# A PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION OF *SUTHERLANDIA FRUTESCENS.*

**By**

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**NOVEMBER 2011**

## DEDICATION

To two of my best friends

## DECLARATION

I, Maria Teresa Faleschini, hereby declare that A phytochemical and biological investigation of *Sutherlandia frutescens* is my own work and that all sources that I have used or quoted have been indicated and acknowledged by means of complete references. The thesis has not been submitted or will not be submitted to a university or any institution for the award of a degree.

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2. Date

3. Date

## ABSTRACT

Since ancient times, indigenous plants have been used by traditional healers for treating various ailments. *Sutherlandia frutescens* is one of the most commonly used medicinal plants of southern Africa. This widely distributed plant has been traditionally used to treat cancer and HIV patients; however scientific validation is still in high demand. This research aimed to phytochemically characterise the various extracts prepared and to determine if any chemotypes were present. Subsequent biological characterisation was carried out to preliminary ascertain whether this medicinal plant could have anti-cancer and/or immune-modulating properties and which compounds might be responsible for these actions.

Various traditional and organic extracts were prepared. Extracts, fractions and compounds generated were analysed and chemical profiles obtained. Column chromatographic techniques were used to isolate and purify compounds and structure elucidation was carried out using various analytical techniques. Sulforhodamine B and cytometric bead array assays were performed to determine the biological activities of samples generated.



**KEY TERMS**

*Sutherlandia frutescens*, traditional uses, anti-cancer, immune-modulating, HIV, phytochemistry, chemotypes, Nuclear Magnetic Resonance, Sulforhodamine B, Cytometric Bead Arrays.

## ACKNOWLEDGEMENTS

"Many discouraging hours will arise before the rainbow of accomplished goals will appear on the horizon." King Haile Sellassie

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| 1D | one dimensional |
| 2D | two dimensional |
| 3D | three dimensional |
| $^{13}C$ | carbon 13 |
| $^{1}H$ | proton |
| δ | NMR chemical shift in ppm |
| $\delta_C$ mult. | delta carbon multiplicity |
| $\delta_H$ mult. | delta proton multiplicity |
| $\lambda_{max}$ | lamda maximum |
| µg/ml | micrograms per mililiter |
| ° C | Degrees Celsius |
| ACD | Advanced chemistry development, Inc. |
| ACN | Acetonitrile |
| AIDS | Acquired Immunodeficiency syndrome |
| APC | Antigen presenting complex |
| BD | Beckton Dickinson Biosiences |
| C-18 | 18 carbon chain |
| CBA | Cytometric bead arrays |
| $CD_3OD$ | deuterated methanol |
| $CHCl_3$ | chloroform |
| CHO | Chinese Hamster Ovary |

| | |
|---|---|
| $CO_2$ | Carbon dioxide |
| COSY | correlation spectroscopy |
| COX-2 | Cyclo-oxygenase-2 |
| CP | chemically pure grade |
| CSIR | Council for scientific and industrial research |
| Ctrl | Control |
| Cyp17 | cytochrome P450 17 |
| Cyp21 | cytochrome P450 21 |
| DBE | Double bond equivalents |
| DCM | dichloromethane |
| DEPT | Distortionless Enhancement by Polarisation Transfer |
| DMSO | dimethyl sulphoxide |
| d-pyr | deuterated pyridine |
| DST | Department of Science and Technology |
| ECACC | European Collection of Cell Culture |
| ECD | Enterprise Creation for Development unit |
| Ech | *Echinacea* |
| EtOH | ethanol |
| ESI | electrospray ionisation |
| $ESI^-$ | electrospray negative mode |
| $ESI^+$ | electrospray positive mode |
| FBS | foetal bovine serum |
| FA | formic acid |

| | |
|---|---|
| FACS | fluorescence-activated cell sorting |
| g | grams |
| GABA | gamma-aminobutyric acid |
| $H_2O$ | distilled water |
| h | hour |
| HCOOH | formic acid adduct |
| HGH | human growth hormone |
| HIV | Human immunodeficiency virus |
| HL60 | Human Leukaemia cell line |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HPLC | High performance liquid chromatography |
| HR | high resolution |
| HSQC | Heteronuclear Single Quantum Coherence |
| Hz | hertz |
| $IC_{50}$ | Inhibitory concentration of 50 % |
| IFN | interferon |
| IL | Interleukin |
| IR | Immune response |
| *J* | spin-spin coupling constant in Hz |
| kg | kilograms |
| $KHCO_3$ | potassium hydrogen carbonate |
| L | liters |
| LPS | lipopolysaccharide |

| | |
|---|---|
| M | base peak mass |
| MCF7 | human breast adenocarcinoma cell line |
| mDa | milidaltons |
| MeOH | methanol |
| $MgSO_4$ | magnesium sulphate |
| MHz | mega hertz |
| MRC | Medical Research Council |
| MS | Mass spectrometry |
| *m/z* | mass to charge ratio |
| NCI | National Cancer Institute |
| ng/ml | nanograms per mililiter |
| nm | nanometers |
| NMR | Nuclear magnetic resonance |
| OH | hydroxy |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PDA | photo diode array |
| PE | phycoerythrin-conjugated anti-human cytokine antibodies |
| PMA | Phorbol 12-myristate 13-acetate |
| PP | polypropylene |
| ppm | parts per million |
| PRR | pattern recognition receptors |
| QTOF | Quadrupole time of flight |

| | |
|---|---|
| Rf | Retention factor |
| RPMI | Roswell Park Memorial Institute |
| SANBI | South African National Biodiversity Institute |
| SD | Standard deviation |
| SRB | sulforhodamine B |
| SU1 | sutherlandioside B |
| SU2 | sutherlandioside A |
| SQD | Single Quadruple Detector |
| TB | Tuberculosis |
| TCA | trichloroacetic acid |
| TLC | thin layer chromatography |
| TNF | tumour necrosis factor |
| $T_0$ | time zero |
| TOF | time of flight |
| UACC62 | melanoma caner cell line |
| UPLC | ultra performance liquid chromatography |
| UV | Ultraviolet |
| UV-VIS | Ultraviolet-visible region |
| V | viability |

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# CHAPTER 1

# INTRODUCTION

## 1.1 MEDICINAL PLANTS

Although one can be gifted genetically with a powerful immune system nutrients help to ensure optimal development of one's immune system. Plants constitute the base of the food chain and provide the most valuable source of natural nutrients; in addition, constituents of medicinal importance are also present in plants (Anonymous, 2000).

Plants used for medicinal purposes contribute significantly to the development of major medical drugs that are used today. Most of our common medicines have compounds extracted from plants as their primary active ingredients and many have also provided blueprints for synthetic or partially synthetic drugs (Simpson and Ogorzaly, 2001).

A major part of using plants as medicines involves the use of plant extracts or their active principles. Medicinal plants and plant-derived medicines are widely used in different traditions all over the world and they are becoming increasingly popular in modern scientific communities as natural alternatives to synthetic chemicals (Van Wyk and Wink, 2004). Many plants synthesize substances that are useful to the maintenance of health in humans and other animals. These include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins. In many cases, substances such as alkaloids serve as plant defence mechanisms against predation by microorganisms, insects and herbivores. In general many herbs and spices are used for food seasoning which also underlies useful medicinal properties (Lai, 2004).

A single plant can be viewed from a purely scientific perspective as a biosynthetic facility manufacturing a large number of molecules from simple nutrients, water, carbon dioxide and solar energy. The resulting chemistry is quite complex and includes all the final molecules in the biosynthetic pathway, together with their precursors. Molecules that are produced when an alternative pathway has been activated and are not required in primary metabolic processes are known as secondary metabolites, and some are believed to have evolved to form part of the plant's defence mechanism. Many of these secondary metabolites have biological activities that can be assayed in the laboratory, providing a scientific rationale for

the use of the particular plant. In some cases isolated active compounds are subsequently channelled into drug development regimes and eventually commercialised. In this regard, it has been estimated that about a quarter of all modern drugs were originally derived from plant sources with relatively complex or advanced characteristics (Kinghorn and Balandrin, 1993).

Well-known examples of plant-derived medicines include quinine (from *Chincona* species), morphine and codeine (from O*pium* species), colchicines (from *Colchicum autumnale),* atropine (from the Solanaceae family), reserpine (from *Rauwolfa serpentine),* salicin (from *Salix alba*) and digoxin (from *Digitalis purpurea).* Important advances in anti-cancer drugs such as taxol (from *Taxus brevifolia)* and vincristine (from *Catharanthus roseus)* have been developed from plants (Dewick, 2002; Simpson and Ogorzaly, 2001; Van Wyk and Wink, 2004).

The vast knowledge of herbal remedies in traditional cultures is believed to have developed through trial and error over many centuries, with the most important cures being carefully passed on via the verbal route from one generation to the next (Van Wyk and Wink, 2004). One of the spin-offs derived from such a plethora of traditional knowledge, is that many new and important remedies are still being discovered.

## 1.2 MEDICINAL PLANTS OF SOUTHERN AFRICA

Traditional medicine in Africa is perhaps the oldest and most diverse of all medicine systems (Van Wyk and Wink, 2004). Southern Africa is rich in plant and cultural diversity and various endemic and foreign plants today are used for a variety of medicinal purposes. The study of the use of plants by local people is widely dispersed in southern Africa and the interactions of these plants with biological systems needs to be continuously investigated. Collection and documentation of this indigenous knowledge are required to prevent loss to future generations (Van Wyk and Gericke, 2000) and to guide future medicinal investigations. The use of traditional medicines by Africans is widespread and promoted by Ministries of Health in several African nations, including South Africa. Two herbal remedies *(Hypoxis hemerocallidea* and *Sutherlandia frutescens*), are currently recommended by the South Africa Ministry of Health for management of many ailments, including cancer and infection with human immunodeficiency virus (HIV) (Mills, Cooper *et al.,* 2005). Both of

these remedies have shown the potential for drug interactions, but efforts should be made by health professionals to provide validated information to traditional healers and their patients on the careful use of herbal medicines (Mills, Foster *et al.,* 2005). To date no pure isolated compound from S*utherlandia* has been developed into a marketable drug. However, a number of phytopharmaceuticals (pharmaceuticals made from plant extracts standardised using active compounds) have been developed and commercialised (such as 'Power-Your-Life' products from Parceval pharmaceuticals) (www.parceval.co.za).

Extracts from around 4 000 species of plants have been used for medicinal purposes in southern Africa. Tonic plants have multiple functions in the human body, and act in healthy individuals by maintaining and supporting general physical and mental health. When individuals are ill, or on the way to recovery, tonics can assist in restoring their health and are believed to tone the body's organ systems as well as improving immune function. These substances act by enhancing body metabolism, including secretion and excretion of waste products, improving digestion and may also have a mild purgative effect. Some tonics are effective adaptogens, significantly enhancing the body's ability to adapt to physical and emotional stress (Van Wyk and Gericke, 2000).

In general, most of these herbal and traditional medicines are not well researched, poorly regulated and may even contain contaminated products which could produce adverse effects (Mills, Cooper *et al.,* 2005). There is a growing appreciation for the potential of natural tonics in maintaining and supporting health, particularly where modern allopathic medicine is concerned. This has come about mainly as a result of scientific investigations into the activities, pharmacology and chemistry of some well known adaptogenic plants, including ginseng (*Panax ginseng*), American ginseng (*Panax quinquefolium*), Siberian ginseng (*Eleutherococcus senticosus*) and Indian ginseng (*Withania somnifera*). One of the most significant adaptogenic plants from southern Africa belongs to the *Sutherlandia* genus. Affordable natural tonics, supported by formal clinical studies, could play an important role in improving the quality of life in patients with cancer, tuberculosis, HIV/AIDS and other serious health conditions. In spite of their broad utility and common use, tonics should be used responsibly in appropriate doses, as well as for defined durations (Van Wyk and Gericke, 2000).

Indigenous knowledge systems are dynamic and adaptive, and over the last few hundred years many introduced plants have been extensively used in southern African medicine,

including rue (*Ruta graveolens*) for a variety of ailments, camphor (*Cinnamomum camphora*) for fever, colds, antiseptic and mild analgesic activity, plus bluegum (*Eucalyptus globulus*) for its use in decongestant medicines (Van Wyk and Gericke, 2000). There are many other plants which have an important use in traditional medicines, but the main research focus of this study is on one of the above mentioned indigenous plants - *Sutherlandia frutescens*.

## 1.3 S*UTHERLANDIA FRUTESCENS*

The *Sutherlandia* plant occurs endemically in South Africa, being widely spread over the South Western and Northern Cape Provinces, essentially being found along road verges in the drier areas of the country, as indicated in **Figure 1.1**. The term 'sutherlandia' refers to a shrubby plant that is known by many different names, including cancer bush, balloon pea (English); umnwele (Xhosa); kankerbos, blaasbossie, blaas-ertjie, eendjies, gansiekeurtjie, klappers, hoenderbelletjie (Afrikaans); Unwele and Insiswa (Zulu); Musa-Pelo, Motlepelo and Phethola (Sotho) (Jackson, 1990).

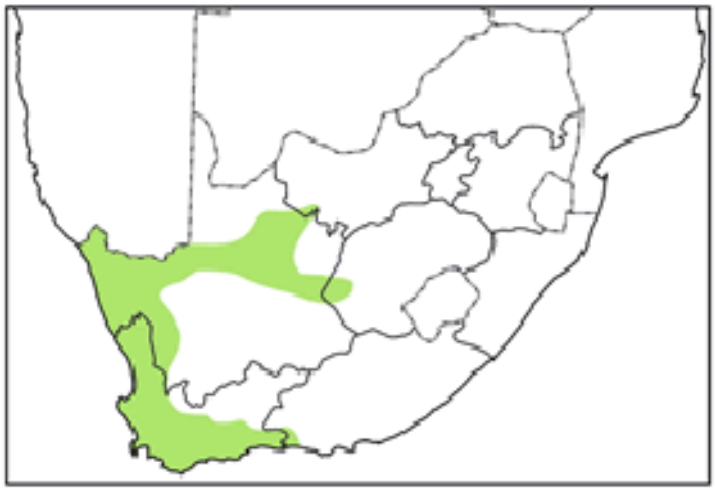


**Figure 1.1 Geographical distributions of *Sutherlandia* species throughout South Africa (sahealthinfo.org).**

*Sutherlandia* belongs to the family Fabaceae (pea and bean or pod-bearing family) which is the second largest flowering plant family in the world. This family contains more than 600

genera and 1200 species. In southern Africa, this family is represented by 134 genera and more than 1300 species (Jackson, 1990). There are five *Sutherlandia* species namely, *S. frutescens*, *S. microphylla*, *S. montana*, *S. tomentosa*, and *S. humilis*. The genus took its name from James Sutherland, first Director of the Edinburgh Botanic Garden (Duncan, 2009). While field studies over a period of many years have shown that only two species should be recognised, along with several geographically separated subspecies (Moshe *et al.*, 1998), this classification has not been implemented and many chemical variations are believed to occur, involving differences in biological activity and major compounds present in the plant.

Ecologically, legumes are known for fixing nitrogen in the soil through a symbiotic relationship with bacteria. The bacteria infect the roots, forming small growths or nodules. Inside the nodules, atmospheric nitrogen, which the plants cannot use, is converted to ammonia, which plants can use. The plant supplies sugars for the bacteria; while the bacteria provide the biologically useful nitrogen that the plant absorbs (Jackson, 1990).

*Sutherlandia frutescens* (see **Figure 1.2**) is one of the best-known multi-purpose medicinal plants. Used as an adaptogenic tonic, the plant's therapeutic properties can be attributed to subsequent effects on the human body to mobilise immunological and physiological resources to help combat diseases, as well as helping to combat physical and mental stress (Ojewole, 2008; www.sutherlandia.org). The recommended dose of *Sutherlandia frutescens* in humans is 9 mg per kg body weight per day (Seier *et al.*, 2002). Phyto Nova, of South Africa, is a major distributor of both powdered and encapsulated forms of this herb, as well as in a gel preparation. Parceval Pharmaceuticals has produced 'Power-Your-Life' products varying from tablets (Reclaim+Protect) to Vaseline (Defend+Protect) and immune-boosting syrup with added vitamins (Support+Protect). All are made from *Sutherlandia* extracts that are said to contain the active ingredient SU1 (sutherlandioside B - a cycloartane glycoside) (www.parceval.co.za).

**Figure 1.2 *S. frutescens* growing in the wild at Goegap Nature Reaserve, Springbok, Northern Cape (photo taken by Nial Harding).**

### 1.3.1 A historical aspect of medicinal uses of *Sutherlandia*

This plant has been used for hundreds of years by the original inhabitants of the Cape, being the Khoi San and Nama people, who used it mainly as a decoction for the washing of wounds and also take it internally to reduce fevers. For many hundreds of years, Zulu warriors returning from battle used the plant to relax themselves. Grieving widows used it as an anti-depressant to help them through their loss. The old Zulu word of "insiswa" means "that which takes away the dark" refers to the ability of the plant to help with depression and sadness (www.plantzafrica.com - *Sutherlandia frutescens* (L.) R. Br.; Van Wyk and Albrecht, 2008).

*Sutherlandia* has long been known and respected as a medicinal plant in southern Africa and is regarded as the African adaptogens *par excellence*. The early colonists regarded it as giving successful results in the treatment of chicken pox, stomach problems, internal cancers and eye troubles (Watt and Breyer-Brandwjik, 1962). It continues to be used to this day as a remedy for the above-mentioned ailments. According to Van Wyk and Gericke (2000), tinctures, infusions and decoctions of the leaves and young stems of *Sutherlandia* have been

used to treat colds, flu, asthma, TB, bronchitis, rheumatism, rheumatoid arthritis and osteo-arthritis, liver problems, haemorrhoids, piles, bladder, uterus and 'women's' complaints, diarrhoea and dysentery, stomach ailments, heartburn, peptic ulcers, backache, diabetes, varicose veins and inflammation. It is also used in the treatment of mental and emotional stress, including irritability, anxiety and depression and is used as a gentle tranquillizer. This plant is said to be a useful bitter tonic and that a little taken before meals will aid digestion and improve the appetite.

The Rastafarians also recommend *Sutherlandia* as a treatment for these medical conditions (Dyson, Ashwell and Loedolft, 1998). There is preliminary clinical evidence that it has a direct anti-cancer effect in some cancers and that it acts as an immune stimulant (www.sutherlandia.org).

Based on preliminary safety studies, the South African Ministry of Health has concluded that extracts from this plant are fit for human consumption. While certain extracts from *Sutherlandia* are said to have immune-boosting activities, their mechanism of action has not been comprehensively documented. According to Mills, Cooper *et al., (*2005), extracts from hot water displayed superoxide- as well as hydrogen peroxide scavenging activities that ostensibly could account for the anti-oxidant potential and anti-inflammatory properties of *S. frutescens.* Ethanolic extracts were also shown to have an anti-proliferative effect on several human tumour cell lines, but did not show significant anti-oxidant activity.

### 1.3.2 Pre-isolated compounds from *Sutherlandia* species

A number of biologically active compounds occur in high concentrations in extracts from *Sutherlandia* species, such as L-canavanine, D-pinitol, and γ-amino butyric acid (GABA) (Van Wyk and Gericke, 2000), including, a novel triterpenoid glycoside (sutherlandioside B) (Fu *et al.,* 2008) also known as ‘SU1’ (Olivier *et al.,* 2009; Van Wyk and Albrecht, 2008), as well as various flavonol glycosides (Fu *et al.,* 2010) have been isolated and characterised. Plant extracts have also been shown to contain amino acids, small amounts of saponins, but no alkaloids (Van Wyk and Gericke, 2000). A more detailed discussion of these compounds, together with some of their activities, follows below.

**1.3.2.1 High levels of free and protein-bound amino acids** are common with many members of the family Fabaceae (Moshe, 1998). Analyses of leaf extracts from *Sutherlandia* contain high levels of amino acids such as asparagine, proline and arginine. The presence of

L-arginine is important, because it acts as an antagonist of L-canavanine that attenuates the anti-proliferative activity of canavanine (Van Wyk and Albrecht, 2008).

**1.3.2.2 Canavanine:** is frequently found in the seeds of Fabaceae and therefore its discovery at high concentrations in extracts from the leaves of *Sutherlandia* by Moshe (1998), was of considerable interest. Canavanine (see **Figure 1.3**), which is a potent L-arginine competitor and the L-2-amino-4-guanidino-oxy, is a structural analogue of arginine (Mills, Cooper *et al.,* 2005). It has also been shown to have anti-cancer as well as anti-viral activity, including inhibition of influenza virus and retroviruses (Green, 1988). As such, this finding justifies the traditional use of this plant against cancer; in addition, this also appears to be the first known case of a canavanine-containing plant having a well-recorded history of use against cancer ( Bence *et al.,* 2002; Crooks and Rosenthal, 1994; Rosenthal, 1997; Swaffar, 1995; Van Wyk and Albrecht, 2008). Between 30-40 mg of canavanine can be found per dry gram of the leaves (Gericke *et al.,* 2001). Canavanine is an inhibitor of nitric oxide synthase, which may be beneficial in certain forms of heart failure (Van Wyk and Gericke, 2000).

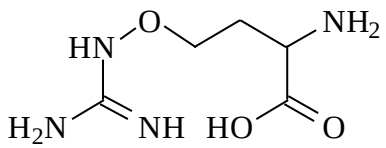



**Figure 1.3 Compound 1: Canavanine.**

**1.3.2.3 Pinitol:** is a type of sugar found in many types of legumes and is classified as chiro-inositol. It was first found by Snyders (1965), as well as Viljoen (1969) and Brummerhoff (1969) in *Sutherlandia microphylla* leaves. It is also known as 3-O-methyl-D-chiro-inositol, or 3-0-methyl-1,2,4 *cis*-3,5,6-*trans*-hexahydroxy-cyclohexanol (Mills, Cooper *et al.,* 2005). 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). It has been used for the treatment of wasting in cancer as well as AIDS and is a known anti-diabetic agent (Narayanan *et al.,* 1987; Ostlund and Sherman, 1996). Pinitol has also been shown to exert an insulin-like effect (hypoglycaemic effect), resulting in lower blood sugar levels and increased availability of glucose for cell metabolism (Bates, Jones and

Bailey, 2000). It has also been shown to enhance the retention of creatinine by muscle cells (Greenwood *et al.*, 2001), as well as increasing the ability of the muscle cells to absorb glucose, resulting in benefits for sporting enthusiasts, who have concomitant requirements for strength and endurance, together with the added benefits of shortened physiological recovery times. Pinitol (see **Figure 1.4**), has also been shown to have a positive effect on muscles being able to store carbohydrates, thereby allowing for muscle growth, whilst permitting the burning of fat (www.sutherlandia.com). 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). This effect to increase muscle mass, thus has major applications in the treatment of wasting syndrome in cancer, HIV/AIDS and tuberculosis. It is not surprising therefore, that *Sutherlandia* has been used on the African continent for generations, to treat sufferers of tuberculosis, with impressive results (www.sutherlandia.com).

It is not only the pinitol present in *Sutherlandia* that results in all its notable properties. Current research has indicated that there is strong evidence to support the interaction of pinitol with the other bio-active compounds (www.sutherlandia.com).

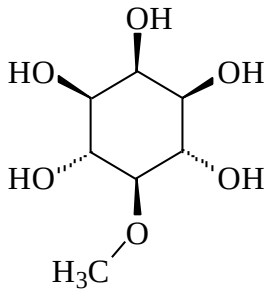



**Figure 1.4 Compound 2: Pinitol.**

**1.3.2.4 Gamma Amino Butyric Acid (GABA):** is a non-essential amino acid and inhibitory neurotransmitter that is found in the leaves of *Sutherlandia*. It is primarily synthesized by decarboxylation of glutamate by the enzyme L-glutamic acid-1-decarboxylase (Ebadi, 2007). Together with niacinamide (vitamin B3) and inositol, GABA prevents anxiety and stress-related neuron messages from reaching the motor centres of the brain. It is thus essential for brain metabolism, where it also decreases neuron activity, thus preventing them from over-firing (www.vitamins-supplements.org). The presence of GABA also justifies the use of *S. frutescens* in treating anxiety and stress. According to Van Wyk and Gericke (2000),

the seeds and leaves have been smoked by labourers and teenagers as a dagga substitute in Namaqualand, with the leaves invoking a strong GABA-induced sedative effect when smoked.

According to Braveman and Pfeiffer (1987); Petty *et al.* (1993) and Petty (1995), GABA has been used in the treatment of depression, bipolar disorder, seizures, premenstrual dysphoric (feeling depressed) disorder, and anxiety. GABA (see **Figure 1.5**) also improves sleep cycles, as well as having a powerful stabilizing effect on blood pressure. It is a very effective analgesic, eliminating pain from chronic conditions such as arthritis and lower back pain. It has also been used to treat epilepsy and hypertension, where it is thought to induce tranquillity in individuals who have a high activity of manic behaviour and acute agitation (Denver Naturopathic Clinic[1]).

GABA signals the pituitary gland to naturally release Human Growth Hormone (HGH), which is widely known for its powerful anabolic (muscle building) effects as well as its lipotropic (breakdown and utilization of body fat) effects (Di Luigi *et al.,* 1999). GABA has also been found to inhibit tumour cell migration (Ortega, 2003).

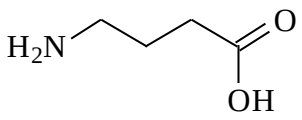


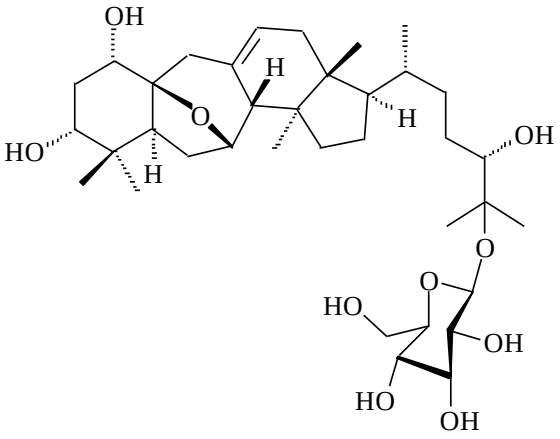

**Figure 1.5 Compound 3: GABA.**

**1.3.2.5 Sutherlandiosides A to D:** The major triterpene, in commercially produced *Sutherlandia* material, is a cycloartane-type triterpene glycoside called sutherlandioside B. At least 56 different triterpene glycosides have been detected. According to Van Wyk and Albrecht (2008), mixtures of cycloartane-type triterpenoid glycosides have extensive geographical variation in South Africa. A study done by Fu *et al.* (2008), found four cycloartane-type triperpenoids which have a bitter tonic activity that stimulates appetite and

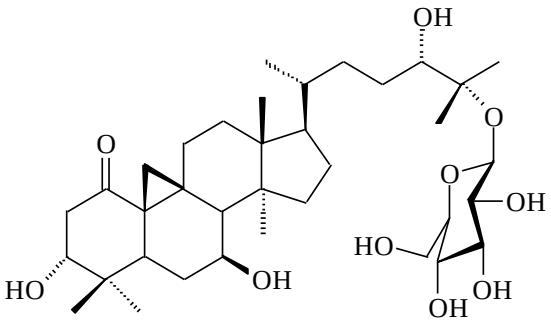
[1] 1181 S Parker Rd # 101, Denver, CO 80231-2152, United States

may contribute to the adaptogenic and immune-boosting effects of *Sutherlandia*. These four compounds are called sutherlandioside A (SU2), B (SU1), C and D.

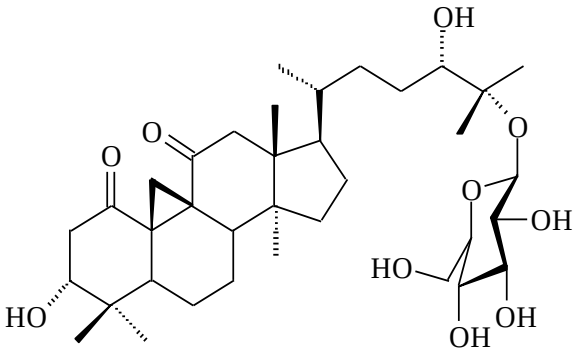
Powerful inhibitory effects of cycloartanes with hydroxylation at C-24 and with a 3-oxo group have been recorded in an *in vivo* mouse skin carcinogenesis test performed by Kikuchi *et al.* (2007). This arrangement is found in sutherlandioside B. The structures of the four sutherlandiosides A to D are shown in **Figure 1.6** as structures **4** to **7**.



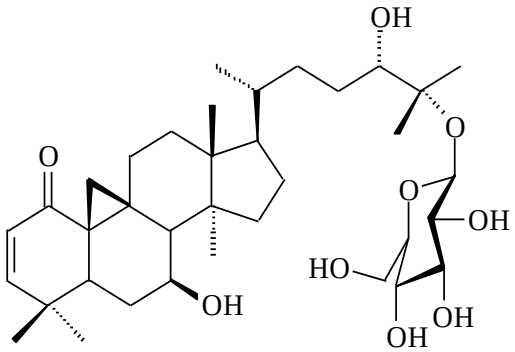

**Compound 4: sutherlandioside A**




**Compound 5: sutherlandioside B**



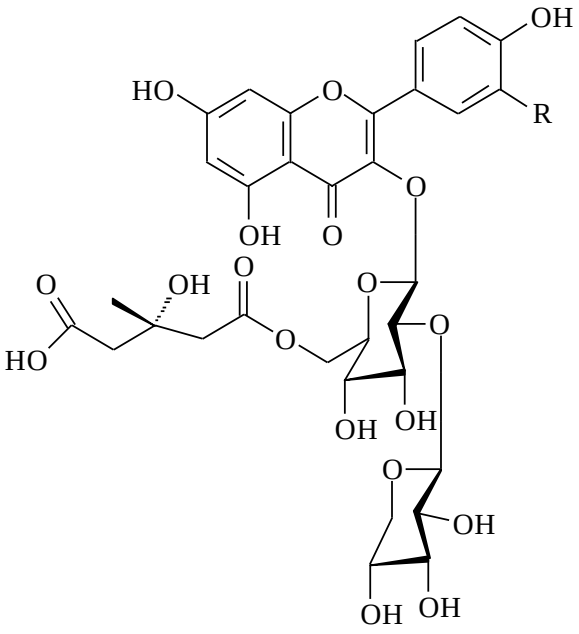

**Compound 6: sutherlandioside C**



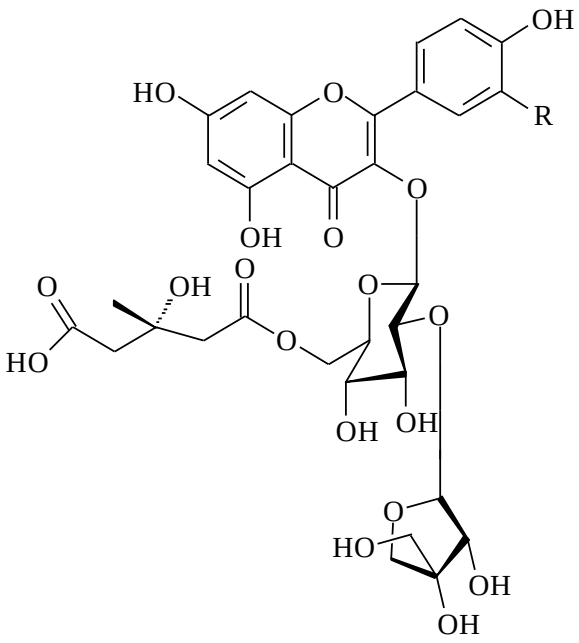

**Compound 7: sutherlandioside D**

**Figure 1.6 Structures of sutherlandiosides A to D are respectively referred to as Compounds 4 to 7.**

**1.3.2.6 Flavonol glycosides:** *Sutherlandia* leaves are known to contain at least six flavonoids (Moshe, 1998). A study done by Fu *et al.* (2010) identified four of these to be flavonol glycosides named sutherlandin A, B, C and D as shown in **Figure 1.7** below. Flavonoids are said to have anti-oxidant activity and help to eliminate mutagen and carcinogens - which are of value in cancer prevention (Tai *et al.*, 2004).




A: R = OH
C: R = H




B: R = OH
D: R = H

**Compound 8: sutherlandin A** **Compound 9: sutherlandin B**

**Compound 10: sutherlandin C** **Compound 11: sutherlandin D**

**Figure 1.7 Structures of sutherlandin A to D are referred to as Compounds 8 to 11.**

**1.3.2.7 Other compounds:** hexadecanoic acid, γ-sitosterol, stigmast-4-en-3-one and at least three long chain fatty acids have been reported in *Sutherlandia* (Tai *et al.*, 2004). High levels of unidentified polysaccharides have also been reported in aqueous extracts (Van Wyk and Albrecht, 2008).

### 1.3.3 Various activities attributed to *Sutherlandia* extracts

**1.3.3.1 Anti-oxidant potential:** Phagocyte-derived reactive oxygen species, such as hydrogen peroxide and superoxide radicals, are responsible for pathogenesis of various inflammatory conditions. According to Tai *et al.* (2004), *Sutherlandia* extracts have been shown to contain superoxide- as well as hydrogen peroxide-scavenging activities at low concentrations, which account for the anti-inflammatory properties. The anti-inflammatory agents have shown to exert chemo-preventive activity by targeting cyclo-oxygenase (COX-2), a rate-limiting enzyme involved in the inflammatory process. They have also reported that ethanolic extracts show anti-proliferative effects on several human tumour cells lines, but no anti-oxidant effects.

**1.3.3.2 Analgesic and hypoglycaemic properties:** Extracts have been shown to contain these properties, which account for the use in type-2 diabetes mellitus (Ojewole, 2004).

**1.3.3.3 Immune-modulator:** *Sutherlandia* extracts have also been reported to have immune-boosting functions in the treatment of HIV/AIDS (Goldblatt and Manning, 2000), although the mechanism for this activity has not yet been elucidated. As such, insufficient scientific data at present has confounded any attempts to explain the mechanism by which extracts from this plant could act on cells of the immune system.

**1.3.3.4 Pharmacological effects and toxicity attributed to *Sutherlandia*:** There has been much work done to assess the pharmacokinetics of this medicinal plant. Van Wyk, Van Oudtshoorn and Gericke (2009) have indicated that extracts with anti-diabetic effects may be due to the presence of pinitol. Similarly, triterpenoids, which have a bitter tonic (amarum) activity in stimulating appetite, may also contribute to possible adaptogenic, anti-cachexia, immune-boosting and cortico-mimetic effects of *Sutherlandia* extracts. As previously mentioned, Van Wyk, Van Oudtshoorn and Gericke (2009) indicated that canavanine may also contribute to the anti-cancer activity. Recently, a new product has been released on the market for diabetic patients, containing *Sutherlandia* plant material in tablet form (Albrecht, 2011).

Prevoo, Swart and Swart (2008) have shown that *S. frutescens* extracts also contain bioactive compounds capable of inhibiting the binding of natural substrates (pregnenolone and

progesterone), as well as inhibiting the catalytic activity of CYP17 and CYP21 enzymes involved in the biosynthesis of steroid hormones.

In general, from a toxicity perspective, an effective toxicological screen of *Sutherlandia* extracts using a primate model, with doses up to 9 times greater than the recommended dosage, did not identify any clinical, haematological or physiological toxicity of this plant (MRC and NRF, 2002).

Johnson *et al.* (2007) completed a phase I clinical trial in healthy adults to evaluate the safety of the dried plant material. It was noted that no side effects were found during or 3 months after the trial period. A statistically significant increase in appetite was noted in the treatment group, although remained in the normal physiological range.

#### 1.3.3.5 Anti-cancer effects attributed to *Sutherlandia* extracts

Clinical evidence shows that *Sutherlandia* extracts have direct anti-cancer effects in some cancers, together with immune-stimulating properties. Goldblatt and Manning (2000) considered these extracts not as a universal cure for cancer, but rather as an improvement-of-life tonic, wherein the body is assisted to mobilise its own resources to cope with the illness.

In general, *Sutherlandia* extracts have been reported to decrease anxiety and irritability, thus elevating the mood. Appropriate doses have been reported to dramatically improve appetite and weight gain in cancer-wasted patients. Energy levels and exercise tolerance of these patients were improved, including an enhancement of their sense of well being. The dose administered was generally one tablet (300 mg) twice a day, after meals, but not to be taken during pregnancy (http://www.sutherlandia.org).

Chinkwo (2005) reported that the aqueous extracts could induce apoptosis in cultured carcinoma cells and cytotoxicity in neoplastic cells (cervical carcinoma) and also in CHO (Chinese Hamster Ovary) cell lines. Induced apoptosis was confirmed by flow cytometric analysis.

#### 1.3.3.6 Anti-HIV effects of *Sutherlandia* extracts

Mills, Foster *et al.* (2005) have reported that the herbal remedy has been recommended for HIV management, which was shown to cause an improvement in $CD_4$ counts together with a decrease of viral loads in AIDS patients. It is hoped that this treatment regime will delay the progression of HIV into AIDS. They further reported that *Sutherlandia* contained inhibitory

compounds active against HIV target enzymes. Canavanine, which was found to be present in the extracts, has also been reported to have antiviral activity against influenza and retroviruses. *Sutherlandia* extracts have also been reported to have effects on cytochrome P450 3A4 metabolism, together with activation of the pregnane X-receptor, which are involved in anti-retroviral metabolism. Mills, Foster *et al.* (2005), have further indicated that factors which need to be taken into consideration with HIV patients are risk of treatment failure, induced viral resistance or subsequent drug toxicity. They also considered that uncontrolled human consumption of *Sutherlandia* extracts could affect anti-retroviral drug metabolism, leading to bi-directional drug interactions and loss of therapeutic efficacy.

*Sutherlandia* has also recently been shown to interact with the permeability glycoprotein (P-gp) receptor, to allow for increased absorption of anti-retroviral drugs (such as Amprenavir) into the cell system, which could lead to drug intoxication, but had no significant interaction with the drug itself (Katerere and Rewerts, 2011).

## 1.4 PROBLEM STATEMENT

Although there has been a recent boom in research on *Sutherlandia*, many shortcomings in scientific evidence on *S. frutescens* have appeared in the literature, both from a phytochemical, pharmacological, as well as from a medical point of view. These shortcomings have now been intensified by the discovery of variation in chemical composition between different plants from the same species.

Field studies over a period of many years have shown that only two species should be recognised, along with several geographically separated subspecies (Moshe *et al.*, 1998), this classification has not been implemented. In addition, many chemical variations are believed to occur, which could affect the variations in biological activity of various compounds present in these plants, as noted by Chinkwo (2005).

Investigations into the medicinal properties of *S. frutescens* have tended to focus mainly on *in vitro* anti-cancer properties of extracts from this plant on different tumour cell lines (Chinkwo, 2005; Stander *et al.*, 2007; Tai *et al.*, 2004). Accordingly, very little documentation exists on *in vivo* studies of *Sutherlandia* extracts, their mechanism of action, or of the various compounds relating to these activities. Ngcobo (2008) has reported on the plant's immune-stimulating abilities on a T cell lymphoma cell line, H9, as well as on

Peripheral Blood Mononuclear Cells (PBMC), together with various cytokines produced during stimulation with certain *Sutherlandia* extracts. While studies by Ngcobo (2008) might suggest some kind of clinical evidence to support a direct anti-cancer effect of *Sutherlandia* extracts in some cancers, together with possible immune-stimulating/modulatory mechanisms, they have not been very well characterised and are subsequently not well understood. Furthermore, to date, no evidence of the anticancer and stimulated cytokine expression on a macrophage cell line and isolated macrophage cells has been investigated and its usefulness in fighting various diseases.

In general, most biological assays on *Sutherlandia* extracts have tended to focus on the organic or traditional preparations of the plant material or commercial products (Van Wyk and Albrecht, 2008). No conclusive study of the compounds isolated and purified from the plant material, have been rigorously connected to biological activities. The mechanisms of action have not been extensively studied and it is not known how this highly appreciated medicinal plant functions or which compounds present in this plant are responsible for these actions.

## 1.5 AIMS OF THE STUDY

In view of the current lack of evidence, the objectives of this study were to investigate the chemical composition and biological activities of various preparations of one batch of plant material, as well as for the isolation and purification of compounds from these extracts.

For the isolation and phytochemical characterisation of the extracts various solvents along with a variety of analytical techniques, spectral and chemical analysis were used; such as column chromatography, NMR spectroscopy (1D and 2D data), HPLC-MS as well as UPLC-TOF-MS, before screening for biological activity.

The biological screening assays were setup to determine the role *S. frutescens* plays in its anticancer and immune modulating activities by using a 3-cancer cell line panel (consisting of melanoma, breast and leukaemia cell lines) and a cytokine stimulation model (consisting of pro- and anti-inflammatory cytokines) in a leukaemia cell line. This was done to specifically link certain extracts and/or compounds that might be responsible for these activities, as a preliminary approach for further investigations.

As the study progressed, it also became necessary to investigate potential variations in the chemical profiles of various *S. frutescens* plant material, received from a commercial supplier.

## 1.6 ORGANISATION OF THE DISSERTATION

The dissertation was organised into two separate parts. Part One, which consists of Chapters 2 to 5 (pages 19 to 66), describes the phytochemical investigations of plant extracts, while Part Two mainly focuses on the biological investigations on some of these extracts, comprising Chapters 6 to 8 (pages 67 to 107).

**Part 1 Phytochemical studies**

**Chapter 2** contains the phytochemical techniques used for extraction procedures of the plant material, isolation of compounds, equipment used for this purpose and analysis techniques for chemotypes, extracts prepared and isolated compounds.

**Chapter 3** presents a detailed discussion of the phytochemical results of the various extract and fractions of *S. frutescens,* as well as the characterisation of the compounds isolated with their structures and identification procedures.

**Chapter 4** contains a discussion of the differences and/or similarities found in extracts from six samples of plant material identified as *Sutherlandia frutescens,* obtained from a commercial supplier, that were analysed using HPLC-MS.

**Chapter 5** presents a conclusion from the observed phytochemical results from Chapters 3 and 4.

**Part 2 Biological investigations**

**Chapter 6** contains the materials and methods of the biological techniques used to determine the anti-cancer potential of selected extracts, cytokine release assay techniques, as well as instrumental descriptions used for these purposes.

**Chapter 7** contains a detailed account of a preliminary investigation into the bio-activities of selected extracts, fractions and compounds isolated from *S. frutescens*. Their anti-cancer

potential, stimulated release of cytokines by target cells, together with potential immune responses correlated with their chemical profiles, will be presented and discussed.

In **Chapter 8**, the observed results (from Chapters 6 and 7) will be summarised with their concluding remarks. Future plans and suggestions regarding the investigation of medicinal values of extracts from *S. frutescens* and other medicinal plants will be given.

# PART 1

# PHYTOCHEMICAL STUDIES

# CHAPTER 2

# MATERIALS AND METHODS

Medicinal properties associated with plants can be verified by phytochemical and biological studies. The discovery of certain compounds present in plants could explain their use as traditional medicines against certain ailments. It has become an important aspect to study the plant from a phytochemical point of view in order to identify compounds that are related to its therapeutic properties that could be used to justify its use as a traditional medicine and for the discovery of new drugs for various diseases. The plant extracts, analysis of the chemical profiles of these preparations and the pharmacological assays play a very important role in drug discovery. Traditionally, a tea infusion of the leaves is used for the preparation of the plant material. In this study general extraction procedures were followed: some of which mimic the traditional preparations and other more scientifically accepted extraction procedures (i.e., various organic preparations) for standardisation and comparison of the activities present in the different preparations. Thus, this chapter detailed a discussion of the materials and methods used in the extraction of plant material, High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis and isolation and purification of compounds from the extracts.

## 2.1 EXTRACT PREPARATIONS

### 2.1.1 Materials

All plant material (aerial parts) was obtained from a community-based project in Petrusburg in the Free State, South Africa (29° 6.774′ S; 25° 24.305′ E; 1249m above sea level). This project is fully funded by the Department of Science and Technology (DST) and implemented by the Council for Scientific and Industrial Research (CSIR), Enterprise Creation for Development Unit (ECD) in Pretoria. The seeds were supplied by a commercial-based nursery in Wellington in the Western Cape, South Africa. Solvents (CP grade) that

were used in the laboratory were purchased from Merck and distilled before use. Solid phase extraction C-18 cartridges (Supelco 140ml PP tubes), Lipophilic Sephadex LH-20 and silica gel 60 were purchased from Sigma Aldrich. Distilled water ($H_2O$) was used for all procedures.

### 2.1.2 Plant Collection

820 kg of fresh *Sutherlandia* aerial parts (leaves and stems) were received from Petrusburg in the Free State (seeds planted in September 2008, plants harvested in July 2009). Pilot scale drying was conducted in open air, which proved critical to bio-burden load. A twig containing a flower was sent to the South African National Biodiversity Institute (SANBI) for identification and was identified as *Sutherlandia frutescens (L.) R. B.r.* (SANBI Genspec number: 462 1) See **Figure 2.1** below.

A batch of 2.36 kg of the fresh stems and leaves were taken before drying and used for traditional preparations. This was placed in a fabric bag and stored in the cold room at 4 °C until processing. Once the remaining plant material (817.64 kg) had dried, the leaves and stems were separated and 6.5 kg of the dried leaves was taken and used for preparation of various organic and traditional extracts. Four kilograms of the dried leaves were used for a large scale production of a spray dried extract (seeds planted in September 2007, harvested in July 2008). Six samples of dry plant material (leaves) of *S. frutescens* were obtained from a commercial supplier for HPLC-MS profiling.

**Figure 2.1 Photo of *Sutherlandia frutescens* (L.) R. Br. plant material received.**

### 2.1.3 Extraction procedures

#### 2.1.3.1 Fresh plant material extraction based on traditional procedures

Fresh stems and leaves (2.36 kg) were used for the traditional extraction procedures. The stems (1.694 kg) and leaves (0.488 kg) were processed separately and boiled in 12.5L of distilled water ($H_2O$) each, for 1 hour using a hot plate and steel extraction vessel, with occasional stirring. The suspension was then filtered through cheese cloth and collected in a bucket. The aqueous extracts were then freeze-dried and a powder subsequently obtained. This was stored in an airtight container in the cold room at 4 °C until processing. See **Figure 2.2** below for flow diagram of the extraction process and percentage yields of extracts with their sample numbers. The aqueous extract from the fresh leaves was labelled **Extract A**, while the aqueous extract from the fresh stems was labelled **Extract B**.

