Use of antioxidant activity and flavonoid levels to assess the quality of commercially available solid dose *Sutherlandia frutescens* products

By

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KEYWORDS

Sutherlandia frutescens,
Antioxidant activity,
Flavonoids,
HPLC,
DPPH,
FRAP

SUMMARY

There are various preparations of *Sutherlandia frutescens* commercially available on the market, the quality and consistency of which have not been scientifically investigated.

The overall aims of this project were to assess the pharmaceutical quality and consistency of commercially available solid dose *Sutherlandia frutescens* containing products (viz. tablets & capsules) by exploring the use of monitoring the pharmaceutical presentation, flavonoid profile and antioxidant activity levels and to develop/or adapt methods and specifications that may be used for the quality control of such products.

Seven solid dose *Sutherlandia frutescens* containing products (SCP) were purchased from various stores around the Western Cape in this study. The physical appearance, information on the packaging, the presentation of the package insert, the organoleptic properties of the dosage form of the selected products were assessed as a means to determine the possible compliance of the products with regulatory requirements.

Total phenol concentrations of each of the SCP were analyzed using the Folin-Ciocalteu reagent assay. Antioxidant tests were conducted on all the SCP using two assays, viz ferric reducing antioxidant power (FRAP) and diphenyl picrylhydrazyl (DPPH). Chromographic fingerprints of all of the SCP were generated using high performance liquid chromatography (HPLC) to identify and isolate individual flavonoids. Fractions of these flavonoids were collected and further analyzed using LC-MS to confirm identification of compounds.

Stability tests were conducted on all of the selected SCP. The products were stored under elevated temperatures and environmental humidity conditions and total phenol, antioxidant and

chromatographic analysis was conducted on these samples. Samples of each of the SCP were hydrolyzed using HCL and then analyzed using HPLC to test the stability of the flavonoids present in each product.

The SCP investigated in this study physically appeared to be of quite good "pharmaceutical" quality, but generally lacked information on the date of manufacture and lacked package inserts, or when these were present they contained insufficient information.

All the SCP had antioxidant activity which ranged, in descending order as follows: Promune® >> Big Tree 350® >> *Sutherlandia* Pinnacle® > *Sutherlandia* Health Connection Wholefoods® > Bio-*Sutherlandia*® > Big Tree Pro® >>Phyto Nova SU1®. The two assays used i.e. ferric reducing antioxidant power (FRAP) and diphenyl picrylhydrazyl (DPPH) were useful in comparing the relative potencies of the SCP. The SCP capsules possessed greater antioxidant power than the tablets.

The total phenol concentration of the selected SCP varied from 4.162 to 13.930 mg gallic acid equivalence, in descending order: Promune® >> Big Tree 350® >> Sutherlandia Pinnacle® > Big Tree Pro® > Phyto Nova SU1® > Sutherlandia Health Connection Wholefoods® > Bio-Sutherlandia®, which correlated with the antioxidant activity results.

All the selected SCP contained the flavonoids, sutherlandins A to D in varying amounts. The SCP capsules having far greater sutherlandin levels than the tablet dosage form. The chromatographic fingerprints generated were compared to existing data. The peaks that resembled sutherlandins A to D were collected and sent for mass spectrometry analysis to confirm their identity.

Elevated temperature and humidity significantly affected the aesthetic, pharmaceutical quality and the chemical stability of the SCP. The SCP appeared to be particularly affected by the elevated humidity, which caused a decrease in the total phenol content of the products.

The SCP are very susceptible to acid hydrolysis. Under these conditions the 4 sutherlandins are significantly degraded to derivatives of quercetin and kaempferol, which in turn are also susceptible to acid hydrolysis but not to the same extent.

Based on the results obtained, it is recommended that, the manufacturers of SCP pay more attention to the information provided on the package inserts and the storage conditions for their products. Further the levels of antioxidant activity, total phenols and flavonoid (sutherlandins A to D) be used as specifications to control the quality of commercially available solid dose *Sutherlandia frutescens* containing prepara ndividual basis.

DEDICATION

In memory of my deceased father Errol John Hess for his love and support. I also dedicate this thesis to my mother, Berenice Lydia Hess, for her love and encouragement.

DECLARATION

I declare that the thesis Use of antioxidant activity and flavonoid levels to assess the quality of commercially available solid dose *Sutherlandia frutescens* products is my own work, that it has not been submitted before for any degree or examination in any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

M.S. Hess

September 2010

Signed:

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Chapter 1

INTRODUCTION

Sutherlandia frutescens (*Fabaceae/Leguminosa*) is a traditionally used phytomedicine, indigenous to South Africa (Fu *et al*., 2008). It is commonly known as "cancer bush", "*kankerbos*" and "*umnwele*" (Van, Wyk, 1997) and is used or claimed to be useful in the treatment of internal cancers, inflammation and viral diseases (Rood, 1994, Humphries *et al*., 2006) as well as a supportive treatment for anxiety, convulsion, depression, hot flushes and irritability during menopause (Gericke *et al*., 2001; Mills *et al*., 2005; Prevoo *et al*., 2004; Ojewole, 2008). The most commonly used ethnomedicinal method of preparing *S. frutescens* for treatment of internal disease is by making a hot water extract from the dried twigs and leaves, similar to preparing a tea (Humphries *et al*., 2006). *S* is however increasingly commercially available in various dosage forms, namely tablets, caps s, creams, gels and herbal teas. The quality and consistency of these commercially available phytomedicinal preparations are however not known nor easily verifiable.

Many factors determine the quality of herbal products. As briefly discussed by Kroll and Cordes (2006) in order to have a high quality product of an individual herbal species one must comply with the guidelines for Good Agricultural Practice (GAP) during the cultivation of the plant, as well as Good Manufacturing Practice (GMP) standards during the manufacture of the final product. In addition, the finished product must comply with packaging, labelling and regulatory requirements set by the South African Medicine Control Council (MCC). Bandaranayake (2006) defines the term, "quality" as the status of a drug that is determined by identity, purity, content, chemical, physical, and biological properties, or by the manufacturing processes. Upon reading the packaging labels of current commercially available *S. frutescens* (*Sutherlandia*) preparations, the names of the products and manufacturers are often visible and

easily identifiable. The treatment or claimed therapeutic uses are frequently given somewhere in fine print and expiry dates are not always present. Often, package inserts or information regarding the chemical constituents, possible side-effects, dose recommendations and physical appearance (e.g. white tablet) found in synthetic pharmaceutical products are omitted in herbal preparations. Moreover none of these products are registered with the Medical Control Council (MCC) which is the regulatory authority of all medicines available in South Africa. Thus there is no guarantee that the present commercially available herbal products are of good pharmaceutical quality, safe for use and effective.

The quality of the commercially available *Sutherlandia* preparations can be judged on the levels of its chemical constituents. *Sutherlandia frutescens* is said to contain gamma (γ)-amino butyric acid (GABA), D-pinitol, L-canavanine, triterpenoids, and flavonoids (Gericke *et al*., 2001; Mills *et al*., 2005). Flavonoids are water-soluble polyphenols and over 4000 have been isolated from plants. These compounds are good free radical scavengers (1999; Chu *et al*., 2000; Hsu *et al*., 2005) and as such, may be used to prevent inflammation a atment of tumours and cancers (Atawodi, 2004; Fernandes *et al*., 2004). Flavonoids may therefore be responsible for, or contribute to most of the therapeutic properties which *Sutherlandia* is claimed to possess and should thus be good markers to use when monitoring the quality of commercially available *Sutherlandia* preparations. Since flavonoids are polyphenols, one could simply measure the level of total polyphenols in the *Sutherlandia* preparation as a measure of their quality. Individual flavonoids (glycosides and aglycones) present in the plant preparation can be assayed using high performance liquid chromatography (HPLC), while total phenol levels could be estimated using tests such as the Folin-Ciocalteu reagent technique (Singleton *et al*., 1999). The profile and levels of flavonoids and the total polyphenol content of *Sutherlandia*, and the factors which may influence their levels in the finished herbal product have however not yet been fully studied.

The quality of commercially available *Sutherlandia* preparations can perhaps also be judged on the levels of activity they should possess. For example, as for many other herbal preparations *Sutherlandia* is expected to have antioxidant activity (Atawodi, 2005). The antioxidant activity of the *Sutherlandia* preparations may be quantified from their ability to scavenge free radicals. For the latter several tests are available, one being the diphenyl-2-picrylhydrazyl radical (DPPH) assay. Diphenyl-2-picrylhydrazyl is a stable organic free radical with an absorption range between 515-528nm, and one that freely accepts electrons. When DPPH accepts an electron it loses its absorption ability, and a visual discolouration from purple to transparent/yellow is observable (Hsu *et al*., 2005). Another colourimetric antioxidant activity assay is the ferric reducing antioxidant power (FRAP) assay (Benzie *et al*., 1996). The use of the level of antioxidant activity to compare the quality of *Sutherlandia* product has however, to our knowledge, not yet been employed.

Given the afore mentioned arguments, the 

s of this project were to explore the use of monitoring flavonoid profile and antioxidan vels to assess the pharmaceutical quality and consistency of commercially available solid dose *Sutherlandia frutescens* containing products (viz. tablets & capsules) and to develop/or adapt methods and specifications that may be used for the quality control of such products.

The specific objectives of the study were:

- To assess and describe the quality and regulatory compliance of selected representative solid dose *Sutherlandia*-containing products,
- To determine the total phenol and flavonoid levels of selected commercially available solid dose *Sutherlandia* products,
- To determine the profile and levels of specific flavonoids in the selected *Sutherlandia* products,
- To determine the levels of antioxidant activities of the selected *Sutherlandia* products,

- To determine the effect of temperature, environmental humidity and chemical-induced (or acid-induced) degradation on the flavonoid profile and levels and the antioxidant activities of the selected *Sutherlandia* products, and
- To formulate quality control specifications that may be used for the selected *Sutherlandia* preparations.

It was hypothesized that when solid dosage forms of *Sutherlandia frutescens* are subjected to quality transforming conditions such as elevated temperature, humidity and acidity;

- The antioxidant activity will decrease,
- Total flavonoid and polyphenol levels may decrease or remain unchanged,
- Levels of flavonoid glycosides will decrease and
- Levels of free aglycone flavonoids will decrease.

The present study thus focussed on the qual  ercially available pharmaceutical solid dose *S. frutescens* containing products and the use ofctivity, total and free flavonoid levels to assess the quality of such products.

Chapter 2

LITERATURE REVIEW

2.1. Introduction

This chapter provides an overview of the *Sutherlandia frutescens* herb, focusing on the description of the plant, its traditional uses, dosage forms and the known phytochemicals. A discussion on the flavonoids, methods used to evaluate antioxidant activity, quality control of herbal preparations as well as the parameters used to assess quality of solid dose *Sutherlandia* products are also included.

2.2 *Sutherlandia frutescens* the p

Sutherlandia frutescens (L.) R. Br. is a member of the *Fabaceae/Leguminosa* family (Rood, 1994; Van Wyk *et al*., 1997; Roberts 1990; Manning, 2003). It is a small perennial woody shrub, which grows to the approximate height of 1meter. The leaves are pinnately compound made up of small oblong leaflets that are rounded at the tips (Van Wyk *et al*., 1997; Manning, 2003). These leaflets have a silvery appearance but are essentially grey-green in colour with fine hairs on the top surface. The flowers bloom in spring to mid-summer (September to December), they are orange-red in colour and are carried in short racemes in the leaf axils at the tips of the branches. Large, bladder-like, papery inflated seed pods that are almost transparent (Xaba *et al*., 2003) are also present (figure 1).

Figure 1: Flowering *Sutherlandia frutescens* plant. (Xaba, 2003)

Sutherlandia frutescens, also known as *Lessertia frutescens* (Van Wyk 2008), is indigenous to the dry regions of South Africa and mainly distributed in the Western Cape and the Karoo regions of South Africa (Van Wyk *et al*., 1997; Fernandes *et al*., 2004). Due to the popularity of *Sutherlandia* amongst different ethnic groups, it is known by many different names, some of which are listed in table 1.

Table 1: Common names of *Sutherlandia frutescens* amongst different ethnic groups in South Africa

Language	Common name/s	Reference
English	"Duck plant", "Cancer Bush", "Bladder Senna" and the "Scarlet balloon pea"	(Humphries *et al*., 2006; Manning, 2003)
Afrikaans	"Kankerbos", "Blaasbossie", "Blaas-ertjie", "Eendjies", "Gansiekeurtjie", "Klappers" and "Hoenderbelletjie"	(Katerere *et al*., 2005; Xaba, 2003)
Tswana	"Phettola"	
North-Sotho	"Lerumo-Lamandi"	
Sotho	"Motelepe"	
Ancient Zulu	"Insiswa"	(Gericke *et al*., cited 2008)
Xhosa/Zulu	"Umnwele"	(Xaba *et al*., 2003)

The medicinal uses for *Sutherlandia* are ve[illegible]nd include the treatment of internal cancers, inflammation and viral diseases (Rood, 1994 [illegible]*et al*., 2004). It is also used in the treatment of anxiety, convulsion, depression, as well as hot flushes and irritability during menopause (Gericke *et al*., 2001; Mills *et al*., 2005; Prevoo *et al*., 2004; Ojewole, 2008). *Sutherlandia* is regarded as an immunomodulator since it appears to have the ability to regulate the immune system in chronic inflammatory diseases e.g. osteoarthritis, rheumatoid arthritis and inflammatory bowl disease (Gericke *et al*., 2001; Mills *et al*., 2005; Prevoo *et al*., 2004; Humphries *et al*., 2006).

Sutherlandia frutescens is used in a variety of medicinal dosage forms e.g. gels, creams, tablets and capsules. However, the most commonly used ethnomedicinal method of preparing *S. frutescens* for systemic use is by making a hot water extract from the dried twigs and leaves, similar to preparing a tea (Humphries *et al*., 2006). Moreover, *S. frutescens* prepared in this way has been shown to possess reactive oxygen species (ROS) scavenging abilities (Fernandes *et al*., 2004; Atawodi, 2005).

Sutherlandia frutescens contains many active compounds. One such compound is the non-protein α-amino acid, L-canavanine which is said to have anti-tumour and antiviral activity that may serve as a marker in the treatment of HIV/AIDS (Fernandes *et al*., 2004; Humphries *et al*., 2006). Other chemical actives of the plant include pinnitol, which is used traditionally as an anti-diabetic and in the treatment of cachexia (Fernandes *et al*., 2004 and Humphries *et al*., 2006), while small amounts of saponins, gamma-amino butyric acid (GABA), asparagine and triterpenoid glucosides, known as sutherlandiosides, are also present (Gericke *et al*., 2007). In addition, *S. frutescens* also contains flavonoids, in the form of sutherlandins, which are well known constituents of most plants that possess antioxidant properties (Fu *et al*., 2009; Mills *et al*., 2005).

2.3 Flavonoids

Flavonoids are water soluble polyphenols (L[illegible] 2006) with a characteristic structure, as illustrated in [illegible]nerally the chemical architecture is based on a three ring structure, with the phenyl rings having a variety of functional groups (i.e. R_1-R_6) such as hydroxyl and carboxylic groups. Flavonoids exist as free aglycones, and as *O*- or *C*-glycosides, differing only in their type of bonds (de Sousa *et al*., 2008). The position of the functional groups helps to distinguish between the different flavonoids and contributes to their different functions (Lai *et al*., 2007).

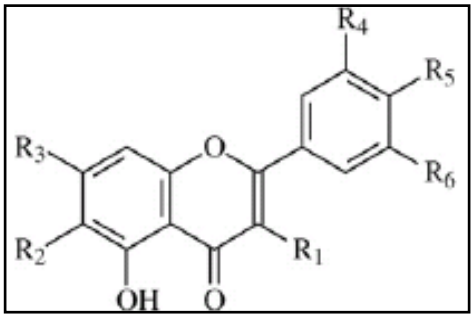

Figure 2: Chemical structure of a general flavonoid aglycone. (Lai *et al*., 2006)

Over 4000 flavonoids have been identified to date in different higher and lower plant species and it is estimated that 2% of all carbon photosynthesized by plants is converted into flavonoids which makes them excellent marker compounds to detect in plants (Marinova *et al*., 2005; Vijayakumar *et al*., 2008). These compounds are widely distributed in plants and add important sensory value, i.e. colour, flavour and

fragrance, as well as nutritional value (Li *et al*., 2006) among others. Flavonoids have been shown to demonstrate anti-inflammatory and anti-tumorial properties (Lai *et al*., 2007). Plant flavonoids are usually bound to a sugar molecule, in the form of glycosides, which allows for several combinations of sugar(s) for a single aglycone (Harborne, 1973). These flavonoids are further divided into five sub-classes, namely flavonols, flavones, anthocyanidins, catechins and flavanones as illustrated in figure 3 (Fang *et al*., 2007).

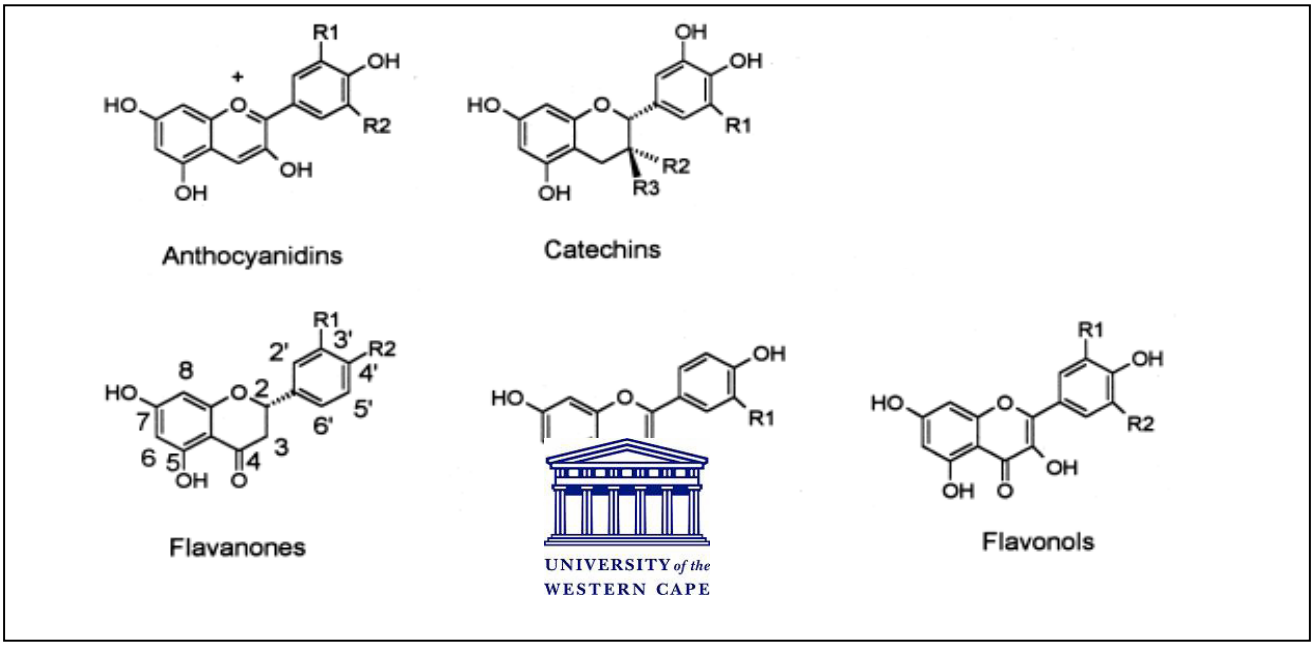

Figure 3: The sub-classes of flavonoids. (Merken *et al*., 2000)

Various methods may be used to quantify the levels of phenols and flavonoids in plants. These include: high-performance liquid chromatography (HPLC), liquid chromatography with diode array detection or atmospheric pressure chemical ionization/mass spectrometry (i.e. LC/DAD-APCI/MS). The HPLC and LC/DAD-APCI/MS methods generate chromatographic fingerprints of compounds present in an extract which is detected either by diode array or fluorescence detectors and these chromatographic fingerprints can be used to identify and isolate individual flavonoids (Lai *et al*., 2007; Germanǒ *et al*., 2006). Alkaline and acid hydrolysis may be used to release bound phenolic acids (flavonoids with sugar moieties attached) before these chromatographic tests are conducted (Germanǒ *et al*., 2006). While providing excellent information, these methods however require expensive and sophisticated equipment and much simpler cost effective methods may be worth considering.

One method used to identify total phenols (total flavonoids) from natural products is known as the Folin-Ciocalteu Reagent (FCR) assay (Roura *et al*., 2006). The first phase of the FCR assay consists of making up a liquid filtrate of the material or substance which is to be evaluated. This is followed by a purification step which removes all sugars and organic acids that may contribute to the absorbance measurements in the FCR assay (Li *et al*., 2006). In the final phase of the assay, one would follow a typical standard Folin-Ciocalteu assay protocol such as the one developed by Wildenrant and Singleton in 1974, where a standard curve of total phenol concentration *versus* absorbance is constructed using gallic acid as standard and then the concentration of total phenols in the unknown solution established from the absorbance readings of the gallic acid solutions, in terms of gallic acid equivalence (Singleton *et al*., 1999). It is also important to note that different phenolic compounds show different colorimetric responses when using FCR (Amarowicz *et al*., 2004).

Different classes of phenolics are likely to have different antioxidant strengths, synergism of polyphenolics with one another and other components present in an extract may contribute to the overall antioxidant activity, thus antioxidant activity cannot be predicted solely on the basis of total phenolic content (Amarowicz *et al*., 2004).

2.4 Antioxidants and their purpose

There is a considerable amount of evidence that suggests that cellular damage arises from the accumulation of reactive oxygen species (ROS), especially in chronic inflammatory diseases such as asthma, Alzheimer's disease, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), atherogenesis and diabetes mellitus (Atawodi, 2005). The accumulation of ROS can be lessened by using antioxidants which displace electrons that contribute to chronic inflammatory diseases.

Inflammation is one of many specific immune responses induced by the innate immune system, which only provides early defence against pathogens that invade the body. It is characterized by an increase in blood flow and permeable vasculature, which increases the accumulation of fluid, leukocytes and inflammatory mediators, i.e. cytokines, to the injured tissue (Feghali *et al*., 1997). When the immune cells are activated, they accumulate and penetrate into the tissue where they undergo a respiratory explosion, resulting in an increase in their oxygen use, an increase production of cytokines, reactive oxygen species (ROS), and other inflammatory mediators. The ROS produced can initiate and continue inflammatory cascades which cause subsequent tissue damage. It is apparent that ROS does this through the up-regulation of a number of different genes involved in immune and inflammatory response (Kaplan *et al*., 2006). One such ever-present transcription factor that may be implicated in regulating these genes is the nuclear transcription factor-kappa B (NF-κB), whose primary role is to regulate the innate and adaptive immune responses (Kaplan *et al*., 2006; 4). By interacting with or by reducing the accumulation of ROS e.g. with use of antiox nmune and inflammatory response can thus be significantly modulated.

Most antioxidants which are non-enzymatic defences against the accumulation of ROS are introduced into the body via our diets (Berker *et al*., 2007). A variety of herbs have been identified as possessing anti-inflammatory and antioxidant properties, among them, *S. frutescens*. These herbs are often used to treat inflammatory disorders including those caused by excessive accumulation of ROS. It has been suggested that since oxidants are capable of activating nuclear factor κB (NF- κB) involved in inducing apoptotic cell death, a group of structurally diverse antioxidants should be capable of inhibiting NF-κB activation (Kaplan *et al*., 2006; Dirsch *et al*., 1998). Given the potential usefulness of the antioxidant activity of *S. frutescens* it would thus be desirable to quantitate the levels of such activity in the different solid dose SCP and explore the methods which may be used to do so.

2.5 Methods used to evaluate antioxidant activity

There are many methods that can be used to determine the antioxidant activity of herbal products. They include methods based on measuring radical scavenging, luminol and lucigenin enhanced chemoluminescence of neutrophils and the ferric reducing antioxidant power (FRAP).

Sutherlandia contains flavonoids and phenols which are able to act as free radical scavengers that terminate radical chain reactions during triglyceride oxidation. Thus the antioxidant activity of phenols can be tested based on their ability to scavenge the stable diphenylpicrylhydrazyl (DPPH) free radical (Li *et al*., 2007). The free radical DPPH test method is commonly used to determine the ability of flavonoids to transfer hydrogen (H) atoms to radicals (Tsimogiannis *et al*., 2006). Using distilled deionized water as a standard, one measures the absorbance of the antioxidant activity of DPPH by the crude extract at 515nm (Li *et al*., 2007), which is the ideal wavele sure DPPH absorption (Brand-Williams *et al*., 1994). A visual colour change from purple to served and the absorbance at 515nm decreases when/if the extract is able to reduce the stable $DPPH^{\cdot}$ (Li *et al*., 2007; Brand-Williams *et al*., 1994). The advantages of using the DPPH test are that one can see a visual change in colour when a reaction has occurred, one can evaluate many samples in a short period and it is sensitive enough to detect active ingredients at low concentrations (Hsu *et al*., 2005).

A second antioxidant activity test is one that focuses on luminol and lucigenin enhanced chemoluminescence of neutrophils stimulated with L-formyl-L-methionyl-L-phenylalanine (FMLP) and can also be used in *in-vitro* studies (Fernandes *et al*., 2004). This test is however very time consuming and requires a lot of precision.

A third test for antioxidant activity is the ferric reducing antioxidant power (FRAP) assay. In this assay changes in absorbance of test mixtures at 593nm are monitored and compared to that for mixtures containing known concentrations of ferrous ions (Benzie *et al*., 1996). This assay measures the reduction of ferric-tripiridyltriazine (TPTZ) to ferrous iron (Fe^{3+}/Ferricyanide complex to Ferrous Fe^{2+}) in the presence of antioxidants with a notable change in absorbance and colour from yellow/clear to Prussian blue or shades of green (Cao *et al*., 1998; Amarowicz *et al*., 2004; Hadi *et al*., 2004). This method is also very simple, inexpensive and convenient.

The one disadvantage of using the DPPH and FRAP antioxidant tests to assess herbal products is that one cannot assess the direct effect of a specific chemical compound present in the herbal preparation that contributes to the action. Thus further tests, e.g. HPLC fingerprinting, etc, to identify the phenolic compound responsible for the free radical scavenging must be conducted.

2.6 Quality of commercially available *Sutherlandia frutescens* preparations

Sutherlandia frutescens containing products are commercially available in a variety of dosage forms, such as gels and creams which are topically applied to the skin, tonics, tablets and capsules which are consumed orally, and the dried herb/plant material which can be made into a tea. In modern society it is important that such products are of acceptable quality, safety and efficacy for consumers.

It is however extremely difficult to assess the quality of these preparations since there are few universally accepted standard parameters and validated procedures (tests) by which the herbal remedies, especially traditionally used herbal remedies, can be tested. There are many websites and health shops which can be visited either electronically or physically to purchase *S. frutescens* containing products which are claimed to have miraculous healing abilities, ranging from treating mild asthma to being an immune modulator.

There are many companies which invest a lot of money in producing these herbal remedies but few who actually test whether, or provide evidence that, their claims of efficacy are true. As a result of this lack of scientific research on the identification, quality and effects of all (or even just some of) the active ingredients in *S. frutescens* products by their manufacturers, these products will not generally qualify as registered medicines.

At a national level, the Medicines Control Council (MCC) plays a vital role in regulating the registration of any medicines produced in South Africa. Any products sold with a stated medical purpose have to satisfy the MCC standards and regulations pertaining to safety, efficacy and quality (Mander, 1998). These regulatory standards are very time consuming and costly to comply with and therefore many indigenous herbal "medicines" can only be traded as remedies because they have not met the standards set by the MCC for registration as medicines. At an international level a few guidelines and standards are available to ensure quality of traditional herbal and natural food supplements. Such guidelines may be used to identify tests and criteria on which the *Sutherlandia* products could also be assessed.

