STABILITY OF FREEZE-DRIED AQUEOUS AND OTHER MODIFIED EXTRACTS OF *LEONOTIS LEONURUS*

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KEYWORDS

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Marrubiin

Leonurine

Stability

Freeze-dried aqueous extract

Hygroscopicity

HPLC

Shelf-life

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SUMMARY

Stability of freeze-dried aqueous and other modified extracts of *Leonotis leonurus*

Leonotis leonurus, a South African indigenous medicinal plant, is frequently used in the form of a tea. However, this dosage form has many disadvantages. Consequently three *L. leonurus* solid extract preparations were prepared and explored as possible replacements of the tea form, but very little was known about their physical and chemical stability during storage.

The specific objectives were to: (i) prepare a freeze dried aqueous extract (FDAE), 20 % aqueous ethanol (Aq EtOH) extract and calcium alginate beads of the FDAE form of *L. leonurus*, (ii) characterize the extracts using parameters of select physical and chemical features and, (iii) determine the long-term stability of the extracts. It was hypothesised that the Aq EtOH extract would contain higher levels of chemical marker compounds (marrubiin and leonurine) than the FDAE and calcium alginate FDAE beads of *L. leonurus* and, that the calcium alginate FDAE beads would have greater stability (i.e. longer shelf-life) than the FDAE and the Aq EtOH extract.

The three *L. leonurus* solid extracts were prepared using accepted published methods. For the physical characterization of the extracts, the organoleptic properties were determined using the natural senses (e.g. sight, smell, taste, etc.) and for chemical characterization, total phenol content (TPC; using the Folin-Ciocalteu reagent method), total flavonoid content (TFC; using aluminium chloride-methanol solution) and antioxidant activity (using the -diphenyl-2-picryl-hydrazyl (DPPH) assay). To establish the long-term stability of the preparations, encapsulated *L. leonurus* solid extracts was stored in sealed standard plastic containers at four conditions: (A), room temperature of 24 ˚C ± 5 ˚C; (B), fixed temperature of 30˚C ± 5 ˚C and (C), elevated temperature of 40˚C ± 5 ˚C for 6 months, and (D), accelerated stability test conditions of 40˚C ± 5 ˚C / 75 % RH for 4 weeks. Samples of the stored encapsulated preparations were collected periodically and assessed for changes in organoleptic properties, TPC, TFC, antioxidant activity levels and marker compound (i.e. marrubiin and leonurine) levels. The latter was determined by validated HPLC assay.

Yields of 19.9, 12.82 and 10.7 % of FDAE, Aq EtOH extract and calcium alginate FDAE beads were obtained, respectively. Physically the calcium alginate beads contained less moisture (1.86 %) than the FDAE (3.77 %) and Aq EtOH (2.91 %). Chemically the FDAE, Aq EtOH extract and calcium alginate

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FDAE beads respectively had appreciable and similar TPC (i.e.7.86, 7.52 &, 6.94 mg GAE/g; $p > 0.05$; Anova) and TFC (i.e. 4.30, 4.47 & 3.67 mg QE/g; $p > 0.05$; Anova) levels, but variable amounts of marrubiin (i.e. 22.5, 17.5, and 0.4 ug/mg plant extract) and leonurine (i.e. 2.0, 1.4 and 0.7 ug/mg plant extract), respectively. The antioxidant activity levels were also different i.e. EC_{50} values of 7.71, 6.66 and 11.53 mg/mL (student t-test p-value of < 0.0001; ANOVA-test; $p< 0.05$) for the FDAE, Aq EtOH extract and calcium alginate FDAE beads, respectively.

During storage (i.e. stability study) the *L. leonurus* solid extracts generally remained physically unaffected by temperature (i.e. no significant change in organoleptic features), but when exposed to humidity the FDAE and Aq EtOH extracts showed clear signs of physical degradation i.e. changed from being flaky powders to sticky melted masses, while the calcium alginate beads remained unchanged. Within 1 month storage at RT, 30 °C, 40 °C and 1 week at 40 °C / 75 % RH the TPC of the encapsulated FDAE decreased significantly by 61, 60, 58 and 52 %, respectively, that for the encapsulated Aq EtOH extract by 61, 54, 46 and 50 %, respectively, and for calcium alginate FDAE beads by 66, 71, 59 and 57 %, respectively. Using TPC as a stability parameter all three encapsulated extracts had very short shelf-lives ranging from 1.24 weeks (0.31 months) to 3.72 weeks (0.93 months). Under the same conditions and storage periods (i.e. 1 month & 1 week) the TFC of the encapsulated FDAE decreased significantly by 25, 25, 29 and 66 %, respectively, for encapsulated Aq EtOH extract by 26, 26, 23 and 70 %, respectively, and the calcium alginate FDAE beads by 55, 55, 52 and 64 %, respectively. The results obtained for TFC was thus similar to that obtained for the TPC data. Based on the TFC data all three encapsulated extracts had very short shelf-lives ranging, from 1.56 weeks (0.39 months) to 6.76 weeks (1.69 months). Under the same conditions and storage periods (i.e. 1 month & 1 week) as that used to determine TPC and TFC, the antioxidant activity of the extracts changed little, i.e. decreased by 0.2, 0.1, 0.8 and 2 %, respectively for FDAE, by 0.7 %, 1 %, 0.1 % and 5.3 %, respectively for the Aq EtOH and by 2, 2, 1.4 and 0.8 %, respectively for the calcium alginate FDAE beads. Moreover, based on antioxidant activity, all three encapsulated extracts had relatively long shelf-lives ranging from 15.6 weeks (3.9 months) to 22.4 weeks (5.6 months).

Finally, the determination of the stability of the encapsulated *L. leonurus* extracts stored under stress conditions (i.e. 40 °C / 75 % RH) and based on marker compound levels was unresolved. Between the time of extract preparation and characterisation until start of the stability study the marrubiin levels in the FDAE, Aq. ETOH and calcium beads had decreased from 22.5, 17.5, and 0.4 ug/mg plant extract, respectively, to 0.30, 0.11, 0.30 µg/mg, respectively, and the leonurine levels from 2.0, 1.4 and 0.7 to

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0.46, 0.38 and 0.09 µg/mg, respectively and was too low to conduct a meaningful stability study with the developed validated assay.

Overall, all three the encapsulated *L. leonurus* solid extracts studied were clearly very unstable and did not have suitable long-term storage stability**.** The modification of the freeze-dried aqueous extract of *L. leonurus* into a calcium alginate bead form seemed to combat physical instability but did not improve the chemical instability of the aqueous extract. It is therefore recommended that the addition of excipients or other post extract modification (e.g. production of phytosomes) be explored to combat the hygroscopicity of *L. leonurus* FDAE and ultimately improve its overall product stability.

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DECLARATION

I declare that the thesis "Stability of freeze-dried aqueous and other modified extracts of *Leonotis leonurus*" is my own work that it had not been submitted before for any degree or examination in any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Ilana Alison Basson

Signed: 

Date: 14 March 2017

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DEDICATION

I dedicate my thesis to my mom Ilona Basson and dad Alistair Basson, not forgetting my little sister Ashante Basson and late brother Eldrid Basson, for their love, support, encouragement, sacrifices and always believing in me.

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CHAPTER 1: INTRODUCTION

Traditional medicine (TM), a term used to denote the indigenous health traditions of the world, have over the past two decades claimed an increasing share in the public's awareness and the agenda of medical research. According to the World Health Organization (WHO), as many as 65 % of the world's people depend on traditional medicine for their healthcare needs (Fabricant, *et al*., 2001). Popular use of traditional medicine has been accompanied by a growth in research and associated literature with an increase in evidence-based approaches. Studies have documented that about half the population of many industrialized countries now use traditional medicine (Bodeker, *et al*., 2002).

There are considerable economic benefits in the development of indigenous herbal medicines in appropriate high quality dosage forms for the treatment of various diseases (Muthu, *et al*., 2006). With about 27 million consumers, the current African market value of traditional medicine is an estimated R29 billion (Mander, *et al*., 2007). Diseases such as arthritis, backache, kidney and bladder disorders, cancer, colds, influenza, diabetes, headache, heart rheumatism, high blood pressure and stomach ailments could possibly be treated with these traditional medicines (Thring, *et al*., 2006). Traditionally, most of the herbal therapies involve the use of plant extracts in the form of aqueous solutions ("herbal teas") (Powell, *et al*., 2003). These herbal preparations are typically prepared by seeping or heating crude plant material, a practice that has prevailed for centuries, and has led to herbal teas which the modern healthcare providers sometimes prescribe (McKay, *et al*., 2006).

Leonotis leonurus (L.) R. Br. is a well-known medicinal plant, indigenous to South Africa, widely used in various dosage forms for a variety of traditional uses. Various parts of this plant are commonly used in the treatment of chronic conditions such as diabetes, hypertension and epilepsy, etc. (Maphosa, *et al*., 2008). However, few of these traditional uses may have been scientifically proven or validated. Traditionally, *L. leonurus* leaves are most often administered in the form of a tea, an excellent beverage with some effective pharmaceutical properties. This dosage form has been used since the discovery of traditional medicine (Thring, *et al.*, 2006). The advantage of using medicinal plants as a tea is that it contains and rapidly releases antioxidants, phenols and flavonoids which protect the body against free radicals, thus possibly facilitating cancer prevention, strengthening immunity and reducing the risk of various chronic

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diseases e.g. cardiovascular disease (Rice-Evans, *et al.*, 1996). However, the tea dosage form of *L. leonurus* also have many inherent disadvantages.

The disadvantages of the tea as a dosage form include the facts that, unless the leaves are dried and packaged in a teabag, its preparation is time consuming and inconvenient, it is not easy to keep the preparation free from microbial contamination and traditional dose measurements (e.g. take half a cupful, take one spoonful, etc.) are found to be inconsistent or imprecise. Furthermore, teas may contain a limited amount of plant active compounds as not all its chemical constituents are water soluble. Moreover, the compounds extracted could, over time, undergo possible degradation resulting in poor pharmaceutical quality. This in turn may result in an inappropriately short shelf-life of the brewed tea. These short-comings may however be remedied by using appropriate dosage forms of the plant material.

Solid dosage forms of medicinal plants, such as *L. leonurus*, may offer many advantages. And, possible appropriate oral dosage forms to replace the tea (i.e. liquid form) include tablets and capsules made from an appropriate dry aqueous extract of the plant.

The solid dosage form must, however, as closely as possible, mimic the tea in terms of similar active constituents, good pharmaceutical quality and stability (i.e. shelf-life). This can be achieved by preparing a freeze-dried aqueous extract (FDAE) that can mimic the traditional tea dosage form. But, FDAE's of plants were found to have their own disadvantages and are particularly prone to being hygroscopic (Ma, 2006). However, hygroscopicity of FDAE can be remedied by preparing modified forms of the extract by, for instance, using calcium alginate to coat the FDAE (Egieyeh, 2011). Another means of circumventing the hygroscopicity and stability problems of FDAE might be the use of an alcohol-water extract instead of the traditional aqueous tea extract. The viability of these options have however not yet been investigated for *L. leonurus*. For such an investigation, suitable chemical marker compounds found in the plant material and dosage are required. Compounds associated with bioactivity or therapeutic effects are usually present in all medicinal plants (Ahmad, *et al*., 2001) and could also be used as markers in monitoring the stability and pharmaceutical quality of FDAE and modified forms of *L. leonurus*. The active compounds allow the plant to be easily assayed and therefore serve to be monitored as indicators of product deterioration (Ahmad, *et al*., 2001).

A variety of chemical compounds has been cited as being potentially responsible for the therapeutic effects of *L. leonurus* and it contains at least 2 classes of chemical compounds that have been identified.

The first of these classes of compounds noted to be present in *L. leonurus* is the diterpenoids. These compounds contain 20 carbon atoms and 4 branched methyl groups and the diterpenoid labdane lactone marrubiin is a major marker compound found in *L. leonurus* (Mnonopi, *et al*., 2012). Indeed, marrubiin was isolated and quantified in *L. leonurus* FDAE which was observed to have anticoagulant, antiplatelet and anti-inflammatory effects in *in vitro* rat studies (Mnonopi, *et al*., 2011) and anti-diabetic effect (i.e. increased insulin secretion) in obese rat models (Mnonopi, *et al*., 2012). A second important class of active compounds found in *L. leonurus* is alkaloids. This class of compounds contains nitrogen and heterocyclic ring structures and exhibits powerful pharmacological effects. For example, the alkaloid leonurine (4-guanidino-n-butyl syringate) was found in the plant tissue of *L. leonurus* (Liu, *et al*., 2010) and showed anti-oxidative, anti-apoptotic and cardio-protective effects in both *in vitro* and *in vivo* models (Liu, *et al*., 2012). Leonurine and marrubiin may thus be ideal candidates for use as chemical markers to establish the quality and stability of solid extract forms of *L. leonurus,* but this has not yet been fully established.

Finally, an important product quality requirement for any herbal medicinal product, including *L. leonurus*, is its appropriate stability on storage and long term use. Stability of a product entails the non-changing of physical, chemical and microbiological test characteristics of the product over time and ultimately decides its shelf-life (Roberts, *et al*., 2002). Stability will also be a crucial parameter when the suitability of any solid dosage form of *L. leonurus* is considered Indeed, both the physical and chemical quality of the *L. leonurus* plant extracts and its modified forms as well as the stability of any identified marker compounds that might contribute to its therapeutic activity are essential to ensure the quality, safety and efficacy of a marketable product and guarantee its shelf-life (Gafner, *et al*., 2005). Therefore this study also investigated the stability of 3 forms of *L. leonurus* plant extracts to establish whether the FDAE (i.e. a solid form that can mimic the traditional tea dosage form) and other modified forms may, compared to the tea form, offer advantages with respect to enhanced long term product stability.

Given the above arguments, the objectives of this study consequently were to, first, prepare various solid extract forms of *L. leonurus*, then compare them in terms of their physical and chemical profiles and, finally, determine and compare their long-term stability using a variety of physical and chemical characteristics and the potential active constituents, marrubiin and leonurine, as marker compounds.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In this chapter an overview of traditional medicinal plants is given. Specifically focussing on *L. leonurus*, its description, traditional uses, phytochemistry, pharmacology and available traditional dosage forms. The shortcomings such as physical and chemical stability of traditional preparations will also be highlighted, and proposed methods of addressing them discussed. Parameters used to access the physical and chemical stability profiles of *L. leonurus* solid extract preparations are also included.

2.2 Traditional medicinal plants

Plants used as medicine are known as medicinal plants (Fabricant, *et al*., 2001). Medicinal plants form the bases of many sophisticated medicines as we know it today, because of its wealth in bioactive compounds (Elujoba, *et al*., 2005).

Bioactive compounds are often extracted from various parts of the plant (i.e. leaves, root, stem, bark etc.) using a number of extraction procedures. The extraction procedures for volatile oils can include maceration, percolation and distillation. For fluid extracts, ethanol or water can be used or a combination of both. However, for powders the solvents used in the fluid extract process is usually evaporated (Calixto, 2000). These extracts are what we know to be herbal medicines, and can take the form of crude drugs such as tinctures, teas, powders, poultices and other herbal formulations (Balunas, *et al.*, 2005) which are often used in the treatment of coughs, colds, influenza, bronchitis, hypertension, headaches, delayed menstruation, diabetes mellitus, intestinal worms, constipation, spider bites, scorpion stings, snake bites, etc. (Oyedemi, *et al*., 2010).

About 75-80 % of the world's population uses medicinal plants for their primary health care needs because of its better cultural acceptability and compatibility with the human body (Kamboj, 2000). Medicinal plants have played a key role in world health despite advances in modern medicines (Calixto, 2000), but needs to undergo a series of processes in order for its safety, efficacy and pharmacokinetic profile to be established. This would usually require the identification and isolation of bioactive compounds, which are assayed and formulated into suitable dosage forms. The dosage forms would then need to undergo clinical trials (Iwu, *et al*.,

1999). This process has however not been established or followed for most medicinal plants despite their cultural acceptability.

2.3 *Leonotis leonurus* – A traditionally important medicinal plant

2.3.1 Vernacular names

English:	Wild dagga
Afrikaans:	Wilde dagga
Xhosa:	Umfincamfincane
Zulu:	Umunyane
Sotho/Tswana:	Lebake
Shona:	Umhlahlampetu

2.3.2 Botanical classification and morphology of *Leonotis leonurus*

Leonotis leonurus belongs to:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Sub class:	Asteridae
Order:	Lamiales
Family:	Lamiaceae
Genus:	*Leonotis*
Species:	*L. leonurus*

Fig.2.1 *Leonotis leonurus*

Leonotis leonurus (L.) R. Br. is a very well-known medicinal plant indigenous to South Africa. It belongs to the Lamiaceae (mint) family (Ascensão, *et al*., 1995) and is made-up of about 3200 species in 200 genera (Mazimba, 2015). This plant has been used for centuries by various South African tribes, namely the Xhosa, Zulu, Sotho and the Shona, and therefore has a variety of vernacular names (see 2.3.1). The hairy flowers resemble a lion's ear giving it its specie name *leonurus* meaning lion's ear (Oyedemi *et al.*, 2010). *L. leonurus* (figure 2.1) is a robust

shrub with a bright orange flowering top growing up to 2-5 m in height and 1.5 m in width with a thick woody base and pale brown branches (Maphosa, *et al*., 2008). The leaves are bright yellow-green in colour, shaped narrow with serrate edges growing opposite each other, rough in texture with a highly aromatic-pungent mint odour (Mazimba, 2015; Oyedemi, et al., 2010). *L. leonurus* is easily cultivated with the ability to withstand drought and frost, is fast growing and blooms in the late summer and early autumn (Nsuala, *et al*, 2015).

2.3.3 Traditional uses of *L. leonurus*

Leonotis leonurus has many traditional medicinal uses and is mainly taken orally, applied topically or administered per rectum in the treatment of many ailments. A decoction of the plant leaves are generally prepared by which water soluble compounds are extracted by boiling the leaves in water and allowing it to cool. This is traditionally referred to as a tea. Once the tea has cooled, it can be used internally for the treatment of coughs, colds, influenza, bronchitis, hypertension, headaches, delayed menstruation, diabetes mellitus, intestinal worms, constipation, spider bites, scorpion stings and snake bites. Teas made from the leaves and the stem can be topically applied for the treatment of haemorrhoids, boils, eczema, skin disease, rashes, sores and muscular cramps (Maphosa, *et al*, 2008; Oyedemi, *et al*, 2010; Mazimba, 2015). The teas are also said to have both a hypnotic and diuretic effect (Mazimba, 2015).

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Teas made from the flowers and seeds, leaves or stems are commonly used as purgatives and tonics in the treatment of tuberculosis, jaundice, muscular cramps, high blood pressure, diabetes, viral hepatitis, dysentery and diarrhoea. Fresh stem juice is also used as a tea in the treatment of blood impurity (Mazimba, 2015). Teas made from the whole plant is used for the treatment of arteritis, piles, bladder, kidney disorders, obesity, cancer and rheumatism (Nsuala, *et al*, 2015).

Leonotis leonurus dried leaves or flowers, also referred to as "wild cannabis", and is often smoked. The flowers are known to be hallucinogenic and is said to have a marijuana-like effect, but is less potent (Mazimba, 2015; Nsuala, *et al*, 2015). Smoking "wild cannabis" flowers has been found to have a direct effect on the user's mental and emotional condition, in which the user experiences intense feelings of well-being, elation, happiness and joy. This can have side effects such as visual changes, nausea, dizziness, sedation, sweating and light headaches that can cause physical discomfort such as lung and throat irritation (Mazimba, 2015; Nsuala, *et al*, 2015).

2.3.4 Traditional dosage forms and preparation

Traditional dosage forms essentially refer to the form in which herbal products are either used or marketed for use. Suitable traditional dosage forms must be properly formulated for patients regardless of their age. In addition to that, the controlled delivery of an exact dosage is frequently key to its efficacy for the patient. Herbal products are traditionally dispensed in the form of liquids (e.g. infusions, decoctions, elixirs, and tinctures), semi-solids (e.g. pastes, creams and ointments), solids (e.g. whole or powdered plant parts, pills and tablets), and gasses (e.g. incense, fumigants and inhalants). Most of these dosage forms are given orally or applied externally to the affected areas on the body. Liquid dosage forms, also known as teas, are by far the most popular dosage forms used when it comes to traditional medicine (Ma, 2006).

Traditional dosage forms are prepared in various ways. Generally, infusions are prepared from the more tender plants, leaves and delicate herbs. An infusion is more like a tea. First the water is boiled and added to approximately a ¼ cup of plant leaves or fresh flowers. This is then left to stand and seep for 5 minutes before it is strained. In some cases honey is added to sweeten the infusion, making it less bitter and more pleasant to drink. Infusions are prepared according to the concentrations needed and should be taken the same day (Roberts, 1990).

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Decoctions are usually prepared using the more woody parts of the plant such as the stems, seeds or course leaves. Extraction for these plant parts are usually more difficult, therefore the plant material is boiled in water for a longer period of time, thus allowing it to soften and active compounds to be extracted. Decoctions are one of the oldest traditional medicine preparation forms and its preparation is inconvenient as well as time consuming. A single preparation can easily be cooked for about an hour (Oyedemi, *et al*., 2009).

Tinctures are alcohol only or alcohol and water extracts. This type of extract is used when plants contain compounds that are unable to be extracted using water only. During the extraction process volatile oils from plants are released making the extracts more concentrated exhibiting stronger activity (Zampini *et al*., 2009).

Maceration is said to be the easiest preparation method. The plant material used can either be fresh or dried. During this extraction process the plant material is soaked overnight in cool water using a covered container. The herb mixture is then strained and the extract drunk. This method is used for plants containing heat sensitive or alcohol sensitive compounds (Ma, 2006).

2.3.5 Shortcomings of the traditional dosage-form preparation

The aforementioned traditional dosage forms of *L. leonurus* has several disadvantages arising from inconsistent preparation, methods of administration and poor stability during storage.

Firstly, wet leaves should not be used when preparing the tea, as moisture present in the leaves may promote bacterial growth which may lead to deterioration of the product (McCutcheon, 2002).

Secondly, directions for dosage preparations are often generally obscure. For example, the use of inexact measures such as a quarter cup, a handful, tumblerful and calabashful. These preparation instructions are very often vague and incorrect. This leads to lack of mass and content uniformity as it is difficult to accurately quantify a handful or measure a quarter cup consistently (Sofowora, 1982; Williamson *et al*., 1996). Thus, it may be anticipated that such variability in the preparation may lead to variations in dose each time a treatment is prepared, therefore altering the amount of active constituents extracted.

Thirdly, *L. leonurus* teas (infusions or decoctions) are often smelly and awful tasting for oral use. Sweeteners such as honey and sugar gets added to make the tea more palatable, improving patient acceptability. However, these additives in turn act as good media for the growth of microorganisms like bacteria and fungi (Burlage *et al*., 1963).

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Lastly, incorrect storage of plant material causes chemical and biological activity changes. Previous research findings suggest that antibacterial activity in fresh plants increase after 90 days of storage and stays consistent for up to 5 years when stored at 20 ºC. Antibacterial activity in fresh plants deteriorates after 15 days when stored at 55 ºC and 100 % humidity. It was concluded that temperature, light, pH, microbes and plant enzymes are the main cause of chemical and biological activity deterioration (Mazimba, 2015).

2.3.6 Phytochemical constituents of *L. leonurus*

Extensive research had been conducted on the phytochemical constituents of *L. leonurus*. It was found that terpenoids (mono-, sesqui and diterpenoids) known for their biologically active nature, are the main compounds in the *L. leonurus* plant (Nsuala, *et al.*, 2015). Of these terpenoids, labdane diterpenoids extracted from plant leaves were the most abundant compounds present in the plant (Nsuala, *et al.*, 2015).

2.3.6.1 Marrubiin

Phytochemical studies have revealed that marrubiin, a labdane diterpenoid produced by *Leonotis leonurus, Leonotis nepetifolia,* and *Marrubium vulgare,* is the most active diterpenoid lactone present and is therefore responsible for the therapeutic properties observed in these plant species (Mnonopi *et al*., 2011). Pre-furanic (premarrubiin) and furanic (marrubiin) labdanoids are widespread in the family Lamiaceae. Furanic labdanoids are made of a C-9 hydroxyl group with a furan ring and side chain, have the same marrubiin structure and is considered to be the final product of the biosynthetic pathway (Popoola *et al.,* 2013). However, other researchers suggest that they are artefacts which arise from their corresponding pre-furan labdanoids during or after the extraction or isolation process due to cleavage of the 9,13-epoxide bridge. Recent literature findings report the detection of marrubiin in fresh plant material. The data obtained from these studies confirms that it is a natural product produced at the end of a biosynthetic pathway (Popoola *et al*., 2013). Marrubiin was found to be responsible for dose-related anti-nociceptive and cardio-protective effects as well as the inhibition of gastric acid secretion in animal models (Mnonopi *et al*., 2011; Mnonopi, *et al*., 2012: Popoola *et al*., 2013). This compound has been used as a valuable marker compound in the investigation of *L. leonurus*.

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2.3.6.2 Leonurine

Leonotis leonurus also contain pharmacologically active guanidine alkaloid leonurine (4-hydroxy-3,5-dimethoxybenzoic acid 4-guanidinobutyl ester) which is considered one of its major active ingredients. This active compound mainly exerting cardiovascular, hypotensive, uterotonic and neuroprotective effects (Kuchta, *et al*., 2012). Research findings on leonurine show that it has a protective effect against myocardial and cerebral ischemia both *in vitro* and *in vivo* (Zhu *et al*., 2012). The underlying mechanism of action may be associated with its anti-oxidative and anti-apoptotic effects and its ability to protect mitochondrial function. The results of previous studies suggest that leonurine has become a novel promising cardiovascular drug candidate (Zhu *et al*., 2012).

According to literature both marrubiin (figure 2.2) and leonurine (figure 2.3) are present in *L. leonurus* at considerable levels and can be assayed by simple extraction and HPLC analytical procedures (Mnonopi *et al*., 2011; Chao, *et al*., 2004). Moreover, based on the pharmacological

activities of marrubiin and leonurine (as mentioned above), it is hypothesized that the presence of these compounds in *L. leonurus* is responsible for the therapeutic activities of the plant.

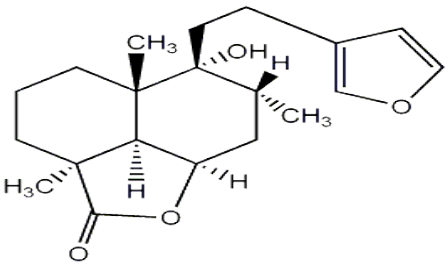

Fig. 2.2: Marrubiin structure

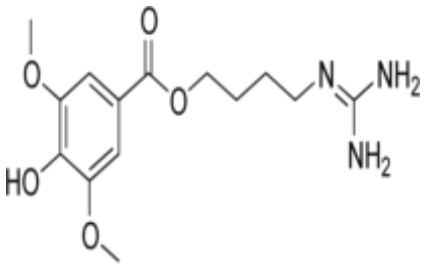

Fig. 2.3: Leonurine structure

2.4 *Leonotis leonurus* extracts, short-comings and possible solutions to short-comings

2.4.1 Freeze-dried extracts

In order to improve the physical and chemical stability of a product, water has to be removed from it (Wolkers *et al*., 2001). Freeze-drying therefore involves the removal of water or other solvent from a product (i.e. plant material, food etc.), ensuring a final product of the highest quality when compared to other drying techniques. It is the most preferred technique commonly used to covert solutions or suspensions into solids of sufficient stability, for distribution and storage (Wolkers *et al*., 2001). Freeze-drying is based on the dehydration and sublimation of a frozen product. This occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase. Due to the absence of water and the low temperatures required for the process, most of the deterioration and microbial processes are stopped, ensuring good product quality. The solid state of water during freeze-drying ensures that the primary structure and shape is retained with minimal reduction in volume (Ratti, 2001).

