THE IMPACT OF STORAGE TIME AND SEASONAL HARVESTING ON BIOMARKER LEVELS OF *LESSERTIA FRUTESCENS*

James Campbell

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

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Supervisor:

Prof W Mabusela

Co-supervisor:

Prof R Henkel

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ABSTRACT

THE IMPACT OF STORAGE TIME AND SEASONAL HARVESTING ON BIOMARKER LEVELS OF *LESSERTIA FRUTESCENS*

In South Africa, it is estimated that approximately 70% of the population frequently make use of traditional medicinal plants for their health care needs. The use of *Lessertia frutescens* by the various cultural groups in South Africa dates back to the earlier civilizations and continues to be used today to treat a multitude of ailments. To get the best results from a medicinal plant, one would need to ensure that the crude material is of good quality through interventions like being properly grown, well dried and correctly processed. This would add a measure of quality assurance, which will contribute towards the safety and efficacy aspect of herbal medicine.

The aim of this study was to investigate what impact a particular season of harvest and the time in storage would have on the flavonoid and triterpenoid marker levels of *Lessertia frutescens*. To achieve this, the following was investigated: (1) storage variation of *Lessertia frutescens* leaves by comparing the results obtained from the High Performance Liquid Chromatography (HPLC) analysis of the flavonoids and triterpenoids, (2) seasonal variation of *Lessertia frutescens* leaves by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids, (3) leaf and stem variation of *Lessertia frutescens* by comparing the results obtained from HPLC analysis of the flavonoids and triterpenoids. The hypotheses were: (1) the stored sample would indicate the same level of the biomarkers for the flavonoids and triterpenoids, as that of the freshly prepared sample, (2) the sample that was harvested during the summer season would indicate higher levels of the biomarkers of flavonoids and triterpenoids than the other three seasons, (3) the leaf sample would indicate the same level of the biomarkers for the flavonoids and triterpenoids, as that of the stem sample.

An Agilent 1200 series HPLC was used for the determination of the flavonoids sutherlandin A and sutherlandin D as well as the triterpenoids sutherlandioside B and sutherlandioside D. Results show that for both sutherlandin A (summer: 3.67 ± 2.88 mg/ml; storage:

4.07 ± 2.88 mg/ml) and D (summer: 4.10 ± 1.06 mg/ml; storage: 4.25 ± 1.06 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the storage samples. For both sutherlandioside B (summer: 3.01 ± 0.39 mg/ml; storage: 2.82 ± 0.39 mg/ml) and D (summer: 5.82 ± 0.42 mg/ml; storage: 4.66 ± 0.42 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the fresh summer samples.

For the seasonal comparison, results show that for sutherlandin A (summer: 3.67 ± 12.49 mg/ml; autumn: 4.75 ± 12.49 mg/ml; winter: 4.23 ± 12.49 mg/ml; spring: 6.56 ± 12.49 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the spring sample. For sutherlandin D (summer: 4.10 ± 10.32 mg/ml; autumn: 6.37 ± 10.32 mg/ml; winter: 5.25 ± 10.32 mg/ml; spring; 6.08 ± 10.32 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the autumn sample. For both sutherlandioside B (summer: 3.01 ± 7.19 mg/ml; autumn: 2.15 ± 7.19 mg/ml; winter: 2.89 ± 7.19 mg/ml; spring: 1.47 ± 7.19 mg/ml) and D (summer: 5.82 ± 14.48 mg/ml; autumn: 3.33 ± 14.48 mg/ml; winter: 4.23 ± 14.48 mg/ml; spring: 2.50 ± 14.48 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the autumn sample.

For the summer leaf/stem comparison, results show that for sutherlandin A (leaf: 3.67 ± 8.18 mg/ml; stem: 4.67 ± 8.18 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the stem sample. For the sutherlandin D (leaf: 4.10 ± 4.81 mg/ml; stem: 3.31 ± 4.81 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the summer leaf sample. For both the sutherlandioside B (leaf: 3.01 ± 4.24 mg/ml; stem: 3.62 ± 4.24 mg/ml) and D (leaf: 5.82 ± 0.42 mg/ml; stem: 5.80 ± 0.42 mg/ml) show significantly ($P < 0.0001$) higher concentrations in the case of the stem samples.

Results demonstrate that the production of secondary metabolites are influenced by environmental factors like seasonal harvesting, as indicated by the variation in the chemical constituent composition of *Lessertia frutescens* depending on the season collected in. Moreover, the storage of *Lessertia frutescens* for a period of one year resulted in an increase of two of the four constituents being monitored. There was slight variations in the chemical constituents, depending on whether the leaf or stem material of *Lessertia frutescens* was being used. Finally, the type of chemical constituent being monitored was also important in the consideration of this study. Therefore, this study can be seen as a starting point to further investigations of these aspects, which are of clinical, pharmacological and economic importance.

DECLARATION

I declare that *The impact of storage time and seasonal harvesting on biomarker levels of Lessertia frutescens* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all of the sources I have used or quoted have been indicated and acknowledged as complete references.

James Campbell   November 2012

Signed:

DEDICATION

This is dedicated to my wife Janine and daughter Jesse for their love, support and understanding. It is also dedicated to my parents Arthur and Betty Campbell, Valerie Smorenburg and Connie Meyer.

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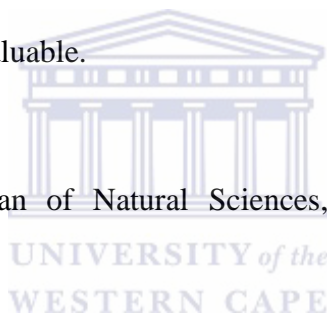
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Prof Gail Hughes for her assistance in securing financial support through the South African Herbal Science and Medicine Institute (SAHSMI).

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CHAPTER 1

INTRODUCTION

1.1 General information

Since the beginning of time, the human species has relied on plant-based materials for the physical survival, health and wellbeing of the species (Chevallier, 1996; Bown, 2002; Springfield *et al*., 2005). It has been over thousands of years that this reliance, on plant-based materials as a source of food, medicine, fuel, shelter and clothing, has led to the human species acquiring an evolved knowledge of the plant world surrounding them (Bown, 2002). Not only were plants used as food for sustenance, but also for the medicinal benefits that certain of them offered (Chevallier, 1996). According to Emboden (1997), during humankind's initial contact with plants, humans would have discovered that some of the plants were good for food, some that were poisonous and others that had mind altering effects, resulting in the relief of pain or disease. This knowledge was handed down from generation to generation laying the foundations to various traditional based medicine systems throughout the world (Vogel, 1991).

Due to growth in international trade, information about traditional medicinal plants, previously only available to the region's cultural groups, started becoming

more sought after throughout the world, leading to an increase in supply and demand (Van Staden, 1999; Joshi *et al*., 2004; Singh, 2008). It is estimated that the world market for medicinal plants has grown annually at a rate of between 5% and 15%, and still continues to grow (Joshi *et al*., 2004). The report from the World Health Organization (1998a) estimates that 80% of the people in the developing nations make use of traditional medicinal plants for their health care needs. While according to Bown (2002), it is estimated that about 80% of the world's medicinal plants are still being harvested from the wild, causing concerns as many of the species are becoming rare due to over collection, slash and burn techniques, land clearance and other environmental issues. In order to meet these demands, one would need to consider where future supplies would come from and therefore put in place measures to establish that what plant material consumers are purchasing, is really what it is being sold as (Bown, 2002).
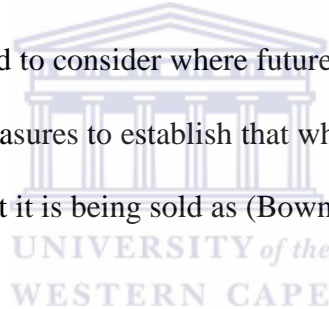
1.2 Problem: setting and background

South Africa has a rich biodiversity of over 22 000 higher plant species, of which 9 000 are indigenous, and represent 10% of the total of the world's plant species, occupying less than 1% of the earth's space (Goldblatt and Manning, 2000). It is estimated that over 3000 indigenous plants are used and traded as traditional medicinal plants throughout Southern Africa, with 350 species being frequently used (Van Wyk *et al*., 1997; Van Wyk and Gericke, 2000). Nevertheless, very little research has been carried out to verify the medicinal plants quality, safety and efficacy (Fennell *et al*., 2004a; Springfield *et al*., 2005; Johnson *et al*., 2007).

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In South Africa, it is estimated that approximately 70% of the population frequently make use of traditional medicinal plants for their health care needs (Springfield *et al*., 2005). Mander and Le Breton (2006) ascribe the use of traditional medicinal plants by such a large number of the population, to factors such as fairly easy availability of the plants, the relatively cheap price, that the traditional based knowledge concerning the use of plants as medicines is good, the lack of access to allopathic medicinal services and cultural pride.

The use of *Lessertia frutescens* by the various cultural groups in South Africa dates back to the earlier civilizations (Van Wyk and Albrecht, 2008). It continues to be used to treat a multitude of ailments affecting the integumentary, respiratory, cardiovascular, gastrointestinal, nervous, urinary, musculoskeletal, endocrine and immune systems (Van Wyk and Wink, 2004; Van Wyk, 2006; Van Wyk and Albrecht, 2008). Traditionally, the leaves and stems of *Lessertia frutescens* were considered the parts to be used for their medicinal benefits. Research verified that the leaves and stems do indeed yield the biologically active constituents, namely the secondary metabolites, which contribute to its medicinal properties (Moshe, 1998; Van Wyk and Albrecht, 2008). *Lessertia frutescens* contains the amino acids alanine, arginine, aspartic acid, asparagine, leucine, phenylanine, proline and tryptophan (Tai *et al*., 2004). Moshe (1998) indicated the presence of elevated levels of protein-bound amino acids and free amino acids. The non-protein amino acid L-canavanine, as well as pinitol and γ-aminobutyric acid (GABA) were isolated from *Lessertia frutescens* (Moshe, 1998).

More recently, four cycloartanol glycosides were isolated from the leaves of *Lessertia frutescens* namely; sutherlandiosides A, sutherlandiosides B, sutherlandiosides C and sutherlandiosides D (Fu *et al*., 2008). In addition to the discovery of the four cycloartanol glycosides, another study led to the isolation of four flavonoid glycosides, also from the leaf material of *Lessertia frutescens,* namely; sutherlandin A, sutherlandin B, sutherlandin C and sutherlandin D (Fu *et al*., 2010). Avula *et al*. (2010) developed an analytical method of determination of these four cycloartanol glycosides and four flavonoid glycosides.

In South Africa in the past, and still applicable in the present, many of the traditional medicinal plants were harvested from the wild. This procedure, however, is not sustainable in the current economic climate where the increased demand for medicinal plants serve not only a local, but international market as well (Van Staden, 1999; Street *et al*., 2008). Small scale farming of traditional medicinal plant crops may be a way of addressing the growing needs (Van Staden, 1999). Van Wyk and Albrecht (2008) reported that cultivation and commercialization of *Lessertia frutescens* was started in 1990.

In order to produce herbal medicines of quality, it is important to be able to correctly identify the crude material, thus adding a measure of quality assurance, which will contribute towards the safety and efficacy aspect of the herbal medicine (Joshi *et al*., 2004; Springfield *et al*., 2005). To get the best results from a medicinal plant one would need to ensure that the crude material is of good

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quality through interventions such as being properly grown, well dried and correctly processed (Chevallier, 1996; Fennell *et al*., 2004a; Street *et al*., 2008). Through the use of good agricultural methods that are well understood by those countries that have been growing food crops for many years, one could ensure quality, safety and efficacy of medicinal plant material (Fennell *et al*., 2004a). Fingerprinting could add further validation to the process by indicating the chemical profile that represents the complex makeup of chemical constituents that occur in each species. This could be used as a marker for quality control (Springfield *et al*., 2005).

Yanivie and Palevitch (1982), and Bopana and Saxena (2007) indicated that besides the genetic involvement, environmental factors also influenced the production of secondary metabolites. According to McGimpsey *et al*. (1994) and Badi *et al*. (2003) the time of harvest is one of the most important factors that influence the quality and quantity of secondary metabolites. Furthermore, Fennell *et al*. (2004a) have reported that despite its importance, storage of medicinal plant material and its effect on the constituents is very poorly researched.

1.3 *Lessertia frutescens* – an indigenous medicinal plant

In Southern Africa, predominantly in the drier parts of South Eastern Botswana, Southern Namibia, Lesotho and particularly in South Africa, *Lessertia frutescens* can be found growing naturally throughout these regions (Van Wyk *et al*., 1997; Van Wyk and Albrecht, 2008). The geographical diversity of *Lessertia frutescens*

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and its use by various cultural groups living within these geographical locations has resulted in numerous vernacular names for the medicinal plant (Van Wyk and Albrecht, 2008). *Lessertia frutescens* is a traditional medicinal plant commonly referred to as cancer bush, duck plant, sutherlandia, ballon-pea, turkey flower (English); kankerbos, wildekeur, rooikeurtjie, kalkoenbos, belbos, keurtjie, gansies, blaasbossie, blaas-ertjie (Afrikaans); unwele (Zulu and Xhosa); insiswa (Zulu); musa-pelo, motlepelo, phethola (Sesotho and Setswana); lerumo-lamadi (North Sesotho), throughout South Africa (Van Wyk *et al*., 1997; Xaba and Notten, 2003; Powrie, 2004; Van Wyk and Albrecht, 2008).

Lessertia frutescens is a shrublet (Figure 1 A), with greyish-green leaflets (Figure 1 B), that can grow up to one metre in height (Van Wyk *et al*., 1997; Van Wyk and Gericke, 2000; Xaba and Notten, 2003; Manning, 2007).
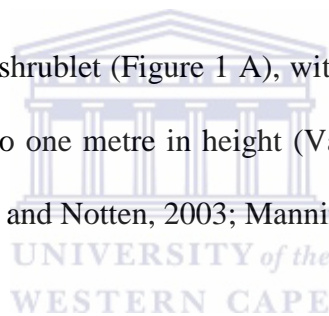
Figure 1: A: *Lessertia frutescens* whole plant (Van Wyk and Albrecht, 2008).

B: *Lessertia frutescens* leaves (Van Wyk and Albrecht, 2008).

Van Wyk (2006) pointed out the complexity of the *Lessertia* species in that it has many regional forms and chemotypes. Originally, the classification (Table 1) of the genus *Sutherlandia* was considered to be divided into six species as per the taxonomy described by Phillips and Dyer (1934). The six species were named; *Sutherlandia frutescens*, *Sutherlandia microphylla*, *Sutherlandia montana*, *Sutherlandia humilis*, *Sutherlandia speciosa* and *Sutherlandia tormenlosa* (Philips and Dyer, 1934). However, according to research done by Moshe (1998) there is only evidence to suggest two distinct species namely, *Sutherlandia frutescens* and *Sutherlandia tormenlosa*, while the others are considered to be subspecies of the two. In 2000, Goldblatt and Manning proposed that the genus *Sutherlandia* be relocated under the genus *Lessertia*. Although acknowledging that there may be merit in the reclassification, Van Wyk and Albrecht (2008) do question whether the inclusion of *Sutherlandia* into the *Lessertia* genus can truly be demonstrated from a morphological or genetic point of view. The relocation resulted in the need to rename *Sutherlandia tormentosa* to *Lessertia canescens* (Van Wyk and Albrecht, 2008). *Sutherlandia frutescens* became *Lessertia frutescens*, with reference being made to both names, indicating the same plant (Van Wyk., 2006; Van Wyk and Albrecht, 2008).

Table 1: Taxonomic classification of *Lessertia frutescens.*

Taxonomic categories	
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnohopsida
Order	Fabels
Family	Fabaceae (alt. Leguminosae)
Genus	*Lessertia*
Species	*L. frutescens*

1.4 Botanical description

Lessertia frutescens is a woody perennial shrublet that can grow up to one metre in height (Van Wyk *et al.*, 1997; Van Wyk and Gericke, 2000; Xaba and Notten, 2003; Manning, 2007). Yet, according to Van Wyk and Albrecht (2008) *Lessertia frutescens* is a short-lived shrub that can grow between 0.2 to 2.5 metres in height. Some of its stems may grow along the ground, while others grow upright. The shrub has compound petiolate, stipulate and pinnate leaves which are oval to oblong in shape, being longer than they are wide (Manning, 2007; Van Wyk and Albrecht, 2008). The appearance of the leaflets vary in that they may range from hairless to slightly or completely hairy and have a slightly silvery/grayish green colour (Van Wyk *et al.*, 1997; Van Wyk *et al.*, 2000; Van Wyk *et al.*, 2004; Manning, 2007).

Red-orange coloured flowers (Figure 2), between 25-35mm in length, are produced during the spring to midsummer months of September, October,

November and December (Van Wyk *et al*., 1997; Van Wyk *et al*., 2000; van Wyk *et al*., 2004; Manning, 2007). These flowers, after being pollinated, develop into large puffed up pods (Figure 2), papery and hairless in nature, wherein tiny black seeds develop (Van Wyk and Albrecht, 2008). Figure 3 shows a commercial plantation growing in South Africa.

Figure 2: *Lessertia frutescens* showing the leaves, flowers and pods (Van Wyk, 2008).

Figure 3: Commercial plantation of *Lessertia frutescens* (Van Wyk and Albrecht, 2008).

1.5 Origin and distribution

Lessertia frutescens, although now commonly found in many gardens throughout the world, was originally native to the drier parts of Southern Africa only (Van Wyk *et al*., 1997; Xaba and Notten, 2003). Within South Africa, the shrub is prevalent in the Western Cape, up the West Coast into the Northern Cape, throughout the Western Karoo and also found in parts of the Eastern Cape (Xaba and Notten, 2003). Figure 4 indicates the geographical locations of *Lessertia frutescens.*
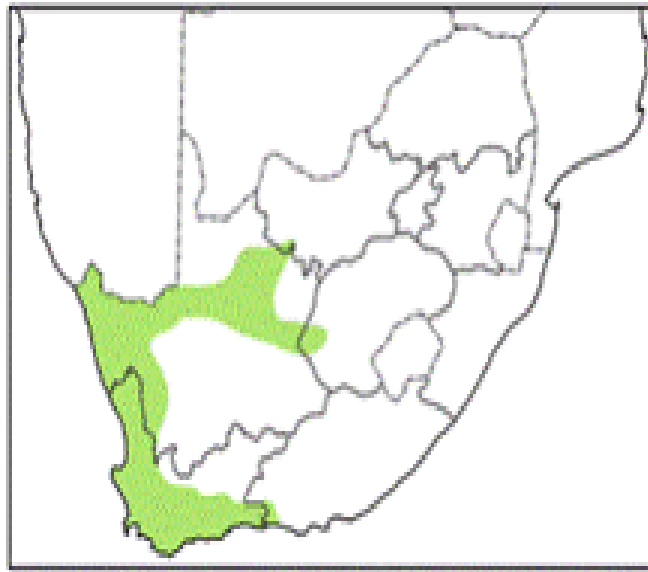
Figure 4: Map illustrating the distribution of *Lessertia frutescens* throughout South Africa (sahealthinfo.org).

1.6 Traditional and current uses

The use of *Lessertia frutescens* (whole plant material but mainly leaf and stem) as a traditional medicinal remedy has its origins dating back in history to where it was used by various cultural groups in South Africa; for instance the Khoi San, Nama people, Zulu, Xhosa and Cape Dutch (Van Wyk and Albrecht, 2008). Today, it is still considered a very popular medicinal plant and is used for the treatment of a multitude of health problems affecting the integumentary, respiratory, cardiovascular, gastrointestinal, nervous, urinary, musculoskeletal, endocrine and immune systems (Roberts, 1990; Van Wyk *et al*., 1997; Van Wyk and Gericke, 2000; Fernandes *et al*., 2004; Van Wyk and Wink, 2004; Ojewole, 2008; Van Wyk and Albrecht, 2008).

