundant protein in human blood plasma, where its function is the transport and deposition of ligands (Yamasaki *et al.*,

1996). It has a three-dimensional structure with multiple coordination sites for different ligands, including fatty acids, amino acids, drugs and hormones (Carter and Ho, **1994**). Binding affinity towards HSA determines the ligands' overall distribution, metabolism and efficacy; therefore a study into their binding capacity to HSA is very important. In chemical and biological sciences, MNPs owe their wide applications to three properties: suspension stability, easy solid-liquid separation, and easy surface modification. Ligand fishing based on MNPs has broad applications in the purification of proteins.

Qing *et al*. (**2010**) discovered that three saponin ligands from a *Dioscorea nipponica* ethanol extract demonstrated a binding affinity to HSA. Of the five saponins previously detected in the extract, two showed no affinity at all towards HSA. Qing *et al*. (**2010**) concluded that binding affinity towards HSA is highly dependent on the chemical structure of the ligand and, therefore, medicinal applications, since it may be imagined that possibly, only those ligands with a capacity to bind to HSA are likely to be distributed in the blood stream, and hence able to reach the remote tissues or organs where they are expected to express activity.

1.6 Plant species studied in this thesis

1.6.1 *Myrothamnus flabellifolius*

There are some plant species that have, over the years, acquired a rare ability to tolerate regular periods of severe dehydration (desiccation), and yet resume full metabolic activity on rehydration (Gaff, **1971**). These plants are generally called resurrection plants. Southern Africa has a rich diversity of resurrection plants. Among these, *Myrothamnus flabellifolius* (resurrection bush in English, Moritela Tshwene in Setswana, Opstandingsplant/Bergboegoe in Afrikaans, Vukwabafile in Zulu) is a woody shrub which stands at 0.4 m in height, with

strong, rigid branches. The plant has very small aromatic leaves that are toothed on the margins, and the plant usually grows in rocky areas as shown in Figure 5.

Figure 5: *Myrothamnus flabellifolius* in habitat on a: A) dry season and B) rainy season.

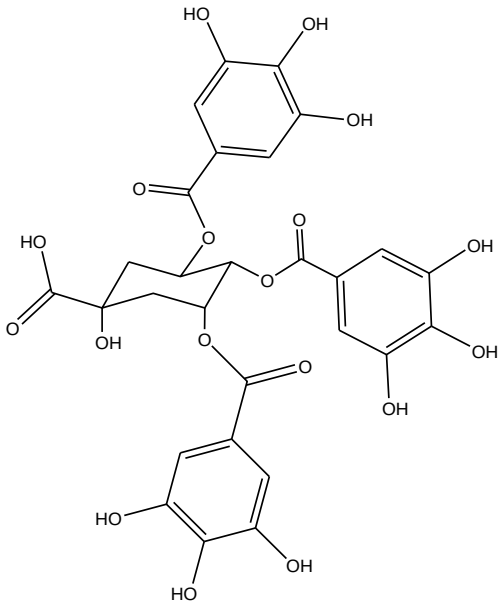
Myrothamnus is the only genus that belongs to the family Myrothamnaceae, a family of flowering plants. There are two currently known *Myrothamnus* species, namely: *Myrothamnus flabellifolius* and *Myrothamnus moschatus*.

Myrothamnus flabellifoolius is distributed throughout the rocky areas of Namibia, Botswana, South Africa (North Western part of the North West Province and Limpopo) and Zimbabwe. The leaves of the plant have a strong aromatic character, and together with the twigs are used in many medicinal preparations. Traditionally, the leaves of *M. flabellifolius* are burnt and the

smoke inhaled to treat chest pains and asthma, or the leaves are smoked in pipes. Some people chew the aromatic leaves for the treatment of mouth ailments, and some traditional healers use the plant to treat epilepsy, madness and coughs. *M. flabellifolius* has long been used as a nutritional herbal tea by the community of the Mokgola Village near Zeerust (North West Province, South Africa). The plant is taken as a tea with some reported minor health benefits. However, little is known about the nutritional content, the safety, the antioxidant properties, and the carbohydrate content of the plant. Studies on these topics, and more, could assist with the development of a branded commercial herbal tea which may be marketed both locally and internationally. In turn, commercialization of this tea should contribute to the economy of the Mokgola community (and that of the country), thus alleviating poverty in the area.

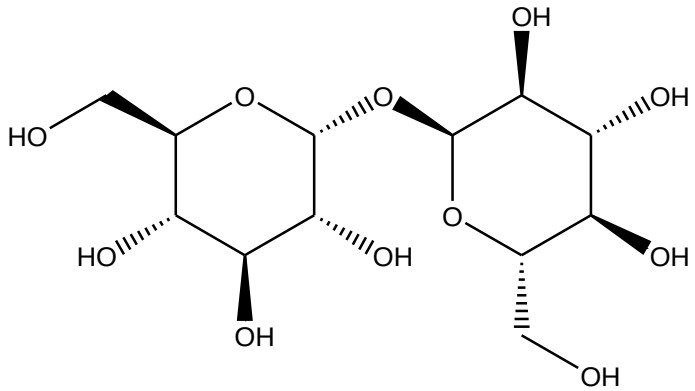
1.6.1.1 Previously isolated compounds from *M. flabellifolius*

High polyphenol (tannins) levels have been reported to be present in the leaves of *Myrothamnus flabellifolius* (Moore *et al*., **2007**). The most predominant polyphenol was found to be 3,4,5 tri-*O*-galloylquinic acid (Moore *et al.*, **2005**).



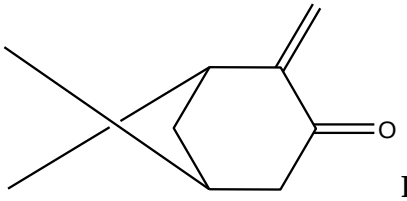
3,4,5 tri-*O*-galloylquinic acid

Moore *et al.* (**2005**) further suggested that 3,4,5 tri-*O*-galloylquinic acid is the main compound that is responsible for providing protection to the plant against dying from desiccation, in addition to its free radical scavenging properties. The leaves of the plant have also been reported to contain a disaccharide called trehalose, which is also known to provide protection against desiccation, due to its high water retention capabilities (Moore *et al.*, **2007**).

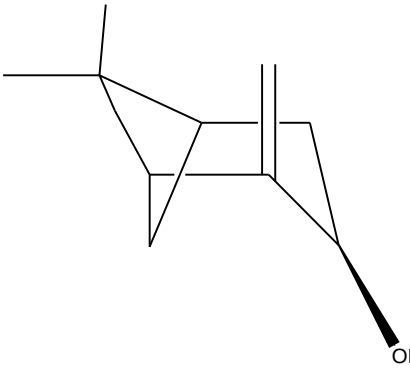


Trehalose

Van Wyk *et al.* (**2002**) also reported about 85 compounds contained in the hydro-distilled essential oils, with pinocarvone and *trans*-pinocarveol being identified as the major constituents in the oil.



Pinocarvone



***trans*-pinocarveol**

A qualitative phytochemical screening conducted by Molefe-Khamanga *et al.* (**2012a**), on the leaves of *M. flabellifolius*, revealed the presence of phenolics, tannins, alkaloids, flavanoids, reducing sugars, steroids, proteins and amino acids in both the methanol and water extracts. Such chemical composition, as observed in *M. flabellifolius*, may be implicated in a number of biological activities exhibited by the plant (see Table 2). In a separate study, Molefe-Khamanga *et al.* (**2012b**) reported that extracts from *M. flabellifolius* do not contain any caffeine, and hence the plant may be classified as a source of naturally caffeine free tea, thus making it suitable as a health tea.

However, *M. flabellifolius* shows differences in its chemical composition depending on the location, the nature of the soil and climate in which the plant grows. Previous studies have shown differences in the polyphenol composition between the South African and Namibian populations (Moore *et al.*, **2007**).

Table 2: **Reported biological activities of compounds and extracts from *M. flabellifolius*.**

Biological activity	**Part(s) of the plant**	**Compound(s)**	**References**
Anti-HIV reverse transcriptase	Leaves and twigs	3,4,5tri-*O*-galloylquinic acid	Moore *et al.*, **2005**
Antioxidant	Leaves Water extract	Trehalose	Moore *et al.*, **2007** Motlhanka and Mathapa, **2012**

1.6.2 *Sutherlandia frutescens*

Sutherlandia frutescens (syn. *Lessertia frutescens* (L.), *Colutea frutescens* L.) is a flowering plant which belongs to the family Fabaceae, the second largest flowering plant family in the world (see Figure 6). In nature, *S. frutescens* is a woody, shrubby plant which possesses red flowers and slightly hairy leaves that appear silvery (Van Wyk *et al*., **1997**). The plant grows to an approximate height of 1m. *S. frutescens* is widely distributed in the dry regions of South Africa (Western Cape and the Karoo region), Namibia and Botswana. The plant is very popular amongst different cultural groups, hence it is known by a variety of vernacular names such as: “cancer bush, *Sutherlandia*, scarlet balloon pea” (English), “kankerbossie, gansies, grootgansies” (Afrikaans), “umnwele” (Xhosa), “insiswa” (Zulu), “lerumo-lamadi, musapelo, motlepelo” (Sesotho), “phetola” (Tswana).

Figure 6: Leaves and flowers of the *S. frutescens* plant (www.treknature.com).

The Zulu name, "insiswa", which means "dispels darkness", was conceived due to the ability of *S. frutescens* to alleviate depression and sadness (Van Wyk and Albrecht, **2008)**. Some hundred years ago, Zulu women used this plant as a gentle tranquilizer/antidepressant when grieving the loss of their warrior spouses in wars. The warriors themselves used it to relax and recover. The Tswana name, "phetola", which means "to change", also alludes to the ability to alleviate stress related conditions. The first people to use the plant for medicinal purposes, however, were the Khoi San and Nama people, who used it primarily as a decoction for washing wounds and also took it internally to reduce fevers. Traditional healers have long used the plant in the treatment of a number of ailments, such as asthma, cancer (prevention and treatment), poor appetite, HIV/AIDS, cachexia (muscle wasting), diabetes, fever, colds, cough, influenza, indigestion, heartburn, heart failure, peptic ulcers, kidney and liver ailments, urinary tract infections, etc (Van Wyk and Albrecht, **2008**). The leaves of the

plant are the most commonly used, but other aerial parts can also be used in making decoctions (Van Wyk, **1997**).

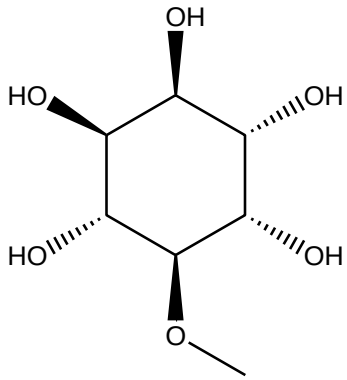
The commercially available *S frutescens* contains only selected leaves and tender stems (Brevoort, **1998**). Other parts of the plant such as flowers, pods and seeds are discarded. During processing, the leaves and stems are dried and ground into a powder. In retail shops, *S. frutescens* may be found as capsules which have been gamma irradiated so as to eliminate microbiological contamination. The recommended daily therapeutic dosage of *S. frutescens* is 9.0 mg/kg body weight per day in humans (Seier *et al*., **2002**). In South Africa, a pharmaceutical company called Phyto Nova is the major distributor of commercial products derived from *S. frutescens*.

1.6.2.1 Previously isolated compounds from *S. frutescens*

The biological activities of *S. frutescens* may be attributed to the presence of a variety of biologically active compounds. Some of the compounds which have been isolated from *S.frutescens* include; pinitol, *L*-canavanine, gamma-aminobutyric acid (GABA), triterpenoid glycosides, flavanol glycosides, and more (Fu *et al*., **2010**; Van Wyk and Albrecht, **2008**).

Pinitol

Early studies by Snyders (**1965**), Viljoen (**1969**) and Brummerhoff (**1969**) on *Sutherlandia microphylla* leaves, showed the presence of a mono-methylated form of D-chiro-inositol called pinitol (also called 3-O-methyl-chiro-inositol) for the first time.

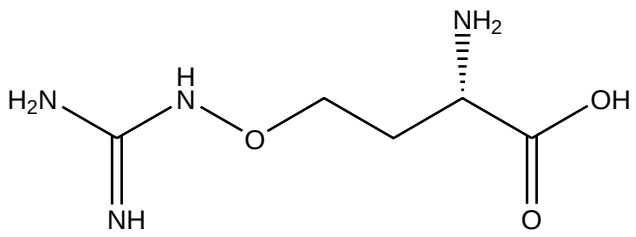


Pinitol

The recorded bio-activities of pinitol make it a potentially important compound in the context of the traditional uses of *Sutherlandia* against diabetes and inflammation (Moshe, **1998**). Besides acting as an anti-diabetic agent, pinitol is also used in the treatment of wasting in cancer and AIDS patients (Ostlund and Sherman, **1996**). It has also been documented that pinitol exerts an acute and chronic insulin-like effect (hypoglycaemic effect), resulting in lower blood sugar levels and increased availability of glucose for cell metabolism (Bates *et al*., **2000**), and enhances the retention of creatinine by muscle cells (Greenwood *et al*., **2001**). Overall, pinitol therefore seems to play a role in regulating cellular energy, resulting in increased energy levels and a reduction in fatigue (Van Wyk and Albrecht, **2008**).

L-canavanine

L-canavanine is a non-protein amino acid, most commonly found in the seeds of the family Fabaceae.

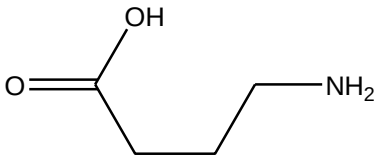


***L*-canavanine**

The detection of *L*-canavanine in high concentrations in the *Sutherlandia* leaf extracts (Moshe, **1998**), provided some justification for the traditional use of *Sutherlandia* against cancer. *L*-canavanine has been reported to have anti-cancer as well as anti-viral activity, including inhibition of the influenza virus and retroviruses, such as HIV (Green, **1988**). *Sutherlandia* is the first known canavanine-containing plant for which anticancer activity has been documented (Bence *et al*., **2002**; Van Wyk and Albrecht, **2008**).

Gamma-aminobutyric acid (GABA)

Gaba is an amino acid which is a product of decarboxylation of glutamate by the enzyme *L*-glutamic acid-1-decarboxylase (Ebadi, **2007**).

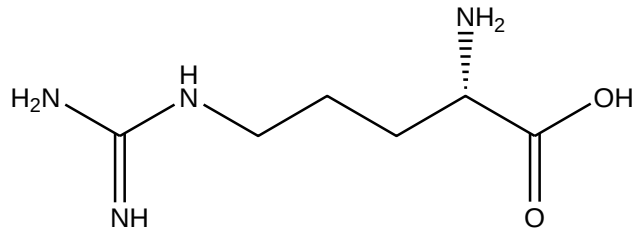


GABA

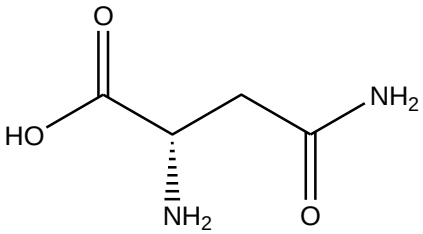
Gaba acts as an inhibitory neurotransmitter which, together with inositol and nicotinamide, is responsible for blocking anxiety and stress related impulses from reaching the motor centers of the brain. In cases of epilepsy and hypertension, Gaba induces calmness in patients (www.anyvitamins.com). As such, Namaqualand residents are reported to smoke *Sutherlandia* seeds and leaves as a dagga substitute, with the leaves invoking a strong GABA-induced sedative effect when smoked (Van Wyk and Gericke, **2000**). GABA has also been found to inhibit tumour cell migration (Ortega, **2003**).

Free Amino Acids

The leaf extracts of *Sutherlandia* contain high concentrations of free and protein-bound amino acids, such as *L*-arginine, *L*-asparagine and proline (Van Wyk and Albrecht, **2008**), whose structures are shown below. The presence of *L*-arginine is particularly important, because it acts as an antagonist of *L*-canavanine that reduces the anti-proliferative activity of canavanine.



***L*-arginine**

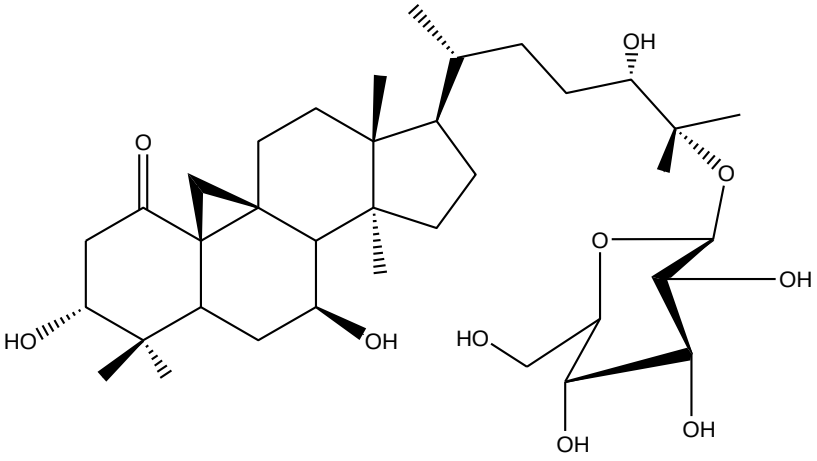


***L*-asparagine**

Saponins

Brummerhof (1969) and Viljoen (1969) detected the presence of triterpene glycosides in *Sutherlandia microphylla*, but the compounds were not characterized. A phytochemical study of *S. frutescens* conducted by Fu *et al*. (**2008**), led to the isolation and identification of four cycloartane-type triterpene glycosides, namely; Sutherlandioside A, B, C and D. These saponins have a bitter taste that may stimulate appetite and contribute to the adaptogenic and immune-boosting effects of *Sutherlandia*. Sutherlandioside B, C and D are the first examples of naturally occurring cycloartanes with a C-1 ketene function, but the exact role they play in

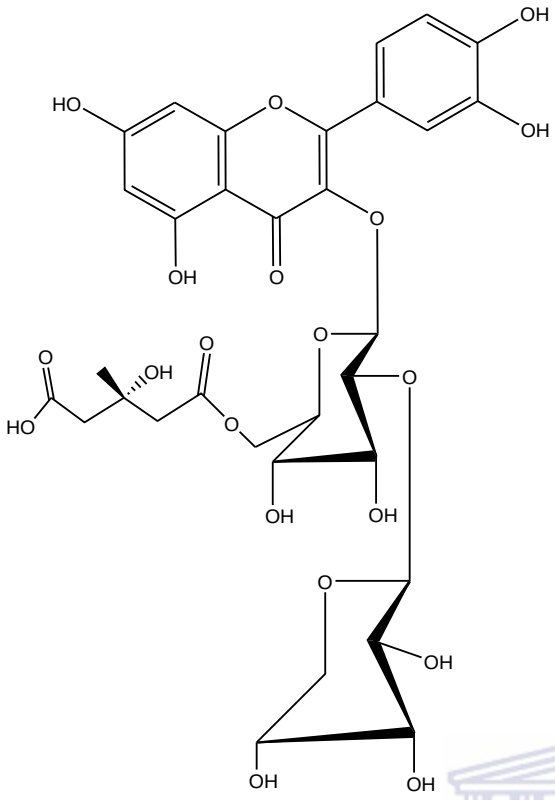
phytotherapy is still unknown (Fu *et al*., **2008**). The major triterpene in commercially available *S. frutescens*, is sutherlandioside B shown below.



Sutherlandioside B

Flavonol glycosides

In a study conducted by Moshe (**1998**), at least six flavonoids were detected in the leaf extracts of *Sutherlandia*. Four of these were subsequently identified as flavonol glycosides; Sutherlandin A, B, C and D (Fu *et el*., **2010**). Flavonoids are important in phytotherapy because they possess anti-oxidant activity, and may play a critical role in cancer prevention through the ability to eliminate mutagens and carcinogens (Tai *et al*., **2004**). A typical structure of one of the flavonol glycosides isolated from *S. frutescens* is shown below.



Sutherlandin A

Other compounds

High concentrations of unidentified polysaccharides have been reported in aqueous leaf extracts from *S. frutescens* (Van Wyk and Albrecht, **2008**). Hexadecanoic acid, γ-sitosterol, stigmast-4-en-3-one and at least three long chain fatty acids have also been reported (Tai *et al*., **2004**).

1.6.2.2 Biological properties of *S. frutescens* extracts.

Most medicinal plants owe their toxicity to one or a combination of the following factors; type of metabolites present in the plants, contaminants, adulterants, and their interaction with other plant constituents or drugs. In terms of traditional use, history shows that *S. frutescens* is one of the safest plants to use, with only a few reported side effects associated with its use.

Some of the reported side effects include dry mouth, occasional mild diarrhea, dizziness and mild dieresis (Mills *et al*., **2005**).

In a study conducted by Ojewole (**2004**), it was demonstrated that the crude extracts from *S. frutescens* are relatively safe to use in mammals. In addition, an extensive toxicology study which was carried out on vervet monkeys using higher doses than the recommended daily dose (9.0 mg/kg) clearly showed that *S. frutescens* did not exhibit any significant clinical or physiological toxicity (Seier *et al*., **2002**). However, studies on the long term use of plant extracts have yet to be documented. It is advised that during pregnancy, use of *S. frutescens* extracts should be avoided.

The pre-isolated compounds from *S. frutescens* are known to evoke a few pharmacological effects and recent scientific interest has led to the identification of several biological targets (Table 3).

Table 3: **Some reported biological activities of compounds and extracts from *S. frutescens*.**

Biological activity	**Part(s) of the plant**	**Compound(s) or extract**	**References**
Anti-diabetic	Leaves	Pinitol *L*-canavanine and *L*-arginine Aqueous extract	Moshe *et al.*, **1998**; Bates *et al*., **2000;** Sia, **2004**; Ojewole, **2004**

Biological activity	**Part(s) of the plant**	**Compound(s) or extract**	**References**
Anti-cancer	Leaves	*L*-canavanine Triterpenoids Ethanolic extract Dichloromethane (DCM) extract	Van Wyk and Gericke, **2000**; Seier *et al*., **2002**; Tai *et al*., **2004**; Reid *et al*., **2006**; Kikuchi *et al*., **2007**
Anti-HIV	Leaves	Aqueous and Organic extracts	Gericke *et al*., **2001**; Chaffy and Stokes, **2002**; Harnett *et al*., **2005**; Bessong *et al*., **2006**; Johnson *et al*., **2007**; Katerere and Rewerts, **2011**
Anti-inflammatory and antibacterial	Leaves	Aqueous and hexane extracts Flavonol glycosides *L*-canavanine	Green, **1988**; Fernandez *et al*., **2004**; Ojewole, **2004**; Katerere and Eloff, **2005**
Stress	Leaves	Gaba	Prevoo *et al*., **2004**; Sia, **2004**; Smith and Myburgh, **2004**;Tai *et al*., **2004**

1.7 Problem statement

Africa is one of the richest continents with respect to biodiversity, blessed with well over 50 000 different plant species. Many of these plants have been found to be very useful in traditional medicine, on which about 80% of the continent's population fully or partly relies for primary health care needs. However, only a few of these medicinal plant species have been commercialized as branded medical products. According to Van Wyk and Wink (**2004**), Africa has only 83 medicinal plants that have been fully or partly commercialized. This can be attributed to a lack of extensive research studies and development activity conducted on most of the plants. As such, safety issues on how to handle or administer the crude extracts from these plants are not fully understood, and this problem extends to their pharmacological efficacy and activity. *M. flabellifolius* is just one of the plant species that has not been commercialized. Traditionally, *M. flabellifolius* has been used as a nutritional herbal tea, and in the treatment of a variety of ailments including asthma, epilepsy, etc. Thus, chemical studies on *M. flabellifolius* may lead to the isolation of some bioactive natural products, which may be used as precursors in the synthesis of new drugs.

An example of a commercialized medicinal plant is *S. frutescens*, a southern African plant popularly used in the treatment and prevention of cancer and HIV. The plant contains compounds which are believed to have anticancer and anti-HIV properties. However, the mode of action related to these activities is not well understood. Since it is believed that bioactive compounds ought to be transportable in the blood stream and hence have the capacity to bind to human serum albumin (HSA), a part of this study was designed to investigate the use of magnetic nanoparticles on which HSA is anchored, in order to examine whether these compounds would readily bind to HSA.

1.8 Hypothesis

As a result of the high cost of drugs, and the alarming rate at which drug-resistant pathogens are emerging, medicinal plants may be used as an alternative source to conventional drugs, thus taking advantage of the natural product constituents present in such plants. Hence, isolation and characterization of such compounds may contribute to a better understanding of their role or otherwise in relevant pharmacological activities.