Freeze-drying is superior to other drying methods and is regarded as one of the most important steps in the preservation of medicinal properties, found in plant material. The freeze-dried (lyophilized) extract is usually prepared before the analysis of various active compounds present in plant material. Freeze-dried extracts contain hydrophilic characteristics, but there is no information available on whether or not this will affect the shelf-life of products after the consumer packaging is opened and the contents exposed to oxygen and humidity (Abascal, *et al*,. 2005).

2.4.2 Short-comings of freeze-dried extracts

According to a study by Egieyeh (2009), hygroscopicity is the key degradation factor of freeze-dried aqueous extracts. Hygroscopicity is a term widely used in the pharmaceutical community that describes the ability of a substance to attract and hold water molecules from the surrounding environment. The hygroscopic ability of a plant can be greatly influenced by increasing temperature and relative humidity (RH) values. According to a study conducted by Callahan (1982), solid extracts were classified into various groups based on their water uptake after storage for one week at various conditions: Class 1 would be regarded non-hygroscopic (no water sorption below 90 % RH, and < 20 % at 90 % RH); Class II would be slightly hygroscopic (no water sorption below 80 % RH, and 40 % at 80 % RH); Class III would be moderately hygroscopic (5 % below 60 %, and < 50 % at 80 % RH) and Class IV would be very hygroscopic (> 5 % below 60 % RH). These criteria may not directly apply to this investigation, but is considered a good indicator of various hygroscopic classes. Hygroscopicity in solid plant extracts facilitate physical as well as chemical degradation, thus allowing plants to be less effective and unable to execute its therapeutic purposes.

2.4.3 Alginate coating

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With hygroscopicity being the key degradation factor in solid plant extracts, an innovative approach is needed to combat it. One approach to combatting hygroscopicity is by coating the solid plant extract (i.e. freeze-dried extract) with alginate.

Alginate is a naturally occurring biopolymer that is finding increasing applications as it has been used successfully in the food and beverage industry as a thickening agent, gelling agent and a colloidal stabilizer. Alginate properties allow it to be used as a matrix for the entrapment and delivery of a variety of proteins to cells. These properties include: (i) a relatively inert aqueous environment within the matrix; (ii) a mild room temperature encapsulation process free of organic solvents; (iii) a high porosity which allows for high diffusion rates of macromolecules; (iv) the ability to control porosity with simple coating procedures and (v) dissolution and biodegradation system under normal physiological conditions (Gombotz, *et al*., 2012).

There are various sources of alginate. Commercial alginate can be extracted from three species of brown algae (kelp). These include *Laminaria hyperborea, Ascophyllum nodosum and*

Microcystis pyrifera. Other sources include *Laminaria japonica, Eclonia maxima*, *Lesonia negrescens* and *Sargassum* species. In all of these algae alginate is the primary polysaccharide present. Alginate is found in the intracellular matrix where it exists as a mixed salt of various cations found in sea water such as Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and Na^{+}. The native alginate is mainly present as an insoluble Ca^{2+} cross-linked gel. Bacterial alginates have also been isolated form *Azotobacter vinelandii* and several *Pseudomonas* species (Gombotz, *et al*., 2012).

Alginate beads can be prepared by extruding a solution of sodium alginate containing the desired protein as droplets, into a divalent crosslinking solution such as Ca^{2+}, Sr^{2+} or Br^{2+}. Monovalent cations and Mg^{2+} ions do not induce gelatin while Ba^{2+} and Sr^{2+} ions produce stronger alginate gels than Ca^{2+}. The gelatin and crosslinking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of this guluronic groups to form a characteristic egg-box structure. The divalent cations bind to the α-L-guluronic acid blocks in a highly cooperative manner and the size of the cooperative unit is more than 20 monomers. This allows each alginate chain to dimerize and form junctions with many other chains and as a result gel networks are formed rather than insoluble precipitants (Gombotz, *et al*., 2012).

2.5 Quality evaluation of solid dosage forms

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2.5.1 Stability testing

Stability is an essential part of drug development (Lusina *et al*. 2005). It demonstrates physical and chemical stability of a drug product exposed to a variety of environmental conditions such as temperature, humidity and light (Allinson, *et al*., 2001). A well-designed stability study should include testing of those attributes that are susceptible to change during storage and are likely to influence the quality, safety and efficacy of a product (Lusina *et al*., 2005).

The challenge of a moisture sensitive compound is to demonstrate stability at accelerated conditions of 40 ˚C/ 75 % RH over a period of time. Many compounds are moisture sensitive resulting in significant product degradation (Allinson, *et al*., 2001). Moisture is frequently associated with physical and/ or chemical instability of a pharmaceutical drug product. Moisture sensitive products should be adequately protected during shelf-life (Badawy, *et al*., 2001).

Stability testing of crude drugs is a fundamental requirement of industry and other organizations in the assurance of product quality. It should be noted that product stability is directly affected by the complexity and inconsistency of the active pharmaceutical ingredient (API) (Mukherjee *et al*., 2008). Degradation of the API is the most important cause of plant product stability changes. This can be caused by hydrolysis, oxidation, photolysis and thermal decomposition (Bhinge, *et al*., 2008).

API of herbal products should be standardized since crude drugs have been seriously criticized for their lack of dosage precision, standardization and short shelf-life (Onunkwo, *et al*., 1996). Two main aspects that play an important role in shelf-life determination are assaying of the API and the degradents generated during the stability study (Naidu, *et al*., 2005).

2.5.2 Total phenol content evaluation

Phenolic compounds have been recognized as the major source of natural antioxidants (Kähkönen *et al*., 1999). Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways in plants. Phenolic compounds is one of the most frequently occurring groups of phytochemicals and are of considerable physical and morphological importance in plants. These compounds play an important role in growth and reproduction, providing protection against pathogens and predators, besides contributing towards the colour and sensory characteristics of fruits and vegetables. Phenolics exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-microbial, anti-inflammatory, anti-oxidant, anti-thrombotic, cardio-protective and vasodilatory effects. The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity. Phenolic compounds could be a major determinant of antioxidant potentials of foods and could therefore be a natural source of antioxidants (Nookabkaew, *et al*., 2006).

Colour development using Folin-Ciocalteu reagent (Folin- Ciocalteu assay) is the generally preferred method for measuring phenolics, because most plant derived antioxidants contain large amounts of polyphenols (Katsube *et al*., 2004). The Folin- Ciocalteu assay was developed in 1927 for the measurement of tyrosine. The reagent consists of a mixture of sodium molybdate, sodium tangstate, and other reagents. Upon reaction it produces a blue colour which absorbs at a wavelength of 765 nm UV light. The assay has been used for many years by food and agricultural industries to determine the phenolic content of plant products (Everette *et al*., 2010).

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2.5.3 Total flavonoid content evaluation

Flavonoids are polyphenolic compounds also known as low molecular weight phenolics. These properties include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions (Bahorun *et al*., 2004).

Flavonoids can act as vasodilators and platelet disaggregates and also possess antioxidant and free radical scavenging abilities (Bahorun *et al*., 2004). They exist readily in the plant kingdom and are especially common in leaves, flowering tissues and pollen. Plant flavonoids are an important part of the diet because of their effects on human nutrition. These phytochemicals can modulate lipid peroxidation involved in artherogenesis, thrombosis and carcinogenesis (Zhishen, *et al*., 1999).

Known properties of flavonoids include free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Some evidence suggests that the pharmacological effects of flavonoids are correlated with their antioxidant activities (Zhishen, *et al.*, 1999). Moreover, it is suggested that the overall antioxidant effect of flavonoids on lipid peroxidation may be related to their hydroxide and oxygen scavenging properties and their reaction with peroxy radicals. Flavonoids can be used directly to scavenge oxygen and hydroxide by single electron transfer, this scavenging process can be followed by means of electron spin resonance but the expense of such instruments hinders their use by the average laboratory (Zhishen, *et al.*, 1999).

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Flavonoids contain a highly conjugated aromatic system and therefore exhibit intense, characteristic absorption or florescence spectra. Chelation with other metals causes spectral shifts that can be correlated with flavonoid structures. The UV/visible absorption spectra of flavonoids in the presence of Al_3 have been used to distinguish flavonoids that contain either a free 5- or 3-hydroxyl group. Flavonoids that do not contain these groups do not form complexes with Al_3 and therefore their spectra are unaltered. Among those flavonoids that do complex Al_3, morin, quercetin, and kaempferol have been used in the flueorometric determination of Al_3. In a neutral or acidic medium (typically ethanol), the characteristic florescence of the chelat provides detection levels for Al_3 as much as few parts per billion (Deng, *et al*., 1998).

2.5.4 Antioxidant activity evaluation

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Antioxidant activity is a fundamental property important for life (Velioglu *et al*., 1998). Many of the biological functions, such as anti-mutagenicity, anti-carcinogenicity, and anti-aging, among others originate from this property. There are two basic categories of antioxidants namely synthetic and natural. Synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen acids (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid (Velioglu *et al*., 1998). Many naturally occurring oxidative compounds from plant sources have been identified as free radical inhibitors or active oxygen scavengers (Duh, *et al*., 1999).

In recent years much attention has been devoted to plants containing natural antioxidants and their association with health benefits. Plants produce various anti-oxidative compounds to counteract reactive oxygen species in order to survive (Huda-Faujan *et al*., 2009). Reactive oxygen species which include free radicals such as superoxide anion radicals (O_2), hydroxyl radicals (OH) and non-free radical species such as H_2O_2 and singled oxygen (O_2) are various forms of activated oxygen. These molecules are exacerbating factors to cell injury and aging processes (Huda-Faujan *et al*., 2009).

There are several methods used to determine the antioxidant activity of biological material. The two most widely used being the 2,2'-azino-bis(3-ethyl benzothiazoline -6-sulphonic acid) (ABTS) and 2,2-Diphenyl-1picrylhydrazyl (DPPH) radicals. Both present excellent stability in certain assay conditions but also show several important differences in their response to antioxidants and in their manipulation. DPPH is a free radical that is required directly without preparation, while ABTS must be generated by enzymatic or chemical reactions. Another difference is that ABTS can be solubilized in aqueous and organic media in which the antioxidant activity can be measured due to hydrophilic and lyophilic nature of the compounds in samples. In contrast to that DPPH can be absorbed in organic media. DPPH is a very stable absorbance radical and is therefore widely used to characterize plant material (Arnao, 2000).

To evaluate the oxidative activity of specific compounds or extracts, plant extracts are allowed to react with a stable radical DPPH in a methanol solution. The reduction of DPPH is followed by monitoring the decrease in its absorbance at a characteristic wavelength of light during its

reaction. In its radical form DPPH absorbs light at 515 nm, but upon reduction by an antioxidant or radical species the reduction disappears. DPPH is thus used to determine antiradical activity (Brand-Williams, *et al*., 1995).

2.5.5 HPLC fingerprinting evaluation

HPLC fingerprinting has the ability to provide timely, accurate, and reliable data. It is central to the role of analytical chemists, especially in the discovery, development and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release (Green, 1996). The quality of analytical data is a key factor in the success of the drug development program. The process of method development and validation has a direct impact on the quality of this data. Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones (Green, 1996).

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. HPLC validation methods for regulatory submission must include studies on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit and robustness. This approach should be viewed with the understanding that validation requirements are continually changing and vary widely, depending on the type of drug being tested, the stage of drug development and the regulatory group that will review the drug application. During each validation study, key parameters are determined and then used for all consecutive validation steps. HPLC fingerprints is therefore used to determine product stability. If the products are not stable, storage conditions or additives should be identified to improve stability (Green, 1996). HLPC fingerprinting is considered the method of choice for the quantitative determination of drugs. (Matuszewski, *et al*., 2003).

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CHAPTER 3: PLAN OF WORK

In this chapter, the objectives pursued, the hypotheses tested and the study approach was outlined.

3.1 Aims

The overall aims of the study were to test whether: (i) the solid dosage form of *L. leonurus* solid extracts is a viable replacement for the traditional tea form, (ii) to establish whether they had suitable long-term storage stability, and (iii) to establish which stability parameters (including the determination of marker compound levels, marrubiin and leonurine), were suitable to use as product stability indicators for these extracts.

3.2 Objectives

Consequently, the specific objectives included: (i) the preparation and characterization of a FDAE, Aq EtOH extract and calcium alginate FDAE beads forms of *L. leonurus*, (ii) determination and comparison of their long-term stability using physical characteristics (e.g. organoleptic properties) and chemical parameters (e.g. TPC, TFC, antioxidant activity levels and marker compound levels) as stability-indicating parameters.

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3.3 Hypotheses

The hypotheses to be tested were that:

(i) The Aq EtOH extract of *L. leonurus* plant material would contain higher levels of chemicals (TPC, TFC) and chemical marker compounds (marrubiin and leonurine) than the FDAE and calcium alginate FDAE beads of *L. leonurus* and,

(ii) Calcium alginate FDAE beads would have greater stability (i.e. longer shelf-life) than the FDAE and the Aq EtOH extract.

3.4 Study approach and motivation

To realize the above objectives, crude extracts was to be prepared from the dried *L. leonurus* leaves. This included a freeze-dried aqueous extract (FDAE) that would closely mimic the traditional tea and a 20 % aqueous ethanolic (Aq EtOH) extract and calcium alginate FDAE beads. Since it was expected that the FDAE would be hygroscopic in nature, the calcium

alginate coating was primarily intended to remedy the hygroscopicity of the FDAE, while the Aq EtOH extract was expected to contain higher levels of chemical constituents than the FDAE. The physical and chemical characteristics of all three solid *L. leonurus* forms would need to be determined and compared to one another in order to establish the stability profiles of each form. All three solid *L. leonurus* forms would then be encapsulated, the capsules placed in plastic containers and subjected to different conditions, including storage at elevated temperature and humidity. This would be followed by periodic sampling and using various parameters to determine changes in both physical and chemical long-term stability of the encapsulated solid extract forms. The stability profiles of *L. leonurus* solid extracts would be established and compared.

3.5 Why stability testing and the specific test parameters used?

Because of the known hygroscopic nature of the FDAE and expected hygroscopic nature of the Aq EtOH extract, the modification of FDAE into calcium alginate FDAE beads was therefore explored. Though the calcium alginate FDAE beads were expected to be more physically stable, overall physical and chemical stability of all three *L. leonurus* solid extracts needed to be investigated in order to produce a marketable product with suitable long-term storage stability.

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Stability tests can be done using various parameters. In this study, parameters based on the physical, chemical and activity characteristics of the products were to be used to determine physical stability changes in organoleptic features as parameters. This method involves the use of the natural senses making it a very easy method to apply (Ma, 2006).

Chemical stability levels of total phenol content were to be determined using the Folin-Ciocalteu reagent method. Most plant derived antioxidants contain large amounts of polyphenols and the selected method is a commonly used and preferred one for measuring phenolics (Katsube *et al*., 2004). The reagent consists of a mixture of sodium molybdate, sodium tangstate, and other reagents and upon reaction a colour change from yellow to blue/green is observed indicating the presence of phenols.

Another chemical parameter to be measured was the total phenol content and an aluminium (Al_3) chloride-methanol solution method to test total flavonoid content. *L. leonurus*, like most plants, is known to contain flavonoids (Oyedemi, *et al.*, 2011) and the UV/visible absorption spectra of flavonoids in the presence of Al_3 have been used to distinguish flavonoids that

contain either a free 5- or 3-hydroxyl group. Flavonoids that do not contain these groups do not form complexes with Al_3 and therefore their spectra are unaltered. This method was therefore found best suited for the study.

As a further chemical parameter, levels of two marker compounds, viz. marrubiin and leonurine, was also to be monitored. For this an HPLC assay was to be validated and used for their quantification in the *L. leonurus* solid forms. The process of HPLC method development and validation has a direct impact on the quality of data from this section of the study. Although a thorough validation cannot rule out all potential problems, the process of method development and validation is expected to address the most common ones (Green, 1996). Following the validation of an HPLC assay, HPLC fingerprints obtained can then be used to determine product stability. Indeed, HPLC fingerprinting is considered the method of choice for the quantitative determination of drugs (Matuszewski, *et al*., 2003).

In addition to using physical and chemical parameters to monitor the stability of the *L. leonurus* preparations, the antioxidant activity was also determined. For this the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was to be used to determine antioxidant activity because DPPH is a very stable absorbance radical, is easily absorbed in organic media and is therefore widely used to characterize plant material (Arnao, 2000). To evaluate the oxidative activity of specific compounds or extracts, plant extracts are normally allowed to react with the stable radical DPPH in a methanol solution and the reduction of DPPH followed by monitoring the decrease in its absorbance at a characteristic wavelength of light during this reaction (Brand-Williams, *et al*., 1995).

Finally, to demonstrate physical and chemical stability of the *L. leonurus* solid forms they were to be exposed to varying environmental conditions such as temperature and humidity ranging from room temperature (moderate) to accelerated conditions (room temperature, 24 ˚C ± 5 ˚C; fixed temperature of 30˚C ± 5 ˚C; elevated temperature of 40˚C ± 5 ˚C and accelerated conditions of 40 ± 5 ˚C / 75 % relative humidity (RH)). This is usually done as an essential part of product development, and entails testing those attributes that are susceptible to change during storage and are likely to influence the quality, safety and efficacy of the product and the afore-mentioned commonly used storage conditions (Allinson, *et al*., 2001 & Lusina *et al*., 2005)

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CHAPTER 4: PREPARATION AND CHARACTERIZATION OF *L. LEONURUS* EXTRACTS

4.1 Introduction

In this chapter, the preparation and characterization of *L. leonurus* solid extracts was investigated and discussed. The aim of this part of the study was to determine whether *L. leonurus* solid extracts was a viable replacement for the traditional tea form and the objective was to prepare and characterize the *L. leonurus* solid extracts. It was hypothesized that freeze-dried 20 % aqueous ethanol extract (Aq EtOH) of *L. leonurus* plant material would contain higher levels of chemical marker compounds than the freeze-dried aqueous extract (FDAE) and calcium alginate FDAE beads. It was also expected that FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* might differ in organoleptic features, chemical constituent profile and levels, and anti-oxidant activity.

Furthermore, the chemicals, materials, equipment and experimental procedures used to prepare and characterize the FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* were presented and the results obtained reported and discussed.

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4.2 Chemicals, Materials and Equipment

The chemicals and materials used included:

Leonotis leonurus leaves (*Parceval Pharmaceuticals (Pty) Limited, South Africa*), sodium carbonate, ascorbic acid, gallic acid, 1-diphenyl-2-picryl-hydrazyl (DPPH), Folin Ciocalteu reagent, aluminium chloride *(AnalytiCals Carlo Erba, France),* absolute ethanol, methanol *(KIMIX, South Africa)* and distilled water *(Saarchem, South Africa).*

The equipment used included:

A hot plate and stirrer *(MH-4, 1586, FRIED*), halogen moisture analyser HR73 *(Mettler toled, South Africa),* scale *(AR2140, Adventurer, OHAUS),* filter *(CH-9230, V-500, Buchi Laboratories AG),* filter paper *(Whatman no.3, Whatman England,* vacuum pump *(V-700, Buchi Laboratories AG),* rotor evaporator (RII, *Buchi Laboratories AG*), -86 °C freezer *(Ultra flow freezer, NUAIRE),* freeze-drier *(Sentry 2.0, Vir Tis SPScientific),* photomicroscope *(VMS-004 Delux, Veho, Discovery),* incubator *(220, Scientific Incubator),* spectrophotometer *(Chemi*

HR 410 BioSpectrum Imaging System UV spectrophotometer), microplate reader *(BMG LABTECH GMBH, SPECTROstar Nano 601-0040 UV/Vis)*, water bath *(WMR 14, Memmer)* and a vortex mixer *(VM-300, Vortex mixer, Gemmy Industrial Corp).*

4.2.1 Experimental Procedures

4.2.2 Preparation of freeze-dried aqueous extract (FDAE)

Dried leaves of *L. leonurus* were obtained from Parceval Pharmaceuticals (Pty) Limited, a commercial supplier in Wellington, Western Cape, South Africa. The leaves were stored in sealed plastic bags in a cool place, away from direct light. The extraction procedure used simulated the traditional preparation of medicinal herbs (Egieyeh, 2011). Commonly, a quarter cup of *L. leonurus* leaves added to a cup of boiling water is allowed to stand and seep for 10 minutes. For this study, 4.42 L of boiled distilled water was added to 221 g of dried plant leaves (approx. 1:20 ratio of dried plant material to solvent), stirred for 10 minutes with a magnetic stirrer and then allowed to cool down to room temperature. Thereafter the extract was filtered through Whatman no. 1 filter paper using a Buchner funnel and a vacuum pump, the filtrate poured into tarred round bottom flasks, quickly frozen to a thin layer using liquid nitrogen and then kept in a freezer at - 80 °C for 48 hours. The frozen extract was dried under vacuum in a freeze-dryer at – 44 °C for 72 hours. The freeze-dried powder was weighed for calculation of the percentage yield and then transferred to an amber glass bottle, sealed and stored in a desiccator until further testing. The yield was calculated using formula:

% yield = [Wt FDAE powder / Wt dried plant leaves] * 100 %

4.2.3 Preparation of 20 % aqueous ethanol extract

The extraction procedure used to prepare the ethanol extract of *L. leonurus* was similar to that for a traditional herbal tea preparation, except that the extracting solvent consisted of a 20 % ethanol in distilled water (dH_2O) solution. For the ethanol extract, 1 L solution of 0.8 L distilled water plus 0.2 L 99 % ethanol was boiled and added to 50 g of dried *L. leonurus* leaves (i.e. approx. 1:20 ratio of dried plant material to solvent), the mixture stirred with a magnetic stirrer for 10 minutes and allowed to cool to room temperature. Once cool the extract was filtered through Whatman no. 1 filter paper using a Buchner funnel and a vacuum pump, the filtrate collected and transferred to tarred round bottom flasks in which it was then evaporated to dryness with a rotor evaporator at 40 °C. When all the ethanol had evaporated, the flasks were placed in a freezer at - 80 °C for 48 hours to freeze the residue. The frozen extract was then

dried under vacuum at – 44 °C for 72 hours, the freeze-dried extract powder was collected, weighed for calculation of the yield using the formula below and thereafter transferred to an amber glass bottle, sealed and stored in a desiccator until further testing.

% yield = [Wt 20% EtOH extract powder / Wt dried plant leaves] * 100

4.2.4 Preparation of calcium alginate FDAE beads

The calcium alginate FDAE beads were prepared according to the method of Egieyeh (2011) (figure 4.1). First, a 2 % (w/v) solution of sodium alginate was prepared by mixing 2 g sodium alginate **(A)** in 0.1 L of dH_2O. Then 2 g of FDAE was added to this 2 % sodium alginate solution. Separately, 4 % (w/v) calcium chloride was prepared by dissolving 4 g calcium chloride **(B)** in 0.1 L dH_2O. The 2 % alginate solution containing the solid plant extract was drawn-up into a 20 mL syringe and then added, drop-wise through a 23 G needle, to the 4 % calcium chloride solution. The beads formed in this process were allowed to soak for 5 minutes before the suspension was passed through a sieve and the beads collected. The beads were then rinsed with distilled water to remove the excess calcium chloride, oven-dried at 60 °C and weighed for the percentage yield calculation. Finally, the prepared calcium alginate FDAE beads were transferred to an amber glass bottle, sealed and stored in a desiccator until further testing.

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$$\% \text{ yield} = \frac{[(\text{Wt Ca alginate FDAE beads}) * \% \text{ FDAE}]}{\text{FDAE + AB}} * 100$$

where AB = A (sodium alginate) + B (calcium chloride)

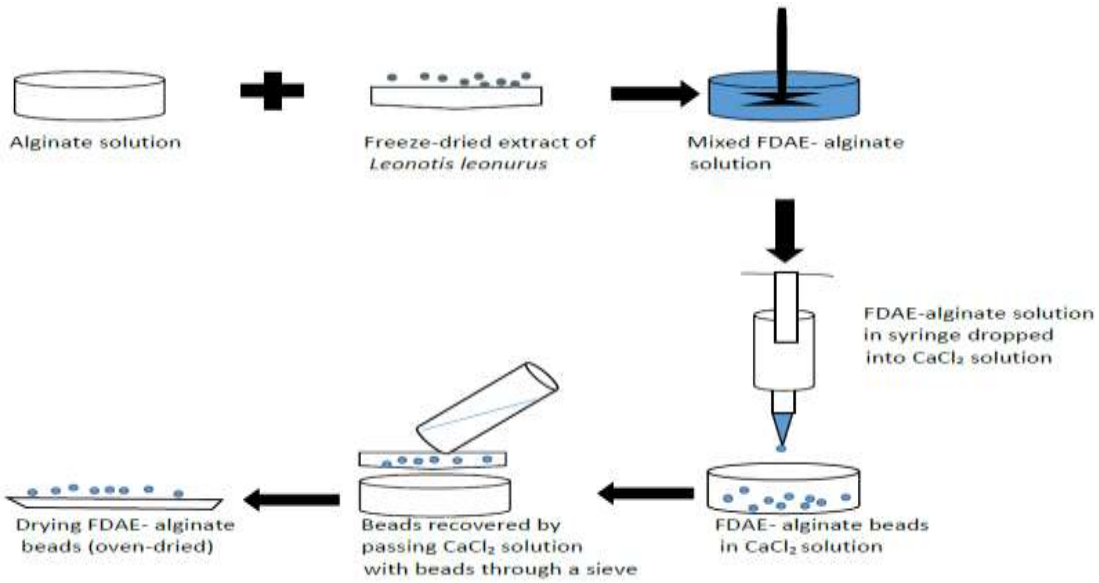

Fig. 4.1 A schematic representation of the production of calcium alginate FDAE beads using *L. leonurus* (Egieyeh, 2011)

4.3 Characterisation of *L. leonurus* extract forms

4.3.1 Determination of organoleptic properties

Organoleptic properties refer to the appearance, colour, odour and taste of a substance. It is the first step in the description of a drug substance during a pre-formulation or formulation development program. For this study, the appearance, colour, scent, taste and texture of the FDAE and other modified forms of *L. leonurus* was characterized using the natural senses (e.g. sight, smell, taste, etc.) according to the method used by Ma (2006). The results obtained for the different extracts were recorded, tabulated and compared in table 4.1.

4.3.2 Determination of moisture content

Approximately 300 mg of each of the solid extracts was accurately weighed and the weight recorded as wet weight of sample **(A)**. The sample was then dried at 115 °C, allowed to cool and then weighed again to give the dry weight of the sample **(B)**. This was all done with the aid of a Halogen Moisture Analyser HR73 (fig 4.2). The percentage moisture content (% MC) of the prepared solid extracts was then calculated using the following equation (Biking, 2003):

% MC = [(A-B)/B] * 100

where: % MC = Percentage of moisture in the sample

A = Weight of wet sample (grams), and

B = Weight of dry sample (grams)

The average moisture content of the freshly prepared solid extracts was recorded and compared.

Fig 4.2: Halogen Moisture Analyser HR73

4.3.3 Determination of total phenol content (TPC)

To determine the total phenol content of the freshly prepared *L. leonurus* extracts, the method of Oyedemi *et al*., (2011) was used with some modifications. A volume of 2.5 mL 10 % Folin-Ciocalteu reagent and 2 mL Na_2CO_3 (75 % w/v) was added to 0.5 mL plant extract (i.e. FDAE, Aq EtOH extract and calcium alginate FDAE beads) material (10 mg/mL) in a vial. The resulting mixture was vortexed for 15 seconds and incubated for 30 minutes at 40 °C in a water bath for colour development. Thereafter the solution was allowed to cool to room temperature and the absorbance measured at wavelength 765 nm with a UV spectrophotometer. A standard curve of absorbance at 765 nm *versus* concentration of gallic acid was plotted and the line of best fit determined by linear regression using GraphPad Prism. The TPC, in terms of gallic acid equivalents (mg/mg), for each of the extracts was then determined from this standard curve (appendix 2A) and compared.