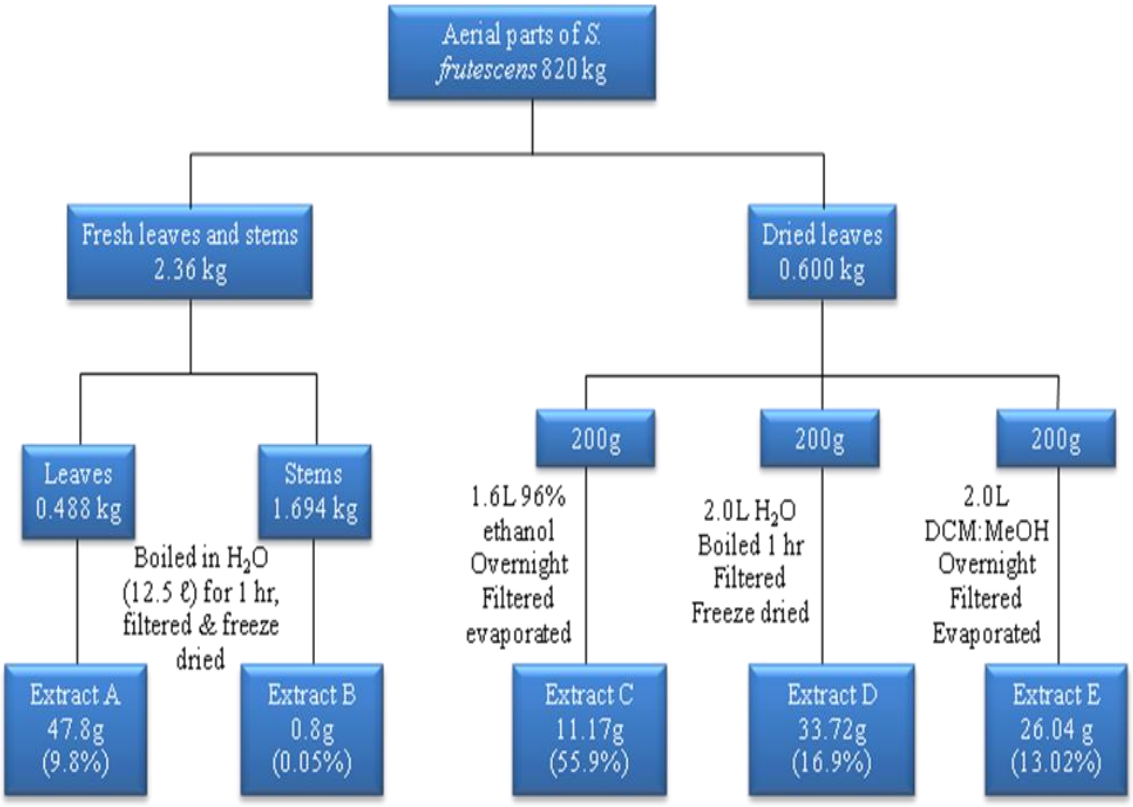



**Figure 2.2 Flow diagrams for fresh and dry plant material processing showing sample numbers and percentage yield of each resulting extract.**

### 2.1.3.2 Dry plant material extraction based on organic and traditional procedures

The dried leaves of *S. frutescens* (600 g) were separated into 3 x 200g each, labelled C, D, and E. Ethanol (EtOH; 1.6 L of 96% v/v) was added to 200 g of plant material. This was stirred, left overnight and then filtered and evaporated to give **extract C**. **Extract D** was obtained by boiling 200 g of plant material in 2.0L of distilled $H_2O$ for 1 hour with occasional stirring. This solution was filtered after it cooled down and freeze-dried. **Extract E** was obtained by adding a 1:1 dichloromethane: methanol (DCM: MeOH; 2.0 L) solution to 200 g of plant material, which was stirred overnight, filtered and evaporated. See **Figure 2.2** above for flow diagram of the extraction procedures and percentage yields obtained.

#### 2.1.3.2.1 Liquid-liquid partitioning of dried leaves

A total of 1.7 kg of dried leaves was ground to a powder and extracted three times with 10.0L of methanol (MeOH) and stirred for 24 hours. Filtration of the extract was done with a vacuum pump before 12 g of anhydrous magnesium sulphate ($MgSO_4$) was added and left for 30 min. to remove water and filtered. The MeOH was evaporated (**extract F**). A portion (100g) of **extract F** was suspended in water and extracted sequentially with hexane, chloroform and *n*-butanol (each phase saturated with water and shaken for optimised extraction). All resulting filtrates were evaporated and labelled **extract G, H, I and J** (hexane, chloroform, butanol and water portions, respectively). See **Figure 2.3** below for flow diagram of extraction procedure.

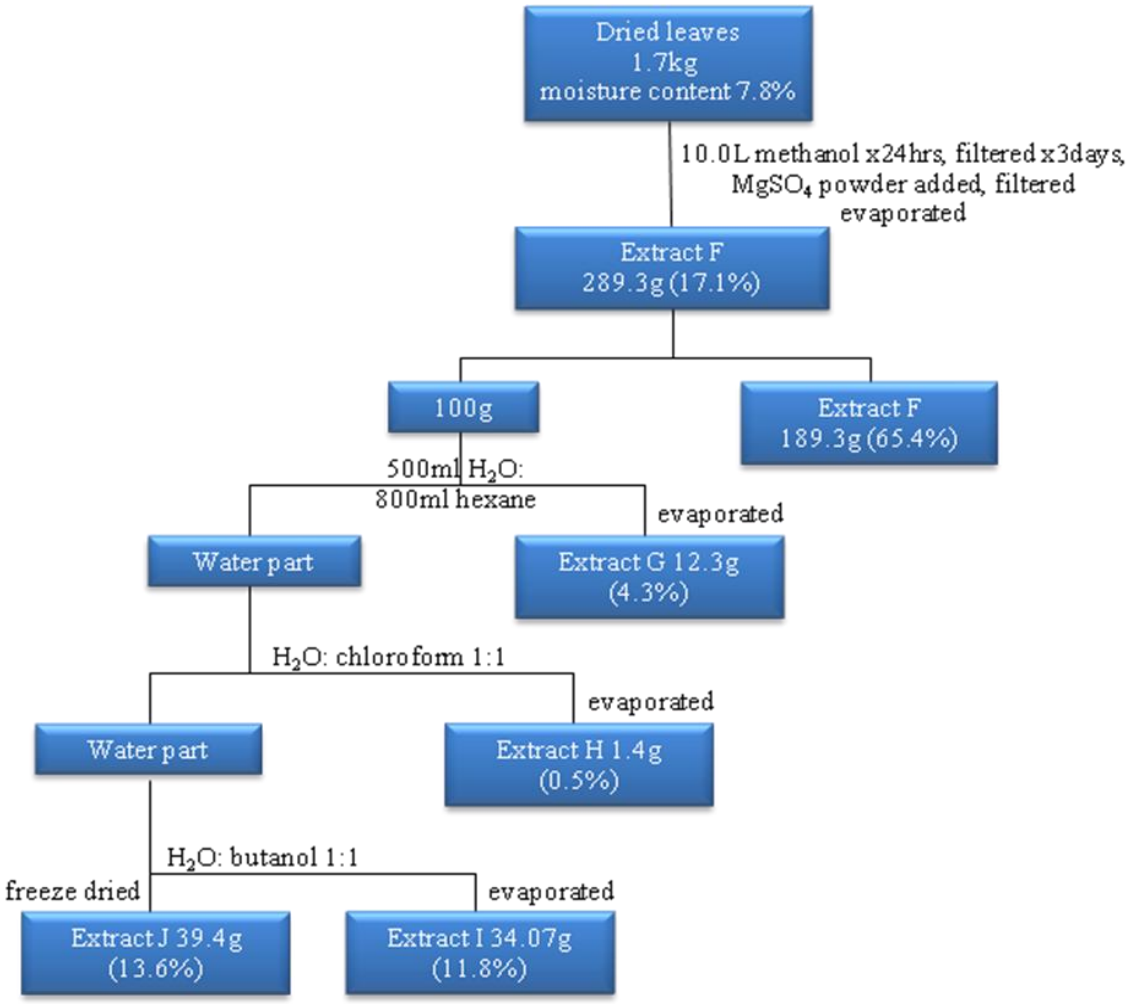



**Figure 2.3 Flow diagram for liquid-liquid partitioning of dry plant material (1.7 kg) showing sample numbers and percentage yields of resulting extracts.**

#### 2.1.3.2.2 Spray dried extract

Extraction: Distilled water (60 L) was added to 4 kg of the dried leaves in an extraction vessel. This was then boiled for 1 hour. The extract was cooled by cooling the jacket of the extraction vessel.

Filtration: A hydraulic press was used for filtration with a filter pad (PY07010 @ 185cm wide, WHATMAN) and two filter bags (FT557 @ 170cm wide, WHATMAN), one on the inside of the hydraulic press and one in the collection bucket.

Spray drying: The Nitro Spray Dryer was operated with the following parameters: process gas 380 Kg/Hr; inlet temperature 180 °C; outlet temperature 88 °C; wall sweep temperature 80 °C; pneumatic hammer 1 bar; wall sweep process gas 1 bar; atomizer 2600 rpm and filter clean pressure 5 bar. The spray dryer was run for 3 hours and 40 minutes. Pre-cleaning of the feed system was done. The spray-dried extract was collected, weighed and labeled **extract K**.

#### 2.1.3.2.3 Solid Phase Extraction (SPE) for extract C

A C-18 (Supelco 140 ml PP tubes, Sigma Aldrich) cartridge was activated with 1:1 $H_2O$: MeOH, 100 % MeOH and 100 % $H_2O$. A 10 g sample of extract C was dissolved in 20 ml $H_2O$ and placed on the activated cartridge. This was run with 200 ml 100 % $H_2O$, 160 ml 20 % MeOH, 110 ml 40 % MeOH, 160 ml 60 % MeOH, 160 ml 80 % MeOH, 200 ml 100 % MeOH and 200 ml 100 % Acetonitrile (ACN). The resulting fractions were evaporated and freeze dried and combined to give **fractions I to III**.

### 2.1.4 Immune-boosting standard

#### *Echinacea* preparation

*Echinacea* Premium Syrup (1 ml) from Medi Herb was used in this study as an immune-boosting control and dried-off on the Genevac speedvac to remove the ethanol used in the formulation of the syrup. Each ml of syrup contains 1.5 mg of alkylamides (the active immune-boosting compounds). The tincture is made up of 60 % *E. purpurea* root (1:2 dilution) and 40 % *E. angustifolia* root (1:2 dilution). The evaporated extract was given a sample code of **Ech**.

## 2.2 ISOLATION AND PURIFICATION OF COMPOUNDS

In the following section of this chapter, details will be given of the various chromatographic techniques used to purify cycloartane and flavonol glycosides.

### 2.2.1 Isolation and purification of cycloartane glycosides

In this study, the butanol extract (17.2 g; **extract I**) was used to purify the cycloartane glycosides, which was subsequently subjected to silica gel column chromatography (particle size 0.063 - 0.2 mm), hereafter referred to as **column a**. The purification was done using a stepwise gradient mixture of chloroform ($CHCl_3$): MeOH, starting from 100:0 until 70:30, as eluent to give an initial number of 24 fractions. From **column a**, five fractions (in the more non-polar region) were combined (6.33 g) and subjected to further silica gel column chromatographic purification, hereafter referred to as **column b**, and subsequently eluted using a stepwise gradient mixture of $CHCl_3$: MeOH, starting from 95:5 until 75:25, to give another set of 20 fractions. Upon visual inspection of thin layer chromatography (TLC) plates, certain fractions were run on HPLC (using the method described below in section 2.3) to determine their purity. From these results, one non-polar fraction that eluted with 10 % MeOH, from **column b**, was found to be a fairly pure compound and was further crystallised, hereafter referred to as **compound 4**. **Compound 4** was further analysed using the UPLC-QTOF and Nuclear Magnetic Resonance spectroscopy (1D and 2D experiments) for its structure elucidation. Another set of more polar fractions - from **column b** - were combined (612.3 mg) and subjected to Sephadex (Lipophilic Sephadex LH-20) column chromatography, hereafter referred to as **column c**.

Sephadex was pre-washed with 100 % $H_2O$, 100 % MeOH and again with 100 % $H_2O$. The washed Sephadex was then left overnight in $H_2O$ to allow swelling and then packed in a glass column. **Column c** was first eluted with $H_2O$, followed by a stepwise gradient of increasing MeOH concentration. Small volumes (5 ml) were collected and evaporated on the Genevac. Collected test tubes were analysed with TLC and chemically similar samples were subsequently combined to give another set of 22 fractions. Two non-polar fractions - from **column c** (38.2 mg) - which were found to be fairly pure according to HPLC analysis, were then packed on a third short silica gel column for further purification, hereafter referred to as **column d**. Separation on this column was achieved with 100 ml of 100 % $CHCl_3$, with increasing MeOH concentration in 1 % increments, every 100 ml, until 7 % MeOH was reached, followed by washing with 10 % MeOH. Test tube samples collected were

evaporated on the Genevac. Collected samples were analysed using TLC and chemically similar fractions were combined to give 3 resulting fractions. HPLC analysis was done on the second fraction (that eluted with 6 % MeOH) to determine its purity and it was found to be pure, hereafter referred to as **compound 7**. **Compound 7** was further analysed using 600 MHz Varian NMR (1D and 2D experiments) and UPLC-QTOF for structure elucidation. Finally, 7 of the non-polar fractions (37.0 mg) from **column c**, produced another set of fractions from a fourth silica gel column, hereafter referred to as **column e**. These fractions were purified using 100 ml of 100 % $CHCl_3$, increasing the MeOH concentration by 1 % every 100 ml, up to 8 % MeOH and subsequently washed out with 10 % MeOH. TLC was run on the collected fractions and chemically similar ones were combined to give 4 fractions. From this, the second fraction (eluting with 5 % MeOH), from **column e**, hereafter referred to as **fraction IV**, was analysed using HPLC-MS to characterise the compounds present. A brief summary of the cycloartane glycosides purification process, incorporating the various runs and recombinations of **columns a** to **e**, are presented in **Figure 2.4** below.

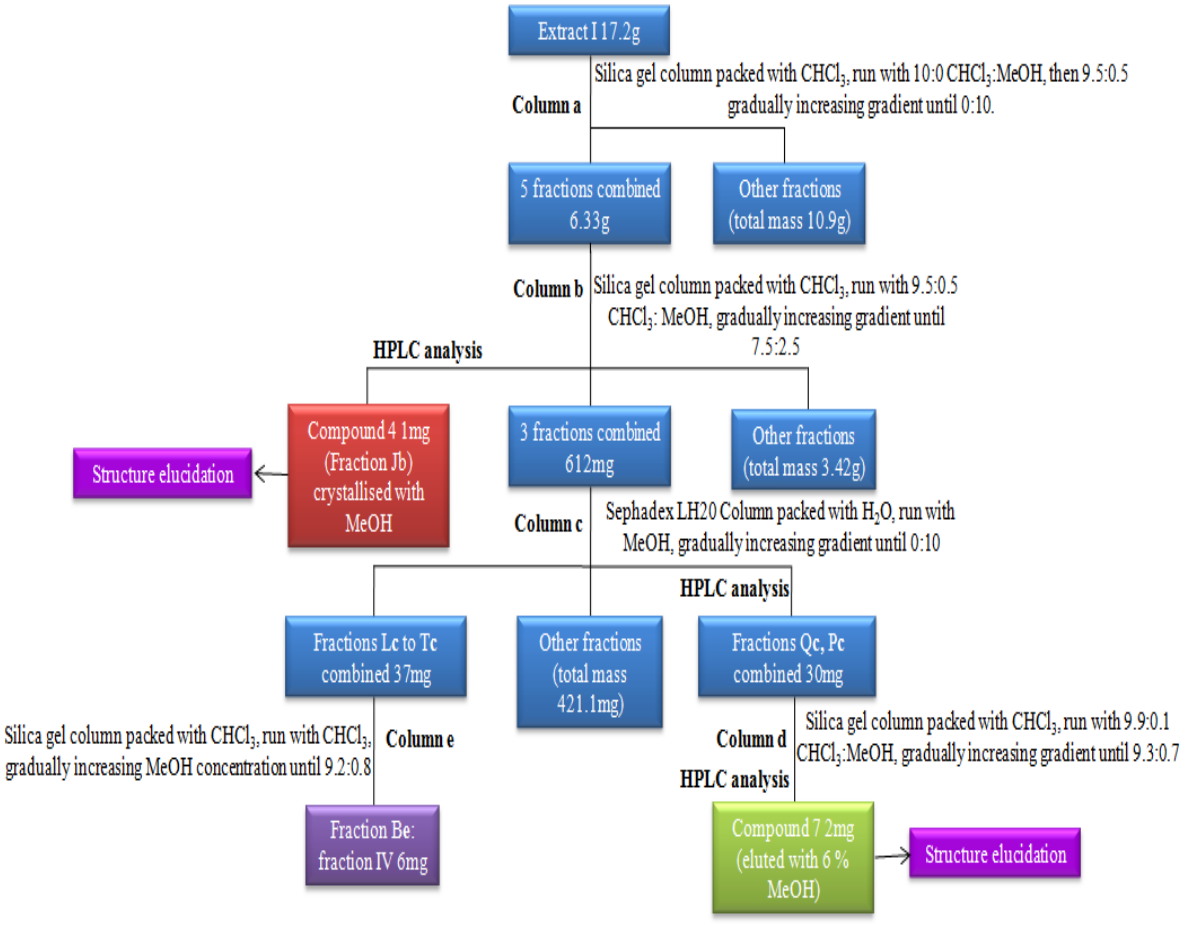



**Figure 2.4 Flow diagram of cycloartane glycosides column chromatography and resulting compounds and fractions.**

### 2.2.2 Isolation and purification of flavonol glycosides

For the isolation of the flavonol glycosides several column chromatographic techniques were employed, beginning with 10 g of **extract K** dissolved in 20 ml $H_2O$ and packed on a 50 g C-18 cartridge (the cartridge was first activated as explained above just with a 1:1 dilution of $H_2O$: MeOH, 100% MeOH and 100 % $H_2O$, sequentially). The extract was packed on the C-18 cartridge and eluted with 250 ml of 100 % $H_2O$, 160 ml 20 % MeOH, 110 ml 40 % MeOH, 160 ml 60 % MeOH, 160 ml 80 % MeOH, 200 ml 100 % MeOH and washed with 100 ml 1:1 MeOH: ACN and 100 ml 100 % ACN. This was repeated three times with the same quantities and extract. All resulting fractions were freeze dried, evaporated and combined. Flavonoid detection was done on the resulting fractions from **column f** using 1 g of ferric (iii) chloride hexahydrate ($FeCl_3.6H_2O$) dissolved in 1 ml of $H_2O$ and 1 ml of each fraction. A colour change from yellow to blue-green confirmed the presence of flavonoids in one of the polar fractions (B**f**) only, which was taken for further purification.

Thereafter a Discovery DPA-6S (60 ml Tube; 5 g) polyamide cartridge was activated with 100 % MeOH and 30 % MeOH. This polar fraction (500 mg) was dissolved in 3:7 MeOH: $H_2O$ and packed on the activated cartridge, hereafter referred to as **column g**. Fraction B**f** was chromatographed employing a solvent system of increasing MeOH concentration starting from 30 % until 70 %. The column was then washed with 95.5: 0.5 MeOH: ammonia ($NH_3$) and 85:15 dimethylformamide: $H_2O$. Each solvent system ratio was collected separately in vials labelled and evaporated to give 7 fractions. Fraction 4 (70 mg), from **column g**, was subsequently packed on a silica gel column and eluted with 90:10:1 ($CHCl_3$:MeOH:Formic acid); 85:15:1 and washed with 100 % MeOH, hereafter referred to as **column h**. TLC analyses on the collected test tubes, from **column h** were conducted, and chemically similar test tubes were combined to give 8 fractions. From **column h**, fractions B**h** and D**h**, hereafter referred to as **fractions V and VI**, were used for screening of flavonoid activity. A brief summary of the purification of the flavonol glycosides is shown in **Figure 2.5** below.

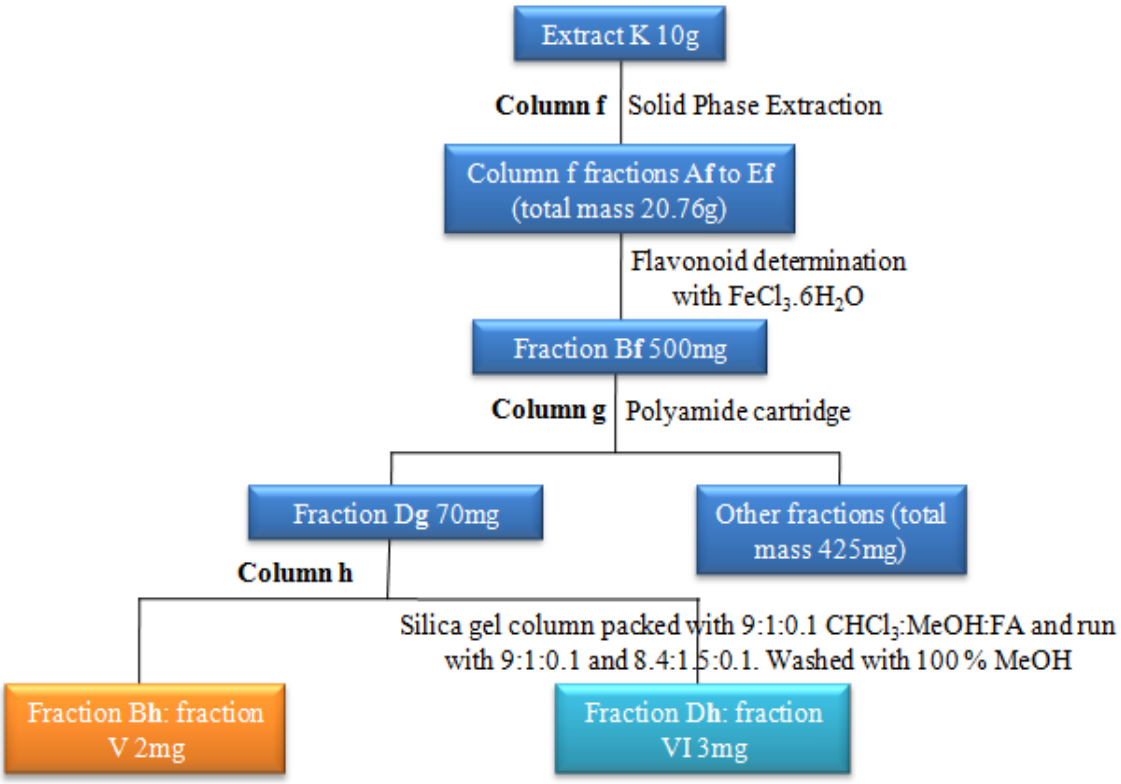



**Figure 2.5 Flow diagram of flavonoid column chromatography and resulting fractions.**

## 2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY (HPLC-MS)

The various extracts, fractions and compounds were then analysed using a High Performance Liquid Chromatography-Single Quadrupole Detector-Mass Spectrometry (HPLC-SQD-MS) instrument to determine their chemical profiles. Ultra Performance Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry (UPLC-QTOF-MS) was used for structure elucidation and accurate mass determination of the isolated compounds. HPLC and UPLC are highly developed powerful analytical techniques with a wide range of variations available mainly used as preparative tools and to obtain chemical fingerprints of plant material (Holme and Peck, 1998), while MS and QTOF, coupled to these techniques, allow for faster analysis time, being used for the identification and structure elucidation of the components therein. In the drug development process, MS has been used for lead compound discovery, structural analysis, synthetic development, combinatorial chemistry, pharmacokinetics and drug metabolism (Pavia *et al.*, 2009). QTOF allows for a more accurate mass analysis, which is based on principle that the velocities of two ions vary based on the mass of the two ions

(Pavia *et al.*, 2009). This gives us indication of the quantity of compounds present, and together with their molecular masses, allows identification of intrinsic differences and similarities between compounds present in the plant extracts.

### 2.3.1 Sample preparation

**Commercial samples**: For each sample approximately 2 g of the dried, ground leaves was weighed out into a volumetric flask and 5 ml of HPLC-grade methanol was then added. This was then put in the ultrasonic bath for 30 minutes, transferred to a vial and centrifuged for 10 min. at 2500 rpm. The supernatant was then removed, with the whole process being twice repeated.

**Extracts and fractions:** For each extract and fraction, approximately 25 mg was weighed out into a vial and 2 ml of HPLC-grade solvent (methanol, acetonitrile or water depending on the solubility of the sample) was added. This mixture was then put in an ultrasonic bath for 10 minutes and filtered through Acrodisc GHP syringe filters, before being placed into 2 ml HPLC vials.

**Compounds:** For each compound isolated approximately 1 mg was weighed out into a vial and 2 ml of HPLC-grade solvent was added (solvent choice depended on compound solubility). This mixture was then put in an ultrasonic bath for 10 minutes and filtered through a 0.2 μm GHP filter membrane filter, before being injected into HPLC vials using a Norm-jet Luer Lock syringe. These compounds were also run on UPLC-QTOF to obtain the accurate mass of each.

### 2.3.2 HPLC-MS analysis

The various samples were analysed using a WATERS 2695 HPLC separation module. Two Atlantis T3 columns (10 x 250 mm, 5 μ particle size) connected in series, were used for the separation. UV-VIS detection was done on a WATERS PDA scanning from 200 – 600 nm. The mobile phase used was 0.1 % (v/v) formic acid in water (A), methanol (B) and acetonitrile (C). The ratio of mobile phase components that were optimised for the extracts prepared (see section 2.2.1) is given in the **Table 2.1** below. Additionally, mass spectrometry detection was performed using a WATERS SQD scanning from 100 – 1200 *m/z* with polarity (+/-) switching with a scan time of 0.20 seconds. The operating conditions in the ESI source were as follows: source temperature, 150 °C; desolvation temperature, 450 °C; capillary

voltage, 3.00 kV; cone voltage, 30.0 V. Gas flow ($N_2$): desolvation, 800 L/hr; cone gas, 10 L/hr.

**Table 2.1 The Gradient timetable for the HPLC-SQD method.**

| Time (min) | Flow rate (ml/min) | % A FA $H_2O$ | % B MeOH | % C ACN |
|---|---|---|---|---|
| 0.00 | 0.3 | 95.0 | 5.0 | 0.0 |
| 4.00 | 0.3 | 95.0 | 5.0 | 0.0 |
| 5.00 | 0.3 | 75.0 | 25.0 | 0.0 |
| 27.00 | 0.3 | 55.0 | 45.0 | 0.0 |
| 30.00 | 0.3 | 42.0 | 58.0 | 0.0 |
| 70.00 | 0.3 | 22.0 | 78.0 | 0.0 |
| 80.00 | 0.3 | 12.0 | 88.0 | 0.0 |
| 85.00 | 0.3 | 12.0 | 88.0 | 0.0 |
| 90.00 | 0.3 | 0.0 | 100.0 | 0.0 |
| 100.00 | 0.3 | 0.0 | 100.0 | 0.0 |
| 107.00 | 0.3 | 0.0 | 0.0 | 100.0 |
| 113.00 | 0.3 | 0.0 | 0.0 | 100.0 |
| 115.00 | 0.3 | 95.0 | 5.0 | 0.0 |

## 2.4 GENERAL EQUIPMENT USED FOR COMPOUND AND EXTRACT ANALYSIS

HPLC analysis on the extracts and fractions was done on a WATERS 2695 HPLC separation module with a PDA and a SQD, with the help of Nial Harding. Two Atlantis T3 columns (10 x 250 mm, particle size: 5 µ) were used.

For structure elucidation of the compounds, NMR spectroscopy was performed using a 600 MHz Varian NMR, using deuterated solvents depending on the solubility of the samples (pyridine-$d_5$ or methanol-$d_4$). The duration of the experiments that were run, depended on the purity and quantity of the samples. All samples were run at a set temperature of 25 °C.

Accurate mass analysis was performed on a WATERS Synapt G1 UPLC-TOF-MS system by Paul Steenkamp. Two column types were used namely, WATERS Acquity CSH C18 (150 x 2.1 mm, particle size: 1.7 µ) for the apolar compounds and WATERS Acquity HSS T3 (150 x 2.1 mm, particle size: 1.8 µ) for the more polar compounds.

# CHAPTER 3

# RESULTS AND DISCUSSION

# OF EXTRACTS AND COMPOUNDS

HPLC-MS is an analytical chemistry technique that uses the physical separation capabilities of liquid chromatography and the mass analysis capabilities of mass spectrometry. It is used for the specific detection, potential identification, stability and purity studies of chemicals present in a complex mixture. Various scientific studies have found, that determining the chemical profile of plant extracts, helped in identifying some of the chemical components present and also provided guidance for further investigations in pharmacological activity in support of traditional uses. Nuclear Magnetic Resonance (NMR) spectroscopy and Quadrupole Time-of-Flight (QTOF) have proved to be useful techniques for the structural elucidation of compounds isolated from plant material. NMR exploits the magnetic properties of nuclei present in the atoms, which results in a characteristic spectrum that can be used for determining the structure of the isolated compounds. Various 1D and 2D experiments were used (such as proton - $H^1$, carbon - $C^{13}$, Heteronuclear Multiple Bond Correlation - HMBC, Heteronuclear Single Quantum Coherence - HSQC, Correlation SpectroscopY - COSY and Distortionless Enhancement by Polarisation Transfer - DEPT) to determine the proton to carbon relation and the chemical environment they are in for structure elucidation (Silverstein *et al.*, 2005).

QTOF provides a measurement of the accurate mass of the compound/s under investigation and the resulting fragmentation pattern which was used, in conjunction with the NMR data, for structure elucidation. QTOF is a technique used to determine the mass of the compound under investigation using the principle that smaller (lighter) ions will travel faster through a flight tube than larger (heavier) ions. The velocity of an ion depends on its mass-to-charge ratio. The velocity that an ion obtains in the TOF analyser is therefore used to determine the mass of the ion and is a more accurate estimate (Silverstein *et al.*, 2005; Pavia *et al.*, 2009). In this chapter, the chemical profiles of the extracts made, together with fractions obtained, along with characterisation of the isolated compounds, will be presented. The extracts and fractions were analysed using the WATERS HPLC-PDA-SQD system (HPLC), while the

compounds were analysed on the WATERS Synapt G1 UPLC-TOF-MS system (UPLC) for accurate mass detection.

### 3.1 CHEMICAL PROFILES OF EXTRACTS

The HPLC chromatograms of the various extracts, fractions and compounds can be found in **Appendix 2**. Selected chromatograms however, have been included in this portion of the Dissertation, and show the chemical profiles of some of the extracts made from the leaves of *S. frutescens* (see Chapter 2, section 2.1.3, page 21). The chemical profile of **extract C** (ethanol extract) is shown below (see also Appendix Figure 2.1, page 122). In the extracts, the major cycloartane glycoside found in *S. frutescens*, **compound 5**, was found to elute at 43.51 minutes in ESI negative mode. The other compounds isolated were found to elute at 57.24 and 59.25 minutes in ESI negative mode for **compound 7** and **4**, respectively.

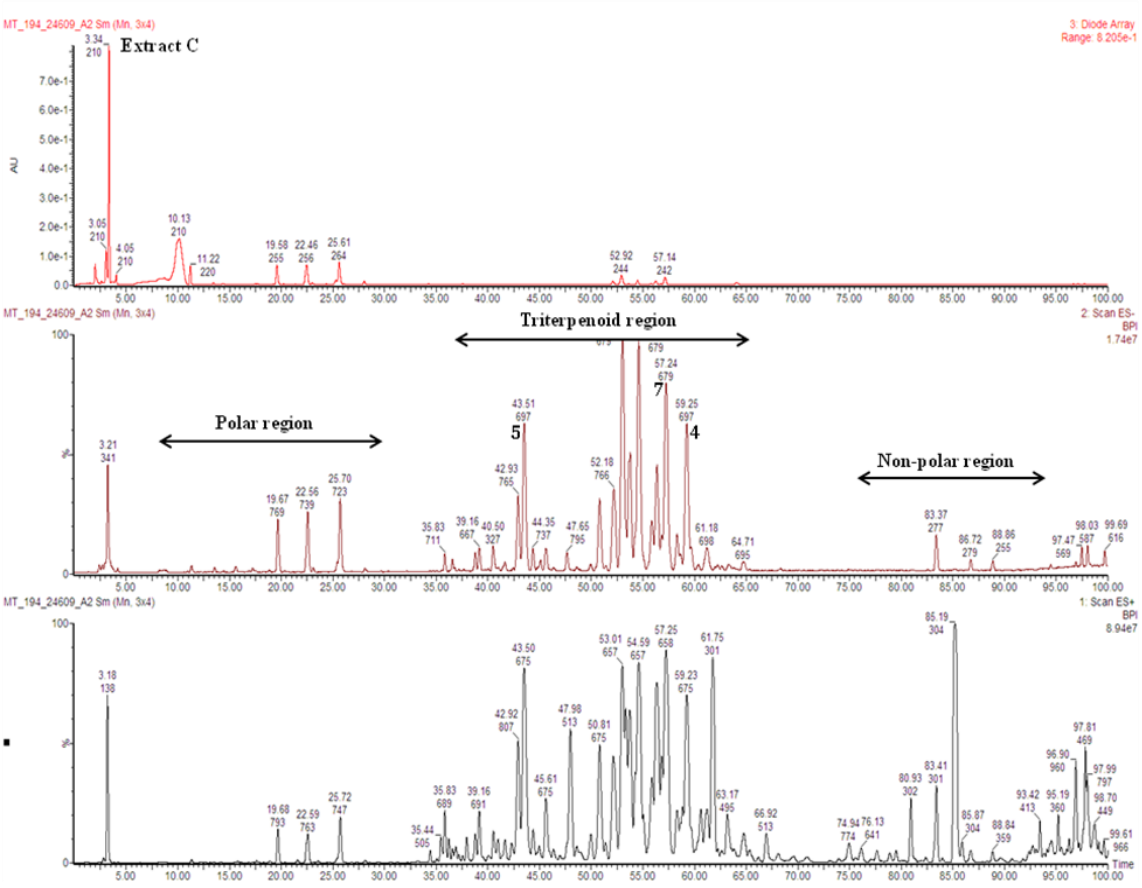



**Figure 3.1 The ESI$^+$, ESI$^-$ and UV chromatograms for extract C with the regions and compounds 4, 5 and 7 labelled in negative mode. UV-VIS was detected in the range from 200 to 600 nm.**

From **Figure 3.1** above, it can be seen that the polar compounds are found to elute from 10 to 30 minutes, with a characteristic UV pattern. As the less polar region of the extract starts to elute (from 40 to 90 minutes), there is a great decline in UV activity, reflecting very little, or lack of aromatic rings and/or conjugated double bonds in the less polar compounds present in this region. As such, *S. frutescens* can be regarded as being one of the very few herbal plants whose extracts cannot be successfully separated or chemically profiled just on the basis of UV activity. This is possibly due to the presence of a complex mixture of compounds with little or no UV activity, as can be seen in the $ESI^+$, $ESI^-$ and UV chromatograms above. The cycloartane glycosidic compounds are found to elute from 40 to 60 minutes and display very low UV activity. The very non-polar compounds (from 80 to 90 minutes) have not been identified from *Sutherlandia* as of yet, and also show a lack in UV absorbing abilities. The flavonol glycosides are more polar in nature and therefore are found to elute from 20 to 30 minutes, with characteristic UV profiles, showing the presence of more aromatic rings and conjugated double bonds found in these compounds.

For the purpose of comparison of the chemical profiles from the various extracts made from *S. frutescens*, specific focus will be placed on the $ESI^-$ and $ESI^+$ chromatograms (depending on which show better ionisation of the compounds) due to a lack of UV activity. The $ESI^-$ chromatograms of the aqueous preparations of the fresh leaves and stems, namely **extracts A** and **B** respectively, together with the spray dried aqueous extract of the dried leaves (**extract K**), are discussed below. Corresponding chromatograms can be found in **Appendix 2** (Appendix Figure 2.2, page 123).

From these chromatograms, the presence of more polar compounds, eluting from 15 to 27 minutes and majority of the triterpenoid type compounds eluting from 40 to 60 minutes can clearly be seen. It is apparent that there is very little variation in the chromatographic profiles of the polar compounds. However, variations in the triterpenoid region of the different aqueous preparations were observed. This result might be due to their different solubility characteristics and could also be attributed to the natural drying process in the initial preparation of plant material that could have resulted in the degradation of some of the unstable triterpenoid compounds, thereby causing a variation. The difference in the triterpenoid region might also be due to the different drying processes used to absorb the water from the extracts after filtration. For example, freeze drying was employed on the aqueous extracts from the fresh plant material, while spray drying was used on the aqueous extracts from the dry plant material. The freeze drying process might be more suitable for

controlling instability of these thermally labile compounds, where low temperatures were used to absorb the water, while the spray drying process used high temperatures (ranging from 60 to 160 °C) to evaporate the water, which might have further caused the degradation of the compounds present in the triterpenoid region of the chromatogram.

In addition, the aqueous extract of the fresh stems contained a lower concentration of the compounds when compared to the aqueous extract of the fresh leaves. One can therefore conclude that the stems of *Sutherlandia* do not contain many of the compounds found in other parts of the plant and are probably in rapid transit from the roots to the leaves via the stems. As such, the compounds are probably stored in the leaves, confirming the presence of a higher concentration of the compounds which were found in extracts from the leaf material.

The next part of the results and discussion will be around the chemical profiles of the methanol extract and resulting phases from it (**extracts F, G, H, I** and **J**, see Appendix Figure 2.3, page 124).

**Extract I** contained the majority of the compounds found in the triterpenoid region. For this reason, most of the purification was done on this portion, to isolate the triterpenoids found in *Sutherlandia* species. The hexane soluble part of the methanol extract (**extract G**) mostly contained the fatty acids and other non-polar compounds, due to their solubility affinities. The chloroform soluble portion of the methanol extract (**extract H**) dissolved some compounds eluting in the triterpenoid region with very little UV activity. **Extract I** contained mainly the triterpenoid region and some of the less polar compounds that were not soluble in the water portion (**extract J**). **Extract J** contained the rest of the compounds present from **extract F** that were not soluble in any of the other phases. The very polar compounds remained in high concentrations in this extract. **Extracts C, D** and **E**'s (ethanol, water and DCM:MeOH extracts, respectively) $ESI^-$ chromatograms of the extracts of the dried leaves from *S. frutescens* are discussed below (see Appendix Figure 2.4, page 125).

The ethanol extract (**extract C**) was fairly similar to the butanol extract (**extract I**) with a high concentration of compounds in the triterpenoid region and a lower concentration in the polar region. In general, it was found that a low concentration of the flavonoids dissolved in the ethanol extract. The aqueous extract of the dried leaf material (**extract D**) contained a higher concentration of the polar compounds (from 15.00 to 30.00 minutes), while the 1:1 DCM:MeOH extract (**extract E**) displayed a higher concentration of compounds found in the triterpenoid region (from 40.00 to 60.00 minutes) when compared to the other extracts.

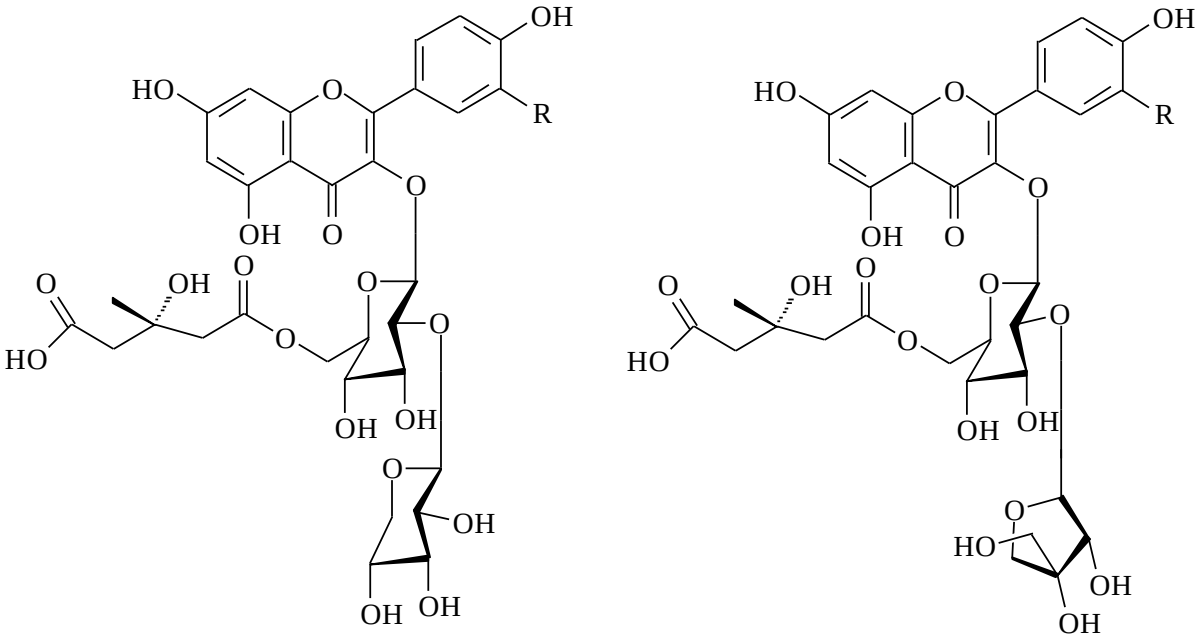
## 3.2 CHEMICAL PROFILES OF FRACTIONS

The following section presents the chemical profiles of each of the resulting **fractions I** to **VI** obtained from the further purification of the extracts above (see Chapter 2, sections 2.1.3 and 2.2, pages 21 and 25). The chromatograms of each fraction can be found in **Appendix 3** (page 126 to 131). **Extract C** was further purified (using SPE, Chapter 2 section 2.1.3.2) into three fractions namely, **fractions I, II** and **III**, while the resulting triterpenoid fraction (**fraction IV**) was purified from **extract I** and **fraction**s **V** and **VI** (the flavonoid rich fractions) were purified from **extract K**. These fractions were discussed based on their $ESI^+$ chromatograms due to better ionisation of compounds than in negative mode.

**Fraction I** was found to contain more polar compounds, while **fraction II** contained mainly the glycosidic triterpenoid type compounds that were more polar in nature due to the presence of a sugar moiety attached to the structure. **Fraction III** contained the non-polar compounds. **Fraction IV** contained **compounds 4** and **7** (identified using retention time and mass spectral data) in very minor quantities, while **compound 5** was not found to be present in this fraction. The major peaks eluting in this fraction have similar fragmentation patterns characteristic of the cycloartane glycosides isolated from *S. frutescens* (**compounds 4** to **7**). There are still many more of these compounds to be isolated from the plant material and further investigations need to be done on different purification techniques, thereby making it easier to achieve higher yields of the compounds. Two purified fractions, which contained the characteristic flavonol glycosides isolated from *S. frutescens*, were obtained. The compounds were not isolated in a pure form due to their low quantities as well as their inclination to degrade. The compounds present in the chromatograms showed a mass fragmentation and UV pattern characteristic of **compounds 8** to **11**. For a full chromatogram of **fraction V** and **VI** see Appendix Figure 3.2 (page 127). In **fraction V,** the peaks eluting at 24.73 and 25.51 minutes displayed similar mass fragmentation and UV spectra to that of **compounds 10** and **11**, whereas in **fraction VI** the peaks eluting at 21.21 and 22.23 minutes displayed similar mass fragmentation and UV spectra to that of **compounds 8** and **9** (Fu *et al.*, 2010; Avula *et al.,* 2010). These results are therefore indicative that these compounds could possibly be **compounds 8** to **11** present in the fractions, however this cannot be confirmed based on mass spectral data alone, due to the closely related structures (mass spectral and UV data can be found in Appendix Figures 3.3 to 3.6, pages 128 to 130). Further investigations on different purification techniques should be done, in order to obtain more compounds from *Sutherlandia* in high enough quantities for more detailed NMR analysis. There are many

other peaks found in the chromatograms that exhibit similar fragmentation patterns, but with different maximum UV absorptions, that are suggestive of the presence of closely related flavonol glycosides. Further work needs to be done to isolate these compounds in purer and higher concentrations so that better comparisons can be made with commercial standards as well. Appendix Figures 3.5 and 3.6 show the UV absorption spectra of the two peaks in **fractions V** and **VI** which could correspond to **compounds 10** and/ or **11** in **fraction V** and **8** and/ or **9** in **fraction VI**.

Appendix Figure 3.5 shows the UV spectra from **fraction V** with a similar UV absorption pattern to that of **compounds 10** or **11** (Avula *et al.,* 2010) suggesting that this compound could possibly be that of **compound 10** or **11** i.e. sutherlandin C or D. The mass spectral data (shown in Appendix Figure 3.3, page 128) corresponds to that found in the study done by Avula *et al.* (2010). However, the structure cannot be confirmed, due to the lack of NMR supporting data, because of insufficient quantities for NMR analysis. The UV data of peaks found in **fraction VI** can be found in Appendix Figure 3.6. The two peaks that elute at 21.14 and 22.16 minutes in **fraction VI**, shows a comparable UV absorbing pattern to that found for **compounds 8** and **9** by Avula *et al.* (2010), suggesting that these compounds could possibly be that of **compounds 8** or **9**, namely - sutherlandin A or B, present in the fractions. The mass spectral data (shown in Appendix Figure 3.4, page 129) corresponds to that found in the study done by Avula *et al.* (2010). However, the structures cannot be confirmed due to the lack of NMR supporting data. Further purification of the compounds is needed to confirm the structure with NMR analysis. **Figure 3.2** below shows the structures of the flavonol glycosides (**compounds 8** to **11**) that have been previously isolated from *S. frutescens*.



A: R = OH
C: R = H

B: R = OH
D: R = H

**Figure 3.2 Structures of compounds 8 (A), 9 (B), 10 (C) and 11 (D) from Fu *et al.*, 2010 and Avula *et al.*, 2010.**

In order to provide some reasonable explanation as to the order of elution of the four flavonoids, further analysis was done. Various UPLC-MS and UPLC-MS-MS experiments were performed and it was shown that the first pair - the di-hydroxy flavonoids (**compounds 8** and **9** - quercetin 3-O-diglycosides) - was found to elute first (*m/z* 740.1) and the mono-hydroxy flavonoid pair (**compounds 10** and **11** - kaempferol 3-O-diglycosides) being less hydrophilic eluted after (*m/*z 725.1). Further attempts to determine which flavonoid elutes first from the pairs were done [i.e., from the di-hydroxy pair - **compound 8** or **9** - which would elute first; the one containing the 6-membered sugar (pyranose) attachment (**8** and **10**) or the one with the 5-membered sugar (furanose) attachment (**9** and **11**)]. A 3D structure evaluation was performed. The structures were drawn in ChemSketch 12[2] and optimised for 3D viewing. All the settings for 3D optimisation were default settings supplied by the software.

[2] Software downloaded from ACD labs (Advanced Chemistry Development, Inc.), which is freely available at <http://www.acdlabs.com/resources/freeware/chemsketch/>.