1.9 Aim of the study

The aim of the study was to isolate and characterize the natural products from the leaves of *M. flabellifolius* and to use *S. frutescens* as a model plant to assess the capacity of selected compounds to bind to human serum albumin (HSA).

1.10 Objectives of the study

The specific objectives were to:

1) Perform solvent extraction on the leaves of both *M. flabellifolius* and *S. frutescens*.
2) Isolate and characterize natural products from the crude extracts of *M. flabellifolius* using chromatographic and spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS).
3) To examine the capacity of selected compounds to bind to HSA.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and general methods.

2.1.1 Reagents

Solvents: *n*-hexane, dichloromethane, ethyl acetate, butanol, methanol, deuterated methanol and deuterated chloroform were purchased from Sigma-Aldrich, South Africa. $FeCl_3$, Na_2SO_3, tetraethyl orthosilicate (TEOS), 3-aminopropyltrimethoxysilane (APTMS), coupling buffer (10mM pyridine, pH 6.0), human serum albumin (HSA), reaction-stop buffer (1M glutaric acid, pH 8.0), the wash buffer (10mM Tris, pH 7.4) and ammonium 10mM acetate buffer (pH 7.4) were all purchased from Sigma-Aldrich, South Africa. Dextran standards with molecular weights 10 000Da, 70 000 Da, 110 000 Da, 150 000 Da and 500 000 Da were purchased from Amersham Biosciences, Sweden.

2.1.2 General methods

2.1.2.1 Solvent Evaporation

Solvent evaporation was performed on a Buchi Rotavapor RE 111, with the temperature of the water bath maintained at 45^0C.

2.1.2.2 Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Varian-200 NMR, Agilent-600 NMR at the University of Stellenbosch, and Bruker-600 NMR at the University of the Free State. The chemical shifts were expressed in δ (ppm), and coupling constants (*J*) in Hz.

Fourier Transform -Infrared Spectroscopy (FT-IR Spectroscopy)

IR spectra of powdered material were recorded on a Perkin–Elmer paragon 1000 spectrometer that had been corrected against an air background, at a resolution of $3cm^{-1}$.

2.1.2.3 Chromatography

Column chromatography (CC)

Silica gel 60 (0.040-0.063mm) 230-400 mesh particle size (Merck) was packed in glass columns (20-25mm diameter) for column chromatography. Size-exclusion chromatography was performed using Sephadex® LH-20 (Pharmacia), eluting with MeOH:CH_2Cl_2 (1:1). Ion exchange chromatography of polysaccharide fractions was carried out using DEAE Sepharose CL-6B (Sigma Aldrich), eluting with 0.1-1.0M NaCl. Gel filtration chromatography was carried out using Sephacryl S-400 HR (Pharmacia), eluting with 1.0M NaCl.

Thin layer chromatography (TLC)

Thin layer chromatography was carried out on pre-coated silica gel 60 F_{254} plates (Merck) with a 0.2 mm layer thickness. Visualisation of the TLC spots was carried out under UV light at 254nm and/or 366nm, and further detection of compounds was achieved by spraying with

vanillin spray reagent (prepared by dissolving 15g of vanillin in 250ml ethanol followed by the addition of 2.5ml concentrated sulphuric acid). After spraying, the TLC plates were heated on a hot plate until spots became visible.

2.1.2.4 Mass Spectrometry (MS)

Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analyses were performed at the University of Stellenbosch Central Analytical Facility.

LC-MS method

LC-MS analysis was conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer. The instrument was connected to a Waters Acquity ultra-performance liquid chromatography (UPLC) and Acquity photo diode array (PDA) detector. Ionisation was achieved with an electrospray source using a cone voltage of 15V and capillary voltage of 2.5 kV and positive mode was utilized. Nitrogen was used as the desolvation gas at 650 l/h and the desolvation temperature was set to 275˚C. A Walters UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size) was used and 3µl was injected for each analysis. The gradient started with 100% using 0.1% (v/v) formic acid (solvent A) and this was kept 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B for an additional 2 min. The column was then re-equilibrated over 1 min to yield a total run of 25 min. A flow rate of 0.4 ml/min was applied

GC and GC-MS method

Separation of the monosaccharide derivatives was achieved on an Agilent 6890N GC instrument with CTC CombiPAL Autosampler and Agilent 5975B MS spectrometer with a

DB 225 capillary column (30 m x 0.25 mm internal diameter x 0.25 μm film thickness), Model Number: J&W 122-2232, at 215°C isothermal temperature, FID detector at 300°C, injector temperature at 250°C and injector split 20:1. Positive ionisation mode was utilized and the flow rate was set at 2 ml/min.

2.2 Plant material

Powdered plant material of *Myrothamnus flabellifolius* was provided by the Medical Research Council (MRC) following collection from the Mokgola village, near Zeerust in the North West Province, South Africa.

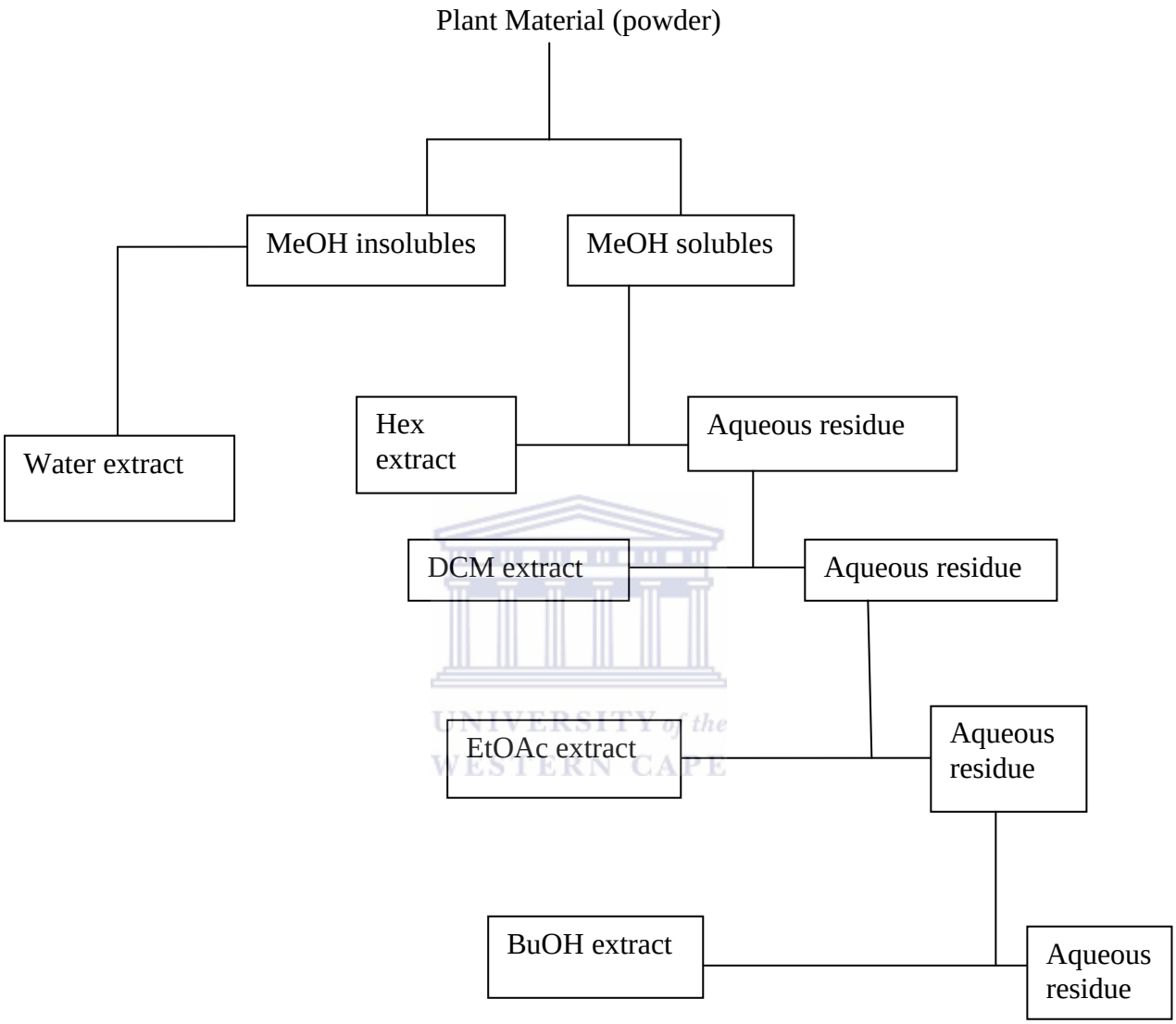
Sutherlandia frutescens powder was supplied by Afriplex, a commercial manufacturing company located in Paarl, South Africa.

2.3 Preparation of extracts

Extraction of both plant species, *Myrothamnus flabellifolius* (200g) and *Sutherlandia frutescens* (50g), was carried out as illustrated in Scheme 1. The powdered leaf material was extracted with methanol (MeOH). The methanol extract was evaporated to dryness under reduced pressure on a rotary evaporator. The dried extract was suspended in water and partitioned successively with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and butanol (BuOH). Each extract was concentrated to dryness under reduced pressure.

The methanol insoluble material was extracted with water, and then lyophilized to yield the water extract. Purification and isolation of natural products was achieved through one or a combination of chromatographic techniques.

Scheme 1: A flow diagram representing preparation of extracts.



2.4 Isolation of natural products from *M. flabellifolius*

2.4.1 Fractionation of the BuOH extract

The BuOH extract (1 g) was dissolved in a mixture of methanol: dichloromethane (1:1), and then chromatographed on Sephadex LH-20 column. A mixture of methanol: dichloromethane

(1:1) [500ml] was used as an eluent. Fractions were collected and analyzed by TLC using a $CHCl_3$:MeOH:H_2O (200:52:6) mixture as eluent. Fractions labelled 4-9 showed similar TLC profiles, and thus were pooled together for further purification on the Sephadex column, using the same eluent as above. Fractions were collected and analyzed by TLC as previously described. Pooling of the relevant fractions resulted, after evaporation of the solvent, in the isolation of pure compound **1** (8.5 mg) as a faint yellow powder.

Acetylation of compound 1

Compound **1** (8.5 mg) was dissolved in a mixture of pyridine and acetic anhydride (2:1 v/v), and then stirred overnight at room temperature. To the mixture, an ice-water mixture (50ml) was added, followed by vigorous stirring for 0.5-1.0 hour. The mixture was extracted with 70ml DCM, and the extract was washed twice with 50ml of water, alternating with 50ml of 1.0M HCl each time, and then twice with water alone until free of pyridine and acetic acid. The DCM layer was dried over anhydrous Na_2SO_4, followed by solvent evaporation. The acetylated product was analyzed by NMR spectroscopy

2.4.2 Fractionation of the EtOAc extract

The EtOAc extract (10 g) was pre-adsorbed on silica gel before loading on a column. Fractionation was achieved through gravity column chromatography with the use of gradient elution. Eluents employed were 500ml volumes of DCM: EtOAc mixtures at ratios; (100:0), 90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90), (0:100); followed by 500ml volumes of EtOAc: MeOH mixtures at ratios; (95:5), (90:10), (85:15), (80:20), (75:25), (70:30), (65:35), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90), (0:100). Fractions were collected and analyzed by TLC using $CHCl_3$:MeOH:H_2O (200:52:6)

as an eluent mixture. Fractionation resulted in the isolation of pure compound **2** (23 mg) as a white crystalline material.

2.4.3 Purification and fractionation of H_2O extract

Ethanol precipitation

The water extract (10 g) was dissolved in water, and then ethanol was added to a final concentration of 80%. The mixture was centrifuged at 4000rpm for 20min. The centrifugate was redissolved in water and subjected to three more ethanol precipitations. The precipitate was washed with ethanol, centrifuged, and then dissolved in water and dialyzed. To obtain the crude polysaccharide (3.7 g), the precipitate was freeze dried. The crude polysaccharide (about 1 mg) was analyzed using FT-IR spectroscopy by incorporating the powdered material into KBr disc. The IR spectrum was recorded within the range of 4000-300 cm^{-1}.

Determination of Uronic Acid content

The uronic acid content of the crude polysaccharide was determined by the carbazole assay. An aliquot of the polysaccharide solution (0.5 ml) at (100 µg/ml), standard solutions (5, 10, 20, 40, 50 µg/ml of uronic acid) and control (distilled water) were placed in a test tube. Reagent A (1.8 mg sodium tetraborate decahydrate; 20 ml distilled water; 180 ml of 98% concentrated H_2SO_4 that was cooled in ice) (3 ml) was added to each and mixed. The closed tubes were shaken gently at first and then vigorously with constant cooling in an ice bath. The test tubes were heated at 100ºC for 10 minutes in a boiling water bath and then cooled rapidly in an ice-bath. Reagent B (100 mg carbazole; 100 ml absolute EtOH) (100 µl) was

then added. The samples were re-heated at 100ºC for 15 minutes, cooled rapidly to room temperature and the absorbance was measured at 525 nm on a spectrophotometer.

Composition analysis of polysaccharides

A standard mixture of known monosaccharides: rhamnose, arabinose, xylose, mannose, galactose and glucose (5 mg each) was dissolved in distilled water (3 ml). The mixture was reduced with 100mg of sodium borohydride ($NaBH_4$). The mixture was converted into alditol acetates similarly to the polysaccharide rich fraction as described in (i).

(i). The crude polysaccharide (5-50 mg) was hydrolyzed with 2M Trifluoroacetic acid (TFA) (2-5 ml) at 100°C. TFA was removed by successive addition and evaporation (5X) of MeOH. The residue was dissolved in 5 ml (4 H_2O + 1 MeOH). 25% Aqueous ammonia solution (2 drops) was added to neutralise residual TFA and then $NaBH_4$ (~100 mg) was added. The contents were mixed by gentle swirling and allowed to stand overnight. The reaction mixture was acidified to ~pH 6 with drop wise addition of acetic acid followed by evaporation with MeOH as described above. To the resulting dry white/colourless residue, was added acetic anhydride (3-5 ml), and the mixture was heated at 100°C (steam bath) for 2 hours with occasional swirling.

Crushed ice (~30 ml) was added, followed by vigorous stirring for 0.5-1 hour in order to destroy excess acetic anhydride. Addition of $CHCl_3$ (~30 ml) was followed by vigorous shaking of the contents for about a minute, which were then transferred into a separating flask. Removal of the aqueous layer was followed by washing of the $CHCl_3$ layer with distilled water (~20 ml) three times. Anhydrous Na_2SO_4 was added to dry the $CHCl_3$ layer

(by allowing it to stand for 10-15 minutes with occasional swirling) and the Na_2SO_4 was filtered off.

(ii) An aliquot of the reconstituted residue in $CHCl_3$ was analysed using GC and GC-MS. A $CHCl_3$ solution of the standard alditol acetates was injected in order to determine their retention times and mass.

Methylation

The following procedure was performed under nitrogen gas, using dry glassware, in order to minimize interaction with moisture. The crude polysaccharide (5-40mg) was dissolved in 2-5ml of dimethyl sulfoxide (DMSO). To the mixture, NaOH powder (200-500mg) was added, followed by stirring for 1 hour. The sample mixture was frozen before the introduction of MeI (3-4ml). Upon addition of MeI, the flow rate of nitrogen gas was slowed down. The reaction mixture was then sealed with silicon septum and stirred overnight.

The sample mixture was partitioned with water (7.5ml) and DCM (7.5ml) by shaking a separating funnel. The partitioning was repeated 3 times, and the organic layer was dried over Na_2SO_4. The methylation process was repeated by adding a mixture of Ag_2O and 4ml of MeI followed by refluxing for two days at 40^0C in order to ensure complete methylation. The resulting sample mixture was partitioned as described above. The organic phase was dried, and the methylated product was hydrolysed, reduced, acetylated and injected into a GC and GC-MS.

Ion exchange chromatography

The crude polysaccharide (1g) was chromatographed on a polyvinylpolypyrrolidone (PVPP) column and the resulting tannin-reduced fraction (100 mg) was purified on a DEAE Sepharose CL-6B ion exchange column, eluting stepwise with 70ml volumes of distilled

water and a NaCl gradient (0.1-1.0M) at a flow rate of 0.8 ml/min. Fractions (4ml) were collected, and for each fraction, the carbohydrate content was determined using the phenol-sulfuric acid assay (Dubois *et al*.,1956). The carbohydrate containing fractions were pooled together, dialyzed and freeze-dried.

Size exclusion chromatography

The carbohydrate-rich fraction was further purified on a Sephacryl S-400 gel permeation column, eluting with NaCl (1M) and collecting 4mL fractions. For each fraction, the carbohydrate content was determined using the phenol-sulfuric acid assay. The column was calibrated using a mixture of Dextran standards [Amersham Biosciences] with known molecular weights (10,000 Da; 70,000 Da; 110,000 Da; 150,000 Da and 500,000 Da).

2.5 Preparation of human serum albumin functionalized magnetic nanoparticles (HSA-MNPs) (Lou *et al*, **2006**)

A mixture of 1M $FeCl_3$ (8ml) and 0.5M Na_2SO_3 (4ml) was stirred under nitrogen. After a colour change from orange to yellow, 25% ammonia solution was added until pH 10. The colour of the resulting Fe_3O_4 solution changed to black. The solution was vigorously stirred for 15min, followed by separation and several washings with MeOH and de-ionized H_2O. The Fe_2O_3 solution (2ml) was dispersed in a mixture of ethanol (80ml), de-ionized H_2O (18ml) and concentrated ammonia solution (2.5ml), followed by addition of 0.5ml of tetraethyl orthosilicate (TEOS) under continuous stirring for about 8 hours. The silica coated nanoparticles were obtained through magnetic separation, with repeated washings (x4) with distilled water. The nanoparticles were then mixed in an ethanol solution (100ml) containing

0.1ml of 3-aminopropyltrimethoxysilane (APTMS). The mixture was stirred for 12 hours at 60^{o}C. The amino functionalized nanoparticles were obtained by magnetic separation, following repeated washings (x4) with ethanol. In a conical flask, the nanoparticles (5ml) were mixed with 15ml of the coupling buffer (10mM pyridine, pH 6.0), vortexed and followed by magnetic separation. The supernatant was discarded. The resulting beads were mixed with 20ml of glutaraldehyde solution containing the coupling buffer, followed by vortexing and gentle rotation for 3 hours. Magnetic separation led to the removal of the supernatant, followed by repeated washings (x3) of the beads with the coupling buffer. To the activated beads, HSA solution (50mg dissolved in 8ml of the coupling buffer) was introduced, followed by 24 hour gentle rotation at room temperature. The supernatant was removed through magnetic separation. An introduction of 20ml of the reaction-stop buffer (1M glutaric acid, pH 8.0) to the flask was followed by a 30min gentle rotation. The supernatant was removed through magnetic separation. The resulting nanoparticles were washed (x3) with the wash buffer (10mM Tris, pH 7.4) containing 0.15M NaCl, 1mM EDTA, 0.1% BSA, 0.1% sodium azide. The resulting HSA-MNPs were stored in ammonium 10mM acetate buffer (pH 7.4) until further use.

Ligand fishing from *S. frutescens* BuOH extract

Ligand fishing was carried out following the procedure described by Qing *et al.* (**2010**). A 1.3mg/mL solution of the BuOH extract (S0) was prepared in 10mM ammonium acetate buffer (pH 7.4). A portion of S0 (1ml) and 100µl HSA-MNPs were transferred to a 4ml Eppendorf tube. The tube was vortexed for 30 min, and then put on a magnet for 5min to achieve a liquid–solid separation. The supernatant (S1) was carefully transferred to a test tube and saved. The HSA-MNPs was washed three times (using 1mL buffer each time) with

vigorously shaking for 2min. After liquid–solid separation, the supernatants were carefully collected and saved as solutions S2, S3 and S4, respectively. The fourth wash of the HSA-MNPs was carried out with 1mL buffer containing 50% ACN for 2min. The supernatant was collected and saved as solution S5. In order to monitor the change of the components binding to HSA-MNPs, all the solutions were analyzed by LC-MS.

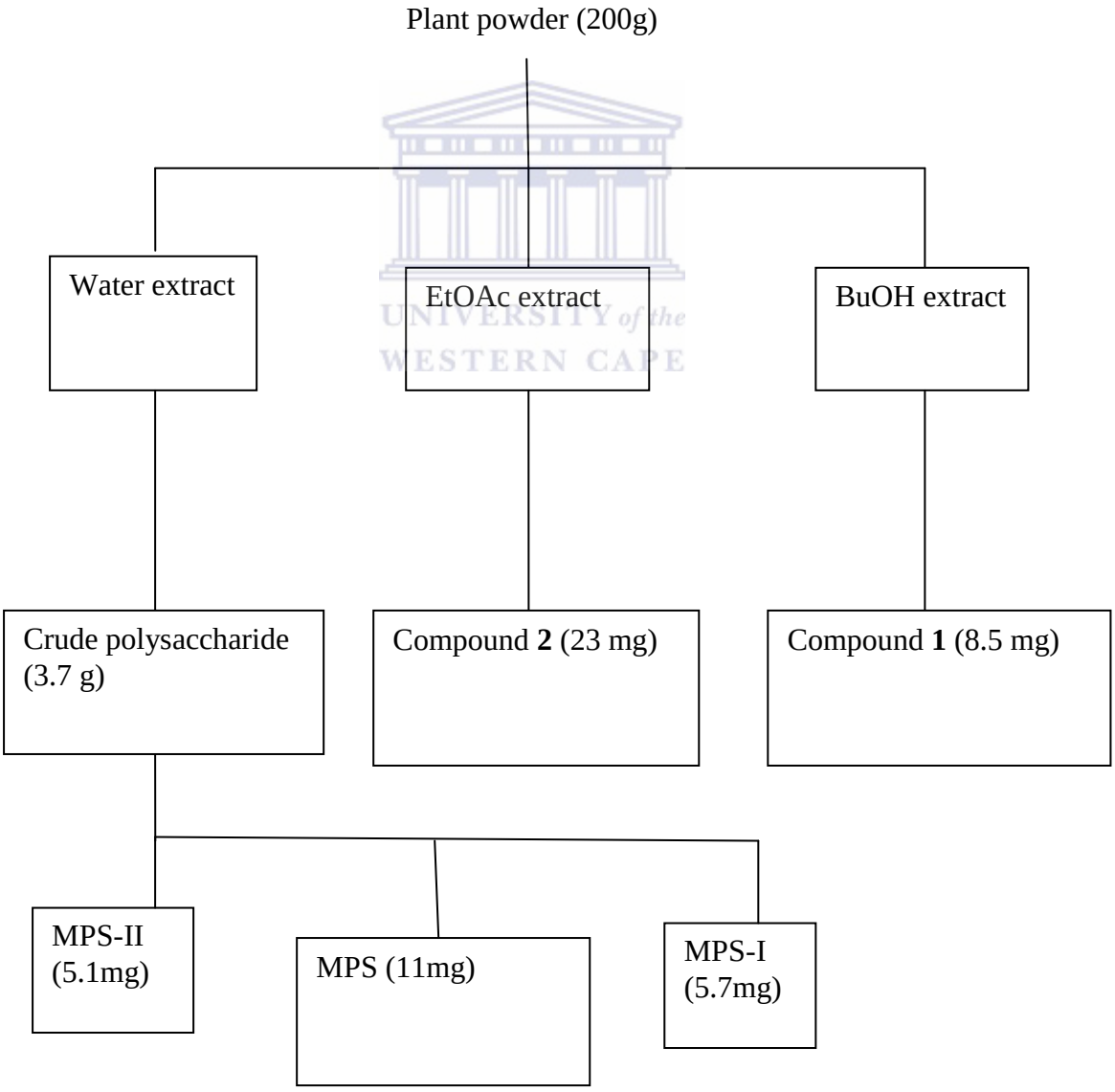
RESULTS AND DISCUSSION

CHAPTER 3

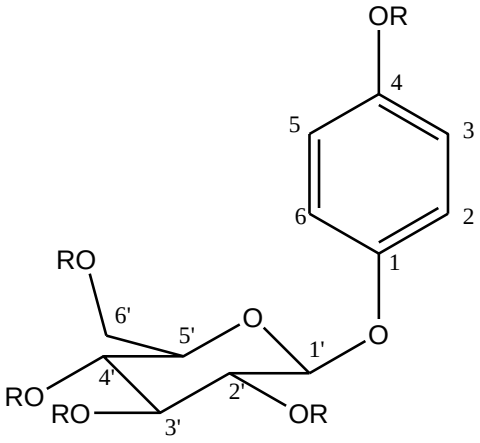
IDENTIFICATION OF NATURAL PRODUCTS FROM *Myrothamnus flabellifolius*

The isolation of natural products was carried out through a fractionation process as summarized in Scheme 2.