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4.3.4 Determination of total flavonoid content (TFC)

To determine the flavonoid content of the freshly prepared *L. leonurus* extracts, the method of Mbaebie *et al.*, (2012) was used with some modifications. A volume of 0.5 mL 2 % aluminium chloride-methanol solution was mixed with 0.5 mL plant extract (i.e. FDAE, Aq EtOH extract or calcium alginate FDAE beads) material in methanol (MeOH) (10 mg/mL) in a vial. The resultant mixture was incubated at room temperature for 1 hour for yellow colour development indicating the presence of flavonoids. The absorbance was measured at a wavelength of 420 nm with a UV spectrophotometer. A standard curve of absorbance at 420 nm *versus* concentration of quercetin was plotted and the line of best fit determined by linear regression using GraphPad Prism. The TFC, in terms of quercetin equivalents (mg/mg), for each of the extracts was then determined from this standard curve (Appendix 2B) and compared.

4.3.5 Determination of antioxidant activity

The method of More *et al*., (2013) was used with some modifications. Free-radical scavenging activity was measured using the 1-diphenyl-2-picryl-hydrazyl (DPPH) assay. A 0.25 mM working solution was prepared by dissolving 1 mg of 1-diphenyl-2-picryl-hydrazyl (DPPH) in 10 mL of MeOH. Methanolic solutions of the FDAE and Aq EtOH *L. leonurus* extracts (10 mg/mL) was prepared, while the alginate bead extract solution (10 mg/mL) was prepared using dH_2O because of its inability to dissolve in MeOH. As a positive control 1 mg/mL ascorbic acid in methanol was prepared. For the assay a 96-well plate was used and 200 µl of MeOH added to the first row of wells and 110 µl MeOH in the remaining wells. Then 20 µl volumes of either plant extract or ascorbic acid solution was added to the first row of wells (3 wells per sample), the plate gently shaken to mix the solution and then 110 µl of the solution transferred to the next row of corresponding wells containing the 110 µl of MeOH. This process was repeated in subsequent wells to obtain serially diluted solutions of extract or ascorbic acid. Thereafter, 90 µl of methanolic solution of DPPH (90 mM) was added to all the wells. The final concentrations of the extract/compounds ranged from 1 to 0.008 mg/mL for ascorbic acid and 10 to 0.08 mg/ml for the plant extract preparations. The microplates were incubated at 37 °C for 2 hours, thereafter cooled down to room temperature and the absorbance read at a wavelength of 517 nm using the microplate reader. The percent radical scavenging activity of the *L. leonurus* preparations was determined by comparison with MeOH blank and calculated as follows:

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% DPPH radical-scavenging = ((AC – AS) / AC) x 100

Where **AC** is the absorbance of the control solution (containing only DPPH solution), and **AS** is the absorbance of the sample (i.e. plant extract or ascorbic acid) in DPPH solution. Using GraphPad Prism 5®, the percentage of DPPH scavenging activity *versus* plant extract or ascorbic acid concentrations (mg/mL) was plotted and the concentration of extract or ascorbic acid required to scavenge DPPH by 50 % (i.e. EC_{50}), determined by nonlinear regression analysis. This parameter was then used to compare the antioxidant activity of the *L. leonurus* preparations.

4.4 Results and discussion

4.4.1 Percentage yields of *L. leonurus* solid extract preparations

The percentage yields of *L. leonurus* solid extract preparations was obtained using the procedure described in 4.3 and were displayed in table 4.1 and appendices 1A, 1B & 1C. Although their preparation was time consuming, the solid extracts were fairly easy to prepare. The yield obtained for the FDAE was 19.9 % which was similar to that obtained by other investigators i.e. Mukinda and Syce (2007), the 20.4 % of Egieyeh (2010) and 21.96 % of Dube (2006). For the Aq EtOH extract the yield obtained was 12.82 %, and lower than that of the FDAE powder. The Aq EtOH extract was quite flaky in appearance, suggesting that any volatile oils extracted was probably removed during the rotary evaporation and freeze-drying processes. An average of 3.42 ± 0.01 g of *L. leonurus* calcium alginate beads was obtained from the 2 g of FDAE used, clearly indicating the incorporation of the calcium alginate (presumably mainly as a coating) in the final product. Based on the total constituent ingredients used, the estimated yield for the calcium alginate FDAE beads was low (10.7 %) and it was not clear how much of FDAE the beads contained. Previous studies on alginate bead formation also do not provide records of the percentage yields obtained. At this stage it is also not clear whether the yield of the beads can be improved (e.g. by small changes in the method of preparation), but further investigation into options to improve the yield of the beads might be warranted.

Table 4.1: Summary of *L. leonurus* solid extract preparations characteristics

Characteristics	FDAE	Aq EtOH extract	Calcium alginate FDAE beads
% Yield (mean ± SD; n = 3)	19.9±0.35	12.82±0.02	10.70 ± 0.02
Organoleptic properties			
Appearance	Free flowing powder	Flaky powder	rounded balls
Colour	medium brown	medium brown	dark brown
Scent	highly aromatic	highly aromatic	odourless
Taste	intensely bitter	intensely bitter	tasteless
Texture	fairly course	very course	smooth
Moisture content (%) (mean ± SD; n = 3)	3.77±0.05	2.91±0.12	1.86±0.06
Antioxidant ability			
R_2 (non-linear)	0.9974	0.9284	0.9240
EC50	0.8868	0.7525	1.062
Total phenol content (Gallic acid equivalent; mg/mg; Mean ± SD; n = 3)	7.86±0.013	7.52±0.008	6.94±0.009
Total flavonoid content (Quercetin equivalent; mg/mg; Mean ± SD; n = 3)	4.30±0.010	4.47±0.009	3.67±0.014

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4.4.2 Organoleptic properties of *L. leonurus* solid extract preparations

The organoleptic properties of the three *L. leonurus* solid extract preparations were obtained using the procedure described in 4.4.1 and were summarized in table 4.1 and shown in figures 4.3a to 4.3c. Generally the three solid extracts differed in appearance with the FDAE being a free flowing powder, the Aq EtOH appearing flaky and the calcium alginate FDAE beads taking the form of small round balls. While both FDAE and Aq EtOH extract were medium brown in colour, had highly aromatic scents and intensely bitter tastes, they differed in texture. In addition, the FDAE powder was fairly coarse while the Aq EtOH powder was very coarse. The calcium alginate FDAE beads, on the other hand, differed completely from the afore-mentioned powders, in colour, scent, taste and texture. It was dark drown, odorless, tasteless and had a smooth texture. The differences in organoleptic features were clearly the result of the various preparation methods used.

a b c

Fig. 4.3: Photomicrographs of (a) FDAE, (b) 20 % Aq EtOH extract and (c) calcium alginate FDAE beads

4.4.3 Moisture content of *L. leonurus* solid extract preparations

The moisture content levels of the three *L. leonurus* solid extract preparations were obtained using the procedure described in 4.4.2 and summarized in table 4.1. The FDAE, Aq EtOH extract and calcium alginate FDAE beads contained 3.77±0.05, 2.91±0.12 and 1.86±0.06 % moisture, respectively. These moisture content values did not specifically reflect the hygroscopic nature of the solid dosage forms; instead it reflected the amount of moisture present in the product immediately after manufacturing. Nevertheless, the fact that the alginate beads contained the least and significantly less moisture (t-test; $p < 0.05$) when compared to the FDAE (p value = 0.0001) and 20 % Aq EtOH (p value = 0.0002) strongly suggested that such bead formation might be able to solve any hygroscopicity problem associated with the FDAE.

The latter suggestion must however still be conclusively proven with hygroscopicity evaluations.

4.4.4 Total phenol content (TPC) of *L. leonurus* solid extract preparations

The amount of total phenols in the prepared solid extracts was determined according to the procedure described in 4.4.3 and calculated from the standard curve of the gallic acid concentration *vs* UV/VIS absorbance as shown in appendix 2A. The standard curve was linear with equation Y = 0.02239x and regression coefficient, $R^2 = 0.9989$ and was used to determine the TPC levels in the extracts.

The levels obtained were recorded in table 4.2 and were 7.86 ± 0.013, 7.52 ± 0.008 and 6.94 ± 0.009 mg/g gallic acid equivalent for the FDAE, the Aq EtOH extract and the calcium alginate FDAE beads, respectively. The TPC of the preparations were in a reasonably similar (ANOVA; $p > 0.05$) range but the calcium alginate FDAE bead extract clearly contained significantly lower levels of total phenols when compared to the FDAE and Aq EtOH extracts (t-test; $p < 0.05$). The only other information available on the TPC levels in *L. leonurus* preparations is that reported by Oyedemi *et al*., (2011), who found 48 mg/g tannic acid equivalent total phenols in *L. leonurus* aqueous extracts. In the FDAE of *Artemisia afra* a value of 258.39 mg/g tannic acid equivalent total phenols was found (Sunmona, *et al*, 2012). This was however much higher than that found for the FDAE of *L. Leonurus*, possibly because of the different plant and standard used.

Overall, it is clear that use of different extract preparations of *L leonurus*, and especially the preparation or more sophisticated modified forms such as alginate beads of the FDAE, which in turn was prepared to solve pharmaceutical (e.g. high hygroscopicity) or biopharmaceutical (e.g. low bioavailability) problems, can be expected to affect the levels of active compounds (e.g. TPC's) in the final product.

Table 4.2: Total phenol content levels in *L. leonurus* solid extracts

Solid plant extract	Absorbance at 765 nm (AUF's) (Mean ± SD; n = 3)	Gallic acid equivalence (mg/g) (Mean ± SD; n = 3)
FDAE	0.327±0.013	7.86±0.013
Aq EtOH extract	0.313±0.008	7.52±0.008
Calcium alginate FDAE beads	0.289±0.009	6.94±0.009

4.4.5 Total flavonoid content of *L. leonurus* solid extract preparations

The amount of total flavonoids in the prepared solid extracts was determined according to the procedure described in 4.4.4 and from the standard curve of the quercetin concentration *vs* UV absorbance shown in appendix 2B. The standard curve was linear with Y= 0.02970x, R^2 = 0.9805 and was used to determine the TFC levels in the extracts.

The TFC levels were recorded in table 4.3 and were 4.30±0.010, 4.49±0.009 and 3.67±0.014 mg/g quercetin equivalent FDAE, Aq EtOH extract and calcium alginate FDAE beads, respectively. The flavonoid levels of calcium alginate FDAE beads being slightly lower than that of both the FDAE and Aq EtOH extract. This was most likely due to the effect of the calcium alginate coating. The levels of flavonoid in the FDAE and Aq EtOH was fairly similar (t-test; $p < 0.05$) to each other and also to that found by Oyedemi *et al.,* (2011) in an aqueous extract of *L. leonurus* (4.8 mg/g quercetin equivalent). These flavonoid levels were however much more than that found in the acetone (0.77 ± 0.003), methanol (0.46 ± 0.02) and water (0.69 ± 0.00) quercetin equivalent (mg/g) of *L. leonurus* FDAE (Jimoh, *el al.*, 2010).

Overall, the TFC level did not appear to be much affected by the type of solid *L. leonurus* extract material prepared. Moreover, since the TFC and TPC level findings were fairly similar it is very likely that most of the phenolics in the preparations were flavonoid compounds.

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Table 4.3: Total flavonoid content levels in *L. leonurus* solid extracts

Solid plant extract	Absorbance at 420 nm (AUFS) (Mean ± SD; n = 3)	Quercetin equivalents (mg/ g) (Mean ± SD; n = 3)
FDAE	0.284±0.010	4.30±0.010
Aq EtOH extract	0.296±0.009	4.49±0.009
Calcium alginate FDAE beads	0.242±0.014	3.67±0.014

4.4.6 Antioxidant activity of *L. leonurus* solid extract preparations

The antioxidant activities of the *L. leonurus* solid extract preparations and ascorbic acid reference standard was determined according to the procedure described in 4.4.5 and is summarized in table 4.4 and displayed in figure 4.4.

From the curves in figures 4.4 the EC_{50}, a slope co-efficient and a non-linear correlation coefficient, shown as R_2 (non-linear) was calculated and presented in table 4.4. Generally the non-linear correlation coefficients of the % DPPH radical-scavenging ability *vs* concentration

curves for ascorbic acid reference standard and all three *L. leonurus* solid extract preparations showed a good fit with R_2 (non-linear) values of 0.9885, 0.9974, 0.9284 and 0.9240 for ascorbic acid, FDAE, Aq EtOH extract and calcium alginate FDAE beads, respectively. More importantly, based on the EC_{50} values the calcium alginate FDAE bead preparations 11.53 mg/mL (1.062) differed significantly from the FDAE 7.71 mg/mL (0.8868) and the Aq EtOH extract 5.66 mg/mL (0.7525) in its antioxidant ability (student t-test p-value of < 0.0001; ANOVA-test; $p< 0.05$). Clearly the solid *L. leonurus* preparations contained antioxidants but differed in free-radical scavenging ability and, presumably, overall levels of antioxidants. When comparing the EC_{50} values of *L. leonurus* FDAE preparations (0.8868) to that of the ascorbic acid standard (0.005) it was clear that these preparations did not have very remarkable antioxidant efficacy. Former studies had reported slightly higher antioxidant ability for ascorbic acid (0.0025) (Nkobole, *et al,* 2011) and popular South African plants such as *Aloe ferrox* (0.04853) and *Artemisia afra* (0.01695) (More, *et al*, 2012). When comparing the *L. leonurus* FDAE to that of the aforementioned *Aloe ferrox* and *Artemisia afra* it was found not to possess substantial antioxidant activity. This significant difference in activity between the three plants is possibly a reflection of the difference in levels of active constituents, e.g. flavonoids, also found in this study (see 4.5.5 discussion).

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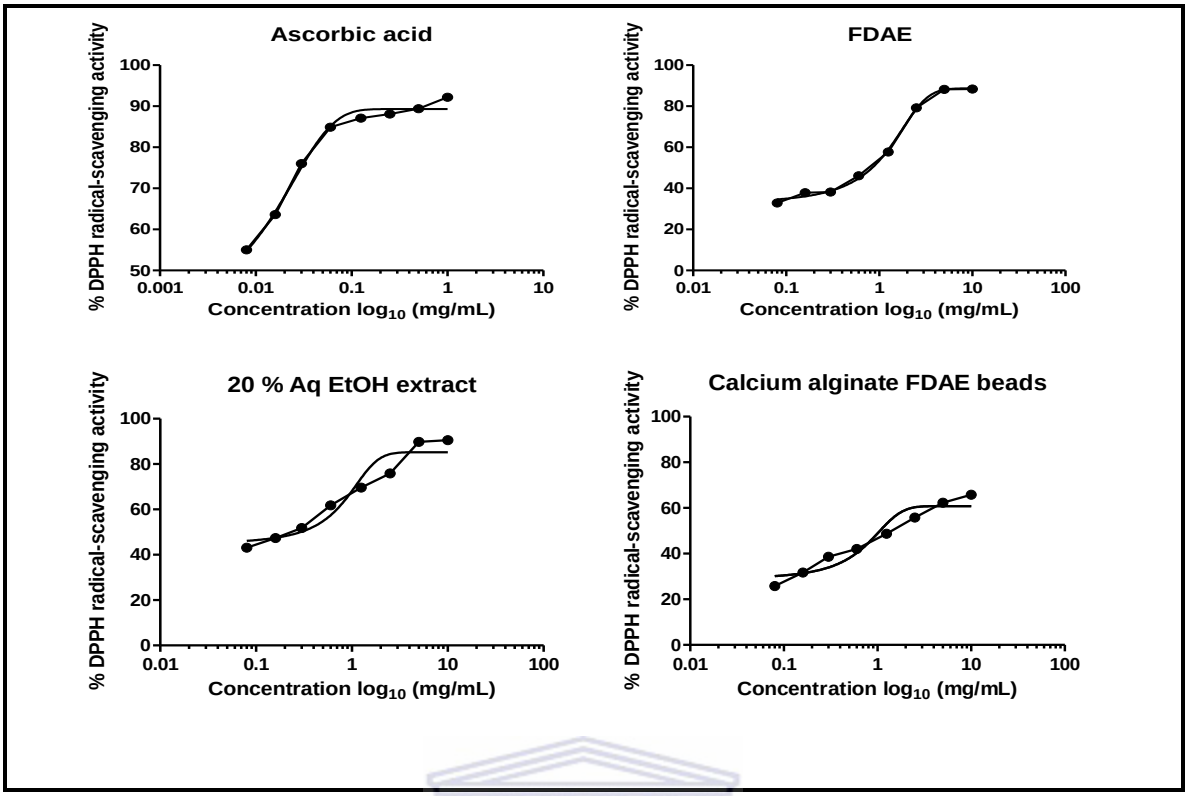

Fig. 4.4: Antioxidant response curves depicting % DPPH radical scavenging activity *verses* concentration of ascorbic acid standard, FDAE, Aq EtOH extract and alcium alginate FDAE beads. The data represents the percentage DPPH inhibition. Each value represents, mean ± S.D (n=3).

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Table 4.4: Antioxidant activities of the solid plant extracts of *L. leonurus* and ascorbic acid standard expressed as EC_{50} values

Solid plant extract	EC_{50} (log_{10} mg/mL)	*R_2
Ascorbic acid	0.005	0.9885
FDAE	0.8868	0.9974
Aq EtOH extract	0.7525	0.9284
Calcium alginate FDAE beads	1.062	0.9240

***R_2 = nonlinear correlation coefficient for % DPPH scavenging ability *vs* log concentration curve**

4.5 Conclusion

Overall, the results obtained indicated that the solid extracts of *L. leonurus* could be easily prepared, and can therefore be viable practical replacements for the traditional tea form.

The three *L. leonurus* solid extracts differed in organoleptic properties and the fact that the alginate beads contained the least moisture strongly suggested that such bead formation might be able to solve any hygroscopicity problem associated with the FDAE.

Chemically, the total phenol levels for all three *L. leonurus* preparations were very similar and so was the TFC levels of the three extracts, possibly indicating that most of the phenolics in the *L. leonurus* preparations were flavonoid compounds. However, the antioxidant activities for the three extracts were not similar (ANOVA-test; $p < 0.05$). The antioxidant activity was slightly lower for the calcium alginate beads than that of the FDAE and Aq EtOH extract, thus clearly indicating that all the solid *L. leonurus* preparations contained antioxidants but differed in free-radical scavenging ability and, presumably, overall levels (and/or types) of antioxidants.

Clearly the hypothesis that the Aq EtOH extract contained higher levels of chemical compounds compared to the FDAE and calcium alginate FDAE beads was not met, at least not in terms of TPC, TFC and antioxidant (activity) levels. Whether it would hold in terms of marker compound levels was considered in chapter 5.

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CHAPTER 5: VALIDATION OF HPLC ASSAY METHOD FOR *L. LEONURUS*

5.1 Introduction

In this chapter, the validation of high-performance liquid chromatography (HPLC) assay method for *L. leonurus* is investigated and discussed. The aim of this part of the study was to establish whether the marker compounds, marrubiin and leonurine, were suitable to use as product stability indicators. This was to validate the HPLC assay method for *L. leonurus* solid extracts and determine the chemical marker profiles and content levels of the solid extract forms by HPLC fingerprinting.

Furthermore, the chemicals, materials, equipment and experimental procedures used for the validation of the HPLC assay for *L. leonurus* was presented and the results obtained reported and discussed.

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5.2 Chemicals, Materials and Equipment

The chemicals and materials used included:

Rutin, quercetin, marrubiin, leonurine, sodium carbonate, ascorbic acid, gallic acid, acetonitrile, formic acid *(all from Sigma-Aldrich, Germany)*, absolute ethanol, methanol HPLC grade *(KIMIX)* and distilled water *(Saarchem, South Africa).*

The equipment used included:

An HPLC system Agilent 1200 system consisting of a degassing system *(G1322A, Japan)*, quaternary pump *(G1311A, Germany)*, auto loading sampler *(G1329A, Germany)*, TCC *(G1316A, Germany)*, diode array detector *(G1315B, Germany)*, fluorescence detector *(G1521A, Germany)*, analyte fraction collector *(G164C, Germany)*, Agilent ChemStation software *(G2173-60101L, Germany*); column (*Luna C18 column, 5 µm, 4.6 x 250 mm*); vortex mixer *(VM-300, Gemmy Industrial Corp)* and a scale (*Adventurer OHAUS, Model AR2140, USA*).

5.3 Experimental procedure

5.3.1 HPLC conditions and validation of HLPC assay for marker compounds marrubiin & leonurine

The method of Bienvenu *et al.*, (2002), was used with some modifications, to separate, detect and quantitate the marrubiin, leonurine and other compounds in the *L. leonurus* samples. Specifically, an Agilent 1200 Series HPLC system equipped with a quaternary pump, photodiode array (PDA) detector, in-line degasser, column oven and a PC with ChemStation software was used. Separation of the compounds in the test samples were obtained by using a C18 column (Phenomenex Luna, 5 µm, 4.6 x 250 mm) maintained at a column temperature of 25 ˚C, gradient elution with a mobile phase solvent system consisting of H_2O with 0.01 % formic acid (solvent A) and acetonitrile containing 0.01 % formic acid (solvent B) and a mobile phase flow rate of 0.8 mL/min. The peaks were eluted using the following solvent gradient: at 0 min, solvent B: 15 %; 3 min, solvent B: 15 %; 26 min, solvent B: 100 %; 30 min, solvent B: 100 %; 31 min, solvent B: 15 % and at 36 min (i.e. the end of run), solvent B: 15 %. The injection volume used was 20 µL and the peaks were detected on a PDA detector set at wavelengths 214, 254 and 280 nm.

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For the validation of the assay, the following parameters were determined: linearity of the standard curve, precision, accuracy, lowest limit of detection and lowest limit of quantification. In performing the validation, stock solutions of marrubiin and leonurine was prepared in 50 % methanol/water and stored in a refrigerator at -80 °C. Standard solutions were prepared by diluting the stock solution of marrubiin in 50 % methanol/water to a concentration range of 14 – 140 µg/ml and leonurine in 50 % methanol/water to obtain a concentration range of 6 – 38 µg/ml. These standard solutions were used to establish the standard curve. For each standard, 20 µl was injected onto the column and the marrubiin and leonurine peaks detected at wavelengths 214 and 280 nm, respectively. Thereafter the marrubiin and leonurine peak areas were plotted against corresponding concentrations of the injected standard sample. GraphPad™ prism was used for plotting and determination of the standard curve, assessing its linearity (R^2) and extrapolation of the concentrations of the test samples.

The precision of the analytical method was determined by assaying three replicates of the low (i.e. the low concentration quantitation or LCQ), medium (MCQ) and high (HCQ) concentrations of the marrubiin and leonurine solutions on three consecutive days. On each

occasion the average, standard deviation and percent relative standard deviation (RSD) was calculated and compared. The accuracy of the method was determined from the mean concentrations obtained for the replicates and the percentage difference. The limits of detection (LOD) and limits of quantitation (LOQ) for marrubiin and leonurine were determined from the detector response (peak area) with LOD defined as the concentration giving an analyte response that is three times that of the noise (signal to noise ratio 3:1) and the LOQ as the lowest concentration where an accuracy better than 20 % was achieved. Lastly, the percentage bias was determined as the difference between the concentration measured (and determined from the standard curve linear regression equation) and the concentration of the prepared standard and expressed as a percentage of the prepared standard concentration.

5.3.2 Identification and comparison of marrubiin and leonurine levels in *L. leonurus* plant extract preparations

For this part of the study the validated HPLC assay described above was used. Replicate samples of 10 mg were accurately weighed for all three *L. leonurus* solid extracts. These samples were individually dissolved in 1 ml of methanol (FDAE & Aq EtOH extract) or 1 mL dH2O (in the case of the calcium alginate FDAE beads which was insoluble in methanol). The samples were vortexed for 15 seconds, sonicated for 30 min, then withdrawn and filtered through a 0.45 micron Millipore™ filter using a syringe and the filtrate transferred to amber HPLC vials for analysis. Twenty microliters of the samples were injected onto the HPLC column and the HPLC chromatographic fingerprint obtained, analysed for total numbers and profile of peaks. The peaks of active constituents were identified and the fingerprints of plant preparations were compared. Finally, the retention times, heights and peak areas of the marrubiin and leonurine peaks in the chromatograms of the different samples were determined and the levels of the actives determined from their respective calibration and compared for the three preparations.

5.4 Results and discussion

5.4.1 Validation of HPLC assay for marrubiin

Under the HPLC conditions described in section 5.3.1, good symmetrical peaks with retention time of 23.75 ± 0.25 minutes were found for marrubiin at a wavelength of 214 nm (figure 5.1; table 5.1). For the marrubiin assay the standard curve was linear (r^2 = 0.9991) (figure 5.2) over

the concentration range of 4.75 to 40.0 µg / ml marrubiin (table 5.2) and the assay had a LOD and LOQ of 2.38 and 4.75 ug/ml, respectively (table 5.3).

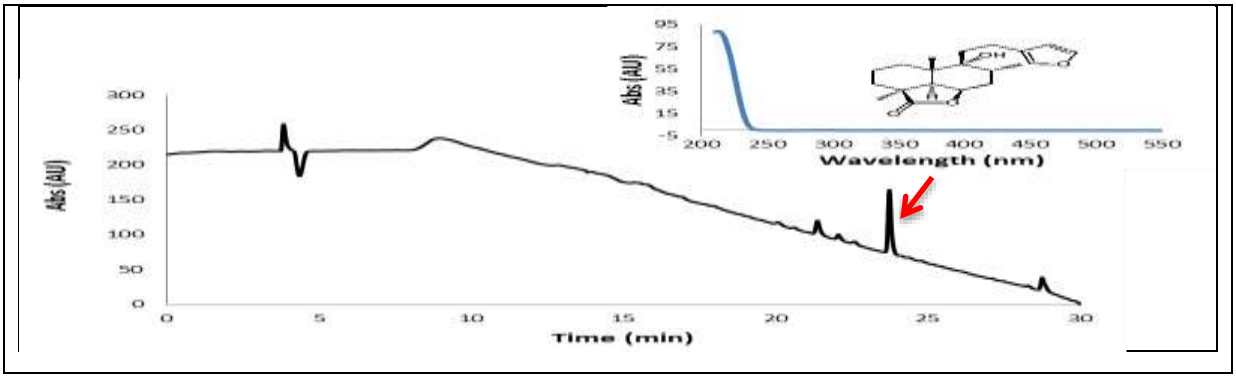

Fig 5.1: HPLC chromatogram of marrubiin reference standard at 214 nm Inset: UV/Vis spectrum for the reference compound at ~23.75 min (indicated by red arrow on chromatogram)

Table 5.1: HPLC retention times and selected system suitability parameters for marrubiin detected at 214 nm

Compound	Retention time (min)	k'*	R#	T&
Marrubiin	23.75	59.95624	193.486	1.29508

Where

*k' the capacity factor is calculated from equation $k' = (t_a/t_\alpha) - 1$. Peaks should be well resolved from the void volume and k' > 2 is acceptable. The Agilent ChemStation software was used to calculate these.

#Resolution: $R_s = 2 \times (t_2 + t_1) / (W_2 + W_1)$. $R_s > 2$ between the peak of interest and the closest potential interfering peak are desirable.

&Tailing factor: T = W0.05 / 2f. T < 2 is acceptable. The Agilent ChemStation software was used to calculate these.