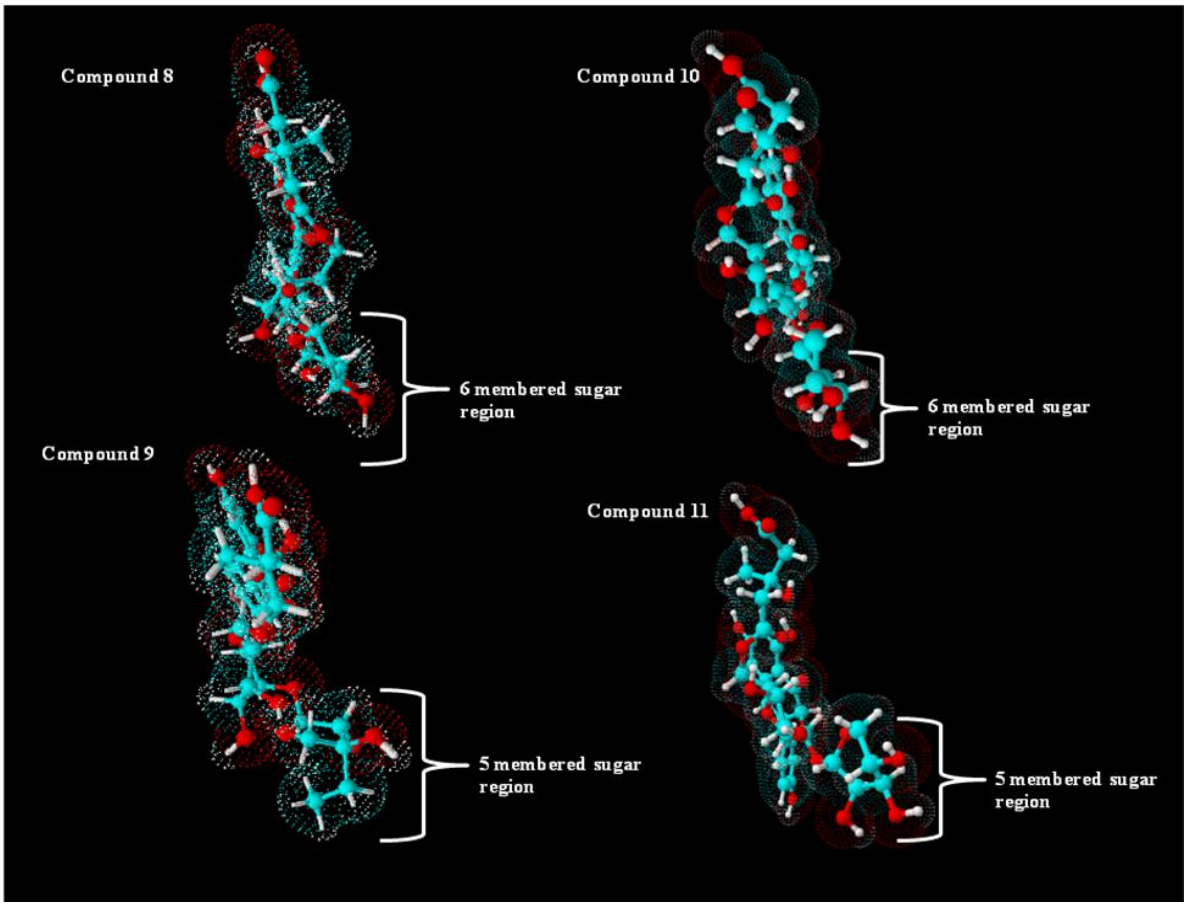



**Figure 3.3 3D representations of compounds 8 to 11 showing the sugar regions and the space occupied by each. This figure was originated using 3D ChemSketch software version 12.0.**

From the structure representations of the di-hydroxy flavonoid pair (**compounds 8** and **9**) it was shown that the 5-membered sugar region (of compound 9) occupied more space thus encountering a higher level of interactions with the stationary phase, resulting in a longer elution time. The same occurs for the mono-hydroxy flavonoid pair (**compounds 10** and **11**). **Compound 11** occupies more space and therefore allowing more interaction with the stationary phase to take place thus eluting at a later time. From these observations, the proposed order of elution would thus be as follows: compound 8, 9, 10 and 11 (sutherlandin A, B, C and D respectively). This order of elution is in agreement with the published information from Avula and co-workers (2010). Appendix Figures 3.7 and 3.8 (page 131) provide the proposed structure analysis and order of elution as investigated on the UPLC system.

## 3.3 CHARACTERISATION OF COMPOUNDS

Natural compounds have been used extensively in the treatment of many infections and are of interest to researchers both in their natural forms and as templates for synthetic modification. Natural compounds currently used in medicine exhibit a very wide chemical diversity, and together with their analogues and several other natural products, they demonstrate the importance of compounds from natural sources in modern drug discovery efforts.

Scientific studies have shown triterpenoids to be potential anti-inflammatory and anti-cancer agents (Yadav *et al.*, 2010). A structure-activity relationship study by Kikuchi *et al.* (2007) has shown that the most powerful inhibitory effects were found in cycloartanes with hydroxylation at C-24 and a 3-oxo group in an *in vivo* mouse skin carcinogenesis test. Such a configuration was found in one of the cycloartane glycosides from *Sutherlandia* namely, **compound 5** (Van Wyk and Albrecht, 2008).

At least 56 different triterpene glycosides have been detected in *Sutherlandia* (Van Wyk and Albrecht, 2008). The mixture of cycloartane-type triterpenoid glycosides varies geographically in South Africa (Van Wyk and Albrecht, 2008). A study done by Fu *et al.* (2008) found four cycloartane-type triperpenoids (see chapter 1 **compounds 4** to **7**, page 11) that are said to have a bitter tonic activity that stimulates appetite and may contribute to the adaptogenic and immune-boosting effects of *Sutherlandia*. Two of these compounds are also known as SU1 (**compound 5**) and SU2 (**compound 4**) (Van Wyk and Gericke, 2000).

*Sutherlandia* leaves are also known to contain at least six flavonoids (Moshe, 1998). A study done by Fu *et al.* (2010) identified four of these to be flavonol glycosides named sutherlandin A, B, C and D (see Chapter 1, **Figure 1.7 compounds 8** to **11**, page 12). Flavonoids are said to have anti-oxidant activity and help to eliminate mutagen and carcinogens - which are of value in cancer prevention (Tai *et al.*, 2004). They exert their anti-oxidant effects by neutralizing or by chelating different types of oxidizing radicals which includes the superoxide and hydroxyl radicals (Baghat, www.scribd.com).

Following the procedures discussed in Chapter 2 section 2.3 (page 28), the *n*-butanol phase (**extract I**) from the methanol extract was used for isolation of triterpenoid compounds as this phase was found to mainly contain types of compounds associated with immune-boosting activities. The spray dried water extract (**extract K**) was used to isolate the flavononol glycosidic compounds, which were present in high concentrations. This section will focus on

the compounds which were isolated and subsequently characterised, using NMR spectroscopy and UPLC-QTOF spectrometry.

### 3.3.1 Compound 5 standard

**Compound 5** is said to be a major triterpene found in commercial *Sutherlandia* material; it is also said to be the active compound for the anti-cancer properties related to *Sutherlandia* (Van Wyk and Gericke, 2000). A standard of the compound was kindly supplied by Prof. C. Albrecht[3]. This was used for NMR and spectroscopic analysis for comparison purposes only and was not isolated by the author. The structure was corroborated on a 600 MHz Varian NMR using deuterated pyridine (d-pyr) and a WATERS Synapt G1 UPLC-TOF-MS supported initial findings on the WATERS HPLC-PDA-SQD system. **Compound 5** was not visible on a Thin Layer Chromatographic (TLC) plate, under either long (360 nm) or short (254 nm) wavelength UV light. However, after spraying the plate with vanillin reagent and heating it at 100 °C for a few minutes, the compound became visible as a purple spot with an $R_f$ of 0.1 (solvent system 9:1:0.1 chloroform: methanol: formic acid).

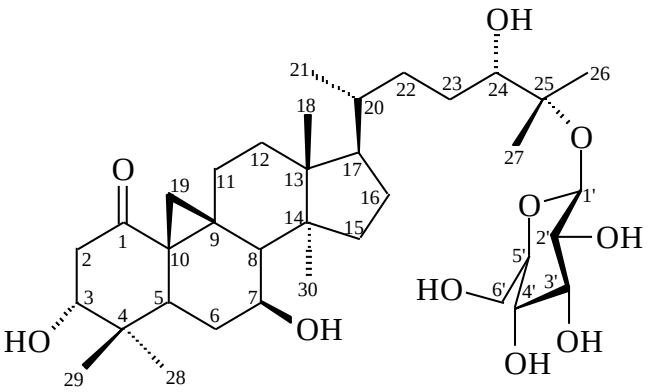



**Figure 3.4 Structure of compound 5.**

Following the HPLC method as discussed in Chapter 2 section 2.3.2 (page 29), the standard was analysed to determine its retention time and profile. The chromatogram from the UPLC-

[3] Prof. C. Albrecht. Head of Research. Cancer Association of South Africa, Durbanville. calbrecht@cansa.org.za

TOF-MS system for the standard of **compound 5** can be found in **Appendix 4** (Appendix Figure 4.1, page 132). In this chromatogram, **compound 5** was found to elute at 1.73 minutes in ESI negative mode. Further NMR analysis was carried out on this compound to confirm its structure. The Table below shows NMR data for **compound 5** in pyridine-$d_5$ (d-pyr).

**Table 3.1 $^{13}C$ and $^{1}H$ (600 MHz) data for compound 5 in pyridine-$d_5$ (d-pyr) being compared to literature.**

| Position | Compound 5 | | Literature * | |
|---|---|---|---|---|
| | $\delta_C$ mult. | $\delta_H$ ($J$ = Hz) | $\delta_C$ mult. | $\delta_H$ ($J$ = Hz) |
| 1 | 210.7 | | 210.7 | |
| 2a | 49 | 2.81 dd (13.7, 4.3) a | 48.8 | 2.81 dd (13.6, 4.4) |
| 2b | | 3.23 dd (13.7; 4.3) b | | 3.23 dd (13.8, 4.2) |
| 3 | 78.7 | 4.04 m | 78.5 | 4.03 |
| 4 | 40.1 | | 39.9 | |
| 5 | 38.9 | 2.93 dd (13.8, 3.4) | 38.7 | 2.90 dd (13.4, 3.4) |
| 6 | 31.7 | 1.65 m; 2.19 m | 31.5 | 1.66; 2.17 |
| 7 | 68.5 | 4.04 m | 68.4 | 3.97 |
| 8 | 51.8 | 2.43 d (4.2) | 51.5 | 2.43 d (4.0) |
| 9 | 28.8 | | 28.7 | |
| 10 | 39.5 | | 39.2 | |
| 11a | 29.4 | 2.01 m | 29.2 | 1.99 |
| 11b | | 2.38 br d (14.1) | | 2.37 dd (12.8, 2.8) |
| 12 | 33.8 | 1.55 m; 1.88 m | 33.6 | 1.54; 1.84 |
| 13 | 46 | | 45.9 | |
| 14 | 50.2 | | 50.1 | |
| 15 | 34.7 | 1.55 m; 1.88 m | 34.5 | 1.55, 1.86 |
| 16 | 28.6 | 1.35 m; 1.88 m | 28.4 | 1.36 t (14.0), 1.84 |
| 17 | 52.6 | 1.65 m | 52.4 | 1.6 |
| 18 | 16.2 | 1.0 s | 16 | 0.99 s |
| 19a | 24.6 | 0.86 d (4.7) | 24.4 | 0.86 d (4.4) |
| 19b | | 1.65 m | | 1.60 d (4.0) |
| 20 | 37.5 | 1.47 m | 37.4 | 1.46 |
| 21 | 19.4 | 0.96 d (6.5) | 19.3 | 0.95 d (6.0) |
| 22 | 35.1 | 1.14 m; 2.19 m | 34.9 | 1.12; 2.17 |
| 23 | 29.7 | 1.47 m; 1.88 m | 29.5 | 1.46; 1.83 |
| 24 | 78.9 | 3.85 br d (7.0) | 78.7 | 3.84 br d (9.2) |
| 25 | 81.4 | | 81.3 | |
| 26 | 21.7 | 1.58 s | 21.6 | 1.57 s |
| 27 | 24.8 | 1.55 s | 24.6 | 1.54 s |
| 28 | 25.8 | 1.27 s | 25.6 | 1.26 s |
| 29 | 21.9 | 1.05 s | 21.7 | 1.04 s |
| 30 | 19.1 | 1.10 s | 18.9 | 1.09 s |
| 1' | 99.2 | 5.25 br s | 99 | 5.24 d (7.6) |
| 2' | 75.9 | 4.04 m | 75.7 | 4.04 t (7.4) |
| 3' | 79.2 | 4.28 m | 79 | 4.28 |
| 4' | 72.2 | 4.28 m | 72 | 4.22 |
| 5' | 78.6 | 4.04 m | 78.4 | 4.03 |
| 6'a | 63.2 | 4.38 m | 63 | 4.34 dd (11.6, 5.2) |
| 6'b | | 4.56 br d (11.7) | | 4.54 br d (10.8) |

*** Literature data from Fu *et al.*, 2008.**

NMR data of the **compound 5** (see **Table 3.1** above) revealed thirty six resonance signals in the $^{13}C$ spectrum, confirming that the compound has a triterpenoid glycoside skeleton. Structural elucidation of triterpenoids is challenging, especially to characterise the complex saturated alkyl region. In the $^{13}C$ spectrum, there is evidence for a carbonyl function ($\delta_C$ 210.7 ppm), a cyclopropane ($\delta_C$ 28.8, 39.5 and 24.6 ppm), three oxygen-containing methine groups ($\delta_C$ 68.5, 78.7 and 78.9 ppm) and the presence of a sugar ($\delta_C$ 63.2, 72.2, 75.9, 78.6, 79.2 and 99.2 ppm).

From the proton spectrum, the anomeric proton from the sugar is seen at $\delta_H$ 5.25 ppm as a broad singlet, while the other five protons from the sugar are found at $\delta_H$ 4.28, 4.28 4.08, 4.38 and 4.56 ppm. Six singlet resonances show the confirmation of the six methyl groups attached to quaternary carbons ($\delta_H$ 1.58, 1.55, 1.27 1.05, 1.10 and 1.1 ppm). One doublet resonance at $\delta_H$ 0.95 ppm shows the presence of another methyl group attached to a methine carbon. The $^{13}C$ and $^{1}H$ spectra of compound 5 can be found in **Appendix 5**, pages 139 and 140.

High resolution Time-of-Flight Mass Spectroscopy (HRTOFMS) can be used to confirm the molecular mass of the compound. The spectrum below (**Figure 3.5**) shows the mass spectrum of **compound 5** with its fragmentation pattern that was obtained from UPLC-QTOF analysis.

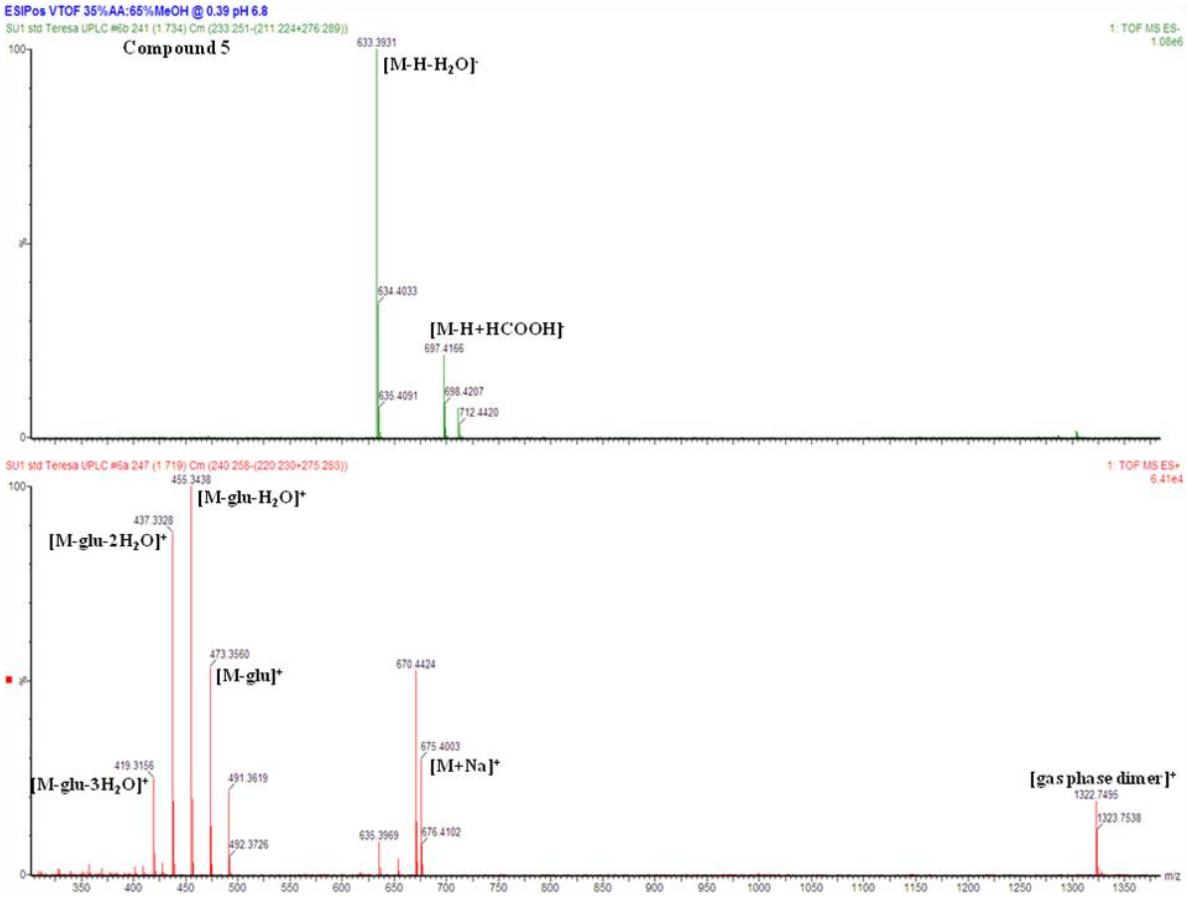



**Figure 3.5 HRTOFMS ($ESI^-$ and $ESI^+$) spectra for compound 5.**

The mass spectrum from HRTOFMS (**Figure 3.5** also found in Appendix Figure 4.2, page 133) is similar to that found in literature (Fu *et al.*, 2008) and shows *m/z* 675.4003 $(C_{36}H_{60}O_{10} + Na)^+$ in $ESI^+$ mode, *m/z* 697.4166 $(C_{36}H_{60}O_{10} + HCOOH)^-$ as well as *m/z* 633.3931 $(C_{36}H_{60}O_{10} - H_2O)^-$ in $ESI^-$ mode thereby confirming the accurate mass of the compound 652.4073 ($C_{36}H_{60}O_{10}$). A gas phase dimer is shown to be formed at *m/z* 1322.7495 $(2M + Na)^+$ in $ESI^+$ mode. The compound has a $\lambda_{max}$ of 219 nm (see Appendix Figure 4.10, page 138). An elemental composition analysis was done on the *m/z* 697.4166 which gave the formula $C_{37}H_{61}O_{12}$ (which fits the formula $M + HCOO^-$) with an i-FIT value of 0.0 (a value closer to zero shows a perfect fit), a mass error of 0.3 (value should be < 5) and a calculated DBE value of 7 (double bond equivalents and ring structures which fit the structure and double bond count of the compound and possible adducts calculated on the molecular formula $C_{36}H_{60}O_{10}$). All the above mentioned data confirmed the structure of the standard obtained to be that of **compound 5** (3R,7S,24S,25S-tetrahydroxycycloartan-1-one 25-*O*-β-D-glucopyranoside). Once the compound's structure and its purity have been confirmed, it can

then be tested in various biological assays to determine its activity. The graphs for the activity of **compound 5** in the cancer cells and cytokine expression assays will be presented in Chapter 7, section 7.1.3.4 and 7.2 (page 84 to 87).

### 3.3.2 Characterisation of compound 4

**Compound 4** was isolated from the *n*-butanol soluble portion (**extract I**) of the methanol extract of the leaves of *S. frutescens* by column chromatography on silica gel and was crystallised using methanol (see Chapter 2 section 2.2.1, page 25). **Compound 4** was not visible on a TLC plate under either long (360 nm) or short (254 nm) wavelength UV light. However, after spraying the plate with vanillin reagent and heating it at 100 °C for a few minutes, the compound showed up as a purple spot with an $R_f$ of 0.2 (solvent system 9:1:0.1 chloroform: methanol: formic acid). Only a 0.006 % yield was obtained from the 17.2 g of **extract I** that was used, whereas a 0.2 % yield was obtained from 29.75 g of the same extract from the study done by Fu *et al.,* 2008.

**Compound 4** was analysed using UPLC to determine its retention time and chemical profile. The resulting chromatogram can be found in **Appendix 4** (Appendix Figure 4.3, page 134). **Compound 4** displayed a retention time of 2.89 and 2.91 minutes in ESI negative and positive modes, respectively. The resulting chromatogram confirmed the purity of **compound 4**, which was subsequently subjected to NMR analysis, to determine the structure of the compound. **Table 3.2** below shows the NMR data for **compound 4** in methanol-$d_4$ ($CD_3OD$), which was compared to that found in literature to determine its structure (Fu *et al.,* 2008).

**Table 3.2 $^{13}C$ data (600 MHz) for compound 4 in methanol-$d_4$ ($CD_3OD$) being compared to literature in d-pyr.**

| | Compound 4 | Literature * |
|---|---|---|
| **Position** | **δc mult.** | **δc mult.** |
| 1 | 71.8 | 72 |
| 2 | 31.5 | 31.8 |
| 3 | 77.8 | 77 |
| 4 | 38.3 | 37.7 |
| 5 | 46.7 | 46.1 |
| 6 | 31.3 | 30.9 |
| 7 | 77.1 | 75.8 |
| 8 | 53.9 | 53.1 |
| 9 | 135.8 | 135.9 |
| 10 | 85.3 | 84.8 |
| 11 | 122.4 | 121.1 |
| 12 | 38 | 37.6 |
| 13 | 46.5 | 45.7 |
| 14 | 46.2 | 45.4 |
| 15 | 34.8 | 34.4 |
| 16 | 29 | 28.8 |
| 17 | 51.7 | 51 |
| 18 | 15.6 | 15.5 |
| 19 | 42.8 | 42.8 |
| 20 | 37.2 | 37.2 |
| 21 | 18.9 | 19.2 |
| 22 | 35 | 35 |
| 23 | 29.3 | 29.6 |
| 24 | 78 | 78.7 |
| 25 | 81.6 | 81.3 |
| 26 | 22.8 | 21.7 |
| 27 | 23.9 | 24.6 |
| 28 | 27 | 27.4 |
| 29 | 23.1 | 24 |
| 30 | 20.3 | 20 |
| 1' | 98.1 | 99 |
| 2' | 75.5 | 75.7 |
| 3' | 78.4 | 79 |
| 4' | 72.8 | 72.1 |
| 5' | 77.9 | 78.6 |
| 6' | 62.9 | 63.1 |

*** Literature data from Fu *et al.*, 2008.**

From the NMR data in **Table 3.2** the first dominant feature of **compound 4** is that it lacks a carbonyl carbon at position 1 (as seen in the structure of **compound 5** at $\delta_C$ 213 ppm); this is substituted by the presence of an oxygen-containing methine group ($\delta_C$ 71.8 ppm), thus exhibiting three oxygen-containing methine groups ($\delta_C$ 71.8, 77.8 and 78 ppm), which is also found in literature of **compound 4** (at $\delta_C$ 72, 77 and 78.7 ppm). The next dominant feature is the absence of the cyclopropane structure, which is substituted by an oxygen-bridge between carbon 10 and 7 ($\delta_C$ 85.3 and 77.1 ppm). **Compound 4** also has an additional double bond between carbon 9 and 11 ($\delta_C$ 135.8 and 122.4 ppm). Both these features are found in literature of **compound 4** as well (at $\delta_C$ 84.8 and 75.8 ppm for carbon 10 and 7; and $\delta_C$ 135.9 and 121.1 ppm for carbon 9 and 11). The remaining features, like the sugar moiety and cycloartane type triterpenoid structure, is similar to that of compound 5 and 4, thus confirming the skeleton structure of **compound 4** and its structure to be that of **compound 4** (sutherlandioside A); which is closely related to **compound 5** with the absence of the carbonyl carbon (at position 1) and the presence of a double bond between carbon number 9 and 11 (see **Figure 3.9** for structure). The $^{13}C$ and $^{1}H$ spectra of compound 4 can be found in Appendix Figure 5.3 and 5.4, page 141. HRTOFMS was used to confirm the molecular mass of the compound and its characteristic fragmentation patterns. The mass spectrum below (**Figure 3.6** below, relating to **Appendix 4** Figure 4.4, page 134) of **compound 4** displays its fragmentation pattern as observed in HRTOFMS $ESI^-$ and $ESI^+$ modes.

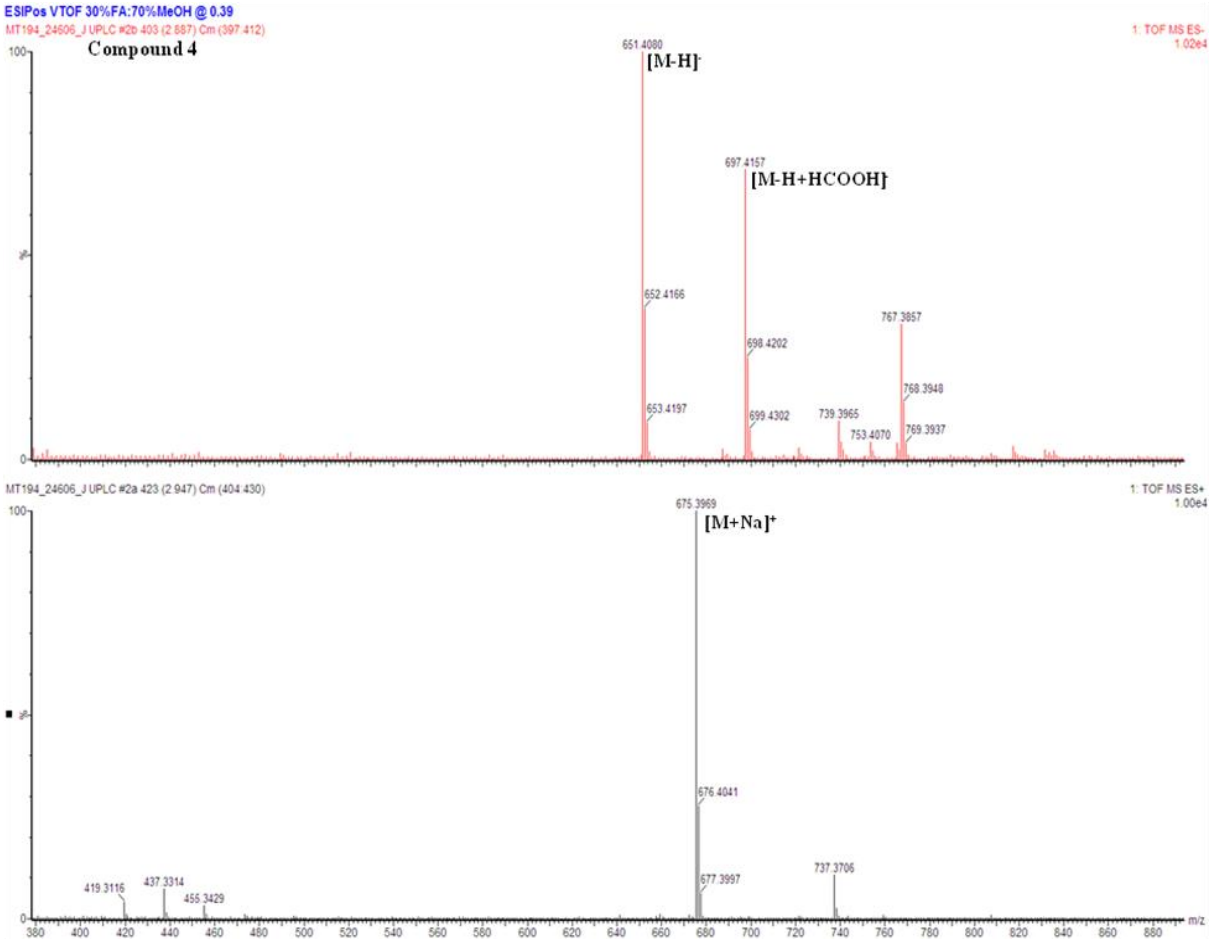



**Figure 3.6 HRTOFMS ($ESI^-$ and $ESI^+$) for compound 4.**

The HRTOFMS $ESI^-$ (negative mode) gave a pseudo molecular ion at *m/z* 651.4080 $(C_{36}H_{60}O_{10} - H)^-$, with a $\lambda_{max}$ of 203 nm. The mass ion at *m/z* 697.4157 shows the presence of a formic acid adduct $(C_{36}H_{60}O_{10} - H + HCOOH)^-$ due to its presence in the source. An elemental composition analysis was done on the *m/z* 697.4157 and 651.4080 mass ions which gave the formulae $C_{37}H_{61}O_{12}$ and $C_{36}H_{59}O_{10}$ (which fits the formula M + $HCOO^-$ and M - H) with an i-FIT value of 0.0 for both fragments, a mass error of -0.6 mDa and -2.8 mDa, respectively, and a calculated DBE value of 7 for both fragments (calculated based on the molecular formula $C_{36}H_{60}O_{10}$). The HRTOFMS $ESI^+$ (positive mode) shows a pseudo molecular peak at *m/z* 675.3969 $(C_{36}H_{60}O_{10} + Na)^+$. To confirm the presence of the sodium adduct at *m/z* 675.3969 another experiment on the HPLC-MS system was conducted, where formic acid was replaced with potassium hydrogen carbonate ($KHCO_3$) in the mobile phase. In the mass spectral data below (obtained from the HPLC-SQD), the formation of sodium (in positive mode, see **Figure 3.8**) and formic acid (in negative mode, see **Figure 3.7**) adducts

when the formic acid was used, can be seen, together with the formation of potassium adducts when $KHCO_3$ was used (in positive mode, see **Figure 3.8**) in the ESI negative and positive modes.

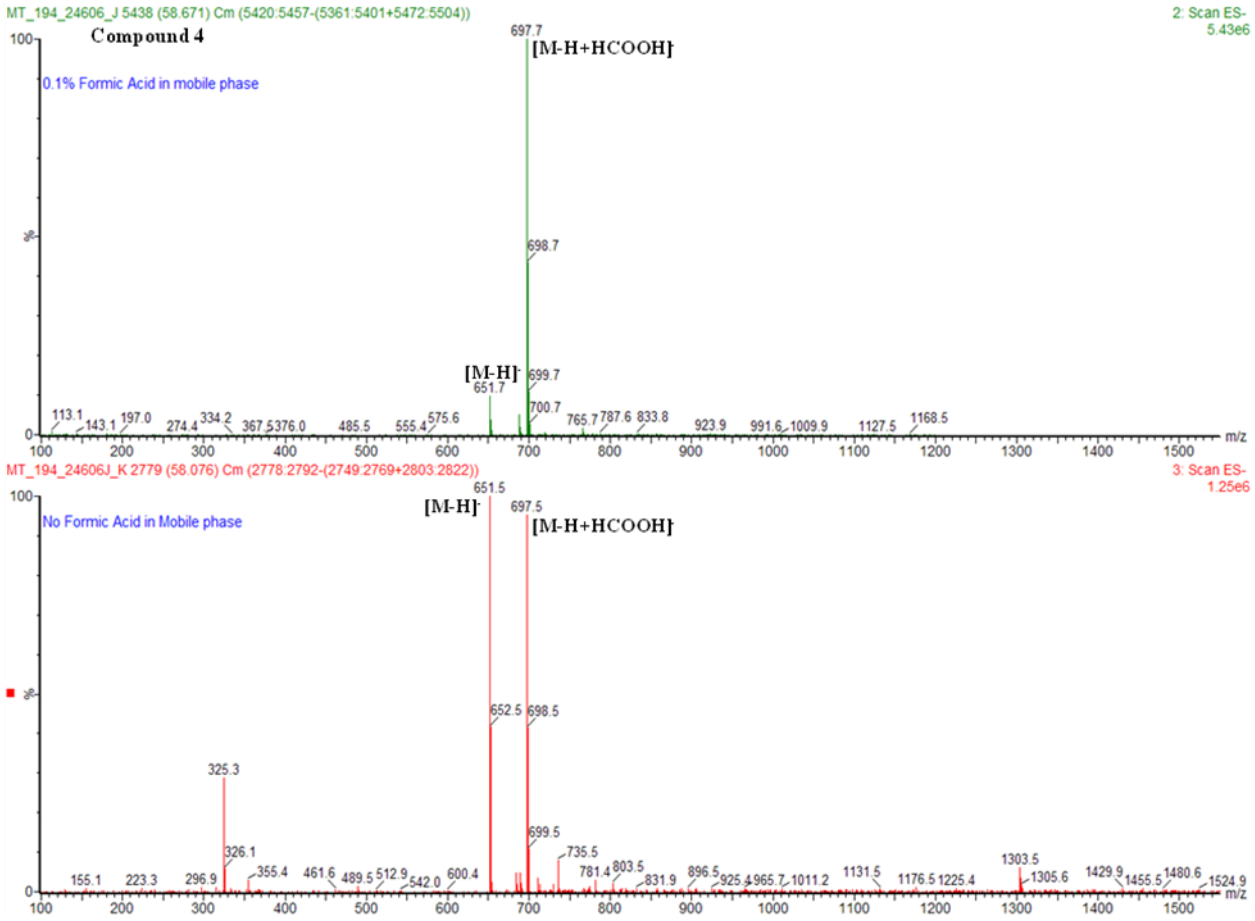



**Figure 3.7 ESI mass spectra (ESI$^-$) for compound 4 showing the formic acid adduct from the SQD system (also found in Appendix 4 Figure 4.5, page 135).**

Thus the formation of sodium, formic acid and potassium adducts depended on the ionisation mode as well as the mobile phase additives present in the mobile phase.

In the ESIMS, the pseudo molecular ion at *m/z* 697.7 (ESI$^-$) shows the presence of a formic acid adduct $(M\text{-}H+COOH)^-$. This formation is due to the presence of 0.1 % formic acid in the mobile phase (**Figure 3.7** top spectrum). This is substantiated by removing the formic acid from the mobile phase, thus showing *m/z* 651.5 (**Figure 3.7** bottom spectrum). The formation

of the formic acid ion (*m/z* 697.5) in this case is most likely due to its presence in the source, as residual formic acid was not washed out from the source before the following injection. The ratio of the molecular ion to the formic acid ion shows the formic acid ion being produced from the source. **Figure 3.8** below confirms the presence of sodium and potassium adducts when the mobile phase is supplemented with formic acid and $KHCO_3$, respectively.

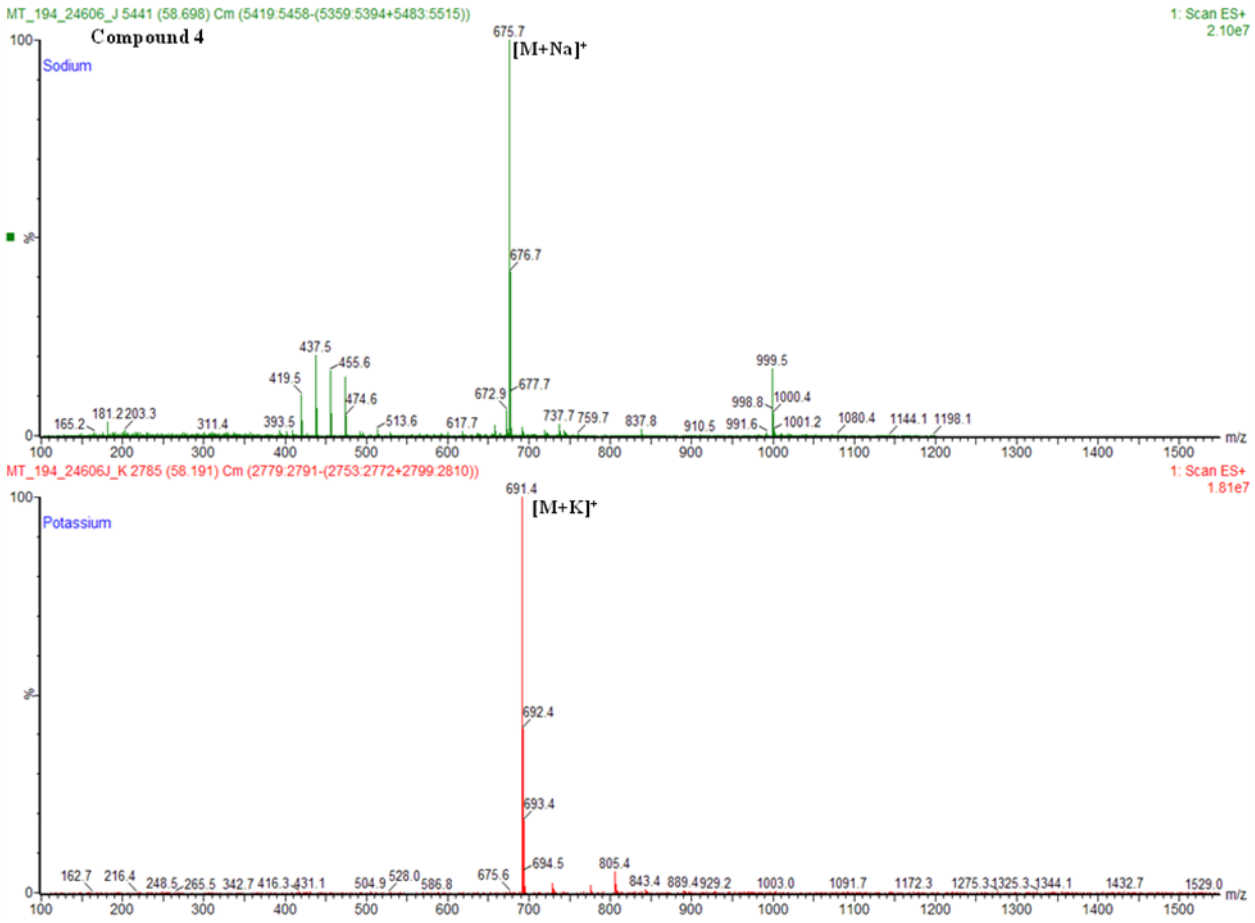



**Figure 3.8 ESI mass spectra (ESI$^+$) for compound 4 showing sodium and potassium adducts from the SQD system (also found in Appendix Figure 4.6, page 135).**

In the ESI ESI$^+$ the molecular ion at *m/z* 675.7 (**Figure 3.8** top spectrum) shows the presence of a sodium adduct $(C_{36}H_{60}O_{10} + Na)^+$. To confirm this, a potassium buffer (bottom spectrum) was used instead of sodium formate, therefore now producing a potassium adduct (*m/z* 691.4). This confirms the molecular ion is 652.4206 ($C_{36}H_{60}O_{10}$), which justifies the claim that the isolated **compound 4** has the same structure as literature of **compound 4**, which is sutherlandioside A (1S,3R,24S,25-tetrahydroxy-7S,10S-epoxy-9,10-seco-9,19-cyclolanost-

9(11)-ene 25-*O*-β-D-glucopyranoside). A typical fragmentation pattern is seen with these types of compounds in the MS thus confirming their structures to be related to that of **compound 5**.

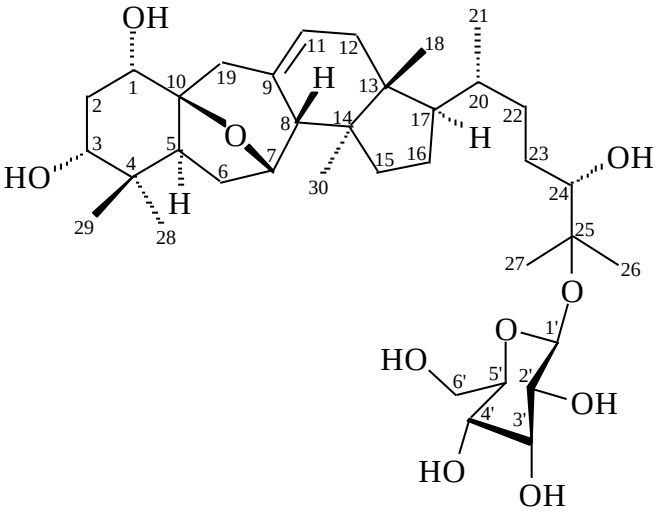



**Figure 3.9 The structure of compound 4 i.e. sutherlandioside A.**

Sutherlandioside A was present in very sparse amounts to continue with the biological investigations; therefore the activity related to triterpenoids can be seen in **fraction IV**, where four of these related compounds (showing a similar fragmentation pattern in their mass spectra) are present in relatively high concentrations.

### 3.3.3 Characterisation of compound 7

**Compound 7** was also isolated from **extract I** from the dried leaves *S. frutescens* by column chromatography on silica gel and sephadex (see Chapter 2 section 2.2.1, page 25). **Compound 7** displayed a slightly visible spot on a TLC plate under short (254 nm) wavelength but not under long (360 nm) wavelength UV light. After the plate was sprayed with vanillin reagent and heated at 100 °C for a few minutes, the compound appeared as a purple spot with an $R_f$ of 0.2 (solvent system 9:1:0.1 chloroform: methanol: formic acid). Only a 0.012 % yield was obtained from the 17.2 g of **extract I** that was used, whereas a

0.019 % yield was obtained from 29.75 g of the same extract from the study done by Fu *et al.*, 2008.

To confirm **compound 7**'s chemical profile and obtain its retention time – UPLC-HRTOFMS was used. The resulting chromatogram is presented in **Appendix 4** (Appendix Figure 4.7, page 136). **Compound 7** had a retention time of 2.46 minutes in ESI negative and positive modes. Further NMR analysis was conducted on the isolated **compound 7** to determine its structure. The resulting NMR data for **compound 7** in methanol-$d_4$ ($CD_3OD$) is presented in **Table 3.3** below and was compared with data of compounds 5 and 7 found in literature (Fu *et al.*, 2008) to determine its structure.

**Table 3.3 $^{13}C$ (600 MHz) data for compound 7 in $CD_3OD$ being compared to literature data in d-pyr.**

| Position | Compound 7 | Literature * |
|---|---|---|
| | $\delta_C$ mult. | $\delta_C$ mult. |
| 1 | 201.9 | 200.9 |
| 2 | 126 | 129.6 |
| 3 | 161.1 | 159.1 |
| 4 | 38.8 | 37 |
| 5 | 41.8 | 42.5 |
| 6 | 34.1 | 31.5 |
| 7 | 70.9 | 68 |
| 8 | 55.4 | 52.8 |
| 9 | 34 | 31.3 |
| 10 | 38.1 | 35.9 |
| 11 | 27.2 | 25.8 |
| 12 | 37.1 | 34.9 |
| 13 | 45.7 | 45.8 |
| 14 | 51.3 | 49.8 |
| 15 | 35.6 | 33.4 |
| 16 | 32.8 | 29.6 |
| 17 | 53.7 | 52.5 |
| 18 | 14.8 | 16.3 |
| 19 | 30.1 | 29.1 |
| 20 | 40.1 | 37.5 |
| 21 | 18.3 | 19.3 |
| 22 | 36.6 | 34.9 |
| 23 | 28.5 | 28.5 |
| 24 | 77.2 | 78.7 |
| 25 | 80.8 | 81.3 |
| 26 | 21.9 | 21.6 |
| 27 | 22.3 | 24.6 |
| 28 | 28.2 | 28 |
| 29 | 18.8 | 21.4 |
| 30 | 18.2 | 19.1 |
| 1' | 97.3 | 99 |
| 2' | 74.6 | 75.7 |
| 3' | 77.5 | 79 |
| 4' | 71.6 | 72 |
| 5' | 77 | 78.6 |
| 6' | 64.2 | 63 |

***Literature data from Fu *et al.*, 2008.**

The NMR data of the isolated **compound 7** revealed thirty six resonance signals in the $^{13}C$ spectrum suggesting that this compound could be **compound 5** or closely related. Thus, the compound was analysed in deuterated methanol ($CD_3OD$) and compared to what was found in literature. **Compound 7** showed the absence of one methine group at position 3 ($\delta_C$ 78.1 ppm found in **compound 5**), which is substituted with a double bond ($\delta_C$ 126 and 161.1 ppm). The double bond at position 3 is also found in literature of **compound 7** ($\delta_C$ 129.6 and 159.1 ppm). The remaining NMR assignments for **compound 7** are in good agreement with literature data of **compounds 5** and **7**, thus confirming the cycloartane glycosidic structure (see Table 3.3 above for $^{13}C$ shifts). Due to the presence of the double bond at position 3, the carbonyl group ($\delta_C$ 201.9 ppm), a cyclopropane ($\delta_C$ 34.0, 38.1 and 30.1 ppm), two oxygen-containing methine groups ($\delta_C$ 70.9 and 77.2 ppm) and a sugar moiety ($\delta_C$ 64.2, 71.6, 74.6, 77.0, 77.5 and 97.3 ppm); and the absence of a methine group at position 3, in the resulting NMR data of **compound 7**, corroborates the structure to be that of **compound 7** (i.e. sutherlandioside D). The $^{13}C$ and $^{1}H$ spectra of compound 7 can be found in **Appendix 5**, page 142. HRTOFMS was used to confirm the molecular mass of the compound. The spectrum below (**Figure 3.10** below, as well as **Appendix 4** Figure 4.8, page 137) shows the mass spectrum of **compound 7** with its fragmentation pattern.

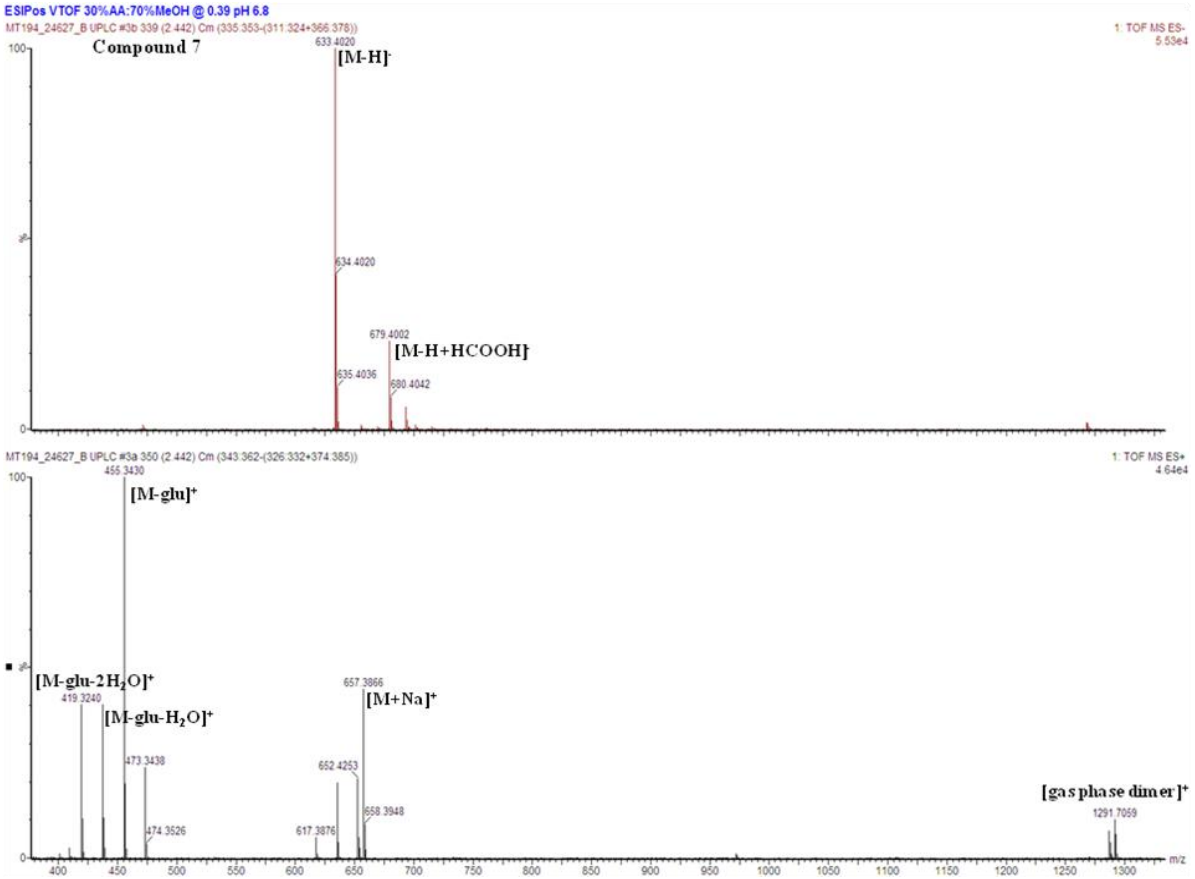



**Figure 3.10 HRTOFMS ($ESI^-$ and $ESI^+$) for compound 7.**

The HRTOFMS $ESI^-$ (negative mode) gave a pseudo molecular ion at *m/z* 633.4020 (calculated for $C_{36}H_{58}O_9$ - H)$^-$ thereby confirming the accurate mass 634.4093 ($C_{36}H_{58}O_9$) found in literature (Fu *et al.*, 2008). There is also the presence of a formic acid adduct ion at *m/z* 679.4002 (M-H+HCOOH)$^-$. In HRTOFMS $ESI^+$ (positive mode) a distinct fragmentation pattern is seen typical of these compounds. Firstly the compound undergoes a loss of about *m/z* 179 which corresponds to the subsequent loss of the hydroxyl groups of the sugar moiety resulting in the fragment at *m/z* 473.3438 and subsequently the loss of the sodium adduct (22.9897) which results in the *m/z* 455.3430. The second (*m/z* 437.3317) and third (*m/z* 419.3214) fragmentations are due to the loss of two water (18.0152 each) molecules (attached to carbon 24 and 7). The compound has a $\lambda_{max}$ of 227 nm. As was done with **compound 4**, the presence of the sodium and potassium adducts was also demonstrated for **compound 7**. The *m/z* at 657.3832 represents ($C_{36}H_{58}O_9$ + Na)$^+$ in the HRTOFMS $ESI^+$. The mass spectral data that displays the presence of a sodium adduct (*m/z* 657.7) and a potassium adduct (*m/z* 673.4) in HRESI $ESI^+$ for **compound 7**, can be found in **Appendix 4** (Appendix Figure 4.9,

page 137). An elemental composition analysis was done on the *m/z* 633.4020 which gave the formula $C_{36}H_{57}O_9$ (which fits the formula M - H) with an i-FIT value of 0.0 and a mass error of 1.9 mDa (value should be < 5) and a calculated DBE value of 8 (which fits the six rings and two double bonds of the structure on the molecular formula of $C_{36}H_{58}O_9$). All the above data confirmed the structure of the isolated **compound 7** to be that of **compound 7** (i.e. sutherlandioside D - 7S,24S,25-trihydroxycycloart-2-en-1-one 25-*O*-β-D-glucopyranoside).