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The traditional use of *Lessertia frutescens* by the diverse cultural groups within South Africa, offers treatments for a variety of health problems (Table 2) manifesting within the various systems throughout the human body (Roberts, 1990; Van Wyk, *et al*., 1997; Van Wyk and Gericke, 2000; Fernades *et al*., 2004; Van Wyk and Wink, 2004; Mills, *et al*., 2005; Ojewole, 2008; Van Wyk and Albrecht, 2008).

Table 2: Traditional and current use of *Lessertia frutescens* for health problems in relation to the major systems of the human body.

Major systems of the human body	Health problems
Integumentary (skin)	Minor burns, minor wounds and inflammation
Respiratory	Colds and flu, bronchitis, coughs, asthma and tuberculosis
Cardiovascular	Varicose veins, piles and heart failure
Gastrointestinal	Poor appetite, indigestion, gastritis, diarrhoea, constipation, dysentery and peptic ulcers
Nervous	Depression, anxiety, stress, shock and irritability
Urinary	Kidney problems, uterine troubles and urinary tract infections
Musculoskeletal	Arthritis, osteoarthritis, rheumatoid arthritis and general backache
Endocrine	Good adaptogen and diabetes
Immune	Fever, chickenpox, tumors, wasting in aids, wasting in cancer, internal cancers and tonic

In recent years, there has been a lot of research being carried out concerning the use of *Lessertia frutescens*, these either confirming traditional uses or establishing new uses. In this regard, recent *in vitro* and *in vivo* studies have shown anti-diabetic, anti-proliferative, anti-inflammatory, antibacterial, anti-HIV, analgesic, anticonvulsant and antithrombotic activities (Van Wyk and Albrecht, 2008).

1.7 Constituents of *Lessertia frutescens*

Secondary metabolites in medicinal plants are referred to as active principles, bioactive compounds or more commonly as constituents (Mills and Bone, 2000; Singh, 2008). Compared to primary metabolites, the secondary metabolites are not as abundant (Deswick, 2002). These secondary metabolites are not there by chance but part of a plant's evolutionary process that contributes towards factors like, the plants defence mechanism, attraction of pollinators or as a colouring agent (Deswick, 2002; Gurib-Fakim, 2006; Street *et al*., 2008). It is the impact these secondary metabolites have, from a pharmacological perspective, which is of interest for their use as medicines (Gurib-Fakim, 2006).

Herbal preparations of *Lessertia frutescens* are mainly made from the leaves of the plant, although the twigs and stems are often included as these also contain the active ingredients (Van Wyk *et al*., 1997; Tai *et al*., 2004). There are a number of constituents that are present in *Lessertia frutescens* namely, L-canavanine, alanine, arginine, aspartic acid, asparagine, leucine, phenylanine, proline, tryptophan, pinitol, GABA, flavonoids and triterpenoids (Bell, 1958; Snyders,

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1965; Viljoen, 1969; Brummerhof, 1969; Bell *et al*., 1978; Moshe, 1998; Tai *et al*., 2004; Van Wyk and Albrecht, 2008; Fu *et al*., 2008; Avula *et al*., 2010).

Research by Moshe (1998) revealed the presence of protein-bound and free amino acids in the leaves of the *Lessertia* genus. These levels of protein-bound and free amino acids were recognized as being high, according to the authors (Moshe, 1998; Van Wyk and Albrecht, 2008). In another study, *Sutherlandia frutescens* leaves were used to analyse the presence of free amino acids that was commercially grown in different areas (Van Wyk *et al*. (unpublished); Van Wyk and Albrecht, 2008). According to the authors, high levels of arginine, asparagine and proline were isolated from the plants analysed. Van Wyk and Albrecht (2008) go on to indicate the importance of L-arginine present, as this constituent acts as an antagonist of L-canavanine, potentiating the anticancer activity.
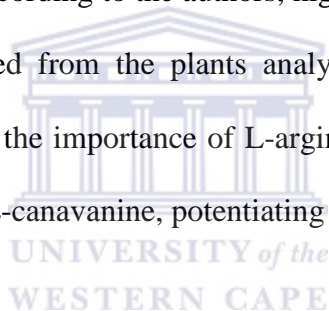
Although non-proteinogenic amino acids were discovered in the seeds of the *Lessertia* genus by Bell (1958) and Bell *et al*. (1978), it was the discovery of the presence of L-canavanine in the *Lessertia* genus leaves by Moshe (1998) that was of interest. Tai *et al*. (2004) confirmed the presence of L-canavanine, alanine, arginine, aspartic acid, asparagine, leucine, phenylanine, proline and tryptophan in commercial samples. Van Wyk and Albrecht (2008) highlighted the discovery of L-canavanine as the possible explanation for the anticancer effect of the *Lessertia* genus in traditional medicine. There is documented evidence for the anticancer and antiviral activity of L-canavanine (Figure 5), as well as its effect on

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retroviruses and influenza viruses (Green, 1988; Crooks and Rosenthal, 1994; Swaffar, 1995; Rosenthal, 1997; Bence *et al*., 2002).

COOH
|
H₂N─C─H
|
CHCH₂ONHCNH₂
||
NH
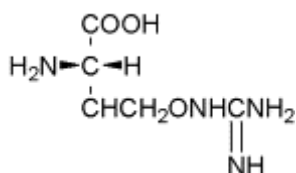
Figure 5: Structure of chemical constituent - L-canavanine.

The discovery of γ-aminobutyric acid (Figure 6) in the *Lessertia* genus possibly explains its use in cases relating to anxiety and stress (Van Wyk and Albrecht, 2008). GABA is considered an inhibitory neurotransmitter (Sia, 2004; Mills *et al*., 2005). Van Wyk and Albrecht (2008) note some interesting traditional uses of *Lessertia frutescens* for depression, fits and shock which were indicated in the research carried out by Moteetee and Van Wyk (2007). The Zulu and Tswana names for *Lessertia frutescens* are 'insiswa' and 'phetola' respectively meaning 'the one that dispels darkness' and 'it changes', both suggesting their inclination for use in stress related conditions (Van Wyk and Albrecht, 2008). Ortega (2003) indicated the use of GABA (Figure 6) to inhibit tumor cell migration.

COOH
|
CH₂
|
CH₂CH₂NH₂
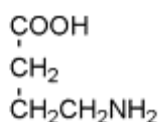
Figure 6: Structure of chemical constituent - γ-aminobutyric acid (GABA).

A cyclitol called ino-inicytol, commonly referred to as pinitol (Figure 7), was discovered in the leaves of the *Lessertia* genus by Snyders (1965) and later by Viljoen (1969) and Brummerhof (1969). Moshe (1998) confirmed the presence of pinitol in the *Lessertia* genus samples by HPLC analysis. The importance of pinitol as an anti-diabetic agent and in cachexia resulting from cancer and AIDS is well researched (Ostlund and Sherman, 1996; Bates *et al*., 2000).
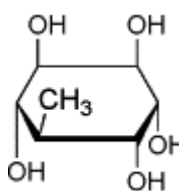
Figure 7: Structure of chemical constituent – pinitol.

Flavonoids were detected in the *Lessertia* genus by Moshe in 1998. According to this study, the *Lessertia* genus leaves contained a minimum of at least six flavonoids. Flavonoids may occur in a free state or as glycosides, two constituents known as the largest group of naturally occurring polyphenolic compounds (Evans, 1989). The use of flavonoids for a variety of ailments can be attributed to the wide range of activities namely; antioxidant, anti-inflammatory, anti-allergic, anti-platelet, vasoprotective, antiviral, antimicrobial and anti-cancer (Evans, 1989; Mills and Bone, 2000).

More recent research using the leaves of *Sutherlandia frutescens* led to the discovery of four cycloartanol glycosides (Figure 9) were isolated from the leaves

of *Lessertia frutescens* namely; sutherlandiosides A, sutherlandiosides B, sutherlandiosides C and sutherlandiosides D (Fu *et al*., 2008). In addition to this discovery, another study led to the isolation of four flavonoid glycosides (Figure 8) namely; sutherlandin A, sutherlandin B, sutherlandin C and sutherlandin D (Fu *et al*., 2010). Avula *et al*. (2010) developed an analytical method of determination of these four cycloartanol glycosides and four flavonoid glycosides.
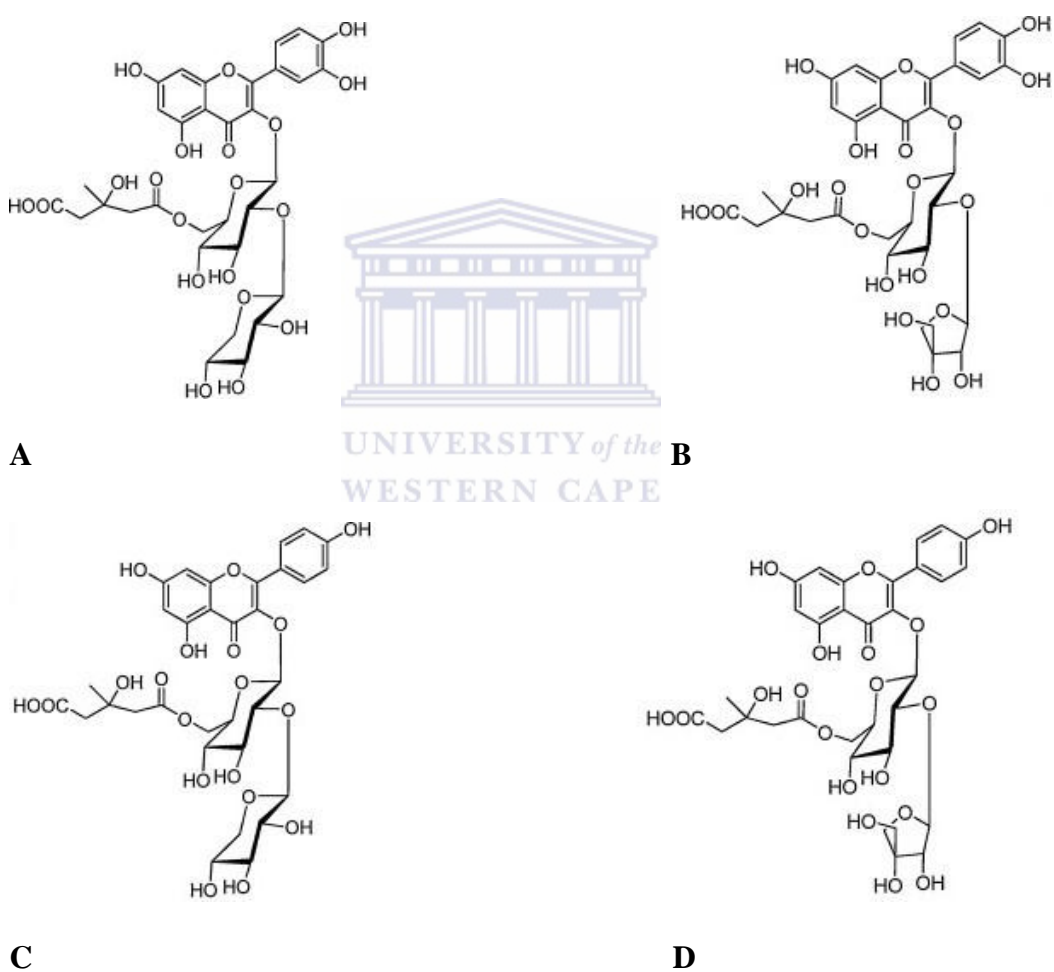
Figure 8: Structure of chemical constituents – sutherlandin A (**A**), sutherlandin B (**B**), sutherlandin C (**C**) and sutherlandin D (**D**).
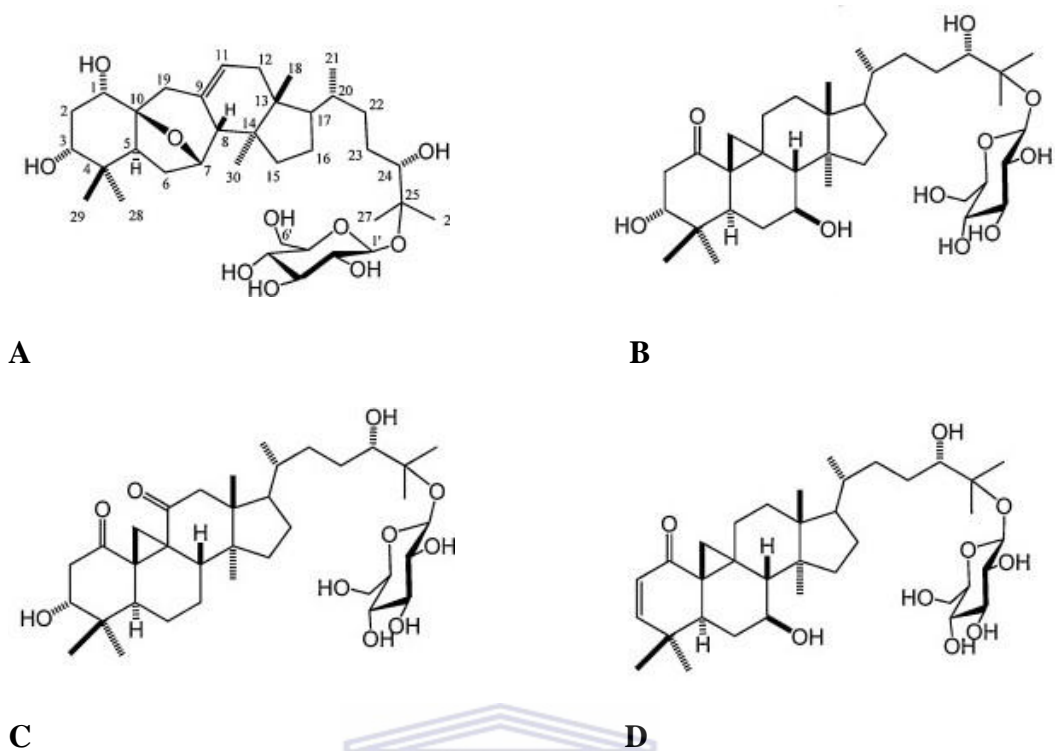
Figure 9: Structure of chemical constituents – sutherlandioside A (**A**), sutherlandioside B (**B**), sutherlandioside C (**C**) and sutherlandioside D (**D**).

A safety study done on *Lessertia frutescens* by the Medical Research Council of South Africa, reported no signs of toxicity (Seier *et al*., 2002). This study involved the monitoring of possible toxicity in adult male vervet monkeys, after consumption of the powdered leaf, over a period of three months. The biochemical, haematological, physiological and physical variables were monitored for the purpose of the study and Seier *et al* (2002) concluded, that even at 9 times the recommended dose, no adverse effects were noted. In a clinical (phase 1) trial involving adults with no known illnesses or allergic conditions,

participants were given 400mg capsules containing *Sutherlandia* leaf powder, twice daily, over a period of three months (Johnson *et al*., 2007). The physical, vital, blood and biomarkers variables were used and Johnson *et al* (2007) indicated no toxicity from the use of *Lessertia frutescens* at a dose of up to 800mg per day. Although there was no toxicity reported, there were minor side effects in some individuals ranging from occasional mild diarrhoea, dry mouth, mild diuresis and dizziness in patients presenting with cachexia (Mills *et al*., 2005).

1.8 Methods of identification

In order to ensure the quality, safety and efficacy of traditional plant medicines, regulatory guidelines were set up by the World Health Organization (WHO, 2000). Worldwide there would be a need to set up pharmacopoeias accurately recording the data for the quality, safety and efficacy of the country's traditional medicinal plants, as per these WHO's regulatory guidelines. Most of these guidelines suggest macroscopic, microscopic and chemical profiling as a measure of ensuring quality control (Evans, 1989; BHP, 1996; WHO, 1998; Joshi *et al*., 2004). Joshi *et al*. (2004) also go on to mention the parameters into which these evaluations were divided.

Macroscopic parameters used for evaluation are colour, odour, shape, size, taste, texture, amongst others, while in microscopy one would consider the microscopic inspection of the plant material (Evans, 1989; Joshi *et al*., 2004). The limitations of the reliance on these methods as the only methods for evaluation are that the

sample may contain substitutes or adulterants that look like the authentic material. Adding chemical profiling as an evaluation technique allows for the chemical fingerprint of each plant to be established, thus ensuring that the crude material is authentic (Evans, 1989; BHP, 1996; WHO, 1998; Joshi *et al*., 2004; Springfield *et al*., 2005).

The use of analytical techniques such as Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) is effective in establishing quality control parameters (BHP, 1996; WHO, 1998; Joshi *et al*., 2004; Springfield *et al*., 2005). Springfield *et al*. (2005) also reiterated the use of HPLC as an effective analytical tool for 'fingerprinting' and when this was combined with online UV spectrum detection via a diode array configuration, in order to 'establish a code of practice for the quality control of herbal products'. HPLC-UV is recognized as a simple, specific and effective analytical method for profiling the active constituents in herbal medicines (Foukaridis *et al*., 1994; BHP, 1996; WHO, 1998; Amabeoku *et al*., 2001; Springfield, *et al*., 2005). With this in mind, the South African Traditional Medicines Research Group (SATMERG) was initiated within the School of Pharmacy at the University of the Western Cape (UWC), and by using the identification techniques of taxonomy, microscopy, Thin-layer Chromotography (TLC) and HPLC linked to a diode array detector (DAD), monographs for 60 traditional medicinal plants have been established (Springfield *et al*., 2005).

1.9 Factors influencing quality, safety and efficacy

With the large majority of the world's population relying on traditional medicines for their health care needs, quality assurance of the basic material is essential in order to establish quality, safety and efficacy (Fennell *et al*., 2004a; Joshi *et al*., 2004; Springfield *et al*., 2005; Johnson *et al*., 2007; Street *et al*., 2008., Avula *et al*., 2010). Springfield *et al*. (2005) pointed out that the trade and use of traditional medicinal plants are largely unregulated.

There are many factors that may influence the biological activity or chemical profile of traditional medicinal plants (Fennell *et al*., 2004a; Joshi *et al*., 2004; Street *et al*., 2008). These may be grouped under intrinsic and extrinsic factors (Joshi *et al*., 2004). Genetics would fall under the heading intrinsic, while cultivation, harvesting, drying and storage would be examples of extrinsic factors (Joshi *et al*., 2004).

When considering choosing the correct plant material, factors such as the selection of the correct chemotype, as well as the identification of the correct plant species, are important when trying to establish quality, safety and efficacy (WHO, 1998; Fennell *et al*., 2004a; Joshi *et al*., 2004; Springfield *et al*., 2005). Joshi *et al*. (2004) notes the difficulty in identifying a species when a plant has various botanical names, depending on the region it is growing in.

Street *et al*. (2008) refers to the influences of industrialization on the environmental and water resources, namely contamination of these two and, thus the quality, safety and efficacy of the basic plant material from these areas. Factors like mining, industrial waste, dry sewage sludge, polluted waters, heavy metal contamination, pesticides, chemical fertilizers and microbial contaminants all contribute towards a negative impact on quality, safety and efficacy of the plant material (Naicker *et al*., 2003; Roychoudhury and Starke, 2006).

Biological activity may also be influenced by factors like the plant's age, geographical harvest site, seasonal variation and growth conditions (Taylor *et al*., 2003; Buwa and Van Staden, 2007). Fennell *et al*. (2004a) also draw attention to other factors like harvesting practices and storage conditions. Ramakrischnappa (2002) relates to factors like climate, drying and proper storage conditions often being neglected by the growers of traditional medicinal plants.

Quite a few factors have so far been established as having a considerable influence on the quality, safety and efficacy of traditional medicinal plant material and all are considered important, but for the purpose of this study only the seasonal and storage conditions have been considered.

1.10 Seasonal harvest influences on constituents

The impact seasonal harvesting had on the constituents of a plant was an important factor for the various cultures throughout the world to consider, notably

taking into account the thousands of years of coexistence human beings have had with plants as a source of food and medicine (Chevallier, 1996; Taylor *et al*., 2003; Shale *et al*., 2004; Buwa and Van Staden, 2007).