And generally: t_α = elution time of the void volume or non-retained compounds; t_a = the retention time from the time of injection to the time of the elution of the peak maximum; and t_2 and t_1 = the retention times of the two components being separated

Table 5.2: Peak area values for 20 µL aliquots of fixed concentrations of standard marrubiin injected on column and measured at 214 nm

Concentration (ng/µL)	Mean peak area (mAU)	%RSD
4.75	83.74	1.63
7.9	143.56	1.87
15.8	277.80	1.13
23.8	410.01	0.22
31.7	531.47	1.34
39.6	685.84	0.13

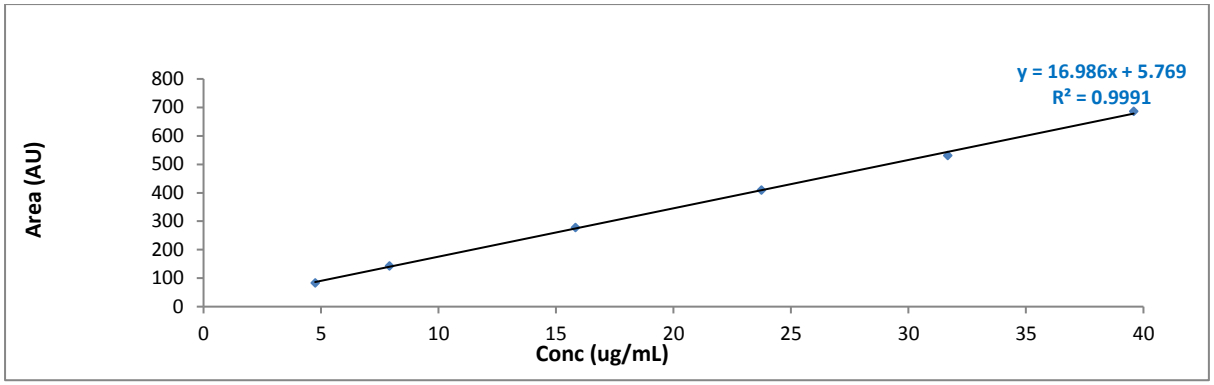

Fig. 5.2: Standard curve of marrubiin peak areas *vs*. concentration using the method described in section 5.3.1

In addition, the accuracy and precision for the quantitation of marrubiin (table 5.4) at 7.9 ng/µL was found to be 7.98 ng/µL and at 31.7 ng/µL to be 30.70 ng/µL which were both highly acceptable with percentage relative standard deviations (% RSD) of 0.67 and 1.68 %, respectively. Overall, these values indicated good validity and reproducibility of the assay.

Table 5.3: LOD and LOQ data of marrubiin over the 4.8 – 40.0 ug/mL range at 214 nm

Prepared concentration (µg/mL)	Mean peak area (units)	Concentration found (µg/mL)	% RSD	% Bias[&]	LOD[#]	LOQ
7.9	143.56	7.11	1.87	2.4644	Accept	Accept
4.75	83.74	4.59	1.63	-3.3587	Accept	Accept
2.38	23.45	1.04	1.08	-56.2742	Accept	Reject
0.79	6.77	0.06	4.13	-92.5746	Reject	Reject

Bias[&]: The difference between the mean concentration measured and the prepared concentrations as a percentage of the prepared concentration shown in table 5.3.

LOD[#]: Determined using an analyte response 3 times that of the noise (signal-to-noise ratio of 3:1) with the mean baseline noise = 0.4 a.u., n=3.

*Concentration could not be determined since the peak area was less than the regression equation intercept.

Table 5.4: Assay precision and accuracy data for quantification of marrubiin at 214 nm

Concentration (ng/µL)	*Concentration found(ng/µL), Mean (n=6)	%RSD
7.9	7.98	1.68
31.7	30.70	0.67

*Calculated using the linear regression equation given in figure 5.2

5.4.2 Validation of HPLC assay for leonurine

Under the HPLC conditions described in section 5.3.1, good symmetrical peaks with retention time of 10.11 ± 0.35 minutes was found for leonurine at a wavelength of 280 nm (figure 5.3; table 5.5). Moreover, the leonurine standard curve was linear (r^2 = 0.9960) (figure 5.4) over the concentration range of 2.0 to 12.0 µg / ml leonurine (table 5.6).

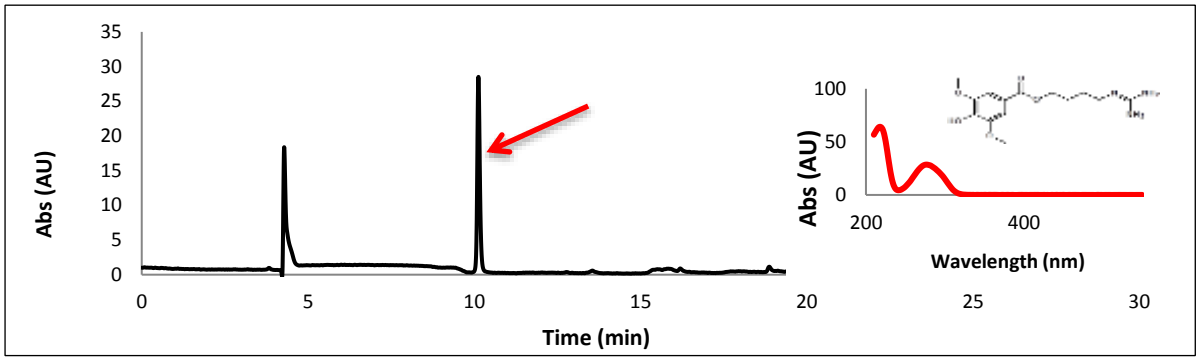

Fig. 5.3: HPLC chromatogram of leonurine reference standard at wavelength 280 nm. Inset: UV/Vis spectrum for the reference compound at ~10.11 min (indicated with red on chromatogram)

Table 5.5: HPLC retention times and selected system suitability parameters of leonurine at 280 nm

Compound	Retention time (min)	k'*	R_s#	T&
Leonurine	(10.11)	(24.94592)	-	(2.62766)
	10.11	24.94613	193.486	1.22034

k'*, the capacity factor, where k' = ($t_a/t_□$) – 1. Peaks should be well resolved from the void volume and k' > 2 is acceptable. The Agilent ChemStation software was used to calculate these.

Resolution#: $R_s = 2 \times (t_2 + t_1) / (W_2 + W_1)$. $R_s > 2$ between the peak of interest and the closest potential interfering peak are desirable.

Tailing factor: T& = W0.05 / 2f. T < 2 is acceptable. The Agilent ChemStation software was used to calculate these.

And $t_α$ = elution time of the void volume or non-retained compounds; t_a = is the retention time from the time of injection to the time of the elution of the peak maximum; t_2 and t_1 = are the retention times of the two components; W_1 and W_2 = are the corresponding widths of the bases of the peaks, obtained by extrapolating the sides of the peaks to the baseline; and f = distance from the front edge of the peak to the perpendicular at the peak maximum.

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Table 5.6: Peak area values for fixed concentrations of standard leonurine injected on column

Concentration (ng/µL)	Mean peak area (mAU)	%RSD
2.00	23.67	17.85
4.00	47.53	1.37
6.00	70.50	0.28
8.00	95.17	0.34
10.00	128.63	0.76
12.00	154.37	1.37

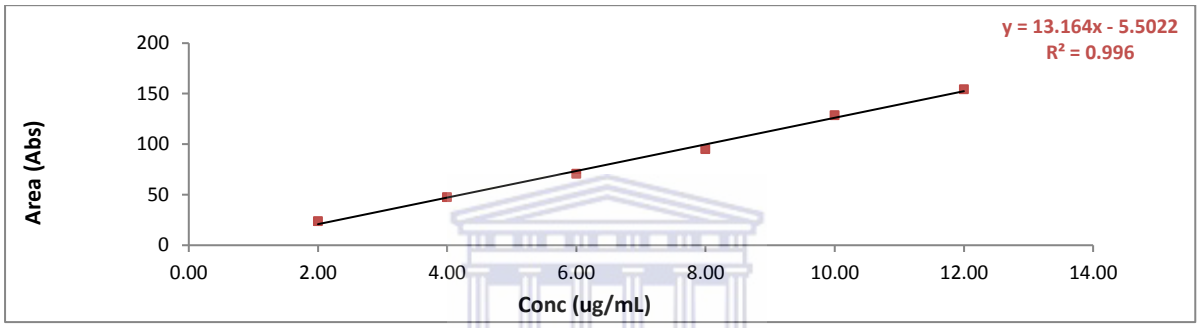

Fig. 5.4: Standard curve of leonurine peak areas vs. concentration

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As for the marrubiin, the accuracy and precision of the leonurine assay (table 5.7) was also highly acceptable with the 4.0 ng/µL standard being determined as 4.08 ng/µL, the10 ng/µL standard unchanged as 10 ng/µL and the percentage relative standard deviations (%RSD) being 2.11 and 2.35 %, respectively. In addition, the assay had a LOD and LOQ of 2.0 and 4.0 ug/mL, respectively (table 5.8). Overall, these values indicated good validity and reproducibility for the leonurine assay.

Table 5.7: Precision and Accuracy data for quantification of leonurine at 280 nm

Concentration (ng/µL)	Concentration found (ug/mL), Mean (n=6)	%RSD
10.0	4.08	2.11
	10.00	2.35

Table 5.8: LOD and LOQ data of leonurine over the 2.0 – 12.0 ug/mL range at 280 nm

Prepared concentration (µg/mL)	Mean peak area (units)	Concentration found (µg/mL)	% RSD	% Bias[&]	LOD[#]	LOQ
4.00	4.03	3.57	1.37	0.7204	Accept	Accept
2.00	2.39	1.07	1.46	19.5255	Accept	Reject
0.80	0.96	0.51	3.73	19.6635	Reject	Reject
0.20	-*	-*	-*	-*	Reject	Reject

Bias[&]: The difference between the mean concentration measured and the prepared concentrations as a percentage of the prepared concentration shown in table 5.7.

LOD[#]: Determined using an analyte response 3 times that of the noise (signal-to-noise ratio of 3:1) with the mean baseline noise = 0.4 a.u., n=3.

*Concentration could not be determined since the peak area was less than the regression intercept.

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Based on the above data the assays were considered to be specific, reproducible enough and linear over suitable concentration ranges to quantify marrubiin and leonurine in the *L. leonurus* preparations.

5.4.3 Identification and comparison of levels of marrubiin and leonurine in *L. leonurus* plant preparations

The above described and validated an HPLC assay which was then used to identify and compare the levels of marrubiin and leonurine in the *L. leonurus* plant extract preparations. The marrubiin and leonurine in the plant preparations was identified by comparing the retention times and UV spectra of those peaks with that obtained with the standards and their levels were determined from the standard curves. Figures 5.5, 5.6 and 5.7 are copies of the chromatograms obtained after the HPLC analysis of solutions of the FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus.* Several peaks were evident in these chromatograms clearly indicating the presence of many compounds in the *L. leonurus* preparations but marrubiin eluted at a retention time of 27.9 min (using the 214 nm UV detection wavelength). From the peak areas and standard curve analysis the FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* appeared to contain 22.5±1.241 (2.25 %), 17.5±1.033 (1.75 %) and 0.4±0.112 (0.04 %) µg marrubiin /mg plant, respectively (table 5.9). The FDAE

thus contained the highest level of marrubiin, with the Aq EtOH extract having a slightly lower level and the calcium alginate FDAE beads containing the least amount of marrubiin. Marrubiin was thus indeed present in all three of the *L. leonurus* plant preparations, but at different levels. Thus far there has been few reports on the quantitative level of marrubiin in *L. leonurus.* In one study, detection and the quantification of *L. leonurus* leaf extract yielded as much as 5 % marrubiin (Mnonopi *et al*., 2011). This was more than double the marrubiin quantified in the present *L. leonurus* FDAE, and could be a result of acetone (instead of water) being used as solvent during the extraction process, resulting in higher levels of marrubiin being quantified. Clearly different forms of the *L. leonurus* preparations can be expected to have different marrubiin levels depending on how they were prepared.

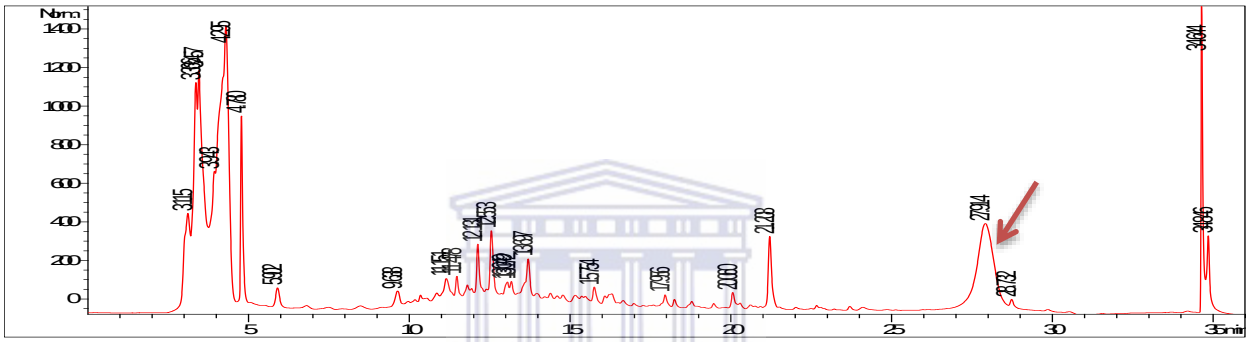

Fig. 5.5: HPLC fingerprint of FDAE at UV detection wavelength 214 nm. The marrubiin is indicated by the arrow at retention time 27.9 min

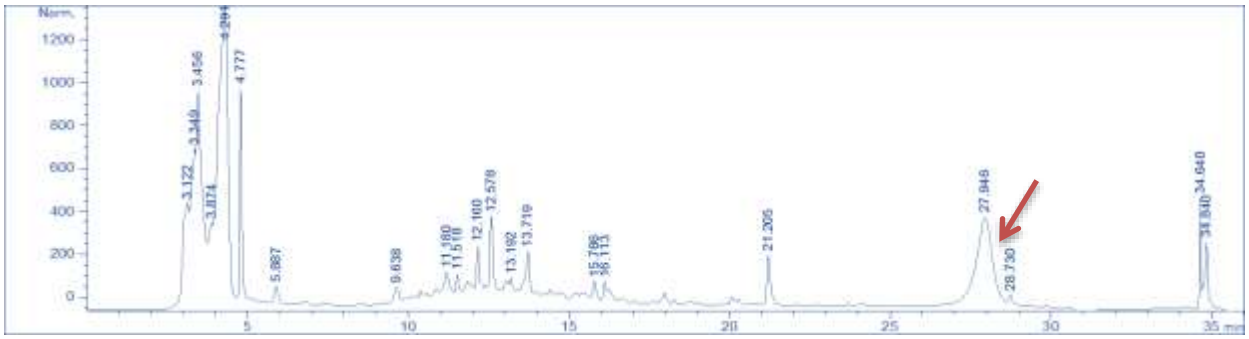

Fig. 5.6: HPLC chromatogram of Aq EtOH at UV detection wavelength 214 nm. The marrubiin peak is indicated by the arrow at retention time 27.9 min

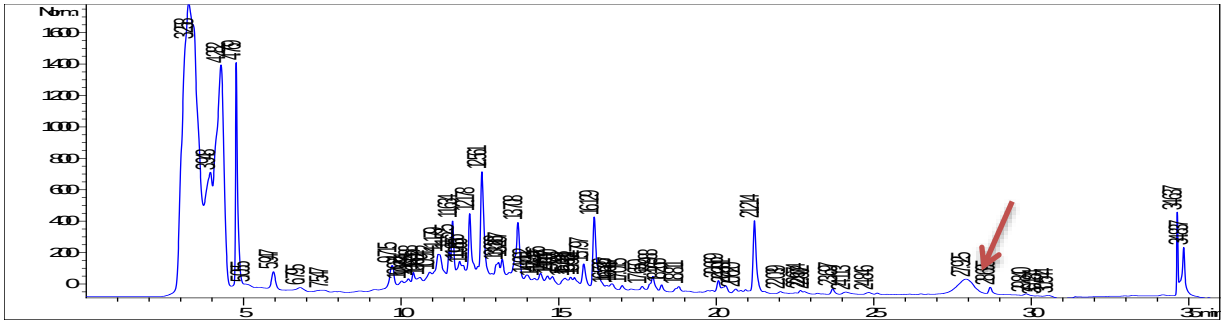

Fig. 5.7: HPLC chromatogram of calcium alginate FDAE beads at UV detection wavelength 214 nm. The low levels of marrubiin peak is indicated by the arrow at retention time 27.9 min

Figures 5.8 to 5.10 are copies of HPLC chromatograms of the *L leonurus* preparations obtained at UV detection wavelength of 280 nm and, along with several other peaks, indicating the presence of leonurine at a retention time of 10.11 min. Moreover, the FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* contained 2±0.436 (i.e. 0.2 %), 1.4±0.155 (0.14 %) and 0.7±0.150 (0.07 %) µg leonurine /mg plant, respectively (table 5.9). When compared to marrubiin the *L. leonurus* preparations contained fairly low levels of leonurine. Again the FDAE contained the highest level of leonurine, followed by the Aq EtOH extract having a slightly lower level of leonurine and the calcium alginate FDAE beads containing the least amount of leonurine. Clearly, leonurine was present in all three *L. leonurus* plant preparations, but in significantly different levels. Despite leonurine being detected and quantified, a former study reported that it could not be detected in the *L. leonurus* plant (Kuchta, *et al*., 2012). Leonurine has however been quantified in other plants such as *Leonuri herba* which was reported to contain 2.0 µg leonurine /mg plant (0.2 %) (Pang1 *et al.*, 2001), i.e. approximately the same amount as that found in the *L. leonurus* FDAE in this study. Leonurine was also present in *Chinese motherwort* which was reported to contain 0.15 µg leonurine /mg plant (0.015 %) (Chen1, *el at.*, 2010), less than that found in the calcium alginate FDAE beads prepared in the present study. It is clear that a limited amount of research has thus far been done on the quantification of leonurine from *L. leonurus* plants. Quantified levels vary depending on the type of plant preparation (e.g. type of extract) used. This however serves as important topic for further investigation.

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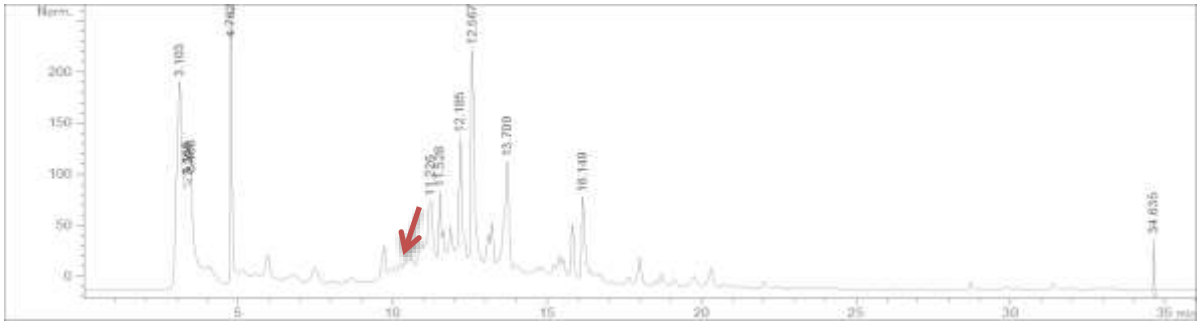

Fig. 5.8: HPLC chromatogram of FDAE at UV detection wavelength 280 nm. The low levels of leonurine peak is indicated by the arrow at retention time 10.11 min

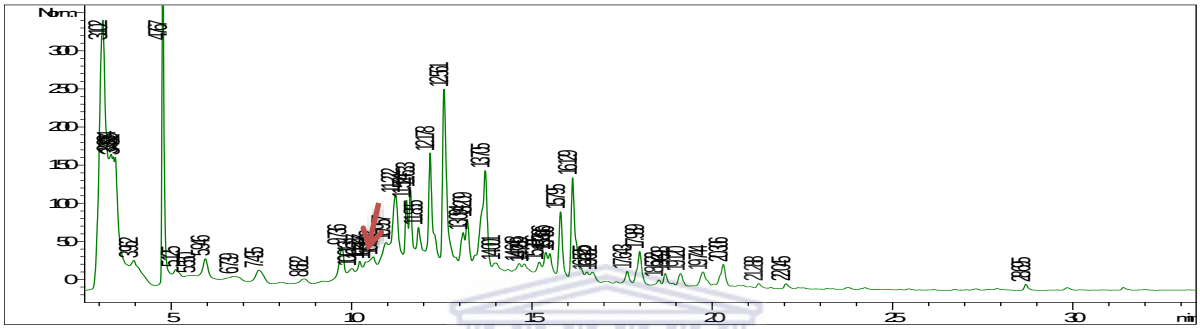

Fig. 5.9: HPLC chromatogram of Aq EtOH extract at UV detection wavelength 280 nm. The low levels of leonurine peak is indicated by the arrow at retention time 10.11 min

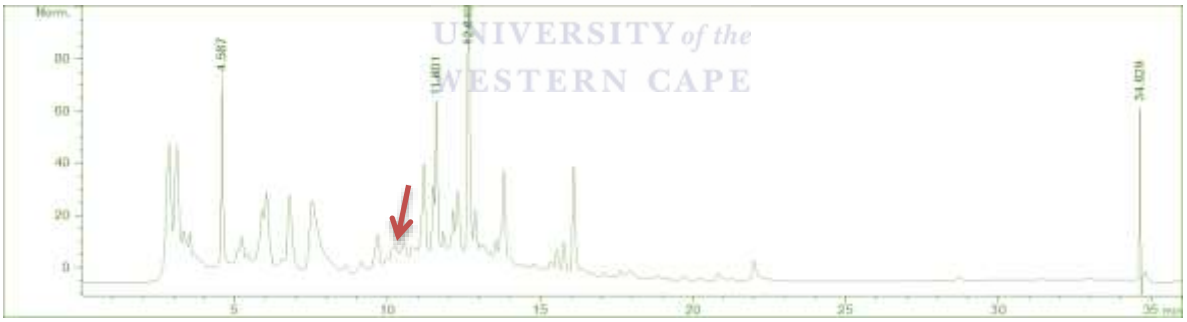

Fig. 5.10: HPLC chromatogram of calcium alginate FDAE beads at UV detection wavelength 280 nm. The low levels of leonurine peak is indicated by the arrow at retention time 10.11 min.

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Table 5.9: Level of marrubiin and leonurine present in *L. leonurus* plant preparations

	***L. Leonurus* plant preparations**		
Characteristics	**FDAE**	**Aq EtOH extract**	**Calcium alginate FDAE beads**
Marrubiin (concentration µg/mg of plant extract; SD; n=3)	22.5±0.241	17.5±0.033	0.4±0.112
Leonurine (concentration µg/mg of plant extract; SD; n=3)	2.0±0.436	1.4±0.155	0.7±0.150

5.5 Conclusion

In summary, it can be said that the developed validated HPLC method was reliable and reproducible enough to identify and quantitate leonurine and marrubiin present in *L. leonurus.* The method could therefore also be used in the further stability testing. In addition, the findings of this part of the study confirmed that both marrubiin and leonurine was present in the *L. leonurus* plant extract preparations and could therefore be used as marker compounds for quality control of such preparations. Moreover, the FDAE contained higher levels of marrubiin and leonurine than the Aq EtOH extract and calcium alginate FDAE beads. This may impact the pharmacological activities of the individual preparations. Clearly the hypothesis that the Aq EtOH extract contained higher levels of the chemical marker compounds compared to the FDAE was not proven.

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CHAPTER 6: STABILITY OF AQUEOUS AND OTHER MODIFIED EXTRACTS OF *L. LEONURUS*

6.1 Introduction

Another major main aim of the study was to test whether the *L. leonurus* solid extracts had suitable long-term storage stability. Within this aim the specific objectives was to assess the stability of encapsulated *L. leonurus* solid extracts stored under different storage conditions by monitoring changes in their organoleptic features, chemical components (i.e. total phenol, flavonoid and marker compound, viz. marrubiin and leonurine, levels) and potential therapeutic activity (i.e. antioxidant activity). It was hypothesized that the calcium alginate FDAE beads would have greater stability (i.e. longer shelf-life) than the FDAE and the Aq EtOH extract preparations.

In this chapter, the chemicals, materials, equipment and experimental procedures used to determine the stability of the FDAE, Aq EtOH extract, and calcium alginate FDAE beads of *L. leonurus* was presented and the results obtained presented and discussed.

6.2 Chemicals, Materials and Equipment

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The chemicals and materials used included:

Rutin, quercetin, marrubiin, leonurine, sodium carbonate, ascorbic acid, gallic acid, 1-diphenyl-2-picryl-hydrazyl (DPPH), Folin Ciocalteu reagent, acetonitrile, formic acid *(all from Sigma-Aldrich, Germany)*, aluminium chloride *(AnalytiCals Carlo Erba, France)*, absolute ethanol, methanol HPLC grade *(KIMIX, South Africa)*, distilled water *(Saarchem, South Africa)* and hard gelatine capsule shells *(Size 0; Colour: green and white, Cape Town)*.

The equipment used included:

HPLC system (*Agilent 1200 system consisting of: degassing system (G1322A, Japan)*, quaternary pump *(G1311A, Germany)*, auto loading sampler *(G1329A, Germany)*, thermostatted column holder TCC *(G1316A, Germany)*, diode array detector *(G1315B, Germany)*, fluorescence detector *(G1521A, Germany)*, analyte fraction collector *(G164C, Germany)*, Agilent ChemStation software *(G2173-60101L, Germany*), HPLC column (*Luna column, 5 µm, 4.6 x 250 mm*), vortex mixer *(VM-300 Vortex mixer, Gemmy Industrial Corp)*,

scale (*Adventurer OHAUS, Model AR2140, USA*), hot plate and stirrer *(MH-4, 1586, FRIED*), incubator *(220, Scientific Incubator),* spectrophotometer *(Chemi HR 410 BioSpectrum Imaging System UV spectrophotometer),* microplate reader *(BMG LABTECH GMBH, SPECTROstar Nano 601-0040 UV/Vis)* and a water bath *(WMR 14, Memmer)* and manual capsule filling apparatus with tamper *(Cap-M-Quick).*

6.3 Experimental Procedures

6.3.1 Manufacture of capsules & determination of capsule mass uniformity

Encapsulation of the three *L. leonurus* solid extracts was done using a manual capsule filling machine (i.e. the plate method using the *Cap-M-Quick* hand operated capsule filler, figure 6.1). Empty "0" size capsules were placed by hand into the holes of the capsule filling plate, with the bodies fitting snuggly into the plate. The capsule caps were then removed and the capsule bodies filled with *L. leonurus* solid extract (FDAE, Aq EtOH extract or calcium alginate FDAE beads, respectively) by placing *L. leonurus* solid extracts onto the surface of the plate containing the capsule bodies and spreading it evenly with a *Cap-M-Quick* card so that the powder flowed into all the empty capsule bodies. Thereafter the plate was gently shaken to remove any possible air pockets and the process repeated, i.e. more of the respective *L. leonurus* solid extract spread onto the surface and filled into the capsule body. Once filled to the brim, the cap of each capsule was repositioned over the extract-filled capsule body, and the two capsule parts re-joined together using gentle pressure as per method used by Ma (2006).

