Effects of environmental growth conditions on the levels of sutherlandins 3 and 4 and sutherlandiosides B and D, in *Sutherlandia frutescens* (L.) R. Br.

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A thesis submitted in partial fulfillment of the requirements for the degree of Magister Scientiae at the South African Herbal Science and Medicine Institute, University of the Western Cape.

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Keywords

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Sutherlandiosides

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HPLC

LC-MS

ABSTRACT

Effects of environmental growth conditions on the levels of sutherlandins 3 and 4 and sutherlandiosides B and D, in *Sutherlandia frutescens* (L.) R. Br.

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M.Sc. Thesis South African Herbal Science and Medicine Institute, University of the Western Cape.

Sutherlandia frutescens (L.) R. Br. (*Fabaceae*), indigenous to the Western Cape region of South Africa, is found in a Mediterranean-type climate known for its many environmental stressors that can influence the levels of metabolites found in plants. *Sutherlandia frutescens* contains many known potential active constituents among them, flavonoids such as sutherlandins 3 and 4 (Su3 and Su4) and terpenoids such as sutherlandiosides B and D (SuB and SuD). Whether the profiles and levels of Su3, Su4, SuB and SuD are significantly affected by the environmental factors found in this area is however, unknown.

iBatech[TM] is an ethanolic plant extract that is manufactured by researchers in the Department of Medical Biosciences, UWC, for use as a pesticide. HPLC analysis performed on *Lycopersicon species* treated with the iBatech[TM] product have shown that it also caused an increase in the concentrations of total polyphenols in the plant (Klaasen *et.al., unpublished data*). Whether the treatment with iBatech[TM] might also

cause an increase in the polyphenols such as sutherlandins 3 and 4 and sutherlandiosides B and D is also unknown.

The objectives of this study were to determine the concentrations of sutherlandins 3 and 4 (Su3 and Su4) and sutherlandiosides B and D (SuB and SuD) in *S. frutescens* collected from different sites and after the treatment with the iBatech^{TM} product. The specific objectives were: *a)* to locate and categorize sites where *S. frutescens* is grown, based on a selection of pertinent environmental growth factors, *b)* to determine and compare the concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens* collected from the different environmental growth sites and after treatment with the iBatech^{TM} product.

To realize these objectives, *S. frutescens* samples were collected from eight different sites and broadly categorized into three environmental categories. A high-performance liquid chromatography (HPLC) method using diode array ultraviolet detection (HPLC-DAD) for the simultaneous analysis of flavonoids and terpenoids was developed and validated, and used for the profiling and determination of the average levels of sutherlandins 3 and 4 and sutherlandiosides B and D in the samples from the sites and that treated with the iBatech^{TM} product.

The Kruskal-Wallis test was used to determine statistically significant differences among the environmental categories. The post ANOVA, Dunn's Multiple Comparison test was performed to determine which groups were significantly different. The Mann-Whitney, two-tail, t-test was used to compare each environmental category to the standard and the column statistics of the raw data was

analyzed to determine significant differences among samples from the same environmental category.

In the samples collected from the sites, the values represent the average levels of metabolites for each environmental category whereas the significance values indicated were among samples from the same environmental category. The levels for sutherlandin 3 were Afriplex^{TM} (Std.) 2495.08, the natural field (NF) 2810.33 *(P=0.0005),* the cultivated field (CF) 2519.81 and the greenhouse (GH) 2580.25. The levels for sutherlandin 4 were significantly different when comparing the (NF) 1495.67 *(P=0.0001),* (CF) 3114.42 *(P=0.0140)* and (GH) 2361.72 *(P=0.0001),* with the CF group showing the highest levels of Su4 and the NF showing the lowest. The levels for sutherlandioside B were (NF) 189.7 *(P=0.0189),* (CF) 594.56 *(P=0.0140)* and (GH) 326.72 *(P=0.0001),* however, the CF group showed the highest average levels for SuB. The levels for sutherlandioside D were (NF) 144.1 *(P=0.0192),* (CF) 544.37 *(P=0.0308)* and (GH) 387.49 *(P=0.0001),* with the NF category having the lowest average levels.

In the iBatech^{TM} treated samples, the values indicate the average levels of three samples in each treatment group. The levels for sutherlandin 3 were (control) 9758.43, the (50%) 2232.63 and the (100%) 2031.97 treatment groups. The levels for sutherlandin 4) were (control) 2241.63, the (50%) 2247.47 and the (100%) 2392.60, with the 100% treatment group having the highest levels. The levels for sutherlandioside B were (control) 289.66, the (50%) 284.93 and the (100%) 332.30. The levels for sutherlandioside D were (control) 282.77, the (50%) 280.60 and the (100%) 315.13 treatment groups, with the 100% treatment group having the highest

levels. The levels of Su3, Su4, SuB and SuD were significantly different *(P=0.0001)* among all treatment groups.

In conclusion, the data shows that only sutherlandin 4 (Su4) was significantly different when comparing the environmental groups. Due to the significant differences in the Su3, Su4, SuB and SuD levels among samples from the same group the levels of these metabolites cannot be correlated with the environmental groups.

DECLARATION

I, Darryn Whisgary, declare that "Effects of environmental growth conditions on the levels of sutherlandins 3 and 4 and sutherlandiosides B and D, in *Sutherlandia frutescens* (L.) R. Br." is my own work, that it has not been submitted for any degree or examination to any other University, and that all sources that I have used or quoted have been indicated and acknowledged by means of complete references.
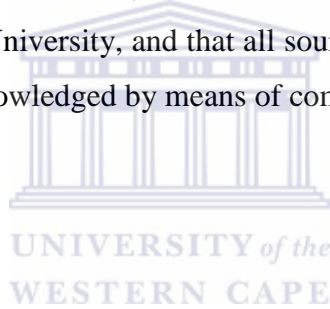
Signature   **August 2011**

I hope that my achievements in life shall be these -- that I will have fought for what was right and fair, that I will have risked for that which mattered, and that I will have given help to those who were in need, that I will have left the earth a better place for what I've done and who I've been.

C. Hoppe

ACKNOWLEDGEMENTS

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CHAPTER 1

Introduction

Sutherlandia frutescens (L.) R. Br. (*Fabaceae*) synonymous with *Lessertia frutescens*, indigenous to the Western Cape region of South Africa, has been used extensively, by many cultures inhabiting this area. *Sutherlandia* has over 25 vernacular names but some of the most prominent are: "Cancer Bush"; "Kanker-Bos"; "Spear of the blood" and "Unwele". The natural environment in which *Sutherlandia* is found is in the Mediterranean-type climate of South Africa. This climate has long warm summers and cool moist winters (Aschmann, 1973). Some of the environmental stresses experienced by *Sutherlandia* in its natural environment include: water-deficit stress, nutrient stress and salt stress, among others. In general such stressors affect the plant's phenology and physiology, which in-turn affects the production/ synthesis of chemical constituents (secondary metabolites) in the plant. A previous study which tested whether water-deficit stress affected *Sutherlandia's* physiology, indicated a decrease in carbon assimilation and nitrogen fixation, suggesting that the concentrations of secondary metabolites in this medicinal plant can indeed vary among the same species grown under different conditions (Whisgary *et. al.,* unpublished data).

Sutherlandia species contains many known constituents and as the plant is being more intensely investigated for therapeutic use new constituent molecules such as flavonoids and terpenoids are still being discovered. It is commonly known that *Sutherlandia* contains: free-amino acids such as asparagine, non-protein amino acids like canavanine,

pinitol, flavonoid glycosides and triterpenoid saponins, among others. The focus of this study is on the flavonoid glycosides and triterpenoid saponins found in *Sutherlandia frutescens* samples.

Saponins are *amphiphilic*, non-volatile, surface-active triterpene glycosides that are found in a large number of *Fabaceae* species (Lasztity, *et. al.,* 1998), and are bitter in taste (Price *et. al.,* 1985). Triterpene saponins have attracted great interest due to their pharmacological properties, namely anti-HIV, anti-cancer, anti-tumor, anti-diabetic, anti-inflammatory and hepatoprotective properties, among others (Dini *et. al*., 2008). Triterpenoid saponins were first detected in *S. microphylla* in (1969) by Brümmerhoff and isolated to a pure compound from *S. frutescens* by Gabrielse (1996). Moshe (1998) has reported a complex pattern of triterpene profiles in the various species, but limited variations in the same population. Fu *et. al.,* (2008) recently discovered four new cycloartane glycosides respectively known as sutherlandiosides A, B, C and D. Cycloartane glycosides are believed to have immunostimulatory, anti-bacterial (Calis *et. al*., 1997), anti-tumor (Abdallah *et. al*., 1993), as well as chemopreventative effects (Tian *et. al*., 2005). Although Moshe (1998) has reported limited variations in triterpene profiles in the same population, it is however expected that the quantitative and qualitative compositions of these compounds in the plants, in addition to species variations, can also be affected by a number of environmental factors such as micro-climate, season and plant development stage. Whether changes in environmental growth conditions affect the levels of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens* is unknown.

Due to the multitude of factors that affect the chemical composition, and thus the potency of herbal medicines, the development and implementation of standardized herbal preparations are challenging. Whether the environmental growth conditions might affect the profile and level of the potential active constituents of *S. frutescens,* should enable one to produce better quality preparations of this widely used indigenous medicine. Various attempts have been made to increase the synthesis of secondary metabolites in plants (Bourgaud *et. al.,* 2001).

iBatech[TM] is an ethanolic plant extract that is manufactured by researchers in the Department of Medical Biosciences, UWC, for use as a pesticide to protect the plants from various pathogenic conditions. HPLC analysis performed on *Lycopersicon species* treated with the iBatech[TM] product have shown that it also caused an increase in the concentrations of total polyphenols in the plant (Klaasen *et.al., unpublished data*). Whether treatment with iBatech[TM] might also cause an increase in the polyphenols such flavonoid and cycloartane glycosides found in *Sutherlandia* is also unknown.

This study was aimed at determining the effects of environmental conditions and treatment with iBatech[TM] on the concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens* plants. The specific objectives were:

- to locate sites where *S. frutescens* is grown, broadly categorize the sites into environmental groups based on the pertinent environmental growth factors,

- to develop and validate a high-performance liquid chromatography – diode array detector method for Su3, Su4, SuB and SuD analysis,

- to determine and compare the profiles and levels of Su3, Su4, SuB and SuD *S. frutescens* collected from the different environmental growth sites and

- to determine whether the application of the iBatech[TM] product increases the number and concentrations of secondary metabolites in *S. frutescens*.

It was hypothesized that:

- the profiles and concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens*, collected from different sites, would vary and

- the application of the iBatech[TM] product will increase the number and concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens*.
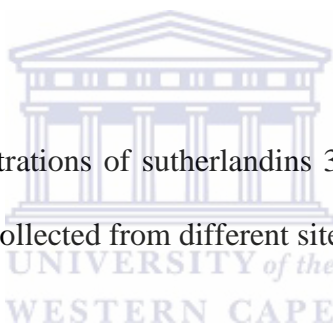
CHAPTER 2

Literature review

The botany, classification of *Sutherlandia*, and its use in disease management, its primary and secondary metabolites, terpenoids and its use in disease management, the synthesis and chemistry of terpenoids, factors affecting metabolite synthesis, cycloartane glycosides and sutherlandiosides, scientific strategies to improve the synthesis of secondary metabolites, the environmental conditions in which *S. frutescens* is found, the intended uses of the iBatech^{TM} product and the use of high performance liquid chromatography for the assaying of terpenoids are reviewed and discussed here.

2.1 Botany and classification of *Sutherlandia*

Sutherlandia frutescens (L.) R. Br. (synonymous with *Lessertia frutescens*) is a perennial shrub, reaching a height of approximately two meters. It has a pinnately compound leaf structure with large bright-red flowers. The fruits are very distinctive and appear as inflated, bladdery/ papery pods (Phillips *et. al*., 1934). *Sutherlandia* is a pioneer plant, the first inhabitant plant in a new ecosystem, where no other plants seem to grow, and thus, can withstand harsh environmental conditions.
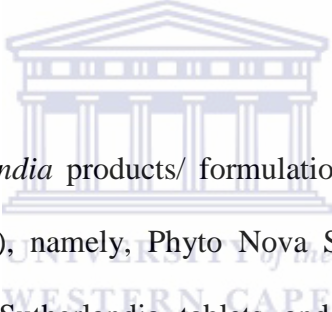
Figure 2.1: Freshly harvested *Sutherlandia frutescens*

With characteristic bright-orange flowers and papery seedpods.

In classification, prominent geographical variations have been recorded within the genus *Sutherlandia,* with two species *S. frutescens* and *S. tomentosa,* comprising several regional forms (van Wyk *et. al.,* 1997). *Sutherlandia* is also morphologically and chemically similar to the genera *Lessertia*, *Astragalus* and some other members of the Galegeae tribe but the genetic evidence to prove that it is in fact the same as *Lessertia,* is insufficient. A study conducted by van Wyk *et. al.,* (1997) using enzyme electrophoresis concluded that even though there are genetic differences in *Sutherlandia*, it is however difficult to distinguish these differences.

2.2 The use of *Sutherlandia* in disease management

Human immunodeficiency virus, acquired immune-deficiency syndrome (HIV/ AIDS), tuberculosis, diabetes mellitus, stress and cancers are all forms of diseases. They place a significant burden on the healthcare system and are becoming increasingly difficult to treat, cure and manage. All South Africans are either directly or indirectly affected by a number of infectious diseases. It is on a constant increase, due to factors such as; a poor economy; lack of education; lack of hope; lack of disease curative-effect and low patient drug compliance. *Sutherlandia* is used traditionally for the treatment of HIV/ AIDS, diabetes mellitus, tuberculosis, internal cancers, hypertension, as an anti-analgesic and anti-inflammatory.

Many pharmaceutical *Sutherlandia* products/ formulations are available on the market (Hess *et.al., unpublished data*), namely, Phyto Nova Sutherlandia Su1™ tablets and capsules, African Botanicals Sutherlandia tablets and gels, Big Tree Sutherlandia/ African Ginseng™ tablets and capsules, Biogenesis Anti-aging Sutherlandia capsules, Terra Treatment Sutherlandia tablets, gels and capsules and Bioharmony Bio-Sutherlandia tablets but unfortunately evidence supporting the use of *Sutherlandia* in disease management is lacking.

Very little is known about how *Sutherlandia* shows efficacy in HIV/ AIDS patients. A clinical trial by Johnson *et. al.* (2007) concluded that diseased patients showed improved levels of energy and fitness, improved sleep patterns, an increase in appetite and a

significant increase in weight gain only among ill individuals. Other results report an increase in the count of CD4 cells and a decrease in the viral load in AIDS patients.

A number of studies have been done to test the anti-oxidative, anti-inflammatory, anti-analgesic and antibacterial effects of *Sutherlandia*. Anti-oxidative studies done by Tai *et. al.* (2004) and Fernandes *et. al.* (2004) showed the inhibition of the production of nitrous oxide (NO) by lipopolysaccharide-stimulated cells, and the aqueous extract of *Sutherlandia* captured a significant number of superoxide and hydrogen peroxide radicals and converted them to nontoxic molecules. Anti-inflammatory studies by Tai *et. al*. (2004), showed that the administration of *Sutherlandia* did not significantly inhibit the production of anti-inflammatory cytokines, i.e. TNF and IL-1, while Ojewole (2004) suggests that *Sutherlandia* administered during inflammation, has similar effects to that of the non-steroidal diclofenac, which inhibits prostaglandin and cyclooxygenases. Ojewole (2004) also showed that *Sutherlandia* significantly affected thermal and chemical pain in mice but these effects were dosage-dependant. An increase in dose resulted in an increase in the effect.

Sutherlandia is believed to be an adaptogen. An adaptogen is any substance or chemical compound, which acts by regulating the body's defences and plays an important role in homeostasis of the body's metabolic functions (Brekhman *et. al.,* 1969). It assists the body in defending itself against invading stressors and allows adaptation to sudden pressures more easily. Smith *et. al.* (2004) suggests a significant reduction in the stress levels of patients taking *Sutherlandia*. The administration of *Sutherlandia* has shown to

significantly reduce the percentage of corticosteroid (Smith *et. al.,* 2004), with no effects on testosterone levels. This could be the main cause for mood enhancement and general improvement in health (Van Wyk *et. al.,* 2004) in AIDS and cancer patients. The fact that testosterone was unaffected by *Sutherlandia* could also relate to the weight gain and general strength seen in these chronically ill patients as described by Johnson *et. al.* (2007).

Sutherlandia frutescens "*Cancer Bush*" has been used traditionally for the treatment of all the above-mentioned disease states. Due to this, the Medical Research Council (SAMRC) of South Africa has declared this plant safe for use in clinical trials (Johnson *et. al.,* 2007), to assess and substantiate its use in the treatment of disease. Results obtained from clinical trials on *Sutherlandia* show an increase in CD4 T-cell count, believed to be due to the chemical L-canavanine, and an overall improvement in the quality of life in these patients due to the remaining actives. This knowledge has brought us to examine the means of increasing the active chemicals found in *Sutherlandia,* both primary and secondary, to improve *Sutherlandia*'s therapeutic ability.

2.3 Primary and secondary metabolites found in *Sutherlandia frutescens*

Intensive *Sutherlandia* research shows that the leaves contain a complex mixture of both primary and secondary metabolites namely the primary non-protein amino acids such as L-canavanine, L-arginine, pinitol, amino butyric acid (GABA), asparagines (Tai. *et. al.,* 2004) and the secondary flavonoids and terpenoids such as sutherlandins 1,2,3 and 4 and sutherlandiosides A,B,C and D respectively (Fu *et. al.,* 2008). Many of the primary

metabolites have already been studied and a fair knowledge of their possible pharmacological properties gained. The secondary metabolites have however not been investigated intensely enough, but is believed to have a wide range of pharmacological properties.