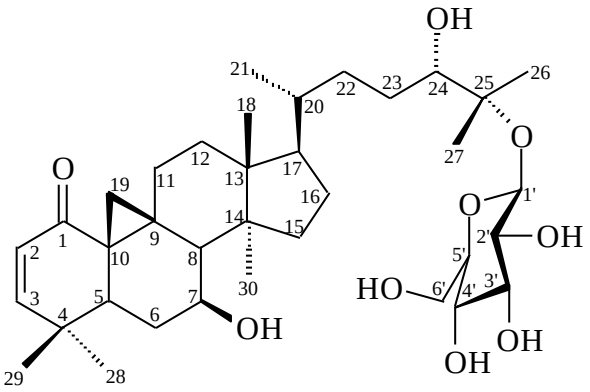



**Figure 3.11 The structure of compound 7 i.e. sutherlandioside D.**

The activity of sutherlandioside D in the biological investigations was not determined due to its limited amount. In Chapter 6 the activity of **fraction IV** containing four cycloartane glycosides in relatively high concentrations is presented. Sutherlandioside D is also present in this fraction in very low concentrations.

The Table below is a summary of **compounds 4**, **5** and **7** with their mass spectral data to show their correlation with literature data. The UV absorption spectra of all three compounds can be found in Appendix Figure 4.10, page 138. Various 2D NMR experiments that were run can be found in **Appendix 5** pages 143 to 144.

**Table 3.4 Comparison of mass spectral data of compounds isolated to literature data.**

| Samples | $\lambda_{max}$ (nm) | $ESI^-$ $(M - H + HCOOH)^-$ *m/z* | $ESI^+$ $(M + Na)^+$ *m/z* | Calculated mass (*m/z*) | Literature mass (*m/z*)* |
|---|---|---|---|---|---|
| **Compound 5 (Sutherlandioside B)** | 219 | 697.4166 | 675.4003 | 652.3997 | 652.4186 |
| **Compound 4 (Sutherlandioside A)** | 203 | 697.4114 | 675.4020 | 652.3945 | 652.4186 |
| **Compound 7 (Sutherlandioside D)** | 227 | 679.4014 | 657.3832 | 634.4093 | 634.4081 |

* Literature data from Fu *et al.*, 2008.

# CHAPTER 4

# COMPARISON OF CHEMOTYPES

Six samples of plant material of *Sutherlandia frutescens* were received from a commercial supplier in the Western Cape. These samples were all identified as *S. frutescens* however, upon subsequent growth their morphological appearances differed. In this regard it was suspected that there might be different chemotypes of *S. frutescens* present; resulting in subtle chemical differences within the plant species. *S. frutescens* has been found to be chemically, genetically and geographically extremely variable (Van Wyk and Albrecht, 2008). According to Moshe *et al.* (1998), it should be divided into three subspecies and several geographical regional forms. The extent to which different chemotypes of *S. frutescens* exist also makes it difficult in selecting the correct plant for research purposes, particularly with regard to reproducibility and standardisation of research results. Reliable chemical characterisation needs to be in place, in order to avoid incorrect or misleading identification of possible species and chemotypes.

With this background in mind, the purpose of this part of the study was to determine the extent of the differences and/or similarities of *Sutherlandia frutescens* plant material collected in one geographical area using HPLC-MS analytical techniques. As previously mentioned (page 29), this technique allows for an indication of the compounds present based on their molecular masses, which can be subsequently used to identify the structure of the compounds. In this chapter, six different samples of *Sutherlandia frutescens* were investigated, by comparing their HPLC-MS chemical profiles, based on similarities and/or differences of retention times and mass spectral data.

## 4.1 UV CHROMATOGRAMS OF CHEMOTYPES

According to the methodology for generating HPLC-MS-UV data (previously discussed in Chapter 2 page 29), the following UV chromatograms for **clone** samples **I** to **VI** are given in **Figure 4.1** below.

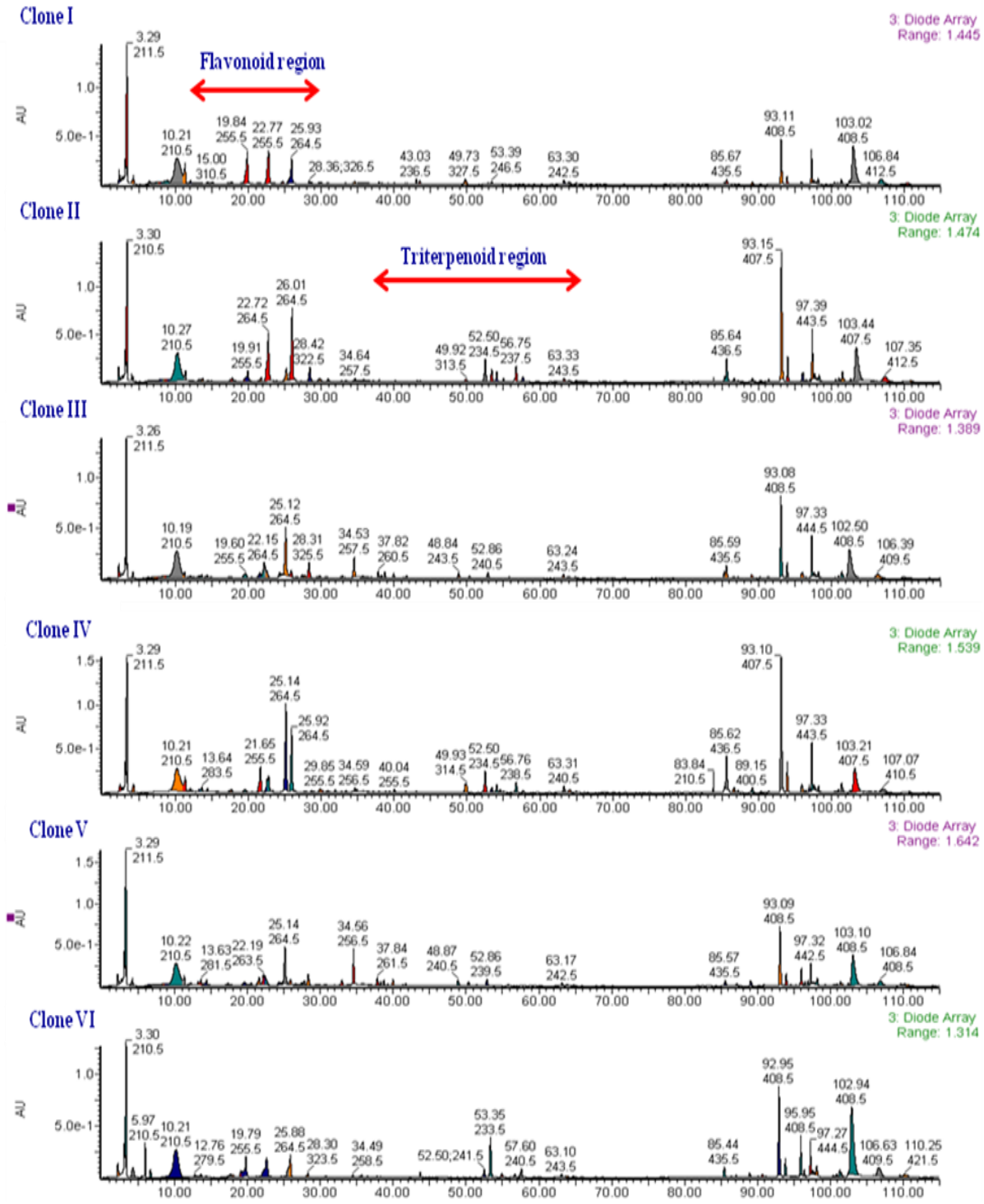



**Figure 4.1 UV chromatograms of clone samples I to VI. UV-VIS detection was done on a WATERS PDA scanning from 200 – 600 nm on the SQD system.**

Some of the similarities and/or differences are outlined in **Table 4.1** below, which point out the differences based on retention time and mass spectral data of the major peaks for each of the samples.

**Table 4.1 Retention times of the major peaks (and base peak values) found in each chromatogram in the UV-VIS range from 200 to 600 nm.**

| Sample | Retention time (minutes) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Clone I** | 10.21 | 19.84 | 22.77* (255) | - | - | 25.93 | - | - | 93.11 |
| **Clone II** | 10.27 | - | - | 22.72* (264) | - | - | 26.01 | - | 93.15 |
| **Clone III** | 10.19 | - | - | - | 25.12 | - | - | 34.53 | 93.08 |
| **Clone IV** | 10.21 | - | - | - | 25.14 | 25.92 | - | - | 93.10 |
| **Clone V** | 10.22 | - | - | - | 25.14 | - | - | 34.56 | 93.09 |
| **Clone VI** | 10.21 | 19.79 | - | 22.60* (264) | - | 25.88 | - | - | 92.95 |

***Compounds eluting at similar retention times, but have different base peak values (255 and 264 respectively).**

The data in **Table 4.1** above shows that there are clear differences between the various samples. For example, the peak eluting at 19.84 minutes and 19.79 minutes in **clone I** and **clone VI** respectively, is absent from the rest of the samples. The similarities in the clone samples can also be seen from **Table 4.1**, such as the peak eluting at 93.11 minutes that occurs in high concentrations in all of the samples. Another peak that is present in all the samples is the one eluting at 10.21 minutes. This compound appears to be present in all of the samples, but in varying quantities, as was reflected by different intensities of the peaks in each chromatogram particularly in **clones I** and **V**, which had the highest intensities (major peaks), whereas in the rest of the samples, lesser peak intensities were observed. It is also apparent that there are compounds present that have different base peak values, but with the same retention times, thus confirming the presence of compounds with the same polarity, but different masses, such as the compounds eluting at 22.77 minutes in clone samples **I**, **II** and

**VI** which have a base peak value of 255 and 264, respectively. Based on the data presented in **Table 4.1** it is clear that there are major differences between the chromatograms. To further illustrate differences between the six samples, masses of the eight compounds previously isolated from *Sutherlandia* (**compounds 4** to **11** from Fu *et al.*, 2008 and Fu *et al.*, 2010) were extracted from the full $ESI^-$ data set obtained from the HPLC system and resulting chromatograms are displayed below.

## 4.2 IDENTIFICATION OF SIMILARITIES AND/OR DIFFERENCES OF COMPOUNDS 4 TO 11 PRESENT IN THE CHEMOTYPES.

**Figure 4.2** below shows the $ES^-$ chromatograms for each of the samples. Differences and/or similarities found in the major cycloartane and flavonol glycosides of *S. frutescens* are apparent between samples. The Figure below includes **Extract F** (MeOH) as a reference sample.

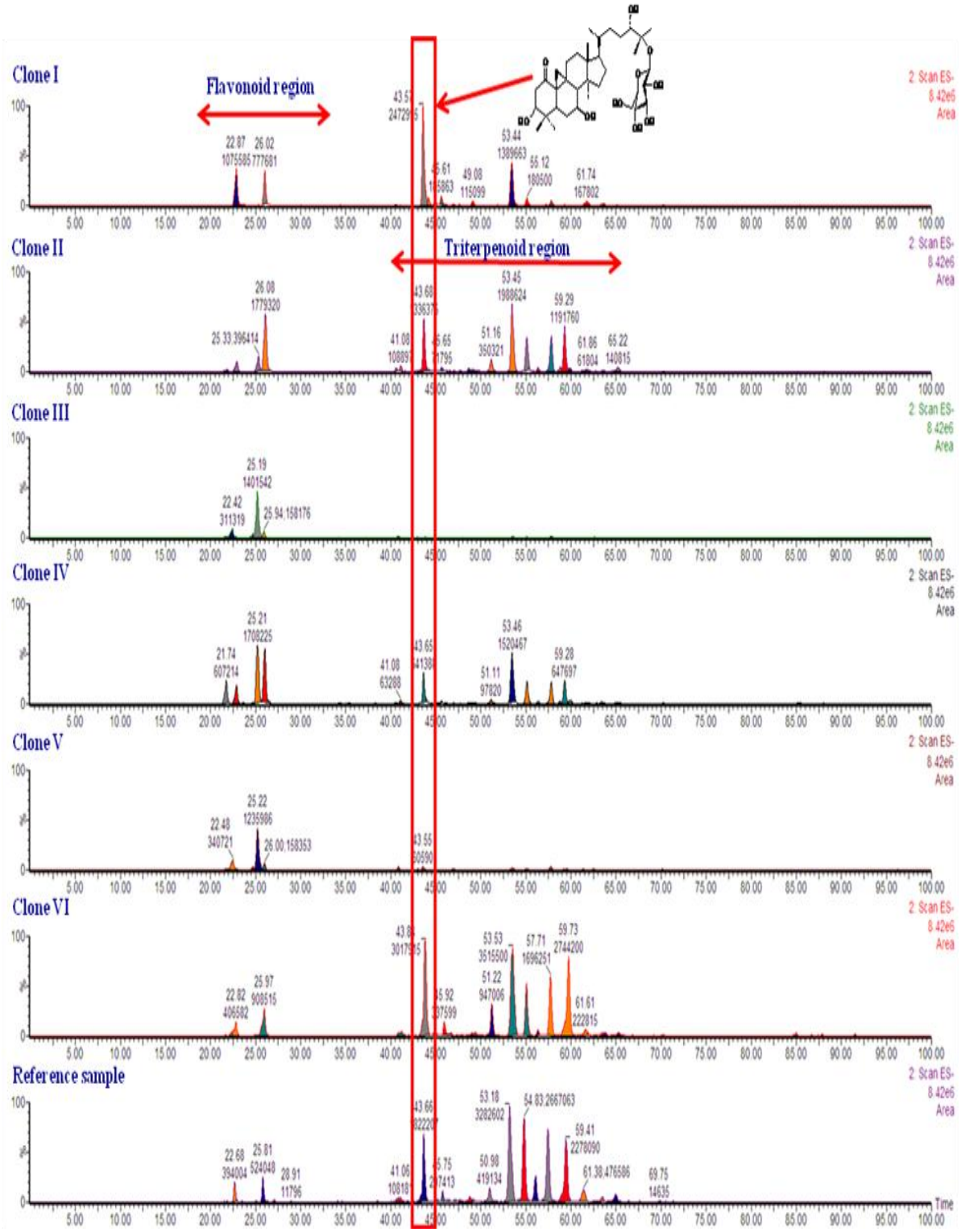



**Figure 4.2 ESI⁻ chromatograms of clone samples I to VI and the reference sample obtained from the HPLC SQD system. Extracted masses in ESI⁻ mode: 739_740+723_724+697_698+679_680+695_696. The peak corresponding to compound 5 is highlighted in the red block with its structure.**

From the above chromatograms, variation in the amounts of some of the major compounds previously isolated from *S. frutescens* can be discerned. Differences, based on retention time and mass spectral data of these compounds, for each of the samples are shown in **Table 4.2** below.

**Table 4.2 Retention times of the major compounds (and base peak values) found in each chromatogram.**

| **Sample** | | **Retention time (min.)** | | | | | |
|---|---|---|---|---|---|---|---|
| **Clone I** | 22.87 | - | 43.58 | - | 53.44* (695) | - | - |
| **Clone II** | 22.85 | 25.32 | 43.68 | 51.15 | - | 53.45* (679) | 59.29 |
| **Clone III** | 22.43 | 25.21 | - | - | - | - | - |
| **Clone IV** | 22.81 | 25.21 | 43.65 | 51.11 | - | 53.46* (679) | 59.29 |
| **Clone V** | 21.75 | 25.22 | 43.65 | - | - | - | - |
| **Clone VI** | 22.82 | 25.97 | 43.82 | 51.21 | 53.53* (695) | - | 59.73 |
| **Reference sample** | 22.68 | 25.81 | 43.66 | - | 53.18* (695) | - | 59.73 |

***Compounds eluting at similar retention time, but have different base peak values (679 and 695 respectively).**

The compound eluting at 43.58 minutes in **clone** sample **I** was identified to be that of **compound 5** (based on the standard of **compound 5** which was analysed on the same method and resulting mass spectral data from the HPLC SQD system). From the chromatograms above (in **Figure 4.2**) clear differences were apparent between some of the flavonoid and triterpenoid compounds. The flavonoids were seen to elute from the column between 20.00 and 30.00 minutes, while the triterpenoids eluted between 40.00 and 65.00 minutes.

This pattern of elution was also confirmed by mass spectral data. In **Figure 4.2** differential concentrations of **compound 5** occur relative to each sample. **Clone** samples **I** and **VI** were seen to contain higher concentrations of **compound 5** (with 2298223.16 and 2638843.18 counts respectively) which was completely missing from **clone III** and very small amounts in

**clone V** (with only 60590 counts) All the clone samples contain the flavonoid compound eluting at 22.87 minutes, which has a *m/z* of 763, corresponding to either **compounds 8** or **9**.

More variation was seen to occur with the other two flavonoids (**compounds 10** and **11**). **Clone** samples **I** and **VI** and the reference sample have similar profiles for these compounds, with **clone VI** demonstrating more complexity. Compounds eluting at 53.36 minutes in **clones I**, **II**, **IV** and **VI** all have the same retention times, but different base peak values implying that they are different compounds. **Clones I** and **VI** and the reference sample contain the same compound (with a base peak of 695), while **clones II** and **IV** have a compound with a base peak value of 679.

Based on the data presented in the **Table 4.1** and **4.2**, it is clear that there are major differences between the detected peaks observed in these chromatograms. On the basis of having comparable quantities of compound 5, but differing amounts of the other compounds, three groups can be identified, namely **clones I** and **VI**, **clones III** and **V,** and **clones II** and **IV**. The compounds found in the reference sample were found to be more complex than the clone samples, but was similar to **clone** sample **VI**. This was also confirmed in the literature, where Chinkwo (2005) noted differences in the anti-cancer activity of the aqueous extracts of the plant material that was collected from different locations, all being identified as *S. frutescens*. This corroborates our findings on the variations found in the plant material. The full chemical profiles of each of the samples can be found in **Appendix 6** Figure 6.1, page 145.

# CHAPTER 5

# PHYTOCHEMISTRY CONCLUSION

From the analyses done on the extracts, it was concluded that the aqueous extract of the fresh leaves (**extract A**) contained more compounds than the aqueous extract of the dried leaves (**extract D**). The freeze drying process was found to be more suitable for controlling stability of some compounds, compared to the spray drying process, which degraded some of the compounds present (**extract D** vs. **extract K**). The organic preparations were found to contain more of a variety of compounds, especially from 40.00 through to 90.00 minutes (the triterpenoid and non-polar regions). The HPLC SQD and UPLC TOFMS analysis of the compounds displayed a characteristic pattern that was challenging to elucidate. The characteristic cycloartane glycosides formed a sodium adduct on the pseudo molecular ion in positive mode, plus a formic acid adduct in $ESI^-$ mode, if formic acid was present in the mobile phase or source. The structures of **compounds 4** and **7** that were isolated (in low yields) from the butanol extract (**Extract I**) were confirmed to be sutherlandioside A and D using NMR and UPLC-TOF-MS data analyses [1S,3R,24S,25-tetrahydroxy-7S,10S-epoxy-9,10-seco-9,19-cyclolanost-9(11)-ene 25-*O*-β-D-glucopyranoside (**4**) and 7S,24S,25-trihydroxycycloart-2-en-1-one 25-*O*-β-D-glucopyranoside (**7**)]. Although Fu and co-workers (2008) obtained higher yields of the cycloartane glycosides, it could be possible that the batches of plant material might be that of different subspecies, thus containing varying amounts of the compounds. Further investigations on different purification techniques for the various subspecies should be done, in order to investigate a better way of purifying these compounds to give higher yields and also to provide standards for the market. Mass directed fractionation (for the cycloartane glycosides) and/or counter-current chromatography (for the flavonol glycosides) might have provided for easier purification methods. The flavonol glycosides need to be handled with care due to their observed tendency to degrade. Higher volumes of extract material should ideally be subject to optimised purification techniques in order to obtain better yields of the resulting compounds.

From the comparison of the six chemical profiles of the *S. frutescens* samples (presented in **Tables 4.1** and **4.2**) it was clear that major differences between the various peaks in the

chromatograms were present. As such, this showed clear differences in the plant material collected from a single geographical location, supporting the morphological differences seen. These results point to the fact that there are significant chemical differences within this plant species, as was reflected in the plant material of the different cultivars, obtained from the same commercial supplier, which have manifested as different chemotypes. Based on the presence and amounts of **compound 5**, three similar groups were identified namely, **clone samples I** and **VI**, **clone samples III** and **V**, and **clone samples II** and **IV**.

On the basis of these initial findings, it is recommended that care should be taken when selecting *S. frutescens* plant samples for research and commercialization purposes, etc. For example, correct identification of subspecies needs to be done and commercial products need to be standardised on a single subspecies. Mixing of samples should be avoided. Documentation to trace back the source of the plant material should be well recorded. All future research should identify the plant material according to subspecies and database the related activities to avoid ambiguous information. Further investigations on the activities of the different chemotypes, together with a qualitative analysis of the compounds present, should be carried out in future, so as to obtain the most suitable chemotype to be used for subsequent cultivation, research and standardisation purposes.

# PART 2

# BIOLOGICAL INVESTIGATIONS

# CHAPTER 6

# MATERIALS AND METHODS

The use of medicinal plants and the investigation of their pharmacological activities is a very important aspect in drug discovery. Very often various scientific studies have found that a plant extract or isolated chemical compound from the plant has pharmacological activity which supports traditional uses. However, in order to provide scientific credibility on the plant extracts or isolated compounds' role in our immune system or on foreign microorganisms, along with their optimal dose and duration, further investigations on their pharmacological impacts need to be undertaken.

One of the ways to discover possible mechanisms associated with medicinal plants is via various pharmacological assay systems. Once protocols for various preparations of these plants have been established, an important aspect for the discovery of new drugs, for various diseases, would be to analyse these preparations for different kinds of biological activity. In this study, various biological activities were performed on the extracts of the aerial parts of *S. frutescens* prepared in Part 1. In particular, anti-cancer and cytokine release assays were performed using *in vitro* assay systems on the resulting extracts, fractions and compounds. This chapter presents a detailed discussion of the materials and methods used to biologically investigate the extracts prepared and compounds isolated from *S. frutescens*.

## 6.1 TISSUE CULTURE TECHNIQUES

Tissue culture is a general term used to cover methodologies for the removal of cells, tissues, or organs from an animal or plant and their successive placement into an artificial environment favourable for growth (Ryan, 2008). These methodologies form an important part of molecular biology and are crucial to building our understanding of a range of disease mechanisms and their subsequent treatment. According to Ryan (2008), these methodologies are also as important in forming an *in vitro* model system for rational drug discovery and

development. In this study, HL60 (human promyelocytic leukaemia) cells were used to study the effects of extracts made from *S. frutescens* on the cancer cell line and subsequent release of cytokines were measured. To extend the range of potential effects of selected extracts on other cancer cells, two additional cell lines, namely MCF7 and UACC62 were also used. Primary macrophage cells isolated from whole blood were used to determine toxicity.

### 6.1.1 Materials

RPMI-1640, Fetal Bovine Serum (FBS) and L-Glutamine were purchased from Whitehead Scientific. Gentamicin, Phorbol 12-Myristate 13-Acetate (PMA) and Etoposide standards were purchased from Sigma Aldrich. The anti-cancer screening assay involved Sulforhodamine B staining of treated cells developed by Skehan *et al.* (1990). The Cytometric Bead Array™ Human Inflammation Cytometric Bead array (CBA) kit (551811) was purchased from BD Biosciences and analysed using BD Biosciences' equipment and software. The protocol for the isolation of macrophages from whole blood was followed according to Rice and Hermann Laboratory protocols, which was performed by Dr. S. Barichievy[4].

### 6.1.2 Tissue culturing

#### 6.1.2.1 Pre-preparations of laboratory

Aseptic techniques were adhered to, as well as personal protective equipment was worn at all times during culturing of cells in the tissue culture laboratory. To minimise contamination of cell cultures, all work surface areas and materials were sterilised with 70 % ethanol before and after any procedure. Where appropriate, all materials used were sterilised by autoclaving. Cell culture work was performed in a Biosafety II cabinet equipped with a vertical laminar flow hood and UV light.

#### 6.1.2.2 Maintenance of cells

Commercially available cell lines, being HL60, MCF7 and UACC62 cells, originally isolated from leukaemia, breast adenocarcinoma and melanoma cancer patients, respectively, were used. The cancer cell lines UACC62 and MCF7 were obtained from the National Cancer Institute (NCI) in a framework of collaborative research program between the Council for

[4] Dr. Samantha Barichievy, Emerging Health Technologies, Biosciences, CSIR, Pretoria SBarichievy@csir.co.za

Scientific and Industrial Research (CSIR) Biosciences and NCI. The HL60 cell line was obtained from the European Collection of Cell Culture (ECACC). The macrophage cells obtained from the Gulf Coast Blood Regional Blood Centre are pathogen-negative leukopacks (which contain 50 ml leukocytes enriched from 450 ml whole blood).

The derived cell lines used in this study, as well as other isolated cells, required specialised environments for their successful culture and growth. The essential requirements for an optimum cell culture environment include temperature, pH, gas phases and media which contained necessary nutrients. For this study, RPMI-1640, supplemented with foetal bovine serum (FBS), L-glutamine and gentamicin was used for culturing of the cell lines. Glutamine is essential for cells that have a high energy demand and synthesise large amounts of proteins and nucleic acids. It is an alternative energy source for rapidly dividing cells that use glucose inefficiently. Although L-glutamine is synthesised by all normal cells, transformed cancerous cells are dependent on exogenous sources (ECACC handbook, 2006). Gentamicin was included to control bacterial contamination of the cultures.

Cell lines were routinely maintained, either as a monolayer cell culture (UACC62 and MCF7) or in suspension (HL60), at 37 °C in 5 % carbon dioxide ($CO_2$) and 100 % relative humidity in RPMI-1640 supplemented with 5 % FBS, 2 mM L-glutamine and 50 µg/ml gentamicin. Splitting of cells was done at least twice weekly. UACC62 cells are long elongated cells and can form round cells at confluence. They have a doubling time of 31.3hrs, while MCF 7 cells are small round cells and fast growing with a doubling time of 25.4hrs. HL60 cells have a doubling time of 28.6 hours. Sub-culturing was performed on the cell lines while growing in the exponential growth phase, which was dependent on the confluence of cells and their growth rate in each flask.

The process of sub-culturing for HL60 cells (in suspension) involved centrifugation (at 1 000g for 2 min.), to remove the media and subsequently splitting the cells into two new flasks with newly prepared RPMI-1640 media. A small quantity (100 µl) of the cell suspension was used for cell quantification and determining seeding density (see section 6.1.2.3 below). The process of sub-culturing for UACC62 and MCF7 cells (adherent cells) involved decanting of spent media, adding trypsin to detach cells from the surface and subsequently collecting the cells by centrifugation. The collected cells were then split into two new flasks with fresh culture media.

The preparation of primary human macrophages from the leukopacks was followed according to the standard Rice and Hermann Laboratory protocols and was performed by Dr. S. Barichievy. Thereafter, the viable macrophage cells were subjected to the standard protocols followed with the cancer cell lines. This included cell quantification and seeding density as well as the standard NCI protocol for cancer cell viability (see sections 6.1.2.3 and 6.2 below, pages 71 and 72).

#### 6.1.2.3 Cell quantification and seeding density

This was done with a haemocytometer and viewed under a light microscope (20x magnification). A 100 µl cell suspension was mixed with an equal volume of trypan blue and applied to the haemocytometer slide, where viable cells appear bright and dead cells are stained blue. To determine seeding density the following calculation was used:

**Total number of viable cells/ml = Average count per square x dilution factor x $10^4$**

**Dilution factor = 2 (volume of cell suspension and trypan blue 1:1)**

**$10^4$ = correction factor (supplied by manufacturer)**

**For seeding 10 000 cells/well in a volume of 100 µl = total number of viable cells/ml / 100 000**

#### 6.1.2.4 Cryopreservation of cells

For cryopreservation of cells, a flask was identified and viewed under the microscope to assess the degree of cell density and cell viability. Trypsinised cells were then sedimented at 1 000g for 2 min. to remove the culture medium which was then replaced with 6 ml of cryopreservation medium (10% DMSO in RPMI). One ml each of this suspension was aliquotted into sterile vials (approx. 1 000 000 cells / vial) and placed in the freezer at -20 °C for 2 to 3 hours, then subsequently removed and placed in the -70 °C freezer and finally in a liquid nitrogen container, to ensure a slow temperature decline to prevent cell damage. When cells were needed, they were quickly thawed from a cryovial and immediately placed in warm media and incubated overnight. The old media was removed after this incubation period and cells were placed in fresh RPMI-1640 media.

## 6.2 ANTI-CANCER AND MACROPHAGE CELL SCREENING

### Standard NCI Protocol for Cancer Cell Viability Assay

The SRB assay was performed in collaboration with the Pharmacology group at the CSIR in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute (NCI) (Monks *et al.,* 1991; Kuo *et al.,* 1993; Leteurtre *et al.,* 1994). Following the protocol developed by Skehan *et al.* (1990) as a standard NCI protocol for anti-cancer drug screening. Its principle is based on the ability of the protein dye sulforhodamine B to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions, the dye binds to the fixed cellular proteins, while under mild basic conditions; it can be extracted and solubilized for spectrophotometric measurement at 540 nm.

For screening purposes the cells (3 to 19 passages) were inoculated in a 96-well microtiter plate at plating densities of 7 to 10 000 cells/well and incubated for 24 hours. After 24 h, one plate was fixed with 50 % trichloroacetic acid (TCA) to represent the measurement of the cell population for each cell line at the time of drug addition ($T_0$). The other plates with cells were treated with experimental drugs which were previously dissolved in dimethylsulfoxide (DMSO) and diluted in media to the desired final test concentrations (100, 50 and 25 µg/ml). The final concentration of DMSO in the cell culture media of all of the wells i.e., control plus experiment did not exceed 1 % (v/v). This approach, with residual DMSO being present in the cell culture media, was in line with the protocol developed by Skehan *et al.* (1990).

Cells without drug addition served as a control. The blank contained complete cell culture media without cells. Etoposide was used as a positive control for the anti-cancer assay. The effect of PMA on the cancer cells was determined for a 6 and 48 hour incubation period at 12.5 and 6.25 ng/ml. The plant extracts were screened at 100, 50 and 25 µg/ml for a 48 hour incubation period. See **Table 6.1** below for plate setup for cancer cells and samples.

The plates were then incubated for 48 hours; viable cells were fixed to the bottom of each well with cold 50 % TCA (for UACC62, MCF7 and macrophages) and 80 % TCA (for HL60), for approximately 1 hour. The TCA was washed off with tap water and left to dry completely in the fume hood for 15 min. After this, the SRB dye (100 µl) was added to each well and left for 10 min. Unbound SRB dye was washed off with 1 % acetic acid and protein-

bound SRB dye was extracted with 10 mM Tris. The optical density was determined at a wavelength of 540 nm, using a multiwell spectrophotometer.

**Cell viability (V) was calculated with the following formula and expressed as a percentage:**

**% V = (T - blank) / (Ctrl - blank) * 100**

**Where T = Optical density of the test well after 48 hours of exposure to the test drug, and**

**Ctrl = Optical density of the control (untreated cells after 48 h incubation)**

The control (**Ctrl**) is set at 100 % viability of cells. For cells > 100 %, the drug favours cell growth and for cells < 100 %, the drug causes a reduction of proliferation of cells (cell death). $IC_{50}$ is found when a drug reduces the growth of the cells by 50 % less than the amount of the control. As this assay was based on a standard viability cell assay used by the NCI according to Skehan *et al.* (1990), no additional controls were included to determine the effect of any residual organic solvents that might have been present in the plant extracts. From a cell culture quality control perspective, the direct effect of DMSO [at 1 % (v/v) or less] on the cancer cells has previously been shown to have a negligible effect, as validated by NCI and the CSIR Pharmacology group. DMSO at higher concentrations has been shown to affect cell viability (Cheah *et al.*, 2009).

**Table 6.1 96-Well microtiter plate setup for cancer screen.**

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-No cells |
| **B** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-UACC62 |
| **C** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-UACC62 |
| **D** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-MCF7 |
| **E** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-MCF7 |
| **F** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-HL60 |
| **G** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-HL60 |
| **H** | Control | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | <-No cells |

## 6.3 QUANTIFICATION OF CYTOKINE RELEASE

Cytokines are small molecular weight messenger proteins that are produced by cells of the immune system in response to a stimulus (Lydyard, Whelan and Fanger, 2004; O'Gorman and Donnenberg, 2008). They mediate chemical communication between the cells as well as a variety of other induced functions, including modulation of many biological pathways controlling growth, differentiation, activation of cells and/or programmed cell death. Cytokine production in HL60 cells only was analysed in supernatants of cells cultured for 48 h with test samples using Cytometric Bead Arrays™ (BD Biosciences). The samples were analysed on the BD FACSCalibur™ platform which offered a unique modular approach to flow cytometry. The BD FACSCalibur flow cytometer was designed with multicolour applications to provide flexibility for a wide variety of research and clinical applications (www.bdbiosciences.com). The HL60 cells were counted and seeded (100 µl/well) at the recommended density (10 000 cells/well) in a 96-well microtiter plate and incubated for 24 h. Media was added to some wells as a control. The test samples were prepared in media for a final concentration of 25 µg/ml (in the wells) and added to the wells (50 µl). The plate was then incubated for 42 h. PMA at 12.5 ng/ml was used as a stimulant for some samples and only added to the 42 h plate for a 6 hour incubation period. Each well contained a final volume of 200 µl. See **Table 6.2** below for plate setup. After a total of 48 hrs incubation time, the plate was removed and centrifuged for 2 min. at 1 000g, supernatants (50 µl) from each well were removed and placed in Eppendorf tubes separately and frozen (at -80 °C) until use.

The IL12p70, TNF, IL10, IL6, IL1β and IL8 cytokines were detected using the human inflammation CBA kit (551811; BD Biosciences). Tests were performed according to the manufacturer's instructions. Briefly, 50 µl of the supernatants was mixed with 50 µl of all 6 cytokine capture beads (IL12p70, TNF, IL10, IL6, IL1β and IL8) and 50 µl of PE detection reagent (phycoerythrin-conjugated anti-human cytokine antibodies). The six bead populations are mixed together to form the BD CBA, which is resolved in a red channel (i.e. FL3 or FL4) of a BD flow cytometer. For each set of experiments, a standard curve was generated. After a 3 hour incubation period, samples were rinsed, resuspended in phosphate buffered saline (PBS) and analysed by flow cytometry (FACScan with the CBA Analysis Software; BD Biosciences). Flow cytometry was performed with a BD technician at University of Pretoria on a BD FACSCalibur flow cytometer. The results were expressed as pg/ml and then

analysed for their relative expression (control versus treated samples). The lower limit of detection for each cytokine was determined as 10 pg/ml.

**Table 6.2 96-Well microtiter plate setup for CBA.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | Drug11 | <-HL 60 |
| **B** | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | Drug11 | <-HL 60 |
| **C** | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | Drug11 | <-HL 60 |
| **D** | Control | Drug1 | Drug2 | Drug3 | Drug4 | Drug5 | Drug6 | Drug7 | Drug8 | Drug9 | Drug10 | Drug11 | <-HL 60 |
| **E** | PMA | Drug1 +PMA | Drug2 +PMA | Drug3 +PMA | Drug4 +PMA | Drug5 +PMA | Drug6 +PMA | Drug7 +PMA | Drug8 +PMA | Drug9 +PMA | Drug10 + PMA | Drug11 + PMA | <-HL 60 |
| **F** | PMA | Drug1 +PMA | Drug2 +PMA | Drug3 +PMA | Drug4 +PMA | Drug5 +PMA | Drug6 +PMA | Drug7 +PMA | Drug8 +PMA | Drug9 +PMA | Drug10 + PMA | Drug11 + PMA | <-HL 60 |
| **G** | PMA | Drug1 +PMA | Drug2 +PMA | Drug3 +PMA | Drug4 +PMA | Drug5 +PMA | Drug6 +PMA | Drug7 +PMA | Drug8 +PMA | Drug9 +PMA | Drug10 + PMA | Drug11 + PMA | <-HL 60 |
| **H** | PMA | Drug1 +PMA | Drug2 +PMA | Drug3 +PMA | Drug4 +PMA | Drug5 +PMA | Drug6 +PMA | Drug7 +PMA | Drug8 +PMA | Drug9 +PMA | Drug10 + PMA | Drug11 + PMA | <-HL 60 |

# CHAPTER 7

# RESULTS AND DISCUSSION

# FOR

# ANTI-CANCER AND CYTOKINE ASSAYS

The use of medicinal plants and the investigation of their pharmacological activities is a very important aspect in drug discovery. In order to provide preliminary scientific credibility on the role *S. frutescens* plays with its natural aspects, specific focus on the extracts, fractions and compounds of *S. frutescens*; with their influence on cancer cells and release in cytokines with phorbol 12-myristate 13-acetate (PMA) as a co-stimulant, as well as a brief look into the effect of one extract on macrophage cells was investigated. A variety of factors govern the study of medicinal values of plant extracts, including solutes used in the extraction procedure, the concentration of the extracts being applied, together with the exposure time (Ngcobo, 2008). As a result, a number of organic and aqueous extracts, prepared from *S. frutescens* in Part 1, with varying concentrations on cultured cells were investigated for biological activities and discussed in this chapter.

## 7.1 CANCER CELL SCREENING

In order to investigate the viability (**V**) effects of the extracts on the cancer cells in a 3-cell line panel consisting of UACC62 (melanoma), MCF7 (breast) and HL60 (leukemia) cells; the SRB assay was performed, in collaboration with the Pharmacology group at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI (Monks *et al.*, 1991; Kuo *et al.*, 1993; Leteurtre *et al.*, 1994). The SRB assay was developed by Skehan *et al.* (1990) to measure drug-induced cytotoxicity and cell proliferation. The discussion that follows below will look at selected extracts, fractions and compound's response on cell viability for each of the UACC62, MCF7 and HL60 cell lines.

### 7.1.1 Control

The graphs below (i.e., **Figure 7.1**) express the three cancer cell's viability (in percentage) relative to their control. The control (**Ctrl**) contains untreated cancer cells that undergo normal incubation conditions and its values are set as 100 % to represent their natural growth.

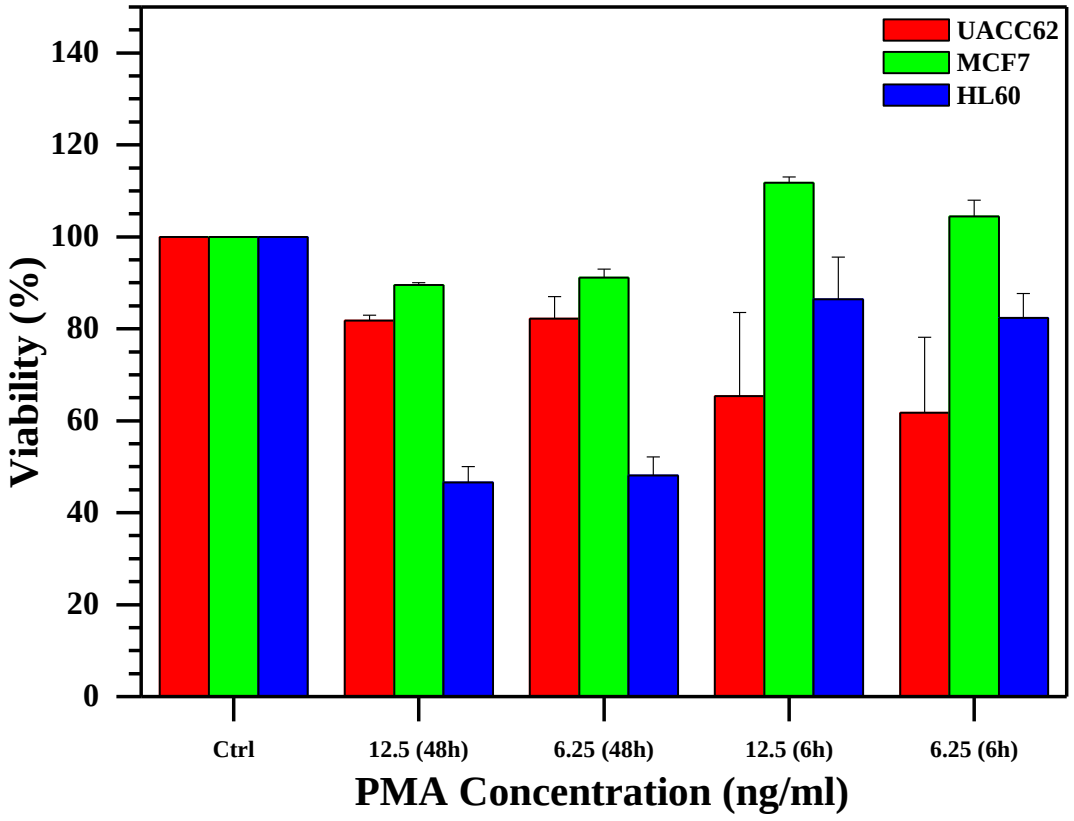



**Figure 7.1 Viability of UACC62, MCF7 and HL60 treated with PMA dilutions at 12.5 and 6.25 ng/ml over a 48 and 6 hour incubation period.**

### 7.1.2 Phorbol 12-Myristate 13-Acetate (PMA)

Varying the PMA exposure time on the cancer cells (i.e., for 48 and 6 hour incubation period) and concentration (i.e., for 12.5 and 6.25 ng/ml), its effect on the cancer cells viability has been studied and presented in **Figure 7.1** above. In this study, a marginal decrease in cell viability, when cultured in the presence of PMA, was observed. Irrespective of the concentration of PMA used on the target cells, no differential effect was observed in the assay system. Over the course of a 6 hour incubation period, the MCF7 cells were observed

to increase in viability, but when the culture system was exposed for a longer duration (48 hours), there was about a 10 % decrease in cell viability, irrespective of the PMA concentration. This could possibly be a hermetic effect exhibited by the MCF7 cells.

Both the UACC62 and HL60 cell lines decreased in cell viability for the 6 and 48 hour incubation periods at both concentrations of PMA. It was interesting to note that the effect of PMA on UACC62 cells increased the viability over a longer duration (i.e., at 48 hrs where **V** decreased only by about 20 %) as compared to the 6 hour incubation period (where **V** decreased by about 37 %) at both concentrations of PMA (6.25 and 12.5 ng/ml). This indicated that the UACC62 cells were able to develop some mechanisms that resisted the effect of PMA through time or that some of these cells contained a PMA resistance mechanism that allowed their survival with subsequent increase in total number per culture well over time. In the case of the HL60 cells, there was a reduction in cell numbers over time, with respectively 20 and 50 % decrease in cell viability for the 6 and 48 hour incubation periods (with an $IC_{50}$ on the HL60 cells, for the 48 hrs).

From these observations, PMA showed a time-dependant outcome on cell viability, rather than a dose-dependent consequence (**V** variation for time varied from 13 to 40 % and **V** variation for dose varied from 0.3 to 7 %). One of the limitations of this experiment was that it did not reflect the kinetics of cell growth (and death) over the full 48 h incubation period. As such, the kinetics of cell death were not followed throughout, therefore no conclusions can be made as to whether the increased cell growth at 48 h was due to a resistant clone of cells or whether a resistance mechanism was induced over this period to counteract possible toxic/negative agents in the medium.

PMA, which was used as a mitogen, has been known to inhibit MCF7 cell growth, but simultaneously stimulate cell survival (Fortino *et al.*, 2008). A study done by Wright and Meyer (1986) showed the binding affinity of soluble immune complexes (on phagocytosis-promoting receptors) was intensively decreased upon stimulation of polymorphonuclear leukocytes (PMN) with PMA. Wright and Meyer attributed this result to a dephosphorylation event initiated by PMA. Another study done by Bigby *et al.* (1990), illustrated the down-modulation of CD4 expression induced by PMA in various cell types, including the human lymphoblastic leukaemia cell line (MOLT-3), which resulted in a down regulation of an immune response and coincided with a decrease in cell viability. In addition, they indicated that PMA activated protein kinase C could cause a cascade of down regulation in the

expression of CD4 and CD8 cells, which are required for further initiation of an immune response and ultimately resulted in a decrease in cell viability. Responses obtained from the addition of the plant extracts on the cancer cells will be discussed in the following sections.

### 7.1.3 Effect of plant extracts on cancer cell growth

The experimental cancer cells that were exposed to selected preparations of *S. frutescens* showed either a decrease or increase in cell viability relative to the control. An increase in **V** above 100 % showed that the extracts stimulated cancer cell growth to a greater extent than that of the normal incubation conditions.