Scheme 2: A flow diagram representing a summarized fractionation process that led to isolation of natural products.



3.1 Compound 1 (4-hydroxyphenyl-β-D-glucopyranoside; Arbutin)



1: R=H

1a: R=Ac

Compound **1** was obtained as a faint yellow powder. The negative ESIMS revealed a molecular ion $[M-H]^-$ peak at m/z 271.0800, corresponding to the molecular formula $C_{12}H_{16}O_7$. Compound **1** was identified as a phenolic glycoside on the basis of ^1H and ^{13}C NMR spectra (see Table 4, Appendix 1). The ^1H NMR (600 MHz, CD_3OD) spectrum revealed the presence of two symmetric 2-proton doublets at δ 6.71 (H-3, H-5, *J*=8.8 Hz) and 6.96 (H-2, H-6, *J*=8.8 Hz), representing a para-disubstituted aromatic ring. The assignment of chemical shifts as H-2 and H-6 was made due to these being the more deshielded protons, as a result of their close proximity to the electron-deficient environment (created by the positioning of the glycosidic bond oxygen close to the sugar ring oxygen). H-3 and H-5 are less deshielded as a result of their close proximity to an electron-rich environment created by the aromatic ring hydroxyl group. Chemical shifts of a typical sugar moiety were also observed at δ 3.38 - 3.90 (6H), as well as a single-proton doublet at δ 4.75 (H-1', *J*=6.0 Hz) representing an anomeric proton. H-1' is the most deshielded proton of the sugar moiety protons since it is bonded to an anomeric carbon that is attached to two oxygen atoms, thus

positioned in an electron-deficient environment. The ^{13}C NMR spectrum showed the presence of an anomeric carbon at δ 103.6 (C-1'). Four signals were observed in the aromatic region, C-1 at δ 153.7 and C-4 at δ 152.4, and the other two at δ 119.4 (C-2 and C-6) and δ 116.6 (C-3 and C-5). The ^{1}H and ^{13}C NMR spectral data of compound **1** was similar to the data that had been reported for a known compound, arbutin, in the literature (Wiedenfeld *et al*., **2007**).

Table 4: ^{1}H and ^{13}C NMR Data (600 MHz, CD_3OD) for compound 1

Position	δ C	δ H
1	153.7	
2/6	119.4	6.96 (d, J = 8.8 Hz)
3/5	116.6	6.71 (d, J = 8.8 Hz)
4	152.4	
1'	103.6	4.75 (d, J = 6.0 Hz)
2'	74.9	3.38-3.45 (m)
3'	78.0	3.38-3.45 (m)
4'	71.4	3.38-3.45 (m)
5'	77.9	3.38-3.45 (m)
6'a	62.5	3.90 (d, J = 12.0 Hz)
6'b	62.5	3.62 (d, J = 7.0 Hz)

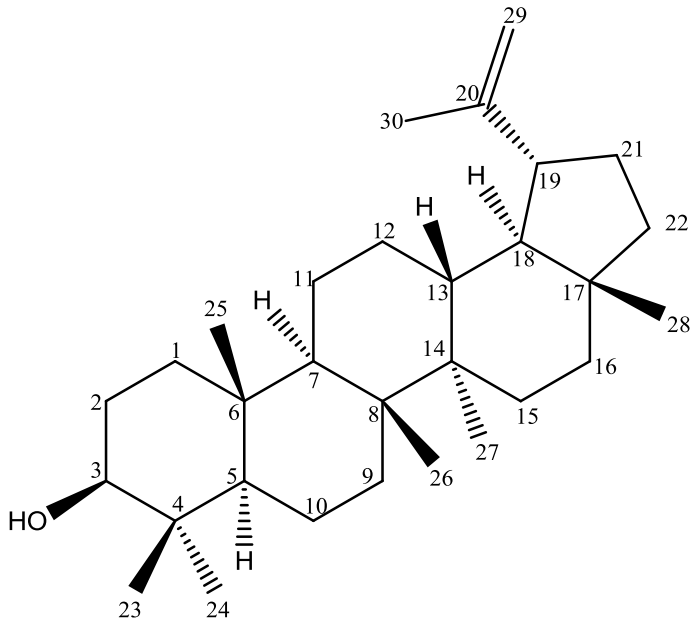
The ^{1}H NMR (200 MHz, $CDCl_3$) spectrum of the acetylated compound **1** showed a 3-proton singlet at δ 2.27 which could be attributed to the phenolic acetate protons. Four more 3-

proton singlets were observed at δ 2.06, 2.04, 2.03 and 2.01, and all could be attributed to the monosaccharide acetyl groups protons. In the aromatic region, the spectrum showed a 4-proton singlet at δ 6.98 which could be assigned to the chemically equivalent aromatic protons. Acetylation of compound **1** led to a noticeable downfield shift of the proton signals as a result of the presence of electron-withdrawing acetyl groups, thus becoming more deshielded. The ^{1}H NMR data: δ6.98 (4H, *s*), 5.28-4.99 (4H, *m*), 4.27 (lH, *dd*, *J*=12.4 and 5.4 Hz), 4.13 (lH,*dd*, *J*=12.4 and 2.7Hz), 2.27, 2.06, 2.04, 2.03, 2.01 (each 3H, *s*, 5 x OAc).

The ^{1}H NMR spectrum of the acetylated compound **1** was in agreement with that of an acetylated arbutin which was prepared from a commercially available sample. Therefore, compound **1** was confirmed to be arbutin. Arbutin had been isolated before from *Myrothamnus flabellifolius* in an acetylated form, i.e. as arbutin pentaacetate (Suau *et al*., **1991**)

Arbutin is a naturally occurring hydroquinone derivative that is used as a skin lightening agent and has been reported to decrease the melanin formation by inhibiting tyrosinase activity (Maeda and Fukuda, **1996**). It has also been reported that arbutin has antioxidant properties (Gerich, **2001**), as well as antihyperglycaemic and antihyperlipidemic (Shahaboddin *et al*, **2011**), antibacterial and antifungal activities (Kundakovic, **2014**). The anti-ulcer activity of *Turnera diffusa* was attributed to arbutin's immunomodulatory properties as well as lipid peroxidation inhibitory properties (Taha *et al*., **2012**).

3.2 Compound 2 (Lupeol; 20(29) lupen-3β-ol)



Compound **2** was obtained as a white crystalline material. It was identified as a pentacyclic triterpenoid on the basis of ^{1}H and ^{13}C NMR spectra (see Table 5, Appendix 2). The ^{1}H NMR spectrum revealed two olefinic protons that appeared as broad singlets at δ 4.67 (1H, *br s*, H-29b) and δ 4.54 (1H, *br s*, H-29a), which represent the exomethylene group. A doublet of doublet was observed at δ 3.12 (1H, *dd*, *J*=11.0 and 5.4 Hz, H-3) representative of the proton attached to the secondary carbon that is bonded to the hydroxyl group. A single proton signal was observed at δ 2.21 (1H, *m*, H-19). A broad 3-proton singlet was observed at δ 1.62 (3H, *br s*, H-30) indicating an isopropenyl function. The ^{1}H NMR spectrum also showed six 3-proton singlets at δ 0.75, 0.77, 0.83, 0.89, 0.90, 1.18 representatives of six tertiary methyl groups. The rest of the protons could be observed between δ 1.23 and δ 2.52 as complex multiplets.

The ^{13}C NMR spectrum of compound **2** showed that thirty signals, typical of a lupine-type triterpenoid skeleton. Two olefinic carbons of the exomethylene group appeared at δ 150.6

(C-20) and δ 109.8 (C-29), of which the olefinic carbon C-20 was the most deshielded. The spectrum also showed a signal at δ 79.1 (C-3) due to the carbon attached to a hydroxyl group.

Table 5: ^{13}C NMR Data (600 MHz, $CDCl_3$) for compound 2

Carbon	δ C	Carbon	δ C	Carbon	δ C
1	37.0	13	38.2	25	16.0
2	29.7	14	42.3	26	15.7
3	79.1	15	27.7	27	14.6
4	38.2	16	35.5	28	18.3
5	55.4	17	49.3	29	109.8
6	18.3	18	46.7	30	19.2
7	34.2	19	48.0		
8	40.9	20	150.6		
9	50.4	21	28.0		
10	42.3	22	35.6		
11	20.9	23	27.7		
12	25.7	24	14.6		

Compound **2** was thus identified and confirmed to be lupeol after comparing its ^{1}H and ^{13}C NMR spectral data with the spectral data of the reported compound (Abdullahi *et al*, **2013**; Prachayasittikul *et al*, **2010**; Ravi and Venkatachalapathi, **2012**; Venkata *et al*, **2012**). To our knowledge, this is the first time that lupeol has been isolated from *Myrothamnus flabellifolius*.

Lupeol has been reported to possess a wide range of health benefits, either as a preventive or therapeutic agent. Lupeol is known to exhibit antitumor, anti-inflammatory, antiprotozoal, antimicrobial and chemopreventive properties (Gallo and Sarachine, **2009**). It has also been shown to be an active antiarthritic agent (Agarwal and Rangari, **2003**), while its derivatives are active antimalarial agents (Kumar *et al*, **2008**).

3.3 Isolation and characterization of *M. flabellifolius* polysaccharides

The water extract from *M. flabellifolius* was investigated for the presence of polysaccharides. Following ethanol precipitation, crude polysaccharides were obtained at 37% yield. The low yield suggests that the water extract predominantly contained low molecular weight material, such as disaccharides/oligosaccharides, which were removed during dialysis. The crude polysaccharides were examined by FT-IR spectroscopy. The FT-IR spectrum (see Fig 7) of the crude polysaccharides showed stretching frequencies at 1594 and 1375 cm^{-1}, which are frequencies characteristic of the presence of the non-protonated carboxylate ion (COO^{-}). The presence of the non-protonated carboxylate ion implies that the polysaccharides are possibly pectic in nature as suggested in literature (Moore *et al*., **2006**). It also implies that the carboxylate ion could be associated with any of the following ions: Na^{+}, K^{+}, Ca^{2+}, etc., since plants contain salts.

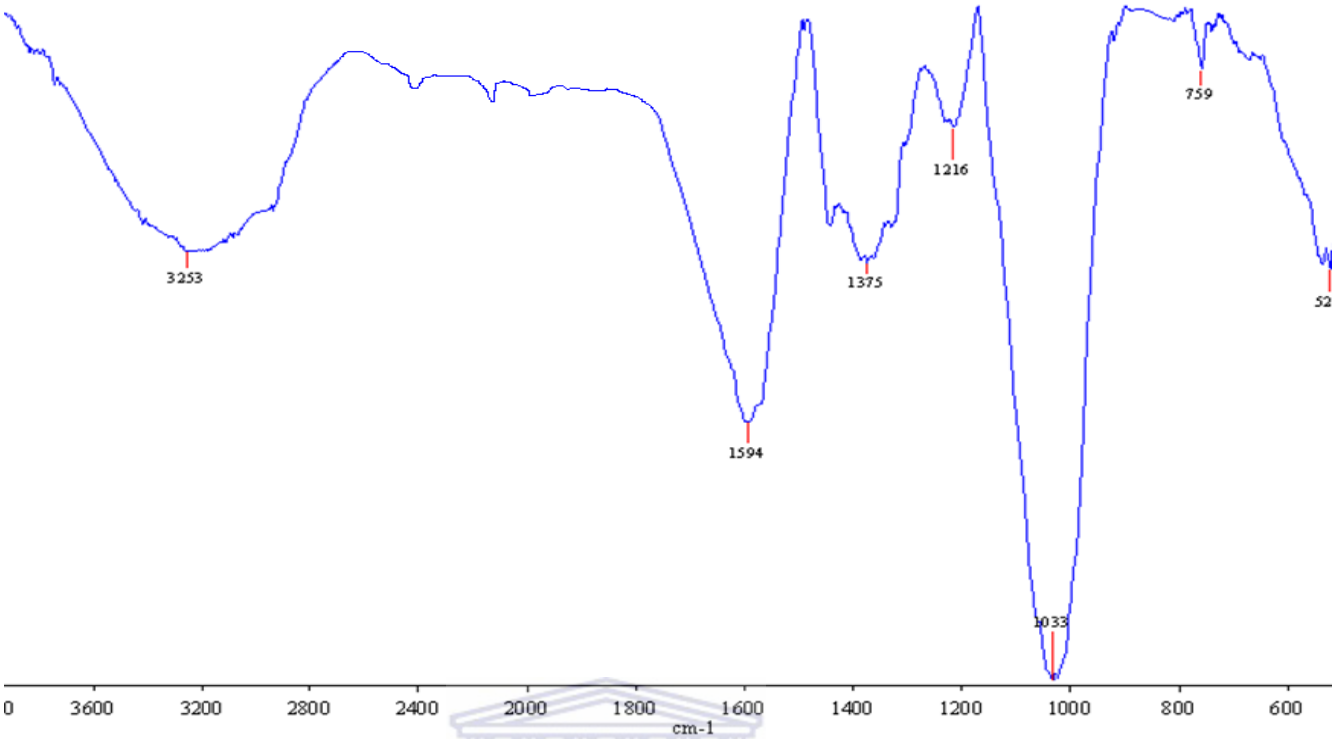


Figure 7: FT-IR spectrum of the crude polysaccharides.

Confirmation of the presence of uronic acids was achieved through carbazole assay (see Fig 8). The carbazole assay showed that the absorbance of the crude polysaccharides was 0.1021, which equated to approximately 15.7% of uronic acid content present in the crude polysaccharides, thus further confirming the possible pectic nature of the polysaccharides.

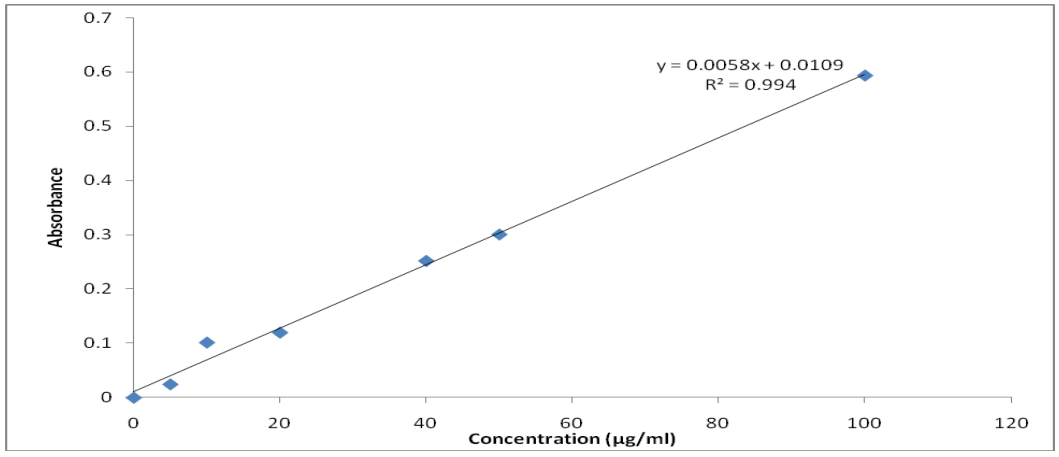


Figure 8: Carbazole assay calibration curve at λ =525nm

Following the quantification of the uronic acid content, neutral sugar composition of the crude polysaccharide was determined through acid hydrolysis and GC analysis. Table 6 highlights the neutral sugar composition of the crude polysaccharide. Glucose (Glc) is the most predominant sugar, accounting for 47.5% (relative mol percentage) of the total crude polysaccharide. The crude polysaccharide also consisted of other neutral sugars such as rhamnose (Rha), arabinose (Ara), xylose (Xyl), mannose (Man) and galactose (Gal) in concentrations of 1.6%, 7.5%, 3.3%. 3.3% and 21.1% respectively.

Table 6: Neutral sugar composition of the crude polysaccharide

Sugar	**Relative sugar composition (mol %)**
Ara	7.5
Rha	1.6
Xyl	3.3
Man	3.3
Gal	21.1
Glc	47.5

The crude polysaccharide was subjected to methylation and GC-MS analysis (see Appendix 3) so as to determine the characteristic monosaccharide linkages. The monosaccharide linkage results are tabulated in Table 7.

Table 7: Linkage analysis of the crude polysaccharide

Sugar	Type of linkage		Relative sugar composition (mol %)
Ara	1,2	[→1)-Ara*f*-(2→]	3.2
	1,5	[→1)-Ara*f*-(5→]	4.3
Rha	T	[Rha*p*-(1→]	0.4
	1,2	[→1)-Rha*p*-(2→]	0.9
	1,2,4	[→1,4)-Rha*p*-(2→]	0.3
Xyl	1,2	[→1)-Xyl*f*-(2→]	3.3
Man	T	[Man*p*-(1→]	1.3
	1,2	[→1)-Man*p*-(2→]	1.4
	1,2,4	[→1,4)-Man*p*-(2→]	0.6
Gal	T	[Gal*p*-(1→]	7.2
	1,3	[→1)-Gal-*p*(3→]	3.2
	1,2	[→1)-Gal-*p*(2→]	1.7
	1,4	[→1)-Gal-*p*(4→]	3.9
	1,6	[→1)-Gal-*p*(6→]	3.5
	1,3,6	[→1,3)-Gal*p*-(6→]	1.6

Table 7: continued

Sugar	Type of linkage		Relative sugar composition (mol %)
Glc	T	[Glc*p*-(1→]	14.2
	1,3	[→1)-Glc*p*-(3→]	5.7
	1,6	[→1)-Glc*p*-(6→]	12.2
	1,4	[→1)-Glc*p*-(4→]	11.8
	1,4,6	[→1,6)-Glc*p*-(4→]	3.6
Uronic acid	Nf		Nf

nf = not found

According to Table 7, the crude polysaccharide is made up of galactose units, of which 34% are terminal. About 58% of galactose units are 1,2-linked, 1,3-linked, 1,4-linked and 1,6-linked. The remaining small portion of galactose units is 1,3,6-linked, thus showing some branching. Arabinose is present mainly as 1,2-linked and 1,5-linked. This suggests that there is a possible presence of arabinogalactans as a major constituent of the crude polysaccharide.

Table 7 also shows that the crude polysaccharide also contained terminal, 1,2-linked and 1,2,4-linked rhamnose units, a composition indicative of the presence of pectic polysaccharide. Xylose is found only as 1,2-linked residue, while mannose is present as terminal, 1,2-linked and 1,2,4-linked units. The presence of significant amounts of terminal, 1,4-linked and 1,6-linked glucose units may suggest the presence of starch.

The linkages of the structural units with substituent uronic acids were not determined, the reason being that uronic acids will be converted into methyl esters during the methylation exercise, and the subsequent acid hydrolysis steps will convert the methyl esters into non-volatile acids. The stability of the uronic acid-sugar linkages is such that the sugars linked with substituent uronic acids will not be fully released and therefore under estimated in both composition and linkage analysis. This led to an attempt to perform carboxyl reduction on the crude polysaccharide, followed by methylation and GC-MS analysis. Unfortunately, the PMAA's derived from the carboxyl reduced sample could not be analysed in time to generate useful results to complement the structural elucidation because by the time they were analysed they had decomposed, and there was not sufficient time to repeat the sequence of the relevant experiments.

The crude polysaccharide was purified on an ion exchange column, DEAE Sepharose CL-6B (calibrated with commercial pectin), and the resulting elution profile of the crude polysaccharide is shown in Fig 9. The elution profile revealed three polysaccharide containing fractions (labelled MPS, MPS-I and MPS-II) eluting at different NaCl concentrations, which suggests that the each of the fractions had a distinct ionic character. The fractions MPS, MPS-I and MPS-II eluted at 0.1-0.2M, 0.4-0.5M and 0.5-0.6M NaCl concentrations, respectively. The pectin standard eluted at 0.1-0.3M NaCl concentrations, and so it appears as though the early eluting MPS contains the pectin. The combined yields of the purified fractions; MPS (11% yield), MPS-I (5.7% yield) and MPS-II (5.1% yield), added up to about 22% of the polysaccharide content. It could be speculated that the low polysaccharide yield may be due to interactions between tannins and polysaccharides or between proteins and polysaccharides. Moore *et al* (**2007**) reported that *M. flabellifolius* contains high levels of tannins. The free tannins were removed through fractionation of crude

polysaccharide (brown in colour) on a PVPP column. Following fractionation on PVPP, it was expected that the colour of the crude polysaccharide would change from brown to white. Instead, the crude polysaccharide appeared to be a faint orange colour, an indication that the crude polysaccharide could have been associated with either tannins or proteins. It has been reported that tannin-polysaccharide complexes are very stable and their structures depend on the tannin degree of polymerization (Carn *et al*, **2012**). In a study by Chen *et al* (**2008**), a polysaccharide-protein complex was successfully isolated from a medicinal plant *Lycium barbarum*. Upon purification on the DEAE sepharose column, there was a visible tinge of brown colour in the column after eluting with 1.0M NaCl which was indicative of material that was tightly bound to the DEAE sepharose. Therefore, the yield of the crude polysaccharide and that of the pure polysaccharide fractions could have been under estimated during the removal of free tannins and upon purification, respectively.

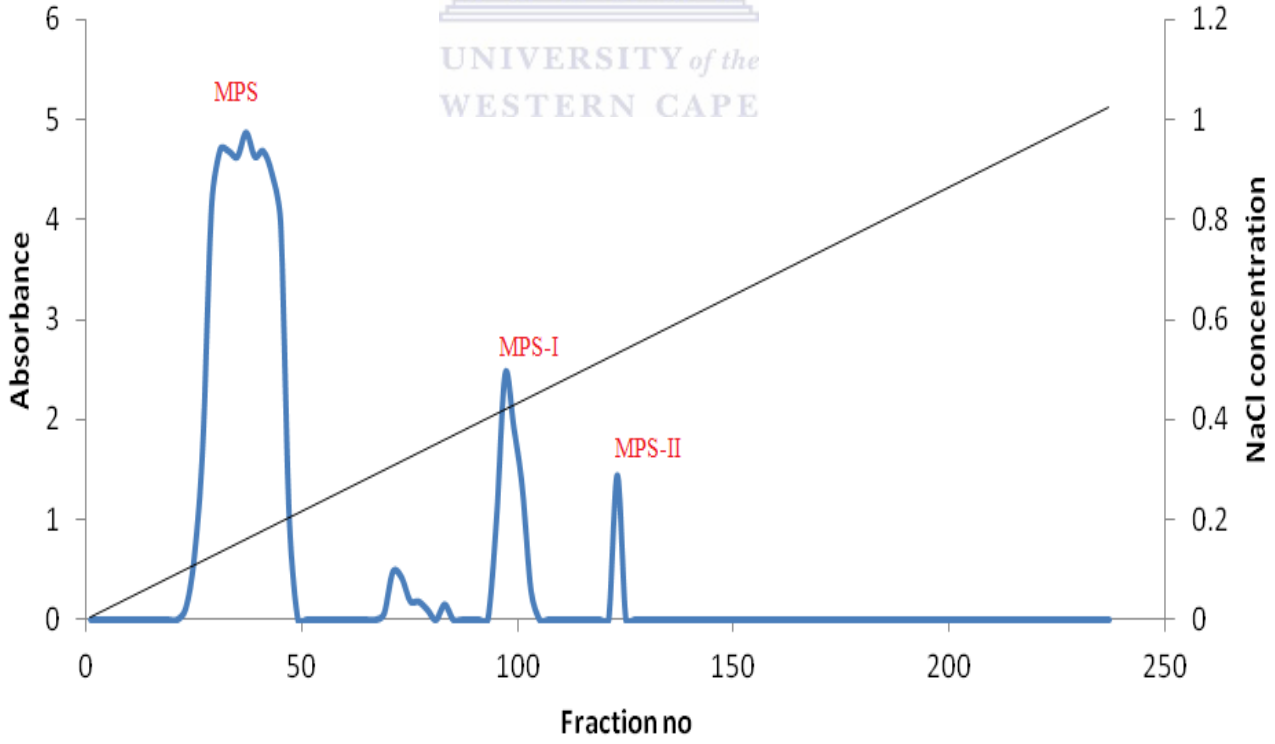


Figure 9: Elution curve of the crude polysaccharide on DEAE Sepharose CL-6B achieved via phenol-sulfuric acid assay.