L-canavanine is a non-protein amino acid and is derived from hydroxylamine. It is only present in plants from the *Fabaceae* family (Bell, 1958), and to date 60% of plants tested from this plant family have shown to contain canavanine. *Sutherlandia* was found to contain an average of 2.2 – 3 mg/g to dried plant material of canavanine. It is one of the most active ingredients found in *Sutherlandia* as it has many medicinal attributes (Swaffar, 1995 and Green, 1998) and is also an analogue to the amino acid L-argenine (van Wyk *et. al*., 2008).
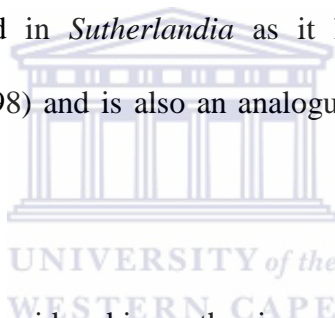
L-arginine is an alkaline amino acid and is another important constituent of *Sutherlandia*, found in concentrations of 3mg/g of dried plant material (Van Wyk *et. al*., unpublished data). L-arginine is involved in many fundamentality important bodily functions. It can be synthesized from many different proteins and is particularly rich in nitrogen concentrations. L-arginine acts as a precursor for nitrogen oxide (Andrew *et.al.,* 1999), which is essential for blood vessels, circulation and for the transmission of neurons in the brain. It is also said that L-arginine improves the function of the heart as it improves pumping action and also ensures the formation of various hormones. It is involved in the release of growth hormones from the pituitary gland, insulin from the pancreas and noradrenalin from the adrenal glands.
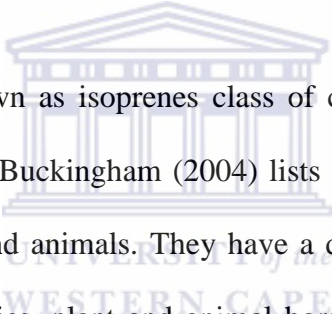
L-arginine also plays a fundamental role in the immune system. It improves cellular immune response, contributes to T-lymphocyte formation, minimizes their malfunction and stimulates phagocytosis. It also promotes the healing (Stechmiller *et.al.,* 2005 and Witte *et.al.,* 2003) of wounds through its involvement in protein synthesis and assists the body in excretion of excess nitrogen during the ureic cycle, when the liver splits L-arginine into urea and the amino acid ornithine.

D-pinitol is a glycan present in *Sutherlandia* (Snyders, 1965), and many other plants of the *Fabaceae* family. In the body, D-pinitol is converted into D-chiro-inositol, and the latter is involved in a number of metabolic processes, namely blood sugar metabolism (Bates *et. al*, 2000), as its role as a second messenger.

Gamma aminobutyric acid (GABA) is a by-product of the decarboxylization of glutamic acid. GABA and glycine are two of the most important neurotransmitter inhibitors in the central nervous system (van Wyk *et. al.,* 2008). GABA acts by binding to specific receptors in the plasma membrane of both pre-synaptic and postsynaptic neurons. GABA binding to these receptors causes the opening of ion channels to allow either the flow of negatively-charged chloride ions into the cell or positively-charged potassium ions out of the cell. This results in a negative charge in the transmembrane potential, causing hyper-polarization. GABA is modulated by numerous chemical substances which bind to different receptors, one of which affects the action of benzodiazepines, a group of substances with hypnotic, anxiolytic and anticonvulsive effects (Foster *et.al.,* 2006 and

Chapouthier, *et.al.,* 2001) thus *Sutherlandia*'s mood enhancement effects could also possibly be attributed to GABA (Sia, 2004).

Asparagine, the acid amide of asparaginic acid, is a non-essential hydrophilic amino acid present throughout the body. Asparagines and glycine cause postprandial glucagons secretion while argentine and leucine stimulate release of insulin. Insulin is responsible for blood-sugar regulation and promotes the synthesis of muscle protein whereas glucagon activates gluconeogenesis from amino acids in the liver, resulting in an efficient reduction of amino acid concentration in plasma.

Finally, the terpenes also known as isoprenes class of compounds, is one of the most diverse classes of metabolites. Buckingham (2004) lists over 30 000 forms in this class originating from both plants and animals. They have a diverse range of functions from flavours to fragrances, antibiotics, plant and animal hormones, membrane lipids, insect attractants and insect-deterrents, and also acts as mediators of the essential electron-transport processes during plant photosynthesis and respiration. Terpenes form part of a number of bioactive natural plant products, from the phytol side-chain of chlorophylls and the diterpenoid skeleton, and make this a very important class of compounds in drug discovery and human health. The terpenes found in *S. frutescens,* specifically sutherlandiosides B and D, are the main focus of this study.

2.4 Terpenoids and its use in disease management

Cycloartane glycosides are a form of triterpene glycoside. Reports suggest they have apoptotic activity due to cytotoxicity (Xiao *et. al.,* 2005) and this could be invaluable in anti-cancer research. Manners *et. al.,* (1999) developed a good understanding on the properties of terpenes and studies show that an increased amount of triterpenes are shown to have anti-malarial, anti-bacterial, anti-cancer, anti-HIV/AIDS (Kashiwada *et. al.,* 1998), anti-diabetic, anti-inflammatory (Banno *et. al.,* 2004), hepatoprotective (Visen, 1996) and anti-oxidant (Somova, 2003) among other properties. They also act on various stages of tumor development, including tumorigenesis inhibition (Lee *et. al.,* 2001 and Folkman, 1995), tumor promotion (Liu, 1995) and induction of tumour cell differentiation (Lee *et. al.,* 1994) with relatively high tumour cytotoxicity and low hepatotoxicity (Xiao *et. al.,* 2005). Terpenoids also have a high bioavailability due to their abundance in our diets (Crowell, 1999 and Fraser *et. al.,* 2004), which make these particularly attractive compounds for potential therapeutic agents.

2.5 Terpenoids

2.5.1 Terpenoid synthesis

Terpenoids are typically synthesized through 2 possible modes. The dimethylallyl pyrophosphate pathway (DMAPP), or biosynthesis of isopentenyl pyrophosphate pathway (IPP). The dimethylallyl pyrophosphate (DMAPP) and isopentenyl pysosphosphate (IPP) are the universal 5-carbon precursor molecules (Ainsworth, 2006) which combine to form the basic terpene structure. In green plants (Shah *et. al.,* 1969), algae (Schwender *et. al.,* 1996) and some bacteria (Pandian *et. al*., 1981) an alternative

pathway namely the 1-deoxyxylulose phosphate (1-DXP) pathway, has been discovered. This pathway synthesizes terpenes in the plastids of green tissues and oil gland cells.

The 1-DXP pathway, operating in plasmids, is responsible for the formation of essential oil monoterpenes (Eisenreich *et. al*., 1997), some sesquiterpenes (Adam *et. al.,* 1999), diterpenes (Eisenreich, 1996) and carotenoids and phytol (Lichtenthaler *et. al*., 1997). The mevalonate pathway is responsible for the synthesis of triterpenes, sterols and most sesquiterpenes (Lichenthaler, 1999). A study in lima bean by Piel *et. al.,* (1998) suggests that a limited degree of "cross-talk" occurs whereby the two pathways cross during the production of terpenes. This "cross-talk" is believed to be advantageous to the plant during nutrient stress.
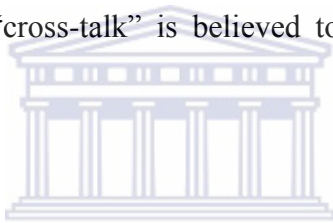
2.5.2 Factors affecting terpenoid synthesis

Generally, secondary metabolites are thought to be synthesized during adverse environmental conditions (Passam *et. al.,* 2007) as a protective measure taken by the plant, whereas other reports suggest the exact opposite. The argument being, that during adverse environmental conditions, essential soil nutrients often become unavailable to the plant. Often the syntheses of secondary metabolites rely on the availability of nutrients such as carbon and nitrogen molecules and if they are unavailable during adverse environmental conditions, how then can secondary metabolites be synthesized? A study conducted by Mosaleeyanon *et. al.* (2005) concluded that secondary metabolite concentrations were dramatically higher in plants grown in a controlled environment compared to the same plant species grown in the field. This could be as a result of

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photosynthesis-limiting factors such as temperatures that exceed the optimum for plant growth or wind, which reduces the relative humidity.

Secondary metabolite synthesis depends mainly on the physiological metabolism of many of the macronutrients, namely carbon and nitrogen. A previous study by Whisgary *et. al.* (*unpublished data*), indicated by, the rate of photosynthesis, the rate of transpiration, stomatal conductance as well as the assimilation of carbon and nitrogen, that water-deficit stress affects the physiology of *Sutherlandia frutescens* seedlings, possibly due to the unavailability of carbon and nitrogen. When macronutrients such as these become unavailable, the plant's physiology and metabolism is compromised, and could therefore affect the synthesis of secondary metabolites. A study by Haefele *et. al.* (2008) suggests that the efficiency and use of supplementary nitrogen is not affected by water-deficit stress, but depends greatly on species and cultivar. On the contrary, a study by Ercoli *et. al.,* (2007) suggests that the accumulation and remobilization of nitrogen is adversely affected during water-deficit stress and thereby affects plant biomass. Carbon molecules are one of the major components of the terpenoid structure and when carbon accumulation and assimilation is compromised it could have an effect on terpenoid synthesis. Bourgaud *et. al.,* (2001) explains that during the earlier growth stages the carbon assimilated is allocated to primary metabolism (the production of cell structures) and later when plant growth plateaus, carbon will no longer be required in large quantities and secondary compounds can be more actively synthesized.

2.6 Terpenoid chemistry

Terpenes are very widely spread in nature and occur in many plant and animal species including humans (Crozier *et. al.,* 2006), with a chemical structure based on multiples of C_5 units, and are often attached to a glycoside. Terpenes contribute to many of the aromatic properties of plants and range structurally from simpler to more complex molecules from C_5 units to C_{30} units (Ainsworth, 2006). Their structural complexity determines the chemical molecule they form e.g. latex, rubber and leaf waxes. They come from a class of compounds that are highly structurally diverse from one another and have extensive functional group chemistry. The simplest terpenes are called hemiterpenes (C_5) and are produced by modification reactions to either dimethylallyl pyrophosphate (DMAPP) or isopentenyl pysosphosphate (IPP) and include simple acids (isomers) such as tiglic acid and angelic acid, which form esters with many natural products. Monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes and steroids (C_{30}) and tetraterpenes (carotenoids, C_{40}) are all medicinally important.

Triterpenes (C30-derived terpenoids) has exceptionally wide distribution in humans, plants, fungi, bacteria, soft corals and amphibians. They include very important molecules such as steroids (Dharmananda, 2000), e.g. testosterone/ oestrogen, which are degraded triterpenes with fundamental importance in mammals, such as pheromones. Triterpenes are also components of resins and resinous exudates from plants and are often bitter in taste, a characteristic displayed by many triterpenes. They are usually produced as a physical barrier in injured plants, to prevent microbial attack on plants and therefore

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exhibit anti-bacterial and anti-fungal activity by either killing off invading microbes or down-regulating microbial growth, allowing the plant to heal itself (Crozier *et. al.,* 2006).

2.7 Cycloartane glycosides and sutherlandiosides

Glycoside is the collective name given to molecules that attach themselves to other molecules making them more structurally complex. The term "glycoside" is a generic term for a natural product that is chemically bound to a sugar. Thus, glycosides are made-up of two parts: *(i)* the aglycone and *(ii)* sugar (Dharmananda, 2000). The sugar can be bound to any type of molecule namely: flavonoids, terpenoids and coumarins among others. thus making the compound more hydrophilic. Glycosides come from one of two classes; *C-glycosides* or *O-glycosides* differing only by the type of bonds. *C-glycosides* have carbon-carbon bonding whereas *O-glycosides* have oxygen-carbon bonding types.
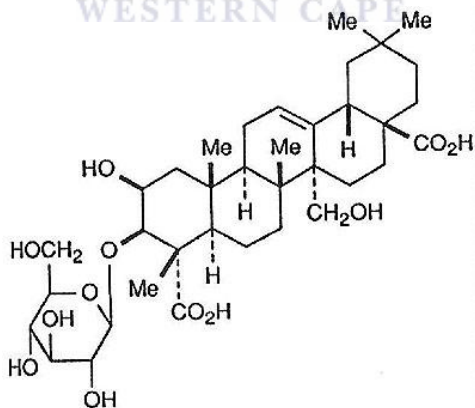
Figure 2.2: The basic chemical structure of a triterpene glycoside (Dharmananda, 2000)

Triterpene glycosides have a widespread distribution throughout the plant kingdom and are commonly/ collectively referred to as saponins due to their ability to form foams in solution. Triterpene glycosides have steroid-like structures and can have similar effects as steroids during overuse such as: hypertension and thrombosis.
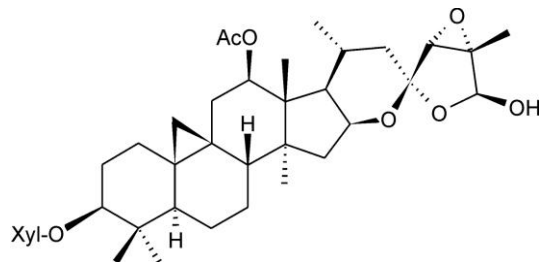
Figure 2.3: The basic chemical structure of a cycloartane glycoside (Gaube *et.al.* 2007**)**

Brümmerhoff (1969) first detected triterpene glycosides in *Sutherlandia microphylla* leaves but the chemical structures were not elucidated. Gabrielse (1996) isolated and purified the first reported triterpene glycoside from *S. microphylla* and this was later called sutherlandioside 1 (SU1). Moshe (1998) found that various *Sutherlandia* species contained complex patterns on triterpenes with limited variations with the same population but much larger variations between populations. Albrecht (*unpublished data*) also reported that cycloartane-type glycosides vary dramatically among geographic regions in South Africa and to date; at least 56 triterpene glycosides have been reported in *Sutherlandia.* The major triterpene in commercial *Sutherlandia* material is SU1 and has been structurally elucidated (Figure 2.4) by Fu *et. al.,* (2008) and Olivier *et. al.,* (2009).
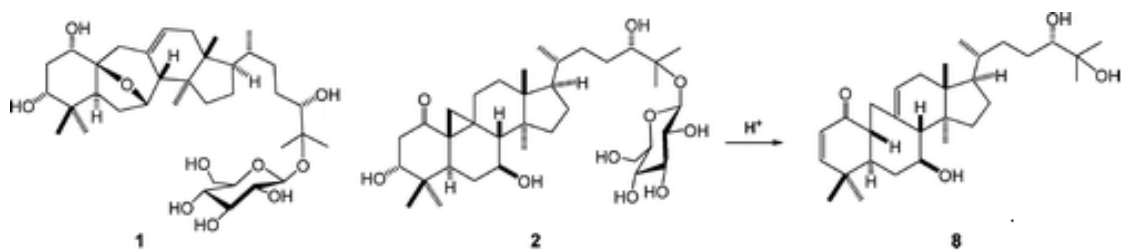
Figure 2.4: Structural elucidations of sutherlandiosides by Fu *et. al*. (2008)

2.8 Strategies to improve secondary metabolite synthesis

Over the last 50 years, various strategies have been extensively investigated to improve the production of secondary metabolites. Plant cell culture technology, plant physiology, plant anatomy, DNA sequencing and gene selection being a few of the prominent approaches explored. Despite great technological advances, biotechnological approaches to this have proven to be costly with few commercial successes (Bourgaud *et. al.,* 2001). Some researchers believe that plants grown in the natural field, exposed to abiotic and biotic factors can adversely affect the safety of these medicinal plants and could be harmful to consumers (Southwell *et. al*., 2001). Others such as Zobayed *et. al.,* (2004), report high secondary metabolite yields from medicinal plants grown in a controlled environment with high levels of CO_2 and light irradiation. However, with studies like these it is not totally clear whether the increase in secondary metabolites are due to the freely available CO_2 and light which maximizes photosynthesis or whether the plant is reacting to CO_2 and light radiation stress.

A study by Chinkwo (2005) to determine the effectiveness of *Sutherlandia* against cancer confirmed that *Sutherlandia* extracts has apoptotic inducing properties when tested

against Chinese hamster ovary (CHO), Caski and Jurkat T-lymphoma cell lines but are population, dose and location dependant. Tai *et. al.,* (2004) in a study using *Sutherlandia* ethanolic extract against human tumour cell lines showed anti-tumor properties but these results were dosage dependant and samples obtained from the Western Province area showed greater apoptotic inducing capabilities compared to samples from the Northern Province and Orange Free State. These observations suggest that the location where *Sutherlandia* is grown could affect the chemical composition, due to factors such as soil composition, percentage of annual rainfall and UV light intensity, which also leads to the synthesis of secondary metabolites (Wink, 1999). These factors can therefore either promote or compromise the synthesis of secondary metabolites.

2.9 Growth environments in which *Sutherlandia* is found

The Cape floristic region of South Africa is classified as a Mediterranean-type climate. This climate is comprised of long dry summers and cooler moist winters, with up to 60% of the annual rainfall occurring during the winter months (Aschmann, 1973). These Mediterranean-type biogeographical regions comprise of some of the most diverse flora in the world (Cowling *et. al.,* 1996) and are particularly threatened by global climate change (McCarthy *et. al*., 2001 and Lovette *et. al.,* 2005). The evergreen shrubs occurring in the Mediterranean-type climate of South Africa is collectively known as *Fynbos*. Structurally, they are generally smaller shrubs growing between 20 cm – 1.5 m in height with hairy leaves. With the predictable summer dry period (Cowling *et. al.,* 2005), this flora needs to be able to cope with negative xylem pressures while still transporting water to the leaves and actively expanding shoots (Agenbag, 2006). Mediterranean-type

climates contain from seasonal dimorphics to evergreen schlerophylls. These differ both physiologically as well as structurally in their ability to cope with adverse environmental conditions such as stress (Mooney *et. al*., 1970 and Werner *et. al*., 1999). Plants grown in warm climates such as these may be water stressed especially during late spring and summer (Haefele *et. al.,* 2008). The availability of water is generally an important factor essential for plant growth; however, it can often exceed threshold values before affecting phenology. Phenological development is only really affected under severe drought stress levels (Rassaa *et. al.,* 2008).

