UNIVERSITY OF KWAZULU-NATAL

SCHOOL OF BIOLOGICAL & CONSERVATION SCIENCES

IN VITRO STUDIES AND PHYTOCOMPOUND ANALYSIS IN *Lessertia frutescens* (FABACEAE)

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A thesis submitted to the School of Biological and Conservation Sciences, Faculty of Science and Agriculture, University of KwaZulu-Natal, Westville Campus, for the degree of Doctor of Philosophy.

This thesis has been prepared according to **Format 4** as outlined in the guidelines from the Faculty of Science and Agriculture which states: This is a thesis in which the chapters are written as a set of discrete research papers, with an overall Introduction and a final Discussion, where some of the chapters have already been published in internationally-recognised, peer-reviewed journals.

AUTHENTICATION

As the candidate's supervisors, we have approved this thesis for submission.

Supervisor:

Signed: …… Name: …… Date: ……...

Co-Supervisor:

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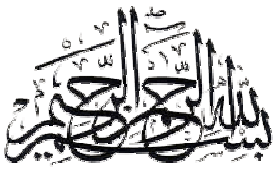
DEDICATION



To my parents, Daya and Preshella,
my husband Junaid,
and my two sons, Shuayb and Shaheen,
with all my love.

ABSTRACT

The cancer bush (*Lessertia frutescens* L.) is an important leguminous perennial native to southern Africa and has been used for centuries in traditional medicine by the continent's diverse cultural groups. Like many other legumes, the seeds of this species exhibit dormancy. Moreover, woody plants are typically difficult to propagate in in vitro culture systems. But in vitro shoot cultures are valuable in providing an alternative means of deriving desired secondary metabolites or phytocompounds, under controlled conditions. This study describes novel protocols for breaking seed dormancy, rapid and efficient in vitro propagation, bioreactor culture, and comprehensive phytochemical data following screening and analysis of in vitro and field extracts of *L. frutescens*. Experiments using physical, mechanical and chemical pre-sowing treatments were conducted to determine the germination response of this species. The results indicated that seeds of *L. frutescens* exhibited exogenous dormancy due to the inhibitory effect of the hard coat on germination. Seed dormancy was released by mechanical scarification in which 100 % germination was achieved. In vitro propagation studies using single node explants in Murashige and Skoog (MS) medium supplemented with combinations of different concentrations of benzyladenine and naphthaleneacetic acid revealed a maximum number of 10 shoots per explant in solid medium, and 12.9 shoots per explant in liquid medium inside a temporary immersion bioreactor. Indirect shoot organogenesis and plant regeneration using rachis and stem segments was achieved with the highest percentage of explants forming shoots (88.8 %) from rachis explants cultured onto MS medium supplemented with thidiazuron. Direct shoot organogenesis from hypocotyl and cotyledon segments was also achieved in *L. frutescens*. The highest shoot regeneration using hypocotyls (83 %) was obtained in MS medium supplemented with kinetin

whilst the highest shoot regeneration using cotyledons (46 %) was obtained in MS medium supplemented with kinetin in combination with benzyladenine. Successful rooting (up to 80 %) and acclimatization (up to 90 % survival rate) was attained. Spectrophotometric and gravimetric methods indicated that saponins were the most abundant, followed by phenolics, flavonoids and then alkaloids in in vitro leaf extracts then in field leaf extracts and seed extracts, respectively. After qualitative analysis these extracts were also found to contain tannins, phlobatannins and cardiac glycosides of medicinal interest. By using gas and liquid chromatography the presence of the medicinally important L-canavanine, gamma amino-butyric acid and D-pinitol was verified in in vitro leaf, field leaf and seed extracts. In vitro leaves had higher quantities of all compounds, except for D-pinitol. Phytocompound analysis of shoots derived from several of the cytokinin-enhanced media showed that these organs contained higher quantities of L-canavanine compared to the control. This study, therefore, highlights the potential techno-economic production of medicinal phytocompounds from in vitro leaves of *L. frutescens* following large scale production using the protocols described in this study.

DECLARATIONS

DECLARATION 1 – PLAGIARISM

I, ……., declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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   a. Their words have been re-written but the general information attributed to them has been referenced.
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

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DECLARATION 2 – PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published, and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1

Shaik, S., Dewir, Y.H., Singh, N., Nicholas, A. 2008. Influences of pre-sowing seed treatments on germination of the cancer bush (*Sutherlandia frutescens*), a reputed medicinal plant in arid environments. *Seed Science and Technology,* 36:795–80.

Contributions: SS carried out experimental work, recorded data and wrote the manuscript. NS and YHD assisted with experimental design and statistical analysis. NS edited the manuscript. NS and AN were the supervisors.

Publication 2

Shakira Shaik, Yaser Hassan Dewir, Nisha Singh and Ashley Nicholas. 2010. Micropropagation and bioreactor studies of the medicinally important plant *Lessertia (Sutherlandia) frutescens* L. *South African Journal of Botany,* 76:180–186. DOI:10.1016/j.sajb.2009.10.005.

Contributions: SS carried out experimental work, recorded data, performed statistical analyses and wrote the manuscript. NS and YHD assisted with experimental design and manuscript editing. NS and AN were the supervisors.

Publication 3

Yaser Hassan Dewir, Nisha Singh, Shakira Shaik and Ashley Nicholas. 2010. Indirect regeneration of the Cancer bush (*Sutherlandia frutescens* L.) and detection of L-canavanine in in vitro plantlets using NMR. *In Vitro Cellular and Developmental Biology – Plant,* 46:41–46. DOI: 10.1007/s11627-009-9260-4.

Contributions: YHD conducted the tissue culture experiments, recorded data, performed statistical analyses, and wrote the manuscript. NS supervised the chemical analysis and edited the manuscript. SS assisted with manuscript writing and editing, and carried out SEM work. NS and AN were the supervisors.

Publication 4

Shakira Shaik, Nisha Singh and Ashley Nicholas. 2010. Comparison of selected secondary metabolites in extracts of the cancer-bush *Lessertia (Sutherlandia) frutescens* L. *African Journal of Traditional, Complementary and Alternative Medicines, In Press*.

SS designed experiments, carried out experimental work, recorded data, performed statistical analyses and wrote manuscript. NS assisted with experimental design and edited the manuscript. NS and AN were the supervisors.

Publication 5

Shakira Shaik, Nisha Singh, Ashley Nicholas. 2010. HPLC and GC analyses of in vitro-grown leaves of the cancer bush *Lessertia (Sutherlandia) frutescens* L. reveal higher yields of bioactive compounds. *Plant Cell Tissue and Organ Culture.* [Online]. DOI: 10.1007/s11240-010-9884-4.

SS designed experiments, carried out experimental work, recorded data, performed statistical analyses and wrote manuscript. NS assisted with experimental design and edited the manuscript. NS and AN were the supervisors.

Publication 6

Shakira Shaik, Nisha Singh, Ashley Nicholas. 2010. Cytokinin-induced organogenesis in *Lessertia (Sutherlandia) frutescens* L. using hypocotyl and cotyledon explants affects yields of L-canavanine in shoots. *Plant Cell Tissue and Organ Culture*. [Online]. DOI: 10.1007/s11240-010-9885-3.

SS designed experiments, carried out experimental work, recorded data, performed statistical analyses and wrote the manuscript. NS assisted with experimental design and edited the manuscript. NS and AN were the supervisors.

Signed:

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I would like to thank the Lord, Allah, for giving me the strength and ability to persevere throughout the duration of this study.

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CHAPTER 1

INTRODUCTION

In recent years, the pharmacological value of *Lessertia frutescens* L. *(Sutherlandia frutescens)* has gained rapid recognition because of its proclaimed effectiveness and safety in treating debilitating diseases such as AIDS and cancer. Consequently, there is an enormous demand on fresh material which could result in the attrition of natural populations to the point of extinction of this species. By using tissue culture techniques it is possible to generate large quantities of in vitro plants, tissues and organs which can be optimized for phytocompound production in the laboratory. Literature on in vitro studies of *L. frutescens* is lacking. This project therefore aims to ascertain whether a laboratory population of this plant species can be obtained via tissue culture for the purpose of phytochemical production on a large scale. By optimizing growth conditions it is envisaged that bulk quantities of the desired plant tissues and the phytocompound/s of interest can be generated.

L. frutescens is an important leguminous perennial native to southern Africa and has been used for centuries in traditional medicine by the continent's diverse cultural groups. Like many other legumes, the seeds of this species exhibit dormancy. Moreover, woody plants are typically difficult to propagate in in vitro culture systems (Singh et al. 2002). In vitro shoot cultures, on the other hand, are valuable in providing an alternative means of deriving desired secondary metabolites or phytocompounds, under controlled conditions. This study describes novel protocols for breaking seed dormancy, rapid and efficient in vitro propagation, bioreactor culture, and comprehensive phytochemical data following screening and analysis of in vitro and field extracts of *L. frutescens*.