MPS was found to be the major polysaccharide fraction, followed by MPS-I and MPS-II. The fractions were compared for their sugar composition (see Table 8). The purified fractions revealed a significant decrease in the glucose molar concentration, when compared to that of the crude polysaccharide shown in Table 6, and an increase in molar concentrations of other sugars. This suggests that the purified polysaccharide fractions are free of salts and starch, a polysaccharide which is present in most plants. Neutral sugar composition of MPS shows that Rha (22.2%), Ara (26.8%) and Gal (26.1%) are the predominant sugars, accounting for approximately 75% of the neutral sugar content in the purified polysaccharide (see Table 8). The rest of the neutral sugar content of MPS is made up of Glc (5.7%), Xyl (14.8%) and Man (4.4%). The uronic acid content of MPS was determined (through carbazole assay) to be 15.2%, almost the same as that of the crude polysaccharide. The MPS sugar composition is similar to that of the arabinose-rich pectic polysaccharides reported in literature (Moore *et al*., **2006**). Table 8 shows a significant increase in the Glc (14.2%), Man (10.6%) and Gal (35.3%) sugar concentrations in the purified polysaccharide MPS-I relative to those of MPS. However, MPS-I has a lower Rha (5.0%) and Xyl (7.2%) content. The highest Glc (21.7%), Rha (30.4%) and Man (28.2%) sugar content was observed in MPS-II. Table 8 also highlights that MPS-II did not contain any galactose, therefore MPS-II is a galactose-free polysaccharide. The uronic acid content of MPS-I and MPS-II could not be determined due to the low quantities of both polysaccharide fractions.

Table 8: Neutral sugar composition of purified polysaccharide fractions MPS, MPS-I and MPS-II

Sugar	**Relative sugar composition (mol %)**		
	MPS	MPS-I	MPS-II
Glc	5.7	14.2	21.7
Rha	22.2	5.0	30.4
Ara	26.8	27.7	10.5
Xyl	14.8	7.2	9.2
Man	4.4	10.6	28.2
Gal	26.1	35.3	-

The significant differences in the ionic character and neutral sugar composition between the purified polysaccharide fractions, confirm that three different polysaccharide types have been successfully isolated from *M. flabellifolius*. The low quantities of MPS-I and MPS-II made it impossible to further characterize these two polysaccharides. MPS was further fractionated on gel permeation chromatography, Sephacryl S-400, which was calibrated with Dextrans of known molecular weights. During fractionation, the small molecules (low molecular weight) get trapped in the gel pores and the larger molecules (higher molecular weight) flow through the gel. Thus, larger molecules elute first, followed by the smaller molecules in the order of their sizes (Garrett and Grisham, **1999**). Fractions (2 ml) were collected and their carbohydrate content was determined through phenol-sulfuric acid assay. Figure 10 shows

that the fractionation of MPS led to the separation of a major peak, MPS-A, which eluted first. The void volume of the column (V_0) was 43 ml. Several minor fractions of different molecular weights eluted later. The last peak, at 354 ml, represents the sugar with the lowest molecular weight. The total volume of the column was 383 ml.

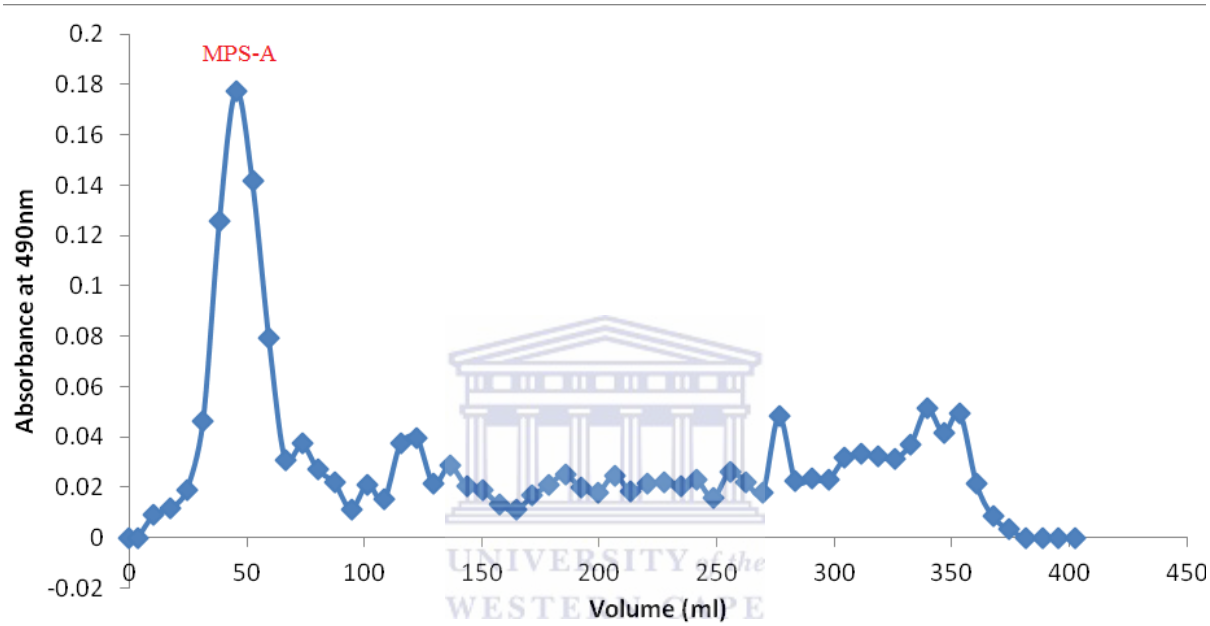


Figure 10: Elution curve of MPS on Sephacryl S-400 achieved via phenol-sulfuric acid assay

The molecular weight distribution of polysaccharide-containing fraction, MPS-A, was determined using the Dextran calibration curve (see Fig 11). Using the formula: Log (Mw) = -0.0227x + 6.3424; where Mw and x represent the molecular weight and the elution volume, respectively, the molecular weight of MPS-A was estimated at 2 X 10^5 Da.

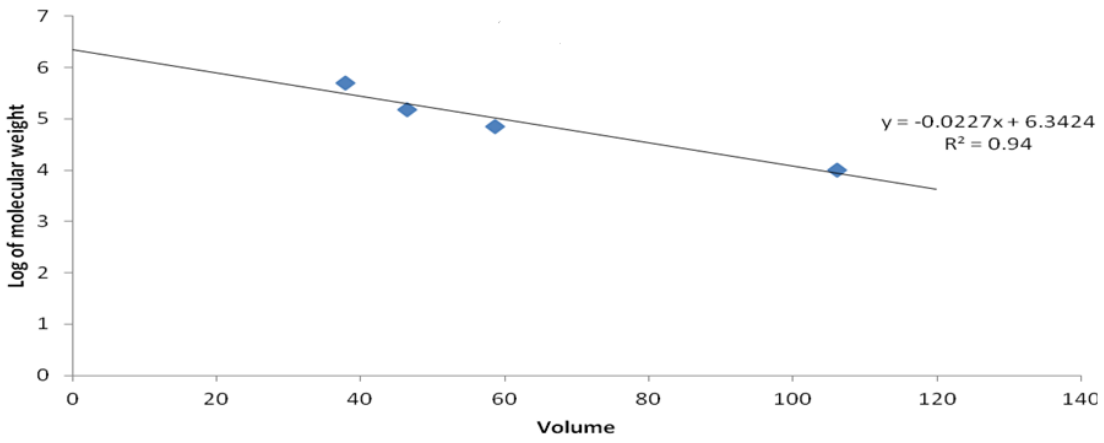


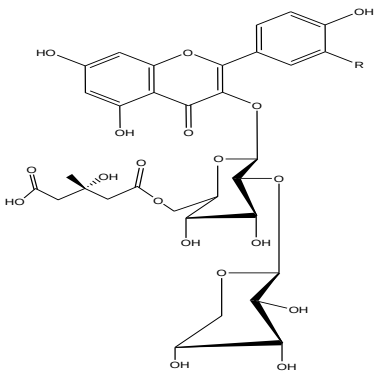
Figure 11: Dextran Calibration curve for determining molecular weight distribution.

Determination of molecular weight distribution in polysaccharides is of utmost importance because molecular size of polysaccharides is often closely related to their biological activity. It was reported that the polysaccharide molecular weight determines the levan antitumour activities and that a specific class of molecular weight may be responsible for this effect (Calazans *et al.*, **2000**). The ionic character in some polysaccharides is also correlated with their biological activity. It was reported that the more flexible pectin hairy regions were responsible for the biological activities (Ognyanov *et al.*, **2013**). Therefore, molecular weight distribution and uronic acid composition are important for quality control of polysaccharides.

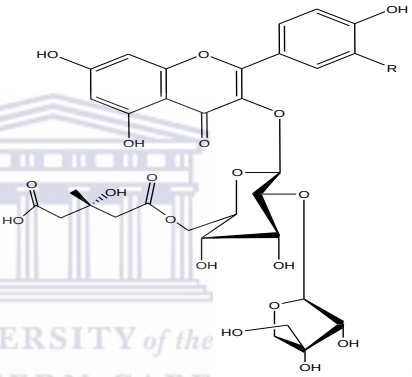
CHAPTER 4

Ligand fishing from *Sutherlandia frutescens* extract

The four flavonol glycosides and four saponins (see Fig 12) which had been isolated following lengthy and laborious chromatographic separation techniques, from the butanol extracts of *S frutescens* (Fu *et al*., **2010**; Fu *et al*., **2008**) were targeted for this particular study.



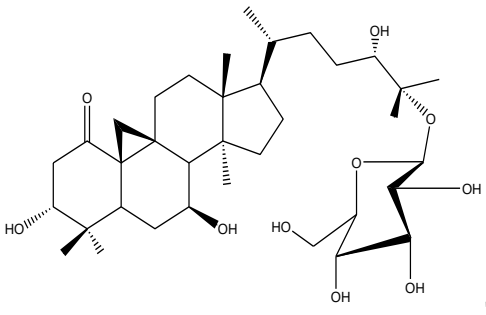
1 R=OH



2 R=OH

3 R=H

4 R=H



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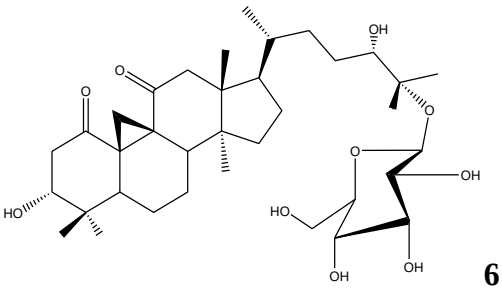

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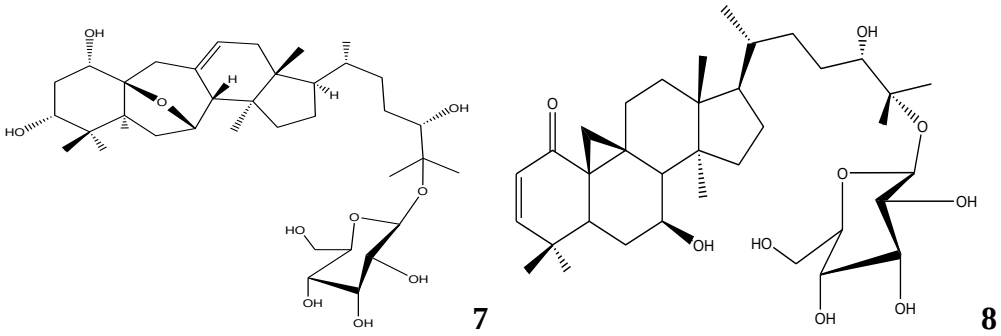

Figure 12: Chemical structures of the leaf constituents isolated from *S. frutescens*. (**1**) sutherlandin A, (**2**) sutherlandin B, (**3**) sutherlandin C, (**4**) sutherlandin D, (**5**) sutherlandioside B, (**6**) sutherlandioside C, (**7**) sutherlandioside A and (**8**) sutherlandioside D.

It is believed that the flavonol glycosides and saponins isolated from *S. frutescens* are responsible for the plant's wide usage in the prevention and treatment of cancer and HIV (Kikuchi *et al*., **2007**; Tai *et al*., **2004**).

In this study, human serum albumin functionalized magnetic nanoparticles (HSA-MNPs) were used in order to investigate the possible isolation of flavonol glycosides and saponins from *S. frutescens* butanol extract. The analysis of the extract was performed using LC-MS (ESI-MS positive mode). ESI-MS analysis of the butanol extract (solution S0) revealed the presence of a large number of compounds (see Fig 13).

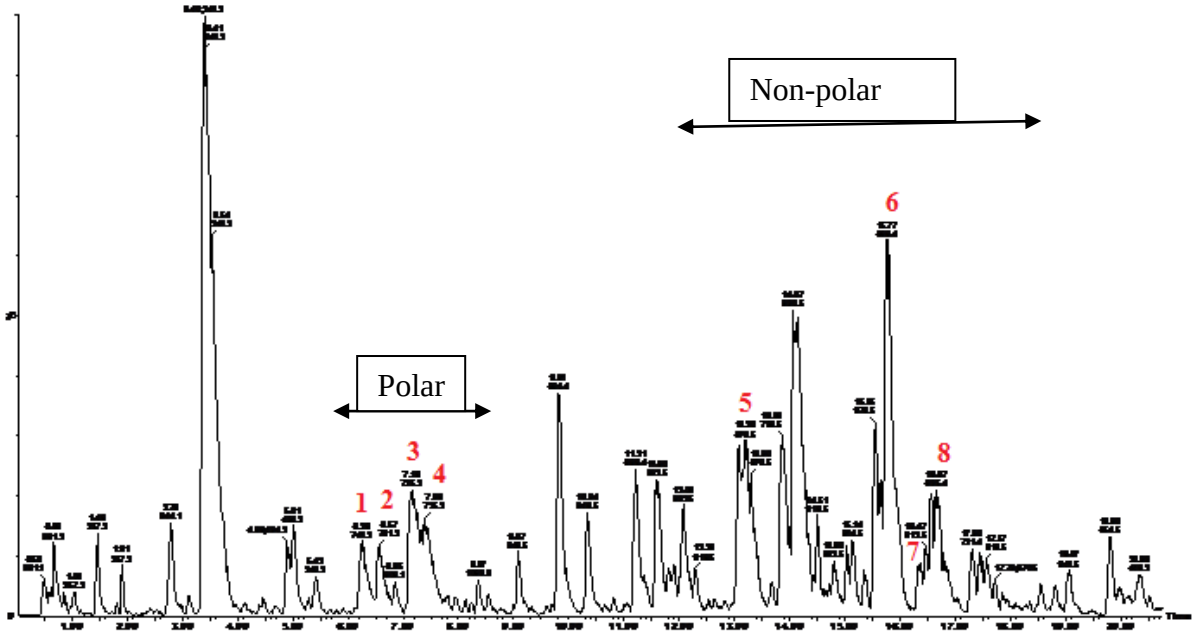

Figure 13: LC-ESI-MS profile of *S. frutescens* butanol extract (S0). Peak identification was done in accordance to the numbering of chemical structures in Fig 12.

As shown in the chromatogram (Fig 13), eight peaks could be assigned as follows: *m/z* 741.2 $[M+H]^+$ sutherlandin A (**1**), *m/z* 741.2 $[M+H]^+$ sutherlandin B (**2**), *m/z* 725.2 $[M+H]^+$ sutherlandin C (**3**), *m/z* 725.2 $[M+H]^+$ sutherlandin D (**4**), *m/z* 670.5 $[M+NH_4]^+$ sutherlandioside B (**5**), *m/z* 668.4 $[M+NH_4]^+$ sutherlandioside C (**6**), *m/z* 670.5 $[M+NH_4]^+$ sutherlandioside A (**7**) and *m/z* 635.4 $[M+H]^+$ sutherlandioside D (**8**). Previous studies on *S. frutescens* did not show the presence of any isomers of the compounds in Fig 12. Furthermore, Fig 13 shows that the identified compounds do not have any isomers. The identification of flavonol glycosides and saponins in the butanol extract of *S. frutescens* could be developed as a quality control protocol by using LC-MS.

Following the first liquid-solid separation of HSA-MNPs, ESI-MS analysis of solution S1 resulted, as judged qualitatively, in a similar chromatographic profile to that of solution S0. Thus all the peaks that were observed in Fig 13 could also be found to be present in the

chromatogram representing solution S1 (see Fig 14). The similarity in chromatographic profiles could be attributed to the HSA-MNPs being saturated with the constituents present in solution S0.

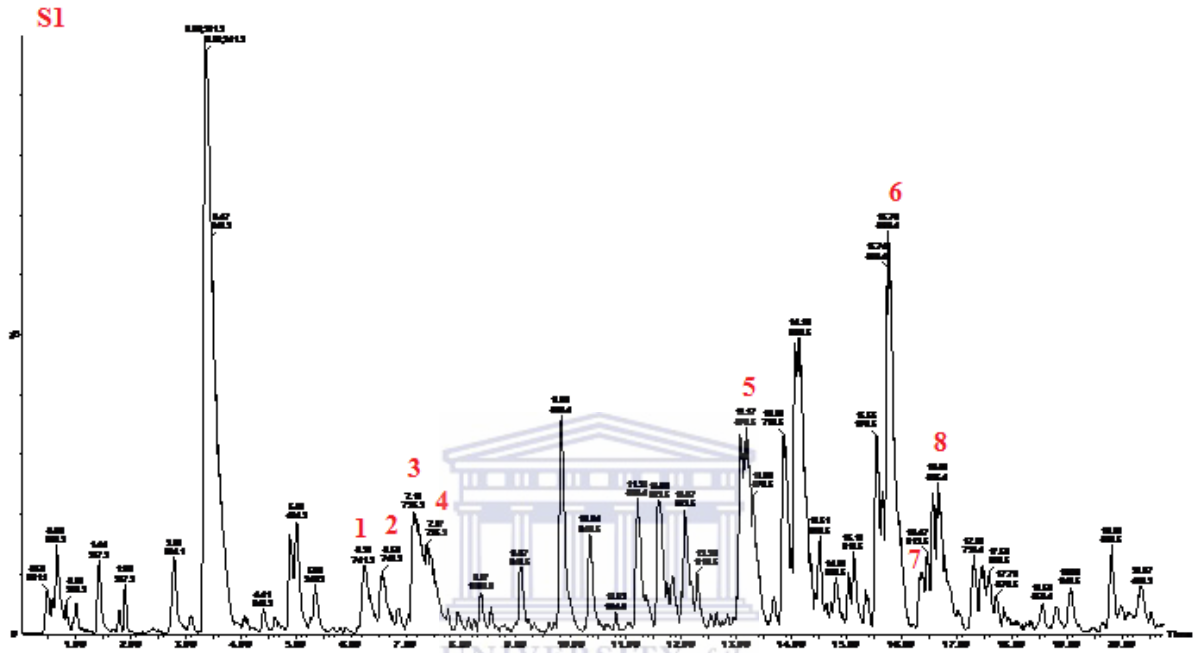

Figure 14: LC-ESI-MS profile of solution S1 from HSA-MNPs

From the chromatographic profiles of solutions S0 and S1, it could be observed that in the polar and non-polar regions, the most prominent peaks were those representing compounds **3** and **6**, respectively. A similar pattern was also observed after the analysis of solution S2 (see Fig 15).

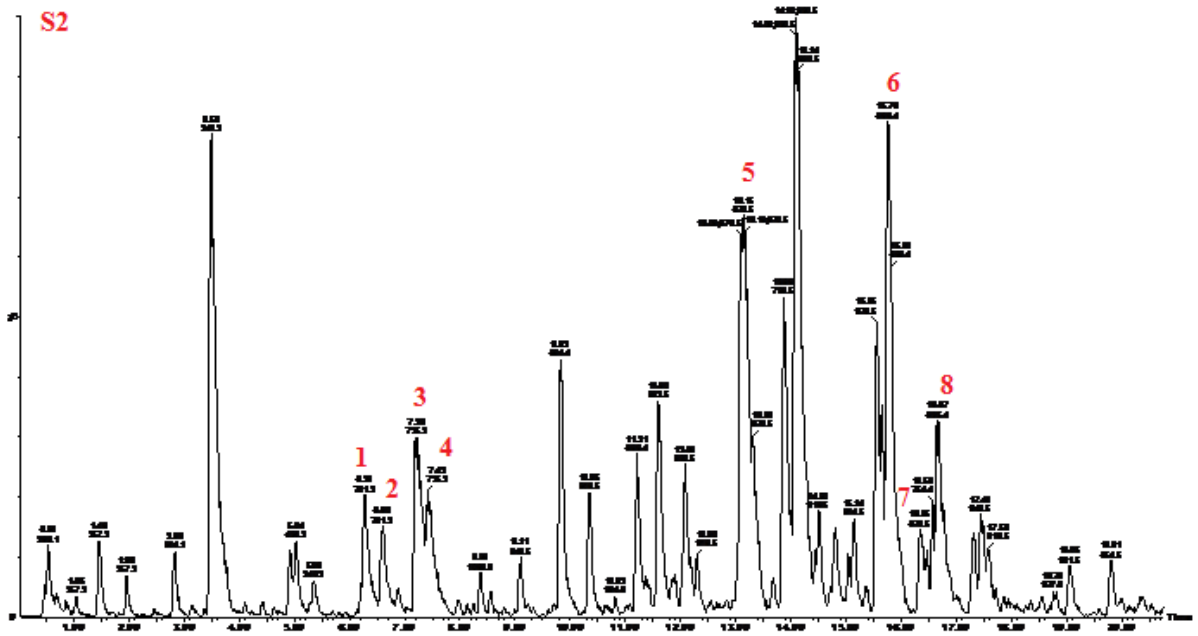

Figure 15: LC-ESI-MS profile of solution S2 from HSA-MNPs

Following the second washing of HSA-MNPs with the buffer, analysis of solution S3 showed a noticeable change in the pattern in the non-polar region. The peak representing compound **5** was greatly enhanced to such an extent that it had become the most prominent peak (see Fig 16). The peak representing compound **6** was slightly reduced.

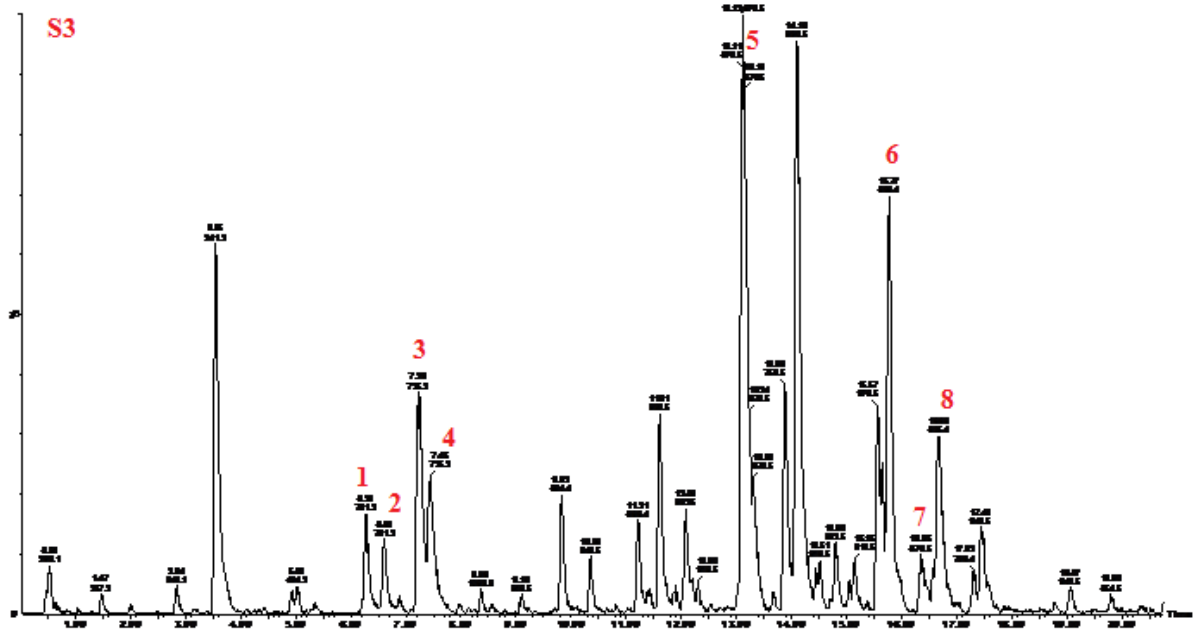

Figure 16: LC-ESI-MS profile of solution S3 from HSA-MNPs

Analysis of solution S4 showed further reduction in the compound **6** peak, and a disappearance of the peak representing compound **7** (see Fig 17).