In severe stress conditions, plants synthesize various chemical compounds (Passam *et. al.,* 2007) to cope with stresses. Some of these stresses for which plants synthesize chemicals could be: pest; drought; saline soils; nutrient starvation; UV light intensity or any other adverse environmental conditions. Examples of such chemicals found in *Sutherlandia* are the primary metabolites L-canavanine, L-argenine, pinitol, GABA and asparagines and the secondary metabolites like flavonoids and terpenoids among others. This has lead us to examine the various environments in which *Sutherlandia* is grown and determine which environment is best suited for the production of metabolite-rich medicinal *Sutherlandia* plants.

2.9.1 Natural field conditions

There is no control over temperature, humidity, wind or light intensity, water regimes and any supplementary fertilization or pest control management. The survival of these plants are solely subjective to the micro-climatic conditions under which they are found and are

usually well adapted to suit those specific environmental conditions, often due to natural selection and survival of the strongest genes (Simões *et. al.,* 2007). These plants are often more acclimatized to harsher environmental conditions than plants grown in greenhouses or shade houses but are still at risk if one of the environmental conditions is drastically altered. If so, an entire plant population can be destroyed.

2.9.2 Cultivated field conditions

Usually water regime and supplementary fertilization or pest control is practiced but due to the open-air environment, wind, light intensity and humidity cannot be controlled. The major advantage of field cultivation is relatively low management costs when compared to the greenhouse but the disadvantage with field cultivation is that atmospheric temperatures cannot be maintained. The plants therefore need to withstand high midday temperatures and often drastically cooler night-time temperatures, during the summer months. During winter, plants often have to withstand temperatures below zero degrees Celsius while still maintaining an active metabolism. Plants cultivated in fields such as these are mostly species specific and depends greatly on the climatic conditions of the region.

2.9.3 Greenhouse conditions

Plants are grown in greenhouses for various reasons. One of the main reasons are to control the environmental conditions, namely irrigation, humidity, temperature and light intensity. Greenhouses are typically divided into two major sections, the glasshouse section, where seedlings and environmentally-sensitive plants are grown, and the shade-

house section, where the plants are placed to acclimatize before being introduced to the outside environment (Janick, 1963). The shade-house section is also used as a storage facility for plants waiting to be sold or moved out into the field. A shade-house is typically made of landscape shade netting and allows you to control wind and light intensity, water regime when coupled with an irrigation system and the use of supplementary fertilization and pest control. The three major shade net colours used for this purpose are black, green and white and inhibit 70%, 60% and 40% of light respectively. The major disadvantages of shade-houses are that neither humidity nor temperature can be controlled.

2.10 iBatech[TM] product

Klaasen *et. al*. (2009) patented a plant derived extract, which is used as an adjuvant. It is an ethanolic extract (patent ref. no. PA149863/P) of an indigenous South African plant and is believed to promote the synthesis of secondary plant metabolites. Some testing has been done using this product as an adjuvant spray application on agricultural crops resulting in an increase in total polyphenolics (Klaasen *et. al.,* unpublished data) when analyzed using HPLC. No literature has as yet been published on the iBatech[TM] product and no studies have been done on its possible mechanisms of action to date.

2.11 Techniques used to assay for terpenoids

There is a great need for standardizing commercial plant products by conducting quality control studies. Various assays have been reported, based on a wide variety of analytical techniques, including high performance liquid chromatography (HPLC), or capillary

electrophoresis with ultraviolet (UV) absorbance detection, HPLC with refractive index (RI) detection, HPLC with evaporative light scattering detection (ELSD), gas chromatography (GC) with flame ionization detection (FID), GC-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and liquid chromatography-mass spectrometry (LC-MS) (van Breemen *et. al.,* 2007). These means of assay have a considerable amount of advantages but also have limitations. It is thus important to understand the chemistry of the compound of interest to select the best-suited method for analysis.

Refractive index detection lacks both sensitivity and selectivity and is more suitable for monitoring preparative HPLC separations than for quantitative analysis. Evaporative light scattering detection responds to both terpenoids and flavonoids, although the standard curves are non-linear. However, like UV and refractive index detection, ELSD lacks selectivity. Although both GC with flame ionization detection and GC-MS are highly sensitive analytical techniques, GC-based methods require complicated and time-consuming sample preparation and derivatization. NMR-based assays often lack sensitivity and are slow compared to LC-MS assays (van Breemen *et. al.,* 2007).

HPLC assaying facilitates the rapid quantitative analysis of various plant and dietary supplements with excellent recovery, reproducibility, accuracy, and sensitivity, and does not require sample derivatization prior to analysis, however, there are limitations. Plant extracts usually contain considerable quantities of flavonoids, which have strong UV absorption and terpenoids, which lack UV chromophores thus making detection using

UV difficult. Even trace amounts of co-eluting compounds such as the abundant flavonoids can interfere with the detection of terpenoids. Therefore assays based on UV absorption lack sensitivity and selectivity for the quantitative analysis of terpenoids in complex plant extracts. However this can be overcome by the development of a suitable HPLC method, developed specifically for the analysis of the compound of interest like the method developed by Avula *et. al*., (2010) for the analysis of sutherlandiosides B and D. Reversed-phase HPLC coupled with photodiode array detection has become one of the most widely used techniques for the identification, and quantification of plant compounds and allows individual compounds to be separated by their polarity, which cause them to elute at different times and can be quantified with the use of an external standard. HPLC is therefore a suitable technique to determine with reasonable accuracy the presence and concentrations of secondary metabolites in *Sutherlandia frutescens* grown under varying environmental conditions.
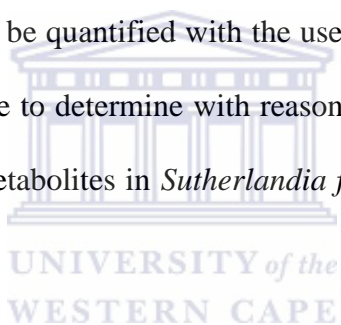
2.12 Summary

A review of the available literature on the botany, uses and metabolite synthesis of *Sutherlandia frutescens* shows that this plant has been fairly well investigated. The fact that it is used as a phytotherapeutic requires the plant to meet strict drug compliance regulations as set out by the Medicines Control Council of South Africa (MCC). Although studies are being carried out on *S. frutescens* to investigate the medicine production-stage requirements of the MCC, little research is focussed on the effects that the plant growth-stage have on the final *Sutherlandia* medicinal product. Numerous studies suggest that primary and secondary metabolite synthesis in plants can be

significantly affected by environmental conditions. To our knowledge no work has as yet been done to determine the effects of environmental growth conditions on the synthesis of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens.*

CHAPTER 3

Aim, objectives, hypothesis and approach

3.1 Aim, objectives, hypothesis and approach

3.1.1 The aim of the study

This study was aimed at determining the effects of environmental conditions, on the levels of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens.*

3.1.2 The objectives of the study

The specific objectives were:

- to locate sites where *S. frutescens* is grown, broadly categorize the sites into environmental groups based on the pertinent environmental growth factors,

- to develop and validate a high-performance liquid chromatography – diode array detector method for Su3, Su4, SuB and SuD analysis,

- to determine and compare the profiles and levels of Su3, Su4, SuB and SuD *S. frutescens* collected from the different environmental growth sites and

- to determine whether the application of the iBatech^{TM} product increases the number and concentrations of secondary metabolites in *S. frutescens*.

3.1.3 Hypothesis

It was hypothesized that:

- *S. frutescens* grown in the natural field and cultivated field groups will have an increase in the number and concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D compared to that of the greenhouse group.

- The application of the iBatech^{TM} product will increase the concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D.

3.2 The study approach

To realize the above-mentioned objectives, the following was done.

3.2.1 Identification of sites where *S. frutescens* is grown and the pertinent growth factors

Various sites where *S. frutescens* is grown where visited and broadly categorized into environmental categories, based on the pertinent environmental growth factors. The weather conditions at the time of harvest, the factors that we assumed could limit the synthesis of secondary metabolites, and plant heights were documented. This environmental growth factor identification was done to be able to correlate the environmental categories with the concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D.

3.2.2 Determination of sutherlandins 3 and 4 and sutherlandiosides B and D profiles and concentrations in *S. frutescens* plants from different growth sites.

To determine the sutherlandins 3 and 4 and sutherlandiosides B and D profiles and concentrations HPLC was used. It is appropriate, accurate and precise and has the ability to provide scientifically acceptable results. It is sensitive, to easily detect compounds and allows for the simultaneous analysis of flavonoids and terpenoids. A new method will be developed based on a method developed by Avula *et. al.,* (2010) and will be validated according to the guidelines as set out by the International Conference for Harmonization (ICH), to assay for sutherlandins 3 and 4 and sutherlandiosides B and D using HPLC - DAD. This new HPLC method will be used to determine the profiles and average levels of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* collected from the different sites, and that treated with the iBatech^{TM} product. The *Sutherlandia frutescens* Afriplex^{TM} sample was used as the standard plant material.

3.2.3 Determination of the effects of iBatech^{TM} treatment on the profiles of sutherlandins 3 and 4 and sutherlandiosides B and D

According to the manufacturers, the iBatech^{TM} product, in previous studies has shown to increase overall polyphenol count in *Lycopersicon* agricultural crops. In these studies, general polyphenols were assayed for but specific classes of compounds were not. Only one HPLC assay was run on samples treated with the iBatech^{TM} product and thus, a thorough understanding of the chemical changes taking place in these plant samples are not fully understood. The use of HPLC-DAD would provide a better understanding of the

type of chemical changes taking place in the plants when treated with the iBatech[TM] product at varying concentrations.

CHAPTER 4

Methods

This chapter describes the chemicals, materials, equipment and methods used. The identification of the sites where *Sutherlandia* is grown, with their relative pertinent growth factors, are documented, the collection, preparation and storage of plant material are described. The iBatech[TM] treatment experiment, the development and validation of an HPLC assay for the identification and profiling of sutherlandins 3 and 4 and sutherlandiosides B and D and the concentrations of the latter of *S. frutescens* from the varying environmental growth categories are presented here.

4.1 Chemicals, products and equipment

4.1.1 Chemicals and products

The methanol used for extractions and acetonitrile (HPLC grade) used for HPLC assays were purchased from Merck chemicals, SA. The distilled water also used for the HPLC assays was prepared in the pharmacology laboratory and purified using a (Glass Chem WS8Lc, Waterstill 8 L/H, 220 V). The plant material was collected from the wild, donated by Kirstenbosch botanical gardens, the University of the Western Cape greenhouse and local farmlands from the Western Cape area growing *S. frutescens* for medicinal use. The patent holder, Dr. J. Klaasen Department of Medical BioSciences, University of the Western Cape, provided the iBatech[TM] product used in this experiment (Patent reference number PA149863/P, batch number IF/UWC – T70023). The sutherlandiosides B and D ratio used as the standard, to construct the calibration curves,

were obtained from the University of Mississippi. The consumables used were; test tubes *(Cellstar 15 ml, PP, graduated, sterile, blue cap),* airtight plastic bags *(Packit 1 BRC 2536, SA)*, millipore filters *(Millipore Millex-HV, hydrophilic PVDF 0.45 um),* syringes *(K 12 5ml)* and amber HPLC vials *(Supelco, conv pack 8/425, PTFE/ Silicone/PTFE Septa, USA).*

4.1.2 Equipment

A ventilated oven *(Memmert, 854 Schwabach, Western Germany),* a coffee grinder *(Philips Cucina coffee grinder Type HR 1737, Brazil)*, a weighing balance *(AR 2140 Max cap. 210 g, China),* a vortex *(Vortex Genie 2, model no. G-560E, Scientific industries, USA),* a centrifuge *(Labofuge 200 Heraeus Sepatech 1,2 kg/dm^3 Germany)* and a pipette *(Finnpipette 773676 thermo Electronic 100-1000 ul)* were used in this experiment.

4.2 Location of the sites in which *S. frutescens* is grown in the Western Cape area, collection, preparation and storage of plant material

Sites in the Western Cape area where *S. frutescens* are grown were visited during a thirty-day period from September to October 2009 during early spring. Eventually eight *S. frutescens* sites were identified and broadly categorized into natural field, cultivated field and greenhouse based on the location and the conditions under which the plants were growing. For each site the pertinent environmental growth factors, which we assume may affect the synthesis of secondary metabolites were identified and documented.

To determine the effects of the growth factors collectively on sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens,* plant samples were collected in the following order. The natural field and cultivated field categories plant samples were collected from three sites each, while for the greenhouse category, which is not common practise for the growth of *S. frutescens*, samples were only collected from two sites. Thus, plant samples from eight *Sutherlandia* sites were collected for this study. From each of the eight, selected *Sutherlandia* sites, a minimum of three samples of leaf and stem material per site were collected. All samples were oven-dried at 40 ˚C for 72 hours after collection and thereafter the flowers and seedpods removed. The leaves and stems were then ground into a powder and put into appropriately labelled airtight plastic bags. In order to prevent moisture, light and oxidative stress from compromising the quality of the *S. frutescens* powdered material; the bags were placed inside a brown cardboard box and placed inside a cool, dry cupboard for storage until analysis.
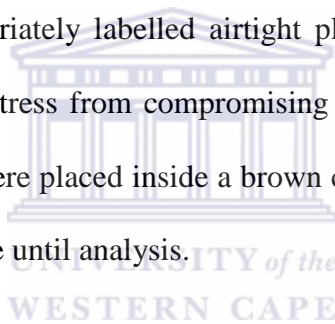
In preparation for HPLC analysis, the three samples from each site were weighed individually to obtain 1 g per sample, these were then combined, shaken, and 1 g of this homogenized material taken as a representative sample for the site. In addition, one further sample from each site was collected and used by a botanist, for species verification. At the time of sampling, the size of the plant, the weather conditions, the presence of flowers and/ or seedpods were also determined and recorded.
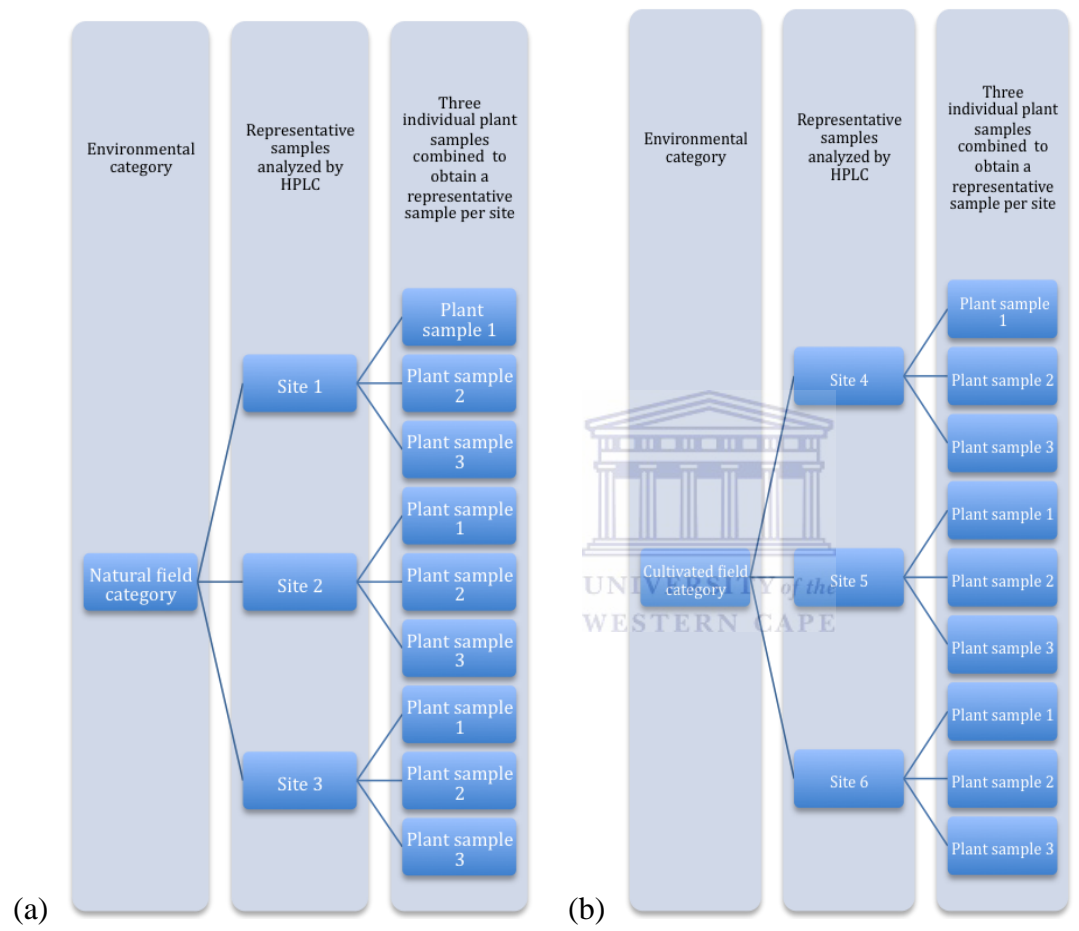
Figures 4.1: The collection of plant samples from (a) the natural field, (b) cultivated field and (c) greenhouse environmental categories
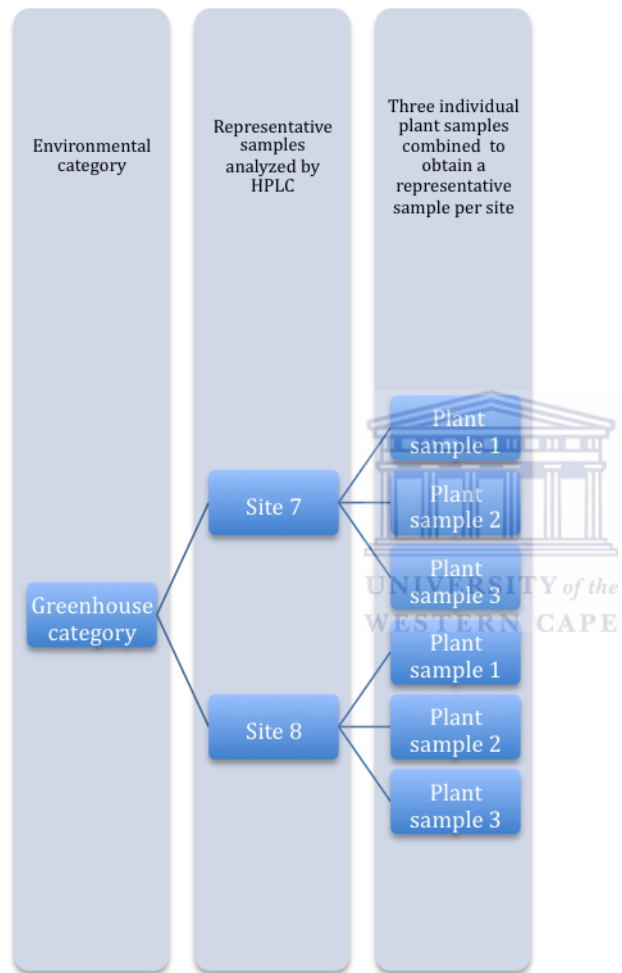
(c)