2.6.1 Parameters to assess quality of solid dose *Sutherlandia* products

As mentioned in chapter 1, the variability of the medicinal value of a plant can be influenced by the different conditions of growth, harvesting, drying and storing of the material (Gulati *et al*., 2006) as well as how it is manufactured and packaged. Thus, various guidelines such as Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP) and the guidelines from various regulatory authorities, such as the Food and Drug Administration (FDA) of the USA, the European Agency for the Evaluation of Medicinal Products (EMEA), and the Therapeutic Goods Administration (TGA) of Australia to mention a few, are available to help establish the quality of herbal products. These guidelines set standards for the cultivation and manufacture of herbal and other pharmaceutical preparations and provides information

pertaining to a variety of parameters that has a bearing on product quality. As established by the European Union (EMEA, 2006), some of the parameters that could be used to assess the quality of indigenous herbal products such as *Sutherlandia,* are as follows:

1. **Botanical source** (i.e. identity, scientific and common names, part of plant used, origin, contamination, and which part of the plant is most toxic),
2. **Growth conditions** (GAP, whether wild or cultivated, site of collection, time of harvest, stage of growth, storage conditions, as well as pre- and post- harvest treatments),
3. **Raw Materials** (specifications should follow preset standards, quantitative tests that should be conducted to determine constituents relevant to the plants significance and stability data should be recorded),
4. **Processes applied to the starting material** (extraction process, solvents, method used and specific precautions that should be followed),
5. **Botanical preparations** (established stan[UNIVERSITY of the WESTERN CAPE]ourity and specification criteria which includes; physiochemical properties, biological actives, formulation methodology and storage conditions),
6. **End product** (dosage form for consumption and stability data should be included), and
7. **Herbal product specifications** (the standards on which the scientific quality, quality control/assurance and appropriate use can be guaranteed. Such specifications should comply with existing standards, with human safety data given top priority and non-clinical tests concentrating on toxicology (Gulati *et al*., 2006)).

Since no *Sutherlandia frutescens* preparations have thus far, mainly due to insufficient available scientific evidence, been approved by the MCC, this thesis aims to investigate the feasibility of evaluating the quality and consistency of selected *Sutherlandia*-containing products based on their antioxidant activity, total phenol and flavonoid levels. The results obtained could convince the MCC that sufficient scientific

data on at least these parameters should be available if a manufacturer wishes to claim that their products are of suitable quality to be classified as a medicine.

Chapter 3

PLAN OF WORK

3.1 Introduction

In this chapter the objectives of, and the approach used in this study to assess the pharmaceutical quality of commercially available solid oral dosage forms of *Sutherlandia frutescens* are presented.

3.2 Objectives of study

The overall aims of this project were to expl[UNIVERSITY of the WESTERN CAPE]of monitoring flavonoid profile and antioxidant activity levels to compare the pharmaceutica[UNIVERSITY of the WESTERN CAPE] consistency of commercially available tablets and capsules of *Sutherlandia frutescens* and to develop or adapt methods and specifications that may be used for the quality control of such products.

The specific objectives of the study were:

- To assess and describe the quality and regulatory compliance of selected representative solid dose *S. frutescens*-containing products,
- To compare the total phenol levels of selected representative solid dose *S. frutescens*-containing products,
- To determine and compare the antioxidant activity of selected commercially available solid dose *S. frutescens* products,
- To determine the profile and specific flavonoids in the above mentioned products,

- To determine the effect of various environmental stresses [i.e. elevated temperature, environmental humidity and chemically-induced (or acid-induced) degradation] on the selected products, and
- To formulate quality control specifications that may be used for the selected *Sutherlandia frutescens* preparations.

3.3 Study Approach

To realize the above mentioned objectives the following were done:

3.3.1 Selection and collection of commercially available solid dose *Sutherlandia* containing products

In order to establish what *S. frutescens* contai 

s were commercially available, a select number of local pharmacies, health stores and sup ere visited to access the availability of any *Sutherlandia*-containing solid dose preparations. Products listed on the internet which appear to be available in more than one province in South Africa as well as exported internationally will be included in this study if they are readily available. From this, approximately 5 tablet and 5 capsule preparations were to be purchased and included in this study. It was postulated that this sampling procedure and sample size would best represent the *Sutherlandia* solid dose preparations that would be the most frequently used and whose ongoing quality would be an important consideration.

3.3.2 Assessment of regulatory compliance of selected *Sutherlandia* dosage forms

Although at present there are no regulatory processes by which herbal preparations are formally registered in South Africa it can still be assumed that the physical appearance of herbal dosage forms, the quality of

the packaging and labeling of the preparation and any information that accompanies such preparations should, as for normal pharmaceuticals, be useful as indicators of the quality and possible regulatory compliance of such herbal preparations. Thus, in this study the physical appearance of, information on the packaging, the presence and content of the packaging insert and the organoleptic properties of the dosage form of the selected products were to be assessed as a means to determine its possible compliance with regulatory requirements.

Upon reading the criteria which the EU has established when differentiating between a herbal supplement and herbal medicine, the following regulatory compliance items on which the quality assessment could be based were selected.

1. **Physical appearance of the packaged preparation**: e.g. the container choice of selection would be plastic or amber glass securitainers with screw-on caps. The containers should serve its function of protecting the product from de environmental conditions such as sunlight and humidity. Also allows for opening and re-sealing of the product. A safety seal (tac seal) should be fully intact on all newly purchased products to ensure that the product has not been tampered with.
2. **Label:** The labeling should be clear and visible. All printed information should be legible. The product name, manufacturer, composition, an expiratory date and storage conditions should be stipulated on the label.
3. **Product:** At least one month's supply (approximately 30) of tablets or capsules should be available in each of the selected *S. frutescens* containing products. The colour, texture and odour are also important. Uniformity of weight and size of each preparation would be another factor to consider.
4. **Package insert**: The information contained on the insert should comply with set guidelines.

The above items and criteria were selected on the basis that they would probably be part of the set criteria required by most regulatory authorities and would be the minimum to which any *Sutherlandia*-containing

product of acceptable quality should comply. Thus before any tests were to be conducted on any *Sutherlandia*-containing product it had to meet the set criteria recorded in appendix A1.

3.3.3 Comparison of total phenol content of the dosage forms

Sutherlandia, like most plants, are rich in polyphenols which probably also contribute to their activities, especially antioxidant activity. The level of polyphenols will not specifically indicate the quality of *S. frutescens* containing products, but will only indicate the presence of other polyphenols which may be due to inclusion of other plant materials or even synthetic molecules. Consequently, the Folin-Ciocalteu test was used to measure the total polyphenol levels of each of the selected solid dose *Sutherlandia* containing products, using gallic acid as standard. This test offered several advantages. Firstly, the Folin-Ciocalteu Reagent (FCR) which consists of sodium tungstate, sodium molybate, distilled water and hydrochloric acid (Singleton *et al*., 1999), can be purchase  xed. Secondly, the test is quick, a visual colour change from intense yellow to green/blue ochenols are added. Thirdly, in this study it was intended to conduct the FCR test using smaller quantities than usually prescribed so that the assay could be done on a 96well microplate and in so doing allow many samples to be tested simultaneously. Finally, because the identity of all the phenols in the plant material was not known the total phenol levels of the plant materials were to be expressed in terms of gallic acid equivalents. Gallic acid is a popular comparison standard used to test total phenol content. Historically tannins were first used as standards to test total phenol content in wines and spirits, however due to the varying methods of preparation and origins of tannins, too wide a range of colour yield per unit weight was covered. Gallic acid is a phenolic unit in tannic acid from oak galls, it is inexpensive, water soluble, easily recrystallized from water, readily dried and is stable in the dry form (Singleton *et al*., 1999) and was thus deemed suitable for use in this study.

3.3.4 Determination and comparison of antioxidant activity of the dosage forms

Herbal medicines, as for conventional medicines, are taken for some or other therapeutic effect. While the exact therapeutic effects of *Sutherlandia* are not known it is expected to have antioxidant activities (Atawodi, 2005). Antioxidants have gained popularity particularly for their ability to prevent the detrimental effects that free radicals have on the human body. Antioxidant activities will not only indicate the quality of *S. frutescens* containing products, but will also indicate the presence of molecules that are capable of giving an antioxidant activity which may be due to the inclusion of other plant material or even synthetic molecules.

Although the products to be tested were solid dosage forms it was noted that indigenously *S. frutescens* is usually consumed orally as a hot water infusion for internal ailments such as cancers (Fernandes *et al*., 2004; Humphries *et al*., 2006). Such teas are known to contain water-soluble compounds such as flavonoids (phenolics) which possess antioxidant activity (Marinova *et al*., 2005) and are the compounds of interest in the present study. Thus it was decided, for the antioxidant tests, to use aqueous extracts of the solid dosage form of the *Sutherlandia* that more closely mimic the traditional tea preparation.

In this study the diphenylpicrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays were used because these tests are less expensive and more robust than other antioxidant assays.

The DPPH test is based upon observing an antioxidant's ability to transfer hydrogen atoms to a stable DPPH free radical (Tsimogiannis *et al*., 2005; Molyneux, 2004). The reduced DPPH can be observed by a visual decolourization from purple to transparent or yellow which can be further analyzed by testing the absorbance using UV detectors (Molyneux, 2004). The popular standard used in the DPPH assay is ascorbic acid (vitamin C) (Molyneux, 2004), but since *S. frutescens* contains flavonoids that are known

antioxidants the use of the flavonoid, quercetin, was considered as a better positive control for the present study. In addition, with a slight modification of the existing assay (Singleton *et al*., 1999) the DPPH assay could also be converted to a micro-plate assay, with the added advantages of speed of analysis and increased reproducibility.

The FRAP assay measures the reduction of ferric-tripiridyltriazine (TPTZ) to ferrous iron (Fe^{3+}/Ferricyanide complex to Fe^{2+}/Ferrous) in the presence of antioxidants. A notable change in absorbance and colour from yellow/clear to Prussian blue is observed (Cao *et al*., 1998; Amarowicz *et al*., 2004; Hadi *et al*., 2004). As has been done in previous plant studies (Molyneux, 2004) ascorbic acid was used as a positive control in this study and the product's antioxidant activity expressed in terms of ascorbic acid equivalents. This assay was also conducted on a multi well micro-plate which allowed for simultaneous analysis as is required by the strict time dependency of colour development.

3.3.5 Determination of the flavonoid pr[illegible]osage forms

Flavonoids are present in *S. frutescens* and thus must also be in products of *Sutherlandia*, and contribute to the activity, and thus quality, of such products. The qualitative flavonoid levels in such preparations can thus be used to monitor and compare the quality of such products, provided a reliable assay is used.

In this study high performance liquid chromatography (HPLC) was used to obtain chromatographic fingerprints of compounds present in, and thus the flavonoid profile of, the aqueous solutions of the products. The method used was to be based, with a few modifications, on the analytical method used by Avula, *et al*. (2010). HPLC allows for the separation of compounds present in the aqueous extracts of *S. frutescens*, and indicates individual compounds as peaks in the chromatogram that can be further analyzed by observing the purity of such peaks at different wavelengths and by collecting them via a fraction

collector connected to the HPLC system and thereafter analyzing the fractions further using mass spectrophotometry. The HPLC method offered a sensitive, quick and accurate means to determine the flavonoid profiles of the different product and allowed comparison with previous (i.e. Avula *et al*., 2010) findings.

3.3.6 Determination of the effect of temperature and humidity on the stability of the dosage forms

Although some of the *Sutherlandia* solid dosage forms that are commercially available have expiry dates it is unclear how those dates or shelf-lives have been determined. Since temperature and humidity are the main factors that could affect the stability of herbal products the effect of these factors on the stability of the different *Sutherlandia* products were investigated. For this the products were to be subjected to ambient and elevated temperature and humidi ic chamber) conditions and thereafter tested for total phenol and antioxidant activity and th ained for the products compared. Accelerated stability tests are routinely recommended for conventional pharmaceutical products (Lieberman *et al*., 1998) and herbal products (Ababalle *et al*., 2008; EMEA, 2006) and the results used to determine shelf-lives, expiry dates, etc. Ideally such stability tests should be based on the level(s) of specific or select chemical constituents but this was not to be done in this study. Instead, the viability of using total phenol and antioxidant levels was to be investigated as potential alternate methods.

3.3.7 Determination of the effect of acid hydrolysis on the stability of the dosage forms

The *Sutherlandia* products contain constituents such as flavonoid glycosides, etc. that are expected to be sensitive to acid hydrolysis. Depending on the degree of susceptibility to acid the various solid dosage form products may vary in their release of active compounds in acidic environment (e.g., stomach) leading

to variable absorption and bioavailability and, consequently, variable *in vivo* effects that can be expected from the dosage form. In this study the hydrolysis of the products by hydrochloric acid was to be assessed as another means to compare the quality of the various products. Thus the HPLC fingerprints of the unhydrolysed and acid hydrolyzed products were assessed to compare the relative stability of the products.

3.3.8 Determination of the quality control specifications that may be used for the selected *Sutherlandia frutescens* preparations

In order to determine the specifications which can be used for quality control specifications of SCP the results of the above mentioned assays (DPPH, FRAP, FCR and HPLC) were used.

Chapter 4

METHODS AND PROCEDURES

In this chapter the methods and procedures used to assess the quality and consistency of selected solid oral dose *Sutherlandia frutescens* containing products are presented. The information is arranged under the following sub-headings: Chemicals, materials and equipment, determination of quality-indicating properties of selected *Sutherlandia frutescens* solid dosage forms, determination of antioxidant activity of the dosage forms, determination of total phenol content of the dosage forms, determination of the flavonoid profiles of the dosage forms, determination of the effect of temperature and humidity on the stability of the dosage forms, determination of the effect of acid hydrolysis on the stability of the dosage forms and data analysis.

4.1 Chemicals, materials and equipment

4.1.1 Chemicals and materials

The following chemicals and materials were used in this study:

- Commercial solid dose ***Sutherlandia* containing products** (see figure 4 in results section),
- **Distilled water** (distilled using a Fi-streem 2901 manufactured in England),
- **2.2-diphenyl-1-picrylhydrazyl (DPPH), free radical powder** (Sigma, D9132, USA),
- **methanol** (analysed grade, Saarchem, 4116408ME, South Africa),
- **gallic acid** (Purum, Fluka, 48630, Switzerland),
- **sodium carbonate** (general purpose reagent, BDH Chemicals Ltd., 30121, England),
- **Folin-Ciocalteu Reagent** (Sigma-Aldrich, 47641, USA),
- **quercetin dehydrate** (HPLC grade, Sigma, 085K0720, USA),
- **ethanol** (analysed reagent, KIMIX, 09665 27706, South Africa),
- **ascorbic acid** (99% pure, Saarchem, 1022315, South Africa),

- **formic acid** (analysed reagent, Saarchem, A/7A570484, South Africa),
- **acetonitrile** (HPLC grade, Honeywell. Burdick & Jackson, 10071743, USA),
- **nitrogen** (99.95%, AFROX, 8007258270508, SA),
- **dimethyl sulfoxide** (Grade 1, Sigma, D-5879, Germany),
- **sodium acetate** anhydrous powder (99%, Analytical reagent),
- **glacial acetic acid** (analysed reagent, Merck, 1021020LC, South Africa),
- **hydrochloric acid 32%(HCL)** (analytical reagent, Saarchem, 1028782, South Africa),
- **2,4,6-tri[2-pyridyl]-s-triazine (TPTZ)** (puriss ≥99%, Fluka, 93285, Switzerland),
- **iron (III) chloride hexahydrate ($FeCl_3$)** (reagent grade, Sigma, 035K0180, USA),
- **helium** (99.95%, Afrox, 8007258, South Africa).

4.1.2 Equipment

The following equipment was used in this study:

- **balance** (*Adventurer OHAUS, Model AR2140, USA*),
- **centrifuge** (*Heraeus Sepatech*, *Labofuge 20 ıany)*,
- **vortex** (*Scientific industries, G-560E, USA*),
- **micro-plates** (*Nunclon 96well plate*),

- **spectrophotometer** (*Labsystem, Labsystem Multiskan ® MCC/340, Finland*),
- **hot plate** (*Heidolph, model 50301, Germany*),
- **grinder tubes** (*15ml Screw cap plastic test tubes*),
- **digital thermometer** (*French Cooking -50°C to 200°C/-58°F to 392°F, France*),
- **micro-pore filter paper** (*47mm filter membrane hydrophilic 0.45µm PVDF, Millipore Millex-HV*),
- **hydrophillic polypropylene membrane filters 47mm, 0.45µm** (*Pall corporation, 66548*),
- **syringes** (*10ml Sterile Syringe for single use, 060830*),
- **recorder** (*Epson Quiet colour upgradeable LX-300 printer*),
- **micropipettes and tips, mortar and pestle, epindorf tubes**,
- **paper** (A4 white sheets of paper),
- **beakers** (glass of various sizes),
- glass **graduated measuring cylinders** of various sizes,
- **high performance liquid chromatography (HPLC) apparatus**

(*Agilent 1200 system consisting of; degassing system (G1322A, Japan), quaternary pump (G1311A, Germany), auto loading sampler (G1329A, Germany), TCC (G1316A, Germany), column C18 column 15cm X 46mm, 5µm (Discovery, BL6357 HS, USA), diode array detector (G1315B, Germany), fluorescence detector (G1521A, Germany), analyte fraction collector (G164C, Germany), and the Agilent ChemStation software G2173-60101L, Germany*),

- **climatic chamber** (*Labcon, FSIE-H2O humidity, L17030, South Africa*),
- **filtration system**; **vacuum pump** (*ABM diaphragm vacuum pump, serial number 1740891095), rubber tubing, Erlenmeyer flask (1000ml Millipore), side arm glass filter (Millipore, positioned above Erlenmeyer flask), Volumetric cup which is placed above the glass filter (Millipore approx. 250ml), metal clip (Millipore, 1004703*),
- **pH meter** (*Thermo Orion 2000, 074012, USA*),
- **hot water bath** (*Gefran 500, 09/700, Italy*),
- **desiccator** (*2atc, Gepruft, Germany*),
- **magnetic stirrer and hot plate** (*Fried electric, MH.4, Israel*)
- **thermo hygrometer** (*Tinytag TH-2500, Gemini Data Loggers, UK*)
- **LC-MS system** (*Waters API Q-TOF Ultima [illegible]s*).

4.2 Methods and procedures

In order to determine the set objectives the following methods were used;

4.2.1 Determination of quality-indicating properties of selected *Sutherlandia* solid dosage forms

Local pharmacies and retail outlets were visited between March and July 2008 and various internet sites searched to identify commercially available, oral solid dose *Sutherlandia*-containing products (SCP). Eventually, 7 SCP that were readily available were selected, purchased and included in this study. For each selected SCP the product name, supplier, package description, manufacturer, organoleptic properties,

dosage form, unit dose weight and presence or absence of package insert was tabulated. This information was used to make an assessment of the pharmaceutical quality of the preparations.

4.2.2 Determination of antioxidant activity of the dosage forms

To determine antioxidant activity of the SCP two popular techniques, viz. the DPPH (Brand-Williams et *al.*., 1995; Molyneux, 2004) and FRAP (Berker, *et al*., 2007) tests were used. The precise methods used are briefly described in the next few paragraphs.

4.2.2.1 The di-phenyl-picryl-hydrazyl (DPPH) test

First, a 20µM DPPH working solution was made by dissolving 7.88mg DPPH powder in 20ml methanol which was stored in an amber bottle in a [illegible]set at 2ºC until analysis. Methanolic standard solutions of quercetin dihydrate (1mg/ml) an[illegible]id (0.176mg/ml) were made and appropriately diluted with methanol to obtain several standard solutions. These standard solutions were used to determine the free radical scavenging ability of quercetin and ascorbic acid over the concentrations ranges of 1.5-10µg/ml and 0-176µg/ml, respectively. These concentration ranges were established from preliminary experiments. Each designated well on the micro-plate received 50µl of DPPH and 150µl methanol, quercetin or ascorbic acid standard solutions at various concentrations. All samples of the standards were run in triplicate. After 30 minutes the absorbance of each well was read on a spectrophotometer at 540nm and 492nm wavelengths. These wavelengths were chosen because they were the closest filters that the plate reader had to the recommended 515nm and 528nm, recommended in the literature (Li *et al*., 2007; Brand–Williams *et al*., 1994). The absorbance readings were recorded and used to calculate the percentage inhibition using the following equation:

$$\text{Percent inhibition (\%)} = [(A_0 - A_S) / A_0] \times 100 \qquad \text{equation 1.}$$

Where (A_s) = the absorbance of DPPH with the sample (ascorbic acid or quercetin), (A_o) = the absorbance of DPPH with the vehicle (distilled water or methanol).

To determine the radical scavenging ability of the SCP' aqueous extracts were made as follows. If the product was in a tablet form they were first ground into a fine powder using a mortar and pestle. For the capsule products the powder contents were used as such. For each product, 1g of powder was placed in marked grinder tubes (designated 1 to 7), 10ml of boiled distilled water added, the mixture vortexed for 2minutes, sonicated for 35minutes, allowed to steep for approximately 2hours, thereafter re-vortexed for another 2minutes, centrifuged at 3500rpm for 10minutes and the supernatants pipetted into clean correspondingly labelled grinder tubes. The concentration of each product's aqueous extract was then taken as being 100mg/ml and these were stored in the freezer at -18°C until examination (for up to 9months). The aqueous extracts of the SCP samples were thawed and diluted to obtain a series of 6 concentrations within a suitable concentration range which was previously determined for each product. The ideal free radical inhibition concentration ranges were found to be: product 1, 0 to 15mg/ml; product 2, 0 to 18mg/ml; product 3, 0 to 20mg/ml; product 4, 0 to 12.5mg/ml; product 5, 0 to 1.5mg/ml; product 6, 0 to 6mg/ml and product 7, 0 to 6mg/ml. For the assay 100µl DPPH working solution followed by 100µl diluted product aqueous extract was added to the respective wells. After 30minutes, the absorbance of each well was measured at 540nm and 492nm wavelengths. The absorbance readings at these 2 wavelengths were used to calculate the percentage inhibition and the latter values plotted *versus* concentration for each SCP. The better of the 2 curves at 540 and 492nm i.e. the one indicating more than 50% inhibition and/or the best linear regression fit of the data was then used, i.e. data recorded at 540nm. The inhibitor concentration at which 50% DPPH had been scavenged (IC_{50}) was then interpolated from the selected curve for each SCP and this value used to compare the antioxidant powers the various SCP.

4.2.2.2 The Ferric Reducing Antioxidant Power (FRAP) assay

Firstly, a 300mM acetate buffer was made by dissolving 1.55g sodium acetate in 8ml acetic acid and diluting it with distilled water up to a volume of 500ml. The acetate buffer had a pH of 3.6 and was stored at room temperature over a period of 1 month. A 40mM HCL solution was made by mixing 25ml HCL with 175ml distilled water. Using 3ml of the 40mM HCL and 0.0093g TPTZ powder a 10mM TPTZ solution was made which could be stored in a fridge for up to two weeks. On the day of the FRAP test a fresh solution of 20mM $FeCl_3$ was made by dissolving 54mg iron (III) chloride in 10ml distilled water. The FRAP reagent was made by placing 30ml acetate buffer into a amber vile to which 3ml $FeCl_3.6H_2O$ solution, 3ml TPTZ solution and 6ml distilled water were added. A fresh solution of FRAP reagent was made on the day of analysis.

For the positive control a 1mM ascorbic acid solution was made by dissolving 8.8mg powder in 50ml distilled water and used to prepare solutions ranging from 0 to 0.176mg/ml in concentration.

Generally the 100mg/ml aqueous extracts of the SCP (see 4.2.2.1) were too concentrated to use as such in the FRAP test but after several dilution tests the ideal concentration for each product was found to be 15mg/ml. This concentration was then used in the FRAP test.

The FRAP assay was conducted on a 96well micro-plate where 25µl of the test sample, water or ascorbic acid was pipetted into individual wells, 200µl FRAP reagent added and, after a 10minute development period, the absorbance at 540nm wavelength measured on a multi-scan spectrophotometer. Each test was run in triplicate.

A standard curve of the 540nm absorbance *versus* concentration of the ascorbic acid was constructed using GraphPad Prism 5®, the line of best fit obtained by least squares linear regression (r^2=0.9961) and from this the ascorbic acid equivalents (AAE) of the SCP were calculated using the absorbance values obtained for the individual SCP. The AAE for each SCP was tabulated and used to compare the antioxidant activity of the various SCP.

4.2.3 Determination of total phenol content of the dosage forms

To determine the total phenol content of the selected SCP the Folin-Ciocalteu reagent (FCR) assay (Roura *et al*., 2006) was conducted.

The first requirement in conducting a total phenol test using FCR was to make up a sodium carbonate solution as follows: 25g anhydrous sodium [illegible]d in 100ml distilled water, brought to the boil, allowed to cool to room temperature, a few [illegible]ls added, the solution was allowed to stand for 24hours, thereafter filtered and distilled water added to the filtrate to make up a volume of 500ml. This solution was stored in a sealed bottle in a cool dark cupboard until use.

For the positive control a stock solution of gallic acid (GA) was made by dissolving 0.5g GA in 10ml ethanol which was then diluted with distilled water to 100ml. The flask was sealed with parafilm and stored in the refrigerator until needed or discarded after 2days. At the time of the assay, calibration solutions of GA over the concentration range of 0 to 18.750mg/ml were made by appropriately diluting the GA stock solution. Two hundred micro-litre of each solution was added to the micro-plate well, 10µl FCR and 30µl $NaCO_4$ added, and after a 2hr plate development period the absorbance was read at 690nm wavelength. The results were used to construct a standard curve of the GA concentrations *versus*

absorbance from which an equation (y=mx+c) was extrapolated by linear regression and used to determine the total phenol concentration of each solid dose SCP in terms of gallic acid equivalents (GAE).

To determine the total phenol content of the SCP, 200µl of 1.875mg/ml SCP' aqueous extract solutions were added to 10µl FCR and 30µl $NaCO_4$ and the absorbance at 690nm obtained. Using these absorbance values the GAE for each individual SCP were obtained from the gallic acid (GA) concentration *versus* 690 nm absorbance standard curve. Finally, the GAE values obtained were used to compare the total phenol content for the SCP.

4.2.4 Determination of flavonoid profile of the dosage forms

To determine the flavonoid profile of the dosage forms a high performance liquid chromatography (HPLC) technique was used to create chroma[UNIVERSITY of the WESTERN CAPE]gerprints of compounds present in the aqueous solutions of the products.

The HPLC method of Avula *et al.* (2010), with a few modifications, was used to generate the chromatographic fingerprints. Briefly, 50µl of 100mg/ml aqueous solution and 50µl of 100mg/ml methanol extracts of each SCP was injected into the Agilent HPLC system (see 4.1.2 for HPLC apparatus used). Before injection the solutions were filtered using a syringe and micro-pore filters of *0.45*µm pore size. Separation of the various plant constituents was achieved on a reverse phase C18 column (Supelco, 15cm X 46mm, 5µm) operated at 20ºC. The mobile phase consisted of 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in distilled water (solvent B) and a flow rate of 1.000ml/min and the following gradient: 85% [B] and 15% [A] to 35% [B] and 65% [A] over a 20min period, each run was followed by a 100% [A] wash phase of 10mins and a 5min equilibration period, giving a total run time of

35mins, were used. A diode array detector was used to detect the eluted compounds at a 260nm and 360nm wavelengths.

Data acquisition was performed by the ChemStation® software operated on a HP Compaq® microprocessor. The chromatographic fingerprints for each SCP was inspected and the retention times and heights of all the SCP peaks present recorded.

To identify some of the peaks the chromatographic fingerprints of the methanolic extract of product 5 was inspected and peaks that occurred at retention times between 8.5mins to 20mins, and eluted over a 0.5mins period with a threshold greater than 17.8mAU's were individually collected using the fraction collector (G1364C Analyt). The collected fractions were divided into 2 batches: batch 1 fractions were injected onto the HPLC column using the same gradient as described above and each peak seen in the chromatographic fingerprint was tested for p[illegible]d spectral profile over 180nm to 400nm using the DAD and ChemStation® software facilit[illegible]PLC. The batch 2 fractions were subjected to liquid chromatography mass spectrometric (LC-MS) analysis to confirm whether these fractions were sutherlandins or not. The LC-MS was conducted on a Waters API Q-TOF Ultima LC-MS instrument, the injection volume of the samples were 2µl (diluted 10 times with 1ml 0.1% formic acid 50% acetonitrile using an ultrasonic bath for 20mins prior to injection), the column a Waters BEH C18, (2.1x50mm) reverse phase column while the mobile phase consisted of solvent A: water and B: acetonitrile, with a flow rate of 0.35ml/min, and a run time of 15minutes. The following solvent gradient was used to effect the peak separation: at 0mins to 0.5mins solvent A was 100%, from 0.5mins to 6mins solvent A decreased linearly to 0%, from 6mins to 8mins solvent B remained at 100% and at 8.01mins the system was re-equilibrated to 100% solvent A until the end of the run time. The following detection conditions were used: source ESI+, capillary voltage set at 3.5kV, cone voltage 35, RF1 40, source temperature 100°C, desolvation temperature of 350°C, desolvation gas flow 350 L/h and cone gas flow 50 L/h.