#### 7.1.3.1 Organic extracts

Ethanol and butanol extracts were tested for their anti-cancer response at 100, 50 and 25 µg/ml over a 48 hour incubation period, as discussed in Chapter 6 section 6.2 (page 72).

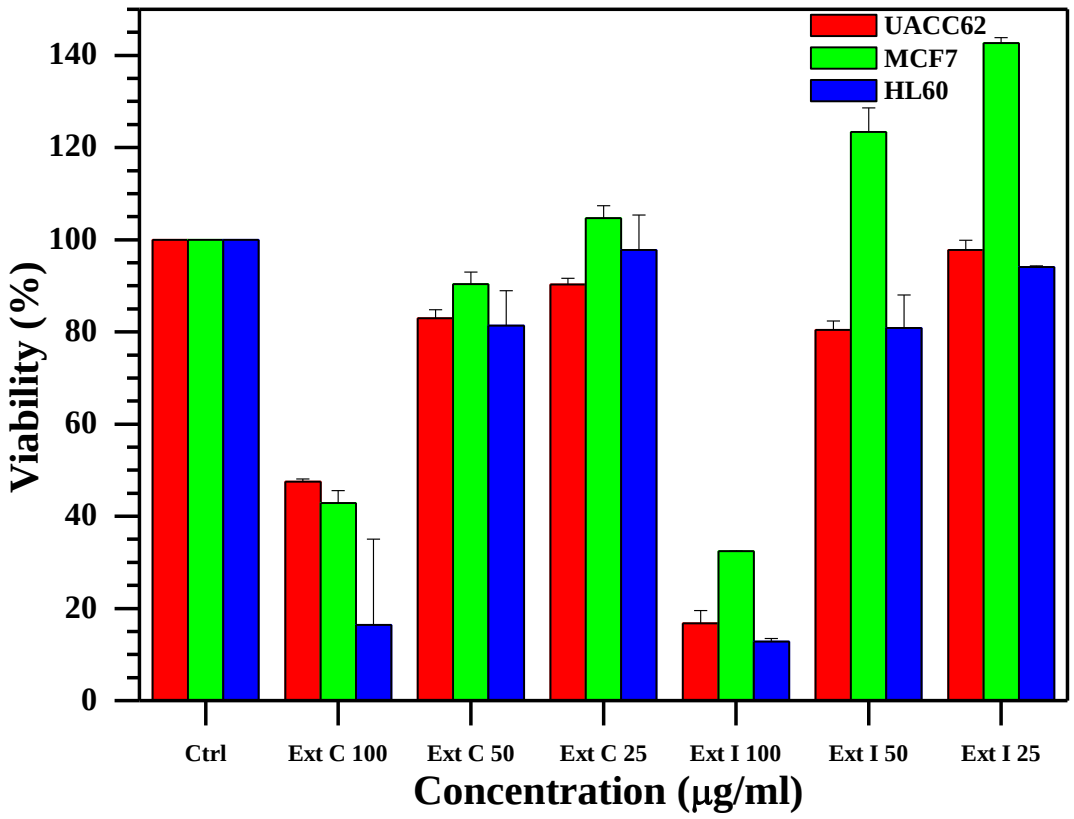



**Figure 7.2 The effect of extracts C and I on cancer cell viability at 100, 50 and 25 µg/ml over a 48 hour incubation period.**

In **Figure 7.2** an inversely proportional relationship was observed between increasing concentration of extracts and cell viability for both ethanol (**Ext C**) and butanol (**Ext I**) extracts. A variable sensitivity was observed for each cell line, with the HL60 cells being more susceptible to variations in concentration of the two extracts than that of MCF7 and UACC62 cells (with UACC62 being relatively the least sensitive).

Cells cultured in the presence of the ethanol extracts, irrespective of the concentration, displayed a decrease in viability, with the exception of MCF7 at 25 µg/ml (approx. **V ≈ Ctrl**), demonstrating the effective anti-cancer properties of this preparation. Stander *et al.,* (2007) reported that an ethanolic preparation of the leaves of *S. frutescens* inhibited cell growth of MCF7 cells with an $IC_{50}$ of 1.5 mg/ml over 24 hours, as well as throughout a 72 hour time-dependant study, with intervals of 24, 36, 48 and 72 h. Their morphological analysis demonstrated an increase in apoptotic cells, suggesting that *Sutherlandia* might induce its growth inhibitory effects through an initiation of apoptosis on the MCF7 cells.

Furthermore, the interpolation of experimental data points of the ethanol extract showed that for MCF7 and HL60 cell lines, the $IC_{50}$ corresponded to 93 and 74 µg/ml, being respectively, lower than those found by Stander *et al.* (2007). However, for UACC62 cells the $IC_{50}$ values were different for the ethanol (97 µg/ml) and butanol (75 µg/ml) extracts (the interpolation graph is not shown). Both extracts showed a large decrease in **V** (more than the $IC_{50}$) at 100 µg/ml, with the butanol extract being more toxic. This finding might be due to the presence of an increased concentration of cycloartane-type triterpenoid compounds, compared to the ethanol extract. These compounds are believed to have potent anti-cancer properties, as was shown in the study done by Kikuchi *et al.* 2007.

For all three concentrations of the butanol extract, there was an additional increase in MCF7 cell viability at 50 and 25 µg/ml, relative to the **Ctrl**. The increase in viability of MCF7 cells cultured in the presence of less saturated butanol extracts provided some evidence that the breast cancer cells were able to develop some regulating mechanisms that were able to promote their growth in the presence of the extracts over and above that of the normal environmental conditions. Kundu and co-workers (2005) have shown that a methanolic extract of *Sutherlandia* (at 100 and 200 µg/ml) inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cyclooxygenase-2 (COX-2) expression in mouse skin cells, thereby providing additional evidence that the extracts of *S. frutescens* exhibit anti-tumour potential.

Aqueous extracts, in this study, displayed a less toxic effect on cancer cell **V** at lower concentrations than the organic preparations, as can be seen in **Figure 7.3** below.

### 7.1.3.2 Aqueous extracts

A traditional extraction procedure was followed, resulting in **extracts A**, **B**, **D** and **K** which were prepared using different plant parts (leaves and stems) and comparing fresh to dry plant material (Part 1 pages 19 to 24). Customarily, a tea infusion of the dried leaves is used by the traditional healers. For comparative purposes, different extracts were prepared to help determine the most effective preparation with the most efficient biological activity for further use and further investigations. The influence of these extracts (at 100, 50 and 25 µg/ml) on different cancer cell's viabilities is shown below in **Figure 7.3**.

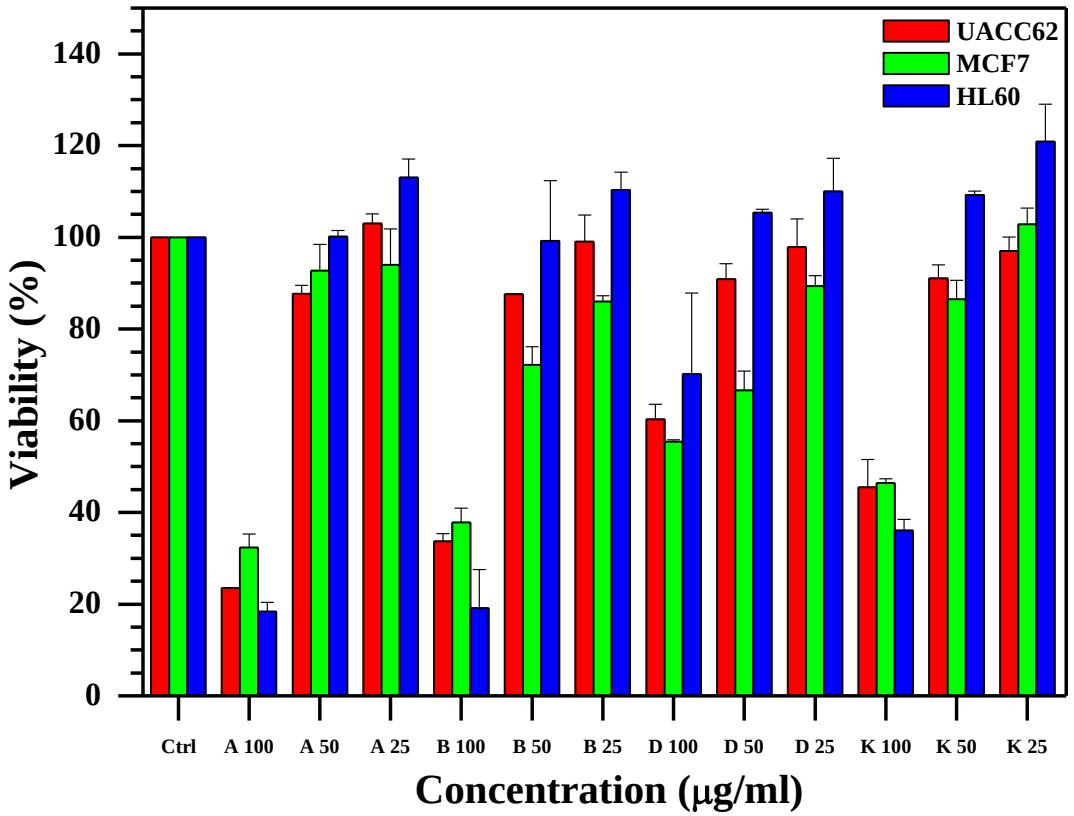



**Figure 7.3 The effect of extracts A, B, D and K on cancer cell viability at 100, 50 and 25 µg/ml over a 48 hour incubation period.**

For the aqueous extracts an inversely proportional relation was also observed between increasing concentration of extracts and cell viability. The aqueous extracts (**A**, **B**, **D** and **K**), at 25 µg/ml, all favoured the growth of the HL60 cells more than that of the other cancer cells (**V** = 113, 110, 109 and 120 %, respectively for HL60 cells). However, the aqueous extracts of the fresh plant material (**extracts A** and **B**) were found to be more toxic to the cancer cells at 100 µg/ml (showing on average a decrease in **V** for all three cell lines of about 75 and 69 % for **A** and **B** respectively) than the aqueous extracts of the dry plant material (**D**), which exhibited an average decrease in **V** by about 40 %. Aqueous extracts prepared from the fresh plant material were shown to have significant growth retarding effects on the cancer cell lines at concentrations of 100 µg/ml, irrespective of the part of the plant used (leaves or stems). At lower concentrations of the aqueous extracts (e.g. at 25 µg/ml) stimulation of cell growth was observed. Compared to the other two cell lines used in this study, the HL60 cells were found to be more sensitive to a change in concentration of the extracts. This might have been due to the nature of the cells, together with the greater solubility of the more polar compounds extracted when these extracts were prepared. In support of this contention, chemical profiles of the extracts shown in **Appendix 2** (page 122), displayed a higher concentration of polar compounds being extracted compared to their non-polar counterparts.

In contrast, there was a loss in anti-cancer activity in the aqueous extract of the dry plant material (**extract D**), compared to the aqueous extracts of the fresh plant material. This loss in activity could be due to the unstable nature of some of the active compounds extracted with water, as well as the natural drying process used in preparing these extracts, which might have changed the active chemical structures, resulting in a higher concentration of dormant compounds. The spray dried aqueous extract (**K**) in **Figure 7.3** was shown to be less toxic (with an average cell viability of around 42.6% for all three cell lines) compared to the aqueous extracts of the fresh plant material (which were freeze dried), but with slightly more potent effects than the freeze dried extract (**D**) at 100 µg/ml. A time and dose dependant study by Chinkwo (2005) showed differences in the anti-cancer potential of the aqueous extracts of *S. frutescens* on cervical carcinoma cell lines, but also noted differences in activity of plant material collected from different locations.

#### 7.1.3.3 *Echinacea* tincture (Ech)

*Echinacea* (see **Figure 7.4** below), which was used as a positive control for immune-modulating abilities on cultured cells, was also examined for its anti-cancer properties, to

compare with the *Sutherlandia* preparations. Etoposide, which was used as an anti-cancer standard, displayed an $IC_{50}$ at 46 µg/ml for the HL60 cells.

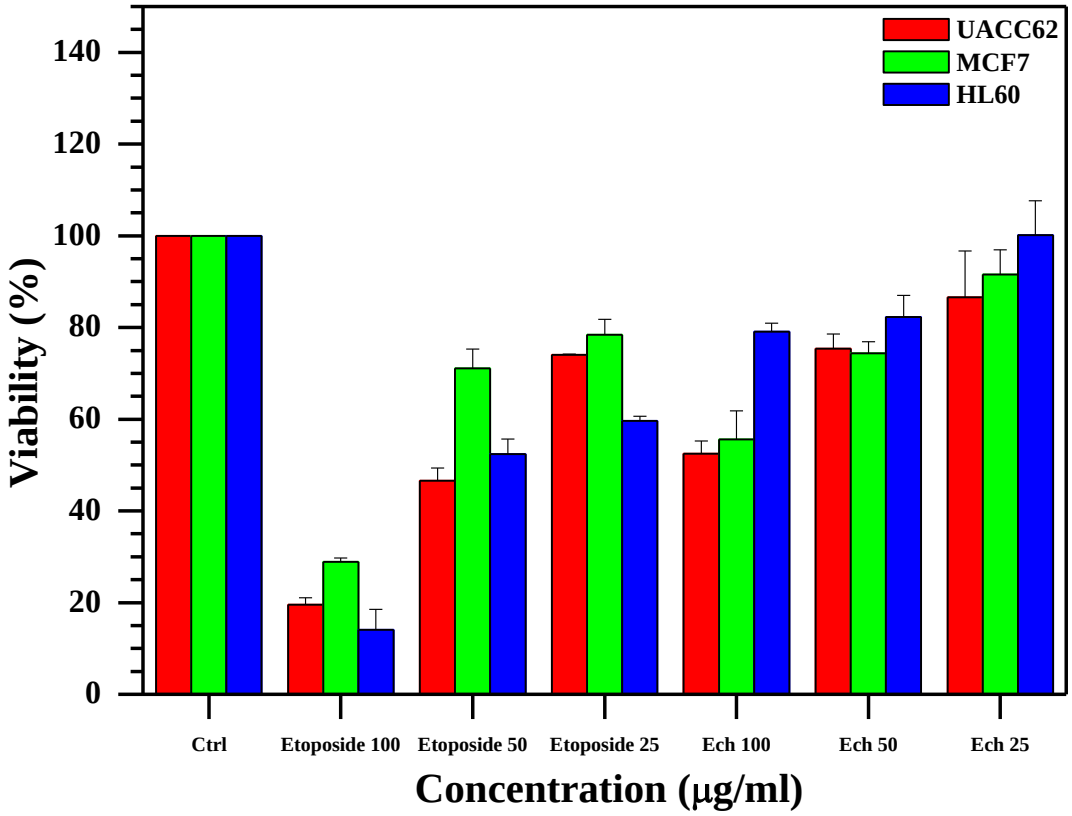



**Figure 7.4 The effect of etoposide and *Echinacea* on cancer cell viability at 100, 50 and 25 µg/ml over a 48 hour incubation period.**

As seen with the other traditional and organic plant preparations, *Echinacea* also exhibited a negative impact on cancer cell **V** as the concentration increased, which was also cell dependant. Nevertheless, its negative impact on cancer cell viability was lower than the *Sutherlandia* preparations. The impact of **Ech** on the HL60 cells was less than the other cancer cell lines (average **V** = 87 % at all three concentrations for HL60 cells). Also, the interpolation of experimental data points showed that for UACC62 and MCF7 cells the $IC_{50}$ values corresponded to about 100 µg/ml for both cell lines (the interpolation graph is not shown) higher than that of the *Sutherlandia* extracts.

The active compounds in *Echinacea* are known as alkylamides and are responsible for its immuno-modulatory properties (Raduner *et al.*, 2006). The activity of *Echinacea* could possibly be attributed to the interaction of the active alkylamide compounds with the cancer cells. From these results, it can be concluded that the *S. frutescens* preparations exhibited more potent anti-cancer potential compared to when the cancer cells were cultured with *Echinacea.* This finding could probably be due to the complex nature of the compounds present in *S. frutescens*, thereby providing further validation for the traditional uses of *S. frutescens* as an herbal remedy.

#### 7.1.3.4 Fractions

The ethanol extract was further fractionated into three fractions using a C-18 cartridge namely, **fractions I**, **II** and **III**, representing a polar fraction, a fraction containing triterpenoid-type compounds (which are less polar in nature than the polar fraction) and a fraction containing the non-polar compounds that are found in the ethanol extract (see Part 1 page 25 as well as **Appendix 3**, page 127 for chemical profiles). Further fractionation was performed in order to determine the effects of the different polarities of the compounds present in the ethanol extract on the three cancer cell lines at a standardised concentration of 25 µg/ml. A purified fraction (Chapter 2 section 2.2.1 page 25**, fraction IV**) comprising of four triterpenoid (cycloartane) compounds in major quantities and **compounds 4** and **7** in minor quantities, as well as purified flavonoid **fractions V** and **VI** were also investigated at 5 µg/ml for their anti-cancer activities.

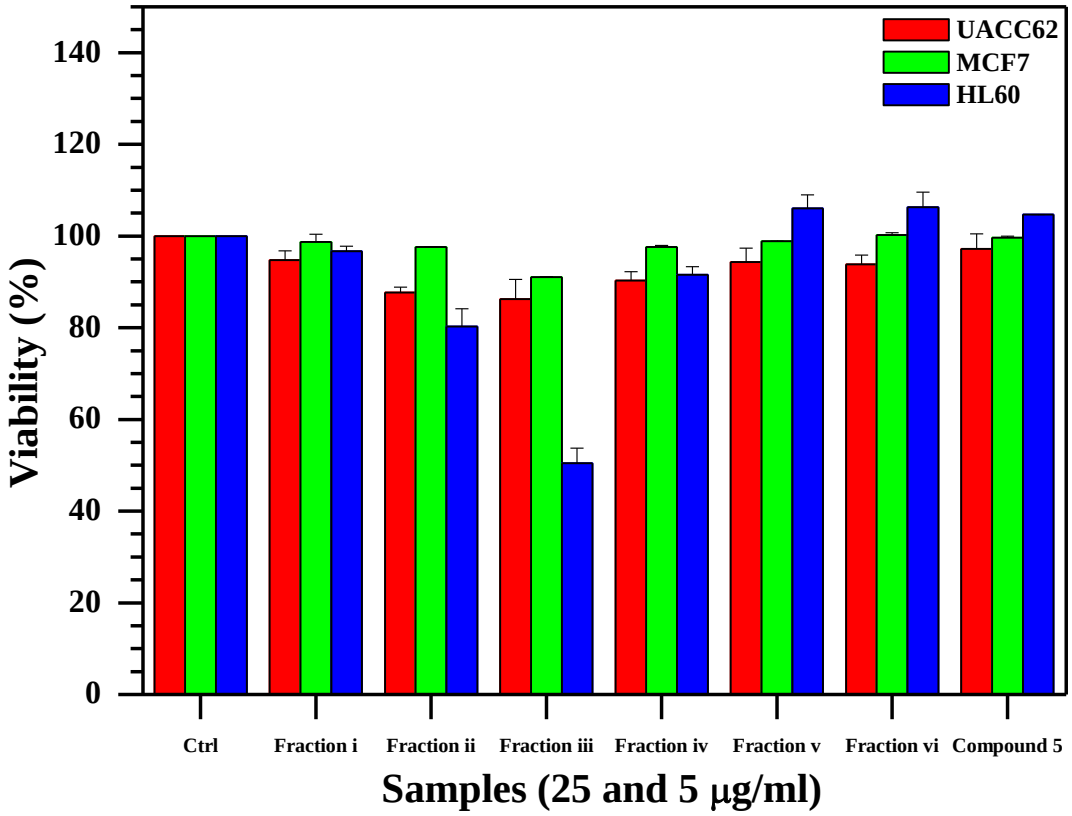



**Figure 7.5 The effect of fractions I to III (at 25 µg/ml), IV to VI (at 5 µg/ml) and compound 5 (at 5 µg/ml) on cancer cell viability over a 48 hour incubation period.**

The results of **fractions I**, **II** and **III** showed that a decrease in polarity of the compounds, found in the ethanol extract, also resulted in an increase of anti-cancer potential. On average the viability decreased by about 4 and 12% for the different cell lines for **fraction I** and **fraction II**, respectively. The non-polar compounds in **fraction III** (from the ethanol extract) exhibited an $IC_{50}$ at 25 µg/ml for HL60 (more potent than that of etoposide which has an $IC_{50}$ at 46 µg/ml), whereas UACC62 and MCF7 cells demonstrated only a 14 and 9 % decrease in cell viability, respectively. When **extract C** was further fractionated, inhibitory effects of the compounds present in these fractions and the role these compounds play in the extract as a whole on the cancer cell lines could be observed. As such, this approach would help to establish if there was any chemically-mediated selection mechanism on the part of the cancer cells, for increasing their survival rates. This becomes apparent if effects of the fractions, on the three cell lines, are compared to the effects of the non-fractionated ethanol extract (see

**Figures 7.2** – **extract C** and **7.4** – **fraction I, II** and **III**) - along with the aid of the chemical profiles in **Appendix 2** page 122 and **Appendix 3** page 126. There is an apparent dominant effect of the polar compounds (**fraction I**) over the non-polar compounds (**fraction III**), which could be responsible for the observed activities of the original non-fractionated ethanol extract (i.e., the polar compounds inhibit the activity of the non-polar compounds present in the ethanol extract).

Purified **fraction IV** (at 5 µg/ml) had a similar effect on the three cancer cell lines as did **fraction II** (at 25 µg/ml) and the butanol extract. Purified **fractions V** and **VI** (at 5 µg/ml) exhibited a similar effect on these cells as for **fraction I** (at 25 µg/ml) and the aqueous extracts (**V ≈ Ctrl**). Thus, these observed effects could be related to the compounds (cycloartane- and flavonol- glycosides) present in the fractions and extracts.

The interaction of the flavonol-type compounds found in **fractions I**, **V** and **VI** with the cancer cells, might be responsible for the increase in cancer cell viability comparable to that found with the aqueous extracts (see **Figure 7.3**, page 81). A synergistic effect of the cycloartane glycosides, on the decrease in cancer cell viability for HL60 and UACC62 cells, was observed at low concentrations; however MCF7 cells did not seem to exhibit a suppression of growth related to their survival in the presence of these compounds (**V ≈ Ctrl**). It has already been demonstrated that the triterpenoids (cycloartane glycosides) were found to be more potent anti-cancer agents than the flavonol-type compounds (as shown in **Figure 7.5**, page 85).

**Figure 7.5** also depicts the cancer cell viability response for **compound 5**, which displayed a slight increase in viability (**V = 104.7 %**) for HL60 cells, while MCF7 and UACC62 display on average a **V = 98.4 %**. A study done by Kikuchi and co-workers (2007), demonstrated the potent chemo-preventative effects of cycloartane-type triterpenoids on Epstein-Barr virus early antigen inhibition in TPA-induced Raji cells. The most potent inhibitory effects were found with the compounds that had a hydroxylated side chain on C-24 and a 3-oxo group. This configuration was found to be present in **compounds 4, 5 and 6** of *S. frutescens*. Although at a low concentration of **compound** 5, a toxic chemo-preventative effect was not observed (**Figure 7.5**).

#### 7.1.3.5 Summary

In general, excluding **fraction III** for HL60 cells, the results showed that at 50 or 25 µg/ml, none of the extracts exhibited a profound impact on cancer cell viability; since the **V ≥ 70 %**, (i.e., these results did not show an Inhibitory Concentration of 50 %; $IC_{50}$). These results therefore indicate that most of these extracts would not be effective as anti-cancer agents at these particular concentrations. The traditional extracts (**A**, **B**, **D** and **K**), have shown a greater tendency to favour the growth of the leukaemia cells at lower concentrations, compared to the organic extracts - which supported the growth of breast cancer cells. The non-polar compounds present in the ethanol extract displayed more potent anti-cancer activities than that of etoposide and could therefore be further examined for potential anti-cancer drug applications against leukaemia. The triterpenoid and non-polar compounds present in *S. frutescens* were shown to have effected greater reduction in viability of cultured cancer cells than the flavonol glycosides (polar region), which are said to have anti-oxidant properties, rather than anti-cancer potential. In general, further targeted purification of selected fractions could result in more potent compounds being found. However the purification procedure needs to be optimised in order to obtain sufficient amounts of resulting compounds.

## 7.2 QUANTIFICATION OF CYTOKINE RELEASE

In order for immune cells to work effectively they need to be recruited to the sites of inflammation and appropriately activated. This is achieved by cellular receptors and associated cytokines that bind to these receptors. Cytokines are small molecular weight messenger proteins that are produced by cells of the immune system in response to a stimulus. They mediate interactions between cells, for example, communication between cells, may induce or suppress growth, differentiation and activation of cells, along with a variety of other functions. Generally, their role in health and diseases provides a key to the physiological importance of cytokines in cell-to-cell communication and their ability to regulate a wide spectrum of biological functions, including an immune response (Lydyard, Whelan and Fanger 2004; O'Gorman and Donnenberg 2008; Parkin and Cohen, 2001).

Cytokines can be classified according to the cells that produce them. These include interferons (IFN), tumour necrosis factor (TNF), interleukins (IL), lymphokines and

chemokines, which regulate the response to inflammation via the various cells of the immune system and their associated response to infections. Moreover, changes in cytokine profiles may serve as indicators of pathological conditions associated with diseases. Although the role of cytokines in the pathogenesis of human diseases is not yet fully understood, assays for cytokines have become a common feature in research and clinical laboratories (Lydyard, Whelan and Fanger, 2004; O'Gorman and Donnenberg, 2008; Parkin and Cohen, 2001).

With this background in place, a similar approach was followed to characterise the biological activity of selected extracts from *S. frutescens.* As such, this part of the study was focussed on measuring the effects of extracts made from *S. frutescens* on cytokine release by a HL60 cell line at 25 µg/ml. The HL60 cell line was chosen due to its related characteristics with white blood cells associated with cytokines being released during an immune response (IR) to an invading pathogen. The amount of different cytokines released from these cells was measured using a commercial Cytometric Bead Array™ (BD Biosciences) kit. This kit was specifically developed to measure amounts of inflammatory cytokines, such as of IL1β, IL6, IL8, IL10, TNF and IL12p70, in the supernatants of stimulated immune cells. In this model system, an enhanced or suppressed plant extract driven trigger for cytokine release from HL60 cells *in vitro* would then be expected to parallel a similar situation of cytokines being released from counterpart immune cells *in vivo*.

In order to control for cell viability factors, which may have influenced the outcome of cytokine release by stimulated cells, the results of cytokine release were also correlated with the cancer cell viability effects of *S. frutescens* extracts on cell lines UACC62, MCF7 and HL60 (see section 7.1 for cell viability and section 7.3 for cytokine and cell viability relation, pages 76 and 100). In order to simplify reference to the various plant extracts used from *S. frutescens* in this part of the study, extracts were numbered as followed:

**Table 7.1 New codes representing the extracts, fractions and compound 5 tested in the assay for the release of cytokines.**

| Number | Extract code |
|---|---|
| **1** | Control (**Con.**) |
| **2** | PMA (12.5 ng/ml) |
| **3** | Ethanol (**extract C**) |
| **4** | **C** + PMA |
| **5** | Fresh leaves (**extract A**) |
| **6** | **A** + PMA |
| **7** | Fresh stems (**extract B**) |
| **8** | **B** + PMA |
| **9** | Dry leaves (**extract D**) |
| **10** | **D** + PMA |
| **11** | Butanol (**extract I**) |
| **12** | **I** + PMA |
| **13** | DCM:MeOH (**extract E**) |
| **14** | **E** + PMA |
| **15** | **Fraction I** |
| **16** | **I** + PMA |
| **17** | **Fraction II** |
| **18** | **II** + PMA |
| **19** | **Fraction III** |
| **20** | **III** + PMA |
| **21** | *Echinacea* (**Ech**) |
| **22** | **Ech** + PMA |
| **23** | 24 hour incubation **extract C** |
| **24** | 24H **C** + PMA |
| **25** | **Compound 5** Std. |
| **26** | **5** + PMA |
| **27** | **Fraction IV** |
| **28** | **IV** + PMA |
| **29** | **Fraction V** |
| **30** | **V** + PMA |
| **31** | **Fraction VI** |
| **32** | **VI** + PMA |

The cytokines IL1β, IL6, IL10, IL12p70, IL8 and TNF were tested in this assay due to their role in various pro- & anti-inflammatory actions involved in the first step of the IR i.e. inflammation. The subsequent graphs below present the amounts of the six cytokines released by the samples in the table above.

### 7.2.1 The release of IL1β, IL6, IL10 and IL12p70

The graphs below graphically present the effect the *S. frutescens* samples had on the release of IL1β, IL6, IL10 and IL12p70. The control (1) represented untreated HL60 cells and their

baseline level of cytokines being released into the cell culture supernatant under normal growth conditions i.e. without any additional chemical stimulation.

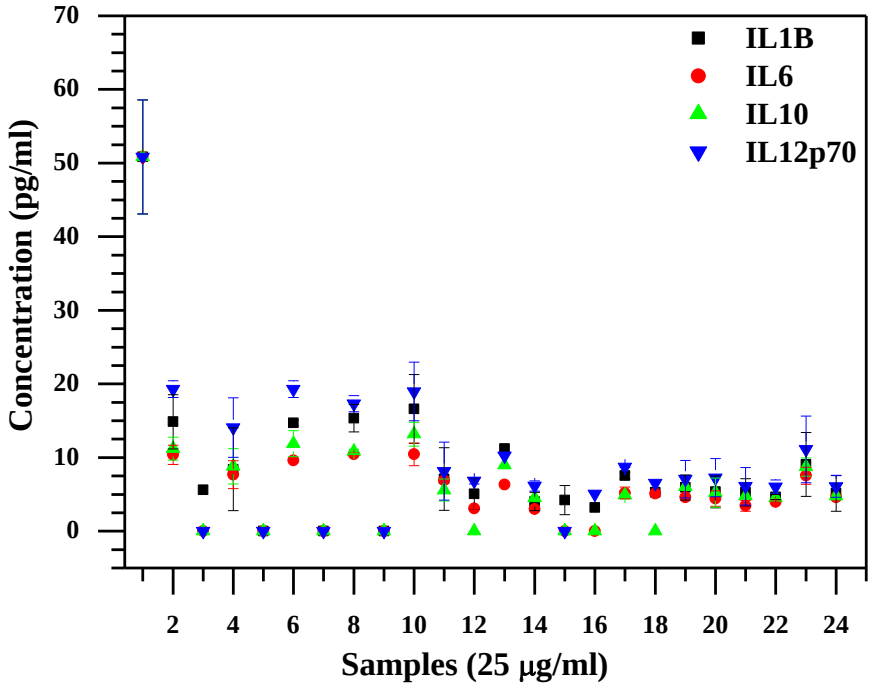



**Figure 7.6a Release of IL1β, IL6, IL10 and IL12p70 cytokines for extracts and fractions I to III at 25 µg/ml for a 48 hour incubation period.**

In **Figure 7.6b** below, the reduced amount of cytokines IL1β, IL6, IL10 and IL12p70 (which vary from about 3 to 20 pg/ml) being released into the culture supernatant was also observed for samples 25 to 32 (**fractions IV**, **V**, **VI** and **compound 5**) similar to that of the extracts in **Figure 7.6a** above.

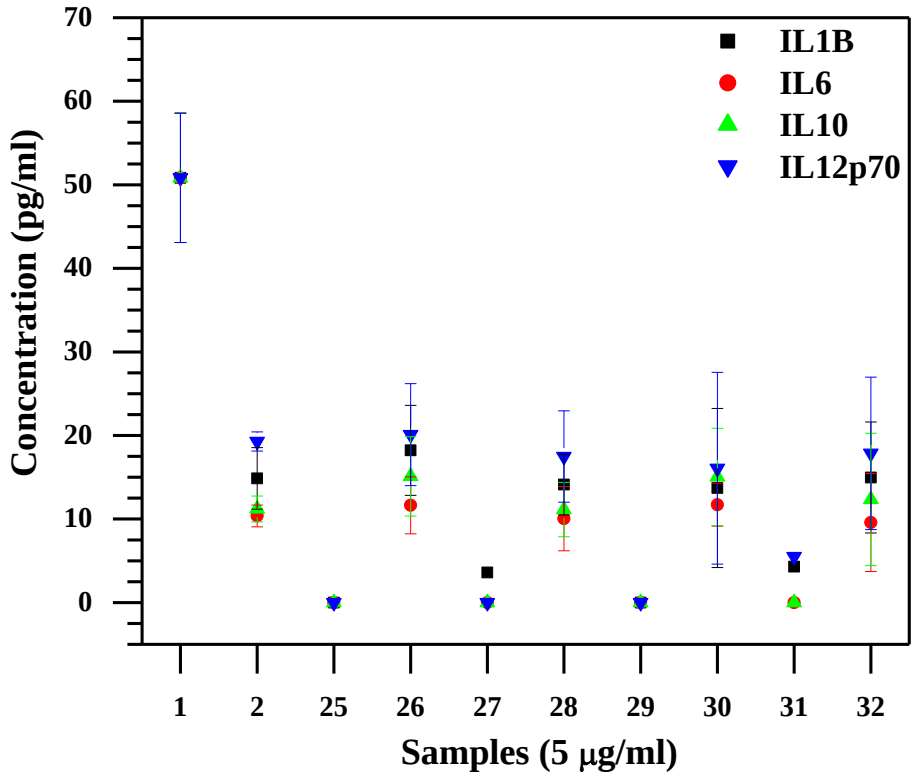



**Figure 7.6b Release of IL1β, IL6, IL10 and IL12p70 cytokines for fractions IV, V, VI and compound 5 at 5 µg/ml for a 48 hour incubation period.**

The above graphs reflect the role the samples play in the release of four of the cytokines (IL1β, IL6, IL10 and IL12p70). Similar amounts of IL1β, IL6, IL10 and IL12p70 were released into the culture supernatant, relative to the control supernatant which had a concentration of 50.83 ± 7.74 pg/ml after a 48 hour incubation period. By contrast, when HL60 cells were exposed to different extracts from *S. frutescens*, there was a much lower amount of these cytokines released into the culture supernatant compared to the control and varied from 3 to 40 pg/ml. In addition, it was also observed that the *S. frutescens* samples, together with PMA, did not affect subsequent release of the four cytokines in the culture system. A study done by Ngcobo (2008) showed that high concentrations of aqueous and ethanol extracts of *S. frutescens* could reduce the production of IL1β and TNFα, which are regarded as being helpful in fighting muscle wasting in cancer and HIV/AIDS patients. However, where TNF and IL8 were concerned, there was a difference in the amounts released with and/or without co-stimulation by PMA.

It was observed that only in the cases when PMA was added to the cell culture system was there a higher production of IL8 and improvement of TNF being released into the supernatant. In light of these observations, the main focus of this discussion will be centred around the synergistic effects of the extracts of *S. frutescens*, co-stimulated with PMA, in HL60 cells on the subsequent release of IL8 and TNF cytokines.

### 7.2.2 The release of TNF and IL8 induced by PMA

Mitogens (PMA) are often used to stimulate lymphocytes and therefore can be used to assess immune responses in cells. According to Fortino *et al.* (2008) PMA was found to have either growth stimulatory or inhibitory effects on cultured cells, depending on which kind of cell was involved, including certain cancer cells. PMA has also been shown to induce expression of IL8, which in turn, can inhibit cell growth of PMA-induced HL60 cells (Oda *et al.*, 2007). By this proposed mechanism, PMA could cause cells to undergo mitosis, thereby causing them to differentiate into the appropriate effector cells required for an appropriate IR. This kind of IR is usually exacerbated by the Pattern Recognition Receptors (PRR) present on the HL60 cells. This recognition mechanism is antigen-specific for a particular immunoglobulin released, but can also activate B cells, regardless of their antigenic specificity. The effect of PMA on the HL60 cells, after 6 hours of co-incubation, showed a decrease in cell viability, which could possibly be attributed to associated toxic effects of PMA itself, together with the lack of a proper Antigen Presenting Complex (APC) in the HL60 cell culture system used in this study, therefore resulting in a non-specific activation of anti-toxic defence mechanisms.

As shown below in bar line 2 of **Figures 7.7** and **7.8** (pages 95 and 97), PMA co-stimulated the release of TNF (130 ± 19.21 pg/ml) and IL8 (4552 ± 410.85 pg/ml) well above that of the baseline level. PMA forms part of the lipopolysaccharide (LPS) of Gram negative bacterial cells and can therefore, also be recognised by PRR present on the target cells used in this study. As such, the expression of TNF is stimulated by LPS interaction with PRR on the HL60 cell line. TNF activation is subsequently coupled with programmed cell death (apoptosis) initiated by infection with a particular pathogen and also coincides with release of inflammatory mediators which stimulate recruitment of other immune cells to the site of infection (Lydyard, Whelan and Fanger, 2004). The highest production of IL8 expression was found to be induced by interaction of PMA with PRR in monocytic cells, together with activated release of TNF. In general, this mechanism is believed to help the immune system to increase chemotaxis for neutrophils (as well as for T cells and basophils) at the site of

inflammation. With this principle in place, responses obtained from the addition of the plant extracts on the HL60 cells, with their subsequent cytokines released, will be discussed.

### 7.2.3 Amounts of TNF and IL8 released by *S. frutescens* extracts with co-stimulation of PMA

The following section presents the amounts of TNF and IL8 released in the presence of the samples 1 to 32. TNF release can be seen in **Figures 7.7a** and **b**, while IL8 release is presented in **Figures 7.8a** and **b** below, where all even numbers represent the samples with the addition of PMA and odd numbers represent the samples alone (**Table 7.1**, p**age 89**).

No significant increment in the release of all six cytokines (i.e., initiation of an immune response) was observed when the extracts of *S. frutescens* were applied to the HL60 cells without the co-stimulation of PMA. These observations could be related to the presence of GABA and pinitol (compounds 3 and 2, pages 10 and 9) found in *Sutherlandia* species. These compounds have been said to contribute to the improvement of wasting conditions experienced by cancer and HIV/AIDS patients, by the inhibition of the release of certain pro-inflammatory cytokines, specifically TNF and IL1β (Tai *et al.*, 2004; Ostlund and Sherman, 1996; Mombereau *et al.*, 2004).

However, when the HL60 cells were co-stimulated with PMA, the *S. frutescens* extracts exhibited a greater release of TNF and IL8, relative to the baseline levels. Thus, the results below highlight the variation of TNF and IL8 concentrations relative to the baseline level for the release of cytokines by the HL60 cells, particularly when co-stimulated with PMA.

#### 7.2.3.1 Amounts of TNF release

**Figures 7.7a** and **b** below depict the differential release of TNF co-stimulated by PMA in the presence of extracts of *S. frutescens.* In all the cases where the extract, together with PMA was added to the cells, a resultant increased release of TNF was observed. This contrasted with the situation of TNF release by the cells cultured in the presence of PMA without extract being present. As such, the effect of *S. frutescens* extracts, on the target cells, was seen to occur mainly in association with extracts plus PMA. In essence, this observed effect, together with the release of specific cytokines into the culture supernatant, could be taken to mimick a possible *in vivo* situation of a bacterial infection in a given host, being combated by the release of appropriate cytokines. Sample number 20 (**fraction III** + PMA) exhibited the greatest increase in the release of TNF (approx. 230 pg/ml).

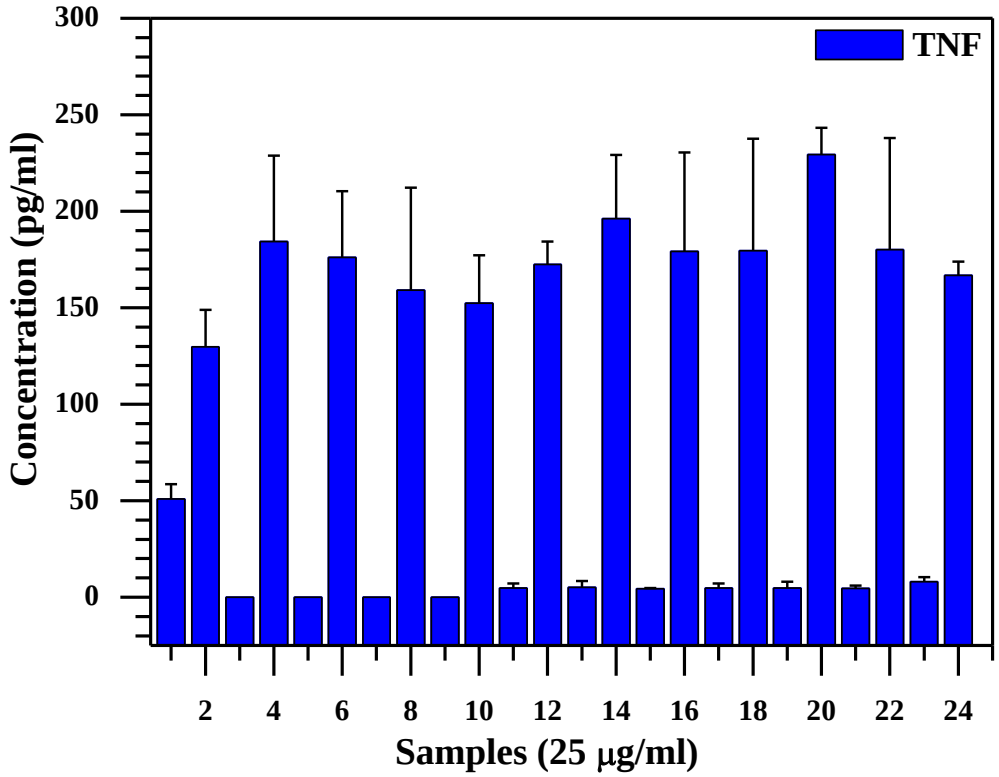



**Figure 7.7a Release of TNF for extracts and fractions I to III at 25 μg/ml for a 48 hour incubation period.**

As with the similar pattern of the extracts as mentioned above (**Figure 7.7a**), it was observed that co-stimulation with PMA, resulted in an increase in the amount of TNF released with samples 25 to 32 (**fractions IV**, **V**, **VI** and **compound 5**) in **Figure 7.7b** below.

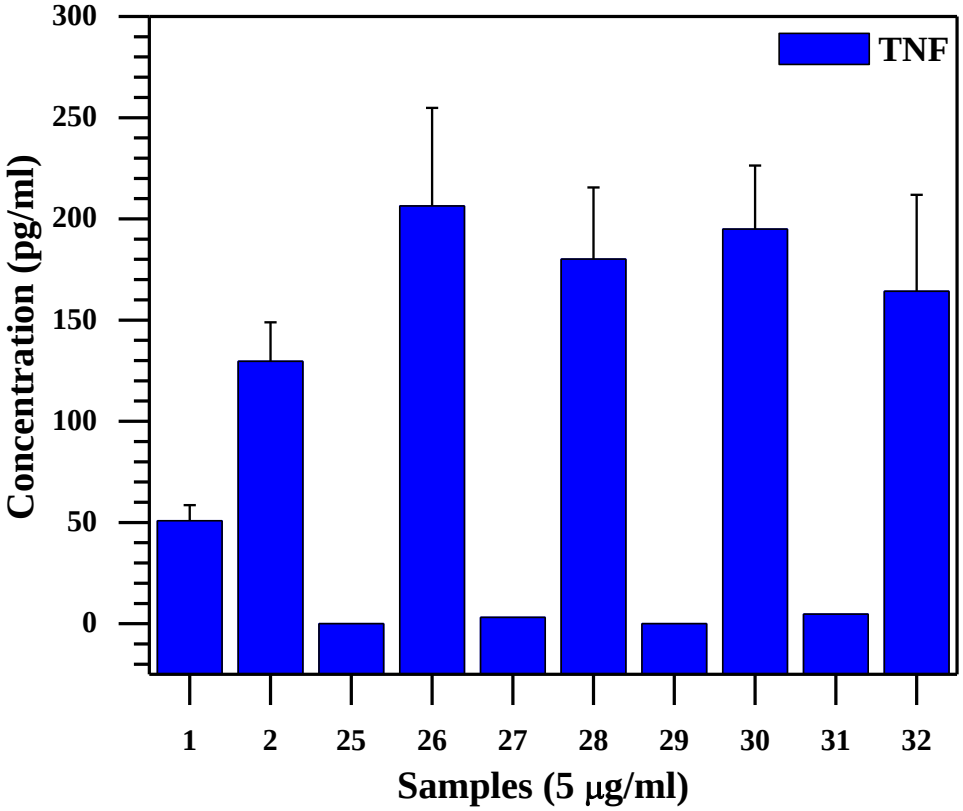



**Figure 7.7b Release of TNF for fractions IV to VI and compound 5 at 5 μg/ml for a 48 hour incubation period.**

The presence of PMA in the culture system coincided with the release of TNF (130 ± 19.21 pg/ml), which might have been due to its related characteristics connected with a bacterial infection. All *S. frutescens* samples did not exhibit much of a response by themselves on subsequent TNF release by the culture system, but when PMA was present, a significant increase in the release of TNF was found.

Addition of samples **26** to **32** to the cell culture system resulted in more TNF being released, similar to that obtained with **fraction III**, compared to other samples, but at a much lower relative concentration. The flavonoid fractions displayed a similar response in the *in vitro* culture system as was obtained with the aqueous extracts, together with PMA. As such, this activity could possibly be related to the presence of flavonol glycosides and their polar interactions with cells of the *in vitro* cell culture system. From these observations, it can

therefore be concluded that all compounds present in *S. frutescens* have an additive effect on the amount of TNF being released by the cultured cells in this system. This has provided further preliminary evidence to support the contention that extracts from *S. frutescens* could amplify the release of specific, immune-modulating cytokines by cells already stimulated by a pathogenic microorganism, such as a gram negative bacterium. As such, these extracts were observed to have an additive effect in helping the cells release more of a particular cytokine, thereby further assisting the elimination of a potential invasion with a bacterial pathogen. These results also suggested that the cultured cells needed to be intrinsically releasing certain cytokines, so that when the *S. frutescens* extracts were subsequently added to the HL60 cells, they were able to up regulate and release more of the particular cytokines involved in the IR.

#### 7.2.3.2 Amounts of IL8 released

A similar trend of IL8 release, as for the release of TNF, just discussed, is depicted in the series of graphs (**Figures 7.8a** and **b**) below. The release of IL8 induced by the presence of the plant samples in the cell culture system together with PMA are represented by even numbers in the graphs below, while odd numbers represent the effect on the cultured cells in the presence of the samples without PMA (**Table 7.1**, page 89).