4.3 Determination of profiles and peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens* plants from different growth sites

To identify and quantify the sutherlandins 3 and 4 and sutherlandiosides B and D, an HPLC method was developed according to a method adapted and modified from the method developed by Avula *et. al.,* (2010). The method was validated according to the International Conference on Harmonization (ICH) guidelines on the validation of analytical methods (Baber, 1994 and ICH, 1996). Triplicate analyses were conducted on three *Sutherlandia frutescens* samples purchased from Afriplex^{TM} (Paarl 7620, South Africa) for use as the standard plant material. Calibration curves were constructed by preparing serial dilutions of the standard SuB/ SuD compound ratio and the peak area values and the best-fit method was used to determine linearity. Inter and intra-day samples were prepared as all other plant samples previously described. Intra-day assay accuracy was determined from three *S. frutescens* Afriplex^{TM} samples analyzed in triplicate, whereas inter-day accuracy involved the calculation of the mean values of three samples analyzed in triplicate and also quantified in terms of percentage RSD over three different days.

4.3.1 HPLC system

An Agilent 1200 series HPLC system was used. It consisted of a degassing system (*G1322A, Japan*), a quaternary pump (*G1311A, Germany*), a auto sampler (*G1329A, Germany*), a C18 Discovery^{TM} column 150mm x 4.60mm, 5*u*m (*Discovery, BL6357 HS, USA),* coupled with a diode-array detector (*G164C, Germany*), a fluorescence detector

(*G1521A, Germany*) and a fraction collector (*G164C, Germany*), managed by the Agilent Chemstation software.

4.3.2 HPLC conditions

The plant samples were analyzed for sutherlandins 3 and 4 and sutherlandiosides B and D, according to a method adapted and modified from a method developed by Avula *et. al.,* (2010). Ten micro litre (10 µL) samples were injected onto the HPLC C18 Discovery^{TM} column, maintained at 25 ˚C, and the compounds eluted using the following solvent system and gradient: of acetonitrile (A) and distilled water (B) both with 0.1 % formic acid; and gradient: A:B (20:80) at time 0; A:B (60: 40) at 20 minutes; A:B (60:40) from time 20 – 25 minutes; A:B (100:0) from 25 – 30 minutes and A:B (80:20) from 30.1 – 40 minutes, with a flow rate of 1.000 ml / minute. The compounds in the eluent were detected by UV at 254 nm, with the use of a diode-array detector and the SuB and SuD fractions collected for liquid chromatography – mass spectrometric (LC-MS) analysis, based on the retention times and threshold values.

4.3.3 Preparation of plant samples for HPLC analysis

The samples of plants collected from the various sites and those included in the iBatech^{TM} product experiment, were analyzed for the presence, or absence, of sutherlandins 3 and 4 and sutherlandiosides B and D, using HPLC. One gram of each representative sample was placed into a test tube and combined with 10 ml of methanol, vortexed for 2 minutes, placed into a sonicating bath at room temperature for 20 minutes, vortexed for a further two minutes, then centrifuged at 3500 rpm for 10 minutes. The

supernatant was removed, filtered through a millipore filter using a syringe and placed into an amber HPLC vial for analysis. Ten micro litres (10 µL) were injected onto the HPLC column and the fingerprints obtained were used to compare sutherlandins 3 and 4 and sutherlandiosides B and D profiles and peak area values. In the case of sutherlandiosides B and D, a standard curve of concentration versus the peak area was set-up, analyzed by linear regression and used to quantify the levels of sutherlandiosides B and D in all the plant samples.

4.4 iBatech^{TM} experiment

Sutherlandia frutescens plants grown under shade-house conditions were maintained under these conditions for the duration of the experiment and watered at the nursery manager's discretion, as prior to the experiment. The plants were divided into three groups of five plants each i.e. group-A control; group-B 50% treatment group and group-C) 100% treatment group. The control group had no treatment and was kept in the same location as other groups to minimise environmental variations. The iBatech^{TM} product was diluted according to the manufacturers specifications i.e. 100 ml of product diluted into one litre of water, used as the 100% treatment concentration and 50 ml diluted into one litre of water, as the 50% treatment concentration. The product dilutions were applied to the plant leaf and stem surface, using a standard 500 ml plant spray bottle. During iBatech^{TM} product applications, the plants were moved to a well-ventilated area to prevent product mist residues from coming into contact with the other plant groups. The plants were treated with the iBatech^{TM} product once per week and harvested after 30 days of treatment (with a total number of four treatments). The leaves and stems from each

individual plant, of three plants of each experimental group were taken as an individual

sample, resulting in nine samples. These nine samples were dried and stored in the same

manner as previously described in section 4.2.
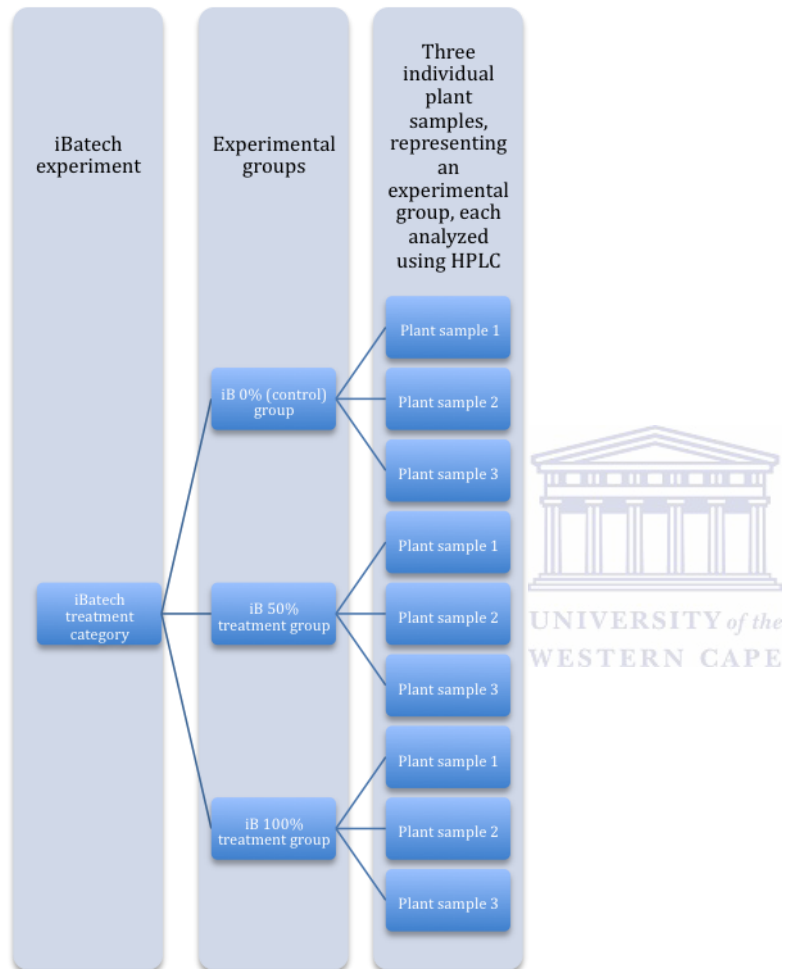
Figure 4.2: Plant sample collection for the iBatech^{TM} experiment

4.5 Confirmation of the identified peaks, using Liquid Chromatography – Mass Spectrometry

Fractions of the sutherlandiosides B and D were collected and pooled together, then subjected to liquid chromatography - mass spectrometric (LC-MS) analysis to confirm their identity. The LC-MS was performed at Stellenbosch University, Central Analytical Facility, on a Waters API Q-TOF Ultima LC-MS instrument. The sample was diluted 10 times with 1 ml 0.1% formic acid and 50% acetonitrile using an ultrasonic bath for 20 minutes then a 2 µL injection volume was injected onto the column (Waters BEH C18, 2.1x50 mm). The mobile phase consisted of solvent A: water and solvent B: acetonitrile, with a gradient of 0 minutes to 0.5 minutes solvent A was 100%, from 0.5 to 6 minutes solvent A decreased steadily to 0% from 6 minutes to 8 minutes, solvent B remained at 100% at 8.01 minutes and the system was re-equilibrated to 100% of solvent A with a total run-time of 15 minutes and a flow rate of 0.35 ml /minute.

The detection conditions were as follows: source ESI +, capillary voltage set at 3.5 kV, cone voltage 35, RF1 40, source temperature 100 °C, desolvation temperature 350 °C, desolvation gas flow 350 L/h and cone gas flow 50 L/h. The mass to charge ratio (*m/z*) of the LC-MS data was noted and compared to existing data in the literature to confirm the identity of the compounds.

4.6 Identification and quantification of sutherlandiosides B and D.

The sutherlandiosides B and D from the samples were identified based on retention times and spectral analysis, and compared to that obtained from a standard mixture of

sutherlandiosides B and D, isolated and purified by the University of Mississippi. The serial dilutions containing 0.465 mg /ml, 0.931 mg /ml, 1.862 mg /ml, 3.725 mg /ml, 7.45 mg /ml, were injected in triplicate. Standard curves of the concentrations vs. peak area were obtained and used to quantify sutherlandiosides B and D in all plant samples, using linear regression and the Graphpad prism5 software.

4.7 Statistical analysis

The statistical analysis was performed using the GraphPad Prism 5 software at a significance level of 0.05, in the following manner. The Kruskal-Wallis test was used to determine if there were any significant differences in the mean levels of sutherlandins 3 and 4 and sutherlandiosides B and D, among the experimental groups and was applied to both the environmental group data and iBatech[TM] experiment data. Furthermore, groups that showed to be significantly different, was subjected to the post ANOVA, Dunn's Multiple Comparison test. The Mann-Whitney, two-tail, t-test was used to compare the mean levels of each experimental group to the standard and was only applied to the environmental group data. Lastly, the column statistics was done to determine whether there were any significant differences among samples from the same group.

CHAPTER 5

Results and discussion

The results obtained in the location of the sites where *Sutherlandia* is grown, the determination of the profiles and the average peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D, the effects of the iBatech^{TM} product treatment on the profiles and average peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D, and the quantification of sutherlandiosides B and D, are presented and discussed here.

5.1 Growth environments and locations in which *Sutherlandia* is grown in the Western Cape area used in this study

To determine the growth environments in which *Sutherlandia* is grown in the Western Cape area, various sites were visited and analyzed for their relative collective growth conditions, broadly categorized into the 1. natural field, 2. cultivated field or 3. greenhouse environmental categories and allocated a site number which represents a specific location from which the plant material was collected and will be referred to from here forward. The weather conditions at the time of collection, the presence or absence of flowers and/ or seedpods and the height were documented and are presented in Table 5.1 below. Site 5 and 6 samples were donated and therefore the variables are unknown.

Table 5.1: The environmental category, sample name, weather conditions at time of collection, presence or absence of flowers or seedpods and height

Environmental category	Samples	Conditions at collection	Presence of flowers	Presence of seed pods	Height cm
Natural field	Site 1	Sunny/ windy	No	Yes	10-20
	Site 2	Cold/ snowy	Yes	No	10-20
	Site 3	Sunny	Yes	Yes	20-30
Cultivated field	Site 4	Sunny	No	No	40-60
	Site 5	Unknown	Unknown	Unknown	Unknown
	Site 6	Unknown	Unknown	Unknown	Unknown
Greenhouse	Site 7	Shady	No	No	40-60
	Site 8	Shady	No	No	30-40

From the range of weather conditions at the time of collection, the greenhouse category appears more uniform. As covered in the literature, environmental growth conditions play a fundamental role in the synthesis of secondary metabolites (Mosaleeyanon *et. al.*, 2005). The natural field category samples i.e. Site 1, Site 2, and Site 3, were collected from the Western Cape's natural *Fynbos* locations. From the literature reviewed, the Western Cape *Fynbos* region should be experiencing the summer drought period, after the long wet winter (Aschmann, 1973). From the conditions at harvest column, it is evident that the weather patterns are not true to that described in the literature. The observations made here were only performed on the day of collection and therefore cannot be generalized for the season, however, these environmental factors, when experienced for a prolonged period, could affect the concentrations (Wink, 1999) of sutherlandins 3 and 4, and sutherlandiosides B and D.
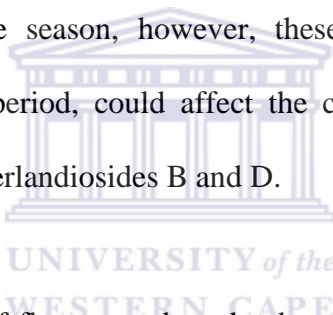
The presence and or absence of flowers and seedpods provide an indication as to which stage of development the plant may be in. Previous studies suggest that the stage of development also greatly affects the synthesis of secondary metabolites (Whisgary *et. al.*, unpublished data), as it is believed that during the early stages of development the plant allocates assimilated carbon and nitrogen to primary growth (Bourgaud *et. al.*, 2001) and once growth plateaus, assimilated carbon and nitrogen can be allocated to secondary growth and the synthesis of secondary metabolites. In the case of flowering, the plant has reached a secondary growth stage, however, assimilated carbon and nitrogen is now allocated to flowering and the development of seeds, thus reducing the concentrations of secondary metabolites. In the natural field flowers and seedpods are present and therefore

the plant is in the developmental stage to be able to produce secondary metabolites such as sutherlandins 3 and 4 and sutherlandiosides B and D. The fact that flowers and seedpods are present could also mean that the concentrations of these secondary metabolites could have been compromised at the time of collection and may be increased in the stages before and after flowering. The absence of flowers and seedpods in the greenhouse category can be attributed to the controlled environment under which these plants are grown (Zobayed *et. al*., 2004). In the natural field and cultivated field categories plants are exposed to natural seasonal cycles and therefore should be flowering as per normal, at the time of harvest (early spring) (Pienaar, 2001). The greenhouse category however, maintains fairly constant growth conditions throughout the year, regardless of season, thus the plant no longer follows its natural growth cycle.
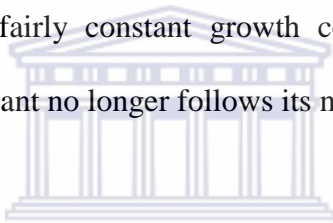
The two samples in the greenhouse category are both shady and this could explain for the height being greater than that of the other categories. When plants are in competition for light, they tend to invest more energy into longitudinal growth than latitudinal growth; however, plant biomass cannot necessarily be used as an indication for secondary metabolite concentrations. It is often observed, that smaller, more compact plants have higher secondary metabolite yields compared to that of taller plants. Overall, the greenhouse category shows more uniform characteristics whereas the natural field and cultivated field categories have considerably varying characteristics.

5.2 Determination of the profiles and peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the three environmental categories, the *S. frutescens* Afriplex™ standard and the iBatech™ treatment

The aim was to determine if there were any variations in the profiles and average peak area values of Su3, Su4, SuB and SuD in the plant samples collected from the natural field, cultivated field, greenhouse categories when compared to the *S. frutescens* Afriplex™ standard sample, and to determine any variations in average peak area values among the iBatech™ treatment groups, using HPLC.