In India, traditional healers advocated the decoction of *Alstonia sholaris* to be consumed only during the monsoon season, as it was less toxic to the consumer than when collected during the other seasons (Jagetia and Baliga, 2005). In an *in vitro* study, the same authors (Jagetia and Baliga, 2005) explored the effect of seasonal variation on the anti-neoplastic activity and cytoxicity of *Alstonia sholaris* (Jagetia and Baliga, 2005). According to the results of this study, samples had been collected during the monsoon, winter and summer seasons and showed a dose dependent increase in anti-neoplastic activity of *Alstonia sholaris* where the highest cell killing effect had been observed for the extract prepared from the summer collections, and the study had also demonstrated a marked difference in potency of anti-neoplastic activity of extracts prepared from the winter and monsoon season collections (Jagetia and Baliga, 2005).

The production of secondary metabolites, besides being genetically controlled, is also influenced by environmental factors (Yanivie and Palevitch, 1982; Bopana and Saxena, 2007). The above mentioned determinants, of which time of harvest is amongst the most important, are able to influence the quality and quantity of the metabolites (Badi *et al*., 2003). According to Mc Gimpsey *et al*. (1994), a plant
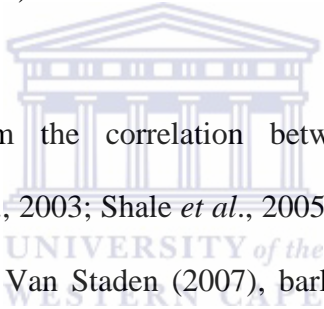
during its flowering period yields a greater volume of essential oil compared to any other period, therefore indicating a preferred time of harvest.

The relationship between oil composition, toxicity and seasonal changes were investigated in an *in vivo* study for *Salvia libanotica* (Farhat *et al*., 2001). The leaves of *Salvia libanotica* from the four seasons were processed for extraction of the oils and compared. High levels of camphor (12.3%), camphene (4.8%) and beta-thujone (1.9%) had been found in the winter (Farhat *et al*., 2001) extracts, indicating the highest levels of toxicity compared to the other seasons. The least toxic extract was that of spring. The highest yield of oil had been found during the dry summer months, conditions that where favourable to high oil production (Pitarevic *et al*., 1985). Variations in the chemical composition of essential oils from *Ocimum selloi* were associated with the particular season of harvest (Moraes *et al*., 2002).

The effects of seasonal variation on the central nervous system activity of *Ocimum gratissimum* essential oil was investigated by monitoring the pharmacological changes (Freire *et al*., 2006). This was done by testing the sleep inducing activity that results from the use of the essential oils (Freire *et al*., 2006). Samples were collected that were representative of the four seasons. The greatest effect had been obtained with the autumn preparation, and the least effect was observed with the winter preparation (Freire *et al*., 2006). The autumn preparation contained 16.81% 1.8-cineole, in comparison with the higher amount

in winter of 33.61%. The 1.8-cineole is a monoterpene which acts as a central nervous system (CNS) stimulant (Burkard *et al*., 1999; Umezu *et al*., 2001). Thus, it had been possible to suggest that the decrease in the amount of this compound facilitated an increased sleeping time.

A correlation study was carried out considering the main active constituent for the traditional antifungal activity of *Polygonom acuminatum* and the season of the year. The study concluded that the constituent, polygodial had been the most concentrated sequiterpene with antifungal behavior and this had been collected in autumn (Derita *et al*., 2009).

Various studies confirm the correlation between season of harvest and constituents (Taylor *et al*., 2003; Shale *et al*., 2005; Buwa and Van Staden, 2007). According to Buwa and Van Staden (2007), bark samples collected during the summer months from *Harpephyllum caffrum*, yielded bioactive constituents that had a higher antibacterial activity than any of the other samples collected that were representative of the other three seasons. The effect seasonal influence can have on constituent levels may be even more diversified. A study done by De Vasconcelos *et al*. (1999) noted that there were variations in constituent levels of the essential oils from the *Ocimum gratissimum* leaves during day time. The result indicated that sunlight has an influence on the production of eugenol (De Vasconcelos *et al*., 1999).

1.11 Duration of storage influences on constituents

Regardless of the influence duration of time in storage may have on the pharmacological activity of medicinal plants, not much research has been done in this field worldwide, especially in Southern Africa (Fennell *et al*., 2004a). An understanding of the factors that affect the constituent levels of medicinal plants are important as they would affect the plants efficacy in treatment of disease, and its cytotoxic activity (Fennell *et al*., 2004a).

It is generally understood that there are several factors that may influence chemical changes of plant material being stored such as; temperature, light, pH and enzymes (Fennell *et al*., 2004a). At the pre-storage stage, factors like drying heat, cooling and packaging help prevent the degradation (Fennell *et al*., 2004a). Other factors like cultivation, harvesting, drying and the duration of storage may also lead to degradation but this is not as easily detected visually or olfactorially. Therefore, scientific investigation would be the most suitable method (Fennell *et al*., 2004a; Joshi *et al*., 2004).

The possible variation in chemical composition of dried plant material samples stored for many years under different storage conditions has been investigated (Houba *et al*., 1995). Reference samples that were certified and stored under laboratory conditions for twenty years had shown no changes in the results for almost all elements analysed (Houba *et al*., 1995). Samples obtained from a continuous inter-laboratory exchange program for dried material had shown

constant composition during storage, independent of the way the samples had been stored (Houba *et al*., 1995). These authors also reported on lucerne samples that were stored in paper bags for four years under normal laboratory conditions and summer barley stored in sealed glass for thirteen years, this at room temperature. The results of the elements had not changed significantly during storage and the same conclusion was made from a sample of tea leaves that had been stored for eight years in sealed glass bottles and thereafter for two years at -20°C (Houba *et al*., 1995). Houba *et al*. (1995) concluded that dried plant samples could be stored for a very long time without any significant changes in their chemical composition and that storage conditions seemed of less importance (Houba *et al*., 1995).
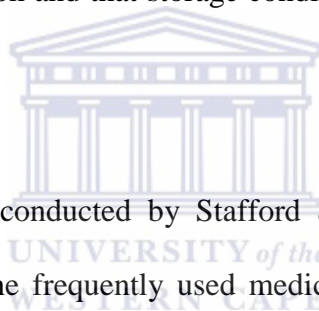
An *in vitro* study was conducted by Stafford *et al*. (2005) to determine the biological activity of nine frequently used medicinal plants of South Africa by monitoring the results after variations in storage times. The nine plants investigated were *Alepedia amatymbica, Leonotis leonurus, Drimia robusta, Vernonia colorata, Merwilla natalensis, Eucomis autumnalis, Bowiea volubilis, Helichrysum cymosum* and *Siphonochilus aethiopicus*. The 90 day old and the one year old material were assayed for antibacterial activity and COX-1 inhibition. Thin Layer Chromatography (TLC) fingerprints of fresh and stored extracts were generated in order to document chemical changes during storage (Stafford *et al*., 2005). Results indicated that chemical breakdown had occurred during storage of certain species. It also appeared that in some cases certain

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compounds underwent degradation as a result of the drying process. Compounds that had previously not been detected in the fresh extracts were found in the extracts stored for a year (Stafford *et al*., 2005).

The major antibacterial compounds found in *Leonotis leonorus* appeared to be stable as they were found in the fresh, one and five year old material (Stafford *et al*., 2005). A second group of antibacterial compounds were observed in the five year old material that had not been detected in the fresh and one year old material. This suggested that the quantities of those compounds might have been increasing as a result of storage (Stafford *et al*., 2005). Extracts of *Veronia colorata* and *Leonotis leonorus* had shown an increase in activity against *Escherichia coli* after one year (Stafford *et al*., 2005). The five year old *Leonotis leonorus* material had shown an increased antibacterial activity against all bacteria except *Klebsiella pneumonia*, against which it appeared to have lost activity. Since then there had been an increase in the biological activity, as was observed with the antibacterial activity, it was deduced that the precursor was less active than the product of the chemical change, or the ageing process had resulted in greater proportions of the active compound. Antibacterial compounds appeared to be either stable or converted into more active compounds during storage (Stafford *et al*., 2005).

An interesting observation with regards to the anti-inflammatory bioassay results was that in most situations the water and ethanol extracts indicated an increased activity after storage (Stafford *et al*., 2005). This study by Stafford *et al*. (2005)

concluded that ideally, the chemical composition of plant material, as well as its biological activity should be determined directly after drying. Thus, the effect of drying and storage of plant material should be dealt with separately. In some cases it appeared that higher levels of compounds had been extracted as a result of the drying process (Stafford *et al*., 2005). The authors concluded that if a specific plant retained its activity for one to five years after harvesting then it would not be necessary to collect fresh material all the time but rather store the dried material until it was needed. Stafford *et al*. (2005) did indicate that the effect of storage would be species-specific because the chemical composition of each species would be different and thus it would not simply be a matter of assuming one particular shelf-life recommendation could be applied to all plant material.

In other research, phenolic compounds, gallic acid and gallotannins in *Mangifera indica*, were found to naturally decline during storage, which resulted in a loss of astringency (El Ansai *et al*., 1971; Kim Lounds-Singleton and Talcott, 2009; Lakshminarayana *et al*., 1970; Mitra and Balwin, 1997; Shale *et al*., 1975).

1.12 Rationale for the use of *Lessertia frutescens*

The rationale was motivated by the long history of the use of *Lessertia frutescens* by the various cultural groups in South Africa as a popular traditional medicinal plant to treat a variety of ailments (Roberts, 1990; Van Wyk *et al*., 1997; Van Wyk and Gericke, 2000; Fernandes *et al*., 2004; Van Wyk and Wink, 2004; Ojewole, 2008; Van Wyk and Albrecht, 2008). Also that today, *Lessertia*

frutescens is still considered a very popular medicinal plant used for the treatment of a multitude of ailments (Roberts, 1990; Van Wyk *et al*., 1997; Van Wyk and Gericke, 2000; Fernandes *et al*., 2004; Van Wyk and Wink, 2004; Van Wyk and Albrecht, 2008).

In recent years there has been a lot of research being carried out concerning the use of *Lessertia frutescens*, these either confirming traditional uses or establishing new uses. The flavonoids and triterpenoids of *Lessertia frutescens* have been part of very recent research (Fu *et al*., 2008; Avula *et al*., 2010).

The report from the World Health Organization (WHO) (1998a) that estimates that 80% of the people in the developing nations make use of traditional medicinal plants for their health care needs. To get the best results from a medicinal plant one would need to ensure that the crude material is of good quality through interventions like being properly grown, well dried and correctly processed, and then validate this by scientific research to establish safety and efficacy data.

1.13 Research design and rationale

Chemical analysis was performed using the HPLC technique to create fingerprints of the flavonoids and triterpenoids of *Lessertia frutescens* plant material to investigate what impact, if any, the duration of storage and a particular season of harvest would have on the flavonoid and triterpenoid marker levels of *Lessertia frutescens*.

According to Joshi *et al*. (2004) chromatographic techniques and molecular markers are important in herbal drug technology. The biologically active components; L-canavanine, GABA and pinitol were found in *Sutherlandia frutescens* by experimental analysis (Moshe, Van Wyk *et al*., 1997; Tai *et al*., 2004). Springfield *et al*. (2005) used High Performance Liquid Chromatography with diode array detection as a means of "fingerprinting" *Chironia baccifera*, to contribute to establishing quality standards for indigenous medicinal plants in South Africa. In research done to provide chemical markers for *Sutherlandia frutescens*, phytochemical investigation led to the discovery of four flavonoids and four triterpenoids (Fu *et al*., 2008; Avula *et al*., 2010).
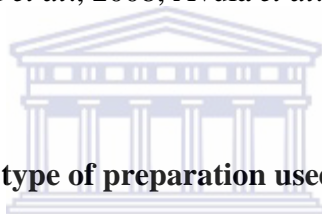
1.13.1 Rationale for the type of preparation used

Ethanolic extracts were chosen because it is a popular means of administering medicinal plants in the modern world (Mills, 1991; Mills and Bone, 2000). In this way, soluble herbal constituents can be separated from the fibrous material (Mills, 1991; Mills and Bone, 2000). In addition, ethanol strength above 20% can ensure sterility, thus acting as a preservative, so increasing the shelf life of the extract (Mills, 1991; Mills and Bone, 2000). The ethanol concentration also has an effect on the type of chemical constituents, which end up getting extracted (Mills, 1991; Mills and Bone, 2000). Ethanol-water mixtures can be used to extract a variety of chemical constituents including flavonoids and triterpenoids.

1.13.2 Rationale for the monitoring of the flavonoids and triterpenoids

Moshe (1998) reported that *Lessertia frutescens* leaves contained at least six flavonoids. More recent research done by Fu *et al*. (2008) in order to establish other chemical constituents for *Lessertia frutescens*, resulted in the discovery of the four flavonoids; sutherlandin A, sutherlandin B, sutherlandin C, sutherlandin D and four triterpenoids; sutherlandioside A, sutherlandioside B, sutherlandioside C, sutherlandioside D. Then, Avula *et al*. (2010) developed the first analytical method for the determination of these flavonoids and triterpenoids.

1.13.3 Rationale for the use of High Performance Liquid Chromatography

HPLC, is being increasingly used and it has become standard practice to use this technique as an important tool for qualitative assessment of various chemical constituents (Foukaridis *et al*., 1994; Springfield *et al*., 2005; Joshi, *et al*., 2007). HPLC is able to perform separation of chemical constituents within a relatively fast time and can detect low concentration levels (Harris, 2003). There is also the added advantage of easily being able to repeat and reproduce experiments.

1.14 Research questions

Within this context, it can be hypothesized that further investigations into factors that ensure that the crude material is of good quality, would be of relevance and is beneficial in order to ensure safety and efficacy. The questions that arose in the planning of this study were:

- Would a particular season show a greater yield of biomarker/s than any of the other seasons?

- Which marker/s would most suitably represent *Lessertia frutescens* in monitoring the more favourable season of harvest?

- Would the flavonoids and triterpenoids be suitable markers?

- Would the quality of fresh dried *Lessertia frutescens* deteriorate while being stored after a period of one year?

- Which marker/s would most suitably represent *Lessertia frutescens* in monitoring the impact of time in storage?

- How would the constituent levels in the stems compare to those in the leaves?

1.15 Aim of the study

The aim of this study was to investigate what impact, if any, the duration of storage and a particular season of harvest would have on the flavonoid and triterpenoid marker levels of *Lessertia frutescens*.

1.16 Objectives of the study

- To investigate storage variation of *Lessertia frutescens* leaves by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids.

- To investigate seasonal variation of *Lessertia frutescens* leaves by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids.

- To investigate leaf and stem variation of *Lessertia frutescens* by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids.

1.17 Hypotheses

The following hypotheses were tested:

- The stored sample would indicate the same level of the biomarkers for the flavonoids and triterpenoids, as that of the freshly prepared sample. The null hypothesis states that there would be no changes in the storage variation of *Lessertia frutescens* leaves.

- The sample that was harvested during the summer season would indicate higher levels of the biomarkers of flavonoids and triterpenoids than the other three seasons. The null hypothesis states that there would be no changes in the seasonal variation of *Lessertia frutescens* leaves.

- The leaf sample would indicate the same level of the biomarkers for the flavonoids and triterpenoids, as that of the stem sample. The null hypothesis states that there would be no changes in the leaf and stem variation of *Lessertia frutescens*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals, reagents, materials and equipment

The following chemicals, reagents, materials and items of equipment were used for the preparation and analysis of the *Lessertia frutescens* samples.

2.1.1 Chemicals, reagents and materials

Freshly harvested leaves of *Lessertia frutescens* (Ezibusisweni farm, Stellenbosch, South Africa); distilled water that was obtained from Durbell Pharmacy, manufactured by Reitzer Pharmaceuticals (Pty) Ltd, Johannesburg, South Africa; ethanol 99% obtained from Azanin Pharmaceuticals, manufactured by Huletts, Pietermaritizburg, South Africa; qualitative filter paper, (Whatman No. 40, Whatman, Kent, England); high vacuum grease (Dow Corning, Seneffe, Belgium); helium (99.95%, Afrox, Johannesburg, South Africa); acetonitrile - HPLC grade (Honeywell, Burdick and Jackson, Michigan, USA); methanol - HPLC grade (Thermo Scientific Pierce, Rocklands, USA); formic acid - HPLC grade (Sigma-Aldrich, St. Louis, USA); Quercetin - HPLC grade (Sigma-Aldrich, St. Louis, USA); 1ml glass vial (Anatech Instruments (Pty) Ltd, Cape Town, South Africa.

2.1.2 Equipment

Ventilated oven (Memmert, model 854, Schwabach, Germany); rotary evaporator (Buchi, Labortechnik, Flawil, Switzerland); balance (Mettler, model PE 6000, Gottingen, Germany); freeze-drier (Virtis, Freeze Mobile model 125L, The Virtis Company Gardener, New York, USA); freezer (-85°C Lozone CFC Freezer, model U855360, New Brunswick Scientific, New Jersey, USA). For the chromatographic analysis an Agilent 1200 series High Performance Liquid Chromatography (HPLC) system was used, the system is listed under HPLC analysis.

2.2 Authentication, collection and preparation of plant material

Ezibusisweni farm (Spier Wine Estate) was identified as the site for the collection of freshly harvested plant material of *Lessertia frutescens*. The farm is located in Stellenbosch (33° 55' 43.88" South and 18° 48' 11.76" East), which is situated in the Western Cape Province of South Africa. On this farm, a commercial plantation of *Lessertia frutescens* was being specifically grown for a company that produces herbal tinctures. As part of the agreement between the supplier and purchasing company, no artificial fertilizers, pesticides or herbicides were to be used during the growing and harvesting of these plant crops. Well matured cow manure was used during the growing phase and the plantation was watered three times weekly, specifically during the hot dry months of summer. This particular *Lessertia frutescens* plantation was two years old.

2.2.1 Authentication of plant material

For authentication of the plant, material was collected from Ezibusisweni farm during the summer month of January 2010. The material was authenticated by the botanist and curator of the herbarium at the University of the Western Cape (UWC), Mr F Weitz, as *Lessertia frutescens*. The specimen was dried, prepared as per herbarium guidelines and finally stored in the herbarium of the Biodiversity and Conservation Biology department at the University of the Western Cape, under voucher number UWC 6971.

2.2.2 Collection of plant material

After authentication was established, an area of 16 m² was demarcated, which had nine *Lessertia frutescens* plants growing within this space allocation on the Ezibusisweni farm. Throughout the year, four sets of plant material samples were collected from this area, representative of the seasons; summer (January), autumn (April), winter (July) and spring (October), as listed in Table 3. Each sample collected consisted of freshly harvested plant material (leaves, twigs and stems) from all nine *Lessertia frutescens* plants. The first sample (subdivided as LF1A, LF1B and LF1C), was collected during the summer season of South Africa at 10.00 am on the 29th January, 2010. The rest of the seasonal samples, representative of autumn (LF2), winter (LF3) and spring (LF4), were also collected at 10.00 am on the 29th day of the indicated months. Collection details such as the parts used, dates, times and weather conditions are indicated in Table 4. All the samples were collected within the same year, 2010.

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Table 3: Plant material samples representative of the four seasons and their months of collection.

Plant material sample	Abbreviation	Season	Month
Lessertia frutescens 1A	LF1A	Summer	January
[a] *Lessertia frutescens* 1B	LF1B	Summer	January
[b] *Lessertia frutescens* 1C	LF1C	Summer	January
Lessertia frutescens 2	LF2	Autumn	April
Lessertia frutescens 3	LF3	Winter	July
Lessertia frutescens 4	LF4	Spring	October

[a] Collected at the same time as LF1A and stored for one year.

[b] Collected at the same time as LF1A and is representative of the twig and stem material.

Table 4: Plant material sample collection details.