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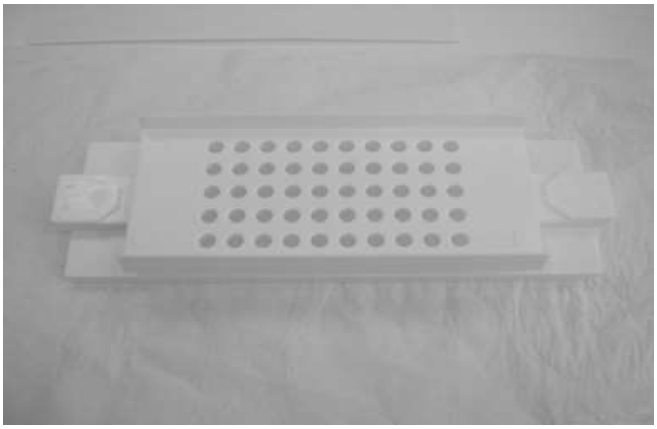

Fig. 6.1: *Cap-M-Quick* hand operated capsule filler

After the capsule filling, the mass uniformity of the respected capsules were determined using the British Pharmacopoeia (BP, 2000) method, with some modifications. According to this

method, individual weights (masses) of not more than two of the capsules may deviate by more than 7.5 % from the average weight (mass) and none by more than twice that percentage for the capsules to have acceptable mass uniformity. For this uniformity of weight determination, ten randomly selected filled capsules from each of the manufactured batches were weighed, each then completely emptied of its contents, the empty shell brushed to remove any remaining particles, and the empty capsule reweighed. The mass of the capsule content was then calculated by subtracting the mass of the empty capsule shell from the initial mass of the full capsule. The values for the 10 capsules were averaged, the percent deviation calculated and the results tabulated and compared.

6.3.2 Determination of stability profile of *L. leonurus* solid extracts

For the stability study, storage conditions suggested in the current guidelines of the South African Medicines Regulatory Authority, viz. the Medicines Control Council (MCC, 2012), was used, with some modifications. The encapsulated *L. leonurus* solid extracts, prepared as described in section 4.3.1, were placed in sealed standard plastic capsule containers and stored at three temperature conditions: **(A)** room temperature, 24 ˚C ± 5 ˚C; **(B)** fixed temperature of 30˚C ± 5 ˚C and **(C)** elevated temperature of 40˚C ± 5 ˚C. Furthermore, an additional set of each of the *L. leonurus* solid extract capsules were stored in sealed standard capsule containers under stressed condition **(D)**, viz. 40 ± 5 ˚C / 75 % relative humidity (RH). Condition **A** was attained in a controlled laboratory environment (having RH > 50 %), and both **B** and **C** by using ovens (with RH > 20 %), while condition **D** was attained using a climatic chamber. The manufactured capsules were stored under the aforementioned conditions **A**, **B** and **C** for a period of 6 months and every month samples of capsules and their content were taken from each site and assessed for organoleptic properties (i.e. gross physical nature, appearance, texture, odour, scent, and appearance of the capsules and their contents), total phenol content (TPC), total flavonoid content (TFC) and antioxidant activity levels. For the organoleptic properties, TPC, TFC and antioxidant activity levels the methods described in sections 4.4.1, 4.4.3, 4.4.4 and 4.4.5, respectively, was used and the retrieved data recorded and compared at the end of the 6 months.

For storage condition **D**, the manufactured capsules was stored for a period of 4 weeks and were assessed using the same parameters i.e. organoleptic properties, TPC, TFC and antioxidant activity levels as those used for storage conditions **A**, **B** and **C**. At the end of the 4

weeks the retrieved data were recorded and compared to the data obtained for storage conditions A, B and C.

6.3.3 Quantification of extent of degradation of *L. leonurus* solid extracts during storage

The degradation profiles of all three *L. leonurus* solid extracts, in terms of TPC and TFC, was quantified as follows. First, the levels of TPC and TFC for each set of extract-containing capsules stored from baseline (i.e. t = 0) to 6 months or 4 weeks were recorded (as GAE i.e. gallic acid equivalents, or QE i.e. quercetin equivalents). These values were then converted to percentages of the baseline values (i.e. baseline GAE or QE = 100%) and plotted *vs* storage time. Since the latter plot was distinctly nonlinear, the data was then fitted assuming at least first or second order degradation kinetics (Pugh, *et al.*, 2002 & Murphy, *et al*,, 1997) and further analysed using GraphPad Prism 5.

For first order degradation reaction the relevant equations were:

$$-d[A]/dt = k[A]^2$$

$$[A]_t = [A]_0 . e^{-K1t}$$

$$K^1 = (\ln [A]_{t6} - \ln[A]_{t0}) / (t_6 - t_0)$$

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where $[A_{t0}$ and $[A]_{t6}$ = TPC or TFC levels initially (t0) and at 6 months (or 4 weeks for the test under stressed conditions), and K^1 = first order rate constant. The graph of ln [A] vs t, fitted by linear regression then gave the slope as an estimate of K^1 and this could be used to calculate the shelf-life from

$$\text{Shelf-life } (t_{90}) = 0.105 / K^1$$

For second order reaction the relevant equations were:

$$-d[A]/dt = k[A]^2$$

with plot of 1/ [A] *vs* t giving the slope as an estimate of the second order rate constant k, and

$$\text{Shelf-life } (t_{90}) = 0.0011 / k$$

Finally, the relative stabilities of the encapsulated *L. leonurus* solid extracts under the different storage conditions were compared using the calculated shelf-life values.

6.3.4 Determination of stability profile of *L. leonurus* solid extracts based on marker compound levels

For this part of the study the *L. leonurus* capsules containing the solid extract preparations were stored under stressed conditions of 40˚C ± 5 ˚C / 75 % RH (as described in section 6.3.2) for 1 month, 10 mg samples was drawn every week and these assayed for marrubiin and leonurine content. The validated HPLC assay method described in section 5.3.1 was used for the latter purpose. From the chromatographic fingerprints of the weekly samples the peaks for the marrubiin and leonurine compounds were identified, the peak areas recorded, and their concentrations determined using the calibration curves presented in chapter 5. Change in marker compound concentrations for the specific extracts over the 4 week period was then assessed to compare the chemical stability of the test capsules under the stressed storage conditions.

6.4 Results and discussion

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6.4.1 Mass content uniformity of capsules containing *L. leonurus* solid extracts

The average weights of *L. leonurus* solid extracts that were loaded into the capsules are given in table 6.1 and appendices 3A, 3B & 3C. According to the results obtained the average solid extract content mass was 369 ± 0.018, 386 ± 0.025 and 599 ± 0.018 mg for the FDAE, Aq EtOH extract and calcium alginate FDAE beads containing capsules, respectively. The average mass of encapsulated FDAE and Aq EtOH extracts was similar (t-test; $p < 0.05$) but significantly ($p < 0.05$) higher than the solid extract content of 320 ± 0.01 mg of Phela, another herbal leaf product, that was also previously packed in size “0” capsules and tested in the present laboratory (Sehume, 2010).

Table 6.1 Mass content uniformity of *L. leonurus* solid extract capsules

Characteristic	FDAE	Aq EtOH extract	Calcium alginate FDAE beads
Mass of capsule content (g) (mean± SD; n = 10)	0.369±0.018	0.386±0.025	0.599±0.018
Mass uniformity (RSD %) (mean± SD; n = 10)	0.048±0.018	0.064±0.025	0.030±0.018

As expected, the capsules containing calcium alginate FDAE beads was however significantly heavier (t-test; $p < 0.05$) than the other two *L. leonurus* extract-filled capsules, presumably because of the heavier calcium alginate coating and more compact filling of the capsules. Finally, the % RSD for the mass of 10 capsules for all three *L. leonurus* solid extract forms was reasonable (i.e. < 6.5%) with the content masses of only two (but not more than two) of the FDAE and two of the ten Aq EtOH extract-containing capsules deviating by > 7.5 % from the average mass. The three sets of capsules thus met the content uniformity acceptance criterion, i.e. that "not more than two of the individual weights (masses) should deviate from the average weight (mass) by more than 7.5 % and none of them by more than twice that percentage" (BP, 2000e). However, the flaky nature of the Aq EtOH extract clearly affected the consistent filling of the capsules and this must be borne in mind when the practicality and final formulation of this *L. leonurus* solid extract is considered for use in a capsule solid dosage form.

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6.4.2 Stability profile of encapsulated *L. leonurus* solid extracts based on organoleptic properties

The organoleptic properties was determined using the procedure described in 4.4.1 and the stability test results obtained for the *L. leonurus* solid extract preparations were summarized and tabulated in appendices 4A and 4B.

From the findings (in appendix 4A and 4B) it was clear that, in the absence of moisture (i.e. in presence of low humidity), temperature (i.e. 30 and 40 ˚C) in itself did not appear to have any significant macroscopic effect on either the organoleptic properties of the *L. leonurus* FDAE, Aq EtOH extract or the calcium alginate FDAE beads. However, when exposed to a relative humidity (RH) of 54 %, the FDAE and Aq EtOH extracts, after a period of 4 months, started

showing signs of physical degradation. They changed in physical appearance from being powders to being melted masses and their texture had changed from being fairly coarse to sticky. When exposed to the stressed conditions (i.e. 40 ± 5 ˚C / 75 % RH) the appearance of the FDAE changed even more significantly, from being flaky to a clumped mass, by week 1 and completely melted by week 4 while its colour changed from medium to dark brown and the texture from fairly course to sticky. Previous studies have reported similar organoleptic property changes in the FDAE of *A. afra* as those displayed by the *L. leonurus* FDAE (and Aq EtOH) extracts used in the present study (Dube, 2006). Moreover, the Aq EtOH *L. leonurus* extract showed gross physical degradation from the very first week of storage. Its appearance changed from flaky to being completely melted, colour changed from medium to dark brown and texture from being fairly course to sticky. The physical appearance of both the FDAE and Aq EtOH extracts were thus particularly affected by the moisture level during storage with the deterioration being accelerated when temperature is also elevated. The storage conditions for capsules containing these two *L. leonurus* extracts must therefore preferably include a maximum storage temperature specification of < 30 ˚C (e.g. store below 25 ˚C) and protection from high humidity (e.g. in tightly closed storage containers, inclusion of silica moisture adsorbents, etc.). On the other hand, the organoleptic features of the calcium alginate FDAE beads was, irrespective of the storage conditions and storage duration, not affected by either temperature or humidity making it the most physically stable *L. leonurus* solid extract preparation of the three, i.e. compared to the FDAE and Aq EtOH extract preparations. Particularly restrictive storage and packaging conditions should therefore not be required for capsules containing this extract of *L. leonurus*. It was however also important to know whether the storage condition-induced changes in the organoleptic features of the three *L. leonurus* solids were matched by chemical changes in the products and this is addressed in the next section.

6.4.3 Stability profile of encapsulated *L. leonurus* solid extracts based on total phenol content (TPC)

It was expected that some of the active constituents of *L. leonurus* may be phenolic compounds and therefore the amounts of total phenols in the stored encapsulated *L. leonurus* solid extracts was determined. This was done using the procedures described in 4.4.3, 6.3.2 and 6.3.3 and the levels quantitated using a standard curve of the gallic acid concentration *vs* UV/VIS absorbance, as shown in appendix 2A. The curve was linear with equation Y = 0.02239x and

regression coefficient, R^2 = 0.9989 and was used to determine the TPC levels in the extracts stored from baseline to 6 months and/or 4 weeks (in case the of accelerated storage conditions). The TPC levels (expressed in gallic acid equivalents (mg/g)) of the *L. leonurus* solid extract preparations stored over time under the different temperature conditions, were recorded in appendices 5A to 5E, and displayed graphically in figure 6.1.

The high TPC baseline levels in all three *L. leonurus* plant preparations decreased significantly during subsequent storage at the different conditions. The TPC of encapsulated FDAE, which contained 7.86 mg/g GAE of total phenols at baseline, decreased significantly to 3.05, 3.15, 3.27 and 3.75 mg/g GAE (i.e. by 61, 60, 58 and 52 %) over 1 month storage at RT, 30 °C, 40 °C and 1 week storage at 40 °C / 75 % RH, respectively. Similarly the TPC of encapsulated Aq EtOH extract, which had contained 7.52 mg/g GAE total phenols at baseline, decreased significantly to 2.93, 3.45, 4.03 and 3.77 mg/g GAE (i.e. by 61, 54, 46 and 50 %) over 1 month when stored at RT, 30 °C and 40 °C and over 1 week when stored at 40 °C / 75 % RH, respectively. Finally, the TPC in the calcium alginate FDAE beads, which contained 6.94 mg/g GAE total phenols at baseline, decreased significantly to 2.34, 2.01, 2.83 and 2.99 mg/g GAE (i.e. by 66, 71, 59 and 57 %) over the 1 month when stored at RT, 30 °C and 40 °C and over 1 week when stored at 40 °C / 75 % RH, respectively (appendix 5 and figure 6.1). For all three extracts, the bulk of the TPC decreases (i.e. between 46 % to 71 % on average) occurred within the first month of storage at RT, 30 °C and 40 °C, and thereafter at much slower rates. This firstly indicated that, based on TPC, the shelf-lives (i.e. t_{90} = time for level to decrease by 10 %) for all three extracts were very short (i.e. < 1 month). The data also clearly indicated the presence of at least a 2 phase decay rate process (i.e. a second order degradation reaction) being responsible for the decline in polyphenol levels in the tested *L. leonurus* plant preparations.

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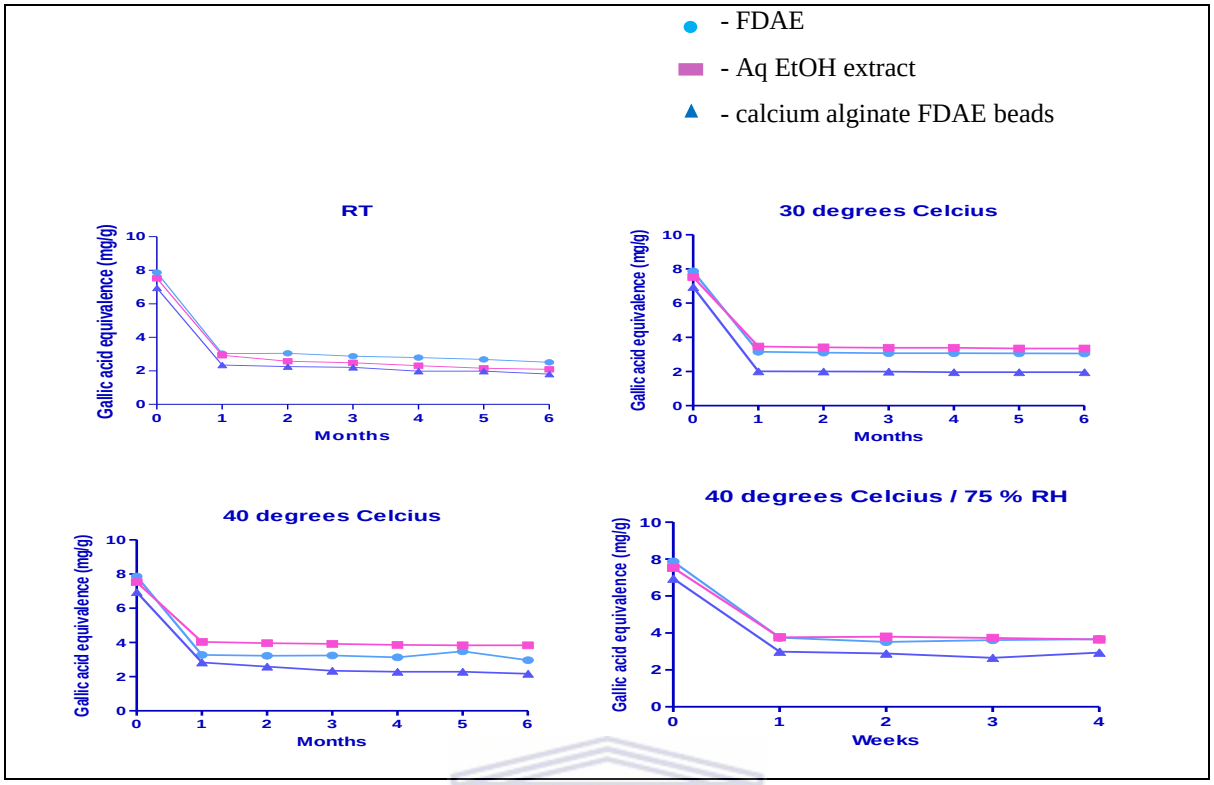

Fig. 6.1: Profile of total phenol content levels (mg/g) of *L. leonurus* solid extracts stored at RT (room temperature, 24 ± 2 ˚C / 54 % RH); 30 °C (30 ± 5 ˚C) ; 40 °C (40 ± 5 ˚C) and 40 ± 5 ˚C / 75 % RH for 6 months and 4 weeks

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However, when the data was fitted to both first and second order degradation equations (i.e. ln [A] *versus* time and 1/ [A] *vs* t plots), the linear regression fits were not very good (see R^2 values in table 6.2 of results assuming second order degradation). There were simply not enough data points over the first month to describe the first rapid decay process (over month 1) accurately and obtain reliable rate constants (to use to calculate t_{90}) for that part of the curve. Nevertheless, if the k values obtained from the poor second order reaction fit were used (i.e. see table 6.2) k = 0.0023, the calculated shelf-lives was no more than 0.47 months (1.9 weeks) confirming that all three extracts were very unstable if TPC is used as the marker for stability. Overall, it was also hard to tell which of the three *L. leonurus* solid extracts were, in terms of their TPC, the most or least stable overtime as they all retained approximately the same level of TPC and thus had the same degree of instability.

Table 6.2 Second order degradation rate constants and shelf-lives for TPC *in L. leonurus* solid extracts stored under different conditions

Stability storage conditions	FDAE			Aq EtOH extract			Calcium alginate FDAE beads		
	R_2	K	T_{90}	R_2	K	T_{90}	R_2	K	T_{90}
24 ± 2 ˚C / 54 % RH	0.6178	0.0025	0.43	0.7603	0.0036	0.31	0.6647	0.0035	0.31
30 ± 5 ˚C	0.4388	0.0018	0.60	0.3750	0.0013	0.86	0.3750	0.0027	0.41
40 ± 5 ˚C	0.4706	0.0018	0.62	0.4932	0.0012	0.93	0.6528	0.0028	0.39
40 ± 5 ˚C / 75 % RH	0.4953	0.0023	0.48	0.5708	0.0022	0.50	0.5748	0.0031	0.35

R^2 = Linear regression coefficient for 1/[A] vs time plot

K = 1 / GAE (mg/g) solid plant extract vs. time (months at RT, 30 °C and 40 °C or weeks at 40 °C/ 75 % RH)

T_{90} = 0.0011 / K (expressed in months at RT, 30 °C and 40 °C or in weeks at 40 °C/ 75 % RH)

With this assumption, the TPC data were also used to estimate the shelf-life of the encapsulated extracts and the results obtained were summarised in table 6.2. Using TPC as stability parameter, all 3 encapsulated extracts had very short, shelf-lives ranging, from 1.24 weeks (0.31 months) to 3.72 weeks (0.93 months) (e.g. compare samples stored at RT *vs* 30 °C and 40 °C) (see table 6.2). Unfortunately not enough data (i.e. only 1 data point) was collected over the first month of the 6 month stability test periods to quantitate the effects of temperature and humidity on shelf-lives. When stored under the officially recommended stressed conditions for stability testing (i.e. 40 ± 5 ˚C / 75 % RH) the shelf-lives for the encapsulated extracts were even shorter i.e. 3.10 days (i.e. (0.44) weeks x 7 days/week), confirming the instability of these encapsulated *L. leonurus* extracts. Presently there are no reports in literature on the stability of the polyphenols in *L. leonurus* plant extracts. However, this instability and short shelf-lives of the 3 extracts is problematic and needs to be addressed if high quality therapeutically effective *L. leonurus* preparations are to be made. Moreover, conversion of the FDAE into the calcium alginate beads also did not alleviate the clear instability of the *L. leonurus* aqueous extract.

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6.4.4 Stability profile of encapsulated *L. leonurus* solid extracts in terms of total flavonoid content (TFC)

It was expected that some of the active constituents of *L. leonurus* may be flavonoid compounds and therefore the amount of total flavonoids in the stored encapsulated *L. leonurus* solid extracts was determined. This was done using the procedure described in 4.4.4, 6.3.2 and 6.3.3 and the levels quantitated using a standard curve of the quercetin concentration *vs* UV

absorbance as shown in appendix 2B. The curve was linear with equation Y= 0.02970x and regression co-efficient, R^2 = 0.9805 and was used to determine the TFC levels in the extracts from baseline to 6 months and/or 4 weeks (in case of accelerated storage conditions). The TFC levels (expressed in quercetin equivalent (mg/g)) of the *L. leonurus* solid extract preparations stored over time under different temperature conditions, were recorded in appendices 6A to 6E, and displayed in figure 6.2.

The high TFC baseline levels in all three *L. leonurus* plant preparations decreased significantly during subsequent storage at the different conditions. In fact, the TFC of encapsulated FDAE, which contained 4.30 mg/g quercetin equivalents of total flavonoids at baseline, decreased significantly to 3.02, 3.34, 1.89 and 2.69 mg/g (i.e. by 25, 25, 29 and 66 % %) after 1 month storage at RT, 30 °C and 40 °C and 1 week storage at 40 °C / 75 % RH, respectively. Similarly the TFC of encapsulated Aq EtOH extract, which contained 4.49 mg/g quercetin equivalence total flavonoids at baseline, had decreased significantly to 3.24, 3.49, 1.65 and 2.28 mg/g (i.e. by 26, 26, 23 and 70 %) after 1 month storage at RT, 30 °C and 40 °C and 1 week storage at 40 °C / 75 % RH, respectively. Finally, the TFC in the calcium alginate FDAE beads, which contained 3.69 mg/g quercetin equivalence total flavonoids at baseline, also decreased significantly to 3.07, 3.47, 1.77 and 2.53 (i.e. by 55, 55, 52 and 64 %) after 1 month storage at RT, 30 °C and 40 °C and 1 week storage at 40 °C / 75 % RH, respectively (appendix 6 and figure 6.2). The results obtained for TFC was thus similar to that obtained for the TPC data. Again the decrease in TFC in all three *L. leonurus* plant preparations were biphasic with a very rapid drop in flavonoid levels during month 1 followed by a slower decline thereafter for all the storage conditions, clearly suggesting that a second order degradation process was involved.

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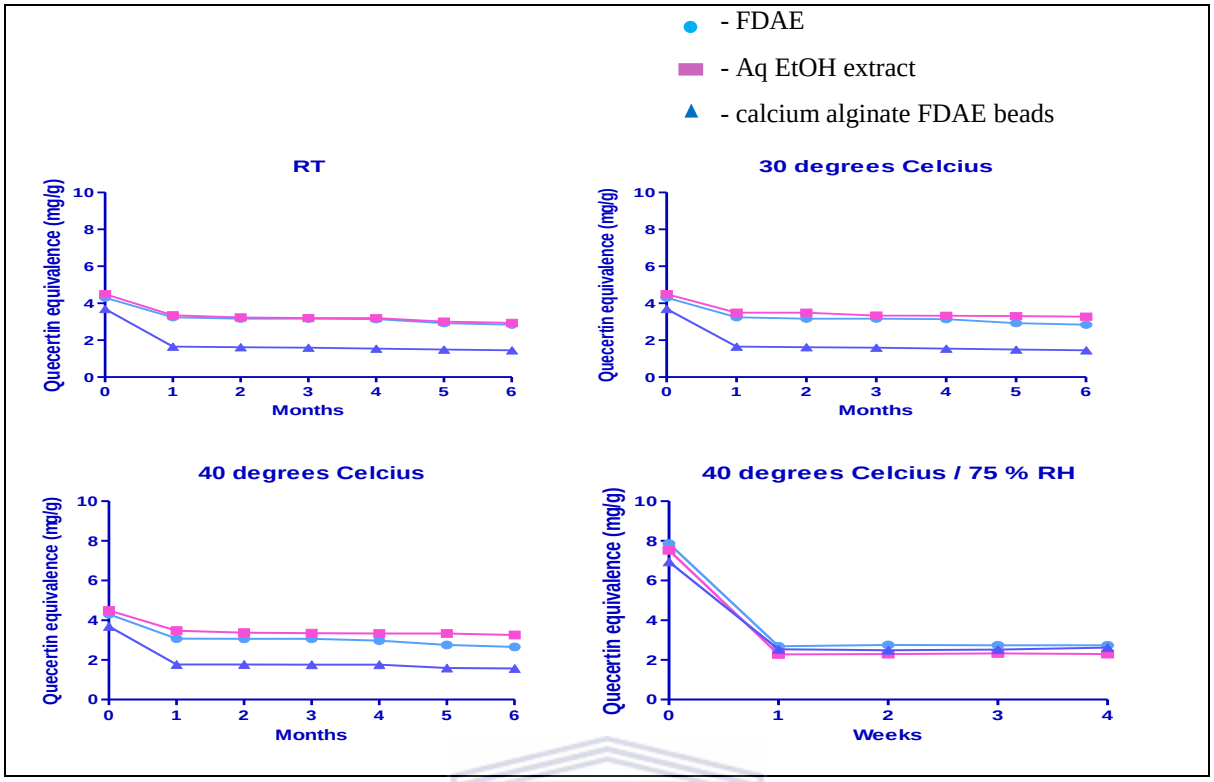

Fig. 6.2: Profile of total flavonoid content levels (mg/g) of *L. leonurus* solid extracts stored at room temperature (RT) 24 ± 2 ˚C / 54 % RH; 30 °C (30 ± 5 ˚C); 40 °C (40 ± 5 ˚C) and 40 ± 5 ˚C / 75 % RH for 6 months and 4 weeks

Indeed, for all three extracts in the current study, the bulk of the TFC decreases (i.e. between 23 % to 70 % on average) occurred within the first month of storage at RT, 30 °C and 40 °C, and thereafter at much slower rates (i.e. 0.12, 0.07 and 0.13 QE mg/ g /month), confirming a 2 phase decay rate process for the flavonoids (as was found for the total polyphenols) in the tested *L. leonurus* plant preparations. These preparations were therefore not very stable in terms of its flavonoid content.

Shelf-lives of the encapsulated extracts were also estimated from the TFC data and the results obtained were summarised in table 6.3. Based on the TFC data all three encapsulated extracts had very short, shelf-lives ranging, from 2.28 weeks (0.57 months) to 8.8 weeks (2.2 months) that was further shortened with an increased temperature (e.g. compare samples stored at RT *vs* 30 °C and 40 °C) and addition of humidity (see table 6.5). Unfortunately not enough data (i.e. only 1 data point) was collected over the first month of the 6 month stability test periods to quantitate the effects of temperature and humidity on the shelf-lives of the extracts. When stored under the officially recommended stressed conditions for accelerated stability testing (i.e. 40 ± 5 ˚C / 75 % RH) the shelf-lives for the encapsulated extracts were, as expected, even shorter i.e. 1.96 days (i.e. (0.28 weeks x 7 days/week), confirming the instability of these encapsulated *L. leonurus* extracts.