5.2.1 HPLC assay validation

To validate the HPLC assay used for the analysis of Su3, Su4, SuB and SuD in the *S. frutescens* samples, inter-day variations, intra-day variations and the construction of calibration curves were used according to the ICH guidelines for analytical methods. In Figure 5.1 is a representative chromatogram of *S. frutescens* obtained from the analysis of the Afriplex™ sample, which is being used in this study, as a standard to compare the natural field, cultivated field and greenhouse categories to. The fingerprint obtained, illustrates the presence of a number of metabolites however for this study only sutherlandins 3 and 4 (Su3 and Su4) and sutherlandiosides B and D (SuB and SuD) were monitored. For inter-day and intra-day analysis (Appendix Table 1), the *S. frutescens* Afriplex™ standard plant material was used, whereas the isolated standard SuB/ SuD ratio was used to construct the calibration curves.
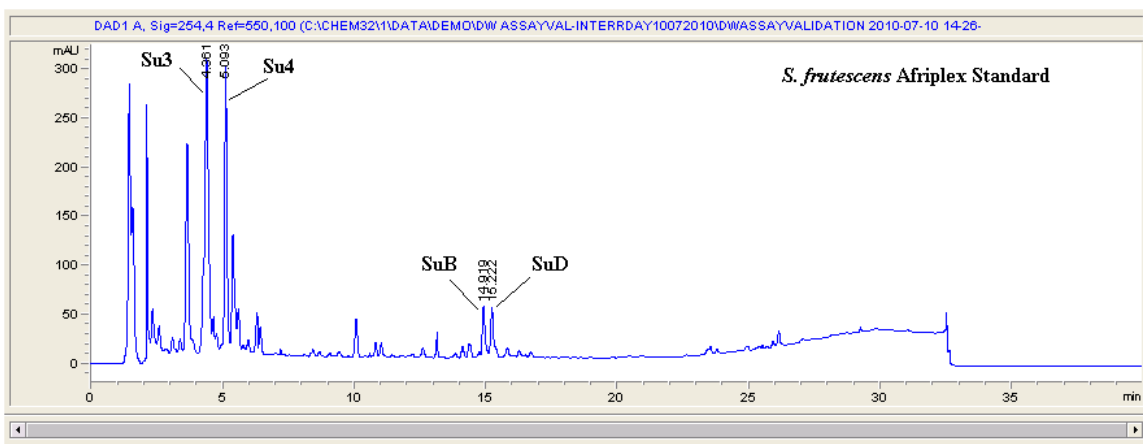
Figure 5.1: HPLC chromatographic fingerprint of *S. frutescens* Afriplex^TM sample used as plant sample standard

The spectral data was obtained (Figure 5.2) and found to conform to those of flavonoids and terpenoids. The flavonoids Su3 and Su4, were compared to the spectral data obtained by Hess *et. al., (unpublished data)* with the relative retention times of 4.361 and 5.093 respectively and the terpenoids SuB and SuD, were compared to the spectral data obtained by Avula *et. al.,* (2010) with the relative retention times of 14.919 and 15.222 respectively, to verify their identity.
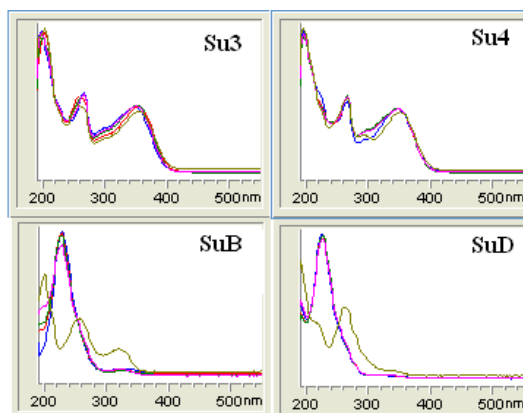
Figure 5.2: The spectral data for Su3, Su4, SuB and SuD

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Serial dilutions of the sutherlandiosides B and D standard were prepared and a $10u$L injection was made onto the HPLC column. The following chromatogram (Figure 5.3) is a representation of the SuB and SuD standard ratio and also provides the spectral data as well as the relative retention times. This was then used for accurate identification of sutherlandiosides B and D in all the plant samples analyzed with HPLC.

Figure 5.3: Representative chromatogram of SuB and SuD standard ratio

5.2.2 Profiles and average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the Afriplex^{TM} standard, natural field, cultivated field and greenhouse environmental categories

The aim was to determine the qualitative variations, if any, in the profiles of sutherlandins 3 and 4 and sutherlandiosides B and D. The following chromatograms serve as representatives for all of the samples analyzed from the same environmental category. The samples collected from the various sites and categorized into the environmental categories, were allocated a name with the relative environmental category in brackets e.g. Site 1 (NF), and will be referred to from here forth. One gram of representative plant sample from each site was extracted with 10 ml MeOH. A 10 µL sample was injected to

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Serial dilutions of the sutherlandiosides B and D standard were prepared and a $10u$L injection was made onto the HPLC column. The following chromatogram (Figure 5.3) is a representation of the SuB and SuD standard ratio and also provides the spectral data as well as the relative retention times. This was then used for accurate identification of sutherlandiosides B and D in all the plant samples analyzed with HPLC.

Figure 5.3: Representative chromatogram of SuB and SuD standard ratio

5.2.2 Profiles and average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the Afriplex[TM] standard, natural field, cultivated field and greenhouse environmental categories

The aim was to determine the qualitative variations, if any, in the profiles of sutherlandins 3 and 4 and sutherlandiosides B and D. The following chromatograms serve as representatives for all of the samples analyzed from the same environmental category. The samples collected from the various sites and categorized into the environmental categories, were allocated a name with the relative environmental category in brackets e.g. Site 1 (NF), and will be referred to from here forth. One gram of representative plant sample from each site was extracted with 10 ml MeOH. A 10 µL sample was injected to

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obtain the chromatograms as described in method 4.3. Sutherlandin 3 (Su3), sutherlandin 4 (Su4), sutherlandioside B (SuB) and sutherlandioside D (SuD) were monitored.

Figure 5.4 represents the natural field (NF) group. Sutherlandins 3 and 4 quantities are minimal in the Site 1 (NF) sample and considerably increased in the Site 2 (NF) and Site 3 (NF) samples. In Site 2 (NF) sutherlandin 3 is greater than 4 and vice versa in the Site 3 (NF) sample. Sutherlandiosides B and D are also in greater quantity in the Site 3 (NF) sample and unidentifiable in the Site 1 (NF) and Site 2 (NF) samples.

Figure 5.5 shows dramatic variations in the quantities of Su3, Su4, SuB and SuD among all samples. The Site 4 (CF) sample shows the lowest quantities whereas the Site 5 (CF) shows higher quantities and Site 6 (CF) dramatically higher quantities especially in SuB and SuD when compared to all other samples analyzed across all environmental categories. Figure 5.6 shows Su3, Su4, SuB and SuD profiles with no visible variations in quantities between the Site 7 (GH) and Site 8 (GH) samples.

a)

Natural Field Category
Site 1
Representative chromatogram

b)

Natural Field Category
Site 2
Representative chromatogram

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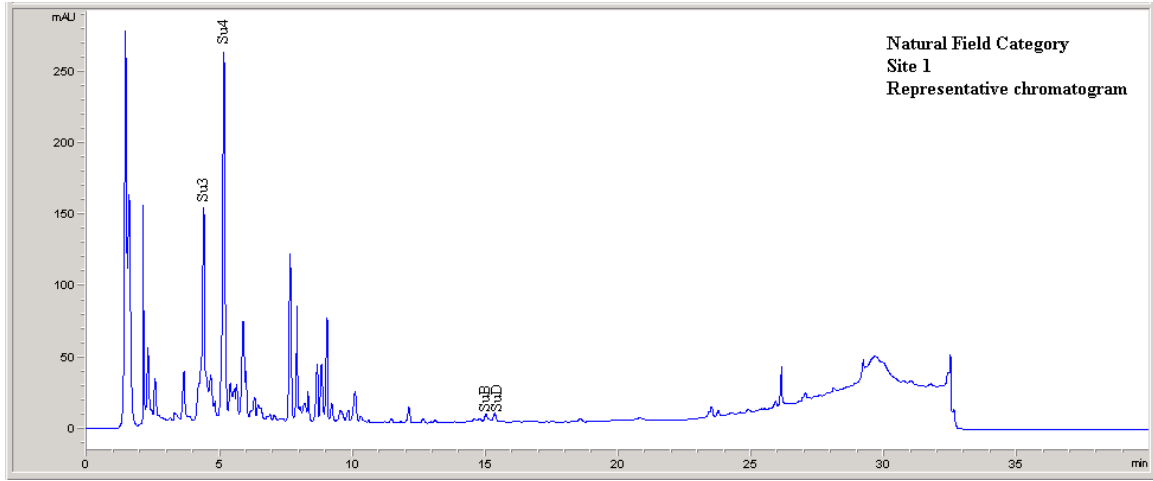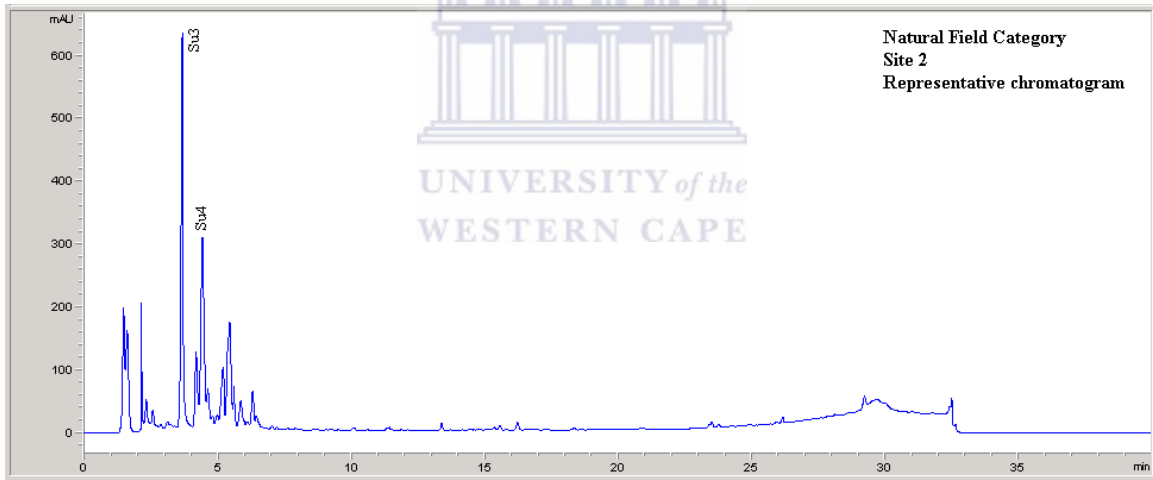
c)
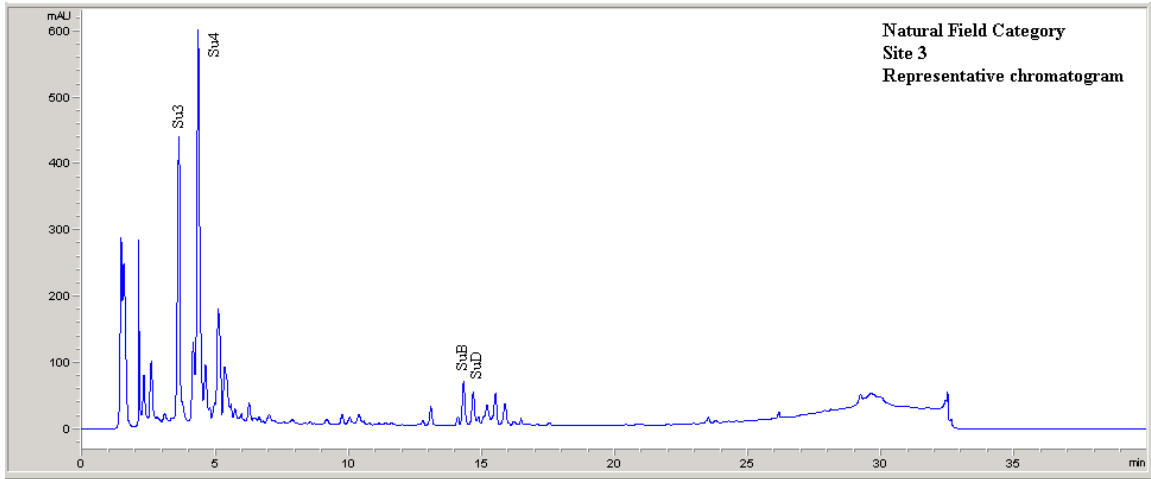
Figures 5.4 a , b and c: Representative HPLC chromatograms for the natural field category samples.

a)
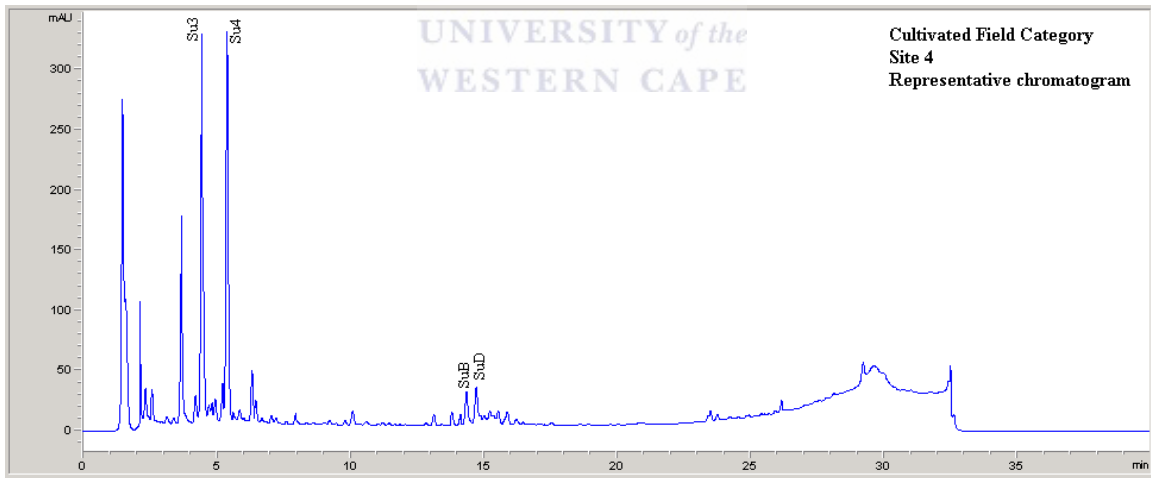
b)
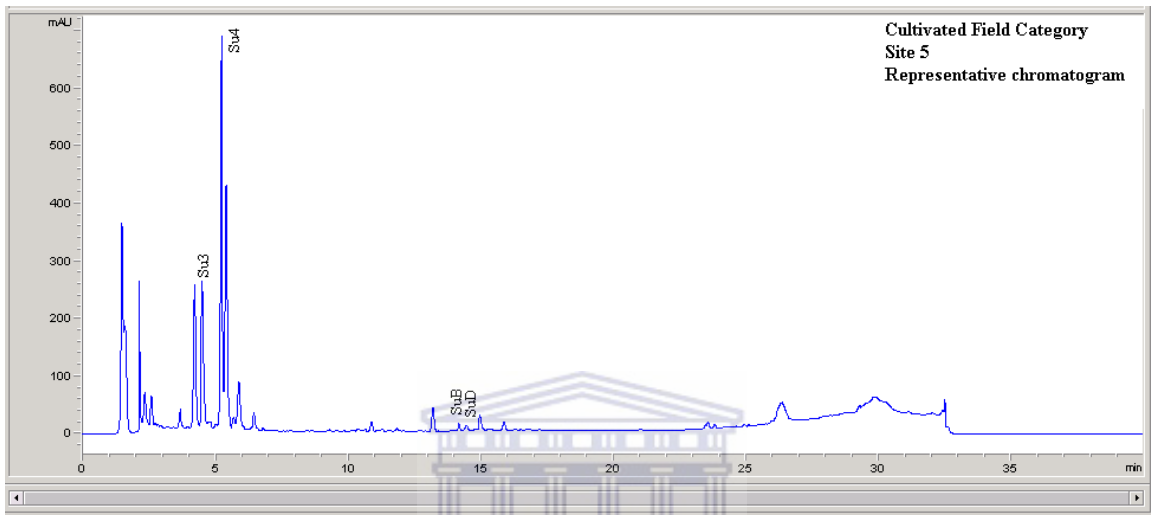
c)
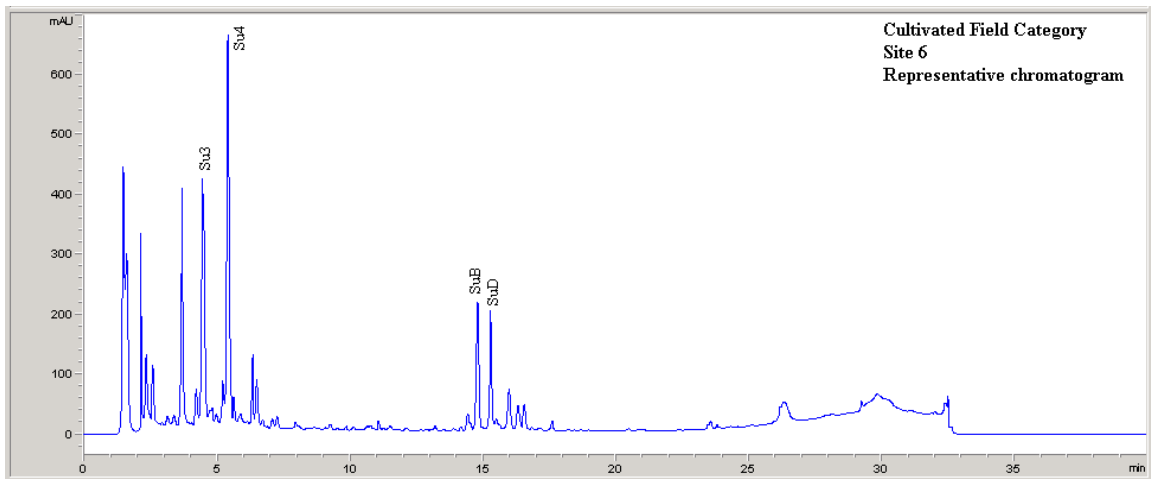
Figures 5.5 a, b and c: Representative HPLC chromatograms for the cultivated field category samples.