Sample	Parts used	Collection Date	Collection time	Weather conditions
LF1A	Leaves	29/01/2010	10 am	Clear
[a] LF1B	Leaves	29/01/2010	10 am	Clear
[b] LF1C	Twigs/stems	29/01/2010	10 am	Clear
LF2	Leaves	29/04/2010	10 am	Misty
LF3	Leaves	29/07/2010	10 am	Raining
LF4	Leaves	29/10/2010	10 am	Misty

[a] Collected at the same time as LF1A, stored for a year and prepared for HPLC analysis on 29/01/2011.

[b] Collected at the same time as LF1A and is representative of the twig and stem material.

2.2.3 Preparation of plant material

All freshly collected plant material was rinsed with distilled water to remove any foreign particles, as per Springfield *et al*. (2005), and then the leaf material was separated from the bits of twigs and stems. Once sorted, the plant material was weighed and measured. For the summer season sample (subdivided as LF1A, LF1B and LF1C), a total weight of 330g was calculated, which was made up of 110g of twig and stem material for LF1C, and 220g leaf material. The leaf material was equally divided to provide 110g of fresh plant material for LF1A and 110g for LF1B. For the autumn (LF2), winter (LF3) and spring (LF4) seasonal samples, only the leaf material was weighed, 110g for each sample. All fresh material was dried in a ventilated oven at a temperature of 35°C for three days.

In the case of the summer season sample (for the storage component of this study), LF1B was placed into an amber glass container, which was sealed with a lid and placed into a cupboard, at room temperature. This was stored for one year, after which it was prepared and analyzed as per the preparation method for all the other samples.

2.2.4 Preparation of ethanolic extract

All the dried plant material samples were prepared as ethanolic extracts, done according to the information in the British Herbal Pharmacopoeia (BHP) (1993), the British Pharmacopoeia (BP) (1923) and the British Pharmaceutical Codex (BPC) (1968).

The preparation was done as follows:

Dried plant material was weighed to measure a total of 50 g and placed into a one litre glass jar. Then, 400ml of 45% (v/v) ethanol was prepared and added to the 50 g of dried plant material. The jar was sealed with a lid in order to prevent any loss of alcohol and placed in a dark cupboard, at room temperature, for 14 days (Mills, 1991; Mills and Bone, 2000). Throughout the two week period, the mixture was hand stirred daily so as to turn the contents in order to facilitate the dissolution of the soluble constituents (Mills, 1991). After the determined time (14 days), the liquid was drained from the marc (solid material left after extraction) and left to stand for a few hours until it settled, after which it was filtered using filter paper.
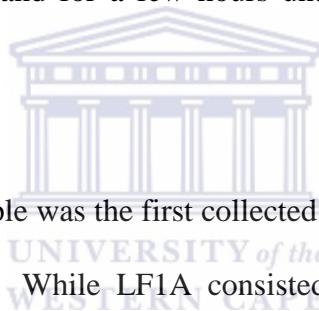
The summer season sample was the first collected and thus the first to be prepared as an ethanolic extract. While LF1A consisted of dried leaf material, LF1B consisted of dried twig and stem material. They were prepared simultaneously. In turn, the leaf material for the autumn (LF2), winter (LF3) and spring (LF4) seasonal samples, were prepared as ethanolic extracts, as per the preparation method described in the paragraph above. The last ethanolic extract prepared was that of LF1B after having being stored for one year.

Once the filtration process was complete, the filtrate was transferred into round bottom flasks and rotary evaporated at a temperature of 40°C, until dry (Springfield, *et al*., 2005). This was done in order to remove all traces of solvents

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from the sample. Once dried, the extract powder was removed from the round bottom flask, weighed to calculate yield and transferred into an amber glass bottle with a cap and frozen at -85°C in a freezer. Storage in the freezer lasted for four weeks due to the freeze-drier being out of commission (for the sake of consistency and accurately replicating the method, all the other samples were also frozen for the four week period). Then, the cap was removed and the amber glass bottle with the frozen extract was placed into the freeze-drier at -40°C for a period of four days. Once this stage was completed the freeze-dried extract powder was removed and weighed to calculate yield.

2.2.5 Preparation of the freeze-dried extract powder for HPLC analysis

Freeze-dried extract powder was weighed (0.2g), placed into a 10ml glass vial and reconstituted with 12ml of 45% (v/v) ethanol. A lid was placed on the vial. The mixture was vortex-mixed for 2 minutes, after which an aliquot of 1ml was taken out and placed into a 1ml glass vial. The above method was repeated to establish an HPLC analysis sample size of 12 (n=12). This was done for all the seasonal samples, in the case of the summer sample (LF1A, LF1B and LF1C), autumn (LF2), winter (LF3) and spring (LF4). These were labeled in the case of LF1A as; LF1A-1, LF1A-2, LF1A-3, LF1A-4, LF1A-5, LF1A-6, LF1A-7, LF1A-8, LFA1-9, LF1A-10, LF1A-11, LF1A-12. The same labeling format was used for all other samples. These HPLC samples were now ready to be placed in the auto loading sampler for HPLC analysis.

2.3 High Performance Liquid Chromatography (HPLC) analysis

For the chromatographic analysis an Agilent 1200 series HPLC system was used to separate, identify and quantify compounds. It was comprised of a degassing system (Agilent Technologies, system part no: G1322A, Tokyo, Japan); a quaternary pump (Agilent Technologies, system part no: G1311A, Waldbronn, Germany); an auto loading sampler (Agilent Technologies, system part no: G1329A, Waldbronn, Germany); a C18 Discovery™ column 150mm x 4.60mm, 5µm (Sigma-Aldrich, St. Louis, USA), coupled with a diode-array detector (Agilent Technologies, system part no: G1315B, Waldbronn, Germany), a fluorescence detector (Agilent Technologies, system part no: G1521A, Waldbronn, Germany) and an analyte fraction collector (Agilent Technologies, system part no: G164C, Waldbronn, Germany). The whole setup was managed by the Agilent ChemStation software 'online and offline' (Agilent Technologies, system part no: G2173-60101L, Waldbronn, Germany).

2.3.1 HPLC method

An Agilent 1200 series HPLC system was used for the chromatographic determination of the flavonoids, sutherlandin A (1) and sutherlandin D (2), as well as the triterpenoids; sutherlandioside B (3) and sutherlandioside D (4). The analytical method applied was adapted from the method developed by Avula *et al.* (2010). Ten microlitres (10µL) of sample were injected into the column to obtain the separation of peaks. For the stationary phase, a C18 Discovery™ reversed-phase hydrophobic column (25mm x 4.6mm; 5µm particle size) was

used and the temperature was maintained at 25°C. While the mobile phase consisted of: acetonitrile containing 0.1% formic acid (A), and distilled water containing 0.1% formic acid (B), at a flow rate of 1.000 ml/minute. The following gradient elution was used to do the analysis: from 0 min, A:B (15%: 85%) to A:B (65%: 35%) over the period of 35 minutes. Each run was followed by a 5 minute wash using 100% (A) and a 15 minute equilibration period, bringing the total run time to 55 minutes. To ensure conditions remained the same, after every third sample, a wash phase was carried out using 100% (A).

Table 5 lists the running times of the HPLC gradient and the composition of solvents. The flavonoid and triterpenoid compounds were detected by ultraviolet (UV) at 260nm and 360nm, respectively, with the use of a diode-array detector, based on the retention times and UV spectra.
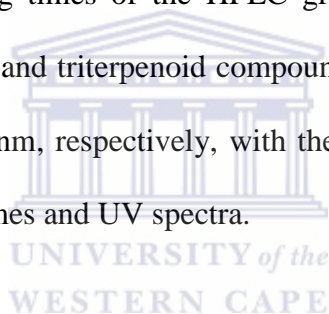
Table 5: Running times of HPLC gradient and composition of solvents.

Time (min)	A% (Acetonitrile)	B% (Distilled water)
0	15	85
35	65	35
35.1	100	0
40	100	0
40.1	15	85
55	15	85

A = Acetonitrile containing 0.1% formic acid.

B = Distilled water containing 0.1% formic acid.

2.3.2 Validation of HPLC

To validate the HPLC fractionation that was used, the following analytical parameters were used: precision, accuracy and linearity. These parameters were selected from the European Medicine Agency (EMEA) (1994 and 1996) quality guidelines, which are the internationally accepted documents that determine the validation of analytical methods. Quercetin (HPLC grade) and methanol (HPLC grade) were used as standards for control and calibration purpose.

For the determination of the precision and accuracy of the assay, an intra-day and inter-day approach was used. This was achieved by analysing three samples ($n = 3$) per day, over a period of three days.

For the linearity of the assay, the correlation coefficient (R^2) for the standard curve of the peak height (Y) versus the concentration (X) of the flavonoids and triterpenoids were calculated by analysing three samples ($n = 3$), diluted at five different concentrations. Serial dilutions of the extract in 45% ethanol with distilled water were carried out to obtain 100%, 50%, 25%, 12.5% and 6.25%.

2.4 Data and statistical analysis

To interpret the results obtained from the analysis of the various samples by the HPLC method, statistical methods were used for data transformation and analysis. To determine the flavonoid and triterpenoid levels of the samples, data was entered into an Excel Spreadsheet (Microsoft Office Excel 2010, Redmond,

Washington, USA) computer software package, which was used for the raw data transformation and analysis. The flavonoid and triterpenoid area versus concentration data was used to determine linear regression analysis, the precision and accuracy by an intra-day and inter-day approach analysis.

Statistical evaluation was performed using the MedCalc statistical software (MedCalc version 12.3.0, Mariakerke, Belgium). Normal distribution of data was checked by means of the Kolmogorov-Smirnov test. Depending on the normal distribution, parametric (independent t test) or non-parametric (Mann-Whitney test) tests were applied. Data were then expressed as mean $\pm$ standard deviation (SD) and 95% confidence intervals (CI). For the calculation of correlations, Pearson correlations were performed. A p-value of less than 0.05 was considered significant.
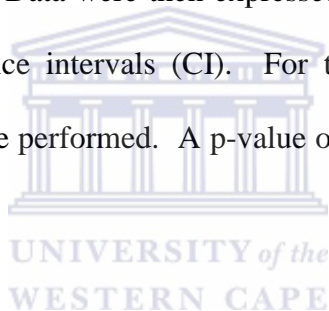
CHAPTER 3

RESULTS

3.1 Organoleptic characteristics

The organoleptic characteristics of the plant materials were incorporated as some pharmacopoeias include the colour of the material as part of the monograph (European Pharmacopoeia, 2002a; European Pharmacopoeia, 2002b; European Pharmacopoeia, 2002c). Upon visual inspection of the fresh and dried plant material, it was observed that the leaf material retained its silvery-green-grey appearance after being dried (Figure 10 B), but was lighter in colour compared to that of the fresh material (Figure 10 A). The fresh twig and stem material (Figure 11 A) had a silvery-green-grey appearance but was a light brown colour after being dried (Figure 11 B). While in the case of the ethanol extract, it was noted that the leaf material retained its silvery-green-grey appearance while submerged in the solvent, and that the solvent took on a yellowish-brown appearance (Figure 12). In the case of the stored dried leaf material, it appeared slightly more yellow in colour (Figure 10 C). Both the rotary evaporated and the freeze-dried extract powders were dark brown in appearance. The results of the organoleptic characteristics of the plant material are shown in Table 6.

In the literature, the bitter taste and the use of *Lessertia frutescens* as a bitter tonic is often mentioned (Roberts, 1990; Van Wyk *et al*., 1997; Van Wyk and Wink, 2004; Van Wyk, 2008). The characteristic bitter taste and strong bitter aromatic smell of the dried material gave the impression of being much stronger in the dried material than that of the fresh material. The characteristic bitter taste was definitely stronger in the ethanol extract, than that of the fresh or dried material. In the case of the rotary evaporated and freeze-dried extract powders, both were much stronger tasting than either the fresh, dried or ethanolic extract comparisons.

Table 6: Organoleptic characteristics of *Lessertia frutescens,* visual identification details.

[a] 5 g of material was placed against a white background and its colour observed.

[b] 5 ml of liquid was placed in a white porcelain dish and its colour observed.

Sample	Appearance
[a] Fresh leaf material	Silvery-green-grey
[a] Dried leaf material	Slightly lighter silvery-green-grey
[a] Stored dried leaf material	Slightly yellowish
[a] Fresh twig and stem material	Silvery-green-grey
[a] Dried twig and stem material	Light brown
[b] Ethanol extract	Yellowish-brown
Rotary evaporated extract powder	Dark brown
Freeze-dried extract powder	Dark brown

Figure 10: A: Fresh leaves of *Lessertia frutescens*.

 B: Dried leaves of *Lessertia frutescens*.

 C: Stored dried leaves of *Lessertia frutescens*.

Figure 11: A: Fresh twig and stem material of *Lessertia fruescens*.

         B: Dried twig and stem material of *Lessertia fruescens*.

Figure 12: Ethanolic extract of *Lessertia frutescens* in a porcelain dish.

3.2 Yield of plant preparation and extraction

The drying of the plant material reduced the weight of the fresh leaf material by an average of 52.3%, which produced an average yield of 52.1 g per sample. However, in the case of the twig and stem material, drying reduced the weight of the fresh material by only 31.8%, to produce a yield of 75 g dried material. The average weight and percentage yields after drying are presented in Table 7.

Table 7: Average yields of *Lessertia frutescens* leaves, twigs and stem material after drying, in a ventilated oven at 35°C, for three days. Average was calculated for leaf material only.

Sample	Parts used	Fresh material (g)	Yield of dried material (g)	Yield of dried material (%)
LF1A	Leaves	110	50.5	45.9
LF1B	Leaves	110	51	46.4
LF2	Leaves	110	53	48.2
LF3	Leaves	110	54	49.1
LF4	Leaves	110	52	47.3
Average			**52.1**	**47.4**
LF1C	Twigs/stems	110	75	68.2

The separation of ethanolic extract from the marc (solid material left after extraction) reduced the volume of liquid by an average of 30.2%, which produced an average yield of 279.2 ml per sample. In the case of the twig and stem material, the separation reduced the volume of liquid by 35%, to produce a yield of 260 ml ethanolic extract. There was no significant difference in the yield

between summer, autumn, winter and spring extractions. The lowest yield was that of the summer sample (LF1A), being 68.5% and the highest yield was that of spring, 71%, in comparison with the average of 69.5%. The average volume of liquid and percentage yields after separation are presented in Table 8.

Table 8:   Average volume of liquid and percentage yields after separation of the ethanolic extract from the marc. Average was calculated for leaf material only.

Sample	Parts used	Dried material (g)	Ethanol material (ml)	Yield on separation (ml)	Yields on separation (%)
LF1A	Leaves	50	400	274	68.5
LF1B	Leaves	50	400	276	69
LF2	Leaves	50	400	280	70
LF3	Leaves	50	400	282	70.5
LF4	Leaves	50	400	284	71
Average				**279.2**	**69.8**
LF1C	Twigs/stems	50	400	260	65

The rotary evaporation of the ethanolic extract made from 50 g of leaf matter produced an average of 3.99 g of extract powder, which was an average yield of 7.98%. For the twig and stem material, 3.80 g was produced, reflecting a yield of 7.60%.   No significant difference was noted in the yield between summer, autumn, winter and spring extractions. The lowest yield was that of the summer sample (LF1A), being 7.88% and the highest yield was that of winter and spring,

8.04%, in comparison with the average of 7.98%. Table 9 indicates the average weight and percentage yields after rotary evaporation.

Table 9: Average yields of *Lessertia frutescens* leaves, twigs and stem material, after rotary evaporation of the ethanolic extracts, at 40°C, for three days. Average was calculated for leaf material extracts only.

Sample	Parts used	Dried material (g)	Extract powder (g)	Loss on evaporation (g)	Yield on separation (%)
LF1A	Leaves	50	3.94	46.06	7.88
LF1B	Leaves	50	3.99	46.01	7.98
LF2	Leaves	50	3.99	46.01	7.98
LF3	Leaves	50	4.02	45.98	8.04
LF4	Leaves	50	4.02	45.98	8.04
Average				**46.01**	**7.98**
LF1C	Twigs/stems	50	3.80	46.20	7.60

3.3 HPLC analysis

The calibration curve of the HPLC analysis for the leaf material indicated a linear correlation between peak area and concentration in the range of 0.0104125 to 0.166 g/ml. This was described by the regression equation being $Y = 166442 X + 3584.1$ and the correlation coefficient of $R^2 = 0.9812$. The results from the HPLC analysis for accuracy of the summer sample, as an example, are presented in Figure 13.
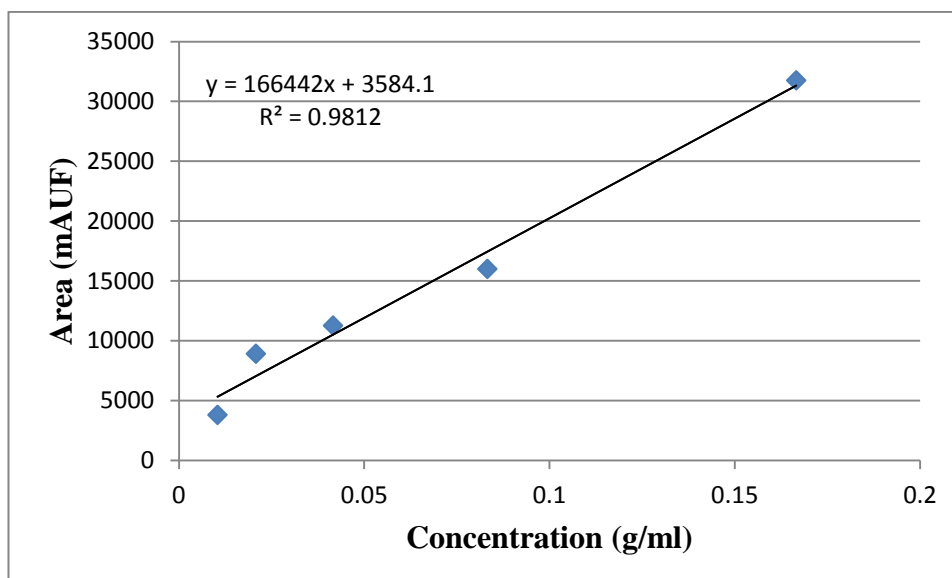
Figure 13: Calibration curve and linear regression line for the determinations of the respected concentrations of *Lessertia frutescens* leaf material.