1.1 Medicinal plant biotechnology

Plants afford an assortment of natural products or secondary metabolites which exhibit extensive chemistry and activities (Vanisree and Tsay 2007). Products of secondary metabolism are also called phytocompounds and are acknowledged for playing a central role in the adaptation of plants to their changing environment. Many of these compounds have medicinal applications,

and as such, have received global awareness, with about 40 % of pharmaceuticals originating from plant organs (Rout et al. 2000). Moreover, in developing countries the vast majority of people are still dependent on local medicinal plants for primary health care (WHO 1995). In both cases, the source materials, usually mass quantities of whole plants, are obtained from various geographical biotopes which are typically faced with disease, drought, grazing pressure and other negative environmental factors (DiCosmo and Misawa 1995). The extended phases between planting and harvesting, further complicate the selection of high-yielding varieties. Overall, the consistent collection of healthy plant materials for medicinal usage remains a challenge.

Tissue culture has developed into a widely used, rapid and efficient alternative for the commercial propagation of medicinal plant species (Rout et al. 2000). Tissue culture technology dates back approximately 240 years when Duhamel first demonstrated the formation of undifferentiated cells and tissues (callus) in elm plants (Gautheret 1985). The science behind this discovery expanded after the cell theory was exemplified by Schlieden and Schwann in the 19th century (Allan 1991). With the concurrent use of nutrients and synthetic plant growth regulators, the cell can regenerate into a complete organism, a concept known as totipotency (Allan 1991). The phytocompound yield of regenerated plants is dependent on in vitro culture conditions such as the type, concentration and combination of plant growth regulators, mineral and carbon sources, and several culture environment factors (Stafford et al. 1986) such as light, temperature and humidity. Plant tissue culture systems are therefore a means of competitive production compared to whole plant extraction, especially if it is possible to screen, select and propagate the hyper-producing varieties. The enhancement and application of good analytical techniques such as gas and liquid chromatography and nuclear magnetic resonance spectroscopy can greatly facilitate in vitro phytocompound research. Importantly, the authentication and quantification of known phytocompounds or identification of unknown ones can be accurately performed.

1.2 Biotechnological approaches used for medicinal plant propagation

Various in vitro experimental approaches can be utilized for the successful regeneration of medicinal plants. Plant regeneration via in vitro seed germination is one way of increasing the production of medicinal plants, especially when the seeds exhibit dormancy and have very low chances of germination and growth in the natural environment (Kermode 2004). In the plant

kingdom, seeds of the Fabaceae generally exhibit dormancy, a condition thought to be imposed by the hard coat (Tsiantis 2005). Different in vitro pre-sowing treatments of the seed coat can optimise the germination response followed by excision of the embryo from the testa for subsequent culture, growth and development.

Other simple in vitro techniques, such as proliferation through axillary buds and organogenesis, may also be used. The former, also known as clonal propagation, utilizes plant growth regulators to release the dormancy of preformed meristems in axillary buds to generate lateral shoots (Phillips and Hubstenberger 1995). Apical dominance of the axillary bud is suppressed via the application of hormones, usually cytokinin, thereby stimulating lateral branching. It is a rapid technique for mass propagation of medicinal plants which could result in a tenfold increase in shoot number in thirty days (Phillips and Hubstenberger 1995). Thereafter, shoot tips and axillary buds from these cultures can be used to produce clumps of shoots which can be separated, rooted and further propagated to increase the biomass of the medicinal plant species concerned. Autonomous plants can then be produced by gradually acclimatizing the newly regenerated plants to growth in a chamber or glasshouse followed by field transplantation.

Organogenesis may be defined as the regeneration of plant organs inclusive of shoots and roots from an adventitious origin i.e. not from a preformed meristem (Phillips and Hubstenberger 1995). Following hormonal intervention, adventitious meristems can be induced (Warren 1991) from leaf blades, stems, petioles, rachis, internodes and cotyledons. When adventitious meristems are induced directly from the explant tissue, this is known as direct organogenesis, whilst those derived subsequent to an intervening callus phase is called indirect organogenesis. The resultant shoots can be induced for root development and further propagated to increase the production of the desired medicinal species.

The main advantages of regenerating medicinal plants through in vitro culture are a significant reduction in the overall production time and the obliteration of seasonal and climatic variation.

1.3 Applications of medicinal tissue culture systems

Tissue culture systems have extensive applications inclusive of embryo, anther, microspore and protoplast culture, germplasm storage, bioreactor technology, secondary metabolite or phytocompound production, electron microscopy studies, horticulture, agricultural crop improvement, genetic engineering and biotransformations, pathogen-free cultures, industrial processing, pharmaceutical applications and biodiversity conservation. Only the applications used in this study will be given a brief overview.

1.3.1 Bioreactor technology

A bioreactor or fermenter is a large vessel in which biological cells or tissues are grown in an active environment. Various types of bioreactors are available but balloon type bubble bioreactors (BTBB) have been reported as superior for biomass growth of several medicinal plant species (Choi et al. 2006). Through the use of bioreactor technology it is possible to closely regulate and monitor the conditions inside the chamber, and up–scale the cultures to develop a marketable process (Scragg 1991).

1.3.2 Scanning electron microscopy

Scanning electron microscopy (SEM) utilises an electron beam to scan the surfaces of appropriately prepared specimens and therefore is an invaluable tool for studying the external morphology of plant cells, tissues and organs (Fowke 1995). SEM may therefore be used to examine and record the sequence of events during organ development as high resolution images at high magnification.

1.3.3 Biodiversity conservation

Human activities, particularly urbanization and deforestation, have had a devastating impact on species diversity, and as such, the primary concern now centres on the permanent loss of this biological diversity (Sharma 2002). The preservation of plant resources and biodiversity has become both public and scientific focal points following an increased realization of the importance of genetic variation to the well-being of the global ecosystem (Callow et al. 1997). Conservation of field plants is challenging and exacerbated by natural disasters, microbial pathogen attack, pest interference and the high costs of labour (Engelmann 1997). Through the

use of modern biotechnology, elite medicinal plant varieties have been conserved, with disease-free stocks and cost effectiveness being the main benefits. As a result, the pharmaceutical industry has benefitted as has the traditional medicine trade. Nonetheless, many important species are still recorded on the red danger list, hence the preservation of biodiversity is considered a fundamental prerequisite for the continued production, development and exploitation of high-yielding medicinal cultures. This provides an opportunity to link conservation and the sustainable application of important medicinal species, which in turn, may have economic value.

1.4 Rationale and motivation for research carried out in this study

South Africa comprises one of the leading biodiversities in the world and has approximately 25 000 known plant species (Golding 2002). Over the last few decades severe concerns have been articulated regarding the fast-diminishing supplies of many of these species due to habitat loss, habitat degradation and over-exploitation, with some 3500 species threatened with extinction. The loss of valuable indigenous medicinal plant species is also a serious environmental concern in South Africa. About 3000 species are utilized for medicinal purposes by approximately 200 000 traditional healers (van Wyk and Gericke 2003) established within a large informal trade business. The indigenous medicinal plant industry depends almost exclusively on the extreme harvesting of wild populations, many illegally, to create low-cost phyto-pharmaceutical preparations, which has resulted in the depletion of these populations. According to Mander et al. (1995), the use of indigenous plants in primary health care may be maintained if sustainable quantities of raw medicinal plant materials are made available. Therefore, by using the tools of in vitro bio-processing technology, efficient protocols for sustainable mass propagation of threatened, vulnerable and medicinal species can be developed, thereby eliminating the enormous strain on natural resources, and conserving indigenous biodiversity.

The indigenous plant selected for this study was *Lessertia frutescens* L., commonly known as cancer bush or its synonym *Sutherlandia frutescens.* It belongs to the large pod-bearing family, the Fabaceae, and is a perennial shrub that grows in arid environments in southern Africa. The plant is also a popular ornamental, sometimes found in gardens because of its eye-catching scarlet flowers. Its medicinal benefits, however, render it more widely utilized by traditional

healers. The ethnobotanical literature of *L. frutescens* is well documented. According to the local consumers, the leaves of the plant, usually ingested as a bitter tea, are effective in the treatment of a variety of medical ailments which include cancer, ulcers, stomach ailments, fever, diabetes, colds, cough, asthma, bronchitis, kidney and liver infections, stress, anxiety, heart and urinary tract conditions, and rheumatism (van Wyk et al. 1997). Scientifically validated reports (cited in the chapters that follow) show that leaf extracts of *L. frutescens* play a significant role in the management of HIV/AIDS, cancer and diabetes, in addition to exhibiting anti-inflammatory, anti-oxidant and anti-convulsant properties. The plant's reputed medicinal properties have been attributed to its active ingredients, L-canavanine, D-pinitol, γ-amino-butyric acid (GABA), and flavonoids and cycloartanol glycosides, recently characterised as sutherlandins and sutherlandiosides, respectively (Avula et al. 2010, Fu et al. 2008, Fu et al. 2010).

Although ethno-botanical records and published information on the plant's medicinal properties are accessible, the literature indicates a general lack of data on the use of plant biotechnology for the cultivation, conservation and phytocompound production of *L. frutescens*. The medicinal benefits of *L. frutescens* are characterized by its powerful phytocompounds. However, chemical and genetic variability in genotypes from different geographical biotopes (Chinkwo 2005) could complicate the standardization of the commercially available end product. In addition, the bioactive ingredients are usually found in low quantities in natural populations (Vanisree and Tsay 2007). Compounded to this, reports show that many herbal preparations generally demonstrate the presence of bacterial and fungal contaminants (Govender et al. 2006, Khanyile et al. 2009). Therefore, consideration was given to the potential economy and efficiency of tissue culture for the production of a sustainable source of elite, contamination-free progeny of *L. frutescens*. Furthermore, the production of higher-yielding clones, can occur consistently, speedily and economically. No scientific reports exist on the phytocompound composition of the seeds of this species. Therefore a comparative analysis of phytocompounds in seeds, field leaves and in vitro leaves of *L. frutescens* was undertaken.