Table 6.3 Degradation rate constant (shelf-life) of TFC *in L. leonurus* solid extracts

Stability storage conditions	FDAE			Aq EtOH extract			Calcium alginate FDAE beads		
	R_2	K	T_{90}	R_2	K	T_{90}	R_2	K	T_{90}
24 ± 2 ˚C / 54 % RH	0.7278	0.0007	1.57	0.7278	0.0007	1.57	0.5768	0.0019	0.57
30 ± 5 ˚C	0.7278	0.0007	1.57	0.6696	0.0005	2.2	0.5768	0.0019	0.57
40 ± 5 ˚C	0.7202	0.0008	1.38	0.5455	0.0004	2.2	0.5865	0.0016	0.68
40 ± 5 ˚C / 75 % RH	0.5000	0.0038	0.29	0.4882	0.0045	0.24	0.4980	0.0035	0.31

QE = Quercetin equivalent (mg/g) (Mean ± SD; n = 3))

K = 1 / QE (mg/g) solid plant extract vs. time (months at RT, 30 °C and 40 °C or weeks at 40 °C/ 75 % RH)

T_{90} = 0.0011 / K (expressed in months at RT, 30 °C and 40 °C or in weeks at 40 °C/ 75 % RH)

Overall, these results were similar to that of Ogangole (2007) who measured luteolin levels (flavonoid) in freeze dried *A. afra* extract stored under ambient room and accelerated (i.e. 40 C /75 % RH) storage conditions. He found that, based on total luteolin levels, the FDAE of A. afra had shelf-lives (t90) of 7.57 and 2.27 days when stored under ambient and elevated conditions, respectively. On the other hand Heigl & Franz (2003), who investigated the TFC of flavonoid containing herbal drugs (i.e. Marigold flowers, Elder flower and Birch leaves) stored under moderate climatic conditions (room temperature) 25 °C / 60 RH for 24 months and stressed conditions 40 °C/ 75 % RH for 6 months, found that no significant changes occurred in the TFC levels of the herbal drugs during storage at 25 °C / 60 RH, but a significant decrease at the 40 °C/ 75 % RH storage condition. They in fact concluded that the stability of flavonoids in herbal drugs or plant material could be guaranteed for a period of two years (Heigl & Franz, 2003), but clearly that won't be the case for freeze dried extracts (such as *A. afra* in the Ogangole (2007)) or the *L. leonurus* plant preparations investigated in the present study.

6.4.5 Stability profile of encapsulated *L. leonurus* solid extracts based on antioxidant properties

Another way to monitor the stability of *L. leonurus* solid extracts might be via measurement of their antioxidant activity. In this study, for antioxidant activity, the radical-scavenging ability of the stored extracts was monitored using the procedure described in sections 4.4.5 and 6.3.2 and the results obtained were summarized in appendices 7A to 7D and displayed in figure 6.3.

The antioxidant activity of encapsulated FDAE, which contained 100 % DPPH scavenging activity at baseline, decreased to 99.8, 99.7, 98.2 and 98 % (i.e. by only 0.2, 0.3 , 1.2 and 2 %)

after 1 month storage at RT, 30 °C and 40 °C, and 1 week storage, at 40 °C / 75 % RH, respectively. The data showed that the increased temperature and exposure to humidly during storage hardly affected the stability of encapsulated FDAE, the product remained relatively stable. The results were similar for the encapsulated Aq EtOH extracts which had 100 % DPPH scavenging activity at baseline and had that decreased to 99.3, 98.7, 99.9 and 94.7% (i.e. by 0.7 %, 1.3 %, 0.1 % and 5.3 %) after 1 month storage at RT, 30 °C and 40 °C, and 1 week storage at 40 °C / 75 % RH, respectively. In addition, the encapsulated calcium alginate beads which had 100 % DPPH scavenging activity at baseline also only had it decreased to 98, 97.7, 98.6 and 99.2 % (i.e. only by 0.2, 3.3, 2.4 and 0.8 %) after 1 month storage at RT, 30 °C and 40 °C, and 1 week storage at 40 °C / 75 % RH, respectively. All three *L. leonurus* solid extracts showed the same degree of antioxidant stability and therefore displayed reasonably good overall antioxidant activity stability profiles under the various storage temperature and humidity conditions used in the present study. No previous studies appear to have been done on specifically the antioxidant activity of stored *L. leonurus* solid extracts, but Amoo (2012) had performed a stability study on *L. leonurus* and 20 other medicinal plants in which they oven-dried the plants at 50 °C, stored them in brown paper bags at 25 °C for a period of 12-16 years and then tested it for antioxidant activity. They found that a 50 % methanolic extract of the *L. leonurus* leaves had a maximum radical-scavenging activity of 93.7 % which agrees somewhat with the levels found in the present study. Overall, based on the above-mentioned results obtained for the antioxidant study and when that is compared to the results obtained for the TPC and TFC, it was clear that antioxidant activity level was not a viable parameter to use to monitor and quantitate the stability of the *L. leonurus* extracts.

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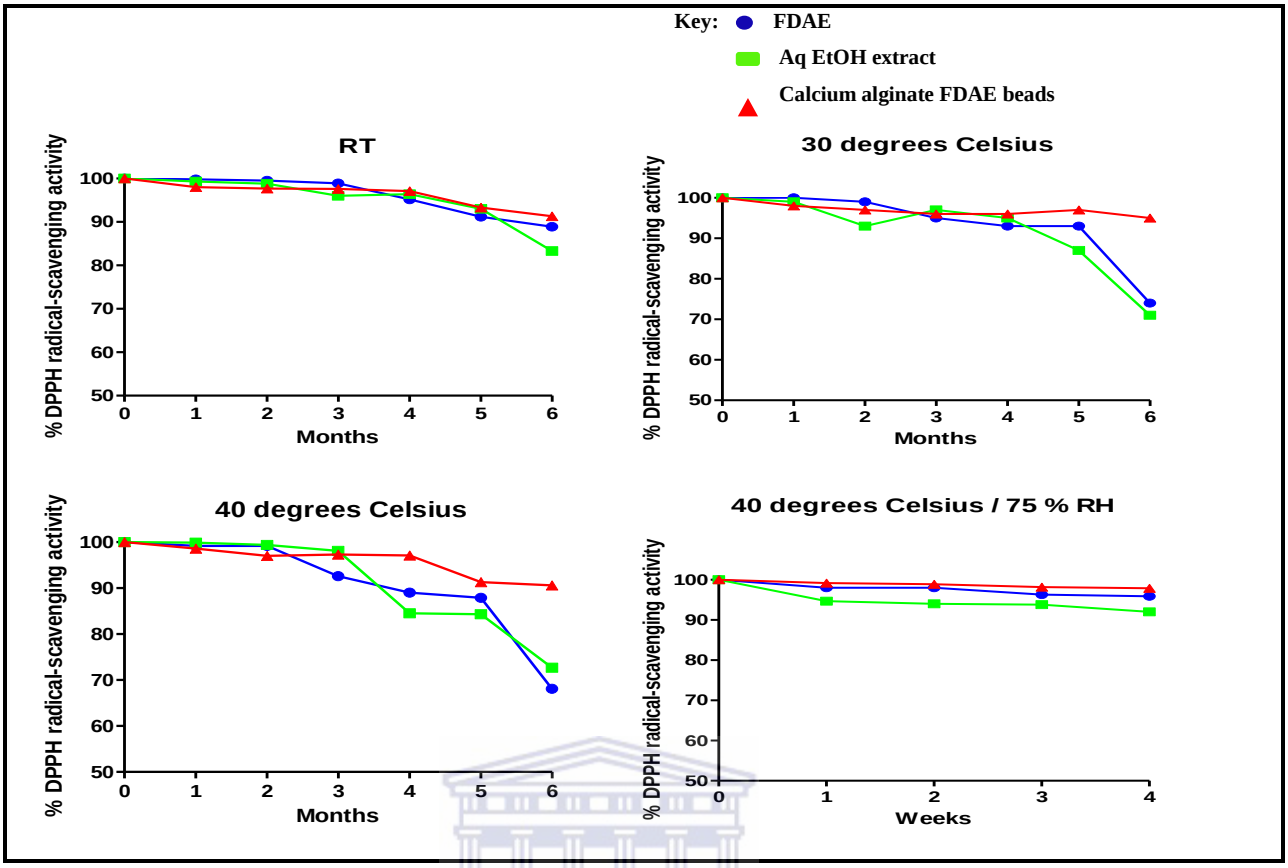

Fig. 6.3: Profiles of antioxidant activity (expressed as % DPPH radical-scavenging activity) of *L. leonurus* solid extracts stored at room temperature (RT) 24 ± 2 ˚C / 54 % RH; 30 °C (30 ± 5 ˚C); 40 °C (40 ± 5 ˚C) and 40 ± 5 ˚C / 75 % RH for 6 months or 4 weeks.

Based on the antioxidant test data all 3 encapsulated extracts had fairly long shelf-lives ranging, from 22.4 weeks (5.6 months) to 18.8 weeks (4.7 months) and 15.6 weeks (3.9 months) when stored at RT, 30 °C and 40 °C respectively. When stored under the officially recommended stressed conditions for accelerated stability testing (i.e. 40 ± 5 ˚C / 75 % RH) the shelf-lives for the encapsulated extracts were even longer i.e. 28 days (i.e. (4 weeks x 7 days/week), confirming the instability of these encapsulated *L. leonurus* extracts.

6.4.6 Stability profile of encapsulated *L. leonurus* solid extracts based on marrubiin and leonurine levels

The stability of the *L. leonurus* solid extracts stored under stress conditions was also monitored in terms of levels of 2 marker compounds, viz. marrubiin and leonurine**,** using the procedures described in sections 5.3.1 and 6.3.2, and the chromatographic fingerprints obtained were given

in appendices 8A to 8F and the marker compound level v*ersus* storage time results in tables 6.5 and 6.6.

6.4.6.1 Stability of L. leonurus solid extracts based on marrubiin levels

The concentration of marrubiin in the FDAE (table 6.5) after preparation was 22.5 µg/mg and at the start of the stability study was 0.46 µg/mg, marrubiin levels decreased rapidly and significantly to 0.25 µg/mg within the first week under the accelerated (stressed) storage conditions. By the end of the second week of storage the concentration of marrubiin in the FDAE was non-detectable (0 µg/mg). The marrubiin in the FDAE was thus very unstable and degraded rapidly under this storage condition. For the *L. leonurus* Aq EtOH extract the initial concentration of marrubiin upon preparation was 17.5 µg/mg at t=0 and at the beginning of the stability study had degraded to 0.38 µg/mg, but had completely degraded after week 1 of storage, i.e. no detectable amounts present (Appendix 8C 8.2 to 8C 8.5). As for the FDAE, the marrubiin in the Aq EtOH extract was also very unstable. On the other hand, much lower marrubiin levels were detected in the stored calcium alginate FDAE beads, compared to the FADE and Aq EtOH extract, before storage the marrubiin concentration was 0.4 µg/mg and at time zero 0.09 µg/mg was detected (Appendix 8E 8.2 to 8E 8.5) for at least 3 weeks. After 4 weeks storage the levels were however also not quantifiable with the present assay, suggesting degradation even at such low levels. Overall, all three products contained marrubiin which was rapidly degraded under the stressed storage conditions. With the current assay method and because of its low content of marrubiin this marker compound was however not likely to be an effective marker to monitor the stability of calcium alginate FDAE beads but may be a suitable marker for the FDAE and Aq EtOH extract. Alternatively, a much more sensitive assay needs to be developed and used.

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Table 6.5: Levels of marrubiin in encapsulated *L. leonurus* solid extracts stored under stressed conditions (40 ˚C/ 75 % RH) and measured at 214 nm

Plant extract	Sample storage time in weeks	Marrubiin peak area (mAU)	Marrubiin Concentration (µg/mg)
FDAE	week 0	550	0.46
	week 1	250	0.25
	week 2	0	0
	week 3	0	0
	week 4	0	0
Aq EtOH extract	week 0	450	0.38
	week 1	0	0
	week 2	0	0
	week 3	0	0
	week 4	0	0
Calcium alginate beads	week 0	100	0.09
	week 1	100	0.09
	week 2	100	0.09
	week 3	100	0.09
	week 4	0	0

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6.4.6.2 Stability of *L. leonurus* solid extracts based on leonurine levels

The HPLC fingerprint chromatograms and results for the leonurine levels was shown in appendix 8 and table 6.6 and were generally not very good i.e. the leonurine peak was small and not very well separated from surrounding peaks. Thus generally, unless a more selective and sensitive HPLC method is developed this compound may not be a very useful marker to assess the stability of the encapsulated *L. leonurus* solid extracts.

Also, the concentration of leonurine in FDAE prior to the stability study was 0.30 µg/mg (appendix 8B 8.1) and then decreased inconsistently to 0.23 µg/mg by the third week and below detection limit (0 mg/mg) at 4th week of storage, while there was no change in leonurine levels and therefore no obvious degradation of the marker compound, over the 4 weeks the

encapsulated Aq EtOH extract was stored at 40 ˚C/ 75 % RH. On the other hand the leonurine level in the calcium alginate FDAE beads dropped rapidly from 0.30 µg/mg to 0.11 µg/mg at week 1 (table 6.6) and then remained constant at this level for the remaining 3 weeks. No meaningful conclusions on the stability profile of the three extracts could thus be drawn from the data on this marker compound.

Overall, the main finding on the marrubiin and leonurine levels was that indeed both were present in all three *L. leonurus* solid extract preparations. The levels of marrubiin were initially higher compared to that for leonurine but the latter took much longer to decrease (i.e. leonurine took much longer to breakdown) and was therefore the more stable of the 2 marker compounds. When comparing the *L. leonurus* solid extract preparations to one another, the

Table 6.6: Levels of leonurine in encapsulated *L. leonurus* solid extracts stored under stressed conditions (40 ˚C/ 75 % RH) and measured at 214 nm

Plant extract	Sample storage time in weeks	Leonurine peak area (mAU)	Leonurine Concentration (µg/mg)
FDAE	week 0	40	0.30
	week 1	40	0.30
	week 2	30	0.23
	week 3	30	0.23
	week 4	0	0
Aq EtOH extract	week 0	15	0.11
	week 1	15	0.11
	week 2	15	0.11
	week 3	15	0.11
	week 4	15	0.11
Calcium alginate beads	week 0	40	0.30
	week 1	15	0.11
	week 2	15	0.11
	week 3	15	0.11
	week 4	15	0.11

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low levels of both marrubiin and leonurine detected in the calcium alginate FDAE bead preparations also possibly reflecting that this *L. leonurus* extract preparation contained less of the plant material (and hence marker compounds). Collectively, the leonurine and marrubiin data obtained strongly suggests that a more sensitive and selective assay (e.g. using LC MS detector and/or including solid phase extraction or sample clean up, etc.) will be required if the stability of *L. leonurus* preparations is to be monitored using these marker compounds.

6.4.7 Conclusions

A major goal of this study was to see if different encapsulated *L. leonurus* solid extract preparations would vary in their stability upon storage. Based on results of previous studies (Ogangole, 2007) done on aqueous extracts of other plants (e.g. *A. afra*) it was expected that the FDAE and even a Aq EtOH extract of *L. leonurus* would not be very stable upon storage and that a calcium alginate FDAE bead preparation of the FDAE of *L. leonurus* might have improved shelf-life (Egieyeh, 2011). To answer these questions it was however also important to, first, prepare capsule dosage forms of the *L. leonurus* extracts that were of acceptable pharmaceutical quality and, secondly determine which of the different stability-indicating parameters could be used to actually effectively monitor/assess the stability profile of the test preparations.

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Generally, the monitoring of the parameters of physical characteristics (e.g. organoleptic features) and chemical characteristics (e.g. TPC, TFC, individual marker compound levels) provided similar results, viz, that all three preparations were very unstable, while the results based on the activity parameter (i.e. antioxidant activity) showed no or very little instability. Quite possibly, the degradation of the preparations (as evidenced by changes in the physicochemical characteristics and esp. the TPC & TFC profiles) simply did not involve compounds responsible for antioxidant activity and/or simply lead to the production of other chemical compounds which also had antioxidant activity. Whatever the reason, clearly the latter parameter (i.e. monitoring antioxidant activity) should preferably not be used to establish the stability profile or shelf-life of *L. leonurus* preparations. Moreover, monitoring of TPC and TFC showed the most clear and dramatic stability profiles and appeared to be particularly good parameters to use to accurately, inexpensively and rapidly establish (and confirm) the stability profile and shelf-life of *L. leonurus* preparations (provided sufficient attention is given to using correct sample collection time points e.g. sufficient time points during first month of storage). Finally, the use of leonurine and marrubiin levels as possible stability indicating parameters for

these preparations was not very convincing but might still work provided a more sensitive and selective assay than the HPLC assay used in the present study, is developed and utilized.

Overall, all three the encapsulated *L. leonurus* solid extracts studied were clearly very unstable and did not have suitable long-term storage stability. Indeed with estimated shelf-lives of less than 4 weeks, even under ambient room conditions, the encapsulated FDAE, Aq EtOH and calcium alginate FDAE beads of *L. leonurus* was not suitable for use as they are at present and will require substantial additional pharmaceutical formulation and/or packaging to become products having acceptable pharmaceutical quality and shelf-life.

Finally, modification of the freeze dried aqueous extract of *L. leonurus* into a calcium alginate bead form seemed to combat physical instability but did not improve the known chemical instability of the aqueous extract i.e. the postulated hypothesis was not proven. Clearly a different solution must be sought.

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CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Objectives of the study

Overall, the aims of this study were to determine whether the FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* were viable replacements for the traditional tea form, whether they had suitable long-term storage stability and, finally, to establish which stability parameters (including the determination of marker compound levels, marrubiin and leonurine), were suitable to use as product stability indicators for these extracts.

The specific objectives of the study were:

(i) To prepare and characterize the different solid extract forms of *L. leonurus* (FDAE, Aq EtOH extract and calcium alginate FDAE beads) and

(ii) To determine and compare their long-term stability using physical characteristics (e.g. organoleptic properties) and chemical parameters (e.g. TPC, TFC, antioxidant activity levels and marker compound levels)

It was hypothesised that the Aq EtOH extract of *L. leonurus* plant material would contain higher levels of chemicals (TPC, TFC) and chemical marker compounds (marrubiin and leonurine) than the FDAE and calcium alginate FDAE beads of *L. leonurus* and, that the calcium alginate FDAE beads would have greater stability (i.e. longer shelf-life) than the FDAE and the Aq EtOH extract.

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7.2 Conclusions

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

1. The *L. leonurus* solid extracts was fairly easy to prepare and the percentage yield obtained for the FDAE was consistent with that obtained by other investigators.

2. Physically the calcium alginate beads contained the least moisture, while chemically all three *L. leonurus* extract preparations contained appreciable and similar TPC and TFC levels but variable amounts of marrubiin and leonurine.

3. All three encapsulated *L. leonurus* solid extract (i.e. FDAE, Aq EtOH and calcium alginate FDAE bead) preparations studied were very unstable and did not have suitable long-term storage stability (i.e. shelf-lives of < 2 weeks)**.**

4. An array of parameters of physical characteristics (e.g. organoleptic features) and chemical characteristics (e.g. TFC, TPC, individual marker compound levels), but not antioxidant activity, can be used to effectively monitor the quality and stability of the three *L. leonurus* forms. Of these the TFC and TPC appear to be particularly good parameters to use to accurately, inexpensively and rapidly establish (and confirm) the stability profile and shelf-life of such *L. leonurus* preparations.

5. Assessment of the stability of the *L. leonurus* preparations by monitoring the levels of the specific chemical marker compounds, marrubiin and leonurine, was not successful in the present study; a more sensitive assay was required.

6. Finally, the modification of the freeze dried aqueous extract of *L. leonurus* into a calcium alginate bead form, although less hygroscopic, also did not improve the known and expected instability of the aqueous extract, i.e. the postulated hypothesis was not proven and clearly a different solution must be sought.

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7.3 Recommendations

Based on the findings of this study it is consequently recommended that:

1) The addition of excipients to the *L. leonurus* extract prior to freeze-drying should be explored to combat the hygroscopicity problem and ultimately improve overall product stability of the *L. leonurus* extract forms;
2) TPC and TFC can be used to accurately, inexpensively and rapidly establish (and confirm) the stability profile and shelf-life of *L. leonurus* preparations, and possibly other herbal products known to contain flavonoids and polyphenols, and
3) A more sensitive and selective assay will be required if the stability of *L. leonurus* preparations is to be monitored using marker compounds marrubiin and leonurine.

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APPENDICES

APPENDIX 1: Yield of *L. leonurus* solid extracts

Appendix 1A: Yield of freeze-dried aqueous extract (FDAE) of *L. leonurus*

Solid extract	Weight of dried plant leaves (g)	Volume of dH_2O (mL)	Weight of FDAE (g)	% Yield o plant extract
FDAE	221	4.42	45	20.3
FDAE	221	4.42	43	19.6
FDAE	221	4.42	44	19.9
Mean & SD			**44±1**	**19.9±0.35**

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Appendix 1B: Yield of Aq EtOH extract of *L. leonurus*

Solid extract	Weight of dried plant leaves (g)	Volume of 20 % EtOH in dH_2O (mL)	Weight of 20 % Aq EtOH extract (g)	% Yield of plant extract
20 % Aq EtOH	50	100	6.43	12.86
20 % Aq EtOH	50	100	6.41	12.82
20 % Aq EtOH	50	100	6.40	12.80
Mean & SD			**6.41±0.15**	**12.82±0.02**

Appendix 1C: Yield of calcium alginate FDAE beads of *L. leonurus*

Solid extract	Weight of FDAE (g)	Weight of sodium alginate (g)	Weight of calcium chloride (g)	Volume of dH_2O (mL)	Weight of calcium alginate FDAE beads (g)	% Yield of plant extract
Calcium alginate FDAE beads	2	2	4	100	3.43	10.72
Calcium alginate FDAE beads	2	2	4	100	3.42	10.69
Calcium alginate FDAE beads	2	2	4	100	3.42	10.69
Mean & SD					**3.42±0.01**	**10.70±0.02**

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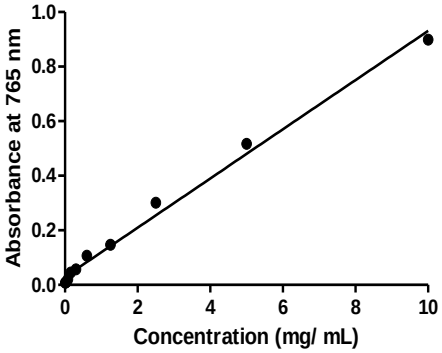
APPENDIX 2: Standard curves

Appendix 2A: Gallic acid standard curve



Best-fit values	
Slope	0.09016 ± 0.002796
Y-intercept when X=0.0	0.02924 ± 0.009732
X-intercept when Y=0.0	-0.3243
1/slope	11.09
95% Confidence Intervals	
Slope	0.08384 to 0.09649
Y-intercept when X=0.0	0.007227 to 0.05125
X-intercept when Y=0.0	-0.5925 to -0.07728
Goodness of Fit	
R square	0.9914
Sy.x	0.02755

Fig. 2A 2.1: Standard curve of gallic acid concentration *vs* absorbance at 765 nm

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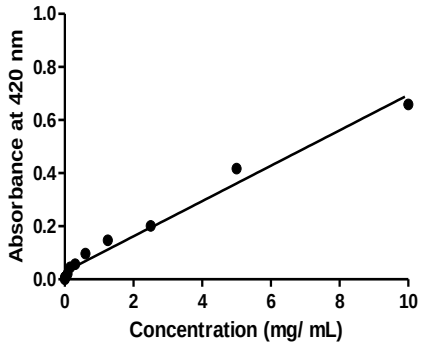
Appendix 2B: Quercetin standard curve



Best-fit values	
Slope	0.06660 ± 0.002968
Y-intercept when X=0.0	0.02850 ± 0.009893
X-intercept when Y=0.0	-0.4280
1/slope	15.02
95% Confidence Intervals	
Slope	0.05998 to 0.07321
Y-intercept when X=0.0	0.006460 to 0.05054
X-intercept when Y=0.0	-0.8053 to -0.09233
Goodness of Fit	
R square	0.9805
Sy.x	0.02970

Fig. 2B 2.2: Standard curve of quercetin concentration *vs* absorbance at 420 nm

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Appendix 2C: Antioxidant activity of *L. leonurus* solid extract preparations

Cpd	Conc (mg/mL)	*AS AUFS	**AC AUFS	***DPPH Scavenging %	REGRESSION ANALYSIS EC50	R^2
Ascorbic Acid	1	0.094	1.205	92.2	0.005	0.9885
	0.5	0.128	1.205	89.4		
	0.25	0.143	1.205	88.1		
	0.125	0.155	1.205	87.1		
	0.063	0.182	1.205	84.9		
	0.031	0.289	1.205	76.0		
	0.016	0.439	1.205	63.6		
	0.008	0.542	1.205	55.0		
FDAE	10	0.140	1.205	88.4	0.8868	0.9974
	5	0.142	1.205	88.2		
	2.5	0.251	1.205	79.2		
	1.25	0.510	1.205	57.7		
	0.63	0.649	1.205	46.1		
	0.31	0.745	1.205	38.2		
	0.16	0.748	1.205	37.9		
	0.08	0.809	1.205	32.9		
Aq EtOH	10	0.114	1.205	90.5	0.7525	0.9284
	5	0.123	1.205	89.8		
	2.5	0.290	1.205	75.9		
	1.25	0.366	1.205	69.6		
	0.63	0.460	1.205	61.8		
	0.31	0.581	1.205	51.8		
	0.16	0.634	1.205	47.4		
	0.08	0.686	1.205	43.1		
Calcium alginate FDAE beads	10	0.412	1.205	65.8	1.062	0.9240
	5	0.454	1.205	62.3		
	2.5	0.533	1.205	55.8		
	1.25	0.618	1.205	48.7		
	0.63	0.699	1.205	42.0		
	0.31	0.740	1.205	38.6		
	0.16	0.823	1.205	31.7		
	0.08	0.894	1.205	25.8		

Where

*AS = the absorbance of the sample (i.e. plant extract or ascorbic acid) in DPPH solution

*AC = the absorbance of the control solution (containing only DPPH solution)

***DPPH Scavenging = (AC – AS) / AC x100 %

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APPENDIX 3: Content uniformity of *L. leonurus* solid extract capsules

Appendix 3A: Content uniformity data of capsules containing *L. leonurus* FDAE

Capsule number	Mass of whole capsule (g)	Empty shell mass (g)	Capsule content mass (g)	Deviation from average (%)
Capsule 1	0.464	0.094	0.370	0.27
Capsule 2	0.492	0.095	0.397	**7.59***
Capsule 3	0.431	0.095	0.336	**-8.94***
Capsule 4	0.467	0.094	0.373	1.08
Capsule 5	0.468	0.094	0.374	1.36
Capsule 6	0.476	0.094	0.382	3.52
Capsule 7	0.479	0.094	0.385	4.34
Capsule 8	0.451	0.093	0.358	-2.98
Capsule 9	0.443	0.094	0.349	-5.42
Capsule 10	0.462	0.096	0.366	-0.81
Mean & SD	**0.463±0.017**	**0.094±0.001**	**0.369±0.018**	**0.17±3.22**

Deviation form average = (Capsule mass content - Mean of capsule mass content) / Capsule mass content * 100

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Appendix 3B: Content uniformity data for capsules containing *L. leonurus* Aq EtOH extract

Capsule number	Mass of whole capsule (g)	Empty shell mass (g)	Capsule content mass (g)	Deviation from average (%)
Capsule 1	0.444	0.094	0.350	**-10.29***
Capsule 2	0.504	0.093	0.411	6.08
Capsule 3	0.493	0.094	0.399	3.26
Capsule 4	0.458	0.092	0.366	-5.46
Capsule 5	0.482	0.095	0.387	0.26
Capsule 6	0.479	0.095	0.384	-0.52
Capsule 7	0.498	0.096	0.402	3.98
Capsule 8	0.440	0.096	0.344	**-12.21***
Capsule 9	0.504	0.095	0.409	5.62
Capsule 10	0.511	0.094	0.417	7.43
Mean & SD	**0.481±0.025**	**0.094±0.001**	**0.386±0.025**	**2.58±2.46**

Deviation form average = (Capsule mass content - Mean of capsule mass content) / Capsule mass content * 100

Appendix 3C: Content uniformity data for capsules containing *L. leonurus* calcium alginate FDAE beads