The calibration curves for the flavonoids (sutherlandin A and sutherlandin D) and triterpenoids (sutherlandioside B and sutherlandioside D) showed the following regression equations and correlation coefficients (R^2) between peak area and concentration depicted in Table 10 and Figures 14 to 17, where Y = peak area and X = concentration.
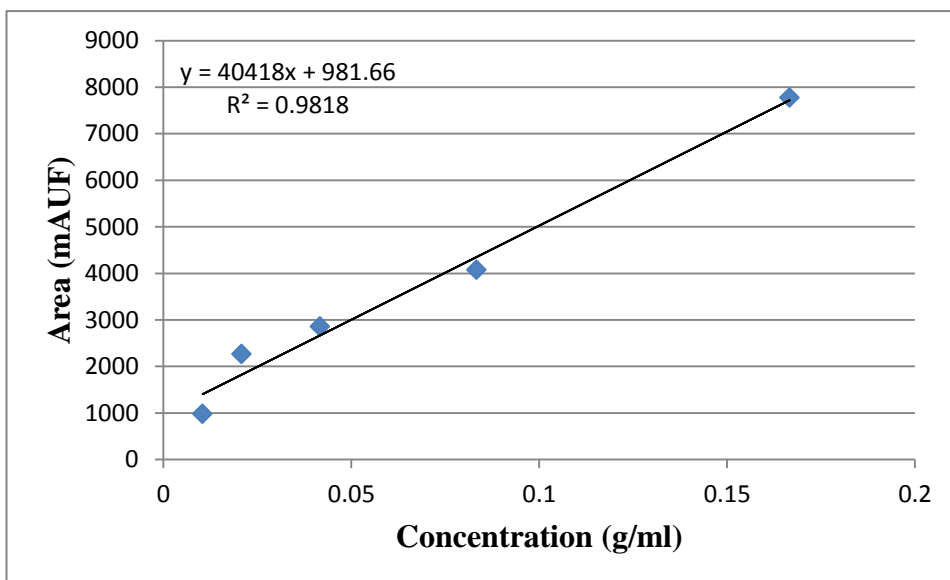
Figure 14: Calibration curve and linear regression line for the determinations of the sutherlandin A concentrations in *Lessertia frutescens*.
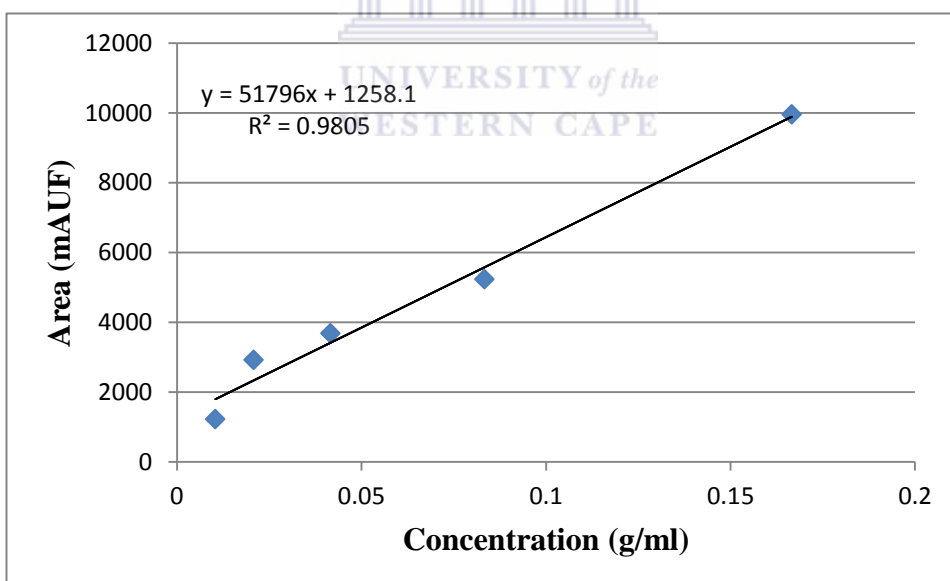
Figure 15: Calibration curve and linear regression line for the determinations of the sutherlandin D concentrations in *Lessertia frutescens*.
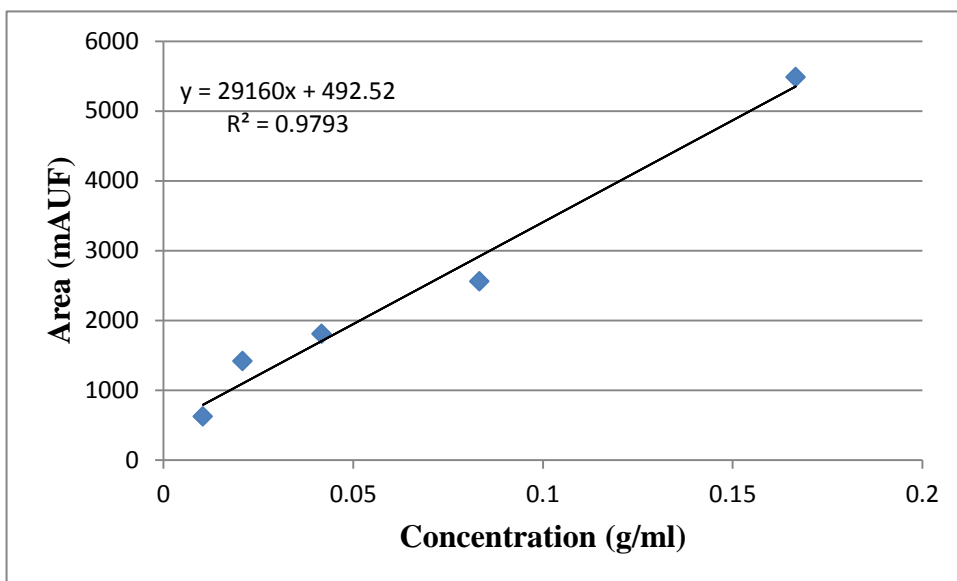
Figure 16: Calibration curve and linear regression line for the determinations of the sutherlandioside B concentrations of *Lessertia frutescens*.
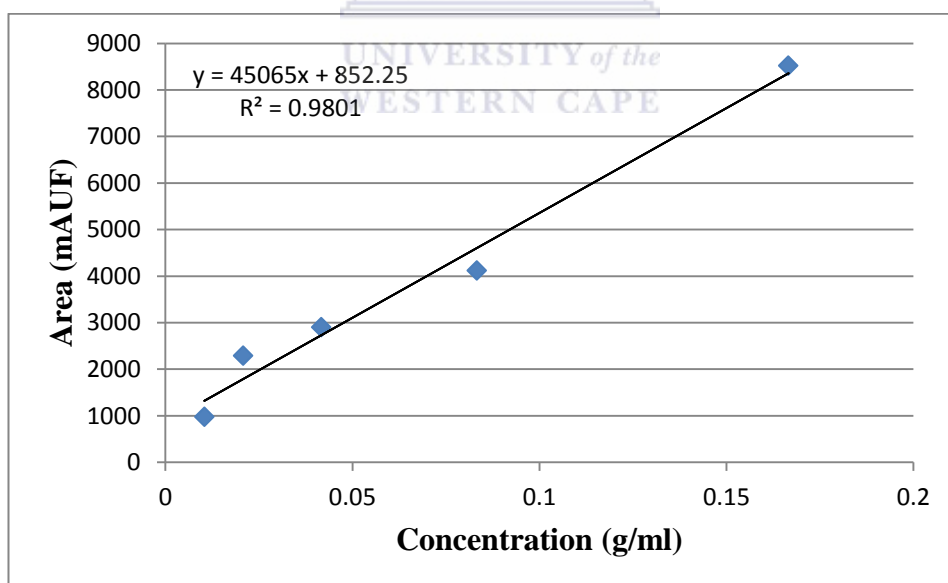
Figure 17: Calibration curve and linear regression line for the determinations of the sutherlandioside D concentrations in *Lessertia frutescens*.

Table 10: Regression equation and correlation efficient for sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

Chemical constituent	Regression equation	Correlation coefficient (R^2)
Sutherlandin A	Y = 40418 X + 981.66	0.9818
Sutherlandin D	Y = 51796 X + 1258.1	0.9805
Sutherlandioside B	Y = 29160 X + 492.52	0.9793
Sutherlandioside D	Y = 45065 X + 852.25	0.9801

The results for the intra- and inter-day precision of the HPLC analysis are shown in Table 11. These were achieved by injecting 10µL into the column, corresponding to a concentration of 0.166 g/ml of the extracts. Results are expressed as mean ± SD. The average intra-day precision was 1789.96 ± 16.25 mAUF for sutherlandin A, 1549.58 ± 525.10 mAUF for sutherlandin D, 1810.35 ± 30.00 mAUF for sutherlandioside B and 2600.65 ± 34.56 mAUF for sutherlandioside D. While for the average inter-day precision was 1789.95 ± 16.49 mAUF for sutherlandin A, 1640.84 ± 482.30 mAUF for sutherlandin D, 1810.36 ± 34.41 mAUF for sutherlandioside B and 2600.65 ± 42.48 mAUF.

Table 11: Intra- and inter-day precision of samples for the validation of the HPLC method of the constituents, sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

Chemical constituent	Intra-day (mAUF) (mean ± SD) (n=3)			Inter-day (mAUF) (mean ± SD) (n=3)
	Day 1	Day 2	Day 3	
Sutherlandin A	1789.07 ± 22.11	1789.47 ± 17.62	1791.33 ± 9.02	1789.95 ± 16.49
Sutherlandin D	1571.07± 609.41	1822.72± 365.65	1254.94± 600.23	1640.84± 482.3
Sutherlandioside B	1802.21 ± 30.21	1796.30 ± 17.40	1832.55 ± 42.38	1810.36 ± 34.41
Sutherlandioside D	2615.93 ± 41.51	2619.45 ± 20.08	2566.58 ± 42.10	2600.65 ± 42.48
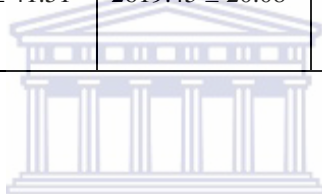
3.4 Flavonoid and triterpenoid profile of the ethanolic extracts

Retention times (mins) were used in order to identify individual flavonoids (sutherlandin A and sutherlandin D) and triterpenoids (sutherlandioside B and sutherlandioside D) (Figure 18), while the peak areas milli-Absorbance Units (mAUF) were the parameters used to determine the level of flavonoids and triterpenoids in the samples (Table 12). An example of a chromatogram fingerprint representative of the summer season is presented in Figure 19 A and 20 A. These peak areas had average retention times of 9.186 ± 0.024 min, 13.128 ± 0.030 min, 35.559 ± 0.048 min and 36.139 ± 0.042 min; the autumn season (Figure 19 B) of 9.234 ± 0.005 min, 13.171 ± 0.008 min, 35.574 ± 0.018 min and 36.141 ± 0.002 min; the winter season (Figure 19 C) of 9.243 ± 0.005

min, 13.169 ± 0.006 min, 35.651 ± 0.002 min and 36.232 ± 0.003 min; the spring season (Figure 19 D) of 9.249 ± 0.013 min, 13.196 ± 0.013 min, 35.619 ± 0.030 min and 36.200 ± 0.021 min; the stem sample (Figure 20 B) of 9.261 ± 0.010 min, 13.227 ± 0.011 min, 35.615 ± 0.020 min and 35.860 ± 1.152 min; the storage sample (Figure 20 C) of 9.260 ± 0.033 min, 13.208 ± 0.029 min, 35.611 ± 0.042 min and 36.185 ± 0.041 min.
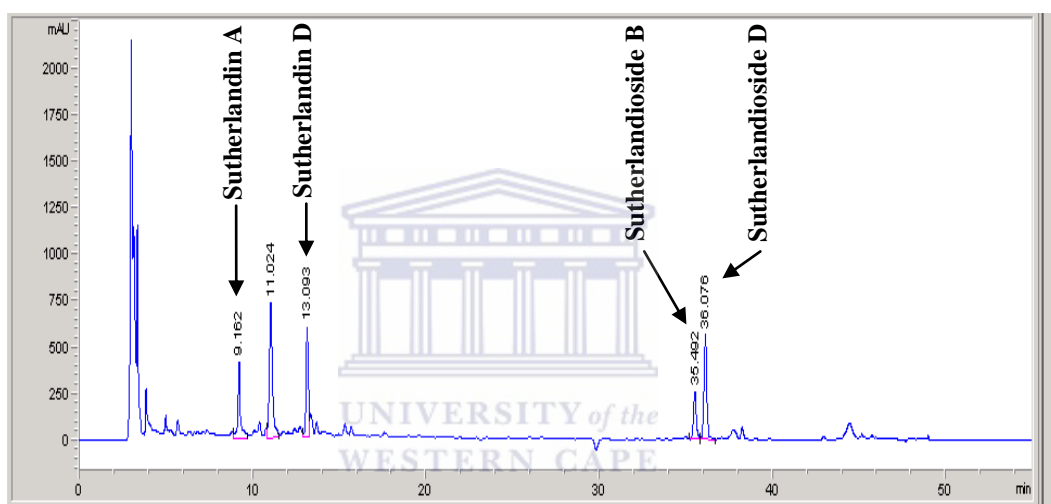
Figure 18: HPLC chromatogram for the summer sample (LF1A) indicating retention times of sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.
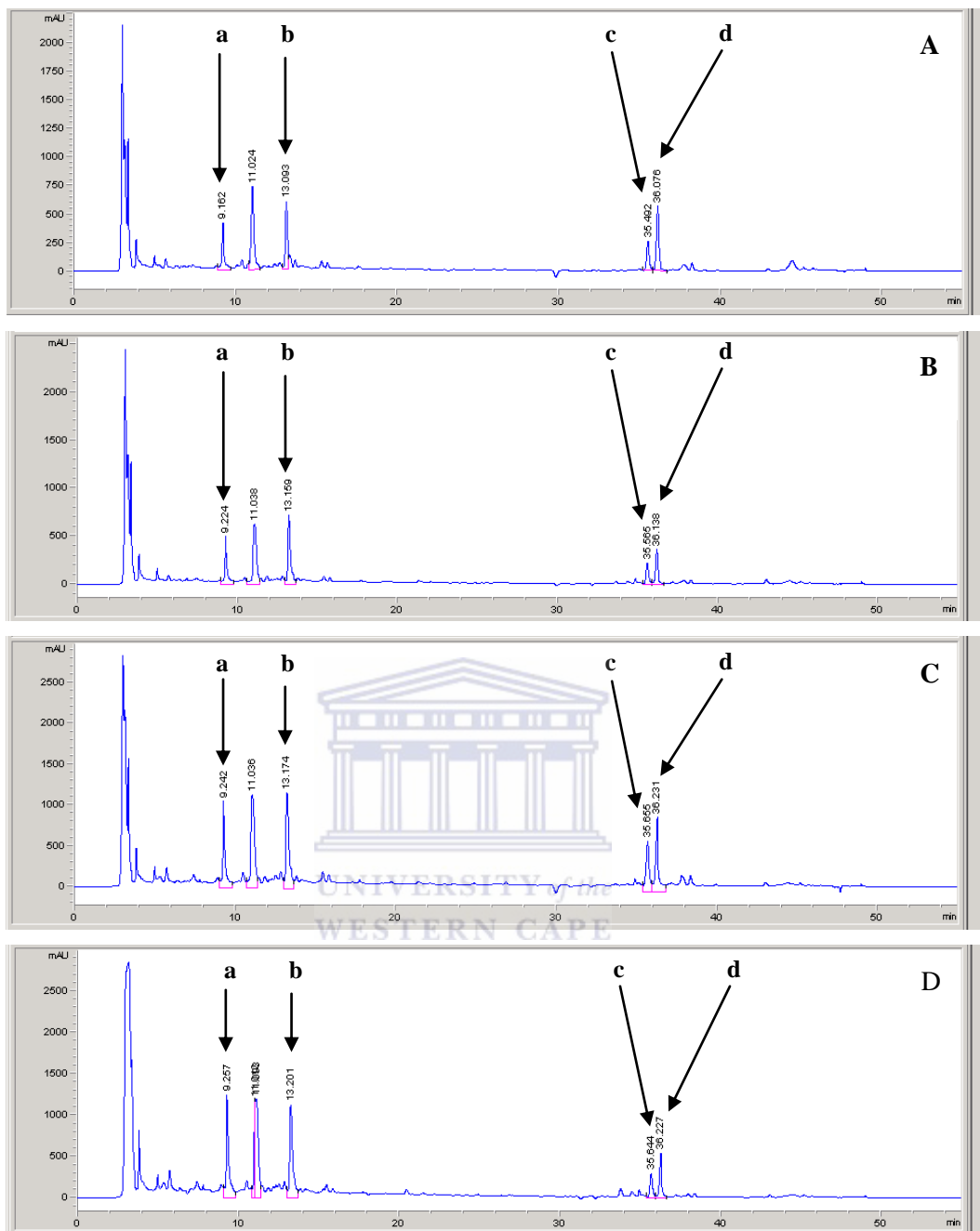
Figure 19: Sample HPLC chromatograms from material collected in different seasons

       A: HPLC chromatogram for the summer sample (LF1A)

       B: HPLC chromatogram for the autumn sample (LF2)

       C: HPLC chromatogram for the winter sample (LF3)

       D: HPLC chromatogram for the spring sample (LF4)

Peaks indicate the following:

a: sutherlandin A; b: sutherlandin D; c: sutherlandioside B; d: sutherlandioside D
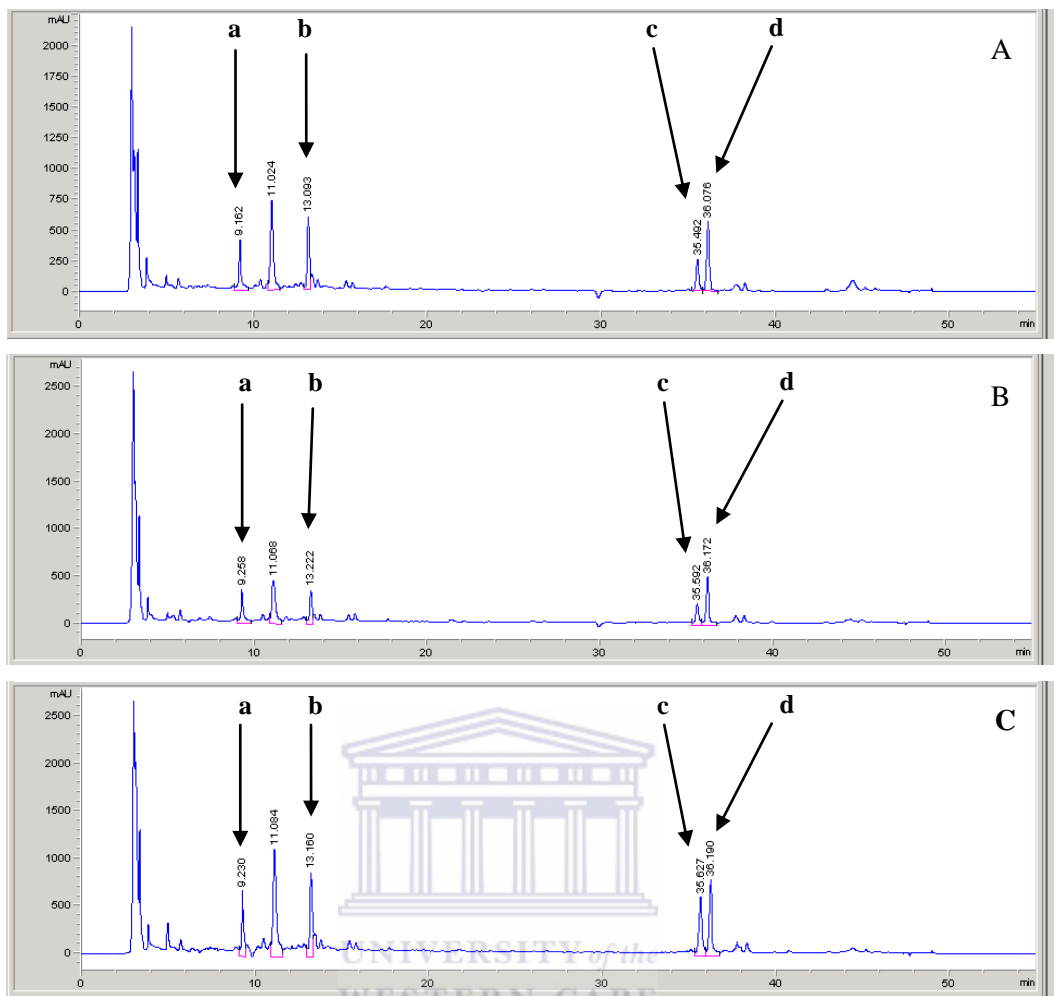
Figure 20: Sample HPLC chromatograms from leave and stem material

      A: HPLC chromatogram for the summer sample (LF1A)

      B: HPLC chromatogram for the stem sample (LF1C)

      C: HPLC chromatogram for the storage sample (LF1B)

Peaks indicate the following:

a: sutherlandin A; b: sutherlandin D; c: sutherlandioside B; d: sutherlandioside D

Table 12: Calculated concentrations of flavonoids and triterpenoids after HPLC analysis of leave material from different seasons, storage and stem samples of *Lessertia frutescens.* Concentrations were calculated according to peak areas.