1.5 Aims and objectives

Aim 1: To develop efficient and effective protocols for the micropropagation of *L. frutescens.*

To achieve this aim, the following objectives were identified:

- To generate in vitro plantlets of *L. frutescens* as stock material, suitable for further experimentation.
- To develop efficient in vitro protocols for the micropropagation of *L. frutescens* in solid culture.
- To investigate the growth and yields of *L. frutescens* in liquid culture using bioreactor technology.
- To investigate direct and indirect organogenesis in *L. frutescens*.
- To investigate in vitro rooting in *L. frutescens*.
- To acclimatize regenerated plantlets in a growth chamber.
- To examine morphological organ development using scanning electron microscopy.
- To evaluate the impact of various pre-sowing seed treatments on in vitro germination of *L. frutescens*.

Aim 2: To analyse the phytocompounds in extracts of *L. frutescens*.

To achieve this aim, the following objectives were identified:

- To prepare extracts of in vitro leaves, field leaves and seeds of *L. frutescens* using organic solvents.
- To verify the presence of L-canavanine, GABA, D-pinitol and arginine in the extracts using gas and liquid chromatography.
- To quantify and then compare the levels of L-canavanine, GABA, D-pinitol and arginine in field and in vitro extracts.
- To verify, quantify and compare saponin, alkaloid, phenolic and flavonoid content in prepared extracts using spectrophotometry and gravimetry.
- To qualitatively evaluate the presence of tannins, phlobatannins and cardiac glycosides in prepared extracts.

1.6 Outline of research presented in this thesis

In order to achieve the study aims and objectives, different biotechnological techniques were utilized to develop novel methods for the micropropagation, regeneration and improvement of *L. frutescens*. In addition, the extraction, identification and quantification of important medicinal phytocompounds were undertaken using various analytical chemistry techniques such as thin layer, gas and liquid chromatography, and spectrophotometry.

Following the findings of the investigations, this thesis is outlined as follows: Chapter 2 evaluates the impact of various pre-sowing seed treatments on the germination response of *L. frutescens* and outlines the development of an effective method of breaking exogenous seed dormancy. Chapter 3 covers the development of efficient in vitro protocols for the micropropagation of *L. frutescens* using nodal explants followed by successful acclimatization. Chapter 4 establishes a protocol for inducing indirect shoot organogenesis in *L. frutescens* using rachis and stem segments. Chapter 5 compares the phenolic, flavonoid and alkaloid content using spectrophotometry, and saponin content using gravimetric analysis, in in vitro leaves, field leaves and seeds of *L. frutescens*. In addition, qualitative tests for the presence of tannins, phlobatannins and cardiac glycosides in these extracts are described. The quantification of L-canavanine, GABA, D-pinitol and arginine content in seeds, field leaves and in vitro leaves of *L. frutescens* using gas and liquid chromatography is presented in Chapter 6. The use of hypocotyls and cotyledons as explants for direct shoot organogenesis and plantlet regeneration is described in Chapter 7. In addition, extracts of shoots derived from these explants were analysed for L-canavanine and arginine content. An overall discussion based on the synthesis of the previous chapters is presented in Chapter 8. Chapter 9 includes future research and recommendations.

Due to the format adopted for this thesis each chapter has been presented according to the referencing style required by the respective journal wherein the manuscript has been published/submitted. However, in the Introduction, Discussion and final References section, the Harvard referencing style was used. For better clarity, the concentrations of plant growth regulators have been expressed in μM (molar concentration) as well as mg L^{-1} (M/V) in the overall discussion (Chapter 8).

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CHAPTER 2

JOURNAL ARTICLE FOR PUBLICATION 1:

INFLUENCES OF PRE-SOWING SEED TREATMENTS ON GERMINATION OF THE CANCER-BUSH (*Sutherlandia frutescens*), A REPUTED MEDICINAL PLANT IN ARID ENVIRONMENTS

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MICROPROPAGATION AND BIOREACTOR STUDIES OF THE MEDICINALLY IMPORTANT PLANT *Lessertia (Sutherlandia) frutescens* L.

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JOURNAL ARTICLE FOR PUBLICATION 3:

INDIRECT REGENERATION OF THE CANCER BUSH (*Sutherlandia frutescens* L.) AND DETECTION OF L-CANAVANINE IN IN VITRO PLANTLETS USING NMR

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CHAPTER 5

COMPARISON OF SELECTED SECONDARY METABOLITES IN EXTRACTS OF THE CANCER-BUSH *Lessertia (Sutherlandia) frutescens* L.

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Abstract

Extracts of in vitro leaves, field leaves and seeds of *Lessertia frutescens*, a leguminous plant, were analyzed using spectrophotometric and gravimetric methods for quantitative comparison of their phenolic, flavonoid, alkaloid and saponin content. Saponins were found to be most abundant, followed by phenolics, flavonoids and then alkaloids in in vitro leaves than field leaves and seeds. The extracts were also qualitatively analyzed to evaluate the presence of other phytochemicals of medicinal interest. This qualitative analysis indicated the presence of tannins, phlobatannins and cardiac glycosides. Due to the documented therapeutic use of these types of phytochemicals, the results of this study offer a strong rationale for further animal and clinical investigations of *L. frutescens* extracts.

Key words: alkaloids, flavonoids, phenolics, phytochemicals, saponins, spectrophotometry

INTRODUCTION

Complex pathways of secondary metabolism in higher plants result in the production of a vast assortment of chemical complexes known as secondary metabolites (Yazaki, 2006) or

phytochemicals. Although these natural compounds may not be essential for growth and reproduction (Starmans and Nijhuis, 1996), recent technological advancements, particularly in the fields of molecular biology and biochemistry, have provided evidence of their allelochemical functions. Being sessile, a plant's ability to avoid attack by herbivores and bacterial, viral and fungal pathogens is limited. Consequentially, adaptive traits such as chemical defense compounds evolved in plants to aid in their survival, by warding off, inhibiting and destroying predators and diseases. According to Verpoorte and Memelink (2002) the primary function of secondary metabolites is plant defense.

Secondary metabolites are structurally diverse and their classification is mainly derived according to their biosynthetic pathways (Harborne, 1999). In pharmacognosy, phenolics (comprising flavonoids, tannins, coumarins, quinones and anthocyanins) are regarded as the most widespread phytochemical group, the alkaloids (containing one or more nitrogen atoms) are more specific to distinct genera and species (Bourgaud, 2001) and the terpenoids (comprising triterpenes, steroids, saponins and cardiac glycosides) have the most diverse chemical structure (Yazaki, 2006).

Phenolic compounds range from simple structures containing one aromatic ring to very complex polymeric structures (Trease and Evans, 1996). They have been documented to inhibit UV and carcinogenic tumours (Scalbert et al., 2005) and also exhibit anti-mutagenic, anti-bacterial, anti-viral and anti-inflammatory effects (Middelton et al., 2000). Flavonoids contain free hydroxyl groups attached to aromatic rings. Flavonoids such as rutin, in certain buckwheat species, are known to inhibit lipid oxidation by scavenging radicals (Jiang et al., 2007). These protect against coronary heart ailments, and also have anti-microbial, anti-tumour and anti-inflammatory effects (Harborne and Williams, 2000). Phenolics and flavonoids in eggplant are also beneficial for controlling glucose absorption in type 2 diabetes by inhibiting intestinal α-glucosidase (Kwon et al., 2008).

Alkaloids are heterocyclic compounds which have powerful physiological effects in mammals (Shamsa et al., 2008). Pyridine alkaloids like trigonelline in *Trigonella foenum-graecum*, have been reported to be useful in diabetes management (Liu, et al. 2010). Saponins (a class of

terpenoids) are amphipathic compounds composed of a saccharide attached to a steroid or tripterpene. Saponins such as quercetine and soyasaponin in certain *Trifolium* species, are beneficial in human nutrition (Oleszek and Stochmal, 2002). Others, like bidesmosidic saponins in *Mimusops laurifolia*, have found use in cosmetics and detergents because of their foaming ability (Eskander et al., 2006).

For the purposes of this study, the medicinally important plant *Lessertia frutescens* was selected for phytochemical profiling. This plant has been used for centuries in traditional medicine by the diverse cultural groups residing in southern Africa to manage a wide variety of ailments including gynaecological, gastrointestinal, urogenital and musculoskeletal system disorders; with no adverse results recorded (Xaba and Notten, 2003). Numerous reports document the anti-oxidant (Tai et al., 2004), stress-relieving (Prevoo et al., 2008), hypoglycaemic (Chadwick et al., 2007), anti-mutagenic (Reid et al., 2006), and anti-tumour (Stander et al., 2007) properties of extracts of *L. frutescens*. Extracts are also reported to inhibit the action of HIV target enzymes (Hartnett et al., 2005). However little is known about the bioactive compounds that facilitate these remedies. Aqueous and methanol extracts of field leaves of *L. frutescens* have been shown to contain flavonoids and saponins (Van Wyk & Albrecht, 2008; Avula et al., 2010). However, quantitative and qualitative assessment of alkaloids, phenolics, saponins and flavonoids in the seeds or in in vitro cultures of this species are very rare. Spectrophotometry has become a useful technique for screening crude plant extracts for the detection and verification of distinct classes of compounds. Data that will contribute to the medicinal properties of the health-enhancing components of *L. frutescens* will vastly enhance the commercial value of the extracts. Therefore the objective of this study was to compare the phenolic, flavonoid and alkaloid content using spectrophotometry, and saponin content using gravimetric analysis, in in vitro leaves, field leaves and seeds of *L. frutescens*. In addition, qualitative tests were carried out to evaluate the presence of tannins, phlobatannins and cardiac glycosides in the extracts.