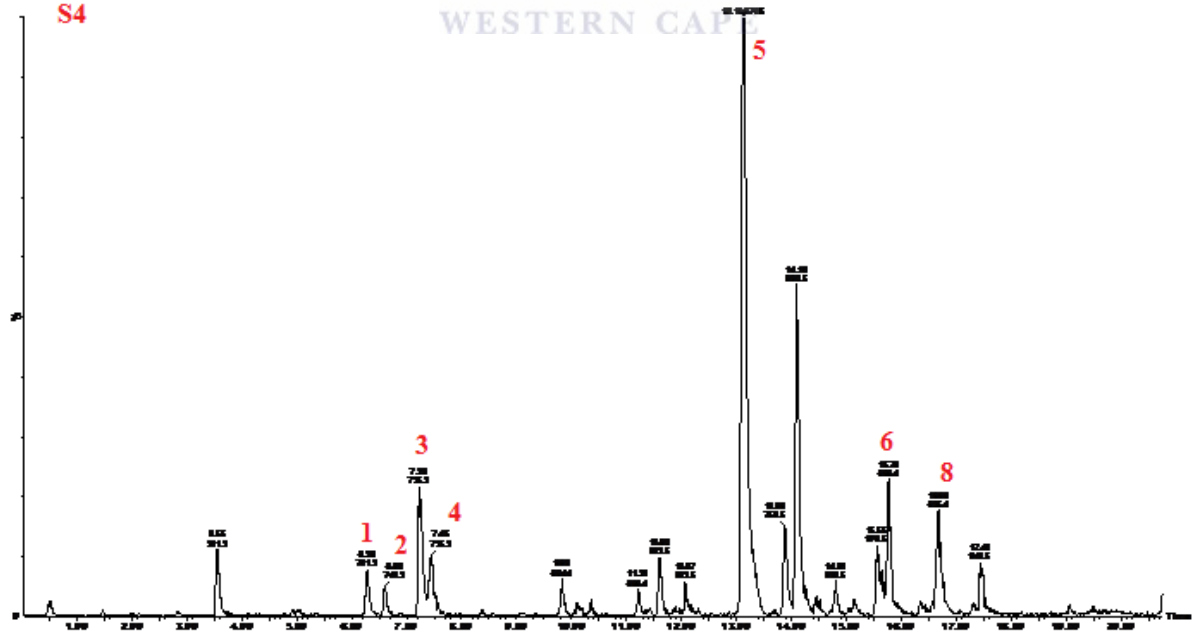

Figure 17: LC-ESI-MS profile of solution S4 from HSA-MNPs

Following the washing of HSA-MNPs with a mixture of the buffer containing 50% ACN, the chromatogram of solution S5 showed the presence of five compounds (see Fig 18) which were identified as; sutherlandin C (**3**), sutherlandioside B (**5**), sutherlandioside C (**6**), sutherlandioside A (**7**) and sutherlandioside D (**8**). Thus, the five compounds showed an affinity to bind strongly but reversibly to HSA immobilized on MNPs. The other three compounds identified in the butanol extract, i.e. sutherlandin A (**1**), sutherlandin B (**2**) and sutherlandin D (**4**) were not detected.

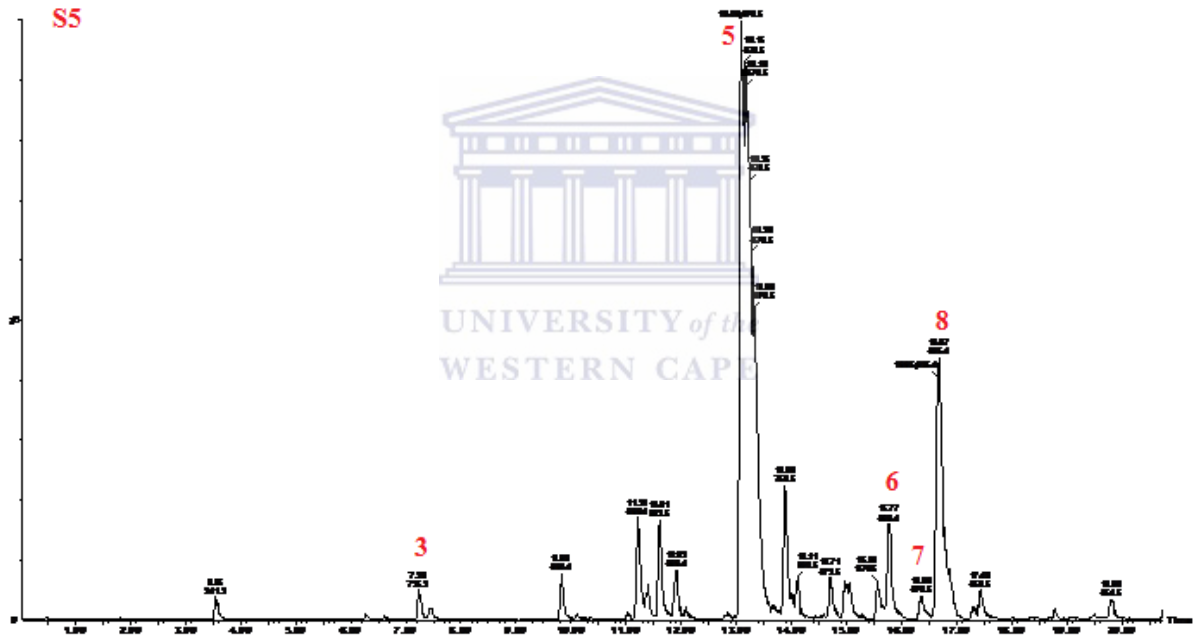

Figure 18: LC-ESI-MS (positive mode) analysis of the 50% ACN eluent (solution S5) from HSA-MNPs.

The three flavonol glycosides (sutherlandin A, B and D) may have not been detected because of one or a combination of the following;

i. Present in very low concentrations

ii. Possibility of irreversible binding

Comparing the chromatograms from solution S0 through to solution S5, it is evident that the peak abundance is greatly enhanced for sutherlandiosides B (**5**) and D (**8**). These results may suggest that sutherlandiosides B and D may have a higher affinity to bind reversibly to HSA. Sutherlandioside B is the major saponin isolated from *S. frutescens* (Fu *et al.*, **2008**). Fig 18 shows that it is also the most predominant after ligand fishing. The molecular formula of sutherlandioside B was determined to be $C_{36}H_{60}O_{10}$ by mass spectroscopy (*m/z* 652.4186). The MS^2 spectrum of sutherlandioside B gave fragment ions at *m/z* 635.4126, indicating the loss of a water molecule (see Fig 19). The MS^3 spectrum of the *m/z* 635.4126 showed fragment ions at *m/z* 491.3742, indicating the loss of a glucosyl moiety.

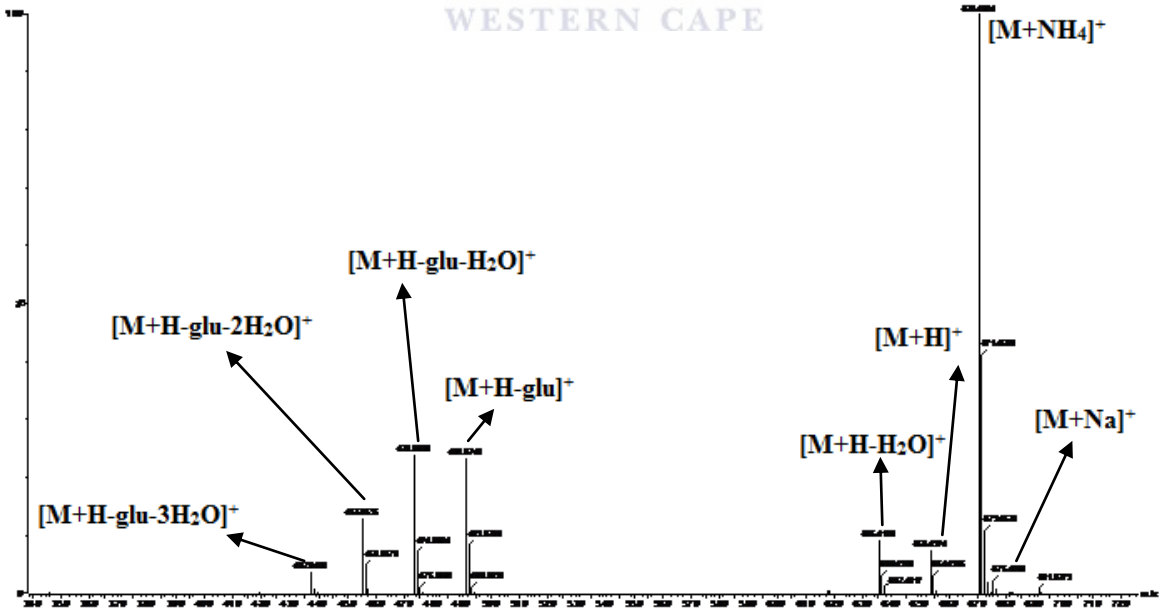

Figure 19: Fragmentation pathways for sutherlandioside B.

These results show that the use of HSA as a ‘receptor’ to extract ligands from *S. frutescens* is depended on the structural characteristics of the bioactive components present. As such, the procedure proved to be biased towards specific structural characteristics, as evidenced by the simple spectrum (Fig 18) compared to that of the crude butanol extract. Therefore, this procedure could be used as a pre-treatment of crude extracts to prepare for MS characterization.

Because the HSA binders were identified to be saponins (triterpenoid glycosides), the ligand fishing procedure was attempted on the butanol extract of *M. flabellifolius* since arbutin, a phenolic glycoside, was isolated from it. Unlike saponins, arbutin did not show any binding affinity towards HSA.

CHAPTER 5

CONCLUSIONS

Isolation of some natural products from *Myrothamnus flabellifolius* was carried out successfully. Two compounds, arbutin, a phenolic glycoside, and lupeol, a triterpenoid, were isolated and fully characterized. The water extract was found to contain a heterogeneous mixture of polysaccharides which included those of the pectic type, where the latter could be separated into three polysaccharide fractions, MPS, MPS-I and MPS-II. The isolated natural products have been reported to exhibit some important biological activities, some of which include; antibacterial, antifungal, immunomodulatory, antioxidant, antihyperglycaemic, antihyperlipidemic, antitumor, anti-inflammatory, antiprotozoal, antimicrobial, chemopreventive, antimalarial, antiarthritic, antitumor, etc. Therefore, the isolated bioactive compounds validate the use of *M. flabellifolius* as a nutritious herbal tea. Furthermore, there is a great possibility that more bioactive compounds may still be isolated from *M. flabelliffolius*, with the use of a variety of organic extracts. Biological activity tests of the various extracts will also provide very useful information, and so should toxicology studies.

It has been reported in literature that natural products such as 3,4,5-tri-*O*-galloylquinic acid, trehalose, pinocarvone and trans-pinocarveol were isolated from *M. flabellifolius*. However, such natural product composition differs from what has been reported in this thesis. This difference in natural product composition might be due to differences in geographical location, the nature of the soil, seasonal collection and/or climate in which the plant grows. Or it might be due to differences in plant extraction techniques employed.

The use of human serum albumin (HSA) following its anchoring on magnetic nanoparticles, for the purpose of isolating identified saponins from *Sutherlandia frutescens* was shown to be a potentially useful method for further exploration towards purification of the individual saponins for pharmacological studies, given that the four reported saponins, namely sutherlandiosides A, B, C and D showed a good affinity to bind to the HSA. An investigation of association/dissociation constants between HSA and these saponins would probably provide a better insight into their importance in pharmacological activity, and so should a statistical analysis of their binding capacity. Molecular modelling might as well provide valuable information. Exploring different buffer systems may help improve drug-protein binding, e.g. the case where arbutin did not show any binding affinity towards HSA, because the protein binding behaviour is influenced by the type of buffer system in solution with the protein.

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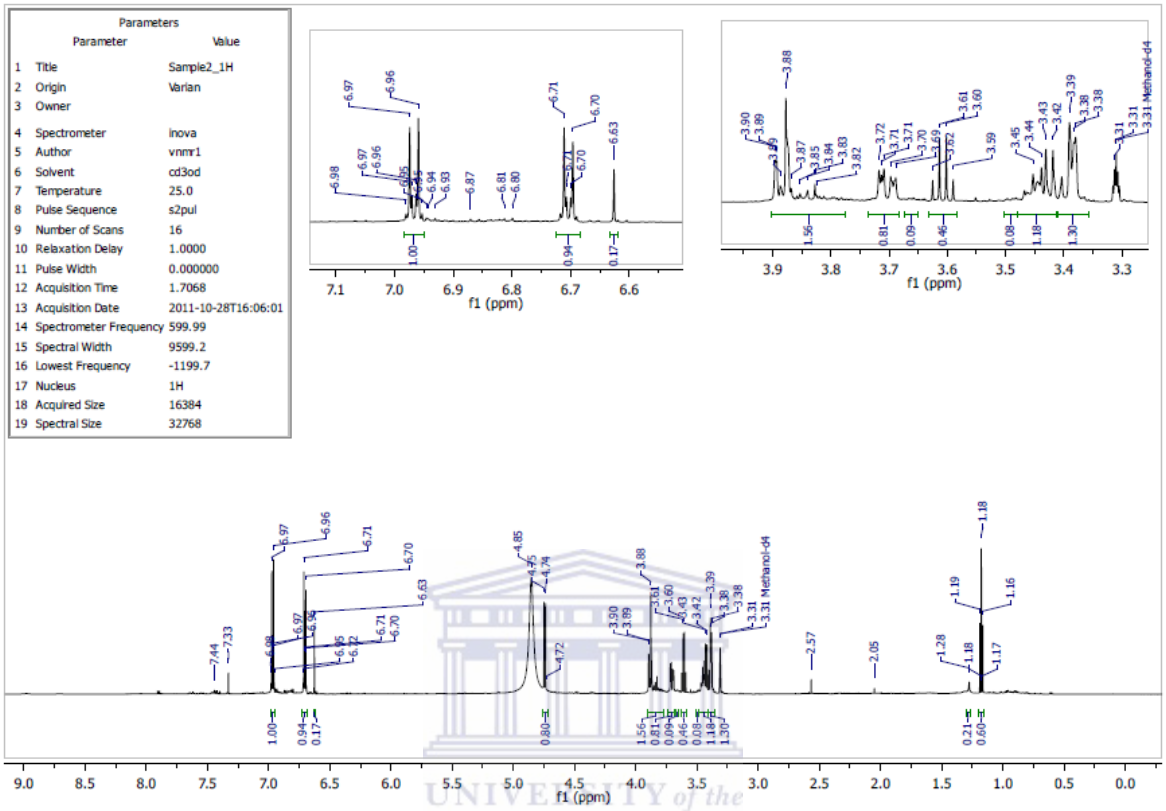
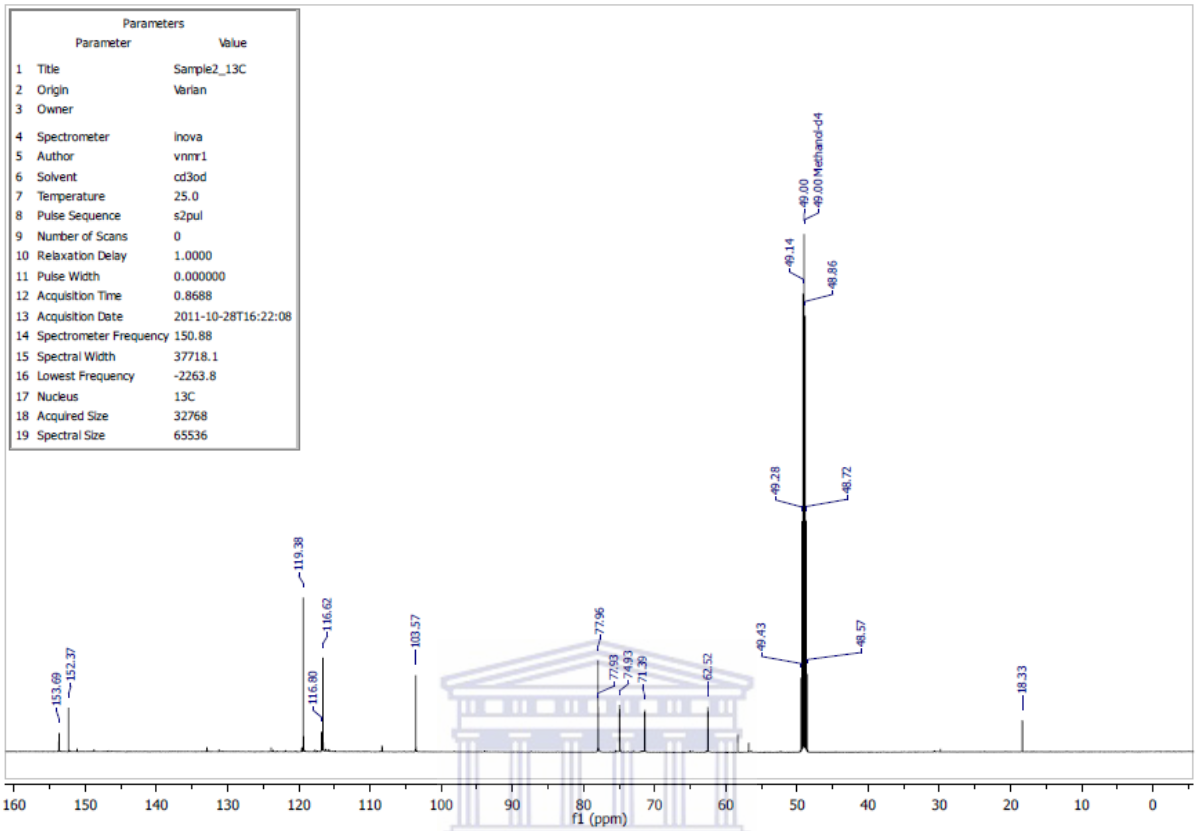
APPENDIX

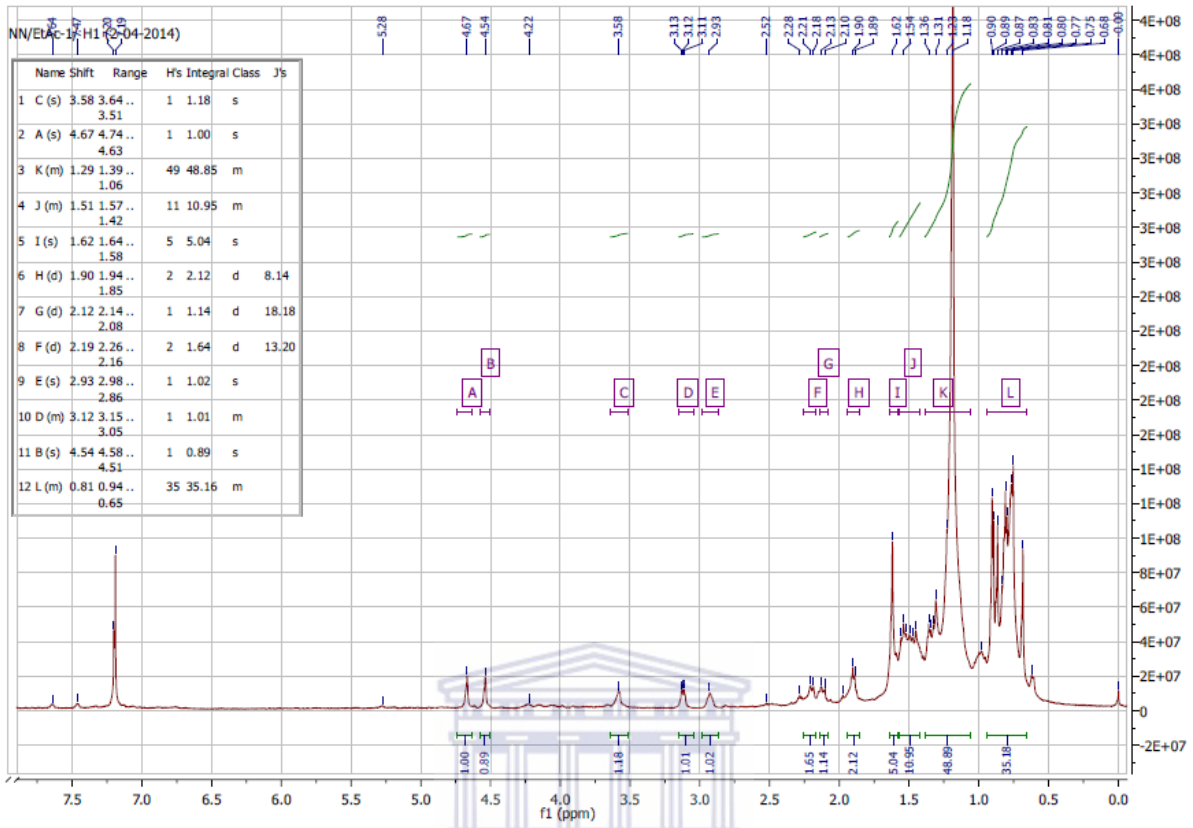
Appendix 1a ^{1}H NMR of compound **1**



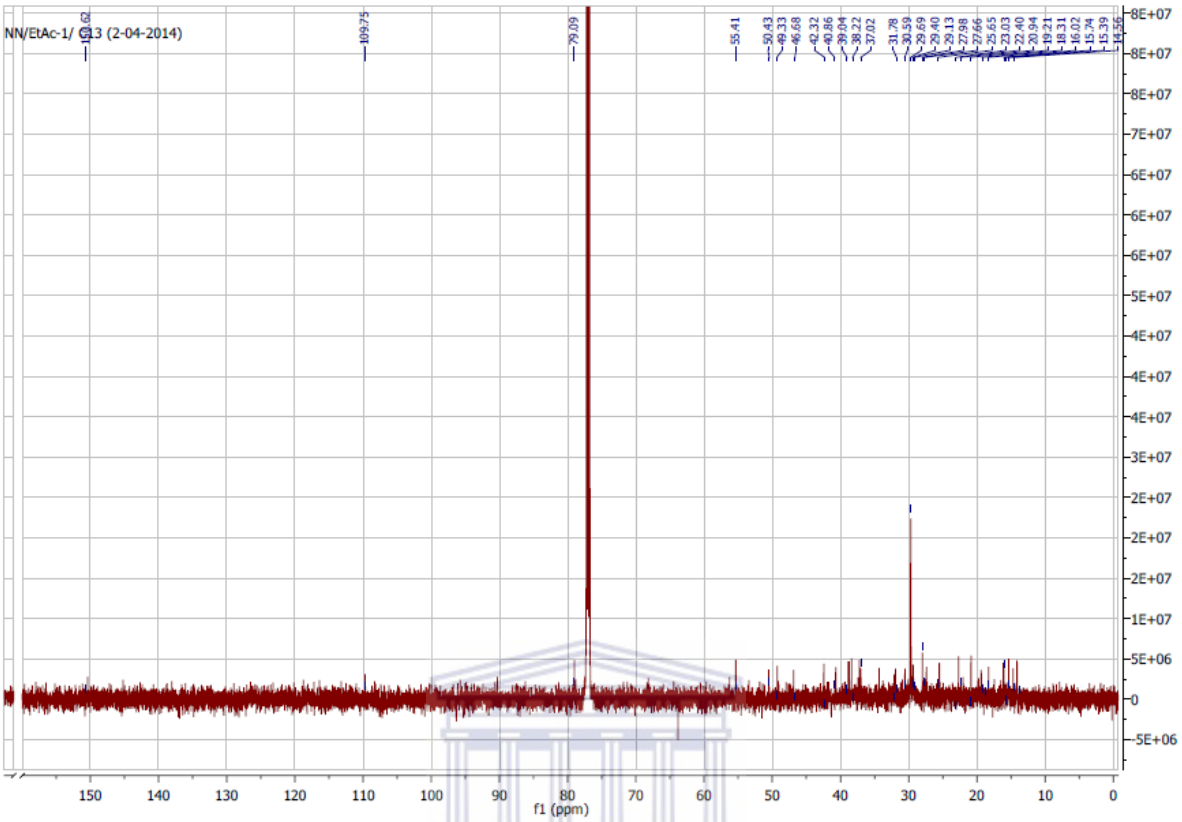
Appendix 1b ^{13}C NMR of compound **1**