Capsule number	Mass of whole capsule (g)	Empty shell mass (g)	Capsule content mass (g)	Deviation from average (%)
Capsule 1	0.664	0.094	0.570	-5.09
Capsule 2	0.704	0.093	0.611	1.96
Capsule 3	0.693	0.094	0.599	0.00
Capsule 4	0.658	0.092	0.566	-5.83
Capsule 5	0.682	0.095	0.587	-2.04
Capsule 6	0.679	0. 095	0.584	-2.57
Capsule 7	0.698	0.096	0.602	0.50
Capsule 8	0.704	0.096	0.608	1.48
Capsule 9	0.714	0.095	0.619	3.23
Capsule 10	0.711	0.094	0.617	2.92
Mean & SD	**0.691±0.019**	**0.094±0.001**	**0.599±0.018**	**-0.54±3.21**

Deviation form average = (Capsule mass content - Mean of capsule mass content) / Capsule mass content * 100

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APPENDIX 4: Organoleptic features of encapsulated *L. leonurus* solid extracts

Appendix 4A: Organoleptic features of encapsulated FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* stored at room temperature (RT) 24 ± 2 ˚C / 54 % RH; 30 ± 5 ˚C and 40 ± 5 ˚C for 6 months

Plant extract	Storage temperature	Organoleptic feature	At baseline	After 6 months
FDAE	Room temperature	Appearance	Free- flowing powder	**Melted**
		Colour	Medium brown	Medium brown
		Scent	Highly aromatic	Highly aromatic
		Taste	Intensely bitter	Intensely bitter
		Texture	Fairly course	**Sticky**
FDAE	30 ± 5 ° C	Appearance	Free- flowing powder	Unchanged
		Colour	Medium brown	Unchanged
		Scent	Highly aromatic	Unchanged
		Taste	Intensely bitter	Unchanged
		Texture	Fairly course	Unchanged
FDAE	40 ± 5 ° C	Appearance	Free- flowing powder	Unchanged
		Colour	Medium brown	Unchanged
		Scent	Highly aromatic	Unchanged
		Taste	Intensely bitter	Unchanged
		Texture	Fairly course	Unchanged
Aq EtOH	Room temperature	Appearance	Flaky powder	**Melted**
		Colour	Medium brown	Medium brown
		Scent	Highly aromatic	Highly aromatic
		Taste	Intensely bitter	Intensely bitter
		Texture	Very course	**Sticky**
Aq EtOH	30 ± 5 ° C	Appearance	Flaky powder	Unchanged
		Colour	Medium brown	Unchanged
		Scent	Highly aromatic	Unchanged
		Taste	Intensely bitter	Unchanged
		Texture	Very course	Unchanged
Aq EtOH	40 ± 5 ° C	Appearance	Flaky powder	Unchanged
		Colour	Medium brown	Unchanged
		Scent	Highly aromatic	Unchanged
		Taste	Intensely bitter	Unchanged
		Texture	Very course	Unchanged
Alginate beads	Room temperature	Appearance	Rounded balls	Unchanged
		Colour	Dark balls	Unchanged
		Scent	Odourless	Unchanged
		Taste	Tasteless	Unchanged
		Texture	Smooth	Unchanged
Alginate beads	30 ± 5 ° C	Appearance	Rounded balls	Unchanged
		Colour	Dark balls	Unchanged
		Scent	Odourless	Unchanged
		Taste	Tasteless	Unchanged
		Texture	Smooth	Unchanged
Alginate beads	40 ± 5 ° C	Appearance	Rounded balls	Unchanged
		Colour	Dark balls	Unchanged
		Scent	Odourless	Unchanged
		Taste	Tasteless	Unchanged
		Texture	Smooth	Unchanged

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Appendix 4B: Organoleptic features of encapsulated FDAE, Aq EtOH extract and calcium alginate FDAE beads of *L. leonurus* stored under stressed conditions, 40 ±5 ˚C / 75 % RH for 4 weeks

Plant extract	Organoleptic features	At baseline	After 4 weeks
FDAE	Appearance	Free- flowing powder	**Melted**
	Colour	Medium brown	**Dark brown**
	Scent	Highly aromatic	Highly aromatic
	Taste	Intensely bitter	Intensely bitter
	Texture	Fairly course	**Sticky**
Aq EtOH	Appearance	Flaky powder	**Melted**
	Colour	Medium brown	**Dark brown**
	Scent	Highly aromatic	Aromatic
	Taste	Intensely bitter	Intensely bitter
	Texture	Very course	**Sticky**
Alginate beads	Appearance	Rounded balls	Unchanged
	Colour	Dark balls	Unchanged
	Scent	Odourless	Unchanged
	Taste	Tasteless	Unchanged
	Texture	Smooth	Unchanged

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APPENDIX 5: Total phenol content of encapsulated *L. leonurus* solid extracts

Appendix 5A: Total phenol content of encapsulated *L. leonurus* solid extracts stored at room temperature (RT) 24 ± 2 ˚C / 54 % RH for 6 months

Stability storage period	Total phenol content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	GAE	%	GAE	%	GAE	%
Month 0	7.86 ± 0.013	100	7.52 ± 0.008	100	6.94 ± 0.009	100
Month 1	3.05 ± 0.012	39	2.93 ± 0.007	39	2.34 ± 0.007	34
Month 2	3.05 ± 0.012	39	2.57 ± 0.004	34	2.25 ± 0.002	32
Month 3	2.87 ± 0.024	37	2.49 ± 0.021	33	2.21 ± 0.012	32
Month 4	2.80 ± 0.013	36	2.31 ± 0.001	31	1.98 ± 0.004	28
Month 5	2.68 ± 0.012	34	2.15 ± 0.003	29	1.98 ± 0.004	28
Month 6	2.51 ± 0.004	32	2.09 ± 0.003	28	1.81 ± 0.003	26

GAE = Gallic acid equivalents (mg/g) (Mean ± SD; n = 3))

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Appendix 5B: Total phenol content of encapsulated *L. leonurus* solid extracts stored at 30 ±5 ˚C for 6 months

Stability storage period	Total phenol content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	GAE	%	GAE	%	GAE	%
Month 0	7.86 ± 0.013	100	7.52 ± 0.008	100	6.94 ± 0.009	100
Month 1	3.15 ± 0.005	40	3.45 ± 0.005	46	2.01 ± 0.003	29
Month 2	3.10 ± 0.011	39	3.41 ± 0.005	45	2.00 ± 0.002	29
Month 3	3.07 ± 0.008	39	3.39 ± 0.018	45	1.99 ± 0.012	29
Month 4	3.07 ± 0.008	39	3.39 ± 0.005	45	1.96 ± 0.003	28
Month 5	3.06 ± 0.013	39	3.35 ± 0.016	45	1.96 ± 0.003	28
Month 6	3.05 ± 0.002	39	3.35 ± 0.002	45	1.96 ± 0.001	28

GAE = Gallic acid equivalents (mg/g) (Mean ± SD; n = 3))

Appendix 5C: Total phenol content of encapsulated *L. leonurus* solid extracts stored at 40 ±5 ˚C for 6 months

Stability storage period	Total phenol content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	GAE	%	GAE	%	GAE	%
Month 0	7.86 ± 0.013	100	7.52 ± 0.008	100	6.94 ± 0.009	100
Month 1	3.27 ± 0.012	42	4.03 ± 0.007	54	2.83 ± 0.007	41
Month 2	3.22 ± 0.012	41	3.96 ± 0.004	53	2.59 ± 0.002	37
Month 3	3.24 ± 0.024	41	3.91 ± 0.021	52	2.34 ± 0.012	34
Month 4	3.14 ± 0.013	40	3.86 ± 0.001	51	2.29 ± 0.004	33
Month 5	3.48 ± 0.012	44	3.83 ± 0.003	51	2.29 ± 0.004	33
Month 6	2.96 ± 0.004	38	3.83 ± 0.003	51	2.17 ± 0.003	31

GAE = Gallic acid equivalents (mg/g) (Mean ± SD; n = 3))

Appendix 5D: Total phenol content levels of encapsulated *L. leonurus* solid extracts stored under stressed conditions (i.e. at 40 ± 5 ˚C / 75 % RH) for 1 month

Stability storage period	Total phenol content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	GAE	%	GAE	%	GAE	%
Week 0	7.86 ± 0.013	100	7.52 ± 0.008	100	6.94 ± 0.009	100
Week 1	3.75 ± 0.020	48	3.77 ± 0.003	50	2.99 ± 0.005	43
Week 2	3.51 ± 0.029	45	3.80 ± 0.013	51	2.89 ± 0.040	42
Week 3	3.61 ± 0.007	46	3.73 ± 0.006	50	2.65 ± 0.011	38
Week 4	3.66 ± 0.013	47	3.65 ± 0.018	49	2.94 ± 0.015	42

GAE = Gallic acid equivalents (mg/g) (Mean ± SD; n = 3))

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Appendix 5E: Profile of 1 / [A] *vs* time

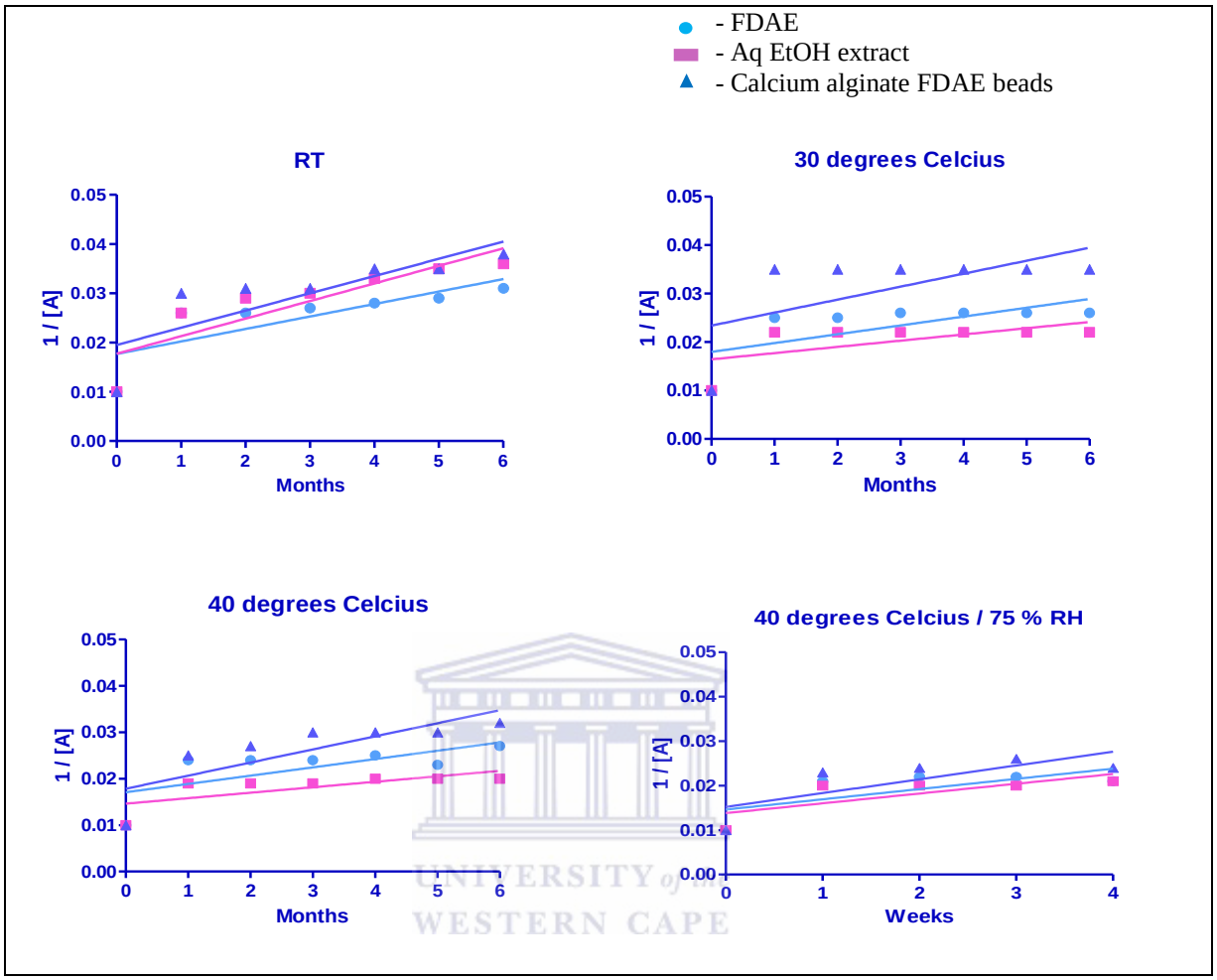

Fig. 5A 5.1: Profile of 1/ [A] *vs* t giving the slope as an estimate of the second order rate constant k of *L. leonurus* solid extracts stored at RT (room temperature, 24 ± 2 ˚C / 54 % RH); 30 °C (30 ± 5 ˚C) ; 40 °C (40 ± 5 ˚C) and 40 ± 5 ˚C / 75 % RH for 6 months and 4 weeks

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APPENDIX 6: Total flavonoid content of encapsulated *L. leonurus* solid extracts

Appendix 6A: Total flavonoid content levels of encapsulated *L. leonurus* solid extracts stored at room temperature (RT) 24 ± 2 ˚C / 54 % RH

Stability storage period	Total flavonoid content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	QE	%	QE	%	QE	%
Month 0	4.30 ± 0.010	100	4.49 ± 0.009	100	3.67 ± 0.014	100
Month 1	3.02 ± 0.008	75	3.34 ± 0.014	74	1.89 ± 0.008	45
Month 2	2.97 ± 0.027	73	3.32 ± 0.025	72	1.76 ± 0.009	44
Month 3	2.53 ± 0.003	73	3.19 ± 0.008	71	1.76 ± 0.011	43
Month 4	2.53 ± 0.003	73	3.19 ± 0.005	71	1.76 ± 0.011	42
Month 5	2.43 ± 0.002	68	3.01 ± 0.008	67	1.57 ± 0.004	40
Month 6	2.33 ± 0.002	66	2.29 ± 0.002	65	1.43 ± 0.002	39

QE = Quercetin equivalents (mg/g) (Mean ± SD; n = 3))

Appendix 6B: Total flavonoid content levels of encapsulated *L. leonurus* solid extracts stored at 30 ± 5 ˚C

Stability storage period	Total flavonoid content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	QE	%	QE	%	QE	%
Month 0	4.30 ± 0.010	100	4.49 ± 0.009	100	3.67 ± 0.014	100
Month 1	3.24 ± 0.023	75	3.49 ± 0.001	74	1.65 ± 0.002	45
Month 2	3.16 ± 0.016	73	3.49 ± 0.008	72	1.62 ± 0.016	44
Month 3	3.16 ± 0.022	73	3.33 ± 0.016	71	1.59 ± 0.015	43
Month 4	3.14 ± 0.007	73	3.32 ± 0.021	71	1.54 ± 0.001	42
Month 5	2.92 ± 0.003	68	3.30 ± 0.021	67	1.49 ± 0.006	40
Month 6	2.84 ± 0.002	66	3.27 ± 0.004	65	1.45 ± 0.003	39

QE = Quercetin equivalents (mg/g) (Mean ± SD; n = 3))

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Appendix 6C:Total flavonoid content levels of encapsulated *L. leonurus* solid extracts stored at 40 ± 5 ˚C

Stability storage period	Total flavonoid content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	QE	%	QE	%	QE	%
Month 0	4.30 ± 0.010	100	4.49 ± 0.009	100	3.67 ± 0.014	100
Month 1	3.07 ± 0.005	71	3.47 ± 0.001	77	1.77 ± 0.003	48
Month 2	3.06 ± 0.015	71	3.37 ± 0.008	75	1.77 ± 0.005	48
Month 3	3.06 ± 0.015	71	3.35 ± 0.016	75	1.76 ± 0.006	48
Month 4	2.97 ± 0.007	69	3.33 ± 0.016	74	1.76 ± 0.005	48
Month 5	2.75 ± 0.002	64	3.33 ± 0.021	74	1.59 ± 0.009	43
Month 6	2.65 ± 0.003	62	3.25 ± 0.004	72	1.57 ± 0.002	43

QE = Quercetin equivalents (mg/g) (Mean ± SD; n = 3))

Appendix 6D: Total flavonoid content levels of encapsulated *L. leonurus* solid extracts stored under stressed conditions (i.e. 40 ± 5 ˚C / 75 % RH)

Stability storage period	Total flavonoid content of capsules containing					
	FDAE		Aq EtOH extract		Calcium alginate FDAE beads	
	QE	%	QE	%	QE	%
Week 0	4.30 ± 0.010	100	4.49 ± 0.009	100	3.67 ± 0.014	100
Week 1	2.69 ± 0.020	34	2.28 ± 0.006	30	2.53 ± 0.006	36
Week 2	2.75 ± 0.004	35	2.30 ± 0.007	31	2.50 ± 0.007	36
Week 3	2.74 ± 0.009	35	2.33 ± 0.003	31	2.52 ± 0.010	36
Week 4	2.74 ± 0.040	35	2.30 ± 0.002	31	2.62 ± 0.005	38

QE = Quercetin equivalents (mg/g) (Mean ± SD; n = 3))

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Appendix 6E: Profile of 1 / [A] *vs* time

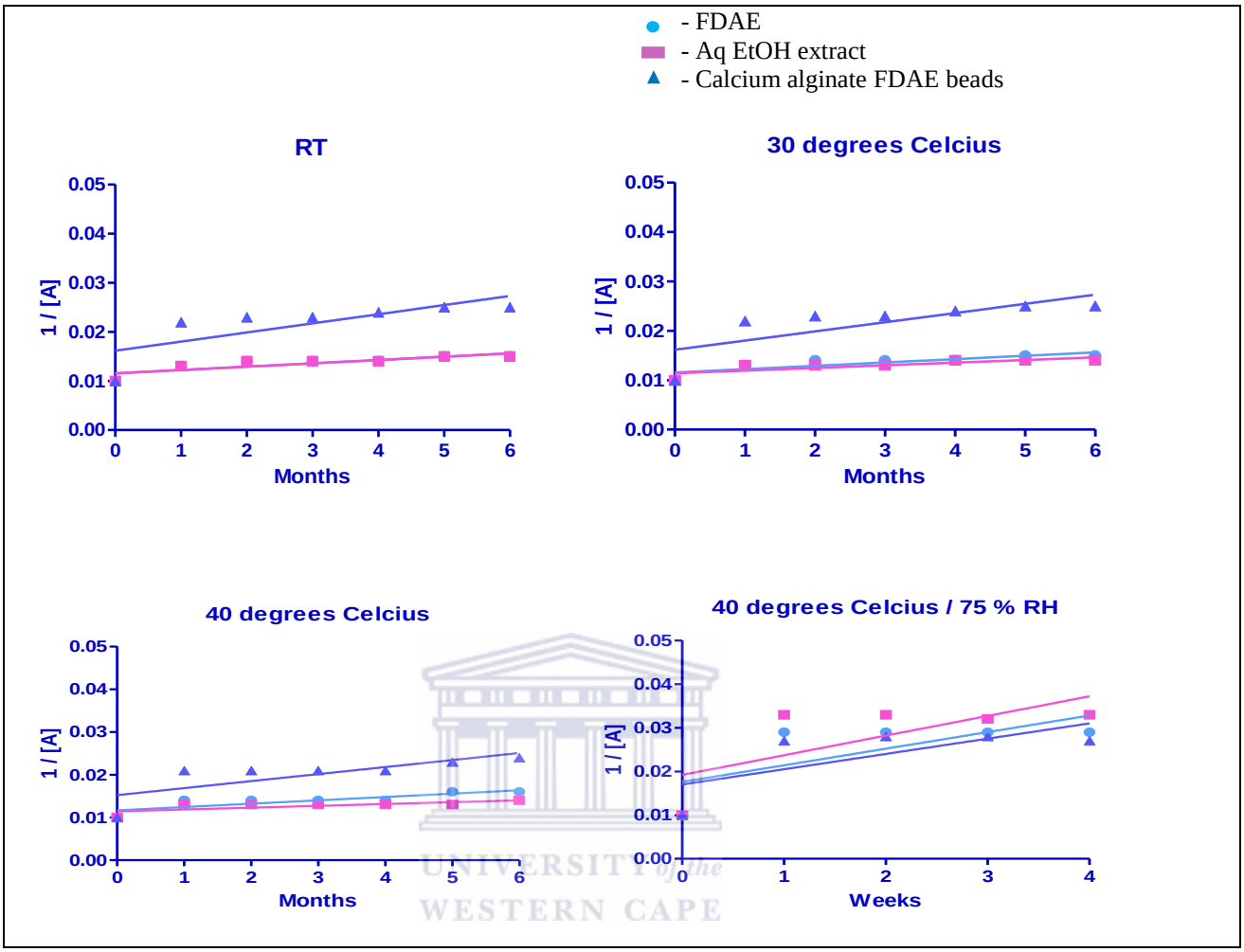

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Fig. 6A 6.1: **Profile of 1/ [A] *vs* t giving the slope as an estimate of the second order rate constant k of *L. leonurus* solid extracts stored at RT (room temperature, 24 ± 2 ˚C / 54 % RH); 30 °C (30 ± 5 ˚C) ; 40 °C (40 ± 5 ˚C) and 40 ± 5 ˚C / 75 % RH for 6 months and 4 weeks**

APPENDIX 7: Antioxidant activity of encapsulated *L. leonurus* solid extracts

Appendix 7A: Antioxidant activity of encapsulated *L. leonurus* FDAE under different storage conditions

Stability study storage conditions	Storage period	Concentration of solid extract solution (mg/ mL)	*AS (AUFS)	**AC (AUFS)	***DPPH Scavenging
24 ± 2 ˚C / 54 % RH	Month 0	10	0.140	1.205	100.0
	Month 1	10	0.142	1.205	99.8
	Month 2	10	0.145	1.205	99.5
	Month 3	10	0.152	1.205	98.9
	Month 4	10	0.190	1.205	95.2
	Month 5	10	0.234	1.205	91.2
	Month 6	10	0.258	1.205	88.9
30 ± 5 ˚C	Month 0	10	0.140	1.205	100
	Month 1	10	0.143	1.205	99.9
	Month 2	10	0.155	1.205	98
	Month 3	10	0.189	1.205	95
	Month 4	10	0.212	1.205	93
	Month 5	10	0.216	1.205	93
	Month 6	10	0.416	1.205	74
40 ± 5 ˚C	Month 0	10	0.140	1.205	100
	Month 1	10	0.148	1.205	99.2
	Month 2	10	0.148	1.205	99.2
	Month 3	10	0.218	1.205	92.6
	Month 4	10	0.257	1.205	89
	Month 5	10	0.269	1.205	87.9
	Month 6	10	0.480	1.205	68.1

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Appendix 7B: Antioxidant activity of encapsulated *L. leonurus* Aq EtOH extract

Stability study storage conditions	Stability period	Concentration of solid extract solution (mg/ mL)	*AS (AUFS)	**AC (AUFS)	***DPPH Scavenging
24 ± 2 ˚C / 54 % RH	Month 0	10	0.114	1.205	100
	Month 1	10	0.143	1.205	99.3
	Month 2	10	0.155	1.205	98.8
	Month 3	10	0.189	1.205	96
	Month 4	10	0.212	1.205	96.4
	Month 5	10	0.216	1.205	93
	Month 6	10	0.293	1.205	83.3
30 ± 5 ˚C	Month 0	10	0.114	1.205	100
	Month 1	10	0.114	1.205	99
	Month 2	10	0.129	1.205	93
	Month 3	10	0.186	1.205	97
	Month 4	10	0.149	1.205	95
	Month 5	10	0.167	1.205	87
	Month 6	10	0.254	1.205	71
40 ± 5 ˚C	Month 0	10	0.114	1.205	100
	Month 1	10	0.141	1.205	99.9
	Month 2	10	0.143	1.205	99.4
	Month 3	10	0.208	1.205	98.1
	Month 4	10	0.307	1.205	84.5
	Month 5	10	0.401	1.205	84.3
	Month 6	10	0.447	1.205	72.7

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Appendix 7C: Antioxidant activity of encapsulated *L. leonurus* calcium alginate FDAE beads

Stability study storage conditions	Stability period	Concentration of solid extract solution (mg/mL)	*AS (AUFS)	**AC (AUFS)	***DPPH Scavenging
24 ± 2 ˚C / 54 % RH	Month 0	10	0.412	1.205	100
	Month 1	10	0.427	1.205	98
	Month 2	10	0.43	1.205	97.7
	Month 3	10	0.431	1.205	97.6
	Month 4	10	0.435	1.205	97.1
	Month 5	10	0.465	1.205	93.3
	Month 6	10	0.48	1.205	91.3
30 ± 5 ˚C	Month 0	10	0.412	1.205	100
	Month 1	10	0.429	1.205	98
	Month 2	10	0.432	1.205	97
	Month 3	10	0.442	1.205	96
	Month 4	10	0.44	1.205	96
	Month 5	10	0.438	1.205	97
	Month 6	10	0.45	1.205	95
40 ± 5 ˚C	Month 0	10	0.412	1.205	100
	Month 1	10	0.422	1.205	98.6
	Month 2	10	0.436	1.205	97
	Month 3	10	0.433	1.205	97.3
	Month 4	10	0.435	1.205	97.1
	Month 5	10	0.476	1.205	91.3
	Month 6	10	0.486	1.205	90.6

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Appendix 7D: Antioxidant activity of encapsulated *L. leonurus* solid extracts stored under stressed conditions, i.e. 40 ± 5 ˚C / 75 % RH

Stability study storage conditions	Stability period	Concentration of solid extract solution (mg/mL)	*AS (AUFS)	**AC (AUFS)	***DPPH Scavenging
FDAE	Week 0	10	0.140	1.205	100.0
	Week 1	10	0.161	1.205	98.0
	Week 2	10	0.161	1.205	98.0
	Week 3	10	0.180	1.205	96.3
	Week 4	10	0.182	1.205	95.9
Aq EtOH extract	Week 0	10	0.140	1.205	100.0
	Week 1	10	0.172	1.205	94.7
	Week 2	10	0.180	1.205	94.0
	Week 3	10	0.182	1.205	93.8
	Week 4	10	0.201	1.205	92.0
Calcium alginate FDAE beads	Week 0	10	0.412	1.205	100.0
	Week 1	10	0.417	1.205	99.2
	Week 2	10	0.42	1.205	98.9
	Week 3	10	0.426	1.205	98.2
	Week 4	10	0.428	1.205	97.9

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Where:

*AS = the UV absorbance of the sample (i.e. plant extract or ascorbic acid) in DPPH solution

**AC = the UV absorbance of the control solution (containing DPPH only)

***DPPH Scavenging = (AC – AS) / AC *100 %

APPENDIX 8: HPLC fingerprints of encapsulated *L. leonurus* solid extracts

Appendix 8A: HPLC fingerprints of encapsulated *L. leonurus* FDAE stored under stressed conditions and developed at 214nm to illustrate marrubiin

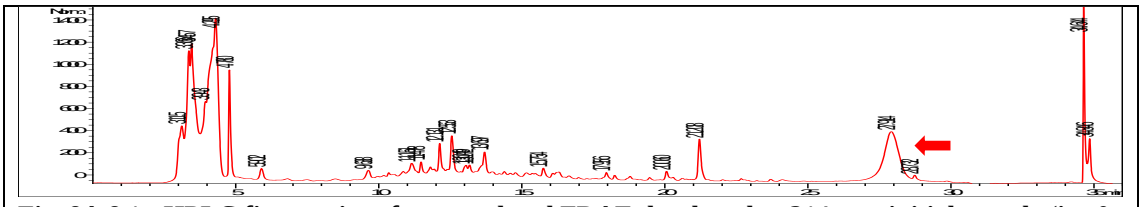

Fig. 8A 8.1: HPLC fingerprint of encapsulated FDAE developed at 214 nm: initial sample (i.e. 0 storage)