MATERIALS AND METHODS

Plant materials

Three different types of material, viz. in vitro leaves, field leaves and seeds were used for chemical profiling. In vitro leaves (Sample A) were obtained from cultures in our laboratory (Shaik et al., 2010), where stock plants of *L. frutescens* were verified against specimens (W.J. Louw 2876 and R. Erasmus 198) in Ward Herbarium, University of KwaZulu-Natal, South Africa. Commercially available powdered leaves (Bee-Med Natural Herbs, South Africa) were used as a source of field leaves (Sample B), and seeds (Sample C) were obtained from Silverhill Seeds and Books, Kenilworth, South Africa. Samples A and C were dried at 60ºC for 48 h in a laboratory oven followed by grinding into fine powder using a pestle and mortar.

Quantitative determination of phenolics, flavonoids, alkaloids and saponins

A Buchi rotary evaporator (Switzerland) was used to concentrate the extracts, and a Beckman DU 530 UV/VIS spectrophotometer was used for spectrophotometric measurements. All analyses were done in duplicate.

Phenolics

An adaptation of the method published by Biglari et al. (2008) was used to determine phenolic content. Five grams each of samples A, B and C were separately extracted in 15 ml of methanol at room temperature for 5 h using a mechanical shaker (Labcon 3100 E, South Africa). The extracts were then filtered through Whatman No. 1 filter paper and centrifuged using an Eppendorf 5810 R (Germany) centrifuge at 5400 x *g* for 10 min. The supernatant was concentrated for 15 min under reduced pressure at 40 ºC using the rotary evaporator to obtain the methanolic crude extract. The dry weights of the extracts after evaporation were 0.51, 1.00 and 0.30 g for A, B and C, respectively. Each evaporated extract was diluted with 5 ml methanol. Forty µl of each sample were mixed with 1.8 ml Folin-Ciocalteu reagent which was prediluted 10-fold with distilled water and allowed to stand at room temperature for 5 min. Thereafter, 1.2 ml of 7.5 % sodium bicarbonate was added to each mixture. After standing for 60 min at room temperature, the absorbance of these solutions was measured at 765 nm. Gallic acid (Sigma) was used as a standard. The concentration of total phenolic compounds was expressed as mg of gallic

acid equivalents per g of dry weight of plant through a calibration curve with gallic acid. The calibration curve range was 1-20 µg/ml. All gallic acid solutions were assayed in the same way as the samples.

Flavonoids

An adaptation of the method published by Wang et al. (2008) was used to determine flavonoid content. Two and a half grams each of samples A, B and C were separately extracted in 8 ml of methanol on a GFL 1083 water bath (Germany) shaking under reflux for 12 h at 70 ºC. The extracts were then filtered through Whatman No. 1 filter paper and evaporated to dryness in the rotary vacuum evaporator at 40 ºC for 10 min. The dry weights of the extracts after evaporation were 0.26, 0.50 and 0.15 g for A, B and C, respectively. This was followed by the addition of 20 ml of methanol to dissolve each extract. Thereafter, 1 ml of each methanolic solution was transferred separately to 10 ml volumetric flasks, to which 0.3 ml of 5 % sodium nitrite was added, and left at room temperature for 6 min. After addition of 0.3 ml of 10 % aluminium nitrate to each flask, the mixtures were incubated at room temperature for another 6 min. Following the addition of 4 ml of 1N sodium hydroxide to each flask the final volume was attained using methanol. Further incubation for 15 min at room temperature for colour development was followed by measurement of absorbance at 510 nm. Rutin (Sigma) was used as the standard. Total flavonoid content was expressed as mg of rutin equivalents per g of dry weight of plant through a calibration curve with rutin. The calibration curve range was 10-100 µg/ml. All rutin solutions were assayed in the same way as the samples.

Alkaloids

An adaptation of the method published by Shamsa et al. (2008) was used to determine alkaloid content. Five grams each of samples A, B and C were separately extracted in 15 ml methanol for 24 h on a mechanical shaker (Labcon 3100 E, South Africa). The extracts were filtered through Whatman No. 1 filter paper and the methanol was evaporated under vacuum at 45 ºC for 15 min to dryness. The dry weights of the extracts after evaporation were 0.55, 0.80 and 0.20 g for A, B and C, respectively. Thereafter, 20 mg of each residue was dissolved in 10 ml of 2N hydrochloric acid and then filtered as before. One ml of the resulting solutions were transferred

to separatory funnels and washed three times with 10 ml chloroform, each time. The pH of each solution was then adjusted to neutral using 0.1 N sodium hydroxide followed by the addition of 5 ml bromocresol green solution (69.8 µg/ml) and 5 ml phosphate buffer (pH 4.7). After vigorous shaking each solution was extracted separately in1, 2, 3 and 4 ml chloroform. Each extract was collected in a 10 ml volumetric flask and diluted to final volume with chloroform. Absorbance was measured at 470 nm. Atropine (Sigma) was used as the standard. The concentration of total alkaloid compounds was expressed as mg of atropine equivalents per g of dry weight of plant through a calibration curve with atropine. The calibration curve range was 1-20 µg/ml. All atropine solutions were assayed in the same way as the samples.

Saponins

An adaptation of the method published by Shiau et al. (2009) was used to determine saponin content. Five grams each of samples A, B and C were separately extracted by maceration in 50 ml methanol at 70 ºC for 6 h. The cooled extracts were filtered through Whatman No. 1 filter paper and the methanol was evaporated under vacuum at 45 ºC for 30 min to dryness. The dry weights of the extracts after evaporation were 0.20, 0.30 and 0.03 g for A, B and C, respectively. The residues were each suspended in 50 ml distilled water and extracted successively, three times, with 100 ml ethyl ether, each time. After removing the remaining ethyl ether in the aqueous layer by evaporation, the solutions were further extracted with 100 ml and 50 ml of *n*-butanol respectively. The *n*-butanol fractions were dried by evaporation. The resultant products constituted crude saponin and were expressed as mg of saponin per g of dry weight of plant material.

Qualitative determination of other phytochemical compounds

Phytochemical tests for tannins, phlobatannins and cardiac glycosides as described by Trease and Evans (1978, 1996) were carried out for samples A, B and C. Each of the tests was qualitatively expressed as negative (-) or positive (+).

Ferric chloride test for tannins

Half a gram each of the dried samples of A, B and C were placed in separate test tubes. Twenty mls of distilled water were added to each test tube and boiled for 10 min. After cooling, each

extract was separately filtered through Whatman No. 1 filter paper. Thereafter, 3 drops of 0.1% ferric chloride was added to each extract and observed for colouration.

Hydrochloric acid test for phlobatannins

Half a gram each of the dried samples of A, B and C were placed in separate test tubes. Twenty ml of distilled water were added to each test tube and boiled for 10 min. After cooling, each extract was separately filtered through Whatman No. 1 filter paper. Thereafter, 2 ml of 1% aqueous hydrochloric acid was added to each extract and observed for colouration.

Keller-Killiani test for cardiac glycosides

Half a gram each of the dried samples of A, B and C were placed in separate test tubes. Twenty ml of distilled water were added to each test tube. After 24 h, each extract was separately filtered through Whatman No. 1 filter paper. Thereafter, 5 ml of each extract was treated with 2 ml concentrated glacial acetic acid and 2 drops of 0.1% ferric chloride solution. This mixture was then carefully added to 1 ml of concentrated sulphuric acid. The interface was observed for colouration.

Statistical analysis

Data are represented as the mean standard deviation of duplicate determinations. Data were subjected to Duncan's multiple range test using the SAS program (Version 6.12, SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Quantitative analysis of total saponins, alkaloids, phenolics and flavonoids in different *L. frutescens* extracts were performed in duplicate and the results are presented in Table 1. In all phytochemical groups profiled, saponins were found to be the highest (53.34, 60.00 and 6.00 mg/g) followed by phenolics (30.18, 15.09 and 4.89 mg/g) and then alkaloids (4.52, 1.58 and 0.47 mg/g) in in vitro leaves, field leaves and seeds respectively. Smaller amounts of flavonoids were found in the above samples (7.18, 5.55 and 1.87 mg/g respectively).