Season, storage, twigs/stem	Sutherlandin A (*n* = 12) (mg/ml)	Sutherlandin D (*n* = 12) (mg/ml)	Sutherlandioside B (*n* = 12) (mg/ml)	Sutherlandioside D (*n* = 12) (mg/ml)
Summer	3.67 ± 0.01	4.10 ± 0.01	3.01 ± 0.03	5.82 ± 0.04
Autumn	4.75 ± 0.04	6.37 ± 0.03	2.15 ± 0.03	3.33 ± 0.03
Winter	4.23 ± 0.01	5.25 ± 0.01	2.89 ± 0.02	4.23 ± 0.02
Spring	6.56 ± 0.02	6.08 ± 0.02	1.47 ± 0.01	2.50 ± 0.02
Storage	4.07 ± 0.05	4.25 ± 0.02	2.82 ± 0.11	4.66 ± 0.03
Twigs / stem	4.67 ± 0.08	3.31 ± 0.06	3.62 ± 0.01	5.80 ± 0.14

3.5 Comparison of the flavonoid and triterpenoid levels

The peak area was the parameter used in order to compare the levels of flavonoids (sutherlandin A and sutherlandin D) and triterpenoids (sutherlandioside B and sutherlandioside D). Sets of results were established for each of the four constituents, representative of the four seasons, as well as for the stem and storage component of the study, collected during the summer season, and thus were compared to the summer season sample.

The results of the chromatogram fingerprint peak area calculated concentrations for the seasonal samples ranged between 3.67 and 6.56 mg/ml for sutherlandin A, 4.10 and 6.37 mg/ml for sutherlandin D, 1.47 and 3.01 mg/ml for sutherlandioside B, and 2.50 and 5.82 mg/ml for sutherlandioside D. This indicated that the

highest seasonal concentrations for sutherlandin A were 6.56 ± 12.49 mg/ml, for sutherlandin D 6.37 ± 10.32 mg/ml, for sutherlandioside B 3.01 ± 7.19 mg/ml and for sutherlandioside D 5.82 ± 14.48 mg/ml.

For the summer leaf and storage samples concentrations of the constituents ranged between 3.67 and 4.07 mg/ml for sutherlandin A, 4.10 and 4.25 mg/ml for sutherlandin D, 3.01 and 3.62 mg/ml for sutherlandioside B, and 4.66 and 5.82 mg/ml for sutherlandioside D. This indicated that the highest summer/storage concentrations for sutherlandin A were 4.07 ± 2.88 mg/ml, for sutherlandin D 4.25 ± 1.06 mg/ml, for sutherlandioside B 3.62 ± 0.39 mg/ml and for sutherlandioside D 5.82 ± 0.42 mg/ml.

For the summer leaf and stem samples, the readings ranged between 3.67 and 4.67 mg/ml for sutherlandin A, 3.31 and 4.10 mg/ml for sutherlandin D, 2.82 and 3.01 mg/ml for sutherlandioside B, and 5.80 and 5.82 mg/ml for sutherlandioside D. This indicated that the highest summer/stem concentrations for sutherlandin A were 4.67 ± 8.18 mg/ml, for sutherlandin D 4.10 ± 4.81 mg/ml, for sutherlandioside B 3.01 ± 4.24 mg/ml and for sutherlandioside D was 5.82 ± 0.42 mg/ml.

3.5.1 Flavonoid and triterpenoid levels of the seasonal samples

The season with the highest yield of sutherlandin A levels was spring (6.56 ± 12.49 mg/ml), followed by autumn (4.75 ± 12.49 mg/ml), winter (4.23 ± 12.49 mg/ml) and then summer (3.67 ± 12.49 mg/ml) having the lowest levels. This accounted for 34.00 % of the total yield for sutherlandin A (Table 13). The highest yield of sutherlandin D levels was autumn (6.37 ± 10.32 mg/ml), followed by spring (6.08 ± 10.32 mg/ml), winter (5.25 ± 10.32 mg/ml) and then summer (4.10 ± 10.32 mg/ml) having the lowest. This accounted for 29.22 % of the total yield for sutherlandin D (Table 13). The highest yield of sutherlandioside B levels was summer (3.01 ± 7.19 mg/ml), followed by winter (2.89 ± 7.19 mg/ml), autumn (2.15 ± 7.19 mg/ml) and then spring (1.47 ± 7.19 mg/ml) having the lowest. This accounted for 31.61 % of the total yield for sutherlandioside B (Table 13). The highest yield of sutherlandioside D levels was summer (5.82 ± 14.48 mg/ml), followed by winter (4.23 ± 14.48 mg/ml), autumn (3.33 ± 14.48 mg/ml) and then spring (2.50 ± 14.48 mg/ml) having the lowest. This accounted for 36.78 % of the total yield for sutherlandioside D (Table 13).

Table 13: Percentage portion of the comparison of the four seasons relating to sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

[a] The percentage portion of the total yield for a particular constituent across the four seasons.

[b] The percentage proportion for a particular season from the total yield of sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

Constituent	Summer (mg/ml) [a] (%) [b] (%)	Autumn (mg/ml) [a] (%) [b] (%)	Winter (mg/ml) [a] (%) [b] (%)	Spring (mg/ml) [a] (%) [b] (%)
Sutherlandin A	3.67 (19.13) (22.05)	4.75 (24.96) (28.73)	4.23 (21.91) (25.38)	6.56 (34.00) (39.33)
Sutherlandin D	4.10 (18.81) (24.59)	6.37 (29.22) (38.31)	5.25 (24.08) (31.56)	6.08 (27.89) (36.58)
Sutherlandioside B	3.01 (31.61) (18.17)	2.15 (22.59) (12.97)	2.89 (30.26) (17.49)	1.47 (15.54) (8.97)
Sutherlandioside D	5.82 (36.78) (35.19)	3.33 (20.91) (19.99)	4.23 (26.57) (25.57)	2.50 (15.74) (15.12)

Independent *t* tests were performed to compare the concentrations averages of sutherlandin A found in summer, autumn, winter and spring, with one another. The same procedure was repeated for the comparison with regards to sutherlandin D, sutherlandioside B and sutherlandioside D. The sutherlandin A concentration (6.56 ± 12.49 mg/ml) in material harvested in spring differs from all other seasons significantly ($P < 0.0001$). For the sutherlandin D concentration (6.37 ± 10.32 mg/ml) in material harvested in autumn differs from all other

seasons significantly (P < 0.0001). For the sutherlandioside B concentration (3.01 ± 7.19 mg/ml) in material harvested in summer differs from all other seasons significantly (P < 0.0001). For the sutherlandioside D concentration (5.82 ± 14.48 mg/ml) in material harvested in summer differs from all other seasons significantly (P < 0.0001). The seasonal variations for the four constituents, sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D are presented in Figures 21 to 24.

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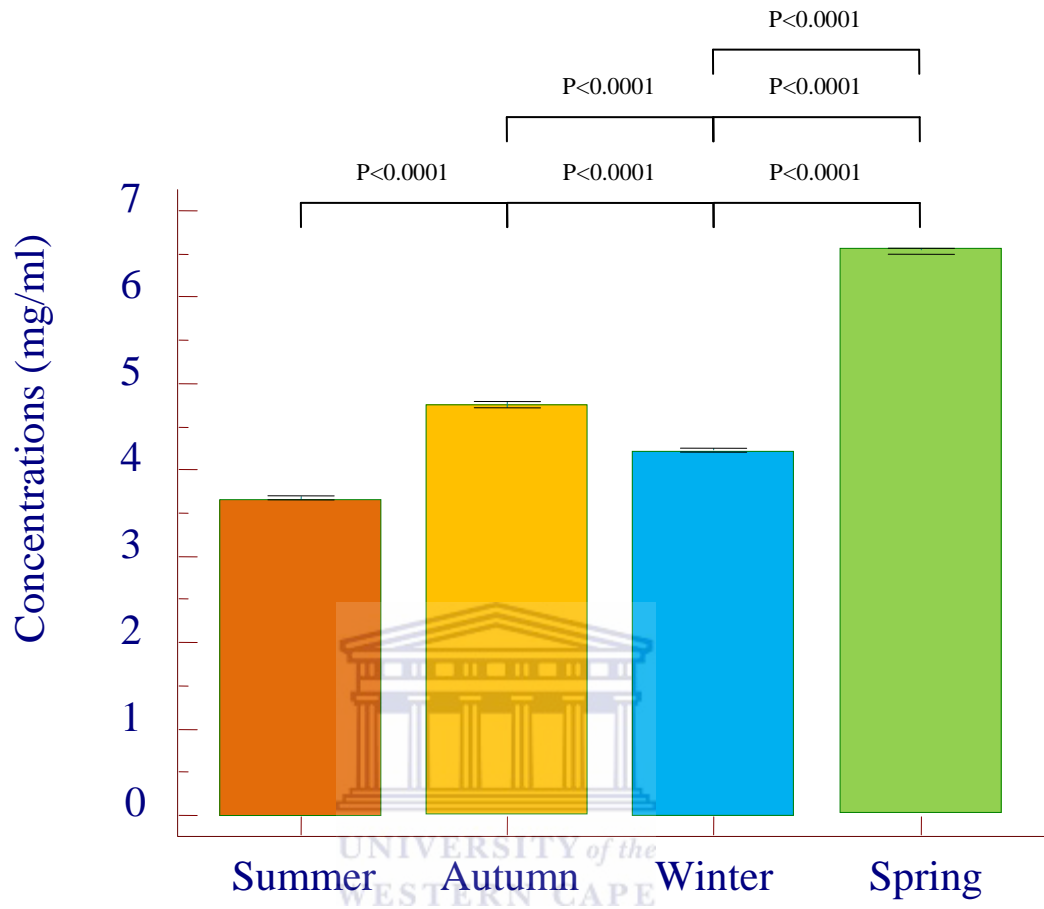
Figure 21: Concentrations of sutherlandin A in leaf material of summer, autumn, winter and spring. The sutherlandin A concentration (6.56 ± 12.49 mg/ml) harvested in spring is the highest and differs from all other seasons significantly (P<0.0001).
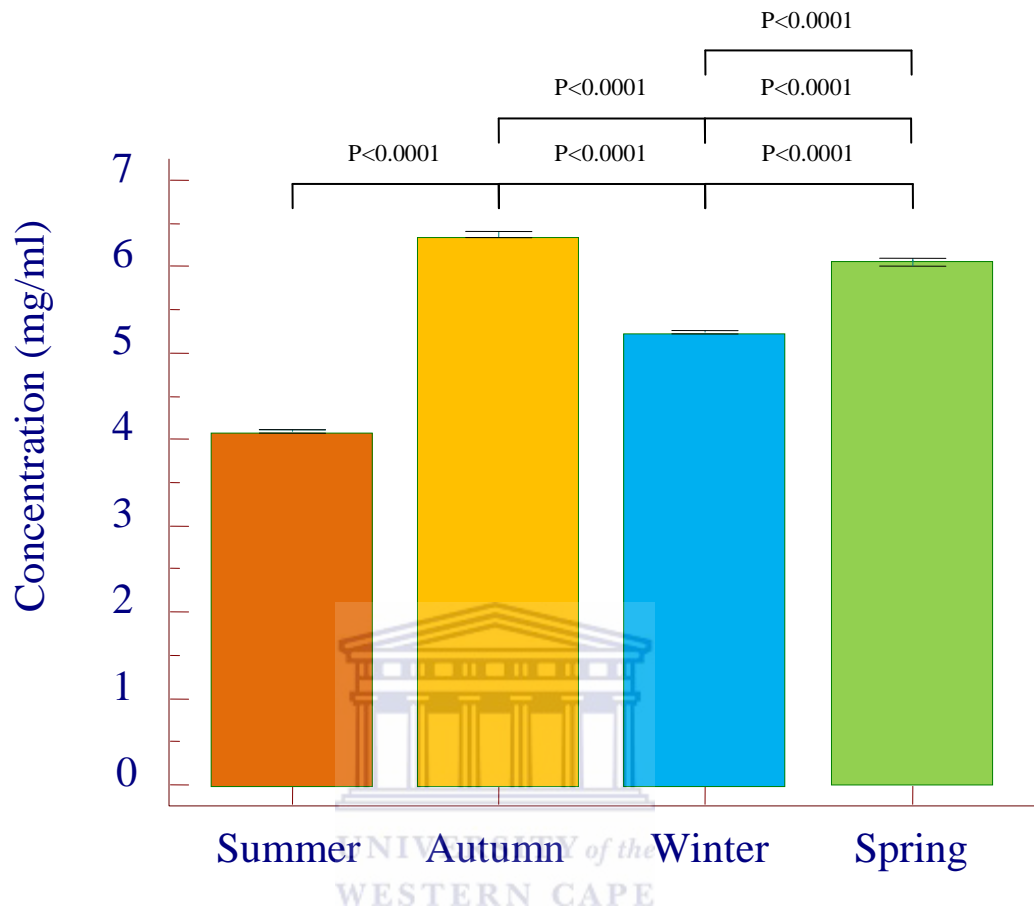
Figure 22: Concentrations of sutherlandin D in leaf material of summer, autumn, winter and spring. The sutherlandin D concentration (6.37 ± 10.32 mg/ml) harvested in autumn is the highest and differs from all other seasons significantly (P<0.0001).
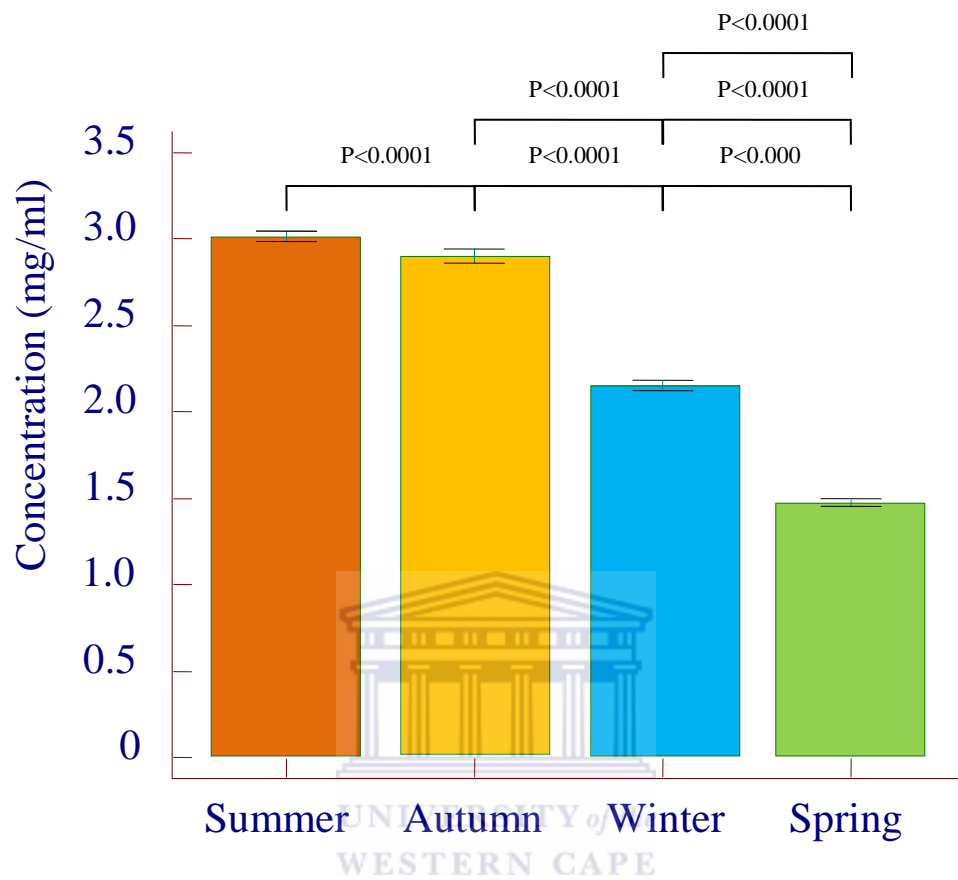
Figure 23: Concentrations of sutherlandioside B in leaf material of summer, autumn, winter and spring. The sutherlandioside B concentration (3.01 ± 7.19 mg/ml) harvested in summer is the highest and differs from all other seasons significantly (P<0.0001).
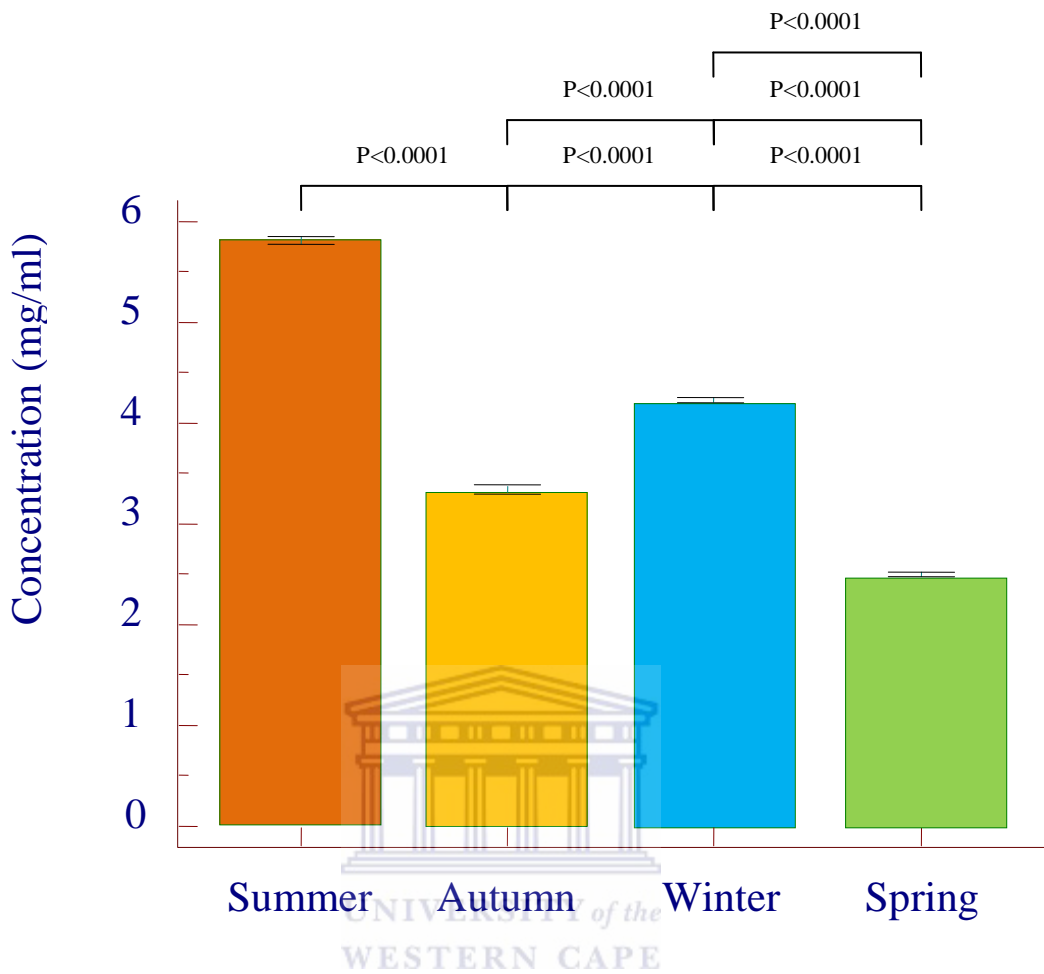
Figure 24: Concentrations of sutherlandioside D in leaf material of summer, autumn, winter and spring. The sutherlandioside D concentration (5.82 ± 14.48 mg/ml) harvested in summer is the highest and differs from all other seasons significantly (P<0.0001).