The mass to charge ratio (*m/z*) for each peak in the collected fractions was recorded and compared with data existing in the literature to identify the isolated compounds.

4.3.1 Determination of the effect of temperature and humidity on the stability of the dosage forms

To determine the effects of temperature and humidity on the quality of the selected SCP, the products were exposed to ambient and elevated temperature and humidity conditions.

First, to ascertain whether elevated environmental temperatures (40°C to 45°C) and humidity levels (70% to 79% RH) could induce gross physical changes in SCP stored exposed and sealed (i.e. enclosed in package), samples of each product were stored (for 7 to 21 days) under varied conditions in a make-shift chamber and the organoleptic features of the tablets, capsules and capsule powders monitored regularly. The make-shift climatic chamber consisted 

ıtor in which a saturated aqueous solution of sodium chloride was placed to create a stable 75% (Rockland, 1960). The lid of the desiccator was sealed with silica gel and the entire desiccator then placed in a hot water bath where the temperature was maintained at 45ºC. The relative humidity and temperature inside this chamber was monitored daily using a thermohygrometer (TinyTag). Two sets of samples of the products were placed in this chamber; the batch "**Sealed**" samples were placed in air- and water-tight containers while the "**Exposed**" samples were placed in plastic containers open and exposed to the controlled climatic conditions of this chamber. The samples in the chamber were monitored every 2days and at the end of the 14day period and visually checked for organoleptic changes to determine the effect of storage in air-and humidity-tight containers on the quality of the SCP. The original intention was also to measure and compare the total phenol level, antioxidant activity and flavonoid profile (as described in 4.2.2 to 4.2.4) of the 2 sets of samples (Sealed and exposed groups) but this was not done (see results and discussion 5.2.5.4).

For the main data 5 samples (n=5) of each intact SCP (i.e. intact tablet or capsule) were left exposed to ambient environmental conditions in a petri dish on a bench top in the laboratory and monitored over a 2month period. The ambient temperature ranged between 18°C to 28ºC and the relative humidity was below 35%. A further quintuplet samples (n=5, designated Elevated group) were placed in individual snap-cap airtight transparent plastic containers in a climatic chamber maintained at 40ºC and a relative humidity of 70% for 14-days. The chamber was checked daily to verify the temperature and humidity levels (i.e. machine digital display). The SCP samples stored under the two sets of conditions (Ambient & Elevated groups) were monitored twice a week and removed for tests after the designated 14-day period. Aqueous extracts of the samples were prepared as described in 4.2.2 and analyzed for total phenol level, antioxidant activity and flavonoid profile as described in 4.2.3 to 4.2.5. The data of the products stored under the 2 sets of conditions i.e. ambient and elevated temperature and humidity conditions were compared to determine the effect of temperature and humidity of the quality of the SCP. The student t-test with p = 0.05 was used to determine the statistical significance of any change in the antioxidant activities (measured as IC_{50} or mg/ml AAE), total phenol levels (measured as mg/ml GAE) and flavonoid levels (peak heights) caused by the different storage conditions.

4.3.2 Determination of the effect of acid hydrolysis on the stability of the dosage forms

The solid dose *Sutherlandia* products contain constituents such as flavonoid glycosides, that may be sensitive to acid hydrolysis and the levels of such susceptible compounds and the degree of susceptibility to acid may vary from product to product. Comparison of the acid stability of various SCPs may thus be a useful criterion on which the quality of such SCP can be compared (esp. since this may affect in an acidic environment (e.g. stomach) the conversion of glycosides to aglycones etc. and complement the release of active compounds from the products leading to variable absorption and bioavailability and, consequently, variable *in vivo* effects that can be expected from the dosage form). In this study the hydrolysis of the SCP

by hydrochloric acid was therefore assessed as another means to compare the quality of the various products. Two milliliter of the 100mg/ml aqueous extract of each product was refluxed with 2ml of 0.6M HCL for 1hour at 80ºC in a water bath and left to cool overnight. The next day the solution was filtered and dried using nitrogen gas (Audu *et al*., 2007). The dried hydrolyzed sample residues were then re-suspended in 2ml distilled water, micro-pore filtered and analyzed by HPLC as described in section 4.2.5. The chromatographic fingerprints obtained for each product was analyzed for the presence or absence and size of peaks suspected to be flavonoid compounds (see section 4.2.5) to determine the relative acid susceptibility of the various *Sutherlandia* containing products.

4.4 Data Analysis

To ascertain whether there were significant differences between the antioxidant activity, total phenol content and flavonoid levels for the different [illegible]verage and standard deviations were calculated and the student t test applied to determine wh[illegible]nces were statistically significant with the level of significance being set at p equal to or less than 0.05.

Chapter 5

RESULTS AND DISCUSSION

5.1 Introduction

In this chapter the findings on the regulatory compliance of the selected commercially available *S. frutescens* products together with their antioxidant activity, total phenol, flavonoid levels and the effect that elevated temperature, environmental humidity and acid hydrolysis have on these properties are discussed.

5.2.1 Regulatory compliance of the selec*ndia* solid dosage forms

In the period of March till the end of July 2008 there were approximately 22 products containing *S. frutescens* commercially available in pharmacies and health shops in the city of Cape Town. Of these the 7 shown in figure 4 were selected for inclusion in this study and various details (i.e. product name, manufacturer, organoleptic properties, dosage form, dosage weight, supplier and presence of a package insert, to mention but a few) of these are recorded, in table 2, and assessed in order to establish and describe the quality and regulatory compliance of solid dose *Sutherlandia*-containing products (SCP) in general.

Figure 4: Selected *Sutherlandia frutescens* containing products (SCP). Product 1: Bio harmony, Bio-*Sutherlandia*® tablets, 2: Phyto Nova *Sutherlandia* SU1® tablets, 3:Health Wholefoods Connection *Sutherlandia*® tablets, 4:Pinnacle *Sutherlandia*® tablets, 5:Adjuvant Promune® *Sutherlandia* Extract capsules, 6:Big Tree *Sutherlandia* Pro® .capsules and 7:Big Tree *Sutherlandia* 350®

Table 2: Quality indicating properties of selected commercially available solid dose *Sutherlandia* containing products.

Quality Indicating Property or Item	PRODUCTS						
	Bio*Sutherlandia*® Bio harmony **1**	*Sutherlandia* SU1® Phyto Nova **2**	*Sutherlandia*® Health Wholefoods Connection **3**	*Sutherlandia*® Pinnacle **4**	Promune® Adjuvant **5**	*Sutherlandia* Pro® Big Tree **6**	*Sutherlandia* 350® Big Tree **7**
Batch number	52488	28941	0609SL03	0180022	P12210207022	5316	6E01113
Package insert	Absent	Present	Absent	Absent	Present	Absent	Absent
Description of container & packaging	White plastic container with a transparent green screw-on lid.	White plastic container with an orange screw-on lid.	White securitainers and white snap-on cap.	White plastic container with a blue screw-on lid.	White securitainers and white snap-on cap.	White plastic container with a white screw-on lid.	Transparent amber glass container with a black screw-on lid.
	Lime green label on container with black font and an image of the top half of a golden apple on a green background.	Yellow label with green, red and black print. Stylized *Sutherlandia* plant image.	Pale and dark blue label with black, navy and white print.	Yellow label with blue, white and black print and an emblem of a pinnacle, i.e. triangle on the top quarter of the label.	Lime-green label with dark green, black and white print	White label which fades into light purple, with black and purple font. Stylized image of a lime green yellow tree on the top half of the label.	White label which fades into green, with black font. Stylized image of a lime green yellow tree on the top half of the label.
	Enclosed in a box with the same colouring						
Amount in container	60	60	60	60	60	60	60
Dosage form & Physical dimensions	Tablet, Convex-round, D = 15mm T = 2mm C = 41mm	Tablet, Convex-round, D = 12mm T = 2mm C = 41mm	Tablet, round flat, D = 12mm T = 3mm C = 45mm	Tablet, round convex, [illegible]m [illegible]m [illegible]m	Capsule Size 2: D = 5mm 15mm long	Capsule Size 000: D = 10mm 23mm long	Capsule Size 00: D = 7mm 20mm long
Organoleptic properties	Uncoated compound powder tablets. Pale-green colour with fine dark green and yellow specks with a faint odour	Uncoated compound powder tablets. Pale- green colour with distinct odour	Uncoated compound [illegible] tablets. Pale-gray-green colour with faint odour	[illegible]ed [illegible]nd [illegible] tablets. Pale-brownish green colour with specks and a distinct odour	White body & dark green cap, Whitish-gray fluffy fine powder with a distinct odour	Large sized transparent casing, Pale green fluffy fibrous powder with a strong distinct odour.	Medium sized transparent casing, Light green fine-fluffy-fibrous powder and a strong distinct odour.
Date of Manufacture	-	-	-	-	-	-	-
Expiratory date	2009-09	2011-04	-	2010-01	2009-02	2008-09	2009-10
Weight (g) RSD	0.770 ±0.045 5.844%	0.516 ±0.003 0.581%	0.608 ±0.014 2.303%	0.505 ±0.006 1.188%	0.287 ±0.001 0.348%	0.567 ±0.033 5.820	0.406 ±0.036 8.867%
Dosage	1 tablet daily *	1 tablet twice daily after meals	1-2 tablet(s) twice daily with meals *	1-2 tablet(s) twice daily after meals *	2 capsules once daily after meals	1capsule twice daily	1capsule twice daily *
Constituents	*S. frutescens* subspecies Microphylla herb. 300mg Tableted with dicalcium phosphate, starch and Magnesium stearate	*S. frutescens* subspecies Microphylla SU1. 300mg Guar gum, magnesium stearate, microcrystalline cellulose, starch (maize), sodium starch glycolate	*Sutherlandia* 300mg	*Sutherlandia* 300mg	*S. frutescens* extract 90mg Microcystalline cellulose 120mg	*S. frutescens* subspecies Microphylla SU1. 300mg	*S. frutescens.* 350mg

Key:
* As recommended by a practitioner
RSD Relative Standard deviation

The selected products comprised of 4 compressed powder tablets and 3 capsule preparations. All the products looked like typical conventional, registered and pharmaceutically acceptable dosage forms. The name of each product was clearly visible and all the products except Promune® Adjuvant were easily identifiable as *Sutherlandia*-containing products. The packaging of the SCP clearly served to protect the product from deterioration by environmental conditions and the safety seals on all products were fully intact upon purchase which ensured that the products had not been tampered with.

Besides the name, the rest of the label information of the SCP was clear, visible and legible. The labels of all the products contained details of the product name, manufacturer, composition, expiratory date and storage conditions except the Health Wholefood Connections® product where an expiratory date was absent. None of the 7 products had any indication of a manufacturing date, this appears suspect since there is no means on whether to test the reliability of the expiratory date.

The amount of *Sutherlandia frutescens* contained in the selected SCP ranged between 90mg to 350mg per dosage form and generally the products were uniform in mass, having a relative standard deviation (RSD) less than 9%, an acceptable value that is well within the limit of the Food and Drug administration (FDA) (Abballe *et al*., 2008).

Five of the seven products did not contain package inserts although the recommended dosage was indicated some where on the packaging. When present the package insert did not contain all the necessary information required of a registered drug. The required information should include: scheduling status, composition, pharmacological classification, pharmacological action, indications, contra-indications, warnings, dosage and directions for use, side-effects and special precautions, known symptoms of overdose and particulars of its treatment, identification, presentation, storage instructions, registration

number, name and business address of applicant and, lastly, the date of publication of the package insert. Two of the package inserts only contained approximately 65% of this required information.

Although not being registered most of the selected SCP aesthetically appeared to comply with many of the specifications that would be set for a registered pharmaceutical product. In short, based on the physical assessment they appeared to be of quite good "pharmaceutical" quality. The South African Medical Control Council (MCC) regulates any medicines with a stated or implicit medical purpose that is traded and produced nationally by setting standards regarding safety, efficacy and quality (Mander, 1998). Herbal products such as the SCP investigated in this study are not yet formally included in this registration process, mainly because the stated or implicit medical purpose of SCP is not yet clinically proven or scientifically accepted. The registering process is also complicated, expensive and it takes a long period to satisfy all the set criteria (Marchetti *et al*., 2007). Nevertheless it should be feasible that the *Sutherlandia*-containing tablet or capsule dosage forms i  n this study could, with relatively little extra information (e.g. stability data and validation es, etc), easily meet most of the product quality criteria needed for their registration by the MCC. The MCC should thus insist that manufacturers of these SCP do the necessary tests, collect and provide the relevant information to confirm and assure the pharmaceutical quality of the products and then apply for drug registration based on pharmaceutical quality. The inclusion of information from additional tests e.g. measurement of antioxidant activity and total phenol levels, etc. could even strengthen the evidence of pharmaceutical quality of the herbal product.

In conclusion, the *Sutherlandia*-containing tablet and capsule dosage forms investigated in this study, physically appeared to be of quite good "pharmaceutical" quality, but generally lacked information on the date of manufacture and lacked package inserts, or when these were present they contained insufficient information. It should however be feasible that present commercially available *Sutherlandia*-containing

solid dosage forms could, with relatively little extra information, easily meet most of the product quality criteria needed for their registration by the MCC.

5.2.2 Antioxidant activity of the selected *Sutherlandia* products

To compare the quality of the selected SCP their antioxidant abilities were determined by two tests, viz., the diphenyl picrylhydrazyl (DPPH) scavenging and ferric reducing antioxidant power (FRAP) assays.

5.2.2.1 The DPPH scavenging ability of SCP

Firstly the conditions for the test with regard to the reaction time, optimal wavelength and concentration range for the various products to be tested were established. To do this, serial dilutions of quercetin dihydrate or ascorbic acid were added to a solution of DPPH and the inhibition of the scavenger monitored by observing the colour loss and recording the absorbance at 492nm and 540nm after 30mins, 45mins, and 60mins and calculating the percent inhibition of the DPPH absorbance. Sample results of these experiments are recorded in tables A.1 and A.2, in the appendices. As the concentration of the standard solutions increased, the DPPH absorbance decreased and the percent inhibition increased. A 10mg/ml solution of quercetin produced a 52% and 71.9% inhibition of the DPPH absorbance at 492nm and 540nm, respectively and 0.176mg/ml solution of ascorbic acid caused 57% and 33.1% inhibition, respectively. From this data the ideal concentration range over which each of the selected SCP could be tested against DPPH was established and was found to be 0 to 15mg/ml for product 1, 0 to 18mg/ml for product 2, 0 to 20mg/ml for product 3, 0 to 10mg/ml for product 4, 0 to 1.5mg/ml for product 5, 0 to 6mg/ml for product 6 and 0 to 6mg/ml for product 7.

The percent inhibition of DPPH absorbance produced by various concentrations of each of the SCP is given in tables A.3 to A.9 in appendix A. Figure 5, summarizes the results of these tables.

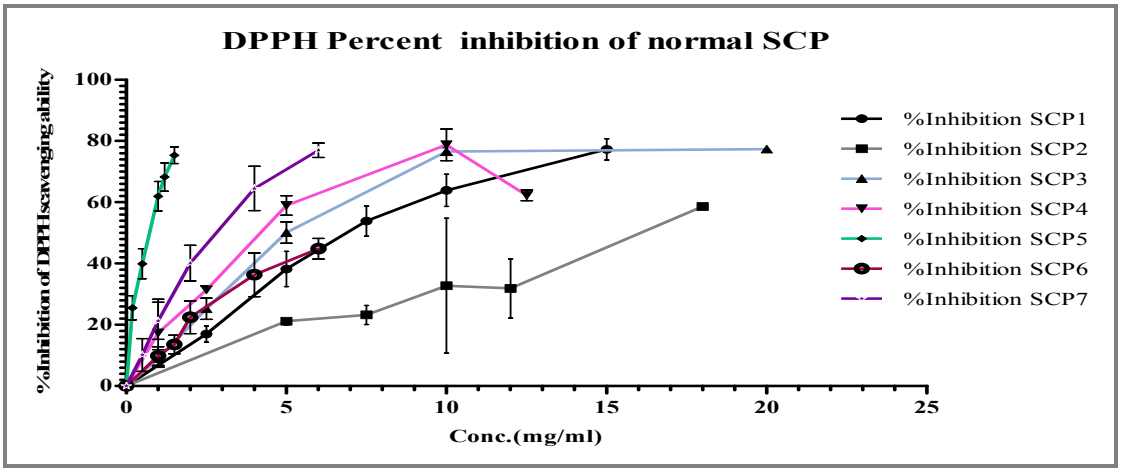

Figure 5: Percent inhibition of DPPH by various concentrations of SCP.

Generally, as the concentrations of SCP increased so too did the extent of their ability to scavenge DPPH free radicals (i.e. their inhibition of DPPH absorbance). Product 5 (Promune®), represented by the green line, had the steepest % inhibition *vs.* concentration gradient with the maximum percent inhibition occurring at a relatively low concentration (that of the other SCP, while the gradient for product 2 (Phyto Nova SU1®), represented b ne, had the lowest of the SCP, with maximum percent inhibition occurring at much higher concentrations. The IC_{50}, i.e. the concentration of the SCP at which 50% of the DPPH was reduced, was extrapolated from the graphs and the values obtained are recorded in table 3.

Table 3: **IC_{50} values (i.e. Concentration at which 50% of DPPH is scavenged) of commercially available SCP 1 to 7.**

IC_{50} of SCP		
	Product	IC_{50} conc. (mg/ml)
1	**Bio-*Sutherlandia*®**	6.90
2	***Sutherlandia* SU1®**	16.20
3	**Health Wholefoods Connection *Sutherlandia*®**	5.00
4	**Pinnacle *Sutherlandia*®**	4.20
5	**Promune®**	0.80
6	**Big Tree *Sutherlandia* Pro®.**	8.20
7	**Big Tree *Sutherlandia* 350®**	2.80
	Quercetin	5.765
	Ascorbic acid	0.219

The DPPH IC_{50} values for the 7 products ranged from 0.8 to 16.2mg/ml with product 5 (Promune®) having the lowest and product 2 (Phyto Nova SU1®) the highest IC_{50}. The lower the IC_{50} value, the greater the antioxidant power of that product. Thus the selected SCP ranged, in descending order of greatest to lowest antioxidant power, as follows: Promune® >> Big Tree 350® >> *Sutherlandia* Pinnacle® > *Sutherlandia* Health Connection Wholefoods® > Bio-*Sutherlandia*® > Big Tree Pro® >>>Phyto Nova SU1®. Moreover, in terms of dosage form the capsules seemed to possess a greater antioxidant power (and possibly potency?) than the tablets.

5.2.2.2 The ferric reducing antioxidant power of SCP

For the FRAP test ascorbic acid was used as the control antioxidant and the standard curve of varying concentrations of ascorbic acid *versus* the absorbance of the reaction with the FRAP reagent at 540nm obtained is given in figure 6. The colour cha[UNIVERSITY of the WESTERN CAPE]FRAP-ascorbic acid reaction was from clear to Prussian blue, and the change in absorban[UNIVERSITY of the WESTERN CAPE]scorbic acid concentration was linear over a concentration range of 0 to 100ug/ml with an r^2 value of 0.9961.

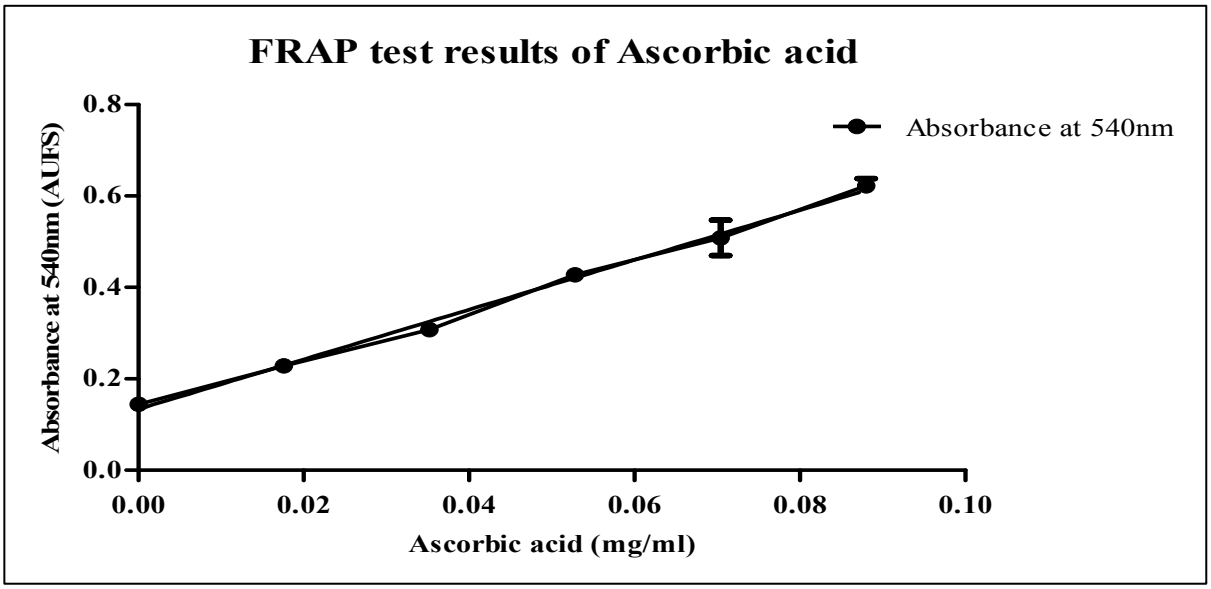

Figure 6: Standard curve of ascorbic acid concentrations *versus* absorbance at 540nm in the FRAP test

The ascorbic acid equivalents (AAE) for each SCP was calculated using the equation, y = (5.440 ± 0.1698) x + (0.1338 ± 0.009049) and the results obtained are shown in table 4.

Table 4: Antioxidant activity (in ascorbic acid equivalents) of *Sutherlandia* containing products 1 to 7 as determined in the FRAP assay.

	Product	Antioxidant activity (in Ascorbic acid equivalents (AAE))
		AAE (mg/ml)
1	**Bio-*Sutherlandia*®**	0.004508
2	***Sutherlandia* SU1®**	0.007265
3	**Health Wholefoods Connection *Sutherlandia*®**	0.009717
4	**Pinnacle *Sutherlandia*®**	0.014741
5	**Promune®**	0.061433
6	**Big Tree *Sutherlandia* Pro®.**	0.009839
7	**Big Tree *Sutherlandia* 350®**	0.029018

Based on the FRAP test results (table 4) the SCP[illegible]n antioxidant activity, in descending order from greatest to lowest AAE, as follows (and >> [illegible]ignificance, where p>0.05): Promune®>> Big Tree 350® >> *Sutherlandia* Pinnacle® >> Big Tree Pro® >> *Sutherlandia* Health Connection Wholefoods® >> Bio-*Sutherlandia*® > Phyto Nova SU1®. This was the exact order that was obtained with the DPPH test, and again the capsules had the highest activity. The FRAP antioxidant activity results thus corresponded directly to that of the DPPH test, confirming that Promune® had the greatest antioxidant ability of all the selected commercially available *Sutherlandia* containing products and Phyto Nova SU1® the lowest.

Although there are no product specific data available, Fernandes, *et al*. (2004), reported that hot water extracts of *S. frutescens* possessed significant antioxidant activity. The present results confirmed that such activity is also found in solid dose SCP. It is noteworthy that the Promune® capsule contains *S. frutescens* freeze dried extract and that it had the highest antioxidant activity compared to the other products (2

capsules and 4 tablets) which contained the dried whole *S. frutescens* plant material. Overall the antioxidant activity results suggest that such tests may be useful to compare the relative potencies of SCP.

5.2.3 Total phenol content of selected *Sutherlandia* products

For the determination and comparison of the total phenol content of the SCP a standard curve of gallic acid concentration reacting with FCR *versus* absorbance at 690nm was constructed, see figure B.1 in appendix B. The equation y= (0.03757 ± 0.002156) x + (0.06127 ± 0.022035) was taken from the graph and then used to calculate the total phenol content, in terms of gallic acid equivalents (GAE), of the SCP as a whole product and in terms of the stated amount of *S. frutescens* contained in each product. The results obtained from this calculation are summarized in table 5.

Table 5: Average mass and total phenol c[illegible]TPC) of *Sutherlandia* containing products

Product number and name	Average [illegible] unit d[illegible] (mg) (n=3)	[illegible]PC in total Product Powder (mg *GAE)	TPC per mg *Sutherlandia frutescens* in product (mg *GAE / mg)
1 **Bio-*Sutherlandia*®**	770.43 ± 0.045	4.162	0.005
2 ***Sutherlandia* SU1®**	516.00 ± 0.003	5.209	0.010
3 **Health Wholefoods Connection *Sutherlandia*®**	608.17 ± 0.014	5.404	0.009
4 **Pinnacle *Sutherlandia*®**	505.37 ± 0.006	8.279	0.016
5 **Promune®**	225.2 ± 0.001	13.930	0.062
6 **Big Tree *Sutherlandia* Pro®.**	439.87 ± 0.033	5.715	0.013
7 **Big Tree *Sutherlandia* 350®**	309.93 ± 0.036	6.637	0.021

*** GAE = gallic acid equivalent**

Product 5 (Promune®) had the greatest total phenol concentration of 13.930mg GAE per dose unit dose, compared to that of the other SCP which ranged between 4.162 to 8.279mg GAE. The Promune® also had the highest level of TPC in terms of the stated amount of the *S. frutescens* present in each SCP while the

TPC per mg plant material for the other products ranged from 5 to 20µg/mg plant material. Since Promune® is made from a freeze-dried aqueous extract of Sutherlandia this extraction process most likely contributed to the high levels of TPC in this product compared to the other SCP which contained the whole dried plant material.

Overall the SCP varied in total phenol concentration in descending order (and >> represents significance, where $p \leq 0.05$): Promune® >> Big Tree 350® > *Sutherlandia* Pinnacle® >> Big Tree Pro® > Phyto Nova SU1® > *Sutherlandia* Health Connection Wholefoods® > Bio-*Sutherlandia*®, which correlated exactly with the antioxidant activity results for the selected SCP. The latter finding is however not unexpected since many phenolic compounds contained in plants have been shown to have antioxidant activity (Kirakosyan *et al*., 2004; Li *et al*., 2006; Amarowicz *et al*., 2004). Both the antioxidant activity and TPC results thus gave a good indication of the relative potencies of the SCP. A great portion of the TPC of the SCP probably consisted of flavonoids, compounds known to have antioxidant activity but the identity of the exact compounds responsible for the antioxidant activity of the SCP was not pursued further in the present study.

5.2.4 Flavonoid profile of selected *Sutherlandia* products

To identify and compare the flavonoid levels in each of the SCP, high performance liquid chromatography (HPLC) analysis was conducted on the aqueous extracts of the SCP and the chromatographic fingerprints obtained for all the SCP are presented in figures 7 to 13. Separate individual flavonoid compounds were identified by the retention time and the UV spectrum of the peak and the levels of the flavonoids are determined by the peak heights.

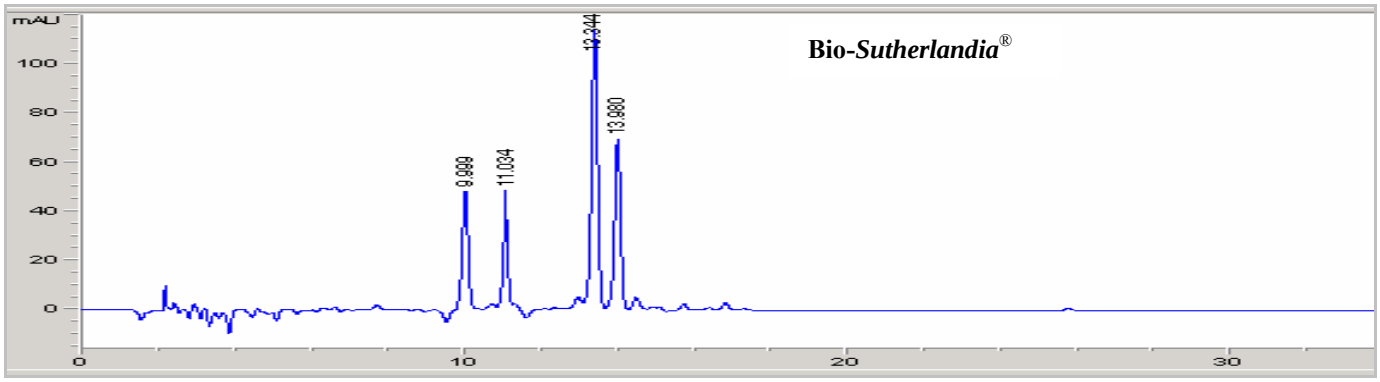

Figure 7: HPLC chromatogram of 100mg/ml aqueous extract of SCP 1.