a)
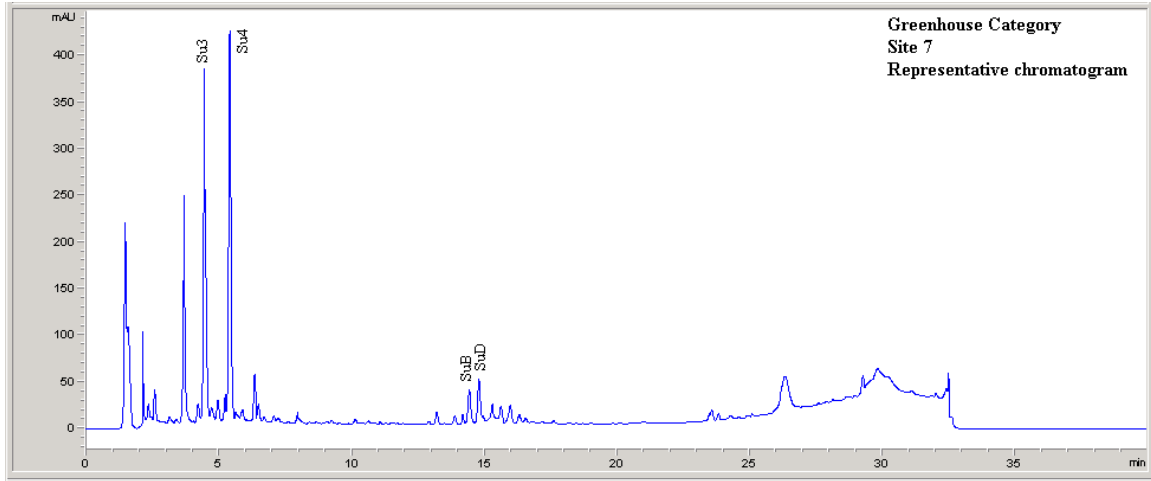
b)
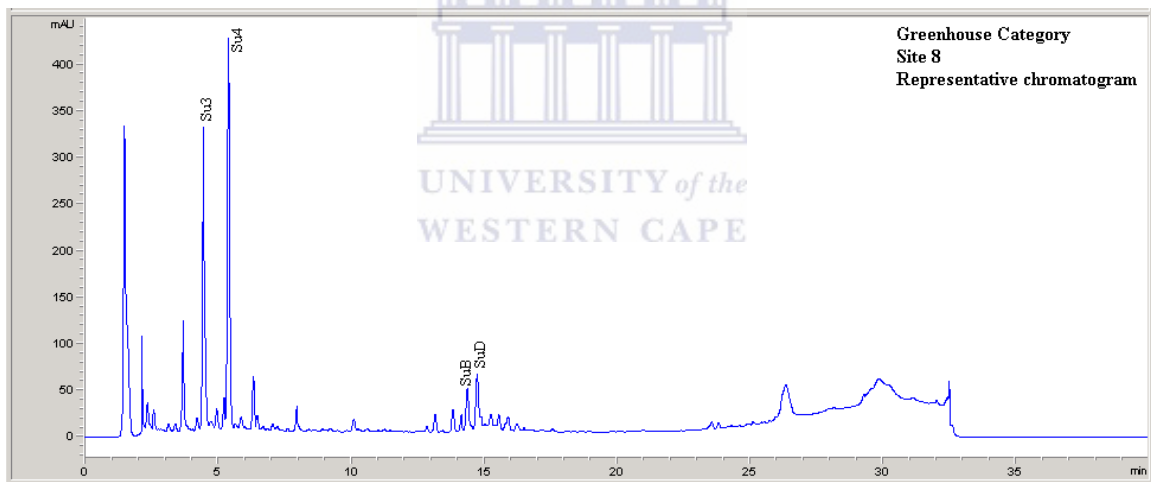
Figures 5.6 a and b: Representative HPLC chromatograms for the greenhouse category samples.

Figures 5.7, 5.8, 5.9, and 5.10, represent the average peak area values of Su3, Su4, SuB and SuD respectively and compares the average peak area values for the natural field (NF), cultivated field (CF) and greenhouse (GH) environmental categories to that of the

Afriplex[TM] *S. frutescens* plant material (Std.) used as the standard. The error bars plotted illustrate the standard deviation among three homogenized samples collected from the different sites but in the same environmental category. Due to the unavailability of a third sample from the greenhouse category, the error bar represents the standard deviation of two samples.
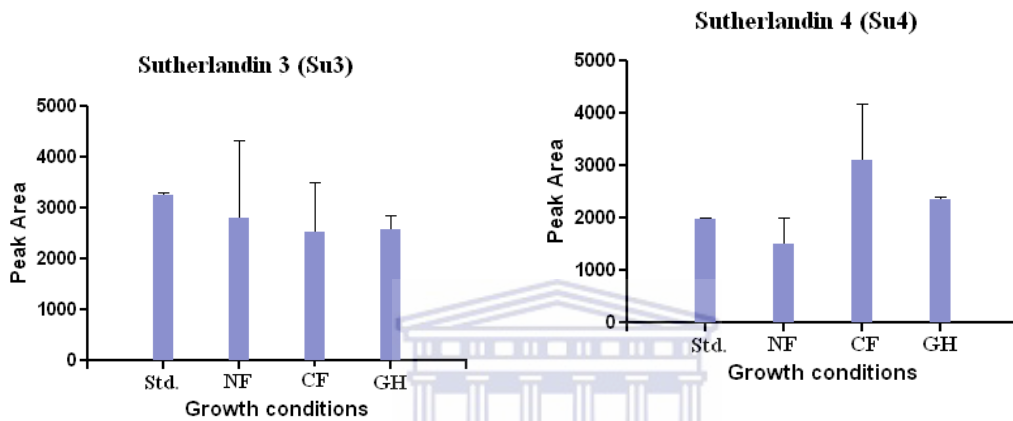
Figure 5.7: Sutherlandin 3 (Su3)   **Figure 5.8: Sutherlandin 4 (Su4)**
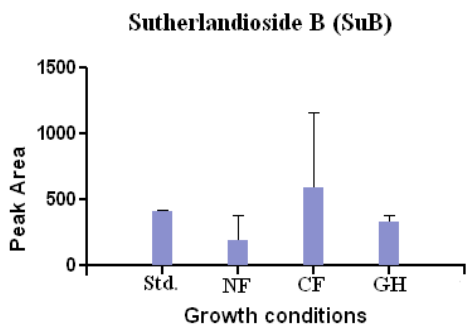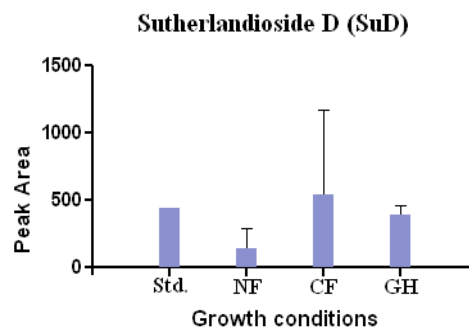
Figure 5.9: Sutherlandioside B (SuB)   **Figure 5.10: Sutherlandioside D (SuD)**

Figure 5.7 (sutherlandin 3) illustrates the average peak area values of Su3 for the Afriplex[TM] (Std.) 2495.08, the natural field (NF) 2810.33, the cultivated field (CF)

2519.81 and the greenhouse (GH) 2580.25 categories. Figure 5.8 (sutherlandin 4) illustrates the average peak area values of (Std.) 2065.18, (NF) 1495.67, (CF) 3114.42 and (GH) 2361.72, with the CF group showing the highest average peak area values of Su4 and the NF showing the lowest. The GH group shows higher average peak area values of sutherlandin 4 than that of the Std. Figure 5.9 (sutherlandioside B) illustrates the average peak area values of (Std.) 236.95, (NF) 189.7, (CF) 594.56 and (GH) 326.72, however, the CF group shows the highest average peak area values for SuB. Figure 5.10 (sutherlandioside D) illustrates the average peak area values of the (Std.) 255.38, (NF) 144.1, (CF) 544.37 and (GH) 387.49 groups for SuD, with the NF category having the lowest average peak area values.
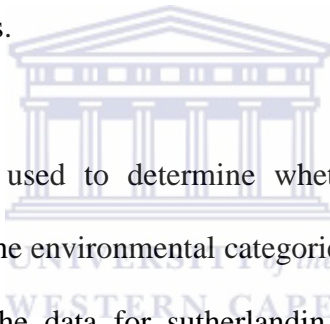
The Kruskal-Wallis test was used to determine whether there are any statistically significant differences among the environmental categories, for sutherlandins 3 and 4 and sutherlandiosides B and D. The data for sutherlandin 4 (Su4) was significant *(P = 0.0021)* and therefore the post ANOVA, Dunn's Multiple Comparison test was performed to determine which groups were significantly different. The results showed that the natural field group was significantly different to both the cultivated field and greenhouse groups.

The Mann-Whitney, two-tail, t-test was used to analyze the data for Su3, Su4, SuB and SuD, to compare each environmental category to the standard, however, no significant differences were found.

The column statistics of the raw data was analyzed to determine if there were any significant differences among samples from the same environmental category. Sutherlandin 3 (Figure 5.7), illustrated significant differences in Su3 concentrations among the samples from the same environmental category i.e. NF ($P = 0.0005$), CF ($P = 0.0001$) and GH ($P = 0.0001$). Sutherlandin 4 (figure 5.8), illustrated significant differences in Su4 concentrations among samples when analyzing the NF ($P = 0.0001$), CF ($P = 0.0001$) and GH ($P = 0.0001$) groups. Sutherlandioside B (figure 5.9), illustrated significant differences in SuB concentrations for NF ($P = 0.0189$), CF ($P = 0.0140$) and GH ($P = 0.0001$) among samples from the same group. Sutherlandioside D (figure 5.10), had significant differences in SuD concentrations among samples for the NF ($P = 0.0192$), CF ($P = 0.0308$) and GH ($P = 0.0001$) groups.

In summary, the data shows that only sutherlandin 4 (Su4) was significantly different. The natural field group was significantly different to both the cultivated field and greenhouse groups. When comparing all groups to the standard, no significance was found, however, significant differences were found among samples from the same environmental group. These significant differences among samples from the same group can therefore be responsible for the lack of significant differences among environmental categories due to the wide range of data.

5.2.3 Profiles and average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the iBatech[TM] treatment experiment

The chromatograms presented in Figure 5.11 illustrate minimal visual differences in Su3, Su4, SuB and SuD profiles when comparing the control group, 50% treatment group and 100% treatment group. However, with closer examination of the average peak area values, a concentration dependant increase in Su3, Su4, SuB and SuD are evident. One gram of representative plant sample for each site was extracted with 10 ml MeOH. A 10 µL sample was injected to obtain the chromatograms as described in method 4.3. Sutherlandin 3 (Su3), sutherlandin 4 (Su4), sutherlandioside B (SuB) and sutherlandioside D (SuD) were monitored.

a)
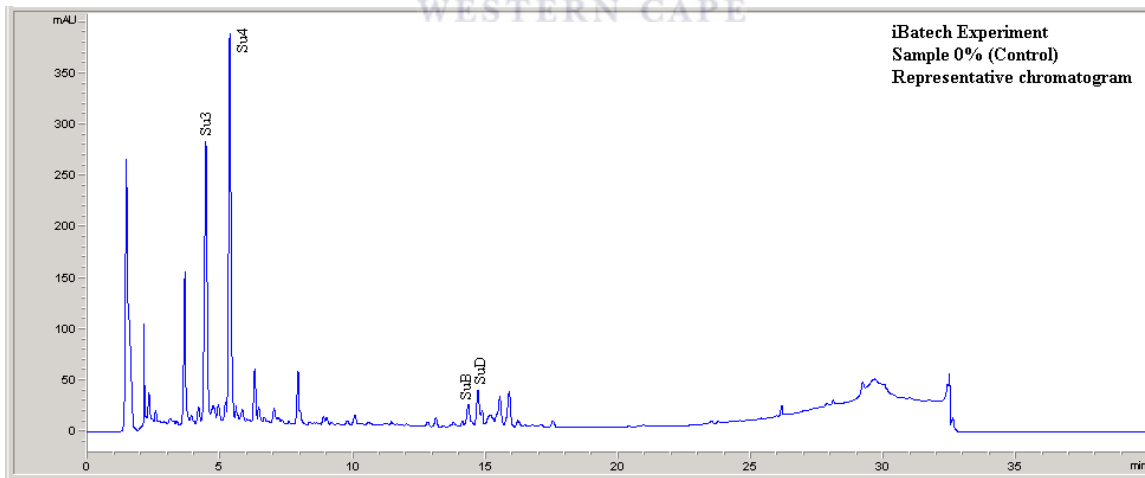
b)
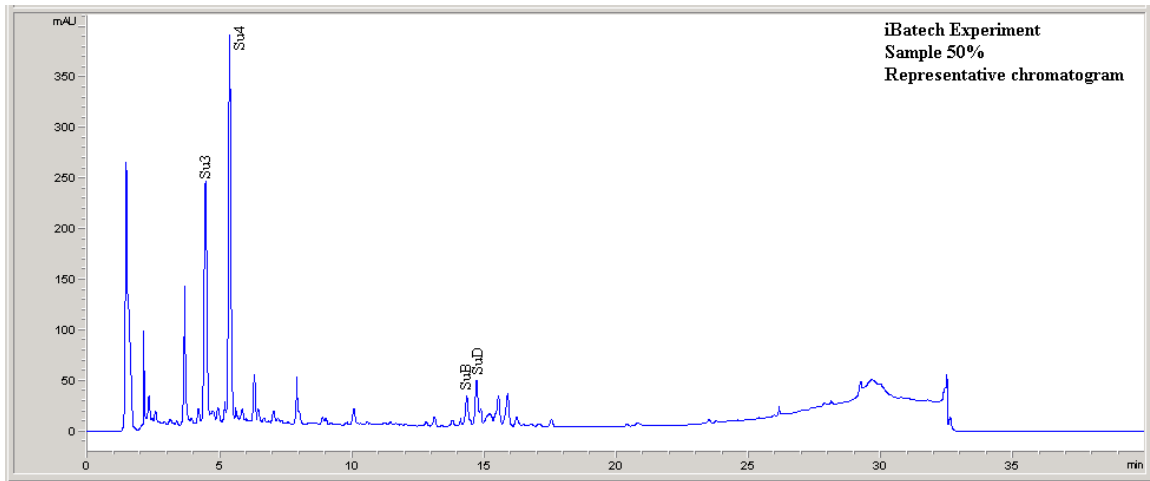
c)
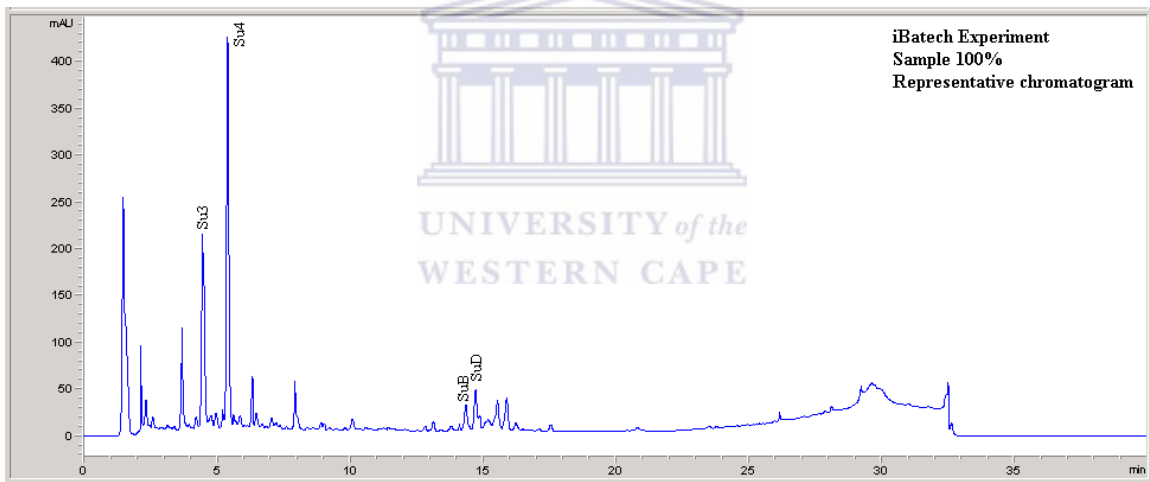
Figures 5.11 a, b and c: Representative HPLC chromatograms for the iBatech^{TM} samples.