3.5.2 Flavonoid and triterpenoid levels of the storage samples

For the comparison of the levels of flavonoids and triterpenoids in the summer leaf material and storage samples, the highest yield of sutherlandin A levels was for that of the storage sample (4.07 ± 2.88 mg/ml) and then summer (3.67 ± 2.88 mg/ml) having the lowest levels. The storage sample accounted for 52.60 % of the total yield for sutherlandin A (Table 14). The highest yield of sutherlandin D levels was that of the storage sample (4.25 ± 1.06 mg/ml) and then summer (4.10 ± 1.06 mg/ml) having the lowest. The storage sample accounted for 50.89 % of the total yield for sutherlandin D (Table 14). The highest yield of sutherlandioside B levels was summer (3.01 ± 0.39 mg/ml) and then storage (2.82 ± 0.39 mg/ml) having the lowest. The summer sample accounted for 50.83 % of the total yield for sutherlandioside B (Table 14). The highest yield of sutherlandioside D levels was summer (5.82 ± 0.42 mg/ml) and then storage (4.66 ± 0.42 mg/ml) having the lowest. The summer sample accounted for 55.65 % of the total yield for sutherlandioside D (Table 14).

Table 14: Percentage portion of the comparison of the summer leaf sample to that of the storage sample, relating to sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

[a] The percentage portion of the total yield for a particular constituent.

[b] The percentage proportion from the total yield of sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

Constituent	Summer (mg/ml) [a] (%) [b] (%)	Storage (1 year) (mg/ml) [a] (%) [b] (%)
Sutherlandin A	3.67 (47.40) (22.05)	4.07 (52.60) (25.61)
Sutherlandin D	4.10 (49.11) (24.60)	4.25 (50.89) (26.67)
Sutherlandioside B	3.01 (50.83) (18.16)	2.82 (49.17) (18.38)
Sutherlandioside D	5.82 (55.65) (35.19)	4.66 (44.35) (29.34)

Concentration averages of sutherlandin A found in summer leaf samples were compared with that of the storage sample. The same procedure was repeated for the comparison with regards to sutherlandin D, sutherlandioside B and sutherlandioside D. The sutherlandin A concentration (4.07 ± 2.88 mg/ml) of the storage sample was higher than that of the summer leaf sample (3.67 ± 2.88 mg/ml), which was a significant difference (P < 0.0001). The sutherlandin

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D concentration (4.25 ± 1.06 mg/ml) of the storage sample was significantly (P < 0.0001) higher than that of the summer leaf sample (4.10 ± 1.06 mg/ml). For the sutherlandioside B concentration (2.82 ± 0.39 mg/ml) of the storage sample was significantly (P < 0.0001) higher than that of the summer leaf sample (3.01 ± 0.39 mg/ml). For the sutherlandioside D concentration (5.82 ± 0.42 mg/ml) of the summer leaf sample was significantly (P < 0.0001) higher than that of the storage sample (4.66 ± 0.42 mg/ml). The variations for the four constituents, sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D are presented in Figures 25 to 28.

3.5.3 Flavonoid and triterpenoid levels of the stem samples

For the comparison of the level of flavonoids and triterpenoids in the summer leaf and stem samples, the highest yield of sutherlandin A levels were obtained in the stem material (4.67 ± 8.18 mg/ml) compared to leaves harvested in summer (3.67 ± 8.18 mg/ml). This difference is highly significant (P < 0.0001). The stem sample accounted for 56.76 % of the total yield for sutherlandin A (Table 15). The highest (P < 0.0001) yield of sutherlandin D levels was in summer samples (4.10 ± 4.81 mg/ml) compared to the stems (3.31 ± 4.81 mg/ml). The summer sample accounted for 54.49 % of the total yield for sutherlandin D (Table 15). The highest yield of sutherlandioside B levels were measured in stems (3.62 ± 4.24 mg/ml), while leaves harvested in summer (3.01 ± 4.24 mg/ml) having the lowest (P < 0.0001). The stem sample accounted for 54.48 % of the total yield for sutherlandioside B (Table 15). The highest

(P < 0.0001) yield of sutherlandioside D levels was found in the summer leaf sample (5.82 ± 0.42 mg/ml) compared to the stem samples (5.80 ± 0.42 mg/ml). The summer leaf sample accounted for 50.25 % of the total yield for sutherlandioside D (Table 15).

Table 15: Percentage portion of the comparison of the summer leaf sample to that of the stem sample, relating to sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

[a] The percentage portion of the total yield for a particular constituent.

[b] The percentage proportion from the total yield of sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D.

Constituent	Leaves (summer) (mg/ml) [a] (%) [b] (%)	Stem (summer) (mg/ml) [a] (%) [b] (%)
Sutherlandin A	3.67 (43.24) (22.05)	4.67 (56.76) (27.11)
Sutherlandin D	4.10 (54.49) (24.59)	3.31 (45.51) (19.24)
Sutherlandioside B	3.01 (45.52) (18.17)	3.62 (54.48) (20.36)
Sutherlandioside D	5.82 (50.25) (35.19)	5.80 (49.75) (33.29)

Concentration averages of sutherlandin A found in summer leaf sample were compared with those of the stem samples. The same procedure was repeated for the comparison with regards to sutherlandin D, sutherlandioside B and sutherlandioside D. The sutherlandin A concentration (4.67 ± 8.18 mg/ml) of the stem sample were significantly higher ($P < 0.0001$) than that of the summer leaf sample (3.67 ± 8.18 mg/ml). The sutherlandin D concentration (4.10 ± 4.81 mg/ml) of the summer leaf sample showed significantly ($P < 0.0001$) higher values compared to the stem sample (3.31 ± 4.81 mg/ml). Similar results were obtained for sutherlandioside B (summer leaf: 3.01 ± 4.24 mg/ml versus stem: 3.62 ± 4.24 mg/ml) and sutherlandioside D (summer leaf: 5.82 ± 0.42 mg/ml versus stem: 5.80 ± 0.42 mg/ml), which was of significant difference ($P < 0.0001$). The variations for the four constituents, sutherlandin A, sutherlandin D, sutherlandioside B and sutherlandioside D are presented in Figures 25 to 28.

When comparing the flavonoid and triterpenoid concentrations in the stems with those of stored leaves results reveal significantly higher levels of sutherlandin A (stem: 4.67 ± 8.18 mg/ml; storage: 4.07 ± 2.88 mg/ml) and D (stem: 3.31 ± 4.81 mg/ml; storage: 4.25 ± 1.06 mg/ml), which was of significant difference ($P < 0.0001$). In contrast, the effect of storage on the sutherlandioside B (stem: 3.62 ± 4.24 mg/ml; storage: 2.82 ± 0.39 mg/ml; $P < 0.0001$) and D (stem: 5.80 ± 0.42 mg/ml; storage: 4.66 ± 0.42 mg/ml; $P < 0.0001$) had the opposite effect (Figures 25 to 28).
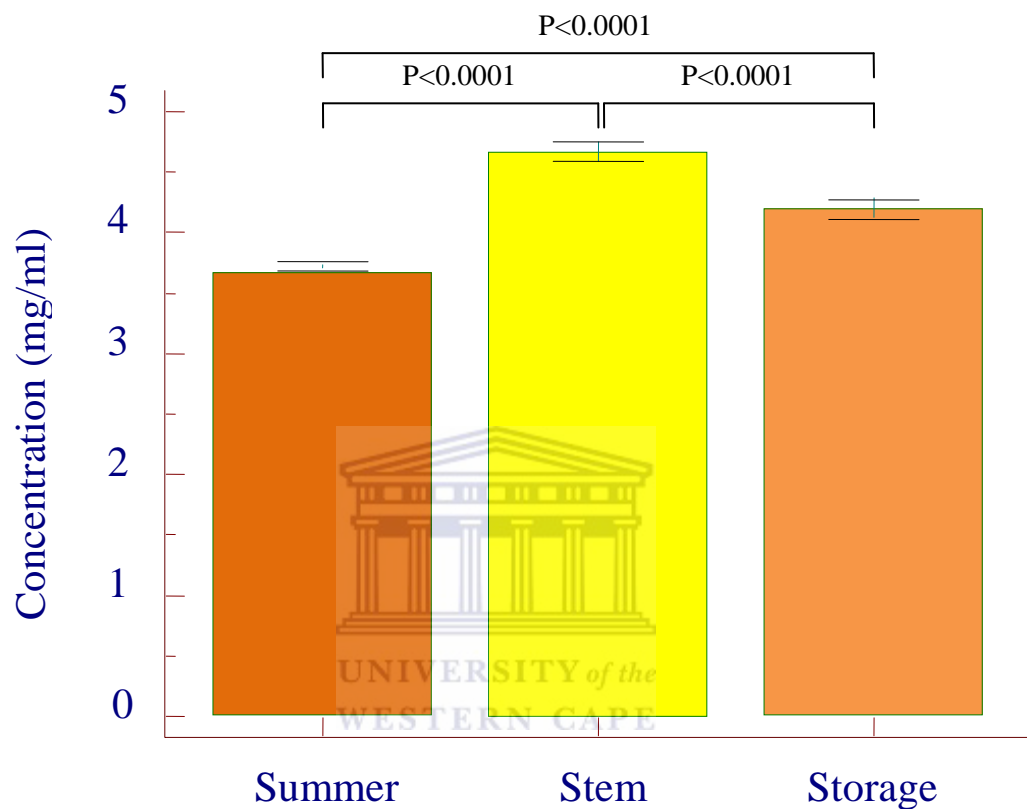
Figure 25: Concentrations of sutherlandin A in the summer, stem and storage samples. The sutherlandin A concentration (4.67 ± 8.18 mg/ml) from the stem sample was the highest, compared to that of the summer sample, which was of significant difference (P<0.0001). The sutherlandin A concentration (4.07 ± 2.88 mg/ml) from the storage sample was the highest, compared to that of the summer sample, which was of significant difference (P<0.0001).
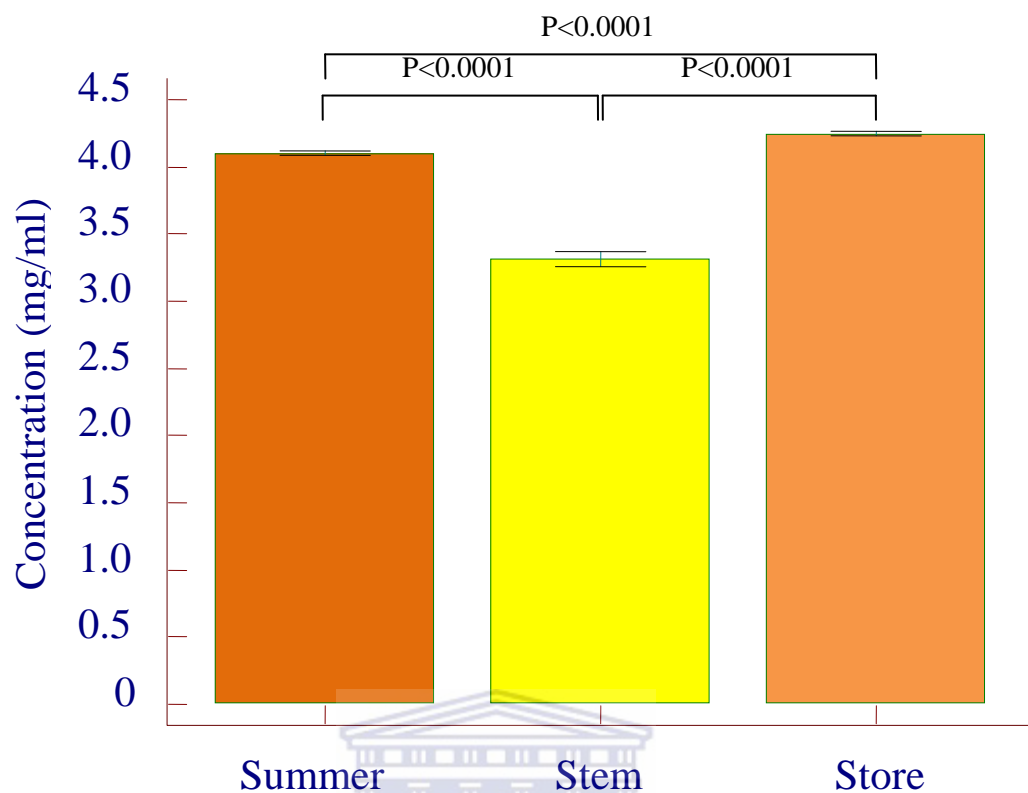
Figure 26: Concentrations of sutherlandin D in the summer, stem and storage samples. The sutherlandin D concentration (4.10 ± 4.81 mg/ml) from the summer sample was the highest, compared to that of the stem sample, which was of significant difference (P<0.0001). The sutherlandin D concentration (4.25 ± 1.06 mg/ml) from the storage sample was the highest, compared to that of the summer sample, which was of significant difference (P<0.0001).
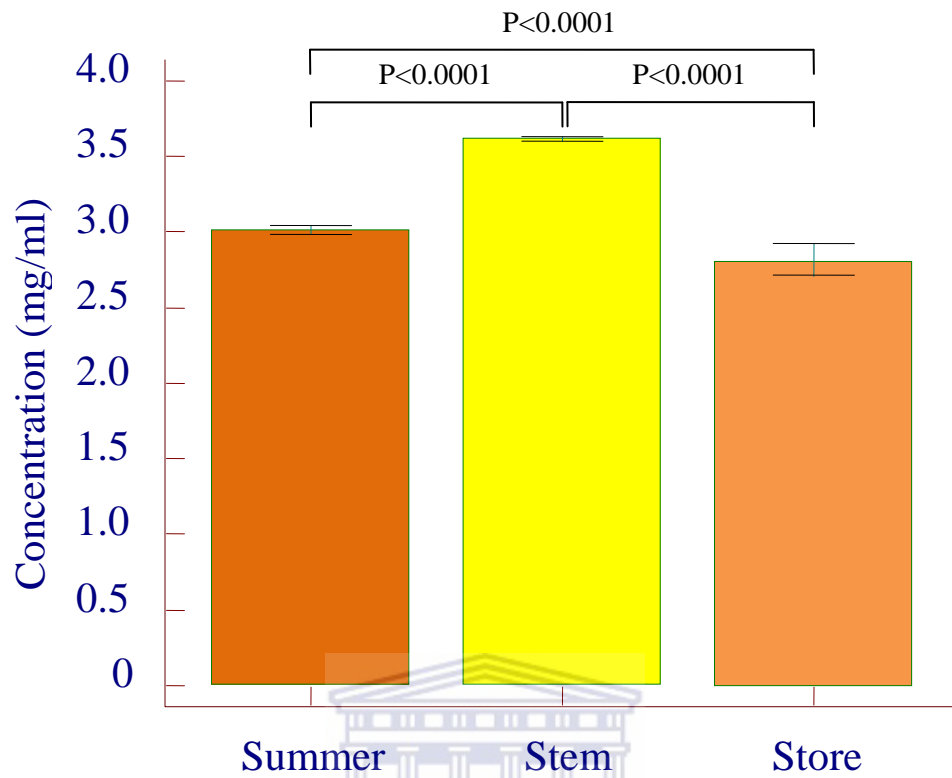
Figure 27: Concentrations of sutherlandioside B in the summer, stem and storage samples. The sutherlandioside B concentration (3.62 ± 4.24 mg/ml) from the stem sample was the highest, compared to that of the summer sample, which was of significant difference (P<0.0001). The sutherlandioside B concentration (2.82 ± 0.39 mg/ml) from the storage sample was the highest, compared to that of the summer sample, which was of significant difference (P<0.0001).
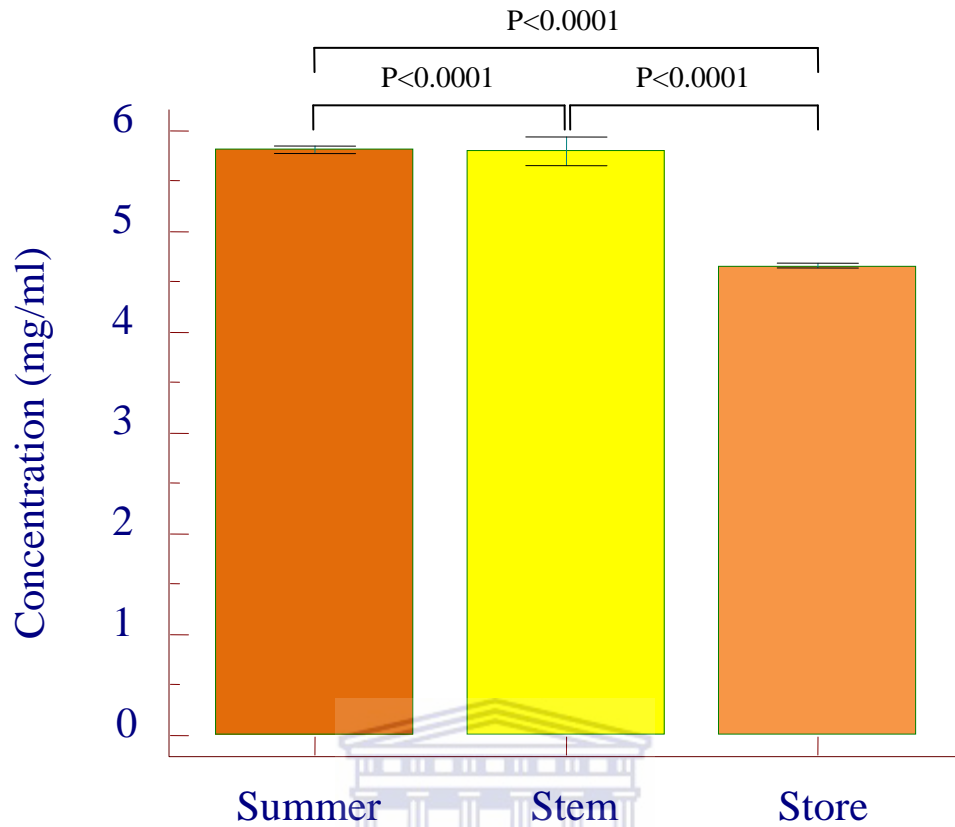
Figure 28: Concentrations of sutherlandioside D in the summer, stem and storage samples. The sutherlandioside D concentration (5.82 ± 0.42 mg/ml) from the summer leaf sample was the highest, compared to that of the stem sample, which was of significant difference (P<0.0001). The sutherlandioside D concentration (5.82 ± 0.42 mg/ml) from the summer sample was the highest, compared to that of the storage sample, which was of significant difference (P<0.0001).

CHAPTER 4

DISCUSSION

4.1 Overview

The aim of this study was to investigate what impact, if any, the duration of storage and a particular season of harvest would have on the flavonoid and triterpenoid compounds levels of *Lessertia frutescens*. In addition, the summer sample was used to compare the results from the leaf material, with that of the stem. Three objectives were needing to be addressed, 1) to investigate storage variation of *Lessertia frutescens* leaves by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids, 2) to investigate seasonal variation of *Lessertia frutescens* leaves by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids, and 3) to investigate leaf and stem variation of *Lessertia frutescens* by comparing the results obtained from the HPLC analysis of the flavonoids and triterpenoids.

What impact would the duration in storage have on the levels of flavonoids or triterpenoids, and would the summer season sample indicate a higher level of the above mentioned biomarkers, compared to the other three seasons? There was mixed opinion from the suppliers and retailers of products containing *Lessertia*

frutescens, as to which part of the plant would have a higher concentration of biologically active constituents, the majority said the leaves (personal information). According to traditional use, the parts of *Lessertia frutescens* used for its medicinal properties, had always been the leaves, along with the bits of stalks and stems, as reported by van Wyk *et al*. (1997), Moshe (1998), and Van Wyk and Albrecht (2008).

The comparison of the four seasonal samples (LF1A, LF2, LF3 and LF4), the summer leaf (LF1A) sample with the storage sample (LF1B), and the summer leaf sample (LF1A) with the stem sample (LF1C) indicated some expected and some unexpected results with regard to the hypotheses of this study. The expectation was that for the four seasonal samples, the spring sample would yield the highest levels of chemical constituents, while the storage sample would yield the same level of constituents as that of the summer leaf sample, and finally that the leaf sample would yield the same levels of constituents as that of the stem sample.