The differences in quantity of the studied phytochemicals between in vitro and field leaf extracts may be attributed to genetic differences in the plant material, environmental conditions, harvesting season and storage (Santos-Gomes et al., 2002; Biglari et al., 2008; Jiang et al., 2007). It has been reported that metabolic reactions in the cells may become repressed before the phytochemical analysis is conducted (Bourgaud et al., 2001). However, the in vitro leaf extract was shown to contain significantly higher quantities of phenolics, flavonoids and alkaloids. Bourgaud et al. (2001) reported that this effect may be due to the slowed down allocation of carbon for cell construction, and the available carbon is then used for increased phytochemical production. Furthermore, in this study, all extraction parameters were kept constant, but the drying method of the field leaves used by the commercial company is unknown.

Saponins are widespread in all cells of legumes and often occur in large quantities in the aerial parts of healthy plants (Palazón et al., 2006). Saponin content in this study (Table 1) mirror these conclusions. Saponins appear to be stable in *L. frutescens*, do not degrade easily and therefore were found in large quantities in in vitro (53.34 mg/g) and field leaves (60.00 mg/g) despite environmental and collection differences.

Phenolic content was higher in the leaf extracts (30.18 and 15.09 mg/g in in vitro and field leaves respectively) compared to the seed extract (4.89 mg/g). Shoots and leaves were also reported to have higher phenolic content in comparison to other plant parts (Bernardi et al., 2008). The results of this study revealed that the in vitro leaf extract yielded twice as much phenolics as the field leaf extract. Reasons for the variations in phenolic quantity in in vitro and field leaves are said to be as a result of endogenous degradation of some of the phenolic compounds after exposure to air, and an increase in temperature or light during field sampling (Santos-Gomes et al., 2002). Phenolic composition is also dependent on cultivation season, contamination by insects and other agents, different physiological phases and genetic profile (Bernardi et al., 2008), which may explain the lower yield in field leaves.

Flavonoid content in seeds is characteristically low (Oleszek and Stochmal, 2002) as was evident in this study (1.87 mg/g). Flavonoids in the in vitro leaf extract (7.18 mg/g) was significantly higher than that of the field leaf extract (5.55 mg/g) which may be attributed to differences in

light exposure (Hernandez et al., 2008) and warm temperature characteristic of the growth chamber conditions used in in vitro plant culture (Shaik et al., 2010). As is indicated in the literature, it is likely that abiotic stresses due to low light intensity and temperature differences during in vitro culture may have stimulated the increased production of flavonoids as a response mechanism (Hernandez et al., 2008). Furthermore, the synthesis and accumulation of flavonoids can be influenced by other factors such as genotype (species and variety), and ecological conditions such as locality and harvesting period (Jiang et al., 2007).

Alkaloid yields were the lowest in this study, since this group of compounds is sparsely distributed and is more specific to genera and species (Bourgaud et al., 2001). The alkaloid content of seeds (0.47 mg/g) was significantly lower than that of in vitro leaves (4.52 mg/g) and field leaves (1.58 mg/g), probably because the alkaloids are biosynthesized in the leaves and occur in very small amounts in seeds as a consequence of decreased translocation. In the wild, plants are susceptible to a variety of pathogens, and this too has contributed to reduced alkaloid production (Zehra et al., 1998). Increased alkaloid production is reported to be the result of favourable culture conditions such as pH, temperature and nutrient supply (Liu et al., 2010) coupled with specialized spatial and temporal controls (Yazaki, 2006) which are characteristic of in vitro culture.

Qualitative analysis of tannins, phlobatannins and cardiac glycosides in *L. frutescens* extracts was performed according to standard phytochemical screening tests and the results are presented in Table 2. All three phytochemical classes were found in all extracts studied. Tannins occur widely in higher plants and are present as two types i.e. condensed tannins (or proanthocyanidins) and hydrolysable tannins. In this study, the tannins determined from the qualitative test were evaluated to be condensed tannins as verified by the formation of red insoluble compounds called phlobatannins (or phlobaphenes) when condensed tannins are treated with acid (Trease and Evans, 1996). This finding also supports other studies which show that condensed tannins are usually associated with woody plants (Trease and Evans 1978, 1996). The results also revealed the presence of cardenolides, a type of cardiac glycoside that has been used in the treatment of congestive heart failure (Braga et al., 1997). Both tannins and cardiac glycosides are found in plants and are used as protection against herbivory (Chavan et al., 2001).

The literature (mentioned above) is abundant with reports that validate the importance of *L. frutescens* extracts for use as a medicine. Anecdotal information (Xaba and Notten, 2003) outlines the usefulness and efficacy of these extracts. This study provides evidence of some of the actual compounds that may contribute to the medicinal value of *L. frutescens* extracts.

The aim of the present study was to validate and quantify phenolics, flavonoids, alkaloids and saponins and to authenticate the presence of tannins, phlobatannins and cardiac glycosides in in vitro leaves, field leaves and seeds of *L. frutescens*. Due to the variability of many factors including environmental conditions, harvesting season, sampling techniques, nutrient disparity and genetic differences, the extracts studied showed great differences in phytochemical yield. One of the major findings was that the in vitro extracts contained the largest quantities of all studied phytochemicals, except for saponins. In addition the presence of tannins, phlobatannins and cardiac glycosides was confirmed. Because of the concerns about depletion of indigenous plants in the wild, extracts of in vitro grown plants provide a possible alternative means of bio-production. Further research is required to evaluate the potential of in vitro biomass production for optimized yield of medicinal compounds seeing as environmental, nutritional and growth factors can easily be maintained in the laboratory. The establishment of these types of protocols for cultivation of medicinal plants with consistent yield of bioactive compounds will render invaluable commercial and research applications. The clear presence and apparent stability of phenolics, flavonoids, alkaloids and saponins in the in vitro extracts indicate that they could be produced in suitable quantities biotechnologically.

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Table 1 Total content[y] of selected secondary metabolites in different extracts of *L. frutescens*

Extract	Quantity of phytochemical compounds (mg/g dry weight)			
	Phenolics	Flavonoids	Alkaloids	Saponins
In vitro leaves	30.18 a[z] ± 1.16	7.18 a ± 0.05	4.52 a ± 0.31	53.34 a± 1.89
Field leaves	15.09 b ± 0.56	5.55 b ± 0.08	1.58 b ± 0.07	60.00 a ± 2.83
Seeds	4.89 c ± 0.00	1.87 c ± 0.38	0.47 c ± 0.08	6.00 b ± 1.41

[y]Values are the mean ± standard deviation (n = 2)

[Z] Mean separation within columns by Duncan's multiple range test ($P < 0.05$)

Table 2 Qualitative determination of selected secondary metabolites in different samples of *L. frutescens*

Sample	Tannin test for brownish-green colour	Phlobatannin test for bright red colour	Cardiac glycoside test for brown ring at interface
In vitro leaves	+	+	+
Field leaves	+	+	+
Seeds	+	+	+

CHAPTER 6

JOURNAL ARTICLE FOR PUBLICATION 5:

HPLC AND GC ANALYSES OF IN VITRO-GROWN LEAVES OF THE CANCER BUSH *Lessertia (Sutherlandia) frutescens* L. REVEAL HIGHER YIELDS OF BIOACTIVE COMPOUNDS

(pages 51-59)

CHAPTER 7

JOURNAL ARTICLE FOR PUBLICATION 6:

CYTOKININ-INDUCED ORGANOGENESIS IN *Lessertia (Sutherlandia) frutescens* L. USING HYPOCOTYL AND COTYLEDON EXPLANTS AFFECTS YIELDS OF L-CANAVANINE IN SHOOTS

(pages 60-68)

CHAPTER 8

DISCUSSION

Advances in plant biotechnology have contributed to the development of various protocols for the conservation and utilization of medicinal plants. The demands, on and for, medicinal plants are ever-changing. Many scientists, therefore, regard the tools of biotechnology as important alternatives for successful management of valuable plant species. In this thesis, the tools of in vitro biotechnology were extensively utilized to establish simple, rapid and efficient protocols for the sustainable propagation of *L. frutescens*, a species of both medicinal and economic importance. Following extraction, the quantitative and qualitative assessment of the important phytocompounds in in vitro and field materials, were compared.

The experimental approach for the propagation of *L. frutescens* in this study included in vitro seed germination, shoot tip and axillary bud culture, and organogenesis. In vitro seed germination was studied to determine the nature of dormancy exhibited by seeds of *L. frutescens.* Typically, legumes display exogenous dormancy, a trait which is entirely imposed by the toughness of the seed coat (Tsiantis 2005). This attribute may be regarded as an adaptation to unfavourable conditions such as high temperature, grazing, drought and mechanical damage, in the natural habitat. The ecological advantage is that by being dormant, seeds accumulate in the environment, and the result is that the chances of germination are thereby increased due to the large number of seeds that are available. However, the findings of this study showed that removal of the testa by physical, chemical and mechanical scarification to render the seed water-permeable, resulted in high in vitro germination rates. One of the most favourable pre-sowing treatments was achieved by soaking intact seeds in concentrated H_2SO_4 for 30 minutes. However, complete release of seed dormancy which was achieved in 2 days, was through mechanical scarification. These results confirmed that seeds of *L. frutescens* are exogenously dormant. In this study, the successful propagation of this invaluable plant species was attained by breaking seed dormancy and then optimizing the conditions for seed germination. By using these techniques the supply of fresh material to traditional healers and pharmaceutical companies may be improved.