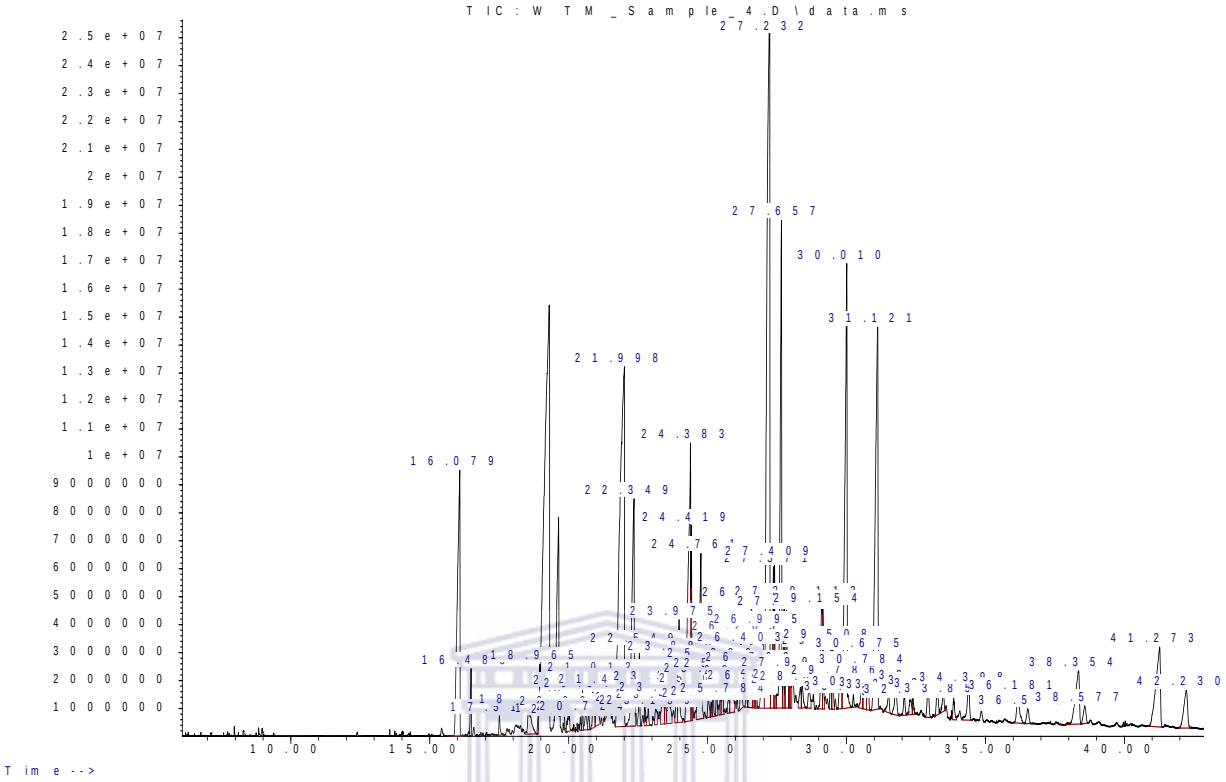
Appendix 2a ^{1}H NMR of compound **2**



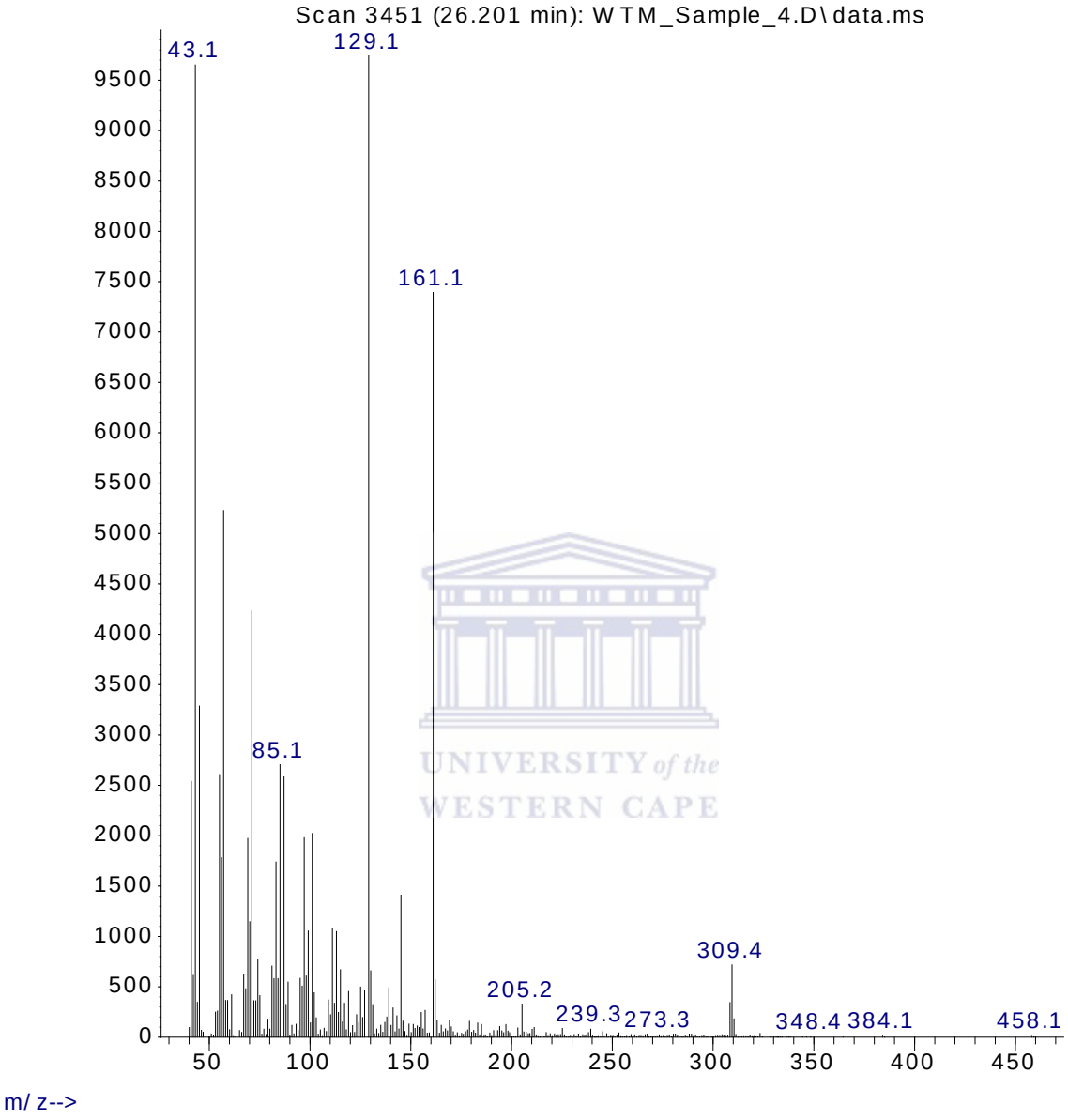
Appendix 2b ^{13}C NMR of compound **2**



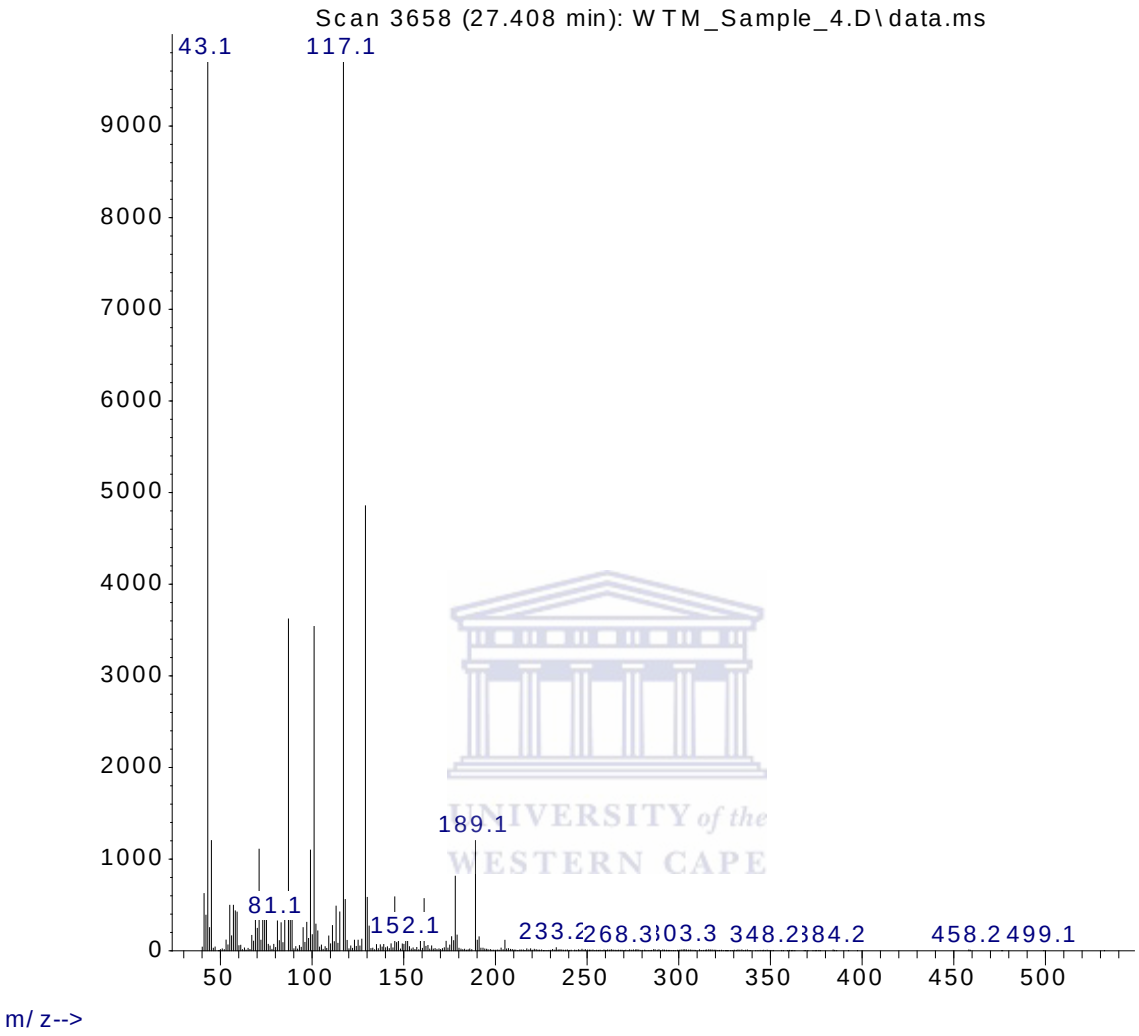
Appendix 3a Chromatogram of the partially methylated alditol acetates



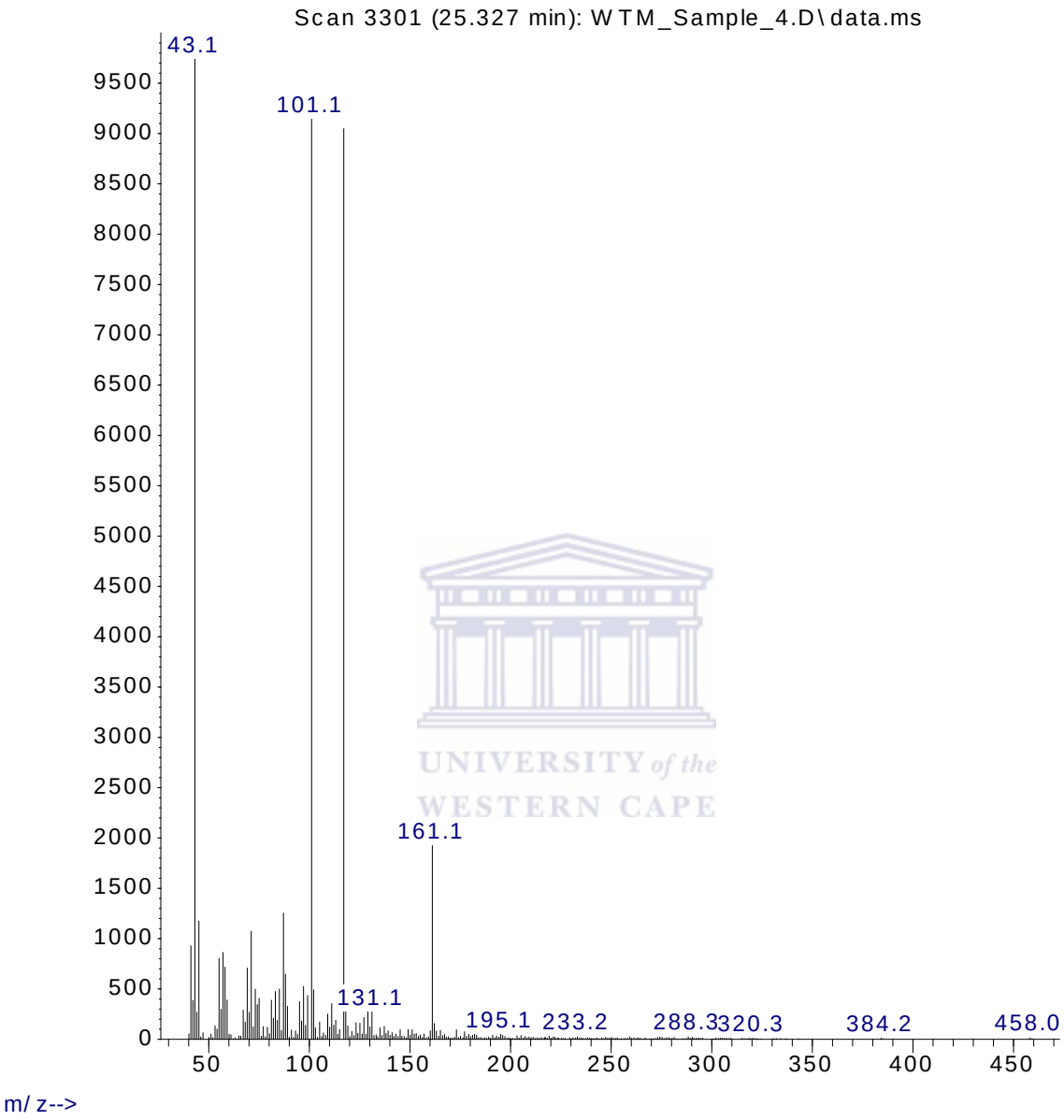
Appendix 3b Mass spectrum of →1)-Ara*f*-(2→



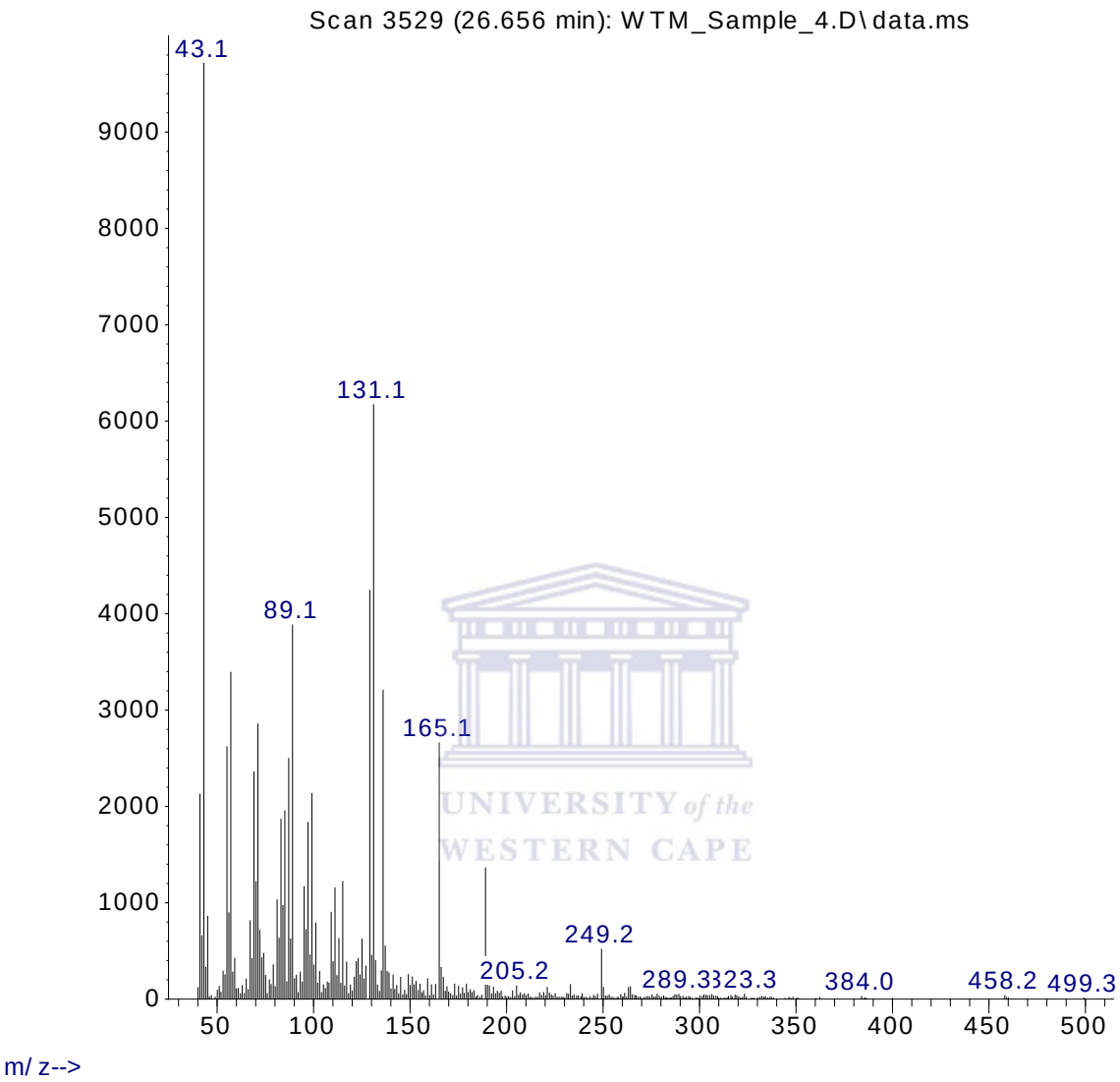
Appendix 3c Mass spectrum of →1)-Ara*f*-(5→



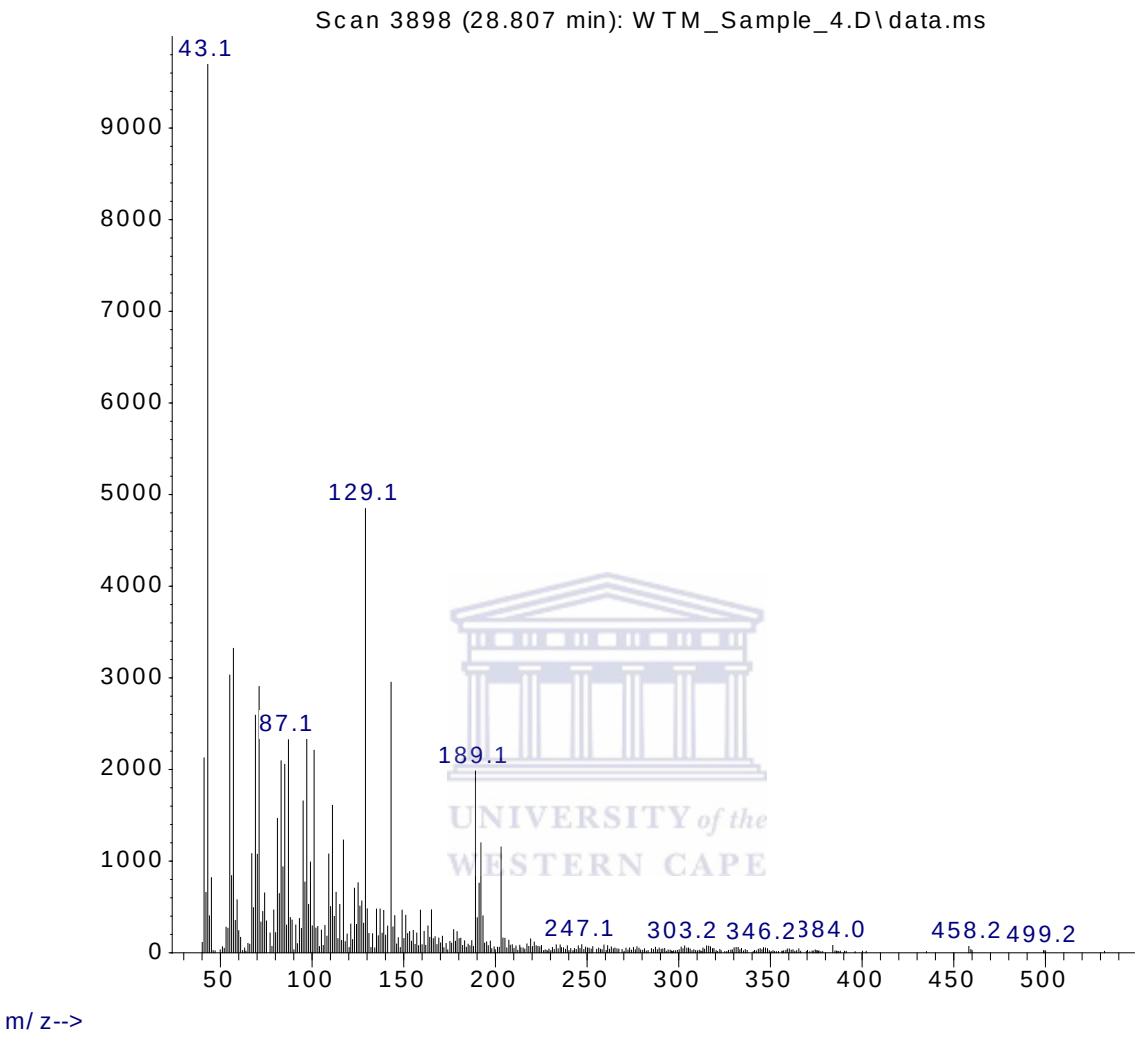
Appendix 3d Mass spectrum of Rha*p*-(1→



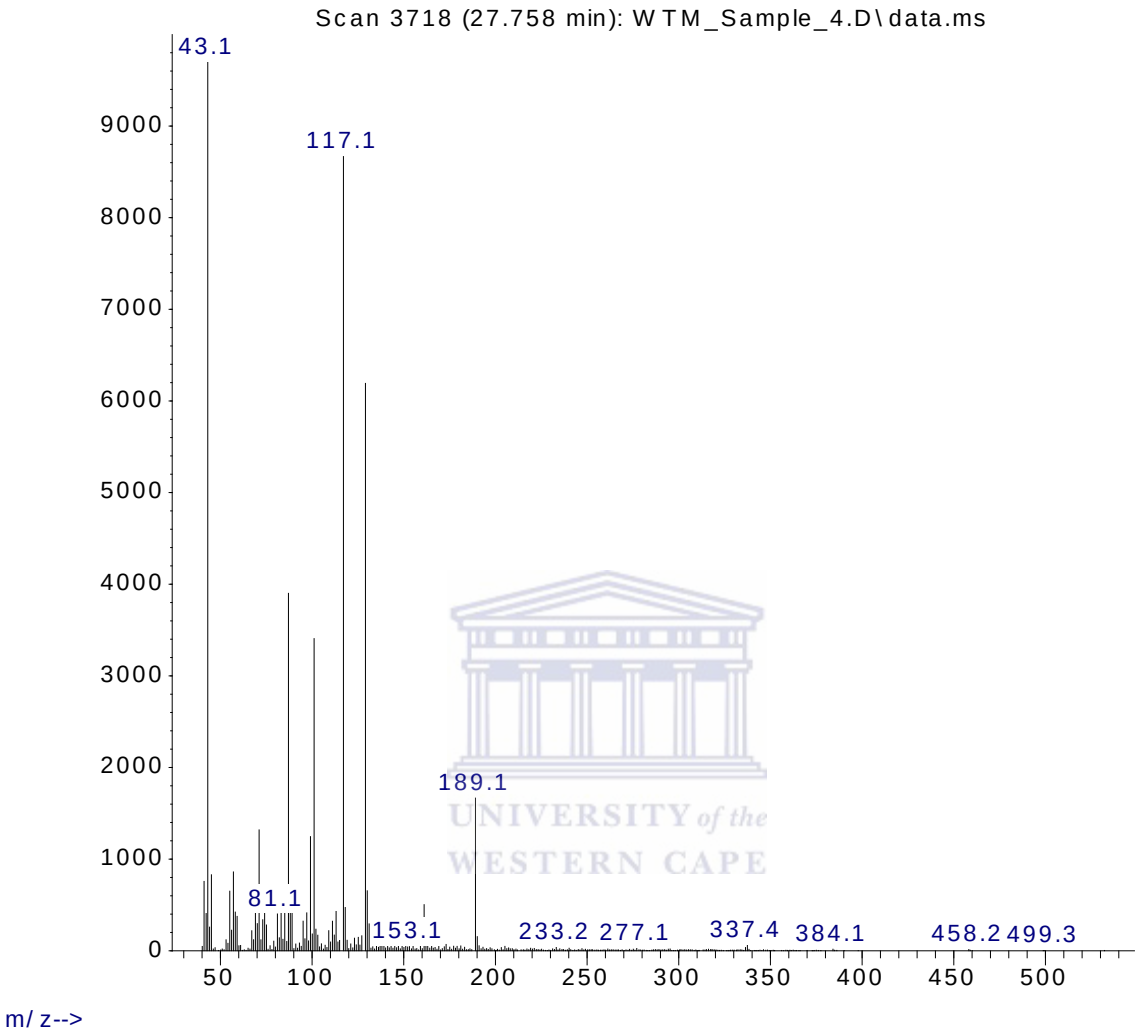
Appendix 3e Mass spectrum of →1)-Rha*p*-(2→