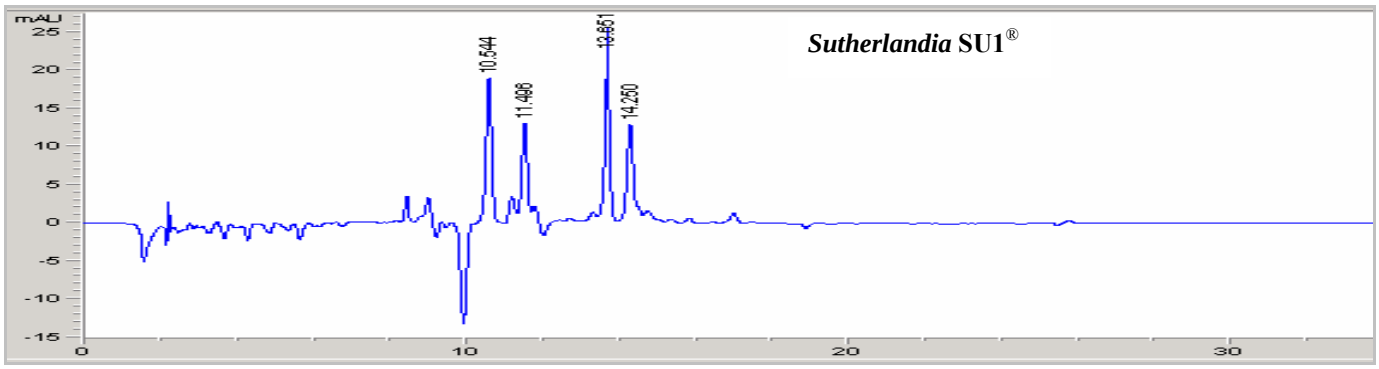

Figure 8: HPLC chromatogram of 100mg/ml aqueo[illegible]CP 2.

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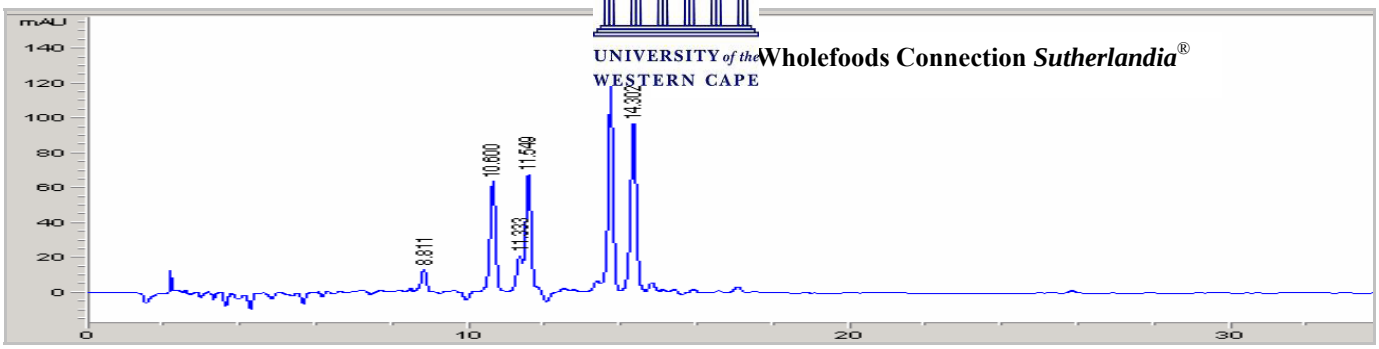

Figure 9: HPLC chromatogram of 100mg/ml aqueous extract of SCP 3.

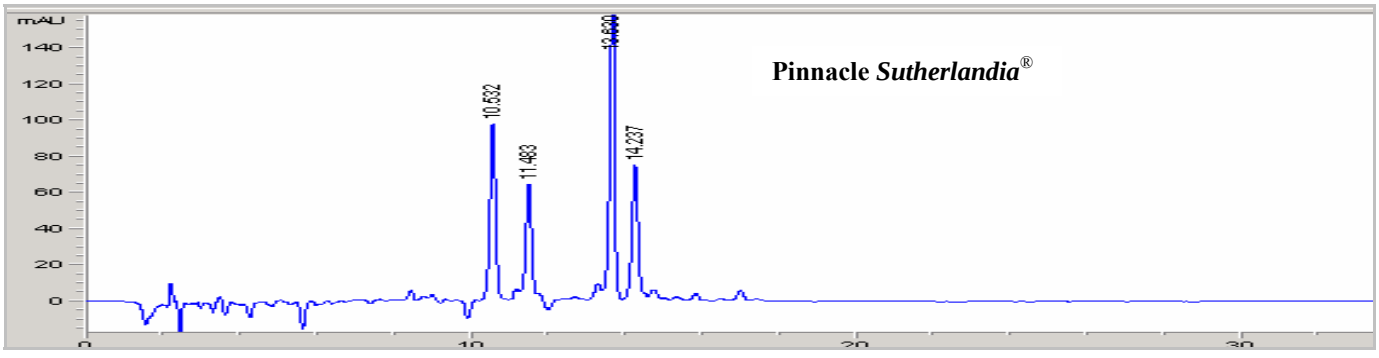

Figure 10: HPLC chromatogram of 100mg/ml aqueous extract of SCP 4.

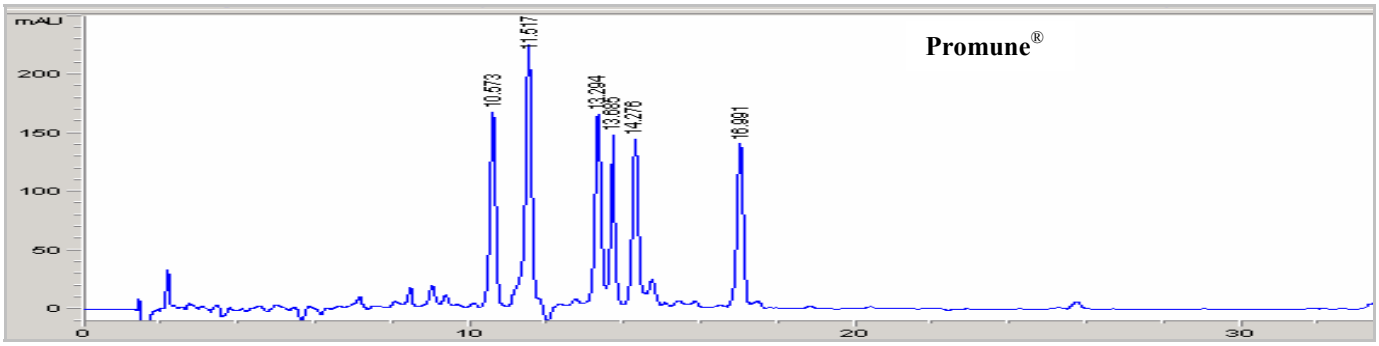

Figure 11: **HPLC chromatogram of 100mg/ml aqueous extract of SCP 5.**

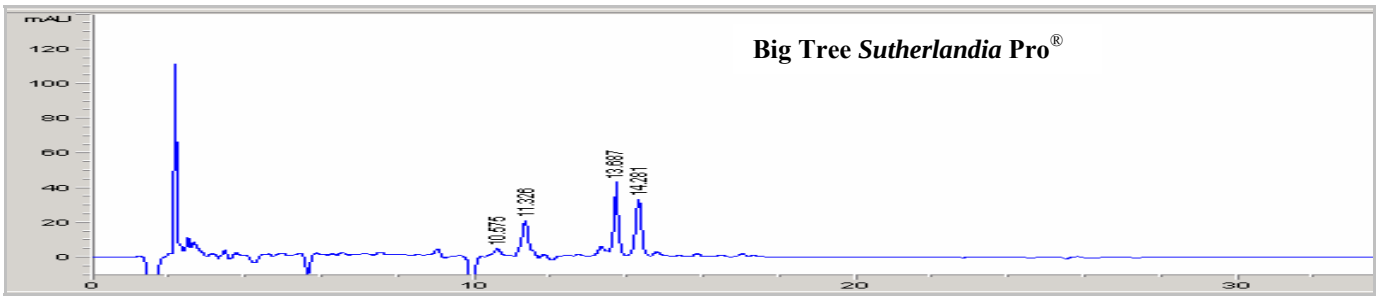

Figure 12: **HPLC chromatogram of 100mg/ml aqueous extract of SCP 6.**

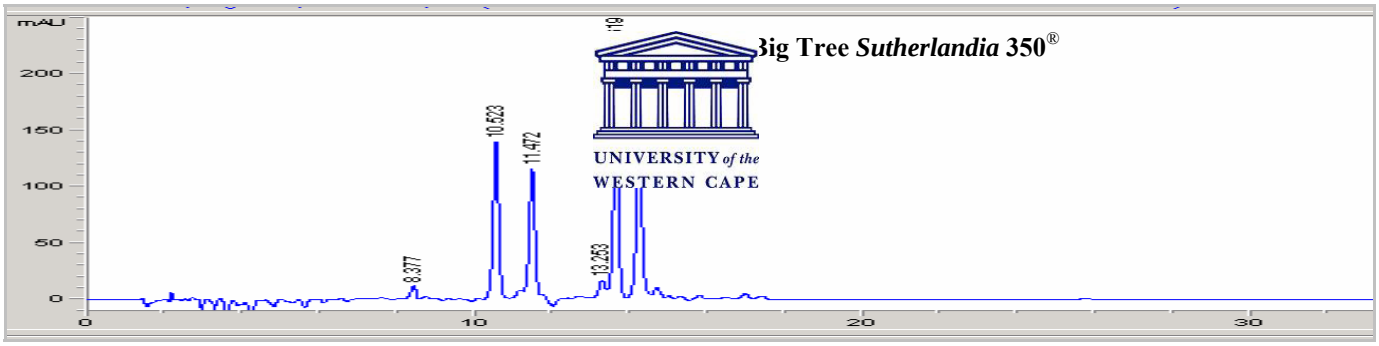

Figure 13: **HPLC chromatogram of 100mg/ml aqueous extract of SCP 7.**

From the chromatographic fingerprints (figures 7 to 13) there is a clear pattern of four dominant peaks of compounds that are present in all the SCP. These peaks had average retention times of 10.48 ± 0.21, 11.41 ± 0.18, 13.62 ± 0.12 and 14.22 ± 0.11mins, but the peak heights varied between the different SCP as shown in table 6.

Table 6: Retention times and peak heights of reoccurring peaks of the *Sutherlandia* containing products.

Product	Peak 1		Peak 2		Peak 3		Peak 4	
Name and number	RT (min.)	Height (mAUFs)	RT (min.)	Height (mAUFs)	RT (min.)	Height (mAUFs)	RT (min.)	Height (mAUFs)
1 **Bio-*Sutherlandia*®**	10.00	52.70	11.03	52.40	13.34	116.70	13.98	71.70
2 ***Sutherlandia* SU1®**	10.54	29.40	11.47	19.30	13.65	25.40	14.25	12.70
3 **Health Wholefoods Connection *Sutherlandia*®**	10.60	64.80	11.55	70.20	13.70	149.30	14.30	96.20
4 **Pinnacle *Sutherlandia*®**	10.53	100.80	11.49	67.10	13.63	179.10	14.24	74.40
5 **Promune®**	10.57	170.50	11.52	233.00	13.69	146.60	14.28	141.10
6 **Big Tree *Sutherlandia* Pro®.**	10.58	10.80	11.33	21.30	13.69	42.50	14.28	32.70
7 **Big Tree *Sutherlandia* 350®**	10.52	139.70	11.47	118.60	13.62	219.50	14.23	123.60
Average	10.48	81.24	11.41	83.13	13.62	125.59	14.22	78.91
Standard deviation	0.21	58.45	0.18	74.14	0.12	70.36	0.11	46.11
%RSD	2.00		1.58		0.88		0.77	

Key
RT= retention time

For product 5 (Promune®) three of the 4 pea[UNIVERSITY of the WESTERN CAPE]more dominating (i.e. had higher peak heights) than that for the other SCP and the fingerprint[UNIVERSITY of the WESTERN CAPE]duct also contained additional peaks at retention times 13.294 and 16.991mins. This implies that the SCP contained varying amounts of the compounds represented by the 4 peaks, and that the Promune® contained 2 extra compounds. The data also suggested that the extent of this variation in the amounts of the 4 peaks among the SCP could be quite substantial (93.67, 91.72, 88.43 and 91% respectively). In general the heights of the peaks in the fingerprints of the capsule SCP were also higher than that for the tablet products, a finding that correlated well with the total phenol and antioxidant level results.

The spectral profile of each of the 4 peaks, plus the extra 2 identified in the chromatographic fingerprints of the Promune and other SCP were determined using the diode array detector and spectral analysis software of the HPLC system. In addition, the 6 identified peaks were collected with a fraction collector and analysed by HPLC and the spectral profiles of 5 of the fractions are given in appendix E. The peaks (retention times of 10.51 ± 0.21, 11.53 ± 0.25, 13.32 ± 0.32, 13.74 ± 0.36, 14.41 ± 0.48 and 17.13 ±

0.54mins (n=4)) appeared to be that of single compounds and had distinct flavonoid type spectral profiles (e.g. maximum absorbance between 200nm to 360nm). Indeed, these fingerprints had excellent purity and the spectra of each were similar, if not identical, to that of sutherlandins recorded by Avula *et al*. (2010). The results thus suggested that all of the peaks were those of flavonoids.

The isolated fractions were also subjected to LC-MS analysis and the chromatographic fingerprints and LC-MS data obtained are given in Appendix F and the results summarized in table 7.

Table 7: The mass of the fragments (with > 30% abundance) in the collected HPLC fractions of Promune®

HPLC fraction # and retention time (min)	Mass spectra positive ion $(M + H)^+$ (*m/z*)			
1, 10.51 ± 0.21	609.2	741.2	-	-
2, 11.53 ± 0.25	609.2	741.2	-	-
3, 13.32 ± 0.32	609.2		-	-
4, 13.74 ± 0.36	593.2		726.2	-
5, 14.41 ± 0.48	593.2		725.2	726.2
6, 17.13 ± 0.54	593.2	-	-	-

The positive ion mass of the main compound in fractions 1 and 2 was 741.2 which correspond to that of analytes 1 and 2 reported by Avula *et al.* (2010) and were identified as sutherlandin A and sutherlandin B, respectively. Fractions 4 and 5 had an m/z value of 725.2 which corresponded to the analytes 3 and 4 also reported by Avula *et al*. (2010) and designated sutherlandin C and sutherlandin D, respectively. Thus the HPLC collected fractions 1, 2, 4 and 5, with the retention times of 10.928, 11.969, 14.373 and 15.040mins, could be identified as sutherlandins A to D, respectively. The structures of these compounds are given in figure 14 and according to Fu *et al.* (2010) sutherlandin A (**peak 1**) is quercetin 3-O-β-D-pylopyranosyl(1→2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside, sutherlandin B (**peak 2**) is quercetin 3-O-β-D-apiofuranosyl(1→2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside, sutherlandin C (**peak 3**) is kaempferol 3-O-β-D-xylopyranosyl(1→2)-[6-O-(3-hydroxy-3-

methylglutaroyl)]-β-D-glucopyranoside and sutherlandin D (**peak 4**) is kaempferol 3-O-β-D-apiofuranosyl(1→2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside. Moreover the other 2 prominent peaks in the Promune® fingerprint are, with m/z of 609.2 and 593.2, most likely diglycoside derivatives of quercetin and kaempferol, respectively.

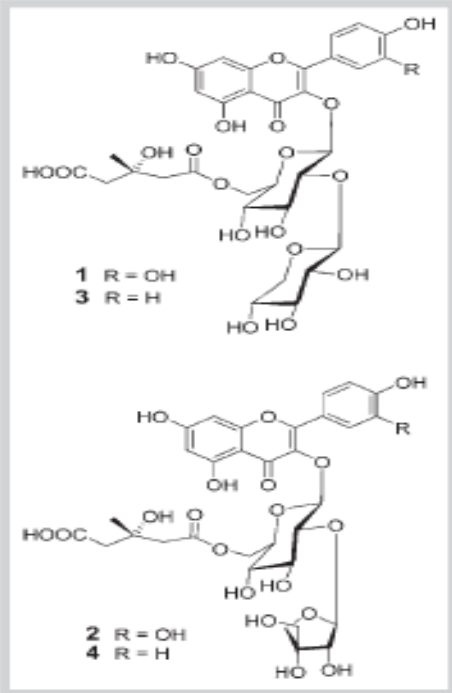

Figure 14: Schematic diagrams of sutherlandins A to D. (Fu, *et al.* 2010)

Collectively, the results of the present study thus showed that all the SCP contained 4 flavonoids (sutherlandins A to D) in varying amounts the levels of which can be quantified and used to compare the relative potency of each product. Finally, as far as the dosage forms were concerned the capsules again had far greater sutherlandin peak heights than that of the tablet dosage form, suggesting that the capsules had noid potency.

5.2.5 Effect of elevated temperature and environmental humidity on the organoleptic features, antioxidant activity, total phenol content (TPC) and flavonoid profiles of *Sutherlandia* containing products.

The effects of elevated temperature and environmental humidity on the quality of the SCP were determined by storing the products for a period of time under ambient and elevated temperature and humidity conditions and then testing for their antioxidant activity, total phenol concentrations and flavonoid levels.

First, to ascertain whether elevated environmental temperature (40°C) and relative humidity level (very high > 70% RH, medium = 55 to 70% (RH) and low < 55% RH) could induce gross physical changes in

the SCP, samples of each product were stored exposed and enclosed in its package for 7 to 21 days under these varied conditions in a make-shift chamber and the organoleptic features of the tablets, capsules and capsule powders monitored regularly (table 8). The results obtained for antioxidant activity after 21 days are summarized in table 9.

Table 8: Organoleptic properties of *Sutherlandia* containing products after exposure to high environmental temperature (40° C) and humidity conditions in makeshift chamber for 21 days (see section 4.3.1)

***Sutherlandia*-containing product (SCP)**	**Organoleptic properties**		
	Pre-stored sample (from table 2)	Sealed batch	Exposed batch
1 **Bio-*Sutherlandia*®**	Pale-green colour with fine dark green and yellow specks with a faint odour	Light-green colour with fine dark green- yellow specks with a damp straw odour. Tablet slightly swollen	Straw colour with yellow specks. A slight white mould-like growth present. Sharp distinct odour.
2 ***Sutherlandia* SU1®**	Pale- green colour with distinct odour	Pale-brownish green colour with sharp distinct damp straw-like odour. Tablet slightly swollen.	Brittle tablet with a darker shade of brownish green colour. Sharp distinct odour.
3 **Health Wholefoods Connection *Sutherlandia*®**	Pale-gray-green colour with faint odour	Pale-gray-green colour with faint damp-straw-like odour. swollen.	Darker shade of grey green. Damp odour…mould like.
4 **Pinnacle *Sutherlandia*®**	Pale-brownish green colour with specks and a distinct odour	green colour with istinct odour	Darker shade of brownish green with sharp distinct odour.
5 **Promune®**	White body & dark green cap, Whitish-gray fluffy fine powder with a distinct odour	White body & dark green cap, rubbery texture, Whitish-gray clumpy powder with a distinct straw-like odour	Pale yellow body and dark green cap when present, otherwise sticky green and yellow capsule coating residue. The powders inside the whole capsules were whitish-gray and clumpy.
6 **Big Tree *Sutherlandia* Pro®**	Large sized transparent casing, Pale green fluffy fibrous powder with a strong distinct odour	Large sized transparent casing, with rubbery and sticky texture, Light green damp and clumpy fibrous powder with a strong damp-straw-like odour	Capsule casing disintegrated. Container consisted of black gunk. Foul pungent odour.
7 **Big Tree *Sutherlandia* 350®**	Medium sized transparent casing, Light green fine-fluffy-fibrous powder and a strong distinct odour.	Medium sized transparent casing, with rubbery and sticky texture, Light green fine-fluffy-fibrous powder and a strong distinct odour.	Pale yellow capsules with fragile casing. Pungent distinctive odour.

Generally the high temperature and humidity made the capsules fragile (product 7), change colour (product 2), caused them to disintegrate (product 6) and/or induced changes in the colour and odour of the tablets (products 1 to 4) and capsule powders (products 6 & 7). In at least one case (i.e. product 1) mould

growth was also evident after 6 days. Clearly the elevated temperature and humidity significantly affected the aesthetic and pharmaceutical quality and, most probably, the chemical stability of the SCP.

The SCP appeared to be particularly affected by the elevated humidity. The inclusion of silicon gel packs and use of airtight containers (i.e. SCP1, 2, 4, 6 and 7) are thus well warranted for these products. On the other hand the vulnerability to humidity would make the use of elevated humidity in the accelerated stability testing (an important part of pharmaceutical quality control) of herbal products such as SCP quite problematic.

It must however be noted that although the changes in organoleptic features of the SCP indicate gross deterioration in the aesthetic quality of the products, whether they also mimic changes in the chemical constituents and possible efficacy of the products is not known. For this more sensitive tests as discussed below are required.

It was hypothesized that when solid dosage forms of *Sutherlandia frutescens* are subjected to storage under elevated temperature and humidity conditions their antioxidant activity would decrease. To assess the effect of elevated temperature and environmental humidity on the antioxidant activity of SCP the DPPH and FRAP assays were used. For the DPPH assay the percent inhibition of DPPH absorbance produced by various concentrations of SCP was determined, the percent inhibition *vs.* SCP concentration plotted (as shown in appendix C) and fitted with a polynomial equation, the IC_{50} (i.e. concentration of SCP causing 50% inhibition of the DPPH absorbance) calculated and the results that were obtained are summarised in table 8 and figure 15.

The IC_{50} values (table 9) for the control group (stored in original packaging in cupboard) of SCP (*Sutherlandia* containing products) ranged between no value for product 6 (Big Tree *Sutherlandia* Pro®) to 0.650 mg/ml for Product 5 (Promune®) and 16.114 mg/ml for product 2 (*Sutherlandia* SU1®). According

to Amarowicz *et a*l. (2004) the more rapidly the decrease in absorbance of DPPH occurs and the lower the IC_{50} value, the greater the antioxidant activity. No IC_{50} value could be obtained for product 6 (Big Tree *Sutherlandia* Pro®) because instead of the purple DPPH solution going transparent, a yellow colour developed, even at low concentrations of the product. The maximum percent inhibition of the DPPH absorbance by this product was lower than the 50% inhibition level (see graphs in appendix C). It thus had a very high level of antioxidant activity which could not be established at the concentrations and experimental conditions used in this project. With an IC_{50} value of 0.650 mg/ml Product 5 (Promune®) also had substantial antioxidant activity and was much more potent (ca. 25 fold) than the *Sutherlandia* SU1 in this respect. In general the SCP thus varied considerably in their levels of antioxidant activity (see control C in fig 14). It must be noted that for the antioxidant results given in section 5.2.3 the IC_{50} was determined by extrapolation from the percent inhibition *versus* concentration curve while the IC_{50} values in table 9 were obtained by fitting the data with the most suitable polynomial model (Appendix C). Despite the difference in the method of data a[illegible]general trend for the antioxidant activity among the SCP stored under controlled conditions w[illegible]essentially the same as that reported in figure 5 and table 3 of section 5.2.2.1.

Table 9: Antioxidant activity of *Sutherlandia*-containing products after exposure to elevated temperature and environmental humidity using the DPPH assay

Product Number and Name	Storage Condition	Antioxidant activity			polynomial	Goodness of fit
		IC50 (mg/ml)	CI upper limit	CI lower limit	Order	r^2
1. Bio-*Sutherlandia*®	C	7.035	7.798	6.348	2nd	0.951
	A	4.196	4.694	3.749		0.935
	E	No value	NA	NA		0.686
2 *Sutherlandia* SU1®	C	16.114	no value	12.936	3rd	0.766
	A	5.415	7.185	3.867		0.821
	E	5.875	no value	3.737		0.821
3 Health Wholefoods Connection *Sutherlandia*®	C	5.246	5.569	4.954	2nd	0.986
	A	3.756	4.742	2.988		0.793
	E	7.471	11.367	5.682		0.739
4 Pinnacle *Sutherlandia*®	C	3.794	4.270	3.405	2nd	0.956
	A	2.359	3.091	1.789		0.746
	E	4.955	no value	3.319		0.556
5 Promune®	C	0.650	0.773	0.535	3rd	0.952
	A	0.512	0.565	0.466		0.989
	E	(	0.295	0.224		0.949
6 Big Tree *Sutherlandia* Pro®.	C	N(	NA	NA	2nd	0.942
	A	N(	NA	NA		0.016
	E	N(	NA	NA		0.376
7 Big Tree *Sutherlandia* 350®	C	2.706	3.171	2.322	2nd	0.937
	A	1.277	1.756	0.941		0.700
	E	1.773	2.121	1.517		0.891

Key
A: Ambient conditions; SCP stored exposed in laboratory
C: Control conditions; SCP in original package in cupboard
E: Elevated climatic chamber conditions; 40°C & 70% RH
CI: 90% Confidence Interval
IC_{50}: Concentration of aqueous extract of SCP producing 50% inhibition of DPPH

In figure 15 the antioxidant activities of the SCP under the 3 storage conditions (i.e. control, ambient and elevated temperature and humidity conditions) are shown. Storage under ambient (18°C to 28°C & 25 to 35% RH) as opposed to controlled (stored in original packaging in dark and dry cupboard) conditions caused an increase in the antioxidant activity of the SCP. For instance, the antioxidant activity of product 2 (*Sutherlandia* SU1®) increased from 16.114mg/ml for the control group to 5.415mg/ml after storage in ambient conditions. The increased antioxidant abilities were significant for all the SCP, except for product 5. Product 5, Promune® is the only freeze dried *Sutherlandia* plant extract product among the selected

SCP and the change in its IC_{50} values form 0.650mg/ml (control) to 0.512mg/ml (ambient) was statistically insignificant (p=0.075). This could mean that freeze dried extracts are less affected by ambient storage conditions. In conclusion, when stored unprotected under ambient conditions the SCP, except the capsule containing the freeze dried aqueous extract (Promune®), changed in organoleptic features (i.e. visibly) and exhibited greater antioxidant activity.

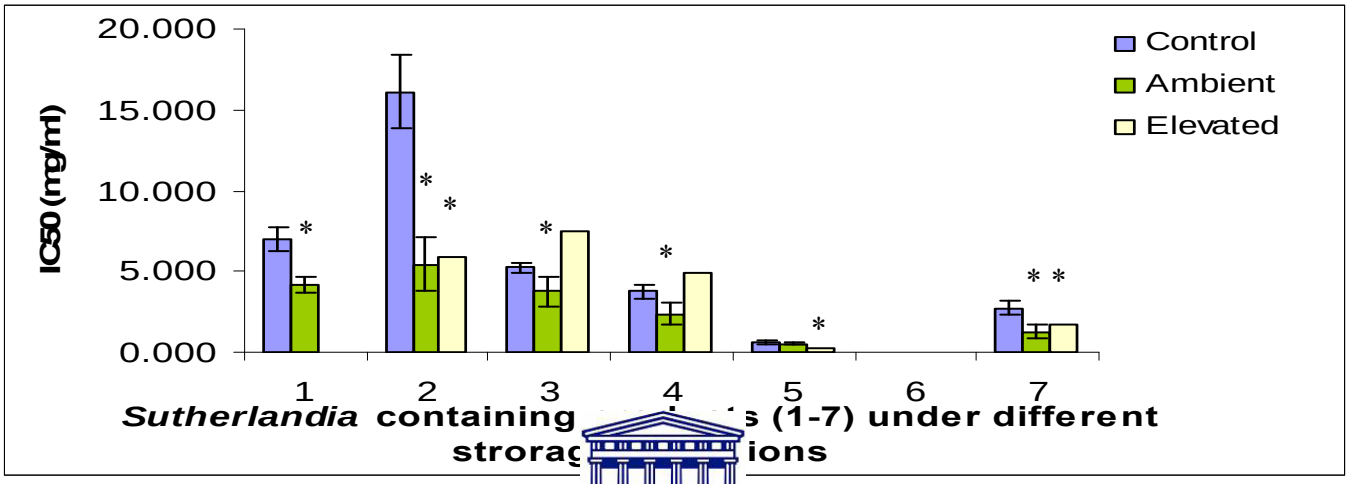

Figure 15: Comparative levels of antioxidant act *rlandia* containing products under different storage conditions. IC50 = concentration of aqueous extracts of SCP that inhibits UV absorbance of DPPH at 560nm; SCP 1 to 7 = 7 solid dose *Sutherlandia* containing products; ambient (A) =18°C to 28°C and 25 to 37% RH range, elevated (E) =40°C & 70% RH and Control (C) = stored in original packaging in dark cupboard. N = 3 and * =significant difference from control (p≤0.05, student t-test)

When the SCP were stored at 40°C and 70% RH the outcomes were more variable. For SCP 2, 5 & 7 the antioxidant activity was statistically significantly increased. While SCP 3 (Health Wholefoods Connection *Sutherlandia*®) & 4 (Pinnacle *Sutherlandia*®) decreased in antioxidant activity from their control group samples, these decreases were however not statistically significant (p>0.05). It is possible that this inconsistency arose because the SCP stored in the climatic chamber were tested after 2 weeks, which may have been insufficient time for some products to have changed, and if left longer, e.g. 2 months, all may have changed.