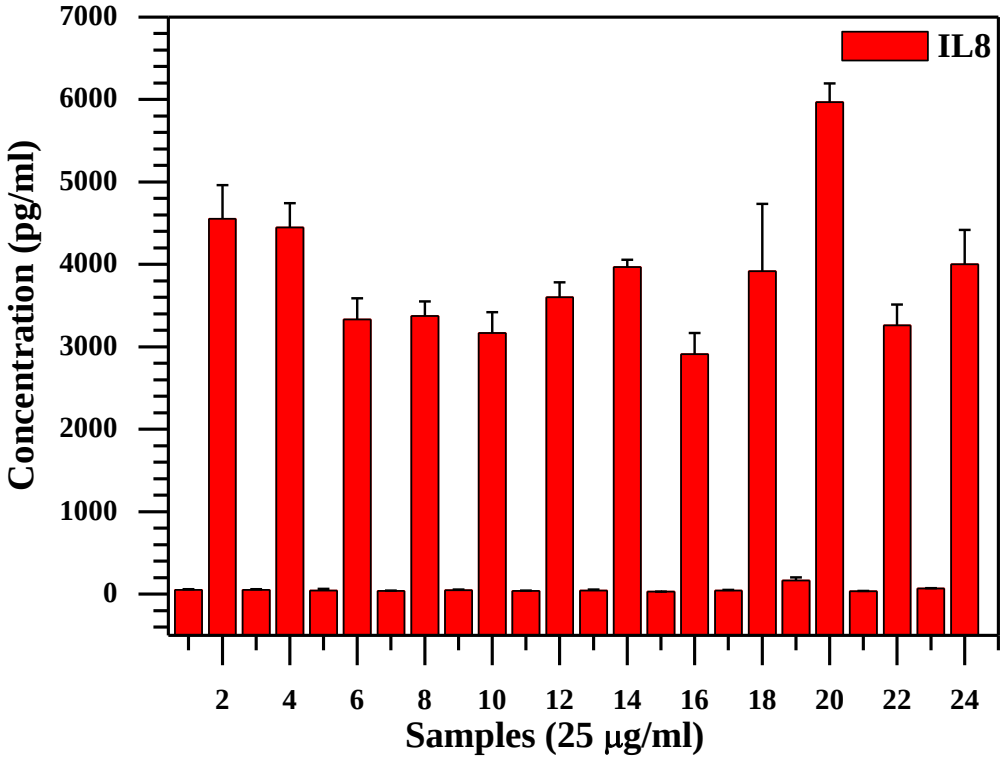



**Figure 7.8a Release of IL8 for extracts and fractions I to III at 25 μg/ml for a 48 hour incubation period.**

As with TNF release, the highest amount of IL8 released was caused by sample number 20 (**Fraction III** + PMA), which showed a release of 5967.93 ± 226.86 pg/ml. The release of IL8 caused by sample numbers **25** to **32** are depicted below.

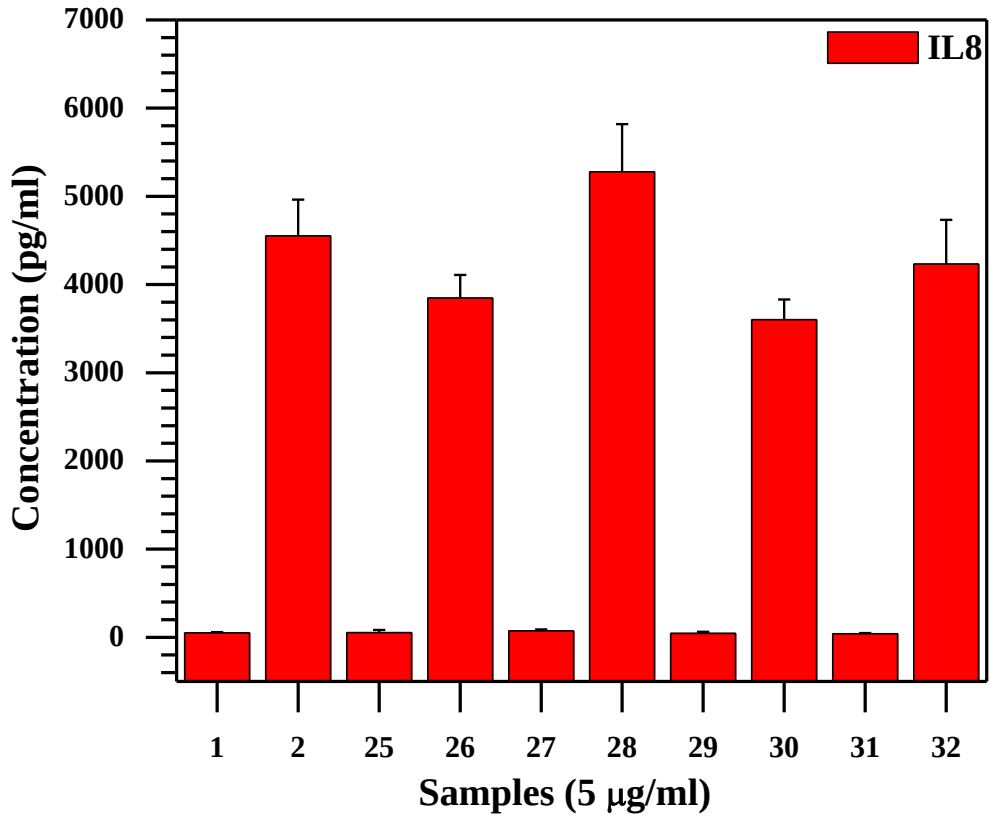



**Figure 7.8b Release of IL8 for fractions IV to VI and compound 5 at 5 μg/ml for a 48 hour incubation period.**

An increase in IL8 will help in the recruitment of immune cells to the site of invasion by a pathogen in the process called chemotaxis. All of the test samples displayed a increase in the amounts of IL8 released relative to that of the control (1), however the amounts were less than that of the cells co-stimulated by PMA (number 2) alone (except number 20). The aqueous preparations (numbers 6, 8 and 10) and polar fractions (numbers 16, 30 and 32) displayed lower amounts of IL8 released, compared to the effect of the organic extracts. In comparison, sample number 20 (fraction containing non-polar compounds present in the ethanol extract in conjunction with PMA) and 28 (triterpenoid fraction with co-stimulation of PMA) caused a greater cytokine release. From the chromatogram of **fraction IV** (see **Appendix 3** Figure 3.3, page 128) there was evidence for some non-polar and triterpenoid compounds occurring in this fraction, which might have caused a synergistic effect in

enhancing the response of the *in vitro* model system. The cycolartane glycosides, in a purified form, demonstrated a slightly lower amount (as seen with the response of **compound 5**). The results from Tian and co-workers (2005) implied that triterpenoids have potential anti-tumour properties by exerting their cytotoxic effects via apoptosis and G2/M cell cycle arrest.

Addition of **Fraction V** to the culture system caused a release of IL8 at a concentration of 3601.96 ± 229.4 pg/ml, while **fraction VI** showed a release of IL8 at a concentration of 4231.76 ± 500.79 pg/ml. **Fractions V** and **VI** could thus be of value, boosting the IR, particularly when invading microorganisms need to be driven off, but they were not found to be as effective as the non-polar or triterpenoid compounds present in *S. frutescens,* due to the lower expression of IL8, which is necessary for chemotaxis of the immune cells, such as neutrophils, to the site of infection (Lydyard, Whelan and Fanger, 2004).

A study done by Katerere and Eloff (2005) showed that free radical scavenging activity was present in the more polar extracts from *S. frutescens* after 30 minutes exposure. The release of free radicals has been reported by Haraguchi (2001) to play an adverse role in tissue injury and diseases. These scavenging activities of *S. frutescens* extracts may possibly explain its usefulness as a health tonic (Katerere and Eloff, 2005). Compounds with anti-oxidant activities have also been shown to enhance the immune system, by assisting the production and activity of cytotoxic immune cells, including enhanced expression of cancer suppressor gene products and associated inhibition of tumour angiogenesis (Shklar, 1998). Likewise, the inhibitory action of some compounds present in *S. frutescens* extracts on the release of certain pro-inflammatory cytokines, was also demonstrated by Tai *et al.* (2004); Ostlund and Sherman (1996) and Mombereau *et al.* (2004), all of which have been shown to be of value in the subsequent treatment of cachexia and wasting syndrome in cancer and HIV/AIDS patients. The decreased influence of *S. frutescens* extracts on the release of IL8, together with the co-stimulation by PMA in the *in vitro* cell culture system, provides further evidence related to the potential anti-oxidant attributes of the *Sutherlandia* extracts.

### 7.2.4 Summary

An absence in the release of certain pro-inflammatory cytokines, as measured in the *in vitro* cell culture system, provided further justification for anti-inflammatory and anti-oxidant effects related to *Sutherlandia* extracts alone. By inhibiting the action of TNF and IL1β, certain primary metabolites in *S. frutescens*, are said to contribute a reduction of the characteristic wasting syndrome experienced by cancer and HIV/AIDS patients (Ngcobo,

2008; Tai *et al.,* 2004; Ostlund and Sherman 1996; Mombereau *et al.,* 2004). Anti-inflammatory agents have been shown to exhibit chemo-preventative activity (Surh *et al.,* 2001; Surh, 2002); therefore, the anti-inflammatory properties which have also been attributed to *S. frutescens* could possibly be used in a chemo-preventative setting as well. However, the differential increase in TNF and IL8, together with the co-stimulation by PMA, could also contribute to possible immune-stimulating effects. For this reason, preparations of *Sutherlandia* seem to exhibit many attributes that could be related to the complex nature of compounds present in the raw plant material. Possible synergistic effects between these compounds present in the extracts of *S. frutescens*, most likely justify its various traditional uses.

**Fraction III** and **IV** exhibited the greatest increase in TNF and IL8 (with the co-stimulation of PMA) which has provided evidence that the *in vitro* immune-stimulating properties of *S. frutescens* extracts could possibly be due to the presence of non-polar compounds found in the ethanol extract and the triterpenoid compounds. According to the results, these fractions could be considered a good target for future investigations into possible *in vivo* immune-modulating properties, particularly in view of the observed decrease in cancer cell viability (although marginal for **fraction IV**) but also associated with an increase in the release of cytokines, as measured in the *in vitro* cell culture system. The compounds present in these fractions displayed a synergistic action on the resultant increased release of cytokines by the HL60 cells. Further purification might cause a reduction in its anti-cancer properties but an increase in cytokine release should not be affected as seen with the response of **compound 5**. (a slight increase in cancer cell viability, together with release of cytokines).

## 7.3 INTEGRATED DISCUSSION OF ANTI-CANCER AND CYTOKINE RELEASE IN THE PRESENCE OF PLANT EXTRACTS

In this section the net total affects of the *S. frutescens* extracts, with and/or without PMA co-stimulation on cancer cell viability and release in cytokines, simultaneously will be presented. The cytokine release assay was tested only, at 25 µg/ml, thus the comparison of the corresponding extracts influence on cancer cell viability will be done for the same concentration.

### 7.3.1 Immune-stimulating properties

Vaccination of foreign microorganisms and/or adjuvants into the body sets the immune system in action to organise defensive mechanisms for the host's survival. Thus, the PMA treatment showed an increase in the release of cytokines (see **Figures 7.7** and **7.8**, pages 94 to 98). From the results of PMA (**Figures 7.1**, **7.7** and **7.8**) it was observed that PMA could play a dual role in a cellular system by indicating anti-cancer properties (which might be due to the toxicity of PMA and lack of APC on the cancer cells) and increasing cytokine expressions.

The most active extract that was observed, at 25 µg/ml, for possible *in vitro* immune-modulating properties was the ethanol extract (**Ext C**) with PMA co-stimulation (**Figures 7.7a** and **7.8a** number **4**). Further fractionation of this extract showed the non-polar portion of the ethanol extract with PMA co-stimulation (see number **20** in **Figures 7.7b** and **7.8b**) exhibited the maximum amount of release of TNF and IL8 (even higher than that of the net ethanol extract's impact; see **Figures 7.2** and **7.5**, pages 79 and 85). This provided preliminary evidence that the active compounds which displayed the most profound *in vitro* immune-stimulating response and anti-cancer influence in the ethanol extract were part of the non-polar fraction of this extract. The triterpenoid-rich fraction (**Fraction IV**) also displayed a slight decrease in cancer cell viability and increased amount of the release in cytokines when co-stimulated with PMA at a lower concentration, therefore these compounds could also contribute to the activity related to *S. frutescens*.

The aqueous preparations were shown to exhibit an increase in cell viability (see **Figure 7.3**, page 81) and release in cytokines with the co-stimulation of PMA (see **Figures 7.7a** and **7.8a** numbers **6**, **8**, **10** and **14**) on the various cancer cell lines. The polar compounds present in these preparations might suppress the replication of PMA and thus resulted in less PMA in the body so the *in vitro* IR was not as high as the ethanol extract and only a small amount of immune cells were recruited therefore a decrease in the release of cytokines relative to PMA co-stimulation was observed. This showed a good response for helping the immune system in conserving energy. In contrast, as shown in **Figure 7.3**, due to the unique characteristics of the cancer cells and/or the non toxic nature of these compounds, these extracts also promoted cancer cell growth which might have been further caused by the multifunctional role of IL8 in the cancer cells i.e., neutrophils that are attracted by IL8 could lead to the release of proteases, heparin and enzymes that are involved in the degradation of the extracellular

matrix, resulting in metastasis of the tumour. This, in turn, may have contributed towards tumour aggressiveness through the promotion of invasion and angiogenesis (Yao *et al.*, 2007). Due to these observations, there is preliminary evidence that these extracts should probably not be taken when a patient has cancer as they may contribute to the aggressiveness of the cancer (at low concentrations) instead of helping the immune system to fight off the cancer. These extracts did still increase the expression of TNF, therefore, there was still an initiation of inflammation but due to the possible enhancement of tumour aggressiveness and angiogenesis, the cancer cells would probably overpower the effect of TNF thus resulting in a net increase in cell viability. The butanol and *Echinacea* extracts exhibited some anti-cancer and immune-stimulating properties but were not as effective as the non-polar portion of the ethanol extract. The table below provides a brief summary of the cancer cell viability and amounts of cytokines released to clearly compare the effects as discussed above.

**Table 7.2 A summary of cancer cell viability and amount of cytokines released for all extracts, fractions and compound 5.**

| Extracts | Concentration (µg/ml) | Incubation time (hours) | Viability for HL60 cells (%) | Cytokine expression for extracts with PMA (pg/ml) | |
|---|---|---|---|---|---|
| | | | | TNF | IL8 |
| Control | No drug | 48 | 100 | 50.83 ± 7.7 | 50.83 ± 7.7 |
| PMA | 12.5 ng/ml | 6 | 86.4 ± 9.22 | 129.7 ± 19.2 | 4551.9 ± 410.8 |
| Extract A | 25 | 48 | 113.0 ± 4.0 | 176.1 ± 34.3 | 3333.9 ± 254.6 |
| Extract B | 25 | 48 | 110.3 ± 4.0 | 159.1 ± 53.1 | 3372.8 ± 180.2 |
| Extract I | 25 | 48 | 94.1 ± 0.2 | 172.5 ± 11.7 | 3600 ± 184.8 |
| Extract C | 25 | 48 | 97.7 ± 7.6 | 184.4 ± 44.5 | 4449.6 ± 291.6 |
| Extract D | 25 | 48 | 110 ± 7.2 | 152.4 ± 24.8 | 3168.3 ± 252.5 |
| Extract E | 25 | 48 | 115.3 ± 4.9 | 196.1 ± 33.1 | 3968.6 ± 87.4 |
| Fraction I | 25 | 48 | 96.7 ± 1.1 | 179.2 ± 51.2 | 2909.5 ± 257.1 |
| Fraction II | 25 | 48 | 80.3 ± 3.4 | 179.5 ± 58.1 | 3916.6 ± 817.2 |
| Fraction III | 25 | 48 | 50.5 ± 3.3 | 229.4 ± 13.9 | 5967.9 ± 226.9 |
| Ech | 25 | 48 | 100.2 ± 7.4 | 180.1 ± 57.8 | 3260.9 ± 250.4 |
| Fraction IV | 5 | 48 | 91.5 ± 1.8 | 180.2 ± 35.4 | 5277.7 ± 541.9 |
| Fraction V | 5 | 48 | 106.1 ± 2.9 | 194.9 ± 31.4 | 3602 ± 229.4 |
| Fraction VI | 5 | 48 | 106.3 ± 3.3 | 164.3 ± 47.6 | 4231.8 ± 500.8 |
| Compound 5 | 5 | 48 | 104.7 ± 0.04 | 206.4 ± 48.4 | 3846.8 ± 262.1 |

## 7.4 CYTOTOXICITY EFFECTS OF *SUTHERLANDIA*

There is a fine line between inflammation and cancer progression. Studies have shown that chronic inflammation is a progenitor of tumour progression and that many cancers have been found to arise from sites of infection, chronic irritation and inflammation. Factors such as TNF and the interleukins may also serve as connecting links between inflammation and cancer. Dysregulation and overproduction of these can lead to cancer and other diseases (Yadav *et al.*, 2010). This raises a few questions as to whether *S. frutescens* preparations will contribute to the progression of tumour cells while being administered to cancer patients in clinical trials. In order to answer this essential question, the study continued with a

preliminary cytotoxicity experiment, which was done in collaboration with Dr S. Barichievy from the Synthetic Biology group of CSIR. Macrophages from whole blood were isolated and one extract, chosen on the basis of its cancer cell viability and amount of cytokines released, was added to the macrophage cells to determine the cell viability after *S. frutescens* addition using the SRB assay. This was conducted using the same procedure as the cell viability experiment for the cancer cells.

**Extract A** (aqueous extract of the fresh leaves) was chosen because it showed an increase in cancer cell viability at 25 µg/ml and a decrease in cancer cell viability at 100 µg/ml for all three cell lines (see **Figure 7.3**, page 81) and at 25 µg/ml it shows an increase in TNF and IL8 expressions (see **Figures 7.7a** and **7.8a**, pages 94 and 97). To determine the effects of this extract on macrophage immune cells would provide evidence as to whether *S. frutescens* is cytotoxic to the immune cells at the same concentrations. The graph below shows the macrophage and HL60 cell viabilities for 25 and 100 µg/ml.

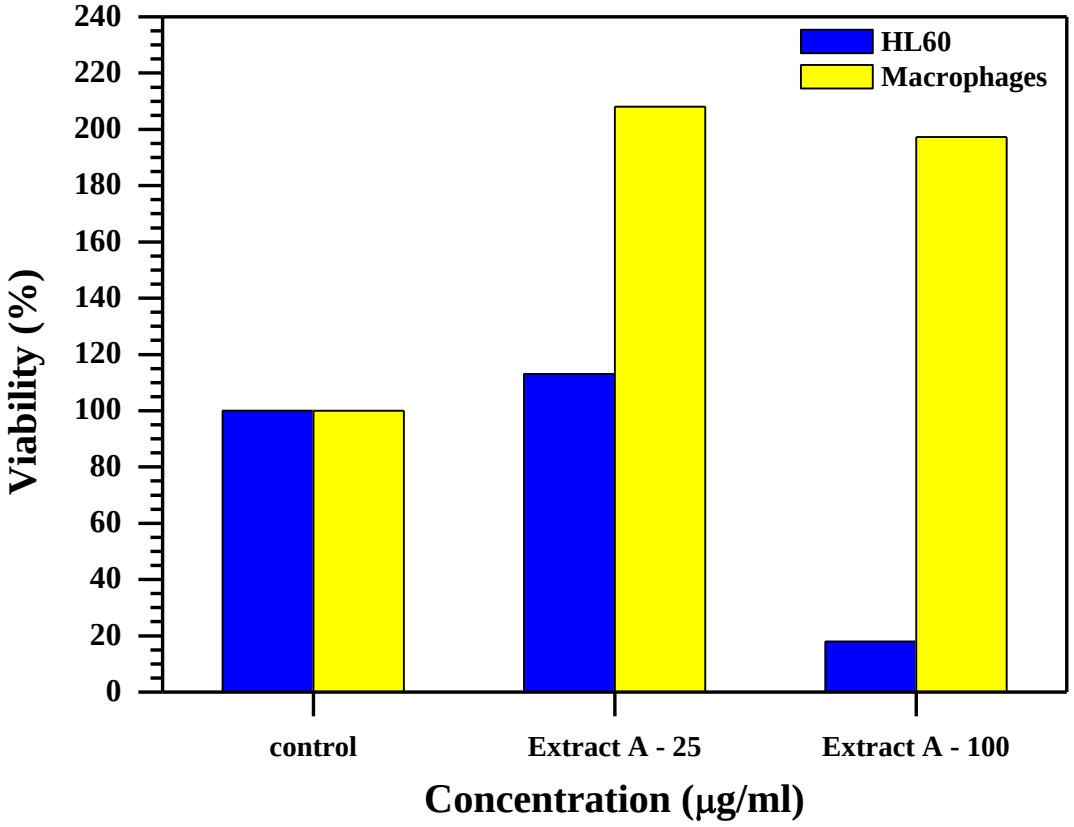



**Figure 7.9 Macrophage and HL60 cell viability after exposure with extract A at 100 and 25 µg/ml for a 48 h incubation period.**

**Extract A** illustrated an increase in cancer cell viability at 25 µg/ml and a decrease at 100 µg/ml but the effect on macrophage cell viability increased at both concentrations (**V = 208.03** and **197.28 %** at 25 and 100 µg/ml respectively). From these observations it can be stated that *S. frutescens* was not cytotoxic to the macrophage cells, favoured their survival and growth and displayed selectivity for immune cells. These results are supported by Ngcobo (2008) who also found that aqueous extracts were not cytotoxic to normal T cells. Nbcobo (2008) also showed that the ethanol extract induced proliferation in the T cells after 24 hours but had the opposite effect after 48 hours, while the aqueous extract significantly increased the T cell viability only after 48 hours of exposure. The results concluded that higher concentrations could be toxic to the normal T cells while lower concentrations of *S. frutescens* could stimulate the immune cells. To conclude, the above observations give reason to suggest that if *S. frutescens* was to be administered to cancer patients, at the correct dosages and time intervals, it could possibly have an additive effect on the immune cells of the body and increase their numbers so that there would be more cells to help fight off the invading microorganisms.

# CHAPTER 8

# CONCLUSION

*Sutherlandia frutescens* has a long history of medicinal use in southern Africa and has been widely used for many various ailments including diabetes, stress, fever, internal cancers and more recently HIV/AIDS (Van Wyk and Albrecht, 2008; Van Wyk, Van Oudtshoorn and Gericke, 2009; Ojewole, 2008). The primary aim of this research was to phytochemically characterise the various extracts made from *S. frutescens*. Due allowance was made for various extract preparation methodologies, which ranged from traditional methods to other more scientifically acceptable methods using organic solvents. The chemical profiles were obtained by HPLC analysis where the various regions were identified and two compounds were isolated and purified from the plant material (using various column chromatographic techniques). The structures of these two compounds, along with one of the standards were successfully elucidated using the NMR spectroscopic and mass spectrometric techniques. Using UPLC-TOF-MS techniques and ChemSketch optimised for 3D viewing, a structural analysis prediction was achieved for the flavonoid fractions that were not purified completely.

The chemical profiles of the six *S. frutescens* plant samples were analysed on the HPLC instrument and it was noted that various differences were present pointing to the possible presence of chemotypes.

The purpose of the second part of the study was to connect the compounds present in the extracts to the possible immune-modulating and anti-cancer abilities of *S. frutescens*. Using a three cell line panel screen with melanoma, breast and leukaemia cell lines and a cytokine up regulating assay could help to preliminary ascertain whether this traditional medicinal plant could possibly have anti-cancer and/or immune-modulating properties and which compounds (present in the extracts and singularly) could be responsible for these actions.

With the background chemistry of the extracts and compounds in place, the SRB assay was used to determine cancer cell viability for the *Sutherlandia* preparations. The effects of the extracts were studied over a 48 hour incubation period and it was found that the organic extracts were shown to be more effective at decreasing the cancer cell viability than the

traditional extracts. From the ethanol preparation, the non-polar portion contributed more to the potency on the cancer cell lines displaying an $IC_{50}$ at a concentration of 25 µg/ml, which was more potent than etoposide ($IC_{50}$ 46 µg/ml) against the leukaemia cell lines.

HPLC-MS provided confirmation that the ethanol extract contained a higher concentration of the non-polar compounds and triterpenoids together as compared to the other extracts, therefore its strong anti-cancer activities could be related to the presence of these compounds. The aqueous extracts of the fresh and dry plant material contained a higher concentration of the polar compounds, which were found to exhibit lower anti-cancer properties at lower concentrations. For the aqueous extracts to be effective against cancer the optimal dose and duration should be increased. The flavonol glycosides were found in the polar regions of the extracts and exhibit anti-oxidant properties. The butanol portion of the methanol extract contained the highest concentration of the cycloartane glycosides found in *S. frutescens* and exhibited similar anti-cancer properties to the ethanol extract.

From the cytokine expression assay, only TNF and IL8 were increased with the addition of PMA and the extracts together (relative to the control). All the *Sutherlandia* preparations increased the release of TNF and IL8 (with co-stimulation by PMA), but the greatest increase was that of the ethanol extract. Furthermore the non-polar fraction from the ethanol extract was mainly due to this activity. The interaction of these non-polar compounds with our immune system was favourable and was able to preliminary assist the cells of the immune system to increase the amount of the necessary cytokines being released. Organic preparations of *S. frutescens* were more effective than the traditional preparations. *S. frutescens* alone did not affect the *in vitro* model system in assisting the release of cytokines, but rather when the body was undergoing an IR, the addition of an ethanolic preparation of *S. frutescens* to the diet could have possibly assisted the body in fighting the disease. This could be of great interest for treatment of diseases that specifically attack the immune system, such as HIV/AIDS and cancer, as the body only needs to recognise the need for an IR and supplementation with *Sutherlandia* could possibly increase the production of the molecules necessary for the immune response to be effective. This resulted in a more rapid expulsion of the invader organism and limited the likelihood of the infectious organism from spreading to other cells.

From these observations an ethanolic preparation would probably been the best choice as a herbal remedy for stimulating the immune system. In essence, this *in vitro* cell culture system

has proved useful in demonstrating the nature of the cells in relation to their interactions with the compounds present in the plant extracts and associated solvent systems, particularly with regard to differential cell survival patterns.

HPLC-MS analysis made it possible to determine that the non-polar and triterpenoid compounds were mainly present in high concentrations in the extracts and fractions that were effective against the cancer cell lines and at stimulating the *in vitro* immune system.

Some semi-synthetic analogues of anti-cancer drugs that have been isolated from plants were active against numerous cancer cells. However, most of these compounds failed as effective drugs because of their non-specific toxicity to both, normal and cancer cells, as well as bioavailability problems (Ayres and Loike, 1990). *S. frutescens* has shown potential as an anti-cancer remedy due to the selectivity index present when tested on macrophage cells derived from whole blood. A single extract was tested and exhibited no cytotoxicity towards the macrophage cells while at the same time showed potential anti-cancer abilities against all three cell lines.

If *Sutherlandia* could be administered to cancer patients there is reason to suggest that it could possibly have an additive effect on the immune cells of the body by increasing their numbers and amount of cytokines produced so that there would be more cells activated and produced to help fight off infectious microorganisms. To conclude, *S. frutescens* could be of value for developing an anti-cancer remedy due to the increase in viability of macrophage cells as compared to the cancer cells. However, it was shown that *Sutherlandia* did not illicit an IR in the absence of an immunological stimulus.

**RECOMMENDATIONS:**

In order to clearly determine the effective concentration of these extracts, fractions and compounds to achieve optimal anti-cancer and immune-stimulating effects, a dose response curve should ideally have been done on all samples to determine the $IC_{50}$ values. To determine if a single compound could be responsible for the anti-cancer and immune-stimulating properties, a targeted purification approach should be followed in future. Due to the complex nature of the compounds, it is of utmost importance to determine an effective isolation and purification technique to optimise the phytochemical process.

Further investigations into the role *S. frutescens* plays in its medicinal properties, both *in vitro* and *in vivo*, should be done. Further cytotoxicity assays should be carried out on the extracts and isolated compounds to determine the therapeutic index. A study on drug interactions of *Sutherlandia* should also be investigated to determine the efficiency when taken in collaboration with drugs used for HIV/AIDS and cancer patients, such as anti-retrovirals and chemo-preventative drugs.

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# APPENDICES

## APPENDIX 1

## PLANT IDENTIFICATION

**South African National Biodiversity Institute**

Ref:
Addr: 405

Page 1 of 1

**PLANT IDENTIFICATION DISPATCH LIST**

**Final List**

**Batch no**: 10056 **Date received**: 30 September 2010 **Date**: 27 October 2010

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

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

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

| | SPECIMEN NO | GENSPEC | NO | PLANT NAME | ID CODE |
|---|---|---|---|---|---|
| **T.Faleschini** | | | | | |
| * | 194-24637A | 462 | 1 | Sutherlandia frutescens (L.) R.Br. | |
| * | 194-24637B | 462 | 1 | Sutherlandia frutescens (L.) R.Br. | |

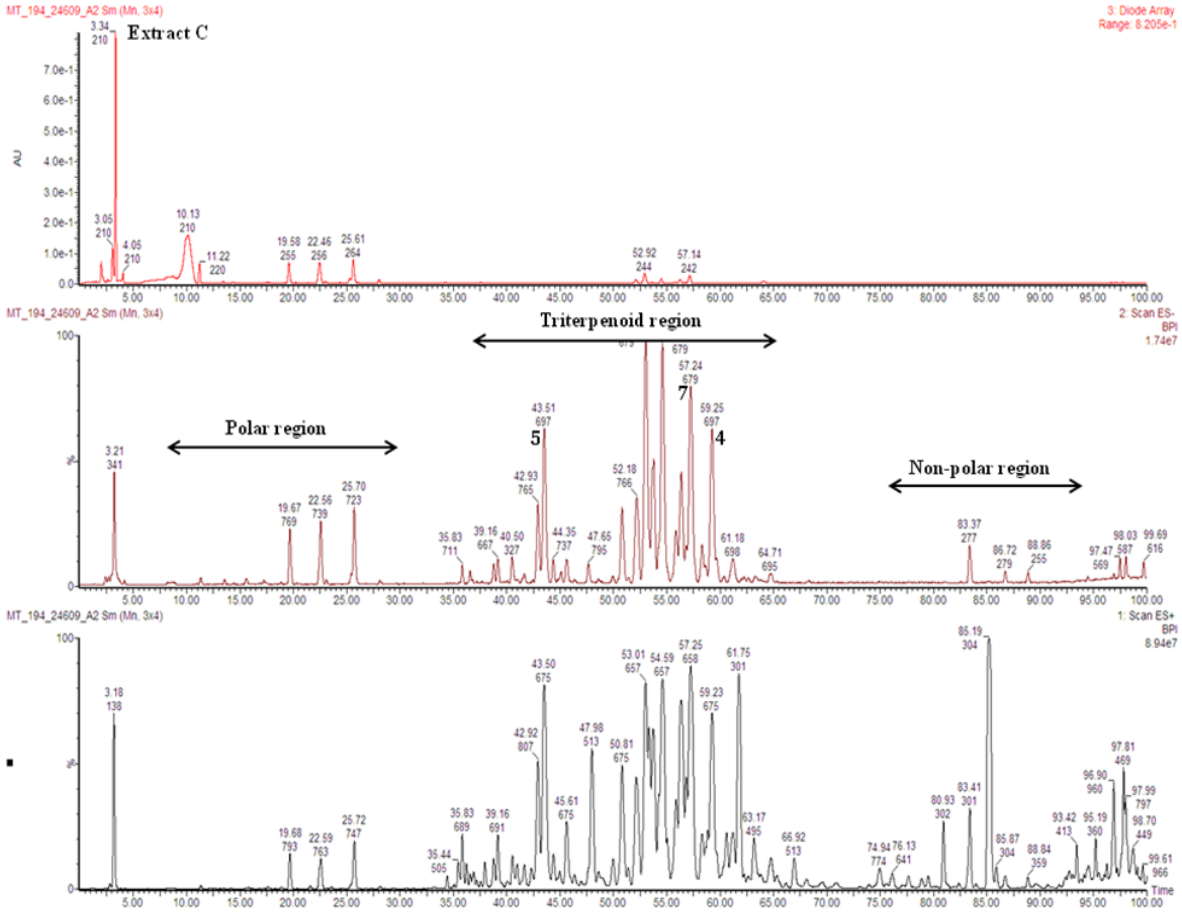
**SUMMARY**: **Received** = 2 **(Requested back (*)** = 5 **)** **Completed** = 2
**Insystem** = 0

Page 1

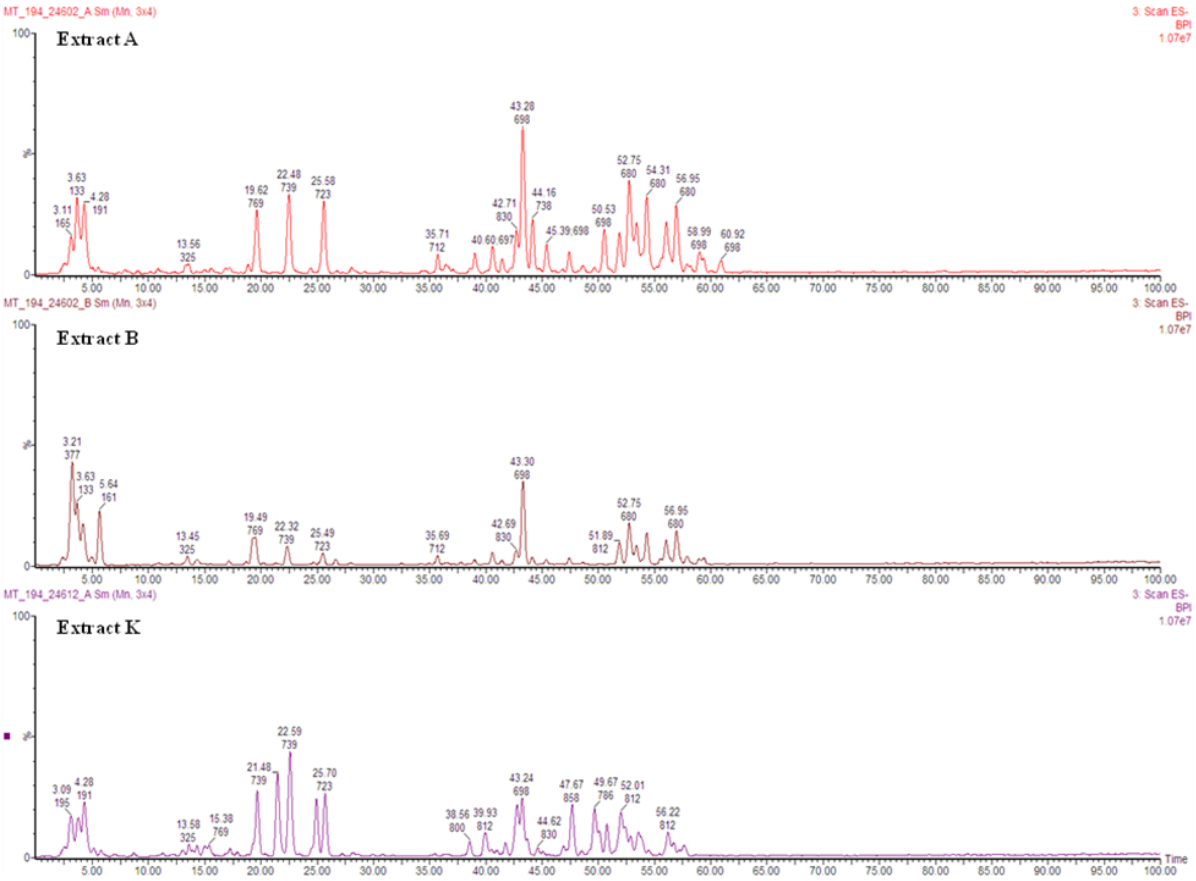
**Appendix Figure 1.1 SANBI Identification.**

# APPENDIX 2

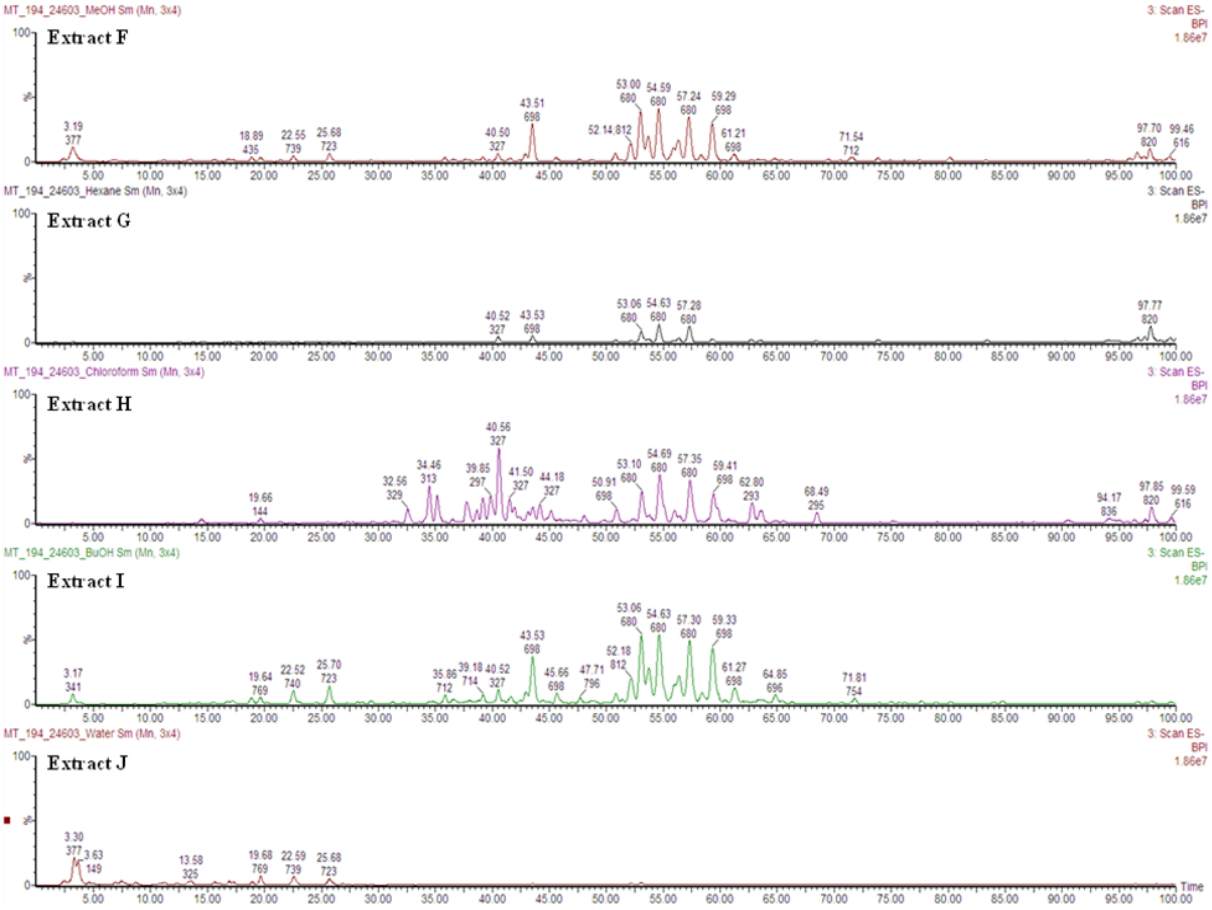
## EXTRACT CHROMATOGRAMS



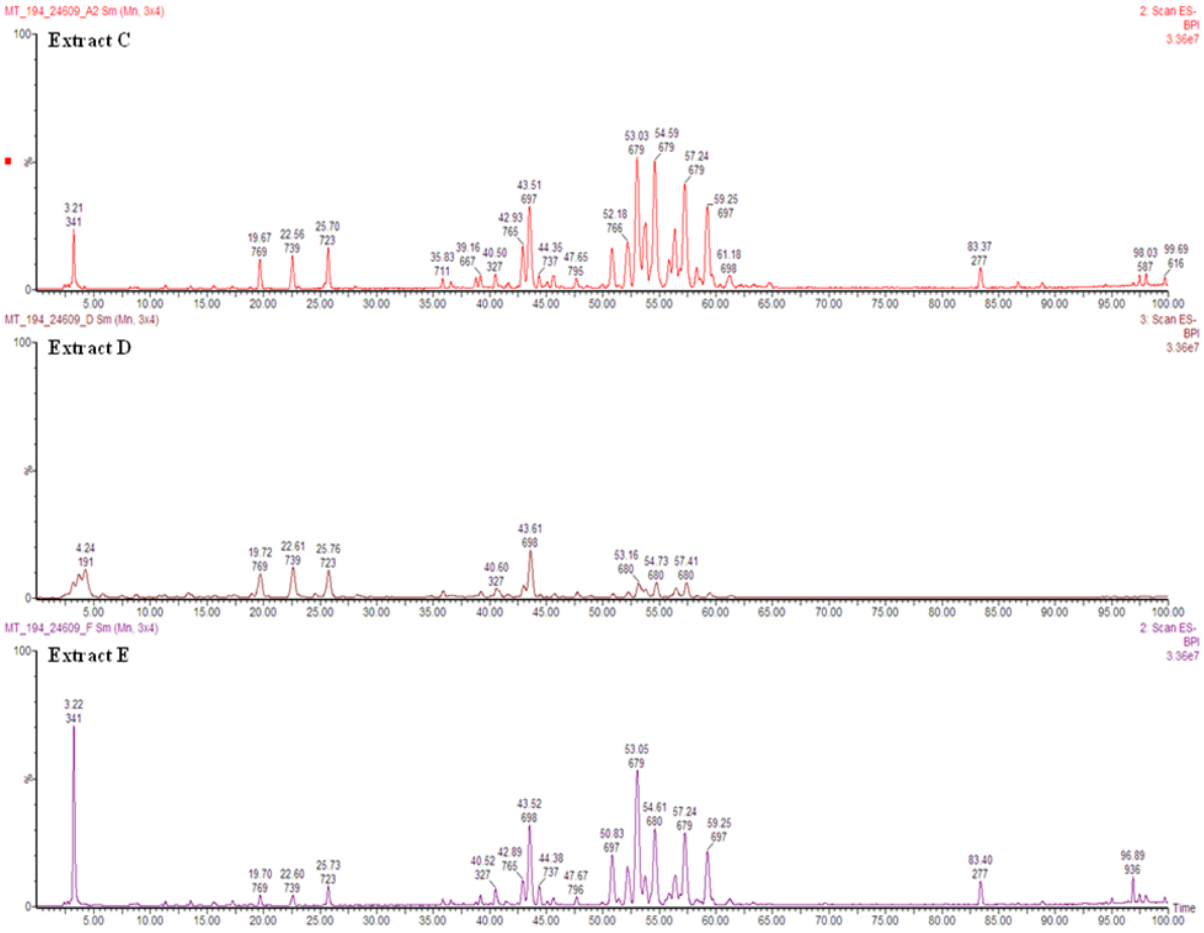

**Appendix Figure 2.1 The ESI$^+$, ESI$^-$ and UV chromatograms for extract C with the regions and compounds 4, 5 and 7 labelled in negative mode (refer to page 33). UV-VIS detection done scanning from 200 to 600 nm.**