The in vitro regeneration of *L. frutescens* was successfully achieved using shoot tips and axillary buds as explants, as well as through direct and indirect organogenesis, and bioreactor culture. The totipotency of *L. frutescens* was evident after optimization of the culture environments, growth media and regulators. The type, quantity and combination of exogenous plant growth regulators, and strength of micro- and macro-nutrients in the various media, critically influenced the developmental pathways of cells and tissues. This was reflected in the solid and bioreactor cultures, where the addition of cytokinins in the growth medium, either alone, or in combination with auxins, enhanced the multiplication of shoots from nodal explants. Benzyladenine (BA) was found to be the most effective cytokinin for shoot multiplication at a concentration of 2.22 µM (0.5 mg L^{-1}). Shoot multiplication was further increased by combining 2.22 µM BA (0.5 mg L^{-1}) with 0.54 µM (0.1 mg L^{-1}) of the auxin, 1-naphthaleneacetic acid (NAA). At these concentrations, more shoots were produced using full strength Murashige & Skoog (MS) medium compared to other media types.

The different types of exogenous cytokinins, as well their concentration and combinations were vital in determining the *de novo* pathways of shoot formation in *L. frutescens*. During indirect organogenesis it was observed that the inclusion of thidiazuron (TDZ) in the growth media was necessary for callus induction and subsequent shoot organogenesis. Callus formation was triggered by physical wounding of the explant tissue. Thereafter, it became apparent that the culture conditions may have caused the differentiation of callus cells into meristematic regions where shoot formation was initiated. Media that contained TDZ at a concentration of 45.41 µM (10 mg L^{-1}) or 22.71 µM (5 mg L^{-1}) formed the highest percentage of callus and shoots in rachis and stem explants, respectively. Scanning electron microscopy confirmed the early development of adventitious shoots derived from callus cells.

Media that contained different exogenous cytokinins at various concentrations were necessary for shoot induction during direct organogenesis. The formation of shoots occurred without an intervening callus phase, organogenesis was therefore direct. The inclusion of kinetin (K) and BA in the growth media stimulated the differentiation of wounded cells of hypocotyl and cotyledon explants into new shoots. The best shoot regeneration using hypocotyls was obtained in MS medium supplemented with 1 mg L^{-1} (4.65 µM) K whilst the best shoot regeneration using

cotyledons was obtained in MS medium supplemented with 1 mg L^{-1} (4.65 µM) K in combination with 1 mg L^{-1} (4.44 µM) BA.

A bioreactor system was also successfully used for the micropropagation of *L. frutescens*. Enhanced shoot formation from nodal explants was favoured by temporary immersion. This most likely was due to the periodic supply of the necessary nutrients and oxygen during occasional contact between the explants and the liquid medium. However, high humidity, poor gaseous exchange and accumulation of ethylene may have caused 50 % of the shoots to exhibit features of hyperhydricity. The natural habitat of *L. frutescens* is hot, dry and arid. Therefore, the propagation of this plant using bioreactors requires further investigation as the parameters that affect cell growth can be effectively manipulated to increase healthy shoot formation.

The inclusion of exogenous auxins was found to be necessary for in vitro root induction in *L. frutescens*. The rooting potential of *L. frutescens* from shoot tips varied according to the type and strength of auxin used, whether on its own or in combination with others. The strength of the MS salts was also important. Shoot tips derived from micropropagation and direct organogenesis were successfully rooted on half MS supplemented with 19.6 µM (4 mg L^{-1}) IBA, whilst those derived from indirect organogenesis were successfully rooted on half MS supplemented with 24.6 µM (5 mg L^{-1}) IBA. The survival rate of acclimatized plants varied from 85-90 % in a growth chamber.

In this study, both tissues at an early stage of development, and more mature tissues, were effectively used as explants for shoot and root formation. The younger, more rapidly-growing tissues such as axillary buds (nodal explants), hypocotyls, cotyledons and shoot tips, were efficient in shoot and root initiation, respectively. In addition, the mature tissues of stems and rachises were also effective in shoot initiation.

The success of the tissue culture procedures described in this thesis, depended, in part, on the type, concentration and combination of plant growth regulators, and strength of micro- and macro-nutrients in the various media. Other parameters that were crucial for successful culture included environmental factors such as light, temperature and relative humidity. Both the

intensity and quality of light were important for the successful morphogenetic processes of shoot and root initiation and development. The most effective conditions for in vitro experimentation in the culture room were exposure to light for 16-18 hours per day under 40-55 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps and temperature at 25º C. The most effective conditions for hardening and acclimatization in the growth room were exposure to light for 16 hours per day under 100 μmol m^{-2} s^{-1} provided by halide lamps, temperature at 25º C and relative humidity between 40-50 %.

Like many other traditionally used plants, *L. frutescens* produces various biochemical compounds that are of importance in healthcare. These phytocompounds, which are products of secondary metabolism, have been identified as L-canavanine, GABA, D-pinitol, sutherlandins and sutherlandiosides. This research focused on the presentation of scientific evidence for the occurrence of selected active natural products, not only in traditionally-used field plants, but also in in vitro progeny. In this thesis various analytical techniques, including chromatography, spectroscopy and spectrophotometry, have been outlined.

At the outset, the presence of L-canavanine in field leaf and in vitro leaf extracts was qualitatively determined by TLC and confirmed after isolation via column fractionation and NMR spectroscopy. This was an important finding and laid the groundwork for the analyses to follow. In methanol extracts of field leaves, in vitro leaves and seeds, quantification of L-canavanine and arginine was achieved by using tandem mass spectrometry while quantification of GABA and D-pinitol was carried out using gas chromatography. The most significant finding was that the in vitro leaves were shown to contain higher quantities of L-canavanine, arginine and GABA. The higher content of these phytocompounds in the in vitro leaf extracts could be attributed to a range of environmental and nutritional factors which are known to influence the biosynthetic pathways of natural products (DiCosmo and Misawa 1995). This indicates the value of tissue culture as an alternative means of producing desired secondary compounds.

More rapid forms of analysis i.e. spectrophotometry and gravimetry were used for quantitative comparison of other groups of medicinal phytocompounds in in vitro leaves, field leaves and seeds of *L. frutescens.* The phenolic, flavonoid and alkaloid contents were determined by UV

spectroscopy while the saponin content was determined using gravimetric analysis, in strict accordance to recent protocols. The significant finding was that the in vitro leaf extracts contained the largest quantities of phenolics, flavonoids and alkaloids. Once again the in vitro culture environment favoured higher phytocompound yields. In both quantitative phtyocompound studies, no plant growth regulators were used, hence the higher yields were probably the consequence of favourable in vitro culture conditions such as pH, temperature, light, nutrient supply, high nitrogen content in the nutrient medium, and the obliteration of seasonal variation.

Interestingly, when K was applied to the culture media, the derived shoots, following direct organogenesis, contained higher quantities of L-canavanine compared to the plant growth regulator-free control. This indicates that the media composition can be optimised, using adequate quantities of appropriate cytokinins, to enhance biomass and to stimulate the biosynthetic pathways of desired phytocompounds in *L. frutescens*.

The standard phytocompound screening tests of the prepared extracts revealed the presence of tannins, phlobatannins and cardiac glycosides. These results, not reported before, may provide evidence of other key compounds that contribute to the medicinal significance of *L. frutescens* extracts.

CONCLUSION

This study has demonstrated the application of tissue culture technology as an effective alternative system of propagating large biomass of *L. frutescens*. It was shown that the seeds of *L. frutescens* exhibit exogenous dormancy which was easily overcome through mechanical scarification for quick and consistent seed germination.

The cells and tissues of *L. frutescens* demonstrated morphogenetic capacity in response to appropriate exogenous stimuli. The protocols established in this study showed that plant growth regulators, mineral nutrients, carbohydrates, and optimized environmental culture conditions were required to attain high in vitro regeneration rates in this species. The use of a bioreactor

system was also effective for enhancing the shoot multiplication response in liquid media using temporary immersion.

Indirect shoot organogenesis and plant regeneration of *L. frutescens* was established using rachis and stem segments. Scanning electron microscopy demonstrated the early development of adventitious shoots derived from callus cultures. A rapid and efficient direct organogenesis protocol using hypocotyl and cotyledon segments of *L. frutescens* was also described in this study. Scanning electron microscopy revealed that shoots regenerated directly from the wounded epidermal tissue. Apart from the initiation and proliferation of shoots, the exogenous application of cytokinins in the medium also enhanced the yield of important phytocompounds in this species. Regenerated plantlets were successfully rooted and acclimatized and were morphologically similar to the parent plant.

Scientific verification of the presence of important phytocompounds in the in vitro progeny of *L. frutescens* was attained through this study using GC and HPLC. Moreover, the compounds used in ethnomedicine, have been established by means of rapid, modern and accurate methods of analysis such as spectrophotometry, gas chromatography, liquid chromatography and nuclear magnetic resonance spectroscopy. It was shown that in vitro leaf extracts contained significantly higher quantities of most compounds, with or without the use of plant growth regulators. In addition the presence of tannins, phlobatannins and cardiac glycosides was confirmed. The synthesis of these metabolites depended on the composition of the culture medium especially in respect of salt type and strength, cytokinins (where used), and abiotic culture conditions.