Overall the data thus suggested, contrary to the postulated hypothesis, the level of antioxidant activity in the SCP increased when these preparations were stored (exposed) under elevated temperature and humid environmental conditions. It therefore appears that the monitoring of antioxidant activity could be a reliable method to monitor, control and/or compare the pharmaceutical quality of SCP over time. However, the DPPH assay may not be a very viable method to use for the SCP since reliable IC_{50} values could not be produced for all the SCP when this method was used.

To confirm that monitoring of antioxidant activity can be used to compare the pharmaceutical quality of SCP over time, the data obtained using the ferric reducing antioxidant power (FRAP) assay was also assessed. A standard curve of ascorbic acid concentration *vs.* absorbance at 540nm described by the equation, y = (5.440±0.1698)x + (0.1338±0.009049), was obtained and used to calculate the ascorbic acid antioxidant equivalents of each SCP stored under the different environmental temperature and humidity conditions, see table C.1. The results obtained are summarized in figure 16.

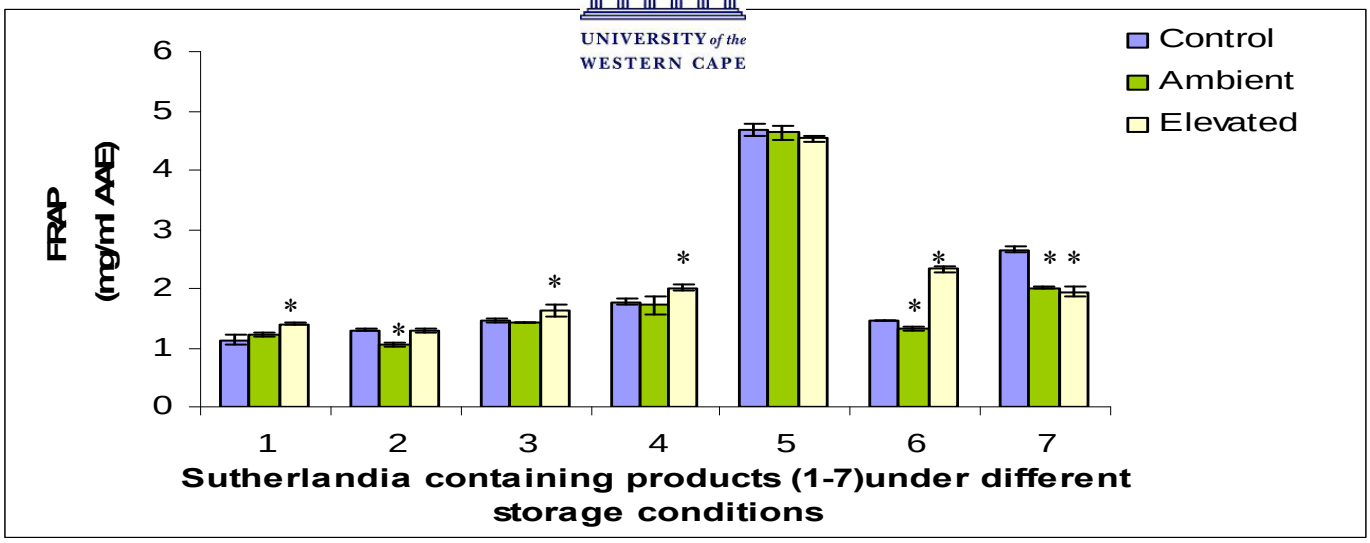

Figure 16: Comparative levels of antioxidant activities (FRAP) of aqueous extracts of *Sutherlandia* containing products under different storage conditions. FRAP = Ferric Reducing antioxidant power; SCP 1 to 7 = 7 solid dose *Sutherlandia* containing products; ambient (A) =18°C to 28°C and 25 to 37% RH range, elevated (E) =40°C & 70% RH and Control (C) = stored in original packaging in dark cupboard. N = 3 and * =significant difference from control ($p \leq 0.05$, student t-test)

The lower the ascorbic acid equivalence (AAE) result of the SCP, the greater their ability to convert ferric acid to ferrous at lower concentrations and thus, the greater the antioxidant ability. The results for SCP 2 to 7 all increased in antioxidant activity after 2months of storage under ambient conditions. The increase was however only statistically significant ($p \leq 0.05$) for SCP 2 (*Sutherlandia* SU1®), 6 (Big Tree *Sutherlandia* Pro®) and SCP 7 (Big Tree *Sutherlandia* 350®). On the other hand, the antioxidant activity of SCP 1 actually decreased (i.e. from 1.133789 to 1.209806mg/ml AAE) although this decrease was not statistically significant.

The samples stored under elevated temperature and humidity seemed to follow the trend of a decrease in antioxidant activity, SCP 1, 3, 4 and 6 had statistically significant ($p \leq 0.05$) decreased in antioxidant levels. The SCP 5 (Promune®) and 7 (Big Tree *Sutherlandia* 350®) seemed to increase in antioxidant activity after storage in elevated temperature and humidity conditions, however only SCP 7 had a significant difference. One can assume that, climatic cl itions decreases the ability of each product to transform these irons at lower concentrations.

From figure 16, product 5 (Promune®) appeared to be the least affected when exposed to extreme (40°C and 70%RH) storage conditions, the antioxidant ability p values between the control and ambient, and control and elevated temperature and humidity stored samples were 0.775 and 0.251mg/ml AAE respectively. Thus the difference in antioxidant ability between these storage conditions had no statistically significant effect on SCP 5 when using the FRAP assay.

Thus, climatic chamber storage (elevated group) decreased the antioxidant activity of most of the SCP, whereas the ambient stressed samples either had little effect, or actually increased the antioxidant ability of the SCP samples. As in nature, according to Ksouri *et al*. (2008), secondary metabolites, such as phenols which contribute to antioxidant activity, are 1.4 fold higher in medicinal plants growing in arid environments compared to those found in humid environments. Research conducted by Stafford *et al*.

(2005) concluded that anti-bacterial activity, which is also a result of secondary metabolite synthesis, was either stable or increased during storage of dried medicinal plants under ambient conditions, whereas accelerated aging (55ºC and 100% RH) decreased the anti-inflammatory effects of certain medicinal plants. Stafford *et al.* (2005) also stated that changes in biological material cannot be contributed to storage conditions alone, but also upon the drying process, which could affect the chemical make up of the plant material. These arguments together with the results of this study suggests that antioxidant activity will either increase when in warm and dry storage conditions or decrease when stored in warm and moist/humid storage conditions. Thus antioxidant activity assays, mainly the FRAP assay, may be used to monitor, control and compare pharmaceutical quality of SCP over time, provided that storage humidity conditions are noted.

To test and compare the effect that storage under ambient and elevated temperatures and humidity conditions had on the total phenol content of *Sutherlandia* containing products, the Folin-Ciocalteu assay was conducted on all samples. The results obtained are recorded in Appendix D and summarized in figure 17.

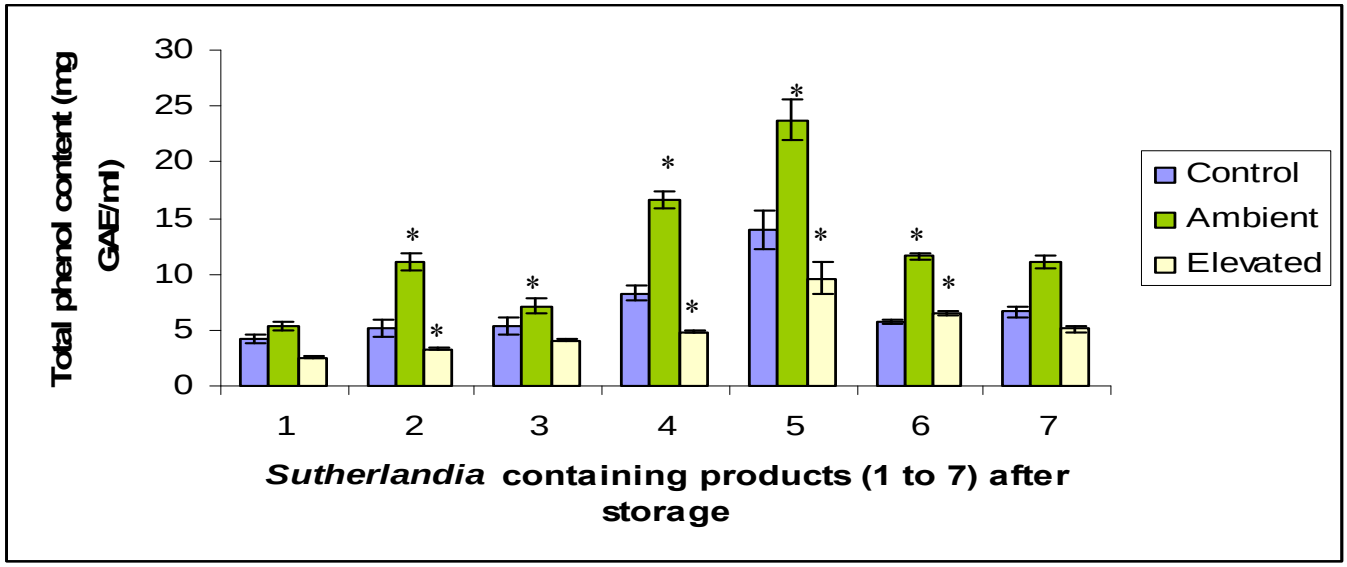

Figure 17: Comparative levels of total phenol levels of aqueous extracts of *Sutherlandia* containing products under different storage conditions. GAE = gallic acid equivalence, SCP1 to 7 = 7 solid dose *Sutherlandia* containing products; ambient (A) =18°C to 28°C and 25 to 37% RH range, Elevated (E) =40°C & 70% RH and Control (C) = stored in origina dark cupboard. N = 3 and * =significant difference from control (p≤0.05)

In general, storage under ambient conditions (i.e.18°C to 30°C and 10 to 30% RH range) caused an increase in the total phenol content of the SCP constant with that of the "control" samples (i.e. sample stored in original package in cupboard). All the products except, SCP 2 and 7 had significant ($P<0.05$) increases in their TPC when stored under ambient conditions.

Upon storage under climatic chamber stress conditions (i.e. 40°C & 70% RH) the total phenol levels of all the SCP decreased compared to the levels of the control group. The ambient stored samples of SCP 2, 4, 5 and 6 decreased significantly ($p<0.05$) compared to that of the samples stored under control conditions. This implies that under these conditions the phenols in the SCP are overwhelmingly degraded, evidently, just 2 weeks storage in a climatic chamber under 40°C and 70% RH was sufficient to produce degradation of not only the existing free phenols but also those that may initially have formed from phenol conjugates.

The results of both the ambient and elevated environmental temperature and humidity storage conditions show that high relative humidity decreases total phenol content and increase in temperature alone increases total phenol content. This result was contrary to the set hypothesis that the total flavonoid and polyphenol levels may decrease or remain unchanged.

According to Ksouri *et al.* (2008) and Kirakosyan *et al*. (2003), the secondary metabolites of plants are enhanced when subjected to elevated and dry environments. This proves true for the dried plant material/products too (Stafford *et al*., 2005). These secondary metabolites, (i.e. phenols) contribute to antioxidant activity, thus at higher concentrations of SCP it is anticipated that the TPC and antioxidant activity will increase when subjected to high temperatures and low humidity for a long period. However as stated by Stafford *et al.* (2005) storage conditions alone do not contribute to the chemical and biological properties of the medicinal plant, the manner in which the product is prepared, i.e. drying process will also effect these total phenol and antioxidant le storage under ambient conditions could have removed some of the moisture of the "dried" ial of the solid dosage forms of the SCP, thus concentrating the TPC and elevating the antioxidant ability of that product.

In general the trends in the change in total phenol content among the 7 SCP stored under the different environmental storage conditions were similar to that of the antioxidant results. This strongly suggests that the use of total phenol content monitoring may be a useful tool to assess the long term quality, stability and shelf life of commercially available *Sutherlandia* containing pharmaceutical dosage forms.

Some of the most important phenols with antioxidant activity in the SCP may be the flavonoids. To test and compare the effect that storage under ambient and elevated temperatures and environmental relative humidity conditions had on the flavonoid profile of SCP HPLC analysis was conducted. The peaks that were most prominent in all the 7 SCP were identified as Sutherlandins A to D as previously recorded by Avula *et al*. (2010), were used to quantitate the effect of the different storage conditions on the SCP.

The sutherlandins had retention times of 10.928, 11.969, 14.373 and 15.040mins, respectively and these were used to analyse the HPLC fingerprints of SCP that had been subjected to storage under ambient (18 to 30°C) and 40°C and 70 % RH (i.e. Elevated climatic chamber storage). Samples of these fingerprints are given in Appendix G and H and the changes in the height of the 4 relevant peaks (sutherlandins A to D) in the chromatograms of the SCP stored under the different environmental conditions are summarized in figures 17 to 20 below.

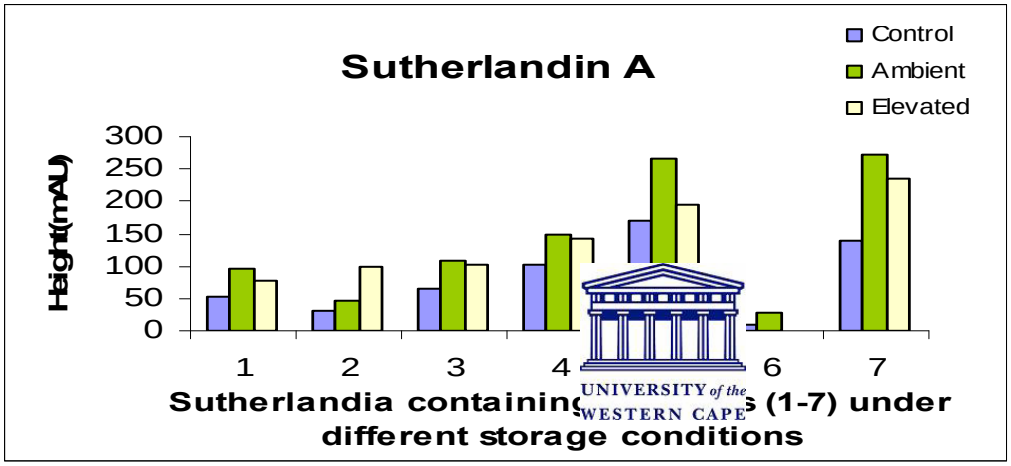

Figure 18: **The effect of different environmental storage conditions on the height of the Sutherlandin A peak (retention time =10.928min) obtained for 7 SCP.**

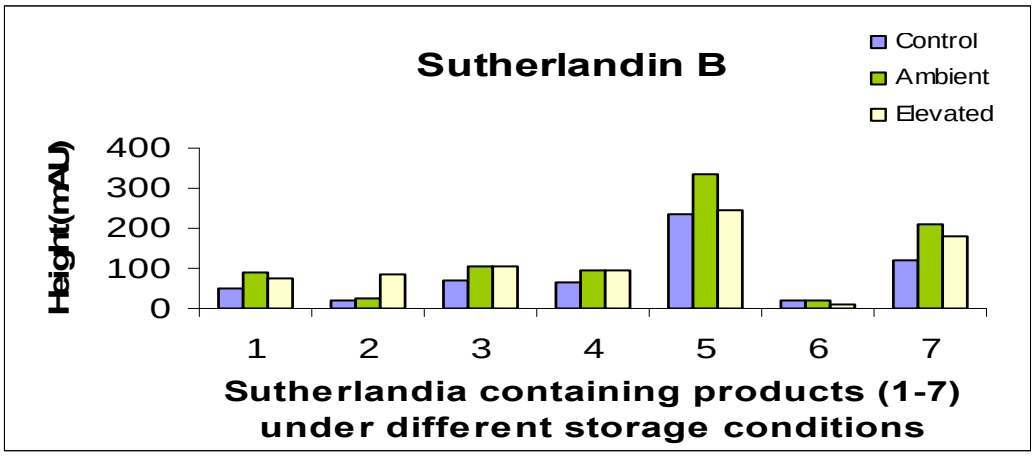

Figure 19: **The effect of different environmental storage conditions on the height of the Sutherlandin B peak (retention time =11.969min) obtained for 7 SCP.**

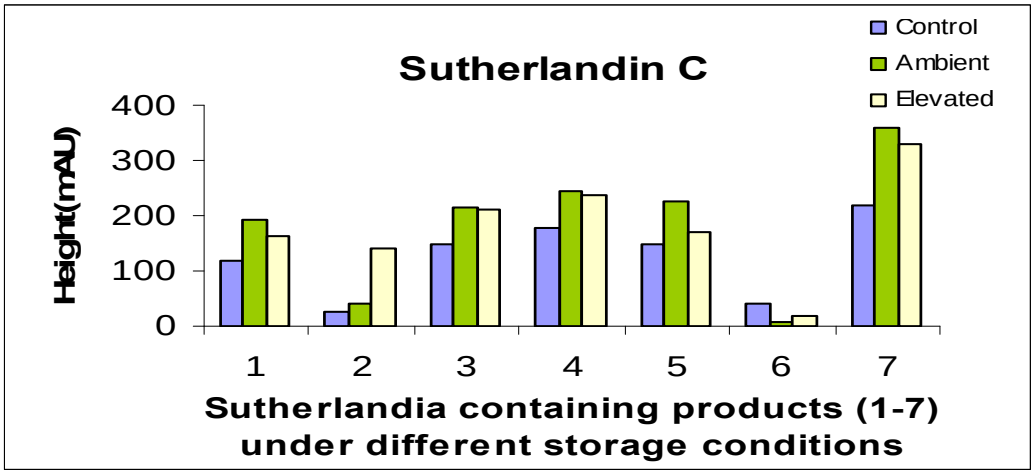

Figure 20: **The effect of different environmental storage conditions on the height of the Sutherlandin C peak (retention time = 14.373min) obtained for 7 SCP.**

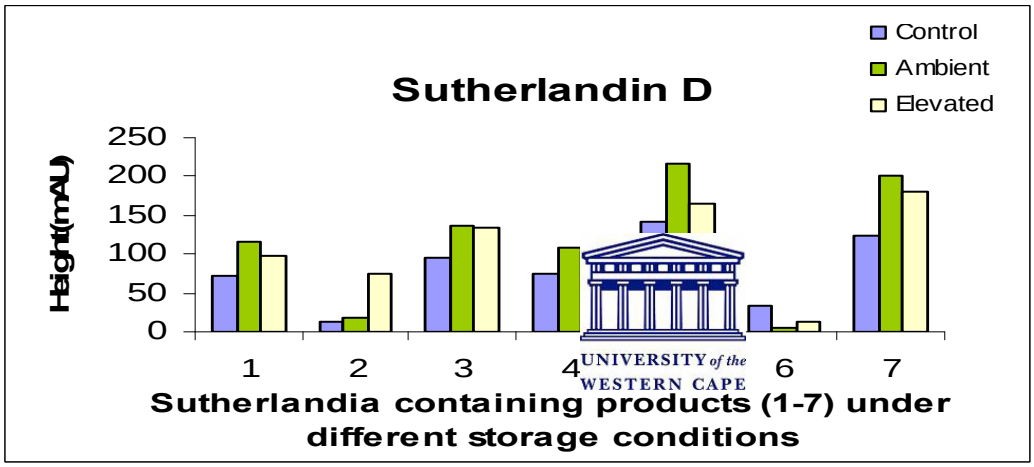

Figure 21: **The effect of different environmental storage conditions on the height of the Sutherlandin D peak (retention time =15.040min) obtained for 7 SCP.**

Figures 18 to 21 simplifies whether the peak heights of sutherlandins A to D increased or decreased after the SCP were stored under different environmental conditions (i.e. Control, Ambient and Elevated). All SCP samples had increased sutherlandin peak heights after storage in elevated and ambient environmental conditions, all except product 6 (*Sutherlandia* Pro®).

When conducting statistical student t-test on all the SCP stored under different environmental conditions, sutherlandin A had the most significant increase in peak heights between the control and ambient stored group, where p=0.0136, and the control and elevated conditions stored group p=0.0072. The peak heights

of sutherlandins B, C and D also increased significantly ($p \leq 0.05$), between the control and ambient, and the control and elevated stored samples, however sutherlandin D had the least significant ($p=0.0418$) change between the control and ambient stored samples.

These results are conflicting with those of the total phenol content assay test, where the TPC decreased when exposed to elevated temperature and humidity. However the individual sutherlandins increasing when stored in ambient conditions, this increase correlates with the TPC results. This could suggest that the sutherlandins are not the only phenols which contribute to the total phenol content of the SCP. For the exact values of the heights see table I.1 in the appendices. These results are contrary to the earlier hypothesis which states that levels of flavonoid glycosides will decrease after exposure to elevated environmental temperature and humidity conditions.

Another interesting find from these results during storage conditions, sutherlandins actually had greater concentrations than when stored in elevated environmental temperature and humidity conditions, providing compelling evidence that high humidity decreases the amount of secondary metabolites being expressed by the SCP (Stafford *et al.*, 2005). As a further study it would be best to have HPLC chromatic fingerprints of SCP which have been exposed to a climatic chamber for two months and compare them to two month ambient exposure.

5.2.6 The effect of acid hydrolysis on solid dose *Sutherlandia frutescens* containing products

Solid dose *Sutherlandia* products contain constituents such as flavonoid glycosides that may vary in their susceptibility to acid hydrolysis and in their release of active compounds in acidic environment (e.g. stomach) which in turn may lead to variable absorption, bioavailability and consequently, variable *in-vivo* effects from the dosage form. Variability in susceptibility to acid hydrolysis can thus be used to ascertain inconsistency in quality among SCP.

To determine the effect of acid hydrolysis on the quality of the SCP the products were exposed to 0.6N hydrochloric acid solution for up to 24hours and then analyzed using HPLC. The chromatographic fingerprints obtained for the hydrolyzed and unhydrolysed (control) solutions of SCP groups are given in figures 21 to 27.

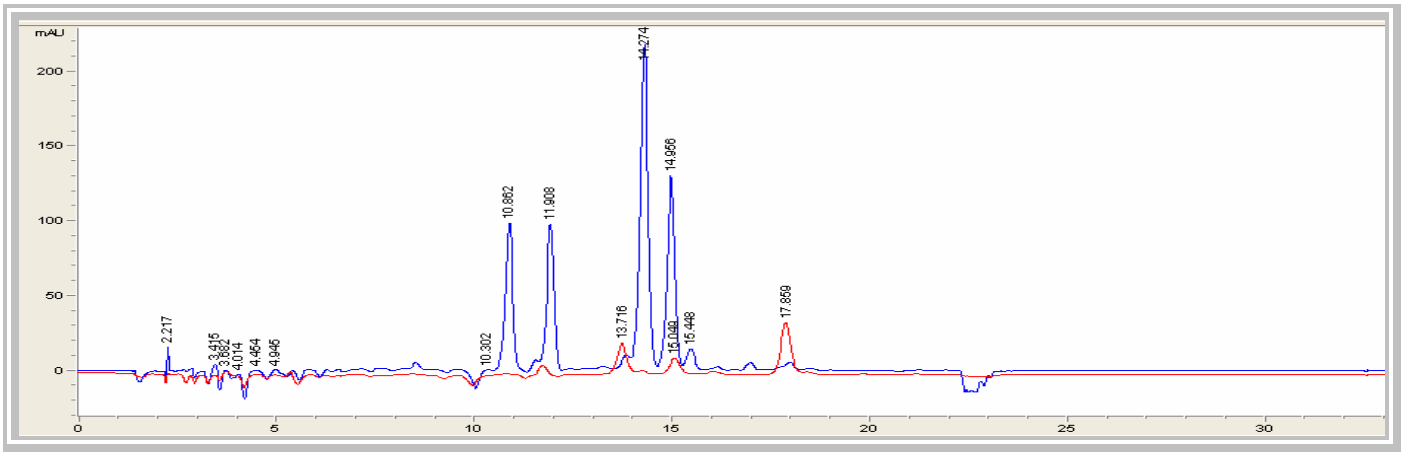

Figure 22: Chromatographic fingerprints of ur blue line) and hydrolysed (red line) solution of *Sutherlandia* containing product 1.

After acid hydrolysis of SCP1, the chromatographic peaks eluting in the first 10minutes of the HPLC run (figure 22) were essentially the same as those for the unhydrolysed sample. However, the flavonoid compounds eluting at the retention times of 10.802, 11.908, 14.274, and 14.956mins (& previously identified as sutherlandin A to D, see section 5.2.4) for the un-hydrolysed sample disappeared completely from the chromatographic fingerprint of the acid-hydrolyzed sample while the peaks at the retention time of 13.771±0.055 and 17.909±0.05mins with flavonoid-like spectrum appeared to be slightly higher (9.4 to 20.8 and 5.4 to 33.7mAU's respectively) after hydrolysis.

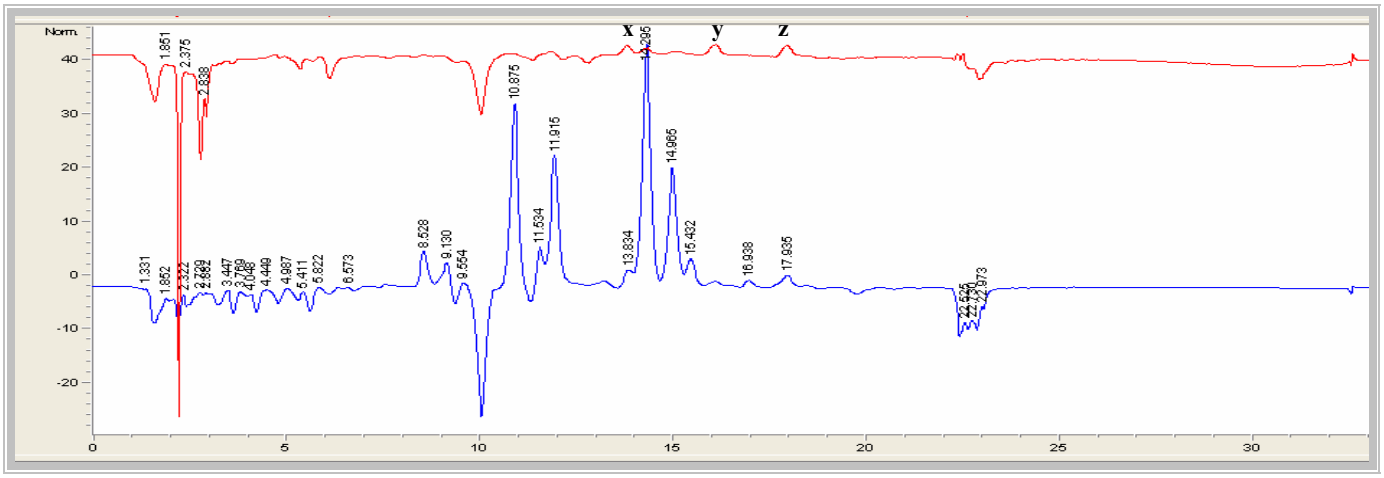

Figure 23: Chromatographic fingerprints of unhydrolysed (blue line) and hydrolysed (red line) solution of *Sutherlandia* containing product 2. (Note that the scale for the red and blue traces are not the same; height of peak at 17.935min in red and blue traces are <0.01mAU's, and 1.7mAUs, respectively)

After acid hydrolysis, the 4 sutherlandin peaks at 10.875, 11.915, 14.295 and 14.965mins in the SCP 2 fingerprint (figure 23) also disappeared while the height of the peaks at retention times of 13.84 and 17.935mins (also seen in SCP 1) was reduced. It is noted that the scale for the red and blue traces in figure 23 is not the same i.e. the heights for the 17.935mins peaks in the hydrolysed and un-hydrolysed samples were <0.01, and 1.7mAUs, respectively. Similar results were obtained after the acid hydrolysis of SCP 3, 4, 6 and 7 (figures 24 to 27), except that the peak at 17.805±0.15mins did not change much in peak height. It is suspected, from the presently available MS data, that the compound at 17805±0.15mins is a breakdown product of sutherlandin C and D i.e. a kaempferol derivative.