4.2 Comparison of seasonal samples

While for the comparison of the four seasonal samples the results did indicate that the spring sample yielded the highest levels of sutherlandin A, it was the autumn sample that yielded the highest levels of sutherlandin D, and the summer samples for sutherlandioside B and sutherlandioside D.

Previous studies have reported variations in secondary metabolites depending on the season and time of harvest. Jerkovic *et al*. (2001) demonstrated that although *Origanum vulgare* (oregano, wild majoram) samples were harvested from the same geographical location, the different seasons had an effect on the qualitative and quantitative composition of the essential oils, with only a negligible amount of quality loss after one year in storage. However, efficacy of medicinal plants relies on the selection of the correct chemotype. In a study carried out on *Withania somnifera* (ashwaganda), which has three chemotypes, it was noticed that differences varied depending on genotype, time of collection and the environment (Joshi *et al*., 2004). A study on *Bacopa monneri* (brahami), reported a higher level of the active constituent bacoside A, from September to March and then for the month of June (Mathur *et al*., 2002). Active constituents and efficacy vary according to the season or time that medicinal plants are harvested (Singh, 2008). In the case of *Lessertia frutescens* the seasonal variations are in accordance with literature concerning seasonal harvesting in relation to other plant species.

The molecular formula of the two flavonoid glycosides, sutherlandin A ($C_{32}H_{36}O_{20}$) and sutherlandin D ($C_{32}H_{36}O_{19}$) both have the ester 3-hydroxy-3-methylglutaroyl (HMG) moiety attached at C-6 (Fu *et al*., 2010). The esterification at the glucosyl unit may lead to shift changes for C-5 and C-6. The authors thus refer to the two flavonols, sutherlandin A as the aglycone, quercetin and sutherlandin D as the aglycone, kaempferol. Although two flavonoids have

two different sugar moiety's attached, namely β-D-xylospyranose and β-D-glucose, quercetin and kaempferol differ in that the latter has an extra hydroxyl group. Thus, it is clear that both sutherlandin A and sutherlandin D are from the same biosynthetic pathway, in common with naturally occurring polyphenolics bearing hydroxyls on the same aromatic ring system. It could be speculated that that both sutherlandin A and sutherlandin D would oxidize with time, in the case of sutherlandin A to ortho-quinone and sutherlandin D to meta-quinone.

The naturally occurring cycloartane glycosides with C-1 functionality group, sutherlandioside B ($C_{36}H_{60}O_{10}$) and sutherlandioside D ($C_{36}H_{58}O_9$) are very similar (Fu *et al*., 2008). The biosynthetic pathway considerations suggest that the presence of the C-1 ketone in sutherlandioside B may facilitate the ring opening of the strained cyclopropane system. It could be speculated that that both sutherlandioside B and sutherlandioside D undergo changes though dehydration at the drying stage already, combined with possible oxidizing while being stored over time.

The quality of a medicinal plant is preserved by drying. However, different species of plants have varying sensitivities to temperature (Rocha *et al*., 2011). Thus, by drying the fresh plant material to remove water, the concept of an enzymatic or microbial activity on the plant material, is basically ruled out. Consideration is made that all moisture is never entirely removed and although the

European Pharmacopedia (2005) does indicate maximum final moisture allowed to still be regarded as preserved, in the case of *Lessertia frutescens* this has not been listed.

In the case of this study, all fresh *Lessertia frutescens* material was dried and immediately placed into glass containers and sealed to prevent any moisture or insects from entering the material during storage. As the container with the dried material was placed into a dark cupboard the chance of degradation due to light was excluded as well. Harbourne *et al*. (2009) studied the impact of drying *Filependula ulmaria* (meadowsweet) at 30°C and 70°C, the higher temperature resulted in a reduction of the plants flavonoid content. The *Lessertia frutescens* material was dried at 35°C in an attempt to preserve the flavonoid content in light of the literature but acknowledge that no fresh sample was analysed for grounds of a comparison.

In this study, the seasons of spring and autumn yielded the highest levels of flavonoids, while the summer season yielded the highest levels of the triterpenoids. In light of the literature (Yanivie and Palevitch, 1982; Bopana and Saxena, 2007) concerning the impact the environment has on secondary metabolite production, it is speculated that the milder weather gives rise to the production of the flavonoids, sutherlandin A in Spring, followed by sutherlandin D in autumn, while in the case of much warmer weather the production of the

triterpenoids, sutherlandioside B and sutherlandioside D is required by *Lessertia frutescens* plants.

4.3 Comparison of summer and storage samples

Comparing the freshly prepared summer sample with the sample that was in storage for one year, the stored sample indicated the highest levels of sutherlandin A and sutherlandin D. However, in the case of the freshly prepared summer sample, sutherlandioside B and sutherlandioside D indicated higher levels.

Previous studies (Houba *et al*., 1995; Fennell *et al*., 2004a) have reported that secondary metabolites can be influenced by duration in storage, depending on the type of constituent. Harborne and Williams (2000) and Joshi *et al*. (2004) report that the factors effecting the chemical profile of plants are divided into two groups, intrinsic (genetics) and extrinsic (cultivation, drying, storage) factors. According to Bottcher *et al*. (2011) the extrinsic factor, post-harvest storage is important as well. In their work, the post-harvest storage of *Matricaria recutita* (chamomile) resulted in deterioration of the external quality of Matricaria flowers in a much shorter time than other flowers. The higher the temperature, the more deterioration occurred. Plant quality was maintained as good quality for up to 70 hours after harvest, if the plant material was stored at 10°C. To maintain maximum essential oils, the drying temperature of 35°C was selected (Bottcher *et al.*, 2011).

Eloff (1999) discovered that despite herbarium specimens (*Helichrysum pedunculatum*) being 100 years old, the plant material still displayed antibacterial activity. In line with these observations, Houba *et al.* (1995) reported no significant change in chemical composition in plant material (lucerne, barley and tea leaves) after 10-20 years of storage. *Eucomis autumnalis* (pineapple flower) was monitored for its anti-inflammatory activity in a study carried out by Taylor and Van Staden (2001) that monitored the impact of age, season and growth conditions on the anti-inflammatory activity of this plant. The authors reported differences depending on time of harvest in relation to before or after growing season, with the highest activity just before dormancy. In addition, mature plant material indicated higher anti-inflammatory activity than that of younger plants.

The pharmacological activity of medicinal plants could be influenced by storage but this was noted as being species-and temperature-dependent (Fennell *et al.*, 2004a). When comparing the ethanolic extract of fresh and dried leaves of *Sisphonochilus aethiopicus* (wild ginger) for COX-1 inhibition and antibacterial activity against *Staphylococcus aureus*, a significant decrease of inhibition, by as much as 49% in the dried extract was obvious (Fennell *et al.*, 2004a).

Factors that influence degradation of plant material are chemical breakdown, decomposition, microbial and insect attack (Fennell *et al.*, 2004a). The biological activity of the plant material will be influenced by chemical changes and these reactions are in return influenced by a number of factors, enzymes being one of

them (Fennell *et al*., 2004a). In the case of storage of essential oils of *Zingiber officinalis* (ginger), Sukamura (1987) reported that the chemical composition of certain oils increased, while other oils decreased drastically, even to undetectable amounts. Stafford *et al*. (2005) reported that the anti-bacterial constituents remained stable while being stored or in some cases changed into compounds that demonstrated more activity. This would definitely have an impact on the efficacy of the medicinal plant material. In terms of sustainability, storage of certain plant materials could be extended, making it financially more viable and therefore fewer plants being harvested from the wild (Griggs *et al*., 2001).

In the case of this study, the storage variations indicated an increase in the case of the flavonoids, sutherlandin A and sutherlandin D but no increase in the case of the triterpenoids, sutherlandioside B and sutherlandioside D. Nishikikawa *et al*. (2005) bring attention to the essential roles sugars play in the biochemical metabolism in plant material especially during post-harvest of perishable commodities. *Lessertia frutescens* would not have such high sugar content, but the sugar substituents would be higher in the flavonoids than in the triterpenpoids.

4.4 Comparison of the leaf and stem samples

For the leaves versus stem comparison, the leaf sample yielded the highest levels of sutherlandin D, while the stems yielded the highest levels of sutherlandin A, sutherlandioside B and sutherlandioside D.

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Research carried out on *Glycine max* (soybean) in order to establish the occurrence of pinitol in the developing soybean seed, revealed interesting information concerning levels of constituents during developmental stages (Kuo *et al*., 1997). The leaves, petioles, stems, roots, nodules, bean and seeds were used. Concentrations of pinitol, *myo*-inisitol and raffinose saccharids were regularly effected during seed development. *Myo*-inositol increased during seed fill then decreased rapidly as seeds accumulated raffinose saccharids (soluble carbohydrates), whereas when pinitol increased, *myo*-inisitol levels decreased (Kuo *et al*., 1997). The authors suggested that the possible occurrence of pinitol in leaves of plants resulted as a response to water or salinity stress. It was noted that as the axis of leaves turned yellow, due to temperature stress, the pinitol levels increased (Guo and Oosterhuis, 1995). The research thus indicates that secondary metabolites may vary throughout the various parts of a plant.
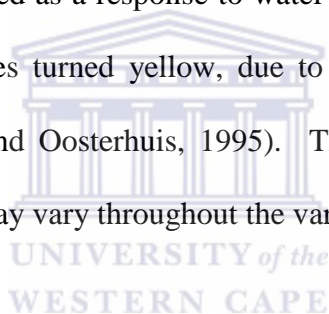
Plant developmental stages influenced the secondary metabolite production and Guo and Oosterhuis (1995) found that secondary metabolites produced as a form of defence were more concentrated and diverse when plants were young, decreasing with age. These authors suggested that young plants were more appealing to herbivores than older plants. Letchamo (1998) reported that alkalmide composition in *Echinacea palla* varied qualitatively and quantitatively throughout the growth and developmental stages. Alkamide levels decreased after 34-58 days in the roots of the control plants, while ketoalkynes levels increased as the roots aged (Binns *et al*. 2001).

In the case of *Lessertia frutescens*, the leaf versus stem comparison reflects typically as to the literature (Van Wyk and Gericke, 2000; Van Wyk and Wink, 2004) that traditional healers used both the leaf and stem material as medicine. As to why there are mostly higher levels of the flavonoids and triterpenoids in the stem material compared to the leaves, could be considered from the perspective that production of the constituents being monitored are higher in the older material (stem) of the plant, than the leaves. Or that the drying temperature on softer material like the leaves contributed to increased degradation of the constituents compared to harder material of the stems.

With regards to efficacy of *Lessertia frutescens*, Tai *et al*. (2004) demonstrated the anti-proliferative activities of Sutherlandia in an *in vitro* study carried out on human breast and leukemia tumor cell lines. Results indicated a difference in antiproliferative effect on breast and leukemia cell lines, with the activity being higher in the breast cell lines. With different cell lines being arrested at different phases, it was suggested that this may be due to different constituents having an effect. The authors do mention that there is uncertainty as to exactly which components are responsible for this anti-proliferative effect.

Crooks and Rosenthal (1994) have been investigating L-canavanine as an important constituent with regards to pancreatic cancer. It is noted that L-canavanine changes to canavanine-containing proteins, which disrupts RNA and DNA synthesis, *in vitro* (Bence *et al*., 2002). *Sutherlandia* is a member of the

leguminous family, which are able to produce and store L-canavanine as a means of protection (Rosenthal, 1997).

All these studies indicate that the efficacy of medicinal plants is far more complicated than merely administering the correct therapeutic dosage and the plant material attributed to a particular disease. The extrinsic factors influence plants in very specific ways depending on the species, leading to the development/accumulation of secondary metabolites that become crucial for the survival of the plants. These secondary metabolite pools seem to be able to change depending on the situation the plant is experiencing; for instance depending on whether a plant is starting to produce seed or the particular season of the year that the plant is growing in. Albrecht (2008) suggests that the Sutherlandia species seems to be subject to continuous variations of the secondary metabolites like the flavonoids and triterpenoids, which contributes to the capability of tumour-cell death. When looking at efficacy of medicinal plants in relation to human beings, one would need to integrate this knowledge with the findings related to the extrinsic factors effecting plant secondary metabolite production in order to be able really understand the offering plants can make in benefiting health in human beings.

The results obtained from this study did reflect similarly to what much of the literature regarding seasonal, storage and parts of medicinal plants being used has been indicating. No doubt that the matter pertaining to seasonal harvesting,

storage and parts being used is more diversified and complex than expected, one would need to consider a much broader range of parameters in order to be more specific. Therefore, this study can be seen as a starting point to further investigations of these aspects, which are of clinical, pharmacological and economic importance. Thus, a number of limitations of this study are acknowledged.

4.5 Scope and limitations

Regardless of the influence seasonal harvesting and the duration of time in storage may have on the pharmacological activity of medicinal plants, not much research has been done in this field worldwide and especially in Southern Africa (Griggs *et al*., 2001; Fennell *et al*., 2004a., Springfield *et al*., 2005). It is for this reason that a considerable part of the literature review of this study was devoted to the seasonal and storage time influences on constituents of plant material other than *Lessertia frutescens*. This was done in order to establish a base upon which information relevant to *Lessertia frutescens* and eventually other indigenous medicinal plants could be created. This study shows the importance of seasonal changes. It shows the changes during storage. Thus, it might be used as a guideline for *Lessertia frutescens*. However, the study has limitations, these being:

The geographical context, as these samples were grown in Stellenbosch and the preference to a particular climatic environment may result in variations in

constituent levels. Geographical deviation often indicates biological activity variations (Taylor and Van Staden, 2001; Shale *et al*., 2005; Buwa and Van Staden, 2007).

Other factors like altitude, competition, soil type and nutrients, moisture stress, rain, temperature, microclimate, light and growth stages were not considered for the purpose of this study. The impact of environmental factors and their effect on the secondary metabolites are known to have an influence on the quality of medicinal plants (Fennell *et al*., 2004a; Ncube *et al*., 2012).

This was a commercially farmed crop, which was irrigated and organically composted. Plant material harvested from the wild could possibly indicate different levels of the secondary metabolites. Bopana and Saxena (2007) indicate that factors like ecological, environmental and genetics differences result in variations of biologically active constituents between plants harvested in the wild compared to commercially harvest plants.

The *Lessertia frutescens* plants used in this study were all two years of age, what would the results be from younger or older plant material? In addition, the samples were collected at 10h00 in the morning on each selected seasonal collection date; would a late afternoon collection time have yielded a different result? A phenomenon most likely for essential oils since they are located on the

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surface but less likely for solvent extracted materials but possibly worth additional investigation.

Post-harvest temperature was not monitored as the plant material was dried within a couple of hours of harvesting, but in the hotter months chemical degradation of the plant material could be increased. Physiological post-harvest responses are considered very important in being able to prevent additional deterioration of the plant material before processing (Bottcher *et al*., 1999; Bottcher *et al*., 2001; Bottcher *et al*., 2003).

Other constituents not used as markers in this study that could possibly reveal different data are alanine, arginine, aspartic acid, asparagine, leucine, phenylanine, proline, tryptophan, L-canavine and γ-aminobutyric acid (GABA). Various constituents (e.g. L-canavanine for certain cancers and pinitol in cases of diabetes) found in *Lessertia frutescens* have been reported and considered important in the efficacy of the plant (Bell, 1958; Snyders, 1965; Viljoen, 1969; Brummerhof, 1969; Bell *et al*., 1978; Moshe, 1998; Tai *et al*., 2004; VanWyk and Albrecht, 2008; Fu *et al*., 2008; Avula *et al*., 2010).

The duration of storage was selected as one year, while a longer period could or may not have a different outcome. Houba *et al*. (1995) concluded that plant material could be stored for long periods of time with no changes to the constituent levels or composition.

Furthermore, one would have to consider that the findings of this study based are on an ethanolic extract. However, what would the results be from an aqueous extract of *Lessertia frutescens*? Aqueous extracts would closely resemble the traditional way of preparation of the medicine for oral ingestion. Infusions and decoctions were the most common dosage forms recorded under traditional practices (Van Wyk, 2008).

4.6 Conclusion and recommendations
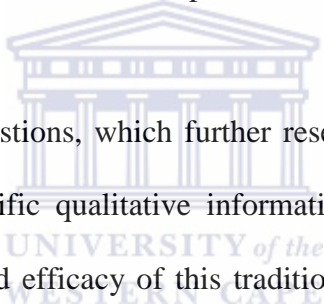
With the renewed interest in medicinal plants, increased demand on the world markets and reliance for healthcare needs of traditional medicines, comes a great opportunity for trade and commerce (Joshi *et al*., 2004, Fennell *et al*., 2004a). Consumers, manufacturers, growers, researchers, amongst others, are able to benefit from the many opportunities resulting from the acquiring of new knowledge and understanding of the diverse plant world (Binns *et al*., 2001). The authors express the importance of identifying chemotypes as a means of possible prediction of phytochemical content. In order to benefit from the range of herbal medicines, pharmaceuticals, phytochemicals, nutraceuticals, and cosmetics require correct identification, quality assurance, standardization and quality control in order to contribute towards safety and efficacy (Joshi *et al*., 2004).

Databases would play a vital role in establishing drug diversity, development and therapeutics (Joshi *et al*., 2004). Van Wyk (1996) proposes a multidisciplinary approach that would involve the fields of biosystematics, ethanobotany,

horticulture, organic chemistry and pharmacology as means to best practice for expanding commercial opportunities. Van Wyk (1996) also suggests that the biological understanding of the species be the starting point, which would include the areas of chemical variation, genetics, phylogeny, reproductive biology and taxonomy.

The plant world offers an abundance of chemical constituents that are produced as secondary metabolites as a direct relation to the environment they are in (Dey and Harborne, 1989). Ncube *et al*. (2012) considers the evidence of environment and genetics on the production of secondary metabolites, as a means of questioning the possibility of being able to manipulate the environment, in so doing increasing the production of the secondary metabolites desired for commercial markets. Plants are unable to avoid interaction with the environment, these external factors impact on the metabolic processes of the plant, effecting plant development, growth rates and thus the secondary metabolite production (Lommen *et al*., 2008). Chemical constituents in medicinal plants may act individually, additively or in synergy to improve health (Ncube, *et al*., 2012). Van Wyk and Wink (2004) highlight the importance of considering that even small changes in the chemistry of a plant, would have an impact on the pharmacological activity. The above gives a greater insight into the complexities of the plant world and secondary metabolite production. By understanding these diverse factors it may contribute considerably to the development of commercial products.

From the results of this study, one can conclude that the production of secondary metabolites are influenced by environmental factors like seasonal harvesting, as indicated by the variation in the chemical constituent composition of *Lessertia frutescens* depending on the season collected in. Moreover, that the storage of *Lessertia frutescens* for a period of one year under the conditions described, did appear to result in an increase of two of the four constituents being monitored. As well as the slight variations in the chemical constituents in the leaves and stem material of *Lessertia frutescens.* Finally, besides the seasonal influence, storage and part (leaves or stem) of *Lessertia frutescens* being used, the type of chemical constituent being monitored was also important in the consideration of this study.

The results did raise questions, which further research would need to answer in order to establish scientific qualitative information for *Lessertia frutescens* to ensure quality, safety and efficacy of this traditional medicinal plant. Once the relevant data are established, commercially cultivated plant material could replace wild harvested plant material, to ensure sustainability as the supply and demand for traditional medicinal plant material increases.

In conclusion, the results of this study provides initial data on the impact duration of storage and seasonal harvesting have on selected biomarker levels for *Lessertia frutescens,* and this should be useful in further research for developing a complete profile for *Lessertia frutescens* as a useful traditional medicine. In addition, it could provide a foundation upon which similar research could be conducted on

other commonly used traditional medicinal plants, in order to establish quality assurance, safety and efficacy. Thus, these data are of importance for clinical, pharmacological and economic aspects.

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