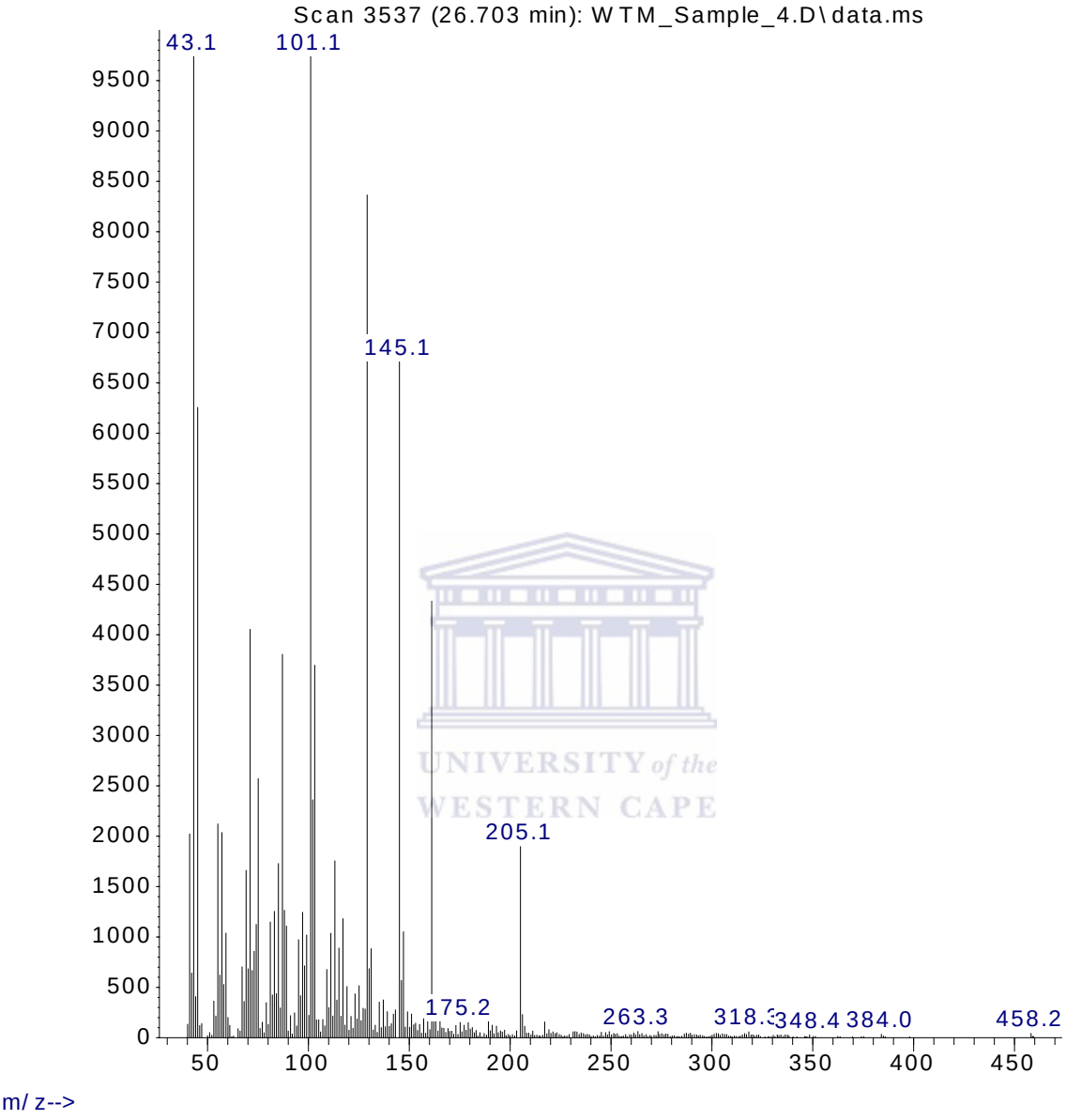
Appendix 3f Mass spectrum of →1,4)-Rha*p*-(2→



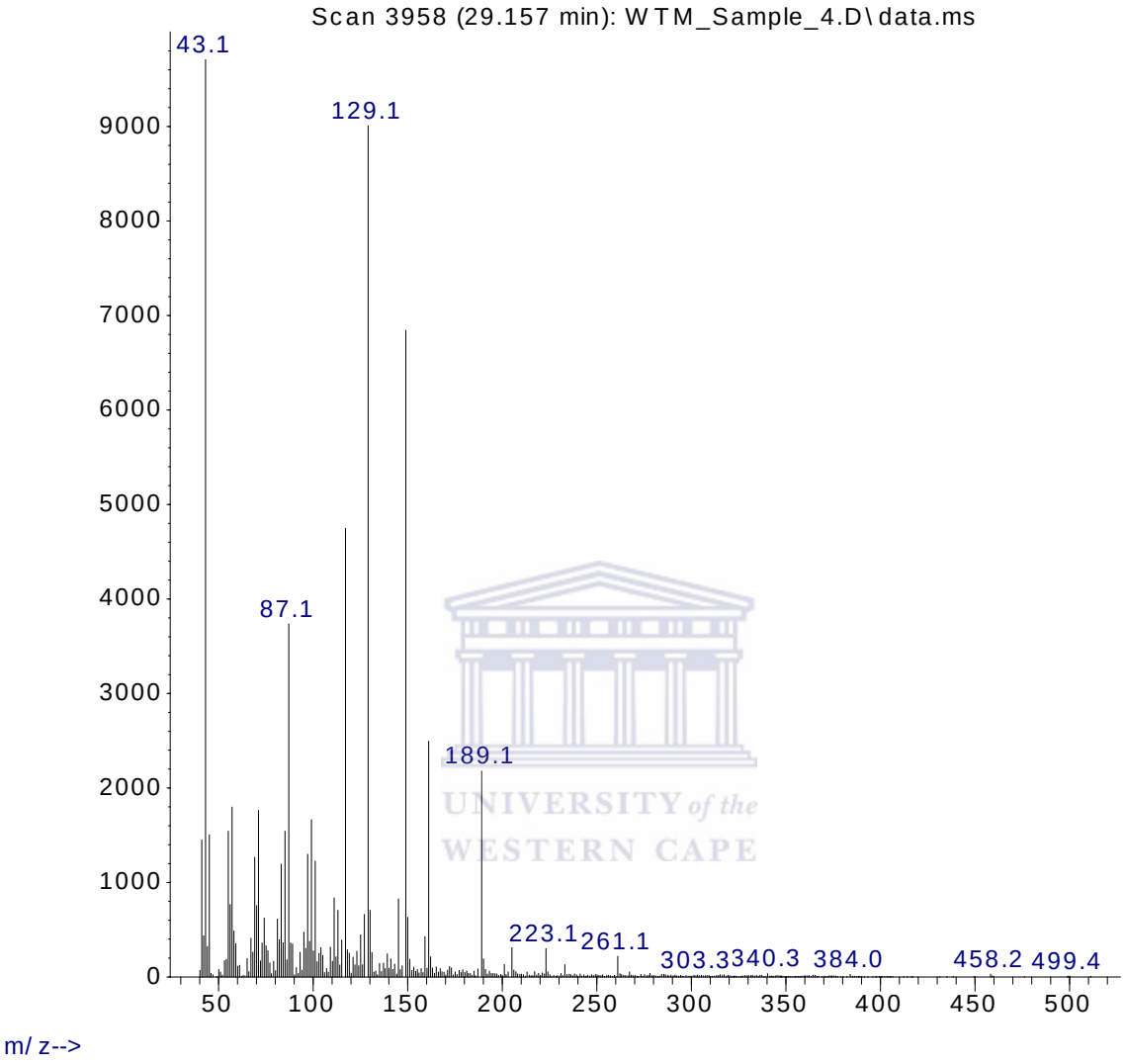
Appendix 3g Mass spectrum of →1)-Xyl*f*-(2→



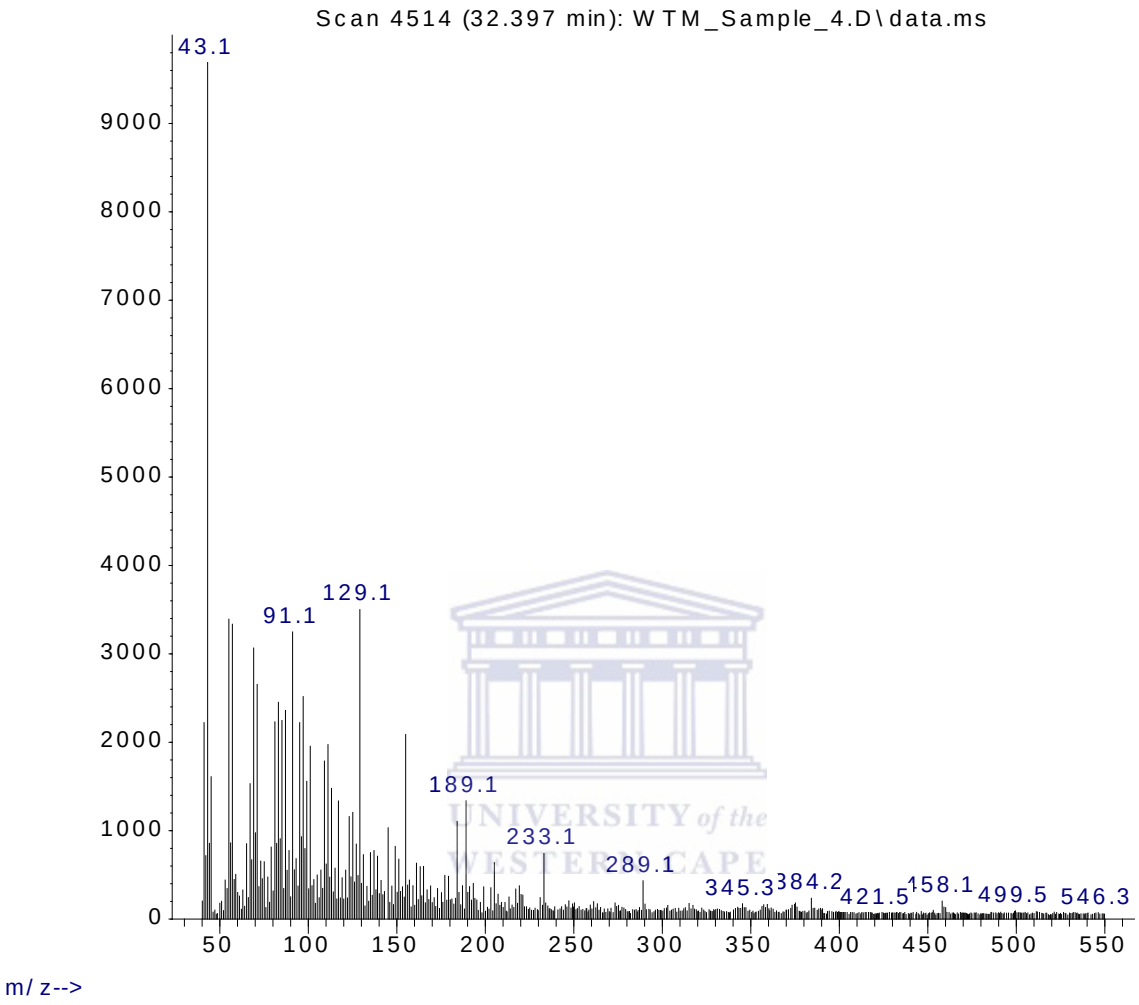
Appendix 3h Mass spectrum of Man*p*-(1→



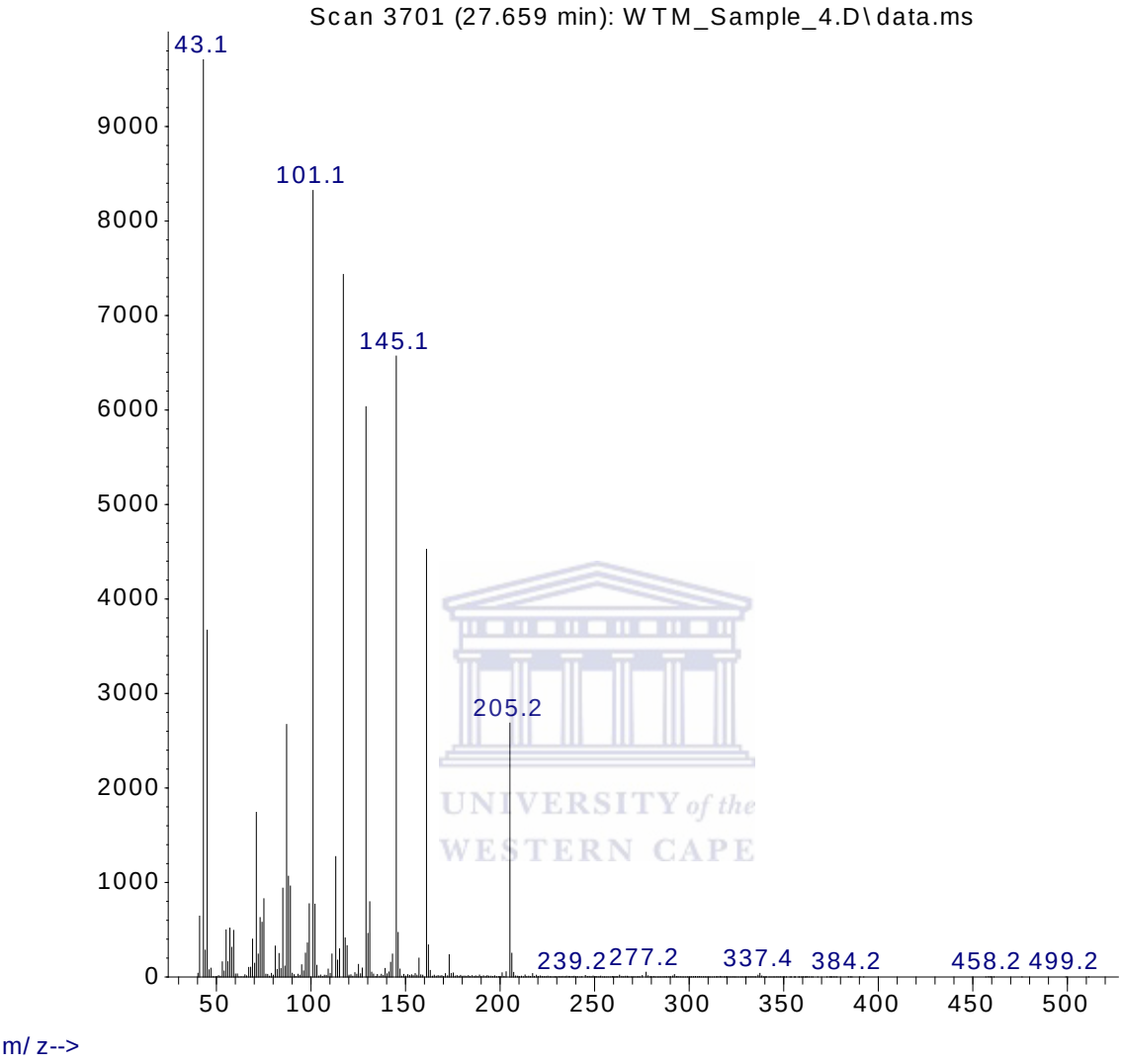
Appendix 3i Mass spectrum of →1)-Man*p*-(2→



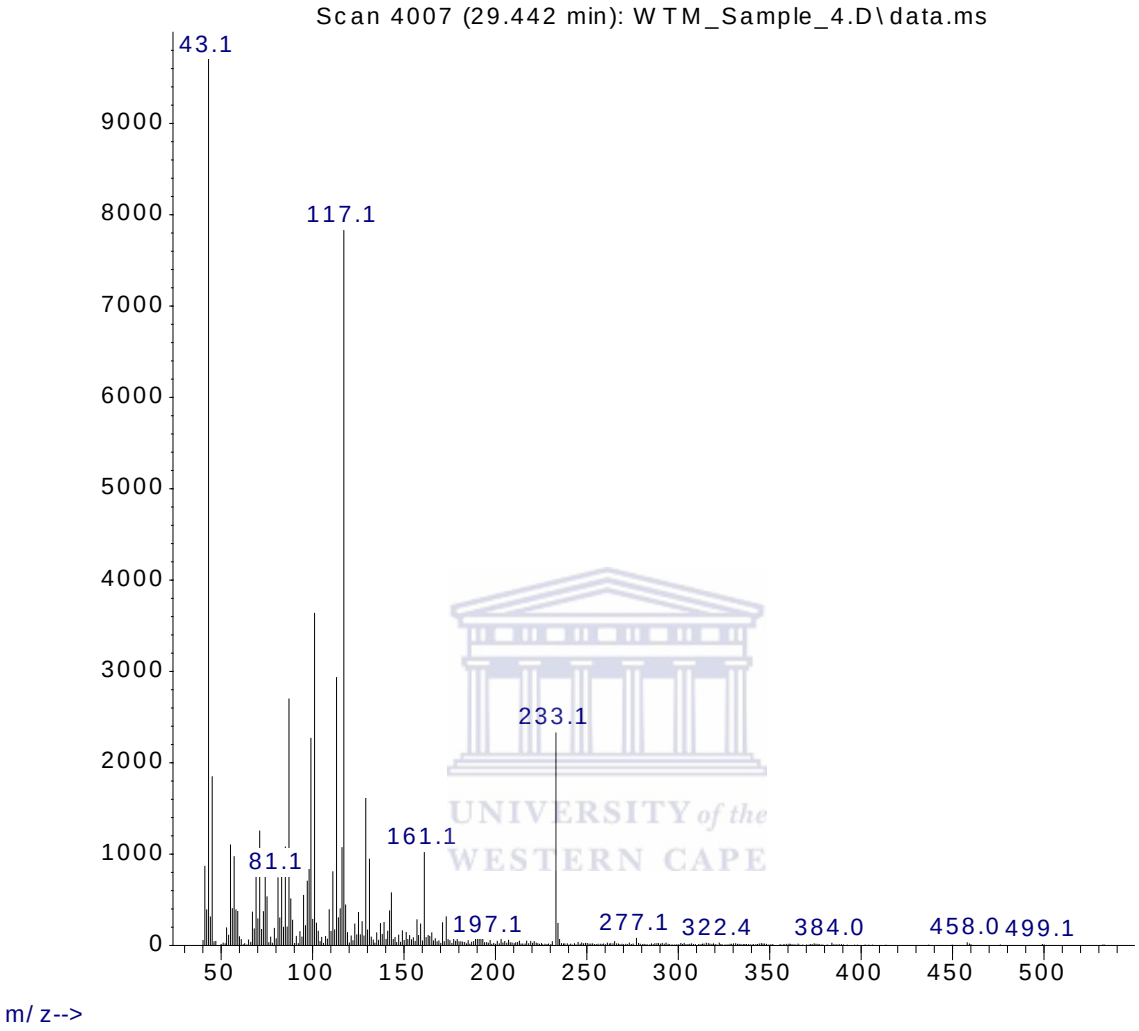
Appendix 3j Mass spectrum of →1,4)-Man*p*-(2→



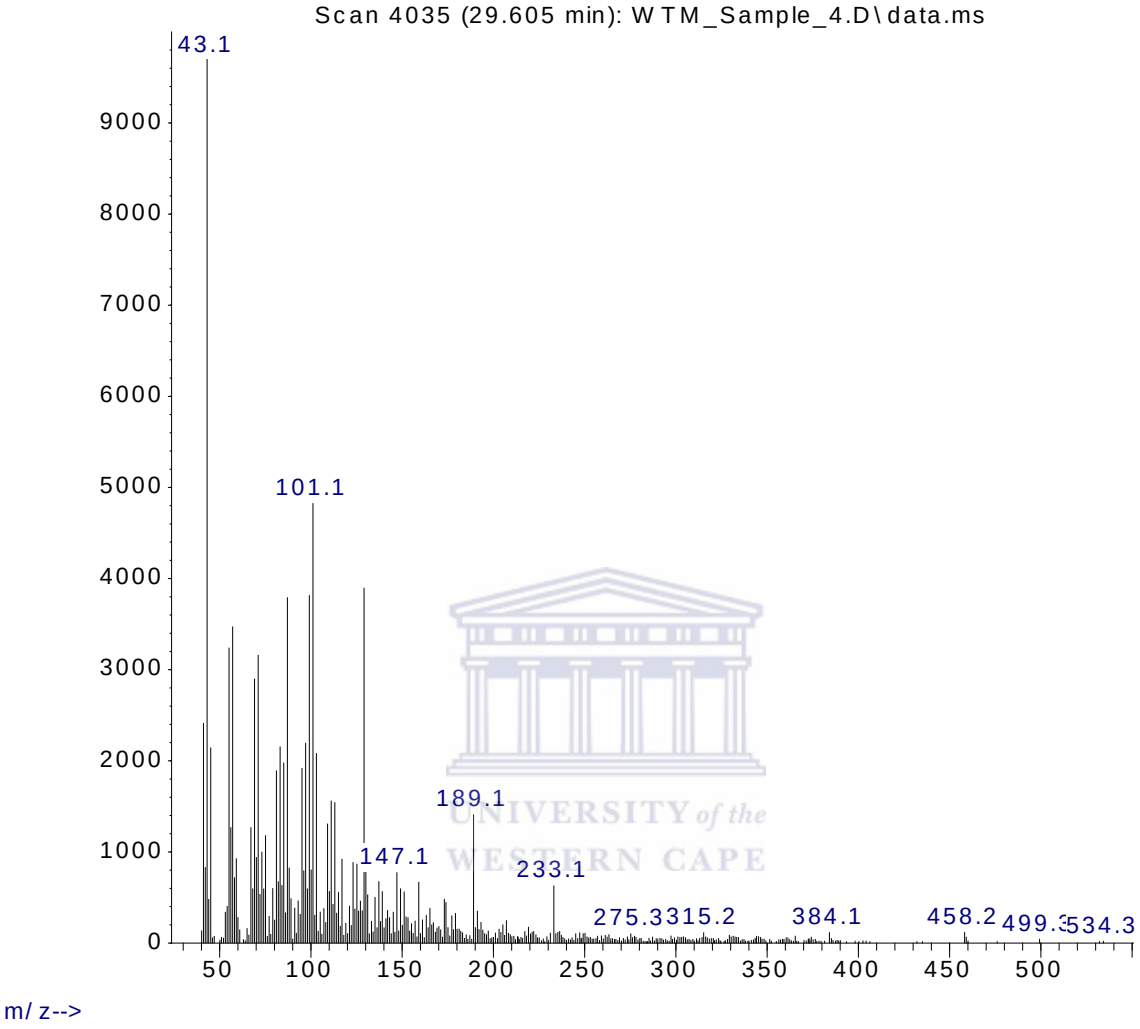
Appendix 3k Mass spectrum of Gal*p*-(1→



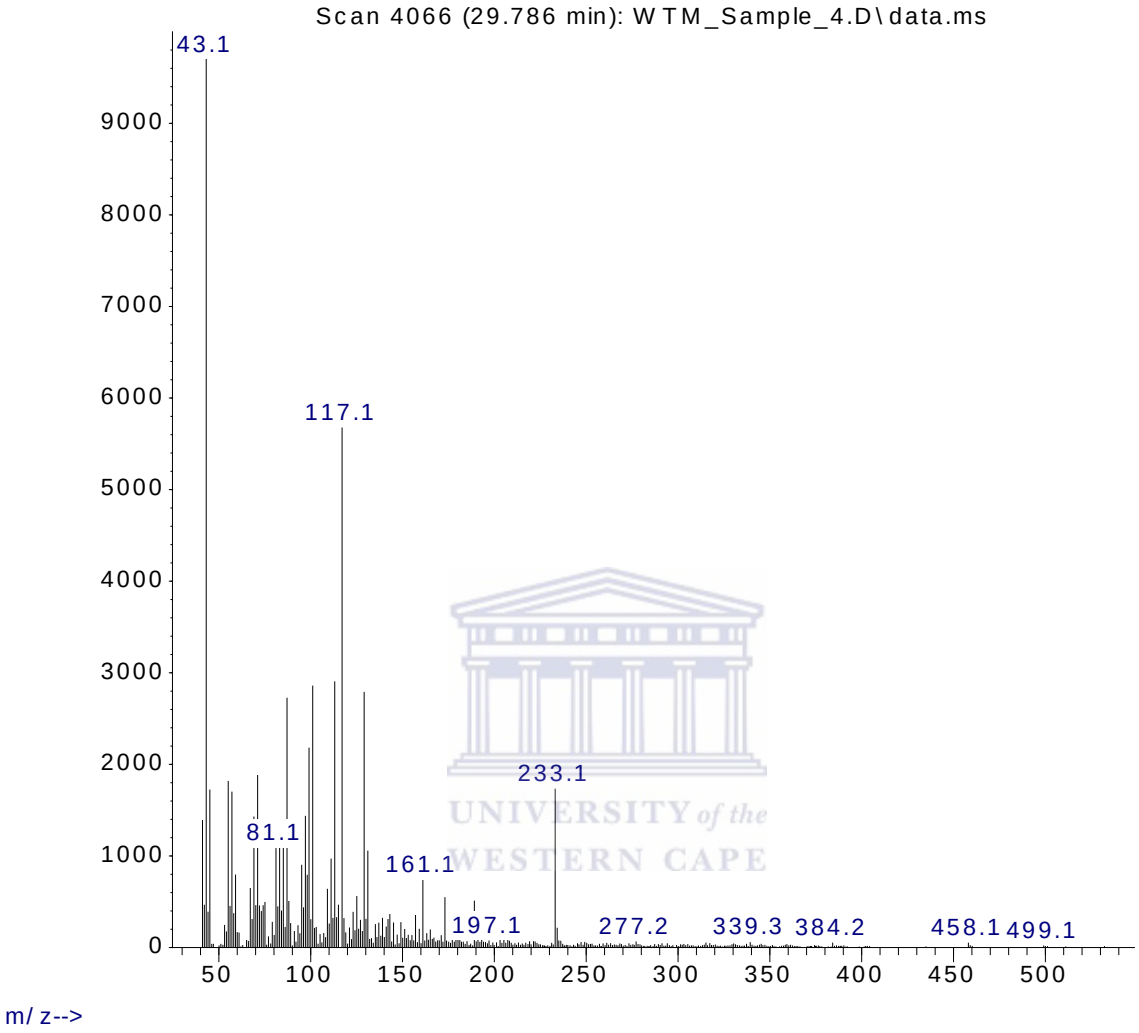
Appendix 3l Mass spectrum of →1)-Gal-*p*(3→