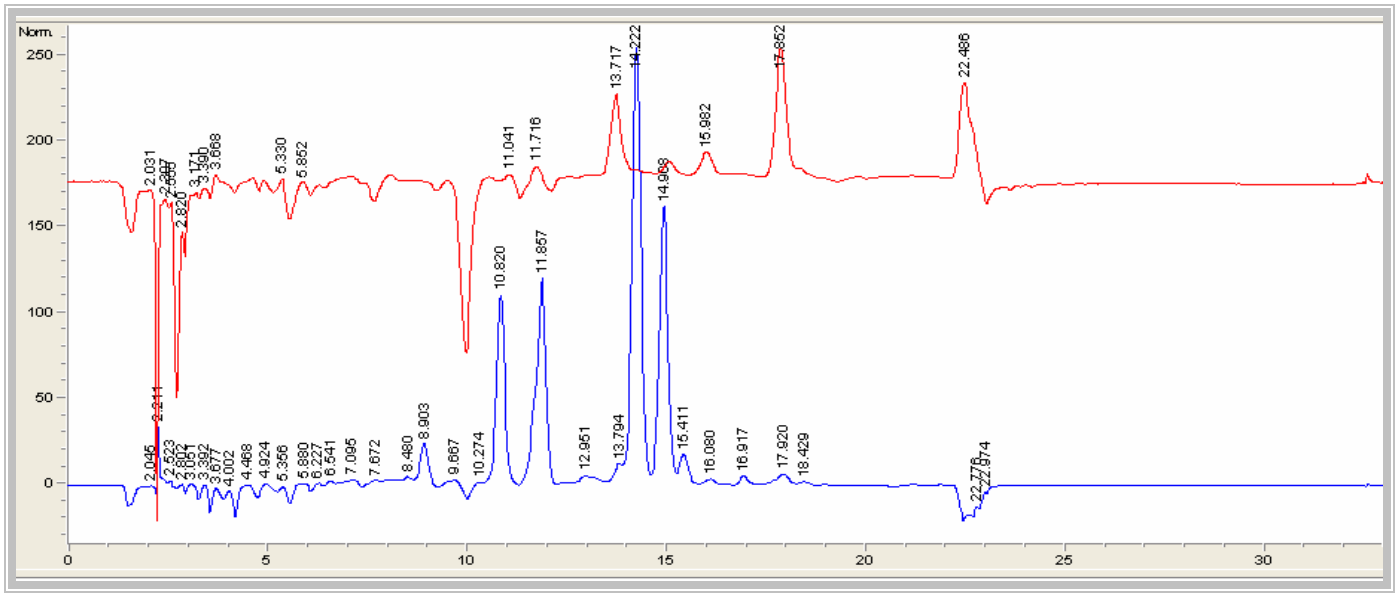

Figure 24: Chromatographic fingerprints of unhydrolysed (blue line) and hydrolysed (red line) solution of *Sutherlandia* containing product 3. (Note that the scales for the red and blue traces are not the same; heights of peak at 17.886±0.034min in red and blue traces are 7.9, and 6.4 mAUs, respectively)

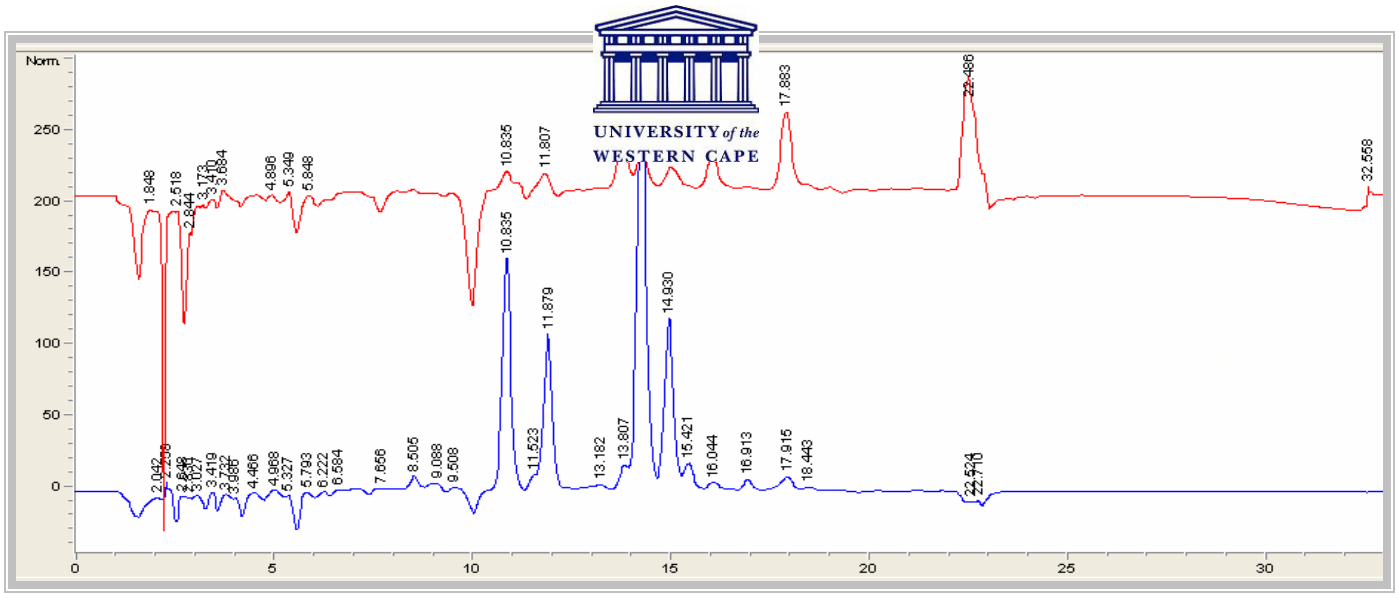

Figure 25: Chromatographic fingerprints of unhydrolysed (blue line) and hydrolysed (red line) solution of *Sutherlandia* containing product 4. (Note that the scales for the red and blue traces are not the same; heights of peak at 17.899±0.016min in red and blue traces are 6.9, and 9.4 mAUs, respectively)

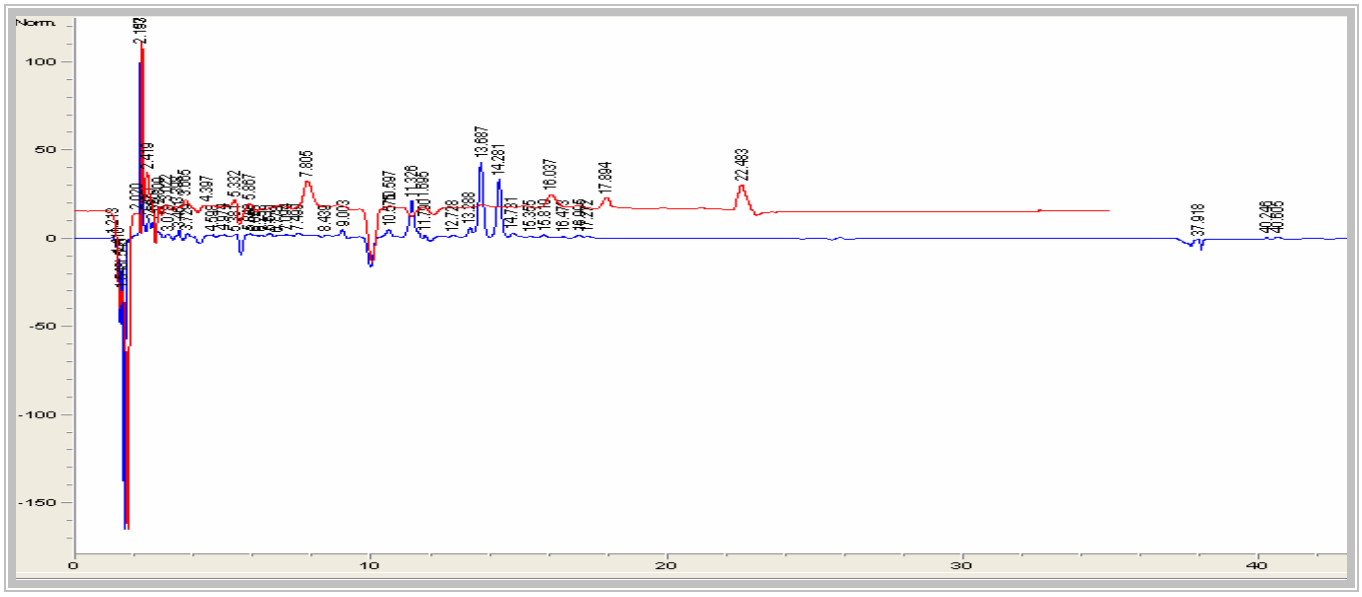

Figure 26: Chromatographic fingerprint of hydrolysed *Sutherlandia* containing product 6. (Note that the scales for the red and blue traces are not the same; height of peak at 17.894min in red and 16.790min in the blue traces are 3.2, and 3.3mAUs, respectively)

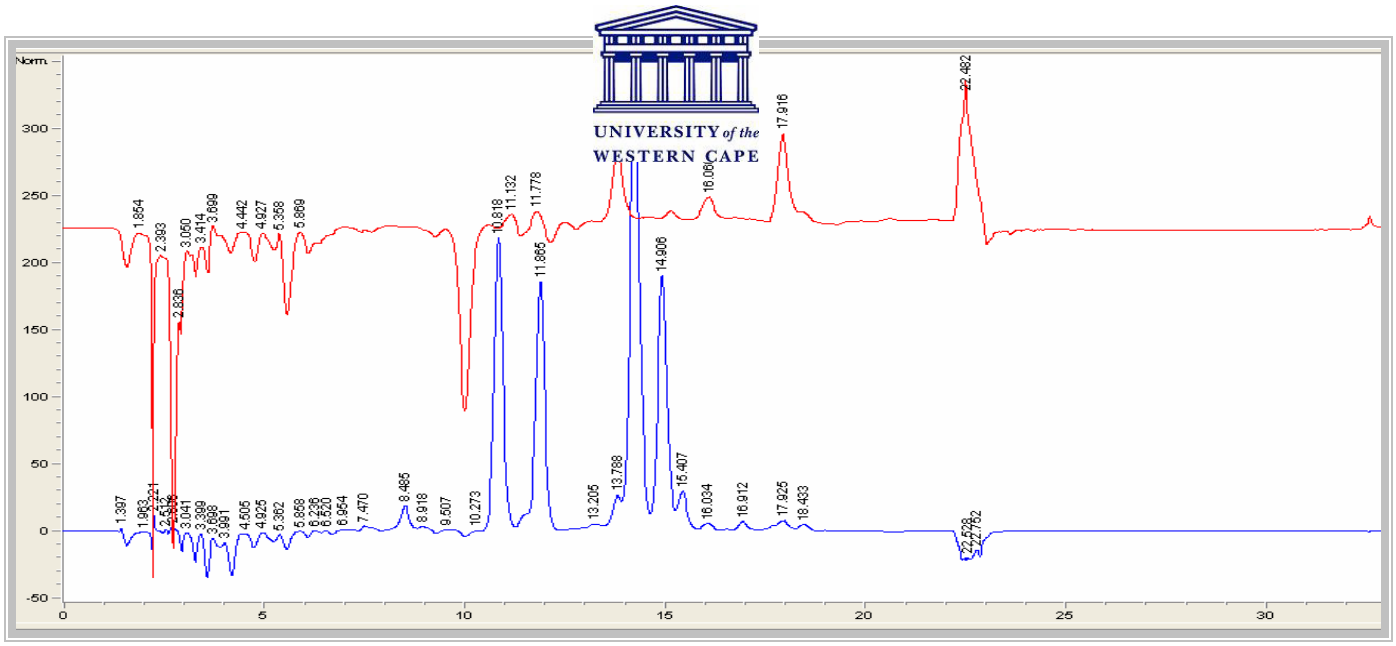

Figure 27: Chromatographic fingerprints of unhydrolysed (blue line) and hydrolysed (red line) solution of *Sutherlandia* containing product 7. (Note that the scales for the red and blue traces are not the same; heights of peak at 17.921±0.005min in red and blue traces are 6.8, and 7.6mAUs, respectively)

It thus appears that acid hydrolysis causes the degradation of at least 4 flavonoid-like compounds (sutherlandins) in the SCP 1 to 4 and 6 & 7. From the present results it is however not clear what break

down products resulted from this degradation. If they are converted to the aglycones it is very likely, from the present data, that such aglycones were also susceptible to acid-hydrolysis.

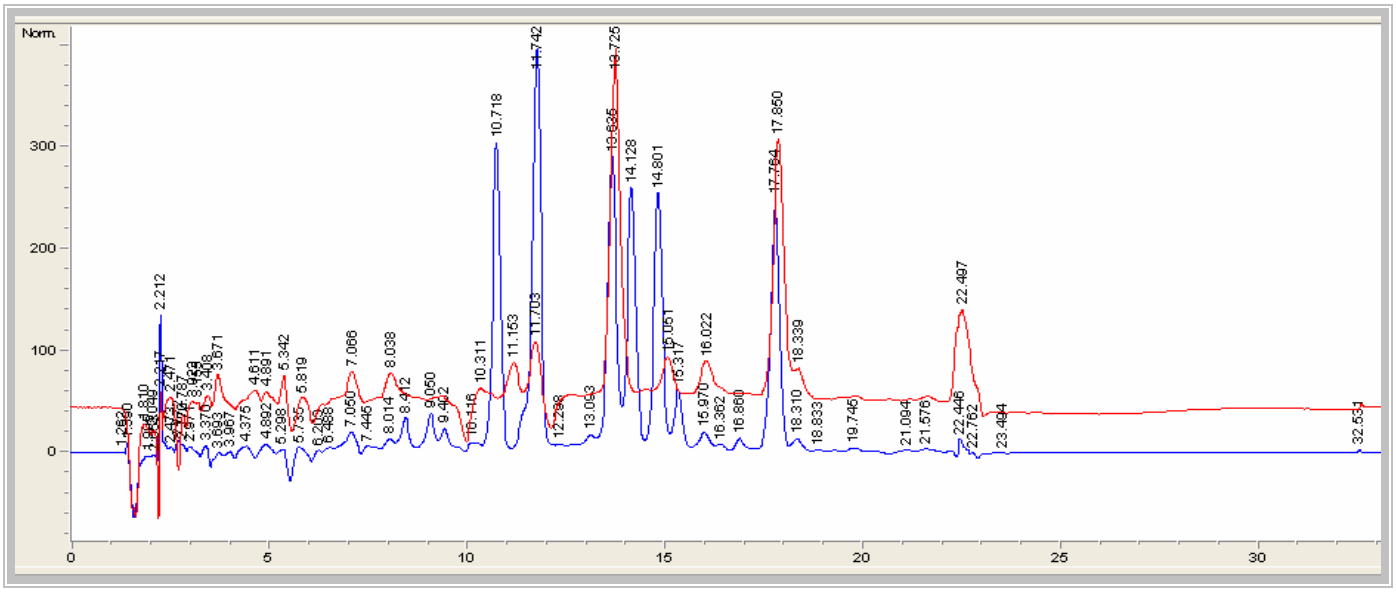

Figure 28: Chromatographic fingerprints of unhydrolysed (blue line) and hydrolysed (red line) solution of *Sutherlandia* containing product 5.

As reported earlier (section 5.2.4) the fingerp -hydrolysed aqueous solution of Product 5 also had, as seen in the other products, the 4 pr erlandin peaks at retention times 10.51±0.21, 11.53±0.25, 13.74±0.36, and 14.41±0.48mins. These flavonoids also disappeared or were reduced after acid hydrolysis (figure 28). For product 5, as was the case for the other SCP, the peak at 17.13±0.54mins was also present and decreased in height from 268.5 to 36.8mAU's after acid hydrolysis. The results for product 5 also differed slightly from that for the other SCP in that it the flavonoid-like peak at 13.725mins which was much more prominent in the Promune® than the other products also decreased (from 321.3 to 49.2mAU) after acid hydrolysis. It is suspected, from the presently available MS data, that the compound at 13.7mins is a breakdown product of sutherlandin A and B i.e. a quercetin derivative.

Under the same HPLC conditions the aglycone quercetin had retention times of 10.271±0.02min (n= 3). The chromatograms for the SCP after acid hydrolysis contained no peaks at this retention time suggesting that even aglycones was susceptible to acid hydrolysis.

Overall, it was clear that the 4 sutherlandins (section 5.2.4) identified in the SCP investigated in this study were significantly degraded under acidic conditions. The flavonoids-like peaks at the retention times around 13.69±0.114, and 17.805±0.153mins, were most likely those of derivatives of quercetin and kaemferol, respectively, and break down products of sutherlandins A & B and C & D, respectively. These were also susceptible to acid hydrolysis but not to the same extent. It is likely that the sutherlandins (i.e. flavonoid glycosides) are hydrolysed to their aglycone forms but since no evidence of the presence of the latter was obtained in the present study it is likely that even the aglycones were also susceptible to the acid hydrolysis (Nuutila *et al.*, 2002). The hypothesis that the levels of free aglycone flavonoids will decrease after environmental elevation was accepted. These results mean that the sutherlandins contained in the SCP may not be very stable in acidic pH (as in the stomach) and this may affect their bioavailability after oral administration. A more thorough investigation of the acid induced hydrolysis of the sutherlandins in the SCP (i.e. determination of the actual rate wn, the identity and stability of the breakdown products, etc.) is warranted.

5.2.7 Quality control specifications that may be used for the selected *Sutherlandia frutescens* preparations

Based on the results of the set objectives, antioxidant activity is an effective means of comparing the different SCP quality and could be used in industry as a golden standard. It is inexpensive and robust, and when performed on micro-plates many replicates can be run simultaneously. For comparing product quality antioxidant activity and TPC are excellent markers, however longer periods of environmental stress should be conducted on these products. Promune® or rather aqueous freeze dried extracts of *S. frutescens* could be used to isolate large amounts of sutherlandins A to D, which can be used as standards in HPLC analysis to quantify the levels of each flavonoid (sutherlandin) in each SCP. Of the methods used

to test product quality and comparison, the DPPH assay (taken into far lower concentrations) and HPLC analysis, are excellent means of testing solid dose *Sutherlandia frutescens* containing product quality.

Antioxidant activity and flavonoid levels can be used to assess the quality of commercially available solid *Sutherlandia frutescens* products, however both these properties decrease when exposed to elevated environmental humidity conditions. The only possible way to compare SCP optimally is by analysing the contents of each product on the date of manufacture and then storing it under elevated environmetal temperature and humidity to establish an appropriate expiration date. As a further study it would be feasible to establish which sutherlandin contributes to the most antioxidant ability of *Sutherlandia frutescens,* this would shorten the quality assessment of each solid dose SCP by only using HPLC analysis to quantify that specific sutherlandin.

Chapter 6

CONCLUSION AND RECOMMENDATION

The overall aims of this project were to explore the use of monitoring flavonoid profile and antioxidant activity levels to assess the pharmaceutical quality and consistency of commercially available solid dose *Sutherlandia frutescens* containing products (viz. tablets & capsules) and to develop/or adapt methods and specifications that may be used for the quality control of such products.

The objectives of this study were:

- To assess and describe the quality and re 

pliance of selected representative solid dose *S. frutescens* containing products,
- To determine and compare the antioxidant activity of selected commercially available solid dose *S. frutescens* containing products,
- To compare the total phenol level of selected representative solid dose *S. frutescens* containing products,
- To determine the profile and level of specific flavonoids in the above mentioned products,
- To determine the effect of various environmental stresses [i.e. elevated temperature, environmental humidity and chemically-induced (or acid-induced) degradation] on the selected products, and
- To formulate quality control specifications that may be used for the selected *Sutherlandia frutescens* preparations.

6.1 Conclusion and Recommendations

Based on the results obtained the following conclusions could be drawn:

1. The *Sutherlandia*-containing tablet and capsule dosage forms investigated in this study, physically appeared to be of quite good "pharmaceutical" quality, but generally lacked information on the date of manufacture and lacked package inserts, or when these were present they contained insufficient information. It should be feasible that present commercially available solid dosage *Sutherlandia*-containing products could, with relatively little extra information, easily meet most of the product quality criteria needed for their registration by the MCC.
2. All the SCP had antioxidant activity which ranged, in descending order of greatest to lowest antioxidant power, as follows: Promune® >> Big Tree 350® > *Sutherlandia* Pinnacle® > *Sutherlandia* Health Connection Wholefoods® > Bio-*Sutherlandia*® > Big Tree Pro® >>Phyto Nova SU1®. The capsules possessed greater antioxidant power than the tablets and the two assays (FRAP and DPPH) were useful in comparing the relative potencies of the selected SCP.
3. The SCP varied in total phenol concentration in descending order: Promune® >> Big Tree 350® >> *Sutherlandia* Pinnacle® > Big Tree Pro® > Phyto Nova SU1® > *Sutherlandia* Health Connection Wholefoods® > Bio-*Sutherlandia*®, which correlated with the antioxidant activity results for the selected SCP. Both the antioxidant activity and TPC results gave good indications of the relative potencies and chemical integrity of the SCP.
4. All the selected SCP contained the flavonoids sutherlandins A to D in varying amounts with the sutherlandin C being the most concentrated in all the SCP and the capsules having far greater levels of all these flavonoids than the tablet dosage form. These flavonoids can thus be used as marker compounds and be quantified to compare and monitor the relative pharmaceutical quality (e.g. content uniformity, expiry date, etc) of *Sutherlandia* containing solid dose products

5. Elevated temperature and humidity significantly affected the aesthetic, pharmaceutical quality and the chemical stability of the SCP. The SCP appeared to be particularly affected by the elevated humidity which caused a decrease in the total phenol content of the products. Careful consideration of the storage conditions, particularly those that reduce the effects of humidity (e.g. use of moisture-resistant containers or packaging, inclusion of silica sachets, etc.) is thus required if the long term stability of SCP is to be ensured.
6. The SCP were very susceptible to acid hydrolysis. Under these conditions the 4 sutherlandins are ssignificantly degraded to derivatives of quercetin and kaempferol, which in turn are also susceptible to acid hydrolysis but not to the same extent. In this process aglycones are either not formed or very rapidly degraded. The sutherlandins contained in the SCP are thus not very stable in acidic pH (as in the stomach) and this may affect their bioavailability after oral administration. A more thorough investigation of the acid induced hydrolysis of the sutherlandins in the SCP (i.e. determination of the actual rates of breakdown, the identity and stability of the br ducts, etc.) is warranted.

Based on the results obtained it is recommended that,

1) the manufacturers of SCP pay more attention to the information provided on the package inserts and the storage conditions for their products and
2) the levels of antioxidant activity, total phenols and flavonoids (sutherlandins A to D) be used as specifications to control the quality of commercially available solid dose *Sutherlandia frutescens* containing preparations on an individual basis.

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Appendix A1

SELECTION CRITERIA OF SOLID DOSE *SUTHERLANDIA FRUTESCENS* PRODUCTS

List of selection criteria of solid dose *Sutherlandia frutescens* products

1. Solid dosage form

The dosage form for the *S. frutescens* product selection should either be in the form of a capsule or tablet.

2. Composition

The selected product must contain only *Sutherlandia frutescens* as a single active ingredient (90-300mg/dosage form). There should be no signs on the packaging label indicating the presence of other actives. Special note should be taken of the preservatives added to the product as these may contribute to the effectiveness of the drug.

3. Manufacturer

The manufactures name as well as their contact details should be visible on the packaging label.

4. Supplier/Purchased at

Note the appearance and location of the store where the product is to be purchased. Store should be clean and product should be easy to locate on the sh lish whether a supplier chain exists.

5. Packaging

The container choice of selection would b ainers with snap-on or screw-on caps. The containers should serve its function of protecting the product from deterioration by environmental conditions. This also allows for opening and resealing of the product. A safety seal (tac seal) should be fully intact on all newly purchased products to ensure that the product has not been tampered with.

6. Label

The labeling should be clear and visible. All printed information should be legible to the consumer. The products name, manufacturer and an expiratory date should also be present on the label.

7. Batch number and expiratory date (manufacture date)

The batch number should always be present as this provides a record of the production history of the product. The expiratory date indicates when the product is expected to have deteriorated. No product is to be bought if the expiratory date falls within the next 7 months of this year (2008).

8. Storage conditions

The storage conditions should be stipulated on the container, whether the product is to be stored in specific conditions, i.e. temperature, humidity and light.

9. Popularity

The number of a specific *S. frutescens* containing product on the shelves of various stores.

10. Quantity

At least one months supply (approximately 30) tablets or capsules should be available in each of the selected *S. frutescens* containing products.

Appendix A

ANTIOXIDANT ACTIVITY OF *SUTHERLANDIA* CONTAINING PRODUCTS

Table A.1: The percent inhibition of DPPH by varying concentrations of quercetin.

Quercetin (mg/ml)	Absorbance at 492nm after 30mins	% Inhibition	Absorbance at 540nm after 40mins	% Inhibition
0.0000	0.6150	0.0000	0.7260	0.0000
1.0000	0.5550	9.7561	0.6350	12.5344
2.0000	0.5550	9.7561	0.6100	15.9780
5.0000	0.4680	23.9024	0.4610	36.5014
10.0000	0.2940	52.1951	0.2040	71.9008

Table A.2: The percent inhibition of DPPH by varying concentrations of ascorbic acid.

Ascorbic Acid (mg/ml)	Absorbance at 492nm after 30mins	% Inhibition	Absorbance at 540nm after 40mins	% Inhibition
0.0000	0.4780	0.0000	0.4200	0.0000
0.0176	0.5250	[illegible]6	0.4460	-6.1905
0.0352	0.4510	[illegible]5	0.4460	-6.1905
0.0880	0.3080	[illegible]9	0.3370	19.7619
0.1170	0.2410	[illegible]6	0.2800	33.3333
0.1760	0.2050	57.1130	0.2810	33.0952

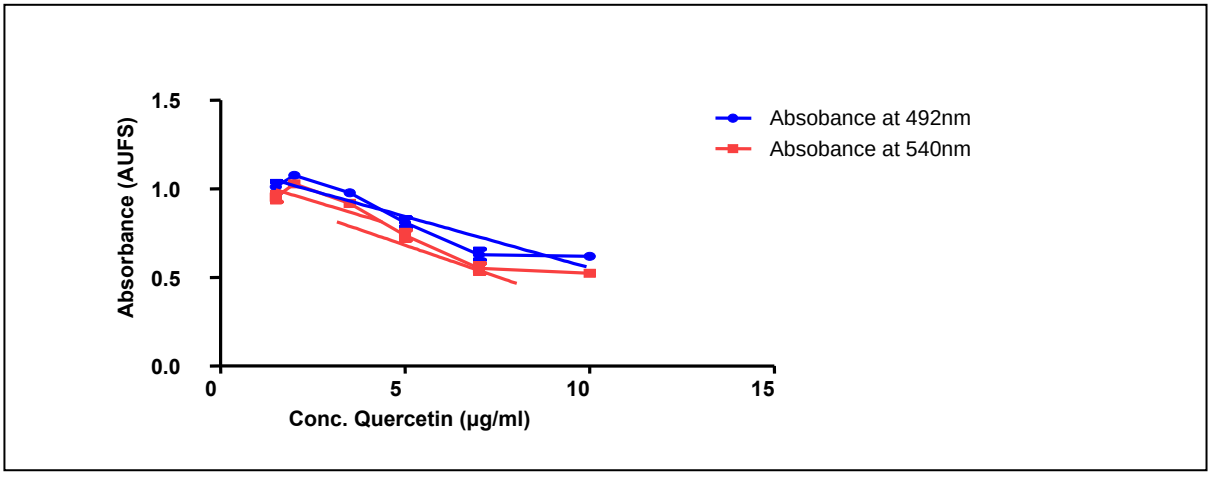

Figure A.1: Absorbance of DPPH after the addition of 200ul of various concentrations of quercetin.