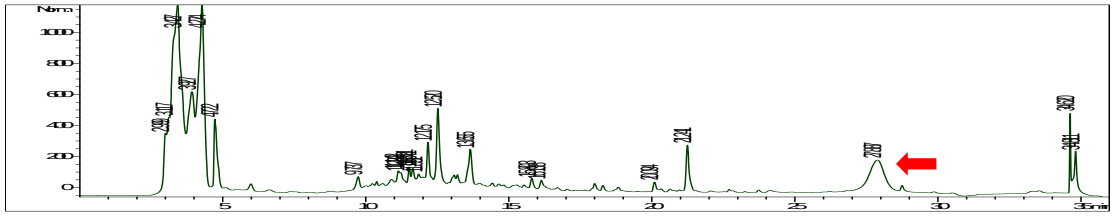

Fig. 8A 8.2: HPLC fingerprint of encapsulated FDAE stored at 40 °C / 75 % RH and developed at 214 nm: sample after 1 week storage

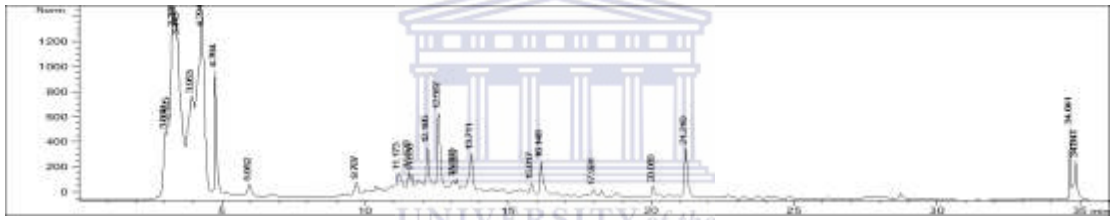

Fig. 8A 8.3: HPLC fingerprint of encapsulated FDAE stored at 40 °C / 75 % RH and developed at 214 nm: sample after 2 week storage

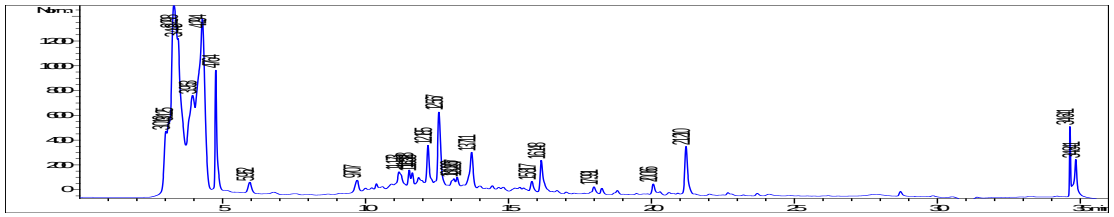

Fig. 8A 8.4: HPLC fingerprint of encapsulated FDAE stored at 40 °C / 75 % RH and developed at 214 nm: sample after 3 week storage

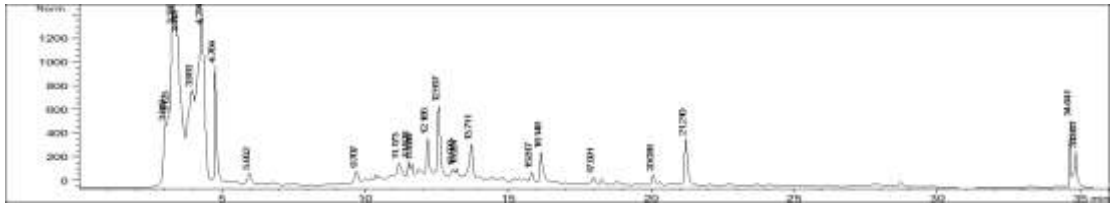

Fig. 8A 8.5: HPLC fingerprint of encapsulated FDAE stored at 40 °C / 75 % RH and developed at 214 nm: sample after 4 week storage

Appendix 8B: HPLC fingerprints of encapsulated *L. leonurus* FDAE stored under stressed conditions and developed at 280 nm to illustrate leonurine

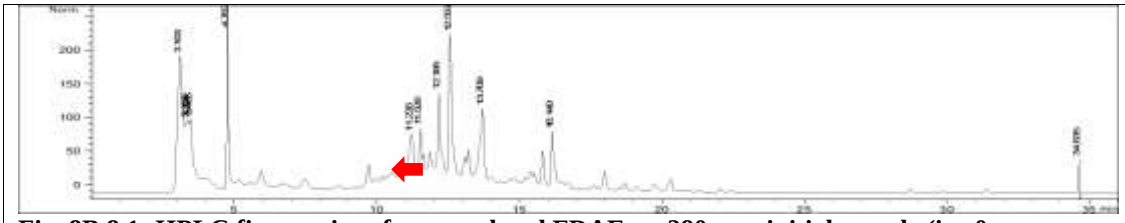

Fig. 8B 8.1: HPLC fingerprint of encapsulated FDAE at 280 nm: initial sample (i.e. 0 storage)

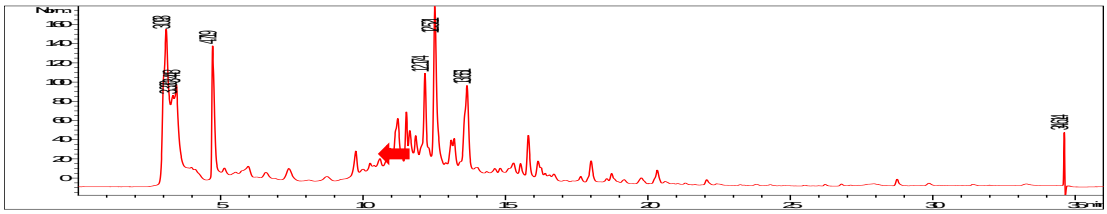

Fig. 8B 8.2: HPLC fingerprint of FDAE stored at 40 °C / 75 % RH and developed at 280 nm: sample after 1 week storage

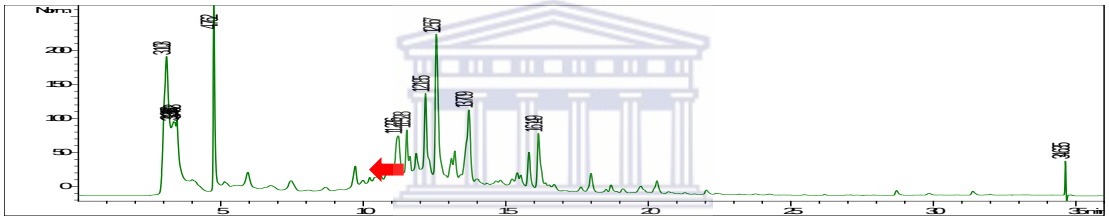

Fig. 8B 8.3: HPLC fingerprint of FDAE stored at 40 °C / 75 % RH and developed at 280 nm: sample after 2 week storage

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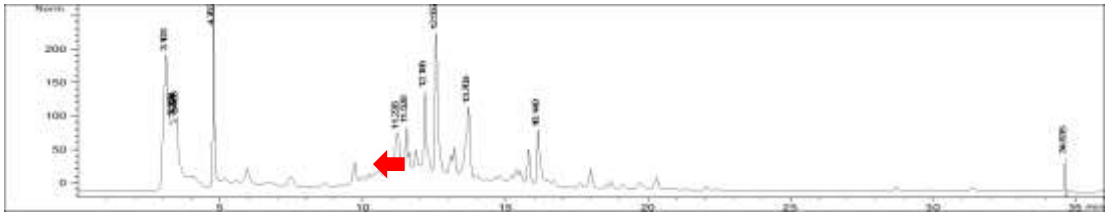

Fig. 8B 8.4: HPLC fingerprint of FDAE stored at 40 °C / 75 % RH and developed at 280 nm: sample after 3 week storage

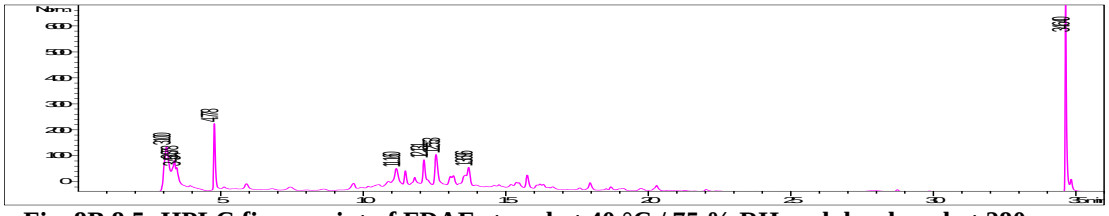

Fig. 8B 8.5: HPLC fingerprint of FDAE stored at 40 °C / 75 % RH and developed at 280 nm: sample after 4 week storage

Appendix 8C: HPLC fingerprints of encapsulated *L. leonurus* Aq EtOH extract stored under stressed conditions and developed at 214nm to illustrate marrubiin.

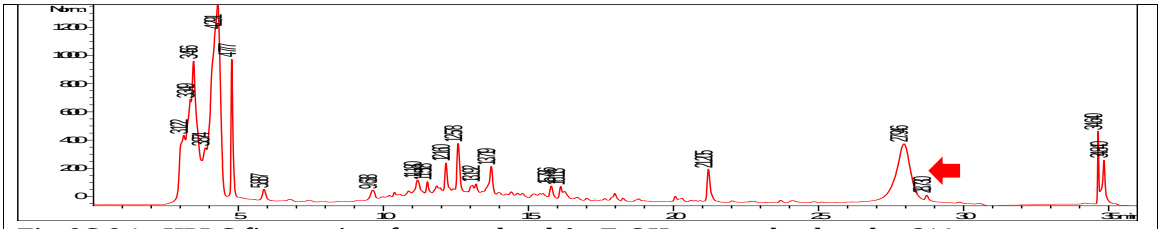

Fig. 8C 8.1: HPLC fingerprint of encapsulated Aq EtOH extract developed at 214 nm: initial sample (i.e. 0 storage)

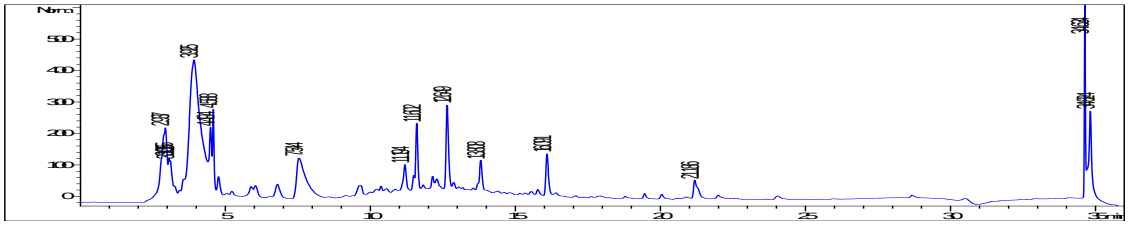

Fig. 8C 8.2: HPLC fingerprint of encapsulated Aq EtOH extract stored at 40 °C / 75 % RH and developed at 214 nm: sample after 1 week storage

Fig. 8C 8.3: HPLC fingerprint of encapsulated Aq EtOH extract stored at 40 °C / 75 % RH and developed at 214 nm: sample after 2 week storage

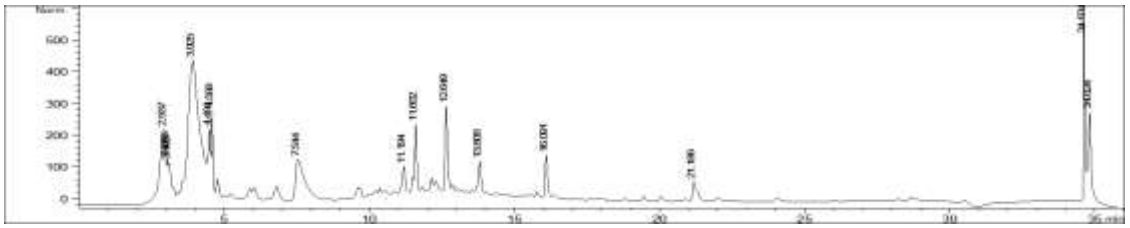

Fig. 8C 8.4: HPLC fingerprint of encapsulated Aq EtOH extract stored at 40 °C / 75 % RH and developed at 214 nm: sample after 3 week storage

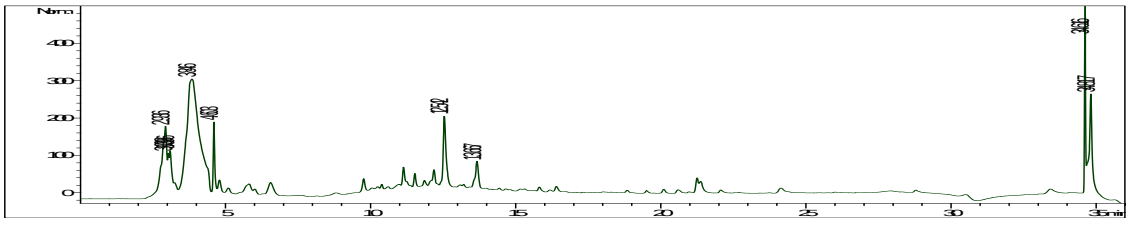

Fig. 8C 8.5: HPLC fingerprint of encapsulated Aq EtOH extract stored at 40 °C / 75 % RH and developed at 214 nm: sample after 4 week storage

Appendix 8D: HPLC fingerprints of encapsulated *L. leonurus* Aq EtOH extract stored under stressed conditions and developed at 280 nm to illustrate Leonurine

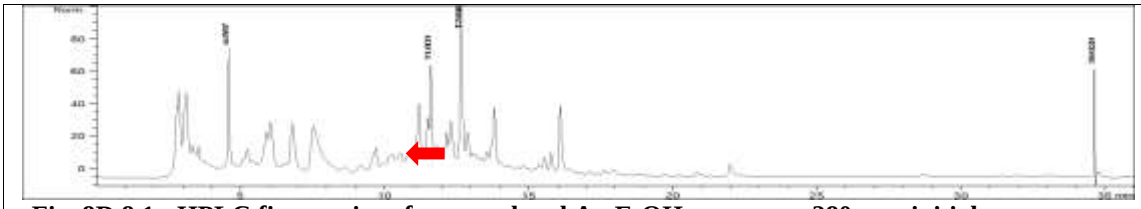

Fig. 8D 8.1: HPLC fingerprint of encapsulated Aq EtOH extract at 280 nm: initial sample (i.e. 0 storage)

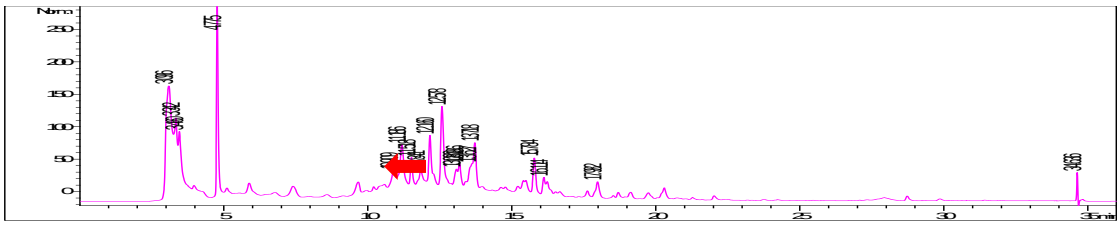

Fig. 8D 8.2: HPLC fingerprint of Aq EtOH extract stored at 40 °C / 75 % RH and developed at 280 nm: sample after 1 week storage

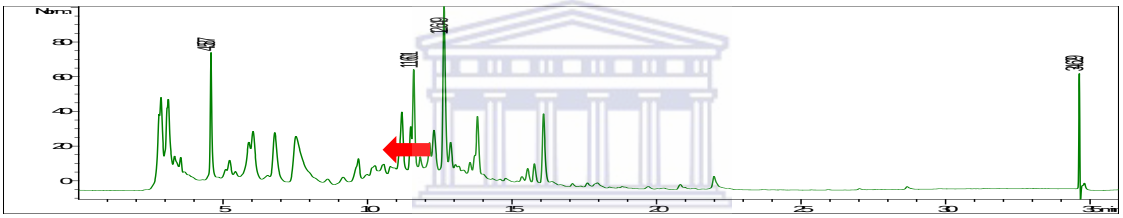

Fig. 8D 8.3: HPLC fingerprint of Aq EtOH extract stored at 40 °C / 75 % RH and developed at 280 nm: sample after 2 week storage

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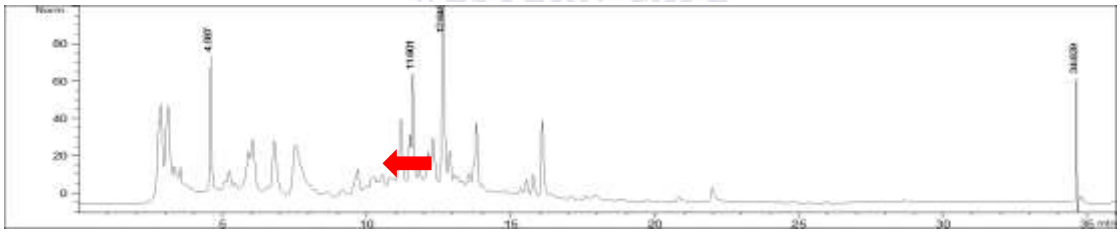

Fig. 8D 8.4: HPLC fingerprint of Aq EtOH extract stored at 40 °C / 75 % RH and developed at 280 nm: sample after 3 week storage

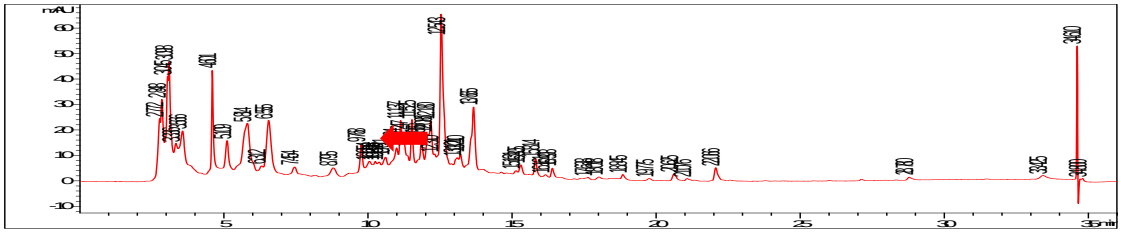

Fig. 8D 8.5: HPLC fingerprint of Aq EtOH extract stored at 40 °C / 75 % RH and developed at 280 nm: sample after 4 week storage

Appendix 8E: HPLC fingerprints of encapsulated *L. leonurus* calcium alginate FDAE beads stored under stressed conditions and developed at 214nm to illustrate marrubiin

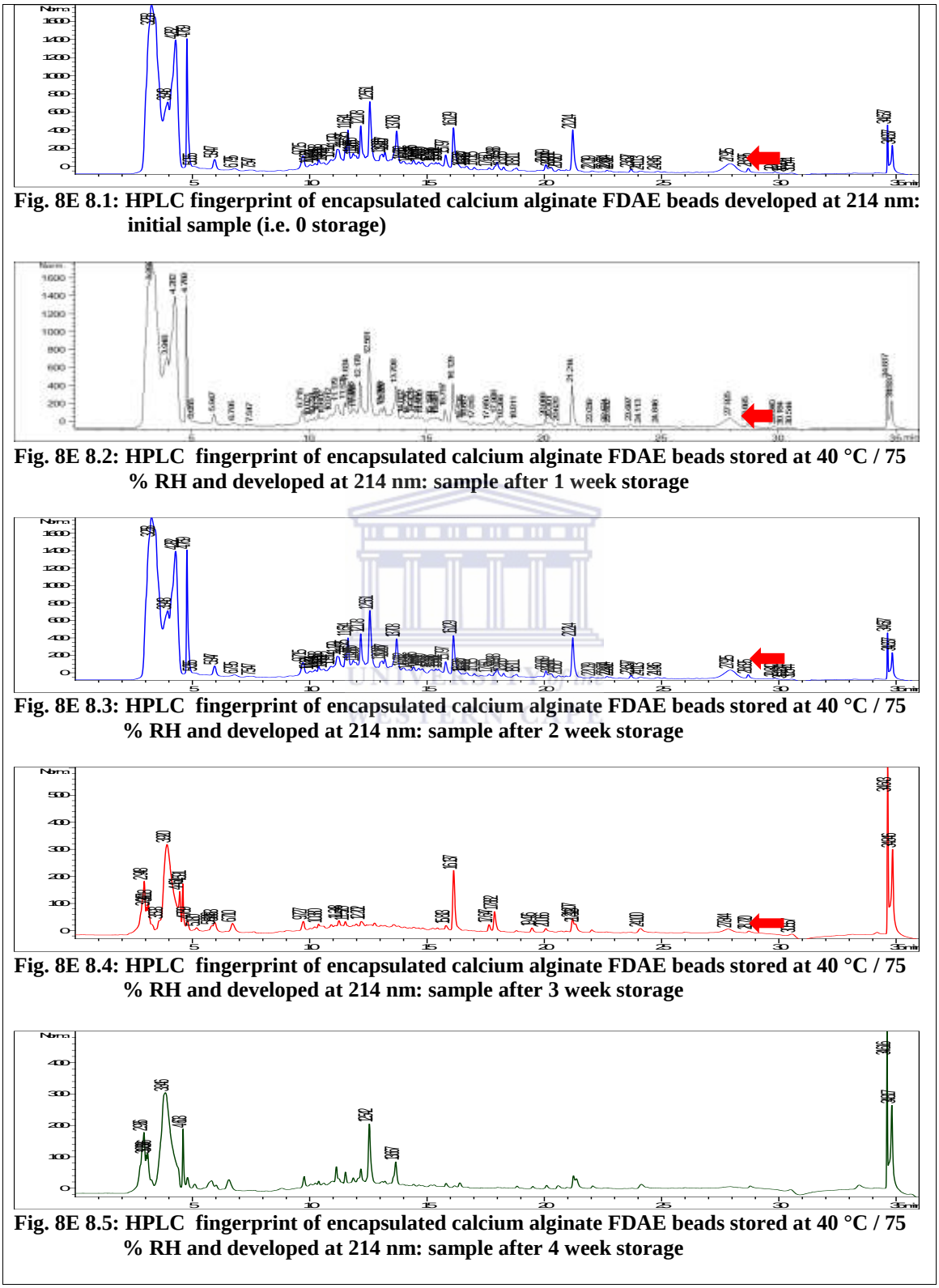

Fig. 8E 8.1: HPLC fingerprint of encapsulated calcium alginate FDAE beads developed at 214 nm: initial sample (i.e. 0 storage)

Fig. 8E 8.2: HPLC fingerprint of encapsulated calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 214 nm: sample after 1 week storage

Fig. 8E 8.3: HPLC fingerprint of encapsulated calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 214 nm: sample after 2 week storage

Fig. 8E 8.4: HPLC fingerprint of encapsulated calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 214 nm: sample after 3 week storage

Fig. 8E 8.5: HPLC fingerprint of encapsulated calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 214 nm: sample after 4 week storage

Appendix 8F: HPLC fingerprints of encapsulated *L. leonurus* calcium alginate FDAE beads stored under stressed conditions and developed at 280 nm to illustrate leonurine

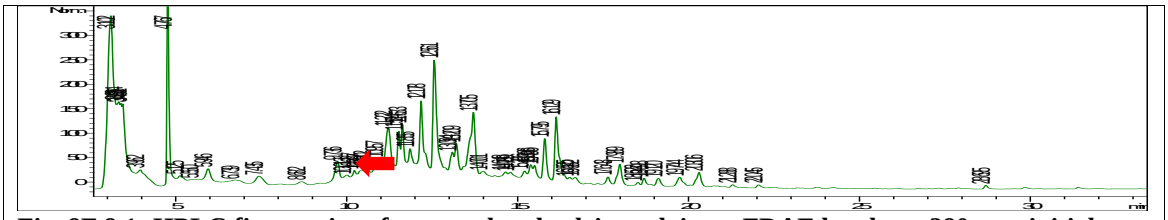

Fig. 8F 8.1: HPLC fingerprint of encapsulated calcium alginate FDAE beads at 280 nm: initial sample (i.e. 0 storage)

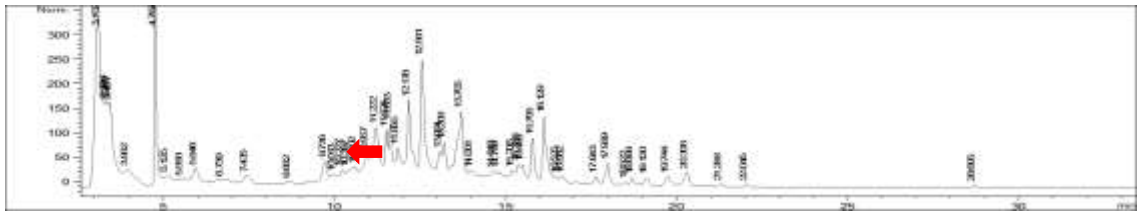

Fig. 8F 8.2: HPLC fingerprint of calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 280 nm: sample after 1 week storage

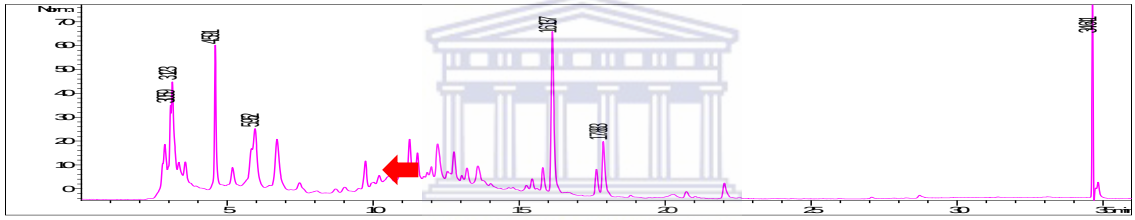

Fig. 8F 8.3: HPLC fingerprint of calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 280 nm: sample after 2 week storage

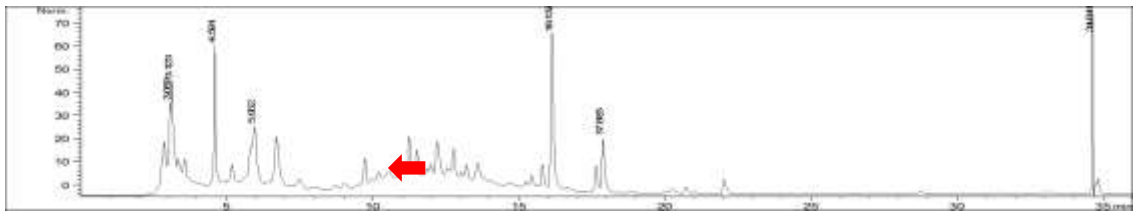

Fig. 8F 8.4: HPLC fingerprint of calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 280 nm: sample after 3 week storage

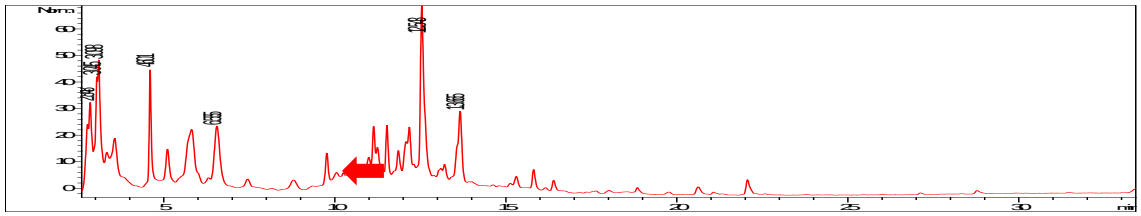

Fig. 8F 8.5: HPLC fingerprint of calcium alginate FDAE beads stored at 40 °C / 75 % RH and developed at 280 nm: sample after 4 week storage

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