The different in vitro proliferation techniques used in this study were shown to have potential in increasing yields to meet commercial demands of *L. frutescens*. These in vitro techniques have thus made an invaluable contribution to biodiversity conservation in South Africa by providing opportunities to prevent the attrition of natural populations to the point of extinction. In addition, the clear presence and apparent stability of the important phytocompounds in the in vitro extracts indicate that plant tissue culture holds much promise as an alternative tool for the in vitro accumulation of desired compounds in elite, high-yielding *L. frutescens* plants.

Medicinal plant biotechnology, a multi-disciplinary field including botany, biochemistry, pharmacognosy, phytochemistry and medicine, is a current and exciting area of research. I remain optimistic that *L. frutescens* will continue to play an integral role in traditional and allopathic healthcare in South Africa.

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CHAPTER 9

RECOMMENDATIONS FOR FUTURE RESEARCH

The methodologies developed in this study can be used to further standardize the production of the active phytocompounds in *L. frutescens* and to assess their toxicity. By using these techniques it would be possible to continuously monitor the derived phytocompounds and select the hyper-producing clones. Furthermore, since regenerated clonal plants can be produced in a variety of ways, analyses on ex vitro material could provide additional sources of desired phytocompounds.

The elicitation of culture media with biotic, abiotic elicitors or pre-cursors, and cell culture are recommended for the enhanced production of desired phytocompounds. The use of hairy roots or shooty teratomas of *L. frutescens* following transformation with *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*, respectively, provide further prospects for increased phytocompound yield. A study of the genes that control the biosynthetic pathways of metabolite production is also recommended. If the secondary metabolite pathways can be unraveled, then successful commercial application would be possible. Successful utilization of these technologies to enhance production of desired metabolites may also decrease the dependence on natural populations as a source of active ingredients.

Potential opportunities in the health sector as applications in the pharmaceutical, neutraceutical and cosmetic industries, are indicated for the medicinal phytocompounds of *L. frutescens*. The chemical verification and quantification of the compounds used in ethnomedicine is empowering for the advancement of empirical knowledge, the facilitation of partnership development and the promotion of Indigenous Knowledge Systems.

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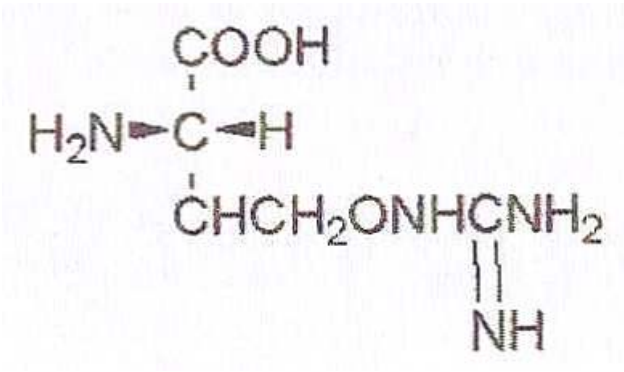
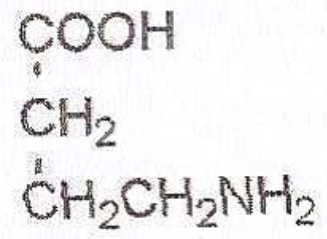
APPENDIX 1

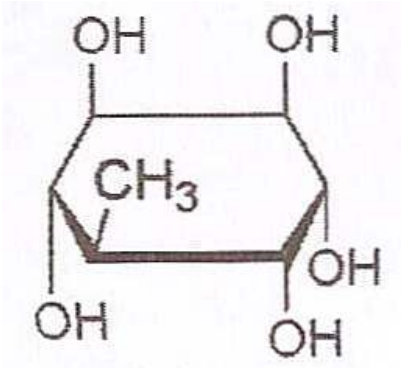
Chemical structures of L-canavanine, GABA and D-pinitol



L-canavanine



γ-amino butyric acid (GABA)

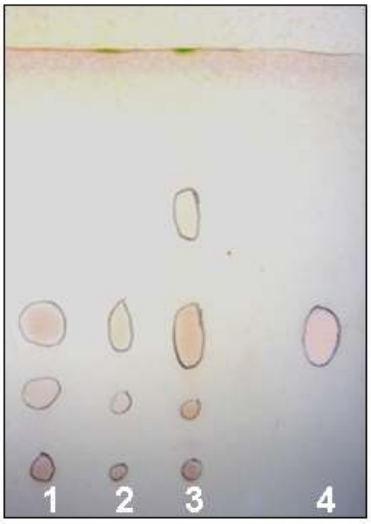


D-pinitol

Adapted from van Wyk and Albrecht (2008)