Figures 5.12, 5.13, 5.14 and 5.15 illustrate the average peak area values of Su3, Su4, SuB and SuD for the plants subjected to the iBatech^{TM} product treatment, with a control 0%, 50% and 100% treatment concentrations. The error bars plotted represent three plant samples from the same product treatment concentration.
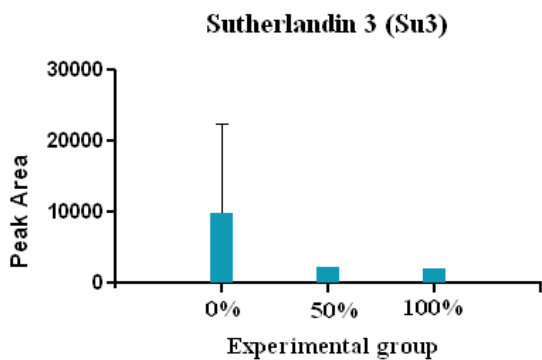
Figure 5.12: Sutherlandin 3 (Su3)   **Figure 5.13: Sutherlandin 4 (Su4)**
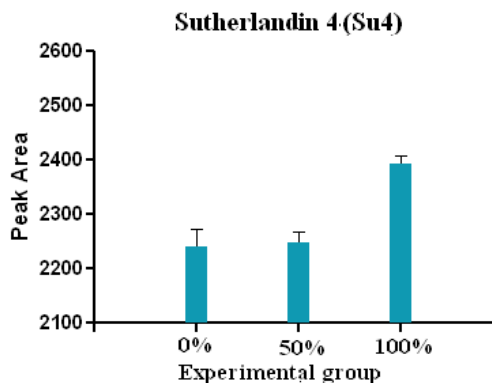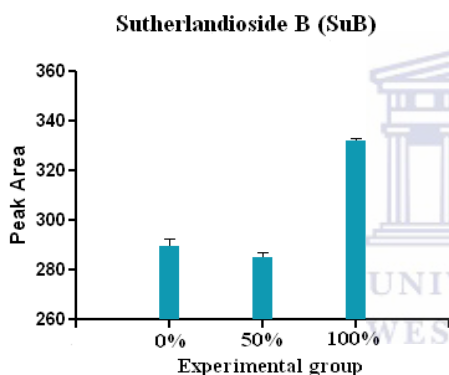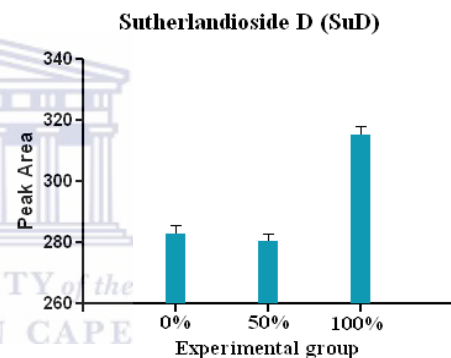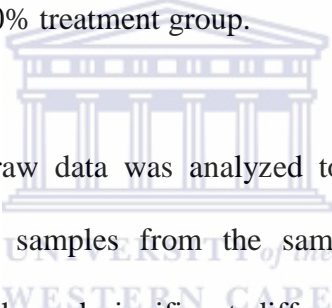
Figure 5.14: Sutherlandioside B (SuB)   Figure 5.15: Sutherlandioside D (SuD)

Figure 5.12 (sutherlandin 3) illustrates the average peak area values of the (control) 9758.43, the (50%) 2232.63 and the (100%) 2031.97 treatment groups. Figure 5.13 (sutherlandin 4) illustrates the average peak area values of the (control) 2241.63, the (50%) 2247.47 and the (100%) 2392.60 treatment groups, with the 100% treatment group having the highest average peak area values of Su4. Figure 5.14 (sutherlandioside B) illustrates the average peak area values of the (control) 289.66, the (50%) 284.93 and the (100%) 332.30 treatment groups. Figure 5.15 (sutherlandioside D) illustrates the average

peak area values of the (control) 282.77, the (50%) 280.60 and the (100%) 315.13 treatment groups, with the 100% treatment group having the highest average peak area values for SuD.

The Kruskal-Wallis test was used to determine whether there are any variations among the iBatech™ experimental groups for sutherlandins 3 and 4 and sutherlandiosides B and D. The data for sutherlandioside B (SuB) was significant *(P = 0.0390)* and therefore the post ANOVA, Dunn's Multiple Comparison test was performed to determine which groups were significantly different. The results showed that the 50% treatment group was significantly different to the 100% treatment group.

The column statistics of the raw data was analyzed to determine if there were any significant differences among samples from the same iBatech™ treatment group. Sutherlandin 3 (Figure 5.12) showed significant differences among samples from the 50% (*P = 0.0001*) and 100% (*P = 0.0001*) treatment groups but not from the control (*P = 0.3158*) group. Sutherlandin 4 (Figure 5.13) had significant differences among samples from the control (*P = 0.0001*), 50% (*P = 0.0001*) and 100% (*P = 0.0001*) treatment groups. Sutherlandioside B (Figure 5.14) had significant differences among the samples from the control (*P = 0.0001*), 50% (*P = 0.0001*) and 100% (*P = 0.0001*) treatment groups. Sutherlandioside D (Figure 5.15) had significant differences among the samples from the control, 50% and 100% treatment groups.

In summary, sutherlandioside B was found to have significant differences when comparing the 50% and 100% treatment groups. It is important to note that there were significant differences among all samples from all of the iBatech™ treatment groups, except for sutherlandin 3 in the control group. This means that the data had a wide range and could explain why significant differences could not be found when comparing the different treatment groups.

5.3 Liquid Chromatography – Mass Spectrometry

HPLC was performed on *Sutherlandia frutescens* Afriplex™ plant material as described in section 4.2 and fractions of the SuB and SuD collected and pooled. Due to the nature of sutherlandiosides B and D i.e. the compounds elute with less than a minute apart, which complicates collecting them as separate fractions. The pooled collected fractions were used for liquid chromatography – mass spectrometric (LC-MS) analysis. The standard SuB and SuD fraction obtained from the University of Mississippi was also a ratio between the two compounds.

The fractions collected from the *Sutherlandia frutescens* Afriplex™ sample with the spectral data conforming to those of sutherlandiosides B and D (SuB and SuD) as described by Avula *et. al.* (2010), were subjected to mass spectrometry for verification of their identities. The prominent compound in the collected fraction (Figure 5.16 and Figure 5.17) was eluted at retention time 7.23 minutes and had a mass of 635.4412.
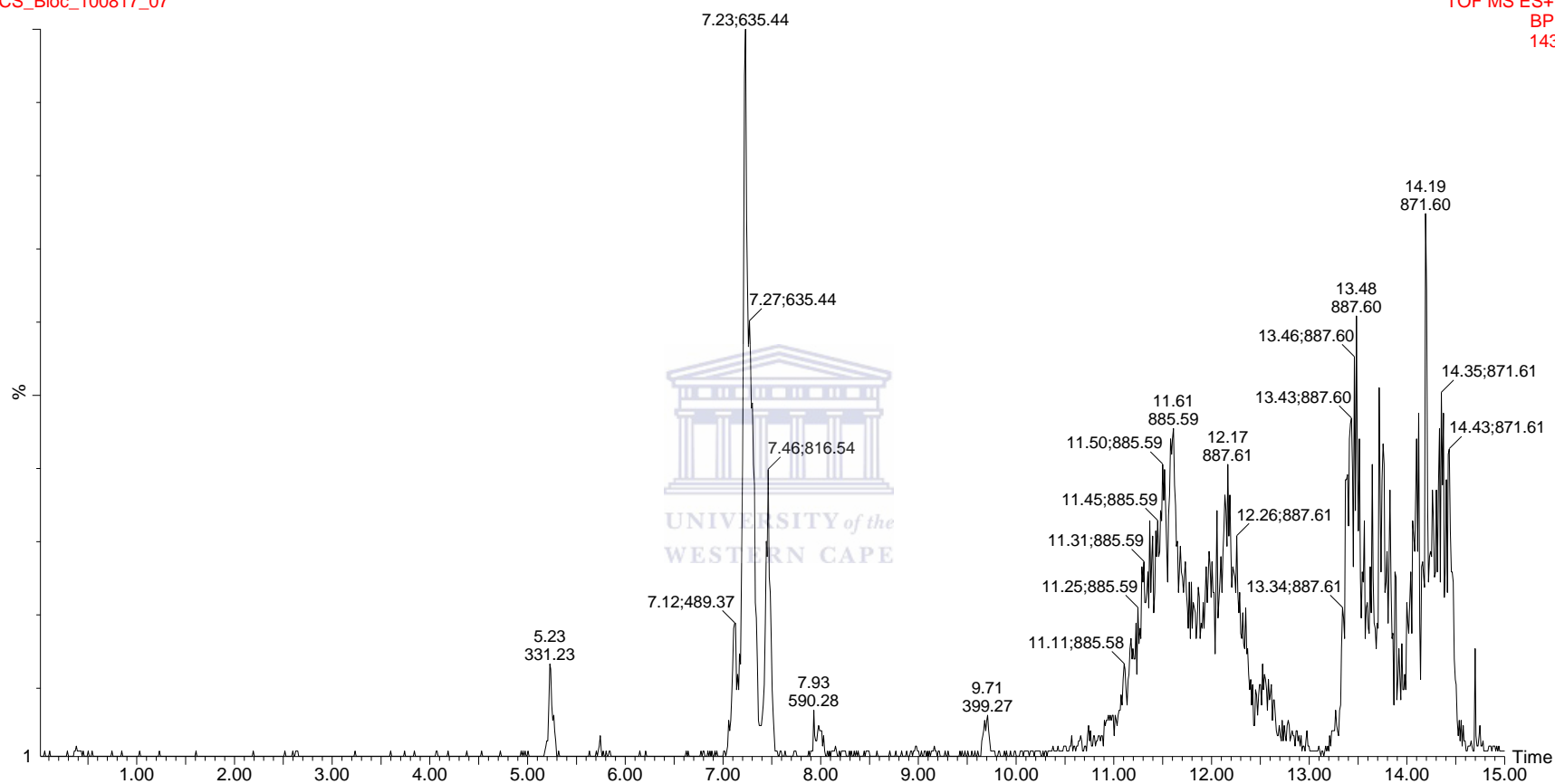
Figure 5.16: An LC-MS chromatogram of the collected SuB/ SuD pooled fraction
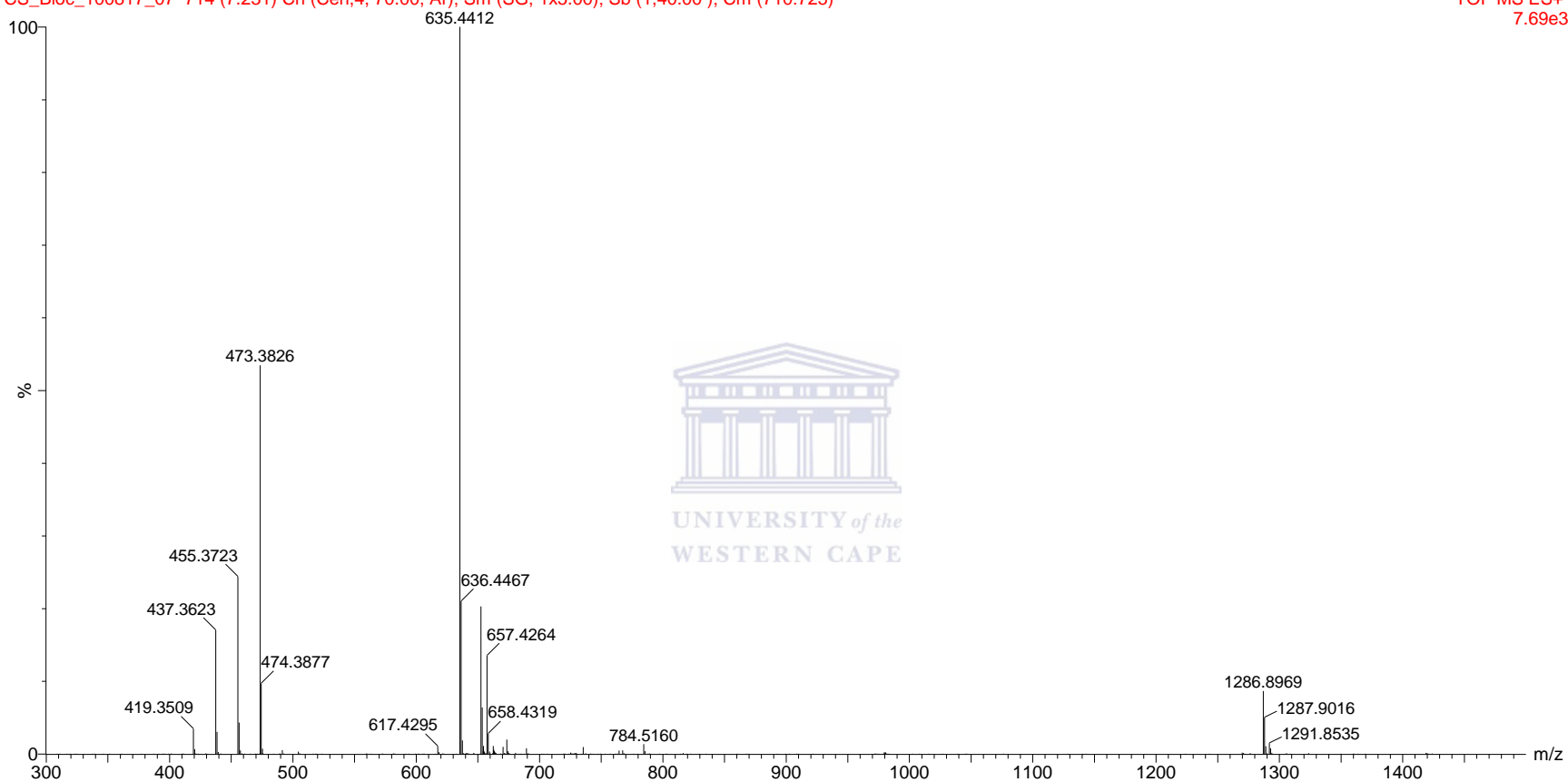
Figure 5.17: LC-MS mass spectral chromatogram for the collected SuB/ SuD fraction

Table 5.2: The concentrations of sutherlandiosides B and D in mg/ml in all plant samples

Environmental category	Sample Name	SuB		SuD	
		Peak Area	Concentration mg/ml	Peak Area	Concentration mg/ml
Natural field category	Site 1	53.566	0.15	43.066	0.09
	Site 2	67.233	0.19	48.100	0.11
	Site 3	448.300	1.43	341.133	1.15
Cultivated field category	Site 4	216.433	0.68	231.800	0.76
	Site 5	212.733	0.66	32.766	0.05
	Site 6	1354.500	4.36	1368.533	4.80
Greenhouse category	Site 7	275.500	0.87	321.900	1.08
	Site 8	377.933	1.20	453.066	1.55
iBatech experiment	iBatech 0% (control)	289.666	0.91	282.766	0.94
	iBatech 50% treatment	284.933	0.90	280.600	0.93
	iBatech 100% treatment	332.300	1.05	315.133	1.06

5.4 Quantification: concentrations of sutherlandiosides B and D in samples collected from the natural field group, cultivated field group, greenhouse group and the iBatech^{TM} experiment

In both the NF and CF groups (Table 5.2) there appears to be dramatic variations in the concentrations of SuB and SuD. The Site 3 (CF) and Site 6 (CF) samples have a five and six fold increase respectively in SuB and SuD concentrations when compared to the Site 1 (NF), Site 2 (NF), Site 4 (CF) and Site 5 (CF) samples. The GH group also had differences among samples from the same group but appear relatively uniform when compared to the iBatech^{TM} control group and iBatech^{TM} 50% treated group that were also grown in a greenhouse. The iBatech^{TM} 100% treated group shows an increase in SuB and SuD concentrations to that of the iBatech^{TM} control and iBatech^{TM} 50% treated groups.

In conclusion, there were dramatic variations in the environmental factors of samples from the same environmental category; however the environmental factors in the greenhouse group, appeared more uniform. Significant differences were found in sutherlandin 4 when comparing the different environmental categories and sutherlandioside B when comparing the 50% and 100% treatment groups in the iBatech^{TM} experiment. Furthermore, significant differences were found in metabolite profiles and peak area values of Su3, Su4, SuB and SuD, among samples from the same environmental category as well as among samples from the iBatech^{TM} experiment. The LC-MS results obtained on the collected fractions confirm that it was SuB and SuD as described by Avula *et. al.* (2010) and the SuB and SuD quantification results suggest no

relationship between environmental category or iBatech[TM] treatment group and the synthesis of these metabolites.

Chapter 6

Conclusions and recommendations

This study was aimed at to locate sites where *S. frutescens* is grown, broadly categorize the sites into environmental groups based on the pertinent environmental growth factors, to develop and validate a high-performance liquid chromatography method for Su3, Su4, SuB and SuD analysis, to determine and compare the profiles and levels of Su3, Su4, SuB and SuD *S. frutescens* grown in different environmental growth sites and to determine whether the application of the iBatech^{TM} product increases the number and concentrations of secondary metabolites in *S. frutescens*. To achieve the aim of this study, a minimum of three *S. frutescens* samples (homogenized) per site, were collected from eight different sites and broadly categorized into three environmental categories viz. 1. natural field, 2. cultivated field and 3. greenhouse. *S. frutescens* plants were also grown in a greenhouse and treated with the iBatech^{TM} product, with a 0% control, 50% product concentration and 100% product concentration. A high performance liquid chromatography (HPLC) method was developed and validated, using the ICH guidelines, for the simultaneous analysis of flavonoids and terpenoids in *S. frutescens* and used for the profiling and determination of the average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D. Liquid chromatography − mass spectrometry (LC-MS) was used to confirm the identity of the isolated and collected SuB and SuD pooled fractions. Furthermore, HPLC was also used for the construction of calibration curves, for the quantification of sutherlandiosides B and D (SuB and SuD) in all samples.

The specific objectives were: to determine and describe sites where *Sutherlandia frutescens* is grown, which could be broadly categorized into, 1. natural field, 2. cultivated field and 3. greenhouse and the pertinent growth factors which we assume could affect secondary metabolite synthesis, to determine sutherlandins 3 and 4 (Su3 and Su4) and sutherlandiosides B and D (SuB and SuD) profiles and average peak area values in *S. frutescens* collected from the eight sites, to determine the effects of the iBatech^{TM} product treatment on sutherlandins 3 and 4 sutherlandiosides B and D profiles and average peak area values, and finally, to quantify the concentrations of sutherlandiosides B and D in all samples.
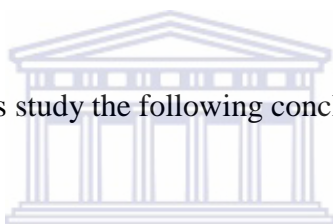
From the results obtained in this study the following conclusions can be drawn:

1. The sites that were categorized into three environmental categories illustrate a range of conditions at the time of collection, possible secondary metabolite synthesis-limiting factors and variations in plant size. The Su3, Su4, SuB and SuD profiles, average peak area values and concentrations of SuB and SuD can therefore not be correlated with the environmental categories.

2. The sutherlandins 3 and 4 and sutherlandiosides B and D profiles and average peak area values vary among the different environmental growth categories. Variations in the profiles and peak area values are also visible among sites from the same environmental growth category. These variations can be attributed to the specific prevailing environmental conditions (micro-climates) at each site. The greenhouse (GH) group,

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although not having significantly different, or the highest values for average peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D, appear more uniform in the synthesis of these metabolites and can be attributed to the control of the environmental growth factors, which is the nature of a greenhouse.