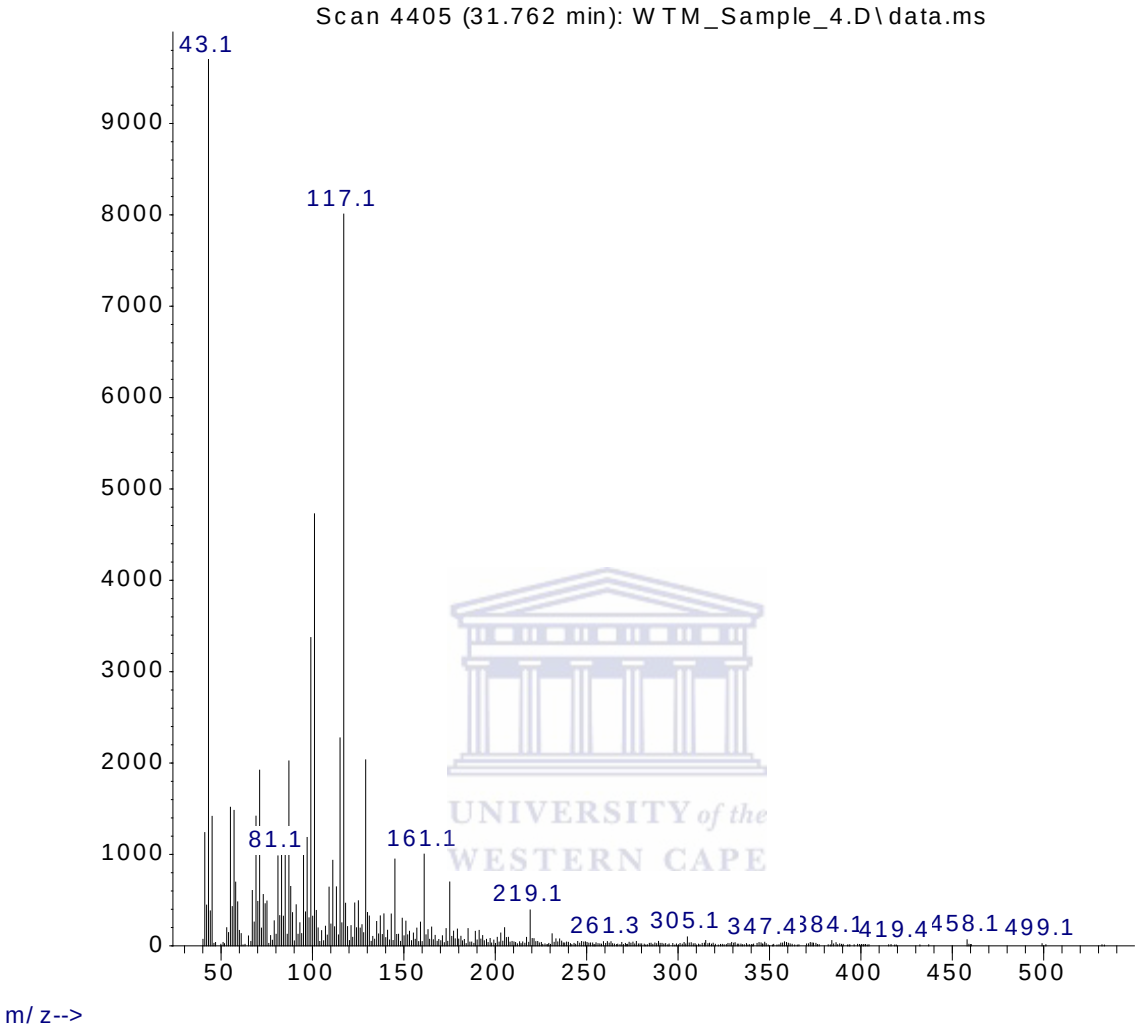
Appendix 3m Mass spectrum of →1)-Gal-*p*(2→



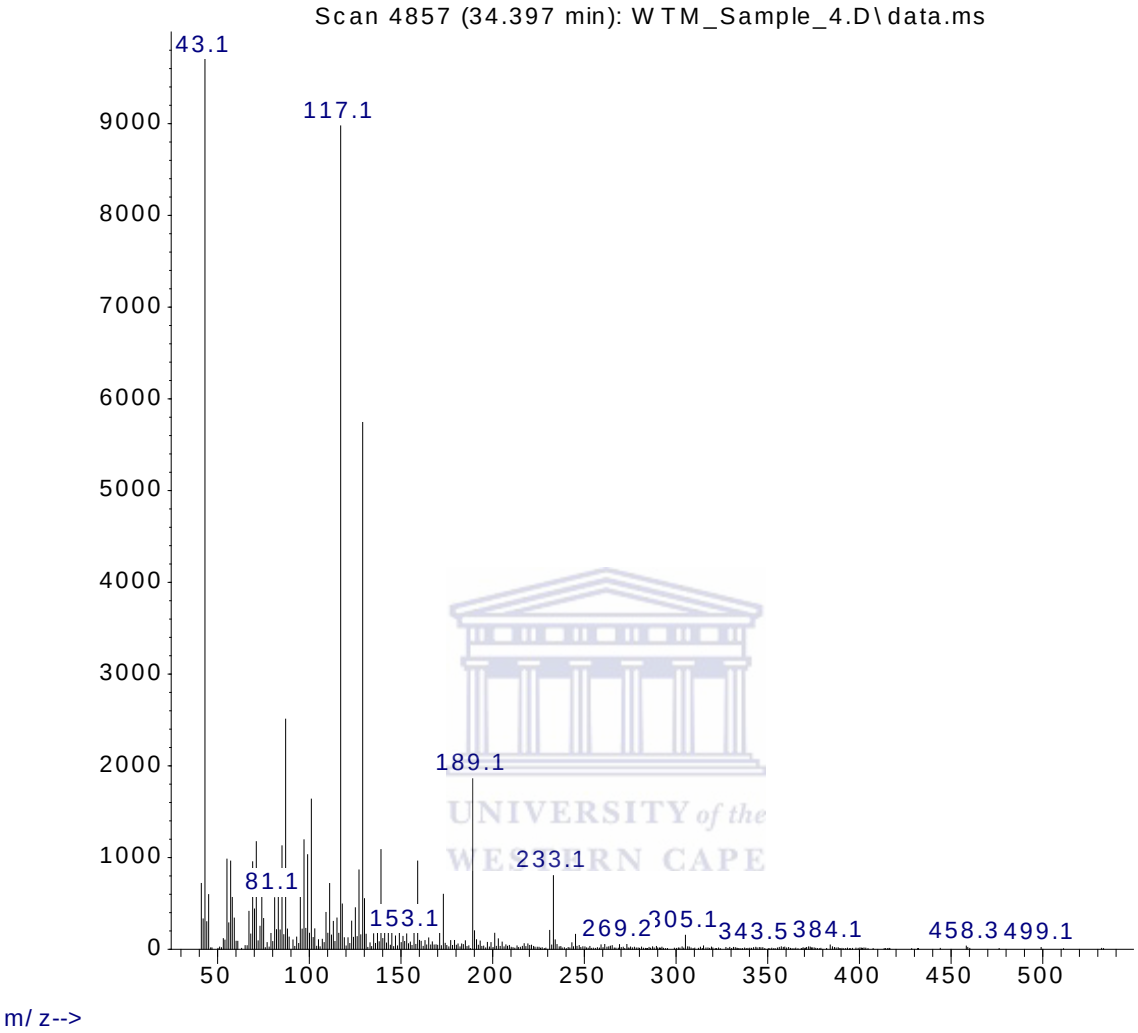
Appendix 3n Mass spectrum of →1)-Gal-*p*(4→



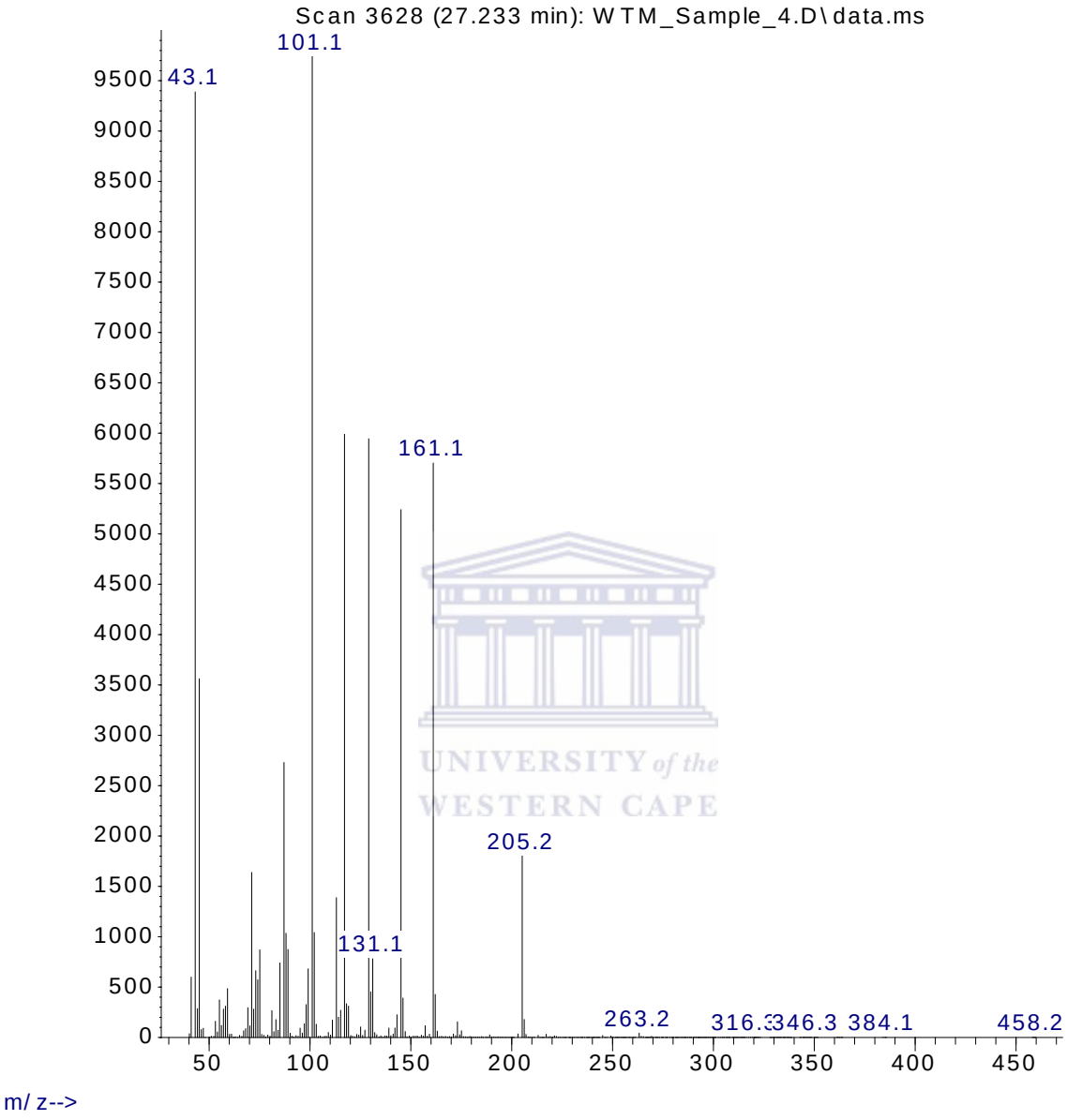
Appendix 3o Mass spectrum of →1)-Gal-*p*(6→



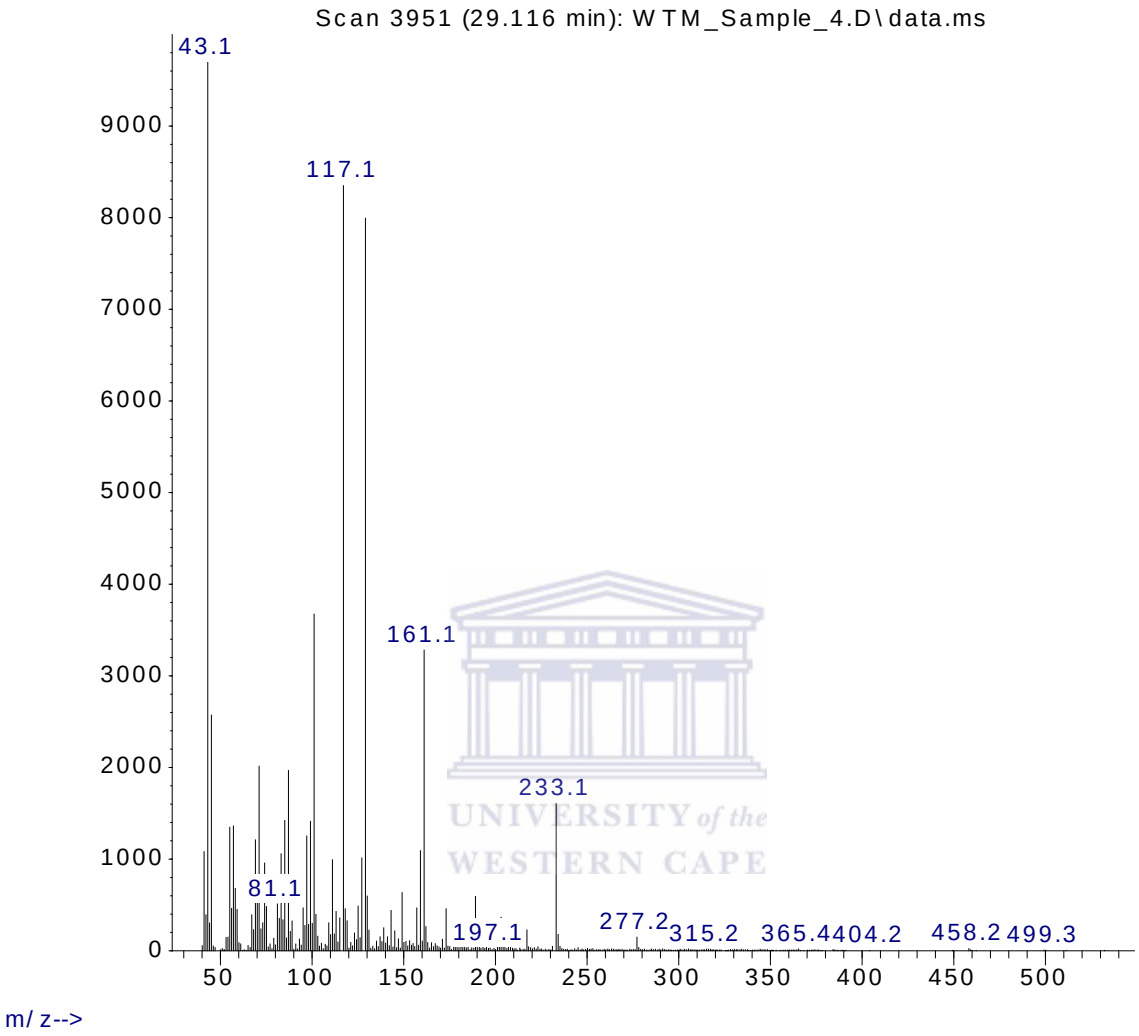
Appendix 3p Mass spectrum of →1,3)-Gal*p*-(6→



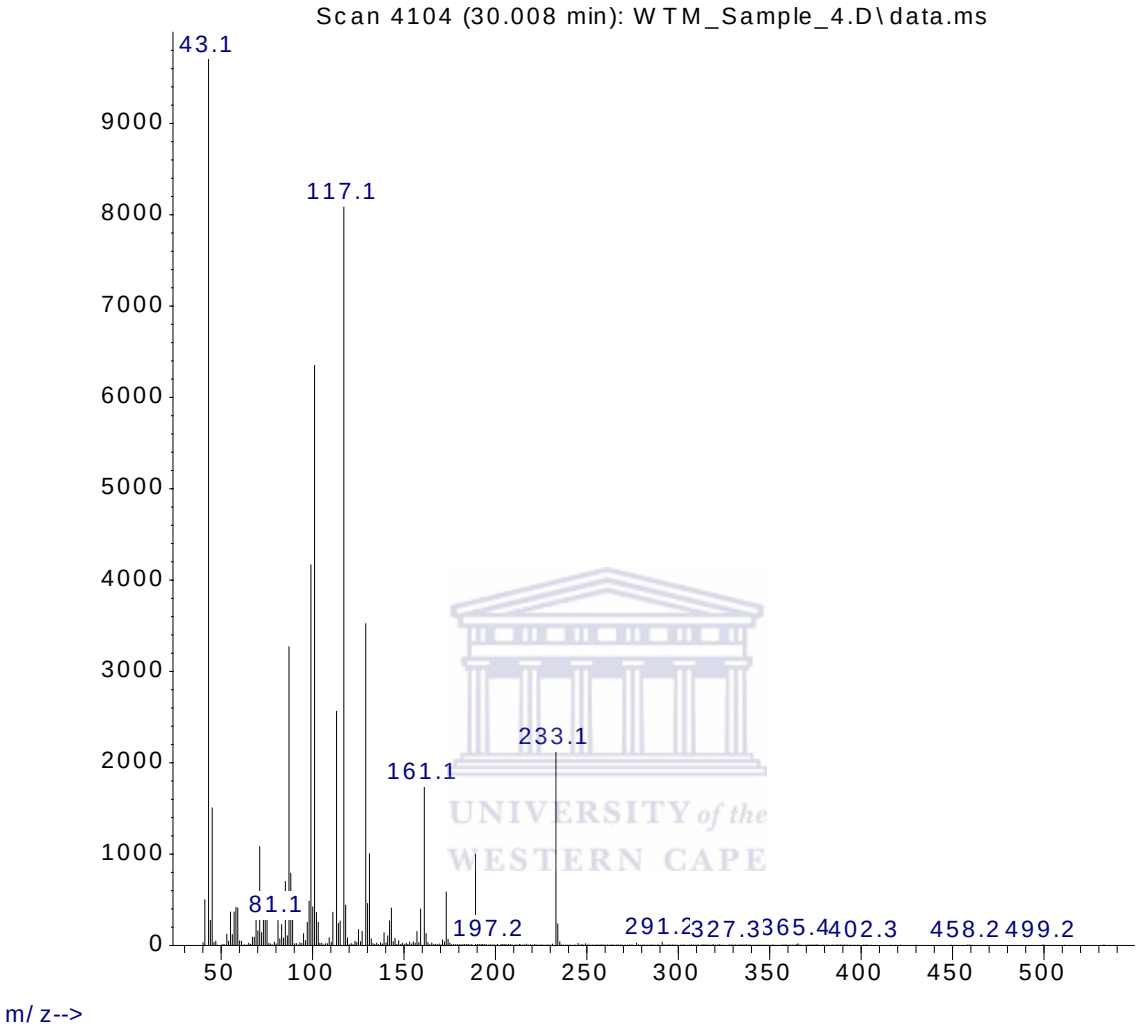
Appendix 3q Mass spectrum of Glc*p*-(1→



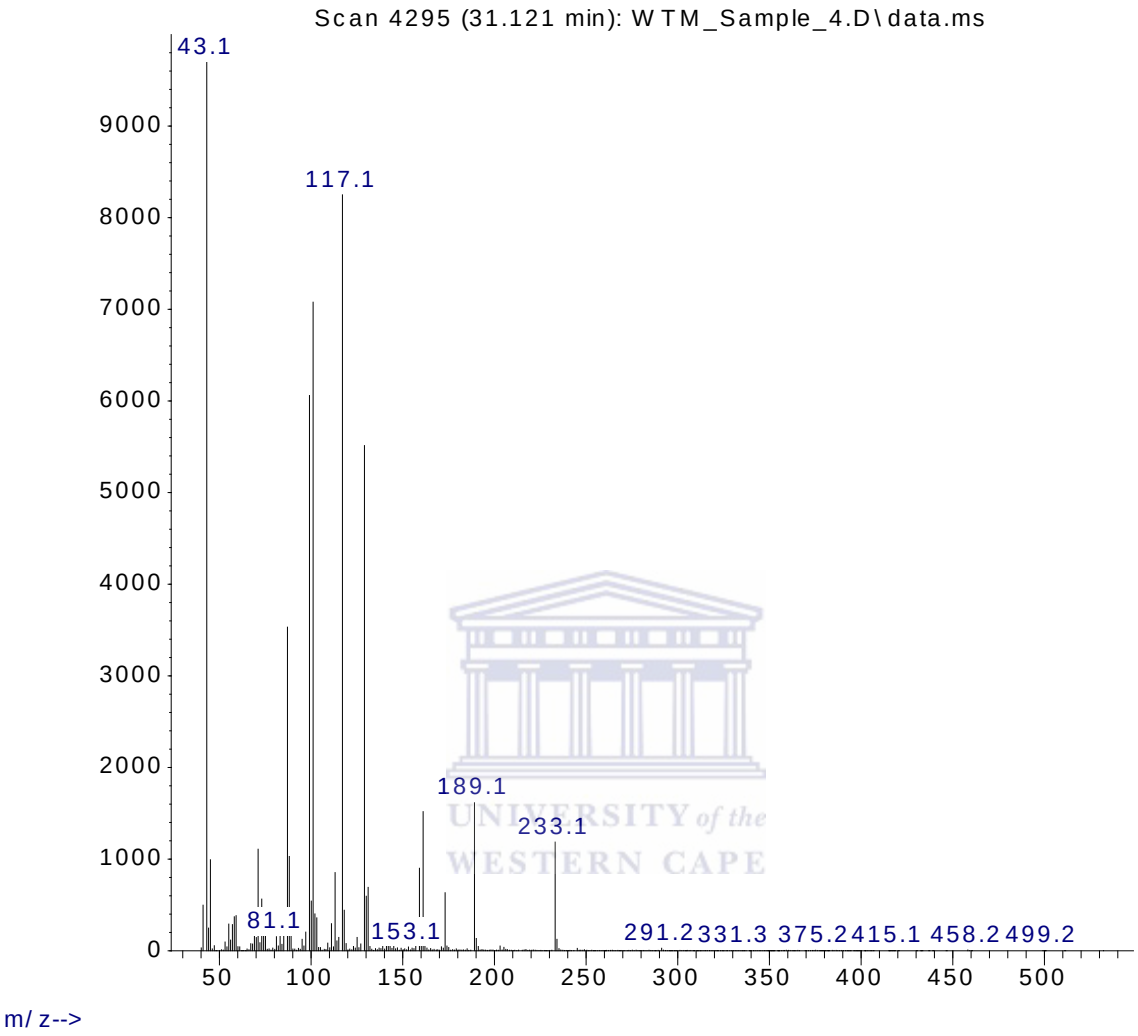
Appendix 3r Mass spectrum of →1)-Glc*p*-(3→



Appendix 3s Mass spectrum of →1)-Glc*p*-(6→



Appendix 3t Mass spectrum of →1)-Glc*p*-(4→



Appendix 3u Mass spectrum of →1,6)-Glc*p*-(4→