Table A.3 **Percent inhibition of DPPH by SCP1 at varying concentrations**

Conc.	Normal	
(mg/ml)	%IC	Stdev.
0.00	0.00	0.00
2.50	16.96	4.51
5.00	38.21	9.93
7.50	53.85	8.57
10.00	63.83	9.09
15.00	77.29	6.03

Table A.4 **Percent inhibition of DPPH by SCP2 at varying concentrations**

Conc.	Normal	
(mg/ml)	%IC	Stdev.
0.00	0.00	0.00
5.00	21.16	1.76
7.50	23.20	4.34
10.00	37.13	23.29
12.00	26.40	13.54
18.00	58.65	1.16

Table A.5 **Percent inhibition of DPPH by SCP3 at varying concentrations**

Conc.	Normal	
(mg/ml)	%IC	Stdev.
0.00	0.00	0.00
1.00	8.96	5.00
2.50	25.22	6.03
5.00	50.12	6.01
10.00	76.59	2.22
20.00	77.34	1.84

Table A.6 **Percent inhibition of DPPH by SCP4 at varying concentrations**

Conc.	Normal	
(mg/ml)	%IC	Stdev.
0.00	0.00	0.00
1.00	17.31	15.55
2.50	31.40	1.76
5.00	58.95	4.52
10.00	72.22	16.55
12.50	68.78	6.78

Table A.7 **Percent inhibition of DPPH by SCP5 at varying concentrations**

Conc.	Normal	
(mg/ml)	%IC	Stdev.
0.00	0.00	0.00
0.20	25.53	6.87
0.50	39.90	8.54
1.00	61.94	8.32
1.20	68.24	8.05
1.50	75.37	4.67

Table A.8 **Percent inhibition of DPPH by SCP6 at varying concentrations**

Conc.	Normal	
(mg/ml)	%IC	Stdev.
0.00	0.00	0.00
1.00	9.80	4.25
1.50	13.55	4.24
2.00	22.45	7.51
4.00	36.31	10.05
6.00	44.85	4.77

Table A.9 **Percent inhibition of DPPH by SCP7 at varying concentrations**

Conc.		
(mg/ml)		ev.
0.00		0.00
0.50	10.16	9.27
1.00	21.27	10.49
2.00	40.11	10.09
4.00	64.51	12.53
6.00	77.01	4.06

Appendix B

TOTAL PHENOL CONTENT STANDARD

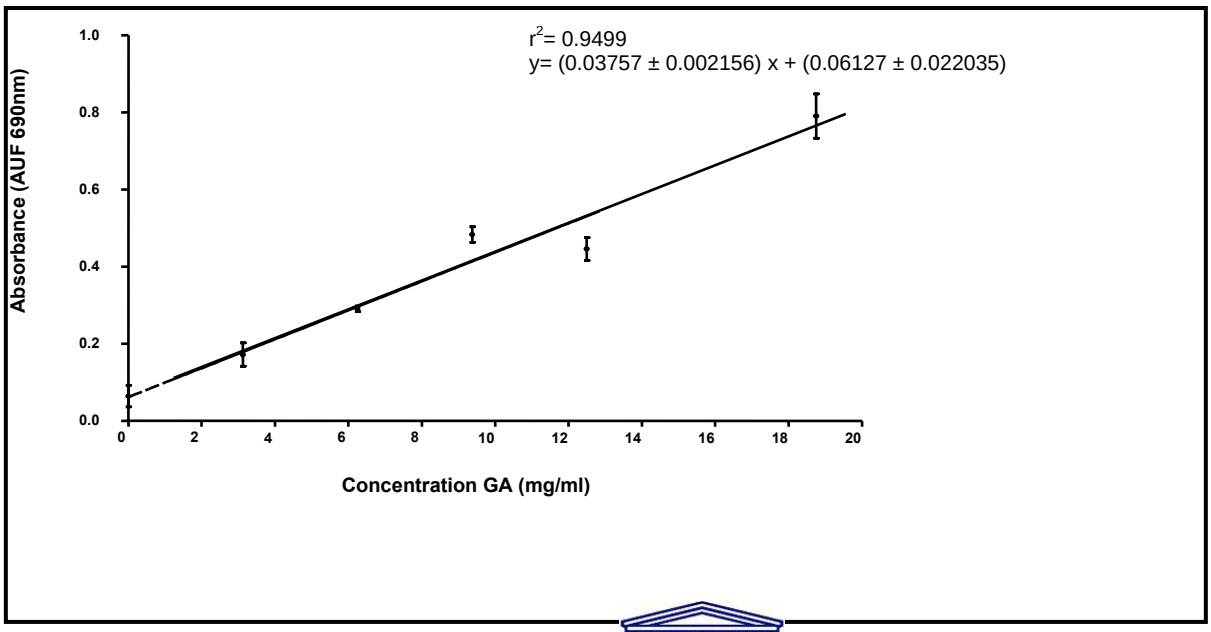

Figure B.1: Gallic acid concentration versus absorbance at 765nm

Appendix C
EFFECT OF AMBIENT AND ELEVATED TEMPERATURE AND HUMID ENVIRONMENTAL CONDITIONS ON THE ANTIOXIDANT ACTIVITY OF SCP AS DETERMINED BY DPPH AND FRAP ASSAYS

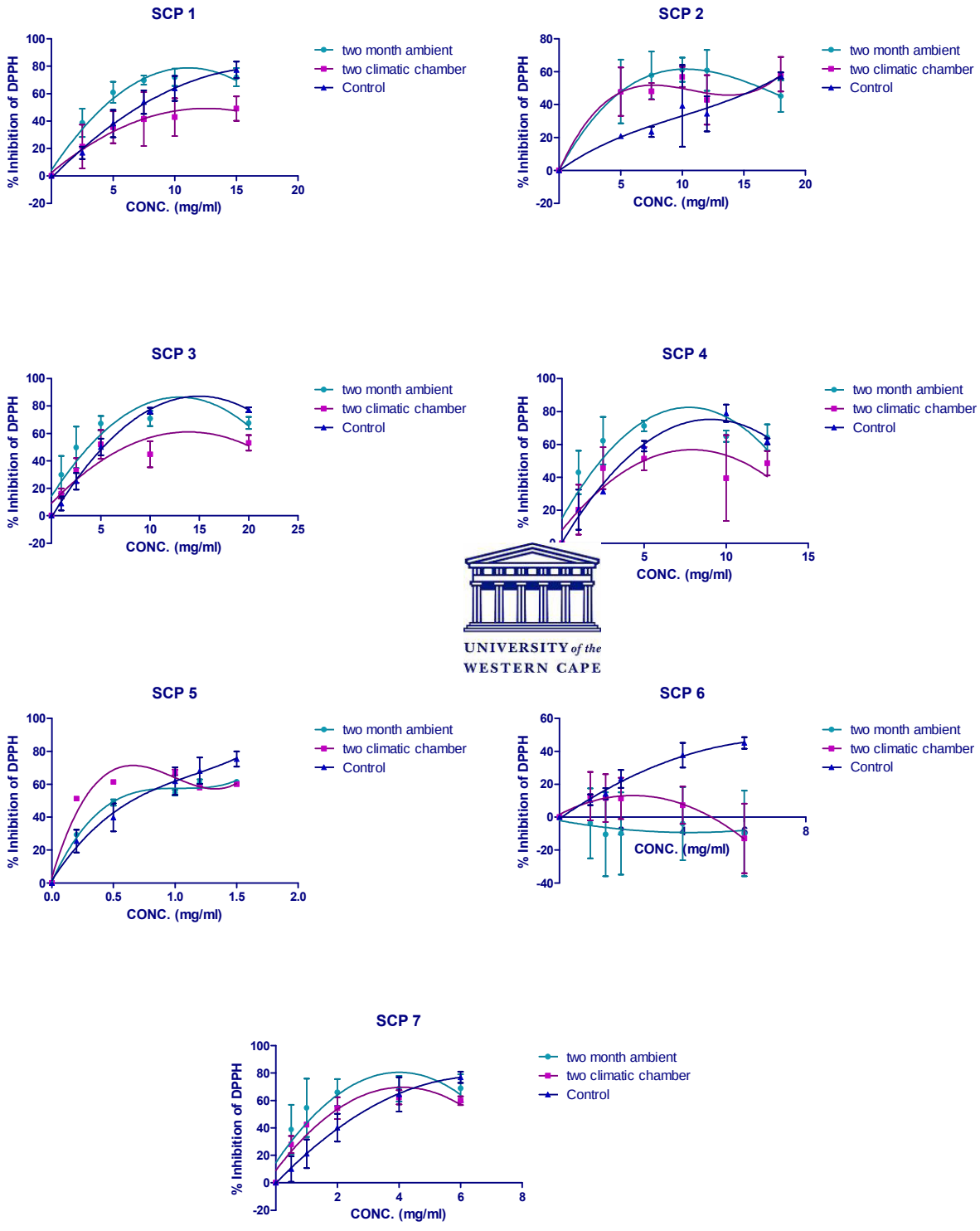

Figure C.1: The DPPH antioxidant activity of *Sutherlandia* containing products enduring the effect of elevated temperature and environmental humidity

Table C.1: Ferric reducing antioxidant power of stressed and unstressed *Sutherlandia* containing products

Product	Antioxidant activity (FRAP, ascorbic acid equivalence mg/ml))					
	Control	Ambient	Climatic chamber	STDEV	Average	RSD%
1 **Bio-*Sutherlandia*®**	1.133789	1.209806	1.403648803	0.139151	1.24908	11.14025
2 ***Sutherlandia* SU1®**	1.304827	1.042569	1.289623717	0.147222	1.21234	12.14363
3 **Health Wholefoods Connection *Sutherlandia*®**	1.456861	1.434055	1.624097301	0.103766	1.505	6.89472
4 **Pinnacle *Sutherlandia*®**	1.768529	1.72672	2.011782592	0.153938	1.83568	8.385885
5 **Promune®**	4.664766	4.630559	4.527936146	0.071209	4.60775	1.545409
6 ***Sutherlandia* Pro®**	1.464462	1.32003	995	0.544635	1.70391	31.96373
7 ***Sutherlandia* 350®**	2.654124	2.011783	1.947168377	0.390846	2.20436	17.7306

Appendix D

EFFECT OF AMBIENT AND ELEVATED TEMPERATURE AND HUMID ENVIRONMENTAL CONDITIONS ON THE TOTAL PHENOL CONTENT OF SCP

Table D.1: **Total phenol content of *Sutherlandia* containing products (SCP) after storage in original packaging in dark cupboard.**

Product	Abs 1	Abs 2	Abs 3	mg GAE/ml				
1mg/ml	690nm	690nm	690nm	1	2	3	Average	Stdev
1	0.174	0.231	0.248	24.986	3.001	4.518	4.162	12.279
2	0.233	0.282	0.256	51.603	4.571	5.875	5.209	0.652
3	0.320	0.253	0.220	78.220	6.887	5.103	5.404	0.955
4	0.341	0.397	0.379	104.837	7.446	8.936	8.279	0.747
5	0.540	0.650	0.564	131.454	12.742	15.670	13.930	1.473
6	0.279	0.278	0.271	158.071	5.795	5.769	5.715	0.041
7	0.272	0.278	0.385	184.688	5.609	5.769	6.637	0.553

Table D.2: **Total phenol content of *Sutherlandia* ducts (SCP) after storage for 2 months in ambient conditions**

Product	Abs 1	Abs 2	Abs 3	mg GAE/ml				
1mg/ml	690nm	690nm	690nm		2	3	Average	Stdev
1	0.277	0.277	0.261	5.742	5.316	5.050	5.369	0.349
2	0.481	0.481	0.452	11.172	10.400	11.891	11.153	0.745
3	0.356	0.356	0.332	7.845	7.206	6.381	7.143	0.734
4	0.715	0.715	0.662	17.400	15.990	16.415	16.600	0.724
5	0.968	0.968	1.014	24.134	25.359	21.845	23.777	1.783
6	0.500	0.500	0.502	11.678	11.731	11.278	11.561	0.247
7	0.477	0.477	0.497	11.065	11.598	10.613	11.091	0.493

Table D.3: **Total phenol content of *Sutherlandia* containing products (SCP) after storage for 2 weeks in elevated temperature and humidity conditions.**

Product	Abs 1	Abs 2	Abs 3	mg GAE/ml				
1mg/ml	690nm	690nm	690nm	1	2	3	Average	Stdev
1	0.155	0.154	0.159	2.495	2.468	2.601	2.521	0.070
2	0.182	0.187	0.191	3.213	3.347	3.453	3.337	0.120
3	0.210	0.214	0.217	3.959	4.065	4.145	4.056	0.093
4	0.241	0.240	0.249	4.784	4.757	4.997	4.845	0.131
5	0.359	0.464	0.447	7.925	10.719	10.267	9.636	1.500
6	0.304	0.297	0.310	6.461	6.274	6.620	6.451	0.173
7	0.243	0.261	0.256	4.837	5.316	5.183	5.112	0.247

Appendix E

CHROMATOGRAPHIC FINGERPRINTS AND SPECTRAL AND PEAK PURITY PROFILES OF COLLECTED FRACTIONS OF SCP

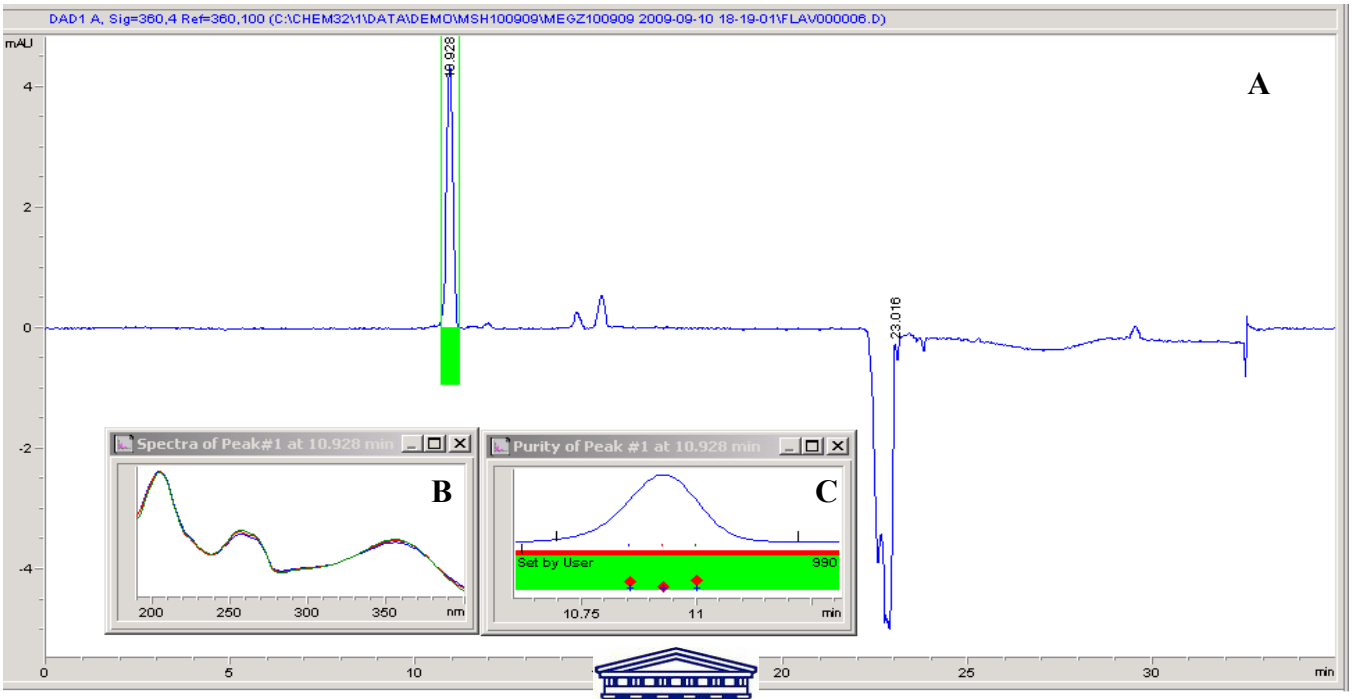

Figure E.1: HPLC chromatogram (A), and spectra[illegible]B) and purity (C) profiles of collected fraction 1, with peak at retention time of 10.92[illegible]

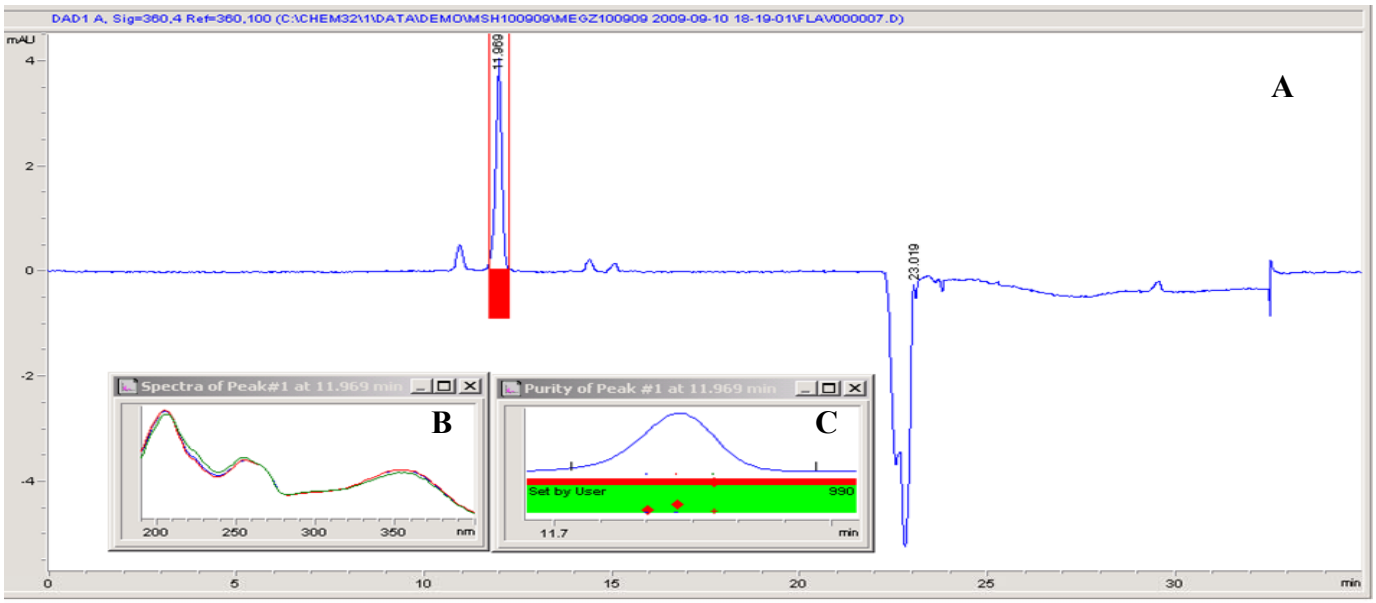

Figure E.2: HPLC chromatogram (A), and spectral absorbance (B) and purity (C) profiles of collected fraction 2, with peak at retention time of 11.969 minutes.

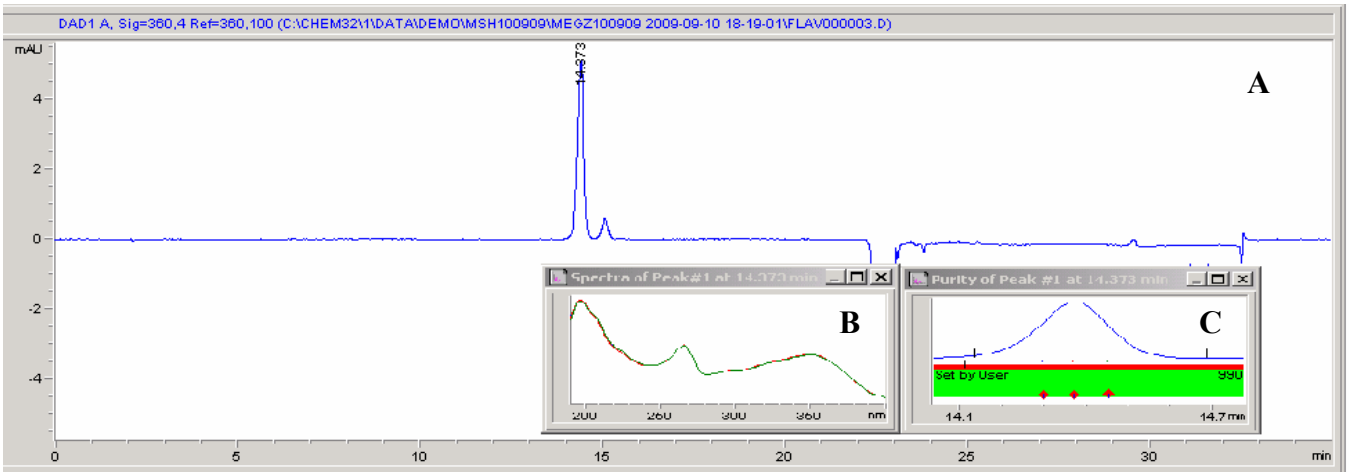

Figure E.3: **HPLC chromatogram (A), and spectral absorbance (B) and purity (C) profiles of collected fraction 3, with peak at retention time of 14.373 minutes.**

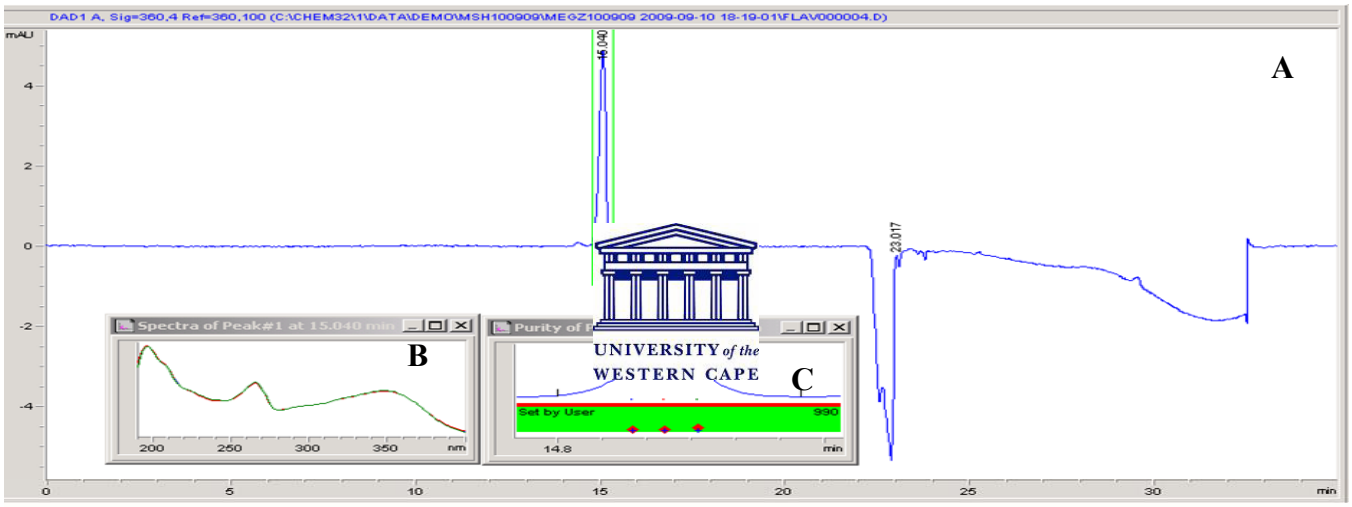

Figure E.4: **HPLC chromatogram (A), and spectral absorbance (B) and purity (C) profiles of collected fraction 4, with peak at retention time of 15.040 minutes.**

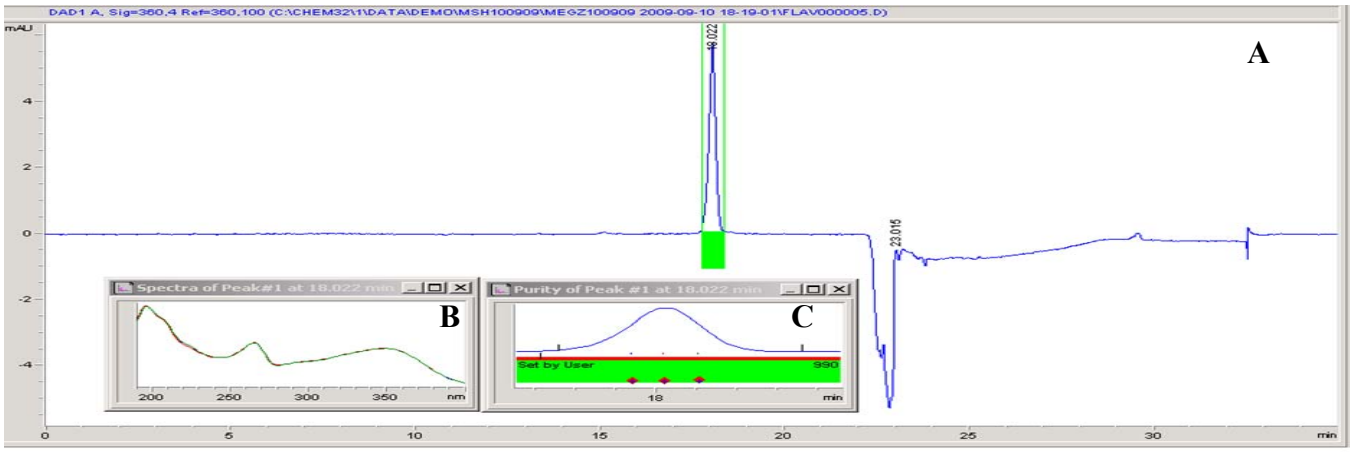

Figure E.5: **HPLC chromatogram (A), and spectral absorbance (B) and purity (C) profiles of collected fraction 5, with peak at retention time of 18.022 minutes.**

Appendix F

CHROMATOGRAPHIC FINGERPRINTS AND LCMS DATA OF HPLC COLLECTED FRACTIONS

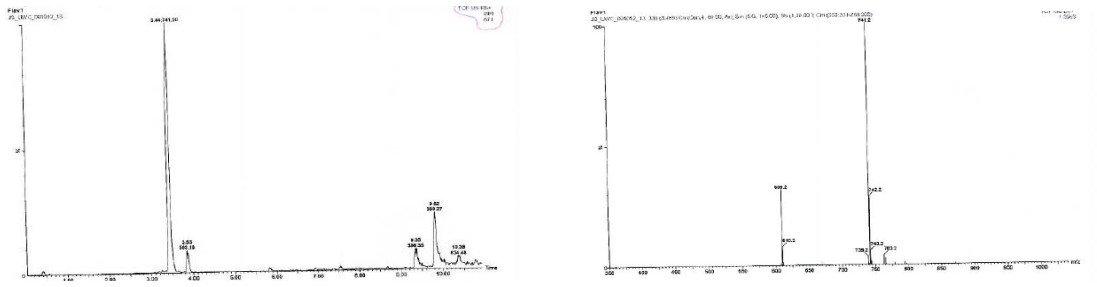

Figure F.1and F.2: Chromatographic fingerprint and LCMS data of fraction 1 (retention time = 10.928min) collected by HPLC (see fig. E.1).

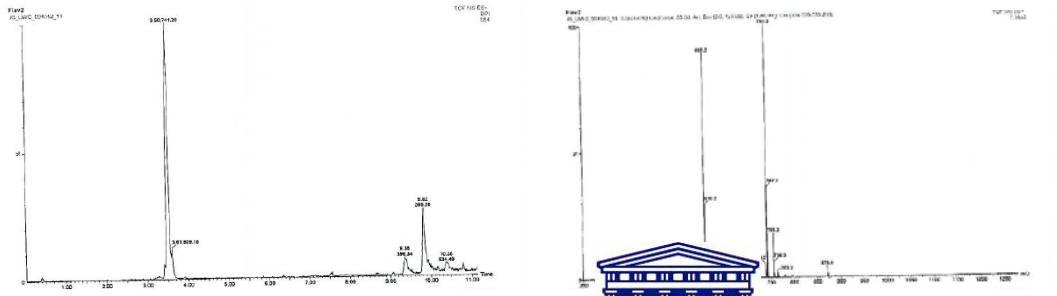

Figure F.3 and F.4: Chromatographic fingerprint and LCMS data of fraction 1 (retention time = 11.969min) collected by HPLC (see fig. E.2).