**Appendix Figure 2.2 The ESI⁻ chromatograms for extracts A, B and K (refer to page 34).**



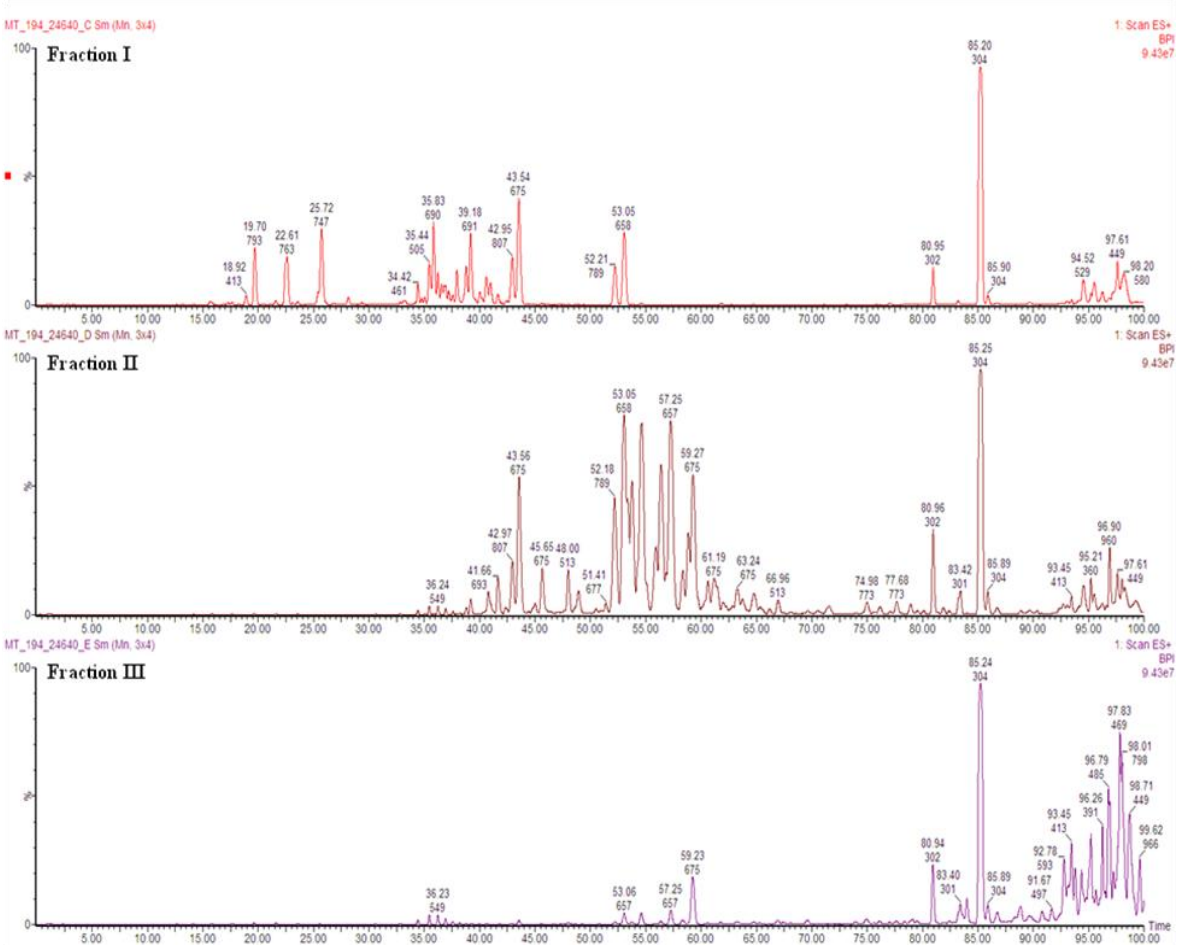

**Appendix Figure 2.3 The ESI⁻ chromatograms for extracts F, G, H, I and J. Refer to page 35 for explanation.**



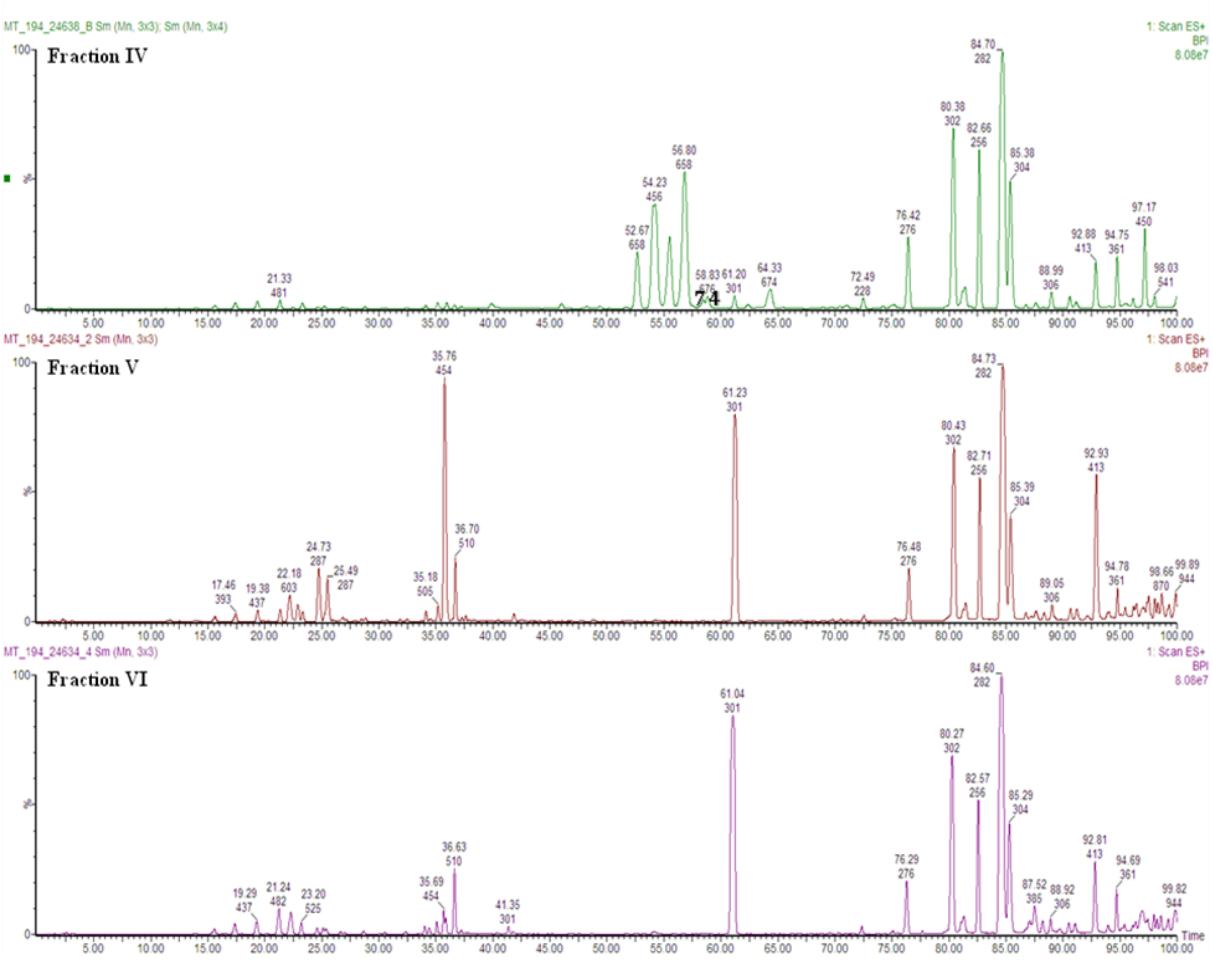

**Appendix Figure 2.4 The ESI⁻ chromatograms for extracts C, D and E. Refer to page 35 for explanation.**

# APPENDIX 3

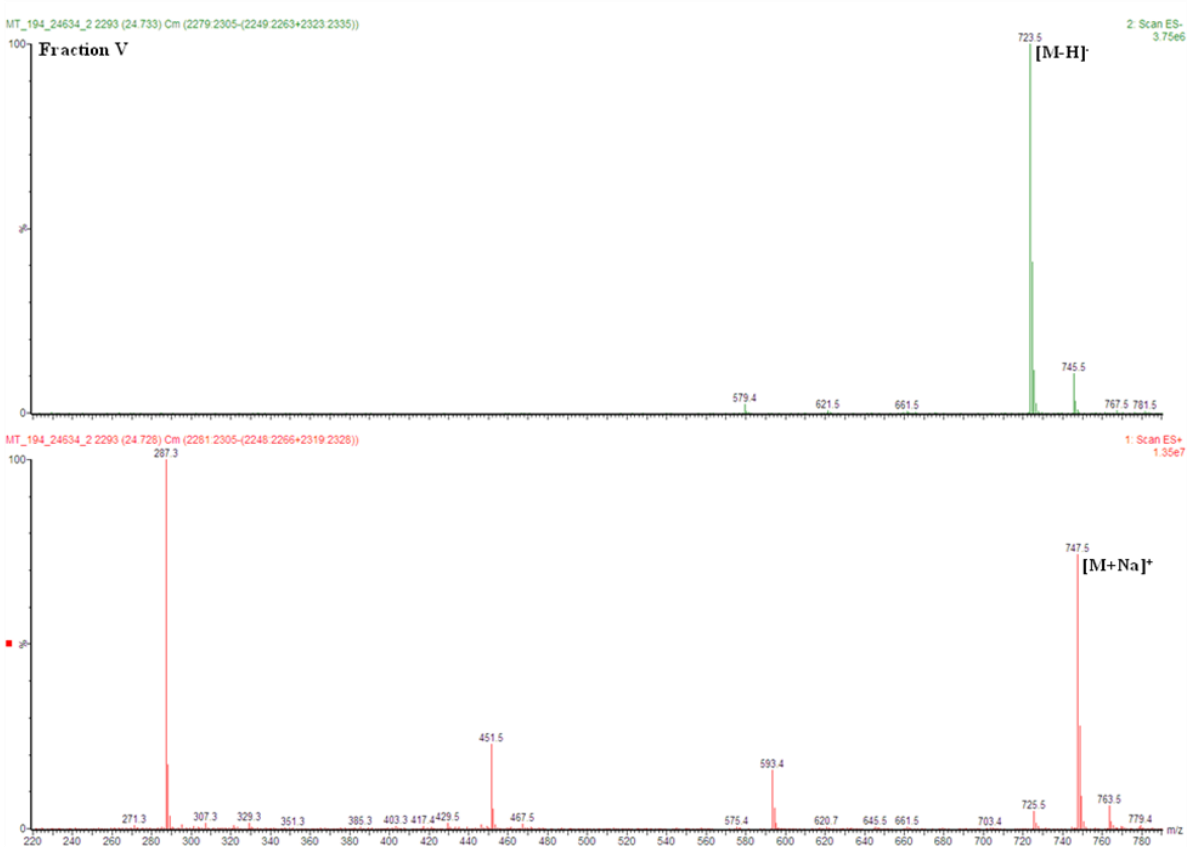
## FRACTION DATA



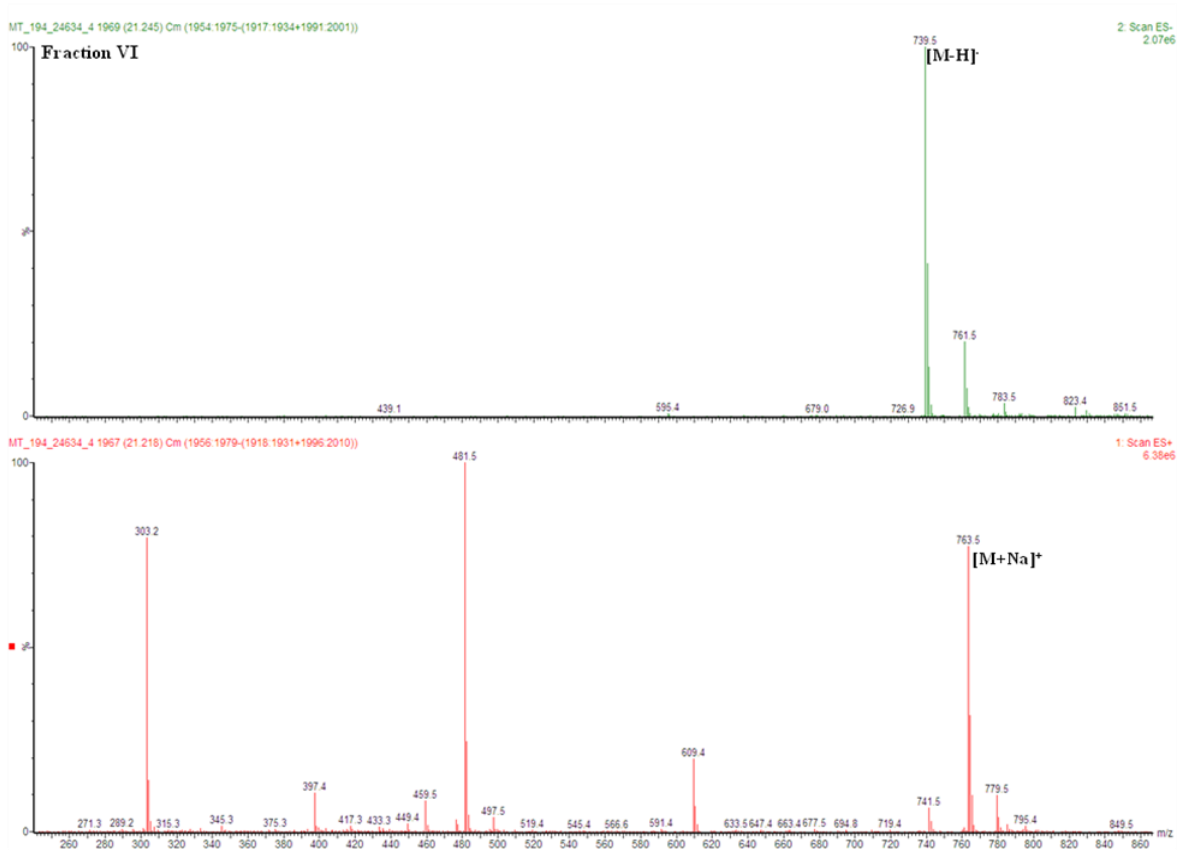

**Appendix Figure 3.1 The ESI$^+$ chromatograms for fractions I, II and III. refer to page 36.**



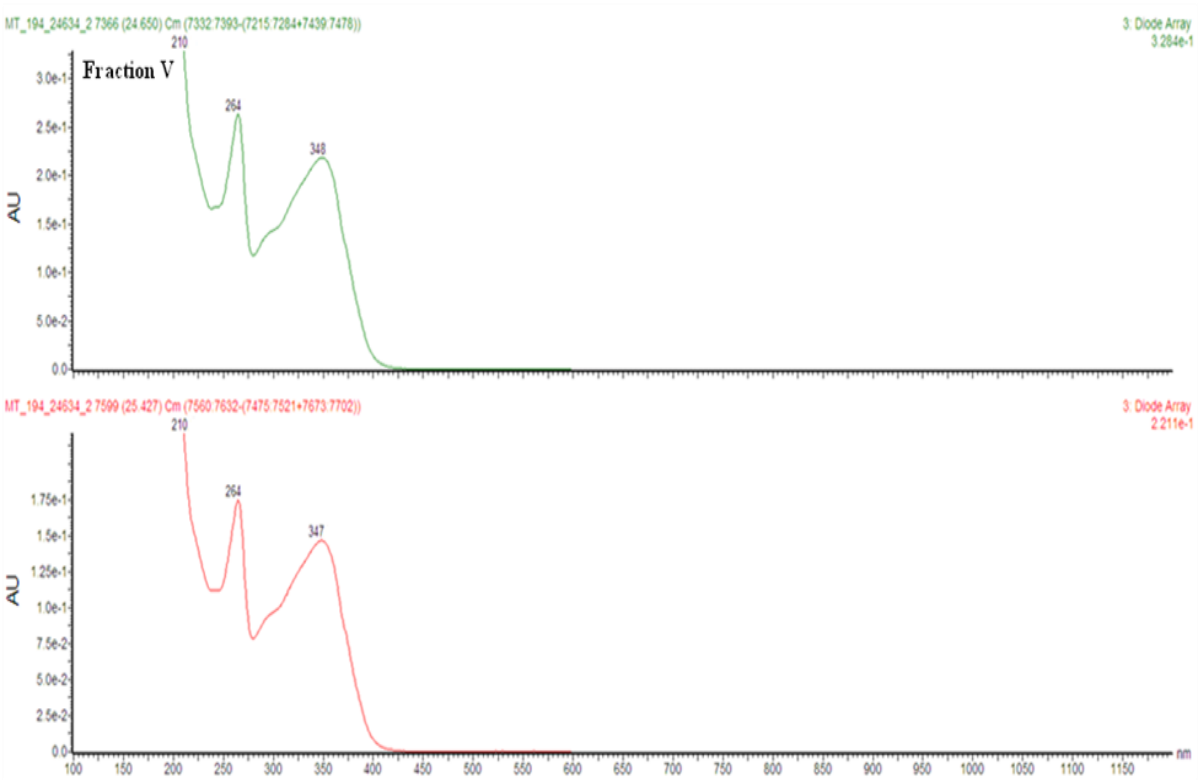

**Appendix Figure 3.2 The ESI$^+$ chromatograms for fractions IV, V and VI. Refer to page 36.**



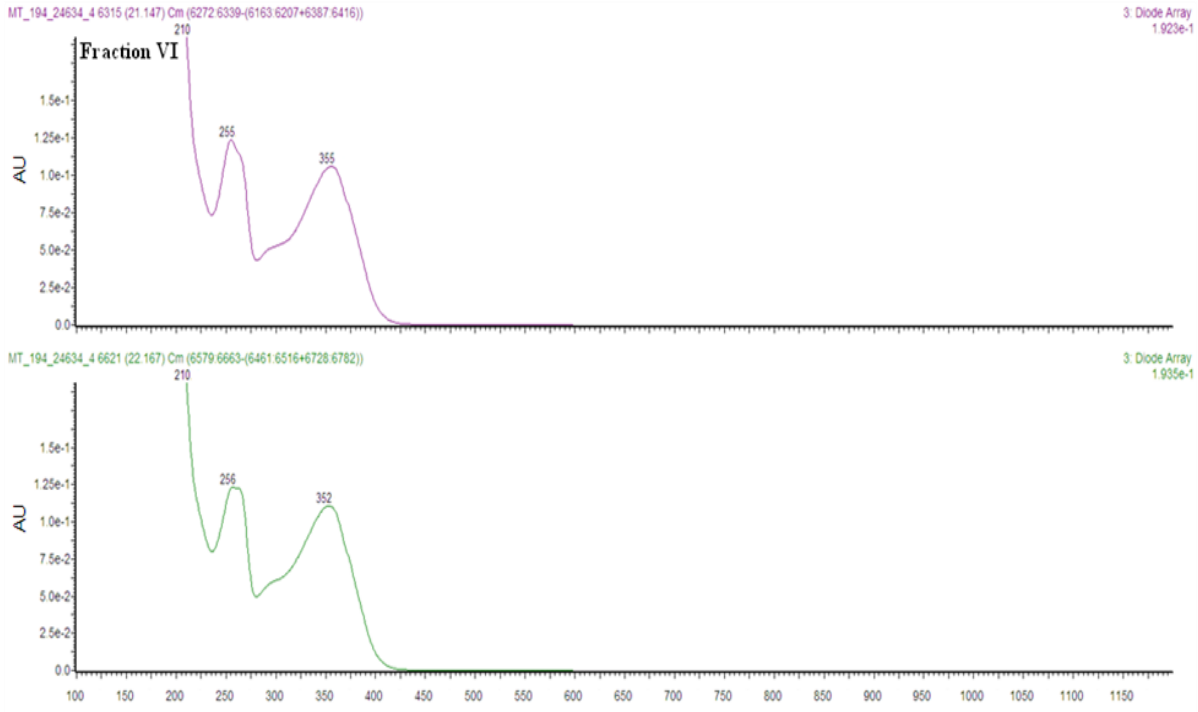

**Appendix Figure 3.3 Mass spectral data ($ESI^-$ and $ESI^+$) of peak eluting at 24.73 min. from fraction V which corresponds to that of compounds 10 or 11 is displayed above from the SQD system. Refer to pages 36 and 37 for explanations.**



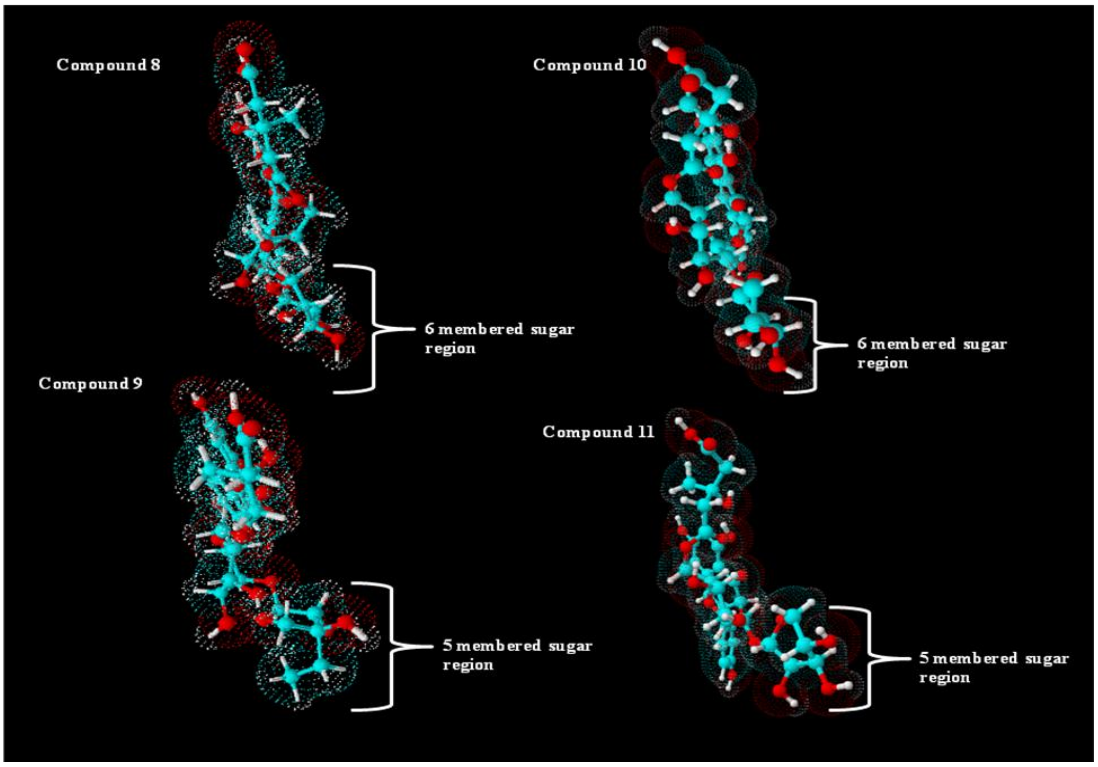

**Appendix Figure 3.4 Mass spectral data ($ESI^-$ and $ESI^+$) of peak eluting at 21.25 min. from fraction VI which corresponds to that of compounds 8 or 9 from the SQD system. Refer to pages 36 and 37 for explanations.**



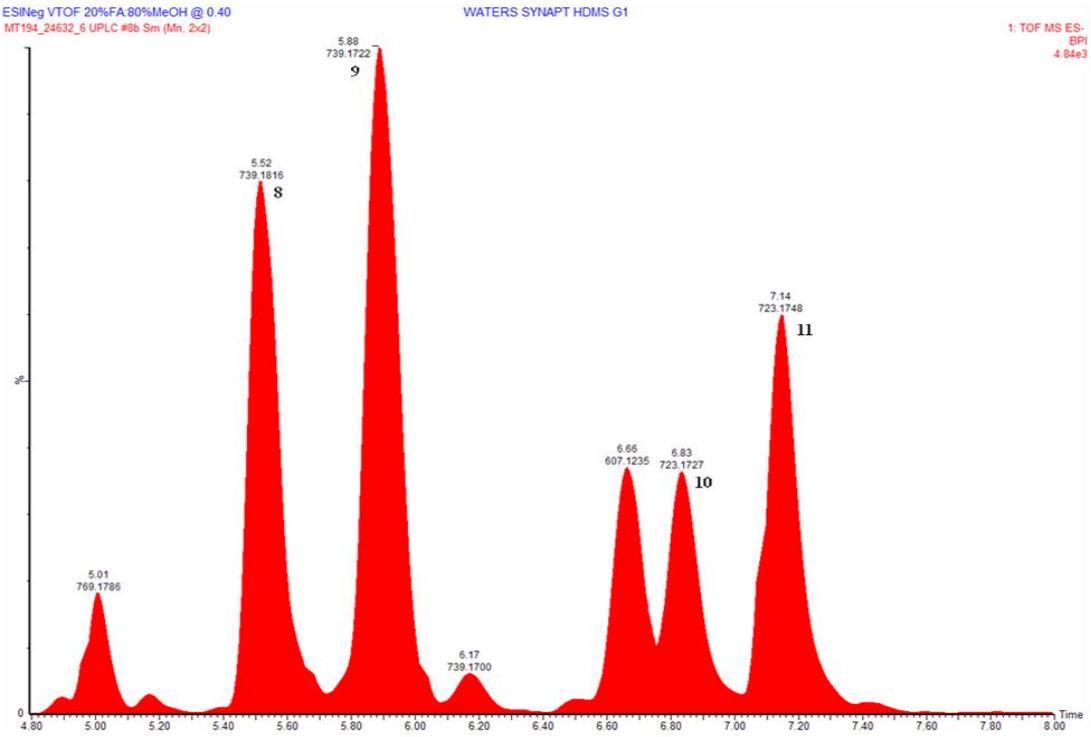

**Appendix Figure 3.5 UV absorption spectra of peaks eluting at 24.65 and 25.43 minutes in fraction V from the SQD system (scanning from 200 to 600 nm). Refer to page 36.**




**Appendix Figure 3.6 UV absorption spectra of peaks eluting at 21.15 and 22.17 minutes in fraction VI (scanning from 200 to 600 nm). Refer to page 36.**




**Appendix Figure 3.7 3D representations of compounds 8 to 11 showing the sugar regions and the space occupied by each using ChemSketch 12 (refer to page 39).**

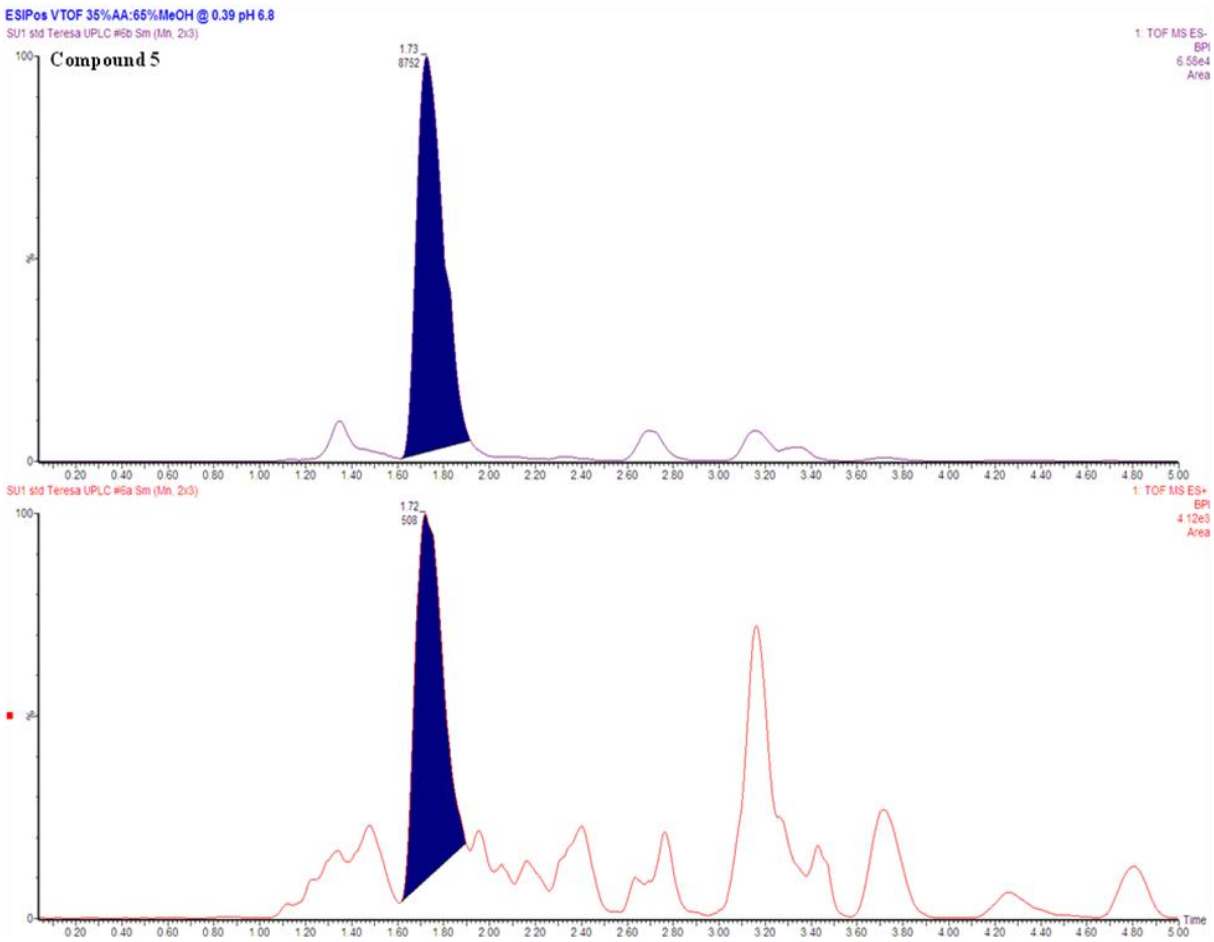



**Appendix Figure 3.8 WATERS synapt UPLC chromatogram (HRTOF MS ESI⁻ mode) with proposed order of elution of the four flavonoid compounds 8 to 11 (refer to page 39).**

# APPENDIX 4

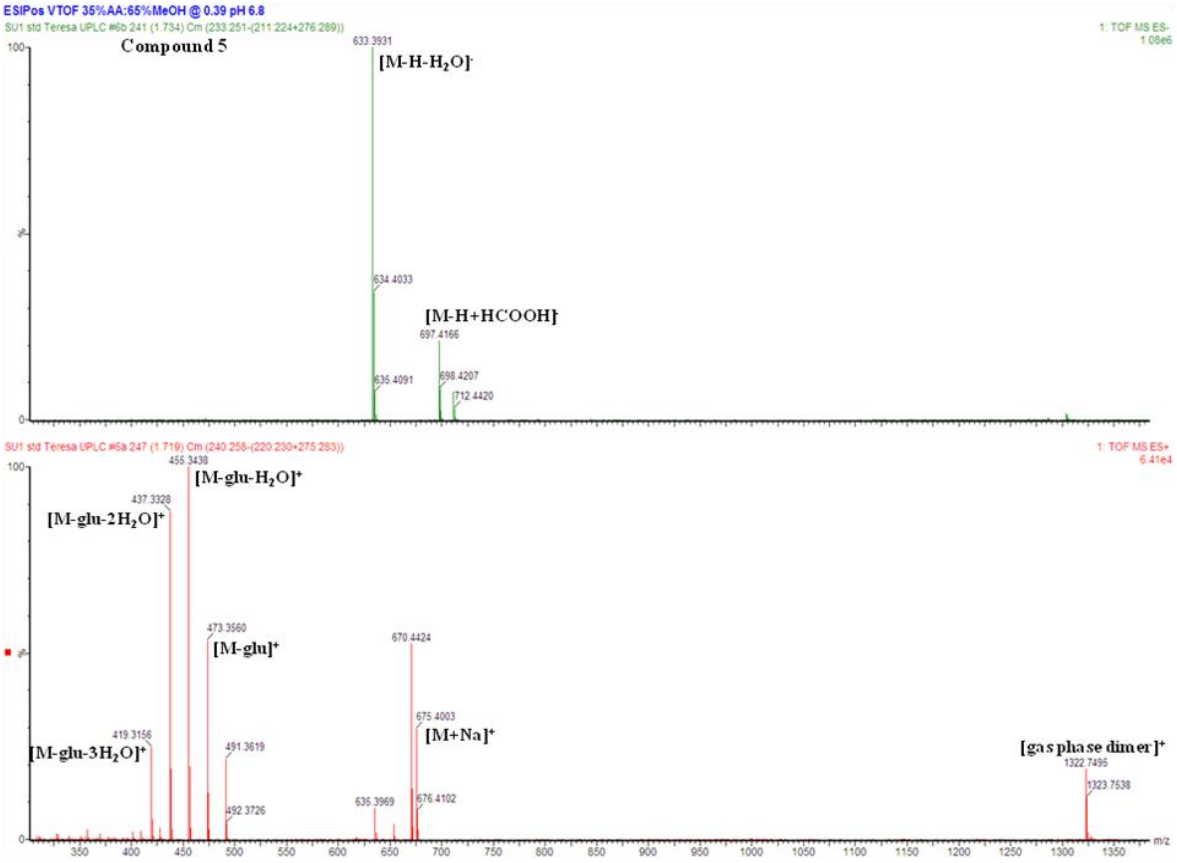
# COMPOUND DATA

## Compound 5



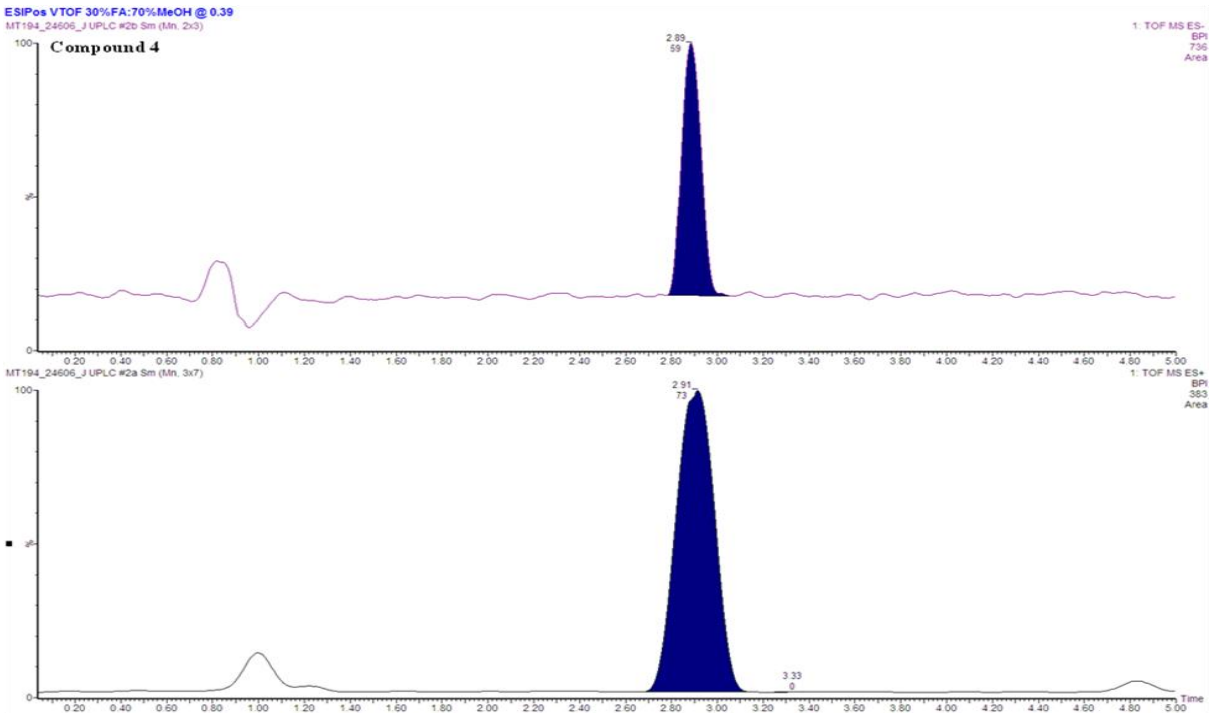

**Appendix Figure 4.1 UPLC chromatogram for compound 5 ($HRESI^-$ and $HRESI^+$).**

**Refer to page 42.**

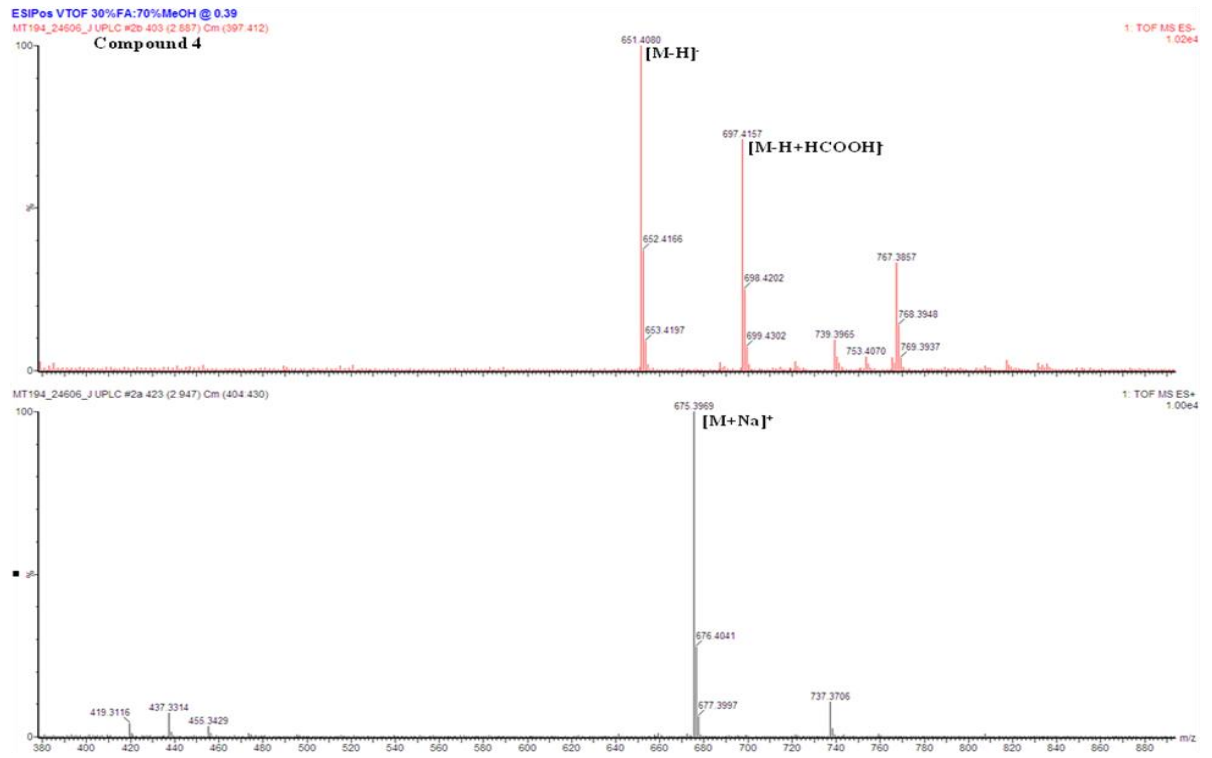



**Appendix Figure 4.2 HRTOFMS ($ESI^-$ and $ESI^+$) spectra for the standard of compound 5 from the QTOF system. Refer to page 45.**

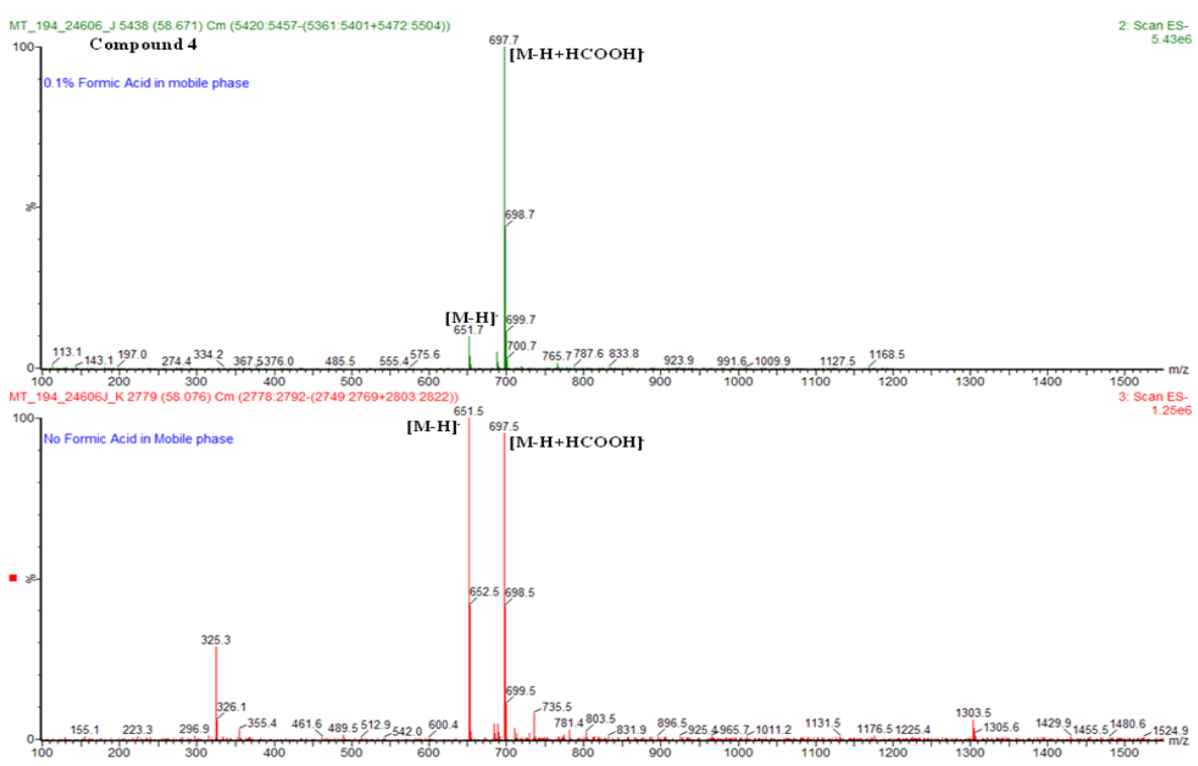
## Compound 4



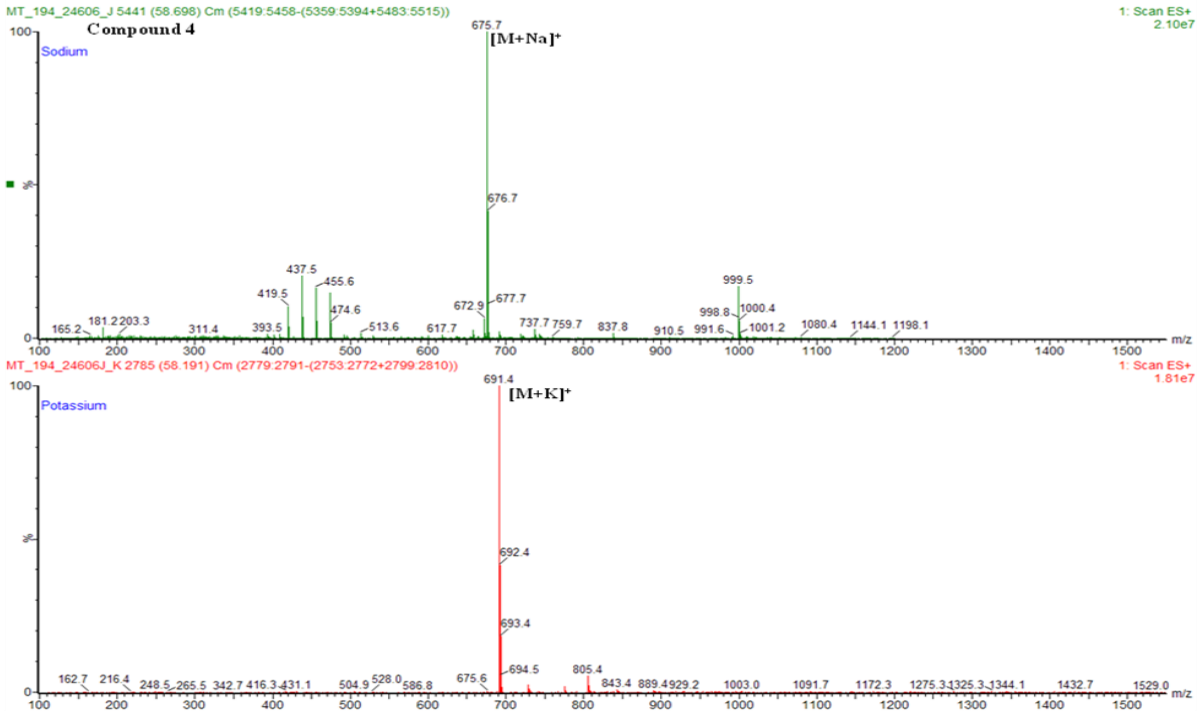

**Appendix Figure 4.3 UPLC chromatogram for compound 4 (HRESI$^-$ and HRESI$^+$). Refer to page 46.**




**Appendix Figure 4.4 HRTOFMS (ESI$^-$ and ESI$^+$) for compound 4 from the QTOF system. Refer to page 48.**

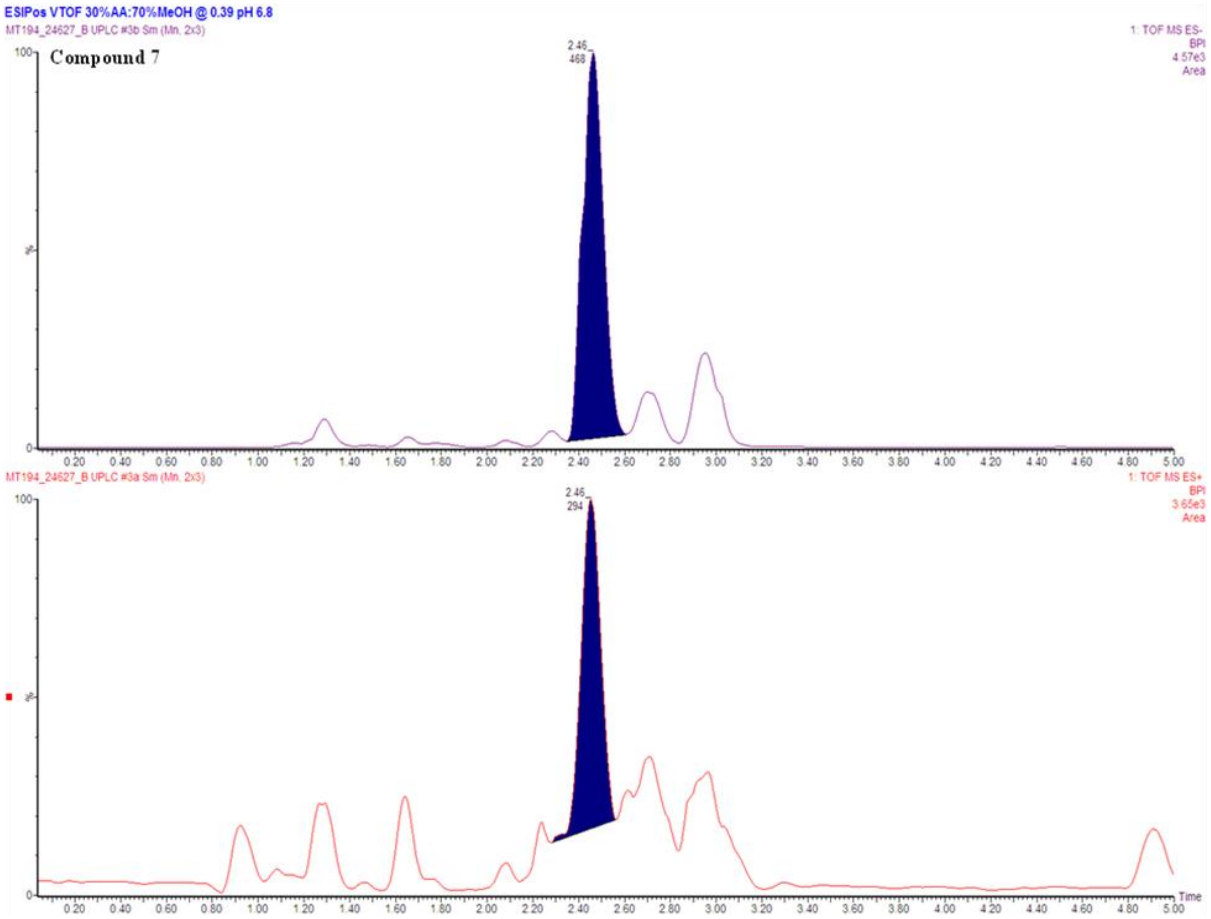



**Appendix Figure 4.5 ESI mass spectra (ESI$^-$) for compound 4 showing the formic acid adduct from the SQD system. Refer to page 50.**



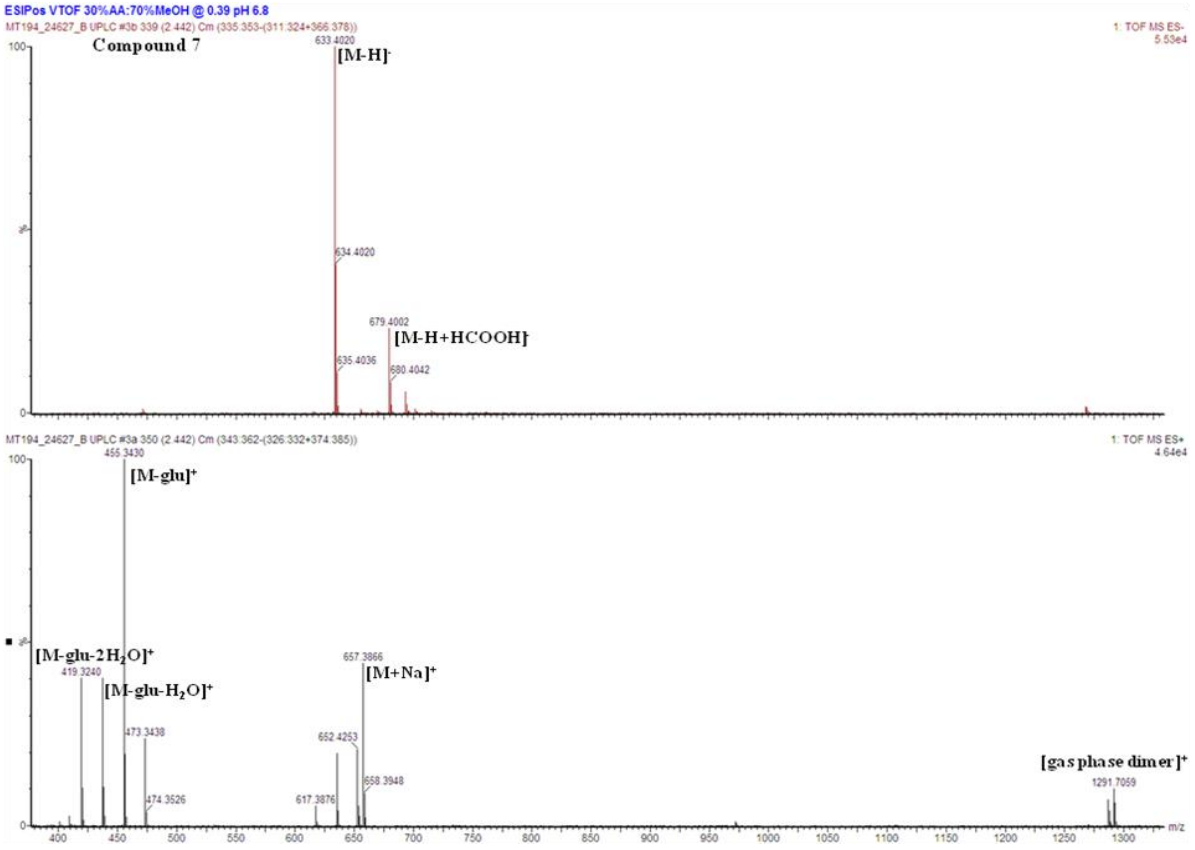

**Appendix Figure 4.6 ESI mass spectra (ESI$^+$) for compound 4 showing the sodium and potassium adducts from the SQD system. Refer to page 51.**