APPENDIX 2

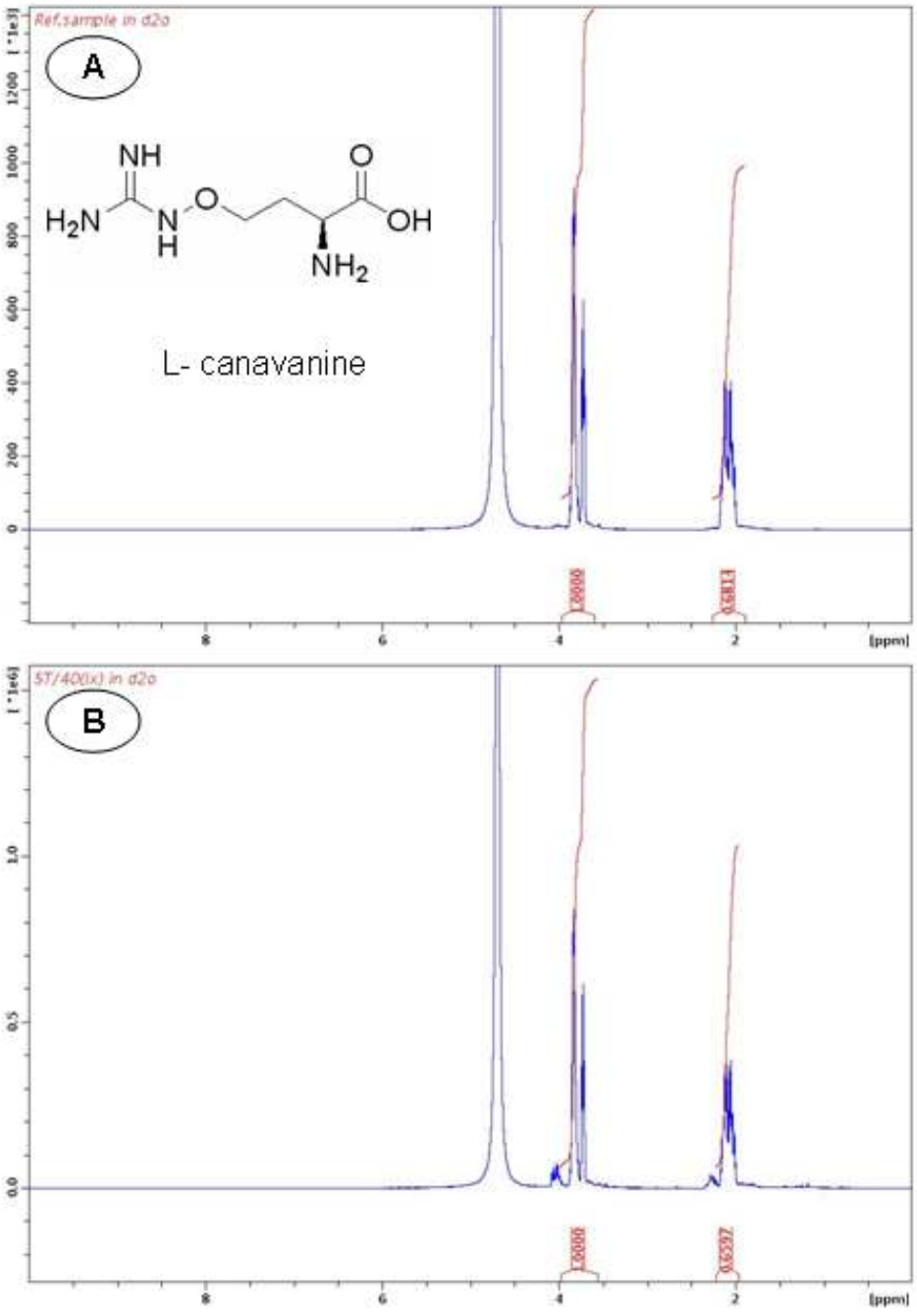
TLC plate of L-canavanine



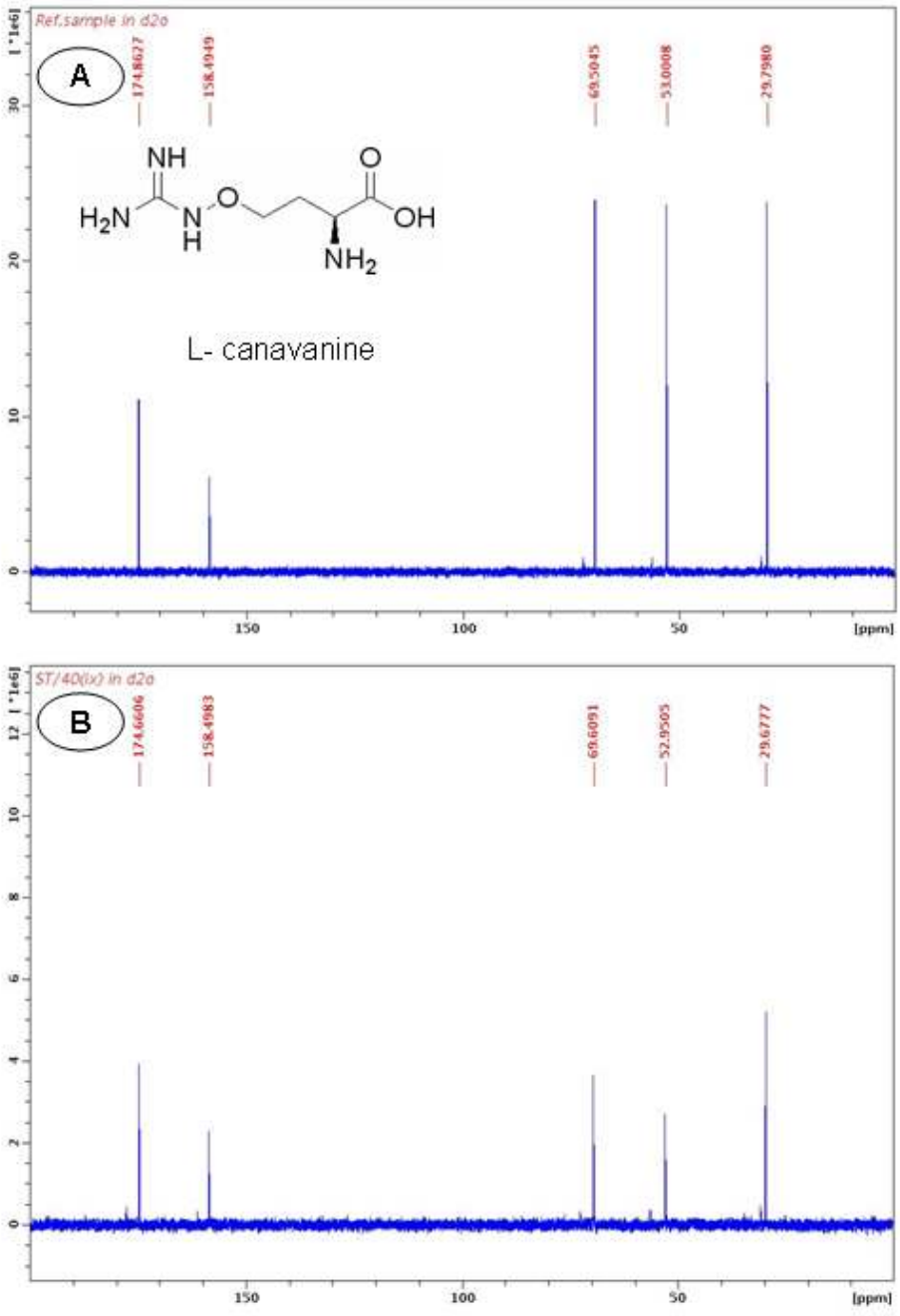
TLC analysis of L-canavanine in *in vitro* leaves of *L. frutescens* (1) extract with boiled water; (2) extract with ethanol; (3) extract with methanol (4) L - canavanine standard in water.

APPENDIX 3

NMR spectra of L-canavanine



[1]H NMR Spectrum. A) L-canavanine standard (Sigma) B) Methanol extract of *Lessertia* leaves



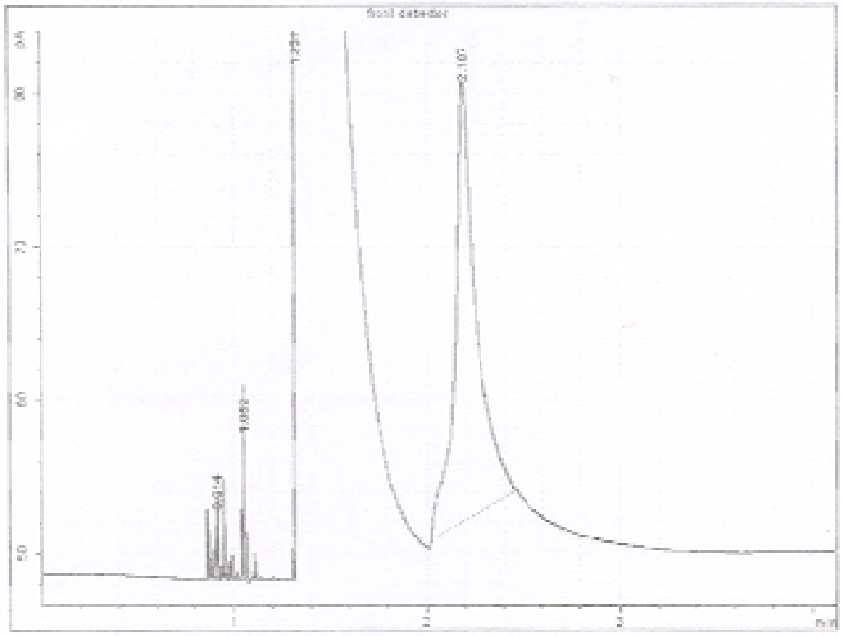
^{13}C NMR Spectrum. A) L-canavanine standard (Sigma) B) Methanol extract of *Lessertia* leaves

APPENDIX 4

GC chromatogram of GABA standard

Agilent Cerity QA/QC Report

Sample name:	*Reprocessed: 1mg 2 ul std
Sample note:	
Submission time:	08 February 2010 11:17
Operator:	
Injection date:	08 February 2010 11:18
GC Description:	inst1 - SN. CN10851002
Signal description:	FID1 A, front detector
Method:	gaba MeOH 4
Method last saved:	01 February 2010 15:32



Area Percent Report

Calibration last saved:	
Multiplier:	1.0000
Dilution:	1.0000
Sample amount:	0.0000 µL
Sample type:	Sample
Sampling source:	Manual

Signal	Retention Time [min]	Type	Width [min]	Area [pA*s]	Area %
1	0.914	BP	0.011	3.16849	0.00251
1	1.052	BP	0.006	3.54583	0.00280
1	1.331	PB S	0.023	1.2623e+005	99.81794
1	2.187	PB	0.105	223.52540	0.17675

Total Area = 126401.47875

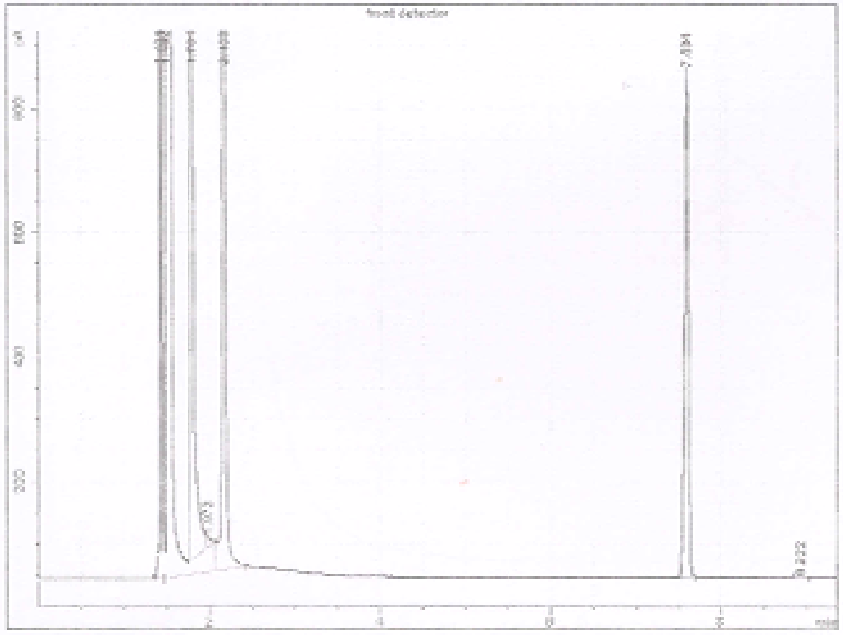
Report summary:
Warning(s): Sample amount is zero. Absolute amounts calculated

APPENDIX 5

GC chromatogram of D-pinitol standard

Agilent Cerity QA/QC Report

Sample name:	*Reprocessed: std 5mg/ml1 0.5ul
Sample note:	
Submission time:	27 January 2010 14:22
Operator:	
Injection date:	27 January 2010 14:22
GC Description:	inst1 - SN: CN10651002
Signal description:	FID1 A, front detector
Method:	galss MeOH 4
Method last saved:	01 February 2010 15:32



Area Percent Report

Calibration last saved:	
Multiplier:	1.0000
Dilution:	1.0000
Sample amount:	0.0000 µL
Sample type:	Sample
Sampling source:	Manual

Signal	Retention Time [min]	Type	Width [min]	Area [pA*s]	Area %
1	1.431	BP S	0.011	3900.78751	1.40135
1	1.492	VV S	0.030	2.4466e+005	87.89216
1	1.784	BV I	0.036	5618.72025	2.01852
1	1.973	VV T	0.052	66.71384	0.02397
1	2.168	VB S	0.026	2.1634e+004	7.77196
1	7.584	BB	0.046	2474.89640	0.88910
1	8.922	PP	0.054	8.16869	0.00293

Total Area = 278358.93040

Report summary:
Warning(s): Sample amount is zero. Absolute amounts calculated