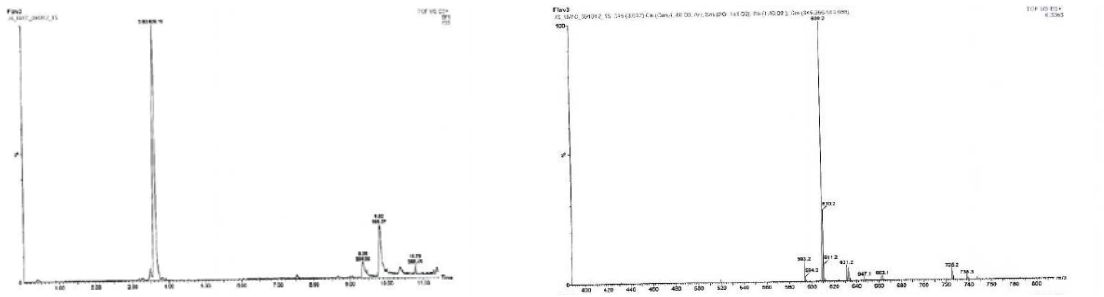

Figure F.5 and F.6: Chromatographic fingerprint and LCMS data of fraction 1 (retention time = 14.373min) collected by HPLC (see fig. E.3).

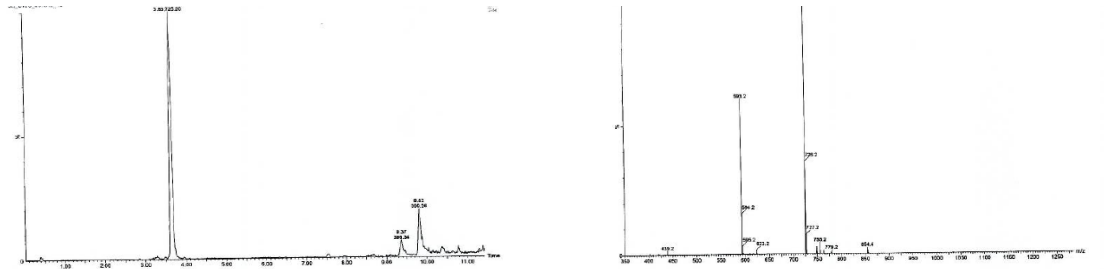

Figure F.7 and F.8: Chromatographic fingerprint and LCMS data of fraction 1 (retention time = 15.040min) collected by HPLC (see fig. E.4).

Appendix G

CHROMATOGRAPHIC FINGERPRINTS OF AMBIENT STORED SCP

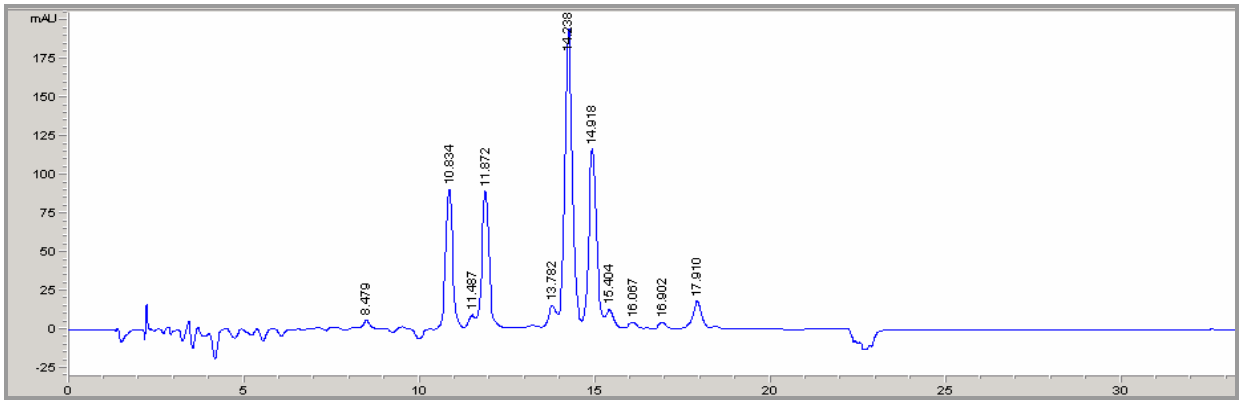

Figure G.1: **Chromatogram of ambient stressed *Sutherlandia* containing product 1.**

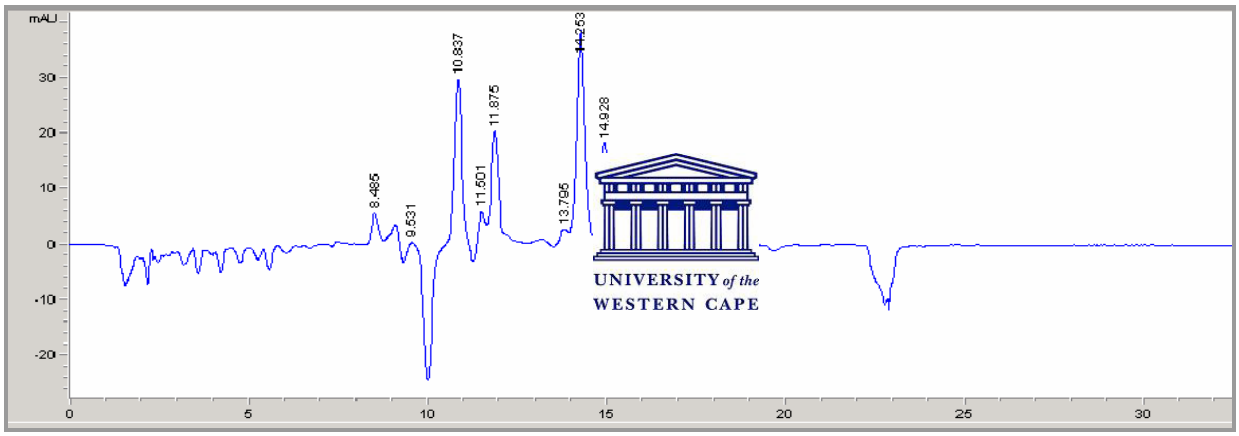

Figure G.2: **Chromatogram of ambient stressed *Sutherlandia* containing product 2.**

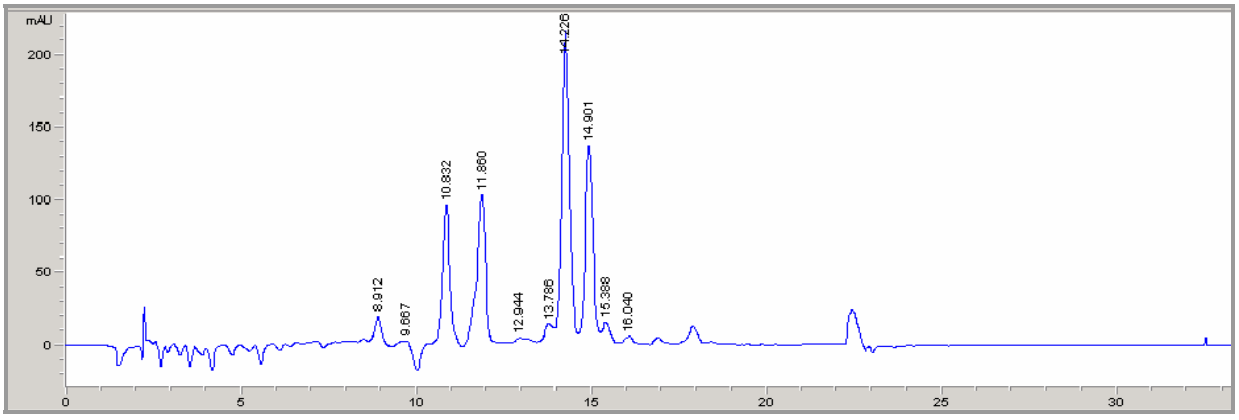

Figure G.3: **Chromatogram of ambient stressed *Sutherlandia* containing product 3.**

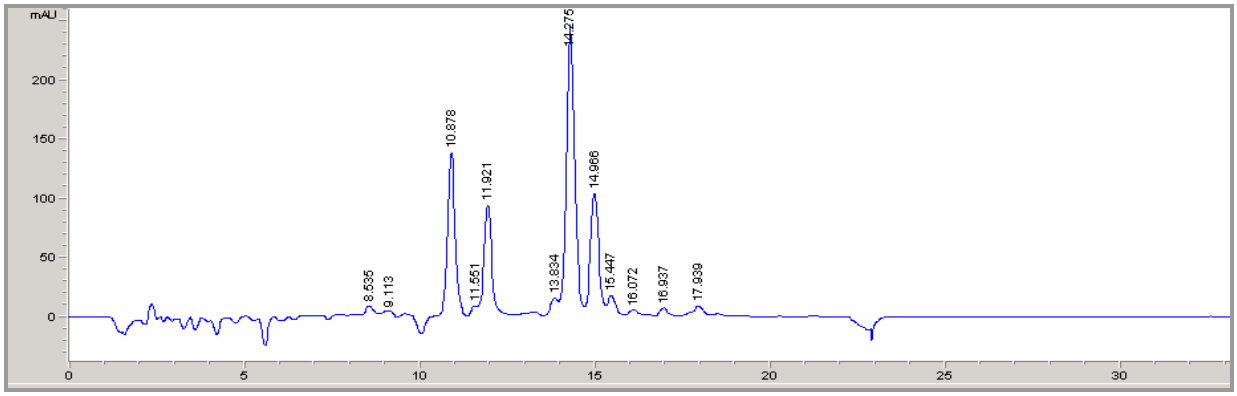

Figure G.4: **Chromatogram of ambient stressed *Sutherlandia* containing product 4.**

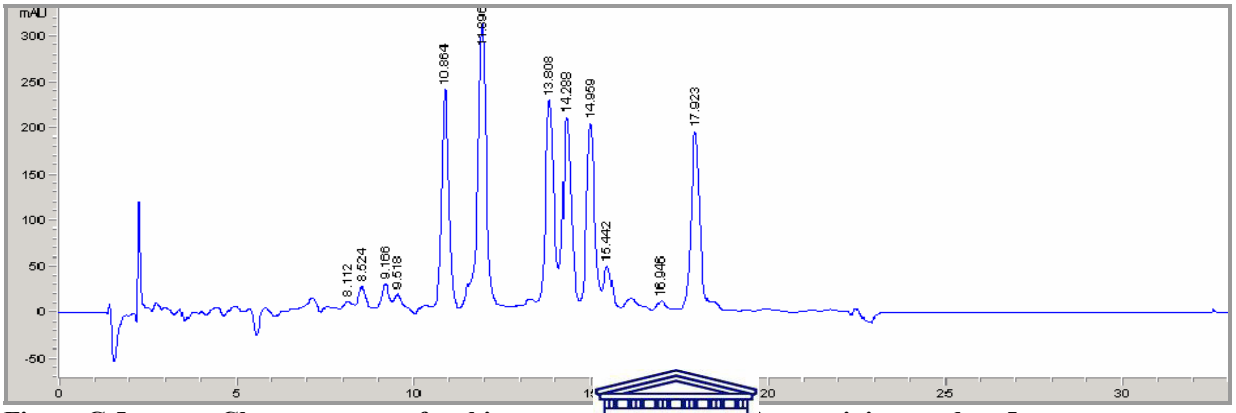

Figure G.5: **Chromatogram of ambient stress a containing product 5.**

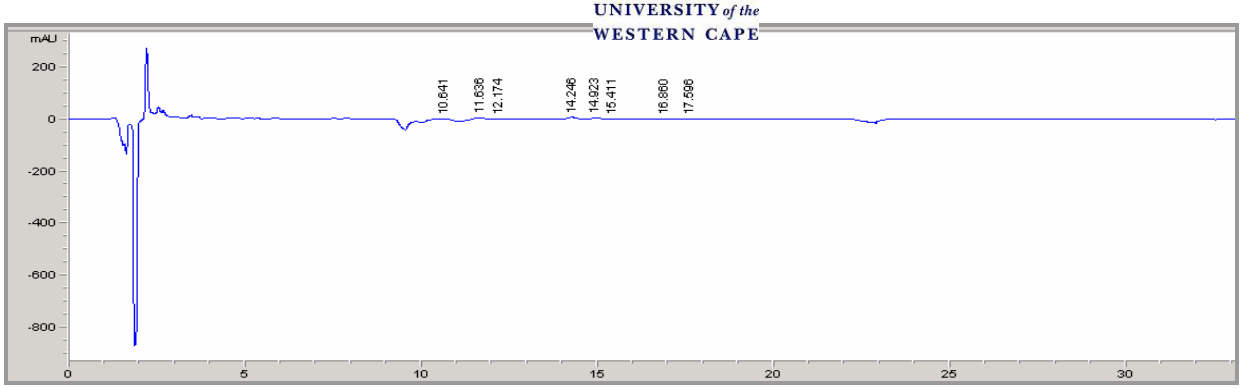

Figure G.6: **Chromatogram of ambient stressed *Sutherlandia* containing product 6.**

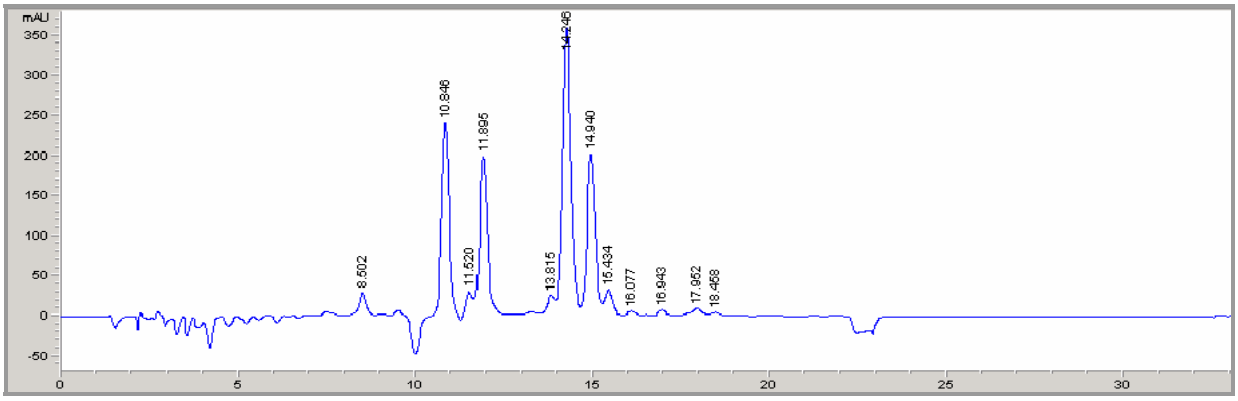

Figure G.7: **Chromatogram of ambient stressed *Sutherlandia* containing product 7.**

Appendix H

CHROMATOGRAPHIC FINGERPRINTS OF CLIMATIC CHAMBER STORED SCP

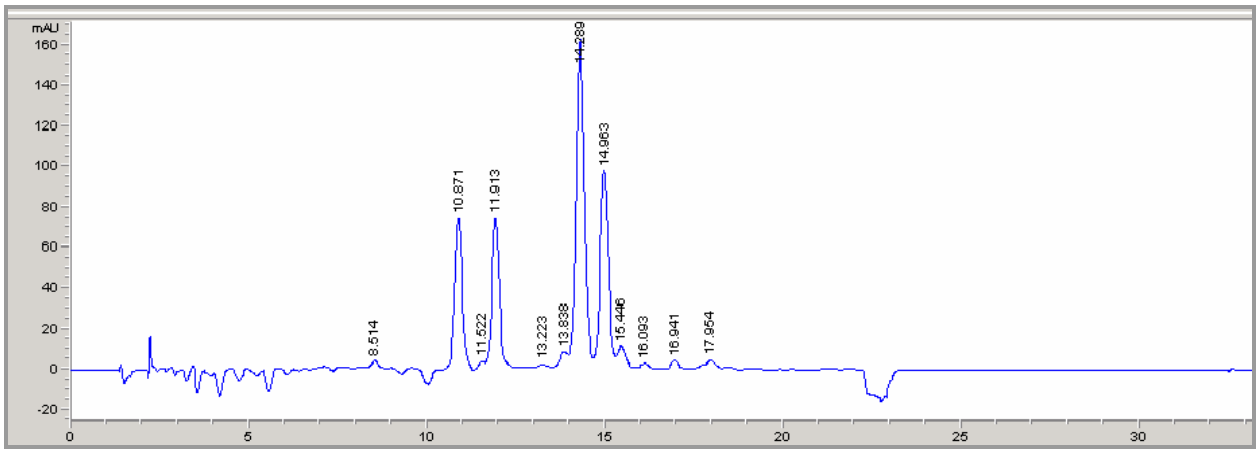

Figure H.1: **Chromatogram of climatic chamber stressed *Sutherlandia* containing product 1.**

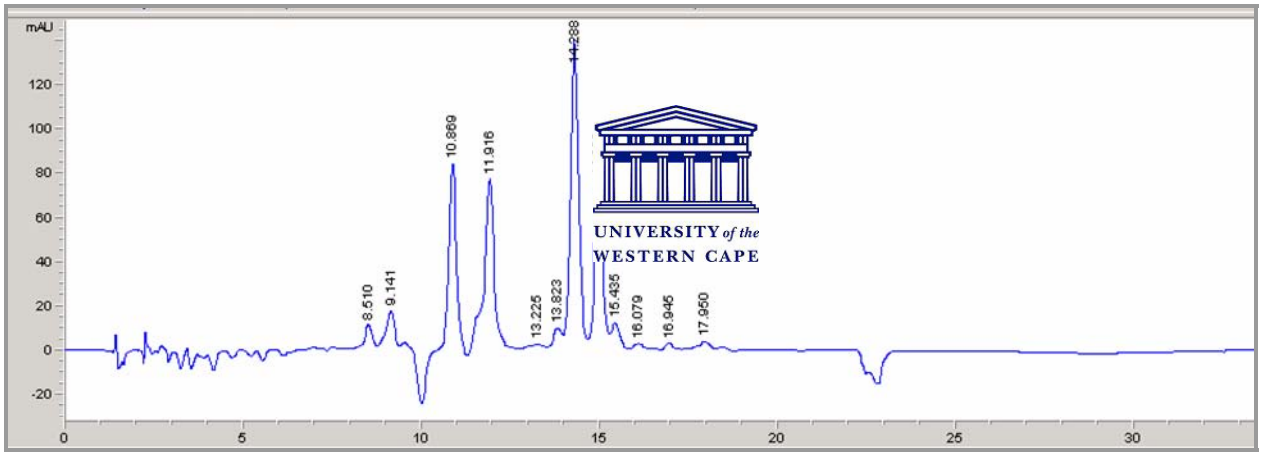

Figure H.2: **Chromatogram of climatic chamber stressed *Sutherlandia* containing product 2.**

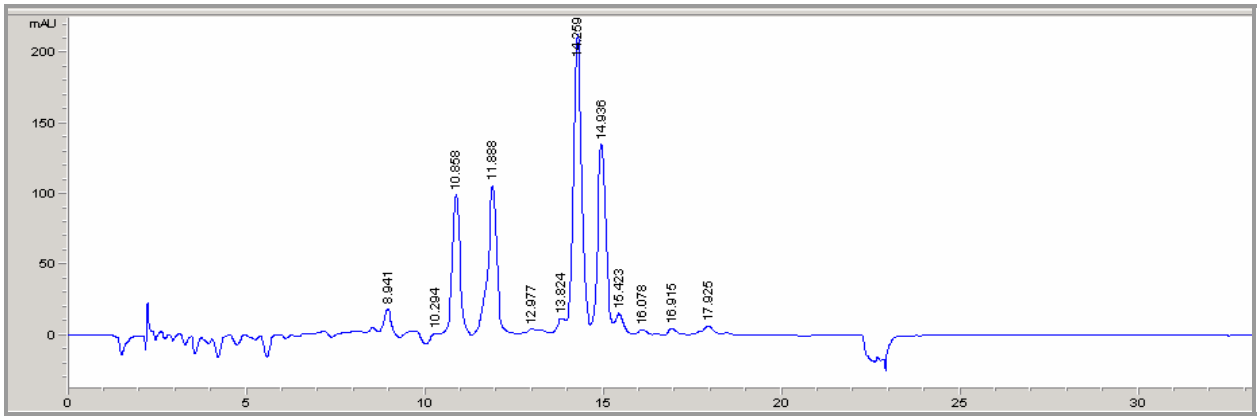

Figure H.3: **Chromatogram of climatic chamber stressed *Sutherlandia* containing product 3.**

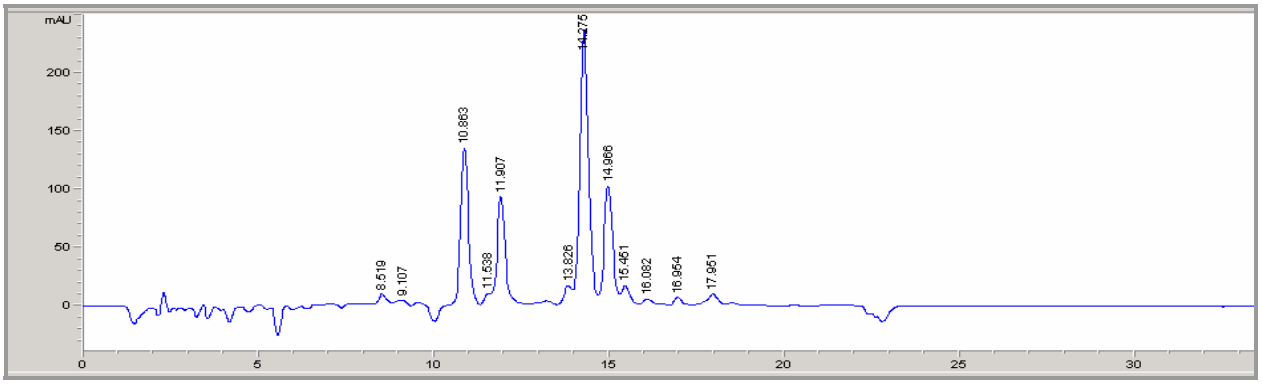

Figure H.4: Chromatogram of climatic chamber stressed *Sutherlandia* containing product 4.

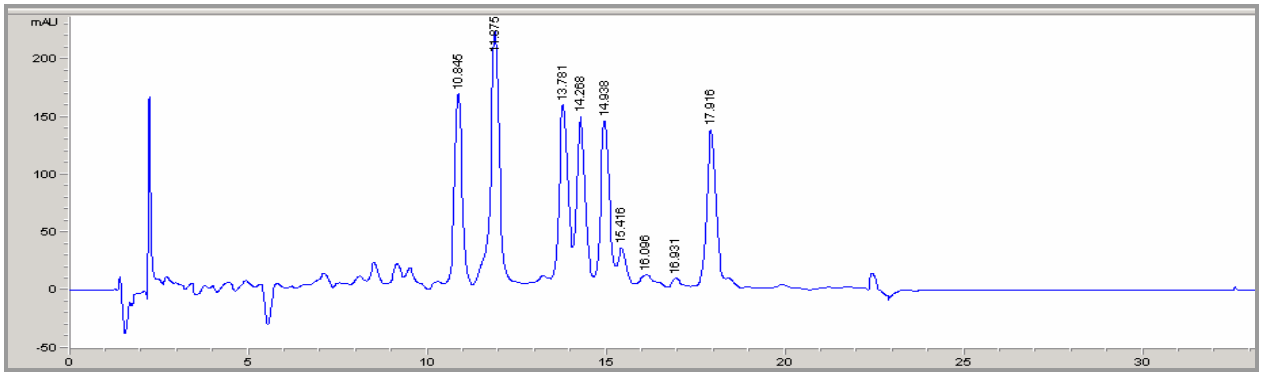

Figure H.5: Chromatogram of climatic chamber stressed *Sutherlandia* containing product 5.

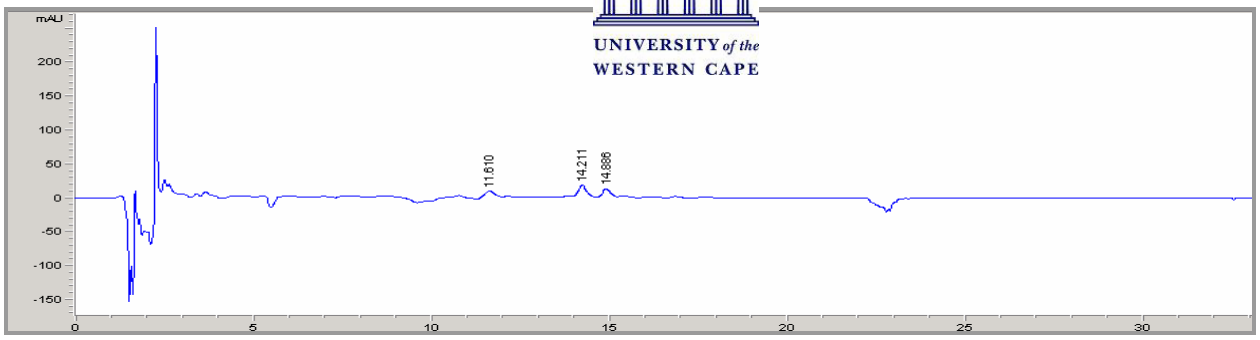

Figure H.6: Chromatogram of climatic chamber stressed *Sutherlandia* containing product 6.

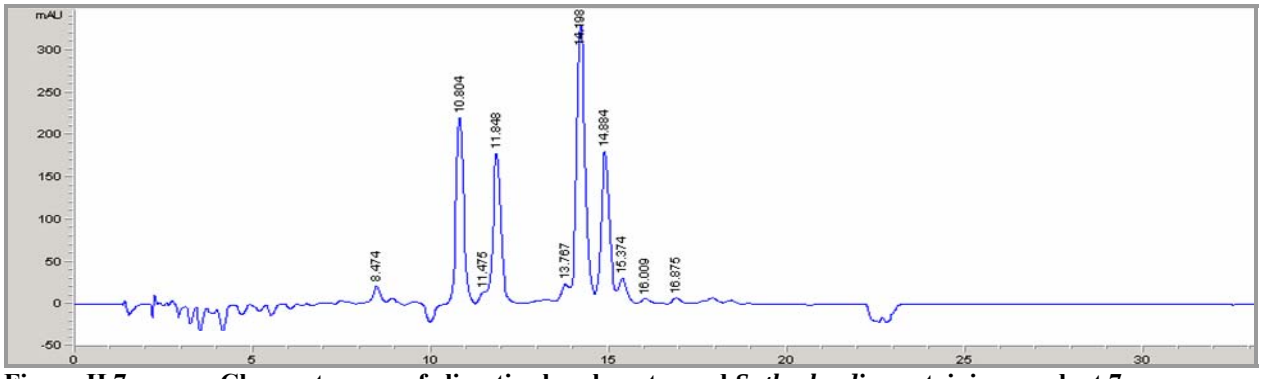

Figure H.7: Chromatogram of climatic chamber stressed *Sutherlandia* containing product 7.

Appendix I

TABLE OF PEAK HEIGHT VALUES AFTER DIFFERENT ENVIRONMENTAL STORAGE CONDITIONS

Table I.1: Difference in peak heights of *Sutherlandia* solid dose products' sutherlandins after a period of storage in elevated temperature and environmental humidity.

Product	Sutherlandin A		Sutherlandin B		Sutherlandin C		Sutherlandin D	
	Ambient	Climatic chamber	Ambient	Climatic chamber	Ambient	Climatic chamber	Ambient	Climatic chamber
1 **Bio-*Sutherlandia*®**	94.4	78.8	90	75.4	193.8	162.1	116	97.2
2 ***Sutherlandia* SU1®**	45.7	100	25.9	82.6	39	140.3	18.5	75.5
3 **Health Wholefoods Connection *Sutherlandia*®**	107.4	102.9	106.2	106.5	215.7	212.4	136.1	134.6
4 **Pinnacle *Sutherlandia*®**	147.8	143.8			245.9	236.1	109.5	102
5 **Promune®**	266.5	194.6	335.5	247.5	226.3	170.2	217.1	165.8
6 ***Sutherlandia* Pro®**	27.1	-	19.7	12	7.8	17.7	6.4	12.5
7 ***Sutherlandia* 350®**	272.4	234.1	211	182.4	358.5	329	201.4	179.6
P values	0.0136	0.0072	0.0283	0.0190	0.0309	0.0230	0.0418	0.0240