## Compound 7

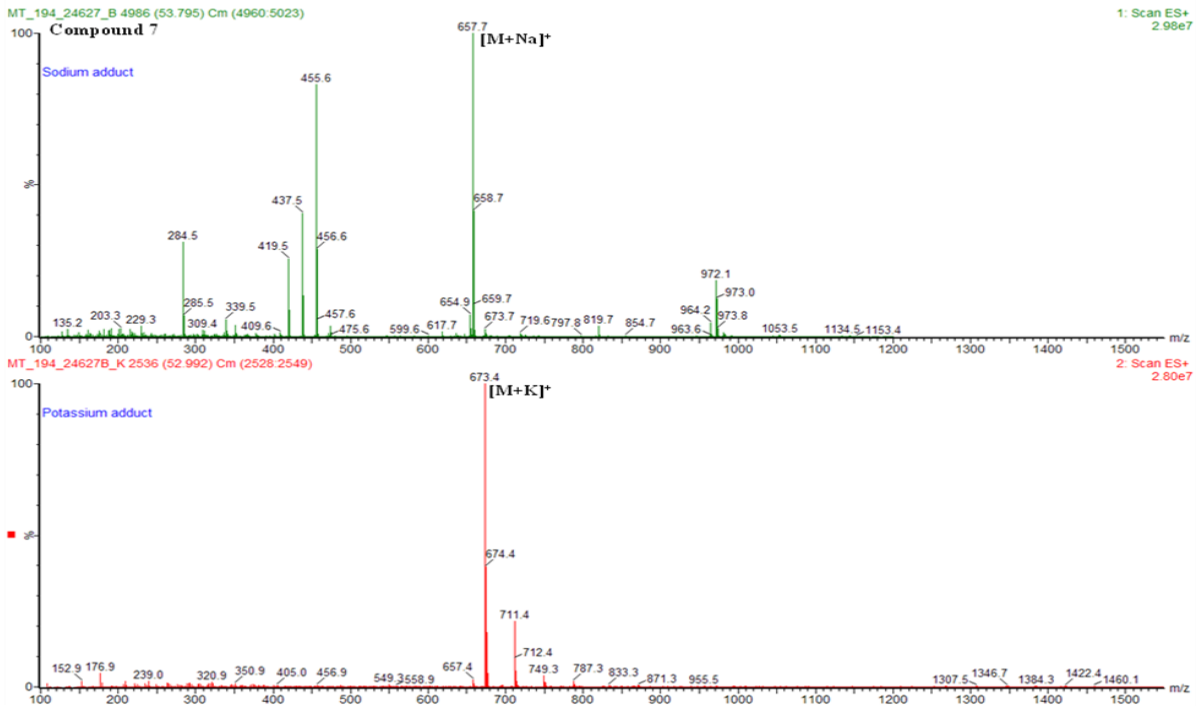



**Appendix Figure 4.7 UPLC chromatogram for compound 7 (HRESI$^-$ and HRESI$^+$).**

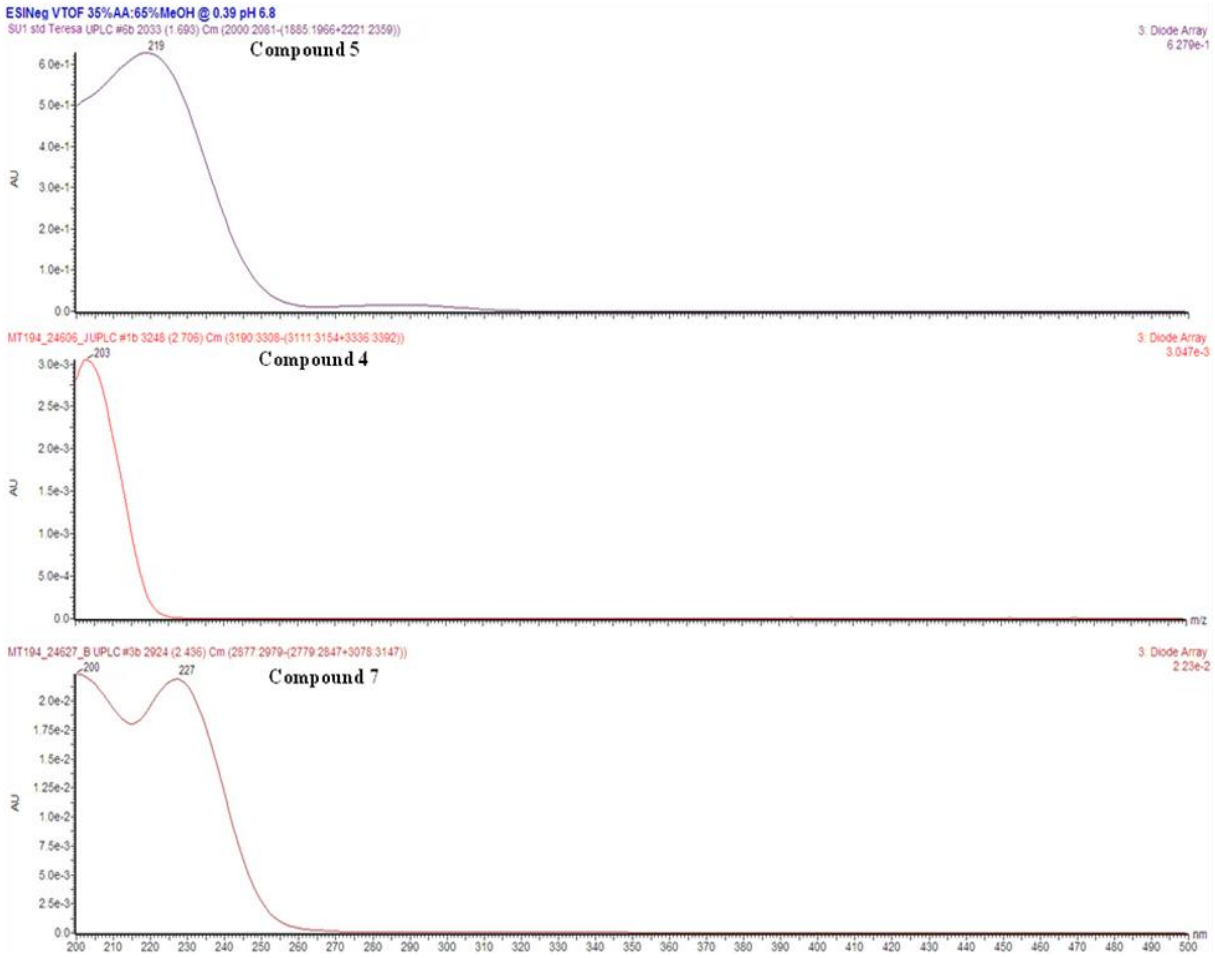
**Refer to page 53.**




**Appendix Figure 4.8 HRTOFMS ($ESI^-$ and $ESI^+$) for compound 7 from the QTOF system. Refer to page 55.**




**Appendix Figure 4.9 Mass spectral data for compound 7 showing the sodium and potassium adducts formed in positive mode of ESI from the SQD system. Refer to page 55.**

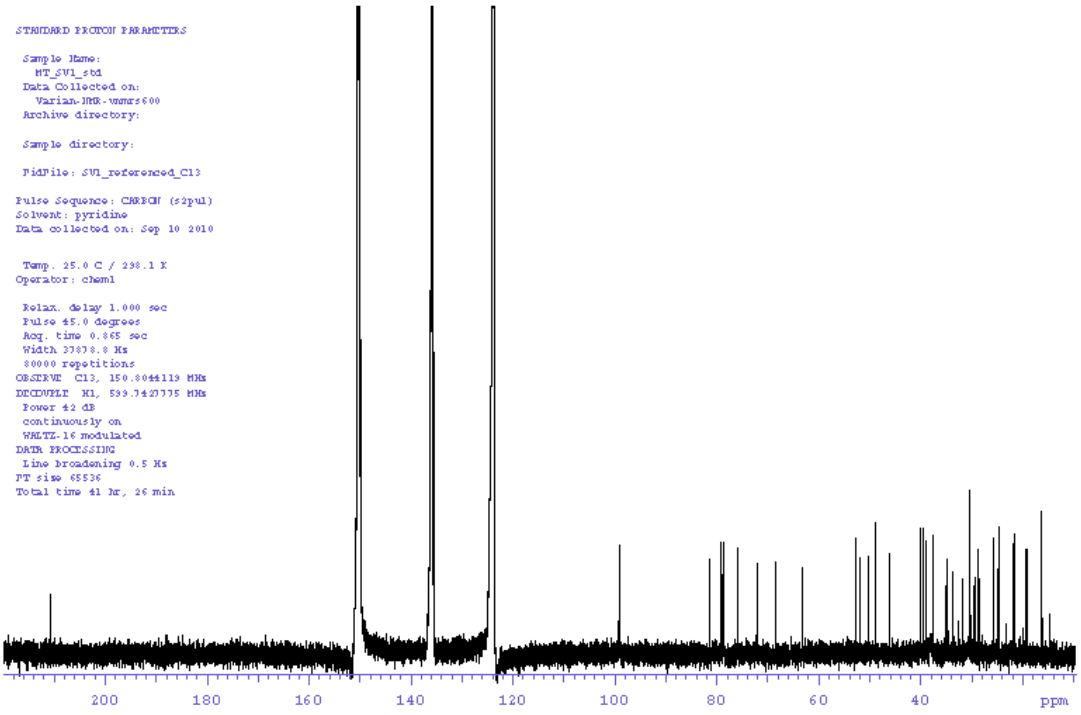



**Appendix Figure 4.10 UV data for compounds 5, 4 and 7 from the QTOF system (scanning from 200 to 600 nm). Refer to page 57.**

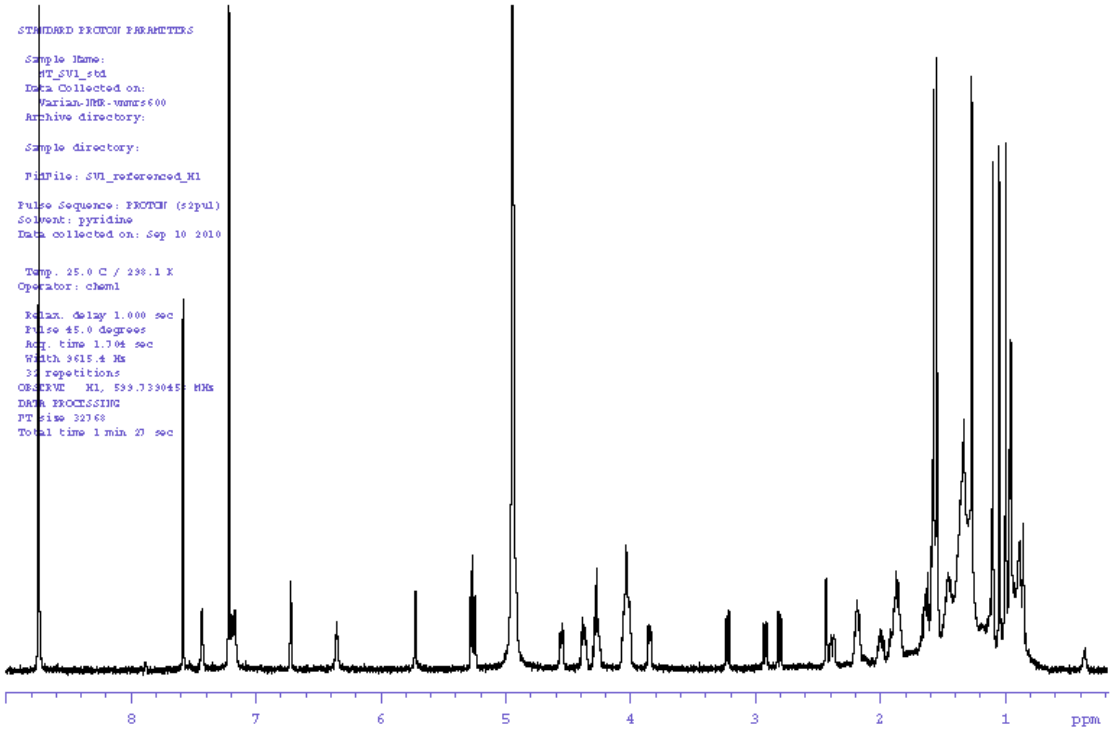
# APPENDIX 5

# NMR DATA

## Compound 5

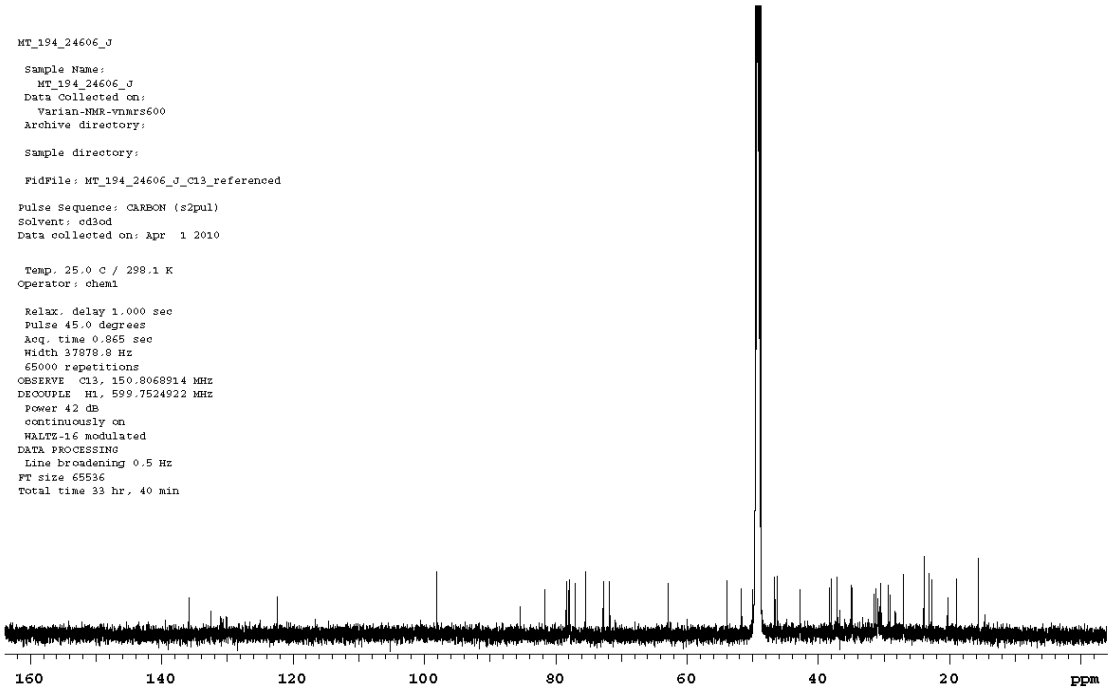



**Appendix Figure 5.1 $C^{13}$ NMR spectrum for compound 5 (600 MHz Varian NMR in d-pyridine). Refer to page 44.**

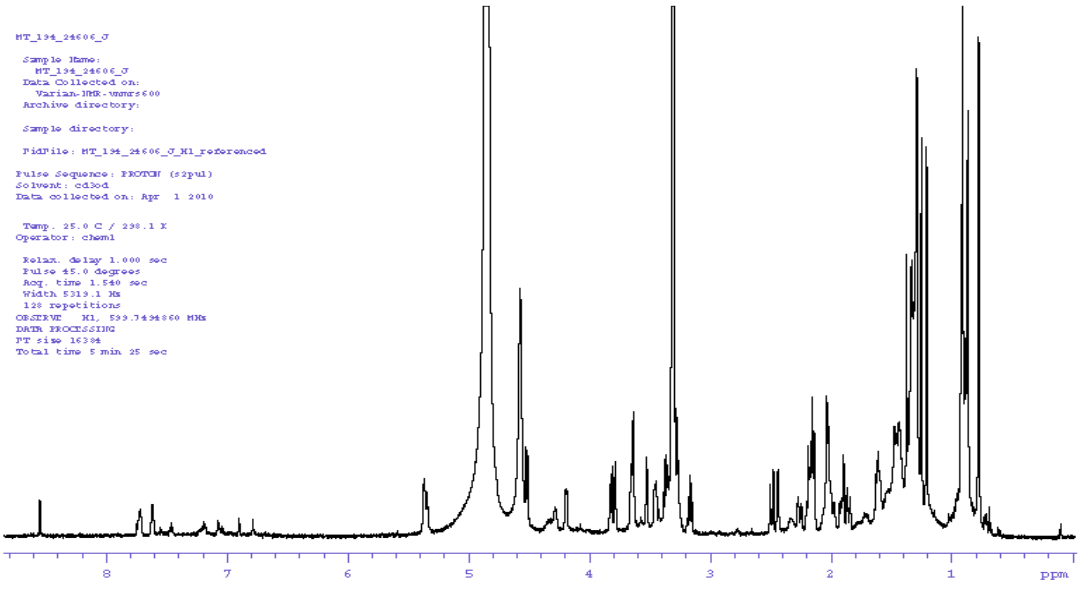



**Appendix Figure 5.2 $H^1$ NMR spectrum for compound 5 (600 MHz Varian NMR in d-pyridine).**

## Compound 4

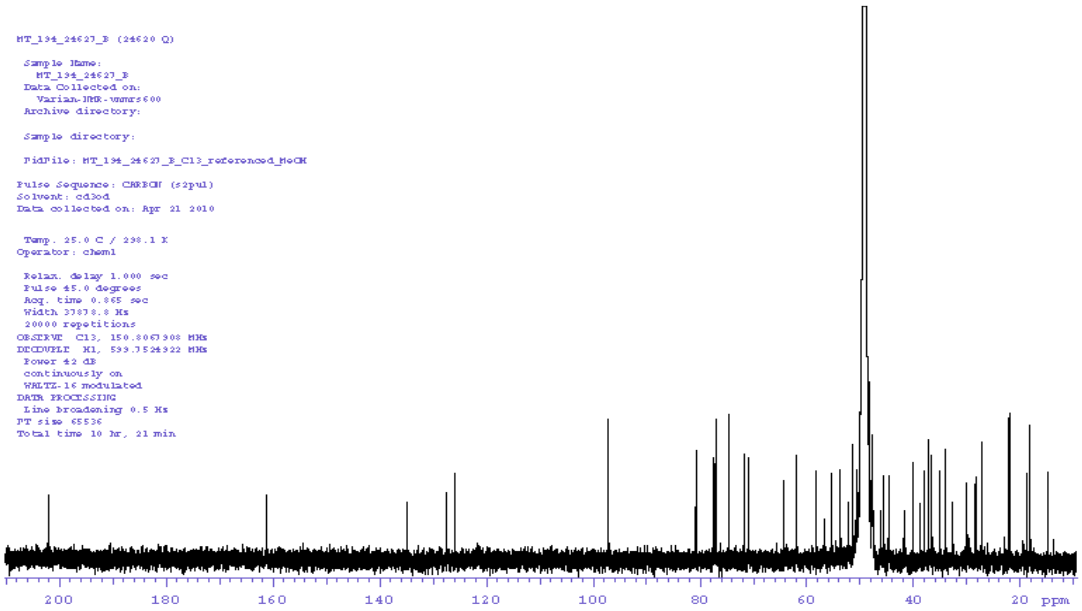



**Appendix Figure 5.3 $C^{13}$ NMR spectrum for compound 4 (600 MHz Varian NMR in *d-methanol*). Refer to page 48.**

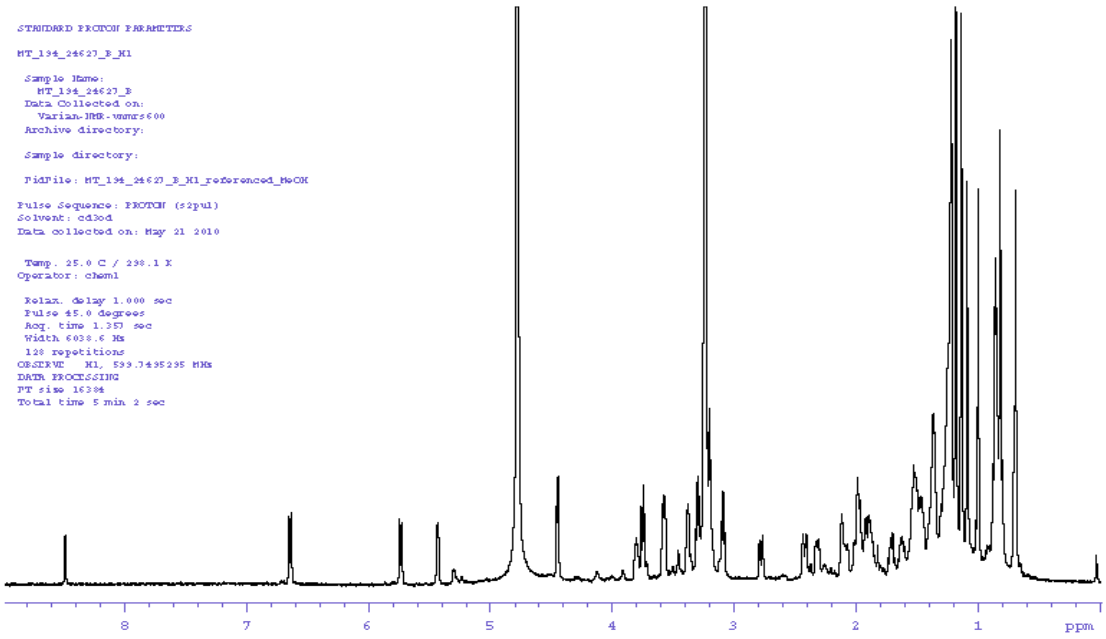



**Appendix Figure 5.4 $H^{1}$ NMR spectrum for compound 4 (600 MHz Varian NMR in *d-methanol*).**

## Compound 7



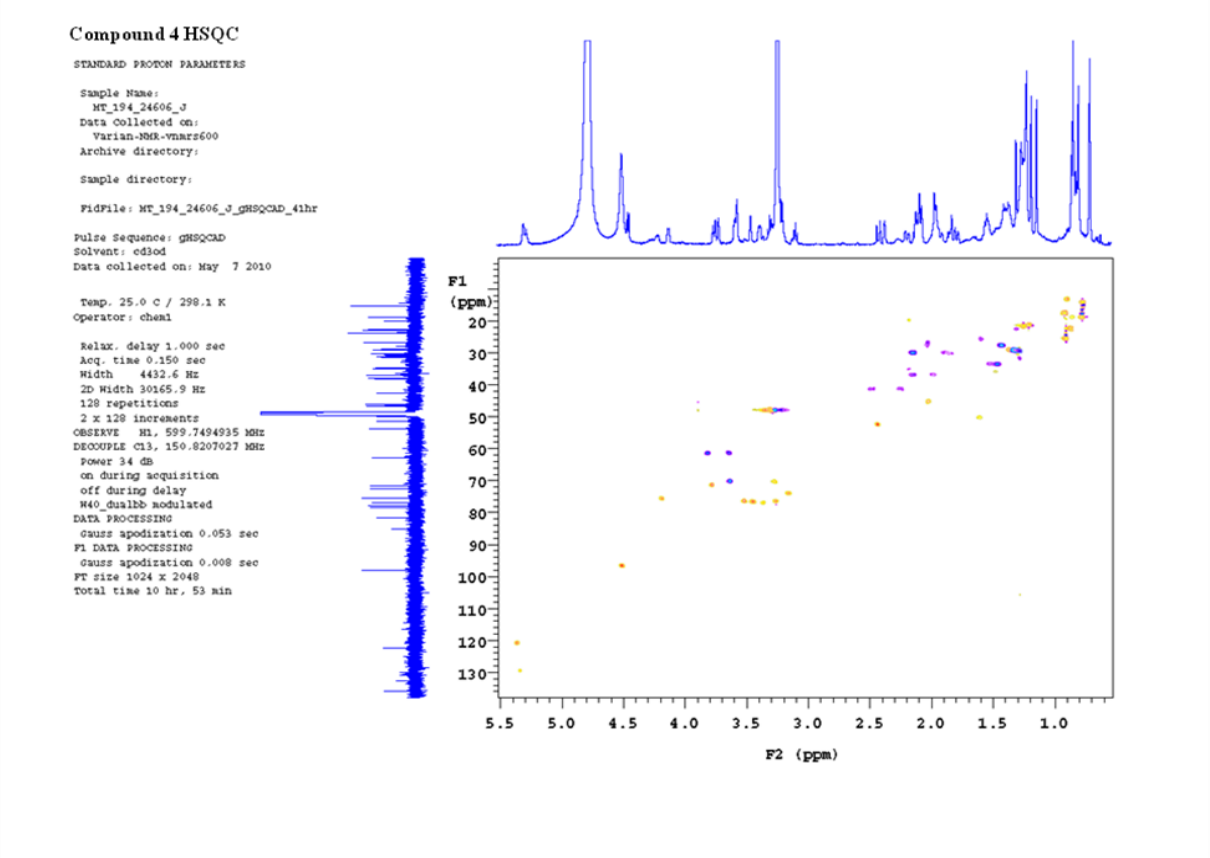

**Appendix Figure 5.5 $C^{13}$ NMR spectrum for compound 7 (600 MHz Varian NMR in *d-methanol*). Refer to page 55.**



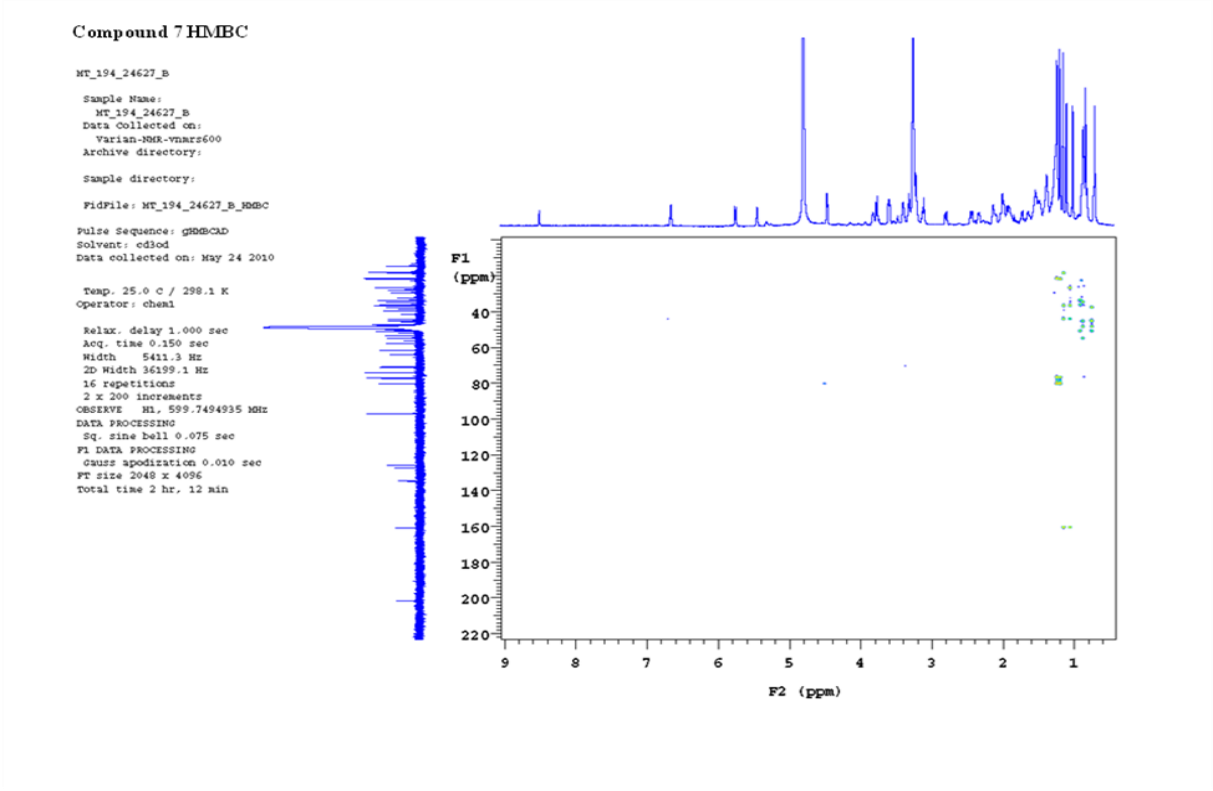

**Appendix Figure 5.6 $H^{1}$ NMR spectrum for compound 7 (600 MHz Varian NMR in *d-methanol*).**

## Various 2D data processed

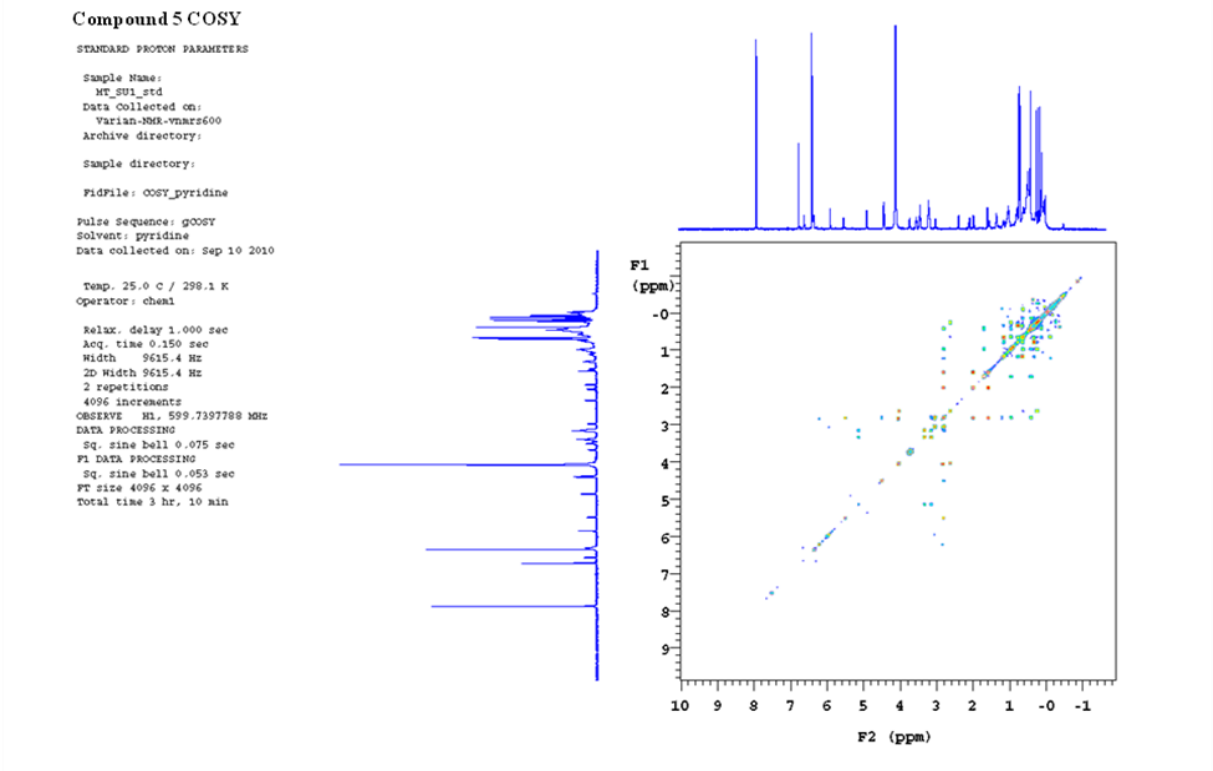
## HSQC of compound 4



## HMBC of compound 7

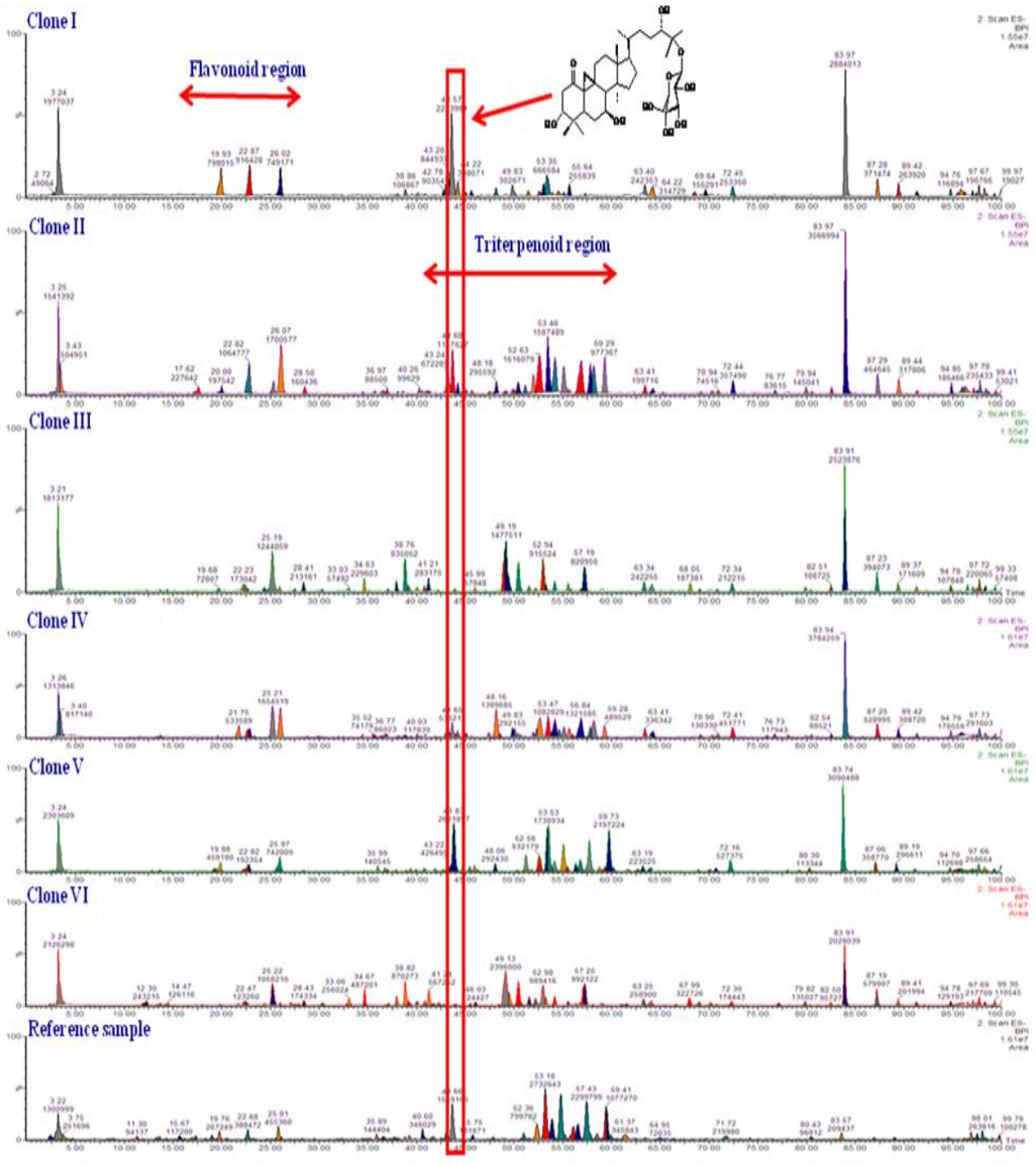



## COSY of compound 5



## APPENDIX 6

## SIX *S. FRUTESCENS* SAMPLE DATA




**Appendix Figure 6.1 Full chemical profiles ($ESI^-$ chromatograms) for clone samples I to VI and the reference material (MeOH extract). Refer to page 65.**

# APPENDIX 7

# DATA GENERATED FOR BIOLOGICAL SCREENINGS

## Appendix Table 7.1 Anti-cancer assay data results calculated with standard deviations (SD). Statistical analysis n = 2. Refer to pages 76 to 87.

| % Viability results | | | | | | |
|---|---|---|---|---|---|---|
| **SAMPLES (µg/ml)** | **UACC62** | **SD** | **MCF7** | **SD** | **HL60** | **SD** |
| **Ctrl** | 100 | 0 | 100 | 0 | 100 | 0 |
| **Extract A (100)** | 23.48407 | 0.04494 | 32.37519 | 2.89024 | 18.35066 | 2.05154 |
| Extract A (50) | 87.6565 | 1.85875 | 92.69469 | 5.7216 | 100.11704 | 1.38385 |
| Extract A (25) | 102.96765 | 2.10564 | 94.01483 | 7.78995 | 113.01877 | 4.01386 |
| **Extract B (100)** | 33.69003 | 1.71352 | 37.83866 | 3.11077 | 19.13639 | 8.44171 |
| Extract B (50) | 87.58415 | 0.07181 | 72.16467 | 3.9936 | 99.19836 | 13.13688 |
| Extract B (25) | 99.07113 | 5.80248 | 85.95438 | 1.34174 | 110.29158 | 3.95349 |
| **Extract C (100)** | 47.53136 | 0.55679 | 42.83491 | 2.69765 | 16.38217 | 18.63402 |
| Extract C (50) | 82.98204 | 1.86738 | 90.33509 | 2.61133 | 81.31711 | 7.65688 |
| Extract C (25) | 90.28356 | 1.33388 | 104.71191 | 2.69874 | 97.74518 | 7.63003 |
| **Extract D (100)** | 60.28127 | 3.28024 | 55.43637 | 0.39913 | 70.15985 | 17.71078 |
| Extract D (50) | 90.83639 | 3.38421 | 66.65241 | 4.17172 | 105.32235 | 0.80622 |
| Extract D (25) | 97.844 | 6.12998 | 89.34703 | 2.29034 | 109.99995 | 7.24951 |
| **Extract E (50)** | 86.44057 | 1.48451 | 81.84864 | 1.65842 | 95.65806 | 4.78073 |
| Extract E (25) | 99.07443 | 2.20003 | 105.14393 | 3.6159 | 115.27623 | 4.89714 |
| **Extract F (100)** | 59.15935 | 5.75346 | 62.19129 | 10.45318 | 32.64957 | 4.22293 |
| Extract F (50) | 77.91523 | 3.67983 | 117.92915 | 8.09439 | 96.7739 | 4.64393 |
| Extract F (25) | 85.33684 | 8.19225 | 126.50605 | 18.94846 | 133.17079 | 5.09545 |
| **Extract G (100)** | 48.93725 | 7.94231 | 44.38272 | 0.15353 | 22.85188 | 6.00007 |
| Extract G (50) | 85.84547 | 0.75865 | 114.04423 | 0.62541 | 76.60875 | 7.03748 |
| Extract G (25) | 95.41544 | 1.77026 | 128.96535 | 2.61178 | 87.97124 | 6.52025 |
| **Extract H (100)** | 7.41766 | 0.01602 | 5.45407 | 2.4754 | 5.00611 | 2.25057 |
| Extract H (50) | 72.79403 | 3.46097 | 58.45799 | 3.58834 | 53.95347 | 2.2427 |
| Extract H (25) | 95.11126 | 7.55796 | 122.70151 | 3.40399 | 84.05935 | 5.15066 |
| **Extract I (100)** | 16.78041 | 2.75994 | 32.43819 | 0.02848 | 12.7739 | 0.6705 |
| Extract I (50) | 80.40379 | 2.00504 | 123.35906 | 5.2609 | 80.87556 | 7.1187 |
| Extract I (25) | 97.76921 | 2.13162 | 142.66677 | 1.21836 | 94.0724 | 0.24455 |
| **Extract J (100)** | 58.85738 | 2.37813 | 48.87008 | 1.20663 | 13.85412 | 11.66793 |
| Extract J (50) | 90.97793 | 2.01284 | 67.77294 | 2.35168 | 81.01717 | 0.89438 |
| Extract J (25) | 100.78209 | 2.11408 | 88.57625 | 1.23345 | 91.26911 | 3.37189 |
| **Extract K (100)** | 45.50335 | 6.06549 | 46.43662 | 0.91167 | 36.01832 | 2.49817 |
| Extract K (50) | 91.0577 | 2.93531 | 86.49719 | 4.12504 | 109.21898 | 0.88115 |
| Extract K (25) | 97.03052 | 3.0347 | 102.87426 | 3.49163 | 120.85974 | 8.18933 |
| **Fraction i (25)** | 94.79116 | 1.95379 | 98.68143 | 1.68232 | 96.71377 | 1.06085 |
| **Fraction ii (25)** | 87.66729 | 1.20318 | 97.59378 | 0.10452 | 80.29478 | 3.86201 |
| **Fraction iii (25)** | 86.28014 | 4.26566 | 91.00992 | 0.11693 | 50.46192 | 3.28457 |
| **Fraction iv (5)** | 90.28384 | 1.91369 | 97.57541 | 0.3921 | 91.51932 | 1.81187 |
| **Fraction v (5)** | 94.35947 | 3.00776 | 98.87651 | 0.01843 | 106.05186 | 2.90202 |
| **Fraction vi (5)** | 93.82518 | 1.99376 | 100.20876 | 0.48751 | 106.27041 | 3.3049 |
| **Compound 5 (5)** | 97.16235 | 3.28054 | 99.66922 | 0.31989 | 104.6595 | 0.03862 |

**Appendix Table 7.2 Cytokine assay data calculated with standard deviations (SD). Statistical analysis n = 4. Refer to pages 87 to 99.**

| | | IL1β | SD | IL6 | SD | IL10 | SD | IL12p70 | SD | TNF | SD | IL8 | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | **Control** | 50.83 | 7.73706 | 50.83 | 7.73706 | 50.83 | 7.73706 | 50.83 | 7.73706 | 50.83 | 7.73706 | 50.83 | 7.73706 |
| **2** | **PMA** | 14.86 | 3.6911 | 10.37 | 1.30108 | 11.21 | 1.54149 | 19.29 | 1.14551 | 129.69 | 19.2106 | 4551.95 | 410.8487 |
| **3** | **Ext C ethanol** | 5.62 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50.4125 | 8.72949 |
| **4** | **C + PMA** | 8.40333 | 5.61513 | 7.67 | 1.89505 | 8.815 | 2.41123 | 14.075 | 4.02344 | 184.3675 | 44.50132 | 4449.638 | 291.5709 |
| **5** | **Ext A (aq fresh leaf)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 43.965 | 18.3664 |
| **6** | **A + PMA** | 14.69 | 0.39598 | 9.615 | 0.07778 | 11.9 | 1.76777 | 19.29 | 1.14551 | 176.1425 | 34.28128 | 3333.85 | 254.626 |
| **7** | **Ext B (aq fresh stem)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 38.72 | 6.61508 |
| **8** | **B + PMA** | 15.36 | 1.86676 | 10.465 | 0.16263 | 10.85 | 0.28284 | 17.305 | 1.09602 | 159.12 | 53.0709 | 3372.813 | 180.1947 |
| **9** | **Ext D (aq dry leaf)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 47.5375 | 9.56774 |
| **10** | **D + PMA** | 16.605 | 4.65983 | 10.435 | 1.54856 | 13.175 | 1.63342 | 18.97 | 3.97394 | 152.3975 | 24.77091 | 3168.28 | 252.5174 |
| **11** | **Ext I (butanol)** | 7.09667 | 4.25187 | 6.92 | 0 | 5.585 | 1.492 | 8.15333 | 3.92826 | 4.8125 | 2.23284 | 38.145 | 6.08923 |
| **12** | **I + PMA** | 5.085 | 2.11425 | 3.07 | 0.07071 | 0 | 0 | 6.84 | 0.42426 | 172.4875 | 11.73381 | 3600.035 | 184.7743 |
| **13** | **Ext E (DCM:MeOH)** | 11.2 | 0 | 6.33 | 0 | 8.96 | 0 | 10.25 | 0 | 5.02667 | 3.33708 | 43.95 | 10.18165 |
| **14** | **E + PMA** | 4.01667 | 1.24134 | 3.02 | 0 | 4.53 | 0 | 6.14333 | 0.68705 | 196.1375 | 33.12863 | 3968.628 | 87.37321 |
| **15** | **Fraction i** | 4.215 | 1.98697 | 0 | 0 | 0 | 0 | 0 | 0 | 4.385 | 0.36062 | 29.50333 | 2.59643 |
| **16** | **i + PMA** | 3.22 | 0 | 0 | 0 | 0 | 0 | 5.05 | 0 | 179.23 | 51.20867 | 2909.5 | 257.0757 |
| **17** | **Fraction ii** | 7.55 | 0.45255 | 5.185 | 0.79903 | 4.885 | 0.16263 | 8.745 | 0.10607 | 4.79 | 2.40504 | 42.24 | 10.62793 |
| **18** | **ii + PMA** | 5.29 | 0 | 5.09 | 0 | 0 | 0 | 6.54 | 0 | 179.5175 | 58.06748 | 3916.568 | 817.1514 |
| **19** | **Fraction iii** | 5.93 | 1.70994 | 4.62 | 0 | 6.05 | 0 | 7.07333 | 2.54449 | 4.72 | 3.22308 | 163.5725 | 40.50969 |
| **20** | **iii + PMA** | 5.35333 | 2.16569 | 4.435 | 1.05359 | 5.235 | 1.98697 | 7.27333 | 2.59257 | 229.445 | 13.89247 | 5967.925 | 226.8568 |
| **21** | **Echinacea** | 5.265 | 1.85969 | 3.445 | 0.74246 | 4.77 | 0 | 6.1 | 2.54558 | 4.54667 | 1.51398 | 33.54 | 3.564 |
| **22** | **Ech + PMA** | 4.63333 | 0.33501 | 3.965 | 0.13435 | 4.53 | 0 | 6.04333 | 0.91106 | 180.1225 | 57.76763 | 3260.92 | 250.3971 |
| **23** | **24H EtOH** | 9.08 | 4.3428 | 7.54 | 1.15966 | 8.72 | 1.24451 | 11.12 | 4.52607 | 7.96 | 2.46246 | 69.27 | 2.28171 |
| **24** | **24H EtOH + PMA** | 5.13 | 2.43358 | 4.62 | 0 | 4.77 | 0 | 6.095 | 1.47785 | 166.78 | 7.15367 | 4002.553 | 414.8947 |
| **25** | **Compound 5** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 53.745 | 29.64469 |
| **26** | **5 + PMA** | 18.22 | 5.38815 | 11.64 | 3.40825 | 15.105 | 4.75883 | 20.1 | 6.1094 | 206.405 | 48.44754 | 3846.77 | 262.1111 |
| **27** | **Fraction iv** | 3.59 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.1 | 0 | 73.9075 | 16.00801 |
| **28** | **iv + PMA** | 14.115 | 3.62746 | 10.045 | 3.83959 | 11.135 | 3.25976 | 17.48 | 5.47301 | 180.1725 | 35.36543 | 5277.695 | 541.9048 |
| **29** | **Fraction v** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 44.9325 | 19.09687 |
| **30** | **v + PMA** | 13.71333 | 9.52696 | 11.715 | 2.5668 | 15.035 | 5.81949 | 16.07667 | 11.47788 | 194.93 | 31.41209 | 3601.96 | 229.3982 |
| **31** | **Fraction vi** | 4.3 | 0 | 0 | 0 | 0 | 0 | 5.5 | 0 | 4.72 | 0 | 39.9775 | 9.05988 |
| **32** | **vi + PMA** | 14.97 | 6.64017 | 9.58667 | 5.86989 | 12.34333 | 7.91975 | 17.86333 | 9.1225 | 164.28 | 47.64315 | 4231.76 | 500.7884 |