3. The iBatech[TM] product appears to have had a concentration related effect; however these differences were not significant. To date, the iBatech[TM] product has not been thoroughly investigated, thus its mechanism of action is unknown. Mass spectrometry results on the iBatech[TM] product performed by the manufacturers reveal that although containing a wide variety of flavonoids and terpenoids it does not contain the specific compounds evaluated in this study. However, some of the compounds revealed by mass spectrometric analysis are known to act as precursor compounds. It is therefore plausible to deduce that in this experiment, these compounds acted as precursors and promoted the synthesis of sutherlandins 3 and 4 and sutherlandiosides B and D.

4. The results obtained from the sutherlandiosides B and D (SuB and SuD) quantification, show two samples from the natural field (NF) and the cultivated field (CF) groups each with higher concentrations of SuB and SuD, when compared to the other samples. The greenhouse (GH), iBatech[TM] control and iBatech[TM] 50% treatment groups, which were all grown under greenhouse environmental conditions, show more stable synthesis of SuB and SuD.

From these results, we can conclude that the greenhouse growth environment, although not producing the significantly highest concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D, have more consistent/ uniform results with regard to environmental factors, metabolite profiles, average peak area values and the concentrations of sutherlandiosides B and D. I therefore recommend that *S. frutescens* grown for medicinal purposes, for the yield of sutherlandins 3 and 4 and sutherlandiosides B and D, be grown under greenhouse conditions in conjunction with the application of the iBatech^{TM} product to produce consistent concentrations of Su3, Su4, SuB and SuD. As we have covered in the literature review, the environmental growth factors can be controlled in the greenhouse. It would therefore be beneficial to more closely investigate the environmental conditions in the sites from the natural field (NF) and cultivated field (CF) groups, which show the highest concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D, and mimic those conditions in the greenhouse.
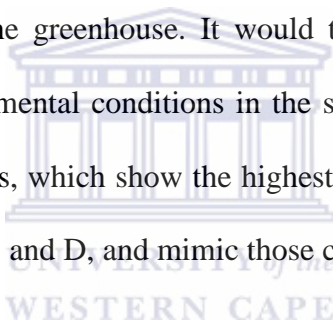
The results of this study are important in understanding the best suited environmental growth factors, applied collectively, in which to grow *S. frutescens* for greater yields of sutherlandins 3 and 4 and sutherlandiosides B and D, when growing *S. frutescens* for medicinal purposes.

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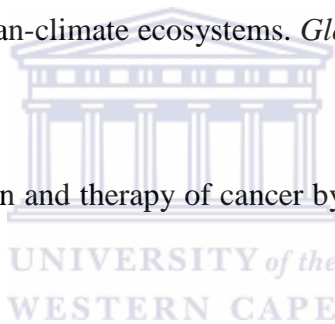
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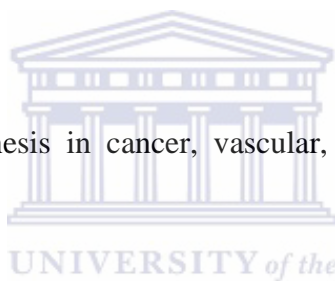
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Appendixes

Appendix 1:

Assay validation data: SuB std. curve linear regression

Std. curve SUB

$r^2 = 0.9990$

$y = (-7.813 \pm 10.28) \pm (308.7 \pm 2.674)$
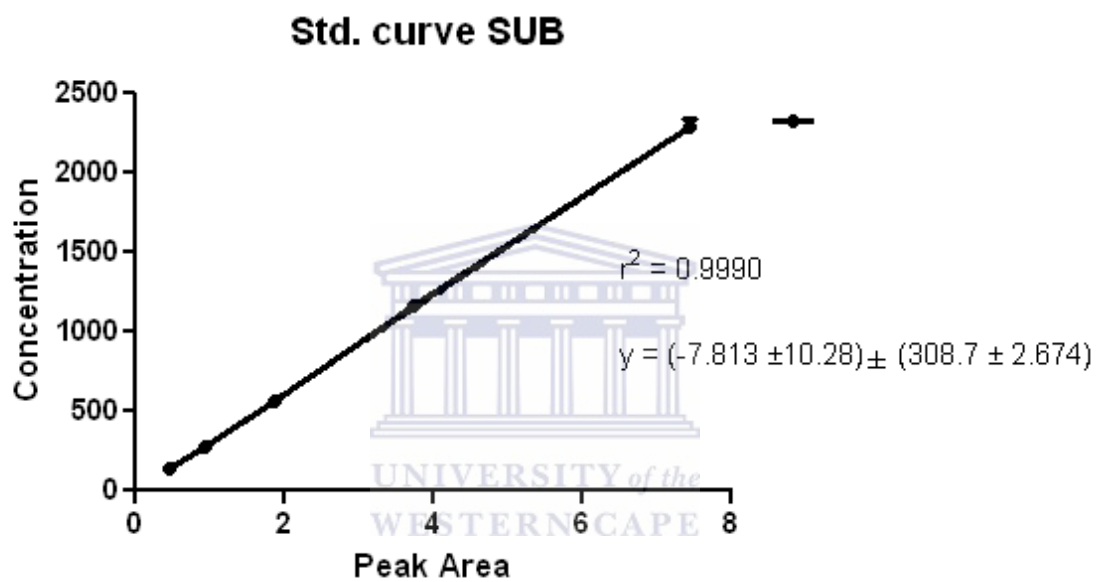
Appendix 2:

Assay validation data: SuD std. curve linear regression

Std. curve for SUD

$r^2 = 0.9999$

$y = (-17.60 \pm 5.900) \pm (281.4 \pm 1.534)$

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WESTERN CAPE
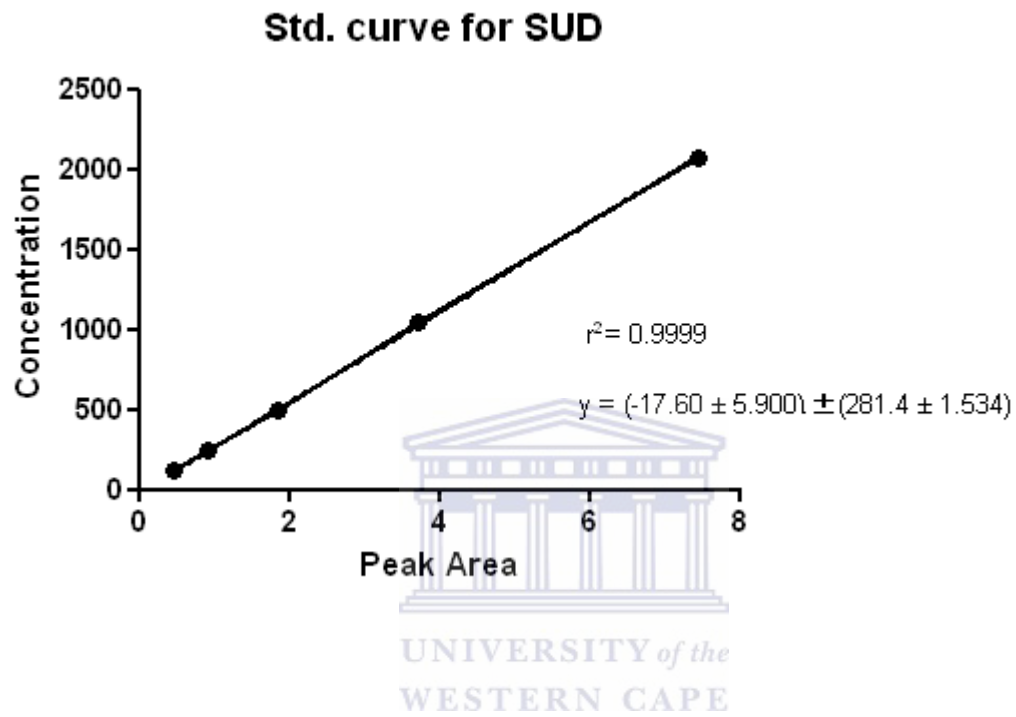
Appendix 3: The values for standard deviation, the averages and percentage RSD of the intra and inter-day variations for assay validation

		Su3	Su4	SuB	SuD
Intra-day	STDeviation	46.56798	21.30078	5.345789	6.5292
	Average	3220.178	1981.207	402.6148	427.3259
	%RSD	1.44	1.07	1.32	1.52
		Su3	Su4	SuB	SuD
Inter-day	STDeviation	48.15179	22.38726	9.00924	35.61073
	Average	3250.373	1991.1	426.0444	406.2852
	%RSD	1.48		2.11	8.76

Appendix 4: Retention time and peak area averages and standard deviation values for Sites 1-8 and iBatech[TM] experiment.

Plant Samples		sutherlandin 3		sutherlandin 4		sutherlandioside B		sutherlandioside D	
		Rt (mins)	Area (mAU)	Rt (mins)	Area (mAU)	Rt (mins)	Area (mAU)	Rt (mins)	Area (mAU)
Natural field	Site 1	4.355 ± 0.014	1527.9 ± 6.286	5.115 ± 0.013	2045.03 ± 6.785	15.005 ± 0.005	53.567 ± 0.251	15.341 ± 0.003	43.067 ± 0.450
	Site 2	4.312 ± 0.128	2330.17 ± 1314.352	5.1567 ± 0.009	907.867 ± 6.092	15.326 ± 0.003	67.23 ± 2.112	15.533 ± 0.003	48.1 ± 0.7
	Site 3	4.338 ± 0.013	4572.93 ± 2.055	5.098 ± 0.016	1534.1 ± 2.951	14.329 ± 0.020	448.3 ± 2.271	14.692 ± 0.019	341.133 ± 2.490
Cultivated field	Site 4	4.417 ± 0.025	2444.5 ± 23.612	5.3777 ± 0.027	1876.5 ± 16.802	14.357 ± 0.027	216.433 ± 2.350	14.725 ± 0.027	231.8 ± 1.824
	Site 5	4.49 ± 0.019	1747.27 ± 29.286	5.254 ± 0.126	3433 ± 847.971	14.944 ± 0.027	212.733 ± 2.335	15.299 ± 0.023	32.767 ± 0.230
	Site 6	4.430 ± 0.036	3367.67 ± 1360.339	5.377 ± 0.017	4033.77 ± 5.834	14.756 ± 0.027	1354.5 ± 2.497	15.262 ± 0.026	1368.53 ± 2.948
Green-house	Site 7	4.407 ± 0.008	2826.2 ± 3.716	5.369 ± 0.011	2390.43 ± 0.550	14.364 ± 0.034	275.5 ± 6.337	14.731 ± 0.034	321.9 ± 3.704
	Site 8	4.406 ± 0.004	2334.3 ± 3.831	5.363 ± 0.001	2333 ± 2.951	14.350 ± 0.006	377.933 ± 5.481	14.721 ± 0.007	453.067 ± 3.444
iBatech experiment	iBatech 0%	4.463 ± 0.017	9758.433 ± 12738.012	5.3555 ± 0.021	2241.633 ± 29.885	15.525 ± 0.009	289.667 ± 3.008	15.880 ± 0.008	282.767 ± 2.843
	iBatech 50%	4.45 ± 0.010	2232.633 ± 22.292	5.346 ± 0.009	2247.467 ± 21.096	15.518 ± 0.007	284.933 ± 2.150	15.873± 0.008	280.6 ± 2.151
	iBatech 100%	4.406 ± 0.011	2031.967 ± 12.859	5.364 ± 0.005	2392.6 ± 15.561	15.521 ± 0.010	332.3 ± 0.624	15.875 ± 0.010	315.133 ± 2.676

Appendix 5: Unprocessed HPLC data for the iBatech^{TM} experiment and Sites 1-8.

SAMPLE	sutherlandin 3				sutherlandin 4				sutherlandioside B				sutherlandioside D			
	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
iBatech Control	4.453	2426.9	283.5	0.1179	5.343	2250.2	387.8	0.0857	15.52	291.6	30.6	0.1332	15.875	285.1	35.8	0.117
iBatech Control	4.452	2381.4	278.5	0.1198	5.344	2208.4	378.3	0.0861	15.519	286.2	29.9	0.1338	15.874	279.6	35.1	0.117
iBatech Control	4.483	24467	286.4	0.1177	5.38	2266.3	390.6	0.0857	15.536	291.2	30.7	0.1328	15.89	283.6	35.8	0.1164
iBatech 50%	4.459	2258.3	391.7	0.0877	5.354	2271.7	391.7	0.0877	15.514	287.1	31.2	0.1295	15.868	282.7	33.4	0.1207
iBatech 50%	4.452	2221.5	239.4	0.1262	5.348	2237.5	392.2	0.0846	15.527	284.9	30.7	0.1322	15.882	280.7	32.9	0.1236
iBatech 50%	4.439	2218.1	239.7	0.1239	5.336	2233.2	388.4	0.0871	15.513	282.8	30.5	0.13	15.868	278.4	32.8	0.1232
iBatech 100%	4.414	2019.4	215	0.1256	5.369	2377.4	425.7	0.0852	15.517	332.1	34.2	0.1353	15.869	318.1	37.2	0.1237
iBatech 100%	4.412	2031.4	217.5	0.127	5.366	2391.9	425.6	0.0856	15.513	331.8	34.1	0.1355	15.868	312.9	37.2	0.1221
iBatech 100%	4.394	2045.1	213.6	0.1297	5.358	2408.5	425.6	0.086	15.532	333	34.5	0.1346	15.887	314.4	37.3	0.1202
SAMPLE	sutherlandin 3				sutherlandin 4				sutherlandioside B				sutherlandioside D			
	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
NA	4.365	1529.1	154.2	0.1357	5.125	2048.8	263.1	0.1109	15.01	53.6	5.4	0.1418	15.344	43.5	6.1	0.1094
NA	4.338	1521.1	153	0.138	5.099	2037.2	261.9	0.1107	14.999	53.3	5.3	0.1455	15.337	42.6	5.9	0.1116
NA	4.362	1533.5	154.2	0.136	5.12	2049.1	265.4	0.1101	15.007	53.8	5.4	0.1426	15.343	43.1	6	0.1116
NB	4.376	3072.7	310.2	0.1376	5.148	900.9	103.3	0.1218	15.328	68.3	5	0.1904	15.535	48.1	8.2	0.0928
NB	4.395	3105.2	314	0.1374	5.167	910.5	106	0.1202	15.328	68.6	5	0.1887	15.536	48.8	8.3	0.0907
NB	4.164	812.6	128.5	0.0938	5.155	912.2	105.5	0.1228	15.322	64.8	4.9	0.1834	15.529	47.4	8.2	0.0912

SAMPLE	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
NC	4.324	4575.3	602.3	0.1086	5.082	1532.6	179.6	0.1356	14.306	447.3	67.7	0.0991	14.669	339.9	51.3	0.1014
NC	4.351	4571.6	599.6	0.1089	5.114	1537.5	178.9	0.1382	14.342	450.9	67.9	0.0995	14.704	344	51.8	0.1015
NC	4.338	4571.9	602.2	0.1086	5.099	1532.2	179.2	0.1378	14.34	446.7	68	0.1007	14.702	339.5	51.4	0.099

SAMPLE	sutherlandin 3				sutherlandin 4				sutherlandioside B				sutherlandioside D			
	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
CA	4.404	2418.1	328.9	0.1038	5.365	1857.5	330.7	0.0855	14.334	216.4	28.3	0.1113	14.702	230.9	32.2	0.1076
CA	4.401	2451.8	334.6	0.1035	5.359	1882.6	336.8	0.0832	14.349	214.1	28.3	0.1124	14.719	230.6	32.3	0.1073
CA	4.446	2463.6	339.6	0.1026	5.409	1889.4	338.5	0.0851	14.387	218.8	28.7	0.113	14.755	233.9	32.6	0.1077
CB	4.483	1754	265.6	0.097	5.189	3959.5	691.1	0.0868	14.96	213.2	27.5	0.1124	15.225	32.9	4.4	0.1065
CB	4.475	1715.2	264.6	0.0956	5.173	3884.7	679.4	0.0867	14.912	210.2	27.3	0.112	15.208	32.5	4.4	0.1081
CB	4.512	1772.6	271.8	0.0961	5.399	2454.8	434.5	0.0839	14.959	214.8	27.9	0.1119	15.254	32.9	4.5	0.1061
CC	4.416	4146.6	426	0.1316	5.379	4027.6	666.3	0.0905	14.77	1352.5	216.5	0.0949	15.275	1365.2	202.4	0.1027
CC	4.471	1796.9	400	0.0749	5.393	4034.5	676.7	0.0896	14.774	1357.3	218.2	0.0966	15.279	1369.6	204.1	0.1023
CC	4.402	4159.5	423.5	0.1326	5.359	4039.2	666.8	0.0907	14.724	1353.7	216.8	0.0948	15.232	1370.8	202	0.1032

SAMPLE	sutherlandin 3				sutherlandin 4				sutherlandioside B				sutherlandioside D			
	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
GA	4.416	2829.7	384.4	0.1059	5.382	2389.9	425	0.0856	14.404	268.2	37.7	0.1069	14.77	317.7	48.2	0.0989
GA	4.406	2822.3	384.2	0.1037	5.364	2390.4	426.8	0.0853	14.341	279.6	38.7	0.1084	14.705	323.3	47.7	0.1031
GA	4.4	2826.6	384	0.1039	5.362	2391	428.2	0.0851	14.348	278.7	37.9	0.1079	14.719	324.7	48.5	0.102
GB	4.409	2331.7	331.6	0.102	5.362	2331.1	426.9	0.0817	14.345	371.7	47.9	0.1146	14.715	449.1	63	0.1071
GB	4.407	2338.7	335.3	0.1014	5.363	2336.4	422.2	0.0846	14.357	380.1	48.4	0.1155	14.729	454.8	62.8	0.1085
GB	4.401	2332.5	334.2	0.0994	5.364	2331.5	419.4	0.0849	14.347	382	48.4	0.1162	14.718	455.3	62.7	0.1087